COMPENDIUM OF INTERNATIONAL METHODS OF ANALYSIS FOR VINEGARS

INTERNATIONAL ORGANISATION
OF VINE AND WINE

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Type II method

Determination of total acidity content in vinegars

(OENO 52/2000

OIV-OENO 597-2018)

1. Definition

The total acidity refers to a vinegar whose acidity can be titled in the presence of phenolphthalein in an alcoholic solution, used as indicator.

2. Principle

Neutralization of acids in sample by alkali solution.

3. Reagents

3.1. Solution of sodium hydroxide 0.5M

3.2. Indicator – phenolphthalein alcoholic solution at 1 g per 100 ml.

In a calibrated flask, capacity 100 ml, dissolve 1 g of phenolphthalein with a sufficient quantity of ethanol at 95% (v/v) and bring up to the line.

4. Equipment and ustensils

Standard laboratory equipment.

5. Preparation of sample

Thoroughly mix the sample by stirring and filter if necessary.

6. Technique[1]

In a 250-mL conical flask, add 10 mL of vinegar. Add water, free of carbon dioxide, so that the solution is barely coloured. Add a few drops of the indicator (3.2) and titrate with the sodium hydroxide solution (3.1) until a persistent pink coulour is obtained.

Note: Titration may also be monitored by potentiometry, taking into consideration the respective equivalence point.

7. Results

7.1. Calculation

Considering

- **V** the volume in ml of the sodium hydroxide solution using in titling.

The total acidity content expressed in grams of acetic acid per l of sample will be given by

$$3\,V.$$

7.2. Presentation

Round off the results in grams of acetic acid per liter to the nearest decimal.

[1] CPIV has described a method, using the potentiometric titration.

8. Interlaboratory validation (hitos et al., 2000)

Units : %(m/V)

Sample	**r**	**S_r**	**RSD_r**	**R**	**S_R**	**RSD_R**	**RSD_R (Horwitz)**	**Horrat Index**
1 – 0.17% (m/v)	0.0628	0.022	0.27	0.1570	0.560	0.67	2.90	0.23
2 – 0.17% (m/v)	0.0742	0.026	0.23	0.2127	0.076	0.67	2.78	0.24
3 – 0.08% (m/v)	0.0617	0.022	0.20	0.2197	0.078	0.70	2.78	0.25
4 – 0.07% (m/v)	0.0559	0.020	0.17	0.1543	0.055	0.46	2.75	0.17
5 – 0.08% (m/v)	0.0738	0.026	0.23	0.3544	0.0127	1.13	2.78	0.41

9. Bibliography

1. Anonymous, 1993, Métodos Oficiales de Análisis, Tomo II, Ministério de Agricultura, Pesca y Alimentación, Madrid, Spain.
2. AOAC, 1984, Official Methods of the Ass. Offic. Agric. Chem., 14th edit., Arlington USA.
3. Curvelo-Garcia A.S. and Laureano O., 1993. Organization of collaborative studies and comparison of various statistical models, Commission II Report (III.A) of the 73rfd General Meeting of the OIV, San Francisco, USA.
4. AOF/WHO – Commission of Codex Alimentarius, Methods of analyzing the European regional standard for vinegar, Alinorm 83/19 and 85/19.
5. Hitos P., Pons A., Martin de la Hinojosa, I, Gomez R., Hernandez A. and Muñoz J., 2000. Validation of analysis methods for total, fixed and volatile acidity of non-volatile reducing substances, copper and zinc in wine vinegars, Green Sheet of OIV No. 115.

Method OIV-MA-VI-02 : R2000

Type II method

Determination of the fixed acidity content in vinegars

(OENO 53/2000)

1. Definition

The fixed acidity of a vinegar refers to all the fixed (non-volatile) acids titled in the presence of phenolphthalein in an alcoholic solution, used as indicator.

2. Principle

Elimination of volatile substances from the vinegar by evaporation. Neutralization of the (non-volatile) acids of the residue in an aqueous solution using an alkali solution.

3. Reagents

3.1. Sodium hydroxide solution 0.1 M

3.2. Indicator - alcoholic solution of phenolphthalein at 1 g per 100 ml.

In a calibrated 100 ml flask, dissolve 1 g of phenolphthalein with a sufficient quality of ethanol at 95% (v/v) and bring up to the line.

4. Equipment and utensils

Standard laboratory equipment including:

4.1. Water bath at 100 °C

4.2. 200 ml capacity porcelain capsules.

5. Preparation of sample

Homogenize the sample by stirring and filter if necessary.

6. Technique

In a 200 ml porcelain capsule, add 10 ml of vinegar. In a water bath at 100 °C, evaporate until dry. Add 5 to 10 ml of water. Evaporate again until dry. Repeat this step five times, add approximately 180 ml of recently boiled and cooled water, add a few drops of indicator (3.2) and title with the sodium hydroxide solution (3.1) until a persistent pink color is obtained.

7. Results

7.1. Calculation

Considering:

- V to be the volume in ml of the sodium hydroxide solution using in titling.

The fixed acidity content expressed in grams of acetic acid per l of sample is given by

- 0.6 V.

7.2. Presentation

Round off the results expressed in grams of acetic acid by L, to the nearest decimal.

8. Interlaboratory validation (Hitos *et al.*, 2000)

Units: % (m/V)

Sample	r	S_r	RSDr	R	S_R	RSD_R	RSD_R (Horwitz)	Horrat Index
1 – 0.17% (m/v)	1.0125	0.004	2.69	0.0428	0.015	9.18	5.22	1.76
2 – 0.17% (m/v)	0.0103	0.004	2.19	0.0431	0.015	9.15	5.22	1.75
3 – 0.08% (m/v)	0.0103	0.004	4.88	0.0201	0.007	9.57	5.85	1.64
4 – 0.07% (m/v)	0.0083	0.003	4.20	0.0246	0.009	12.38	5.97	2.07
5 – 0.08% (m/v)	0.0077	0.003	3.26	0.0285	0.010	12.11	5.85	2.07

9. Bibliography

1. Anonymous, 1993, *Métodos Oficiales de Análisis*, Tomo II, Ministério de Agricultura, Pesca y Alimentación, Madrid, Spain.
2. AOAC, 1984, *Official Methods of the Ass. Offic. Agric. Chem.*, 14th edit., Arlington USA.
3. Hitos P., Pons A., Martin de la Hinojosa, I, Gomez R., Hernandez A. and Muñoz J., 2000. Validation of analysis methods for total, fixed and volatile acidity of non-volatile reducing substances, copper and zinc in wine vinegars, *Green Sheet of* OIV No. 115.

4. Llaguno C. et Polo M.G., 1991. *El Vinagre de Vino*, Consejo Superior de Investigaciones Cientificas, Madrid, Spain.

Method OIV-MA-VI-03 : R2000

Type II method

Determination of the volatile acid content in vinegars

(OENO 54/2000)

1. Definition

By convention, the volatile acidity of vinegar refers to the difference between the total acidity and the fixed acidity.

2. Principle

Calculation of difference between total acidity and fixed acidity, expressed in grams of acetic acid per L.

3. References

See the methods I (determination of total acidity content) and II (determination of the fixed acidity content).

4. Results

4.1. Calculation

Considering:

- A_t to be the total acidity content (expressed in grams of acetic acid per L of sample) and
- A_f to be the fixed acidity content (expressed in grams of acetic acid per L of sample).

The volatile acidity content expressed in grams of acetic acid per L of sample is given by:

$$A_t - A_f$$

4.2. Presentation

The results expressed in grams of acetic acid per liter are given to the first decimal.

5. Interlaboratory validation (Hitos et *al.*2000)

Units: % (m/V)

Sample	**r**	**S_r**	**RSD_r**	**R**	**S_R**	**RSD_R**	**RSD_R (Horwitz)**	**Horrat Index**
1 – 8.24% (m/v)	0.0445	0.016	0.19	0.1632	0.058	0.71	2.91	0.24
2 – 11.17% (m/v)	0.0438	0.016	0.14	0.1967	0.070	0.63	2.78	0.23
3 – 11.20% (m/v)	0.0595	0.021	0.19	0.2076	0.074	0.66	2.78	0.24
4 – 11.94% (m/v)	0.0473	0.017	0.14	0.1652	0.059	0.49	2.75	0.18
5 – 11.16% (m/v)	0.0518	0.019	0.17	0.3577	0.0128	1.14	2.78	0.41

6. Bibliography

1. Anonymous, 1993, *Métodos Oficiales de Análisis*, Tomo II, Ministério de Agricultura, Pesca y Alimentación, Madrid, Spain.
2. AOAC, 1984, *Official Methods of the Ass. Offic. Agric. Chem.*, 14th edit., Arlington USA.
3. Hitos P., Pons A., Martin de la Hinojosa, I, Gomez R., Hernandez A. and Muñoz J., 2000. Validation of analysis methods for total, fixed and volatile acidity of non-volatile reducing substances, copper and zinc in wine vinegars, *Green Sheet of* OIV No. 115.

Method OIV-MA-VI-04 : R2000

Type IV method

Detection and quantification of the presence of synthetic acetic acid in vinegars

(OENO 55/2000)

1. Introduction

The presence of synthetic acetic acid (detection and possibly quantification) in a vinegar is now determined by the content in ^{14}C. Natural vinegars (obtained without the addition of synthetic acetic acid) have ^{14}C contents that are accurately determined (according to the year of production). Values less than the characteristic contents of the assumed year of production represent:

- either a mixture with products from more recent years,
- or, if they are less than the natural radioactive contents ^{14}C of approximately 15 dpmg (disintegration per minute and per gram of carbon), the addition of all or part of the synthetic acetic acid (whose radioactivity ^{14}C is 0 dpmg).

2. Principle

After extracting the acetic acid using sodium hydroxide, complete by liquid scintillation the reactivity ^{14}C of the product converted into benzene.

3. Reagents

3.1. Concentrated solution of sodium hydroxide

3.2. Phenolphthalein, alcoolic solution at 1g for 100 ml

3.3. Synthetic benzene (0 dpmg of ^{14}C)

3.4. 0.01 g of 2-(4-tert-Butylphenyl)-5-(4-biphenylyl)-1.3.4-oxadiazole (Butyl PDB) and of 1.4 Bis (2-methylstyryl)benzene (bis-MSB) for 4 ml of benzene

3.5. International radiocarbon standard: oxalic acid N.B.S:

- 13.56 dpmg.

4. Equipment and utensils

4.1. 1-liter balloon flasks

4.2. Vigreux column combined with coolant

4.3. Balloon flask heater

4.4. Reactor for burning chemical products at 5 bars of oxygen

4.5. Chemical bench for synthesis of benzene from CO_2,

4.6. Liquid scintillation spectrometer

4.7. Glass counting cells (with low content and ^{40}K) and provided with highly sealed plastic covers

5. Preparation of sample

Homogenize the sample by stirring and filter if necessary.

6. Technique

6.1. Extraction of acetic acid

Distill 1 liter of vinegar having a total acid content of 60 g/L (or the corresponding quantity if the vinegar has any other total acidity). Recover the water-acetic acid mixture in the receiving balloon flask.

Recover the distillate, add to it 100 µl of phenolphthalein solution (3.2) and neutralize rapidly with sodium hydroxide concentrated solution (3.1) that has been filtered. Then distill the product obtained again to eliminate the water. The sodium acetate thus formed is recovered in the balloon flask, then dried.

The dry acetate is placed in the combustion reactor, which is raised to 5 bars of oxygen. Combustion is initiated by heating a filament, and the carbon gas formed in this way is trapped in the liquid nitrogen.

On the benzene synthesis bench, the CO_2 is reduced by lithium, then transformed into carbide which, through the addition of H_20, produces acetylene. A vanadium aluminum catalyst enables trimerization into benzene.

6.2. Spectrometric measurements

To determine the natural background noise at the metering flask, add 4 ml of synthetic benzene with the scintillating mixture and count until a background noise statistical margin of less than 0.01 dpmg is obtained. This gives us A_{bdf}

To determine the efficiency of the apparatus, count 4 ml of benzene prepared from international standard ^{14}C using the same concentration of scintillating mixture until a statistical margin of 0.1 dpmg is obtained to provide A_{st}

Then add to the meter, the flask containing the sample to be measured in the form of benzene, again using the same quantity of scintillation mixture. Count the number of times 1,000 minutes needed to obtain a statistical margin of 0.1 dpmg. In this way, we obtain A_{mes}.

7. Results

7.1. Calculation of specific activity

The specific activity ^{14}C of the sample (A_{ech}) compared to standard ^{14}C can be calculated from measured activity A_{ech} from which the background noise of the metering flask is removed.

$$A_{ech} = (A_{mes} - A_{bdf})/A_{st}$$

Where A_{ech} and A_{st} are raised to an efficiency of 100 bar so that they can be compared with the reference values.

7.2. Presentation

This specific activity is expressed in dpm of ^{14}C per gram of carbon and is rounded off to the first decimal.

8. Interpretation of results

Interpretation presupposes that the laboratory has natural vinegar radioactivity data (obtained by acetic fermentation) according to the year of production, starting from the year 1955, the first year when the radiocarbon enrichment due to thermonuclear bombs was detectable.

9. Bibliography

1. Lecoq R., 1965: Volatile acidity in wines: In "Manuel d'analyses alimentaires et d'expertises usuelles", Doin et Deren Edit, Tome II, pp. 2001-2007.
2. Llaguno C & Polo M.C;, 1991: El vinagre de vino - Consejo Superior de Investigacionaes Cientificas, Madrid.

3. Mongereau N. & Evin J., 1993: The applications of radiocarbon and expertise: Legal expertise records, Vol. 5 No. 3-4, pp. 105-110.
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Method OIV-MA-VI-05 : R2000

Type II method

Determination of the residual alcohol content in vinegars

(OENO 56/2000)

1. Definition

The residual alcohol content is the percentage by volume of ethanol still contained in the vinegar after acetic fermentation.

2. Principle[1]

Distillation of vinegar, oxidization of ethanol by potassium dichromate and determination of its content by titling the excess potassium dichromate by a solution of iron sulfate (11) and ammonium.

3. Reagents

3.1. Sulfuric acid ρ20 = 1.84 g/ml.

3.2. Sulfuric acid solution at 50% (v/v).

3.3. Solution of potassium dichromate

[1] CPIV has described another method (distillation of the sample and determination of the density of the distillate).

In a 1 l calibrated flask, dissolve 33.6 g (to 0.001 g) of potassium dichromate in water. Bring up to the line with water.

1 ml of this oxide solution 7.8924 mg of ethanol.

The solution must be preserved in a flask with a ground glass stopper.

3.4. Solution of iron sulfate (II) and ammonium (Mohr's salt).

In a 1 l calibrated flask, dissolve 135 g of iron sulfate (II) and ammonium in 20 ml of sulfuric acid (3.1) and bring up to the mark with water.

1 ml of this solution, recently prepared, corresponds to approximately 2.5 ml of potassium dichromate solution.

Given that this solution oxidizes easily, it must be titled frequently with potassium dichromate as described in 6.2 and 6.3, but replacing 10 ml of distillate by 10 ml water.

3.5. potassium permanganate solution

In a 1 l calibrated flask, dissolve 1.088 g of potassium permanganate in water and bring up to the line.

3.6. orthophenantroline solution

In a 100 ml calibrated flask, dissolve in water 0.695 g of $FeSO_4$ $7H_20$ and 1. 485 of mono-hydrated orthophenantroline.

Heat to facilitate distillation, cool and bring up to the line with water.

3.7. Calcium hydroxide solution at 120 of CaO per L.

4. Equipment and utensils

Standard laboratory equipment including:

4.1. Distillation apparatus comprising a 1 l calibrated flask connected to a ground glass union with a rectification column at least 20 cm long or another equivalent device, and a condensation column.

4.2. 250 ml conical flasks with ground glass stoppers.

5. Preparation of sample

Homogenize the sample by stirring and filter if necessary.

6. Technique

6.1. Distillation

Add 200 ml of vinegar to a distillation flask. Add calcium hydroxide solution (3.7) until alkalization is observed by litmus paper contact.

Add a few pieces of pumice stone, porcelain or glass balls and one drop of silicone aqueous solution to prevent the formation of foam. Heat to boiling point and gather approximately 90 ml in a 100 ml flask. Allow to cool and bring up to the line with water.

6.2. Oxidization

In a conical flask with a ground glass stopper, add 200 ml of the potassium dichromate (3.3) solution and 20 ml of sulfuric acid. Stir.

Add 10 ml of distillate. Wet the stopper with a drop of sulfuric acid, stop the flask and shake.

Wait at least 30 min. and shake from time to time.

6.3. Titling

Title the excess potassium dichromate with the iron sulfate solution (II) and ammonium (3.4). When the green color turns to greenish-blue, add 4 drops of orthophenantroline (3.6) solution. Titling is terminated when the solution turns to green-brown.

If the change point is slightly exceeded, come back to the exact point of change of color using the potassium permanganate (3.5) solution. One-tenth of the volume of the solution used must be deducted from the volume of the iron sulfate (II) and ammonium solution used.

6.4. Specimen test

Carry out a specimen test with 10 ml of water instead of 10 ml of distillate.

7. Results

7.1. Calculation

Considering:

- V the volume of ml of the iron sulfate solution (II) and of aluminum used in titling (6.3).
- V' the volume in ml of the iron sulfate solution (II) and of ammonium used in the specimen test (6.4).

The residual alcohol content expressed as a percentage (v/v) at 20°C, as given by:

$$(V' - V)/V'$$

7.2. Presentation

Round off the results to the nearest decimal.

8. Inter-laboratory validation (Curvelo-Garcia, 1996)

- F < 0.02% (v/v)
- R < 0.04% (v/v)

9. Bibliography

1. Anonymous, 1993, *Métodos Oficiales de Análisis*, Tomo II, Ministério de Agricultura, Pesca y Alimentación, Madrid, Spain.
2. Curvelo-Garcia A.S. 1996. *Wine vinegars*, *Analysis Methods (Part Two)*, Green Book of OIV No. 1033.
3. AOF / WHO - Commission of Codex Alimentarius, *Methods of analyzing the European regional standard for vinegar*, Alinorm 83/19 and 85/19.
4. Llaguno C. et Polo M.G., 1991. *El Vinagre de Vino*, Consejo Superior de Investigaciones Cientificas, Madrid, Spain.

