RESOLUTION OIV-OENO 599-2018

DETERMINATION OF L-MALIC ACID IN WINES BY AUTOMATED ENZYMATIC METHOD (Type III method)

WARNING: this resolution amends the following resolution:
- OIV-OENO 391-2010

THE GENERAL ASSEMBLY,

IN VIEW of Article 2, paragraph 2 iv of the Agreement of 3 April 2001 establishing the International Organisation of Vine and Wine,

CONSIDERING the proposal of the "Methods of Analysis" Sub-Commission,

CONSIDERING the interest expressed by some SCMA experts in including automated methods in the *Compendium of International Methods of Wine and Must Analysis*,

DECIDES to remove the practical example of the analyte in the resolution OIV-OENO 391/2010 when an automatic method concerning the same analyte is adopted

DECIDES to add the following method to the *Compendium of International Methods of Wine and Must Analysis*:

Determination of L-malic acid in wine by automated enzymatic method

Type III method

1. Scope of application

This method makes it possible to determine L-malic acid in wine by specific enzyme analysis using an automatic sequential analyser.

In this document, a collaborative study is reported which demonstrates application of the method for measurement of L-malic acid in the range from 0.12 to 2.3 g/L.

Note: Where necessary, each laboratory using this method may refine, and potentially widen, this range through a validation study.

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2. Standard references

- OIV Compendium of International Methods of Analysis: L-malic acid – enzymatic method, OIV-MA-AS313-11,
- ISO 78-2: Chemistry – Layouts for standards.

3. Reaction principles

In the presence of nicotinamide adenine dinucleotide (NAD), L-malic acid is oxidised to oxaloacetate in a reaction catalysed by L-malate dehydrogenase (L-MDH). Since the equilibrium reaction is in favour of the malate, it is necessary to remove the oxaloacetate formed which is converted into L-aspartate in the presence of L-glutamate. This reaction is catalysed by glutamate oxaloacetate transaminase (GOT).

$$L-malate + NAD^{+} \overset{L-MDH}{\longleftrightarrow} oxaloacetate + NADH + H^{+}$$

$$oxaloacetate + L-glutamate \overset{GOT}{\longleftrightarrow} L-aspartate + \propto -ketoglutarate$$

The reduced nicotinamide adenine dinucleotide (NADH) produced is measured based on its absorption at 340 nm. It is proportional to the quantity of L-malic acid.

4. Reagents and working solutions

During the analysis – unless stated otherwise – only use reagents of recognised analytical grade and water that is distilled, demineralised or of equivalent purity.

4.1. Reagents

4.1.1. Quality I or II water for analytical usage (ISO 3696 standard);
4.1.2. Glycylglycine (CAS no. 556-50-3);
4.1.3. Glutamic acid (CAS no. 56-86-0);
4.1.4. NAD (nicotinamide adenine dinucleotide) (CAS no. 53-84-9);
4.1.5. L-MDH (L-malate dehydrogenase) (CAS no. 9001-64-3);
4.1.6. GOT (glutamate oxaloacetate transaminase) (CAS no. 9000-97-9);

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4.1.7. L-malic acid, purity ≥ 95% (CAS no. 97-67-6);

4.1.8. Optional: polyvinylpyrrolidone (PVP) (CAS no. 9003-39-8) or potentially PVPP (CAS no. 25249-54-1);

4.1.9. Sodium hydroxide (CAS no. 1310-73-2).

Note 1: There are commercial kits for the determination of L-malic acid. The user needs to check the composition to ensure it contains the above-indicated reagents.

Note 2: The use of PVP is recommended to eliminate any possible negative effect of tannins in wine on the enzyme protein molecules. Should the use of PVP not prove effective, the laboratory should ensure that the wine tannins do not interfere with the enzymes.

4.2. Working solutions

4.2.1. A pH 10 buffer (0.60 M glycylglycine, 0.1 M L-glutamic acid).

The preparation may be as follows:

- glycylglycine (4.1.2): 4.75 g,
- glutamic acid (4.1.3): 0.88 g,
- PVP (4.1.8): 1 g,
- water for analytical usage (4.1.1): 50 mL.

The mixture is adjusted to pH 10 using a 10 M sodium hydroxide solution, then made up to 60 mL with water for analytical usage. This solution is stable for at least 4 weeks at 2-8 °C (approx.).

