# COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS


INTERNATIONAL ORGANISATION
OF VINE AND WINE

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# *COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS*

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# Foreword

The *Compendium of International Methods of Wine Analysis* was first published in 1962 and re-published in 1965, 1972, 1978, 1990 and 2000; each time it included additional material as approved by the General Assembly and produced each year by the Sub-Commission.

This edition of *Compendium of International Methods of Wine and Must Analysis* includes all material as approved by the General Assembly of representatives of the member governments of the OIV, revised and amended since 2000.

The Compendium plays a major part in harmonising methods of analysis. Many vine-growing countries have introduced its definitions and methods into their own regulations.

Regulation (EC) No 479/2008 lays down that *the analysis methods for establishing the composition of the products covered by that Regulation and the rules for checking whether those products have been subjected to processes in violation of authorised oenological practice are those recommended and published by the OIV in the Compendium of International Methods of Analysis of Wines and Musts*. In Regulation (EC) No 606/2009 to ensure greater transparency, it was stated to publish at Community level (C Series of the *Official Journal of the European Union*) the list and description of the analysis methods described in the Compendium of International Methods of Analysis of Wines and Musts of the International Organisation of Vine and Wine and applicable for the control of vitivinicultural products.
In this way the European Union recognises all of the methods in the Compendium and makes them binding in all Member States, confirming the close collaboration established between the EU and the OIV.

Thus, through its leading role in the harmonisation of methods of analysis, the Compendium contributes to facilitating international trade. With the *International Code of Oenological Practices* and the *International Oenological Codex*, it constitutes a body of considerable scientific, legal and practical benefit.

**General organization of the Compendium**

***NB: Each method has its references into the brackets (referred resolutions, reference methods of the "Recueil des méthodes internationales d'analyse des vins et des moûts" 1990 = Recueil OIV ed. 1990; methods of the "Recueil des méthodes internationales d'analyse des vins" 1978 = A number i.e. A1, A2 …)***

# Layout and wording of OIV method of analysis

Extract of ISO 78-2:1999 standard

## 1. Title

## 2. Introduction

optional

## 3. Scope

This clause shall state succintly the method of chemical analysis and specifically the product to which applies.

## 4. Définitions

## 5. Principle

This optional clause indicates the essential steps in the method used, the basic principles.

## 6. Reagents and materials

This clause shall list all the reagents and materials used during the test, together with their essential characteristics, and shall specify, if necessary, their degree of purity.
Shall be given :
Products used in their commercially available form
Solutions of defined concentration
Standard volumetric solution
Standard reference solution
Standard solution
Standard matching solution
Note : each reagent shall be mentioned by a specific reference number

## 7. Apparatus

This cluse shall list the names and significant characteristics of all the apparatus and equipment to be used during the analysis or test.

## 8. Sampling (Preparation of the sample)

Shall be given :
Sampling procedure
Preparation of the test sample

## 9. Procedure

Each sequence of operations shall be described unambiguously and concisely.
This clause shall normally include the following subclauses :
Test portion (this subclause shall give all the information necessary for the preparation of the test portion from the test sample).
Determination(s), or test(s) (this subclause shall be described accurately in order to facilitate the description, the understanding and the application of the procedure).
Calibration (if necessary).

## 10. Calculation (Results)

This clause shall indicate the method for calculating the results. Shall be precised the units, the equation used, the meanings of the algebraic symbols, the number of decimal places to which the results is to be given.

## 11. Precision (if interlaboratory validation)

The precision data shall be indicated:
The number of laboratoriese
The mean value of the concentration
The repeatability and the reproducibility
The repeatability and reproducibility standard deviation
A reference to the document containing the published results of the interlaboratory tests.

## 12. Annex

Annex related to precision clauses
Annex concerning statistical and other data derived from the results of interlaboratory tests.

## 13. Bibliography

**Annex related to precision clauses**

This annex shall indicate in particular
- repeatability statements
- reproducibility statements

Annex concerning statistical and other data derived from the results of interlaboratory tests.

Statistical and other data derived from the results of interlaboratory tests may be given in an informative annex.

Example of table giving statistical results

| **Sample identification** | **A** | **B** | **C** |
|---|---|---|---|
| Number of participating laboratories | | | |
| Number of accepted test results | | | |
| Mean values (g/100g sample) | | | |
| True or accepted value (g/100g) | | | |
| Repeatability standard deviation ($S_r$) | | | |
| Repeatability coefficient of variation | | | |
| Repeatability limit (r) (2,8 x $S_r$) | | | |
| Reproducibility standard deviation ($S_R$) | | | |
| Reproducibility coefficient of variation | | | |
| Reproducibility limit (R) (2,8 x $S_R$) | | | |

Whilst it may not be considered necessary to include all the data shown in the table, it is recommended that at least the following data be included:

- The number of laboratories
- The mean value of the concentration
- The repeatability standard deviation
- The reproducibility standard deviation
- A reference to the document containing the published results of the interlaboratory tests.

# General Remarks

1/ Clear wine or must, must be used for chemical and physical analysis. If the wine or the must is cloudy, it is first filtered through filter paper in a covered funnel or centrifuged in a closed container. This operation must be stated on any required documentation.

2/ The reference of the method employed for each determination must be on any required documentation.

3/ Units of measure for the various magnitudes (volume, mass, concentration, temperature, pressure, etc.) shall be in accordance with the recommendations of the IUPAC (International Union for Pure and Applied Chemistry).

4/ In respect of reagents and titration solutions used, unless otherwise required in the text, the chemicals used are to be of "analytical grade" and the water is to be distilled or of equivalent purity.

5/ Enzyme methods, and the determination of a number of parameters, are to be based on absolute measurements of absorbance, which requires spectrophotometers to be calibrated for wavelengths and absorbance. Wavelength may be calibrated by use of Hg lines: 239.94, 248.0, 253.65, 280.4, 302.25, 313.16, 334.15, 365.43, 404.66, 435.83, 546.07, 578.0, and 1014.0 nm. Absorbance may be calibrated by means of commercial reference solutions, obtained from suitable suppliers, or neutral density filters.

6/ The essential bibliographical references are given. The references to working documents of the Sub-Commission are marked '*F.V., O.I.V.*' (feuillets verts or 'green pages'), followed by the year of publication and the number of the document.

# Annex A

# Methods of analysis of wines and musts

# Classification of analytical methods

(Resolution Oeno 9/2000)

CATEGORY I* (CRITERION BENCHMARK METHOD): A method which determines a value that can be arrived at only by implementing the method *per se* and which serves, by definition, as the only method for establishing the accepted value of the parameter measured (e.g., alcoholometric content, total acidity, volatile acidity).

CATEGORY II* (BENCHMARK METHOD): A category II method is designated as the Benchmark Method in cases where category I methods cannot be used. It should be selected from category III methods (as defined below). Such methods should be recommended for use in cases of disputes and for calibration purposes. (e.g., potassium, citric acid).

CATEGORY III* (APPROVED ALTERNATIVE METHODS): A category III Method meets all of the criteria specified by the Sub-Committee on Methods of Analysis and is used for monitoring, inspection and regulatory purposes (e.g., enzymatic determinations of glucose and fructose).

CATEGORY IV (AUXILIARY METHOD): A category IV Method is a conventional or recently-implemented technique, with respect to which the Sub-Committee on Methods of Analysis has not as yet specified the requisite criteria (e.g., synthesized coloring agents, measurement of oxidation-reduction potential).

* Methods requiring formal approval in accordance with the procedures in force at the Sub-Commission of Methods of Analysis.

# Matrix effect for metals content analysis using atomic absorption

(Resolution oeno 5/2000)

The GENERAL ASSEMBLY,

In consideration of Article 5, Paragraph 4 of the International Standardization Convention on Methods of Wine Analysis and Rating of October 13, 1954,

Action on the proposal of the Sub-Committee on International Methods of Analysis and Rating of Wines,

CONSIDERING that the methods described in the Compendium of International Methods of Wine and Must Analysis and entailing the use of reference solutions are implemented for dry wines,

DRAWS the attention of users to the fact that deviations may be observed in other cases involving the presence of sugars or sugar derivatives,

DECIDES that it is therefore necessary to undertake analyses using the quantified additions method. A minimum of three aliquot portions of the sample containing various additions should be used.

DECIDES to supplement the methods for analyzing metals (iron, lead, zinc, silver, cadmium) and arsenic with a description of the quantified additions technique, when the matrix effect so requires.

# Principle of validation of routine methods with respect to reference methods

(Resolution Oeno 7/98)

The OIV acknowledges the existence of methods of analysis of wines in addition to those described in the Summary of International Methods of Analysis of Wines and Musts, of common methods most often automated. These methods are economically and commercially important because they permit maintaining a complete and efficient analytical framework around the production and marketing of wine. Moreover, these methods allow the use of modern means of analysis and the development and adaptation of techniques of analysis.

In order to allow laboratories to use these methods and to insure their linkage to methods described within the Summary, the OIV decides to establish a plan of evaluation and validation by a laboratory of an alternative, common method, mechanized or not with respect to a reference method described in the Summary of International Methods of Analysis of Wines and Musts.

This principle, which will be adapted to the particular situation of the analysis of wines and musts, will take its inspiration from international standards in current use and allow the laboratory to assess and validate its alternative method in two ways:

# Collaborative Study

The purpose of the collaborative study is to give a quantified indication of the precision of method of analysis, expressed as its repeatability *r* and reproducibility R.

***Repeatability***: the value below which the absolute difference between two single test results obtained using the same method on identical test material, under the same conditions (same operator, same apparatus, same laboratory and a short period of time) may be expected to lie within a specified probability.

***Reproducibility***: the value below which the absolute difference between two single test results obtained using the same method on identical test material, under different conditions (different operators, different apparatus and/or different laboratories and/or different time) may be expected to lie within a specified probability.

The term "individual result" is the value obtained when the standardized trial method is applied, once and fully, to a single sample. Unless otherwise stated, the probability is 95%.

**General Principles**

- The method subjected to trial must be standardized, that is, chosen from the existing methods as the method best suited for subsequent general use.
- The protocol must be clear and precise.
- The number of laboratories participating must be at least ten.
- The samples used in the trials must be taken from homogeneous batches of material.
- The levels of the analyte to be determined must cover the concentrations generally encountered.
- Those taking part must have a good experience of the technique employed.
- For each participant, all analyses must be conducted within the same laboratory by the same analyst.
- The method must be followed as strictly as possible. Any departure from the method described must be documented.
- The experimental values must be determined under strictly identical conditions: on the same type of apparatus, etc.
- They must be determined independently of each other and immediately after each other.
- The results must be expressed by all laboratories in the same units, to the same number of decimal places.
- Five replicate experimental values must be determined, free from outliers. If an experimental value is an outlier according to the Grubbs test, three additional measurements must be taken.

**Statistical Model**

The statistical methods set out in this document are given for one level (concentration, sample). If there are a number of levels, the statistical evaluation must be made separately for each. If a linear relationship is found ($y = bx$ or $y = a + bx$) as between the repeatability (r) or reproducibility (R) and the concentration ($\bar{\bar{x}}$), a regression of r (or R) may be run as a function of $\bar{\bar{x}}$

The statistical methods given below suppose normally-distributed random values.

The steps to be followed are as follows:

A/ Elimination of outliers within a single laboratory by Grubbs test. Outliers are values which depart so far from the other experimental values that these deviations cannot be regarded as random, assuming the causes of such deviations are not known.

B/ Examine whether all laboratories are working to the same precision, by comparing variances by the Bartlett test and Cochran test. Eliminate those laboratories for which statistically deviant values are obtained.

C/ Track down the systematic errors from the remaining laboratories by a variance analysis and by a Dixon test identify the extreme outlier values. Eliminate those laboratories for which the outlier values are significant.

D/ From the remaining figures, calculate standard deviation of repeatability); *Sr.*, and repeatability *r* standard deviation of reproducibility $S_R$ and reproducibility R.

*Notation:*

The following designations have been chosen:

| | |
|---|---|
| $m$ | Number of laboratories |
| $i (i = 1, 2... m)$ | Index (No. of the laboratory) |
| $n_i$ | Number of individual values from the i*th* laboratory |
| $N=\sum_{i=1}^{m} n_i$ | Total number of individual values |
| $x (i = 1, 2... n_i)$ | Individual value of the i*th* laboratory |
| $\bar{x}_i=\frac{1}{n_i}\sum_{i=1}^{n_i} x_i$ | Mean value of the i*th* laboratory |
| $\bar{\bar{x}}=\frac{1}{N}\sum_{i=1}^{m}\sum_{i=1}^{n_i} x_i$ | Total mean value |
| $s_i=\sqrt{\frac{1}{n_i-1}\sum_{i=1}^{n_i}\left(x_i-\bar{x}_i\right)^2}$ | Standard deviation of the i*th* laboratory |

A/ ***Verification of outlier values within one laboratory***

After determining five individual values $x_i$, a Grubbs test is performed at the laboratory, to identify the outliers' values.

Test the null hypothesis whereby the experimental value with the greatest absolute deviation from the mean is not an outlier observation.

Calculate PG = $\frac{\left| x_i^* - \bar{x}_i \right|}{s_i}$

$x_i^*$ = suspect value

Compare PG with the corresponding value shown in Table 1 for P = 95%.

If PG < value as read, value $x_i^*$ is not an outlier and $s_i$ can be calculated.

If PG > value as read, value $x_i^*$ probably is an outlier therefore make a further three determinations.

Calculate the Grubbs test for $x_i^*$ with the eight determinations.

If PG > corresponding value for P = 99%, regard $x_i^*$ as a deviant value and calculate $s_i$ without $x_i^*$.

B/ ***Comparison of variances among laboratories***
***- Bartlett Test***

The Bartlett test allows us to examine both major and minor variances. It serves to test the null hypothesis of the equality of variances in all laboratories, as against the alternative hypothesis whereby the variances are not equal in the case of some laboratories.

At least five individual values are required per laboratory.

Calculate the statistics of the test:

$$PB = \frac{1}{C}\left[ (N - \mathrm{m}) \ln S_r^2 - \sum_{\mathrm{i}=1}^{\mathrm{m}} \mathrm{f_i} \ln \mathrm{s_i^2} \right]$$

$$C = \frac{\sum_{\mathrm{i}=1}^{\mathrm{m}} \frac{1}{\mathrm{f_i}} - \frac{1}{N - \mathrm{m}}}{3(\mathrm{m} - 1)} + 1$$

$$S_r^2 = \frac{\sum_{i=1}^{m} \mathrm{f_i} s_I^2}{N - \mathrm{m}}$$

$f_i = n_i$ - 1 degrees of freedom of $s_i$.

Compare PB with the value $x^2$ indicated in table 2 at $m$ - 1 degrees of freedom.

If PB > the value in the table, there are differences among the variances.

The Cochran test is used to confirm that the variance from one laboratory is greater than that from other laboratories.

Calculate the test statistics:

$$PC = \frac{s_i^2 \max}{\sum_{i=1}^{m} s_i^2}$$

Compare PC with the value shown in table 3 for $m$ and $n_i$ at P = 99%.

If PC > the table value, the variance is significantly greater than the others.

If there is a significant result from the Bartlett or Cochran tests, eliminate the outlier variance and calculate the statistical test again.

In the absence of a statistical method appropriate to a simultaneous test of several outlier values, the repeated application of the tests is permitted, but should be used with caution.

If the laboratories produce variances that differ sharply from each other, an investigation must be made to find the causes and to decide whether the experimental values found by those laboratories are to be eliminated or not. If they are, the coordinator will have to consider how representative the remaining laboratories are.

If statistical analysis shows that there are differing variances, this shows that the laboratories have operated the methods at varying precisions. This may be due to inadequate practice or to lack of clarity or inadequate description in the method.

C/ ***Systematic errors***

Systematic errors made by laboratories are identified using either Fischer's method or Dixon's test.

*R .A. Fischer variance analysis*

This test is applied to the remaining experimental values from the laboratories with an identical variance.

The test is used to identify whether the spread of the mean values from the laboratories is very much greater than that for the individual values expressed by the variance among the laboratories ($s_Z^2$) or the variance within the laboratories ($s_I^2$).

Calculate the test statistics:

$$PF = \frac{s_Z^2}{s_I^2}$$

$$s_Z^2 = \frac{1}{m\text{-}1} \sum_{i=1}^{m} n_i \left( \bar{x}_i - \bar{\bar{x}} \right)^2$$

$$s_I^2 = \frac{1}{N - m} \sum_{i=1}^{m} \sum_{i=1}^{n_i} \left( x_i - \bar{x}_i \right)^2$$

Compare PF with the corresponding value shown in table 4 (distribution of F) where $f_1 = f_Z = m$ - 1 and $f_2 = f_1 = N - m$ degrees of freedom.

If PF > the table value, it can be concluded that there are differences among the means, that is, there are systematic errors.

*Dixon test*

This test enables us to confirm that the mean from one laboratory is greater or smaller than that from the other laboratories.

Take a data series Z(*h*), *h* = 1,2,3...H, ranged in increasing order.

Calculate the statistics for the test:

| | | | |
|---|---|---|---|
| 3 to 7 | $Q_{10} = \frac{Z(2) - Z(1)}{Z(H) - Z(1)}$ | or | $\frac{Z(H) - Z(H-1)}{Z(H) - Z(1)}$ |
| 8 to 12 | $Q_{11} = \frac{Z(2) - Z(1)}{Z(H-1) - Z(1)}$ | or | $\frac{Z(H) - Z(H-1)}{Z(H) - Z(2)}$ |
| 13 plus | $Q_{22} = \frac{Z(3) - Z(1)}{Z(H-2) - Z(1)}$ | or | $\frac{Z(H) - Z(H-2)}{Z(H) - Z(3)}$ |

Compare the greatest value of Q with the critical values shown in table 5.

If the test statistic is > the table value at P = 95%, the mean in question can be regarded as an outlier.

If there is a significant result in the R A Fischer variance analysis or the Dixon test, eliminate one of the extreme values and calculate the test statistics again with the remaining values. As regards repeated application of the tests, see the explanations in paragraph (B).

If the systematic errors are found, the corresponding experimental values concerned must not be included in subsequent computations; the cause of the systematic error must be investigated.

D/***Calculating repeatability (r) and reproducibility (R).***

From the results remaining after elimination of outliers, calculate the standard deviation of repeatability $s_r$ and repeatability $r$, and the standard deviation of reproducibility $s_R$ and reproducibility R, which are shown as characteristic values of the method of analysis.

$$s_r=\sqrt{\frac{1}{N-m}\sum_{i=1}^{m} f_i s_i^2} \qquad r=s_r \cdot 2\sqrt{2}$$

$$s_R=\sqrt{\frac{1}{a}\left[s_Z^2+(a-1)s_I^2\right]} \qquad R=s_R \cdot 2\sqrt{2}$$

$$a=\frac{1}{m-1}\left[\left(N-\sum_{i=1}^{m}\frac{n_i^2}{N}\right)\right]$$

If there is no difference between the means from the laboratories, then there is no difference between $s_r$ and $s_R$ or between $r$ and R. But, if we find differences among the laboratory means, although these may be tolerated for practical considerations, we have to show $s_r$ and $s_R$, and $r$ and R.

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Table 1 - Critical values for the Grubbs test

| $n_i$ | P = 95% | P 99% |
|---|---|---|
| 3 | 1,155 | 1,155 |
| 4 | 1,481 | 1,496 |
| 5 | 1,715 | 1,764 |
| 6 | 1,887 | 1,973 |
| 7 | 2,020 | 2,139 |
| 8 | 2,126 | 2,274 |
| 9 | 2,215 | 2,387 |
| 10 | 2,290 | 2,482 |
| 11 | 2,355 | 2,564 |
| 12 | 2,412 | 2,636 |

Table 2 – Critical values for the Bartlett test (P = 95%)

| *f(m - 1)* | $X^2$ | *f(m - 1)* | $X^2$ |
|---|---|---|---|
| 1 | 3,84 | 21 | 32,7 |
| 2 | 5,99 | 22 | 33,9 |
| 3 | 7,81 | 23 | 35,2 |
| 4 | 9,49 | 24 | 36,4 |
| 5 | 11,07 | 25 | 37,7 |
| 6 | 12,59 | 26 | 38,9 |
| 7 | 14,07 | 27 | 40,1 |
| 8 | 15,51 | 28 | 41,3 |
| 9 | 16,92 | 29 | 42,6 |
| 10 | 18,31 | 30 | 43,8 |
| 11 | 19,68 | 35 | 49,8 |
| 12 | 21,03 | 40 | 55,8 |
| 13 | 22,36 | 50 | 67,5 |
| 14 | 23,69 | 60 | 79,1 |
| 15 | 25,00 | 70 | 90,5 |
| 16 | 26,30 | 80 | 101,9 |
| 17 | 27,59 | 90 | 113,1 |
| 18 | 28,87 | 100 | 124,3 |
| 19 | 30,14 | | |
| 20 | 31,41 | | |

Table 3 – Critical values for the Cochran test

| *m* | $n_i$ = 2 | | $n_i$= 3 | | $n_i$ = 4 | | $n_i$ = 5 | | $n_i$ = 6 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 99% | 95% | 99% | 95% | 99% | 95% | 99% | 95% | 99% | 95% |
| 2 | - | - | 0.995 | 0.975 | 0.979 | 0.939 | 0.959 | 0.906 | 0.937 | 0.877 |
| 3 | 0.993 | 0.967 | 0.942 | 0.871 | 0.883 | 0.798 | 0.834 | 0.746 | 0.793 | 0.707 |
| 4 | 0.968 | 0.906 | 0.864 | 0.768 | 0.781 | 0.684 | 0.721 | 0.629 | 0.676 | 0.590 |
| 5 | 0.928 | 0.841 | 0.788 | 0.684 | 0.696 | 0.598 | 0.633 | 0.544 | 0.588 | 0.506 |
| 6 | 0.883 | 0.781 | 0.722 | 0.616 | 0.626 | 0.532 | 0.564 | 0.480 | 0.520 | 0.445 |
| 7 | 0.838 | 0.727 | 0.664 | 0.561 | 0.568 | 0.480 | 0.508 | 0.431 | 0.466 | 0.397 |
| 8 | 0.794 | 0.680 | 0.615 | 0.516 | 0.521 | 0.438 | 0.463 | 0.391 | 0.423 | 0.360 |
| 9 | 0.754 | 0.638 | 0.573 | 0.478 | 0.481 | 0.403 | 0.425 | 0.358 | 0.387 | 0.329 |
| 10 | 0.718 | 0.602 | 0.536 | 0.445 | 0.447 | 0.373 | 0.393 | 0.331 | 0.357 | 0.303 |
| 11 | 0.684 | 0.570 | 0.504 | 0.417 | 0.418 | 0.348 | 0.366 | 0.308 | 0.332 | 0.281 |
| 12 | 0.653 | 0.541 | 0.475 | 0.392 | 0.392 | 0.326 | 0.343 | 0.288 | 0.310 | 0.262 |
| 13 | 0.624 | 0.515 | 0.450 | 0.371 | 0.369 | 0.307 | 0.322 | 0.271 | 0.291 | 0.246 |
| 14 | 0.599 | 0.492 | 0.427 | 0.352 | 0.349 | 0.291 | 0.304 | 0.255 | 0.274 | 0.232 |
| 15 | 0.575 | 0.471 | 0.407 | 0.335 | 0.332 | 0.276 | 0.288 | 0.242 | 0.259 | 0.220 |
| 16 | 0.553 | 0.452 | 0.388 | 0.319 | 0.316 | 0.262 | 0.274 | 0.230 | 0.246 | 0.208 |
| 17 | 0.532 | 0.434 | 0.372 | 0.305 | 0.301 | 0.250 | 0.261 | 0.219 | 0.234 | 0.198 |
| 18 | 0.514 | 0.418 | 0.356 | 0.293 | 0.288 | 0.240 | 0.249 | 0.209 | 0.223 | 0.189 |
| 19 | 0.496 | 0.403 | 0.343 | 0.281 | 0.276 | 0.230 | 0.238 | 0.200 | 0.214 | 0.181 |
| 20 | 0.480 | 0.389 | 0.330 | 0.270 | 0.265 | 0.220 | 0.229 | 0.192 | 0.205 | 0.174 |
| 21 | 0.465 | 0.377 | 0.318 | 0.261 | 0.255 | 0.212 | 0.220 | 0.185 | 0.197 | 0.167 |
| 22 | 0.450 | 0.365 | 0.307 | 0.252 | 0.246 | 0.204 | 0.212 | 0.178 | 0.189 | 0.160 |
| 23 | 0.437 | 0.354 | 0.297 | 0.243 | 0.238 | 0.197 | 0.204 | 0.172 | 0.182 | 0.155 |
| 24 | 0.425 | 0.343 | 0.287 | 0.235 | 0.230 | 0.191 | 0.197 | 0.166 | 0.176 | 0.149 |
| 25 | 0.413 | 0.334 | 0.278 | 0.228 | 0.222 | 0.185 | 0.190 | 0.160 | 0.170 | 0.144 |
| 26 | 0.402 | 0.325 | 0.270 | 0.221 | 0.215 | 0.179 | 0.184 | 0.155 | 0.164 | 0.140 |
| 27 | 0.391 | 0.316 | 0.262 | 0.215 | 0.209 | 0.173 | 0.179 | 0.150 | 0.159 | 0.135 |
| 28 | 0.382 | 0.308 | 0.255 | 0.209 | 0.202 | 0.168 | 0.173 | 0.146 | 0.154 | 0.131 |
| 29 | 0.372 | 0.300 | 0.248 | 0.203 | 0.196 | 0.164 | 0.168 | 0.142 | 0.150 | 0.127 |
| 30 | 0.363 | 0.293 | 0.241 | 0.198 | 0.191 | 0.159 | 0.164 | 0.138 | 0.145 | 0.124 |
| 31 | 0.355 | 0.286 | 0.235 | 0.193 | 0.186 | 0.155 | 0.159 | 0.134 | 0.141 | 0.120 |
| 32 | 0.347 | 0.280 | 0.229 | 0.188 | 0.181 | 0.151 | 0.155 | 0.131 | 0.138 | 0.117 |
| 33 | 0.339 | 0.273 | 0.224 | 0.184 | 0.177 | 0.147 | 0.151 | 0.127 | 0.134 | 0.114 |
| 34 | 0.332 | 0.267 | 0.218 | 0.179 | 0.172 | 0.144 | 0.147 | 0.124 | 0.131 | 0.111 |
| 35 | 0.325 | 0.262 | 0.213 | 0.175 | 0.168 | 0.140 | 0.144 | 0.121 | 0.127 | 0.108 |
| 36 | 0.318 | 0.256 | 0.208 | 0.172 | 0.165 | 0.137 | 0.140 | 0.119 | 0.124 | 0.106 |
| 37 | 0.312 | 0.251 | 0.204 | 0.168 | 0.161 | 0.134 | 0.137 | 0.116 | 0.121 | 0.103 |
| 38 | 0.306 | 0.246 | 0.200 | 0.164 | 0.157 | 0.131 | 0.134 | 0.113 | 0.119 | 0.101 |
| 39 | 0.300 | 0.242 | 0.196 | 0.161 | 0.154 | 0.129 | 0.131 | 0.111 | 0.116 | 0.099 |
| 40 | 0.294 | 0.237 | 0.192 | 0.158 | 0.151 | 0.126 | 0.128 | 0.108 | 0.114 | 0.097 |

Table 4 – Critical values for the F-Test (P=99%)

| $f_2$ \ $f_1$ | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 4052 | 4999 | 5403 | 5625 | 5764 | 5859 | 5928 | 5981 | 6023 | 6056 | 6083 | 6106 | 6126 | 6143 | 6157 |
| 2 | 98.5 | 99.0 | 99.2 | 99.3 | 99.3 | 99.3 | 99.4 | 99.4 | 99.4 | 99.4 | 99.4 | 99.4 | 99.4 | 99.4 | 99.4 |
| 3 | 34.1 | 30.8 | 29.4 | 28.7 | 28.2 | 27.9 | 27.7 | 27.5 | 27.3 | 27.2 | 27.1 | 27.1 | 27.0 | 26.9 | 26.9 |
| 4 | 21.2 | 18.0 | 16.7 | 16.0 | 15.5 | 15.2 | 15.0 | 14.8 | 14.7 | 14.5 | 14.5 | 14.4 | 14.3 | 14.2 | 14.2 |
| 5 | 16.3 | 13.3 | 12.1 | 11.4 | 11.0 | 10.7 | 10.5 | 10.3 | 10.2 | 10.1 | 9.96 | 9.89 | 9.82 | 9.77 | 9.72 |
| 6 | 13.7 | 10.9 | 9.78 | 9.15 | 8.75 | 8.47 | 8.26 | 8.10 | 7.98 | 7.87 | 7.79 | 7.72 | 7.66 | 7.60 | 7.56 |
| 7 | 12.2 | 9.55 | 8.45 | 7.85 | 7.46 | 7.19 | 6.99 | 6.84 | 6.72 | 6.62 | 6.54 | 6.47 | 6.41 | 6.36 | 6.31 |
| 8 | 11.3 | 8.65 | 7.59 | 7.01 | 6.63 | 6.37 | 6.18 | 6.03 | 5.91 | 5.81 | 5.73 | 5.67 | 5.61 | 5.56 | 5.52 |
| 9 | 10.6 | 8.02 | 6.99 | 6.42 | 6.06 | 5.80 | 5.61 | 5.47 | 5.35 | 5.26 | 5.18 | 5.11 | 5.05 | 5.01 | 4.96 |
| 10 | 10.0 | 7.56 | 6.55 | 5.99 | 5.64 | 5.39 | 5.20 | 5.06 | 4.94 | 4.85 | 4.77 | 4.71 | 4.65 | 4.60 | 4.56 |
| 11 | 9.64 | 7.20 | 6.21 | 5.67 | 5.31 | 5.07 | 4.88 | 4.74 | 4.63 | 4.54 | 4.46 | 4.39 | 4.34 | 4.29 | 4.25 |
| 12 | 9.33 | 6.93 | 5.95 | 5.41 | 5.06 | 4.82 | 4.64 | 4.50 | 4.39 | 4.30 | 4.22 | 4.16 | 4.10 | 4.05 | 4.01 |
| 13 | 9.07 | 6.70 | 5.74 | 5.21 | 4.86 | 4.62 | 4.44 | 4.30 | 4.19 | 4.10 | 4.02 | 3.96 | 3.90 | 3.86 | 3.82 |
| 14 | 8.86 | 6.51 | 5.56 | 5.04 | 4.69 | 4.46 | 4.28 | 4.14 | 4.03 | 3.94 | 3.86 | 3.80 | 3.75 | 3.70 | 3.66 |
| 15 | 8.68 | 6.36 | 5.42 | 4.89 | 4.56 | 4.32 | 4.14 | 4.00 | 3.89 | 3.80 | 3.73 | 3.67 | 3.61 | 3.56 | 3.52 |
| 16 | 8.53 | 6.23 | 5.29 | 4.77 | 4.44 | 4.20 | 4.03 | 3.89 | 3.78 | 3.69 | 3.62 | 3.55 | 3.50 | 3.45 | 3.41 |
| 17 | 8.40 | 6.11 | 5.18 | 4.67 | 4.34 | 4.10 | 3.93 | 3.79 | 3.68 | 3.59 | 3.52 | 3.46 | 3.40 | 3.35 | 3.31 |
| 18 | 8.29 | 6.01 | 5.09 | 4.58 | 4.25 | 4.01 | 3.84 | 3.71 | 3.60 | 3.51 | 3.43 | 3.37 | 3.32 | 3.27 | 3.23 |
| 19 | 8.18 | 5.93 | 5.01 | 4.50 | 4.17 | 3.94 | 3.77 | 3.63 | 3.52 | 3.43 | 3.36 | 3.30 | 3.24 | 3.19 | 3.15 |
| 20 | 8.10 | 5.85 | 4.94 | 4.43 | 4.10 | 3.87 | 3.70 | 3.56 | 3.46 | 3.37 | 3.29 | 3.23 | 3.18 | 3.13 | 3.09 |
| 21 | 8.02 | 5.78 | 4.87 | 4.37 | 4.04 | 3.81 | 3.64 | 3.51 | 3.40 | 3.31 | 3.24 | 3.17 | 3.12 | 3.07 | 3.03 |
| 22 | 7.95 | 5.72 | 4.82 | 4.31 | 3.99 | 3.76 | 3.59 | 3.45 | 3.35 | 3.26 | 3.18 | 3.12 | 3.07 | 3.02 | 2.98 |
| 23 | 7.88 | 5.66 | 4.76 | 4.26 | 3.94 | 3.71 | 3.54 | 3.41 | 3.30 | 3.21 | 3.14 | 3.07 | 3.02 | 2.97 | 2.93 |
| 24 | 7.82 | 5.61 | 4.72 | 4.22 | 3.90 | 3.67 | 3.50 | 3.36 | 3.26 | 3.17 | 3.09 | 3.03 | 2.98 | 2.93 | 2.89 |
| 25 | 7.77 | 5.57 | 4.68 | 4.18 | 3.85 | 3.63 | 3.46 | 3.32 | 3.22 | 3.13 | 3.06 | 2.99 | 2.94 | 2.89 | 2.85 |
| 26 | 7.72 | 5.53 | 4.64 | 4.14 | 3.82 | 3.59 | 3.42 | 3.29 | 3.18 | 3.09 | 3.02 | 2.96 | 2.90 | 2.86 | 2.81 |
| 27 | 7.68 | 5.49 | 4.60 | 4.11 | 3.78 | 3.56 | 3.39 | 3.26 | 3.15 | 3.06 | 2.99 | 2.93 | 2.87 | 2.82 | 2.78 |
| 28 | 7.64 | 5.45 | 4.57 | 4.07 | 3.75 | 3.53 | 3.36 | 3.23 | 3.12 | 3.03 | 2.96 | 2.90 | 2.84 | 2.79 | 2.75 |
| 29 | 7.60 | 5.42 | 4.54 | 4.04 | 3.73 | 3.50 | 3.33 | 3.20 | 3.09 | 3.00 | 2.93 | 2.87 | 2.81 | 2.77 | 2.73 |
| 30 | 7.56 | 5.39 | 4.51 | 4.02 | 3.70 | 3.47 | 3.30 | 3.17 | 3.07 | 2.98 | 2.91 | 2.84 | 2.79 | 2.74 | 2.70 |
| 40 | 7.31 | 5.18 | 4.31 | 3.83 | 3.51 | 3.29 | 3.12 | 2.99 | 2.89 | 2.80 | 2.73 | 2.66 | 2.61 | 2.56 | 2.52 |
| 50 | 7.17 | 5.06 | 4.20 | 3.72 | 3.41 | 3.19 | 3.02 | 2.89 | 2.78 | 2.70 | 2.62 | 2.56 | 2.51 | 2.46 | 2.42 |
| 60 | 7.07 | 4.98 | 4.13 | 3.65 | 3.34 | 3.12 | 2.95 | 2.82 | 2.72 | 2.63 | 2.56 | 2.50 | 2.44 | 2.39 | 2.35 |
| 70 | 7.01 | 4.92 | 4.07 | 3.60 | 3.29 | 3.07 | 2.91 | 2.78 | 2.67 | 2.59 | 2.51 | 2.45 | 2.40 | 2.35 | 2.31 |
| 80 | 6.96 | 4.88 | 4.04 | 3.56 | 3.25 | 3.04 | 2.87 | 2.74 | 2.64 | 2.55 | 2.48 | 2.42 | 2.36 | 2.31 | 2.27 |
| 90 | 6.92 | 4.85 | 4.01 | 3.53 | 3.23 | 3.01 | 2.84 | 2.72 | 2.61 | 2.52 | 2.45 | 2.39 | 2.33 | 2.29 | 2.24 |
| 100 | 6.89 | 4.82 | 3.98 | 3.51 | 3.21 | 2.99 | 2.82 | 2.69 | 2.59 | 2.50 | 2.43 | 2.37 | 2.31 | 2.27 | 2.22 |
| 200 | 6.75 | 4.71 | 3.88 | 3.41 | 3.11 | 2.89 | 2.73 | 2.60 | 2.50 | 2.41 | 2.34 | 2.27 | 2.22 | 2.17 | 2.13 |
| 500 | 6.69 | 4.65 | 3.82 | 3.36 | 3.05 | 2.84 | 2.68 | 2.55 | 2.44 | 2.36 | 2.29 | 2.22 | 2.17 | 2.12 | 2.07 |
| □ | 6.63 | 4.61 | 3.78 | 3.32 | 3.02 | 2.80 | 2.64 | 2.51 | 2.41 | 2.32 | 2.25 | 2.18 | 2.13 | 2.08 | 2.04 |

Table 4 – Critical values for the F-Test (P=99%) [Continued]

| $f_2$ \ $f_1$ | 16 | 17 | 18 | 19 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 100 | 200 | 500 | ∞ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 6169 | 6182 | 6192 | 6201 | 6209 | 6261 | 6287 | 6303 | 6313 | 6320 | 6326 | 6335 | 6350 | 6361 | 6366 |
| 2 | 99.4 | 99.4 | 99.4 | 99.4 | 99.5 | 99.5 | 99.5 | 99.5 | 99.5 | 99.5 | 99.5 | 99.5 | 99.3 | 99.5 | 99.5 |
| 3 | 26.8 | 26.8 | 26.8 | 26.7 | 26.7 | 26.5 | 26.4 | 26.4 | 26.3 | 26.3 | 26.3 | 26.2 | 26.2 | 26.1 | 26.1 |
| 4 | 14.2 | 14.1 | 14.1 | 14.0 | 14.0 | 13.8 | 13.7 | 13.7 | 13.7 | 13.6 | 13.6 | 13.6 | 13.5 | 13.5 | 13.5 |
| 5 | 9.68 | 9.64 | 9.61 | 9.58 | 9.55 | 9.38 | 9.29 | 9.24 | 9.20 | 9.18 | 9.16 | 9.13 | 9.08 | 9.04 | 9.02 |
| 6 | 7.52 | 7.48 | 7.45 | 7.42 | 7.40 | 7.23 | 7.14 | 7.09 | 7.06 | 7.03 | 7.01 | 6.99 | 6.93 | 6.90 | 6.88 |
| 7 | 6.28 | 6.24 | 6.21 | 6.18 | 6.16 | 5.99 | 5.91 | 5.86 | 5.82 | 5.80 | 5.78 | 5.75 | 5.70 | 5.67 | 5.65 |
| 8 | 5.48 | 5.44 | 5.41 | 5.38 | 5.36 | 5.20 | 5.12 | 5.07 | 5.03 | 5.01 | 4.99 | 4.96 | 4.91 | 4.88 | 4.86 |
| 9 | 4.92 | 4.89 | 4.86 | 4.83 | 4.81 | 4.65 | 4.57 | 4.52 | 4.48 | 4.46 | 4.44 | 4.41 | 4.36 | 4.33 | 4.31 |
| 10 | 4.52 | 4.49 | 4.46 | 4.43 | 4.41 | 4.25 | 4.17 | 4.12 | 4.08 | 4.06 | 4.04 | 4.01 | 3.96 | 3.93 | 3.91 |
| 11 | 4.21 | 4.18 | 4.15 | 4.12 | 4.10 | 3.94 | 3.86 | 3.81 | 3.77 | 3.75 | 3.73 | 3.70 | 3.65 | 3.62 | 3.60 |
| 12 | 3.97 | 3.94 | 3.91 | 3.88 | 3.86 | 3.70 | 3.62 | 3.57 | 3.54 | 3.51 | 3.49 | 3.47 | 3.41 | 3.38 | 3.36 |
| 13 | 3.78 | 3.74 | 3.72 | 3.69 | 3.66 | 3.51 | 3.42 | 3.37 | 3.34 | 3.32 | 3.30 | 3.27 | 3.22 | 3.19 | 3.17 |
| 14 | 3.62 | 3.59 | 3.56 | 3.53 | 3.51 | 3.35 | 3.27 | 3.22 | 3.18 | 3.16 | 3.14 | 3.11 | 3.06 | 3.03 | 3.00 |
| 15 | 3.49 | 3.45 | 3.42 | 3.40 | 3.37 | 3.21 | 3.13 | 3.08 | 3.05 | 3.02 | 3.00 | 2.98 | 2.92 | 2.89 | 2.87 |
| 16 | 3.37 | 3.34 | 3.31 | 3.28 | 3.26 | 3.10 | 3.02 | 2.97 | 2.93 | 2.91 | 2.89 | 2.86 | 2.81 | 2.78 | 2.75 |
| 17 | 3.27 | 3.24 | 3.21 | 3.19 | 3.16 | 3.00 | 2.92 | 2.87 | 2.83 | 2.81 | 2.79 | 2.76 | 2.71 | 2.68 | 2.65 |
| 18 | 3.19 | 3.16 | 3.13 | 3.10 | 3.08 | 2.92 | 2.84 | 2.78 | 2.75 | 2.72 | 2.70 | 2.68 | 2.62 | 2.59 | 2.57 |
| 19 | 3.12 | 3.08 | 3.05 | 3.03 | 3.00 | 2.84 | 2.76 | 2.71 | 2.67 | 2.65 | 2.63 | 2.60 | 2.55 | 2.51 | 2.49 |
| 20 | 3.05 | 3.02 | 2.99 | 2.96 | 2.94 | 2.78 | 2.69 | 2.64 | 2.61 | 2.58 | 2.56 | 2.54 | 2.48 | 2.44 | 2.42 |
| 21 | 2.99 | 2.96 | 2.93 | 2.90 | 2.88 | 2.72 | 2.64 | 2.58 | 2.55 | 2.52 | 2.50 | 2.48 | 2.42 | 2.38 | 2.36 |
| 22 | 2.94 | 2.91 | 2.88 | 2.85 | 2.83 | 2.67 | 2.58 | 2.53 | 2.50 | 2.47 | 2.45 | 2.42 | 2.36 | 2.33 | 2.31 |
| 23 | 2.89 | 2.86 | 2.83 | 2.80 | 2.78 | 2.62 | 2.54 | 2.48 | 2.45 | 2.42 | 2.40 | 2.37 | 2.32 | 2.28 | 2.26 |
| 24 | 2.85 | 2.82 | 2.79 | 2.76 | 2.74 | 2.58 | 2.49 | 2.44 | 2.40 | 2.38 | 2.36 | 2.33 | 2.27 | 2.24 | 2.21 |
| 25 | 2.81 | 2.78 | 2.75 | 2.72 | 2.70 | 2.54 | 2.45 | 2.40 | 2.36 | 2.34 | 2.32 | 2.29 | 2.23 | 2.19 | 2.17 |
| 26 | 2.78 | 2.75 | 2.72 | 2.69 | 2.66 | 2.50 | 2.42 | 2.36 | 2.33 | 2.30 | 2.28 | 2.25 | 2.19 | 2.16 | 2.13 |
| 27 | 2.75 | 2.71 | 2.68 | 2.66 | 2.63 | 2.47 | 2.38 | 2.33 | 2.29 | 2.27 | 2.25 | 2.22 | 2.16 | 2.12 | 2.10 |
| 28 | 2.72 | 2.68 | 2.65 | 2.63 | 2.60 | 2.44 | 2.35 | 2.30 | 2.26 | 2.24 | 2.22 | 2.19 | 2.13 | 2.09 | 2.06 |
| 29 | 2.69 | 2.66 | 2.63 | 2.60 | 2.57 | 2.41 | 2.33 | 2.27 | 2.23 | 2.21 | 2.19 | 2.16 | 2.10 | 2.06 | 2.03 |
| 30 | 2.66 | 2.63 | 2.60 | 2.57 | 2.55 | 2.39 | 2.30 | 2.25 | 2.21 | 2.18 | 2.16 | 2.13 | 2.07 | 2.03 | 2.01 |
| 40 | 2.48 | 2.45 | 2.42 | 2.39 | 2.37 | 2.20 | 2.11 | 2.06 | 2.02 | 1.99 | 1.97 | 1.94 | 1.87 | 1.85 | 1.80 |
| 50 | 2.38 | 2.35 | 2.32 | 2.29 | 2.27 | 2.10 | 2.01 | 1.95 | 1.91 | 1.88 | 1.86 | 1.82 | 1.76 | 1.71 | 1.68 |
| 60 | 2.31 | 2.28 | 2.25 | 2.22 | 2.20 | 2.03 | 1.94 | 1.88 | 1.84 | 1.81 | 1.78 | 1.75 | 1.68 | 1.63 | 1.60 |
| 70 | 2.27 | 2.23 | 2.20 | 2.18 | 2.15 | 1.98 | 1.89 | 1.83 | 1.78 | 1.75 | 1.73 | 1.70 | 1.62 | 1.57 | 1.54 |
| 80 | 2.23 | 2.20 | 2.17 | 2.14 | 2.12 | 1.94 | 1.85 | 1.79 | 1.75 | 1.71 | 1.69 | 1.65 | 1.58 | 1.53 | 1.49 |
| 90 | 2.21 | 2.17 | 2.14 | 2.11 | 2.09 | 1.92 | 1.82 | 1.76 | 1.72 | 1.68 | 1.66 | 1.62 | 1.55 | 1.50 | 1.46 |
| 100 | 2.19 | 2.15 | 2.12 | 2.09 | 2.07 | 1.89 | 1.80 | 1.74 | 1.69 | 1.66 | 1.63 | 1.60 | 1.52 | 1.47 | 1.43 |
| 200 | 2.09 | 2.06 | 2.03 | 2.00 | 1.97 | 1.79 | 1.69 | 1.63 | 1.58 | 1.55 | 1.52 | 1.48 | 1.39 | 1.33 | 1.28 |
| 500 | 2.04 | 2.00 | 1.97 | 1.94 | 1.92 | 1.74 | 1.63 | 1.56 | 1.52 | 1.48 | 1.45 | 1.41 | 1.31 | 1.23 | 1.16 |
| ∞ | 2.00 | 1.97 | 1.93 | 1.90 | 1.88 | 1.70 | 1.59 | 1.52 | 1.47 | 1.43 | 1.40 | 1.36 | 1.25 | 1.15 | 1.00 |

Table 5 – Critical values for the Dixon test

| Test criteria | | Critical values | |
|---|---|---|---|
| | *m* | 95% | 99% |
| $Q_{10} = \frac{Z(2) - Z(1)}{Z(H) - Z(1)}$ ou $\frac{Z(H) - Z(H-1)}{Z(H) - Z(1)}$ The greater of the two values | 3 | 0,970 | 0,994 |
| | 4 | 0,829 | 0,926 |
| | 5 | 0,710 | 0,821 |
| | 6 | 0,628 | 0,740 |
| | 7 | 0,569 | 0,680 |
| $Q_{11} = \frac{Z(2) - Z(1)}{Z(H-1) - Z(1)}$ ou $\frac{Z(H) - Z(H-1)}{Z(H) - Z(2)}$ The greater of the two values | 8 | 0,608 | 0,717 |
| | 9 | 0,564 | 0,672 |
| | 10 | 0,530 | 0,635 |
| | 11 | 0,502 | 0,605 |
| | 12 | 0,479 | 0,579 |
| $Q_{22} = \frac{Z(3) - Z(1)}{Z(H-2) - Z(1)}$ ou $\frac{Z(H) - Z(H-2)}{Z(H) - Z(3)}$ The greater of the two values | 13 | 0,611 | 0,697 |
| | 14 | 0,586 | 0,670 |
| | 15 | 0,565 | 0,647 |
| | 16 | 0,546 | 0,627 |
| | 17 | 0,529 | 0,610 |
| | 18 | 0,514 | 0,594 |
| | 19 | 0,501 | 0,580 |
| | 20 | 0,489 | 0,567 |
| | 21 | 0,478 | 0,555 |
| | 22 | 0,468 | 0,544 |
| | 23 | 0,459 | 0,535 |
| | 24 | 0,451 | 0,526 |
| | 25 | 0,443 | 0,517 |
| | 26 | 0,436 | 0,510 |
| | 27 | 0,429 | 0,502 |
| | 28 | 0,423 | 0,495 |
| | 29 | 0,417 | 0,489 |
| | 30 | 0,412 | 0,483 |
| | 31 | 0,407 | 0,477 |
| | 32 | 0,402 | 0,472 |
| | 33 | 0,397 | 0,467 |
| | 34 | 0,393 | 0,462 |
| | 35 | 0,388 | 0,458 |
| | 36 | 0,384 | 0,454 |
| | 37 | 0,381 | 0,450 |
| | 38 | 0,377 | 0,446 |
| | 39 | 0,374 | 0,442 |
| | 40 | 0,371 | 0,438 |

Table 6 – Results of the collaborative study

| Analysis | | | | | | | | | | Sample | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lab n° | Individual values $x_1$ | | | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | | $n_1$ | $x_1$ | $s_1$ | $s^2_1$ | |
| 1 | 548 | 556 | 558 | 553 | 542 | | | | | 5 | 551 | 6,47 | 41,8 | |
| 2 | 300 | 299 | 304 | 308 | 300 | | | | | 5 | 302 | 3,83 | 14,7 | $x_{1,} < x$ |
| 3 | 567 | 558 | 563 | 532* | 560 | 560 | 563 | 567 | | 7 | 563 | 3,51 | 12,3 | |
| 4 | 557 | 550 | 555 | 560 | 551 | | | | | 5 | 555 | 4,16 | 17,3 | |
| 5 | 569 | 575 | 565 | 560 | 572 | | | | | 5 | 568 | 5,89 | 34,7 | |
| - 6 = | 550 | 546 | 549 | 557 | 588 | 570 | 576 | 568 | | 8 | 563 | 14,92 | 222,6 | $s_1 > s_1$ |
| 7 | 557 | 560 | 560 | 552 | 547 | | | | | 5 | 555 | 5,63 | 31,7 | |
| 8 | 548 | 543 | 560 | 551 | 548 | | | | | 5 | 550 | 6,28 | 39,5 | |
| 9 | 558 | 563 | 551 | 555 | 560 | | | | | 5 | 556 | 5,63 | 31,7 | |
| 10 | 554 | 559 | 551 | 545 | 557 | | | | | 5 | 553 | 5,5 | 30,2 | |

*Statistical Figures:*

*Within laboratory:* $s_1 = \pm 5.37$ $f_{1=34}$

*Between laboratory:* $s_z = \pm 13.97$ $fz = 7$

$s_r = \pm 5.37$ $r = 15$ $s_R = \pm 7.78$ $R = 22$

*Bartlett Test:*

PB = 3.16 < 15.51 (95%; $f = 8$)

*Analysis of variance*:

PF = 6.76 > 3.21 (99%; $f_1 = 7$; $f_2 = 34$)

# Reliability of analytical results

(Resolution Oeno 5/99)

Data concerning the reliability of analytical methods, as determined by collaborative studies, are applicable in the following cases:

1) Verifying the results obtained by a laboratory with a reference method
2) Evaluating analytical results which indicate a legal limit has been exceeded
3) Comparing results obtained by two or more laboratories and comparing those results with a reference value
4) Evaluating results obtained from a non-validated method

## 1) VERIFICATION OF THE ACCEPTABILITY OF RESULTS OBTAINED WITH A REFERENCE METHOD

The validity of analytical results depends on the following:

- the laboratory should perform all analyses within the framework of an appropriate quality control system which includes the organization, responsibilities, procedures, etc.
- as part of the quality control system, the laboratory should operate according to an internal Quality Control Procedure
- results should be obtained in accordance with the acceptability criteria described in the internal Quality Control Procedure

Internal quality control shall be established in accordance with internationally recognized standards, such those of the IUPAC document titled, "Harmonized Guidelines for Internal Quality Control in Analytical Laboratories."

Internal Quality Control implies an analysis of the reference material.

Reference samples should consist of a template of the samples to be analyzed and should contain an appropriate, known concentration of the substance analyzed which is similar to that found in the sample.

To the extent possible, reference material shall be certified by an internationally recognized organization.

However, for many types of analysis, there are no certified reference materials. In this case, one could use, for example, material analyzed by several laboratories in a competence test and considering the average of the results to be the value assigned to the substance analyzed.

One could also prepare reference material by formulation (model solution with known components) or by adding a known quantity of the substance analyzed to a

sample which does not contain (or not yet contain) the substance by means of a recovery test (dosed addition) on one of the samples to analyze.

Quality Control is assured by adding reference material to each series of samples, and analyzing these pairs (test samples and reference material). This verifies correct implementation of the method and should be independent of the analytical calibration and protocol as its goal is to verify the aforementioned.

Series means a number of samples analyzed under repeatable conditions. Internal controls serve to ensure the appropriate level of uncertainty is not exceeded.

If the analytical results are considered to be part of a normal population whose mean is m and standard deviation is s, only around 0.3% of the results will be outside the limits m ± 3s. When aberrant results are obtained (outside these limits), the system is considered to be outside statistical control (unreliable data).

The control is graphically represented using Shewhart Control Graphs. To produce these graphical results, the measured values obtained from the reference material are placed on the vertical axis while the series numbers are placed on the horizontal axis. The graph also includes horizontal lines representing the mean, m, m ± 2 (warning limits) and m ± 3 (action limits) (Figure 1).

To estimate the standard deviation, a control should be analyzed, in pairs, in at least 12 trials. Each analytical pair shall be analyzed under repeatable conditions and randomly inserted in a sample series. Analyses will be duplicated on different days to reflect reasonable changes from one series to another. Variations can have several causes: modification of the reactants composition, instrument re-calibration and even different operators. After eliminating aberrant data using the Grubbs test, calculate the standard deviation to construct the Shewhart graphs. This standard deviation is compared to that of the reference method. If a published precision level is not obtained for the reference method, caused should be investigated.

The precision limits of the laboratory should be periodically revised by repeating the indicated procedure.

Once the Quality Control graph is constructed, graph the results obtained from each series for the control material.

A series is considered outside statistical control if:

I) a value is outside the action limit,
II) the current and previous values are situated outside the attention limits even in within the action limits,
III) nine successive values lie on the same side of the mean.

The laboratory response to "outside control" conditions is to reject the results for the series and perform tests to determine the cause, then take action to remedy the situation.

A Shewhart Control Graph can also be produced for the differences between analytical pairs in the same sample, especially when reference material does not exist. In this case, the absolute difference between two analyses of the same sample is graphed. The graph's lower line is 0 and the attention limit is $1.128S_w$ while the action limit is 3.686Sw where $S_w$ = the standard deviation of a series.

This type of graph only accounts for repeatability. It should be no greater than the published repeatability limit for the method.

In the absence of control material, it sometimes becomes necessary to verify that the reproducibility limit of the reference method is not exceeded by comparing the results obtained to those of obtained by an experimental laboratory using the same sample.

Each laboratory performs two tests and the following formula is used:

$$C_r D_{95}(\bar{y}_1 - \bar{y}_2) = \sqrt[2]{R^2 - \frac{r^2}{2}}$$

| | | |
|---|---|---|
| $C_rD_{95}$ | = | Critical difference (P=0,95) |
| $\bar{y}_1$ | = | Means of 2 results obtained by lab 1 |
| $\bar{y}_2$ | = | Means of 2 results obtained by lab 2 |
| R | = | Reproducibility of reference method |
| r | = | Repeatability of reference method |

If the critical difference has been exceeded, the underlying reason is to be found and the test is to be repeated within one month.

## 2) EVALUATION OF ANALYTIC RESULTS INDICATING THAT A LEGAL LIMIT HAS BEEN EXCEEDED.

When analytical results indicated that a legal limit has been exceeded, the following procedure should be followed:

1) In the case of an individual result, conduct a second test under repeatable conditions. If it is not possible to conduct a second test under repeatable conditions, conduct a double analysis under repeatable conditions and use these data to evaluate the critical difference.
2) Determine the absolute value of the difference between the mean of the results obtained under repeatable conditions and the legal limit. An absolute value of the difference which is greater than the critical distance indicates that the sample does not fit the specifications.

Critical difference is calculated by the formula:

$$C_r D_{95}(\bar{y} - m_0) = \frac{1}{\sqrt[2]{2}} \sqrt[2]{R^2 - r^2 \frac{n-1}{n}}$$

$\bar{y}$ = Mean of results obtained
$m_0$ = Limit
n = Number of analyses
R = reproducibility
r = repeatability

In other words, this is a maximal limit where the average of the results obtained should not be greater than:

$$m_0 + C_r D_{95}(y - m_0)$$

If the limit is a minimum, the average of the results obtained should not be less than:

$$m_0 - C_r D_{95}(y - m_0)$$

## 3) COMPARING RESULTS OBTAINED USING TWO OR MORE LABORATORIES AND COMPARING THESE RESULTS TO A REFERENCE VALUE

To determine whether or not data originating in two laboratories are in agreement, calculate the absolute difference between the two results and compare to the critical difference:

$$C_r D_{95}(\bar{y}_1 - \bar{y}_2) = \sqrt{2}\sqrt{R^2 - r^2\left(1 - \frac{1}{2n_1} - \frac{1}{2n_2}\right)}$$

$\bar{y}_1$ = Mean of 2 results obtained by lab 1
$y_2$ = Mean of 2 results obtained by lab 2
$n_1$ = number of analyses in lab 1 sample
$n_2$ = number of analyses in lab 2 sample
R = Reproducibility of reference method
r = Repeatability of reference method

If the result is the average of two tests, the equation can be simplified to:

$$C_r D_{95}(\bar{y}_1 - \bar{y}_2) = \sqrt{2}\sqrt{R^2 - \frac{r^2}{2}}$$

If the data are individual results, the critical difference is R.
If the critical difference is not exceeded, the conclusion is that the results of the two laboratories are in agreement.
Comparing results obtained by several laboratories with a reference value:
Suppose p laboratories have made n1 determinations, whose mean for each laboratory is $y_1$ and whose total mean is:

$$\bar{y} = \frac{1}{p}\sum \bar{y}_i$$

The mean of all laboratories is compared with the reference value. If the absolute difference exceeds the critical difference, as calculated using the following formula, we conclude the results are not in agreement with the reference value:

$$C_r D_{95}(\bar{y} - m_0) = \frac{1}{\sqrt[2]{2p}} \sqrt[2]{R^2 - r^2\left(1 - \frac{1}{p}\sum\frac{1}{n_1}\right)}$$

$C_rD_{95}$ = Critical difference, calculated as indicated in point 2, for the reference method.

For example, the reference value can be the value assigned to a reference material or the value obtained by the same laboratory or by a different laboratory with a different method.

## 4) EVALUATING ANALYTICAL RESULTS OBTAINED USING NON-VALIDATED METHODS

A provisional reproducibility value can be assigned to a non-validated method by comparing it to that of a second laboratory:

$$R_{prov} = \sqrt[2]{(\bar{y}_1 - \bar{y}_2)^2 + \frac{r^2}{2}}$$

$\bar{y}_1$ = Mean of 2 results obtained by lab 1
$y_2$ = Mean of 2 results obtained by lab 2
r = Repeatability of reference method

Provisional reproducibility can be used to calculate critical difference.

If provisional reproducibility is less than twice the value of repeatability, it should be set to 2r.

A reproducibility value greater than three times repeatability or twice the value calculated using the Horwitz equation is not acceptable.

Horwitz equation:

$$RSD_R\% = 2^{1-0,5\log_{10}C}$$

$RSD_R$ % = Standard deviation for reproducibility (expressed as a percentage of the mean)

C = concentration, expressed as a decimal fraction (for example, 10g/100g = 0.1)

This equation was empirically obtained from more than 3000 collaborative studies including a diverse group of analyzed substances, matrices and measurement techniques. In the absence of other information, $RSD_R$ values that are lower or equal to the $RSD_R$ values calculated using the Horwitz equation can be considered acceptable.

**$RSD_R$ values calculated by the Horwitz equation:**

| Concentration | $RSD_R$ % |
|---|---|
| $10^{-9}$ | 45 |
| $10^{-8}$ | 32 |
| $10^{-7}$ | 23 |
| $10^{-6}$ | 16 |
| $10^{-5}$ | 11 |
| $10^{-4}$ | 8 |
| $10^{-3}$ | 5,6 |
| $10^{-2}$ | 4 |
| $10^{-1}$ | 2,8 |
| 1 | 2 |

If the result obtained using a non-validated method is close to the limit specified by legislation, the decision on the limit shall be decided as follows (for upper limits):

$$S = m_0 + \{(R_{rout}/R_{ref})-1\}\times C_rD_{95}$$

and, for lower limits,

$$S = m_0 - \{(R_{rout}/R_{ref})-1\}\times C_rD_{95}$$

$S$ = decision limit
$m_0$ = legal limit
$R_{rout}$ = provisional reproducibility for non-validated method
$R_{ref}$ = reproducibility for reference method
$C_rD_{95}$ = critical difference, calculated as indicated in point 2, for the reference method

The result which exceeds the decision limit should be replaced with a final result obtained using the reference method.

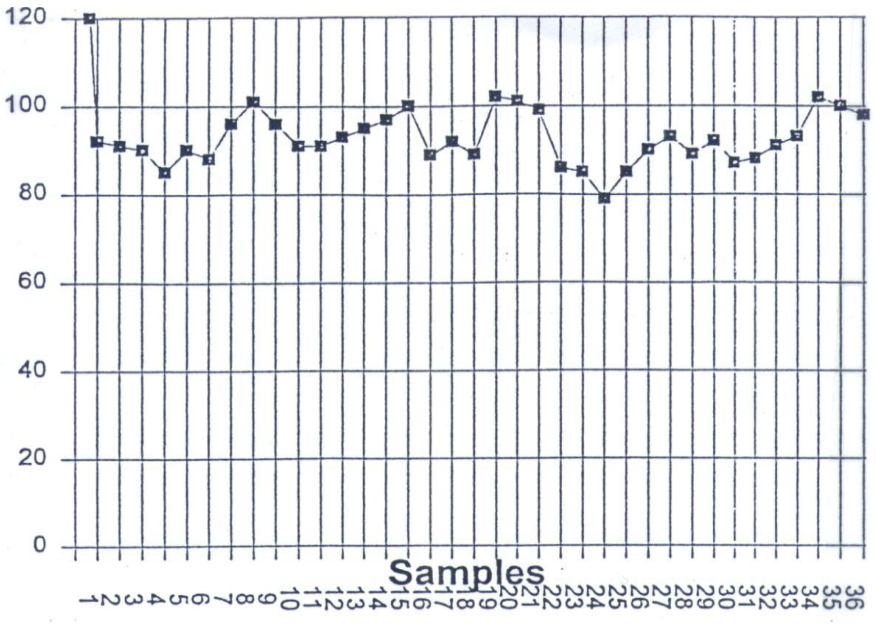
Critical differences for probability levels other than 95%

This difference can be determined by multiplying the critical differences at the 95% level by the coefficients shown in Table 1.

Table 1 - Multiplicative coefficients allowing the calculation of critical differences for probability levels other than 95%

| Probability level P | Multiplicative coefficient |
|---|---|
| 90 | 0,82 |
| 95 | 1,00 |
| 98 | 1,16 |
| 99 | 1,29 |
| 99,5 | 1,40 |

SHEWHART CONTROL GRAPH



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  of their performance characteristics". IUPAC. Pure an App. Chem., Vol. 62, nº 1, 149-162. 1990.

# Protocol for the design, conducts and interpretation of collaborative studies

(Resolution Oeno 6/2000)

## INTRODUCTION

After a number of meetings and workshops, a group of representatives from 27 organizations adopted by consensus a "Protocol for the design, conducts and interpretation of collaborative studies" which was published in Pure & Appl. Chem. 60, 855-864, 1995. A number of organizations have accepted and used this protocol. As a result of their experience and the recommendations of the Codex Committee on Methods of Analysis and Sampling (Joint FAO/WHO Food Standards Programme, Report of the Eighteenth Session, 9-13 November, 1992; FAO, Rome Italy, ALINORM 93/23, Sections 34-39), three minor revisions were recommended for incorporation into the original protocol. These are: (1) Delete the double split level design because the interaction term it generates depends upon the choice of levels and if it is statistically significant, the interaction cannot be physically interpreted. (2) Amplify the definition of "material". (3) Change the outlier removal criterion from 1% to 2.5%.

The revised protocol incorporating the changes is reproduced below. Some minor editorial revisions to improve readability have also been made. The vocabulary and definitions of the document 'Nomenclature of Interlaboratory Studies (Recommendations 1994)' [published in Pure Appl Chem., 66, 1903-1911 (1994)] has been incorporated into this revision, as well as utilizing, as far as possible, the appropriate terms of the International Organization for Standardization (ISO), modified to be applicable to analytical chemistry.

## PROTOCOL

### 1 Preliminary work

Method-performance (collaborative) studies require considerable effort and should be conducted only on methods that have received adequate prior testing. Such within-laboratory testing should include, as applicable, information on the following:

#### 1.1 Preliminary estimates of precision

Estimates of the total within-laboratory standard deviation of the analytical results over the concentration range of interest as a minimum at the upper and lower limits

of the concentration range, with particular emphasis on any standard or specification value.

NOTE 1: The total within-laboratory standard deviation is a more inclusive measure of imprecision that the ISO repeatability standard deviation, §3.3 below. This standard deviation is the largest of the within-laboratory type precision variables to be expected from the performance of a method; it includes at least variability from different days and preferably from different calibration curves. It includes between-run (between-batch) as well as within-run (within-batch) variations. In this respect it can be considered as a measure of within-laboratory reproducibility. Unless this value is well within acceptable limits, it cannot be expected that the between-laboratory standard deviation (reproducibility standard deviation) will be any better. This precision term is not estimated from the minimum study described in this protocol.

NOTE 2: The total within-laboratory standard deviation may also be estimated from ruggedness trials that indicate how tightly controlled the experimental factors must be and what their permissible ranges are. These experimentally determined ranges should be incorporated into the description of the method.

1.2 Systematic error (bias)

Estimates of the systematic error of the analytical results over the concentration range and in the substances of interest, as a minimum at the upper and lower limits of the concentration range, with particular emphasis on any standard or specification value.
The results obtained by applying the method to relevant reference materials should be noted.

1.3 Recoveries

The recoveries of "spikes" added to real materials and to extracts, digests, or other treated solutions thereof.

1.4 Applicability

The ability of the method to identify and measure the physical and chemical forms of the analyte likely to be present in the materials, with due regard to matrix effects.

1.5 Interference

The effect of other constituents that are likely to be present at appreciable concentrations in matrices of interest and which may interfere in the determination.

1.6 Method comparison

The results of comparison of the application of the method with existing tested methods intended for similar purposes.

1.7 Calibration Procedures

The procedures specified for calibration and for blank correction must not introduce important bias into the results.

1.8 Method description

The method must be clearly and unambiguously written.

1.9 Significant figures

The initiating laboratory should indicate the number of significant figures to be reported, based on the output of the measuring instrument.

NOTE: In making statistical calculations from the reported data, the full power of the calculator or computer is to be used with no rounding or truncating until the final reported mean and standard deviations are achieved. At this point the standard deviations are rounded to 2 significant figures and the means and related standard deviations are rounded to accommodate the significant figures of the standard deviation. For example, if $S_R$ = 0.012, c is reported as 0.147, not as 0. 1473 or 0. 15, and $RSD_R$ is reported as 8.2%. (Symbols are defined in Appendix L) If standard deviation calculations must be conducted manually in steps, with the transfer of intermediate results, the number of significant figures to be retained for squared numbers should be at least 2 times the number of figures in the data plus 1.

## 2. Design of the method-performance study

2.1 Number of materials

For a single type of substance, at least 5 materials (test samples) must be used; only when a single level specification is involved for a single matrix may this minimum required number of materials to be reduced to 3. For this design parameter, the two portions of a split level and the two individual portions of blind replicates per laboratory are considered as a single material.

NOTE 1: A material is an 'analyte/matrix/concentration' combination to which the method-performance parameters apply. This parameter determines the applicability of a method. For application to a number of different substances, a sufficient

number of matrices and levels should be chosen to include potential interferences and the concentration of typical use.

NOTE 2: The 2 or more test samples of blind or open replicates statistically, are a single material (they are not independent).

NOTE 3: A single split level (Youden pair) statistically analyzed as a pair is a single material; if analyzed statistically and reported as single test samples, they are 2 materials. In addition, the pair can be used to calculate the within-laboratory standard deviation, $s_r$ as

$$s_r = \sqrt{(\Sigma d_i^{\,2}) / 2n} \qquad \text{(for duplicates, blind or open),}$$

$$s_r = \sqrt{(\Sigma(d_i^{\,2}) \ / 2(n - 1)} \qquad \text{(for Youden pairs),}$$

where $d_i$, the difference between the 2 individual values from the split level for each laboratory and n is the number of laboratories. In this special case, $S_R$, the among laboratories standard deviation, is merely the average of the two $S_R$ values calculated from the individual components of the split level, and it is used only as a check of the calculations.

NOTE 4: The blank or negative control may be a material or not depending on the usual purpose of the analysis. For example, in trace analysis, where very low levels (near the limit of quantitation) are often sought, the blanks are considered as materials and are necessary to determine certain 'limits of measurement.' However, if the blank is merely a procedural control in macro analysis (e.g., fat in cheese), it would not be considered a material.

2.2 Number of laboratories

At least 8 laboratories must report results for each material; only when it is impossible to obtain this number (e.g., very expensive instrumentation or specialized laboratories required) may the study be conducted with less, but with an absolute minimum of 5 laboratories. If the study is intended for international use, laboratories from different countries should participate. In the case of methods requiring the use of specialized instruments, the study might include the entire population of available laboratories. In such cases, "n" is used in the denominator for calculating the standard deviation instead of "(n - 1)". Subsequent entrants to the field should demonstrate the ability to perform as well as the original participant.

2.3 Number of Replicates

The repeatability precision parameters must be estimated by using one of the following sets of designs (listed in approximate order of desirability):

2.3.1 Split Level

For each level that is split and which constitutes only a single material for purposes of design and statistical analysis, use 2 nearly identical test samples that differ only slightly in analyte concentration (e.g., <1-5%). Each laboratory must analyse each test sample once and only once.

NOTE: The statistical criterion that must be met for a pair of test samples to constitute a split level is that the reproducibility standard deviation of the two parts of the single split level must be equal.

2.3.2 Combination blind replicates and split level

Use split levels for some materials and blind replicates for other materials in the same study (single values from each submitted test sample).

2.3.3 Blind replicates

For each material, use blind identical replicates, when data censoring is impossible (e.g., automatic input, calculation, and printout) non-blind identical replicates may be used.

2.3.4 Known replicates

For each material, use known replicates (2 or more analyses of test portions from the same test sample), but only when it is not practical to use one of the preceding designs.

2.3.5 Independent analyses

Use only a single test portion from each material (i.e., do not perform multiple analyses) in the study, but rectify the inability to calculate repeatability parameters by quality control parameters or other within-laboratory data obtained independently of the method-performance study.

## 3. Statistical analysis (See Flowchart, A.4. 1)

For the statistical analysis of the data, the required statistical procedures listed below must be performed and the results reported. Supplemental, additional procedures are not precluded.

3.1 Valid data

Only valid data should be reported and subjected to statistical treatment. Valid data are those data that would be reported as resulting from the normal performance of laboratory analyses; they are not marred by method deviations, instrument malfunctions, unexpected occurrences during performance, or by clerical, typographical and arithmetical errors.

3.2 One-way analysis of variance

One-way analysis of variance and outlier treatments must be applied separately to each material (test sample) to estimate the components of variance and repeatability and reproducibility parameters.

3.3 Initial estimation

Calculate the mean, c (= the average of laboratory averages), repeatability relative standard deviation, $RSD_r$, and reproducibility relative standard deviation, $RSD_R$ with no outliers removed, but using only valid data.

3.4 Outlier treatment

The estimated precision parameters that must also be reported are based on the initial valid data purged of all outliers flagged by the harmonized 1994 outlier removal procedure. This procedure essentially consists of sequential application of the Cochran and Grubbs tests (at 2.5% probability (P) level, 1-tail for Cochran and 2-tail for Grubbs) until no further outliers are flagged or until a drop of 22.2% (= 219) in the original number of laboratories providing valid data would occur.

NOTE: Prompt consultation with a laboratory reporting suspect values may result in correction of mistakes or discovering conditions that lead to invalid data, 3.1. Recognizing mistakes and invalid data per se is much preferred to relying upon statistical tests to remove deviate values.

3.4.1 Cochran test

First apply Cochran outlier test (1-tail test a P = 2.5%) and remove any laboratory whose critical value exceeds the tabular value given in the tale, Appendix A.3. 1, for the number of laboratories and replicates involved.

3.4.2 Grubbs tests

Apply the single value Grubbs test (2 tail) and remove any outlying laboratory. If no laboratory is flagged, then apply the pair value tests (2 tail) - - 2 at the same end and 1 value at each end, P = 2.5% overall. Remove any laboratory(ies) flagged by these tests whose critical value exceeds the tabular value given in the appropriate column of the table Appendix A.3.3. Stop removal when the next application of the test will flag as table, A outliers more that 22.2% (2 of 9) of the laboratories.

NOTE: The Grubbs tests are to be applied one material at a time to the set of replicate means from all laboratories, and not to the individual values from replicated designs because the distribution of all the values taken together is multimodal, not Caussian, i.e., their differences from the overall mean for that material are not independent.

3.4.3 Final estimation

Recalculate the parameters as in §3.3 after the laboratories flagged by the preceding procedure have been removed. If no outliers were removed by the Cochran-Grubbs sequence, terminate testing. Otherwise, reapply the Cochran-Grubbs sequence to the data purged of the flagged outliers until no further outliers are flagged or until more than a total of 22.2% (2 of 9 laboratories) would be removed in the next cycle. See flowchart A.3.4.

## 4. Final report

The final report should be published and should include all valid data. Other information and parameters should be reported in a format similar (with respect to the reported items) to the following, as applicable:

[x] Method-performance tests carried out at the international level in [year(s)] by [organisation] in which [y and z] laboratories participated, each performing [k] replicates, gave the following statistical results:

TABLE OF METHOD-PERFORMANCE PARAMETERS

Analyte; Results expressed in [units]
Material [Description and listed in columns across top of table in increasing order of magnitude of means]
Number of laboratories retained after eliminating outliers
Number of outlying laboratories
Code (or designation) of outlying laboratories
Number of accepted results
Mean
True or accepted value, if known
Repeatability standard deviation ($S_r$)
Repeatability relative standard deviation ($RSD_R$)
Repeatability limit, r (2.8 x $S_r$)
Reproducibility standard deviation ($S_R$)
Reproducibility relative standard deviation ($RSD_R$)
Reproducibility limit, R (2.8 X $S_R$)

### 4.1 Symbols

A set of symbols for use in reports and publications is attached as Appendix 1 (A.1.).

### 4.2 Definitions

A set of definitions for use in study reports and publications is attached as Appendix 2 (A.2.).

### 4.3 Miscellaneous

#### 4.3.1 Recovery

Recovery of added analyte as a control on method or laboratory bias should be calculated as follows:

[Marginal] Recovery, %=
(Total analyte found - analyte originally present) x 100/(analyte added)

Although the analyte may be expressed as either concentration or amount, the units must be the same throughout. When the quantity of analyte is determined by analysis, it must be determined in the same way throughout.

Analytical results should be reported uncorrected for recovery. Report recoveries separately.

4.3.2 When $S_r$, is negative

By definition, $S_R$ is greater than or equal to $S_r$ in method-performance studies; occasionally the estimate of $S_r$ is greater than the estimate of $S_R$ (the average of the replicates is greater than the range of laboratory averages and the calculated $S_L^2$ is then negative). When this occurs, set $S_L = 0$ and $S_R = S_r$.

## 5. REFERENCES

Horwitz, W. (1988) Protocol for the design, conduct, and interpretation of method performance studies. Pure & Appl. Chem. 60, 855-864.

Pocklington, W.D. (1990) Harmonized protocol for the adoption of standardized analytical methods and for the presentation of their performance characteristics. Pure and Appl. Chem. 62, 149-162.

International Organization for Standardization. International Standard 5725-1986. Under revision in 6 parts; individual parts may be available from National Standards member bodies.

## A. APPENDICES

### APPENDIX 1. - SYMBOLS

Use the following set of symbols and terms for designating parameters developed by a method-performance study.

| | |
|---|---|
| Mean (of laboratory averages) | x |
| Standard deviations: | s (estimates) |
| Repeatability | $S_r$ |
| 'Pure' between-laboratory | $S_L$ |
| Reproducibility | $S_R$ |
| Variances: $S_R^2 = S_L^2 + S_r^2$ | $S^2$ (with subscripts, r, L, and R) |
| Relative standard deviations: | RSD (with subscripts, r, L, and r) |
| Maximum tolerable differences (as defined by ISO 5725-1986); See A.2.4 and A.2.5) | |
| Repeatability limit | $r = (2.8 \times S_r)$ |
| Reproducibility limit | $R = (2.8 \times S_R)$ |
| Number of replicates per laboratory | k (general) |
| Average number of replicates per laboratory i | k (for a balanced design) |
| Number of laboratories | L |
| Number of materials (test samples) | m |

Total number of values in a given assay n (= kL for a balanced design)
Total number of values in a given study N (= kLm for an overall balanced design)

_____

If other symbols are used, their relationship to the recommended symbols should be explained fully.

## **APPENDIX 2. -** DEFINITIONS

Use the following definitions. The first three definitions utilize the 1UPAC document "Nomenclature of Interlaboratory Studies" (approved for publication 1994). The next two definitions are assembled from components given in ISO 3534-1:1993. All test results are assumed to be independent, i.e., 'obtained in a manner not influenced by any previous result on the same or similar test object. Quantitative measures of precision depend critically on the stipulated conditions. Repeatability and reproducibility conditions are particular sets of extreme stipulated conditions.'

A.2.1 Method-performance studies

An interlaboratory study in which all laboratories follow the same written protocol and use the same test method to measure a quantity in sets of identical test items [test samples, materials]. The reported results are used to estimate the performance characteristics of the method. Usually these characteristics are within-laboratory and among-laboratories precision, and when necessary and possible, other pertinent characteristics such as systematic error, recovery, internal quality control parameters, sensitivity, limit of determination, and applicability.

A.2.2. Laboratory-performance study

An interlaboratory study that consists of one or more analyses or measurements by a group of laboratories on one or more homogeneous, stable test items, by the method selected or used by each laboratory. The reported results are compared with those of other laboratories or with the known or assigned reference value, usually with the objective of evaluating or improving laboratory performance.

A.2.3 Material certification stud

An interlaboratory study that assigns a reference value ('true value') to a quantity (concentration or property) in the test item, usually with a stated uncertainty.

A.2.4 Repeatability limit (r)

When the mean of the values obtained from two single determinations with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time, lies within the range of the mean values cited in the Final Report, 4.0, the absolute difference between the two test results obtained should be less than or equal to the repeatability limit (r) [= 2.8 x s,) that can generally be inferred by linear interpolation of $s_r$ from the Report.

NOTE: This definition, and the corresponding definition for reproducibility limit, has been assembled from five cascading terms and expanded to permit application by interpolation to a test item whose mean is not the same as that used to establish the original parameters, which is the usual case in applying these definitions. The term 'repeatability [and reproducibility] limit' is applied specifically to a probability

of 95% and is taken as 2.8 x s, [or SRI. The general term for this statistical concept applied to any measure of location (e.g., median) and with other probabilities (e.g., 99%) is "repeatability [and reproducibility] critical difference".

A.2.5 Reproducibility limit (R)

When the mean of the values obtained from two single determinations with the same method on identical test items in different laboratories with different operators using different equipment, lies within the range of the mean values cited in the Final Report, 4.0, the absolute difference between the two test results obtained should be less than or equal to the reproducibility limit (R) [= 2.8 x $s_R$] that can generally be inferred by linear interpolation of $S_R$ from the Report.

NOTE 1: When the results of the interlaboratory test make it possible, the value of r and R can be indicated as a relative value (e.g., as a percentage of the determined mean value) as an alternative to the absolute value.

NOTE 2: When the final reported result in the study is an average derived from more than a single value, i.e., k is greater than 1, the value for R must be adjusted according to the following formula before using R to compare the results of a single routine analyses between two laboratories.

$R' = (R^2 + r^2 (1 - [l /k])^{1/2}$

Similar adjustments must be made for replicate results constituting the final values for $S_R$ and $RSD_R$, if these will be the reported parameters used for quality control purposes.

NOTE 3: The repeatability limit, r, may be interpreted as the amount within which two determinations should agree with each other within a laboratory 95% of the time. The reproducibility limit, R, may be interpreted as the amount within which two separate determinations conducted in different laboratories should agree with each other 95% of the time.

NOTE 4: Estimates Of $S_R$ can be obtained only from a planned, organized method performance study; estimates of $S_r$ can be obtained from routine work within a laboratory by use of control charts. For occasional analyses, in the absence of control charts, within-laboratory precision may be approximated as one half $S_R$ (Pure and Appl. Chem., 62, 149-162 (1990) , Sec. L3, Note.).

A.2.6 One-way analysis of variance

One-way analysis of variance is the statistical procedure for obtaining the estimates of within laboratory and between-laboratory variability on a material-by-material basis. Examples of the calculations for the single level and single-split-level designs can be found in ISO 5725-1986.

## **APPENDIX 3. -** CRITICAL VALUES

A.3.1 Critical values for the Cochran maximum variance ratio at the 2.5% (1 -tail) rejection level, expressed as the percentage the highest variance is of the total variance; r = number of replicates.

| **No. of Labs** | **r=2** | **r = 3** | **r=4** | **r = 5** | **r = 6** |
|---|---|---|---|---|---|
| 4 | 94.3 | 81.0 | 72.5 | 65.4 | 62.5 |
| 5 | 88.6 | 72.6 | 64.6 | 58.1 | 53.9 |
| 6 | 83.2 | 65.8 | 58.3 | 52.2 | 47.3 |
| 7 | 78.2 | 60.2 | 52.2 | 47.3 | 42.3 |
| 8 | 73.6 | 55.6 | 47.4 | 43.0 | 38.5 |
| 9 | 69.3 | 51.8 | 43.3 | 39.3 | 35.3 |
| 10 | 65.5 | 48.6 | 39.9 | 36.2 | 32.6 |
| 11 | 62.2 | 45.8 | 37.2 | 33.6 | 30.3 |
| 12 | 59.2 | 43.1 | 35.0 | 31.3 | 28.3 |
| 13 | 56.4 | 40.5 | 33.2 | 29.2 | 26.5 |
| 14 | 53.8 | 38.3 | 31.5 | 27.3 | 25.0 |
| 15 | 51.5 | 36.4 | 29.9 | 25.7 | 23.7 |
| 16 | 49.5 | 34.7 | 28.4 | 24.4 | 22.0 |
| 17 | 47.8 | 33.2 | 27.1 | 23.3 | 21.2 |
| 18 | 46.0 | 31.8 | 25.9 | 22.4 | 20.4 |
| 19 | 44.3 | 30.5 | 24.8 | 21.5 | 19.5 |
| 20 | 42.8 | 29.3 | 23.8 | 20.7 | 18.7 |
| 21 | 41.5 | 28.2 | 22.9 | 19.9 | 18.0 |
| 22 | 40.3 | 27.2 | 22.0 | 19.2 | 17.3 |
| 23 | 39.1 | 26.3 | 21.2 | 18.5 | 16.6 |
| 24 | 37.9 | 25.5 | 20.5 | 17.8 | 16.0 |
| 25 | 36.7 | 24.8 | 19.9 | 17.2 | 15.5 |
| 26 | 35.5 | 24.1 | 19.3 | 16.6 | 15.0 |
| 27 | 34.5 | 23.4 | 18.7 | 16.1 | 14.5 |
| 28 | 33.7 | 22.7 | 18.1 | 15.7 | 14.1 |
| 29 | 33.1 | 22.1 | 17.5 | 15.3 | 13.7 |
| 30 | 32.5 | 21.6 | 16.9 | 14.9 | 13.3 |
| 35 | 29.3 | 19.5 | 15.3 | 12.9 | 11.6 |
| 40 | 26.0 | 17.0 | 13.5 | 11.6 | 10.2 |
| 50 | 21.6 | 14.3 | 11.4 | 9.7 | 8.6 |

Tables A.3.1 and A.3.3 were calculated by R. Albert (October, 1993) by computer simulation involving several runs of approximately 7000 cycles each for each value, and then smoothed. Although Table A.3.1 is strictly applicable only to a balanced design (same number of replicates from all laboratories), it can be applied to an unbalanced design without too much error, if there are only a few deviations.

A.3.2 Calculation of Cochran maximum variance outlier ratio

Compute the within-laboratory variance for each laboratory and divide the largest of these variances by the sum of the all of the variances and multiply by 100. The resulting quotient is the Cochran statistic which indicates the presence of a removable outlier if this quotient exceed the critical value listed above in the Cochran table for the number of replicates and laboratories specified.

A.3.3 Critical values for the Grubbs extreme deviation outlier tests at the 2.5% (2-tail), 1.25% (1tail) rejection level, expressed as the percent reduction in standard deviations caused by the removal of the suspect value(s).

| No. of labs | One highest or lowest | Two highest or two lowest | One highest and one lowest |
|---|---|---|---|
| 4 | 86.1 | 98.9 | 99.1 |
| 5 | 73.5 | 90.9 | 92.7 |
| 6 | 64.0 | 81.3 | 84.0 |
| 7 | 57.0 | 73.1 | 76.2 |
| 8 | 51.4 | 66.5 | 69.6 |
| 9 | 46.8 | 61.0 | 64.1 |
| 10 | 42.8 | 56.4 | 59.5 |
| 11 | 39.3 | 52.5 | 55.5 |
| 12 | 36.3 | 49.1 | 52.1 |
| 13 | 33.8 | 46.1 | 49.1 |
| 14 | 31.7 | 43.5 | 46.5 |
| 15 | 29.9 | 41.2 | 44.1 |
| 16 | 28.3 | 39.2 | 42.0 |
| 17 | 26.9 | 37.4 | 40.1 |
| 18 | 25.7 | 35.9 | 38.4 |
| 19 | 24.6 | 34.5 | 36.9 |
| 20 | 23.6 | 33.2 | 35.4 |
| 21 | 22.7 | 31.9 | 34.0 |
| 22 | 21.9 | 30.7 | 32.8 |
| 23 | 21.2 | 29.7 | 31.8 |
| 24 | 20.5 | 28.8 | 30.8 |
| 25 | 19.8 | 28.0 | 29.8 |
| 26 | 19.1 | 27.1 | 28.9 |
| 27 | 18.4 | 26.2 | 28.1 |
| 28 | 17.8 | 25.4 | 27.3 |
| 29 | 17.4 | 24.7 | 26.6 |
| 30 | 17.1 | 24.1 | 26.0 |
| 40 | 13.3 | 19.1 | 20.5 |
| 50 | 11.1 | 16.2 | 17.3 |

A.3.4 Calculation of the Grubbs test values

To calculate the single Chubbs test statistic, compute the average for each laboratory and then calculate the standard deviation (M) of these L averages (designate as the original s). Calculate the SD of the set of averages with the highest average removed (SH); calculate the SD of the set of averages with the lowest average removed (SL). The calculate the percentage decrease in SD for both as follows:

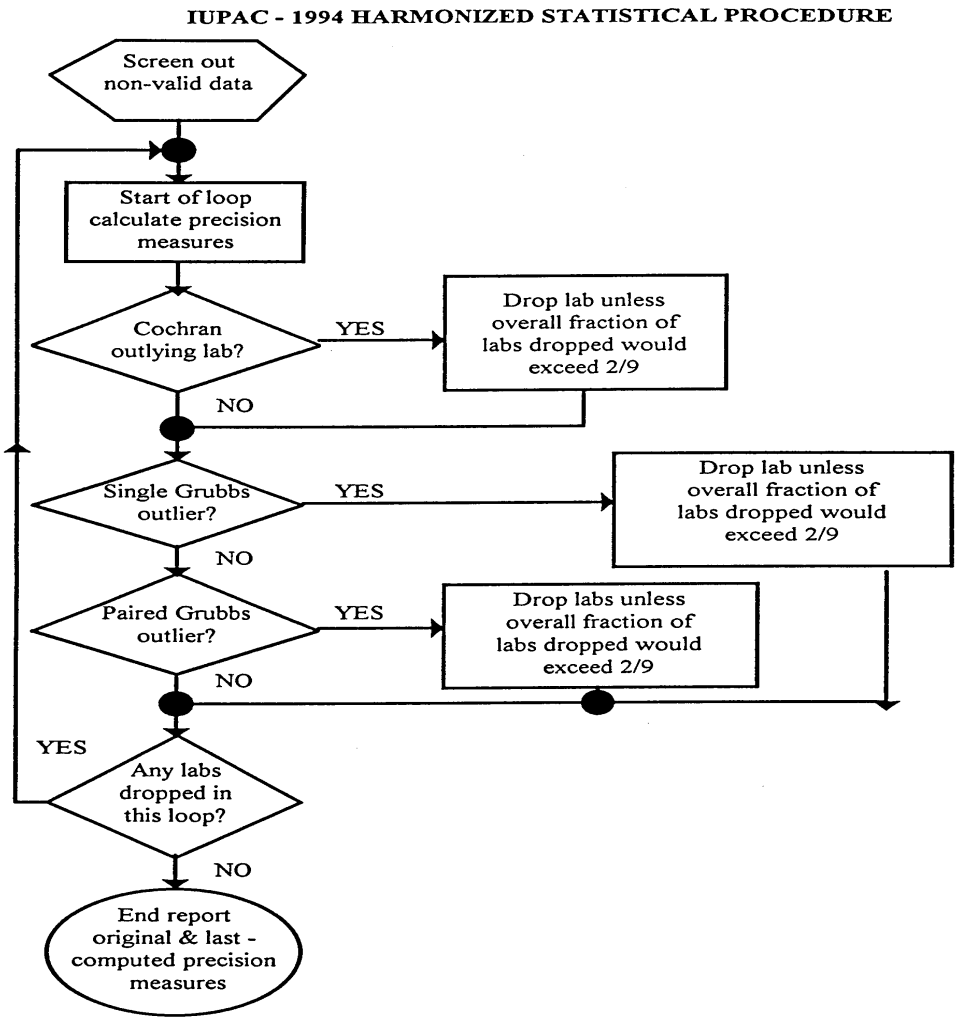
100 x [ 1 - (sL/s] and 100 x [ 1 - (sH/s)].

The higher of these two percentage decreases is the singe Grubbs test statistic, which signal the presence of an outlier to be omitted at the P = 2.5% level, 2–tail, if it exceeds the critical value listed in the single value column, Column 2, of Table A.3.3 , for the number of laboratory averages used to calculate the original s.

To calculate the paired Grubbs test statistics, calculate the percentage decrease in standard deviation obtained by dropping the two highest averages and also by dropping the two lowest averages, as above. Compare the higher of the percentage changes in standard deviation with the tabular values in column 3 and proceed with (1) or (2): (1) If the tabular value is exceeded, remove the responsible pair. Repeat the cycle again, starting at the beginning with the Cochran extreme variance test again, the Grubbs extreme value test, and the paired Grubbs extreme value test. (2) If no further values are removed, then calculate the percentage change in standard deviation obtained by dropping both the highest extreme value and the lowest extreme value together, and compare with the tabular values in the last column of A.3.3. If the tabular value is exceeded, remove the high-low pair of averages, and start the cycle again with the Cochran test until no further values are removed. In all cases, stop outlier testing when more than 22.2% (2/9) of the averages are removed.

## APPENDIX 4

A.4.1. Flowchart for outlier removal



# Estimation of the detection and quantification limits of a method of analysis

(Resolution Oeno 7/2000)

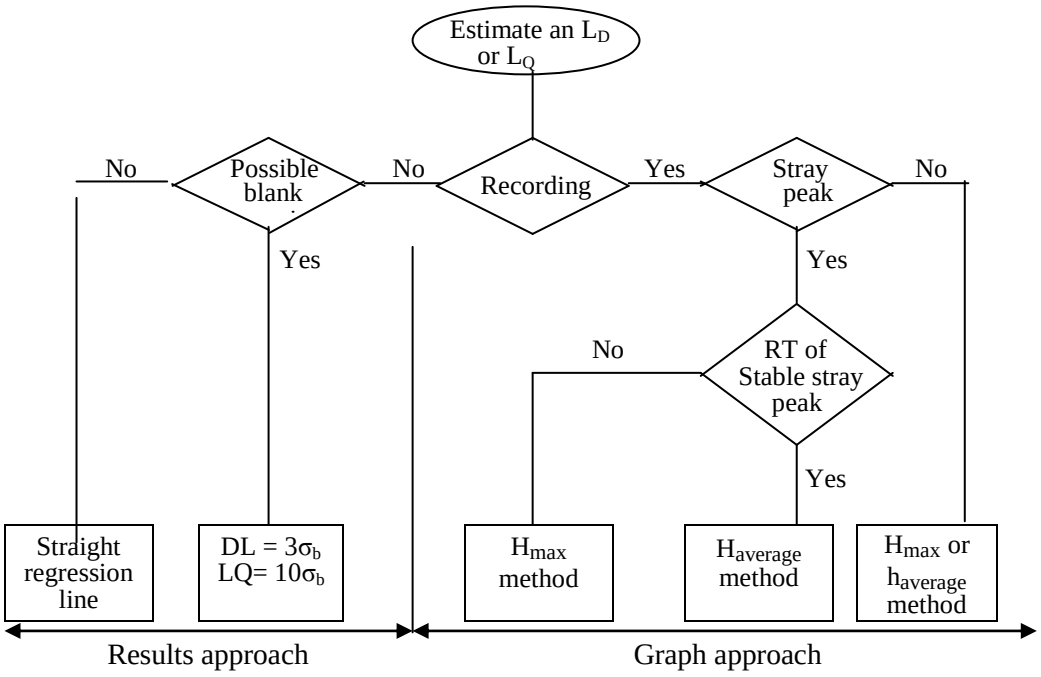
**1 - Purpose:** to establish the detection and quantification limits of a method

**N.B. : The proposed calculation procedure sets « detection and quantification limiting » values with respect to the instrumental response. For a given method, the final calculation of these values must take cognizance of factors arising from the preparation of the sample.**

**2 - Definitions**

- Detection limit: the smallest concentration or proportion of the analyzed substance that can be detected with an acceptable level of uncertainty, but that is not quantified under the experimental conditions described in the method
- Quantification limit: the smallest concentration or proportion of the analyzed substance that can be quantified with an acceptable level of uncertainty, under the experimental conditions described in the method.

**3 – Logic Diagram for Decision-Making**



## 4 - Methodology

### 4.1 "Results" approach

When the analytical method produces no recorded graph, but only numerical values (i.e., colorimetry), the detection limit ($L_D$) and the quantification limit ($L_Q$) are estimated using one of the two following methods.

4.1.1 - Method 1:

Directly read n measurements (analyte quantity or response) of separate analytic « blank » samples that contain all of the constituents, with the exception of the substance to be tested for.

$$L_D = m_{blank} + 3S_{blank} \text{ and}$$
$$L_Q = m_{blank} + 10S_{blank}$$

where $m_{blank}$ and $S_{blank}$ are the mean and standard deviation for n measurements.

*Note: A multiplication factor of 3 corresponds to a 0.13% chance of concluding that the substance sought is present, when, in fact, it is lacking. 10 corresponds to a 0.5% chance.*

4.1.2 - Method 2:

Using the straight calibration line: $Y = a + bX$

The detection limit is the smallest concentration of a substance that can be distinguished from the blank, with a 0.13% risk of retaining samples containing nothing ; in other words, the value beginning at which a statistical test comparing the response to 0 becomes significant with an error level α of 0.13%. Hence:

$$Y_{DL} = a + 3S_a$$
$$X_{DL} = (a + 3S_a)/b$$

Where Sa is the standard deviation on the ordinate at the origin of the straight regression line. The logic is the same for $L_{Q,,}$ where the multiplication factor is 10 (risk of 0.5%).

4.2 - "*Graph" Approach*

For analytical methods which generate graphs (i.e., chromatography), the detection limit is estimated based on the ground noise of the analytic blank recording for a given sample.

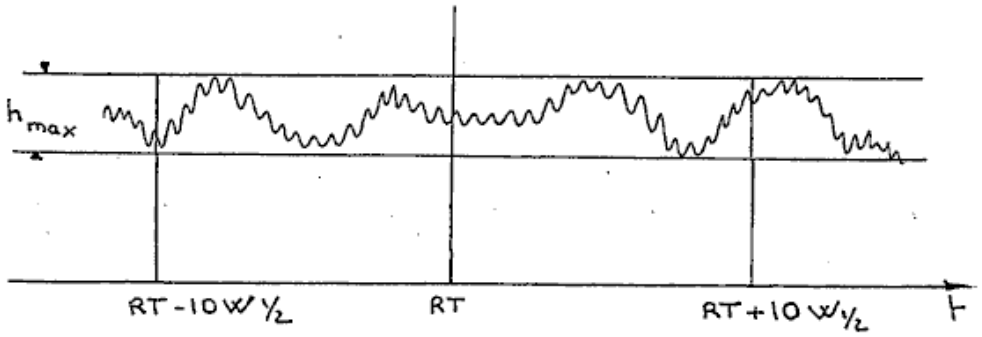
$L_D$ = 3 x h x R (associated risk is below 0.13%) and
$L_Q$ = 10 x h x R (associated risk is below 0.5%), where

- h is the average or maximum amplitude of the signal window corresponding to 10 width s of the mid-height peak on either side of the retention time, as a function of stability.

- R is the quantity/signal response factor expressed as a function of the quantity of substance/height.

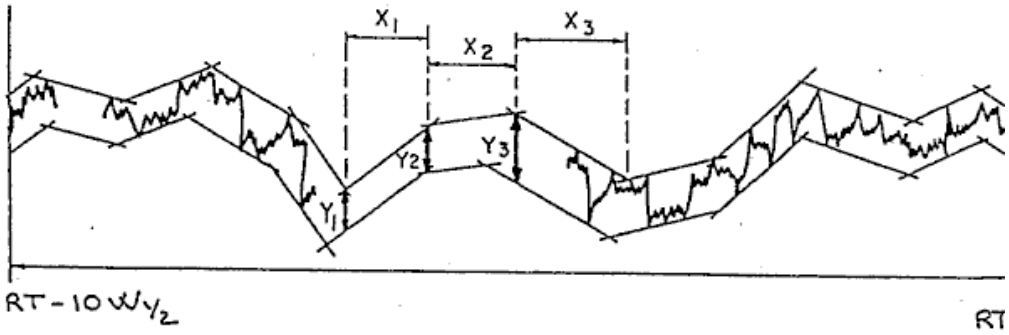
On each occasion, three series of three injections each are performed on test blanks at an interval of several days.

4.2.1 $h_{max}$ method



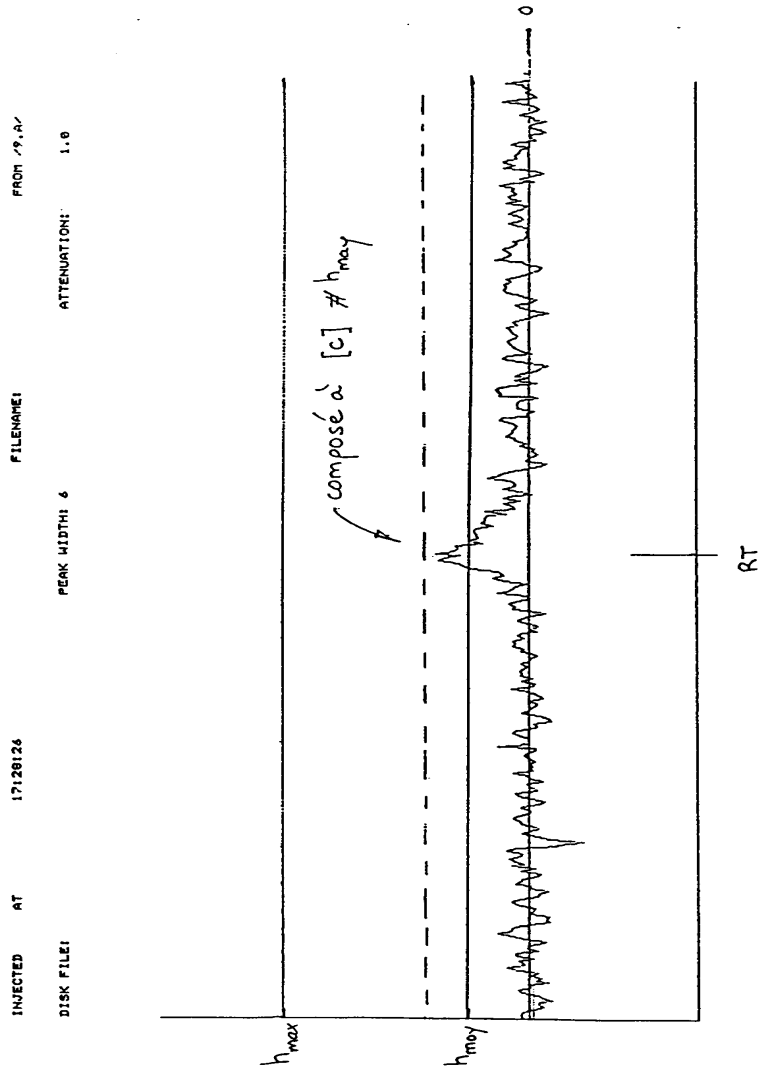

- Increase ground noise to the maximum (Fig. 1 above) ;
- center around the retention time (RT) of the product ;
- draw a window of 10 widths of the mid-height peak (W1/2) on either side of the RT ;
- draw two parallel lines, one running through the highest point of the highest peak, the other through the base of the deepest trough ;
- evaluate height -> $h_{max}$ ;
- calculate the response factor (R factor) ;
- $L_{Dmax} = 3 \times h_{max} \times R$
- $L_{Qmax} = 10 \times h_{max} \times R$

4.2.2 $h_{average}$ Method




- increase the ground noise to the maximum (Fig. 2 above) ;
- center around the retention time (RT) of the product ;
- draw a window of 10 widths of the mid-height peck (W1/2) on either side of the RT ;
- divide into 20 equal sections (x) ;
- draw two parallel lines in each block, one running through the highest point of the highest peak, the other through the base of the deepest trough ;
- measure the heights, y ;
- calculate the average ($y = h_{average}$);
- calculate the response factor (R factor);
- $L_{Daverage} = 3 \times h_{average} \times R$ ;
- $L_{Qaverage} = 10 \times h_{average} \times R$

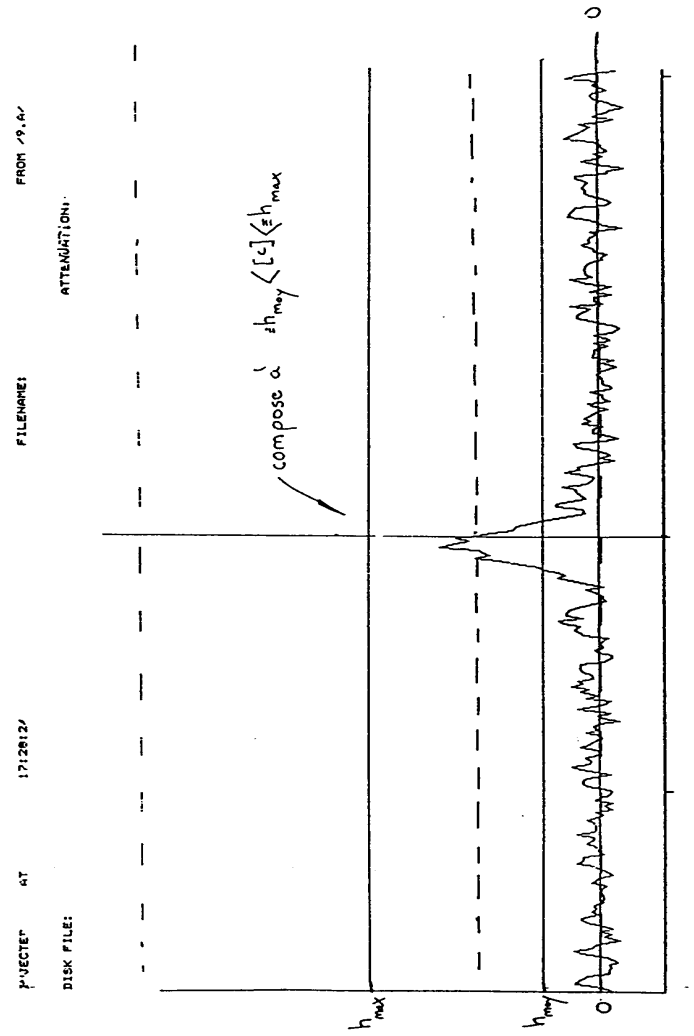
These estimates can themselves be validated by injecting quantities of solute that are close to the calculated limits (Figures 3 and 4).




Compound at [c] # $h_{max}$

Figure No. 3: Validating calculations of limits.
Concentration of the compound approaches $H_{average}$

N.B. : The dotted line corresponds to the real injected value however, since this figure is provided as an example, it may be deleted from the final text.




Compound at $h_{average} < [c] < \approx h_{max}$

Figure No. 4: Validating calculations of limits.
Concentration of compound between $H_{average}$ and $H_{max}$

N.B. : The dotted line corresponds to the real injected value; however, since this figure is provided as an example, it may be deleted from the final text.

# Harmonized guidelines for internal quality control in analytical chemistry laboratories

(Resolution Oeno 19/2002)

## CONTENTS

# 1. INTRODUCTION

## 1.1 Basic concept

This document sets out guidelines for the implementation of internal quality control (IQC) in analytical laboratories. IQC is one of a number of concerted measures that analytical chemists can take to ensure that the data produced in the laboratory are fit for their intended purpose. In practice, fitness for purpose is determined by a comparison of the accuracy achieved in a laboratory at a given time with a required level of accuracy. Internal quality control therefore comprises the routine practical procedures that enable the analytical chemist to accept a result or group of results as fit for purpose, or reject the results and repeat the analysis. As such, IQC is an important determinant of the quality of analytical data, and is recognised as such by accreditation agencies.

Internal quality control is undertaken by the inclusion of particular reference materials, here called "control materials", into the analytical sequence and by duplicate analysis. The control materials should, wherever possible, be representative of the test materials under consideration in respect of matrix composition, the state of physical preparation and the concentration range of the analyte. As the control materials are treated in exactly the same way as the test materials, they are regarded as surrogates that can be used to characterise the performance of the analytical system, both at a specific time and over longer intervals.

Internal quality control is a final check of the correct execution of all of the procedures (including calibration) that are prescribed in the analytical protocol and all of the other quality assurance measures that underlie good analytical practice. IQC is therefore necessarily retrospective. It is also required to be as far as possible independent of the analytical protocol, especially the calibration, that it is designed to test.

Ideally both the control materials and those used to create the calibration should be traceable to appropriate certified reference materials or a recognised empirical reference method. When this is not possible, control materials should be traceable at least to a material of guaranteed purity or other well characterised material. However, the two paths of traceability must not become coincident at too late a stage in the analytical process. For instance, if control materials and calibration standards were prepared from a single stock solution of analyte, IQC would not detect any inaccuracy stemming from the incorrect preparation of the stock solution.

In a typical analytical situation several, or perhaps many, similar test materials will be analysed together, and control materials will be included in the group. Often determinations will be duplicated by the analysis of separate test portions of the same material. Such a group of materials is referred to in this document as an analytical "run". (The words "set", "series" and "batch" have also been used as synonyms for "run".) Runs are regarded as being analysed under effectively constant conditions. The batches of reagents, the instrument settings, the analyst, and the laboratory environment will, under ideal conditions, remain unchanged during analysis of a run. Systematic errors should therefore remain constant during a run, as should the values of the parameters that describe random errors. .As the monitoring of these errors is of concern, the run is the basic operational unit of IQC.

A run is therefore regarded as being carried out under repeatability conditions, *i.e.*, the random measurement errors are of a magnitude that would be encountered in a "short" period of time. In practice the analysis of a run may occupy sufficient time for small systematic changes to occur. For example, reagents may degrade, instruments may drift, minor adjustments to instrumental settings may be called for, or the laboratory temperature may rise. However, these systematic effects are, for the purposes of IQC, subsumed into the repeatability variations. Sorting the materials making up a run into a randomised order converts the effects of drift into random errors.

### 1.2 Scope of this document

This document is a harmonisation of IQC procedures that have evolved in various fields of analysis, notably clinical biochemistry, geochemistry and environmental studies, occupational hygiene and food analysis[3-9]. There is much common ground in the procedures from these various fields. Analytical chemistry comprises an even wider range of activities and the basic principles of IQC should be able to encompass all of these. The present document provides guidelines that will be applicable in most instances. This policy necessarily excludes a number of IQC practices that are restricted to individual sectors of the analytical community. In addition in some sectors it is common to combine IQC as defined here with other aspects of quality assurance practice. There is no harm in such combination, but it must remain clear what are the essential aspects of IQC.

In order to achieve a harmonisation and provide basic guidance on IQC, some types of analytical activity have been excluded from this document. Issues specifically excluded are as follows.

(i) *Quality control of sampling.* While it is recognised that the quality of the analytical result can be no better than that of the sample, quality control of sampling is a separate subject and in many areas is not fully developed. Moreover, in many instances analytical laboratories have no control over sampling practice and quality.

(ii) *In-line analysis and continuous monitoring*. In this style of analysis there is no possibly of repeating the measurement, so the concept of IQC as used in this document is inapplicable.

(iii) *Multivariate IQC*. Multivariate methods in IQC are still the subject of research and cannot be regarded as sufficiently established for inclusion here. The current document regards multianalyte data as requiring a series of univariante IQC tests. Caution is necessary in the interpretation of this type of data to avoid inappropriately frequent rejection of data.

(iv) *Statutory and contractual requirements*.

(v) *Quality assurance measures* such as checks on instrumental stability before and during analysis, wavelength calibration, balance calibration, tests on resolution of chromatography columns, and problem diagnostics are not included. For present purposes they are regarded as part of the analytical protocol, and IQC tests their effectiveness together with the other aspects of the methodology.

### 1.3 Internal quality control and uncertainty

A prerequisite of analytical chemistry is the recognition of "fitness for purpose", the standard of accuracy that is required for an effective use of the analytical data. This standard is arrived at by consideration of the intended uses of the data although it is seldom possible to foresee all of the potential future applications of analytical results. For this reason in order to prevent inappropriate interpretation, it is important that a statement of the uncertainty should accompany analytical results, or be readily available to those who wish to use the data.

Strictly speaking, an analytical result cannot be interpreted unless it is accompanied by knowledge of its associated uncertainty at a stated level of confidence. A simple example demonstrates this principle. Suppose that there is a statutory requirement that a foodstuff must not contain more than 10 $\mu g\ g^{-1}$ of a particular constituent. A manufacturer analyses a batch and obtains a result of 9 $\mu g\ g^{-1}$ for that constituent. If the uncertainty of the result expressed as a half range (assuming no sampling error) is 0.1 $\mu g\ g^{-1}$ (i.e. the true result falls, with a high probability, within the range 8.9-9.1) then it may be assumed that the legal limit is not exceeded. If, in contrast, the uncertainty is 2 $\mu g\ g^{-1}$ then there is no such assurance. The interpretation and use that may be made of the measurement thus depends on the uncertainty associated with it.

Analytical results should therefore have an associated uncertainty if any definite meaning is to be attached to them or an informed interpretation made. If this requirement cannot be fulfilled, the use to which the data can be put is limited. Moreover, the achievement of the required measurement uncertainty must be tested as a routine procedure, because the quality of data can vary, both in time within a single laboratory and between different laboratories. IQC comprises the process of checking that the required uncertainty is achieved in a run.

## 2. DEFINITIONS

### 2.1 International definitions

**Quality assurance**. All those planned and systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality(10).

**Trueness**: closeness of the agreement between the average value obtained from a large series of test results and an accepted reference value(11).

**Precision**: closeness of agreement between independent test results obtained under prescribed conditions(12).

**Bias**: difference between the expectation of the test results and an accepted reference value(11).

**Accuracy**: closeness of the agreement between the result of a measurement and a true value of the measurand(13).
*Note 1*. Accuracy is a qualitative concept.
*Note 2*. The term *precision* should not be used for *accuracy*.

**Error**: result of a measurement minus a true value of the measurand(13).

**Repeatability conditions**. conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time(11).

**Uncertainty of measurement**: parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand(14).

*Note 1*. The parameter may be, for example, a standard deviation (or a given multiple of it), or the half-width of an interval having a stated level of confidence.

*Note 2*. Uncertainty of measurement comprises, in general, many components. Some of these components may be evaluated from the statistical distribution of results of a series of measurements and can be characterised by experimental standard deviations. The other components, which can also be characterised by standard deviations, are evaluated from assumed probability distributions based on experience or other information.

*Note 3*. It is understood that the result of a measurement is the best estimate of the value of a measurand, and that all components of uncertainty, including those arising from systematic effects, such as components associated with corrections and reference standards, contribute to the dispersion.

**Traceability**: property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties(13).

**Reference material**: material or substance one of whose property values are sufficiently homogeneous and well established to be used for the

calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials(13).

**Certified reference material**: reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realisation of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence(13).

### 2.2 Definitions of terms specific to this document

**Internal quality control**: set of procedures undertaken by laboratory staff for the continuous monitoring of operation and the results of measurements in order to decide whether results are reliable enough to be released.

**Control material**: material used for the purposes of internal quality control and subjected to the same or part of the same measurement procedure as that used for test materials.

**Run (analytical run)**: set of measurements performed under repeatability conditions.

**Fitness for purpose**: degree to which data produced by a measurement process enables a user to make technically and administratively correct decisions for a stated purpose

**Analytical system**: range of circumstances that contribute to the quality of analytical data, including equipment, reagents, procedures, test materials, personnel, environment and quality assurance measures.

## 3. QUALITY ASSURANCE PRACTICES AND INTERNAL QUALITY CONTROL

### 3.1 Quality assurance

Quality assurance is the essential organisational infrastructure that underlies all reliable analytical measurements. It is concerned with achieving appropriate levels in matters such as staff training and management, adequacy of the laboratory environment, safety, the storage, integrity and identity of samples, record keeping, the maintenance and

calibration of instruments, and the use of technically validated and properly documented methods . Failure in any of these areas might undermine vigorous efforts elsewhere to achieve the desired quality of data. In recent years these practices have been codified and formally recognised as essential. However, the prevalence of these favourable circumstances by no means ensures the attainment of appropriate data quality unless IQC is conducted.

### 3.2 Choice of analytical method

It is important that laboratories restrict their choice of methods to those that have been characterised as suitable for the matrix and analyte of interest. The laboratory must possess documentation describing the performance characteristics of the method, estimated under appropriate conditions.

The use of a method does not in itself guarantee the achievement of its established performance characteristics. There is, for a given method, only the potential to achieve a certain standard of reliability when the method is applied under a particular set of circumstances. It is this collection of circumstances, known as the "analytical system", that is therefore responsible for the accuracy of analytical data. Hence it is important to monitor the analytical system in order to achieve fitness for purpose. This is the aim of the IQC measures undertaken in a laboratory.

### 3.3 Internal quality control and proficiency tests

Proficiency testing is a periodic assessment of the performance of individual laboratories and groups of laboratories that is achieved by the distribution by an independent testing body of typical materials for unsupervised analysis by the participants(2). Although important, participation in proficiency testing schemes is not a substitute for IQC measures, or *vice versa*.

Proficiency testing schemes can be regarded as a routine, but relatively infrequent, check on analytical errors. Without the support of a well-developed IQC system, the value of participation in a proficiency test is negligible. Probably the main beneficial effect of proficiency tests is that of encouraging participants to install effective quality control systems. It has been shown that laboratories with effective IQC systems performed better in a proficiency testing scheme(15).

## 4. INTERNAL QUALITY CONTROL PROCEDURES

### 4.1 Introduction

Internal quality control involves the practical steps undertaken to ensure that errors in analytical data are of a magnitude appropriate for the use to which the data will be put. The practice of IQC depends on the use of two strategies, the analysis of reference materials to monitor trueness and statistical control, and duplication to monitor precision.

The basic approach to IQC involves the analysis of control materials alongside the test materials under examination. The outcome of the control analyses forms the basis of a decision regarding the acceptability of the test data. Two key points are worth noting in this context.

(i) The interpretation of control data must be based on documented, objective criteria, and on statistical principles wherever possible.

(ii) The results of control analyses should be viewed primarily as indicators of the performance of the analytical system, and only secondarily as a guide to the errors associated with individual test results. Substantial changes in the apparent accuracy of control determinations can sometimes be taken to imply similar changes to data for contemporary test materials, but correction of analytical data on the basis of this premise is unacceptable.

### 4.2 General Approach - Statistical Control

The interpretation of the results of IQC analyses depends largely on the concept of statistical control, which corresponds with stability of operation. Statistical control implies that an IQC result $x$ can be interpreted as arising independently and at random from a normal population with mean $\mu$ and variance $\sigma^2$.

Under these constraints only about 0.27% of results ($x$) would fall outside the bounds of $\mu \pm 3\sigma$. When such extreme results are encountered they are regarded as being "out-of- control" and interpreted to mean that the analytical system has started to behave differently. Loss of control therefore implies that the data produced by the system are of unknown accuracy and hence cannot be relied upon. The analytical system therefore requires investigation and remedial action before further analysis is undertaken. Compliance with statistical control can be monitored graphically with Shewhart control charts (see Appendix 1). An equivalent

numerical approach, comparing values of $z = (x-\mu)/\sigma$ against appropriate values of the standard normal deviate, is also possible.

### 4.3 Internal quality control and fitness for purpose.

For the most part, the process of IQC is based on a description in terms of the statistical parameters of an ongoing analytical system in normal operation. Control limits are therefore based on the estimated values of these parameters rather than measures derived from considerations of fitness for purpose. Control limits must be narrower than the requirements of fitness for purpose or the analysis would be futile.

The concept of statistical control is inappropriate, however, when the so-called *ad hoc* analysis is being undertaken. In *ad hoc* analysis the test materials may be unfamiliar or rarely encountered, and runs are often made up of only a few such test materials. Under these circumstances there is no statistical basis for the construction of control charts. In such an instance the analytical chemist has to use fitness for purpose criteria, historical data or consistency with the visual properties of the test material for judging the acceptability of the results obtained.

Either way, agreed methods of establishing quantitative criteria to characterise fitness for purpose would be desirable. Unfortunately, this is one of the less-developed aspects of IQC. In specific application areas guidelines may emerge by consensus. For example, in environmental studies it is usually recognised that relative uncertainties of less than ten percent in the concentration of a trace analyte are rarely of consequence. In food analysis the Horwitz curve[16] is sometimes used as a fitness for purpose criterion. Such criteria have been defined for clinical analysis[17,18]. In some areas of applied geochemistry a systematic approach has given rise to fitness for purpose criteria for sampling and analytical precisions. However, it is not practicable here to give guidelines in these areas, and at present no general principles can be advanced that would allow specific applications to be addressed.

### 4.4 The nature of errors

Two main categories of analytical error are recognised, namely random errors and systematic errors, which give rise to imprecision and bias respectively. The importance of categorising errors in this way lies in the fact that they have different sources, remedies and consequences for the interpretation of data.

*Random errors* determine the precision of measurement. They cause random positive and negative deviations of results about the underlying mean value. *Systematic errors* comprise displacement of the mean of many determinations from the true value. For the purposes of IQC two levels of systematic error are worth consideration.

(i) *Persistent bias* affects the analytical system (for a given type of test material) over a long period and affects all data. Such bias, if small in relation to random error, may be identifiable only after the analytical system has been in operation for a long time. It might be regarded as tolerable, provided it is kept within prescribed bounds.

(ii) *The run effect* is exemplified by a deviation of the analytical system during a particular run. This effect, where it is sufficiently large, will be identified by IQC at the time of occurrence as an out-of-control condition.

The conventional division of errors between the random and the systematic depends on the timescale over which the system is viewed. Run effects of unknown source can be regarded in the long-term as the manifestation of a random process. Alternatively, if a shorter-term view is taken, the same variation could be seen as a bias-like change affecting a particular run.

The statistical model used for IQC in this document is as follows[1]. The value of a measurement ($x$) in a particular run is given by:

$x$ = true value + persistent bias + run effect + random error (+ gross error).

The variance of $x$ ($\sigma_x^2$) in the absence of gross errors is given by:

$$\sigma_x^2 = \sigma_0^2 + \sigma_1^2$$

where

$\sigma_0^2$ = variance of the random error (within run) and
$\sigma_1^2$ = variance of the run effect.

[1] The model could be extended if necessary to include other features of the analytical system

The variances of the true value and the persistent bias are both zero. An analytical system in control is fully described by $\sigma_0^2$ , $\sigma_1^2$ and the value of the persistent bias. Gross errors are implied when the analytical system does not comply with such a description.

## 5 IQC AND WITHIN-RUN PRECISION

### 5.1 Precision and duplication

A limited control of within-run precision is achieved by the duplication within a run of measurements made on test materials. The objective is to ensure that the differences between paired results are consistent with or better than the level implied by the value of $\sigma_0$ used by a laboratory for IQC purposes[2]. Such a test alerts the user to the possibility of poor within-run precision and provides additional information to help in interpreting control charts. The method is especially useful in *ad hoc* analysis, where attention is centred on a single run and information obtained from control materials is unlikely to be completely satisfactory.

As a general approach all of the test materials, or a random selection from them, are analysed in duplicate. The absolute differences $|d| = |x_1 - x_2|$ between duplicated analytical results $x_1$ and $x_2$ are tested against an upper control limit based on an appropriate value of $\sigma_0$. However, if the test materials in the run have a wide range of concentration of analyte, no single value of $\sigma_0$ can be assumed(19).

Duplicates for IQC must reflect as far as possible the full range of variation present in the run. They must not be analysed as adjacent members of the run, otherwise they will reveal only the smallest possible measure of analytical variability. The best placing of duplicates is at random within each run. Moreover the duplication required for IQC requires the complete and independent analysis (preferably blind) of separate test portions of the test material. A duplication of the instrumental measurement of a single test solution would be ineffective because the variations introduced by the preliminary chemical treatment of the test material would be absent.

[2] There is no intention here of estimating the standard deviation of repeatability $\sigma_r$ from the IQC data or of comparing estimates: there would usually be too few results for a satisfactory outcome. Where such an estimate is needed the formula $s_r = \sqrt{\sum d^2 / 2n}$ can be used.

### 5.2 Interpretation of duplicate data

*5.2.1 Narrow concentration range.* In the simplest situation the test materials comprising the run have a small range of analyte concentrations so that a common within-run standard deviation $\sigma_0$ can be applied.

A value of this parameter must be estimated to provide a control limit. The upper 95% bound of $|d|$ is $2\sqrt{2}\,\sigma_0$ and on average only about three in a thousand results should exceed $3\sqrt{2}\,\sigma_0$.A group of *n* duplicated results can be interpreted in several ways.

For example, the standardised difference

$$z_d = d/\sqrt{2}\,\sigma_0$$

should have a normal distribution with zero mean and unit standard deviation. The sum of a group of *n* such results would have a standard deviation of $\sqrt{n}$ so only about three runs in a thousand would produce a value of $|\Sigma z_d| > 3\sqrt{n}$ . Alternatively a group of *n* values of $z_d$ from a run can be combined to form $\Sigma z_d^2$ and the result interpreted as a sample from a chi-squared distribution with *n* degrees of freedom, ($\chi_n^2$). Some caution is needed in the use of this statistic, however, as it is sensitive to outlying results.

*5.2.2 Wide concentration range.* If the test materials comprising a run have a wide range of analyte concentrations, no common standard of precision ($\sigma_0$) can be assumed. In such an instance $\sigma_0$ must be expressed as a functional relationship with concentration. The value of concentration for a particular material is taken to be $(x_1 + x_2)/2$, and an appropriate value of $\sigma_0$ obtained from the functional relationship, the parameters of which have to be estimated in advance.

## 6. CONTROL MATERIALS IN IQC

### 6.1 Introduction

Control materials are characterised substances that are inserted into the run alongside the test materials and subjected to exactly the same treatment. A

control material must contain an appropriate concentration of the analyte, and a value of that concentration must be assigned to the material. Control materials act as surrogates for the test materials and must therefore be representative, *i.e.*, they should be subject to the same potential sources of error. To be fully representative, a control material must have the same matrix in terms of bulk composition, including minor constituents that may have a bearing on accuracy. It should also be in a similar physical form, *i.e.*, state of comminution, as the test materials. There are other essential characteristics of a control material. It must be adequately stable over the period of interest. It must be possible to divide the control material into effectively identical portions for analysis. It is often required in large amounts to allow its use over an extended period.

Reference materials in IQC are used in combination with control charts that allow both persistent bias and run effects to be addressed (Appendix 1). Persistent bias is evident as a significant deviation of the centre line from the assigned value. The variation in the run effect is predictable in terms of a standard deviation when the system is under statistical control, and that standard deviation is used to define action limits and warning limits at appropriate distances from the true value.

**6.2 The role of certified reference materials**

Certified reference materials (CRM) as defined in Section 2 (*i.e.*, with a statement of uncertainty and traceability), when available and of suitable composition, are ideal control materials in that they can be regarded for traceability purposes as ultimate standards of trueness[20]. In the past CRMs were regarded as being for reference purposes only and not for routine use. A more modern approach is to treat CRMs as consumable and therefore suitable for IQC.

The use of CRMs in this way is, however, subject to a number of constraints.

(i) Despite the constantly increasing range of CRMs available, for the majority of analyses there is no closely matching CRM available.

(ii) Although the cost of CRMs is not prohibitive in relation to the total costs of analysis, it may not be possible for a laboratory with a wide range of activities to stock every relevant kind of reference material.

(iii) The concept of the reference material is not applicable to materials where either the matrix or the analyte is unstable.

(iv) CRMs are not necessarily available in sufficient amounts to provide for IQC use over extended periods.

(v) It must be remembered that not all apparently certified reference materials are of equal quality. Caution is suggested when the information on the certificate is inadequate.

If for any of the above reasons the use of a CRM is not appropriate it falls on individual laboratories or groups of laboratories to prepare their own control materials and assign traceable[3] values of analyte concentration to them. Such a material is sometimes referred to as a "house reference material" (HRM). Suggestions for preparing HRMs are listed in Section 6.3. Not all of the methods described there are applicable to all analytical situations.

### 6.3 Preparation of control materials

*6.3.1 Assigning a true value by analysis*. In principle a working value can be assigned to a stable reference material simply by careful analysis. However, precautions are necessary to avoid biases in the assigned value. This requires some form of independent check such as may be provided by analysis of the materials in a number of laboratories and where possible, the use of methods based on different physico-chemical principles. Lack of attention to independent validation of control materials has been shown to be a weakness in IQC systems(15).

One way of establishing a traceable assigned value in a control material is to analyse a run comprising the candidate material and a selection of matching CRMs, with replication and randomisation. This course of action would be appropriate if only limited amounts of CRMs were available. The CRMs must be appropriate in both matrix composition and analyte concentration. The CRMs are used directly to calibrate the analytical procedure for the analysis of the control material. An appropriate analytical method is a prerequisite for this approach. It would be a dangerous approach if, say, a minor and variable fraction of the analyte

[3] Where a CRM is not available traceability only to a reference method or to a batch of a reagent supplied by a manufacturer may be necessary.

were extracted for measurement. The uncertainty introduced into the assigned value must also be considered.

*6.3.2 Materials validated in proficiency testing* comprise a valuable source of control materials. Such materials would have been analysed by many laboratories using a variety of methods. In the absence of counter-indications, such as an obvious bias or unusual frequency distribution of results, the consensus of the laboratories could be regarded as a validated assigned value to which a meaningful uncertainty could be attached. (There is a possibility that the consensus could suffer from a bias of consequence, but this potential is always present in reference values.) There would be a theoretical problem of establishing the traceability of such a value, but that does not detract from the validity of the proposed procedure. The range of such materials available would be limited, but organisers of proficiency tests could ensure a copious supply by preparing batches of material in excess of the immediate requirements of the round. The normal requirements of stability would have to be demonstrable.

*6.3.3 Assigning a true value by formulation*. In favourable instances a control material can be prepared simply by mixing constituents of known purity in predetermined amounts. For example, this approach would often be satisfactory in instances where the control material is a solution. Problems are often encountered in formulation in producing solid control materials in a satisfactory physical state or in ensuring that the speciation and physical distribution of the analyte in the matrix is realistic. Moreover an adequate mixing of the constituents must be demonstrable.

*6.3.4 Spiked control materials*. "Spiking" is a way of creating a control material in which a value is assigned by a combination of formulation and analysis. This method is feasible when a test material essentially free of the analyte is available. After exhaustive analytical checks to ensure the background level is adequately low, the material is spiked with a known amount of analyte. The reference sample prepared in this way is thus of the same matrix as the test materials to be analysed and of known analyte level - the uncertainty in the assigned concentration is limited only by the possible error in the unspiked determination. However, it may be difficult to ensure that the speciation, binding and physical form of the added analyte is the same as that of the native analyte and that the mixing is adequate.

*6.3.5 Recovery Checks*. If the use of a reference material is not practicable then a limited check on bias is possible by a test of recovery. This is especially useful when analytes or matrices cannot be stabilised or when *ad*

*hoc* analysis is executed. A test portion of the test material spiked with a known amount of the analyte and analysed alongside the original test material. The recovery of the added analyte (known as the "marginal recovery") is the difference between the two measurements divided by the amount that is added. The obvious advantages of recovery checks are that the matrix is representative and the approach is widely applicable - most test materials can be spiked by some means. However, the recovery check suffers from the disadvantage previously noted regarding the speciation, binding and physical distribution of the analyte. Furthermore, the assumption of an equivalent recovery of the analyte added as a spike and of the native analyte may not be valid. However, it can normally be assumed that a poor performance in a recovery check is strongly indicative of a similar or worse performance for the native analyte in the test materials.

Spiking and recovery testing as an IQC method must be distinguished from the method of standard additions, which is a measurement procedure: a single spiking addition cannot be used to fulfil the roles of both measurement and IQC.

### 6.4 Blank determinations

Blank determinations are nearly always an essential part of the analytical process and can conveniently be effected alongside the IQC protocol. The simplest form of blank is the "reagent blank", where the analytical procedure is executed in all respects apart from the addition of the test portion. This kind of blank, in fact, tests more than the purity of the reagents. For example it is capable of detecting contamination of the analytical system originating from any source, *e.g.*, glassware and the atmosphere, and is therefore better described as a "procedural blank". In some instances, better execution of blank determinations is achieved if a simulated test material is employed. The simulant could be an actual test material known to be virtually analyte-free or a surrogate (e.g., ashless filter paper used instead of plant material). Where it can be contrived, the best type of blank is the "field blank", which is a typical matrix with zero concentration of analyte.

An inconsistent set of blanks in a run suggests sporadic contamination and may add weight to IQC evidence suggesting the rejection of the results. When an analytical protocol prescribes the subtraction of a blank value, the blank value must be subtracted also from the results of the control materials before they are used in IQC.

### 6.5 Traceability in spiking and recovery checks

Potential problems of the traceability of reagents used for spikes and recovery checks must be guarded against. Under conditions where CRMs are not available, traceability can often be established only to the batch of analyte provided by a manufacturer. In such cases, confirmation of identity and a check on purity must be made before use. A further precaution is that the calibration standards and spike should not be traceable to the same stock solution of analyte or the same analyst. If such a common traceability existed, then the corresponding sources of error would not be detected by the IQC.

## 7. RECOMMENDATIONS

The following recommendations represent integrated approaches to IQC that are suitable for many types of analysis and applications areas. Managers of laboratory quality systems will have to adapt the recommendations to the demands of their own particular requirements. Such adaption could be implemented, for example, by adjusting the number of duplicates and control material inserted into a run, or by the inclusion of any additional measures favoured in the particular application area. The procedure finally chosen and its accompanying decision rules must be codified in an IQC protocol that is separate from the analytical system protocol.

The practical approach to quality control is determined by the frequency with which the measurement is carried out and the size and nature of each run. The following recommendations are therefore made. The use of control charts and decision rules are covered in Appendix 1.

In each of the following the order in the run in which the various materials are analysed should be randomised if possible. A failure to randomise may result in an underestimation of various components of error.

(i) *Short (e.g., n<20) frequent runs of similar materials.* Here the concentration range of the
analyte in the run is relatively small, so a common value of standard deviation can be assumed.

Insert a control material at least once per run. Plot either the individual values obtained, or
The mean value, on an appropriate control chart. Analyse in duplicate at least half of the
Test materials, selected at random. Insert at least one blank determination.

(ii) *Longer (e.g., n>20) frequent runs of similar materials*. Again a common level of standard
deviation is assumed.

Insert the control material at an approximate frequency of one per ten test materials. If the run size is likely to vary from run to run it is easier to standardise on a fixed number of insertions per run and plot the mean value on a control chart of means. Otherwise plot individual values.
Analyse in duplicate a minimum of five test materials selected at random. Insert one blank
determination per ten test materials.

(iii) *Frequent runs containing similar materials but with a wide range of analyte concentration.*
Here we cannot assume that a single value of standard deviation is applicable.

Insert control materials in total numbers approximately as recommended above. However, there should be at least two levels of analyte represented, one close to the median level of typical test materials, and the other approximately at the upper or lower decile as appropriate. Enter values for the two control materials on separate control charts. Duplicate a minimum of five test materials, and insert one procedural blank per ten test materials.

(iv) *Ad hoc analysis.* Here the concept of statistical control is not applicable. It is assumed, however, that the materials in the run are of a single type, *i.e.*, sufficiently similar for general conclusions on errors to be made.

Carry out duplicate analysis on all of the test materials. Carry out spiking or recovery tests or use a formulated control material, with an appropriate number of insertions (see above), and with different concentrations of analyte if appropriate. Carry out blank determinations. As no control limits are available, compare the bias and precision with fitness for purpose limits or other established criteria..

## 8. CONCLUSIONS

Internal quality control is an essential aspect of ensuring that data released from a laboratory are fit for purpose. If properly executed, quality control methods can monitor the various aspects of data quality on a run-by-run basis. In runs where performance falls outside acceptable limits, the data produced can be rejected and, after remedial action on the analytical system, the analysis can be repeated.

It must be stressed, however, that internal quality control is not foolproof even when properly executed. Obviously it is subject to "errors of both kinds", *i.e.*, runs that are in control will occasionally be rejected and runs that are out of control occasionally accepted. Of more importance, IQC cannot usually identify sporadic gross errors or short-term disturbances in the analytical system that affect the results for individual test materials. Moreover, inferences based on IQC results are applicable only to test materials that fall within the scope of the analytical method validation. Despite these limitations, which professional experience and diligence can alleviate to a degree, internal quality control is the principal recourse available for ensuring that only data of appropriate quality are released from a laboratory. When properly executed it is very successful.

Finally, it must be appreciated that a perfunctory execution of any quality system will not guarantee the production of data of adequate quality. The correct procedures for feedback, remedial action and staff motivation must also be documented and acted upon. In other words, there must be a genuine commitment to quality within a laboratory for an internal quality control programme to succeed, *i.e.*, the IQC must be part of a total quality management system.

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## APPENDIX 1. SHEWHART CONTROL CHARTS

### 1. INTRODUCTION

The theory, construction and interpretation of the Shewhart chart(1) are detailed in numerous texts on process quality control and applied statistics, and in several ISO standards(2-5). There is a considerable literature on the use of the control chart in clinical chemistry(6,7). Westgard and co-workers have formulated multiple rules for the interpretation of such control charts(8), and the power of these results has been studied in detail(9-10). In this appendix only simple Shewhart charts are considered.

In IQC a Shewhart control chart is obtained when values of concentration measured on a control material in successive runs are plotted on a vertical axis against the run number on the horizontal axis. If more than one analysis of a particular control material is made in a run, either the individual results $x$ or the mean value $\bar{x}$ can be used to form a control chart. The chart is completed by horizontal lines derived from the normal distribution $N(\mu,\sigma^2)$ that is taken to describe the random variations in the plotted values. The selected lines for control purposes are $\mu \pm 2\sigma$ and $\mu \pm 3\sigma$. Different values of $\sigma$ are required for charts of individual values and of means. For a system in statistical control, on average about one in twenty values fall outside the $\mu \pm 2\sigma$ lines, called the "warning limits", and only about three in one thousand fall outside the $\mu \pm 3\sigma$ lines, the "action limits". In practice the estimates $\bar{x}$ and $s$ of the parameters $\mu$ and $\sigma$ are used to construct the chart. A persistent bias is indicated by a significant difference between $\bar{x}$ and the assigned value

### 2. ESTIMATES OF THE PARAMETERS $\mu$ and $\sigma$

An analytical system under control exhibits two sources of random variation, the within-run, characterised by variance $\sigma_0^2$ and the between-run with variance $\sigma_1^2$. The two variances are typically comparable in magnitude. The standard deviation $\sigma_x$ used in a chart of individual values is given by

$$\sigma_x = (\sigma_0^2 + \sigma_1^2)^{1/2}$$

whereas for a control chart of mean values the standard deviation is given by

$$\sigma_{\bar{x}} = (\sigma_0^2/n + \sigma_1^2)^{1/2}$$

where *n* is the number of control measurements in a run from which the mean is calculated. The value of *n* therefore must be constant from run to run, otherwise control limits would be impossible to define. If a fixed number of repeats of a control material per run cannot be guaranteed (*e.g.*, if the run length were variable) then charts of individual values must be used. Furthermore the equation indicates that $\sigma_x$ or $\sigma_{\bar{x}}$ must be estimated with care. An attempt to base an estimate on repeat values from a single run would result in unduly narrow control limits.

Estimates must therefore include the between-run component of variance. If the use of a particular value of *n* can be assumed at the outset, then $\sigma_{\bar{x}}$ can be estimated directly from the *m* means $\bar{x}_i = \sum_{j=1}^{n} x_{ij} / n$

($i$ = 1,.....,$m$) of the *n* repeats in each of *m* successive runs.

Thus the estimate of $\mu$ is

$$\bar{\bar{x}} = \sum_i \bar{x}_i / m$$

and the estimate of $\sigma_{\bar{x}}$ is

$$s_{\bar{x}} = \sqrt{\frac{\sum_i (\bar{x}_i - \bar{\bar{x}})^2}{m-1}}$$

If the value of *n* is not predetermined, then separate estimates of $\sigma_0$and $\sigma_1$ could be obtained by one-way analysis of variance. If the mean squares within- and between- groups are $MS_w$ and $MS_b$ respectively, then

$\sigma_0^2$ is estimated by $MS_w$ and

$\sigma_1^2$ is estimated by $(MS_b - MS_w)/n$

Often in practice it is necessary to initiate a control chart with data collected from a small number of runs, which may be to a degree unrepresentative, as estimates of standard deviation are very variable unless large numbers of observations are used. Moreover, during the initial period, the occurrence of out-of-control conditions are

more than normally likely and will produce outlying values. Such values would bias $\bar{x}$ and inflate *s* beyond its proper value. It is therefore advisable to recalculate $\bar{x}$ and *s* after a further "settling down" period. One method of obviating the effects of outliers in the calculation is to reject them after the application of Dixon's Q or Grubbs'[11] test, and then use the classical statistics given above. Alternatively, the methods of robust statistics could be applied to the data[12, 13].

## 3. THE INTERPRETATION OF CONTROL CHARTS

The following simple rules can be applied to control charts of individual results or of means.

*Single control chart*. An out-of-control condition in the analytical system is signalled if any of the following occur.

(i) The current plotting value falls outside the action limits.
(ii) The current value and the previous plotting value fall outside the warning limits but within the actions limits.
(iii) Nine successive plotting values fall on the same side of the mean line.

*Two control charts.* When two different control materials are used in each run, the respective control charts are considered simultaneously. This increases the chance of a type 1 error (rejection of a sound run) but decreases the chance of a type 2 error (acceptance of a flawed run). An out-of-control condition is indicated if any of the following occur.

(i) At least one of the plotting values falls outside the action limits.
(ii) Both of the plotting values are outside the warning limits.
(iii) The current value and the previous plotting value on the same control chart both fall outside the warning limits.
(iv) Both control charts simultaneously show that four successive plotting values on the same side of the mean line.

(v) One of the charts shows nine successive plotting values falling on the same side of the mean line.

A more thorough treatment of the control chart can be obtained by the application of the full Westgard rules, illustrated in Figure 2.

The analytical chemist should respond to an out-of-control condition by cessation of analysis pending diagnostic tests and remedial action followed by rejection of the results of the run and reanalysis of the test materials.

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# Practical guide for the validation, quality control, and uncertainty assessment of an alternative oenological analysis method

(Resolution 10/2005)

**Contents**

## 1. Purpose

The purpose of this guide is to assist oenological laboratories carrying out serial analysis as part of their validation, internal quality control and uncertainty assessment initiatives concerning the standard methods they use.

## 2. Preamble and scope

International standard ISO 17025, defining the "General Requirements for the Competence of Testing and Calibration Laboratories", states that the accredited laboratories must, when implementing a alternative analytical method, make sure of the quality of the results obtained. To do so, it indicates several steps. The first step consists in defining the customers' requirements concerning the parameter in question, in order to determine, thereafter, whether the method used meets those requirements. The second step includes initial validation for non-standardized, modified or laboratory-developed methods. Once the method is applied, the laboratories must use inspection and traceability methods in order to monitor the quality of the results obtained. Finally, they must assess the uncertainty of the results obtained.

In order to meet these requirements, the laboratories have a significant reference system at their disposal comprising a large number of international guides and standards. However, in practice, the application of these texts is delicate since, because they address every category of calibration and test laboratory, they remain very general and presuppose, on behalf of the reader, in-depth knowledge of the mathematical rules applicable to statistical data processing.

This guide is based on this international reference system, taking into account the specific characteristics of oenology laboratories routinely carrying out analyses on series of must or wine samples. Defining the scope of application in this way enabled a relevant choice of suitable tools to be made, in order to retain only those methods most suitable for that scope. Since it is based on the international reference system, this guide is therefore strictly compliant with it. Readers, however, wishing to study certain points of the guide in greater detail can do so by referring to the international standards and guides, the references for which are given in each chapter.

The authors have chosen to combine the various tools meeting the requirements of the ISO 17025 standard since there is an obvious solution of continuity in their

application, and the data obtained with certain tools can often be used with the others. In addition, the mathematical resources used are often similar.

The various chapters include application examples, taken from oenology laboratories using these tools.

It is important to point out that that this guide does not pretend to be exhaustive. It is only designed to present, in as clear and applicable a way as possible, the contents of the requirements of the ISO 17025 standard and the basic resources that can be implemented in a routine laboratory to meet them. Each laboratory remains perfectly free to supplement these tools or to replace them by others that they consider to be more efficient or more suitable.

Finally, the reader's attention should be drawn to the fact that the tools presented do not constitute an end in themselves and that their use, as well as the interpretation of the results to which they lead, must always be subject to critical analysis. It is only under these conditions that their relevance can be guaranteed, and laboratories will be able to use them as tools to improve the quality of the analyses they carry out.

## 3. General vocabulary

The definitions indicated below used in this document result from the normative references given in the bibliography.

**Analyte**
Object of the analysis method

**Blank**
Test carried out in the absence of a matrix (reagent blank) or on a matrix which does not contain the analyte (matrix blank).

**Bias**
Difference between the expected test results and an accepted reference value.

**Uncertainty budget**
The list of uncertainty sources and their associated standard uncertainties, established in order to assess the compound standard uncertainty associated with a measurement result.

**Gauging (of a measuring instrument)**
Material positioning of each reference mark (or certain principal reference marks only) of a measuring instrument according to the corresponding value of the measurand.

NOTE "gauging" and "calibration" are not be confused

**Repeatability conditions**
Conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.

**Reproducibility conditions (intralaboratory)**
Conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same or different operator(s) using different gauges on different days.

**Experimental standard deviation**
For a series of ***n*** measurements of the same measurand, the quantity ***s*** characterizing the dispersion of the results and given by the formula:

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$$

$x_i$ being the result of the measurement $i^{th}$ and $\bar{x}$ the arithmetic mean of the ***n*** results considered.

**Repeatability standard deviation**
Standard deviation of many repetitions obtained in a single laboratory by the same operator on the same instrument, i.e. under repeatable conditions.

**Internal reproducibility standard deviation (or total intralaboratory variability)**
Standard deviation of repetitions obtained in a single laboratory with the same method, using several operators or instruments and, in particular, by taking measurements on different dates, i.e. under reproducibility conditions.

**Random error**
Result of a measurement minus the mean that would result from an infinite number of measurements of the same measurand carried out under reproducibility conditions.

**Measurement error**
Result of a measurement minus a true value of the measurand.

**Systematic error**
Mean error that would result from an infinite number of measurements of the same measurand carried out under reproducibility conditions minus a true value of the measurand.

NOTE Error is a highly theoretical concept in that it calls upon values that are not accessible in practice, in particular the true values of measurands. On principle, the error is unknown.

**Mathematical expectation**
For a series of ***n*** measurements of the same measurand, if ***n*** tends towards the infinite, the mean $\bar{x}$ tends towards the expectation ***E(x)***.

$$E(x) = n \xrightarrow{\lim} \infty \frac{\sum_{i=1}^{n} x_i}{n}$$

**Calibration**
Series of operations establishing under specified conditions the relation between the values of the quantity indicated by a measuring instrument or system, or the values represented by a materialized measurement or a reference material, and the corresponding values of the quantity measured by standards.

**Intralaboratory evaluation of an analysis method**
Action which consists in submitting an analysis method to an intralaboratory statistical study, based on a standardized and/or recognized protocol, demonstrating that within its scope, the analysis method meets pre-established performance criteria.
Within the framework of this document, the evaluation of a method is based on an intralaboratory study, which includes the comparison with a reference method.

**Precision**
Closeness of agreement between independent test results obtained under prescribed conditions

NOTE 1 Precision depends only on the distribution of random errors and does not have any relationship with the true or specified value.
NOTE 2 The measurement of precision is expressed on the basis of the standard deviation of the test results.
NOTE 3 The expression "independent test results" refers to results obtained such that they are not influenced by a previous result on the same or a similar test

material. Quantitative measurements of precision are critically dependent upon the prescribed conditions. Repeatability and reproducibility conditions are particular sets of extreme conditions.

**Quantity (measurable)**
An attribute of a phenomenon, body or substance that may be distinguished qualitatively and determined quantitatively.

**Uncertainty of measurement**
A parameter associated with the result of a measurement, which characterizes the dispersion of the values that could reasonably be attributed to the measurand.

**Standard uncertainty (*u(xi)*)**
Uncertainty of the result of a measurement expressed in the form of a standard deviation.

**Accuracy**
Closeness of agreement between the mean value obtained starting from a broad series of test results and an accepted reference value.

NOTE The measurement of accuracy is generally expressed in terms of bias.

**Detection limit**
Lowest amount of an analyte to be examined in a test material that can be detected and regarded as different from the blank value (with a given probability), but not necessarily quantified. In fact, two risks must be taken into account:

- the risk $\alpha$ of considering the substance is present in test material when its quantity is null;
- the risk $\beta$ of considering a substance is absent from a substance when its quantity is not null.

**Quantification limit**
Lowest amount of an analyte to be examined in a test material that can be quantitatively determined under the experimental conditions described in the method with a defined variability (given coefficient of variation).

**Linearity**
The ability of a method of analysis, within a certain range, to provide an instrumental response or results proportional to the quality of analyte to be determined in the laboratory sample.
This proportionality is expressed by an a priori defined mathematical expression.

The linearity limits are the experimental limits of concentrations between which a linear calibration model can be applied with a known confidence level (generally taken to be equal to 1%).

**Test material**
Material or substance to which a measuring can be applied with the analysis method under consideration.

**Reference material**
Material or substance one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.

**Certified reference material**
Reference material, accompanied by a certificate, one or more whose property values are certified by a procedure which establishes its traceability to an accurate realization of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence.

**Matrix**
All the constituents of the test material other than the analyte.

**Analysis method**
Written procedure describing all the means and procedures required to carry out the analysis of the analyte, i.e.: scope, principle and/or reactions, definitions, reagents, apparatus, procedures, expression of results, precision, test report.

WARNING The expressions "titration method" and "determination method" are sometimes used as synonyms for the expression "analysis method". These two expressions should not be used in this way.

**Quantitative analysis method**
Analysis method making it possible to measure the analyte quantity present in the laboratory test material.

**Reference analysis method (Type I or Type II methods)**
Method, which gives the accepted reference value for the quantity of the analyte to be measured.

**Non-classified alternative method of analysis**
A routine analysis method used by the laboratory and not considered to be a reference method.

NOTE An alternative method of analysis can consist in a simplified version of the reference method.

**Measurement**
Set of operations having the object of determining a value of a quantity.

NOTE The operations can be carried out automatically.

**Measurand**
Particular quantity subject to measurement.

**Mean**
For a series of ***n*** measurements of the same measurand, mean value, given by the formula:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

$x_i$ being the result of the $i^{th}$ measurement.

**Result of a measurement**
Value assigned to a measurand, obtained by measurement

**Sensitivity**
Ratio between the variation of the information value of the analysis method and the variation of the analyte quantity.
The variation of the analyte quantity is generally obtained by preparing various standard solutions, or by adding the analyte to a matrix.

NOTE 1 Defining, by extension, the sensitivity of a method as its capacity to detect small quantities should be avoided.
NOTE 2 A method is said to be “sensitive" if a low variation of the quantity or analyte quantity incurs a significant variation in the information value.

**Measurement signal**
Quantity representing the measurand and is functionally linked to it.

**Specificity**
Property of an analysis method to respond exclusively to the determination of the quantity of the analyte considered, with the guarantee that the measured signal comes only from the analyte.

**Tolerance**
Deviation from the reference value, as defined by the laboratory for a given level, within which a measured value of a reference material can be accepted.

**Value of a quantity**
Magnitude of a particular quantity generally expressed as a unit of measurement multiplied by a number.

**True value of a quantity**
Value compatible with the definition of a given particular quantity.

NOTE 1 The value that would be obtained if the measurement was perfect
NOTE 2 Any true value is by nature indeterminate

**Accepted reference value**
A value that serves as an agreed-upon reference for comparison and which is derived as:

a) a theoretical or established value, based on scientific principles;
b) an assigned or certified value, based on experimental work of some national or international organization;
c) a consensus or certified value, based on collaborative experimental work under the auspices of a scientific or engineering group;
Within the particular framework of this document, the accepted reference value (or conventionally true value) of the test material is given by the arithmetic mean of the values of measurements repeated as per the reference method.

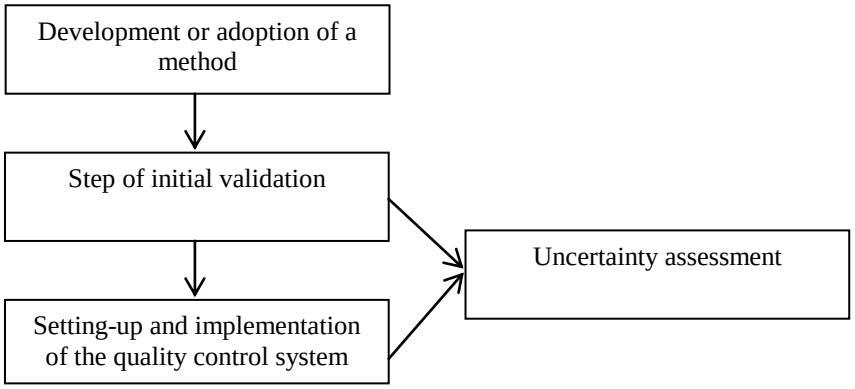
**Variance**
Square of the standard deviation.

## 4. General principles

### 4.1 Methodology

When developing a new alternative method, the laboratory implements a protocol that includes several steps. The first step, applied only once at the initial stage, or on a regular basis, is the validation of the method. This step is followed by permanent quality control. All the data collected during these two steps make it possible to assess the quality of the method. **The data collected during these two**

**steps are used to evaluate the measurement uncertainty**. The latter, which is regularly assessed, is an indicator of the quality of the results obtained by the method under consideration.




All these steps are inter-connected and constitute a global approach that can be used to assess and control measurement errors.

### 4.2 Definition of measurement error

Any measurement carried out using the method under study gives a result which is inevitably associated with a measurement error, defined as being the difference between the result obtained and the true value of the measurand. In practice, **the true value of the measurand is inaccessible** and a value conventionally accepted as such is used instead.

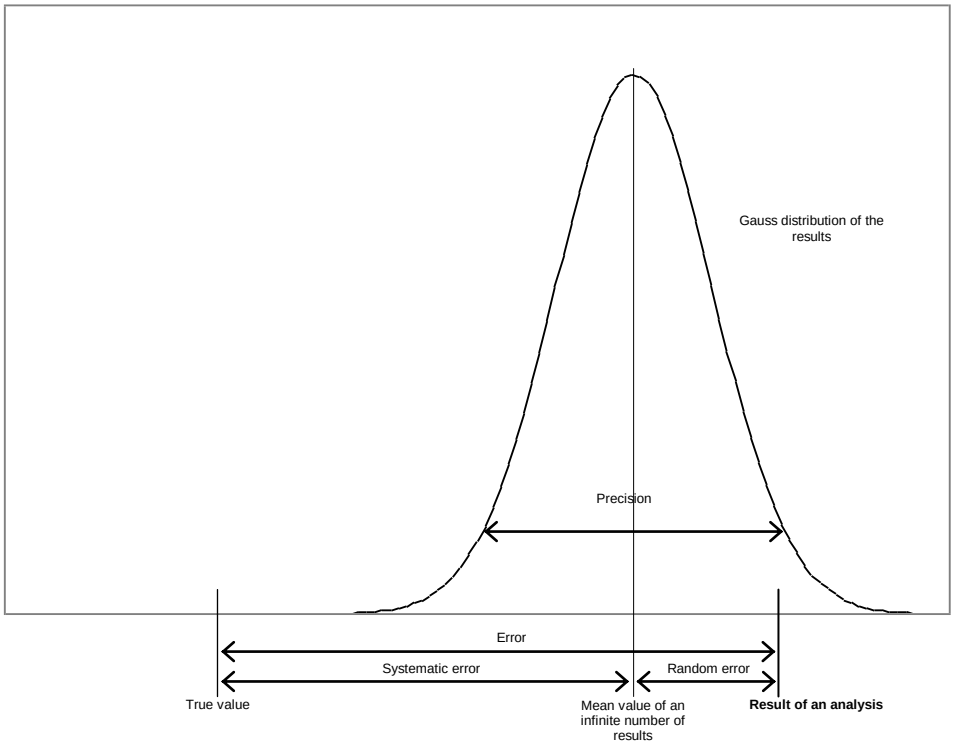
The measurement error includes two components:

Measurement error

**True value = Analysis result + Systematic error + Random error**

In practice, the systematic error results in a bias in relation to the true value, the random error being all the errors associated with the application of the method.

These errors can be graphically represented in the following way:




The validation and quality control tools are used to evaluate the systematic errors and the random errors, and to monitor their changes over time.

## 5. Validating a method

### 5.1 Methodology

Implementing the validation comprises 3 steps, each with objectives. To meet these objectives, the laboratory has validation tools. Sometimes there are many tools for a given objective, and are suitable for various situations. It is up to the laboratory to correctly choose the most suitable tools for the method to be validated.

| Steps | Objectives | Tools for validation |
|---|---|---|
| **Scope of application** | | |
| | - To define the analyzable matrices | |
| | - To define the analyzable range | Detection and quantification limit<br>Robustness study |
| **Systematic error** | | |
| or bias | - Linear response in the scale of analyzable values | Linearity study |
| | - Specificity of the method | Specificity study |
| | - Accuracy of the method | Comparison with a reference method<br>Comparison with reference materials<br>Interlaboratory comparison |
| **Random error** | | |
| | - Precision of the method | Repeatability study<br>Intralaboratory reproducibility study |

## 5.2 Section one: Scope of method

### *5.2.1 Definition of analyzable matrices*

The matrix comprises all constituents in the test material other than the analyte.
If these constituents are liable to influence the result of a measurement, the laboratory should define the matrices on which the method is applicable.

For example, in oenology, the determination of certain parameters can be influenced by the various possible matrices (wines, musts, sweet wines, etc.).

In case of doubt about a matrix effect, more in-depth studies can be carried out as part of the specificity study.

### *5.2.2 Detection and quantification limit*

This step is of course not applicable and not necessary for those methods whose lower limit does not tend towards 0, such as alcoholic strength by volume in wines, total acidity in wines, pH, etc.

#### *5.2.2.1 Normative definition*

The detection limit is the lowest amount of analyte that can be detected but not necessarily quantified as an exact value. The detection limit is a parameter of limit tests.

The quantification limit is the lowest quantity of the compound that can be determined using the method.

#### *5.2.2.2 Reference documents*

- NF V03-110 Standard, intralaboratory validation procedure for an alternative method in relation to a reference method.
- International compendium of analysis methods – OIV, Assessment of the detection and quantification limit of an analysis method (Oeno resolution 7/2000).

#### *5.2.2.3 Application*

In practice, the quantification limit is generally more relevant than the detection limit, the latter being by convention 1/3 of the first.

There are several approaches for assessing the detection and quantification limits:

- Determination on blank
- Approach by the linearity study
- Graphic approach

These methods are suitable for various situations, but in every case they are mathematical approaches giving results of informative value only. It seems crucial, whenever possible, to introduce a check of the value obtained, whether by one of these approaches or estimated empirically, using the checking protocol for a predetermined quantification limit.

#### *5.2.2.4 Procedure*

5.2.2.4.1 Determination on blank

*5.2.2.4.1.1 Scope*

This method can be applied when the blank analysis gives results with a non-zero standard deviation. The operator will judge the advisability of using reagent blanks, or matrix blanks.
If the blank, for reasons related to uncontrolled signal preprocessing, is sometimes not measurable or does not offer a recordable variation (standard deviation of 0), the operation can be carried out on a very low concentration in analyte, close to the blank.

*5.2.2.4.1.2 Basic protocol and calculations*

Carry out the analysis of ***n*** test materials assimilated to blanks, ***n*** being equal to or higher than 10.

- Calculate the mean of the $x_i$ results obtained:

$$\bar{x}_{blank} = \frac{\sum_{i=1}^{n} x_i}{n}$$

- Calculate the standard deviation of the $x_i$ results obtained:

$$S_{blank} = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x}_{blank})^2}{n-1}}$$

- From these results the detection limit is conventionally defined by the formula:

$$L_{d=} \bar{x}_{blank} + (3.S_{blank})$$

- From these results the quantification limit is conventionally defined by the formula:

$$L_{q=} \bar{x}_{blank} + (10.S_{blank})$$

***Example:*** *The table below gives some of the results obtained when assessing the detection limit for the usual determination of free sulfur dioxide.*

| *Test material #* | *X ( mg/l)* |
|---|---|
| *1* | *0* |
| *2* | *1* |
| *3* | *0* |
| *4* | *1.5* |
| *5* | *0* |
| *6* | *1* |
| *7* | *0.5* |
| *8* | *0* |
| *9* | *0* |
| *10* | *0.5* |
| *11* | *0* |
| *12* | *0* |

*The calculated values are as follows:*

$$q = 12$$

$$M_{blank} = 0.375$$

$$S_{blank} = 0.528 \text{ mg/l}$$

$$DL = 1.96 \text{ mg/l}$$

$$QL = 5.65 \text{ mg/l}$$

5.2.2.4.2 Approach by linearity study

*5.2.2.4.2.1 Scope*

This method can be applied in all cases, and is required when the analysis method does not involve background noise. It uses the data calculated during the linearity study.

NOTE This statistical approach may be biased and give pessimistic results when linearity is calculated on a very wide range of values for reference materials, and whose measurement results include variable standard deviations. In such cases, a linearity study limited to a range of low values, close to 0 and with a more homogeneous distribution will result in a more relevant assessment.

*5.2.2.4.2.2 Basic protocol and calculations*

Use the results obtained during the linearity study which made it possible to calculate the parameters of the calibration function $y = a + b.x$

The data to be recovered from the linearity study are (see chapter 5.3.1. linearity study):

- slope of the regression line:

$$b = \frac{\sum_{i=1}^{n}(x_i - M_x)(y_i - M_y)}{\sum_{i=1}^{n}(x_i - M_x)^2}$$

- residual standard deviation:

$$S_{res} = \sqrt{\frac{\sum_{i=1}^{n}\sum_{j=1}^{p}(y_{i,j} - \hat{y}_{i,j})^2}{pn - 2}}$$

- standard deviation at the intercept point (to be calculated):

$$S_a = S_{res}\sqrt{\left(\frac{1}{np} + \frac{M_x^{\,2}}{\sum_{i=1}^{n}p(x_i - M_x)^2}\right)}$$

The estimates of the detection limit **DL** and the quantification limit **QL** are calculated using following formulae:

$DL = \frac{3 \times S_a}{b}$ Estimated detection limit

$QL = \frac{10 \times S_a}{b}$ Estimated quantification limit

**Example**: Estimatation of the detection and quantification limits in the determination of sorbic acid by capillary electrophoresis, based on linearity data acquired on a range from 1 to 20 $mg.L_{-1}$.

| X (ref) | Y1 | Y2 | Y3 | Y4 |
|---|---|---|---|---|
| *1* | *1.9* | *0.8* | *0.5* | *1.5* |
| *2* | *2.4* | *2* | *2.5* | *2.1* |
| *3* | *4* | *2.8* | *3.5* | *4* |
| *4* | *5.3* | *4.5* | *4.7* | *4.5* |
| *5* | *5.3* | *5.3* | *5.2* | *5.3* |
| *10* | *11.6* | *10.88* | *12.1* | *10.5* |
| *15* | *16* | *15.2* | *15.5* | *16.1* |
| *20* | *19.7* | *20.4* | *19.5* | *20.1* |

Number of reference materials
$n = 8$
Number of replicas
$p = 4$
Straight line (y = a + b*x)

$b = 0.9972$

$a = 0.51102$

residual standard deviation:
$S_{res} = 0.588$
Standard deviation on the intercept point
$\boldsymbol{S_a} = 0.1597$

The estimated detection limit is $DL = 0.48\ mg.L^{-1}$
The estimated quantification limit is $QL = 1.6\ mg.L^{-1}$

5.2.2.4.3 Graphic approach based on the background noise of the recording

*5.2.2.4.3.1 Scope*

This approach can be applied to analysis methods that provide a graphic recording (chromatography, etc.) with a background noise. The limits are estimated from a study of the background noise.

*5.2.2.4.3.2 Basic protocol and calculation*

Record a certain number of reagent blanks, using 3 series of 3 injections separated by several days.

Determine the following values:

- $h_{max}$ the greatest variation in amplitude on the y-axis of the signal observed between two acquisition points, excluding drift, at a distance equal to twenty times the width at mid-height of the peak corresponding to the analyte, centered over the retention time of the compound under study.
- R, the quantity/signal response factor, expressed in height.

The detection limit **DL**, and the quantification limit **QL** are calculated according to the following formulae:

$$DL = 3\ h_{max}\ R \qquad QL = 10\ h_{max}\ R$$

5.2.2.4.4 Checking a predetermined quantification limit

This approach can be used to validate a quantification value obtained by statistical or empirical approach.

*5.2.2.4.4.1 Scope*

This method can be used to check that a given quantification limit is *a priori* acceptable. It is applicable when the laboratory can procure at least 10 test materials with known quantities of analyte, at the level of the estimated quantification limit.
In the case of methods with a specific signal, not sensitive to matrix effects, the materials can be synthetic solutions whose reference value is obtained by formulation.

In all other cases, wines (or musts) shall be used whose measurand value as obtained by the reference method is equal to the limit to be studied. Of course, in this case the quantification limit of the reference method must be lower than this value.

*5.2.2.4.4.2 Basic protocol and calculation*

Analyze ***n*** independent test materials whose accepted value is equal to the quantification limit to be checked; ***n*** must at least be equal to 10.

- Calculate the mean of ***n*** measurements:

$$\bar{x}_{LQ} = \frac{\sum_{i=1}^{n} x_i}{n}$$

- Calculate the standard deviation of ***n*** measurements:

$$S_{LQ} = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x}_{LQ})^2}{n-1}}$$

*with $x_i$ results of the measurement of the **i**th test material.*

The two following conditions must be met:

a) the measured mean quantity $\bar{x}_{LQ}$ must not be different from the predetermined quantification limit *QL*:

If $\frac{|QL - \bar{x}_{QL}|}{\frac{S_{QL}}{\sqrt{n}}} < 10$ then quantification limit *QL* is considered to be valid.

NOTE 10 is a purely conventional value relating to the QL criterion.

b) the quantification limit must be other than 0:

If $5\,s_{QL} < QL$ then the quantification limit is other than 0.

A value of 5 corresponds to an approximate value for the spread of the standard deviation, taking into account risk α and risk β to ensure that the QL is other than 0.

This is equivalent to checking that the coefficient of variation for *QL* is lower than 20%.

NOTE1 Remember that the detection limit is obtained by dividing the quantification limit by 3.
NOTE2 A check should be made to ensure that the value of $S_{LQ}$ is not too large (which would produce an artificially positive test), and effectively corresponds to a reasonable standard deviation of the variability of the results for the level under consideration. It is up to the laboratory to make this critical evaluation of the value of $S_{LQ}$.

**Example:** Checking the quantification limit of the determination of malic acid by the enzymatic method.

Estimated quantification limit: 0.1 $g.L^{-1}$

| Wine | Values |
|---|---|
| *1* | *0.1* |
| *2* | *0.1* |
| *3* | *0.09* |
| *4* | *0.1* |
| *5* | *0.09* |
| *6* | *0.08* |
| *7* | *0.08* |
| *8* | *0.09* |
| *9* | *0.09* |
| *10* | *0.08* |

**Mean: *0.090***
**Standard deviation: *0.008***

First condition: $\frac{\left|LQ-\bar{x}_{QL}\right|}{\frac{S_{QL}}{\sqrt{n}}}=3.87<10$ The quantification limit of 0.1 is considered to be valid.

Second condition: $5.S_{LQ}=0.04<0.1$ The quantification limit is considered to be significantly different from 0.

### *5.2.3 Robustness*

#### *5.2.3.1 Definition*

Robustness is the capacity of a method to give close results in the presence of slight changes in the experimental conditions likely to occur during the use of the procedure.

#### *5.2.3.2 Determination*

If there is any doubt about the influence of the variation of operational parameters, the laboratory can use the scientific application of experiment schedules, enabling these critical operating parameters to be tested within the variation range likely to occur under practical conditions. In practice, these tests are difficult to implement.

## 5.3 Section two: systematic error study

### *5.3.1 Linearity study*

#### *5.3.1.1 Normative definition*

The linearity of a method is its ability (within a given range) to provide an informative value or results proportional to the amount of analyte to be determined in the test material.

#### *5.3.1.2 Reference documents*

- NF V03-110 standard. Intralaboratory validation procedure of an alternative method in relation to a reference method.
- ISO 11095 Standard, linear calibration using reference materials.
- ISO 8466-1 Standard, Water quality – Calibration and evaluation of analytical methods and estimation of performance characteristics

#### *5.3.1.3 Application*

The linearity study can be used to define and validate a linear dynamic range.

This study is possible when the laboratory has stable reference materials whose accepted values have been acquired with certainty (in theory these values should have an uncertainty equal to 0). These could therefore be internal reference materials titrated with calibrated material, wines or musts whose value is given by

the mean of at least 3 repetitions of the reference method, external reference materials or certified external reference materials.
In the last case, and only in this case, this study also enables the traceability of the method. The experiment schedule used here could then be considered as a calibration.

In all cases, it is advisable to ensure that the matrix of the reference material is compatible with the method.

Lastly, calculations must be made with the final result of the measurement and not with the value of the signal.

Two approaches are proposed here:

- An ISO 11095 type of approach, the principle of which consists in comparing the residual error with the experimental error using a Fischer's test. This approach is valid above all for relatively narrow ranges (in which the measurand does not vary by more than a factor 10). In addition, under experimental conditions generating a low reproducibility error, the test becomes excessively severe. On the other hand, in the case of poor experimental conditions, the test will easily be positive and will also lose its relevance. This approach requires good homogeneity of the number of measurements over the entire range studied.

- An ISO 8466 type of approach, the principle of which consists in comparing the residual error caused by the linear regression with the residual error produced by a polynomial regression (of order 2 for example) applied to the same data. If the polynomial model gives a significantly lower residual error, a conclusion of nonlinearity could be drawn. This approach is appropriate in particular when there is a risk of high experimental dispersion at one end of the range. It is therefore naturally well-suited to analysis methods for traces. There is no need to work with a homogeneous number of measurements over the whole range, and it is even recommended to increase the number of measurements at the borders of the range.

#### *5.3.1.4 ISO 11095-type approach*

5.3.1.4.1 Basic protocol

It is advisable to use a number ***n*** of reference materials. The number must be higher than 3, but there is no need, however, to exceed 10. The reference materials

should be measured ***p*** times, under **reproducibility conditions**, ***p*** shall be higher than 3, a number of 5 being generally recommended. The accepted values for the reference materials are to be regularly distributed over the studied range of values. The number of measurements must be identical for all the reference materials.

NOTE It is essential that the reproducibility conditions use a maximum of potential sources of variability, with the risk that the test shows non-linearity in an excessive way.

The results are reported in a table presented as follows:

| **Reference materials** | **Accepted reference value material** | **Measured values** | | | | |
|---|---|---|---|---|---|---|
| | | **Replica 1** | **...** | **Replica j** | **...** | **Replica p** |
| *1* | $x_1$ | $y_{11}$ | ... | $y_{1j}$ | ... | $y_{1p}$ |
| ... | ... | ... | ... | ... | ... | ... |
| *i* | $x_i$ | $y_{i1}$ | ... | $y_{ij}$ | ... | $y_{ip}$ |
| ... | ... | ... | ... | ... | ... | ... |
| *n* | $x_n$ | $y_{n1}$ | ... | $y_{nj}$ | ... | $y_{np}$ |

5.3.1.4.2 Calculations and results

*5.3.1.4.2.1 Defining the regression model*

The model to be calculated and tested is as follows:

$$y_{ij} = a + b.x_i + \varepsilon_{ij}$$

where

$y_{ij}$ is the $j^{th}$ replica of the $i^{th}$ reference material.

$x_i$ is the accepted value of the $i^{th}$ reference material.

$b$ is the slope of the regression line.

$a$ is the intercept point of the regression line.

$a+b.x_i$ represents the expectation of the measurement value of the $i^{th}$ reference material.

$\varepsilon_{ij}$ is the difference between $y_{ij}$ and the expectation of the measurement value of the $i^{th}$ reference material.

*5.3.1.4.2.2 Estimating parameters*

The parameters of the regression line are obtained using the following formulae:

- mean of ***p*** measurements of the ***i^th^*** reference material

$$yi = \frac{1}{p}\sum_{j=1}^{p} y_{ij}$$

- mean of all the accepted values of *n* reference materials $M_x = \frac{1}{n}\sum_{i=1}^{n} x_i$

- mean of all the measurements $My = \frac{1}{n}\sum_{i=1}^{n} y_i$

- estimated slope ***b*** $b = \frac{\sum_{i=1}^{n}(x_i - M_x)(y_i - M_y)}{\sum_{i=1}^{n}(x_i - M_x)^2}$

- estimated intercept point ***a*** $a = M_y - b \times M_x$

- regression value associated with the ***i^th^*** reference material $\hat{y}_i$

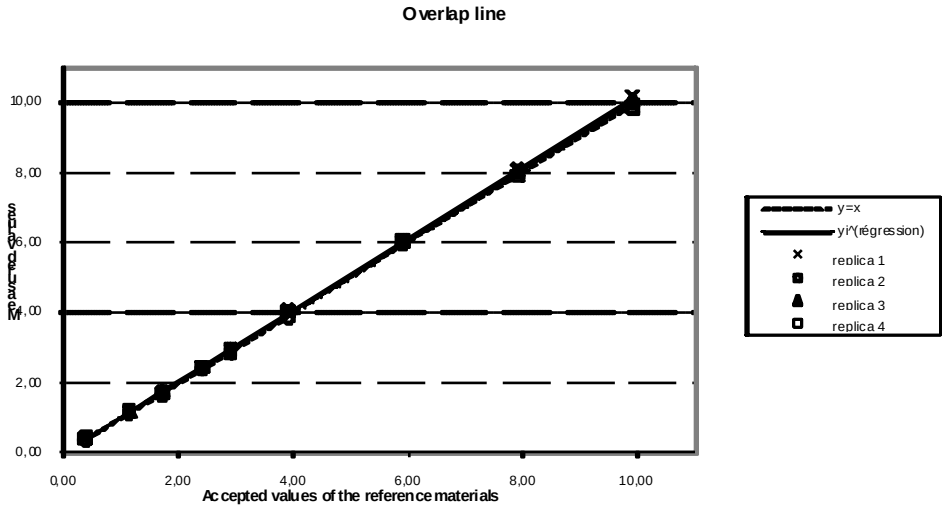
$$\hat{y}_i = a + b \times x_i$$

- residual $e_{ij}$ $e_{ij} = y_{ij} - \hat{y}_i$

*5.3.1.4.2.3 Charts*

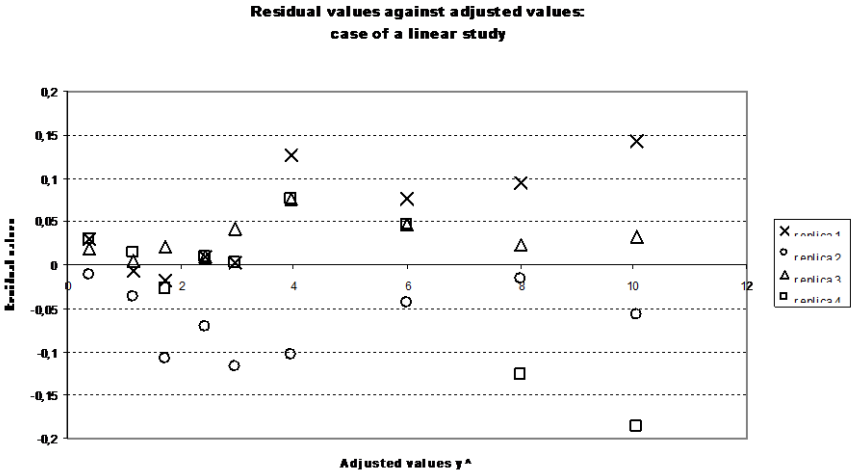
The results can be presented and analyzed in graphic form. Two types of charts are used.

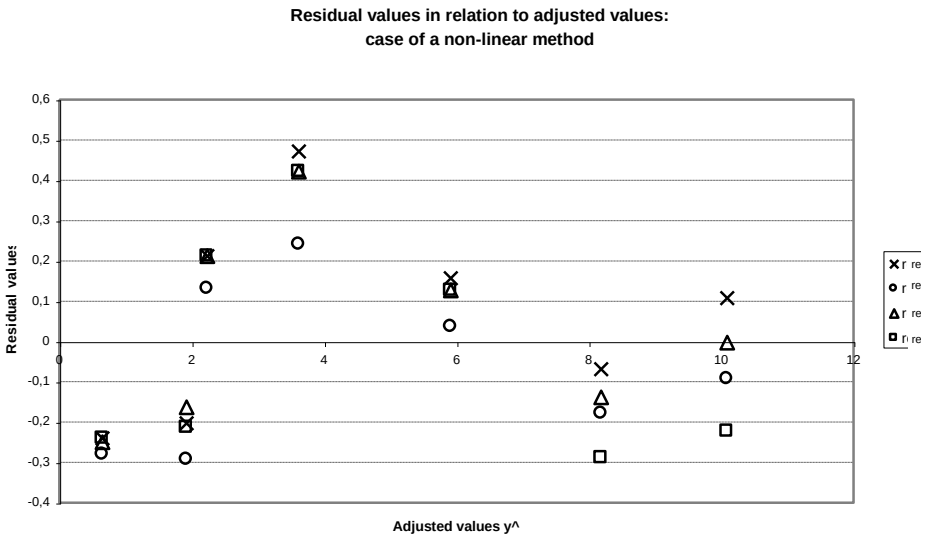
- The first type of graph is the representation of the values measured against the accepted values of reference materials. The calculated overlap line is also plotted.




- The second graph is the representation of the residual values against the estimated values of the reference materials ( $\hat{y}$ ) indicated by the overlap line.

The graph is a good indicator of the deviation in relation to the linearity assumption: the linear dynamic range is valid if the residual values are fairly distributed between the positive and negative values.






In case of doubt about the linearity of the regression, a Fischer-Snedecor test can be carried out in order to test the assumption: "the linear dynamic range is not valid", in addition to the graphic analysis.

*5.3.1.4.2.4 Test of the linearity assumption*

Several error values linked to calibration should be defined first of all: these can be estimated using the data collected during the experiment. A statistical test is then performed on the basis of these results, making it possible to test the assumption of non-validity of the linear dynamic range: this is the Fischer-Snedecor test.

5.3.1.4.2.4.1 Definitions of errors linked to calibration

These errors are given as a standard deviation, resulting from the square root of the ratio between a sum of squares and a degree of freedom.

**Residual error**

The residual error corresponds to the error between the measured values and the value given by the regression line.

The sum of the squares of the residual error is as follows:

$$Q_{res} = \sum_{i=1}^{n}\sum_{j=1}^{p}(y_{ij} - \hat{y}_i)^2$$

The number of degrees of freedom is *np-2*.

The residual standard deviation is then estimated by the formula:

$$S_{res} = \sqrt{\frac{\sum_{i=1}^{n}\sum_{j=1}^{p}(y_{ij} - \hat{y}_i)^2}{np-2}}$$

**Experimental error**

The experimental error corresponds to the reproducibility standard deviation of the experimentation.

The sum of the squares of the experimental error is as follows:

$$Q_{\exp} = \sum_{i=1}^{n}\sum_{j=1}^{p}(y_{ij} - y_i)^2$$

The number of degrees of freedom is *np-n*.

The experimental standard deviation (reproducibility) is then estimated by the formula:

$$S_{\exp} = \sqrt{\frac{\sum_{i=1}^{n}\sum_{j=1}^{p}(y_{ij} - y_i)^2}{np-n}}$$

NOTE This quantity is sometimes also noted $S_R$.

**Adjustment error**

The value of the adjustment error is the experimental error minus the residual error.

The sum of the squares of the adjustment error is:

$$Q_{def} = Q_{res} - Q_{\exp}$$

or

$$Q_{def} = \sum_{i=1}^{n}\sum_{j=1}^{p}(y_{ij} - \hat{y}_i)^2 - \sum_{i=1}^{n}\sum_{j=1}^{p}(y_{ij} - y_i)^2$$

The number of degrees of freedom is n-2

The standard deviation of the adjustment error is estimated by the formula:

$$S_{def} = \sqrt{\frac{Q_{res} - Q_{\exp}}{n-2}}$$

or

$$S_{def} = \sqrt{\frac{\sum_{i=1}^{n}\sum_{j=1}^{p}(y_{ij} - \hat{y}_i)^2 - \sum_{i=1}^{n}\sum_{j=1}^{p}(y_{ij} - y_i)^2}{n-2}}$$

5.3.1.4.2.4.2 Fischer-Snedecor test

The ratio $F_{obs} = \frac{S_{def}^{\ 2}}{S_{\exp}^{\ 2}}$ obeys the Fischer-Snedecor law with the degrees of freedom *n-2, np-n*.

The calculated experimental value $F_{obs}$ is compared with the limit value: $F_{1-\alpha}$ *(n-2,np-n)*, extracted from the Snedecor law table. The value for α used in practice is generally 5%.

If $F_{obs} \geq F_{1-\alpha}$ the assumption of the non-validity of the linear dynamic range is accepted *(with a risk of α error of 5%).*

If $F_{obs} < F_{1-\alpha}$ the assumption of the non-validity of the linear dynamic range is rejected

**Example:** Linearity study for the determination of tartaric acid by capillary electrophoresis. 9 reference materials are used. These are synthetic solutions of tartaric acid, titrated by means of a scale traceable to standard masses.

| Ref. material | Ti (ref) | Y1 | Y2 | Y3 | Y4 |
|---|---|---|---|---|---|
| *1* | *0.38* | *0.41* | *0.37* | *0.4* | *0.41* |
| *2* | *1.15* | *1.15* | *1.12* | *1.16* | *1.17* |
| *3* | *1.72* | *1.72* | *1.63* | *1.76* | *1.71* |
| *4* | *2.41* | *2.45* | *2.37* | *2.45* | *2.45* |
| *5* | *2.91* | *2.95* | *2.83* | *2.99* | *2.95* |
| *6* | *3.91* | *4.09* | *3.86* | *4.04* | *4.04* |
| *7* | *5.91* | *6.07* | *5.95* | *6.04* | *6.04* |
| *8* | *7.91* | *8.12* | *8.01* | *8.05* | *7.9* |
| *9* | *9.91* | *10.2* | *10* | *10.09* | *9.87* |

**Regression line**
Line ( y = a + b*x)
*b = 1.01565*
*a = - 0.00798*

**Errors related to calibration**
Residual standard deviation $S_{res}$ = *0.07161*
Standard deviation of experimental reproducibility $S_{exp}$ = *0.07536*
Standard deviation of the adjustment error $S_{def}$ = *0.0548*

**Interpretation, Fischer-Snedecor test**
$F_{obs} = 0.53 < F_{1-\alpha} = 2.37$
***The assumption of the non-validity of the linear dynamic range is rejected***

### *5.3.1.5 ISO 8466-type approach*

5.3.1.5.1 Basic protocol

It is advisable to use a number ***n*** of reference materials. The number must be higher than 3, but there is no need, however, to exceed 10. The reference materials should be measured several times, under **reproducibility conditions**. The number of measurements may be small at the center of the range studied (minimum = 2) and must be greater at both ends of the range, for which a minimum number of 4 is generally recommended. The accepted values of reference materials must be regularly distributed over the studied range of values.

NOTE It is vital that the reproducibility conditions use the maximum number of potential sources of variability.

The results are reported in a table presented as follows:

| Reference materials | Accepted value of the reference material | Measured values | | | | |
|---|---|---|---|---|---|---|
| | | Replica 1 | Replica 2 | Replica j | ... | Replica p |
| *1* | $x_1$ | $y_{11}$ | $y_{12}$ | $y_{1j}$ | ... | $y_{1p}$ |
| ... | ... | ... | ... | ... | ... | |
| *i* | $x_i$ | $y_{i1}$ | $y_{i2}$ | | | |
| ... | ... | ... | ... | ... | ... | |
| *N* | $x_n$ | $y_{n1}$ | ... | $y_{nj}$ | ... | $y_{np}$ |

5.3.1.5.2 Calculations and results

*5.3.1.5.2.1 Defining the linear regression model*

Calculate the linear regression model using the calculations detailed above.

The residual error of the standard deviation for the linear model $S_{res}$ can then be calculated using the formula indicated in § 5.3.1.4.2.4.1

*5.3.1.5.2.2 Defining the polynomial regression model*

The calculation of the polynomial model of order 2 is given below

The aim is to determine the parameters of the polynomial regression model of order 2 applicable to the data of the experiment schedule.

$$y = a\chi^2 + b\chi + c$$

The purpose is to determine the parameters a, b and c. This determination can generally be computerized using spreadsheets and statistics software.

The estimation formulae for these parameters are as follows:

$$a = \frac{\sum_i x_i^2 y_i \left( N\sum_i x_i^2 - \left[\sum_i xi\right]^2 \right) - \sum_i x_i^3 \left( N\sum_i x_i y_i - \sum_i x_i \sum_i y_i \right) + \sum_i x_i^2 \left( \sum_i x_i y_i \sum_i x_i - \sum_i y_i \sum_i x_i^2 \right)}{\sum_i x_i^4 \left( N\sum_i x_i^2 - \left[\sum_i xi\right]^2 \right) - \sum_i x_i^3 \left( N\sum_i x_i^3 - \sum_i x_i^2 \sum_i x_i \right) + \sum_i x_i^2 \left( \sum_i x_i \sum_i x_i^3 - \left[\sum_i x_i^2\right]^2 \right)}$$

$$b = \frac{\sum_i x_i^4 \left( N\sum_i x_i y_i - \sum_i x_i \sum_i y_i \right) - \sum_i x_i^2 y_i \left( N\sum_i x_i^3 - \sum_i x_i^2 \sum_i x_i \right) + \sum_i x_i^2 \left( \sum_i y_i \sum_i x_i^3 - \sum_i x_i y_i \sum_i x_i^2 \right)}{\sum_i x_i^4 \left( N\sum_i x_i^2 - \left[\sum_i xi\right]^2 \right) - \sum_i x_i^3 \left( N\sum_i x_i^3 - \sum_i x_i^2 \sum_i x_i \right) + \sum_i x_i^2 \left( \sum_i x_i \sum_i x_i^3 - \left[\sum_i x_i^2\right]^2 \right)}$$

$$c = \frac{\sum_i x_i^4\left(\sum_i x_i^2\sum_i y_i - \sum_i x_i\sum_i x_i y_i\right) - \sum_i x_i^3\left(\sum_i x_i^3\sum_i y_i - \sum_i x_i^2\sum_i x_i y_i\right) + \sum_i x_i^2 y_i\left(\sum_i x_i\sum_i x_i^3 - \left[\sum_i x_i^2\right]^2\right)}{\sum_i x_i^4\left(N\sum_i x_i^2 - \left[\sum_i xi\right]^2\right) - \sum_i x_i^3\left(N\sum_i x_i^3 - \sum_i x_i^2\sum_i x_i\right) + \sum_i x_i^2\left(\sum_i x_i\sum_i x_i^3 - \left[\sum_i x_i^2\right]^2\right)}$$

Once the model has been established, the following values are to be calculated:

- regression value associated with the ***i*th** reference material $\hat{y}'_i$

$$\hat{y}'_i = a\chi^2 + b\chi + c$$

- residual $e_{ij}$ $\quad e'_{ij} = y_{ij} - \hat{y}'_i$

Residual standard deviation of the polynomial model

$$S'_{res} = \sqrt{\frac{\sum_{i=1}^{n}\sum_{j=1}^{p}(y_{ij} - \hat{y}'_i)^2}{np-2}}$$

*5.3.1.5.2.3* *Comparing residual standard deviations*

Calculation of

$$DS^2 = (N-2)S_{res}^2 - (N-3)S'^2_{res}$$

Then

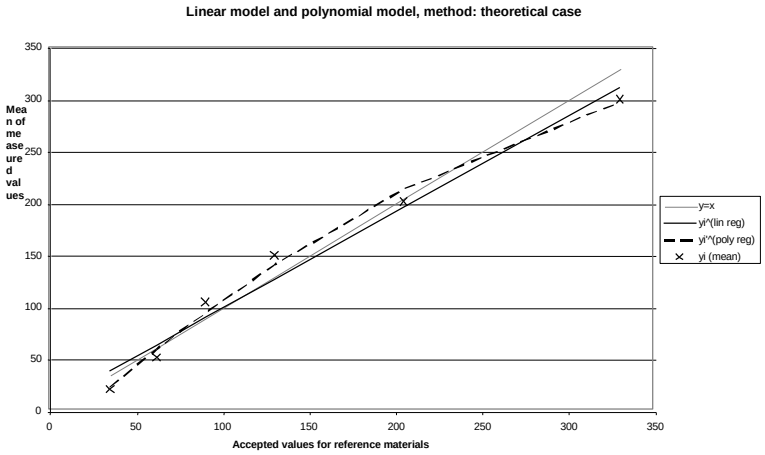
$$PG = \frac{DS^2}{S'^2_{res}}$$

The value PG is compared with the limit value $F_{1-\alpha}$ given by the Fischer-Snedecor table for a confidence level 1-α and a degree of freedom 1 and (N-3).
NOTE In general the α risk used is 5%. In some cases the test may be optimistic and a risk of 10% will prove more realistic.

If PG ≤ $F_{1-\alpha}$: the nonlinear calibration function does not result in an improved adjustment; for example, the calibration function is linear.
If PG > $F_{1-\alpha}$: the work scope must be as narrow as possible to obtain a linear calibration function: otherwise, the information values from the analyzed samples must be evaluated using a nonlinear calibration function.

**Example:** Theoretical case.

| | Ti (ref) | Y1 | Y2 | Y3 | Y4 |
|---|---|---|---|---|---|
| *1* | *35* | *22.6* | *19.6* | *21.6* | *18.4* |
| *2* | *62* | *49.6* | *49.8* | *53* | |
| *3* | *90* | *105.2* | *103.5* | | |
| *4* | *130* | *149* | *149.8* | | |
| *5* | *205* | *203.1* | *202.5* | *197.3* | |
| *6* | *330* | *297.5* | *298.6* | *307.1* | *294.2* |




**Linear regression**

$y = 1.48.x – 0.0015$

$S_{res} = 13.625$

**Polynomial regression**

$y = - 0.0015x^2 + 1.485x – 27.2701$

S'res = 7.407

**Fischer's test**

PG = 10.534 > F(5%) = 10.128

PG>F the linear calibration function cannot be retained

### *5.3.2 Specificity*

#### *5.3.2.1 Normative definition*

The specificity of a method is its ability to measure only the compound being searched for.

#### *5.3.2.2 Application*

In case of doubt about the specificity of the tested method, the laboratory can use experiment schedules designed to check its specificity. Two types of complementary experiments are proposed here that can be used in a large number of cases encountered in the field of oenology.

- The first test is the standard addition test. It can be used to check that the method measures all the analyte.
- The second test can be used to check the influence of other compounds on the result of the measurement.

#### *5.3.2.3 Procedures*

5.3.2.3.1 Standard addition test

*5.3.2.3.1.1 Scope*

This test can be used to check that the method measures all the analyte.
The experiment schedule is based on standard additions of the compound being searched for. It can only be applied to methods that are not sensitive to matrix effects.

*5.3.2.3.1.2 Basic protocol*

This consists in finding a significant degree of added quantities on test materials analyzed before and after the additions.

Carry out variable standard additions on ***n*** test materials. The initial concentration in analyte of test materials, and the standard additions are selected in order to cover the scope of the method. These test materials must consist of the types of matrices called for routine analysis. It is advised to use at least 10 test materials.

The results are reported in a table presented as follows:

| **Test material** | **Quantity before addition** ($x$) | **Quantity added** ($v$) | **Quantity after addition** ($w$) | **Quantity found** ($r$) |
|---|---|---|---|---|
| 1 | $x_1$ | $v_1$ | $w_1$ | $r_1 = w_1 - x_1$ |
| ... | ... | ... | ... | ... |
| $i$ | $x_i$ | $v_i$ | $w_i$ | $r_i = w_i - x_i$ |
| ... | ... | ... | ... | ... |
| $n$ | $X_n$ | $V_n$ | $w_n$ | $r_p = w_n - x_n$ |

NOTE 1 An addition is made with a pure standard solution. It is advised to perform an addition of the same order as the quantity of the test material on which it is carried out. This is why the most concentrated test materials must be diluted to remain within the scope of the method.

NOTE 2 It is advised to prepare the additions using independent standard solutions, in order to avoid any systematic error.

NOTE 3 The quality of values $x$ and $w$ can be improved by using several repetitions.

*5.3.2.3.1.3 Calculations and results*

The principle of the measurement of specificity consists in studying the regression line $\boldsymbol{r = a + b.v}$ and checking that slope $\boldsymbol{b}$ is equivalent to 1 and that intercept point $\boldsymbol{a}$ is equivalent to 0.

5.3.2.3.1.3.1 Study of the regression line $\boldsymbol{r = a + b.v}$

The parameters of the regression line are obtained using the following formulae:

- mean of the added quantities $\bar{v}$ $$\bar{v} = \frac{\sum_{i=1}^{n} v_i}{n}$$

- mean of the quantities found $\bar{r}$ $$\bar{r} = \frac{\sum_{i=1}^{n} r_i}{n}$$

- estimated slope ***b***

$$b = \frac{\sum_{i=1}^{n}(v_i - \bar{v})(r_i - \bar{r})}{\sum_{i=1}^{n}(v_i - \bar{v})^2}$$

- estimated intercept point ***a***

$$a = \bar{r} - b.\bar{v}$$

- regression value associated with the ***i*^th^** reference material $\hat{y}_i$

$$\hat{r}_i = a + b \times v_i$$

- residual standard deviation

$$S_{res} = \sqrt{\frac{\sum_{i=1}^{n}(r_i - \hat{r}_i)^2}{n-2}}$$

- standard deviation on the slope

$$S_b = S_{res}\sqrt{\left(\frac{1}{\sum_{i=1}^{n}(v_i - \bar{v})^2}\right)}$$

- standard deviation on the intercept point

$$S_a = S_{res}\sqrt{\left(\frac{1}{n} + \frac{\bar{v}^2}{\sum_{i=1}^{n}(v_i - \bar{v})^2}\right)}$$

5.3.2.3.1.3.2 Analysis of the results

The purpose is to conclude on the absence of any interference and on an acceptable specificity. This is true if the overlap line $r = a + bv$ is equivalent to the line $y = x$.

To do so, two tests are carried out:

- Test of the assumption that slope b of the overlap line is equal to 1.
- Test of the assumption that intercept point a is equal to 0.

These assumptions are tested using a Student test, generally associated with a risk of error of 1%. A risk of 5% can prove more realistic in some cases.

Let $T_{critical,\ bilateral}$[dof; 1%] be a Student bilateral variable associated with a risk of error of 1% for a number of degrees of freedom (dof).

