Citric acid

UV-method

for the determination of citric acid in foodstuffs and other materials

Cat. No. 10 139 076 035
Test-Combination for 3 × 12 determinations

Principle (Ref. 1)

Citric acid (citrate) is converted to oxaloacetate and acetate in the reaction catalyzed by the enzyme citrate lyase (CL) (1).

1) Citrate $\rightarrow$ oxaloacetate + acetate

In the presence of the enzymes L-malate dehydrogenase (L-MDH) and L-lactate dehydrogenase (L-LDH), oxaloacetate and its decarboxylation product pyruvate are reduced to L-malate and L-lactate, respectively, by reduced nicotinamide-adenine dinucleotide (NADH) (2, 3).

2) Oxaloacetate + NADH + H+ $\rightarrow$ L-malate + NAD+

3) Pyruvate + NADH + H+ $\rightarrow$ L-lactate + NAD+

The amount of NADH oxidized in reactions (2) and (3) is stoichiometric to the amount of citrate. NADH is determined by means of its light absorbance at 334, 340 or 365 nm.

(Note: Free pyruvate in the sample is not measured because of the order of pipetting the reagents.)

The Test-Combination contains

1. Three bottles 1, each with approx. 1.4 g lyophilizate, consisting of: glycylglycine buffer, pH approx. 78; L-malate dehydrogenase, approx. 136 U; L-lactate dehydrogenase, approx. 280 U; NADH, approx. 5 mg
2. Three bottles 2, each with approx. 50 mg lypolizeitate citrate lyase, approx. 12 U
3. Bottle 3 with citric acid assay control solution for assay control purposes

Preparation of solutions for 10 determinations

1. Dissolve contents of one bottle 1 in 12 ml redist. water.
2. Dissolve contents of one bottle 2 in 0.3 ml redist. water.

Stability of reagents

The contents of bottle 1 and 2 are stable at 2-8°C (see pack label).

Solution 1 is stable for 2 weeks at 2-8°C or for 4 weeks at -20 to -25°C.

Solution 2 is stable for 1 week at 2-8°C or for 4 weeks at -20 to -25°C.

Procedure

Wavelength1: 340 nm, Hg 365 nm or Hg 365 nm
Glass cuvette2: 1.00 cm light path
Temperature: 20-25°C
Final volume: 3.020 ml

Read against air (without a cuvette in the light path) or against water.

Sample solution: 1-80 μg of citric acid/assay (in 0.200-2.000 ml sample volume)

<table>
<thead>
<tr>
<th>Pipette into cuvettes</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>solution 1</td>
<td>1.000 ml</td>
<td>1.000 ml</td>
</tr>
<tr>
<td>sample solution*</td>
<td>-</td>
<td>0.200 ml</td>
</tr>
<tr>
<td>redist. water</td>
<td>2.000 ml</td>
<td>1.800 ml</td>
</tr>
</tbody>
</table>

Mix**, read absorbances of the solutions ($A_1$) after approx. 5 min, and start reaction by addition of:

solution 2 | 0.020 ml | 0.020 ml

Mix**, on completion of the reaction (approx. 5 min), read absorbances of the solutions ($A_2$).

* 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)

Determine the absorbance differences ($A_1-A_2$) for both, blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

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

Occasionally a negative value with ($A_1-A_2)_{blank}$ is obtained. This value is then to be added to ($A_1-A_2)_{sample}$ according to the calculation formula.

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

If the absorbance difference of the sample ($\Delta A_{sample}$) is higher than 1.000 (measured at 340 nm, Hg 334 nm) or 0.500 (measured at Hg 365 nm) respectively, the concentration of citric acid in the sample solution is too high. The sample solution is to be diluted according to the dilution table in that case.

