UV assay for the determination of D-malic acid in foodstuffs and other sample materials
Test combination for 50 determinations

For in vitro use only
Lagerung bei 2 - 8 °C

This test was tested with the following matrices: lemonades and soft drinks, fruit juices, tomato juice, white and red grape juice, white and red wine.

Detailed results and further information on the validation data can be found in the validation report.

Other foods or sample materials can be tested and must be validated by the user.

1. Test principle

D-malic acid (D-malate) is oxidized by nicotinamide-adenine-dinucleotide (NAD) in the presence of D-malate-dehydrogenase (D-MDH) to oxaloacetate, which is immediately converted by this enzyme into pyruvate and carbon dioxide (CO_2).

D-Malate + NAD+ — D-MDH→ pyruvate + CO_2 + NADH + H^+

The amount of NADH formed is equivalent to the amount of D-malic acid. NADH can be measured on the basis of its absorption at 340 nm.

2. Reagents

2.1. Content & composition

The test is suitable for manual and automated processing. With manual processing, the reagents are sufficient for 50 determinations. The number of determinations for automated processing is increased by a multiple; however it depends on the device.

- Reagent 1: 2 x 50 mL with buffer, D-MDH
- Reagent 2: 2 x 12.5 mL with buffer, NAD^+

2.2. Reagent preparation

The reagents are ready-to-use and be allowed to reach room temperature (20 - 25 °C) before use. Do not interchange components between kits of different batches.

2.3. Storage & stability

The reagents are stable until the indicated shelf life (see labeling) even after opening at 2 - 8 °C if handled properly. Do not freeze reagents.

2.4. Safety & disposal

This product/test is only suitable for use within the scope of its intended purpose. The instruction for use must be strictly followed.

The general safety rules for working in chemical laboratories should be applied. Do not swallow! Avoid contact with skin and mucous membranes.

This kit may contain hazardous substances. For hazard notes on the contained substances, please refer to the appropriate safety data sheets (SDS) for this product. After use, the reagents can be disposed of with the laboratory waste. Packaging materials may be recycled.

3. Sample preparation

- Sample preparation for manual and automated testing is identical.
- Bring samples to room temperature before measurement.
- Use liquid, clear, and approximately neutral sample solutions directly or after dilution with distilled water to a concentration within the measuring range (see performance data) in the test.
- Adjust strongly acidic or alkaline samples to pH 7 - 8 by adding NaOH/KOH or HCl and incubate for 15 minutes.
- If necessary, adjust colored samples to pH 7 - 8 and measure against a blank sample.
- Filter or centrifuge cloudy solutions.
- Degas carbonated samples.
- Crush and homogenize solid and semi-solid samples and extract with water (e.g. 30 min at 60 °C). Filter, centrifuge, or use Carrez clarification if necessary.
- Weigh highly fatty samples into a measuring flask and extract with hot water; allow the sample solution to cool for fat separation (e.g. 15 min in an ice bath); fill the measuring flask with water up to the mark and filter the aqueous solution before testing.
- If possible, dilute samples with a citric acid or D-*threo*-isocitric acid concentration greater than or equal to 1 g/L or extend the second incubation to 30 minutes.

3.1. Determination of D-malic acid in wine and red grape must

- For precipitation of tartaric acid: Add 125 mg calcium carbonate and 5 mL ethanol (approx. 98 %) to 25 mL wine, stir for 2 minutes and, if necessary, adjust to pH 7 - 8 with KOH (1 M).
- Transfer the water quantitatively to a 50 mL measuring flask using dest. water into a 50 mL volumetric flask, fill to the mark, mix and incubate on ice for 30 minutes. Then filter through a pleated filter.
- Use clear, colorless or slightly colored filtrate in the test.
- Strongly colored filtrates and filtrates that show a slow reaction must be decolorized: Add 2 g of moist PVPP to 10 mL of filtrate, stir for 2 minutes, filter through a pleated filter or centrifuge (5 minutes, 4000 rpm) and use in the test.

3.2. Determination of D-malic acid in white grape must

- Use a syringe filter (PES, 0.22 µm) to filter a suitable amount of white grape must.
- Then add 125 mg calcium carbonate and 5 mL ethanol (approx. 98 %) to 25 ml of the sample, stir for 2 minutes and adjust the pH to 7 - 8 with HCl (2 M) if necessary.
- Transfer quantitatively to a 50 mL volumetric flask, fill with distilled water up to the mark and incubate on ice for 30 minutes.
- Filter and use the clear filtrate for the determination.