Step 1: calculations

Calculation of the comparison criterion on the slope at 1

$$T_{obs}=\frac{|b-1|}{S_b}$$

Calculation of the comparison criterion on the intercept point at 0

$$T'_{obs}=\frac{|a|}{S_a}$$

Calculation of the Student critical value: $T_{critical,\ bilateral}$[ p-2; 1%]

Step 2: interpretation

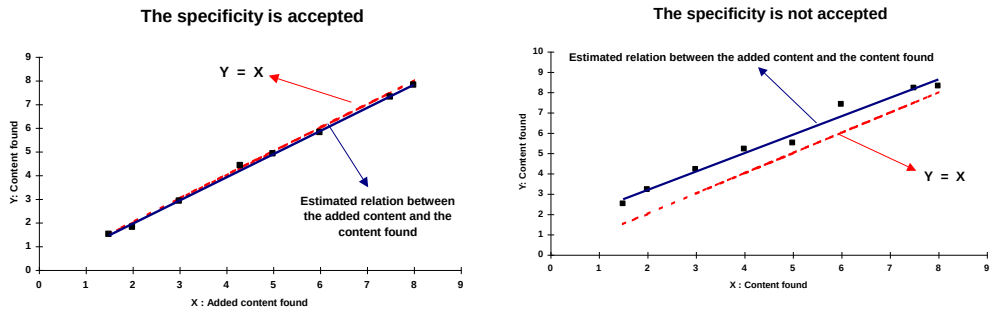
- If $T_{obs}$ is lower than $T_{critical}$, then the slope of the regression line is equivalent to 1
- If $T'_{obs}$ is lower than $T_{critical}$, then the intercept point of the regression line is equivalent to 0.
- If both conditions are true, then the overlap line is equivalent = $y = x$, and the method is deemed to be specific.

NOTE 1 Based on these results, a mean overlap rate can be calculated to quantify the specificity. In no case should it be used to "correct" the results. This is because if a significant bias is detected, the alternative method cannot be validated in relation to an efficiency rate of 100%.

NOTE 2 Since the principle of the test consists in calculating a straight line, at least three levels of addition have to be taken, and their value must be correctly chosen in order to obtain an optimum distribution of the points.

5.3.2.3.1.3.3 Overlap line graphics

**Example of specificity**




5.3.2.3.2 Study of the influence of other compounds on the measurement result

*5.3.2.3.2.1 Scope*

If the laboratory suspects the interaction of compounds other than the analyte, an experiment schedule can be set up to test the influence of various compounds. The experiment schedule proposed here enables a search for the influence of compounds defined *a priori*: thanks to its knowledge of the analytical process and its know-how, the laboratory should be able to define a certain number of compounds liable to be present in the wine and to influence the analytical result.

*5.3.2.3.2.2 Basic protocol and calculations*

Analyze $\boldsymbol{n}$ wines in duplicate, before and after the addition of the compound suspected of having an influence on the analytical result; $\boldsymbol{n}$ must at least be equal to 10.

The mean values $\boldsymbol{Mxi}$ of the 2 measurements $\boldsymbol{x_i}$ and $\boldsymbol{x'_i}$ made before the addition shall be calculated first, then the mean values $\boldsymbol{My_i}$ of the 2 measurements $\boldsymbol{y_i}$ and $\boldsymbol{y'_i}$ made after the addition, and finally the difference $\boldsymbol{d_i}$ between the values $\boldsymbol{Mx_i}$ and $\boldsymbol{My_i}$.
The results of the experiment can be reported as indicated in the following table:

| | ***x***: Before addition | | ***y***: After addition | | Means | | Difference |
|---|---|---|---|---|---|---|---|
| **Samples** | **Rep1** | **Rep2** | **Rep1** | **Rep2** | ***x*** | ***y*** | ***d*** |
| *1* | $x_1$ | $x'_1$ | $y_1$ | $y'_1$ | $Mx_1$ | $My_1$ | $d_1 = Mx_1 - My_1$ |
| ... | ... | ... | ... | ... | ... | ... | ... |
| *i* | $x_i$ | $x'_i$ | $y_i$ | $y'_i$ | $Mx_i$ | $My_i$ | $d_i = Mxi - My_i$ |
| ... | ... | ... | ... | ... | ... | ... | ... |
| *n* | $x_n$ | $x'_n$ | $y_n$ | $y'_n$ | $Mx_n$ | $My_n$ | $d_n = Mx_n - My_n$ |

The mean of the results before addition $\boldsymbol{M_x}$

$$M_x = \frac{1}{n}\sum_{i=1}^{n} Mx_i$$

The mean of the results after addition $\boldsymbol{M_y}$

$$M_y = \frac{1}{n}\sum_{i=1}^{n} My_i$$

Calculate the mean of the differences $M_d$

$$M_d = \sum_{i=1}^{n} \frac{d_i}{n} = My - Mx$$

Calculate the standard deviation of the differences $\boldsymbol{S_d}$

$$S_d = \sqrt{\frac{\sum_{i=1}^{n}(d_i - M_d)^2}{n-1}}$$

Calculate the Z-score

$$Z_{score} = \frac{|M_d|}{S_d}$$

*5.3.2.3.2.3* *Interpretation*

- If the $Z_{score}$ is ≤ 2, the added compound can be considered to have a negligible influence on the result of analysis with a risk of 5%.
- If the $Z_{score}$ is ≥ 2, the added compound can be considered to influence the result of analysis with a risk of 5%.

NOTE Interpreting the $Z_{score}$ is possible given the assumption that the variations obey a normal law with a 95% confidence rate.

**Example:** Study of the interaction of compounds liable to be present in the samples, on the determination of fructose glucose in wines by Fourier transform infrared spectrophotometry (FTIR).

| | Before addition | | + 250 mg.L$^{-1}$ potassium sorbate | | + 1 g. L$^{-1}$ salicylic acid | | Differences | |
|---|---|---|---|---|---|---|---|---|
| vin | rep1 | rep2 | rep1 | rep2 | rep1 | rep2 | sorbate diff | salicylic diff |
| *1* | *6.2* | *6.2* | *6.5* | *6.3* | *5.3* | *5.5* | *0.2* | *-0.8* |
| *2* | *1.2* | *1.2* | *1.3* | *1.2* | *0.5* | *0.6* | *0.05* | *-0.65* |
| *3* | *0.5* | *0.6* | *0.5* | *0.5* | *0.2* | *0.3* | *-0.05* | *-0.3* |
| *4* | *4.3* | *4.2* | *4.1* | *4.3* | *3.8* | *3.9* | *-0.05* | *-0.4* |
| *5* | *12.5* | *12.6* | *12.5* | *12.7* | *11.5* | *11.4* | *0.05* | *-1.1* |
| *6* | *5.3* | *5.3* | *5.4* | *5.3* | *4.2* | *4.3* | *0.05* | *-1.05* |
| *7* | *2.5* | *2.5* | *2.6* | *2.5* | *1.5* | *1.4* | *0.05* | *-1.05* |
| *8* | *1.2* | *1.3* | *1.2* | *1.1* | *0.5* | *0.4* | *-0.1* | *-0.8* |
| *9* | *0.8* | *0.8* | *0.9* | *0.8* | *0.2* | *0.3* | *0.05* | *-0.55* |
| *10* | *0.6* | *0.6* | *0.5* | *0.6* | *0.1* | *0* | *-0.05* | *-0.55* |

Potassium sorbate
*Md* = *0.02*
*Sd* = *0.086*
$Z_{score}$ = *0.23 <2*

Salicylic acid
*Md* = *-0.725*
*Sd* = *0.282*
$Z_{score}$ = *2.57 >2*

In conclusion, it can be stated that potassium sorbate does not influence the determination of fructose glucose by the FTIR gauging studied here. On the other hand, salicylic acid has an influence, and care should be taken to avoid

samples containing salicylic acid, in order to remain within the scope of validity for the gauging under study.

### *5.3.3 Study of method accuracy*

#### *5.3.3.1 Presentation of the step*

5.3.3.1.1 Definition

Correlation between the mean value obtained with a large series of test results and an accepted reference value.

5.3.3.1.2 General principles

When the reference value is output by a certified system, the accuracy study can be regarded a traceability link. This applies to two specific cases in particular:

- Traceability to certified reference materials: in this case, the accuracy study can be undertaken jointly with the linearity and calibration study, using the experiment schedule described for that study.
- Traceability to a certified interlaboratory comparison analysis chain.

The other cases, i.e. which use references that are not based on certified systems, are the most widespread in routine oenological laboratories. These involve comparisons:

- Comparison with a reference method
- Comparison with the results of an uncertified interlaboratory comparison analysis chain.
- Comparison with internal reference materials, or with external uncertified reference materials.

5.3.3.1.3 Reference documents

- NF V03-110 Standard. intralaboratory validation procedure for an alternative method in relation to a reference method.
- NF V03-115 Standard, Guide for the use of reference materials.
- ISO 11095 Standard, linear calibration using reference materials.
- ISO 8466-1 Standard. Water quality – Calibration and evaluation of analytical methods and estimation of performance characteristics

- ISO 57025 Standard, Exactitude of results and methods of measurement

### *5.3.3.2 Comparison of the alternative method with the OIV reference method*

5.3.3.2.1 Scope

This method can be applied if the laboratory uses the OIV reference method, or a traced, validated method, whose performance quality is known and meets the requirements of the laboratory's customers.

To study the comparative accuracy of the two methods, it is advisable first of all to ensure the quality of the repeatability of the method to be validated, and to compare it with the reference method. The method for carrying out the repeatability comparison is described in the chapter on repeatability.

5.3.3.2.2 Accuracy of the alternative method compared with the reference method

*5.3.3.2.2.1 Definition*

Accuracy is defined as the closeness of agreement between the values obtained by the reference method and that obtained by the alternative method, independent of the errors of precision of the two methods.

*5.3.3.2.2.2 Scope*

The accuracy of the alternative method in relation to the reference method is established for a field of application in which the repeatabilities of the two methods are constant.

In practice, it is therefore often advisable to divide the analyzable range of values into several sections or "range levels" (2 to 5), in which we may reasonably consider that the repeatabilities of the methods are comparable to a constant.

*5.3.3.2.2.3 Basic protocol and calculations*

In each range level, accuracy is based on a series of ***n*** test materials with concentration values in analyte covering the range level in question. A minimum number of 10 test materials is required to obtain significant results.

Each test material is to be analyzed in duplicate by the two methods under repeatable conditions.

A calculation is to be made of the mean values $\boldsymbol{Mx_i}$ of the 2 measurements $\boldsymbol{x_i}$ et $\boldsymbol{x'_i}$ made using the alternative method and the mean values $\boldsymbol{My_i}$ of the 2 measurements $\boldsymbol{y_i}$ et $\boldsymbol{y'_i}$ made using the reference method, then the difference $\boldsymbol{d_i}$ is to be calculated between the values $\boldsymbol{Mx_i}$ and $\boldsymbol{My_i}$.

The results of the experiment can be reported as in the following table:

| **Test material** | ***x***: Alternative method | | ***y:*** Reference method | | Means | | Difference |
|---|---|---|---|---|---|---|---|
| | **Rep1** | **Rep2** | **Rep1** | **Rep2** | ***x*** | ***y*** | ***d*** |
| *1* | $x_1$ | $x'_1$ | $y_1$ | $y'_1$ | $Mx_1$ | $My_1$ | $d_1 = Mx_1 - My_1$ |
| ... | ... | ... | ... | ... | ... | ... | ... |
| *i* | $x_i$ | $x'_i$ | $y_i$ | $y'_i$ | $Mx_i$ | $My_i$ | $d_i = Mxi - My_i$ |
| ... | ... | ... | ... | ... | ... | ... | ... |
| *n* | $x_n$ | $x'_n$ | $y_n$ | $y'_n$ | $Mx_n$ | $My_n$ | $d_n = Mx_n - My_n$ |

The following calculations are to be made

- The mean of the results for the alternative method $\boldsymbol{M_x}$

$$M_x = \frac{1}{n}\sum_{i=1}^{n} Mx_i$$

- The mean of the results for the reference method $\boldsymbol{M_y}$

$$M_y = \frac{1}{n}\sum_{i=1}^{n} My_i$$

- Calculate the mean of the differences $\boldsymbol{M_d}$

$$M_d = \sum_{i=1}^{n} \frac{d_i}{n} = Mx - My$$

- Calculate the standard deviation of the differences $S_d$

$$S_d = \sqrt{\frac{\sum_{i=1}^{n}(d_i - M_d)^2}{n-1}}$$

- Calculate the $Z_{score}$

$$Z_{score} = \frac{|M_d|}{S_d}$$

*5.3.3.2.2.4 Interpretation*

- If the $Z_{score}$ is **lower** than or equal to 2.0, it can be concluded that the accuracy of one method in relation to the other is satisfactory, in the range level under consideration, with a risk of error $\alpha = 5\%$.

- If the $Z_{score}$ is **higher** than 2.0, it can be concluded that the alternative method is not accurate in relation to the reference method, in the range level under consideration, with a risk of error $\alpha = 5\%$.

NOTE Interpreting the $Z_{score}$ is possible given the assumption that the variations obey a normal law with a 95% confidence rate.

**Example:** Study of the accuracy of FTIR gauging to determine glucose and fructose in relation to the enzymatic method. The first range level covers the scale from 0 to 5 $g.L^{-1}$ and the second range level covers a scale from 5 to 20 $g.L^{-1}$.

| Wine | FTIR 1 | IRTF2 | Enz 1 | Enz 2 | di |
|---|---|---|---|---|---|
| *1* | *0* | *0.3* | *0.3* | *0.2* | *-0.1* |
| *2* | *0.2* | *0.3* | *0.1* | *0.1* | *0.2* |
| *3* | *0.6* | *0.9* | *0.0* | *0.0* | *0.7* |
| *4* | *0.7* | *1* | *0.8* | *0.7* | *0.1* |
| *5* | *1.2* | *1.6* | *1.1* | *1.3* | *0.2* |
| *6* | *1.3* | *1.4* | *1.3* | *1.3* | *0.0* |
| *7* | *2.1* | *2* | *1.9* | *2.1* | *0.0* |
| *8* | *2.4* | *0* | *1.1* | *1.2* | *0.1* |
| *9* | *2.8* | *2.5* | *2.0* | *2.6* | *0.3* |
| *10* | *3.5* | *4.2* | *3.7* | *3.8* | *0.1* |
| *11* | *4.4* | *4.1* | *4.1* | *4.4* | *0.0* |
| *12* | *4.8* | *5.4* | *5.5* | *5.0* | *-0.2* |

*Md* *0.13*
*Sd* *0.23*
$Z_{score}$ *0.55 < 2*

| Wine | FTIR 1 | IRTF2 | Enz 1 | Enz 2 | di |
|---|---|---|---|---|---|
| *1* | *5.1* | *5.4* | *5.1* | *5.1* | *0.1* |
| *2* | *5.3* | *5.7* | *5.3* | *6.0* | *-0.2* |
| *3* | *7.7* | *7.6* | *7.2* | *7.0* | *0.6* |
| *4* | *8.6* | *8.6* | *8.3* | *8.5* | *0.2* |
| *5* | *9.8* | *9.9* | *9.1* | *9.3* | *0.6* |
| *6* | *9.9* | *9.8* | *9.8* | *10.2* | *-0.1* |
| *7* | *11.5* | *11.9* | *13.3* | *13.0* | *-1.4* |
| *8* | *11.9* | *12.1* | *11.2* | *11.4* | *0.7* |
| *9* | *12.4* | *12.5* | *11.4* | *12.1* | *0.7* |
| *10* | *16* | *15.8* | *15.1* | *15.7* | *0.5* |
| *11* | *17.7* | *18.1* | *17.9* | *18.3* | *-0.2* |
| *12* | *20.5* | *20.1* | *20.0* | *19.1* | *0.7* |

*Md* = *0.19*
*Sd* = *0.63*
$Z_{score}$ = *0.30 < 2*

For the two range levels, the $Z_{score}$ is lower than 2. The FTIR gauging for the determination of fructose glucose studied here, can be considered accurate in relation to the enzymatic method.

### *5.3.3.3 Comparison by interlaboratory tests*

5.3.3.3.1 Scope

Interlaboratory tests are of two types:

1. **Collaborative studies** relate to a single method. These studies are carried out for the initial validation of a new method, mainly in order to define the standard deviation of interlaboratory reproducibility $\boldsymbol{SR_{inter}(method)}$. The mean ***m*** could also be given.

2. Interlaboratory comparison analysis chains, or **aptitude tests**. These tests are carried out for the validation of a method adopted by the laboratory, and the routine quality control (see § 5.3.3.3). The resulting value is the interlaboratory mean ***m***, as well as the standard interlaboratory reproducibility and intermethod deviation ***SRinter***.

By participating in an analysis chain, or in a collaborative study, the laboratory can exploit the results in order to study the accuracy of a method, in order to ensure its validation first of all, and its routine quality control.

If the interlaboratory tests are carried out within the framework of a certified organization, this comparison work can be used for method traceability.

5.3.3.3.2 Basic protocol and calculations

To obtain a sufficient comparison, it is recommended to use a minimum number of 5 test materials over the period.

For each test material, two results are provided:

- The mean of all the laboratories with significant results ***m***

- The standard deviation for interlaboratory reproducibility $S_{R\text{-}inter}$

The test materials are analyzed with ***p*** replicas by the laboratory, these replicas being carried out under repeatable conditions. ***p*** must at least be equal to 2.

In addition, the laboratory must be able to check that the intralaboratory variability (intralaboratory reproducibility) is lower than the interlaboratory variability (interlaboratory reproducibility) given by the analysis chain.

For each test material, the laboratory calculates the $Z_{score}$, given by the following formula:

$$Z_{score} = \frac{|m_{lab} - m|}{S_{R-inter}}$$

The results can be reported as indicated in the following table:

| Test material | Rep1 | ... | Rep j | ... | Rep p | Lab mean | Chain mean | Standard deviation | $Z_{score}$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | $x_{11}$ | ... | $x_{1j}$ | ... | $x_{1p}$ | $m_{lab1}=\frac{\sum_{j=1}^{p} x_{1j}}{p}$ | $m_1$ | $S_{R\text{-}inter(1)}$ | $Z_{score1}=\frac{\lvert m_{lab1}-m_1\rvert}{S_{R-inter(1)}}$ |
| ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| $i$ | $x_{i1}$ | ... | $x_{ij}$ | ... | $x_{ip}$ | $m_{labi}=\frac{\sum_{j=1}^{p} x_{ij}}{p}$ | $m_i$ | $S_{R\text{-}inter(i)}$ | $Z_{scorei}=\frac{\lvert m_{labi}-m_i\rvert}{S_{R-inter(i)}}$ |
| ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| n | $x_{n1}$ | ... | $x_{nj}$ | ... | $x_{np}$ | $m_{labn}=\frac{\sum_{j=1}^{p} x_{nj}}{p}$ | $m_n$ | $S_{R\text{-}inte(n)}$ | $Z_{scoren}=\frac{\lvert m_{labn}-m_n\rvert}{S_{R-inter(n)}}$ |

5.3.3.3.3 Interpretation

If all the $\boldsymbol{Z_{score}}$ results are lower than 2, the results of the method being studied can considered identical to those obtained by the laboratories having produced significant results.

NOTE Interpreting the $Z_{score}$ is possible given the assumption that the variations obey a normal law with a 95% confidence rate.

**Example:** An interlaboratory analysis chain outputs the following results for the free sulfur dioxide parameter, on two samples.

| Samples | $x_1$ | $x_2$ | $x_3$ | $x_4$ | Lab mean | Chain mean | Standard deviation | $Z_{score}$ |
|---|---|---|---|---|---|---|---|---|
| *1* | *34* | *34* | *33* | *34* | *33.75* | *32* | *6* | *0.29* <2 |
| *2* | *26* | *27* | *26* | *26* | *26.25* | *24* | *4* | *0.56* <2 |

It can be concluded that on these two samples, the comparison with the analysis chain is satisfactory.

### *5.3.3.4 Comparison with reference materials*

5.3.3.4.1 Scope

In situations where there is no reference method (or any other method) for a given parameter, and the parameter is not processed by the analysis chains, the only remaining possibility is comparison of the results of the method to be validated with accepted internal or external material reference values.

The reference materials, for example, could be synthetic solutions established with class-A glassware, and/or calibrated metrology apparatus.

In the case of certified reference materials, the comparison constitutes the traceability value, and can be carried out at the same time as the gauging and linearity study.

5.3.3.4.2 Basic protocol and calculations

It is advisable to have ***n*** reference materials for a given range level, in which it can be reasonably estimated that repeatability is comparable to a constant; ***n*** must at least be equal to 10.

Analyze in duplicate each reference material.

Calculate the mean values $Mx_i$ for the 2 measurements $x_i$ and $x'_i$ carried out using the alternative method.

Define $\mathbf{T_i}$ the accepted value for the $i^{th}$ reference material.

The results can be reported as indicated in the following table:

| | ***x***: Alternative method | | | ***T***: Accepted value of the reference material | Difference ***d*** |
|---|---|---|---|---|---|
| **Reference material** | **Rep1** | **Rep2** | **Mean x** | | |
| *1* | $x_1$ | $x'_1$ | $Mx_1$ | $T_1$ | $d_1 = Mx_1-T_1$ |
| ... | | | ... | ... | ... |
| *i* | $x_i$ | $x'_i$ | $Mx_i$ | $T_i$ | $d_i = Mxi-T_i$ |
| ... | | | ... | ... | ... |
| *n* | $x_n$ | $x'_n$ | $Mx_n$ | $T_n$ | $d_n = Mx_n-T_n$ |

The mean of the results of the alternative method $\boldsymbol{M_x}$

$$M_x = \frac{1}{n}\sum_{i=1}^{n} Mx_i$$

The mean of the accepted values of reference materials $\boldsymbol{M_T}$

$$M_T = \frac{1}{n}\sum_{i=1}^{n} T_i$$

Calculate the mean of the differences $M_d$

$$M_d = \sum_{i=1}^{n} \frac{d_i}{n} = Mx - M_T$$

Calculate the standard deviation of the differences $\boldsymbol{S_d}$

$$S_d = \sqrt{\frac{\sum_{i=1}^{n}(d_i - M_d)^2}{n-1}}$$

Calculate the Z-score

$$Z_{score} = \frac{|M_d|}{S_d}$$

5.3.3.4.3 Interpretation

- If the $Z_{score}$ is **lower than** or equal to 2.0, it can be concluded that the accuracy of the alternative method in relation to the accepted values for the reference material is good on the range level under consideration.

- If $Z_{score}$ is **higher** than 2.0, it can be concluded that the alternative method is not accurate in relation to the accepted values for the reference materials in the range level under consideration.

NOTE Interpreting the $Z_{score}$ is possible given the assumption that the variations obey a normal law with a 95% confidence rate.

**Example:** There is no reference method to compare the results of the analysis of Ethyl-4 Phenol (4-EP) by Gas chromatography coupled with mass spectrometry (GC-MS). The results are compared with the accepted values for reference materials, consisting of synthetic solutions formulated by traced equipment.

| Test apparatus | Ti (ref) | Y1 | Y2 | Y3 | Y4 | My | $d_i$ |
|---|---|---|---|---|---|---|---|
| *1* | *4.62* | *6.2* | *6.56* | *4.9* | *5.7* | *5.8* | *1.2* |
| *2* | *12.3* | *15.1* | *10.94* | *12.3* | *11.6* | *12.5* | *0.2* |
| *3* | *24.6* | *24.5* | *18* | *25.7* | *27.8* | *24.0* | *-0.6* |
| *4* | *46.2* | *48.2* | *52.95* | *46.8* | *35* | *45.7* | *-0.5* |
| *5* | *77* | *80.72* | *81.36* | *83.2* | *74.5* | *79.9* | *2.9* |
| *6* | *92.4* | *97.6* | *89* | *94.5* | *99.5* | *95.2* | *2.8* |
| *7* | *123.2* | *126.6* | *129.9* | *119.6* | *126.9* | *125.8* | *2.6* |
| *8* | *246.4* | *254.1* | *250.9* | *243.9* | *240.4* | *247.3* | *0.9* |
| *9* | *385* | *375.8* | *366.9* | *380.4* | *386.9* | *377.5* | *-7.5* |
| *10* | *462* | *467.5* | *454.5* | *433.3* | *457.3* | *453.2* | *-8.9* |

*Md = -0.7*
*Sd = . 4.16*
$Z_{score}$ *= 0.16*

Given these results, the values obtained by the analysis method for 4-EP by GC-MS can be considered accurate compared with the accepted values of reference materials.

## 5.4 Section three: random error study

### *5.4.1 General principle*

Random error is approximated using precision studies. Precision is calculated used a methodology that can be applied under various experimental conditions, ranging between those of repeatability, and those of reproducibility, which constitute the extreme conditions of its measurement.

The precision study is one of the essential items in the study of the uncertainty of measurement.

### *5.4.2 Reference documents*

- ISO 5725 Standard, Exactitude of results and methods of measurement
- NF V03-110 Standard, Intralaboratory validation procedure for an alternative method in relation to a reference method.

### *5.4.3 Precision of the method*

#### *5.4.3.1 Definition*

Closeness of agreement between independent test results obtained under prescribed conditions.

NOTE 1 Precision depends only on the distribution of the random errors and has no relation with the true or specified value.
NOTE 2 Expressing the measurement of precision is based on the standard deviation of the test results.
NOTE 3 The term "independent test results" refers to results obtained such that they are not influenced by a previous result on the same or similar test material. Quantitative measurements of precision are critically dependent on the prescribed conditions. Repeatability and reproducibility conditions are particular sets of extreme conditions.

In practice, precision refers to all the experimental conditions ranging between the conditions of repeatability and those of reproducibility.

#### *5.4.3.2 Scope*

The protocols and calculations are detailed below, from the general theoretical case to the specific cases of repeatability and reproducibility. This exhaustive approach should make it possible to apply the precision study in most laboratory situations.

The precision study can be applied a priori without difficulty to every quantitative method.

In many cases, precision is not constant throughout the validity range for the method. In this case, it is advisable to define several sections or "range levels", in which we may reasonably consider that the precision is comparable to a constant. The calculation of precision is to be reiterated for each range level.

### *5.4.3.3 General theoretical case*

5.4.3.3.1 Basic protocol and calculations

#### *5.4.3.3.1.1 Calculations with several test materials*

***n*** test materials are analyzed over a relatively long period of time with several replicas, $p_i$ being the number of replicas for the $i^{th}$ test material. The properties of the test materials must maintain constant throughout the period in question.
For each replica, the measurement can be made with ***K*** repetitions, (we do not take into account the case here where the number of repetitions ***K*** can vary from one test material to the other, which would complicate the calculations even more).
The total number of replicas must be higher than 10, distributed over all the test materials.

The results can be reported as indicated in the following table, (case in which ***K*** = 2)

| **Replicas** / *Test materials.* | **1** | | **...** | | ***j*** | | $p_1$ | | $p_i$ | | $p_n$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | $x_{11}$ | $x'_{11}$ | ... | ... | $x_{1j}$ | $x'_{1j}$ | $x_{1p1}$ | $x'_{1p1}$ | | | | |
| ... | | | | | | | | | | | | |
| *i* | $x_{i1}$ | $x'_{i1}$ | ... | ... | $x_{ij}$ | $x'_{ij}$ | ... | ... | $x_{ipi}$ | $x'_{ipi}$ | | |
| ... | | | | | | | | | | | | |
| *n* | $x_{n1}$ | $x'_{n1}$ | ... | ... | $x_{nj}$ | $x'_{nj}$ | ... | ... | ... | ... | $x_{npn}$ | $x'_{npn}$ |

In this situation, the standard deviation of total variability (or standard deviation of precision $S_v$) is given by the general expression:

$$S_v = \sqrt{Var(\bar{x}_{ij}) + (1 - \frac{1}{k}) Var(répet)}$$

where:

$Var(\bar{x}_{ij})$ variance of the mean of repeated replicas of all test materials.

$Var(répet)$ variance of the repeatability of all the repetitions.

- If the test materials were analyzed in duplicate with each replica (***K*** = 2), the expression becomes:

$$S_v = \sqrt{Var(\bar{x}_{ij}) + \frac{Var(repeat)}{2}}$$

- When only one measurement of the test material has been carried out with each replica (***K*** = 1), the variance of repeatability is null, the expression becomes:

$$S_v = \sqrt{Var(\bar{x}_{ij})}$$

**- Calculation of** $Var(\bar{x}_{ij})$

The mean of the two replicas $x_{ij}$ and $x'_{ij}$ is:

$$\bar{x}_{ij} = \frac{x_{ij} + x'_{ij}}{2}$$

For each test material, the mean of ***n*** replicas is calculated:

$$Mx_i = \frac{\sum_{j=1}^{p_i} \bar{x}_{ij}}{p_i}$$

The number of different measurements ***n*** is the sum of $p_i$

$$N = \sum_{i=1}^{n} p_i$$

The variance $Var(\bar{x}_{ij})$ is then given by the following equation

$$Var(x_{ij}) = \frac{\sum_{i=1}^{n}\sum_{j=1}^{p_i}(\bar{x}_{ij} - M_{x_i})^2}{N-n}$$

NOTE This variance can also be calculated using the variances of variability of each test material: $\boldsymbol{Var_i\ (x_j)}$. The following relation is then used (it is strictly equivalent to the previous one):

$$Var(x_{ij}) = \frac{\sum_{i=1}^{n}(p_i - 1).Var_i(x_j)}{N-n}$$

**- Calculation of** $Var(repeat)$

The variance of repeatability is calculated as a conventional repeatability equation with ***n*** test materials in duplicate. According to the calculation of repeatability discussed in the section entitled "repeatability", for ***K = 2*** the variance of repeatability is:

$$Var(repeat) = \frac{\sum_{i=1}^{p}\sum_{j=1}^{n_i} w_{ij}^{\ 2}}{2N} \text{ where } \quad w_{ij} = x_{ij} - x'_{ij}$$

Precision ***v*** is calculated according to the formula:

$$v = 2\sqrt{2}.S_v = 2.8.S_v$$

The value of precision ***v*** means that in 95% of the cases, the difference between two values obtained by the method, under the conditions defined, will be lower than or equal to ***v***.

NOTE 1 The use and interpretation of these results is based on the assumption that the variations obey a normal law with a 95% confidence rate.

NOTE 2 One can also define a precision of 99% with $v = 2.58\sqrt{2}.S_v = 3.65.S_v$

*5.4.3.3.1.2* *Calculations with 1 test material*

In this situation, the calculations are simpler. It is advisable to carry out ***p*** measurement replicas of the test material, if necessary with a repetition of the measurement on each replica. ***p*** must at least be equal to 10.

In the following calculations, the measurement is considered to be carried out in duplicate with each replica.

- The variance $Var(\bar{x}_{ij})$ is then given by the following equation:

$$Var(x_{ij}) = \frac{\sum_{i=1}^{p}(\bar{x}_i - M_x)^2}{p-1}$$

where:

| | |
|---|---|
| $\bar{X}i$ | is the mean of the two repetitions of replica ***i*** |
| ***p*** | is the number of replicas |
| $M_x$ | is the mean of all the replicas |

- The variance $Var(repeat)$ is then given by the following equation:

$$Var(repeat) = \frac{\sum_{i=1}^{p} w_i^2}{2p}$$

*where* $w_i$ *: difference between the two repetitions of replica* ***i***

### *5.4.3.4 Repeatability*

5.4.3.4.1 Definitions

Repeatability is the closeness of agreement between mutually-independent analysis results obtained with the method in question on the same wine, in the same laboratory, with the same operator using the same equipment, within a short period of time.

These experimental conditions will be called conditions of repeatability.

The value of repeatability **r** is the value below which the absolute difference between two results of the same analysis is considered to be located, obtained under the conditions of repeatability defined above, with a confidence level of 95%.

The repeatability standard deviation **Sr** is the standard deviation for the results obtained under the conditions of repeatability. It is a parameter of the dispersion of the results, obtained under conditions of repeatability.

5.4.3.4.2 Scope

A priori, the repeatability study can be applied without difficulty to every quantitative method, insofar as the repeatability conditions can be observed.

In many cases, repeatability is not constant throughout the range of validity of the method. It is therefore advisable to define several sections or "range levels", in which we may reasonably consider that the repeatability is comparable to a constant. The repeatability calculation is then to be reiterated for each range level.

5.4.3.4.3 Basic protocol and calculations

*5.4.3.4.3.1 General case*

The number of test materials may vary in relation to the NUMBER of replicas. In practice, we consider that the number of measurements of all test materials must be higher than 20. It is not necessary for the repeatability conditions to be maintained from one test material to another, but all the replicas carried out on the same test material must be carried out under these repeatability conditions.

Repeatability remains a special case of the precision calculation $S_v = \sqrt{Var(\bar{x}_{ij}) + \frac{Var(repeat)}{2}}$. The $Var(repeat)$ part is naturally equal to 0 (only one measurement with each replica), and the calculation is the same as the calculation of $Var(x_{ij})$

$$S_r = \sqrt{Var(x_{ij})} = \sqrt{\frac{\sum_{i=1}^{n}\sum_{j=1}^{p_i}(\bar{x}_{ij} - M_{x_i})^2}{N-n}}$$

The value ***r*** means that in 95% of the cases, the difference between two values acquired under repeatable conditions will be lower than or equal to ***r***.

*5.4.3.4.3.2 Particular case applicable to only 1 repetition*

In practice, the most current situation for automated systems is the analysis of test material with only one repetition. It is advisable to use at least 10 materials in order to reach the 20 measurements required. The two measurement replicas of the same test material must be carried out under repeatable conditions.

In this precise case, the calculation of $S_r$ is simplified and becomes:

$$S_r = \sqrt{\frac{\sum_{i=1}^{q} w_i^2}{2p}}$$

in which:

$\boldsymbol{S_r}$ = the repeatability standard deviation

$\boldsymbol{p}$ = the number of test materials analyzed in duplicate

$\boldsymbol{w_i}$ = the absolute differences between duplicates

Repeatability **r** is calculated according to the formula:

$$\boldsymbol{r = 2.8\ Sr}$$

**Example:** For the alternative determination method of the free sulfur dioxide in question, and for a range of measurements from 0 to 50 mg/l, the operator will seek at least 10 samples with regularly distributed concentrations ranging between these values.

| ***Sample no.*** | ***$x_i$ (in mg/l)*** | ***$x'_i$ (in mg/l)*** | ***$W_i$ (absolute value)*** |
|---|---|---|---|
| *1* | *14* | *14* | *0* |
| *2* | *25* | *24* | *1* |
| *3* | *10* | *10* | *0* |
| *4* | *2* | *3* | *1* |
| *5* | *35* | *35* | *0* |
| *6* | *19* | *19* | *0* |
| *7* | *23* | *23* | *0* |
| *8* | *27* | *27* | *0* |
| *9* | *44* | *45* | *1* |
| *10* | *30* | *30* | *0* |
| *11* | *8* | *8* | *0* |
| *12* | *48* | *46* | *2* |

**Example:** Using the values given in the table above, the following results are obtained:

**Q** = 12

**Sr** = 0.54 mg/l

**R** = 1.5 mg/l

This result can be used to state that, with a probability of 95%, the results obtained by the method under study will have a repeatability rate lower than 1.5 mg/l.

5.4.3.4.4 Comparison of repeatability

*5.4.3.4.4.1 Determination of the repeatability of each method*

To estimate the performance of a method, it can be useful to compare its repeatability with that of a reference method.

Let $S_{r\text{-}alt}$ be the repeatability standard deviation of the alternative method, and $S_{r\text{-}ref.}$ the repeatability standard deviation of the reference method.

The comparison is direct. If the value of repeatability of the alternative method is lower than or equal to that of the reference method, the result is positive. If it is higher, the laboratory must ensure that the result rests compliant with the specification that it accepted for the method concerned. In the latter case, it may also apply a Fischer-Snedecor test to know if the value found for the alternative method is significantly higher than that of the reference method.

*5.4.3.4.4.2 Fischer-Snedecor test*

Calculate the ratio:

$$F_{obs} = \frac{S_{r_{alt}}^{2}}{S_{r_{ref}}^{2}}$$

Use the critical Snedecor value with a risk **α** equal to 0.05 corresponding to the Fischer variable with a confidence level 1 α, in which ν1 = n(x)-n, and ν2 = n(z)-m degrees of freedom: F(N(x)-n, N(y)-m, 1- α). In the case of a calculated repeatability with only one repetition on *p* test materials for the alternative method, and *q* test materials for the reference method, the Fischer variable will have as a degree of freedom ν1 = *p*, and ν2 = *Q*, i.e.: F(p, Q, 1- α).

Interpreting the test:

1/ $\mathbf{F_{obs} > F_{1-\square}}$ **α**, the repeatability value of the alternative method is significantly higher than that of the reference method**.**

2/ $\mathbf{F_{obs} < F_{1-\square}}$ **α**, we cannot state that the repeatability value of the alternative method is significantly higher than that of the reference method**.**

> ***Example:*** *The value of the repeatability standard deviation found for the determination method of free sulfur dioxide is:*
>
> ***Sr*** *= 0.54 mg/l*
>
> *The laboratory carried out the determination on the same test materials using the OIV reference method. The value of the repeatability standard deviation found in this case is:*

***Sref** = 0.39 mg/l*

$$F_{obs} = \frac{0.54^2}{0.39^2} = \frac{0,29}{0,15} = 1,93$$

$\nu_2 = 12$
$\nu_1 = 12$
$F_{1-\alpha} = 2.69 > 1.93$

*The $F_{obs}$ value obtained is lower than the value $F_{1-\alpha}$; we cannot state that the repeatability value of the alternative method is significantly higher than that of the reference method.*

### *5.4.3.5 Intralaboratory reproducibility*

5.4.3.5.1 Definition

Intralaboratory reproducibility is the closeness of agreement between the analysis results obtained with the method under consideration on the same wine, in the same laboratory, with the same operator or different operators using from the different gauging curves, on different days.

5.4.3.5.2 Scope

Reproducibility studies can be implemented on quantitative methods, if the time of analysis is reasonably limited, and if the capacity exists to keep at least one test material stable over time.

In many cases, reproducibility is not constant throughout the validity range of the method. In this case, it is advisable to define several sections or "range levels", in which it can be reasonably considered that reproducibility is comparable to a constant. The reproducibility calculation is then to be reiterated for each range level.

5.4.3.5.3 Basic protocol and calculations

The laboratory chooses one or more stable test materials. It applies the method regularly for a period equal to at least one month and keeps the results obtained ($X_{ij}$, **material *i*, replica *j***). A minimum of 5 replicas is recommended for each test material, the total minimum number of replicas being 10. The replicas can be analyzed in duplicate.

The calculation of precision fully applies to the calculation of reproducibility, integrating $Var(repeat)$ if the measurements are carried out in duplicate.

Reproducibility ***R*** is calculated according to the formula:

$$\boldsymbol{R = 2.8\ S_R}$$

The value ***R*** means that in 95% of the cases, the difference between two values acquired under reproducibility conditions will be lower than or equal to ***R***.

**Example:** Reproducibility study of the determination of the sorbic acid in wines by steam distillation and reading by absorption at 256 Nm.
Two different sorbated wines were kept for a period of 3 months. The determination of the sorbic acid was carried out at regular intervals over this period, with repetition of each measurement.

| | Test material 1 | | Test material 2 | |
|---|---|---|---|---|
| Replicas | **x1** | **x2** | **x1** | **x2** |
| *1* | *122* | *125* | *140* | *139* |
| *2* | *123* | *120* | *138* | *137* |
| *3* | *132* | *130* | *139* | *141* |
| *4* | *121* | *115* | *143* | *142* |
| *5* | *130* | *135* | *139* | *139* |
| *6* | *135* | *142* | *135* | *138* |
| *7* | *137* | *135* | *139* | *139* |
| *8* | *130* | *125* | *145* | *145* |
| *9* | *123* | *130* | *138* | *137* |
| *10* | *112* | *115* | *135* | *134* |
| *11* | *131* | *128* | *146* | *146* |
| *12* | | | *137* | *138* |
| *13* | | | *146* | *147* |
| *14* | | | *145* | *148* |
| *15* | | | *130* | *128* |

$n = 2$
$p_1 = 11$
$p_2 = 15$
$n = 26$

$Var(x_{ij})$=37.8

$Var(repet)$=5.01

$S_R = 6.35$

$R = 17.8$

## 6. Quality control of analysis methods (IQC)

### 6.1 Reference documents

- Resolution OIV Œno 19/2002: Harmonized recommendations for internal quality control in analysis laboratories.
- CITAC/EURACHEM: Guide for quality in analytical chemistry, 2002 Edition
- Standard NF V03-115, Guide for the use of reference materials

### 6.2 General principles

It is recalled that an analysis result can be affected two types of error: systematic error, which translates into bias, and random error. For series analyses, another type of error can be defined, which can be due to both systematic error and random error: this is the series effect, illustrated for example by the deviation of the measuring system during a series.

The IQC is designed to monitor and control these three errors.

### 6.3 Reference materials

The IQC is primarily based on exploiting the measurement results for reference materials. The choice and constitution of the materials are therefore essential steps that it must be controlled in order to provide an efficient basis for the system.

A reference material is defined by two parameters:

- Its matrix
- The assignment of its reference value

Several cases are possible; the cases encountered in oenology are summarized in the following two-dimensional table:

| Reference value | **Synthetic solution**<br>*Synthetic solutions can be used to constitute* | **Natural matrix (wine etc.)**<br>*Natural matrices a priori constitute the most interesting* | **Doped wine**<br>*A doped wine is a wine with an artificial addition of an analyte* |
|---|---|---|---|
| ***Value obtained by formulation*** | The solution must be produced using metrological rules. It is recalled that the formulation value | Not applicable | This method is applicable when the base wine is completely free of analyte. These types of materials are suitable for oenological additives that are not native to the wine. If |
| ***External value to the laboratory*** | The organization supplying the solution must provide guarantees about its quality and be certified if possible. The reference values will be | The external value has been determined on the wine by an interlaboratory analysis chain. Certain organizations propose conditioned wine samples whose values have been determined in | In practice, this involves conditioned wine samples doped and/or chemically stabilized as proposed by organizations. These materials cannot claim to constitute a natural matrix. The reference values are |
| ***Value obtained by a reference method*** | If the synthetic solution has not been obtained with a calibrated material, the reference value can be determined by analyzing the | The measurement is carried out 3 times with the reference method, the selected value is the mean of the 3 results, insofar as they remain within an interval lower than the repeatability of the | The measurement is carried out 3 times with the reference method, the value retained is the mean of the 3 results, insofar as they remain within an interval lower than the repeatability of the method. |
| *of the instrument value as a reference value does not control accuracy* | The reference value is measured by the method to be checked. The material is measured over 10 repetitions, and a check will be made that the differences between these values are lower | The reference value is measured by the method to be checked. The material is measured over 10 repetitions, and a check is to be made that the differences between these values are lower than the repeatability value; the most extreme values can be withdrawn, | The reference value is measured using the method to be checked. The material is measured over 10 repetitions, and a check is made to ensure that the differences between these values are lower than the repeatability value; the most extreme values can be withdrawn, up to a |

## 6.4 Checking the analytical series

### *6.4.1 Definition*

An analytical series is a series of measurements carried out under repeatable conditions.

For a laboratory that mainly uses the analytical series method of analysis, a check must be made to ensure the instantaneous adjustment of the measuring instrument and its stability during the analytical series is correct.

Two complementary approaches are possible:

- the use of reference materials (often called by extension "control materials")
- the use of an internal standard, in particular for separative methods.

### *6.4.2 Checking accuracy using reference materials*

Systematic error can be checked by introducing reference materials, the reference value of which has been assigned using means external to the method being checked.

The measured value of the reference material is associated with a tolerance limit, inside which the measured value is accepted as being valid. The laboratory defines tolerance values for each parameter and for each analytical system. **These values are specific to the laboratory**.

The control materials must be selected so that their reference values correspond to the levels of the values usually found for a given parameter. If the scale of measurement is broad, and the uncertainty of measurement is not constant on the scale, several control materials should be used to cover the various range levels.

### *6.4.3 Intraseries precision*

When the analytical series are rather long, there is a risk of drift of the analytical system. In this case, intraseries precision must be checked using the same reference material positioned at regular intervals in the series. The same control materials as those used for accuracy can be used.

The variation in the measured values for same reference material during the series should be lower than the repeatability value *r* calculated for a confidence level of 95%.

NOTE For a confidence level of 99%, a value of $3.65.S_r$ can be used.

#### *6.4.4 Internal standard*

Certain separative methods enable the introduction of an internal standard into the product to be analyzed.
In this case, an internal standard should be introduced with calibrated material with a known uncertainty of measurement.
The internal standard enables a check to be made both of intraseries accuracy and precision. It should be noted that a drift affects the signals of the analyte and of the internal standard in equal proportions; since the value of the analyte is calculated with the value of the signal of the internal standard, the effect of the drift is cancelled.

The series will be validated if the internal standards are inside the defined tolerance values.

### 6.5 Checking the analysis system

#### *6.5.1 Definition*

This concerns an additional check to the series check. It differs from the latter in that it compiles values acquired over long time scales, and/or compares them with values resulting from other analysis systems.

Two applications will be developed:

- Shewhart charts to monitor the stability of the analysis system
- Internal and external comparison of the analysis system

#### *6.5.2 Shewhart chart*

Shewhart charts are graphic statistical tools used to monitor the drift of measurement systems, by the regular analysis, in practice under reproducibility conditions, of stable reference materials.

##### *6.5.2.1 Data acquisition*

A stable reference material is measured for a sufficiently long period, at defined regular intervals. These measurements are recorded and logged in control charts. The measurements are made under reproducibility conditions, and are in fact

exploitable for the calculation of reproducibility, and for the assessment of measurement uncertainty.

The values of the analytical parameters of the reference materials selected must be within valid measurement ranges.

The reference materials are analyzed during an analytical series, routine if possible, with a variable position in the series from one time to another. In practice, it is perfectly possible to use the measurements of control materials of the series to input the control charts.

#### *6.5.2.2 Presentation of results and definition of limits*

The individual results are compared with the accepted value of the reference material, and with the reproducibility standard deviation for the parameter in question, at the range level in question.

Two types of limits are defined in the Shewhart charts, the limits associated with individual results, and the limits associated with the mean.

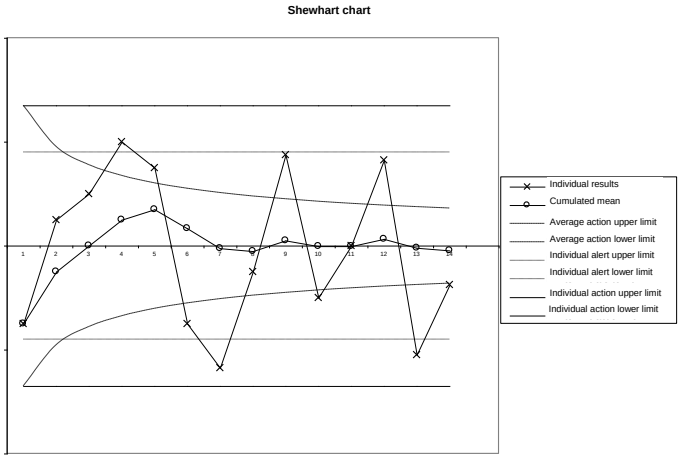
The limits defined for the individual results are usually based on the standard deviation values for intralaboratory reproducibility for the range level in question. They are of two types:

- alert limit: $+/-2.S_R$ .
- action limit: $+/-3.S_R$ .

The limit defined for the cumulated mean narrows as the number of measurements increases.

- This limit is an action limit: $+/-\frac{3.S_R}{\sqrt{n}}$ . ***n*** being the number of measurements indicated on the chart.

NOTE For reasons of legibility, the alert limit of the cumulated mean is only rarely reproduced on the control chart, and has as its value $+/-\frac{2.S_R}{\sqrt{n}}$ .




### *6.5.2.3 Using the Shewhart chart*

Below we indicate the operating criteria most frequently used. It is up to the laboratories to precisely define the criteria they apply.

Corrective action on the method (or the apparatus) will be undertaken:

a) if an individual result is outside the action limits of the individual results.
b) if two consecutive individual results are located outside the alert limits of individual results.
c) if, in addition, *a posteriori* analysis of the control charts indicates a drift in the method in three cases:
- nine consecutive individual result points are located on the same side of the line of the reference values.
- six successive individual result points ascend or descend.
- two successive points out of three are located between the alert limit and the action limit.
d) if the arithmetic mean of ***n*** recorded results is beyond one of the action limits of the cumulated mean (which highlights a systematic deviation of the results).

NOTE The control chart must be revised at ***n*** = 1 as soon as a corrective action has been carried out on the method.

### *6.5.3 Internal comparison of analysis systems*

In a laboratory that has several analysis methods for a given parameter, it is interesting to carry out measurements of the same test materials in order to compare the results. The agreement of the results between the two methods is considered to be satisfactory if their variation remains lower than 2 times the standard deviation of difference calculated during validation, with a confidence level of 95%.

NOTE This interpretation is possible given the assumption that the variations obey a normal law with a 95% confidence rate.

### *6.5.4 External comparison of the analysis system*

#### *6.5.4.1 Analysis chain of interlaboratory comparisons*

The organization of the tests and calculations is given in the chapter "comparison with an interlaboratory analysis chain".

In addition to checking the accuracy by the $\boldsymbol{Z_{score}}$ the results can be analyzed in greater detail, in particular with regard to the position of the values of the laboratory in relation to the mean. If they are systematically on the same side of the mean for several successive analysis chains, this can justify the implementation of corrective action by the laboratory, even if $\boldsymbol{Z_{score}}$ remains lower than the critical value.

NOTE Interpreting the $Z_{score}$ is possible given the assumption that the variations obey a normal law with a 95% confidence rate.

If the intercomparison chain is subject to accreditation, this work of comparison has traceability value.

#### *6.5.4.2 Comparison with external reference materials*

Measuring external reference materials at regular intervals also can be used to supervise the occurrence of a systematic error (bias).

The principle is to measure the external reference material, and to accept or refuse the value in relation to tolerance limits. These limits are defined in relation to the

combination of the uncertainties of the controlled method and the reference value of the reference material.

6.5.4.2.1 Standard uncertainty of reference material

The reference values of these materials are accompanied by confidence intervals. The laboratory must determine the nature of this data, and deduce from them the standard uncertainty value for the reference value $S_{ref}$. A distinction must be made between several cases:

- The case in which uncertainty ***a*** is given in the form of an interval confidence at 95% (expanded uncertainty). This means that a normal law has been adopted. ***a*** therefore constitutes an "expanded uncertainty" and corresponds to 2 times the standard deviation $S_{ref}$ of the standard uncertainty of the reference values of the materials provided.

$$S_{ref} = \frac{a}{2}$$

- The case of a certificate, or another specification, giving limits +/- ***a*** without specifying the confidence level. In this case, a rectangular dispersion has been adopted, and the value of measurement X has the same chance of having an unspecified value in the interval ***ref*****+/- *a***.
-

$$S_{ref} = \frac{a}{\sqrt{3}}$$

- The particular case of glassware giving limits **+/- *a***. This is the framework of a triangular dispersion.
-

$$S_{ref} = \frac{a}{\sqrt{6}}$$

6.5.4.2.2 Defining the validity limits of measuring reference material

To standard uncertainty $S_{ref}$ of the value of the external reference material, is added the standard uncertainty of the laboratory method to be checked, $S_{method}$. These two sources of variability must be taken into account in order to determine the limits.

$S_{method}$ is calculated from the expanded uncertainty of the laboratory method in the following way:

$$S_{method} = \frac{uncertainty}{2}$$

The validity limit of the result (with a confidence level of 95%) =

$$\textbf{\textit{reference value}} \ +/-2.\sqrt{S_{ref}{}^{2} + S_{method}{}^{2}}$$

**Example:** A pH 7 buffer solution is used to check a pH-meter. The confidence interval given by the pH solution is +/- 0.01. It is indicated that this confidence interval corresponds to the expanded uncertainty with a confidence level of 95%. In addition the expanded uncertainty of the pH-meter is 0.024.

The limits will be $+/-2\sqrt{(\frac{0.01}{2})^{2} + (\frac{0.024}{2})^{2}}$

i.e. **+/- 0.026** in relation to the reference value, with a confidence level of 95%.

## 7. Assessment of measurement uncertainty

### 7.1 Definition

Parameter, associated with the result of a measurement, which characterizes the dispersion of the values that can reasonably be allotted to the measurand.

In practice, uncertainty is expressed in the form of a standard deviation called standard uncertainty ***u(x)***, or in an expanded form (generally with k = 2) ***U = +/- k.u***

### 7.2 Reference documents

- AFNOR ENV 13005 Standard: 1999 – Guide for expressing measurement uncertainty
- EURACHEM, 2000. Quantifying Uncertainty in Analytical Measurement, *EURACHEM second edition 2000*
- ISO 5725 Standard: 1994 – Exactitude (accuracy and precision) of results and measurement methods
- ISO 21748 standard: 2004 – Guidelines relating to the use of estimations of repeatability, reproducibility and accuracy in evaluating measurement uncertainty
- Perruchet C and Priel M., Estimating uncertainty, *AFNOR Publications, 2000*

### 7.3 Scope

Uncertainty provides two types of information.

- On the one hand, that intended for the customers of the laboratory, indicating the potential variations to take into account in order to interpret the result of an analysis. It must be indicated, however, that this information cannot be used as an external means of evaluating the laboratory.
- In addition, it constitutes a dynamic in-house tool for evaluating the quality of the laboratory analysis results. Insofar as its evaluation is regular and based on a fixed, well-defined methodology, it can be used to see whether the variations involved in a method change positively or negatively (in the case of an estimate based exclusively on intralaboratory data).

The present guide limits itself to providing a practical methodology for oenological laboratories dealing with series analyses. These laboratories have large volumes of data of a significant statistical scale.

Estimating uncertainties can therefore be carried out in most cases using the data collected as part validation and quality control work (in particular with the data in the Shewhart charts). These data can be supplemented by experiment schedules, in particular to determine the systematic errors.

The reference systems describe two main approaches for determining uncertainty: the intralaboratory approach and the approach interlaboratory. Each provides results that are naturally and significantly different. Their significance and their interpretation cannot be identical.

- **the intralaboratory approach** provides a result specific to the method in question, in the laboratory in question. The uncertainty that results is an indicator of the performance of the laboratory for the method in question. It answers the customer as follows: "what dispersion of results can I expect from the laboratory practicing the method?"

- **the interlaboratory approach** uses results resulting from interlaboratory tests, which provide information about the overall performance of the method.

Laboratories can use the two approaches jointly. It will be interesting to see whether the results obtained using the intralaboratory approach give values lower than the values of the interlaboratory approach.

### 7.4 Methodology

The work of uncertainty assessment involves 3 fundamental steps.

- Definition of the measurand, and description of the quantitative analysis method
- Critical analysis of the measurement process
- Uncertainty assessment.

#### *7.4.1 Definition of the measurand, and description of the quantitative analysis method*

First of all, the following must be specified:

- the purpose of the measurement
- the quantity measured
- If the measurand is to be obtained by calculation based on measured quantities, if possible the mathematical relation between them should be stipulated.
- all the operating conditions.

These items are included in theory in the procedures of the laboratory quality system.

In certain cases the expression of the mathematical relation between the measurand and the quantities can be highly complex (physical methods etc.), and it is neither necessarily relevant nor possible to fully detail them.

### *7.4.2 Critical analysis of the measurement process*

The sources of error influencing the final result should be identified in order to constitute the uncertainty budget. The importance of each source can be estimated, in order to eliminate those that have only a negligible minor influence. This is done by estimating:

- the degree of gravity of the drift generated by poor control of the factor in question
- the frequency of the potential problems
- their detectability.

This critical analysis can, for example, be carried out using the "5M" method.

Labor;
Operator effect

Matter:
Sample effect (stability, homogeneity, matrix effects), and consumables (reagents, products, solutions, reference materials), etc.

Hardware:
Equipment effect (response, sensitivity, integration modes, etc.), and laboratory equipment (balance, glassware etc.).

Method:
Application effect of the procedure (operating conditions, succession of the operations etc.).

Medium:
Environmental conditions (temperature, pressure, lighting, vibration, radiation, moisture etc.).

### *7.4.3 Estimation calculations of standard uncertainty (intralaboratory approach)*

#### *7.4.3.1 Principle*

In the case of laboratories using large series of samples with a limited number of methods, a statistical approach based on intralaboratory reproducibility, supplemented by the calculation of sources of errors not taken into account under

intralaboratory reproducibility conditions, appears to be the most suitable approach.

An analysis result deviated from the true value under the effect of two sources of error: systematic errors and random errors.

**Analysis result = True value + Systematic error + Random error**

Uncertainty characterizes the dispersion of the analysis result. This translates into a standard deviation.

Variability (analysis result) = uncertainty

Variability (true value) = 0

Variability (systematic error) = $\sqrt{\sum S^2_{erreurs\_systématiques}}$

Variability (random error) = $\boldsymbol{S_R}$ (intralaboratory reproducibility standard deviation)

Since standard deviations are squared when added, the estimated standard uncertainty ***u(x)*** takes the following form:

$$u(x) = \sqrt{\sum u^2_{(systematic\_errors)} + S^2_R}$$

Non-integrable sources of errors under the intralaboratory reproducibility conditions, i.e. systematic errors, must be determined in the form of standard deviation to be combined together and with the reproducibility standard deviation.

The laboratory can take action so that the reproducibility conditions applied make it possible to include a maximum number of sources of errors. This is obtained in particular by constituting stable test materials over a sufficiently long period, during which the laboratory takes care to vary all the possible experimental factors. In this way, $\boldsymbol{S_R}$ will cover the greatest number of possible sources of errors (random), and the work involved in estimating the systematic errors, which is often more complex to realize, will be minimized.

It should be noted here that the EURACHEM/CITAC guide entitled "Quantifying uncertainty in analytical measurements" recalls that "In general, the ISO Guide requires that corrections be applied for all systematic effects that are identified and significant". In a method "under control", systematic errors should therefore constitute a minor part of uncertainty.

The following non-exhaustive table gives examples of typical sources of error and proposes an estimation approach for each of them, using integration under reproducibility conditions as much as possible.

| ***Source of error*** | ***Type of error*** | ***Commentary*** | ***Estimation method*** |
|---|---|---|---|
| Sampling (constitution of the sample) | Random | Sampling is one of the "businesses" defined in the ISO 17025 standard. Laboratories stating they do not perform sampling, do not include this source of error in the uncertainty assessment. | Can be including in intralaboratory reproducibility by including sampling in handling. |
| Sub-sampling (sampling a quantity of sample in order to carry out the test) | Random | Is significant if the sample is not homogeneous. This source of error remains minor for wine. | Included in the intralaboratory reproducibility conditions if the test material used is similar to routine test materials. |
| Stability of the sample | Random | Depends on the storage conditions of the sample. In the case of wines, laboratories should pay detailed attention to the losses of sulfur dioxide and ethanol. | Possible changes in the sample can be integrated into the reproducibility conditions. This source of uncertainty can then be evaluated overall. |
| Gauging of the apparatus | Systematic/Random<br>This error is systematic if gauging is established for a long period, and becomes random if gauging is regularly carried out over a time-scale integrated under reproducibility conditions | Source of error to be taken into account in absolute methods. | Error of gauging line § 7.4.2.4.1<br><br>Taken into account under the reproducibility conditions if gauging is regularly revised. |
| Effect of contamination or memory | Random | This effect will be minimized by the proper design of measuring instruments and suitable rinsing operations | The reproducibility conditions take this effect into account, as long as the reference materials are inserted at various positions in the analysis series. |
| Precision of automata | Random | This applies to intraseries drift in particular. This can be controlled in particular by positioning the control materials within the framework of the IQC | The reproducibility conditions take this effect into account, as long as the reference materials are inserted at various positions in the analysis series. |

| | | | |
|---|---|---|---|
| Purity of the reagents | Random | The purity of the reagents has very little effect on the relative methods, insofar as the gauging and analyses are carried out with the same batches of reagents.<br>This effect is to be taken into account in absolute methods. | To be integrated under reproducibility conditions using various batches of reagents. |
| Measurement conditions | Random | Effects of temperature, moisture etc. | Typically taken into account under reproducibility conditions |
| Matrix effect | Random from one sample to another, systematic on the same sample | These effects are to be taken into account in methods whose measured signal is not perfectly specific. | If this effect is regarded as significant, a specific experiment schedule can be used to estimate uncertainty due to this effect § 7.4.2.4.3<br>This effect is not integrated under reproducibility conditions. |
| Gauging effect | Systematic if gauging is constant<br>Random if gauging is regularly renewed | | Taken into account under the reproducibility conditions if gauging is regularly renewed. If the gauging used remains the same one (on the scale of the periods in question within the framework of the reproducibility conditions), it is advisable to implement an experiment schedule in order to estimate the error of the gauging line § 7.4.2.4.1 |
| Operator effect | Random | | To be taken into account in the reproducibility conditions by taking care to utilize all the authorized operators. |
| Bias | Systematic | Must be minimized by the quality control work of the laboratory. | Systematic effect, can be estimated using certified references. |

### *7.4.3.2 Calculating the standard deviation of intralaboratory reproducibility*

The reproducibility standard deviation $S_R$ is calculated using the protocol described in the section entitled "Intralaboratory reproducibility" (cf. § 5.4.3.5).

The calculation can be based on several test materials. In the noteworthy case where $S_R$ is proportional to the size of the measurand, the data collected on several test materials with different values should not be combined: $S_R$ should be expressed in relative value (%).

### *7.4.3.3 Estimating typical sources of systematic errors not taken into account under reproducibility conditions*

7.4.3.3.1 Gauging error (or calibration error)

Whenever the gauging of an instrument (or the calibration of an absolute method) is not regularly redone, its output cannot be integrated in the reproducibility values. An experiment schedule must be carried out in order to estimate it using the residual error of the regression.

*7.4.3.3.1.1 Procedure*

The approach is similar to that carried out in the linearity study of the method.

It is recommended to implement a number ***n*** of reference materials. The number must be higher than 3, but it is not necessary to go beyond 10. The reference materials are to be measured ***p*** times under intralaboratory precision conditions, ***p*** must be higher than 3, a figure of 5 is generally recommended. The accepted values of reference materials must be regularly distributed on the range of values under study. The number of measurements must be the same for all the reference materials.

The results are reported in a table presented as follows:

| **Reference materials** | **Accepted value of the reference material** | **Measured values** | | | | |
|---|---|---|---|---|---|---|
| | | **Replica 1** | **…** | **Replica j** | **…** | **Replica p** |
| *1* | $x_1$ | $y_{11}$ | … | $y_{1j}$ | … | $y_{1p}$ |
| … | … | … | … | … | … | … |
| *i* | $x_i$ | $y_{i1}$ | … | $y_{ij}$ | … | $y_{ip}$ |
| … | … | …. | … | … | … | … |
| *n* | $x_n$ | $y_{n1}$ | … | $y_{nj}$ | … | $y_{np}$ |

*7.4.3.3.1.2 Calculations and results*

The linear regression model is calculated.

$$y_{ij} = a + b.x_i + \varepsilon_{ij}$$

where

$y_{ij}$ is $j^{th}$ replica of the $i^{th}$ reference material.

$x_i$ is the accepted value of the $i^{th}$ reference material.

$b$ is the slope of the regression line.

$a$ is the intercept point of the regression line.

$a+b.x_i$ represent the expectation of the measurement value of the $i^{th}$ reference material.

$\varepsilon_{ij}$ is the difference between $y_{ij}$ and the expectation of the measurement value of the $i^{th}$ reference material.

The parameters of the regression line are obtained using the following formulae:

- mean of ***p*** measurements of the $i^{th}$ reference material

$$yi = \frac{1}{p}\sum_{j=1}^{p} y_{ij}$$

- mean of all the accepted values of *n* reference materials $M_x = \frac{1}{n}\sum_{i=1}^{n} x_i$

- mean of all measurements $My = \frac{1}{n}\sum_{i=1}^{n} y_i$

- estimated slope ***b***

$$b = \frac{\sum_{i=1}^{n}(x_i - M_x)(y_i - M_y)}{\sum_{i=1}^{n}(x_i - M_x)^2}$$

- estimated intercept point ***a***

$$a = M_y - b \times M_x$$

- regression value associated with the ***i*^th^** reference material $\hat{y}_i$

$$\hat{y}_i = a + b \times x_i$$

- residual $e_{ij}$

$$e_{ij} = y_{ij} - \hat{y}_i$$

*7.4.3.3.1.3 Estimating the standard uncertainty associated the gauging line (or calibration line)*

If the errors due to the regression line are constant over the entire field, the standard uncertainty is estimated in a global, single way by the overall residual standard deviation.

$$u_{(gauging)} = S_{res} = \sqrt{\frac{\sum_{i=1}^{n}\sum_{j=1}^{p}(y_{ij} - \hat{y}_i)^2}{np - 2}}$$

If the errors due to the regression line are not constant over the entire field, the standard uncertainty is estimated for a given level by the residual standard deviation for this level.

$$u_{(gauging),i} = S_{res,i} = \sqrt{\frac{\sum_{j=1}^{p}(y_{ij} - \hat{y}_i)^2}{p - 1}}$$

NOTE These estimates of standard deviations can be used if the linear regression model and the gauging (or calibration) domain have been validated (see § 5.3.1)

7.4.3.3.2 Bias error

According to the EURACHEM guide, *"Quantifying uncertainty in analytical measurements",* it is recalled that the ISO guide generally requires that corrections be applied for all identified significant systematic effects. The same applies to the bias of methods for which the laboratory implements its quality control system (see §6), and which tends towards 0 for methods "under control".

In practice, a distinction can be made between two cases:

*7.4.3.3.2.1 Methods adjusted with only one certified reference material*

Bias is permanently adjusted with the same reference material.

The certified reference material (CRM) ensures the metrological traceability of the method. A reference value was allotted to the CRM together with its standard uncertainty $u_{ref}$. This standard uncertainty of the CRM is combined with the compound uncertainty for the method, $u_{comp}$, to determine the overall standard uncertainty of the laboratory method ***u(x)***.

The overall standard uncertainty of the method adjusted with the CRM in question is therefore:

$$u(x) = \sqrt{u_{ref}^{\ 2} + u_{comp}^{\ 2}}$$

NOTE 1 The methodology is identical in the case of methods adjusted with the results of an interlaboratory comparison chain.
NOTE 2 Note the difference between a CRM used to adjust the bias of a method, in which the uncertainty of its reference value combines with that of the method, and a CRM used to control a method adjusted by other means (cf. § 6.5.4.2). In the second case, the uncertainty of the CRM should not be used for the uncertainty assessment of the method.

*7.4.3.3.2.2 Methods adjusted with several reference materials (gauging ranges etc.)*

There is no particular adjustment of bias apart from gauging work.

It is clear that each calibrator introduces bias uncertainty. There is therefore an overall theoretical uncertainty of bias, which is a combination of the uncertainties

of each calibrator. This uncertainty is very delicate to estimate, but it generally proves to be sufficiently low to be ignored, in particular if the laboratory monitors the quality of its calibrators, and the uncertainty of their reference values.

Other than in specific cases, bias uncertainty is ignored here.

7.4.3.3.3 Matrix effect

*7.4.3.3.3.1 Definition*

The matrix effect incurs a repeatable source of error for a given sample, but random from one sample to another. This error is related to the interaction of the compounds present in the product to be analyzed on measuring the required analyte. The matrix effect appears in methods with a nonspecific signal.

The matrix effect often constitutes a small part of uncertainty, particularly in separative methods. In certain other methods, including the infra-red techniques, it is a significant component of uncertainty.

> **Example:** Estimate of the matrix effect on FTIR
>
> The signal for the FTIR, or infra-red spectrum, is not a signal specific to each of the compounds that are measured by this technique. The statistical gauging model can be used to process disturbed, nonspecific spectral data in a sufficiently exact estimate of the value of the measurand. This model integrates the influences of the other compounds of the wine, which vary from one wine to the next and introduce an error into the result. Upstream of the routine analysis work, special work is carried out by the gauging developers to minimize this matrix effect and to make gauging robust, i.e. capable of integrating these variations without reflecting them in the result. Nevertheless the matrix effect is always present and constitutes a source of error at the origin of a significant part of the uncertainty of an FTIR method.
>
> To be completely rigorous, this matrix effect error can be estimated by comparing, on the one hand, the means for a great number of FTIR measurement replicas, obtained on several reference materials (at least 10), under reproducibility conditions, and the true values of reference materials with a natural wine matrix on the other. The standard deviation of the differences gives this variability of gauging (provided that the gauging has been adjusted beforehand (bias = 0)).

This theoretical approach cannot be applied in practice, because the true values are never known, but it is experimentally possible to come sufficiently close to it:

- As a preliminary, the FTIR gauging must be statistically adjusted (bias = 0) in relation to a reference method based on at least 30 samples. This can be used to eliminate the effects of bias in the measurements thereafter.

- The reference materials must be natural wines. It is advisable to use at least 10 different reference materials, with values located inside a range level, the uncertainty of which can be considered to be constant.

- An acceptable reference value is acquired, based on the mean of several measurements by the reference method, carried out under reproducibility conditions. This can be used to lower the uncertainty of the reference value: if, for the reference method used, all the significant sources of uncertainty range within reproducibility conditions, the multiplication of the number p of measurements carried out under reproducibility conditions, enable the uncertainty associated with their mean to be divided by $\sqrt{p}$. The mean obtained using a sufficient number of measurements will then have a low level of uncertainty, even negligible in relation to the uncertainty of the alternative method; and can therefore be used as a reference value. p must at least be equal to 5.

- The reference materials are analyzed by the FTIR method, with several replicas, acquired under reproducibility conditions. By multiplying the number of measurements q under reproducibility conditions on the FTIR method, the variability related to the precision of the method (random error) can be decreased. The mean value of these measurements will have a standard deviation of variability divided by $\sqrt{q}$. This random error can then become negligible in relation to the variability linked to the gauging (matrix effect) that we are trying to estimate. q must at least be equal to 5.

The following example is applied to the determination of acetic acid by FTIR gauging. The reference values are given by 5 measurements under reproducibility conditions on 7 stable test materials. The number of 7 materials is in theory insufficient, but the data here are only given by way of an example.

| | ***Reference method*** | | | | | | ***FTIR*** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Materials | 1 | 2 | 3 | 4 | 5 | Mean Ref | 1 | 2 | 3 | 4 | 5 | Mean FTIR | Diff |
| *1* | *0.3* | *0.3* | *0.3* | *0.3* | *0.3* | *0.30* | *0* | *0.3* | *0.3* | *0.3* | *0.3* | *0.305* | ***-0.004*** |
| *2* | *0.3* | *0.3* | *0.3* | *0.3* | *0.3* | *0.31* | *0* | *0.3* | *0.3* | *0.3* | *0.3* | *0.315* | ***-0.006*** |
| *3* | *0.3* | *0.3* | *0.3* | *0.3* | *0.3* | *0.38* | *0* | *0.3* | *0.3* | *0.3* | *0.3* | *0.37* | ***-0.016*** |
| *4* | *0.2* | *0.2* | *0.2* | *0.2* | *0.2* | *0.24* | *0* | *0.2* | *0.2* | *0.2* | *0.2* | *0.26* | ***0.01*** |
| *5* | *0.3* | *0.3* | *0.4* | *0.4* | *0.3* | *0.39* | *0* | *0.4* | *0.4* | *0.4* | *0.4* | *0.425* | ***0.03*** |
| *6* | *0.2* | *0.2* | *0.2* | *0.2* | *0.2* | *0.26* | *0* | *0.2* | *0.2* | *0.2* | *0.2* | *0.255* | ***-0.008*** |
| *7* | *0.3* | *0.3* | *0.3* | *0.3* | *0.3* | *0.36* | *0* | *0.3* | *0.3* | *0.3* | *0.3* | *0.365* | ***-0.008*** |

Calculation of the differences: ***diff = Mean FTIR – Mean ref.***

The mean of the differences $M_d = 0.000$ verifies (good adjustment of the FTIR compared with the reference method***)***

The standard deviation of the differences, $S_d = 0.015$**.** It is this standard deviation that is used to estimate the variability generated by the gauging, and we can therefore state that:

$$U_f = 0.015$$

NOTE It should be noted that the value of $U_f$ can be over-estimated by this approach. If the laboratory considers that the value is significantly excessive under the operating conditions defined here, it can increase the number of measurements on the reference method and/or the FTIR method.

The reproducibility conditions include all the other significant sources of error, $S_R$ was otherwise calculated: ***SR = 0.017***

The uncertainty of the determination of acetic acid by this FTIR application is:

$$+/-2*\sqrt{0.015^2+0.017^2}$$ or ***+/- 0.045 g.L$^{-1}$***

7.4.3.3.4 Sample effect

In certain cases, the experiment schedules used to estimate uncertainty are based on synthetic test materials. In such a situation, the estimate does not cover the sample effect (homogeneity). The laboratories must therefore estimate this effect.

It should be noted, however, that this effect is often negligible in oenological laboratories, which use homogeneous samples of small quantities.

### *7.4.4 Estimating standard uncertainty by interlaboratory tests*

#### *7.4.4.1 Principle*

The interlaboratory approach uses data output by interlaboratory tests from which a standard deviation of interlaboratory reproducibility is calculated, in accordance with the principles indicated in §5.4.3. The statisticians responsible for calculating the results of the interlaboratory tests can identify "aberrant" laboratory results, by using tests described in the ISO 5725 standard (Cochran test). These results can then be eliminated after agreement between the statisticians and the analysts.

For the uncertainty assessment by interlaboratory approach, the guidelines stated in the ISO 21748 standard are as follows:

1. The reproducibility standard deviation (interlaboratory) obtained in a collaborative study is a valid basis for evaluating the uncertainty of measurement

2. Effects that are not observed as part of the collaborative study must be obviously negligible or be explicitly taken into account.

There are two types of interlaboratory tests:

1. Collaborative studies which relate to only one method. These studies are carried out for the initial validation of a new method in order to define the standard deviation of interlaboratory reproducibility ***$SR_{inter}$ (method)***.

2. Interlaboratory comparison chains, or aptitude tests. These tests are carried out to validate a method adopted by the laboratory, and the routine quality control (see § 5.3.3.3). The data are processed as a whole, and integrate all the analysis methods employed by the laboratories participating in the tests. The results are the interlaboratory mean ***m***, and the standard deviation of interlaboratory and intermethod reproducibility ***$SR_{inter}$***.

***7.4.4.2 Using the standard deviation of interlaboratory and intramethod reproducibility*** $SR_{inter}$ (method)

The standard deviation of intralaboratory reproducibility ***$SR_{inter}$ (method)*** takes into account intralaboratory variability and the overall interlaboratory variability related to the method.

Then must be taken into account the fact that the analysis method can produce a systematic bias compared with the true value.

As part of a collaborative study, whenever possible, the error produced by this bias can be estimated by using certified reference materials, under the same conditions as described in § 7.4.3.3.2, and added to ***$SR_{inter}$ (method)***.

***7.4.4.3 Using the standard deviation of interlaboratory and intermethod reproducibility*** $SR_{inter}$

The standard deviation of intralaboratory reproducibility ***$SR_{inter}$*** takes into account intralaboratory variability and interlaboratory variability for the parameter under study.

The laboratory must check its accuracy in relation to these results (see § 5.3.3).

There is no need to add components associated with method accuracy to the uncertainty budget, since in the "multi-method" aptitude tests, errors of accuracy can be considered to be taken into account in ***$SR_{inter}$***.

#### *7.4.4.4 Other components in the uncertainty budget*

Insofar as the test materials used for the interlaboratory tests are representative of the conventional samples analyzed by laboratories, and that they follow the overall analytical procedure (sub-sampling, extraction, concentration, dilution, distillation etc.), $S_{R\text{-}inter}$ represents the standard uncertainty ***u(x)*** of the method, in the interlaboratory sense.

Errors not taken into account in the interlaboratory tests must then be studied in order to assess their compound standard uncertainty, which will be combined with the compound standard uncertainty of the interlaboratory tests.

### 7.5 Expressing expanded uncertainty

In practice, uncertainty is expressed in its expanded form, is absolute terms for methods in which uncertainty is stable in the scope in question, or relative when uncertainty varies proportionally in relation to the quantity of the measurand:

**Absolute uncertainty:** $U = +/- 2.u(x)$

**Relative uncertainty (in %):** $U = +/- \frac{2.u(x)}{\bar{x}}.100$

where $\bar{x}$ mean represents the reproducibility results.

NOTE This expression of uncertainty is possible given the assumption that the variations obey a normal law with a 95% confidence rate.

These expressions result in a given uncertainty value with a confidence level of **95%**.

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## Annex N°1
### Table A - Law of SNEDECOR

This table indicates values of **F** in function with $\nu_1$ and $\nu_2$ for a risk $\alpha$ of 0,05

*P=0,950*

| $\nu_1$ / $\nu_2$ | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | $\nu_1$ / $\nu_2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 161,4 | 199,5 | 215,7 | 224,6 | 230,2 | 234,0 | 236,8 | 238,9 | 240,5 | 241,9 | **1** |
| **2** | 18,51 | 19,00 | 19,16 | 19,25 | 19,30 | 19,33 | 19,35 | 19,37 | 19,38 | 19,40 | **2** |
| **3** | 10,13 | 9,55 | 9,28 | 9,12 | 9,01 | 8,94 | 8,89 | 8,85 | 8,81 | 8,79 | **3** |
| **4** | 7,71 | 6,94 | 6,59 | 6,39 | 6,26 | 6,16 | 6,09 | 6,04 | 6,00 | 5,96 | **4** |
| **5** | 6,61 | 5,79 | 5,41 | 5,19 | 5,05 | 4,95 | 4,88 | 4,82 | 4,77 | 4,74 | **5** |
| **6** | 5,99 | 5,14 | 4,76 | 4,53 | 4,39 | 4,28 | 4,21 | 4,15 | 4,10 | 4,06 | **6** |
| **7** | 5,59 | 4,74 | 4,35 | 4,12 | 3,97 | 3,87 | 3,79 | 3,73 | 3,68 | 3,64 | **7** |
| **8** | 5,32 | 4,46 | 4,07 | 3,84 | 3,69 | 3,58 | 3,50 | 3,44 | 3,39 | 3,35 | **8** |
| **9** | 5,12 | 4,26 | 3,86 | 3,63 | 3,48 | 3,37 | 3,29 | 3,23 | 3,18 | 3,14 | **9** |
| **10** | 4,96 | 4,10 | 3,71 | 3,48 | 3,33 | 3,22 | 3,14 | 3,07 | 3,02 | 2,98 | **10** |
| **11** | 4,84 | 3,98 | 3,59 | 3,36 | 3,20 | 3,09 | 3,01 | 2,95 | 2,90 | 2,85 | **11** |
| **12** | 4,75 | 3,89 | 3,49 | 3,26 | 3,11 | 3,00 | 2,91 | 2,85 | 2,80 | 2,75 | **12** |
| **13** | 4,67 | 3,81 | 3,41 | 3,18 | 3,03 | 2,92 | 2,83 | 2,77 | 2,71 | 2,67 | **13** |
| **14** | 4,60 | 3,74 | 3,34 | 3,11 | 2,96 | 2,85 | 2,76 | 2,70 | 2,65 | 2,60 | **14** |
| **15** | 4,54 | 3,68 | 3,29 | 3,06 | 2,90 | 2,79 | 2,71 | 2,64 | 2,59 | 2,54 | **15** |
| **16** | 4,49 | 3,63 | 3,24 | 3,01 | 2,85 | 2,74 | 2,66 | 2,59 | 2,54 | 2,49 | **16** |
| **17** | 4,45 | 3,59 | 3,20 | 2,96 | 2,81 | 2,70 | 2,61 | 2,55 | 2,49 | 2,45 | **17** |
| **18** | 4,41 | 3,55 | 3,16 | 2,93 | 2,77 | 2,66 | 2,58 | 2,51 | 2,46 | 2,41 | **18** |
| **19** | 4,38 | 3,52 | 3,13 | 2,90 | 2,74 | 2,63 | 2,54 | 2,48 | 2,42 | 2,38 | **19** |
| **20** | 4,35 | 3,49 | 3,10 | 2,87 | 2,71 | 2,60 | 2,51 | 2,45 | 2,39 | 2,35 | **20** |
| **21** | 4,32 | 3,47 | 3,07 | 2,84 | 2,68 | 2,57 | 2,49 | 2,42 | 2,37 | 2,32 | **21** |
| **22** | 4,30 | 3,44 | 3,05 | 2,82 | 2,66 | 2,55 | 2,46 | 2,40 | 2,34 | 2,30 | **22** |
| **23** | 4,28 | 3,42 | 3,03 | 2,80 | 2,64 | 2,53 | 2,44 | 2,37 | 2,32 | 2,27 | **23** |
| **24** | 4,26 | 3,40 | 3,01 | 2,78 | 2,62 | 2,51 | 2,42 | 2,36 | 2,30 | 2,25 | **24** |
| **25** | 4,24 | 3,39 | 2,99 | 2,76 | 2,60 | 2,49 | 2,40 | 2,34 | 2,28 | 2,24 | **25** |
| **26** | 4,23 | 3,37 | 2,98 | 2,74 | 2,59 | 2,47 | 2,39 | 2,32 | 2,27 | 2,22 | **26** |
| **27** | 4,21 | 3,35 | 2,96 | 2,73 | 2,57 | 2,46 | 2,37 | 2,31 | 2,25 | 2,20 | **27** |
| **28** | 4,20 | 3,34 | 2,95 | 2,71 | 2,56 | 2,45 | 2,36 | 2,29 | 2,24 | 2,19 | **28** |
| **29** | 4,18 | 3,33 | 2,93 | 2,70 | 2,55 | 2,43 | 2,35 | 2,28 | 2,22 | 2,18 | **29** |
| **30** | 4,17 | 3,32 | 2,92 | 2,69 | 2,53 | 2,42 | 2,33 | 2,27 | 2,21 | 2,16 | **30** |
| **40** | 4,08 | 3,23 | 2,84 | 2,61 | 2,45 | 2,34 | 2,25 | 2,18 | 2,12 | 2,08 | **40** |
| **60** | 4,00 | 3,15 | 2,76 | 2,53 | 2,37 | 2,25 | 2,17 | 2,10 | 2,04 | 1,99 | **60** |
| **120** | 3,92 | 3,07 | 2,68 | 2,45 | 2,29 | 2,17 | 2,09 | 2,02 | 1,96 | 1,91 | **120** |
| **∞** | 3,84 | 3,00 | 2,60 | 2,37 | 2,21 | 2,10 | 2,01 | 1,94 | 1,88 | 1,83 | **∞** |
| $\nu_2$ / $\nu_1$ | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | $\nu_2$ / $\nu_1$ |

## Annex N°2

### Table B - Law of STUDENT

This table indicates values of **t** in function with **P** and **ν.**

| *7.5.1.1.* ν | **0,55** | **0,60** | **0,65** | **0,70** | **0,75** | **0,80** | **0,85** | **0,90** | **0,95** | **0,975** | **0,990** | **0,995** | **0,9995** | **P** ν |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 0,158 | 0,325 | 0,510 | 0,727 | 1,000 | 1,376 | 1,963 | 3,078 | 6,314 | 12,706 | 31,821 | 63,657 | 636,619 | **1** |
| **2** | 0,142 | 0,289 | 0,445 | 0,617 | 0,816 | 1,061 | 1,386 | 1,886 | 2,920 | 4,303 | 6,965 | 9,925 | 31,598 | **2** |
| **3** | 0,137 | 0,277 | 0,424 | 0,584 | 0,765 | 0,978 | 1,250 | 1,638 | 2,353 | 3,182 | 4,541 | 5,841 | 12,929 | **3** |
| **4** | 0,134 | 0,271 | 0,414 | 0,569 | 0,741 | 0,941 | 1,190 | 1,533 | 2,132 | 2,776 | 3,747 | 4,604 | 8,610 | **4** |
| **5** | 0,132 | 0,267 | 0,408 | 0,559 | 0,727 | 0,920 | 1,156 | 1,476 | 2,015 | 2,571 | 3,365 | 4,032 | 6,869 | **5** |
| **6** | 0,131 | 0,265 | 0,404 | 0,553 | 0,718 | 0,906 | 1,134 | 1,440 | 1,943 | 2,447 | 3,143 | 3,707 | 5,959 | **6** |
| **7** | 0,130 | 0,263 | 0,402 | 0,549 | 0,711 | 0,896 | 1,119 | 1,415 | 1,895 | 2,365 | 2,998 | 3,499 | 5,408 | **7** |
| **8** | 0,130 | 0,262 | 0,399 | 0,546 | 0,706 | 0,889 | 1,108 | 1,397 | 1,860 | 2,306 | 2,896 | 3,355 | 5,041 | **8** |
| **9** | 0,129 | 0,261 | 0,398 | 0,543 | 0,703 | 0,883 | 1,100 | 1,383 | 1,833 | 2,262 | 2,821 | 3,250 | 4,781 | **9** |
| **10** | 0,129 | 0,260 | 0,397 | 0,542 | 0,700 | 0,879 | 1,093 | 1,372 | 1,812 | 2,228 | 2,764 | 3,169 | 4,587 | **10** |
| **11** | 0,129 | 0,260 | 0,396 | 0,540 | 0,697 | 0,876 | 1,088 | 1,363 | 1,796 | 2,201 | 2,718 | 3,106 | 4,437 | **11** |
| **12** | 0,128 | 0,259 | 0,395 | 0,539 | 0,695 | 0,873 | 1,083 | 1,356 | 1,782 | 2,179 | 2,681 | 3,055 | 4,318 | **12** |
| **13** | 0,128 | 0,259 | 0,394 | 0,538 | 0,694 | 0,870 | 1,079 | 1,350 | 1,771 | 2,160 | 2,650 | 3,012 | 4,221 | **13** |
| **14** | 0,128 | 0,258 | 0,393 | 0,537 | 0,692 | 0,868 | 1,076 | 1,345 | 1,761 | 2,145 | 2,624 | 2,977 | 4,140 | **14** |
| **15** | 0,128 | 0,258 | 0,393 | 0,536 | 0,691 | 0,866 | 1,074 | 1,341 | 1,753 | 2,131 | 2,602 | 2,947 | 4,073 | **15** |
| **16** | 0,128 | 0,258 | 0,392 | 0,535 | 0,690 | 0,865 | 1,071 | 1,337 | 1,746 | 2,120 | 2,583 | 2,921 | 4,015 | **16** |
| **17** | 0,128 | 0,257 | 0,392 | 0,534 | 0,689 | 0,863 | 1,069 | 1,333 | 1,740 | 2,110 | 2,567 | 2,898 | 3,965 | **17** |
| **18** | 0,127 | 0,257 | 0,392 | 0,534 | 0,688 | 0,862 | 1,067 | 1,330 | 1,734 | 2,101 | 2,552 | 2,878 | 3,922 | **18** |
| **19** | 0,127 | 0,257 | 0,391 | 0,533 | 0,688 | 0,861 | 1,066 | 1,328 | 1,729 | 2,093 | 2,539 | 2,861 | 3,883 | **19** |
| **20** | 0,127 | 0,257 | 0,391 | 0,533 | 0,687 | 0,860 | 1,064 | 1,325 | 1,725 | 2,086 | 2,528 | 2,845 | 3,850 | **20** |
| **21** | 0,127 | 0,257 | 0,391 | 0,532 | 0,686 | 0,859 | 1,063 | 1,323 | 1,721 | 2,080 | 2,518 | 2,831 | 3,819 | **21** |
| **22** | 0,127 | 0,256 | 0,390 | 0,532 | 0,686 | 0,858 | 1,061 | 1,321 | 1,717 | 2,074 | 2,508 | 2,819 | 3,792 | **22** |
| **23** | 0,127 | 0,256 | 0,390 | 0,532 | 0,685 | 0,858 | 1,060 | 1,319 | 1,714 | 2,069 | 2,500 | 2,807 | 3,767 | **23** |
| **24** | 0,127 | 0,256 | 0,390 | 0,531 | 0,685 | 0,857 | 1,059 | 1,318 | 1,711 | 2,064 | 2,492 | 2,797 | 3,745 | **24** |
| **25** | 0,127 | 0,256 | 0,390 | 0,531 | 0,684 | 0,856 | 1,058 | 1,316 | 1,708 | 2,060 | 2,485 | 2,787 | 3,725 | **25** |
| **26** | 0,127 | 0,256 | 0,390 | 0,531 | 0,884 | 0,856 | 1,058 | 1,315 | 1,706 | 2,056 | 2,479 | 2,779 | 3,707 | **26** |
| **27** | 0,127 | 0,256 | 0,389 | 0,531 | 0,684 | 0,855 | 1,057 | 1,314 | 1,703 | 2,052 | 2,473 | 2,771 | 3,690 | **27** |
| **28** | 0,127 | 0,256 | 0,389 | 0,530 | 0,683 | 0,855 | 1,056 | 1,313 | 1,701 | 2,048 | 2,467 | 2,763 | 3,674 | **28** |
| **29** | 0,127 | 0,256 | 0,389 | 0,530 | 0,683 | 0,854 | 1,055 | 1,311 | 1,699 | 2,045 | 2,462 | 2,756 | 3,659 | **29** |
| **30** | 0,127 | 0,256 | 0,389 | 0,530 | 0,683 | 0,854 | 1,055 | 1,310 | 1,697 | 2,042 | 2,457 | 2,750 | 3,646 | **30** |
| **40** | 0,126 | 0,255 | 0,388 | 0,529 | 0,681 | 0,851 | 1,050 | 1,303 | 1,684 | 2,021 | 2,423 | 2,704 | 3,551 | **40** |
| **60** | 0,126 | 0,254 | 0,387 | 0,527 | 0,679 | 0,848 | 1,046 | 1,296 | 1,671 | 2,000 | 2,390 | 2,660 | 3,460 | **60** |
| **120** | 0,126 | 0,254 | 0,386 | 0,526 | 0,677 | 0,845 | 1,041 | 1,289 | 1,658 | 1,980 | 2,358 | 2,617 | 3,373 | **120** |
| ∞ | 0,126 | 0,253 | 0,385 | 0,524 | 0,674 | 0,842 | 1,036 | 1,282 | 1,645 | 1,960 | 2,326 | 2,576 | 3,291 | ∞ |
| **ν P** | **0,55** | **0,60** | **0,65** | **0,70** | **0,75** | **0,80** | **0,85** | **0,90** | **0,95** | **0,975** | **0,990** | **0,995** | **0,9995** | **ν P** |

# Harmonised guidelines for single-laboratory validation of methods of analysis (technical report)

(Resolution Oeno 8/2005)

***Synopsis***

Method validation is one of the measures universally recognised as a necessary part of a comprehensive system of quality assurance in analytical chemistry. In the past ISO, IUPAC and AOAC INTERNATIONAL have co-operated to produce agreed protocols or guidelines on the "Design, Conduct and Interpretation of Method Performance Studies"[1] on the "Proficiency Testing of (Chemical) Analytical Laboratories"[2] on "Internal Quality Control in Analytical Chemistry Laboratories"[3] and on "The Use of Recovery Information in Analytical Measurement".[4] ( from the usage of overlapping data in analytical measurements) The Working Group that produced these protocols/guidelines has now been mandated by IUPAC to prepare guidelines on the Single-laboratory Validation of methods of analysis. These guidelines provide minimum recommendations on procedures that should be employed to ensure adequate validation of analytical methods.

A draft of the guidelines has been discussed at an International Symposium on the Harmonisation of Quality Assurance Systems in Chemical Laboratory, the Proceedings from which have been published by the UK Royal Society of Chemistry.

Resulting from the Symposium on Harmonisation of Quality Assurance Systems for Analytical Laboratories, Budapest, Hungary, 4-5 November 1999 held under the sponsorship of IUPAC, ISO and AOAC INTERNATIONAL

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# 1. INTRODUCTION

## 1.1 Background

Reliable analytical methods are required for compliance with national and international regulations in all areas of analysis. It is accordingly internationally recognised that a laboratory must take appropriate measures to ensure that it is capable of providing and does provide data of the required quality. Such measures include:

- using validated methods of analysis;
- using internal quality control procedures;
- participating in proficiency testing schemes; and
- becoming accredited to an International Standard, normally ISO/IEC 17025.

It should be noted that accreditation to ISO/IEC 17025 specifically addresses the establishment of traceability for measurements, as well as requiring a range of other technical and management requirements including all those in the list above.

Method validation is therefore an essential component of the measures that a laboratory should implement to allow it to produce reliable analytical data. Other aspects of the above have been addressed previously by the IUPAC Interdivisional Working Party on Harmonisation of Quality Assurance Schemes for Analytical Laboratories, specifically by preparing Protocols/Guidelines on method performance (collaborative) studies,[1] proficiency testing,[2] and internal quality control.[3]

In some sectors, most notably in the analysis of food, the requirement for methods that have been "fully validated" is prescribed by legislation.[5,6] "Full" validation for an analytical method is usually taken to comprise an examination of the characteristics of the method in an inter-laboratory method performance study (also known as a collaborative study or collaborative trial). Internationally accepted protocols have been established for the "full" validation of a method of analysis by a collaborative trial, most notably the International Harmonised Protocol[1] and the ISO procedure.[7] These protocols/standards require a minimum number of laboratories and test materials to be included in the collaborative trial to validate fully the analytical method. However, it is not always practical or necessary to provide full validation of analytical methods. In such circumstances a "single-laboratory method validation" may be appropriate.

Single-laboratory method validation is appropriate in several circumstances including the following:

- to ensure the viability of the method before the costly exercise of a formal collaborative trial;
- to provide evidence of the reliability of analytical methods if collaborative trial data are not available or where the conduct of a formal collaborative trial is not practicable;
- to ensure that "off-the-shelf" validated methods are being used correctly.

When a method is to be characterised in-house, it is important that the laboratory determines and agrees with its customer exactly which characteristics are to be evaluated. However, in a number of situations these characteristics may be laid down by legislation (e.g. veterinary drug residues in food and pesticides in food sectors). The extent of the evaluation that a laboratory undertakes must meet the requirements of legislation.

Nevertheless in some analytical areas the same analytical method is used by a large number of laboratories to determine stable chemical compounds in defined matrices. It should be appreciated that if a suitable collaboratively studied method can be made available to these laboratories, then the costs of the collaborative trial to validate that method may well be justified. The use of a collaboratively studied method considerably reduces the efforts which a laboratory, before taking a method into routine use, must invest in extensive validation work. A laboratory using a collaboratively studied method, which has been found to be fit for the intended purpose, needs only to demonstrate that it can achieve the performance characteristics stated in the method. Such a verification of the correct use of a method is much less costly than a full single laboratory validation. The total cost to the Analytical Community of validating a specific method through a collaborative trial and then verifying its performance attributes in the laboratories wishing to use it is frequently less than when many laboratories all independently undertake single laboratory validation of the same method.

## 1.2 Existing Protocols, Standards and Guides

A number of protocols and guidelines[8-19] on method validation and uncertainty have been prepared, most notably in AOAC INTERNATIONAL, International Conference on Harmonisation (ICH) and Eurachem documents:

- The Statistics manual of the AOAC, which includes guidance on single laboratory study prior to collaborative testing[13]

- The ICH text[15] and methodology,[16] which prescribe minimum validation study requirements for tests used to support drug approval submission.
- The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics (1998)[12]
- Quantifying Uncertainty in Analytical Measurement (2000)[9]

Method validation was also extensively discussed at a Joint FAO/IAEA Expert Consultation, December 1997, on the Validation of Analytical Methods for Food Controls, the Report of which is available[19].

The present 'Guidelines' bring together the essential scientific principles of the above documents to provide information which has been subjected to international acceptance and, more importantly, to point the way forward for best practice in single-laboratory method validation.

## 2 DEFINITIONS AND TERMINOLOGY

### 2.1 General

Terms used in this document respect ISO and IUPAC definitions where available. The following documents contain relevant definitions:

i) IUPAC: Compendium of chemical terminology, 1987

ii) International vocabulary of basic and general terms in metrology. ISO 1993

### 2.2 Definitions used in this guide only:

*Relative uncertainty*: Uncertainty expressed as a relative standard deviation.

*Validated range*: That part of the concentration range of an analytical method which has been subjected to validation.

## 3 METHOD VALIDATION, UNCERTAINTY, AND QUALITY ASSURANCE

Method validation makes use of a set of tests which both test any assumptions on which the analytical method is based and establish and document the performance characteristics of a method, thereby demonstrating whether the method is fit for a particular analytical purpose. Typical performance characteristics of analytical methods are: applicability; selectivity; calibration; trueness; precision; recovery; operating range; limit of quantification; limit of detection; sensitivity; and ruggedness. To these can be added measurement uncertainty and fitness-for-purpose.

Strictly speaking, validation should refer to an 'analytical system' rather than an 'analytical method', the analytical system comprising a defined method protocol, a defined concentration range for the analyte, and a specified type of test material. For the purposes of this document, a reference to 'method validation' will be taken as referring to an analytical system as a whole. Where the analytical procedure as such is addressed, it will be referred to as 'the protocol'.

In this document method validation is regarded as distinct from ongoing activities such as internal quality control (IQC) or proficiency testing. Method validation is carried out once, or at relatively infrequent intervals during the working lifetime of a method; it tells us what performance we can expect the method to provide in the future. Internal quality control tells us about how the method has performed in the past. IQC is therefore treated as a separate activity in the IUPAC Harmonisation Programme.[3]

In method validation the quantitative characteristics of interest relate to the accuracy of the result likely to be obtained. Therefore it is generally true to say that method validation is tantamount to the task of estimating uncertainty of measurement. Over the years it has become traditional for validation purposes to represent different aspects of method performance by reference to the separate items listed above, and to a considerable extent these guidelines reflect that pattern. However, with an increasing reliance on measurement uncertainty as a key indicator of both fitness for purpose and reliability of results, analytical chemists will increasingly undertake measurement validation to support uncertainty estimation, and some practitioners will want to do so immediately. Accordingly, measurement uncertainty is treated briefly in Annex A as a performance characteristic of an analytical method, while Annex B provides additional guidance on some procedures not otherwise covered.

# 4 BASIC PRINCIPLES OF METHOD VALIDATION

## 4.1 Specification and scope of validation

Validation applies to a defined protocol, for the determination of a specified analyte and range of concentrations in a particular type of test material, used for a specified purpose. In general, validation should check that the method performs adequately for the purpose throughout the range of analyte concentrations and test materials to which it is applied. It follows that these features, together with a statement of any fitness-for-purpose criteria, should be completely specified before any validation takes place.

## 4.2 Testing assumptions

In addition to the provision of performance figures which indicate fitness for purpose and have come to dominate the practical use of validation data, validation studies act as an objective test of any assumptions on which an analytical method is based. For example, if a result is to be calculated from a simple straight line calibration function, it is implicitly assumed that the analysis is free from significant bias, that the response is proportional to analyte concentration, and that the dispersion of random errors is constant throughout the range of interest. In most circumstances, such assumptions are made on the basis of experience accumulated during method development or over the longer term, and are consequently reasonably reliable. Nonetheless, good measurement science relies on *tested* hypotheses. This is the reason that so many validation studies are based on statistical hypothesis testing; the aim is to provide a basic check that the reasonable assumptions made about the principles of the method are not seriously flawed.

There is an important practical implication of this apparently abstruse note. It is easier to check for gross departure from a reliable assumption than to 'prove' that a particular assumption is correct. Thus, where there is long practice of the successful use of a particular analytical technique (such as gas chromatographic analysis, or acid digestion methods) across a range of analytes and matrices, validation checks justifiably take the form of relatively light precautionary tests. Conversely, where experience is slight, the validation study needs to provide strong evidence that the assumptions made are appropriate in the particular cases under study, and it will generally be necessary to study the full range of circumstances in detail. It follows that the extent of validation studies required in a given instance will depend, in part, on the accumulated experience of the analytical technique used.
In the following discussion, it will be taken for granted that the laboratory is well

practised in the technique of interest, and that the purpose of any significance tests is to check that there is no strong evidence to discount the assumptions on which the particular protocol relies. The reader should bear in mind that more stringent checks may be necessary for unfamiliar or less established measurement techniques.

### 4.3 Sources of Error in Analysis

Errors in analytical measurements arise from different sources[*] and at different levels of organisation. One useful way of representing these sources (for a specific concentration of analyte) is as follows[+24]:

- random error of measurement (repeatability);
- run bias ;
- laboratory bias;
- method bias;
- matrix variation effect.

Though these different sources may not necessarily be independent, this list provides a useful way of checking the extent to which a given validation study addresses the sources of error.

The repeatability (within-run) term includes contributions from any part of the procedure that varies within a run, including contributions from the familiar gravimetric and volumetric errors, heterogeneity of the test material, and variation in the chemical treatment stages of the analysis, and is easily seen in the dispersion of replicated analyses. The run effect accounts for additional day-to-day variations in the analytical system, such as changes of analyst, batches of reagents, recalibration of instruments, and the laboratory environment (*e.g.*, temperature changes). In single-laboratory validation, the run effect is typically estimated by

[*] Sampling uncertainty in the strict sense of uncertainty due to the preparation of the laboratory sample from the bulk target is excluded from consideration in this document. Uncertainty associated with taking a test portion from the laboratory sample is an inseparable part of measurement uncertainty and is automatically included at various levels of the following analysis.

[+] Many alternative groupings or 'partitions of error' are possible and may be useful in studying particular sources of error in more detail or across a different range of situations. For example, the statistical model of ISO 5725 generally combines laboratory and run effects, while the uncertainty estimation procedure in the ISO GUM is well suited to assessing the effects of each separate and measurable influence on the result.

conducting a designed experiment with replicated analysis of an appropriate material in a number of separate runs. Between-laboratory variation arises from factors such as variations in calibration standards, differences between local interpretations of a protocol, changes in equipment or reagent source or environmental factors, such as differences in average climatic conditions. Between-laboratory variation is clearly seen as a reality in the results of collaborative trials (method performance studies) and proficiency tests, and between-method variation can sometimes be discerned in the results of the latter.

Generally, the repeatability, run effect and laboratory effect are of comparable magnitude, so none can safely be ignored in validation. In the past there has been a tendency for aspects to be neglected, particularly when estimating and reporting uncertainty information. This results in uncertainty intervals that are too tight. For example, the collaborative trial as normally conducted does not give the complete picture because contributions to uncertainty from method bias and matrix variation are not estimated in collaborative trials and have to be addressed separately (usually by prior single-laboratory study). In single-laboratory validation there is the particular danger that laboratory bias also may be overlooked, and that item is usually the largest single contributor to uncertainty from the above list. Therefore specific attention must be paid to laboratory bias in single-laboratory validation.

In addition to the above-mentioned problems, the validation of a method is limited to the scope of its application, that is, the method as applied to a particular class of test material. If there is a substantial variation of matrix types within the defined class, there will be an additional source of variation due to within-class matrix effects. Of course, if the method is subsequently used for materials outside the defined class (that is, outside the scope of the validation), the analytical system cannot be considered validated: an extra error of unknown magnitude is introduced into the measurement process.

It is also important for analysts to take account of the way in which method performance varies as a function of the concentration of the analyte. In most instances the dispersion of results increases absolutely with concentration and recovery may differ substantially at high and low concentrations. The measurement uncertainty associated with the results is therefore often dependent on both these effects and on other concentration-dependent factors. Fortunately, it is often reasonable to assume a simple relationship between performance and analyte concentration; most commonly that errors are proportional to analyte concentration.[*] However, where the performance of the method is of interest at

[*] This may not be applicable at concentrations less than 10 times the detection limit.

substantially different concentrations, it is important to check the assumed relationship between performance and analyte concentration. This is typically done by checking performance at extremes of the likely range, or at a few selected levels. Linearity checks also provide information of the same kind.

### 4.4 Method and Laboratory effects

It is critically important in single-laboratory method validation to take account of method bias and laboratory bias. There are a few laboratories with special facilities where these biases can be regarded as negligible, but that circumstance is wholly exceptional. (However, that if there is only one laboratory carrying out a particular analysis, then method bias and laboratory bias take on a different perspective). Normally, method and laboratory effects have to be included in the uncertainty budget, but often they are more difficult to address than repeatability error and the run effect. In general, to assess the respective uncertainties it is necessary to use information gathered independently of the laboratory. The most generally useful sources of such information are (i) statistics from collaborative trials (not available in many situations of single-laboratory method validation), (ii) statistics from proficiency tests and (iii) results from the analysis of certified reference materials.

Collaborative trials directly estimate the variance of between-laboratory biases. While there may be theoretical shortcomings in the design of such trials, these variance estimates are appropriate for many practical purposes. Consequently it is always instructive to test single-laboratory validation by comparing the estimates of uncertainty with reproducibility estimates from collaborative trials. If the single-laboratory result is substantially the smaller, it is likely that important sources of uncertainty have been neglected. (Alternatively, it may be that a particular laboratory in fact works to a smaller uncertainty than found in collaborative trials: such a laboratory would have to take special measures to justify such a claim.) If no collaborative trial has been carried out on the particular method/test material combination, an estimate of the reproducibility standard deviation $\sigma_H$ at an analyte concentration $c$ above about 120 ppb can usually be obtained from the Horwitz function, $\sigma_H = 0.02c^{0.8495}$, with both variables expressed as mass fractions. (The Horwitz estimate is normally within a factor of about two of observed collaborative study results). It has been observed that the Horwitz function is incorrect at concentrations lower than about 120 ppb, and a modified function is more appropriate.[21, 25] All of this information may be carried into the single-laboratory area with minimum change.

Statistics from proficiency tests are particularly interesting because they provide

information in general about the magnitude of laboratory and method biases combined and, for the participant, information about total error on specific occasions. Statistics such as the robust standard deviation of the participants results for an analyte in a round of the test can in principle be used in a way similar to reproducibility standard deviations from collaborative trials, *i.e.*, to obtain a benchmark for overall uncertainty for comparison with individual estimates from single-laboratory validation. In practice, statistics from proficiency tests may be more difficult to access, because they are not systematically tabulated and published like collaborative trials, but only made available to participants. Of course, if such statistics are to be used they must refer to the appropriate matrix and concentration of the analyte. Individual participants in proficiency testing schemes can also gauge the validity of their estimated uncertainty by comparing their reported results with the assigned values of successive rounds[26]. This, however, is an ongoing activity and therefore not strictly within the purview of single-laboratory validation (which is a one-off event).

If an appropriate certified reference material is available, a single-laboratory test allows a laboratory to assess laboratory bias and method bias in combination, by analysing the CRM a number of times. The estimate of the combined bias is the difference between the mean result and the certified value.
Appropriate certified reference materials are not always available, so other materials may perforce have to be used. Materials left over from proficiency tests sometimes serve this purpose and, although the assigned values of the materials may have questionable uncertainties, their use certainly provides a check on overall bias. Specifically, proficiency test assigned values are generally chosen to provide a minimally biased estimate, so a test for significant bias against such a material is a sensible practice. A further alternative is to use spiking and recovery information[4] to provide estimates of these biases, although there may be unmeasurable sources of uncertainty associated with these techniques.

Currently the least recognised effect in validation is that due to matrix variation within the defined class of test material. The theoretical requirement for the estimation of this uncertainty component is for a representative collection of test materials to be analysed in a single run, their individual biases estimated, and the variance of these biases calculated. (Analysis in a single run means that higher level biases have no effect on the variance. If there is a wide concentration range involved, then allowance for the change in bias with concentration must be made.) If the representative materials are certified reference materials, the biases can be estimated directly as the differences between the results and the reference values, and the whole procedure is straightforward. In the more likely event that insufficient number of certified reference materials are available, recovery tests with a range of typical test materials may be resorted to, with due caution.

Currently there is very little quantitative information about the magnitude of uncertainties from this source, although in some instances they are suspected of being large.

## 5 Conduct of Validation Studies

The detailed design and execution of method validation studies is covered extensively elsewhere and will not be repeated here. However, the main principles are pertinent and are considered below:

It is essential that validation studies are representative. That is, studies should, as far as possible, be conducted to provide a realistic survey of the number and range of effects operating during normal use of the method, as well as to cover the concentration ranges and sample types within the scope of the method. Where a factor (such as ambient temperature) has varied representatively at random during the course of a precision experiment, for example, the effects of that factor appear directly in the observed variance and need no additional study unless further method optimisation is desirable.

In the context of method validation, "representative variation" means that the factor must take a distribution of values appropriate to the anticipated range of the parameter in question. For continuous measurable parameters, this may be a permitted range, stated uncertainty or expected range; for discontinuous factors, or factors with unpredictable effects such as sample matrix, a representative range corresponds to the variety of types or "factor levels" permitted or encountered in normal use of the method. Ideally, representativeness extends not only to the range of values, but to their distribution. Unfortunately, it is often uneconomic to arrange for full variation of many factors at many levels. For most practical purposes, however, tests based on extremes of the expected range, or on larger changes than anticipated, are an acceptable minimum.

In selecting factors for variation, it is important to ensure that the larger effects are 'exercised' as much as possible. For example, where day to day variation (perhaps arising from recalibration effects) is substantial compared to repeatability, two determinations on each of five days will provide a better estimate of intermediate precision than five determinations on each of two days. Ten single determinations on separate days will be better still, subject to sufficient control, though this will provide no additional information on within-day repeatability.

Clearly, in planning significance checks, any study should have sufficient power to detect such effects before they become practically important (that is, comparable

to the largest component of uncertainty).

In addition, the following considerations may be important:

- Where factors are known or suspected to interact, it is important to ensure that the effect of interaction is accounted for. This may be achieved either by ensuring random selection from different levels of interacting parameters, or by careful systematic design to obtain 'interaction' effects or covariance information.
- In carrying out studies of overall bias, it is important that the reference materials and values are relevant to the materials under routine test.

## 6 Extent of validation studies

The extent to which a laboratory has to undertake validation of a new, modified or unfamiliar method depends to a degree on the existing status of the method and the competence of the laboratory. Suggestions as to the extent of validation and verification measures for different circumstances are given below. Except where stated, it is assumed that the method is intended for routine use.

### 6.1 The laboratory is to use a "fully" validated method

The method has been studied in a collaborative trial and so the laboratory has to verify that it is capable of achieving the published performance characteristics of the method (or is otherwise able to fulfil the requirements of the analytical task). The laboratory should undertake precision studies, bias studies (including matrix variation studies), and possibly linearity studies, although some tests such as that for ruggedness may be omitted.

### 6.2 The laboratory is to use a fully validated method, but new matrix is to be used

The method has been studied in a collaborative trial and so the laboratory has to verify that the new matrix introduces no new sources of error into the system. The same range of validation as the previous is required.

### 6.3 The laboratory is to use a well-established, but not collaboratively studied, method

The same range of validation as the previous is required.

### 6.4 The method has been published in the scientific literature together with some analytical characteristics

The laboratory should undertake precision studies, bias studies (including matrix variation studies), ruggedness and linearity studies.

### 6.5 The method has been published in the scientific literature with no characteristics given or has been developed in-house

The laboratory should undertake precision studies, bias studies (including matrix variation studies), ruggedness and linearity studies.

### 6.6 The method is empirical

An empirical method is one in which the quantity estimated is simply the result found on following the stated procedure. This differs from measurements intended to assess method-independent quantities such as the concentration of a particular analyte in a sample, in that the method bias is conventionally zero, and matrix variation (that is , within the defined class) is irrelevant. Laboratory bias cannot be ignored, but is likely to be difficult to estimate by single-laboratory experiment. Moreover, reference materials are unlikely to be available. In the absence of collaborative trial data some estimate of interlaboratory precision could be obtained from a specially designed ruggedness study or estimated by using the Horwitz function.

### 6.7 The analysis is “ad hoc”

“Ad hoc” analysis is occasionally necessary to establish the general range of a value, without great expenditure and with low criticality. The effort that can go into validation is accordingly strictly limited. Bias should be studied by methods such as recovery estimation or analyte additions, and precision by replication.

### 6.8 Changes in staff and equipment

Important examples include: change in major instruments; new batches of very variable reagents (for example, polyclonal antibodies); changes made in the laboratory premises; methods used for the first time by new staff; or a validated method employed after a period of disuse. Here the essential action is to demonstrate that no deleterious changes have occurred. The minimum check is a

single bias test; a "before and after" experiment on typical test materials or control materials. In general, the tests carried out should reflect the possible impact of the change on the analytical procedure.

## 7 RECOMMENDATIONS

The following recommendations are made regarding the use of single-laboratory method validation:

- Wherever possible and practical a laboratory should use a method of analysis that has had its performance characteristics evaluated through a collaborative trial conforming to an international protocol.
- Where such methods are not available, a method must be validated in-house before being used to generate analytical data for a customer.
- Single-laboratory validation requires the laboratory to select appropriate characteristics for evaluation from the following: applicability, selectivity, calibration, accuracy, precision, range, limit of quantification, limit of detection, sensitivity, ruggedness and practicability. The laboratory must take account of customer requirements in choosing which characteristics are to be determined.
- Evidence that these characteristics have been assessed must be made available to customers of the laboratory if required by the customer.

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# ANNEX A: Notes on the requirements for study of method performance characteristics

The general requirements for the individual performance characteristics for a method are as follows.

## A1 Applicability

After validation the documentation should provide, in addition to any performance specification, the following information:

- the identity of the analyte, including speciation where appropriate (Example: 'total arsenic';
- the concentration range covered by the validation (Example: '0-50 ppm');
- a specification of the range of matrices of the test material covered by the validation (Example: 'seafood');
- a protocol, describing the equipment, reagents, procedure (including permissible variation in specified instructions, e.g., 'heat at 100 ± 5° for 30 ± 5 minutes'), calibration and quality procedures, and any special safety precautions required;
- the intended application and its critical uncertainty requirements (Example: 'The analysis of food for screening purposes. The standard uncertainty $u(c)$ of the result $c$ should be less than $0.1 \times c$.').

## A2 Selectivity

Selectivity is the degree to which a method can quantify the analyte accurately in the presence of interferents. Ideally, selectivity should be evaluated for any important interferent likely to be present. It is particularly important to check interferents which are likely, on chemical principles, to respond to the test. For example, colorimetric tests for ammonia might reasonably be expected to respond to primary aliphatic amines. It may be impracticable to consider or test every potential interferent; where that is the case, it is recommended that the likely worst cases are checked. As a general principle, selectivity should be sufficiently good for any interferences to be ignored.

In many types of analysis, selectivity is essentially a qualitative assessment based on the significance or otherwise of suitable tests for interference. However, there are useful quantitative measures. In particular, one quantitative measure is the selectivity index $b_{an}/b_{int}$, where $b_{an}$ is the sensitivity of the method (slope of the

calibration function) and $b_{int}$ the slope of the response independently produced by a potential interferent, provides a quantitative measure of interference. $b_{int}$ can be determined approximately by execution of the procedure on a matrix blank and the same blank spiked with the potential interferent at one appropriate concentration. If a matrix blank is unavailable, and a typical material used instead, $b_{int}$ can be estimated from such a simple experiment only under the assumption that mutual matrix effects are absent. Note that $b_{int}$ is more easily determined in the absence of the analyte because the effect might be confused with another type of interference when the sensitivity of the analyte is itself affected by the interferent (a matrix effect).

## A3 Calibration and linearity

With the exception of gross errors in preparation of calibration materials, calibration errors are usually (but not always) a minor component of the total uncertainty budget, and can usually be safely subsumed into various categories estimated by "top-down" methods. For example random errors resulting from calibration are part of the run bias, which is assessed as a whole, while systematic errors from that source may appear as laboratory bias, likewise assessed as a whole. Never-the-less, there are some characteristics of calibration that are useful to know at the outset of method validation, because they affect the strategy for the optimal development of the procedure. In this class are such questions as whether the calibration function plausibly (a) is linear, (b) passes through the origin and (c) is unaffected by the matrix of the test material. The procedures described here relate to calibration studies in validation, which are necessarily more exacting than calibration undertaken during routine analysis. For example, once it is established at validation that a calibration function is linear and passes through the origin, a much simpler calibration strategy can be used for routine use (for example, a two point repeated design). Errors from this simpler calibration strategy will normally be subsumed into higher level errors for validation purposes.

### A3.1 Linearity and intercept

Linearity can be tested informally by examination of a plot of residuals produced by linear regression of the responses on the concentrations in an appropriate calibration set. Any curved pattern suggests lack of fit due to a non-linear calibration function. A test of significance can be undertaken by comparing the lack-of-fit variance with that due to pure error. However, there are causes of lack of fit other than nonlinearity that can arise in certain types of analytical calibration, so the significance test must be used in conjunction with a residual plot. Despite its current widespread use as an indication of quality of fit, the correlation coefficient

is misleading and inappropriate as a test for linearity and should not be used.

Design is all-important in tests for lack of fit, because it is easy to confound nonlinearity with drift. Replicate measurements are needed to provide an estimate of pure error if there is no independent estimate. In the absence of specific guidance, the following should apply:

- there should be six or more calibrators;
- the calibrators should be evenly spaced over the concentration range of interest;
- the range should encompass 0-150% or 50-150% of the concentration likely to be encountered, depending on which of these is the more suitable;
- the calibrators should be run at least in duplicate, and preferably triplicate or more, in a random order.

After an exploratory fit with simple linear regression, the residuals should be examined for obvious patterns. Heteroscedasticity is quite common in analytical calibration and a pattern suggesting it means that the calibration data are best treated by weighted regression. Failure to use weighted regression in these circumstances could give rise to exaggerated errors at the low end of the calibration function.

The test for lack of fit can be carried out with either simple or weighted regression. A test for an intercept significantly different from zero can also be made on this data if there is no significant lack of fit.

### A3.2 Test for general matrix effect

It simplifies calibration enormously if the calibrators can be prepared as a simple solution of the analyte. The effects of a possible general matrix mismatch must be assessed in validation if this strategy is adopted. A test for general matrix effect can be made by applying the method of analyte additions (also called “standard additions”) to a test solution derived from a typical test material. The test should be done in a way that provides the same final dilution as the normal procedure produces, and the range of additions should encompass the same range as the procedure-defined calibration validation. If the calibration is linear the slopes of the usual calibration function and the analyte additions plot can be compared for significant difference. A lack of significance means that there is no detectable general matrix effect. If the calibration is not linear a more complex method is needed for a significance test, but a visual comparison at equal concentrations will usually suffice. A lack of significance in this test will often mean that the matrix

variation effect [Section A13] will also be absent.

### A3.3 Final calibration procedure

The calibration strategy as specified in the procedure may also need to be separately validated, although the errors involved will contribute to jointly estimated uncertainties. The important point here is that evaluation uncertainty estimated from the specific designs for linearity etc., will be smaller than those derived from the simpler calibration defined in the procedure protocol.

## A4 Trueness

### A4.1 Estimation of trueness

Trueness is the closeness of agreement between a test result and the accepted reference value of the property being measured. Trueness is stated quantitatively in terms of "bias"; with smaller bias indicating greater trueness. Bias is typically determined by comparing the response of the method to a reference material with the known value assigned to the material. Significance testing is recommended. Where the uncertainty in the reference value is not negligible, evaluation of the results should consider the reference material uncertainty as well as the statistical variability.

### A4.2 Conditions for trueness experiments

Bias can arise at different levels of organisation in an analytical system, for example, run bias, laboratory bias and method bias. It is important to remember which of these is being handled by the various methods of addressing bias. In particular:

- The mean of a series of analyses of a reference material, carried out wholly within a single run, gives information about the sum of method, laboratory and run effect for that particular run. Since the run effect is assumed to be random from run to run, the result will vary from run to run more than would be expected from the observable dispersion of the results, and this needs to be taken into account in the evaluation of the results (for example, by testing the measured bias against the among-runs standard deviation investigated separately).
- The mean of repeated analyses of a reference material in several runs, estimates the combined effect of method and laboratory bias in the

particular laboratory (except where the value is assigned using the particular method).

### A4.3 Reference values for trueness experiments

*A4.3.1 Certified reference materials (CRMs)*

CRMs are traceable to international standards with a known uncertainty and therefore can be used to address all aspects of bias (method, laboratory and within-laboratory) simultaneously, assuming that there is no matrix mismatch. CRMs should accordingly be used in validation of trueness where it is practicable to do so. It is important to ensure that the certified value uncertainties are sufficiently small to permit detection of a bias of important magnitude. Where they are not, the use of CRMs is still recommended, but additional checks should be carried out.

A typical trueness experiment generates a mean response on a reference material. In interpreting the result, the uncertainty associated with the certified value should be taken into account along with the uncertainty arising from statistical variation in the laboratory. The latter term may be based on the within-run, between-run, or an estimate of the between-laboratory standard deviation depending on the intent of the experiment. Statistical or materials. Where the certified value uncertainty is small, a Student's *t* test is normally carried out, using the appropriate precision term.

Where necessary and practicable, a number of suitable CRMs, with appropriate matrices and analyte concentrations, should be examined. Where this is done, and the uncertainties on the certified values are smaller than those on the analytical results, it would be reasonably safe to use simple regression to evaluate the results. In this way bias could be expressed as a function of concentration, and might appear as a non-zero intercept ("transitional" or constant bias) or as a non-unity slope ("rotational" or proportional bias). Due caution should be applied in interpreting the results where the range of matrices is large.

*4.3.2 Reference materials*

Where CRMs are not available, or as an addition to CRMs, use may be made of any material sufficiently well characterised for the purpose (a reference material[10]), bearing in mind always that while insignificant bias may not be proof of zero bias, significant bias on any material remains a cause for investigation. Examples of reference materials include: Materials characterised by a reference material producer, but whose values are not accompanied by an uncertainty statement or are otherwise qualified; materials characterised by a manufacturer of

the material; materials characterised in the laboratory for use as reference materials; materials subjected to a restricted round-robin exercise, or distributed in a proficiency test. While the traceability of these materials may be questionable, it would be far better to use them than to conduct no assessment for bias at all. The materials would be used in much the same way as CRMs, though with no stated uncertainty any significance test relies wholly on the observable precision of results.

*A4.3.3 Use of a reference method*

A reference method can in principle be used to test for bias in another method under validation. This is a useful option when checking an alternative to, or modification of, an established standard method already validated and in use in the laboratory. Both methods are used to analyse a number of typical test materials, preferably covering a useful range of concentration fairly evenly. Comparison of the results over the range by a suitable statistical method (for example, a paired *t*-test, with due checks for homogeneity of variance and normality) would demonstrate any bias between the methods.

*A4.3.4 Use of spiking/recovery*

In the absence of reference materials, or to support reference material studies, bias can be investigated by spiking and recovery. A typical test material is analysed by the method under validation both in its original state and after the addition (spiking) of a known mass of the analyte to the test portion. The difference between the two results as a proportion of the mass added is called the surrogate recovery or sometimes the marginal recovery. Recoveries significantly different from unity indicate that a bias is affecting the method. Strictly, recovery studies as described here only assess bias due to effects operating on the added analyte; the same effects do not necessarily apply to the same extent to the native analyte, and additional effects may apply to the native analyte. Spiking/recovery studies are accordingly very strongly subject to the observation that while good recovery is not a guarantee of trueness, poor recovery is certainly an indication of lack of trueness. Methods of handling spiking/recovery data have been covered in detail elsewhere.[4]

## A5 Precision

Precision is the closeness of agreement between independent test results obtained under stipulated conditions. It is usually specified in terms of standard deviation or relative standard deviation. The distinction between precision and bias is

fundamental, but depends on the level at which the analytical system is viewed. Thus from the viewpoint of a single determination, any deviation affecting the calibration for the run would be seen as a bias. From the point of view of the analyst reviewing a year's work, the run bias will be different every day and act like a random variable with an associated precision. The stipulated conditions for the estimation of precision take account of this change in view point.

For single laboratory validation, two sets of conditions are relevant: (a) precision under repeatability conditions, describing variations observed during a single run as expectation 0 and standard deviation $\sigma_r$, and (b) precision under run-to-run conditions, describing variations in run bias $\delta_{run}$ as expectation 0, standard deviation $\sigma_{run}$. Usually both of these sources of error are operating on individual analytical results, which therefore have a combined precision $\sigma_{tot} = \left(\sigma_r^2 / n + \sigma_{run}^2\right)^{1/2}$, where *n* is the number of repeat results averaged within a run for the reported result. The two precision estimates can be obtained most simply by analysing the selected test material in duplicate in a number of successive runs. The separate variance components can then be calculated by the application of one-way analysis of variance. Each duplicate analysis must be an independent execution of the procedure applied to a separate test portion. Alternatively the combined precision $\sigma_{tot}$ can be estimated directly by the analysis of the test material once in successive runs, and estimating the standard deviation from the usual equation. (Note that observed standard deviations are generally given the symbol *s*, to distinguish them from standard deviations σ).

It is important that the precision values are representative of likely test conditions. First, the variation in conditions among the runs must represent what would normally happen in the laboratory under routine use of the method. For instance, variations in reagent batches, analysts and instruments should be representative. Second, the test material used should be typical, in terms of matrix and (ideally) the state of comminution, of the materials likely to encountered in routine application. So actual test materials or, to a lesser degree, matrix-matched reference materials would be suitable, but standard solutions of the analyte would not. Note also that CRMs and prepared reference materials are frequently homogenised to a greater extent than typical test materials, and precision obtained from their analysis may accordingly under-estimate the variation that will be observed for test materials.

Precision very often varies with analyte concentration. Typical assumptions are i) that there is no change in precision with analyte level, or ii) that the standard deviation is proportional to, or linearly dependent on, analyte level. In both cases,

the assumption needs to be checked if the analyte level is expected to vary substantially (that is, by more than about 30% from its central value). The most economical experiment is likely to be a simple assessment of precision at or near the extremes of the operating range, together with a suitable statistical test for difference in variance. The F-test is appropriate for normally distributed error.

Precision data may be obtained for a wide variety of different sets of conditions in addition to the minimum of repeatability and between-run conditions indicated here, and it may be appropriate to acquire additional information. For example, it may be useful to the assessment of results, or for improving the measurement, to have an indication of separate operator and run effects, between or within-day effects or the precision attainable using one or several instruments. A range of different designs and statistical analysis techniques is available, and careful experimental design is strongly recommended in all such studies.

## A6 Recovery

Methods for estimating recovery are discussed in conjunction with methods of estimating trueness (above).

## A7 Range

The validated range is the interval of analyte concentration within which the method can be regarded as validated. It is important to realise that this range is not necessarily identical to the useful range of the calibration. While the calibration may cover a wide concentration range, the remainder of the validation (and usually much more important part in terms of uncertainty) will cover a more restricted range. In practice, most methods will be validated at only one or two levels of concentration. The validated range may be taken as a reasonable extrapolation from these points on the concentration scale.

When the use of the method focuses on a concentration of interest well above the detection limit, validation near that one critical level would be appropriate. It is impossible to define a general safe extrapolation of this result to other concentrations of the analyte, because much depends on the individual analytical system. Therefore the validation study report should state the range around the critical value in which the person carrying out the validation, using professional judgement, regards the estimated uncertainty to hold true.

When the concentration range of interest approaches zero, or the detection limit, it is incorrect to assume either constant absolute uncertainty or constant relative

uncertainty. A useful approximation in this common circumstance is to assume a linear functional relationship, with a positive intercept, between uncertainty *u* and concentration *c*, that is of the form

$$u(c) = u_0 + \theta c$$

where $\theta$ is the relative uncertainty estimated a some concentration well above the detection limit. $u_0$ is the standard uncertainty estimated for zero concentration and in some circumstances could be estimated as $c_L / 3$. In these circumstances it would be reasonable to regard the validated range as extending from zero to a small integer multiple of the upper validation point. Again this would depend on professional judgement.

## A8 Detection Limit

In broad terms the detection limit (limit of detection) is the smallest amount or concentration of analyte in the test sample that can be reliably distinguished from zero.[22,23] For analytical systems where the validation range does not include or approach it, the detection limit does not need to be part of a validation.

Despite the apparent simplicity of the idea, the whole subject of the detection limit is beset with problems outlined below:

- There are several possible conceptual approaches to the subject, each providing a somewhat different definition of the limit. Attempts to clarify the issue seem ever more confusing.
- Although each of these approaches depends on an estimate of precision at or near zero concentration, it is not clear whether this should be taken as implying repeatability conditions or some other condition for the estimation.

- Unless an inordinate amount of data is collected, estimates of detection limit will be subject to quite large random variation.
- Estimates of detection limit are often biased on the low side because of operational factors.
- Statistical inferences relating to the detection limit depend on the assumption of normality, which is at least questionable at low concentrations.

For most practical purposes in method validation, it seems better to opt for a

simple definition, leading to a quickly implemented estimation which is used only as a rough guide to the utility of the method. However, it must be recognised that the detection limit as estimated in method development, may not be identical in concept or numerical value to one used to characterise a complete analytical method. For instance the "instrumental detection limit", as quoted in the literature or in instrument brochures and then adjusted for dilution, is often far smaller than a "practical" detection limit and inappropriate for method validation.

It is accordingly recommended that for method validation, the precision estimate used ($\hat{\sigma}_0$) should be based on at least 6 independent complete determinations of analyte concentration in a typical matrix blank or low-level material, with no censoring of zero or negative results, and the approximate detection limit calculated as $3\hat{\sigma}_0$. Note that with the recommended minimum number of degrees of freedom, this value is quite uncertain, and may easily be in error by a factor of two. Where more rigorous estimates are required (for example to support decisions based on detection or otherwise of a material), reference should be made to appropriate guidance (see, for example, references 22-23).

## A9 Limit of determination or limit of quantification

It is sometimes useful to state a concentration below which the analytical method cannot operate with an acceptable precision. Sometimes that precision is arbitrarily defined as 10% RSD, sometimes the limit is equally arbitrarily taken as a fixed multiple (typically 2) of the detection limit. While it is to a degree reassuring to operate above such a limit, we must recognise that it is a quite artificial dichotomy of the concentration scale: measurements below such a limit are not devoid of information content and may well be fit for purpose. Hence the use of this type of limit in validation is not recommended here. It is preferable to try to express the uncertainty of measurement as a function of concentration and compare that function with a criterion of fitness for purpose agreed between the laboratory and the client or end-user of the data.

## A10 Sensitivity

The sensitivity of a method is the gradient of the calibration function. As this is usually arbitrary, depending on instrumental settings, it is not useful in validation. (It may be useful in quality assurance procedures, however, to test whether an instrument is performing to a consistent and satisfactory standard.)

## A11 Ruggedness

The ruggedness of an analytical method is the resistance to change in the results produced by an analytical method when minor deviations are made from the experimental conditions described in the procedure. The limits for experimental parameters should be prescribed in the method protocol (although this has not always been done in the past), and such permissible deviations, separately or in any combination, should produce no meaningful change in the results produced. (A "meaningful change" here would imply that the method could not operate within the agreed limits of uncertainty defining fitness for purpose.) The aspects of the method which are likely to affect results should be identified, and their influence on method performance evaluated by using ruggedness tests.
The ruggedness of a method is tested by deliberately introducing small changes to the procedure and examining the effect on the results. A number of aspects of the method may need to be considered, but because most of these will have a negligible effect it will normally be possible to vary several at once. An economical experiment based on fractional factorial designs has been described by Youden[13]. For instance, it is possible to formulate an approach utilising 8 combinations of 7 variable factors, that is to look at the effects of seven parameters with just eight analytical results. Univariate approaches are also feasible, where only one variable at a time is changed.

Examples of the factors that a ruggedness test could address are: changes in the instrument, operator, or brand of reagent; concentration of a reagent; pH of a solution; temperature of a reaction; time allowed for completion of a process etc.

## A12 Fitness for Purpose

Fitness for purpose is the extent to which the performance of a method matches the criteria, agreed between the analyst and the end-user of the data, that describe the end-user's needs. For instance the errors in data should not be of a magnitude that would give rise to incorrect decisions more often than a defined small probability, but they should not be so small that the end-user is involved in unnecessary expenditure. Fitness for purpose criteria could be based on some of the characteristics described in this Annex, but ultimately will be expressed in terms of acceptable total uncertainty.

## A13 Matrix variation

Matrix variation is, in many sectors, one of the most important but least acknowledged sources of error in analytical measurements. When we define the

analytical system to be validated by specifying, amongst other things, the matrix of the test material, there may be scope for considerable variation within the defined class. To cite an extreme example, a sample of the class "soil" could be composed of clay, sand, chalk, laterite (mainly $Fe_2O_3$ and $Al_2O_3$), peat, etc., or of mixtures of these. It is easy to imagine that each of these types would contribute a unique matrix effect on an analytical method such as atomic absorption spectrometry. If we have no information about the type of soils we are analysing, there will be an extra uncertainty in the results because of this variable matrix effect.

Matrix variation uncertainties need to be quantified separately, because they are not taken into account elsewhere in the process of validation. The information is acquired by collecting a representative set of the matrices likely to be encountered within the defined class, all with analyte concentrations in the appropriate range. The material are analysed according to the protocol, and the bias in the results estimated. Unless the test materials are CRMs, the bias estimate will usually have to be undertaken by means of spiking and recovery estimation. The uncertainty is estimated by the standard deviation of the biases. (Note: This estimate will also contain a variance contribution from the repeat analysis. This will have a magnitude $2\sigma_r^2$ if spiking has been used. If a strict uncertainty budget is required, this term should be deducted from the matrix variation variance to avoid double accounting.)

## A14 Measurement Uncertainty

The formal approach to measurement uncertainty estimation calculates a measurement uncertainty estimate from an equation, or mathematical model. The procedures described as method validation are designed to ensure that the equation used to estimate the result, with due allowance for random errors of all kinds, is a valid expression embodying all recognised and significant effects upon the result. It follows that, with one caveat elaborated further below, the equation or 'model' subjected to validation may be used directly to estimate measurement uncertainty. This is done by following established principles, based on the 'law of propagation of uncertainty' which, for independent input effects is

$$u(y(x_1,x_2,...)) = \sqrt{\sum_{i=1,n} c_i^2 u(x_i)^2}$$

where $y(x_1,x_2,....x_n)$ is a function of several independent variables $x_1,x_2...$, and $c_i$ is a sensitivity coefficient evaluated as $c_i=\partial y/\partial x_i$, the partial differential of $y$ with respect to $x_i$. $u(x_i)$ and $u(y)$ are *standard uncertainties*, that is, measurement

uncertainties expressed in the form of standard deviations. Since $u(y(x_1,x_2,...))$ is a function of several separate uncertainty estimates, it is referred to as a *combined standard uncertainty*.

To estimate measurement uncertainty from the equation $y=f(x_1,x_2...)$ used to calculate the result, therefore, it is necessary first, to establish the uncertainties $u(x_i)$ in each of the terms $x_1$, $x_2$ etc. and second, to combine these with the additional terms required to represent random effects as found in validation, and finally to take into account any additional effects. In the discussion of precision above, the implied statistical model is

$$y=f(x_1,x_2...) + \delta_{run} + e$$

where $e$ is the random error for a particular result. Since $\delta_{run}$ and $e$ are known, from the precision experiments, to have standard deviations $\sigma_{run}$ and $\sigma_r$ respectively, these latter terms (or, strictly, their estimates $s_{run}$ and $s_r$) are the uncertainties associated with these additional terms. Where the individual within-run results are averaged, the combined uncertainty associated with these two terms is (as given previously) $s_{tot} = \left(s_r^2/n + s_{run}^2\right)^{1/2}$. Note that where the precision terms are shown to vary with analyte level, the uncertainty estimate for a given result must employ the precision term appropriate to that level. The basis for the uncertainty estimate accordingly follows directly from the statistical model assumed and tested in validation. To this estimate must be added any further terms as necessary to account for (in particular) inhomogeneity and matrix effect (see section A13). Finally, the calculated standard uncertainty is multiplied by a 'coverage factor', $k$, to provide an expanded uncertainty, that is, "an interval expected to encompass a large fraction of the distribution of values that may be attributed to the measurand"[8]. Where the statistical model is well established, the distribution known to be normal, and the number of degrees of freedom associated with the estimate is high, $k$ is generally chosen to be equal to 2. The expanded uncertainty then corresponds approximately to a 95% confidence interval.

There is one important caveat to be added here. In testing the assumed statistical model, imperfect tests are perforce used. It has already been noted that these tests can not prove that any effect is identically zero; they can only show that an effect is too small to detect within the uncertainty associated with the particular test for significance. A particularly important example is the test for significant laboratory bias. Clearly, if this is the only test performed to confirm trueness, there must be some residual uncertainty as to whether the method is indeed unbiased or not. It follows that where such uncertainties are significant with respect to the uncertainty calculated so far, additional allowance should be made.

In the case of an uncertain reference value, the simplest allowance is the stated uncertainty for the material, combined with the statistical uncertainty in the test applied. A full discussion is beyond the scope of this text; reference 9 provides further detail. It is, however, important to note that while the uncertainty estimated directly from the assumed statistical model is the *minimum* uncertainty that can be associated with an analytical result, it will almost certainly be an underestimate; similarly, an expanded uncertainty based on the same considerations and using $k$=2 will not provide sufficient confidence.

The ISO Guide[8] recommends that for increased confidence, rather than arbitrarily adding terms, the value of $k$ should be increased as required. Practical experience suggests that for uncertainty estimates based on a validated statistical model, but with no evidence beyond the validation studies to provide additional confidence in the model, $k$ should not be less than 3. Where there is strong reason to doubt that the validation study is comprehensive, $k$ should be increased further as required.

# ANNEX B. Additional considerations for UNCERTAINTY ESTIMATION IN VALIDATION STUDIES

## B1 Sensitivity analysis

The basic expression used in uncertainty estimation

$$u(y(x_1,x_2,...)) = \sqrt{\sum_{i=1,n} c_i^2 u(x_i)^2}$$

requires the 'sensitivity coefficients' $c_i$. It is common in uncertainty estimation to find that while a given influence factor $x_i$ has a known uncertainty $u(x_i)$, the coefficient $c_i$ is insufficiently characterised or not readily obtainable from the equation for the result. This is particularly common where an effect is not included in the measurement equation because it is not normally significant, or because the relationship is not sufficiently understood to justify a correction. For example, the effect of solution temperature $T_{sol}$ on a room temperature extraction procedure is rarely established in detail.

Where it is desired to assess the uncertainty in a result associated with such an effect, it is possible to determine the coefficient experimentally. This is done most simply by changing $x_i$ and observing the effect on the result, in a manner very similar to basic ruggedness tests. In most cases, it is sufficient in the first instance to choose at most two values of $x_i$ other than the nominal value, and calculate an approximate gradient from the observed results. The gradient then gives an approximate value for $c_i$. The term $c_i.u(x_i)$ can then be determined. (Note that this is one practical method for demonstrating the significance or otherwise of a possible effect on the results).

In such an experiment, it is important that the change in result observed be sufficient for a reliable calculation of $c_i$. This is difficult to predict in advance. However, given a permitted range for the influence quantity $x_i$, or an expanded uncertainty for the quantity, that is expected to result in insignificant change, it is clearly important to assess $c_i$ from a larger range. It is accordingly recommended that for an influence quantity with an expected range of $\pm a$, (where $\pm a$ might be, for example, the permitted range, expanded uncertainty interval or 95% confidence interval) the sensitivity experiment employ, where possible, a change of at least $4a$ to ensure reliable results.

## B2 Judgement

It is not uncommon to find that while an effect is recognised and may be significant, it is not always possible to obtain a reliable estimate of uncertainty. In such circumstances, the ISO Guide makes it quite clear that a professionally considered estimate of the uncertainty is to be preferred to neglect of the uncertainty. Thus, where no estimate of uncertainty is available for a potentially important effect, the analyst should make their own best judgement of the likely uncertainty and apply that in estimating the combined uncertainty. Reference 8 gives further guidance on the use of judgement in uncertainty estimation.

# Recommendations on measurement uncertainty

(Resolution oeno 9/2005)

## INTRODUCTION

It is important that analysts are aware of the uncertainty associated with each analytical result and estimates of uncertainty. The measurement uncertainty may be derived by a number of procedures. Food analysis laboratories are required to be in control, use collaboratively tested methods when available, and verify their application before taking them into routine use. Such laboratories therefore have available to them a range of analytical data which can be used to estimate their measurement uncertainty.

### Terminology

The accepted definition for Measurement Uncertainty[1] is:
"Parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand.

NOTES:

1. The parameter may be, for example, a standard deviation (or a given multiple of it), or the half-width of an interval having a stated level of confidence.

2. Uncertainty of measurement comprises, in general, many components. Some of these components may be evaluated from the statistical distribution of results of a series of measurements and can be characterised by experimental standard deviations. The other components, which can also be characterised by standard deviations, are evaluated from assumed probability distributions based on experience or other information.

3. It is understood that the result of a measurement is the best estimate of the value of a measurand, and that all components of uncertainty, including those arising from systematic effects. Such as components associated with corrections and reference standards, contribute to the dispersion."

[*It is recognised that the term "measurement uncertainty" is the most widely used term by International Organisations and Accreditation Agencies. However The Codex ALIMENTARIUS Committee on Methods of Analysis and Sampling has*

*commented on a number of occasions that the term "Measurement Uncertainty" has some negative associations in legal context and so has noted that an alternative, equivalent, term, "measurement reliability", may be used.]*

**Recommendations**

The following recommendations are made to governments:

1. For OIV purposes the term "measurement uncertainty" or "measurement reliability" shall be used.

2. The measurement uncertainty or "measurement reliability" associated with all analytical results is to be estimated and must, on request be made available to the user (customer) of the results.

3. The measurement uncertainty or "measurement reliability" of an analytical result may be estimated in a number of procedures notably those described by ISO[1] and EURACHEM[2]. These documents recommend procedures based on a component-by-component approach, method validation data, internal quality control data and proficiency test data. The need to undertake an estimation of the measurement uncertainty or "Measurement reliability" using the ISO component-by-component approach is not necessary if the other forms of data are available and used to estimate the uncertainty or reliability. In many cases the overall uncertainty may be determined by an inter-laboratory (collaborative) study by a number of laboratories and a number of matrices by the IUPAC/ISO/AOAC INTERNATIONAL[3] or by the ISO 5725 Protocols[4].

## REFERENCES

1. "Guide to the Expression of Uncertainty in Measurement", ISO, Geneva, 1993.

2. EURACHEM/CITAC Guide Quantifying Uncertainty In Analytical Measurement (Second Edition), EURACHEM Secretariat, HAM, Berlin, 2000. This is available as a free download from http://www.vtt.fi/ket/eurachem.

3. "Protocol for the Design, Conduct and Interpretation of Method Performance Studies", ed. W. Horwitz, Pure Appl. Chem., 1995, 67, 331-343.

4. "Precision of Test Methods", Geneva, 1994, ISO 5725, Previous editions were published in 1981 and 1986.

# Recommendations related to the recovery correction

(Resolution OIV-Oeno 392/2009)

## Recovery

"The OIV recommends the following practice with regards to reporting recovery of analytical results.

- Analytical results are to be expressed on a recovery corrected basis where appropriate and relevant, and when corrected it has to be stated.

- If a result has been corrected for recovery, the method by which the recovery was taken into account should be stated. The recovery rate is to be quoted wherever possible.

- When laying down provisions for standards, it will be necessary to state whether the result obtained by a method used for analysis within conformity checks shall be expressed on a recovery-corrected basis or not."

**Method OIV-MA-AS2-01A** Type I methods

# Density and Specific Gravity at 20°C

## 1. Definition

Density is the mass per unit volume of wine or must at 20°C. It is expressed in grams per milliliter, and denoted by the symbol $\rho_{20°C}$.

Specific gravity at 20°C (or 2°C/2°C relative density) is the ratio, expressed as a decimal number, of the density of the wine or must at 20°C to the density of water at the same temperature, and is denoted by the symbol $d_{20°C}^{20°C}$

## 2. Principle

The density and specific gravity at 20°C are determined on the sample under test:

A. by pycnometry, or
B. by electronic densimetry using an oscillating cell
C. or by densimetry with a hydrostatic balance.

*Note:* For very accurate measurement, the density must be corrected for the presence of sulphur dioxide.

$\rho_{20} = \rho'_{20} - 0.0006 \times S$

$\rho_{20}$ = the corrected density
$\rho'_{20}$ = the observed density
S = total sulphur dioxide in g/l

## 3. Preliminary treatment of sample

If the wine or the must contains appreciable quantities of carbon dioxide, remove most of this by agitating 250 mL of wine in a 1000 mL flask, or by filtering under reduced pressure through 2 g of cotton wool placed in an extension tube.

## 4. Density and Specific Gravity at 20°C by pycnometry (Type I method)

4.1. *Apparatus*

Normal laboratory apparatus and in particular:

4.1.1 Pyrex glass pycnometer of approximately 100 mL capacity with a detachable ground glass thermometer graduated in tenths of a degree from 10 to 30°C. The thermometer must be standardized (fig 1).

Any pycnometer that is technically equivalent may be used.

The pycnometer has a side tube 25 mm in length and 1 mm (maximum) in internal diameter ending in a conical ground joint. The side tube may be capped by a "reservoir stopper" consisting of a conical ground-glass joint tube ending in a tapered section. The stopper serves as an expansion chamber.

The two ground joints of the apparatus should be prepared with care.

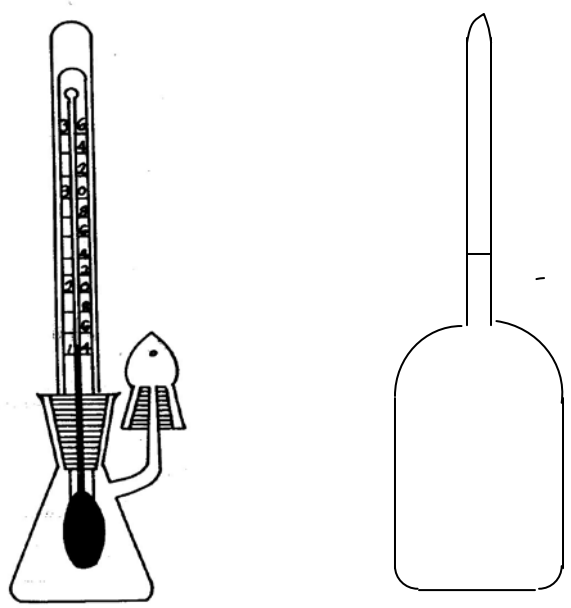

FIGURE 1: Pycnometer with tare flask

4.1.2 A tare flask of the same external volume (to within at least 1 mL) as the pycnometer and with a mass equal to the mass of the pycnometer filled with a liquid of specific gravity 1.01 (sodium chloride solution, 2% (*m/v)*).

A thermally insulated chamber exactly fitting the body of the pycnometer.

4.1.3 A two-pan balance, sensitive to one-tenth milligram, or a single-pan balance, sensitive to one-tenth of a milligram.

4.2. ***Calibration of the Pycnometer***

Calibration of the pycnometer involves determination of the following quantities:

- empty tare;
- volume of pycnometer at 20°C;
- mass of water filled pycnometer at 20°C.

4.2.1 Method using a two-pan balance

Place the tare flask on the left-hand pan of the balance and the pycnometer (clean and dry, with its "receiving stopper" fitted) on the right-hand pan, attain a balance by placing marked weights alongside the pycnometer, to give *p* grams.

Carefully fill the pycnometer with distilled water at ambient temperature. Insert the thermometer. Carefully wipe the pycnometer and place it in the thermally insulated container. Mix by inverting the container until the temperature reading on the thermometer is constant. Accurately adjust the level to the upper rim of the side tube. Wipe the side tube and put on the receiving stopper. Read temperature *t*°C with care and if necessary correct for the inaccuracy of the thermometer scale. Weigh the pycnometer full of water, against the tare and record *p'*, the mass in grams that gives an exact balance.

Calculations: *

Tare of the empty pycnometer:

Tare empty = $p + m$ $m$ = mass of air contained in pycnometer

$$m = 0.0012\ (p - p')$$

Volume at 20°C:

$V_{20°C} = (p + m - p') \times F_t$

$F_t$ = factor obtained from Table I for temperature *t*°C

$V_{20°C}$ must be known to the nearest ± 0.001 mL

Mass of water at 20°C:

$M_{20°C} = V_{20°C} \times 0.998203$

0.998203 = density of water at 20°C.

* A worked example is given in the Annex.

4.2.2 *Using a single-pan balance*

Determine:

- mass of clean dry pycnometer: P,
- mass of pycnometer full of water at *t*°C as described in 4.2.1: $P_1$
- mass of tare flask $T_0$.

Calculations: *

Taring of the empty pycnometer:

Tare empty pycnometer = P – *m*  *m* = mass of air contained in pycnometer

$m = 0.0012 (P_1 - P)$

Volume at 20°C:

$V_{20°C} = [P_1 - (P - m)] \times F_t$

$F_t$ = factor obtained from Table I for temperature *t*°C

$V_{20°C}$ must be known to the nearest ± 0.001 mL

Water mass at 20°C:

$M_{20°C} = V_{20°C} \times 0.998203$

0.998203 = density of water at 20°C.

### *4.3. **Method of measurement*** *

4.3.1 Using a two-pan balance

Weigh the pycnometer filled with the sample prepared for testing (3) as described in 4.2.1.

Let *p*'' be the mass in grams that achieves a balance at *t*°C.

Mass of the liquid in the pycnometer = *p* + *m* - *p*''

Apparent density at *t*°C:

$$\rho_{t^oC} = \frac{p + m - p''}{V_{20^oC}}$$

Calculate the density at 20°C using the appropriate correction table in accordance with the nature of the liquid being measured: dry wine (Table II), natural or concentrated must (Table III), sweet wine (Table IV).

The 20°C/20°C specific gravity of the wine is calculated by dividing the density at 20°C by 0.998203.

4.3.2 Using a single-pan balance *

* A worked example is given in the Annex.

Weigh the tare flask, let its mass be $T_1$;

Calculate $dT = T_1 - T_0$.

Mass of pycnometer empty at time of measurement = $P - m + dT$.

Weigh the pycnometer filled with the sample prepared for the test as described in 4.2.1. Let its mass at $t$°C be $P_2$

Mass of the liquid in the pycnometer at $t$°C = $P_2 - (P - m + dT)$.

Apparent density at $t$°C:

$$\rho_{t°C} = \frac{P_2 - (P - m + dT)}{V_{20°C}}$$

Calculate the density at 20°C of the liquid examined (dry wine, natural or concentrated must or sweet wine) using the correction tables as instructed in 4.3.1.

The 20°C/20°C specific gravity is obtained by dividing the density at 20°C by 0.998203.

4.3.3 *Repeatability* for density measurements
of dry and full bodied wines: $r = 0.00010$
of sweet wines: $r = 0.00018$

4.3.4 *Reproducibility* for density measurements
of dry and full bodied wines: $R = 0.00037$

of sweet wines: $R = 0.00045$*

## 5. Density at 20°C and specific gravity at 20°C measured by electronic densimetry using an oscillating cell

**5.1. Principle**

The density of the wine is measured by electronic densimetry using an oscillating cell. The principle consists of measuring the oscillation frequency of a tube containing the sample and subjected to an electromagnetic field. The density is related to the oscillation frequency by the following equation:

$$\rho = T^2 \times \left( \frac{C}{4\pi^2 V} \right) - \left( \frac{M}{V} \right) \qquad (1)$$

ρ = density of the sample
T = induced oscillation frequency
M = mass of the empty tube
C = spring constant
V = volume of the oscillated sample

This relationship is of the form: $\rho = A\ T^2 - B(2)$, there is therefore a linear relationship between the density and the square of the frequency. The constants A and B are specific for each oscillator and are estimated by measuring the period of fluids of known density.

**5.2. Equipment**

*5.2.1. Electronic oscillating cell densimeter*

The electronic densimeter consists of the following elements:

- a measuring cell containing a measuring tube and a temperature controller,
- a system for oscillating the tube and measuring the oscillation frequency,
- a timer,
- a digital display and if necessary a calculator.

The densimeter is placed on a perfectly stable support, isolated from all vibrations.

**5.3 Reagents and materials**

*5.3.1 Reference fluids*

Two reference fluids are used to adjust the densimeter. The densities of the reference fluids must include those of the wines to be measured. A difference in density between the reference fluids of more than 0.01000 g/ml is recommended.

The density must be known with an uncertainty of less than +/- 0.00005 g/ml, at a temperature of 20.00 +/- 0.05°C.

The reference fluids used to measure the density of the wines by electronic densimetry are:

- dry air (uncontaminated),
- double distilled water, or water of equivalent analytical purity,
- aqueous-alcoholic solutions, or wines whose density has been determined by pycnometry,
- solutions connected to national standards with a viscosity of less than 2 $mm^2/s$.

*5.3.2 Cleaning and drying products*

- detergents, acids, etc.
- organic solvents: ethanol 96% vol., pure acetone, etc.

**5.4 Equipment inspection and calibration**

*5.4.1 Temperature control of measuring cell*

The measuring tube is located in a temperature-controlled device. The variation in temperature must be less than +/- 0.02°C.

When provided as a feature by the densimeter, the temperature of the measuring cell must be controlled since it has a significant impact on the results of the determinations. The density of an aqueous-alcoholic solution with an alcoholic strength by volume (ASV) of 10% vol. is 0.98471 g/ml at 20°C and 0.98447 g/ml at 21°C, i.e. a difference of 0.00024 g/ml.

The test temperature is 20°C. The cell temperature is measured with a thermometer that offers a resolution of less than 0.01°C and connected to national standards. It must ensure that the temperature is measured with an uncertainty of less than +/- 0.07°C.

*5.4.2 Equipment calibration*

The equipment must be calibrated before being used for the first time, then every six months or if the verification is unsatisfactory. The objective is to use two reference fluids to calculate the constants A and B (cf. (2)). For details about the calibration refer to the instructions for the equipment. In principle, this calibration is carried out using dry air (taking atmospheric pressure into consideration) and very pure water (double-distilled and/or microfiltered with a very high resistivity, e.g. > 18 MΩ.cm).

*5.4.3 Verifying the calibration*

The calibration is verified by measuring the density of the reference fluids.

- An air density verification is performed every day. A difference between the theoretical and measured density of more than 0.00008 g/ml may indicate that the tube is soiled. It must then be cleaned. After cleaning, the air density is verified again, and if this verification does not comply then the equipment must be adjusted.
- The density of water must also be verified; if the difference between the theoretical and measured density is greater than 0.00008 g/ml then the apparatus must be adjusted.
    - If the verification of the cell temperature is problematic then the density of a hydroalcoholic solution whose density is comparable with those of the wines analysed can be checked directly.

*5.4.4 Checks*

When the difference between the theoretical density of a reference solution (known with an uncertainty of +/- 0.00005 g/ml) and the measured density is greater than 0.00008 g/ml then the calibration of the device must be checked.

**5.5. Procedure**

The operator must ensure that the temperature of the measuring cell is stable. The wine in the densimeter cell must not contain bubbles of gas and must be homogeneous. If an internal light can be used to check for the absence of bubbles, extinguish it quickly after performing the check since the heat generated by the lamp has an impact on the measured temperature.

If the equipment only gives the frequency, the density is calculated using the constants A and B (refer to the instructions for the equipment).

**5.6 Precision parameters for the density measuring method using an oscillating cell**

| | |
|---|---|
| n | 3800 |
| min | 0.99187 |
| max | 1.01233 |
| r | 0.00011 |
| r% | 0.011 |
| $s_r$ | 0.000038 |
| R | 0.00025 |

| | |
|---|---|
| $s_R$ | 0.000091 |
| R% | 0.025 |

*Key:*
*n*: *number of values selected*
*min*: *lower limit of range of measurement*
*max: upper limit of range of measurement*
*r*: *repeatability*
$s_r$*: Repeatability standard deviation*
*r%*: *Relative repeatability (*$s_r$ *x 100 / mean value)*
*R: reproducibility*
$s_R$: *Reproducibility standard deviation*
*R%*: *Relative reproducibility (*$s_R$ *x 100 / mean value)*

# 6. Density at 20°C and specific gravity at 20°C measured using the hydrostatic balance

## 6.1 Principle

The density of wine may be measured by densimetry with a hydrostatic balance which relies on the phenomenon defined by Archimedes' principle, namely that any object immersed in a fluid experiences an upwards force equal to the weight of the fluid displaced by the object.

## 6.2 Equipment and materials

Standard laboratory equipment, including:

*6.2.1 Single-pan hydrostatic balance with a precision of 1 mg.*

6.2.2 *Float with a volume of at least 20 ml*, specific to the balance, suspended by a thread with a diameter less than or equal to 0.1 mm.

6.2.3 *Measuring cylinder* with a level mark. The float must be capable of being completely contained in the volume below the mark; the surface of the liquid must be penetrated only by the supporting thread. The internal diameter of the measuring cylinder must be at least 6 mm more than that of the float.

6.2.4 *Thermometer* (or temperature probe) with degree and tenth of a degree graduations, from 10 to 40°C, calibrated to ± 0.06°C.

6.2.5 *Weights* calibrated by a recognised certification body.

**6.3 Reagents**

Unless otherwise indicated, only use analytical quality reagents during the analysis with at least class 3 water corresponding to the definition given in standard ISO 3696:1987.

*6.3.1 Washing solution for the float* (sodium hydroxide, 30% m/v).
To prepare 100 ml of solution, dissolve 30 g of sodium hydroxide in ethanol 96% vol.

**6.4 Procedure**

After each measurement, the float and the cylinder must be cleaned with distilled water, wiped with soft laboratory paper which does not shed its fibres and rinsed with the solution whose density is to be determined. The measurements must be performed when the equipment is stable so as to minimise alcohol loss through evaporation.

*6.4.1 Balance calibration*
Although balances usually have an internal calibration system, the hydrostatic balance must be calibrated with weights that are checked by an official certification body.

*6.4.2 Float calibration*
Fill the cylinder up to the mark with double-distilled water (or with water of equivalent purity, e.g. microfiltered water with a conductivity of 18.2 MΩ.cm), whose temperature must be between 15 and 25°C, and ideally at 20°C.

Immerse the float and the thermometer in the liquid, stir, read the density of the liquid indicated by the equipment, and, if necessary, adjust this reading such that it is equal to that of the water at the temperature at which the reading was taken.

*6.4.3.Verification using a solution of known density*

Fill the cylinder up to the mark with a solution of known density, whose temperature is between 15 and 25°C, and ideally at 20°C.

Immerse the float and the thermometer in the liquid, stir, read the density of the liquid indicated by the equipment and record the density and the temperature if the density is measured at t°C (ρ t)

*6.4.4 If necessary, correct ρ* using the table of densities ρ t for water-alcohol mixtures [Table II of Annex II of the OIV's Compendium of international analysis methods].
The density determined in this way must be identical to the previously determined density.

Note: This solution of known density can be used instead of double-distilled water for the calibration of the float.

*6.4.5 Measuring the density of a wine*
Pour the sample under test into the cylinder up to the mark.

Immerse the float and the thermometer in the liquid, stir, read the density of the liquid indicated by the apparatus. Record the temperature if the density is measured at t°C (ρ t).

Correct ρ using the table of densities ρ t for water-alcohol mixtures [Table II of Annex II of the OIV's Compendium of international analysis methods].

*6.4.6 Cleaning the float and the cylinder*.
Immerse the float in the washing solution poured into *the cylinder*.
Leave to soak for one hour, rotating the float frequently.
Rinse thoroughly with tap water, then with distilled water.
Wipe with soft laboratory paper that does not shed fibres.

Perform these operations when the float is used for the first time, and then regularly as required.

### 6.5 Precision parameters for measuring the density using the hydrostatic balance

| | |
|---|---|
| n | 4347 |
| min | 0.99189 |
| max | 1.01229 |
| r | 0.00025 |
| $s_r$ | 0.000090 |
| r% | 0.025 |
| R | 0.00067 |
| $s_R$ | 0.00024 |
| R% | 0.067 |

*Key:*
*n*: *number of values selected*
*min*: *lower limit of range of measurement*
*max: upper limit of range of measurement*
*r*: *repeatability*
$s_r$*: Repeatability standard deviation*
*r%*: *Relative repeatability (*$s_r$ *x 100 /mean value)*
*R: reproducibility*
$s_R$*: Reproducibility standard deviation*
*R%*: *Relative reproducibility (*$s_R$ *x 100 /mean value)*

### 6.6 Comparison of results for the density measuring methods using an oscillating cell or an hydrostatic balance

Using samples with a density between 0.992 and 1.012 g/ml repeatability and reproducibility were measured during an inter-laboratory ring test. The density of different samples as measured using the hydrostatic balance and the electronic densimeter and the repeatability and reproducibility values derived from an extensive multiannual inter-comparison exercise were compared.

*6.6.1. Samples*

Wines of different density and alcoholic strength prepared each month on an industrial scale, taken from a properly stored stock of bottles and delivered as anonymous products to the laboratories.

*6.6.2. Laboratories*

Laboratories participating in the monthly ring test organised by the Unione Italiana Vini (Verona, Italy) according to ISO 5725 (UNI 9225) rules and the International Protocol of Proficiency Testing for chemical analysis laboratories established by AOAC, ISO and IUPAC and ISO 43 and ILAC G13 guidelines. An annual report is supplied by this organisation to all participants.

*6.6.3. Equipment*

6.6.3.1. Electronic hydrostatic balance (accurate to 5 decimal places), if possible with a data processing device:

6.6.3.2. Electronic densimeter, if possible with autosampler.

*6.6.4. Analysis*

According to the rules for the validation of methods, each sample was analysed twice consecutively to determine the alcoholic strength.

*6.6.5. Result*

Table 1 shows the results of the measurements obtained by the laboratories using the hydrostatic balance.

Table 2 shows the results obtained by the laboratories using an electronic densimeter.

*6.6.6. Evaluations of the results*

6.6.6.1. The trial results were examined for evidence of individual systematic error ($p < 0,025$) using Cochran's and Grubb's tests successively, by procedures described in the internationally agreed Protocol for the Design, Conduct and Interpretation of Method-Performance Studies.

6.6.6.2. *Repeatability (r) and reproducibility (R)*

Calculations for repeatability (r) and reproducibility (R) as defined by that protocol were carried out on those results remaining after the removal of outliers. When assessing a new method there is often no validated reference or statutory method with which to compare precision criteria, hence it is useful to compare the precision data obtained from a collaborative trial with 'predicted' levels of precision. These 'predicted' levels are calculated from the Horwitz equation. Comparison of the trial results and the predicted levels give an indication as to whether the method is sufficiently precise for the level of analyte being measured. The Horwitz predicted value is calculated from the Horwitz equation.

$RSDR = 2^{(1-0,5 \log C)}$

where C = measured concentration of analyte expressed as a decimal (e.g. 1 g/100 g = 0.01).

The Horrat value gives a comparison of the actual precision measured with the precision predicted by the Horwitz equation for a method measuring at that particular level of analyte. It is calculated as follows:

HoR = RSDR(measured)/RSDR(Horwitz )

6.6.6.3. Interlaboratory precision

A Horrat value of 1 usually indicates satisfactory inter-laboratory precision, whereas a value of 2 usually indicates unsatisfactory precision, i.e. one that is too variable for most analytical purposes or where the variation obtained is greater than that expected for the type of method employed. Hor is also calculated, and used to assess intra-laboratory precision, using the following approximation:

RSDr(Horwitz) = 0,66 RSDR(Horwitz) (this assumes the approximation r = 0,66 R).

Table 3 shows the differences between the measurements obtained by laboratories using electronic densimetry and those using a hydrostatic balance.

6.6.6.4. *Precision parameters*

Table 4 shows the average overall precision parameters computed from all monthly trials carried out from January 2008 until December 2010.

| Sample | Mean | Total values | Values selected | Repetability | $s_r$ | RSDr | Hor | Reproducibility | $s_R$ | RSDRcalc | HoR | nº replies | CrD95 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 01/08 | 0,995491 | 130 | 120 | 0,0001701 | 0,0000607 | 0,0061016 | 0,0046193 | 0,0005979 | 0,0002135 | 0,0214502 | 0,0107178 | 2 | 0,0004141 |
| 02/08 | 1,011475 | 146 | 125 | 0,0004714 | 0,0001684 | 0,0166457 | 0,0126320 | 0,0008705 | 0,0003109 | 0,0307366 | 0,0153947 | 2 | 0,0005686 |
| 03/08 | 0,992473 | 174 | 161 | 0,0001470 | 0,0000525 | 0,0052898 | 0,0040029 | 0,0004311 | 0,0001540 | 0,0155140 | 0,0077482 | 2 | 0,0002959 |
| 04/08 | 0,993147 | 172 | 155 | 0,0002761 | 0,0000986 | 0,0099274 | 0,0075130 | 0,0005446 | 0,0001945 | 0,0195839 | 0,0097818 | 2 | 0,0003595 |
| 05/08 | 1,004836 | 150 | 138 | 0,0001882 | 0,0000672 | 0,0066905 | 0,0050723 | 0,0007495 | 0,0002677 | 0,0266373 | 0,0133283 | 2 | 0,0005215 |
| 06/08 | 0,993992 | 152 | 136 | 0,0001486 | 0,0000531 | 0,0053391 | 0,0040411 | 0,0005302 | 0,0001894 | 0,0190506 | 0,0095167 | 2 | 0,0003675 |
| 07/08 | 0,992447 | 162 | 150 | 0,0002660 | 0,0000950 | 0,0095709 | 0,0072424 | 0,0006046 | 0,0002159 | 0,0217575 | 0,0108664 | 2 | 0,0004063 |
| 08/08 | 0,992210 | 162 | 151 | 0,0002619 | 0,0000935 | 0,0094281 | 0,0071341 | 0,0006309 | 0,0002253 | 0,0227108 | 0,0113420 | 2 | 0,0004265 |
| 09/08 | 1,002600 | 148 | 131 | 0,0001093 | 0,0000390 | 0,0038920 | 0,0029496 | 0,0007000 | 0,0002500 | 0,0249341 | 0,0124719 | 2 | 0,0004919 |
| 10/08 | 0,994482 | 174 | 152 | 0,0001228 | 0,0000439 | 0,0044105 | 0,0033385 | 0,0004250 | 0,0001518 | 0,0152645 | 0,0076259 | 2 | 0,0002942 |
| 11/08 | 0,992010 | 136 | 125 | 0,0000909 | 0,0000325 | 0,0032742 | 0,0024775 | 0,0004256 | 0,0001520 | 0,0153217 | 0,0076516 | 2 | 0,0002975 |
| 01/09 | 0,994184 | 174 | 152 | 0,0001655 | 0,0000591 | 0,0059435 | 0,0044987 | 0,0005439 | 0,0001942 | 0,0195384 | 0,0097606 | 2 | 0,0003756 |
| 02/09 | 0,992266 | 118 | 101 | 0,0001742 | 0,0000622 | 0,0062682 | 0,0047431 | 0,0005210 | 0,0001861 | 0,0187534 | 0,0093658 | 2 | 0,0003580 |
| 03/09 | 0,991886 | 164 | 135 | 0,0001850 | 0,0000661 | 0,0066603 | 0,0050395 | 0,0004781 | 0,0001707 | 0,0172136 | 0,0085963 | 2 | 0,0003251 |
| 04/09 | 0,993632 | 180 | 150 | 0,0001523 | 0,0000544 | 0,0054754 | 0,0041440 | 0,0004270 | 0,0001525 | 0,0153476 | 0,0076664 | 2 | 0,0002922 |
| 05/09 | 1,011061 | 116 | 100 | 0,0003659 | 0,0001307 | 0,0129234 | 0,0098067 | 0,0008338 | 0,0002978 | 0,0294527 | 0,0147508 | 2 | 0,0005605 |
| 06/09 | 0,992063 | 114 | 105 | 0,0002923 | 0,0001044 | 0,0105238 | 0,0079631 | 0,0005257 | 0,0001877 | 0,0189240 | 0,0094507 | 2 | 0,0003418 |
| 07/09 | 0,992708 | 172 | 155 | 0,0002892 | 0,0001033 | 0,0104040 | 0,0078732 | 0,0006156 | 0,0002199 | 0,0221478 | 0,0110617 | 2 | 0,0004106 |
| 08/09 | 0,993064 | 136 | 127 | 0,0002926 | 0,0001045 | 0,0105224 | 0,0079632 | 0,0007520 | 0,0002686 | 0,0270446 | 0,0135081 | 2 | 0,0005112 |
| 09/09 | 1,005285 | 118 | 110 | 0,0002946 | 0,0001052 | 0,0104661 | 0,0079352 | 0,0007226 | 0,0002581 | 0,0256704 | 0,0128454 | 2 | 0,0004892 |
| 10/09 | 0,992905 | 150 | 132 | 0,0002234 | 0,0000798 | 0,0080358 | 0,0060812 | 0,0004498 | 0,0001607 | 0,0161803 | 0,0080815 | 2 | 0,0002978 |
| 11/09 | 0,994016 | 142 | 127 | 0,0001896 | 0,0000677 | 0,0068114 | 0,0051555 | 0,0004739 | 0,0001693 | 0,0170278 | 0,0085062 | 2 | 0,0003214 |
| 01/10 | 0,994734 | 170 | 152 | 0,0002125 | 0,0000759 | 0,0076288 | 0,0057748 | 0,0005406 | 0,0001931 | 0,0194104 | 0,0096975 | 2 | 0,0003672 |
| 02/10 | 0,993177 | 120 | 110 | 0,0002210 | 0,0000789 | 0,0079467 | 0,0060140 | 0,0005800 | 0,0002071 | 0,0208565 | 0,0104175 | 2 | 0,0003950 |
| 03/10 | 0,992799 | 148 | 136 | 0,0002277 | 0,0000813 | 0,0081923 | 0,0061995 | 0,0015157 | 0,0005413 | 0,0545262 | 0,0272335 | 2 | 0,0010657 |
| 04/10 | 0,995420 | 172 | 157 | 0,0002644 | 0,0000944 | 0,0094866 | 0,0071819 | 0,0006286 | 0,0002245 | 0,0225542 | 0,0112693 | 2 | 0,0004244 |
| 05/10 | 1,002963 | 120 | 108 | 0,0007086 | 0,0002531 | 0,0252330 | 0,0191244 | 0,0013667 | 0,0004881 | 0,0486677 | 0,0243447 | 2 | 0,0008991 |
| 06/10 | 0,992546 | 120 | 113 | 0,0001737 | 0,0000620 | 0,0062506 | 0,0047300 | 0,0005435 | 0,0001941 | 0,0195567 | 0,0097673 | 2 | 0,0003744 |
| 07/10 | 0,992831 | 174 | 152 | 0,0003003 | 0,0001073 | 0,0108031 | 0,0081753 | 0,0006976 | 0,0002492 | 0,0250959 | 0,0125344 | 2 | 0,0004699 |
| 08/10 | 0,993184 | 144 | 130 | 0,0001799 | 0,0000642 | 0,0064674 | 0,0048945 | 0,0005951 | 0,0002125 | 0,0213984 | 0,0106882 | 2 | 0,0004111 |
| 09/10 | 1,012293 | 114 | 103 | 0,0002265 | 0,0000809 | 0,0079907 | 0,0060647 | 0,0014586 | 0,0005209 | 0,0514596 | 0,0257772 | 2 | 0,0010251 |
| 10/10 | 0,992289 | 154 | 136 | 0,0006386 | 0,0002281 | 0,0229860 | 0,0173933 | 0,0007033 | 0,0002512 | 0,0253124 | 0,0126415 | 2 | 0,0003812 |
| 11/10 | 0,994649 | 130 | 112 | 0,0002902 | 0,0001036 | 0,0104200 | 0,0078876 | 0,0005287 | 0,0001888 | 0,0189830 | 0,0094838 | 2 | 0,0003445 |

*Table 1: Hydrostatic balance (HB)*

*Table 2: Electronic densimetry (ED)*

| Sample | Mean | Total values | Values selected | Repetability | $s_r$ | RSDr | Hor | Reproducibility | $s_R$ | RSDRcalc | HoR | n° replies | CrD95 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 01/08 | 0,995504 | 114 | 108 | 0,0000755 | 0,0000270 | 0,0027085 | 0,0020505 | 0,0001571 | 0,0000561 | 0,0056361 | 0,0028162 | 2 | 0,0001045 |
| 02/08 | 1,011493 | 132 | 125 | 0,0001921 | 0,0000686 | 0,0067837 | 0,0051480 | 0,0004435 | 0,0001584 | 0,0156582 | 0,0078426 | 2 | 0,0002985 |
| 03/08 | 0,992491 | 138 | 118 | 0,0000746 | 0,0000266 | 0,0026830 | 0,0020303 | 0,0002745 | 0,0000980 | 0,0098776 | 0,0049332 | 2 | 0,0001905 |
| 04/08 | 0,993129 | 132 | 120 | 0,0001230 | 0,0000439 | 0,0044247 | 0,0033486 | 0,0002863 | 0,0001023 | 0,0102965 | 0,0051429 | 2 | 0,0001929 |
| 05/08 | 1,004892 | 136 | 116 | 0,0000926 | 0,0000331 | 0,0032893 | 0,0024937 | 0,0004777 | 0,0001706 | 0,0169785 | 0,0084955 | 2 | 0,0003346 |
| 06/08 | 0,994063 | 142 | 123 | 0,0000558 | 0,0000199 | 0,0020051 | 0,0015177 | 0,0001776 | 0,0000634 | 0,0063791 | 0,0031867 | 2 | 0,0001224 |
| 07/08 | 0,992498 | 136 | 125 | 0,0000822 | 0,0000294 | 0,0029576 | 0,0022381 | 0,0002094 | 0,0000748 | 0,0075368 | 0,0037641 | 2 | 0,0001423 |
| 08/08 | 0,992270 | 130 | 115 | 0,0000515 | 0,0000184 | 0,0018537 | 0,0014027 | 0,0001665 | 0,0000595 | 0,0059940 | 0,0029935 | 2 | 0,0001149 |
| 09/08 | 1,002603 | 136 | 121 | 0,0000821 | 0,0000293 | 0,0029236 | 0,0022157 | 0,0003328 | 0,0001189 | 0,0118565 | 0,0059306 | 2 | 0,0002318 |
| **10/08** | **0,994493** | **128** | **117** | **0,0000667** | **0,0000238** | **0,0023954** | **0,0018132** | **0,0001429** | **0,0000510** | **0,0051309** | **0,0025633** | **2** | **0,0000954** |
| 11/08 | 0,992017 | 118 | 104 | 0,0000842 | 0,0000301 | 0,0030309 | 0,0022933 | 0,0001962 | 0,0000701 | 0,0070644 | 0,0035279 | 2 | 0,0001322 |
| 01/09 | 0,994216 | 148 | 131 | 0,0000830 | 0,0000297 | 0,0029832 | 0,0022580 | 0,0001551 | 0,0000554 | 0,0055712 | 0,0027832 | 2 | 0,0001015 |
| 02/09 | 0,992251 | 104 | 88 | 0,0000947 | 0,0000338 | 0,0034097 | 0,0025801 | 0,0002846 | 0,0001017 | 0,0102451 | 0,0051165 | 2 | 0,0001956 |
| 03/09 | 0,991875 | 126 | 108 | 0,0001271 | 0,0000454 | 0,0045777 | 0,0034637 | 0,0002067 | 0,0000738 | 0,0074421 | 0,0037165 | 2 | 0,0001316 |
| 04/09 | 0,993654 | 134 | 114 | 0,0001166 | 0,0000416 | 0,0041899 | 0,0031711 | 0,0002043 | 0,0000730 | 0,0073417 | 0,0036673 | 2 | 0,0001322 |
| 05/09 | 1,011035 | 128 | 104 | 0,0002388 | 0,0000853 | 0,0084361 | 0,0064016 | 0,0003554 | 0,0001269 | 0,0125542 | 0,0062875 | 2 | 0,0002211 |
| 06/09 | 0,992104 | 116 | 106 | 0,0001005 | 0,0000359 | 0,0036178 | 0,0027375 | 0,0003169 | 0,0001132 | 0,0114088 | 0,0056976 | 2 | 0,0002184 |
| 07/09 | 0,992720 | 144 | 140 | 0,0001579 | 0,0000564 | 0,0056815 | 0,0042995 | 0,0002916 | 0,0001042 | 0,0104923 | 0,0052404 | 2 | 0,0001905 |
| 08/09 | 0,993139 | 110 | 102 | 0,0001175 | 0,0000420 | 0,0042242 | 0,0031969 | 0,0003603 | 0,0001287 | 0,0129577 | 0,0064721 | 2 | 0,0002479 |
| 09/09 | 1,005276 | 112 | 108 | 0,0001100 | 0,0000393 | 0,0039070 | 0,0029622 | 0,0003522 | 0,0001258 | 0,0125134 | 0,0062617 | 2 | 0,0002429 |
| 10/09 | 0,992912 | 122 | 111 | 0,0000705 | 0,0000252 | 0,0025365 | 0,0019195 | 0,0002122 | 0,0000758 | 0,0076315 | 0,0038117 | 2 | 0,0001458 |
| 11/09 | 0,994031 | 128 | 118 | 0,0000718 | 0,0000256 | 0,0025784 | 0,0019516 | 0,0001639 | 0,0000585 | 0,0058883 | 0,0029415 | 2 | 0,0001102 |
| 01/10 | 0,994752 | 144 | 136 | 0,0000773 | 0,0000276 | 0,0027765 | 0,0021017 | 0,0001787 | 0,0000638 | 0,0064144 | 0,0032046 | 2 | 0,0001203 |
| 02/10 | 0,993181 | 108 | 98 | 0,0001471 | 0,0000525 | 0,0052893 | 0,0040029 | 0,0001693 | 0,0000605 | 0,0060884 | 0,0030410 | 2 | 0,0000945 |
| 03/10 | 0,992665 | 140 | 127 | 0,0001714 | 0,0000612 | 0,0061683 | 0,0046678 | 0,0002378 | 0,0000849 | 0,0085559 | 0,0042732 | 2 | 0,0001447 |
| 04/10 | 0,995502 | 142 | 128 | 0,0001175 | 0,0000419 | 0,0042138 | 0,0031901 | 0,0002320 | 0,0000829 | 0,0083248 | 0,0041596 | 2 | 0,0001532 |
| 05/10 | 1,002851 | 130 | 119 | 0,0001195 | 0,0000427 | 0,0042555 | 0,0032253 | 0,0002971 | 0,0001061 | 0,0105815 | 0,0052930 | 2 | 0,0002014 |
| 06/10 | 0,992607 | 106 | 99 | 0,0001228 | 0,0000438 | 0,0044172 | 0,0033427 | 0,0002226 | 0,0000795 | 0,0080092 | 0,0040001 | 2 | 0,0001449 |
| 07/10 | 0,992871 | 160 | 150 | 0,0001438 | 0,0000513 | 0,0051712 | 0,0039134 | 0,0003732 | 0,0001333 | 0,0134258 | 0,0067057 | 2 | 0,0002539 |
| 08/10 | 0,993235 | 104 | 93 | 0,0000895 | 0,0000320 | 0,0032182 | 0,0024356 | 0,0002458 | 0,0000878 | 0,0088399 | 0,0044154 | 2 | 0,0001680 |
| 09/10 | 1,012328 | 112 | 105 | 0,0000870 | 0,0000311 | 0,0030692 | 0,0023295 | 0,0003395 | 0,0001213 | 0,0119781 | 0,0060001 | 2 | 0,0002361 |
| 10/10 | 0,992308 | 128 | 115 | 0,0000606 | 0,0000216 | 0,0021811 | 0,0016504 | 0,0001635 | 0,0000584 | 0,0058845 | 0,0029388 | 2 | 0,0001116 |
| 11/10 | 0,994683 | 120 | 108 | 0,0001127 | 0,0000402 | 0,0040450 | 0,0030620 | 0,0001597 | 0,0000570 | 0,0057339 | 0,0028647 | 2 | 0,0000979 |

*Table 3: Comparison of results between hydrostatic balance (HB) and electronic densimetry (DE)*

| Density - Hydrostatic balance | | | | Density - Oscillating cell | | | | Comparision |
|---|---|---|---|---|---|---|---|---|
| Sample | Mean value | Total values | Selected values | Échantillon | Mean value | Total values | Selected values | Δ(Bi-DE) |
| 01/08 | 0,995491 | 130 | 120 | 01/08 | 0,995504 | 114 | 108 | -0,000013 |
| 02/08 | 1,011475 | 146 | 125 | 02/08 | 1,011493 | 132 | 125 | -0,000018 |
| 03/08 | 0,992473 | 174 | 161 | 03/08 | 0,992491 | 138 | 118 | -0,000018 |
| 04/08 | 0,993147 | 172 | 155 | 04/08 | 0,993129 | 132 | 120 | 0,000018 |
| 05/08 | 1,004836 | 150 | 138 | 05/08 | 1,004892 | 136 | 116 | -0,000056 |
| 06/08 | 0,993992 | 152 | 136 | 06/08 | 0,994063 | 142 | 123 | -0,000071 |
| 07/08 | 0,992447 | 162 | 150 | 07/08 | 0,992498 | 136 | 125 | -0,000051 |
| 08/08 | 0,992210 | 162 | 151 | 08/08 | 0,992270 | 130 | 115 | -0,000060 |
| 09/08 | 1,002600 | 148 | 131 | 09/08 | 1,002603 | 136 | 121 | -0,000003 |
| 10/08 | 0,994482 | 174 | 152 | 10/08 | 0,994493 | 128 | 117 | -0,000011 |
| 11/08 | 0,992010 | 136 | 125 | 11/08 | 0,992017 | 118 | 104 | -0,000007 |
| 01/09 | 0,994184 | 174 | 152 | 01/09 | 0,994216 | 148 | 131 | -0,000031 |
| 02/09 | 0,992266 | 118 | 101 | 02/09 | 0,992251 | 104 | 88 | 0,000015 |
| 03/09 | 0,991886 | 164 | 135 | 03/09 | 0,991875 | 126 | 108 | 0,000011 |
| 04/09 | 0,993632 | 180 | 150 | 04/09 | 0,993654 | 134 | 114 | -0,000022 |
| 05/09 | 1,011061 | 116 | 100 | 05/09 | 1,011035 | 128 | 104 | 0,000026 |
| 06/09 | 0,992063 | 114 | 105 | 06/09 | 0,992104 | 116 | 106 | -0,000041 |
| 07/09 | 0,992708 | 172 | 155 | 07/09 | 0,992720 | 144 | 140 | -0,000012 |
| 08/09 | 0,993064 | 136 | 127 | 08/09 | 0,993139 | 110 | 102 | -0,000075 |
| 09/09 | 1,005285 | 118 | 110 | 09/09 | 1,005276 | 112 | 108 | 0,000009 |
| 10/09 | 0,992905 | 150 | 132 | 10/09 | 0,992912 | 122 | 111 | -0,000008 |
| 11/09 | 0,994016 | 142 | 127 | 11/09 | 0,994031 | 128 | 118 | -0,000015 |
| 01/10 | 0,994734 | 170 | 152 | 01/10 | 0,994752 | 144 | 136 | -0,000018 |
| 02/10 | 0,993177 | 120 | 110 | 02/10 | 0,993181 | 108 | 98 | -0,000005 |
| 03/10 | 0,992799 | 148 | 136 | 03/10 | 0,992665 | 140 | 127 | 0,000134 |
| 04/10 | 0,995420 | 172 | 157 | 04/10 | 0,995502 | 142 | 128 | -0,000082 |
| 05/10 | 1,002963 | 120 | 108 | 05/10 | 1,002851 | 130 | 119 | 0,000112 |
| 06/10 | 0,992546 | 120 | 113 | 06/10 | 0,992607 | 106 | 99 | -0,000061 |
| 07/10 | 0,992831 | 174 | 152 | 07/10 | 0,992871 | 160 | 150 | -0,000040 |
| 08/10 | 0,993184 | 144 | 130 | 08/10 | 0,993235 | 104 | 93 | -0,000052 |
| 09/10 | 1,012293 | 114 | 103 | 09/10 | 1,012328 | 112 | 105 | -0,000035 |
| 10/10 | 0,992289 | 154 | 136 | 10/10 | 0,992308 | 128 | 115 | -0,000019 |
| 11/10 | 0,994649 | 130 | 112 | 11/10 | 0,994683 | 120 | 108 | -0,000035 |
| | | | | | | average | Δ(Bi-DE) | -0,0000162 |
| | | | | | | Std. dev. | Δ(Bi-DE) | 0,0000447 |

*Table 4: Precision parameters*

| | *hydrostatic balance (HB)* | *electronic densimetry (DE)* |
|---|---|---|
| n° selected values | 4347 | 3800 |
| min | 0,99189 | 0,99187 |
| max | 1,01229 | 1,01233 |
| R | 0,00067 | 0,00025 |
| $s_R$ | 0,00024 | 0,000091 |
| R% | 0,067 | 0,025 |
| r | 0,00025 | 0,00011 |
| $s_r$ | 0,000090 | 0,000038 |
| r% | 0,025 | 0,011 |

ANNEX I

**(worked example)**

**I. Pycnometry with twin-pan balance**

A/ Standardization of the pycnometer

1. Weigh a clean and dry pycnometer:

   Tare = pycnometer + $p$
   $p$= 104.9454 g

2. Weigh pycnometer filled with water at temperature $t$°C:

   Tare = pycnometer + water + $p'$
   $p'$ = 1.2396 g at $t$ = 20.5°C

3. Calculate mass of air within the pycnometer:

   $m = 0.0012\ (p - p')$
   $m = 0.0012\ (104.9454 - 1.2396)$
   $m = 0.1244$

4. Values to record:

   Tare of empty pycnometer: $p + m$
   $p + m = 104.9454 + 0.1244$
   $p + m = 105.0698$ g

   Volume at 20°C = $(p + m - p') \times F_{t°C}$

   $F_{20.50°C} = 1.001900$
   $V_{20°C} = (105.0698 - 1.2396) \times 1.001900$
   $V_{20°C} = 104.0275$ mL

   Mass of water at 20°C = $V_{20°C} \times 0.998203$
   $M_{20°C} = 103.8405$ g

B/. Determination of density at 20°C and 20°C/20°C density for dry wine:

$p''$ = 1.2622 at 17.80°C

$$\rho_{17.80°C} = \frac{105.0698 - 1.2622}{104.0275}$$

$$\rho_{17.80°C} = 0.99788$$

$p_{20°C}$ can be calculated from $\rho_{t°C}$ using Table II and the equation:

$$\rho_{20°C} = \rho_{t°C} \pm \frac{c}{1000}$$

At $t$ = 17.80°C and for an alcoholic strength of 11% vol., $c$ = 0.54:

$$\rho_{20°C} = 0.99788 \pm \frac{0.54}{1000}$$

$$\rho_{20°C} = 0.99734\ \text{g/mL}$$

$$d^{20°C}_{20°C} = \frac{0.99734}{0.998203} + 0.99913$$

**II. Pycnometry with single-pan balance**

A/ Standardization of the pycnometer

1. Mass of clean and dry pycnometer:
   P = 67.7913 g
2. Mass pycnometer filled with water at temperature *t*°C:
   $P_1$ = 169.2715g at 21.65°C
3. Calculate mass of air within the pycnometer:
   *m* = 0.0012 ($P_1$ - P)
   *m* = 0.0012 x 101.4802
   *m* = 0.1218g
4. Values to record:
   Tare of empty pycnometer: P - *m*
   P - *m* = 67.7913 - 0.1218
   P - *m* = 67.6695 g
   Volume at 20°C = [$P_1$ - (P - *m*)] x $F_{t°C}$
   $F_{21.65°C}$ = 1.002140
   $V_{20°C}$ = (169.2715 - 67.6695) x 1.002140
   $V_{20°C}$ = 101.8194 mL

Mass of water at 20°C = $V_{20°C}$ x 0.998203
$M_{20°C}$ = 101.6364 g
Mass of tare flask: $T_0$
$T_0$ = 171.9160 g

B/ *Determination of density at 20°C and 20°C/20°C specific gravity for a dry wine:*

$T_1$ = 171.9178
*d*T = 171.9178 - 171.9160 = +0.0018 g
P - *m* + *d*T = 67.6695 + 0.0018 = 67.6713 g
$P_2$ = 169.2799 at 18°C

$$\rho_{18°C} = \frac{169.2799 - 67.6713}{101.8194}$$

$\rho_{18°C}$ = 0.99793 g/mL

$\rho_{20°C}$ can be calculated from $\rho_{t°C}$ using Table II and the equation:

$$\rho_{20°C} = \rho_{t°C} \pm \frac{c}{1000}$$

For *t* = 18°C and an alcoholic strength of 11% vol., *c* = 0.49:

$$\rho_{20°C} = 0.99793 - \frac{0.49}{1000}$$

$\rho_{20°C}$ = 0.99744 g/mL

$$d_{20C}^{20C} = \frac{0.99744}{0.998203} = 0.99923$$

ANNEX II
Tables

TABLE I

F Factors
by which the mass of the water in the *Pyrex pycnometer* at *t*°C has to be multiplied to calculate the volume of the pycnometer at 20°C.

| *t* °C | F | *t* °C | F | *t* °C | F | *t* °C | F | *t* °C | F | *t* °C | F | *t* °C | F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10.0 | 1.000398 | 13.0 | 1.000691 | 16.0 | 1.001097 | 19.0 | 1.001608 | 22.0 | 1.002215 | 25.0 | 1.002916 | 28.0 | 1.003704 |
| .1 | 1.000406 | .1 | 1.000703 | .1 | 1.001113 | .1 | 1.001627 | .1 | 1.002238 | .1 | 1.002941 | .1 | 1.003731 |
| .2 | 1.000414 | .2 | 1.000714 | .2 | 1.001128 | .2 | 1.001646 | .2 | 1.002260 | .2 | 1.002966 | .2 | 1.003759 |
| .3 | 1.000422 | .3 | 1.000726 | .3 | 1.001144 | .3 | 1.001665 | .3 | 1.002282 | .3 | 1.002990 | .3 | 1.003797 |
| .4 | 1.000430 | .4 | 1.000738 | .4 | 1.001159 | .4 | 1.001684 | .4 | 1.002304 | .4 | 1.003015 | .4 | 1.003815 |
| 10.5 | 1.000439 | 13.5 | 1.000752 | 16.5 | 1.001175 | 19.5 | 1.001703 | 22.5 | 1.002326 | 25.5 | 1.003041 | 28.5 | 1.003843 |
| .6 | 1.000447 | .6 | 1.000764 | .6 | 1.001191 | .6 | 1.001722 | .6 | 1.002349 | .6 | 1.003066 | .6 | 1.003871 |
| .7 | 1.000456 | .7 | 1.000777 | .7 | 1.001207 | .7 | 1.001741 | .7 | 1.002372 | 3 | 1.003092 | .7 | 1.003899 |
| .8 | 1.000465 | .8 | 1.000789 | .8 | 1.001223 | .8 | 1.001761 | .8 | 1.002394 | .8 | 1.003117 | .8 | 1.003928 |
| .9 | 1.000474 | .9 | 1.000803 | .9 | 1.001239 | 9 | 1.001780 | .9 | 1.002417 | .9 | 1.003143 | .9 | 1.003956 |
| 11.0 | 1.000483 | 14.0 | 1.000816 | 17.0 | 1.001257 | 20.0 | 1.001800 | 23.0 | 1.002439 | 26.0 | 1.003168 | 29.0 | 1.003984 |
| .1 | 1.000492 | .1 | 1.000829 | .1 | 1.001273 | .1 | 1.001819 | .1 | 1.002462 | .1 | 1.003194 | .1 | 1.004013 |
| .2 | 1.000501 | .2 | 1.000842 | .2 | 1.001286 | .2 | 1.001839 | .2 | 1.002485 | 1 | 1.003222 | 2 | 1.004042 |
| 3 | 1.000511 | 3 | 1.000855 | 3 | 1.001306 | .3 | 1.001959 | .3 | 1.002508 | .3 | 1.003247 | .3 | 1.004071 |
| .4 | 1.000520 | .4 | 1.000868 | .4 | 1.001323 | .4 | 1.001880 | .4 | 1.002531 | .4 | 1.003273 | .4 | 1.004099 |
| 11.5 | 1.000530 | 14.5 | 1.000882 | 17.5 | 1.001340 | 20.5 | 1.001900 | 23.5 | 1.002555 | 26.5 | 1.003299 | 29.5 | 1.004128 |
| .6 | 1.000540 | .6 | 1.000895 | .6 | 1.001357 | .6 | 1.001920 | .6 | 1.002578 | .6 | 1.003326 | .6 | 1.004158 |
| .7 | 1.000550 | .7 | 1.000909 | .7 | 1.001374 | .7 | 1.001941 | 3 | 1.002602 | .7 | 1.003352 | .7 | 1.004187 |
| .8 | 1.000560 | .8 | 1.000923 | .8 | 1.001391 | .8 | 1.001961 | .8 | 1.002625 | .8 | 1.003379 | . 8 | 1.004216 |
| .9 | 1.000570 | .9 | 1.000937 | .9 | 1.001409 | .9 | 1.001982 | .9 | 1.002649 | .9 | 1.003405 | .9 | 1.004245 |
| 12.0 | 1.000580 | 15.0 | 1.000951 | 18.0 | 1.001427 | 21.0 | 1.002002 | 24.0 | 1.002672 | 27.0 | 1.003432 | 30.0 | 1.004275 |
| .1 | 1.000591 | .1 | 1.000965 | .1 | 1.001445 | .1 | 1.002023 | .1 | 1.002696 | .1 | 1.003459 | | |
| .2 | 1.000601 | .2 | 1.000979 | .2 | 1.001462 | .2 | 1.002044 | .2 | 1.002720 | .2 | 1.003485 | | |
| .3 | 1.000612 | .3 | 1.000993 | .3 | 1.001480 | .3 | 1.002065 | .3 | 1.002745 | .3 | 1.003513 | | |
| .4 | 1.000623 | .4 | 1.001008 | .4 | 1.001498 | .4 | 1.002086 | .4 | 1.002769 | .4 | 1.003540 | | |
| 12.5 | 1.000634 | 15.5 | 1.001022 | 18.5 | 1.001516 | 21.5 | 1.002107 | 24.5 | 1.002793 | 27.5 | 1.003567 | | |
| .6 | 1.000645 | .6 | 1.001037 | .6 | 1.001534 | .6 | 1.002129 | .6 | 1.002817 | .6 | 1.003594 | | |
| .7 | 1.000656 | .7 | 1.0010 52 | .7 | 1.001552 | .7 | 1.002151 | .7 | 1.002842 | .7 | 1.003621 | | |
| .8 | 1.000668 | .8 | 1.001067 | .8 | 1.001570 | .8 | 1.002172 | .8 | 1.002866 | .8 | 1.003649 | | |
| .9 | 1.000679 | .9 | 1.001082 | .9 | 1.001589 | .9 | 1.002194 | .9 | 1.002891 | .9 | 1.003676 | | |

