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

This test was validated for the following matrices: soy sauce, liquid and powdered egg, meat products, vegetable broth powder, fruit juices and wine. For detailed results and further information on validation data, please refer to the validation report.
Other foodstuffs or sample materials can be tested and must be validated 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-veadenine-dinucleotide (NAD) is reduced to NADH. The amount of NADH formed is proportional to the amount of succinic acid formed and is measured at 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. The incubation times for automated processing may deviate and must therefore be verified.

- Reagent 1 1 x 50 mL buffer, NAD
- Reagent 2 1 x 12.5 mL 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) before use. Do not interchange components between kits of different batches.

2.3. Storage & stability

The reagents are stable until the end of the month of the indicated shelf life (see label) even after opening at 2 - 8 °C if handled properly. Do not freeze reagents.

2.4. Safety & disposal

This product/test is only suitable for use within the scope of its intended purpose. The instruction for use must be strictly followed.

The general safety rules for working in chemical laboratories should be applied. Do not swallow! Avoid contact with skin and mucous membranes.

This kit may contain hazardous substances. For hazard notes on the contained substances, please refer to the appropriate safety data sheets (SDS) for this product. After use, the reagents can be disposed of with the laboratory waste. Packaging materials may be recycled.

3. Sample preparation

3.1. General

- Sample preparation for manual and automated testing is identical.
- Bring samples to room temperature before measurement.
- Use liquid, clear and colorless solutions directly or after dilution with dist. water to a concentration within the measuring range (see performance data).
- Turbid solutions have to be centrifuged or filtered.
- Degas samples containing carbonic acid, e.g. by filtration or centrifugation.
- Highly colored and highly concentrated samples should be decolorized with polyvinylpolypyrrolidone (PVPP).
- 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 min in an ice bath); fill the volumetric flask up to the mark with water and filter before testing.
- Sufficiently homogenize and crush solid and semi-solid samples; extract with water or dissolve in dist. water and filter if necessary.
- Adjust strongly acidic samples to approx. pH 8.0 by adding sodium or potassium hydroxide solution.

- **Concentrated** Carrez solutions:
  - Carrez-I: 155 g/L potassium hexacyanoferrate (II) trihydrate
  - Carrez-II: 300 g/L zinc sulphate heptahydrate

- **Diluted** Carrez solutions:
  - Carrez-I: 36 g/L potassium hexacyanoferrate (II) trihydrate
  - Carrez-II: 72 g/L zinc sulphate heptahydrate

3.2. Determination of succinic acid in wine

- White wine can be used directly undiluted.
- Dilute rosé wine 1:2 with dist. water.
- Dilute red wine 1:4 with dist. water.
- If necessary, additionally filter the preparation (white, rosé or red wine) (filter unit with 5 µm pore size) and then use 100 µL sample solution in the test.

3.3. 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) and use 100 µL sample solution in the test.

3.4. Determination of succinic acid in fruit juices

- Colorless fruit juices can be used directly.
- Decolorize colored juices with PVPP. Then use 100 µL sample solution in the test.

3.5. Determination of succinic acid in liquid egg

- Accurately weigh 5 g of homogenized whole egg into a 25 mL volumetric flask.
- Add 10 mL dist. water and 1 drop of *n*-octanol, mix and incubate in a water bath at approx. 100 °C for 15 minutes.
- Allow the flask to cool to 20 - 25 °C.
- 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.
- Then use 100 µL sample solution in the test.

3.6. Determination of succinic acid in powdered egg

- Weigh 5 g of homogenized whole egg accurately into a 25 mL volumetric flask.
- Add 10 mL dist. water and 1 drop of *n*-octanol, mix and incubate in a water bath at approx. 100 °C for 15 minutes.
- Allow the flask to cool to 20 - 25 °C.
- 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.
- Then use 100 µL sample solution in the test.

3.7. Determination of succinic acid in meat products

- Weigh 10 g of homogenized sample accurately into a 50 mL Falcon® tube, add 20 mL dist. water and vortex.
- Fill the total volume to 50 mL with distilled water, close, mix and incubate in a water bath at 70 °C 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 add 100 µL of sample solution to the test.

