Enzymatic UV assay for the determination of succinic acid in foodstuffs and other sample materials
Test combination for 25 determinations

For *in vitro* use only
Store between 2 – 8 °C (36 – 46 °F)

This test was evaluated using selected samples of the following matrices: soy sauce, liquid and powdered egg, meat products, vegetable broth powder, fruit juices and wine.

Detailed results and information regarding associated validation data are found in the Validation Report.

The test may be used with other foods or samples material, provided that these are subjected to individual validation by the user.

1. Test principle

Succinic acid (succinate) is converted to succinyl-CoA in the presence of the enzyme succinyl-CoA synthetase (SCS) by adenosine triphosphate (ATP) and coenzyme A (CoA):

Succinate + ATP + CoA —SCS→ succinyl-CoA + ADP + P

The resulting adenosine diphosphate (ADP) reacts with D-glucose in the presence of an ADP-dependent hexokinase (ADP-HK), whereby D-glucose is phosphorylated to D-glucose-6-phosphate (G-6-P) and adenosine monophosphate (AMP) is formed at the same time:

ADP + D-glucose —ADP-HK→ D-glucose-6-phosphate + AMP

In the presence of glucose-6-phophate-dehydrogenase (G6P-DH, D-glucose-6-phosphate and nicotinamide-adenine-dinucleotide (NAD+) react to D-glucono-δ-lactone-6-phosphate and NADH/H+.

G-6-P + NAD^+ —G6P-DH→ 6-phosphoglucono-δ-lactone + NADH+ H^+

Nicotinamide-adenine-dinucleotide (NAD) is reduced to NADH. The amount of NADH formed is proportional to the amount of succinic acid formed and is measured at a wavelength of 340 nm.

2. Reagents

2.1. Content & composition

The test is suitable for manual and automated processing. With manual processing, the reagents are sufficient for 25 determinations. The number of determinations for automated processing is increased by a multiple; however it depends on the device.

- Reagent 1: 1 x 50 mL with buffer, NAD
- Reagent 2: 1 x 12.5 mL with buffer, SCS, ADP-HK, G6P-DH

2.2. Reagent preparation

The reagents are ready-to-use and be allowed to reach room temperature (20 – 25 °C / 68 – 77 °F) before use. Do not interchange components between kits of different batches.

2.3. Storage & stability

If stored as directed and between 2 – 8 °C (36 – 46 °F), reagents remain stable until the printed expiration date, even after opening. Reagents must not be frozen.

2.4. Safety & disposal

The test is intended solely for the intended use as described. The provided Instructions for Use must be strictly followed.

Follow standard chemical safety procedures when handling this product. Do not swallow. Avoid contact with skin or mucous membranes.

Detail safety information for individual components is available in the corresponding Safety Data Sheets (SDS).

Dispose of used reagents as laboratory waste in compliance with all relevant regulations. Packaging materials are to be recycled according to local regulations.

3. Sample preparation

3.1. General

- Sample preparation for manual and automated testing is the same.
- Samples solutions should be brought to room temperature before measurement.
- Use liquid, clear and almost neutral sample solutions directly or after dilution with distilled water to a concentration within the measuring range (see performance data).
- Adjust **strongly** acidic samples to approx. pH 8.0 by adding KOH or NaOH
- For turbid test samples: Filter by using fluted paper filter or syringe filter or centrifuge the test solution in a reaction tube (e.g. 3000 rpm for at least 5 minutes) until a clear filtrate or supernatant is obtained.
- Degas samples containing carbon dioxide by filtration or centrifugation.
- Decolorize strongly colored samples (such as wine and juices) with polyvinylpolypyrrolidone (PVPP).
- Crush and homogenize solid and semi-solid samples and extract with water or dissolve them in distilled water. Filter or centrifuge if necessary.
- Clarify protein-containing samples with perchloric acid.
- Weigh samples with a high fat content into a volumetric flask and extract with hot water; allow the sample solution to cool for fat separation (e.g. 15 minutes in an ice bath); fill the volumetric flask up to the mark with water and filter before testing.
- In case of higher sample volumes (up to 1000 µL), check the pH value of the test solution and neutralize in case of any doubt.