Table II
Temperature corrections *c*, required for the density of dry wines and dry alcohol free wines, measured in a *Pyrex-glass* pycnometer at *t*°C, in order to correct to 20°C

$$\rho_{20} = \rho_t \pm \frac{c}{1000} \quad \begin{matrix} - \text{ si } t^o \text{ est inférieure à } 20\ ^oC \\ + \text{ si } t^o \text{ est supérieure à } 20\ ^oC \end{matrix}$$

| | | Alcoholic strength | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| Temperatures in °C | 10° | 1,59 | 1,64 | 1,67 | 1,71 | 1,77 | 1,84 | 1,91 | 2,01 | 2,11 | 2,22 | 2,34 | 2,46 | 2,60 | 2,73 | 2,88 | 3,03 | 3,19 | 3,35 | 3,52 | 3,70 | 3,87 | 4,06 | 4,25 | 4,44 |
| | 11° | 1,48 | 1,53 | 1,56 | 1,60 | 1,64 | 1,70 | 1,77 | 1,86 | 1,95 | 2,05 | 2,16 | 2,27 | 2,38 | 2,51 | 2,63 | 2,77 | 2,91 | 3,06 | 3,21 | 3,36 | 3,53 | 3,69 | 3,86 | 4,03 |
| | 12° | 1,36 | 1,40 | 1,43 | 1,46 | 1,50 | 1,56 | 1,62 | 1,69 | 1,78 | 1,86 | 1,96 | 2,05 | 2,16 | 2,27 | 2,38 | 2,50 | 2,62 | 2,75 | 2,88 | 3,02 | 3,16 | 3,31 | 3,46 | 3,61 |
| | 13° | 1,22 | 1,26 | 1,28 | 1,32 | 1,35 | 1,40 | 1,45 | 1,52 | 1,59 | 1,67 | 1,75 | 1,83 | 1,92 | 2,01 | 2,11 | 2,22 | 2,32 | 2,44 | 2,55 | 2,67 | 2,79 | 2,92 | 3,05 | 3,18 |
| | 14° | 1,08 | 1,11 | 1,13 | 1,16 | 1,19 | 1,23 | 1,27 | 1,33 | 1,39 | 1,46 | 1,52 | 1,60 | 1,67 | 1,75 | 1,94 | 1,93 | 2,03 | 2,11 | 2,21 | 2,31 | 2,42 | 2,52 | 2,63 | 2,74, |
| | 15° | 0,92 | 0,96 | 0,97 | 0,99 | 1,02 | 1,05 | 1,09 | 1,13 | 1,19 | 1,24 | 1,30 | 1,36 | 1,42 | 1,48 | 1,55 | 1,63 | 1,70 | 1,78 | 1,86 | 1,95 | 2,03 | 2,12 | 2,21 | 2,30 |
| | 16° | 0,76 | 0,79 | 0,80 | 0,81 | 0,94 | 0,86 | 0,89 | 0,93 | 0,97 | 1,01 | 1,06 | 1,10 | 1,16 | 1,21 | 1,26 | 1,32 | 1,38 | 1,44 | 1,51 | 1,57 | 1,64 | 1,71 | 1,78 | 1,85 |
| | 17° | 0,59 | 0,61 | 0,62 | 0,63 | 0,65 | 0,67 | 0,69 | 0,72 | 0,75 | 0,78 | 0,81 | 0,85 | 0,88 | 0,95 | 0,96 | 1,01 | 1,05 | 1,11 | 1,15 | 1,20 | 1,25 | 1,30 | 1,35 | 1,40 |
| | 18° | 0,40 | 0,42 | 0,42 | 0,43 | 0,44 | 0,46 | 0,47 | 0,49 | 0,51 | 0,53 | 0,55 | 0,57 | 0,60 | 0,63 | 0,65 | 0,68 | 0,71 | 0,74 | 0,77 | 0,81 | 0,84 | 0,87 | 0,91 | 0,94 |
| | 19° | 0,21 | 0,21 | 0,22 | 0,22 | 0,23 | 0,23 | 0,24 | 0,25 | 0,26 | 0,27 | 0,28 | 0,29 | 0,30 | 0,32 | 0,33 | 0,34 | 0,36 | 0,37 | 0,39 | 0,41 | 0,42 | 0,44 | 0,46 | 0,47 |
| | 20° | | | | | | | | | | | | | | | | | | | | | | | | |
| | 21° | 0,21 | 0,22 | 0,22 | 0,23 | 0,23 | 0,24 | 0,25 | 0,26 | 0,27 | 0,28 | 0,29 | 0,30 | 0,31 | 0,32 | 0,34 | 0,36 | 0,37 | 0,38 | 0,40 | 0,41 | 0,43 | 0,44 | 0,46 | 0,48 |
| | 22° | 0,44 | 0,45 | 0,46 | 0,47 | 0,48 | 0,49 | 0,51 | 0,52 | 0,54 | 0,56 | 0,59 | 0,61 | 0,63 | 0,66 | 0,69 | 0,71 | 0,74 | 0,77 | 0,80 | 0,83 | 0,87 | 0,90 | 0,93 | 0,97 |
| | 23° | 0,68 | 0,70 | 0,71 | 0,72 | 0,74 | 0,76 | 0,78 | 0,80 | 0,83 | 0,86 | 0,90 | 0,93 | 0,96 | 1,00 | 1,03 | 1,08 | 1,13 | 1,17 | 1,22 | 1,26 | 1,31 | 1,37 | 1,41 | 1,46 |
| | 24° | 0,93 | 0,96 | 0,97 | 0,99 | 1,01 | 1,03 | 1,06 | 1,10 | 1,13 | 1,18 | 1,22 | 1,26 | 1,31 | 1,36 | 1,41 | 1,47 | 1,52 | 1,58 | 1,64 | 1,71 | 1,77 | 1,84 | 1,90 | 1,97 |
| | 25° | 1,19 | 1,23 | 1,25 | 1,27 | 1,29 | 1,32 | 1,36 | 1,40 | 1,45 | 1,50 | 1,55 | 1,61 | 1,67 | 1,73 | 1,80 | 1,86 | 1,93 | 2,00 | 2,08 | 2,16 | 2,24 | 2,32 | 2,40 | 2,48 |
| | 26° | 1,47 | 1,51 | 1,53 | 1,56 | 1,59 | 1,62 | 1,67 | 1,72 | 1,77 | 1,83 | 1,90 | 1,96 | 2,03 | 2,11 | 2,19 | 2,27 | 2,35 | 2,44 | 2,53 | 2,62 | 2,72 | 2,81 | 2,91 | 3,01 |
| | 27° | 1,75 | 1,80 | 1,82 | 1,85 | 1,89 | 1,93 | 1,98 | 2,04 | 2,11 | 2,18 | 2,25 | 2,33 | 2,41 | 2,50 | 2,59 | 2,68 | 2,78 | 2,88 | 2,98 | 3,09 | 3,20 | 3,31 | 3,42 | 3,33 |
| | 28° | 2,04 | 2,10 | 2,13 | 2,16 | 2,20 | 2,25 | 2,31 | 2,38 | 2,45 | 2,53 | 2,62 | 2,70 | 2,80 | 2,89 | 3,00 | 3,10 | 3,21 | 3,32 | 3,45 | 3,57 | 3,69 | 3,82 | 3,94 | 4,07 |
| | 29° | 2,34 | 2,41 | 2,44 | 2,48 | 2,53 | 2,58 | 2,65 | 2,72 | 2,81 | 2,89 | 2,99 | 3,09 | 3,19 | 3,30 | 3,42 | 3,53 | 3,65 | 3,78 | 3,92 | 4,05 | 4,19 | 4,33 | 4,47 | 4,61 |
| | 30° | 2,66 | 2,73 | 2,77 | 2,81 | 2,86 | 2,92 | 3,00 | 3,08 | 3,17 | 3,27 | 3,37 | 3,48 | 3,59 | 3,72 | 3,84 | 3,97 | 4,11 | 4,25 | 4,40 | 4,55 | 4,70 | 4,85 | 4,92 | 5,17 |

*Note:* This table can be used to convert $d_{20}^{t}$ to $d_{20}^{20}$

Table III

Temperature corrections *c* required for the density of natural or concentrated musts as measured in a *Pyrex-glass* pycnometer at *t* °C to correct to 20°C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$ - if $t^o$ is less than 20 °C + if $t^o$ is more than 20°C

| Temperatures in °C | Density | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1,05 | | 1,0 | 1,0 | 1,0 | 1,0 | 1,1 | 1,1 | 1,1 | 1,1 | 1,1 | 1,1 | 1,1 | 1,18 | 1,2 | 1,2 | 1,2 | 1,2 | 1,2 | 1,3 | 1,3 | 1,3 | 1,3 |
| 10° | | 2,31 | 2,48 | 2,66 | 2,82 | 2,99 | 3,13 | 3,30 | 3,44 | 3,59 | 3,73 | 3,88 | 4,01 | 4,28 | 4,52 | 4,76 | 4,98 | 5,18 | 5,42 | 5,56 | 5,73 | 5,90 | 6,05 |
| 11° | | 2,12 | 2,28 | 2,42 | 2,57 | 2,72 | 2,86 | 2,99 | 3,12 | 3,25 | 3,37 | 3,50 | 3,62 | 3,85 | 4,08 | 4,29 | 4148 | 4,67 | 4,84 | 5,00 | 5,16 | 5,31 | 5,45 |
| 12° | | 1,92 | 2,06 | 2,19 | 2,32 | 2,45 | 2,58 | 2,70 | 2,92 | 2,94 | 3,04 | 3,15 | 3,26 | 3,47 | 3,67 | 3,85 | 4,03 | 4,20 | 4,36 | 4,51 | 4,65 | 478 | 4,91 |
| 13° | | 1,72 | 1,84 | 1,95 | 2,06 | 2,17 | 2,27 | 2,38 | 2,48 | 2,58 | 2,69 | 2,78 | 2,89 | 3,05 | 3,22 | 3,39 | 3,55 | 3,65 | 3,84 | 3,98 | 4,11 | 4:24 | 4,36 |
| 14° | | 1,52 | 1,62 | 1,72 | 1,81 | 1,90 | 2,00 | 2,09 | 2,17 | 2,26 | 2,34 | 2,43 | 2,51 | 2,66 | 2,82 | 2,96 | 3,09 | 3,22 | 3,34 | 3,45 | 3,56 | 3,67 | 3,76 |
| 15° | | 1,28 | 1,36 | 1,44 | 1,52 | 1,60 | 1,67 | 1,75 | 1,82 | 1,89 | 1,96 | 2,04 | 2,11 | 2,24 | 2,36 | 2,48 | 2,59 | 2,69 | 2,79 | 2,88 | 2,97 | 3,03 | 3,10 |
| 16° | | 1,05 | 1,12 | 1,18 | 1,25 | 1,31 | 1,37 | 1,43 | 1,49 | 1,55 | 1,60 | 1,66 | 1,71 | 1,81 | 1,90 | 2,00 | 2,08 | 2,16 | 2,24 | 2,30 | 2,37 | 2,43 | 2,49 |
| 17° | | 0,80 | 0,86 | 0,90 | 0,95 | 1,00 | 1,04 | 1,09 | 1,13 | 1,18 | 1,22 | 1,26 | 1,30 | 1,37 | 1,44 | 1,51 | 1,57 | 1,62 | 1,68 | 1,72 | 1,76 | 1,80 | 1,84 |
| 18° | | 0,56 | 0,59 | 0,62 | 0,66 | 0,68 | 0,72 | 0,75 | 0,77 | 0,80 | 0,83 | 0,85 | 0,88 | 0,93 | 0,98 | 1,02 | 1,05 | 1,09 | 1,12 | 1,16 | 1,19 | 1,21 | 1,24 |
| 19° | | 0,29 | 0,31 | 0,32 | 0,34 | 0,36 | 0,37 | 0,39 | 0,40 | 0,42 | 0,43 | 0,44 | 0,45 | 0,48 | 0,50 | 0,52 | 0,54 | 0,56 | 0,57 | 0,59 | 0,60 | 0,61 | 0:62 |
| 20° | | | | | | | | | | | | | | | | | | | | | | | |
| 21° | | 0,29 | 0,30 | 0,32 | 0,34 | 0,35 | 0,37 | 0,38 | 0,40 | 0,41 | 0,42 | 0,44 | 0,46 | 0,48 | 0,50 | 0,53 | 0,56 | 0,58 | 0,59 | 0,60 | 0,61 | 0,62 | 0,62 |
| 22° | | 0,58 | 0,61 | 0,64 | 0,67 | 0,70 | 0,73 | 0,76 | 0,79 | 0,81 | 0,84 | 0,87 | 0,90 | 0,96 | 1,03 | 1,05 | 1,09 | 1,12 | 1,15 | 1,18 | 1,20 | 1,22 | 1,23 |
| 23° | | 0,89 | 0,94 | 0,99 | 1,03 | 1,08 | 1,12 | 1,16 | 1,20 | 1,25 | 1,29 | 1,33 | 1,37 | 1,44 | 1,51 | 1,57 | 1,63 | 1,67 | 1,73 | 1,77 | 1,80 | 1,82 | 1,94 |
| 24° | | 1,20 | 1,25 | 1,31 | 1,37 | 1,43 | 1,49 | 1,54 | 1,60 | 1,66 | 1,71 | 1,77 | 1,82 | 1,92 | 2,01 | 2,10 | 2,17 | 2,24 | 2,30 | 2,36 | 2,40 | 2,42 | 2,44 |
| 25° | | 1,51 | 1,59 | 1,66 | 1,74 | 1,81 | 1,88 | 1,95 | 2,02 | 2,09 | 2,16 | 2,23 | 2,30 | 2,42 | 2,53 | 2,63 | 2,72 | 2,82 | 2,89 | 2,95 | 2,99 | 3,01 | 3,05 |
| 26° | | 1,84 | 1,92 | 2,01 | 2,10 | 2,18 | 2,26 | 2,34 | 2,42 | 2,50 | 2,58 | 2,65 | 2,73 | 2,87 | 3,00 | 3,13 | 3,25 | 3,36 | 3,47 | 3,57 | 3,65 | 372 | 3,79 |
| 27° | | 2,17 | 2,26 | 2,36 | 2,46 | 2,56 | 2,66 | 2,75 | 2,84 | 2,93 | 3,01 | 3,10 | 3,18 | 3,35 | 3,50 | 3,66 | 3,80 | 3,93 | 4,06 | 4,16 | 4,26 | 4:35 | 4,42 |
| 28° | | 2,50 | 2,62 | 2,74 | 2,85 | 2,96 | 3,07 | 3,18 | 3,28 | 3,40 | 3,50 | 3,60 | 3,69 | 3,87 | 4,04 | 4,21 | 4,36 | 4,50 | 4,64 | 4,75 | 4,86 | 4,94 | 5,00 |
| 29° | | 2,86 | 2,98 | 3,10 | 3,22 | 3,35 | 3,47 | 3,59 | 3,70 | 3,82 | 3,93 | 4,03 | 4,14 | 4,34 | 4,53 | 4,72 | 4,89 | 5,05 | 5,20 | 5,34 | 5,46 | 5,56 | 5,64 |
| 30° | | 3,20 | 3,35 | 3,49 | 3,64 | 3,77 | 3,91 | 4,05 | 4,17 | 4,30 | 4,43 | 4,55 | 4,67 | 4,90 | 5,12 | 5,39 | 5,51 | 5,68 | 5,94 | 5,96 | 6,09 | 6,16 | 6,22 |

*Note:* This table can be used to convert $d_{20}^{t}$ to $d_{20}^{20}$

## TABLE IV

Temperature corrections *c* required for the density of dessert wines measured in a *Pyrex-glass* pycnometer at $t$ °C$_t$ to correct to 20 °C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$

- if $t^o$ is less than 20 °C
+ if $t^o$ is more than 20 °C

| Temperatures in °C | 13% vol. wine | | | | | | | 15% vol. wine | | | | | | | 17% vol. wine | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Density | | | | | | | Density | | | | | | | Density | | | | | | |
| | 1,000 | 1,020 | 1,040 | 1,060 | 1,080 | 1,100 | 1,120 | 1,000 | 1,020 | 1,040 | 1,060 | 1,080 | 1,100 | 1,120 | 1,000 | 1,020 | 1,040 | 1,060 | 1,080 | 1,100 | 1,120 |
| 10° | 2,36 | 2,71 | 3,06 | 3,42 | 3,72 | 3,96 | 4,32 | 2,64 | 2,99 | 3,36 | 3,68 | 3,99 | 4,30 | 4,59 | 2,94 | 3,29 | 3,64 | 3,98 | 4,29 | 4,60 | 4,89 |
| 11° | 2,17 | 2,49 | 2,80 | 2,99 | 3,39 | 3,65 | 3,90 | 2,42 | 2,73 | 3,05 | 3,34 | 3,63 | 3,89 | 4,15 | 2,69 | 3,00 | 3,32 | 3,61 | 3,90 | 4,16 | 4,41 |
| 12° | 1,97 | 2,25 | 2,53 | 2,79 | 3,05 | 3,29 | 3,52 | 2,19 | 2,47 | 2,75 | 3,01 | 3,27 | 3,51 | 3,73 | 2,42 | 2,70 | 2,98 | 3,24 | 3,50 | 3,74 | 3,96 |
| 13° | 1,78 | 2,02 | 2,25 | 2,47 | 2,69 | 2,89 | 3,09 | 1,97 | 2,21 | 2,44 | 2,66 | 2,87 | 3,08 | 3,29 | 2,18 | 2,42 | 2,64 | 2,87 | 3,08 | 3,29 | 3,49 |
| 14° | 1,57 | 1,78 | 1,98 | 2,16 | 2,35 | 2,53 | 2,70 | 1,74 | 1,94 | 2,14 | 2,32 | 2,52 | 2,69 | 2,86 | 1,91 | 2,11 | 2,31 | 2,50 | 2,69 | 2,86 | 3,03 |
| 15° | 1,32 | 1,49 | 1,66 | 1,82 | 1,97 | 2,12 | 2,26 | 1,46 | 1,63 | 1,79 | 1,95 | 2,10 | 2,25 | 2,39 | 1,60 | 1,77 | 1,93 | 2,09 | 2,24 | 2,39 | 2,53 |
| 16° | 1,08 | 1,22 | 1,36 | 1,48 | 1,61 | 1,73 | 1,84 | 1,18 | 1,32 | 1,46 | 1,59 | 1,71 | 1,83 | 1,94 | 1,30 | 1,44 | 1,58 | 1,71 | 1,83 | 1,95 | 2,06 |
| 17° | 0,83 | 0,94 | 1,04 | 1,13 | 1,22 | 1,31 | 1,40 | 0,91 | 1,02 | 1,12 | 1,21 | 1,30 | 1,39 | 1,48 | 1,00 | 1,10 | 1,20 | 1,30 | 1,39 | 1,48 | 1,56 |
| 18° | 0,58 | 0,64 | 0,71 | 0,78 | 0,84 | 0,89 | 0,95 | 0,63 | 0,69 | 0,76 | 0,83 | 0,89 | 0,94 | 1,00 | 0,69 | 0,75 | 0,82 | 0,89 | 0,95 | 1,00 | 1,06 |
| 19° | 0,30 | 0,34 | 0,37 | 0,40 | 0,43 | 0,46 | 0,49 | 0,33 | 0,37 | 0,40 | 0,43 | 0,46 | 0,49 | 0,52 | 0,36 | 0,39 | 0,42 | 0,46 | 0,49 | 0,52 | 0,54 |
| 20° | | | | | | | | | | | | | | | | | | | | | |
| 21° | 0,30 | 0,33 | 0,36 | 0,40 | 0,43 | 0,46 | 0,49 | 0,33 | 0,36 | 0,39 | 0,43 | 0,46 | 0,49 | 0,51 | 0,35 | 0,39 | 0,42 | 0,45 | 0,48 | 0,51 | 0,54 |
| 22° | 0,60 | 0,67 | 0,73 | 0,80 | 0,85 | 0,91 | 0,98 | 0,65 | 0,72 | 0,78 | 0,84 | 0,90 | 0,96 | 1,01 | 0,71 | 0,78 | 0,84 | 0,90 | 0,96 | 1,01 | 1,07 |
| 23° | 0,93 | 1,02 | 1,12 | 1,22 | 1,30 | 1,39 | 1,49 | 1,01 | 1,10 | 1,20 | 1,29 | 1,38 | 1,46 | 1,55 | 1,10 | 1,19 | 1,29 | 1,38 | 1,46 | 1,55 | 1,63 |
| 24° | 1,27 | 1,39 | 1,50 | 1,61 | 1,74 | 1,84 | 1,95 | 1,37 | 1,49 | 1,59 | 1,72 | 1,84 | 1,95 | 2,06 | 1,48 | 1,60 | 1,71 | 1,83 | 1,95 | 2,06 | 2,17 |
| 25° | 1,61 | 1,75 | 1,90 | 2,05 | 2,19 | 2,33 | 2,47 | 1,73 | 1,87 | 2,02 | 2,17 | 2,31 | 2,45 | 2,59 | 1,87 | 2,01 | 2,16 | 2,31 | 2,45 | 2,59 | 2,73 |
| 26° | 1,94 | 2,12 | 2,29 | 2,47 | 2,63 | 2,79 | 2,95 | 2,09 | 2,27 | 2,44 | 2,62 | 2,78 | 2,94 | 3,10 | 2,26 | 2,44 | 2,61 | 2,79 | 2,95 | 3,11 | 3,26 |
| 27° | 2,30 | 2,51 | 2,70 | 2,90 | 3,09 | 3,27 | 3,44 | 2,48 | 2,68 | 2,87 | 3,07 | 3,27 | 3,45 | 3,62 | 2,67 | 2,88 | 3,07 | 3,27 | 3,46 | 3,64 | 3,81 |
| 28° | 2,66 | 2,90 | 3,13 | 3,35 | 3,57 | 3,86 | 4,00 | 2,86 | 3,10 | 3,23 | 3,55 | 3,77 | 3,99 | 4,20 | 3,08 | 3,31 | 3,55 | 3,76 | 3,99 | 4,21 | 4,41 |
| 29° | 3,05 | 3,31 | 3,56 | 3,79 | 4,04 | 4,27 | 4,49 | 3,28 | 3,53 | 3,77 | 4,02 | 4,26 | 4,49 | 4,71 | 3,52 | 3,77 | 4,01 | 4,26 | 4,50 | 4,73 | 4,95 |
| 30° | 3,44 | 3,70 | 3,99 | 4,28 | 4,54 | 4,80 | 5,06 | 3,68 | 3,94 | 4,23 | 4,52 | 4,79 | 5,05 | 5,30 | 3,95 | 4,22 | 4,51 | 4,79 | 5,07 | 5,32 | 5,57 |

TABLE IV (continued)
Temperature corrections *c* required for the density of dessert wines measured in a *Pyrex-glass* pycnometer at *t* °C$_t$ to correct to 20 °C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$

- I f $t^o$ is less than 20 °C
+ if $t^o$ is more than 20 °C

| | | 19% vol. wine | | | | | | | 21% vol. wine | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Density | | | | | | | Density | | | | | | |
| | | 1,000 | 1,020 | 1,040 | 1,060 | 1,000 | 1,100 | 1,120 | 1,000 | 1,020 | 1,040 | 1,060 | 1,080 | 1,100 | 1,120 |
| Temperatures in °C | 10° | 3,27 | 3,62 | 3,97 | 4,30 | 4,62 | 4,92 | 5,21 | 3,62 | 3,97 | 4,32 | 4,66 | 4,97 | 5,27 | 5,56 |
| | 11° | 2,99 | 3,30 | 3,61 | 3,90 | 4,19 | 4,45 | 4,70 | 3,28 | 3,61 | 3,92 | 4,22 | 4,50 | 4,76 | 5,01 |
| | 12° | 2,68 | 2,96 | 3,24 | 3,50 | 3,76 | 4,00 | 4,21 | 2,96 | 3,24 | 3,52 | 3,78 | 4,03 | 4,27 | 4,49 |
| | 13° | 2,68 | 2,96 | 3,24 | 3,50 | 3,76 | 4,00 | 4,21 | 2,96 | 3,24 | 3,52 | 3,78 | 4,03 | 4,27 | 4,49 |
| | 14° | 2,11 | 2,31 | 2,51 | 2,69 | 2,88 | 3,05 | 3,22 | 2,31 | 2,51 | 2,71 | 2,89 | 3,08 | 3,25 | 3,43 |
| | 15° | 1,76 | 1,93 | 2,09 | 2,25 | 2,40 | 2,55 | 2,69 | 1,93 | 2,10 | 2,26 | 2,42 | 2,57 | 2,72 | 2,86 |
| | 16° | 1,43 | 1,57 | 1,70 | 1,83 | 1,95 | 2,08 | 2,18 | 1,56 | 1,70 | 1,84 | 1,97 | 2,09 | 2,21 | 2,32 |
| | 17° | 1,09 | 1,20 | 1,30 | 1,39 | 1,48 | 1,57 | 1,65 | 1,20 | 1,31 | 1,41 | 1,50 | 1,59 | 1,68 | 1,77 |
| | 18° | 0,76 | 0,82 | 0,88 | 0,95 | 1,01 | 1,06 | 1,12 | 0,82 | 0,88 | 0,95 | 1,01 | 1,08 | 1,13 | 1,18 |
| | 19° | 0,39 | 0,42 | 0,45 | 0,49 | 0,52 | 0,55 | 0,57 | 0,42 | 0,46 | 0,49 | 0,52 | 0,55 | 0,58 | 0,61 |
| | 20° | | | | | | | | | | | | | | |
| | 21° | 0,38 | 0,42 | 0,45 | 0,48 | 0,51 | 0,54 | 0,57 | 0,41 | 0,45 | 0,48 | 0,51 | 0,54 | 0,57 | 0,60 |
| | 22° | 0,78 | 0,84 | 0,90 | 0,96 | 1,02 | 1,07 | 1,13 | 0,84 | 0,90 | 0,96 | 1,02 | 1,08 | 1,14 | 1,19 |
| | 23° | 1,19 | 1,28 | 1,38 | 1,47 | 1,55 | 1,64 | 1,72 | 1,29 | 1,39 | 1,48 | 1,57 | 1,65 | 1,74 | 1,82 |
| | 24° | 1,60 | 1,72 | 1,83 | 1,95 | 2,06 | 2,18 | 2,29 | 1,73 | 1,85 | 1,96 | 2,08 | 2,19 | 2,31 | 2,42 |
| | 25° | 2,02 | 2,16 | 2,31 | 2,46 | 2,60 | 2,74 | 2,88 | 2,18 | 2,32 | 2,47 | 2,62 | 2,76 | 2,90 | 3,04 |
| | 26° | 2,44 | 2,62 | 2,79 | 2,96 | 3,12 | 3,28 | 3,43 | 2,53 | 2,81 | 2,97 | 3,15 | 3,31 | 3,47 | 3,62 |
| | 27° | 2,88 | 3,08 | 3,27 | 3,42 | 3,66 | 3,84 | 4,01 | 3,10 | 3,30 | 3,47 | 3,69 | 3,88 | 4,06 | 4,23 |
| | 28° | 3,31 | 3,54 | 3,78 | 4,00 | 4,22 | 4,44 | 4,64 | 3,56 | 3,79 | 4,03 | 4,25 | 4,47 | 4,69 | 4,89 |
| | 29° | 3,78 | 4,03 | 4,27 | 4,52 | 4,76 | 4,99 | 5,21 | 4,06 | 4,31 | 4,55 | 4,80 | 5,04 | 5,27 | 5,48 |
| | 30° | 4,24 | 4,51 | 4,80 | 5,08 | 5,36 | 5,61 | 5,86 | 4,54 | 4,82 | 5,11 | 5,39 | 5,66 | 5,91 | 6,16 |

Table V

Temperature corrections *c* for the density of dry wines and dry wines with alcohol removed, measured with an *ordinary- glass* pycnometer or hydrometer at *t* °C, to correct to 20°C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$ - if $t^o$ is less than 20 °C
+ if $t^o$ is more than 20 °C

| Temperatures in °C | Alcoholic strength | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| 10° | 1,45 | 1,51 | 1,55 | 1,58 | 1,64 | 1,76 | 1,78 | 1,89 | 1,98 | 2,09 | 2,21 | 2,34 | 2,47 | 2,60 | 2,15 | 2,93 | 3,06 | 3,22 | 3,39 | 3,57 | 3,75 | 3,93 | 4,12 | 4,31 |
| 11° | 1,35 | 1,40 | 1,43 | 1,47 | 1,52 | 1,58 | 1,65 | 1,73 | 183 | 1,93 | 2,03 | 2,15 | 2,26 | 2,38 | 2,51 | 2,65 | 2,78 | 2,93 | 3,08 | 3,24 | 3,40 | 3,57 | 3,73 | 3,90 |
| 12° | 1,24 | 1,28 | 1,31 | 1,34 | 1,39 | 1,44 | 1,50 | 1,58 | 1,66 | 1,75 | 1,84 | 1,94 | 2,04 | 2,15 | 2,26 | 2,38 | 2,51 | 2,63 | 2,77 | 2,91 | 3,05 | 3,19 | 3,34 | 3,49 |
| 13° | 1,12 | 1,16 | 1,18 | 1,21 | 1,25 | 1,30 | 1,35 | 1,42 | 1,49 | 1,56 | 1,64 | 1,73 | 1,82 | 1,91 | 2,01 | 2,11 | 2,22 | 2,33 | 2,45 | 2,57 | 2,69 | 2,81 | 2,95 | 3,07 |
| 14° | 0,99 | 1,03 | 1,05 | 1,07 | 1,11 | 1,14 | 1,19 | 1,24 | 1,31 | 1,37 | 1,44 | 1,52 | 1,59 | 1,67 | 1,75 | 1,84 | 1,93 | 2,03 | 2,13 | 2,23 | 2,33 | 2,44 | 2,55 | 2,66 |
| 15° | 0,86 | 0,89 | 0,90 | 0,92 | 0,95 | 0,98 | 1,02 | 1,07 | 1,12 | 1,17 | 1,23 | 1,29 | 1,35 | 1,42 | 1,49 | 1,56 | 1,63 | 1,71 | 1,80 | 1,88 | 1,96 | 2,05 | 2,14 | 2,23 |
| 16° | 0,71 | 0,73 | 0,74 | 0,76 | 0,78 | 0,81 | 0,84 | 0,87 | 0,91 | 0,95 | 0,99 | 1,05 | 1,10 | 1,15 | 1,21 | 1,27 | 1,33 | 1,39 | 1,45 | 1,52 | 1,59 | 1,66 | 1,73 | 1,80 |
| 17° | 0,55 | 0,57 | 0,57 | 0,59 | 0,60 | 0,62 | 0,65 | 0,67 | 0,70 | 0,74 | 0,77 | 0,81 | 0,84 | 0,88 | 0,92 | 0,96 | 1,01 | 1,05 | 1,10 | 1,15 | 1,20 | 1,26 | 1,31 | 1,36 |
| 18° | 0,38 | 0,39 | 0,39 | 0,40 | 0,41 | 0,43 | 0,44 | 0,46 | 0,48 | 0,50 | 0,52 | 0,55 | 0,57 | 0,60 | 0,62 | 0,65 | 0,68 | 0,71 | 0,74 | 0,78 | 0,81 | 0,85 | 0,88 | 0,91 |
| 19° | 0,19 | 0,20 | 0,20 | 0,21 | 0,21 | 0,22 | 0,23 | 0,24 | 0,25 | 0,26 | 0,27 | 0,28 | 0,29 | 0,30 | 0,32 | 0,33 | 0,34 | 0,36 | 0,38 | 0,39 | 0,41 | 0,43 | 0,44 | 0,46 |
| 20° | | | | | | | | | | | | | | | | | | | | | | | | |
| 21° | 0,21 | 0,22 | 0,22 | 0,23 | 0,23 | 0,24 | 0,25 | 0,25 | 0,26 | 0,27 | 0,28 | 0,29 | 0,31 | 0,32 | 0,34 | 0,35 | 0,36 | 0,38 | 0,39 | 0,41 | 0,43 | 0,44 | 0,46 | 0,48 |
| 22° | 0,43 | 0,45 | 0,45 | 0,46 | 0,47 | 0,49 | 0,50 | 0,52 | 0,54 | 0,56 | 0,58 | 0,60 | 0,62 | 0,65 | 0,68 | 0,71 | 0,73 | 0,77 | 0,80 | 0,83 | 0,86 | 0,89 | 0,93 | 0,96 |
| 23° | 0,67 | 0,69 | 0,70 | 0,71 | 0,72 | 0,74 | 0,77 | 0,79 | 0,82 | 0,85 | 0,88 | 0,91 | 0,95 | 0,99 | 1,03 | 1,07 | 1,12 | 1,16 | 1,21 | 1,25 | 1,30 | 1,35 | 1,40 | 1,45 |
| 24° | 0,91 | 0,93 | 0,95 | 0,97 | 0,99 | 1,01 | 1,04 | 1,07 | 1,11 | 1,15 | 1,20 | 1,24 | 1,29 | 1,34 | 1,39 | 1,45 | 1,50 | 1,56 | 1,62 | 1,69 | 1,76 | 1,82 | 1,88 | 1,95 |
| 25° | 1,16 | 1,19 | 1,21 | 1,23 | 1,26 | 1,29 | 1,33 | 1,37 | 1,42 | 1,47 | 1,52 | 1,57 | 1,63 | 1,70 | 1,76 | 1,83 | 1,90 | 1,97 | 2,05 | 2,13 | 2,21 | 2,29 | 2,37 | 2,45 |
| 26° | 1,42 | 1,46 | 1,49 | 1,51 | 1,54 | 1,58 | 1,62 | 1,67 | 1,73 | 1,79 | 1,85 | 1,92 | 1,99 | 2,07 | 2,14 | 2,22 | 2,31 | 2,40 | 2,49 | 2,58 | 2,67 | 2,77 | 2,86 | 2,96 |
| 27° | 1,69 | 1,74 | 1,77 | 1,80 | 1,83 | 1,88 | 1,93 | 1,98 | 2,05 | 2,12 | 2,20 | 2,27 | 2,35 | 2,44 | 2,53 | 2,63 | 2 72 | 2,82 | 2,93 | 3,04 | 3,14 | 3,25 | 3,37 | 3,48 |
| 28° | 1,97 | 2,03 | 2,06 | 2,09 | 2,14 | 2,19 | 2,24 | 2,31 | 2,38 | 2,46 | 2,55 | 2,63 | 2,73 | 2,83 | 2,93 | 3,03 | 3,14 | 3,26 | 3,38 | 3,50 | 3,62 | 3,75 | 3,85 | 4,00 |
| 29° | 2,26 | 2,33 | 2,37 | 2,41 | 2,45 | 2,50 | 2,57 | 2,64 | 2,73 | 2,82 | 2,91 | 2,99 | 3,11 | 3,22 | 3,34 | 3,46 | 3,58 | 3,70 | 3,84 | 3,97 | 4,11 | 4,25 | 4,39 | 4,54 |
| 30° | 2,56 | 2,64 | 2,67 | 2,72 | 2,77 | 2,83 | 2,90 | 2,98 | 3,08 | 3,18 | 3,28 | 3,38 | 3,50 | 3,62 | 3,75 | 3,88 | 4,02 | 4,16 | 4,30 | 4,46 | 4,61 | 4,76 | 4,92 | 5,07 |

*Note:* This table can be used to convert $d^t_{20}$ to $d^{20}_{20}$

Table VI

Temperature corrections c required for the density of natural or concentrated musts, measured with an *ordinary-glass* pycnometer-or hydrometer at $t$ °C, to correct to 20 °C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$

- if $t^o$ is less than 20 °C
+ if $t^o$ is more than 20 °C

| | | Masses volumiques | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1,05 | 1 06 | 1,07 | 1,08 | 1,09 | 1,10 | 1,11 | 1,12 | 1,13 | 1,14 | 1,15 | 1,16 | 1,18 | 1,20 | 1,22 | 1,24 | 1,26 | 1,28 | 1,30 | 1,32 | 1,34 | 1,36 |
| Température en °C | 10° | 2,17 | 2,34 | 2,52 | 2,68 | 2,85 | 2,99 | 3,16 | 3,29 | 3,44 | 3,58 | 3,73 | 3,86 | 4,13 | 4,36 | 4,60 | 4,82 | 5,02 | 5,25 | 5,39 | 5,56 | -5,73 | 5,87 |
| | 11° | 2, 00 | 2,16 | 2,29 | 2,44 | 2,59 | 2,73 | 2,86 | 2,99 | 3,12 | 3,24 | 3,37 | 3,48 | 3,71 | 3,94 | 4,15 | 4,33 | 4,52 | 4,69 | 4,85 | 5,01 | 5,15 | 5,29 |
| | 12° | 1,81 | 1,95 | 2,08 | 2,21 | 2,34 | 2,47 | 2,58 | 2,70 | 2,82 | 2,92 | 3,03 | 3,14 | 3,35 | 3,55 | 3,72 | 3,90 | 4,07 | 4,23 | 4,37 | 4,52 | 4,64 | 4,77 |
| | 13° | 1,62 | 1,74 | 1,85 | 1,96 | 2,07 | 2,17 | 2,28 | 2,38 | 2,48 | 2,59 | 2,68 | 2,77 | 2,94 | 3,11 | 3,28 | 3,44 | 3,54 | 3,72 | 3,86 | 3,99 | 4,12 | 4,24 |
| | 14° | 1,44 | 1,54 | 1,64 | 1,73 | 1,82 | 1,92 | 2,00 | 2,08 | 2,17 | 2,25 | 2,34 | 2,42 | 2,57 | 2,73 | 2,86 | 2,99 | 3,12 | 3,24 | 3,35 | 3,46 | 3,57 | 3,65 |
| | 15° | 1,21 | 1,29 | 1,37 | 1,45 | 1,53 | 1,60 | 1,68 | 1,75 | 1,82 | 1,89 | 1,97 | 2,03 | 2,16 | 2,28 | 2,40 | 2,51 | 2,61 | 2,71 | 2,80 | 2,89 | 2,94 | 3,01 |
| | 16° | 1,00 | 1,06 | 1,12 | 1,19 | 1,25 | 1,31 | 1,37 | 1,43 | 1,49 | 1,54 | 1,60 | 1,65 | 1,75 | 1,84 | 1,94 | 2,02 | 2,09 | 2,17 | 2,23 | 2,30 | 2,36 | 2,42 |
| | 17° | 0,76 | 0,82 | 0,86 | 0,91 | 0,96 | 1,00 | 1,05 | 1,09 | 1,14 | 1,18 | 1,22 | 1,25 | 1,32 | 1,39 | 1,46 | 1,52 | 1,57 | 1,63 | 1,67 | 1,71 | 1,75 | 1,79 |
| | 18° | 0,53 | 0,56 | 0,59 | 0,63 | 0,65 | 0,69 | 0,72 | 0,74 | 0,77 | 0,80 | 0,82 | 0,85 | 0,90 | 0,95 | 0,99 | 1,02 | 1,05 | 1,09 | 1,13 | 1,16 | 1,18 | 1,20 |
| | 19° | 0,28 | 0,30 | 0,31 | 0,33 | 0,35 | 0,36 | 0,38 | 0,39 | 0,41 | 0,42 | 0,43 | 0,43 | 0,46 | 0,48 | 0,50 | 0,52 | 0,54 | 0,55 | 0,57 | 0,58 | 0,59 | 0,60 |
| | 20° | | | | | | | | | | | | | | | | | | | | | | |
| | 21° | 0,28 | 0,29 | 0,31 | 0,33 | 0,34 | 0,36 | 0,37 | 0,39 | 0,40 | 0,41 | 0,43 | 0,44 | 0,46 | 0,48 | 0,51 | 0,54 | 0,56 | 0,57 | 0,58 | 0,59 | 0,60 | 0,60 |
| | 22° | 0,55 | 0,58 | 0,61 | 0,64 | 0,67 | 0,70 | 0,73 | 0,76 | 0,78 | 0,81 | 0,84 | 0,87 | 0,93 | 0,97 | 1,02 | 1,06 | 1,09 | 1,12 | 1,15 | 1,17 | 1,19 | 1,19 |
| | 23° | 0,85 | 0,90 | 0,95 | 0,99 | 1,04 | 1,08 | 1,12 | 1,16 | 1,21 | 1,25 | 1,29 | 1,32 | 1,39 | 1,46 | 1,52 | 1,58 | 1,62 | 1,68 | 1,72 | 1,75 | 1,77 | 1,79 |
| | 24° | 1,15 | 1,19 | 1,25 | 1,31 | 1,37 | 1,43 | 1,48 | 1,54 | 1,60 | 1,65 | 1,71 | 1,76 | 1,86 | 1,95 | 2,04 | 2,11 | 2,17 | 2,23 | 2,29 | 2,33 | 2,35 | 2,37 |
| | 25° | 1,44 | 1,52 | 1,59 | 1,67 | 1,74 | 1,81 | 1,88 | 1,95 | 2,02 | 2,09 | 2,16 | 2,22 | 2,34 | 2,45 | 2,55 | 2,64 | 2,74 | 2,81 | 7,87 | 2,90 | 2,92 | 2,96 |
| | 26° | 1,76 | 1,84 | 1,93 | 2,02 | 2,10 | 2,18 | 2,25 | 2,33 | 2,41 | 2,49 | 2,56 | 2,64 | 2,78 | 2,91 | 3,03 | 3,15 | 3,26 | 3,37 | 3,47 | 3,55 | 3,62 | 3,60 |
| | 27° | 2,07 | 2,16 | 2,26 | 2,36 | 2,46 | 2,56 | 2,65 | 2,74 | 2,83 | 2,91 | 3,00 | 3,07 | 3,24 | 3,39 | 3,55 | 3,69 | 3,82 | 3,94 | 4,04 | 4,14 | 4,23 | 4,30 |
| | 28° | 2,39 | 2,51 | 2,63 | 2,74 | 2,85 | 2,96 | 3,06 | 3,16 | 3,28 | 3,38 | 3,48 | 3,57 | 3,75 | 3,92 | 4,08 | 4,23 | 4,37 | 4,51 | 4,62 | 4,73 | 4,80 | 4,86 |
| | 29° | 2,74 | 2,86 | 2,97 | 3,09 | 3,22 | 3,34 | 3,46 | 3,57 | 3,69 | 3,90 | 3,90 | 4,00 | 4,20 | 4,39 | 4,58 | 4,74 | 4,90 | 5,05 | 5,19 | 5,31 | 5,40 | 5,48 |
| | 30° | 3,06 | 3,21 | 3,35 | 3,50 | 3,63 | 3,77 | 3,91 | 4,02 | 4,15 | 4,28 | 4,40 | 4,52 | 4,75 | 4,96 | 5,16 | 5,35 | 5,52 | 5,67 | 5,79 | 5,91 | 5,99 | 6,04 |

*Note:* This table can be used to convert $d^t_{20}$ to $d^{20}_{20}$

Table VII

Temperature corrections *c* required for the density of dessert wines,
measured in an *ordinary-glass* pycnometer, or hydrometer at *t* °C to correct this to 20 °C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$ - if $t^o$ is less than 20 °C
+ if $t^o$ is more than 20 °C

| | | 13% vol. wine | | | | | | | 15% vol. wine | | | | | | | 17% vol. wine | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Density | | | | | | | Density | | | | | | | Density | | | | | | |
| | | 1,000 | 1,020 | 1,040 | 1,060 | 1,080 | 1,100 | 1,120 | 1,000 | 1,020 | 1,040 | 1,060 | 1,080 | 1,100 | 1,120 | 1,000 | 1,020 | 1,040 | 1,060 | 1,080 | 1,100 | 1,120 |
| Temperature in °C | 10° | 2,24 | 2,58 | 2,93 | 3,27 | 3,59 | 3,89 | 4,18 | 2,51 | 2,85 | 3,20 | 3,54 | 3,85 | 4,02 | 4,46 | 2,81 | 3,15 | 3,50 | 3,84 | 4,15 | 4,45 | 4,74 |
| | 11° | 2,06 | 2,37 | 2,69 | 2,97 | 3,26 | 3,53 | 3,78 | 2,31 | 2,61 | 2,93 | 3,21 | 3,51 | 3,64 | 4,02 | 2,57 | 2,89 | 3,20 | 3,49 | 3,77 | 4,03 | 4,28 |
| | 12° | 1,87 | 2,14 | 2,42 | 2,67 | 2,94 | 3,17 | 3,40 | 2,09 | 2,36 | 2,64 | 2,90 | 3,16 | 3,27 | 3,61 | 2,32 | 2,60 | 2,87 | 3,13 | 3,39 | 3,63 | 3,84 |
| | 13° | 1,69 | 1,93 | 2,14 | 2,37 | 2,59 | 2,80 | 3,00 | 1,88 | 2,12 | 2,34 | 2,56 | 2,78 | 2,88 | 3,19 | 2,09 | 2,33 | 2,55 | 2,77 | 2,98 | 3,19 | 3,39 |
| | 14° | 1,49 | 1,70 | 1,90 | 2,09 | 2,27 | 2,44 | 2,61 | 1,67 | 1,86 | 2,06 | 2,25 | 2,45 | 2,51 | 2,77 | 1,83 | 2,03 | 2,23 | 2,42 | 2,61 | 2,77 | 2,94 |
| | 15° | 1,25 | 1,42 | 1,59 | 1,75 | 1,90 | 2,05 | 2,19 | 1,39 | 1,56 | 1,72 | 1,88 | 2,03 | 2,11 | 2,32 | 1,54 | 1,71 | 1,87 | 2,03 | 2,18 | 2,32 | 2,47 |
| | 16° | 1,03 | 1,17 | 1,30 | 1,43 | 1,55 | 1,67 | 1,78 | 1,06 | 1,27 | 1,40 | 1,53 | 1,65 | 1,77 | 1,88 | 1,25 | 1,39 | 1,52 | 1,65 | 1,77 | 1,89 | 2,00 |
| | 17° | 0,80 | 0,90 | 1,00 | 1,09 | 1,17 | 1,27 | 1,36 | 0,87 | 0,98 | 1,08 | 1,17 | 1,26 | 1,35 | 1,44 | 0,96 | 1,06 | 1,16 | 1,26 | 1,35 | 1,44 | 1,52 |
| | 18° | 0,54 | 0,61 | 0,68 | 0,75 | 0,81 | 0,86 | 0,92 | 0,60 | 0,66 | 0,73 | 0,80 | 0,85 | 0,91 | 0,97 | 0,66 | 0,72 | 0,79 | 0,86 | 0,92 | 0,97 | 1,03 |
| | 19° | 0,29 | 0,33 | 0,36 | 0,39 | 0,42 | 0,45 | 0,48 | 0,32 | 0,36 | 0,39 | 0,42 | 0,45 | 0,48 | 0,51 | 0,35 | 0,38 | 0,41 | 0,45 | 0,48 | 0,51 | 0,53 |
| | 20° | | | | | | | | | | | | | | | | | | | | | |
| | 21° | 0,29 | 0,32 | 0,35 | 0,39 | 0,42 | 0,45 | 0,47 | 0,32 | 0,35 | 0,38 | 0,42 | 0,45 | 0,48 | 0,50 | 0,34 | 0,38 | 0,41 | 0,44 | 0,47 | 0,50 | 0,53 |
| | 22° | 0,57 | 0,64 | 0,70 | 0,76 | 0,82 | 0,88 | 0,93 | 0,63 | 0,69 | 0,75 | 0,81 | 0,87 | 0,93 | 0,99 | 0,68 | 0,75 | 0,81 | 0,87 | 0,93 | 0,99 | 1,04 |
| | 23° | 0,89 | 0,98 | 1,08 | 1,17 | 1,26 | 1,34 | 1,43 | 0,97 | 1,06 | 1,16 | 1,25 | 1,34 | 1,42 | 1,51 | 1,06 | 1,15 | 1,25 | 1,34 | 1,42 | 1,51 | 1,59 |
| | 24° | 1,22 | 1,34 | 1,44 | 1,56 | 1,68 | 1,79 | 1,90 | 1,32 | 1,44 | 1,54 | 1,66 | 1,78 | 1,89 | 2,00 | 1,43 | 1,56 | 1,65 | 1,77 | 1,89 | 2,00 | 2,11 |
| | 25° | 1,61 | 1,68 | 1,83 | 1,98 | 2,12 | 2,26 | 2,40 | 1,66 | 1,81 | 1,96 | 2,11 | 2,25 | 2,39 | 2,52 | 1,80 | 1,94 | 2,09 | 2,24 | 2,39 | 2,52 | 2,66 |
| | 26° | 1,87 | 2,05 | 2,22 | 2,40 | 2,56 | 2,71 | 2,87 | 2,02 | 2,20 | 2,37 | 2,54 | 2,70 | 2,85 | 3,01 | 2,18 | 2,36 | 2,53 | 2,71 | 2,86 | 3,02 | 3,17 |
| | 27° | 2,21 | 2,42 | 2,60 | 2,80 | 3,00 | 3,18 | 3,35 | 2,39 | 2,59 | 2,78 | 2,98 | 3,17 | 3,35 | 3,52 | 2,58 | 2,78 | 2,97 | 3,17 | 3,36 | 3,54 | 3,71 |
| | 28° | 2,56 | 2,80 | 3,02 | 3,25 | 3,47 | 3,67 | 3,89 | 2,75 | 2,89 | 3,22 | 3,44 | 3,66 | 3,96 | 4,07 | 2,97 | 3,21 | 3,44 | 3,66 | 3,88 | 4,09 | 4,30 |
| | 29° | 2,93 | 3,19 | 3,43 | 3,66 | 3,91 | 4,14 | 4,37 | 3,16 | 3,41 | 3,65 | 3,89 | 4,13 | 4,36 | 4,59 | 3,40 | 3,66 | 3,89 | 4,13 | 4,38 | 4,61 | 4,82 |
| | 30° | 3,31 | 3,57 | 3,86 | 4,15 | 4,41 | 4,66 | 4,92 | 3,55 | 3,81 | 4,10 | 4,38 | 4,66 | 4,90 | 5,16 | 3,82 | 4,08 | 4,37 | 4,65 | 4,93 | 5,17 | 5,42 |

Table VII (cont'd)

Temperature corrections *c* required for the density of dessert wines, measured in an *ordinary-glass* pycnometer, or hydrometer at *t* °C to correct this to 20 °C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$ - if $t^o$ is less than 20 °C
+ if $t^o$ is more than 20 °C

| | | 19 % vol. wine | | | | | | | 21 % vol. wine | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Density | | | | | | | Density | | | | | | |
| | | 1,0 | 1,0 | 1,0 | 1,0 | 1,0 | 1,1 | 1,1 | 1,0 | 1,0 | 1,0 | 1,0 | 1,0 | 1,1 | 1,1 |
| Temperatures in °C | 10° | 3,1 | 3,4 | 3,8 | 4,1 | 4,4 | 4,7 | 5,0 | 3,5 | 3,8 | 4,1 | 4,5 | 4,8 | 5,1 | 5,4 |
| | 11° | 2.8 | 3.1 | 3.4 | 3.7 | 4.0 | 4.3 | 4.5 | 3.1 | 3.4 | 3.8 | 4.0 | 4.3 | 4.6 | 4.8 |
| | 12° | 2.5 | 2.9 | 3.1 | 3.3 | 3.6 | 3.8 | 4.1 | 2.8 | 3.1 | 3.4 | 3.6 | 3.9 | 4.1 | 4.3 |
| | 13° | 2.3 | 2.5 | 2.7 | 2.9 | 3.2 | 3.4 | 3.6 | 2.5 | 2.7 | 3.0 | 3.2 | 3.4 | 3.6 | 3.8 |
| | 14° | 2.0 | 2.2 | 2.4 | 2.6 | 2.8 | 2.9 | 3.1 | 2.2 | 2.4 | 2.6 | 2.8 | 3.0 | 3.1 | 3.3 |
| | 15° | 1,6 | 1,8 | 2,0 | 2,1 | 2,3 | 2,4 | 2,6 | 1,8 | 2,0 | 2,1 | 2,3 | 2,5 | 2,6 | 2,8 |
| | 16° | 1.3 | 1.5 | 1.6 | 1.7 | 1.9 | 2.0 | 2.1 | 1.5 | 1.6 | 1.7 | 1.9 | 2.0 | 2.1 | 2.2 |
| | 17° | 1.0 | 1.1 | 1.2 | 1 | 1.4 | 1.5 | 1.6 | 1.1 | 1.2 | 1.3 | 1.4 | 1.5 | 1.6 | 1.7 |
| | 18° | 0.7 | 0.7 | 0.8 | 0.9 | 0.9 | 1.0 | 1.0 | 0.7 | 0.8 | 0.9 | 0.9 | 1.0 | 1.1 | 1.1 |
| | 19° | 0.3 | 0.4 | 0.4 | 0.4 | 0.5 | 0.5 | 0.5 | 0.4 | 0.4 | 0.4 | 0.5 | 0.5 | 0.5 | 0.5 |
| | 20° | | | | | | | | | | | | | | |
| | 21° | 0.3 | 0.4 | 0.4 | 0.4 | 0.5 | 0.5 | 0.5 | 0.4 | 0.4 | 0.4 | 0.5 | 0.5 | 0.5 | 0.5 |
| | 22° | 0.7 | 0.8 | 0.8 | 0.9 | 0.9 | 1.0 | 1.1 | 0.8 | 0.8 | 0.9 | 1.0 | 1.0 | 1.1 | 1.1 |
| | 23° | 1.1 | 1.3 | 1.3 | 1.4 | 1.5 | 1.6 | 1.6 | 1.2 | 1.3 | 1.4 | 1.6 | 1.6 | 1.7 | 1.7 |
| | 24° | 1.5 | 1.6 | 1.7 | 1.8 | 2.0 | 2.1 | 2.2 | 1.6 | 1.8 | 1.9 | 2.0 | 2.1 | 2.2 | 2.3 |
| | 25° | 1,9 | 2,0 | 2,2 | 2,3 | 2,5 | 2,6 | 2,7 | 2,1 | 2,2 | 2,4 | 2,5 | 2,6 | 2,8 | 2,9 |
| | 26° | 2.3 | 2.5 | 2.7 | 2.8 | 3.0 | 3.2 | 3.3 | 2.5 | 2.7 | 2.9 | 3.0 | 3.2 | 3.3 | 3.5 |
| | 27° | 2.7 | 2.9 | 3.1 | 3.3 | 3.5 | 3.7 | 3.9 | 3.0 | 3.2 | 3.4 | 3.5 | 3.7 | 3.9 | 4.1 |
| | 28° | 3.2 | 3.4 | 3.6 | 3.8 | 4.1 | 4.3 | 4.5 | 3.4 | 3.6 | 3.9 | 4.1 | 4.3 | 4.5 | 4.7 |
| | 29° | 3.6 | 3.9 | 4.1 | 4.4 | 4.6 | 4.8 | 5.0 | 3.9 | 4.2 | 4.4 | 4.6 | 4.9 | 5.1 | 5.3 |
| | 30° | 4,1 | 4,3 | 4,6 | 4,9 | 5,2 | 5,4 | 5,7 | 4,4 | 4,6 | 4,9 | 5,2 | 5,5 | 5,7 | 6,0 |

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**Method OIV-MA-AS2-01B** Type IV method

# Density and Specific Gravity at 20°C

## 1. Definition

Density is the mass per unit volume of wine or must at 20°C. It is expressed in grams per milliliter, and denoted by the symbol $\rho_{20°C}$.

Specific gravity at 20°C (or 2°C/2°C relative density) is the ratio, expressed as a decimal number, of the density of the wine or must at 20°C to the density of water at the same temperature, and is denoted by the symbol $d_{20°C}^{20°C}$

## 2. Principle

Density and specific gravity at 20°C are determined on the sample for testing:

- by areometry (hydrometry)

*Note:* For very accurate measurement, the density and relative density must be corrected for the presence of sulfur dioxide.

$\rho_{20}$ = $\rho'_{20}$ - 0.0006 x S

$\rho_{20}$ = the corrected density

$\rho'_{20}$ = the observed density

S = total sulfur dioxide in g/L

## 3. Preliminary treatment of sample

If the wine or the must contains appreciable quantities of carbon dioxide, remove most of this by agitating 250 mL of wine in a 1000 mL flask, or by filtering under reduced pressure through 2 g of cotton wool placed in an extension tube.

## 4. Working Methods

4.1. Hydrometry

4..1.1 *Apparatus*

4..1.1.1 Hydrometer

Hydrometers must meet the AFNOR requirements regarding their dimensions and graduations.

They must have a cylindrical body, a stem of circular cross-section not less than 3 mm in diameter. For dry wines, they must be graduated from 0.983 to

1.003 with graduation marks every 0.0010 and 0.0002; each mark at 0.0010 must be separated from the next corresponding mark by at least 5 mm. For measuring the density of non-alcoholic wines, sweet wines and musts, a set of five hydrometers are to be used, graduated from 1.000 to 1.030, from 1.030 to 1.060, from 1.060 to 1.090, from 1.090 to 1.120 and from 1.120 to 1.150. These hydrometers shall be graduated for density at 20°C by marks every 0.0010 and 0.0005, with each 0.0010 being separated from the next corresponding mark by at least 3 mm. These hydrometers are to be graduated so they are read "at the top of the meniscus". The indication of the graduation in density or specific gravity at 20°C, and of the reading of the top of the meniscus, is to be carried either on the graduated scale or on a strip of paper enclosed on the bulb.

These hydrometers must be checked by an official authority.

4..1.1.2 Thermometer, in intervals of not less than 0.5°C.

4..1.1.3 A measuring cylinder with internal diameter 36 mm and height 320 mm, held vertical by supporting leveling screws.

4.1.2 *Procedure*

Place 250 mL of the prepared sample (3.) in the measuring cylinder 4..1.1.3; insert the hydrometer and thermometer. Mix the sample and wait one minute to allow temperature equilibration; read the thermometer. Remove the thermometer and after a further one minute read the apparent density at *t*°C on the stem of the hydrometer.

Correct the apparent density (as read at *t*°C) for the effect of temperature, using the tables for dry wines (Table V), for musts (Table VI) or for wines containing sugar (Table VII).

The 20°C/20°C specific gravity is obtained by dividing the density at 20°C by 0.998203.

Table V

Temperature corrections *c* for the density of dry wines and dry wines with alcohol removed, measured with an *ordinary- glass* pycnometer or hydrometer at *t* °C, to correct to 20°C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$ - if $t^o$ is less than 20 °C + if $t^o$ is more than 20 °C

| Temperatures in °C | Alcoholic strength | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| 10° | 1,45 | 1,51 | 1,55 | 1,58 | 1,64 | 1,76 | 1,78 | 1,89 | 1,98 | 2,09 | 2,21 | 2,34 | 2,47 | 2,60 | 2,15 | 2,93 | 3,06 | 3,22 | 3,39 | 3,57 | 3,75 | 3,93 | 4,12 | 4,31 |
| 11° | 1,35 | 1,40 | 1,43 | 1,47 | 1,52 | 1,58 | 1,65 | 1,73 | 183 | 1,93 | 2,03 | 2,15 | 2,26 | 2,38 | 2,51 | 2,65 | 2,78 | 2,93 | 3,08 | 3,24 | 3,40 | 3,57 | 3,73 | 3,90 |
| 12° | 1,24 | 1,28 | 1,31 | 1,34 | 1,39 | 1,44 | 1,50 | 1,58 | 1,66 | 1,75 | 1,84 | 1,94 | 2,04 | 2,15 | 2,26 | 2,38 | 2,51 | 2,63 | 2,77 | 2,91 | 3,05 | 3,19 | 3,34 | 3,49 |
| 13° | 1,12 | 1,16 | 1,18 | 1,21 | 1,25 | 1,30 | 1,35 | 1,42 | 1,49 | 1,56 | 1,64 | 1,73 | 1,82 | 1,91 | 2,01 | 2,11 | 2,22 | 2,33 | 2,45 | 2,57 | 2,69 | 2,81 | 2,95 | 3,07 |
| 14° | 0,99 | 1,03 | 1,05 | 1,07 | 1,11 | 1,14 | 1,19 | 1,24 | 1,31 | 1,37 | 1,44 | 1,52 | 1,59 | 1,67 | 1,75 | 1,84 | 1,93 | 2,03 | 2,13 | 2,23 | 2,33 | 2,44 | 2,55 | 2,66 |
| 15° | 0,86 | 0,89 | 0,90 | 0,92 | 0,95 | 0,98 | 1,02 | 1,07 | 1,12 | 1,17 | 1,23 | 1,29 | 1,35 | 1,42 | 1,49 | 1,56 | 1,63 | 1,71 | 1,80 | 1,88 | 1,96 | 2,05 | 2,14 | 2,23 |
| 16° | 0,71 | 0,73 | 0,74 | 0,76 | 0,78 | 0,81 | 0,84 | 0,87 | 0,91 | 0,95 | 0,99 | 1,05 | 1,10 | 1,15 | 1,21 | 1,27 | 1,33 | 1,39 | 1,45 | 1,52 | 1,59 | 1,66 | 1,73 | 1,80 |
| 17° | 0,55 | 0,57 | 0,57 | 0,59 | 0,60 | 0,62 | 0,65 | 0,67 | 0,70 | 0,74 | 0,77 | 0,81 | 0,84 | 0,88 | 0,92 | 0,96 | 1,01 | 1,05 | 1,10 | 1,15 | 1,20 | 1,26 | 1,31 | 1,36 |
| 18° | 0,38 | 0,39 | 0,39 | 0,40 | 0,41 | 0,43 | 0,44 | 0,46 | 0,48 | 0,50 | 0,52 | 0,55 | 0,57 | 0,60 | 0,62 | 0,65 | 0,68 | 0,71 | 0,74 | 0,78 | 0,81 | 0,85 | 0,88 | 0,91 |
| 19° | 0,19 | 0,20 | 0,20 | 0,21 | 0,21 | 0,22 | 0,23 | 0,24 | 0,25 | 0,26 | 0,27 | 0,28 | 0,29 | 0,30 | 0,32 | 0,33 | 0,34 | 0,36 | 0,38 | 0,39 | 0,41 | 0,43 | 0,44 | 0,46 |
| 20° | | | | | | | | | | | | | | | | | | | | | | | | |
| 21° | 0,21 | 0,22 | 0,22 | 0,23 | 0,23 | 0,24 | 0,25 | 0,25 | 0,26 | 0,27 | 0,28 | 0,29 | 0,31 | 0,32 | 0,34 | 0,35 | 0,36 | 0,38 | 0,39 | 0,41 | 0,43 | 0,44 | 0,46 | 0,48 |
| 22° | 0,43 | 0,45 | 0,45 | 0,46 | 0,47 | 0,49 | 0,50 | 0,52 | 0,54 | 0,56 | 0,58 | 0,60 | 0,62 | 0,65 | 0,68 | 0,71 | 0,73 | 0,77 | 0,80 | 0,83 | 0,86 | 0,89 | 0,93 | 0,96 |
| 23° | 0,67 | 0,69 | 0,70 | 0,71 | 0,72 | 0,74 | 0,77 | 0,79 | 0,82 | 0,85 | 0,88 | 0,91 | 0,95 | 0,99 | 1,03 | 1,07 | 1,12 | 1,16 | 1,21 | 1,25 | 1,30 | 1,35 | 1,40 | 1,45 |
| 24° | 0,91 | 0,93 | 0,95 | 0,97 | 0,99 | 1,01 | 1,04 | 1,07 | 1,11 | 1,15 | 1,20 | 1,24 | 1,29 | 1,34 | 1,39 | 1,45 | 1,50 | 1,56 | 1,62 | 1,69 | 1,76 | 1,82 | 1,88 | 1,95 |
| 25° | 1,16 | 1,19 | 1,21 | 1,23 | 1,26 | 1,29 | 1,33 | 1,37 | 1,42 | 1,47 | 1,52 | 1,57 | 1,63 | 1,70 | 1,76 | 1,83 | 1,90 | 1,97 | 2,05 | 2,13 | 2,21 | 2,29 | 2,37 | 2,45 |
| 26° | 1,42 | 1,46 | 1,49 | 1,51 | 1,54 | 1,58 | 1,62 | 1,67 | 1,73 | 1,79 | 1,85 | 1,92 | 1,99 | 2,07 | 2,14 | 2,22 | 2,31 | 2,40 | 2,49 | 2,58 | 2,67 | 2,77 | 2,86 | 2,96 |
| 27° | 1,69 | 1,74 | 1,77 | 1,80 | 1,83 | 1,88 | 1,93 | 1,98 | 2,05 | 2,12 | 2,20 | 2,27 | 2,35 | 2,44 | 2,53 | 2,63 | 2 72 | 2,82 | 2,93 | 3,04 | 3,14 | 3,25 | 3,37 | 3,48 |
| 28° | 1,97 | 2,03 | 2,06 | 2,09 | 2,14 | 2,19 | 2,24 | 2,31 | 2,38 | 2,46 | 2,55 | 2,63 | 2,73 | 2,83 | 2,93 | 3,03 | 3,14 | 3,26 | 3,38 | 3,50 | 3,62 | 3,75 | 3,85 | 4,00 |
| 29° | 2,26 | 2,33 | 2,37 | 2,41 | 2,45 | 2,50 | 2,57 | 2,64 | 2,73 | 2,82 | 2,91 | 2,99 | 3,11 | 3,22 | 3,34 | 3,46 | 3,58 | 3,70 | 3,84 | 3,97 | 4,11 | 4,25 | 4,39 | 4,54 |
| 30° | 2,56 | 2,64 | 2,67 | 2,72 | 2,77 | 2,83 | 2,90 | 2,98 | 3,08 | 3,18 | 3,28 | 3,38 | 3,50 | 3,62 | 3,75 | 3,88 | 4,02 | 4,16 | 4,30 | 4,46 | 4,61 | 4,76 | 4,92 | 5,07 |

*Note:* This table can be used to convert $d^t_{20}$ to $d^{20}_{20}$

Table VI

Temperature corrections c required for the density of natural or concentrated musts, measured with an *ordinary-glass* pycnometer-or hydrometer at $t$ °C, to correct to 20 °C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$

- if $t^o$ is less than 20 °C
+ if $t^o$ is more than 20 °C

| Température en °C | Masses volumiques | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1,05 | 1 ,06 | 1,07 | 1,08 | 1,09 | 1,10 | 1,11 | 1,12 | 1,13 | 1,14 | 1,15 | 1,16 | 1,18 | 1,20 | 1,22 | 1,24 | 1,26 | 1,28 | 1,30 | 1,32 | 1,34 | 1,36 |
| 10° | 2,17 | 2,34 | 2,52 | 2,68 | 2,85 | 2,99 | 3,16 | 3,29 | 3,44 | 3,58 | 3,73 | 3,86 | 4,13 | 4,36 | 4,60 | 4,82 | 5,02 | 5,25 | 5,39 | 5,56 | -5,73 | 5,87 |
| 11° | 2, 00 | 2,16 | 2,29 | 2,44 | 2,59 | 2,73 | 2,86 | 2,99 | 3,12 | 3,24 | 3,37 | 3,48 | 3,71 | 3,94 | 4,15 | 4,33 | 4,52 | 4,69 | 4,85 | 5,01 | 5,15 | 5,29 |
| 12° | 1,81 | 1,95 | 2,08 | 2,21 | 2,34 | 2,47 | 2,58 | 2,70 | 2,82 | 2,92 | 3,03 | 3,14 | 3,35 | 3,55 | 3,72 | 3,90 | 4,07 | 4,23 | 4,37 | 4,52 | 4,64 | 4,77 |
| 13° | 1,62 | 1,74 | 1,85 | 1,96 | 2,07 | 2,17 | 2,28 | 2,38 | 2,48 | 2,59 | 2,68 | 2,77 | 2,94 | 3,11 | 3,28 | 3,44 | 3,54 | 3,72 | 3,86 | 3,99 | 4,12 | 4,24 |
| 14° | 1,44 | 1,54 | 1,64 | 1,73 | 1,82 | 1,92 | 2,00 | 2,08 | 2,17 | 2,25 | 2,34 | 2,42 | 2,57 | 2,73 | 2,86 | 2,99 | 3,12 | 3,24 | 3,35 | 3,46 | 3,57 | 3,65 |
| 15° | 1,21 | 1,29 | 1,37 | 1,45 | 1,53 | 1,60 | 1,68 | 1,75 | 1,82 | 1,89 | 1,97 | 2,03 | 2,16 | 2,28 | 2,40 | 2,51 | 2,61 | 2,71 | 2,80 | 2,89 | 2,94 | 3,01 |
| 16° | 1,00 | 1,06 | 1,12 | 1,19 | 1,25 | 1,31 | 1,37 | 1,43 | 1,49 | 1,54 | 1,60 | 1,65 | 1,75 | 1,84 | 1,94 | 2,02 | 2,09 | 2,17 | 2,23 | 2,30 | 2,36 | 2,42 |
| 17° | 0,76 | 0,82 | 0,86 | 0,91 | 0,96 | 1,00 | 1,05 | 1,09 | 1,14 | 1,18 | 1,22 | 1,25 | 1,32 | 1,39 | 1,46 | 1,52 | 1,57 | 1,63 | 1,67 | 1,71 | 1,75 | 1,79 |
| 18° | 0,53 | 0,56 | 0,59 | 0,63 | 0,65 | 0,69 | 0,72 | 0,74 | 0,77 | 0,80 | 0,82 | 0,85 | 0,90 | 0,95 | 0,99 | 1,02 | 1,05 | 1,09 | 1,13 | 1,16 | 1,18 | 1,20 |
| 19° | 0,28 | 0,30 | 0,31 | 0,33 | 0,35 | 0,36 | 0,38 | 0,39 | 0,41 | 0,42 | 0,43 | 0,43 | 0,46 | 0,48 | 0,50 | 0,52 | 0,54 | 0,55 | 0,57 | 0,58 | 0,59 | 0,60 |
| 20° | | | | | | | | | | | | | | | | | | | | | | |
| 21° | 0,28 | 0,29 | 0,31 | 0,33 | 0,34 | 0,36 | 0,37 | 0,39 | 0,40 | 0,41 | 0,43 | 0,44 | 0,46 | 0,48 | 0,51 | 0,54 | 0,56 | 0,57 | 0,58 | 0,59 | 0,60 | 0,60 |
| 22° | 0,55 | 0,58 | 0,61 | 0,64 | 0,67 | 0,70 | 0,73 | 0,76 | 0,78 | 0,81 | 0,84 | 0,87 | 0,93 | 0,97 | 1,02 | 1,06 | 1,09 | 1,12 | 1,15 | 1,17 | 1,19 | 1,19 |
| 23° | 0,85 | 0,90 | 0,95 | 0,99 | 1,04 | 1,08 | 1,12 | 1,16 | 1,21 | 1,25 | 1,29 | 1,32 | 1,39 | 1,46 | 1,52 | 1,58 | 1,62 | 1,68 | 1,72 | 1,75 | 1,77 | 1,79 |
| 24° | 1,15 | 1,19 | 1,25 | 1,31 | 1,37 | 1,43 | 1,48 | 1,54 | 1,60 | 1,65 | 1,71 | 1,76 | 1,86 | 1,95 | 2,04 | 2,11 | 2,17 | 2,23 | 2,29 | 2,33 | 2,35 | 2,37 |
| 25° | 1,44 | 1,52 | 1,59 | 1,67 | 1,74 | 1,81 | 1,88 | 1,95 | 2,02 | 2,09 | 2,16 | 2,22 | 2,34 | 2,45 | 2,55 | 2,64 | 2,74 | 2,81 | 7,87 | 2,90 | 2,92 | 2,96 |
| 26° | 1,76 | 1,84 | 1,93 | 2,02 | 2,10 | 2,18 | 2,25 | 2,33 | 2,41 | 2,49 | 2,56 | 2,64 | 2,78 | 2,91 | 3,03 | 3,15 | 3,26 | 3,37 | 3,47 | 3,55 | 3,62 | 3,60 |
| 27° | 2,07 | 2,16 | 2,26 | 2,36 | 2,46 | 2,56 | 2,65 | 2,74 | 2,83 | 2,91 | 3,00 | 3,07 | 3,24 | 3,39 | 3,55 | 3,69 | 3,82 | 3,94 | 4,04 | 4,14 | 4,23 | 4,30 |
| 28° | 2,39 | 2,51 | 2,63 | 2,74 | 2,85 | 2,96 | 3,06 | 3,16 | 3,28 | 3,38 | 3,48 | 3,57 | 3,75 | 3,92 | 4,08 | 4,23 | 4,37 | 4,51 | 4,62 | 4,73 | 4,80 | 4,86 |
| 29° | 2,74 | 2,86 | 2,97 | 3,09 | 3,22 | 3,34 | 3,46 | 3,57 | 3,69 | 3,90 | 3,90 | 4,00 | 4,20 | 4,39 | 4,58 | 4,74 | 4,90 | 5,05 | 5,19 | 5,31 | 5,40 | 5,48 |
| 30° | 3,06 | 3,21 | 3,35 | 3,50 | 3,63 | 3,77 | 3,91 | 4,02 | 4,15 | 4,28 | 4,40 | 4,52 | 4,75 | 4,96 | 5,16 | 5,35 | 5,52 | 5,67 | 5,79 | 5,91 | 5,99 | 6,04 |

*Note:* This table can be used to convert $d_{20}^{t}$ to $d_{20}^{20}$

Table VII

Temperature corrections *c* required for the density of dessert wines, measured in an *ordinary-glass* pycnometer, or hydrometer at *t* °C to correct this to 20 °C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$ - if $t^o$ is less than 20 °C + if $t^o$ is more than 20 °C

| | | 13% vol. wine | | | | | | | 15% vol. wine | | | | | | | 17% vol. wine | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Density | | | | | | | Density | | | | | | | Density | | | | | | |
| | | 1,000 | 1,020 | 1,040 | 1,060 | 1,080 | 1,100 | 1,120 | 1,000 | 1,020 | 1,040 | 1,060 | 1,080 | 1,100 | 1,120 | 1,000 | 1,020 | 1,040 | 1,060 | 1,080 | 1,100 | 1,120 |
| Temperature in °C | 10° | 2,24 | 2,58 | 2,93 | 3,27 | 3,59 | 3,89 | 4,18 | 2,51 | 2,85 | 3,20 | 3,54 | 3,85 | 4,02 | 4,46 | 2,81 | 3,15 | 3,50 | 3,84 | 4,15 | 4,45 | 4,74 |
| | 11° | 2,06 | 2,37 | 2,69 | 2,97 | 3,26 | 3,53 | 3,78 | 2,31 | 2,61 | 2,93 | 3,21 | 3,51 | 3,64 | 4,02 | 2,57 | 2,89 | 3,20 | 3,49 | 3,77 | 4,03 | 4,28 |
| | 12° | 1,87 | 2,14 | 2,42 | 2,67 | 2,94 | 3,17 | 3,40 | 2,09 | 2,36 | 2,64 | 2,90 | 3,16 | 3,27 | 3,61 | 2,32 | 2,60 | 2,87 | 3,13 | 3,39 | 3,63 | 3,84 |
| | 13° | 1,69 | 1,93 | 2,14 | 2,37 | 2,59 | 2,80 | 3,00 | 1,88 | 2,12 | 2,34 | 2,56 | 2,78 | 2,88 | 3,19 | 2,09 | 2,33 | 2,55 | 2,77 | 2,98 | 3,19 | 3,39 |
| | 14° | 1,49 | 1,70 | 1,90 | 2,09 | 2,27 | 2,44 | 2,61 | 1,67 | 1,86 | 2,06 | 2,25 | 2,45 | 2,51 | 2,77 | 1,83 | 2,03 | 2,23 | 2,42 | 2,61 | 2,77 | 2,94 |
| | 15° | 1,25 | 1,42 | 1,59 | 1,75 | 1,90 | 2,05 | 2,19 | 1,39 | 1,56 | 1,72 | 1,88 | 2,03 | 2,11 | 2,32 | 1,54 | 1,71 | 1,87 | 2,03 | 2,18 | 2,32 | 2,47 |
| | 16° | 1,03 | 1,17 | 1,30 | 1,43 | 1,55 | 1,67 | 1,78 | 1,06 | 1,27 | 1,40 | 1,53 | 1,65 | 1,77 | 1,88 | 1,25 | 1,39 | 1,52 | 1,65 | 1,77 | 1,89 | 2,00 |
| | 17° | 0,80 | 0,90 | 1,00 | 1,09 | 1,17 | 1,27 | 1,36 | 0,87 | 0,98 | 1,08 | 1,17 | 1,26 | 1,35 | 1,44 | 0,96 | 1,06 | 1,16 | 1,26 | 1,35 | 1,44 | 1,52 |
| | 18° | 0,54 | 0,61 | 0,68 | 0,75 | 0,81 | 0,86 | 0,92 | 0,60 | 0,66 | 0,73 | 0,80 | 0,85 | 0,91 | 0,97 | 0,66 | 0,72 | 0,79 | 0,86 | 0,92 | 0,97 | 1,03 |
| | 19° | 0,29 | 0,33 | 0,36 | 0,39 | 0,42 | 0,45 | 0,48 | 0,32 | 0,36 | 0,39 | 0,42 | 0,45 | 0,48 | 0,51 | 0,35 | 0,38 | 0,41 | 0,45 | 0,48 | 0,51 | 0,53 |
| | 20° | | | | | | | | | | | | | | | | | | | | | |
| | 21° | 0,29 | 0,32 | 0,35 | 0,39 | 0,42 | 0,45 | 0,47 | 0,32 | 0,35 | 0,38 | 0,42 | 0,45 | 0,48 | 0,50 | 0,34 | 0,38 | 0,41 | 0,44 | 0,47 | 0,50 | 0,53 |
| | 22° | 0,57 | 0,64 | 0,70 | 0,76 | 0,82 | 0,88 | 0,93 | 0,63 | 0,69 | 0,75 | 0,81 | 0,87 | 0,93 | 0,99 | 0,68 | 0,75 | 0,81 | 0,87 | 0,93 | 0,99 | 1,04 |
| | 23° | 0,89 | 0,98 | 1,08 | 1,17 | 1,26 | 1,34 | 1,43 | 0,97 | 1,06 | 1,16 | 1,25 | 1,34 | 1,42 | 1,51 | 1,06 | 1,15 | 1,25 | 1,34 | 1,42 | 1,51 | 1,59 |
| | 24° | 1,22 | 1,34 | 1,44 | 1,56 | 1,68 | 1,79 | 1,90 | 1,32 | 1,44 | 1,54 | 1,66 | 1,78 | 1,89 | 2,00 | 1,43 | 1,56 | 1,65 | 1,77 | 1,89 | 2,00 | 2,11 |
| | 25° | 1,61 | 1,68 | 1,83 | 1,98 | 2,12 | 2,26 | 2,40 | 1,66 | 1,81 | 1,96 | 2,11 | 2,25 | 2,39 | 2,52 | 1,80 | 1,94 | 2,09 | 2,24 | 2,39 | 2,52 | 2,66 |
| | 26° | 1,87 | 2,05 | 2,22 | 2,40 | 2,56 | 2,71 | 2,87 | 2,02 | 2,20 | 2,37 | 2,54 | 2,70 | 2,85 | 3,01 | 2,18 | 2,36 | 2,53 | 2,71 | 2,86 | 3,02 | 3,17 |
| | 27° | 2,21 | 2,42 | 2,60 | 2,80 | 3,00 | 3,18 | 3,35 | 2,39 | 2,59 | 2,78 | 2,98 | 3,17 | 3,35 | 3,52 | 2,58 | 2,78 | 2,97 | 3,17 | 3,36 | 3,54 | 3,71 |
| | 28° | 2,56 | 2,80 | 3,02 | 3,25 | 3,47 | 3,67 | 3,89 | 2,75 | 2,89 | 3,22 | 3,44 | 3,66 | 3,96 | 4,07 | 2,97 | 3,21 | 3,44 | 3,66 | 3,88 | 4,09 | 4,30 |
| | 29° | 2,93 | 3,19 | 3,43 | 3,66 | 3,91 | 4,14 | 4,37 | 3,16 | 3,41 | 3,65 | 3,89 | 4,13 | 4,36 | 4,59 | 3,40 | 3,66 | 3,89 | 4,13 | 4,38 | 4,61 | 4,82 |
| | 30° | 3,31 | 3,57 | 3,86 | 4,15 | 4,41 | 4,66 | 4,92 | 3,55 | 3,81 | 4,10 | 4,38 | 4,66 | 4,90 | 5,16 | 3,82 | 4,08 | 4,37 | 4,65 | 4,93 | 5,17 | 5,42 |

Table VII (continued)

Temperature corrections *c* required for the density of dessert wines, measured in an *ordinary-glass* pycnometer, or hydrometer at *t* °C to correct this to 20 °C.

$$\rho_{20} = \rho_t \pm \frac{c}{1000}$$ - if $t^o$ is less than 20 °C
+ if $t^o$ is more than 20 °C

| | | 19 % vol. wine | | | | | | | 21 % vol. wine | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Density | | | | | | | Density | | | | | | |
| | | 1,00 | 1,02 | 1,04 | 1,06 | 1,08 | 1,10 | 1,12 | 1,00 | 1,02 | 1,04 | 1,06 | 1,08 | 1,10 | 1,12 |
| Temperatures in °C | 10° | 3,14 | 3,48 | 3,83 | 4,17 | 4,48 | 4,78 | 5,07 | 3,50 | 3,84 | 4,19 | 4,52 | 4,83 | 5,12 | 5,41 |
| | 11° | 2,87 | 3,18 | 3,49 | 3,78 | 4,06 | 4,32 | 4,57 | 3,18 | 3,49 | 3,80 | 4,09 | 4,34 | 4,63 | 4,88 |
| | 12° | 2,58 | 2,96 | 3,13 | 3,39 | 3,65 | 3,88 | 4,10 | 2,86 | 3,13 | 3,41 | 3,67 | 3,92 | 4,15 | 4,37 |
| | 13° | 2,31 | 2,55 | 2,77 | 2,99 | 3,20 | 3,41 | 3,61 | 2,56 | 2,79 | 3,01 | 3,23 | 3,44 | 3,65 | 3,85 |
| | 14° | 2,03 | 2,23 | 2,43 | 2,61 | 2,80 | 2,96 | 3,13 | 2,23 | 2,43 | 2,63 | 2,81 | 3,00 | 3,16 | 3,33 |
| | 15° | 1,69 | 1,86 | 2,02 | 2,18 | 2,33 | 2,48 | 2,62 | 1,86 | 2,03 | 2,19 | 2,35 | 2,50 | 2,65 | 2,80 |
| | 16° | 1,38 | 1,52 | 1,65 | 1,78 | 1,90 | 2,02 | 2,13 | 1,51 | 1,65 | 1,78 | 1,91 | 2,03 | 2,15 | 2,26 |
| | 17° | 1,06 | 1,16 | 1,26 | 1 ,35 | 1,44 | 1,53 | 1,62 | 1,15 | 1,25 | 1,35 | 1,45 | 1,54 | 1,63 | 1,71 |
| | 18° | 0,73 | 0,79 | 0,85 | 0,92 | 0,98 | 1,03 | 1,09 | 0,79 | 0,85 | 0,92 | 0,98 | 1,05 | 1,10 | 1,15 |
| | 19° | 0,38 | 0,41 | 0,44 | 0,48 | 0,51 | 0,52 | 0,56 | 0,41 | 0,44 | 0,47 | 0,51 | 0,54 | 0,57 | 0,59 |
| | 20° | | | | | | | | | | | | | | |
| | 21° | 0,37 | 0,41 | 0,44 | 0,47 | 0,50 | 0,53 | 0,56 | 0,41 | 0,44 | 0,47 | 0,51 | 0,54 | 0,57 | 0,59 |
| | 22° | 0,75 | 0,81 | 0,87 | 0,93 | 0,99 | 1,04 | 1,10 | 0,81 | 0,88 | 0,94 | 1,00 | 1,06 | 1,10 | 1,17 |
| | 23° | 1,15 | 1,30 | 1,34 | 1,43 | 1,51 | 1,60 | 1,68 | 1,25 | 1,34 | 1,44 | 1,63 | 1,61 | 1,70 | 1,78 |
| | 24° | 1,55 | 1,67 | 1,77 | 1,89 | 2,00 | 2,11 | 2,23 | 1,68 | 1,80 | 1,90 | 2,02 | 2,13 | 2,25 | 2,36 |
| | 25° | 1,95 | 2,09 | 2,24 | 2,39 | 2,53 | 2,67 | 2,71 | 2,11 | 2,25 | 2,40 | 2,55 | 2,69 | 2,83 | 2,97 |
| | 26° | 2,36 | 2,54 | 2,71 | 2,89 | 3,04 | 3,20 | 3,35 | 2,55 | 2,73 | 2,90 | 3,07 | 3,22 | 3,38 | 3,54 |
| | 27° | 2,79 | 2,99 | 3,18 | 3,38 | 3,57 | 3,75 | 3,92 | 3,01 | 3,20 | 3,40 | 3,59 | 3,78 | 3,96 | 4,13 |
| | 28° | 3,20 | 3,44 | 3,66 | 3,89 | 4,11 | 4,32 | 4,53 | 3,46 | 3,69 | 3,93 | 4,15 | 4,36 | 4,58 | 4,77 |
| | 29° | 3,66 | 3,92 | 4,15 | 4,40 | 4,64 | 4,87 | 5,08 | 3,95 | 4,20 | 4,43 | 4,68 | 4,92 | 5,15 | 5,36 |
| | 30° | 4,11 | 4,37 | 4,66 | 4,94 | 5,22 | 5,46 | 5,71 | 4,42 | 4,68 | 4,97 | 5,25 | 5,53 | 5,77 | 6,02 |

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**Method OIV-MA-AS2-02** Type I method

# Evaluation by refractometry of the sugar concentration in grape musts, concentrated grape musts and rectified concentrated grape musts

**(**Oeno 21/2004)
(Revised per Oeno 466/2012)

## 1 Principle

The refractive index at 20°C, expressed either as an absolute value or as a percentage by mass of sucrose, is given in the appropriate table to provide a means of obtaining the sugar concentration in grams per liter and in grams per kilogram for grape musts, concentrated grape musts and rectified concentrated grape musts.

## 2 Apparatus

Abbe refractometer

The refractometer used must be fitted with a scale giving:
- either percentage by mass of sucrose to 0.1%;
- or refractive indices to four decimal places.

The refractometer must be equipped with a thermometer having a scale extending at least from +15°C to +25°C and with a system for circulating water that will enable measurements to be made at a temperature of 20 ± 5°C. The operating instructions for this instrument must be strictly adhered to, particularly with regard to calibration and the light source.

## 3 Preparation of the sample

3.1 *Must and concentrated must*

Pass the must, if necessary, through a dry gauze folded into four and, after discarding the first drops of the filtrate, carry out the determination on the filtered product.

3.2 *Rectified concentrated must*

Depending on the concentration, use either the rectified concentrated must itself or a solution obtained by making up 200 g of rectified concentrated must to 500 g with water, all weighings being carried out accurately.

## 4 Procedure

Bring the sample to a temperature close to 20°C.

Place a small test sample on the lower prism of the refractometer, taking care (because the prisms are pressed firmly against each other) that this test sample covers the glass surface uniformly. Carry out the measurement in accordance with the operating instructions of the instrument used.

Read the percentage by mass of sucrose to within 0.1 or read the refractive index to four decimal places.
Carry out at least two determinations on the same prepared sample. Note the temperature *t*°C.

## 5 Calculation

5.1 *Temperature correction*

- Instruments graduated in percentage by mass of sucrose: use Table I to obtain the temperature correction.
- Instruments graduated in refractive index: find the index measured at *t*°C in Table II to obtain (column 1) the corresponding value of the percentage by mass of sucrose at *t*°C. This value is corrected for temperature and expressed as a concentration at 20°C by means of Table I.

5.2 S*ugar concentration in must and concentrated must*

Find the percentage by mass of sucrose at 20°C in Table II and read from the same row the sugar concentration in grams per liter and grams per kilogram. The sugar concentration is expressed in terms of invert sugar to one decimal place.

5.3 *Sugar concentration in rectified concentrated must*

Find the percentage by mass of sucrose at 20°C in Table III and read from the same row the sugar concentration in grams per liter and grams per kilogram. The sugar concentration is expressed in terms of invert sugar to one decimal place. If the measurement was made on diluted rectified concentrated must, multiply the result by the dilution factor.

5.4 *Refractive index of must, concentrated must and rectified concentrated must*

Find the percentage by mass of sucrose at 20°C in Table II and read from the same row the refractive index at 20°C. This index is expressed to four decimal places.

## Table I

Correction to be made in the case where the percentage by mass of saccharose was determined at a temperature different by 20°C.

| Temperature °C | Percentage by mass measured in % | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 | 65 | 70 | 75 |
| 5 | –0,82 | –0,87 | –0,92 | –0,95 | –0,99 | | | | | | | | | |
| 6 | –0,80 | –0,82 | –0,87 | –0,90 | –0,94 | | | | | | | | | |
| 7 | –0,74 | –0,78 | –0,82 | –0,84 | –0,88 | | | | | | | | | |
| 8 | –0,69 | –0,73 | –0,76 | –0,79 | –0,82 | | | | | | | | | |
| 9 | –0,64 | –0,67 | –0,71 | –0,73 | –0,75 | | | | | | | | | |
| 10 | –0,59 | –0,62 | –0,65 | –0,67 | –0,69 | –0,71 | –0,72 | –0,73 | –0,74 | –0,75 | –0,75 | –0,75 | –0,75 | –0,75 |
| 11 | –0,54 | –0,57 | –0,59 | –0,61 | –0,63 | –0,64 | –0,65 | –0,66 | –0,67 | –0,68 | –0,68 | –0,68 | –0,68 | –0,67 |
| 12 | –0,49 | –0,51 | –0,53 | –0,55 | –0,56 | –0,57 | –0,58 | –0,59 | –0,60 | –0,60 | –0,61 | –0,61 | –0,60 | –0,60 |
| 13 | –0,43 | –0,45 | –0,47 | –0,48 | –0,50 | –0,51 | –0,52 | –0,52 | –0,53 | –0,53 | –0,53 | –0,53 | –0,53 | –0,53 |
| 14 | –0,38 | –0,39 | –0,40 | –0,42 | –0,43 | –0,44 | –0,44 | –0,45 | –0,45 | –0,46 | –0,46 | –0,46 | –0,46 | –0,45 |
| 15 | –0,32 | –0,33 | –0,34 | –0,35 | –0,36 | –0,37 | –0,37 | –0,38 | –0,38 | –0,38 | –0,38 | –0,38 | –0,38 | –0,38 |
| 16 | –0,26 | –0,27 | –0,28 | –0,28 | –0,29 | –0,30 | –0,30 | –0,30 | –0,31 | –0,31 | –0,31 | –0,31 | –0,31 | –0,30 |
| 17 | –0,20 | –0,20 | –0,21 | –0,21 | –0,22 | –0,22 | –0,23 | –0,23 | –0,23 | –0,23 | –0,23 | –0,23 | –0,23 | –0,23 |
| 18 | –0,13 | –0,14 | –0,14 | –0,14 | –0,15 | –0,15 | –0,15 | –0,15 | –0,15 | –0,15 | –0,15 | –0,15 | –0,15 | –0,15 |
| 19 | –0,07 | –0,07 | –0,07 | –0,07 | –0,07 | –0,08 | –0,08 | –0,08 | –0,08 | –0,08 | –0,08 | –0,08 | –0,08 | –0,08 |
| 20 | 0 | | | | RÉFÉREN CE | | | | | | | | 0 | |
| 21 | +0,07 | +0,07 | +0,07 | +0,07 | +0,08 | +0,08 | +0,08 | +0,08 | +0,08 | +0,08 | +0,08 | +0,08 | +0,08 | +0,08 |
| 22 | +0,14 | +0,14 | +0,15 | +0,15 | +0,15 | +0,15 | +0,16 | +0,16 | +0,16 | +0,16 | +0,16 | +0,16 | +0,15 | +0,15 |
| 23 | +0,21 | +0,22 | +0,22 | +0,23 | +0,23 | +0,23 | +0,23 | +0,24 | +0,24 | +0,24 | +0,24 | +0,23 | +0,23 | +0,23 |
| 24 | +0,29 | +0,29 | +0,30 | +0,30 | +0,31 | +0,31 | +0,31 | +0,32 | +0,32 | +0,32 | +0,32 | +0,31 | +0,31 | +0,31 |
| 25 | +0,36 | +0,37 | +0,38 | +0,38 | +0,39 | +0,39 | +0,40 | +0,40 | +0,40 | +0,40 | +0,40 | +0,39 | +0,39 | +0,39 |
| 26 | +0,44 | +0,45 | +0,46 | +0,46 | +0,47 | +0,47 | +0,48 | +0,48 | +0,48 | +0,48 | +0,48 | +0,47 | +0,47 | +0,46 |
| 27 | +0,52 | +0,53 | +0,54 | +0,55 | +0,55 | +0,56 | +0,56 | +0,56 | +0,56 | +0,56 | +0,56 | +0,55 | +0,55 | +0,54 |
| 28 | +0,60 | +0,61 | +0,62 | +0,63 | +0,64 | +0,64 | +0,64 | +0,65 | +0,65 | +0,64 | +0,64 | +0,64 | +0,63 | +0,62 |
| 29 | +0,68 | +0,69 | +0,70 | +0,71 | +0,72 | +0,73 | +0,73 | +0,73 | +0,73 | +0,73 | +0,72 | +0,72 | +0,71 | +0,70 |
| 30 | +0,77 | +0,78 | +0,79 | +0,80 | +0,81 | +0,81 | +0,81 | +0,82 | +0,81 | +0,81 | +0,81 | +0,80 | +0,79 | +0,78 |
| 31 | +0,85 | +0,87 | +0,88 | +0,89 | +0,89 | +0,90 | +0,90 | +0,90 | +0,90 | +0,90 | +0,89 | +0,88 | +0,87 | +0,86 |
| 32 | +0,94 | +0,95 | +0,96 | +0,97 | +0,98 | +0,99 | +0,99 | +0,99 | +0,99 | +0,98 | +0,97 | +0,96 | +0,95 | +0,94 |
| 33 | +1,03 | +1,04 | +1,05 | +1,06 | +1,07 | +1,08 | +1,08 | +1,08 | +1,07 | +1,07 | +1,06 | +1,05 | +1,03 | +1,02 |
| 34 | +1,12 | +1,19 | +1,15 | +1,15 | +1,16 | +1,17 | +1,17 | +1,17 | +1,16 | +1,15 | +1,14 | +1,13 | +1,12 | +1,10 |
| 35 | +1,22 | +1,23 | +1,24 | +1,25 | +1,25 | +1,26 | +1,26 | +1,25 | +1,25 | +1,24 | +1,23 | +1,21 | +1,20 | +1,18 |
| 36 | +1,31 | +1,32 | +1,33 | +1,34 | +1,35 | +1,35 | +1,35 | +1,35 | +1,34 | +1,33 | +1,32 | +1,30 | +1,28 | +1,26 |
| 37 | +1,41 | +1,42 | +1,43 | +1,44 | +1,44 | +1,44 | +1,44 | +1,44 | +1,43 | +1,42 | +1,40 | +1,38 | +1,36 | +1,34 |
| 38 | +1,51 | +1,52 | +1,53 | +1,53 | +1,54 | +1,54 | +1,53 | +1,53 | +1,52 | +1,51 | +1,49 | +1,47 | +1,45 | +1,42 |
| 39 | +1,61 | +1,62 | +1,62 | +1,63 | +1,63 | +1,63 | +1,63 | +1,62 | +1,61 | +1,60 | +1,58 | +1,56 | +1,53 | +1,50 |
| 40 | +1,71 | +1,72 | +1,72 | +1,73 | +1,73 | +1,73 | +1,72 | +1,71 | +1,70 | +1,69 | +1,67 | +1,64 | +1,62 | +1,59 |

It is preferable that the variations in temperature in relation to 20°C do not exceed ± 5°C.

## TABLE II

Table giving the sugar content of musts and concentrated musts in grammes per litre and in grammes per kilogramme, determined using a graduated refractometer, either in percentage by mass of saccharose at 20°C, or refractive index at 20°C. The mass density at 20°C is also given.

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 10.0 | 1.34782 | 1.0391 | 82.2 | 79.1 | 4.89 |
| 10.1 | 1.34798 | 1.0395 | 83.3 | 80.1 | 4.95 |
| 10.2 | 1.34813 | 1.0399 | 84.3 | 81.1 | 5.01 |
| 10.3 | 1.34829 | 1.0403 | 85.4 | 82.1 | 5.08 |
| 10.4 | 1.34844 | 1.0407 | 86.5 | 83.1 | 5.14 |
| 10.5 | 1.34860 | 1.0411 | 87.5 | 84.1 | 5.20 |
| 10.6 | 1.34875 | 1.0415 | 88.6 | 85.0 | 5.27 |
| 10.7 | 1.34891 | 1.0419 | 89.6 | 86.0 | 5.32 |
| 10.8 | 1.34906 | 1.0423 | 90.7 | 87.0 | 5.39 |
| 10.9 | 1.34922 | 1.0427 | 91.8 | 88.0 | 5.46 |
| 11.0 | 1.34937 | 1.0431 | 92.8 | 89.0 | 5.52 |
| 11.1 | 1.34953 | 1.0436 | 93.9 | 90.0 | 5.58 |
| 11.2 | 1.34968 | 1.0440 | 95.0 | 91.0 | 5.65 |
| 11.3 | 1.34984 | 1.0444 | 96.0 | 92.0 | 5.71 |
| 11.4 | 1.34999 | 1.0448 | 97.1 | 92.9 | 5.77 |
| 11.5 | 1.35015 | 1.0452 | 98.2 | 93.9 | 5.84 |
| 11.6 | 1.35031 | 1.0456 | 99.3 | 94.9 | 5.90 |
| 11.7 | 1.35046 | 1.0460 | 100.3 | 95.9 | 5.96 |
| 11.8 | 1.35062 | 1.0464 | 101.4 | 96.9 | 6.03 |
| 11.9 | 1.35077 | 1.0468 | 102.5 | 97.9 | 6.09 |
| 12.0 | 1.35093 | 1.0472 | 103.5 | 98.9 | 6.15 |
| 12.1 | 1.35109 | 1.0477 | 104.6 | 99.9 | 6.22 |
| 12.2 | 1.35124 | 1.0481 | 105.7 | 100.8 | 6.28 |
| 12.3 | 1.35140 | 1.0485 | 106.8 | 101.8 | 6.35 |
| 12.4 | 1.35156 | 1.0489 | 107.8 | 102.8 | 6.41 |
| 12.5 | 1.35171 | 1.0493 | 108.9 | 103.8 | 6.47 |
| 12.6 | 1.35187 | 1.0497 | 110.0 | 104.8 | 6.54 |
| 12.7 | 1.35203 | 1.0501 | 111.1 | 105.8 | 6.60 |
| 12.8 | 1.35219 | 1.0506 | 112.2 | 106.8 | 6.67 |
| 12.9 | 1.35234 | 1.0510 | 113.2 | 107.8 | 6.73 |
| 13.0 | 1.35250 | 1.0514 | 114.3 | 108.7 | 6.79 |
| 13.1 | 1.35266 | 1.0518 | 115.4 | 109.7 | 6.86 |
| 13.2 | 1.35282 | 1.0522 | 116.5 | 110.7 | 6.92 |
| 13.3 | 1.35298 | 1.0527 | 117.6 | 111.7 | 6.99 |
| 13.4 | 1.35313 | 1.0531 | 118.7 | 112.7 | 7.05 |
| 13.5 | 1.35329 | 1.0535 | 119.7 | 113.7 | 7.11 |
| 13.6 | 1.35345 | 1.0539 | 120.8 | 114.7 | 7.18 |
| 13.7 | 1.35361 | 1.0543 | 121.9 | 115.6 | 7.24 |
| 13.8 | 1.35377 | 1.0548 | 123.0 | 116.6 | 7.31 |
| 13.9 | 1.35393 | 1.0552 | 124.1 | 117.6 | 7.38 |
| 14.0 | 1.35408 | 1.0556 | 125.2 | 118.6 | 7.44 |
| 14.1 | 1.35424 | 1.0560 | 126.3 | 119.6 | 7.51 |
| 14.2 | 1.35440 | 1.0564 | 127.4 | 120.6 | 7.57 |
| 14.3 | 1.35456 | 1.0569 | 128.5 | 121.6 | 7.64 |
| 14.4 | 1.35472 | 1.0573 | 129.6 | 122.5 | 7.70 |
| 14.5 | 1.35488 | 1.0577 | 130.6 | 123.5 | 7.76 |
| 14.6 | 1.35504 | 1.0581 | 131.7 | 124.5 | 7.83 |
| 14.7 | 1.35520 | 1.0586 | 132.8 | 125.5 | 7.89 |
| 14.8 | 1.35536 | 1.0590 | 133.9 | 126.5 | 7.96 |
| 14.9 | 1.35552 | 1.0594 | 135.0 | 127.5 | 8.02 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 15.0 | 1.35568 | 1.0598 | 136.1 | 128.4 | 8.09 |
| 15.1 | 1.35584 | 1.0603 | 137.2 | 129.4 | 8.15 |
| 15.2 | 1.35600 | 1.0607 | 138.3 | 130.4 | 8.22 |
| 15.3 | 1.35616 | 1.0611 | 139.4 | 131.4 | 8.28 |
| 15.4 | 1.35632 | 1.0616 | 140.5 | 132.4 | 8.35 |
| 15.5 | 1.35648 | 1.0620 | 141.6 | 133.4 | 8.42 |
| 15.6 | 1.35664 | 1.0624 | 142.7 | 134.3 | 8.48 |
| 15.7 | 1.35680 | 1.0628 | 143.8 | 135.3 | 8.55 |
| 15.8 | 1.35696 | 1.0633 | 144.9 | 136.3 | 8.61 |
| 15.9 | 1.35713 | 1.0637 | 146.0 | 137.3 | 8.68 |
| 16.0 | 1.35729 | 1.0641 | 147.1 | 138.3 | 8.74 |
| 16.1 | 1.35745 | 1.0646 | 148.2 | 139.3 | 8.81 |
| 16.2 | 1.35761 | 1.0650 | 149.3 | 140.2 | 8.87 |
| 16.3 | 1.35777 | 1.0654 | 150.5 | 141.2 | 8.94 |
| 16.4 | 1.35793 | 1.0659 | 151.6 | 142.2 | 9.01 |
| 16.5 | 1.35810 | 1.0663 | 152.7 | 143.2 | 9.07 |
| 16.6 | 1.35826 | 1.0667 | 153.8 | 144.2 | 9.14 |
| 16.7 | 1.35842 | 1.0672 | 154.9 | 145.1 | 9.21 |
| 16.8 | 1.35858 | 1.0676 | 156.0 | 146.1 | 9.27 |
| 16.9 | 1.35874 | 1.0680 | 157.1 | 147.1 | 9.34 |
| 17.0 | 1.35891 | 1.0685 | 158.2 | 148.1 | 9.40 |
| 17.1 | 1.35907 | 1.0689 | 159.3 | 149.1 | 9.47 |
| 17.2 | 1.35923 | 1.0693 | 160.4 | 150.0 | 9.53 |
| 17.3 | 1.35940 | 1.0698 | 161.6 | 151.0 | 9.60 |
| 17.4 | 1.35956 | 1.0702 | 162.7 | 152.0 | 9.67 |
| 17.5 | 1.35972 | 1.0707 | 163.8 | 153.0 | 9.73 |
| 17.6 | 1.35989 | 1.0711 | 164.9 | 154.0 | 9.80 |
| 17.7 | 1.36005 | 1.0715 | 166.0 | 154.9 | 9.87 |
| 17.8 | 1.36021 | 1.0720 | 167.1 | 155.9 | 9.93 |
| 17.9 | 1.36038 | 1.0724 | 168.3 | 156.9 | 10.00 |
| 18.0 | 1.36054 | 1.0729 | 169.4 | 157.9 | 10.07 |
| 18.1 | 1.36070 | 1.0733 | 170.5 | 158.9 | 10.13 |
| 18.2 | 1.36087 | 1.0737 | 171.6 | 159.8 | 10.20 |
| 18.3 | 1.36103 | 1.0742 | 172.7 | 160.8 | 10.26 |
| 18.4 | 1.36120 | 1.0746 | 173.9 | 161.8 | 10.33 |
| 18.5 | 1.36136 | 1.0751 | 175.0 | 162.8 | 10.40 |
| 18.6 | 1.36153 | 1.0755 | 176.1 | 163.7 | 10.47 |
| 18.7 | 1.36169 | 1.0760 | 177.2 | 164.7 | 10.53 |
| 18.8 | 1.36185 | 1.0764 | 178.4 | 165.7 | 10.60 |
| 18.9 | 1.36202 | 1.0768 | 179.5 | 166.7 | 10.67 |
| 19.0 | 1.36219 | 1.0773 | 180.6 | 167.6 | 10.73 |
| 19.1 | 1.36235 | 1.0777 | 181.7 | 168.6 | 10.80 |
| 19.2 | 1.36252 | 1.0782 | 182.9 | 169.6 | 10.87 |
| 19.3 | 1.36268 | 1.0786 | 184.0 | 170.6 | 10.94 |
| 19.4 | 1.36285 | 1.0791 | 185.1 | 171.5 | 11.00 |
| 19.5 | 1.36301 | 1.0795 | 186.2 | 172.5 | 11.07 |
| 19.6 | 1.36318 | 1.0800 | 187.4 | 173.5 | 11.14 |
| 19.7 | 1.36334 | 1.0804 | 188.5 | 174.5 | 11.20 |
| 19.8 | 1.36351 | 1.0809 | 189.6 | 175.4 | 11.27 |
| 19.9 | 1.36368 | 1.0813 | 190.8 | 176.4 | 11.34 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 20.0 | 1.36384 | 1.0818 | 191.9 | 177.4 | 11.40 |
| 20.1 | 1.36401 | 1.0822 | 193.0 | 178.4 | 11.47 |
| 20.2 | 1.36418 | 1.0827 | 194.2 | 179.3 | 11.54 |
| 20.3 | 1.36434 | 1.0831 | 195.3 | 180.3 | 11.61 |
| 20.4 | 1.36451 | 1.0836 | 196.4 | 181.3 | 11.67 |
| 20.5 | 1.36468 | 1.0840 | 197.6 | 182.3 | 11.74 |
| 20.6 | 1.36484 | 1.0845 | 198.7 | 183.2 | 11.81 |
| 20.7 | 1.36501 | 1.0849 | 199.8 | 184.2 | 11.87 |
| 20.8 | 1.36518 | 1.0854 | 201.0 | 185.2 | 11.95 |
| 20.9 | 1.36535 | 1.0858 | 202.1 | 186.1 | 12.01 |
| 21.0 | 1.36551 | 1.0863 | 203.3 | 187.1 | 12.08 |
| 21.1 | 1.36568 | 1.0867 | 204.4 | 188.1 | 12.15 |
| 21.2 | 1.36585 | 1.0872 | 205.5 | 189.1 | 12.21 |
| 21.3 | 1.36602 | 1.0876 | 206.7 | 190.0 | 12.28 |
| 21.4 | 1.36619 | 1.0881 | 207.8 | 191.0 | 12.35 |
| 21.5 | 1.36635 | 1.0885 | 209.0 | 192.0 | 12.42 |
| 21.6 | 1.36652 | 1.0890 | 210.1 | 192.9 | 12.49 |
| 21.7 | 1.36669 | 1.0895 | 211.3 | 193.9 | 12.56 |
| 21.8 | 1.36686 | 1.0899 | 212.4 | 194.9 | 12.62 |
| 21.9 | 1.36703 | 1.0904 | 213.6 | 195.9 | 12.69 |
| 22.0 | 1.36720 | 1.0908 | 214.7 | 196.8 | 12.76 |
| 22.1 | 1.36737 | 1.0913 | 215.9 | 197.8 | 12.83 |
| 22.2 | 1.36754 | 1.0917 | 217.0 | 198.8 | 12.90 |
| 22.3 | 1.36771 | 1.0922 | 218.2 | 199.7 | 12.97 |
| 22.4 | 1.36787 | 1.0927 | 219.3 | 200.7 | 13.03 |
| 22.5 | 1.36804 | 1.0931 | 220.5 | 201.7 | 13.10 |
| 22.6 | 1.36821 | 1.0936 | 221.6 | 202.6 | 13.17 |
| 22.7 | 1.36838 | 1.0940 | 222.8 | 203.6 | 13.24 |
| 22.8 | 1.36855 | 1.0945 | 223.9 | 204.6 | 13.31 |
| 22.9 | 1.36872 | 1.0950 | 225.1 | 205.5 | 13.38 |
| 23.0 | 1.36889 | 1.0954 | 226.2 | 206.5 | 13.44 |
| 23.1 | 1.36906 | 1.0959 | 227.4 | 207.5 | 13.51 |
| 23.2 | 1.36924 | 1.0964 | 228.5 | 208.4 | 13.58 |
| 23.3 | 1.36941 | 1.0968 | 229.7 | 209.4 | 13.65 |
| 23.4 | 1.36958 | 1.0973 | 230.8 | 210.4 | 13.72 |
| 23.5 | 1.36975 | 1.0977 | 232.0 | 211.3 | 13.79 |
| 23.6 | 1.36992 | 1.0982 | 233.2 | 212.3 | 13.86 |
| 23.7 | 1.37009 | 1.0987 | 234.3 | 213.3 | 13.92 |
| 23.8 | 1.37026 | 1.0991 | 235.5 | 214.2 | 14.00 |
| 23.9 | 1.37043 | 1.0996 | 236.6 | 215.2 | 14.06 |
| 24.0 | 1.37060 | 1.1001 | 237.8 | 216.2 | 14.13 |
| 24.1 | 1.37078 | 1.1005 | 239.0 | 217.1 | 14.20 |
| 24.2 | 1.37095 | 1.1010 | 240.1 | 218.1 | 14.27 |
| 24.3 | 1.37112 | 1.1015 | 241.3 | 219.1 | 14.34 |
| 24.4 | 1.37129 | 1.1019 | 242.5 | 220.0 | 14.41 |
| 24.5 | 1.37146 | 1.1024 | 243.6 | 221.0 | 14.48 |
| 24.6 | 1.37164 | 1.1029 | 244.8 | 222.0 | 14.55 |
| 24.7 | 1.37181 | 1.1033 | 246.0 | 222.9 | 14.62 |
| 24.8 | 1.37198 | 1.1038 | 247.1 | 223.9 | 14.69 |
| 24.9 | 1.37216 | 1.1043 | 248.3 | 224.8 | 14.76 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20°C | Mass Density at 20°C | Sugars In g/l | Sugars In g/Kg | ABV % vol At 20°C |
|---|---|---|---|---|---|
| 25.0 | 1.37233 | 1.1047 | 249.5 | 225.8 | 14.83 |
| 25.1 | 1.37250 | 1.1052 | 250.6 | 226.8 | 14.89 |
| 25.2 | 1.37267 | 1.1057 | 251.8 | 227.7 | 14.96 |
| 25.3 | 1.37285 | 1.1062 | 253.0 | 228.7 | 15.04 |
| 25.4 | 1.37302 | 1.1066 | 254.1 | 229.7 | 15.10 |
| 25.5 | 1.37319 | 1.1071 | 255.3 | 230.6 | 15.17 |
| 25.6 | 1.37337 | 1.1076 | 256.5 | 231.6 | 15.24 |
| 25.7 | 1.37354 | 1.1080 | 257.7 | 232.5 | 15.32 |
| 25.8 | 1.37372 | 1.1085 | 258.8 | 233.5 | 15.38 |
| 25.9 | 1.37389 | 1.1090 | 260.0 | 234.5 | 15.45 |
| 26.0 | 1.37407 | 1.1095 | 261.2 | 235.4 | 15.52 |
| 26.1 | 1.37424 | 1.1099 | 262.4 | 236.4 | 15.59 |
| 26.2 | 1.37441 | 1.1104 | 263.6 | 237.3 | 15.67 |
| 26.3 | 1.37459 | 1.1109 | 264.7 | 238.3 | 15.73 |
| 26.4 | 1.37476 | 1.1114 | 265.9 | 239.3 | 15.80 |
| 26.5 | 1.37494 | 1.1118 | 267.1 | 240.2 | 15.87 |
| 26.6 | 1.37511 | 1.1123 | 268.3 | 241.2 | 15.95 |
| 26.7 | 1.37529 | 1.1128 | 269.5 | 242.1 | 16.02 |
| 26.8 | 1.37546 | 1.1133 | 270.6 | 243.1 | 16.08 |
| 26.9 | 1.37564 | 1.1138 | 271.8 | 244.1 | 16.15 |
| 27.0 | 1.37582 | 1.1142 | 273.0 | 245.0 | 16.22 |
| 27.1 | 1.37599 | 1.1147 | 274.2 | 246.0 | 16.30 |
| 27.2 | 1.37617 | 1.1152 | 275.4 | 246.9 | 16.37 |
| 27.3 | 1.37634 | 1.1157 | 276.6 | 247.9 | 16.44 |
| 27.4 | 1.37652 | 1.1161 | 277.8 | 248.9 | 16.51 |
| 27.5 | 1.37670 | 1.1166 | 278.9 | 249.8 | 16.58 |
| 27.6 | 1.37687 | 1.1171 | 280.1 | 250.8 | 16.65 |
| 27.7 | 1.37705 | 1.1176 | 281.3 | 251.7 | 16.72 |
| 27.8 | 1.37723 | 1.1181 | 282.5 | 252.7 | 16.79 |
| 27.9 | 1.37740 | 1.1185 | 283.7 | 253.6 | 16.86 |
| 28.0 | 1.37758 | 1.1190 | 284.9 | 254.6 | 16.93 |
| 28.1 | 1.37776 | 1.1195 | 286.1 | 255.5 | 17.00 |
| 28.2 | 1.37793 | 1.1200 | 287.3 | 256.5 | 17.07 |
| 28.3 | 1.37811 | 1.1205 | 288.5 | 257.5 | 17.15 |
| 28.4 | 1.37829 | 1.1210 | 289.7 | 258.4 | 17.22 |
| 28.5 | 1.37847 | 1.1214 | 290.9 | 259.4 | 17.29 |
| 28.6 | 1.37864 | 1.1219 | 292.1 | 260.3 | 17.36 |
| 28.7 | 1.37882 | 1.1224 | 293.3 | 261.3 | 17.43 |
| 28.8 | 1.37900 | 1.1229 | 294.5 | 262.2 | 17.50 |
| 28.9 | 1.37918 | 1.1234 | 295.7 | 263.2 | 17.57 |
| 29.0 | 1.37936 | 1.1239 | 296.9 | 264.2 | 17.64 |
| 29.1 | 1.37954 | 1.1244 | 298.1 | 265.1 | 17.72 |
| 29.2 | 1.37972 | 1.1248 | 299.3 | 266.1 | 17.79 |
| 29.3 | 1.37989 | 1.1253 | 300.5 | 267.0 | 17.86 |
| 29.4 | 1.38007 | 1.1258 | 301.7 | 268.0 | 17.93 |
| 29.5 | 1.38025 | 1.1263 | 302.9 | 268.9 | 18.00 |
| 29.6 | 1.38043 | 1.1268 | 304.1 | 269.9 | 18.07 |
| 29.7 | 1.38061 | 1.1273 | 305.3 | 270.8 | 18.14 |
| 29.8 | 1.38079 | 1.1278 | 306.5 | 271.8 | 18.22 |
| 29.9 | 1.38097 | 1.1283 | 307.7 | 272.7 | 18.29 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 30.0 | 1.38115 | 1.1287 | 308.9 | 273.7 | 18.36 |
| 30.1 | 1.38133 | 1.1292 | 310.1 | 274.6 | 18.43 |
| 30.2 | 1.38151 | 1.1297 | 311.3 | 275.6 | 18.50 |
| 30.3 | 1.38169 | 1.1302 | 312.6 | 276.5 | 18.58 |
| 30.4 | 1.38187 | 1.1307 | 313.8 | 277.5 | 18.65 |
| 30.5 | 1.38205 | 1.1312 | 315.0 | 278.5 | 18.72 |
| 30.6 | 1.38223 | 1.1317 | 316.2 | 279.4 | 18.79 |
| 30.7 | 1.38241 | 1.1322 | 317.4 | 280.4 | 18.86 |
| 30.8 | 1.38259 | 1.1327 | 318.6 | 281.3 | 18.93 |
| 30.9 | 1.38277 | 1.1332 | 319.8 | 282.3 | 19.01 |
| 31.0 | 1.38296 | 1.1337 | 321.1 | 283.2 | 19.08 |
| 31.1 | 1.38314 | 1.1342 | 322.3 | 284.2 | 19.15 |
| 31.2 | 1.38332 | 1.1346 | 323.5 | 285.1 | 19.23 |
| 31.3 | 1.38350 | 1.1351 | 324.7 | 286.1 | 19.30 |
| 31.4 | 1.38368 | 1.1356 | 325.9 | 287.0 | 19.37 |
| 31.5 | 1.38386 | 1.1361 | 327.2 | 288.0 | 19.45 |
| 31.6 | 1.38405 | 1.1366 | 328.4 | 288.9 | 19.52 |
| 31.7 | 1.38423 | 1.1371 | 329.6 | 289.9 | 19.59 |
| 31.8 | 1.38441 | 1.1376 | 330.8 | 290.8 | 19.66 |
| 31.9 | 1.38459 | 1.1381 | 332.1 | 291.8 | 19.74 |
| 32.0 | 1.38478 | 1.1386 | 333.3 | 292.7 | 19.81 |
| 32.1 | 1.38496 | 1.1391 | 334.5 | 293.7 | 19.88 |
| 32.2 | 1.38514 | 1.1396 | 335.7 | 294.6 | 19.95 |
| 32.3 | 1.38532 | 1.1401 | 337.0 | 295.6 | 20.03 |
| 32.4 | 1.38551 | 1.1406 | 338.2 | 296.5 | 20.10 |
| 32.5 | 1.38569 | 1.1411 | 339.4 | 297.5 | 20.17 |
| 32.6 | 1.38587 | 1.1416 | 340.7 | 298.4 | 20.25 |
| 32.7 | 1.38606 | 1.1421 | 341.9 | 299.4 | 20.32 |
| 32.8 | 1.38624 | 1.1426 | 343.1 | 300.3 | 20.39 |
| 32.9 | 1.38643 | 1.1431 | 344.4 | 301.3 | 20.47 |
| 33.0 | 1.38661 | 1.1436 | 345.6 | 302.2 | 20.54 |
| 33.1 | 1.38679 | 1.1441 | 346.8 | 303.2 | 20.61 |
| 33.2 | 1.38698 | 1.1446 | 348.1 | 304.1 | 20.69 |
| 33.3 | 1.38716 | 1.1451 | 349.3 | 305.0 | 20.76 |
| 33.4 | 1.38735 | 1.1456 | 350.6 | 306.0 | 20.84 |
| 33.5 | 1.38753 | 1.1461 | 351.8 | 306.9 | 20.91 |
| 33.6 | 1.38772 | 1.1466 | 353.0 | 307.9 | 20.98 |
| 33.7 | 1.38790 | 1.1471 | 354.3 | 308.8 | 21.06 |
| 33.8 | 1.38809 | 1.1476 | 355.5 | 309.8 | 21.13 |
| 33.9 | 1.38827 | 1.1481 | 356.8 | 310.7 | 21.20 |
| 34.0 | 1.38846 | 1.1486 | 358.0 | 311.7 | 21.28 |
| 34.1 | 1.38864 | 1.1491 | 359.2 | 312.6 | 21.35 |
| 34.2 | 1.38883 | 1.1496 | 360.5 | 313.6 | 21.42 |
| 34.3 | 1.38902 | 1.1501 | 361.7 | 314.5 | 21.50 |
| 34.4 | 1.38920 | 1.1507 | 363.0 | 315.5 | 21.57 |
| 34.5 | 1.38939 | 1.1512 | 364.2 | 316.4 | 21.64 |
| 34.6 | 1.38958 | 1.1517 | 365.5 | 317.4 | 21.72 |
| 34.7 | 1.38976 | 1.1522 | 366.7 | 318.3 | 21.79 |
| 34.8 | 1.38995 | 1.1527 | 368.0 | 319.2 | 21.87 |
| 34.9 | 1.39014 | 1.1532 | 369.2 | 320.2 | 21.94 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 35.0 | 1.39032 | 1.1537 | 370.5 | 321.1 | 22.02 |
| 35.1 | 1.39051 | 1.1542 | 371.8 | 322.1 | 22.10 |
| 35.2 | 1.39070 | 1.1547 | 373.0 | 323.0 | 22.17 |
| 35.3 | 1.39088 | 1.1552 | 374.3 | 324.0 | 22.24 |
| 35.4 | 1.39107 | 1.1557 | 375.5 | 324.9 | 22.32 |
| 35.5 | 1.39126 | 1.1563 | 376.8 | 325.9 | 22.39 |
| 35.6 | 1.39145 | 1.1568 | 378.0 | 326.8 | 22.46 |
| 35.7 | 1.39164 | 1.1573 | 379.3 | 327.8 | 22.54 |
| 35.8 | 1.39182 | 1.1578 | 380.6 | 328.7 | 22.62 |
| 35.9 | 1.39201 | 1.1583 | 381.8 | 329.6 | 22.69 |
| 36.0 | 1.39220 | 1.1588 | 383.1 | 330.6 | 22.77 |
| 36.1 | 1.39239 | 1.1593 | 384.4 | 331.5 | 22.84 |
| 36.2 | 1.39258 | 1.1598 | 385.6 | 332.5 | 22.92 |
| 36.3 | 1.39277 | 1.1603 | 386.9 | 333.4 | 22.99 |
| 36.4 | 1.39296 | 1.1609 | 388.1 | 334.4 | 23.06 |
| 36.5 | 1.39314 | 1.1614 | 389.4 | 335.3 | 23.14 |
| 36.6 | 1.39333 | 1.1619 | 390.7 | 336.3 | 23.22 |
| 36.7 | 1.39352 | 1.1624 | 392.0 | 337.2 | 23.30 |
| 36.8 | 1.39371 | 1.1629 | 393.2 | 338.1 | 23.37 |
| 36.9 | 1.39390 | 1.1634 | 394.5 | 339.1 | 23.45 |
| 37.0 | 1.39409 | 1.1640 | 395.8 | 340.0 | 23.52 |
| 37.1 | 1.39428 | 1.1645 | 397.0 | 341.0 | 23.59 |
| 37.2 | 1.39447 | 1.1650 | 398.3 | 341.9 | 23.67 |
| 37.3 | 1.39466 | 1.1655 | 399.6 | 342.9 | 23.75 |
| 37.4 | 1.39485 | 1.1660 | 400.9 | 343.8 | 23.83 |
| 37.5 | 1.39504 | 1.1665 | 402.1 | 344.7 | 23.90 |
| 37.6 | 1.39524 | 1.1671 | 403.4 | 345.7 | 23.97 |
| 37.7 | 1.39543 | 1.1676 | 404.7 | 346.6 | 24.05 |
| 37.8 | 1.39562 | 1.1681 | 406.0 | 347.6 | 24.13 |
| 37.9 | 1.39581 | 1.1686 | 407.3 | 348.5 | 24.21 |
| 38.0 | 1.39600 | 1.1691 | 408.6 | 349.4 | 24.28 |
| 38.1 | 1.39619 | 1.1697 | 409.8 | 350.4 | 24.35 |
| 38.2 | 1.39638 | 1.1702 | 411.1 | 351.3 | 24.43 |
| 38.3 | 1.39658 | 1.1707 | 412.4 | 352.3 | 24.51 |
| 38.4 | 1.39677 | 1.1712 | 413.7 | 353.2 | 24.59 |
| 38.5 | 1.39696 | 1.1717 | 415.0 | 354.2 | 24.66 |
| 38.6 | 1.39715 | 1.1723 | 416.3 | 355.1 | 24.74 |
| 38.7 | 1.39734 | 1.1728 | 417.6 | 356.0 | 24.82 |
| 38.8 | 1.39754 | 1.1733 | 418.8 | 357.0 | 24.89 |
| 38.9 | 1.39773 | 1.1738 | 420.1 | 357.9 | 24.97 |
| 39.0 | 1.39792 | 1.1744 | 421.4 | 358.9 | 25.04 |
| 39.1 | 1.39812 | 1.1749 | 422.7 | 359.8 | 25.12 |
| 39.2 | 1.39831 | 1.1754 | 424.0 | 360.7 | 25.20 |
| 39.3 | 1.39850 | 1.1759 | 425.3 | 361.7 | 25.28 |
| 39.4 | 1.39870 | 1.1765 | 426.6 | 362.6 | 25.35 |
| 39.5 | 1.39889 | 1.1770 | 427.9 | 363.6 | 25.43 |
| 39.6 | 1.39908 | 1.1775 | 429.2 | 364.5 | 25.51 |
| 39.7 | 1.39928 | 1.1780 | 430.5 | 365.4 | 25.58 |
| 39.8 | 1.39947 | 1.1786 | 431.8 | 366.4 | 25.66 |
| 39.9 | 1.39967 | 1.1791 | 433.1 | 367.3 | 25.74 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 40.0 | 1.39986 | 1.1796 | 434.4 | 368.3 | 25.82 |
| 40.1 | 1.40006 | 1.1801 | 435.7 | 369.2 | 25.89 |
| 40.2 | 1.40025 | 1.1807 | 437.0 | 370.1 | 25.97 |
| 40.3 | 1.40044 | 1.1812 | 438.3 | 371.1 | 26.05 |
| 40.4 | 1.40064 | 1.1817 | 439.6 | 372.0 | 26.13 |
| 40.5 | 1.40083 | 1.1823 | 440.9 | 373.0 | 26.20 |
| 40.6 | 1.40103 | 1.1828 | 442.2 | 373.9 | 26.28 |
| 40.7 | 1.40123 | 1.1833 | 443.6 | 374.8 | 26.36 |
| 40.8 | 1.40142 | 1.1839 | 444.9 | 375.8 | 26.44 |
| 40.9 | 1.40162 | 1.1844 | 446.2 | 376.7 | 26.52 |
| 41.0 | 1.40181 | 1.1849 | 447.5 | 377.7 | 26.59 |
| 41.1 | 1.40201 | 1.1855 | 448.8 | 378.6 | 26.67 |
| 41.2 | 1.40221 | 1.1860 | 450.1 | 379.5 | 26.75 |
| 41.3 | 1.40240 | 1.1865 | 451.4 | 380.5 | 26.83 |
| 41.4 | 1.40260 | 1.1871 | 452.8 | 381.4 | 26.91 |
| 41.5 | 1.40280 | 1.1876 | 454.1 | 382.3 | 26.99 |
| 41.6 | 1.40299 | 1.1881 | 455.4 | 383.3 | 27.06 |
| 41.7 | 1.40319 | 1.1887 | 456.7 | 384.2 | 27.14 |
| 41.8 | 1.40339 | 1.1892 | 458.0 | 385.2 | 27.22 |
| 41.9 | 1.40358 | 1.1897 | 459.4 | 386.1 | 27.30 |
| 42.0 | 1.40378 | 1.1903 | 460.7 | 387.0 | 27.38 |
| 42.1 | 1.40398 | 1.1908 | 462.0 | 388.0 | 27.46 |
| 42.2 | 1.40418 | 1.1913 | 463.3 | 388.9 | 27.53 |
| 42.3 | 1.40437 | 1.1919 | 464.7 | 389.9 | 27.62 |
| 42.4 | 1.40457 | 1.1924 | 466.0 | 390.8 | 27.69 |
| 42.5 | 1.40477 | 1.1929 | 467.3 | 391.7 | 27.77 |
| 42.6 | 1.40497 | 1.1935 | 468.6 | 392.7 | 27.85 |
| 42.7 | 1.40517 | 1.1940 | 470.0 | 393.6 | 27.93 |
| 42.8 | 1.40537 | 1.1946 | 471.3 | 394.5 | 28.01 |
| 42.9 | 1.40557 | 1.1951 | 472.6 | 395.5 | 28.09 |
| 43.0 | 1.40576 | 1.1956 | 474.0 | 396.4 | 28.17 |
| 43.1 | 1.40596 | 1.1962 | 475.3 | 397.3 | 28.25 |
| 43.2 | 1.40616 | 1.1967 | 476.6 | 398.3 | 28.32 |
| 43.3 | 1.40636 | 1.1973 | 478.0 | 399.2 | 28.41 |
| 43.4 | 1.40656 | 1.1978 | 479.3 | 400.2 | 28.48 |
| 43.5 | 1.40676 | 1.1983 | 480.7 | 401.1 | 28.57 |
| 43.6 | 1.40696 | 1.1989 | 482.0 | 402.0 | 28.65 |
| 43.7 | 1.40716 | 1.1994 | 483.3 | 403.0 | 28.72 |
| 43.8 | 1.40736 | 1.2000 | 484.7 | 403.9 | 28.81 |
| 43.9 | 1.40756 | 1.2005 | 486.0 | 404.8 | 28.88 |
| 44.0 | 1.40776 | 1.2011 | 487.4 | 405.8 | 28.97 |
| 44.1 | 1.40796 | 1.2016 | 488.7 | 406.7 | 29.04 |
| 44.2 | 1.40817 | 1.2022 | 490.1 | 407.6 | 29.13 |
| 44.3 | 1.40837 | 1.2027 | 491.4 | 408.6 | 29.20 |
| 44.4 | 1.40857 | 1.2032 | 492.8 | 409.5 | 29.29 |
| 44.5 | 1.40877 | 1.2038 | 494.1 | 410.4 | 29.36 |
| 44.6 | 1.40897 | 1.2043 | 495.5 | 411.4 | 29.45 |
| 44.7 | 1.40917 | 1.2049 | 496.8 | 412.3 | 29.52 |
| 44.8 | 1.40937 | 1.2054 | 498.2 | 413.3 | 29.61 |
| 44.9 | 1.40958 | 1.2060 | 499.5 | 414.2 | 29.69 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 45.0 | 1.40978 | 1.2065 | 500.9 | 415.1 | 29.77 |
| 45.1 | 1.40998 | 1.2071 | 502.2 | 416.1 | 29.85 |
| 45.2 | 1.41018 | 1.2076 | 503.6 | 417.0 | 29.93 |
| 45.3 | 1.41039 | 1.2082 | 504.9 | 417.9 | 30.01 |
| 45.4 | 1.41059 | 1.2087 | 506.3 | 418.9 | 30.09 |
| 45.5 | 1.41079 | 1.2093 | 507.7 | 419.8 | 30.17 |
| 45.6 | 1.41099 | 1.2098 | 509.0 | 420.7 | 30.25 |
| 45.7 | 1.41120 | 1.2104 | 510.4 | 421.7 | 30.33 |
| 45.8 | 1.41140 | 1.2109 | 511.7 | 422.6 | 30.41 |
| 45.9 | 1.41160 | 1.2115 | 513.1 | 423.5 | 30.49 |
| 46.0 | 1.41181 | 1.2120 | 514.5 | 424.5 | 30.58 |
| 46.1 | 1.41201 | 1.2126 | 515.8 | 425.4 | 30.65 |
| 46.2 | 1.41222 | 1.2131 | 517.2 | 426.3 | 30.74 |
| 46.3 | 1.41242 | 1.2137 | 518.6 | 427.3 | 30.82 |
| 46.4 | 1.41262 | 1.2142 | 519.9 | 428.2 | 30.90 |
| 46.5 | 1.41283 | 1.2148 | 521.3 | 429.1 | 30.98 |
| 46.6 | 1.41303 | 1.2154 | 522.7 | 430.1 | 31.06 |
| 46.7 | 1.41324 | 1.2159 | 524.1 | 431.0 | 31.15 |
| 46.8 | 1.41344 | 1.2165 | 525.4 | 431.9 | 31.22 |
| 46.9 | 1.41365 | 1.2170 | 526.8 | 432.9 | 31.31 |
| 47.0 | 1.41385 | 1.2176 | 528.2 | 433.8 | 31.39 |
| 47.1 | 1.41406 | 1.2181 | 529.6 | 434.7 | 31.47 |
| 47.2 | 1.41427 | 1.2187 | 530.9 | 435.7 | 31.55 |
| 47.3 | 1.41447 | 1.2192 | 532.3 | 436.6 | 31.63 |
| 47.4 | 1.41468 | 1.2198 | 533.7 | 437.5 | 31.72 |
| 47.5 | 1.41488 | 1.2204 | 535.1 | 438.5 | 31.80 |
| 47.6 | 1.41509 | 1.2209 | 536.5 | 439.4 | 31.88 |
| 47.7 | 1.41530 | 1.2215 | 537.9 | 440.3 | 31.97 |
| 47.8 | 1.41550 | 1.2220 | 539.2 | 441.3 | 32.04 |
| 47.9 | 1.41571 | 1.2226 | 540.6 | 442.2 | 32.13 |
| 48.0 | 1.41592 | 1.2232 | 542.0 | 443.1 | 32.21 |
| 48.1 | 1.41612 | 1.2237 | 543.4 | 444.1 | 32.29 |
| 48.2 | 1.41633 | 1.2243 | 544.8 | 445.0 | 32.38 |
| 48.3 | 1.41654 | 1.2248 | 546.2 | 445.9 | 32.46 |
| 48.4 | 1.41674 | 1.2254 | 547.6 | 446.8 | 32.54 |
| 48.5 | 1.41695 | 1.2260 | 549.0 | 447.8 | 32.63 |
| 48.6 | 1.41716 | 1.2265 | 550.4 | 448.7 | 32.71 |
| 48.7 | 1.41737 | 1.2271 | 551.8 | 449.6 | 32.79 |
| 48.8 | 1.41758 | 1.2277 | 553.2 | 450.6 | 32.88 |
| 48.9 | 1.41779 | 1.2282 | 554.6 | 451.5 | 32.96 |
| 49.0 | 1.41799 | 1.2288 | 556.0 | 452.4 | 33.04 |
| 49.1 | 1.41820 | 1.2294 | 557.4 | 453.4 | 33.13 |
| 49.2 | 1.41841 | 1.2299 | 558.8 | 454.3 | 33.21 |
| 49.3 | 1.41862 | 1.2305 | 560.2 | 455.2 | 33.29 |
| 49.4 | 1.41883 | 1.2311 | 561.6 | 456.2 | 33.38 |
| 49.5 | 1.41904 | 1.2316 | 563.0 | 457.1 | 33.46 |
| 49.6 | 1.41925 | 1.2322 | 564.4 | 458.0 | 33.54 |
| 49.7 | 1.41946 | 1.2328 | 565.8 | 458.9 | 33.63 |
| 49.8 | 1.41967 | 1.2333 | 567.2 | 459.9 | 33.71 |
| 49.9 | 1.41988 | 1.2339 | 568.6 | 460.8 | 33.79 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 50.0 | 1.42009 | 1.2345 | 570.0 | 461.7 | 33.88 |
| 50.1 | 1.42030 | 1.2350 | 571.4 | 462.7 | 33.96 |
| 50.2 | 1.42051 | 1.2356 | 572.8 | 463.6 | 34.04 |
| 50.3 | 1.42072 | 1.2362 | 574.2 | 464.5 | 34.12 |
| 50.4 | 1.42093 | 1.2368 | 575.6 | 465.4 | 34.21 |
| 50.5 | 1.42114 | 1.2373 | 577.1 | 466.4 | 34.30 |
| 50.6 | 1.42135 | 1.2379 | 578.5 | 467.3 | 34.38 |
| 50.7 | 1.42156 | 1.2385 | 579.9 | 468.2 | 34.46 |
| 50.8 | 1.42177 | 1.2390 | 581.3 | 469.2 | 34.55 |
| 50.9 | 1.42199 | 1.2396 | 582.7 | 470.1 | 34.63 |
| 51.0 | 1.42220 | 1.2402 | 584.2 | 471.0 | 34.72 |
| 51.1 | 1.42241 | 1.2408 | 585.6 | 471.9 | 34.80 |
| 51.2 | 1.42262 | 1.2413 | 587.0 | 472.9 | 34.89 |
| 51.3 | 1.42283 | 1.2419 | 588.4 | 473.8 | 34.97 |
| 51.4 | 1.42305 | 1.2425 | 589.9 | 474.7 | 35.06 |
| 51.5 | 1.42326 | 1.2431 | 591.3 | 475.7 | 35.14 |
| 51.6 | 1.42347 | 1.2436 | 592.7 | 476.6 | 35.22 |
| 51.7 | 1.42368 | 1.2442 | 594.1 | 477.5 | 35.31 |
| 51.8 | 1.42390 | 1.2448 | 595.6 | 478.4 | 35.40 |
| 51.9 | 1.42411 | 1.2454 | 597.0 | 479.4 | 35.48 |
| 52.0 | 1.42432 | 1.2460 | 598.4 | 480.3 | 35.56 |
| 52.1 | 1.42454 | 1.2465 | 599.9 | 481.2 | 35.65 |
| 52.2 | 1.42475 | 1.2471 | 601.3 | 482.1 | 35.74 |
| 52.3 | 1.42496 | 1.2477 | 602.7 | 483.1 | 35.82 |
| 52.4 | 1.42518 | 1.2483 | 604.2 | 484.0 | 35.91 |
| 52.5 | 1.42539 | 1.2488 | 605.6 | 484.9 | 35.99 |
| 52.6 | 1.42561 | 1.2494 | 607.0 | 485.8 | 36.07 |
| 52.7 | 1.42582 | 1.2500 | 608.5 | 486.8 | 36.16 |
| 52.8 | 1.42604 | 1.2506 | 609.9 | 487.7 | 36.25 |
| 52.9 | 1.42625 | 1.2512 | 611.4 | 488.6 | 36.34 |
| 53.0 | 1.42647 | 1.2518 | 612.8 | 489.5 | 36.42 |
| 53.1 | 1.42668 | 1.2523 | 614.2 | 490.5 | 36.50 |
| 53.2 | 1.42690 | 1.2529 | 615.7 | 491.4 | 36.59 |
| 53.3 | 1.42711 | 1.2535 | 617.1 | 492.3 | 36.67 |
| 53.4 | 1.42733 | 1.2541 | 618.6 | 493.2 | 36.76 |
| 53.5 | 1.42754 | 1.2547 | 620.0 | 494.2 | 36.85 |
| 53.6 | 1.42776 | 1.2553 | 621.5 | 495.1 | 36.94 |
| 53.7 | 1.42798 | 1.2558 | 622.9 | 496.0 | 37.02 |
| 53.8 | 1.42819 | 1.2564 | 624.4 | 496.9 | 37.11 |
| 53.9 | 1.42841 | 1.2570 | 625.8 | 497.9 | 37.19 |
| 54.0 | 1.42863 | 1.2576 | 627.3 | 498.8 | 37.28 |
| 54.1 | 1.42884 | 1.2582 | 628.7 | 499.7 | 37.36 |
| 54.2 | 1.42906 | 1.2588 | 630.2 | 500.6 | 37.45 |
| 54.3 | 1.42928 | 1.2594 | 631.7 | 501.6 | 37.54 |
| 54.4 | 1.42949 | 1.2600 | 633.1 | 502.5 | 37.63 |
| 54.5 | 1.42971 | 1.2606 | 634.6 | 503.4 | 37.71 |
| 54.6 | 1.42993 | 1.2611 | 636.0 | 504.3 | 37.80 |
| 54.7 | 1.43015 | 1.2617 | 637.5 | 505.2 | 37.89 |
| 54.8 | 1.43036 | 1.2623 | 639.0 | 506.2 | 37.98 |
| 54.9 | 1.43058 | 1.2629 | 640.4 | 507.1 | 38.06 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 55.0 | 1.43080 | 1.2635 | 641.9 | 508.0 | 38.15 |
| 55.1 | 1.43102 | 1.2641 | 643.4 | 508.9 | 38.24 |
| 55.2 | 1.43124 | 1.2647 | 644.8 | 509.9 | 38.32 |
| 55.3 | 1.43146 | 1.2653 | 646.3 | 510.8 | 38.41 |
| 55.4 | 1.43168 | 1.2659 | 647.8 | 511.7 | 38.50 |
| 55.5 | 1.43189 | 1.2665 | 649.2 | 512.6 | 38.58 |
| 55.6 | 1.43211 | 1.2671 | 650.7 | 513.5 | 38.67 |
| 55.7 | 1.43233 | 1.2677 | 652.2 | 514.5 | 38.76 |
| 55.8 | 1.43255 | 1.2683 | 653.7 | 515.4 | 38.85 |
| 55.9 | 1.43277 | 1.2689 | 655.1 | 516.3 | 38.93 |
| 56.0 | 1.43299 | 1.2695 | 656.6 | 517.2 | 39.02 |
| 56.1 | 1.43321 | 1.2701 | 658.1 | 518.1 | 39.11 |
| 56.2 | 1.43343 | 1.2706 | 659.6 | 519.1 | 39.20 |
| 56.3 | 1.43365 | 1.2712 | 661.0 | 520.0 | 39.28 |
| 56.4 | 1.43387 | 1.2718 | 662.5 | 520.9 | 39.37 |
| 56.5 | 1.43410 | 1.2724 | 664.0 | 521.8 | 39.46 |
| 56.6 | 1.43432 | 1.2730 | 665.5 | 522.7 | 39.55 |
| 56.7 | 1.43454 | 1.2736 | 667.0 | 523.7 | 39.64 |
| 56.8 | 1.43476 | 1.2742 | 668.5 | 524.6 | 39.73 |
| 56.9 | 1.43498 | 1.2748 | 669.9 | 525.5 | 39.81 |
| 57.0 | 1.43520 | 1.2754 | 671.4 | 526.4 | 39.90 |
| 57.1 | 1.43542 | 1.2760 | 672.9 | 527.3 | 39.99 |
| 57.2 | 1.43565 | 1.2766 | 674.4 | 528.3 | 40.08 |
| 57.3 | 1.43587 | 1.2773 | 675.9 | 529.2 | 40.17 |
| 57.4 | 1.43609 | 1.2779 | 677.4 | 530.1 | 40.26 |
| 57.5 | 1.43631 | 1.2785 | 678.9 | 531.0 | 40.35 |
| 57.6 | 1.43653 | 1.2791 | 680.4 | 531.9 | 40.44 |
| 57.7 | 1.43676 | 1.2797 | 681.9 | 532.8 | 40.53 |
| 57.8 | 1.43698 | 1.2803 | 683.4 | 533.8 | 40.61 |
| 57.9 | 1.43720 | 1.2809 | 684.9 | 534.7 | 40.70 |
| 58.0 | 1.43743 | 1.2815 | 686.4 | 535.6 | 40.79 |
| 58.1 | 1.43765 | 1.2821 | 687.9 | 536.5 | 40.88 |
| 58.2 | 1.43787 | 1.2827 | 689.4 | 537.4 | 40.97 |
| 58.3 | 1.43810 | 1.2833 | 690.9 | 538.3 | 41.06 |
| 58.4 | 1.43832 | 1.2839 | 692.4 | 539.3 | 41.15 |
| 58.5 | 1.43855 | 1.2845 | 693.9 | 540.2 | 41.24 |
| 58.6 | 1.43877 | 1.2851 | 695.4 | 541.1 | 41.33 |
| 58.7 | 1.43899 | 1.2857 | 696.9 | 542.0 | 41.42 |
| 58.8 | 1.43922 | 1.2863 | 698.4 | 542.9 | 41.51 |
| 58.9 | 1.43944 | 1.2870 | 699.9 | 543.8 | 41.60 |
| 59.0 | 1.43967 | 1.2876 | 701.4 | 544.8 | 41.68 |
| 59.1 | 1.43989 | 1.2882 | 702.9 | 545.7 | 41.77 |
| 59.2 | 1.44012 | 1.2888 | 704.4 | 546.6 | 41.86 |
| 59.3 | 1.44035 | 1.2894 | 706.0 | 547.5 | 41.96 |
| 59.4 | 1.44057 | 1.2900 | 707.5 | 548.4 | 42.05 |
| 59.5 | 1.44080 | 1.2906 | 709.0 | 549.3 | 42.14 |
| 59.6 | 1.44102 | 1.2912 | 710.5 | 550.2 | 42.23 |
| 59.7 | 1.44125 | 1.2919 | 712.0 | 551.1 | 42.31 |
| 59.8 | 1.44148 | 1.2925 | 713.5 | 552.1 | 42.40 |
| 59.9 | 1.44170 | 1.2931 | 715.1 | 553.0 | 42.50 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 60.0 | 1.44193 | 1.2937 | 716.6 | 553.9 | 42.59 |
| 60.1 | 1.44216 | 1.2943 | 718.1 | 554.8 | 42.68 |
| 60.2 | 1.44238 | 1.2949 | 719.6 | 555.7 | 42.77 |
| 60.3 | 1.44261 | 1.2956 | 721.1 | 556.6 | 42.85 |
| 60.4 | 1.44284 | 1.2962 | 722.7 | 557.5 | 42.95 |
| 60.5 | 1.44306 | 1.2968 | 724.2 | 558.4 | 43.04 |
| 60.6 | 1.44329 | 1.2974 | 725.7 | 559.4 | 43.13 |
| 60.7 | 1.44352 | 1.2980 | 727.3 | 560.3 | 43.22 |
| 60.8 | 1.44375 | 1.2986 | 728.8 | 561.2 | 43.31 |
| 60.9 | 1.44398 | 1.2993 | 730.3 | 562.1 | 43.40 |
| 61.0 | 1.44420 | 1.2999 | 731.8 | 563.0 | 43.49 |
| 61.1 | 1.44443 | 1.3005 | 733.4 | 563.9 | 43.59 |
| 61.2 | 1.44466 | 1.3011 | 734.9 | 564.8 | 43.68 |
| 61.3 | 1.44489 | 1.3017 | 736.4 | 565.7 | 43.76 |
| 61.4 | 1.44512 | 1.3024 | 738.0 | 566.6 | 43.86 |
| 61.5 | 1.44535 | 1.3030 | 739.5 | 567.6 | 43.95 |
| 61.6 | 1.44558 | 1.3036 | 741.1 | 568.5 | 44.04 |
| 61.7 | 1.44581 | 1.3042 | 742.6 | 569.4 | 44.13 |
| 61.8 | 1.44604 | 1.3049 | 744.1 | 570.3 | 44.22 |
| 61.9 | 1.44627 | 1.3055 | 745.7 | 571.2 | 44.32 |
| 62.0 | 1.44650 | 1.3061 | 747.2 | 572.1 | 44.41 |
| 62.1 | 1.44673 | 1.3067 | 748.8 | 573.0 | 44.50 |
| 62.2 | 1.44696 | 1.3074 | 750.3 | 573.9 | 44.59 |
| 62.3 | 1.44719 | 1.3080 | 751.9 | 574.8 | 44.69 |
| 62.4 | 1.44742 | 1.3086 | 753.4 | 575.7 | 44.77 |
| 62.5 | 1.44765 | 1.3092 | 755.0 | 576.6 | 44.87 |
| 62.6 | 1.44788 | 1.3099 | 756.5 | 577.5 | 44.96 |
| 62.7 | 1.44811 | 1.3105 | 758.1 | 578.5 | 45.05 |
| 62.8 | 1.44834 | 1.3111 | 759.6 | 579.4 | 45.14 |
| 62.9 | 1.44858 | 1.3118 | 761.2 | 580.3 | 45.24 |
| 63.0 | 1.44881 | 1.3124 | 762.7 | 581.2 | 45.33 |
| 63.1 | 1.44904 | 1.3130 | 764.3 | 582.1 | 45.42 |
| 63.2 | 1.44927 | 1.3137 | 765.8 | 583.0 | 45.51 |
| 63.3 | 1.44950 | 1.3143 | 767.4 | 583.9 | 45.61 |
| 63.4 | 1.44974 | 1.3149 | 769.0 | 584.8 | 45.70 |
| 63.5 | 1.44997 | 1.3155 | 770.5 | 585.7 | 45.79 |
| 63.6 | 1.45020 | 1.3162 | 772.1 | 586.6 | 45.89 |
| 63.7 | 1.45043 | 1.3168 | 773.6 | 587.5 | 45.98 |
| 63.8 | 1.45067 | 1.3174 | 775.2 | 588.4 | 46.07 |
| 63.9 | 1.45090 | 1.3181 | 776.8 | 589.3 | 46.17 |
| 64.0 | 1.45113 | 1.3187 | 778.3 | 590.2 | 46.25 |
| 64.1 | 1.45137 | 1.3193 | 779.9 | 591.1 | 46.35 |
| 64.2 | 1.45160 | 1.3200 | 781.5 | 592.0 | 46.44 |
| 64.3 | 1.45184 | 1.3206 | 783.0 | 592.9 | 46.53 |
| 64.4 | 1.45207 | 1.3213 | 784.6 | 593.8 | 46.63 |
| 64.5 | 1.45230 | 1.3219 | 786.2 | 594.7 | 46.72 |
| 64.6 | 1.45254 | 1.3225 | 787.8 | 595.6 | 46.82 |
| 64.7 | 1.45277 | 1.3232 | 789.3 | 596.5 | 46.91 |
| 64.8 | 1.45301 | 1.3238 | 790.9 | 597.4 | 47.00 |
| 64.9 | 1.45324 | 1.3244 | 792.5 | 598.3 | 47.10 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 65.0 | 1.45348 | 1.3251 | 794.1 | 599.3 | 47.19 |
| 65.1 | 1.45371 | 1.3257 | 795.6 | 600.2 | 47.28 |
| 65.2 | 1.45395 | 1.3264 | 797.2 | 601.1 | 47.38 |
| 65.3 | 1.45418 | 1.3270 | 798.8 | 602.0 | 47.47 |
| 65.4 | 1.45442 | 1.3276 | 800.4 | 602.9 | 47.57 |
| 65.5 | 1.45466 | 1.3283 | 802.0 | 603.8 | 47.66 |
| 65.6 | 1.45489 | 1.3289 | 803.6 | 604.7 | 47.76 |
| 65.7 | 1.45513 | 1.3296 | 805.1 | 605.6 | 47.85 |
| 65.8 | 1.45537 | 1.3302 | 806.7 | 606.5 | 47.94 |
| 65.9 | 1.45560 | 1.3309 | 808.3 | 607.4 | 48.04 |
| 66.0 | 1.45584 | 1.3315 | 809.9 | 608.3 | 48.13 |
| 66.1 | 1.45608 | 1.3322 | 811.5 | 609.2 | 48.23 |
| 66.2 | 1.45631 | 1.3328 | 813.1 | 610.1 | 48.32 |
| 66.3 | 1.45655 | 1.3334 | 814.7 | 611.0 | 48.42 |
| 66.4 | 1.45679 | 1.3341 | 816.3 | 611.9 | 48.51 |
| 66.5 | 1.45703 | 1.3347 | 817.9 | 612.8 | 48.61 |
| 66.6 | 1.45726 | 1.3354 | 819.5 | 613.7 | 48.70 |
| 66.7 | 1.45750 | 1.3360 | 821.1 | 614.6 | 48.80 |
| 66.8 | 1.45774 | 1.3367 | 822.7 | 615.5 | 48.89 |
| 66.9 | 1.45798 | 1.3373 | 824.3 | 616.3 | 48.99 |
| 67.0 | 1.45822 | 1.3380 | 825.9 | 617.2 | 49.08 |
| 67.1 | 1.45846 | 1.3386 | 827.5 | 618.1 | 49.18 |
| 67.2 | 1.45870 | 1.3393 | 829.1 | 619.0 | 49.27 |
| 67.3 | 1.45893 | 1.3399 | 830.7 | 619.9 | 49.37 |
| 67.4 | 1.45917 | 1.3406 | 832.3 | 620.8 | 49.46 |
| 67.5 | 1.45941 | 1.3412 | 833.9 | 621.7 | 49.56 |
| 67.6 | 1.45965 | 1.3419 | 835.5 | 622.6 | 49.65 |
| 67.7 | 1.45989 | 1.3425 | 837.1 | 623.5 | 49.75 |
| 67.8 | 1.46013 | 1.3432 | 838.7 | 624.4 | 49.84 |
| 67.9 | 1.46037 | 1.3438 | 840.3 | 625.3 | 49.94 |
| 68.0 | 1.46061 | 1.3445 | 841.9 | 626.2 | 50.03 |
| 68.1 | 1.46085 | 1.3451 | 843.6 | 627.1 | 50.14 |
| 68.2 | 1.46109 | 1.3458 | 845.2 | 628.0 | 50.23 |
| 68.3 | 1.46134 | 1.3464 | 846.8 | 628.9 | 50.33 |
| 68.4 | 1.46158 | 1.3471 | 848.4 | 629.8 | 50.42 |
| 68.5 | 1.46182 | 1.3478 | 850.0 | 630.7 | 50.52 |
| 68.6 | 1.46206 | 1.3484 | 851.6 | 631.6 | 50.61 |
| 68.7 | 1.46230 | 1.3491 | 853.3 | 632.5 | 50.71 |
| 68.8 | 1.46254 | 1.3497 | 854.9 | 633.4 | 50.81 |
| 68.9 | 1.46278 | 1.3504 | 856.5 | 634.3 | 50.90 |
| 69.0 | 1.46303 | 1.3510 | 858.1 | 635.2 | 51.00 |
| 69.1 | 1.46327 | 1.3517 | 859.8 | 636.1 | 51.10 |
| 69.2 | 1.46351 | 1.3524 | 861.4 | 636.9 | 51.19 |
| 69.3 | 1.46375 | 1.3530 | 863.0 | 637.8 | 51.29 |
| 69.4 | 1.46400 | 1.3537 | 864.7 | 638.7 | 51.39 |
| 69.5 | 1.46424 | 1.3543 | 866.3 | 639.6 | 51.48 |
| 69.6 | 1.46448 | 1.3550 | 867.9 | 640.5 | 51.58 |
| 69.7 | 1.46473 | 1.3557 | 869.5 | 641.4 | 51.67 |
| 69.8 | 1.46497 | 1.3563 | 871.2 | 642.3 | 51.78 |
| 69.9 | 1.46521 | 1.3570 | 872.8 | 643.2 | 51.87 |

TABLE II - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 70.0 | 1.46546 | 1.3576 | 874.5 | 644.1 | 51.97 |
| 70.1 | 1.46570 | 1.3583 | 876.1 | 645.0 | 52.07 |
| 70.2 | 1.46594 | 1.3590 | 877.7 | 645.9 | 52.16 |
| 70.3 | 1.46619 | 1.3596 | 879.4 | 646.8 | 52.26 |
| 70.4 | 1.46643 | 1.3603 | 881.0 | 647.7 | 52.36 |
| 70.5 | 1.46668 | 1.3610 | 882.7 | 648.5 | 52.46 |
| 70.6 | 1.46692 | 1.3616 | 884.3 | 649.4 | 52.55 |
| 70.7 | 1.46717 | 1.3623 | 886.0 | 650.3 | 52.65 |
| 70.8 | 1.46741 | 1.3630 | 887.6 | 651.2 | 52.75 |
| 70.9 | 1.46766 | 1.3636 | 889.3 | 652.1 | 52.85 |
| 71.0 | 1.46790 | 1.3643 | 890.9 | 653.0 | 52.95 |
| 71.1 | 1.46815 | 1.3650 | 892.6 | 653.9 | 53.05 |
| 71.2 | 1.46840 | 1.3656 | 894.2 | 654.8 | 53.14 |
| 71.3 | 1.46864 | 1.3663 | 895.9 | 655.7 | 53.24 |
| 71.4 | 1.46889 | 1.3670 | 897.5 | 656.6 | 53.34 |
| 71.5 | 1.46913 | 1.3676 | 899.2 | 657.5 | 53.44 |
| 71.6 | 1.46938 | 1.3683 | 900.8 | 658.3 | 53.53 |
| 71.7 | 1.46963 | 1.3690 | 902.5 | 659.2 | 53.64 |
| 71.8 | 1.46987 | 1.3696 | 904.1 | 660.1 | 53.73 |
| 71.9 | 1.47012 | 1.3703 | 905.8 | 661.0 | 53.83 |
| 72.0 | 1.47037 | 1.3710 | 907.5 | 661.9 | 53.93 |
| 72.1 | 1.47062 | 1.3717 | 909.1 | 662.8 | 54.03 |
| 72.2 | 1.47086 | 1.3723 | 910.8 | 663.7 | 54.13 |
| 72.3 | 1.47111 | 1.3730 | 912.5 | 664.6 | 54.23 |
| 72.4 | 1.47136 | 1.3737 | 914.1 | 665.5 | 54.32 |
| 72.5 | 1.47161 | 1.3743 | 915.8 | 666.3 | 54.43 |
| 72.6 | 1.47186 | 1.3750 | 917.5 | 667.2 | 54.53 |
| 72.7 | 1.47210 | 1.3757 | 919.1 | 668.1 | 54.62 |
| 72.8 | 1.47235 | 1.3764 | 920.8 | 669.0 | 54.72 |
| 72.9 | 1.47260 | 1.3770 | 922.5 | 669.9 | 54.82 |
| 73.0 | 1.47285 | 1.3777 | 924.2 | 670.8 | 54.93 |
| 73.1 | 1.47310 | 1.3784 | 925.8 | 671.7 | 55.02 |
| 73.2 | 1.47335 | 1.3791 | 927.5 | 672.6 | 55.12 |
| 73.3 | 1.47360 | 1.3797 | 929.2 | 673.5 | 55.22 |
| 73.4 | 1.47385 | 1.3804 | 930.9 | 674.3 | 55.32 |
| 73.5 | 1.47410 | 1.3811 | 932.6 | 675.2 | 55.42 |
| 73.6 | 1.47435 | 1.3818 | 934.3 | 676.1 | 55.53 |
| 73.7 | 1.47460 | 1.3825 | 935.9 | 677.0 | 55.62 |
| 73.8 | 1.47485 | 1.3831 | 937.6 | 677.9 | 55.72 |
| 73.9 | 1.47510 | 1.3838 | 939.3 | 678.8 | 55.82 |
| 74.0 | 1.47535 | 1.3845 | 941.0 | 679.7 | 55.92 |
| 74.1 | 1.47560 | 1.3852 | 942.7 | 680.6 | 56.02 |
| 74.2 | 1.47585 | 1.3859 | 944.4 | 681.4 | 56.13 |
| 74.3 | 1.47610 | 1.3865 | 946.1 | 682.3 | 56.23 |
| 74.4 | 1.47635 | 1.3872 | 947.8 | 683.2 | 56.33 |
| 74.5 | 1.47661 | 1.3879 | 949.5 | 684.1 | 56.43 |
| 74.6 | 1.47686 | 1.3886 | 951.2 | 685.0 | 56.53 |
| 74.7 | 1.47711 | 1.3893 | 952.9 | 685.9 | 56.63 |
| 74.8 | 1.47736 | 1.3899 | 954.6 | 686.8 | 56.73 |
| 74.9 | 1.47761 | 1.3906 | 956.3 | 687.7 | 56.83 |

TABLE III

Table giving the sugar concentration in rectified concentrated must
in grams per liter and grams per kilogram.
determined by means of a refractometer graduated
either in percentage by mass of sucrose at 20°C
or in refractive index at 20°C.

TABLE III

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 50.0 | 1.42008 | 1.2342 | 627.6 | 508.5 | 37.30 |
| 50.1 | 1.42029 | 1.2348 | 629.3 | 509.6 | 37.40 |
| 50.2 | 1.42050 | 1.2355 | 630.9 | 510.6 | 37.49 |
| 50.3 | 1.42071 | 1.2362 | 632.4 | 511.6 | 37.58 |
| 50.4 | 1.42092 | 1.2367 | 634.1 | 512.7 | 37.68 |
| 50.5 | 1.42113 | 1.2374 | 635.7 | 513.7 | 37.78 |
| 50.6 | 1.42135 | 1.2381 | 637.3 | 514.7 | 37.87 |
| 50.7 | 1.42156 | 1.2386 | 638.7 | 515.7 | 37.96 |
| 50.8 | 1.42177 | 1.2391 | 640.4 | 516.8 | 38.06 |
| 50.9 | 1.42198 | 1.2396 | 641.9 | 517.8 | 38.15 |
| 51.0 | 1.42219 | 1.2401 | 643.4 | 518.8 | 38.24 |
| 51.1 | 1.42240 | 1.2406 | 645.0 | 519.9 | 38.33 |
| 51.2 | 1.42261 | 1.2411 | 646.5 | 520.9 | 38.42 |
| 51.3 | 1.42282 | 1.2416 | 648.1 | 522.0 | 38.52 |
| 51.4 | 1.42304 | 1.2421 | 649.6 | 523.0 | 38.61 |
| 51.5 | 1.42325 | 1.2427 | 651.2 | 524.0 | 38.70 |
| 51.6 | 1.42347 | 1.2434 | 652.9 | 525.1 | 38.80 |
| 51.7 | 1.42368 | 1.2441 | 654.5 | 526.1 | 38.90 |
| 51.8 | 1.42389 | 1.2447 | 656.1 | 527.1 | 38.99 |
| 51.9 | 1.42410 | 1.2454 | 657.8 | 528.2 | 39.09 |
| 52.0 | 1.42432 | 1.2461 | 659.4 | 529.2 | 39.19 |
| 52.1 | 1.42453 | 1.2466 | 661.0 | 530.2 | 39.28 |
| 52.2 | 1.42475 | 1.2470 | 662.5 | 531.3 | 39.37 |
| 52.3 | 1.42496 | 1.2475 | 664.1 | 532.3 | 39.47 |
| 52.4 | 1.42517 | 1.2480 | 665.6 | 533.3 | 39.56 |
| 52.5 | 1.42538 | 1.2486 | 667.2 | 534.4 | 39.65 |
| 52.6 | 1.42560 | 1.2493 | 668.9 | 535.4 | 39.75 |
| 52.7 | 1.42581 | 1.2500 | 670.5 | 536.4 | 39.85 |
| 52.8 | 1.42603 | 1.2506 | 672.2 | 537.5 | 39.95 |
| 52.9 | 1.42624 | 1.2513 | 673.8 | 538.5 | 40.04 |
| 53.0 | 1.42645 | 1.2520 | 675.5 | 539.5 | 40.14 |
| 53.1 | 1.42667 | 1.2525 | 677.1 | 540.6 | 40.24 |
| 53.2 | 1.42689 | 1.2530 | 678.5 | 541.5 | 40.32 |
| 53.3 | 1.42711 | 1.2535 | 680.2 | 542.6 | 40.42 |
| 53.4 | 1.42733 | 1.2540 | 681.8 | 543.7 | 40.52 |
| 53.5 | 1.42754 | 1.2546 | 683.4 | 544.7 | 40.61 |
| 53.6 | 1.42776 | 1.2553 | 685.1 | 545.8 | 40.72 |
| 53.7 | 1.42797 | 1.2560 | 686.7 | 546.7 | 40.81 |
| 53.8 | 1.42819 | 1.2566 | 688.4 | 547.8 | 40.91 |
| 53.9 | 1.42840 | 1.2573 | 690.1 | 548.9 | 41.01 |
| 54.0 | 1.42861 | 1.2580 | 691.7 | 549.8 | 41.11 |
| 54.1 | 1.42884 | 1.2585 | 693.3 | 550.9 | 41.20 |
| 54.2 | 1.42906 | 1.2590 | 694.9 | 551.9 | 41.30 |
| 54.3 | 1.42927 | 1.2595 | 696.5 | 553.0 | 41.39 |
| 54.4 | 1.42949 | 1.2600 | 698.1 | 554.0 | 41.49 |
| 54.5 | 1.42971 | 1.2606 | 699.7 | 555.1 | 41.58 |
| 54.6 | 1.42993 | 1.2613 | 701.4 | 556.1 | 41.68 |
| 54.7 | 1.43014 | 1.2620 | 703.1 | 557.1 | 41.79 |
| 54.8 | 1.43036 | 1.2625 | 704.7 | 558.2 | 41.88 |
| 54.9 | 1.43058 | 1.2630 | 706.2 | 559.1 | 41.97 |

TABLE III – (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 55.0 | 1.43079 | 1.2635 | 707.8 | 560.2 | 42.06 |
| 55.1 | 1.43102 | 1.2639 | 709.4 | 561.3 | 42.16 |
| 55.2 | 1.43124 | 1.2645 | 711.0 | 562.3 | 42.25 |
| 55.3 | 1.43146 | 1.2652 | 712.7 | 563.3 | 42.36 |
| 55.4 | 1.43168 | 1.2659 | 714.4 | 564.3 | 42.46 |
| 55.5 | 1.43189 | 1.2665 | 716.1 | 565.4 | 42.56 |
| 55.6 | 1.43211 | 1.2672 | 717.8 | 566.4 | 42.66 |
| 55.7 | 1.43233 | 1.2679 | 719.5 | 567.5 | 42.76 |
| 55.8 | 1.43255 | 1.2685 | 721.1 | 568.5 | 42.85 |
| 55.9 | 1.43277 | 1.2692 | 722.8 | 569.5 | 42.96 |
| 56.0 | 1.43298 | 1.2699 | 724.5 | 570.5 | 43.06 |
| 56.1 | 1.43321 | 1.2703 | 726.1 | 571.6 | 43.15 |
| 56.2 | 1.43343 | 1.2708 | 727.7 | 572.6 | 43.25 |
| 56.3 | 1.43365 | 1.2713 | 729.3 | 573.7 | 43.34 |
| 56.4 | 1.43387 | 1.2718 | 730.9 | 574.7 | 43.44 |
| 56.5 | 1.43409 | 1.2724 | 732.6 | 575.8 | 43.54 |
| 56.6 | 1.43431 | 1.2731 | 734.3 | 576.8 | 43.64 |
| 56.7 | 1.43454 | 1.2738 | 736.0 | 577.8 | 43.74 |
| 56.8 | 1.43476 | 1.2744 | 737.6 | 578.8 | 43.84 |
| 56.9 | 1.43498 | 1.2751 | 739.4 | 579.9 | 43.94 |
| 57.0 | 1.43519 | 1.2758 | 741.1 | 580.9 | 44.04 |
| 57.1 | 1.43542 | 1.2763 | 742.8 | 582.0 | 44.14 |
| 57.2 | 1.43564 | 1.2768 | 744.4 | 583.0 | 44.24 |
| 57.3 | 1.43586 | 1.2773 | 745.9 | 584.0 | 44.33 |
| 57.4 | 1.43609 | 1.2778 | 747.6 | 585.1 | 44.43 |
| 57.5 | 1.43631 | 1.2784 | 749.3 | 586.1 | 44.53 |
| 57.6 | 1.43653 | 1.2791 | 751.0 | 587.1 | 44.63 |
| 57.7 | 1.43675 | 1.2798 | 752.7 | 588.1 | 44.73 |
| 57.8 | 1.43698 | 1.2804 | 754.4 | 589.2 | 44.83 |
| 57.9 | 1.43720 | 1.2810 | 756.1 | 590.2 | 44.94 |
| 58.0 | 1.43741 | 1.2818 | 757.8 | 591.2 | 45.04 |
| 58.1 | 1.43764 | 1.2822 | 759.5 | 592.3 | 45.14 |
| 58.2 | 1.43784 | 1.2827 | 761.1 | 593.4 | 45.23 |
| 58.3 | 1.43909 | 1.2832 | 762.6 | 594.3 | 45.32 |
| 58.4 | 1.43832 | 1.2837 | 764.3 | 595.4 | 45.42 |
| 58.5 | 1.43854 | 1.2843 | 766.0 | 596.4 | 45.52 |
| 58.6 | 1.43877 | 1.2850 | 767.8 | 597.5 | 45.63 |
| 58.7 | 1.43899 | 1.2857 | 769.5 | 598.5 | 45.73 |
| 58.8 | 1.43922 | 1.2863 | 771.1 | 599.5 | 45.83 |
| 58.9 | 1.43944 | 1.2869 | 772.9 | 600.6 | 45.93 |
| 59.0 | 1.43966 | 1.2876 | 774.6 | 601.6 | 46.03 |
| 59.1 | 1.43988 | 1.2882 | 776.3 | 602.6 | 46.14 |
| 59.2 | 1.44011 | 1.2889 | 778.1 | 603.7 | 46.24 |
| 59.3 | 1.44034 | 1.2896 | 779.8 | 604.7 | 46.34 |
| 59.4 | 1.44057 | 1.2902 | 781.6 | 605.8 | 46.45 |
| 59.5 | 1.44079 | 1.2909 | 783.3 | 606.8 | 46.55 |
| 59.6 | 1.44102 | 1.2916 | 785.2 | 607.9 | 46.66 |
| 59.7 | 1.44124 | 1.2921 | 786.8 | 608.9 | 46.76 |
| 59.8 | 1.44147 | 1.2926 | 788.4 | 609.9 | 46.85 |
| 59.9 | 1.44169 | 1.2931 | 790.0 | 610.9 | 46.95 |

TABLE III - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 60.0 | 1.44192 | 1.2936 | 791.7 | 612.0 | 47.05 |
| 60.1 | 1.44215 | 1.2942 | 793.3 | 613.0 | 47.15 |
| 60.2 | 1.44238 | 1.2949 | 795.2 | 614.1 | 47.26 |
| 60.3 | 1.44260 | 1.2956 | 796.9 | 615.1 | 47.36 |
| 60.4 | 1.44283 | 1.2962 | 798.6 | 616.1 | 47.46 |
| 60.5 | 1.44305 | 1.2969 | 800.5 | 617.2 | 47.57 |
| 60.6 | 1.44328 | 1.2976 | 802.2 | 618.2 | 47.67 |
| 60.7 | 1.44351 | 1.2981 | 803.9 | 619.3 | 47.78 |
| 60.8 | 1.44374 | 1.2986 | 805.5 | 620.3 | 47.87 |
| 60.9 | 1.44397 | 1.2991 | 807.1 | 621.3 | 47.97 |
| 61.0 | 1.44419 | 1.2996 | 808.7 | 622.3 | 48.06 |
| 61.1 | 1.44442 | 1.3002 | 810.5 | 623.4 | 48.17 |
| 61.2 | 1.44465 | 1.3009 | 812.3 | 624.4 | 48.27 |
| 61.3 | 1.44488 | 1.3016 | 814.2 | 625.5 | 48.39 |
| 61.4 | 1.44511 | 1.3022 | 815.8 | 626.5 | 48.48 |
| 61.5 | 1.44534 | 1.3029 | 817.7 | 627.6 | 48.60 |
| 61.6 | 1.44557 | 1.3036 | 819.4 | 628.6 | 48.70 |
| 61.7 | 1.44580 | 1.3042 | 821.3 | 629.7 | 48.81 |
| 61.8 | 1.44603 | 1.3049 | 823.0 | 630.7 | 48.91 |
| 61.9 | 1.44626 | 1.3056 | 824.8 | 631.7 | 49.02 |
| 62.0 | 1.44648 | 1.3062 | 826.6 | 632.8 | 49.12 |
| 62.1 | 1.44672 | 1.3068 | 828.3 | 633.8 | 49.23 |
| 62.2 | 1.44695 | 1.3075 | 830.0 | 634.8 | 49.33 |
| 62.3 | 1.44718 | 1.3080 | 831.8 | 635.9 | 49.43 |
| 62.4 | 1.44741 | 1.3085 | 833.4 | 636.9 | 49.53 |
| 62.5 | 1.44764 | 1.3090 | 835.1 | 638.0 | 49.63 |
| 62.6 | 1.44787 | 1.3095 | 836.8 | 639.0 | 49.73 |
| 62.7 | 1.44810 | 1.3101 | 838.5 | 640.0 | 49.83 |
| 62.8 | 1.44833 | 1.3108 | 840.2 | 641.0 | 49.93 |
| 62.9 | 1.44856 | 1.3115 | 842.1 | 642.1 | 50.05 |
| 63.0 | 1.44879 | 1.3121 | 843.8 | 643.1 | 50.15 |
| 63.1 | 1.44902 | 1.3128 | 845.7 | 644.2 | 50.26 |
| 63.2 | 1.44926 | 1.3135 | 847.5 | 645.2 | 50.37 |
| 63.3 | 1.44949 | 1.3141 | 849.3 | 646.3 | 50.47 |
| 63.4 | 1.44972 | 1.3148 | 851.1 | 647.3 | 50.58 |
| 63.5 | 1.44995 | 1.3155 | 853.0 | 648.4 | 50.69 |
| 63.6 | 1.45019 | 1.3161 | 854.7 | 649.4 | 50.79 |
| 63.7 | 1.45042 | 1.3168 | 856.5 | 650.4 | 50.90 |
| 63.8 | 1.45065 | 1.3175 | 858.4 | 651.5 | 51.01 |
| 63.9 | 1.45088 | 1.3180 | 860.0 | 652.5 | 51.11 |
| 64.0 | 1.45112 | 1.3185 | 861.6 | 653.5 | 51.20 |
| 64.1 | 1.45135 | 1.3190 | 863.4 | 654.6 | 51.31 |
| 64.2 | 1.45158 | 1.3195 | 865.1 | 655.6 | 51.41 |
| 64.3 | 1.45181 | 1.3201 | 866.9 | 656.7 | 51.52 |
| 64.4 | 1.45205 | 1.3208 | 868.7 | 657.7 | 51.63 |
| 64.5 | 1.45228 | 1.3215 | 870.6 | 658.8 | 51.74 |
| 64.6 | 1.45252 | 1.3221 | 872.3 | 659.8 | 51.84 |
| 64.7 | 1.45275 | 1.3228 | 874.1 | 660.8 | 51.95 |
| 64.8 | 1.45299 | 1.3235 | 876.0 | 661.9 | 52.06 |
| 64.9 | 1.45322 | 1.3241 | 877.8 | 662.9 | 52.17 |

TABLE III - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density at 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 65.0 | 1.45347 | 1.3248 | 879.7 | 664.0 | 52.28 |
| 65.1 | 1.45369 | 1.3255 | 881.5 | 665.0 | 52.39 |
| 65.2 | 1.45393 | 1.3261 | 883.2 | 666.0 | 52.49 |
| 65.3 | 1.45416 | 1.3268 | 885.0 | 667.0 | 52.60 |
| 65.4 | 1.45440 | 1.3275 | 886.9 | 668.1 | 52.71 |
| 65.5 | 1.45463 | 1.3281 | 888.8 | 669.2 | 52.82 |
| 65.6 | 1.45487 | 1.3288 | 890.6 | 670.2 | 52.93 |
| 65.7 | 1.45510 | 1.3295 | 892.4 | 671.2 | 53.04 |
| 65.8 | 1.45534 | 1.3301 | 894.2 | 672.3 | 53.14 |
| 65.9 | 1.45557 | 1.3308 | 896.0 | 673.3 | 53.25 |
| 66.0 | 1.45583 | 1.3315 | 898.0 | 674.4 | 53.37 |
| 66.1 | 1.45605 | 1.3320 | 899.6 | 675.4 | 53.46 |
| 66.2 | 1.45629 | 1.3325 | 901.3 | 676.4 | 53.56 |
| 66.3 | 1.45652 | 1.3330 | 903.1 | 677.5 | 53.67 |
| 66.4 | 1.45676 | 1.3335 | 904.8 | 678.5 | 53.77 |
| 66.5 | 1.45700 | 1.3341 | 906.7 | 679.6 | 53.89 |
| 66.6 | 1.45724 | 1.3348 | 908.5 | 680.6 | 53.99 |
| 66.7 | 1.45747 | 1.3355 | 910.4 | 681.7 | 54.11 |
| 66.8 | 1.45771 | 1.3361 | 912.2 | 682.7 | 54.21 |
| 66.9 | 1.45795 | 1.3367 | 913.9 | 683.7 | 54.31 |
| 67.0 | 1.45820 | 1.3374 | 915.9 | 684.8 | 54.43 |
| 67.1 | 1.45843 | 1.3380 | 917.6 | 685.8 | 54.53 |
| 67.2 | 1.45867 | 1.3387 | 919.6 | 686.9 | 54.65 |
| 67.3 | 1.45890 | 1.3395 | 921.4 | 687.9 | 54.76 |
| 67.4 | 1.45914 | 1.3400 | 923.1 | 688.9 | 54.86 |
| 67.5 | 1.45938 | 1.3407 | 925.1 | 690.0 | 54.98 |
| 67.6 | 1.45962 | 1.3415 | 927.0 | 691.0 | 55.09 |
| 67.7 | 1.45986 | 1.3420 | 928.8 | 692.1 | 55.20 |
| 67.8 | 1.46010 | 1.3427 | 930.6 | 693.1 | 55.31 |
| 67.9 | 1.46034 | 1.3434 | 932.6 | 694.2 | 55.42 |
| 68.0 | 1.46060 | 1.3440 | 934.4 | 695.2 | 55.53 |
| 68.1 | 1.46082 | 1.3447 | 936.2 | 696.2 | 55.64 |
| 68.2 | 1.46106 | 1.3454 | 938.0 | 697.2 | 55.75 |
| 68.3 | 1.46130 | 1.3460 | 939.9 | 698.3 | 55.86 |
| 68.4 | 1.46154 | 1.3466 | 941.8 | 699.4 | 55.97 |
| 68.5 | 1.46178 | 1.3473 | 943.7 | 700.4 | 56.08 |
| 68.6 | 1.46202 | 1.3479 | 945.4 | 701.4 | 56.19 |
| 68.7 | 1.46226 | 1.3486 | 947.4 | 702.5 | 56.30 |
| 68.8 | 1.46251 | 1.3493 | 949.2 | 703.5 | 56.41 |
| 68.9 | 1.46275 | 1.3499 | 951.1 | 704.6 | 56.52 |
| 69.0 | 1.46301 | 1.3506 | 953.0 | 705.6 | 56.64 |
| 69.1 | 1.46323 | 1.3513 | 954.8 | 706.6 | 56.74 |
| 69.2 | 1.46347 | 1.3519 | 956.7 | 707.7 | 56.86 |
| 69.3 | 1.46371 | 1.3526 | 958.6 | 708.7 | 56.97 |
| 69.4 | 1.46396 | 1.3533 | 960.6 | 709.8 | 57.09 |
| 69.5 | 1.46420 | 1.3539 | 962.4 | 710.8 | 57.20 |
| 69.6 | 1.46444 | 1.3546 | 964.3 | 711.9 | 57.31 |
| 69.7 | 1.46468 | 1.3553 | 966.2 | 712.9 | 57.42 |
| 69.8 | 1.46493 | 1.3560 | 968.2 | 714.0 | 57.54 |
| 69.9 | 1.46517 | 1.3566 | 970.0 | 715.0 | 57.65 |

TABLE III - (continued)

| Saccharose % (m/m) | Refractive Index at 20 °C | Mass Density à 20 °C | Sugars in g/l | Sugars in g/kg | ABV % vol at 20 °C |
|---|---|---|---|---|---|
| 70.0 | 1.46544 | 1.3573 | 971.8 | 716.0 | 57.75 |
| 70.1 | 1.46565 | 1.3579 | 973.8 | 717.1 | 57.87 |
| 70.2 | 1.46590 | 1.3586 | 975.6 | 718.1 | 57.98 |
| 70.3 | 1.46614 | 1.3593 | 977.6 | 719.2 | 58.10 |
| 70.4 | 1.46639 | 1.3599 | 979.4 | 720.2 | 58.21 |
| 70.5 | 1.46663 | 1.3606 | 981.3 | 721.2 | 58.32 |
| 70.6 | 1.46688 | 1.3613 | 983.3 | 722.3 | 58.44 |
| 70.7 | 1.46712 | 1.3619 | 985.2 | 723.4 | 58.55 |
| 70.8 | 1.46737 | 1.3626 | 987.1 | 724.4 | 58.66 |
| 70.9 | 1.46761 | 1.3633 | 988.9 | 725.4 | 58.77 |
| 71.0 | 1.46789 | 1.3639 | 990.9 | 726.5 | 58.89 |
| 71.1 | 1.46810 | 1.3646 | 992.8 | 727.5 | 59.00 |
| 71.2 | 1.46835 | 1.3653 | 994.8 | 728.6 | 59.12 |
| 71.3 | 1.46859 | 1.3659 | 996.6 | 729.6 | 59.23 |
| 71.4 | 1.46884 | 1.3665 | 998.5 | 730.7 | 59.34 |
| 71.5 | 1.46908 | 1.3672 | 1000.4 | 731.7 | 59.45 |
| 71.6 | 1.46933 | 1.3678 | 1002.2 | 732.7 | 59.56 |
| 71.7 | 1.46957 | 1.3685 | 1004.2 | 733.8 | 59.68 |
| 71.8 | 1.46982 | 1.3692 | 1006.1 | 734.8 | 59.79 |
| 71.9 | 1.47007 | 1.3698 | 1008.0 | 735.9 | 59.91 |
| 72.0 | 1.47036 | 1.3705 | 1009.9 | 736.9 | 60.02 |
| 72.1 | 1.47056 | 1.3712 | 1012.0 | 738.0 | 60.14 |
| 72.2 | 1.47081 | 1.3718 | 1013.8 | 739.0 | 60.25 |
| 72.3 | 1.47106 | 1.3725 | 1015.7 | 740.0 | 60.36 |
| 72.4 | 1.47131 | 1.3732 | 1017.7 | 741.1 | 60.48 |
| 72.5 | 1.47155 | 1.3738 | 1019.5 | 742.1 | 60.59 |
| 72.6 | 1.47180 | 1.3745 | 1021.5 | 743.2 | 60.71 |
| 72.7 | 1.47205 | 1.3752 | 1023.4 | 744.2 | 60.82 |
| 72.8 | 1.47230 | 1.3758 | 1025.4 | 745.3 | 60.94 |
| 72.9 | 1.47254 | 1.3765 | 1027.3 | 746.3 | 61.05 |
| 73.0 | 1.47284 | 1.3772 | 1029.3 | 747.4 | 61.17 |
| 73.1 | 1.47304 | 1.3778 | 1031.2 | 748.4 | 61.28 |
| 73.2 | 1.47329 | 1.3785 | 1033.2 | 749.5 | 61.40 |
| 73.3 | 1.47354 | 1.3792 | 1035.1 | 750.5 | 61.52 |
| 73.4 | 1.47379 | 1.3798 | 1037.1 | 751.6 | 61.63 |
| 73.5 | 1.47404 | 1.3805 | 1039.0 | 752.6 | 61.75 |
| 73.6 | 1.47429 | 1.3812 | 1040.9 | 753.6 | 61.86 |
| 73.7 | 1.47454 | 1.3818 | 1042.8 | 754.7 | 61.97 |
| 73.8 | 1.47479 | 1.3825 | 1044.8 | 755.7 | 62.09 |
| 73.9 | 1.47504 | 1.3832 | 1046.8 | 756.8 | 62.21 |
| 74.0 | 1.47534 | 1.3838 | 1048.6 | 757.8 | 62.32 |
| 74.1 | 1.47554 | 1.3845 | 1050.7 | 758.9 | 62.44 |
| 74.2 | 1.47579 | 1.3852 | 1052.6 | 759.9 | 62.56 |
| 74.3 | 1.47604 | 1.3858 | 1054.6 | 761.0 | 62.67 |
| 74.4 | 1.47629 | 1.3865 | 1056.5 | 762.0 | 62.79 |
| 74.5 | 1.47654 | 1.3871 | 1058.5 | 763.1 | 62.91 |
| 74.6 | 1.47679 | 1.3878 | 1060.4 | 764.1 | 63.02 |
| 74.7 | 1.47704 | 1.3885 | 1062.3 | 765.1 | 63.13 |
| 74.8 | 1.47730 | 1.3892 | 1064.4 | 766.2 | 63.26 |
| 74.9 | 1.47755 | 1.3898 | 1066.3 | 767.2 | 63.37 |
| 75.0 | 1.47785 | 1.3905 | 1068.3 | 768.3 | 63.49 |

**Method OIV-MA-AS2-03A** Type I method

# Total dry matter

(Resolution Oeno 377/2009 and 387/2009)
(Revised by resolution Oeno 465/2012)

## 1 Definition

The total dry extract or the total dry matter includes all matter that is non-volatile under specified physical conditions. These physical conditions must be such that the matter forming the extract undergoes as little alteration as possible while the test is being carried out.

The sugar-free extract is the difference between the total dry extract and the total sugars. The reduced extract is the difference between the total dry extract and the total sugars in excess of 1 g/L, potassium sulfate in excess of 1 g/L, any mannitol present and any other chemical substances which may have been added to the wine.

The residual extract is the sugar-free extract less the fixed acidity expressed as tartaric acid.

## 2 Principle

The weight of residue obtained when a sample of wine, previously absorbed onto filter paper, is dried in a current of air, at a pressure of 20 - 25 mm Hg at 70°C.

## 3 Method

3.1 *Apparatus*

3.1.1 Oven:

Cylindrical basin (internal diameter: 27 cm, height: 6 cm) made of aluminum with an aluminum lid, heated to 70°C and regulated to 1°C.

A tube (internal diameter: 25 mm) connecting the oven to a vacuum pump providing a flow rate of 50 L/h. The air, previously dried by bubbling through concentrated sulfuric acid, is circulated in the oven by a fan in order to achieve quick homogenous reheating. The rate of airflow is regulated by a tap and is to be 30-40 L per hour and the pressure in the oven is 25 mm of mercury.

The oven can then be used providing it is calibrated as in 3.1.3.

3.1.2 Dishes:

Stainless steel dishes (60 mm internal diameter, 25 mm in height) provided with fitting lids. Each dish contains 4-4.5 g of filter paper, cut into fluted strips 22 mm in length.

The filter paper is first washed with hydrochloric acid, 2 g/L, for 8 h, rinsed five times with water and then dried in air.

3.1.3 Calibration of apparatus and method

*a)* Checking the seal of the dish lids. A dish, containing dried filter paper, with the lid on, after first being cooled in a dessicator containing sulfuric acid, should not gain more than 1 mg/h when left in the laboratory.

*b)* Checking the degree of drying. A pure solution of sucrose, 100 g/L, should give a dry extract of 100 g ± 1 g/L.

*c)* A pure solution of lactic acid, 10 g/L, should give a dry extract of at least 9.5 g/L.

If necessary, the drying time in the oven can be increased or decreased by changing the rate of airflow to the oven or by changing the pressure in order that these conditions should be met.

NOTE - The lactic acid solution can be prepared as follows: 10 mL of lactic acid is diluted to approximately 100 mL with water. This solution is placed in a dish and heated on a boiling water bath for 4 h, distilled water is added if the volume decreases to less than 50 mL (approx). Make up the solution to 1 liter and titrate 10 mL of this solution with alkali, 0.1 M. Adjust the lactic acid solution to 10 g/L.

3.2 *Procedure*

3.2.1 Weighing the dish

Place the dish containing filter paper in the oven for 1 h. Stop the vacuum pump and immediately place the lid on the dish on opening the oven. Cool in a dessicator and weigh to the nearest 0.1 mg: the mass of the dish and lid is $p_O$ g.

3.2.2 Weighing the sample

Place 10 mL of must or wine into the weigh dish. Allow the sample to be completely absorbed onto the filter paper. Place the dish in the oven for 2 h (or for the time used in the calibration of the standard in 3.1.3). Weigh the dish following the procedure 3.2.1 beginning "Stop the vacuum ..." The mass is *p* g.

*Note:* The sample weight should be taken when analyzing very sweet wines or musts.

3.3 *Calculation*

The total dry extract is given by:

$$(p - p_o) \times 100$$

For very sweet wines or musts the total dry extract is given by:

$$(p - p_o) \times \frac{\rho_{20}}{P} \times 1000$$

**P** = mass of sample in grams

$\rho_{20}$ = density of wine or must in g/mL.

3.4 *Expression of results*

The total dry extract is expressed in g/L to one decimal place.

**Note:**

Calculate total dry extract by separately taking into account quantities of glucose and fructose (reducing sugars) and the quantity of saccharose, as follows:

Sugar-free extract = Total dry extract – reducing sugars (glucose + fructose) – saccharose

In the case that the method of analysis allows for sugar inversion, use the following formula for the calculation:

Sugar-free extract = Total dry extract – reducing sugars (glucose + fructose) - [(Sugars after inversion – Sugars before inversion) x 0,95]

*Inversion* refers to the process that leads to the conversion of a stereoisomer into compounds with reverse stereoisomerism. In particular, the process based on splitting sucrose into fructose and glucose, carried out by keeping acidified solutions containing sugars (100 ml solution containing sugars + 5 ml concentrated hydrochloric acid) for at least 15 min at 50°C or above in a water-bath (the water-bath is maintained at 60°C until the temperature of the solution reaches 50°C), is called *sugar inversion*. The final solution is laevo-rotatory due to the presence of fructose, while the initial solution is dextro-rotatory due to the presence of sucrose.

TABLE I
For the calculation of the total dry extract content (g/L)

| Density to 2 decimal places | 3rd decimal place | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| | Extract g/L | | | | | | | | | |
| 1.00 | 0 | 2.6 | 5.1 | 7.7 | 10.3 | 12.9 | 15.4 | 18.0 | 20.6 | 23.2 |
| 1.01 | 25.8 | 28.4 | 31.0 | 33.6 | 36.2 | 38.8 | 41.3 | 43.9 | 46.5 | 49.1 |
| 1.02 | 51.7 | 54.3 | 56.9 | 59.5 | 62.1 | 64.7 | 67.3 | 69.9 | 72.5 | 75.1 |
| 1.03 | 77.7 | 80.3 | 82.9 | 85.5 | 88.1 | 90.7 | 93.3 | 95.9 | 98.5 | 101.1 |
| 1.04 | 103.7 | 106.3 | 109.0 | 111.6 | 114.2 | 116.8 | 119.4 | 122.0 | 124.6 | 127.2 |
| 1.05 | 129.8 | 132.4 | 135.0 | 137.6 | 140.3 | 142.9 | 145.5 | 148.1 | 150.7 | 153.3 |
| 1.06 | 155.9 | 158.6 | 161.2 | 163.8 | 166.4 | 169.0 | 171.6 | 174.3 | 176.9 | 179.5 |
| 1.07 | 182.1 | 184.8 | .187.4 | 190.0 | 192.6 | 195.2 | 197.8 | 200.5 | 203.1 | 205.8 |
| 1.08 | 208.4 | 211.0 | 213.6 | 216.2 | 218.9 | 221.5 | 224.1 | 226.8 | 229.4 | 232.0 |
| 1.09 | 234.7 | 237.3 | 239.9 | 242.5 | 245.2 | 247.8 | 250.4 | 253.1 | 255.7 | 258.4 |
| 1.10 | 261.0 | 263.6 | 266.3 | 268.9 | 271.5 | 274.2 | 276.8 | 279.5 | 282.1 | 284.8 |
| 1.11 | 287.4 | 290.0 | 292.7 | 295.3 | 298.0 | 300.6 | 303.3 | 305.9 | 308.6 | 311.2 |
| 1.12 | 313.9 | 316.5 | 319.2 | 321.8 | 324.5 | 327.1 | 329.8 | 332.4 | 335.1 | 337.8 |
| 1.13 | 340.4 | 343.0 | 345.7 | 348.3 | 351.0 | 353.7 | 356.3 | 359.0 | 361.6 | 364.3 |
| 1.14 | 366.9 | 369.6 | 372.3 | 375.0 | 377.6 | 380.3 | 382.9 | 385.6 | 388.3 | 390.9 |
| 1.15 | 393.6 | 396.2 | 398.9 | 401.6 | 404.3 | 406.9 | 409.6 | 412.3 | 415.0 | 417.6 |
| 1.16 | 420.3 | 423.0 | 425.7 | 428.3 | 431.0 | 433.7 | 436.4 | 439.0 | 441.7 | 444.4 |
| 1.17 | 447.1 | 449.8 | 452.4 | 455.2 | 457.8 | 460.5 | 463.2 | 465.9 | 468.6 | 471.3 |
| 1.18 | 473.9 | 476.6 | 479.3 | 482.0 | 484.7 | 487.4 | 490.1 | 492.8 | 495.5 | 498.2 |
| 1.19 | 500.9 | 503.5 | 506.2 | 508.9 | 511.6 | 514.3 | 517.0 | 519.7 | 522.4 | 525.1 |
| 1.20 | 527.8 | - | - | - | - | - | - | - | - | - |

INTERPOLATION TABLE

| 4th decimal place | Extract g/L | 4th decimal place | Extract g/L | 4th decimal place | Extract g/L |
|---|---|---|---|---|---|
| 1 | 0.3 | 4 | 1.0 | 7 | 1.8 |
| 2 | 0.5 | 5 | 1.3 | 8 | 2.1 |
| 3 | 0.8 | 6 | 1.6 | 9 | 2.3 |

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**Method OIV-MA-AS2-03B** Type IV method

# Total dry matter

(Resolution Oeno 377/2009 and 387/2009)
(Revised by Oeno 465/2012)

## 1 Definition

The total dry extract or the total dry matter includes all matter that is non-volatile under specified physical conditions. These physical conditions must be such that the matter forming the extract undergoes as little alteration as possible while the test is being carried out.

The sugar-free extract is the difference between the total dry extract and the total sugars. The reduced extract is the difference between the total dry extract and the total sugars in excess of 1 g/L, potassium sulfate in excess of 1 g/L, any mannitol present and any other chemical substances which may have been added to the wine.

The residual extract is the sugar-free extract less the fixed acidity expressed as tartaric acid.

## 2 Principle

The total dry extract is calculated indirectly from the specific gravity of the must and, for wine, from the specific gravity of the alcohol-free wine.

This dry extract is expressed in terms of the quantity of sucrose which, when dissolved in water and made up to a volume of one liter, gives a solution of the same gravity as the must or the alcohol-free wine.

## 3 Method

3.1 *Procedure*

Determine the specific gravity of a must or wine.
In the case of wine, calculate the specific gravity of the "alcohol free wine" using the following formula:

$$d_r = d_v - d_a + 1.000$$

where:
$d_v$ = specific gravity of the wine at 20°C (corrected for volatile acidity [1])
$d_a$ = specific gravity at 20°C of a water-alcohol mixture of the same alcoholic strength as the wine obtained using the formula:

$$d_r = 1.00180^{**} (r_v - r_a) + 1.000$$

where :
$r_v$ = density of the wine at 20°C (corrected for volatile acidity [1])
$r_a$ = density at 20°C of the water alcohol mixture of the same alcoholic strength as the wine obtained from Table 1 of chapter *Alcoholic strength by volume* for a temperature of 20°C.

