UV assay for the determination of D-*threo*-isocitric acid and its esters in foodstuff and other sample materials

Test combination for 50 determinations

For *in vitro* use only

Store between 2 - 8 °C

1. Test principle

D-*threo*-isocitric acid (D-*threo*-isocitrate) is converted to oxoglutarate by nicotinamide-adenine-dinucleotide-phosphate (NADP) in the presence of the enzyme isocitrate dehydrogenase (IC-DH):

D-isocitrate + $NADP^+$ — IC-DH → 2-oxoglutarate + CO_2 + NADPH + H^+

In this process, NADP is reduced to NADPH. The amount of NADPH formed in this reaction is equivalent to the converted amount of D-*threo*-isocitric acid and is measured at a wavelength of 340 nm.

Bound D-isocitric acid is determined after alkaline hydrolysis according to the same principle.

D-isocitric acid ester + H_2O — pH 9-10 → D-isocitrate + alcohol

D-isocitric acid lactone + H_2O — pH 9-10 → D-isocitrate

2. Reagents

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, NADP
- Reagent 2: 2 x 12.5 mL with buffer, IC-DH
- Assay control: 1 x 2.0 mL with D-*threo*-isocitric acid (0.05 g/L)

2.1. 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.

2.2. Storage & stability

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

2.3. Safety & disposal

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.
- The samples should be brought to room temperature before measurement.
- Use liquid, clear and almost neutral sample solutions directly or after dilution with dist. water to a concentration within the measuring range (see performance data).
- Filter or centrifuge turbid solutions.
- Degas samples containing carbonic acid.

3.1. Determination of free D-threo-isocitric acid

Strongly colored juices applied undiluted: neutralize 25 mL sample with 2 M NaOH to pH 7 - 7.5, fill up to 50 mL, add 0.5 g PVPP, then filter or centrifuge.

3.2. Determination of total D-isocitric acid according to Wallrauch & Greiner

The determination of D-isocitric acid and its esters in juices and fruit nectars can also be carried out advantageously according to the method of Wallrauch and Greiner. For precise measurements, the use of a suitable quality of activated carbon is necessary.

Reagents:

- Acetone, p. a.
- Ammonia solution, 25 %, p. a.
- Barium chloride, $BaCl_2 \times 2\ H_2O$, p. a.
- Sodium sulfate, p. a.
- Activated carbon
- Tris(hydroxymethyl)aminomethane, Tris
- Ethylendiamintetraacetate, EDTA-$Na_2H_2 \times 2\ H_2O$

Preparation of the solutions:

- Barium chloride solution: dissolve 30 g $BaCl_2 \times 2\ H_2O$ with bidest. water and fill up to 100 mL.
- Sodium sulfate solution: dissolve 71 g Na_2SO_4 with bidest. water and fill up to 1 L.
- Tris buffer solution, pH 7.0: dissolve 2.42 g Tris and 35 mg EDTA with 80 mL bidest. water, adjust to pH 7.0 with hydrochloric acid (1 M) and fill up to 100 mL with bidest. water.

Performance of the determination (precipitation method):

In a 100 mL centrifuge tube, leave 10 mL sample solution, after neutralization if necessary, with 5 mL sodium hydroxide solution (4 M) for 10 min. Then add successively 5 mL hydrochloric acid (4 M), 2 mL ammonia solution (25 %), 3 mL $BaCl_2$ solution and 20 mL acetone. Mix thoroughly and leave to stand for 10 min. Centrifuge the mixture for 5 min. Carefully decant the supernatant liquid, add 20 mL Na_2SO_4 solution to the precipitate and stir the precipitate in the centrifuge tube with a glass stick. Heat in a boiling water bath for 10 min with frequent stirring. After cooling, quantitatively transfer the content of the centrifuge tube into a 50 mL volumetric flask and fill up to the mark with Tris buffer solution. Pour the content of the volumetric flask into an Erlenmeyer flask into which 1 g of activated carbon has previously been weighed and mix, then leave to stand for 5 min and filter. Use the colourless, clear solution with v = 1.000 mL for the test. Take the changed sample volume "v" into account in the calculation formula.