Method OIV-MA-VI-06 : R2000

Type II method

Determination of total dry extract content in vinegars

(OENO 57/2000)

1. Introduction

The main purpose of determining the total dry extract content is to detect certain frauds, for instance the addition of water or an aqueous solution of acetic acid (very low total dry extract value) or the addition of non-volatile substances (very high total dry extract value). To interpret the results accurately, it is necessary to have a database for the type and origin of the analyzed vinegar.

2. Definition

The total dry extract refers to all the substances which, under the conditions described here, do not volatilize and are not affected by alteration.

3. Principle

Evaporation of sample and drying in oven, then weighing.

4. Equipment and utensils

Standard laboratory equipment including:

4.1. Water bath at 100 °C

4.2. Water oven

4.3. Flat base capsules approximately 50 mm in diameter and 20 mm in height of platinum or stainless steel.

5. Preparation of sample

Homogenize the sample by stirring and filter if necessary.

6. Technique

Add 10 ml of the sample to a previously calibrated capsule, evaporate in a water bath at 100°C for 30 min., dry in an oven for 2 hours 30 min., cool in a dryer and weigh.

To obtain conclusive results, always use capsules with the same characteristics and comply strictly with the described drying times.

7. Results

7.1. Calculation

Considering:

- m_1 the mass of the empty capsule in grams
- m_2 the mass of the capsule containing the residue in grams

The total dry extract content, expressed in g/l, given by:

$$100\ (m_2 - m_1)$$

7.2. Presentation

Round off the results given in g/l to the nearest decimal.

8. Inter-laboratory validation (CPIV, 1995, Curvelo-Garcia 1996)

$$r = 0.8\ g/l$$

$$R = 2.8\ g/l$$

9. Bibliography

1. Anonymous, 1993, *Métodos Oficiales de Análisis*, Tomo II, Ministério de Agricultura, Pesca y Alimentación, Madrid, Spain.
2. AOAC, 1984, *Official Methods of the Ass. Offic. Agric. Chem.*, 14th edit., Arlington USA.
3. CPIV, 1995. *Methods of Analysis*, Annex 4 to the minutes of CPIV Technical Committee's Meeting, Stuttgart, Germany.
4. Curvelo-Garcia A.S., 1996. Wine vinegars. Methods of Analysis (Part Two). *Green Sheet of* OIV No. 1033.
5. AOF / WHO - Commission of Codex Alimentarius, *Methods of analyzing the European regional standard for vinegar*, Alinorm 83/19 and 85/19.

Method OIV-MA-VI-07 : R2000

Type II method

Determination of ash content in vinegars

(OENO 58/2000)

1. Introduction

The main purpose of determining the ash content is to detect certain frauds, for instance the addition of water or an acetic acid aqueous solution (very low ash content) or the addition of non-volatile substances (very high ash content) for the correct interpretation of the results, in which case it is necessary to have a database for the type and origin of the vinegar being analyzed.

2. Definition

Vinegar ashes refer to all the incineration products of the evaporation residue of a known volume of vinegar, carried out in such a way as to obtain all the cations (except for ammonium) in the form of carbonates and other anhydrous mineral salts.

3. Principle

Incineration of the vinegar extract between 500°C and 550°C through to complete combustion of the carbon.

4. Equipment and utensils

Standard laboratory equipment including:

4.1. Water bath at 100 °C

4.2. Scales sensitive to within 1/10th of a milligram

4.3. Hot plate or infrared evaporator

4.4. Temperature controlled electric oven

4.5. Platinum (or quartz) capsules, 70 mm in diameter and 25 mm high with a flat bottom.

5. Preparation of sample

Homogenize the sample by stirring, then filter if necessary.

6. Technique

Add 20 ml of the sample to a previously calibrated platinum capsule and evaporator in a water bath at 100°C until a syrupy consistency is obtained. Heat the residue on a hot plate to 200°C or using an infrared evaporator through to carbonization. When the residue no longer gives off vapor, put the capsule in the electric oven brought to 525°C ±25°C. After 5 min. of carbonization, remove the capsule from the oven, allow to cool and add 5 ml of distilled water, which can then be evaporated in the water bath at 100 °C or under the infrared evaporator. Heat again to 525°C for 10 min.

If the carbonated particles do not bur up entirely, repeat the adding water, evaporation and incineration steps.

After cooling in a drying chamber, weigh the capsule (with the ashes).

7. Results

7.1. Calculation

Considering:

- p0 the mass of the empty capsule in grams
- p1 the mass of the capsule containing the ash in grams

The content of ash expressed in g/l is given by

$$50\ (p1 - p0)$$

7.2. Presentation

Round off the results expressed in g/l to the second decimal.

8. Inter-Laboratory validation

$$r = 0.30\ g/l$$

$$R = 1.0\ g/l$$

9. Bibliography

1. Anonymous, 1993, *Métodos Oficiales de Análisis*, Tomo II, Ministério de Agricultura, Pesca y Alimentación, Madrid, Spain.
2. CPIV, 1995. *Methods of Analysis*, Annex 4 to the minutes of CPIV Technical Committee's Meeting, Stuttgart, Germany.
3. Curvelo-Garcia A.S., 1996. Wine vinegars. Methods of Analysis (Part Two). *Green Sheet of* OIV No. 1033.
4. Llaguno C. et Polo M.G., 1991. *El Vinagre de Vino*, Consejo Superior de Investigaciones Cientificas, Madrid, Spain.
5. OIV, 1990. *Collection of international analysis methods of wine and must*, OIV, Paris, France.

Method OIV-MA-VI-08 : R2000

Type II method

Determination of the non-volatile reducing substances content in vinegars

(OENO 59/2000)

1. Definition

Non-volatile reducing substances are all the non-volatile substances having a reducing power in a copper-alkali solution and those that also have this power after hydrolysis.

2. Principles

Evaporation of volatile substances, hydrochloric hydrolysis, oxidization by a copper-alkali solution in excess with titling by iodometry of copper ions.

3. Reagents

3.1. Hydrochloric acid $\rho 20$ = 1.19 g/ml

3.2. Sulfuric acid ($\rho 20$ = 1.84 g/ml).

3.3. Solution of sodium hydroxide 12 M

3.4. Copper-alkali solution

Dissolve separately 25 g of copper sulfate ($CuSO_4, 5H_2O$) in 100 ml of water, 50 g of citric acid ($C_6H_8O_7, H_2O$) in 300 ml of water and 388 g of crystallized sodium carbonate ($Na_3CO_3, 10H_2O$) in approximately 400 ml of hot water. In a calibrated flask, successively add 1000 ml of the citric acid solution and the sodium carbonate solution, then add the copper sulfate solution. Cool, mix and bring up to the gauge line with water.

3.5. Solution of potassium iodine at 30% (m/v). Keep in a tinted glass flask.

3.6. Solution of sulfuric acid at 25% (v/v).

3.7. Starch additive solution at 5 g/l.

Thin out 0.5 g of soluble starch in a small amount of cold water so as to obtain a fluid paste. To 100 ml of boiling water, add, then maintain at the boil for 10 min. Allow to cool.

3.8. Solution of sodium thiosulfate 0.1 M.

3.9. Boiling regulator (glass balls, pumice stone).

4. Equipment and utensils

Laboratory equipment including:

4.1. Reflux apparatus consisting of a 500 ml conical flask connected by a ground seal to a condenser, or any other equivalent apparatus.

4.2. Cooling device consisting of a flow of cold water.

4.3. Water bath at 100 °C.

5. Preparation of sample

Homogenize the sample by stirring and filter if necessary.

6. Technique

6.1. Evaporation of volatile substances

In a 100 ml porcelain capsule, add 50 ml of vinegar and evaporate in a water bath at 100 °C to approximately 1 to 2 ml.

Add 10 ml of water and evaporate again. Repeat the operation with 10 ml of water.

Transfer the evaporation residue into a 100 ml calibrated flask using approximately 50 ml of hot water.

Allow to cool and bring up to the gauge line.

6.2. Hydrolysis

To the reflux apparatus flask, add 10 ml of the solution obtained in 6.1 and 0.3 ml of hydrochloric acid. Heat in a water bath at 100 °C for 2 min.

Cool to room temperature for 15 min. and neutralize with 0.3 ml of the sodium hydroxide solution.

6.3. Oxidization and titling

In the same flask, add 15 ml of water and 25 ml of the copper alkali solution (3.4). Add the boiling regulator and bring to the boil, which should take 2 min. Adapt the condenser to the flask and maintain at the boil for exactly 10 min.

Cool immediately in a flow of cold water without stirring. Successively add 2 ml of the starch solution (3.7), 10 ml of the potassium iodine solution (3.5) and 25 ml of the sulfuric acid solution (3.6). Title with the sodium thiosulfate solution (3.8).

Produce a specimen dose with 25 ml of water and 25 ml of copper-alkali solution.

7. Results

7.1. Calculation

Considering:

- V_1 the volume in ml of the sodium disulfate solution used in titling.
- V_2 the volume in ml of the sodium disulfate solution used in specimen dosing.
- n the quantity of test sample reducing substances in mg, given by the table and as a function of the difference

$$V_2 - V_1$$

The content in non-volatile reducing substances expressed in grams per l of vinegar is given by:

0.2 n

Table

V_2-V_1 (ml)	sub.red (mg)	V_2-V_1 (ml)	sub.red (mg)	V_2-V_1 (ml)	sub.red (mg)
1	2.4	9	22.4	17	44.2
2	4.8	10	25.0	18	47.1
3	7.2	11	27.6	19	50.0

4	9.7	12	30.3	20	53.0
5	12.2	13	33.0	21	56.0
6	14.7	14	35.7	22	59.1
7	17.2	15	38.5	23	62.2
8	19.8	16	41.3		

7.2. Presentation

Round off the results expressed in grams per liter to the nearest decimal.

8. Inter-laboratory validation (Hitos et al, 2000)

Units: % (m/V)

Sample	**r**	**S_r**	**RSD_r**	**R**	**S_R**	**RSD_R**	**RSD_R (Horwitz)**	**Horrat Index**
1 – 2.93 g/L	0.0892	0.032	1.09	1.0267	0.367	12.52	4.81	2.60
2 – 2.82 g/L	0.2635	0.094	3.34	1.4950	0.534	18.94	4.84	3.91
3 – 6.27 g/L	0.1214	0.043	0.69	1.6346	0.584	9.32	4.29	2.17
4 – 8.77 g/L	0.1591	0.057	0.65	1.5054	0.538	6.13	4.08	1.50
5 – 6.22 g/L	0.1019	0.036	0.58	1.2130	0.433	6.96	4.30	1.62

9. Bibliography

1. AOAC, 1984, Official Methods of the Ass. Offic. Agric. Chem., 14th edit., Arlington USA.
2. CT83, 199. Norma Portuguesa NP 3683 (Vinagre. Determinaçã do teor de subtãncias redutoras não valáteis), Instituto Portugués da Qualidade, Lisbon, Portugal.
3. Hitos P., Pons A., Martin de la Hinojosa, I, Gomez R., Hernandez A. and Muñoz J., 2000. Validation of analysis methods for total, fixed

and volatile acidity of non-volatile reducing substances, copper and zinc in wine vinegars, Green Sheet of OIV No. 1115.

4. OIV, 1990. Collection of international analysis methods of wine and must, OIV, Paris, France.

Method OIV-MA-VI-09 : R2008

Type IV method

Determination of the total sulfur dioxide content in vinegars

(OENO 60/2000;

OENO 13/2008)

1. Introduction

In the wine vinegars industry, the addition of sulfur dioxide or salts (E 220 - E 227) is authorized according to defined standards and doses. Accordingly, the applied doses must be checked, above all, their SO_2 contents.

2. Definition

Free sulfur dioxide is that found in the forms H_2SO_3, HSO_3 and SO_3^{2-}.

Combined sulfur dioxide is found in all other forms.

Total sulfur dioxide is the sum of the free sulfur dioxide and the combined sulfur dioxide.

3. Principle[1]

[1] The CPIV has described another method (determination of the absorbance at 550 nm of the solution colored with products from the reaction between sulfur dioxide, formaldehyde and p-rosaniline). The

Free sulfur dioxide - Iodometric direct titling with subtraction of the other oxidizable substances by iodine.

Combined sulfur dioxide - iodometric titling after double alkaline hydrolysis of vinegar whose free sulfur dioxide has been oxidized during the previous determination.

Total sulfur dioxide - sum of the free sulfur dioxide content and the combined sulfur dioxide content.

4. Reagents

4.1. Sulfuric acid (p20 = 1.84 g/ml)

4.2. Sulfuric acid solution to 1/10 (1 + 9 of water by volume)

4.3. Solution of sodium hydroxide 4 M

4.4. Solution of iodine at 0.025 M

4.5. Disodic ethylene diamine tetracetate (EDTA.Na_2)

4.6. Starch solution at 5 g/l.

Dissolve 0.5 g of the soluble starch in a small amount of cold water in order to obtain a fluid base. Add to 100 ml of boiling water and maintain on the boil for 10 min. Allow to cool.

4.7. Ethanal solution at 7 g/l

Instituto da Vinha e do Vinho (Portugal) has developed a continuous flow method based on the same principle.

4.8. Propanal solution at 10 g/l.

5. Equipment and utensils

Standard laboratory equipment.

6. Technique

6.1. Free sulfur dioxide

In a 500 ml conical flask, add 50 ml of vinegar, 3 ml of sulfuric acid solution (4.2), 5 ml of starch solution (4.6) and 30 mg of EDTA Na_2 (4.5).

Title immediately with the iodine solution (4.4) until the blue coloring, first fleeting, becomes persistent for 10 to 15 s.

6.2. Combined sulfur dioxide

To the aforementioned conical flask (6.1), add the solution of sodium hydroxide (4.3) up to pH 11-12 (approximately 18 ml), stir and leave in contact for 5 min. Add, in one go and while shaking thoroughly, 17 ml of sulfuric acid solution (4.2).

Title immediately with the iodine solution (4.4).

Then add 20 ml of sodium hydroxide solution (4.3), stir and leave in contact for 5 min. Dilute with 200 ml of water, as cold as possible, stir thoroughly and add in one go, 30 ml of sulfuric acid solution (4.2). Title the liberated sulfur dioxide immediately using the iodine solution (4.4).

6.3. Interference of other substances

Some other substances may be oxidized by iodine in the acid environment; therefore, it is necessary to determine the quantity of iodine used up by such oxidization.

To do this, it is necessary to combine the free sulfur dioxide by excess ethanal or propanal before iodometric titling. To do this, add 50 ml of vinegar to an Erlenmeyer flask of 300 ml and add 5 ml of ethanal solution or 5 ml of propanal solution. Stop and allow to stand for at least 30 min. Add 3 ml of sulfuric acid solution (4.2) , 5 ml of starch solution (4.6) and the iodine solution (4.4) until a blue color is obtained.

7. Results

7.1. Calculation

Considering:

- **V** the volume in ml of the iodine solution used in 6.1
- $\mathbf{V_1}$ the volume in ml of the iodine solution used for the first titling of 6.2
- $\mathbf{V_2}$ the volume in ml of the iodine solution used for the second titling of 6.2
- $\mathbf{V_3}$ the volume in ml of the iodine solution used in 6.3

The total sulfur dioxide content expressed in milligrams of SO_2 per l of vinegar is:

$$\mathbf{32}\ (\boldsymbol{V + V_1 + V_2 - V_3})$$

7.2. Presentation

Round off the results expressed in milligrams of SO_2 per liter, to the nearest unit.

8. Characteristics of the method

8.1. Repeatability of the iodometry method for determining SO_2 in vinegar

Seven red wine vinegars and five white wine vinegars were analysed in duplicate in order to determine the repeatability parameters (table 1).

Table 1:Total SO2 content in different vinegars in mg/l

	Test 1	Test 2	Difference
Red wine vinegars	14	14	0
	23	27	-4
	64	61	3
	46	50	-4
	119	129	-10
	188	174	14
	38	37	1
White wine vinegars	61	65	-4
	85	85	0
	29	26	3
	96	91	5
	141	150	-9

Mean = 75.54 mg/l

Repeatability: standard deviation=4.4; r limit= 12.38 mg/l

Relative repeatability r = 15%

8.2. Recovery rate of added concentrations

Quantities of SO_2 were added to different vinegars in order to calculate the recovery rate of the iodometry determination method (table 2).

Table 2

Study of recovery rate of known concentrations added to different vinegars

Table 2: Study of recovery rate of known concentrations added to different vinegars

	Initial concentration (mg/l)	Added concentration (mg/l)	Concentration recovered (mg/l)	Recovery rate
Red wine vinegars	5	25	11	**44%**
	5	50	49	**98%**
	38	100	76	**76%**
	38	150	133	**89%**
White wine vinegars	26	25	25	**100%**
	26	50	47	**94%**
	0	100	66	**66%**
	0	150	118	**79%**

The recovery rate varies from 44% to 100%: occasionally it is too low, but is nonetheless more acceptable than the rate noted for the method of drying under a nitrogen stream, which sometimes produces excessive values.

9. Important remark

After studying the application of the reference method for the determination of sulphur dioxide described in the *Compendium of*

International Methods of Analysis of Wine and Must to vinegars, the results produced are unsatisfactory in terms of the recuperation rate of added SO_2 concentrations, which appears to be due to the very high concentration of acetic acid.

10. Bibliography

1. Curvelo-Garcia A.S. and Godinho M.C., 1986. Determinação analítica do dióxido de enxofre em vinagres. Optimização das condições operatórias, *Ciência e Técnica Vitivinícola*, **5**(1): 25 - 29.
2. FAO/OMS, Commission du Codex Alimentarius, 1982. Doc. CX/EURO 82/3, Partie II, Annexe I, Roma.
3. B. Medina: Dosage du SO_2 dans les vinaigres de vin - comparaison de deux méthodes Document OIV, CII-SCMA 03.2006-13.5 - FV 1236

Method OIV-MA-VI-10 : R2000

Type IV method

Determination of total ascorbic acid content in vinegars

(OENO 61/2000)

1. Introduction

In the wine vinegar industry, the technological use of ascorbic acid is regulated in the various producing and consuming countries. The application of this practice must be controlled and its presence quantified if possible.