3.2 *Calculation*
Use the value for specific gravity of the alcohol free wine to obtain the total dry extract (g/L) from table I

3.3 Expression of results
The total dry extract is reported in g/L to one decimal place.

**Note:**
Calculate total dry extract by separately taking into account quantities of glucose and fructose (reducing sugars) and the quantity of saccharose, as follows:

Sugar-free extract = Total dry extract – reducing sugars (glucose + fructose) – saccharose

In the case that the method of analysis allows for sugar inversion, use the following formula for the calculation:

(1) NOTE: Before carrying out this calculation, the specific gravity (or the density) of the wine measured as specified above should be corrected for the effect of the volatile acidity using the formula:

$$d_v = d_{20}^{20} - 0.0000086a \quad \text{or} \quad \rho_v = \rho_{20} - 0.0000086a$$

where *a* is the volatile acidity expressed in milli-equivalents per liter.

** The coefficient 1.0018 approximates to 1 when $r_v$ is below 1.05 which is often the case.

Sugar-free extract = Total dry extract – reducing sugars (glucose + fructose) - [(Sugars after inversion – Sugars before inversion) x 0,95]

*Inversion* refers to the process that leads to the conversion of a stereoisomer into compounds with reverse stereoisomerism. In particular, the process based on splitting sucrose into fructose and glucose, carried out by keeping acidified solutions containing sugars (100 ml solution containing sugars + 5 ml concentrated hydrochloric acid) for at least 15 min at 50°C or above in a water-bath (the water-bath is maintained at 60°C until the temperature of the solution reaches 50°C), is called *sugar inversion*. The final solution is laevo-rotatory due to the presence of fructose, while the initial solution is dextro-rotatory due to the presence of sucrose.

TABLE I
For the calculation of the total dry extract content (g/L)

| Density to 2 decimal places | 3rd decimal place | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| | Extract g/L | | | | | | | | | |
| 1.00 | 0 | 2.6 | 5.1 | 7.7 | 10.3 | 12.9 | 15.4 | 18.0 | 20.6 | 23.2 |
| 1.01 | 25.8 | 28.4 | 31.0 | 33.6 | 36.2 | 38.8 | 41.3 | 43.9 | 46.5 | 49.1 |
| 1.02 | 51.7 | 54.3 | 56.9 | 59.5 | 62.1 | 64.7 | 67.3 | 69.9 | 72.5 | 75.1 |
| 1.03 | 77.7 | 80.3 | 82.9 | 85.5 | 88.1 | 90.7 | 93.3 | 95.9 | 98.5 | 101.1 |
| 1.04 | 103.7 | 106.3 | 109.0 | 111.6 | 114.2 | 116.8 | 119.4 | 122.0 | 124.6 | 127.2 |
| 1.05 | 129.8 | 132.4 | 135.0 | 137.6 | 140.3 | 142.9 | 145.5 | 148.1 | 150.7 | 153.3 |
| 1.06 | 155.9 | 158.6 | 161.2 | 163.8 | 166.4 | 169.0 | 171.6 | 174.3 | 176.9 | 179.5 |
| 1.07 | 182.1 | 184.8 | .187.4 | 190.0 | 192.6 | 195.2 | 197.8 | 200.5 | 203.1 | 205.8 |
| 1.08 | 208.4 | 211.0 | 213.6 | 216.2 | 218.9 | 221.5 | 224.1 | 226.8 | 229.4 | 232.0 |
| 1.09 | 234.7 | 237.3 | 239.9 | 242.5 | 245.2 | 247.8 | 250.4 | 253.1 | 255.7 | 258.4 |
| 1.10 | 261.0 | 263.6 | 266.3 | 268.9 | 271.5 | 274.2 | 276.8 | 279.5 | 282.1 | 284.8 |
| 1.11 | 287.4 | 290.0 | 292.7 | 295.3 | 298.0 | 300.6 | 303.3 | 305.9 | 308.6 | 311.2 |
| 1.12 | 313.9 | 316.5 | 319.2 | 321.8 | 324.5 | 327.1 | 329.8 | 332.4 | 335.1 | 337.8 |
| 1.13 | 340.4 | 343.0 | 345.7 | 348.3 | 351.0 | 353.7 | 356.3 | 359.0 | 361.6 | 364.3 |
| 1.14 | 366.9 | 369.6 | 372.3 | 375.0 | 377.6 | 380.3 | 382.9 | 385.6 | 388.3 | 390.9 |
| 1.15 | 393.6 | 396.2 | 398.9 | 401.6 | 404.3 | 406.9 | 409.6 | 412.3 | 415.0 | 417.6 |
| 1.16 | 420.3 | 423.0 | 425.7 | 428.3 | 431.0 | 433.7 | 436.4 | 439.0 | 441.7 | 444.4 |
| 1.17 | 447.1 | 449.8 | 452.4 | 455.2 | 457.8 | 460.5 | 463.2 | 465.9 | 468.6 | 471.3 |
| 1.18 | 473.9 | 476.6 | 479.3 | 482.0 | 484.7 | 487.4 | 490.1 | 492.8 | 495.5 | 498.2 |
| 1.19 | 500.9 | 503.5 | 506.2 | 508.9 | 511.6 | 514.3 | 517.0 | 519.7 | 522.4 | 525.1 |
| 1.20 | 527.8 | - | - | - | - | - | - | - | - | - |

INTERPOLATION TABLE

| 4th decimal place | Extract g/L | 4th decimal place | Extract g/L | 4th decimal place | Extract g/L |
|---|---|---|---|---|---|
| 1 | 0.3 | 4 | 1.0 | 7 | 1.8 |
| 2 | 0.5 | 5 | 1.3 | 8 | 2.1 |
| 3 | 0.8 | 6 | 1.6 | 9 | 2.3 |

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**Method OIV-MA-AS2-04** Type I method

# Ash

## 1. Definition

The ash content is defined to be all those products remaining after igniting the residue left after the evaporation of the wine. The ignition is carried out in such a way that all the cations (excluding the ammonium cation) are converted into carbonates or other anhydrous inorganic salts.

## 2. Principle

The wine extract is ignited at a temperature between 500 and 550°C until complete combustion (oxidation) of organic material has been achieved.

## 3 Apparatus

3.1 boiling water-bath at 100°C;
3.2 balance sensitive to 0.1 mg;
3.3 hot-plate or infra-red evaporator;
3.4 temperature-controlled electric muffle furnace;
3.5 dessicator;
3.6 flat-bottomed platinum dish 70 mm in diameter and 25 mm in height.

## 4. Procedure

Pipette 20 mL of wine into the previously tared platinum dish (original weight $p_0$ g). Evaporate on the boiling water-bath, and heat the residue on the hot-plate at 200°C or under the infra-red evaporator until carbonization begins. When no more fumes are produced, place the dish in the electric muffle furnace maintained at 525 ± 25°C. After 15 min or carbonization, remove the dish from the furnace, add 5 mL of distilled water, evaporate on the water-bath or under the infra-red evaporator, and again heat the residue to 525°C for 10 min.

If combustion (oxidation) of the carbonized particles is not complete, the following operations are repeated: washing the carbonized particles, evaporation of water, and ignition. For wines with a high sugar content, it is advantageous to add a few drops of pure vegetable oil to the extract before the first ashing to prevent excessive foaming. After cooling in the desiccator, the dish is weighed ($p_1$ g).

The weight of the ash in the sample (20 mL) is then calculated as $p = (p_1 — p_0)$ g.

**5. Expression of results**

The weight P of the ash in grams per liter is given to two decimal places by the expression: $P = 50\ p$.

**Method OIV-MA-AS2-01A** Type IV method

# Alkalinity of Ash

## 1. Definition

The alkalinity of the ash is defined as the sum of cations, other than the ammonium ion, combined with the organic acids in the wine.

## 2. Principle

The ash is dissolved in a known (excess) amount of a hot standardized acid solution; the excess is determined by titration using methyl orange as an indicator.

## 3. Reagents and apparatus

3.1. Sulfuric acid solution, 0.05 M $H_2SO_4$
3.2. Sodium hydroxide solution, 0.1 M NaOH
3.3. Methyl orange, 0.1% solution in distilled water
3.4. Boiling water-bath

## 4. Procedure

Add 10 mL 0.05 M sulfuric acid solution (3.1) to the ash from 20 mL of wine contained in the platinum dish. Place the dish on the boiling water-bath for about 15 min, breaking up and agitating the residue with a glass rod to speed up the dissolution. Add two drops of methyl orange solution and titrate the excess sulfuric acid against 0.1 M sodium hydroxide (3.2) until the color of the indicator changes to yellow.

## 5. Expression of results

5.1. *Method of calculation*

The alkalinity of ash, expressed in milliequivalents per liter to one decimal place, is given by:

$$A = 5\ (10 - n)$$

where $n$ mL is the volume of sodium hydroxide, 0.1 M, used.

5.2. *Alternative expression*

The alkalinity of ash, expressed in grams per liter of potassium carbonate, to two decimal places, is given by:

$$A = 0.345\ (10 - n)$$

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**Method OIV-MA-AS2-06** Type IV method

# Measurement of the oxidation-reduction potential in wines

(Resolution Oeno 3/2000)

## 1. PURPOSE AND SCOPE OF APPLICATION:

The oxidation-reduction potential (EH) is a measure of the oxidation or reduction state of a medium. In the field of enology, oxygen and the oxidation-reduction potential are two important factors in the pre-fermentation processing of the grape harvest, the winemaking process, growing, and wine storage.

Proposals are hereby submitted for equipment designed to measure the Oxidation-reduction Potential in Wines and a working method for taking measurements under normal conditions. This method has not undergone any joint analysis, given the highly variable nature of the oxidation-reduction state of a particular wine, a situation which makes this step in the validation process difficult to implement. As a result, this is a class 4 method[1] intended basically for production.

## 2. UNDERLYING PRINCIPLE

The oxidation-reduction potential of a medium is defined as the difference in potential between a corrosion-proof electrode immersed in this medium and a standard hydrogen electrode linked to the medium. Indeed, only the difference in oxidation-reductions potentials of two linked systems can be measured. Consequently, the oxidation-reduction potential of the hydrogen electrode is considered to be zero, and all oxidation-reduction potentials are compared to it. The oxidation-reduction potential is a measurement value permitting expression of the instantaneous physico-chemical state of a solution. Only potentiometric volumetric analysis of the total oxidation-reduction pairs and an estimate of the oxidizing agent/reducing agent ratio can yield a true quantitative measurement. Oxidation-reduction potential is measured using combined electrodes, whether in wine or in another solution. This system usually involves the use of a platinum electrode (measuring electrode) and a silver or mercurous chloride electrode (reference electrode).

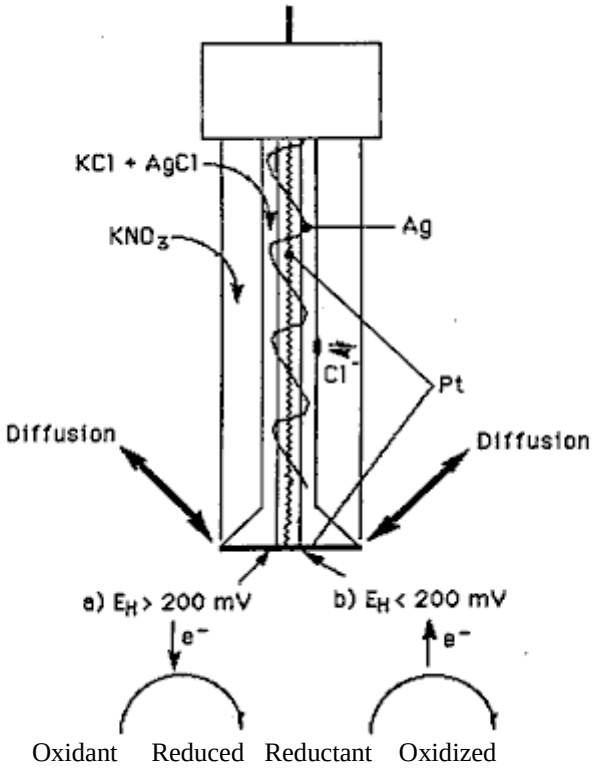
## 3. EQUIPMENT

Although several types of electrodes exist, it is recommended that an electrode adapted for measuring the EH in wine be used. It is recommended that use be made of a double-jacket combined electrode linked to a reference electrode (see figure). This system incorporates a measuring electrode, and a double-jacket reference electrode, both of which are linked to an ion meter. The inner jacket of

[1] In conformity with the classification detailed in the Codex Alimentarius.

the reference electrode is filled with a solution of 17.1% $KNO_3$; trace amounts of AgCl; trace amounts of Triton X-100; 5% KCL; 77.9% de-ionized water; and for the measuring electrode, the solution is made up of <1% AgCL; 29.8% KCL; and 70% de-ionized water.

Modified Combined Electrode




## 4. CLEANING AND CALIBRATION OF THE ELECTRODES

4.1. *Calibration*

The electrodes are calibrated using solutions with known, constant oxidation-reduction potentials. An equimolar solution (10 mM/l) of ferricyanide and potassium ferrous cyanide is used. Its composition is: 0.329g of $K_3Fe(CN)_6$; 0.422g of $K_4Fe(CN)_6$; 0.149g of KCl and up to 1000ml of water. At 20 °C this solution has an oxidation-reduction potential of 406 mV (±5 mV), but this potential changes over time, thus requiring that the solution not be stored for more than two weeks in the dark.

4.2. *Cleaning the Platinum in the Electrode*

The electrode platinum should be cleaned by immersing it in a solution of 30% hydrogen peroxide by volume for one hour, then washing it with water. Complete cleaning in water is required after each series of measurement. The system is normally cleaned after each week of use.

## 5. WORKING METHOD

5.1. *Filling the Inner Jacket*

The composition of the double jacket varies depending on the type of medium for which the EH is being measured (Table below).

Table
Composition of the Filler Solution in the Double Jacket of the Electrode as a Function of the Medium Measured

| Medium to be measured | Solution Composition of the jacket |
|---|---|
| 1 Dry wines | Ethanol 12% by vol., 5g tartaric acid, NaOH N up to pH 3.5, distilled water up to 1000 ml |
| 2 Sweet wines | Solution 1 plus 20 g/l sucrose |
| 3 Special sweet wines | Solution 2 plus 100 mg/l of $SO_2$ ($KHSO_3$) |
| 4 Brandies | Ethanol 50% by vol., acetic acid up to pH 5, distilled water up to 1000 ml. |

### 5.2. Balancing the Electrode with the Medium to Be Measured

Before taking any measurements, the electrodes must be calibrated in Michaelis solution, then stabilized for 15 minutes in a wine, if the measurement s are to be taken in wines. Next, for measurements taken on site, measurements are read after the electrodes have been immersed in the medium for 5 minutes. For laboratory measurements, the stability index is the $\Delta$EH(mV) / T (minutes) ratio ; when this latter is $\leq 0.2$, the potential can be read.

5.3. *Measurements Under Practical Conditions*

Measurements are systematically taken on site without any handling that could change the oxidation-reduction potential values. When taking measurements in storehouses, casks, vats, etc. care should be taken to record temperature, pH and dissolved oxygen content (method under preparation) at the same time as the EH measurement is taken, as these measurements will subsequently be used to interpret results. For wines in bottles, the measurement is taken in the wine after letting it sit

in a room whose temperature is 20 °C, immediately after the container is opened, under a constant flow of nitrogen, and after immersing the entire electrode unit in the bottle.

5.4. *Expression of Results*
Findings are recorded in mV as compared with the standard hydrogen electrode.

**Method OIV-MA-AS2-07A**

# Chromatic Characteristics

## 2. Principle of the methods

A spectrophotometric method which makes it possible to determine the tristimulus values and the three chromaticity coefficients required to specify the color as described by the CIE (*Commission internationale de l'Éclairage*).

WITHDRAWN
(replaced by OIV-MA-AS2-11)

**Method OIV-MA-AS2-07B** Type IV method

# Chromatic Characteristics

## 1. Definitions

The "chromatic characteristics" of a wine are its luminosity and chromaticity. Luminosity depends on transmittance and varies inversely with the intensity of color of the wine. Chromaticity depends on dominant wavelength (distinguishing the shade) and purity.

Conventionally, and for the sake of convenience, the chromatic characteristics of red and rosé wines are described by the intensity of color and shade, in keeping with the procedure adopted as the working method.

## 2. Principle of the methods

(applicable to red and rosé wines)

A spectrophotometric method whereby chromatic characteristics are expressed conventionally, as given below:

- The intensity of color is given by the sum of absorbencies (or optical densities) using a 1 cm optical path and radiations of wavelengths 420, 520 and 620 nm.
- The shade is expressed as the ratio of absorbance at 420 nm to absorbance at 520 nm.

## 3. Method

3.1. *Apparatus*

3.1.1 Spectrophotometer enabling measurements to be made between 300 and 700 nm.

3.1.2 Glass cells (matched pairs) with optical path *b* equal to 0.1, 0.2, 0.5, 1 and 2 cm.

3.2. *Preparation of the sample*

If the wine is cloudy, clarify it by centrifugation; young or sparkling wines must have the bulk of their carbon dioxide removed by agitation under vacuum.

3.3. *Method*

The optical path *b* of the glass cell used must be chosen so that the measured absorbance A, falls between 0.3 and 0.7.

Take the spectrophotometric measurements using distilled water as the reference liquid, in a cell of the same optical path *b*, in order to set the zero on the absorbance scale of the apparatus at the wavelengths of 420, 520 and 620 nm.

Using the appropriate optical path *b*, read off the absorbencies at each of these three wavelengths for the wine.

3.4. *Calculations*

Calculate the absorbencies for a 1 cm optical path for the three wavelengths by dividing the absorbencies found ($A_{420}$, $A_{520}$ and $A_{620}$) by *b*, in cm.

3.5. *Expression of Results*

The color intensity I is conventionally given by:

$$I = A_{420} + A_{520} + A_{620}$$

and is expressed to three decimal places.

The shade N is conventionally given by:

$$N = \frac{A_{420}}{A_{520}}$$

and is expressed to three decimal places.

**TABLE 1**

Converting absorbance into transmittance (T%)

*Method:* find the first decimal figure of the absorbance value in the left-hand column (0-9) and the second decimal figure in the top row (0-9).

Take the figure at the intersection of column and row: to find the transmittance, divide the figure by 10 if absorbance is less than 1, by 100 if between 1 and 2 and by 1000 if between 2 and 3.

*Note*: The figure in the top right hand corner of each box enables the third decimal figure of the absorbance to be determined by interpolation.

| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 23 1000 | 22 977 | 22 955 | 21 933 | 21 912 | 20 891 | 20 871 | 19 851 | 19 932 | 19 813 |
| 1 | 18 794 | 18 776 | 17 759 | 17 741 | 16 724 | 16 708 | 16 692 | 15 676 | 15 661 | 15 646 |
| 2 | 14 631 | 14 617 | 14 603 | 14 589 | 13 575 | 13 562 | 13 549 | 12 537 | 12 525 | 12 513 |
| 3 | 11 501 | 11 490 | 11 479 | 11 468 | 10 457 | 9 447 | 9 436 | 10 427 | 10 417 | 9 407 |
| 4 | 9 398 | 9 389 | 9 380 | 8 371 | 8 363 | 8 355 | 8 347 | 8 339 | 7 331 | 8 324 |
| 5 | 7 316 | 309 71 | 7 302 | 7 295 | 6 288 | 7 282 | 6 275 | 6 269 | 6 263 | 6 257 |
| 6 | 6 251 | 5 245 | 6 240 | 5 234 | 5 229 | 5 224 | 5 219 | 5 214 | 5 209 | 5 204 |
| 7 | 4 199 | 5 195 | 4 190 | 4 186 | 4 182 | 178 | 4 174 | 4 170 | 4 166 | 4 162 |
| 8 | 3 158 | 4 155 | 3 151 | 4 148 | 4 144 | 3 141 | 3 138 | 3 135 | 3 132 | 3 129 |
| 9 | 3 126 | 3 123 | 3 120 | 2 117 | 3 115 | 2 112 | 3 110 | 2 107 | 3 105 | 2 102 |

*Example:*

| Absorbance | | 0.47 | 1.47 | 2.47 | 3.47 |
|---|---|---|---|---|---|
| T% | 33.9% | 3.4% | 0.3% | 0% | |

Transmittance (T%) is expressed to the nearest 0.1%.

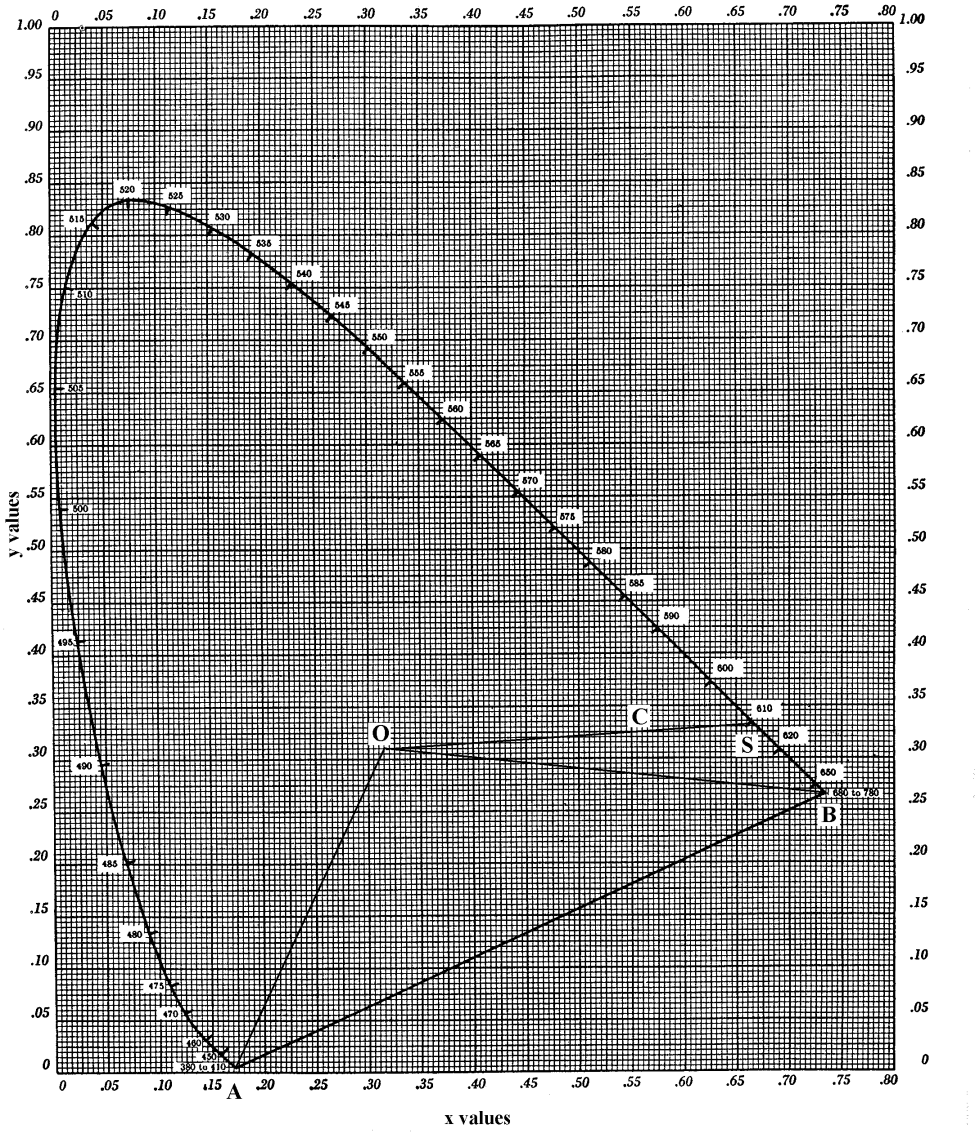



FIGURE 1

Chromaticity diagram, showing the locus of all colors of the spectrum

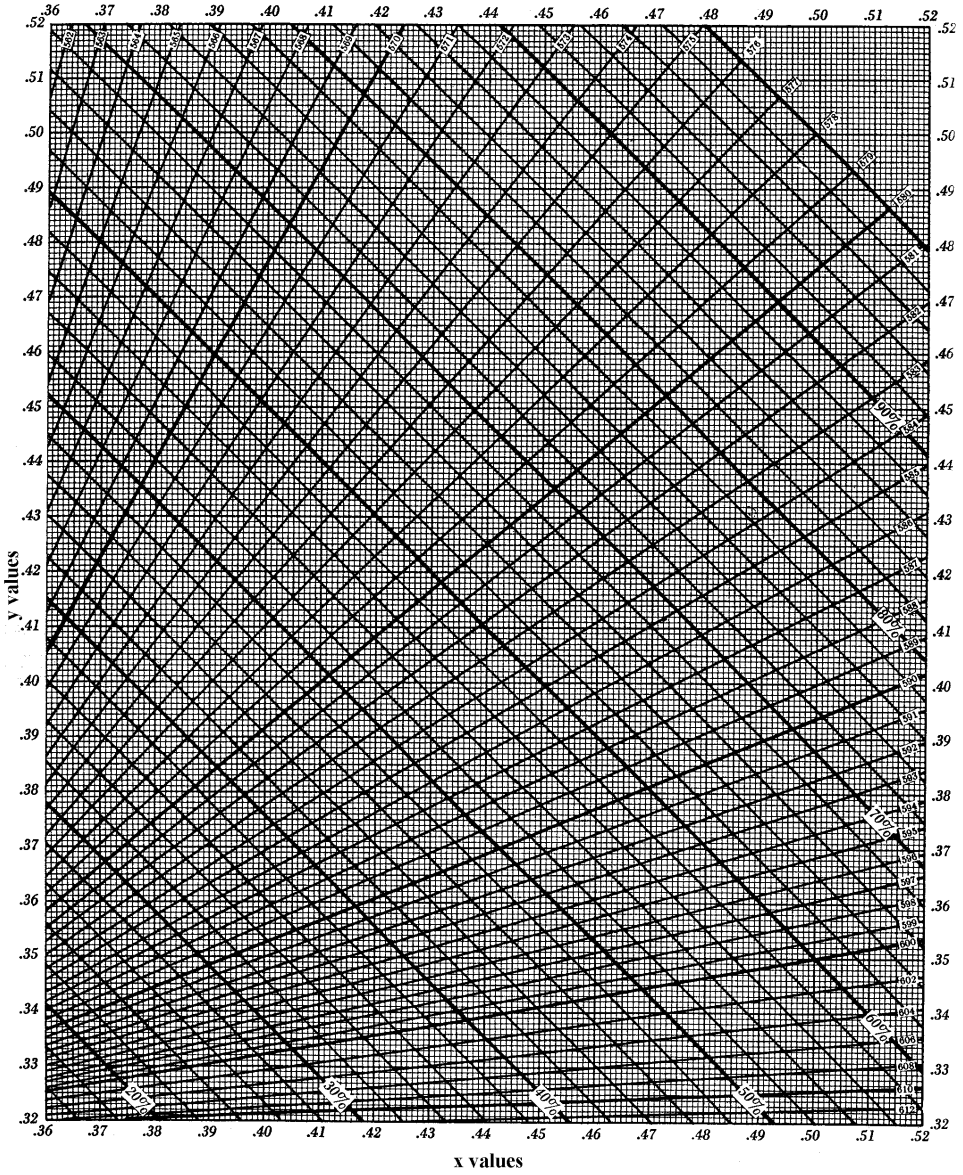



**FIGURE 2**
Chromaticity diagram for pure red wines and brick red wines

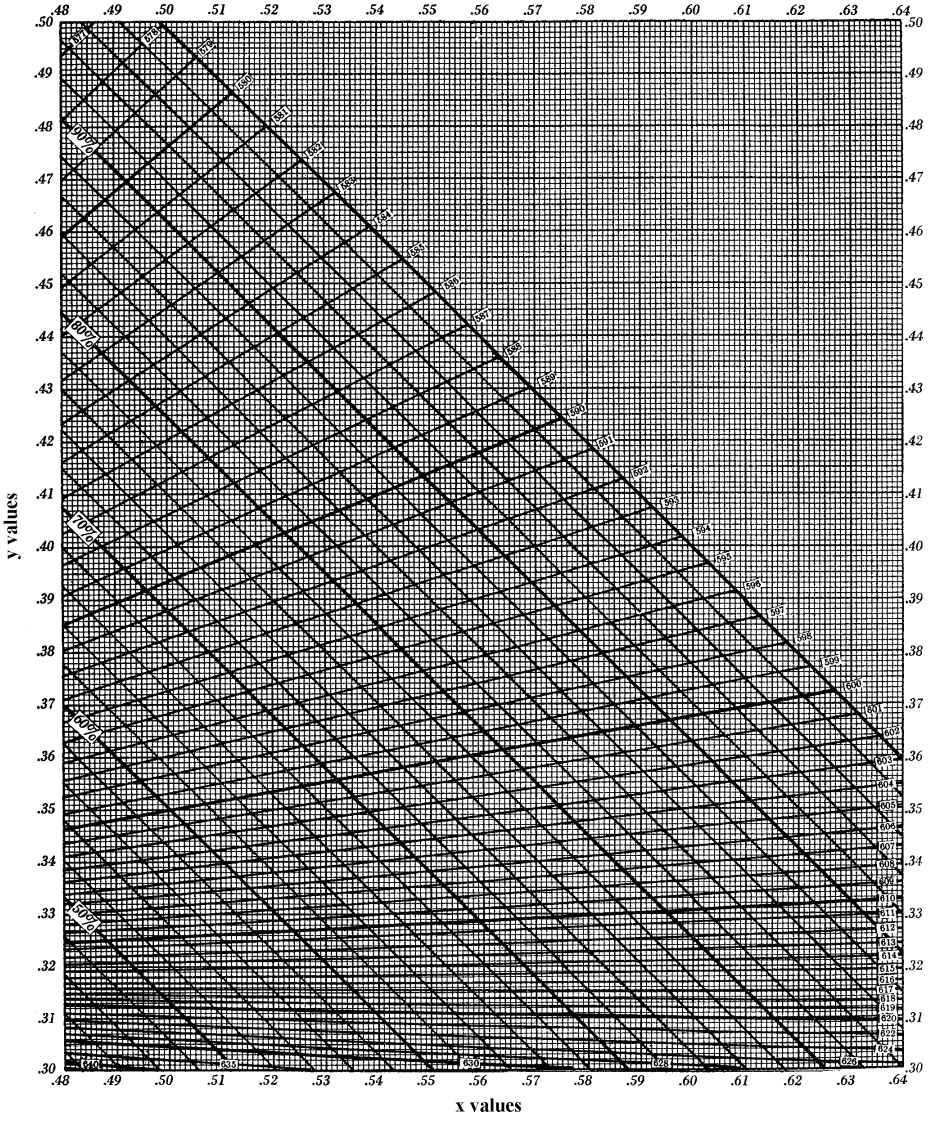



**FIGURE 3**

Chromaticity diagram for pure red wines and brick red wines

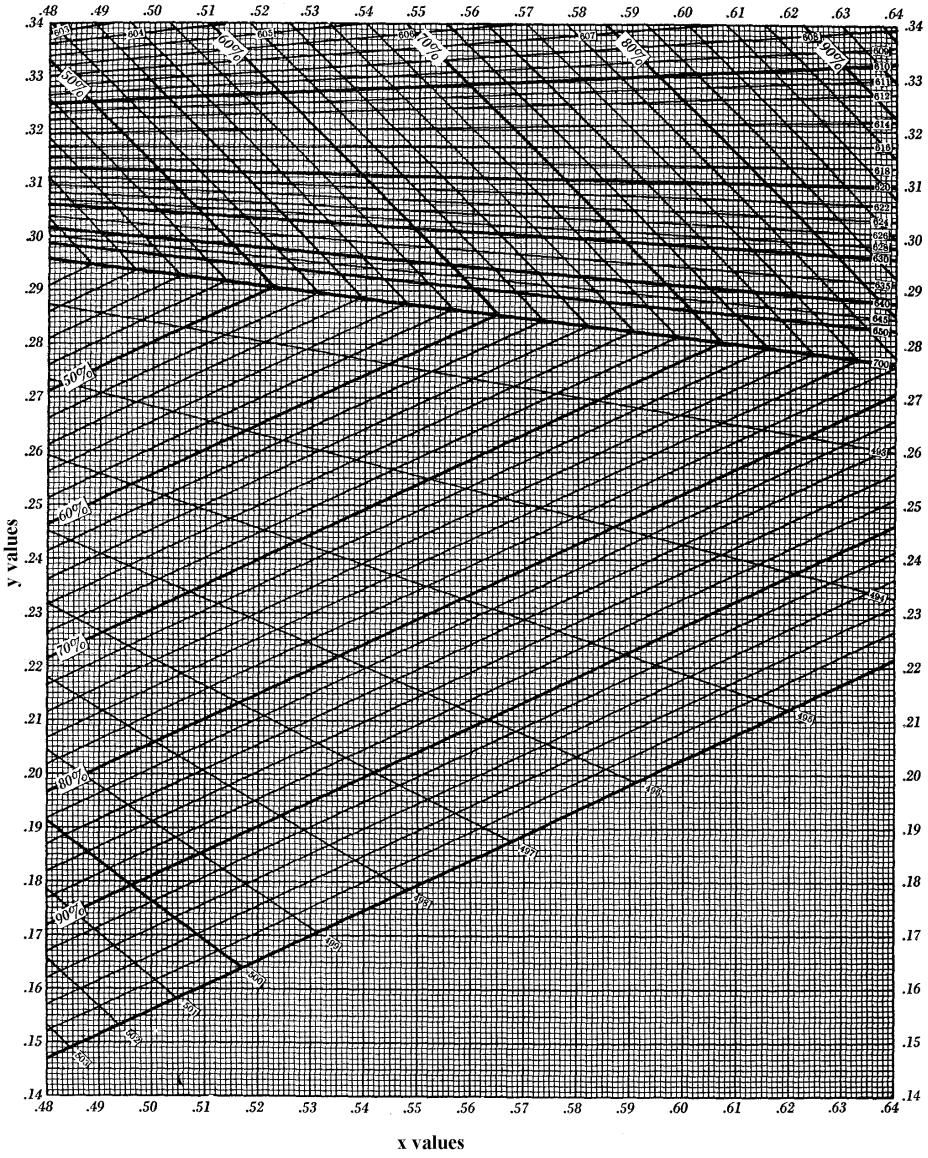



**FIGURE 4**

Chromaticity diagram for pure red wines and purple wines

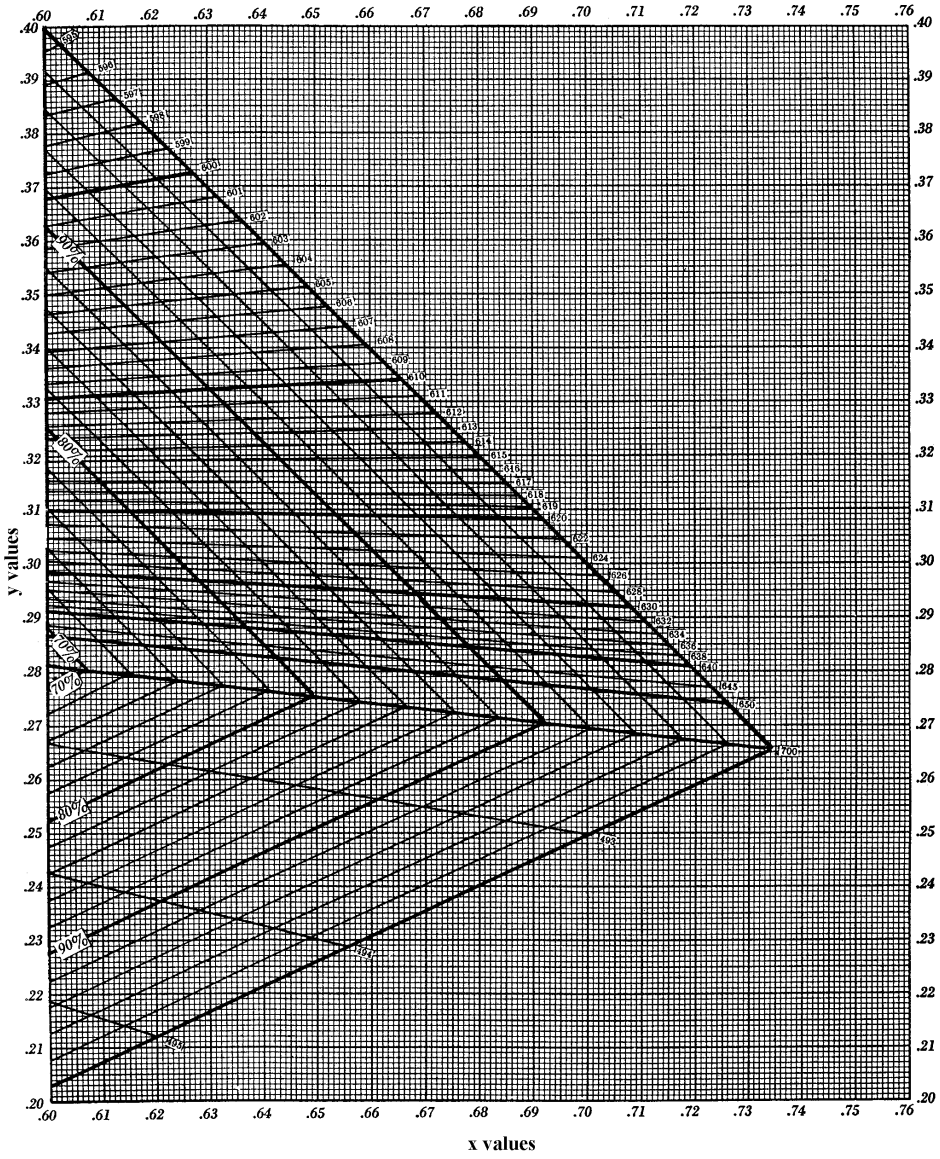



**FIGURE 5**

Chromaticity diagram for pure red wines and purple red wines

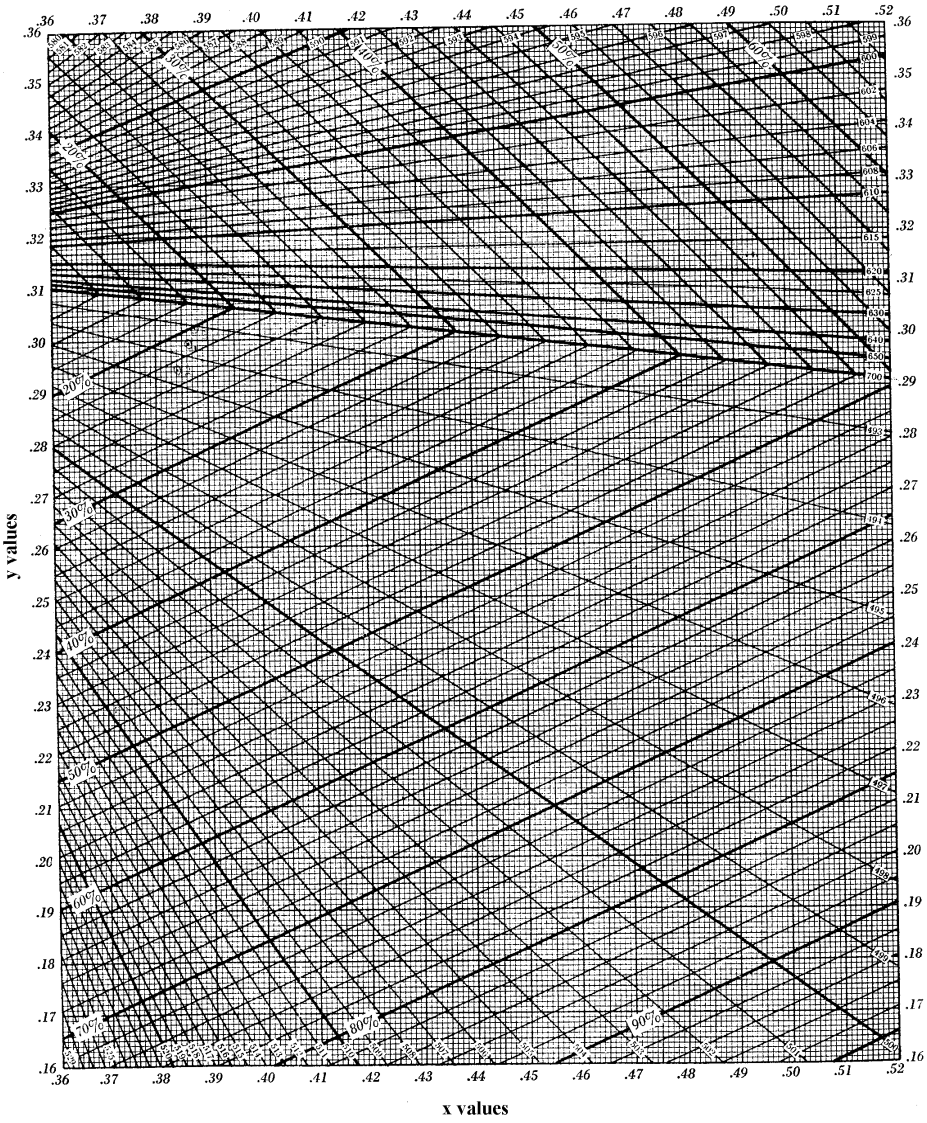



**FIGURE 6**

Chromaticity diagram for brick red wines and purple red wines

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**Method OIV-MA-AS2-08** Type IV method

# Wine turbidity

(Resolution Oeno 4/2000)

**Determination by Nephelometric Analysis**

## 1. Warning

Measurements of turbidity are largely dependent on the design of the equipment used. Therefore, comparative measurements from one instrument to another are not possible unless the same measuring principle is used.

The primary known sources of errors, which are linked to the type of turbidimeter employed, are:

- effect of stray light,
- effect of product color, especially in cases with low cloudiness values,
- electronic shifting due to aging electronic components,
- type of light source, photo detector and the dimensions and type of measurement the cell.

The present method uses a nephelometer incorporating a **double beam with optical compensation** design.

This category of instrument makes it possible to compensate for: electronic shift, fluctuations of mains voltage, and, in part, wine color. Furthermore, calibration is highly stable.

*It should be noted that this method does not lend itself to a collation of data from various sources, given the impossibility of conducting an analysis in collaboration with others.*

## 2. Purpose

The purpose of this document is to describe an optical method capable of measuring the turbidity (or diffusion) index of wine.

## 3. Scope of application

This method is used in the absence of instruments allowing a completely faithful duplication of measurements from one device to another, as well as full compensation for wine color. Therefore, findings are given for informational purposes only, and must be considered with caution.

Above all, this technique is intended for use in production, where it is the most objective criterion of the measurement of clarity.

This method, which cannot be validated accordingly to internationally recognized criteria, will be classified as class **4**[1].

## 4. General principle

Turbidity is an optical effect.

The diffusion index is an intrinsic property of liquids that makes it possible to describe their optical appearance. This optical effect is produced by the presence of extremely fine particles scattered in a liquid dispersion medium. The refraction index of these particles differs from that of the dispersion medium.

If a light is shown through a quantity of optically clean water placed in a container of known volume and the luminous flux diffused with respect to an incident beam is measured, the recorded value of this diffused flux will allow description of the molecular diffusion in the water.

If the value obtained for the water thus analyzed is greater than that of the molecular diffusion, which remains constant for a given wavelength, the same incident flux at the same angle measurement, in a tank of the same shape and at a given temperature, the difference can be attributed to the light diffused by solid, liquid or gaseous particles suspended in the water.

The measurement (taken as described) of the diffused luminous flux constitutes a nephelometric measurement.

## 5. Definitions

5.1. *Turbidity*

Reduction of the transparency of a liquid due to the presence of undissolved substances.

5.2. *Units of Measurement of the Turbidity Index*

The unit of turbidity used is: NTU - NEPHELOMETRIC TURBIDITY UNIT, which is the value corresponding to the measurement of the light diffused by a standard formazine suspension prepared as described under point 6.2.2, at a 90° angle to the direction of the incident beam.

## 6. Preparing the reference Formazine suspension (1)

6.1. *Reagents*

All reagents must be of recognized analytical quality.
They must be stored in glass flasks.

6.1.1 Water for Preparing Control Solutions.

Soak a filter membrane with a pore size of 0.1µm (like those used in bacteriology) for one hour in 100 ml of distilled water. Filter 250 ml distilled water twice through this membrane, and retain this water for preparation of standard solutions.

(1) Care must be given to the precautions for handling, since Formazine is somewhat toxic.

6.1.2. Formazine ($C_2H_4N_2$) Solutions

The compound known as formazine, whose formula is $C_2H_4N_2$, is not commercially available. It can be produced using the following solutions:

**Solution A:** Dissolve 10.0 g hexamethylene-tetramine $(CH_2)_6N_4$ in distilled water prepared according to the instructions in 6.1.1. Then fill to a volume of 100 ml using distilled water.

**Solution B:** Dissolve 1.0 g of hydrazinium sulfate, $N_2H_6SO_4$, in distilled water prepared according to the instruction in 6.1.1. Then fill to a volume of 100 ml using distilled water prepared according to 6.1.1.

**WARNING: Hydrazinium sulfate is poisonous and may be carcinogenic.**

**6.2 Working Method**

Mix 5 ml of Solution A and 5 ml of Solution B. Dilute the solution to a volume of 100 ml with water after 24 hours at 25 °C ± 3 °C (6.1.1).

The turbidity of this standard solution is 400 NTU.

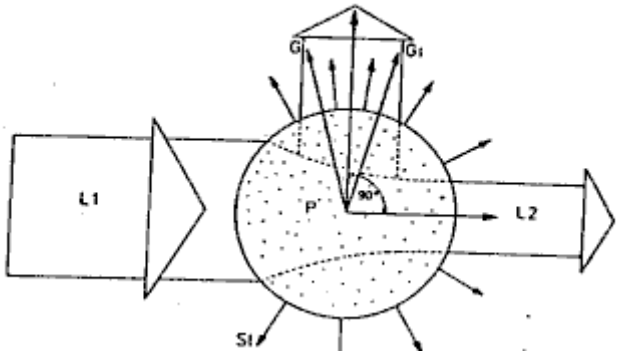
This standard suspension will keep for approximately 4 weeks at room temperature in the dark.

By diluting to 1/400 with recently prepared distilled water, a turbidity of 1 NTU will be obtained.

This solution remains stable for one week only.

**N.B.:** Standard formazine solutions have been compared to standard polymer-based solutions. The differences observed may be considered negligible. Nonetheless, polymer-based standard solutions have the following drawbacks: they are very expensive and they have a limited useful life. They must be handled with care to avoid breaking the polymer particles, as breakage would alter the turbidity value. Polymer use is suggested as an alternative to formazine.

**7. Optical Measurement Principle**



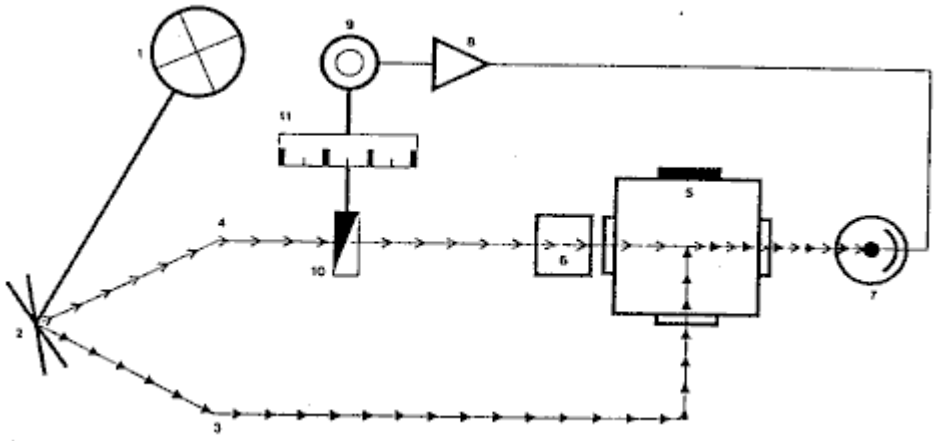

S1

Measurement principle:
L1 = Incident light beam
L2 = Beam after passing through sample
P = Sample
St = diffused light
G/G1 = Limiting rays from the diffused light beam used for measurement

The diffused light should be observed at an angle of 90° to the direction of propagation of the incident beam.

**8. Instrumentation**

8.1. *Optical principle of the dual-beam and optical compensation nephelometer*



A light source (1) powered by the electricity network projects a beam of light onto an oscillating mirror (2) which alternately reflects a measuring beam (3) and a comparison beam (4) at a rate of approximately 600 times per second.

The measuring beam (3) propagates through the fluid to be measured (5) while the comparison beam (4) propagates through an optically stable turbidity-comparison standard fluid (6).

The light diffused by the particles producing turbidity in the fluid (5) and the light diffused by the standard comparison solution (6) are alternately received by a photoelectric cell (7).

Accordingly, this cell receives a measuring beam (3) and a comparison (4) having the same frequency, but different whose luminous intensities.

The photoelectric cell (7) transforms these unequal luminous intensities into electric current which are in turn amplified (8) and fed to a synchronous motor (9) functioning as a servo-motor.

This motor uses a mechanical measuring diaphragm (10) to vary the intensity of the control beam, until the two beams strike the photoelectric cell with equal luminous intensity.
This equilibrium state allows the solid particle content of the fluid to be determined.
The absolute value of the measurement depends on the dimensions of the standard comparison beam and on the position of the diaphragm.

8.2. *Characteristics*

**Note: In order to take these measurements, regardless of the color of the wine, the nephelometer must be equipped with an additional interferential filter allowing measurement at a wavelength of 620 nm. However, the interferential filter is not needed if the light source is an infrared one.**

8.2.1 The width of the spectral band of the incident radiation should be less than or equal to 60 nm.

8.2.2 There should be no divergence in the parallelism of the incident radiation, and convergence must not exceed 1.5°.

8.2.3 The angle of measurement between the optical axis of the incident radiation and that of the diffused radiation should be 90° ± 2.5°.

8.2.4 The apparatus must not cause error due to stray light greater than:

- 0.01 NTU of random light error
within a range of:
- 0 to 0.1 NTU.

**9. Operating Method for measurement**

9.1. *Checking the Apparatus*

Before taking any measurement or series of measurements, check to ensure the proper electrical and mechanical operation of the apparatus in accordance with the recommendations of the manufacturer.

9.2. *Check Measurement Scale Adjustment*

Before taking any measurement or series of measurements, use a previously calibrated instrument to check its measurement scale adjustment consistent with the principle underlying its design.

9.3 *Cleaning the Measuring Unit*

With the greatest care, clean the measuring tank before all analyses. Take all necessary precautions to avoid getting dust in the apparatus and especially in the measuring unit, before and during determination of the turbidity index.

9.4. *Taking Measurements*

- The operating temperature should be between 15° and 25 °C (Take the temperature of the wine to be measured into consideration to ensure proper comparison). Prior to taking the measurement, carefully homogenize the product and, without making any abrupt movement that could create an emulsion, the flask holding the product to be analyze.
- Carefully wash the measuring tank twice with a small amount of the product to be analyzed.

- Carefully pour the product to be analyzed into the measuring tank, taking care to avoid any turbulence in the flow of the liquid, since this would lead to the formation of air bubbles. Carry out the test measurements.
- Wait one minute if the index value is stable.
- Record the resulting turbidity index.

**10. Expressing the results**

The turbidity index of the wine undergoing analysis is recorded and expressed in:

*** NTU**

* if turbidity is less than 1 NTU, round off to 0.01 NTU
* if turbidity is between 1 NTU and 10 NTU, round off to 0.1 NTU
* if turbidity is between 10 NTU and 100 NTU, round off to 1 NTU

**11. Test report**

The test should contain the following information:

a) reference to this method
b) the results, expressed as indicated in 10
c) any detail or occurrence that may have affected the findings.

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***-OIV***

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"Excerpts from technical instructions for nephelometers"

**Method OIV-MA-AS2-09**

# Method for isotopic ratio $^{18}O/^{16}O$ of water content in wines

(Resolution oeno 2/96)

WITHDRAWN
(replaced by OIV-MA-AS2-12)

**Method OIV-MA-AS2-10** Type IV method

# Folin-Ciocalteu Index

## 1. Definition

The Folin-Ciocalteu index is the result obtained by applying the method described below.

## 2. Principle

All phenolic compounds contained in wine are oxidized by Folin-Ciocalteu reagent. This reagent is formed from a mixture of phosphotungstic acid, $H_3PW_{12}O_{40}$, and phosphomolybdic acid, $H_3PMo_{12}O_{40}$, which, after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten, $W_8O_{23}$, and molybdenum, $Mo_8O_{23}$. The blue coloration produced has a maximum absorption in the region of 750 nm, and is proportional to the total quantity of phenolic compounds originally present.

## 3. Apparatus

Normal laboratory apparatus, in particular:

3.1 100 mL volumetric flasks.

3.2 Spectrophotometer capable of operating at 750 nm.

## 4. Reagents

4.1 Folin-Ciocalteu reagent

This reagent is available commercially in a form ready for use.

Alternatively it may be prepared as follows: dissolve 100 g of sodium tungstate, $Na_2WO_4.2H_2O$, and 25 g of sodium molybdate, $Na_2MoO_4.2H_2O$, in 700 mL of distilled water. Add 50 mL phosphoric acid 85% ($\rho_{20}$ = 1.71 g/mL), and 100 mL of concentrated hydrochloric acid ($\rho_{20}$ = 1.19 g/mL). Bring to the boil and reflux for 10 hours. Then add 150 g of lithium sulfate, $Li_2SO_4.H_2O$, and a few drops of bromine and boil for 15 minutes. Allow to cool and make up to one liter with distilled water.

4.2 Anhydrous sodium carbonate, $Na_2CO_3$, made up into a 20% (*m/v*) solution.

## 5. Procedure

5.1 *Red wine*

Introduce the following into a 100 mL volumetric flask (3.1) strictly in the following order:

1 mL of the wine, previously diluted 1/5,
50 mL of distilled water,
5 mL of Folin-Ciocalteu reagent (4.1),
20 mL of sodium carbonate solution (4.2).

Bring to 100 mL with distilled water.

Mix to dissolve. Leave for 30 minutes for the reaction to stabilize. Determine the absorbance at 750 nm through a path length of 1 cm with respect to a blank prepared with distilled water in place of the wine.

If the absorbance is not in the region of 0.3 appropriate dilution should be made.

5.2 *White wine*

Carry out the same procedure with 1 mL of undiluted wine.

**6. Expression of results**

6.1 *Calculation*

The result is expressed in the form of an index obtained by multiplying the absorbance by 100 for red wines diluted 1/5 (or by the corresponding factor for other dilutions) and by 20 for white wines.

6.2 *Precision*

The difference between the results of two determinations carried out simultaneously or very quickly one after the other by the same analyst must not be greater than 1. Good precision of results is aided by using scrupulously clean apparatus (volumetric flasks and spectrophotometer cells).

**Method OIV-MA-AS2-11** Type I method

# Determination of chromatic characteristics according to CIELab

(Resolution Oeno 1/2006)

## 1. Introduction

The colour of a wine is one of the most important visual features available to us, since it provides a considerable amount of highly relevant information.

Colour is a sensation that we perceive visually from the refraction or reflection of light on the surface of objects. Colour is light—as it is strictly related to it—and depending on the type of light (illuminating or luminous stimulus) we see one colour or another. Light is highly variable and so too is colour, to a certain extent.

Wine absorbs a part of the radiations of light that falls and reflects another, which reaches the eyes of the *observer*, making them experience the sensation of colour. For instance, the sensation of very dark red wines is almost entirely due to the fact that incident radiation is absorbed by the wine.

### 1.1. Scope

The purpose of this spectrophotometric method is to define the process of measuring and calculating the *chromatic characteristics* of wines and other beverages derived from *trichromatic components*: X, Y and Z, according to the *Commission Internationale de l'Eclairage* (CIE, 1976), by attempting to imitate real observers with regard to their sensations of colour.

### 1.2. Principle and definitions

The colour of a wine can be described using 3 attributes or specific qualities of visual sensation: tonality, luminosity and chromatism.

*Tonality*—colour itself—is the most characteristic: red, yellow, green or blue. *Luminosity* is the attribute of visual sensation according to which a wine appears to be more or less luminous. However, *chromatism,* or the *level of colouring,* is related to a higher or lower intensity of colour. The combination of these three concepts enables us to define the multiple shades of colour that wines present.

The *chromatic characteristics* of a wine are defined by the *colorimetric* or *chromaticity coordinates* (Fig. 1): *clarity* ($L^*$), *red/green colour component* ($a^*$), and *blue/yellow colour component* ($b^*$); and by its *derived magnitudes*: *chroma* ($C^*$), *tone* ($H^*$) and *chromacity* [($a^*$, $b^*$) or ($C^*$, $H^*$)]. In other words, this CIELab colour or space system is based on a sequential or continuous Cartesian representation of 3 orthogonal axes: $L^*$, $a^*$ and $b^*$ (Fig. 2 and 3). Coordinate $L^*$ represents clarity ($L^* = 0$ black and $L^* = 100$ colourless), $a^*$ green/red colour component ($a^*>0$ red, $a^*<0$ green) and $b^*$ blue/yellow colour component ($b^*>0$ yellow, $b^*<0$ blue).

### 1.2.1. Clarity

Its symbol is $L^*$ and it is defined according to the following mathematical function:

$$L^*=116(Y/Y_n)^{1/3}-16 \qquad \textbf{(I)}$$

Directly related to the visual sensation of luminosity.

### 1.2.2. Red/green colour component

Its symbol is $a^*$ and it is defined according to the following mathematical function:

$$a^*=500[(X/X_n)-(Y/Y_n)] \quad \textbf{(I)}$$

### 1.2.3. Yellow/blue colour component

Its symbol is $b^*$ and it is defined according to the following mathematical function:

$$b^*=200-[(Y/Y_n)^{1/3}-(Z/Z_n)^{1/3}] \quad \textbf{(I)}$$

### 1.2.4. Chroma

The chroma symbol is $C^*$ and it is defined according to the following mathematical function:

$$C^* = \sqrt{(a^{*2} + b^{*2})}$$

### 1.2.5. Tone

The tone symbol is $H^*$, its unit is the sexagesimal degree (º), and it is defined according to the following mathematical function:

$$H^* = tg^{-1} (b^*/a^*)$$

### 1.2.6 Difference of tone between two wines

The symbol is $\Delta H^*$ and it is defined according to the following mathematical function:

$$\Delta H^* = \sqrt{(\Delta E^*)^2 - (\Delta L^*)^2 - (\Delta C^*)^2}$$

**(I) See explanation Annex I**

### 1.2.7. Overall colorimetric difference between two wines

The symbol is $\Delta E^*$ and it is defined according to the following mathematical functions:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} = \sqrt{(\Delta L^*)^2 + (\Delta C^*)^2 + (\Delta H^*)^2}$$

## 1.3. Reagents and products

Distilled water.

**1.4. Apparatus and equipment**

Customary laboratory apparatus and, in particular, the following:

**1.4.1.** Spectrophotometer to carry out transmittance measurements at a wavelength of between 300 and 800 nm, with illuminant D65 and observer placed at 10º. Use apparatus with a resolution equal to or higher than 5 nm and, where possible, with scan.

**1.4.2.** Computer equipment and suitable programme which, when connected to the spectrophotometer, will facilitate calculating colorimetric coordinates ($L^*$, $a^*$ and $b^*$) and their derived magnitudes ($C^*$ and $H^*$).

**1.4.3.** Glass cuvettes, available in pairs, optical thickness 1, 2 and 10 mm.

**1.4.4.** Micropipettes for volumes between 0.020 and 2 ml.

**1.5. Sampling and sample preparation**

Sample taking must particularly respect all concepts of homogeneity and representativity.

If the wine is dull, it must be clarified by centrifugation. For young or sparkling wines, as much carbon dioxide as possible must be eliminated by vacuum stirring or using a sonicator.

**1.6. Procedure**

- Select the pair of cuvettes for the spectrophotometric reading, ensuring that the upper measurement limit within the linear range of the spectrophotometer is not exceeded. By way of indication, for white and rosé wines it is recommended to use cuvettes with 10 mm of optical thickness, and for red wines, cuvettes with 1 mm optical thickness.
- After obtaining and preparing the sample, measure its transmittance from 380 to 780 nm every 5 nm, using distilled water as a reference in a cuvette with the same optical thickness, in order to establish the base line or the white line. Choose illuminant D65 and observer 10º.
- If the optical thickness of the reading cuvette is under 10 mm, the transmittance must be transformed to 10 mm before calculating: $L^*$, $a^*$, $b^*$, $C^*$ and $H^*$.

Summary:

| |
|---|
| Spectral measurements in transmittance from 780 to 380 nm |
| Interval: 5 nm |
| Cuvettes: use appropriately according to wine intensity: 1 cm (white and rosé wines) and 0.1 cm (red wines) |
| Illuminant D65 |
| Observer reference pattern 10º |

## 1.7. Calculations

The spectrophotometer must be connected to a computer programme to facilitate the calculation of the colorimetric coordinates ($L^*$, $a^*$ and $b^*$) and their derived magnitudes ($C^*$ and $H^*$), using the appropriate mathematical algorithms.
In the event of a computer programme not being available, see Annex I on how to proceed.

## 1.8. Expression of results

The colorimetric coordinates of wine will be expressed according to the recommendations in the following table.

| Colorimetric coordinates | Symbol | Unit | Interval | Decimals |
|---|---|---|---|---|
| Clarity | $L^*$ | | 0-100<br>0 black<br>100 colourless | 1 |
| Red/green colour component | $a^*$ | | >0 red<br><0 green | 2 |
| Yellow/blue colour component | $b^*$ | | >0 yellow<br><0 blue | 2 |
| Chroma | $C^*$ | | | 2 |
| Tone | $H^*$ | º | 0-360º | 2 |

## 1.9. Numerical Example

Figure 4 shows the values of the colorimetric coordinates and the chromaticity diagram of a young red wine for the following values:
X = 12.31; Y = 60.03 and Z = 10.24
L* = 29.2
a* = 55.08
b* = 36.10
C* = 66.00
H* = 33.26º

## 2. Accuracy

The above data were obtained from two interlaboratory tests of 8 samples of wine with blind duplicates of progressive chromatic characteristics, in accordance with the recommendations of the harmonized protocol for collaborative studies, with a view to validating the method of analysis.

### 2.1. Colorimetric coordinate L* (clarity, 0-100)

| **Sample Identification** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** |
|---|---|---|---|---|---|---|---|---|
| Year of interlaboratory test | 2004 | 2002 | 2004 | 2004 | 2004 | 2004 | 2002 | 2004 |
| No. of participating laboratories | 18 | 21 | 18 | 18 | 17 | 18 | 23 | 18 |
| No. of laboratories accepted after aberrant value elimination | 14 | 16 | 16 | 16 | 14 | 17 | 21 | 16 |
| Mean value ( $\overline{X}$ ) | 96.8 | 98.0 | 91.6 | 86.0 | 77.4 | 67.0 | 34.6 | 17.6 |
| Repeatability standard deviation ($s_r$) | 0.2 | 0.1 | 0.2 | 0.8 | 0.2 | 0.9 | 0.1 | 0.2 |
| Relative repeatability standard deviation ($RSD_r$) (%) | 0.2 | 0.1 | 0.3 | 1.0 | 0.3 | 1.3 | 0.2 | 1.2 |
| Repeatability limit (r) (2.8 x $s_r$) | 0.5 | 0.2 | 0.7 | 2.2 | 0.7 | 2.5 | 0.2 | 0.6 |
| Reproducibility standard deviation ($s_R$) | 0.6 | 0.1 | 1.2 | 2.0 | 0.8 | 4.1 | 1.0 | 1.0 |
| Relative reproducibility standard deviation ($RSD_R$) (%) | 0.6 | 0.1 | 1.3 | 2.3 | 1.0 | 6.1 | 2.9 | 5.6 |
| Reproducibility limit (R) (2.8 x $s_R$) | 1.7 | 0.4 | 3.3 | 5.5 | 2.2 | 11.5 | 2.8 | 2.8 |

## 2.2. Colorimetric coordinate a* (green/red)

| **Sample Identification** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** |
|---|---|---|---|---|---|---|---|---|
| Year of interlaboratory | 2004 | 2002 | 2004 | 2004 | 2004 | 2004 | 2002 | 2004 |
| No. of participating laboratories | 18 | 21 | 18 | 18 | 17 | 18 | 23 | 18 |
| No. of laboratories accepted after aberrant value elimination | 15 | 15 | 14 | 15 | 13 | 16 | 23 | 17 |
| Mean value ( $\overline{X}$ ) | -0.26 | -0.86 | 2.99 | 11.11 | 20.51 | 29.29 | 52.13 | 47.55 |
| Repeatability standard deviation ($s_r$) | 0.17 | 0.01 | 0.04 | 0.22 | 0.25 | 0.26 | 0.10 | 0.53 |
| Relative repeatability standard deviation ($RSD_r$) (%) | 66.3 | 1.4 | 1.3 | 2.0 | 1.2 | 0.9 | 0.2 | 1.1 |
| Repeatability limit (r) (2.8 x $s_r$) | 0.49 | 0.03 | 0.11 | 0.61 | 0.71 | 0.72 | 0.29 | 1.49 |
| Reproducibility standard deviation ($s_R$) | 0.30 | 0.06 | 0.28 | 0.52 | 0.45 | 0.98 | 0.88 | 1.20 |
| Relative reproducibility standard deviation ($RSD_R$) (%) | 116.0 | 7.5 | 9.4 | 4.7 | 2.2 | 3.4 | 1.7 | 2.5 |
| Reproducibility limit (R) (2.8 x $s_R$) | 0.85 | 0.18 | 0.79 | 1.45 | 1.27 | 2.75 | 2.47 | 3.37 |

## 2.3. Colorimetric coordinate b* (blue/yellow)

| **Sample Identification** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** |
|---|---|---|---|---|---|---|---|---|
| Year of interlaboratory | 2004 | 2002 | 2004 | 2004 | 2004 | 2004 | 2002 | 2004 |
| No. of participating laboratories | 17 | 21 | 17 | 17 | 17 | 18 | 23 | 18 |
| No. of laboratories accepted after aberrant value elimination | 15 | 16 | 13 | 14 | 16 | 18 | 23 | 15 |
| Mean value ( $\overline{X}$ ) | 10.95 | 9.04 | 17.75 | 17.10 | 19.68 | 26.51 | 45.82 | 30.07 |
| Repeatability standard deviation ($s_r$) | 0.25 | 0.03 | 0.08 | 1.08 | 0.76 | 0.65 | 0.15 | 0.36 |
| Relative repeatability standard deviation ($RSD_r$) (%) | 2.3 | 0.4 | 0.4 | 6.3 | 3.8 | 2.5 | 0.3 | 1.2 |
| Repeatability limit (r) (2.8 x $s_r$) | 0.71 | 0.09 | 0.21 | 3.02 | 2.12 | 1.83 | 0.42 | 1.01 |
| Reproducibility standard deviation ($s_R$) | 0.79 | 0.19 | 0.53 | 1.18 | 3.34 | 2.40 | 1.44 | 1.56 |
| Relative reproducibility standard deviation ($RSD_R$) (%) | 7.2 | 2.1 | 3.0 | 6.9 | 16.9 | 9.1 | 3.1 | 5.2 |
| Reproducibility limit (R) (2.8 x $s_R$) | 2.22 | 0.53 | 1.47 | 3.31 | 9.34 | 6.72 | 4.03 | 4.38 |

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## APPENDIX 1

In formal terms, the trichromatic components X, Y, Z of a colour stimulus result from the integration, throughout the visible range of the spectrum, of the functions

obtained by multiplying the relative spectral curve of the colour stimulus by the colorimetric functions of the reference observer. These functions are always obtained by experiment. It is not possible, therefore to calculate the trichromatic components directly by integration. Consequently, the approximate values are determined by replacing these integrals by summations on finished wavelength intervals.

$X = K\sum_{(\lambda)} T_{(\lambda)} S_{(\lambda)} \overline{X}_{10(\lambda)} \Delta_{(\lambda)}$

$Y = K\sum_{(\lambda)} T_{(\lambda)} S_{(\lambda)} \overline{Y}_{10(\lambda)} \Delta_{(\lambda)}$

$Z = K\sum_{(\lambda)} T_{(\lambda)} S_{(\lambda)} \overline{Z}_{10(\lambda)} \Delta_{(\lambda)}$

$K = 100 \Big/ \sum_{(\lambda)} S_{(\lambda)} \overline{Y}_{10(\lambda)} \Delta_{(\lambda)}$

$T_{(\lambda)}$ is the measurement of the transmittance of the wine measured at the wavelength λ expressed at 1 cm from the optical thickness.

$\Delta_{(\lambda)}$ is the interval between the value of λ at which $T_{(\lambda)}$ is measured

$S_{(\lambda)}$: coefficients that are a function of λ and of the illuminant (Table 1).

$\overline{X}_{10(\lambda)}; \overline{Y}_{10(\lambda)}; \overline{Z}_{10(\lambda)}$ : coefficients that are a function of λ and of the observer. (Table 1)

The values of Xn, Yn, and Zn represent the values of the perfect diffuser under an illuminant and a given reference observer. In this case, the illuminant is D65 and the observer is higher than 4 degrees.

$X_n = 94.825$; $Y_n = 100$; $Z_n = 107.381$

This roughly uniform space is derived from the space CIEYxy, in which the trichromatic components X, Y, Z are defined.

The coordinates L*, a* and b* are calculated based on the values of the trichromatic components X, Y, Z, using the following formulae.

**L*** = $116 (Y / Y_n)^{1/3} - 16$ where $Y/Yn > 0.008856$

**L*** = $903.3 (Y / Y_n)$ where $Y / Y_n <$ ó = 0.008856

**a*** = $500 [ f(X / X_n) - f(Y / Y_n) ]$

**b*** = $200 [f(Y / Y_n) - f(Z / Z_n) ]$

$f(X / X_n) = (X / X_n)^{1/3}$ where $(X / X_n) > 0.008856$

$f(X / X_n) = 7.787 (X / X_n) + 16 / 166$ where $(X / X_n) < ó = 0.008856$

$f(Y / Y_n) = (Y / Y_n)^{1/3}$ where $(Y / Y_n) > 0.008856$

$f(Y / Y_n) = 7.787 (Y / Y_n) + 16 / 116$ where $(Y / Y_n) < ó = 0.008856$

$f(Z / Z_n) = (Z / Z_n)^{1/3}$ where $(Z / Z_n) > 0.008856$

$f(Z / Z_n) = 7.787 (Z / Z_n) + 16 / 116$ where $(Z / Z_n) < ó = 0.008856$

The total colorimetric difference between two colours is given by the CIELAB colour difference

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

In the CIELAB space it is possible to express not only overall variations in colour, but also in relation to one or more of the parameters L*, a* and b*. This can be used to define new parameters and to relate them to the attributes of the visual sensation.

Clarity, related to luminosity, is directly represented by the value of L*.

Chroma: $C^* = (a^{*2} + b^{*2})^{1/2}$ defines the chromaticness.

The angle of hue: $H^* = tg^{-1} (b^*/a^*)$ (expressed in degrees); related to hue.

The difference in hue: $\Delta H^* = [(\Delta E^*)^2 - (\Delta L^*)^2 - (\Delta C^*)^2]^{1/2}$

For two unspecified colours, $\Delta C^*$ represents their difference in chroma; $\Delta L^*$, their difference in clarity, and $\Delta E^*$, their overall variation in colour. We thus have:

$$\Delta E^* = [(\Delta L^*)2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} = [(\Delta L^*)^2 + (\Delta C^*)^2 + (\Delta H^*)^2]^{1/2}$$

**Table 1.**

| Wavelength (λ) nm. | $S_{(\lambda)}$ | $\overline{X}_{10(\lambda)}$ | $\overline{Y}_{10(\lambda)}$ | $\overline{Z}_{10(\lambda)}$ |
|---|---|---|---|---|
| 380 | 50.0 | 0.0002 | 0.0000 | 0.0007 |
| 385 | 52.3 | 0.0007 | 0.0001 | 0.0029 |
| 390 | 54.6 | 0.0024 | 0.0003 | 0.0105 |
| 395 | 68.7 | 0.0072 | 0.0008 | 0.0323 |
| 400 | 82.8 | 0.0191 | 0.0020 | 0.0860 |
| 405 | 87.1 | 0.0434 | 0.0045 | 0.1971 |
| 410 | 91.5 | 0.0847 | 0.0088 | 0.3894 |
| 415 | 92.5 | 0.1406 | 0.0145 | 0.6568 |
| 420 | 93.4 | 0.2045 | 0.0214 | 0.9725 |
| 425 | 90.1 | 0.2647 | 0.0295 | 1.2825 |
| 430 | 86.7 | 0.3147 | 0.0387 | 1.5535 |
| 435 | 95.8 | 0.3577 | 0.0496 | 1.7985 |
| 440 | 104.9 | 0.3837 | 0.0621 | 1.9673 |
| 445 | 110.9 | 0.3867 | 0.0747 | 2.0273 |
| 450 | 117.0 | 0.3707 | 0.0895 | 1.9948 |
| 455 | 117.4 | 0.3430 | 0.1063 | 1.9007 |
| 460 | 117.8 | 0.3023 | 0.1282 | 1.7454 |
| 465 | 116.3 | 0.2541 | 0.1528 | 1.5549 |
| 470 | 114.9 | 0.1956 | 0.1852 | 1.3176 |
| 475 | 115.4 | 0.1323 | 0.2199 | 1.0302 |
| 480 | 115.9 | 0.0805 | 0.2536 | 0.7721 |
| 485 | 112.4 | 0.0411 | 0.2977 | 0.5701 |
| 490 | 108.8 | 0.0162 | 0.3391 | 0.4153 |
| 495 | 109.1 | 0.0051 | 0.3954 | 0.3024 |
| 500 | 109.4 | 0.0038 | 0.4608 | 0.2185 |
| 505 | 108.6 | 0.0154 | 0.5314 | 0.1592 |
| 510 | 107.8 | 0.0375 | 0.6067 | 0.1120 |
| 515 | 106.3 | 0.0714 | 0.6857 | 0.0822 |
| 520 | 104.8 | 0.1177 | 0.7618 | 0.0607 |
| 525 | 106.2 | 0.1730 | 0.8233 | 0.0431 |
| 530 | 107.7 | 0.2365 | 0.8752 | 0.0305 |
| 535 | 106.0 | 0.3042 | 0.9238 | 0.0206 |
| 540 | 104.4 | 0.3768 | 0.9620 | 0.0137 |
| 545 | 104.2 | 0.4516 | 0.9822 | 0.0079 |
| 550 | 104.0 | 0.5298 | 0.9918 | 0.0040 |
| 555 | 102.0 | 0.6161 | 0.9991 | 0.0011 |
| 560 | 100.0 | 0.7052 | 0.9973 | 0.0000 |
| 565 | 98.2 | 0.7938 | 0.9824 | 0.0000 |

| | | | | |
|---|---|---|---|---|
| 570 | 96.3 | 0.8787 | 0.9556 | 0.0000 |
| 575 | 96.1 | 0.9512 | 0.9152 | 0.0000 |
| 580 | 95.8 | 1.0142 | 0.8689 | 0.0000 |
| 585 | 92.2 | 1.0743 | 0.8256 | 0.0000 |
| 590 | 88.7 | 1.1185 | 0.7774 | 0.0000 |
| 595 | 89.3 | 1.1343 | 0.7204 | 0.0000 |
| 600 | 90.0 | 1.1240 | 0.6583 | 0.0000 |
| 605 | 89.8 | 1.0891 | 0.5939 | 0.0000 |
| 610 | 89.6 | 1.0305 | 0.5280 | 0.0000 |
| 615 | 88.6 | 0.9507 | 0.4618 | 0.0000 |
| 620 | 87.7 | 0.8563 | 0.3981 | 0.0000 |
| 625 | 85.5 | 0.7549 | 0.3396 | 0.0000 |
| 630 | 83.3 | 0.6475 | 0.2835 | 0.0000 |
| 635 | 83.5 | 0.5351 | 0.2283 | 0.0000 |
| 640 | 83.7 | 0.4316 | 0.1798 | 0.0000 |
| 645 | 81.9 | 0.3437 | 0.1402 | 0.0000 |
| 650 | 80.0 | 0.2683 | 0.1076 | 0.0000 |
| 655 | 80.1 | 0.2043 | 0.0812 | 0.0000 |
| 660 | 80.2 | 0.1526 | 0.0603 | 0.0000 |
| 665 | 81.2 | 0.1122 | 0.0441 | 0.0000 |
| 670 | 82.3 | 0.0813 | 0.0318 | 0.0000 |
| 675 | 80.3 | 0.0579 | 0.0226 | 0.0000 |
| 680 | 78.3 | 0.0409 | 0.0159 | 0.0000 |
| 685 | 74.0 | 0.0286 | 0.0111 | 0.0000 |
| 690 | 69.7 | 0.0199 | 0.0077 | 0.0000 |
| 695 | 70.7 | 0.0138 | 0.0054 | 0.0000 |
| 700 | 71.6 | 0.0096 | 0.0037 | 0.0000 |
| 705 | 73.0 | 0.0066 | 0.0026 | 0.0000 |
| 710 | 74.3 | 0.0046 | 0.0018 | 0.0000 |
| 715 | 68.0 | 0.0031 | 0.0012 | 0.0000 |
| 720 | 61.6 | 0.0022 | 0.0008 | 0.0000 |
| 725 | 65.7 | 0.0015 | 0.0006 | 0.0000 |
| 730 | 69.9 | 0.0010 | 0.0004 | 0.0000 |
| 735 | 72.5 | 0.0007 | 0.0003 | 0.0000 |
| 740 | 75.1 | 0.0005 | 0.0002 | 0.0000 |
| 745 | 69.3 | 0.0004 | 0.0001 | 0.0000 |
| 750 | 63.6 | 0.0003 | 0.0001 | 0.0000 |
| 755 | 55.0 | 0.0002 | 0.0001 | 0.0000 |
| 760 | 46.4 | 0.0001 | 0.0000 | 0.0000 |
| 765 | 56.6 | 0.0001 | 0.0000 | 0.0000 |
| 770 | 66.8 | 0.0001 | 0.0000 | 0.0000 |
| 775 | 65.1 | 0.0000 | 0.0000 | 0.0000 |
| 780 | 63.4 | 0.0000 | 0.0000 | 0.0000 |

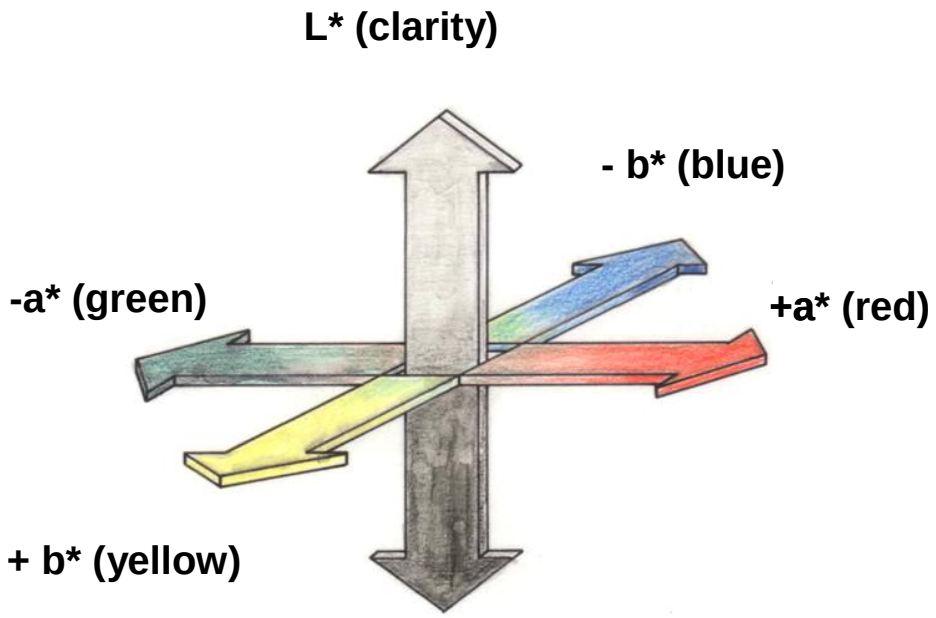



Figure 1. Diagram of colourimetric coordinates according to *Commission Internationale de l'Eclairage* (CIE, 1976)

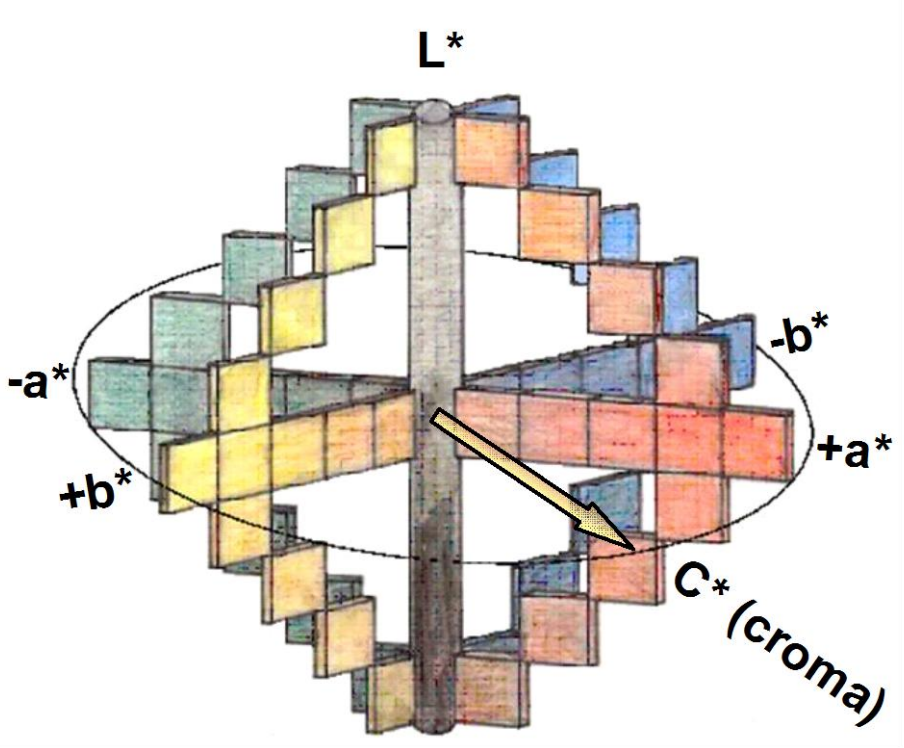



Figure 2. CIELab colourspace, based on a sequential or 3 orthogonal axis continual Cartesian representation $L^*$, $a^*$ y $b^*$

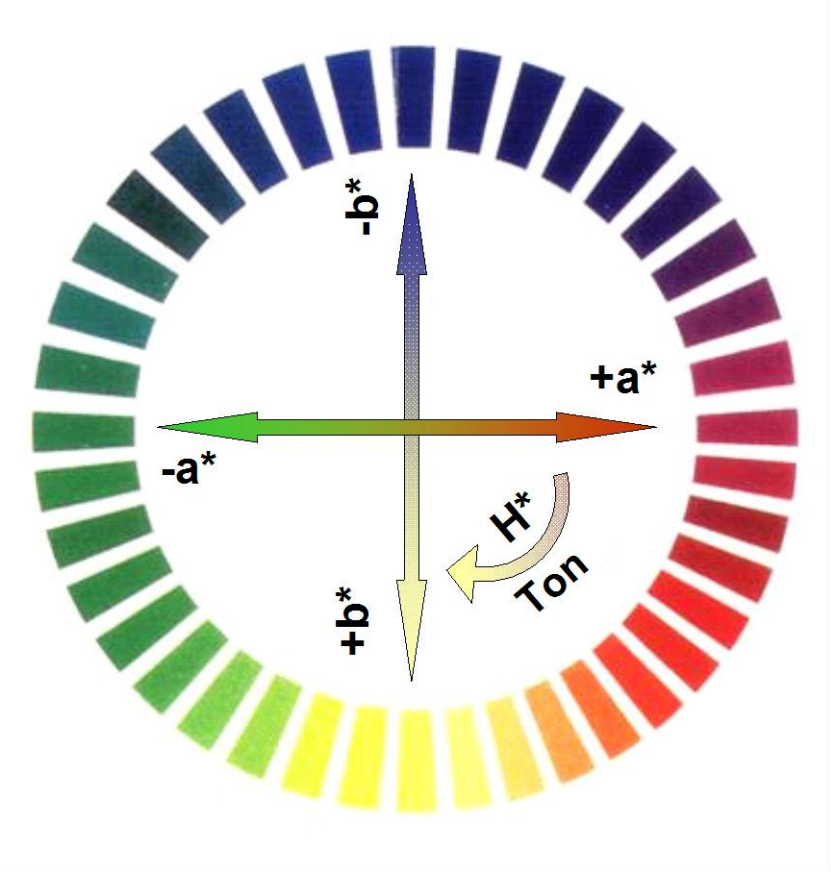
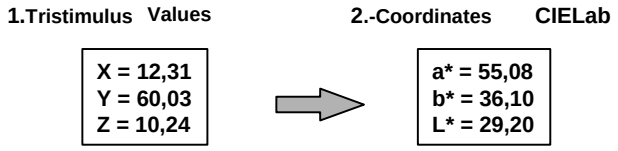



Figure 3. Sequential diagram and/or continuation of a and b colourimetric coordinates and derived magnitude, such as tone (H*)

**Example: Young Red Wine**

❒ **OBTENTION OF ANALYTICAL PARAMETERS:**




❒ **GRAPHIC REPRESENTATION AND ARTICULATION OF RESULTS:**

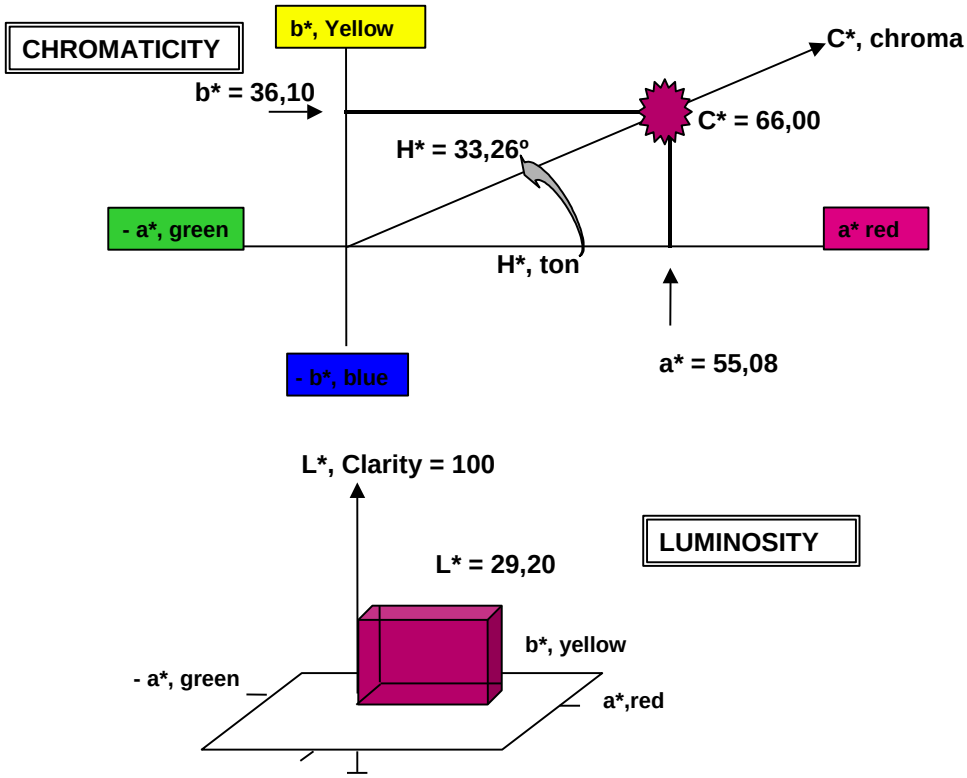



Figure 4. Representation of colour of young red wine used as an example in Chapter 1.8 shown in the CIELab three dimensional diagram.

**Method OIV-MA-AS2-12** Type II method

# Method for $^{18}O/^{16}O$ isotope ratio determination of water in wines and must

(Resolution OIV-Oeno 353/2009)

## 1. SCOPE

The method describes the determination of the $^{18}O/^{16}O$ isotope ratio of water from wine and must after equilibration with $CO_2$, using the isotope ratio mass spectrometry (IRMS).

## 2. REFERENCE STANDARDS

| | |
|---|---|
| ISO 5725:1994: | Accuracy (trueness and precision) of measurement methods and results: Basic method for the determination of repeatability and reproducibility of a standard measurement method. |
| V-SMOW: | Vienna-Standard Mean Ocean Water ($^{18}O/^{16}O = R_{V\text{-}SMOW} = 0.0020052$) |
| GISP | Greenland Ice Sheet Precipitation |
| SLAP | Standard Light Antarctic Precipitation |

## 3. DEFINITIONS

| | |
|---|---|
| $^{18}O/^{16}O$ | Isotope ratio of oxygen 18 to oxygen 16 for a given sample |
| $\delta^{18}O_{V\text{-}SMOW}$ | Relative scale for the expression of the isotope ratio of oxygen 18 to oxygen 16 for a given sample. $\delta^{18}O_{V\text{-}SMOW}$ is calculated using the following equation: |

$$\delta^{18}O_{V-SMOW} = \left[ \frac{\left(\frac{^{18}O}{^{16}O}\right)_{sample} - \left(\frac{^{18}O}{^{16}O}\right)_{standard}}{\left(\frac{^{18}O}{^{16}O}\right)_{standard}} \right] \times 1000 \quad [‰]$$

using the V-SMOW as standard and as reference point for the relative δ scale.

| | |
|---|---|
| BCR | Community Bureau of Reference |
| IAEA | International Atomic Energy Agency (Vienna, Austria) |
| IRMM | Institute for Reference Materials and Measurements |
| IRMS | Isotope Ratio Mass Spectrometry |
| m/z | mass to charge ratio |
| NIST | National Institute of Standards & Technology |
| RM | Reference Material |

## 4. PRINCIPLE

The technique described thereafter is based on the isotopic equilibration of water in samples of wine or must with a $CO_2$ standard gas according to the following isotopic exchange reaction:

$$C^{16}O_2 + H_2{}^{18}O \leftrightarrow C^{16}O^{18}O + H_2{}^{16}O$$

After equilibration the carbon dioxide in the gaseous phase is used for analysis by means of Isotopic Ratio Mass Spectrometry (IRMS) where the $^{18}O/^{16}O$ isotopic ratio is determined on the $CO_2$ resulting from the equilibration.

## 5. REAGENTS AND MATERIALS

The materials and consumables depend on the method used (see chapter 6). The systems generally used are based on the equilibration of water in wine or must with $CO_2$.
The following reference materials, working standards and consumables can be used:

5.1 Reference materials

| Name | issued by | $\delta^{18}O$ versus V-SMOW |
|---|---|---|
| V-SMOW, RM 8535 | IAEA / NIST | 0 ‰ |
| BCR-659 | IRMM | -7.18 ‰ |
| GISP, RM 8536 | IAEA / NIST | -24.78 ‰ |
| SLAP, RM 8537 | IAEA / NIST | -55.5 ‰ |

5.2 Working Standards
5.2.1 Carbon dioxide as a secondary reference gas for measurement (CAS 00124-38-9).
5.2.2 Carbon dioxide used for equilibration (depending on the instrument this gas could be the same as 5.2.1 or in the case of continuous flow systems cylinders containing gas mixture helium-carbon dioxide can also be used)

5.2.3 Working Standards with calibrated $\delta^{18}O_{V\text{-}SMOW}$ values traceable to international reference materials.

5.3 Consumables
Helium for analysis (CAS 07440-59-7)

## 6. APPARATUS

6.1 Isotope ratio mass spectrometry (IRMS)
The Isotope ratio mass spectrometer (IRMS) enables the determination of the relative contents of $^{18}O$ of $CO_2$ gas naturally occurring with an internal accuracy of 0.05‰. Internal accuracy here is defined as the difference between 2 measurements of the same sample of $CO_2$.
The mass spectrometer used for the determination of the isotopic composition of $CO_2$ gas is generally equipped with a triple collector to simultaneously measure the following ion currents:

- m/z = 44 ($^{12}C^{16}O^{16}O$)
- m/z = 45 ($^{13}C^{16}O^{16}O$ and $^{12}C^{17}O^{16}O$)
- m/z = 46 ($^{12}C^{16}O^{18}O$, $^{12}C^{17}O^{17}O$ and $^{13}C^{17}O^{16}O$)

By measuring the corresponding intensities, the $^{18}O/^{16}O$ isotopic ratio is determined from the ratio of intensities of m/z = 46 and m/z = 44 after corrections for isobaric species ($^{12}C^{17}O^{17}O$ and $^{13}C^{17}O^{16}O$) whose contributions can be calculated from the actual intensity observed for m/z= 45 and the usual isotopic abundances for $^{13}C$ and $^{17}O$ in Nature.

The isotope ratio mass spectrometry must either be equipped with:
- a double introduction system (dual inlet system) to alternately measure the unknown sample and a reference standard.
- or a continuous flow system that transfers quantitatively the $CO_2$ from the sample vials after equilibration but also the $CO_2$ standard gas into the mass spectrometer.

6.2 Equipment and Materials
All equipments and materials used must meet stated requirements of the used method / apparatus (as specified by the manufacturer). However, all equipments and materials can be replaced by items with similar performance.
6.2.1 Vials with septa appropriate for the used system
6.2.2 Volumetric pipettes with appropriate tips
6.2.3 Temperature controlled system to carry out the equilibration at constant temperature, typically within ±1 °C
6.2.4 Vacuum pump (if needed for the used system)
6.2.5 Autosampler (if needed for the used system)
6.2.6 Syringes for sampling (if needed for the used system)
6.2.7 GC Column to separate $CO_2$ from other elementary gases (if needed for the used system)
6.2.8 Water removal device (e.g. cryo-trap, selective permeable membranes)

## 7. SAMPLING

Wine and must samples as well as reference materials are used for analysis without any pre-treatment. In the case of the possible fermentation of the sample, benzoic acid (or another anti-fermentation product) should be added or filtered with a with a 0,22 µm pore diameter filter.

Preferably, the reference materials used for calibration and drift-correction should be placed at the beginning and at the end of the sequence and inserted after every ten samples.

## 8. PROCEDURE

The descriptions that follow refer to procedures generally used for the determination of the $^{18}O/^{16}O$ isotopic ratios by means of equilibration of water with a $CO_2$ working standard and the subsequent measurement by IRMS. These procedures can be altered according to changes of equipment and instrumentation provided by the manufacturers as various kind of equilibration devices are available, implying various conditions of operation. Two main technical procedures can be used for introduction of $CO_2$ into the IRMS either through a dual inlet system or using a continuous flow system. The description of all these technical systems and of the corresponding conditions of operation is not possible.

Note: all values given for volumes, temperatures, pressures and time periods are only indicative. Appropriate values must be obtained from specifications provided by the manufacturer and/or determined experimentally.

8.1 Manual equilibration

A defined volume of the sample/standard is transferred into a flask using a pipette. The flask is then attached tightly to the manifold.
Each manifold is cooled down to below – 80 °C to deep-freeze the samples (manifold equipped with capillary opening tubes do not require this freezing step). Subsequently, the whole system is evacuated. After reaching a stable vacuum the gaseous $CO_2$ working standard is allowed to expand into the various flasks. For the equilibration process each manifold is placed in a temperature controlled water-bath typically at 25°C (± 1 °C) for 12 hours (overnight). It is crucial that the temperature of the water-bath is kept constant and homogeneous.
After the equilibration process is completed, the resulting $CO_2$ is transferred from the flasks to the sample side bellow of the dual inlet system. The measurements are performed by comparing several times the ratios of the $CO_2$ contained in the sample side and the standard side ($CO_2$ reference standard gas) of the dual inlet. This approach is repeated till the last sample of the sequence has been measured.

8.2 Use of an automatic equilibration apparatus

A defined volume of the sample/standard is transferred into a vial using a pipette. The sample vials are attached to the equilibration system and cooled down to below – 80 °C to deep-freeze the samples (systems equipped with capillary opening tubes do not require this freezing step). Subsequently, the whole system is evacuated.
After reaching a stable vacuum the gaseous $CO_2$ working standard is expanded into the vials. Equilibrium is reached at a temperature of typically 22 ± 1 °C after a minimum period of 5 hours and with moderate agitation (if available). Since the equilibration duration depends on various parameters (e.g. the vial geometry, temperature, applied agitation ...), the minimum equilibrium time should be determined experimentally.
After the equilibration process is completed, the resulting $CO_2$ is transferred from the vials to the sample side bellow of the dual inlet system. The measurements are performed by comparing several times the ratios of the $CO_2$ contained in the sample side and the standard side ($CO_2$ reference standard gas) of the dual inlet. This approach is repeated till the last sample of the sequence has been measured.

8.3 Manual preparation manual and automatic equilibration and analysis with a dual inlet IRMS

A defined volume of sample / standard (eg. 200 µL) is introduced into a vial using a pipette. The open vials are then placed in a closed chamber filled with the $CO_2$ used for equilibration (5.2.2). After several purges to eliminate any trace of air, the vials are closed and then placed on the thermostated plate of the sample changer. The equilibration is reached after at least 8 hours at 40 °C. Once the process of equilibration completed, the $CO_2$ obtained is dried and then transferred into the sample side of the dual inlet introduction system. The measurements are performed by comparing several times the ratios of the $CO_2$ contained in the sample side and the standard side ($CO_2$ reference standard gas) of the dual inlet. This approach is repeated till the last sample of the sequence has been measured.

8.4 Use of an automatic equilibration apparatus coupled to a continuous flow system

A defined volume of the sample/standard is transferred into a vial using a pipette. The sample vials are placed into a temperature controlled tray.
Using a gas syringe the vials are flushed with mixture of He and $CO_2$. The $CO_2$ remains in the headspace of the vials for equilibration.
Equilibrium is reached at a temperature typically of 30 ± 1 °C after a minimum period of 18 hours.
After the equilibration process is completed the resulting $CO_2$ is transferred by means of the continuous flow system into the ion source of the mass spectrometer. $CO_2$ reference gas is also introduced into the IRMS by means of the continuous flow system. The measurement is carried out according to a specific protocol for each kind of equipment.

## 9. CALCULATION

The intensities for m/z = 44, 45, 46 are recorded for each sample and reference materials analysed in a batch of measurements. The $^{18}O/^{16}O$ isotope ratios are then calculated by the computer and the software of the IRMS instrument according to the principles explained in section 6.1. In practice the $^{18}O/^{16}O$ isotope ratios are measured against a working standard previously calibrated against the V-SMOW. Small variations may occur while measuring on line due to changes in the

instrumental conditions. In such a case the $\delta^{18}O$ of the samples must be corrected according to the difference in the $\delta^{18}O$ value from the working standard and its assigned value, which was calibrated beforehand against V-SMOW. Between two

measurements of the working standard, the variation is the correction applied to the sample results that may be assumed to be linear. Indeed, the working standard must be measured at the beginning and at the end of all sample series. Therefore a correction can be calculated for each sample using linear interpolation between two values (the difference between the assigned value of the working standard and the measurements of the obtained values).
The final results are presented as relative $\delta^{18}O_{V\text{-}SMOW}$ values expressed in ‰.
$\delta^{18}O_{V\text{-}SMOW}$ values are calculated using the following equation:

$$\delta^{18}O_{V-SMOW} = \left[ \frac{\left(\frac{^{18}O}{^{16}O}\right)_{sample} - \left(\frac{^{18}O}{^{16}O}\right)_{V-SMOW}}{\left(\frac{^{18}O}{^{16}O}\right)_{V-SMOW}} \right] \times 1000 \ [‰]$$

The $\delta^{18}O$ value normalized versus the V-SMOW/SLAP scale is calculated using the following equation:

$$\delta^{18}O_{V-SMOW/SLAP} = \left[ \frac{\delta^{18}O_{sample} - \delta^{18}O_{V-SMOW}}{\delta^{18}O_{V-SMOW} - \delta^{18}O_{SLAP}} \right] \times 55.5 \ [‰]$$

The $\delta^{18}O_{V\text{-}SMOW}$ value accepted for SLAP is -55.5‰ (see also 5.1).

## 10. PRECISION

The repeatability (r) is equal to 0.24 ‰.
The reproducibility (R) is equal to 0.50 ‰.

**Summary of statistical results**

| | General average (‰) | | Standard deviation of repeatability (‰) $s_r$ | | Repeatability (‰) r | | Standard deviation of reproducibility (‰) $s_R$ | | Reproducibility (‰) R |
|---|---|---|---|---|---|---|---|---|---|
| **Water** | | | | | | | | | |
| | | | | | | | | | |
| Sample 1 | -8.20 | | 0.068 | | 0.19 | | 0.171 | | 0.48 |
| Sample 2 | -8.22 | | 0.096 | | 0.27 | | 0.136 | | 0.38 |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| **Wine N° 1** | | | | | | | | | |
| | | | | | | | | | |
| Sample 5 | 6.87 | | 0.098 | | 0.27 | | 0.220 | | 0.62 |
| Sample 8 | 6.02 | | 0.074 | | 0.21 | | 0.167 | | 0.47 |
| Sample 9 | 5.19 | | 0.094 | | 0.26 | | 0.194 | | 0.54 |
| Sample 4 | 3.59 | | 0.106 | | 0.30 | | 0.205 | | 0.57 |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| **Wine N° 2** | | | | | | | | | |
| | | | | | | | | | |
| Sample 3 | -1.54 | | 0.065 | | 0.18 | | 0.165 | | 0.46 |
| Sample 6 | -1.79 | | 0.078 | | 0.22 | | 0.141 | | 0.40 |
| Sample 7 | -2.04 | | 0.089 | | 0.25 | | 0.173 | | 0.49 |
| Sample 10 | -2.61 | | 0.103 | | 0.29 | | 0.200 | | 0.56 |

## 11. INTER-LABORATORIES STUDIES

Bulletin de l'O.I.V. janvier-février 1997, 791-792, p.53 - 65.

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**Method OIV-MA-AS311-01A** Type IV method

# Reducing substances

(Resolution Oeno377/2009)

## 1. Definition

Reducing substances comprise all the sugars exhibiting ketonic and aldehydic functions and are determined by their reducing action on an alkaline solution of a copper salt.

## 2. Principle of the method

*Clarification*

The wine is treated with one of the following reagents:
- neutral lead acetate,
- zinc ferrocyanide (II).

## 3. Clarification

The sugar content of the liquid in which sugar is to be determined must lie between 0.5 and 5 g/L.
Dry wines should not be diluted during clarification; sweet wines should be diluted during clarification in order to bring the sugar level to within the limits prescribed in the following table.

| Description | Sugar content (g/L) | Density | Dilution (%) |
|---|---|---|---|
| Musts and mistelles | > 125 | > 1.038 | 1 |
| Sweet wines, whether fortified or not | 25 to 125 | 1.005 to 1.038 | 4 |
| Semi-sweet wines | 5 to 25 | 0.997 to 1.005 | 20 |
| Dry wines | < 5 | < 0.997 | No dilution |

### 3.1. Clarification by neutral lead acetate.

3.1.1. Reagents

- Neutral lead acetate solution (approximately saturated)
  Neutral lead acetate, $Pb(CH_3COO)_2 \cdot 3H_2O$ ..... 250 g
  Very hot water to ..... 500 mL
  Stir until dissolved.
- Sodium hydroxide solution, 1 M
- Calcium carbonate.

3.1.2 Procedure

- Dry wines.

Place 50 mL of the wine in a 100 mL volumetric flask; add 0.5 (n - 0.5) mL sodium hydroxide solution, 1 M, (where n is the volume of sodium hydroxide solution, 0.1 M, used to determine the total acidity in 10 mL of wine). Add, while stirring, 2.5 mL of saturated lead acetate solution and 0.5 g calcium carbonate. Shake several times and allow to stand for at least 15 minutes. Make up to the mark with water. Filter.
1 mL of the filtrate corresponds to 0.5 mL of the wine.

- Musts, mistelles, sweet and semi-sweet wines

Into a 100 mL volumetric flask, place the following volumes of wine (or must or mistelle), the dilutions being given for guidance:

Case 1 - Musts and mistelles: prepare a 10% (*v/v*) solution of the liquid to be analyzed and take 10 mL of the diluted sample.

Case 2 - Sweet wines, whether fortified or not, having a density between 1.005 and 1.038: prepare a 20% (*v/v*) solution of the liquid to be analyzed and take 20 mL of the diluted sample.

Case 3 - Semi-sweet wines having a density between 0.997 and 1.005: take 20 mL of the undiluted wine.
Add 0.5 g calcium carbonate, about 60 mL water and 0.5, 1 or 2 mL of saturated lead acetate solution. Stir and leave to stand for at least 15 minutes, stirring occasionally. Make up to the mark with water. Filter.

*Note:*
Case 1: 1 mL of filtrate contains 0.01 mL of must or mistelle.
Case 2: 1 mL of filtrate contains 0.04 mL of sweet wine.
Case 3: 1 mL of filtrate contains 0.20 mL of semi-sweet wine.

**3.2. Clarification by zinc ferrocyanide (II)**

This clarification process should be used only for white wines, lightly colored sweet wines and musts.

3.2.1 Reagents

Solution I: potassium ferrocyanide (II):

| | |
|---|---|
| Potassium ferrocyanide (II), $K_4Fe(CN)_6 \cdot 3H_2O$ ..... | 150 g |
| Water to ..... | 1000 mL |

Solution II: zinc sulfate:

| | |
|---|---|
| Zinc sulfate, $ZnSO_4 \cdot 7H_4O$ ..... | 300 g |
| Water to ..... | 1000 mL |

3.2.2 Procedure

Into a 100 mL volumetric flask, place the following volumes of wine (or must or mistelle), the dilutions being given for guidance:

Case 1 - Musts and mistelles. Prepare a 10% *(v/v)* solution of the liquid to be analyzed and take 10 mL of the diluted sample.

Case 2 - Sweet wines, whether fortified or not, having a density between 1.005 and 1.038: prepare a 20% *(v/v)* solution of the liquid to be analyzed and take 20 mL of the diluted sample.

Case 3 - Semi-sweet wines having a density at 20°C between 0.997 and 1.005: take 20 mL of the undiluted wine.

Case 4 - Dry wines: take 50 mL of undiluted wine.

Add 5 mL of solution I and 5 mL of solution II. Stir. Make up to the mark with water. Filter.

*Note:*

Case 1: 1 mL of filtrate contains 0.01 mL of must or mistelle.

Case 2: 1 mL of filtrate contains 0.04 mL of sweet wine.

Case 3: 1 mL of filtrate contains 0.20 mL of semi-sweet wine.

Case 4: 1 mL of filtrate contains 0.50 mL of dry wine.

## 4. Determination of sugars

4.1. *Reagents*

- Alkaline copper salt solution:
  - Copper sulfate, pure, $CuSO_4 \cdot 5H_2O$ ..... 25 g
  - Citric acid monohydrate ..... 50 g
  - Crystalline sodium carbonate, $Na_2CO_3 \cdot 10H_2O$ ..... 388 g
  - Water to ..... 1000 mL

  Dissolve the copper sulfate in 100 mL of water, the citric acid in 300 mL of water and the sodium carbonate in 300 to 400 mL of hot water. Mix the citric acid and sodium carbonate solutions. Add the copper sulfate solution and make up to one liter.
- Potassium iodide solution, 30% (*m/v*):
  - Potassium iodide, KI ..... 30 g
  - Water to ..... 100 mL

  Store in a colored glass bottle.
- Sulfuric acid, 25% (*m/v*):

Concentrated sulfuric acid, $H_2SO_4$, $\rho_{20}$ = 1.84 g/Ml …….. 25 g
Water to ……. 100 mL

Add the acid slowly to the water, allow to cool and make up to 100 mL with water.

- Starch solution, 5 g/L:

  Mix 5 g of starch in with about 500 mL of water. Bring to boil, stirring all the time, and boil for 10 minutes. Add 200 g of sodium chloride, NaCl. Allow to cool and then make up to one liter with water.

- Sodium thiosulfate solution, 0.1 M.

- Invert sugar solution, 5 g/L, to be used for checking the method of determination.

  Place the following into a 200 mL volumetric flask:

  Pure dry sucrose ……. 4.75 g
  Water, approximately ……. 100 mL
  Conc. hydrochloric acid ($\rho_{20}$= 1.16 – 1.19 g/mL) ……. 5 mL

  Heat the flask in a water-bath maintained at 60°C until the temperature of the solution reaches 50°C; then keep the flask and solution at 50°C for 15 minutes. Allow the flask to cool naturally for 30 minutes and then immerse it in a cold water-bath. Transfer the solution to a one-liter volumetric flask and make up to one liter. This solution keeps satisfactorily for a month. Immediately before use, neutralize the test sample (the solution being approximately 0.06 M acid) with sodium hydroxide solution.

4.2. *Procedure*

Mix 25 mL of the alkaline copper salt solution, 15 mL water and 10 mL of the clarified solution in a 300 mL conical flask. This volume of sugar solution must not contain more than 60 mg of invert sugar.

Add a few small pieces of pumice stone. Fit a reflux condenser to the flask and bring the mixture to the boil within two minutes. Keep the mixture boiling for exactly 10 minutes.

Cool the flask immediately in cold running water. When completely cool, add 10 mL potassium iodide solution, 30% (*m/v*); 25 mL sulfuric acid, 25% (*m/v*), and 2 mL starch solution.

Titrate with sodium thiosulfate solution, 0.1 M. Let *n* be the number of mL used. Also carry out a blank titration in which the 25 mL of sugar solution is replaced by 25 mL of distilled water. Let *n'* be the number of mL of sodium thiosulfate used.

4.3. *Expression of results*

4.3.1 Calculations

The quantity of sugar, expressed as invert sugar, contained in the test sample is given in the table below as a function of the number ($n'$ - $n$) of mL of sodium thiosulfate used.

The sugar content of the wine is to be expressed in grams of invert sugar per liter to one decimal place, account being taken of the dilution made during clarification and of the volume of the test sample.

Table giving the relationship between the volume of sodium thiosulfate solution: ($n$'-$n$) mL, and the quantity of reducing sugar in mg.

| $Na_2S_2O_3$ (ml 0.1 M) | Reducing sugars (mg) | Diff. | $Na_2S_2O_3$ (ml 0.1 M) | Reducing sugars (mg) | Diff. |
|---|---|---|---|---|---|
| 1 | 2.4 | 2.4 | 13 | 33.0 | 2.7 |
| 2 | 4.8 | 2.4 | 14 | 35.7 | 2.8 |
| 3 | 7.2 | 2.5 | 15 | 38.5 | 2.8 |
| 4 | 9.7 | 2.5 | 16 | 41.3 | 2.9 |
| 5 | 12.2 | 2.5 | 17 | 44.2 | 2.9 |
| 6 | 14.7 | 2.6 | 18 | 47.2 | 2.9 |
| 7 | 17.2 | 2.6 | 19 | 50.0 | 3.0 |
| 8 | 19.8 | 2.6 | 20 | 53.0 | 3.0 |
| 9 | 22.4 | 2.6 | 21 | 56.0 | 3.1 |
| 10 | 25.0 | 2.6 | 22 | 59.1 | 3.1 |
| 11 | 27.6 | 2.7 | 23 | 62.2 | |
| 12 | 30.3 | 2.7 | | | |

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**Method OIV-AS311-01B**

# Reducing sugars

(Resolution Oeno 377/2009)

## Principle of the method

*Clarification*

After neutralization and removal of alcohol, the wine is passed through an anion-exchange resin column in the acetate form, followed by clarification with neutral lead acetate.

WITHDRAWN

**Method OIV-AS311-01C** Type II method

# Reducing sugars

(Resolution Oeno 377/2009)

## Principle of the method

*Determination*

Single method: the clarified wine or must is reacted with a specific quantity of an alkaline copper salt solution and the excess copper ions are then determined iodometrically.

WITHDRAWN

**Method OIV-MA-AS311-02** Type II method

# Glucose and fructose

(Resolution Oeno 377/2009)

## 1. Definition

Glucose and fructose may be determined individually by an enzymatic method, with the sole aim of calculating the glucose/fructose ratio.

## 2. Principle

Glucose and fructose are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalyzed by hexokinase (HK), to produce glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P):

$$\text{glucose} + \text{ATP} \xleftrightarrow{\text{HK}} \text{G6P} + \text{ADP}$$

$$\text{fructose} + \text{ATP} \xleftrightarrow{\text{HK}} \text{F6P} + \text{ADP}$$

The glucose-6-phosphate is first oxidized to gluconate-6-phosphate by nicotinamide adenine dinucleotide phosphate (NADP) in the presence of the enzyme glucose-6-phosphate dehydrogenase (G6PDH). The quantity of reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced corresponds to that of glucose-6-phosphate and thus to that of glucose.

$$\text{G6P} + \text{NADP}^+ \xleftrightarrow{\text{G6PDH}} \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

The reduced nicotinamide adenine dinucleotide phosphate is determined from its absorption at 340 nm.

At the end of this reaction, the fructose-6-phosphate is converted into glucose-6-phosphate by the action of phosphoglucose isomerase (PGI):

$$\text{F6P} \xleftrightarrow{\text{PGI}} \text{G6P}$$

The glucose-6-phosphate again reacts with the nicotinamide adenine dinucleotide phosphate to give gluconate-6-phosphate and reduced nicotinamide adenine dinucleotide phosphate, and the latter is then determined.

**3. Apparatus**

- A spectrophotometer enabling measurements to be made at 340 nm, the wavelength at which absorption by NADPH is at a maximum. Absolute measurements are involved (i.e. calibration plots are not used but standardization is made using the extinction coefficient of NADPH), so that the wavelength scales of, and absorbance values obtained from, the apparatus must be checked.
  If not available, a spectrophotometer using a source with a discontinuous spectrum that enables measurements to be made at 334 nm or at 365 nm may be used.
- Glass cells with optical path lengths of 1 cm or single-use cells.
- Pipettes for use with enzymatic test solutions, 0.02, 0.05, 0.1, 0.2 mL.

**4. Reagents**

Solution 1: buffer solution (0.3 M triethanolamine, pH 7.6, 0.004 M $Mg^{2+}$): dissolve 11.2 g triethanolamine hydrochloride, $(CH_2CH_2OH)_3N.HCl$, and 0.2 g magnesium sulfate, $MgSO_4.7H_2O$, in 150 mL of double-distilled water, add about 4 mL 5 M sodium hydroxide solution to obtain a pH value of 7.6 and make up to 200 mL.
This buffer solution may be kept for four weeks at approx. + 4°C.

Solution 2: nicotinamide adenine dinucleotide phosphate solution (about 0.0115 M): dissolve 50 mg disodium nicotinamide adenine dinucleotide phosphate in 5 mL of double-distilled water.
This solution may be kept for four weeks at approx. +4°C.

Solution 3: adenosine-5'-triphosphate solution (approx. 0.081 M): dissolve 250 mg disodium adenosine-5'-triphosphate and 250 mg sodium hydrogen carbonate, NaHCO3, in 5 mL of double-distilled water.
This solution may be kept for four weeks at approx. +4°C.

Solution 4: hexokinase/glucose-6-phosphate-dehydrogenase: mix 0.5 mL hexokinase (2 mg of protein/mL or 280 U/mL with 0.5 mL glucose-6-phosphate-dehydrogenase (1 mg of protein/mL).
This mixture may be kept for a year at approx. +4°C.

Solution 5: phosphoglucose-isomerase (2 mg of protein/mL or 700 U/mL). The suspension is used undiluted.
This may be kept for a year at approx. +4°C.

*Note:* All solutions used above are available commercially.

## 5. Procedure

5.1. *Preparation of sample*

Depending on the estimated amount of glucose + fructose per liter (g/L) dilute the sample as follows:

| Measurement at 340 and 344 nm (g/L) | Measurement at 365 nm (g/L) | Dilution with water | Dilution factor F |
|---|---|---|---|
| up to 0.4 | 0.8 | - | - |
| up to 4.0 | 8.0 | 1 + 9 | 10 |
| up to 10.0 | 20.0 | 1 + 24 | 25 |
| up to 20.0 | 40.0 | 1 + 49 | 50 |
| up to 40.0 | 80.0 | 1 + 99 | 100 |
| above 40.0 | 80.0 | 1 + 999 | 1000 |

5.2. *Determination*

With the spectrophotometer adjusted to the 340 nm wavelength, make measurements using air (no cell in the optical path) or water as reference.

Temperature between 20 and 25°C.

Into two cells with 1 cm optical paths, place the following:

| | Reference cell | Sample cell |
|---|---|---|
| Solution 1 (taken to 20°C) ..…… .....….. | 2.50 mL | 2.50 mL |
| Solution 2 .....…… | 0.10 mL | 0.10 mL |
| Solution 3 .....…… | 0.10 mL | 0.10 mL |
| Sample to be measured ..... .....…… | | 0.20 mL |
| Double -distilled water..... | | 0.20 mL |

Mix, and after three minutes read the absorbance of the solutions ($A_1$). Start the reaction by adding:

| | | |
|---|---|---|
| Solution 4 ..... | 0.02 mL | 0.02 mL |

Mix, read the absorbance after 15 minutes and after two more minutes check that the reaction has stopped ($A_2$).

Add immediately:

| | | |
|---|---|---|
| Solution 5 ..... | 0.02 mL | 0.02 mL |

Mix; read the absorbance after 10 minutes and after two more minutes check that the reaction has stopped ($A_3$).

Calculate the differences in the absorbance between the reference cell and sample cells.:

$A_2 - A_1$ corresponds to glucose,
$A_3 - A_2$ corresponds to fructose,

Calculate the differences in absorbance for the reference cells ($\Delta A_T$) and the sample cell ($\Delta A_D$) and then obtain:

for glucose: $\Delta A_G = \Delta A_D - \Delta A_T$
for fructose: $\Delta A_F = \Delta A_D - \Delta A_T$

*Note:* The time needed for the completion of enzyme activity may vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch.

5.3. *Expression of results*

5.3.1 Calculation

The general formula for calculating the concentrations is:

$$C = \frac{V \times MV}{\varepsilon \times d \times v \times 10000} \Delta A \text{ (g/L}$$

where:

V = volume of the test solution (mL)
*v* = volume of the sample (mL)
MW = molecular mass of the substance to be determined
*d* = optical path in the cell (cm)
ε = absorption coefficient of NADPH at 340 nm = 6.3 ($mmole^{-1} \times l \times cm^{-1}$)
V = 2.92 mL for the determination of glucose
V = 2.94 mL for the determination of fructose
*v* = 20 mL
PM = 180
*d* = 1

so that:
For glucose : $C(g/L) = 0.417 \times \Delta A_G$
For fructose: $C(g/L) = 0.420 \times \Delta A_F$

If the sample was diluted during its preparation, multiply the result by the dilution factor F.

*Note:* If the measurements are made at 334 or 365 nm, then the following expressions are obtained:

- measurement at 334 nm: $\varepsilon = 6.2$ (mmole $^{-1} \times$ absorbance $\times$ cm$^{-1}$)
  for glucose : $C(g/L) = 0.425 \times \Delta A_G$
  for fructose: $C(g/L) = 0.428 \times \Delta A_F$

- measurement at 365 nm: $\varepsilon = 3.4$ (mmole$^{-1} \times$ absorbance $\times$ cm$^{-1}$)
  for glucose: $C(g/L) = 0.773 \times \Delta A_G$
  for fructose: $C(g/L) = 0.778 \times \Delta A_F$

5.3.2 Repeatability (*r*):
$r = 0.056\ x_i$
$x_i$ = the concentration of glucose or fructose in g/L

5.3.3 Reproducibility (R):
$R = 0.12 + 0.076\ x_i$
$x_i$ = the concentration of glucose or fructose in g/L

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**Method OIV-MA-AS311-03** Type II method[1]

# Dosage of sugars in wine by HPLC

(Resolution Oeno 526/2016)

## 1. SCOPE OF APPLICATION

This method is applicable to the direct quantification of sugars in musts and wines up to 20 g/L and, after dilution, beyond.
Glycerol (between 0.5 and 15 g/L) and sucrose (between 1 and 40 g/L) may also be quantified in the same way.

## 2. PRINCIPLE

Sugars and glycerol are separated by HPLC using an alkylamine column and detected by refractometer.

## 3. REAGENTS

3.1 - Demineralised Type I water (ISO 3696) or equivalent (HPLC grade);
3.2 - acetonitrile [75-05-8] (minimal transmission at 200 nm - purity ≥ 99%);
3.3 - fructose [57-48-7] (purity ≥ 99%);
3.4 - glucose [492-62-6] (purity ≥99%);
3.5 - sucrose [57-50-1] (purity ≥ 99%);
3.6 - glycerol [56-81-5] (purity ≥99%).

## PREPARATION OF REAGENT SOLUTIONS

3.9 - Demineralised water (3.1): filtered through a 0.45 µm cellulose membrane;
3.10 - eluent: acetonitrile (3.2)/water (3.9) with a respective ratio of 80/20.
*Note 2: the water/acetonitrile ratio may be adapted according to the objectives.*

## 4. APPARATUS

4.1. - 0.45 µm Cellulose filtration membrane;
4.2. - silica-based, octadecyl-bonded filter cartridge (e.g. Sep-Pak $C_{18}$);
4.3. - common apparatus for high-performance liquid chromatography;
4.4. - alkylamine column (5 µm, 250 x 4.6 mm);
*Note 3: columns of different lengths, internal diameter and particle size may be used but the type II method refers to the dimensions provided.*
4.5. - refractometric index detector (RID);
4.6. - common laboratory apparatus.

[1] Type II for glucose and fructose. Type IV for sucrose and glycerol.

## 5. SAMPLING

The samples are degassed beforehand if necessary (e.g. with nitrogen or helium, or in an ultrasonic bath).

## 6. PROCEDURE

6.1 - Preparation of the sample

6.1.1 - Dilution

Wines containing less than 20 g/L of (glucose + fructose) are analysed undiluted. Musts and wines containing more than 20 g/L have to be diluted to be within the range of calibration.

6.1.2 - Filtration

The samples must be filtered using a 0.45 µm membrane (4.1) before analysis.

6.1.3 - Elimination of phenolic compounds (if necessary)

For a must or wine, pass over a $C_{18}$ cartridge (4.2).

6.2 - Analyses

6.2.1 - Analytical conditions

*Note 4: The following instructions are mandatory for the type II method.*

*Note 5: Conditions may be adapted by the laboratory with the loss of the type II reference.*

HPLC system (4.3) equipped with column (4.4) and RID (4.5).

Mobile phase: isocratic acetonitrile/water eluent (3.10).

Flow: 1 mL/min.

Injected volume: between 10 and 50 µL, to be adapted according to the material used.

Examples of chromatograms are shown in Annex B (Figures 1 and 2).

The fructose-glucose resolution is recommended to be $\geq 2$.

6.2.2 - External calibration

The calibration solution that applies to all compounds described in this procedure may contain the following:

10 g/L glycerol (3.6) ± 0.01 g/L,

10 g/L fructose (3.3) ± 0.01 g/L,

10 g/L glucose (3.4) ± 0.01 g/L,

10 g/L sucrose (3.5) ± 0.01 g/L.

*Note 6: if quantifying only one of these compounds, a solution that contains only the one required can be prepared.*

6.3 - Calculation of response factors for external calibration used in routine analyses

$RF_i = area_i / C_i$

where

*area i* = peak area of the product in the calibration solution

and $C_i$ = quantity of product present in the calibration solution.

It is also possible to use a calibration curve.

## 7. EXPRESSION OF RESULTS

7.1 - Calculation of concentrations

$C_e = area_e / RF_i$

where

$area_e$ = peak area of product present in the sample.

The results are expressed in g/L.

*Note 7: the results are indicated to a maximum of one decimal place.*

## 8. QUALITY ASSURANCE AND CONTROL

Traceable to the international references through mass, volume and temperature. Synthetic mixtures or samples coming, for instance, from proficiency ring test are used as internal quality control. A control chart may be used

## 9. PERFORMANCE OF THE METHOD

No known compound co-elutes with fructose, glucose or sucrose.

Robustness: the analysis is sensitive to slight variations in temperature. Columns should be protected from temperature variations.

## 10. PRECISION

(See Annex B.3)

10.1 - Glucose (content ≥ 3 g/L)

Repeatability limit ≅ reproducibility limit = 13%

10.2 - Fructose (content ≥ 2 g/L)

Repeatability limit = 7%

Reproducibility limit = 10%

10.3 – Glucose + fructose (content ≥ 5 g/L)

Repeatability limit ≅ Reproducibility limit = 10%

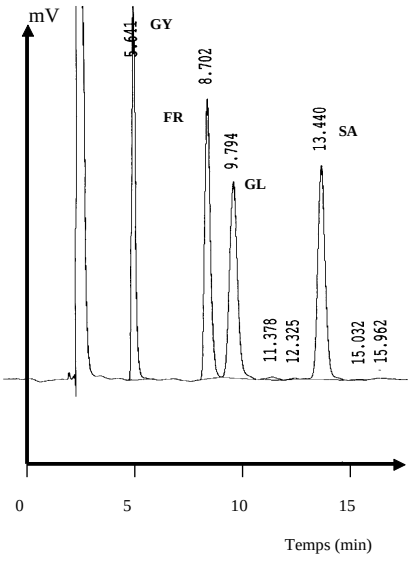



**Figure 1**
**Chromatogram of a calibration solution (sugars and glycerol at 10 g/l.)**

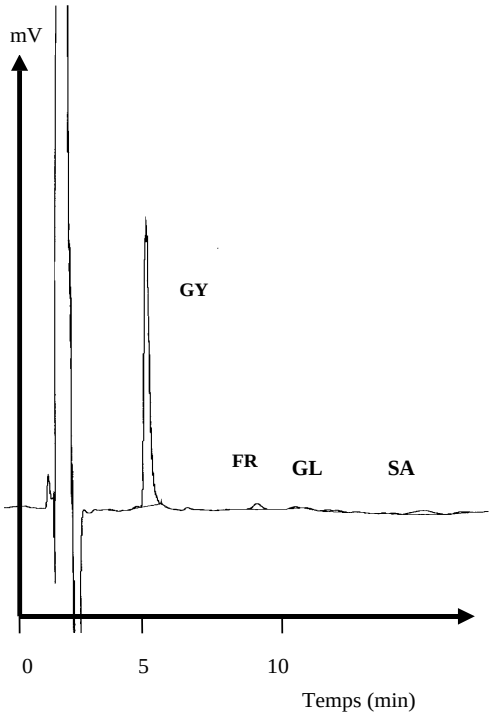
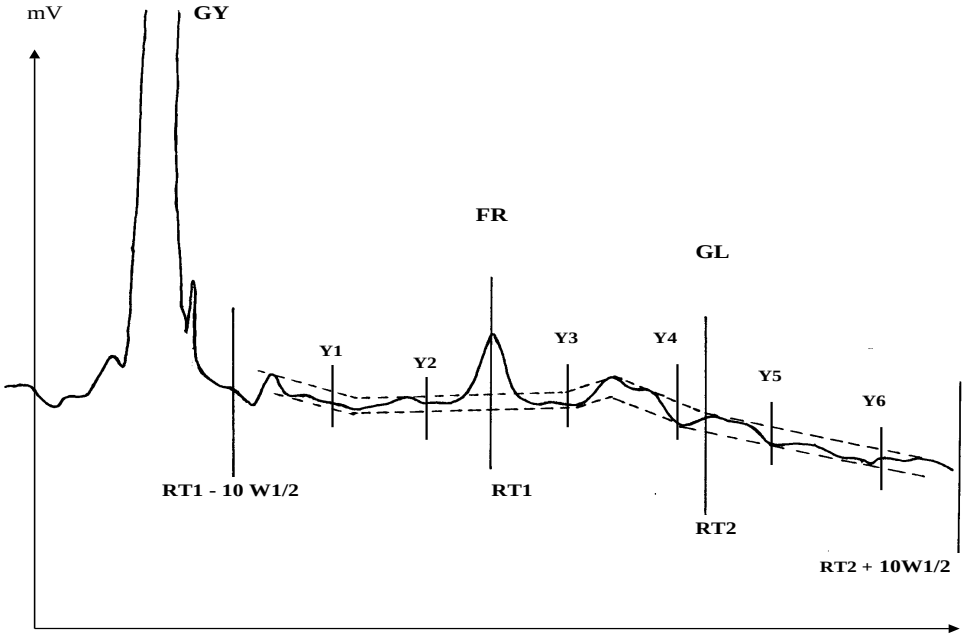



**Figure 2**
**Chromatogram of a rosé wine**

Glycerol (GY), fructose (FR), glucose (GL), saccharose (SA)




fructose (FR), glucose (GL), saccharose (SA) Glycerol (GY),

**Figure 3 - Measure of pitches of noise after enlargement of chromatogram**

RT1: retention time of fructose; RT2: retention time of glucose
W1/2: width of peak at mid-height; Yi: pitch of noise at point i

## Annex B

(informative)

## Precision data

### B.1 - Samples in the interlaboratory test trial

This study was carried out by the Interregional Laboratory of the Répression de Fraudes in Bordeaux. The test trial involved 6 samples in blind duplicates (12 samples in total), identified as A to J (4 white wines and 4 red wines; 2 white Port wines and 2 red Port wines), containing glucose and fructose and whose content of each sugar was between 2 and 65 g/L. The wines from the region of Bordeaux were supplemented with glucose and fructose and stabilised with 100 mg/L of $SO_2$ (TRICARD and MEDINA, 2003).

### B.2 - Chromatographic conditions

Considering the response factors of these two sugars and the scales of the chromatograms, the noise corresponds to a concentration of 0.04 g/L for fructose and of 0.06 g/L for glucose (see Figure A3).
The limits of detection (3 times the noise) and of quantification (10 times the noise) are then obtained:

$LD_{fructose} = 0.12$ g/L,
$LD_{glucose} = 0.18$ g/L,
$LQ_{fructose} = 0.4$ g/L,
$LQ_{glucose} = 0.6$ g/L.

These results are compliant with those determined by TUSSEAU and BOUNIOL (1986) and are repeatable on other chromatograms.

### B.3 - Precision

Nine laboratories participated in the interlaboratory study:
Istituto Sperimentale per l'Enologia, Asti, Italy;
Laboratoire de la DGCCRF de Montpellier, France;
Laboratoire LARA, Toulouse, France;
Instituto do vinho do Porto, Porto, Portugal;
Instituto da Vinha e do Vinho, Unhos, Portugal;
Estación de Viticultura y Enología, Vilafranca del Penedés, Spain;
Comité Interprofessionnel du vin de Champagne, Epernay, France;
Station fédérale de Changins, Switzerland;

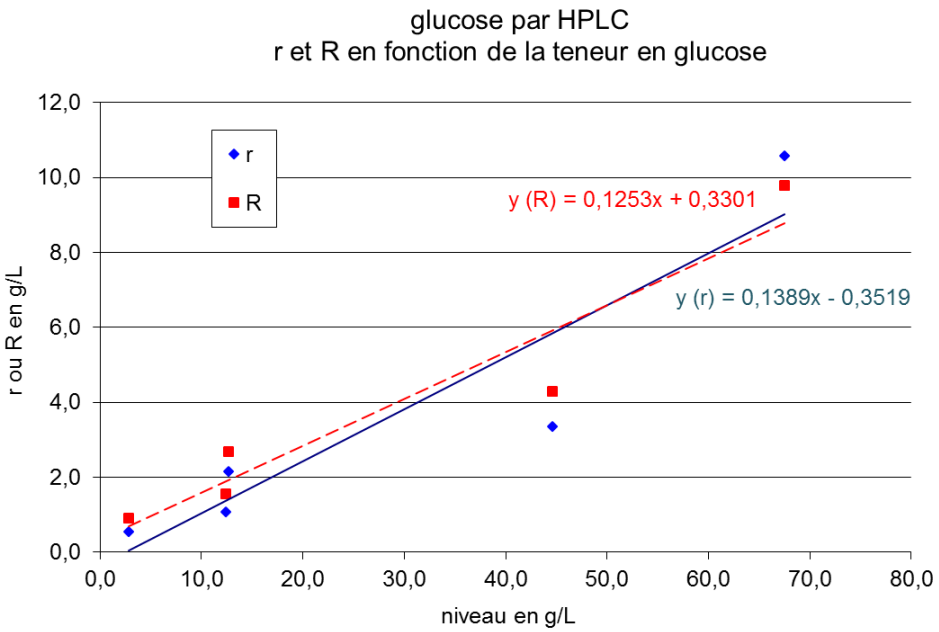
Laboratoire de la DGCCRF de Talence, France.

The analyses of 3 points of the set of calibration solutions and the 12 samples were carried out successively by applying the method of analysis given.
The results were analysed according to the OIV protocol (Validation protocol of methods of analysis – Resolution OENO 6/1999).
This protocol does not require the analyses to be repeated, whereas 4 laboratories gave results of analyses repeated 3 times. A single series was chosen (the first one) for the analysis of the results, in compliance with the OIV protocol.
The calculations of repeatability according to Youden, reproducibility and Cochran and Grubbs tests were performed.
Data on the repetitions made it possible to work out the standard deviations of repeatability in another way (according to ISO 5725).

### B.3.1 – GLUCOSE

| Glucose by HPLC (g/L) | | | | | | |
|---|---|---|---|---|---|---|
| Number of laboratories | 9 | 9 | 9 | 9 | 9 | 9 |
| Number of samples | 2 | 2 | 2 | 2 | 2 | 2 |
| Average value | 2.9 | 2.9 | 12.6 | 12.4 | 44.6 | 67.5 |
| Repeatability standard deviation | 0.44 | 0.17 | 0.67 | 0.34 | 1.05 | 3.31 |
| Repeatability limit | 1.42 | 0.55 | 2.15 | 1.07 | 3.35 | 10.58 |
| Reproducibility standard deviation | 0.78 | 0.30 | 0.90 | 0.52 | 1.43 | 3.28 |
| Reproducibility limit | 2.32 | 0.90 | 2.68 | 1.55 | 4.28 | 9.78 |
| Horrat value | 5.7* | 2.1 | 1.84 | 1.08 | 1.01 | 1.62 |

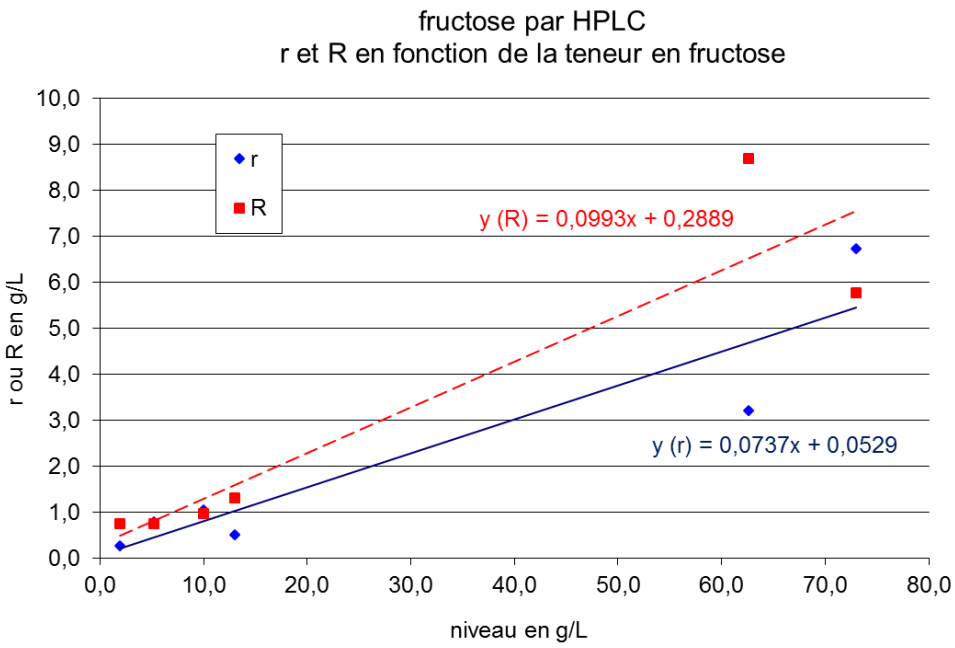
* not taken into account for the expression of precision




Correlation between r and R and the concentration for glucose (ISO 5725)

## B.3.2 – FRUCTOSE

| Fructose by HPLC (g/L) | | | | | | |
|---|---|---|---|---|---|---|
| Number of laboratories | 9 | 9 | 9 | 9 | 9 | 9 |
| Number of samples | 2 | 2 | 2 | 2 | 2 | 2 |
| Average value | 1.9 | 5.2 | 10.0 | 13.0 | 62.6 | 73.0 |
| Repeatability standard deviation | 0.09 | 0.24 | 0.32 | 0.16 | 3.20 | 2.10 |
| Repeatability limit | 0.27 | 0.79 | 1.03 | 0.51 | 3.20 | 6.72 |
| Reproducibility standard deviation | 0.25 | 0.25 | 0.32 | 0.43 | 2.91 | 1.93 |
| Reproducibility limit | 0.75 | 0.75 | 0.96 | 1.30 | 8.68 | 5.77 |
| Horrat value | 2.54 | 1.09 | 0.81 | 0.87 | 1.53 | 0.89 |

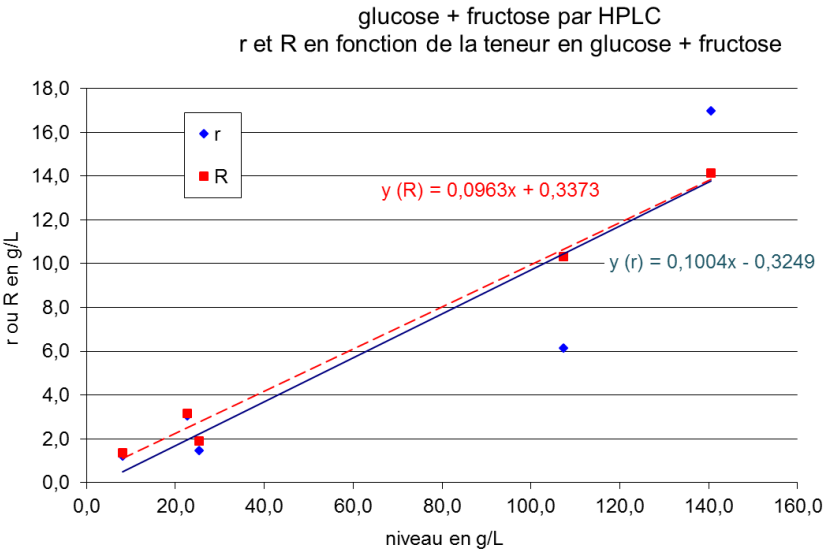



Correlation between r and R and the concentration for fructose (ISO 5725)

### B.3.3 – GLUCOSE + FRUCTOSE

| Glucose + fructose by HPLC (g/L) | | | | | | |
|---|---|---|---|---|---|---|
| Number of laboratories | 9 | 9 | 9 | 9 | 9 | 9 |
| Number of samples | 2 | 2 | 2 | 2 | 2 | 2 |
| Average value | 4.7 | 8.1 | 22.6 | 25.4 | 107.3 | 140.5 |
| Repeatability standard deviation | 0.48 | 0.38 | 1.06 | 0.46 | 1.92 | 5.30 |
| Repeatability limit | 1.52 | 1.21 | 3.07 | 1.48 | 6.13 | 17.0 |
| Reproducibility standard deviation | 0.89 | 0.46 | 1.06 | 0.64 | 3.47 | 4.74 |
| Reproducibility limit | 2.64 | 1.38 | 3.17 | 1.90 | 10.34 | 14.15 |
| Horrat value | 4.17* | 1.39 | 1.33 | 0.72 | 1.15 | 1.26 |

* not taken into account for the expression of precision




Correlation between r and R and the concentration for glucose + fructose (ISO 5725)

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**Method OIV-MA-AS311-04**

# Stabilization of Musts to Detect the Addition of Sucrose

## 1. Principle of the method

The sample is brought to pH 7 with a sodium hydroxide solution and an equal volume of acetone is added.

The acetone is removed by distillation prior to determination of sucrose by TLC (thin-layer chromatography) and HPLC (high-performance liquid chromatography) (see *Sucrose* Chapter).

## 2 Apparatus

Distillation apparatus, with a 100 mL round distillation flask.

## 3 Reagents

3.1 Sodium hydroxide solution, 20% (*m/v*)
3.2 Acetone (propanone).

## 4 Method

4.1 *Stabilizing the samples*

20 mL of must is placed in a 100 mL strong-walled flask and brought to pH 7 with the 20% sodium hydroxide solution (*m/V*) (six to twelve drops). 20 mL of acetone are added. Stopper and store at low temperature.

***WARNING:*** **ACETONE HAS HIGH VAPOUR PRESSURE AND IS HIGHLY INFLAMMABLE.**

4.2 *Preparing the sample* to determine sucrose by TLC or HPLC.

Place the contents of the flask in the 100 mL round flask of the distillation apparatus. Distil and collect approximately 20 mL of distillate, which is discarded. Add 20 mL of water to the contents of the distilling flask and distil again, collecting about 25 mL of distillate, which is discarded.

Transfer the contents of the distillation flask to a graduated 20 mL volumetric flask and make up to the mark with the rinsing water from the round flask. Filter. Analyze the filtrate and (if detected) measure the sucrose using TLC or HPLC.

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**Method OIV-MA-AS311-05** Type II method

# Determination of the deuterium distribution in ethanol derived from fermentation of grape musts, concentrated grape musts, grape sugar (rectified concentrated grape musts) and wines by application of nuclear magnetic resonance (SNIF-NMR/ RMN-FINS [1])

(Oeno 426-2011)

## 1. Introduction

The deuterium contained in the sugars and the water in grape must is redistributed after fermentation in molecules I, II, III and IV of the wine:

| | | | |
|---|---|---|---|
| $CH_2D\ CH_2\ OH$ | $CH_3\ CHD\ OH$ | $CH_3\ CH_2\ OD$ | HOD |
| I | II | III | IV |

## 2. Scope

The method enables measurement of the Deuterium isotope ratios (D/H) in wine ethanol and ethanol obtained by fermentation of products of the vine (musts, concentrated musts, rectified concentrated musts).

## 3. Definitions

$(D/H)_I$ : Isotope ratio associated with molecule I
$(D/H)_{II}$ : Isotope ratio associated with molecule II
$(D/H)^Q_W$ : Isotope ratio of the water in the wine (or in fermented products)
$R = 2(D/H)_{II}/(D/H)_I$

[1] Fractionnement Isotopique Naturel Spécifique étudié par Résonance Magnétique Nucléaire (Site Specific Natural Isotope Fractionation studied by Nuclear Magnetic Resonance). Brevet: France, 8122710; Europe, 824022099; Etats Unis, 854550082; Japon 57123249.

R expresses the relative distribution of deuterium in molecules I and II; R is measured directly from the intensities h (peak heights) of the signals and then $R = 3h_{II}/h_I$.

## 4. Principle

The above defined parameters R, $(D/H)_I$ and $(D/H)_{II}$ are determined by nuclear magnetic resonance of the deuterium in the ethanol extracted from the wine or from the fermentation products of the must, the concentrated must or the grape sugar (rectified concentrated must) obtained under given conditions.

## 5. Reagents and materials

5.1 reagents:

5.1.1 reagents for the determination of water by the Karl Fischer method (when this method is used for the measurement of the alcohol grade of the distillate).

5.1.2 Hexafluorobenzene (C6F6) used as lock substance

5.1.3 Trifluoroacetic acid (TFA, CAS: 76-05-1) or alternatively trifluoroacetic anhydride (TFAA, CAS: 407-25-0)

5.2 Reference Materials (available from the Institute for Reference Materials and Measurements IRMM in Geel (B)):

5.2.1 Sealed NMR tubes CRM-123, used to check the calibration of the NMR instrumentation

5.2.2 Standard N,N-tetramethyl urea (TMU); standard TMU with a calibrated isotope ratio D/H.

5.2.3 Other CRMs available used to check the distillation and preparation steps:

| CRM | | Parameter | Certified value | Uncertainty |
|---|---|---|---|---|
| CRM-656 | Ethanol from wine, 96% vol. | | | |
| | | $t^D$ (ethanol) in % w/w | 94.61 | 0.05 |
| | | $\delta^{13}C$ (ethanol) in ‰ VPDB | -26.91 | 0.07 |
| | | $(D/H)_I$ (ethanol) in ppm | 102.84 | 0.20 |
| | | $(D/H)_{II}$ (ethanol) in ppm | 132.07 | 0.30 |
| | | R (ethanol) | 2.570 | 0.005 |
| CRM-660 | hydro alcoholic solution, 12% vol. | | | |
| | | $t^Q$ (ethanol) in % vol. | 11.96 | 0.06 |
| | | $\delta^{13}C$ (ethanol) in ‰ VPDB | -26.72 | 0.09 |
| | | $(D/H)_I$ (ethanol) in ppm | 102.90 | 0.16 |
| | | $(D/H)_{II}$ (ethanol) in ppm | 131.95 | 0.23 |
| | | R | 2.567 | 0.005 |
| | | (D/H)w (water) in ppm | 148.68 | 0.14 |

5.3 Apparatus

5.3.1 NMR spectrometer fitted with a specific 'deuterium' probe tuned to the characteristic frequency νo of the field Bo (e.g. for Bo = 7.05 T, νo = 46.05 MHz and for Bo = 9.4 T, νo = 61.4 MHz) having a

proton decoupling channel (B2) and field-frequency stabilization channel (lock) at the fluorine frequency. The NMR instrument can possibly be equipped with an automatic sample changer and additional data-processing software for the evaluation of the spectra and computation of the results. The performance of the NMR spectrometer can be checked using the Certified Reference Materials (sealed tubes CRM 123).

5.3.2 10 mm NMR sample tubes

5.3.3 Distillation apparatus

*Note:* Any method for ethanol extraction can be used as long as the alcohol in the wine is recovered without isotopic fractionation.

The Cadiot column shown in figure 1 is an example of a manual distillation system that allows to extract 96 to 98.5% of the ethanol of a wine without isotopic fractionation and obtain a distillate with an alcohol grade of 92 to 93 in % w/w (95% vol.).

Such a system is composed of:

- Electric heating mantle with voltage regulator,
- One-liter round-bottom flask with ground glass neck joint,
- Cadiot column with rotating band (moving part in Teflon),
- conical flasks with ground glass neck joints, for collection of the distillate

Automatic distillation systems are also available.

The performance of the distillation system may be checked periodically for both the yield of extraction as well as for accuracy for the isotopic determination. This control can be done by distillation and measurement of CRM -660.

5.3.4 The following common laboratory equipment and consumables is needed:

-micropipette with appropriate tips,
-balance with 0.1 mg accuracy or better,
-balance with 0.1g accuracy or better
-single use syringe for transfer of liquids,
-precise graduated flasks (50ml, 100 ml, 250ml, …)
-flasks equipped with airtight closing systems and inert septa (for storage of aliquots of wines, distillates and residues until measurement)

-equipment and consumables as specified in the other methods referred to herein.
The laboratory equipment and consumables indicated in the above lists are examples and may be replaced by other equipment of equivalent performance.

## 6. Sampling (Preparation of the sample)

6.1 If not yet available, determine the alcoholic strength of the wine or of the fermented product (tv) to better than the nearest 0.05 % vol. (eg. using the OIV method MA-F-AS312-01-TALVOL).

6.2 Extraction of the ethanol
Using the appropriate graduated flask, introduce a homogeneous sample of a suitable volume V ml of the wine or the fermented product into the round-bottom flask of the distillation apparatus. Place a ground conical flask to receive the distillate. Heat the product to be distilled to obtain a constant reflux ratio at the level of the condenser. Start the collection of the distillate when a stable temperature of the vapours typical of the ethanol-water azeotrope (78 °C) is reached and stop the collection when the temperature increases. The collection of distillate should be continued until the ethanol-water azeotrope is completely recovered.

When using manually a Cadiot column (Figure 1) the following procedure can be applied:
-Collect the boiling liquid corresponding to the ethanol-water azeotrope, when the temperature increases, discontinue collection for five minutes. When the temperature returns to 78 °C, recommence collecting the distillate until the temperature of the vapours increases again. Repeat this operation until the temperature, after discontinuing collection, does not return to 78 °C.
Alternatively, commercially available distillation systems can be used.

The weight $m^D$ of distillate collected is weighed to better than 0.1g.

In order to avoid isotopic fractionation, the distillate should be kept in a tight vial preventing any evaporation until further use for determination of the alcoholic strength (6.3) and preparation of the NMR tube (7.1).

An aliquot of a few ml of the residues is kept. Its isotope ratio $(D/H)^{Q}_{W}$ may be determined if required.

6.3 Determination of the alcoholic strength of the distillate
The alcoholic strength (%w/w) of the distillate must be determined with a precision better than 0.1%.

The water content of the distillate (ρ' g) can be determined by the Karl Fischer method using a sample of about 0.5 ml of alcohol of exactly known mass ρ g .The alcohol strength by mass of the distillate is then given by:
$t_m^D$ % w/w= 100 (1-ρ')/ ρ

Alternatively the alcoholic strength can be determined by densimetry for instance using a electronic densimeter.

6.4 Yield of distillation
The yield of distillation is estimated using the following formula:
Yield of dist.% = $100\ t_m^D\ m^D /(V.tv)$
Given the uncertainty on each term and especially on tv, the yield of distillation is estimated at ±0.5% (in the case of a wine of 10%v/v).
When using the Cadiot column, no significant isotope fractionation effect is expected for yield of extraction higher than 96%. In any case the operator may use a sufficient volume Vml of wine or fermented product for the distillation to ensure that the yield of extraction is sufficient. Typically aliquots of 750, 500, 400 and 300ml of wine sample should be sufficient to obtain a 96% yield when carrying out the above distillation procedure with the Cadiot column on wines or fermented products of respectively tv = 4, 6, 8 and 10% vol.

6.5 Fermentation of musts, concentrated musts and rectified concentrated musts

Prior to use, the yeast can be reactivated in a small volume of must. The fermentation vessel is equipped with a device to keep it airtight and to avoid loss of ethanol.

6.5.1 Musts
Place about one litre of must, whose concentration of fermentable sugars has been previously determined, in the fermentation vessel. Add about 1 g of dry yeast eventually reactivated beforehand. Insert device to keep it airtight. Allow fermentation to proceed until the sugar is used up. The fermented product can then be distilled following the procedure already described for wine in 6.1 to 6.4

Note: Musts preserved by addition of sulphur dioxide have to be de-sulphited by bubbling nitrogen through the must in a water bath at 70 to 80 °C under reflux in order to prevent isotope fractionation through evaporation of water. Alternatively, the sulphur dioxide can be removed by a small addition of a solution of hydrogen peroxide ($H_2O_2$).

6.5.2 Concentrated musts
Place V ml of concentrated must containing a known amount of sugar (approximately 170 g) into the fermentation vessel. Top up to one litre with (1000 - V) ml of water. Add dry yeasts (1 g) and 3 g of Bacto Yeast Nitrogen Base without amino acids. Homogenize and proceed as described in 6.5.1.

6.5.3 Grape sugar (Rectified concentrated musts)
Proceed as described in 6.5.2, topping up to one litre with (1000 - V) ml of water also containing 3 g of dissolved tartaric acid.

Note: Concentrated musts and rectified concentrated musts are diluted in local water having a (D/H) isotope concentration different of that of the original must. By convention, the $(D/H)_I$ and $(D/H)_{II}$ parameters measured on ethanol have to be normalised as if the must had fermented in water having the same deuterium concentration as V-SMOW ( 155.76 ppm).
This normalisation of the data is performed by using the following equations (Martin et al., 1996, J. AOAC, 79, 62-72):

$$\left(\frac{D}{H}\right)_I^{Norm.V-SMOW} = \left(\frac{D}{H}\right)_I - 0.19 \times \left[\left(\frac{D}{H}\right)_W^S - 155.76\right]$$

$$\left(\frac{D}{H}\right)_{II}^{Norm.V-SMOW} = \left(\frac{D}{H}\right)_{II} - 0.78 \times \left[\left(\frac{D}{H}\right)_W^S - 155.76\right]$$

where $\left(\frac{D}{H}\right)_W^S$ is the deuterium isotope ratio of the diluted must. This value can be computed using the equation of the Global Meteoric Water Line (Craig, 1961):

$$\left(\frac{D}{H}\right)_W^S = 155.76 \times \left[\frac{\left(8 \times \delta^{18}O + 10\right)}{1000} + 1\right]$$

Where $\delta^{18}O$ is measured on the diluted must by the method for $^{18}O/^{16}O$ isotope ratio determination of water in wines and must [OIV-MA-AS2-12].

Retain 50 ml of sample of must or sulphur dioxide treated must or concentrated must or rectified concentrated must with a view to the possible extraction of the water and the determination of its isotope ratio $(D/H)_W^Q$.

## 7. Procedure

7.1 Preparation of alcohol sample for NMR measurement

- 10 mm diameter NMR probe: in a previously weighed bottle, collect 3.2 ml of distillate as described in section 6.2 and weigh it to the nearest 0.1 mg ($m_A$); then take 1.3 ml sample of the internal standard TMU (5.2.2) and weigh to the nearest 0.1 mg ($m_{ST}$).

Depending on the type of spectrometer and probe used, add a sufficient quantity of hexafluorobenzene (5.1.2) as a field-frequency stabilization substance (lock):

| | |
|---|---|
| Spectrometer | 10 mm probe |
| 7.05 T | 150 µl |
| 9.4 T | 35 µl |

These figures are indicative and the actual volume to be used should be adjusted to the sensitivity of the NMR instrument. While preparing the tube and until the NMR measurement, the operator should take care to avoid any evaporation of ethanol and TMU since this would cause isotopic fractionation, errors in the weights ($m_A$ and $m_{ST}$) of the components and erroneous NMR results.
The correcteness of the procedure of measurement including this preparation step can be checked using the CRM 656.
Note: the hexafluorobenzene can be added with 10% (v/v) of trifluoroacetic acid (5.1.3) in order to catalyze the fast hydrogen exchange on hydroxyle bond resulting in a single NMR peak for both the hydroxyle and residual water signals.

7.2 Recording of $^2H$ NMR spectra of the alcohol
The homogeneity of the magnetic field $B_0$ in the sample is optimized through the "shimming" procedure maximizing the $^{19}F$ NMR lock signal observed the hexafluorobenzene. Modern NMR spectrometers can perform automatically and efficiently this "shimming" procedure provided that the initial settings are close enough to the optimal magnetic field homogeneity for a given sample as is generally the case for a batch of ethanol samples prepared as described in 7.1. The efficiency of this procedure can be checked through the resolution measured on the spectrum obtained without exponential multiplication (i.e. LB = 0) (Figure 2b) and expressed by the half-width of the methyl and methylene signals of ethanol and the methyl signal of TMU, which must be less than 0.5 Hz in the best conditions. The sensitivity, measured with an exponential multiplying factor LB equal to 2 (Figure 2a) must be greater than or equal to 150 for the methyl signal of ethanol of alcoholic strength 95 % vol (93.5 % mas).

7.2.2 Checking the instrumental settings

Carry out customary standardization for homogeneity and sensitivity according to the manufacturer's specifications.
Use the sealed tubes CRM123 (H: High , M: Medium, L: Low).

Following the procedure described below in 9.3, determine the isotope values of these alcohols, denoting them Hmeas, Mmeas, Lmeas .

Compare them with the given corresponding standard values, denoted by a superscript Hst, Mst, Lst.

Typically, as an indication the standard deviation obtained for 10 repetitions of each spectrum should be of the order of 0.01 for the ratio R and 0.5 ppm for $(D/H)_I$ and 1 ppm for $(D/H)_{II}$.

The average values obtained for the various isotopic parameters (R, $(D/H)_I$, $(D/H)_{II}$) must be within the corresponding standard deviation of repeatability given for those parameters for the CRM123. If they are not, carry out the checks again.

Once the settings have been optimized also other CRM materials can be used to monitor the quality of measurements in routine analysis.

7.3 Conditions for obtaining NMR spectra

Place a sample of alcohol prepared as in 7.1 in a 10 mm tube and introduce it into the probe.

Suggested conditions for obtaining NMR spectra are as follows:

- a constant probe temperature, set to better less than ±0.5°K variation in the range 302 K to 306 K depending on the heating power generated by the decoupling;
- acquisition time of at least 6.8 s for 1200 Hz spectral width (16K memory) (i.e. about 20 ppm at 61.4 MHz or 27 ppm at 46.1 MHz);
- 90° pulse;
- parabolic detection: fix the offset 01 between the OD and CHD reference signals for ethanol and between the HOD and TMU reference signals for water;
- determine the value of the decoupling offset 02 from the proton spectrum measured by the decoupling coil on the same tube. Good decoupling is obtained when 02 is located in the middle of the frequency interval existing between the CH3- and CH2- groups. Use the wide band decoupling mode or

composite pulse sequences (eg. WALTZ16) to ensure homogeneous decoupling on the whole spectrum.

For each spectrum, carry out a number of accumulations NS sufficient to obtain the signal-to-noise ratio indicated as sensitivity in 7.2 and repeat NE times this set of NS accumulations. The values of NS depend on the types of spectrometer and probe used. Examples of the possible choices are:

| Spectrometer | 10 mm probe |
|---|---|
| 7.05 T | NS = 304 |
| 9.4 T | NS = 200 |

The number of repetitions NE should be statistically meaningful and sufficient to achieve the performance and precision of the method as reported below in §9.

In the case that two NMR sample tubes have been prepared following the procedure described in 7.1, five repetitions of NMR spectra (NE=5) can be recorded on each tube. The final result for each isotopic parameter corresponds to the mean value of the measurements obtained on the two NMR sample tubes. In that case, the acceptance criteria for validation of the results obtained with these two tubes are:

$|Mes1(D/H)_I - Mes2(D/H)_I| < 0.5ppm$, $|Mes1(D/H)_{II} - Mes2(D/H)_{II}| < 0.8ppm$

## 8. Expression of results

For each of the NE spectra (see NMR spectrum for ethanol, Figure 2a), determine:

$$R = 3 \cdot \frac{h_{II}}{h_I} = 3 \cdot \frac{\text{height of signal II } (CH_3\ CH_D\ OH)}{\text{height of signal I } (CH_2D\ CH_2\ OH)}$$

$$\left(D/H\right)_I = 1.5866 \cdot T_I \cdot \frac{m_{ST}}{m_A} \cdot \frac{\left(D/H\right)_{ST}}{t_m^D}$$

$$(D/H)_{II} = 2.3799 \cdot T_{II} \cdot \frac{m_{ST}}{m_A} \cdot \frac{(D/H)_{ST}}{t_m^D}$$

with

- $T_I = \frac{\text{height of signal I } (CH_2D\ CH_2\ OH)}{\text{height of signal of internal standard (TMU)}}$
- $T_{II} = \frac{\text{height of signal II } (CH_3\ CHD\ OH)}{\text{height of signal of internal standard (TMU)}}$

- $m_{ST}$ and $m_A$, see 7.1
- $t_m^D$, see 6.3
- $(D/H)_{ST}$ = isotope ratio of internal standard (TMU) indicated on certificate delivered by IRMM.

The use of peak heights instead of peak area, which is less precise, supposes that peak width at half height is identical and is a reasonable approximation if applicable (Figure 2b).

For each of the isotope parameters, calculate the average and the confidence interval for the number of repeated spectra acquired on a given sample.

Optional softwares enable such calculations to be carried out on-line.

## 9. Precision

The repeatability and Reproducibility of the SNIF-NMR method has been studied through collaborative studies on fruit juices as reported in the bibliography here below. However these studies considered only the parameter $(D/H)_I$. In the case of wine data from in-house studies carried out by several laboratories can be considered for establishing the standard deviation of repeatability and the limit of repeatability as presented in Annex I. The results of proficiency testing reported in Annex II provide data that can be used to compute the standard deviation of Reproducibility and the limit of Reproducibility for wines.

These figures can be summarised as follows:

| | $(D/H)_I$ | $(D/H)_{II}$ | R |
|---|---|---|---|
| $S_r$ | 0.26 | 0.30 | 0.005 |
| r | 0.72 | 0.84 | 0.015 |
| $S_R$ | 0.35 | 0.62 | 0.006 |
| R | 0.99 | 1.75 | 0.017 |

with

- $S_r$ : standard deviation of repeatability
- r : limit of repeatability
- $S_R$: standard deviation of reproducibility
- R : limit of Reproducibility

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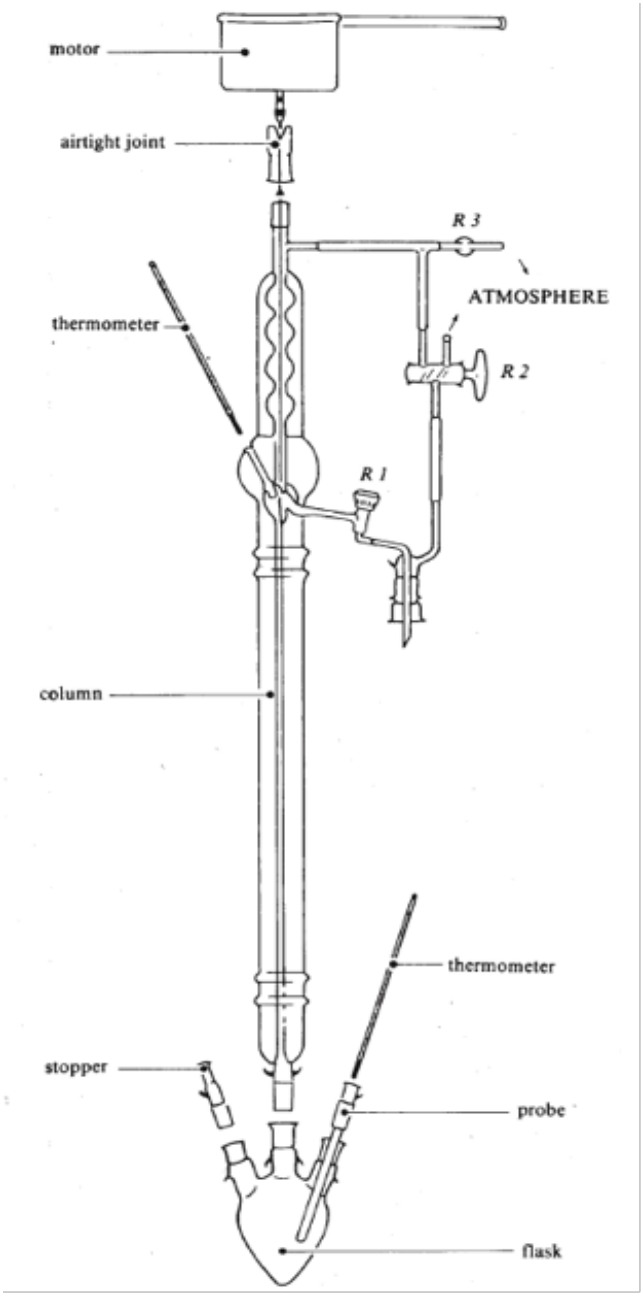



Figure 1 - Apparatus for extracting ethanol

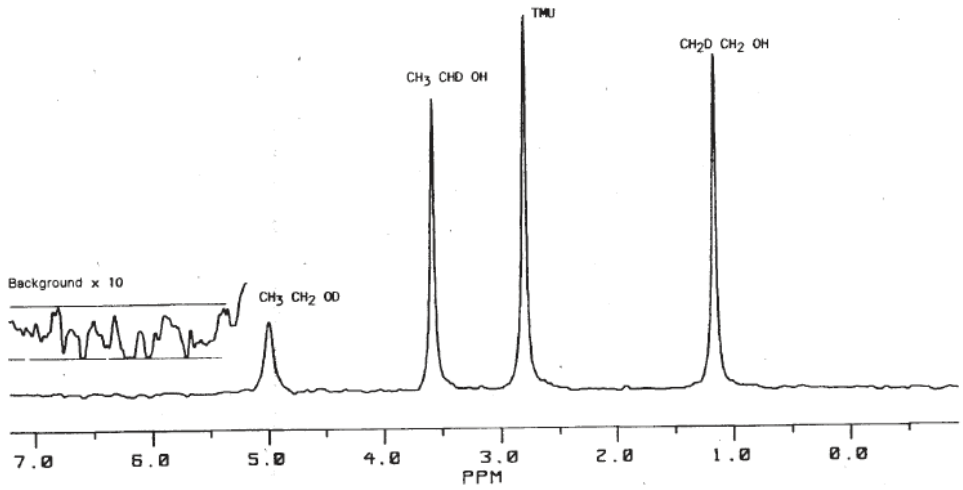



Figure 2a
$^{2}H$ NMR spectrum of an ethanol from wine with an internal standard (TMU: N, N-tetramethylurea)

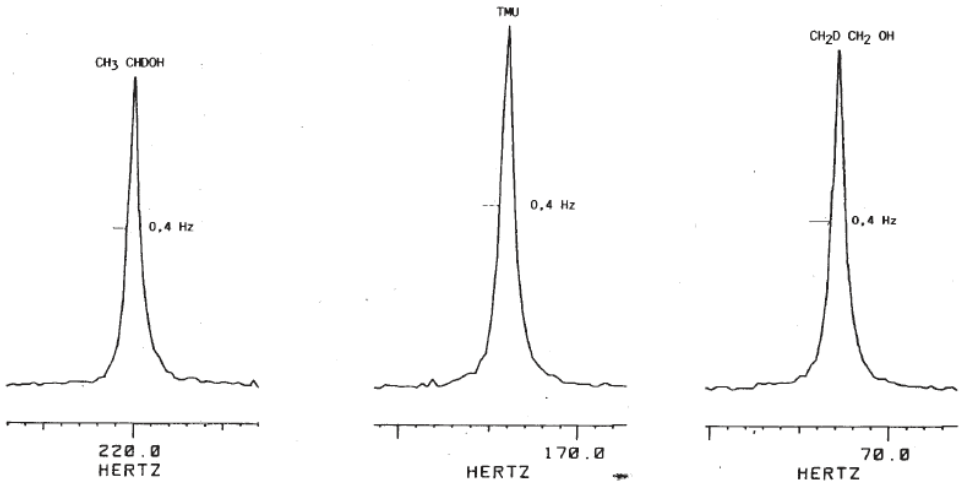



Figure 2b
$^{2}H$ spectrum of ethanol taken under the same conditions as those of Figure 2a, but without exponential multiplication (LB = 0)

## Annex I: Estimation of the repeatability from in-house repeatability studies

The in-house repeatability studies performed in 4 laboratories provide data that allows the estimation of the repeatability of the SNIF-NMR method.
These in-house repeatability studies have been performed by duplicate distillations and measurements of 10, 9 or 15 different wine samples by the laboratories 1, 2 and 3.
Alternatively the laboratory 4 performed 16 distillations and measurements on the same wine in condition of repeatability on a short period of time.

Table I-1: **lab 1 : 10 wines analysed in duplicates**

| | | | | $(D/H)_I$ abs $(\Delta(D/H)_I)$ | Squares | $(D/H)_{II}$ abs $(\Delta(D/H)_{II})$ | Squares | R abs $(\Delta(R))$ | Squares |
|---|---|---|---|---|---|---|---|---|---|
| Sample | $(D/H)_I$ | $(D/H)_{II}$ | R | | | | | | |
| 1 | 103.97 | 130.11 | 2.503 | 0.55 | 0.302 | 0.68 | 0.462 | 0.000 | 0.00000 |
| | 104.52 | 130.79 | 2.503 | | | | | | |
| 2 | 103.53 | 130.89 | 2.529 | 0.41 | 0.168 | 0.32 | 0.102 | 0.016 | 0.00026 |
| | 103.94 | 130.57 | 2.513 | | | | | | |
| 3 | 102.72 | 130.00 | 2.531 | 0.32 | 0.102 | 0.20 | 0.040 | 0.004 | 0.00002 |
| | 103.04 | 130.20 | 2.527 | | | | | | |
| 4 | 105.38 | 132.39 | 2.513 | 0.14 | 0.020 | 0.20 | 0.040 | 0.000 | 0.00000 |
| | 105.52 | 132.59 | 2.513 | | | | | | |
| 5 | 101.59 | 127.94 | 2.519 | 0.48 | 0.230 | 0.20 | 0.040 | 0.016 | 0.00026 |
| | 101.11 | 128.14 | 2.535 | | | | | | |
| 6 | 103.23 | 132.14 | 2.560 | 0.30 | 0.090 | 0.36 | 0.130 | 0.001 | 0.00000 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 102.93 | 131.78 | 2.561 | | | | | | |
| 7 | 103.68 | 130.95 | 2.526 | 0.15 | 0.023 | 0.75 | 0.563 | 0.011 | 0.00012 |
| | 103.53 | 130.20 | 2.515 | | | | | | |
| 8 | 101.76 | 128.86 | 2.533 | 0.24 | 0.058 | 0.42 | 0.176 | 0.003 | 0.00001 |
| | 101.52 | 128.44 | 2.530 | | | | | | |
| 9 | 103.05 | 129.59 | 2.515 | 0.04 | 0.002 | 0.44 | 0.194 | 0.007 | 0.00005 |
| | 103.01 | 129.15 | 2.508 | | | | | | |
| 10 | 101.47 | 132.63 | 2.614 | 0.50 | 0.250 | 0.18 | 0.032 | 0.010 | 0.00010 |
| | 100.97 | 132.45 | 2.624 | | | | | | |
| | | | | Sum of squares: | 1.245 | | 1.779 | | 0.00081 |
| | | | | **Sr** | **0.25** | | **0.30** | | **0.006** |
| | | | | **r** | **0.71** | | **0.84** | | **0.018** |

Table I-2: **lab 2 : 9 wines analysed in duplicates**

| Sample | $(D/H)_I$ | $(D/H)_{II}$ | R | (D/H)I abs ($\Delta(D/H)_I$) | Squares | (D/H)II abs ($\Delta(D/H)_{II}$) | Squares | R abs ($\Delta(R)$) | Squares |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 105.02 | 133.78 | 2.548 | 0.26 | 0.068 | 0.10 | 0.010 | 0.008 | 0.00007 |
| | 104.76 | 133.88 | 2.556 | | | | | | |
| 2 | 102.38 | 130.00 | 2.540 | 0.73 | 0.533 | 0.40 | 0.160 | 0.010 | 0.00011 |
| | 101.65 | 129.60 | 2.550 | | | | | | |
| 3 | 100.26 | 126.08 | 2.515 | 0.84 | 0.706 | 0.64 | 0.410 | 0.008 | 0.00007 |
| | 99.42 | 125.44 | 2.523 | | | | | | |
| 4 | 101.17 | 128.83 | 2.547 | 0.51 | 0.260 | 0.45 | 0.203 | 0.004 | 0.00002 |
| | 100.66 | 128.38 | 2.551 | | | | | | |
| 5 | 101.47 | 128.78 | 2.538 | 0.00 | 0.000 | 0.26 | 0.068 | 0.005 | 0.00003 |
| | 101.47 | 128.52 | 2.533 | | | | | | |
| 6 | 106.14 | 134.37 | 2.532 | 0.12 | 0.014 | 0.04 | 0.002 | 0.002 | 0.00000 |
| | 106.26 | 134.41 | 2.530 | | | | | | |
| 7 | 103.62 | 130.55 | 2.520 | 0.05 | 0.003 | 0.11 | 0.012 | 0.003 | 0.00001 |
| | 103.57 | 130.66 | 2.523 | | | | | | |
| 8 | 103.66 | 129.88 | 2.506 | 0.28 | 0.078 | 0.55 | 0.302 | 0.004 | 0.00001 |
| 9 | 103.38<br>103.50<br>103.93 | 129.33<br>129.66<br>129.44 | 2.502<br>2.506<br>2.491 | 0.43 | 0.185 | 0.22 | 0.048 | 0.015 | 0.00021 |
| | | | | Sum of squares: | 1.846 | | 1.214 | | 0.00053 |
| | | | | **Sr** | **0.32** | | **0.26** | | **0.005** |
| | | | | **r** | **0.91** | | **0.74** | | **0.015** |

Table I-3: **lab 3 : 15 wines analysed in duplicates**

| | | | | **(D/H)I** | | **(D/H)II** | | **R** | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | abs ($\Delta(D/H)_I$) | Squares | abs ($\Delta(D/H)_{II}$) | Squares | abs ($\Delta(R)$) | Squares |
| Sample | **$(D/H)_I$** | **$(D/H)_{II}$** | **R** | | | | | | |
| 1 | 101.63 | 125.87 | 2.477 | 0.06 | 0.004 | 0.46 | 0.212 | 0.007 | 0.00005 |
| | 101.57 | 125.41 | 2.470 | | | | | | |
| 2 | 99.24 | 124.41 | 2.507 | 0.05 | 0.002 | 0.04 | 0.002 | 0.001 | 0.00000 |
| | 99.19 | 124.37 | 2.508 | | | | | | |
| 3 | 101.23 | 125.07 | 2.471 | 0.06 | 0.004 | 0.16 | 0.026 | 0.005 | 0.00002 |
| | 101.17 | 125.23 | 2.476 | | | | | | |
| 4 | 100.71 | 125.29 | 2.488 | 0.07 | 0.005 | 1.16 | 1.346 | 0.024 | 0.00058 |
| | 100.78 | 124.13 | 2.464 | | | | | | |
| 5 | 99.89 | 124.02 | 2.483 | 0.18 | 0.032 | 0.56 | 0.314 | 0.007 | 0.00005 |
| | 99.71 | 123.46 | 2.476 | | | | | | |
| 6 | 100.60 | 124.14 | 2.468 | 0.19 | 0.036 | 0.66 | 0.436 | 0.018 | 0.00032 |
| | 100.41 | 124.80 | 2.486 | | | | | | |
| 7 | 101.47 | 125.60 | 2.476 | 0.23 | 0.053 | 0.14 | 0.020 | 0.003 | 0.00001 |
| | 101.70 | 125.74 | 2.473 | | | | | | |
| 8 | 102.02 | 124.00 | 2.431 | 0.13 | 0.017 | 0.07 | 0.005 | 0.005 | 0.00002 |
| | 102.15 | 123.93 | 2.426 | | | | | | |
| 9 | 99.69 | 124.60 | 2.500 | 0.40 | 0.160 | 0.53 | 0.281 | 0.000 | 0.00000 |
| | 100.09 | 125.13 | 2.500 | | | | | | |
| 10 | 99.17 | 123.71 | 2.495 | 0.30 | 0.090 | 0.19 | 0.036 | 0.004 | 0.00002 |
| | 99.47 | 123.90 | 2.491 | | | | | | |
| 11 | 100.60 | 123.89 | 2.463 | 0.40 | 0.160 | 0.54 | 0.292 | 0.001 | 0.00000 |
| | 101.00 | 124.43 | 2.464 | | | | | | |
| 12 | 99.38 | 124.88 | 2.513 | 0.33 | 0.109 | 0.55 | 0.302 | 0.002 | 0.00000 |
| | 99.05 | 124.33 | 2.511 | | | | | | |
| 13 | 99.51 | 125.24 | 2.517 | 0.44 | 0.194 | 0.01 | 0.000 | 0.011 | 0.00012 |
| | 99.95 | 125.25 | 2.506 | | | | | | |
| 15 | 101.34 | 124.68 | 2.460 | 0.43 | 0.185 | 0.41 | 0.168 | 0.002 | 0.00000 |
| | 101.77 | 125.09 | 2.458 | | | | | | |
| | | | | Sum of squares: | 1.050 | | 3.437 | | 0.00120 |
| | | | | **Sr** | **0.19** | | **0.34** | | **0.006** |
| | | | | **r** | **0.53** | | **0.96** | | **0.018** |

Table I-4

**lab 4 : one wine analysed 16 times**

| Repetition | **$(D/H)_I$** | **$(D/H)_{II}$** | **R** | | **$(D/H)_I$** | **$(D/H)_{II}$** | **R** |
|---|---|---|---|---|---|---|---|
| 1 | 101.38 | 126.87 | 2.503 | Variance: | 0.0703 | 0.0840 | 0.000013 |
| 2 | 101.30 | 126.22 | 2.492 | | | | |
| 3 | 100.98 | 125.86 | 2.493 | **Sr** | **0.27** | **0.29** | **0.004** |
| 4 | 100.94 | 126.00 | 2.497 | | | | |
| 5 | 100.71 | 125.79 | 2.498 | **r** | **0.75** | **0.82** | **0.010** |
| 6 | 100.95 | 126.05 | 2.497 | | | | |
| 7 | 101.17 | 126.30 | 2.497 | | | | |
| 8 | 101.22 | 126.22 | 2.494 | | | | |
| 9 | 100.99 | 125.91 | 2.494 | | | | |
| 10 | 101.29 | 126.24 | 2.493 | | | | |
| 11 | 100.78 | 126.07 | 2.502 | | | | |
| 12 | 100.65 | 125.65 | 2.497 | | | | |
| 13 | 101.01 | 126.17 | 2.498 | | | | |
| 14 | 100.89 | 126.05 | 2.499 | | | | |
| 15 | 101.66 | 126.52 | 2.489 | | | | |
| 16 | 100.98 | 126.11 | 2.498 | | | | |

The pooled data for the standard deviation of repeatability and for the limit of repeatability can thus be estimated as:

| | $(D/H)_I$ | $(D/H)_{II}$ | R |
|---|---|---|---|
| Sr | 0.26 | 0.30 | 0.005 |
| limit of repeatability r | 0.72 | 0.84 | 0.015 |

Data of in-house repeatability studies were provided by (in alphabetic order):

-Bundesinstitut für Risikobewertung,
Thielallee 88-92 PF 330013 D-14195 BERLIN – GERMANY

-Fondazione E. Mach-Istituto Agrario di San Michele all'Adige,
Via E. Mach, 1 - 38010 San Michele all'Adige (TN), ITALY

-Joint Research Centre - Institute for Health and Consumer Protection,
I-21020 ISPRA (VA) – ITALY

-Laboratorio Arbitral Agroalimentario, Carretera de la Coruña, km 10,7
E-28023 MADRID –SPAIN

**Annex II: Evaluation of the Reproducibility from proficiency testing data**

Since December 1994 international proficiency testing exercises on the determination of isotopic parameters on wine and various other food matrices have been regularly organised. These proficiency testing exercises allow participating laboratories to assess their performance and the quality of their analyses. The statistical exploitation of these results obtained on a large number of samples over a long period of time allows the appreciation of the variability of the measurements under conditions of reproductibility. This enables a good estimation of the variance parameters and of the reproducibility limit of the method. The results of 40 rounds of proficiency testing since 1994 until 2010 for various type of wine (red, white, rosé, dry, sweet and sparkling) are summarised in the table II-1 here below.

For $(D/H)_I$ and $(D/H)_{II}$ the pooled $S_R$ can thus be calculated using the following equation:

$$\sqrt{\frac{\sum_i^K (N_i - 1) S_{R,i}^2}{\sum_i^K (N_i - 1)}}$$

with $N_i$ ,and $S_{R,i}$ the number of values and the standard deviation of reproducibility of the $i^{th}$ round, and K the number of rounds.

Considering the definition of the intramolecular ratio R, and applying the standard error propagation rules assuming that $(D/H)_I$ and $(D/H)_{II}$ are uncorrelated (the covariance terms are then zero), one can also estimate the standard deviation of Reproducibility for this parameter.

The following figures can thus be calculated:

| | $(D/H)_I$ | $(D/H)_{II}$ | R |
|---|---|---|---|
| $S_R$: | 0.35 | 0.62 | 0.006 |
| Limit of Reproducibility R | 0.99 | 1.75 | 0.01 |

**Table II-1 : FIT Proficiency Testing – Summary of statistical values observed on wine samples:**

| | | | $(D/H)_I$ | | | $(D/H)_{II}$ | | |
|---|---|---|---|---|---|---|---|---|
| Sample | Year | Round | N | Mean | $S_R$ | N | Mean | $S_R$ |
| Red wine | 1994 | R1 | 10 | 102.50 | 0.362 | 10 | 130.72 | 0.33 |
| Rosé wine | 1995 | R1 | 10 | 102.27 | 0.333 | 10 | 128.61 | 0.35 |
| Red wine | 1995 | R2 | 11 | 101.45 | 0.389 | 11 | 127.00 | 0.55 |
| Red wine | 1996 | R1 | 11 | 101.57 | 0.289 | 11 | 132.23 | 0.34 |
| Rosé wine | 1996 | R2 | 12 | 102.81 | 0.322 | 12 | 128.20 | 0.60 |
| White wine | 1996 | R3 | 15 | 103.42 | 0.362 | 15 | 127.97 | 0.51 |
| Red wine | 1996 | R4 | 15 | 102.02 | 0.377 | 13 | 131.28 | 0.30 |
| Rosé wine | 1997 | R1 | 16 | 103.36 | 0.247 | 16 | 126.33 | 0.44 |
| White wine | 1997 | R2 | 16 | 103.42 | 0.444 | 15 | 127.96 | 0.53 |
| Sweet White Wine | 1997 | R2 | 14 | 99.16 | 0.419 | 15 | 130.02 | 0.88 |
| Wine | 1997 | R3 | 13 | 101.87 | 0.258 | 15 | 132.03 | 0.61 |
| Sweet Wine | 1997 | R3 | 12 | 102.66 | 0.214 | 12 | 128.48 | 0.48 |
| Rosé wine | 1997 | R4 | 16 | 102.29 | 0.324 | 16 | 129.29 | 0.63 |
| Sweet Wine | 1997 | R4 | 15 | 102.04 | 0.269 | 13 | 131.27 | 0.30 |
| White wine | 1998 | R1 | 16 | 105.15 | 0.302 | 16 | 127.59 | 0.59 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sweet Wine | 1998 | R3 | 16 | 102.17 | 0.326 | 16 | 129.60 | 0.56 |
| Red wine | 1998 | R4 | 17 | 102.44 | 0.306 | 17 | 131.60 | 0.47 |
| White wine | 1999 | R1 | 14 | 102.93 | 0.404 | 13 | 129.64 | 0.46 |
| Sweet Wine | 2000 | R2 | 15 | 103.19 | 0.315 | 14 | 129.43 | 0.60 |
| Wine | 2001 | R1 | 12 | 105.28 | 0.264 | 16 | 131.32 | 0.68 |
| Sweet Wine | 2001 | R2 | 14 | 101.96 | 0.249 | 15 | 128.99 | 1.05 |
| Wine | 2002 | R1 | 17 | 101.01 | 0.365 | 16 | 129.02 | 0.74 |
| Wine | 2002 | R2 | 17 | 101.30 | 0.531 | 17 | 129.28 | 0.93 |
| Wine | 2003 | R1 | 18 | 100.08 | 0.335 | 18 | 128.98 | 0.77 |
| Sweet Wine | 2003 | R2 | 17 | 100.51 | 0.399 | 18 | 128.31 | 0.80 |
| Wine | 2004 | R1 | 18 | 102.88 | 0.485 | 19 | 128.06 | 0.81 |
| Sweet Wine | 2004 | R3 | 16 | 101.47 | 0.423 | 16 | 130.10 | 0.71 |
| Wine | 2005 | R1 | 19 | 101.33 | 0.447 | 19 | 129.88 | 0.76 |
| Sweet wine | 2005 | R2 | 15 | 102.53 | 0.395 | 15 | 131.36 | 0.38 |
| Dry wine | 2006 | R1 | 18 | 101.55 | 0.348 | 18 | 131.30 | 0.51 |
| Sweet wine | 2006 | R2 | 18 | 100.31 | 0.299 | 18 | 127.79 | 0.55 |
| Wine | 2007 | R1 | 18 | 103.36 | 0.403 | 18 | 130.90 | 0.90 |
| Sweet wine | 2007 | R2 | 19 | 102.78 | 0.437 | 19 | 130.72 | 0.55 |
| Wine | 2008 | R1 | 24 | 103.20 | 0.261 | 23 | 131.29 | 0.59 |
| Sweet wine | 2008 | R2 | 20 | 101.79 | 0.265 | 19 | 129.73 | 0.34 |
| Dry wine | 2009 | R1 | 24 | 102.96 | 0.280 | 23 | 130.25 | 0.49 |
| Sweet wine | 2009 | R2 | 21 | 101.31 | 0.310 | 21 | 127.07 | 0.50 |
| Dry wine | 2010 | R1 | 21 | 101.80 | 0.350 | 20 | 129.65 | 0.40 |
| Sparkling wine | 2010 | R1 | 11 | 101.51 | 0.310 | 11 | 129.09 | 0.68 |
| Dry wine | 2010 | R2 | 20 | 104.05 | 0.290 | 19 | 133.31 | 0.58 |

**Method OIV-MA-AS311-06** Type IV method

# Determination of polyols derived from sugars and residual sugars found in dry wines by means of gas chromatography

(Resolution Oeno 9/2006)

## 1. Scope

Simultaneous determination of the erythritol, arabitol, mannitol, sorbitol and meso-inositol content of wines.
Because the determination of sugars by gas chromatography (GC) is long and complicated, it is reserved for the determination of traces of sugars and, especially, of sugars for which no other routine enzyme method exists – (Arabinose, Rhamnose, Mannose and Galactose) although it is also applicable to glucose and fructose, the advantage being that it is possible to simultaneously determine all sugar monomers, dimers and even trimers.

*Comment 1 - It is not possible to determine sugars once they have been reduced to alditol form because of the presence of corresponding polyols.*
*Comment 2 - In the form of trimethylsilylated derivatives (TMS), sugars give 2 $\alpha$ and $\beta$ forms and occasionally 3 or 4 (Gamma…) corresponding to the different anomers present in wines.*
*Comment 3 - Without prior dilution, it is difficult to determine glucose and fructose content using this method when it exceeds 5 g/l.*

## 2. Principle

Residual sugars in dry wines can be determined by gas chromatography after the formation of their trimethylsilylated derivatives.

The internal standard is pentaerythritol.

## 3. Reagents

*Silane mixture for example purposes*:
3.1 Pure hexamethyldesilazane (HMDS)
3.2 Pure trifluoroacetic anhydride (TFA)
3.3 Pure pyridine

3.4 Pure pentaerythritol
3.5. Distilled water
3.6 10 g/l pentaerythritol (internal standard solution): dissolve 0.15 g of pentaerythritol (3.4) in 100 ml of water (3.5)
3.7 Pure products that may be used to prepare control solutions, notably glucose, fructose, arabinose, mannitol and sorbitol (non-exhaustive list)
3.8 Control solutions of pure products at 200 mg/l: dissolve 20 mg of each of the products to be determined (3.6) in 100 ml of water.
Comment – Sugar solutions should be prepared immediately prior to use.

**4. Apparatus and Equipment**

4.1 1-ml pipettes, with $1/10^{th}$ ml graduations
4.2 Propipette™ bulbs
4.3 100-µl syringe
4.4 5-ml tubes with screw stoppers fitted with a Teflon-coated sealing cap.
4.5 Rotary vacuum evaporator capable of housing screw-cap test tubes (4.4) in order to evaporate samples to dryness
4.6 Gas chromatograph fitted with a flame ionisation detector x g, and an injector operating in "split" mode - $1/30^{th}$ to $1/50^{th}$ division of the injected volume (1 µl)
4.7 Non-polar capillary column (SE-30, CPSil-5, HP-1, etc.) 50 m x 0.25 mm, 15 mµ stationary phase film thickness (as an example).
4.8 10-µl injection syringe
4.9 Data acquisition system
4.10 Ultra-sonic bath
4.11 Laboratory fume cupboard

**5. Preparation of samples**

5.1 Addition of the internal standard: 1 ml of wine (pipette, 4.1) or of 200 mg/l control solution (3.6) is placed in the screw-cap test tube (4.4)
**Note**: It is possible to operate with lower volumes of wine especially in high content sugar environments.

50 µl of the 10 g/l pentaerythritol solution (3.5) is added by means of the syringe (4.3)

5.2 Obtaining dry solid matter:

The screw-cap test tube is placed on the rotary evaporator, with a water bath kept below 40°C. Evaporation continues until all traces of liquid have disappeared.

5.3 Addition of reagents

5.3.1 Place the tubes containing the dry solid matter and reagents 3.1, 3.2 and 3.3 in the fume cupboard (4.11) and switch on the ventilation.

5.3.2 Using the pipettes (4.1) and Propipette™ bulbs (4.2), add 0.20 ml of pyridine (3.3), 0.7 ml of HMDS (3.1) and 0.1 ml of TFA (3.2) to the test tube one after the other.

5.3.3 Seal the test tube with its stopper.

5.3.4 Put the test tube in the ultra-sonic bath (4.10) for 5 minutes until the dry solid matter has completely dispersed.

5.3.5 Place the test tube in a laboratory kiln at 60°C for two hours in order to obtain the total substitution of the hydroxyl or acid hydrogen by the trimethylsilyl groups (TMS).

*Comment: a single phase only should remain after heating (if not, water would be left in the test tube). Likewise, there should be no brownish deposit, which would indicate an excess of non-derived sugar.*

**6 Chromatographic assay**

6.1 Place the cooled test tube in the ventilated fume cupboard (4.11), remove 1 µl with the syringe (4.8) and inject into the chromatograph in "split" mode (permanent split).

Treat the wine-derived and control sample in the same way.

6.2 Programme the kiln temperature, for example from 60°C to 240°C at a rate of 3°C per minute, such that the complete assay lasts, for example, one hour for complete mannitol and sorbitol separation (resolution higher than 1.5).

**7. Calculations**

Example: calculation of sorbitol concentration

If

| | |
|---|---|
| s | = the peak area of the sorbitol in the wine |
| S | = the peak area of the sorbitol in the control solution |
| i | = the peak area of the internal standard in the wine |
| I | = the peak area of the internal standard in the control solution |

The sorbitol content of the wine (ts) will be

$$ts = 200 \times \frac{s}{S} \times \frac{I}{i} \quad \text{in mg per litre}$$

The same logic makes it possible to calculate the glucose content (tg)

$$tg = 200 \times \frac{g}{G} \times \frac{I}{i} \quad \text{in mg per litre}$$

when g is the sum of the areas of the two peaks of glucose in the wine and G is the sum of the areas of the two peaks of glucose in the control solution.

## 8. Characteristics of the method

Detection threshold approximately 5 mg/l for a polyol (a single chromatographic peak). Average repeatability in the region of 10% for a sugar or polyol concentration in the region of 100 mg/l.

Table 1 Repeatability of the determination of a number of substances found in the dry solid matter of wine after TMS derivatization.

| | Tartaric acid | Fructose | Glucose | Mannitol | Sorbitol | Dulcitol | Meso-inositol |
|---|---|---|---|---|---|---|---|
| Average (mg/l) | 2013 | 1238 | 255 | 164 | 58 | 31 | 456 |
| Typical variance(mg/l) | 184 | 118 | 27 | 8 | 2 | 2 | 28 |
| CV (%) | 9 | 10 | 11 | 5 | 3 | 8 | 6 |

## REFERENCES

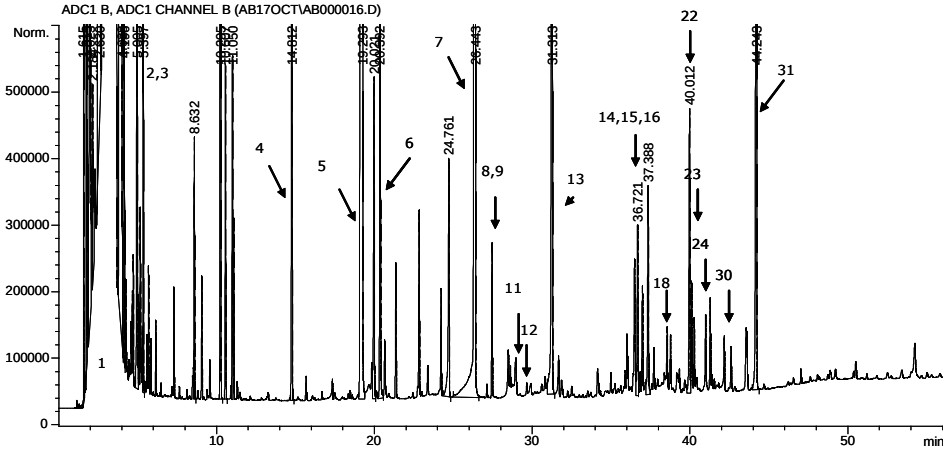
RIBEREAU-GAYON P. and BERTRAND A. 1972, Nouvelles applications de la chromatographie en phase gazeuse à l'analyse des vins *et* au contrôle de leur qualité, Vitis, 10, 318-322.

BERTRAND A**.** (1974), Dosage des principaux acides du vin par chromatographie en phase gazeuse. FV OIV 717—718, 253—274.

DUBERNET M.0. (1974), Application de la chromatographie en phase gazeuse à l'étude des sucres et polyols du vin: thèse 3° Cycle, Bordeaux.

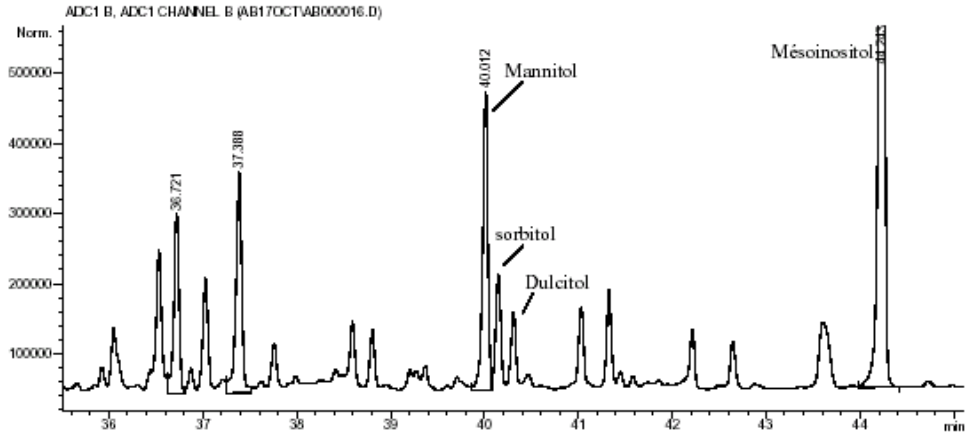
Figure 1

Chromatogram of a white wine following silylation. CPSil-5CB 50 m x 0.25 mm x 0.15 µm column. Split injection, 60°C, 3°C/min, 240°C. Magnification below.