4.2.2. R1 working solution (example):

- water for analytical usage (4.1.1): 12 mL,
- NAD (4.1.4): 420 mg.

This solution is stable for at least 4 weeks at 2-8 °C (approx.).

4.2.3. R2 working solution (example):

- water for analytical usage (4.1.1): 1.2 mL,
- L-MDH (4.1.5): 4800 U,
- GOT (4.1.6): 320 U.

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This solution is stable for at least 4 weeks at 2-8 °C (approx.).

4.3. Calibration solutions

To ensure the closest possible connection to the International System of Units (SI), the calibration range should be created using pure solutions of L-malic acid prepared by weighing and covering the measurement range.

5. Apparatus

5.1. Analyser

5.1.1. Equipment type

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

5.1.2. Absorbance reading

The concentration of the analytes directly relates to the absorbance difference read by the spectrophotometer. The precision of the absorbance reading should be a minimum of 0.1 absorbance unit (AU). It is preferable not to use absorbance values higher than 2.0.

5.1.3. Precision of volumes collected

The precision of the volumes of reagents and samples collected by the pipettes of the analyser influences the measurement result. Quality control of the results using appropriate strategies (e.g. according to the guides published by the OIV) is recommended.

5.1.4. Reaction duration and temperature

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

5.1.5. 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 (blank reagent).

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5.1.6. Wavelength

The wavelength of maximum absorption of the NADH formed by the reaction is 340 nm.

5.2. Balance

This should be calibrated to the International System of Units and have 1 mg precision.

5.3. pH meter

5.4. Measuring glassware

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

6. Sampling

6.1. Preparation of samples of musts and still wines

In most cases, wine and must samples may be analysed without preparation. In some cases, a preparation may be introduced:

- filtration or centrifugation should be used for highly turbid samples,
- sample dilution (manual or automatic) with water for analytical usage should be used for values exceeding the measurement range.

6.2. Preparation of samples of sparkling wine containing CO_2

Sparkling wine samples containing CO_2 may produce bubbling effects. They must be degassed beforehand by stirring under vacuum, ultrasonic processing or any method enabling the required degassing.

7. Procedure

Given that different analysers may be used, it is recommended that the conditions of use provided by the manufacturer be strictly observed. This also applies to the various enzymatic kits available on the market.

The procedure takes place as follows:

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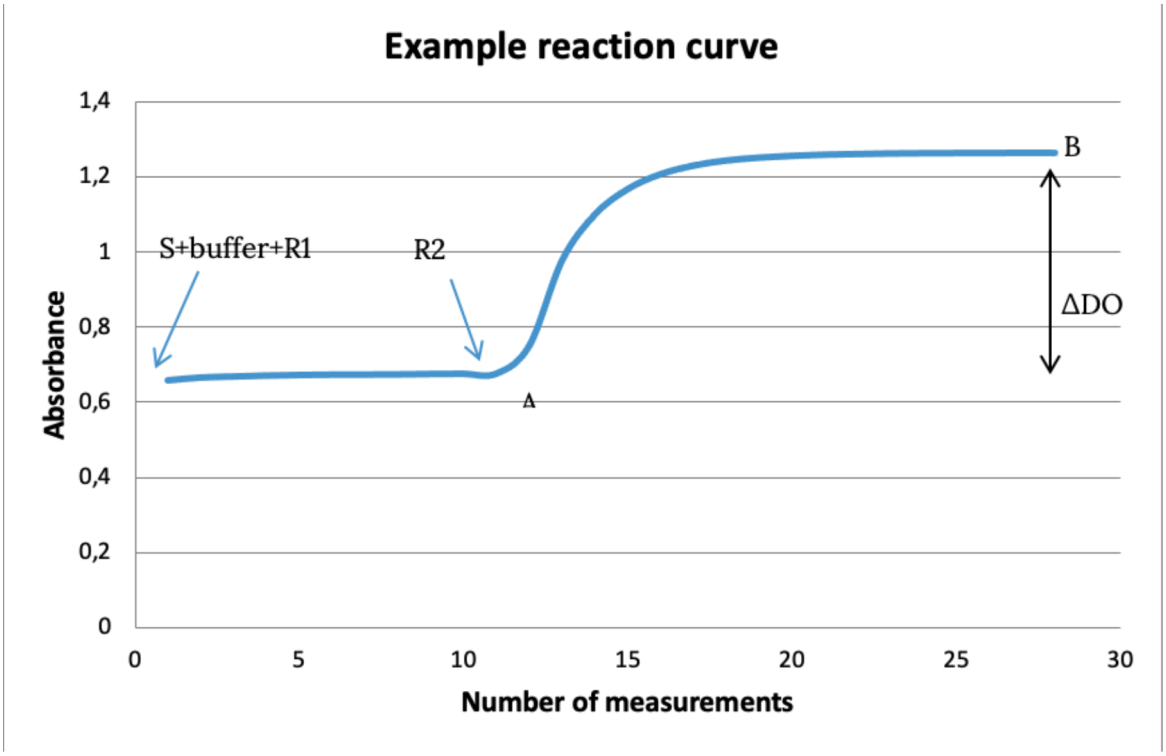
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1. The sample (S) is placed in a reaction cuvette.
2. Working solution R1 (4.2.2) is then added to the cuvette.
3. The two are mixed together. Time is then allowed for a lag period, in order to guarantee absorbance stability. This lag period may last from 1-5 min, and is defined by the laboratory, according to the characteristics of the equipment used.
4. Working solution R2 (4.2.3) is added and the reaction takes place.