2. Principle

Oxidization of ascorbic acid by iodine with transformation into dehydro ascorbic acid, precipitation of this acid by 2.4 - dinitrophenylhydrazine in the form of bis (2.4- dinitrophenylhydrazine). Separation by thin film chromatography, solubilization in acetic medium and colorimetric determination at 500 nm.

3. Reagents

3.1. Solution of metaphosphoric acid at approximately 30 g per 100 ml.

Weigh 30 g of vitreous previously titled metaphosphoric acid.

Wash rapidly while covering in water, then stir. Discard the washing water. Add to a 100 ml flask, dissolve with water, stir and bring up to the mark.

The solution will be kept for 1 week at most in the refrigerator.

3.2. Solution of metaphosphoric acid at 3% (v/v).

At the time of use, prepare a dilution of 3.1.

3.3. Metaphosphoric solution at 1% (v/v).

At the time of use, prepare a dilution of 3.1.

3.4. Polyamide suspension

Weigh 120 g of powdered polyamide for chromatography and add to a 250 ml conical flask. Add 60 ml of water, stir and leave in contact for 2 h (this quantity is enough for 4 determinations).

3.5. Thiourate.

3.6. Iodine solution 0.05 M.

3.7. Glacially cold acetic acid

3.8. Sulfuric acid (p20 = 1.84 g/ml).

3.9. Aceto sulfuric solution of 2.4 dinitrophenylhydrazine

Add to a 6 g flask, 2.4 dinitrophenylhydrazine and 50 ml of glacial acetic acid. A suspension will form. Add 50 ml of sulfuric acid to dissolve the 2.4 dinitrophenylhydrazine.

3.10. Ethyl acetate to which glacial acetic acid has been added (98+2 by volume).

3.11. Chloroform

3.12. Silicagel for chromatography

3.13. Soluble starch solution at 0.5 g/100 ml.

3.14. L-ascorbic acid standard solution

In a 100 ml calibrated flask, add 100 mg of L-ascorbic acid weighed to the nearest 0.1 mg. Dissolve with solution 3.3 and bring up to the gauge line.

3.15. Eluant

Ethyl acetate-chloroform-glacial acetic acid (50 : 60 : 5, v/v/v). Use only 12 h after preparation.

4. Equipment and utensils

Standard laboratory equipment including:

4.1. Development chamber for chromatography

4.2. Equipment appropriate for the preparation of slides

4.3. Glass slides for thin film chromatography, 20 x 20 cm, prepared as follows:

- In a 250 ml conical flask, add 30 g of silicagel, 70 ml of starch solution (3.13) and stir for 1 min. Spread the suspension on the plates to obtain a uniform film 0.3 mm in thickness. Dry the plates in air, then keep them in a dryer containing silicagel. Activate them before use, keeping them in an oven at 105°C for

1 h 30 min. The indicated quantities are sufficient for the preparation of 5 slides.

These sides are also available over the counter.

4.4. Spectrophotometer allowing readings at 500 nm with dishes having a 1 cm optical path.

4.5. 1200 rpm centrifuge, at the least, with 50 ml tubes and screw stoppers.

5. Preparation of sample

Homogenize the sample by stirring, then filter if necessary.

6. Technique

6.1. Oxidization of ascorbic acid

In a 100 ml calibrated phial, add a test sample of 50 ml and 15 ml of polyamide suspension and bring up to the mark with solution 3.2. Leave in contact for 1 h while stirring frequently. Filter with a pleated paper filter.

Add 20 ml of filtrate to a centrifugal tube. Add 1 ml of iodine solution (3.6) and shake after 1 min. Reduce the excess by adding approximately 25 mg of thiurate (3.5).

6.2. Forming an extraction of bis (2.4-dinitrophenylhydrazine)

Place the tube in a bath of water at a temperature included 5 and 10°C inclusive. Add 4 ml of the solution of 2.4 dinitrophenylhydrazine (3.9). Stop the tube and shake with care, taking care not to wet the stopper.

Keep the tube thoroughly stopped in a bath at 20°C for approximately 16 h.

Add 15 ml of solution 3.9. Stop the tube and shake thoroughly for 30 s. Then centrifugate for 5 min. at 1000 - 1200 rpm. Remove 10 ml of the surface solution and add to a conical flask with an emery stopper. Add to the tube 5 ml of solution 3.10, stir again for 30 s then centrifugate for 5 min. at 1000 - 1200 rpm. Remove 5 ml of the surface solution and add to a conical flask to the 10 ml of the initial extraction. Stir.

6.3. Separation of bis (2.4-dinitrophenylhydrazine) by thin film chromatography

Apply chromatographic separation for the 2 h following extractions on a chromatography slide, along a line 2 cm from the lower and side edges; apply 0.2 ml of the extraction solution obtained in 6.2. In a development chamber previously saturated with eluant (3.15), taking up approximately 1 cm of the height, insert the slide and allow the eluant to migrate to the upper edge. Remove the slide and dry for 1 h in a ventilated place.

Maintain the slide in a vertical position on a sheet of gloss non-porous paper, scrape with a spatula perpendicularly to the direction of migration in the reddish coloring zone characteristic of bis (2.4-dinitrophenylhydrazine), away from any drafts.

Transfer all of the powdered products obtained in a small weighing flask with an emery lid and add 4 ml of glacial acetic acid (3.7). Leave in contact for 20 min. while stirring frequently. Filter through a small filter paper folded directly into the bowl of the spectrophotometer and allow to pass the first 25 to 30 droplets of the filtrate through the filter, once again, to obtain total limpidity.

6.4. Reading of absorbants

Using the spectrophotometer, read the absorbance of the filtrate at a wavelength of 500 nm while using glacial acetic acid (3.7) as reference.

6.5. Calibration curve

Into 100 ml calibrated flasks, add respectively 5, 10 and 15 ml of solution 3.14 and bring each flask up to the line with solution 3.3. These solutions contain respectively, 50, 100 and 150 mg of L-ascorbic acid per L. Take a 50 ml sample of each solution instead of the specimen and, for each and successively, perform the operations described from 6.1 to 6.4.

Establish a calibration curve with the concentrations on the abscissas and the absorbances on the ordinates. The graph should be a straight line passing through the origin.

7. Results

7.1. Calculation

Determine the L-ascorbic acid content expressed in milligrams per L of vinegar on the calibration curve and as a function of the absorbance obtained in 6.4.

7.2. Presentation

Round off the results expressed in milligrams per L to the nearest unit.

8. Bibliography

1. AOF / WHO - Commission of Codex Alimentarius, Doc. OX/EURO 82/3, Part II, Rome (1982).

Method OIV-MA-VI-11 : R2000

Type IV method

Measurement of chloride content in vinegars

(OENO 62/2000)

1. Introduction

The main objective of the measurement of chloride content is the detection of the fraudulent increase in the dry extract by the addition of sodium chloride.

2. Principle[1]

Potentiometric titration of Cl^- ions with a solution of silver nitrate, in an acidic environment, after prior measurement of the potential equivalent point of a standard chloride solution.

3. Reagents

3.1. chloride standard solution

Weigh, to an accuracy of ± 0.0001 g, 2.1027 g of potassium chloride (Br content <0.005% Br) dried first for several days in a dessiccator with an appropriate desiccant.

[1]The Instituto da Vinha e do Vinho (Portugal) described a method by continuous flux, according to another principle (Green Leaflet of OIV 949).

Place the KCl into a 1 litre volumetric flask, dissolve in water and make up to the mark.

1 ml of this solution contains 1 mg of Cl^- ion.

3.2. silver nitrate standard solution

Weigh, to an accuracy of ± 0.0001 g, 4.7912 g of silver nitrate.

Place the $AgNO_3$ into a 1 litre volumetric flask, dissolve in a 10% (v/v) aqueous/alcoholic solution and make up to the mark.

1 ml of this solution corresponds to 1 mg of Cl^- ion.

3.3. nitric acid (r_{20} = 1.40 g/ml).

4. Devices and utensils

Standard laboratory material, including:

4.1. graduated potentiometer - 2 by 2 mV, at least.

4.2. Ag/AgCl electrode and mercury sulphate (I) electrode or a combined (Ag/AgCl + reference) electrode.

4.3. micro burette graduated in 0.01 ml.

5. Preparation of sample

Shake the sample to homogenize and filter if necessary.

6. Technique

6.1. Measurement of the potential of the equivalent point

Put 5 ml of standard chloride solution (3.1) into a 200 ml flask, placed on a magnetic

shaker.

Add 95 ml of water and 1 ml of nitric acid (3.3), introduce the electrodes into the flask and, with moderate shaking, add the silver nitrate solution (3.2), using a micro burette. Add the first 4 ml by 1ml parts and read the corresponding values of the potential difference in mV. Add 2 ml by 0.2 ml parts. Then, add fractions of 1 ml until a total volume of 10 ml is reached.

After every addition wait about 30 sec before making the corresponding reading of the potential difference in mV.

Graph on the ordinate axis the potential difference values found (in mV) and on the abscissa axis the corresponding volumes of the silver nitrate solution (in ml). Measure the potential of the equivalent point situated at the point of inflection of the curve.

6.2. Verification of the potential of the equivalent point

In a 200 ml flask, introduce 5 ml of chloride standard solution (3.1), 95 ml of water and 1 ml of nitric acid (3.3). Introduce the electrodes and titrate, while shaking, until the potential of the equivalent point is reached. Repeat this operation until the results are in concordance. This verification must take place before every set of chloride measurements is made on the samples.

6.3. Measurement of the potential of the equivalent point of the sample

In a 200 ml flask, introduce 50 ml of vinegar, 50 ml of water and 1 ml of nitric acid (3.3). Titrate as indicated in 6.2.

7. Results

7.1. Calculation

Taking V as the volume in ml of the silver nitrate solution as in 6.3, the chloride content (expressed as mg of Cl^- ion per L of vinegar) is given by:

$$20\,V^2$$

7.2. Presentation

Round results as mg of Cl^- ion per L to integer values.

8. Bibliography

1. Anonymous, 1993. *Métodos Oficiales de Anàlisis*, (Official Analytical Methods) Tomo II (Part II) Ministério de Agricultura, Pesca y Alimentación, (Ministry for Agriculture, Fishing and Food) Madrid, Spain.
2. FAO/OMS, Commission du Codex Alimentarius (Codex Alimentarius Commission) Doc. CX/EURO 82/3 Part II, Rome (1982).
3. Llaguno C. and Polo M.C., 1991. *El Vinagre de Vino* (The Wine Vinegar) Consejo Superior de Investigaciones Cientificas (High Council of Scientific Research) Madrid, Spain.

Method OIV-MA-VI-12 : R2000

Type IV method

Measurement of sulphate content in vinegars

(OENO 63/2000)

1. Introduction

The main objective of the measurement of the sulphate content in vinegar is, as for the measurement of the chloride content, the detection of frauds (aimed at increasing the total dry extract).

2. Principle

Precipitation of sulphates with barium chloride, drying, calcination and weighing.

3. Reagents

3.1. barium chloride solution ($BaCl_2$, $2H_2O$) at 1% (m/v)

3.2. hydrochloric acid solution M

3.3. silver nitrate solution 1M.

4. Devices and utensils

Standard laboratory material, including:

4.1. flat-bottom platinum crucible (85 mm in diameter)

4.2. filter paper for fine filtering of the precipitate, with an ash content of no more than 0.01%

4.3. water bath at 100 °C

4.4. muffle kiln

4.5. dessiccator

5. Preparation of the sample

Shake the sample to homogenize and filter if necessary.

6. Technique

In a 250 ml conical flask, introduce 100 ml of the sample. Add 2 ml of the hydrochloric acid solution (3.2) and heat to boiling point. Add, drop by drop, 10 ml of the barium chloride solution (3.1), maintain at boiling point for 5 minutes and add hot water to maintain a constant volume. Leave to rest for 10 to 12 hours.

Filter and wash the precipitate with hot water, until the washing waters are free from chlorides, as can be verified by the absence of a precipitate with a silver nitrate solution (3.3).

Carefully transfer the filter with its content into the platinum crucible, previously tared, and kiln in a muffle kiln at 700 °C - 800 °C.

Cool in the dessiccator and weigh.

7. Results

7.1. Calculation

Taking:

- m_1 = the mass, in grams, of the empty platinum crucible
- m_2 = the mass, in grams, of the platinum crucible containing the kilned residue (less the weight of the filter paper ash)

The sulphate content, expressed in grams of potassium sulphate per L of the sample, is given by the expression:

$$7.4651 \times (m_2 - m_1)$$

7.2. Presentation

Round results expressed in grams of potassium sulphate per L, to the first decimal place.

8. Bibliography

1. Anonymous, 1993. *Métodos Oficiales de Anàlisis*, (Official Analytical Methods) Tomo II (Part II) Ministério de Agricultura, Pesca y Alimentación, (Ministry for Agriculture, Fishing and Food) Madrid, Spain.
2. AOAC, *Official Methods of Ass. Offic. Agric. Chern.*, 14th edit., Arlington (1984).
3. Llaguno C. and Polo M.C., 1991. *El Vinagre de Vino* (The Wine Vinegar) Consejo Superior de Investigaciones Cientificas (High Council of Scientific

Method OIV-MA-VI-13 : R2000

Type II method

Measurement of the copper content in vinegars

(OENO 64/2000)

1. Introduction

The presence of copper in vinegars mainly has its origin in contaminations from contact materials during the different phases of their manufacture. Relatively high copper content can cause hazes or even serious alterations in the colour, hence the importance of this measurement.

2. Principle

Direct measurement by atomic absorption spectrophotometry.

3. Reagents

3.1. copper metal

3.2. concentrated nitric acid 65% (p_{20} = 1.38 g/ml).

3.3. diluted nitric acid 1:2 (v/v).

3.4. acetic acid solution 5% (v/v).

3.5. standard solution of copper 1.000 g per L.

Weigh 1.000 g of copper metal, and transfer it entirely into a 1000 ml volumetric flask. Add diluted nitric acid (3.3) in strictly sufficient quantity to dissolve the metal, then add 10 ml of concentrated nitric acid and make up to the mark with doubled distilled water.

This solution can be purchased ready-made.

3.6. standard solutions of copper, from 1 to 10 mg/L.

Prepare standard solutions of 1 to 10 mg/L by diluting the solution (3.5) with the acetic acid solution (3.4).

4. Devices and utensil

Standard laboratory material, plus:

4.1. atomic absorption spectrophotometer

4.2. copper ion hollow cathode lamp

5. Preparation of the sample

Shake the sample to homogenize and filter if necessary.

6. Technique

Select a 324.7 nm wavelength. Adjust the absorbence scale to zero with the acetic acid solution (3.4). Suck the vinegar sample directly into the burner of the spectrophotometer, then successively the standard solutions (3.6). Read the absorbencies. The measurements should be done at least twice.

7. Results

7.1. Calculation

Draw the absorbence variation curve according to the copper concentration of the standard solutions. Write down the absorbencies middle value of the vinegar sample and measure the copper content, expressed in milligrams per L of the sample.

7.2. Presentation

Round the results, expressed in milligrams of copper per L, to the first decimal place.

8. Remarks

For very low copper concentrations in the sample, and to improve the precision and exactness of the measurements, either the method of standard additions, or the method of prior ashing of the sample, should be used. Both are described by OIV (1990) for the measurement of copper content in wine.

Most devices give the concentration of samples directly.

9. Inter-laboratory validation (Hitos et al, 00)

Units: mg/L

Sample	r	S_r	RSD_r	R	S_R	RSD_R	RSD_R (Horwitz)	HORRAT Index
1 - 0.22 mg/L	0.0678	0.024	10.89	0.1905	0.068	30.61	20.10	1.52
2 - 0.03 mg/L	0.0210	0.008	21.82	0.0567	0.020	58.89	27.12	2.17
3 - 0.58 mg/L	0.0895	0.032	5.48	0.3187	0.114	5.48	17.37	0.32

4 - 0.11 mg/L	0.0493	0.018	16.77	0.1567	0.056	53.31	22.31	2.39
5 - 2.78 mg/L	0.2589	0.092	3.33	1.1809	0.422	15.17	13.72	1.11

10. Method of standard additions

10.1. Principle

Copper is measured in vinegar by flame atomic absorption spectrometry (method of standard additions).

10.2. Reagents

10.2.1. ultra pure demineralized water

10.2.2. 1 g/L standard copper solution, ready to use

10.2.3. nitric acid 65% *suprapur*

10.2.4. 10 mg/L copper solution: put 2 mL of the 1 g/L copper solution (10.2.2) in a 200 ml flask; add 1 ml of nitric acid (10.2.3); make up to volume with demineralized water (10.2.1).

10.2.5. to adjust the position of the burner of the spectrophotometer, prepare a standard of copper at 0.4 mg/L: 2 mL of the 10 mg/L copper solution (10.2.4) in a 50 mL volumetric flask; make up to volume with demineralized water (10.2.1).

10.3. Devices and utensils

10.3.1. Glassware

10.3.1.1. 50, 200 ml volumetric flasks (class A)

10.3.1.2. 2, 5 and 10 ml calibrated pipettes (class A)

10.3.1.3. 10 ml cylindrical flasks

10.3.1.4. 200 µl automatic micropipette

10.3.1.5. Atomic absorption spectrophotometer (air-acetylene oxidizing flame, 324.7 nm wavelength, width of the aperture: 0.5 nm, intensity of the hollow cathode lamp: 3.5 mA).

10.4. Preparation of samples

10.4.1. addition of 0.2 mg/L of copper: place 5 ml of vinegar in a cylindrical flask; add a 10 mg/L copper solution with the 100 µl micropipette.

10.4.2. addition of 0.4 mg/L of copper: place 5 ml of vinegar in a cylindrical flask; add a 10 mg/L copper solution with the 200 µl micropipette.

10.4.3. dilution of the vinegar: a dilution of vinegar with demineralized water is only necessary if the copper concentration is higher than 0.5 mg/L.

10.5. Measurements

Make two absorbance readings, each of 10 seconds, for, successively, the blank (demineralized water), vinegar with the added 0.2 mg/L of copper, vinegar with the added 0.4 mg/L of copper (4.2.), and vinegar as is, without addition of copper. The software draws the graph of the

standard additions and gives the vinegar's copper content directly in mg/L. The dilutions resulting from the additions are negligible.

