OIV-MA-AS313-27 Determination of acetic acid in wines by automated enzymatic method

Type II method

1. Scope of application

This method makes it possible to determine acetic acid in wines using an automatic sequential analyser and specific enzyme analysis. The measurement range, which was the object of the current interlaboratory validation, is from 0.2 to 1.14 g/L acetic acid.

NOTE: A range of higher values may be analysed with the introduction of a sample dilution.

2. Standard references

ISO 78-2: Chemistry – Layouts for standards

3. Reaction principles

In the presence of ATP, acetic acid is converted into acetyl phosphate in a reaction catalysed by acetate kinase.

(1) Acetate + ATP $\xrightarrow{Acetate\ kinase}$ $Acetyl\ phosphate + ADP$

The ADP formed by this reaction is reconverted into ATP in a reaction with phosphoenolpyruvate catalysed by pyruvate kinase.

(2) ADP + Phospoenolpyruvate $\xrightarrow{Pyruvate\ kinase}$ $Pyruvate + ATP$

Pyruvate is reduced to L-lactate by reduced nicotinamide adenine dinucleotide (NADH) catalysed by lactate dehydrogenase.

(3) $Pyruvate + NADH + H^{+} \xrightarrow{Lactate\ dehydrogenase} Lactate + NAD^{+} + H_2O$

The quantity of oxidised NADH in reaction (3) is determined by the absorbance measurement at 340 nm, and is proportional to the concentration of acetic acid in the wine.

Note 1:

When this enzymatic analysis is conducted manually, the reading is carried out once the stabilised, final plateau is reached. To achieve this, a fourth reaction makes it

possible to completely push the equilibrium of reaction 1 towards the formation of acetyl phosphate through elimination of the latter.

(4) $Acetyl\ phosphate + CoA \xrightarrow{Phosphotransacetylase} Acetyl - CoA + Inorganic\ phosphate$

In the case of analysis by an automated method, which has a shorter analysis time, it is not necessary to reach a stabilised, final plateau, and this reaction is pointless. The use of phosphotransacetylase is therefore not necessary, and is not described here.

Note 2:

The enzymatic reaction chain involves pyruvate. The low quantities of pyruvate (several tens of mg/L) normally present in wines do not have a significant impact on the result. In the rare cases, the presence in wine of an untypical quantity of pyruvate is likely to produce a method bias.

4. Reagents and working solutions

During analysis – unless otherwise indicated – use only quality, recognised analytical reagents and distilled or demineralised water, or water of equivalent purity.

4.1. Reagents

4.1.1. Quality I or II water for analytical use (ISO 3696 standard)

4.1.2. 3-(N-Morpholino)propanesulfonic acid (MOPS): CAS no. 1132-61-2

4.1.3. Magnesium chloride *hexahydrate*: CAS no. 7791-18-6

4.1.4. Potassium chloride: CAS no. 7447-40-7

4.1.5. □-Nicotinamide adenine dinucleotide (NADH): CAS no. 53-84-9, purity ≥ 98%

4.1.6. Adenosine-5'-triphosphate, disodium salt (ATP): CAS no. 56-65-5

4.1.7. Potassium hydroxide: CAS no. 1310-58-3

4.1.8. Phosphoenolpyruvate tri(cyclohexylammonium) salt: CAS no. 35556-70-8 or monosodium phosphoenolpyruvate: CAS no. 138-08-9 (PEP)

4.1.9. Acetate kinase (AK): CAS no. 9027-42-3

4.1.10. Pyruvate kinase (PK): CAS no. 9001-59-6

4.1.11. Lactate dehydrogenase (LDH): CAS no. 9001-60-9

4.1.12. Polyvinylpyrrolidone (PVP): CAS no. 9003-39-8

4.1.13. Acetic acid: purity ≥ 99.5%; CAS no. 64-19-7

4.1.14. Sodium chloride: CAS no. 7647-14-15

4.1.15. Bovine serum albumin (BSA): CAS no. 9048-46-8

Note 3: There are commercial kits for the determination of acetic acid. The user needs to check the composition to ensure it contains the above- indicated reagents. These

kits are sometimes supplied with phosphotransacetylase (redundant when using an automated method).

