# OIV-MA-AS313-12A D-Malic acid (Enzymatic method)

**Type II method**

## 1. Principle

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

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

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

## 2. Reagents

Reagents that allow 30 determinations to be made are marketed in a set which includes:

- 1/ Flask 1 containing about 30 ml of solution of Hepes buffer acid [N-(2-hydroxyethyl)piperazine-N'-2-ethane sulfonic] pH = 9.0 and stabilizers;
- 2/ Flask 2 containing about 210 mg of NAD lyophilizate;
- 3/ Flask 3 (three flasks), containing D-MDH lyophilizate, with a titer of about 8 units.

*Preparation of the solutions*

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

Bring the solution to a temperature of 20-25 °C before using it.

Stability of the solutions

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

## 3. Apparatus

3.1. Spectrophotometer which is able to measure at the NADH absorption maximum of 340 nm. If this is not available, a spectrophotometer with a discontinuous spectrum source permitting measurements to be made at 334 or 365 nm may be used. Since absolute absorbance measurements are involved (i.e. calibration curves are not used, but standardization is made by consideration of the extinction coefficient of NADH), the wavelength scales and spectral absorbance of the apparatus must be checked.

3.2. Cells with a 1 cm path of glass or single-use cells.

3.3. Micropipettes capable of pipetting volumes between 0.01 and 2 ml.

## 4. Preparation of the sample

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

The quantity of D-malate in the cell must be between 2 µg and 50 µg; wine should be diluted so the malate concentration will be between 0.02 and 0.5 g/L or 0.02 and 0.3 g/L depending on the apparatus used.

Dilution table:

| Estimated quantity of D-malate/liter | Dilution with water | Dilution factor F |
|---|---|---|
| Measured at:<br>340 or 334 nm<br>365 nm | | |
| < 0.3 g<br>< 0.5 g | - | 1 |
| 0.3-3.0 g<br>0.5-5.0 g | 1 + 9 | 10 |

## 5. Procedure

With the spectrophotometer adjusted to a wavelength of 340 nm, determine the absorbance using 1 cm cells, with air as the zero absorbance (reference) standard (no cell in the optical path) or with water as the standard.

Place the following in the 1 cm cells:

| | Reference cell (mL) | Sample cell (mL) |
|---|---|---|
| Solution 1 | 1.00 mL | 1.00 mL |
| Solution 2 | 0.10 mL | 0.10 mL |
| Double-distilled Water | 1.80 mL | 1.70 mL |
| Sample | - | 0.10 mL |

Mix: after approximately 6 minutes, measure the absorbance of the reference and sample solutions ($A_1$).

Add

| | Reference | Sample |
|---|---|---|
| Solution 3 | 0.05 mL | 0.05 mL |

Mix: wait for the end of the reaction (about 20 min.) and measure the absorbance of the reference and sample solutions ($A_2$).

Determine the absorbance differences ($A_2 - A_1$) of the control ($\Delta A_T$) and trial ($\Delta A_D$).

Deduct the control absorbance difference from the trial absorbance difference:

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

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

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

## 6. Expression of the results

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

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

V = volume of the test in ml (here 2.95 mL)

□ = volume of the sample in ml (here 0.1 mL)

PM = molecular mass of the substance to be measured

(here, D-malic acid = 134.09)

d= cell path length in cm (here 1 cm)

□= absorption coefficient of NADH:

- at 340 nm = 6.3 (l $mmol^{-1}$ $cm^{-1}$)
- at 365 nm = 3.4 (l $mmol^{-1}$ $cm^{-1}$)
- at 334 nm = 6.18(l $mmol^{-1}$ $cm^{-1}$).

If a dilution was made during the preparation of the sample, multiply the result by the dilution factor. The concentration in D-malic acid is given in milligrams per liter (mg/L) without decimal.

## 7. Accuracy

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

*7.1. Repeatability*

The absolute difference between individual results obtained on an identical matter submitted to a trial by an operator using the same apparatus, within the shortest time interval, will not exceed the value of repeatability r in more than 5% of the cases. The value is: r = 11 mg/L.