10.6. Quality control

The measurements by flame atomic absorption spectrometry are made manually.

Quality control is achieved by regularly interposing a reference material every 5 measurements, or one after the standard solution, one in the middle and one at the end of the measurements.

A tolerance of two standard variations from the known value of the reference material is acceptable.

11. Bibliography

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2. EEC, 1990. Méthode de référence de dosage de cuivre dans le vin (Reference method for copper measurement in wine), *Journal Officiel des Communautés Européennes* (Official Newspaper of the European Communities), L272,: 145 - 146.
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Method OIV-MA-VI-14 : R2000

Type II method

Measurement of zinc content in vinegars

(OENO 65/2000)

1. Introduction

As for copper, the presence of zinc in vinegars mainly has its origin in contaminations from contact materials during their manufacture, and, likewise, excessive content could cause hazes or even serious alterations in the colour.

2. Principle

Direct measurement by atomic absorption spectrophotometry.

3. Reagents

3.1. zinc metal

3.2. concentrated nitric acid 65% (p_{20}= 1.38 g/ml).

3.3. diluted nitric acid at 1:2 (v/v).

3.4. diluted nitric acid at 1% (v/v).

3.5. acetic acid solution at 5% (v/v).

3.6. standard solution of zinc 1.000 g per L.

Weigh 1.000 g of zinc metal, and transfer it entirely into a 1000 ml volumetric flask. Add diluted nitric acid (3.3) in strictly sufficient quantity to dissolve the metal and make up to the mark with diluted nitric acid (3.4).

This solution can be purchased ready-made.

3.7. standard solutions of zinc, from 0.05 to 2.00 mg/L.

Prepare standard solutions of 0.05 to 2.00 mg/L, by diluting the solution (3.6) with the acetic acid solution (3.5).

4. Devices and utensils

Standard laboratory material, plus:

4.1. atomic absorption spectrophotometer

4.2. zinc ion hollow cathode lamp

5. Preparation of the sample

Shake the sample to homogenize and filter if necessary.

6. Technique

Select a 213.9 nm wavelength. Adjust the absorbence scale to zero with the acetic acid solution (3.5). Suck the vinegar sample directly into the burner of the spectrophotometer, then successively the standard solutions (3.7). Read the absorbencies. The measurements should be done at least twice.

7. Results

7.1. Calculation

Draw the absorbence variation curve according to the zinc concentration of the standard solutions. Write down the absorbencies middle value of the vinegar sample and measure the zinc content, expressed in milligrams per L of the sample.

7.2. Presentation

Round the results expressed in milligrams of zinc per L, to the first decimal place.

8. Inter-laboratory validation (Hitos et al., 00)

Units: mg/L

Sample	R	S_r	RSD_r	R	S_R	RSD_R	RSD_R (Horwitz)	HORRAT Index
1 - 0.39 mg/L	0.0390	0.014	3.55	0.2531	0.090	23.02	18.44	1.25
2 - 0.06 mg/L	0.0454	0.016	25.41	0.0932	0.033	52.22	24.44	2.14
3 - 0.16 mg/L	0.0618	0.022	14.13	0.1800	0.064	41.15	21.08	1.95
4 - 0.25 mg/L	0.0503	0.018	7.28	0.2183	0.078	31.61	19.71	1.60
5 - 0.63 mg/L	0.0689	0.025	3.92	0.4157	0.148	23.64	17.15	1.38

9. Bibliography

1. Anonymous, 1993. *Métodos Oficiales de Anàlisis*, (Official Analytical Methods) Tomo II (Part II) Ministério de Agricultura, Pesca y Alimentación, (Ministry for Agriculture, Fishing and Food) Madrid, Spain.

2. Hitos P., Pons A., Martin de la Hinojosa I., Gomez R., Hernandez A. and Muñoz J., 00. Validation des méthodes d'analyse pour l'acidité totale, fixe et volatile, les substances réductrices non volatiles, le cuivre et le zinc dans les vinaigres de vin (Validation of analytical methods for the total acidity, fixed and volatile, the non volatile reducing substances, the copper and zinc in wine vinegars), *Feuillet Vert de l'*OIV (OIV Green Leaflet) no 1115.
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Method OIV-MA-VI-15 : R2000

Type IV method

Measurement of iron content in vinegars

(OENO 66/2000)

1. Introduction

As for the other metals, the presence of iron in vinegars mainly has its origin in contaminations from contact materials during their manufacture, and of course the iron of the wine itself from which the vinegar has been made. Excessive content could cause hazes or even serious alterations in the colour.

2. Principle

Direct measurement of iron content in the vinegar by flame atomic absorption spectrometry, samples being tested with standard acetic solutions.

3. Reagents

3.1. ultra pure demineralized water

3.2. 90% acetic acid

3.3. ready-made standard iron solution at 1.000 g/l

3.4. iron solution at 100 mg/l: put 10 ml of 1 g/l iron solution (3.3.) in a 100 ml flask; make up to the mark with with demineralized water (3.1).

3.5. standard range: 0, 2, 4, 6, 8 mg of iron per litre

Put successively 0, 1, 2, 3, 4 ml of 100 mg/l iron solution (3.4.) in 4 50 ml flasks (2.1.1.); add 2.5 ml of acetic acid (3.2.); make up to the mark with with demineralized water (3.1).

4. Device and utensils

Standard laboratory material, including:

4.1. 50 and 100 ml volumetric flasks (class A)

4.2. 1, 2, 3, 4, 5, 10 ml calibrated pipettes (class A)

4.3. atomic absorption spectrophotometer

4.4. iron ion hollow cathode lamp

5. Preparation of samples

Shake the sample to homogenize and filter if necessary. In case of an > 8 mg/l iron content, sample should be diluted with an acetic acid solution at 5% (v/v).

6. Technique

6.1. test parameters (atomic absorption spectrometry)

6.1.1. oxidizing air-acetylene flame

6.1.2. 248.3 nm wavelength

6.1.3. width of the aperture: 0.2 nm

6.1.4. intensity of the hollow cathode lamp: 5 mA

6.2. measurements

Suck successively the standard solutions into the burner of the spectrophoto-meter. Read the absorbency for 10 s; make 2 measurements. Then suck the samples. Read the absorbencies. Take into account a possible dilution of the vinegar.

6.3. quality control

Flame atomic absorption spectrometry tests are usually performed manually. Quality control is achieved by regularly interposing a reference material every 5 measurements, or one after the standard solution, one in the middle and one at the end of the measurements.

7. Results

7.1. Calculation

Draw the absorbence variation curve according to the iron concentration made of the standard solutions. Write down the absorbencies middle value of the vinegar sample and measure the iron content, expressed in milligrams per L of the sample. In most devices the iron concentration in the samples is automatically calculated by the software.

7.2. Presentation

Round the results, expressed in milligrams of iron per L, to the first decimal place.

8. Bibliography

1. OIV, 1990. *Recueil des méthodes Internationales d'analyse des vins et des moûts* (Compilation of the international methods of analysis of wines and musts) OIV Paris, France.

Method OIV-MA-VI-16 : R2000

Type IV method

Measurement of lead content in vinegars

(OENO 67/2000)

1. Introduction

As for the other metals, the presence of lead in vinegars mainly has its origin in contaminations from contact materials during their manufacture, and of course the lead of the wine itself from which the vinegar has been made.

2. Principle

Direct measurement of lead content in the vinegar by atomic absorption spectrometry without flame, (electrothermal atomization).

3. Reagents

3.1. ultra pure demineralized water, for example: pre-purification through inverse osmosis; filter to eliminate all particles > 0.22 μm; control of the purified water resistivity (18.2 MΩ)

3.2. nitric acid at 65%: high quality acid

3.3. ammonium dihydrogen-phosphate ($NH_4H_2PO_4$)

3.4. ready-made standard lead solution at 1 g/L

3.5. tantalum powder: > 99.7 % purity

3.6. fluorhydric acid

3.7. anhydrous oxalic acid

3.8. hydrogen peroxide at 30%

4. Devices and utensils

Standard laboratory material, including:

4.1. atomic absorption spectrophotometer

4.2. lead ion hollow cathode lamp

4.3. 50 and 100 mL volumetric flasks (class A).

4.4. calibrated pipettes (class A).

Decontaminate the glassware: rinse with demineralized water, soak for at least 24 hours in a bath of nitric acid at 5% and rinse twice with demineralized water.

5. Preparation of the sample

Shake the sample to homogenize and filter if necessary.

6. Technique (example only using a particular type of equipment)

6.1. Test parameters (atomic absorption spectrometry)

6.1.1. 283.3 nm wavelength

6.1.2. width of the aperture: 0.5 nm

6.1.3. intensity of the hollow cathode lamp: 5 mA

6.1.4. base line correction by the Zeeman effect

6.1.5. introduction of standard solutions and samples when warm in the graphite oven with an automatic feeder (1 drop of Triton in 500 mL of rinsing water)

6.1.6. reading of signal: when highest

6.1.7. length of reading: 1 s.

6.1.8. number of readings per standard solution or sample: 2.

6.2. Pyrolytic graphite tube

6.2.1. pyrolytic graphite oven with a tantalum treated platform of L'Vov

6.2.2. tantalum treatment of a platform: put the platform inside a graphite or used pyrolytic graphite tube, and fit the whole lot onto the atomization unit of the spectrophotometer. Inject 10 µl of the tantalum solution on the platform with an automatic sample feeder. Set the temperature cycle as follows:

drying for 40 s at 100° C, mineralization for 60 s at 900° C, atomization at 2 for 2.5 s at 600° C. Use argon for inert gas.

6.2.3. oven parameters: as per Table 1.

The atomization of lead is performed without a mineralization procedure.

6.3. Preparation of solutions

6.3.1. tantalum solution at 6%: put 3 g of tantalum powder (3.5) in a 100 mL teflon cylindrical flask; add 10 mL of fluorhydric acid (3.6) diluted (1+1), 3 g of anhydrous oxalic acid (3.7) and 0.5 mL of hydrogen peroxide at 30% (3.8); heat carefully to dissolve the metal; add some hydrogen peroxide when the reaction slows down; when the dissolution is complete, add 4 g of oxalic acid (3.7) and approximately 30 mL of demineralized water (3.1); dissolve the acid and make to the mark to 50 mL; store this solution in a plastic flask.

Table I. Oven parameters

temperature (°C)	Duration (s)	gas flow (mL/min)	gas type	reading
150	10.0	3.0	argon	no
150	35.0	3.0	argon	no
150	1.5	0	argon	no
2250	1.1	0	argon	yes
2250	1.5	0	argon	yes
2600	1.5	3.0	argon	no
1250	10.0	3.0	argon	no

75	10.0	3.0	argon	no

6.3.2. matrix modifier ($NH_4H_2PO_4$ at 6%): put 3 g of ammonium dihydrogen-phosphate (3.3) in a 50 mL flask; dissolve and make up with demineralized water (3.1).

6.3.3. lead solution at 10 mg/L: put 1 mL of the 1 g/L lead solution (3.4) in a 100 mL flask; add 1 mL of nitric acid (3.2); make up to the mark with demineralized water (3.1). This solution keeps 1 month at +4° C in a polypropylene flask.

6.3.4. lead solution at 0.l mg/L (100 μg/L): put 1 mL of the 10 mg/L lead solution to (6.3) in a 100 mL flask; add 1 mL of nitric acid (3.2); make up to the mark with demineralized water (3.1). This solution keeps 2 days at +4° C in a polypropylene flask.

6.3.5. range of standard solutions: can be achieved using the automatic feeder cycle with the 100 μg/L lead solution (Table II). These standard lead solutions have the following final concentrations: 0; 16.6; 33.3; 50.0 and 66.6 μg of lead per litre.

6.4. Operating instructions

6.4.1. samples need not be prepared; they are put directly into the pots of the automatic device. The analytical blank is made up of demineralized water with 1% of nitric acid (3.2).

Table II. Automatic sampler parameters	
	injected volumes in μL

	100 µg/L solution	blank	matrix modifier
Blank		5	1
Standard sample n° 1	1	4	1
Standard sample n° 2	2	3	1
Standard sample n° 3	3	2	1
Standard sample n° 4	4	1	1
vinegar sample	2	3	1

6.4.2. measurements: the spectrophotometer software draws the standard sample graph (absorbance according to the lead concentration in the standard solutions) and gives the lead concentration reading of the samples (6.4.1) in µg/L.

6.4.3. results: multiply by 3 the reading given by the software of the device to deduct the lead concentration in the vinegar (2 µL of sample injected in the oven for an end volume of 6 µL). Take into account a possible dilution of the vinegar. The result is expressed in micrograms of lead by litre of vinegar.

7. Detection and quantification limits

7.1. detection limit: set according to a series of 20 analytical blank results of the white and is equal to 3 standard variations.

7.2. quantification limit: equal to 3 times the detection limit.

8. In house quality control

8.1. reference material: a vinegar sample with a carefully measured lead content should be used for quality control.

8.2. in-house quality control mode of use: the lead content of the reference material is measured directly after calibration. If the reading is within a satisfactory bracket (+/- 15% on average), go on with the analysis. Otherwise, the device should be calibrated again. A control quality sample is interposed every 5 pots.

8.3. graphite-oven measurements: some spectrophotometers are equipped with an automatic quality control software. 5 standard solutions, including blank, are used for calibration.

8.4. control card: a control card is drawn up for the reference material. Warning limits are +/-1.96.S_R and control limits +/- 2.58.S_R.

9. Bibliography

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Method OIV-MA-VI-17 : R2000

Type IV method

Measurement of mercury content in vinegars (cold vapour method)

(OENO 68/2000)

1. Principle

Vinegar mineralization: is performed in an acidic environment, through a reflux heating method; complete mineralization with potassium permanganate.

Reduce unused permanganate with hydroxylamine hydrochloride.

Reduce the mercury II to metal mercury with tin (II) chloride.

Drive mercury with an air or argon current, at room temperature.

Measure mercury in a monoatomic vapour state with atomic absorption spectrometry.

2. Reagents

2.1. Ultra pure demineralized water with a 18.2 MΩ resistivity

2.2. High purity 65% nitric acid

2.3. Sulphuric acid ρ20 = 1.84 mg/L

2.4. 5.6 M nitric acid solution: using a test-tube put 100 mL of nitric acid (2.2.) in a 250 mL flask; make up to the mark with demineralized water (2.1.).

2.5. 9 M sulphuric acid solution: using a test-tube put 100 mL of sulphuric acid (2.3.) in a 200 mL flask; cool, then make up to the mark with demineralized water (2.1.).

2.6. Potassium permanganate

2.7. Potassium permanganate solution at 5%: dissolve 10 g of $KMnO_4$ (2.6) in a 100 mL cylindrical flask with demineralized water (2.1.); transfer in a 200 mL flask and make up to the mark.

2.8. Hydroxylamine hydrochloride

2.9. Hydroxylamine hydrochloride solution at 1.5%: dissolve 1.5 g of NH_4OCl (2.8.) in a 100 mL cylindrical flask with demineralized water (2.1.); transfer in a 100 mL flask and make up to the mark.

2.10. Tin II chloride

2.11. Tin chloride solution:

2.11.1. Cold vapours in closed circuit method:

Tin II chloride solution at 10%: dissolve 5 g of $SnCl_2$ .2 H_20 (2.10.) in a 100 mL cylindrical flask with the addition of 5 ml of H_2SO_4 (2.5.) in demineralized water (2.1.); transfer in a 50 mL flask.

2.11.2. Cold vapours in continuous flow method:

25% $SnCl_2$ in 20% HCl: to prepare this tin (II) chloride solution, add concentrated hydrochloric acid directly onto the solid tin (II) chloride and heat on a hotplate to dissolve, then add demineralized water (2.1).

2.12. Mother mercury solution at 1 g/L

2.13. Mercury solution at 10 mg/L: put 1 mL of the mother solution (2.12) in a 100 mL flask; make up to the mark with demineralized water (2.1.).

2.14. Mercury solution at 0.1 mg/L (or 100 µg/L): put 1 mL of the diluted solution (2.13.) in a 100 mL flask; make up to the mark with demineralized water (2.1.).

3. Devices and utensils

3.1. Glassware:

3.1.1. 50, 100, 200, 250 mL volumetric flasks (class A)

3.1.2. 1, 2, 3, 4, 5, 20, 50 mL calibrated pipettes (class A)

3.1.3. 5, 10 mLs calibrated pipettes (class A)

3.1.4. 100 mL calibrated test-tube

3.1.5. 100 mL cylindrical flask.

3.2. Circulation system of the mercury vapours in closed circuit:

3.2.1. Reaction tube

3.2.2. Mineralized sample

3.2.3. Tin (II) chloride

3.2.4. Air compressor

3.2.5. Mercury trap ($KMnO_4$)

3.2.6. Absorption cell

3.2.7. Drier ($CaCl_2$)

3.3. Circulation system of the mercury vapours in continuous flow

3.3.1. Argon

3.3.2. Flow controller

3.3.3. Reducing agent: 25% $SnCl_2$ in 20% HCl

3.3.4. Demineralized water

3.3.5. Mineralized sample

3.3.6. Peristaltic pump

3.3.7. Reagents mixing

3.3.8. To the sink

3.3.9. Gas / liquid separator

3.3.10. To the spectrophotometer

3.4. Atomic absorption spectrophotometer

3.4.1. 253.3 nm wavelength

3.4.2. Width of the aperture: 0.5 nm

3.4.3. Intensity of the lamp: 2.5 mA

3.4.4. Baseline correction with a deuterium lamp

4. Preparation of samples

4.1. Mineralization of vinegars: is performed in a pyrex-glass equipment made of three elements linked with spherical "rodages": a 250 mL flask, a recovery chamber with a cooling device on top.

4.1.1. Water circulation

4.1.2. Cooling device with balls

4.1.3. Reflux

4.1.4. Flask heater

4.1.5. Vinegar + H_2SO_4 + HNO_3

Put 20 mL of vinegar in a 250 mL flask with a pipette; assemble the mineralization equipment. Add very slowly 5 mL of H_2SO_4 (2.3.) and 10 mL of $HN0_3$ (2.4.). Heat slowly with reflux. Recover the condensed vapours in the reaction flask. Leave to cool; rinse with demineralized water (2.1.). Add some $KMn0_4$ (2.7.) until the colour is lasting. Make the $Mn0_2$ precipitate soluble with NH_40CI (2.9.). Transfer the solution in 100 mL flask and make up to the mark with demineralized water. Make a blank without vinegar.