Note 4: The use of PVP is recommended to eliminate any possible negative action of tannins in wine on the enzyme protein molecules. In the event that the use of PVP is not effective, the laboratory should ensure the absence of interference of wine tannins on the enzymes.

Note 5: BSA is an agent used for the stabilisation of enzymes in solution.

4.2. Working solutions

4.2.1. MOPS buffer

The preparation may be as follows:

- 13 g MOPS (3-(N-Morpholino)propanesulfonic acid) (4.1.2),
- 0.5 g magnesium chloride hexahydrate (4.1.3),
- 1.5 g potassium chloride (KCl) (4.1.4),
- 1.3 g PVP (4.1.12),
- 250 mL water for analytical usage (4.1.1).

Adjust the pH to 4.75 with a 1.5 M potassium hydroxide (KOH) solution (4.1.7).

Wait 5 minutes and readjust the pH to 7.45 with a 1.5 M potassium hydroxide (KOH) solution (4.1.7).

Make up to 300 mL with water for analytical use (4.1.1).

The buffer can be kept for at least 60 days at 2-8 °C (approximately).

4.2.2. Working solution 1 (R1)

The preparation may be as follows:

- 100 mL MOPS buffer (4.2.1),
- 300-350 mg adenosine-5'-triphosphate, disodium salt (ATP) (4.1.6),
- 50 mg phosphoenolpyruvate (PEP) tri(cyclohexylammonium) salt (4.1.8),
- 40 mg β-nicotinamide adenine dinucleotide (reduced form) (NADH) (4.1.5).

Working solution R1 can be kept for at least 30 days at 2-8 °C (approximately).

4.2.3. Working solution 2 (R2)

The preparation may be as follows:

- 100 mL MOPS buffer (4.2.1),

- approx. 40 units of pyruvate kinase (PK) (4.1.10),
- approx. 40 units of lactate dehydrogenase (LDH) (4.1.11),
- 50 units of acetate kinase (AK) (4.1.9),
- 300 mg BSA (4.1.15).

Working solution R2 can be kept for approximately 48 hours at 2-8 °C (approximately).

Note: When preparing these solutions, they should be mixed gently to avoid the formation of foam. The life cycle of the working solutions is limited and should be evaluated and respected by the laboratory.

4.3. Calibration solutions

In order to ensure the closest possible connection to the International System of Units (SI), the calibration range should be made up of pure solutions of acetic acid (4.1.13). It is recommended to prepare a stock solution (e.g. 1.5 $g.L^{-1}$ acetic acid) by weight, then the rest of the calibrations are obtained from the stock solution to cover the measurement range.

A "zero" value may be obtained using a 9‰ sodium chloride solution (4.1.14) or equivalent saline solution.

5. Apparatus

5.1. Analyser

5.1.1. Equipment type

Automatic sequential analyser equipped with a spectrophotometer with UV detector. The reaction temperature should be stable (at around 37 °C). The reaction cuvettes are glass, methacrylate or quartz. The equipment is controlled by software that handles its operation, data acquisition and useful calculations.

5.1.2. Absorbance reading

The concentration of the analytes directly relates to the absorbance difference read by the spectrophotometer. The precision of the absorbance reading should be a minimum of 0.1 absorbance unit (AU). The absorbance values should not be saturating for the spectrometer used.

5.1.3. Precision of sampled volumes

The volumes of reagents and samples taken by the pipettes of the analyser should be of sufficient precision so as not to have a significant impact on the measurement

result.

5.1.4. Reaction duration and temperature

In general, the reaction time is 10 minutes and the temperature 37 °C. Some pieces of apparatus may use slightly different values.

5.1.5. Use of a reagent blank

The results are read by comparing the light intensity absorbed at the chosen wavelength between a cuvette in which the reaction is carried out and a cuvette in which the reaction does not take place (reagent blank).

