

Megazyme

SUCCINIC ACID (SUCCINATE)

ASSAY PROCEDURE

K-SUCC 03/11

(20 Assays per Kit)

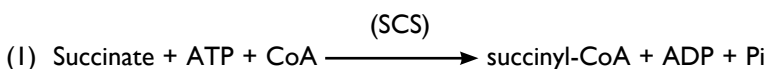


INTRODUCTION:

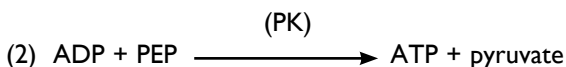
Succinic acid is found in all plant and animal materials as a result of the central metabolic role played by this dicarboxylic acid in the Citric Acid Cycle. Succinic acid concentrations are monitored in the manufacture of numerous foodstuffs and beverages, including wine, soy sauce, soy bean flour, fruit juice and dairy products (e.g. cheese). The ripening process of apples can be followed by monitoring the falling levels of succinic acid. The occurrence of > 5 mg/kg of this acid in egg and egg products is indicative of microbial contamination. Apart from use as a flavouring agent in the food and beverage industries, succinic acid finds many other non-food applications, such as in the production of dyes, drugs, perfumes, lacquers, photographic chemicals and coolants.

PRINCIPLE:

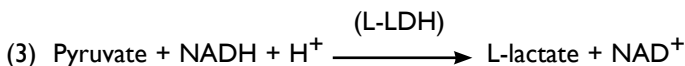
In the presence of adenosine-5'-triphosphate (ATP), succinic acid (succinate) is converted to succinyl-CoA by the enzyme succinyl-CoA synthetase (SCS), with the concurrent formation of adenosine-5'-diphosphate (ADP) and inorganic phosphate (P_i) (1).



In the presence of pyruvate kinase, ADP reacts with phosphoenolpyruvate (PEP) to form pyruvate and ATP (2).



The pyruvate produced is reduced to L-lactate by L-lactate dehydrogenase (L-LDH) in the presence of reduced nicotinamide-adenine dinucleotide (NADH), with the production of NAD⁺ (3).



The amount of NAD⁺ formed in the above coupled reaction pathway is stoichiometric with the amount of succinic acid. It is NADH consumption which is measured by the decrease in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

Analysis of commercial succinic acid should yield recoveries of approx. 100 % (w/w). Other than succinic acid, succinyl-CoA

synthetase also reacts with itaconic acid. However, the level of itaconic acid in foodstuffs is so low that it does not interfere with the analytical results.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.128 mg/L of sample solution at the maximum sample volume of 2.00 mL (or to 2.56 mg/L with a sample volume of 0.1 mL). The detection limit is 0.256 mg/L, which is derived from an absorbance difference of 0.010 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 0.8 to 40 μg of succinic acid per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.00 mL, this corresponds to a succinic acid concentration of approx. 0.128 to 0.256 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of succinic acid has been completed within the time specified in the assay (approx. 5 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding succinic acid (approx. 20 μg in 0.1 mL) to the cuvette on completion of the reaction. A significant decrease in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments i.e. by adding succinic acid to the sample in the initial extraction steps.

SAFETY:

The reagents used in the determination of succinic acid are not hazardous materials in the sense of the Hazardous Substances Regulations. However, the buffer concentrate contains sodium azide (0.02 % w/v) as a preservative. The general safety measures that apply to all chemical substances should be adhered to.

KITS:

Kits suitable for performing 20 determinations are available from Megazyme. The kits contain the full assay method plus:

- Bottle 1:** Buffer (8 mL, pH 8.4) plus sodium azide (0.02 % w/v) as a preservative.
Stable for > 2 years at 4°C.
- Bottle 2: (x2)** NADH plus stabiliser.
Stable for > 2 years at -20°C.
- Bottle 3: (x2)** ATP plus PEP and CoA.
Stable for > 2 years at -20°C.
- Bottle 4:** Pyruvate kinase plus L-lactate dehydrogenase suspension, 0.55 mL.
Stable for > 2 years at 4°C.
- Bottle 5:** Succinyl-CoA synthetase suspension (0.55 mL).
Stable for > 2 years at 4°C.
- Bottle 6:** Succinic acid (~ 2 g).
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.
2. Dissolve the contents of one of bottle 2 in 2.4 mL of distilled water. Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and keep cool during use if possible. **Do not** dissolve the contents of the other bottle until required. Once dissolved, the reagent is stable for > 4 weeks at -20°C.
3. Dissolve the contents of bottle 3 in 2.4 mL of distilled water. Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and keep cool during use if possible. **Do not** dissolve the contents of the other bottle until required. Once dissolved, the reagent is stable for > 4 weeks at -20°C.
- 4 & 5. Use contents of bottles 4 and 5 as supplied. Before opening for the first time, shake the bottles to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position. **Swirl the bottle to mix contents before use.**
Stable for > 2 years at 4°C.
6. Accurately weigh approx. 200 mg of succinic acid to the

nearest 0.1 mg into a 1 L volumetric flask. Fill to the mark with distilled water and mix thoroughly. Store 10 mL aliquots of this solution at -20°C .
Stable for > 2 years at -20°C .

NOTE: The succinic acid standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of succinic acid is determined directly from the extinction coefficient of NADH (see page 5 and 6).

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman[®] (20 μL , 100 μL and 200 μL).
4. Positive displacement pipettor e.g. Eppendorf Multipette[®]
 - with 5.0 mL Combitip[®] (to dispense 0.2 mL aliquots of Buffer I and of NADH and ATP/PEP/CoA solutions.
 - with 25 mL Combitip[®] (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA[®] Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No.1 (9 cm) filter papers.