5. Measurements

5.1. Range of standard solutions:

5.1.1. Cold vapours in closed circuit method:

- Range of standard solutions: 0; 0.1; 0.2; 0.3; 0.4; 05 µg of mercury.
- Put successively 1, 2, 3, 4, 5 mL of the mercury solution at 100 µg/L (2.14.) in the reaction tube; add two drops of $KMnO_4$ (2.7.), 2.5 ml of HNO_3 (2.4.), 2.5 mL of H_2S0_4 (2.5.) and 2.5 mL of NH_40CI (2.9.); make up to a constant volume (70 ml) with demineralized water (2.1.).
- Add 2.5 mL of $SnCl_2$ (2.11.); start immediately the bubbling process. Read the maximal optical densities. Then trap the mercury in $KMn0_4$ (2.7.).

5.1.2. Cold vapours in continuous flow method:

- Range of standard solutions: 0; 2.5; 5; 10 μg/L of mercury.
- Put in 4 100 mL flasks: 0; 2.5; 5; 10 mL of the 100 μg/L diluted mercury solution (2.14.); make up to the mark with demineralized water. The peristaltic pump will suck the reducing solution (25% $SnCl_2$ in 20% HCl), the demineralized water and the standard solution.

5.2. samples:

5.2.1. Cold vapours in closed circuit method:

- Transfer the mineralized sample to the reaction tube with a 50 mL pipette. Make up to a 70 mL constant volume with demineralized water (2.1.). Add 2.5 mL of $SnCl_2$ (2.11.); start immediately the bubbling process. Read the maximal optical densities. Then trap the mercury in the permanganate (2.7.).

5.2.2. Cold vapours in continuous flow method:

- The mineralized sample is sucked by the peristaltic pump together with the tin (II) chloride and the water.

5.3. Results:

5.3.1. Cold vapours in closed circuit method:

- 50 mL of mineralized sample are equivalent to 10 mL of vinegar.
- The concentration given by the software of the device should therefore be multiplied by 100 to have the mercury content of the vinegar in μg/L.

5.3.2. Cold vapours in continuous flow method:

- 20 mL of mineralized vinegar are collected in a 100 mL flask. The software of the device gives the result in μg/L; therefore, to have the mercury content of the vinegar in μg/L, one should multiply the result by 5.

6. Quality control

Quality control is achieved by regularly interposing a reference material of in-house quality control every 10 samples, or, at the most, one after the standard solution, one in the middle and one at the end of the measurements.

A tolerance of two standard variations of the known value is accepted.

7. Bibliography

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2. Varian, 1986. *Spectra* AA-10/20. *Operation Manual.*
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Method OIV-MA-VI-18 : R2000

Type IV method

Measurement of the acetoin content in vinegars

(OENO 69/2000)

1. Introduction

Acetoin ($CH_3COCHOHCH_3$) is always present in wines and in vinegars. According to the bibliography its content in wines is of the order of 10 mg/L. In vinegars, contents can vary with the manufacturing technology between 100 mg/L and over 400 mg/L.

The acetoin content in the wine vinegars is an important reference factor for quality and origin.

2. Principle

Neutralization of the sample at pH 7.00 with calcium hydroxide. Direct measurement of the acetoin via gas chromatography.

3. Reagents

3.1. Purified acetoin. Eliminate any diacetyl via distillation.

3.2. Acetoin reference solutions: dilute acetoin (3.1) with water to prepare 10 to 500 mg/L reference solutions.

3.3. Pentan-1-ol (in-house standard solution)

3.4. Ethanol

3.5. In-house standard solution sample: in a 100 ml volumetric flask, dissolve 2 ml of pentan-1-ol in an aqueous-alcoholic solution at 50%. Make up to the mark with this solution.

3.6. Calcium hydroxide

4. Devices and utensils

Standard laboratory material, plus:

4.1. Gas chromatograph with a flame ionization detector.

4.2. Column for gas chromatography 2 m long and 1/8" in diameter: FFAP 2.5% on G Chromosorb (HP), with the addition of 0.5% of 1500 Carbowax (or any other system able to perform an acceptable separation of acetoin).

5. Technique

Add some in-house standard solution (3.5) to the acetoin reference solutions (3.2), in sufficient quantity for these solutions to have, per L, 15 or 35 μL of pentan-1-ol (according to their acetoin content, that is respectively < or > 50 mg/L).

Neutralize the sample at pH ≡ 7.00 by addition of calcium hydroxide (solid). Add enough of the in-house standard solution (3.5), for the

solution to have, per L, 15 or 35 µL of pentan-1-ol (according to the acetoin content).

Inject into the chromatograph 2 µL of the neutralized sample, the reference solutions, and the in-house standard solution. Temperature of the oven is 70° C, output of the vector gas (nitrogen) is 12.5 ml/min. Temperature of the detector is 180° C.

6. Results

6.1. Calculation

Taking:

- A_1 the surface of the peak of the acetoin in the reference solution 1
- P_1 the surface of the peak of pentan-1-ol in the reference solution 1
- A_X the surface of the peak of the acetoin in the solution to be measured
- P_X the surface of the peak of pentan-1-ol in the solution to be measured

Calculate the ratios A_1 / P_1 for the various reference solutions.

Draw two curves to express graphically these ratios according to the acetoin content of the reference solutions (0 to 50 mg/L and 50 to 500 mg/L).

The acetoin content of the sample, expressed in mg/L, is shown by the ratio A_x/P_x.

6.2. Presentation

Round results as mg per L to integer values.

7. Bibliography

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Method OIV-MA-VI-19 : R2000

Type IV method

Measurement of methanol, superior alcohols and ethyl acetate in vinegars

(OENO 70/2000)

1. Introduction

The method described here allows to measure, at the same time, several major volatile components of vinegars. The interest of this measurement is two-fold, organoleptic and possibly toxicologic.

2. Principle

Neutralization of the sample at pH 7.00 with a sodium hydroxide solution.

Measurement, via gas chromatography, of some volatile components:

- Methanol
- propan-1-ol
- butan-2-ol
- 2-methylpropan-1-ol
- butan-1-ol and 2-methylbutan-1-ol + 3-methylbutan-1-ol.

3. Reagents

3.1. Aqueous-alcoholic solution at 5% v/v.

3.2. In-house standard solution (4-methylpentan-2-ol).

In a 1 L volumetric flask, dissolve 1110.0 mg (to an accuracy of 0.1 mg) of 4-methylpentan2-ol into the aqueous-alcoholic solution (3.1). Make up to the mark with this solution.

3.3. Reference solution

In a 1 L volumetric flask, dissolve, in the aqueous-alcoholic solution (3.1), 152.0 mg of ethyl acetate, 50.0 mg of methanol, 7.7 mg of propan-1-ol, 16.3 mg of butan-2-ol, 17.0 mg of 2-methylpropan-1-ol, 2.5 mg of butan-1-ol, 7.9 mg of 2-methylbutan-1-ol and 8.7 mg of 3-methylbutan-1-ol (to the accuracy of 0.1 mg). Make up to the mark with the solution (3.1).

3.4. Reference solution added from the in-house standard solution. Add 1 ml of the solution (3.2) to 10 ml of the solution (3.3).

3.5. Sodium hydroxide solution at 40% (m/v).

4. Devices and utensils

Standard laboratory material, plus:

4.1. Gas chromatograph with a 'split' type injector and a flame ionization detector

4.2. Supelcowax 10 glass column, 30 m long and 0.75 mm internal diameter (as an example).

5. Technique

Neutralize the sample at pH ≡ 7.00 with the sodium hydroxide solution (3.5), and record the initial and final volumes.

Add 1 ml of the neutralized sample solution at 10 ml (3.2).

Inject 1 µL of each of these two solutions into the chromatograph. Temperatures of the injector and the detector are 250° C. Oven temperatures are: 6 mn at 50° C, 50° C to 70° C to 8° C/mn, 14 mn at 70° C, 70° C to 210° C to 8° C/mn and 16 mn at 210° C. The output of the vector gas (hydrogen) is 10 ml/mn.

6. Results

6.1. Calculation

Taking:

- c_i the component 1 content, in mg/L, in the reference solution (3.4)
- c_e the in-house standard solution content, in mg/L, in the reference solution (3.4)
- s_i the surface of the peak of component 1 of the reference solution (3.4)
- s_e the surface of the peak of the in-house standard solution in the reference solution (3.4)
- S_i the surface of the peak of component 1 in the neutralized sample solution plus the in-house standard solution
- S_e the surface of the peak of the in-house standard solution in the neutralized sample solution plus the in-house standard solution
- C_e the in-house standard solution content, in mg/L, in the neutralized sample solution plus the in-house standard solution

- f the dilution factor resulting from the neutralization of the vinegar sample.

The component i content C_i, expressed in milligrams per L of vinegar, is given by:

$$C_i = \frac{c_i s_e s_i c_e}{c_e s_i s_e} f$$

6.2. Presentation

Round results to the integer value.

7. Bibliography

1. Climaco, M.C., *Estudo de um método de doseamento de compostos voláteis em vinagres por cromatografia em fase gasosa*, Relatório dactil., Estação Vitivinícola Nacional, Dois Portos (1993). (Study of a measurement method for volatile components in vinegars with gaseous chromatography).

Method OIV-MA-VI-20 : R2000

Type IV method

Authentification via fine – NMR® and other isotopic methods in vinegars

(OENO 71/2000)

1. Objective and principle

The main objective for this procedure is to detect and measure synthetic acetic acid in vinegars and also detect any other downgrading of vinegars.

Addition of synthetic acetic acid and other downgrading can be traced because the deuterium concentration of the -CH_3 of this acid is thereby altered.

The method includes the following steps:

- measurement of the acetic acid concentration in the vinegar
- extraction of the acetic acid from the vinegar with ether
- measurement of the percentage of residual water in the purified acetic acid
- measurement of the deuterium in the resulting acetic acid, via NMR.

In order to detect the possible addition of alcohol-vinegar coming from plants whose metabolism is C4, one has also to determine other isotopic parameters, such as the acetic acid $^{13}C/^{12}C$ ratio or the water(D/H), using IRMS (*Isotope Ratio Mass Spectrometry*).

Optical isomers of acetic acid and water which can be detected by this method are:

I	CH_2D-COOH
II	CH_3-COOD
III	HOD

2. Reagents

2.1. Reagents for water measurement using the Karl Fischer method

2.2. N,N- tetramethylurea (TMU); use a TMU reference sample with a given and controlled isotopic ratio

2.3. Trifluoracetic anhydride $(CF_3\text{-}CO)_2O$

2.4. Ethylic ether (HPLC quality)

2.5. Nitrogen gas

3. Devices and utensils

Standard laboratory material, plus:

3.1. Equipment for extracting acetic acid, including:

- a liquid-liquid extractor, with an electric flask-heater and a condenser
- a Cadiot column with rotating strip (mobile, in teflon)
- 125 ml conical flasks with "rodage"
- 250 ml double-"rodage" flask

3.2. Karl Fischer device

3.3. NMR equipment:

- NMR spectrometer with:
- a specific "deuterium" probe set at v_0 frequency characteristic of B_0 field (for example, for B_0 = 9.4 T, v_0 = 61.4 MHz),
- a proton (B_2) decoupler, and a field-frequency stabilizer (*lock*) set at the fluorine frequency.
- automatic sample changer (if necessary)
- data processing software
- 10 mm diameter sample tubes

The resolution measured on the spectrum, transformed without exponential multiplication (LB = 0), and expressed by the width at mid-height of the methyl signal of the TMU, must be lower than 0.7 Hz. The sensitiveness, measured with an exponential multiplication factor LB of 2, must be higher than or equal to 150 for the acetic acid methyl signal of the extract, with a titration of 85% m/m, minimum, of acetic acid.

4. Preparation of the sample

4.1. Extraction of acetic acid in vinegar

Put 350 ml of the vinegar sample into the liquid-liquid extractor. Fill 2/3rd of the 250 ml flask of the extractor with ethylic ether; add a few grains of pumice stone. Heat for 5 hours and bring to boiling point. Collect 100 ml of residual water for analysis of $(D/H)_{water}$ (SMRI).

4.2. Separation of ether from acetic acid

Put the organic phase into the 250 ml double-"rodage" flask (with the pumice stone), and place under the Cadiot column. Heat to bring the ether to boiling point. Install a "rodée" 125 ml conical flask to collect the distillate, collect the boiling liquid between 32° C and 40° C. When the temperature goes over 45° C, stop collecting until the temperature comes back down to 40° C. Collect again the distillate up to 45° C. Repeat this procedure until the temperature, after stopping the collection and operating in closed circuit, doesn't come down again anymore.

Heat to 85° C - 90° C. Introduce the needle into the 250 ml flask, to let nitrogen in. Collect the boiling liquid until 95° C, and stop collecting. Repeat this procedure until temperature goes quickly over 95° C.

The whole distillation process lasts about 4 hours. Then let the flask containing the acetic acid and the water cool down. Transfer to a 100 ml flask.

4.3. Measurement of the percentage of water

From a trial sample close to 0.2 ml of acetic acid solution, of p' mass precisely known, the water content is measured by the Karl Fischer method, that is, p in grams.

The acetic acid mass title is given by:

$$t = 100(p' - p)/p'$$

4.4. Preparation of the sample for the NMR analyses

NMR probe of 10 mm in diameter:

- In a flask which has been tared first, place 1.2 ml of the in-house sample solution (TMU) and weigh to an accuracy of 0.1 mg (m_{st}); then add 3.3 ml of the acetic acid from the distillation procedure and weigh to an accuracy of 0.1 mg (M_A). Add 250 µL of trifluoro-acetic anhydride, to be used as field-frequency

stabilizer (*lock*). Shake to homogenize the compounded solution.

5. Measurement of isotopic parameters

5.1. Adjustments

Perform the usual homogeneity and sensitivity adjustments according to the NMR manufacturer's instructions.

The standard variation of repetitiveness over an average of 10 trials for each spectrum must be inferior to 0.8 ppm on $(D/H)_{CH3}$.

5.2. Conditions of acquisition of the NMR spectra

Put the acetic acid sample prepared in 4.4, while filtering it, in a 10 mm tube and introduce it into the probe.

The conditions of acquisition of the NMR spectra are as follows:

- temperature of the probe (for example, 302 K) must be constant
- acquisition time: 6.8 s at least for 1200 Hz of spectral width (memory 16 K), that is: about 20 ppm at 61.4 MHz or 27 ppm at 46.1 MHz
- impulse: 90°
- adjust the time frame for the acquisition; this value must be close to the sampling time (*dwell time*)
- detection in quadrature: adjust the *offset* 01 between the TMU OD signal and CH_2D signal
- determine the value of the 0_2 decoupling *offset* from the protonic spectrum measured by the decoupling spool on the same tube. Use the large strip decoupling method.

Perform, for each spectrum, a sufficient number of NS accumulations to reach the signal/noise ratio indicated in the specifications of the NMR equipment. Repeat NE = 10 times this NS accumulations series. The NS values will depend on the types of spectrometer and probe used. The values could be, for example:

Spectrometer	10 mm Probe
7.05 T	NS = 304
9.4 T	NS = 200
11.7 T	NS = 104

6. Expression of the results

For each of the 10 spectra, determine:

$$(D/H)_{CH3} = 2.0661 - T - m_{st}/m_A - (D/H)_{st}/t$$

with:

- T = surface of the acetic acid CH_3 signal / surface of the TMU CH_3 signal
- t = purity of the acetic acid
- m_{st} and m_A = mass of the in-house standard solution and the acetic acid (see 4.4)
- $(D/H)_{st}$ = isotopic ratio of the in-house standard solution (TMU)

For $(D/H)_{CH3}$, calculate the average for the 10 measurements and the confidence interval.

The advice is to use the EUROSPEC software with the EUROLIS module in order to get the best results, under as for precision and repetitiveness.

7. Bibliography

1. EUROFINS, Authentification of vinegars SNIF-NMR® by and other isotopic methods, Laboratoires EUROFINS Nantes (1994).
2. OIV, 1990. Recueil des méthodes Internationales d'analyse des vins et des moûts (Compilation of the international methods of analysis of wines and musts) OIV Paris, France.

Method OIV-MA-VI-21 : R2006

Type IV method

Detection of synthetic acid in wine vinegars by measuring the β emissions of ^{14}C in the acetic acid using liquid scintillation

(OENO 12/2006)

1. Introduction

The concentration of the ^{14}C contained in natural vinegars is closely related to the year of production of the corresponding wines.

Levels which are lower than those for a given year indicate that synthetic acetic acid has been added or makes up the entire content.

2. Principle

The acetic acid is first extracted from the vinegar using diethyl ether then mixed with the Scintillation Fluid (SF) and "counted" for 400 minutes using liquid scintillation. Acetic acid of mineral origin (Control) is counted in the same way.

The result of the "net count", obtained by subtracting the reading for the Control from that of the sample represents the β emission value of the ^{14}C in the sample which, after taking statistical corrections into account, is then compared with the average value of the β emissions of ^{14}C found in the ethanol in genuine late harvest wines.

3. Reagents

3.1. Sodium hydroxide pellets

3.2. 40% sodium hydroxide solution

3.3. Pure diethyl ether for analysis.

3.4. Sulphuric acid 10 M

3.5. Anhydrous sodium sulphate

3.6. Decolourising charcoal

3.7. Sodium hydroxide 0.1M

3.8. Scintilling mixture – example : Canberra Packard PicoFluor LLT (SF)

3.9. Hexadecane ^{14}C (Activity $\cong 1 \times 10^6$ dpm/gC)

3.10. Nitromethane

3.11. Synthetic acetic acid

3.12. Phenolphthalein:1% (m/v) solution in 95% vol. alcohol.

4. Equipment

4.1. Hood

4.2. Rotary vacuum evaporator

4.3. Nitrogen bottle with a regulator valve

4.4. Liquid scintillation spectrometer

4.5. Polyethylene counting flasks with plastic lids that can be hermetically sealed

4.6. Laboratory glassware

4.7. Ultrasound bath

5. Preparation of the sample

Homogenise the sample by shaking.