5.1.6. Wavelength

The wavelength of maximum absorption of the NADH formed by the reaction is 340 nm. For spectrophotometers in general use, this wavelength is to be selected.

A secondary wavelength is programmed in order to correct a potential matrix effect; this wavelength is 410 nm.

The measurement is conducted based on the DO_{340} - DO_{410} absorbance difference.

5.2. Balance

This should be calibrated to the SI and have a 1 mg resolution.

5.3. pH meter

5.4. Measuring glassware

The measuring glassware for the preparation of reagents and calibration solutions is class A.

6. Sample preparation

1. Test samples
    1. Preparation of samples of still wines

The majority of wine samples may be analysed without preparation. In some cases, a preparation may be introduced:

Filtration should be used for highly turbid samples.

Sample dilution (manual or automatic) with water for analytical usage (4.1.1) should be used for values higher than the measurement range.

6.1.2. Preparation of samples of sparkling wines

Sparkling wine samples should be subjected to a preliminary degassing by stirring under vacuum, ultrasonic treatment or any method that allows for the required degassing.

7. Procedure

Given that different analysers may be used, it is recommended that the conditions of use provided by the manufacturer are strictly respected. The same goes for different enzymatic kits that are available on the market.

The procedure takes place as follows:

The sample (S) is placed in a reaction cuvette.

Working solution R1 (4.2.2) is then added to the cuvette.

Homogenisation takes place. Time is then allowed for a lag period, in order to guarantee absorbance stability. This lag period may last from 1 to 5 min, and is defined by the laboratory, according to the characteristics of the equipment used.

Working solution R2 (4.2.3) is added and the reaction is triggered.

By way of example, the quantities of the different elements may be as follows:

- sample: 3 µL,
- R1: 120 µL, at T (start of sequence),
- R2: 60 µL, at T_0 + 3 min 40 sec.

The equipment carries out regular measurements that make it possible to obtain a reaction curve, an example of which is given in Figure 1.

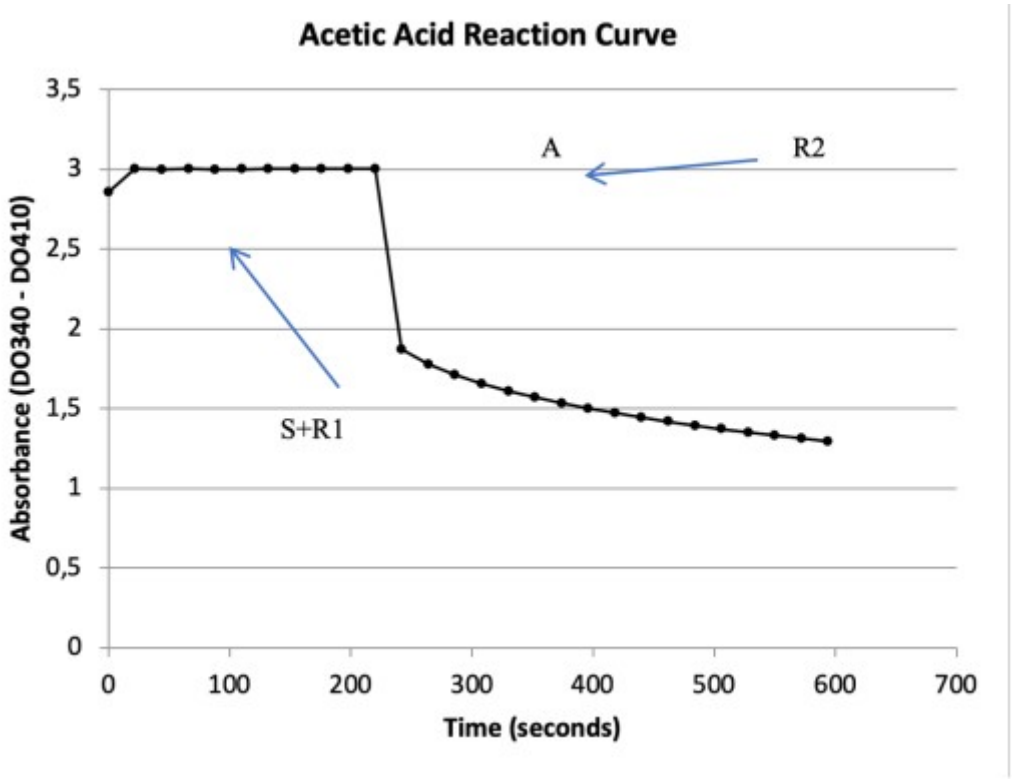

Figure 1: Reaction curve

The equipment makes it possible to choose the reading points for the difference in absorbance sought, for example A and B in Figure 1.

