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
Store between 2 - 8 °C (36 - 46 °F)

This test was evaluated using selected samples of the following matrices: lemonades and soft drinks, fruit and vegetable juices, white and red wine.

Detailed results and information regarding associated validation data are found in the Validation Report.

The test may be used with other foods or samples material, provided that these are subjected to individual validation 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 / 36 - 46 °F) before use. Do not interchange components between kits of different batches.

2.3. Storage & stability

If stored as directed and between 2 - 8 °C (36 - 46 °F), reagents remain stable until the printed expiration date, even after opening. Reagents must not be frozen.

2.4. Safety & disposal

The test is intended solely for the intended use as described. The provided Instructions for Use must be strictly followed.

Follow standard chemical safety procedures when handling this product. Do not swallow. Avoid contact with skin or mucous membranes.

Detail safety information for individual components is available in the corresponding Safety Data Sheets (SDS).

Dispose of used reagents as laboratory waste in compliance with all relevant regulations. Packaging materials are to be recycled according to local regulations.

3. Sample preparation

- Manual and automated sample preparation is the same.
- Samples must be at room temperature for testing.
- Clear and nearly neutral liquid samples may be used directly or after dilution with distilled water to a concentration within the measuring range (see performance data) in the test.
- Neutralize 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.
- Cloudy samples must be filtered or centrifugated.
- Degas carbonated samples.
- Crush and homogenize solid and semi-solid samples and extract with water (e.g. 30 minutes at 60 °C / 140 °F). Filter, centrifuge, or use Carrez clarification if necessary.
- Highly fatty samples must be weighed into a volumetric flask and extracted with hot water. Allow the extract to cool for fat separation (e.g., through a 15 minutes ice bath). Fill the flask to the calibration mark with water and then filter the aqueous solution before testing.
- Dilute samples with a citric acid or D-*threo*-isocitric acid concentration greater than or equal to 1 g/L, if possible, 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.
- 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, if necessary, adjust to pH 7 - 8.
- 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 (68 - 99 °F)
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 minutes at 20 - 37 °C** (68 - 99 °F). **Read absorbance A_1**, then addition of:		
Reagent 2	500 µL	500 µL
Mix, incubate for **25 minutes at 20 - 37 °C** (68 - 99 °F) 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.
- Specified incubation times were verified and established **at 37 °C (99 °F)**. The test may generally perform within a range between **20 - 37 °C (68 - 99 °F)**.
- Use separate tips for each sample extract and the control solutions to avoid cross-contamination; rinse the tip before pipetting.
- A multistepper pipette is recommended for adding reagents 1 and reagent 2. Use a separate tip for each component.

- Stirring spatulas are recommended for mixing each individual cuvette. Remove these from the cuvette immediately before measuring the absorbance.
- Always wait for the reaction to end or for the absorbance to stabilize (at least during the first test runs or validation). If the absorbance has not stopped after the recommended incubation time, continue measuring at 5-minute intervals, for example, until a constant absorbance value is reached.
- If the measured absorbance difference of the samples is too small (< 0.020), the sample solution must be prepared again with a higher weight or a lower dilution.
- If the absorbance difference of the samples is very large (e.g., > 1.500), the sample solution must be diluted if necessary.

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; refer to validation report). **While keeping reagent volumes unchanged**, this requires **conversion of the reagent dilution factor (df)** accordingly.
Increases in the sample volume may influence test performance. Therefore, verify each matrix. Adjust the **reagent blank** value to match the **modified sample volume.**

The concentration of D-malic 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* (Art. No. E8460; 0.250 g/L D-malic acid) or D-malic acid for biochemistry (e.g., from Carl Roth, Art. No. 8633.1 ≥ 99% p.a., 134.09 g/mol. The recovery of this multi-standard *low* and other aqueous control solutions should be 100 ± 5 %.

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.

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, specific test implementation, and laboratory environmental conditions. Detection and quantification limits are dependent on respective sample matrices extraction procedures. Refer toto the current Validation Report for details.

For this test, only the matrices explicitly listed in the documentation were validated, due to the wide variety of food products and other potential sample materials.

When analysing non-validated matrices results should be verified by performing spiking (fortification) experiments. If appropriate or necessary, a suitable sample preparation procedure for the respective matrix must be developed and validated.

The responsibility for validating non-validated matrices and for ensuring the suitability of the assay for its intended use lies solely with the user.

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

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