D-Isocitric acid

UV-method
for the determination of D-isocitric acid and its esters (or lactones, respectively) in foodstuffs and other materials

Determination of total D-isocitric acid according to Wallrauch, see pt. 13
Cat. No. 0 414 433
Test-Combination for approx. 30 determinations

Bottle 2
- 1. Dissolve contents of bottle 2 with the whole contents of bottle 1 (= solution 2).
- Bottle 2 with approx. 60 mg lyophilizate, consisting of:
  (1) D-Isocitric acid (D-isocitrate) + H₂O pH 9-10
  (2) D-Isocitric acid lactone + H₂O pH 9-10
  D-isocitrate + alcohol

Bottle 3
- 2. Dissolve contents of bottle 3 with 1.8 ml redist. water (= solution 3).
- 3. D-Isocitric acid ester + H₂O pH 9-10
- 4. D-Isocitric acid lactone + H₂O pH 9-10

The Test-Combination contains
- 1. Bottle 1 with approx. 30 ml solution, consisting of:
  - imidazole buffer, pH approx. 7.
- 2. Bottle 2 with approx. 30 mg lyophilizate, consisting of:
  - NADP, approx. 45 mg; manganese sulfate
  - imidazole buffer, pH approx. 7.1
- 3. Bottle 3 with lyophilized ICDH, approx. 5 U

Preparation of solutions
- 1. Dissolve contents of bottle 2 with the whole contents of bottle 1 (= solution 2).
- 2. Dissolve contents of bottle 3 with 1.8 ml redist. water (= solution 3).

Stability of reagents
- The contents of bottle 1 are stable at 2-8°C (see pack label).
- Bring solution 1 to 20-25°C before use.
- The contents of bottle 2 are stable at 2-8°C (see pack label).
- Solution 2 is stable for 4 weeks at 2-8°C or for approx. 2 months at -15 to -25°C.
- The contents of bottle 3 are stable at 2-8°C (see pack label).
- Solution 3 is stable for 4 weeks at 2-8°C or for 2 months at -15 to -25°C.

Procedure
- Wavelength: 340 nm, Hg 365 nm or Hg 334 nm
- Glass cuvette: 1.00 cm light path
- Temperature: 20-25°C
- Final volume: 3.050 ml
- Read against air (without a cuvette in the light path) or against water
- Sample solution: 2-100 µg D-isocitric acid/assay (in 0.100-2.00 ml sample volume)
- Pipette into cuvettes: Blank | Sample
  - solution 2 | 1.000 ml | 1.000 ml
  - sample solution* | - | 0.100 ml
  - redist. water | 2.000 ml | 1.900 ml
- Mix**, read absorbances of the solutions (A₁) after approx. 3 min. Start reaction by addition of:
  - solution 3 | 0.050 ml | 0.050 ml
- Mix**, wait for the end of the reaction (approx. 10 min) and read the absorbances of the solutions (A₂).
- If the reaction has not stopped after 10 min, continue to read the absorbances at 2 min intervals until the absorbance increases constantly over 2 min.

1 The absorption maximum of NADPH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm or 334 nm.
2 If desired, disposable cuvettes may be used instead of glass cuvettes.
3 See instructions for performance of assay.
4 Available from Roche Molecular Biochemicals
2. Technical information
The Wallratha precipitation technique (Ref. 3.3 and 2.4) works very well in fruit juice analysis. It is recommended to titrate the hydrochloric acid (HCl; 4 M) to be used against the sodium hydroxide (NaOH; 4 M) by using a pH electrode or a pH indicator paper.
When analyzing "strongly acid" samples (e.g. grapefruit juice), the volume of the ammonia solution (25%) has to be increased: 2.5 ml have to be used instead of 2.0 ml otherwise the results will be too low. Alternatively neutralize "strongly acid" samples before analysis.
For recovery experiments D,L-trisodium-isocitrate, dihydrox, is recommended. When calculating results the molecular weight of D,L-IC-Na₃ × 2 H₂O has to be used; furthermore it has to be taken into consideration that only the D-form reacts in the enzymatic system.