6. Procedure

6.1. Extraction of acetic acid

In a 2 l flask, place 450 ml of vinegar with a total acidity of 60 g/l in acetic acid (or the corresponding quantity if the vinegar presents a different total acidity); add (A×0.1×18) g of Sodium hydroxide pellets (A = number of ml of Sodium hydroxide 0.1M needed to neutralise 1.0 ml of vinegar) and if necessary, a few ml of 40% (3.2) sodium hydroxide solution to bring

the pH to 9-10. Using the rotary evaporator, distil a volume of about 300 ml under vacuum at 50°C until the residue has reached a syrupy consistency and discard the distillate.

After cooling, transfer the residue to a 1 l stoppered flask and extract using diethyl ether (100 ml) in an ultrasound bath.

Transfer to a separatory funnel, separate the phases and discard the ether phase.

Repeat the extraction in an ultrasound bath, using a further 100 ml ether.

Transfer to a separatory funnel, separate the phases and discard the ether phase.

To the aqueous residue, add 25-30 ml sulphuric acid 10 M (pH 2-3) and extract using ether (3 aliquots of 100 ml), for 5 minutes in an ultrasound bath.

Join the ether phases, dry the phases with anhydrous sodium sulphate (approx. 2 g) (3.5), add decolourising charcoal (approx. 3 g) and filter with folded filter paper.

Evaporate the ether at 40°C and remove the last traces by bubbling cold nitrogen through. The acetic acid obtained, which has a slight yellow coloration, is dripped through a column filled with decolourising charcoal. (1,5 g).

The acetic acid obtained, approx. 15 ml, has a concentration ranging from 84 to 87%.

6.2. Assay of the concentration

In a 20 ml graduated flask, use a pipette to drop 2 ml of the extracted acetic acid and add water up to the mark.

Take 1 ml of the diluted solution and titrate using Sodium hydroxide 0.1M (3.7) in the presence of phenolphthalein (3.12).

Calculate the concentration of acetic acid as an average percentage over 4 measurements, using the equation(1)

$$Acetic\ acid\% = a \times M \times 60$$

Where the volume of Sodium hydroxide 0.1M used in titration; M is the molarity of Sodium hydroxide (herein 0.1) and 60 is the weight of an equivalent of acetic acid.

7. Spectrometric measurements

7.1. Quenching curve

7.1.1. Standard solution

In a graduated flask, dissolve an exactly weighed quantity of hexadecane ^{14}C in the scintillation fluid so that the final solution has an activity of approx. 6000 dpm/ml: Standard Solution (S)

7.1.2. Preparation of the vials for the Quenching curve

Prepare 10 vials containing 16.5 ml scintillation fluid and 1 ml standard solution.

Count each vial for 20 minutes using the ET method (Efficiency Tracing) in order to exactly determine the activity.

Choose the 5 vials that have the most comparable activity and add to each vial the quantity of nitromethane indicated in the following table:

flask	Nitromethane µl	Quench level	Activity added
1	0.0	0.0	A_{aj}
2	20.0	+	A_{aj}

3	40.0	++	A_{aj}
4	80.0	+++	A_{aj}
5	100.0	++++	A_{aj}

7.1.3. Counting

Put the flasks in the instrument and count under the following conditions:

Counting time:	10 minutes
Cycles:	4
Nuclide:	Manual
Counting window:	Low Level (LL) = 1.5 kev; Upper Level (UL)= 29 kev
Removal of background noise:	No
Counting method:	Cpm
Automatic correction of "quenching":	No

Calculate the average value of the activity measured A_m (Cpm) and calculate the percentage efficiency (E%) for each vial in relation to the activity added A_{aj} (Dpm):

$$E\% = A_m \times 100/A_{aj}$$

On a diagram, plot the efficiency values calculated (E%) and the corresponding quenching parameters given by the apparatus during the counting and calculate the equation of the curve.

The curve represents all the possible quenching situations which can occur during the course of a measurement for each E% value.

It is therefore possible to calculate the efficiency (E%) of a vial by introducing into the curve equation the quenching parameter given by the apparatus.

8. Counting of the sample

8.1. Preparation of the measurement vials

- In a vial (measurement vial) put 4.5 ml of extracted acetic acid and 17.5 ml of scintillation fluid (measuring flask).
- To determine the noise of each counting flask, prepare a flask with 4.5 ml of synthetic acetic acid and 17.5 ml of scintillating mixture (control flask).
- Put the two flasks in the apparatus and count for 400 minutes (100 minutes for 4 cycles). Cpm_{ef} and Cpm_{fe} are respectively obtained.

9. Results

9.1. Calculating the activity of the sample

The beta emission value of the ^{14}C in the sample (A_{sp}) is calculated from the following:

$$A_{sp} = Cpm_e - Cpm_f) \times 100/E\% \times gC$$

10. Presentation

The beta emission value of the ^{14}C (A_{sp}) is expressed in disintegrations per minute per gramme of carbon (Dpm/gC) or in centiBequerel per gramme of carbon (cBq/gC) and rounded down to one decimal place.

11. Interpretation of the results

The result obtained, taking into account statistical corrections, is compared with the average value of the β emissions of ^{14}C found in the ethanol in genuine late harvest wines.

It should be pointed out, however, that to obtain a more accurate interpretation of the result, the measurement of the vinegar and that of the ethanol should be carried out by the same laboratory and using the same equipment.

12. Characteristics of the method

12.1. Internal validation procedure

Due to the difficulty in finding a suitable number of laboratories which owned the equipment necessary for taking part in the validation checks as set out in the procedures required by the OIV, an internal procedure has been prepared and implemented, as set out in Resolution OENO 8/2005;

12.1.1. Calculating the measurement uncertainty

12.1.2. Procedure:

10 samples of vinegar, 6 from the factory and 6 from sellers were extracted and counted twice.

The standard deviation and uncertainty were calculated on all the counting parameters which contribute to obtaining the final result.

The repeatability and repeatability uncertainty of the Quenching Curve (or Efficiency Curve) were also calculated.

The results are shown in Table 1.

Detection of synthetic acetic acid in wine vinegars by measuring the β emissions of ^{14}C in the acetic acid using liquid scintillation

Table 1

Sample	Cpm B	Gross Cpm	Net Cpm	tSIE	Eff%	Eff	EffCurve	G-H	(G-H)^2	Dpm	gC	Dpm /gC
A1	10.10	28.85	18.75	194.08	77.61	0.77609	0.78177	-0.00568	0.00003	24.16	1.67	14.47
A1	10.25	27.83	17.58	206.76	78.11	0.78112	0.79318	-0.01206	0.00015	22.51	1.54	14.64
A2	10.32	27.52	17.20	217.12	78.43	0.78428	0.80251	-0.01823	0.00033	21.93	1.57	14.01
A2	10.38	28.13	17.75	217.27	78.43	0.78431	0.80264	-0.01833	0.00034	22.63	1.62	13.96
A3	10.21	29.09	18.88	205.99	78.09	0.78085	0.79249	-0.01164	0.00014	24.18	1.62	14.93
A3	10.40	26.67	16.27	217.71	78.44	0.78443	0.80304	-0.01861	0.00035	20.74	1.48	14.01
A4	10.10	27.31	17.21	227.04	78.65	0.78649	0.81144	-0.02494	0.00062	21.88	1.54	14.21
A4	10.21	27.74	17.53	204.88	78.05	0.78046	0.79149	-0.01104	0.00012	22.46	1.62	13.86
A5	10.52	27.7	17.18	195.99	77.69	0.77693	0.78349	-0.00656	0.00004	22.11	1.56	14.17
A5	10.18	27.33	17.15	211.84	78.28	0.78278	0.79776	-0.01498	0.00022	21.91	1.54	14.23
A6	10.33	29.19	18.86	202.26	77.95	0.77948	0.78913	-0.00965	0.00009	24.20	1.66	14.58
A6	10.24	28.31	18.07	210.16	78.23	0.78225	0.79624	-0.01399	0.00020	23.10	1.61	14.35
A7(*)	9.95	27.82	17.87	198.27	77.79	0.77790	0.78554	-0.00765	0.00006	22.97	1.63	14.09

A7(*)	1.01	27.35	17.34	214.25	78.35	0.78349	0.79993	-0.01644	0.00027	22.13	1.59	13.92
A8(*)	1.35	27.63	17.28	204.1	78.02	0.78017	0.79079	-0.01062	0.00011	22.15	1.54	14.38
A8(*)	10.12	26.76	16.64	197.63	77.76	0.77763	0.78497	-0.00734	0.00005	21.40	1.48	14.46
A9(*)	10.18	28.54	18.36	211.14	78.26	0.78256	0.79713	-0.01457	0.00021	23.46	1.58	14.89
A9(*)	9.87	28.53	18.66	202.84	77.97	0.77970	0.78966	-0.00995	0.00010	23.93	1.62	14.77
A10(*)	10.21	28.67	18.46	189.48	77.39	0.77395	0.77763	-0.00368	0.00001	23.85	1.62	14.76
A10(*)	10.15	28.3	18.15	189.46	77.39	0.77394	0.77761	-0.00367	0.00001	23.45	1.67	14.04

(*) Commercial Samples

- Cpm B = Background (disintegration per minute of synthetic acid)
- Gross Cpm = Total number of Counts
- Net Cpm = Gross Cpm - Cpm B
- tSIE = Quench Parameter
- Eff = Efficiency

The efficiency of the count (E) is calculated using equation (1)

$$E = ax^2 + bx + c$$

Where x is a quench parameter (expressed as a tSIE) which is automatically calculated by the instrument.

Given the tSIE values obtained in a significant number of measurements (several hundred) fell within a very narrow range, it was decided to set

the quenching curve as a straight line, represented therefore by equation (2)

$$E = ax + b$$

The efficiency values, which were very similar when calculated using each of the two equations, proved the accuracy of the method adopted.

The following statistical parameters were calculated using the samples from Table 1.

Deviation=	0.04
Repeatability (r) =	0.65
Uncertainty (U) =	0.53 dpm/gC

12.2. Detection Limit (D.L.) and Quantification Limit (Q.L.)

The Detection Limit and the Quantification Limit were calculated by preparing in double 6 vials of synthetic acetic acid and then performing the count.

The results obtained are summarised in Table 2:

Table 2

Cpm B	Average	St Dev	D.L. (Cpm)	Q.L. (Cpm)
10.48	10.60	0.14	0.43	1.40
10.62				
10.45				
10.87				
10.64				
10.65				
10.83				

10.61				
10.45				
10.65				
10.41				
10.57				

The following D.L. and Q.L. values were calculated on the basis of the counts in Table 2:

D.L= 0.43

Q.L= 1.40

12.3. Instrument Consistency

In order to check the consistency of the instruments, 6 vials of biogenic acetic acid were prepared, containing known variable quantities of between 0% and 80% of synthetic acetic acid.

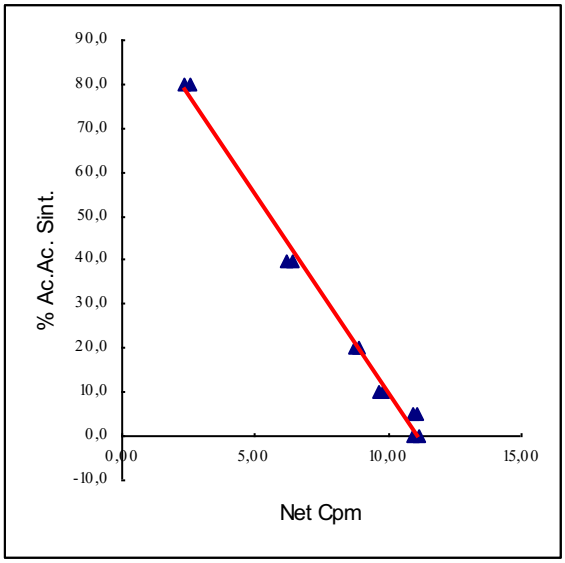
The results of the count in Net Cpm are shown in Table 3

Table 3

%AcH (synth.)	Net Cpm
0.0	10.94
0.0	11.17
5.0	10.96
5.0	11.07
10.0	9.67

10.0	9.81
20.0	8.77
20.0	8.88
40.0	6.20
40.0	6.39
80.0	2.57
80.0	2.33

Fig 1. shows the curve obtained when the values from Table 3 are represented in graph form.



Graph key: Ac.Ac.Sint = synthetic acetic acid

If the Q.L value calculated in 13.1 is introduced into the equation of the straight line in Fig. 1, the % of synthetic acetic acid which can be quantifies with certainty in the example in Table 3 = 13%.

Percentage values of acetic acid ≥ 13 % can therefore be determined in a predictable manner.

Method OIV-MA-VI-22 : R2013

Type II method

Method for $^{13}C/^{12}C$ isotope ratio determination of acetic acid in wine vinegars by isotopic mass spectrometry

(OIV-OENO 510-2013)

1. Object/Scope of application

The method enables the determination of the $^{13}C/^{12}C$ isotope ratio of acetic acid extracted from wine vinegar by isotope ratio mass spectrometry (IRMS).

2. Definitions

$^{13}C/^{12}C$: Isotope ratio of carbon 13 to carbon 12 in a given sample

$\delta^{13}C$: Isotope ratio of carbon 13 (^{13}C) to carbon 12 (^{12}C) expressed in parts per 1000 (‰)

VPDB: Vienna-Pee-Dee Belemnite, or PDB, is the main reference for measuring natural variations in the carbon isotope ratio. Calcium carbonate comes from a cretaceous belemnite from the Pee Dee formation in South Carolina (USA). Its $^{13}C/^{12}C$ isotope ratio or R_{PDB} is R_{PDB}= 0.0112372. PDB reserves have been exhausted for a long time, but it remains the main reference for expressing natural variations in the carbon isotope ratio. Reference materials are calibrated based on PDB and are available at the International Agency of Atomic Energy (IAEA) in Vienna (Austria). The isotopic determinations of naturally occurring carbon are expressed in terms of VPDB, as is standard practice.

m/z: Mass/charge ratio

3. Principle

The $^{13}C/^{12}C$ isotope ratio is determined by the CO_2 resulting from the total combustion of acetic acid extracted from the vinegar sample. An elemental analyser is generally used for the combustion, coupled with a spectrometer to determine the isotope ratios.

4. Reagents and products

The materials and the consumables depend on the apparatus (6) used by the laboratory. The systems used for the combustion of the sample are generally based on elemental analysers. These systems can be equipped to introduce 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 following reference materials, reagents and consumables can be used:

4.1. Reference materials available from the IAEA:

Name	Material	$\delta^{13}C$ according to VPDB
IAEA-CH-6	saccharose	-10.4 ‰
IAEA-CH-7	polyethylene	-32.2 ‰
NBS22	oil	-30.0 ‰
USGS24	graphite	-16.1 ‰

4.2. Working solution

4.2.1. Carbon dioxide (or carbonic anhydride) as a secondary reference gas for the measurement (CAS N° 00124-38-9).

4.2.2. Working and control solution with $\delta^{13}C$ values calibrated according to international reference materials.

4.3. Consumables

A standard list of consumables used in continuous flow systems is as follows:

- helium for analysis (CAS N° 07440-59-7),
- oxygen for analysis (CAS N° 07782-44-7),
- carbon dioxide for analysis, used as a secondary reference gas for the carbon 13 content (CAS N° 00124-38-9).
- oxidation reagent for the oven and the combustion system, such as copper oxide (II) for elemental analysis (CAS N° 1317-38-0),
- drying agent to eliminate water produced by combustion, such as Anhydrone for elemental analysis (magnesium perchlorate) (CAS N° 10034-81-8).

This is not necessary for apparatus equipped with a water-elimination system by cryo-trapping or through selective permeable capillaries.

5. Apparatus

5.1. Isotope ratio mass spectrometry

Isotope ratio mass spectrometry enables the determination of the relative content of 13C with respect to that of 12C in CO2 gas with an internal precision of 0.05‰. Internal precision is defined here as the difference between two measurements of the same sample of CO2.

The mass spectrometer used to determine 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 $^{13}C^{17}O^{16}O$)

When measuring the corresponding intensity, the $^{13}C/^{12}C$ isotope ratio is determined by the m/z=45 and m/z=44 intensity ratios after conducting a series of corrections for the $^{12}C^{17}O^{16}O$ isobaric series, whose contribution may be calculated from the intensity of the current measured by m/z=46 and from the relative abundance of ^{18}O and ^{17}O (Craig correction).

The isotope ratio mass spectrometer must either be equipped with:

- a double entry system (double input system) to alternately measure the sample and a reference standard,
- or a continuous flow system that quantitatively transfers CO_2 resulting from the combustion of the samples and of the working solution into the mass spectrometer.

5.2. Combustion apparatus

Combustion apparatus capable of quantitatively converting acetic acid into carbon dioxide and of eliminating all other combustion products, including water, without any isotopic fractionation. The apparatus can either be an integrated continuous flow system coupled to the mass

spectrometer (6.2.1) or an autonomous combustion system (6.2.2). The apparatus must be as precise as indicated in (11).

5.2.1. Continuous flow system

These consist of an elemental analyser, or of a gas chromatograph equipped with an online combustion system.

The following laboratory materials are used for systems equipped for the introduction of samples contained in metallic capsules:

- volumetric micropipette with appropriate cones,
- analytical balance with 1 μg precision or better,
- pliers for encapsulation,
- tin capsules for liquid samples,
- tin capsules for solid samples.

The following laboratory materials are needed when using an elemental analyser equipped with a liquid injector or when using a gas chromatograph:

- syringe for liquids,
- flasks equipped with a seal-closure system and inert septa.

The laboratory materials indicated are listed as examples and may be replaced with other materials of equivalent performance, depending on the type of combustion and spectrometry apparatus used by the laboratory.

5.2.2. Manual preparation systems

The carbon dioxide samples to be analysed, resulting from the combustion of samples, and the reference samples are collected in bulbs which are then put in a double entry spectrometry system to carry out

isotopic analysis. Several combustion apparatus, which are described in various written works, can be used:

- combustion system with circulating oxygen gas,
- elemental analyser with helium and oxygen flow,
- sealed glass bulb filled with copper oxide (II) used as an oxidation agent.