Identification of peaks: 1 : reactive mixture; 2 and 3: unknown acids; 4: pentaerythriol; 5 and 6: unknown; 7: tartaric acid and arabinose; 8, 10 and 11: rhamnose; 9: arabinose; 12: xylitol; 13: arabitol; 14, 15 and 16: fructose; 17: galactose and unknown; 18: glucose α; 19: galactose and galacturonic acid; 20 and 21: unknown; 22: mannitol; 23: sorbitol; 24: glucose β; 25 and 27: unknown; 26: galacturonic acid; 28 and 30: galactonolactone; 29: mucic acid; 31: meso-inositol.

Chromatogram of a white wine following silylation. CPSil-5CB 50 m x 0.25 mm x 0.15 µm column. Split injection, 60°C, 3°C/min, 240°C. Magnification below.



**Method OIV-MA-AS311-07** Type III method

# Joint determination of the glucose and fructose content in wines by differential ph-metry

(Resolution Oeno 10/2006)

## 1. SCOPE

This method is applicable to the analysis of glucose and fructose in wines between 0 and 60 g/L (average level) or 50 and 270 g/L (high level).

## 2. PRINCIPLE

The joint determination of glucose and fructose content by differential pH-metry consists in the phosphorylation of the glucose and fructose by hexokinase. The $H^+$ ions generated stoechiometrically in relation to the quantities of glucose and fructose are then quantified.

## 3. REACTIONS

The glucose and fructose present are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by hexokinase (HK) (EC. 2.7.1.1)

$$\text{glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP} + H^+$$

$$\text{fructose} + \text{ATP} \xrightarrow{\text{HK}} \text{fructose-6-phosphate} + \text{ADP} + H^+$$

## 4. REAGENTS

**4.1** Demineralised Water (18 MΩ) or bi-distilled

**4.2** 2-Amino-2-(hydroxymethyl)propane-1,3-diol (TRIS) purity ≥ 99%

**4.3** Disodic adenosine triphosphate (ATP, 2Na) purity ≥ 99%

**4.4** Trisodium phosphate with twelve water molecules (Na3PO4·12H2O) purity ≥ 99%

**4.5** Sodium hydroxide (NaOH) purity ≥ 98%

**4.6** Magnesium chloride with six water molecules ($MgCl_2 \cdot 6H_2O$) purity ≥ 99%

**4.7** Triton X 100

**4.8** Potassium chloride (KCl) purity ≥ 99%

**4.9** 2-Bromo-2-nitropropane-1,3-diol (Bronopol) ($C_3H_6BrNO_4$)

**4.10** Hexokinase (EC. 2.7.1.1) 1 mg ≅145 U (e.g. Hofmann La Roche, Mannheim, Germany ref. Hexo-70-1351)

**4.11** Glycerol purity ≥ 98%

**4.12** Glucose purity ≥ 99%

**4.13 Reaction buffer pH 8.0** commercial or prepared according to the following method:
In a graduated 100-ml flask (5.2) pour roughly 70 ml (5.3) of water (4.1), and continuously stir (5.5). Add 0.242 g ± 0.001 g (5.4) of TRIS (4.2), 0.787 g ± 0.001 g (5.4) of ATP (4.3), 0.494 g ± 0.001 g (5.4) of sodium phosphate (4.4), 0.009 mg ± 0.001 g (5.4) of sodium hydroxide (4.5), 0.203 g ± 0.001 g (5.4) of magnesium chloride (4.6), 2.000 ± 0.001 g (5.4) of Triton X 100 (4.7), 0.820 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 ± 0.001 g (4.9) of bronopol. Adjust to volume with water (4.1). The final pH must be 8.0 ± 0.1 (5.6), otherwise adjust it with sodium hydroxide or hydrochloric acid. The buffer thus prepared is stable for two months at 4°C.

**4.14 Enzyme solution** commercial or prepared according to the following method: Using a graduated pipette (5.7) place 5 ml of glycerol (4.11) into a graduated 10-ml flask, adjust to volume with water (4.1) and homogenize. Dissolve 20 mg ±1 mg (5.4) of hexokinase (4.10) and 5 mg of Bronopol (4.9) in 10 ml of the glycerol solution. The activity of the enzyme solution must be 300 U ± 50 U per ml for the hexokinase. The enzyme solution is stable for 6 months at 4°C.

**4.15 Preparation of the calibration solution (average level) if the supposed content is less than 50 g/L of glucose + fructose)**
Place 3.60 g ± 0.01 g (5.4) of glucose (4.12) (desiccated 12 hours beforehand at 40 °C until constant weight), 0.745 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 g ± 0.001 g of bronopol (4.9) in a graduated 100-ml flask (5.2). Add water (4.1). Fully homogenize (5.5). Adjust to volume with water (4.1) after

removing the magnetic bar. The final concentration is 36 g/L of glucose. The solution is stable for 6 months at 4 °C.

**4.16 Preparation of the calibration solution (high level) if the supposed content is above 50 g/L of glucose + fructose)**

Place 18.0 g ± 0.01 g (5.4) of glucose (4.12) (desiccated 12 hours beforehand at 40 °C until constant weight), 0.745 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 g ± 0.001 g of bronopol (4.9) in a graduated 100-ml flask (5.2). Add water (4.1). Fully homogenize (5.5). Adjust to volume with water (4.1) after removing the magnetic bar. The final concentration is 180 g/L of glucose. The solution is stable for 6 months at 4°C.

## 5. APPARATUS

**5.1** Differential pH-metry apparatus (EUROCHEM CL 10 plus, Microlab EFA or equivalent) see appendix A

**5.2** Graduated 100-ml flask, class A

**5.3** Graduated 100-ml test-tube with sole

**5.4** Precision balance to weigh within 1 mg

**5.5** Magnetic stirrer and magnetic Teflon bar

**5.6** pH-meter

**5.7** Graduated 3-mL, 5-mL pipettes, class A

**5.8** Graduated 10-ml flask, class A

**5.9** Automatic syringe pipettes, 25 and 50 µL

## 6. PREPARATION OF SAMPLES

The samples should not be too charged with suspended matter; in the contrary case, centrifuge or filter them. Sparkling wines must be degassed.

## 7. PROCEDURE

The operator must respect the instructions for use of the equipment (5.1). Before any use, the instrument must be stabilized in temperature. The circuits must be rinsed with the buffer solution (4.13) after cleaning, if required.

**7.1 Determination of the blank** (determination of the enzyme signal)
Fill the electrode compartments ($EL_1$ and $EL_2$) of the differential pH-meter (5.1) with the buffer solution (4.13); the potential difference between the two electrodes ($D_1$) must range between ± 150 mpH;
Add 24 µL of enzyme solution (4.14) to the reaction vessel (using the micropipette 5.9 or the preparer) and fill electrode $EL_2$;
Measure the potential difference ($D_2$) between the two electrodes;
Calculate the difference in pH, $\Delta pH_o$ for the blank using the following formula:

$$\Delta pH_o = D_2 - D_1$$

where
$\Delta pH_o$ = the difference in pH between two measurements for the blank;
$D_1$ = the value of the difference in pH between the two electrodes filled with the buffer solution;
$D_2$ = the value of the difference in pH between the two electrodes, one of which is filled with the buffer solution and the other with the buffer solution and enzyme solution.
The value of $\Delta pH_o$ is used to check the state of the electrodes during titration as well as their possible drift over time; it must lie between –30 and 0 mpH and ≤ 1.5 mpH between two consecutive readings. If not, check the quality of the buffer pH and the cleanliness of the hydraulic system and electrodes, clean if necessary and then repeat the blank.

*7.2 Calibration*

*7.2.1 Average level*
Fill the electrode compartments ($EL_1$ and $EL_2$) with the buffer solution (4.13);
Add 25 µL (with the micropipette 5.9 or the preparer) of the standard glucose solution (4.15) to the reaction vessel;
Fill the electrodes $EL_1$ and $EL_2$ with the buffer + standard solution;
Measure the potential difference ($D_3$) between the two electrodes;
Add 24 µL of enzyme solution (4.14) and fill electrode $EL_2$ with the buffer + standard solution + enzyme;
After the time necessary for the enzymatic reaction, measure the potential difference ($D_4$) between the two electrodes;
Calculate the difference in pH, $\Delta pH_c$ for the calibration sample using the following formula:

$$\Delta pH_c = (D_4 - D_3) - \Delta pH_o$$

where
$\Delta pH_c$ = the difference between two measurements $D_3$ and $D_4$ for the calibration sample minus the difference obtained for the blank;
$D_3$ = the value of the difference in pH between the two electrodes filled with the reference buffer/solution mixture;
$D_4$ = the value of the difference in pH between the two electrodes, one of which is filled with the reference buffer/solution and the other with the buffer/ enzyme / reference solution.

Calculate the slope of the calibration line:

$$s = C_u / \Delta pH_c$$

where
$C_u$ is the concentration of glucose in the standard solution expressed in g/L.

Check the validity of the calibration by analysing 25 µL of standard solution (ML) of glucose (4.15) according to the procedure (7.3). The result must range between ± 2% of the reference value. If not, repeat the calibration procedure.

### 7.2.2 High level

Fill the electrode compartments ($EL_1$ and $EL_2$) with the buffer (4.13);
Add 10 µL (with the micropipette 5.9 or the preparer) of standard solution (HL) of glucose (4.16) to the reaction vessel;
Fill the electrodes $EL_1$ and $EL_2$ with the buffer + standard solution mixture;
Measure the potential difference ($D_3$) between the two electrodes;
Add 24 µL of enzyme solution (4.14) and fill electrode $EL_2$ with the buffer + standard solution + enzyme mixture;
After the time required for the enzymatic reaction, measure the potential difference ($D_4$) between the two electrodes;
Calculate the difference in pH, $\Delta pH_c$ for the calibration sample using the following formula:

$$\Delta pH_c = (D_4 - D_3) - \Delta pH_o$$

where
$\Delta pH_c$ = the difference in pH between two measurements $D_3$ and $D_4$ for the calibration sample minus the difference obtained for the blank;
$D_3$ = the value of the difference in pH between the two electrodes filled with the buffer/ reference solution mixture;
$D_4$ = the value of the difference in pH between the two electrodes, one of which is filled with the buffer/ reference solution and the other with the buffer/ reference solution /enzyme.

Calculate the slope of the calibration line:

$$s = C_u/\Delta pH_c$$

where
$C_u$ is the concentration of glucose in the standard solution expressed in g/L.

Check the validity of the calibration by analysing 10 µL of standard solution of glucose (4.16) in accordance with procedure (7.3). The result must range between ± 2% of the reference value. If not, repeat the calibration procedure.

### 7.3 Quantification

Fill the electrode compartments ($EL_1$ and $EL_2$) with the buffer solution (4.13)
Add 10 µL (high level) or 25 µL (mean level) (with the micropipette 5.9 or the preparer) of the sample solution to the reaction vessel;
Fill electrodes $EL_1$ and $EL_2$with the buffer + sample mixture;
Measure the potential difference ($D_5$) between the two electrodes;
Add 24 µL of the enzyme solution (4.14) and fill electrode $EL_2$ with the buffer mixture + sample + enzyme;
Measure the potential difference (D6) between the two electrodes;
Calculate the quantity of aqueous solution in the sample using the following formula:

$$w = s \times [(D_6 - D_5) - \Delta pH_o]$$

where
w = the quantity of aqueous solution in the sample (in g/L);
S is the slope determined by the calibration line;
$\Delta pH_o$ = the difference in pH between two measurements for the blank;

$D_5$ = the value of the difference in pH between the two electrodes filled with the sample/ reference solution;
$D_6$ = the value of the difference in pH between the two electrodes, one of which is filled with the buffer/sample and the other with the buffer/ sample /enzyme.

## 8 EXPRESSION OF RESULTS

The results are expressed in g/L of glucose + fructose with one significant figure after the decimal point.

## 9 PRECISION

The details of the interlaboratory test on the precision of the method are summarized in appendix B.

### 9.1 Repeatability

The absolute difference between two individual results obtained in an identical matter tested by an operator using the same apparatus, in the shortest interval of time possible, shall not exceed the repeatability value *r* in 95% of the cases.
The value is: $r = 0.021x + 0.289$ where x is the content in g/L of glucose + fructose

### 9.2 Reproducibility

The absolute difference between two individual results obtained with an identical matter tested in two different laboratories, shall not exceed the reproducibility value of R in 95% of the cases.
The value is: $R = 0.033x + 0.507$ where w is the content in g/L of glucose + fructose

## 10 OTHER CHARACTERISTICS OF THE ANALYSIS

### 10.1 Detection and quantification limits

10.1.1 Detection limit
The detection limit is determined by using 10 series of three repetitions of an analytical blank and linear regression carried out with the wines of the precision test; it is equal to three standard deviations. In this case, the method gave as a

result a detection limit of 0.03 g/L. Tests by successive dilutions confirmed this value.

10.1.2 Quantification limit

The quantification limit is determined by using 10 series of three repetitions of an analytical blank and linear regression carried out with the wines of the precision test; it is equal to ten standard deviations. In this case, the method gave as a result a quantification limit of 0.10 g/L. Tests by successive dilutions confirmed this value. The quantifications of white and red wine carried out by the laboratories that took part in the interlaboratory analysis also confirm these figures.

### 10.2 Accuracy

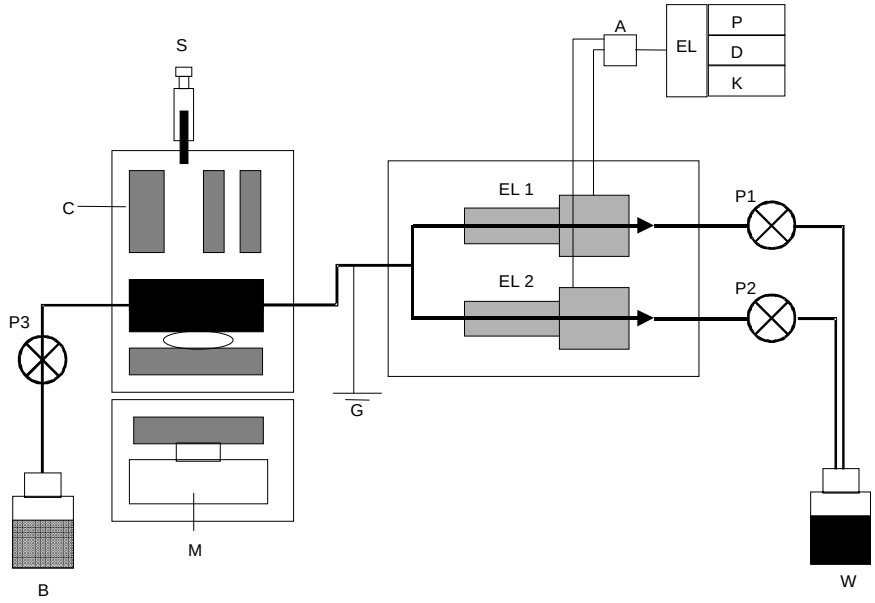
Accuracy is evaluated based on the average coverage rate calculated for the loaded wines analysed double-blind during the interlaboratory test (wines A, B, C, D, F and J). It is equal to 98.9% with a confidence interval of 0.22%.

## 11. QUALITY CONTROL

Quality controls can be carried out with certified reference materials, wines whose characteristics have been determined by consensus, or loaded wines regularly used in analytical series, and by following the related control charts.

## Appendix A

### Diagram of the differential pH-metry apparatus




A: differential amplifier; B: buffer solution; C: mixing chamber; D: indicator; $EL_1$ and $EL_2$ capillary electrodes; EL: electronics; G: ground; K: keyboard; M: magnetic stirrer; P: printer; $P_1$ to $P_3$: peristaltic pumps; S: injection syringe for the sample and enzyme; W: waste.

## Appendix B

### Statistical data obtained with the interlaboratory test results

In accordance with ISO 5725-2:1994, the following parameters were defined during an interlaboratory test. This test was carried out by the laboratory of the Inter-trade Committee for Champagne Wine in Epernay (France).
Year of the interlaboratory test: 2005
Number of laboratories: 13 double blind
Number of samples: 10

| | Wine A | Wine B | Wine C | Wine D | Wine E | Wine F | Wine G | Wine H | Wine I | Wine J |
|---|---|---|---|---|---|---|---|---|---|---|
| Average in g/L | 8.44 | 13.33 | 18.43 | 23.41 | 28.03 | 44.88 | 86.40 | 93.34 | 133.38 | 226.63 |
| Number of laboratories | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 |
| Number of laboratories after elimination of greatest dispersions | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 |
| Standard deviation of repeatability | 0.09 | 0.13 | 0.21 | 0.21 | 0.29 | 0.39 | 0.81 | 0.85 | 1.19 | 1.51 |
| Repeatability limit | 0.27 | 0.38 | 0.61 | 0.62 | 0.86 | 1.14 | 2.38 | 2.51 | 3.52 | 4.45 |
| RSDr, 100% | 1.08 | 0.97 | 1.13 | 0.91 | 1.04 | 0.86 | 0.94 | 0.91 | 0.89 | 0.67 |
| HORRAT r | 0.26 | 0.25 | 0.31 | 0.26 | 0.30 | 0.27 | 0.32 | 0.32 | 0.33 | 0.47 |
| Standard deviation of reproducibility | 0.17 | 0.27 | 0.37 | 0.59 | 0.55 | 0.45 | 1.27 | 1.43 | 1.74 | 2.69 |
| Reproducibility limit | 0.50 | 0.79 | 1.06 | 1.71 | 1.60 | 1.29 | 3.67 | 4.13 | 5.04 | 7.78 |
| RSDR, 100% | 2.05 | 2.05 | 1.99 | 2.54 | 1.97 | 1.00 | 1.47 | 1.53 | 1.31 | 1.19 |
| HORRAT R | 0.50 | 0.54 | 0.55 | 0.72 | 0.58 | 0.31 | 0.51 | 0.53 | 0.48 | 0.47 |

Types of samples:
Wine A: white wine naturally containing sugar, loaded with 2.50 g/L glucose and of 2.50 g/L of fructose;
Wine B: white wine naturally containing sugar (wine A), loaded with 5.00 g/L glucose and 50 g/L of fructose;
Wine C: white wine naturally containing sugar (wine A), loaded with 7.50 g/L glucose and 7,50 g/L of fructose;
Wine D: white wine naturally containing sugar (wine A), loaded with 10.0 g/L glucose and 10.0 g/L of fructose;
Wine E: aromatised wine;
Wine F: white wine naturally containing less than 0.4 g/L of sugar, loaded with 22.50 g/L glucose and 22.50 g/L of fructose;
Wine G: naturally sweet red wine;
Wine H: sweet white wine;
Wine I: basis wine;
Wine J: white wine naturally containing less than 0.4 g/L of sugar, loaded with 115.00 g/L glucose and 115.00 g/L of fructose;

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**Method OIV-MA-AS311-08** Type IV method

# Whole determination of glucose, fructose and saccharose content in wines by differential ph-metry

(Resolution Oeno 11/2006)

## 1. SCOPE

This method is applicable to the analysis of glucose and fructose in wines between 0 and 270 g/L.
This quantification is different from glucose and fructose quantification by its differential pH-metry which can not be substituted.

## 2. PRINCIPLE

The determination by differential pH-metry of glucose, fructose and saccharose content consists in the preliminary hydroloysis of saccharose by invertase, followed by phosphorylation of the glucose and fructose by hexokinase. The $H^+$ ions generated stoechiometrically in relation to the quantities of glucose and fructose are then quantified.

## 3. REACTIONS

Possible traces of saccharose are hydrolysed by invertase (EC 3.2.1.26)

$$\text{Saccharose} \xrightarrow{\text{invertase}} \text{glucose + fructose}$$

The glucose and fructose initially or consecutively present to invertase action are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by hexokinase (HK) (EC. 2.7.1.1)

$$\text{glucose + ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate + ADP + } H^+$$

$$\text{fructose + ATP} \xrightarrow{\text{HK}} \text{fructose-6-phosphate + ADP+ } H^+$$

## 4. REAGENTS

**4.1 Demineralised Water (18 MΩ) or bi-distilled**

**4.2 2-Amino-2-(hydroxymethyl)propane-1,3-diol (TRIS)** purity ≥ 99%

**4.3 Disodic adenosine triphosphate (ATP, 2Na)** purity ≥ 99%

**4.4 Trisodium phosphate with twelve water molecules ($Na_3PO_4.12H_2O$)** purity ≥ 99%

**4.5 Sodium hydroxide (NaOH)** purity ≥ 98%

**4.6 Magnesium chloride with six water molecules ($MgCl_2.6H_2O$)** purity ≥ 99%

**4.7 Triton X 100**

**4.8 Potassium chloride (KCl)** purity ≥ 99%

**4.9 2-Bromo-2-nitropropane-1,3-diol (Bronopol) ($C_3H_6BrNO_4$)**

**4.10 Invertase (EC 3.2.1.26) 1 mg** ≅ 500 U (ex Sigma ref I-4504)

**4.11 Hexokinase (EC. 2.7.1.1)** 1 mg ≅145 U (e.g. Hofmann La Roche, Mannheim, Germany ref. Hexo-70-1351)

**4.12 Glycerol** purity ≥ 98%

**4.13 Saccharose** purity ≥ 99%

**4.14 Reagent buffer pH 8.0** commercial (ex. DIFFCHAMB GEN 644) or prepared according to the following method:
In a graduated 100-ml flask (5.2) pour roughly 70 ml (5.3) of water (4.1), and continuously stir (5.5). Add 0.242 g ± 0.001 g (5.4) of TRIS (4.2), 0.787 g ± 0.001 g (5.4) of ATP (4.3), 0.494 g ± 0.001 g (5.4) of sodium phosphate (4.4), 0.009 mg ± 0.001

g (5.4) of sodium hydroxide (4.5), 0.203 g ± 0.001 g (5.4) of magnesium chloride (4.6), 2.000 ± 0.001 g (5.4) of Triton X 100 (4.7), 0.820 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 ± 0.001 g (4.9) of bronopol. Adjust to volume with water (4.1). The final pH must be 8.0 ± 0.1 (5.6), otherwise

adjust it with sodium hydroxide or hydrochloric acid. The buffer thus prepared is stable for two months at 4°C.

**4.15 Enzyme solution** commercial or prepared according to the following method:
Using a graduated pipette (5.7) place 5 ml of glycerol (4.11) into a graduated 10-ml flask, adjust to volume with water (4.1) and homogenize. Dissolve 300 mg ±1 mg (5.4) of invertase (4.10) 10 mg ± 1 mg (5.4) of hexokinase (4.11) in 3 mL of glycerol solution. Enzyme solution activity must be 50 000 U ± 100 U per ml for intervase and 480 U ± 50 U for hexokinase. The enzyme solution is stable for 6 months at 4°C.

**4.16 PREPARATION OF REFERENCE SOLUTION**

Place 17,100 g ± 0.01 g (5.4) of saccharose (4.13) (desiccated 12 hours beforehand
at 40 °C until constant weight), 0.745 g ± 0.001 g (5.4) of potassium chloride (4.8) and 0.010 g ± 0.001 g (5.4) of bronopol in a graduated 100-ml flask (5.2). Add water (4.1). Fully homogenize (5.5). Adjust to volume with water (4.1) after removing the magnetic bar. The final concentration is 171 g/L of saccharose. The solution is stable for 6 months at 4°C.

## 5. APPARATUS

**5.1 Differential pH-metry apparatus (EUROCHEM CL 10 plus, Microlab EFA or equivalent) see appendix A**

**5.2 Graduated 100-ml flask, class A**

**5.3 Graduated 100-ml test-tube with foot**

**5.4 Precision balance to weigh within 1 mg**

**5.5 Magnetic stirrer and magnetic Teflon bar**

**5.6 pH-meter**

**5.7 Graduated 3-mL, 5-mL pipette, class A**

**5.8 Graduated 10-ml flask, class A**

**5.9 Automatic syringe pipettes, 25 and 50 µL**

## 6. PREPARATION OF SAMPLES

Samples must not contain excessive suspended matter. If this occurs, the solution centrifuge and filter. Sparkling wines must be degassed

## 7. PROCEDURE

The operator must respect the instructions for use of the equipment (5.1). Before any use, the instrument must be stabilized in temperature. The circuits must be rinsed with the buffer solution (4.14) after cleaning, if required.

**7.1 Determination of the blank** (determination of the enzyme signal)

Fill the electrode compartments ($EL_1$ and $EL_2$) of the differential pH-meter (5.1) with the buffer solution (4.14); the potential difference between the two electrodes ($D_1$) must range between ± 150 mpH;

Add 32 µL of enzyme solution (4.15) to the reaction vessel (using the micropipette 5.9 or the preparer) and fill electrode $EL_2$;

Measure the potential difference ($D_2$) between the two electrodes;

Calculate the difference in pH, $\Delta pH_o$ for the blank using the following formula:

$$\Delta pH_o = D_2 - D_1$$

where

$\Delta pH_o$ = the difference in pH between two measurements for the blank;

$D_1$ = the value of the difference in pH between the two electrodes filled with the buffer solution;

$D_2$ = the value of the difference in pH between the two electrodes, one of which is filled with the buffer solution and the other with the buffer solution and enzyme solution.

The value of $\Delta pH_o$ is used to check the state of the electrodes during titration as well as their possible drift over time; it must lie between –30 and 0 mpH and ≤ 1.5 mpH between two consecutive readings. If not, check the quality of the buffer pH and the cleanliness of the hydraulic system and electrodes, clean if necessary and then repeat the blank.

**7.2 Calibration**

Fill the electrode compartments ($EL_1$ and $EL_2$) with the buffer solution (4.14);

Add 10 µL (with the micropipette 5.9 or the preparer) of the standard saccharose solution (5) to the reaction vessel;

Fill the electrodes $EL_1$ and $EL_2$ with the buffer + standard solution;

Measure the potential difference ($D_3$) between the two electrodes;

Add 32 µL of enzyme solution (4.15) and fill electrode $EL_2$ with the buffer + standard solution + enzyme;

After the time necessary for the enzymatic reaction, measure the potential difference ($D_4$) between the two electrodes;

Calculate the difference in pH, $\Delta pH_c$ for the calibration sample using the following formula:

$$\Delta pH_c = (D_4 – D_3) - \Delta pH_o$$

where
$\Delta pH_c$ = the difference between two measurements $D_3$ and $D_4$ for the calibration sample minus the difference obtained for the blank;

$D_3$ = the value of the difference in pH between the two electrodes filled with the reference buffer/solution mixture;
$D_4$ = the value of the difference in pH between the two electrodes, one of which is filled with the reference buffer/solution and the other with the buffer/ enzyme / reference solution.
Calculate the slope of the calibration line:

$$s = C_u / \Delta pH_c$$

where
$C_u$ is the concentration of saccharose in the standard solution expressed in g/L.

Check the validity of the calibration by analysing 10 µL of standard solution (ML) of saccharose (5) according to the procedure (8.3). The result must range between ± 2% of the reference value. If not, repeat the calibration procedure.

### 7.3 Quantification

Fill the electrode compartments ($EL_1$ and $EL_2$) with the buffer solution (4.14)
Add 10 µL (with the micropipette 5.9 or the preparer) of the sample solution to the reaction vessel;
Fill electrodes $EL_1$ and $EL_2$with the buffer + sample mixture;
Measure the potential difference ($D_5$) between the two electrodes;
Add 32 µL of the enzyme solution (4.15) and fill electrode $EL_2$ with the buffer mixture + sample + enzyme;
Measure the potential difference (D6) between the two electrodes;
Calculate the quantity of aqueous solution in the sample using the following formula:

$$w = s \times [(D_6 – D_5) - \Delta pH_o]$$

where
w = the quantity of aqueous solution in the sample (in g/L);
S is the slope determined by the calibration line;
$\Delta pH_o$ = the difference in pH between two measurements for the blank;
$D_5$ = the value of the difference in pH between the two electrodes filled with the sample/ reference solution;
$D_6$ = the value of the difference in pH between the two electrodes, one of which is filled with the buffer/sample and the other with the buffer/ sample /enzyme.

## 8 EXPRESSION OF RESULTS

The results are expressed in g/L of glucose with one significant figure after the decimal point.

## 9 CHARACTERISTICS OF THE ANALYSIS

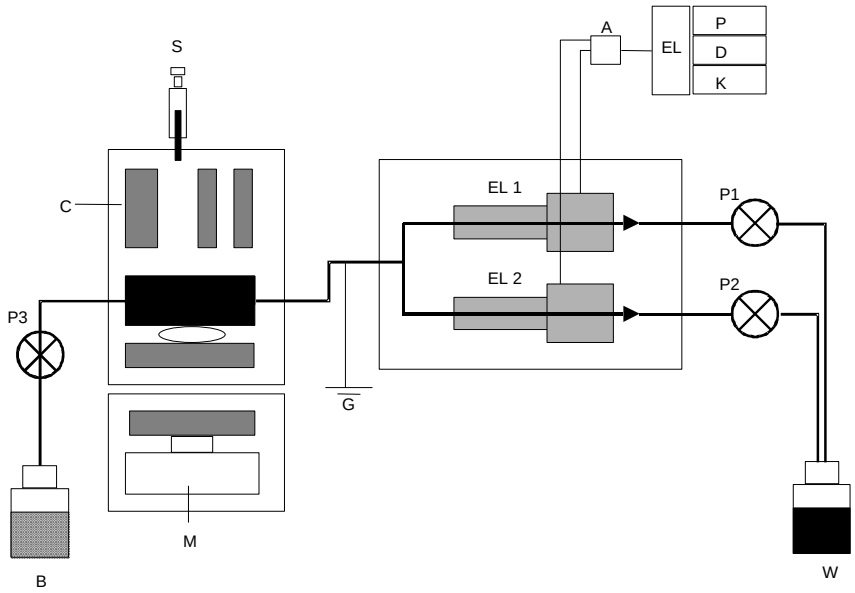
Due to the hydrolysis of saccharose in wines and musts, it is not possible to organise an inter-laboratory analysis according to the OIV protocol.
Inter-laboratory studies of this method demonstrate that for saccharose, the linearity between 0 and 250 g/l, a detection limit of 0.2 g/l, a quantification limit of 0.6 g/l, repeatability of 0.0837x -0.0249 g/l and reproducibility of 0.0935x -0.073 g/l (saccharose content).

## 10 QUALITY CONTROL

Quality controls can be carried out with certified reference materials, wines whose characteristics have been determined by consensus, or loaded wines regularly used in analytical series, and by following the related control charts.

## Appendix A

### Diagram of the differential pH-metry apparatus




A: differential amplifier; B: buffer solution; C: mixing chamber; D: indicator; $EL_1$ and $EL_2$ capillary electrodes; EL: electronics; G: ground; K: keyboard; M: magnetic stirrer; P: printer; $P_1$ to $P_3$: peristaltic pumps; S: injection syringe for the sample and enzyme; W: waste.

## Appendix B

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**Method OIV-MA-AS311-09** Type II and III method

# Determination of the $^{13}C/^{12}C$ isotope ratios of glucose, fructose, glycerol, ethanol in production of vitivinicultural origin by high-performance liquid chromatography coupled to isotope ratio mass spectrometry

(Resolution Oeno 479/2017)

## 1. Scope of application

This method applies to products of vitivinicultural origin.
This method is:

- type II for glucose, fructose and glycerol,
- type III for ethanol.

## 2. Principle

The samples are injected into the HPLC instrument after any necessary dilution and filtration. After oxidation in a liquid interface, the $^{13}C/^{12}C$ isotope ratio of the compounds is determined using isotope ratio mass spectrometry. This liquid interface, symbolised by the acronym "*co"*, permits the chemical oxidation of the organic matter into CO2. HPLC-*co*-IRMS coupling can therefore be used to determine the isotope ratio of the following compounds simultaneously: glucose, fructose, glycerol and ethanol.

## 3. Reagents

3.1 Pure water - resistivity > 18 MΩ cm, HPLC quality
3.2 Ammonium persulfate – analytical purity – [CAS No.: 7727-54-0]
3.3 Orthophosphoric acid (concentration 85%) – analytical purity – [CAS No.: 7664-38-2]
3.4 Analytical-grade helium, used as a carrier gas [CAS No.: 07440-59-7]
3.5 Reference gas: analytical-grade $CO_2$ (carbon dioxide), used as a secondary reference gas [CAS No.: 00124-38-9]
3.6 International standards

## 4. Equipment

4.1 Everyday laboratory equipment
4.2 High-performance liquid chromatography instrument
4.3 Liquid interface for the oxidation of eluted compounds
4.4 Isotope ratio mass spectrometer

## 5. Analysis of the samples

### 5.1 Preparation of the samples

Depending on the sugar, glycerol and ethanol contents, the samples should be diluted with the water (3.1) beforehand in order to obtain a concentration which is observable under the experimental conditions. Depending on the concentrations of the compounds, two measurements are needed with different dilutions.

### 5.2 Example of analytical conditions

Total analysis duration: 20 minutes
As an indication, the dilution of grape juices and wines is around 1:200, while that of concentrated musts is approximately 1:500.

HPLC:

Column: carbohydrate-type column (e.g. 700-CH Carbohydrate column, HyperRez XP Carbohydrate $H^+$)
Injection volume: 25 µl
Mobile phase: water (3.1)
Flowrate: 0.4 mL/min
Column T°: 80 °C

Liquid Interface:

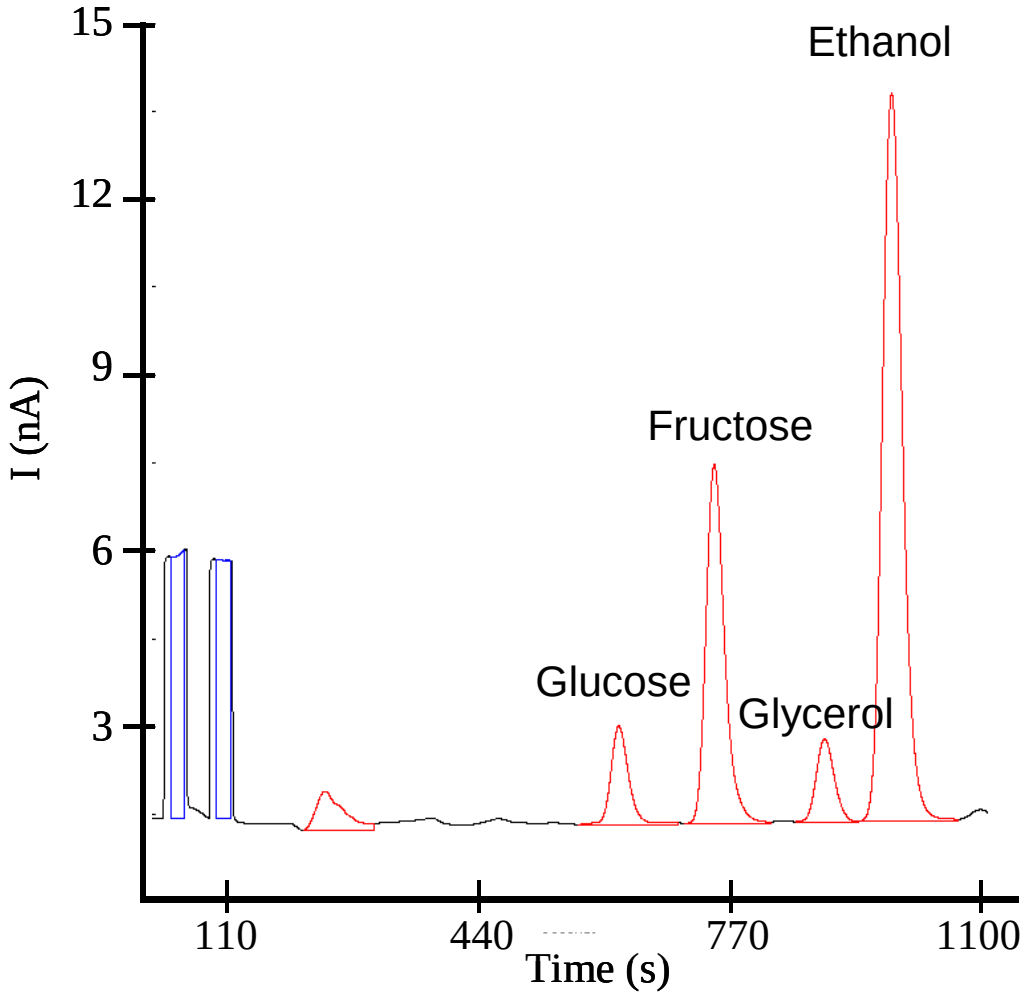
Solution of ammonium persulfate (3.2) (15% in mass) and orthophosphoric acid (2.5% in volume)
Peristaltic pump flow: 0.6 mL/min
Heater temperature: 93 °C
Flow of the helium carrier gas: 15 mL/min
Helium flow for drying: 50 mL/min

IRMS:

Trap current: 300 µA

**5.3 Example chromatogram**

Chromatogram of a sweet wine analysed using HPLC-*co*-IRMS




**6. Determination of isotope ratios**

The reference gas, $CO_2$, is calibrated from international commercial standards. The isotope ratios are expressed in δ ‰ in relation to the Pee Dee Belemnite (PDB) and are defined as:

$$\delta^{13}C_{Sam}\ (‰) = [(R_{Sam} / R_{St}) - 1\ ] * 10^3$$

Where: Sam = sample; St = standard; R = $^{13}C/^{12}C$ isotope ratio

## 7. Method characteristics

The characteristics of the method for the measurement of the $\delta^{13}C$ isotope ratios of glucose, fructose, glycerol and ethanol by HPLC-*co*-IRMS have been determined from the results obtained from an inter-laboratory analysis of four samples (dry wine, sweet wine, grape juice and rectified concentrated must). The results obtained for each compound analysed and each type of matrix are annexed.

## 8. Bibliography

**1**. Cabanero, AI.; Recio, JL.; Rupérez, M. (2008) Isotope ratio mass spectrometry coupled to liquid and gas chromatography for wine ethanol characterization. Rapid Commun. Mass Spectrom. 22: 3111-3118.

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**3**. Guyon, F.; Gaillard, L.; Salagoïty, MH.; Médina, B. (2011) Intrinsic Ratios of Glucose, Fructose, Glycerol and Ethanol $^{13}C/^{12}C$ Isotopic Ratio Determined by HPLC-*co*-IRMS: Toward Determining Constants for Wine Authentication. Anal. Bioanal. Chem. 401:1551-1558

# Annex

## Statistical treatment of the HPLC-*co*-IRMS inter-laboratory analysis for the determination of the precision of the method (repeatability and reproducibility)

**List of laboratories in alphabetical order of country of origin.**

| Country | Laboratory |
|---|---|
| Belgium | IRMM |
| China | CNRIFFI |
| Czech Republic | SZPI |
| France | SCL-33 |
| Germany | INTERTEK |
| Germany | UNI DUE |
| Germany | ELEMENTAR |
| Germany | QSI |
| Germany | LVI |
| Italy | FLORAMO |
| Japan | AKITA Univ. |
| Spain | MAGRAMA |

**Responses:**

12 laboratories / 14 responses

**Treatment of the results of inter-laboratory analyses according to ISO 5725-2**

**Samples:**

1 dry wine (Wine A)
1 sweet wine (Wine B)
1 rectified concentrated must (RCM)
1 grape juice

**Analytical conditions:**

Each sample was analysed in duplicate (repeatability) and double blind (reproducibility)

Expression of results in ‰ vs. PDB

# Precision of the glucose measurement

## Repeatability and reproducibility

| | Wine B | RCM | Grape juice |
|---|---|---|---|
| Number of laboratories | 12 | 12 | 12 |
| Number of responses | 14 | 13 | 14 |
| Number of responses retained (elimination of outliers) | 13 | 13 | 12 |
| | | | |
| Minimum value | -26.33 | -25.04 | -25.78 |
| Maximum value | -23.72 | -23.74 | -24.62 |
| Mean value | -25.10 | -24.24 | -25.19 |
| Repeatability variance | 0.02 | 0.01 | 0.01 |
| Repeatability standard deviation ($S_r$) | 0.14 | 0.10 | 0.09 |
| Repeatability limit (r ‰) | 0.40 | 0.29 | 0.24 |
| Reproducibility variance | 0.39 | 0.14 | 0.11 |
| Reproducibility standard deviation ($S_R$) | 0.62 | 0.38 | 0.33 |
| Reproducibility limit (R ‰) | 1.77 | 1.06 | 0.94 |

# Precision of the fructose measurement

## Repeatability and reproducibility

| | Wine B | RCM | Grape juice |
|---|---|---|---|
| Number of laboratories | 12 | 11 | 12 |
| Number of responses | 14 | 13 | 14 |
| Number of responses retained (elimination of outliers) | 13 | 13 | 13 |
| Minimum value | -25.56 | -24.19 | -25.33 |
| Maximum value | -24.12 | -23.19 | -23.98 |
| Mean value | -24.87 | -23.65 | -24.56 |
| Repeatability variance | 0.02 | 0.03 | 0.02 |
| Repeatability standard deviation ($S_r$) | 0.14 | 0.16 | 0.14 |
| Repeatability limit (r ‰) | 0.40 | 0.46 | 0.39 |
| Reproducibility variance | 0.15 | 0.10 | 0.18 |
| Reproducibility standard deviation ($S_R$) | 0.39 | 0.32 | 0.42 |
| Reproducibility limit (R ‰) | 1.10 | 0.90 | 1.19 |

# Precision of the glycerol measurement

## Repeatability and reproducibility

| | Wine A | Wine B |
|---|---|---|
| Number of laboratories | 12 | 12 |
| Number of responses | 12 | 12 |
| Number of responses retained (elimination of outliers) | 11 | 11 |
| Minimum value | -32.91 | -30.74 |
| Maximum value | -30.17 | -28.27 |
| Mean value | -31.75 | -29.54 |
| Repeatability variance | 0.13 | 0.04 |
| Repeatability standard deviation ($S_r$) | 0.36 | 0.19 |
| Repeatability limit (r ‰) | 1.03 | 0.55 |
| Reproducibility variance | 0.57 | 0.37 |
| Reproducibility standard deviation ($S_R$) | 0.76 | 0.61 |
| Reproducibility limit (R ‰) | 2.14 | 1.72 |

# Precision of the ethanol measurement

## Repeatability and reproducibility

| | Wine A | Wine B |
|---|---|---|
| Number of laboratories | 12 | 12 |
| Number of responses | 11 | 12 |
| Number of responses retained (elimination of outliers) | 10 | 12 |
| Minimum value | -27.85 | -27.60 |
| Maximum value | -26.50 | -26.06 |
| Mean value | -27.21 | -26.82 |
| Repeatability variance | 0.03 | 0.03 |
| Repeatability standard deviation ($S_r$) | 0.16 | 0.17 |
| Repeatability limit (r ‰) | 0.47 | 0.47 |
| Reproducibility variance | 0.16 | 0.23 |
| Reproducibility standard deviation ($S_R$) | 0.40 | 0.47 |
| Reproducibility limit (R ‰) | 1.14 | 1.34 |

**Method OIV-MA-A311-10** Type III method

# Determination of D-glucose and D-fructose in wines by automated enzymatic method

(Résolution OIV-OENO 600/2018)

## 1. Scope of application

This method makes it possible to determine the sum of D-glucose and D-fructose in wine by specific enzyme analysis using an automatic sequential analyser.
In this document a collaborative study is reported which demonstrates application of the method for measurement of D-glucose and D-fructose from 0.1 to 96.31 g/L, taking into account the introduction of a dilution of the sample above 5 g/L.

Note: Where necessary, each laboratory using this method may refine, and potentially widen, this range through a validation study.

## 2. Standard references

- OIV *Compendium of International Methods of Analysis*: Glucose and fructose – enzymatic method, OIV-MA-AS311-02,
- ISO 78-2: Chemistry – Layouts for standards.

## 3. Reaction principles

D-glucose and D-fructose are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by hexokinase (HK) to produce glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P).

$$D-glucose + ATP \overset{HK}{\leftrightarrow} G6P + ADP$$

$$D-fructose\ +\ ATP \overset{HK}{\leftrightarrow} F6P\ +\ ADP$$

Glucose-6-phosphate is first oxidised to gluconate-6-phosphate by nicotinamide adenine dinucleotide phosphate (NADP) in the presence of the enzyme glucose-6-phosphate dehydrogenase (G6PDH). The quantity of reduced nicotinamide adenine dinucleotide phosphate (NADPH) is directly correlated with that of glucose-6-phosphate and thus with that of D-glucose.

$$G6P\ +\ NADP^{+} \overset{G6PDH}{\longleftrightarrow} gluconate-6-phosphate\ +\ NADPH\ +\ H^{+}$$

Fructose-6-phosphate (F6P) is converted into glucose-6-phosphate (G6P) in the presence of phosphoglucose isomerase (PGI):

$$F6P \overset{PGI}{\leftrightarrow} G6P$$

The glucose-6-phosphate thus formed reacts as shown in the above formula.
The reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced is measured based on its absorption at 340 nm.

## 4. Reagents and working solutions

During the analysis – unless stated otherwise – only use reagents of recognised analytical grade and water that is distilled, demineralised or of equivalent purity.

### 4.1. Reagents

4.1.1. Quality I or II water for analytical usage (ISO 3696 standard)

4.1.2. Triethanolamine hydrochloride (CAS no. 637-39-8)

4.1.3. NADP (nicotinamide adenine dinucleotide phosphate) (CAS no. 24292-60-2)

4.1.4. ATP (adenosine-5'-triphosphate) (CAS no. 34369-07-8)

4.1.5. MgSO4 (anhydrous magnesium sulphate) (CAS no. 7487-88-9)

4.1.6. Sodium hydroxide (CAS no. 1310-73-2)

4.1.7. Hexokinase (HK) (CAS no. 9001-51-8)

4.1.8. Glucose-6-phosphate dehydrogenase (G6PDH) (CAS no. 9001-40-5)

4.1.9. Phosphoglucose isomerase (PGI): lyophilised powder, 400-600 units/mg protein (CAS no. 9001-41-6)

*Note: One unit ensures the conversion of 1.0 µmole of D-fructose-6-phosphate into D-glucose-6-phosphate per minute at pH 7.4 and 25 °C*

4.1.10. Polyvinylpyrrolidone (PVP) (CAS no. 9003-39-8)

4.1.11. D-glucose: purity ≥ 99.5% (CAS no. 50-99-7)

4.1.12. D-fructose: purity ≥ 99% (CAS no. 57-48-7)

**Note 1:** There are commercial kits for the determination of D-glucose and D-fructose. The user needs to check the composition to ensure it contains the above-indicated reagents.

**Note 2:** The use of PVP is recommended to eliminate any possible negative effect of tannins in wine on the enzyme protein molecules. This is the case particularly in red wines. Should the use of PVP not prove effective, the laboratory should ensure that the wine tannins do not interfere with the enzymes.

### 4.2. Working solutions

4.2.1. Triethanolamine hydrochloride buffer and magnesium sulphate adjusted to pH 7.6. The preparation may be as follows:

- triethanolamine hydrochloride (4.1.2): 11.2 g,
- magnesium sulphate (4.1.5): 0.2 g,
- PVP (4.1.10): 2 g,
- water for analytical usage (4.1.1): 150 mL.

The mixture is adjusted to pH 7.6 using a 5 M sodium hydroxide solution, then made up to 200 mL with water for analytical usage. The solution is stable for at least 4 weeks at 2-8 °C.

4.2.2. R1 working solution (example):
- triethanolamine buffer (4.2.1): 50 mL,
- NADP (4.1.3): 117 mg,
- ATP (4.1.4): 150 mg.

4.2.3. R2 working solution (example):
- triethanolamine buffer (4.2.1): 2 mL,
- HK (4.1.7): 270 U,
- G6PDH (4.1.8): 340 U,
- PGI (4.1.9): 640 U.

**Note**: Commercial preparations of a HK/6GPDH mixture may be used.

**Note:** When preparing these solutions, they should be mixed gently to prevent foam from forming. The life cycle of the working solutions is limited and should be evaluated and respected by the laboratory.

### 4.3. Calibration solutions

To ensure the closest possible connection to the International System of Units (SI), the calibration range should be created using pure solutions of D-glucose and D-fructose prepared by weighing and covering the measurement range.

## 5. Apparatus

### 5.1. Analyser

5.1.1. Equipment type

Automatic sequential analyser equipped with a spectrophotometer with UV detector. The reaction temperature should be stable (around 37 °C). The reaction cuvettes are glass, methacrylate or quartz. The equipment is controlled by software ensuring its operation, data acquisition and useful calculations.

5.1.2. Absorbance reading

The concentration of the analytes directly relates to the absorbance difference read by the spectrophotometer. The precision of the absorbance reading should be a minimum of 0.1 absorbance unit (AU). It is preferable not to use absorbance values higher than 2.0.

5.1.3. Precision of volumes collected

The precision of the volumes of reagents and samples collected by the pipettes of the analyser influences the measurement result. Quality control of the results using appropriate strategies (e.g. according to the guides published by the OIV) is recommended.

5.1.4. Reaction duration and temperature

In general, the reaction time is 10 minutes and the temperature is 37 °C. Certain pieces of apparatus may use slightly different values.

5.1.5. Wavelength

The wavelength of maximum absorption of the NADPH formed by the reaction is 340 nm. This wavelength will be selected for the spectrophotometers commonly used. Some analysers are equipped with photometers that use a mercury-vapor lamp. In this case, a wavelength with a reading of 365 or 334 nm is to be selected.

### 5.2. Balance

This should be calibrated to the International System of Units and have 1 mg precision.

### 5.3. pH meter

### 5.4. Measuring glassware

The measuring glassware for the preparation of reagents and calibration solutions is class A.

## 6. Sampling

### 6.1 Preparation of samples of musts and wines

The majority of wine and must samples may be analysed without preparation. In some cases, a preparation may be introduced:

filtration should be used for highly turbid samples,
sample dilution (manual or automatic) with water for analytical usage (4.1.1) should be used for values exceeding the measurement range. By way of example, factors of 10x, 20x or 40x are used for musts. Given their impact on the uncertainty budget, these dilutions should be conducted with the utmost care.

### 6.2 Preparation of samples of wines containing $CO_2$

Wine samples containing $CO_2$ may produce bubbling effects. They must be degassed beforehand by stirring under vacuum, ultrasonic processing or any method enabling the required degassing.

## 7. Procedure

Given that different analysers may be used, it is recommended that the conditions of use provided by the manufacturer are strictly observed. This also applies to the different enzymatic kits available on the market.

The procedure takes place as follows:

1. The sample (S) is placed in a reaction cuvette.
2. Working solution R1 (4.2.2) is then added to the cuvette.
3. The two are mixed together. Time is then allowed for a lag period, in order to guarantee absorbance stability. This lag period may last from 1-5 min, and is defined by the laboratory, according to the characteristics of the equipment used.
4. Working solution R2 (4.2.3) is added and the reaction takes place.

By way of example, the quantities of different elements may be as follows:

- sample: 2.0 µL,
- R1: 40 µL,
- R2: 40 µL.

The equipment takes regular measurements (every 12 seconds, for example) that make it possible to obtain a reaction curve, an example of which is given in Figure 1.

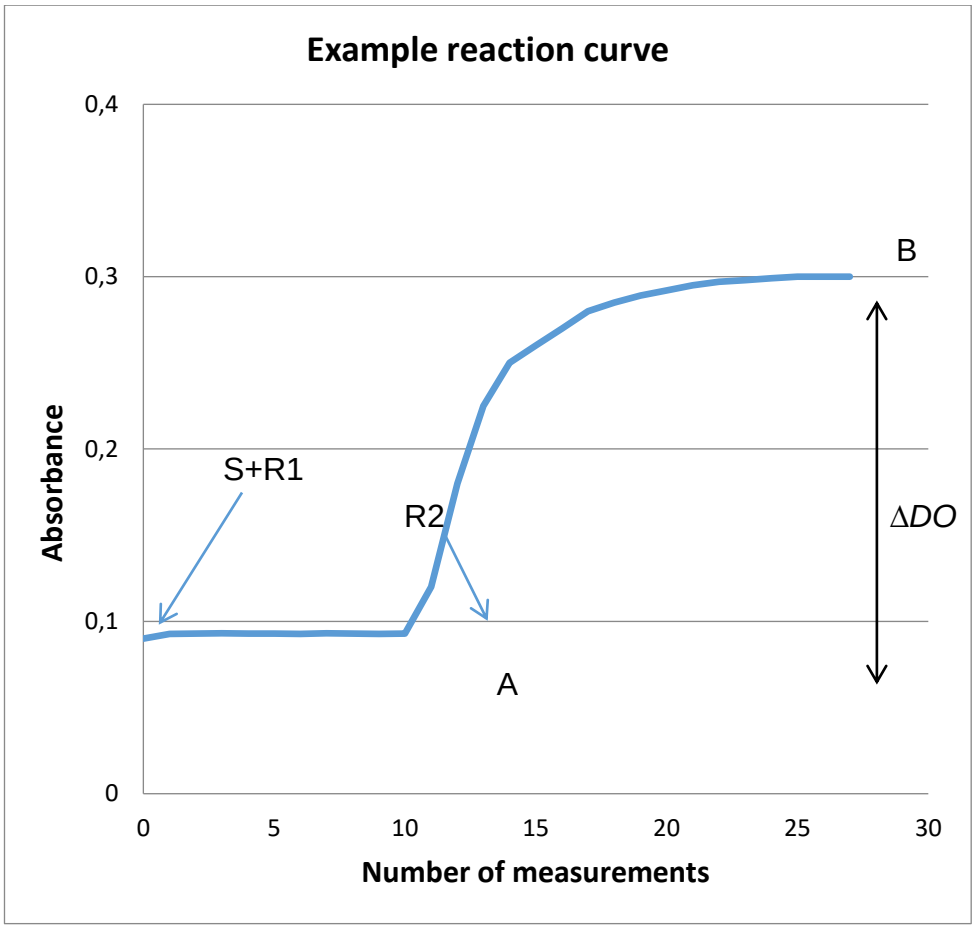

Figure 1: Reaction curve

The equipment makes it possible to choose the reading points for the difference in absorbance sought, for example A and B in Figure 1.

## 8. Calculation of results

The measurement used for the determination of the result is as follows:

$$\Delta DO = (\text{Absorbance } B - \text{Absorbance } A)$$

In order to correlate this ΔDO value with the desired concentration of D-glucose and D-fructose, calibration of the equipment is carried out using the calibration solutions at a minimum of 3 points (§4.3) covering the measurement range. In addition, a

reagent blank is used comprising all of the reagents but no sample (point 0 of the calibration).

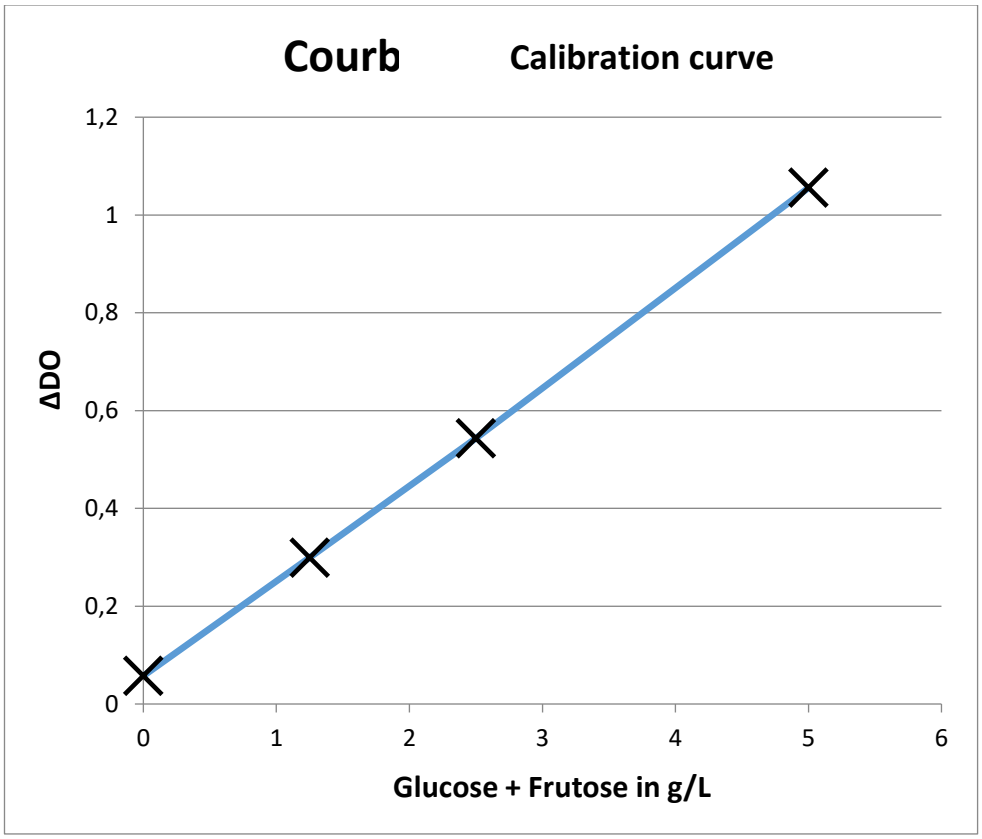


Figure 2: Calibration curve

The calibration curve can be order 1 (*Concentration* = $a.\Delta DO + b$) or even order 2 (*Concentration* = $a.\Delta DO^2 + b.\Delta DO + c$). If using a calibration curve of order 2, the laboratory should take care to limit the calibration domain in order to maintain sufficient sensitivity of the method (risk of crushing the curve).

The final value obtained should be multiplied by any coefficient of dilution used.

## 9. Expression of results

The D-glucose + D-fructose results are expressed in g/L to 2 d.p.

## 10. Precision

**Interlaboratory reproducibility**

$RSD_R$ = 5% (from 1 g/L)
$CV_R$% (k=2) = 2·$RSD_R$= 10%, (from 1 g/L)

**Repeatability**

$RSD_r$= 1.5% (from 1 g/L)
$CV_r$% (k=2) = 2·$RSD_r$ = 3% (from 1 g/L)

**Limit of quantification**

Validated LOQ = 0, 10 g/L
(Concentration where $CV_R$% (k=2) = 60%)

## ANNEX
## Results of the interlaboratory tests

Collaborative study
A total of 17 laboratories from different countries participated in the collaborative study, organised in 2016.

| Labo | Country |
|---|---|
| Miguel Torres S.A.- Finca Mas La Plana | SPAIN |
| Estación Enológica de Castilla y León | SPAIN |
| INGACAL -Consellería do Medio Rural<br>Estación de Viticultura e Enoloxía de Galicia | SPAIN |
| Estación Enológica de Haro | SPAIN |
| Instituto dos Vinhos do Douro e do Porto, IP | PORTUGAL |
| Comissão de Viticultura da Região dos Vinhos Verdes | PORTUGAL |
| Laboratoires Dubernet | FRANCE |
| Laboratoire Diœnos Rhône | FRANCE |
| Laboratoire Natoli | FRANCE |
| SCL Montpellier | FRANCE |
| Agricultural institute of Slovenia | SLOVENIA |

| | |
|---|---|
| Fachbereich: Wein, Weinüberwachung - Chemisches und Veterinärunterchungsamt Karlsruhe | GERMANY |
| HBLAuBA Wein - und Obstbau | AUSTRIA |
| Landesuntersuchungsamt Mainz | GERMANY |
| Hochschule GEISENHEIM University Institut Weinanalytik und Getränkeforschung | GERMANY |
| Unità Chimica Vitienologica e Agroalimentare - Centro Trasferimento Tecnologico - Fondazione Edmund Mach | ITALY |
| Unione Italiana Vini soc. Coop. | ITALY |

For analysis, 2 x 10 blind duplicate samples were used, with 1 repetition. The wines analysed are wines originating from France and Portugal, dry wines and liqueur wines.

| Sample | | A | | B | | C | | D | | E | | F | | G | | H | | I | | J | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Position | | 1 | 9 | 2 | 13 | 3 | 4 | 5 | 15 | 6 | 10 | 16 | 20 | 7 | 11 | 12 | 17 | 8 | 19 | 14 | 18 |
| Labo3 | rep#1 | 94.00 | 96.00 | 3.40 | 3.50 | **0.40** | **0.40** | 0.90 | 1.10 | **2.10** | **2.50** | 0.10 | 0.10 | 1.40 | 1.40 | 5.60 | 5.90 | **4.70** | **4.20** | 17.50 | 17.00 |
| | rep#2 | 96.00 | 98.00 | 3.50 | 3.60 | **0.40** | **0.30** | 1.00 | 1.10 | **2.20** | **2.40** | 0.10 | 0.10 | 1.40 | 1.40 | 5.70 | 6.00 | **4.30** | **4.50** | 17.50 | 17.00 |
| Labo6 | rep#1 | 97.50 | 95.00 | 3.42 | 3.25 | **0.35** | **0.48** | 1.05 | 0.98 | **3.24** | **2.65** | 0.08 | 0.05 | 1.42 | 1.40 | 5.49 | 5.57 | 4.04 | 4.11 | 13.63 | 19.00 |
| | rep#2 | 97.00 | 94.50 | 3.39 | 3.29 | **0.37** | **0.57** | 1.08 | 1.01 | **3.34** | **2.66** | 0.08 | 0.08 | 1.52 | 1.45 | 5.42 | 5.52 | 3.95 | 4.13 | 13.70 | 20.50 |
| Labo7 | rep#1 | 99.22 | 99.53 | 3.46 | 3.56 | 0.31 | 0.34 | 1.00 | 0.98 | 2.50 | 2.58 | 0.04 | 0.04 | 1.49 | 1.39 | 5.77 | 5.75 | 4.26 | 4.35 | 17.66 | 17.35 |
| | rep#2 | 100.30 | 98.90 | 3.53 | 3.53 | 0.31 | 0.32 | 1.02 | 1.02 | 2.48 | 2.50 | 0.04 | 0.02 | 1.48 | 1.34 | 5.89 | 5.79 | 4.23 | 4.40 | 17.21 | 17.94 |
| Labo9 | rep#1 | 92.00 | 94.20 | 3.05 | 3.03 | 0.29 | 0.30 | 0.93 | 0.97 | 2.30 | 2.16 | 0.04 | 0.04 | 1.25 | 1.25 | 5.02 | 5.01 | 3.98 | 3.76 | 15.60 | 15.76 |
| | rep#2 | 95.00 | 97.25 | 3.03 | 3.23 | 0.32 | 0.31 | 0.94 | 0.90 | 2.20 | 2.29 | 0.03 | 0.04 | 1.27 | 1.25 | 5.14 | 5.39 | 3.80 | 4.06 | 16.64 | 16.40 |
| Labo10 | rep#1 | 90.79 | 92.31 | 3.27 | 3.36 | 0.34 | 0.34 | 0.97 | 1.01 | 2.28 | 2.30 | 0.09 | 0.07 | 1.28 | 1.26 | 5.46 | 5.42 | 3.27 | 3.36 | 17.92 | 17.99 |
| | rep#2 | 92.13 | 91.65 | 3.34 | 3.24 | 0.32 | 0.35 | 0.97 | 1.04 | 2.28 | 2.33 | 0.08 | 0.08 | 1.32 | 1.28 | 5.18 | 5.37 | 3.34 | 3.24 | 17.58 | 17.68 |
| Labo11 | rep#1 | 91.40 | 91.28 | 3.06 | 3.12 | 0.57 | 0.30 | 0.95 | 0.93 | 2.15 | 2.18 | 0.07 | 0.05 | 1.16 | 1.22 | 5.19 | 5.34 | 3.70 | 3.86 | 16.22 | 16.47 |
| | rep#2 | 90.13 | 89.94 | 3.10 | 3.14 | 0.56 | 0.30 | 0.93 | 0.93 | 2.14 | 2.18 | 0.07 | 0.06 | 1.16 | 1.20 | 5.28 | 5.18 | 3.76 | 3.86 | 16.13 | 16.33 |
| Labo12 | rep#1 | 100.00 | 100.00 | 3.25 | 3.27 | 0.34 | 0.33 | 1.03 | 1.10 | **2.35** | **2.75** | 0.08 | 0.10 | 1.30 | 1.39 | 5.66 | 5.64 | 4.07 | 4.13 | 17.30 | 17.44 |

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | rep#2 | 101.00 | 97.00 | 3.22 | 3.25 | 0.34 | 0.33 | 1.03 | 1.11 | **2.36** | **2.75** | 0.08 | 0.10 | 1.30 | 1.39 | 5.62 | 5.68 | 4.07 | 4.15 | 17.50 | 17.80 |
| Labo13 | rep#1 | 96.60 | 96.00 | 3.04 | 3.07 | 0.34 | 0.31 | 0.97 | 0.94 | 2.26 | 2.50 | 0.05 | 0.04 | 1.25 | 1.25 | 5.21 | 5.29 | 3.84 | 3.99 | 16.08 | 16.03 |
| | rep#2 | 96.00 | 95.10 | 3.07 | 3.12 | 0.32 | 0.32 | 0.97 | 1.04 | 2.25 | 2.25 | 0.04 | 0.04 | 1.25 | 1.28 | 5.24 | 5.31 | 3.90 | 3.97 | 15.95 | 16.18 |
| Labo14 | rep#1 | **104.00** | **98.00** | 3.19 | 3.16 | 0.33 | 0.33 | 0.97 | 0.96 | 2.47 | 2.44 | 0.05 | 0.05 | 1.34 | 1.32 | 5.77 | 5.81 | 4.20 | 4.21 | 17.76 | 17.04 |
| | rep#2 | **103.00** | **96.00** | 3.18 | 3.17 | 0.33 | 0.33 | 0.97 | 0.97 | 2.48 | 2.44 | 0.05 | 0.05 | 1.34 | 1.32 | 5.77 | 5.78 | 4.20 | 4.14 | 17.44 | 17.24 |
| Labo15 | rep#1 | **110.03** | **99.25** | 3.63 | 3.60 | 0.20 | 0.19 | 0.94 | 0.97 | 2.54 | 2.36 | | | 1.30 | 1.20 | **5.65** | **6.14** | 4.56 | 4.43 | **17.16** | **19.33** |
| | rep#2 | **104.39** | **99.34** | 3.59 | 3.72 | 0.20 | 0.20 | 0.94 | 0.95 | 2.52 | 2.32 | | | 1.32 | 1.20 | **5.62** | **6.19** | 4.39 | 4.54 | **17.41** | **19.29** |
| Labo16 | rep#1 | 95.20 | 94.08 | 3.20 | 3.22 | 0.32 | 0.32 | 0.96 | 0.96 | 2.24 | 2.26 | 0.06 | 0.06 | 1.23 | 1.23 | 5.19 | 5.19 | 3.89 | 3.84 | 17.82 | 17.38 |
| | rep#2 | 96.00 | 94.41 | 3.17 | 3.18 | 0.31 | 0.33 | 0.95 | 0.94 | 2.25 | 2.22 | 0.06 | 0.06 | 1.24 | 1.22 | 5.13 | 5.15 | 3.85 | 3.86 | 17.84 | 17.24 |
| Labo17 | rep#1 | 96.68 | 97.10 | 3.28 | 3.38 | 0.47 | 0.43 | 1.03 | 1.03 | 2.41 | 2.46 | **0.10** | **0.20** | 1.36 | 1.36 | 5.52 | 5.53 | 4.09 | 4.00 | 16.42 | 17.30 |
| | rep#2 | 97.08 | 99.40 | 3.24 | 3.33 | 0.39 | 0.38 | 0.95 | 0.96 | 2.30 | 2.36 | **0.20** | **0.15** | 1.32 | 1.24 | 5.38 | 5.40 | 3.95 | 4.10 | 16.50 | 16.60 |
| Labo18 | rep#1 | 90.23 | 91.39 | 3.14 | 3.26 | 0.46 | 0.47 | 1.12 | 1.10 | 2.30 | 2.44 | **0.23** | **0.24** | 1.38 | 1.30 | 5.19 | 5.49 | 3.91 | 4.10 | 14.83 | 14.89 |
| | rep#2 | 90.02 | 91.74 | 3.18 | 3.31 | 0.47 | 0.47 | 1.07 | 1.07 | 2.31 | 2.40 | **0.23** | **0.24** | 1.38 | 1.32 | 5.23 | 5.45 | 3.94 | 4.04 | 14.82 | 14.85 |
| Labo19 | rep#1 | 99.63 | 103.55 | 3.34 | 3.41 | 0.32 | 0.32 | 0.98 | 0.97 | 2.38 | 2.41 | 0.04 | 0.05 | 1.29 | 1.30 | 5.68 | 5.56 | 4.10 | 4.11 | 17.61 | 17.49 |
| | rep#2 | 100.57 | 103.28 | 3.36 | 3.42 | 0.32 | 0.32 | 0.98 | 0.97 | 2.36 | 2.42 | 0.05 | 0.05 | 1.29 | 1.31 | 5.61 | 5.59 | 4.10 | 4.11 | 17.53 | 17.51 |
| Labo20 | rep#1 | 96.41 | 96.18 | 3.20 | 3.23 | 0.32 | 0.32 | 0.96 | 0.95 | 2.26 | 2.32 | 0.07 | 0.08 | 1.24 | 1.24 | 5.35 | 5.40 | 3.92 | 4.03 | 16.36 | 16.51 |

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | rep#2 | 96.32 | 95.89 | 3.18 | 3.23 | 0.32 | 0.32 | 0.96 | 0.95 | 2.26 | 2.32 | 0.07 | 0.08 | 1.24 | 1.24 | 5.35 | 5.38 | 3.92 | 4.03 | 16.38 | 16.49 |
| Labo21 | rep#1 | 103.60 | 102.02 | 3.37 | 3.60 | 0.23 | 0.25 | 0.95 | 0.98 | 2.41 | 2.49 | 0.05 | 0.05 | 1.27 | 1.33 | 5.95 | 6.12 | **4.02** | **4.53** | **18.41** | **19.70** |
| | rep#2 | 102.50 | 103.02 | 3.34 | 3.51 | 0.23 | 0.26 | 0.92 | 0.98 | 2.45 | 2.45 | 0.03 | 0.05 | 1.26 | 1.27 | 6.02 | 5.99 | **4.09** | **4.42** | **18.96** | **19.90** |
| Labo22 | rep#1 | 96.73 | 96.59 | 3.25 | 3.28 | 0.28 | 0.28 | 0.92 | 0.93 | 2.25 | 2.31 | 0.06 | 0.05 | 1.23 | 1.28 | 5.51 | 5.47 | 4.02 | 3.98 | 17.09 | 17.10 |
| | rep#2 | 97.06 | 96.34 | 3.24 | 3.21 | 0.30 | 0.30 | 0.93 | 0.93 | 2.26 | 2.30 | 0.04 | 0.05 | 1.21 | 1.24 | 5.40 | 5.39 | 4.03 | 4.04 | 17.05 | 17.01 |

**Table of the data obtained.** The values in bold correspond with the values rejected in accordance with the Cochran (variance outliers) test with a 2.5% significance level (one-tailed test), and the Grubbs (outliers from the mean) test with significance levels of 2.5% (two-tailed test).

*Note: The absent values have not been provided by the laboratory in question.*

| Sample | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** |
|---|---|---|---|---|---|---|---|---|---|---|
| No. of laboratories selected | 15 | 17 | 14 | 17 | 14 | 14 | 17 | 16 | 15 | 14 |
| No. of repetitions | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Min. | 90.69 | 3.08 | 0.20 | 0.93 | 2.16 | 0.04 | 1.19 | 5.14 | 3.80 | 14.85 |
| Max. | 102.79 | 3.64 | 0.47 | 1.09 | 2.52 | 0.10 | 1.45 | 6.02 | 4.48 | 17.79 |
| Overall average | 96.31 | 3.29 | 0.32 | 0.98 | 2.34 | 0.06 | 1.30 | 5.50 | 4.05 | 16.86 |
| Repeatability variance | 1.449 | 0.004 | 0.000 | 0.001 | 0.004 | 0.000 | 0.001 | 0.009 | 0.005 | 0.065 |
| Inter-laboratory stand. dev. | 3.60 | 0.16 | 0.06 | 0.05 | 0.10 | 0.02 | 0.07 | 0.26 | 0.17 | 0.83 |
| Reproducibility variance | 14.037 | 0.029 | 0.004 | 0.003 | 0.013 | 0.000 | 0.006 | 0.073 | 0.034 | 0.739 |
| Repeatability variance | 1.20 | 0.06 | 0.01 | 0.04 | 0.06 | 0.01 | 0.04 | 0.09 | 0.07 | 0.26 |
| r limit | 3.40 | 0.17 | 0.04 | 0.10 | 0.17 | 0.02 | 0.11 | 0.26 | 0.21 | 0.72 |
| Repeatability $RSD_r$ | 1.2% | 1.8% | 4.4% | 3.6% | 2.5% | 13.2% | 2.9% | 1.7% | 1.8% | 1.5% |
| Reproducibility stand. dev. | 3.75 | 0.17 | 0.07 | 0.06 | 0.11 | 0.02 | 0.08 | 0.27 | 0.19 | 0.86 |
| R limit | 10.60 | 0.48 | 0.19 | 0.16 | 0.32 | 0.06 | 0.22 | 0.76 | 0.52 | 2.43 |
| Reproducibility $RSD_R$ | 3.9% | 5.1% | 20.4% | 5.7% | 4.8% | 35.3% | 6.1% | 4.9% | 4.6% | 5.1% |
| Horwitz $RSD_r$ | 1.877 | 3.120 | 4.425 | 3.742 | 3.284 | 5.694 | 3.588 | 2.889 | 3.025 | 2.440 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $Horrat_r$ | 0.666 | 0.587 | 1.001 | 0.952 | 0.773 | 2.315 | 0.804 | 0.585 | 0.593 | 0.621 |
| Horwitz $RSD_R$ | 2.84 | 4.73 | 6.70 | 5.67 | 4.98 | 8.63 | 5.44 | 4.38 | 4.58 | 3.70 |
| $Horrat_R$ | 1.368 | 1.086 | 3.036 | 0.997 | 0.965 | 4.087 | 1.123 | 1.122 | 1.000 | 1.378 |

**Table of the results obtained**

*Note: The results from sample F should be taken with caution due to the very low concentration levels, which are below to the laboratories' limit of quantification.*

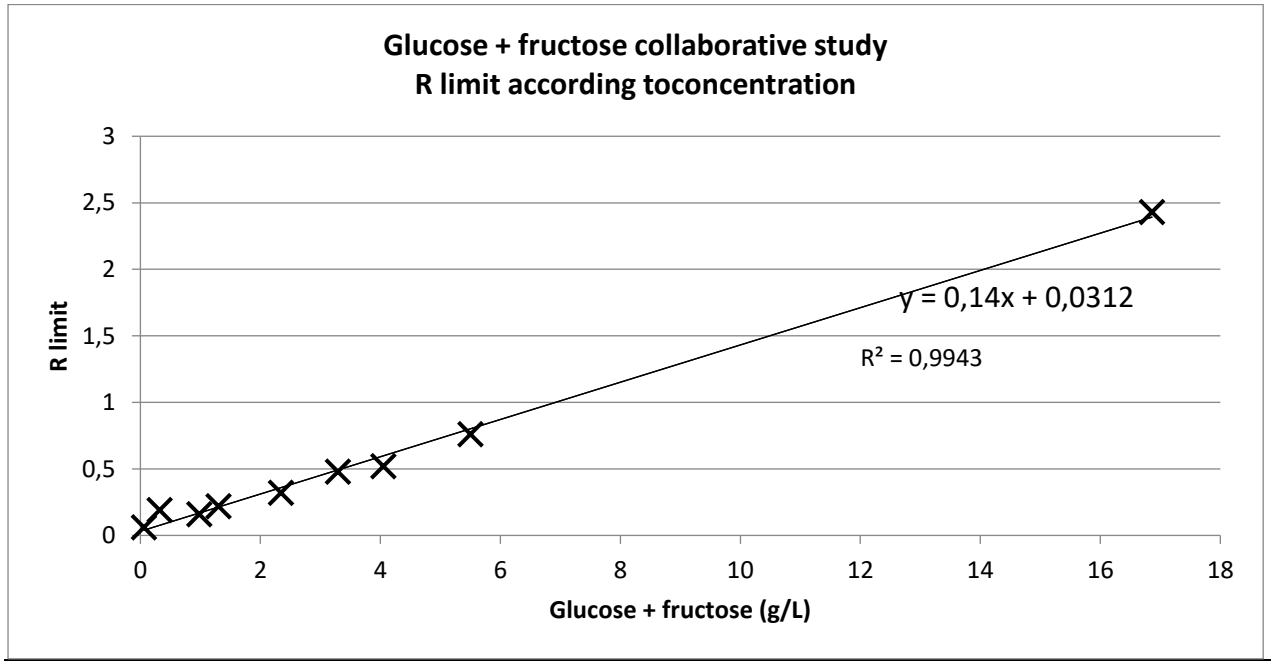
Glucose + fructose collaborative study
R limit according toconcentration
R limit
Glucose + fructose (g/L)
y = 0,14x + 0,0312
R² = 0,9943
0
0,5
1
1,5
2
2,5
3
0
2
4
6
8
10
12
14
16
18

Figure 3: R limit according to concentration

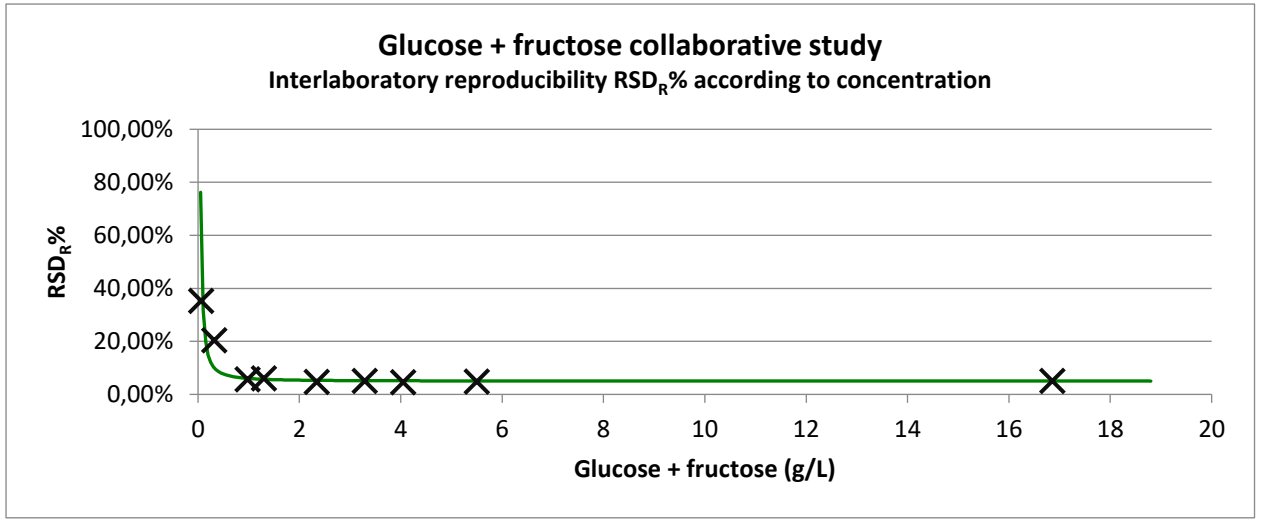


Figure 4: Interlaboratory $RSD_R$ % according to concentration.
Modelling: $\mathbf{RSD_R\% = 1 \cdot C^{(-1.424)} + 5}$

**Method OIV-MA-AS312-01A** Type I methods

# Alcoholic strength by volume

(Resolution Oeno 566/2016)

## 1. DEFINITION

The alcoholic strength by volume is the number of liters of ethanol contained in 100 liters of wine, both volumes being measured at a temperature of 20°C. It is expressed by the symbol '% vol.

*Note:* Homologues of ethanol, together with the ethanol and esters of ethanol homologues are included in the alcoholic strength since they occur in the distillate.

## 2. PRINCIPLE OF METHODS

2.1. *Distillation of wine* made alkaline by a suspension of calcium hydroxide.
Measurement of the alcoholic strength of the distillate:

2.2. *Type I methods:*

A. Measurement of the alcoholic strength of the distillate with a pycnometer
B. Measurement of the alcoholic strength of wine by electronic densimetry using frequency oscillator.
C. Measurement of the alcoholic strength of wine by densimetry using hydrostatic balance.

# 3. Method of obtaining distillate

*3.1. Apparatus*

3.1.1 Distillation apparatus, consisting of:
- a round-bottomed 1-liter flask with ground-glass joints.
- a rectifying column about 20 cm in height or any similar condenser.
- a source of heat; any pyrolysis of extracted matter must be prevented by a suitable arrangement.
- a condenser terminated by a drawn-out tube taking the distillate to the bottom of a graduated receiving flask containing several mL of water.

3.1.2 Steam distillation apparatus consisting of:
- a steam generator
- a steam pipe
- a rectifying column
- a condenser.

Any type of distillation or steam distillation apparatus may be used provided that it satisfies the following test:
Distil an ethanol-water mixture with an alcoholic strength of 10% vol. five times in succession. The distillate should have an alcoholic strength of at least 9.9% vol. after the fifth distillation; i.e. the loss of alcohol during each distillation should not be more than 0.02% vol.

*3.2 Reagent*

Suspension of calcium hydroxide, 2 M
Obtain by carefully pouring 1 liter of water at 60 to 70°C on to 120 g of quicklime, CaO.

3.3. *Preparation of sample*
Remove the bulk of any carbon dioxide from young and sparkling wines by stirring 250 to 300 mL of the wine in a 1000 mL flask.

*3.4. Procedure*

*3.4.1 Procedure for beverages with an ABV grater than 1.5% vol*

Measure out 200 mL of the wine using a volumetric flask. Record the temperature of the wine.

Transfer the wine to the distillation flask and introduce the steam-pipe of the steam distillation apparatus. Rinse the volumetric flask four times with successive 5 mL washings of water added to the flask or the steam-pipe. Add 10 mL of calcium hydroxide. 2 mol/L. and several pieces of inert porous material (pumice etc).

Collect the distillate in the 200 mL graduated flask used to measure the wine.

Collect a volume of about three-quarters of the initial volume if distillation is used and a volume of 198 to 199 mL of distillate if steam distillation is used. Make up to 200 mL with distilled water, keeping the distillate at a temperature within 2°C of the initial temperature.

Mix carefully, using a circular motion.

*Note:* In the case of wines containing particularly large concentrations of ammonium ions, the distillate may be redistilled under the conditions described above, but replacing the suspension of calcium hydroxide with 1 mL sulfuric acid diluted 10 /100.

*3.4.1 Procedure for beverages with an ABV lower than or equal to 1.5% vol*

Take a 200 mL sample of beverage using a calibrated flask. Note the temperature of the beverage. Pour it into the flask of the distillation apparatus or into the bubbler of the steam distillation apparatus. Rinse the calibrated flask four times with 5 mL of water and add this to the apparatus' flask or bubbler.

Add a 10 mL suspension of 2 M calcium hydroxide and, in the case of distillation, if necessary, a boiling regulator (pumice stone, etc.). Collect, in a 100 mL calibrated flask, a volume of distillate equal to around 75 mL in the case of distillation or 98-99 mL in the case of steam distillation. Make up to 100 mL with distilled water while the distillate is within ± 2 °C of the initial temperature. Carefully mix using a circular motion.

Mix carefully, using a circular motion.

*Precautionary safety measures*

Respect the safety guidelines for the usage of distillation apparatuses, the manipulation of hydro-alcoholic and cleaning solutions.

# 4.A. Measurement of the alcoholic strength of the distillate using a pycnometer

**(Method A2/1978 – Resolution 377/2009)**

1. *Apparatus*

Use the standardized pycnometer as described in the chapter *Density and specific gravity* (Annex, chapter 1).

2. *Procedure*

Measure the apparent density of the distillate (3.4) at *t* °C as described in the chapter *Density and specific gravity* (Annex. chapter 1. sections 4.3.1 and 4.3.2). Let this density be $\rho_t$.

3. *Expression of results*

3.1 Method of calculation

Find the alcoholic strength at 20 °C using Table I. In the horizontal line of this table corresponding to the temperature T (expressed as a whole number) immediately below *t* °C, find the smallest density greater than $\rho_t$. Use the tabular difference just below this density to calculate the density ρ at this temperature T.

On the line of the temperature T, find the density ρ' immediately above ρ and calculate the difference between the densities ρ and ρ′. Divide this difference by the tabular difference just to the right of the density ρ′. The quotient gives the decimal part of the alcoholic strength, while the whole number part of this strength is shown at the head of the column in which the density ρ′ is located.
An example of the calculation of an alcoholic strength is given in Annex I of this chapter.

*Note:* This temperature correction has been incorporated in a computer program and might possibly be carried out automatically.

3.2 *Beverages with an ABV lower than or equal to 1.5% vol*

The alcoholic strength by volume of a beverage with low alcohol content, with an ABV of less than 1.5% vol., is given by the following formula: ABV = ABVD/2, ABVD being the alcoholic strength by volume of the distillate.

It is expressed in "% vol.". The result is given to two decimal places.

4.3.2 *Repeatability r:*

$r$ = 0.10 % vol.

4.3.3 *Reproducibility* R:

R = 0.19 % vol.

ANNEX I

**Example of the calculation of the alcoholic strength of a wine**

## I. Measurement by pycnometer on a twin-pan balance

The constants of the pycnometer have been determined and calculated as described in chapter I. *Density and specific gravity*, section 6.1.1.

| **Calculations** | **Example** |
|---|---|
| 1. *Weighing of pycnometer filled with distillate*: | |
| Tare = pycnometer + distillate at $t$ °C + $p''$ | $t$ °C = 18.90°C<br>$t$ °C corrected = 18.70°C<br>$p''$ = 2.8074 g |
| $p + m - p''$ = mass of distillate at $t$ °C | {105.0698 - 2.8074=102.2624 g |
| Apparent density at $t$ °C: $\rho_t = \frac{p + m - p''}{\text{volume of pycnometer at } 20°C}$ | $\rho_{18.7°} = \frac{102.2624}{104.0229} = 0.983076$ |
| 2. *Calculation of alcoholic strength*: | |
| Refer to the table of apparent densities of water-alcohol mixtures at different temperatures, as indicated above | On the line 18 °C of the table of apparent densities, {the smallest density greater than the observed density {of 0.983076 is 0.98398 in column 11% vol.<br><br>The density at 18 °C is:<br>98307.6 + 0.7 x 22) $10^{-5}$ = 0.98323<br>0.98398 - 0.98323 = 0.00075<br><br>The decimal portion of the % vol. of alcoholic strength is<br>75/114 = 0.65<br>The alcoholic strength is:<br>11.65 % vol. |

## II. Measurement by pycnometer on a single pan balance

The constants of the pycnometer have been determined and calculated as described in chapter 1. *Density and specific gravity,* section 6.2.1

### Calculations

1. *Weighing of the pycnometer filled with distillate*:

Weight of tare bottle at the time of measurement in grams: $T_1 = 171.9178$

Pycnometer filled with distillate at 20.50 °C in grams: $P_2 = 167.8438$

Variation in the buoyancy of air: $dT = 171.9178 - 171.9160 = + 0.0018$

Mass of the distillate at 20.50 °C: $L_t = 167.8438 - (67.6695 + 0.0018) = 100.1725$

Apparent density of the distillate: $\rho_{20.50°} = \frac{100.1725}{101.8194} = 0.983825$

2. *Calculation of alcoholic strength*:

Refer to the table of apparent densities of water-alcohol mixtures at different temperatures, as indicated above:

On the line 20°C of the table of apparent {densities, the smallest density greater than {observed density of 0.983825 is 0.98471 in {column 10% vol.

The density at 20°C is:
(98382.5 + 0.5 x 24) $10^{-5}$= 0.983945
(0.98471 - 0.983945 = 0.000765

The decimal portion of the % vol.
76.5/119 = 0.64
The alcoholic strength is:
10.64% vol.

ANNEX II

**Formula from which tables of alcoholic strengths of ethanol-water mixtures are calculated**

The density "ρ" in kilograms per meter cubed ($kg/m^3$) of an ethanol-water mixture at temperature *t* in degrees Celsius is given by the formula below as a function of:

- the alcoholic strength by weight *p* expressed as a decimal; *
- the temperature *t* in °C (EIPT 68);
- the numerical coefficients below.

The formula is valid for temperatures between – 20 °C and + 40 °C.

$$\rho = A_1 + \sum_{k=2}^{12} A_k \, p^{k-1} + \sum_{k=1}^{6} B_k (t - 20°C)^k$$

$$+ \sum_{i=1}^{n} \sum_{k=1}^{m} C_{i,k} \, p^{k(t - 20°C)^i}$$

$$\begin{aligned} n &= 5 \\ m_1 &= 11 \\ m_2 &= 10 \\ m_3 &= 9 \\ m_4 &= 4 \\ m_5 &= 2 \end{aligned}$$

* For example. for an alcoholic strength of 12 % by weight. $q = 0.12$

**Numerical coefficients in the formula**

| *k* | A*k* kg/m$^3$ | B*k* |
|---|---|---|
| 1 | 9.982 012 300 · $10^2$ | – 2.061 851 3 · $10^{-1}$ kg/(m$^3$ · °C) |
| 2 | – 1.929 769 495 · $10^2$ | – 5.268 254 2 · $10^{-3}$ kg/(m$^3$ · °C$^2$) |
| 3 | 3.891 238 958 · $10^2$ | 3.613 001 3 · $10^{-5}$ kg/(m$^3$ · °C$^3$) |
| 4 | – 1.668 103 923 · $10^3$ | – 3.895 770 2 · $10^{-7}$ kg/(m$^3$ · °C$^4$) |
| 5 | 1.352 215 441 · $10^4$ | 7.169 354 0 · $10^{-9}$ kg/(m$^3$ · °C$^5$) |
| 6 | – 8.829 278 388 · $10^4$ | – 9.973 923 1 · $10^{-11}$ kg/(m$^3$ · °C$^6$) |
| 7 | 3.062 874 042 · $10^5$ | |
| 8 | – 6.138 381 234 · $10^5$ | |
| 9 | 7.470 172 998 · $10^5$ | |
| 1 | – 5.478 461 354 · $10^5$ | |
| 1 | 2.234 460 334 · $10^5$ | |
| 1 | – 3.903 285 426 · $10^4$ | |

| *k* | Cl.*k* kg/(m$^3$ . °C) | C2.*k* k g/(m$^3$ . °C2) |
|---|---|---|
| 1 | 1.693 443 461530 087 · $10^{-1}$ | – 1. 193 013 005 057 010 · |
| 2 | – 1.046 914 743 455 169 · $10^1$ | 2.517 399 633 803 46 1 · |
| 3 | 7.196 353 469 546 523 · $10^1$ | – 2.170 575 700 536 993 |
| 4 | – 7.047 478 054 272 792 · $10^2$ | 1.353 034 988 843 029 · $10^1$ |
| 5 | 3.924 090 430 035 045 · $10^3$ | – 5.029 988 758 547 014 · $10^1$ |
| 6 | – 1.210 164 659 068 747 · $10^4$ | 1.096 355 666 577 570 · $10^2$ |
| 7 | 2.248 646 550 400 788 · $10^4$ | – 1.422 753 946 421 155 · $10^2$ |
| 8 | – 2.605 562 982 188 164 · $10^4$ | 1.080 435 942 856 230 · $10^2$ |
| 9 | 1.852 373 922 069 467 · $10^4$ | – 4.414 153 236 817 392 · $10^1$ |
| 1 | – 7.420 201433 430 137 · $10^3$ | 7.442 971 530 188 783 |
| 1 | 1.285 617 841 998 974 · $10^3$ | |

| *k* | C3.*k*<br>kg/(m³ . °C3) | C4.k<br>kg/(m³ . °C4) | C5.k<br>kg/(m³ . °C5) |
|---|---|---|---|
| 1 | – 6.802 995 733 503 803 · $10^{-4}$ | 4.075 376 675 622 027 · $10^{-}$ | – 2.788 074 354 782 409 · |
| 2 | 1.876 837 790 289 664 · $10^{-2}$ | – 8.763 058 573 471 110 · $10^{-}$ | 1.345 612 883493 354 · |
| 3 | – 2.002 561 813734 156 · $10^{-1}$ | 6.515 031 360 099 368 · $10^{-}$ | |
| 4 | 1.022 992 966 719 220 | – 1.515 784 836 987 210 · $10^{-}$ | |
| 5 | – 2.895 696 483 903 638 | | |
| 6 | 4.810 060 584 300 675 | | |
| 7 | – 4.672 147 440 794 683 | | |
| 8 | 2.458 043 105 903 461 | | |
| 9 | – 5.411 227 621 436 812 · $10^{-1}$ | | |

# 4. B. Measurement of the alcoholic strength of wine by electronic densimetry using frequency oscillator
**(Resolution Oeno 8/2000 – 377/2009)**

## 1. Measurement method

1.1. *Strength and introduction*

The alcoholic strength by volume of wine must be measured before being commercialised mainly in order to conform to labelling rules.

The alcoholic strength by volume is equal to the number of litres of ethanol contained in 100 litres of wine; these volumes are both measured at 20 °C. The symbol is " % vol. ".

1.2. *Precautionary safety measures*

Respect the safety guidelines for the usage of distillation apparatuses, the manipulation of hydro-alcoholic and cleaning solutions.

1.3. *Object and field of application*

The method of measurement described is electronic densimetry using a frequency oscillator.

In reference to the provision of the rules in the existing law, the trial temperature is stopped at 20 °C.

1.4. *Principle and definitions*

The principle of the method consists firstly of distilling the wine volume by volume. The distillation procedure is described in the Compendium. This distillation enables the elimination of non-volatile substances. The ethanol counter parts in addition to ethanol and the ethanol counter parts involved in esters are included in the alcoholic strength since they are present in the distillate

The distillate density of the distillate is measured. The density of a liquid at a given temperature is equal to the ratio of its density to its volume.

$\rho = m / V$ , for a wine, it is expressed as g/ml

For hydro-alcoholic solutions such as distillates, given the known temperature, the graphs correspond to the alcoholic strength by volume (OIV, 1990). This alcoholic strength corresponds to that of wine (distillation of volume to volume).

In the present method the distillate density is measured by electronic densimetry using a frequency oscillator. The principle consists of measuring the period of oscillation of a tube containing the sample undergoing an electromagnetic stimulation. The density is thus calculated and is linked to the period of oscillation by the following formula:

$$p = T^2 \times \left(\frac{C}{4\pi^2 V}\right) - \left(\frac{M}{V}\right) \qquad (1)$$

ρ = density of sample
T = period of induced vibration
M = mass of empty tube
C = spring constant
V = volume of vibrating sample

This relation is in the form of, $\rho = A\ T^2 - B$ (2), There is a linear relationship between density and the period squared. The A and B constants specific to each oscillator are estimated by measuring the period of fluids of the known density.

1.5. *Reagents and products*

1.5.1 Reference fluids

Two reference fluids are used to adjust the densimetry. The densities of reference fluids must encompass the densities of the distillates to be measured. A spread between the densities between reference fluids above 0.01000 g/ml is recommended. The density must be known with an uncertainty under +/– 0.00005 g/ml, for a temperature of 20.,00 +/– 0.05 °C.

The measuring of alcoholic strength by volume of wine by electronic densimetry of reference fluids:

- dry air (unpolluted),
- double distilled water or of an equivalent analytical purity,
- hydro alcoholic solution of density determined by pycometry (reference method),
- solutions connected to national standards of viscosity under 2 $mm^2/s$.

*1.5.2 Cleaning and drying products*

- detergents, acids,
- organic solvents: ethanol 96% Vol., pure acetone.

### 1.6. Apparatus

*1.6.1 Electronic densimetry by frequency oscillator*

Electronic densimetry contains the following elements:

- a measuring cell containing a measurement tube and a temperature controlled enclosure,
- a system for setting up an oscillation tube and measurement of the period of oscillation,
- a timer,
- a digital display and possibly a calculator.

The densimetry on a perfectly stable support isolated from all vibrations.

*1.6.2 Temperature control of measuring cell*

The measurement tube is located in the temperature-controlled enclosure. Temperature stability must be better than +/- 0.02 °C.

It is necessary to control the temperature of the measuring cell when the densimetry makes this possible, because this strongly influences .the indication results. Density of this hydro alcoholic solution with an alcoholic strength by volume of 10% Vol., and is at 0.98471 g/ml at 20°C and at 0.98447 g/ml at 21°C or a spread of 0.00024 g/ml.

The trial temperature is stopped at 20°C. The temperature is taken at the cell level and done with a resolution thermometer 0.01°C and connected to national standards. This must enable a temperature measurement with an uncertainty of under +/- 0.07°C.

*1.6.3 Calibration of the apparatus*

The apparatus must be calibrated before using for the first time, then every six months or is the verification is not satisfactory. The objective is to use two reference fluids to calculate the constants A and B (cf. (2)). To carry out the calibration refer to the user's manual of the apparatus. In principle, this calibration is carried out with dry air (take into account the atmospheric pressure) and very pure water (double distilled and/or very high micro filtered resistance, for example > 18 M Ω

*1.6.4 Calibration verification*

In order to verify the calibration we measure the density of the reference fluids.

- Every day, a density check of the air is carried out. A difference between the theoretical density and the observed density of more than 0.00008 g/ml may indicate that the tube is clogged. In that case, it must be cleaned. After cleaning, verify the air density again. If the verification is not conclusive adjust the apparatus.
- Check the density of water, if the difference between the theoretical density and the density observed is greater than 0.00008 g/ml, adjust the apparatus.
- If the verification of cell temperature is difficult, it is possible to directly check hydro alcoholic density of the alcoholic strength by volume compared to the distillates analysed.

*1.6.5 Check*

When the difference between the theoretical density of the reference solution (known with an uncertainty of +/- 0.00005 g/ml) and the measurement is above 0.00008 g/ml the temperature of the cell must be taken.

1.7. *Sampling and preparation of samples*

(Cf. Compendium if International methods of wine and musts 1990, page 59, Obtaining distillate)

1.8. *Operating procedure*

After obtaining a distillate, (OIV, 1990) we measure the density or the alcoholic strength by volume by densimetry.

The operator must ensure the stability and the temperature of the measuring cell. The distillate in the densimetry cell must not contain air bubble and must be homogeneous. If there is an available lighting system, turn off quickly after checking because the heat generated by the lamp can influence the measuring temperature.

If the apparatus only provides the period, density can be calculated by the A and B constants (cf. A.4 c). If the apparatus does not provide the alcoholic strength by volume directly, we can obtain the alcoholic strength by volume using the (OIV, 1990) tables if we know the density.

1.9. *Expression of results*

The alcoholic strength by volume is obtained from the distillate. This is expressed as " % vol. ".

If the temperature conditions are not respected, a correction must be made to express the temperature at 20°C. The result is quoted to two decimal places

1.9.1 *Beverages with an ABV lower than or equal to 1.5% vol*

The alcoholic strength by volume of a beverage with low alcohol content, with an ABV of less than 1.5% vol., is given by the following formula: ABV = ABVD/2, ABVD being the alcoholic strength by volume of the distillate.

It is expressed in "% vol.". The result is given to two decimal places.

1.10. *Comments*

The volume introduced into the cell must be sufficient enough to avoid possible contamination caused from the previous sample. It is thus necessary to carry out two testing. If this does not provide results included in the repeatability limits, a third testing may be necessary. In general, results from the last two testing are homogeneous and we then eliminate the first factor.

1.11 *Reliability*

For alcoholic strength by volume samples between 4 to 18% Vol.

Repeatability (r) = 0.067 (% vol.),

Reproducibility (R) = 0.0454 + 0.0105 x alcoholic strength by volume.

## 2. Interlaboratory Tests. Reliability and accuracy on additions

2.1. *Samples*

The samples used for this joint study are described in Table 1.

**Table 1: Samples for joint study**

| Num | Nature | Approx alcoholic strength by volume (% vol.) |
|---|---|---|
| C0 | Cider (filtered through membrane to remove $CO_2$) | 5 |
| V0 | Filtered wine | 10 |
| V1 | Filtered wine then doped | 11 |
| V2 | Filtered wine then doped | 12 |
| V3 | Filtered wine then doped | 13 |
| P0 | Liqueur wine | 16 |

All samples are homogenised before filling the bottles to be sent to the participants. For wine, 40 litres of wine are homogenised before sending and carrying out the additions

For the additions, pour absolute ethanol into a 5 litre volumetric flask and then fill up to the line with filtered wine. This operation is repeated two times. The volumes of ethanol are respectively 50, 100 and 150 ml for the V1, V2 and V3 samples.

2.2. *Participating laboratories*

The participating laboratories in the joint study are outlined in Table 2.

| Laboratory | Zip Code | City | Contact |
|---|---|---|---|
| ALKO Group LTD | FIN-00101 | Helsinki | Monsieur Lehtonen |
| Bénédictine | 76400 | Fécamp | Madame Pillon |
| Casanis | 18881 | Gemenos | Madame Cozon |
| CIVC | 51200 | Epernay | Monsieur Tusseau |
| Cointreau | 49181 | St Barthélémy d'Anjou | Madame Guerin |
| Courvoisier | 16200 | Jarnac | Monsieur Lavergne |
| Hennessy | 16100 | Cognac | Monsieur Calvo |
| IDAC | 44120 | Vertou | Madame Mars |
| Laboratoire Gendrot | 33000 | Bordeaux | Madame Gubbiotti |
| Martell | 16100 | Cognac | Monsieur Barboteau |
| Ricard | 94320 | Thiais | Monsieur Boulanger |
| SOEC Martin Vialatte | 51319 | Epernay | Madame Bertemes |

In order not to introduce a methodological angle, the *Station Viticole du Bureau National Interprofessionnel du Cognac,* the joint study organiser, will not be taken into account.

2.3. *Analyses*

The C0 and P0 products are distilled two times, the V0, V1, V2 and V3 products three times. Three alcoholic strength by volume tests were done for each distillate. The results were carried over to the results table.

2.4. *Results*

The second testing (out of the three carried out) is kept of the accuracy study (Table 3).

*Table 3: Results (second testing per distillate) (% vol.)*

| Laboratory | C0 | V0 | V1 | V2 | V3 | P0 |
|---|---|---|---|---|---|---|
| 1 | 6,020 | 9,500 | 10,390 | 11,290 | 12,100 | 17,080 |
| | 5,970 | 9,470 | 10,380 | 11,260 | 12,150 | 17,080 |
| | | 9,450 | 10,340 | 11,260 | 12,150 | |
| 2 | 6,040 | 9,500 | 10,990 | 11,270 | 12,210 | 17,050 |
| | 6,040 | 9,500 | 10,390 | 11,280 | 12,210 | 17,050 |
| | | 9,510 | 10,400 | 11,290 | 12,200 | |
| 3 | 5,960 | 9,460 | 10,350 | 11,280 | 12,170 | 17,190 |
| | 5,910 | 9,460 | 10,360 | 11,280 | 12,150 | 17,200 |
| | | 9,450 | 10,340 | 11,260 | 12,170 | |
| 4 | 6,020 | 9,470 | 10,310 | 11,250 | 12,160 | 16,940 |
| | 6,020 | 9,450 | 10,350 | 11,250 | 12,120 | 17,070 |
| | | 9,450 | 10,330 | 11,210 | 12,130 | |
| 5 | 5,950 | 9,350 | 10,250 | 11,300 | 12,050 | 17,000 |
| | 5,950 | 9,430 | 10,250 | 11,300 | 12,050 | 17,000 |
| | | 9,430 | 10,250 | 11,300 | 12,050 | |
| 6 | 6,016 | 9,513 | 10,370 | 11,275 | 12,222 | 17,120 |
| | 6,031 | 9,513 | 10,336 | 11,266 | 12,222 | 17,194 |
| | | 9,505 | 10,386 | 11,275 | 12,220 | |
| 7 | 5,730 | 9,350 | 10,230 | 11,440 | 12,080 | 17,010 |
| | 5,730 | 9,430 | 10,220 | 11,090 | 12,030 | 16,920 |
| | | 9,460 | 10,220 | 11,080 | 11,930 | |
| 8 | 5,990 | 9,400 | 10,340 | 11,160 | 12,110 | 17,080 |
| | 6,000 | 9,440 | 10,320 | 11,150 | 12,090 | 17,110 |
| | | 9,440 | 10,360 | 11,210 | 12,090 | |
| 9 | 6,031 | 9,508 | 10,428 | 11,289 | 12,180 | 17,089 |
| | 6,019 | 9,478 | 10,406 | 11,293 | 12,215 | 17,084 |
| | | 9,509 | 10,411 | 11,297 | 12,215 | |
| 10 | 6,030 | 9,500 | 10,380 | 11,250 | 12,150 | 17,130 |
| | 6,020 | 9,510 | 10,380 | 11,250 | 12,150 | 17,100 |
| | | 9,510 | 10,380 | 11,250 | 12,160 | |
| 11 | 6,020 | 9,480 | 10,400 | 11,260 | 12,150 | 17,040 |
| | 6,000 | 9,470 | 10,390 | 11,260 | 12,140 | 17,000 |
| | | 9,490 | 10,370 | 11,240 | 12,160 | |

2.5. *Repeatability and reproducibility calculations*

Repeatability and reproducibility calculations are carried out in compliance with the standard NF X 06-041, September 1983, ISO 5725.Table 4 presents the standard deviation per cell (laboratory x sample).

*Table 4: Dispersion table (standard deviation in % vol.)*

| Laboratory | C0 | V0 | V1 | V2 | V3 | P0 |
|---|---|---|---|---|---|---|
| 1 | 0,0354 | 0,0252 | 0,0265 | 0,0173 | 0,0289 | 0,0000 |
| 2 | 0,0000 | 0,0058 | 0,3436 | 0,0100 | 0,0058 | 0,0000 |
| 3 | 0,0354 | 0,0058 | 0,0100 | 0,0115 | 0,0115 | 0,0071 |
| 4 | 0,0000 | 0,0115 | 0,0200 | 0,0231 | 0,0208 | 0,0919 |
| 5 | 0,0000 | 0,0462 | 0,0000 | 0,0000 | 0,0000 | 0,0000 |
| 6 | 0,0106 | 0,0046 | 0,0255 | 0,0052 | 0,0012 | 0,0523 |
| 7 | 0,0000 | 0,0569 | 0,0058 | 0,2050 | 0,0764 | 0,0636 |
| 8 | 0,0071 | 0,0231 | 0,0200 | 0,0321 | 0,0115 | 0,0212 |
| 9 | 0,0085 | 0,0176 | 0,0115 | 0,0040 | 0,0202 | 0,0035 |
| 10 | 0,0071 | 0,0058 | 0,0000 | 0,0000 | 0,0058 | 0,0212 |
| 11 | 0,0141 | 0,0100 | 0,0153 | 0,0115 | 0,0100 | 0,0283 |

Three cells presented strong dispersions (probability with Cochran test under 1%). These cells are represented in grey (Table 4).

For laboratory 7 and the V3 product, the standard deviation of 0.0764 is maintained despite the Cochran test because it is on the same high level as that observed at the same laboratory on the V0 product.

An examination of figures for each distillate leads us to eliminate (Table 3):

- laboratory 2, product V1, value 10.990,
- laboratory 7, product V2, value 11.440.

After eliminating these two values, the cell averages are calculated (laboratory x sample) (Table 5).

Table 5: Table of averages (averages in % vol.)

| Laboratory | C0 | V0 | V1 | V2 | V3 | P0 |
|---|---|---|---|---|---|---|
| 1 | 5,9950 | 9,4733 | 10,3700 | 11,2700 | 12,1333 | 17,0800 |
| 2 | 6,0400 | 9,5033 | 10,3950 | 11,2800 | 12,2067 | 17,0500 |
| 3 | 5,9350 | 9,4567 | 10,3500 | 11,2733 | 12,1633 | 17,1950 |
| 4 | 6,0200 | 9,4567 | 10,3300 | 11,2367 | 12,1367 | 17,0050 |
| 5 | 5,9500 | 9,4033 | 10,2500 | 11,3000 | 12,0500 | 17,0000 |
| 6 | 6,0235 | 9,5103 | 10,3640 | 11,2720 | 12,2213 | 17,1570 |
| 7 | 5,7300 | 9,4133 | 10,2233 | 11,0850 | 12,0133 | 16,9650 |
| 8 | 5,9950 | 9,4267 | 10,3400 | 11,1733 | 12,0967 | 17,0950 |
| 9 | 6,0250 | 9,4983 | 10,4150 | 11,2930 | 12,2033 | 17,0865 |
| 10 | 6,0250 | 9,5067 | 10,3800 | 11,2500 | 12,1533 | 17,1150 |
| 11 | 6,0100 | 9,4800 | 10,3867 | 11,2533 | 12,1500 | 17,0200 |

The figures given by laboratory 7 are generally low (Table 5). In the case of cider the average for this laboratory is very far from the figures of the other laboratories (associated probability to the Dixon test under 1 %). The results of this laboratory for this product are eliminated.

Table 6 presents the calculated repeatability and reproducibility.

*Table 6: Calculation of repeatability and reproducibility*

| Sample | P | n | TAV | S2r | S2L | r | R |
|---|---|---|---|---|---|---|---|
| C0 | 10 | 20 | 6,002 | 0,000298 | 0,001033 | 0,049 | 0,103 |
| V0 | 11 | 33 | 9,466 | 0,000654 | 0,001255 | 0,072 | 0,124 |
| V1 | 11 | 32 | 10,344 | 0,000255 | 0,003485 | 0,045 | 0,173 |
| V2 | 11 | 32 | 11,249 | 0,000219 | 0,003113 | 0,042 | 0,163 |
| V3 | 11 | 33 | 12,139 | 0,000722 | 0,003955 | 0,076 | 0,194 |
| P0 | 11 | 22 | 17,070 | 0,001545 | 0,004154 | 0,111 | 0,214 |

*Key:*

*p* : *number of laboratories retained*
*n* : *number of values retained*
*TAV* : *average alcoholic strength by volume (% vol.)*
*S2r* : *repeatability variation (% vol.)*$^2$
*S2L* : *interlaboratory variation (% vol.)* $^2$

*r* : *repeatability (% vol.)*
*R* : *reproducibility (% vol.)*

Reproducibility increases with the samples' alcoholic strength by volume (Figure 1). The increase in repeatability according to alcoholic strength by volume is less noticeable and global repeatability is calculated according to the average repeatability variation. As such, for the alcoholic strength by volume samples between 4 and 18% vol.,

Repeatability (r) = 0.067 (% vol.),

Reproducibility (R) = 0.0454 + 0.0105 x alcoholic strength by volume.

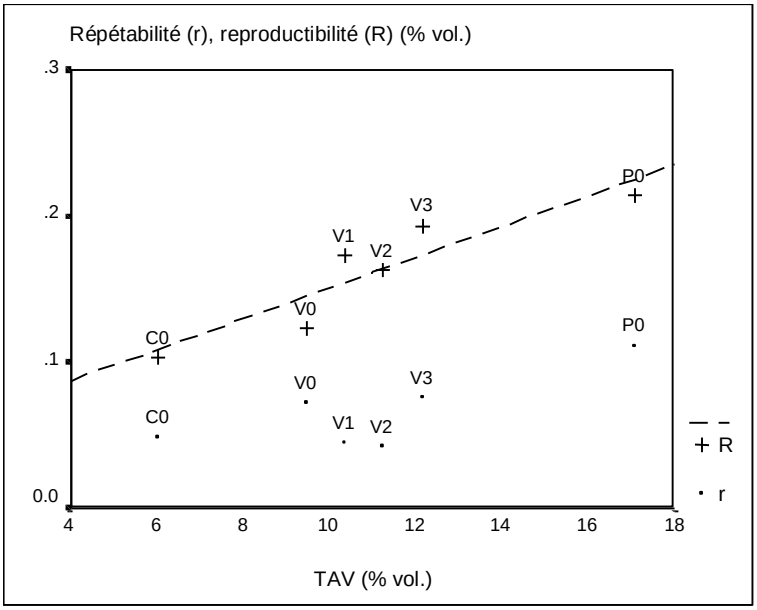


*Figure 1: Repeatability and reproducibility according to alcoholic strength by volume*

2.6. *Accuracy on additions carried out on wine*

The regression line of alcoholic strength after the addition according to the volume of ethanol supplied, for a volume of 0 ml, an estimation of the initial alcoholic strength of product (Figure 2). This regression is carried out with average values for each laboratory (Table 5).

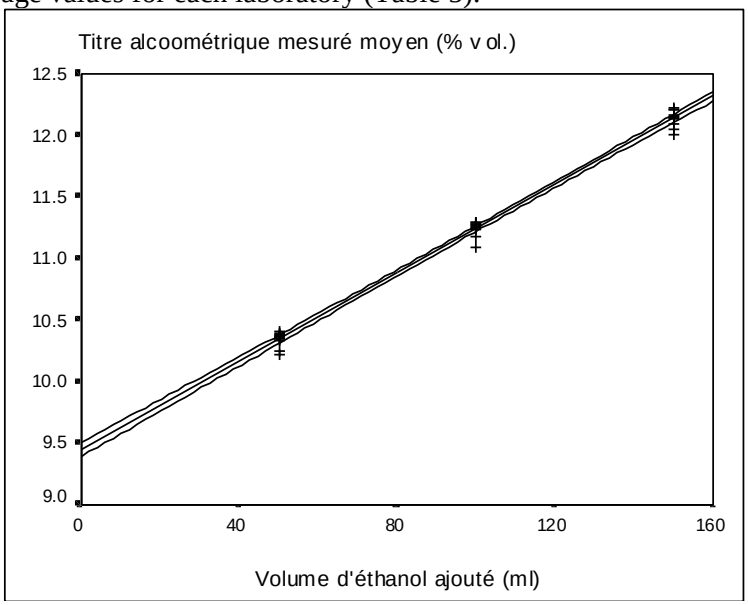


*Figure 2: Regression of measures alcoholic strength by volume of added ethanol*

Measurements carried out on initial products are not included in this estimation. This estimation is made up of the average of measurements taken on this product before additions; the intervals of relative confidence on these two estimations are calculated (Table 7).

**Table 7: Additions on products**

| BI | Average measurements | BS | BI | estimation with measurements on products + additions | BS |
|---|---|---|---|---|---|
| 9,440 | 9,466 | 9,492 | 9,392 | 9,450 | 9,508 |

*Key: BI : lower bound of confidence interval at 95%*
BS : upper bound of confidence interval at 95%

The two confidence intervals cover a large overlapping spreading centre. Thanks to the measurements on doped samples, the alcoholic strength by volume of the initial product can be found.

2.7. Conclusion of interlaboratory trials

The repeatability and the reproducibility indications by interlaboratory trials provide the following equations, for alcoholic strength by volume products between 4 to 18% vol.:

Repeatability (r) = 0.67 (% vol.),
Reproducibility (R) = 0.454 + 0.0105 x alcoholic strength by volume (% vol.).

The Horwitz indicators, Hor and HoR are weak (Table 8). These indicators provide good details of the method compared to the level of analyte measured.

**Table 8: Table summary of method reliability**

| Samples | C0 | V0 | V1 | V2 | V3 | P0 |
|---|---|---|---|---|---|---|
| n | 20 | 33 | 32 | 32 | 33 | 22 |
| p | 10 | 11 | 11 | 11 | 11 | 11 |
| Alcoholic strength by volume | 6,0019 | 9,4662 | 10,3443 | 11,2492 | 12,1389 | 17,0699 |
| r | 0,0489 | 0,0724 | 0,0452 | 0,0419 | 0,0760 | 0,1113 |
| sr | 0,0173 | 0,0256 | 0,0160 | 0,0148 | 0,0269 | 0,0393 |
| RSDr | 0,2878 | 0,2702 | 0,1543 | 0,1316 | 0,2214 | 0,2303 |
| RSDrH | 2,0159 | 1,8822 | 1,8573 | 1,8340 | 1,8131 | 1,7224 |
| Hor | 0,1428 | 0,1436 | 0,0831 | 0,0718 | 0,1221 | 0,1337 |
| R | 0,1033 | 0,1237 | 0,1731 | 0,1634 | 0,1935 | 0,2136 |
| sR | 0,0365 | 0,0437 | 0,0612 | 0,0577 | 0,0684 | 0,0755 |
| RSDR | 0,6080 | 0,4616 | 0,5912 | 0,5131 | 0,5634 | 0,4423 |
| RSDRH | 3,0543 | 2,8519 | 2,8141 | 2,7788 | 2,7471 | 2,6097 |
| HoR | 0,1991 | 0,1619 | 0,2101 | 0,1847 | 0,2051 | 0,1695 |

*Key:*

*n* : *number of values retained*
*p* : *number of laboratories retained*
*Alcoholic strength by volume: average rate (% vol.)*
*r* : *repeatability (% vol.)*
*sr* : *Standard deviation of repeatability (% vol.)*
*RSDr* : *Repeatability coefficient of variation ( sr x 100 / TAV) (%)*
*RSDrH* : *Horwitz repeatability coefficient of variation ( .0.66 x RSDRH) (%)*
*Hor* : *Horrat repeatability value (RSDr/RSDrH)*
*R* : *Reproducibility (% vol.)*
*sR* : *Reproducibility standard deviation (% vol.)*
*RSDR* : *Reproducibility coefficient of variation ( sR x 100 / TAV) (%)*
*RSDRH* : *Horwitz reproducibility coefficient of variation* $( 2^{(1-0,5\log(TAV))} ) (\%)$
*HoR* : *Horrat reproducibility value ( RSDR/RSDRH)*

Interlaboratory trials' measurements carried out on wine with additions enable us to find the value obtained before the addition. We respectively find 9.45 and 9.47% vol.

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Standard ISO 5725, page 7

# 4. C. Measurement of the alcoholic strength of wine by densimetry using hydrostatic balance
**(Resolution Oeno 24/2003 – 377/2009)**

## 1. METHOD OF MEASUREMENT

### 1.1 Strength and introduction

Measurement of alcoholic strength by volume should be determined before marketing notably to be in compliance with labelling rules.
Alcoholic strength by volume is equal to the number of litres of ethanol contained in 100 litres of wine measured at 20°C, referred to as "% vol.".

### 1.2 Safety precaution

Respect safety measures concerning the use of distillation apparatuses, manipulation of hydro-alcoholic solutions and cleaning products.

### 1.3 Object and field of application

The method of measurement is densimetry using a hydrostatic balance.
In reference to regulatory provisions in force the trial temperature is set at 20°C.

### 1.4 Principle and definitions

The principle of this method involves firstly distilling wine volume by volume. The distilling method is described in the Compendium. Non volatile substances can be eliminated through distillation. Ethanol counterparts and ethanol found in esters are included in the alcoholic strength as they are found in the distillate.
Secondly, the volumetric weight of the distillate obtained is measured. The volumetric weight of a liquid at a given temperature is equal to the ratio of the weight over its volume: $\rho=m/V$, for wine, it is expressed in g/ml.

The alcoholic strength of wine can be measured by densimetry using a hydrostatic balance following the Archimedes principle by which any body plunged into a fluid undergoes a vertical push, from the bottom to the top, equal to the weight of the displaced fluid.

### 1.5 Reagents

Unless other wise indicated, only recognised analytical quality reagents should be used during the analysis with at least class 3 water corresponding to the definition of the standard ISO 3696:1987.

1.5.1 *Solution for washing float device (sodium hydroxide , 30% m/v).*
To prepare a 100 ml solution, weigh 30 g of sodium hydroxide and fill using 96% vol. ethanol.

### 1.6 Apparatus and material

current laboratory apparatus including:

1.6.1 Single-plate hydrostatic balance with 1 mg precision.

1.6.2 Floater with at least 20 ml volume, specifically adapted for the balance, suspended by a thread with a diameter less than or equal to 0.1 mm.

1.6.3 Cylindrical test tube with level indicator. The floater must entirely fill the test tube volume above the marker, only the slinging wire goes through the surface of the liquid. The cylindrical test tube should have an inside diameter at least above 6 mm of the floater.

1.6.4 Thermometer (or temperature measurement pipette) with degree and 10th of degree graduations, from 10°C to 40°C, calibrated to ± 0.05°C.

1.6.5 Calibrated weight by a recognized certification body.

### 1.7 Procedure

After each measurement, the floater and the test tube must be cleaned with distilled water, wiped with soft laboratory paper which doesn't loose its fibres and rinsed with solution whose volumetric weight is to be determined. These measurements must be carried out once the apparatus has reached a stable level in order to limit alcohol loss through evaporation.

1.7.1 Balance calibration

While balances usually have internal calibration systems, hydrostatic balances must be calibrated with controlled weights by an official certification body.

1.7.2 Floater calibration

1.7.2.1 Fill cylindrical test tube up to marker with bidistilled water (or an equivalent purity, for example microfiltered water with a conductivity of

18.2 MΩ/cm), whose temperature between 15°C to 25°C, but preferably at 20°C.

1.7.2.2 Plunge the floater and the thermometer into the liquid, shake, note down the volumetric weight on the apparatus and, if necessary, adjust the reading in order for it to be equal to the water measurement temperature.

1.7.3 Control using a hydroalcoholic solution

1.7.3.1 Fill the cylindrical test tube up to the marker with a known titre of hydroalcoholic solution at a temperature between 15°C to 25°C, preferably at 20°C.

1.7.3.2 Plunge the floater and the thermometer into the liquid, shake, note down the volumetric weight on the apparatus (or the alcoholic strength if possible). The established alcoholic strength must be equal to the previously determined alcoholic strength.

Note 2: This alcoholic strength solution can be replaced by bidistilled water for floater calibration.

1.7.4 Measure volumetric weight of the distillate (or alcoholic strength if possible)

1.7.4.1 Pour the sample for the trial in the cylindrical test tube up to the marker level.

1.7.4.2 Plunge the floater and the thermometer into the liquid, shake, note down the volumetric weight on the apparatus (or the alcoholic strength if possible. Note the temperature if the volumetric mass is measured at t°C ($\tilde{n}_t$).

1.7.4.3 Correct $\tilde{n}_t$ using a volumetric weight table $\tilde{n}_t$ of hydroalcoholic mixtures [Table II of Annex II of the Compendium of methods of analysis of the OIV].

1.7.5 Clean the floater and cylindrical test tube.

1.7.5.1 Plunge the floater into the wash solution in the test tube.

1.7.5.2 Allow to soak 1 hour while turning the floater regularly.

1.7.5.3 Rinse with tap water, then with distilled water.

1.7.5.4 Wipe with soft laboratory paper which doesn't loose its fibres.

Carry out these operations when the floater is used for the first time and then on a regular basis when necessary.

1.7.6 Result

Using $\tilde{n}_{20}$, volumetric weight, calculate real alcoholic strength by using the table indicating volumetric alcoholic strength (% vol.) at 20°C according to volumetric weight at 20°C of hyrdoalcoholic mixtures. This is the

international table adopted by the International Organisation of Legal Metrology in its recommendation number 22.

1.7.6.1 *Beverages with an ABV lower than or equal to 1.5% vol*

The alcoholic strength by volume of a beverage with low alcohol content, with an ABV of less than 1.5% vol., is given by the following formula: ABV = ABVD/2, ABVD being the alcoholic strength by volume of the distillate.

It is expressed in "% vol.". The result is given to two decimal places.

## 2. COMPARISON OF MEASUREMENTS CARRIED OUT

using a hydrostatic balance with measurements obtained using an electronic density-meter (Annex A of the Compendium of International Methods of Analysis).

From samples whose alcoholic strength is between 4% vol. and 18% vol. the measurements of repeatability and reproducibility were performed after an inter-laboratory ring test. It is the comparison of the measurements of wine alcoholic strength of different samples using the hydrostatic balance and the electronic density-meter, including the repeatability and reproducibility values derived from pluri-annual intercomparison test trials performed on a large scale.

2.1 ***Samples****:* wines of different density and alcoholic strengths prepared monthly on an industrial scale, taken from a bottled stock stored under normal conditions, and supplied as anonymous products to laboratories.

2.2 ***Laboratories****:* laboratories participating into the monthly ring test organised by Unione Italiana Vini Verona, (Italy) according to ISO 5725 (UNI 9225) regulation and the 'International Protocol of Proficiency test for chemical analysis laboratories' established by AOAC, ISO and IUPAC (J. AOAC Intern., **1993**, *74/4*) and according to guidelines ISO 43 and ILAC G13. An annual report is supplied by the cited company to all participants.

2.3 ***Apparatus****:*

2.3.1 Electronic hydrostatic balance (whose precision allows to give the $5^{th}$ decimal of density) eventually equipped with a data treatment device.

2.3.2 Electronic density-meter eventually equipped with an autosampler.

2.4 ***Analyses***

According to method validation rules (resolution OENO 6/99), each sample is analysed twice consecutively to determine the alcoholic strength.

2.5 ***Results***

Table 1 shows the results of the measurements obtained by the laboratories using the hydrostatic balance.
Table 2 shows the results obtained by the laboratories using an electronic densimeter.

***2.6 Evaluations of the results***

2.6.1 The trial results were examined for evidence of individual systematic error ($p<0.025$) using Cochran's and Grubbs' tests successively, by procedures described in the internationally agreed *["Protocol for the Design, Conduct and Interpretation of Method-Performance Studies" Ed W Horwitz, Pure and Applied Chemistry, 1995, 67, (2), 331-343.*].

2.6.2 Repeatability (r) and reproducibility (R)

Calculations for repeatability (r) and reproducibility (R) as defined by the protocol were carried out on the results remaining after the removal of outliers. When assessing a new method there is often no validated reference or statutory method with which to compare precision criteria, hence it is useful to compare the precision data obtained from collaborative trials with "predicted" levels of precision. These "predicted" levels are calculated from the Horwitz formula. Comparison of the trial results and the predicted levels indicate as to whether the method is sufficiently precise for the level of analyte being measured.

The predicted Horwitz value is calculated from the Horwitz formula.

$RSD_R = 2^{(1-0.5 \log C)}$

where C = measured concentration of analyte expressed in decimals.
(e.g. 1 g/100g = 0.01) *[Horwitz, W., Analytical Chemistry, 1982, 54, 67A-76A.*].

The Horrat value gives a comparison of the actual precision measured with the precision predicted by the Horwitz formula for the method and at that particular level of concentration of the analyte. It is calculated as follows:
$Ho_R = RSD_R(\text{measured})/RSD_R(\text{Horwitz})$

2.6.3 Interlaboratory precision
A Horrat value of 1 usually indicates satisfactory inter-laboratory

precision, whereas a value of more than 2 usually normally indicates unsatisfactory precision, i.e. one that is too variable for most analytical purposes or where the variation obtained is greater than that expected for the type of method employed. $Ho_r$ is also calculated, and used to assess intra-laboratory precision, using the following approximation:
$RSD_r(Horwitz) = 0.66\ RSD_R(Horwitz)$ (this assumes the approximation $r = 0.66\ R$).

Table 3 shows the differences between the measurements obtained by laboratories using an electronic densimeter and those using a hydrostatic balance. Excluding the sample of 2000/3 with very low alcohol strength and for which both techniques show poor reproducibility, a very good concordance is generally observed for the other samples.

2.6.4 Fidelity parameters

Table 4 shows the averaged overall fidelity parameters computed from all monthly trials carried out from January 1999 until May 2001.
In particular:
Repeatability (r)= 0.074 (% vol.) for the hydrostatic balance and 0.061 (% vol.) for electronic densitometry;
Reproducibility (R)= 0.229 (% vol.) for the hydrostatic balance and 0.174 (% vol.) for electronic densimetry, this latter value is concordant to the value estimated for the electronic densimetry from the OIV Compendium of International Methods of Analysis;

## 2.7 Conclusion

The results concerning the determination of the alcoholic strength of a large range of wines show that the measurements carried out with the hydrostatic balance are concordant with those carried out by electronic densimetry using a flexion resonator and that the validation parameter values are similar for both methods.

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Legend:

*mean* *the mean of all the data used in the statistical analysis*

*n* *total number of sets of data submitted*

*nc* *number of results excluded from statistical analysis due to non-compliance*

*outliers* *number of results excluded from statistical analysis due to determination as outliers by either Cochran's or Grubbs' tests*

$n_1$ *number of results used in statistical analysis*

*r* *repeatability limit*

$S_r$ *the standard deviation of the repeatability*

$RSD_r$ *the relative standard deviation of the repeatability ($S_r$ x 100/MEAN).*

$Ho_r$ *the HORRAT value for repeatability is the observed $RSD_r$ divided by the $RSD_r$ value estimated from the Horwitz formula using the approximation r = 0.66R*

*R* *reproducibility limit*

$S_R$ *the standard deviation of the reproducibility*

$Ho_R$ *the HORRAT value for reproducibility is the observed $RSD_R$ value divided by the $RSD_R$ value calculated from*

$Ho_R = RSD_R(measured)/RSD_R$

## Table 1: Hydrostatic Balance (HB)

| | MEAN | n | outliers | n1 | r | sr | RSDr | Hor | R | sR | RSDR | HoR | no. of replicates | critical difference CrD95 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1999/1 | 11.043 | 17 | 1 | 16 | 0.0571 | 0.0204 | 0.1846 | 0.1004 | 0.1579 | 0.0564 | 0.5107 | 0.18 | 2 | 0.1080 |
| 1999/2 | 11.247 | 14 | 1 | 13 | 0.0584 | 0.0208 | 0.1854 | 0.1011 | 0.1803 | 0.0644 | 0.5727 | 0.21 | 2 | 0.1241 |
| 1999/3 | 11.946 | 16 | 0 | 16 | 0.0405 | 0.0145 | 0.1211 | 0.0666 | 0.1593 | 0.0569 | 0.4764 | 0.17 | 2 | 0.1108 |
| 1999/4 | 7.653 | 17 | 1 | 16 | 0.0502 | 0.0179 | 0.2344 | 0.1206 | 0.1537 | 0.0549 | 0.7172 | 0.24 | 2 | 0.1057 |
| 1999/5 | 11.188 | 17 | 0 | 17 | 0.0871 | 0.0311 | 0.2780 | 0.1515 | 0.2701 | 0.0965 | 0.8622 | 0.31 | 2 | 0.1860 |
| 1999/6 | 11.276 | 19 | 0 | 19 | 0.0846 | 0.0302 | 0.2680 | 0.1462 | 0.2957 | 0.1056 | 0.9365 | 0.34 | 2 | 0.2047 |
| 1999/7 | 8.018 | 17 | 0 | 17 | 0.0890 | 0.0318 | 0.3964 | 0.2054 | 0.2573 | 0.0919 | 1.1462 | 0.39 | 2 | 0.1764 |
| 1999/9 | 11.226 | 17 | 0 | 17 | 0.0580 | 0.0207 | 0.1846 | 0.1423 | 0.2796 | 0.0999 | 0.8896 | 0.45 | 2 | 0.1956 |
| 1999/10 | 11.026 | 17 | 0 | 17 | 0.0606 | 0.0216 | 0.1961 | 0.1066 | 0.2651 | 0.0947 | 0.8588 | 0.31 | 2 | 0.1850 |
| 1999/11 | 7.701 | 16 | 1 | 15 | 0.0643 | 0.0229 | 0.2980 | 0.1535 | 0.2330 | 0.0832 | 1.0805 | 0.37 | 2 | 0.1616 |
| 1999/12 | 10.987 | 17 | 2 | 15 | 0.0655 | 0.0234 | 0.2128 | 0.1156 | 0.1258 | 0.0449 | 0.4089 | 0.15 | 2 | 0.0827 |
| 2000/1 | 11.313 | 16 | 0 | 16 | 0.0986 | 0.0352 | 0.3113 | 0.1699 | 0.2577 | 0.0920 | 0.8135 | 0.29 | 2 | 0.1754 |
| 2000/2 | 11.232 | 17 | 0 | 17 | 0.0859 | 0.0307 | 0.2731 | 0.1489 | 0.2535 | 0.0905 | 0.8060 | 0.29 | 2 | 0.1740 |
| 2000/3 | 0.679 | 10 | 0 | 10 | 0.0680 | 0.0243 | 3.5773 | 1.2783 | 0.6529 | 0.2332 | 34.3395 | 8.10 | 2 | 0.4604 |
| 2000/4 | 11.223 | 18 | 0 | 18 | 0.0709 | 0.0253 | 0.2257 | 0.1230 | 0.2184 | 0.0780 | 0.6951 | 0.25 | 2 | 0.1503 |
| 2000/5 | 7.439 | 19 | 1 | 18 | 0.0630 | 0.0225 | 0.3023 | 0.1549 | 0.1522 | 0.0544 | 0.7307 | 0.25 | 2 | 0.1029 |
| 2000/6 | 11.181 | 19 | 0 | 19 | 0.0536 | 0.0191 | 0.1710 | 0.0932 | 0.2783 | 0.0994 | 0.8890 | 0.32 | 2 | 0.1950 |
| 2000/7 | 10.858 | 16 | 0 | 16 | 0.0526 | 0.0188 | 0.1731 | 0.0939 | 0.1827 | 0.0653 | 0.6011 | 0.22 | 2 | 0.1265 |
| 2000/9 | 12.031 | 17 | 1 | 16 | 0.0602 | 0.0215 | 0.1787 | 0.0985 | 0.2447 | 0.0874 | 0.7263 | 0.26 | 2 | 0.1704 |
| 2000/10 | 11.374 | 18 | 0 | 18 | 0.0814 | 0.0291 | 0.2555 | 0.1395 | 0.2701 | 0.0965 | 0.8482 | 0.31 | 2 | 0.1866 |
| 2000/11 | 7.644 | 18 | 0 | 18 | 0.0827 | 0.0295 | 0.3863 | 0.1988 | 0.2289 | 0.0817 | 1.0694 | 0.36 | 2 | 0.1565 |
| 2000/12 | 11.314 | 19 | 1 | 18 | 0.0775 | 0.0277 | 0.2447 | 0.1336 | 0.2421 | 0.0864 | 0.7641 | 0.28 | 2 | 0.1667 |
| 2001/1 | 11.415 | 19 | 0 | 19 | 0.0950 | 0.0339 | 0.2971 | 0.1623 | 0.2410 | 0.0861 | 0.7539 | 0.27 | 2 | 0.1636 |
| 2001/2 | 11.347 | 19 | 0 | 19 | 0.0792 | 0.0283 | 0.2493 | 0.1361 | 0.1944 | 0.0694 | 0.6119 | 0.22 | 2 | 0.1316 |
| 2001/3 | 11.818 | 16 | 0 | 16 | 0.0659 | 0.0235 | 0.1990 | 0.1093 | 0.2636 | 0.0941 | 0.7965 | 0.29 | 2 | 0.1834 |
| 2001/4 | 11.331 | 17 | 0 | 17 | 0.1067 | 0.0381 | 0.3364 | 0.1836 | 0.1895 | 0.0677 | 0.5971 | 0.22 | 2 | 0.1229 |
| 2001/5 | 8.063 | 19 | 1 | 18 | 0.0782 | 0.0279 | 0.3465 | 0.1797 | 0.1906 | 0.0681 | 0.8442 | 0.29 | 2 | 0.1290 |

## Table 2: Electronic Densimetry (ED)

| | MEAN n1 | n | outliers | n1 | r | sr | RSDr | Hor | R | sR | RSDR | HoR | no. of replicates | critical difference CrD95 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D1999/1 | 11.019 | 18 | 1 | 17 | 0.0677 | 0.0242 | 0.2196 | 0.1193 | 0.1996 | 0.0713 | 0.6470 | 0.23 | 2 | 0.1370 |
| D1999/2 | 11.245 | 19 | 2 | 17 | 0.0448 | 0.0160 | 0.1423 | 0.0776 | 0.1311 | 0.0468 | 0.4165 | 0.15 | 2 | 0.0900 |
| D1999/3 | 11.967 | 21 | 0 | 21 | 0.0701 | 0.0250 | 0.2091 | 0.1151 | 0.1552 | 0.0554 | 0.4631 | 0.17 | 2 | 0.1040 |
| D1999/4 | 7.643 | 19 | 1 | 18 | 0.0610 | 0.0218 | 0.2852 | 0.1467 | 0.1340 | 0.0479 | 0.6262 | 0.21 | 2 | 0.0897 |
| D1999/5 | 11.188 | 21 | 3 | 18 | 0.0260 | 0.0093 | 0.0829 | 0.0452 | 0.2047 | 0.0731 | 0.6536 | 0.24 | 2 | 0.1442 |
| D1999/6 | 11.303 | 21 | 0 | 21 | 0.0652 | 0.0233 | 0.2061 | 0.1125 | 0.1466 | 0.0523 | 0.4631 | 0.17 | 2 | 0.0984 |
| D1999/7 | 8.026 | 21 | 0 | 21 | 0.0884 | 0.0316 | 0.3935 | 0.2039 | 0.1708 | 0.0610 | 0.7600 | 0.26 | 2 | 0.1124 |
| D1999/9 | 11.225 | 17 | 0 | 17 | 0.0372 | 0.0133 | 0.1183 | 0.0645 | 0.1686 | 0.0602 | 0.5366 | 0.19 | 2 | 0.1178 |
| D1999/10 | 11.011 | 19 | 0 | 19 | 0.0915 | 0.0327 | 0.2969 | 0.1613 | 0.1723 | 0.0615 | 0.5588 | 0.20 | 2 | 0.1129 |
| D1999/11 | 7.648 | 21 | 1 | 20 | 0.0615 | 0.0220 | 0.2872 | 0.1478 | 0.1538 | 0.0549 | 0.7183 | 0.24 | 2 | 0.1043 |
| D1999/12 | 10.999 | 16 | 1 | 15 | 0.0428 | 0.0153 | 0.1389 | 0.0755 | 0.2015 | 0.0720 | 0.6541 | 0.23 | 2 | 0.1408 |
| D2000/1 | 11.248 | 22 | 1 | 21 | 0.0697 | 0.0249 | 0.2212 | 0.1206 | 0.1422 | 0.0508 | 0.4516 | 0.16 | 2 | 0.0944 |
| D2000/2 | 11.240 | 19 | 3 | 16 | 0.0448 | 0.0160 | 0.1424 | 0.0776 | 0.1619 | 0.0578 | 0.5145 | 0.19 | 2 | 0.1123 |
| D2000/3 | 0.526 | 12 | 1 | 11 | 0.0327 | 0.0117 | 2.2185 | 0.7630 | 0.9344 | 0.3337 | 63.4009 | 14.39 | 2 | 0.6605 |
| D2000/4 | 11.225 | 19 | 1 | 18 | 0.0476 | 0.0170 | 0.1514 | 0.0825 | 0.1350 | 0.0482 | 0.4295 | 0.15 | 2 | 0.0924 |
| D2000/5 | 7.423 | 21 | 0 | 21 | 0.0628 | 0.0224 | 0.3019 | 0.1547 | 0.2635 | 0.0941 | 1.2677 | 0.43 | 2 | 0.1836 |
| D2000/6 | 11.175 | 23 | 2 | 21 | 0.0606 | 0.0217 | 0.1938 | 0.1056 | 0.1697 | 0.0606 | 0.5424 | 0.20 | 2 | 0.1161 |
| D2000/7 | 10.845 | 21 | 5 | 16 | 0.0440 | 0.0157 | 0.1449 | 0.0786 | 0.1447 | 0.0517 | 0.4766 | 0.17 | 2 | 0.0999 |
| D2000/9 | 11.983 | 22 | 1 | 21 | 0.0841 | 0.0300 | 0.2507 | 0.1380 | 0.2410 | 0.0861 | 0.7183 | 0.26 | 2 | 0.1651 |
| D2000/10 | 11.356 | 22 | 1 | 21 | 0.0635 | 0.0227 | 0.1997 | 0.1090 | 0.1865 | 0.0666 | 0.5866 | 0.21 | 2 | 0.1280 |
| D2000/11 | 7.601 | 27 | 0 | 27 | 0.0521 | 0.0186 | 0.2448 | 0.1258 | 0.1685 | 0.0602 | 0.7916 | 0.27 | 2 | 0.1162 |
| D2000/12 | 11.322 | 25 | 1 | 24 | 0.0476 | 0.0170 | 0.1503 | 0.0820 | 0.1594 | 0.0569 | 0.5028 | 0.18 | 2 | 0.1102 |
| D2001/1 | 11.427 | 29 | 0 | 29 | 0.0706 | 0.0252 | 0.2207 | 0.1206 | 0.1526 | 0.0545 | 0.4771 | 0.17 | 2 | 0.1020 |
| D2001/2 | 11.320 | 29 | 1 | 28 | 0.0675 | 0.0241 | 0.2128 | 0.1161 | 0.1570 | 0.0561 | 0.4952 | 0.18 | 2 | 0.1057 |
| D2001/3 | 11.826 | 34 | 1 | 33 | 0.0489 | 0.0175 | 0.1476 | 0.0811 | 0.1762 | 0.0629 | 0.5322 | 0.19 | 2 | 0.1222 |
| D2001/4 | 11.339 | 31 | 2 | 29 | 0.0639 | 0.0228 | 0.2012 | 0.1099 | 0.1520 | 0.0543 | 0.4788 | 0.17 | 2 | 0.1026 |
| D2001/5 | 8.058 | 28 | 0 | 28 | 0.0473 | 0.0169 | 0.2098 | 0.1088 | 0.2025 | 0.0723 | 0.8976 | 0.31 | 2 | 0.1412 |

## Table 3: Comparison of results between hydrostatic balance and electronic densimetry

| | MEAN (HB) | n | outliers | n1 | | MEAN (ED) | n | outliers | n1 | | ΔTAV(HB-ED) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1999/1 | 11.043 | 17 | 1 | 16 | D1999/1 | 11.019 | 18 | 1 | 17 | | 0.024 |
| 1999/2 | 11.247 | 14 | 1 | 13 | D1999/2 | 11.245 | 19 | 2 | 17 | | 0.002 |
| 1999/3 | 11.946 | 16 | 0 | 16 | D1999/3 | 11.967 | 21 | 0 | 21 | | -0.021 |
| 1999/4 | 7.653 | 17 | 1 | 16 | D1999/4 | 7.643 | 19 | 1 | 18 | | 0.010 |
| 1999/5 | 11.188 | 17 | 0 | 17 | D1999/5 | 11.188 | 21 | 3 | 18 | | 0.000 |
| 1999/6 | 11.276 | 19 | 0 | 19 | D1999/6 | 11.303 | 21 | 0 | 21 | | -0.028 |
| 1999/7 | 8.018 | 17 | 0 | 17 | D1999/7 | 8.026 | 21 | 0 | 21 | | -0.008 |
| 1999/9 | 11.226 | 17 | 0 | 17 | D1999/9 | 11.225 | 17 | 0 | 17 | | 0.002 |
| 1999/10 | 11.026 | 17 | 0 | 17 | D1999/10 | 11.011 | 19 | 0 | 19 | | 0.015 |
| 1999/11 | 7.701 | 16 | 1 | 15 | D1999/11 | 7.648 | 21 | 1 | 20 | | 0.052 |
| 1999/12 | 10.987 | 17 | 2 | 15 | D1999/12 | 10.999 | 16 | 1 | 15 | | -0.013 |
| 2000/1 | 11.313 | 16 | 0 | 16 | D2000/1 | 11.248 | 22 | 1 | 21 | | 0.065 |
| 2000/2 | 11.232 | 17 | 0 | 17 | D2000/2 | 11.240 | 19 | 3 | 16 | | -0.008 |
| *2000/3* | *0.679* | *10* | *0* | *10* | *D2000/3* | *0.526* | *12* | *1* | *11* | * | *0.153* |
| 2000/4 | 11.223 | 18 | 0 | 18 | D2000/4 | 11.225 | 19 | 1 | 18 | | -0.002 |
| 2000/5 | 7.439 | 19 | 1 | 18 | D2000/5 | 7.423 | 21 | 0 | 21 | | 0.016 |
| 2000/6 | 11.181 | 19 | 0 | 19 | D2000/6 | 11.175 | 23 | 2 | 21 | | 0.006 |
| 2000/7 | 10.858 | 16 | 0 | 16 | D2000/7 | 10.845 | 21 | 5 | 16 | | 0.013 |
| 2000/9 | 12.031 | 17 | 1 | 16 | D2000/9 | 11.983 | 22 | 1 | 21 | | 0.049 |
| 2000/10 | 11.374 | 18 | 0 | 18 | D2000/10 | 11.356 | 22 | 1 | 21 | | 0.018 |
| 2000/11 | 7.644 | 18 | 0 | 18 | D2000/11 | 7.601 | 27 | 0 | 27 | | 0.043 |
| 2000/12 | 11.314 | 19 | 1 | 18 | D2000/12 | 11.322 | 25 | 1 | 24 | | -0.008 |
| 2001/1 | 11.415 | 19 | 0 | 19 | D2001/1 | 11.427 | 29 | 0 | 29 | | -0.012 |
| 2001/2 | 11.347 | 19 | 0 | 19 | D2001/2 | 11.320 | 29 | 1 | 28 | | 0.027 |
| 2001/3 | 11.818 | 16 | 0 | 16 | D2001/3 | 11.826 | 34 | 1 | 33 | | -0.008 |
| 2001/4 | 11.331 | 17 | 0 | 17 | D2001/4 | 11.339 | 31 | 2 | 29 | | -0.008 |
| 2001/5 | 8.063 | 19 | 1 | 18 | D2001/5 | 8.058 | 28 | 0 | 28 | | 0.004 |
| | | | | | Average difference/ Δ TAV (HB-ED) | | | | | | 0.014 |
| | | | | | standard deviation on difference | | | | | | 0.036 |
| | | | | * | round 2000/3 is not taken into account (very low grade) | | | | | | |

## Table 4: Fidelity parameters

| MEAN | Hydrostatic balance | Electronic densimeter |
|---|---|---|
| n1 | 441 | 557 |
| Weighted repeatability variance | 0.309 | 0.267 |
| r | 0.074 | 0.061 |
| sr | 0.026 | 0.022 |
| Weighted reproducibility variance | 2.948 | 2.150 |
| R | 0.229 | 0.174 |
| sR | 0.082 | 0.062 |

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# ANNEXE

## validation parameters relating to the measurement of the ABV of beverages with low alcohol content

This document presents the results of the validation study corresponding to the method for beverages with low alcohol content (updated).

The study was carried out in accordance with the OIV documents MA-F-AS1-08-FIDMET and MA-F-AS1-09-PROPER.

**1/ Samples**

| Sample no. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Nature | Grape juice | Beverage obtained by dealcoholisation of wine | Beverage obtained by partial dealcoholisation of wine | Partially fermented grape juice | Cider | Wine-based beverage |
| Approximate value of ABV (% vol.) | < 0.5 | 0.5 | 1.5 | 2.5 | 4.5 | 6.5 |

The samples were sent to the participating laboratories, applying the double-blind principle.

**2/ Analyses**

Each of the 12 samples received by the laboratories was analysed by simple distillation or steam distillation, according to the two following procedures:

- OIV reference method involving the use of 200 mL and recovery of 200 mL of distillate,
- alternative method involving the use of 200 mL and recovery of 100 mL of distillate.

**3/ Participating laboratories**

19 laboratories from different countries took part:

| | | |
|---|---|---|
| Laboratório CVRVV | 4050-501 Porto | Portugal |
| Laboratório de Análises da CVRA | 7006-806 Évora | Portugal |
| Testing Laboratory CAFIA | 603 00 Brno | Czech Republic |
| Laboratório ASAE - LBPV | 1649-038 Lisbon | Portugal |
| Agroscope - Site de Changins | 1260 Nyon 1 | Switzerland |
| Labo SCL de Bordeaux | 33608 Pessac | France |
| Labo SCL de Montpellier | 34196 Montpellier | France |
| Laboratorio Arbitral Agroalimentario | 28023 Madrid | Spain |
| Estación Enológica de Haro | 26200 Haro La Rioja | Spain |
| Instituto dos Vinho do Douro do Porto | 4050-253 Porto | Portugal |
| IVICAM | 13700 Tomelloso, Ciudad Real | Spain |
| INCAVI | 08720 Vilafranca del Penedès | Spain |
| ICQRF Laboratorio di Conegliano/Susegana | 31058 Susegana (TV) | Italy |
| ICQRF Laboratorio di Catania | 95122 Catania | Italy |
| ICQRF Laboratorio di Modena | 41100 Modena | Italy |
| ICQRF laboratorio di Perugia | 06128 Perugia | Italy |
| ICQRF laboratorio di Salerno | 84098 Salerno | Italy |
| ICQRF Laboratorio centrale di Roma | 00149 Rome | Italy |
| Laboratoires DUBERNET | 11100 Narbonne | France |

## 4/ Results

| | Sample N° 1 | | Sample N° 2 | | Sample N° 3 | | Sample N° 4 | | Sample N° 5 | | Sample N° 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | POSITION : | | | | | | | | | | | |
| LAB | 2 | 7 | 4 | 11 | 6 | 12 | 5 | 8 | 9 | 10 | 1 | 3 |
| A | 0,21 | 0,21 | 0,55 | 0,55 | 1,34 | 1,34 | 2,58 | 2,58 | 4,59 | 4,60 | 6,54 | 6,50 |
| B | 0,11 | 0,14 | 0,49 | 0,50 | 1,32 | 1,38 | 2,60 | 2,57 | 4,68 | 4,72 | 6,52 | 6,55 |
| C | 0,33 | 0,28 | 0,68 | 0,61 | 1,43 | 1,35 | 2,63 | 2,60 | 4,63 | 4,66 | 6,58 | 6,51 |
| D | | | 0,62 | 0,62 | 1,38 | 1,36 | 2,68 | 2,67 | 4,69 | 4,73 | 6,62 | 6,64 |
| E | 0,20 | 0,21 | 0,55 | 0,56 | 1,36 | 1,40 | 2,61 | 2,62 | 4,67 | 4,68 | 6,56 | 6,55 |
| F | 0,18 | 0,12 | 0,52 | 0,51 | 1,31 | 1,30 | 2,56 | 2,56 | 4,70 | 4,66 | 6,51 | 6,54 |
| G | 0,22 | 0,22 | 0,55 | 0,56 | 1,37 | 1,37 | 2,62 | 2,62 | 4,68 | 4,68 | 6,58 | 6,57 |
| H | | | 0,41 | 0,42 | 1,25 | 1,27 | 2,46 | 2,49 | 4,57 | 4,56 | 6,39 | 6,40 |
| I | 0,20 | 0,13 | 0,54 | 0,48 | 1,32 | 1,28 | 2,60 | 2,58 | 4,62 | 4,62 | 6,57 | 6,55 |
| J | 0,24 | 0,24 | 0,58 | 0,60 | 1,41 | 1,37 | 2,63 | 2,63 | 4,69 | 4,67 | 6,55 | 6,55 |
| K | 0,22 | 0,22 | 0,56 | 0,55 | 1,35 | 1,35 | 2,63 | 2,63 | 4,67 | 4,68 | 6,59 | 6,58 |
| L | 0,22 | 0,23 | 0,56 | 0,57 | 1,38 | 1,36 | 2,63 | 2,61 | 4,66 | 4,67 | 6,56 | 6,57 |
| M | 0,18 | 0,18 | 0,53 | 0,53 | 1,33 | 1,29 | | | 4,66 | 4,65 | 6,53 | 6,52 |
| N | 0,22 | 0,23 | 0,56 | 0,57 | 1,38 | 1,41 | 2,26 | 2,61 | 4,67 | 4,67 | 6,51 | 6,57 |
| O | 0,12 | 0,19 | 0,53 | 0,52 | 1,33 | 1,33 | 2,64 | 2,62 | 4,67 | 4,67 | 6,51 | 6,55 |
| P | 0,25 | 0,25 | 0,57 | 0,58 | 1,39 | 1,41 | 2,66 | 2,65 | 4,70 | 4,68 | 6,62 | 6,62 |
| Q | 0,22 | 0,20 | 0,55 | 0,59 | 1,34 | 1,33 | 2,61 | 2,63 | 4,65 | 4,63 | 6,52 | 6,54 |
| R | 0,21 | 0,21 | 0,55 | 0,52 | 1,29 | 1,28 | 2,52 | 2,55 | 4,62 | 4,56 | 6,50 | 6,53 |
| S | 0,18 | 0,17 | 0,41 | 0,42 | 1,38 | 1,37 | 2,61 | 2,58 | 4,63 | 4,58 | 6,51 | 6,48 |

**Results table obtained for a distillation of 200 mL with a recovery volume of 200 mL.** The values in bold correspond to the values rejected by the Cochran test (variance outliers) with a significance level of 2.5% (1-tail test) and by the Grubbs test (means outliers) with a significance level of 2.5% (2-tail test).

*Note: The values absent were not provided by the laboratory in question.*

| | Sample N° 1 | | Sample N° 2 | | Sample N° 3 | | Sample N° 4 | | Sample N° 5 | | Sample N° 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | POSITION : | | | | | | | | | | | |
| LAB | 2 | 7 | 4 | 11 | 6 | 12 | 5 | 8 | 9 | 10 | 1 | 3 |
| A | | | | | | | | | | | | |
| B | 0,17 | 0,18 | 0,52 | 0,53 | 1,34 | 1,36 | 2,62 | 2,62 | 4,62 | 4,60 | 6,48 | 6,52 |
| C | 0,25 | 0,25 | 0,56 | 0,62 | 1,35 | 1,36 | 2,50 | 2,46 | 4,48 | 4,44 | 6,12 | 6,19 |
| D | 0,29 | 0,29 | 0,63 | 0,63 | 1,43 | 1,42 | 2,66 | 2,65 | 4,68 | 4,69 | 6,58 | 6,59 |
| E | 0,24 | 0,24 | 0,58 | 0,58 | 1,39 | 1,39 | 2,64 | 2,64 | 4,66 | 4,67 | 6,55 | 6,57 |
| F | 0,21 | 0,18 | 0,53 | 0,53 | 1,31 | 1,27 | 2,41 | 2,48 | 4,30 | 4,31 | 6,22 | 5,89 |
| G | 0,24 | 0,24 | 0,56 | 0,57 | 1,35 | 1,36 | 2,58 | 2,57 | 4,57 | 4,56 | 6,46 | 6,43 |
| H | 0,19 | 0,18 | 0,48 | 0,55 | 1,33 | 1,32 | 2,51 | 2,55 | 4,59 | 4,54 | 6,38 | 6,42 |
| I | 0,25 | 0,18 | 0,56 | 0,53 | 1,34 | 1,33 | 2,62 | 2,61 | 4,64 | 4,64 | 6,25 | 6,28 |
| J | 0,24 | 0,24 | 0,55 | 0,56 | 1,31 | 1,32 | 2,49 | 2,53 | 4,37 | 4,34 | 6,14 | 6,12 |
| K | 0,25 | 0,25 | 0,57 | 0,57 | 1,37 | 1,38 | 2,60 | 2,61 | 4,60 | 4,61 | 6,48 | 6,38 |
| L | 0,24 | 0,24 | 0,55 | 0,55 | 1,35 | 1,31 | 2,52 | 2,47 | 4,38 | 4,31 | 6,09 | 6,06 |
| M | 0,19 | 0,20 | 0,55 | 0,55 | 1,34 | 1,31 | | | 4,68 | 4,67 | 6,52 | 6,54 |
| N | 0,28 | 0,26 | 0,58 | 0,59 | 1,28 | 1,28 | 2,52 | 2,47 | 4,44 | 4,32 | 6,01 | 6,15 |
| O | 0,19 | 0,25 | 0,57 | 0,57 | 1,39 | 1,39 | 2,63 | 2,64 | 4,66 | 4,66 | 6,57 | 6,57 |
| P | 0,25 | 0,26 | 0,57 | 0,57 | 1,36 | 1,36 | 2,58 | 2,56 | 4,54 | 4,53 | 6,34 | 6,38 |
| Q | 0,24 | 0,24 | 0,57 | 0,57 | 1,38 | 1,38 | 2,63 | 2,62 | 4,66 | 4,67 | 6,56 | 6,56 |
| R | 0,23 | 0,23 | 0,54 | 0,55 | 1,32 | 1,30 | 2,54 | 2,56 | 4,56 | 4,52 | 6,40 | 6,35 |
| S | 0,27 | 0,26 | 0,55 | 0,57 | 1,34 | 1,34 | 2,46 | 2,43 | 4,53 | 4,51 | 6,36 | 6,36 |

**Results table obtained for a distillation of 200 mL with a recovery volume of 100 mL.** The values in bold correspond to the values rejected by the Cochran test (variance outliers) with a significance level of 2.5% (1-tail test) and by the Grubbs test (means outliers) with a significance level of 2.5% (2-tail test).

*Note: The values absent were not provided by the laboratory in question.*

| | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
|---|---|---|---|---|---|---|
| No. of laboratories considered | 17 | 19 | 19 | 17 | 19 | 18 |
| No. of repetitions | 2 | 2 | 2 | 2 | 2 | 2 |
| Minimum | 0.11 | 0.41 | 1.25 | 2.46 | 4.56 | 6.48 |
| Maximum | 0.33 | 0.68 | 1.43 | 2.68 | 4.73 | 6.64 |
| Overall average | 0.20 | 0.54 | 1.35 | 2.60 | 4.65 | 6.55 |
| Repeatability variance | 0.00052 | 0.00033 | 0.00050 | 0.00019 | 0.00036 | 0.00047 |
| Reproducibility variance | 0.00211 | 0.00345 | 0.00190 | 0.00229 | 0.00181 | 0.00147 |
| Inter-laboratory standard deviation | 0.043 | 0.057 | 0.041 | 0.047 | 0.040 | 0.035 |
| Repeatability standard deviation | 0.02 | 0.02 | 0.02 | 0.01 | 0.02 | 0.02 |
| r limit | 0.06 | 0.05 | 0.06 | 0.04 | 0.05 | 0.061 |
| Repeatability CV | 11.1 | 3.3 | 1.7 | 0.5 | 0.4 | 0.3 |
| Reproducibility standard deviation | 0.046 | 0.059 | 0.044 | 0.048 | 0.043 | 0.038 |
| R limit | 0.130 | 0.166 | 0.123 | 0.135 | 0.120 | 0.109 |
| Reproducibility CV | 22.5 | 10.9 | 3.2 | 1.8 | 0.9 | 0.6 |
| Horwitz $RSD_r$ | 3.36 | 2.90 | 2.52 | 2.29 | 2.09 | 1.99 |
| $Horrat_r$ | 3.3 | 1.1 | 0.7 | 0.2 | 0.2 | 0.2 |
| Horwitz $RSD_R$ | 5.10 | 4.39 | 3.82 | 3.46 | 3.17 | 3.01 |
| $Horrat_R$ | 4.4 | 2.5 | 0.8 | 0.5 | 0.3 | 0.2 |

Table: Data obtained for a 200 mL distillate from a 200 mL sample.

| | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
|---|---|---|---|---|---|---|
| No. of laboratories considered | 16 | 15 | 18 | 17 | 17 | 17 |
| No. of repetitions | 2 | 2 | 2 | 2 | 2 | 2 |
| Minimum | 0.17 | 0.52 | 1.27 | 2.41 | 4.30 | 6.01 |
| Maximum | 0.29 | 0.63 | 1.43 | 2.66 | 4.69 | 6.59 |
| Overall average | 0.24 | 0.56 | 1.35 | 2.56 | 4.55 | 6.38 |
| Repeatability variance | 0.00006 | 0.00003 | 0.00016 | 0.00050 | 0.00039 | 0.00135 |
| Inter-laboratory standard deviation | 0.03209 | 0.02496 | 0.03752 | 0.07013 | 0.12167 | 0.17621 |
| Reproducibility variance | 0.001 | 0.001 | 0.001 | 0.005 | 0.015 | 0.031 |
| Repeatability standard deviation | 0.01 | 0.01 | 0.01 | 0.02 | 0.02 | 0.04 |
| r limit | 0.02 | 0.02 | 0.04 | 0.06 | 0.06 | 0.104 |
| Repeatability CV | 3.2 | 1.0 | 0.9 | 0.9 | 0.4 | 0.6 |
| Reproducibility standard deviation | 0.033 | 0.025 | 0.039 | 0.072 | 0.122 | 0.178 |
| R limit | 0.092 | 0.071 | 0.109 | 0.203 | 0.347 | 0.504 |
| Reproducibility CV | 13.8 | 4.5 | 2.9 | 2.8 | 2.7 | 2.8 |
| Horwitz $RSD_r$ | 3.27 | 2.88 | 2.52 | 2.29 | 2.10 | 2.00 |
| $Horrat_r$ | 1.0 | 0.4 | 0.4 | 0.4 | 0.2 | 0.3 |
| Horwitz $RSD_R$ | 4.96 | 4.36 | 3.82 | 3.47 | 3.18 | 3.03 |
| $Horrat_R$ | 2.8 | 1.0 | 0.8 | 0.8 | 0.9 | 0.9 |

Table: Data obtained for a 100 mL distillate from a 200 mL sample.

**Method OIV-MA-AS312-01B** Type IV methods

# Alcoholic strength by volume

(Resolution Oeno 377/2009)

## 1. DEFINITION

The alcoholic strength by volume is the number of liters of ethanol contained in 100 liters of wine, both volumes being measured at a temperature of 20°C. It is expressed by the symbol '% vol.

*Note:* Homologues of ethanol, together with the ethanol and esters of ethanol homologues are included in the alcoholic strength since they occur in the distillate.

## 2. PRINCIPLE OF METHODS

2.1. *Distillation of wine* made alkaline by a suspension of calcium hydroxide.
Measurement of the alcoholic strength of the distillate:

2.3. *Type IV methods:*

A. Measurement of the alcoholic strength of the distillate with a hydrometer
B. Measurement of the alcoholic strength of the distillate by refractometry.

# 3. METHOD OF OBTAINING DISTILLATE

## 3.1. Apparatus

3.1.1 Distillation apparatus, consisting of:

- a round-bottomed 1-liter flask with ground-glass joints.
- a rectifying column about 20 cm in height or any similar condenser.
- a source of heat; any pyrolysis of extracted matter must be prevented by a suitable arrangement.
- a condenser terminated by a drawn-out tube taking the distillate to the bottom of a graduated receiving flask containing several mL of water.

3.1.2 Steam distillation apparatus consisting of:

- a steam generator
- a steam pipe
- a rectifying column
- a condenser.

Any type of distillation or steam distillation apparatus may be used provided that it satisfies the following test:

Distil an ethanol-water mixture with an alcoholic strength of 10% vol. five times in succession. The distillate should have an alcoholic strength of at least 9.9% vol. after the fifth distillation; i.e. the loss of alcohol during each distillation should not be more than 0.02% vol.

## 3.2. Reagents

Suspension of calcium hydroxide, 2 M

Obtain by carefully pouring 1 liter of water at 60 to 70°C on to 120 g of quicklime, CaO.

## 3.3. Preparation of sample

Remove the bulk of any carbon dioxide from young and sparkling wines by stirring 250 to 300 mL of the wine in a 1000 mL flask.

## 3.4. Procedure

Measure out 200 mL of the wine using a volumetric flask. Record the temperature of the wine.

Transfer the wine to the distillation flask and introduce the steam-pipe of the steam distillation apparatus. Rinse the volumetric flask four times with successive 5 mL washings of water added to the flask or the steam-pipe. Add

10 mL of calcium hydroxide. 2 mol/L. and several pieces of inert porous material (pumice etc).

Collect the distillate in the 200 mL graduated flask used to measure the wine.

Collect a volume of about three-quarters of the initial volume if distillation is used and a volume of 198 to 199 mL of distillate if steam distillation is used. Make up to 200 mL with distilled water, keeping the distillate at a temperature within 2°C of the initial temperature.

Mix carefully, using a circular motion.

*Note:* In the case of wines containing particularly large concentrations of ammonium ions, the distillate may be redistilled under the conditions described above, but replacing the suspension of calcium hydroxide with 1 mL sulfuric acid diluted 10 /100.

*Precautionary safety measures*

Respect the safety guidelines for the usage of distillation apparatuses, the manipulation of hydro-alcoholic and cleaning solutions.

# 4. Measurement of the alcoholic strength of the distillate with a hydrometer or by refractometry
## (Type IV Methods)

### 4.1. *Hydrometer*

4.1.1 Apparatus

- Alcoholmeter

The alcoholmeter must conform to the specification for class I or class II equipment defined in International Recommendation No 44. *Alcoholmeters and Alcohol Hydrometers,* of the OIML (Organisation Internationale de Métrologie Légale).

- Thermometer graduated in degrees and in 0.1°C from 0 to 40°C certified to within 1/20th degree.
- Measuring cylinder. 36 mm diameter and 320 mm height, held vertically by supporting leveling screws.

4.1.2 Procedure

Pour the distillate (3.4) into the measuring cylinder. Ensure that the cylinder is kept vertical. Insert the thermometer and alcoholmeter. Read the temperature on the thermometer one minute after stirring to equilibrate the temperature of the measuring cylinder, the thermometer, the alcoholmeter and the distillate. Remove the thermometer and read the apparent alcoholic strength after one minute. Take at least three readings using a magnifying glass. Correct the apparent strength measure at *t*°C for the effect of temperature using Table II.

The temperature of the liquid must differ very little from ambient temperature (at most, by 5°C).

### 4.2. *Refractometry*

4.2.1 *Apparatus*

- Refractometer enabling refractive indices to be measured in the range 1.330 to 1.346.

Depending on the type of equipment, measurements are made:

- either at 20°C with a suitable instrument.
- or at ambient temperature *t*°C by an instrument fitted with a thermometer enabling the temperature to be determined to within at least 0.05°C. A table giving temperature corrections will be provided with the instrument.

4.2.2 *Procedure*

The refractive index of the wine distillate obtained as in 3.3 above is measured by following the procedure prescribed for the type of instrument used.

4.2.3 *Expression of results*

Table IV is used to find the alcoholic strength corresponding to the refractive index at 20°C.

*Note:* Table IV gives the alcoholic strengths corresponding to refractive indices for both pure alcohol-water mixtures and for wine distillates. In the case of wine distillates, it takes into account the presence of impurities in the distillate (mainly higher alcohols). The presence of methanol lowers the refractive index and thus the alcoholic strength.

*Note:* To obtain the alcoholic strength from the density of the distillate, use Tables I, II and III in Annex II to this section of this Chapter. These have been calculated from the *International Tables of Alcoholic Strength* published in 1972 by the International Legal Metrology Organization in its Recommendation No. 22 and adopted by the OIV (General Assembly. 1974). Annex I gives the general formula relating the alcoholic strength by volume and the density of alcohol-water mixtures as a function of temperature.

