Succinic acid

UV-method
for the determination of succinic acid in foodstuffs and other materials
Cat. No. 10 176 281 035
Test-Combination for 11 determinations

Principle (Ref. 1)
Succinic acid (succinate) is converted to succinyl-CoA by the enzyme succinyl-CoA synthetase (SCS), also known as succinate thiokinase, and inorganic phosphate (P_i) (1). Succinate + ITP + CoA \rightarrow succinyl-CoA + Pi + IDP

1. Bottle 1 with approx. 830 mg lyophilizate, consisting of:
   - glycylglycine buffer, pH approx. 6.4; NADH, approx. 6 mg
2. Bottle 2 with approx. 10 tablets; each tablet contains:
   - CoA, approx. 0.75 mg; ITP, approx. 0.7 mg; PK, approx. 230 U; L-LDH, approx. 230 U
3. Bottle 3 with approx. 0.5 ml suspension, containing:
   - Suspension 3
4. Bottle 4 with approx. 10 tablets; each tablet contains:
   - Suspension 4

Preparation of solutions
1. Dissolve contents of bottle 1 with 13 ml distilled water.
2. Dissolve one tablet of bottle 2 with one ml solution of bottle 1, depending on the number of determinations for each assay (sample and blank) in a beaker or reaction tube (use forceps for removing tablets from bottle 2). This results in the reaction mixture 2*.
3. Use contents of bottle 3 undiluted.
4. Use contents of bottle 4 undiluted.

Stability of reagents
The contents of bottle 1 are stable for at 2-8°C (see pack label). Solution 1 is stable for 4 weeks at 2-8°C.

Procedures
Wavelength1: 340 nm, Hg 365 nm or Hg 334 nm
Temperature: 37°C
Final volume: 1.000 ml
Read against air (without a cuvette in the light path) or against water
Sample solution: 1-40 mg succinic acid/assay (in 0.100-2.000 ml sample volume)

Calculation
According to the general equation for calculating the concentration:

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

where:
- \( c \) = concentration of succinic acid [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]
- \( \varepsilon \) = extinction coefficient of NADH at:
  - 340 nm = 6.3 \text{ [l × mmol}^{-1} × \text{cm}^{-1}]
  - Hg 365 nm = 3.4 \text{ [l × mmol}^{-1} × \text{cm}^{-1}]
  - Hg 334 nm = 6.18 \text{ [l × mmol}^{-1} × \text{cm}^{-1}]

It follows for succinic acid:

\[ c = \frac{3.070 \times 118.09}{\varepsilon \times 1,000} \times \frac{\Delta A}{3,625} \times \Delta A \text{ [g succinic acid/l sample solution]} \]

1 The absorption maximum of NADH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectrally selective photometers are 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 The reaction is completed when sample and blank show equal changes in absorbance.

1. Instructions for performance of assay
The amount of succinic acid present in the assay has to be between 1 mg and 40 μg. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a succinic acid concentration between 0.05 and 0.4 g/l.

For in vitro use only

Store at 2-8°C

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

<table>
<thead>
<tr>
<th>Estimated amount of succinic acid per liter</th>
<th>Dilution with water</th>
<th>Dilution factor F</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.4 g</td>
<td>-</td>
<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>
<td>1000</td>
</tr>
</tbody>
</table>

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 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 Sample preparation with concentrated Carrez-solutions has proved beneficial in the analysis of liquid whole egg and whole egg powder (see ref. 2.1). The sample solution can also be used for the determination of D-3-hydroxybutyric acid and L-lactic acid.

2.2 In carrying out the calculation, a clear indication should be given as to whether the results are to be given as succinic acid (molar mass 118.09 g/mol) or as succinate (molar mass 116.07 g/mol). (In enzymatic determinations, the succinate ion is measured.)

3. Specificity (Ref. 1)

Succinyl-CoA synthetase reacts not only with succinic acid but also with itaconic acid. In comparison to succinic acid the content of itaconic acid in foodstuffs is very low. Therefore, this fact is unimportant for the determination of succinic acid.

In the analysis of commercial succinic acid, results of approx. 100 % have to be expected.

4. Sensitivity and detection limit (Ref. 1.2)

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 succinic concentration of 0.15 mg/l sample solution (if v = 0.100 ml, this corresponds to 3mg/l sample solution). The detection limit of 0.6 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 approx. 1 µg succinic acid/assay (0.6 mg succinic acid/l sample solution; sample volume v = 2.000 ml) to 40 µg succinic acid/assay (0.4 g succinic 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 succinic acid concentration of approx. 3–6 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 0.03–0.08 g/l/100 g can be expected.)

