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 (36 – 46 °F)

This test was evaluated using selected samples of the following matrices: fruit and vegetable juices.

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-*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

Note: The Enzytec™ *Liquid* D-Isocitric acid test specifically targets Ds-(+)-threo-isocitric acid, the only naturally occurring isomer in plants and animals.

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, 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)

2.2. Reagent preparation

The reagents are ready-to-use and be allowed to reach room temperature (20 – 25 °C / 68 – 77 °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

3.1. General

- Sample preparation for manual and automated testing is the same.
- Bring samples to room temperature before measurement.
- Use liquid, clear and almost neutral sample solutions directly or after dilution with distilled water to a concentration within the measuring range (see performance data).
- Neutralize **strongly** acidic or alkaline samples by adding KOH/NaOH or HCl, respectively (pH approx. 7 – 7.5).
- For turbid test samples (e.g., juices): filter the test solution through a pleated paper or syringe filter. Alternatively, centrifuge the sample in a test tube at (3000 g for at least 5 minutes is recommended) until a clear filtrate or supernatant is obtained.
- Degas samples containing carbonic acid, e.g. by stirring in a beaker, filtration or centrifugation.
- Crush and homogenize solid and semi-solid samples and extract or dissolve them in water. Filter if necessary.
- Weigh samples with high fat content into a volumetric flask and extract with hot water (extraction temperature above the melting point of the fat in question); allow to cool to separate the fat; fill the flask with water to the mark, place it in an ice bath for 15 minutes, and filter before testing.
- In case of higher sample volumes (up to 1000 µL), check the pH value of the test solution and neutralize in case of any doubt.

3.2. Determination of free D-threo-isocitric acid in juices and wines

- Decolorize **strongly** colored juices and wines, that are measured undiluted, using polyvinylpolypyrrolidone (PVPP).
- Neutralize 25 mL sample with 2 M NaOH to a pH value of approx. 7 – 7.5 and fill up to 50 mL with distilled water.
- Let the solution stand for about 10 minutes at 20 – 25 °C (68 – 77 °F).
- Add 0.5 g of PVPP and stir for 1 minute, then filter or centrifuge.
- Use the clear, possibly slightly colored solution for the assay.
- Strongly acidic sample solutions, that are used undiluted in the test may need to be neutralized beforehand.

3.3. 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 & Greiner*. For precise measurements, the use of a suitable quality of activated carbon is necessary.

3.3.1. Reagents

- Acetone, p. a.
- Ammonia solution, 25 %, p. a.
- Barium chloride, $BaCl_2$ × 2 H2O, p. a.
- Sodium sulfate, p. a.
- Activated carbon
- Tris(hydroxymethyl)aminomethane, Tris
- Ethylendiamintetraacetate, EDTA-Na_2H_2 × 2 H_2O

3.3.2. Preparation of the solutions

- Barium chloride solution: dissolve 30 g $BaCl_2$ × 2 H_2O with distilled water and fill up to 100 mL.
- Sodium sulfate solution: dissolve 71 g Na_2SO_4 with distilled 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 distilled water, adjust to pH 7.0 with HCl (1 M) and fill up to 100 mL with distilled water.

3.3.3. Procedure of the determination (precipitation method)

- Leave a 10 mL sample solution in a 100 mL centrifuge tube for 10 minutes, after neutralization with 5 mL of 4 M NaOH solution if necessary.
- Add 5 mL of hydrochloric acid (4 M), 2 mL of ammonia solution (25 %), 3 mL of $BaCl_2$ solution, and 20 mL of acetone, one after the other. Mix thoroughly and leave to stand for 10 minutes.
- Centrifuge the mixture for 5 minutes. Carefully decant the supernatant liquid and add 20 ml of sodium sulphate solution to the precipitate in the centrifuge tube. Stir the precipitate with a glass stick.
- Heat in a boiling water bath for 10 minutes, stirring frequently.
- Let the solution cool, transfer the content quantitatively into a 50 ml volumetric flask and fill up to the mark with Tris buffer solution.
- Pour the contents of the volumetric flask into an Erlenmeyer flask containing 1 g of activated carbon, mix, leave to stand for 5 minutes and filter.
- Use the colorless, clear solution with a volume of v = 1000 µL for the test (consider the change in the calculation formula).