In order to correlate this value with the concentration of acetic acid, regular calibration of the apparatus is carried out using the calibration solutions at a minimum of 3 points covering the measurement range used. The calibration curve obtained is near to a straight line. Nevertheless, a second-degree equation may be used. An example is given in Figure 2.

In addition, a reagent blank is used comprising all of the reagents but no sample (point 0 of calibration).

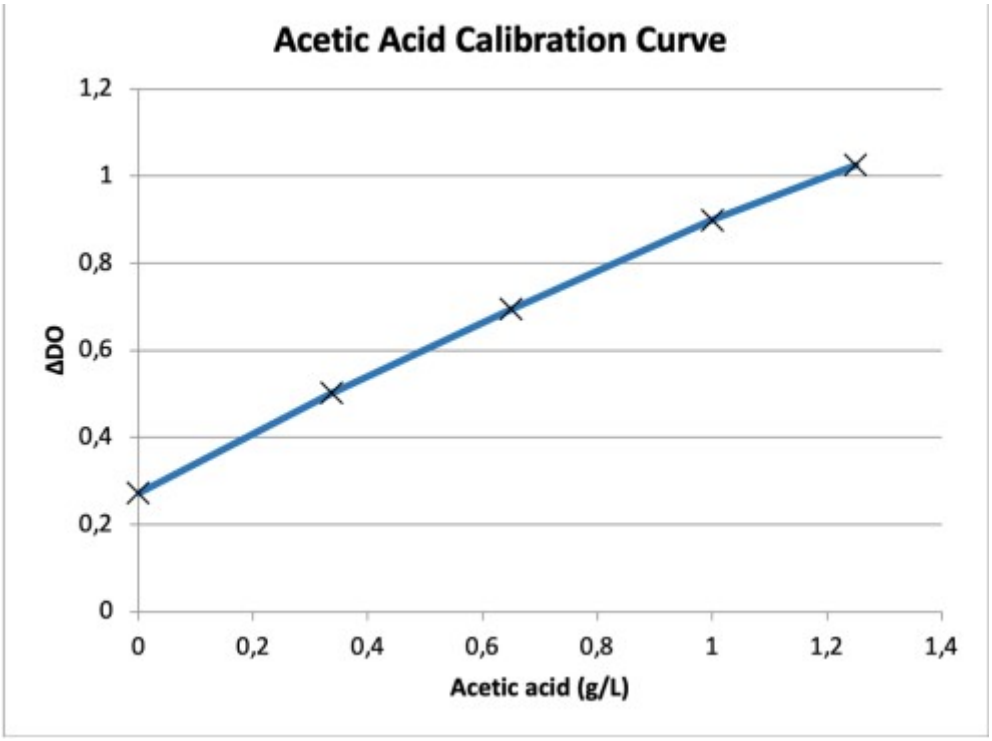

Figure 2: Calibration curve

8. Calculations

For each measurement, the result is given according to the following formula:

$$R = |Absorbance\ B - Absorbance\ A|$$

The values thus obtained are recorded on the calibration curve to obtain the acetic acid concentration. The final value obtained should be multiplied by any coefficient of dilution used.

9. Expression of results

The results for acetic acid are expressed in g/L of acetic acid, calculated to two decimal places, or in another unit according to usage (meq/L). The expression of the result should be consistent with the measurement uncertainty.