Calculation

According to the general equation for calculating the concentration:

$\frac{c}{\epsilon \times d \times v \times 1000} = \frac{\Delta A}{\epsilon} \times \frac{V}{MW}$

$V$ = final volume [ml]
$v$ = sample volume [ml]
$MW$ = molecular weight of the substance to be assayed [g/mol]
$\epsilon$ = extinction coefficient of NADH at:

- $340$ nm = 6.3 [l × mmol$^{-1}$ × cm$^{-1}$]
- $365$ nm = 3.4 [l × mmol$^{-1}$ × cm$^{-1}$]
- $334$ nm = 6.18 [l × mmol$^{-1}$ × cm$^{-1}$]

It follows for citric acid (calculated as the anhydrous):

$\frac{c}{\epsilon \times 1.00 \times 0.200 \times 1000} = \frac{2.900}{\epsilon} \times \Delta A \ [g \text{ citric acid/l sample solution}]$

for citric acid (calculated as monohydrate):

$\frac{c}{\epsilon \times 1.00 \times 0.200 \times 1000} = \frac{3.173}{\epsilon} \times \Delta A \ [g \text{ citric acid mono-}

hydrate/l sample solution]

If the sample has been diluted during 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:

Contentcitric acid = $\frac{\text{citric acid [g/l sample solution]}}{\text{weight sample in g/l sample solution}} \times 100 \ [g/100 \text{ g}]

1 Instructions for performance of assay

The amount of citric acid present in the assay has to be between 1 μg and 80 μg. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a citric acid concentration between 0.04 and 0.4 g/l.

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

For in vitro use only

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

For in vitro use only

Store at 2-8°C

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Test-Combination for 3 × 12 determinations For recommendations for methods and standardized procedures see references (2)
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. 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.

2. Technical information

2.1 In carrying out the calculation, a clear indication should be given as to whether the results are to be given as citric acid (molar mass 192.1 g/mol), as citric acid monohydrate (molar mass 210.1), or as citrate (molar mass 189.1 g/mol). (In enzymatic determinations, the citrate ion is measured.)

2.2 In evaluating the analytical results, it should be taken into account that in the acidimetric determination of "total acid calculated as citric acid" protons are measured and in enzymatic determinations the citrate ion is measured. It is thus not possible to compare such results directly.

3. Specificity (Ref. 1)

The method is specific for citric acid.

In the analysis of commercial citric acid monohydrate, results of >100% are obtained if the crystal water is lost during storage and the results are calculated with the molecular weight of citric acid monohydrate (210.1).

4. Sensitivity and detection limit (Ref. 1.4)

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 nm of a citric acid concentration of 0.25 mg/l sample solution (if v = 0.200 ml, this corresponds to 2.5 mg/l sample solution).

The detection limit of 0.5 mg/l is derived from the absorbance difference of 0.010 (as measured at 340 nm) and a maximum sample volume v = 2.000 ml.

5. Linearity

Linearity of the determination exists from 1 μg citric acid/assay (0.5 mg citric acid/l sample solution; sample volume v = 2.000 ml) to 80 μg citric acid/assay (0.4 g citric acid/l sample solution; sample volume v = 0.200 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 = 0.200 ml and measurement at 340 nm, this corresponds to a citric acid concentration of approx. 3-5 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 0.03-0.05 g/100 g can be expected.)

The following data have been published in the literature:

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<td>&lt; 0.4 g</td>
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<td>1</td>
</tr>
<tr>
<td>0.4-4.0 g</td>
<td>1 + 9</td>
<td>10</td>
</tr>
<tr>
<td>4.0-40 g</td>
<td>1 + 99</td>
<td>100</td>
</tr>
<tr>
<td>&gt; 40 g</td>
<td>1 + 999</td>
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If the sample solution contains free pyruvic acid, NADH is already consumed before the measuring of A3. In this case it is recommended to add NADH to the sample additionally (e.g. 0.100 ml NADH solution, 5 mg/ml)², and to use less redist. water, appropriately.

8. Recognizing interference during the assay procedure

8.1 If the conversion of citric 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 citric acid or sodium citrate (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 citric acid contain hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulations 67/548 and 99/45 and subsequent alterations, supplementation and adaptation guidelines. Please refer to the safety data sheet or the labels of the affected vials for further information.

10. General information on sample preparation

In carrying out the assay:

- Use 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 8 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min;
- Treat "strongly colored" samples that are used undiluted or with a higher sample volume with polyvinylpolyglycolidone (PVPP) or with polyclamide (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;
- Deproteinize samples containing protein with perchloric acid;
- 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.