3.2. Additional reagents

- Carrez reagents:
  - Carrez-I: 36 g/L potassium hexacyanoferrate (II) trihydrate
  - Carrez-II: 72 g/L zinc sulphate heptahydrate
- Concentrated Carrez reagents:
  - Carrez-I: 155 g/L potassium hexacyanoferrate (II) trihydrate
  - Carrez-II: 300 g/L zinc sulphate heptahydrate

3.3. Determination of succinic acid in wine

- White wine can be used directly undiluted.
- Dilute rosé wine 1:2 with distilled water.
- Dilute red wine 1:4 with distilled water.
- If necessary, decolorize red wine with PVPP: Add 0.4 g PVPP to 20 mL of wine, vortex and shake for 10 minutes.
- If necessary, filter the sample (white, rosé, or red wine) again (using a filter with a pore size of 5 µm).
- Use 100 µL sample solution (clear filtrate) in the test.

3.4. Determination of succinic acid in soy sauce

- Mix 1 mL of soy sauce with 2 mL of diluted Carrez I solution and swirl gently.
- Add 2 mL diluted Carrez II solution and swirl gently.
- Filter the preparation (filter unit with 5 µm pore size).
- Use 100 µL sample solution in the test.

3.5. Determination of succinic acid in fruit juices

- Colorless fruit juices can be used directly.
- Decolorize strongly colored juices with PVPP: Add 0.4 g PVPP to 20 mL of juice, vortex and shake for 10 minutes.
- Then filter using paper filter or syringe filter.
- Use 100 µL sample solution (clear filtrate) in the test.

3.6. Determination of succinic acid in liquid egg

- Accurately weigh 5 g of homogenized whole egg into a 25 mL volumetric flask.
- Add 10 mL distilled water and 1 drop of *n*-octanol, mix and incubate in a water bath at approx. 100 °C (212 °F) for 15 minutes.
- Allow the flask to cool to 20 – 25 °C (68 – 77 °F).
- Add 1 mL of concentrated Carrez I solution and swirl vigorously.
- Add 1 mL of concentrated Carrez II solution and swirl vigorously.
- Fill to the 25 mL mark with 0.1 M NaOH, mix and filter through a pleated filter.
- Use 100 µL sample solution in the test.

3.7. Determination of succinic acid in powdered egg

- Weigh 5 g of homogenized whole egg accurately into a 25 mL volumetric flask.
- Add 10 mL distilled water and 1 drop of *n*-octanol, mix and incubate in a water bath at approx. 100 °C (212°°F) for 15 minutes.
- Allow the flask to cool to 20 – 25 °C (68 – 77 °F).
- Add 1 mL of concentrated Carrez I solution and swirl vigorously.
- Add 1 mL of concentrated Carrez II solution and swirl vigorously.
- Adjust to pH 8 – 9 with 1 M NaOH.
- Fill to the 25 mL mark with 0.1 M NaOH, mix and filter through a pleated filter.
- Use 100 µL sample solution in the test.

3.8. Determination of succinic acid in meat products

- Weigh 10 g of homogenized sample accurately into a 50 mL Falcon® tube, add 20 mL distilled water and vortex.
- Fill the total volume to 50 mL with distilled water, close, mix and incubate in a water bath at 70 °C (158 °F) for 15 minutes.
- Carefully add a drop of concentrated sulphuric acid, close and vortex again.
- Transfer the extract to a 100 mL volumetric flask and allow to cool to room temperature.
- Fill to the mark with distilled water so that the fat layer is above the mark.
- Filter through a pleated filter and then use 100 µL of sample solution in the test.

3.9. Determination of succinic acid in vegetable broth powder

- Weigh 1 g homogenized sample accurately into a 50 mL Falcon® tube, add 15 mL warm distilled water and vortex.
- Fill the total volume to 50 mL with distilled water, close, mix and incubate in a water bath at 70 °C (158 °F) for 15 minutes.
- Transfer the extract to a 100 mL volumetric flask and place on ice for 15 minutes.
- Fill to the 100 mL mark with distilled water, filter through a pleated filter.
- Use 1000 µL sample solution in the test.