6. Preparation of samples

Acetic acid must be extracted from vinegar and purified in order to be analysed by IRMS. At least 6 mL of pure acetic acid must be recovered at the end of the extraction.

To extract and purify the acetic acid, any method that does not involve isotopic fractionation may be used. The following method (including the reagents used) and Cadiot column in Figure 1 are given as an example.

6.1. Principle

The acetic acid from vinegar is first extracted with diethyl ether. It is then purified by distillation.

6.2. Reagents

- Diethyl ether (purity ≥99.8)

6.3. Laboratory apparatus

- Liquid–liquid extractor,
- Cadiot-type extraction column,
- round-bottom flask,
- condenser,

- heating mantle.

6.4. Experimental determinations

6.4.1. Liquid–liquid extraction

Pour around 125 mL of diethyl ether into a 250 mL round-bottom flask. Use a 400 mL to 800 mL liquid–liquid extractor; depending on the acetic acid content of the vinegar (at least 6 mL of pure acetic acid must have been recovered at the end of the extraction).

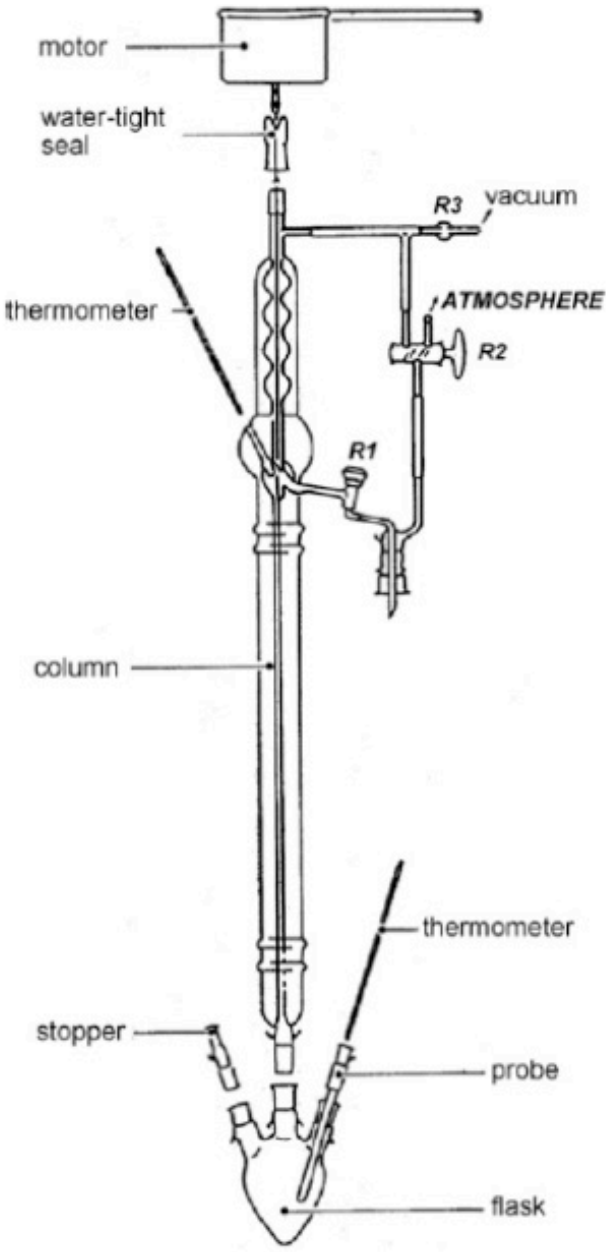
Pour the vinegar into the extractor and top up with diethyl ether. Place the round-bottom flask in the heating mantle, connect the extractor and open the water for the condenser, which is located in the upper part of the extractor. The extraction must last at least 5 hours.

After this time period, separate the aqueous and organic solutions. Add the organic solution to the extract in the round bottom flask.

6.4.2. Purification of the extract

The acetic acid contained in the round bottom flask in diethyl ether solution is distilled on a column which avoids isotopic fractionation of acetic acid, as shown in Fig. 1.

Fig. 1: Diagram of the distillation device (from Thomas and Jamin, 2009)


motor
water-tight seal
R3
vacuum
thermometer
ATMOSPHERE
R2
R1
column
thermometer
stopper
probe
flask

An appropriate 250 mL flask is used to collect the distillate.

Open the water for the condenser and switch the heating mantle on. Take care to ensure that the temperature is moderate during the distillation of the diethyl ether (which has a boiling point of 34 °C).

When most of the diethyl ether has been distilled (there is no more vapour at the top of the column), increase the temperature.

The distillation is complete when the internal temperature at the top of the column is stable at about 90°C (pure acetic acid distils at 116-117°C).

The remaining traces of diethyl ether in the acetic acid must be removed by blowing N_2 for 10 minutes on the residue at room temperature.

7. Procedure

All preparation steps must be carried out without any significant acetic acid loss through evaporation, which would change the isotopic composition of the sample.

The following description makes reference to the procedures generally used for acetic acid sample combustion using commercial automatic combustion systems. Any other method may be used, provided that it ensures that samples are converted quantitatively into carbon dioxide without losses by evaporation

7.1. Placing the samples in capsules

- Use capsules, tweezers and a clean preparation tray,
- take an appropriate-size capsule using tweezers,
- introduce the necessary amount of liquid into the capsule using a micropipette.

Note: The appropriate quantity of sample must be calculated according to the quantity of carbon necessary, depending on the mass spectrometry instrument sensitivity.

- Close the capsule using pliers,
- each capsule must be completely sealed. If not, it must be discarded and a new capsule must be prepared,
- two capsules must be prepared for every sample,
- place the capsules in an appropriate place on the samples drum of the elemental analyser. Every capsule must be clearly numbered in order,
- systematically place capsules containing the working solution at the beginning and at the end of the sample series,
- regularly insert a control sample in between the sample series.

7.2. Checking and adjusting the elemental analysis and mass spectrometry instruments

- Adjust the temperature of the elemental analyser ovens and the helium and oxygen gas flows for an optimal combustion of the sample,
- check the elemental analysis system and the mass spectrometry system for leaks (for example, by checking the ion current for m/z = 28 corresponding to N_2.).
- adjust the mass spectrometer to measure the intensities of ion currents for m/z = 44, 45 and 46,
- check the system using reference standards before starting to measure the samples.

7.3. Carrying out a series of measurements

The samples are successively introduced into the elemental analyser. The carbon dioxide resulting from the combustion of each sample is transferred to the mass spectrometer, which measures the ion current. The computer, which is connected to the apparatus, records the ion-current intensities and calculates the δ values for each sample (9).

8. Calculations

The objective of the method is to measure the $^{13}C/^{12}C$ isotope ratio of acetic acid extracted from vinegar. The $^{13}C/^{12}C$ isotope ratio is expressed as the deviation in relation to a working solution. The carbon ($\delta^{13}C$) isotope ratio is calculated on a delta scale per thousand by comparing the results obtained for the sample to those obtained for the working solution calibrated beforehand, based on the primary international reference (VPDB). The $\delta^{13}C$ values are expressed compared to the working solution:

$$\delta^{13}C_{sam/ref}\% = 1000 \times (R_{sam} - R_{ref})/R_{ref}$$

where R_{sam} and R_{ref} are, respectively, the $^{13}C/^{12}C$ isotope ratio of the sample and of the internal standard.

The values $\delta^{13}C$ are thus expressed according to VPDB:

$$\delta^{13}C_{sam/VPDB}\% = \delta^{13}C_{sam/ref} + \delta^{13}C_{ref/VPDB} + (\delta^{13}C_{sam/ref} \times \delta^{13}C_{ref/VPDB})/1000$$

where $\delta^{13}C_{ref/VPDB}$ is the isotope value determined beforehand for the working solution to VPDB.

Small variations may occur while measuring online due to changes in the instrumental conditions. In this case, the $\delta^{13}C$ of the samples must be corrected according to the difference between the $\delta^{13}C$ value from the working solution and its real value, which must be calibrated beforehand against VPDB by comparison with one of the international reference

materials. The correction that must be applied to the samples varies in a linear fashion according to the value of the two working solutions which precede and follow the samples. The working reference 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 value assigned to the working solution and the obtained measurements).

9. Quality control of the analyses

Check that the ^{13}C value of the control standard fits the acceptancy criteria defined by the control card of the document OIV-MA-AS1-11. If not, the spectrometer instrument adjustments must be checked and possibly readjusted.

For each sample, verify that the difference in the result between the two capsules measured successively is under 0.3‰. The final result for a given sample is the average value for the two capsules. If the deviation is higher than 0.3‰, the measurement should be repeated.

Measurement condition monitoring can be based on the ion-current intensity for $m/z = 44$ and is proportional to the quantity of carbon injected in the elemental analyser. Under standard conditions, the ion-current intensity should be almost constant for the samples analysed. A significant deviation could be indicative of acetic acid evaporation (an imperfect seal on a capsule), or of instability of the elemental analyser or the mass spectrometer.

10. Precision

An international collaborative study was carried out (Thomas and Jamin, 2009) for validation of the method.

The repeatability limit (r) is, on average, 0.51‰.

The reproducibility limit (R) is, on average, 0.93‰.

The data and results of the international collaborative study are presented in Annex A.

11. Bibliography

1. OIV-MA-AS1-08: Reliability of analytical results (Resolution 5/99)
2. OIV-MA-AS1-09: Protocol for the design, conducts and interpretation of collaborative studies (Resolution 6/2000)
3. OIV-MA-AS1-11: Harmonised guidelines for internal quality control in analytical chemistry laboratories
4. OIV-MA-AS312-06: R2009 Determination by isotope ratio mass spectrometry $^{13}C/^{12}C$ of wine ethanol or that obtained through the fermentation of musts, concentrated musts or grape sugar.
5. IUPAC Protocol in W. Horwitz, *Pure Appl. Chem*. 67 (2) (1995) 331
6. Thomas, F., Jamin, E. (2009). (2)H NMR and (13)C-IRMS analyses of acetic acid from vinegar, (18)O-IRMS analysis of water in vinegar: International collaborative study report. *Analytica Chimica Acta*, 649, 98-105.

Annex A: Results of the international collaborative study

This document sets out the results of the study for the validation of the method of analysis of the $^{13}C/^{12}C$ ratio in acetic acid extracted from wine vinegar (Thomas and Jamin 2009).

The study was carried out in accordance with documents OIV-MA-AS1-08 and OIV-MA-AS1-09.

1. Participating laboratories

14 laboratories participated in the study:

Bundesinstitut für Risikobewertung, Berlin	Germany
Central Science Laboratory, York	United Kingdom
Custom Technical Laboratory, Prague	Czech Republic
Chemical Institute of the Hungarian Customs and Finance Guard, Budapest	Hungary
Eurofins, Nantes	France
FEM-IASMA, San Michele all'Adige, Trento	Italy
Joint Research Center, Ispra	Italy
Arbitral Agroalimentario Del MAPA, Madrid	Spain
Landesuntersuchungsamt, Speyer	Germany
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Wurzburg	Germany
Service Commun des Laboratoires, Bordeaux	France
Service Commun des Laboratoires, Montpellier	France
Service Commun des Laboratoires, Paris	France
Unione Italiana Vini, Verona	Italy

2. Samples

The study was carried out on 5 double blind samples: three wine-vinegar samples (B, C, D), an alcohol-vinegar sample (A) and a wine-vinegar sample with 20% of A (Thomas and Jamin, 2009).

For each sample the acetic acid was extracted and the $\delta^{13}C$ was analysed. The results are indicated in the following table.

3. Statistical evaluation

Statistical calculations were performed according to the OIV-MA-AS1-09 protocol, which is based on Horwitz' IUPAC protocol. Outliers were removed in the following way: Cochran tests for removal of the laboratories with the highest variance; single and pair value Grubbs tests for individual or paired individual outliers; then more Cochran tests, etc., keeping a proportion of outliers <2/9. The standard deviations of repeatability (sr) and of reproducibility (sR) were then calculated for each material from valid results pairs from the blind duplicates.

For more information, refer to the publication by Thomas and Jamin, 2009.

Description of the sample	N° of valid results	N° of replicates	$\delta^{13}C$ average (‰)	s_r (‰)	Repeatability limit r (2.8×S_r) ‰	s_R (‰)	Reproducibility limit R (2,8× SR) ‰
A	10	2	-29.52	-0.14	0.39	0.34	0.95
B	11	2	-26.66	0.11	0.31	0.25	0.70
C	10	2	-27.51	0.20	0.56	0.33	0.92
B+20%A	11	2	-27.15	0.14	0.39	0.29	0.81
D	7	2	-27.19	0.33	0.92	0.46	1.29

The average repeatability limit (*r*) was 0.51‰, and the average reproducibility limit (*R*) was 0.93‰, comparable to the values observed for wine alcohol (OIV-MA-AS312-06).

Method OIV-MA-VI-23 : R2013

Type II method

Method for $^{18}O/^{16}O$ isotope ratio determination of water in wine vinegar using isotopic mass spectrometry

(OIV-OENO 511-2013)

1. Object/Scope of application

The method enables the determination of the $^{18}O/^{16}O$ isotope ratio of water in wine vinegar after equilibration with CO_2, using isotope ratio mass spectrometry (IRMS).

2. Definitions

$^{18}O/^{16}O$:Isotope ratio of oxygen 18 to oxygen 16 in a given sample.

$\delta^{18}O_{V\text{-}SMOW}$:Relative scale for the expression of the isotope ratio of oxygen 18 to oxygen 16 in a given sample. $\delta^{18}O_{V\text{-}SMOW}$ is calculated according to the following equation:

$$\delta^{18}O_{V-SMOW} = \left[\frac{(\frac{^{18}O}{^{16}O})_{sample} - (\frac{^{18}O}{^{16}O})_{standard}}{(\frac{^{18}O}{^{16}O})_{standard}}\right] \times 1000\ [,,]$$

using the V-SMOW standard as a reference 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: U.S. National Institute of Standards & Technology
- RM: Reference Material
- 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. Principle

The technique described below is based on the isotopic equilibration of water in samples of wine vinegar with a CO_2 gas standard, 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 to analyse the $^{18}O/^{16}O$ isotopic ratio by means of Isotopic Ratio Mass Spectrometry (IRMS).

4. Reagents and materials

The materials and consumables depend on the method used (see point 6).

The following reference materials, working solutions and consumables can be used:

4.1. Reference materials

Name	Issuing organisation	$\delta^{18}O$ according to 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 ‰

4.2. Working solutions

4.2.1. Carbon dioxide (or carbonic anhydride) as a secondary reference gas for measurement (CAS N° 00124-38-9).

4.2.2. Carbon dioxide used for equilibration (depending on the instrument this gas could be the same as that which is listed in 5.2.1 or, in the case of cylinders with continuous flow systems, a helium-carbon dioxide mixture can also be used).

4.2.3. Working solutions with $\delta^{18}O_{V\text{-}SMOW}$ values calibrated according to international reference materials.

4.3. Consumables

Helium for analysis (CAS N° 07440-59-7)

5. Apparatus

5.1. Isotope ratio mass spectrometer

The Isotope ratio mass spectrometer (IRMS) enables the determination of the relative contents of $^{18}0$ and ^{16}O in CO_2 gas with an internal precision of 0.05‰. Internal precision is defined here as the difference between two measurements of the same sample of CO_2.

The mass spectrometer designed for the determination of the isotopic composition of CO_2 gas is generally equipped with a triple collector capable of simultaneously measuring 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 $^{13}C^{17}O^{16}O$)

When measuring the corresponding intensities, the $^{18}O/^{16}O$ isotopic ratio is determined from the m/z = 46 and m/z = 44 intensity ratios after conducting a series of corrections for the $^{12}C^{17}O^{17}O$ and $^{13}C^{17}O^{16}O$ isobaric species, whose contributions can be calculated from the actual intensity observed for m/z= 45 and from the usual isotopic abundances for ^{13}C and ^{17}O in nature.

The isotope ratio mass spectrometer must either be equipped with:

- a double entry system (dual inlet system) to alternately measure the sample and a reference sample,
- or a continuous flow system that transfers quantitatively the CO_2 from the sample vials after equilibration and the CO_2 standard gas into the mass spectrometer.

5.2. Equipment and materials

All equipment and materials used must satisfy the stated requirements of the method or of the apparatus used (as specified by the

manufacturer). However, other equipment and materials of equivalent performance can also be used.

5.3. Vials with septa appropriate for the system used

5.4. Volumetric pipettes with appropriate tips

5.5. Temperature control system to carry out the isotopic exchange reaction at a constant temperature (typically within $\pm$1°C)

5.6. Vacuum pump (if needed for the system used)

5.7. Autosampler (if needed for the system used)

5.8. Syringes for sampling (if needed for the system used)

5.9. GC Column to separate CO_2 from other elementary gases (if needed for the system used)

5.10. Water removal device (e.g. cryo-trap or selective permeable membranes)

6. Preparation of the samples

Wine vinegar samples as well as reference materials are used for analysis without any pre-treatment. If the sample contains impurities, it should be filtered with a 0.22 μm pore diameter filter.

Preferably, the reference materials used for calibration and data correction should be placed at the beginning and at the end of each series and inserted after every ten samples.

7. Procedure

The procedure generally used for the determination of the $^{18}O/^{16}O$ isotope ratio by means of equilibration of water with a CO_2 working solution and subsequent analysis by IRMS is presented below. These procedures can be altered according to the apparatus and equipment used, given that various kinds of equilibration devices with different functions are available. The two main technical procedures for introduction of CO_2 into the IRMS provide a dual inlet system and a continuous flow system respectively.

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 or determined experimentally.

7.1. Manual equilibration

A defined volume of the sample/working solution 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 (manifolds equipped with capillary opening tubes do not require this freezing phase). Subsequently, the whole system is evacuated.

After reaching a stable vacuum, the CO_2 resulting from the working solution in the round bottom flasks is distributed. For the equilibration process, each manifold is placed in a water-bath at 25°C ($\pm$ 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 complete, 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 the isotope ratios of the CO_2 contained in the sample side with those of the CO_2 contained in the working solution side (gas

reference standard: CO_2) of the dual inlet. This approach is repeated till the last sample of the sequence has been measured.