Important note

The Carrez-clarification cannot be used in the sample preparation for citric acid determination due to a too low recovery rate (adsorption of citric acid).

If, in addition to free citric acid, esterified citric acid is to be determined - e.g. citric acid esters of polyphenols or anthocyans - the esters must be converted to the free acid by alkaline hydrolysis. Proceed as stated under "wine".

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Determination of citric acid esters in wine

Mix 10 ml wine and 0.1 g polyamide or polyvinylpolypyrrolidone (PVPP), stir for 1 min, and filter. Use the clear or slightly colored solution for the assay.

Determination of citric acid in beer

For removal of carbonic acid, stir approx. 5-10 ml beer for 1 min with a glass rod or filter. The largely CO₂-free sample of beer is used for the assay without further dilution.

Determination of citric acid in margarine, edible oil and salves

Grind approx. 20-50 g sample material (using e.g. a mortar, a meat grinder or a press) and homogenize it. Alternatively, deproteinization can be carried out with perchloric acid. See Tables of the European Economic Community (A.I.J.N.) respectively, can be determined in the presence of free citric acid (citrates) if the sample is extracted with chloroform and the esters are subsequently saponified with potassium hydroxide. In this case proceed as follows:

Boil the well minced and homogenized samples which contain up to approx. 120 mg monoglyceride citric acid ester (e.g. monooleylcitryl glyceride ester, MW approx. 550) or up to 170 mg diglyceride citric acid ester (e.g. dioleoylcitryl glyceride ester, MW approx. 810) with approx. 50 ml chloroform under reflux condenser for approx. 2 h in a 250 ml round bottomed flask. Filter and rinse with chloroform. Evaporate the chloroform in a rotary evaporator. Boil the nearly dry residue with 25 ml methanolic potassium hydroxide (1 M) for 10 min under a reflux condenser. Allow to cool to 20-25°C and neutralize or weakly acidify respectively with approx. 5 ml hydrochloric acid (5 M). Transfer the solution quantitatively into a 100 ml volumetric flask, make up to the mark with water, mix and filter. Use the nearly clear solution for the assay.

For determination of the content the molecular weight of the glyceride must be taken into account.

12. Further applications

The method may also be used in the examination of paper, cosmetics, detergents (Ref. 3.6), pharmaceuticals, as well as in research when analyzing biological materials.

For details of sampling, treatment and stability of the sample see Ref. 1.3 and 1.5.

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ung des Gehaltes an Citronensäure (Citrat) in Gemüsesäften, spektralphotometrische
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eatables of the European Economic Community (A.I.J.N.)
Citric acid assay control solution (Bottle 3)

Concentration*: see bottle label

Citric acid assay control solution is a stabilized aqueous solution of citric acid in foodstuffs and other materials. It serves as an assay control solution for the enzymatic determination of citric acid in foodstuffs and other materials.

**Application:**

1. **Addition of citric acid assay control solution to the assay mixture:**
   Instead of sample solution the assay control solution is used for the assay.

2. **Restart of the reaction, quantitatively:**
   After completion of the reaction with sample solution and measuring of \( A_2 \) add 0.100 ml assay control solution to the assay mixture. Read absorbance \( A_3 \) after the end of the reaction (approx. 10 min). Calculate the concentration from the difference of \( (A_2-A_3) \) 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 addition of the assay control solution, the result differs insignificantly from the data stated on the bottle label.

* Stated as anhydrous citric acid

3. **Internal standard:**
   The assay control solution can be used as an 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:

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

Also available:

**Test-Combination D-Isocitric acid, Cat. No. 10 414 433 035**

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2.20 International Standard ISO 2963 (März 1997) Cheese and processed cheese products - Determination of citric acid content - Enzymatic method


3.8 Seppi, A. & Sperandio, A. (1983) L’acido citrico nei vini, determinazione con metodo enzimatico e con metodo chimico ufficiale, La Rivista della Societa Italiana di Scienza dell’Alimentazione 12, 479–482