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TABLE I International alcoholic strength at 20°C

**Table of apparent densities of ethanol-water mixtures - Pyrex pycnometer** Densities at *t*°C. corrected for air buoyancy

| | Alcohol % by volume | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *t*° | **0** | | **1** | | **2** | | **3** | | **4** | | **5** | | **6** | | **7** | | **8** | | **9** | | **10** | | **11** | |
| 0° | 999.64 | 1.50 | 998.14 | 1.44 | 996.70 | 1.40 | 995.30 | 1.35 | 993.95 | 1.30 | 992.65 | 1.24 | 991.41 | 1.19 | 990.22 | 1.14 | 989.08 | 1.10 | 987.98 | 1.05 | 986.93 | 1.00 | 985.93 | 0.95 |
| | -0.07 | | -0.06 | | -0.06 | | -0.06 | | -0.06 | | -0.06 | | -0.06 | | -0.05 | | -0.04 | | -0.03 | | -0.02 | | -0.01 | |
| 1° | 999.71 | 1.51 | 998.20 | 1.44 | 996.76 | 1.40 | 995.36 | 1.35 | 994.01 | 1.30 | 992.71 | 1.24 | 991.47 | 1.20 | 990.27 | 1.15 | 989:12 | 1.11 | 988.01 | 1.06 | 986.95 | 1.01 | 985.94 | 0.97 |
| | -0.05 | | -0.05 | | -0.04 | | -0.04 | | -0.04 | | -0.04 | | -0.03 | | -0.03 | | -0.02 | | -0.02 | | -0.01 | | 0.00 | |
| 2° | 999.76 | 1.51 | 998.25 | 1.45 | 996.80 | 1.40 | 995.40 | 1.35 | 994.05 | 1.30 | 992.75 | 1.25 | 99150 | 1.20 | 990.30 | 1.16 | 989.14 | 1.11 | 988.03 | 1.07 | 986.96 | 1.02 | 985.94 | 0.98 |
| | -0.03 | | -0.03 | | 4.03 | | -0.02 | | -0.02 | | -0.02 | | -0.02 | | -0.01 | | -0.01 | | 0.00 | | 0.01 | | 0.02 | |
| 3° | 999.79 | 1.51 | 998.28 | 1.45 | 996.83 | 1.41 | 995.42 | 1.35 | 994.07 | 1.30 | 992.77 | 1.25 | 991.52 | 1.21 | 990.31 | 1.16 | 989.15 | 1.12 | 988.03 | 1.08 | 986.95 | 1.03 | 985.92 | 1.00 |
| | -0.02 | | -0.02 | | -0.01 | | -0.02 | | -0.01 | | -0.01 | | 0.00 | | 0.00 | | 0.01 | | 0.02 | | 0.03 | | 0.04 | |
| 4° | 999.81 | 1.51 | 998.30 | 1.46 | 996.84 | 1.40 | 995.44 | 1.36 | 994.08 | 1.30 | 992.78 | 1.26 | 991.52 | 1.21 | 990.31 | 1.17 | 989.14 | 1.13 | 988:01 | 1.09 | 986.92 | 1.04 | 985.88 | 1.00 |
| | 0.00 | | 0.00 | | 0.00 | | 0.00 | | 0.01 | | 0.02 | | 0.02 | | 0.02 | | 0.02 | | 0.03 | | 0.04 | | 0.05 | |
| 5° | 999.81 | 1.51 | 998.30 | 1.46 | 996.84 | 1.40 | 995.44 | 1.37 | 994.07 | 1.31 | 992.76 | 1.26 | 991.50 | 1.21 | 990.29 | 1.17 | 989.12 | 1.14 | 987.98 | 1.10 | 986.88 | 1.05 | 985.83 | 1.01 |
| | 0.01 | | 0.01 | | 0.01 | | 0.02 | | 0.01 | | 0.02 | | 0.03 | | 0.04 | | 0.05 | | 0.05 | | 0.05 | | 0.06 | |
| 6° | 999.80 | 1.51 | 998.29 | 1.46 | 996.83 | 1.41 | 995.42 | 1.36 | 994.06 | 1.32 | 992.74 | 1.27 | 991.47 | 1.22 | 990.25 | 1.18 | 989:07 | 1.14 | 987.93 | 1.10 | 986.83 | 1.06 | 985.77 | 1.03 |
| | 0.03 | | 0.03 | | 0.03 | | 0.03 | | 0.04 | | 0.04 | | 0.04 | | 0.05 | | 0.06 | | 0.07 | | 0.08 | | 0.09 | |
| 7° | 999.77 | 1.51 | 998.26 | 1.46 | 996.80 | 1.41 | 995.39 | 1.37 | 994.02 | 1.32 | 99270 | 1.27 | 99143 | 1.23 | 99020 | 1.19 | 989.01 | 1.15 | 987:86 | 1.11 | 986.75 | 1.07 | 985.68 | 1.03 |
| | 0.05 | | 0.04 | | 0.04 | | 0.05 | | 0.05 | | 0.05 | | 0.05 | | 0.06 | | 0.06 | | 0.07 | | 0.08 | | 0.09 | |
| 8° | 999.72 | 1.50 | 998.22 | 1.46 | 996.76 | 1.42 | 995.34 | 1.37 | 993.97 | 1.32 | 992.65 | 1.27 | 991.38 | 1.24 | 990.14 | 1.19 | 988.95 | 1.16 | 987.79 | 1.12 | 986.67 | 1.08 | 985.59 | 1.05 |
| | 0.05 | | 0.06 | | 0.06 | | 0.06 | | 0.06 | | 0.06 | | 0.07 | | 0.07 | | 0.08 | | 0.09 | | 0.10 | | 0.11 | |
| 9° | 999.67 | 1.51 | 998.16 | 1.46 | 996.70 | 1.42 | 995.28 | 1.37 | 993.91 | 1.32 | 992:59 | 1.28 | 99131 | 1.24 | 990.07 | 1.20 | 988.87 | 1.17 | 987.70 | 1.13 | 986.57 | 1.09 | 985.48 | 1.06 |
| | 0.07 | | 0.07 | | 0.07 | | 0.07 | | 0.07 | | 0.08 | | 0.08 | | 0.09 | | 0.09 | | 0.10 | | 0.11 | | 0.12 | |
| 10° | 999.60 | 1.51 | 998.09 | 1.46 | 996.63 | 1.42 | 995.21 | 1.37 | 993.84 | 1.33 | 992.51 | 1.28 | 991.23 | 1.25 | 989.98 | 1.20 | 988.78 | 1.17 | 987.60 | 1.14 | 986.46 | 1.10 | 985.36 | 1.06 |
| | 0.09 | | 0.09 | | 0.09 | | 0.08 | | 0.09 | | 0.09 | | 0.10 | | 0.10 | | 0.11 | | 0.11 | | 0.12 | | 0.13 | |
| 11° | 999.51 | 1.51 | 998.00 | 1.46 | 996.54 | 1.41 | 995.13 | 1.38 | 993.75 | 1.33 | 992.42 | 1.29 | 991.13 | 1.25 | 989.88 | 1.21 | 988.67 | 1.18 | 987.49 | 1.15 | 986.34 | 1.11 | 985.23 | 1.07 |
| | 0.10 | | 0.09 | | 0.09 | | 0.10 | | 0.10 | | 0.11 | | 0.11 | | 0.11 | | 0.12 | | 0.13 | | 0.13 | | 0.14 | |
| 12° | 999.41 | 1.50 | 997.91 | 1.46 | 996.45 | 1.42 | 995.03 | 1.38 | 993.65 | 1.34 | 992.31 | 1.29 | 991.02 | 1.25 | 989.77 | 1.22 | 988.55 | 1.19 | 987.36 | 1.15 | 986.21 | 1.12 | 98.509 | 1.09 |
| | 0.11 | | 0.11 | | 0.11 | | 0.11 | | 0.11 | | 0.11 | | 0.12 | | 0.12 | | 0.13 | | 0.14 | | 0.15 | | 0.16 | |
| 13° | 999.30 | 1.50 | 997.80 | 1.46 | 996.34 | 1.42 | 994.92 | 1.38 | 993.54 | 1.34 | 992.20 | 1.30 | 990.90 | 1.25 | 989.65 | 1.23 | 988.42 | 1.20 | 987.22 | 1.16 | 986.06 | 1.13 | 984.93 | 1.09 |
| | 0.12 | | 0.12 | | 0.13 | | 0.13 | | 0.13 | | 0.13 | | 0.14 | | 0.14 | | 0.15 | | 0.16 | | 0.16 | | 0.16 | |
| 14° | 999.18 | 1.50 | 997.68 | 1.46 | 996.22 | 1.43 | 994.79 | 1.38 | 993.41 | 1.34 | 992.07 | 1.30 | 990.77 | 1.26 | 989.51 | 1.23 | 988.28 | 1.21 | 987.07 | 1.17 | 985.90 | 1.13 | 984.77 | 1.11 |
| | 0.14 | | 0.14 | | 0.13 | | 0.13 | | 0.14 | | 0.14 | | 0.15 | | 0.16 | | 0.16 | | 0.17 | | 0.18 | | 0.18 | |
| 15° | 999.05 | 1.51 | 997.54 | 1.46 | 996.08 | 1.42 | 994.66 | 1.38 | 993.28 | 1.35 | 991.93 | 1.30 | 990.63 | 1.27 | 989.36 | 1.24 | 988.12 | 1.21 | 986.91 | 1.18 | 985.73 | 1.14 | 984.59 | 1.12 |
| | 0.14 | | 0.14 | | 0.15 | | 0.15 | | 0.15 | | 0.16 | | 0.16 | | 0.17 | | 0.17 | | 0.18 | | 0.19 | | 0.19 | |
| 16° | 998.90 | 1.50 | 997.40 | 1.46 | 995.94 | 1.43 | 994.51 | 1.38 | 993.13 | 1.35 | 991.78 | 1.31 | 990.47 | 1.27 | 989.20 | 1.25 | 987.95 | 1.21 | 986.74 | 1.19 | 985.55 | 1.15 | 984.40 | 1.13 |
| | 0.16 | | 0.16 | | 0.16 | | 0.16 | | 0.17 | | 017 | | 0.18 | | 0.18 | | 0.19 | | 0.19 | | 0.20 | | 0.20 | |
| 17° | 998.74 | 1.50 | 997.24 | 1.46 | 995.78 | 1.43 | 994.35 | 1.38 | 992.97 | 1.36 | 991.61 | 1.31 | 990.30 | 1.28 | 989.02 | 1.25 | 987.17 | 1.22 | 986.55 | 1.19 | 985.36 | 1.16 | 984.20 | 1.14 |
| | 0.17 | | 0.17 | | 0.16 | | 0.17 | | 0.17 | | 0.18 | | 0.18 | | 0.19 | | 0.20 | | 0.21 | | 0.22 | | 0.22 | |
| 18° | 998.57 | 1.50 | 997.07 | 1.46 | 995.61 | 1.42 | 994.19 | 1.39 | 992.80 | 1.36 | 991.44 | 1.32 | 990.12 | 1.28 | 988.84 | 1.26 | 987.58 | 1.23 | 986.35 | 1.20 | 985.15 | 1.17 | 983.98 | 1.14 |
| | 0.18 | | 0.18 | | 0.19 | | 0.19 | | 0.19 | | 0.19 | | 0.20 | | 0.20 | | 0.20 | | 0.21 | | 0.21 | | 0.22 | |
| 19° | 998.39 | 1.50 | 996.89 | 1.46 | 995.43 | 1.43 | 994.00 | 1.39 | 992.61 | 1.36 | 991.25 | 1.32 | 989.93 | 1.29 | 988.64 | 1.26 | 987.38 | 1.23 | 986.15 | 1.21 | 984.94 | 1.10 | 983.76 | 1.16 |
| | 0.19 | | 0.19 | | 0.19 | | 0.19 | | 0.19 | | 0.20 | | 0.20 | | 0.21 | | 0.22 | | 0.23 | | 0.24 | | 0.24 | |
| 20° | 998.20 | 1.50 | 996.70 | 1.46 | 995.24 | 1.43 | 993.81 | 1.39 | 992.42 | 1.36 | 991.06 | 1.33 | 989.73 | 1.29 | 988.44 | 1.27 | 987.17 | 1.24 | 985.93 | 1.22 | 984.71 | 1.19 | 983.52 | 1.16 |

TABLE I (continued) International alcoholic strength at 20°C

**Table of apparent densities of ethanol-water mixtures - Pyrex pycnometer** Densities at *t*°C. corrected for air buoyancy

| | Alcohol % by volume | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *t*° | **0** | | **1** | | **2** | | **3** | | **4** | | **5** | | **6** | | **7** | | **8** | | **9** | | **10** | | **11** | |
| 20° | 998.20 | 1.50 | 996.70 | 1.46 | 995.24 | 1.43 | 993.81 | 1.39 | 992.42 | 1.36 | 991.06 | 1.33 | 989.73 | 1.29 | 988.44 | 1.27 | 987.17 | 1.24 | 985.93 | 1.22 | 984.71 | 1.19 | 983.52 | 1.16 |
| | 0.20 | | 0.20 | | 0.20 | | 0.20 | | 0.21 | | 0.21 | | 0.21 | | 0.22 | | 0.22 | | 0.23 | | 0.24 | | 0.24 | |
| 21° | 998.00 | 1.50 | 996.50 | 1.46 | 995.04 | 1.43 | 993.61 | 1.40 | 992.21 | 1.36 | 990.85 | 1.33 | 989.52 | 1.30 | 988.22 | 1.27 | 986.95 | 1.25 | 985.70 | 1.23 | 984.47 | 1.19 | 983.28 | 1.18 |
| | 0.21 | | 0.21 | | 0.21 | | 0.21 | | 0.21 | | 0.22 | | 0.22 | | 0.23 | | 0.24 | | 0.24 | | 0.24 | | 0.26 | |
| 22° | 997.79 | 1.50 | 996.29 | 1.46 | 994.83 | 1.43 | 993.40 | 1.40 | 992.00 | 1.37 | 990.63 | 1.33 | 989.30 | 1.31 | 987.99 | 1.28 | 986.71 | 1.25 | 985.46 | 1.23 | 984.23 | 1.21 | 983.02 | 1.18 |
| | 0.22 | | 0.22 | | 023 | | 0.23 | | 0.23 | | 0.23 | | 0.24 | | 0.24 | | 0.24 | | 0.25 | | 0.26 | | 0.25 | |
| 23° | 997.57 | 1.50 | 996.07 | 1.47 | 994.60 | 1.43 | 993.17 | 1.40 | 991.77 | 1.37 | 990.40 | 1.34 | 989.06 | 1.31 | 987.75 | 1.28 | 986.47 | 1.26 | 985.21 | 1.24 | 983.97 | 1.20 | 982.77 | 1.20 |
| | 0.24 | | 0.23 | | 0.23 | | 0.23 | | 0.24 | | 0.24 | | 0.24 | | 0.25 | | 0.26 | | 0.26 | | 0.27 | | 0.29 | |
| 24° | 997.33 | 1.49 | 995.94 | 1.47 | 994.37 | 1.43 | 992.94 | 1.41 | 991.53 | 1.37 | 990.16 | 1.34 | 988.82 | 1.32. | 987.50 | 1.29 | 986.21 | 1.26 | 984.95 | 1.25 | 983.70 | 1.22 | 982.48 | 1.20 |
| | 0.24 | | 0.25 | | 0.24 | | 0.25 | | 0.24 | | 0.25 | | 0.26 | | 0.26 | | 0.26 | | 0.27 | | 0.28 | | 0.28 | |
| 25° | 997.09 | 1.50 | 995.59 | 1.46 | 994.13 | 1.44 | 992.69 | 1.40 | 991.29 | 1.38 | 989.91 | 1.35 | 988.56 | 1.32 | 987.24 | 1.29 | 985.95 | 1.27 | | 1.26 | 983.42 | 1.22 | 982.20 | 1.21 |
| | 0.25 | | 0.25 | | 0.26 | | 0.25 | | 0.26 | | 0.26 | | 0.26 | | 0.26 | | 0.28 | | 0.28 | | 0.28 | | 0.30 | |
| 26° | 996.84 | 1.50 | 995.34 | 1.47 | 993.87 | 1.43 | 992.44 | 1.41 | 991.03 | 1.38 | 989.65 | 1.35 | 988.30 | 1.32 | 986.98 | 1.31 | 985.67 | 1.27 | 984.40 | 1.26 | 983.14 | 1.24 | 981.90 | 1.22 |
| | 0.26 | | 0.26 | | 0.26 | | 0.27 | | 0.27 | | 0.27 | | 0.27 | | 0.28 | | 0.28 | | 0.29 | | 0.30 | | 0.30 | |
| 27° | 996.58 | 1.50 | 995.68 | 1.47 | 993.61 | 1.44 | 992.17 | 1.41 | 990.76 | 1.38 | 989.38 | 1. 35 | 988.03 | 1.33 | 986.70 | 1.31 | 985.39 | 1.28 | 984.11 | 1.27 | 982.84 | 1.24 | 981.60 | 1.23 |
| | 0.27 | | 0.27 | | 0.27 | | 0.27 | | 0.28 | | 0.28 | | 0.29 | | 0.29 | | 0.29 | | 0.30 | | 0.31 | | 0.32 | |
| 28° | 996.31 | 1.50 | 994.81 | 1.47 | 993.34 | 1.44 | 991.90 | 1.42 | 990.48 | 1.38 | 989.10 | 1.36 | 987.74 | 1.33 | 986.41 | 1.31 | 985.10 | 1.29 | 983.81 | 1.28 | 982.53 | 1.25 | 981.28 | 1.23 |
| | 0.28 | | 0.28 | | 0.28 | | 0.29 | | 0.28 | | 0.29 | | 0.29 | | 0.30 | | 0.31 | | 0.31 | | 0.31 | | 0.32 | |
| 29° | 996.03 | 1.50 | 994.53 | 1.47 | 993.06 | 1.45 | 991.61 | 1.41 | 990.20 | 1.39 | 988.81 | 1.36 | 987.45 | 1.34 | 986.11 | 1.32 | 984.79 | 1.29 | 983.50 | 1.28 | 982.22 | 1.26 | 980.96 | 1.24 |
| | 0.28 | | 0.29 | | 0.29 | | 0.29 | | 0.30 | | 0.30 | | 0.31 | | 0.31 | | 0.31 | | 0.32 | | 0.32 | | 0.33 | |
| 30° | 995.75 | 1.51 | 994.24 | 1.47 | 992.77 | 1.45 | 991.32 | 1.42 | 989.90 | 1.39 | 988.51 | 1.37 | 987.14 | 1.34 | 985.80 | 1.32 | 984.48 | 1.30 | 983.18 | 1.28 | 981.90 | 1.27 | 980.63 | 1.25 |
| | 0.30 | | 0.30 | | 0.30 | | 0.30 | | 0.31 | | 0.31 | | 0.31 | | 0.31 | | 0.32 | | 0.33 | | 0.34 | | 0.34 | |
| 31° | 995.45 | 1.51 | 993.94 | 1.47 | 992.47 | 1.45 | 991.02 | 1.43 | 989.59 | 1.39 | 988.20 | 1.37 | 986.83 | 1.34 | 985.49 | 1.33 | 984.16 | 1.31 | 982.85 | 1.29 | 981.56 | 1.27 | 980.29 | 1.26 |
| | 0.31 | | 0.31 | | 0.31 | | 0.32 | | 0.31 | | 0.32 | | 0.32 | | 0.33 | | 0.33 | | 0.34 | | 0.35 | | 0.36 | |
| 30° | 995.14 | 1.51 | 993.63 | 1.47 | 992.16 | 1.46 | 990.70 | 1.42 | 989.28 | 1.40 | 987.88 | 1.37 | 986.51 | 1.35 | 985.16 | 1.33 | 983.83 | 1.32 | 982.51 | 1.30 | 981.21 | 1.28 | 979.93 | 1.26 |
| | 0.31 | | 0.31 | | 0.32 | | 0.32 | | 0.32 | | 0.33 | | 0.33 | | 0.34 | | 0.35 | | 0.35 | | 0.35 | | 0.35 | |
| 33° | 994.93 | 1.51 | 993.32 | 1.48 | 991.84 | 1.46 | 990.38 | 1.42 | 988.96 | 1.41 | 987.55 | 1.37 | 986.18 | 1.36 | 984.82 | 1.34 | 983.48 | 1.32 | 982.16 | 1.30 | 980.86 | 1.28 | 979.58 | 1.28 |
| | 0.32 | | 0.33 | | 0.33 | | 0.33 | | 0.35 | | 0.34 | | 0.35 | | 0.35 | | 0.34 | | 0.35 | | 0.36 | | 0.37 | |
| 34° | 994.51 | 1.52 | 992.99 | 1.48 | 991.51 | 1.46 | 990.05 | 1.44 | 988.61 | 1.40 | 987.21 | 1.38 | 985.83 | 1.36 | 984.47 | 1.33 | 983.14 | 1.33 | 981.81 | 1.31 | 980.50 | 1.29 | 979.21 | 1.28 |
| | 0.33 | | 0.33 | | 0.34 | | 0.35 | | 0.34 | | 0.35 | | 0.35 | | 0.35 | | 0.36 | | 0.36 | | 0.36 | | 0.37 | |
| 35° | 994.18 | 1.52 | 992.66 | 1.49 | 991.17 | 1.47 | 989.70 | 1.43 | 988.27 | 1.41 | 986.86 | 1.38 | 985.48 | 1.36 | 984.12 | 1.34 | 982.78 | 1.33 | 981.45 | 1.31 | 980.14 | 1.30 | 978.84 | 1.29 |
| | 0.34 | | 0.35 | | 0.35 | | 0.35 | | 0.35 | | 0.35 | | 0.35 | | 0.36 | | 0.36 | | 0.37 | | 0.37 | | 0.38 | |
| 36° | 993.84 | 1.53 | 992.31 | 1.49 | 990.82 | 1.47 | 989.35 | 1.43 | 987.92 | 1.41 | 986.51 | 1.38 | 985.13 | 1.37 | 983.76 | 1.34 | 982.42 | 1.34 | 981.08 | 1.31 | 979.77 | 1.31 | 978.46 | 1.29 |
| | 0.35 | | 0.35 | | 0.36 | | 0.35 | | 0.36 | | 0.36 | | 0.37 | | 0.37 | | 0.38 | | 0.37 | | 0.39 | | 0.39 | |
| 37° | 993.49 | 1.53 | 991.96 | 1.50 | 990.46 | 1.46 | 989.00 | 1.44 | 987.56 | 1.41 | 986.15 | 1.39 | 984.76 | 1.37 | 983.39 | 1.35 | 982.04 | 1.33 | 980.71 | 1.33 | 97938 | 1.31 | 978.07 | 1.30 |
| | 0.36 | | 0.36 | | 0.36 | | 0.37 | | 0.37 | | 0.37 | | 0.37 | | 0.37 | | 0.38 | | 0.39 | | 0.38 | | 0.39 | |
| 38° | 993.13 | 1.53 | 991.60 | 1.50 | 990.10 | 1.47 | 988.63 | 1.44 | 987.19 | 1.41 | 985.78 | 1.39 | 984.39 | 1.37 | 983.02 | 1.36 | 981.66 | 1.34 | 980.32 | 1.32 | 979.00 | 1.32 | 977.68 | 1.31 |
| | 0.36 | | 0.37 | | 0.37 | | 0.37 | | 0.38 | | 0.38 | | 0.38 | | 0.39 | | 0.38 | | 0.39 | | 0.40 | | 0.40 | |
| 39° | 992.77 | 1.54 | 991.23 | 1.50 | 989.73 | 1.47 | 988.26 | 1.45 | 986.81 | 1.41 | 985.40 | 1.39 | 994.01 | 1.38 | 982.63 | 1.35 | 981.28 | 1. 35 | 979.93 | 1.33 | 978.60 | 1.32 | 977.28 | 1.32 |
| | 0.37 | | 0.37 | | 0.38 | | 0.39 | | 0.38 | | 0.39 | | 0.39 | | 0.39 | | 0.40 | | 0.39 | | 0.40 | | 0.41 | |
| $40^0$ | 992.40 | 1.54 | 990.86 | 1.51 | 989.35 | 1.48 | 987.87 | 1.44 | 986.43 | 1.42 | 985.01 | 1.39 | 983.62 | 1.38 | 982.24 | 1.36 | 980.88 | 1.34 | 979.54 | 1.34 | 978.20 | 1.33 | 976.87 | 1.32 |

TABLE I (continued) International alcoholic strength at 20°C

**Table of apparent densities of ethanol-water mixtures - Pyrex pycnometer** Densities at *t*°C. corrected for air buoyancy

| | Alcohol % by volume | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $t^o$ | 10 | | 11 | | 12 | | 13 | | 14 | | 15 | | 16 | | 17 | | 18 | | 19 | | 20 | | 21 | |
| 0 | 986.93 | 1.00 | 985.93 | 0.95 | 984.98 | 0.92 | 984.0 | 0.88 | 983.1 | 0.84 | 982.34 | 0.80 | 981.54 | 0.78 | 980.76 | 0.75 | 980.01 | 0.73 | 979.28 | 0.72 | 978.56 | 0.70 | 977.86 | 0.70 |
| | -0.02 | | -0.01 | | 0.01 | | 0.01 | | 0.03 | | 0.04 | | 0.07 | | 0.08 | | 0.10 | | 0.12 | | 0.14 | | 0.17 | |
| 1 | 986.95 | 1.01 | 995.94 | 0.97 | 984.97 | 0.92 | 984.0 | 0.90 | 983.1 | 0.85 | 982.30 | 0.83 | 981.47 | 0.79 | 980.68 | 0.77 | 979.91 | 0.75 | 979.16 | 0.74 | 978.42 | 0.73 | 977.69 | 0.72 |
| | -0.01 | | 0.00 | | 0.01 | | 0.03 | | 0.04 | | 0.07 | | 0.08 | | 0.10 | | 0.12 | | 0.14 | | 0.16 | | 0.18 | |
| 2 | 986.96 | 1.02 | 985.94 | 0.98 | 984.96 | 0.94 | 984.0 | 0.91 | 983.1 | 0.98 | 982.23 | 0.84 | 981.39 | 0.81 | 980.58 | 0.79 | 979.79 | 0.77 | 979.02 | 0.76 | 978.26 | 0.75 | 977.51 | 0.74. |
| | 0.01 | | 0.02 | | 0.04 | | 0.05 | | 0.06 | | 0.07 | | 0.09 | | 0.11 | | 0.13 | | 0.15 | | 0.17 | | 0.19 | |
| 3 | 986 95 | 1.03 | 985.92 | 1.00 | 984.92 | 0.95 | 983.9 | 0.92 | 983.0 | 0.89 | 982.16 | 0.86 | 981.30 | 0.83 | 980.47 | 0.81 | 979.66 | 0.79 | 978.87 | 0.78 | 978.09 | 0.77 | 977.32 | 0.77 |
| | 0.03 | | 0.04 | | 0.04 | | 0.06 | | 0.07 | | 0.09 | | 0.10 | | 0.12 | | 0.14 | | 0.16 | | 0.18 | | 0.20 | |
| 4 | 986 92 | 1.04 | 985.88 | 1.00 | 984.88 | 0.97 | 983.9 | 0.93 | 982.9 | 0.91 | 982.07 | 0.87 | 981.20 | 0.85 | 980.35 | 0.83 | 979.52 | 0.81 | 978.71 | 0.80 | 977.91 | 0.79 | 977.12 | 0.79 |
| | 0.04 | | 0.05 | | 0.06 | | 0.07 | | 0.09 | | 0.10 | | 0.12 | | 0.14 | | 0.15 | | 0.17 | | 0.19 | | 0.22 | |
| 5 | 986.88 | 1.05 | 985.83 | 1.01 | 984.82 | 0.98 | 983.8 | 0.95 | 982.8 | 0.92 | 981.97 | 0.89 | 981.08 | 0.87 | 980.21 | 0.84 | 979.37 | 0.83 | 978.54 | 0.82 | 977.72 | 0.82 | 976 90 | 0.80 |
| | 0.05 | | 0.06 | | 0.08 | | 0.09 | | 0.10 | | 0.12 | | 0.13 | | 0.14 | | 0.17 | | 0.19 | | 0.21 | | 0.22 | |
| 6 | 986.93 | 1.06 | 985.77 | 1.03 | 984.74 | 0.99 | 983.7 | 0.96 | 982.7 | 0.94 | 981.85 | 0.90 | 980.95 | 0.88 | 980.07 | 0.87 | 979.20 | 0.85 | 978.35 | 0.84 | 977.51 | 0.83 | 976.68 | 0.83 |
| | 0.08 | | 0.09 | | 0.09 | | 0.10 | | 0.12 | | 0.13 | | 0.15 | | 0.16 | | 0.18 | | 0.19 | | 0.21 | | 0.23 | |
| 7 | 986.75 | 1.07 | 995.68 | 1.03 | 984.65 | 1.00 | 983.6 | 0.98 | 982.6 | 0.95 | 981.72 | 0.92 | 980.80 | 0.89 | 979.91 | 0.89 | 979.02 | 0.86 | 978.16 | 0.86 | 977.30 | 0.85 | 976.45 | 0.85 |
| | 0.08 | | 0.09 | | 0.11 | | 0.13 | | 0.13 | | 0.14 | | 0.15 | | 0.18 | | 0.19 | | 0.21 | | 0.23 | | 0.25 | |
| 8 | 986.67 | 1.08 | 985.59 | 1.05 | 984.54 | 1.02 | 983.5 | 0.98 | 982.5 | 0.96 | 981.58 | 0.93 | 980.65 | 0.92 | 979.73 | 0.90 | 978.83 | 0.88 | 977.95 | 0.88 | 977.07 | 0.87 | 976.20 | 0.87 |
| | 0.10 | | 0 11 | | 0.12 | | 0.12 | | 0.14 | | 0.16 | | 0.18 | | 0.19 | | 0.21 | | 0.22 | | 0.24 | | 0.26 | |
| 9 | 986.57 | 1.09 | 985.48 | 1.06 | 984.42 | 1.02 | 983.4 | 1.00 | 982.4 | 0.98 | 981.42 | 0.95 | 980.47 | 0.93 | 979.54 | 0.92 | 978.62 | 0.89 | 977.73 | 0.90 | 976.83 | 0.89 | 975.94 | 0.89 |
| | 0.11 | | 0.12 | | 0.12 | | 0.14 | | 0.16 | | 0.17 | | 0.18 | | 0.20 | | 0.20 | | 0.23 | | 0.24 | | 0.26 | |
| 10 | 986.46 | 1.10 | 985.36 | 1.06 | 984.30 | 1.04 | 983.2 | 1.02 | 982.2 | 0.99 | 981.25 | 0.96 | 980.29 | 0.95 | 979.34 | 0.92 | 978.42 | .0.92 | 977.50 | 0.91 | 976.59 | 0.91 | 975.68 | 0.91 |
| | 0.12 | | 0.13 | | 0.14 | | 0.16 | | 0.16 | | 0.17 | | 0.19 | | 0.20 | | 0.23 | | 0.25 | | 0.27 | | 0.29 | |
| 11 | 986.34 | 1.11 | 985.23 | 1.07 | 984.16 | 1.06 | 983.1 | 1.02 | 982.0 | 1.00 | 981.08 | 0.98 | 980.10 | 0.96 | 979.14 | 0.95 | 978.19 | 0.94 | 977.25 | 0.93 | 976.32 | 0.93 | 975.39 | 0.92 |
| | 0.13 | | 0.14 | | 0.16 | | 0.16 | 1 | 0.18 | | 0.19 | | 0.21 | | 0.22 | | 0 24 | | 0.25 | | 0.27 | | 0.28 | |
| 12 | 986.21 | 1.12 | 985.09 | 1.09 | 984.00 | 1.06 | 982.9 | 1.04 | 981.9 | 1.01 | 980.89 | 1.00 | 979.89 | 0.97 | 978.92 | 0.97 | 977.95 | 0.95 | 977.00 | 0.95 | 976.05 | 0.94 | 975.11 | 0.95 |
| | 0.15 | | 0.16 | | 0.16 | | 0.18 | | 0.19 | | 0.20 | | 0.21 | | 0.23 | | 0 24 | | 0.26 | | 0.28 | | 0.30 | |
| 13 | 986.06 | 1.13 | 984.93 | 1.09 | 983.84 | 1.08 | 982.7 | 1.05 | 981.7 | 1.02 | 980.69 | 1.01 | 979.68 | 0.99 | 978.69 | 0.98 | 977.71 | 0.97 | 976.74 | 0.97 | 975.77 | 0.96 | 974.81 | 096 |
| | 0.16 | | 0.16 | | 0.18 | | 0.18 | | 0.20 | | 0.22 | | 0.23 | | 0.24 | | 0.26 | | 0.27 | | 0.28 | | 0.30 | |
| 14 | 985.90 | 1.13 | 994.77 | 1.11 | 983.66 | 1.08 | 982.5 | 1.07 | 981.5 | 1.04 | 980.47 | 1.02 | 979.45 | 1.00 | 978.45 | 1.00 | 977.45 | 0.98 | 976.47 | 0.98 | 975.49 | 0.98 | 975.51 | 0.98 |
| | 0.17 | | 0.18 | | 0.19 | | 0.20 | | 0.21 | | 0.22 | | 0.24 | | 0.25 | | 0.26 | | 0.28 | | 0.30 | | 0.32 | |
| 15 | 985.73 | 1.14 | 994.59 | 1.12 | 983.47 | 1.09 | 982.3 | 1.08 | 981.3 | 1.05 | 960.25 | 1.04 | 979.21 | 1.01 | 978.20 | 1.01 | 977.19 | 1.00 | 976.19 | 1.00 | 975.19 | 1.00 | 974.19 | 1.00 |
| | 0.18 | | 0.19 | | 0.20 | | 0.22 | | 0.22 | | 0.24 | | 0.24 | | 0.27 | | 0.28 | | 0.30 | | 0.31 | | 0.32 | |
| 16 | 985.55 | 1.15 | 984.40 | 1.13 | 983.27 | 1.11 | 982.1 | 1.08 | 981.0 | 1.07 | 980.01 | 1.04 | 978.97 | 1.04 | 977.93 | 1.02 | 976.91 | 1.02 | 975.89 | 1.01 | 974.88 | 1.01 | 973.87 | 1.02 |
| | 0.19 | | 0.20 | | 0.21 | | 0.22 | | 0.23 | | 0.24 | | 0.26 | | 0.27 | | 0.29 | | 0.30 | | 0.32 | | 0.33 | |
| 17 | 985.13 | 1.16 | 984.20 | 1.14 | 983.06 | 1.12 | 981.9 | 1.09 | 980.8 | 1.08 | 979.77 | 1.06 | 978.71 | 1.05 | 977.66 | 1.04 | 976.62 | 1.03 | 975.59 | 1.03 | 974.56 | 1.02 | 973.54 | 1.04 |
| | 0.21 | | 0.22 | | 0.22 | | 0 23 | | 0.25 | | 0.26 | | 0.27 | | 0.28 | | 0.29 | | 0.31 | | 0.32 | | 0.35 | |
| 18 | 985.15 | 1.17 | 983.76 | 1.14 | 982.84 | 1.13 | 981.7 | 1.11 | 980.6 | 1.09 | 979.51 | 1.07 | 978.44 | 1.06 | 977.38 | 1.05 | 976.33 | 1.05 | 975.28 | 1.04 | 974.24 | 1.05 | 973.19 | 1.05 |
| | 0.21 | | 0.22 | | 0.24 | | 0.24 | | 0.25 | | 0.26 | | 0.28 | | 0.29 | | 0.31 | | 0.32 | | 0.34 | | 0.35 | |
| 19 | 984.94 | 1.18 | 983.76 | 1.16 | 982.60 | 1.13 | 981.4 | 1.12 | 980.3 | 1.10 | 979.25 | 1.09 | 978.16 | 1.07 | 977.09 | 1.07 | 976.02 | 1.06 | 974.96 | 1.06 | 973.90 | 1.06 | 972.84 | 1.06 |
| | 0.23 | | 0.24 | | 0.24 | | 0.26 | | 0.27 | | 0.28 | | 0.29 | | 0.30 | | 0.31 | | 0.33 | | 0.34 | | 0.36 | |
| 20 | 984.71 | 1.19 | 983.52 | 1.16 | 982.36 | 1.15 | 981.2 | 1.13 | 980.0 | 1.11 | 978.97 | 1.10 | 977.87 | 1.08 | 1976.7 | 1.08 | 1975.7 | 1.08 | 974.63 | 1.07 | 973.56 | 1.08 | 972.48 | 1.08 |

TABLE I (continued) International alcoholic strength at 20°C

**Table of apparent densities of ethanol-water mixtures - Pyrex pycnometer** Densities at *t*°C. corrected for air buoyancy

| | Alcohol % by volume | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $t^o$ | 10 | | 11 | | 12 | | 13 | | 14 | | 15 | | 16 | | 17 | | 18 | | 19 | | 20 | | 21 | |
| 20° | 984.71 | 1.19 | 983.52 | 1.16 | 982.36 | 1.15 | 981.21 | 1.13 | 980.08 | 1.11 | 978.97 | 1.10 | 977.87 | 1.08 | 976.79 | 1.08 | 975.71 | 1.08 | 974.63 | 1.07 | 973.56 | 1.08 | 972.48 | 1.08 |
| | 0.24 | | 0.24 | | 0.26 | | 0.26 | | 0.27 | | 0.28 | | 0.29 | | 0.31 | | 0.33 | | 0.34 | | 0.36 | | 0.37 | |
| 21° | 994.47 | 1.19 | 983.28 | 1.18 | 982.10 | 1.15 | 980.95 | 1.14 | 978.81 | 1.12 | 978.69 | 1.11 | 977.58 | 1.10 | 976.48 | 1.10 | 975.38 | 1.09 | 974.29 | 1.09 | 973.20 | 1.09 | 972.11 | 1.09 |
| | 0.24 | | 0.26 | | 0.28 | | 0.29 | | 0.30 | | 0.31 | | 0.33 | | 0.33 | | 0.35 | | 0.35 | | 0.36 | | 0.37 | |
| 22° | 984.23 | 1.21 | 983.02 | 1.18 | 981.84 | 1.17 | 980.67 | 1.15 | 979.52 | 1.13 | 978.39 | 1.12 | 977.27 | 1.12 | 976.15 | 1.10 | 975.05 | 1.11 | 973.94 | 1.10 | 972.84 | 1.10 | 971.74 | 1.12 |
| | 0.26 | | 0.26 | | 0.27 | | 0.28 | | 0.29 | | 0.31 | | 0.32 | | 0.33 | | 0.35 | | 0.35 | | 0.37 | | 0.39 | |
| 23° | 983.97 | 1.20 | 982.77 | 1.20 | 981.57 | 1.18 | 980.39 | 1.16 | 979.23 | 1.15 | 978.08 | 1.13 | 976.95 | 1.13 | 975.82 | 1.12 | 974.70 | 1.11 | 973.59 | 1.12 | 972.47 | 1.12 | 971.47 | 1.12 |
| | 0.27 | | 0.29 | | 0.29 | | 0.29 | | 0.30 | | 0.31 | | 0.33 | | 0.33 | | 0.35 | | 0.37 | | 0.38 | | 0.40 | |
| 24° | 983.70 | 1.22 | 982.48 | 1.20 | 981.28 | 1.18 | 980.10 | 1.17 | 978.93 | 1.16 | 977.77 | 1.15 | 976.62 | 1.13 | 975.49 | 1.14 | 974.35 | 1.13 | 973.22 | 1.13 | 972.09 | 1.14 | 970.95 | 1.14 |
| | 0.28 | | 0.28 | | 0.29 | | 0.31 | | 0.32 | | 0.33 | | 0.33 | | 0.35 | | 0.36 | | 0.37 | | 0.39 | | 0.40 | |
| 25° | 983.42 | 1.22 | 982.20 | 1.21 | 980.99 | 1.20 | 979.79 | 1.18 | 978.61 | 1.17 | 977.44 | 1.15 | 976.29 | 1.15 | 975.14 | 1.15 | 973.99 | 1.14 | 972.85 | 1.15 | 971.70 | 1.15 | 970.55 | 1.16 |
| | 0.28 | | 0.30 | | 0.31 | | 0.31 | | 0.32 | | 0.33 | | 0.35 | | 0.36 | | 0.37 | | 0.39 | | 0.40 | | 0.41 | |
| 26° | 983.14 | 1.24 | 981.90 | 1.22 | 980.68 | 1.20 | 979.48 | 1.19 | 978.29 | 1.18 | 977.11 | 1.17 | 975.94 | 1.16 | 974.78 | 1.16 | 973.62 | 1.16 | 972.46 | 1.16 | 971.30 | 1.16 | 970.14 | 1.17 |
| | 0.30 | | 0.30 | | 0.31 | | 0.32 | | 0.33 | | 0.34 | | 0.35 | | 0.36 | | 0.38 | | 0.39 | | 0.40 | | 0.42 | |
| 27° | 982.84 | 1.24 | 981.60 | 1.23 | 980.37 | 1.21 | 979.16 | 1.20 | 977.96 | 1.19 | 976.77 | 1.18 | 975.59 | 1.17 | 974.42 | 1.18 | 973.24 | 1.17 | 972.07 | 1.17 | 970.90 | 1.18 | 969.72 | 1.18 |
| | 0.31 | | 0.32 | | 0.32 | | 0.33 | | 0.34 | | 0.35 | | 0.36 | | 0.38 | | 0.38 | | 0.40 | | 0.41 | | 0.43 | |
| 28° | 982.53 | 1.25 | 981.28 | 1.23 | 980.05 | 1.22 | 978.83 | 1.21 | 977.62 | 1.20 | 976.42 | 1.19 | 975.23 | 1.19 | 974.04 | 1.18 | 972.86 | 1.19 | 971.67 | 1.18 | 970.49 | 1.20 | 969.29 | 1.20 |
| | 0.31 | | 0.32 | | 0.33 | | 0.34 | | 0.35 | | 0.36 | | 0.37 | | 0.38 | | 0.40 | | 0.40 | | 0.42 | | 0.43 | |
| 29° | 982.22 | 1.26 | 980.96 | 1.24 | 979.72 | 1.23 | 978.49 | 1.22 | 977.27 | 1.21 | 976.06 | 1.20 | 974.86 | 1.20 | 973.66 | 1.20 | 972.46 | 1.19 | 971.27 | 1.20 | 970.07 | 1.21 | 968.86 | 1.22 |
| | 0.32 | | 0.33 | | 0.34 | | 0.35 | | 0.36 | | 0.37 | | 0.38 | | 0.40 | | 0.41 | | 0.43 | | 0.44 | | 0.45 | |
| 30° | 981.90 | 1.27 | 980.63 | 1.25 | 979.38 | 1.24 | 978.14 | 1.23 | 976.91 | 1.22 | 975.69 | 1.21 | 974.48 | 1.22 | 973.26 | 1.21 | 972.05 | 1.21 | 970.84 | 1.21 | 969.63 | 1.22 | 968.41 | 1.23 |
| | 0.34 | | 0.34 | | 0.35 | | 0.36 | | 0.37 | | 0.38 | | 0.40 | | 0.40 | | 0.41 | | 0.42 | | 0.44 | | 0.45 | |
| 31° | 981.56 | 1.27 | 980.29 | 1.26 | 979.03 | 1.25 | 977.78 | 1.24 | 976.54 | 1.23 | 975.31 | 1.23 | 974.08 | 1.22 | 972.86 | 1.22 | 971.64 | 1.22 | 970.42 | 1.23 | 969.19 | 1.23 | 967.96 | 1.24 |
| | 0.35 | | 0.36 | | 0.36 | | 0.37 | | 0.38 | | 0.39 | | 0.39 | | 0.40 | | 0.42 | | 0.43 | | 0.44 | | 0.46 | |
| 32° | 981.21 | 1.28 | 979.93 | 1.26 | 978.67 | 1.26 | 977.41 | 1.25 | 976.16 | 1.24 | 974.92 | 1.23 | 973.69 | 1.23 | 972.46 | 1.24 | 971.22 | 1.23 | 969.99 | 1.24 | 968.75 | 1.25 | 967.50 | 1.25 |
| | 0.35 | | 0.35 | | 0.37 | | 0.37 | | 0.38 | | 0.39 | | 0.40 | | 0.42 | | 0.42 | | 0.44 | | 0.45 | | 0.46 | |
| 33° | 980.86 | 1.28 | 979.58 | 1.28 | 978.30 | 1.26 | 977.04 | 1.26 | 975.78 | 1.25 | 974.53 | 1.24 | 973.29 | 1.25 | 972.04 | 1.24 | 970.80 | 1.25 | 969.55 | 1.25 | 968.30 | 1.26 | 967.04 | 1.27 |
| | 0.36 | | 0.37 | | 0.37 | | 0.38 | | 0.39 | | 0.40 | | 0.41 | | 0.42 | | 0.43 | | 0.44 | | 0.46 | | 0.47 | |
| 34° | 980.50 | 1.29 | 979.21 | 1.28 | 977.93 | 1.27 | 976.66 | 1.27 | 975.39 | 1.26 | 974.13 | 1.25 | 972.88 | 1.26 | 971.62 | 1.25 | 970.37 | 1.26 | 969.11 | 1.27 | 967.84 | 1.27 | 966.57 | 1.29 |
| | 0.36 | | 0.37 | | 0.38 | | 0.39 | | 0.39 | | 0.40 | | 0.42 | - | 0.42 | | 0.44 | | 0.46 | | 0.46 | | 0.48 | |
| 35° | 980.14 | 1.30 | 978.94 | 1.29 | 977.55 | 1.28 | 976.27 | 1.27 | 975.00 | 1.27 | 973.73 | 1.27 | 972.46 | 1.26 | 971.20 | 1.27 | 969.93 | 1.28 | 968.65 | 127 | 967.38 | 1.29 | 966.09 | 1.30 |
| | 0.37 | | 0.38 | | 0.38 | | 0.39 | | 0.40 | | 0.41 | | 0.42 | | 0.44 | | 0.45 | | 0.45 | | 0.47 | | 0.48 | |
| 36° | 979.77 | 1.31 | 978.46 | 1.29 | 977.17 | 1.29 | 975.88 | 1.28 | 974.60 | 1.28 | 973.32 | 1.28 | 972.04 | 1.28 | 970.76 | 1.28 | 969.48 | 1.28 | 968.20 | 1.29 | 966.91 | 1.30 | 965.61 | 1.32 |
| | 0.39 | | 0.39 | | 0.40 | | 0.40 | | 0.41 | | 0.42 | | 0.43 | | 0.44 | | 0.45 | | 0.47 | | 0.48 | | 0.49 | |
| 37° | 978.38 | 1.31 | 978.07 | 1.30 | 976.77 | 1.29 | 975.48 | 1.29 | 974.19 | 1.29 | 972.90 | 1.29 | 971.61 | 1.29 | 970.32 | 1.29 | 969.03 | 1.30 | 967.73 | 1.30 | 966.43 | 1.31 | 965.12 | 1.33 |
| | 0.38 | | 0.39 | | 0.40 | | 0.41 | | 0.42 | | 0.43 | | 0.44 | | 0.45 | | 0.46 | | 0.47 | | 0.49 | | 0.50 | |
| 38° | 979.00 | 1.32 | 977.68 | 1.31 | 976.37 | 1.30 | 975.07 | 1.30 | 973.77 | 1.30 | 972.47 | 1.30 | 971.17 | 1.30 | 969.87 | 1.30 | 968.57 | 1.31 | 967.26 | 1.32 | 965.94 | 1.32 | 964.62 | 1.34 |
| | 0.40 | | 0.40 | | 0.41 | | 0.42 | | 0.42 | | 0.43 | | 0.44 | | 0.45 | | 0.47 | | 0.48 | | 0.49 | | 0.50 | |
| 39° | 978.60 | 1.32 | 977.28 | 1.32 | 975.96 | 1.31 | 974.65 | 1.30 | 973.35 | 1.31 | 972.04 | 1.31 | 970.73 | 1.31 | 969.42 | 1.32 | 968.10 | 1.32 | 966.78 | 1.33 | 965.45 | 1.33 | 964.12 | 1.36 |
| | 0.40 | | 0.41 | | 0.41 | | 0.42 | | 0.43 | | 0.44 | | 0.45 | | 0.46 | | 0.47 | | 0.48 | | 0.49 | | 0.51 | |
| 40° | 978.20 | 1.33 | 976.87 | 1.32 | 975.55 | 1.32 | 974.23 | 1.31 | 972.92 | 1.32 | 971.60 | 1.52 | 970.28 | 1.32 | 968.96 | 1.33 | 967.63 | 1.33 | 966.30 | 1.34 | 964.96 | 1.35 | 963.61 | 1.37 |

TABLE I (continued) International alcoholic strength at 20°C

**Table of apparent densities of ethanol-water mixtures - Pyrex pycnometer** Densities at *t*°C. corrected for air buoyancy

| | Alcohol % by volume | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $t^o$ | 20 | | 21 | | 22 | | 23 | | 24 | | 25 | | 26 | | 27 | | 28 | | 29 | | 30 | | 31 | |
| 0 | 978.56 | 0.70 | 977.8 | 0.70 | 977.16 | 0.69 | 976.47 | 0.71 | 975.76 | 0.71 | 975.05 | 0.72 | 974.33 | 0.75 | 973.58 | 0.77 | 972.81 | 0.80 | 972.01 | 0.83 | 971.18 | 0.87 | 970.31 | 0.90 |
| | 0.14 | | 0.17 | | 0.19 | | 0.22 | | 0.24 | | 0.26 | | 0.29 | | 0.31 | | 0.34 | | 0.36 | | 0.39 | | 0.41 | |
| 1 | 978.42 | 0.73 | 977.6 | 0.72 | 976.97 | 0.72 | 976.25 | 0.73 | 975.52 | 0.73 | 974.79 | 0.75 | 974.04 | 0.77 | 973.27 | 0.80 | 972.47 | 0182 | 971.65 | 0.86 | 970.79 | 0.89 | 969.90 | 0.92 |
| | 0.16 | | 0.18 | | 0.20 | | 0.23 | | 0.25 | | 0.28 | | 0.30 | | 0.32 | | 0.34 | | 0.37 | | 0.39 | | 0.41 | |
| 2 | 978.26 | 0.75 | 977.5 | 0.74 | 976.77 | 0.75 | 976.02 | 0.75 | 975.27 | 0.76 | 974.51 | 0.77 | 973.74 | 0.79 | 972.95 | 0.82 | 972.13 | 0.85 | 971.28 | 0.88 | 970.40 | 0.91 | 969.49 | 0.95 |
| | 0.17 | | 0.19 | | 0.22 | | 0.23 | | 0.26 | | 0.28 | | 0.31 | | 0.33 | | 0.36 | | 0.38 | | 0.40 | | 0.42 | |
| 3 | 978.09 | 0.77 | 977.3 | 0.77 | 976.55 | 0.76 | 975.79 | 0.78 | 975.01 | 0.78 | 974.23 | 0.80 | 973.43 | 0.81 | 972.62 | 0.85 | 971.77 | 0.87 | 970.90 | 0.90 | 970.00 | 0.93 | 969.07 | 0.98 |
| | 0.18 | | 0.20 | | 0.22 | | 0.25 | | 0.27 | | 0.29 | | 0.31 | | 0.34 | | 0.36 | | 0.38 | | 0.40 | | 0.43 | |
| 4 | 977.91 | 0.79 | 977.1 | 0.79 | 976.33 | 0.79 | 975.54 | 0.80 | 974.94 | 0.80 | 973.94 | 0.82 | 973.12 | 0.84 | 972.28 | 0.87 | 971.41 | 0.89 | 970.52 | 0.92 | 969.60 | 0.96 | 968.64 | 1.00 |
| | 0.19 | | 0.22 | | 0.23 | | 0.26 | | 0.27 | | 0.30 | | 0.33 | | 0.35 | | 0.37 | | 0.39 | | 0.42 | | 0.44 | |
| 5 | 977.72 | 0.82 | 976.9 | 0.80 | 976.10 | 0.82 | 975.28 | 0.81 | 974.47 | 0.83 | 973.64 | 0.85 | 972.79 | 0.86 | 971.93 | 0.89 | 971.04 | 0.91 | 970.13 | 0.95 | 969.18 | 0.98 | 968.20 | 1.01 |
| | 0.21 | | 0.22 | | 0.25 | | 0.26 | | 0.29 | | 0.31 | | 0.33 | | 0.35 | | 0.37 | | 0.40 | | 0.42 | | 0.44 | |
| 6 | 977.51 | 0.83 | 976.6 | 0.83 | 975.85 | 0.83 | 975.02 | 0.84 | 974.18 | 0.85 | 973.33 | 0.87 | 972.46 | 0.86 | 971.58 | 0.91 | 970.67 | 0.94 | 969.73 | 0.97 | 968.76 | 1.00 | 967.76 | 1.03 |
| | 0.21 | | 0.23 | | 0.25 | | 0.28 | | 0.30 | | 0.32 | | 0.34 | | 0.36 | | 0.36 | | 0.40 | | 0.42 | | 0.44 | |
| 7 | 977.30 | 0.85 | 976.4 | 0.85 | 975.60 | 0.86 | 974.74 | 0.86 | 973.88 | 0.87 | 973.01 | 0.89 | 972.12 | 0.90 | 971.22 | 0.93 | 970.20 | 0.96 | 969.33 | 0.99 | 968.34 | 1.02 | 967.32 | 1.06 |
| | 0.23 | | 0.25 | | 0.27 | | 0.28 | | 0.31 | | 0.33 | | 0.35 | | 0.37 | | 0.40 | | 0.42 | | 0.43 | | 0.46 | |
| 8 | 977.07 | 0.87 | 976.2 | 0.87 | 975.33 | 0.87 | 974.46 | 0.89 | 973.57 | 0.89 | 972.68 | 0.91 | 971.77 | 0.92 | 970.85 | 0.96 | 969.89 | 0.98 | 968.91 | 1.00 | 967.91 | 1.05 | 966.86 | 1.07 |
| | 0.24 | | 0.26 | | 0.28 | | 0.30 | | 0.31 | | 0.34 | | 0.35 | | 0.38 | | 0.39 | | 0.41 | | 0.44 | | 0.46 | |
| 9 | 976.83 | 0.89 | 97.59 | 0.89 | 97.505 | 0.89 | 974.16 | 0.90 | 973.26 | 0.92 | 972.34 | 0.92 | 971.42 | 0.95 | 970.47 | 0.97 | 969.50 | 1.00 | 968.50 | 1.03 | 967.47 | 1.07 | 966.40 | 1.09 |
| | 0.24 | | 0.26 | | 0.28 | | 0.30 | | 0.33 | | 0.34 | | 0.37 | | 0.39 | | 0.41 | | 0.43 | | 0.45 | | 0.46 | |
| 10 | 976.59 | 0.91 | 975.6 | 0.91 | 974.77 | 0.91 | 973.86 | 0.93 | 972.93 | 0.93 | 972.00 | 0.95 | 971.05 | 0.97 | 970.08 | 0.99 | 969.09 | 1.02 | 968.07 | 1.05 | 967.02 | 1.08 | 965.94 | 1.12 |
| | 0.27 | | 0.29 | | 0.30 | | 0.33 | | 0.34 | | 0.36 | | 0.38 | | 0.40 | | 0.42 | | 0.44 | | 0.46 | | 0.47 | |
| 11 | 976.32 | 0.93 | 975.3 | 0.92 | 974.47 | 0.94 | 973.53 | 0.94 | 972.59 | 0.95 | 971.64 | 0.97 | 970.67 | 0.99 | 969.68 | 1.01 | 968.67 | 1.04 | 967.63 | 1.07 | 966.56 | 1.09 | 965.47 | 1.13 |
| | 0.27 | | 0.28 | | 0.31 | | 0.32 | | 0.34 | | 0.36 | | 0.38 | | 0.40 | | 0.42 | | 0.44 | | 0.45 | | 0.48 | |
| 12 | 976.05 | 0.94 | 975.1 | 0.95 | 974.16 | 0.95 | 973.21 | 0.96 | 972.25 | 0.97 | 971.28 | 0.99 | 970.29 | 1.01 | 969.28 | 1.03 | 968.25 | 1.06 | 967.19 | 1.08 | 966.11 | 1.12 | 964.99 | 1.15 |
| | 0.28 | | 030 | | 0.31 | | 0.33 | | 0.35 | | 0.37 | | 0.39 | | 0.41 | | 0.43 | | 0.45 | | 0.47 | | 0.49 | |
| 13 | 975.77 | 0.96 | 974.8 | 0.96 | 973.85 | 0.97 | 972.88 | 0.98 | 971.90 | 0.99 | 970.91 | 1.01 | 969.90 | 1.03 | 968.87 | 1.05 | 967.82 | 1.08 | 966.74 | 1.10 | 965.64 | 1.14 | 964.50 | 1.17 |
| | 0.28 | | 0.30 | | 0.32 | | 0.34 | | 0.36 | | 0.38 | | 0.40 | | 0.41 | | 0.43 | | 0.45 | | 0.47 | | 0.49 | |
| 14 | 975.49 | 0.98 | 974.5 | 0.98 | 973.53 | 0.99 | 972.54 | 1.00 | 971.54 | 1.01 | 970.53 | 1.03 | 969.50 | 1.04 | 968.46 | 1.07 | 967.39 | 1.10 | 966.29 | 1.12 | 965.17 | 1.16 | 964.01 | 1.19 |
| | 0.30 | | 0.32 | | 0.34 | | 0.35 | | 0.37 | | 0.39 | | 0.40 | | 0.42 | | 0.44 | | 0.46 | | 0.48 | | 0.49 | |
| 15 | 975.19 | 1.00 | 974.1 | 1.00 | 973.19 | 1.00 | 972.19 | 1.02 | 971.17 | 1.03 | 970.14 | 1.04 | 969.10 | 1.06 | 968.04 | 1.09 | 966.95 | 1.12 | 965.83 | 1.14 | 964.69 | 1.17 | 963.52 | 1.21 |
| | 0.31 | | 0.32 | | 0.34 | | 0.36 | | 0.37 | | 0.39 | | 0.41 | | 0.43 | | 0.45 | | 0.46 | | 0.48 | | 0.51 | |
| 16 | 974.88 | 1.01 | 973.8 | 1.02 | 972.85 | 1.02 | 971.83 | 1.03 | 970.80 | 1.05 | 969.75 | 1.06 | 968.69 | 1.08 | 967.61 | 1.11 | 966.50 | 1.13 | 965.37 | 1.16 | 964.21 | 1.20 | 963.01 | 1.22 |
| | 0.32 | | 0.33 | | 0.35 | | 0.37 | | 0.39 | | 0.40 | | 0.42 | | 0.44 | | 0.45 | | 0.48 | | 0.50 | | 0.50 | |
| 17 | 974.56 | 1.02 | 973.5 | 1.04 | 972.50 | 1.04 | 971.46 | 1.05 | 970.41 | 1.06 | 969.35 | 1.08 | 968.27 | 1.10 | 967.17 | 1.12 | 966.05 | 1.16 | 964.89 | 1.18 | 963.71 | 1.20 | 962.51 | 1.24 |
| | 0.32 | | 0.35 | | 0.36 | | 0.37 | | 0.39 | | 0.41 | | 0.43 | | 0.45 | | 0.47 | | 0.48 | | 0.49 | | 0.52 | |
| 18 | 974.24 | 1.05 | 973.1 | 1.05 | 972.14 | 1.05 | 971.09 | 1.07 | 970.02 | 1.08 | 968.94 | 1.10 | 967.84 | 1.12 | 966.72 | 1.14 | 965.58 | 1.17 | 964.41 | 1.19 | 963.22 | 1.23 | 961.99 | 1.25 |
| | 0.34 | | 0.35 | | 0.36 | | 0.39 | | 0.40 | | 0.42 | | 0.43 | | 0.45 | | 0.47 | | 0.48 | | 0.50 | | 0.52 | |
| 19 | 973.90 | 1.06 | 972.8 | 1.06 | 971.78 | 1.08 | 970.70 | 1.08 | 969.62 | 1.10 | 968.52 | 1.11 | 967.41 | 1.14 | 966.27 | 1.16 | 965.11 | 1.18 | 963.93 | 1.21 | 962.72 | 1.25 | 961.47 | 1.27 |
| | 0.34 | | 0.36 | | 0.38 | | 0.39 | | 0.41 | | 0.42 | | 0.45 | | 0.46 | | 0.47 | | 0.49 | | 0.51 | | 0.52 | |
| 20 | 973.56 | 1.08 | 972.4 | 1.08 | 971.40 | 1.09 | 970.31 | 1.10 | 969.21 | 1.11 | 968.10 | 1.14 | 966.96 | 1.15 | 965.81 | 1.17 | 964.64 | 1.20 | 963.44 | 1.23 | 962.21 | 1.26 | 960.95 | 1.29 |

TABLE I (continued) International alcoholic strength at 20°C

**Table of apparent densities of ethanol-water mixtures - Pyrex pycnometer** Densities at *t*°C. corrected for air buoyancy

| | Alcohol % by volume | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *t* ° | 20 | | 21 | | 22 | | 23 | | 24 | | 25 | | 26 | | 27 | | 28 | | 29 | | 30 | | 31 | |
| 20 | 973.56 | 1.08 | 972.48 | 1.08 | 971.40 | 1.09 | 970.31 | 1.10 | 969.21 | 1.11 | 968.10 | 1.14 | 966.96 | 1.15 | 965.81 | 1.17 | 964.64 | 1.20 | 963.44 | 1.23 | 962.21 | 1.26 | 960.95 | 1.29 |
| | 0.36 | | 0.37 | | 0.38 | | 0.40 | | 0.42 | | 0.44 | | 0.45 | | 0.46 | | 0.49 | | 0.50 | | 0.52 | | 0.53 | |
| 21 | 973.20 | 1.09 | 972.11 | 1.09 | 971.02 | 1.11 | 969.91 | 1.12 | 968.79 | 1.13 | 967.66 | 1.15 | 966.51 | 1.16 | 965.35 | 1.20 | 964.15 | 1.21 | 962.94 | 1.25 | 961.69 | 1.27 | 960.42 | 1.31 |
| | 0.36 | | 0.37 | | 0.40 | | 0.41 | | 0.42 | | 0.44 | | 0.45 | | 0.48 | | 0.49 | | 0.51 | | 0.52 | | 0.54 | |
| 22 | 972.84 | 1.10 | 971.74 | 1.12 | 970.62 | 1.12 | 969.50 | 1.13 | 968.37 | 1.15 | 967.22 | 1.16 | 966.06 | 1.19 | 964.87 | 1.21 | 963.66 | 1.23 | 962.43 | 1.26 | 961.17 | 1.29 | 959.88 | 1.32 |
| | 0.37 | | 0.39 | | 0.40 | | 0.42 | | 0.43 | | 0.45 | | 0.47 | | 0.48 | | 0.49 | | 0.51 | | 0.53 | | 0.55 | |
| 23 | 972.47 | 1.12 | 971.35 | 1.13 | 970.22 | 1.14 | 969.08 | 1.14 | 967.94 | 1.17 | 966.77 | 1.18 | 965.59 | 1.20 | 964.39 | 1.22 | 963.17 | 1.25 | 961.92 | 1.28 | 960.64 | 1.31 | 959.33 | 1.33 |
| | 0.38 | | 0.40 | | 0.41 | | 0.42 | | 0.44 | | 0.45 | | 0.47 | | 0.49 | | 0.51 | | 0.52 | | 0.54 | | 0.55 | |
| 24 | 972.09 | 1.14 | 970.95 | 1.14 | 969.81 | 1.15 | 968.66 | 1.16 | 967.50 | 1.18 | 966.32 | 1.20 | 965.12 | 1.22 | 963.90 | 1.24 | 962.66 | 1.26 | 961.40 | 1.30 | 960.10 | 1.32 | 958.78 | 1.35 |
| | 0.39 | | 0.40 | | 0.42 | | 0.43 | | 0.45 | | 0.47 | | 0.48 | | 0.49 | | 0.51 | | 0.53 | | 0.54 | | 0.55 | |
| 25 | 971.70 | 1.15 | 970.55 | 1.16 | 969.39 | 1.16 | 968.23 | 1.18 | 967.05 | 1.20 | 965.85 | 1.21 | 964.64 | 1.23 | 963.41 | 1.26 | 962.15 | 1.28 | 960.87 | 1.31 | 959.56 | 1.33 | 958.23 | 1.37 |
| | 0.40 | | 0.41 | | 0.42 | | 0.44 | | 0.46 | | 0.47 | | 0.49 | | 0.50 | | 0.51 | | 0.53 | | 0.54 | | 0.57 | |
| 26 | 971.30 | 1.16 | 970.14 | 1.17 | 968.97 | 1.18 | 967.79 | 1.20 | 966.59 | 1.21 | 965.38 | 1.23 | 964.15 | 1.24 | 962.91 | 1.27 | 961.64 | 1.30 | 960.34 | 1.32 | 959.02 | 1.36 | 957.66 | 1.38 |
| | 0.40 | | 0.42 | | 0.43 | | 0.45 | | 0.46 | | 0.48 | | 0.49 | | 0.51 | | 0.53 | | 0.54 | | 0.56 | | 0.56 | |
| 27 | 970.90 | 1.18 | 969.72 | 1.18 | 968.54 | 1.20 | 967.34 | 1.21 | 966.13 | 1.23 | 964.90 | 1.24 | 963.66 | 1.26 | 962.40 | 1.29 | 961.11 | 1.31 | 959.80 | 1.34 | 958.46 | 1.36 | 957.10 | 1.40 |
| | 0.41 | | 0.43 | | 0.45 | | 0.46 | | 0.47 | | 0.48 | | 0.50 | | 0.52 | | 0.54 | | 0.56 | | 0.57 | | 0.59 | |
| 28 | 970.49 | 1.20 | 969.29 | 1.20 | 968.09 | 1.21 | 966.88 | 1.22 | 965.66 | 1.24 | 964.42 | 1.26 | 963.16 | 1.28 | 961.88 | 1.31 | 960.57 | 1.33 | 959.24 | 1.35 | 957.89 | 1.38 | 956.51 | 1.41 |
| | 0.42 | | 0.43 | | 0.45 | | 0.47 | | 0.49 | | 0.50 | | 0.52 | | 0.53 | | 0.53 | | 0.55 | | 0.56 | | 0.58 | |
| 29 | 970.07 | 1.21 | 968.86 | 1.22 | 967.64 | 1.23 | 966.41 | 1.24 | 965.17 | 1.25 | 963.92 | 1.28 | 962.64 | 1.29 | 961.35 | 1.31 | 960.04 | 1.35 | 958.69 | 1.36 | 957.33 | 1.40 | 955.93 | 1.42 |
| | 0.44 | | 0.45 | | 0.46 | | 0.47 | | 0.49 | | 0.50 | | 0.51 | | 0.53 | | 0.55 | | 0.55 | | 0.58 | | 0.58 | |
| 30 | 969.63 | 1.22 | 968.41 | 1.23 | 967.18 | 1.24 | 965.94 | 1.26 | 964.68 | 1.26 | 963.42 | 1.29 | 962.13 | 1.31 | 960.82 | 1.33 | 959.49 | 1.35 | 958.14 | 1.39 | 956.75 | 1.40 | 955.35 | 1.44 |
| | 0.44 | | 0.45 | | 0.46 | | 0.48 | | 0.49 | | 0.51 | | 0.52 | | 0.53 | | 0.55 | | 0.57 | | 0.58 | | 0.60 | |
| 31 | 969.19 | 1.23 | 967.96 | 1.24 | 966.72 | 1.26 | 965.46 | 1.27 | 964.19 | 1.28 | 962.91 | 1.30 | 961.61 | 1.32 | 960.29 | 1.35 | 958.94 | 1.37 | 957.57 | 1.40 | 956.17 | 1.42 | 954.75 | 1.44 |
| | 0.44 | | 0.46 | | 0.47 | | 0.48 | | 0.50 | | 0.51 | | 0.53 | | 0.54 | | 0.55 | | 0.57 | | 0.58 | | 0.59 | |
| 32 | 968.75 | 1.25 | 967.50 | 1.25 | 966.25 | 1.27 | 964.98 | 1.29 | 963.69 | 1.29 | 962.40 | 1.32 | 961.08 | 1.33 | 959.75 | 1.36 | 958.39 | 1.39 | 957.00 | 1.41 | 955.59 | 1.43 | 954.16 | 1.46 |
| | 0.45 | | 0.46 | | 0.48 | | 0.49 | | 0.50 | | 0.52 | | 0.53 | | 0.55 | | 0.57 | | 0.57 | | 0.59 | | 0.61 | |
| 33 | 968.30 | 1.26 | 967.04 | 1.27 | 965.77 | 1.28 | 964.49 | 1.30 | 963.19 | 1.31 | 961.88 | 1.33 | 960.55 | 1.35 | 959.20 | 1.38 | 957.82 | 1.39 | 956.43 | 1.43 | 955.00 | 1.45 | 953.55 | 1.47 |
| | 0.46 | | 0.47 | | 0.49 | | 0.50 | | 0.51 | | 0.53 | | 0.54 | | 0.56 | | 0.56 | | 0.59 | | 0.59 | | 0.60 | |
| 34 | 967.84 | 1.27 | 966.57 | 1.29 | 965.28 | 1.29 | 963.99 | 1.31 | 962.68 | 1.33 | 961.35 | 1.34 | 960.01 | 1.37 | 958.64 | 1.38 | 957.26 | 1.42 | 95584 | 1.43 | 954.41 | 1.46 | 952.95 | 1.49 |
| | 0.46 | | 0.48 | | 0.49 | | 0.51 | | 0.52 | | 0.53 | | 0.55 | | 0.56 | | 0.58 | | 0.58 | | 0.60 | | 0.62 | |
| 35 | 967.38 | 1.29 | 966.09 | 1.30 | 964.79 | 1.31 | 963.48 | 1.32 | 962.16 | 1.34 | 960.82 | 1.36 | 959.46 | 1.38 | 958.08 | 1.40 | 956.68 | 1.42 | 955.26 | 1.45 | 953.81 | 1.48 | 952.33 | 1 50 |
| | 0.47 | | 0.48 | | 0.50 | | 0.51 | | 0.53 | | 0.54 | | 0.55 | | 0.57 | | 0.58 | | 0.60 | | 0.61 | | 0.62 | |
| 36 | 966.91 | 1.30 | 965.61 | 1.32 | 964.29 | 1.32 | 962.97 | 1.34 | 961.63 | 1.35 | 960.28 | 1.37 | 958.91 | 1.40 | 957.51 | 1.41 | 956.10 | 1.44 | 954.66 | 1.46 | 953.20 | 1.49 | 951.71 | 1.51 |
| | 0.48 | | 0.49 | | 0.50 | | 0.52 | | 0.53 | | 0.55 | | 0.56 | | 0.57 | | 0.59 | | 0.60 | | 0.61 | | 0.62 | |
| 37 | 966.43 | 1.31 | 965.12 | 1.33 | 963.79 | 1.34 | 962.45 | 1.35 | 961.10 | 1.37 | 959.73 | 1.38 | 958.35 | 1.41 | 956.94 | 1.43 | 955.51 | 1.45 | 954.06 | 1.47 | 952.59 | 1.50 | 951.09 | 1.53 |
| | 0.49 | | 0.50 | | 0.51 | | 0.52 | | 0.54 | | 0.55 | | 0.57 | | 0.58 | | 0.59 | | 0.60 | | 0.62 | | 0.63 | |
| 38 | 965.94 | 1.32 | 964.62 | 1.34 | 963.28 | 1.35 | 961.93 | 1.37 | 960.56 | 1.38 | 959.18 | 1.40 | 957.78 | 1.42 | 956.36 | 1.44 | 954.92 | 1.46 | 953.46 | 1.49 | 951.97 | 1.51 | 950.4 | 1.54 |
| | 0.49 | | 0.50 | | 0.52 | | 0.53 | | 0.54 | | 0.56 | | 0.57 | | 0.58 | | 0.60 | | 0.61 | | 0.62 | | 0.64 | |
| 39 | 965.45 | 1.33 | 964.12 | 1.36 | 962.76 | 1.36 | 961.40 | 1.38 | 960.02 | 1.40 | 958.62 | 1.41 | 957.21 | 1.43 | 955.78 | 1.46 | 954.32 | 1.47 | 952.85 | 1.50 | 951.35 | 1.53 | 949.82 | 1.55 |
| | 0.49 | | 0.51 | | 0.52 | | 0.54 | | 0.55 | | 0.56 | | 0.58 | | 0.59 | | 0.60 | | 0.62 | | 0.63 | | 0.64 | |
| 40 | 964.96 | 1.35 | 963.61 | 1.37 | 962.24 | 1.38 | 960.86 | 1.39 | 959.47 | 1.41 | 958.06 | 1.43 | 956.63 | 1.44 | 955.19 | 1.47 | 953.72 | 1.49 | 952.23 | 1.51 | 950.72 | 1.54 | 949.18 | 1.57 |

TABLE II International alcoholic strength at 20°C

Table of Corrections to be applied to the apparent alcoholic strength to correct for the effect of temperature

Add or subtract from the apparent alcoholic strength at *t*°C (ordinary glass alcohol meter) the correction indicated below

| | | | Apparent alcoholic strength at *t*°C | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Temperatures | 0 | To add | 0.76 | 0.77 | 0.82 | 0.87 | 0.95 | 1.04 | 1.16 | 1.31 | 1.49 | 1.70 | 1.95 | 2.26 | 2.62 | 3.03 | 3.49 | 4.02 | 4.56 |
| | 1° | | 0.81 | 0.83 | 0.87 | 0.92 | 1.00 | 1.09 | 1.20 | 1.35 | 1.52 | 1.73 | 1.97 | 2.26 | 2.59 | 2.97 | 3.40 | 3.87 | 4.36 |
| | 2° | | 0.85 | 0.87 | 0.92 | 0.97 | 1.04 | 1.13 | 1.24 | 1.38 | 1.54 | 1.74 | 1.97 | 2.24 | 2.54 | 2.89 | 3.29 | 3.72 | 4.17 |
| | 3° | | 0.88 | 0.91 | 0.95 | 1.00 | 1.07 | 1.15 | 1.26 | 1.39 | 1.55 | 1.73 | 1.95 | 2.20 | 2.48 | 2.80 | 3.16 | 3.55 | 3.95 |
| | 4° | | 0.90 | 0.92 | 0.97 | 1.02 | 1.09 | 1.17 | 1.27 | 1.40 | 1.55 | 1.72 | 1.92 | 2.15 | 2.41 | 2.71 | 3.03 | 3.38 | 3.75 |
| | 5° | | 0.91 | 0.93 | 0.98 | 1.03 | 1.10 | 1.17 | 1.27 | 1.39 | 1.53 | 1.69 | 1.87 | 2.08 | 2.33 | 2.60 | 2.89 | 3.21 | 3.54 |
| | 6° | | 0.92 | 0.94 | 0.98 | 1.02 | 1.09 | 1.16 | 1.25 | 1.37 | 1.50 | 1.65 | 1.82 | 2.01 | 2.23 | 2.47 | 2.74 | 3.02 | 3.32 |
| | 7° | | 0.91 | 0.93 | 0.97 | 1.01 | 1.07 | 1.14 | 1.23 | 1.33 | 1.45 | 1.59 | 1.75 | 1.92 | 2.12 | 2.34 | 2.58 | 2.83 | 3.10 |
| | 8° | | 0.89 | 0.91 | 0.94 | 0.98 | 1.04 | 1.11 | 1.19 | 1.28 | 1.39 | 1.52 | 1.66 | 1.82 | 2.00 | 2.20 | 2.42 | 2.65 | 2.88 |
| | 9° | | 0.86 | 0.88 | 0.91 | 0.95 | 1.01 | 1.07 | 1.14 | 1.23 | 1.33 | 1.44 | 1.57 | 1.71 | 1.97 | 2.05 | 2.24 | 2.44 | 2.65 |
| | 10° | | 0.82 | 0.84 | 0.87 | 0.91 | 0.96 | 1.01 | 1.08 | 1.16 | 1.25 | 1.35 | 1.47 | 1.60 | 1.74 | 1.89 | 2.06 | 2.24 | 2.43 |
| | 11° | | 0.78 | 0.79 | 0.82 | 0.86 | 0.90 | 0.95 | 1.01 | 1.08 | 1.16 | 1.25 | 1 36 | 1.47 | 1.60 | 173 | 1.88 | 2.03 | 2.20 |
| | 12° | | 0.72 | 0.74 | 0.76 | 0.79 | 0.83 | 0.88 | 0.93 | 0.99 | 1.07 | 1.15 | 1.24 | 1.34 | 1.44 | 1.56 | 1.69 | 1.82 | 1.96 |
| | 13° | | 0.66 | 0.67 | 0.69 | 0.72 | 0.76 | 0.80 | 0.84 | 0.90 | 0.96 | 1.03 | 1.11 | 1.19 | 1.28 | 1.38 | 1.49 | 1.61 | 1.73 |
| | 14° | | 0.59 | 0.60 | 0.62 | 0.64 | 0.67 | 0.71 | 0.74 | 0.79 | 0.85 | 0.91 | 0.97 | 1.04 | 1.12 | 1.20 | 1.29 | 1.39 | 1.49 |
| | 15° | | 0.51 | 0.52 | 0.53 | 0.55 | 0.58 | 0.61 | 0.64 | 0.68 | 0.73 | 0.77 | 0.83 | 0.89 | 0995 | 1.02 | 1.09 | 1.16 | 1.24 |
| | 16° | | 0.42 | 0.43 | 0.44 | 0.46 | 0.48 | 0.50 | 0.53 | 0956 | 0.60 | 0963 | 0.67 | 0.72 | 0.77 | 0.82 | 0.88 | 0.94 | 1.00 |
| | 17° | | 0.33 | 0.33 | 0.34 | 0.35 | 0.37 | 0.39 | 0.41 | 0.43 | 0.46 | 0.48 | 0.51 | 0.55 | 0.59 | 0.62 | 0.67 | 0.71 | 0.75 |
| | 18° | | 0.23 | 0.23 | 0.23 | 0.24 | 0.25 | 0.26 | 0.27 | 0.29 | 0.31 | 0.33 | 0.35 | 0.37 | 0.40 | 0.42 | 0.45 | 0.48 | 0.51 |
| | 19° | | 0.12 | 0.12 | 0.12 | 0.12 | 0.13 | 0.13 | 0.14 | 0.15 | 0.16 | 0.17 | 0.18 | 0.19 | 0.20 | 0.21 | 0.23 | 0.24 | 0.25 |

TABLE II (continued)

International alcoholic strength at 20°C

Table of Corrections to be applied to the apparent alcoholic strength to correct for the effect of temperature

Add or subtract from the apparent alcoholic strength at *t*°C (ordinary glass alcohol meter) the correction indicated below

| Temperatures | | To subtract | Apparent alcoholic strength at *t*°C | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| | 21° | | | 0.13 | 0.13 | 0.13 | 0.14 | 0.14 | 0.15 | 0.16 | 0.17 | 0.18 | 0.19 | 0.19 | 0.20 | 0.22 | 0.23 | 0.25 | 0.26 |
| | 22° | | | 0.26 | 0.27 | 0.28 | 0.29 | 0.30 | 0.31 | 0.32 | 0.34 | 0.36 | 0.37 | 0.39 | 0.41 | 0.44 | 0.47 | 0.49 | 0.52 |
| | 23° | | | 0.40 | 0.41 | 0.42 | 0.44 | 0.45 | 0.47 | 0.49 | 0.51 | 0.54 | 0.57 | 0.60 | 0.63 | 0.66 | 0.70 | 0.74 | 0.78 |
| | 24° | | | 0.55 | 0.56 | 0.58 | 0.60 | 0.62 | 0.64 | 0.67 | 0.70 | 0.73 | 0.77 | 0.81 | 0.85 | 0.89 | 0.94 | 0.99 | 1.04 |
| | 25° | | | 0.69 | 0.71 | 0.73 | 0.76 | 0.79 | 0.82 | 0.85 | 0.89 | 0.93 | 0.97 | 1.02 | 1.07 | 1.13 | 1.19 | 1.25 | 1.31 |
| | 26° | | | 0.85 | 0.87 | 0.90 | 0.93 | 0.96 | 1.00 | 1.04 | 1.08 | 1.13 | 1.18 | 1.24 | 1.30 | 1.36 | 1.43 | 1.50 | 1.57 |
| | 27° | | | | 1.03 | 1.07 | 1.11 | 1.15 | 1.19 | 1.23 | 1.28 | 1.34 | 1.40 | 1.46 | 1.53 | 1.60 | 1.68 | 1.76 | 1.84 |
| | 28° | | | | 1.21 | 1.25 | 1.29 | 1.33 | 1.38 | 1.43 | 1.49 | 1.55 | 1.62 | 1.69 | 1.77 | 1.85 | 1.93 | 2.02 | 2.11 |
| | 29° | | | | 1.39 | 1.43 | 1.47 | 1.52 | 1.58 | 1.63 | 1.70 | 1.76 | 1.84 | 1.92 | 2.01 | 2.10 | 2.19 | 2.29 | 2.39 |
| | 30° | | | | 1.57 | 1.61 | 1.66 | 1.72 | 1.78 | 1.84 | 1.91 | 1.98 | 2.07 | 2.15 | 2.25 | 2.35 | 2.45 | 2.56 | 2.67 |
| | 31° | | | | 1.75 | 1.80 | 1.86 | 1.92 | 1.98 | 2.05 | 2.13 | 2.21 | 2.30 | 2.39 | 2.49 | 2.60 | 2.71 | 2.83 | 2.94 |
| | 32° | | | | 1.94 | 2.00 | 2.06 | 2.13 | 2.20 | 2.27 | 2.35 | 2.44 | 2.53 | 2.63 | 2.74 | 2.86 | 2.97 | 3.09 | 3.22 |
| | 33° | | | | | 2.20 | 2.27 | 2.34 | 2.42 | 2.50 | 2.58 | 2.67 | 2.77 | 2.88 | 2.99 | 3.12 | 3.24 | 3.37 | 3.51 |
| | 34° | | | | | 2.41 | 2.48 | 2.56 | 2.64 | 2.72 | 2.81 | 2.91 | 3.02 | 3.13 | 3.25 | 3.38 | 3.51 | 3.65 | 3.79 |
| | 35° | | | | | | 2.62 | 2.70 | 2.78 | 2.86 | 2.95 | 3.05 | 3.16 | 3.27 | 3.39 | 3.51 | 3.64 | 3.78 | 3.93 | 4.08 |
| | 36° | | | | | | 2.83 | 2.91 | 3.00 | 3.09 | 3.19 | 3.29 | 3.41 | 3.53 | 3.65 | 3.78 | 3.91 | 4.05 | 4.21 | 4.37 |
| | 37° | | | | | | | 3.13 | 3.23 | 3.33 | 3.43 | 3.54 | 3.65 | 3.78 | 3.91 | 4.04 | 4.18 | 4.33 | 4.49 | 4.65 |
| | 38° | | | | | | | 3.36 | 3.47 | 3.57 | 3.68 | 3.79 | 3.91 | 4.03 | 4.17 | 4.31 | 4.46 | 4.61 | 4.77 | 4.94 |
| | 39° | | | | | | | 3.59 | 3.70 | 3.81 | 3.93 | 4.05 | 4.17 | 4.44 | 4.58 | 4.74 | 4.90 | 5.06 | 5.06 | 5.23 |
| | 40° | | | | | | | 3.82 | 3.94 | 4.06 | 4.18 | 4.31 | 4.44 | 4.57 | 4.71 | 4.86 | 5.02 | 5.19 | 5.36 | 5.53 |

TABLE II (continued)
International alcoholic strength at 20°C

Table of Corrections to be applied to the apparent alcoholic strength to correct for the effect of temperature
Add or subtract from the apparent alcoholic strength at *t*°C (ordinary glass alcohol meter) the correction indicated below

| | | | Apparent alcoholic strength at *t*°C | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| Temperatures | 0° | To add | 3.49 | 4.02 | 4.56 | 5.11 | 5.65 | 6.16 | 6.63 | 7.05 | 7.39 | 7.67 | 7.91 | 8.07 | 8.20 | 8.30 | 8.36 | 8.39 | 8.40 |
| | 1° | | 3.40 | 3.87 | 4.36 | 4.86 | 5.35 | 5.82 | 6.26 | 6.64 | 6.96 | 7.23 | 7.45 | 7.62 | 7.75 | 7.85 | 7.91 | 7.95 | 7.96 |
| | 2° | | 3.29 | 3.72 | 4.17 | 4.61 | 5.05 | 5.49 | 5.89 | 6.25 | 6.55 | 6.81 | 7.02 | 7.18 | 7.31 | 7.40 | 7.47 | 7.51 | 7.53 |
| | 3° | | 3.16 | 3.55 | 3.95 | 4.36 | 4.77 | 5.17 | 5.53 | 5.85 | 6.14 | 6.39 | 6.59 | 6.74 | 6.86 | 6.97 | 7.03 | 7.07 | 7.09 |
| | 4° | | 3.03 | 3.38 | 3.75 | 4.11 | 4.48 | 4.84 | 5.17 | 5.48 | 5.74 | 5.97 | 6.16 | 6.31 | 6.43 | 6.53 | 6.59 | 6.63 | 6.66 |
| | 5° | | 2.89 | 3.21 | 3.54 | 3.86 | 4.20 | 4.52 | 4.83 | 5.11 | 5.35 | 5.56 | 5.74 | 5.89 | 6.00 | 6.10 | 6.16 | 6.20 | 6.23 |
| | 6° | | 2.74 | 3.02 | 3.32 | 3.61 | 3.91 | 4.21 | 4.49 | 4.74 | 4.96 | 5.16 | 5.33 | 5.47 | 5.58 | 5.67 | 5.73 | 5.77 | 5.80 |
| | 7° | | 2.58 | 2.83 | 3.10 | 3.36 | 3.63 | 3.90 | 4.15 | 4.38 | 4.58 | 4.77 | 4.92 | 5.05 | 5.15 | 5.24 | 5.30 | 5.34 | 5.37 |
| | 8° | | 2.42 | 2.65 | 2.88 | 3.11 | 3.35 | 3.59 | 3.81 | 4.02 | 4.21 | 4.38 | 4.52 | 4.64 | 4.74 | 4.81 | 4.87 | 4.92 | 4.95 |
| | 9° | | 2.24 | 2.44 | 2.65 | 2.86 | 3.07 | 3.28 | 3.48 | 3.67 | 3.84 | 3.99 | 4.12 | 4.23 | 4.32 | 4.39 | 4.45 | 4.50 | 4.53 |
| | 10° | | 2.06 | 2.24 | 2.43 | 2.61 | 2.80 | 2.98 | 3.16 | 3.33 | 3.48 | 3.61 | 3.73 | 3.83 | 3.91 | 3.98 | 4.03 | 4.08 | 4.11 |
| | 11° | | 1.88 | 2.03 | 2.20 | 2.36 | 2.52 | 2.68 | 2.83 | 2.98 | 3.12 | 3.24 | 3.34 | 3.43 | 3.50 | 3.57 | 3.62 | 3.66 | 3.69 |
| | 12° | | 1.69 | 1.82 | 1.96 | 2.10 | 2.24 | 2.38 | 2.51 | 2.64 | 2.76 | 2.87 | 2.96 | 3.04 | 3.10 | 3.16 | 3.21 | 3.25 | 3.27 |
| | 13° | | 1.49 | 1.61 | 1.73 | 1.84 | 1.96 | 2.08 | 2.20 | 2.31 | 2.41 | 2.50 | 2.58 | 2.65 | 2.71 | 2.76 | 2.80 | 2.83 | 2.85 |
| | 14° | | 1.29 | 1.39 | 1.49 | 1.58 | 1.68 | 1.78 | 1.88 | 1.97 | 2.06 | 2.13 | 2.20 | 2.26 | 2.31 | 2.36 | 2.39 | 2.42 | 2.44 |
| | 15° | | 1.09 | 1.16 | 1.24 | 1.32 | 1.40 | 1.48 | 1.56 | 1.64 | 1.71 | 1.77 | 1.83 | 1.88 | 1.92 | 1.96 | 1.98 | 2.01 | 2.03 |
| | 16° | | 0.88 | 0.94 | 1.00 | 1.06 | 1.12 | 1.19 | 1.25 | 1.31 | 1.36 | 1.41 | 1.46 | 1.50 | 1.53 | 1.56 | 1.58 | 1.60 | 1.62 |
| | 17° | | 0.67 | 0.71 | 0.75 | 0.80 | 0.84 | 0.89 | 0.94 | 0.98 | 1.02 | 1.05 | 1.09 | 1.12 | 1.14 | 1.17 | 1.18 | 1.20 | 1.21 |
| | 18° | | 0.45 | 0.48 | 0.51 | 0.53 | 0.56 | 0.59 | 0.62 | 0.65 | 0.68 | 0.70 | 0.72 | 0.74 | 0.76 | 0.78 | 0.79 | 0.80 | 0.81 |
| | 19° | | 0.23 | 0.24 | 0.25 | 0.27 | 0.28 | 0.30 | 0.31 | 0.33 | 0.34 | 0.35 | 0.36 | 0.37 | 0.38 | 0.39 | 0.40 | 0.41 | 0.41 |

TABLE II (continued)
International alcoholic strength at 20ºC

Table of Corrections to be applied to the apparent alcoholic strength to correct for the effect of temperature
Add or subtract from the apparent alcoholic strength at *t*ºC (ordinary glass alcohol meter) the correction indicated below

| Temperatures | To subtract | Apparent alcoholic strength at *t*ºC | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| 21º | | 0.23 | 0.25 | 0.26 | 0.28 | 0.29 | 0.30 | 0.31 | 0.33 | 0.34 | 0.35 | 0.35 | 0.37 | 0.38 | 0.38 | 0.39 | 0.39 | 0.40 |
| 22º | | 0.47 | 0.49 | 0.52 | 0.55 | 0.57 | 0.60 | 0.62 | 0.65 | 0.67 | 0.70 | 0.72 | 0.74 | 0.75 | 0.76 | 0.78 | 0.79 | 0.80 |
| 23º | | 0.70 | 0.74 | 0.78 | 0.82 | 0.86 | 0.90 | 0.93 | 0.97 | 1.01 | 1.04 | 1.07 | 1.10 | 1.12 | 1.15 | 1.17 | 1.18 | 1.19 |
| 24º | | 0.94 | 0.99 | 1.04 | 1.10 | 1.15 | 1.20 | 1.25 | 1.29 | 1.34 | 1.39 | 1.43 | 1.46 | 1.50 | 1.53 | 1.55 | 1.57 | 1.59 |
| 25º | | 1.19 | 1.25 | 1.31 | 1.37 | 1.43 | 1.49 | 1.56 | 1.62 | 1.68 | 1.73 | 1.78 | 1.83 | 1.87 | 1.90 | 1.94 | 1.97 | 1.99 |
| 26º | | 1.43 | 1.50 | 1.57 | 1.65 | 1.73 | 1.80 | 1.87 | 1.94 | 2.01 | 2.07 | 2.13 | 2.19 | 2.24 | 2.28 | 2.32 | 2.35 | 2.38 |
| 27º | | 1.68 | 1.76 | 1.84 | 1.93 | 2.01 | 2.10 | 2.18 | 2.26 | 2.34 | 2.41 | 2.48 | 2.55 | 2.61 | 2.66 | 2.70 | 2.74 | 2.77 |
| 28º | | 1.93 | 2.02 | 2.11 | 2.21 | 2.31 | 2.40 | 2.49 | 2.58 | 2.67 | 2.76 | 2.83 | 2.90 | 2.98 | 3.03 | 3.08 | 3.13 | 3.17 |
| 29o | | 2.19 | 2.29 | 2.39 | 2.50 | 2.60 | 2 70 | 2.81 | 2.91 | 3.00 | 3.09 | 3.18 | 3.26 | 3.34 | 3.40 | 3.46 | 3.51 | 3.55 |
| 30o | | 2.45 | 2.56 | 2.67 | 2.78 | 2.90 | 3.01 | 3.12 | 3.23 | 3.34 | 3.44 | 3.53 | 3.62 | 3.70 | 3.77 | 3.84 | 3.90 | 3.95 |
| 31º | | 2.71 | 2.83 | 2.94 | 3.07 | 3.19 | 3.31 | 3.43 | 3.55 | 3.67 | 3.78 | 3.88 | 3.98 | 4.07 | 4.15 | 4.22 | 4.28 | 4.33 |
| 32o | | 2.97 | 3.09 | 3.22 | 3.36 | 3.49 | 3.62 | 3.74 | 3.87 | 4.00 | 4.11 | 4.22 | 4.33 | 4.43 | 4.51 | 4.59 | 4.66 | 4.72 |
| 33o | | 3.24 | 3.37 | 3.51 | 3.65 | 3.79 | 3.92 | 4.06 | 4.20 | 4.33 | 4.45 | 4.57 | 4.68 | 4.79 | 4.88 | 4.97 | 5.04 | 5.10 |
| 34º | | 3.51 | 3.65 | 3.79 | 3.94 | 4.09 | 4.23 | 4.37 | 4.52 | 4.66 | 4.79 | 4.91 | 5.03 | 5.15 | 5.25 | 5.34 | 5.42 | 5.49 |
| 35º | | 3.78 | 3.93 | 4.08 | 4.23 | 4.38 | 4.53 | 4.69 | 4.84 | 4.98 | 5.12 | 5.26 | 5.38 | 5.50 | 5.61 | 5.71 | 5.80 | 5.87 |
| 36º | | 4.05 | 4.21 | 4.37 | 4.52 | 4.68 | 4.84 | 5.00 | 5.16 | 5.31 | 5.46 | 5.60 | 5.73 | 5.86 | 5.97 | 6.08 | 6.17 | 6.25 |
| 37º | | 4.33 | 4.49 | 4.65 | 4.82 | 4.98 | 5.15 | 5.31 | 5.48 | 5.64 | 5.80 | 5.95 | 6.09 | 6.22 | 6.33 | 6.44 | 6.54 | 6.63 |
| 38º | | 4.61 | 4.77 | 4.94 | 5.12 | 5.29 | 5.46 | 5.63 | 5.80 | 5.97 | 6.13 | 6.29 | 6.43 | 6.57 | 6.69 | 6.81 | 6.92 | 7.01 |
| 39º | | 4.90 | 5.06 | 5.23 | 5.41 | 5.59 | 5.77 | 5.94 | 6.12 | 6.30 | 6.47 | 6.63 | 6.78 | 6.93 | 7.06 | 7.18 | 7.29 | 7.39 |
| 40º | | 5.19 | 5.36 | 5.53 | 5.71 | 5.90 | 6.08 | 6.26 | 6.44 | 6.62 | 6.80 | 6.97 | 7.13 | 7.28 | 7.41 | 7.54 | 7.66 | 7.76 |

TABLE III International alcoholic strength at 20°C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at $t^o$C corrected for air buoyancy

| | Alcoholic strength in % | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $t^o$ | **0** | | **1** | | **2** | | **3** | | **4** | | **5** | | **6** | | **7** | | **8** | | **9** | | **10** | | **11** | |
| 0 | 999.34 | 1.52 | 997.82 | 1.45 | 996.37 | 1.39 | 994.98 | 1.35 | 993.63 | 1.29 | 992.34 | 1.24 | 991.10 | 1.18 | 989.92 | 1.15 | 988.77 | 1.09 | 987.68 | 1.05 | 986.63 | 1.00 | 985.63 | 0.96 |
| | --0.09 | | -0.09 | | -0.09 | | -0.08 | | -0.08 | | -0.08 | | -0.07 | | -0.05 | | -0.05 | | -0.04 | | -0.03 | | -0.02 | |
| 1 | 999.43 | 1.52 | 997.91 | 1.45 | 996.46 | 1.40 | 995.06 | 1.35 | 993.71 | 1.29 | 992.42 | 1.25 | 991.17 | 1.20 | 989.97 | 1.15 | 988.82 | 1.10 | 987.72 | 1.06 | 986.66 | 1.01 | 985.65 | 0.97 |
| | --0.06 | | -0.06 | | -0.06 | | -0.06 | | -0.06 | | -0.05 | | -0.05 | | -0.04 | | -0.03 | | -0.02 | | 0.02 | | -0.01 | |
| 2 | 999.49 | 1.52 | 997.97 | 1.40 | 996.52 | 1.40 | 995.12 | 1.35 | 993.77 | 1.30 | 992.47 | 1.25 | 991.22 | 1.21 | 990.01 | 1.16 | 988.85 | 1.11 | 987.74 | 1.06 | 986.68 | 1.02 | 985.66 | 0.98 |
| | -0.05 | | -0.05 | | -0.04 | | -0.04 | | -0.04 | | -0.04 | | -0.03 | | -0.03 | | -0.03 | | -0.02 | | 0.00 | | 0.01 | |
| 3 | 999.54 | 1.52 | 998.02 | 1.46 | 996.56 | 1.40 | 995.16 | 1.35 | 993.81 | 1.30 | 992.51 | 1.26 | 991.25 | 1.21 | 990.04 | 1.16 | 988.88 | 1.12 | 987.76 | 1.08 | 986.68 | 1.03 | 985.65 | 0.99 |
| | -0.03 | | -0.03 | | -0.03 | | -0.03 | | -0.02 | | -0.02 | | -0.02 | | -0.01 | | 0.00 | | 0.01 | | 0.01 | | 0.02 | |
| 4 | 999.57 | 1.52 | 998.05 | 1.46 | 996.59 | 1.40 | 995.19 | 1.36 | 993.83 | 1.30 | 992.53 | 1.26 | 991.27 | 1.22 | 990.05 | 1.17 | 98888 | 1.13 | 987.75 | 1.08 | 986.67 | 1.04 | 985.63 | 1.00 |
| | -0.02 | | -0.02 | | -0.02 | | -0.02 | | -0.02 | | -0.01 | | 0.00 | | 0.00 | | 0.00 | | 0.01 | | 0.02 | | 0.03 | |
| 5 | 999.59 | 1.52 | 998.07 | 1.46 | 996.61 | 1.40 | 995.21 | 1.36 | 993.85 | 1.31 | 992.54 | 1.27 | 991.27 | 1.22 | 990.05 | 1.17 | 988.88 | 1.14 | 987.74 | 1.09 | 986.65 | 1.05 | 985.60 | 1.02 |
| | 0.00 | | 0.00 | | 0.00 | | 0.01 | | 0.01 | | 0.01 | | 0.01 | | 0.02 | | 0.03 | | 0.03 | | 0.04 | | 0.06 | |
| 6 | 999.59 | 1.52 | 998.07 | 1.46 | 996.61 | 1.41 | 995.20 | 1.36 | 993.84 | 1.31 | 992.53 | 1.27 | 991.26 | 1.23 | 990.03 | 1.18 | 988.85 | 1.14 | 987.71 | 1.10 | 986.61 | 1.07 | 985.54 | 1.02 |
| | 0.01 | | 0.01 | | 0.01 | | 0.01 | | 0.01 | | 0.02 | | 0.02 | | 0.02 | | 0.03 | | 0.04 | | 0.05 | | 0.06 | |
| 7 | 999.58 | 1.52 | 998.06 | 1.46 | 996.60 | 1.41 | 995.19 | 1.36 | 99383 | 1.32 | 992.51 | 1.27 | 991.24 | 1.23 | 990.01 | 1.19 | 988.82 | 1.15 | 987.67 | 1.11 | 986.56 | 1.08 | 985.48 | 1.04 |
| | 0.03 | | 0.03 | | 0.03 | | 0.03 | | 0.04 | | 0.04 | | 0.05 | | 0.05 | | 0.06 | | 0.07 | | 0.07 | | 0.08 | |
| 8 | 999.55 | 1.52 | 998.03 | 1.46 | 996.57 | 1.41 | 995.16 | 1.37 | 993.79 | 1.32 | 992.47 | 1.28 | 991.19 | 1.23 | 989.96 | 1.20 | 988.76 | 1.16 | 987.60 | 1.11 | 986.49 | 1.09 | 985.40 | 1.05 |
| | 0.04 | | 0.04 | | 0.04 | | 0.04 | | 0.04 | | 0.04 | | 0.05 | | 0.06 | | 0.06 | | 0.06 | | 0.08 | | 0.08 | |
| 9 | 99951 | 1.52 | 997.99 | 1.46 | 996.53 | 1.41 | 995.12 | 1.37 | 993.75 | 1.32 | 992.43 | 1.29 | 991.14 | 1.24 | 989.90 | 1.20 | 988.70 | 1.16 | 987.54 | 1.13 | 986.41 | 1.09 | 985 32 | 1.06 |
| | 0.06 | | 0.06 | | 0.06 | | 0.06 | | 0.06 | | 0.07 | | 0.07 | | 0.07 | | 0.08 | | 0.09 | | 0.10 | | 0.11 | |
| 10 | 999.45 | 1.52 | 997.93 | 1.46 | 996.47 | 1.41 | 995.06 | 1.37 | 993.69 | 1.33 | 992.36 | 1.29 | 991.07 | 1.24 | 989.83 | 1.21 | 988.62 | 1.17 | 987.45 | 1.14 | 986.31 | 1.10 | 985.21 | 1.07 |
| | 0.07 | | 0.06 | | 0.06 | | 0.07 | | 0.07 | | 0.07 | | 0.07 | | 0.08 | | 0.09 | | 0.10 | | 0.10 | | 0.11 | |
| 11 | 999.38 | 1.51 | 997.87 | 1.46 | 996.41 | 1.42 | 994.99 | 1.37 | 993.62 | 1.33 | 992.29 | 1.29 | 991.00 | 1.25 | 989.75 | 1.22 | 988.53 | 1.18 | 987.35 | 1.14 | 986.21 | 1.11 | 985.10 | 1.08 |
| | 0.09 | | 0.09 | | 0.09 | | 0.09 | | 0.09 | | 0.09 | | 0.10 | | 0.11 | | 0.11 | | 0.11 | | 0.1 2 | | 0.13 | |
| 12 | 999.29 | 1.51 | 997.78 | 1.46 | 996.32 | 1.42 | 994.90 | 1.37 | 993.53 | 1.33 | 992.20 | 1.30 | 990.90 | 1.26 | 989.64 | 1.22 | 988.42 | 1.18 | 987.24 | 1.15 | 986.109 | 1.12 | 984.97 | 1.09 |
| | 0.09 | | 0.09 | | 0.09 | | 0.09 | | 0.10 | | 0.10 | | 0.10 | | 0.10 | | 0.11 | | 0.12 | | 0.13 | | 0.14 | |
| 13 | 999.20 | 1.51 | 997.69 | 1.46 | 996.23 | 1.42 | 994.81 | 1.38 | 993.43 | 1.33 | 992.10 | 1.30 | 990.80 | 1.26 | 989.54 | 1.23 | 988.31 | 1.19 | 987.12 | 1.16 | 985.96 | 1.13 | 984.83 | 1.10 |
| | 0.11 | | 0.11 | | 0.11 | | 0.11 | | 0.11 | | 0.12 | | 0.12 | | 0.13 | | 0.13 | | 0 14 | | 0.15 | | 0.16 | |
| 14 | 999.09 | 1.51 | 997.58 | 1.46 | 996.12 | 1.42 | 994.70 | 1.38 | 993.32 | 1.34 | 991.98 | 1.30 | 990.68 | 1.27 | 989.41 | 1.23 | 988.18 | 1.20 | 986.98 | 1.17 | 985.81 | 1.14 | 984.67 | 1.11 |
| | 0.12 | | 0.12 | | 0.12 | | 0.12 | | 0.12 | | 0.12 | | 0.13 | | 0.13 | | 0.14 | | 0.14 | | 0.15 | | 0.16 | |
| 15 | 998.97 | 1.51 | 997.46 | 1.46 | 996.00 | 1.42 | 994.58 | 1.38 | 993.20 | 1.34 | 991.86 | 1.31 | 990.55 | 1.27 | 989.28 | 1.24 | 988.04 | 1.20 | 986.94 | 1.18 | 985.66 | 1.15 | 984.51 | 1.12 |
| | 0.13 | | 0.13 | | 0.13 | | 0.13 | | 0.14 | | 0.14 | | 0.14 | | 0.15 | | 0.15 | | 0.17 | | 0.17 | | 0.18 | |
| 16 | 998.84 | 1.51 | 997.33 | 1.46 | 995.87 | 1.42 | 994.45 | 1.39 | 993.06 | 1.34 | 991.72 | 1.31 | 990.41 | 1.28 | 989.13 | 1.24 | 987.89 | 1.22 | 986.67 | 1.18 | 985.49 | 1.16 | 984.33 | 1.13 |
| | 0.14 | | 0.14 | | 0.14 | | 0.14 | | 0.14 | | 0.15 | | 0.15 | | 0.15 | | 0.16 | | 0.17 | | 0.17 | | 0.18 | |
| 17 | 998.70 | 1.51 | 997.19 | 1.46 | 995.73 | 1.42 | 994.31 | 1.39 | 992.92 | 1.35 | 991.57 | 1.31 | 990.26 | 1.28 | 988.98 | 1.25 | 987.73 | 1.22 | 986.50 | 1.18 | 985.32 | 1.17 | 984.15 | 1.14 |
| | 0.15 | | 0.15 | | 0.16 | | 0.16 | | 0.16 | | 0.16 | | 0.17 | | 0.17 | | 0.18 | | 0.18 | | 0.19 | | 0.19 | |
| 18 | 998.55 | 1.51 | 997.04 | 1.47 | 995.57 | 1.42 | 994.15 | 1.39 | 992.76 | 1.35 | 991.41 | 1.32 | 990.09 | 1.28 | 988.81 | 1.26 | 987.55 | 1.23 | 986.32 | 1.19 | 985.13 | 1.17 | 983.96 | 1.15 |
| | 0.17 | | 0.16 | | 0.16 | | 0.16 | | 0.16 | | 0.16 | | 0.17 | | 0.18 | | 0.18 | | 0.19 | | 0.20 | | 0.21 | |
| 19 | 998.38 | 1.50 | 996.88 | 1.47 | 995.41 | 1.42 | 993.99 | 1.39 | 992.60 | 1.35 | 991.25 | 1.33 | 989.92 | 1.29 | 988.63 | 1.26 | 987.37 | 1.24 | 986 13 | 1.20 | 98493 | 1.18 | 983.75 | 1.16 |
| | 0.18 | | 0.18 | | 0.18 | | 0.18 | | 0.19 | | 0.19 | | 0.19 | | 0.20 | | 0.21 | | 0.22 | | 0.22 | | 0.23 | |
| 20 | 998.20 | 1.50 | 996.70 | 1.47 | 995.23 | 1.42 | 993.81 | 1.40 | 992.41 | 1.35 | 991.06 | 1.33 | 989.73 | 1.30 | 988.43 | 1.27 | 987.16 | 1.24 | 985 92 | 1.21 | 984.71 | 1.19 | 983 52 | 1.17 |

TABLE III (continued) International alcoholic strength at 20°C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at *t*°C corrected for air buoyancy

| | Alcoholic strength in % | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| t° | **0** | | **1** | | **2** | | **3** | | **4** | | **5** | | **6** | | **7** | | **8** | | **9** | | **10** | | **11** | |
| 20 | 998.20 | 1.50 | 996.70 | 1.47 | 995.23 | 1.42 | 993.81 | 1.40 | 992.41 | 1.35 | 991.06 | 1.33 | 989.73 | 1.30 | 988.43 | 1.27 | 987.16 | 1.24 | 985.92 | 1.21 | 984.71 | 1.19 | 983.52 | 1.17 |
| | 0.19 | | 0.19 | | 0.19 | | 0.19 | | 0.19 | | 0.20 | | 0.20 | | 0.21 | | 0.21 | | 0.22 | | 0.23 | | 0.23 | |
| 21 | 998.01 | 1.50 | 996.51 | 1.47 | 995.04 | 1.42 | 993.62 | 1.40 | 992.22 | 136 | 990.86 | 1.33 | 989.53 | 1.31 | 988.22 | 1.27 | 986.95 | 1.25 | 985.70 | 1.22 | 984.48 | 1.19 | 983.29 | 1.17 |
| | 0.20 | | 0.20 | | 0.19 | | 0.20 | | 0.20 | | 0.20 | | 0.21 | | 0.21 | | 0.22 | | 0.22 | | 0.23 | | 0.24 | |
| 22 | 987.81 | 1.50 | 996.31 | 1.46 | 994.85 | 1.43 | 993.42 | 1.40 | 992.02 | 1.36 | 990.66 | 1.34 | 989.32 | 1.31 | 988.01 | 1.28 | 986.73 | 1.25 | 985.48 | 1.23 | 984.25 | 1.20 | 983.05 | 1.18 |
| | 0.21 | | 0.21 | | 0.21 | | 0.21 | | 0.21 | | 0.22 | | 0.22 | | 0.22 | | 0.23 | | 0.24 | | 0.24 | | 0.25 | |
| 23 | 997.60 | 1.50 | 996.10 | 1.46 | 994.64 | 1.43 | 993.21 | 1.40 | 991.81 | 1.37 | 990.44 | 1.34 | 989.10 | 1.31 | 987.79 | 1.29 | 986.50 | 1.26 | 985.24 | 1.23 | 984.01 | 1.21 | 982.80 | 1.19 |
| | 0.21 | | 0.21 | | 0.22 | | 0.22 | | 0.22 | | 0.22 | | 0.23 | | 0.23 | | 0.23 | | 0.24 | | 0.25 | | 0.26 | |
| 24 | 997.39 | 1.50 | 995.89 | 1.47 | 994.42 | 1.43 | 992.99 | 1.40 | 991.59 | 1.37 | 990.22 | 1.35 | 988.87 | 1.31 | 987.56 | 1.29 | 986.27 | 1.27 | 985.00 | 1.24 | 98376 | 1.22 | 982.54 | 1.20 |
| | 0.23 | | 0.23 | | 0.23 | | 0.23 | | 0.24 | | 0.24 | | 0.24 | | 0.25 | | 0.25 | | 0.25 | | 0.26 | | 0.27 | |
| 25 | 997.16 | 1.50 | 995.66 | 1.47 | 994.19 | 1.43 | 992.76 | 1.41 | 991 35 | 1.37 | 989.98 | 1.35 | 988.63 | 1.32 | 987.31 | 1.29 | 986.02 | 1.27 | 984.75 | 1.25 | 983.50 | 1.23 | 982.27 | 1.21 |
| | 0.23 | | 0.23 | | 0.23 | | 0.24 | | 0.24 | | 0.24 | | 0.24 | | 0.25 | | 0.26 | | 0.27 | | 0.27 | | 0.28 | |
| 26 | 996.93 | 1.50 | 995.43 | 1.47 | 993.96 | 1.44 | 992.52 | 1.41 | 991.11 | 1.37 | 989.74 | 1.35 | 988.39 | 1.33 | 987.06 | 1.30 | 985.76 | 1.28 | 984.48 | 1.25 | 983.23 | 1.24 | 981.99 | 1.22 |
| | 0.25 | | 0.25 | | 0.25 | | 0.25 | | 0.25 | | 0.26 | | 0.26 | | 0.26 | | 0.27 | | 0.28 | | 0.29 | | 0.29 | |
| 27 | 996.68 | 1.50 | 995.18 | 1.47 | 993.71 | 1.44 | 992.27 | 1.41 | 990.86 | 1.38 | 989.48 | 1.35 | 988.13 | 1.33 | 986.80 | 1.31 | 985.49 | 1.29 | 994.20 | 1.26 | 982.94 | 1.24 | 981.70 | 1.23 |
| | 0.25 | | 0.25 | | 0.26 | | 0.26 | | 0.26 | | 0.26 | | 0.27 | | 0.28 | | 0.28 | | 0.28 | | 0.29 | | 0.30 | |
| 28 | 996.43 | 1.50 | 994.93 | 1.48 | 993.45 | 1.44 | 992.01 | 1.41 | 990.60 | 1.38 | 989.22 | 1.36 | 987.86 | 1.34 | 986.52 | 1.31 | 985.21 | 1.29 | 983.92 | 1.27 | 982.65 | 1.25 | 981.40 | 1.23 |
| | 0.26 | | 0.27 | | 0.27 | | 0.27 | | 027 | | 0.28 | | 0.28 | | 0.28 | | 0.29 | | 0.29 | | 0.30 | | 0.31 | |
| 29 | 996 17 | 1.51 | 994.66 | 1.48 | 993 18 | 1.44 | 991.74 | 1.41 | 990.33 | 1.39 | 988.94 | 1.36 | 98758 | 1.34 | 98624 | 1.32 | 984.92 | 1.29 | 983.63 | 1.28 | 98235 | 1.26 | 98109 | 1.24 |
| | 0.27 | | 0.27 | | 0.27 | | 0.28 | | 0.28 | | 0.28 | | 0.28 | | 0.29 | | 0.29 | | 0.30 | | 0.31 | | 0.32 | |
| 30 | 995.90 | 1.51 | 994.39 | 1.48 | 992.91 | 1.45 | 991.46 | 1.41 | 990.05 | 1.39 | 988.66 | 1.37 | 987.29 | 1.34 | 985.95 | 1.32 | 984.63 | 1.30 | 983.33 | 1.29 | 982.04 | 1.27 | 980.77 | 1.25 |
| | 0.29 | | 0.29 | | 0.29 | | 0.29 | | 0.30 | | 0.30 | | 0.30 | | 0.31 | | 0.31 | | 0.32 | | 0.32 | | 0.32 | |
| 31 | 995.61 | 151 | 994.10 | 1.48 | 992.62 | 1.45 | 991.17 | 1.42 | 989.75 | 1.39 | 988.36 | 1.37 | 986.99 | 1.35 | 985.64 | 1.33 | 984.31 | 1.30 | 983.01 | 1.29 | 981.72 | 1.27 | 980.45 | 1.26 |
| | 0.29 | | 0.29 | | 0.29 | | 0.29 | | 0.30 | | 0.31 | | 0.31 | | 0.31 | | 0.31 | | 0.32 | | 0.33 | | 0.34 | |
| 32 | 995.32 | 1.51 | 993.81 | 1.48 | 992.33 | 1.45 | 990.88 | 1.42 | 989.45 | 1.40 | 988.05 | 1.37 | 986.68 | 1.35 | 985.33 | 1.33 | 984.00 | 1.31 | 982.69 | 1.30 | 981.39 | 1.28 | 980.11 | 1.26 |
| | 0.30 | | 0.31 | | 0.31 | | 0.31 | | 0.31 | | 0.31 | | 0.31 | | 0.32 | | 0.33 | | 0.33 | | 0.34 | | 0.34 | |
| 33 | 995.02 | 1.52 | 993.50 | 1.48 | 992.02 | 1.45 | 990.57 | 1.43 | 989.14 | 1.40 | 987.74 | 1.37 | 986.37 | 1.36 | 985.01 | 1.34 | 983.67 | 1.31 | 982.36 | 1.31 | 981.05 | 1.28 | 979.77 | 1 27 |
| | 0.30 | | 0.31 | | 0.31 | | 0.31 | | 0.31 | | 0.32 | | 0.33 | | 0.33 | | 0.33 | | 0.34 | | 0.34 | | 0.35 | |
| 34 | 994.72 | 1.53 | 993.19 | 1.48 | 991.71 | 1.45 | 990.26 | 1.43 | 988.83 | 1.41 | 987.42 | 1.38 | 98604 | 1.36 | 984.68 | 1.34 | 983.34 | 1.32 | 982.02 | 1.31 | 980.71 | 1.29 | 979.42 | 1.28 |
| | 0.32 | | 0.32 | | 0.32 | | 0.33 | | 0.33 | | 0.33 | | 0.33 | | 0.33 | | 0.33 | | 0.34 | | 0.34 | | 0.35 | |
| 35 | 994.40 | 1.53 | 992.87 | 1.48 | 991.39 | 1.46 | 989.93 | 1.43 | 988.50 | 1.41 | 987.09 | 1.38 | 985.71 | 1.36 | 984.35 | 1.34 | 983.01 | 1.33 | 981.68 | 1.31 | 980.37 | 1.30 | 979.07 | 1.29 |
| | 0.32 | | 0.32 | | 0.33 | | 0.33 | | 0.33 | | 0.33 | | 0. 34 | | 0.34 | | 0.35 | | 0.35 | | 0.36 | | 0.37 | |
| 36 | 994.08 | 1.53 | 992.55 | 1.49 | 991.06 | 1.46 | 989.60 | 1.43 | 988.17 | 1.41 | 986.76 | 1.39 | 985.37 | 1.36 | 984.01 | 1.35 | 982.66 | 1.33 | 981.33 | 1.32 | 980.01 | 1.31 | 978.70 | 1.29 |
| | 0.33 | | 0.34 | | 0.34 | | 0.34 | | 0.35 | | 0.35 | | 0.35 | | 0.35 | | 0.36 | | 0.36 | | 0.36 | | 0.37 | |
| 37 | 993.75 | 1.54 | 992.21 | 1.49 | 990.72 | 1.46 | 989.26 | 1.44 | 987.82 | 1.41 | 986.41 | 1.39 | 985.02 | 1.37 | 983.65 | 1.35 | 982.30 | 1.33 | 980.97 | 1.32 | 979.65 | 1.32 | 978.33 | 1.30 |
| | 0.34 | | 0.34 | | 0.35 | | 0.36 | | 0.36 | | 0.36 | | 0.36 | | 0.36 | | 0.37 | | 0.38 | | 0.38 | | 0.38 | |
| 38 | 993.41 | 1.54 | 991.87 | 1.50 | 990.37 | 1.47 | 988.90 | 1.44 | 87.46 | 1.41 | 986.05 | 1.39 | 984.66 | 1.37 | 983.29 | 1.36 | 981.93 | 1.34 | 980.59 | 1.32 | 979.27 | 1.32 | 977.95 | 1.31 |
| | 0.35 | | 0.35 | | 0.36 | | 0.36 | | 0.36 | | 0.37 | | 0.37 | | 0.37 | | 0.37 | | 0.38 | | 0.38 | | 0.39 | |
| 39 | 993.06 | 1.54 | 991.52 | 1.51 | 990.01 | 1.47 | 98854 | 1.44 | 987.10 | 1.41 | 98568 | 1.39 | 984.29 | 1.37 | 982.92 | 1.36 | 981.56 | 1.34 | 980.22 | 1.33 | 978.89 | 1.33 | 977.56 | 1.31 |
| | 0.35 | | 0.36 | | 0.36 | | 0.37 | | 0.38 | | 0.38 | | 0.38 | | 0.38 | | 0.38 | | 0.39 | | 0.39 | | 0.39 | |
| 40 | 992.71 | 1.55 | 991.16 | 1.51 | 989.65 | 1.48 | 988.17 | 1.45 | 986.72 | 1.42 | 985.30 | 1.39 | 983.91 | 1.37 | 982.54 | 1.36 | 981.18 | 1.35 | 979.83 | 1.33 | 978.50 | 1.33 | 977.17 | 1.32 |

TABLE III (continued) International alcoholic strength in 20°C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at *t*°C corrected for air buoyancy

| | Alcoholic strength in % | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| t° | 10 | | 11 | | 12 | | 13 | | 14 | | 15 | | 16 | | 17 | | 18 | | 19 | | 20 | | 21 | |
| 0 | 986.63 | 1.00 | 985.63 | 0.96 | 984.67 | 0.92 | 983.75 | 0.87 | 982.88 | 0.84 | 982.04 | 0.81 | 981.23 | 0.77 | 980.46 | 0.75 | 979.71 | 0.73 | 978.98 | 0.72 | 978.26 | 0.70 | 977.56 | 0.70 |
| | -0.03 | | -0.02 | | -0.01 | | 0.00 | | 0.02 | | 0.04 | | 0.05 | | 0.07 | | 0.09 | | 0.11 | | 0.13 | | 0.15 | |
| 1 | 986.66 | 1.01 | 985.65 | 0.97 | 984.68 | 0.93 | 983.75 | 0.89 | 982.86 | 0.86 | 982.00 | 0.82 | 981.18 | 0.79 | 98039 | 0.77 | 979.62 | 0.75 | 978.87 | 0.74 | 978.13 | 0.72 | 977.41 | 0.72 |
| | -0.02 | | -0.01 | | 0.00 | | 0.01 | | 0.03 | | 0.04 | | 0.06 | | 0.08 | | 0.10 | | 0.12 | | 0.14 | | 0.17 | |
| 2 | 986.68 | 1.02 | 985.66 | 0.98 | 984.68 | 0.94 | 983.74 | 0.91 | 982.83 | 0.87 | 981.96 | 0.84 | 981.12 | 0.81 | 980.3l | 0.79 | 979.52 | 0.77 | 978.75 | 0.76 | 977.99 | 0.75 | 977.24 | 0.74 |
| | 0.00 | | 0.01 | | 0.02 | | 0.04 | | 0.05 | | 0.06 | | 0.08 | | 0.10 | | 0.12 | | 0.14 | | 0.16 | | 0.18 | |
| 3 | 986.68 | 1.03 | 985.65 | 0.99 | 984.66 | 0.96 | 983.70 | 0.92 | 982.78 | 0.88 | 981.90 | 0.86 | 981.04 | 0.83 | 980.21 | 0.81 | 979.40 | 0.79 | 978.61 | 0.78 | 977.83 | 0.77 | 977.06 | 0.76 |
| | 0.01 | | 0.02 | | 0.03 | | 0.04 | | 0.05 | | 0.07 | | 0.08 | | 0.10 | | 0.12 | | 0.14 | | 0.16 | | 0.18 | |
| 4 | 986.67 | 1.04 | 985.63 | 1.00 | 984.63 | 0.97 | 983.66 | 0.93 | 982.73 | 0.90 | 981.83 | 0.87 | 980.96 | 0.85 | 980.11 | 0.83 | 979.28 | 0.81 | 978.47 | 0.80 | 977.67 | 0.79 | 976.88 | 0.79 |
| | 0.02 | | 0.03 | | 0.05 | | 0.06 | | 0.08 | | 0.09 | | 0.11 | | 0.13 | | 0.14 | | 0.16 | | 0.18 | | 0.20 | |
| 5 | 986.65 | 1.05 | 985.60 | 1.02 | 984.58 | 0.98 | 983.60 | 0.95 | 982.65 | 0.91 | 981.74 | 0.89 | 980.85 | 0.87 | 979.98 | 0.84 | 979.11 | 0.83 | 978.31 | 0.82 | 977.49 | 0.81 | 976.68 | 0.81 |
| | 0.04 | | 0.06 | | 0.06 | | 0.07 | | 0.08 | | 0.10 | | 0.11 | | 0.13 | | 0.15 | | 0.17 | | 0.19 | | 0.21 | |
| 6 | 986.61 | 1.07 | 985.54 | 1.02 | 984.52 | 0.99 | 983.53 | 0.96 | 982.57 | 0.93 | 981.64 | 0.90 | 980.74 | 0.89 | 979.85 | 0.86 | 978.99 | 0.85 | 978.14 | 0.84 | 977.30 | 0.83 | 976.47 | 0.83 |
| | 0.05 | | 0.06 | | 0.08 | | 0.09 | | 0.10 | | 0.12 | | 0.14 | | 0.15 | | 0.17 | | 0.19 | | 0.20 | | 0.22 | |
| 7 | 986.56 | 1.08 | 985.48 | 1.04 | 994.44 | 1.00 | 983.44 | 0.97 | 982.47 | 0.95 | 981.52 | 0.92 | 980.60 | 0.90 | 979.70 | 0.88 | 978.82 | 0.87 | 977.95 | 0.85 | 977.10 | 0.85 | 976.25 | 0.85 |
| | 0.07 | | 0.08 | | 0.09 | | 0.10 | | 0.11 | | 0.12 | | 0.14 | | 0.16 | | 0.18 | | 0.19 | | 0.21 | | 0.23 | |
| 8 | 986.49 | 1.09 | 985.40 | 1.05 | 984.35 | 1.01 | 983.34 | 0.98 | 982.36 | 0.96 | 981.40 | 0.94 | 980.46 | 0.92 | 979.54 | 0.90 | 978.64 | 0.88 | 977.76 | 0.87 | 976.89 | 0.87 | 976.02 | 0.97 |
| | 0.08 | | 0.08 | | 0.09 | | 0.1l | | 0.13 | | 0.14 | | 0.15 | | 0.16 | | 0.18 | | 0.20 | | 0.22 | | 0.24 | |
| 9 | 986.41 | 1.09 | 985.32 | 1.06 | 984.26 | 1.03 | 983.23 | 1.00 | 982.23 | 0.97 | 981.26 | 0.95 | 980.31 | 0.93 | 979.38 | 0.92 | 978.48 | 0.90 | 977.56 | 0.89 | 976.67 | 0.89 | 975.78 | 0.89 |
| | 0.10 | | 0.11 | | 0.12 | | 0.13 | | 0.14 | | 0.16 | | 0.17 | | 0.18 | | 0.19 | | 0.21 | | 0.23 | | 0.25 | |
| 10 | 986.31 | 1.10 | 985.21 | 1.07 | 984.14 | 1.04 | 983.10 | 1.01 | 982.09 | 0.99 | 981.10 | 0.96 | 980.14 | 0.94 | 979.20 | 0.93 | 918.27 | 0.92 | 977.35 | 0.91 | 976.44 | 0.91 | 975.53 | 0.91 |
| | 0.10 | | 0.11 | | 0.12 | | 0.13 | | 0.15 | | 0.16 | | 0.17 | | 0.19 | | 0.21 | | 0.23 | | 0.25 | | 0.27 | |
| 11 | 986.21 | 1.11 | 985.10 | 1.08 | 984.02 | 1.05 | 982.97 | 1.03 | 981.94 | 1.00 | 980.94 | 0.97 | 979.97 | 0.96 | 979.01 | 0.95 | 978.06 | 0.94 | 977.12 | 0.93 | 976.19 | 0.93 | 975.26 | 0.92 |
| | 0.12 | | 0.13 | | 0.14 | | 0.15 | | 0.16 | | 0.17 | | 0.19 | | 0.21 | | 0.22 | | 0.24 | | 0.26 | | 0.27 | |
| 12 | 986.09 | 1.12 | 984.97 | 1.09 | 983.88 | 1.06 | 982.82 | 1.04 | 981.78 | 1.01 | 980.77 | 0.99 | 979.78 | 0.98 | 978.80 | 0.96 | 977.84 | 0.96 | 976.88 | 0.95 | 975.93 | 0.94 | 974.99 | 0.94 |
| | 0.13 | | 0.14 | | 0.15 | | 0.16 | | 0.17 | | 0.19 | | 0.20 | | 0.21 | | 0.23 | | 0.24 | | 0.26 | | 0.28 | |
| 13 | 985.96 | 1.13 | 984.83 | 1.10 | 983.73 | 1.07 | 982.66 | 1.05 | 981.61 | 1.03 | 980.58 | 1.00 | 979.58 | 0.99 | 978.59 | 0.98 | 977.61 | 0.97 | 976.64 | 0.97 | 975.67 | 0.96 | 974.71 | 0.96 |
| | 0.15 | | 0.16 | | 0.17 | | 0.18 | | 0.19 | | 0.20 | | 0.22 | | 0.23 | | 0.24 | | 0.26 | | 0.27 | | 0.29 | |
| 14 | 985.81 | 1.14 | 984.67 | 1.11 | 983.56 | 1.08 | 982.48 | 1.06 | 981.42 | 1.04 | 980.38 | 1.02 | 979.36 | 1.00 | 978.36 | 0.99 | 977.37 | 0.99 | 976.38 | 0.98 | 975.40 | 0.98 | 974.42 | 0.98 |
| | 0.15 | | 0.16 | | 0.17 | | 0.18 | | 0.19 | | 0.20 | | 0.22 | | 0.24 | | 0.26 | | 0.27 | | 0.28 | | 0.30 | |
| 15 | 985.66 | 1.15 | 984.51 | 1.12 | 983.39 | 1.09 | 982.30 | 1.07 | 981.23 | 1.05 | 980.18 | 1.04 | 979.14 | 1.02 | 978.12 | 1.01 | 977.11 | 1.00 | 976.11 | 0.99 | 975.12 | 1.00 | 974.12 | 1.00 |
| | 0.17 | | 0.18 | | 0.19 | | 0.20 | | 0.21 | | 0.22 | | 0.23 | | 0.25 | | 0.26 | | 0.28 | | 0.30 | | 0.31 | |
| 16 | 985.49 | 1.16 | 984.33 | 1.13 | 983.20 | 1.10 | 982.10 | 1.08 | 981.02 | 1.06 | 979.96 | 1.05 | 978.91 | 1.04 | 977.87 | 1.02 | 976.85 | 1.02 | 975.83 | 1.01 | 974.82 | 1.01 | 973.81 | 1.02 |
| | 0.17 | | 0.18 | | 0.19 | | 0.20 | | 0.21 | | 0.23 | | 0.24 | | 0.25 | | 0.27 | | 0.29 | | 0.30 | | 0.31 | |
| 17 | 985.32 | 1.17 | 984.15 | 1.14 | 98.301 | 1.11 | 981.90 | 1.09 | 980.81 | 1.08 | 979.73 | 1.06 | 978.67 | 1.05 | 977.62 | 1.04 | 976.58 | 1.04 | 975.54 | 1.02 | 974.52 | 1.02 | 973.95 | 1.04 |
| | 0.19 | | 0.19 | | 0.20 | | 0.22 | | 0.24 | | 0.25 | | 0.26 | | 0.27 | | 0.28 | | 0.29 | | 0.31 | | 0.33 | |
| 18 | 985.13 | 1.17 | 983.96 | 1.15 | 982.81 | 1.13 | 981.68 | 1.11 | 980.57 | 1.09 | 979.48 | 1.07 | 978.41 | 1.06 | 977.35 | 1.05 | 976.30 | 1.05 | 975.25 | 1.04 | 974.21 | 1.04 | 973.17 | 1.05 |
| | 0.20 | | 0.21 | | 0.22 | | 0.23 | | 0.24 | | 0.25 | | 0.26 | | 0.27 | | 0.29 | | 0.30 | | 0.32 | | 0.34 | |
| 19 | 984.93 | 1.18 | 983.75 | 1.16 | 982.59 | 1.14 | 981.45 | 1.12 | 980.33 | 1.10 | 979.23 | 1.08 | 978 15 | 107 | 977.08 | 1.07 | 976.01 | 1.06 | 974.94 | 1.05 | 973.89 | 1.06 | 972.83 | 1.06 |
| | 0.22 | | 0.23 | | 0.24 | | 0.24 | | 0.25 | | 0.26 | | 0.28 | | 0.29 | | 0.30 | | 0.31 | | 0.33 | | 0.35 | |
| 20 | 984.71 | 1.19 | 983.52 | 1.17 | 982.35 | 1.14 | 981.21 | 1.13 | 980.08 | 1.11 | 978.97 | 1.10 | 977.87 | 1.08 | 976.79 | 1.08 | 975.71 | 1.08 | 974.63 | 1.07 | 973.56 | 1.08 | 972.48 | 1.08 |

TABLE III (continued) International alcoholic strength in 20$^{O}$C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at $t^{o}$C corrected for air buoyancy

| | Alcoholic strength in % | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $t^{o}$ | **10** | | **11** | | **12** | | **13** | | **14** | | **15** | | **16** | | **17** | | **18** | | **19** | | **20** | | **21** | |
| 20 | 984.71 | 1.19 | 983.52 | 1.17 | 982.35 | 1.14 | 981.21 | 1.13 | 980.08 | 1.11 | 978.97 | 1.10 | 977.87 | 1.08 | 976.79 | 1.08 | 975.71 | 1.08 | 974.63 | 1.07 | 973.56 | 1.08 | 972.48 | 1.08 |
| | 0.23 | | 0.23 | | 0.23 | | 0.25 | | 0.26 | | 0.28 | | 0.29 | | 0.31 | | 0.32 | | 0.33 | | 0.35 | | 0.36 | |
| 21 | 984.48 | 1.19 | 983.29 | 1.17 | 982.12 | 1.16 | 980.96 | 1.14 | 979.82 | 1.13 | 978.69 | 1.11 | 97758 | 1.10 | 976.48 | 1.09 | 975.39 | 1.09 | 974.30 | 1.09 | 973.21 | 1.09 | 972.12 | 1.09 |
| | 0.23 | | 0.24 | | 0.25 | | 0.26 | | 0.27 | | 0.28 | | 0.29 | | 0.31 | | 0.32 | | 0.33 | | 0.35 | | 0.36 | |
| 22 | 984.25 | 1.20 | 983.05 | 1.18 | 981.97 | 1.17 | 980.70 | 1.15 | 979.55 | 1.14 | 978.41 | 1.12 | 977.29 | 1.12 | 976.17 | 1.10 | 975.07 | 1.10 | 973.97 | 1.10 | 972.86 | 1.10 | 971.76 | 1.11 |
| | 0.24 | | 0.25 | | 0.26 | | 0.27 | | 0.28 | | 0.29 | | 0.30 | | 0.31 | | 0.33 | | 0.34 | | 0.35 | | 0.37 | |
| 23 | 984.01 | 1.21 | 982.80 | 1.19 | 981.61 | 1.18 | 980.43 | 1.16 | 979.27 | 1.15 | 978.12 | 1.13 | 976.99 | 1.13 | 975.86 | 1.12 | 974.74 | 1.11 | 973.63 | 1.12 | 972.51 | 1.12 | 971.39 | 1.13 |
| | 0.25 | | 0.26 | | 0.27 | | 0.28 | | 0.29 | | 0.30 | | 0.31 | | 0.32 | | 0.33 | | 0.35 | | 0.36 | | 0.38 | |
| 24 | 983.76 | 1.22 | 982.54 | 1.20 | 981.34 | 1.19 | 980.15 | 1.17 | 978.98 | 1.16 | 977.82 | 1.14 | 976.68 | 1.14 | 97554 | 1.13 | 974.41 | 1.13 | 97328 | 1.13 | 972.15 | 1.14 | 971.01 | 1.14 |
| | 0.26 | | 0.27 | | 0.28 | | 0.29 | | 0.30 | | 0.31 | | 0.32 | | 0.33 | | 0.35 | | 0.36 | | 0.38 | | 0.39 | |
| 25 | 983.50 | 1.23 | 982.27 | 1.21 | 981.06 | 1.20 | 979.86 | 1.18 | 978.68 | 1.17 | 977.51 | 1.16 | 976.36 | 1.15 | 975.21 | 1.15 | 974.06 | 1.14 | 972.92 | 1.15 | 971.77 | 1.15 | 970.62 | 1.15 |
| | 0.27 | | 0.28 | | 0.29 | | 0.29 | | 0.30 | | 0.31 | | 0.33 | | 0.34 | | 0.35 | | 0.37 | | 0.38 | | 0.39 | |
| 26 | 983.23 | 1.24 | 981.99 | 1.22 | 980.77 | 1.20 | 979.57 | 1.19 | 978.38 | 1.18 | 977.20 | 1.17 | 976.03 | 1.16 | 974.87 | 1.16 | 973.71 | 1.16 | 972.55 | 1.16 | 971.39 | 1.16 | 970.23 | 1.17 |
| | 0.29 | | 0.29 | | 0.30 | | 0.31 | | 0.32 | | 0.33 | | 0.34 | | 0.36 | | 0.37 | | 0.38 | | 0.39 | | 0.41 | |
| 27 | 982.94 | 1.24 | 981.70 | 1.23 | 980.47 | 1.21 | 979.26 | 1.20 | 978.06 | 1.19 | 976.87 | 1.18 | 975.69 | 1.18 | 974.51 | 1.17 | 973.34 | 1.17 | 972.17 | 1.17 | 971.00 | 1.18 | 969.82 | 1.18 |
| | 0.29 | | 0.30 | | 0.30 | | 0.31 | | 0.32 | | 0.33 | | 0.35 | | 0.36 | | 0.38 | | 0.39 | | 0.40 | | 0.41 | |
| 28 | 982.65 | 1.25 | 981.40 | 1.23 | 980.17 | 1.22 | 978.95 | 1.21 | 977.74 | 1.20 | 976.54 | 1.20 | 975.34 | 1.19 | 974.15 | 1.19 | 972.96 | 1.18 | 971.78 | 1.18 | 970.60 | 1.19 | 969.41 | 1.20 |
| | 0.30 | | 0.31 | | 0.32 | | 033 | | 0.34 | | 0.35 | | 0.36 | | 0.37 | | 0.38 | | 0.39 | | 0.40 | | 0.42 | |
| 29 | 982.35 | 1.26 | 981.09 | 1.24 | 979.85 | 1.23 | 978.62 | 1.22 | 977.40 | 1.21 | 976.19 | 1.21 | 974.98 | 1.20 | 973.78 | 1.20 | 972.58 | 1.19 | 971.39 | 1.19 | 970.20 | 1.21 | 968.99 | 1.21 |
| | 0.31 | | 0.32 | | 0.33 | | 0.34 | | 0.35 | | 0.36 | | 0.37 | | 0.38 | | 0.38 | | 0.40 | | 0.42 | | 0.43 | |
| 30 | 982.04 | 1.27 | 980.77 | 1.25 | 979.52 | 1.24 | 978.28 | 1.23 | 977.05 | 1.22 | 975.83 | 1.21 | 974.62 | 1.21 | 973.41 | 1.21 | 972.20 | 1.21 | 970.99 | 1.21 | 969.78 | 1.22 | 968.56 | 1.23 |
| | 0.32 | | 0.32 | | 0.33 | | 0.34 | | 0.35 | | 0.36 | | 0.37 | | 0.38 | | 0.39 | | 0.40 | | 0.42 | | 0.43 | |
| 31 | 981.72 | 1.27 | 980.45 | 1.26. | 979.19 | 1.25 | 977.94 | 1.24 | 976.70 | 1.23 | 975.47 | 1.22 | 974.25 | 1.22 | 973.03 | 1.22 | 971.81 | 1.22 | 970.59 | 1.23 | 969.36 | 1.23 | 968.13 | 1.24 |
| | 0.33 | | 0.34 | | 0.34 | | 0.35 | | 0.36 | | 0.37 | | 0.38 | | 0.39 | | 0.40 | | 0.42 | | 0.43 | | 0.45 | |
| 32 | 981.39 | 1.28 | 980.11 | 1.26 | 978.95 | 1.26 | 977.59 | 1.25 | 976.34 | 1.24 | 975.10 | 1.23 | 973.87 | 1.23 | 972.64 | 1.23 | 971.41 | 1.24 | 970.17 | 1.24 | 968.93 | 1.25 | 967.68 | 1.26 |
| | 0.34 | | 0.34 | | 0.35 | | 0.35 | | 0.36 | | 0.37 | | 0.39 | | 0.40 | | 0.41 | | 0.42 | | 0.43 | | 0.45 | |
| 33 | 981.05 | 1.28 | 979.77 | 1.27 | 978.50 | 1.26 | 977.24 | 1.26 | 975.78 | 1.25 | 974.73 | 1.25 | 973.48 | 1.24 | 972.24 | 1.24 | 971.00 | 1.25 | 969.75 | 1.25 | 968.50 | 1.27 | 967.23 | 1.27 |
| | 0.34 | | 0.35 | | 0.36 | | 0.37 | | 0.38 | | 0.39 | | 0.40 | | 0.41 | | 0.42 | | 0.43 | | 0.45 | | 0.45 | |
| 34 | 98071 | 1.29 | 979.42 | 1.28 | 978.14 | 1.27 | 976.97 | 1.27 | 975. 60 | 1.26 | 974.34 | 1.26 | 973.08 | 1.25 | 971.83 | 1.25 | 970.58 | 1.26 | 969.32 | 1.27 | 968.05 | 1.27 | 966.78 | 1.29 |
| | 0.34 | | 0.35 | | 0.36 | | 0.37 | | 0.38 | | 0.39 | | 0.40 | | 0.41 | | 0.43 | | 0.44 | | 0.45 | | 0.47 | |
| 35 | 980.37 | 1.30 | 979.07 | 1.29 | 977.78 | 1.28 | 976.50 | 1.28 | 975.22 | 1.27 | 973.95 | 1.27 | 972.68 | 1.26 | 971.42 | 1.27 | 970.15 | 1.27 | 968.88 | 1.28 | 967.60 | 1.29 | 966.31 | 1.30 |
| | 0.36 | | 0.37 | | 0.37 | | 0.38 | | 0.38 | | 0.39 | | 0.40 | | 0.42 | | 0.43 | | 0.44 | | 0.45 | | 0.47 | |
| 36 | 980.01 | 1.31 | 978.70 | 1.29 | 977.41 | 1.29 | 976.12 | 1.28 | 974.84 | 1.28 | 973.56 | 1.28 | 972.28 | 1.28 | 971.00 | 1.28 | 969.72 | 1.28 | 968.44 | 1.29 | 967.15 | 1.31 | 965.84 | 1.31 |
| | 0.36 | | 0.37 | | 0.38 | | 0.39 | | 0.40 | | 0.41 | | 0.42 | | 0.43 | | 0.44 | | 0.45 | | 0.46 | | 0.47 | |
| 37 | 979.65 | 1.32 | 978.33 | 1.30 | 977.03 | 1.30 | 975.73 | 1.29 | 974.44 | 1.29 | 973.15 | 1.29 | 971.86 | 1.29 | 970.57 | 1.29 | 969.28 | 1.29 | 967.99 | 1.30 | 966.69 | 1.32 | 965.37 | 1.32 |
| | 0.38 | | 0.38 | | 0.39 | | 0.39 | | 0.40 | | 0.41 | | 0.42 | | 0.43 | | 0.44 | | 0.46 | | 0.47 | | 0.48 | |
| 38 | 979.27 | 1.32 | 977.95 | 1.31 | 976.64 | 1.30 | 975.34 | 1.30 | 974.04 | 1.30 | 972.74 | 1.30 | 971.44 | 1.30 | 970.14 | 1.30 | 968.84 | 1.31 | 967.53 | 1.31 | 966.22 | 1.33 | 964.89 | 1.34 |
| | 0.38 | | 0.39 | | 0.39 | | 0.40 | | 0.41 | | 0.42 | | 0.43 | | 0.44 | | 0.45 | | 0.46 | | 0.48 | | 0.49 | |
| 39 | 978.89 | 1.33 | 977.56 | 1.31 | 976.25 | 1.31 | 974.94 | 1.31 | 973.63 | 1.31 | 972.32 | 1.31 | 971.01 | 1.31 | 969.70 | 1.31 | 968.39 | 1.32 | 967.07 | 1.33 | 965.74 | 1.34 | 964.40 | 1.36 |
| | 0.39 | | 0.39 | | 0.40 | | 0.41 | | 0.42 | | 0.42 | | 0.43 | | 0.45 | | 0.47 | | 0.48 | | 0.49 | | 0.50 | |
| 40 | 978.50 | 1.33 | 977.17 | 1.32 | 975.85 | 1.32 | 974.53 | 1.32 | 973.21 | 1.31 | 971.90 | 1.32 | 970.58 | 1.33 | 969.25 | 1.33 | 967.92 | 1.33 | 966.59 | 1.34 | 565.25 | 1.35 | 963.90 | 1.37 |

TABLE III (continued) International alcoholic strength in 20°C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at *t*°C corrected for air buoyancy

| | Alcoholic strength in % | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *t*° | **20** | | **21** | | **22** | | **23** | | **24** | | **25** | | **26** | | **27** | | **28** | | **29** | | **30** | | **31** | |
| 0 | 978.26 | 0.70 | 977.56 | 0.70 | 976.86 | 0.69 | 976.17 | 0.70 | 975.47 | 0.72 | 974.75 | 0.72 | 974.03 | 0.74 | 973.29 | 0.77 | 972.52 | 0.80 | 971.72 | 0.83 | 970.89 | 0.87 | 970.02 | 0.90 |
| | 0.13 | | 0.15 | | 0.17 | | 0.20 | | 0.22 | | 0.24 | | 0.27 | | 0.30 | | 0.32 | | 0.35 | | 0.37 | | 0.39 | |
| 1 | 978.13 | 0.72 | 977.41 | 0.72 | 976.69 | 0.72 | 975.97 | 0.72 | 975.25 | 0.74 | 974.51 | 0.75 | 973.76 | 0.77 | 972.99 | 0.79 | 972.20 | 0.83 | 971.37 | 0.85 | 970.52 | 0.89 | 969.63 | 0.93 |
| | 0.14 | | 0.17 | | 0.19 | | 0.21 | | 0.24 | | 0.26 | | 0.29 | | 0.31 | | 0.34 | | 0.36 | | 0.38 | | 0.41 | |
| 2 | 977.99 | 0.75 | 977.24 | 0.74 | 976.50 | 0.74 | 975.76 | 0.75 | 975.01 | 0.76 | 974.25 | 0.78 | 973.47 | 0.79 | 972.68 | 0.82 | 971.86 | 0.85 | 971.01 | 0.87 | 970.14 | 0.92 | 960.22 | 0.96 |
| | 0.16 | | 0.18 | | 0.20 | | 0.23 | | 0.25 | | 0.27 | | 0.29 | | 0.32 | | 0.34 | | 0.36 | | 0.38 | | 0.40 | |
| 3 | 977.83 | 0.77 | 977.06 | 0.76 | 976.30 | 0.77 | 975.53 | 0.77 | 974.76 | 0.78 | 973.98 | 0.80 | 973.18 | 0.82 | 972.36 | 0.84 | 971.52 | 0.87 | 970.65 | 0.89 | 969.76 | 0.94 | 968.82 | 0.98 |
| | 0.16 | | 0.18 | | 0.21 | | 0.23 | | 0.25 | | 0.28 | | 0.30 | | 0.32 | | 0.34 | | 0.36 | | 039 | | 0.42 | |
| 4 | 977.67 | 0.79 | 976.98 | 0.79 | 976.09 | 0.79 | 975.30 | 0.79 | 974.51 | 0.81 | 973.70 | 0.82 | 972.88 | 0.84 | 972.04 | 0.86 | 971.18 | 0.89 | 970.29 | 0.92 | 969.37 | 0.96 | 968.40 | 1.00 |
| | 0.18 | | 0.20 | | 0.22 | | 0.24 | | 0.26 | | 0.28 | | 0.30 | | 0.33 | | 0.35 | | 0.38 | | 0.40 | | 0.41 | |
| 5 | 977.49 | 0.81 | 976.68 | 0.81 | 975.87 | 0.81 | 975.06 | 0.81 | 974.25 | 0.83 | 973.42 | 0.84 | 972.58 | 0.86 | 971.71 | 0.88 | 970.83 | 0.92 | 969.91 | 0.94 | 968.97 | 0.98 | 967.99 | 1.02 |
| | 0.19 | | 0.21 | | 0.23 | | 0.25 | | 0.27 | | 0.30 | | 0.33 | | 0.34 | | 0.37 | | 0.39 | | 0.41 | | 0.43 | |
| 6 | 977.30 | 0.83 | 976.47 | 0.83 | 975.64 | 0.83 | 974.81 | 0.84 | 973.97 | 0.85 | 973.12 | 0.87 | 972.25 | 0.88 | 971.37 | 0.91 | 970.46 | 0.94 | 969.52 | 0.96 | 968.56 | 1.00 | 967.56 | 1.04 |
| | 0.20 | | 0.22 | | 0.24 | | 0.26 | | 0.28 | | 0.30 | | 0.32 | | 0.35 | | 0.37 | | 0.39 | | 0.41 | | 0.43 | |
| 7 | 976.10 | 0.85 | 976.25 | 0.85 | 975.40 | 0.85 | 974.55 | 0.96 | 973.69 | 0.87 | 972.82 | 0.89 | 971.93 | 0.91 | 971.02 | 0.93 | 970.09 | 0.96 | 969.13 | 0.98 | 968.15 | 1.02 | 967.13 | 1.06 |
| | 0.21 | | 0.23 | | 0.25 | | 0.27 | | 0.29 | | 0.31 | | 0.33 | | 0.35 | | 0.37 | | 0.39 | | 0.42 | | 0.44 | |
| 8 | 976.89 | 0.87 | 976.02 | 0.87 | 975.15 | 0.87 | 974.28 | 0.88 | 973.40 | 0.89 | 972.51 | 0.91 | 971.60 | 0.93 | 970.67 | 0.95 | 969.72 | 0.98 | 968.74 | 1.01 | 967.73 | 1.04 | 966.69 | 1.08 |
| | 0.22 | | 0.24 | | 0.26 | | 0.28 | | 0.30 | | 0.32 | | 0.34 | | 0.36 | | 0.39 | | 0.41 | | 0.43 | | 0.45 | |
| 9 | 976.67 | 0.89 | 975.78 | 0.89 | 974.89 | 0.89 | 974.00 | 0.90 | 973.10 | 0.91 | 972.19 | 0.93 | 971.26 | 0.95 | 970.31 | 0.98 | 969.33 | 1.00 | 968.33 | 1.03 | 967.30 | 1.06 | 966.24 | 1.09 |
| | 0.23 | | 0.25 | | 0.27 | | 0.29 | | 0.31 | | 0.33 | | 0.35 | | 0.37 | | 0.39 | | 0.41 | | 0.43 | | 0.45 | |
| 10 | 976.44 | 0.91 | 975.53 | 0.91 | 974.62 | 0.91 | 973.71 | 0.92 | 972.79 | 0.93 | 971.86 | 0.95 | 970.91 | 0.97 | 969.94 | 1.00 | 968.94 | 1.02 | 967.92 | 1.05 | 966.87 | 1.08 | 965.79 | 1.11 |
| | 0.25 | | 0.27 | | 0.28 | | 0.30 | | 0.32 | | 0.34 | | 0.36 | | 0.38 | | 0.40 | | 0.42 | | 0.44 | | 0.45 | |
| 11 | 976.11 | 0.93 | 975.26 | 0.92 | 97434 | 0.93 | 973.41 | 0.94 | 972.47 | 0.95 | 971.52 | 0.97 | 970.55 | 0.99 | 969.56 | 1.02 | 968.54 | 1.04 | 967.50 | 1.07 | 966.43 | 1.09 | 965.34 | 1.13 |
| | 0.26 | | 0.27 | | 0.29 | | 0.31 | | 0.33 | | 0.35 | | 0.37 | | 0.39 | | 0.40 | | 0.42 | | 0.44 | | 0.46 | |
| 12 | 975.93 | 0.94 | 974.99 | 0.94 | 974.05 | 0.95 | 973.10 | 0.96 | 972.14 | 0.97 | 971.17 | 0.99 | 970.18 | 1.01 | 969.17 | 1.03 | 968.14 | 1.06 | 967.08 | 1.09 | 965.99 | 1.11 | 964.88 | 1.15 |
| | 0.26 | | 0.28 | | 0.30 | | 0.32 | | 0.34 | | 0.36 | | 0.38 | | 0.39 | | 0.41 | | 0.43 | | 0.45 | | 0.47 | |
| 13 | 975.67 | 0.96 | 974.71 | 0.96 | 973.75 | 0.97 | 972.78 | 0.98 | 971.80 | 0.99 | 970.81 | 1.01 | 969.80 | 1.02 | 968.78 | 1.05 | 967.73 | 1.08 | 966.65 | 1.11 | 965.54 | 1.13 | 964.41 | 1.17 |
| | 0.27 | | 0.29 | | 0.31 | | 0.33 | | 0.35 | | 0.37 | | 0.38 | | 0.40 | | 0.42 | | 0.44 | | 0.45 | | 0.47 | |
| 14 | 975.40 | 0.98 | 974.42 | 0.98 | 973.44 | 0.99 | 972.45 | 1.00 | 971.45 | 1.01 | 970.44 | 1.02 | 969.42 | 1.04 | 968.38 | 1.07 | 967.31 | 1.10 | 966.21 | 1.12 | 965.09 | 1.15 | 963.94 | 1.19 |
| | 0.28 | | 0.30 | | 0.32 | | 0.33 | | 0.35 | | 0.37 | | 0.39 | | 0.41 | | 0.43 | | 0.45 | | 0.47 | | 0.49 | |
| 15 | 975.12 | 1.00 | 974.12 | 1.00 | 973.12 | 1.00 | 972.12 | 1.02 | 971.10 | 1.03 | 970.07 | 1.04 | 969.03 | 1.06 | 967.97 | 1.09 | 966.88 | 1.12 | 965.76 | 1.14 | 964.62 | 1.17 | 963.45 | 1.20 |
| | 0.30 | | 0.31 | | 0.33 | | 0.35 | | 0.36 | | 0.38 | | 0.40 | | 0.42 | | 0.44 | | 0.45 | | 0.47 | | 0.49 | |
| 16 | 974.82 | 1.01 | 973.81 | 1.02 | 972.79 | 1.02 | 971.77 | 1.03 | 970.74 | 1.05 | 969.69 | 1.06 | 968.63 | 1.08 | 967.55 | 1.11 | 966.44 | 1.13 | 965.31 | 1.16 | 964.15 | 1.19 | 962.96 | 1.22 |
| | 0.30 | | 0.31 | | 0.33 | | 0.35 | | 0.37 | | 0.38 | | 0.40 | | 0.42 | | 0.43 | | 0.45 | | 0.47 | | 0.49 | |
| 17 | 974.52 | 1.02 | 973.50 | 1.04 | 972.46 | 1.04 | 971.42 | 1.05 | 970.37 | 1.06 | 969.31 | 1.08 | 968.23 | 1.10 | 967.13 | 1.12 | 966.01 | 1.15 | 964.86 | 1.18 | 963.68 | 1.21 | 962.47 | 1.24 |
| | 0.31 | | 0.33 | | 0.34 | | 0.36 | | 0.38 | | 0.40 | | 0.42 | | 0.43 | | 0.45 | | 0.47 | | 0.48 | | 0.50 | |
| 18 | 974.21 | 1.04 | 973.17 | 1.05 | 972.12 | 1.06 | 971.06 | 1.07 | 969.99 | 1.08 | 968.91 | 1.10 | 967. 81 | 1.11 | 966.70 | 1.14 | 965.56 | 1.17 | 964.39 | 1.19 | 963.20 | 1.23 | 961.97 | 1.26 |
| | 0.32 | | 0.34 | | 0.35 | | 0.36 | | 0.38 | | 0.40 | | 0.42 | | 0.44 | | 0.46 | | 0.47 | | 0.49 | | 0.50 | |
| 19 | 973.89 | 1.06 | 972.83 | 1.06 | 971.77 | 1.07 | 970.70 | 1.09 | 969.61 | 1.10 | 968.51 | 1.11 | 967.39 | 1.13 | 966.26 | 1.16 | 965.10 | 1.18 | 963.92 | 1.21 | 962.71 | 1.24 | 961.47 | 1.28 |
| | 0.33 | | 0.35 | | 0.37 | | 0.39 | | 0.40 | | 0.41 | | 0.42 | | 0.45 | | 0.46 | | 0.48 | | 0.51 | | 052 | |
| 20 | 973.56 | 1.08 | 972.48 | 1.08 | 971.40 | 1.09 | 979.31 | 1.10 | 969.21 | 1.11 | 968.10 | 1.13 | 966.97 | 1.14 | 965.81 | 1.17 | 964.64 | 1.20 | 963.44 | 1.23 | 962.21 | 1.26 | 960.95 | 1.29 |

TABLE III (continued) International alcoholic strength in 20°C

Table of apparent densities of ethanol-water mixtures – Ordinary glass apparatus Densities at *t*°C corrected for air buoyancy

| t° | Alcoholic strength at % | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | | 21 | | 22 | | 23 | | 24 | | 25 | | 26 | | 27 | | 28 | | 29 | | 30 | | 31 | |
| 20 | 973.56 | 1.08 | 972.48 | 1.08 | 971.40 | 1.09 | 970.31 | 1.10 | 969.21 | 1.11 | 968.10 | 1.13 | 966.97 | 1.16 | 965.81 | 1.17 | 964.64 | 1.20 | 963.44 | 1.23 | 962.21 | 1.26 | 960.95 | 1.2 |
| | 0.35 | | 0.36 | | 0.37 | | 0.39 | | 0.40 | | 0.42 | | 0.44 | | 0.45 | | 0.47 | | 0.49 | | 0.50 | | 0.52 | |
| 21 | 973.21 | 1.09 | 972.12 | 1.09 | 971.03 | 1.11 | 969.92 | 1.11 | 968.81 | 1.13 | 967.68 | 1.15 | 966.53 | 1.17 | 965.36 | 1.19 | 964.17 | 1.22 | 962.95 | 1.24 | 961.71 | 1.28 | 960.43 | 1.3 |
| | 0.35 | | 0.36 | | 0.38 | | 0.39 | | 0.41 | | 0.43 | | 0.44 | | 0.46 | | 0.48 | | 0.49 | | 0.51 | | 0.52 | |
| 22 | 972.86 | 1.10 | 971.76 | 1.11 | 970.65 | 1.12 | 969.53 | 1.13 | 968.40 | 1.15 | 967.25 | 1.16 | 966.09 | 1.19 | 964.90 | 1.21 | 963.69 | 1.23 | 962.46 | 1.26 | 961.20 | 1.29 | 959.91 | 1.3 |
| | 0.35 | | 0.37 | | 0.39 | | 0.40 | | 0.42 | | 0.43 | | 0.45 | | 0.46 | | 0.48 | | 0.50 | | 0.52 | | 0.53 | |
| 23 | 972.51 | 1.12 | 971.39 | 1.13 | 970.26 | 1.13 | 969.13 | 1.15 | 967.98 | 1.16 | 966.82 | 1.18 | 965.64 | 1.20 | 964.44 | 1.23 | 963.21 | 1.25 | 961.96 | 1.28 | 960.68 | 1.30 | 959.38 | 1.3 |
| | 0.36 | | 0.38 | | 0.39 | | 0.41 | | 0.42 | | 0.44 | | 0.46 | | 0.48 | | 0.49 | | 0.51 | | 0.53 | | 0.54 | |
| 24 | 972.15 | 1.14 | 971.01 | 1.14 | 969.87 | 1.15 | 968.72 | 1.16 | 967.56 | 1.18 | 966.38 | 1.20 | 965.18 | 1.22 | 963.96 | 1.24 | 962.72 | 1.27 | 961 45 | 1.29 | 960.16 | 1.32 | 958.84 | 1.3 |
| | 0.38 | | 0.39 | | 0.40 | | 0.42 | | 0.44 | | 0.45 | | 0.46 | | 0.48 | | 0.50 | | 0.51 | | 0.53 | | 0.54 | |
| 25 | 971.77 | 1.15 | 970.62 | 1.15 | 969.47 | 1.17 | 968.30 | 1. 18 | 96 | 1.19 | 965.93 | 1.21 | 964.72 | 1.24 | 963.48 | 1.26 | 962.22 | 1.28 | 960.94 | 1.31 | 959.63 | 1.33 | 958.30 | 1.3 |
| | 0.38 | | 0.39 | | 0.41 | | 0.42 | | 0.44 | | 0.46 | | 0.48 | | 0.49 | | 0.50 | | 0.52 | | 0.53 | | 0.55 | |
| 26 | 971.39 | 1.16 | 970.23 | 1.17 | 969.06 | 1.18 | 967.88 | 1.20 | 966.68 | 1.21 | 965.47 | 1.23 | 964.24 | 1.25 | 962.99 | 1.27 | 961.72 | 1.30 | 960.42 | 1.32 | 959.10 | 1.35 | 957.75 | 1.3 |
| | 0.39 | | 0.41 | | 0.42 | | 0.44 | | 0.45 | | 0.46 | | 0.48 | | 0.50 | | 0.51 | | 0.52 | | 0.53 | | 0.55 | |
| 27 | 971.00 | 1.18 | 969.82 | 1.18 | 968.64 | 1.20 | 967.44 | 1.21 | 966.23 | 1.22 | 965.01 | 1.25 | 963.76 | 1.27 | 962.49 | 1.28 | 961.21 | 1.31 | 959.90 | 1.33 | 958.57 | 1.37 | 957.20 | 1.4 |
| | 0.40 | | 0.41 | | 0.43 | | 0.44 | | 0.46 | | 0.48 | | 0.49 | | 0.50 | | 0.52 | | 0.53 | | 0.55 | | 0.56 | |
| 28 | 970.60 | 1.19 | 969.41 | 1.20 | 968.21 | 1.21 | 967.00 | 1.23 | 965.77 | 1.24 | 964.53 | 1.26 | 963.27 | 1.28 | 961.99 | 1.30 | 960.69 | 1.32 | 959.37 | 1.35 | 958.02 | 1.38 | 956.64 | 1.4 |
| | 0.40 | | 0.42 | | 0.43 | | 0.45 | | 0.46 | | 0.48 | | 0.49 | | 0.50 | | 0.52 | | 0.54 | | 0.55 | | 0.56 | |
| 29 | 970.20 | 1.21 | 968.99 | 1.21 | 96778 | 1.23 | 966.55 | 1.24 | 965.31 | 1.26 | 964.05 | 1.27 | 962.78 | 1.29 | 961.49 | 1.32 | 960.17 | 1.34 | 958.83 | 1.36 | 957.47 | 1.39 | 956.08 | 1.4 |
| | 0.42 | | 0.43 | | 0.45 | | 0.46 | | 0.47 | | 0.48 | | 0.50 | | 0.52 | | 0.53 | | 0.54 | | 0.56 | | 0.58 | |
| 30 | 969.78 | 1.22 | 968.56 | 1.23 | 967.33 | 1.24 | 966.09 | 1.25 | 964.84 | 1.27 | 963.57 | 1.29 | 962.28 | 1.31 | 960.97 | 1.33 | 959.64 | 1.35 | 958.29 | 1.38 | 956.91 | 1.41 | 955.50 | 1.4 |
| | 0.42 | | 0.43 | | 0.44 | | 0.45 | | 0.47 | | 0.49 | | 0.51 | | 0.52 | | 0.53 | | 0.55 | | 0.56 | | 0.58 | |
| 31 | 969.36 | 1.23 | 968.13 | 1.24 | 966.89 | 1.25 | 965.64 | 1.27 | 964.37 | 1.29 | 963.08 | 1.31 | 961.77 | 1.32 | 960.45 | 1.34 | 959.11 | 1.37 | 957.74 | 1.39 | 956.35 | 1.43 | 954.92 | 1.4 |
| | 0.43 | | 0.45 | | 0.46 | | 0.48 | | 0.49 | | 0.50 | | 0.51 | | 0.52 | | 0.54 | | 0.56 | | 0.57 | | 0.58 | |
| 32 | 968.93 | 1.25 | 967.68 | 1.25 | 966.43 | 1.27 | 965.16 | 1.28 | 963.88 | 1.30 | 962.58 | 1.32 | 961.26 | 1.33 | 959.93 | 1.36 | 958.57 | 1.39 | 957.18 | 1.40 | 955.78 | 1.44 | 954.34 | 1.4 |
| | 0.43 | | 0.45 | | 0.47 | | 0.48 | | 0.50 | | 0.51 | | 0.52 | | 0.54 | | 0.55 | | 0.56 | | 0.58 | | 0.59 | |
| 33 | 968.50 | 1.27 | 967.23 | 1.27 | 965.96 | 1.28 | 964.68 | 1.30 | 963.38 | 1.31 | 962.07 | 1.33 | 960.74 | 1.35 | 959.39 | 1.37 | 958.02 | 1.40 | 956.62 | 1.42 | 955.20 | 1.45 | 953.75 | 1.4 |
| | 0.45 | | 0.45 | | 0.47 | | 0.49 | | 0.50 | | 0.51 | | 0.52 | | 0.54 | | 0.55 | | 0.56 | | 0.58 | | 0.60 | |
| 34 | 968.05 | 1.27 | 966.78 | 1.29 | 965.49 | 1.30 | 964 19 | 1.31 | 962.88 | 1.32 | 961.56 | 1.34 | 960.22 | 1.37 | 958.85 | 1.38 | 957.47 | 1.41 | 956.06 | 1.44 | 954.62 | 1.47 | 953.15 | 1.4 |
| | 0.45 | | 0.47 | | 0.48 | | 0.49 | | 0.50 | | 0.52 | | 0.54 | | 0.55 | | 0.57 | | 0.58 | | 0.59 | | 0.60 | |
| 35 | 967.60 | 1.29 | 996.31 | 1.30 | 965.01 | 1.31 | 963.70 | 1.32 | 962.38 | 1.34 | 961.04 | 1.36 | 959.68 | 1.38 | 958. 0 | 1.40 | 956.90 | 1.42 | 955.48 | 1.45 | 954.03 | 1.48 | 952.55 | 1.5 |
| | 0.45 | | 0.47 | | 0.48 | | 0.49 | | 0.51 | | 0.53 | | 0.54 | | | | 0.57 | | 0.59 | | 0.60 | | 061 | |
| 36 | 967.15 | 1.31 | 965.84 | 1.31 | 964.53 | 1.32 | 963.21 | 1.34 | 961.87 | 1.36 | 960.51 | 1.37 | 959.14 | 1.39 | 957.75 | 1.42 | 956.33 | 1.44 | 954.89 | 1.46 | 953.43 | 1.49 | 951.94 | 1.5 |
| | 0.46 | | 0.47 | | 0.48 | | 0.50 | | 0.52 | | 0.53 | | 0.55 | | 0.56 | | 0.57 | | 0.58 | | 0.60 | | 0.61 | |
| 37 | 966.69 | 1.32 | 965.37 | 1.32 | 964.05 | 1.34 | 962.71 | 1.36 | 961.35 | 1.37 | 959.98 | 1.39 | 958.59 | 1.40 | 957.19 | 1.43 | 955.76 | 1.45 | 954.31 | 1.48 | 952.83 | 1.50 | 951.33 | 1.5 |
| | 0.47 | | 0.48 | | 0.50 | | 0.51 | | 0.52 | | 0.54 | | 0.55 | | 0.57 | | 0.58 | | 0.59 | | 0.60 | | 0.61 | |
| 38 | 966.22 | 1.33 | 964.89 | 1.34 | 963.55 | 1.35 | 962.20 | 1.37 | 960.83 | 1.39 | 959.44 | 1.40 | 958.04 | 1.42 | 956.62 | 1.44 | 955.18 | 1.46 | 953.72 | 1.49 | 952.23 | 1.51 | 950.72 | 1.5 |
| | 0.48 | | 0.49 | | 0.51 | | 0.52 | | 0.53 | | 0.54 | | 0.56 | | 0.57 | | 0.58 | | 0.60 | | 0.61 | | 0.62 | |
| 39 | 965.74 | 1.34 | 964.40 | 1.36 | 963.04 | 1.36 | 961.68 | 1.38 | 960.30 | 1.40 | 958.90 | 1.42 | 957.48 | 1.43 | 956.05 | 1.45 | 954.60 | 1.48 | 953 12 | 1.50 | 951.62 | 1.52 | 950.10 | 1.5 |
| | 0.49 | | 0.50 | | 0.51 | | 0.53 | | 0.54 | | 055 | | 0.56 | | 058 | | 0.60 | | 0.61 | | 0.62 | | 0.64 | |
| 40 | 965.25 | 1.35 | 963.90 | 1.37 | 962.53 | 1.38 | 961.15 | 1.39 | 959.76 | 1.41 | 958.35 | 1.43 | 956.92 | 1.45 | 955.47 | 1.47 | 954.00 | 1.49 | 952.51 | 1.51 | 951.00 | 1.54 | 949.49 | 1.5 |

TABLE IV

Table giving the refractive indices of pure ethanol-water mixtures and distillates at 20ºC and the corresponding alcoholic strengths at 20ºC

| Refractive index at 20ºC | Alcoholic strength at 20ºC | | | | Refractive index at 20ºC | Alcoholic strength at 20ºC | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Water-ethanol mixtures | | Distillates | | | Water-ethanol mixtures | | Distillates | |
| 1.33628 | 6.54 | 0.25 | 6.48 | 0.26 | 1.34222 | 16.76 | 0.23 | 16.65 | 0.23 |
| 1.33642 | 6.79 | 0.26 | 6.74 | 0.26 | 1.34236 | 16.99 | 0.23 | 16.88 | 0.24 |
| 1.33656 | 7.05 | 0.25 | 7.00 | 0.27 | 1.34250 | 17.22 | 0.22 | 17.12 | 0.22 |
| 1.33670 | 7.30 | 0.28 | 7.27 | 0.27 | 1.34264 | 17.44 | 0.24 | 17.34 | 0.22 |
| 1.33685 | 7.58 | 0.25 | 7.54 | 0.25 | 1.34278 | 17.68 | 0.21 | 17.56 | 0.22 |
| 1.33699 | 7.83 | 0.26 | 7.79 | 0.26 | 1.34291 | 17.89 | 0.23 | 17.78 | 0.23 |
| 1.33713 | 8.09 | 0.25 | 8.05 | 0.25 | 1.34305 | 18.12 | 0.24 | 18.01 | 0.22 |
| 1.33727 | 8.34 | 0.28 | 8.30 | 0.26 | 1.34319 | 18.36 | 0.23 | 18.23 | 0.23 |
| 1.33742 | 8.62 | 0.25 | 8.56 | 0.25 | 1.34333 | 18.59 | 0.23 | 18.46 | 0.24 |
| 1.33756 | 8.87 | 0.25 | 8.81 | 0.25 | 1.34347 | 18.82 | 0.23 | 18.70 | 0.22 |
| 1.33770 | 9.12 | 0.24 | 9.06 | 0.24 | 1.34361 | 19.05 | 0.23 | 18.92 | 0.25 |
| 1.33784 | 9.36 | 0.27 | 9.30 | 0.25 | 1.34375 | 19.28 | 0.23 | 19.17 | 0.23 |
| 1.33799 | 9.63 | 0.24 | 9.55 | 0.26 | 1.34389 | 19.51 | 0.24 | 19.40 | 0.22 |
| 1.33813 | 9.87 | 0.25 | 9.81 | 0.24 | 1.34403 | 19.75 | 0.23 | 19.62 | 0.24 |
| 1.33827 | 10.12 | 0.23 | 10.05 | 0.24 | 1.34417 | 19.98 | 0.24 | 19.86 | 0.23 |
| 1.33841 | 10.35 | 0.26 | 10.29 | 0.25 | 1.34431 | 20.22 | 0.22 | 20.09 | 0.24 |
| 1.33856 | 10.61 | 0.25 | 10.54 | 0.24 | 1.34445 | 20.44 | 0.21 | 20.33 | 0.21 |
| 1.33870 | 10.86 | 0.24 | 10.78 | 0.24 | 1.34458 | 20.65 | 0.24 | 20.54 | 0.22 |
| 1.33884 | 11.10 | 0.23 | 11.02 | 0.24 | 1.34472 | 20.89 | 0.22 | 20.76 | 0.23 |
| 1.33898 | 11.33 | 0.24 | 11.26 | 0.24 | 1.34486 | 21.11 | 0.23 | 20.99 | 0.22 |
| 1.33912 | 11.47 | 0.24 | 11.50 | 0.24 | 1.34500 | 21.34 | 0.21 | 21.21 | 0.23 |
| 1.33926 | 11.81 | 0.24 | 11.74 | 0.24 | 1.34513 | 21.55 | 0.23 | 21.44 | 0.21 |
| 1.33940 | 12.05 | 0.25 | 11.98 | 0.24 | 1.34527 | 21.78 | 0.22 | 21.65 | 0.22 |
| 1.33955 | 12.30 | 0.23 | 12.22 | 0.24 | 1.34541 | 22.00 | 0.23 | 21.87 | 0.23 |
| 1.33969 | 12.53 | 0.23 | 12.46 | 0.23 | 1.34555 | 22.23 | 0.21 | 22.10 | 0.21 |
| 1.33983 | 12.76 | 0.24 | 12.69 | 0.23 | 1.34568 | 22.44 | 0.23 | 22.31 | 0.23 |
| 1.33997 | 13.00 | 0.23 | 12.92 | 0.23 | 1.34582 | 22.67 | 0.23 | 22.54 | 0.21 |
| 1.34011 | 13.23 | 0.24 | 13.15 | 0.25 | 1.34596 | 22.90 | 0.23 | 22.75 | 0.21 |
| 1.34025 | 13.47 | 0.23 | 13.40 | 0.22 | 1.34610 | 23.13 | 0.20 | 22.96 | 0.21 |
| 1.34039 | 13.70 | 0.23 | 13.62 | 0.24 | 1.34623 | 23.33 | 0.24 | 23.17 | 0.23 |
| 1.34053 | 13.93 | 0.23 | 13.86 | 0.23 | 1.34637 | 23.57 | 0.24 | 23.40 | 0.21 |
| 1.34067 | 14.16 | 0.25 | 14.09 | 0.23 | 1.34651 | 23.81 | 0.23 | 23.61 | 0.24 |
| 1.34081 | 14.41 | 0.25 | 14.32 | 0.25 | 1.34665 | 24.04 | 0.22 | 23.85 | 0.24 |
| 1.34096 | 14.66 | 0.23 | 14.57 | 0.24 | 1.34678 | 24.26 | 0.22 | 24.09 | 0.22 |
| 1.34110 | 14.89 | 0.24 | 14.81 | 0.25 | 1.34692 | 24.48 | 0.24 | 24.31 | 0.25 |
| 1.34124 | 15.13 | 0.23 | 15.06 | 0.22 | 1.34706 | 24.72 | 0.23 | 24.56 | 0.22 |
| 1.34138 | 15.36 | 0.23 | 15.28 | 0.22 | 1.34720 | 24.95 | 0.21 | 24.78 | 0.22 |
| 1.34152 | 15.59 | 0.24 | 15.50 | 0.24 | 1.34733 | 25.16 | 0.24 | 25.00 | 0.23 |
| 1.34166 | 15.83 | 0.23 | 15.74 | 0.22 | 1.34747 | 25.40 | 0.22 | 25.23 | 0.22 |
| 1.34180 | 16.06 | 0.23 | 15.96 | 0.23 | 1.34760 | 25.62 | 0.24 | 25.45 | 0.25 |
| 1.34194 | 16.29 | 0.23 | 16.19 | 0.22 | 1.34774 | 25.86 | 0.24 | 25.70 | 0.23 |
| 1.34208 | 16.52 | 0.24 | 16.41 | 0.24 | 1.34788 | 26.10 | 0.22 | 25.93 | 0.22 |

**Method OIV-MA-AS312-03A** Type II method

# Methanol

(Resolution Oeno 377/2009, Revised by OIV-OENO 480/2014)

## 1. Scope of application

This method is applicable to the determination of methanol in wine for concentrations between 50 and 500 mg/L.

## 2. Principle

Methanol is determined in the distillate, to which an internal standard is added, using gas chromatography with a flame ionisation detector (FID).

## 3. Reagents and materials

3.1. Type II water, according to ISO standard 3696

3.2. Ethanol: purity ≥ 96 % (CAS no. 64-17-5)

3.3. Hydrogen: minimum specifications: 99.999% purity (CAS no. 1333-74-0)

3.4. Helium: minimum specifications: 99.999% purity (CAS no. 7440-59-7)

3.5. Methanol: purity ≥ 99 % (CAS no. 67-56-1)

3.6. 4-Methyl-2-pentanol (internal standard): purity ≥ 98 % (CAS no. 108-11-2). Internal standard used in the validation.

*Note 1: Other internal standards can be used, such as:*

- 3-pentanol: purity ≥ 98% (CAS no. 584-02-1)
- 4-methyl-1-pentanol: purity ≥ 98% (CAS no. 626-89-1)
- Methyl nonanoate: purity ≥ 98% (CAS no. 1731-84-6)

3.7. Reference materials: these may be, for example, wines from laboratory proficiency tests.

3.8. Preparation of working solutions (by way of example):

*3.8.1. Approximately 10% v/v aqueous-alcoholic mixture*

This mixture should be as close as possible to the alcohol content of the wine to be analysed. Pour 100 mL of ethanol (3.2) into a 1 L calibrated flask (4.2), make up to volume with demineralised water (3.1) and mix.

*3.8.2. 10 g/L Internal standard solution*

Using an analytical balance (4.1), weigh approximately 1 g of internal standard (3.6) into a 100 mL calibrated flask (4.3) that contains around 60 mL of 10% ethanol solution (3.8.1), so as to minimise evaporation of the internal standard. Make up to volume with the ethanol solution (3.8.1) and mix.

*3.8.3. 1 g/L Internal standard solution*

Add 10 mL of the 10 g/L internal standard solution (3.8.2) using a pipette (4.8) and make up to 100 mL (4.3) using the 10% v/v hydroalcoholic mixture (3.8.1).

*3.8.4. 5 g/L Methanol stock solution*

Using an analytical balance (4.1), weigh approximately 500 mg of methanol (3.5) into a 100 mL calibrated flask (4.3) that contains about 60 mL of 10% ethanol solution (3.8.1), so as to minimise evaporation of the methanol. Make up to volume with the ethanol solution (3.8.1) and mix.

*3.8.5. Working calibration solutions*

By way of example, a method for plotting a calibration curve is outlined below.

Each solution should be prepared with the 10% aqueous-alcoholic mixture (3.8.1).

*3.8.5.1. 500 mg/L Methanol standard solution*

Add 10 mL of the 5 g/L stock solution (3.8.4) to a 100 mL calibrated flask (4.3) using a pipette (4.8) and make up to volume with the 10% v/v ethanol solution (3.8.1).

*3.8.5.1.1. 250 mg/L Methanol standard solution*

Add 10 mL of the 500 mg/L methanol solution (3.8.5.1) to a 20 mL calibrated flask (4.5) using a pipette (4.8) and make up to volume with the 10% v/v ethanol solution (3.8.1).

*3.8.5.1.2. 200 mg/L Methanol standard solution*

Add 20 mL of the 500 mg/L methanol solution (3.8.5.1) to a 50 mL calibrated flask (4.4) using a pipette (4.7) and make up to volume with the 10% v/v ethanol solution (3.8.1).

*3.8.5.1.3. 150 mg/L Methanol standard solution*

Add 6 mL of the 500 mg/L methanol solution (3.8.5.1) to a 20 mL calibrated flask (4.5) using a pipette (4.9) and make up to volume with the 10% v/v ethanol solution (3.8.1).

*3.8.5.1.4. 100 mg/L Methanol standard solution*

Add 4 mL of the 500 mg/L methanol solution (3.8.5.1) to a 20 mL calibrated flask (4.5) using a pipette (4.10) and make up to volume with the 10% v/v ethanol solution (3.8.1).

*3.8.5.1.5. 50 mg/L Methanol standard solution*

Add 2 mL of the 500 mg/L methanol solution (3.8.5.1) to a 20 mL calibrated flask (4.5) using a pipette (4.11) and make up to volume with the 10% v/v ethanol solution (3.8.1).

**4. Apparatus**

4.1. Analytical balance (1 mg precision)

4.2. 1 L Class A calibrated flasks

4.3. 100 mL Class A calibrated flasks

4.4. 50 mL Class A calibrated flasks

4.5. 20 mL Class A calibrated flasks

4.6. 10 mL Class A calibrated flasks

4.7. 20 mL Class A pipettes with two marks

4.8. 10 mL Class A pipettes with two marks

4.9. 6 mL Class A pipettes with two marks

4.10. 4 mL Class A pipettes with two marks

4.11. 2 mL Class A pipettes with two marks

4.12. 1 mL Class A pipettes with two marks or 1 mL micropipettes

4.13. Temperature-programmable gas chromatograph with a flame ionisation detector and a data processing system capable of calculating areas or measuring peak heights

4.14. Fused silica capillary column coated with a Carbowax 20M-type polar stationary phase (for example):

- Chrompack CP-wax 57 CB, 50 m x 0.32 mm x 0.45 µm
- DB-WAX 52, 30 m x 25 mm x 0.2 µm

**5. Sample preparation**

Sparkling and/or young wines must be pre-degassed, for example, by mixing 200 mL of wine in a 1 L flask. Subsequently, the samples are distilled according to the method for determining alcoholic strength by volume (OIV-MA-AS312-01A). The distillation can be carried out without adding calcium hydroxide in this case.

5.1. Addition of internal standard (by way of example)

Pour 10 mL of distillate into a 10 mL calibrated flask (4.6), add 1 mL (4.12) of internal standard solution (3.8.3) and mix.

## 6. Procedure

The calibration curve standards are treated in the same way as the samples (point 5.1).

It is recommended that the aqueous-alcoholic mixture (3.8.1) is injected at the start of the sequence in order to verify that it does not contain methanol.

*6.1. Operating conditions (as a guide):*

Carrier gas: helium or hydrogen

Carrier gas flow: 7 mL/min

Injection: split (ratio: 7:50)

Injection volume: 1 or 2 µL

Injector temperature: 200-260 ºC

Detector temperature: 220-300 ºC

Temperature programme: from 35 ºC (for 2 minutes) to 170 ºC, at 7.5 ºC/min

## 7. Calculations

Calculate the concentration of methanol ($C_i$), using the following equation:

$$C_i = \frac{C_p}{m}\left(\frac{A_i}{A_p} - b\right)$$

$A_i$ – Peak area of methanol

$A_p$ – Peak area of internal standard

$C_p$ – Concentration of internal standard

m - Slope of the calibration curve

b - Y-intercept of the calibration curve

**8. Expression of the results**

The concentration of methanol may be expressed in mg/L or in mg/100 mL absolute alcohol; in the latter case, the alcohol content by volume of the wine should be determined.

*Note 2:* mg/100 mL absolute alcohol = mg/L x 10/alcohol content by volume

**9. Precision**

The data from the international interlaboratory test is outlined in Annex A.

**10. Quality control**

Internal quality control may be carried out using certified reference materials or wines whose characteristics have been determined from a consensus (3.7). These should be prepared as for the samples (point 5). Participation in proficiency tests is recommended.

**11. Report of the results**

The results are expressed to the nearest whole number (in accordance with the uncertainty).

**12. Bibliography**

Compendium of international methods of wine and must analysis. Method OIV-MA-AS312-01A (Alcoholic strength).

**Annex A**
**Statistical results of the interlaboratory test**

**Design of validation study**

The validation study was conducted with 10 samples: 2 white wines, one dry and one sweet, 2 red wines, one of which was oaked, and 1 fortified wine (Port), including blind duplicates, according to OIV recommendations. The approximate concentration of methanol is shown in the following table.

| Sample | White wine Dry | White wine Sweet | Red wine | Red wine oaked | Fortified wine port |
|---|---|---|---|---|---|
| Methanol (mg/L) | 50 | 150* | 270 | 400* | 120 |

(*) In this particular indicated case, methanol was added to the wine to cover a greater range of concentrations. The wine was then mixed, stabilised and bottled.

**Participating laboratories:**

Samples were sent to 17 laboratories in 9 different countries.

Laboratorios Agroalimentarios, Madrid (Spain)

Estación de Viticultura y Enología de Galicia, EVEGA (Spain)

Estació de Viticultura i Enologia de Vilafranca del Penedès, (Spain)

Estación Enológica de Haro, La Rioja (Spain)

Estación de Viticultura y Enología de Galicia (Spain)

Lab. Bordeaux, Service Commun des Lab., Pessac (France)

Laboratoire d'Ile-de-France, Paris (France)

Laboratoires Inter Rhône (France)

Comité Interprof. du Vin de Champagne (CIVC) (France)

Bfr-Bundesinst. f. Risikobewertung (Germany)

Landesuntersuchungsamt Mainz (Germany)

Instituto Nacional de Vitivinicultura, Mendoza (Argentina)

ALKO Inc., Alcohol Control Lab. (ACL) (Finland)

Instituto dos Vinhos do Douro e do Porto (Portugal)

Czech Agriculture and Food Inspection Authority (CAFIA), Brno (Czech Republic) CZ

National Food Safety Office, Directorate of Oenology and Alcoholic Beverages (NÉBIH BAII), Budapest (Hungary)

Lehr- und Forschungszentrum, Klosterneuburg (Austria)

**Collaborative study on methanol**

| | Dry white | | Sweet white | | Red | | Oaked red | | Port | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Laboratory code** | A | G | B | H | C | I | D | J | E | K |
| A | 39.99 | 38.13 | 127.42 | 136.25 | 144.80 | 145.71 | 496.53 | 513.00 | 192.13 | 219.39 |
| B | 41.20 | 40.90 | 157.60 | 160.50 | 150.40 | 146.90 | 484.90 | 477.80 | 222.40 | 219.60 |
| C | 36.80 | 35.60 | 133.50 | 129.20 | 119.10 | 134.10 | 454.10 | 478.40 | 197.00 | 174.80 |
| D | 36.00 | 39.60 | **177.40** | **145.50** | 160.80 | 138.00 | **302.00** | **494.50** | 216.10 | 248.50 |
| E | **68.00** | **70.00** | 163.00 | 169.00 | 178.00 | 177.00 | 503.00 | 495.50 | 225.00 | 227.00 |
| F | 37.00 | 37.10 | 148.30 | 148.20 | 143.40 | 142.40 | 484.10 | 474.00 | 206.30 | 206.90 |
| G | 41.40 | 42.30 | 152.60 | 152.40 | 149.70 | 150.50 | 489.60 | 491.10 | 216.60 | 217.20 |
| H | 36.80 | 32.40 | 140.80 | 129.10 | 128.00 | 137.70 | 440.60 | 429.30 | 187.50 | 192.80 |
| I | 42.90 | 43.30 | 153.50 | 155.50 | 139.70 | 147.40 | 468.30 | 456.10 | 225.30 | 225.60 |
| J | 40.90 | 40.60 | 155.50 | 154.60 | 148.50 | 149.10 | 496.40 | 499.80 | 217.10 | 217.00 |
| K | 39.30 | 36.20 | **103.10** | **143.10** | 131.90 | 115.90 | **437.90** | **334.00** | 156.10 | 172.60 |
| L | 35.00 | 39.00 | 164.00 | 167.00 | 157.00 | 160.00 | 492.00 | 508.00 | 249.00 | 220.00 |
| M | 43.60 | 43.40 | 157.30 | 154.90 | 155.50 | 158.90 | 506.80 | 496.10 | 217.70 | 219.50 |
| N | 34.20 | 33.60 | 126.50 | 125.70 | 125.90 | 133.60 | 429.10 | 429.00 | 192.10 | 188.90 |
| O | 34.00 | 35.70 | 149.00 | 154.80 | 144.20 | 141.80 | 482.80 | 473.60 | 210.40 | 218.10 |
| P | 44.70 | 43.70 | 151.60 | 146.90 | 140.70 | 147.60 | 451.20 | 472.80 | 205.40 | 205.80 |
| Q | 40.70 | 38.80 | 153.00 | 149.80 | 158.20 | 153.40 | 498.20 | 497.50 | 225.50 | 217.20 |

Note: The values in bold correspond to values rejected according to the Cochran (variance outliers) and Grubbs (mean outliers) tests.

| Indicators | Dry white | Sweet white | Red | Oaked red | Port |
|---|---|---|---|---|---|
| Number of accepted laboratories | 16 | 15 | 17 | 15 | 17 |
| Number of repetitions | 2 | 2 | 2 | 2 | 2 |
| Minimum | 32.40 | 125.70 | 115.90 | 429.00 | 156.10 |
| Maximum | 44.70 | 169.00 | 178.00 | 513.00 | 249.00 |
| Repeatability variance $s_r^2$ | 2.2466 | 12.1330 | 39.0164 | 76.3567 | 105.3390 |
| Intergroup variance $s_r^2$ | 9.61893 | 146.39249 | 151.90249 | 535.61827 | 292.14282 |
| Reproducibility variance $s_r^2$ | 11.8655 | 158.5254 | 190.9189 | 611.9750 | 397.4819 |
| Overall mean | 38.90 | 148.92 | 145.76 | 478.97 | 210.37 |
| Repeatability standard deviation | 1.50 | 3.48 | 6.25 | 8.74 | 10.26 |
| r Limit | 4.242 | 9.858 | 17.677 | 24.729 | 29.046 |
| Repeatability CV | 3.9 | 2.3 | 4.3 | 1.8 | 4.9 |
| Reproducibility standard deviation | 3.44 | 12.59 | 13.82 | 24.74 | 19.94 |
| R Limit | 9.748 | 35.632 | 39.103 | 70.009 | 56.422 |
| Reproducibility CV | 8.9 | 8.5 | 9.5 | 5.2 | 9.5 |
| Horwitz RSD | 6.09 | 4.97 | 4.99 | 4.17 | 4.72 |
| Horrat r | 0.6 | 0.5 | 0.9 | 0.4 | 1.0 |
| Horwitz RSD | 9.22 | 7.53 | 7.56 | 6.32 | 7.15 |
| Horrat R | 1.0 | **1.1** | **1.3** | 0.8 | **1.3** |

**According to the Horrat values, the repeatability and reproducibility of the method are acceptable**

**Z-scores obtained by the participants: of the 85 Z-scores, 3 are unsatisfactory and 4 are questionable**

| Laboratory code | Z-score Dry white wine | Z-score Sweet white wine | Z-score Red wine | Z-score Oaked red wine | Z-score Port |
|---|---|---|---|---|---|
| A | 0.05 | -1.36 | -0.04 | 1.04 | -0.23 |
| B | 0.62 | 0.80 | 0.21 | 0.10 | 0.53 |
| C | -0.78 | -1.40 | -1.39 | -0.51 | -1.23 |
| D | -0.32 | 1.00 | 0.26 | **-3.26** | 1.10 |
| E | **8.74** | 1.36 | **2.30** | 0.81 | 0.78 |
| F | -0.54 | -0.05 | -0.21 | 0.00 | -0.19 |
| G | 0.86 | 0.28 | 0.31 | 0.46 | 0.33 |
| H | -1.25 | -1.11 | -0.93 | -1.78 | -1.01 |
| I | 1.22 | 0.44 | -0.16 | -0.68 | 0.76 |
| J | 0.54 | 0.49 | 0.22 | 0.77 | 0.34 |
| K | -0.33 | **-2.05** | -1.58 | **-3.76** | **-2.31** |
| L | -0.55 | 1.32 | 0.92 | 0.85 | 1.21 |
| M | 1.34 | 0.57 | 0.83 | 0.91 | 0.41 |
| N | -1.45 | -1.81 | -1.16 | **-2.02** | -1.00 |
| O | -1.18 | 0.24 | -0.20 | -0.03 | 0.19 |
| P | 1.54 | 0.03 | -0.12 | -0.69 | -0.24 |
| Q | 0.25 | 0.20 | 0.73 | 0.76 | 0.55 |

**Method OIV-MA-AS312-03B** Type IV method

# Methanol

(Resolution Oeno 377/2009)

## 1. Principle

The wine distillate is diluted to an ethanol content of 5% (*v/v*). Methanol is oxidized to formaldehyde (methanol) by potassium permanganate (acidified by phosphoric acid). The amount of formaldehyde is determined by the violet color formed by the reaction of chromotropic acid in a sulfuric medium. The intensity of the color is determined by spectrophotometry at 575 nm.

## 2.. Method

2.1 *Reagents*

2.1.1 Chromotropic Acid

4,5–Dihydroxy–2,7–naphthalenedisulfonic acid, ($C_{10}H_8O_8S_2 \cdot 2H_2O$), (MW 356.34 g)

White or light brown powder, soluble in water. The *di*-sodium salt of this acid that forms a yellow or light brown substance, and is very soluble in water can also be used.

*Purification* - The chromotropic acid must be pure and give a negligible color in the blank tests of reagents prepared with it. If this is not the case, proceed with purification using the following procedure:

Dissolve 10 g of chromotropic acid or its salt in 25 mL of distilled water. If the salt has been used, add 2 mL of concentrated sulfuric acid ($\rho_{20}$ = 1.84 g/mL) to release the acid. Add 50 mL of methanol, heat to boiling and filter. Add 100 mL of *iso*-propanol to precipitate the pure crystals of chromotropic acid, allow the crystals formed to drain and cold dry.

*Reaction* - The addition of ferric chloride (1 drop) to 10 mL of a 0.1 g/L solution should give a green color.

*Sensitivity test* - Dilute 0.5 mL of analytical grade formaldehyde to 1 L with water. To 5 mL of 0.05%chromotropic acid solution in sulfuric acid, 75% (*v/v*), add 0.1 mL of formaldehyde solution and heat to 70°C for 20 min. A violet color should be produced.

2.1.2 Chromotropic acid solution, 0.05%, in sulfuric acid solution, 75% (*v/v*).
Dissolve 50 mg chromotropic acid (2.1.1) or its sodium salt in 35 mL of distilled water. Cool this solution with iced water and add carefully 75 mL of concentrated sulfuric acid ($\rho_{20}$= 1.84 g/mL) in small portions, while mixing.
This solution must be prepared just before use.

2.1.3 Methanol, 5 g/L, standard solution in alcohol 5%, (*v/v*)

| | |
|---|---|
| Pure methanol ($E_{760}$ = 64.7 ± 0.2) ..... | 0.5 g |
| Absolute alcohol (without methanol) ..... | 50 mL |
| Distilled water to ..... | 1 liter |

2.1.4 Dilution solution

| | |
|---|---|
| Absolute alcohol (without methanol) ..... | 50 mL |
| Distilled water to ..... | 1 liter |

2.1.5 Phosphoric acid solution, 50% (*m/v*)

2.1.6 Potassium permanganate solution, 5% (*m/v*)

2.1.7 Neutral sodium sulfite solution, 2% (*m/v*)

Solution rapidly oxidizes in air. Determine the exact strength by iodometric titration.

2.2 *Procedure*

Dilute the wine distillate (see chapter *Alcoholic strength*) to reduce the alcoholic strength to 5% vol.

Into a ground-glass stopper test tube place 0.5 mL of the diluted distillate, add 1 drop of phosphoric acid, 50%, 2 drops of potassium permanganate solution, 5%, shake and allow to stand for 10 minutes.

Decolorize the permanganate by adding a few drops, usually 4, of neutralized 2% sodium sulfite solution, (avoid any excess). Add 5 mL 0.05% chromotropic acid. Place in a water bath at 70°C for 20 min. Allow to cool.

Determine the absorbance $A_S$, at 570 nm, the zero of the absorbance being adjusted on the control sample prepared with 0.5 mL of the dilution solution.

*Calibration curve*

In a series of 50 mL volumetric spherical flasks, place 2.5, 5, 10, 15, 20, 25 mL respectively of the methanol, 0.5 g/L, solution in ethanol 5%. Make up to volume with a 5% ethanol solution. These solutions contain 25, 50, 100, 150, 200 and 250 mg of methanol per liter.

Treat simultaneously 0.5 mL of the dilution solution and 0.5 mL of each of the standard solutions, with the same technique as used to bring the wine distillate to an ethanol concentration of 5%.

Determine the absorbance of these solutions at 570 nm, in the conditions described above.

The graph of absorbance of the standard solutions as a function of concentration should be a straight line.

2.3 *Calculations*

Determine the methanol concentration, expressed in mg/L of the wine distillate brought to an alcoholic strength of 5% vol., and plotted as $A_S$ on the calibration line.

Express the concentration in wine in mg/L taking into account the dilution performed to bring the strength to 5% vol.

**Method OIV-MA-AS312-04** Type IV method

# Glycerol and 2,3-Butanediol

(Resolution Oeno 377/2009)

## 1. Principle

Glycerol and 2,3-butanediol are oxidized by periodic acid after treatment through an anion exchange resin column to fix the sugars and a large proportion of mannitol and sorbitol. The product obtained by the action of phloroglucinol on formaldehyde (by glycerol oxidation) is determined colorimetrically at 480 nm. The product formed by the action of piperidine solution and sodium nitroferricyanide solution with the ethanol (by oxidation of 2,3-butanediol) is determined colorimetrically at 570 nm.

## 2. Apparatus

2.1 Glass column 300 mm long and approximately 10-11 mm internal diameter fitted with a stopcock.

2.2 Spectrophotometer allowing measurement to be made between 300 and 700 nm and glass cells with optical path length of 1 cm.

## 3. Reagents

3.1 Glycerol, $C_3H_8O_3$

3.2 2,3-Butanediol, $C_4H_{10}O_2$

3.3 A strongly basic anion exchange resin e.g. anion exchange resin of Merck strength III or Amberlite IRA 400.

3.4 Polyvinylpolypyrrolidone (PVPP) (see *International Oenological Codex*).

3.5 Periodic acid, 0.1 M, in sulfuric acid, 0.05 M.

Weigh 10.696 g of sodium periodate, $NaIO_4$, place into a 500 mL volumetric flask, dissolve with 50 mL of sulfuric acid, 0.5 M, and make up to 500 mL with distilled water.

3.6 Periodic acid, 0.05 M, in sulfuric acid, 0.025 M.

The above solution (3.5) is diluted 1 : 1 with distilled water.

3.7 Sulfuric acid, 0.5 M.

3.8 Sodium hydroxide solution, 1 M.

3.9 Sodium hydroxide solution, 5% (*m/v*).

3.10 Ethanol, 96% (*v/v*).

3.11 Phloroglucinol solution, 2% (*m/v*), to be prepared fresh daily.

3.12 Sodium acetate solution, 27% (*m/v*), prepared from anhydrous sodium acetate, $CH_3COONa$.

3.13 Sodium nitroferricyanide solution, $Na_2Fe(CN)_5NO.2H_2O$, 2% (*m/v*), to be prepared fresh daily

3.14 Piperidine solution, $C_5H_{11}N$ 25% (*v/v*), to be prepared fresh daily.

3.15 Standard glycerol solution

Prepare a solution containing 250 g glycerol in 100 mL and determine the glycerol content by the enzymatic or periodimetric method (see section 6).

Prepare the standard glycerol solution as follows: weigh in a 100 mL volumetric flask a mass "*m*" corresponding to 250 mg of pure glycerol, make up to 100 mL with water.

3.16 Standard 2,3-butanediol solution

Prepare a solution containing 250 mg of 2,3-butanediol sample in 100 mL and determine the 2,3-butanediol content by the periodimetric method (see section 6).

Prepare the standard solution of 2,3-butanediol by weighing in a 100 mL volumetric flask a mass "*m*" corresponding to 250 mg of pure 2,3-butanediol; make up to 100 mL with water.

3.17 Alkaline copper solution:

Copper Solution A

Copper sulfate, $CuSO_4.5H_20$ ..... 40 g
Sulfuric acid (r=1.84 g/mL) ..... 2 mL
Make up to 1000 mL with water

Alkaline tartaric solution B

Potassium sodium tartrate tetrahydrate
$KNaC_4H_4O_6.4H_2O$ ..... 200 g
Sodium hydroxide ..... 150 g

Make up to 1000 mL with water

The copper alkaline solution is obtained by mixing solution A and B in equal quantities at the time of use.

**4. Procedure**

4.1 *Preparation of an anion exchange column*

The anion exchange resin ($Cl^-$) must be kept in a flask filled with decarbonated distilled water.

Put 30 mL of anion exchange resin (3.3) in the column (2.1), place a wool plug on the top of the column to stop air contact with the resin. Pass 150 mL of 5% sodium hydroxide (3.9) through the column at a flow rate of 3.5 to 5 mL per minute followed by a similar quantity of decarbonated distilled water at the

same flow rate until the eluent is neutral or slightly alkaline to phenolphthalein. The resin is then ready for use.

The anion exchange resin can only be used once. It can be regenerated by treating with 5% hydrochloric acid for a few hours and then rinsed with water until free of chloride. (Check for absence of chloride).

4.2*Preparation of sample*

The wine sample is diluted 10/50.

In case of strongly colored wines, first decolorize with PVPP (3.4): place 10 mL wine in a 50 mL volumetric flask, dilute with water (20 mL) and add 300 mg of PVPP (3.4). Leave for 20 min. stirring occasionally, make to the mark and filter through fluted filter paper. Take 10 mL of diluted wine (treated or untreated with PVPP) and place on the anion exchange column. Allow to drain, drop by drop, at flow rate not exceeding 2 mL per minute. When the level of diluted wine reaches 5-10 mm above the glass wool plug, add decarbonated distilled water to bring the volume of the eluent to 100 mL at a flow rate 2-3 mL per minute. The eluate must be free of sugars. To ensure this, boil rapidly 5 mL of eluate with 5 mL of alkaline copper solution (3.17). There should not be any discoloration or precipitation.

4.3 *Determination of glycerol*

4.3.1 Photometric determination

Place into a 100 mL conical ground necked vessel:

10 mL eluate and add successively

10 mL distilled water and

10 mL periodic acid solution, 0.05 M (3.6).

Stir carefully; leave exactly 5 min. for the oxidation to take place. Add 10 mL sodium hydroxide solution, (3.8), and 5 mL 96% ethanol (*v/v*) (3.10).

Stir after each addition, then add 10 mL phloroglucinol solution (3.11)

Mix rapidly and transfer the solution into a 1 cm cell. The purple coloration is obtained very quickly. Its intensity reaches a maximum after 50 to 60 seconds, then decreases. Note the maximal absorbance. The measurement is carried out at 480 nm using air as a reference.

4.3.2 Preparation of the calibration curve

Pipette into 100 mL volumetric flasks:

3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 mL glycerol standard solution (3.15) and make up to volume with distilled water.

These solutions correspond, according to the conditions in 4.2, to the following concentrations:

3.75, 5.00, 6.25, 7.50, 8.75 and 10.00 g/L of glycerol.

Proceed with the determination as described in 4.3.1, replacing the eluate by the same volume of each of the standard solutions.

4.4 *Determination of 2,3-butanediol*

4.4.1 Photometric determination

Place into a conical 100 mL ground stoppered vessel:

20 mL eluate and add successively

5 mL sodium acetate solution (3.12) and

5 mL 0.1 M periodic acid solution (3.5).

Stir to mix, leave for 2 min exactly for oxidation to take place

Add:

5 mL sodium nitroferricyanide solution (3.13) and

5 mL piperidine solution (3.14).

Transfer the solution into a 1 cm cell. The purple color is obtained very rapidly; its intensity reaches a maximum after 30-40 sec then diminishes. Note the maximal absorbance. The measurement is carried out at 570 nm using air as a reference.

4.4.2 Preparation of the calibration curve

Put 10.0 mL of 2,3-butanediol standard solution (3.16) in a 100 mL volumetric flask and make up with distilled water. From this solution prepare standard solutions by pipetting respectively into 100 mL volumetric flasks:

2.0, 4.0, 6.0, 8.0 and 10.0 mL, make up with distilled water

These solutions correspond, according to the conditions described in 4.2 to the following concentrations: 0.25, 0.50, 0.74, 1.00 and 1.25 g/L of 2,3-butanediol.

Proceed with the determination as described in 4.4.1, replacing the eluate by the same volume of each of the standard solutions. The straight line of the calibration graph should pass through the origin.

## 5. Calculation and expression of results

5.1 *Glycerol*

5.1.1 Method of calculation

Read the glycerol content from the calibration curve. The result is expressed in g/L to one decimal place.

5.1.2 Repeatability

5.1.3 Reproducibility

5.2 *2,3-Butanediol*

5.2.1 Method of calculation

Read the 2,3-butanediol content on the calibration. The result is expressed in g/L to two decimal places.

5.2.2 Repeatability

5.2.3 Reproducibility

**6. Glycerol and 2,3-butanediol by periodimetric titration**

6.1 *Reagents*

6.1.1 Sodium hydroxide solution, 1 M.

6.1.2 Sulfuric acid solution, 0.5 M.

6.1.3 Periodic acid solution, 0.025 M.

6.1.4 Sodium bicarbonate solution, $NaHCO_3$, 8% (*m/v*).

6.1.5 Sodium arsenate solution, 0.025 M.

In a 1000 mL volumetric flask, dissolve 2.473 g of arsenic III oxide, $As_2O_3$, with 30 mL 1 M sodium hydroxide, (6.1.1) add 35 mL 0.5 M sulfuric acid (6.1.2), and make up to the mark with distilled water.

6.1.6 Iodine solution, 0.025 M.

6.1.7 Potassium iodide, 10% (*m/v*).

6.1.8 Starch paste, 2% (*m/v*).

6.2 *Procedure*

In a 300 mL conical flask add:
5 mL glycerol sample solution (3.15)
45 mL distilled water
or
25 mL 2,3-butanediol sample solution (3.16)
25 mL distilled water

Add:
20 mL periodic acid, 0.025 M (6.1.3), leave for 15 min, shaking from time to time
10-20 mL sodium bicarbonate solution (6.1.4)
20 mL sodium arsenate solution (6.1.5)

Leave for 15 min shaking from time to time and add:
5 mL potassium iodide solution (6.1.7)
2 mL starch paste (6.1.8)

Titrate the excess sodium arsenate with 0.025 M iodine solution (6.1.6).
Prepare at the same time a blank test using 50 mL distilled water and the same quantity of reagents.

6.3 *Method of calculation*

6.3.1 Glycerol

1 mL periodic acid, 0.025 M, oxidizes 1.151 mg glycerol.

The glycerol content in g/L of the glycerol standard solution (3.15) is:

$$G = \frac{(X - B) \times 1{,}151}{\alpha}$$

The percentage of glycerol used in the standard glycerol solution (3.15) is:

$$\frac{G}{2{,}5} \times 100$$

X = mL of the iodine solution, 0.025 M, used up by the standard solution (3.15)

B = mL of the iodine solution, 0.025 M, in the blank test

*a* = mL of the solution test (3.15) (equal to 5 mL)

6.3.2 2,3-Butanediol

1 mL periodic acid, 0.025 M, oxidizes 2.253 mg of 2,3-butanediol.

The 2,3-butanediol content in g/L of the 2,3-butanediol standard solution (3.16) is:

$$BD = \frac{(X' - B') \times 2{,}253}{b}$$

The percentage of 2,3-butanediol used in the 2,3-butanediol standard solution (3.2) is:

$$\frac{BD}{2{,}5} \times 100$$

X' = mL of iodine solution, 0.025 M, used up by the standard solution (3.16)

B' = mL of iodine solution, 0.025 M, used in blank test

*b* = mL of the solution test (3.16) (equal to 25 mL)

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**Method OIV-MA-AS312-05** Type IV method

# Glycerol

(Resolution Oeno 377/2009)

## 1 Principle

The glycerokinase (GK) catalyses the phosphorylation of glycerol to glycerol-3-phosphate by adenosine-5'-triphosphate (ATP) (1):

(1) $$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate + ADP}$$

The adenosine-5'-diphosphate (ADP) is then converted into ATP by phosphoenol-pyruvate (PEP) in presence of pyruvate-kinase (PK) with pyruvate (2) formation:

(2) $$\text{ADP + PEP} \xrightarrow{\text{PK}} \text{ATP + pyruvate}$$

Pyruvate is converted into lactate by reduced nicotinamide-adenine dinucleotide NADH) in presence of lactate-dehydrogenase (LDH) (3):

(3) $$\text{pyruvate + NADH + H}^+ \xrightarrow{\text{LDH}} \text{lactate + NAD}^+$$

The quantity of $NAD^+$ formed during the reaction is proportional to the quantity of glycerol. The NADH oxidization is measured by the decrease of its extinction at wavelengths of 334 nm, 340 nm or 365 nm.

## 2. Apparatus

2.1 Spectrophotometer enabling measurements to be made at 340 nm, at which absorption by NADH is at a maximum.

If not available, a photometer using a source with a discontinuous spectrum enabling measurements to be made at 334 nm or at 365 nm, may be used.

2.2 Glass cells of 1 cm optical path length or single-use cells.

2.3 Micropipettes enabling the selection of volumes from 0.02 to 2 mL.

## 3. Reagents

3.1 Buffer solution (0.75 M glycylglycine, $Mg^{2+}$ $10^{-3}$ M, pH = 7.4)

Dissolve 10.0 g of glycylglycine and 0.25 g of magnesium sulfate ($MgSO_4 \cdot 7 H_2O$) in about 80 mL of double distilled water, add about 2.4 mL of 5 M sodium hydroxide solution to obtain a pH of 7.4 and make up to 100 mL. This buffer solution may be kept for 3 months at + 4°C.

3.2 (NADH $8.2.10^{-3}$ M, ATP $33.10^{-3}$ M, PEP $46.10^{-3}$ M)

Dissolve:

42 mg of nicotinamide-adenine-dinucleotide reduced - $Na_2$
120 mg of adenosine-5'-triphosphate, $Na_2H_2$
60 mg of phosphoenol pyruvate, Na and
300 mg of sodium bicarbonate ($NaHCO_3$)
in 6 mL of double distilled water.
This may be kept for 2-3 days at + 4°C.

3.3 Pyruvate-kinase/lactate-dehydrogenase (PK/LDH)

(PK 3 mg /mL, LDH 1 mg /mL)
Use the suspension without diluting it.
This may be kept for a year at about + 4°C.

3.4 Glycerokinase (GK 1 mg/mL)

The suspension may be kept for a year at about + 4°C.

*Note:* All reagents needed for the above are available commercially.

**4. Preparation of sample**

The determination of glycerol is generally made directly on the wine, which is diluted with double distilled water so that the resulting glycerol concentration is between 30 and 500 mg/L. Wine diluted 2 /100 is usually sufficient.

**5. Procedure**

With spectrophotometer adjusted to 340 nm wavelength the absorbance measurements are made in the glass cells with optical path length of 1 cm, with air as a reference.

Into cells with 1 cm optical paths place the following:

| | Reference cell | Sample cell |
|---|---|---|
| Solution 3.1 | 1.00 Ml | 1.00 mL |
| Solution 3.2 | 0.10 mL | 0.10 mL |
| Sample to be measured | - 0.10 mL | |
| Water | 2.00 mL | 1.90 mL |
| Suspension 3.3 | 0.01 mL | 0.01 mL |

Mix, and after about 5 min, read the absorbances ($A_1$). Start the reaction by adding:

| | | |
|---|---|---|
| Suspension 3.4 ……. | 0.01 mL | 0.01 mL |

Mix, wait until the end of the reaction (5 to 10 min), read the absorbance of the solutions ($A_2$). Read the absorbance after 10 min and check every 2 min until the absorbance is constant for 2 min.
Calculate the differences in the absorbance:

$$A_2 - A_1$$

for the reference and sample cells.

Calculate the differences in absorbance between the reference cell ($\Delta A_T$) and the sample cell ($\Delta A_D$) using the equation:

$$\Delta A = \Delta A_D - \Delta A_T$$

**6. Expression of results**

6.1 *Calculation*

The general formula for calculating the concentration is:

$$C = \frac{V \ x \ PM}{\varepsilon \ x \ d \ x \ v \ x \ 1000} X \Delta A$$

*V* = volume of the test in mL (3.12 mL)
*v* = volume of the sample mL (0.1 mL)
*PM* = molecular weight of the substance to be determined (glycerol = 92.1)
*d* = optical path length of the cell (1 cm)
ε = absorption coefficient of NADH at 340 nm

$$\varepsilon = 6.3 \ (mmol^{-1} \times l \times cm^{-1})$$

When using the amounts given in brackets this reduces to:

$$C = 0.456 \times \Delta A \times F$$

F = dilution factor

*Note*:
— Measurement at 334 nm, ε = 6.2 ($mmol^{-1}$ x *l* x $cm^{-1}$)

$$C = 0.463 \times \Delta A \times F$$

— Measurement at 365 nm, ε = 3.4 ($mmol^{-1}$ x *l* x $cm^{-1}$)

$$C = 0.845 \times \Delta A \times F$$

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**Method OIV-MA-AS312-06** Type II method

# Determination by isotope ratio mass spectometry $^{13}C/^{12}C$ of wine ethanol or that obtained through the fermentation of musts, concentrated musts or grape sugar.

(Resolution Oeno 17/2001)

## 1. FIELD OF APPLICATION

The method enables the measuring of isotope ratio $^{13}C/^{12}C$ of ethanol in wine and ethanol obtained after fermentation of products derived from the vine (musts, concentrated musts, grape sugar).

## 2. REFERENCE STANDARDS

ISO 5725 :1994 «Accuracy (trueness and precision) of measurement methods and results: Basic method for the determination of repeatability and reproducibility of a standard measurement method»

V-PDB : Vienna-Pee-Dee Belemnite ($R_{PDB}$ = 0.0112372).

Method OIV «Detection of enriching musts, concentrated musts, grape sugar and wine by application of nuclear magnetic deuterium resonance (RMN-FINS): »

## 3. TERMS AND DEFINITIONS

$^{13}C/^{12}C$ : Carbon 13 and carbon 12 isotope ratio for a given sample

$\delta^{13}C$ : Carbon 13 contents ($^{13}C$) expressed in parts per 1000 (‰)

RMN-FINS : Fractioning the specific natural isotope studied by nuclear magnetic resonance

V-PDB : Vienna-Pee-Dee Belemnite. or PDB, is the main reference for measuring natural variations of carbon 13 isotopic contents. Calcium carbonate comes from a Cretaceous belemnite from the Pee Dee formation in South Carolina (USA). Its isotopic ratio $^{13}C/^{12}C$ or $R_{PDB}$

is $R_{PDB}$ = 0.0112372. PDB reserves have been exhausted for a long time, but it has natural variations of Carbon 13 isotopic contents. Reference material is calibrated based on this content and is available at the International Agency of Atomic Energy (IAEA) in Vienna (Austria). The isotopic indications of naturally occurring carbon 13 are expressed by V-PDB, as is the custom.

m/z: Mass to charge ratio

## 4. PRINCIPLE

During photosynthesis, the assimilation of carbonic gas by plants occurs according to 2 principle types of metabolism that are: metabolism $C_3$ (Calvin cycle) and $C_4$ (Hatch and Slack). These two means of photosynthesis present a different type of isotope fractionation. Products, such as sugars and alcohol, derived from $C_4$ plants and fermentation, have higher levels of Carbon 13 than from $C_3$ .plants. Most plants, such as vines and sugar beets belong to the $C_3$.group. Sugar cane and corn belong to the group $C_4$. Measuring the carbon 13 content enables the detection and the quantification of $C_4$ (sugar cane or corn isoglucose) origin sugars which are added to products derived from grapes (grape musts, wines). The combined information on carbon 13 content and information obtained from RMN-FINS enable the quantification of mixed sugars added or alcohol of plant origin $C_3$ and $C_4$.

The carbon 13 content is determined by carbonic gas resulting from the complete combustion of the sample. The abundance of main mass isotopomers 44 ($^{12}C^{16}O_2$), 45 ($^{13}C^{16}O_2$ et $^{12}C^{17}O^{16}O$) and 46 ($^{12}C^{16}O^{18}O$), resulting from different possible combinations of isotopes $^{18}O$, $^{17}O$, $^{16}O$, $^{13}C$ et $^{12}C$, are determined from ionic currents measured by three different collectors of mass isotopic spectrometers. The contributions of isotopomers $^{13}C^{17}O^{16}O$ et $^{12}C^{17}O_2$ are sometimes neglected because of their small presence. The ionic current for $m/z = 45$ is corrected for the contribution of $^{12}C^{17}O^{16}O$ which is calculated according to the current intensity measured for $m/z = 46$ while considering the relative abundance of $^{18}O$ et $^{17}O$ (Craig adjustment). The comparison with the calibrated reference and the international reference V-PDB enable the calculation of carbon 13 content on a relative scale of $\delta^{13}C$.

## 5. REAGENTS

The material and the consumables depend on the apparatus (6) used by the laboratory. The systems generally used are based on elementary analysers. These systems can be equipped to introduce the samples placed in sealed metal capsules or for the injection of liquid samples through a septum using a syringe.

Depending on the type of instrument used, the reference material, reagents, and consumables can be used:

- Reference material

available from the IAEA:

| Name | Material | $\delta^{13}C$ versus V-PDB (9) |
|---|---|---|
| - IAEA-CH-6 | saccharose | -10.4 ‰ |
| -IAEA-CH-7 | polyethylene | -31.8 ‰ |
| - NBS22 | oil | -29.7 ‰ |
| - USGS24 | graphite | -16.1 ‰ |

available from the IRMM de Geel (B) (Institut des Matériaux et Mesures de Référence) :

| Name | Material | $\delta^{13}C$ versus V-PDB (9) |
|---|---|---|
| - CRM 656 | Wine alcohol | -26.93 ‰ |
| - CRM 657 | glucose | -10.75 ‰ |
| - CRM 660 | hydroalcoholic solution (TAV 12%) | -26.72 ‰ |

Standard work sample with a known calibrated $^{13}C/^{12}C$ ratio with international reference materials.

A standard list of consumables established for continuous flow systems follows here under :

- Helium for analysis (CAS 07440-59-7)
- Oxygen for analysis (CAS 07782-44-7)
- Carbon dioxide for analysis, used as a secondary reference gas for the content of carbon

  13 (CAS 00124-38-9)
- Oxidation reagent for the oven and the combustion system as follows:

  copper oxide (II) for elementary analyzed (CAS 1317-38-0)
- Drying agent to eliminate water produced by combustion. For example:

  anhydrone for elementary analysis (magnesium perchlorate) (CAS 10034-81-8).

*This is not necessary for apparatuses equipped with a water elimination system by cryo-trapping or through selective permeable capillaries.*

## 6. APPARATUS AND MATERIAL

6.*1.. Isotope ratio mass spectometry (IRMS)*

Isotope ratio mass spectometry (IRMS) enables the determination the relative contents of $^{13}C$ of $CO_2$ gas naturally occurring with an internal accuracy of 0.05‰ or expressed in relative value (9). Internal accuracy here is defined as the difference between 2 measurements of the same sample of $CO_2$. the mass spectrometer used to measure isotope ratios is generally equipped with a triple collector to simultaneously measure m/z = 44, 45 and 46 intensities. The isotope ratio mass spectrometry must either be equipped with a double introduction system to alternately measure the unknown sample and a reference sample, or use an integrated system that carries out quantitative combustion on samples and separates the carbon dioxide from the other combustion products before measuring the mass spectrum.

*6.2. Combustion apparatus*

Combustion apparatus able to quantitively convert ethanol in carbon dioxide and able of eliminating all other combustion products including water, without any isotopic fractioning. The apparatus can be either an integrated continual flow system integrated with mass spectometry (6.2.1), of an autonomous combustion system (6.2.2). The apparatus must be as precise as indicated in (11).

6.2.1. Continual flow system

These are made up by an elementary analyser, either by chromatography in gaseous state equipped with an online combustion system.

The following laboratory material is used for systems equipped for the introduction of samples contained in metallic capsules :

- volumetric micropipette with appropriate cones
- scale with μg accuracy or better
- pliers for encapsulation
- tin capsules for liquid samples
- tin capsules for solid samples

The following laboratory material is needed when using an elementary analyser equipped with a liquid injector or in the case of a preparation system for combustion chromatography:

- syringe for liquids
- flasks equipped with sealed closing system and inert septa

The laboratory material indicated in the lists are examples that are susceptible of being replaced by other equivalent performance material depending on the type of combustion apparatus and of mass spectometry used by the laboratory.

6.2.2 Autonomous preparation system

The samples of carbon dioxide resulting from the combustion of samples to be analyzed and the reference sample are collected in bulbs which are then put in a double entry spectometry system to carry out isotopic analyses. Several combustion apparatuses described in writings can be used:

- Closed combustion system filled with oxygen gas circulating
- Elementary analyser with helium and oxygen flow
- Bulb sealed in glass filled with copper oxide (II) used as an oxidation agent

## 7. PREPARATION OF SAMPLES FOR TRIALS

Ethanol must be extracted from wine before isotopic testing. This is carried out by distilling wine as described in §3.1 using the RMN-FINS method.

Sugars must be fermented in ethanol first as described in the RMN-FINS method in the case of grape musts, concentrated rectified grape musts (grape sugar).

## 8. PROCEDURE

All preparation steps must be carried out without any significant ethanol loss through evaporation, which would change the isotopic composition of the sample.

The description that follows makes reference to the procedure generally used for ethanol sample combustion using commercial automatic combustion systems. All other methods, ensuring that ethanol samples are converted by quantity in carbon dioxide without the evaporation of ethanol, can use the preparation of carbon dioxide for isotopic analyses. An experimental procedure based on the usage of an elementary analyser:

a) Placing the samples in capsules

- use capsules, a tweezers and a clean preparation tray
- take an appropriate sized capsule using a tweezers
- introduce an appropriate amount of liquid into the capsule using a micropipette

Note: 3.84 mg of absolute ethanol or 4.17 mg of distillate with an alcohol content of 92% m/m are necessary to obtain 2 mg of carbon. The appropriate quantity of distillate must be calculated in the same way according to the quantity of carbon necessary based on the mass spectometry instruments' sensitivity.

- close the capsule with the tweezers.
- each capsule must be completely sealed. If not, it must be discarded and the capsule must be repaired.
- two capsules must be prepared for every sample
- place the capsules in an appropriate place on the tray elementary analyser sample. Every capsule must be carefully identified in order by number .
- systematically place capsules containing work references at the beginning and the end of the sample series
- regularly insert a check sample in the sample series.

b) check and adjust the elementary analysis and mass spectometry instruments

- adjust the temperature of the elementary analyzer ovens and the helium and oxygen gas flow for an optimal combustion of the sample;
- check the elementary analysis system and the mass spectometry system for leaks (for example by checking the ionic current where m/z = 28 corresponding to $N_2$.);
- adjust the mass spectrometer to measure the intensities of ionic current where m/z = 44, 45 and 46;
- check the system using known reference samples before starting to measure the samples.

c) To carry out a series of measurements

The samples that are placed under the elementary or chromatography are introduced successively. The carbon dioxide for each sample combustion is eluted towards the mass spectrometer which measures the ionic current. The interface computer records the ionic current intensities and calculates the values δ for each sample (9).

