UV method for approx. 32 assays

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

The method is contained in the German, Swiss and Spanish food laws. Recommended e. g. by IFU, AIJN. Standardized by DIN, EN, NF, NEN, GOST.

Principle

D-Isocitrate + $NADP^+$ ——— ICDH ⟶ 2-oxoglutarate + CO_2 + NADPH + H^+
D-Isocitric acid esters + H_2O ——— pH 9-10 ⟶ D-isocitrate + alcohol
D-Isocitric acid lactone + H_2O ——— pH 9-10 ⟶ D-isocitrate

Ref.: Beutler, H.-O. (1985) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 3rd ed., vol VII, pp. 13-19, 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: 3.050 ml
Measurement: against air or against water
Sample solution: 2 to 100 µg D-Isocitric acid in 0.100 to 2.000 ml sample solution

Reagents

1: Approx. 33 ml Imidazole buffer, pH approx. 7.1 (for stability see pack label). *The solution is ready for use*.

2: NADP, lyophilizate, approx. 50 mg (for stability see pack label). Dissolve contents of bottle # 2 with the whole contents of bottle # 1 (= solution # 2). The solution is stable for 1 month at +2 to +8 °C, resp. for 2 months at -15 to -25 °C.

3: Approx. 1.8 ml ammonium sulphate containing isocitrate dehydrogenase (ICDH, approx. 10 U). For stability see pack label. The suspension is ready for use. Swirl bottle carefully before the suspension is pipetted..

In addition (not contained in the kit):
Standard solution D-isocitric acid, 0.5 g/l, for test control only.

The reagents 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]**
Imidazole buffer with NADP, solution # 2	1.000 ml	1.000 ml	1.000 ml	1.000 ml	1.000 ml	1.000 ml
Sample solution[6] (e.g. 0.05 to 0.5 g D-isocitric acid/l)	-	-	**0.100 ml**	**0.200 ml**	**0.100 ml**	**2.000 ml**
Standard solution[6] (e.g. 0.5 g D-isocitric acid/l)	-	0.100 ml	-	-	0.100 ml	-
Redist. Water	2.000 ml	1.900 ml	1.900 ml	1.800 ml	1.800 ml	-
Mix[7], after approx. 3 min read the absorbances (A_1). Add:						
ICDH, suspension # 3	0.050 ml	0.050 ml	0.050 ml	0.050 ml	0.050 ml	0.050 ml
Mix[7], after completion of the reaction (approx. 10 min) read the absorbance (A_2). 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. 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 2.000 ml (0.001 to 0.05 g D-isocitric acid / l).
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 the absorbances increase constantly over 2 min. Extrapolate absorbances to the time of the addition of ICDH (suspension # 3)

Calculation

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

c = (V x MW x ΔA) / (ε x d x v x 1000) [g D-isocitric acid/l sample solution]

c = (3.050 x 192.1 x ΔA) / (6.3 x 1.00 x 0.100 x 1000) **= 0.9300 x ΔA [g/l D-isocitric acid in sample solution]**

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

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

$$Content_{D\text{-isocitric acid}} = \frac{c_{D\text{-isocitric acid}}\ [g/l\ sample\ solution]}{weight_{sample}\ [in\ g/l\ sample\ solution]} \times 100\ [g/100\ g]$$

Sample preparation

1. Dilute *clear, colorless and almost neutral liquid* samples *to get a sample* solution with 0.05 to 0.5 g D-isocitric 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 colored) solutions* with KOH or NaOH to approx. pH 7 to 7.5, incubate a few minutes, or dilute (see pt. 1) without pH adjustment in the case of colorless samples.
5. Measure *"colored samples"* (adjusted to pH 8) against a sample blank.
6. Treat *"strongly colored solutions"* used undiluted with PVPP or with polyamide, 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 (see pt. 1) if necessary.
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. *Sample preparation acc. to Wallrauch:*
   Incubate 10 ml of *neutralized* fruit juice with 5 ml NaOH (4 M) for 10 min. Add successively 5 ml HCl (4 M), 2 ml ammonia (25 %), 3 ml $BaCl_2$ (30 g/100 ml), 20 ml acetone. Mix and incubate for 10 min. Centrifuge for 5 min.
   Decant supernatant, add 20 ml Na_2SO_4 (71 g/l), suspend precipitate with a glass rod, heat in boiling water for 10 min while stirring. Adjust to +20 to +25 °C, transfer into a 50 ml volumetric flask, fill up to mark with Tris solution (2.42 g/100 ml, adjusted to pH 7.0 with HCl (1 M); contains 35 mg EDTA). Transfer into an Erlenmeyer flask containing 1 g activated charcoal, mix, incubate for 5 min, filter. Use 1.000 ml of the filtrate for the assay.

Assay characteristics

1. *Specific* — for D-isocitric acid. In the analysis of commercial tri-sodium-D,L-isocitrate dihydrate (MW = 294.1) results of approx. 50 % have to be expected (only the D-isocitrate reacts).
2. *Sensitivity:* — 0.25 mg/l (ΔA = 0.005; v = 2.000 ml; V = 3.050 ml)
3. *Detection limit:* — 1 mg/l (ΔA = 0.020; v = 2.000 ml; V = 3.050 ml)
4. *Linearity:* — 2 µg/assay (v = 2.000 ml; V = 3.050 ml)
   to 100 µg/assay (v = 0.100 ml; V = 3.050 ml)
5. *Precision:* — ΔA = +/- 0.005 to 0.010 absorbance units
   CV = approx. 1 to 2 % (CV is higher in trace level compound analysis)
   Fruit juice: r = 2.5 mg/l
   s(r) = +/- 0.8944 mg/l
   R = 4.4 mg/l
   s(R) = +/- 1.5665 mg/l
6. *Interferences:* — Sulfite (> 30 µg/assay) causes a slight creep reaction because of the decomposition of NADPH. Extrapolate absorbance A_2 to the time of the addition of ICDH (suspension # 3) to the assay.
7. *Technical information:* the "Wallrauch sample preparation technique" is often used in fruit juice analysis.