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

- sample: 2.5 µL,
- mixture of 80% buffer (3.2.1) and 20% R1 (3.2.2): 120 µL,
- R2 (3.2.3): 15 µL.

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



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Figure 1: Reaction curve

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

8. Calculation of results

The measurement used for the determination of the result is as follows:

ΔDO = (Absorbance B – Absorbance A)

In order to correlate this ΔDO value with the desired concentration of L-malic acid, regular calibration of the apparatus is carried out using the calibration solutions (§4.3) at a minimum of 3 points covering the measurement range. In the example given in Figure 2, the calibration curve obtained is a straight line for values between 0 and 2 g/L L-malic acid. In this case, for higher values, it is preferable to dilute the sample. In addition, a reagent blank is used comprising all of the reagents but no sample (point 0 of the calibration).

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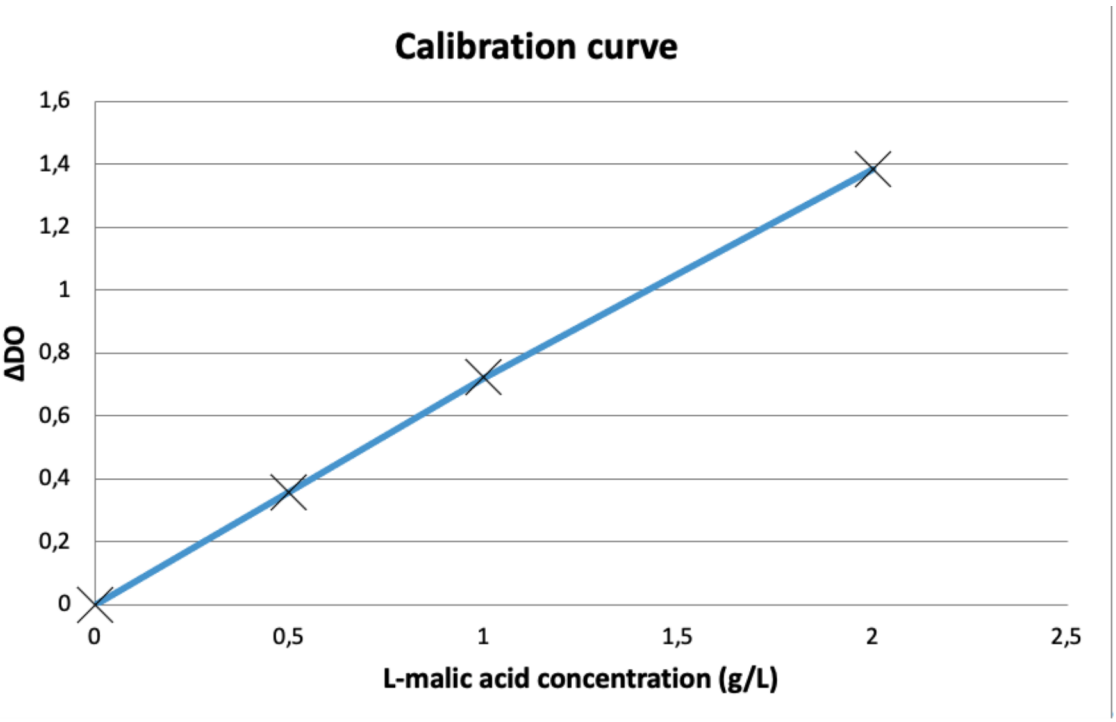