PROCEDURE:

Wavelength:	340 nm
Cuvette:	1 cm light path (glass or plastic)
Temperature:	~ 25°C
Final volume:	2.74 mL
Sample solution:	0.8-40 µg of succinic acid per cuvette (in 0.10-2.0 mL sample volume)

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

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.10 mL	2.00 mL
sample	-	0.10 mL
solution 1 (buffer)	0.20 mL	0.20 mL
solution 2 (NADH)	0.20 mL	0.20 mL
solution 3 (ATP/PEP/CoA)	0.20 mL	0.20 mL
suspension 4 (PK/L-LDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
suspension 5 (SCS)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 6 min). If the reaction has not stopped after 6 min, continue to read the absorbances at 2 min intervals until the absorbances either remain the same, or decrease constantly over 2 min**.		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

** if this “creep” rate is greater for the sample than for the blank, extrapolate the absorbances (sample and blank) back to the time of addition of suspension 5.

CALCULATION:

Determine the absorbance difference ($A_1 - A_2$) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{succinic acid}}$. The value of $\Delta A_{\text{succinic acid}}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of succinic acid can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{\text{succinic acid}} \quad [\text{g/L}]$$

where:

V = final volume [mL]

MW = molecular weight of succinic acid [g/mol]

ε = extinction coefficient of NADH at 340 nm
= 6300 [$\text{l} \times \text{mol}^{-1} \times \text{cm}^{-1}$]

d = light path [cm]

v = sample volume [mL]

It follows for succinic acid:

$$c = \frac{2.74 \times 118.09}{6300 \times 1.0 \times 0.1} \times \Delta A_{\text{succinic acid}} \quad [\text{g/L}]$$

$$= 0.5136 \times \Delta A_{\text{succinic acid}} \quad [\text{g/L}]$$

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

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of succinic acid

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

NOTE: These calculations can be simplified by using the Megazyme **Mega-Calc™**, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).

SAMPLE PREPARATION:

1. Sample dilution.

The amount of succinic acid present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.8 and 40 μg . The sample solution must therefore be diluted sufficiently to yield a succinic acid concentration between 0.008 and 0.40 g/L.

Dilution Table

Estimated concentration of succinic acid (g/L)	Dilution with water	Dilution factor (F)
< 0.40	No dilution required	1
0.40-4.0	1 + 9	10
4.0-40	1 + 99	100
> 40	1 + 999	1000

If the value of $\Delta A_{\text{succinic acid}}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

2. Sample clarification.

(a) Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}\}$ (Sigma cat. no. P-9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) (Sigma cat. no. Z-4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

(b) Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

3. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if 0.1 mL of an acidic sample is to be used undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 8.4 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing significant amounts of carbon dioxide, such as beer, should be degassed by increasing the pH to approx. 8.4 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no SCS, may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of PVPP/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask. Adjust to room temperature and fill the volumetric flask to the mark with distilled water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate, and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge at 1,500 g for 10 min and neutralise the supernatant with 1 M KOH. Alternatively use Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of succinic acid in white wine.

No sample preparation is required. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(b) Determination of succinic acid in red wine.

Dispense 25 mL of sample into a 100 mL beaker and adjust the pH to 7-8 using 1M NaOH. Adjust the volume to 50 mL with distilled water and add 0.5 g of PVPP. Stir for 5 min and filter an aliquot of the suspension through Whatman No. 1 (9 cm) filter paper. *Typically, a dilution of 1:2 and a sample volume of 0.1 mL are satisfactory.*

(c) Determination of succinic acid in whole liquid egg.

Accurately weigh approx. 5 g of homogenised whole egg into a 50 mL volumetric flask, add 25 mL of distilled water and 1 drop of *n*-octanol. Mix and incubate for 15 min in a water bath (approx. 100°C). Cool to 20-25°C and carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter. *Typically, no further dilution is required and a sample volume of 0.2-1.0 mL is satisfactory.*

(d) Determination of succinic acid in whole egg powder.

Accurately weigh approx. 1 g of whole egg powder into a 50 mL volumetric flask, add 30 mL of distilled water and 1 drop of *n*-octanol. Mix and incubate for 15 min in a water bath (approx. 100°C). Cool to 20-25°C and carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter. *Typically, no dilution is required and a sample volume of 0.2-1.0 mL is satisfactory.*

(e) Determination of succinic acid in cheese.

Accurately weigh approx. 5 g of cheese into a 100 mL volumetric flask, add approx. 80 mL of distilled water and incubate at 60°C for 15 min. Shake the flask several times. Cool to 20-25°C and adjust the volume to 100 mL. For the separation of fat, place the flask in a refrigerator or on ice for approx. 20 min and centrifuge an aliquot of the lower aqueous phase. *Typically, no dilution is required and a sample volume of 1.0 mL is satisfactory.*

(f) Determination of succinic acid in soy sauce.

Add 5 mL of soy sauce to a 100 mL volumetric flask and adjust to volume with distilled water, transfer the solution to a 200 mL beaker and add 4 g activated charcoal and stir for 2 min. Filter an aliquot of the solution through Whatman GF/A glass fiber filter paper. *Typically, no further dilution is required and a sample volume of 1.0 mL is satisfactory.*

REFERENCE:

Beutler, H. -O. (1989). Succinate. In *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VII**, pp. 25-33, VCH Publishers (UK) Ltd., Cambridge, UK.



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