UV method for approx. 32 assays

For laboratory use only
Store between +2 and +8°C

This method is contained in the German, Italian, Swiss food laws and in European regulations. Recommended e.g. by IFU (International Federation of Fruit Juice Producers), AIJN (Association of the Industry of Juices and Nectars), MEBAK (Mitteleuropäische Brautechnische Analysen-Kommission), OIV (Office International de la Vigne et du Vin). Standardized by DIN (Germany), EN (Europe), GOST (Russia), NEN (Holland), NF (France). Approved by AOAC (Association of Analytical Communities, USA).

Principle

L-Malate + NAD^+ ——— L-MDH ⟶ oxaloacetate + NADH + H^+
Oxaloacetate + L-glutamate ——— GOT ⟶ L-aspartate + 2-oxoglutarate

Ref.: Möllering, H. (1985) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 3rd ed., vol VII, pp. 39-47, Verlag Chemie, Weinheim, Deerfield Beach/Florida, Basel.

Assay performance

Wavelength:	340 nm (NADH), ε = 6.3 l x $mmol^{-1}$ x cm^{-1}
Light path:	1.00 cm (glass or plastic cuvettes)
Temperature:	+20 to +25°C
Assay volume:	2.220 ml
Measurement:	against air or against water
Sample solution:	0.5 to 30 µg L-malic acid in 0.100 to 1.000 ml sample solution

Reagents

1: Approx. 34 ml Glycylglycine buffer, pH approx. 10.0, approx. 490 mg L-glutamic acid (for stability see pack label). *The solution is ready for use.*

2: NAD, lyophilizate, approx. 250 mg (for stability see pack label). *Dissolve contents of bottle # 2 with 7 ml redist. water.* The solution is stable for 1 month at +2 to +8°C, resp. for 2 months at -15 to -25°C.

3: Approx. 0.4 ml glutamate oxaloacetate transaminase (GOT) suspension (approx. 160 U) in ammonium sulfate (for stability see pack label). *The suspension is ready for use.* Swirl bottle carefully before the suspension is pipetted.

4: Approx. 0.4 ml L-malate dehydrogenase (L-MDH) solution (approx. 2400 U) in glycerol (for stability see pack label). T*he solution is ready for use.*

In addition (not contained in the kit):
Standard solution L-malic acid at 0.15 g/l, for test control only.

The reagents for the determination of L-malic acid are not hazardous. The general safety rules for the work in chemical laboratories should be applied. After use the reagents can be disposed of with the laboratory waste. Packaging materials may be recycled.

Procedure

Pipette into cuvettes	**Blank**	**Standard[1]**	**Sample[2]**	**Rerun assay[3]**	**Assay with internal standard[4]**	**High sensitive assay[5]**
Glycylglycine buffer # 1	1.000 ml	1.000 ml	1.000 ml	1.000 ml	1.000 ml	1.000 ml
NAD solution # 2	0.200 ml	0.200 ml	0.200 ml	0.200 ml	0.200 ml	0.200 ml
GOT suspension # 3	0.010 ml	0.010 ml	0.010 ml	0.010 ml	0.010 ml	0.010 ml
Sample solution[6] (e.g. 0.02 to 0.15 g/l L-malic acid)	**-**	**-**	**0.100 ml**	**0.200 ml**	**0.100 ml**	**1.000 ml**
Standard solution[6] (e.g. 0.15 g/l L-malic acid)	-	0.100 ml	-	-	0.100 ml	-
Redist. Water	1.000 ml	0.900 ml	0.900 ml	0.800 ml	0.800 ml	-
Mix[7], after approx. 3 min read the absorbances (A_1). Add:						
L-MDH solution # 4	0.010 ml	0.010 ml	0.010 ml	0.010 ml	0.010 ml	0.010 ml
Mix[7], after completion of the reaction (approx. 5 to 10 min) read the absorbances of blank and the other assays (A_2) immediately one after the other. Repeat absorbance reading after another 2 min[8].						