7.2. Use of automatic equilibration apparatus

A defined volume of the sample/working solution is transferred into a round bottom flask using a pipette. The flasks containing the sample are attached to the equilibration apparatus 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 CO_2 resulting from the working solution in the round bottom flasks is distributed. Equilibrium is reached at a temperature of 22 $\pm$ 1°C after a minimum period of 5 hours with moderate stirring (if possible). Since the equilibration duration depends on various parameters (e.g. the flask geometry, temperature, amount of stirring), the minimum equilibrium time required should be determined experimentally.

After the equilibration process is complete, the resulting CO_2 is transferred from the flasks to the sample -side bellows of the dual inlet system. Analysis is carried out by comparing the isotope ratios of the CO_2 contained in the sample side with those of the CO_2 contained in the working-solution side of the dual inlet. This approach is repeated till the last sample of the sequence has been measured.

7.3. Manual preparation and automatic equilibration and analysis using a dual inlet system coupled to a continuous flow IRMS

A defined volume of sample/working solution (e.g. 200 μL) is transferred to 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 thermal tray of the autosampler. The equilibration is reached after at least 8 hours at 40°C. Once the process of equilibration is complete, the CO_2 obtained is dried and then transferred into the sample side of the dual inlet system. The measurements are performed by comparing the isotope ratios of the CO_2 contained in the sample side and the working solution side (CO_2 reference standard gas) of the dual inlet several times. This process is repeated until the last sample of the sequence has been measured.

7.4. Use of automatic equilibration apparatus by means of a continuous flow system

A defined volume of the sample/working solution is transferred into a vial using a pipette. The sample vials are placed into a temperature controlled tray.

A mixture of He and CO_2 is added to the vials using a gas syringe. The CO_2 remains in the top of the vials for equilibration.

Equilibrium is reached at a typical temperature of 30 ± 1°C after a minimum period of 18 hours.

After the equilibration process is complete, the resulting CO_2 is transferred by means of the continuous flow system into the mass spectrometer. The CO_2 gas standard is also introduced into the IRMS by

means of the continuous flow system. The measurement is carried out according to the specific protocol for each kind of equipment.

8. Calculations

The intensities for m/z = 44, 45, 46 are recorded for each sample and reference material analysed in a series of measurements. The $^{18}O/^{16}O$ isotope ratios are then calculated by a computer, using the IRMS apparatus' software, according to the principles explained in point 6.1. In practice, the $^{18}O/^{16}O$ isotope ratios are measured against a working solution previously calibrated against the V-SMOW. Changes in the instrumental conditions while measuring on line may entail small variations. If this occurs, the $\delta\,^{18}O$ of the samples must be corrected according to the difference in the $\delta\,^{18}O$ value from the working solution and its assigned value, which was calibrated beforehand against V-SMOW. The correction that is applied to the samples varies in a linear fashion with respect to the values of the two working solutions that precede and follow the samples. Indeed, the working solution must be measured at the beginning and at the end of all sample series. A correction can therefore be calculated for each sample using linear interpolation between two values (the difference between the assigned value and the measured value of the working solution).

The final results are presented as $\delta^{18}O_{V\text{-}SMOW}$ values expressed in ‰.

$\delta^{18}O_{V\text{-}SMOW}$ values are calculated according to the following equation:

$$\delta^{18}O_{V-SMOW} = \left[\frac{(\frac{^{18}O}{^{16}O})_{sample} - (\frac{^{18}O}{^{16}O})_{standard}}{(\frac{^{18}O}{^{16}O})_{standard}}\right] \times 1000\ [,,]$$

The $\delta^{18}O$ value normalized with respect to the V-SMOW/SLAP scale is calculated according to 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 point 5.1).

9. Validation of the method

An international collaborative study was carried out (Thomas and Jamin, 2009) for validation of the method.

The repeatability limit (r) is, on average, 0.15‰.

The reproducibility limit (R) is, on average, 0.54‰.

The data and results of the international collaborative study are presented in Annex A.

10. Bibliograph

1. OIV-MA-AS1-08: Reliability of analytical results (Resolution 5/99)
2. OIV-MA-AS1-09: Protocol for the design, conducts and interpretation of collaborative studies (Resolution 6/2000)
3. OIV-MA-AS2-12: Method for $^{18}O/^{16}O$ isotope ratio determination of water in wines and must
4. IUPAC Protocol in W. Horwitz, *Pure Appl. Chem*. 67 (2) (1995) 331
5. Thomas, F., Jamin, E. (2009). (2)H NMR and (13)C-IRMS analyses of acetic acid from vinegar, (18)O-IRMS analysis of water in vinegar: International collaborative study report. *Analytica Chimica Acta*, 649, 98-105

Annex A: Results of the international collaborative study

This document sets out the results of the study for the validation of the method of analysis of the $^{18}O/^{16}O$ ratio of water in wine vinegar (Thomas and Jamin 2009).

The study was carried out in accordance with documents OIV-MA-AS1-08 and OIV-MA-AS1-09.

1. Participating laboratories

14 laboratories participated in the study:

Bundesinstitut für Risikobewertung, Berlin	Germany
Central Science Laboratory, York	United Kingdom
Custom Technical Laboratory, Prague	Czech Republic
Chemical Institute of the Hungarian Customs and Finance Guard, Budapest	Hungary
Eurofins, Nantes	France
FEM-IASMA, San Michele all'Adige, Trento	Italy
Joint Research Center, Ispra	Italy
Arbitral Agroalimentario Del MAPA, Madrid	Spain
Landesuntersuchungsamt, Speyer	Germany
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Wurzburg	Germany
Service Commun des Laboratoires, Bordeaux	France
Service Commun des Laboratoires, Montpellier	France

Service Commun des Laboratoires, Paris	France
Unione Italiana Vini, Verona	Italy

2. Samples

The study was carried out on 5 double-blind samples: three wine vinegar samples (B, C, D), an alcohol vinegar sample (A) and a wine vinegar sample with 20% of A (Thomas and Jamin, 2009).

For each sample, the $\delta^{18}C$ of the water was analysed. The results are indicated in the following table.

3. Statistical evaluation

Statistical calculations were performed according to the OIV-MA-AS1-09 protocol, which is based on Horwitz' IUPAC protocol. Outliers were removed in the following way: Cochran tests for removal of the laboratories with the highest variance; single- and pair-value Grubbs tests for individual or paired individual outliers; then more Cochran tests, etc., keeping a proportion of outliers <2/9. The standard deviations of repeatability (sr) and of reproducibility (sR) were then calculated for each material from valid results pairs from the blind duplicates.

For more information, refer to the publication by Thomas and Jamin, 2009.

Description of the sample	N° of valid results	N° of replicates	$\delta^{13}C$ average (‰)	s_r (‰)	Repeatability limit r ($2.8 \times S_r$) ‰	s_R (‰)	Reproducibility limit R ($2.8 \times SR$) ‰
A	8	2	-6.96	0.06	0.17	0.22	0.62

B	8	2	-0.86	0.08	0.22	0.21	0.59
C	8	2	-1.38	0.02	0.06	0.2	0.56
B+20%A	8	2	-1.79	0.05	0.14	0.2	0.56
D	5	2	-1.23	0.05	0.14	0.14	0.39

The average repeatability limit (*r*) was 0.15‰, and the average reproducibility limit (*R*) was 0.54‰, comparable to the values observed for wine alcohol (OIV-MA-AS2-12).

Method OIV-MA-VI-24 : R2015

Type IV method

Determination of the distribution of deuterium in acetic acid extracted from wine vinegar using nuclear magnetic resonance (NMR)

(OIV-OENO 527-2015)

1. Scope of application

This method makes it possible to analyse the isotope ratio of hydrogen $(D/H)_{CH3}$ at the methyl site of acetic acid extracted from wine vinegar according to the procedure described in the method OIV-OENO 510-2013. The analysis is carried out by ^{1}H-NMR and ^{2}H-SNIF-NMR using a composite NMR experiment (Fauhl et al., 1996; Hermann, 2001). The ^{1}H-NMR experiment is used to determine the ratio between tetramethylurea (TMU) and acetic acid, which is then used, together with the results from the ^{2}H-SNIF-NMR experiment, to calculate the $(D/H)_{CH3}$ isotope ratio. If the acetic acid extracted is pure (> 99%) or if the exact content of acetic acid in the extract is known, the ^{1}H-NMR experiment is not necessary.

2. Definition

$(D/H)_{CH3}$: isotope ratio associated with the molecule CH_2D COOH

3. Principle

The deuterium contained in the sugars and the water in grape must will be redistributed after alcoholic fermentation in molecules I, II, III and IV

of the wine ethanol and after acetic fermentation in molecules V and VI of the acetic acid:

$CH_2D\ CH_2\ OH$	$CH_3\ CHD\ OH$	$CH_3\ CH_2\ OD$	HOD	$CH_2D\ COOH$	$CH_3\ COOD$
I	II	III	IV	V	VI

The parameter $(D/H)_{CH3}$ expresses the D/H ratio of the sugars in the must.

4. Reagents and products

- Hexafluorobenzene (C_6F_6), used as a field-frequency stabilisation substance (lock),
- or, alternatively trifluoroacetic acid (TFA, CAS: 76-05-1) or trifluoroacetic anhydride (TFAA, CAS: 407-25-0), standard N,N-tetramethylurea (TMU); standard TMU with a calibrated D/H isotope ratio,
- 10 mm NMR tubes (probes),
- 0.45 μm filter.

5. Apparatus

NMR spectrometer fitted with a specific deuterium probe tuned to a frequency ν0, characteristic of the field B0 (e.g. where B0 = 7.05 T, ν0 = 46.05 MHz and where B0 = 9.4 T, ν0 = 61.4 MHz) with a proton decoupling channel (B2) and a field-frequency stabilization channel (lock) at the fluorine frequency. The second channel, B2, is also used for the 1H-NMR experiment. The resolution measured on the spectrum, transformed without exponential multiplication (i.e. LB = 0) and expressed as the width at half height of the methyl signal of acetic acid and the methyl signal of TMU, must be less than 0.5 Hz. The sensitivity (signal-to-noise

ratio), measured with an exponential multiplying factor LB equal to 2, must be greater than or equal to 150 for the methyl signal of acetic acid containing less than 25% water. For example, using an NMR spectrometer of field B0 = 7.05 T, 200 scans are required to reach this value.

- balance with a precision of 0.1 mg or greater,
- balance with a precision of 0.1 g or greater.

The laboratory apparatus and consumables set out in sections 5 and 6 are examples and may be replaced by other materials with equivalent performance.

6. Preparation of samples

Acetic acid must be extracted from the vinegar and purified in order to be analysed by SNIF-NMR. At least 6 mL of pure acetic acid must be recovered at the end of the extraction.

The extraction and purification procedure is described in the method OIV 510-2013.

7. Procedure

Preliminary note: the amount and type of reagents and the instrumental conditions depend on the type of apparatus used. The procedure described here is merely an example.

All the preparatory stages must be carried out without any significant evaporation of acetic acid, which would change the isotopic composition of the sample.

7.1. Preparation of the acetic acid sample for NMR measurement

NMR tube 10 mm in diameter: in a pre-weighed glass vial, collect 3.25 g of the solution containing acetic acid obtained in step 6 and weigh to the nearest 0.1 mg (m_A); then add 1.1 g of internal standard TMU and reweigh the flask to the nearest 0.1 mg (m_{ST}).

Depending on the type of spectrometer and probe used, add a sufficient amount of hexafluorobenzene 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 according to the sensitivity of the NMR apparatus. During the probe preparation operations and until the NMR measurement is carried out, the operator must ensure that the acetic acid and TMU do not evaporate, as this would result in isotope fractionation.

The sample is then poured into the NMR probe and, if necessary, filtered using a syringe fitted with a 0.45 µm filter.

N.B.: It is recommended that the NMR probe be prepared under a fume hood, for which gloves and protective goggles should be worn.

7.2. Acquisition of the NMR spectrum

The following conditions are recommended when acquiring the ^{2}H-SNIF-NMR spectrum:

- probe temperature must be constant (e.g. 303 K),
- the sample should be rotated (e.g. 15-20 Hz,)
- acquisition time of at least 5.5 s at a spectral width of 1200 Hz (memory 16 K) (i.e. about 20 ppm at 61.4 MHz or 27 ppm at 46.1 MHz),

- 90° pulse angle,
- adjustment of acquisition time: its value must be of the same order as the dwell time,
- parabolic detection: fix the offset 01 between the OD and CH2D reference signals for acetic acid,
- 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 between the CH3- and TMU groups. Use the wide band decoupling mode or composite pulse sequences (e.g. WALTZ16) to ensure uniform decoupling across the entire spectrum.

For each spectrum, carry out a set of NS accumulations sufficient to obtain the signal-to-noise ratio indicated in step 6, and repeat the set of NS accumulations five times. The NS values will depend on the type of spectrometer and probe used.

The conditions suggested for obtaining the ^{1}H-NMR spectrum are as follows:

- probe temperature must be constant (e.g. 303 K),
- the sample should be rotated (e.g. 15-20 Hz),
- acquisition time of at least 4.1 s for an 8000 Hz spectral width (16 K memory),
- pulse angle of 30° or lower,
- D1=7s at least,
- parabolic detection: fix the offset 01 between the OH and CH3 reference signals for acetic acid,
- absence of decoupling.

For ^{1}H-NMR, 16 scans and 4 tests are sufficient to get an appropriate signal-to-noise ratio. 5 repetitions will also be needed.

8. Calculation of results

Appropriate software based on a complex signal processing algorithm determined by the method of least squares must be adopted to determine the area of the signal (phase and baseline are sensitive parameters that must be properly adjusted). If there are no significant phase and baseline errors (e.g. using appropriate corrections), then other software may be adopted.

For each of the ^{1}H-NMR spectra, calculate the RH ratio as follows:

$$RH = S_{TMU}/S_{acetic\ acid}$$

where S is the area of the ^{1}H-NMR signal, provided by the data processing software according to the Fourier-transformed Free Induction Decay, with a bandwidth equal to 0.5 Hz.

Calculate the mean number of repetitions and standard deviation.

Determine for each of the ^{2}H-SNIF-NMR spectra:

the RD ratio as follows:

$$RD = S_{acetic\ acid}/S_{TMU}$$

Where S' is the area of the ^{2}H-SNIF-NMR signal, provided by the data processing software according to the Fourier-transformed Free Induction Decay, with a bandwidth equal to 2 Hz;

the D/H_{CH3} (ppm) as follows:

$$(D/H)_{CH3} = RH \times RD \times D/H_{TMU}$$

where $(D/H)_{TMU}$ is the isotope ratio of the internal standard (TMU) indicated on the certificate issued by IRMM.

Calculate the mean number of repetitions and standard deviation.

Optional software enables these calculations to be performed online.

9. Quality control of the analyses

The sensitivity and resolution of the spectrometer must be checked in accordance with the specifications of the apparatus (5).

10. Method characteristics

A preliminary study was carried out to characterize the method.

The standard deviation of the repeatability (Sr) averages 0.4 ppm and the mean repeatability limit (r) is 1.0 ppm.

The standard deviation of the reproducibility (SR) averages 0.5 ppm and the mean reproducibility limit (R) is 1.4 ppm.

The details and results of the study are presented in Annex A.

The mean repeatability limit (r) and the mean reproducibility limit (R) are comparable to those observed for alcohol and wine (OIV 426-2011).

11. Bibliography

1. Hermann ,A. (2001) Determination of D/H isotope ratio in acetic acid from vinegars and pickled products by H-2-NMR spectroscopy. Eur Food Res Techn, 212 683-686
2. Fauhl, C., Wittkowski , R. (1996) On-line 1H-NMR to facilitate tube preparation in SNIF-NMR analysis. Z Lebensm Unters Forsch (1996), 541-545
3. Thomas, F., Jamin, E. (2009) (2)H NMR and (13)C-IRMS analyses of acetic acid from vinegar, (18)O-IRMS analysis of water in vinegar: International collaborative study report. *Analytica Chimica Acta*, 649, 98-105

4. Method OIV-OENO 510-2013: Method for $^{13}C/^{12}C$ isotope ratio determination of acetic acid in wine vinegar by isotopic mass spectrometry
5. Method OIV-OENO 426-2011: 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/).

Annex A: Results of the method characterisation

This document sets out the results of the method characterisation study into the method of analysis of the $(D/H)_{CH3}$ ratio in acetic acid extracted from wine vinegar.

An internal repeatability study was performed on 2 samples of vinegar. The results are reported in the table below.

Internal repeatability of 2 samples extracted and analysed 10 times

Repetition	Sample 1 $(D/H)_{CH3}$	Sample 2 $(D/H)_{CH3}$
1	100.8	104.9
2	101.7	104.9
3	100.9	104.7
4	101.3	104.4
5	101.0	104.9
6	102.1	104.8
7	101.8	104.5
8	101.2	104.6
9	101.6	104.3

10	101.0	104.8
Sr	0.4	0.2
Repeatability limit r ($2.8 \times S_r$)	1.2	0.6

An international collaborative study was carried out in 2012 involving 3 laboratories.

1. Participating laboratories

The study involved three laboratories:

Eurofins, Nantes	France
FEM-IASMA, San Michele all'Adige, Trento	Italy
Landesuntersuchungsamt, Speyer	Germany

2. Samples

The study included 4 samples of wine vinegar (Nos. 1-4) and 6 (Nos. 5-10) samples taken from a wine supplemented with increasing percentages (10, 14, 20, 33, 40, 42%) of sugar cane alcohol and then subjected to acetic fermentation. The acetic acid was extracted from each sample and the $(D/H)_{CH3}$ ratio analysed. The results are shown in the table below.

	$(D/H)_{CH3}$			SR	Reproducibility limit R ($2.8 \times S_R$)
SAMPLE	lab1	lab2	lab3		
1	104.8	105.4	104.8	0.4	1.1
2	105.0	105.0	104.5	0.3	0.8
3	106.0	106.0	104.8	0.7	2.0
4	106.2	106.5	106.9	0.3	0.8

5	106.9	106.6	106.6	0.2	0.6
6	107.7	108.2	107.6	0.3	0.8
7	107.3	107.9	107.3	0.3	0.8
8	108.5	107.5	108.6	0.6	1.7
9	109.2	109.2	107.9	0.8	2.2
10	108.9	109.5	107.5	0.1	2.8
mean					
SR				0.5	1.4

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