RESOLUTION OENO 6/98

D-MALIC ACID DOSAGE BY ENZYMATIC METHOD

THE GENERAL ASSEMBLY,

ON THE PROPOSAL of Commission II « Oenology », after study of the works of the Sub-Commission of methods of analysis,

DECLARES null and void the method described in the Compendium of international methods of analysis of wines and musts,

PROPOSES to replace it by the following measuring process, taking into account the fact that the enzymatic reaction is « slippery » and that the quantification threshold of this method will have to be established,

PROPOSES that the chromatographic methods, using a « chiral » stationary phase, of D-malic acid methilic ester be considered as usual methods after validation.

D-MALIC ACID - Dosage by enzymatic method

1. Principle

In the presence of D-malate-dehydrogenase (D-MDH), D-malic acid (D-malate) is oxidized into oxalo-acetate by nicotinamide-adenine-dinucleotide (NAD). The formed oxalo-acetate is transformed into pyruvate and carbon dioxide.

$$(1)\ D-malate + NAD \overset{D-MDH}{\Longleftrightarrow} pyruvate + CO_2 + NADH + H^+$$

The formation of NADH, mesured by the increase of absorbance for 334, 340 ou 365 nm wave lengths, is proportional to the quantity of D-malate present.

2. Reactives

Reactives allowing for 30 determinations to be made are marketed in a set which includes:

1. Flask 1 containing about 30 ml of solution of Hepes buffer acid [N-(2-hydroxyethyl)piperazine-N'-2-ethane sulfonic] pH = 9,0 and stabilizers ;

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2. Flask 2 containing about 210 mg of NAD lyophilisat;
3. Flask 3 (three flasks), containing D-MDH lyophilisat, with a titration of about 8 units.

Preparation of the solutions

1. Use the content of flask 1 without dilution.Bring the solution to a temperature of 20-25°C before using it.
2. Dissolve the content of flask2 in 4 ml of bi-distilled water.
3. Dissolve the content of one the flasks 3 in 0,6 ml of bi-distilled water. Bring the solution to a temperature of 20-25 °C before using it.

Stability of the solutions

The content of flask 1 can be kept for at least one year at + 4°C ; solution 2 can be kept about 3 weeks at + 4 °C and 2 months at - 20 °C ; solution 3 can be kept 5 days at + 4 °C.

3. Apparatus

3.1. Spectrophotometer which allows to carry out measures at a maximum of 340 nm of NADH absorption.

If this is not available: photometer with a discontinuous spectrum allowing to carry out measures at 334 nm at 365 nm. Concerning absolute absorbance measures (no range of standardization, but reference to the NADH extinction coefficient), the scales of wave lengths and absorbance of the apparatus must be controled.

3.2. Vats with a 1 cm optical course made of glass or single-use vats.

3.3. Micropipettes allowing to take volumes comprised between 0,01 et 2 ml.

4. Preparation of the sample

The dosage of D-malate is generally carried out directly on the wine without preliminary decoloration.

The quantity of D-malate in the vat must be comprised between 2 µg and 50 µg, wine should be diluted in such a way as for malate concentration to be included between 0,02 and 0,5 g/l or 0,02 and 0,3 g/l depending on the apparatus used).

Dilution table:

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Astimated quantity of D-malate/litre Dilution with water Dilution factor F

Astimated quantity of D-malate/litre		Dilution with water	Dilution factor F
Measured at : 340 or 334 nm	365 nm		
< 0,3 g	< 0,5 g	-	1
0,3-3,0 g	0,5-5,0 g	1 + 9	10

5. Operational mode

The spectrophotometer is set at a wave length of 340 nm, the absorbance measures are carried out in vats with a 1 cm optical course, zero absorbance is set in function of the air (no vat on the optical course) or in function of water.

In the vats with a 1 cm optical course, introduce:

	Control	Trial
Solution 1	1,00 ml	1,00 ml
Solution 2	0,10 ml	0,10 ml
Bi-distilled Water	1,80 ml	1,70 ml
Sample	-	0,10 ml

Mix: after approximately 6 minutes, measure the absorbance of the control and trial solutions (A1).

Add		Control	Trial
	Solution 3	0,05 ml	0,05 ml

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Mix : wait for the end of the reaction (about 20 min.) and measure the absorbance of the control and trial solutions (A_2).

Determine the absorbance differences ($A_2 - A_1$) of the control (ΔA_T) and trial (ΔA_D).

Deduct the control absorbance difference from the trial absorbance difference:

$$\Delta A = \Delta A_D - \Delta A_T$$

Comment : the time required for the enzymes' action can vary from one batch to the other. It is given here only as an indication. It is recommended to determine it for each batch.

D-malic acid reacts rapidly. An extra activity of the enzyme also transforms L-tartaric acid even though it is not as quick. This is the reason why there is a small side reaction which may be corrected by means of extrapolation (see annex 1).

6. Expression of the results

The concentration in miligrammes per liter is calculated with the general formula:

$$C = \frac{V \times PM}{\varepsilon \times d \times v} \times \Delta A$$

V = volume of the test in ml (ici 2,95 ml)

v = volume of the sample in ml (ici 0,1 ml)

PM = molecular mass of the substance to be measured (here, D-malic acid = 134.09)

d = optical course of the vat in cm (here 1 cm)

ε = absorption coefficient of NADH:

- at 340 nm = 6,3 ($l\ mmol^{-1}\ cm^{-1}$)
- at 365 nm = 3,4 ($l\ mmol^{-1}\ cm^{-1}$))
- at 334 nm = 6,18($l\ mmol^{-1}\ cm^{-1}$).

If a dilution was made during the preparation of the sample, multiply the result by the dilution factor.