3.8. Determination of succinic acid in vegetable broth powder

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

4. Assay performance

Wavelength: 340 nm
Temperature: 20 - 37 °C (during the measurement)
Measurement: against air (without cuvette)
Measuring range: 3 - 400 mg/L (for 100 µL sample)

	Reagent blank	Samples / controls
Reagent 1	2000 µL	2000 µL
Sample / control	-	100 µL
Dist. water	100 µL	-
Mix, incubate for 3 min at 20 - 37 °C. Read absorbance A_1, then addition of:		
Reagent 2	500 µL	500 µL
Mix, incubate for 12 min at 37 °C or 17 min at 20 °C and read absorbance A_2.		

- The reagent blank (RB) must be determined **once** for each parameter in each run and subtracted from each sample result for the corresponding parameter.
- Reagent blank and sample must be measured **in the same run** and under the same conditions.
- To increase sensitivity, the sample volume can be increased by up to 1000 µL (see validation report) with **unchanged** reagent volumes.
- The volume of the reagent blank must be adjusted to the changed sample volume.
- Increasing the sample volume may affect the test system. In general, this must be checked depending on the matrix.

4.1. Important notes for assay performance

- The specified incubation times may vary depending on the prevailing laboratory conditions and the pipetting accuracy. It is therefore recommended to wait for the end of the reaction during the first runs and to adjust the times if necessary.
- If the reaction has not stopped after the specified incubation time, the absorbances should be measured at 2 min intervals until a constant increase in absorbance per 2 min is achieved. If constant absorbance increases were observed, the absorbances A_2 are extrapolated to the time of addition of reagent 2.
- To obtain a sufficiently precise result, the measured absorbance differences should usually be at least 0.050 - 0.100 absorbance units.
- The use of a multistepper pipette is recommended for the addition of reagents. Use a separate tip for each component and rinse the tip with the respective reagent before pipetting.
- The use of stirring spatulas for each individual cuvette is recommended for mixing. Only remove this from the cuvette immediately before the absorbance measurements.
- If the measured absorbance difference is too small (e. g. $\Delta A < 0.02$), increase the sample volume (v) to a maximum of 1000 µL or prepare the sample solution again (higher weight or less dilution).
  In this case, the volume of water in the reagent blank must be adjusted accordingly so that the same test volume is present in the sample and blank preparations.
  The changed sample volume must be used accordingly in the calculation formulae.

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:

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

df: dilution factor
RB: reagent blank

$$df_{100\mu L} = \frac{\text{sample volume + volume R1}}{\text{test volume}} = 0.808$$

The stated df value of 0.808 applies to a basic application of 100 µL. **Increasing the sample volume requires conversion of the reagent dilution factor (df).**

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

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

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)

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

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:

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

Example:

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

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

5.3. Controls & acceptance criteria

Controls or reference samples should be carried along for quality control during each run. Recovery of aqueous standard solutions should be within 100 ± 5 %.

We recommend the use of reference materials or standard solutions, for example:

- Enzytec™ *Liquid* Multi-acid Standard 2 *low* (E8470) with 0.25 g/L succinic acid as control for direct use

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 the usual food concentrations.

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 400 mg/L succinic acid. The recommended measuring range for 100 µL is 3 - 400 mg/L or 0.4 - 40 mg/L for 1000 µL sample volume.

The limit of detection (LoD) was determined according to the DIN 32645:2008-11 method in stabilized aqueous solution. This results in an LoD of 1 mg/L succinic acid for a sample volume of 100 µL and 0.1 mg/L for 1000 µL sample volume.

The limit of quantification (LoQ) was determined using a precision profile and confirms a concentration of 3 mg/L for 100 µL and 0.4 mg/L for 1000 µL sample volume.

7. Supporting documents

On request, we offer the following documents:

- Enzytec™ *Liquid* Validation reports
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* Excel templates for results calculation
- Enzytec™ *Liquid* Technical information
- Enzytec™ *Liquid* Troubleshooting guide

Safety data sheets (SDS) and certificates of analysis (CoA) are available in digital form at the following link:

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

8. Limits of this method

Test results may vary depending on the sample matrix, the individual test procedure and the laboratory environment. Detection and quantification limits depend on the respective sample matrix and the extraction method. For detailed results and further information, please refer to the current validation report.
For the present enzymatic test, only stated, exemplary matrices could be validated due to the large number of foodstuffs and other sample materials.
When analyzing a non-validated matrix, it is recommended to verify the results obtained by means of spike experiments. If necessary, a suitable sample preparation validation for the sample matrix of interest will need to be performed and validated.

9. Services & technical support

On request, we offer the following services:

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

10. Disclaimer

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