## 9. CALCULATION

The objective of the method is to measure the isotopic ratio $^{13}C/^{12}C$ ethanol extract from wine or from products derived from grapes following fermentation. The isotopic ratio $^{13}C/^{12}C$ can be expressed by its deviation compared to the reference work. Carbon 13 ($\delta\ ^{13}C$)'s isotopic ratio is calculated on a delta scale per thousand

by comparing the results obtained for the sample to be measured to the reference work calibrated before based on the primary international reference (V-PDB). The values $\delta\ ^{13}C$ are expressed compared to reference work:

$$\delta^{13}C_{ech/ref}‰ = 1000 \times (R_{ech}-R_{ref})/R_{ref}$$

where $R_{ech}$ and $R_{ref}$ are respectively the isotopic ratio $^{13}C/^{12}C$ of the sample and the work reference.

The values $\delta\ ^{13}C$ are thus expressed using V-PDB:

$$\delta^{13}C_{ech/V\text{-}PDB}‰ = \delta^{13}C_{ech/ref} + \delta^{13}C_{ref/V\text{-}PDB} + (\delta^{13}C_{ech/ref} \times \delta^{13}C_{ref/V\text{-}PDB})/1000$$

where $\delta^{13}C_{ref/V\text{-}PDB}$ is the isotopic deviation determined beforehand for the work reference to V-PDB.

Small variations may occur while measuring on line due to changes in the instrumental conditions. In this case the $\delta\ ^{13}C$ samples must be corrected according to the difference in the value $\delta^{13}C$ from the work reference and the real value, which was calibrated beforehand against V-PDB by comparison with one of the international reference materials. Between two measurements of the reference work, the variation is the correction applied to the sample results that may be assumed to be linear. The reference work must be measured at the beginning and at the end of all sample series. A correction can be calculated for each sample using linear interpolation between two values ( the difference between the assigned value of the reference work and the measurements of obtained values).

## 10. QUALITY INSURANCE AND CONTROL

Check that the value $^{13}C$ for the reference work does not differ by more than 0.5‰ of the admitted value. If not, the spectrometer instrument adjustments must be checked and possibly readjusted.

For each sample, verify that the difference in result between the 2 capsules measured successively is under 0.3‰. The final result for a given sample is the average value between the 2 capsules. If the deviation is higher than 0.3‰ the measurement should be repeated.

Measurement condition monitoring can be based on the ionic current intensity where m/z = 44 and is proportional to the quantity of carbon injected in the elementary analyzer. Under standard conditions, the ionic current intensity should be almost constant for the samples analysed. A significant deviation could be indicative of ethanol evaporation (an imperfect seal on a capsule), an instability of the elementary analyser or the mass spectrometer.

## 11. METHOD PERFORMANCE TRAITS (Accuracy)

One joint analysis (11.1) was carried out on distillates containing alcohol of vinous origin and cane and beet alcohol, in addition to different mixtures of these three origins. This study did not take into account the distillation step, further information from other joint laboratory studies on wine (11.2) and namely circuits of aptitude tests (11.3) for isotopic measurements were also considered. The results show that different distillation systems under satisfactory conditions, and in particular those used to measure RMN-FINS, do not have significant varieties for determining $\delta^{13}C$ of ethanol in wine. The precision parameters observed for wine are almost identical to those obtained in the joint study on distillates (11.1) sur les distillats.

*11.1. Joint study on distillates*

Year of joint laboratory study: 1996

Number of laboratories: 20

Number of samples: 6 samples in double-blind comparison

Analysis: $\delta\ ^{13}C$ ethanol

| Sample code | Vinous origin alcohol | Beet alcohol | Sugar cane alcohol |
|---|---|---|---|
| A & G | 80% | 10% | 10% |
| B & C | 90% | 10% | 0% |
| D & F | 0% | 100% | 0% |
| E & I | 90% | 0% | 10% |
| H & K | 100% | 0% | 0% |
| J & L | 0% | 0% | 100% |

| Samples | A / G | B / C | D / F | E / I | H / K | J / L |
|---|---|---|---|---|---|---|
| Number of laboratories retained after eliminating aberrant results | 19 | 18 | 17 | 19 | 19 | 19 |
| Number of results accepted | 38 | 36 | 34 | 38 | 38 | 38 |
| Average value ($\delta\ ^{13}C$) ‰ | -25.32 | -26.75 | -27.79 | -25.26 | -26.63 | -12.54 |
| $Sr^2$ | 0.0064 | 0.0077 | 0.0031 | 0.0127 | 0.0069 | 0.0041 |
| Repeatability standard deviation (Sr) ‰ | 0.08 | 0.09 | 0.06 | 0.11 | 0.08 | 0.06 |
| Repeatability limit r ($2,8 \times S_r$) ‰ | 0.22 | 0.25 | 0.16 | 0.32 | 0.23 | 0.18 |
| $S_R^2$ | 0.0389 | 0.0309 | 0.0382 | 0.0459 | 0.0316 | 0.0584 |
| Reproductability standard deviation ($S_R$) ‰ | 0.20 | 0.18 | 0.20 | 0.21 | 0.18 | 0.24 |
| Reproductability limit R ($2,8 \times S_R$) ‰ | 0.55 | 0.9 | 0.55 | 0.60 | 0.50 | 0.68 |

*11.2. Joint laboratory study on two wines and one alcohol*

Year of joint laboratory trial: 1996

Number of laboratories: 14 for distillation of wine and 7 for also measuring $\delta\ ^{13}C$ of ethanol in wine

8 for measuring $\delta\ ^{13}C$ in alcohol sample

Number of samples 3 (White wine TAV 9.3% vol., White wine TAV 9.6% Alcohol strength 93% m/m)

Analysis: $\delta\ ^{13}C$ of ethanol

| Samples | Red wine | White wine | Alcohol |
|---|---|---|---|
| Number of laboratories | 7 | 7 | 8 |
| Number of accepted results | 7 | 7 | 8 |
| Average value ($\delta\ ^{13}C$) ‰ | -26.20 | -26.20 | -25.08 |
| Reproductability variance $S_R^2$ | 0.0525 | 0.0740 | 0.0962 |
| Reproductability standard deviation ($S_R$) ‰ | 0.23 | 0.27 | 0.31 |
| Reproductability limit R (2,8× $S_R$) ‰ | 0.64 | 0.76 | 0.87 |

Different distillation systems were used by the participating laboratories. The isotopic indications $\delta\ ^{13}C$ carried out in one laboratory on the whole number of distillates returned by the participants, does not reveal any absurd values or significant distinct average values. The variation in results ($S^2 = 0.0059$) is comparable to repeatability variances $Sr^2$ from the joint study on distillates (11.1).

*11.3. Results from the exercises of aptitude circuits to isotopic trials*

Since December 1994 international aptitude exercises to determine the isotopic measurements for wine and alcohol (TAV distillates 96% vol.) have been regularly organized. The results enable participating laboratories to check the quality of their analyses. Statistical results enable the appreciation of the variety of derterminants

under the reproductability conditions. This enables the estimating the variance parametres and the reproductability limit. The results obtained for the wine and distillate ethanol $\delta\ ^{13}C$ determants are summarized in the table below:

| | Wine | | | | Distillates | | | |
|---|---|---|---|---|---|---|---|---|
| Date | N | $S_R$ | $S^2_R$ | R | N | $S_R$ | $S^2_R$ | R |
| Dec. 1994 | 6 | 0.210 | 0.044 | 0.59 | 6 | 0.151 | 0.023 | 0.42 |
| June 1995 | 8 | 0.133 | 0.018 | 0.37 | 8 | 0.147 | 0.021 | 0.41 |
| Dec. 1995 | 7 | 0.075 | 0.006 | 0.21 | 8 | 0.115 | 0.013 | 0.32 |
| March 1996 | 9 | 0.249 | 0.062 | 0.70 | 11 | 0.278 | 0.077 | 0.78 |
| June 1996 | 8 | 0.127 | 0.016 | 0.36 | 8 | 0.189 | 0.036 | 0.53 |
| Sept. 1996 | 10 | 0.147 | 0.022 | 0.41 | 11 | 0.224 | 0.050 | 0.63 |
| Dec. 1996 | 10 | 0.330 | 0.109 | 0.92 | 9 | 0.057 | 0.003 | 0.16 |
| March 1997 | 10 | 0.069 | 0.005 | 0.19 | 8 | 0.059 | 0.003 | 0.16 |
| June 1997 | 11 | 0.280 | 0.079 | 0.78 | 11 | 0.175 | 0.031 | 0.49 |
| Sept 1997 | 12 | 0.237 | 0.056 | 0.66 | 11 | 0.203 | 0.041 | 0.57 |
| Dec. 1997 | 11 | 0.127 | 0.016 | 0.36 | 12 | 0.156 | 0.024 | 0.44 |
| March 1998 | 12 | 0.285 | 0.081 | 0.80 | 13 | 0.245 | 0.060 | 0.69 |
| June 1998 | 12 | 0.182 | 0.033 | 0.51 | 12 | 0.263 | 0.069 | 0.74 |
| Sept 1998 | 11 | 0.264 | 0.070 | 0.74 | 12 | 0.327 | 0.107 | 0.91 |
| Weighted average | | 0.215 | 0.046 | 0.60 | | 0.209 | 0.044 | 0.59 |

N : number of participating laboratories

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Method OIV-AS312-07 Type of the method **IV**

# Method for the determination of the $^{13}C/^{12}C$ isotope ratio of glycerol in wines by Gas Chromatography Combustion or High performance Liquid Chromatography coupled to Isotopic Ratio Mass Spectrometry (GC-C-IRMS or HPLC-IRMS)

**(OIV-Oeno 343-2010)**

## 1. SCOPE

The present methods, based on gas chromatography [1] or liquid chromatography [2] coupled to an isotope ratio mass spectrometer (GC-C-IRMS or HPLC-IRMS), permit measurements of the $^{13}C/^{12}C$ ratio of glycerol. If its quantification is required simultaneously with the $^{13}C/^{12}C$ isotope ratio, GC-IRMS may be used.
The use of 1,5-pentanediol, as internal standard, also allows the determination of the glycerol concentration during the same analysis of the 13C/12C ratio.

## 2. DEFINITIONS

- $^{13}C/^{12}C$: ratio of carbon-13 ($^{13}C$) to carbon-12 ($^{12}C$) isotopes for a given sample.
- $\delta^{13}C$: carbon-13 content ($^{13}C$) expressed in parts per 1000 (‰, per mil).
- GC-C-IRMS: hyphenated technique of gas chromatography coupled to a combustion interface and isotope ratio mass spectrometry.
- V-PDB: Vienna-Pee-Dee-Belemnite. PDB is the primary reference material for measuring natural variations of carbon-13 isotope content, consisting of calcium carbonate from a Cretaceous belemnite rostrum from the Pee Dee Formation in South Carolina (USA). Its $^{13}C/^{12}C$ isotope ratio or $R_{PDB}$ is 0.0112372. PDB reserves have been exhausted for a long time, but it has remained the primary reference for expressing natural variations of carbon-13 isotope content and against which the reference material available at the IAEA (*International Atomic Energy Agency*) in Vienna (Austria) is calibrated. Isotopic indications of naturally occurring carbon-13 are conventionally expressed in relation to V-PDB.

## 3. PRINCIPLE

A significant difference exists between the carbon-13 content of sugars from plants following the different photosynthetic $C_3$ (Calvin cycle) and $C_4$ (Hatch-Slack) cycles. Most plants, such as the vine and beet, belong to the $C_3$ group, whilst maize and cane belong to the $C_4$ group. The carbon-13 contents of the sugar and of the corresponding metabolites obtained by fermentation (ethanol, glycerol) are correlated.

The measurement of the carbon-13 content of glycerol may enable possible detection of addition of glycerol from maize ($C_4$ plant) or from synthesis (fossil sources) to wines or to spirit drinks.

The separation of glycerol from the wine matrix is achieved using gas or liquid chromatography.

In GC-C-IRMS, after the chromatographic separation the effluent undergoes a combustion and a reduction step, passing through the oxidation and the reduction ovens of a combustion interface. Components other than the glycerol, namely the solvent, are vented with a back-flush valve during the run, to avoid oven soiling and interferences in chromatograms. The carbon-13 content is determined on the carbon dioxide gas resulting from the oxidation of the glycerol contained in the sample. Once the glycerol is oxidized, $CO_2$ and $H_2O$ are produced. Water produced during the combustion is eliminated by a water-removing trap, consisting of a Nafion® membrane. The carbon dioxide is eluted by a helium stream to the IRMS source for $^{13}C/^{12}C$ analysis.

In HPLC-IRMS, after the chromatographic separation the sample is oxidized while still in the mobile phase at the interface. The $CO_2$ formed is removed on-line from the solvent stream through a gas-exchange membrane into a stream of He. This He stream passes through a water trap consisting of a Nafion® membrane, and is then admitted to the ion source of the IRMS via an open split.

The various possible combinations of the $^{18}O$, $^{17}O$, $^{16}O$ and $^{13}C$, $^{12}C$, isotopes lead to the mass 44 corresponding to the $^{12}C^{16}O_2$ isotopomer, the mass 45 corresponding to $^{13}C^{16}O_2$ and $^{12}C^{17}O^{16}O$ species and the mass 46 to the $^{12}C^{16}O^{18}O$ isotopomer ($^{13}C^{17}O^{16}O$ and $^{12}C^{17}O_2$ can be neglected due to their very low abundance). The corresponding ion currents are determined on three different collectors. The ion current m/z 45 is corrected for the contribution of $^{12}C^{17}O^{16}O$ which is computed from the current intensity measured for m/z 46 by considering the relative abundance of $^{18}O$ and $^{17}O$ (Craig correction). The comparison with a reference calibrated against the international standard V-PDB permits the calculation of the carbon-13 content on the $\delta^{13}C$ ‰ relative scale.

## 4. REAGENTS

The following reagents and working standards should be used:

4.1 Anhydrous ethanol (CAS number 64-17-5).
4.2 Pure glycerol ≥ 99 % (CAS 56-81-5).
4.3 1,5-pentanediol (CAS 111-29-5).
4.4 Bulk solution of 1,5-pentanediol (4.3) in ethanol (4.1). This solution prepared at a precisely known concentration, in the range of 0.5 to 1.0 g $L^{-1}$ is used to dilute wine samples.
4.5 Orthophosphoric acid
4.6 Sodium peroxodisulfate, used as oxidation reagent
4.7 Helium for analysis, used as carrier gas (CAS 07440-59
4.8 Oxygen for analysis, used as regenerating gas for the combustion reactor (CAS 07782-44-7).
4.9 Cylinder of carbon dioxide for analysis, used as a secondary reference gas for the carbon-13 content (CAS 00124-38-9).
4.10 Working standard samples of glycerol with a known $^{13}C/^{12}C$ ratio calibrated against international reference materials.
4.11 Working standard samples of 1,5-pentanediol with a known $^{13}C/^{12}C$ ratio calibrated against international reference materials.

## 5. APPARATUS AND EQUIPMENT

**5.1.** Isotope ratio mass spectrometer

Isotope ratio mass spectrometer (IRMS) capable of determining the relative $^{13}C$ content of naturally-occurring $CO_2$ gas with an internal accuracy of 0.05 ‰ or better expressed as a relative value (point 8. Calculation). Internal accuracy here is defined as the difference between two measurements of the same sample of $CO_2$. The mass spectrometer used to measure isotope ratios is equipped with a triple collector to simultaneously measure intensities for m/z = 44, 45 and 46. The IRMS is equipped with software for running the analysis, acquisition of data and processing of analytical results for computation of isotope ratios.

**5.2.** Gas chromatograph

Gas chromatograph (GC) coupled through a combustion interface to an isotope ratio mass spectrometer (5.1).

The gas chromatograph must be equipped with a polar capillary column enabling the chromatographic separation of glycerol from the other wine components (e.g. Chrompack WCOT fused silica capillary column filled with bonded polyethylene glycol CP-Wax-57 CB, 25 m, 0.25 mm id, 0.20 μm film thickness).

Combustion interface generally made up of an oxidation reactor (a ceramic tube containing nickel, platinum and copper wires) and of a reduction reactor (ceramic tube containing copper wires).

**5.3.** Liquid chromatograph

Liquid chromatograph (LC) coupled through a LC Isolink interface to an isotope ratio mass spectrometer (5.1).

The liquid chromatograph must be equipped with a column enabling the chromatographic separation of glycerol from the other wine components without using organic solvents or additives (e.g. HyperREZ Carbohydrate $H^+$, 30 cm, 8 mm).

Isolink interface made up of a capillary oxidation reactor and a membrane exchanger (three membranes).

**5.4.** Equipment

Usual laboratory equipment and in particular the following:

- Sample injection syringes or autosampler

- Volumetric flasks, 0.2 μm filters, chromatographic vials and 10 μL syringe for liquids.

The laboratory equipment indicated in the above list is an example and may be replaced by other equipment of equivalent performance.

## 6. PREPARATION OF TEST SAMPLES

**6.1.** $^{13}C/^{12}C$ determination of glycerol by GC-C-IRMS

Each wine sample is filtered on a 0.2 µm filter and then an aliquot is diluted (in the ratio 1:4) with ethanol. Each sample is then transferred to an appropriate chromatographic vial which is then tightly closed and stored at T ≤ 4 °C until analysis.

**6.2.** $^{13}C/^{12}C$ ratio of glycerol and its quantification by GC-C-IRMS

Each wine sample is filtered on a 0.2 µm filter and then an aliquot is diluted (in the ratio 1:4) with the bulk solution of 1,5-pentanediol (4.4). Each sample is then transferred to an appropriate chromatographic vial which is then tightly closed and stored at T ≤ 4 °C until analysis.

**6.3.** $^{13}C/^{12}C$ determination of glycerol by HPLC-IRMS

Each wine sample is filtered on a 0.2 µm filter and then an aliquot is diluted with water. Each sample is then transferred to an appropriate chromatographic vial which is then tightly closed and stored at T ≤ 4 °C until analysis

## 7. PROCEDURE

### 7.1. GC-C-IRMS

The following description refers to the procedures generally used for glycerol $^{13}C/^{12}C$ isotope-ratio determination using commercial automated GC-C-IRMS systems.

Procedures may be adapted according to changes introduced by the manufacturers. Note: volumes, temperature, flows and times are indicative. The correct values should be optimized according to the manufacturer's instructions.

**7.1.1** Working conditions

Using the column and combustion interface described as an example in 5.2 the following parameters can be applied:
**A.** The injector temperature is set to 270 °C.
**B.** The temperature program is set as follows: initial column temperature of 120 °C; a holding time of 2 min; then a temperature increase at a rate of 10 °C $min^{-1}$, up to the final value of 220 °C, with a final holding time of 2 min.
Each run takes 14 min, not taking into account the time needed for cooling.
**C**. He is used as the carrier gas.
**D.** The temperatures of the combustion and reduction reactors of the GC combustion interface are set to 960 and 640°C respectively.
**E.** In each injection 0.3 µL of sample solution is introduced into the column using a high-split mode (split flow 120 mL $min^{-1}$).
At regular intervals (e.g. once a week) re-oxidation of the oxidation reactor with $O_2$ is required (the intervals depend on the total amount of substances that has passed through the reactor).

**7.1.2**. $^{13}C/^{12}C$ ratio of glycerol

During each $^{13}C/^{12}C$ analysis, at least two pulses of reference $CO_2$ gas (4.9) from the cylinder are introduced. This $CO_2$ is previously calibrated against other V-PDB-calibrated international standards, themselves calibrated against international IAEA standards. The reference $CO_2$ gas may also be calibrated against in-house standards.
Each wine sample (6.1) is injected 3 times. Suitable control references must be included in each batch.
A typical batch is as follows:
• Control Sample
• Control Sample
• Sample 1
• Sample 1
• Sample 1
• Sample 2
Each sample is measured 3 times
• …..
• Sample 6
• Sample 6
• Sample 6
• Control sample
• Control sample

The control sample is an ethanol solution of glycerol with a known accurately-measured $\delta^{13}C$ value (by an elemental analyser-IRMS for instance) and enables possible drift during the sequence of measurements to be checked and the correction of results.

**7.1.3** $^{13}C/^{12}C$ ratio of glycerol and its quantification

If quantification of glycerol is required at the same time as $^{13}C/^{12}C$ isotope ratio measurement, the previous procedure (7.1.2) is applied to the samples prepared as described in 6.2.
The 1,5-pentanediol (4.3) permits the determination of the concentration of glycerol. Furthermore, $\delta^{13}C$ values for the internal reference can be used to assess the correctness of the injections and the quality control of the isotopic determinations and of the combustion reaction step.
The concentration of glycerol in wine samples is determined using the internal-standard method. To do this, a calibration curve must be produced, using a constant known concentration for the internal standard, 1,5-pentanediol, and five glycerol solutions at different known concentrations, from 0.50 to 10 g $L^{-1}$. These solutions are prepared by weighing and dissolving glycerol (4.2) and 1,5-pentanediol in ethanol (4.1), using volumetric flasks. Ensure that the response is linear by successively analysing in triplicate each of the linearity standard solutions containing the internal standard.

**7.2. HPLC-IRMS**

The following description refers to the procedures generally used for glycerol $^{13}C/^{12}C$ isotope ratio determination using commercial automated HPLC-IRMS systems.

Procedures may be adapted according to changes introduced by the manufacturers. Note: volumes, temperature, flows and times are indicative. The correct values should be optimized according to the manufacturer's instructions.

**7.2.1** Working conditions

Using the column and interface described as an example in 5.3 the following parameters can be applied:

**A.** Flow rate of the eluent is set at 400 μL $min^{-1}$
**B.** Flow rate of the acid and oxidant reagents in the LC interface is set at 40 and 30 μL $min^{-1}$, respectively
**C**. The temperatures of the interface reactor and the column are set at 99.9 and 65 °C, respectively
**D.** Helium flow rate of the separation unit is set at 1 μL $min^{-1}$

The reagent bottles are degassed with helium during the complete chromatographic run.

**7.2.2**. $^{13}C/^{12}C$ ratio of glycerol

During each $^{13}C/^{12}C$ analysis, at least two pulses of reference $CO_2$ gas (4.9) from the cylinder are introduced (see example of chromatogram in 11.2). This $CO_2$ is previously calibrated against other V-PDB-calibrated international standards, themselves calibrated against international IAEA standards. The reference $CO_2$ gas may also be calibrated against in-house standards.
Each wine sample (6.3) is injected 3 times. Suitable control references must be included in each batch.
A typical batch is as follows:
• Control sample
• Control sample
• Sample 1
• Sample 1
• Sample 1
• Sample 2
Each sample is measured 3 times
• …..
• Sample 6
• Sample 6
• Sample 6
• Control sample
• Control sample
The control sample is a solution of glycerol with a known accurately measured $\delta^{13}C$ value (by an elemental analyser-IRMS for instance) and enables possible drift during the sequence of measurements to be checked and the correction of results.

## 8. CALCULATION

**8.1**. $^{13}C/^{12}C$ ratio

The $^{13}C/^{12}C$ isotope ratio can be expressed by its deviation from a working reference.
The isotopic deviation of carbon-13 ($\delta^{13}C$) is then calculated on a delta scale per thousand ($\delta$/1000 or $\delta$ ‰) by comparing the results obtained for the sample to be measured with those for a working reference, previously calibrated on the basis of the primary international reference (V-PDB). During $^{13}C/^{12}C$ analyses, a reference $CO_2$ gas is introduced, which is calibrated against other PDB-calibrated international standards.
The $\delta\ ^{13}C$ values are expressed in relation to the working reference as follows:

$$\delta^{13}C_{\text{sample/ref}}‰ = (R_{\text{sample}}/R_{\text{ref}} - 1) \times 1000$$

where $R_{sample}$ and $R_{ref}$ are respectively the $^{13}C/^{12}C$ isotope ratios of the sample and of the carbon dioxide used as the reference gas (4.9).
The $\delta\ ^{13}C$ values are expressed in relation to V-PDB as follows:

$$\delta^{13}C_{\text{sample/V-PDB}}‰ = \delta^{13}C_{\text{sample/ref}} + \delta^{13}C_{\text{ref/V-PDB}} + (\delta^{13}C_{\text{sample/ref}} \times \delta^{13}C_{\text{ref/V-PDB}})/1000$$

where $\delta^{13}C_{\text{ref/V-PDB}}$ is the previously determined isotopic deviation of the working reference from V-PDB

Small variations may occur while measuring on-line due to changes in the instrumental conditions. In this case the $\delta\ ^{13}C$ values of the samples must be corrected according to the difference between the measured $\delta^{13}C$ value of the standard working sample and its true value, previously calibrated against V-PDB by comparison with one of the international reference materials. Between two measurements of the standard working sample, the variation, and therefore the correction to be applied to the results obtained from the samples, may be assumed to be linear. The standard working sample must be measured at the beginning and at the end of all sample series. A correction can then be calculated for each sample using linear interpolation.

8.2. Glycerol concentration by GC-IRMS

When producing the calibration curve, for each injection, the measured parameter which is taken into account is the area S (in V*s) given by the spectrometer.

Calculate the ratio R as expressed in equation 1 below, and plot a graph of R versus the concentration ratio of glycerol to the internal standard (IS), C.
A linear plot should be obtained, with a correlation coefficient of at least 0.99.

Equation 1 $$R = \frac{\text{Peak area glycerol}}{\text{Peak area of IS}}$$

Using the analytical conditions described (7.1.1), 1,5-pentanediol being less polar than glycerol shows a retention time of around 310 sec, while that of glycerol is 460 sec ((see example of a chromatogram in 11.1).
The concentration of glycerol in each injection is calculated using the following equation:

Equation 2 $$C_{glyc_{Sample}} = K \cdot C_{1,5PD_{Sample}} \cdot \frac{S_{glyc_{Sample}}}{S_{1,5PD_{Sample}}} \times \text{dilution factor}$$

Where:

$Cx_{Sample}$ is the concentration in g $L^{-1}$ of the species in the sample;

**$SX_{sample}$ is the area of the peaks produced;**

K ( the response factor) is calculated as follows:

$$K = \frac{C_{glyc_{St}}}{C_{1,5PD_{St}}} \cdot \frac{S_{1,5PD_{St}}}{S_{glyc_{St}}}$$ Equation 3 (see 8.2)

The St suffix indicates the concentrations and the areas of 1,5-pentandiol and glycerol in the five standard solutions prepared for the calibration (7.1.3);

Dilution factor: considering the sampling conditions described above (7), the dilution factor is 4.

The concentration value in g $L^{-1}$ of each sample is the mean of the three injections

## 9. QUALITY ASSURANCE AND CONTROL

### 9.1. GC-C-IRMS

For each sample, check that the standard deviation (SD) in three vials measured successively is less than 0.6 ‰. The final result for a given sample is the average value for the three measurements. If the deviation is greater than 06 ‰, the measurement must be repeated.

Checks on correct measurement can be based on the ion current of m/z = 44, which is proportional to the quantity of carbon injected into the system. Under standard conditions, the ion current should be almost constant for the samples analysed. A significant deviation could be indicative of imperfect separation and oxidation of glycerol or instability of the mass spectrometer.

### 9.2. HPLC-IRMS

Check that the $^{13}C$ value for the working reference does not differ by more than 0.5 ‰ from the admissible value. If not, the spectrometer settings should be checked and, if necessary, adjusted.

For each sample, check that the standard deviation (SD) in three vials measured successively is less than 0.6 ‰. The final result for a given sample is the average value for the three measurements. If the deviation is greater than 0.6 ‰, the measurement must be repeated.

Checks on correct measurement can be based on the ion current of m/z = 44, which is proportional to the quantity of carbon injected into the system. Under standard conditions, the ion current should be almost constant for the samples analysed. A significant deviation could be indicative of imperfect separation and oxidation of glycerol or instability of the mass spectrometer.

## 10. PERFORMANCE CHARACTERISTICS OF THE METHOD

### 10.1. GC-C-IRMS

**10.1.1** Precision

Preliminary studies have been performed on 4 synthetic wine solutions (water-ethanol-glycerol), prepared using glycerol samples of different origins and with a $\delta^{13}C$ value already determined by EA-IRMS. For the 3 repetitions, n=3, using the GC-C-IRMS technique a standard deviation≤ SD 0.6 ‰ was considered acceptable.
Precision can be affected by overlapping between 1,5-PD and other wine components or by-products when measuring sweet wines.

**10.1.2.** Determination of the concentration of glycerol

For the validation of this method, 2 glycerol solutions were used. Assuming that the typical concentration of glycerol is 4 to 10 g $L^{-1}$ in dry wine, the 2 solutions represent this range. The first solution was 4.0 g $L^{-1}$ and gave an experimental concentration of 3.6 g $L^{-1}$ (SD=0.2, n=8). The second solution, 8.0 g $L^{-1}$, gave a value of 7.9 g $L^{-1}$ (SD=0.3, n=8).
Furthermore, 5 wine samples (A-E) already analysed for their glycerol concentration using other methods* through the BIPEA proficiency–testing scheme were injected to test the method.

**Table 1:** Comparison with the concentration of 5 dosed wines.

| Sample | A | B | C | D | E |
|---|---|---|---|---|---|
| **Type** | White | Rosé | White | Red | White |
| **Given range** | 6.2 - 8.4 | 4.8 - 6.6 | 5.7 - 7.7 | 6.3 - 8.5 | 4.6 - 6.2 |
| **Mean value** | 7.3 | 5.4 | 6.7 | 7.4 | 5.4 |
| **by GC-C-IRMS** | 6.4 | 5.4 | 6.7 | 7.8 | 5.4 |

* BIPEA determinations were performed by HPLC and/or enzymatic analysis.
Concentrations are given in g $L^{-1}$. n>3 and SD < 0.6.

The concentrations of glycerol found by GC-C-IRMS are consistent with the values obtained using other analytical techniques such as enzymatic determination or HPLC.

### 10.2. HPLC-IRMS

**Internal validation of HPLC-IRMS method**

For the validation of the HPLC-IRMS method, the following samples have been used: a glycerol standard, three synthetic wines (glycerol concentrations ranged within typical concentration found in wines) and a wine.
The precision of the measurement for glycerol was determined by repeating the analysis ten times on each sample, under repeatable conditions, and by performing ten independent analyses on the same sample on three different days, under reproducible conditions (Table 2).

**Table 2.** Accuracy and precision of δ13C values of glycerol obtained by HPLC-IRMSa

| | | HPLC-IRMS | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Day 1 | | Day 2 | | Day 3 | | Precision | |
| Sample | Repetitions per sample | Mean $\delta^{13}C$ (‰) | SD (‰) | Mean $\delta^{13}C$ (‰) | SD (‰) | Mean $\delta^{13}C$ (‰) | SD (‰) | r (‰) | R (‰) |
| Glycerol (standard)[b] | 10 | -27.99 | 0.05 | -27.94 | 0.04 | -27.95 | 0.08 | 0.17 | 0.18 |
| Synthetic wine (6 g/l) | 10 | -28.06 | 0.13 | -28.14 | 0.12 | -28.14 | 0.11 | 0.34 | 0.35 |
| Synthetic wine (8 g/l) | 10 | -28.11 | 0.12 | -28.18 | 0.07 | -28.21 | 0.07 | 0.25 | 0.28 |
| Synthetic wine (10 g/l) | 10 | -28.06 | 0.06 | -28.06 | 0.09 | -28.05 | 0.09 | 0.23 | 0.24 |
| Wine | 10 | -28.88 | 0.10 | -28.85 | 0.27 | -28.72 | 0.23 | 0.60 | 0.62 |

**[a]Values of $\delta^{13}C$ are expressed in ‰ vs V-PDB**

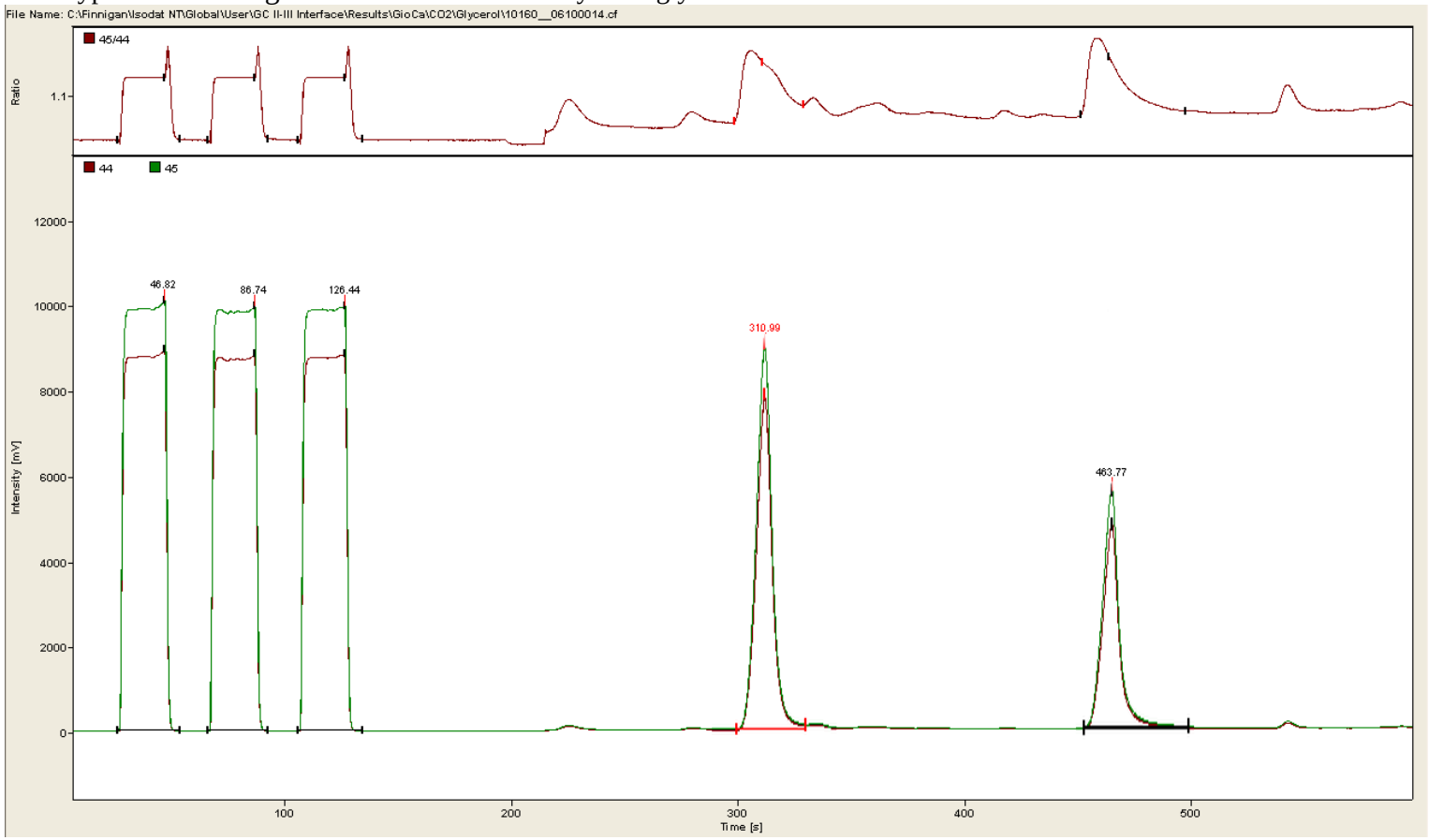
**[b]EA-IRMS glycerol (standard) result: -28.02 ± 0.09 ‰**

The following performance parameters for determining the $\delta^{13}C$ of glycerol were obtained from a wine sample:

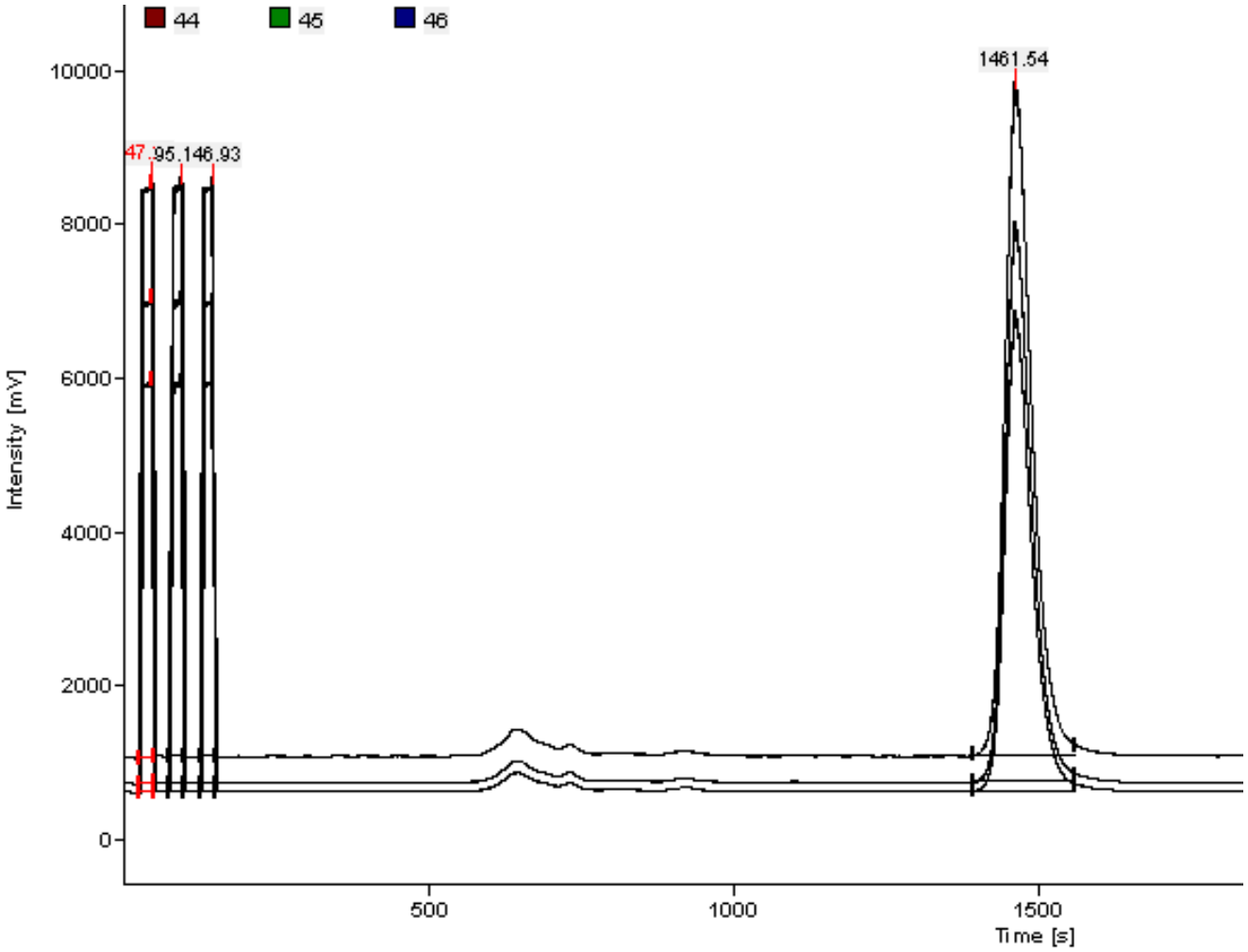
- Repeatability **r: 0,60 ‰**
- Reproducibility **R: 0,62 ‰**

## 11. ANNEX

11.1 Typical chromatogram of a GC-C-IRMS analysis of glycerol in wine



**11.2** Typical chromatogram of a HPLC-IRMS analysis of glycerol



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**Method OIV-MA-AS313-01** Type I method

# Total acidity

## 1. Definition

The total acidity of the wine is the sum of its titratable acidities when it is titrated to pH 7 against a standard alkaline solution. Carbon dioxide is not included in the total acidity.

## 2. Principle

Potentiometric titration or titration with bromothymol blue as indicator and comparison with an end-point color standard.

## 3. Apparatus

3.1 *Water vacuum pump*.

3.2 *Vacuum flask, 500 mL*.

3.3 *Potentiometer* with scale graduated in pH values, and electrodes. The glass electrode must be kept in distilled water. The calomel/saturated potassium chloride electrode must be kept in a saturated potassium chloride solution.

3.4 *Beakers of 12 cm diameter or any appropriate recipient*

## 4. Reagents

4.1 *Buffer solution pH 7.0*:

| | |
|---|---|
| potassium *di*-hydrogen phosphate, $KH_2PO_4$ | 107.3 g |
| sodium hydroxide solution, NaOH, 1 mol/L | 500 mL |
| water to | 1000 mL |

Alternatively, ready-made buffer solutions are available commercially.

4.2 Sodium hydroxide solution, NaOH, 0.1 mol/L.

4.3 Bromothymol blue indicator solution, 4 g/L.

| | |
|---|---|
| bromothymol blue | 4 g |
| neutral ethanol, 96% (*v/v*) | 200 mL |

Dissolve and add:

| | |
|---|---|
| water free of $CO_2$ | 200 mL |

sodium hydroxide solution, 1 mol/L, sufficient to produce

| | |
|---|---|
| blue green color (pH 7) | ~ 7.5 mL |
| water to | 1000 mL |

## 5. Procedure

5.1 *Preparation of sample:* elimination of carbon dioxide.

Place approximately 50 mL of wine in a vacuum flask; apply vacuum to the flask using a water pump for one to two min, while shaking continuously. Other CO2 elimination systems may be used if the CO2 elimination is guaranteed.

5.2 *Potentiometric titration*

5.2.1 Calibration of pH meter

The pH meter is calibrated for use at 20°C, according to the manufacturer's instructions, with the pH 7 buffer solution at 20°C.

5.2.2 Method of measurement

Into a beaker, introduce a volume of the sample, prepared as described in 5.1, equal to 10 mL in the case of wine and 50 mL in the case of rectified concentrated must. Add about 10 mL of distilled water and then add sodium hydroxide solution, 0.1 mol/L, from a burette until the pH is equal to 7 at 20°C. The sodium hydroxide must be added slowly and the solution stirred continuously. Let *n* mL be the volume of sodium hydroxide, 0.1 mol/L, added.

5.3 *Titration with indicator (bromothymol blue)*

5.3.1 Preliminary test: end-point color determination.

Into a beaker (3.4) place 25 mL of boiled distilled water, 1 mL of bromothymol blue solution and a volume prepared as in 5.1 equal to 10 mL in the case of wine and 50 mL in the case of rectified concentrated must. Add sodium hydroxide solution, 0.1 mol/L, until the color changes to blue-green. Then add 5 mL of the pH 7 buffer solution.

5.3.2 Measurement

Into a beaker (3.4) place 30 mL of boiled distilled water, 1 mL of bromothymol blue solution and a volume of the sample, prepared as described in 5.1, equal to 10 mL in the case of wine and 50 mL in the case of rectified concentrated must. Add sodium hydroxide solution, 0.1 mol/L, until the same color is obtained as in the preliminary test above (5.3.1). Let *n* mL be the volume of sodium hydroxide solution, 0.1 mol/L, added.

## 6. Expression of results

6.1 *Method of calculation*

- The total acidity expressed in milliequivalents per liter is given by:

$$A = 10\ n.$$

It is recorded to one decimal place.

- The total acidity expressed in grams of tartaric acid per liter is given by:

$$A' = 0.075 \times A$$

The result is quoted to two decimal places.

- The total acidity expressed in grams of sulfuric acid per liter is given by:

$$A' = 0.049 \times A$$

The result is quoted to two decimal places.

6.2 *Repeatability (r)* for titration with the indicator:(5.3):

$r$ = 0.9 meq/L
$r$ = 0.04 g sulfuric acid/L
$r$ = 0.07 g tartaric acid/L

6.3 *Reproducibility (*R*)* for titration with the indicator (5.3):

For white and rosé wines:

R = 3.6 meq/L
R = 0.2 g sulfuric acid/L
R = 0.3 g tartaric acid/L

For red wines:

$R$ = 5.1 meq/L
$R$ = 0.3 g sulfuric acid/L
$R$ = 0.4 g tartaric acid/L

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**Method OIV-MA-AS313-02** Type I method

# Volatile Acidity

## 1. Definition

The volatile acidity is derived from the acids of the acetic series present in wine in the free state and combined as salts.

## 2. Principle

Carbon dioxide is first removed from the wine. Volatile acids are separated from the wine by steam distillation and titrated using standard sodium hydroxide.

The acidity of free and combined sulfur dioxide distilled under these conditions should be subtracted from the acidity of the distillate.

The acidity of any sorbic acid, which may have been added to the wine, must also be subtracted.

*Note:* Part of the salicylic acid used in some countries to stabilize the wines before analysis is present in the distillate. This must be determined and subtracted from the acidity. The method of determination is given in the Annex of this Chapter.

## 3. Apparatus

3.1 Steam distillation apparatus consisting of:

- a steam generator; the steam must be free of carbon dioxide;
- a flask with steam pipe;
- a distillation column;
- a condenser.

This equipment must pass the following three tests:

(a) Place 20 mL of boiled water in the flask. Collect 250 mL of the distillate and add to it 0.1 mL sodium hydroxide solution, 0.1 M, and two drops of phenolphthalein solution. The pink coloration must be stable for at least 10 sec (i.e. steam to be free of carbon dioxide).

(b) Place 20 mL acetic acid solution, 0.1 M, in the flask. Collect 250 mL of the distillate. Titrate with the sodium hydroxide solution, 0.1 M: the volume of the titer must be at least 19.9 mL (i.e. at least 99.5% of the acetic acid entrained with the steam).

(c) Place 20 mL lactic acid solution, 1 M, in the flask. Collect 250 mL of the distillate and titrate the acid with the sodium hydroxide solution, 0.1 M.
The volume of sodium hydroxide solution added must be less than or equal to 1.0 mL (i.e. not more than 0.5% of lactic acid is distilled).

Any apparatus or procedure which passes these tests satisfactorily fulfils the requirements of official international apparatus or procedures.

3.2 Water aspirator vacuum pump.

3.3 Vacuum flask.

**4. Reagents**

4.1 Tartaric acid, crystalline.

4.2 Sodium hydroxide solution, 0.1 M.

4.3 Phenolphthalein solution, 1%, in neutral alcohol, 96% (*m/v*).

4.4 Hydrochloric acid ($\rho_{20}$ = 1.18 to 1.19 g/mL) diluted 1/4 with distilled water.

4.5 Iodine solution, 0.005 M.

4.6 Potassium iodide, crystalline.

4.7 Starch solution, 5 g/L.

Mix 5 g of starch with about 500 mL of water. Bring to the boil, stirring continuously and boil for 10 min. Add 200 g sodium chloride. When cool, make up to one liter.

4.8 Saturated solution of sodium tetraborate, $Na_2B_4O_7.10H_2O$, about 55 g/L at 20°C.

4.9 Acetic acid, 0.1 M.

4.10 Lactic acid solution, 0.1 M

100 mL of lactic acid is diluted in 400 mL of water. This solution is heated in an evaporating dish over a boiling water bath for four hours, topping up the volume occasionally with distilled water. After cooling, make up to a liter. Titrate the lactic acid in 10 mL of this solution with 1 M sodium hydroxide solution. Adjust the solution to 1 M lactic acid (90 g/L).

**5. Procedure**

5.1 *Preparation of sample***:** elimination of carbon dioxide. Place about 50 mL of wine in a vacuum flask; apply vacuum to the flask with the water pump for one to two min while shaking continuously. Other $CO_2$ elimination systems may be used if the $CO_2$ elimination is guaranteed.

5.2 *Steam distillation*

Place 20 mL of wine, freed from carbon dioxide as in 5.1, into the flask. Add about 0.5 g of tartaric acid. Collect at least 250 mL of the distillate.

5.3 *Titration*

Titrate with the sodium hydroxide solution, (4.2), using two drops of phenolphthalein (4.3) as indicator. Let *n* mL be the volume of sodium hydroxide used.

Add four drops of the dilute hydrochloric acid (4.4), 2 mL starch solution (4.7) and a few crystals of potassium iodide (4.6). Titrate the free sulfur dioxide with the iodine solution, 0.005 M (4.5). Let *n'* mL be the volume used.

Add the saturated sodium tetraborate solution (4.8) until the pink coloration reappears. Titrate the combined sulfur dioxide with the iodine solution, 0.005 M (4.5). Let *n''* mL be the volume used.

**6. Expression of results**

6.1 *Method of calculation*

The volatile acidity, expressed in milliequivalents per liter to one decimal place, is given by:

$$5\ (n - 0.1\ n' - 0.05\ n'').$$

The volatile acidity, expressed in grams of sulfuric acid per liter to two decimal places, is given by:

$$0.245\ (n - 0.1\ n' - 0.05\ n'').$$

The volatile acidity, expressed in grams of acetic acid per liter to two decimal places, is given by:

$$0.300\ (n - 0.1\ n' - 0.05\ n'').$$

6.2 *Repeatability (r)* $r = 0.7$ meq/L
$r = 0.03$ g sulfuric acid/L
$r = 0.04$ g acetic acid/L.

6.3 *Reproducibility* (R) R = 1.3 meq/L
R = 0.06 g sulfuric acid/L
R = 0.08 g acetic acid/L.

6.4 *Wine with sorbic acid present*

Since 96% of sorbic acid is steam distilled with a distillate volume of 250 mL, its acidity must be subtracted from the volatile acidity, knowing that 100 mg of sorbic acid corresponds to an acidity of 0.89 milliequivalents or 0.053 g of acetic acid and knowing the concentration of sorbic acid in mg/L as determined by other methods.

ANNEX

**Determination of Salicylic Acid entrained in the distillate from the volatile acidity**

1. *Principle*

After the determination of the volatile acidity and the correction for sulfur dioxide, the presence of salicylic acid is indicated, after acidification, by the violet coloration that appears when an iron (III) salt is added.

The determination of the salicylic acid entrained in the distillate with the volatile acidity is carried out on a second distillate having the same volume as that on which the determination of volatile acidity was carried out. In this distillate, the salicylic acid is determined by a comparative colorimetric method. It is subtracted from the acidity of the volatile acidity distillate.

**2** ***Reagents***

- Hydrochloric acid, HCl, ($\rho_{20}$ = 1.18 to 1.19 g/L).
- Sodium thiosulfate solution, $Na_2S_2O_3.5H_2O$, 0.1 M.
- Iron (III) ammonium sulfate solution, $Fe_2(SO_4)_3(NH_4)_2SO_4.24H_2O$, 10% (*m/v*)
- Sodium salicylate solution, 0.01 M: containing 1.60 g/L sodium salicylate, $NaC_7H_5O_3$.

**3.** ***Procedure***

3.1 *Identification of salicylic acid in the volatile acidity distillate*

Immediately after the determination of the volatile acidity and the correction for free and combined sulfur dioxide, introduce into a conical flask 0.5 mL hydrochloric acid, 3 mL of the sodium thiosulfate solution, 0.1 M, and 1 mL of the iron (III) ammonium sulfate solution. If salicylic acid is present, a violet coloration appears.

3.2 *Determination of salicylic acid*

On the above conical flask, indicate the volume of the distillate by a reference mark. Empty and rinse the flask. Subject a new test sample of 20 mL wine to steam distillation and collect the distillate in the conical flask up to the reference mark. Add 0.3 mL concentrated hydrochloric acid, and 1 mL of the iron (III) ammonium sulfate solution. The contents of the conical flask turn violet.

Into a conical flask identical to that carrying the reference mark, introduce distilled water up to the same level as that of the distillate. Add 0.3 mL concentrated hydrochloric acid and 1 mL of the iron (III) ammonium sulfate solution. From the burette run in the sodium salicylate solution, 0.01 M, until the violet coloration obtained has the same intensity as that of the conical flask containing the wine distillate.

Let *n'''* mL be the volume of solution added from the burette.

4. *Correction to the volatile acidity*

Subtract the volume 0.1 x *n''''* mL from the volume *n* mL of sodium hydroxide solution, 0.1 M, used to titrate the acidity of the distillate during the determination of volatile acidity.

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**Method OIV-MA-AS313-03** Type I method

# Fixed acidity

## 1. Principle

The fixed acidity is calculated from the difference between total acidity and volatile acidity.

## 2. Expression of results

The fixed acidity is expressed in:

— milliequivalents per liter.
— grams of sulfuric acid per liter.
— grams of tartaric acid per liter.

**Method OIV-MA-AS313-04** Type IV method

# Organic acids

Wine organic acids may be separated and simultaneously determined by *high performance liquid chromatography* (HPLC).

## 1. Principle of method

Wine organic acids may be separated using two stationary phases: octyl-bonded silica and ion exchange resin columns. The acids are detected by spectrophotometric absorbance in ultraviolet.

For the determination of malic and tartaric acids, it is advisable to use an octyl-bonded silica column and for citric and lactic acids, an ion exchange resin column. The determination of these acids is performed with reference to an external standard analyzed under similar conditions.

This method is also able to give an evaluation of contents of shikimic, acetic, succinic and fumaric acids.

*Note*: other types of columns may also give a good separation. The columns and operating conditions given below are given as examples.

## 2. Apparatus

2.1. *Cellulose membrane filtration apparatus* (*diameter of pores: 0.45 µm*)

2.2. *Octadecyl-bonded silica fitted cartridges* (*e.g. Sep Pak - Waters Assoc.*)

2.3. *High Performance Liquid Chromatograph equipped with:*

— a 10 µL loop injector,
— a temperature control apparatus,
— spectrophotometer detector capable of making absorbance measurements at 210 nm,
— a chart recorder, or integrator.

*Operating conditions*

2.3.1 In the case of citric, lactic and acetic acid separation:

— a column containing a strong cation ($H^+$) exchange resin (300 mm length, 7.8 mm internal diameter, 9 µm particle size) (e.g. HPX-87 H BIO-RAD),
— mobile phase: sulfuric acid solution, 0.0125 mol/L,
— flow rate: 0.6 mL/min,
— temperature: 60 - 65°C. (Depending on the type of resin).

2.3.2 In the case of fumaric, succinic, shikimic, lactic, malic and tartaric acid separation.

— two columns (250 mm length, 4 mm internal diameter) placed in series, fitted with octyl-bonded silica, spherical particles of 5 µm diameter,
— mobile phase: potassium *di*-hydrogen phosphate solution, 70 g/L, ammonium sulfate, 14 g/L, and adjusted to pH 2.1 by adding phosphoric acid,
— flow rate: 0.8 mL/min,
— temperature: 20°C.

**3. Reagents**

3.1. Distilled water of HPLC quality

3.2. Distilled methanol

3.3. Tartaric acid

3.4. Malic acid

3.5. Sodium lactate

3.6. Shikimic acid

3.7. Sodium acetate

3.8. Succinic acid

3.9. Citric acid

3.10. Fumaric acid

3.11. Sulfuric acid ($\rho_{20}$ = 1.84 g/mL)

3.12. Sulfuric acid solution, 0.0125 mol/L

3.13. Potassium *di*-hydrogen *ortho*-phosphate, $KH_2PO_4$

3.14. Ammonium sulfate, $(NH_4)_2SO_4$

3.15. O*rtho*-phosphoric acid, 85% ($\rho_{20}$ = 1.71 g/mL)

3.16. Reference solution made of: tartaric acid, 5 g/L, malic acid, 5 g/L, sodium lactate, 6.22 g/L, shikimic acid, 0.05 g/L, sodium acetate, 6.83 g/L, succinic acid, 5 g/L, fumaric acid, 0.01 g/L and citric acid, 5 g/L.

**4. Procedure**

4.1. *Preparation of sample*

First wash cartridge (2.2) with 10 mL methanol (3.2) then with 10 mL water (3.1).

Remove gas from wine or must sample. Filter through membrane (0.45 µm) (2.1). Put 8 mL of filtered sample into a syringe already rinsed with the sample; pass through the cartridge. Disregard the first 3 mL and collect the following 5 mL (prevent the cartridge from becoming dry).

4.2. *Chromatography*

Inject successively into the chromatograph 10 µL reference solution and 10 µL sample solution prepared according to 4.1. Repeat these injections three times in the same order.

**5. Calculation**

5.1. *Qualitative analysis*

Determine the respective times of retention for each of the eluates.

The organic acids of the reference solution are divided in order of elution as follows:

— citric, tartaric, malic, succinic + shikimic, lactic, fumaric and acetic acids in the technique 2.3.1.

— tartaric, malic, shikimic, lactic, acetic, citric, succinic and fumaric acids in the technique 2.3.2.

5.2. *Quantitative analysis*

Measure the area of each of the peaks and determine the average of the three answers for the reference and sample solutions to be analyzed. Deduct the sample concentration from the organic acids.

**6. Expression of results**

The concentrations are expressed as follows:

— grams per liter to one decimal place for the tartaric, malic, lactic and succinic acids

— milligrams per liter for the citric, acetic and fumaric acids.

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**Method OIV-MA-AS313-05A** Type IV method

# Tartaric acid

## 1. Principle

Tartaric acid is precipitated in the form of calcium (±)tartrate and determined gravimetrically. This determination may be completed using a volumetric procedure for comparison. The conditions for precipitation (pH, total volume used, concentrations of precipitating ions) are such that precipitation of the calcium (±)tartrate is complete whereas the calcium D(–) tartrate remains in solution.

When *meta*-tartaric acid has been added to the wine, which causes the precipitation of the calcium (±)tartrate to be incomplete, it must first be hydrolyzed.

## 2. Method

2.1. *Gravimetric method*

2.1.1. Reagents

- Calcium acetate solution containing 10 g of calcium per liter:

| | |
|---|---|
| Calcium carbonate, $CaCO_3$ | 25 g |
| Acetic acid, glacial, $CH_3COOH$ ($\rho_{20}$= 1.05 g/mL) | 40 mL |
| Water to | 1000 mL |

- Calcium (±)tartrate, crystallized: $CaC_4O_6H_4 \cdot 4H_2O$.

Place 20 mL of L(+) tartaric acid solution, 5 g/L, into a 400 mL beaker.

Add 20 mL of ammonium D(–) tartrate solution, 6.126 g/L, and 6 mL of calcium acetate solution containing 10 g of calcium per liter.

Allow to stand for two hours to precipitate. Collect the precipitate in a sintered glass crucible of porosity No 4, and wash it three times with about 30 mL of distilled water. Dry to constant weight in the oven at 70°C. Using the quantities of reagent indicated above, about 340 mg of crystallized calcium (±) tartrate is obtained. Store in a stoppered bottle.

- Precipitation solution (pH 4.75):

| | |
|---|---|
| D(–) ammonium tartrate | 150 mg |
| Calcium acetate solution, 10 g calcium/L | 8.8 mL |
| Water to | 1000 mL |

Dissolve the D(-) ammonium tartrate in 900 mL water; add 8.8 mL calcium acetate solution and make up to 1000 mL. Since calcium (±)tartrate is

slightly soluble in this solution, add 5 mg of calcium (±)tartrate per liter, stir for 12 hours and filter.

*Note*: The precipitation solution may also be prepared from D(-) tartaric acid.

D(–) tartaric acid ..... 122 mg
Ammonium hydroxide solution ($\rho_{20}$= 0.97 g/mL), 25 % (*v/v*) ..... 0.3 mL

Dissolve the D(–) tartaric acid, add the ammonium hydroxide solution and make up to about 900 mL; add 8.8 mL of calcium acetate solution, make up to a liter and adjust the pH to 4.75 with acetic acid. Since calcium (±)tartrate is slightly soluble in this solution, add 5 mg of calcium (±)tartrate per liter, stir for 12 hours and filter.

2.1.2. Procedure

— Wines with no added *meta*-tartaric acid

Place 500 mL of precipitation solution and 10 mL of wine into a 600 mL beaker. Mix and initiate precipitation by rubbing the sides of the vessel with the tip of a glass rod. Leave to precipitate for 12 hours (overnight).

Filter the liquid and precipitate through a weighed sintered glass crucible of porosity No. 4 fitted on a clean vacuum flask. Rinse the vessel in which precipitation took place with the filtrate to ensure that all precipitate is transferred.

Dry to constant weight in an oven at 70°C. Weigh. Let *p* be the weight of crystallized calcium (±)tartrate, $CaC_4O_6H_4 \cdot 4H_2O$, obtained.

— Wines to which *meta*-tartaric acid has been added.

When analyzing wines to which *meta*-tartaric acid has been or is suspected of having been added, proceed by first hydrolyzing this acid as follows:

Place 10 mL of wine and 0.4 mL of glacial acetic acid, $CH_3COOH$, ($\rho_{20}$= 1.05 g/mL) into a 50 mL conical flask. Place a reflux condenser on top of the flask and boil for 30 min. Allow to cool and then transfer the solution in the conical flask to a 600 mL beaker. Rinse the flask twice using 5 mL of water each time and then continue as described above.

*Meta*-Tartaric acid is calculated and included as tartaric acid in the final result.

2.1.3. Expression of results

One molecule of calcium (±)tartrate corresponds to half a molecule of L(+) tartaric acid in the wine.

- The quantity of tartaric acid per liter of wine, expressed in milliequivalents, is equal to:

$$384.5\ p.$$

It is quoted to one decimal place.

- The quantity of tartaric acid per liter of wine, expressed in grams of tartaric acid, is equal to:

$$28.84\ p.$$

It is quoted to one decimal place.

- The quantity of tartaric acid per liter of wine, expressed in grams of potassium tartrate, is equal to:

$$36.15\ p.$$

It is quoted to one decimal place.

2.2. *Comparative volumetric analysis*

2.2.1. Reagents

- Hydrochloric acid ($\rho_{20}$ = 1.18 to 1.19 g/mL) diluted 1:5 with distilled water
- EDTA solution, 0.05 M:

EDTA (ethylenediaminetetraacetic acid disodium salt)..... 18.61 g
Water to ..... 1000 mL

- Sodium hydroxide solution, 40% (*m/v*):

Sodium hydroxide, NaOH ..... 40 g
Water to ..... 100 mL

- Complexometric indicator: 1% (*m/m*)

2-hydroxy-1-(2-hydroxy-4-sulpho-1-naphthylazo)
-3-naphthoic acid ..... 1 g
Sodium sulfate, $Na_2SO_4$ (anhydrous) ..... 100 g

2.2.2. Procedure

After weighing, replace the sintered glass crucible containing the precipitate of calcium (±)tartrate on the vacuum flask and dissolve the precipitate with 10 mL of dilute hydrochloric acid. Wash the sintered glass crucible with 50 mL of distilled water.

Add 5 mL 40% sodium hydroxide solution and about 30 mg of indicator. Titrate with EDTA solution, 0.05 M. Let the number of mL used be *n*.

2.2.3. Expression of results

- The quantity of tartaric acid per liter of wine, expressed in milliequivalents, is equal to:

$$5\ n.$$

It is quoted to one decimal place.

- The quantity of tartaric acid per liter of wine, expressed in grams of tartaric acid, is equal to:

$$0.375\ n.$$

It is quoted to one decimal place.

- The quantity of tartaric acid per liter of wine, expressed in grams of potassium acid tartrate, is equal to:

$$0.470\ n.$$

It is quoted to one decimal place.

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**Method OIV-MA-AS313-05B**

# Tartaric acid

## 1. Principle

The tartaric acid, separated using an ion exchange column, is determined colorimetrically in the eluate by measurement of the red color produced on reaction with vanadic acid. The eluate also contains lactic and malic acids that do not interfere.

WITHDRAWN
(resolution Oeno 377/2009)

**Method OIV-MA-AS313-06**

# Lactic acid

## 1. Principle

The lactic acid, separated by passage through an ion exchange resin column, is oxidized to acetaldehyde (ethanol) and determined by colorimetry after reacting with sodium nitroprusside and piperidine.

WITHDRAWN
(Resolution 377/2009)

**Method OIV-MA-AS313-07** Type II method

# Lactic acid

Enzymatic method

## 1. Principle

Total lactic acid (L-lactate and D-lactate) is oxidized by nicotinamide adenine dinucleotide (NAD) to pyruvate in a reaction catalyzed by L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH).

The equilibrium of the reaction normally lies more strongly in favor of the lactate. Removal of the pyruvate from the reaction mixture displaces the equilibrium towards the formation of pyruvate.

In the presence of L-glutamate, the pyruvate is transformed into L-alanine in a reaction catalyzed by glutamate pyruvate transaminase (GPT):

(1) L-lactate + $NAD^+$ $\xrightleftharpoons{\text{L-LDH}}$ pyruvate + NADH + $H^+$

(2) D-lactate + $NAD^+$ $\xrightleftharpoons{\text{L-LDH}}$ pyruvate + NADH + $H^+$

(3) Pyruvate + L-glutamate $\xrightleftharpoons{\text{L-GPT}}$ L-alanine + $\alpha$-ketoglutarate

The amount of NADH formed, measured by the increase in absorbance at the wavelength of 340 nm, is proportional to the quantity of lactate originally present.

*Note:* L-lactic acid may be determined independently by using reactions (1) and (3), while D-lactic acid may be similarly determined by using reactions (2) and (3).

## 2. Apparatus

2.1. A spectrophotometer permitting measurements to be made at 340 nm, the wavelength at which the absorbance of NADH is a maximum.
Failing that, a spectrophotometer with a discontinuous spectrum source permitting measurements to be made at 334 or 365 nm may be used.

2.2. Glass cells with optical path lengths of 1 cm or single-use-cells.

2.3. Micropipettes for pipetting sample volumes in the range 0.02 to 2 mL.

## 3. Reagents

Double-distilled water

3.1. Buffer solution, pH 10 (glycylglycine, 0.6 M; L-glutamate, 0.1 M):
Dissolve 4.75 g of glycylglycine and 0.88 g of L-glutamic acid in approximately 50 mL of double distilled water; adjust the pH to 10 with a few

milliliters sodium hydroxide, 10 M, and make up to 60 mL with double distilled water.
This solution will remain stable for at least 12 weeks at 4°C.

3.2. Nicotinamide adenine dinucleotide (NAD) solution, approximately 40 x $10^{-3}$ M: dissolve 900 mg of NAD in 30 mL of double distilled water. This solution will remain stable for at least four weeks at 4°C.

3.3. Glutamate pyruvate transaminase (GPT) suspension, 20 mg/mL.
The suspension remains stable for at least a year at 4°C.

3.4. L-lactate dehydrogenase (L-LDH) suspension, 5 mg/mL.
This suspension remains stable for at least a year at 4°C.

3.5. D-lactate dehydrogenase (D-LDH) suspension, 5 mg/mL.
This suspension remains stable for at least a year at 4°C.
It is recommended that, prior to the determination, the enzyme activity should be checked.

*Note:* All the reagents are available commercially.

## 4. Preparation of the sample

Lactate determination is normally carried out directly on the wine, without prior removal of pigmentation (coloration) and without dilution provided that the lactic acid concentration is less than 100 mg/L. However, if the lactic acid concentration lies between:

100 mg/L and 1 g/L, dilute 1/10 with double distilled water, 1 g/L and 2.5 g/L, dilute 1/25 with double distilled water, 2.5 g/L and 5 g/L, dilute 1/50 with double distilled water.

## 5. Procedure

*Preliminary note:*

No part of the glassware that comes into contact with the reaction mixture should be touched with the fingers, since this could introduce L-lactic acid and thus give erroneous results.

The buffer solution must be at a temperature between 20 and 25°C before proceeding to the measurement.

5.1. *Determination of total lactic acid*

With the spectrophotometer adjusted to a wavelength of 340 nm, determine the absorbance using 1 cm cells, with air as the zero absorbance (reference) standard; (no cell in the optical path) or with water as the standard.
Place the following in the 1 cm cells:

| | Reference cell (mL) | Sample cell (mL) |
|---|---|---|
| Solution 3.1. ..... | 1.00 | 1.00 |
| Solution 3.2. ..... | 0.20 | 0.20 |
| Double distilled water ..... | 1.00 | 0.80 |

| | | |
|---|---|---|
| Suspension 3.3. ..... | 0.02 | 0.02 |
| Sample to be measured ..... | — | 0.20 |

Mix using a glass stirrer or a rod of synthetic material with a flattened end; after about five min, measure the absorbencies of the solutions in the reference and sample cells ($A_1$).

Add 0.02 mL of solution 3.4 and 0.05 mL of solution 3.5, homogenize, wait for the reaction to be completed (about 30 min) and measure the absorbencies of the solutions in the reference and sample cells ($A_2$).

Calculate the differences ($A_2 - A_1$) in the absorbencies of the solutions in the reference and sample cells, $\Delta A_S$ and $\Delta A_R$.

Finally, calculate the difference between those differences:

$$\Delta A = \Delta A_S - \Delta A_R$$

5.2. *Determination of L-lactic acid and D-lactic acid*

Determination of the L-lactic acid or D-lactic acid can be carried out independently by applying the procedure for total lactic acid up to the determination of $A_1$ and then continuing as follows:

Add 0.02 mL of solution 3.4, homogenize, wait until the reaction is complete (about 20 min) and measure the absorbencies of the solutions in the reference and sample cells ($A_2$).

Add 0.05 mL of solution 3.5, homogenize, wait until the reaction is complete (about 30 min) and measure the absorbencies of the solutions in the reference and sample cells ($A_3$).

Calculate the differences ($A_2 - A_1$) for L-lactic acid and ($A_3 - A_2$) for D-lactic acid between the absorbencies of the solutions in the reference and sample cells, $\Delta A_S$ and $\Delta A_R$.

Finally, calculate the difference between those differences:

$$\Delta A = A_S - \Delta A_R.$$

*Note:* The time needed for the completion of enzyme activity can vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch. When determining the L-lactic acid alone, the incubation time may be reduced to 10 min.

**6. Expression of results**

Lactic acid concentration is given in grams per liter (g/L) to one decimal place.

6.1. *Method of calculation*

The general formula for calculating the concentration in g/L is:

$$C = \frac{V \times M}{\varepsilon \times \delta \times \nu \times 1000} \times \Delta A$$

where

$V$ = volume of test solution in mL (V = 2.24 mL for L-lactic acid, V = 2.29 mL for D-lactic acid and total lactic acid)
$v$ = volume of the sample in mL (0.2 mL)
$M$ = molecular mass of the substance to be determined (for DL-lactic acid, M = 90.08)
$\delta$ = optical path in the cell in cm (1 cm)
$\varepsilon$ = absorption coefficient of NADH, at 340 nm
($\varepsilon$ = 6.3 $mmol^{-1}$ x l x $cm^{-1}$).

6.1.1 Total lactic acid and D-lactic acid

$$C = 0.164 \times \Delta A$$

If the sample was diluted during its preparation, multiply the result by the dilution factor.

*Note:*

- Measurement at 334 nm: $C = 0.167 \times \Delta A$, ($\varepsilon$ = 6.2 $mmol^{-1}$ x 1 x $cm^{-1}$).
- Measurement at 365 nm: $C = 0.303 \times \Delta A$, ($\varepsilon$ = 3.4 $mmol^{-1}$ x 1 x $cm^{-1}$).

6.1.2 L-lactic acid

$$C = 0.160 \times \Delta A$$

If the sample was diluted during its preparation, multiply the result by the dilution factor.

*Note:*

- Measurement at 334 nm: $C = 0.163 \times \Delta A$, ($\varepsilon$ = 6.2 $mmol^{-1}$ x 1 x $cm^{-1}$).
- Measurement at 365 nm: $C = 0.297 \times \Delta A$, ($\varepsilon$ = 3.4 $mmol^{-1}$ x 1 x $cm^{-1}$).

6.2 *Repeatability (r)*

$$r = 0.02 + 0.07\, x_i$$

$x_i$ is the lactic acid concentration in the sample in g/L.

6.3. *Reproducibility (R)*

$$R = 0.05 + 0.125\, x_i$$

$x_i$ is the lactic acid concentration in the sample in g/L.

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**Method OIV-MA-AS313-08** Type IV method

# Citric acid

Chemical method

## 1. Principle

Citric acid is fixed with other wine acids onto an anion exchange column. The citramalic acid is obtained by fractionating the elute.

The citric acid is oxidized to acetone, which is separated by distillation. The acetaldehyde (ethanol) is oxidized to acetic acid and acetone is determined by iodometry.

## 2. Apparatus

2.1. *Anion exchange column*

In a 25 mL burette with tap, place a glass wool plug and pour 20 mL of Dowex resin 1 x 2.

Initially the resin goes through two complete regeneration cycles with alternate passages of hydrochloric acid solution, 1 M, and sodium hydroxide solution, 1 M. Rinse with 50 mL distilled water [(1)]. Saturate the resin with acetate ions by adding 250 mL acetic acid solution, 4 M. Wash with 100 mL distilled water.

The sample is passed through a column conforming to the description below. After the elution of the acids, rinse with 50 mL of distilled water and proceed once more to saturate the resin with acetic acid solution, 4 M. Rinse with 100 mL water. The resin is then ready for re-use.

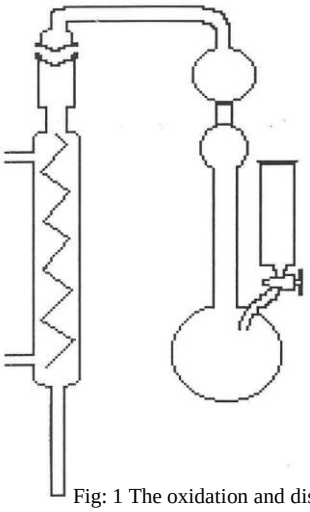

Fig: 1 The oxidation and distillation apparatus for the determination of citric acid

2.2 *Oxidation apparatus*

The use of a distillation apparatus with oxidation round bottom flask, see drawing Fig. 1 facilitates the introduction of potassium permanganate, with a very regular flow.

If unavailable, use a 500 mL round bottom flask and a funnel fitted with a tap and a tapered end,

[(1)] The passage of the sodium hydroxide causes a contraction that, followed by a swelling during washings, stops the flow. It is recommended to stir the resin as soon as the first few mL of water pass through the column to stop the resin from sticking to the bottom of the burette.

to ensure that there is as regular flow of potassium permanganate as possible.

**3. Reagents**

- Dowex resin 1 x 2 (50 - 100 mesh)
- Acetic acid solution, 4 M
- Acetic acid solution, 2.5 M
- Sodium hydroxide solution, 2 M
- Sulfuric acid ($\rho_{20}$ = 1.84 g/mL) diluted $^1/_5$ (*v*/*v*)
- Buffer solution of pH 3.2 - 3.4

Potassium *di*-hydrogen phosphate $KH_2PO_4$ ..... 150 g
Concentrated phosphoric acid ($p_{20}$ = 1.70 g/mL) ..... 5 mL
Water to: ..... 1000 mL

- Manganese sulfate solution, $MnSO_4.H_2O$, 50 g/L
- Pumice stone
- Potassium permanganate solution, 0.01 M
- Sulfuric acid ($\rho_{20}$ = 1.84 g/mL) diluted $^1/_3$ (*v*/*v*)
- Potassium permanganate solution, 0.4 M
- Iron (II) sulfate, $FeSO_4.7H_2O$, 40% (*m*/*v*)
- Sodium hydroxide solution, 5 M
- Iodine solution, 0.01 M
- Sodium thiosulfate solution, 0.02 M
- Thiodene or starch

**4. Method**

4.1 *Separation of citramalic and citric acids*

Pass 25 mL wine through the ion exchange Dowex 1 x 2 resin column (in an acetate form) at a flow rate of 3 mL every 2 minutes. Rinse the column three times with 20 mL distilled water. Elute the acids with 200 mL acetic acid solution, 2.5 M, at the same flow rate. This eluate fraction contains succinic, lactic, galacturonic, citramalic acids and nearly all of the malic acid.

Proceed with the elution of citric and tartaric acids by passing 100 mL sodium hydroxide solution, 2 M, through the column. Collect the eluate in the oxidation flask.

4.2. *Oxidation*

In the flask containing this second eluate, add sulfuric acid diluted 1/5 (about 20 mL) to bring the pH to between 3.2 and 3.8. Add 25 mL of pH 3.2-3.4 buffer solution, 1 mL of manganese sulfate solution and few grains of pumice stone.

Bring to the boil and distil over 50 mL, which is discarded.

Put the potassium permanganate solution, 0.01 M, in the funnel and introduce at 1 drop per second into the boiling eluate. The distillate is collected in a 500 mL ground glass stoppered flask containing few millimeters of water. The oxidation is

carried on until a brown coloration of the liquid appears indicating an excess of permanganate.

4.3. *Separation of the acetone*

If the volume of the distillate is less than 90 mL, make up with distilled water, add 4.5 mL of sulfuric acid diluted $^1/_3$, and 5 mL potassium permanganate solution, 4.4 M. If the collected distillate largely exceeds this volume, complete to 180 mL and double the amounts of the reagents.

Under those conditions (i.e. sulfuric acid, 0.25 M, and potassium permanganate, 0.02 M), acetaldehyde (ethan0l) is oxidized into acetic acid while acetone remains intact.

The stoppered flask is left to rest for 45 minutes at room temperature. After which the excess of permanganate is destroyed by addition of iron (II) sulfate solution.

Distillate and collect about 50 mL of distillate in a ground glass stoppered flask containing 5 mL sodium hydroxide solution, 5 M.

4.4. *Determination of acetone*

Add 25 mL iodine solution, 0.01 M, to the flask [*]. Leave for 20 minutes. Add 8 mL of sulfuric acid diluted 1/5. Titrate the excess of iodine by sodium thiosulfate, 0.02 M, in the presence of thiodene or starch, *n* mL.

Under the same conditions make a blank determination replacing 50 mL of distillate by 50 mL of distilled water.

*n'* mL of thiosulfate used.

**5. Calculations**

1 mL iodine, 0.01 M, corresponds to 0.64 mg of citric acid.

Under the same given conditions, the quantity of citric acid in milligrams per liter corresponds to:

$$(n' - n) \times 25.6$$

**6. Expression of results**

The concentration of citric acid is expressed in milligrams per liter.

[*] This amount is suitable for citric acid contents not exceeding 0.5 to 0.6 g/L. For higher contents the volume of the iodine solution prescribed is not sufficient and the solution does not take a yellow color which is typical of an iodine excess. In this case double or triple the quantity of iodine until the solution becomes really yellow. However, in exceptional cases where the amount of citric acid in wine exceeds 1.5 g/L, it is recommended to restart the analysis on 10 mL of wine.

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**Method OIV-MA-AS313-09** Type II method

# Citric acid

Enzymatic method

## 1. Principle

Citric acid is converted into oxaloacetate and acetate in a reaction catalyzed by citratelyase (CL):

$$\text{Citrate} \xrightleftharpoons{\text{CL}} \text{oxaloacetate} + \text{acetate}$$

In the presence of malate dehydrogenase (MDH) and lactate dehydrogenase (LDH), the oxaloacetate and its decarboxylation derivative, pyruvate, are reduced to L-malate and L-lactate by reduced nicotinamide adenine dinucelotide (NADH):

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{MDH}} \text{L-malate} + \text{NAD}^+$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$

The amount of NADH oxidized to NAD+ in these reactions is proportional to the amount of citrate present. The oxidation of NADH is measured by the resultant decrease in absorbance at a wavelength of 340 nm.

## 2. Apparatus

2.1 A spectrophotometer permitting measurement to be made at 340 nm, the wavelength at which absorbance of NADH is a maximum.

Alternatively, a spectrophotometer, with a discontinuous spectrum source permitting measurements to be made at 334 nm or 365 nm, may be used.

Since absolute absorbance measurements are involved (i.e. calibration curves are not used but standardization is made by consideration of the extinction coefficient of NADH), the wavelength scales and spectral absorbance of the apparatus must be checked.

2.2 Glass cells with optical path lengths of 1 cm or single-use cells.

2.3 Micropipettes for pipetting volumes in the range 0.02 to 2 mL.

**3. Reagents**

3.1 Buffer solution pH 7.8 (glycylglycine, 0.51 M; pH 7.8; $Zn^{+}$(0.6 x $10^{-3}$ M): dissolve 7.13 g of glycylglycine in approximately 70 mL of double distilled water. Adjust the pH to 7.8 with approximately 13 mL sodium hydroxide solution, 5 M, add 10 mL of zinc chloride, $ZnCl_2$, (80 mg in 100 mL double distilled water) solution and make up to 100 mL with double distilled water.

3.2 Reduced nicotinamide adenine dinucleotide, NADH, solution (approximately 6 x $10^{-3}$M): dissolve 30 mg NADH and 60 mg $NaHCO_3$ in 6 mL of double distilled water.

3.3 Malate dehydrogenase/lactate dehydrogenase solution (MDH/LDH) (0.5 mg MDH/mL; 2.5 mg LDH/mL): mix together 0.1 mL MDH (5 mg MDH/mL), 0.4 mL ammonium sulfate solution, 3.2 M, and 0.5 mL LDH (5 mg/mL).
This suspension remains stable for at least a year at 4°C.

3.4 Citrate-lyase (CL, 5 mg protein/mL): dissolve 168 mg lyophilisate in 1 mL ice-cold water. This solution remains stable for at least a week at 4°C and for at least four weeks if frozen.
It is recommended that, prior to the determination, the enzyme activity should be checked.

3.5 Polyvinylpolypyrrolidone (PVPP).

*Note:* All the reagents above are available commercially.

**4. Preparation of the sample**

Citrate determination is normally carried out directly on wine, without preliminary removal of pigmentation (coloration) and without dilution, provided that the citric acid content is less than 400 mg/L. If not, dilute the wine until the citrate concentration lies between 20 and 400 mg/L (i.e. between 5 and 80 µg of citrate in the test sample).

With red wines that are rich in phenolic compounds, preliminary treatment with PVPP is recommended:

Form a suspension of about 0.2 g of PVPP in water and allow to stand for 15 min. Filter using a fluted filter.

Place 10 mL of wine in a 50 mL conical flask, add the moist PVPP removed from the filter with a spatula. Shake for two to three minutes. Filter.

**5. Procedure**

With the spectrophotometer adjusted to a wavelength of 340 nm, determine the absorbance using the 1 cm cells, with air as the zero absorbance (reference) standard (no cell in the optical path). Place the following in the 1 cm cells:

| | Reference cell (mL) | Sample cell (mL) |
|---|---|---|
| Solution 3.1 | 1.00 | 1.00 |
| Solution 3.2 | 0.10 | 0.10 |
| Sample to be measured | - | 0.20 |
| Double distilled water | 2.00 | 1.80 |
| Solution 3.3 | 0.02 | 0.02 |

Mix, and after about five min read the absorbance of the solutions in the reference and sample cells ($A_1$).

Add:

| | | |
|---|---|---|
| Solution 3.4 ..... | 0.02 mL | 0.02 mL |

Mix; wait until the reaction is completed (about five min) and read the absorbance of the solutions in the reference and sample cells ($A_2$).

Calculate the absorbance difference ($A_1$- $A_2$) for the reference and sample cells, $\Delta A_S$ and $\Delta A_R$.

Finally, calculate the difference between those differences:

$$\Delta A = \Delta A_S - \Delta A_R.$$

Note: The time needed for the completion of enzyme activity can vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch.

**6. Expression of results**

Citric acid concentration is given in milligrams per liter to the nearest whole number.

6.1 *Method of calculation*

The general formula for calculating the concentration in mg/L is:

$$C=\frac{VxM}{exdxv}x\Delta A$$

where:

V = volume of test solution in mL (3.14 mL)

*v* = volume of the sample in mL (0.2 mL)

M = molecular mass of the substance to be determined
(for anhydrous citric acid, M = 192.1)

*d* = optical path in the cell in cm (1 cm)

ε = absorption coefficient of NADH, (at 340 nm, ε = 6.3 $mmol^{-1}$ x l x $cm^{-1}$).

so that:

$$C = 479 \times \Delta A$$

If the sample was diluted during its preparation, multiply the result by the dilution factor.

*Note:*

- At 334 nm: C = 488 x Δ*A* (ε= 6.3 $mmol^{-1}$ x l x $cm^{-1}$).
- At 365 nm: C = 887 x Δ*A* (ε= 3.4 $mmol^{-1}$ x l x $cm^{-1}$).

6.2 *Repeatability (r)*

Citric acid concentration less than 400 mg/L: *r* = 14 mg/L.
Citric acid concentration greater than 400 mg/L: *r* = 28 mg/L.

6.3 *Reproducibility (R)*

Citric acid concentration less than 400 mg/L: *R* = 39 mg/L.
Citric acid concentration greater than 400 mg/L: *R* = 65 mg/L.

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**Method OIV-MA-AS313-10** Type IV method

# Total Malic Acid

## 1. Principle

Malic acid, separated by means of an anion exchange column, is determined colorimetrically in the eluent by measuring the yellow coloration it forms with chromotropic acid in the presence of concentrated sulfuric acid. A correction for interfering substances is made by subtracting the absorbance, obtained using 86% sulfuric and chromotropic acid respectively (malic acid does not react at these acid concentrations), from the absorbance obtained from using 96% strength acids.

## 2. Apparatus

2.1 Glass column 250 mm approximately in length and 35 mm internal diameter, fitted with drain tap.

2.2 Glass column approximately 300 mm in length and 10 to 11 mm internal diameter, fitted with drain tap.

2.3 Thermostatically controlled water bath at 100°C.

2.4 Spectrophotometer set to measure absorbance at 420 nm using cells of 1 cm optical path.

## 3. Reagents

3.1 A strongly basic anion exchanger (e.g. Merck III)

3.2 Sodium hydroxide, 5% (*m/v*).

3.3 Acetic acid, 30% (*m/v*).

3.4 Acetic acid, 0.5% (*m/v*).

3.5 Sodium sulfate solution, 10% (*m/v*).

3.6 Concentrated sulfuric acid, 95-97% (*m/m*).

3.7 Sulfuric acid, 86% (*m/m*).

3.8 Chromotropic acid, 5% (*m/v*).

Prepare fresh solution before each determination by dissolving 500 mg sodium chromotropate, $C_{10}H_6Na_2O_8S_2.2H_2O$, in 10 mL distilled water

3.9 0.5 g DL-malic acid per liter solution

Dissolve 250 g malic acid ($C_4H_6O_5$) in sodium sulfate solution, 10%, to obtain 500 mL.

## 4. Procedure

4.1 *Preparation of ion exchanger*

Place a plug of cotton impregnated with distilled water in a 35 x 250 mm glass column. Pour a suspension of the anion exchange resin into the glass column. The level of the liquid should be 50 mm above the top of the resin. Rinse with 1000 mL of distilled water. Wash the column with sodium hydroxide solution, 5%, allow to drain to approximately 2 to 3 mm of the top of the resin and repeat with two further washings of sodium hydroxide, 5%, and leave for one hour. Wash the column with 1000 mL of distilled water. Refill the column with acetic acid, 30%, allow to drain to approximately 2 to 3 mm above the top of the resin and repeat with two further washings of acetic acid, 30%. Leave for at least 24 hours before use. Keep the ion exchange resin in acetic acid, 30%, for the subsequent analysis.

4.2 *Preparation of ion exchange column.*

Place a plug of cotton wool at the bottom of the column measuring 11 x 300 mm above the tap. Pour in the ion exchanger prepared as described above in 4.1 to a height of 10 cm. Open the tap and allow the acetic acid solution, 30%, to drain to approximately 2 to 3 mm above the surface of the exchanger. Wash the exchanger with a 50 mL acetic acid solution, 0.5%.

4.3 *Separation of DL-Malic acid*

Pour onto the column (4.2) 10 mL of wine or must. Allow to drain drop by drop (average rate of one drop per second) and stop the flow 2 to 3 mm from the top of the resin. Wash the column with 50 mL acetic acid, 0.5% (*m/v*), then with 50 mL of distilled water and allow to drain at the same rate as previously, stopping the flow 2 to 3 mm from the top of the resin.

Elute the acids absorbed on the exchange resin with sodium sulfate solution, 10%, at the same rate as in the previous steps (1 drop/sec). Collect the eluate in a 100 mL volumetric flask. The ion exchange column can be regenerated using the procedure described in 4.1

4.4 *Determination of malic acid*

Take two wide necked 30 mL tubes fitted with ground glass stoppers, A and B. In each tube add 1.0 mL of the eluate and 1.0 mL chromotropic acid solution, 5%. Add to tube A 10.0 mL sulfuric acid, 86% (*m/m*), (reference) and to the tube B 10.0 mL sulfuric acid, 96% (*m/m*), (sample). Stopper and shake to homogenize carefully, without wetting the glass stopper. Immerse the tubes in a boiling water bath for exactly 10 min. Cool the tubes in darkness at 20 C for exactly 90 min. Immediately measure the absorbance of tube B relative to the sample tube A at 420 nm in 1 cm cells.

4.5 *Plotting the calibration curve*

Pipette 5, 10, 15 and 20 mL of the DL-malic acid solution (0.5g/L) into separate 50 mL volumetric flasks. Make up to the mark with sodium sulfate solution, 10%.

These solutions correspond to eluates obtained from wines containing 0.5, 1.0, 1.5 and 2.0 g DL-malic acid per liter.

Continue as indicated in 4.4. The graph of the absorbencies of these solutions verses their malic acid concentration should appear as a straight line passing through the origin.

The intensity of the coloration depends to a large extent on the strength of the sulfuric acid used. It is necessary to check the calibration curve to see if the concentration of the sulfuric acid has changed.

**5. Expression of results**

Plot the absorbance on calibration graph to obtain the content of DL-malic acid in grams per liter. This content is expressed with 1 decimal.

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**Method OIV-MA-AS313-11** Type II method

# L-Malic acid

## 1. Principle of the method

L-malic acid (L-malate) is oxidized by nicotinamide adenine dinucleotide (NAD) to oxaloacetate in a reaction catalysed by L-malate dehydrogenase (L-MDH). The equilibrium of the reaction normally lies more strongly in favour of the malate. Removal of the oxaloacetate from the reaction mixture displaces the equilibrium towards the formation of oxaloacetate. In the presence of L-glutamate, the oxaloacetate is transformed into L-aspartate in a reaction catalysed by glutamate oxaloacetate transaminase (GOT):

(1) L-malate + $NAD^+$ $\overset{\text{L-MDH}}{\rightleftharpoons}$ oxaloacetate + NADH + $H^+$

(2) Oxaloacetate + L-glutamate $\overset{\text{GOT}}{\rightleftharpoons}$ L-aspartate + $\alpha$-ketoglutarate

The amount of NADH formed, measured by the increase in absorbance at the wavelength of 340 nm, is proportional to the quantity of L-malate originally present.

## 2. Apparatus

2.1. A spectrophotometer permitting measurement to be made at 340 nm, the wavelength at which absorption by NADH is at a maximum. Failing that, a spectrophotometer, with a discontinuous spectrum source permitting measurements to be made at 334 or 365 nm, may be used.

Since absolute measurements of absorbance are involved (i.e. calibration curves are not used, but standardization is made by consideration of the extinction coefficient of NADH), the wavelength scales and spectral absorbance of the apparatus must be checked.

2.2. Glass cells with optical path lengths of 1 cm or single-use cells.

2.3. Micropipettes for pipetting sample volumes in the range 0,01 to 2 ml.

## 3. Reagents

Doubly distilled water

3.1. *Buffer solution, pH 10*

(glycylglycine 0,6 M; L-glutamate 0,1 M):

dissolve 4,75 g of glycylglycine and 0,88 g of L-glutamic acid in approximately 50 ml of doubly distilled water; adjust the pH to 10 with about 4,6 ml of 10 M sodium hydroxide and make up to 60 ml with doubly distilled

water. This solution will remain stable for at least 12 weeks at 4 °C.

3.2. Nicotinamide adenine dinucleotide (NAD) solution, approximately 47 × 10 3 M: dissolve 420 mg of NAD in 12 ml of doubly distilled water. This solution will remain stable for at least four weeks at 4 °C.

3.3. Glutamate oxaloacetate transaminase (GOT) suspension, 2 mg/ml. The suspension remains stable for at least a year at 4 °C.

3.4. L-malate dehydrogenase (L-MDH) solution, 5 mg/ml. This solution remains stable for at least a year at 4 °C.

Note: All the reagents above are available commercially.

## 4. Preparation of the sample

L-malate determination is normally carried out directly on the wine, without prior removal of pigmentation (colouration) and without dilution provided that the L–malic acid concentration is less than 350 mg/l (measured at 365 mg/l). If this is not so, dilute the wine with doubly distilled water until the L-malate concentration lies between 30 and 350 mg/l (i.e. amount of L-malate in the test sample lies between 3 and 35 µg).

If the malate concentration in the wine is less than 30 mg/l, the volume of the test sample may be increased up to 1 ml. In this case, the volume of water to be added is reduced in such a way that the total volumes in the two cells are equal.

## 5. Procedure

With the spectrophotometer adjusted to a wavelength of 340 nm, determine the absorbance using the cells having optical paths of 1 cm, with air as the zero absorbance (reference) standard (no cell in the optical path) or with water as the standard.

Place the following in the cells having 1 cm optical paths:

| | Reference cell (ml) | Sample cell (ml) |
|---|---|---|
| Solution 3.1 | 1,00 | 1,00 |
| Solution 3.2 | 0,20 | 0,20 |
| Doubly distilled water | 1,00 | 0,90 |
| Suspension 3.3 | 0,01 | 0,01 |
| Sample to be measured | - | 0,10 |

Mix; after about three minutes, measure the absorbances of the solutions in the reference and sample cells (A1).

Add:

Solution 3.4 ..... 0,01 ml

0,01 ml

Mix; wait for the reaction to be completed (about 5 to 10 minutes) and measure the absorbances of the solutions in the reference and sample cells ($A_2$).

Calculate the differences ($A_2 - A_1$) in the absorbances of the solutions in the reference and sample cells, $\Delta A_R$ and $\Delta A_S$.

Finally, calculate the difference between those differences: $\Delta A = \Delta A_S - \Delta A_R$

Note: The time needed for the completion of enzyme activity can vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch.

**6. Expression of results**

L-malic acid concentration is given in grams per litre to one decimal place.

6.1. *Method of calculation*

The general formula for calculating the concentration in g/l is:

$$C = \frac{V \times PM}{\varepsilon \times \delta \times 1000} \times \Delta A$$

where:

V = volume of test solution in ml (here 2,22 ml)

v = volume of the sample in ml (here 0,1 ml)

M = molecular mass of the substance to be determined (here, for L-malic acid, M=134,09)

$\delta$ = optical path in the cell in cm (here, 1 cm)

$\varepsilon$ = absorption coefficient of NADH, (at 340 nm

$\varepsilon$ = 6,3 m mol 1 × l × cm 1),

so that for L-malate:

$$C = 0{,}473 \times \Delta A \text{ g/l}$$

If the sample was diluted during its preparation, multiply the result by the dilution factor.

Note:

- Measurement at 334 nm, $\varepsilon$ = 6,2 ($mmole^{-11}$ x 1 x $cm^2$)
  $C = 0{,}482 \times \Delta A$

- Measurement at 365 nm, $\varepsilon$ = 6,2 ($mmole^{-1\text{-}1}$x l x $cm^2$)
  $C = 0{,}876 \times \Delta A$

6.2. *Repeatability (*r*)*

$r = 0{,}03 + 0{,}034\ x_i$

$x_i$ is the malic acid concentration in the sample in g/l.

6.3. *Reproducibility (*R*)*

$R = 0{,}05 + 0{,}071\ x_i$

$x_i$ is the malic acid concentration in the sample in g/l.

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**Method OIV-MA-AS313-12A** Type II method

# D-Malic acid

Enzymatic method

## 1. Principle

In the presence of D-malate-dehydrogenase (D-MDH), D-malic acid (D-malate) is oxidized to oxalo-acetate by nicotinamide-adenine-dinucleotide (NAD). The formed oxalo-acetate is transformed into pyruvate and carbon dioxide.

(1) D-malate + NAD $\overset{+\text{ D-MDH}}{\rightleftharpoons}$ pyruvate + $CO_2$ + NADH + $H^+$

The formation of NADH, measured by the increase of absorbance for 334, 340 or 365 nm wave lengths, is proportional to the quantity of D-malate present.

## 2. Reagents

Reagents that allow 30 determinations to be made are marketed in a set which includes:

1/ Flask 1 containing about 30 ml of solution of Hepes buffer acid [N-(2-hydroxyethyl)piperazine-N'-2-ethane sulfonic] pH = 9.0 and stabilizers;
2/ Flask 2 containing about 210 mg of NAD lyophilizate;
3/ Flask 3 (three flasks), containing D-MDH lyophilizate, with a titer of about 8 units.

*Preparation of the solutions*

1/ Use the content of flask 1 without dilution. Bring the solution to a temperature of 20-25°C before using it.
2/ Dissolve the content of flask 2 in 4 ml of double-distilled water.
3/ Dissolve the content of one the flasks 3 in 0,6 ml of double-distilled water. Bring the solution to a temperature of 20-25 °C before using it.

*Stability of the solutions*

The contents of flask 1 can be kept for at least one year at + 4°C; solution 2 can be kept about 3 weeks at + 4 °C and 2 months at - 20 °C; solution 3 can be kept 5 days at + 4 °C.

## 3. Apparatus

3.1. Spectrophotometer which is able to measure at the NADH absorption maximum of 340 nm. If this is not available, a spectrophotometer with a discontinuous spectrum source permitting measurements to be made at 334 or 365 nm may be used. Since absolute absorbance measurements are involved (i.e. calibration curves are not used, but standardization is made by

consideration of the extinction coefficient of NADH), the wavelength scales and spectral absorbance of the apparatus must be checked.

3.2. Cells with a 1 cm path of glass or single-use cells.

3.3. Micropipettes capable of pipetting volumes between 0.01 and 2 ml.

## 4. Preparation of the sample

The analysis of D-malate is generally carried out directly on the wine without preliminary decoloration.

The quantity of D-malate in the cell must be between 2 µg and 50 µg; wine should be diluted so the malate concentration will be between 0.02 and 0.5 g/L or 0.02 and 0.3 g/L depending on the apparatus used.

Dilution table:

| Estimated quantity of D-malate/liter | | Dilution with water | Dilution factor F |
|---|---|---|---|
| Measured at: | | | |
| 340 or 334 nm | 365 nm | | |
| < 0.3 g | < 0.5 g | - | 1 |
| 0.3-3.0 g | 0.5-5.0 g | 1 + 9 | 10 |

## 5. Procedure

With the spectrophotometer adjusted to a wavelength of 340 nm, determine the absorbance using 1 cm cells, with air as the zero absorbance (reference) standard (no cell in the optical path) or with water as the standard.

Place the following in the 1 cm cells:

| | Reference cell (mL) | Sample cell (mL) |
|---|---|---|
| Solution 1 | 1.00 mL | 1.00 mL |
| Solution 2 | 0.10 mL | 0.10 mL |
| Double-distilled Water | 1.80 mL | 1.70 mL |
| Sample | - | 0.10 mL |

Mix: after approximately 6 minutes, measure the absorbance of the reference and sample solutions ($A_1$).

Add

| | Reference | Sample |
|---|---|---|
| Solution 3 | 0.05 mL | 0.05 mL |

Mix: wait for the end of the reaction (about 20 min.) and measure the absorbance of the reference and sample solutions ($A_2$).

Determine the absorbance differences ($A_2$ - $A_1$) of the control ($\Delta A_T$) and trial ($\Delta A_D$).

Deduct the control absorbance difference from the trial absorbance difference:

$$\Delta A = \Delta A_D - \Delta A_T$$

Comment: the time required for the enzymes' action can vary from one batch to the other. It is given here only as an indication. It is recommended it be determined for each batch.
D-malic acid reacts rapidly. An additional activity of the enzyme also transforms L-tartaric acid even though it is not as rapid. This is the reason why there is a small side reaction which may be corrected by means of extrapolation (see annex 1).

**6. Expression of the results**

The concentration in milligrams per liter is calculated with the general formula:

$$C = \frac{V \times PM}{\varepsilon \times d \times \nu} \times \Delta A$$

V = volume of the test in ml (here 2.95 mL)
ν = volume of the sample in ml (here 0.1 mL)
PM = molecular mass of the substance to be measured
(here, D-malic acid = 134.09)
d = cell path length in cm (here 1 cm)
ε = absorption coefficient of NADH:
at 340 nm = 6.3 ($l\ mmol^{-1}\ cm^{-1}$)
at 365 nm = 3.4 ($l\ mmol^{-1}\ cm^{-1}$)
at 334 nm = 6.18($l\ mmol^{-1}\ cm^{-1}$).

If a dilution was made during the preparation of the sample, multiply the result by the dilution factor. The concentration in D-malic acid is given in milligrams per liter (mg/L) without decimal.

**7. Accuracy**

The details of the interlaboratory trial on the accuracy of the method are summarized in annex 2. The derived values of the interlaboratory study may not be applicable to ranges of concentration of the analyte and to other matrices other than those given in annex 2.

7.1. *Repeatability*

The absolute difference between individual results obtained on an identical matter submitted to a trial by an operator using the same apparatus, within the shortest time interval, will not exceed the value of repeatability r in more than 5% of the cases. The value is: r = 11 mg/L.

7.2. *Reproducibility*

The absolute difference between individual results obtained on an identical material submitted to a test in two laboratories will not exceed the value of reproducibility R in more than 5% of the cases. The value is: R = 20 mg/L.

## 8. Comments

Taking into account the method's accuracy, the values of D-malic acid less than 50 mg/L must be confirmed by another analytical method using another measuring principle such as that of PRZYBORSKI et al, (1993). Values of D-malic acid less than 100 mg/L must not be interpreted as an addition of D, L-malic acid to wine.

The wine content in the cuvette must not exceed 0.1mL to avoid a possible inhibition of enzymatic activity by polyphenols.

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ANNEX 1

**How to treat side reactions**

Side reactions are generally due to secondary reactions of the enzyme, in the presence of other enzymes in the sample's matrix, or the interaction of one or several elements of the matrix with a co-factor of the enzymatic reaction.

With a normal reaction, absorbance reaches a constant value after a certain time, generally between 10 min and 20 min, according to the speed of the specific enzymatic reaction. However, when secondary reactions occur, the absorbance does not reach a constant value, but increases regularly with time; this type of process is commonly called a « side reaction ».

When this problem arises, one should measure the solution's absorbance at regular intervals (2 min to 5 min), after the required time for the standard solution to reach its final absorbance. When the absorbance increases regularly, carry out 5 or 6 measurements, than establish a graphic or calculated extrapolation, in order to obtain what the solution's absorbance would have been when the final enzyme was added (T0). The difference in extrapolated absorbance at this time (Af-Ai) is used for the calculation of the substrate concentration.

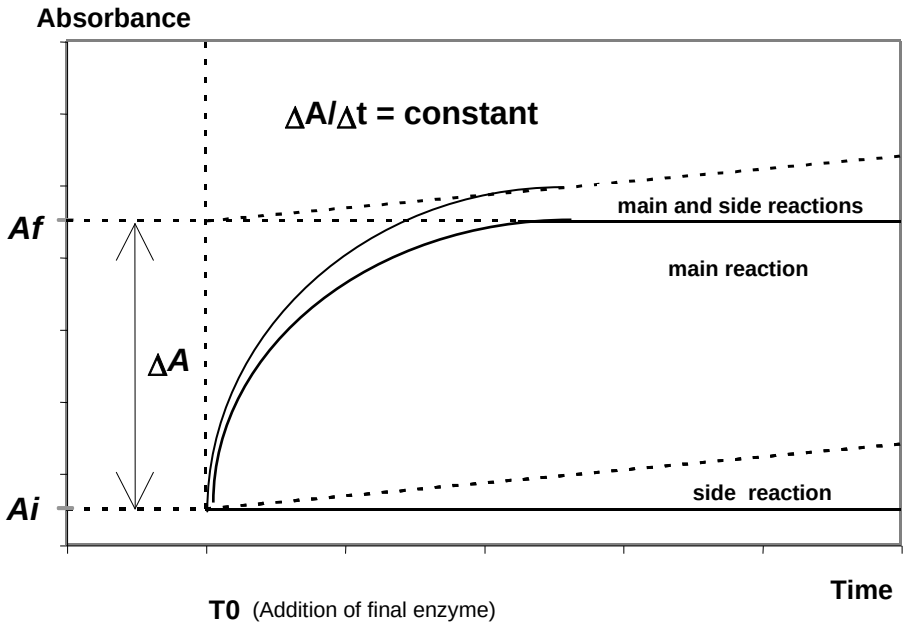


**Figure 1:** Side reaction

## ANNEX 2

**Interlaboratory trials statistical results**

Year of the interlaboratory trial 1995
Number of laboratories 8
Number of samples 5 with addition of D-malic acid

| Sample | A | B | C | D | E |
|---|---|---|---|---|---|
| Number of laboratories retained after elimination of laboratories presenting aberrant results | 7 | 8 | 7 | 8 | 7 |
| Number of laboratories presenting aberrant results | 1 | - | 1 | - | 1 |
| Number of accepted results | 35 | 41 | 35 | 41 | 36 |
| Average value($\xi$) (mg/L) | 161.7 | 65.9 | 33.1 | 106.9 | 111.0 |
| Standard deviation of repeatability ($s_r$) (mg/L) | 4.53 | 4.24 | 1.93 | 4.36 | 4.47 |
| Relative standard deviation of repeatability ($RSD_r$) (%) | 2.8 | 6.4 | 5.8 | 4.1 | 4.00 |
| Limit of repeatability (r) (mg/L) | 12.7 | 11.9 | 5.4 | 12.2 | 12.5 |
| Standard deviation of reproducibility ($s_R$) (mg/L) | 9.26 | 7.24 | 5.89 | 6.36 | 6.08 |
| Relative standard deviation of reproducibility ($RSD_R$) (%) | 5.7 | 11 | 17.8 | 5.9 | 5.5 |
| Limit of reproducibility (R) (mg/L) | 25.9 | 20.3 | 16.5 | 17.8 | 17.0 |

Types of samples:

| | | | |
|---|---|---|---|
| A | red wine | C | white wine |
| B | red wine | D | white wine |
| | | E | white wine |

**Method OIV-MA-AS313-12B** Type IV method

# Determination of d-malic acid in wines at low concentrations using the enzymatic method

(Resolution Oeno 16/2002)

## 1. FIELD OF APPLICATION

*The method described is applied to dosage, by the enzymatic means, of malic acid D of wines with contents under 50 mg/l.*

## 2. PRINCIPLE

The principle of the method is based on malic acid D(+) oxidation (D-malate) by nicotinamide-adenine-dinucleotide (NAD) in oxaloacetate that is transformed into pyruvate and carbon dioxide; the formation of NADH, measured by the increase of absorbance in wave length at 340 nm, is proportional to the quantity of D-malate present (principle of the method described for malic acid D determination for concentrations above 50 mg/l), after introducing a quantity of malic acid D of 50 mg/l in a cuvette.

## 3. REAGENTS

*Malic acid D solution of 0.199 g/l, above reagents indicated in the methods described for contents above 50 mg/l.*

## 4. APPARATUS

*Apparatus indicated in the method described for concentration above 50 mg/l.*

## 5. SAMPLE PREPARATION

Sample preparation is indicated in the method described for concentrations above 50 mg/l.

## 6. PROCEDURE

The procedure is indicated in the method described for concentrations above 50 mg/l. (Resolution Oeno 6/98), but with the introduction in the tank of a quantity of malic acid D equivalent to 50 mg/l. (Introduction of 0.025 mL of malic acid D at 0.199 g/l, substituting the equivalent volume of water); the values obtained are decreased by 50 mg/l.

## 7. INTERNAL VALIDATION

Summary of the internal validation file on the dosage of malic acid D(+)- after the addition of 50 mg/l of this isomer

| | |
|---|---|
| Work level | 0 mg of 70 mg of malic acid D(+)-per liter.<br>Within these limits, the method is linear with a correlation coeffiency between 0.990 and 0.994 |
| Setting limit | 24.4 mg/l |
| Detection limit | 8.3 mg/l |
| Sensitivity | 0.0015 abs / mg/l |
| Recovery percent range | 87.5 to 115.0% for white wines and 75 to 105% for red wines |
| Repeatability | =12.4 mg/l for white wines<br>(according to the OIV method =12,5 mg/l)<br>=12.6 mg/l for red wines<br>(according to OIV method=12,7 mg/l) |
| Percentage standard deviation | 4.2% to 7.6% (white wines and red wines) |
| Intralaboratory variability | CV=7.4% (s=4.4mg/l; X average=59.3 mg/l) |

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**Method OIV-MA-AS313-13A** Type IV method

# L-Ascorbic acid

(Resolution Oeno 377/2009)

## 1. Principle

The following methods enable the presence of L-ascorbic acid and dehydroascorbic acid in wines or musts to be determined.

Ascorbic acid is converted on activated carbon to dehydroascorbic acid. The latter forms a fluorescent compound on reaction with orthophenylenediamine (OPDA). A control prepared in the presence of boric acid enables spurious fluorescence to be determined (by the formation of a boric acid/dehydroascorbic acid complex). The sample and the control are analyzed fluorometrically and the concentration of dehydroascorbic acid calculated.

## 2. Method (fluorimetric method)

2.1 *Apparatus*

2.1.1 Fluorometer.

A spectrofluorometer equipped with a lamp giving a continuous spectrum and using it at minimum power.

The optimum excitation and emission wavelengths for the test are to be determined beforehand and depend on the equipment used. As a guide, the excitation wavelength will be approximately 350 nm and the emission wavelength approximately 430 nm. Cells of 1 cm path length.

2.1.2 Sintered glass filter of porosity 3.

2.1.3 Test tubes (diameter approximately 10 mm).

2.1.4 Stirring rods for test tubes.

2.2 *Reagents*

2.2.1 Orthophenylenediamine dihydrochloride solution ($C_6H_{10}Cl_2N_2$), 0.02 % (*m/v*), prepared just before use.

2.2.2 Sodium acetate trihydrate solution ($CH_3COONa \cdot 3H_2O$), 500 g/L.

2.2.3 Mixed solution of boric acid and sodium acetate:

Dissolve 3 g of boric acid, ($H_3BO_3$) in 100 mL of a 500 g/L sodium acetate solution. This solution must be prepared just before use.

2.2.4 Acetic acid solution ($CH_3COOH$) 56%: glacial acetic acid ($\rho_{20}$= 1.05 g/mL), diluted to 56% (*v/v*), pH approximately 1.2.

2.2.5 L-Ascorbic acid standard solution, 1 g/L.

Just before use, dissolve 50 mg of L-ascorbic acid previously dehydrated in a desiccator and protected against light, in 50 mL of acetic acid solution (2.2.4).

2.2.6 Very pure analytical grade activated carbon.

Place 100 g of activated carbon into a 2-liter conical flask and add 500 mL aqueous hydrochloric acid solution, 10% (*v/v*), ($\rho_{20}$= 1.19 g/mL). Bring to a boil, and filter through a sintered glass filter of porosity 3. Collect the carbon treated in this way in a 2-liter conical flask. Add 1 liter of water, shake and filter using a sintered glass filter of porosity 3. Repeat this operation two more times. Place the residue in an oven controlled to 115°C ± 5 °C for 12 hours (or overnight).

2.3 *Procedure*

2.3.1 Preparation of the sample of wine or must

Take a volume of the wine or must and dilute to 100 mL in a graduated flask with the acetic acid solution, 56% (2.2.4), in order to obtain a solution with an ascorbic acid concentration between 0 and 60 mg/L. Thoroughly mix the contents of the flask by shaking. Add 2 g of activated carbon and allow to stand for 15 minutes, shaking occasionally. Filter using ordinary filter paper, discarding the first few milliliters of filtrate.

Pipette 5 mL of the filtrate into two 100 mL graduated flasks. Add to the first 5 mL of the mixed solution of boric acid and sodium acetate solution (2.2.3) (sample blank) and to the second 5 mL of the sodium acetate solution (2.2.2) (sample). Allow to stand for 15 minutes, stirring occasionally. Make to 100 mL with distilled water. Pipette 2 mL from the contents of each flask into a test tube and add 5 mL of orthophenylenediamine solution. Stir with the stirring rod and allow the reaction proceed for 30 minutes in the dark and then make the spectrofluorometric measurements.

2.3.2.Preparation of the calibration curve.

Into three 100 mL graduated flasks pipette 2, 4, and 6 mL respectively of the standard ascorbic acid solution (2.2.5), make to 100 mL with acetic acid solution and thoroughly mix by stirring. The standard solutions prepared in this way contain 2, 4 and 6 mg per 100 mL of L-ascorbic acid respectively.

Add 2 g of activated carbon to each of the flasks and allow to stand for 15 minutes, stirring occasionally. Filter through ordinary filter paper, discarding the first few milliliters. Pipette 5 mL of each filtrate into three 100 mL graduated flasks (first series). Repeat the operation and obtain a second series of three graduated flasks. To each of the flasks in the first series

(corresponding to the blank test) add 5 mL of the mixed solution of boric acid and sodium acetate (2.2.3), and to each of the flasks in the second series add 5 mL of the sodium acetate solution (2.2.2). Let stand for 15 minutes, stirring occasionally. Make up to 100 mL with distilled water. Take 2 mL of the contents of each flask; add 5 mL of orthophenylenediamine solution. Stir and allow the reaction to proceed for 30 minutes in the dark and then make the spectrofluorometric measurements.

2.3.3 Fluorometric determination

Set the zero on the scale of measurement using the corresponding control test sample for each solution. Measure the intensity of the fluorescence for each solution over the calibration range and for the solution to be determined. Plot the calibration curve, which should be a straight line passing through the origin. From the graph determine the concentration C of ascorbic acid and dehydroascorbic acid in the solution analyzed.

2.4 *Expression of results*

The concentration of L-ascorbic acid and the dehydroascorbic acid in the wine in milligrams per liter is given by:

$$C \times F$$

where F is the dilution factor.

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**Method OIV-AS313-13B** Type IV method

# L-Ascorbic acid

(Resolution Oeno 377/2009)

WITHDRAWN

**Method OIV-MA-AS313-14A** Type IV method

# Sorbic acid

## 1. Principle of Method

*Determination using ultraviolet absorption spectrophotometry*

Sorbic acid (*trans, trans*, 2,4-hexadienoic acid) extracted by steam distillation is determined in wine distillate by ultraviolet absorption spectrophotometry. Substances that interfere with the measure of absorption in ultraviolet are removed by evaporation to dryness using a slightly alkaline calcium hydroxide solution. Samples with less than 20 mg/L are confirmed using thin layer chromatography (sensitivity: 1 mg/L).

## 2. Determination by ultraviolet absorption spectrophotometry

2.1 *Apparatus*

2.1.1 Steam distillation apparatus (see chapter "Volatile Acidity")

2.1.2 Water bath 100 °C

2.1.3 Spectrophotometer allowing absorbance measurements to be made at a wavelength of 256 nm and having a quartz cell with a 1 cm optical path

2.2 *Reagents*

2.2.1 Crystalline tartaric acid

2.2.2 Calcium hydroxide solution, approx. 0.02 M

2.2.3 Sorbic acid standard solution, 20 mg/L:

Dissolve 20 mg sorbic acid in approximately 2 mL 0.1 M sodium hydroxide solution. Pour into a 1 L volumetric flask, and make up to volume with water. Alternatively dissolve 26.8 mg of potassium sorbate, $C_6H_7KO_2$, in water and make up to 1 L with water.

2.3 *Procedure*

2.3.1 Distillation

Place 10 mL of wine in the bubbler of the steam distillation apparatus and add about 1 g tartaric acid. Collect 250 mL of distillate.

2.3.2 Preparation of the calibration curve

Prepare, by dilution of the standard solution (2.2.3) with water, four dilute standard solutions containing 0.5, 1.0, 2.5 and 5 mg of sorbic acid per liter.

Measure their absorbance with the spectrophotometer at 256 nm using distilled water as a blank. Plot a curve showing the variation of absorbance as a function of concentration. The relationship is linear.

2.3.3 Determination

Place 5 mL of the distillate in an evaporating dish of 55 mm diameter, add 1 mL of calcium hydroxide solution (2.2.2). Evaporate to dryness on a boiling water bath. Dissolve the residue in several mL of distilled water, transfer completely to a 20 mL volumetric flask and bring to volume with the rinsing water. Measure the absorbance at 256 nm using a solution obtained by diluting 1 mL of calcium hydroxide solution to 20 mL with water as the blank. Plot the value of the absorbance on the calibration curve and from this interpolate the concentration *C* of sorbic acid in the solution.

*Note:* In this method the loss due to evaporation is negligible and the absorbance is measured on the treated distillate diluted 1/4 with distilled water.

2.4 *Expression of results*

2.4.1 Calculation

The sorbic acid concentration in the wine expressed in mg/L is given by:

$$100 \times C$$

*C* = concentration of sorbic acid in the solution obtained in 2.3.3 expressed in mg/L.

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**Method OIV-MA-AS313-14B** Type IV method

# Sorbic acid

## 1. Principle of Methods

*Determination by gas chromatography*

Sorbic acid extracted in diethyl ether is determined by gas chromatography using an internal standard.

## 2. Determination by gas chromatography

2.1 *Apparatus*

2.1.1. Gas chromatograph fitted with a flame ionization detector and a stainless steel column (4 m x 1/8 inch) previously treated with dimethyldichlorosilane and packed with a stationary phase consisting of a mixture of diethyleneglycol succinate, 5%, and phosphoric acid, 1%, (DEGS - $H_3PO_4$), or of a mixture of diethyleneglycol adipate, 7%, and phosphoric acid, 1%, (DEGA - $H_3PO_4$) bonded on Gaschrom Q 80 - 100 mesh.

Treatment of column with dimethyldichlorosilane (DMDCS): pass a solution containing 2 to 3 g of (DMDCS) in toluene through the column.

Immediately wash with methanol, followed by nitrogen and then wash with hexane followed by more nitrogen. The column is now ready to be packed.

Operating conditions:

- Oven temperature: 175 °C
- Temperature of the injector and detector: 230 °C.
- Carrier gas: nitrogen (flow rate = 200 mL/min)

*Note:* Other types of columns can also give a good separation, particularly capillary columns (e.g. FFAP). The working method described below is given as an example.

2.1.2 Microsyringe, 10 µL capacity graduated in 0.1 µL.

2.2 *Reagents*

2.2.1 Diethyl ether distilled just before use

2.2.2 Internal standard: solution of undecanoic acid, $C_{11}H_{22}O_2$, 1 g/L in ethanol, 95% (*v/v*)

2.2.3. Aqueous solution of sulfuric acid, $H_2SO_4$, ($\rho_{20}$ = 1.84 g/mL) diluted 1/3 (*v/v*)

2.3 *Procedure*

2.3.1 Preparation of sample to be analyzed

Into a glass test tube of approximately 40 mL capacity and fitted with a ground glass stopper, place 20 mL of wine, 2 mL of the internal standard (2.2.2) and 1 mL of dilute sulfuric acid.

After mixing the solution by repeatedly turning the tube over, add 10 mL of diethyl ether (2.2.1). Extract the sorbic acid into the organic phase by shaking the tube for five minutes. Allow to settle.

2.3.2 Preparation of the spiked sample

Select a wine for which the chromatogram of the ether extract shows no peak corresponding to the elution of sorbic acid. Fortify this wine with sorbic acid at a concentration of 100 mg/L. Treat 20 mL of the sample prepared in this way according to the procedure described in 2.3.1.

2.3.3. Chromatography

Inject 2 µL of the ether-extract phase obtained in 2.3.2, into the chromatograph using a microsyringe, followed by 2 µL of the ether-extracted phase obtained in 2.3.1.

Record the respective chromatograms: check the identity of the respective retention times of the sorbic acid and the internal standard. Measure the height (or area) of each of the recorded peaks.

2.4 *Expression of results*

2.4.1 Calculation

The concentration of sorbic acid in the analyzed wine, expressed in mg/L, is given by:

$$100 \times \frac{h}{H} \times \frac{I}{i}$$

where

H = height of the sorbic acid peak in the spiked solution
*h* = height of the sorbic acid peak in the sample for analysis
I = height of the internal standard peak in the spiked solution
*i* = height of the internal standard peak in the sample for analysis

*Note:* The sorbic acid concentration may be determined in the same way from measurements of the respective peak areas.

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**Method OIV-MA-AS313-14C** Type IV method

# Sorbic acid

## 1. Principle of Methods

*Identification of traces by thin-layer chromatography*

Sorbic acid extracted in ethyl ether is separated by thin layer chromatography and its concentration is evaluated semi-quantitatively.

## 2. Identification of traces of sorbic acid by thin layer chromatography

2.1 *Apparatus*

2.1.1 Precoated 20 x 20 cm plates for thin layer chromatography coated with polyamide gel (0.15 mm thick) with the addition of a fluorescence indicator

2.1.2 Chamber for thin layer chromatography

2.1.3 Micropipette or microsyringe for delivering volumes of 5 µL to within ± 0.1 µL

2.1.4 Ultraviolet lamp (254 nm)

2.2. *Reagents*

2.2.1 Diethyl ether, $(C_2H_5)_2$ O

2.2.2 Aqueous sulfuric acid solution: sulfuric acid ($\rho_{20}$= 1.84 g/mL), diluted 1/3 (*v*/*v*)

2.2.3 Standard solution of sorbic acid, approximately 20 mg/L, in a 10% (*v/v*) ethanol/water mixture.

4.2.4Mobile phase: hexane + pentane + acetic acid (20:20:3).

4.2.5

2.3 *Procedure*

2.3.1 Preparation of sample to be analyzed

Into a glass test tube of approximately 25 mL capacity and fitted with a ground glass stopper, place 10 mL of wine; add 1 mL of dilute sulfuric acid (2.2.2) and 5 mL of diethyl ether (2.2.1). Mix by repeatedly inverting the tube. Allow to settle.

2.3.2 Preparation of dilute standard solutions

Prepare five dilute standard solutions from the solution in 2.2.3. containing 2, 4, 6, 8 and 10 mg sorbic acid per liter.

2.3.3 Chromatography

Using a microsyringe or micropipette, deposit 5 µL of the ether-extracted phase obtained in 2.3.1 and 5 µL each of the dilute standard solutions (2.3.2) at points 2 cm from the lower edge of the plate and 2 cm apart from each other.

Place the mobile phase in the chromatograph tank to a height of about 0.5 cm and allow the atmosphere in the tank to become saturated with solvent vapor. Place the plate in the tank. Allow the chromatogram to develop over 12 to 15 cm (development time approximately 30 minutes). Dry the plate in a current of cool air. Examine the chromatogram under a 254 nm ultraviolet lamp.

The spots indicating the presence of sorbic acid will appear dark violet against the yellow fluorescent background of the plate.

2.4 *Expression of the results*

A comparison of the intensities of the spots produced by the test sample and by the standard solutions will enable a semi-quantitative assessment of a sorbic acid concentration between 2 and 10 mg/L. A concentration equal to 1 mg/L may be determined by using a 10 µL sample size.

Concentrations above 10 mg/L may be determined using a sample volume of less than 5 µL (measured out using a microsyringe).

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**Method OIV-MA-AS313-15** Type I method

# pH

(A31, Oeno 438-2011)

## 1. Principle

The difference in potential between two electrodes immersed in the liquid under test is measured. One of these two electrodes has a potential that is a function of the pH of the liquid, while the other has a fixed and known potential and constitutes the reference electrode.

## 2. Apparatus

2.1 pH meter with a scale calibrated in pH units and enabling measurements to be made to at least ±0.01 pH units.

2.2 Electrodes:

- glass electrode, kept in distilled water;
- calomel-saturated potassium chloride reference electrode, kept in a saturated solution of potassium chloride; or,
- a combined electrode, kept in distilled water.

## 3. Reagents

– Buffer solutions:

- Saturated potassium hydrogen tartrate solution, containing 5.7 g/L potassium hydrogen tartrate ($CO_2HC_2H_4O_2CO_2K$) at 20°C. (This solution may be kept for up to two months by adding 0.1 g of thymol per 200 mL.)

$$\text{pH} \begin{cases} 3.57 \text{ at } 20\ ^{o}\text{C} \\ 3.56 \text{ at } 25\ ^{o}\text{C} \\ 3.55 \text{ at } 30\ ^{o}\text{C} \end{cases}$$

- Potassium hydrogen phthalate solution, 0.05 M, containing 10.211 g/L potassium hydrogen phthalate, $CO_2HC_6H_4CO_2K$, at 20°C. (This solution may be kept for up to two months.)

$$\text{pH} \begin{cases} 3.999 \text{ at } 15\ ^{o}\text{C} \\ 4.003 \text{ at } 20\ ^{o}\text{C} \\ 4.008 \text{ at } 25\ ^{o}\text{C} \\ 4.015 \text{ at } 30\ ^{o}\text{C} \end{cases}$$

Solution containing:
potassium *di*-hydrogen phosphate, $KH_2PO_4$ ...……..... 3.402 g
*di*-potassium hydrogen phosphate, $K_2HPO_4$ ....……..... 4.354 g
water to .....… …./....……. 1 litre
(This solution may be kept for up to two months)

pH { 6.90 at 15 °C
6.88 at 20 °C
6.86 at 25 °C
6.85 at 30 °C

Note: commercial reference buffer solutions traceable to the SI may be used.

For example: pH 1.679 ±0.01 at 25°C
pH 4.005 ±0.01 at 25°C
pH 7.000 ±0.01 at 25°C

**4. Procedure**

4.1 *Zeroing of the apparatus*

Zeroing is carried out before any measurement is made, according to the instructions provided with the apparatus used.

4.2 *Calibration of the pH meter*

The pH meter must be calibrated at 20°C using standard buffer solutions connected to the SI. The pH values selected must encompass the range of values that may be encountered in musts and wines. If the pH meter used is not compatible with calibration at sufficiently low values, a verification using a standard buffer solution linked to the SI and which has a pH value close to the values encountered in the musts and wines may be used.

4.3 *Determination*

Dip the electrode into the sample to be analyzed, the temperature of which should be between 20 and 25°C and as close as possible to 20°C. Read the pH value directly off the scale.

Carry out at least two determinations on the same sample.

The final result is taken to be the arithmetic mean of two determinations.

**5. Expression of results**

The pH of the must or the wine is reported to two decimal places.

**Method OIV-MA-AS313-16** Type IV method

# Determination of organic acids and mineral anions in wines by ionic chromatography

(Resolution Oeno 23/2004)

## Preamble

The development of high performance ionic chromatography in laboratories has enabled the study the determination of organic acids and mineral anions in alcoholic and non alcoholic beverages by this technique.
Particularly concerning the analysis of wines, the results of intercomparison test trials and the measurements of recovery rates have enabled us to validate an analytical methodology.
The major interest of this method is that the ion exchange columns allow the separation of most organic acids and anions, and the detection by conductimetry frees the analysis from interferences due to the presence of phenolic compounds. This type of interference is very notable in chromatographic methods that include detection in ultra-violet radiation at 210 nm.

## 1 - OBJECT AND FIELD OF APPLICATION

This method for mineral anions and organic acids by ionic chromatography is applicable to alcoholic beverages (wines, wine spirits and liqueurs). It enables the determination of organic acids in the ranges of concentration listed in table 1; these concentrations are obtained by diluting samples.

Table 1: range of concentration of anions for their analysis by ionic chromatography

| | | |
|---|---|---|
| Sulfate | 0.1 to 10 | mg/l |
| Ortho-phosphate | 0.2 to 10 | mg/l |
| Malic acid | 1 to 20 | mg/l |
| Tartaric acid | 1 to 20 | mg/l |
| Citric acid | 1 to 20 | mg/l |
| Isocitric acid | 0.5 to 5 | mg/l |

The ranges of the above-mentioned work are given as an example. They include the methods of calibration commonly practiced and are therefore adaptable

according to the type of apparatus used (nature of column, sensitivity of the detector, etc.) and procedure (volume of sample injected, dilution, etc.).

## 2- PRINCIPLE

Separation of mineral and organic anions on an ion exchanger resin.
Detection by conductimetry.
Identification after the retention time and quantification using the calibration curve.

## 3 - REAGENTS

All the reagents used during the analysis must be of analytical quality. The water used for the preparation of solutions must be distilled or deionised water of a conductivity lower than 0.06 µS, free from anions determined at thresholds compatible with the detection limits of the apparatus used.

### 3.1 Eluant

The composition of the eluant depends on the nature of the separation column and the nature of the sample to be analysed. Nevertheless it is always prepared using aqueous solutions of sodium hydroxide.
The performances of the chromatographic analysis are alternated by carbonation of the sodium hydroxide solution; consequently, the mobile phase flasks are swept with helium before adding sodium hydroxide and all precautions should be taken in order to avoid contaminating them with room air.
Lastly, commercial concentrated sodium hydroxide solutions will be used.

Remark

The table in chapter 9 mentions the main interferents susceptible of being present in the samples.
It is therefore necessary to know beforehand if they coelute with the ions to be determined and if they are present at such a concentration that the analysis is disrupted.

Fermented drinks contain succinic acid which can interfere with the malic acid determination. To this effect, it is necessary to add methanol to the eluant in order to improve the resolution of the column for these two substances (20% of methanol).

### 3.2 Calibration reference solutions

Prepare calibration reference solutions of precise concentrations close to those indicated in the following table. Dissolve in water, quantities of salts or corresponding acids in 1000 ml volumetric flasks. (Table 2)

Table 2: Concentration of anions determined in calibration reference solutions

| Anions and acids | Compounds weighed | Concentration final (mg/l) | Quantity weighed (mg) |
|---|---|---|---|
| Sulphate | Na2SO4 | 500 | 739.5 |
| Orthophosphate | KH2PO4 | 700 | 1003.1 |
| Malic acid | Malic acid | 1000 | 1000.0 |
| Tartaric acid | Tartaric acid | 1000 | 1000.0 |
| Citric acid | Citric acid, $H_2O$ | 1000 | 1093.8 |
| Isocitric acid | Isocitrate 3Na, $2H_2O$ | 400 | 612.4 |

Remark

The laboratory must take the necessary precautions regarding the hygroscopic character of certain salts.

### 3.3 Calibration solutions

The calibration solutions are obtained by diluting the reference solutions of each ion or acid in water.
These solutions should contain all the ions or acids determined in a range of concentrations covering those corresponding to the samples to be analysed. They must be prepared the day of their use.

At least two calibration solutions and a blank must be analysed so as to establish, for each substance, the calibration curves using three points (0, maximum semi-concentration, maximum concentration).

Remark

Table 1 gives indications on the maximum concentrations of anions and acids in calibration solutions but the performances of the chromatographic columns are better with very diluted solutions.
So the best adequation possible between the performances of the column and the level of dilution of the samples should be looked for.

In general, the sample is diluted between 50 and 200 times maximum except for particular cases.
For prolonging the life span of the dilution solutions, it is preferable to prepare them in a water/methanol solution (80/20).

## 4 - APPARATUS

**4.1 Instrument system for ionic chromatography including:**

4.1.1 Eluant reservoir(s)

4.1.2 Constant-stroke pump, without pulsing action

4.1.3 Injector, either manual or automatic with a loop sampling valve (for example 25 or 50 µl).

4.1.4 Separation columns

System made up of an anion exchanger column of controlled performance, possibly a precolumn of the same type as the main column. For example, it is possible to use the AS11 columns and DIONEX® AG11 precolumn.

4.1.5 Detection system

Circulation conductivity cell of very low volume connected to a conductivity meter with several ranges of sensitivity.
In order to lower the conductivity of the eluant, a chemical suppression mechanism, a cation exchanger is installed in front of the conductivity cell.

4.1.6 Recorder, integrator or other device for the treatment of signals.

**4.2 Precise balance to 1 mg**

**4.3 Volumetric flasks from 10 to 1000 ml**

**4.4 Calibrated pipettes from 1 to 50 ml**

**4.5 Filtrating membranes with an average pore diameter of 0.45 µm.**

## 5 - SAMPLING

The samples are diluted while taking into account the mineral anions and organic acids that are to be determined.
If their concentration is very variable in the sample, two levels of dilution will be necessary in order to respect the ranges of concentration covered by the calibration solutions.

## 6 - PROCEDURE

Turn on the apparatus by following the manufacturer's instructions.
Adjust the pumping (eluant flux) and detection conditions so as to obtain good separations of the peaks in the range of concentrations of ions to be analysed.

Allow the system to balance until a stable base line is obtained.

**6.1 Calibration**
Prepare the calibration solutions as indicated in 3.3.
Inject the calibration solutions so that the volume injected is at least 5 times that of the sampling loop to allow the rinsing of the system.
Trace the calibration curves for each ion. These must normally be straight.

**6.2 Blank trial**
Inject the water used for the preparation of the calibration solutions and samples.
Control the absence of parasite peaks and quantify the mineral anions present (chloride, sulphate, etc.).

**6.3 Analysis**
Dilute the sample possibly at two different levels as indicated in 5, so that the anions and acids to be determined are present in the range of concentrations of the calibration solutions.
Filter the diluted sample on a filtrating membrane (4.5) before injection.
Then proceed as for the calibration (6.1).

## 7 - REPEATABILITY, REPRODUCIBILITY

An interlaboratory circuit tested this method, but this does not constitute a formal validation according to The OIV protocol (Oeno 6/99).
A repeatability limit and a reproducibility limit for the determination of each ion in wine were calculated according to the ISO 5725 standard.
Each analysis was repeated 3 times.

Number of participating laboratories: 11; the results were as follows:

White wine

| | **No labs** | **Average** (mg/l) | **Repeatability** (mg/l) | **Reproducibility** (mg/l) |
|---|---|---|---|---|
| Malic acid | 11/11 | 2745 | 110 | 559 |
| Citric acid | 9/11 | 124 | 13 | 37 |
| Tartaric acid | 10/11 | 2001 | 96 | 527 |
| Sulphate | 10/11 | 253 | 15 | 43 |
| O.phosphate | 9/11 | 57 | 5 | 18 |

Red wine

| | **No labs** | **Average** (mg/l) | **Repeatability** (mg/l) | **Reproducibility** (mg/l) |
|---|---|---|---|---|
| Malic acid | 8/11 | 128 | 16 | 99 |
| Citric acid | 8/10 | 117 | 8 | 44 |
| Tartaric acid | 9/11 | 2154 | 48 | 393 |
| Sulphate | 10/11 | 324 | 17 | 85 |
| O.phosphate | 10/11 | 269 | 38 | 46 |

## 8 – CALCULATION OF RECOVERY RATE

**The supplemented sample is a white wine.**

| **Determination** | **No labs** | **Concentration initial (mg/l)** | **Real addition (mg/l)** | **Measured addition (mg/l)** | **Recovery rate (%)** |
|---|---|---|---|---|---|
| Citric acid | 11/11 | 122 | 25.8 | 24.2 | 93.8 |
| Malic acid | 11/11 | 2746 | 600 | 577 | 96.2 |
| Tartaric acid | 11/11 | 2018 | 401 | 366 | 91.3 |

## 9 - RISKS OF INTERFERENCES

Any substance whose retention time coincides with that of one of the ions analysed can constitute an interference.
The most common interference include the following:

| **Anions or** | **Interferents acids** |
|---|---|
| Nitrate | bromide |
| Sulphate | oxalate, maleate |
| Orthophosphate | phtalate |
| Malic acid | Succinic acid, Citramalic acid |
| Tartric acid | Malonic acid |
| Citric acid | - |
| Isocitric acid | - |

Remark: The addition of methanol in the mobile phase can resolve certain analytical problems.

**Method OIV-MA-AS313-17** Type II method

# Determination of shikimic acid in wine by HPLC and UV-detection

(Resolution Oeno 33/2004)

## 1. INTRODUCTION

Shikimic acid (3,4,5-Trihydroxy-1-cyclohexene-1-carboxylic acid) is biosynthetically synthesized from chinic acid by dehydration and plays a major role as a precursor of phenylanaline, tyrosine, tryptophan and plant alkaloids [1]. As a minor carboxylic acid shikimic acid is naturally found in a wide range of fruits [2].

Member states are encouraged to continue research in this area to avoid any non scientific evaluation of the results.

This method has been validated in an international collaborative study via the analyses of wine samples with naturally occurring amounts of shikimic acid ranging from about 10 to 150 mg/l. The trueness has been proved by an interlaboratory comparison using HPLC and GC/FID and GC/MS respectively [3].

## 2. SCOPE

This paper specifies an isocratic routine method for the quantitative determination of shikimic acid in red, rosé and white wine (included sparkling and special wines) at concentration levels ranging from 1 mg/l up to 300 mg/l by high performance liquid chromatography. When the method is applied to sparkling wine the samples must be previously degassed (for instance by sonication).

## 3. PRINCIPLE

Shikimic acid is determined directly without previous sample preparation by high performance liquid chromatography using a coupled column system. In a first step the organic acids in wine are pre-separated with a $C_{18}$ reversed phase column followed by a cation exchange column at 65 °C performing the final separation. By using slightly acidified water as elution solvent a baseline resolution of shikimic acid is achieved without any interferences from the wine matrix . Due to the double bond within the cyclohexene ring

system shikimic acid has a strong absorption and can therefore be detected easily with an UV-detector at its absorption maximum at 210 nm.

## 4. REAGENTS AND MATERIALS

**4.1** Shikimic acid (CAS 138-59-0) , at least 98 % pure

**4.2** Sulfuric acid 0,5 M

**4.3** Bidestilled water

**4.4** Preparation of the elution solvent ( 0,01 M $H_2SO_4$ )
Pipette 20 ml of the 1 N sulfuric acid (4.2) to a 1000 ml volumetric flask, fill up with bidestilled water (4.3) to about 900 ml, shake and adjust to 1000 ml. Filter the elution solvent with a filter of a pore size less than or equal to 0,45 µm and degas.

**4.5** Preparation of stock standard solution (500 mg/l shikimic acid)
Weigh exactly 50 mg shikimic acid (4.1), transfer them without loss to a 100 ml volumetric flask, fill up with bidestilled water (4.3) to about 90 ml, shake and adjust to 100 ml. At –18 °C the stock standard solution can be stored for months.

**4.6** Preparation of working standard solutions ( 5 , 25 , 50 , 100, 150 mg/l shikimic acid) Dilute stock solution 500 mg/l (4.5) appropriately with bidestilled water (4.3) to give five working standards of 5 , 25 , 50 , 100, 150 mg/l shikimic acid. Prepare working standard solutions daily.

## 5. APPARATUS

Usual laboratory equipment, in particular, the following:

**5.1** HPLC system capable of achieving baseline resolution of shikimic acid

**5.1.1** High-performance liquid chromatograph with a six-way injection valve fitted with a 5 µl loop or any other device, either automatic or manual, for a reliable injection of microvolumes

**5.1.2** Isocratic pumping system enabling one to achieve and maintain a constant or programmed rate of flow with great precision.

**5.1.3** Column heater enabling one to heat a 300 mm column to 65 °C

**5.1.4** UV-VIS detector with a flow cell and wavelength set of 210 nm

**5.1.5** Computational integrator or other data collection system

**5.2** HPLC column system of stainless steel

**5.2.1** Guard column

It is recommended that a suitable pre-column is attached in front of the analytical column system.

**5.2.2** Analytical column system

1. Reversed Phase Column (ambient)
   Material: stainless steel
   Internal diameter: 4 - 4,6 mm
   Length : 200 - 250 mm
   Stationary phase: spherical $C_{18}$ reversed phase material, particles 5µ in diameter*)
   *coupled with*

2. Cation exchange column (heated up to 65 ° C)
   Material: stainless steel
   Internal diameter: 4 - 7,8 mm
   Length : 300 mm
   Stationary phase: Sulfonated sterene-divinylbenzene gel type resin (S-DVB),containing a hydrogen packing, cross linked 8 %**)

## 6. SAMPLING

Clear samples are filled directly into sample vials and supplied to chromatography without any sample preparation. Cloudy wine samples are filtered through a 0,45 µm membrane filter before injection, while the first fractions of filtrates are rejected.

## 7. PROCEDURE

**7.1** Operating conditions of HPLC analysis

Inject 5 µL of wine into the chromatographic apparatus by full loop injection system.
Flow rate: 0,4 ml/min (if internal diameter of the cation exchange column is 4 mm)
0,6 ml/min (if internal diameter of the cation exchange column is 7,8 mm)
Mobile Phase: 0,01 M $H_2SO_4$
Column heater for cation exchange column: 65 °
Run time: 40 min

*) Lichrospher™ 100 RP-18 , Hypersil™-ODS or Omnichrom™ YMC-ODS-A are examples of suitable columns available commercially

**) Aminex™ HPX 87-H or Rezex™ ROA-Organic Acid are examples of suitable columns available commercially

Equilibration time: 20 min (to ensure that all substances from the wine matrix are completely eluted)

Detection wavelength: 210 nm

Injection volume: 5 µL

***Note:*** *Due to the different separation properties of various columns and different dead volumes of various HPLC-equipments the absolute retention time (min) for the shikimic acid peak may vary more or less significantly. Even though shikimic acid can be identified easily by calculating the a relative retention (r) related to a reference peak, here tartaric acid, a major organic acid naturally occurring in wine and the first and dominant peak in the chromatogram . By trying different* $C_{18}$ reversed phase columns and various cation exchange *columns a relative retention (r) of 1*.33 (± 0.2) *has been calculated.*

**7.2.** Detection limit

The detection limit of this method calculated according to the OIV protocol was estimated to 1 mg/l.

## 8. CALCULATION

Prepare a 5-point calibration curve from the working standard solutions (4.6).

Following the method of external standard the quantification of shikimic acid is performed by measuring the peak areas at shikimic acid retention time and comparing them with the relevant calibration curve. The results are expressed in mg/l shikimic acid at 1 decimal place.

## 9. PRECISION

The method was tested in a collaborative study with 19 international laboratories participating. Design and assessment followed O.I.V. Resolution Oeno 8/2000 "Validation Protocol of Analytical Methods". The study included 5 different samples of red and white wines. The samples covered concentration levels from 10 to 120 mg/l (see Annex 3).

The Standard Deviations of Repeatability and Reproducibility correlated with the shikimic acid concentration (see Annex 2). The actual performance parameters can be calculated by

$s_r = 0{,}0146 \cdot x + 0{,}2716$
$s_R = 0{,}0286 \cdot x + 1{,}4883$

x: shikimic acid concentration (mg/l)

Example:

shikimic acid: 50 mg/l
$s_r = \pm 1{,}0$ mg/l
$s_R = \pm 2{,}92$ mg/l

## 10. ANNEX

A typical separation of shikimic acid from other organic acids in wine is given in the Annex 1.

The correlationship of shikimic acid concentration and the standard deviation of repeatability and reproducibility is given in Annex 2.

The statistical data derivated from the results of the interlaboratory study is given in Annex 3.

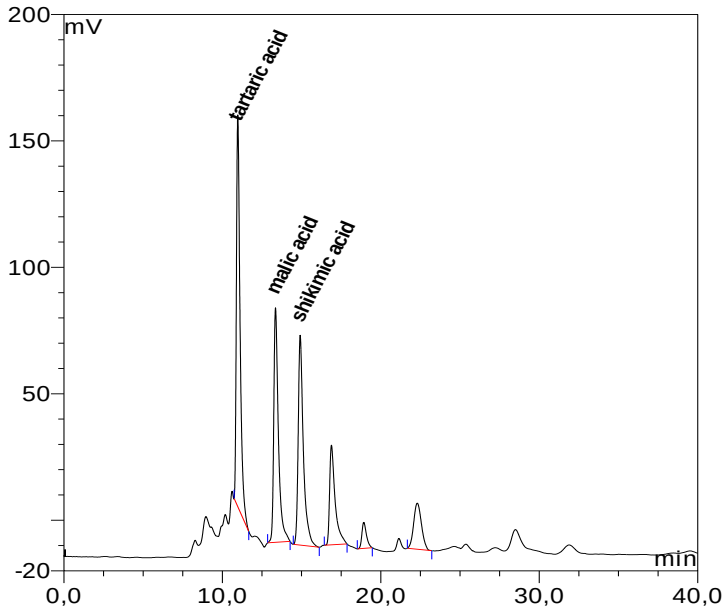
## 11. BIBLIOGRAPHY

**[1]** Römpp Lexikon Chemie-Version 2.0, Stuttgart/New York, Georg Thieme Verlag 1999

**[2]** Wallrauch S., Flüssiges Obst 3, 107 – 113 (1999)

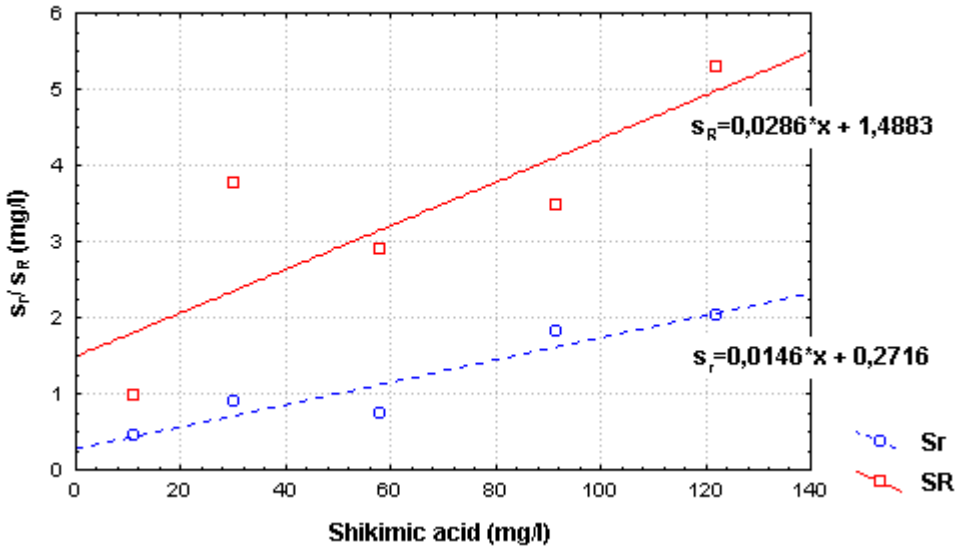
**[3]** 44th Session SCMA, 23-26 march 2004, Comparison of HPLC-, GC- and GC-MS-Determination of Shikimic Acid in Wine, FV 1193

Annex 1: Chromatogram of organic acids in wine




Annex 2: Correlationship of shikimic acid concentration and standard deviation of repeatability and reproducibility respectively

.



Annex 3: Table of method performance parameters

| **sample identification** | **A** | **B** | **C** | **D** | **E** |
|---|---|---|---|---|---|
| Number of participating laboratories | 19 | 19 | 19 | 19 | 19 |
| Number of accepted laboratories | 17 | 18 | 17 | 18 | 18 |
| mean | 58.15 | 30.05 | 11.17 | 122.17 | 91.20 |
| $s_r^2$ | 0.54588 | 0.84694 | 0.19353 | 4.32417 | 2.67306 |
| $s_r$ | 0.73884 | 0.92030 | 0.43992 | 2.07946 | 1.63495 |
| $RSD_r$ (%) | 1.27 | 3.06 | 3.93 | 1.70 | 1.79 |
| r | 2.07 | 2.58 | 1.23 | 5.82 | 4.58 |
| $s_L^2$ | 8.45221 | 13.27078 | 0.73013 | 24.62737 | 8.55508 |
| $s_R^2$ | 8.99809 | 14.11773 | 0.92366 | 28.95154 | 11.22814 |
| $s_R$ | 2.99968 | 3.75736 | 0.96107 | 5.38066 | 3.35084 |
| $RSD_R$ (%) | 5.16 | 12.50 | 8.60 | 4.40 | 3.67 |
| R | 8.40 | 10.52 | 2.69 | 15.07 | 9.38 |

| | |
|---|---|
| $s_r^2$ | variance of repeatability |
| $s_r$ | standard deviation of repeatability |
| $RSD_r$ (%) | relative standard deviation of repeatability |
| r | repeatability |
| $s_L^2$ | variance between laboratory |
| $s_R^2$ | variance of reproducibility |
| $s_R$ | variance of reproducibility |
| $RSD_R$ (%) | relative standard deviation of reproducibility |
| R | reproducibility |

**Method OIV-MA-AS313-18** Type IV method

# Determination of sorbic acid in wines by capillary electrophoresis

(Resolution Oeno 4/2006)

## 1 Scope

The present method is used to determine the sorbic acid in wines in a range from 0 to 300 mg/l.

## 2 Principle

The negatively charged sorbate ion naturally enables easy separation by capillary electrophoresis. At the capillary outlet, detection is carried out in the ultraviolet spectrum at 254 Nm.

## 3 Reagents and products

### 3.1 Reagents

3.1.1 Sodium dihydrogenophosphate [10049-21-5] purity > 96%
3.1.2 Sodium hydrogenophosphate [10028-24-7] purity > 99%
3.1.3 Sodium hydroxide [1310-73-2] purity > 97%
3.1.4 Hippuric sodium [532-94-5] purity > 99%
3.1.5 Demineralised water (< 15 MOHMS) or double-distilled

### 3.2 Migration buffer solution

The migration buffer is made up in the following way:

Sodium dihydrogenophosphate (3.1.1): 5 mM
Sodium hydrogenophosphate(3.1.2) 5 mM

### 3.3 Internal standard

Hippuric spdium (3.1.4) in an aqueous solution 0.5 g.L-1

### 3.4 Rinse solutions

3.4.1 Sodium hydroxide (3.1.3) N/10
3.4.2 Sodium hydroxide (3.1.3) N

## 4 Sample preparation

The wine samples are prepared as follows, which involves a 1/20 dilution:

| | | |
|---|---|---|
| Wine to be analyzed: | 0.5 ml | |
| Sodium hydroxide (3.1.3): | | 0.5 ml |
| Internal standard (3.1.4) with 0.5 g. $L^{-1}$: | | 0.5 ml |
| Qsp 10 ml with demineralized water (3.1.5) | | |

## 5 Operating conditions

### 5.1 Conditioning the capillary

Before its first use, and as soon as the migration times increase, the capillary must be conditioned according to the following process:

5.1.1 Rinse with sodium hydroxide solution 1N (3.4.2) at 20 psi (140 kPA) for 8 min.
5.1.2 Rinse with sodium hydroxide solution (3.4.1) 0.1 N at 20 psi (140 kPA) for 12 min.
5.1.3 Rinse with water (3.1.5) at 20 psi (140 kPA) for 10 min.
5.1.4 Rinse with the migration buffer (3.2) at 20 psi (140 kPA) for 30 min.

### 5.2 Migration conditions

*These conditions may be slightly changed depending on the equipment used.*

5.2.1 The molten silica capillary is 31 cm long, with a diameter of 50 microns.
5.2.2 Migration temperature: 25°C
5.2.3 Reading wavelength: 254 nm.
5.2.4 Reading of the signal in direct mode (sorbic acid absorbs in the UV spectrum).
5.2.5 First Pre-rinse under pressure 30 psi (210 kPA) with sodium hydroxide solution 0.1 N (3.4.1) for 30 seconds
5.2.6 Second Pre-rinse under pressure 30 psi (210 kPA) with the migration buffer (3.2) for 30 seconds.
5.2.7 The injection is done under a pressure of 0.3 psi (2.1 kPA) for 10 seconds.
5.2.8 The migration lasts approximately 1.5 to 2 minutes under a potential difference of + 25 kV, in normal polarity (cathod at the exit).
5.2.9 Certain capillary electrophoresis apparatus propose large-capacity vials for migration buffer solutions. This is preferable when several analyses are carried out in series, because the electrolytic properties are maintained longer.

### 5.3 Reading the results

The absorption peaks for the internal standard and the sorbic acid are obtained on average 1 to 1.5 minutes after the start of the migration phase live. Migration time is fairly constant, but can slightly vary according to the state of the capillary. If the migration time degrades, reconditioning of the capillary is necessary, and if the nominal conditions are not restored, the capillary must be replaced.

## 6 Characteristics of the method

The different validation steps described were carried out according to the OIV resolution OENO 10/2005.

### 6.1 Intralaboratory repeatability

| | |
|---|---|
| Standard repeatability deviation **Sr** | **1.6 mg/** $L^{-1}$ |
| Repeatability **r** | **4.6 mg/** $L^{-1}$ |

### 6.2 Linearity

| | |
|---|---|
| Regression line | Y = **0,99491 X + 2,52727** |
| Correlation coefficient **r** | **0,9997** |
| Residual standard deviation **Sxy** | **1,6 mg.**$L^{-1}$ |
| Standard deviation slope **Sb** | **0,008 mg.**$L^{-1}$ |

### 6.3 Intralaboratory reproducibility

| | |
|---|---|
| Standard reproductibility deviation **Sr** | **2.1 mg/** $L^{-1}$ |
| Reproductibility **R** | **5.8 mg/** $L^{-1}$ |

### 6.4 Detection and quantification limits

| | |
|---|---|
| Detection limit **Ld** | **1.8 mg/** $L^{-1}$ |
| Quantification limit **Lq** | **4.8 mg/** $L^{-1}$ |

### 6.5 Robustness

6.5.1 Determination

Since the method is relative, any slight variations in the analysis conditions will have no effect on the final result, but will primarily influence the migration time.

### 6.6 Method specificity

Possible influence of principle oenological additives were tested. None of them modify the results obtained.

### 6.7 Correlating the method with the OIV reference method

The OIV reference method is determination by ultraviolet absorption spectrometry. The sorbic acid, extracted by steam distillation, is determined in the wine distillate by ultraviolet absorption spectrometry at 256 Nm.

6.7.1 Comparison of repeatabilities

| | Capillary electrophoresis | OIV reference method |
|---|---|---|
| Standard deviation of repeatability $\mathbf{S_r}$ | **1.6 mg/l** | **2.5 mg/** $L^{-1}$ |
| Repeatability **r** | **4.6 mg/l** | **7.0 mg/** $L^{-1}$ |

6.7.2 Accuracy of the usual method in relation to the reference method

| | |
|---|---|
| Coefficient of correlation **r** | **0.999** |
| Average bias **Md** | **0.03 mg** $L^{-1}$ |
| Average bias standard deviation **Sd** | **3.1 mg** $L^{-1}$ |
| Z-score (Md/Sd) | **0.01** |

**Method OIV-MA-AS313-19** Method Type: II for organic acids
III for sulphate

# Determination of the principal organic acids of wines and sulphates by capillary electrophoresis

(Oeno 5/2006, extended by Oeno 407-2011)

## 1. Introduction

Tartaric, malic and lactic acids and sulphates are separated and assayed by capillary electrophoresis after simple dilution and addition of an internal standard.

## 2. Title

**Determination of the principal organic acids of wines and sulphates by capillary electrophoresis**

## 3. Scope

Capillary electrophoresis can be used to assay the tartaric and malic acid in musts, as well as the tartaric, malic and lactic acids and sulphates in wines that have been diluted, degassed and filtered beforehand if need be.

## 4. Définitions

4.1 Capillary electrophoresis

Capillary electrophoresis: all the techniques that use a capillary tube of very small diameter with an appropriate buffer solution to effectively separate small and large electrically charged molecules in the presence a high-voltage electric current.

4.2 Buffer for electrophoresis

Solution containing one or more solvents and aqueous solutions with suitable electrophoretic mobilities to buffer the pH of the solution.

4.3 Electrophoretic mobility

Aptitude of an ion to move quickly under the effect of an electric field.

4.4 Electroosmotic flow

Flow of solvent in the buffer solution along the internal wall of the capillary tube due to displacement of the solvated ions under the effects of the field and the electric charges of the silica.

## 5. Principle

Separations of the aqueous solutions of a mixture by capillary electrophoresis are obtained by differential migrations in a buffered electrolyte referred to as a buffer. The electrophoresis takes place in a silica tube with an inside diameter ranging between 25 and 75 µm. The aqueous solutions to be separated are simultaneously driven by 2 forces that can act in the same direction or in the opposite direction. These two forces are caused by the **electric field** and the **electroosmotic flow**.

The electric field is represented by the voltage in volts applied between the electrodes brought to within one centimetre of the capillary tube, and is expressed in $V.cm^{-1}$. Mobility is a characteristic of ions. The smaller the molecules, the greater their electrophoretic mobility.

If the internal wall of the capillary tube is not coated, the negative electric charges of the silica fix part of the cations of the buffer. The solvation and displacement towards the cathode of part of the cations of the buffer create the electroosmotic flow. The pH of the buffer and additives can be chosen in order to control the direction and the intensity of the electroosmotic flow.

The addition of a chromophoric ion in the buffer can be used to obtain negative peaks that quantitatively represent the solutions to be separated which do not absorb at the used wavelength.

## 6. Reagents and products

6.1 Chemically pure grade products for analysis at least at 99%

6.1.1 Sodium sulphate or Potassium sulphate

6.1.2 L-tartaric acid

6.1.3 D,L- malic acid

6.1.4 Monohydrated citric acid

6.1.5 Succinic acid

6.1.6 D,L Lactic acid

6.1.7 Sodium dihydrogenophosphate

6.1.8 Sodium gluconate

6.1.9 Sodium chlorate

6.1.10 Dipicolinic acid

6.1.11 Cethyltrimethyl ammonium bromure

6.1.12 Acetonitrile for HPLC

6.1.13 Deionized ultra filtered pure water

6.1.14 Sodium hydroxide

6.2 Solutions

6.2.1 Calibration stock solution

- Solution in pure water (6.1.13) of different acids and sulphates to be measured (6.1.1 to 6.1.6) at exact known concentrations ranging between 800 and 1200 mg $l^{-1}$
- Solution to be kept at +5° C for a maximum of 1 month

6.2.2 Internal standard solution

- Solution of sodium chlorate (6.1.9) at approximately 2 g $l^{-1}$ in pure water (6.1.13)
- Solution to be kept at +5° C for a maximum of 1 month

6.2.3 Calibration solution to be injected

In a graduated 50-ml class "A" flask using class "A" pipettes, deposit:

- 2 ml of calibration solution (6.2.1)
- 1 ml of internal standard solution (6.2.2)
- Adjust solution to 50 ml with pure water (6.1.13)

Homogenize by agitation

Solution to be prepared each day

6.2.4 Sodium hydroxide solutions

6.2.4.1 Sodium hydroxide solution M

In a 100-ml flask place 4g of sodium hydroxide (6.1.14)

Adjust with pure water (6.1.13)

Shake until completely dissolved.

6.2.4.2 sodium hydroxide solution 0.1M

In a 100 ml flask place 10 ml of sodium hydroxide M (6.2.4.1)

Adjust with pure water (6.1.13)

Homogenise.

6.2.5 Electrophoretic buffer solution

In a graduated 200-ml class "A" flask, place:

- 0.668 g of dipicolinic acid (6.1.10)
- 0.364 g of cethyltrimethyl-ammonium bromide. (6.1.11)
- 20 ml of acetonitrile (6.1.12)
- Approximately 160 ml of pure water (6.1.13)
- Shake until complete dissolution (if need be, place in ultrasound bath to eliminate any aggregated material)
- Bring M sodium hydroxide solution M (6.2.4.1) to pH 5.64 and then 0.1M sodium hydroxide (6.2.4.2)
- Make up to 200 ml with pure water (6.1.13)

- Homogenize by agitation
- Solution to be prepared each month.
- Store at laboratory temperature.

This buffer can be replaced by equivalent commercial product.

## 7. Apparatus

The capillary electrophoresis apparatus required for these determinations basically comprises:

- A sample changer
- Two bottles (phials) containing the buffer
- A non-coated silica capillary tube, internal diameter 50 µm, length 60 cm, between the inlet of the capillary tube and the detection cell. Depending on the apparatus, an additional 7 to 15 cm are required so that the outlet of the capillary tube is immersed in the centre of another bottle
- A high voltage DC power supply capable of outputting voltages of -30 to + 30 kV. The electrodes immersed in the two bottles where the outlets of the capillary tube emerge are connected to the terminals of the generator
- A pressurization system capable of circulating the buffer in the capillary tube and enabling the injection of the test specimen
- A UV detector
- A data acquisition system

## 8. Preparation of samples for tests

### 8.1 Degassing and filtration

The samples rich in carbon dioxide are degassed for 2 min with ultra-sound. Turbid samples are filtered on a membrane with an average pore diameter of 0.45 µm.

### 8.2 Dilution and addition of internal standard

Place 2 ml of sample in a graduated flask of 50 ml. Add 1 ml of internal standard solution (6.2.2). Adjust to 50 ml with pure water (6.1.13)

Homogenize.

## 9. Procedure

9.1 Conditioning of a new capillary tube (for example)

- Circulate pure water (6.1.13) in the opposite direction (from the outlet of the capillary tube towards the inlet flask) for 5 min at a pressure of approximately 40 psi (2.76 bar or 276 kPa)
- Circulate 0.1M sodium hydroxide (6.2.4.2) in the opposite direction for 5 min at the same pressure
- Circulate pure water (6.1.13) in the opposite direction (from the outlet of the capillary tube towards the inlet flask) for 5 min at the same pressure
- Repeat the cycle of circulating pure water, 0.1M sodium hydroxide , pure water
- Circulate electrophoretic buffer (6.2.5) in the opposite direction for 10 min

9.2. Reconditioning a capillary tube in the course of use (optional)

When the quality of the separations becomes insufficient, new conditioning of the capillary tube is essential. If the results obtained are still not satisfactory, change capillary tube and condition it.

9.3. Checking the quality of the capillary tube (optional)

Analyse 5 times the calibration solution under the recommended analysis conditions.

9.4. Separation and detection conditions (for example)

- Light the detector lamp 1 hour before the start of the analyses
- Rinse the capillary tube by circulating the buffer for 3 min in the opposite direction at a pressure of 40 psi
- Pressure inject the samples (prepared at 8.1) at 0.5 psi for 6 to 15 seconds
- The polarity is regulated such that the anode is on the detector side
- Apply a voltage from 0 to 16 kV in 1 min then 16 kV for approximately 18 min (the duration of separation can slightly vary depending on the quality of the capillary tube)
- Maintain the temperature at + 25 C°
- Detection in the ultraviolet is at 254 Nm

- Rinse the capillary tube by circulating the electrophoretic buffer (6.2.5) for 2 min in the opposite direction at a pressure of 40 psi
- Change the electrophoretic buffer (6.2.5) contained in the inlet and outlet flasks at least every 6 injections

9.5 Order that the analyses are to be carried out (for example)
Change the electrophoretic buffer (6.2.5) for every new series of analyses

- The sequence of analysis in order contains:Analysis of reference material (external concentration sample known for different acids to be measured)
- Analysis of samples prepared in 8.2,chromatograms should look like those presented in appendix A
- At the end of analysis, rinse with pure water (6.1.13) 10 mm in opposite direction (outlet of capillary tube toward the inlet)
- Switch off detector lamp

## 10. Calculation of results

The calculations are based on the surface areas of the peaks obtained after integration.
The surface areas of the peaks of the aqueous solutions of the calibration solution (6.2.3) are corrected by taking into account the variations in the surface areas of the peaks of the internal standard. The response factor for each acid is calculated.

The surface areas of the peaks of the internal standard and the peaks of the aqueous solutions are read off for each sample. The surface areas of the aqueous solutions to be assayed are recalculated by taking into account variations in the surface areas of the peaks of the internal standard a second time in order to obtain "corrected" surface areas.
The corrected surface areas are then multiplied by the value of the corresponding response factor.
It is possible to use an automatic data management system, so that they can be controlled in accordance with the principles described above as well as with the best practices (calculation of response factor and / or establishment of a calibration curve).

CALCULATION FORMULA

The abbreviations used to calculate the concentration in an acid are given in the following table:
Surfaces are expressed by the whole numbers of integration units.
The concentrations are given in g/L (only indicate to two decimal places).

| ABBREVIATIONS | | |
|---|---|---|
| | **REFERENCE SOLUTION** | **SAMPLE** |
| **SURFACE AREAS OF TITRATED PEAKS** | **$S_{AR}$** | **$S_{AE}$** |
| **INTERNAL STANDARD PEAKS** | **$S_{EIR}$** | **$S_{EIE}$** |
| **CONCENTRATION** | **$C_{AR}$** | **$C_E$** |

The calculation formula is:

$$C_E = \frac{C_{AR} \times S_{AE} \times S_{EIR}}{S_{AR} \times S_{EIE}}$$

Whenever possible, a duplicate analysis is used to highlight a possible error in the recognition of the peaks or inaccuracy of integration. The sample changer makes it possible to carry out the analyses in automatic mode day and night.

## 11. Precision

### 11.1 Organization of the tests

Interlaboratory trials and correspondent results are described in appendix B1 and B2

### 11.2 Measurement of precision

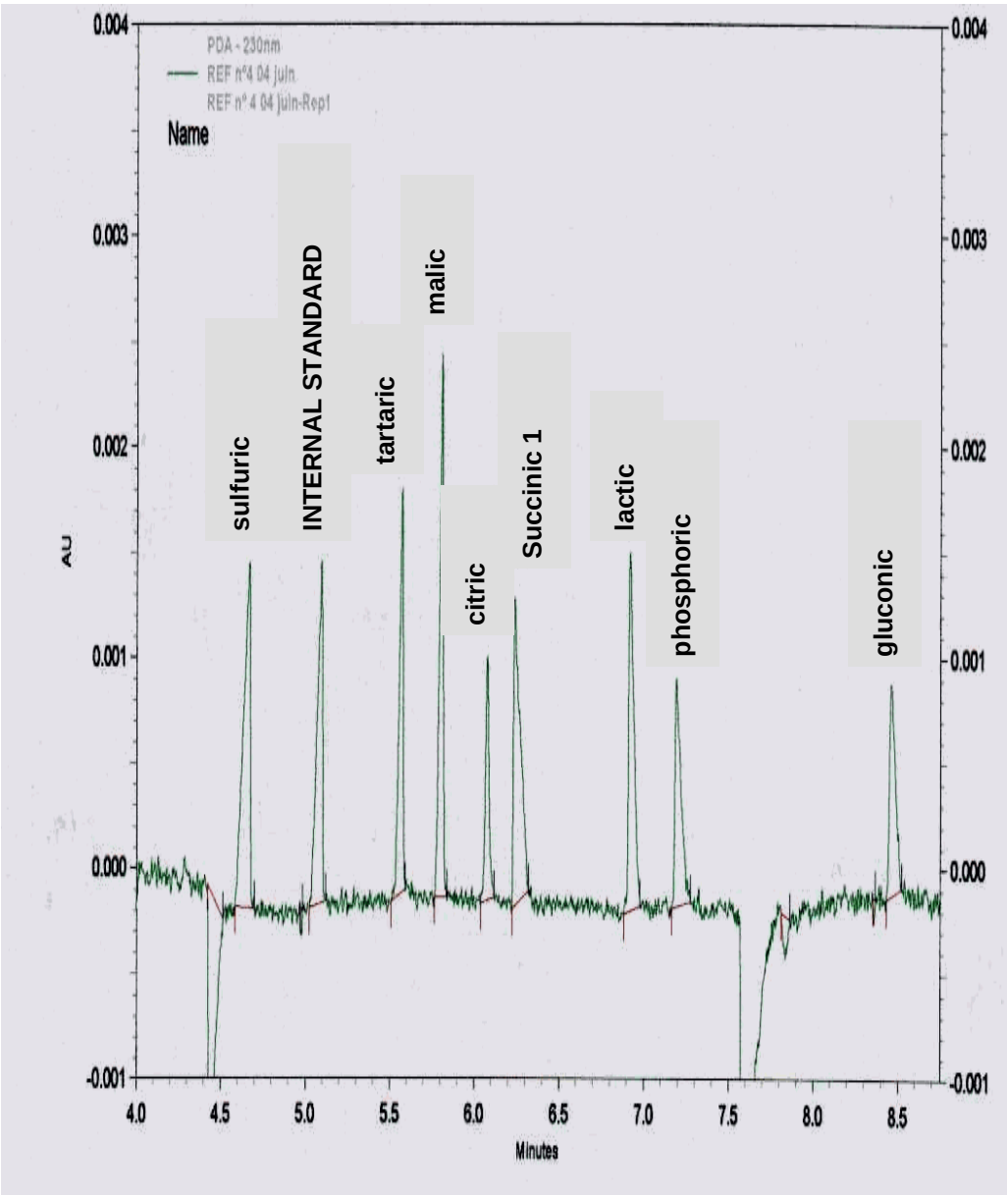
ASSESSEMENT OF PRECISION BY INTERLABORATORY TRIALS
Number of laboratories involved: 5

Results expressed in mg / L

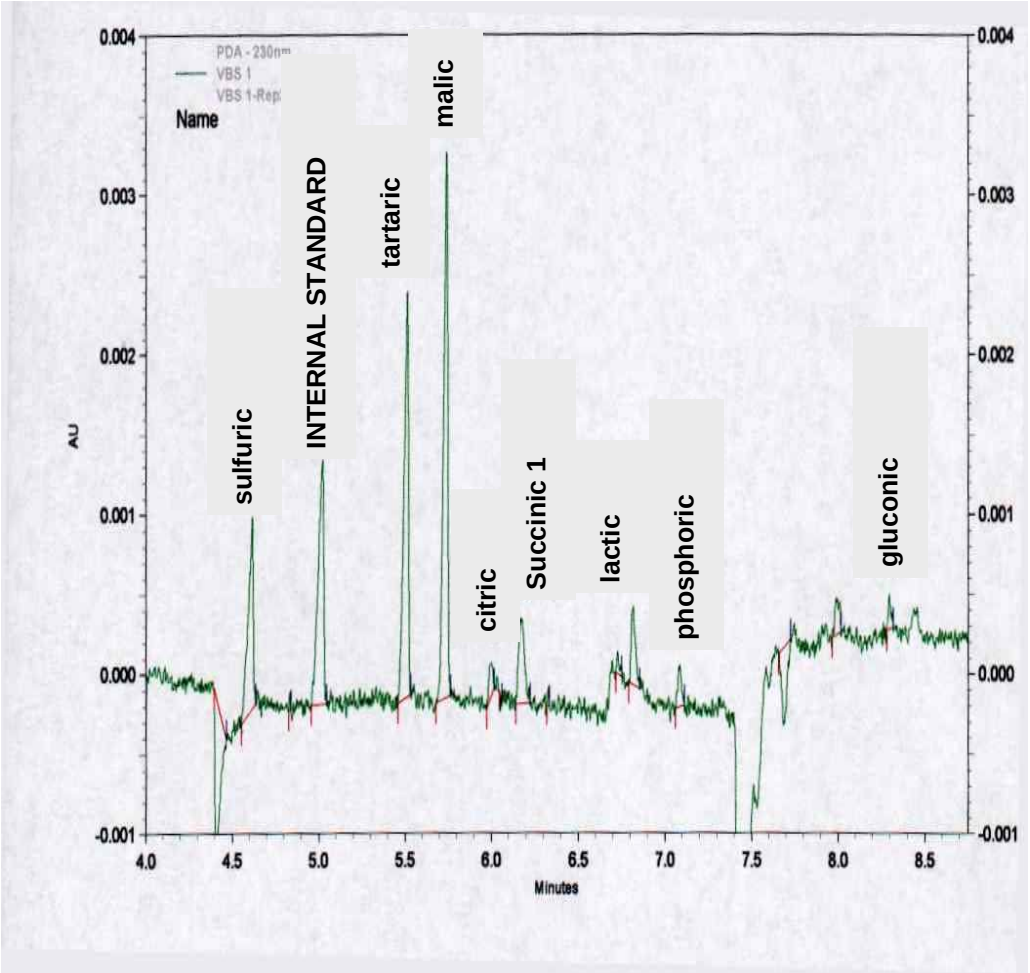
| | TARTRIC ACID | MALIC ACID | LACTIC ACID |
|---|---|---|---|
| Average values of concentrations | 1395 | 1884 | 1013 |
| Average values of standard deviations in repeatability | 38 | 54 | 42 |
| Average values of standard deviations in reproducibility | 87 | 113 | 42 |

## 12. APPENDICES

### APPENDIX A
### ELECTROPHOREGRAM OF A STANDARD SOLUTION OF ACI



## ELECTROPHEROGRAM OF A WINE



# APPENDIX B1

## Statistic data obtained from the results of the interlaboratory trials (2006)

According to ISO 5725-2:1994, the following parameters have been defined during an interlaboratory trial. This trial has been conducted by the laboratory « Direction Générale de la Consommation et de la Répression des Fraudes de Bordeaux (France). »

Year of interlaboratory trial: 2006
Number of laboratories: 5
Number of samples: 8 double-blind (2 dry white wines, 2 sweet white wines, 2 rosé wines and 2 red wines)

INTERLABORATORY TESTS

Determination of TARTRIC ACID by capillary electrophoresis

| **Identification of the sample** | dry white wines | | liquorous white wines | | rosé wines | | red wines | |
|---|---|---|---|---|---|---|---|---|
| | **A + D** | **B + C** | **E + F** | **G + H** | **I + J** | **K + L** | **M + N** | **O + P** |
| Number of laboratories taking part | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Number of results accepted | 5 | 5 | 4 | 5 | 5 | 5 | 4 | 4<br>5 |
| Average value in mg/l | 1943 | 2563 | 1440 | 255 | 553 | 1885 | 1373 | 1148 |
| Accepted value in mg/l | 1943 | 2563 | 1387 | 2217 | 1877 | 1593 | 1370 | 1830 |
| Standard deviation of repeatability (Sr) | 27 | 25 | 106 | 23 | 40 | 31 | 25 | 24 |
| Repeatability coefficient of variation | 1.4 | 1,0 | 7.7 | 1,0 | 2.2 | 1.9 | 1.8 | 1.3 |
| Limit of repeatability ( r ) | 77 | 70 | 298 | 65 | 113 | 86 | 70 | 66 |
| Standard deviation of reproducibility (SR) | 96 | 128 | 174 | 80 | 57 | 55 | 52 | 53 |
| Reproducibility coefficient of variation in % | 4.9 | 5 | 12.6 | 3.6 | 3 | 3.5 | 3.8 | 2.9 |
| Reproducibility limit ( R ) | 268 | 359 | 488 | 223 | 160 | 154 | 145 | 148 |

INTERLABORATORY TESTS

Determination of MALIC ACID by capillary electrophoresis

| **Identification of the sample** | dry white wines | | liquorous white wines | | rosé wines | | red wines | |
|---|---|---|---|---|---|---|---|---|
| | **A + D** | **B + C** | **E + F** | **G + H** | **I + J** | **K + L** | **M + N** | **O + P** |
| Number of laboratories taking part | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Number of results accepted | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 4<br>5 |
| Average value in mg/l | 2571 | 1602 | 1680 | 2539 | 3524 | 2109 | 173 | 869 |
| Accepted value in mg/l | 2571 | 1602 | 1680 | 2539 | 3524 | 2109 | 177 | 869 |
| Standard deviation of repeatability (Sr) | 54 | 19 | 113 | 35 | 61 | 109 | 7 | 32 |
| Repeatability coefficient of variation | 2.1 | 1.2 | 6.7 | 1.4 | 1.7 | 5.2 | 4.1 | 3.7 |
| Repeatability limit ( r ) | 151 | 54 | 315 | 99 | 170 | 305 | 20 | 89 |
| Standard deviation of reproducibility (SR) | 90 | 51 | 171 | 97 | 279 | 142 | 21 | 53 |
| Reproducibility coefficient of variation in % | 13.6 | 9.8 | 41 | 39.6 | 14.7 | 9 | 14.1 | 7.6 |
| Limit of reproducibility ( R ) | 252 | 142 | 479 | 273 | 782 | 397 | 59 | 148 |

INTERLABORATORY TESTS

Determination of LACTIC ACID by capillary electrophoresis
results in mg/l

| **Sample identification** | dry white wines | | liquorous white wines | | rosé wines | | red wines | |
|---|---|---|---|---|---|---|---|---|
| | **A + D** | **B + C** | **E + F** | **G + H** | **I + J** | **K + L** | **M + N** | **O + P** |
| Number of laboratories taking part | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Number of results accepted | 4 | 5 | 5 | 5 | 5 | 5 | 4 | 4<br>5 |
| Average value in mg/l | 659 | 1324 | 258 | 255 | 553 | 1885 | 2066 | 1148 |
| Accepted value in mg/l | 650 | 1324 | 258 | 255 | 553 | 1885 | 2036 | 1148 |
| Standard deviation of repeatability (Sr) | 20 | 42 | 20 | 39 | 27 | 99 | 75 | 16 |
| Repeatability coefficient of variation | 3.1 | 3.2 | 7.8 | 15.1 | 4.8 | 5.3 | 3.7 | 16,0 |
| Repeatability limit ( r ) | 57 | 117 | 56 | 108 | 75 | 278 | 211 | 46 |
| Standard deviation of reproducibility (SR) | 20 | 42 | 20 | 39 | 27 | 99 | 75 | 16 |
| Reproducibility coefficient of variation in % | 13,6 | 9,8 | 41 | 39,6 | 14,7 | 9 | 14,1 | 7,6 |
| Reproducibility limit ( R ) | 247 | 363 | 296 | 283 | 227 | 475 | 802 | 243 |

## APPENDIX B2

### Statistic data obtained from the results of the interlaboratory trials (sulphates 2010)

According to ISO 5725-2:1994, the following parameters have been defined during an interlaboratory trial. This trial has been conducted by the laboratory "Instituto dos Vinhos do Douro e do Porto (Portugal)"

Year of interlaboratory trial: 2010-2011
Number of laboratories: 7 (one laboratorysent two sets of results obtained by means of two different instruments)
Number of samples: 6 double-blind

| Indicators | White wine (A/G) | Rosé (B/F) | Rosé (C/O) | Red wine (D/M) | Liquor wine (E/N) | Liquor wine (I/K) | White wine (H/Q) | Red wine (J/P) | Liquor wine (L) |
|---|---|---|---|---|---|---|---|---|---|
| Number of groups | 7 | 7 | 6 | 7 | 8 | 7 | 7 | 7 | 8 |
| Number of repetitions | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Minimum (g/L $K_2SO_4$) | 0,71 | 0,34 | 0,40 | 0,62 | 1,79 | 1,06 | 1,38 | 1,96 | 2,17 |
| Maximum (g/L $K_2SO_4$) | 0,88 | 0,54 | 0,52 | 0,75 | 2,40 | 1,35 | 1,70 | 2,30 | 2,85 |
| Repeatability variation $s_r^2$ | 0,0012 | 0,0011 | 0,0001 | 0,0016 | 0,0063 | 0,0013 | 0,0036 | 0,0015 | 0,0053 |
| Intergroup variation $s_L^2$ | 0,00148 | 0,00230 | 0,00163 | 0,00055 | 0,01952 | 0,01082 | 0,00668 | 0,01744 | 0,03552 |
| Reproducibility variation $s_R^2$ | 0,0027 | 0,0034 | 0,0018 | 0,0022 | 0,0258 | 0,0122 | 0,0103 | 0,0189 | 0,0408 |
| Mean (g/L $K_2SO_4$) | **0,78** | **0,43** | **0,44** | **0,69** | **2,01** | **1,19** | **1,49** | **2,15** | **2,41** |
| Standard deviation of Repeatability (g/L$K_2SO_4$) | **0,04** | **0,03** | **0,01** | **0,04** | **0,08** | **0,04** | **0,06** | **0,04** | **0,07** |
| Limit r (g/L $K_2SO_4$) | 0,100 | 0,093 | 0,031 | 0,115 | 0,224 | 0,103 | 0,170 | 0,109 | 0,206 |
| Repeatability CV | 5% | 8% | 3% | 6% | 4% | 3% | 4% | 2% | 3% |
| Standard deviation of Reproducibility (g/L $K_2SO_4$) | **0,05** | **0,06** | **0,04** | **0,05** | **0,16** | **0,11** | **0,10** | **0,14** | **0,20** |
| Limit R (g/L $K_2SO_4$) | 0,148 | 0,165 | 0,118 | 0,132 | 0,454 | 0,312 | 0,287 | 0,389 | 0,572 |
| Reproducibility CV | 7% | 14% | 10% | 7% | 8% | 9% | 7% | 6% | 8% |
| HORRAT | 1,1 | 2,1 | 1,5 | 1,1 | 1,6 | 1,7 | 1,3 | 1,3 | 1,7 |

## 13. Bibliography

ARELLANO M., COUDERC F. and PUIG .L (1997): Simultaneous separation of organic and inorganic acids by capillary zone electrophoresis. Application to wines and fruit juices. Am. J. Enol. Vitic., 48, 408-412.

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KLAMPF C.F. (1999): Analysis of organic acids and inorganic anions in different types of beer using capillary zone electrophoresis. J. Agric. Food Chem., 47, 987-990.

**Method OIV-MA-AS313-20** Type IV method

# Determination of sorbic, benzoic and salicylic acid content in wine by the use of high-performance liquid chromatography

(Resolution Oeno 6/2006)

## 1. Introduction

Sorbic acid and its potassium salt constitute an antiseptic that can be used in wine-making, although some countries will not tolerate even traces of it, the main reason being the smell of geraniums that develops when sorbic acid is broken down by lactic acid bacteria. Benzoic acid and salicylic acid are still prohibited in wine, but are used in other beverages.

## 2. Scope

All wines and grape musts, especially those likely to contain only traces of sorbic, benzoic or salicylic acid (demonstration from 1 mg/l).

## 3. Principle

The antiseptics are determined using HPLC by direct injection of the sample into a column functioning by isocratic reversed-phase partition chromatography with ultraviolet detection at a wavelength of 235 nm.

## 4. Products

4.1 Micro-filtered fresh water (e.g. resistivity greater than 18.2 MΩ)
4.2 Pure tetrahydrofuran
4.3 Pure methanol
4.4 0.1 M hydrochloric acid (prepared by means of dilution funnels)
4.5 Water with a pH of 2: adjust the pH of 650 ml of water (4.1) to pH2 using a pH meter (5.5) and by adding 0.1 M hydrochloric acid drop by drop without stirring (4.4)
4.6 Elution solution: mix 650 ml of water at pH2 (4.5) with 280 ml of methanol (4.3) and 7 ml of tetrahydrofuran (4.2)

Note: it is likewise possible to use other elution solvents, for example: 80% ammonium acetate 0.005M (0.38 g/l) adjusted to pH 4 with pure acetic acid + 20% acetonitrile.

4.7 Pure sorbic acid
4.8 Pure benzoic acid
4.9 Pure salicylic acid
4.10 Absolute alcohol
4.11 50% vol. hydro-alcohol solution: put 500 ml of absolute alcohol (4.10) into a 1-litre flask and dilute to volume with distilled water (4.1)
4.12 Stock solution of sorbic acids at 500 mg/l: dissolve 50 mg of sorbic acids (4.7), benzoic (4.8) and salicylic (4.9) acids in 100 ml of the 50% vol. hydro-alcohol solution (4.11)
4.13 Sorbic, benzoic and salicylic acid surrogate solutions: dilute the stock solution (4.12) in the hydro-alcohol solution (4.11) in such a way as to obtain the final concentrations required. For example, for a solution of

- 200 mg/l: put 20 ml of stock solution (4.12) into a 50-ml flask and top up to the filling mark with 4.11.
- 1 mg/l: put 2 ml of stock solution (4.12) into a 50-ml flask and top up to the filling mark with 4.11.

Intermediate solutions may be produced in the same way to satisfy calibration requirements.

## 5. Apparatus

5.1 Laboratory glassware, especially pipette and volumetric flasks
5.2 Ultrasonic bath
5.3 Vacuum filtration device for large volumes (1 litre) using membrane filters with a pore diameter of under 1 µm (generally 0.45 µm)
5.4 Mini-filter for samples (1 to 2 ml) using membrane filters with a pore diameter of under 1 µm (generally 0.45 µm)
5.5 pH meter
5.6 Isocratic-mode liquid phase chromatograph equipped with an injection system for small volumes (for example), 10 or 20-µl loop valve.
5.7 Detector capable of functioning at an ultraviolet rating of 235 nm and fitted with a circulating tank for HPLC (for example, 8 µl for 1 cm of optical thickness)

5.8 A 5-µm stationary phase HPLC column of the silica-type with immobilisation by octadecyl groups (C18), length 20 cm, inside diameter 4 mm
5.9 Data acquisition system

## 6. Preparation of samples and the elution solvent

6.1 Filter the samples to be analysed using the mini-filter (5.4)
6.2 Degas the elution solvent (4.6) for 5 minutes using the ultrasonic bath (5.2)

6.3 Filter the solvent using the device in (5.4)

## 7. Procedure

7.1 Column conditioning. Prior to injection, start the pump and rinse the column with the solvent for at least 30 minutes.

7.2 Inject one of the surrogate solutions (4.13) to check system sensitivity and ensure the resolution of the peaks of the substances to be analysed is satisfactory.

7.3 Inject the sample to be analysed. It is possible to analyse an identical sample, to which the acids sought have been added (adapt the amount added to the quantity observed during the previous analysis - for 1 mg present, add 1 mg, and so on).

Check the resolution of the peaks of the acids sought with the peaks of the wines (normally, there are none in this zone)

## 8. Calculation

Having located the peaks of the acids to be determined in the sample, compare the peak area with those of the acids of a surrogate solution (4.13) with a known concentration C.

For example, let s be the peak area of the acid to be determined, and S is the peak area of the solution (4.13) with concentration C

$$X_{\text{in the sample}} = C \times \frac{s}{S} \qquad \text{in mg/l}$$

## 9. Characteristics of the method

| | Sorbic acid | Benzoic acid | Salicylic acid |
|---|---|---|---|
| Linearity range | 0 to 200 mg/l | 0 to 200 mg/l | 0 to 200 mg/l |
| Accuracy (rate of recuperation) | > 90 % | > 90 % | > 90 % |
| Répétabilité : r* | 2% | 3% | 8% |
| Reproducibility: R* | 8% | 9% | 12% |
| Detection limit | 3 mg/l | 3 mg/l | 3 mg/l |
| Quantification limit | 5 mg/l | 6 mg/l | 7 mg/l |
| Uncertainty | 11% | 12% | 13% |

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**Method OIV-MA-AS313-21** Type IV method

# Determination of the presence of metatartaric acid

(Resolution Oeno 10/2007)

## 1. Introduction

Metatartaric acid added to the wine to avoid tartaric precipitation is traditionally proportioned by the difference between the total tartaric acid following hot hydrolysis of metatartaric acid and natural tartaric acid preceding hydrolysis. However, taking into account the precision of the determination of tartaric acid, traces of metatartaric acid are not detectable by this method, and the additive, which is not accepted in certain countries, must therefore be characterised using a more specific method.

## 2. Scope

Wines likely to contain traces of metatartaric acid.

## 3. Principle

In relatively acid mediums, metatartaric acid forms an insoluble precipitate with cadmium acetate; it is the only one of all the elements present in must and wine to give such a precipitate .

Note: Tartaric acid is also precipitated with cadmium acetate, but only in the presence of an alcohol content greater than 25% vol. The precipitate redissolves in water, unlike the precipitate obtained with metatartaric acid.

The cadmium precipitate of metatartaric acid breaks down by heating with sodium hydroxide and releases tartaric acid. The latter produces a specific orange colour with ammonium metavanadate.

## 4. Reagents

4.1 Cadmium acetate solution at 5 p.100
- 4.1.1 Dihydrated cadmium acetate at 98%
- 4.1.2 Pure acetic acid
- 4.1.3 Distilled or demineralized water

4.1.4 Cadmium acetate solution: dissolve 5 g of cadmium acetate (4.1.1) in 99 mL of water (4.1.3) add 1 mL of pure acetic acid (4.1.2)

4.2 Sodium hydroxide 1M

4.3 Sulfuric acid 1M

4.4 Solution of ammonium metavanadate 2% w/v

4.4.1 Ammonium metavanadate

4.4.2 Trihydrated sodium acetate at 99%

4.4.3 Sodium acetate solution at 27 p. 100: dissolve 478 g of sodium acetate (4.4.2) in 1 liter of water (4.1.3)

4.4.4 Solution of ammonium metavanadate: dissolve 10 g of ammonium metavanadate (4.4.1) in 150 mL of sodium hydroxide 1 M (4.2) add 200 of the sodium acetate solution at 27 p. 100 (4.4.3) and fill to 500 mL with water (4.1.3)

4.5 Ethanol at 96% vol.

## 5. Apparatus

5.1 Centrifuge with a rotor capable of housing 50-mL bottles

5.2 Spectrometer capable of operating in the visible spectrum and of housing cuvets with an optical thickness of 1 cm.

## 6. Operating method

6.1 Centrifuge 50 mL of wine for 10 minutes at 11000 rpm

6.2 Take 40 mL of limpid wine using a test-tube and place the sample in a centrifuge flask

6.3 Add 5 mL of ethanol at 96% vol (4.5)

6.4 Add 5 mL of the cadmium acetate solution (4.1.4)

6.5 Mix and leave to rest for 10 minutes

6.6 Centrifuge for 10 minutes at 11000 rpm

6.7 Decant by completely reversing the flask (once) and throw away the supernatant.

In the presence of metatartaric acid, a lamellate precipitate is formed at the bottom of the tube.

In the absence of any precipitate, the sample will be regarded as free from metatartaric acid. In the contrary case, or if the presence of a light precipitate is to be established with certainty, proceed as follows:

6.8 Wash the precipitate once with 10 mL of water (4.1.3) in the form of an energetic jet towards the bottom of the tube in order to detach the precipitate from the bottom
6.9 Add 2 mL of cadmium acetate solution (4.1.4)
6.10 Centrifuge at 11000 rpm for 10 minutes then throw away the supernatant by completely reversing the tube (once)
6.11 After adding one mL of sodium hydroxide 1M (4.2), plunge the tube to be centrifuged for 5 minutes in a water bath at 100° C
6.12 After cooling, add 1 mL of sulfuric acid 1M (4.3) and 1 mL of ammonium metavanadate solution (4.4.4)
6.13 Wait 15 minutes
6.14 Centrifuge for 10 minutes at 11000 rpm
6.15 Pour the supernatant into a spectrophotometer tank and measure the absorbance at 530 nm, after determining the zero point with water (4.1.3)

i.e. $Abs_E$

Standard. In parallel, produce a standard comprising the same wine as that analyzed but heated beforehand for 2.5 minutes using a microwave generator set to maximum power or with a water bath at 100° C for 5 minutes.

i.e. $Abs_T$

## 7. Calculation

The presence of metatartaric acid in the wine is established when, at 530 nm:

$$Abs_E - Abs_T > 0.050$$

**Method OIV-MA-AS313-22** Type II method

# Simultaneous determination of L-ascorbic acid and D-iso-ascorbic acid (erythorbic acid) in wine by HPLC and UV-detection

(Resolution Oeno 11/2008)

## 1. Introduction

Ascorbic acid is an antioxidant that is naturally occurring in a wide range of foods. The natural amount of ascorbic acid in grapes decreases during must and wine production, but it can be added to musts and to wines within certain limits.
The method described has been validated in a collaborative study by the analyses of wine samples with spiked amounts of 30 mg/L to 150 mg/l for L-ascorbic acid and 10 mg/L to 100 mg/l for D-isoascorbic acid respectively.

## 2. Scope

This method is suitable for the simultaneous determination of L-ascorbic acid and D-iso-ascorbic acid (erythorbic acid) in wine by high performance liquid chromatography and UV-detection in a range of 3 mg/L to 150 mg/l.
For contents above 150 mg/l, sample dilution is necessary.

## 3. Principle

The samples are directly injected into the HPLC system after membrane filtration. The analytes are separated on a reversed phase column and UV-detection at 266 nm. The quantification of L-ascorbic acid and D-iso-ascorbic acid is done with reference to an external standard.

Note: The columns and operating conditions are given as example. Other types of columns may also give a good separation.

## 4. Reagents and Material

### 4.1 Reagents

4.1.1. N-octylamine, puriss. ≥ 99.0 %
4.1.2. Sodium acetate, 3 $H_2O$, puriss ≥ 99.0 %
4.1.3. Pure acetic acid, 100 %
4.1.4. Phosphoric acid, approx. 25%

4.1.5. Oxalic acid, puriss. ≥ 99.0 %
4.1.6. Ascorbate oxidase
4.1.7. L-ascorbic acid, ultra ≥ 99.5 %
4.1.8. D-iso-ascorbic acid, puriss. ≥ 99.0 %
4.1.9. Bi-distilled water
4.1.10. Methanol, p.A. 99.8 %

### 4.2 Preparation of the mobile phase

4.2.1 Solutions for the mobile phase

For the mobile phase prepare the following solutions:

4.2.1.1 12.93 g n-octylamine in 100 ml methanol
4.2.1.2 68.05 g sodium acetate, 3 $H_2O$ in 500 ml bi-distilled water
4.2.1.3 12.01 g pure acetic acid in 200 ml bi-distilled water
4.2.1.4 Buffer solution (pH 5.4) : 430 ml sodium acetate solution (4.2.1.2) and 70 ml acetic acid solution (4.2.1.3)

4.2.2 Preparation of the mobile phase

Add 5 ml of n-octylamine solution (4.2.1.1) to approximately 400 ml bi-distilled water in a beaker. Adjust this solution to a pH of 5.4 to 5.6 by adding 25% phosphoric acid (4.1.4) drop by drop. Add 50 ml of the buffer solution (4.2.1.4), transfer the composite mix to a 1000 ml volumetric flask and fill up with bi-distilled water. Before use, the mobile phase has to be filtered through a membrane (regenerated cellulose, 0.2 µm) and if possible degassed with helium (approximately 10 minutes) depending on the needs of the HPLC system used.

### 4.3 Preparation of the standard solution

Note: All standard solutions (stock solution 4.3.1. and working solutions 4.3.2) have to be prepared daily and preferably stored cold in a refrigerator prior to injection.

4.3.1 Preparation of the stock solution (1 mg/ml)

Prepare a 2% aqueous oxalic acid solution and eliminate dissolved oxygen by blowing through nitrogen.

Weigh exactly 100 mg each of L-ascorbic acid and D-iso-ascorbic acid in a 100 ml volumetric flask and make to the mark with the 2% aqueous oxalic acid solution.

4.3.2 Preparation of the working solutions

For the working solutions dilute the stock solution (4.3.1) to the desired concentrations with the 2% oxalic acid solution. Concentrations between 10 mg/l and 120 mg/l are recommended, e.g. 100 µl, 200 µl, 400 µl, 800 µl, 1200µl to 10 ml, corresponding to 10, 20, 40, 80 and 120 mg/l.

**5. Apparatus**

Usual laboratory equipment, in particular the following:

**5.1 HPLC-pump**

**5.2 Loop injector, 20 µl**

**5.3 UV-detector**

**6. Sampling**

Wine samples are fltered through a membrane with pore size 0.2 µm before injection.

For contents above 150 mg/L, it is necessary to dilute the sample.