3.3. Simplified preparation method for the determination of total D-isocitric acid and its esters in juices and fruit nectars:

- 2.5 mL sample + 1.25 mL 2 M NaOH, mix, let stand for 10 min at room temperature.
- Then add 2.5 mL of a Tris-HCl solution (2 M HCl solution and 4 M Tris buffer solution, pH 7.5, mix 1:1) and mix.
- Centrifuge for 5 min at 4000 rpm (corresponds to approx. 3450 x *g*).
- Use 100 µL of the clear supernatant in the test (note: take the dilution factor of 2.5 into account when evaluating!).

4. Assays performance

Wavelength: 340 nm
Temperature: 20 - 37 °C (during the measurement)
Measurement: against air (without cuvette) or water
Measuring range: 6 - 1500 mg/L

	Reagent blank	Sample / control
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 15 min at 20 - 37 °C and read absorbance A_2.		

The reagent blank value must be determined once for each run and subtracted from each sample result.

5. Calculation of results

5.1. Calculation of sample solutions

5.1.1. Total concentration of D-threo-isocitric acid

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

df: Dilution factor
RB: Reagent blank

$$df = \frac{\text{sample volume + R1}}{\text{test volume}} = 0.808$$

Increasing the sample volume (up to max. 1000 µL) with unchanged reagent volumes requires conversion of the reagent dilution factor (df). If the volume is increased, the test system may be affected. In general, this must be checked depending on the matrix.

$$\mathbf{C_{D\text{-}threo\text{-}isocitrate}\ [g/L]} = \frac{(V \times MW \times \Delta A)}{(\varepsilon \times d \times v \times 1000)} = \mathbf{0.7929 \times \Delta A}$$

V:	Test volume basic application [mL]	= 2.600
MW:	Molecular weight [g/mol]	= 192.13
d:	Optical path [cm]	= 1.00
v:	Sample volume [mL]	= 0.100
ε:	Extinction coefficient NADH [L/mmol x cm]	= 6.3 (at 340 nm)

5.2. Calculation of solid samples

$$\text{Content}_{D\text{-}threo\text{-}isocitrate}\ [g/100\ g] = \frac{C_{D\text{-}threo\text{-}isocitrate}\ [g/L\ \text{sample solution}]}{\text{weight}_{sample}\ [g/L\ \text{sample solution}]} \times 100$$

5.3. Controls & acceptance criteria

Controls or reference samples should be carried along for quality control during each run. For this purpose, we recommend the use of the enclosed D-*threo*-isocitric acid assay control.
The recovery of the assay control should be within 100 ± 5 %.

6. Performance data

6.1. Specificity, side activities, interferences

The test is specific for D-*threo*-isocitric acid and shows no side activities or interferences with other relevant acids. SO_2 interferes at concentrations below 50 mg/L.

6.2. Linearity, measuring range & sensitivity

Linearity is given up to 1900 mg/L D-*threo*-isocitrate, with the recommended measuring range between 6 and 1500 mg/L (sample volume of 100 µL). If this range is exceeded, the samples should be diluted with dist. water to a concentration within the measuring range. The dilution factor must be taken into account in the calculation.

The lower limit of detection (LoD) and the limit of quantification (LoQ) were determined according to the method DIN 32645:2008-11 in buffered aqueous solution:

- Sample volume v = 100 µL: LoD = 1.0 mg/L
  LoQ = 6.0 mg/L
- Sample volume v = 1000 µL: LoD = 0.15 mg/L
  LoQ = 0.40 mg/L

7. Supporting documents

On request, we offer the following documents:

- Enzytec™ *Liquid* Validation reports
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* Excel templates for results calculation
- Enzytec™ *Liquid* Troubleshooting guide

Safety data sheets (SDS) und certificates of analysis (CoA) are available in digital form under the following link
https://eifu.r-biopharm.com/

8. Services & technical support

On request, we offer the following services:

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

9. Disclaimer

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