3. Specificity (Ref. 1)
3.1 The enzyme isocitrate dehydrogenase catalyzes specifically the oxidative decarboxylation of D-isocitrate.
3.2 Preparations of ICDH may contain traces of L-malate dehydrogenase and aconitase. In spite of this, the L-malic acid in 100-fold excess and citric acid in 200-fold excess do not interfere with the determination of D-isocitric acid. In the presence of larger amounts of L-malic and citric acid, a sample-dependent "creep reaction" appears which may be eliminated by extrapolation (see procedure, pipetting scheme).
In the analysis of commercial tri-sodium-D,L-isocitrate dihydrox (molecular weight 294.1) results of approx. 50% have to be expected. (Only the D-form is measured enzymatically.)

4. Sensitivity and detection limit
The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume v = 2.000 ml.

5. Linearity
Linearity of the determination exists from 2 µg D-isocitric acid/assay to 1 mg D-isocitric acid/l sample solution; sample volume v = 2.000 ml to 100 µg D-isocitric acid/assay; sample volume v = 0.100 ml.

6. Precision
In a double determination using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to 5 mg/l sample solution.

7. Interference/sources of error
Iron ions at high concentrations (> 0.8 µg/assay) interfere with the assay because of causing turbidity. If iron ions are present adjust sample solution to the alkaline range of pH > 8.0 and incubate for approx. 5 min. Filter solution and adjust to pH 7.0-7.5, if necessary.
Sulfite ions in high concentrations (> 30 µg/assay) cause a slight creep reaction because of decomposition of NADPH. The exact absorption value can be calculated by extrapolation of absorbance (A₀) to the time of addition of ICDH (solution 3) to the assay.

8. Recognizing interference during the assay procedure
8.1 If the conversion of D-isocitric acid has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.
8.2 On completion of the reaction, the determination can be restarted by adding D,L-isocitrate (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.
8.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml); the measured differences in absorbance should be proportional to the sample volumes used. When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.
8.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample and assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.
8.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

9. Reagent hazard
The reagents used in the determination of D-isocitric acid are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to. After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

10. General information on sample preparation
In carrying out the assay:
Use clear, colorless and practically neutral liquid samples directly, or after dilution according to the dilution table, and of a volume up to 2.000 ml; Filter turbid solutions;
Degas samples containing carbon dioxide (e.g. by filtration);
Adjust acid samples to approx. pH 8 by adding sodium or potassium hydroxide solution;
Adjust acid and weakly colored samples to approx. pH 7.75 by adding sodium or potassium hydroxide solution and incubate for approx. 30 min;
Measure "colored" samples (if necessary adjusted to pH 7-7.5) against a sample blank (= buffer or redist. water + sample), adjust the photometer to 0.000 with the blank in the beam;
Treat "strongly colored" samples that are used undiluted or with a higher sample volume with polyvinylpolypyrrolidone (PVPP) or with polyanime, e.g. 1 g/100 ml;
Crush or homogenize solid or semi-solid samples, extract with water or dissolve in water and filter if necessary.
Extract samples containing fat with hot water (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter.

11. Application examples
Determination of D-isocitric acid in colored juices
Adjust 25 ml filtered sample solution with sodium hydroxide solution (2 M) to pH 20-25 and dilute with redist. water to 50 ml (volumetric flask). Incubate at 20-25°C for approx. 10 min. Add 0.5 g polyvinylpolypyrrolidone (PVPP) or bentonite to the diluted sample solution. Stir for 1 min and filter. Use the clear, possibly slightly colored solution for the assay.
Neutralize strongly acid sample solutions which are used undiluted for the assay.
12. Determination of total D-isocitric acid

(free D-isocitric acid + bound D-isocitric acid, e.g. esterified or lactonized)

Adjust 25 ml sample solution in an Erlenmeyer flask with sodium hydroxide solution (2 M) to pH 10-11. In the presence of reducing substances add 0.01 ml hydrogen peroxide (30%, w/v), if necessary, and incubate for 20 min in a boiling water-bath. Check pH from time to time and adjust pH with sodium hydroxide solution, if necessary. Allow to cool to 20-25°C and adjust with hydrochloric acid (1 M) to pH 6.9-7.2. Transfer sample solution quantitatively into a 50 ml volumetric flask, add 0.2 g bentonite, stir for approx. 1 min (magnetic stirrer) and fill up to the mark with redist. water. Mix, filter and use the clear solution for the assay, diluted, if necessary.

