

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

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Enzymatic BioAnalysis / Food Analysis

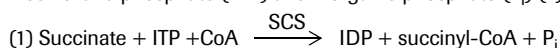
For *in vitro* use only

Store at 2-8°C

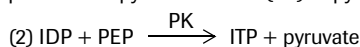
For recommendations for methods and standardized procedures see references (2)

Principle (Ref. 1)

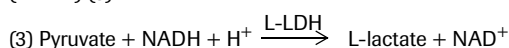
Succinic acid (succinate) is converted to succinyl-CoA by the enzyme succinyl-CoA synthetase (SCS), also known as succinate thiokinase, and inosine-5'-triphosphate (ITP) and coenzyme A (CoA) with a simultaneous formation of inosine-5'-diphosphate (IDP) and inorganic phosphate (P_i) (1).



Inosine-5'-diphosphate reacts with phosphoenolpyruvate (PEP) in the presence of pyruvate kinase (PK) to pyruvate and ITP (2).



Pyruvate is reduced by NADH in the presence of L-lactate dehydrogenase (L-LDH) (3).



The amount of NADH oxidized in the above reaction is stoichiometric to the amount of succinic acid. NADH is measured by its light absorbance at 334, 340 or 365 nm.

The Test-Combination contains

1. Bottle 1 with approx. 830 mg lyophilizate, consisting of: glycyglycine buffer, pH approx. 8.4; NADH, approx. 6 mg
2. Bottle 2 with approx. 10 tablets; each tablet contains: CoA, approx. 0.75 mg; ITP, approx. 0.7 mg; PEP-CHA, approx. 0.7 mg
3. Bottle 3 with approx. 0.5 ml suspension, containing: PK approx. 250 U; L-LDH approx. 230 U
4. Bottle 4 with approx. 0.25 ml suspension containing succinyl-CoA synthetase approx. 12 U

Preparation of solutions

1. Dissolve contents of bottle 1 with 13 ml redist. 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 reagent 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.

Bring solution 1 to 37°C before use.

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

Prepare reaction mixture immediately before use.

Bring reaction mixture 2 to 37°C before use.

The contents of bottles 3 and 4 are stable at 2-8°C (see pack label).

Procedure

Wavelength¹: 340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette²: 1.00 cm light path
Temperature: 37°C
Final volume: 3.070 ml
Read against air (without a cuvette in the light path) or against water
Sample solution: 1-40 µg succinic acid/assay³ (in 0.100-2.000 ml sample volume)

Pipette into cuvettes	Blank	Sample
reaction mixture 2* (warmed to 37°C)	1.000 ml	1.000 ml
suspension 3	0.050 ml	0.050 ml
sample solution**	-	0.100 ml
redist. water	2.000 ml	1.900 ml
Mix***, after approx. 5 min incubation at 37°C read absorbances of the solutions (A ₁). Start reaction by addition of:		
suspension 4	0.020 ml	0.020 ml
Mix***, after completion of the reaction (approx. 20 min at 37°C) read absorbances of blank and sample immediately one after the other (A ₂) ⁴ (see pt. 7).		

* For simplification of the assay performance it is also possible to pipette directly 1.000 ml of solution 1 into the cuvette and add 1 tablet from bottle 2. After dissolution of the tablet with the aid of a spatula continue working as described in the procedure. The difference in volume of ca. 1% (increase of volume by 1 tablet per 3.070 ml assay volume) has to be taken into account in the calculation by multiplication of the result with 1.01.

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

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

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 (ΔA_{sample}) is higher than 0.850 (measured at 340 nm or Hg 334 nm respectively) or 0.470 (measured at 365 nm), the concentration of succinic acid in the sample solution is too high. The sample is to be diluted according to the dilution table in that case.

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 NADH at:

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

$$\text{Hg } 365 \text{ nm} = 3.4 \text{ [l} \times \text{mmol}^{-1} \times \text{cm}^{-1}\text{]}$$

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

It follows for succinic acid:

$$c = \frac{3.070 \times 118.09}{\epsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A = \frac{3.625}{\epsilon} \times \Delta A \text{ [g succinic acid/l sample solution]}$$

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:

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

1. Instructions for performance of assay

The amount of succinic acid present in the assay has to be between 1 µg 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.