10. Automated enzymatic method characteristics

Interlaboratory reproducibility

RSD_R = 10%

CV_R% (k=2) = 2·RSD_R= 20%

Repeatability

RSD_r = 4%

CV_r% (k=2) = 2·RSD_r = 8%

Limit of quantification

Validated LQ < 0.2 g/L

Not determined in the collaborative study

11. Bibliography

- McCLOSKEY Leo P., 'An Improved enzymatic assay for acetate in juice and wine', *Am. J. Enol. Vitic.*, Vol. 31, No. 21980.

AnnexMethod performance studies

Collaborative study

In total, 11 laboratories from 5 different countries took part in the collaborative study.

Laboratory	Country
Miguel Torres S.A.- Finca Mas La Plana	Spain
INGACAL -Consellería do Medio Rural Estación de Viticultura e Enoloxía de Galicia	Spain
Estación Enológica de Haro	Spain
Laboratoires Dubernet	France
Laboratoire Dicœnos Rhône	France
Laboratoire Natoli	France
SCL Montpellier	France

Fachbereich: Wein, Weinüberwachung - Chemisches und Veterinärunterchungsamt Karlsruhe	Germany
HBLAuBA Wein - und Obstbau	Austria
Hochschule GEISENHEIM University Institut Weinanalytik und Getränkeforschung	Germany
Unione Italiana Vini soc. Coop.	Italy

Table 1. Participating laboratories

In total, 2 x 10 samples prepared as blind duplicates were analysed, with 1 repetition. The wines analysed were dry wines, sweetened wines, and liqueur wines, wines originating from France and Portugal.

Sample		A		B		C		D		E		F		G		H		I		J	
		Port wine		Sweetened wine		Dry wine		Dry wine		Sweetened wine		Dry wine		Dry wine		Sweetened wine		Sweetened wine		Sweetened wine	
Position		1	9	2	13	3	4	5	15	6	10	16	20	7	11	12	17	8	19	14	18
Lab3	rep#1	0.24	0.27	0.20	0.21	0.65	0.65	0.47	0.49	0.54	0.52	*1.28*	*1.30*	0.64	0.63	0.29	0.31	0.39	0.37	0.63	0.62
	rep#2	0.25	0.26	0.20	0.21	0.67	0.65	0.46	0.50	0.56	0.53	*1.29*	*1.33*	0.65	0.67	0.29	0.28	0.36	0.37	0.65	0.61
Lab7	rep#1	0.20	0.20	0.22	0.23	0.62	0.62	0.45	0.46	0.50	0.50	*1.25*	*1.30*	0.61	0.62	0.28	0.28	0.34	0.35	0.62	0.60
	rep#2	0.20	0.21	0.21	0.22	0.63	0.64	0.45	0.46	0.53	0.52	*1.20*	*1.20*	0.61	0.64	0.29	0.28	0.35	0.37	0.60	0.61
Lab9	rep#1	0.17	0.18	0.18	0.19	0.57	0.52	0.40	0.40	0.41	0.43	*1.18*	*1.18*	0.57	0.54	0.24	0.29	0.36	0.32	0.53	0.51
	rep#2	0.17	0.19	0.16	0.17	0.59	0.57	0.39	0.43	0.44	0.41	*1.16*	*1.14*	0.55	0.55	0.25	0.29	0.30	0.33	0.55	0.51
Lab12	rep#1	0.17	0.18	0.20	0.20	0.56	0.53	0.40	0.41	0.44	0.44	*1.02*	*1.01*	0.53	0.53	0.27	0.28	0.36	0.33	0.49	0.51
	rep#2	0.17	0.18	0.20	0.21	0.55	0.54	0.40	0.41	0.44	0.44	*1.02*	*1.01*	0.52	0.52	0.28	0.29	0.36	0.34	0.48	0.51
Lab13	rep#1	0.22	0.19	0.23	0.20	0.50	0.51	0.40	0.40	0.42	0.44	*0.95*	*0.97*	0.48	0.49	0.27	0.28	0.32	0.32	0.48	0.50
	rep#2	0.20	0.19	0.23	0.21	0.52	0.52	0.39	0.39	0.43	0.42	*0.97*	*0.96*	0.51	0.48	0.28	0.28	0.32	0.33	0.50	0.51
Lab14	rep#1	0.17	0.17	0.20	0.19	0.56	0.57	0.42	0.41	0.46	0.45	*1.10*	*1.14*	0.55	0.54	0.27	0.26	0.34	0.32	0.53	0.51
	rep#2	0.17	0.17	0.20	0.19	0.56	0.57	0.42	0.41	0.45	0.44	*1.12*	*1.10*	0.53	0.55	0.26	0.26	0.33	0.31	0.53	0.53
Lab15	rep#1	0.22	0.23	0.28	0.27	0.68	0.68	0.52	0.52	0.56	0.56	*1.20*	*1.23*	0.69	0.73	0.35	0.34	**0.47**	**0.42**	0.60	0.62
	rep#2	0.22	0.22	0.26	0.26	0.68	0.63	0.53	0.50	0.52	0.54	*1.18*	*1.13*	0.65	0.67	0.34	0.34	**0.42**	**0.41**	0.59	0.64
Lab17	rep#1	0.20	0.19	0.26	0.25	0.54	0.52	0.41	0.42	0.39	0.39	*1.01*	*1.00*	**0.49**	**0.45**	0.32	0.29	0.34	0.35	**0.44**	**0.43**
	rep#2	0.20	0.20	0.27	0.27	0.53	0.55	0.43	0.43	0.43	0.43	*1.03*	*1.05*	**0.49**	**0.49**	0.31	0.32	0.37	0.38	**0.44**	**0.46**

