

D-Isocitric acid

UV-method

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

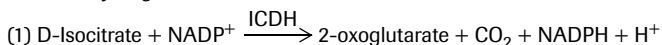
Determination of total D-isocitric acid according to Wallrauch, see pt. 13

Cat. No. 10 414 433 035

Test-Combination for 33 determinations

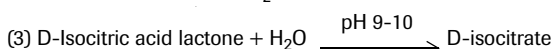
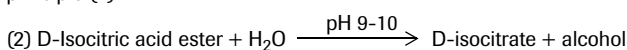
Principle (Ref. 1)

D-Isocitric acid (D-isocitrate) is oxidatively decarboxylated by nicotinamide-adenine dinucleotide phosphate (NADP) in the presence of the enzyme isocitrate dehydrogenase (ICDH) (1).



The amount of NADPH formed in reaction (1) is stoichiometric to the amount of D-isocitrate. NADPH is determined by means of its light absorbance at 334, 340 or 365 nm.

The bound D-isocitric acid (esters, lactones) is determined after alkaline hydrolysis (2,3) (see instructions pt. 12 and pt. 13) according to the same principle (1).



The Test-Combination contains

- Bottle 1 with approx. 30 ml solution, consisting of: imidazole buffer, pH approx. 7.1
- Bottle 2 with approx. 60 mg lyophilizate, consisting of: NADP, approx. 45 mg; manganese sulfate
- Bottle 3 with lyophilizate ICDH, approx. 5 U

Preparation of solutions

- Dissolve contents of bottle 2 with the whole contents of bottle 1 (= solution 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

Wavelength¹: 340 nm, Hg 365 nm or Hg 334 nm

Glass cuvette²: 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/assay³ (in 0.100-2.000 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
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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.

* Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.

** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

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For *in vitro* use only

Store at 2-8°C

For recommendations for methods and standardized procedures see references (2)

Determine the absorbance differences (A₂-A₁) for both, blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$$\Delta A = (A_2 - A_1)_{\text{sample}} - (A_2 - A_1)_{\text{blank}}$$

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions of performance of assay" and "Sensitivity and detection limit", pt 4).

Calculation

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\epsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = final volume [ml]

v = sample volume [ml]

MW = molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ε = extinction coefficient of NADPH at:

$$340 \text{ nm} = 6.3 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

$$\text{Hg } 365 \text{ nm} = 3.5 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

$$\text{Hg } 334 \text{ nm} = 6.18 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

It follows for D-isocitric acid:

$$c = \frac{3.050 \times 192.1}{\epsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A = \frac{5.859}{\epsilon} \times \Delta A \text{ [g D-isocitric acid/l sample solution]}$$

If the sample has been diluted on preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

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

1. Instructions for performance of assay

The amount of D-isocitric acid present in the assay has to be between 3 µg and 100 µg (measurement at 365 nm) or 2 µg and 50 µg (measurement at 340, 334 nm), respectively. In order to obtain a sufficient absorbance difference, the sample solution is diluted to yield a concentration of D-isocitric acid between 0.2 and 1.0 g/l or 0.1 and 0.5 g/l, respectively.

Dilution table

Estimated amount of D-isocitric acid per liter measurement at		Dilution with water	Dilution factor F
340 or 334 nm	365 nm		
< 0.5 g	< 1 g	-	1
0.5-5.0 g	1.0-10.0 g	1 + 9	10
> 5.0 g	> 10.0 g	1 + 99	100

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) or the sample volume to be pipetted into the cuvette can be increased up to 2.000 ml. Neutralize the sample, if necessary. The volume of water added must then be reduced so as to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

- 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.
- If desired, disposable cuvettes may be used instead of glass cuvettes.
- See instructions for performance of assay
- Available from Roche Applied Science



2. Technical information

The Wallrauch 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, dihydrate, 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, 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 dihydrate (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 and measurement at 340 of a D-isocitric acid concentration of 0.25 mg/l sample solution (if $v = 0.100$ ml, this corresponds to 5 mg/l sample solution).

The detection limit of 1 mg/l is derived from the absorbance difference of 0.020 (as measured at 340 nm) and a maximum sample volume $v = 2.000$ ml.

5. Linearity

Linearity of the determination exists from 2 µg D-isocitric acid/ assay (1 mg D-isocitric acid/l sample solution; sample volume $v = 2.000$ ml) to 100 µg D-isocitric acid/assay (1 g D-isocitric acid/l sample solution; 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 a D-isocitric acid concentration of approx. 5-10 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.05-0.1 g/100 g can be expected.)

The following data have been published in the literature:

$x = 40$ mg/l orange juice CV = 6 % (Ref. 1.1)

$x = 33$ µg/assay CV = 0.70 % $n = 16$
 $x = 67$ µg/assay CV = 0.58 % $n = 16$
 $x = 133$ µg/assay CV = 0.69 % $n = 16$ (Ref. 1.2)

Fruit juice:

$r = 2.5$ mg/l $s_{(r)} = \pm 0.8944$ mg/l
 $R = 4.4$ mg/l $s_{(R)} = \pm 1.5665$ mg/l (Ref. 2.4)

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 (A_2) 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 7-7.5 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 polyvinylpyrrolidone (PVPP) or with polyamide, 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 7.0-7.5 and dilute with redist. water to 50 ml (volumetric flask). Incubate at 20-25°C for approx. 10 min. Add 0.5 g polyvinylpyrrolidone (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, $\text{BaCl}_2 \times 2 \text{H}_2\text{O}$, A. R.

Sodium sulfate, A. R.

Activated charcoal

Tris (hydroxymethyl)-aminomethane, Tris, Cat. No. 127 434⁴

Ethylenediamine tetra acetate, $\text{EDTA Na}_2\text{H}_2 \times 2 \text{H}_2\text{O}$

Preparation of solutions

Barium chloride solution:

Dissolve 30 g $\text{BaCl}_2 \times 2 \text{H}_2\text{O}$ with redist. water and fill up to 100 ml.

Sodium sulfate solution:

Dissolve 71 g Na_2SO_4 with redist. 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 redist. water, adjust to pH 7.0 with hydrochloric acid (1 M) and fill up to 100 ml with redist. water.

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 BaCl_2 solution and 20 ml acetone. Mix thoroughly and incubate for 10 min. Centrifuge mixture for 5 min.

Decant the supernatant solution carefully, add 20 ml Na_2SO_4 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 Tris 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 ($v = 1.000 \text{ ml}$).

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.

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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:

Pipette into cuvettes	Blank	Sample	Standard	Sample + Standard
solution 2	1.000 ml	1.000 ml	1.000 ml	1.000 ml
redist. water	2.000 ml	1.900 ml	1.900 ml	1.900 ml
sample sln.	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml

Mix, and read absorbances of the solutions (A_1) 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:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100 [\%]$$

4. Recovery experiments with original samples:

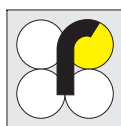
For checking sample preparation and assay, recovery experiments may be carried out. For this, either the a. m. assay control solution is used or another assay control solution with a suitable concentration is prepared.

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

- 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.

Also available:

**Test-Combination Citric acid,
Cat. No. 10 139 076 035**



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