Figure 2: Calibration curve

The calibration curve can be order 1 (*Concentration = a.ΔDO + b*) or even order 2 (*Concentration = a.ΔDO + b.ΔDO + c*). If using a calibration curve of order 2, the laboratory should take care to limit the calibration domain in order to maintain sufficient sensitivity of the method (risk of crushing the curve with high concentrations).

The final value obtained should be multiplied by any coefficient of dilution used.

9. Expression of results

The results are expressed in g/L L-malic acid to 2 d.p.

10. Precision

Interlaboratory reproducibility

- RSD_R = 5% (from 1 g/L)

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- CV_R% (k=2) = 2·RSD_R= 10%, (from 1 g/L)

Repeatability

- RSD_r = 2% (from 1 g/L)
- CV_r% (k=2) = 2·RSD_r = 4% (from 1 g/L)

Limit of quantification

Validated LOQ = 0,12 g/L

(Concentration where CV_R% (k=2) = 60%)

ANNEX

Results of the interlaboratory tests

Collaborative study

A total of 16 Laboratories from different countries participated in the collaborative study organised in 2016.

Labo	Country
Miguel Torres S.A.- Finca Mas La Plana	SPAIN
Estación Enológica de Castilla y León	SPAIN
INGACAL -Consellería do Medio Rural Estación de Viticultura e Enoloxía de Galicia	SPAIN
Estación Enológica de Haro	SPAIN
Comissão de Viticultura da Região dos Vinhos Verdes	Portugal
Laboratoires Dubernet	France

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Laboratoire Diœnos Rhône	France
Laboratoire Natoli	France
SCL Montpellier	France
Agricultural institute of Slovenia	Slovenia
Fachbereich: Wein, Weinüberwachung - Chemisches und Veterinärunterchungsamt Karlsruhe	Germany
HBLAuBA Wein - und Obstbau	aUSTRIA
Landesuntersuchungsamt Mainz	Germany
Hochschule GEISENHEIM University Institut Weinanalytik und Getränkeforschung	Germany
Unità Chimica Vitienologica e Agroalimentare - Centro Trasferimento Tecnologico - Fondazione Edmund Mach	ItalY
Unione Italiana Vini soc. Coop.	ItalY

For analysis, 2 x 10 blind duplicate samples were used, with 1 repetition. The wines analysed are wines originating from France and Portugal, dry wines and liqueur wines.

Sample		A		B		C		D		E		F		G		H		I		J	
Position		1	9	2	13	3	4	5	15	6	10	16	20	7	11	12	17	8	19	14	18
Labo3	rep#1	1.12	1.09	2.16	2.21	**0.08**	**0.08**	1.38	1.39	**0.16**	**0.16**	*0.05*	*0.04*	0.07	0.06	1.31	1.35	**1.24**	**1.27**	0.07	0.07
	rep#2	1.15	1.11	2.18	2.11	**0.08**	**0.09**	1.35	1.41	**0.16**	**0.16**	*0.05*	*0.04*	0.06	0.06	1.32	1.30	**1.23**	**1.24**	0.07	0.07
Labo6	rep#1	0.99	0.95	2.28	2.31	**0.04**	**0.02**	1.44	1.47	**0.05**	**0.08**	*0.04*	*0.04*	0.06	0.04	1.38	1.46	1.34	1.19	**0.04**	**0.03**
	rep#2	0.93	0.93	2.15	2.16	**0.05**	**0.03**	1.23	1.37	**0.08**	**0.09**	*0.03*	*0.05*	0.03	0.04	1.28	1.30	1.20	1.28	**0.03**	**0.03**
Labo7	rep#1	1.10	1.10	2.28	2.33	0.01		1.44	1.52	0.12	0.10		*0.01*			1.38	1.41	1.33	1.30	0.01	0.02
	rep#2	1.13	1.13	2.35	2.33	0.03	0.04	1.47	1.49	0.14	0.13				0.02	1.40	1.43	1.32	1.33	0.02	0.03
Labo9	rep#1	1.14	1.10	2.27	2.21	0.06	0.10	1.43	1.48	0.19	0.19	*0.02*	*0.02*	0.05	0.06	1.35	1.38	1.27	1.28	0.05	0.08
	rep#2	1.15	1.20	2.20	2.23	0.10	0.06	1.50	1.51	0.15	0.14	*0.03*	*0.01*	0.09	0.11	1.31	1.37	1.22	1.28	0.04	0.06
Labo12	rep#1	1.12	1.12	2.34	2.32	**0.06**	**0.02**	1.47	1.54	0.14	0.14	*0.04*	*0.03*	0.03	0.03	1.37	1.37	1.29	1.31	0.06	0.04
	rep#2	1.12	1.12	2.38	2.34	**0.07**	**0.01**	1.46	1.46	0.16	0.19	*0.04*	*0.05*	0.03	0.04	1.32	1.33	1.28	1.30	0.06	0.04