The bound D-isocitric acid, e.g. esterified or lactonized, corresponds to the difference between the total D-isocitric acid and free D-isocitric acid.

13. Further instructions for the determination of total D-isocitric acid acc. to Wallrauch

The determination of D-isocitric acid and its esters may also be performed favorably in colored juices according to the method of Wallrauch and Greiner (Ref. 3.3). It is necessary to use a suitable quality of activated charcoal for reliable measurements.

Reagents

Acetone, A. R.
Ammonia solution, 25%, A. R.
Barium chloride, BaCl₂ × 2 H₂O, A. R.
Sodium sulfate, A. R.
Activated charcoal
Tria (hydroxymethyl)-aminomethane, Tria, Cat. No. 127 4344
Ethylenediamine tetra acetate, EDTA Na₂H₂ × 2 H₂O

Preparation of solutions

Barium chloride solution: Dissolve 30 g BaCl₂ × 2 H₂O with redist. water and fill up to 100 ml.
Sodium sulfate solution: Dissolve 71 g Na₂SO₄ with redist. water and fill up to 1 l.

Procedure (precipitation method, s. Ref. 2.4 and 3.3)

Incubate 10 ml sample solution (after neutralization, if necessary; see pt. 2) with 5 ml sodium hydroxide (4 M) for 10 min in a 100 ml centrifuge tube. Add successively 5 ml hydrochloric acid (4 M), 2 ml ammonia solution (25%), 3 ml BaCl₂ solution and 20 ml acetone. Mix thoroughly and incubate for 10 min. Centrifugation mixture for 5 min.

Decant the supernatant solution carefully, add 20 ml Na₂SO₄ solution to the precipitate and stir the precipitate in the centrifuge tube with a glass rod. Heat for 10 min in a boiling water-bath while stirring rigorously. After cooling transfer the contents of the centrifuge tube quantitatively into a 50 ml volumetric flask and fill up to the mark with Tria buffer solution. Weigh 1 g activated charcoal into an Erlenmeyer flask, transfer the contents of the volumetric flask into this Erlenmeyer flask, mix, incubate for 5 min and filter. Use the colorless and clear solution for the assay (v = 1.000 ml).

The altered sample volume v must be taken into account in the calculation formula.

14. Further applications

The method may also be used in research when analyzing biological samples. For details of sampling, treatment and stability of the sample see Ref. 1.1 and 1.2.

References

2.2 Schweizerisches Lebensmittelbuch, Kapitel 61B (Enzymatische Bestimmungen)3.2/ (1981), Kapitel 26A (Frucht- und Gemüsesäfte a.u.)7.16 (1988), Kapitel 34 (Gärungs- essig)4.6 (1994)
2.4 Ämliche Sammlung von Untersuchungsverfahren nach 185 LMBG; Untersuchung von Lebensmitteln: Bestimmung von D-Isocitronensäure in Fruchtsäften, 31.00-9 (November 1983); Enzymatische Bestimmung des Gehaltes an D-Isocitronensäure in Frucht- und Gemüsesäften, 31.00-9 (Januar 1997); Enzymatische Bestimmung des Gehaltes an D-Isocitronensäure in Gemüsesäften, 29.26-9 (Januar 1997)
2.5 Niederländische Norm NEN 2844 (De ruk, maart 1984) Vruchtesappen: Beplanting van het totale D-isocitronensuurgehalte; Enzymatichte methodo (Fruit juices – Determination of the total D-isocitric acid content - Enzymatic method)
2.9 Deutsche Norm DIN EN 1139 (1994) Frucht- und Gemüsesäfte; Enzymatische Bestimmung des Gehaltes an D-Isocitronensäuren; Spektrophotometrische Bestimmung von NADPH (Fruit and vegetable juices; Enzymatic determination of D-isocitric acid content - NADPH spectrophotometric method)
2.10 European Standard EN 1139 (Dec 1994) Fruit and vegetable juices; Enzymatic determination of D-isocitric acid content by the NADPH spectrophotometric method
2.13 Bergner-Lang, B. (1977) Neue Ergebnisse zur Bestimmung der Isocitronensäure in Zitrusfrüchten, Deutsche Lebensmittel-Rundschau 73, 211-216
D-Isocitric acid assay control solution