The following data have been published in the literature:

CV = 0.98–1.4 % n = 15 in series (Ref. 1.2)

Whole egg powder:

x = 11 mg/kg r = 0.68 mg/kg s<sub>0</sub> = ± 2.4 mg/kg
R = 12.1 mg/kg s<sub>R0</sub> = ± 4.3 mg/kg (Ref. 2.1)

7. Interference/sources of error

Fumaric acid reacts with SCS, although very slowly with a creep reaction under the aforementioned conditions. This can be eliminated by mathematical extrapolation as usual.

The presence of "NADH oxidases" in the assay system as well as the tendency of succinyl-CoA for hydrolysis (especially under alkaline conditions) leads to creep reactions. If the absorbance of blank and sample is read immediately ones after the other, extrapolation of the absorbance values back to the time of the addition of suspension 4 (SCS) is not necessary.

8. Recognizing interference during the assay procedure

8.1 If the conversion of succinic 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 succinic acid (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 succinic acid are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent amendment, standardization 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 and weakly colored samples to pH 8-9 by adding sodium or potassium hydroxide solution;

Adjust acid and weakly colored samples to pH 8-9 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 polyvinylpolypyrrolidone (PVPP) - (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; resp. remove turbidities or dyestuffs by cartridge clarification;

Deproteinize samples containing protein with perchloric acid, alternatively clearly with Carrez reagent;

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; alternatively clarify with Carrez-solutions after the extraction with hot water.

Carrez clarification:

Pipe the liquid sample into a 100 ml volumetric flask which contains approx. 60 ml redist. water, or weigh sufficient quantity of the sample into a 100 ml volumetric flask and add approx. 60 ml redist. water. Subsequently, carefully add 5 ml Carrez-I-solution (potassium hexacyanoferrate(II) (ferrocyanide), 85 mM = 3.60 g K₄[Fe(CN)₆] × 3 H₂O/100 ml) and 5 ml Carrez-II-solution (zinc sulfate, 250 mM = 720 g ZnSO₄ × 7 H₂O/100 ml). Adjust to pH 7.5-8.5 with sodium hydroxide (0.1 M; e.g. 10 ml). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

Preparation of egg and egg product samples is dealt with in pt. 11 (application examples). Note: Treatment with concentrated Carrez-solutions has proved beneficial in routine analysis. In Germany, the method has been standardized and published in § 64 of the Foodstuffs and Consumer Goods Law (Lebensmittel- and Bedarfsgegenständegesetz, LMBG). The sample solution resulting from Carrez clarification can also be used for the determination of D-3-hydroxybutyric acid and of L-lactic acid.

11. Application examples

Determination of succinic acid in wine

Succinic acid can be determined in white or red wine normally without previous dilution or decolorization. Use 0.100 ml of the sample for the assay.

Determination of succinic acid in soy sauce

Add 2 ml diluted Carrez-I-solution (3.60 g potassium hexacyanoferrate(II), K₄[Fe(CN)₆] × 3 H₂O/100 ml) to 1 ml soy sauce and swirl gently. After addi-
The assay control solution serves as a control for the enzymatic determination of succinic acid in foodstuffs and other materials.

**Reagents**

Succinic acid, AR grade

**Preparation of the assay control solution**

Accurately weigh approx. 40 mg succinic acid to the nearest 0.1 mg into a 100 ml volumetric flask, fill up to the mark with redist. water, and mix thoroughly.

Prepare assay control solution freshly before use. The assay control solution may be frozen in portions.

**Application:**

1. **Addition of succinic acid assay control solution to the assay mixture.** Instead of sample solution the assay control solution is used for the assay. (The measurement of the assay control solution is not necessary for the calculation of the results.)

2. **Restart of the reaction, quantitatively:** After completion of the reaction with sample solution and measuring A2, add 0.050 ml assay control solution to the assay mixture. Read absorbance A3 after 3 min of the reaction (approx. 30 min at 37°C). Calculate the concentration from the following equation (A3-A2) 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 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.

**Pipette into cuvettes**

<table>
<thead>
<tr>
<th>Blank</th>
<th>Sample</th>
<th>Standard</th>
<th>Sample + Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>reaction mixture 2</td>
<td>1.000 ml</td>
<td>1.000 ml</td>
<td>1.000 ml</td>
</tr>
<tr>
<td>suspension 3</td>
<td>0.050 ml</td>
<td>0.050 ml</td>
<td>0.050 ml</td>
</tr>
<tr>
<td>sample solution</td>
<td>0.100 ml</td>
<td>0.100 ml</td>
<td>0.100 ml</td>
</tr>
<tr>
<td>assay control sln.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>redist. water</td>
<td>2.000 ml</td>
<td>1.900 ml</td>
<td>1.900 ml</td>
</tr>
</tbody>
</table>

Mix, after approx. 5 min incubation at 37°C read absorbances of the solutions (A3). 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:

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

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 succinic acid. The amount of added succinic acid is either the same as expected to be present in the original sample, or corresponds to that amount of succinic acid which is allowed to be contained in the sample e.g. according to standards or other regulations.