- 1 The absorption maximum of NADH 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 The reaction is completed when sample and blank show equal changes in absorbance.



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Dilution table

Estimated amount of succinic acid per liter	Dilution with water	Dilution factor F
< 0.4 g	-	1
0.4-4.0 g	1 + 9	10
4.0-40 g	1 + 99	100
> 40 g	1 + 999	1000

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 116.07g/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 nm of a succinic acid 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 0.03-0.06 g/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 $r = 06.8$ mg/kg $s_{(r)} = \pm 2.4$ mg/kg
 $R = 12.1$ mg/kg $s_{(R)} = \pm 4.3$ 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 one 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 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 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 polyvinylpyrrolidone (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 Carrez clarification;

Deproteinize **samples containing protein** with perchloric acid, alternatively clarify 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:

Pipette 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 $K_4[Fe(CN)_6] \times 3 H_2O/100$ ml) and 5 ml Carrez-II-solution (zinc sulfate, 250 mM = 720 g $ZnSO_4 \times 7 H_2O/100$ 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 § 35 of the Foodstuffs and Consumer Goods Law (Lebensmittel- und 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), $K_4[Fe(CN)_6] \times 3 H_2O/100$ ml) to 1 ml soy sauce and swirl gently. After addi-

tion of 2 ml diluted Carrez-II-solution (7.20 g zinc sulfate, $ZnSO_4 \times 7 H_2O/100$ ml) swirl gently and filter (do not neutralize, otherwise the solution becomes black again!). Use $v = 0.100$ ml of the filtrate for the assay. Dilution factor $F = 5$ is to be taken into account in the calculation.

Determination of succinic acid in fruit juices

Use colorless fruit juices directly for the assay. Because of the low content of succinic acid the sample volume is mostly larger than 0.100 ml. Acidic juices have to be neutralized (the dilution by addition of alkali must be taken into account). Colored juices have to be treated with PVPP according to the above mentioned procedure.

Determination of succinic acid in protein-containing samples

Add to protein-containing sample solutions perchloric acid (1 M) in a ratio of 1+2 to 1+3, mix and centrifuge. Neutralize an aliquot volume of the supernatant solution with KOH (2 M), dilute to a certain volume in the volumetric flask, place in a refrigerator for 20 min in order to precipitate the $KClO_4$ and filter. Use the clear solution, which may be diluted, if necessary, for the assay.

Determination of succinic acid in Swiss cheese

Accurately weigh approx. 5 g of ground cheese into a 100 ml volumetric flask, add about 80 ml redist. water and incubate at 60°C for 15 min. Shake flask from time to time. After cooling to 20-25°C, fill up to the mark with water. For separation of fat, place in a refrigerator for approx. 20 min and centrifuge. Use 0.100 to 0.200 ml for the assay.

Determination of succinic acid in liquid whole egg (Ref. 2.1)

Accurately weigh approx. 5 g homogenized whole egg into a 25 ml-volumetric flask, add 10 ml redist. water, one drop n-octanol, mix and heat for 15 min in a water-bath (approx. 100°C). Allow to cool to 20-25°C, and add one after the other and shake after each addition: 1 ml concentrated Carrez-I-solution (15.0 g potassium hexacyanoferrate(II), $K_4[Fe(CN)_6] \times 3 H_2O/100$ ml), 1 ml concentrated Carrez-II-solution (30.0 g zinc sulfate, $ZnSO_4 \times 7 H_2O/100$ ml). Fill up to the mark with NaOH (0.1 M), mix and filter with a fluted filter paper in a glass funnel. Use filtrate for the assay ($v = 0.100$ ml, when using microbial contaminated egg; $v = 1.000$ ml, when using fresh egg). The altered sample volume must be taken into account in the calculation.

