Megazyme

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SUCCINIC ACID (SUCCINATE)

ASSAY PROCEDURE

K-SUCC 06/18

(*20 Manual Assays per Kit) or
(270 Auto-Analyser Assays per Kit) or
(200 Microplate Assays per Kit)

** The number of tests per kit can be doubled if all volumes are halved*

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 COA, 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).

$$\text{(1)}\quad \text{Succinate} + \text{ATP} + \text{CoA} \xrightarrow{\text{(SCS)}} \text{succinyl-CoA} + \text{ADP} + P_i$$

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

$$\text{(2)}\quad \text{ADP} + \text{PEP} \xrightarrow{\text{(PK)}} \text{ATP} + \text{pyruvate}$$

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).

$$\text{(3)}\quad \text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{(L-LDH)}} \text{L-lactate} + NAD^+$$

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 general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 20 assays in manual format (or 270 assays in auto-analyser format or 200 assays in microplate format) 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 below -10°C.

Bottle 3: (x2) ATP plus PEP and CoA.
Stable for > 2 years below -10°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. **Stable for > 1 year at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).
Do not dissolve the contents of the other bottle until required.

3. Dissolve the contents of one of bottle 3 in 2.4 mL of distilled water. Divide into appropriately sized aliquots and store in polypropylene tubes below -10°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 below -10°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 below -10°C.
Stable for > 2 years below -10°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 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 1 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.

A. MANUAL ASSAY 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 0.10-2.00 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* and 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:

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

where:

V = final volume [mL]
MW = molecular weight of succinic acid [g/mol]
ε = extinction coefficient of NADH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for succinic acid:

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

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

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

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

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).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for succinic acid can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of succinic acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
distilled water	20.55 mL
solution 1 (buffer)	2.40 mL
solution 2 (NADH)	2.40 mL (after adding 2.4 mL of H_2O to bottle 2)
solution 3 (ATP/PEP/CoA)	2.40 mL (after adding 2.4 mL of H_2O to bottle 3)
suspension 4 (PK/L-LDH)	0.24 mL
Total volume	27.99 mL

Preparation of R2:

Component	Volume
distilled water	3.55 mL
suspension 5 (SCS)	0.26 mL
Total volume	3.81 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 6 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	decrease
Linearity:	up to 343 mg/L of succinic acid using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for succinic acid can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of succinic acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.274 mL
Linearity: 0.1-4 µg of succinic acid per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.020 mL	0.020 mL	0.020 mL
solution 2 (NADH)	0.020 mL	0.020 mL	0.020 mL
solution 3 (ATP/PEP/CoA)	0.020 mL	0.020 mL	0.020 mL
suspension 4 (PK/L-LDH)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:			
suspension 5 (SCS)	0.002 mL	0.002 mL	0.002 mL
Mix* and 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 5 min intervals until the absorbances increase constantly over 5 min**.			

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 5.

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L\ standard \times F$$

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

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) {$K_4[Fe(CN)_6].3H_2O$} (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) 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 1 M 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.*

(g) Determination of succinic acid in whole blood samples.

a. Solutions:

Concentrated Carrez I solution. Dissolve 30 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P9387) in 200 mL of distilled water. Store at room temperature.

Concentrated Carrez II solution. Dissolve 60 g of zinc sulphate $\{ZnSO_4.7H_2O\}$ (Sigma cat. no. Z4750) in 200 mL of distilled water. Store at room temperature.

b. Procedure:

Heat 1 mL of whole blood sample at approx. 80°C for 20 min in a microfuge tube then centrifuge at 13,000 x *g* for 10 min and recover the supernatant. Add 20 µL Carrez Reagent II and mix thoroughly, then add 20 µL Carrez Reagent I and mix thoroughly. Centrifuge the sample again at 13,000 x *g* for 10 min and recover the clarified

supernatant for use in the assay. If required, dilute the sample appropriately in distilled water for the assay.

NOTE: The final volume of the clarified supernatant will be approx. one quarter of the starting volume of the original sample. Therefore, adjust the volume of the starting material as required to obtain sufficient volume of clarified sample for the test.

(h) Determination of succinic acid in biological tissue samples.

Accurately weigh approx. 5 g of representative biological tissue into a 100 mL Duran® bottle. Add 20 mL of 1 M perchloric acid and homogenise for 2 min using an Ultra-turrax® or Polytron® homogeniser (or equivalent). Quantitatively transfer to a 40 mL glass beaker and adjust the pH to approx. 8.0 using 2 M KOH. Quantitatively transfer to a 100 mL volumetric flask and adjust to the mark with distilled water (ensuring the fat containing layer is "above" the mark, and the aqueous layer is "at" the mark). Store on ice for 20 min to precipitate potassium perchlorate and allow separation of the fat (if present). Centrifuge an appropriate volume of the sample at 13,000 x *g* for 10 min and recover the clarified supernatant for use in the assay, alternatively filter through Whatman No. 1 filter paper, discarding the first 3-5 mL, and use the clear filtrate for the assay. If required, dilute the sample appropriately in distilled water for the assay.

NOTE: The amount of starting material and volumes used can be adjusted accordingly depending on the amount of analyte present in the sample.

(i) Determination of succinic acid in biological fluid samples (e.g. urine and serum).

For some biological fluid samples it may be sufficient to test them directly without any sample preparation other than appropriate dilution in distilled water. If this is not adequate then deproteinisation with either perchloric acid or trichloracetic acid may be required.

Deproteinise biological samples by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge an appropriate volume of the sample at 1,500 x *g* for 10 min and recover the supernatant for use in the assay, alternatively filter through Whatman No. 1 filter paper, discarding the first 3-5 mL, and use the filtrate for the assay. If required, dilute the sample appropriately in distilled water for the assay. Alternatively, use 50% (w/v) trichloroacetic acid instead of perchloric acid.

REFERENCE:

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

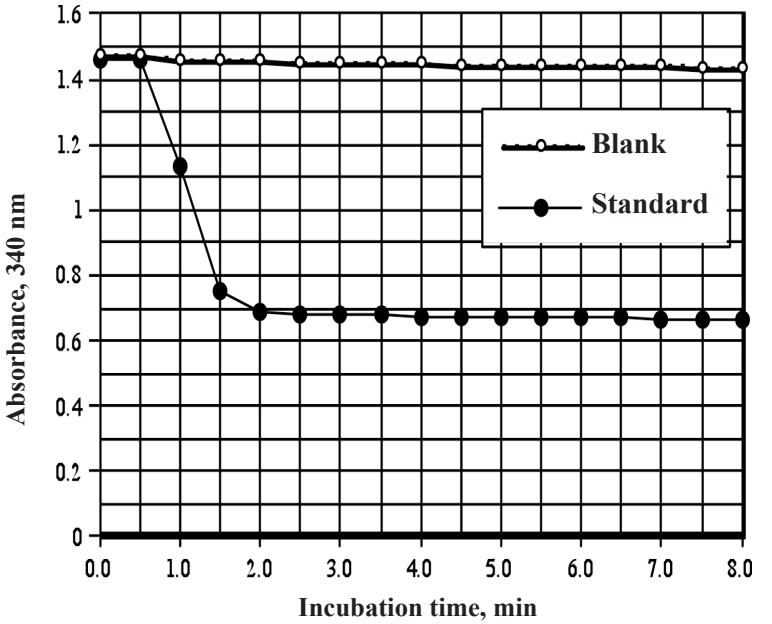

Figure 1. Decrease in absorbance at 340 nm on incubation of 40 µg of succinic acid with succinyl-CoA synthetase, pyruvate kinase and L-lactate dehydrogenase in the presence of NADH.

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