7.2. *Reproducibility*

The absolute difference between individual results obtained on an identical material submitted to a test in two laboratories will not exceed the value of reproducibility R in more than 5% of the cases. The value is: R = 20 mg/L.

## 8. Comments

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

The wine content in the cuvette must not exceed 0.1mL to avoid a possible inhibition of enzymatic activity by polyphenols.

**Bibliogaphy**

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

**Annex 1 :How to treat side reactions**

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

With a normal reaction, absorbance reaches a constant value after a certain time, generally between 10 min and 20 min, according to the speed of the specific enzymatic reaction. However, when secondary reactions occur, the absorbance does not reach a constant value, but increases regularly with time; this type of process is commonly called a « side reaction ».

When this problem arises, one should measure the solution's absorbance at regular intervals (2 min to 5 min), after the required time for the standard solution to reach its final absorbance. When the absorbance increases regularly, carry out 5 or 6 measurements, than establish a graphic or calculated extrapolation, in order to obtain what the solution's absorbance would have been when the final enzyme was added (T0). The difference in extrapolated absorbance at this time (Af-Ai) is used for the calculation of the substrate concentration.

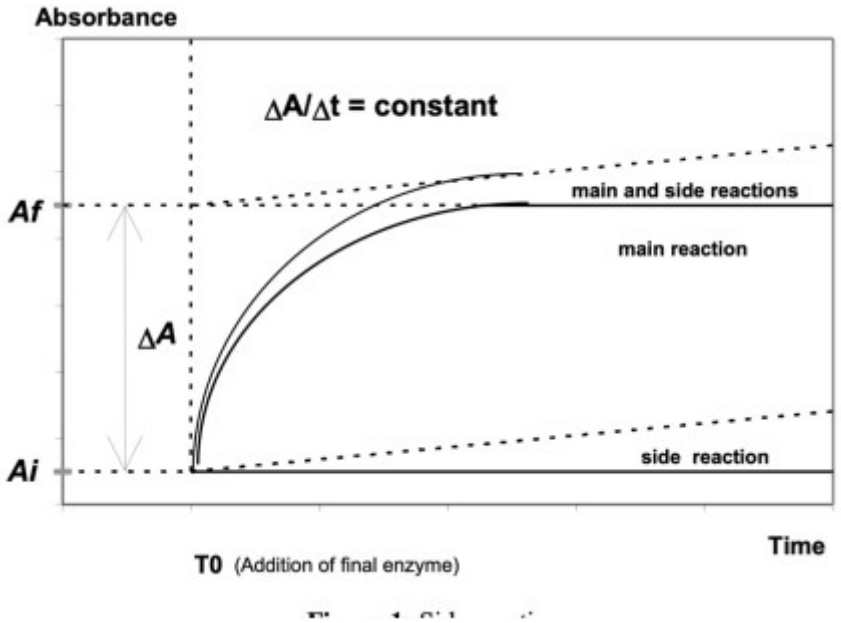


Figure 1: Side reaction

**Annex 2**

**Interlaboratory trials statistical results**

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

| Sample | A | B | C | D | E |
|---|---|---|---|---|---|
| Number of laboratories retained after elimination of laboratories presenting aberrant results<br>Number of laboratories presenting aberrant results<br>Number of accepted results | 7<br>1<br>35 | 8<br>-<br>41 | 7<br>1<br>35 | 8<br>-<br>41 | 7<br>1<br>36 |
| Average value(x□ (mg/L) | 161.7 | 65.9 | 33.1 | 106.9 | 111.0 |
| Standard deviation of repeatability ($s_r$) (mg/L)<br>Relative standard deviation of repeatability ($RSD_r$) (%) | 4.53<br>2.8 | 4.24<br>6.4 | 1.93<br>5.8 | 4.36<br>4.1 | 4.47<br>4.00 |
| Limit of repeatability (r) (mg/L) | 12.7 | 11.9 | 5.4 | 12.2 | 12.5 |
| Standard deviation of reproducibility ($s_R$) (mg/L)<br>Relative standard deviation of reproducibility ($RSD_R$) (%) | 9.26<br>5.7 | 7.24<br>11 | 5.89<br>17.8 | 6.36<br>5.9 | 6.08<br>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 |
| E | White wine |