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Labo13	rep#1	1.11	1.09	2.12	2.26	0.04	0.02	1.44	1.45	**0.12**	**0.14**	*0.01*	*0.01*	0.03	0.03	1.45	1.33	1.29	1.29	0.02	0.02
	rep#2	1.18	1.17	2.20	2.29	0.03	0.01	1.47	1.50	**0.11**	**0.12**			0.02	0.03	1.39	1.38	1.32	1.33		0.01
Labo14	rep#1	1.32	1.30	2.66	2.68			1.69	1.67	0.08	0.08					1.65	1.60	1.51	1.52		
	rep#2	1.30	1.30	2.63	2.68			1.67	1.67	0.08	0.08					1.61	1.60	1.51	1.53		
Labo15	rep#1	**1.18**	**1.19**	2.29	2.42	0.06	0.05	1.41	1.50	0.16	0.16	*0.04*	*0.04*	0.08	0.08	1.33	1.39	1.26	1.30	0.07	0.07
	rep#2	**1.13**	**1.20**	2.21	2.42	0.05	0.05	1.49	1.52	0.16	0.16	*0.04*	*0.04*	0.08	0.08	1.38	1.39	1.27	1.28	0.07	0.07
Labo16	rep#1	**1.22**	**1.22**	2.52	2.48			1.63	1.62	0.09	0.09					**1.50**	**1.53**	1.47	1.45		
	rep#2	**1.21**	**1.20**	2.45	2.55			1.62	1.61	0.09	0.09		*0.03*			**1.52**	**1.51**	1.44	1.45		
Labo17	rep#1	1.14	1.14	2.22	2.22	0.07	0.08	1.51	1.50	0.17	0.17	*0.07*	*0.07*	0.11	0.11	1.34	1.35	1.28	1.27	0.09	0.09
	rep#2	1.13	1.15	2.17	2.20	0.08	0.08	1.46	1.47	0.18	0.17	*0.08*	*0.07*	0.12	0.12	1.31	1.34	1.28	1.29	0.09	0.09
Labo18	rep#1	1.10	1.11	2.14	2.13	0.18	0.18	1.42	1.52	0.28	0.28	***0.16***	***0.16***	0.19	0.19	1.33	1.38	1.27	1.31	0.17	0.18
	rep#2	1.10	1.13	2.19	2.16	0.18	0.18	1.44	1.51	0.27	0.27	***0.16***	***0.16***	0.19	0.19	1.33	1.38	1.29	1.33	0.17	0.18
Labo19	rep#1	1.13	1.19	2.20	2.23	0.08	0.08	1.47	1.44	0.17	0.19	***0.07***	***0.08***	0.11	0.11	1.36	1.30	1.27	1.26	0.10	0.10
	rep#2	1.15	1.22	2.21	2.23	0.08	0.09	1.48	1.45	0.18	0.19	***0.07***	***0.08***	0.12	0.11	1.33	1.30	1.28	1.24	0.09	0.10
Labo20	rep#1	1.17	1.20	2.27	2.27	0.04	0.05	1.50	1.48	0.17	0.17	*0.04*	*0.03*	0.07	0.07	1.33	1.34	1.28	1.29	0.07	0.06
	rep#2	1.17	1.20	2.27	2.27	0.04	0.05	1.50	1.48	0.17	0.17	*0.04*	*0.03*	0.07	0.07	1.34	1.34	1.28	1.29	0.07	0.06
Labo21	rep#1	1.10	1.12	2.30	2.47	0.05	0.05	1.45	1.49	0.11	0.10	*0.05*	*0.05*	0.05	0.05	1.46	1.44	1.33	1.41	0.05	0.05
	rep#2	1.09	1.16	2.28	2.50	0.05	0.05	1.49	1.51	0.09	0.11	*0.05*	*0.05*	0.05	0.05	1.43	1.46	1.34	1.37	0.05	0.05
Labo22	rep#1	1.08	1.07	2.35	2.31	0.04	0.03	1.46	1.45	0.11	0.12	*0.01*	*0.02*	0.03	0.03	1.35	1.35	**1.26**	**1.31**	**0.03**	**0.02**
	rep#2	1.06	1.08	2.30	2.31	0.04	0.04	1.51	1.46	0.12	0.12	*0.01*	*0.03*	0.04	0.03	1.35	1.34	**1.28**	**1.25**	**0.01**	**0.01**