4. Manual assay procedure

Wavelength:	340 nm
Temperature:	20 - 37 °C (during the measurement)
Photometer alignment:	against air (without cuvette)
Measuring range:	14 - 500 mg/L (for 100 µL sample)

	Reagent blank	Samples / controls
Reagent 1	2000 µL	2000 µL
Sample / control	-	100 µL
Dist. water	100 µL	-
Mix, incubate for **3 min at 20 - 37 °C**. **Read absorbance A_1**, then addition of:		
Reagent 2	500 µL	500 µL
Mix, incubate for **25 min at 20 - 37 °C** and **read absorbance A_2**.		

4.1. Important notes for assay procedure

- The reagent blank value (water sample) **must be determined once for each run** and subtracted from **each** sample result.
- The test can generally be performed at temperatures between 20 and 37 °C. Please note that the incubation times for the test were determined and validated exclusively at 25 °C (manual processing) and 37 °C (automated processing). For measurements above or below the validated temperatures, it is recommended to carry out the measurements to the end point, provided that this range is specified in the instruction for use.
- The incubation times specified may vary depending on the prevailing laboratory conditions and pipetting accuracy. It is therefore recommended to wait until the end of the reaction during the first few runs and adjust the times if necessary.

- If the reaction has not come to a standstill after the specified incubation time, the extinctions should continue to be measured at 2-minute intervals until a constant increase in extinction per 2 minutes is achieved. If constant increases in absorbance are observed, the absorbance values A_2 are extrapolated to the time of addition of reagent 2.
- To achieve a sufficiently accurate result, the measured absorbance differences should normally be at least 0.050 - 0.100 absorbance units.
- If the measured absorbance difference is too small (e.g. $\Delta A < 0.02$), increase the sample volume (v) to a maximum of 1000 µl or prepare the sample solution again (higher initial weight or less dilution).
- Use separate tips for each sample extract and the control solutions to avoid cross-contamination; rinse the tip before pipetting.
- Stirring spatulas are recommended for mixing each individual cuvette. Remove these from the cuvette immediately before the absorbance measurements.
- A multistepper pipette is recommended for adding reagents 1 and reagent 2. Use a separate tip for each component.

5. Calculation of results

5.1. Calculation of sample solutions

5.1.1. Concentration of D-Malic acid

The extinction difference ΔA must be calculated for each sample:

$$\Delta A = (A_2 - A_1 \times df)_{\text{sample or control}} - (A_2 - A_1 \times df)_{\text{RB}}$$

df: dilution factor
RB: reagent blank

$$df_{100\mu L} = \frac{\text{sample volume + volume R1}}{\text{test volume}} = 0.808$$

The specified df value of 0.808 **applies to a base application of 100 µL**. An increase in sample volume is possible (max. 1000 µL; see validation report). **If reagent volumes remain constant**, this requires **conversion of the reagent dilution factor (df)**.
Increasing the sample volume may affect the test system. In general, this should be checked depending on the matrix. **The reagent blank value must be adjusted to the changed sample volume.**

The concentration of succinic acid is calculated using Lambert-Beer's law:

$$\mathbf{C_{D\text{-}Malic\ acid}\ [g/L]} = \frac{(V \times MW \times \Delta A)}{(\varepsilon \times d \times v \times 1000)} = \mathbf{0.5534 \times \Delta A} \times F$$

If the sample solution was diluted before measurement, this result has to be multiplied with the **sample pre-dilution factor F**.

V:	Test volume basic application [mL]	= 2.600
MW:	Molecular weight D-Malic acid [g/mol]	= 134.09
d:	Optical path [cm]	= 1.00
v:	Sample volume (basic application) [mL]	= 0.100
ε:	Extinction coefficient NADH [L/mmol x cm]	= 6.3 (at 340 nm)

5.2. Calculation for solid samples

When analyzing solid and semi-solid samples that have to be weighed in for the extraction of the sample, the content is related to the sample weight:

$$\text{Content}_{\text{D-Malic acid}}\ [g/100\ g] = \frac{C_{\text{D-Malic acid}}\ [g/L\ \text{sample solution}]}{\text{weigh-in}_{\text{sample}}\ \text{in g/L sample sol.}} \times 100$$

5.3. Controls & acceptance criteria

Control or reference samples should be included in each run for quality control purposes. For this purpose, we recommend Enzytec™ *Liquid* Multi-Acid Standard *low* (E8460) with 0.250 g/L D-malic acid or D-malic acid, Carl Roth (item no. 8633.1) ≥ 99% p.a. 134.09 g/mol.