4. Manual test procedure

Wavelength:	340 nm
Temperature (measurement):	20 – 37 °C (68 – 99 °F)
Photometer alignment:	against air (without cuvette)
Measuring range:	3 – 200 mg/L (for 100 µL)

	Reagent blank	**Samples / controls**
Reagent 1	2000 µL	2000 µL
Sample / control	-	100 µL
Dist. water	100 µL	-
Mix, incubate for **3 minutes at 20 – 37 °C (68 – 99 °F)**. **Read absorbance A_1**, then addition of:		
Reagent 2	500 µL	500 µL
Mix, incubate for **12 minutes at 25 – 37 °C (77 – 99 °F) or at least 17 minutes at 20 °C (68 °F)** and **read absorbance A_2**.		

4.1. Important notes for assay procedure

- The reagent blank value (water sample) must be determined **in each series of measurement** and subtracted from **each** sample result.
- Specified incubation times were validated at 37 °C (99 °F). The test may generally perform within a range between **20 – 37 °C (68 – 99 °F)**.
- Use separate tips for each sample extract and the control solutions to avoid cross-contamination; rinse the tip before pipetting.
- A multistepper pipette is recommended for adding reagents. Use a separate tip for each component.
- Stirring spatulas are recommended for mixing each individual cuvette. Remove these from the cuvette immediately before measuring the absorbance
- Always wait for the reaction to end or for the absorbance to stabilize (at least during the first test runs or validation). If the absorbance has not stopped after the recommended incubation time, continue measuring at 2- or 5-minute intervals, for example, until a constant absorbance value is reached.
- If a creep reaction occurs, the reaction will not have finished after stated incubation times and will typically show a constant increase of ΔA. Calculate the analyte-specific ΔA value by plotting the absorbance values against time and performing a linear regression to determine the rate of increase in ΔA per minute related to the creep reaction. Then, extrapolate the absorbance to the time at which reagent 2 is added.
- If the measured absorbance difference of the samples is too small (< 0.020), the sample solution must be prepared again with a higher weight or a lower dilution.
- If the absorbance difference of the samples is very large (e.g., > 1.500), the sample solution must be diluted if necessary.

5. Calculation of results

5.1. Calculation of sample solutions

5.1.1. Total concentration of succinate

The extinction difference ΔA must be calculated for each sample:

$$\Delta A = (A_2 - df \times A_1)_{\text{sample or control}} - (A_2 - df \times A_1)_{RB}$$

df: Dilution factor
RB: Reagent blank

$$df = \frac{\text{sample volume + R1}}{\text{test volume}} = 0.808$$

The specified df value of **0.808 applies to a base application of 100 µL**. An increase in sample volume is possible (max. 1000 µL; refer to validation report). **While keeping reagent volumes unchanged**, this requires **conversion of the reagent dilution factor (df)** accordingly.

Increasing the sample volume may influence test performance. This must generally be checked depending on the matrix. **The reagent blank value must be adjusted to the changed sample volume.**

The concentration of succinic acid is calculated using Lambert-Beer's law:

$$\mathbf{C}_{\text{succinate}}\ \mathbf{[g/L]} = \frac{(V \times MW \times \Delta A)}{(\varepsilon \times d \times v \times 1000)} = \mathbf{0.4874 \times \Delta A} \times F$$

If the sample solution was diluted before measurement, this result has to be multiplied with the **sample pre-dilution factor F**.