Lab18	rep#1	0.27	0.25	**0.35**	**0.33**	0.69	0.68	**0.53**	**0.56**	**0.59**	**0.59**	*1.24*	*1.21*	0.66	0.68	**0.43**	**0.41**	**0.50**	**0.51**	**0.65**	**0.63**
	rep#2	0.28	0.27	**0.36**	**0.36**	0.68	0.69	**0.55**	**0.57**	**0.60**	**0.60**	*1.26*	*1.23*	0.68	0.71	**0.44**	**0.43**	**0.50**	**0.52**	**0.63**	**0.65**
Lab20	rep#1	0.23	0.20	0.29	0.29	0.58	0.57	0.49	0.47	0.47	0.47	*1.15*	*1.13*	0.55	0.58	0.34	0.35	0.39	0.40	0.55	0.52
	rep#2	0.23	0.20	0.29	0.29	0.58	0.57	0.49	0.47	0.47	0.47	*1.15*	*1.13*	0.55	0.58	0.34	0.36	0.39	0.40	0.55	0.52
Lab22	rep#1	0.17	0.16	0.20	0.18	0.60	0.60	0.43	0.44	0.47	0.47	*1.20*	*1.20*	0.58	0.58	0.26	0.26	0.32	0.32	0.55	0.58
	rep#2	0.17	0.17	0.19	0.19	0.61	0.61	0.43	0.43	0.48	0.47	*1.21*	*1.22*	0.59	0.58	0.26	0.27	0.31	0.33	0.54	0.59

Table 2. Table of data obtained (in g·L^{-1} of acetic acid). The values in bold correspond to the values rejected by the Cochran (variance outliers) test with a 2.5% significance level (one-tailed test), and the Grubbs (outliers from the mean) test with a significance level of 2.5% (two-tailed test).