Determination of succinic acid in whole egg powder (Ref. 2.1)

Accurately weigh approx. 1 g whole egg powder into 25 ml volumetric flask, add 12 ml redist water and one drop n-octanol, mix and heat for 15 min in a water-bath (approx. 100°C). Allow to cool to 20-25°C, add one after the other and shake rigorously after each addition: 1 ml concentrated Carrez-I-solution

(15.0 g potassium hexacyanoferrate(II), $K_4[Fe(CN)_6] \times 3 H_2O/100$ ml), 1 ml concentrated Carrez-II-solution (30.0 g zinc sulfate, $ZnSO_4 \times 7 H_2O/100$ ml). Adjust to pH 8-9 with NaOH (1 M), fill up to the mark with redist. water, mix and filter with a fluted filter paper in a glass funnel. Use 0.100-1.000 ml filtrate for the assay. The altered sample volume must be taken into account in the calculation.

Determination of succinic acid in pastes containing soy bean flour

Weigh approx. 5 g pasty material (accuracy 1 mg) into a 50 ml-volumetric flask and add 40 ml redist. water. Heat for 10 min in a water-bath (approx. 70°C). Allow to cool to 20-25°C and fill up to the mark with redist. water. Mix and filter. Discard the first few ml of the filtrate. Use 0.100 ml of the filtrate for the assay.

12. Further applications

The method may also be used in research for the analysis of biological materials. For details of sampling, treatment and stability of the sample see Ref. 1.2.

References

- 1.1 Michal, G., Beutler, H.-O., Lang, G. & Güntner, U. (1976) Enzymatic determination of succinic acid in foodstuffs, Z. Anal. Chem. **279**, 137-138
- 1.2 Beutler, H.-O. (1985) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 3rd ed., vol. VII, pp. 25-33, Verlag Chemie Weinheim, Deerfield Beach/Florida, Basel
- 2.1 Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG; Untersuchung von Lebensmitteln: Bestimmung von L-Milchsäure, Bernsteinsäure und D-3-Hydroxybuttersäure in Ei und Eiprodukten, 05.00-2 (November 1987)
- 2.2 European Communities: Council Directive of 20 June 1989 on hygiene and health problems affecting the production and the placing on the market of egg products (89/437/EEC), Official Journal No. L 212, 22/07/89 P. 0087
- 2.3 Schweizerisches Lebensmittelbuch, Kapitel 30A (Wein aus Trauben)/6.8 (1994)
- 3.1 Sponholz, W.R. & Dittrich, H.H. (1977) Enzymatische Bestimmung von Bernsteinsäure in Mosten und Weinen, Weinwissenschaft **32**, 38-47
- 3.2 Wagner, K. & Kreutzer, P. (1977) Zusammensetzung und Beurteilung von Auslesen, Beeren- und Trockenbeerenauslesen, Die Weinwirtschaft **10**, 272-275
- 3.3 Joyeux, A. & Lafon-Lafourcade, S. (1979) Dosage de l'acide succinique dans les vins par méthode enzymatique, Ann. Fals. Exp. Chim **72**, 317-320
- 3.4 Beutler, H.-O., Schneider, H., Schütz, A. & Henniger, G. (1986) Succinic Acid - A New Test Kit for Enzymatic Determination, Poster-Präsentation bei Association of Official Analytical Chemists Annual International Meeting, Scottsdale, AZ, USA
- 3.5 Littmann-Nienstedt, S. & Beutler, H.-O. (1988) Auswertung des Ringversuches der Arbeitsgruppe "Ei-Analytik" zur Bestimmung von (L-)Milchsäure, 3-Hydroxybuttersäure und Bernsteinsäure in Eiprodukten, Deutsche Lebensmittel-Rundschau **84**, 320-322

Succinic acid assay control solution

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 A_2 , add 0.050 ml assay control solution to the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 30 min at 37°C). Calculate the concentration from the difference ($A_2 - A_3$) 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 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	Blank	Sample	Standard	Sample + Standard
reaction mixture 2	1.000 ml	1.000 ml	1.000 ml	1.000 ml
suspension 3	0.050 ml	0.050 ml	0.050 ml	0.050 ml
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml
redist. water	2.000 ml	1.900 ml	1.900 ml	1.900 ml

Mix, after approx. 5 min incubation at 37°C read adsorbances of the solutions (A_1). 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 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.



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