**7. Procedure**

**7.1 Operating conditions for HPLC**

Inject 20 µl of the membrane-filtered sample into the chromatographic apparatus.

| | |
|---|---|
| Precolumn: | e.g. Nucleosil 120 C18 (4cm x 4 mm x 7 µm) |
| Column: | e.g. Nucleosil 120 C18 (25 cm x 4 mm x 7 µm) |
| Injection Volume: | 20 µl |
| Mobile Phase: | see 4.2.2, isocratic |
| Flow rate: | 1ml/min |
| UV-detection: | 266 nm |
| Rinse cycle: | at least 30ml bi-distilled water followed by 30ml methanol and 30ml acetonitrile |

**7.2 Identification/Confirmation**

Identification of peaks is done by the comparison of retention times between standards and samples. With the chromatographic system described as an example, the retention times are: for L-ascorbic acid 7.7 min. and for D-iso-ascorbic 8.3 min. respectively. (See figure 1, chromatogram A).

For further confirmation of positive findings these samples should be treated with a spatula of ascorbate oxidase and measured again (see figure 1, chromatogram B).

As a result of the degradation of L-ascorbic acid and D-iso-ascorbic acid caused by the ascorbate oxidase, no signal should be found at the retention time of L-ascorbic acid and D-iso-ascorbic acid. If interfering peaks are detected, their peak area should be taken into account for the calculation of the concentration of the analytes.

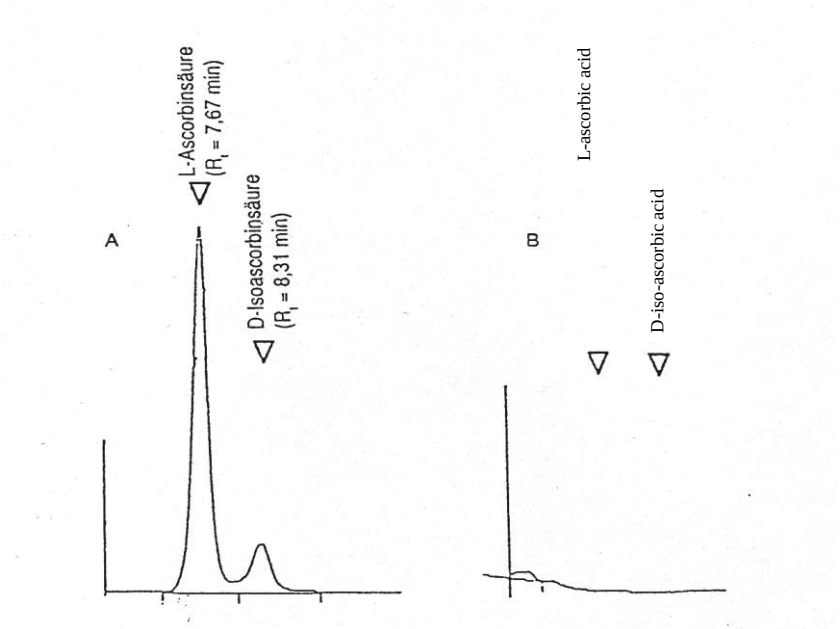



Figure 1: Example of a chromatogram of white wine: A: prior to treatment with ascorbate oxidase; B: after treatment

**Note:** It is recommended to analyse the ascorbate oxidase treated samples at the end of a sequence, followed by the rinse cycle for removing remaining ascorbate oxidase from the column. Otherwise the L-ascorbic acid and the D-iso-ascorbic acid may be converted by the remaining ascorbate oxidase during the HPLC-measurement and the result may be altered.

**8 Calculation**

Prepare a calibration curve from the working solutions (4.3.2). Following the method of external standard the quantification of L-ascorbic acid and D-isoascorbic acid is performed by measuring the peak areas and comparing them with the relevant concentration in the calibration curve.

**Expression of results**

The results are expressed in mg/l L-ascorbic acid and D-isoascorbic acid respectively with one decimal (e.g. 51,3 mg/l).
For contents above 150 mg/L, take into account the dilution.

**9. Precision**

The method was tested in a collaborative study with 27 laboratories participating, organised by the former Bundesgesundheitsamt (Germany) in 1994. The design of the
collaborative trial followed the § 35 of the German Food Law that has been accepted by the O.I.V until the new protocol (OENO 6/2000) was introduced.
The study included four different samples of wine - two white wines and two red wines - of which five repetitions of each were requested. Due to the fact that it was not possible to prepare samples with a sufficient stability of the analytes (different degradation rates) it was decided to send defined amounts of pure standard substances together with the wine samples to the participants. The laboratories were advised to transfer the standards quantitatively to the wine samples and to analyse them immediately. Amounts of 30 to 150 mg/l for L-ascorbic acid and 10 to 100 mg/l for D-iso-ascorbic acid were analysed. In the Annex the detailed study results are presented. Evaluation was done following the DIN/ISO 5725 (Version 1988) standard.

The standard deviations of repeatability ($s_r$) and reproducibility ($s_R$) were coherent with the L-ascorbic acid and D-iso-ascorbic acid concentrations. The actual precision parameters can be calculated by the following equations:

**L-ascorbic acid**

$s_r = 0.011\ x + 0.31$
$s_R = 0.064\ x + 1.39$

x: L-ascorbic acid concentration (mg/l)

**D-iso-ascorbic acid**

$s_r = 0.014\ x + 0.31$
$s_R = 0.079\ x + 1.29$

x: D-iso-ascorbic acid concentration (mg/l)

Example:
D-iso-ascorbic acid 50 mg/l $s_r = 1.0$ mg/l
$s_R = 5.2$ mg/l

## 10. Other characteristics of the analysis

### 10.1 Limit of detection

The limit of detection of this method was estimated at 3mg/l for L-ascorbic acid and D-iso-ascorbic acid

### 10.2. Trueness

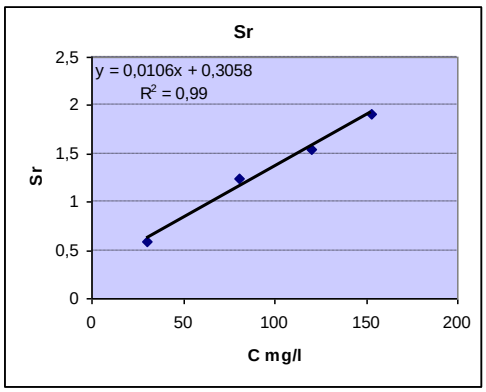
The mean recovery calculated from the collaborative trial over four samples (see Annex) was:

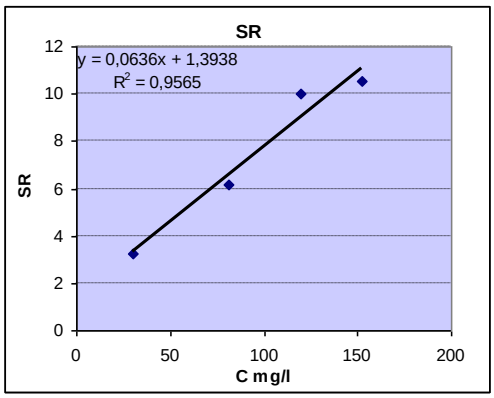
100.6 % for L-ascorbic acid
103.3 % for D-iso-ascorbic acid

## 11. ANNEX: Collaborative Trial

### L-Ascorbic Acid

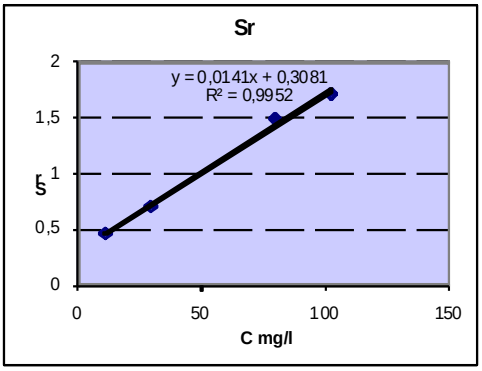
| | | Red Wine I | White Wine II | Red Wine III | White Wine IV |
|---|---|---|---|---|---|
| X | mg/l | **152.7** | **119.8** | **81.0** | **29.9** |
| Amount spiked | mg/l | 150 | 120 | 80 | 30 |
| Recovery | % | 101.8 | 99.8 | 101.3 | 99.7 |
| n | | 25 | 23 | 25 | 23 |
| Outliers | | 1 | 3 | 1 | 3 |
| Repeatability $s_r$ | mg/l | 1.92 | 1.55 | 1.25 | 0.58 |
| $RSD_r$ | % | 1.3 | 1.3 | 1.5 | 1.9 |
| HorRat | | **0.17** | **0.17** | **0.19** | **0.20** |
| r | mg/l | 5.4 | 4.3 | 3.5 | 1.6 |
| Reproducibility $S_R$ | mg/l | 10.52 | 10.03 | 6.14 | 3.26 |
| $RSD_R$ | % | 6.9 | 8.4 | 7.6 | 10.9 |
| Horwitz $RSD_R$ | % | 7.5 | 7.8 | 8.3 | 9.6 |
| HorRat | | **0.92** | **1.08** | **0.92** | **1.14** |
| R | mg/l | 29.5 | 28.1 | 17.2 | 9.1 |

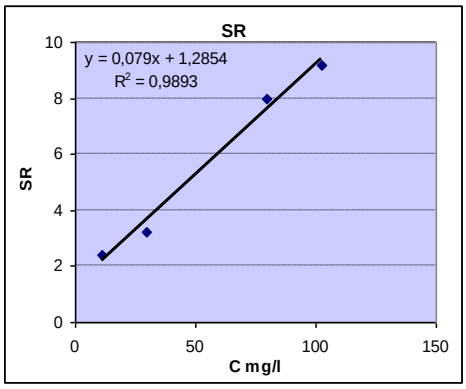


**D-Isoascorbic Acid**

| | | Red Wine I | White Wine II | Red Wine III | White Wine IV |
|---|---|---|---|---|---|
| X | mg/l | **102.4** | **79.8** | **11.3** | **29.4** |
| Amount Spiked | mg/l | 100 | 80 | 10 | 30 |
| Recovery | % | 102.4 | 99.8 | 113.0 | 98.0 |
| n | | 25 | 23 | 24 | 22 |
| Outliers | | 1 | 3 | 2 | 4 |
| Repeatability $s_r$ | mg/l | 1.71 | 1.49 | 0.47 | 0.70 |
| $RSD_r$ | % | 1.7 | 1.9 | 4.1 | 2.4 |
| HorRat | | **0.21** | **0.23** | **0.37** | **0.25** |
| r | mg/l | 4.8 | 4.2 | 1.3 | 2.0 |
| Reproducibility $S_R$ | mg/l | 9.18 | 7.96 | 2.394 | 3.23 |
| $RSD_R$ | % | 9.0 | 10.0 | 21.2 | 11.0 |
| Horwitz $RSD_R$ | % | 8.0 | 8.3 | 11.1 | 9.6 |
| HorRat | | **1.12** | **1.21** | **1.91** | **1.14** |
| R | mg/l | 25.7 | 22.3 | 6.7 | 9.0 |



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**Method OIV-MA-AS313-23** Type IV method

# Identification of L- tartaric acid as being of plant or fossil origin by measuring its $^{14}C$ activity

(Resolution Oeno 12/2008)

## 1. PURPOSE AND SCOPE

The method can be used to identify tartaric acid as being of plant or fossil origin, and in cases of a mixture of the two, to determine the respective proportions of the two types. In these situations, the method enables the detection of fossil-derived L(+)-tartaric acid quantities below 10%.

## 2. PRINCIPLE

In the majority of cases, commercially available tartaric acid of plant origin is a product of winemaking. The potassium hydrogénotartrate present in the lees is extracted and marketed in the form of L-tartaric acid. The $^{14}C$ concentration in the acid is therefore related, as with ethanol from wine, to the $^{14}C$ concentration in the carbon dioxide in wines from the same year of production. This concentration is relatively high as a result of the human activity involved.
Synthetic tartaric acid on the other hand, derived from fossil fuel by-products, has a much lower or even negligible concentration of $^{14}C$.
Measuring the $^{14}C$ activity in DPM/gram of carbon (Disintegrations Per Minute) using liquid scintillation therefore allows the origin to be determined as well as any combination of the types.

## 3. REAGENTS AND PRODUCTS

### 3.1 Reagents

3.1.1 Scintillation fluid such as Instagel Plus
3.1.2 $^{14}C$ toluene reference with activity certified by laboratory for callibration, for calculating the sensitivity and efficiency of the machine by the definition of a quench curve
3.1.3 $^{14}C$ and $^{3}H$ standards and $^{12}C$ toluene for the background noise, for calibrating the scintillation counter

3.1.4 Nitromethane 99%
3.1.5 Ultrapure water (>18 MΩ )
3.1.6 $^{14}C$ toluene solution with activity of approx. 430 DPM/ml obtained by diluting stock $^{14}C$ reference solution in $^{12}C$ toluene.

**3.2 Standards**

3.2.1 Defining the quench curve

Once the scintillator has been calibrated using the three certified $^{14}C$, $^{3}H$ and $^{12}C$ toluene standards, plot a quench curve using the following procedure.

Prepare a dozen vials with 10 ml of a solution of 500 g/l of fossil-derived L-tartaric acid in water, then add the quantity of toluene $^{14}C$ standard needed for approx. 400-1000 DPM in total per vial (if necessary, make up an intermediate solution of standard solution in toluene), then add increasing quantities of nitromethane, e.g. for 12 vials: 0, 0, 0, 5, 10, 15, 20, 35, 50, 100, 200 and 400 µL followed by 10 m of scintillation fluid. There must be at least 3 samples containing no nitromethane.

Define a quench curve once a year, analysing the vials in increasing order of nitromethane content.

The quench curve can then be used to determine the sensitivity or mean efficiency.

3.2.2 Determination of background noise (test blank)

Using fossil-derived L-tartaric acid, such as that used for calculating the efficiency, determine the background noise, or test blank value. This test should be performed immediately after defining the quench curve, then roughly every three months.

3.2.3 Defining the calibration curve

The purity of the plant and fossil-derived L-tartaric acids must be checked using HPLC before the scintillation test is done.

Calibration using a mixture of tartaric acid (which is known with certainty to be of plant origin) containing between 0% and 100 % of this type in combination with the fossil-derived type.

| Preparation of 500 g/l solutions | | | |
|---|---|---|---|
| | Blank or background noise | Standards | Internal standard |
| Weighing | respectively in 50 ml volumetric flasks | | |
| | 25 g fossil-derived L-tartaric acid | 25 g known combinations of fossil and plant L-tartaric acid | Use the blank |
| Dissolution | Seal | | |
| | Homogenise the mixture well by shaking and/or tumbling | | |
| Preparation of scintillation mixtures | | | |
| | In plastic vials, add respectively | | |
| Sample taken from the 500 g/l solutions | 10 ml using volumetric pipettes | | |
| Added concentration | ////////////////////////// | ////////////////////////// | 100 µL |
| Added scintillation fluid | 10 ml using an automatic burette | | |
| | Screw the cap on | | |
| | Wait 5 min. then analyse for 500 min. | | |

**3.3. Internal control**

3.3.1 Nature of product used for internal control

A 500 g/l solution of fossil-derived L-tartaric acid is enriched with a quantity of toluene $^{14}C$ (DPM<100)
The background noise should be determined using the same fossil-derived L-tartaric acid solution.

3.3.2 Nature of internal control

Measurement of the added concentration provides verification that there is no spectral interference in the medium being studied.

3.3.3 Internal control limits

The control limits depend on the equipment used: a 5% value is acceptable.

3.3.4 Inspection frequency and procedure

Once a month during frequent use, or at each analysis sequence, an internal control is performed on the scintillator. The same check is also carried out at every change of scintillation fluid batch or after a new quench curve has been defined.

3.3.5 Decision rules to be taken depending on the results of the internal control

If the results fall outside the internal control limits, calibrate the scintillator after checking the protocol, then repeat the internal control.
If the calibration is accurate but the new internal control measurement is not, make a new quench curve and carry out a new control.

## 4. APPARATUS

4.1 Liquid scintillation spectrometre with computer and printer previously callibrated with quenching curve established with nitromethane

4.2 Low content potassium identical bottles (40K) with screw top stopper, and low background noise

4.3 10 mL 2 graduations pipettes

4.4 Automatic distribution burette adapted to screw top for liquid scintillating bottle

4.5 Glass laboratory

## 1) 5. SAMPLES

The purity of the samples can be checked using HPLC if required, before running the scintillation analysis.
Make up a 500 g/solution of the sample to be analysed in ultra-pure water.

| Preparation of 500 g/l solutions | | | | |
|---|---|---|---|---|
| | Test blank or background noise | Standards | Internal standard | Sample |
| Weighing | respectively in 50 ml volumetric flasks | | | |
| | 25 g fossil-derived L-tartaric acid | 25 g known combinations of fossil-derived and plant L-tartaric acid | Use the blank | 25 g |
| Dissolution | Seal | | | |
| | Homogenise the mixture well by shaking and/or tumbling | | | |
| Preparation of scintillation mixtures | | | | |
| | In plastic vials, add respectively | | | |
| Sample taken from the 500 g/l solutions | 10 ml using volumetric pipettes | | | |
| Added concentration | ////////////////////// | ///////////////// | 100 µL | ////////////// |
| Added scintillatic fluid | 10 ml using an automatic burette | | | |
| | Screw the cap on and shake hard | | | |
| | Wait 5 min. then analyse for 500 min. | | | |
| ***Notes*** | Every 5 to 10 test samples, run a sample with 0 % plant tartaric acid, i.e. 10 ml fossil tartaric acid and 10 ml scintillation fluid. | | | |
| | Measure the background noise at the end of each analysis sequence | | | |

## 6. CALCULATION

Measurements are given directly in Counts Per Minute CPM, but these must be converted to DPM/gram of carbon.

### 6.1 Results:

Calculation of the specific $^{14}C$ radioactivity of the sample in DPM/gram of carbon:

$$A = \frac{(X - X') \text{ x } 100 \text{ x } 3.125_{(1)}}{Rm \text{ x } m}$$

- **A**: radioactivity in disintegrations/minute and per gram of carbon
- **X**: CPM of the sample
- **X':** CPM for the fossil L-tartaric acid used for the background noise
- **m:** mass of the tartaric acid in the 10 ml sample from the 500 g/l solution, i.e. in 5 g of acid
- **Rm:** the mean efficiency expressed as a percentage

(1) There are 3.125 grams of tartaric acid to each gram of carbon (ratio of the molar mass of the acid (150 g/mol) to the total mass of carbon (or 4 *x 12 = 48 g/mol)

The result is expressed to one decimal place.

**6.2 Verification of the results using internal controls:**

The check should be carried out by comparing the value obtained at § 3.5.1 with the result given by the added concentration method. If the difference is significant (> 5 %), recalculate the DPM value from the CPM value as below:

$$\text{Recalculated DPM} = \frac{CPM}{Rm}$$

with the mean efficiency being obtained from the quench curve.

The two results must not differ by more than 5% from their mean value. If they do, repeat the analysis on the sample, doubling the quantity of the internal standard. Compare the 2 results obtained with the standards: if they do not differ by more than 5 % from the mean of the 2, give the mean result.

Note: in this case, that would mean that the quenching of the sample is so great that direct analysis cannot be used.

**6.3 Uncertainty**

The uncertainty value obtained under standard test conditions is +/- 0.7 DPM/gram of carbon.

## 7. VALIDATION BY COMPARISON WITH A REFERENCE METHOD

### 7.1 Principle

Tartaric acid is converted to $CO_2$ by combustion then converted to benzene; Measurement is then carried out using liquid scintillation.

After undergoing a pre-treatment designed to eliminate any contamination, the $CO_2$ from the sample is converted to benzene following the reaction chain below:

$C + O_2 \rightarrow CO_2$ (1)

$CaCO_3 + 2\ HCl \rightarrow CO_2 + H_2O + Ca^{2+} + 2Cl^-$ (2)

$$2\ CO_2 + 10\ Li \xrightarrow{800°C} Li_2C_2 + 4\ Li_2O \quad (3)$$

$Li_2C_2 + 2\ H_2O \rightarrow C_2H_2 + 2\ LiOH$ (4)

$$3\ C_2H_2 \xrightarrow{Al_2O_3\ avec\ Cr^{3+}\ et\ V} C_6H_6 \quad (5)$$

**(1)** Organic sample: the carbon flushed with oxygen plus a heat source (or by combustion in the presence of pressurised oxygen) produces carbon dioxide from the sample ($CO_2$).

**(2)** Mineral sample (marine or continental carbonates, water, etc.): The carbonate is attacked by pure hydrochloric acid (HC) to produce the carbon dioxide ($CO_2$) from the sample plus water and ionised calcium.

**(3)** The action of the $CO_2$ on lithium metal heated to between +600°C and +800°C produces lithium carbide and lithium oxide (-$Li_2$ O).

**(4)** The action of water (hydrolysis) on the lithium carbide produces acetylene ($C_2H_2$), lithium hydroxide,. Non-tritiated, radon-free water must be used.

**(5)** Trimerisation of the acetylene over a chrome-plated aluminium-based catalyst support at approx. 185 °C produces benzene ($C_6H_6$).

**7.2 Procedure:**

The carbon dioxide ($CO_2$) from a sample, obtained either by burning, combustion or acid attack, is preserved in a storage cylinder. The necessary quantity of lithium (lithium = catalyst for a chemical transformation) is placed in a nickel capsule, which is then placed at the bottom of a heat reaction chamber. A vacuum is created inside the chamber and its lower part is heated while its upper part is cooled at the sides with the help of a water circulation partition.

7.2.1 Carburisation.

After approximately one hour of heating, the temperature reaches 650°C. The $CO_2$ can then be brought into contact with melted lithium. The quantity of lithium is always higher in relation to the quantity of carbon in the sample. The excess amount of lithium to use in relation to the **stoechiometric** conditions varies from 20% to 100% according to different sources.

The chemical reaction (carburisation or "pickup") is almost instantaneous and the first few minutes of pickup are the most crucial in the carburisation process.

The reaction is **exothermic** (an increase of 200°C). Carburisation is quite rapid and is considered to be at the carburised stage after the first 20 minutes, but heating continues for 45 to 50 minutes in order to any eliminate traces of **radon** (a by-product of uranium), which could be mixed in with the carbon dioxide.

7.2.2 Cooling

Once the treatment period (heating) is complete, the reaction chambers are allowed to cool until they reach room temperature (25-30°C).

7.2.3 Hydrolysis of Lithium Carbide

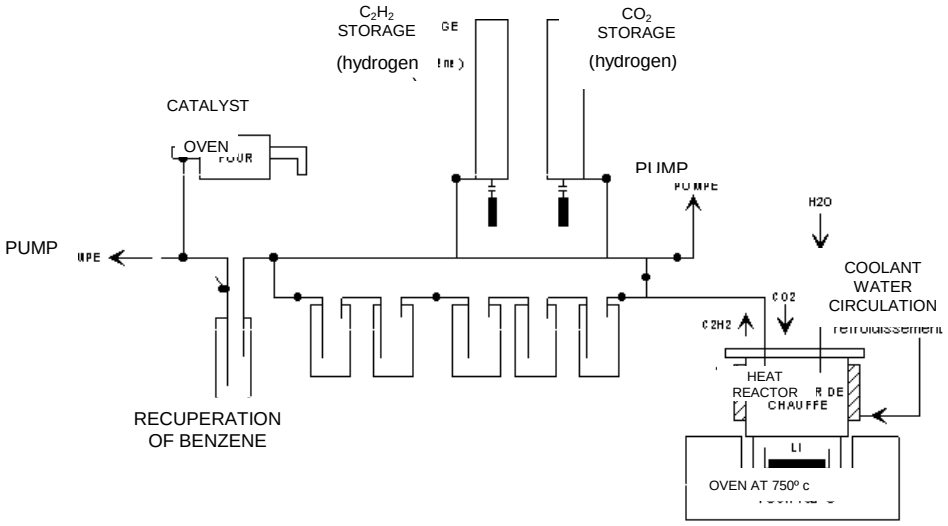
Water is introduced into the reaction chambers, in a much higher quantity than that required by the reaction (1.5 L). The chemical reaction is instantaneous and the acetylene is released at the same time. This reaction is also exothermic (temperature increase between +80°C and +100°C).

The acetylene produced is then brought to a vapour state (sublimation) and trapped over the chrome-plated (Cr3+) aluminium catalyst support. This is previously air dried for a minimum of three hours, then vacuum dried for two hours under heat at +380°C. Drying is vital in order to eliminate any water remaining in the catalyst support balls.

7.2.4 Trimerisation - Polymerisation of acetylene to benzene by catalysis

Before trimerisation, the temperature of the catalyst support must have dropped to between +60°C and +70°C, and since this reaction is also exothermic, automatic temperature maintenance is needed. The catalyst support is then reheated to +180°C for 1½ hours and the vaporised **benzene** is desorbed then trapped in a trap tube surrounded by liquid nitrogen. Desorption takes place under dynamic vacuum. At the end of the experiment, the crystallised benzene is left to reheat to room temperature so that it regains its liquid state before being used for the counting.

### 7.3 Benchtop arrangement for the synthesis of Benzene




### 7.4 Reference Chemical solution for the Counting

A **solution volume set at 4 ml** is used as the reference for the liquid scintillation counting.

The solution comprises a target base of 3.52g benzene from the sample (solvent) + the scintillation fluid (solute) made up of 2 scintillation fluids, one main and one secondary.

Since the mass per volume of benzene is 0.88 g/litre, **0.88 x 4ml = 3.52 g**.

| | |
|---|---|
| **Main scintillation fluid** | **Buthyl-PBD** |
| Chemical composition | **(2-(4-Biphenylyl)-5-(4-tert-buthyl-phenyl)-1,3,4-oxadiazole)** |
| Maximum wavelength fluorescence | **367 nanometers** |
| **Secondary scintillation fluid** | **bis-MSB** |
| Chemical composition | **1,4-Di-(2-Methylstyryl)-Benzene** |
| Maximum wavelength fluorescence | **415 nanometers** |
| **Optical absorption and coupling emission of the two fluids:** | |
| Maximum absorption wavelength | **409 nanometers** |
| Maximum absorption wavelength | **412 nanometers** |

### 7.5 Delta $^{13}C$ correction for Isotope Fractionation

The measurement involves a correction for isotope fractionation using the standardisation procedure with a stand PDB $^{13}C$ **with a value of - 25 o/oo**.

## 8. CHARACTERISTICS OF THE METHOD

### 8.1 Procedure

One sample of wine-derived tartaric acid and one sample of synthetic acetic acid were used to prepare test tartaric acid solutions at 500 g/l.

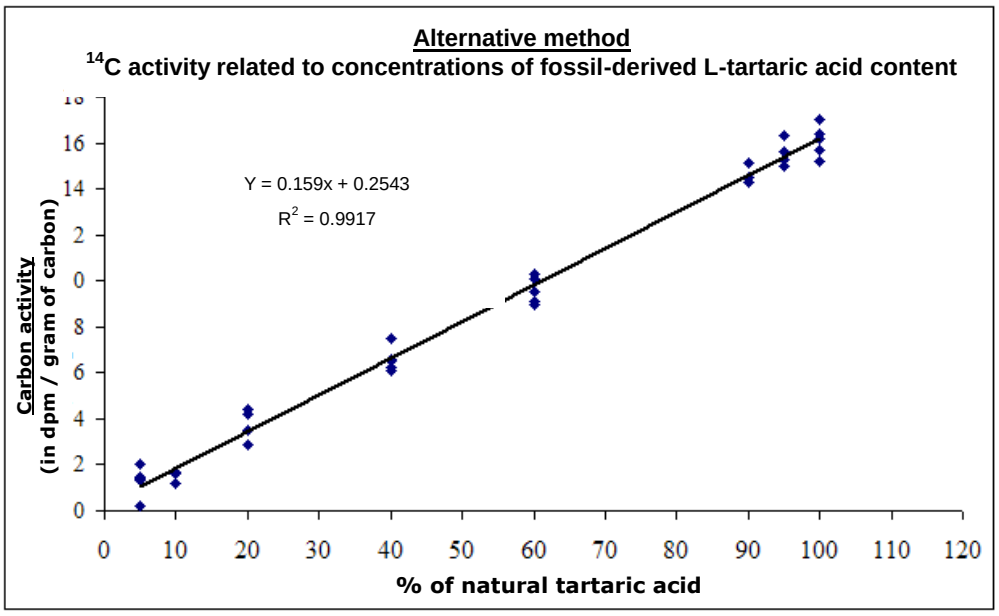
The concentrations of the wine-derived tartaric acid in the solutions varied between 0°C and 100%.

The origin and purity of the two starter samples had been previously checked using the reference method.

**8.2 Results:**

The results are given in the table and diagram below:

| % OF WINE-DERIVED TARTARIC ACID | | |
|---|---|---|
| ACTUAL CONCENTRATIONS | RESULTS FROM THE ALTERNATIVE METHOD | RESULTS FROM THE REFERENCE METHOD |
| 0 | 0 and 0 | 0 |
| 10 | 3.5 and 6.0 | 12 |
| 20 | 11.4 and 12 | 22 |
| 30 | 24.6 and 25.4 | 31 |
| 40 | 34.7 and 38 | 40 |
| 50 | 41.4 and 50.6 | 50 |
| 60 | 57.8 and 58.8 | 63 |
| 70 | 60 and 63.3 | 70 |
| 80 | 81 | 81 |
| 85 | 84 | 86 |
| 90 | 88 | 91 |
| 95 | 94 | 96 |
| 100 | 100 | 100 |



**8.3 Accuracy,trueness:**

Accuracy is 6.9%.
The standard deviation of repeatability for the alternative method is: 2.86 % of plant tartaric acid.

## 9. BIBLIOGRAPHY

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S. COHEN, B. CRESTO, S. NACHAMPASSAK, T. PAYOT, B. MEDINA, S. CHAUVET, Détermination de l'origine de l'acide tartrique L(+): naturelle ou fossile par la détermination de son activité $C^{14}$ - Document OIV FV 1238, 2006

**Method OIV-MA-AS313-27** Type II method

# DETERMINATION OF ACETIC ACID IN WINES BY AUTOMATED ENZYMATIC METHOD

OIV-OENO 621-2019

## *1. Scope of application*

This method makes it possible to determine acetic acid in wines using an automatic sequential analyser and specific enzyme analysis. The measurement range, which was the object of the current interlaboratory validation, is from 0.2 to 1.14 g/L acetic acid.

NOTE: A range of higher values may be analysed with the introduction of a sample dilution.

## *2. Standard references*

- ISO 78-2: Chemistry – Layouts for standards

## *3. Reaction principles*

In the presence of ATP, acetic acid is converted into acetyl phosphate in a reaction catalysed by acetate kinase.

**(1) Acetate + ATP** $\xrightarrow{\text{Acetate kinase}}$ **Acetyl phosphate + ADP**

The ADP formed by this reaction is reconverted into ATP in a reaction with phosphoenolpyruvate catalysed by pyruvate kinase.

**(2) ADP + Phosphoenolpyruvate** $\xrightarrow{\text{Pyruvate kinase}}$ **Pyruvate + ATP**

Pyruvate is reduced to L-lactate by reduced nicotinamide adenine dinucleotide (NADH) catalysed by lactate dehydrogenase.

**(3) Pyruvate + NADH + $H^+$ —Lactate dehydrogenase→ Lactate + $NAD^+$ + $H_2O$**

The quantity of oxidised NADH in reaction (3) is determined by the absorbance measurement at 340 nm, and is proportional to the concentration of acetic acid in the wine.

**Note 1:**

When this enzymatic analysis is conducted manually, the reading is carried out once the stabilised, final plateau is reached. To achieve this, a fourth reaction makes it possible to completely push the equilibrium of reaction 1 towards the formation of acetyl phosphate through elimination of the latter.

**(4) Acetyl phosphate + CoA —Phosphotransacetylase→ Acetyl-CoA + Inorganic phosphate**

In the case of analysis by an automated method, which has a shorter analysis time, it is not necessary to reach a stabilised, final plateau, and this reaction is pointless. The use of phosphotransacetylase is therefore not necessary, and is not described here.

**Note 2:**

The enzymatic reaction chain involves pyruvate. The low quantities of pyruvate (several tens of mg/L) normally present in wines do not have a significant impact on the result. In the rare cases, the presence in wine of an untypical quantity of pyruvate is likely to produce a method bias.

## *4. Reagents and working solutions*

During analysis – unless otherwise indicated – use only quality, recognised analytical reagents and distilled or demineralised water, or water of equivalent purity.

4.1. Reagents

4.1.1. Quality I or II water for analytical use (ISO 3696 standard)

4.1.2. 3-(N-Morpholino)propanesulfonic acid (MOPS): CAS no. 1132-61-2

4.1.3. Magnesium chloride hexahydrate: CAS no. 7791-18-6

4.1.4. Potassium chloride: CAS no. 7447-40-7

4.1.5. ß-Nicotinamide adenine dinucleotide (NADH): CAS no. 53-84-9, purity ≥ 98%

4.1.6. Adenosine-5'-triphosphate, disodium salt (ATP): CAS no. 56-65-5

4.1.7. Potassium hydroxide: CAS no. 1310-58-3

4.1.8. Phosphoenolpyruvate tri(cyclohexylammonium) salt: CAS no. 35556-70-8 or monosodium phosphoenolpyruvate: CAS no. 138-08-9 (PEP)

4.1.9. Acetate kinase (AK): CAS no. 9027-42-3

4.1.10. Pyruvate kinase (PK): CAS no. 9001-59-6

4.1.11. Lactate dehydrogenase (LDH): CAS no. 9001-60-9

4.1.12. Polyvinylpyrrolidone (PVP): CAS no. 9003-39-8

4.1.13. Acetic acid: purity ≥ 99.5%; CAS no. 64-19-7

4.1.14. Sodium chloride: CAS no. 7647-14-15

4.1.15. Bovine serum albumin (BSA): CAS no. 9048-46-8

**Note 3:** There are commercial kits for the determination of acetic acid. The user needs to check the composition to ensure it contains the above-indicated reagents. These kits are sometimes supplied with phosphotransacetylase (redundant when using an automated method).
**Note 4:** The use of PVP is recommended to eliminate any possible negative action of tannins in wine on the enzyme protein molecules. In the event that the use of PVP is not effective, the laboratory should ensure the absence of interference of wine tannins on the enzymes.
**Note 5:** BSA is an agent used for the stabilisation of enzymes in solution.

### 4.2. Working solutions

#### 4.2.1. MOPS buffer

The preparation may be as follows:

- 13 g MOPS (3-(N-Morpholino)propanesulfonic acid) (4.1.2),
- 0.5 g magnesium chloride hexahydrate (4.1.3),
- 1.5 g potassium chloride (KCl) (4.1.4),
- 1.3 g PVP (4.1.12),
- 250 mL water for analytical usage (4.1.1).

Adjust the pH to 4.75 with a 1.5 M potassium hydroxide (KOH) solution (4.1.7).

Wait 5 minutes and readjust the pH to 7.45 with a 1.5 M potassium hydroxide (KOH) solution (4.1.7).

Make up to 300 mL with water for analytical use (4.1.1).

The buffer can be kept for at least 60 days at 2-8 °C (approximately).

#### 4.2.2. Working solution 1 (R1)

The preparation may be as follows:

- 100 mL MOPS buffer (4.2.1),
- 300-350 mg adenosine-5'-triphosphate, disodium salt (ATP) (4.1.6),
- 50 mg phosphoenolpyruvate (PEP) tri(cyclohexylammonium) salt (4.1.8),
- 40 mg β-nicotinamide adenine dinucleotide (reduced form) (NADH) (4.1.5).

Working solution R1 can be kept for at least 30 days at 2-8 °C (approximately).

#### 4.2.3. Working solution 2 (R2)

The preparation may be as follows:

- 100 mL MOPS buffer (4.2.1),
- approx. 40 units of pyruvate kinase (PK) (4.1.10),

- approx. 40 units of lactate dehydrogenase (LDH) (4.1.11),
- 50 units of acetate kinase (AK) (4.1.9),
- 300 mg BSA (4.1.15).

Working solution R2 can be kept for approximately 48 hours at 2-8 °C (approximately).

**Note:** When preparing these solutions, they should be mixed gently to avoid the formation of foam. The life cycle of the working solutions is limited and should be evaluated and respected by the laboratory.

### 4.3. Calibration solutions

In order to ensure the closest possible connection to the International System of Units (SI), the calibration range should be made up of pure solutions of acetic acid (4.1.13). It is recommended to prepare a stock solution (e.g. 1.5 $g.L^{-1}$ acetic acid) by weight, then the rest of the calibrations are obtained from the stock solution to cover the measurement range.
A "zero" value may be obtained using a 9‰ sodium chloride solution (4.1.14) or equivalent saline solution.

## *5. Apparatus*

### 5.1. Analyser

#### 5.1.1. Equipment type

**Automatic** sequential analyser equipped with a spectrophotometer with UV detector. The reaction temperature should be stable (at around 37 °C). The reaction cuvettes are glass, methacrylate or quartz. The equipment is controlled by software that handles its operation, data acquisition and useful calculations.

#### 5.1.2. Absorbance reading

The concentration of the analytes directly relates to the absorbance difference read by the spectrophotometer. The precision of the absorbance reading should be a minimum of 0.1 absorbance unit (AU).

The absorbance values should not be saturating for the spectrometer used.

#### 5.1.3. Precision of sampled volumes

The volumes of reagents and samples taken by the pipettes of the analyser should be of sufficient precision so as not to have a significant impact on the measurement result.

#### 5.1.4. Reaction duration and temperature

In general, the reaction time is 10 minutes and the temperature 37 °C. Some pieces of apparatus may use slightly different values.

#### 5.1.5. Use of a reagent blank

The results are read by comparing the light intensity absorbed at the chosen wavelength between a cuvette in which the reaction is carried out and a cuvette in which the reaction does not take place (reagent blank).

#### 5.1.6. Wavelength

The wavelength of maximum absorption of the NADH formed by the reaction is 340 nm. For spectrophotometers in general use, this wavelength is to be selected.
A secondary wavelength is programmed in order to correct a potential matrix effect; this wavelength is 410 nm.
The measurement is conducted based on the $DO_{340}$ - $DO_{410}$ absorbance difference.

### 5.2. Balance

**This should be calibrated to the SI and have a 1 mg resolution.**

### 5.3. pH meter

### 5.4. Measuring glassware

The measuring glassware for the preparation of reagents and calibration solutions is class A.

## *6. Sample preparation*

### 6.1. Test samples

#### 6.1.1. Preparation of samples of still wines

The majority of wine samples may be analysed without preparation. In some cases, a preparation may be introduced:

- Filtration should be used for highly turbid samples.
  Sample dilution (manual or automatic) with water for analytical usage (4.1.1) should be used for values higher than the measurement range.

#### 6.1.2. Preparation of samples of sparkling wines

Sparkling wine samples should be subjected to a preliminary degassing by stirring under vacuum, ultrasonic treatment or any method that allows for the required degassing.

## *7. Procedure*

Given that different analysers may be used, it is recommended that the conditions of use provided by the manufacturer are strictly respected. The same goes for different enzymatic kits that are available on the market.
The procedure takes place as follows:

1. The sample (S) is placed in a reaction cuvette.
2. Working solution R1 (4.2.2) is then added to the cuvette.
3. Homogenisation takes place. Time is then allowed for a lag period, in order to guarantee absorbance stability. This lag period may last from 1 to 5 min, and is defined by the laboratory, according to the characteristics of the equipment used.
4. Working solution R2 (4.2.3) is added and the reaction is triggered.

   By way of example, the quantities of the different elements may be as follows:
   - sample: 3 µL,
   - R1: 120 µL, at $T_0$ (start of sequence),
   - R2: 60 µL, at $T_0$ + 3 min 40 sec.

The equipment carries out regular measurements that make it possible to obtain a reaction curve, an example of which is given in Figure 1.

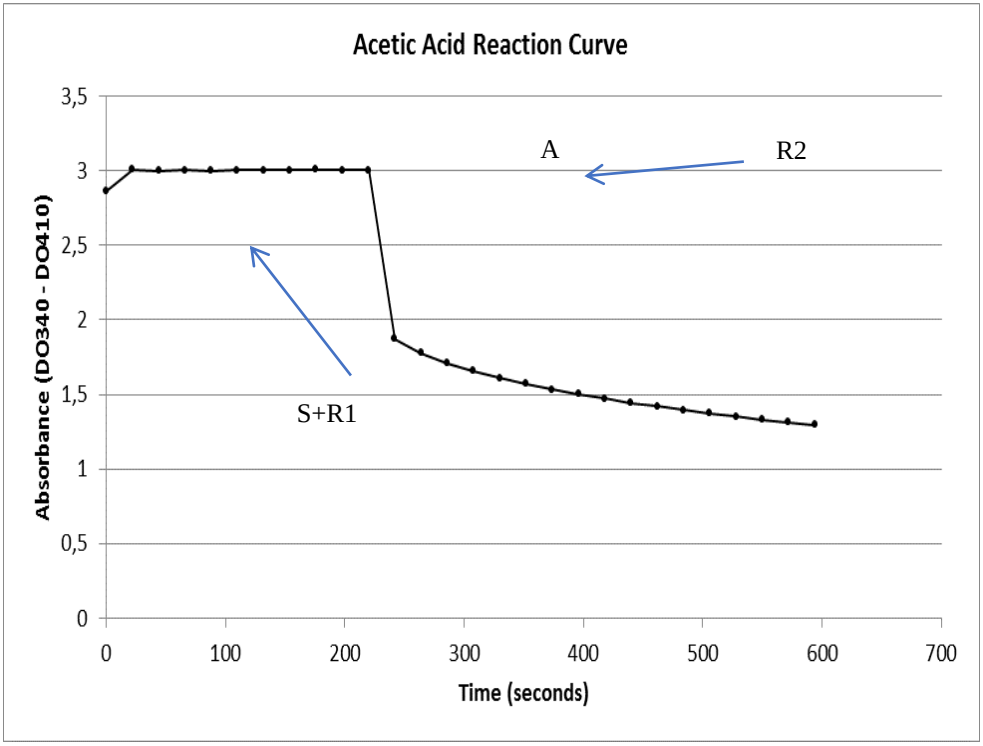


Figure 1. Reaction curve

The equipment makes it possible to choose the reading points for the difference in absorbance sought, for example A and B in Figure 1.

In order to correlate this value with the concentration of acetic acid, regular calibration of the apparatus is carried out using the calibration solutions at a minimum of 3 points covering the measurement range used. The calibration curve obtained is near to a straight line. Nevertheless, a second-degree equation may be used. An example is given in Figure 2.

In addition, a reagent blank is used comprising all of the reagents but no sample (point 0 of calibration).

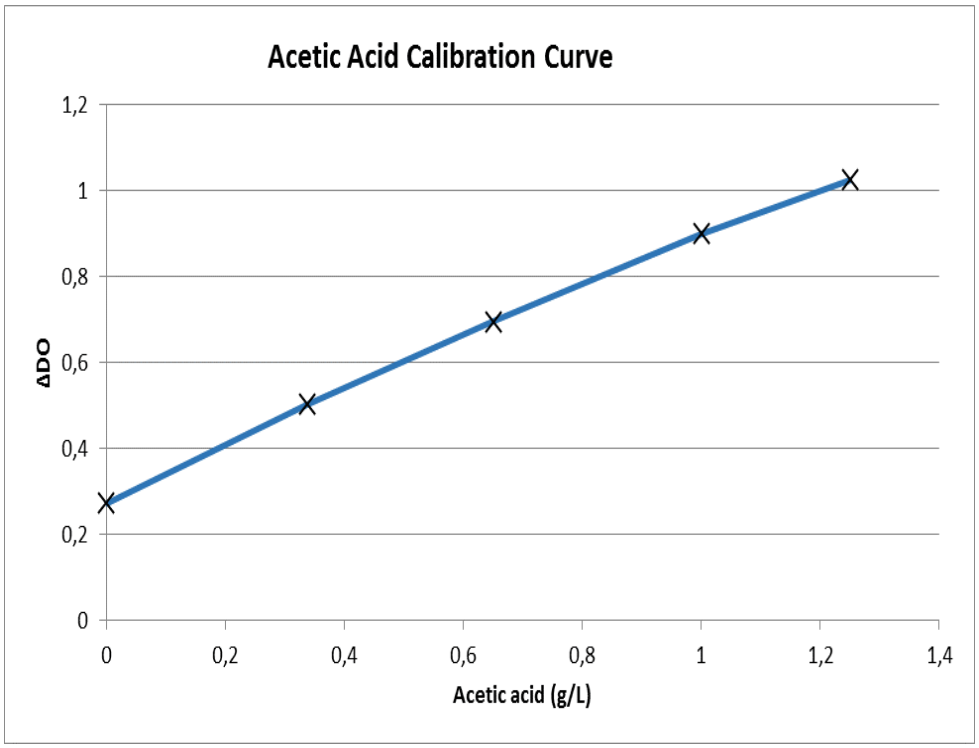


Figure 2. Calibration curve

## *8. Calculations*

For each measurement, the result is given according to the following formula:

$$R = |Absorbance\ B - Absorbance\ A|$$

The values thus obtained are recorded on the calibration curve to obtain the acetic acid concentration. The final value obtained should be multiplied by any coefficient of dilution used.

## *9. Expression of results*

The results for acetic acid are expressed in g/L of acetic acid, calculated to two decimal places, or in another unit according to usage (meq/L). The expression of the result should be consistent with the measurement uncertainty.

## *10. Automated enzymatic method characteristics*

**Interlaboratory reproducibility**

$RSD_R$ = 10%
$CV_R$% (k=2) = 2·$RSD_R$= 20%

**Repeatability**

$RSD_r$ = 4%
$CV_r$% (k=2) = 2·$RSD_r$ = 8%

**Limit of quantification**

Validated LQ < 0.2 g/L
Not determined in the collaborative study

## *11. Bibliography*

- McCLOSKEY Leo P., 'An Improved enzymatic assay for acetate in juice and wine', *Am. J. Enol. Vitic.*, Vol. 31, No. 21980.

## ANNEX

Method performance studies

Collaborative study

In total, 11 laboratories from 5 different countries took part in the collaborative study.

| Laboratory | Country |
|---|---|
| Miguel Torres S.A.- Finca Mas La Plana | Spain |
| INGACAL -Consellería do Medio Rural<br>Estación de Viticultura e Enoloxía de Galicia | Spain |
| Estación Enológica de Haro | Spain |
| Laboratoires Dubernet | France |
| Laboratoire Dicœnos Rhône | France |
| Laboratoire Natoli | France |
| SCL Montpellier | France |
| Fachbereich: Wein, Weinüberwachung - Chemisches und Veterinärunterchungsamt Karlsruhe | Germany |
| HBLAuBA Wein - und Obstbau | Austria |
| Hochschule GEISENHEIM University Institut Weinanalytik und Getränkeforschung | Germany |
| Unione Italiana Vini soc. Coop. | Italy |

Table 1. Participating laboratories

In total, 2 x 10 samples prepared as blind duplicates were analysed, with 1 repetition. The wines analysed were dry wines, sweetened wines, and liqueur wines, wines originating from France and Portugal.

| Sample | | A | | B | | C | | D | | E | | F | | G | | H | | I | | J | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Port wine | | Sweetened wine | | Dry wine | | Dry wine | | Sweetened wine | | Dry wine | | Dry wine | | Sweetened wine | | Sweetened wine | | Sweetened wine | |
| Position | | 1 | 9 | 2 | 13 | 3 | 4 | 5 | 15 | 6 | 10 | 16 | 20 | 7 | 11 | 12 | 17 | 8 | 19 | 14 | 18 |
| Lab3 | rep#1 | 0.24 | 0.27 | 0.20 | 0.21 | 0.65 | 0.65 | 0.47 | 0.49 | 0.54 | 0.52 | *1.28* | *1.30* | 0.64 | 0.63 | 0.29 | 0.31 | 0.39 | 0.37 | 0.63 | 0.62 |
| | rep#2 | 0.25 | 0.26 | 0.20 | 0.21 | 0.67 | 0.65 | 0.46 | 0.50 | 0.56 | 0.53 | *1.29* | *1.33* | 0.65 | 0.67 | 0.29 | 0.28 | 0.36 | 0.37 | 0.65 | 0.61 |
| Lab7 | rep#1 | 0.20 | 0.20 | 0.22 | 0.23 | 0.62 | 0.62 | 0.45 | 0.46 | 0.50 | 0.50 | *1.25* | *1.30* | 0.61 | 0.62 | 0.28 | 0.28 | 0.34 | 0.35 | 0.62 | 0.60 |
| | rep#2 | 0.20 | 0.21 | 0.21 | 0.22 | 0.63 | 0.64 | 0.45 | 0.46 | 0.53 | 0.52 | *1.20* | *1.20* | 0.61 | 0.64 | 0.29 | 0.28 | 0.35 | 0.37 | 0.60 | 0.61 |
| Lab9 | rep#1 | 0.17 | 0.18 | 0.18 | 0.19 | 0.57 | 0.52 | 0.40 | 0.40 | 0.41 | 0.43 | *1.18* | *1.18* | 0.57 | 0.54 | 0.24 | 0.29 | 0.36 | 0.32 | 0.53 | 0.51 |
| | rep#2 | 0.17 | 0.19 | 0.16 | 0.17 | 0.59 | 0.57 | 0.39 | 0.43 | 0.44 | 0.41 | *1.16* | *1.14* | 0.55 | 0.55 | 0.25 | 0.29 | 0.30 | 0.33 | 0.55 | 0.51 |
| Lab12 | rep#1 | 0.17 | 0.18 | 0.20 | 0.20 | 0.56 | 0.53 | 0.40 | 0.41 | 0.44 | 0.44 | *1.02* | *1.01* | 0.53 | 0.53 | 0.27 | 0.28 | 0.36 | 0.33 | 0.49 | 0.51 |
| | rep#2 | 0.17 | 0.18 | 0.20 | 0.21 | 0.55 | 0.54 | 0.40 | 0.41 | 0.44 | 0.44 | *1.02* | *1.01* | 0.52 | 0.52 | 0.28 | 0.29 | 0.36 | 0.34 | 0.48 | 0.51 |
| Lab13 | rep#1 | 0.22 | 0.19 | 0.23 | 0.20 | 0.50 | 0.51 | 0.40 | 0.40 | 0.42 | 0.44 | *0.95* | *0.97* | 0.48 | 0.49 | 0.27 | 0.28 | 0.32 | 0.32 | 0.48 | 0.50 |
| | rep#2 | 0.20 | 0.19 | 0.23 | 0.21 | 0.52 | 0.52 | 0.39 | 0.39 | 0.43 | 0.42 | *0.97* | *0.96* | 0.51 | 0.48 | 0.28 | 0.28 | 0.32 | 0.33 | 0.50 | 0.51 |
| Lab14 | rep#1 | 0.17 | 0.17 | 0.20 | 0.19 | 0.56 | 0.57 | 0.42 | 0.41 | 0.46 | 0.45 | *1.10* | *1.14* | 0.55 | 0.54 | 0.27 | 0.26 | 0.34 | 0.32 | 0.53 | 0.51 |
| | rep#2 | 0.17 | 0.17 | 0.20 | 0.19 | 0.56 | 0.57 | 0.42 | 0.41 | 0.45 | 0.44 | *1.12* | *1.10* | 0.53 | 0.55 | 0.26 | 0.26 | 0.33 | 0.31 | 0.53 | 0.53 |
| Lab15 | rep#1 | 0.22 | 0.23 | 0.28 | 0.27 | 0.68 | 0.68 | 0.52 | 0.52 | 0.56 | 0.56 | *1.20* | *1.23* | 0.69 | 0.73 | 0.35 | 0.34 | **0.47** | **0.42** | 0.60 | 0.62 |
| | rep#2 | 0.22 | 0.22 | 0.26 | 0.26 | 0.68 | 0.63 | 0.53 | 0.50 | 0.52 | 0.54 | *1.18* | *1.13* | 0.65 | 0.67 | 0.34 | 0.34 | **0.42** | **0.41** | 0.59 | 0.64 |
| Lab17 | rep#1 | 0.20 | 0.19 | 0.26 | 0.25 | 0.54 | 0.52 | 0.41 | 0.42 | 0.39 | 0.39 | *1.01* | *1.00* | **0.49** | **0.45** | 0.32 | 0.29 | 0.34 | 0.35 | **0.44** | **0.43** |
| | rep#2 | 0.20 | 0.20 | 0.27 | 0.27 | 0.53 | 0.55 | 0.43 | 0.43 | 0.43 | 0.43 | *1.03* | *1.05* | **0.49** | **0.49** | 0.31 | 0.32 | 0.37 | 0.38 | **0.44** | **0.46** |
| Lab18 | rep#1 | 0.27 | 0.25 | **0.35** | **0.33** | 0.69 | 0.68 | **0.53** | **0.56** | **0.59** | **0.59** | *1.24* | *1.21* | 0.66 | 0.68 | **0.43** | **0.41** | **0.50** | **0.51** | **0.65** | **0.63** |
| | rep#2 | 0.28 | 0.27 | **0.36** | **0.36** | 0.68 | 0.69 | **0.55** | **0.57** | **0.60** | **0.60** | *1.26* | *1.23* | 0.68 | 0.71 | **0.44** | **0.43** | **0.50** | **0.52** | **0.63** | **0.65** |
| Lab20 | rep#1 | 0.23 | 0.20 | 0.29 | 0.29 | 0.58 | 0.57 | 0.49 | 0.47 | 0.47 | 0.47 | *1.15* | *1.13* | 0.55 | 0.58 | 0.34 | 0.35 | 0.39 | 0.40 | 0.55 | 0.52 |
| | rep#2 | 0.23 | 0.20 | 0.29 | 0.29 | 0.58 | 0.57 | 0.49 | 0.47 | 0.47 | 0.47 | *1.15* | *1.13* | 0.55 | 0.58 | 0.34 | 0.36 | 0.39 | 0.40 | 0.55 | 0.52 |
| Lab22 | rep#1 | 0.17 | 0.16 | 0.20 | 0.18 | 0.60 | 0.60 | 0.43 | 0.44 | 0.47 | 0.47 | *1.20* | *1.20* | 0.58 | 0.58 | 0.26 | 0.26 | 0.32 | 0.32 | 0.55 | 0.58 |
| | rep#2 | 0.17 | 0.17 | 0.19 | 0.19 | 0.61 | 0.61 | 0.43 | 0.43 | 0.48 | 0.47 | *1.21* | *1.22* | 0.59 | 0.58 | 0.26 | 0.27 | 0.31 | 0.33 | 0.54 | 0.59 |

**Table 2. Table of data obtained (in g·L$^{-1}$ of acetic acid).** The values in bold correspond to the values rejected by the Cochran (variance outliers) test with a 2.5% significance level (one-tailed test), and the Grubbs (outliers from the mean) test with a significance level of 2.5% (two-tailed test).

| Sample | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| No. of laboratories selected | 11 | 10 | 11 | 10 | 10 | 11 | 10 | 9 | 9 | 9 |
| No. of repetitions | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Min. | 0.17 | 0.18 | 0.51 | 0.40 | 0.41 | 0.96 | 0.49 | 0.26 | 0.32 | 0.50 |
| Max. | 0.27 | 0.29 | 0.69 | 0.52 | 0.55 | 1.30 | 0.69 | 0.35 | 0.39 | 0.63 |
| Overall average | 0.20 | 0.22 | 0.59 | 0.44 | 0.47 | 1.14 | 0.59 | 0.30 | 0.35 | 0.55 |
| Repeatability variance | 0.0001 | 0.0001 | 0.0002 | 0.0001 | 0.0002 | 0.0006 | 0.0003 | 0.0001 | 0.0003 | 0.0003 |
| Inter-lab. stand. dev. | 0.03 | 0.04 | 0.06 | 0.04 | 0.05 | 0.11 | 0.07 | 0.03 | 0.03 | 0.05 |
| Reproducibility variance | 0.001 | 0.001 | 0.003 | 0.002 | 0.002 | 0.012 | 0.005 | 0.001 | 0.001 | 0.003 |
| Repeatability stand. dev. | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.02 | 0.02 | 0.01 | 0.02 | 0.02 |
| r limit | 0.03 | 0.03 | 0.04 | 0.03 | 0.04 | 0.07 | 0.05 | 0.02 | 0.05 | 0.05 |
| Repeatability $RSD_r$ | 4.5% | 4.5% | 2.4% | 2.7% | 2.9% | 2.1% | 3.1% | 2.8% | 4.8% | 3.2% |
| Reproducibility stand. dev. | 0.03 | 0.04 | 0.06 | 0.04 | 0.05 | 0.11 | 0.07 | 0.03 | 0.03 | 0.05 |
| R limit | 0.10 | 0.11 | 0.17 | 0.12 | 0.14 | 0.31 | 0.19 | 0.09 | 0.08 | 0.15 |
| Reproducibility $RSD_R$ | 16.8% | 17.4% | 9.9% | 9.5% | 10.6% | 9.5% | 11.7% | 11.1% | 8.5% | 9.5% |
| Horwitz RSD | 4.74 | 4.68 | 4.04 | 4.22 | 4.18 | 3.66 | 4.04 | 4.49 | 4.38 | 4.08 |
| $HorRat_r$ | 0.96 | 0.97 | 0.60 | 0.63 | 0.70 | 0.58 | 0.77 | 0.62 | 1.10 | 0.79 |
| Horwitz RSD | 7.18 | 7.09 | 6.12 | 6.40 | 6.34 | 5.54 | 6.13 | 6.80 | 6.63 | 6.18 |
| $HorRat_R$ | 2.34 | 2.45 | 1.63 | 1.49 | 1.67 | 1.71 | 1.91 | 1.63 | 1.29 | 1.53 |

**Table 3. Table of results obtained**

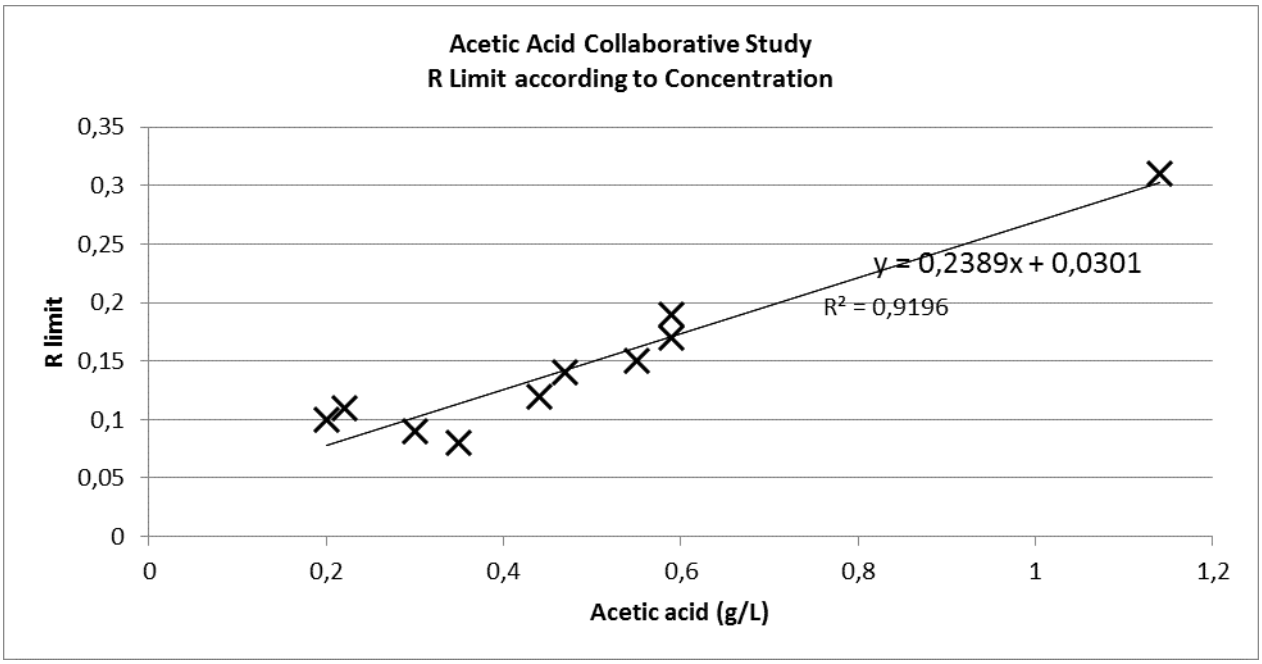


Figure 3. R limit according to concentration

**Method OIV-MA-AS313-28** Type II method

# Determination of D-gluconic acid in wines and musts by automated enzymatic method

OIV-OENO 622-2019

## *1. Scope of application*

This method makes it possible to determine D-gluconic acid in wines and musts by specific enzymatic analysis using an automatic sequential analyser, with concentrations of 0.06 g/L to 5.28 g/L of analyte (taking into account that the sample may be diluted).

## *2. Principle*

The D-gluconate present in the sample is phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by gluconate kinase (GK), to produce D-gluconate 6-phosphate and adenosine diphosphate (ADP).

$$\text{D-gluconate} + \text{ATP} \xrightarrow{\text{GK}} \text{D-Gluconate-6-P} + \text{ADP}$$

In the presence of nicotinamide adenine dinucleotide phosphate (NADP), D-gluconate 6-phosphate oxidises to form ribulose 5-phosphate through the action of enzyme 6-phosphogluconate dehydrogenase (6-PGDH). The quantity produced of reduced nicotinamide adenine dinucleotide phosphate (NADPH) corresponds to that of D-gluconate-6-phosphate and, as such, of D-gluconic acid.

$$\text{D-gluconate-6-P} + \text{NADP}^{+} \xrightarrow{\text{6-PGDH}} \text{Ribulose-5-P} + \text{NADPH} + CO_2 + H^{+}$$

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is determined by spectrophotometry at 340 nm (the wavelength of maximum absorption of NADPH). The NADPH concentration is proportional to the concentration of D-gluconic acid.

## *3. Reagents and working solutions*

3.1. Reagents:

3.1.1. Distilled water for laboratory use, certified to the EN ISO 3696 standard

3.1.2. PIPES (Piperazine-1,4-bis[ethanesulfonic acid]) (CAS No. 5625-37-6)

3.1.3. β-NADP-$Na_2$ (β-Nicotinamide adenine dinucleotide phosphate, disodium salt) (CAS No. 24292-60-2)

3.1.4. $MgCl_2 \cdot 6H_2O$ (Magnesium chloride hexahydrate) (CAS No. 7791-18-6)

3.1.5. ATP-$Na_2$ (Adenosine 5'-triphosphate disodium salt) (CAS No. 987-65-5)

3.1.6. Gluconate kinase (GK) (EC 2.7.1.12)

3.1.7. 6-phosphogluconate dehydrogenase (6-PGDH) (EC 1.1.1.44)

3.1.8. D-gluconic acid sodium salt (CAS No.527-07-1), minimum purity ≥ 99%

3.1.9. NaOH (Sodium hydroxide) (CAS No. 1310-73-2)

3.1.10 . PVP K-90 (Polyvinylpyrrolidone K-90) (CAS No. 9003-39-8)

3.2. Working solutions

3.2.1. Reagent 1: dissolve 30.2 g PIPES (3.1.2) (100 mmol/L), 1 g β-NADP-$Na_2$ (3.1.3) (1.3 mmol/L), 5.28 g NaOH (3.1.9) and 5 g PVP K-90 (3.1.10) in 1 L distilled water (3.1.1). The pH should be in the 6.3-6.4 range. This solution is stable for at least 4 weeks at 2-8 °C.

3.2.2. Reagent 2: dissolve 30.2 g PIPES (3.1.2) (100 mmol/L), 1 g $MgCl_2 \cdot 6H_2O$ (3.1.4) (1.3 mmol/L), 4.84 g ATP-$Na_2$ (3.1.5) and 7.6 g NaOH (3.1.9) in 1 L distilled water (3.1.1). The pH should be in the 7.0-7.2 range. Add 10 KU kinase glutonate (3.1.6) and 10 KU 6-phosphogluconate dehydrogenase (3.1.7). This solution is stable for at least 4 weeks at 2-8 °C.

3.3. Calibration solutions

Calibration solutions are prepared from the D-gluconic acid sodium salt (3.1.8), by weighing, in concentrations that cover the linear range of the method (0.06-2 g/L).

**Note 1**: The formulations described above are for preparing 1 L of reagent. Other volumes may be prepared according to the needs of the laboratory.

**Note 2**: Commercial kits are available for the determination of D-gluconic acid. The user should check that the kit includes the reagents mentioned above.

## *4. Apparatus*

4.1. Sequential automatic analyser with temperature control (approximately 37 °C), adjusted to measure absorbance at 340 nm. The apparatus should have software that facilitates data acquisition and carries out the necessary calculations.
4.2. Spectrophotometer or photometer to measure absorbance at 340 nm
4.3. Glass, quartz or methacrylate cuvettes
4.4. Class-A glassware for regular laboratory use (flasks, pipettes, etc.)
4.5. Micropipettes
4.6. Analytical balance with a resolution of ± 0.0001 g
4.7. pH meter

## *5. Sample preparation*

If necessary, follow the procedure for preparation of the corresponding sample:

5.1. Filter or centrifuge the samples if they contain suspended particles.
5.2. Degas samples that contain carbon dioxide through stirring under vacuum, an ultrasonic bath or any other means that makes it possible to reach the required level of degasification.

5.3. Samples with a concentration higher than the specified limit of linearity (2 g/L) should be diluted with distilled water (3.1.1). Multiply the concentration obtained by the dilution factor.

## *6. Procedure*

Given that different types of analysers may be used, it is recommended to strictly follow the manufacturer's instructions. This is also applicable to commercial enzymatic kits.

The procedures are those detailed below (volumes are given by way of example).

6.1. Manual procedure

6.1.1. Preheat the reagents and photometer to 37 °C.

6.1.2. Add the following to a cuvette using a pipette:

| | Reagent blank (RB) | Standard / Sample |
|---|---|---|
| Standard / Sample | - | 33 µL |
| Distilled water | 33 µL | - |
| Reagent 1 | 800 µL | 800 µL |

6.1.3. Mix and incubate for 1 min at 37 °C. Read the absorbance (A1) at 340 nm.

6.1.4. Add the following to the cuvette using a pipette:

| | | |
|---|---|---|
| Reagent 2 | 200 µL | 200 µL |

6.1.5. Mix and incubate for 10 min at 37 °C. Read the absorbance (A2) of the reagent blank, standard and sample at 340 nm.

6.2. Automated procedure

6.2.1 Introduce the following parameters into the automatic analyser (which complies with the requirements in paragraph 4.1):

| | |
|---|---|
| Wavelength: | 340 nm |
| Temperature: | 37 °C |
| Analysis mode: | 2 points (differential) |
| Sample volume: | 10 □L |
| Volume of Reagent 1: | 240 □L |
| Volume of Reagent 2: | 60 □L |

6.2.2 Programme an application in the analyser so that it performs the following sequence:

| | Reagent blank (RB) | Standard / Sample |
|---|---|---|
| Standard / Sample | - | 10 µL |
| Distilled water | 10 µL | - |
| Reagent 1 | 240 µL | 240 µL |

Mix, incubate for 1-5 min and read the absorbance (A1). Then add:

| | | |
|---|---|---|
| Reagent 2 | 60 µL | 60 µL |

Mix, incubate for 10 min and read the absorbance (A2).

The apparatus takes regular measurements, which makes it possible to obtain reaction kinetics (Fig. 1).

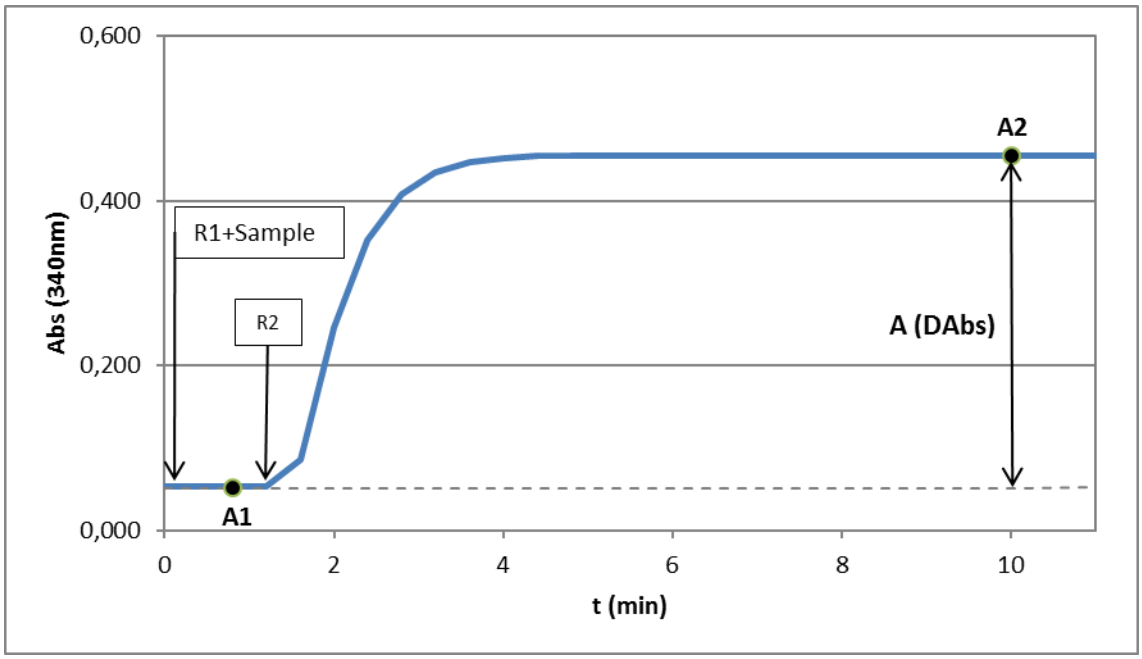


Figure 1: Example of reaction kinetics

6.2.3. It is advisable to check the calibration by carrying out three controls spread out over the measurement range. Each laboratory should establish its own internal quality-control programme, as well as correction procedures in case the controls do not comply with the acceptable tolerance levels.

### *7. Calculations*

Calculate the D-gluconic acid concentration using the following formula:

- If the calibration is carried out with one point (standard) and the blank:

$$\frac{(A2 - 0.81 \times A1)_{Sample} - (A2 - 0.81 \times A1}{(A2 - 0.81 \times A1)_{Standard} - (A2 - 0.81 \times A_{\_,RB}} \times F \times g/L_{Standard} = g/L_{Sample}$$

- If the calibration is with a calibration line:

$$A = (A2 - 0.81 \times A1)_{Sample} - (A2 - 0.81 \times A1)_{RB}$$

The absorbance calculated (A) is interpolated on the calibration line (Fig. 2) to obtain the D-gluconic acid concentration. Multiply the concentration obtained by the dilution factor (F).

A1: absorbance of the Blank/Standard/Sample + Reagent 1
A2: absorbance of the Blank/Standard/Sample + Reagent 1 + Reagent 2
RB: reagent blank
0.81: factor of correction of the dilution of Reagent 1 (this may vary depending on the volumes used according to the formula [Sample vol. + Reagent 1] / [Sample vol. + Reagent 1 + Reagent 2]).
F: factor of dilution of the sample (to be applied if necessary)

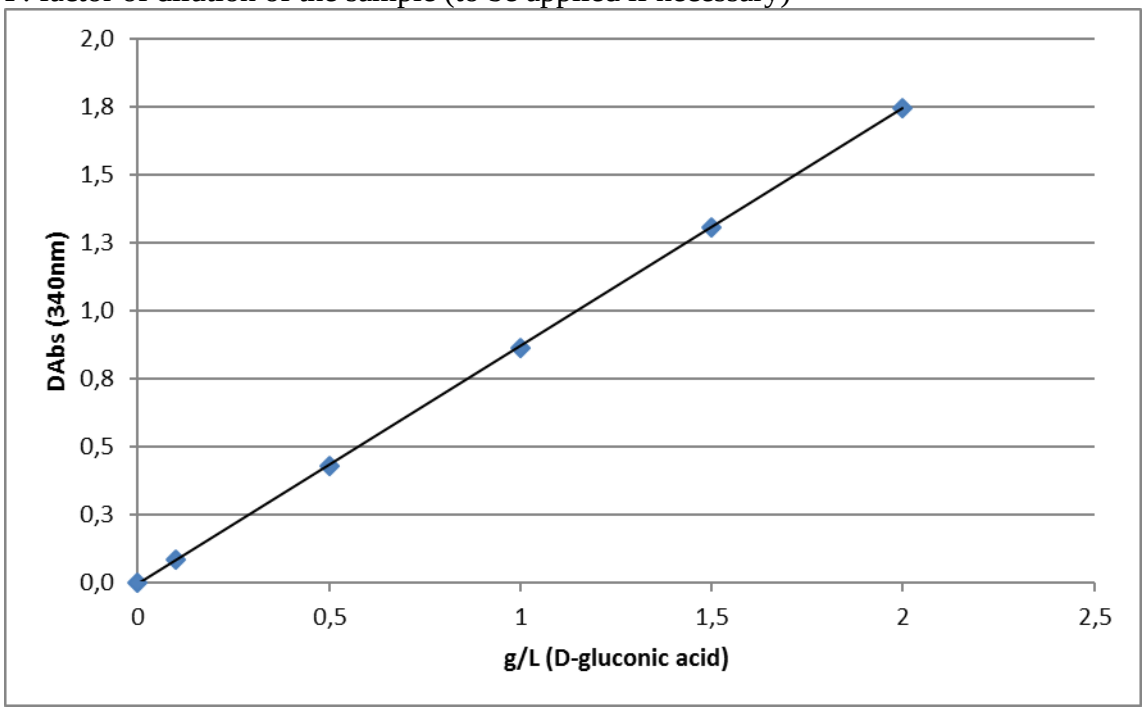


Figure 2: Example of a calibration line

### *8. Expression of results*

The results are expressed in g/L to 2 decimal points, or in accordance with the uncertainty.

## *9. Automated enzymatic method characteristics*

9.1. Repeatability

$r = 0.0396x + 0.0098$

With *x* representing the concentration of gluconic acid in g/L.

9.2 Reproducibility
$R = 0.1226x + 0.0237$
With *x* representing the concentration of gluconic acid in g/L.

9.3 Limit of quantification
Validated LoQ = 0.06 g/L

## ANNEX

Results of the inter-laboratory study

***1. Collaborative study***

1.1. Participating laboratories: 19 laboratories participated from 6 different countries.

| Laboratory | Country |
|---|---|
| Agroscope | Switzerland |
| Biosystems S.A | Spain |
| Bundesamt für Weinbau | Austria |
| Bundesinstitut für Risikobewertung (BfR) | Germany |
| Centrolab 2006, S.L | Spain |
| Comité Champagne Comité Interprofessionnel du vin de Champagne (CIVC) | France |
| Estación de Viticultura y Enología de Navarra (EVENA) | Spain |
| Estación de Viticultura y Enología Alcázar de San Juan | Spain |
| Estación Enológica de Castilla y León (ITACyL) | Spain |
| Estación Enológica de Haro | Spain |
| Federal College and Research Institute for Viticulture and Pomology (HBLA) | Austria |
| Freixenet S.A | Spain |
| Institut Català de la Vinya i el Vi (INCAVI) | Spain |
| Instituto dos Vinhos do Douro e do Porto (IVDP) | Portugal |
| Laboratoires Dicœnos Rhône | France |
| Laboratoires Dubernet | France |
| Laboratorio Arbitral Agroalimentario | Spain |
| Landesuntersuchungsamt, Institut für Lebensmittelchemie und Arzneimittelprüfung | Germany |
| Miguel Torres, SA | Spain |

For analysis, use 2 x 10 blind duplicate samples, with 1 repetition.

1.2. Samples

| Sample | Vial | Type of sample |
|---|---|---|
| A | 1 / 12 | Moscatel |
| B | 2 / 11 | Concentrated must |
| C | 3 / 13 | Sulphited must |
| D | 4 / 15 | White wine |
| E | 5 / 14 | White wine |
| F | 6 / 16 | Rosé wine |
| G | 7 / 10 | Red wine |
| H | 8 / 19 | Red wine |
| I | 9 / 18 | Red wine |
| J | 17 / 20 | Synthetic matrix |

1.3. Automated method results

| | | A | | B | | C | | D | | E | | F | | G | | H | | I | | J | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **1** | **12** | **2** | **11** | **3** | **13** | **4** | **15** | **5** | **14** | **6** | **16** | **7** | **10** | **8** | **19** | **9** | **18** | **17** | **20** |
| 1 | Rep #1 | 2.10 | 2.00 | 1.00 | 1.00 | 0.23 | 0.23 | 0.29 | 0.28 | 0.10 | 0.11 | 2.72 | 2.72 | 5.20 | 5.25 | 0.15 | 0.15 | 0.49 | 0.48 | 0.06 | 0.06 |
| | Rep #2 | 2.01 | 2.02 | 0.99 | 1.00 | 0.22 | 0.23 | 0.30 | 0.29 | 0.11 | 0.10 | 2.70 | 2.68 | 5.13 | 5.18 | 0.14 | 0.14 | 0.48 | 0.49 | 0.05 | 0.06 |
| | (1) | **2.06** | **2.01** | **1.00** | **1.00** | **0.23** | **0.23** | **0.30** | **0.29** | **0.11** | **0.11** | **2.71** | **2.70** | **5.17** | **5.22** | **0.15** | **0.15** | **0.49** | **0.49** | **0.06** | **0.06** |
| | Rep #1 | 1.95 | 2.02 | 1.02 | 0.99 | 0.23 | 0.23 | 0.29 | 0.30 | 0.10 | 0.09 | 2.79 | 2.72 | 5.27 | 5.24 | 0.13 | 0.13 | 0.47 | 0.46 | 0.05 | 0.05 |
| 2 | Rep #2 | 2.00 | 2.10 | 1.03 | 1.01 | 0.23 | 0.23 | 0.29 | 0.30 | 0.11 | 0.10 | 2.75 | 2.80 | 5.30 | 5.20 | 0.13 | 0.13 | 0.47 | 0.45 | 0.05 | 0.05 |
| | (2) | **1.97** | **2.06** | **1.03** | **1.00** | **0.23** | **0.23** | **0.29** | **0.30** | **0.11** | **0.10** | **2.77** | **2.76** | **5.28** | **5.22** | **0.13** | **0.13** | **0.47** | **0.45** | **0.05** | **0.05** |
| | Rep #1 | 2.19 | 2.19 | 1.06 | 1.07 | 0.27 | 0.28 | 0.34 | 0.33 | *0.13* | *0.13* | 2.95 | 3.06 | 5.54 | 5.63 | 0.08 | 0.08 | 0.51 | 0.50 | 0.06 | 0.06 |
| 3 | Rep #2 | 2.21 | 2.30 | 1.09 | 1.07 | 0.27 | 0.28 | 0.34 | 0.34 | *0.13* | *0.13* | 2.95 | 2.99 | 5.51 | 5.68 | 0.07 | 0.08 | 0.49 | 0.50 | 0.06 | 0.06 |
| | (3) | **2.20** | **2.24** | **1.07** | **1.07** | **0.27** | **0.28** | **0.34** | **0.34** | ***0.13*** | ***0.13*** | **2.95** | **3.02** | **5.53** | **5.66** | **0.07** | **0.08** | **0.50** | **0.50** | **0.06** | **0.06** |
| | Rep #1 | 2.10 | 2.10 | 1.02 | 1.05 | 0.23 | 0.24 | 0.28 | 0.29 | 0.10 | 0.10 | 2.70 | 2.80 | *3.78* | *3.94* | 0.13 | 0.12 | 0.45 | 0.46 | *0.10* | *0.10* |
| 4 | Rep #2 | 2.08 | 2.12 | 1.03 | 1.04 | 0.24 | 0.25 | 0.29 | 0.29 | 0.10 | 0.11 | 2.72 | 2.82 | *3.80* | *3.98* | 0.13 | 0.13 | 0.45 | 0.45 | *0.10* | *0.10* |
| | (4) | **2.09** | **2.11** | **1.03** | **1.05** | **0.24** | **0.25** | **0.29** | **0.29** | **0.10** | **0.11** | **2.71** | **2.81** | ***3.79*** | ***3.96*** | **0.13** | **0.13** | **0.45** | **0.46** | ***0.10*** | ***0.10*** |
| | Rep #1 | 1.88 | 1.94 | 0.98 | 0.96 | *0.22* | *0.33* | 0.27 | 0.26 | 0.10 | 0.09 | 2.68 | 2.58 | 4.95 | 4.90 | 0.11 | 0.08 | 0.43 | 0.42 | 0.04 | 0.05 |
| 5 | Rep #2 | 2.06 | 2.12 | 0.99 | 1.00 | *0.24* | *0.23* | 0.29 | 0.29 | 0.11 | 0.09 | 3.08 | 2.84 | 5.40 | 5.30 | 0.12 | 0.15 | 0.47 | 0.48 | 0.07 | 0.06 |
| | (5) | **1.97** | **2.03** | **0.99** | **0.98** | ***0.23*** | ***0.28*** | **0.28** | **0.28** | **0.11** | **0.09** | **2.88** | **2.71** | **5.18** | **5.10** | **0.12** | **0.12** | **0.45** | **0.45** | **0.06** | **0.06** |
| | Rep #1 | 2.06 | 2.02 | 1.00 | 1.01 | 0.22 | 0.23 | 0.28 | 0.29 | 0.10 | 0.10 | 2.80 | 2.78 | 5.22 | 5.22 | 0.12 | 0.12 | 0.46 | 0.46 | 0.05 | 0.05 |
| 6 | Rep #2 | 1.98 | 2.01 | 0.99 | 1.00 | 0.22 | 0.22 | 0.27 | 0.28 | 0.09 | 0.10 | 2.75 | 2.75 | 5.22 | 5.22 | 0.12 | 0.11 | 0.45 | 0.46 | 0.05 | 0.05 |
| | (6) | **2.02** | **2.02** | **0.99** | **1.00** | **0.22** | **0.23** | **0.28** | **0.29** | **0.09** | **0.10** | **2.78** | **2.77** | **5.22** | **5.22** | **0.12** | **0.12** | **0.45** | **0.46** | **0.05** | **0.05** |
| | Rep #1 | 2.02 | 2.02 | 0.98 | 0.99 | 0.23 | 0.23 | 0.29 | 0.29 | 0.11 | 0.11 | 2.74 | 2.75 | 5.28 | 5.16 | 0.13 | 0.13 | 0.46 | 0.47 | 0.05 | 0.06 |
| 7 | Rep #2 | 2.01 | 2.01 | 0.98 | 0.99 | 0.23 | 0.23 | 0.30 | 0.29 | 0.11 | 0.12 | 2.75 | 2.74 | 5.28 | 5.22 | 0.14 | 0.14 | 0.46 | 0.47 | 0.05 | 0.05 |
| | (7) | **2.02** | **2.02** | **0.98** | **0.99** | **0.23** | **0.23** | **0.30** | **0.29** | **0.11** | **0.12** | **2.75** | **2.75** | **5.28** | **5.19** | **0.14** | **0.14** | **0.46** | **0.47** | **0.05** | **0.06** |
| | Rep #1 | 2.09 | 2.11 | 1.02 | 1.01 | 0.24 | 0.24 | 0.29 | 0.29 | 0.10 | 0.09 | 2.83 | 2.84 | 5.20 | 5.25 | 0.10 | 0.10 | 0.47 | 0.46 | 0.05 | 0.05 |
| 8 | Rep #2 | 2.10 | 2.09 | 1.00 | 1.01 | 0.24 | 0.23 | 0.29 | 0.29 | 0.10 | 0.10 | 2.80 | 2.78 | 5.18 | 5.18 | 0.10 | 0.08 | 0.47 | 0.47 | 0.05 | 0.05 |
| | (8) | **2.10** | **2.10** | **1.01** | **1.01** | **0.24** | **0.24** | **0.29** | **0.29** | **0.10** | **0.10** | **2.82** | **2.81** | **5.19** | **5.22** | **0.10** | **0.09** | **0.47** | **0.47** | **0.05** | **0.05** |
| | Rep #1 | 1.94 | 2.02 | 1.00 | 1.00 | 0.24 | 0.24 | 0.29 | 0.29 | 0.11 | 0.11 | 2.74 | 2.74 | 5.15 | 5.25 | 0.15 | 0.15 | 0.47 | 0.48 | 0.06 | 0.06 |
| 9 | Rep #2 | 1.94 | 2.00 | 1.00 | 1.00 | 0.24 | 0.24 | 0.29 | 0.29 | 0.11 | 0.11 | 2.76 | 2.74 | 5.10 | 5.10 | 0.14 | 0.15 | 0.47 | 0.47 | 0.06 | 0.06 |
| | (9) | **1.94** | **2.01** | **1.00** | **1.00** | **0.24** | **0.24** | **0.29** | **0.29** | **0.11** | **0.11** | **2.75** | **2.74** | **5.13** | **5.18** | **0.15** | **0.15** | **0.47** | **0.48** | **0.06** | **0.06** |
| | Rep #1 | 1.95 | 2.05 | 1.00 | 1.00 | 0.23 | 0.23 | 0.29 | 0.29 | 0.11 | 0.10 | 2.70 | 2.65 | 5.20 | 5.30 | 0.14 | 0.14 | 0.48 | 0.45 | 0.05 | 0.05 |
| 10 | Rep #2 | 2.00 | 2.00 | 0.99 | 1.00 | 0.23 | 0.25 | 0.28 | 0.29 | 0.10 | 0.11 | 2.70 | 2.75 | 5.40 | 5.30 | 0.13 | 0.13 | 0.48 | 0.46 | 0.05 | 0.05 |
| | (10) | **1.98** | **2.03** | **1.00** | **1.00** | **0.23** | **0.24** | **0.29** | **0.29** | **0.11** | **0.11** | **2.70** | **2.70** | **5.30** | **5.30** | **0.14** | **0.14** | **0.48** | **0.46** | **0.05** | **0.05** |
| | Rep #1 | *0.10* | *<0.05* | *<0.05* | *<0.05* | 0.23 | 0.25 | 0.29 | 0.33 | 0.08 | 0.10 | *0.13* | *0.26* | *<0.05* | *<0.05* | *<0.05* | *<0.05* | *<0.05* | *<0.05* | 0.06 | 0.05 |
| 11 | Rep #2 | *0.10* | *<0.05* | *<0.05* | *<0.05* | 0.25 | 0.24 | 0.28 | 0.27 | 0.08 | 0.10 | *0.13* | *0.26* | *<0.05* | *<0.05* | *<0.05* | *<0.05* | *<0.05* | *<0.05* | 0.05 | 0.05 |
| | (11) | ***0.10*** | - | - | - | **0.24** | **0.25** | **0.29** | **0.30** | **0.08** | **0.10** | ***0.13*** | ***0.26*** | - | - | - | - | - | - | **0.06** | **0.05** |
| | Rep #1 | *2.49* | *2.47* | *1.17* | *1.16* | 0.29 | 0.27 | *0.38* | *0.38* | *0.14* | *0.13* | *3.51* | *3.42* | *6.37* | *6.12* | 0.18 | 0.18 | *0.61* | *0.60* | 0.06 | 0.05 |
| 12 | Rep #2 | *2.56* | *2.53* | *1.16* | *1.20* | 0.30 | 0.28 | *0.38* | *0.37* | *0.14* | *0.13* | *3.43* | *3.32* | *6.28* | *6.24* | 0.18 | 0.18 | *0.61* | *0.60* | 0.06 | 0.05 |
| | (12) | ***2.53*** | ***2.50*** | ***1.17*** | ***1.18*** | **0.30** | **0.28** | ***0.38*** | ***0.38*** | ***0.14*** | ***0.13*** | ***3.47*** | ***3.37*** | ***6.33*** | ***6.18*** | **0.18** | **0.18** | ***0.61*** | ***0.60*** | **0.06** | **0.05** |
| | 13a | 2.05 | 2.06 | 0.98 | 0.98 | 0.22 | 0.22 | 0.28 | 0.27 | 0.10 | 0.10 | 2.76 | 2.74 | 5.27 | 5.18 | 0.13 | 0.15 | 0.46 | 0.45 | 0.05 | 0.05 |
| 13 | 13b | 2.05 | 2.06 | 0.98 | 0.98 | 0.22 | 0.22 | 0.28 | 0.27 | 0.10 | 0.10 | 2.76 | 2.74 | 5.27 | 5.18 | 0.13 | 0.15 | 0.46 | 0.45 | 0.05 | 0.05 |
| | (13) | **2.05** | **2.06** | **0.98** | **0.98** | **0.22** | **0.22** | **0.28** | **0.27** | **0.10** | **0.10** | **2.76** | **2.74** | **5.27** | **5.18** | **0.13** | **0.15** | **0.46** | **0.45** | **0.05** | **0.05** |
| | Rep #1 | 1.86 | 1.86 | 0.96 | 0.96 | 0.23 | 0.24 | *0.29* | *0.32* | 0.11 | 0.10 | 2.57 | 2.64 | 4.99 | 5.19 | *0.17* | *0.11* | *0.50* | *0.39* | 0.05 | 0.09 |
| 14 | Rep #2 | 1.88 | 1.86 | 0.95 | 0.97 | 0.23 | 0.23 | *0.29* | *0.32* | 0.15 | 0.1 | 2.56 | 2.65 | 5.00 | 5.10 | *0.15* | *0.11* | *0.45* | *0.34* | 0.06 | 0.05 |
| | (14) | **1.87** | **1.86** | **0.96** | **0.97** | **0.23** | **0.24** | ***0.29*** | ***0.32*** | **0.13** | **0.10** | **2.57** | **2.65** | **5.00** | **5.15** | ***0.16*** | ***0.11*** | ***0.48*** | ***0.37*** | **0.06** | **0.07** |
| | Rep #1 | 1.91 | 1.98 | 1.11 | 1.12 | 0.30 | 0.31 | 0.32 | 0.33 | 0.10 | 0.11 | 3.23 | 3.13 | 5.88 | 6.01 | *0.13* | *0.16* | 0.50 | 0.52 | *0.02* | *0.04* |
| 15 | Rep #2 | 1.93 | 1.99 | 1.12 | 1.13 | 0.31 | 0.32 | 0.32 | 0.34 | 0.09 | 0.12 | 3.24 | 3.14 | 5.90 | 6.08 | *0.12* | *0.17* | 0.51 | 0.53 | *0.02* | *0.04* |
| | (15) | **1.92** | **1.99** | **1.12** | **1.13** | **0.31** | **0.32** | **0.32** | **0.34** | **0.10** | **0.12** | **3.24** | **3.14** | **5.89** | **6.05** | ***0.13*** | ***0.17*** | **0.51** | **0.53** | ***0.02*** | ***0.04*** |
| | Rep #1 | 1.98 | 1.99 | 1.00 | 1.00 | 0.23 | 0.23 | 0.28 | 0.28 | 0.10 | 0.10 | 2.78 | 2.82 | 5.27 | 5.34 | 0.13 | 0.13 | 0.47 | 0.47 | 0.05 | 0.05 |
| 16 | Rep #2 | 2.04 | 2.08 | 0.99 | 1.00 | 0.23 | 0.23 | 0.28 | 0.28 | 0.10 | 0.10 | 2.79 | 2.81 | 5.30 | 5.28 | 0.14 | 0.14 | 0.47 | 0.47 | 0.05 | 0.05 |
| | (16) | **2.01** | **2.04** | **1.00** | **1.00** | **0.23** | **0.23** | **0.28** | **0.28** | **0.10** | **0.10** | **2.79** | **2.82** | **5.29** | **5.31** | **0.14** | **0.14** | **0.47** | **0.47** | **0.05** | **0.05** |
| | Rep #1 | 2.27 | 2.22 | *1.17* | *1.20* | 0.27 | 0.27 | 0.30 | 0.29 | 0.12 | 0.10 | 2.75 | 2.72 | 5.20 | 4.95 | 0.16 | 0.17 | 0.46 | 0.47 | 0.07 | 0.07 |
| 17 | Rep #2 | 2.24 | 2.21 | *1.19* | *1.19* | 0.29 | 0.29 | 0.29 | 0.28 | 0.12 | 0.11 | 2.77 | 2.79 | 5.05 | 4.90 | 0.16 | 0.12 | 0.46 | 0.42 | 0.08 | 0.06 |
| | (17) | **2.26** | **2.22** | ***1.18*** | ***1.20*** | **0.28** | **0.28** | **0.30** | **0.29** | **0.12** | **0.11** | **2.76** | **2.76** | **5.13** | **4.93** | **0.16** | **0.15** | **0.46** | **0.45** | **0.08** | **0.07** |
| | Rep #1 | 2.08 | 2.08 | 1.02 | 1.00 | 0.24 | 0.23 | 0.29 | 0.28 | 0.11 | 0.10 | 2.80 | 2.82 | 5.40 | 5.37 | 0.14 | 0.15 | 0.48 | 0.49 | 0.06 | 0.05 |
| 19 | Rep #2 | 2.08 | 2.08 | 1.01 | 1.00 | 0.23 | 0.24 | 0.28 | 0.29 | 0.10 | 0.11 | 2.82 | 2.80 | 5.28 | 5.40 | 0.14 | 0.14 | 0.47 | 0.47 | 0.05 | 0.05 |
| | (19) | **2.08** | **2.08** | **1.02** | **1.00** | **0.24** | **0.24** | **0.29** | **0.29** | **0.11** | **0.11** | **2.81** | **2.81** | **5.34** | **5.39** | **0.14** | **0.15** | **0.48** | **0.48** | **0.06** | **0.05** |

**Table of data obtained.** The values in italics are the results removed due to outliers from individual values according to the simple 2-tail Grubbs test and the double Grubbs test (2-tail, P = 2.5%), and according to the Cochran test (1-tail test where P = 2.5%).

| Sample | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| Accepted labs | 16 | 15 | 17 | 16 | 16 | 16 | 15 | 15 | 15 | 16 |
| Repetitions | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Minimum value | 1.87 | 0.96 | 0.22 | 0.28 | 0.09 | 2.61 | 5.03 | 0.07 | 0.45 | 0.05 |
| Maximum value | 2.24 | 1.12 | 0.31 | 0.34 | 0.12 | 2.81 | 5.97 | 0.18 | 0.52 | 0.07 |
| Mean value (g/L) | **2.04** | **1.01** | **0.25** | **0.29** | **0.10** | **2.79** | **5.28** | **0.13** | **0.47** | **0.06** |
| $S_r$ | 0.03 | 0.01 | 0.01 | 0.01 | 0.01 | 0.05 | 0.08 | 0.01 | 0.01 | 0.01 |
| r limit = 2□2* $S_r$ | 0.09 | 0.02 | 0.02 | 0.02 | 0.03 | 0.13 | 0.22 | 0.02 | 0.02 | 0.02 |
| $RSD_r$ | 1.48% | 0.76% | 2.13% | 1.93% | 8.53% | 1.70% | 1.50% | 3.99% | 1.70% | 9.86% |
| S reproducibility ($S_R$) | 0.09 | 0.04 | 0.03 | 0.02 | 0.01 | 0.13 | 0.24 | 0.03 | 0.02 | 0.01 |
| R limit = 2□2* $S_R$ | 0.28 | 0.11 | 0.07 | 0.05 | 0.06 | 0.38 | 0.67 | 0.07 | 0.05 | 0.02 |
| $RSD_R$ | 4.63% | 3.96% | 10.57% | 5.89% | 8.91% | 4.81% | 4.50% | 19.21% | 4.09% | 12.49% |
| Horwitz $RSD_r$ (%) | 3.39% | 3.77% | 4.66% | 4.54% | 5.31% | 3.23% | 2.94% | 5.12% | 4.22% | 5.84% |
| $HorRat_r$ | 0.44 | 0.20 | 0.46 | 0.43 | 1.61 | 0.53 | 0.51 | 0.78 | 0.40 | 1.69 |
| Horwitz $RSD_R$ (%) | 5.08% | 5.65% | 6.99% | 6.81% | 7.96% | 4.85% | 4.40% | 7.68% | 6.34% | 8.75% |
| $HorRat_R$ | 0.91 | 0.70 | 1.51 | 0.86 | 1.12 | 0.99 | 1.02 | 2.50 | 0.65 | 1.43 |

S: Standard deviation / RSD: Relative standard deviation / r: Repeatability limit / R: Reproducibility limit

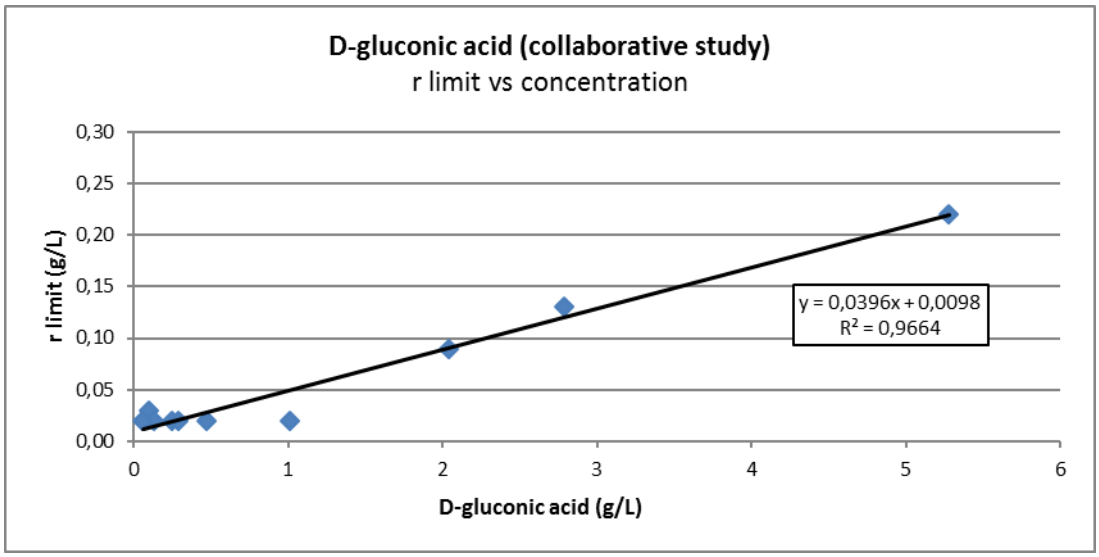


Figure 3: Repeatability limit according to concentration

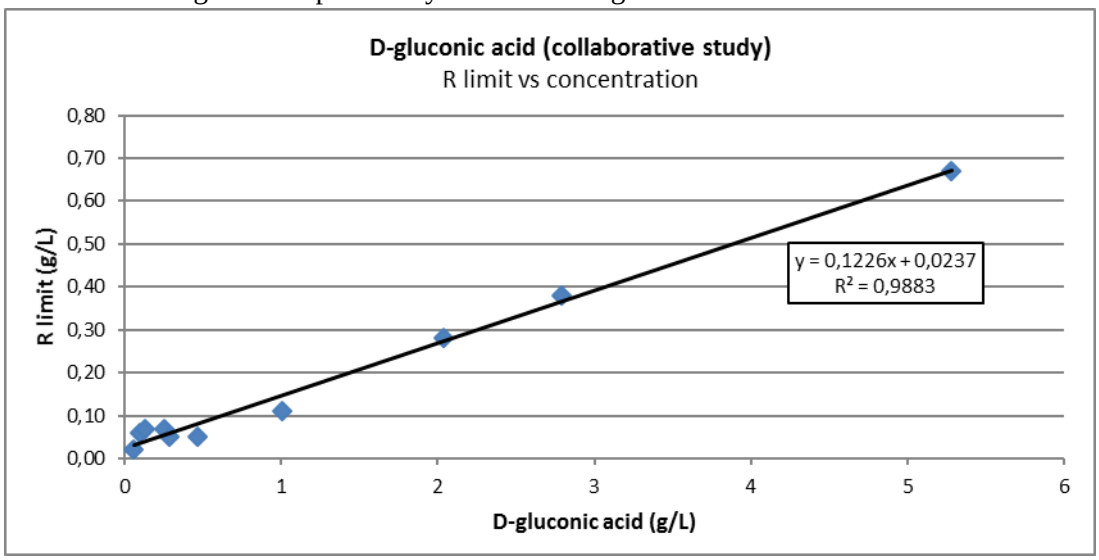


Figure 4: Reproducibility limit according to concentration

1.4. Manual method results

| | | A | | B | | C | | D | | E | | F | | G | | H | | I | | J | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **1** | **12** | **2** | **11** | **3** | **13** | **4** | **15** | **5** | **14** | **6** | **16** | **7** | **10** | **8** | **19** | **9** | **18** | **17** | **20** |
| | Rep #1 | 2.05 | 2.09 | 1.06 | 0.99 | 0.25 | 0.25 | 0.34 | 0.33 | *0.10* | *0.12* | 2.85 | 2.84 | 5.32 | 5.34 | 0.14 | 0.13 | 0.45 | 0.46 | 0.05 | 0.05 |
| 2 | Rep #2 | 2.08 | 2.10 | 1.03 | 1.02 | 0.23 | 0.26 | 0.35 | 0.32 | *0.09* | *0.10* | 2.83 | 2.86 | 5.34 | 5.36 | 0.15 | 0.13 | 0.44 | 0.45 | 0.05 | 0.05 |
| | □ (2) | **2.07** | **2.10** | **1.05** | **1.01** | **0.24** | **0.26** | **0.35** | **0.33** | **0.10** | **0.11** | **2.84** | **2.85** | **5.33** | **5.35** | **0.15** | **0.13** | **0.45** | **0.46** | **0.05** | **0.05** |
| | Rep #1 | 2.24 | 2.11 | 1.01 | 1.04 | 0.26 | 0.26 | 0.34 | 0.33 | 0.11 | 0.11 | 3.05 | 3.19 | 5.64 | 5.68 | 0.14 | 0.16 | 0.34 | 0.41 | 0.05 | 0.05 |
| 10 | Rep #2 | 2.37 | 2.24 | 1.01 | 1.06 | 0.25 | 0.26 | 0.35 | 0.34 | 0.12 | 0.11 | 3.10 | 3.02 | 5.65 | 5.78 | 0.14 | 0.15 | 0.33 | 0.42 | 0.05 | 0.05 |
| | □ (10) | **2.31** | **2.18** | **1.01** | **1.05** | **0.26** | **0.26** | **0.35** | **0.34** | **0.12** | **0.11** | **3.08** | **3.11** | **5.65** | **5.73** | **0.14** | **0.16** | **0.34** | **0.42** | **0.05** | **0.05** |
| | Rep #1 | 2.61 | 2.54 | 1.04 | 0.99 | 0.27 | 0.28 | 0.34 | 0.34 | 0.13 | 0.12 | 3.44 | 3.38 | 5.97 | 6.22 | 0.21 | 0.23 | 0.44 | 0.47 | 0.05 | 0.05 |
| 18 | Rep #2 | 2.57 | 2.54 | 0.97 | 1.01 | 0.28 | 0.28 | 0.35 | 0.35 | 0.12 | 0.12 | 3.32 | 3.42 | 6.04 | 6.31 | 0.21 | 0.21 | 0.51 | 0.53 | 0.05 | 0.05 |
| | □ (18) | **2.59** | **2.54** | **1.00** | **1.00** | **0.28** | **0.28** | **0.34** | **0.34** | **0.12** | **0.12** | **3.38** | **3.40** | **6.00** | **6.26** | **0.21** | **0.22** | **0.48** | **0.50** | **0.05** | **0.05** |

**Table of data obtained.** The values in italics are the results removed due to outliers from individual values according to the simple 2-tail Grubbs test and the double Grubbs test (2-tail, P = 2.5%), and according to the Cochran test (1-tail test where P = 2.5%).

| Sample | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| Accepted labs | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Repetitions | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Minimum value | 2.05 | 0.97 | 0.23 | 0.32 | 0.09 | 2.83 | 5.32 | 0.13 | 0.33 | 0.05 |
| Maximum value | 2.61 | 1.06 | 0.28 | 0.35 | 0.13 | 3.44 | 6.31 | 0.23 | 0.53 | 0.05 |
| Mean value (g/L) | **2.29** | **1.02** | **0.26** | **0.34** | **0.11** | **3.11** | **5.72** | **0.17** | **0.44** | **0.05** |
| $S_r$ | 0.06 | 0.02 | 0.01 | 0.01 | 0.01 | 0.02 | 0.11 | 0.01 | 0.03 | - |
| r limit = 2□2* $S_r$ | 0.16 | 0.07 | 0.02 | 0.03 | 0.02 | 0.04 | 0.31 | 0.03 | 0.10 | - |
| $RSD_r$ | 0.03% | 0.02% | 0.03% | 0.03% | 0.06% | 0.01% | 0.02% | 0.06% | 0.08% | - |
| S reproducibility ($S_R$) | 0.25 | 0.02 | 0.02 | 0.01 | 0.01 | 0.27 | 0.41 | 0.04 | 0.06 | - |
| R limit = 2□2* $S_R$ | 0.70 | 0.07 | 0.05 | 0.03 | 0.03 | 0.77 | 1.14 | 0.12 | 0.17 | - |
| $RSD_R$ | 0.11% | 0.02% | 0.06% | 0.03% | 0.10% | 0.09% | 0.07% | 0.26% | 0.14% | - |
| Horwitz $RSD_r$ (%) | 3.33% | 3.76% | 4.62% | 4.44% | 5.24% | 3.18% | 2.90% | 4.94% | 4.27% | - |
| $HorRat_r$ | 0.77 | 0.60 | 0.55 | 0.61 | 1.09 | 0.16 | 0.67 | 1.21 | 1.82 | - |
| Horwitz $RSD_R$ (%) | 4.99% | 5.64% | 6.92% | 6.66% | 7.86% | 4.77% | 4.35% | 7.41% | 6.41% | - |
| $HorRat_R$ | 2.18 | 0.42 | 0.93 | 0.47 | 1.30 | 1.85 | 1.63 | 3.46 | 2.22 | - |

S: Standard deviation / RSD: Relative standard deviation / r: Repeatability limit / R: Reproducibility limit. The statistical parameters were calculated taking into account the results of the 3 laboratories.

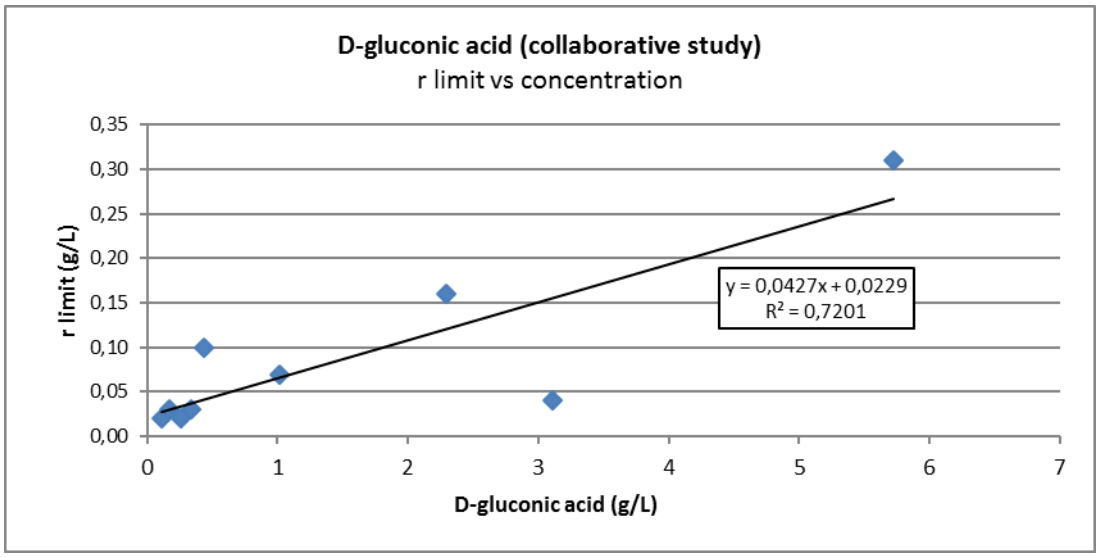


Figure 5: Repeatability limit according to concentration

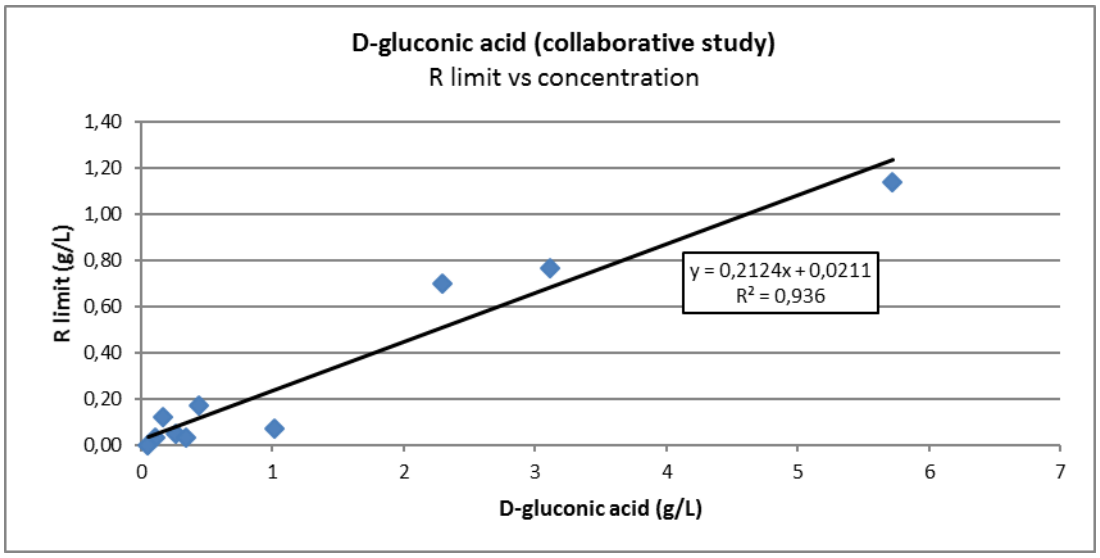


Figure 6: Reproducibility limit according to concentration

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Zoecklein, B. W., Fugelsang, K. C., Gump, B. H. & Nury, F.S., *Wine analysis and production*, Van Nostrand Reinhold, 1st edition, 31 December, 1990.

**Method OIV-MA-AS314-01** Type II method

# Carbone Dioxide

With a range of concentration up to 1.5 g/L
(A 39 modified by oeno 21/2003 and completed by resolution Oeno 3/2006)

## 1. Principle

1.1 Still wines ($CO_2$ over pressure ≤ 0.5 x $10^5$ Pa*)

The volume of wine taken from the sample is cooled to around 0°C and mixed with a sufficient quantity of sodium hydroxide to give a pH of 10-11. Titration is carried out with an acid solution in the presence of carbonic anhydrase. The carbon dioxide content is calculated from the volume of acid needed to change the pH from 8.6 (bicarbonate form) to 4.0 (carbonic acid). A blank titration is carried out in the same conditions on decarbonated wine in order to take account of the volume of sodium hydroxide solution taken up by the wine acids.

1.2 Sparkling and semi-sparkling wines

The sample of wine to be analyzed is cooled near to the freezing point. After removal of a sub-sample to be used as a blank after decarbonation, the remainder of the bottle is made alkaline to fix all the carbon dioxide in the form of $Na_2CO_3$. Titration is carried out with an acid solution in the presence of carbonic anhydrase. The carbon dioxide content is calculated from the volume of acid solution needed to change the pH from 8.6 (bicarbonate form) to 4.0 (carbonic acid). A blank titration is carried out in the same conditions in decarbonated wine in order to take account of the volume of sodium hydroxide taken up by the wine acids.

## 2. Description of the method

2.1 *Still Wines*

($CO_2$ over pressure ≤ 0.5 x $10^5$ Pa)

2.1.1 Apparatus

- Magnetic stirrer
- pH meter

2.1.2 Reagents

* 1 bar = $10^5$ Pascal (Pa)

- Sodium hydroxide solution, 0.1 M
- Sulfuric acid solution, 0.05 M
- Carbonic anhydrase solution, 1 g/L

2.1.3 Procedure

Cool the wine sample together with the 10 mL pipette used for sampling to approximately 0°C.

Place 25 mL sodium hydroxide solution, 0.1 M, in a 100 mL beaker; add two drops of carbonic anhydrase solution, 1 g/L. Introduce 10 mL of wine using the pipette cooled to 0°C.

Place the beaker on the magnetic stirrer, immerse the pH electrode and magnetic rod, and stir moderately.

When the liquid has reached room temperature, titrate slowly with the sulfuric acid solution, 0.05 M, until the pH reaches 8.6. Note the burette reading.

Continue titrating with the sulfuric acid until the pH reaches 4.0. Let *n* mL be the volume used between pH 8.6 and 4.0.

Remove $CO_2$ from approximately 50 mL of the wine sample by shaking under vacuum for three minutes, the flask being heated in a water bath to about 25 °C.

Carry out the above procedure on 10 mL of the decarbonated wine. Let *n*' mL be the volume used.

2.1.4 Expression of results

1 mL of the titrated sodium hydroxide solution, 0.05 M, corresponds to 4.4 mg of $CO_2$. The quantity of $CO_2$ in grams per liter of wine is given by:

$$0.44\ (n - n')$$

The result is quoted to two decimal places.

*Note:* For wines which contain little $CO_2$ ($CO_2$ < 1 g/L), the addition of carbonic anhydrase to catalyze the hydration of $CO_2$ is unnecessary.

2.2 *Sparkling and semi-sparkling wines*

2.2.1 Apparatus

- Magnetic stirrer
- pH meter

2.2.2 Reagents

- Sodium hydroxide, 50% (*m/m*)
- Sulfuric acid solution, 0.05 M
- Carbonic anhydrase solution, 1 g/L

2.2.3 Procedure

Mark the level of wine in the bottle and then cool until freezing begins.
Allow the bottle to warm up slightly, while shaking, until ice crystals disappear.
Remove the stopper rapidly and place 45 to 50 mL of wine in a measuring cylinder for blank titration. The exact volume removed, *v* mL, is determined by reading on the measuring cylinder after it has returned to room temperature.
Immediately after the blank sample has been removed, add 20 mL of the sodium hydroxide solution for a 750 mL bottle.

Allow the wine to reach room temperature.

Place 30 mL of boiled distilled water and two drops of the carbonic anhydrase solution into a 100 mL beaker. Add 10 mL of wine that has been made alkaline.

Place the beaker on the magnetic stirrer, set up the electrode and magnetic rod and stir moderately.

Titrate with the sulfuric acid solution, 0.05 M, slowly until the pH reaches 8.6. Note the burette reading.

Continue titrating slowly with the sulfuric acid, 0.05 M, until the pH reaches 4.0. Let *n* mL be the volume added between pH 8.6 and 4.0.

Remove $CO_2$ from the *v* mL of wine placed on one side for the blank titration by agitating under vacuum for three minutes, the flask being heated in a water bath at about 25 °C. Remove 10 mL of decarbonated wine and add to 30 mL of boiled distilled water, add two to three drops of sodium hydroxide solution, 50%, to bring the pH to 10 to 11. Then follow the above procedure. Let *n*' mL be the volume of sulfuric acid added, 0.05 M.

2.2.4 Expression of results

1 mL sulfuric acid, 0.05 M, corresponds to 4.4 mg of $CO_2$.

Empty the bottle of wine which has been made alkaline and determine to within 1 mL the initial volume of wine by making up to the mark with water, say V mL. The quantity of $CO_2$ in grams per liter of wine is given by the following formula:

$$0.44\ (n - n')\ \times\ \frac{V - v + 20}{V - v}$$

The result is quoted to two decimal places.

2.3 *Expression of Results*

The excess pressure at 20°C ($Paph_{20}$) expressed in Pascals is given by the formula:

$$Paph_{20} = \frac{Q}{1{,}951 \times 10^{-5} (0{,}86 - 0{,}01A)(1 - 0{,}00144S)} - Patm$$

where:

Q = $CO_2$ content in g/L of wine,

A = the alcoholic strength of wine at 20 °C,

S = the sugar content of the wine in g/L,

Patm= the atmospheric pressure, expressed in Pascals.

2.4 *Note*

The procedure described below can be used as the usual method for wines containing less than 4 g per liter of carbon dioxide.

Prepare two samples of wine for analysis.

Open one of the samples after it has been cooled to approximately 5°C and immediately add 5 mL of a sodium hydroxide solution, 50% (*m/m*), for 375 mL of sample. Stopper immediately and mix. Place 10 mL of wine so processed into a beaker containing 40 mL of water and add 3 drops of carbonic anhydrase solution, 0.1 mg/mL. Titrate with a sulfuric acid solution, 0.02275 M, until reaching a pH of 8.6, then continue titrating to a pH of 4.0. The volume used to change the pH from 8.6 to 4.0 is *n* mL.

Remove the carbon dioxide from about 25 mL of wine, taken from the second sample, by agitation under a vacuum for about 1 min. into a 500 mL flask containing 3 drops of carbonic anhydrase solution. Add 0.33 mL of sodium hydroxide, 50% (*m/m*). Apply the above titration procedure to 10 mL decarbonated wine. Let *n*' mL be the volume of $H_2SO_4$, 0.02275 M used. 1mL corresponds to 200 mg of carbon dioxide per liter. The amount of wine analyzed for carbon dioxide, in milligrams per liter:

$$(n - n') \times 200 \times 1.013$$

## BIBLIOGRAPHY

*Reference method:*

CAPUTI A, UEDA M., WALTER P. & BROWN T., *Amer. J. Enol. Vitic.*, 1970, **21,** 140-144.
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BRUN S. & TEP Y., *F.V., O.I.V.*, 1981, n° 736 & 1982, n° 736 (bis).

**- Collaborative Study -**

**Titrimetric determination of carbon dioxide in sparkling and semi-sparkling wines**
**- Report on Results -**

## Goal of the study

The objective of the study is to determine the repeatability and reproducibility characteristics of the reference method (MA-E-AS314-01-DIOCAR) for the titrimetric $CO_2$ determination in sparkling and semi-sparkling wine.
O.I.V. definitions and limits for the $CO_2$ content are given with resolution OENO 1/2002.

## Needs and purpose of the study

The reference method for the $CO_2$ determination includes no precision data. This collaborative trial was thus conducted.
Due to the analytical particularity, theconventional validation protocol was not able to be completely respected. Out of one bottle of sample only one independent determination could be done. Each bottle had to be considered as

individual. Therefore homogeneity testing within the pre-investigations for collaborative studies was impossible. In order to provide homogenous test material close co-operation with producers was necessary. Samples were obtained during the filling of the bottles on the filling line in a very short time space, thus that it must be assumed that the $CO_2$ is homogeneously distributed in all bottles.
This study was designed to be a blind duplicate test. The complete anonymity of the samples could not be guaranteed because the partners involved used different types of bottles and/or stoppers for the different samples. Therefore we had to rely on the honesty of the participating laboratories which were requested to perform the data analysis independently without any data modification.

## Scope and applicability

1. The method is quantitative.
2. The method is applicable for the determination of $CO_2$ in sparkling and semi-sparkling wines to check that standards are respected.

**Materials and matrices**

The collaborative study included 6 different samples. All were sent in blind duplicate, so that in total 12 bottles were distributed to the participants.

**Table 1. Samples and coding.**

| Sample | Bottle Code | Type |
|---|---|---|
| SAMPLE A | (Code 1 + 9) | sparkling wine |
| SAMPLE B | (Code 2 + 5) | semi-sparkling wine ("petillant") |
| SAMPLE C | (Code 3 + 4) | sparkling wine |
| SAMPLE D | (Code 6 + 10) | semi- sparkling wine ("petillant") |
| SAMPLE E | (Code 7 + 11) | semi- sparkling wine ("petillant") |
| SAMPLE F | (Code 8 + 12) | sparkling wine (red) |

**Control measures**

The method considered is already approved in practice. Only the missing precision data had to be determined within the collaborative study. A pre-trial was not required because most of the laboratories had been already using the reference method in routine analysis.

**Method to be followed and supporting documents**

. Supporting documents were given to the participants (Covering letter Reference for method of analysis, Sample Receipt Form and Result Sheet).
. The determination of $CO_2$ content in g/l should be expressed in g/l.

**Data analysis**

1. Determination of outliers was assessed by Cochran, Grubbs and paired Grubbs tests.
2. Statistical analysis was performed to obtain repeatability and reproducibility data.
3. HORRAT values were calculated.

**Participants**

13 laboratories from several different countries participated in the collaborative study. Lab-Code numbers were given to the laboratories. The participating laboratories have proven experience in the analysis of $CO_2$ in sparkling wine.

**Table 2. List of participants.**

Landesuntersuchungsamt
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GERMANY

Landesuntersuchungsamt
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GERMANY

Institut für Lebensmittel, Arzneimittel und Tierseuchen
D-10557 BERLIN
GERMANY

Servicio Central de Viticultura y Enologia
E-08720 Villafranca Del Pendes
SPAIN

Landesuntersuchungsamt
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Landesuntersuchungsamt
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GERMANY

Chemisches und Veterinäruntersuchungsamt
D-70736 Fellbach
GERMANY

**Results**

The uncertainty data are directly calculated for the $CO_2$ determination from the results submitted. For the assessment of the collaborative trial the Horrat-ratio is of relevance. For all samples a ratio of < 2 was obtained for r and R, convincing for a collaborative study. Table 3 shows the results of the $CO_2$ titration for each sample.

**Table 3. Summarised results of the $CO_2$ determination.**

| **$CO_2$** | **SAMPLE A** | **SAMPLE B** | **SAMPLE C** | **SAMPLE D** | **SAMPLE E** | **SAMPLE F** |
|---|---|---|---|---|---|---|
| **Mean** [g/l] | 9.401 | 3.344 | 9.328 | 4.382 | 4.645 | 8.642 |
| **r** [g/l] | 0.626 | 0.180 | 0.560 | 0.407 | 0.365 | 0.327 |
| **sr** [g/l] | 0.224 | 0.064 | 0.200 | 0.145 | 0.130 | 0.117 |
| **RSDr** % | 2.379 | 1.921 | 2.145 | 3.314 | 2.803 | 1.352 |
| **Hor** | 0.893 | 0.617 | 0.804 | 1.109 | 0.946 | 0.501 |
| **R** [g/l] | 1.323 | 0.588 | 0.768 | 0.888 | 0.999 | 0.718 |
| **sR** [g/l] | 0.473 | 0.210 | 0.274 | 0.317 | 0.357 | 0.256 |
| **RSDR** % | 5.028 | 6.276 | 2.942 | 7.239 | 7.680 | 2.967 |
| **HoR** | 1.245 | 1.331 | 0.728 | 1.599 | 1.711 | 0.726 |

**Method OIV-MA-AS314-02** Type I method

# Overpressure measurement of sparkling wines

(Resolution Oeno 21/2003)

## 1. PRINCIPLE

After thermal stabilisation and agitation of the bottle, the overpressure is measured using an aphrometer (pressure gauge). It is expressed in Pascals (Pa) (type 1 method).

## 2. APPARATUS

The apparatus, which measures the overpressure in bottles of sparkling and semi-sparkling wines, is called an aphrometer. It can be in different forms depending on the stopper of the bottle (metal capsule, crown, plastic or cork stopper).

### 2. 1. Bottles with capsules

It is made up of three parts (figure 1):
- The top part (a screw needle holder) is made up of a manometer, a manual tightening ring, an endless screw, which slips into the middle part, and a needle, which goes through the capsule. The needle has a lateral hole that transmits pressure to the manometer. A joint ensures the tightness of the whole thing on the capsule of the bottle.
- The middle part (or the nut) enables the centring of the top part. It is screwed into the lower part, which strongly holds onto the bottle.
- The lower part (clamp) is equipped with a spur, that slips under the ring of the bottle in order to hold the whole thing together. There are rings adaptable to every kind of bottle.

### 2. 2. Bottles with corks

It is made up of two parts (figure 2):
- The top part is identical to the previous apparatus, but the needle is longer. It is made up of a long empty tube with a pointer on one end to aid in going through the cork. This pointer can be moved and it falls in the wine once the cork has been pierced.

- The lower part is made up of a nut and a base sitting on the stopper. This is equipped with four tightening screws used to maintain everything on the stopper.

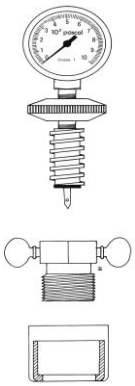


Figure1
Aphrometer for capsules

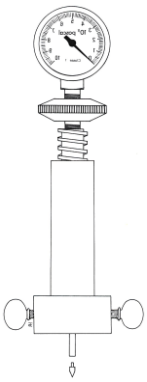


Figure 2
Aphrometer for stoppers

*Remarks concerning the manometers that equip these two types of apparatuses:*

- *They can be either a mechanical Bourdon tube or digital piezoelectrical captors. In the first case, the Bourdon tube must be made of stainless steel.*
- *It is graduated in Pascals (Pa). For sparkling wine, it is more practical to use $10^5$ Pascals ($10^5$ Pa) or kilopascal (kPa) as the unit of measurement.*
- *Aphrometers can be from different classes. The class of a manometer is the reading precision compared to the full scale expressed in percentages (e.g. manometer 1000 kPa class 1, signifies the maximum usable pressure 1000 kPa, reading at ± 10 kPa). Class 1 is recommended for precise measurements.*

## 3. PROCEDURE

Measurements can be carried out on bottles if the temperature has stabilised for at least 24 hours.
After piercing the crown, the cork or plastic stopper, the bottle must be vigorously shaked to reach a constant pressure in order to make a reading.

### 3.1. Capsuled bottles

Slip the clamp's spur binders under the ring of the bottle. Tighten the nut until the whole thing is tight on the bottle.
The top part is screwed on the nut. To avoid loosing gas, piercing the capsule should be done as quickly as possible in order to bring the joint in contact with the capsule. The bottle must be shaken vigorously to reach a constant pressure in order to make a reading.

### 3.2. Bottles with stopper

Place a pointer at the end of the needle. Position this fixture on the cork. Tighten the four screws on the stopper.
Tighten the top part (the needle goes through the cork). The pointer should fall in the bottle so that the pressure can be transmitted to the manometer. Make a reading after shaking the bottle until reaching constant pressure. Recuperate the pointer after the reading.

## 4. EXPRESSION OF RESULTS

The overpressure at 20°C ($Paph_{20}$) is expressed in Pascals (Pa) or in kilopascals (kPa).
This must be in accordance with the precision of the manometer (for example: 6.3 $10^5$ Pa or 630 kPa and not 6.33 $10^5$ Pa or 633 kPa for the manometer 1000 kPa full scale, of class 1).
When the temperature measurement is other than 20°C, it is necessary to correct this by multiplying the pressure measured by an appropriate coefficient (see Table 1).

| | | | |
|---|---|---|---|
| 0 | 1.85 | 13 | 1.24 |
| 1 | 1.80 | 14 | 1.20 |
| 2 | 1.74 | 15 | 1.16 |
| 3 | 1.68 | 16 | 1.13 |
| 4 | 1.64 | 17 | 1.09 |
| 5 | 1.59 | 18 | 1.06 |
| 6 | 1.54 | 19 | 1.03 |
| 7 | 1.50 | 20 | 1.00 |
| 8 | 1.45 | 21 | 0.97 |
| 9 | 1.40 | 22 | 0.95 |
| 10 | 1.36 | 23 | 0.93 |
| 11 | 1.32 | 24 | 0.91 |
| 12 | 1.28 | 25 | 0.88 |

TABLE 1: Relationship of $Paph_{20}$ excess pressure of semi-sparkling and sparkling wine at 20°C with the $Paph_t$ excess pressure at temperature *t*

## 5. CONTROL OF RESULTS

Direct determination method of physical parameters (type 1 criteria method)

Verification of aphrometers

The aphrometers should be verified on a regular basis (at least once a year).
Test beds are used for verification. This enables the comparison of the manometer to be tested and the reference manometer, of higher class, connected to national standards set up. The control is used to check the values indicated by the two apparatuses and increasing and decreasing pressures against each other. If there is a difference between the two , an adjustment can be made to make the necessary changes.
Laboratories and authorised bodies are equipped with such test beds, which are likewise available from manufacturers of manometers.

**Method OIV-MA-AS314-03** Type II method

# Determination of the carbon isotope ratio $^{13}C/^{12}C$ of $CO_2$ in sparkling wines
# Method using isotope ratio mass spectrometry (IRMS)

(Resolution Oeno 7/2005, Revised by OIV-OENO 512-2014)

## Foreword

The following standard method has been prepared with the agreement of all the laboratories participating in the OIV Collaborative study: $^{13}C$-IRMS analyses of $CO_2$ in sparkling wine (2003-2004).

## Introduction

The headspace in a bottle of sparkling wines contains a $CO_2$-rich gaseous phase in equilibrium with the $CO_2$ dissolved in the liquid phase. This gas evolves during the second fermentation, induced by the addition of sugar from grape, beet, sugar cane or maize. However, the $CO_2$ content of sparkling wines may also be increased artificially with industrial $CO_2$.

In 1997, an off-line method for the determination of the $^{13}C/^{12}C$ isotopic ratio of $CO_2$ from sparkling wines by isotope mass spectrometry (IRMS) was presented to the OIV. This method led on to new procedures based on automated on-line techniques, developed in some European laboratories. One of these procedures was presented to the OIV in 2001. Technical progress in the next few years may well lead to new procedures for determining reliably and rapidly the $^{13}C/^{12}C$ isotopic ratio of numerous samples of $CO_2$. An exhaustive description of all applicable procedures for different techniques runs the risk of the method being rapidly superseded. The following method takes this into account and describes the basic principles for the correct measurement of the carbon-13 content in $CO_2$ from sparkling wine and includes a brief description of the procedures used nowadays and, by way of examples, some exhaustive descriptions of procedures based on off-line and on-line techniques.

## 1. Scope

This method determines by isotope mass spectrometry (IRMS) the stable carbon isotope ratio ($^{13}C/^{12}C$) of $CO_2$ in sparkling wines. The method includes a range of procedures whose use depends on the instruments available.

## 2. Normative references

ISO 5725-2:1994 "Accuracy (trueness and precision) of measurement methods and results. Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method".

ISO 78-2:1999 "Chemistry - Layouts for standards - Part 2: Methods of chemical analysis".

## 3. Definitions

$^{13}C/^{12}C$: Isotope ratio of carbon 13 to carbon 12 for a considered sample;

$\delta^{13}C$: Carbon 13 ($^{13}C$) content expressed in parts per mill (‰);

V-PDB: Vienna-Pee-Dee Belemnite. The PDB standard is a fossil calcium carbonate from South Carolina in USA, with an isotope ratio ($^{13}C/^{12}C$ or $R_{PDB}$) = 0.0112372. This value is the reference point for the common international PDB scale for $\delta^{13}C$ values expressed in parts per mill (‰).

m/z: mass to charge relationship

$S_r$: Repeatability standard deviation. The standard deviation of test results obtained under repeatability conditions (conditions where independent test results are obtained with the same method on identical test samples in the same laboratory by the same operator using the same equipment within short intervals of time).

r: Repeatability limit. Value less than or equal to which the absolute difference between two test results obtained under repeatability conditions may be expected to be with a probability of 95%; $r=2.8 \cdot S_r$.

$S_R$: Reproducibility standard deviation. The standard deviation of test results obtained under reproducibility conditions (conditions where test results are obtained with the same method on identical test samples in different laboratories with different operators using different equipment).

R: Reproducibility limit. Value less than or equal to which the absolute difference between two test results obtained under reproducibility conditions may be expected to be with a probability of 95%; $R=2.8 \cdot S_R$

## 4. Principle

Plants are classified as C3 and C4 depending on the route followed for sugar synthesis. The sugar from C3 plants, such as grape and beet, has lower $^{13}C$ content than the sugar from C4 plants like cane sugar and maize. This difference is

maintained in the $^{13}C$ content of the fermentation products of sugars such as ethanol and $CO_2$. Moreover, the industrial $CO_2$ used in the food industry and that comes from the combustion of fossil fuels or from the thermal treatment of carbonates has $^{13}C$ content different from the products of C3 and C4 plants. Consequently, the $^{13}C/^{12}C$ isotope ratio of $CO_2$ from sparkling wine is governed by the type of sugar used in the second fermentation (C3 or C4) or by the isotopic composition of the industrial $CO_2$ added.

The studies performed till now on the $^{13}C$ content of $CO_2$ from sparkling wine have shown that the $CO_2$ obtained by fermentation of sugar from C3 plants has $\delta^{13}C$ in the range of -17‰ to -26‰, whereas $CO_2$ obtained by fermentation of sugar from C4 plants has $\delta^{13}C$ in the range of -7‰ to -10‰. Gasified wines have their $^{13}C/^{12}C$ isotope ratio below -29‰ or above -10‰, depending on the carbon dioxide source[1-4]. Therefore, the measurement of the stable carbon isotope ratio ($^{13}C/^{12}C$) of $CO_2$ from sparkling wines can be a good method for finding the origin of the gas.

$^{13}C$ content is determined from carbon dioxide gas obtained from sparkling wine. The various possible combinations of the $^{18}O$, $^{17}O$, $^{16}O$ and $^{13}C$, $^{12}C$ isotopes lead to mass 44 corresponding to the $^{12}C^{16}O_2$ isotopomer, mass 45 corresponding to $^{13}C^{16}O_2$ and $^{12}C^{17}O^{16}O$ species, and mass 46 for the $^{12}C^{16}O^{18}O$ isotopomer ($^{l3}C^{17}O^{16}O$ and $^{12}C^{17}O_2$ can be ignored due to their very low abundance). The corresponding ion currents are determined on the three different collectors. The ionic current m/z 45 is corrected for the contribution of $^{12}C^{17}O^{16}O$ which is computed from the intensity current measured for m/z 46 by including the relative abundance of $^{18}O$ and $^{17}O$ (Craig correction). Comparison with a reference calibrated against the international standard V-PDB then allows the calculation of the $^{13}C$ content on the $\delta^{l3}C$‰ relative scale.

## 5. Reagents and material

The materials and consumables depend on the equipment used in the laboratory.

When the separation and purification of the $CO_2$ samples is performed by cryotrapping in a vacuum line the following reagents are used:

- Liquid nitrogen
- Ethanol
- Solid $CO_2$

In general, the following consumables are used for the analysis with any Continuous Flow system (EA-IRMS or GC-C-IRMS). Other materials of similar quality can replace any product on this list:

- Helium for analysis (CAS 07440-59-7)
- Oxygen for analysis (CAS 07782-44-7)
- Carbon dioxide for analysis used as a secondary reference gas for carbon-13 content (CAS 00124-38-9).
- Oxidising reagent for the furnace of the combustion system, such as cupper oxide for microanalysis (CAS 1317-38-0).
- Desiccant to remove water produced by combustion: for example, magnesium perchlorate for microanalyses (CAS 10034-81-4). This is not necessary when the EA-IRMS or the GC-C-IRMS systems remove water by cryotrapping.
- Capillary column and the Naphion membrane to remove water produced by combustion in GC-C-IRMS systems.

The Reference Gas used in the measurements can be a certified gas or a working reference gas calibrated compared to international references with known delta values (certified gases or reference materials). Some international reference materials that can be used for gas reference calibration and for control of the gas reference calibration are the following:

| Code sample | Material | $\delta^{13}C_{PDB}$ | |
|---|---|---|---|
| IMEP-8-A | $CO_2$ | -6.40‰ | from Messer Griesheim |
| ISO-TOP | $CO_2$ | -25.7‰ | " |
| BCR-656 | Ethanol | -20.91‰ | from IRMM |
| BCR-657 | Glucose | -10.76‰ | " |
| SAI-692C | $CO_2$ | -10.96‰ | from Oztech Trading Coorporation (USA) |
| NBS-22 | Oil | -29.7‰ | from IAEA |
| IAEA-CH-6 (ANU) | Sucrose | -10.4‰ | " |
| NBS-18 | Calcite | -5.1‰ | " |
| NBS-19 | TS-limestone | +1.95‰ | " |
| FID-Mix | mixture of n-alkanes in isooctanol | | from Varian |
| C14 | -29.61‰ | | |
| C15 | -25.51‰ | | |
| C16 | -33.39‰ | | |

## 6. Apparatus

The usual laboratory apparatus for carbon isotope ratio measurements and, in particular, the following:

- Isotopic ratio mass spectrometry (IRMS), with the ability to determine the $^{13}C$ content of $CO_2$ gas at natural abundance with an internal precision of 0.05 ‰ or better (expressed in relative □ value). The internal precision is defined here as the difference between two measurements of the same $CO_2$ sample.

  The mass spectrometer will generally be fitted with a triple collector to measure simultaneously the current intensities for m/z 44, 45 and 46. The mass spectrometer should either be fitted with a dual-inlet system, for alternating measurement of the unknown sample and a standard, or use a continuous-flow technique (CF-IRMS).

- Continuous-flow systems (CF-IRMS). Continuous-flow systems with an automated gas sampling system can be used. Several commercially available CF-IRMS techniques suitable for the scope of the present method are:

  - GC-C-IRMS (Gas chromatography – combustion- IRMS)
  - EA-IRMS (Elemental analyser equipped for liquid or solid injection)

  These systems separate and purify $CO_2$ and elute the resulting carbon dioxide to the ionisation chamber of the spectrometer.

- Gas Sampler-IRMS**.** A peripheral system may be used for the on-line gas preparation, isolation of $CO_2$ and introduction of $CO_2$ into the isotope ratio mass spectrometer.

- Glass or steel vacuum line, with cryogenic traps and connected to a pump able to obtain a pressure lower than $5.10^{-3}$ mbar.

- Gas sampling devices, commercially available (such as syringe for gas samples) or designed in-house, able to extract a $CO_2$ aliquot from the sparkling wine without isotopic fractionation.

- Sealed vials for gas samples, adaptable on gas autosampler to the continuous-flow systems.

- Sealed vials for sparkling wine aliquots, adaptable on vacuum line and/or on gas autosampler to the continuous-flow systems.

## 7. Procedure

The proposed method includes three steps: $CO_2$ sampling, $CO_2$ purification and separation, and $^{13}C/^{12}C$ ratio measurement. These steps can be totally independent

(off-line system) or fully or partially connected on-line (on-line system). Any procedure that avoids isotopic fractionation of the $CO_2$ sample during the three steps of the method may be used. Details on particular procedures based on off-line and CF systems are given in Annexes A, B and C.

The following description refers to the procedures used for the participant laboratories in the inter-laboratory test.

### 7.1. $CO_2$ sampling procedures:

a. Sampling the $CO_2$ at room temperature from the headspace of the bottle by plugging a special device through the cork, or

b. Sampling the $CO_2$ from the headspace of the bottle after removing the cork and sealing the bottle with a gas-tight precision lock connected to a sampling device. The sparkling wine bottle should be cooled to under 0ºC before changing the locking device and then warmed to room temperature. An aliquot of gas collected in the sampling device is removed by a gas-tight syringe and injected into a sealed GC-vial, or

c. Sampling the $CO_2$ from an aliquot of sparkling wine. The sparkling wine bottle should be cooled to 4º-5ºC before removing the cork. The wine aliquots are placed in a special bottle adaptable to a glass vacuum line or to a gas autosampler.

d. Refrigerate the sample at 4-5 °C, before quickly transferring the liquid into a vial and sealing it with a Teflon-silicone septum cap. Then 50 µL of liquid is then transferred into a 10 mL vial and analysed. If necessary, the vial should be filled with helium in order to remove the atmospheric $CO_2$.

e. After refrigerating the sample, the bottle is opened at room temperature and a sample of 200 µL of liquid is taken using a pipette and placed in suitable vials. The vials are immediately resealed then placed in an ultrasonic bath for 10 min prior to analysis.

The statistical results of the inter-laboratory test for sampling procedures 7.1.d and 7.1.e are given in ANNEX E.

### 7.2. $CO_2$ purification and separation procedures

a. Uncondensed gases and water present in the gas sample are removed in a vacuum line by use of cryogenic traps, or

b. Gas samples are purified and $CO_2$ separated by different on-line systems, which are connected to the IRMS by means of continuous-flow or a cryogenic trap. Some of the on-line systems that can be used are the following:

- a water cryogenic trap on-line with a continuous-flow system

- a water trap (magnesium perchlorate) followed by a gas chromatograph

- a gas chromatograph connected either directly to the IRMS or by means of a combustion interface.

### 7.3. $^{13}C/^{12}C$ ratio measurement:

The carbon isotope ratio of $CO_2$ obtained from sparkling wine is measured by using an isotopic ratio mass spectrometer.

## 8. Calculation

Express the $^{13}C/^{12}C$ isotope ratio of the $CO_2$ from sparkling wine as the deviation from a working standard ($\delta^{13}C$) previously calibrated in relation to the international standard PDB (Pee Dee Belemnite). This parameter is defined as the relative difference per thousand between the $^{13}C$ and $^{12}C$ ratios of a sample in relation to the PDB Standard. The PDB standard is a fossil calcium carbonate from South Carolina in USA, with an isotope ratio ($R_{PDB}$) = 0.0112372. This value is the reference point of the common international PDB scale for $\delta^{13}C$ values expressed in parts per mill (‰).

The $\delta^{13}C$ values expressed in relation to the working standard are calculated with the following equation:

$$\delta^{13}C_{sam/ref}\ (‰) = 1000 \times (R_{sam} - R_{ref}) / R_{ref}$$

where

$R_{sam}$ is the $^{13}C/^{12}C$ isotope ratio of the test portion;

$R_{ref}$ is the $^{13}C/^{12}C$ isotope ratio of the working standard.

The $\delta^{13}C$ values expressed in relation to the PDB standard are calculated using the following equation:

$$\delta^{13}C_{sam/V\text{-}PDB}\ (‰) = \delta^{13}C_{sam/ref} + \delta^{13}C_{ref/V\text{-}PDB} + (\delta^{13}C_{sam/ref} \times \delta^{13}C_{ref/V\text{-}PDB}) / 1000$$

where

$\delta^{13}C_{ref/V\text{-}PDB}$ is the isotopic deviation of the working standard previously determined from the PDB standard expressed in parts per mill (‰).

Express the results to two decimal places.

## 9. Precision

*Details of the inter-laboratory test on precision of the method are given in annex D and E.*

### 9.1. Repeatability

The absolute difference between two single results found on identical test sample by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit *r* in no more than 5% of the cases.
The accepted mean values of the standard deviation of repeatability ($S_r$) and repeatability limit (r) are equal to:

$S_r$ = 0.21‰ r = 0.58‰
Characteristics of sampling procedures 7.1.a-c

$S_r$ = 0.21‰ r = 0.56‰
Characteristics of sampling procedures 7.1d and 7.1e

### 9.2. Reproducibility

The absolute difference between two single results found on identical test sample reported by two laboratories will exceed the reproducibility *R* in not more than 5% of the cases.
The accepted mean values of the standard deviation of reproducibility ($S_R$) and reproducibility limit (R) are equal to:

$S_R$ = 0.47‰ R = 1.33‰
Characteristics of sampling procedures 7.1.a-c

$S_R$ = 0.68‰ R = 1.91‰
Characteristics of sampling procedures 7.1d and 7.1e

## 10. Test report

The test report shall contain the following data:

- all the information necessary for the identification of the sample tested;
- a reference to the International Standard Method;
- the method used, including the procedure for sampling and measurement and the instrument system used;
- the results of the test and units, including the results of the individual determinations and their mean, calculated as specified in clause 8 ("Calculation");
- any deviations from the procedure specified;
- any unusual features observed during the test;
- the date of the test;
- whether repeatability has been verified;
- a description of the procedure for the reference gas calibration used to measure the test portions.

**Annexes** (A,B,C,D, E)

## 11. Bibliography

1. *Mesure du rapport isotopique $^{13}C/^{12}C$ du gaz carbonique des vins mousseux et des vins gazéifiés. J. Merin and S. Mínguez. Office International de la Vigne et du Vin. Paris. F.V. 1039, 2426/200297 (1997).*
2. *Examination of the $^{13}C/^{12}C$ isotopes in sparkling and semi-sparkling wine with the aid of simple on-line sampling. M. Boner and H. Förstel. Office International de la Vigne et du Vin. Paris. F.V. 1152. (2001).*
3. *Use of $^{13}C/^{12}C$ ratios for studying the origin of $CO_2$ in sparkling wines. J.Dunbar. Fresenius Z. Anal. Chem., 311, 578-580 (1982).*
4. *Contribution to the study of the origin of $CO_2$ in sparkling wines by determination of the $^{13}C/^{12}C$ isotope ratio. I. González-Martin, C. González-Pérez, E. Marqués-Macías. J. Agric. Food Chem. 45, 1149-1151 (1997).*
5. *Protocol for Design, Conduct and Interpretation of Method-Performance studies. Pure Appl. Chem., 1995, 67, 331-343.*

**ANNEX A**

**Experimental procedure based on off-line systems for sampling and measurement**
**("in-house" sampling device, off-line vacuum line and dual-inlet IRMS)**

## 1. Material

- Sampling device. The device that will be used to extract gas aliquots from the bottle consists of a hollow punch (steel needle) with three lateral orifices through which the gas enters. It is connected to a valve system composed of two valves connected in sequence and has a capacity of about 1 mL. One valve is attached to the punch (Valve 1) and the other is attached to a steel tube (Valve 2), which connects the device to a vacuum line. For a glass vacuum line an adapter with a flexible steel tube will be necessary. Figure shows the device for gas collection.
- Off-line vacuum line with two cryogenic traps (P<0.05 mbar). Two types of vacuum line can be used, a glass or steel vacuum line.
- Dual-inlet - Isotope ratio mass spectrometer with the ability to determine the $^{13}C$ content of $CO_2$ gas at natural abundance with an internal precision of 0.05‰ or better (expressed in relative δ value). Internal precision is here defined as the difference between two measurements of the same $CO_2$ sample.

## 2. Procedure (see Figure)

### 2.1. $CO_2$ sampling:

1. Connect the sampling device to vacuum line and test its seal capacity.
2. Punch the sampling device with the valves closed into the bottle cork by means of a circular movement whilst maintaining the device vertical.
3. Connect the sampling device–wine bottle assembly to the vacuum line and evacuate the line and the reservoir delimited by the two valves (Valve 2 opened and Valve 1 closed).
4. Once a vacuum has been created in the reservoir, close valve 2, open valve 1 and maintain this configuration for 1 min. After the equilibration time, close valve 1. The gas retained in the reservoir is then purified.

### 2.2. $CO_2$ purification and separation:

1. Transfer the $CO_2$ collected in the reservoir to the first cryogenic trap by liquid nitrogen for at least 1 min, then pump the uncondensed gas until a pressure of less than 0.05 mbar is reached.
2. Transfer the $CO_2$ sample to the measurement device by using liquid nitrogen in the second cryogenic trap and by changing the liquid nitrogen in the first cryogenic trap for a water trap at –80 ± 5 ºC. Maintain this for at least 1 min.
3. Pump the uncondensed gas (until a pressure of less than 0.05 mbar is reached) before closing the measurement device.

### 2.3. $^{13}C/^{12}C$ ratio measurement

The carbon isotope ratio of $CO_2$ obtained is measured by using a dual-inlet IRMS.

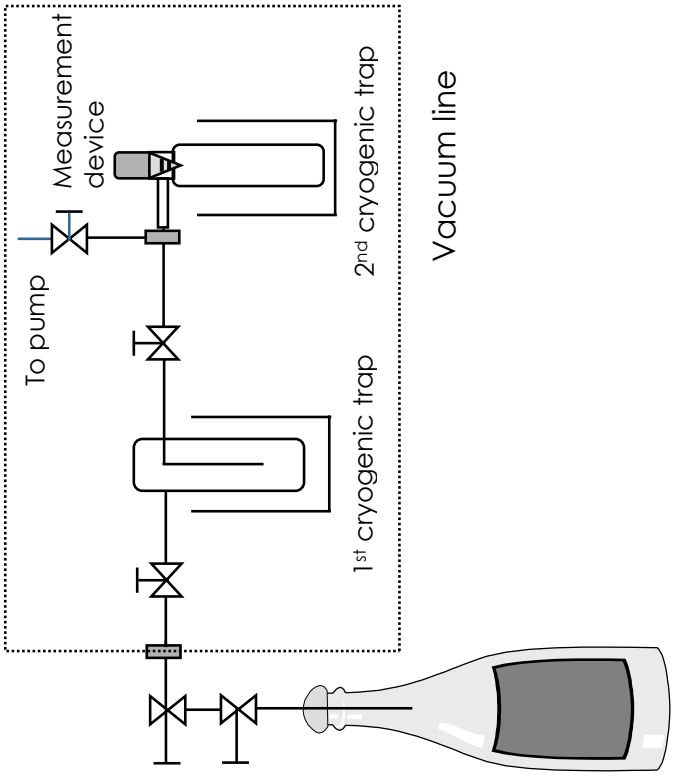
## 3. Reference

Mesure du rapport isotopique $^{13}C/^{12}C$ du gaz carbonique des vins mousseux et des vins gazeifiés. J.Merín, S.Mínguez. Office International de la Vigne et du Vin, F.V. 1039, 2426/200297.

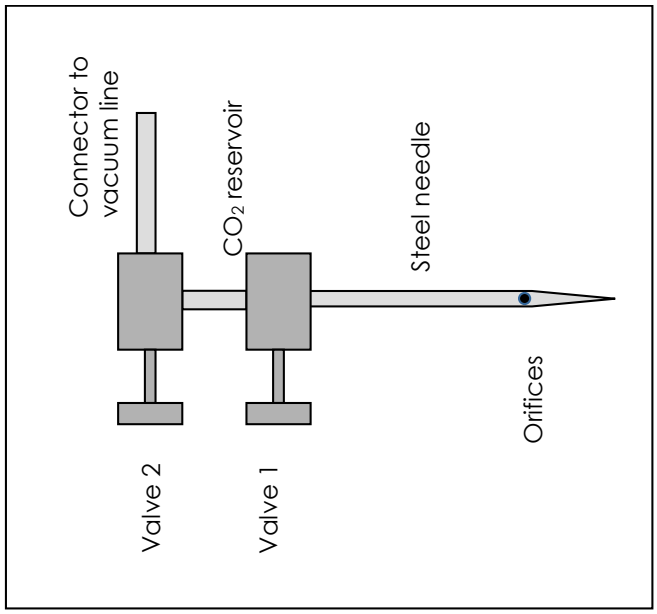
**ANNEX A**

Diagrams of the off-line system

**Gas Sampling and $CO_2$ purification**




**Gas sampling device**



## ANNEX B

## Experimental procedure based on the on-line systems for sampling and measurement (CF-IRMS)

### 1. Sampling technique

At first the sampling system is evacuated, the carbon dioxide is extracted from the bottle using a "sampling device", and a specific quantity is transferred to the storage vessel. After applying an overpressure, a small quantity of sample gas is introduced into the on-line helium flow with the aid of a restrictor. The sampling system is illustrated in Figure 2.

There is now a continuous carbon dioxide flow present in the helium flow (sample flow). The remaining helium flow is free from carbon dioxide and acts as the zero flow. Artificial "switching peaks" are generated by temporarily switching from the zero flow to the sample flow (switching time: 2 seconds), which are measured in the MS for their isotopic ratio.

**2. Procedure** (see Figure):

**2.1. Evacuation of the sampling system**

The entire sampling system is evacuated to a negative pressure of 1 mbar (V3 closed)

**2.2. Sampling**

The closure is pierced with a "sampling device" and the bottle atmosphere is transferred into the gas storage vessel (GV) with the aid of the negative pressure (pressure increase to approx. after 50 mbar). The fine adjustment valve (VF) permits a controlled and slow transfer of the gas. The gas is purified in the cryotrap during transfer.

**2.3. Feeding**

After sampling (V3, V2 closed, V4 open), an overpressure of 1,5 bar is built up with the aid of helium. The gas to be measured is fed to the CF-IRMS by opening V3. The measurement can be performed after a pre-run of 150 seconds. A capillary is integrated as a restrictor which only allows the feeding of a very small carrier gas quantity (10mL/min).

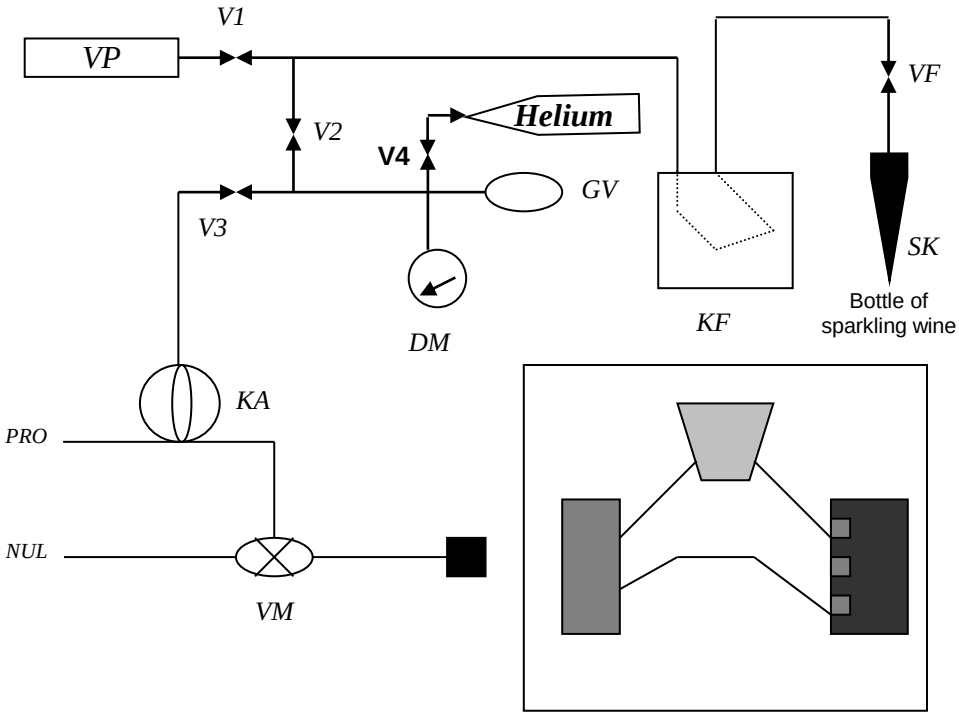
**2.4. Measurement**

A carbon dioxide flow is now continuously present in the helium sample flow (PRO). Switching from the sample flow (PRO) to the pure helium flow (NUL) permits the generation of artificial switching peaks.
Switching on the sample side: 2 seconds (zero side: 10-30 seconds).

## 3. Reference

Examination of the $^{13}C/^{12}C$ isotopes in sparkling and semi-sparkling wine with the aid of simple on-line sampling. M. Boner and H. Förstel. Office International de la Vigne et du Vin, FV 1152.

Diagram of the on-line system




| | |
|---|---|
| V1-V4 | check valve |
| VP | vacuum pump |
| VF | fine adjustment valve |
| SK | sampling device |
| PRO | helium sample flow (50 mL/min) |
| NUL | helium (zero) flow (60mL/min) |
| KF | water trap propanol at – 90ºC |
| GV | 250 ml gas storage vessel |
| DM | pressure gauge |
| KA | restrictor capillary (10cm, 150µm) |
| VM | 2/4-way valve |

## ANNEX C

## Experimental procedure based on the GC-C-IRMS technique

### 1. Instrument characteristics

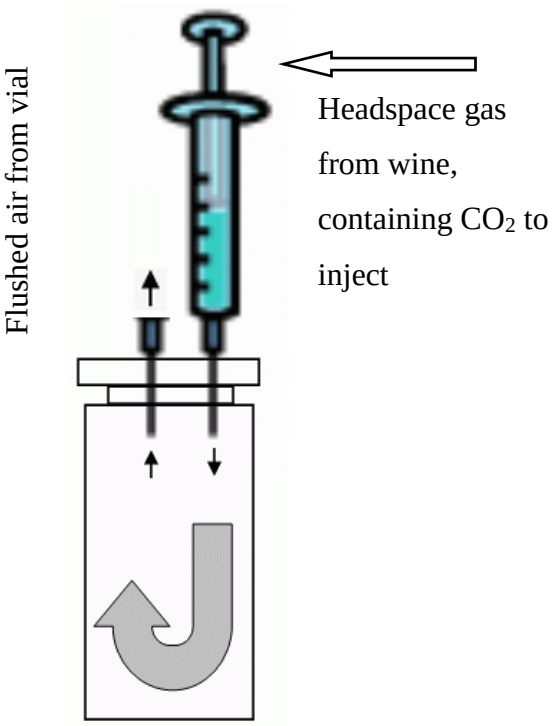
- Gas Chromatograph: GC Varian 3400
- Capillary Column: HP-INNOWax (Crosslinked Polyethylene Glycol), 30 m x 0.25 mm ID, film thickness 0.5 µm
- Combustion interface by ThermoFinnigan-MAT, with oxidation oven set at 940°C or off; reduction oven at 640°C or off
- Mass Spectrometer: DeltaPlus ThermoFinnigan-MAT.
-

### 2. Procedure

### 2.1. $CO_2$ sampling:

1. Aliquots of gas were collected through a 25cc syringe, by plugging a long iron needle through the cork. $CO_2$ pressure filled the syringe with the headspace gas spontaneously.
2. Transfer the gas in already crimped vials for subsequent analysis. The vials used to store the gas are previously crimped with Teflon-silicone septum caps. To flush out the air inside – and thus the atmospheric $CO_2$ – a second needle is plunged into the septum, to guarantee that headspace gas from wine pushes out the air in vial. See figure below.

NOTE: A bigger syringe is used, in line with vial volume, to make sure the vial is clean. In our case, a 25cc (or even bigger) syringe for a 2 ml vial.

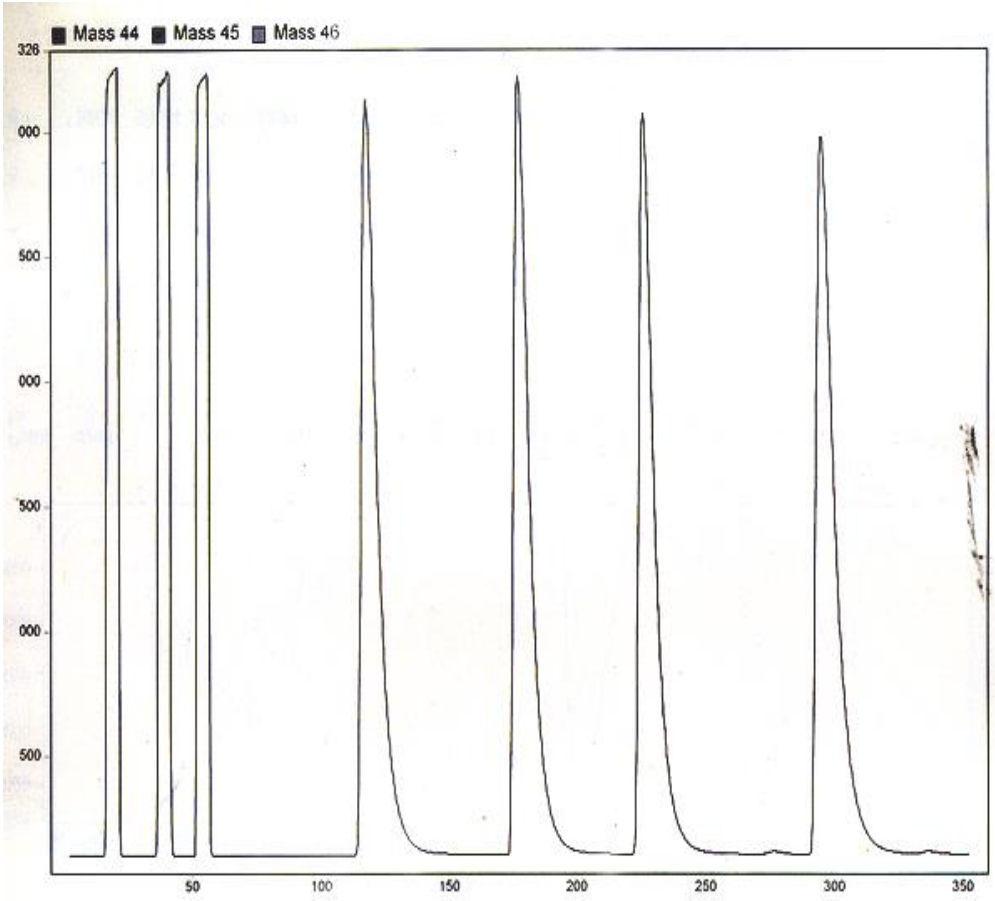



* Note that vial is not in scale with syringe.

### 2.2. GC-IRMS analyses: $CO_2$ injection and $^{13}C/^{12}C$ ratio measurement

A very few µL of gas were directly injected into the column with a 10 µL cemented-needle Hamilton syringe. Split conditions of high flow were set up. The carrier helium was at 20 PSI.

4 injections were carried out in each run for each sample. Total run time for the analysis was 6 minutes. See chromatogram below.




### 2.3. Processing of results

The software used to record and elaborate signals from the mass spectrometer, was version 1.50 of Isodat NT, from ThermoFinnigan-Bremen, running under MS-Windows NT OS.

For each sample, the mean $\delta^{13}C$ value is calculated as the average value of the last 3 injections. The $\delta^{13}C$ value of the first injection is systematically discarded.

## ANNEX D
(informative)

### Statistical results of the inter-laboratory test

In accordance with ISO 5725:1994, the following parameters were defined in an inter-laboratory test conducted by 11 European laboratories and a Mexican laboratory.

| | |
|---|---|
| Year of the inter-laboratory test | 2003-2004 |
| Number of laboratories | 12 |
| Number of samples | 5 in blind duplicates |
| Parameter | $\delta^{13}C$ of $CO_2$ |

| Sample identification | **A** | **B** | **C** | **D** | **E** |
|---|---|---|---|---|---|
| Number of participating laboratories | 12 | 12 | 12 | 12 | 12 |
| Number of laboratories retained after eliminati outliers | 12 | 11 | 12 | 12 | 12 |
| Number of replicates per laboratory | 2 | 2 | 2 | 2 | 2 |
| Number of accepted test results | 24 | 22 | 24 | 24 | 24 |
| Mean ($\delta^{13}C$) ‰ | -9.92 | -20.84 | -23.66 | -34.80 | -36.43 |
| $s_r^2$ | 0.057 | 0.031 | 0.119 | 0.006 | 0.044 |
| Repeatability standard deviation ($S_r$) ‰ | 0.24 | 0.18 | 0.35 | 0.08 | 0.21 |
| Repeatability value, r (2.8 x $S_r$) ‰ | 0.67 | 0.49 | 0.97 | 0.21 | 0.58 |
| $S_R^2$ | 0.284 | 0.301 | 0.256 | 0.140 | 0.172 |
| Reproducibility standard deviation ($S_R$) ‰ | 0.53 | 0.55 | 0.51 | 0.37 | 0.41 |
| Reproducibility value, R (2.8 x $S_R$) ‰ | 1.49 | 1.54 | 1.42 | 1.05 | 1.16 |

Sample types:
- A Sparkling wine - $C_4$ sugar
- B Sparkling wine - $C_3$ sugar
- C Sparkling wine - $C_3$ sugar
- D Gasified wine
- E Gasified wine

## ANNEX E

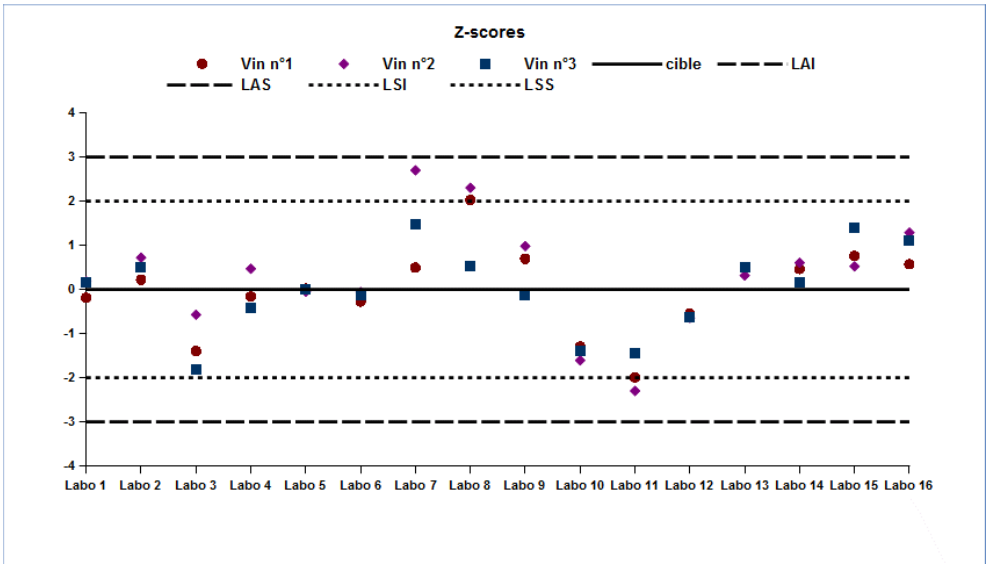
Statistical results of the inter-laboratory test on sparkling and gasified wines
Sampling procedures 7.1.d and 7.1.e

In accordance with method **OIV-MA-AS1-09: R2000**, the following parameters were defined as part of an inter-laboratory test conducted with 16 laboratories.

Year of the inter-laboratory test: 2013-2014
Number of laboratories: 16
Type of samples: Sparkling and gasified wines
Number of samples: 3, as blind duplicates
Parameter measured: $\delta^{13}C$

| INDICATORS | WINE NO. 1 | WINE NO. 2 | WINE NO. 3 |
|---|---|---|---|
| Number of laboratories | 16 | 14 | 16 |
| Number of repetitions | 2 | 2 | 2 |
| Minimum | -32.90 | -33.10 | -23.64 |
| Maximum | -29.83 | -30.97 | -20.57 |
| Repeatability variance $s_r^2$ | 0.0467 | 0.0118 | 0.0648 |
| Inter-group variance $s_L^2$ | 0.43853 | 0,29762 | 0.51616 |
| Reproducibility variance $s_R^2$ | 0.4852 | 0.3094 | 0.5810 |
| Overall average | -31.42 | -31.83 | -22.15 |
| Repeatability standard deviation | 0.22 | 0.11 | 0.25 |
| r limit | 0.612 | 0.307 | 0.720 |
| Reproducibility standard deviation | 0.70 | 0.56 | 0.76 |
| R limit | 1.971 | 1.574 | 2.157 |

| Laboratory Code | A | B | A | B | A | B | Wine No. 1 | Wine No. 2 | Wine No. 3 |
|---|---|---|---|---|---|---|---|---|---|
| Lab 1 | -31.40 | -31.69 | -31.56 | -31.88 | -21.93 | -22.12 | -0.18 | -0.19 | 0.16 |
| Lab 2 | -31.23 | -31.29 | -31.43 | -31.41 | -21.46 | -22.04 | 0.23 | -0.73 | 0.52 |
| Lab 3 | -32.65 | -32.12 | -32.15 | -32.13 | -23.41 | -23.64 | -1.39 | -0.56 | -1.81 |
| Lab 4 | -31.55 | -31.50 | -31.46 | -31.66 | -22.40 | -22.54 | -0.15 | 0.48 | -0.42 |
| Lab 5 | -31.50 | -31.30 | -31.80 | -31.90 | -22.00 | -22.30 | 0.03 | -0.04 | 0.00 |
| Lab 6 | -31.46 | -31.75 | -31.96 | -31.75 | -22.39 | -22.10 | -0.27 | -0.05 | -0.13 |
| Lab 7 | -31.48 | -30.66 | -31.29 | -29.35 | -21.47 | -20.57 | 0.50 | 2.71 | 1.48 |
| Lab 8 | -29.83 | -30.17 | -29.73 | -31.35 | -21.50 | -21.96 | 2.04 | 2.31 | 0.55 |
| Lab 9 | -30.96 | -30.90 | -31.34 | -31.21 | -22.22 | -22.27 | 0.70 | 0.99 | -0.13 |
| Lab 10 | -32.34 | -32.29 | -32.68 | -32.75 | -23.25 | -23.14 | -1.29 | -1.60 | -1.37 |
| Lab 11 | -32.90 | -32.70 | -33.10 | -33.10 | -23.00 | -23.50 | -1.98 | -2.29 | -1.45 |
| Lab 12 | -31.91 | -31.68 | -32.22 | -32.14 | -22.58 | -22.66 | -0.54 | -0.63 | -0.62 |
| Lab 13 | -31.03 | -31.10 | -31.61 | -31.68 | -21.78 | -21.74 | 0.51 | 0.33 | 0.51 |
| Lab 14 | -31.25 | -30.93 | -31.43 | -31.54 | -22.01 | -22.02 | 0.57 | 0.62 | 0.17 |
| Lab 15 | -30.89 | -30.88 | -31.59 | -31.47 | -21.08 | -21.07 | 0.76 | 0.53 | 1.41 |
| Lab 16 | -31.05 | -30.98 | -31.24 | -30.97 | -21.090 | -21.490 | 0.58 | 1.30 | 1.13 |




**Biblipgraphy**

1. Ana I. Cabañero, Tamar San-Hipólito and Mercedes Rupérez, GasBench/isotope ratio mass spectrometry: a carbon isotope approach to detect exogenous CO2 in sparkling drinks Rapid Commun. Mass Spectrom. 2007; 21: 3323–3328.

2. Laetitia Gaillard, Francois Guyon ⁄, Marie-Hélène Salagoïty, Bernard Médina, Authenticity of carbon dioxide bubbles in French ciders through multiflow-isotope ratio mass spectrometry measurements. Food Chemistry. 2013, 141: 2103–2107

**Method OIV-MA-AS314-04** Type II method

# Determination of carbon dioxide in wine by manometric method

## For a range of concentration from 0.5 g/L to 7 g/L

(Resolution Oeno 2/2006)

### 1. PRINCIPLE

The carbon dioxide in the sample is bound with 10 M sodium hydroxide. An Erlenmeyer flask with a side arm is connected to a manometer and the carbon dioxide is released with sulphuric acid from the prepared sample. The resultant increase in pressure is measured. It allows quantifying carbon dioxide content.

### 2. REAGENTS

**2.1. Freshly distilled or deionised water**;
**2.2. Sodium hydroxide** (purity >98%);
**2.3. Sulphuric acid** (purity >95-97%);
**2.4. Sodium carbonate** anhydrous (purity >99%).

Preparation of the reagents

**2.5. 10 M** Sodium hydroxide: dissolve 100 g of sodium hydroxide (2.2) in 200 ml water (2.1) and make up to 250 ml in a volumetric flask.

**2.6. Sulphuric acid, about 50% (v/v)**: cautiously add concentrated sulphuric acid (2.3) to an equal volume of water (2.1). Mix well by stirring. Cool to room temperature.

**2.7. Carbon dioxide standard solution 10 g/l**: dry anhydrous sodium carbonate (2.4) in an oven at 260°C-270°C over night, and cool to room temperature in a desiccator. Dissolve 6.021 g of dry sodium carbonate in water (2.1) and make up to 250 ml in a volumetric flask.

**2.8. Carbon dioxide calibration solutions 0.4; 1; 2; 4 and 6 g/l**: with pipettes take 2, 5, 10, 20 and 30 ml of the standard solution (2.7) in separate 50 ml volumetric flasks and make up to 50 ml with water (2.1).

## 3. APPARATUS

**3.1. 250 ml and 50 ml volumetric flasks;**
**3.2.Oven;**
**3.3. Dessicator;**
**3.4. Balance with an accuracy of ± 0.1 mg;**
**3.5. Refrigerator or water-ethylene glycol bath, -4°C;**
**3.6. Electronic density meter or pycnometer and thermostatic water bath, 20°C;**
**3.7. Pipettes 0.5, 2, 3, 5, 10, 20 and 30 ml;**
**3.8. 100 ml cone-shaped vial**, large ground-glass mouth;
**3.9. Digital manometer (allowing measures up to 200 kPa with an accuracy of 0.1kPa);**
**3.10. Reaction flask: 25 ml Erlenmeyer flask with a 3 ml side arm and a three-way valve (see figure 1);**
**3.11. Vacuum system** (i.e. water suction pump).
**3.12 Separation funnel**

## 4. PROCEDURE

### 4.1. Sample preparation

Prepare the sample in duplicate. Cool the sample in a refrigerator overnight or in a -4°C water-ethylene glycol bath for 40 min. Place 3 ml of 10 M sodium hydroxide solution (2.5) in a 100 ml cone-shaped vial. Weigh the flask with contents at an accuracy of 0.1 mg. Pour approximately 75 ml of the cooled sample in the cone-shaped vial containing the sodium hydroxide solution. Weigh the flask with contents at an accuracy of 0.1 mg. Mix and allow to warm up to room temperature.

**4.2. Determination of carbon dioxide content**

Transfer 2 ml of the prepared sample (4.1) into the reaction flask. Connect the flask to the manometer via the open three-way valve. Pipette 0.5 ml of 50% sulphuric acid (2.6) into the side arm. Secure the three-way valve and the side arm stopper with clips. Note the air pressure. Close the three-way valve. Mix the contents by tilting and shaking vigorously. Note the pressure. The prepared sample can be diluted with water
if necessary.

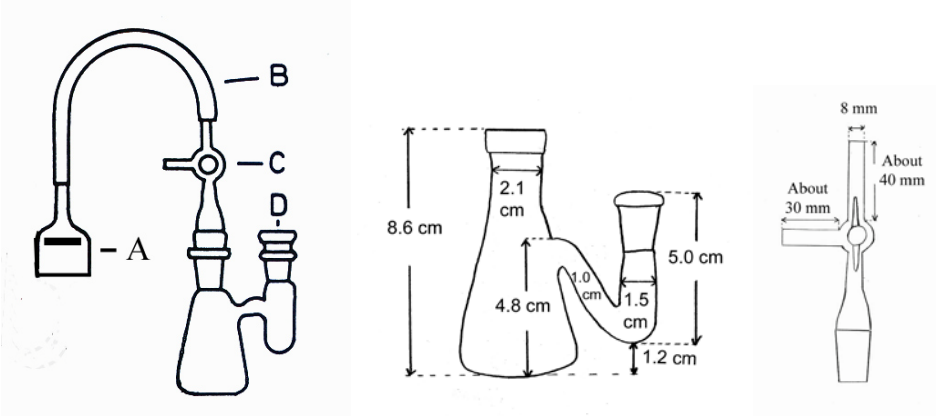



**Fig.1 Apparatus. A manometer, B rubber hose, C three-way valve, D reaction flask (left) and approximate measures of the glassware (centre and right).**

**4.3. Calibration**

Determine the carbon dioxide content of the calibration solutions as described above (4.2). Measure three calibration solutions which are within the expected concentration range of the sample. These calibration solutions are measured in duplicate.

### 4.4. Measurement of the density of the sample

Remove carbon dioxide from the sample by shaking the sample first in a separation funnel and then for 3 min in a vacuum generated by a water suction pump. Measure the density of the sample either with an electronic density meter or a pycnometer.

## 5. CALCULATION

Calculate the pressure increase caused by the carbon dioxide released from each calibration solution and construct a calibration graph.

Calculate the slope (a) and bias (b) of the calibration graph.

Volume V (ml) of the prepared sample:

$V = [(m2-m1) \times 1000]/d$ (1)
where
m1 (g)= weight of (flask + 3 ml NaOH);
m2 (g) = weight of (flask + 3 ml NaOH + sample);
d ($kg/m^3$) = density of sample.

Pressure increase $p_i$ caused by the carbon dioxide released from the prepared sample:

$$pi = ps - pap \quad (2)$$

where
$p_s$ = manometer reading after releasing the carbon dioxide from the sample
$p_{ap}$ = manometer reading before addition of $H_2SO_4$ (i.e. air pressure)

Concentration of carbon dioxide, C, in the sample (g/l) is given by:

$$C = [( pi - b) / a] \times [(V + 3)/V] \times L \quad (3)$$

where
$p_i$ = increase of pressure ( equation 2)
a = slope of calibration graph
b = bias of calibration graph
V = sample volume (equation 1)
L = dilution factor in case the sample is diluted after sample preparation

Content of carbon dioxide in % by weight:

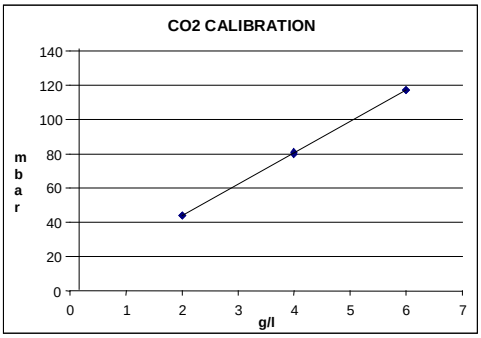
$$CO2\ \%\ (w/w) = C \times 100/d \qquad (4)$$

*Example of the calculation of the content of carbon dioxide:*

**Calibration**

| Conc of STD g/l | Air pressure mbar | Pressure std mbar | Pressure increase mbar |
|---|---|---|---|
| 2 | 1021 | 1065 | 44 |
| 2 | 1021 | 1065 | 44 |
| 4 | 1021 | 1101 | 80 |
| 4 | 1021 | 1102 | 81 |
| 6 | 1021 | 1138 | 117 |
| 6 | 1021 | 1138 | 117 |

| | |
|---|---|
| **slope** | 18.25000 |
| **intercept** | 7.50000 |
| **correlation** | 0.99995 |




**Calculation of the content of $CO_2$**

| SAMPLE | Density d (kg/m$^3$) | Flask + NaOH m1 (g) | Flask NaOH+ sampe m2 (g) | Air pressure $p_{ap}$ (mbar) | Sample pressure $p_s$ (mbar) | $p_s$-$p_{ap}$ | Sample V (ml) | CO2 g/l | Mean CO2 g/l |
|---|---|---|---|---|---|---|---|---|---|
| Sparkling wine 1 | 1027.2 | 84.6287 | 156.162 | 1021 | 1112 | 91 | 69.64 | **4.77** | |
| Sparkling wine 1 | 1027.2 | 84.6287 | 156.162 | 1021 | 1113 | 92 | 69.64 | **4.83** | **4.80** |
| Sparkling wine 2 | 1025.3 | 86.1066 | 153.4407 | 1021 | 1118 | 97 | 65.67 | **5.13** | |
| Sparkling wine 2 | 1025.3 | 86.1066 | 153.4407 | 1021 | 1118 | 97 | 65.67 | **5.13** | **5.13** |

## 6. VALIDATION

### 6.1. Performance criteria

- Standard deviation estimated from duplicates, $s_o$ = 0.07 g/l
- Relative standard deviation, RSD = 1.9%
- Repeatability, r = 5.6 %
- Expanded measurement uncertainty (k = 2), U = 3.8%
- Calibration range 0.4-6 g/L
- Determination range 0.3 -12 g/L (samples with concentration above 6 g/L should be diluted 1:2 with water to fit the calibration range)
- Detection limit 0.14 g/L
- Quantification limit 0.48 g/L

**Annex A**

**Literature**

European Brewery Convention Analytica-EBC, Fourth edition, 1987, 9.15 Carbon dioxide.

OIV, SCMA 2002, FV N° 1153, determination of carbon dioxide in alcoholic beverages by a modified EBC method

OIV, SCMA 2004, FV N° 1192, determination of carbon dioxide in alcoholic Beverages by a modified EBC method, Statistical results of the collaborative study

OIV, SCMA 2005, FV N° 1222, comparison of the titrimetric method and the modified EBC method for the determination of carbon dioxide in alcoholic beverages

Ali-Mattila, E. and Lehtonen, P., Determination of carbon dioxide in alcoholic beverages by a modified EBC method, Mitteilungen Klosterneuburg 52 (2002): 233-236

**Annex B**

**Statistical results of the collaborative study**

**DETERMINATION OF CARBON DIOXIDE IN ALCOHOLIC BEVERAGES BY A MODIFIED EBC METHOD**

**1. Goal of the study**

The objective of the study was to determine the repeatability and reproducibility of the modified EBC method for the determination of carbon dioxide in wines, sparkling wines, ciders and beers.

**2. Needs and purpose of the study**

Fermentation produces carbon dioxide in alcoholic beverages. In the production of sparkling wines, carbon dioxide is one of the most essential products and it can also be added to certain alcoholic beverages. Carbon dioxide modifies the taste and aroma and is a preserving agent in alcoholic beverages.

In accordance with the definitions of the International Code of Oenological practices, sparkling wine should have an excess pressure of not less than 3 bar due to carbon dioxide in solution, when kept at a temperature of 20°C in closed containers. Correspondingly semi-sparkling wine should have an excess pressure of not less than 1 bar and not more than 2,5 bar. Excess pressure of, 3 bar, 2.5 bar and 1 bar correspond at 20°C about, 5.83 g/L, 5.17 g/L and 3.08 g/L of carbon dioxide in solution, respectively.

There is currently no practical and reliable method for the determination of carbon dioxide in alcoholic beverages. The wide variation in carbon dioxide results in international proficiency tests is a clear indication of the fact that there is a need for a reliable method.

**3. Scope and applicability**

The proposed method is quantitative and it is applicable for the determination of carbon dioxide in alcoholic beverages. This method was validated in a collaborative study for the determination of carbon dioxide in wine, beer, cider and sparkling wine via the analyses at levels ranging approximately from 0.4 g/L to 12 g/L (Note: the actual calibration level ranges from 0,4 g/L to 6 g/L. The samples should be diluted with water to this level in case the carbon dioxide content is higher than 6 g/L).

**4. Materials and matrices**

The collaborative study consisted of 6 different samples. All except the beer samples were sent in blind duplicate, so that in total 12 bottles were distributed to the participants: two beers, two ciders, two red wines, two white wines, two pearl wines and two sparkling wines. Each bottle was coded individually for each participant. All samples were delivered in original bottles and the labels were removed from all samples except the sparkling wine samples. Measuring the amount of carbon dioxide in 10 bottles of the same lot number tested the homogeneity of the samples.

**5. Practice samples**

Four control samples were sent to participants to familiarize them with the method. . These samples included one beer, one wine, one pearl wine and one sparkling wine sample.

**6. Method to be followed and supporting documents**

The method and an Excel table for the calculation of results were sent to participants.

Supporting documents were also given, including the covering letter, sample receipt form, and result sheets.

**7. Data analysis**

7.1. Determination of outliers was assessed by Cochran's test, Grubbs' test and bilateral Grubbs test.

7.2. Statistical analysis was performed to obtain repeatability and reproducibility data.

**8. Participants**

Nine laboratories in different countries participated in the collaborative study. Lab-codes were given to the laboratories. The participating laboratories have proven experience in the analysis of alcoholic beverages.

Alcohol Control Laboratory
Alko Inc.
P.O.Box 279
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Altia Ltd
Valta-akseli
Rajamäki
Finland

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0503 Oslo

ARETO Ltd
Mere pst 8a
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Estonia

Norway

Bundesamt für Weinbau
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A-7000 Eisenstadt
Austria

Comité Interprofessionnel du
Vin de Champagne
5, rue Henri MARTIN
BP 135
51204 EPERNAY CEDEX
France

High-Tec Foods Ltd
Ruomelantie 12 B
02210 Espoo
Finland

Institut für Radioagronomie
Forschungszentrum
Jülich GMBH
Postfach 1913
52425 JÜLICH
Germany

Systembolagets laboratorioum
Armaturvägen 4,
S-136 50 HANINGE
Sweden

## 9. Results

The homogeneity of the samples was determined by measuring the carbon dioxide content in 10 bottles of the same lot number at the Alcohol Control Laboratory (Finland). Samples with the corresponding lot numbers were sent to the participants:

| $CO_2$ g/L | Beer 1 | Beer 2 | Cider | White wine | Red Wine | Pearl wine | Sparkling wine |
|---|---|---|---|---|---|---|---|
| Mean | 5.191 | 5.140 | 4.817 | 1.337 | 0.595 | 5.254 | 7.463 |
| s | 0.020 | 0.027 | 0.025 | 0.036 | 0.038 | 0.022 | 0.046 |

According to the homogeneity test the $CO_2$ content in the two beers was the same and therefore they were considered as blind duplicates.

The individual results for all samples and laboratories of the collaborative study are given below.

| Lab code | Beer 1 | Beer 2 | Cider 1 | Cider 2 | White wine 1 | White wine 2 | Red wine 1 | Red wine 2 | Pearl wine 1 | Pearl wine 2 | Sparkling wine 1 | Sparkling wine 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 5,39 | 5,08 | 4,75 | 4,91 | 1,25 | 1,11 | 0,54 | 0,54 | 5,15 | 5,22 | 6,93 | 6,91 |
| B | 4,76 | 5,53 | 4,71 | 4,70 | 1,90[3] | 1,78[3] | 0,73[2] | 1,19[2] | 5,85[3] | 5,93[3] | 7,66[3] | 7,72[3] |
| C | 5,15 | 5,14 | 4,93 | 4,94 | 1,36 | 1,41 | 0,51 | 0,48 | 5,23 | 5,33 | 7,33 | 7,36 |
| D | 3,13[1] | 3,95[1] | 4,36[1] | 0,38[1] | 1,11[1] | 1,11[1] | 0,43[1] | 0,38[1] | 4,47[1] | 4,29[1] | 5,54[1] | 5,52[1] |
| E | 4,87 | 4,73 | 4,96 | 4,78 | 1,52 | 1,52 | 0,78[3] | 0,80[3] | 4,98 | 4,94 | 5,83 | 6,17 |
| F | 5.34 | 4.91 | 4.71 | 5.01 | 1.33 | 1.40 | 0.46 | 0.57 | 5.22 | 4.95 | 6.52 | 6.67 |
| G | 5,18 | 5,15 | 4,82 | 4,86 | 1,37 | 1,36 | 0,56 | 0,59 | 5,22 | 5,27 | 7,54 | 7,47 |
| H | 5,42 | 5,40 | 5,05 | 5,12 | 1,15 | 1,30 | 0,52 | 0,53 | 5,12 | 5,10 | 7,25 | 7,34 |
| I | 5,14 | 5,13 | 4,65 | 4,76 | 1,16 | 1,19 | 0,47 | 0,61 | 5,16 | 5,06 | 6,88 | 6,48 |

1. Removed because of large systematic error obviously due to poor calibration
2. Outlier by Cochran's test
3. Outlier by Grubbs' test

Statistical results of the collaborative test are summarised below.

| | Beer | Cider | White wine | Red Wine | Pearl wine | Sparkling wine |
|---|---|---|---|---|---|---|
| **Mean** (g/L) | 5.145 | 4.859 | 1.316 | 0.532 | 5.139 | 6.906 |
| **Mean rep. 1** (g/L) | 5.156 | 4.833 | 1.306 | 0.510 | 5.154 | 6.897 |
| **Mean rep 2** (g/L) | 5.134 | 4.885 | 1.327 | 0.553 | 5.124 | 6.914 |
| $s_r$ (g/L) | 0.237 | 0.089 | 0.060 | 0.053 | 0.086 | 0.149 |
| $s_R$ (g/L) | 0.237 | 0.139 | 0.135 | 0.059 | 0.124 | 0.538 |
| $SDR_r$ (%) | 4.597 | 1.821 | 4.562 | 9.953 | 1.663 | 2.163 |
| $RSD_R$ (%) | 4.611 | 2.855 | 10.22 | 11.07 | 2.407 | 7.795 |
| **r (2,8*$s_r$)** (g/L) | 0.662 | 0.248 | 0.168 | 0.148 | 0.239 | 0.418 |
| **R (2,8*$s_R$)** (g/L) | 0.664 | 0.388 | 0.377 | 0.165 | 0.346 | 1.507 |
| **HORRAT R** | 1.043 | 0.640 | 1.883 | 1.779 | 0.544 | 1.843 |

**Conclusion**

The Horrat values are < 2 indicating an acceptable method. The Horrat values are, however, a little bit high. In five of the nine participating laboratories these tests were made almost with no previous experience. Therefore the results can be considered at least as very satisfactory.

The method gives the results in g/L but the results can be converted to pressure units. [1]

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1. Troost, G. and Haushofer, H., Sekt, Schaum- und Perlwein, Eugen Ulmer Gmbh & Co., 1980, Klosterneuburg am Rhein, ISBN 3-8001-5804-3, Diagram 1 on the page 13.

## Annex C

## Validation at low carbon dioxide levels

### 1. The detection and the determination limit

A sample of white wine was analysed in duplicate ten times. The statistical data was as follows:

| | |
|---|---|
| Replicates | 10 |
| Mean $CO_2$ (g/L) | 0.41 |
| Standard deviation of the mean, s (g/L) | 0.048 |
| Detection limit 3 x s | 0.14 |
| Determination limit 6 x s | 0.48 |

### 2. Standard addition

Standard additions in five different concentrations in duplicates were made into the same wine which was used for the determination of the detection and determination limits. The corresponding concentrations of $CO_2$ were also added to water. The linear regressions of these two experiments were compared.

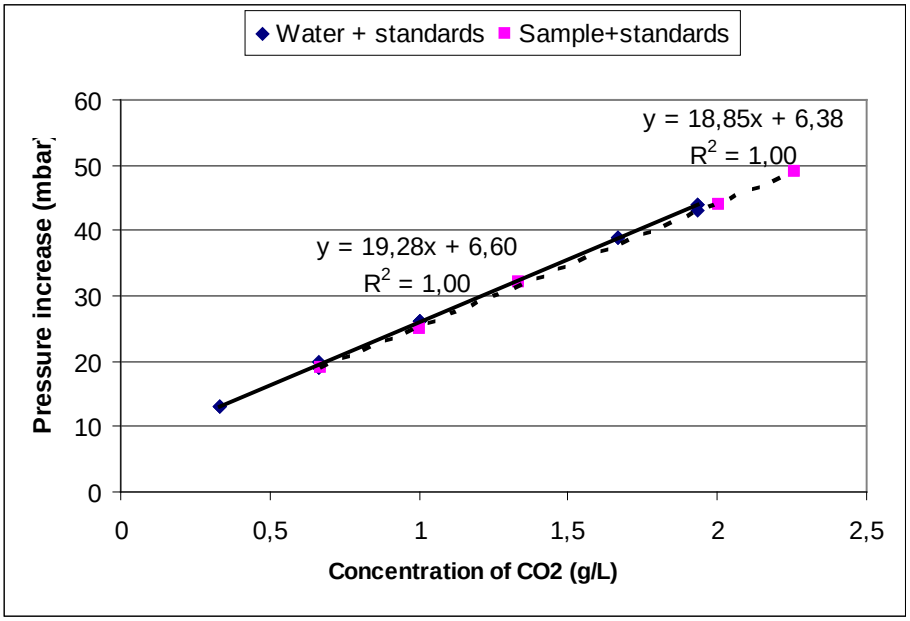


Fig. 1 Standard additions to the sample and to water.

Statistical data of the plots:

| | Water+ standards | Sample+standards |
|---|---|---|
| Slope | 19.3 | 18.9 |
| Uncertainty of the slope | 0.3 | 0.3 |
| Intercept | 6.6 | 6.4 |
| Uncertainty of the intercept | 0.4 | 0.5 |
| Residual standard deviation | 0.4 | 0.3 |
| number of samples | 15 | 10 |

According to statistical data the two regression lines are similar.

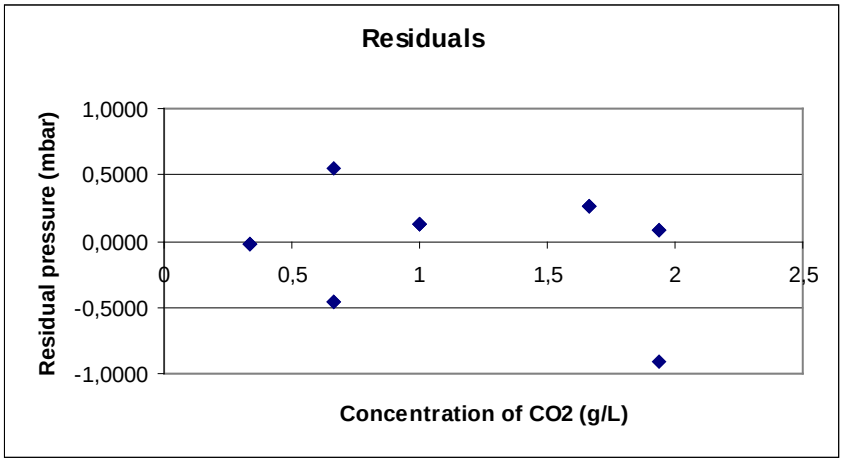


Fig. 2. The residuals of the "water+standards" equation

The residuals are dispatched on both sides of zero indicating that the regression line is linear.

**Annex D**

**Comparison with other techniques and laboratories**

**1. Comparison of the modified EBC method with the commercial Anton Paar CarboQC instrument**

| **Sample** | **Modified EBC method (**g/L) | **Anton Paar CarboQC** (g/L) | **Difference** |
|---|---|---|---|
| Sparkling wine | 9.14 | 9.35 | -0.21 |
| Cider | 4,20 | 4,10 | 0.1 |
| White wine | 1,18 | 1,10 | 0.08 |
| Red wine | 1,08 | 0,83 | 0.25 |
| Beer 1 | 5,26 | 5,15 | 0.11 |
| Beer 2 | 4,89 | 4,82 | 0.07 |
| Beer 3 | 4,90 | 4,92 | -0.02 |
| Non-alcohol Beer 1 | 5,41 | 5,33 | 0.08 |
| Non-alcohol beer 2 | 5,39 | 5,36 | 0.03 |
| | | | **Mean 0.06** |

According to t-test there is no systematic difference in the measurements.

**2. Comparison between Bfr, Germany and ACL, Finland**

Bfr sent four samples to ACL, and ACL sent five samples to Bfr. These nine samples were analysed independently both by ACL using the method presented in this paper and in Germany at Bfr using the titrimetric method. Statistics of the results were as follows:

| | |
|---|---|
| Mean of the difference | 0.14 g/L |
| Std. of the difference | 0.13 g/L |
| Z-score | 1.04 |

The method presented here and the titrimetric method were also compared by Bundesamt für Weinbau in Austria using 21 samples of their own. Statistical data was as follows:

| | |
|---|---|
| Mean of the difference | -0.01 g/L |
| Std. of the difference | 0.26 g/L |
| Z-score | -0.03 |

**Conclusion**

According to this paper as well as earlier experiments this method is universal. It is suitable for the determination of the carbon dioxide content in all kinds of alcoholic beverages, e.g. beers, wines, fruit wines, ciders, pearl wines and sparkling wines with the concentration level of 0.3 g/L and higher.

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