The concentration in D-malic acid is given in milligrammes per litre (mg/l) without

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decimal.

7. Accuracy

The details of the interlaboratory trial on the accuracy of the method are summarized in annex 2. The derived values of the interlaboratory study may not be applicable to the ranges of concentration in analyte and to other matrix than those given in annex 2.

7.1. Repeatabilit

The absolute difference between individual results obtained on an identical matter submitted to a trial by an operator using the same apparatus, within the shortest time interval, will not exceed the value of repeatability r in more than 5% of the cases.

The value is : r =11 mg/l.

7.2. Reproducibility

The absolute difference between individual results obtained on an identical matter submitted to a trial in two laboratories will not exceed the value of reproducibility R in more than 5% of the cases.

The value is : R = 20 mg/l.

8. Comments

Taking into account the method's accuracy, the values of D-malic acid inferior to 50 mg/L must be confirmed by another analytical method using another measuring pronciple such as that of PRZYBORSKI et al, (1993) and the values of D-malic acid inferior to 100 mg/L must not be interpreted as an addition of D, L-malic acid to wine.

The wine content in the dish must not exceed 0,1ml to avoid a possible inhibition of enzymatic activity by polyphenols..

ANNEX 1 How to treat side reactions

Side reactions are generally due to secondary reactions of the enzyme, in the presence of other enzymes in the sample's matrix, or the interaction of one or several elements of the matrix with a co-factor of the enzymatic reaction.

With a normal reaction, absorbance reaches a constant value after a certain time, generally after 10 min and 20 min, according to the speed of the specific enzymatic reaction. However, when secondary reactions occur, the absorbance does not reach a

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constant value, but increases regularly with time, this type of process is commonly called « side reaction ».

When this problem arises, one should measure the solution's absorbance at regular intervals (2 min to 5 min), after the required time for the standard solution to reach its final absorbance. When the absorbance increases regularly, carry out 5 or 6 measurements, than establish a graphic or calculated extrapolation, in order to obtain what the solution's absorbance would have been when the final enzyme was added (TO). The difference in extrapolated absorbance at this time (Af-Ai) is used for the calcultation of the substrate concentration.

Bibliography:

1. PRZYBORSKI et al. Mitteilungen Klosterneuburg 43, 1993; 215-218.

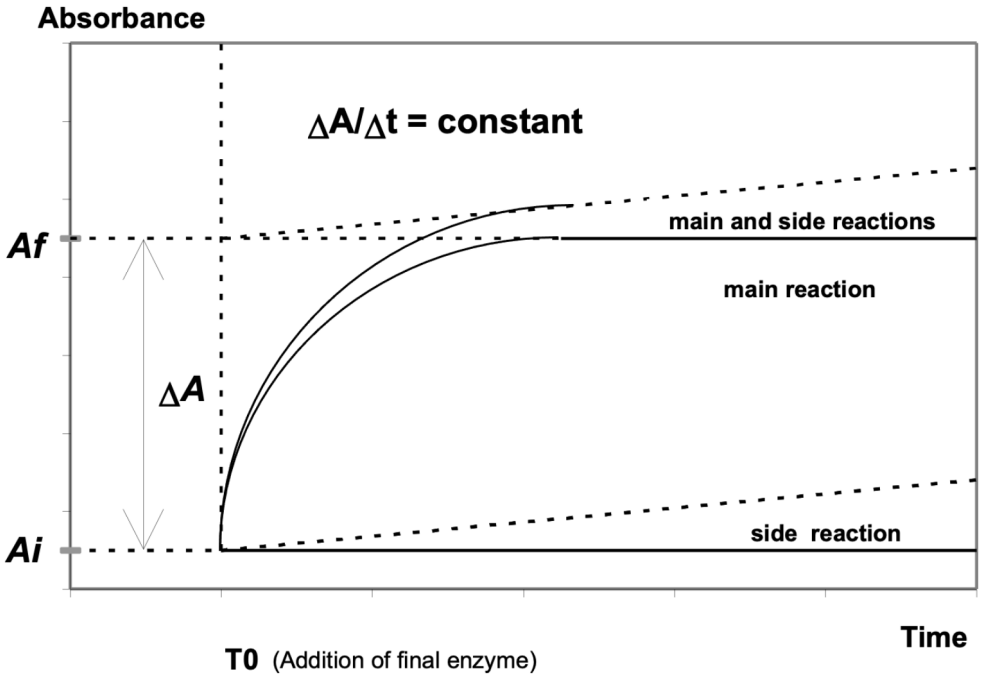

Figure 1: Side reaction

ANNEX 2 Interlaboratories trials statistical results

Year of the interlaboratory trial	1995
Number of laboratories	8
Number of samples	5 with addition of D-malic acid

Sample	A	B	C	D	E
Number of laboratories retained after elimination of laboratories presenting aberrant results Number of laboratories presenting aberrant results Number of accepted results	7 1 35	8 - 41	7 1 35	8 - 41	7 1 36
Average value (x) (mg/l)	161,7	65,9	33,1	106,9	111,0
Standard deviation of repeatability(sr) (mg/l) Relative standard deviation of repeatability(RSD_r) (%)	4,53 2,8	4,24 6,4	1,93 5,8	4,36 4,1	4,47 4,00
Limit of repeatability (r) (mg/l)	12,7	11,9	5,4	12,2	12,5
Standard deviation of reproducibility (sR) (mg/l) Relative standard deviation of reproducibility(RSD_R) (%)	9,26 5,7	7,24 11	5,89 17,8	6,36 5,9	6,08 5,5
Limit of reproducibility (R) (mg/l)	25,9	20,3	16,5	17,8	17,0

Types of samples

A	Red wine
B	Red wine
C	White wine
D	White wine

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E White wine

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