3.4. 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, leave for 10 minutes at room temperature.
- 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 minutes at 4000 rpm (approx. 3450 g).
- Use 100 µL of the clear supernatant in the test.
- Take the dilution factor of 2.5 into account when evaluating.

4. Manual test procedure

Wavelength:	340 nm
Temperature (measurement):	20 – 37 °C (68 – 99 °F)
Photometer alignment:	against air (without cuvette)
Measuring range:	6 – 1500 mg/L (for 100 µL)

	Reagent blank	Samples / controls
Reagent 1 **Sample / control** **Dist. water**	2000 µL - 100 µL	2000 µL 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 **15 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 **in each series of measurement** and subtracted from **each** sample result.
- Specified incubation times were validated at 37 °C (77 °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. 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 a creep reaction occurs, the reaction will not have finished after stated incubation times and will typically show a constant increase of ΔA. Calculate the analyte-specific ΔA value by plotting the absorbance values against time and performing a linear regression to determine the rate of increase in ΔA per minute related to the creep reaction. Then, extrapolate the absorbance to the time at which reagent 2 is added.
- 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. Total concentration of D-threo-isocitric acid

The extinction difference ΔA must be calculated for each sample:

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

df: Dilution factor
RB: Reagent blank

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

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**.

Increasing the sample volume may influence test performance. This must generally be checked depending on the matrix. **The reagent blank value must be adjusted to the changed sample volume.**

The concentration of D-*threo*-isocitric acid is calculated using Lambert-Beer's law:

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

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 isocitric acid [g/mol]	= 192.13
d:	Optical path [cm]	= 1.00
v:	Sample volume [mL]	= 0.100
ε:	Extinction coefficient NADPH [L/mmol x cm]	= 6.3 (at 340 nm)

5.2. Calculation of 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:

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

5.3. Controls & acceptance criteria

Control or reference samples should be included in each run for quality control purposes. For this purpose, we recommend the included assay control solution containing 0.050 g/L D-*threo*-isocitric acid.

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

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

- NIST Standard Reference Material 3282, *Low Calorie Cranberry Juice Cocktail*

6. Performance data

6.1. Specificity & side activities

The enzyme isocitrate dehydrogenase specifically catalyzes the oxidation of D-*threo*-isocitrate. The test shows no side-activity with other relevant acids.

6.2. Interferences

An interference study was conducted in the presence of 0.5 g/L isocitric acid and equal volumes of investigated substances with different concentrations. The tested substances included: L-malic acid and citric acid (75 g/L each), glyoxylate, succinate, 2-oxoglutarate (12.5 g/L each), L-ascorbic acid, D-fructose, D-glucose, D-galactose, lactose, maltose, and sucrose (10 g/L each) and glycerol (1 g/L). These substances do not have an interfering effect in the determination of isocitric acid.

Sulfur dioxide (SO_2) – if present in a fruit or vegetable juice – is interfering with the system and showed an influence of the A1 measurement at high concentrations between 1.25 up to 10 g/L in the presence of 0.5 g/L isocitric acid.

6.3. Linearity, measuring range & sensitivity

Linearity is given up to 1900 mg/L D-isocitric acid (sample volume of 100 µL) with a recommended measuring range of 6 – 1500 mg/L.

If this range is exceeded, the samples should be diluted with distilled water to a concentration within the measuring range. The dilution factor F 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* D-Isocitric acid Validation reports
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* D-Isocitric acid Excel template for results
- Enzytec™ Liquid D-Isocitric 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

On request, we offer the following services:

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

10. Disclaimer

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