To prepare a 5 g/L D-malic acid control solution, weigh the required amount of the substance (e.g. 125 mg D-malic acid) into a 25 mL volumetric flask, dissolve with water and fill the flask to the mark with water.

Other concentrations (e.g. for spiking experiments) can be prepared by diluting this stock solution with distilled water. The aliquots can be stored at 2 - 8 °C for one month.

The recovery of this multi-standard low and other aqueous control solutions should be 100 ± 5 %.

As a certified (standard) reference material, we recommend:

- LGC Fruit juice organic acid mixture (DRE-GS09000056WA); c = 1.998 ± 0.11 g/L (k = 2) D/L-malic acid.
- Standard wine of the Germanwine analysts (Standardwein der deutschen Weinanalytiker);
  https://www.weinanalytiker.de/standard-testloesung/
  - Label “orange” lot 1081608: c = 0.821 ± 0.0714 g/L D-malic acid; k = 1
  - Label “moosgrün“ lot 1071505: c = 0.397 ± 0.023 g/L D-malic acid: k = 1
- D-Malic acid analytical standard, Supelco (Sigma Aldrich) (Art. No. 46940-U), ampule of 100 mg

6. Performance data

6.1. Specificity & side activities

The determination is specific for D-malic acid. However, the enzyme exhibits a creeping reaction in the presence of L-tartaric acid, which occurs in large quantities in grape juice and in smaller quantities in wine.

The precipitation of L-tartaric acid with ethanol and calcium hydroxide, as described in section *3.1. Determination of D-malic acid in wine and red grape must* and section *3.2. Determination of D-malic acid in white grape must*, can prevent this creep reaction. A residual amount of L-tartaric acid remaining after precipitation may lead to a slight stray reaction, but this can be taken into account by mathematical extrapolation.

Tested concentrations of 0.2 g/L or 5 g/L benzoic acid, citric acid, acetic acid, glycolic acid, hydroxybutyric acid, isocitric acid, L-malic acid, D-lactic acid, L-lactic acid and D-tartaric acid showed no side activities or, in the case of L-ascorbic acid, a negligible side activity.

6.2. Interferences

α-Ketoglutaric acid and sulfite do not interfere at concentrations up to 0.5 g/L.

Similarly, pyrogallol at or below 0.05 g/L and *meso*-tartaric acid at or below 0.2 g/L showed no relevant interference.

6.3. Linearity, measuring range & sensitivity

Linearity is given up to 500 mg/L D-malic acid (100 µL sample volume), with the recommended measuring range between 14 and 500 mg/L and for a sample volume of 1000 µL at 1.2 - 50 mg/L.

The limit of detection (LoD) was determined according to the DIN 32645:2008-11 method in a buffered aqueous solution. This results in an LoD of 3.7 mg/L for a sample volume of 100 µL and 0.38 mg/L for a sample volume of 1000 µL.

The limit of quantification (LoQ) was determined using a precision profile and is 14 mg/L for a sample volume of 100 µL and 1.2 mg/L for a sample volume of 1000 µL.

The smallest difference in absorbance that the method can distinguish is ΔA = 0.005. For a sample volume of v = 1000 µL, this results in a calculated LoD of 0.37 mg/L. Based on ΔA = 0.010, an LoQ of 0.74 mg/L was calculated.

7. Supporting documents

On request, we offer the following documents:

- Enzytec™ *Liquid* D-Malic acid Validation report
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* D-Malic acid Excel template for results
- Enzytec™ *Liquid* D-Malic acid Technical information
- Enzytec™ *Liquid* Troubleshooting guide

Safety data sheets (SDS) and certificates of analysis (CoA) are available in digital form, quoting the batch number, via the following link:

https://eifu.r-biopharm.com/

8. Limits of this method

Test results may vary depending on the sample matrix, the individual test procedure and the laboratory environment. Detection and quantification limits depend on the respective sample matrix and the extraction method. For detailed results and further information, please refer to the current validation report.

For the present enzymatic test, only stated, exemplary matrices could be validated due to the large number of foodstuffs and other sample materials.

When analyzing a non-validated matrix, it is recommended to verify the results obtained by means of spike experiments. If necessary, a suitable sample preparation validation for the sample matrix of interest will need to be performed and validated.

9. Services & technical support

Upon request, we offer the following services, among others:

- Customized troubleshooting
- Workflow analysis
- Data & results analysis
- Customer workshops & webinars
- Automation: application support and technical service

10. Disclaimer

This information corresponds to our present state of technology and provides information on our products and their uses. R-Biopharm makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. Defective products will be replaced. There is no warranty of merchantability of this product, or of the fitness of the product for any purpose. R-Biopharm shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.