Notes

1 Run a "standard" to see "accidents" in analysis. The measurement of the standard is not necessary for calculating results.
2 This assay together with the blank is a single determination.
3 In the case of a double determination, run two assays with different sample volumes. The absorbance differences measured have to be proportional to the sample volumes. Calculate with the resp. volume v.
4 Recovery = $[(\Delta A_{sample+standard} - \Delta A_{sample}) / \Delta A_{standard}] \times 100$ [%]
5 Assay recommended in the case of trace level compound analysis with sample volume increased up to 1.000 ml (0.0005 to 0.015 g/l of L-malic acid).
6 Before dispensing, rinse the enzyme pipette, resp. the tip of the piston pipette with sample resp. with standard solution.
7 e.g. with a plastic spatula, or after closing the cuvette with Parafilm (trademark of American Can Co., Greenwich Ct., USA)
8 The reaction has stopped when the absorbance is constant. If the reaction has not stopped in assays containing sample solution, continue to read absorbances until they increase constantly over e. g. 2 min. Extrapolate absorbances to the time of the addition of L-MDH (# 4).
9 Results from acidimetric determination of 'total acid' calculated as malic acid (protons are measured) cannot be compared with enzymatic analysis

Sample preparation

1. Dilute *clear, colourless and almost neutral liquid samples* to get a sample solution with 0.02 to 0.15 g L-malic acid /l.
2. Filter or centrifuge *turbid solutions*, dilute (see pt. 1).
3. Degas *samples containing carbon dioxide*, e.g. by filtration, or add $NaHCO_3$ till the solution is slightly alkaline, dilute (see pt. 1).
4. Adjust *acid (esp. slightly coloured) solutions* with KOH or NaOH to approx. pH 8 to 10, incubate a few minutes, or dilute (see pt. 1) without pH adjustment in the case of colourless samples.
5. Measure *"coloured samples"* (adjusted to pH 8) against a sample blank.
6. Treat *"strongly coloured solutions"* used undiluted with PVPP, e. g. 1 g/100 ml, mix, incubate a few minutes, filter.
7. Crush (corn size < 0.3 mm) or homogenize *solid or semi-solid (pasty) samples*, extract with water, or dissolve in water, filter and dilute if necessary (see pt. 1).
8. Extract *fat containing samples* with hot water at a temperature above the melting point of fat, e.g. in a 100 ml volumetric flask. Adjust to +20°C, fill volumetric flask to the mark. Store in ice or in refrigerator for approx. 15 resp. 30 min, filter.
9. Clarification with Carrez reaction or deproteinization with perchloric acid is not recommended for L-malic acid, because of low recoveries.

Calculation[9]

$\Delta A = (A_2 - A_1)_{sample,\ resp.\ standard} - (A_2 - A_1)_{blank}$

$c = (V \times MW \times \Delta A) / (\varepsilon \times d \times v \times 1000)$ [g L-malic acid/l sample solution]

$c = (2.220 \times 134.09 \times \Delta A) / (6.3 \times 1.00 \times 0.100 \times 1000) =$ 0.4725 x Δ A [g/l L-malic acid]

If the sample has been diluted during preparation, multiply the result with the dilution factor F.

When analyzing samples which are weighed out for sample preparation, calculate the content from the amount weighed:

$$\text{Content}_{\text{L-malic acid}} = \frac{c_{\text{L-malic acid}}\ [\text{g/l sample solution}]}{\text{weight}_{\text{sample}}\ [\text{in g/l sample solution}]} \times 100\ [\text{g/100 g}]$$

Assay characteristics

1. *Specific* for L-malic acid (the L-malate ion reacts). In the analysis of commercial L-malic acid results of approx. 99 % have to be expected.
2. *Sensitivity:* 0.25 mg/l (Δ A = 0.005; v = 1.000 ml; V = 2.220 ml)
3. *Detection limit*: 0.5 mg/l (Δ A = 0.010; v = 1.000 ml; V = 2.220 ml)
4. *Linearity:* 0.5 µg/assay (v = 1.000 ml; V = 2.220 ml)
   to 30 µg/assay (v = 0.100 ml; V = 2.220 ml)
5. *Precision:* Δ A = +/- 0.005 to 0.010 absorbance units
   CV = approx. 1 to 2 %

   Fruit juice: $r = 0.014 + 0.030 \times c_{\text{L-malic acid in g/l}}$ [g/l]
   $R = 0.032 + 0.070 \times c_{\text{L-malic acid in g/l}}$ [g/l]

   Wine: $r = 0.03 + 0.034 \times c_{\text{L-malic acid in g/l}}$ [g/l]
   $R = 0.05 + 0.071 \times c_{\text{L-malic acid in g/l}}$ [g/l]
6. *Interferences*: There may be a reagent dependent creep reaction after the conversion of L-malate at A_2. An extrapolation of the absorbances back to the time of the addition of L-MDH may not necessary when the absorbances of blank and sample assays are measured immediately one after the other.