Table of the data obtained. The values in bold correspond with the values rejected in accordance with the Cochran (variance outliers) test with a 2.5% significance level (one-tailed test), and the Grubbs (outliers from the mean) test with significance levels of 2.5% (two-tailed test).

Note: The absent values have not been provided by the laboratory in question.

Sample	A	B	C	D	E	F	G	H	I	J
No. of laboratories selected	15	15	12	14	13	13	12	12	12	12
No of repetitions	4	4	4	4	4	4	4	4	4	4
Min.	0.95	2.15	0.03	1.38	0.08	0.01	0.02	1.32	1.25	0.02
Max.	1.31	2.66	0.08	1.68	0.28	0.08	0.19	1.45	1.36	0.10

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Overall average	1.14	2.30	0.05	1.49	0.14	0.04	0.07	1.36	1.29	0.05
Repeatability variance	0.001	0.003	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.000
Inter-laboratory stand. dev.	0.08	0.14	0.02	0.07	0.05	0.02	0.05	0.04	0.03	0.03
Reproducibility variance	0.006	0.021	0.001	0.006	0.003	0.000	0.002	0.002	0.001	0.001
Repeatability stand. dev.	0.03	0.05	0.01	0.03	0.01	0.01	0.01	0.03	0.02	0.01
r limit	0.07	0.15	0.04	0.08	0.02	0.02	0.01	0.07	0.06	0.02
Repeatability RSD_r	2.2%	2.4%	25.0%	2.0%	6.2%	15.4%	7.4%	1.9%	1.6%	14.5%
Reproducibility stand. dev.	0.08	0.14	0.02	0.08	0.05	0.02	0.05	0.04	0.04	0.03
R limit	0.22	0.41	0.07	0.21	0.16	0.06	0.14	0.12	0.10	0.08
Reproducibility RSD_R	6.9%	6.2%	46.9%	5.0%	39.1%	57.5%	71.2%	3.2%	2.8%	53.1%
Horwitz RSD_r	3.66	3.29	5.81	3.51	5.02	6.13	5.57	3.56	3.59	5.81
$Horrat_r$	0.61	0.72	4.30	0.57	1.24	2.52	1.33	0.53	0.45	2.49
Horwitz RSD_R	5.55	4.99	8.80	5.32	7.60	9.29	8.44	5.40	5.44	8.81
$Horrat_R$	1.25	1.25	5.33	0.95	5.14	6.19	8.43	0.59	0.51	6.02

Table of the results obtained

Note: The results from samples C, F, G and J should be taken with caution due to the very low concentration levels, which are below the laboratories' limit of quantification.