V:	Test volume basic application [mL]	= 2.600
MW:	Molecular weight succinate [g/mol]	= 118.09
d:	Optical path [cm]	= 1.00
v:	Sample volume [mL]	= 0.100
ε:	Extinction coefficient NADH [L/mmol x cm]	= 6.3 (at 340 nm)

5.2. Calculation for solid samples

When analyzing solid and semi-solid samples that have to be weighed in for the extraction of the sample, the content is related to the sample weight:

$$\text{Content}_{\text{succinate}}\,[\text{g}/100\,\text{g}] = \frac{C_{\text{succinate}}\,[\text{g/L sample}]}{\text{weigh-in}_{\text{sample}}\,\text{in g/L sample}} \times 100$$

Example:

$C_{\text{succinate}}$ = 0.454 g/L Weigh-in = 5.02 g/100 mL ≙ 50.2 g/L

$$\text{Content}_{\text{succinate}} = \frac{0.454\ \text{g/L}}{50.2\ \text{g/L}} \times 100 = 0.904\ \text{g}/100\ \text{g (or \%)}$$

5.3. Controls & acceptance criteria

Control or reference samples should be included in each run for quality control purposes. Therefore, we recommend Enzytec™ *Liquid* Multi-Acid Standard *low* (Art. No. E8470; 0.250 g/L succinic acid). Note that this standard must first be diluted 1:2 within the measuring range of this test to a final concentration of 0.125 g/L succinic acid.

The recovery of this multi-standard low and other aqueous control solutions should be 100 ± 5 %.

As a certified (standard) reference material, we recommend:

- Succinate Standard for Ion Chromatography, TraceCert, 1000 mg/L ± 5 mg/L (k=2); 100 mL, mat. no. 43057-100 mL; Sigma-Aldrich, St. Louis, MO, USA

6. Performance data

6.1. Specificity & side activities

The succinyl-CoA synthetase is specific for succinic acid. For itaconic acid and D-/L-malic acid, minor side activities above the LoQ of the test could be identified. These activities are reflected in a creep reaction, which can, however, be eliminated by calculation (see validation report).

6.2. Interferences

For glycerol, sucrose, D-fructose, D-glucose, citric acid, L-ascorbic acid, 2-oxoglutaric acid, L-tartaric acid, malonic acid and NaCl, no interferences could be identified at usual concentrations in foodstuffs.

Oxalic acid shows a slight interference at 25 g/L, which is no longer present at 3.13 g/L. D-/L-malic acid and sulphite show no interference at or below 0.3 g/L. Sulphite as a redox-reactive substance does not interfere at ≤ 2 g/L.

6.3. Linearity, measuring range & sensitivity

With a sample volume of 100 µL, linearity is given up to 200 mg/L succinic acid. The recommended measuring range for 100 µL is 3 – 200 mg/L or 0.4 - 20 mg/L for 1000 µL sample volume.

The limit of detection (LoD) was determined according to the DIN 32645:2008-11 method in buffered aqueous solution. For a sample volume of 100 µL, the calculated LoD is 1.0 mg/L and 0.1 mg/L for 1000 µL sample volume, respectively.

The limit of quantification (LoQ) was determined by precision profile. The calculated LoQ is 3.0 mg/L for a sample volume of 100 µL and 0.4 mg/L for 1000 µL sample volume.

7. Supporting documents

On request, we offer the following documents:

- Enzytec™ *Liquid* Succinic acid Validation Report
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* Succinic acid Excel template for results
- Enzytec™ *Liquid* Succinic acid Technical information
- Enzytec™ *Liquid* Troubleshooting guide

Safety data sheets (SDS) and certificates of analysis (CoA) are available in digital form, quoting the batch number, via the following link:

https://eifu.r-biopharm.com/

8. Limits of this method

Test results may vary depending on the sample matrix, specific test implementation, and laboratory environmental conditions. Detection and quantification limits are dependent on respective sample matrices extraction procedures. Refer toto the current Validation Report for details.

For this test, only the matrices explicitly listed in the documentation were validated, due to the wide variety of food products and other potential sample materials.

When analysing non-validated matrices results should be verified by performing spiking (fortification) experiments. If appropriate or necessary, a suitable sample preparation procedure for the respective matrix must be developed and validated.

The responsibility for validating non-validated matrices and for ensuring the suitability of the assay for its intended use lies solely with the user.

9. Services & technical support

Upon request, we offer the following services, among others:

- Customized troubleshooting
- Workflow analysis
- Data & results analysis
- Customer workshops & webinars
- Automation: application support & technical service

10. Disclaimer

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