Sample	A	B	C	D	E	F	G	H	I	J
No. of laboratories selected	11	10	11	10	10	11	10	9	9	9
No. of repetitions	4	4	4	4	4	4	4	4	4	4
Min.	0.17	0.18	0.51	0.40	0.41	0.96	0.49	0.26	0.32	0.50
Max.	0.27	0.29	0.69	0.52	0.55	1.30	0.69	0.35	0.39	0.63
Overall average	0.20	0.22	0.59	0.44	0.47	1.14	0.59	0.30	0.35	0.55
Repeatability variance	0.0001	0.0001	0.0002	0.0001	0.0002	0.0006	0.0003	0.0001	0.0003	0.0003
Inter-lab. stand. dev.	0.03	0.04	0.06	0.04	0.05	0.11	0.07	0.03	0.03	0.05
Reproducibility variance	0.001	0.001	0.003	0.002	0.002	0.012	0.005	0.001	0.001	0.003
Repeatability stand. dev.	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.02	0.02
r limit	0.03	0.03	0.04	0.03	0.04	0.07	0.05	0.02	0.05	0.05
Repeatability RSD_r	4.5%	4.5%	2.4%	2.7%	2.9%	2.1%	3.1%	2.8%	4.8%	3.2%
Reproducibility stand. dev.	0.03	0.04	0.06	0.04	0.05	0.11	0.07	0.03	0.03	0.05
R limit	0.10	0.11	0.17	0.12	0.14	0.31	0.19	0.09	0.08	0.15

Reproducibility RSD_R	16.8%	17.4%	9.9%	9.5%	10.6%	9.5%	11.7%	11.1%	8.5%	9.5%
Horwitz RSD	4.74	4.68	4.04	4.22	4.18	3.66	4.04	4.49	4.38	4.08
$HorRat_r$	0.96	0.97	0.60	0.63	0.70	0.58	0.77	0.62	1.10	0.79
Horwitz RSD	7.18	7.09	6.12	6.40	6.34	5.54	6.13	6.80	6.63	6.18
$HorRat_R$	2.34	2.45	1.63	1.49	1.67	1.71	1.91	1.63	1.29	1.53

Table 3. Table of results obtained

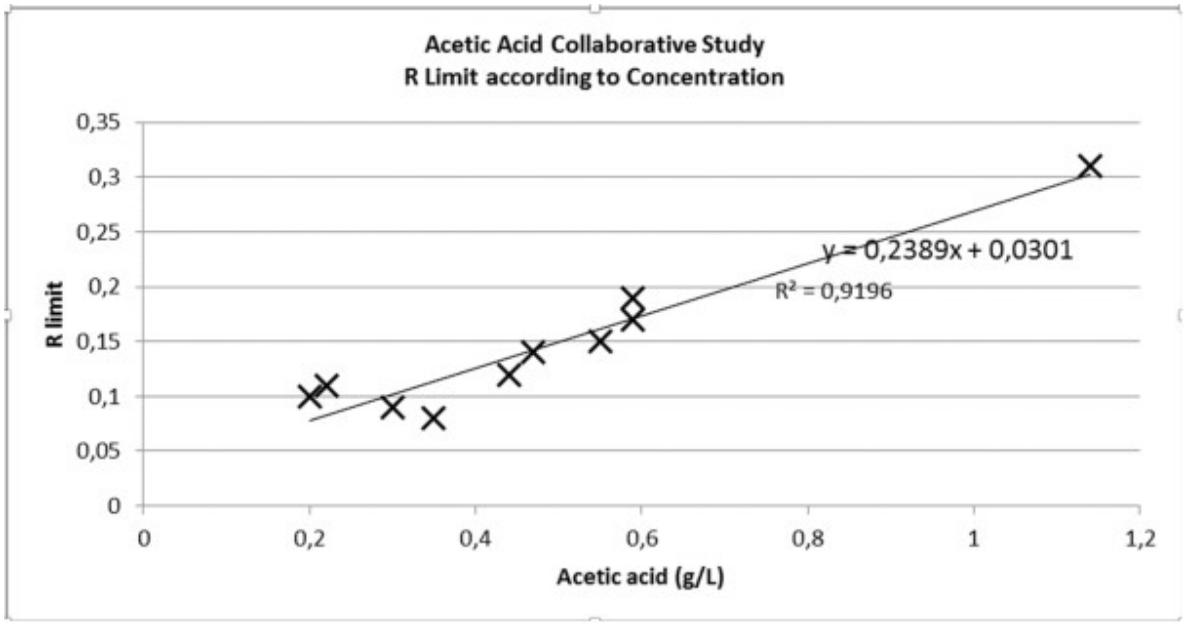

Figure 3. R limit according to concentration