The assay control solution serves as a control for the enzymatic determination of D-isocitric acid in foodstuffs and other materials.

**Reagents**

D,L-Isocitric acid, tri-sodium salt, di-hydrate, AR grade

**Preparation of the assay control solution**

Accurately weigh approx. 30 mg D,L-tri-sodium isocitrate, di-hydrate to the nearest 0.1 mg into a 20 ml volumetric flask, fill up to the mark with redist. water, and mix thoroughly (this corresponds approx. 0.5 g D-isocitric acid/l). Prepare assay control solution freshly before use. The assay control solution may be frozen in portions.

**Application:**

1. **Addition of D,L-isocitric acid assay control solution to the assay mixture:**
   Instead of sample solution the assay control solution is used for the assay. (It has to be considered when calculating results that in the enzymatic determination only the D-form is measured.)

   The measurement of the assay control solution is not necessary for calculating the results.

2. **Restart of reaction, quantitatively:**
   After completion of the reaction with sample solution and measuring $A_2$, add 0.050 ml assay control solution to the assay mixture. Read absorbance $A_3$ after the end of the reaction (approx. 15 min). Calculate the concentration from the difference ($A_3 - A_2$) according to the general equation for calculating the concentration. The altered total volume must be taken into account. Because of the dilution of the assay mixture by the addition of the assay control solution, the result differs insignificantly from the result got according to pt. 1.

3. **Internal standard:**
   The assay control solution can be used as internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

   
<table>
<thead>
<tr>
<th>Pipette into</th>
<th>Blank</th>
<th>Sample</th>
<th>Standard</th>
<th>Sample + Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>cuvettes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>solution 2</td>
<td>1.000 ml</td>
<td>1.000 ml</td>
<td>1.000 ml</td>
<td>1.000 ml</td>
</tr>
<tr>
<td>redist. water</td>
<td>2.000 ml</td>
<td>1.900 ml</td>
<td>1.900 ml</td>
<td>1.900 ml</td>
</tr>
<tr>
<td>sample sln.</td>
<td>-</td>
<td>0.100 ml</td>
<td>-</td>
<td>0.100 ml</td>
</tr>
<tr>
<td>assay control sln.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.050 ml</td>
</tr>
</tbody>
</table>

   Mix and read absorbances of the solutions ($A_3$) after approx. 3 min. Continue as described in the pipetting scheme under “Procedure”. Follow the instructions given under “Instructions for performance of assay” and the footnotes.

   The recovery of the standard is calculated according to the following formula:

   $$\text{recovery} = \frac{2 \times (A_{\text{sample + standard}} - A_{\text{sample}})}{A_{\text{standard}}} \times 100\%$$

4. **Recovery experiments with original samples:**
   For checking sample preparation and assay, recovery experiments may be carried out. For this, either the a. m. assay control solution is used or another assay control solution with a suitable concentration is prepared.

   The original sample is measured with and without added D-isocitric acid.

   - either the same as expected to be present in the original sample,
   - or the added D-isocitrate corresponds to that amount of D-isocitrate which should be contained in the sample e.g. according to standards or other regulations.

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**Also available:**

Test-Combination Citric acid,
Cat. No. 0 139 076

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