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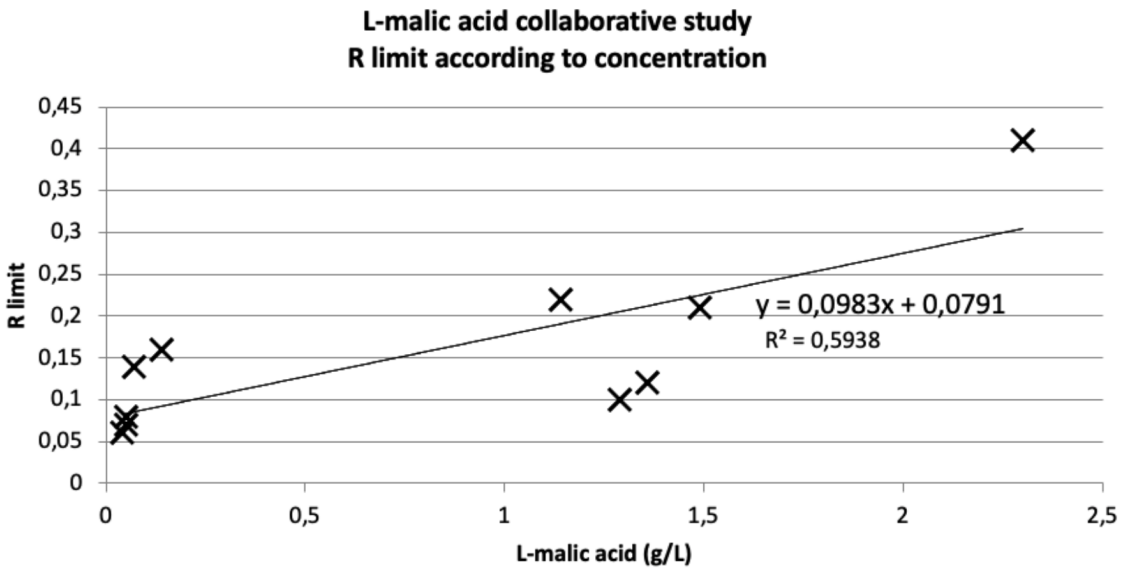


Figure 3: R limit according to concentration

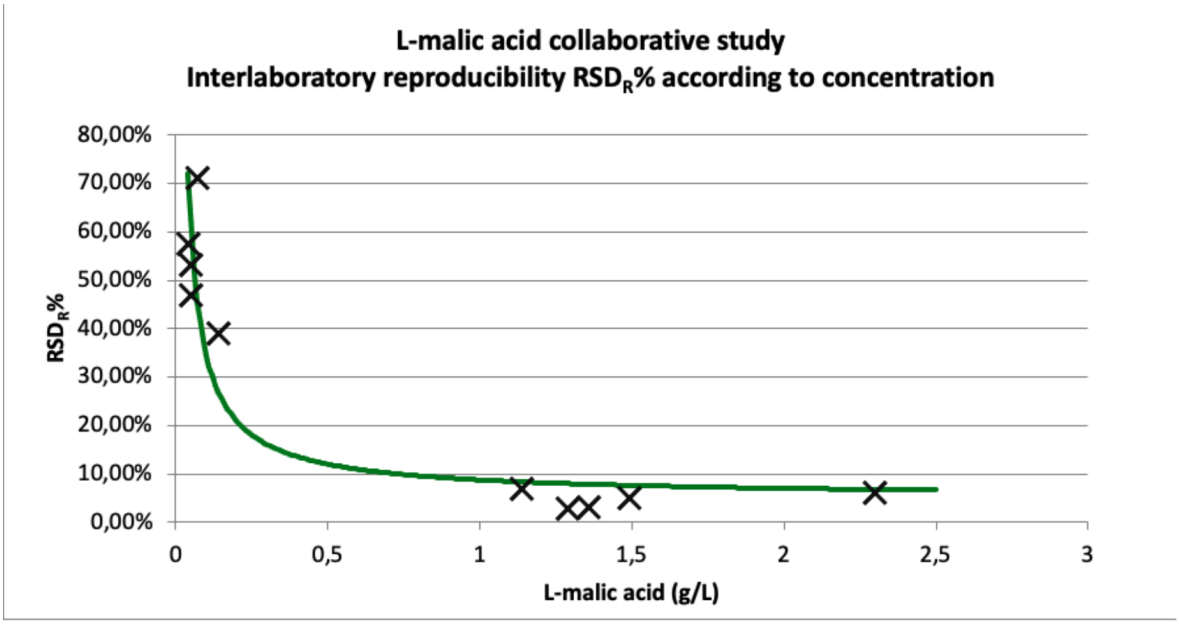


Figure 4: RSDR% according to concentration
Modelling: CV% = 3.763·C(-0.895)+ 5

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