Enzytec^{TM} *Liquid* D-3-Hydroxybutyric acid — Art. No. E8540

Version 2 / 2026-05-07

Enzymatic UV assay for the determination of D-3-hydroxybutyric acid in foodstuffs and other sample materials
Test combination for 50 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: whole liquid egg, whole egg powder and pasta.

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

D-3-Hydroxybutyric acid and D-3-hydroxybutyrate reacts in the presence of nicotinamide-adenine-dinucleotide (NAD^+) and the enzyme 3-hydroxybutyric acid dehydrogenase (3-HBDH) to acetoacetate, where NAD^+ is simultaneously reduced to NADH:

$$\text{D-3-Hydroxybutyrate} + NAD^+ \xrightarrow{\text{3-HBDH}} \text{acetoacetate} + NADH/H^+$$

The enzyme diaphorase catalyzes the conversion of $NADH/H^+$ and tetrazolium blue (tetrazoliumchloride, TTC) to NAD^+ and formazan, whereby NADH is oxidized again to NAD^+:

$$\text{Tetrazolium blue} + NADH + H^+ \xrightarrow{\text{Diaphorase}} \text{Formazan} + NAD^+$$

The amount of formazan formed is equimolar to the concentration of D-3-hydroxybutyric acid converted. Due to its specific absorption in the visible range, formazan is measured at 492 nm.

2. Reagents

2.1. Content & composition

The test is suitable for manual and automated processing. With manual processing, the reagents are sufficient for 50 determinations. The number of determinations for automated processing is increased by a multiple; however it depends on the device.

- Reagent 1: 2 x 50 mL with buffer, 3-HBDH, diaphorase
- Reagent 2: 2 x 12.5 mL with buffer, tetrazolium blue, NAD

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 identical.
- Bring samples to room temperature before measurement.
- Use liquid, clear and colorless solutions directly or after dilution with distilled water to a concentration within the measuring range (see performance data).
- Filter turbid solutions using a pleated or syringe filter to obtain a clear test solution. Alternatively, centrifuge at 3000 g for at least 5 minutes until a clear supernatant is obtained.
- Degas samples containing carbonic acid, e.g. by stirring in a beaker, filtration or centrifugation.
- Highly colored and highly concentrated samples should be decolorized with polyvinylpolypyrrolidone (PVPP).
- Sufficiently homogenize and crush solid and semi-solid samples, extract with water or dissolve in dist. water and filter if necessary.
- 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.

3.2. Simplified extraction with PEG 8000 (recommended)

For the determination of D-3-hydroxybutyric acid in whole liquid egg and whole egg powder, a simplified extraction with PEG 8000 is recommended below, in which proteins and fats are precipitated by emulsion separation and salting-out effects. The result is a clear aqueous test solution.

Please also refer to section *5.2. Note on calculation for extraction with PEG 8000.*

3.2.1. Determination of D-3-hydroxybutyric acid in whole liquid egg after extraction with PEG 8000

- Weigh approx. 10 g homogenized whole liquid egg **accurately** into a 50 mL Falcon® tube (alternatively: volumetric flask).
- Add 1.4 g PEG 8000, shake manually to solve the PEG; mix 10 min using an automatic multi vortex.
- Centrifuge at 3000 g for 10 min and transfer 1 to 3 mL of the clear supernatant in a new vial (no solid particles should remain in this supernatant).
- Use 100 µL of the clear extract for measurement.
- Do not store these extracts for more than 12 hours.

3.2.2. Determination of D-3-hydroxybutyric acid in whole egg powder after extraction with PEG 8000

- Weigh approx. 2.5 g homogenized whole egg powder **accurately** into a 50 mL Falcon® tube (alternatively: volumetric flask).
- Add 7.5 g dist. water, shake manually for 15 seconds and afterwards 10 seconds by vortexing to reconstitute the egg powder.
- Add 1.4 g of PEG 8000 and shake manually to solve the PEG; mix 10 min using e.g. an automatic multi vortex.
- Centrifuge at 3000 g for 10 min and transfer 1 to 3 mL of the clear supernatant in a new vial (no solid particles should remain in this supernatant).
- Use 100 µL of the clear extract for measurement.
- Do not store these extracts for more than 12 hours.

3.3. Determination of D-3-hydroxybutyric acid in pasta

- Weigh approx. 1 g homogenized pasta **accurately** into a 50 mL Falcon® tube (alternatively: volumetric flask).
- Add 12 mL dist. water and one drop of *n*-octanol, mix manually and incubate for 15 min in a boiling water bath.
- Let the extract cool down to room temperature.
- Add 1 mL **concentrated** Carrez-I solution (155 g/L potassium hexacyanoferrate (II) trihydrate) and mix.
- Add 1 mL **concentrated** Carrez-II solution (300 g/L zinc sulphate heptahydrate) and mix.

- Adjust the pH value between 8 and 9 with 0.1 M NaOH.
- Transfer quantitatively into a 25 mL volumetric flask and fill up with dist. water up to the mark.
- Centrifuge at 3000 g for 2 min and filter the supernatant through a fluted paper filter or use a syringe filter.
- Use 100 µL up to max. 500 µL of the clear solution for determination.

4. Manual test procedure

Wavelength:	492 nm
Temperature (measurement):	20 – 37 °C (68 – 99 °F)
Photometer alignment:	against air (without cuvette)
Measuring range:	0.5 – 50 mg/L (for 100 µL)

	Reagent blank	Samples / controls
Reagent 1 **Sample / control** **Dist. water**	2000 µL - 100 µL	2000 µL 100 µL -
Mix, incubate for **3 minutes at 20 – 37 °C (68 – 99 °F)**. **Read** absorbance **A₁** at **492 nm**, then add:		
Reagent 2	500 µL	500 µL
Mix, incubate for **15 minutes at 20 – 37 °C (68 – 99 °F)** and **read** absorbance **A₂** at **492 nm**.		

4.1. Important notes for assay performance

- The reagent blank value (water sample) must be determined **in each series of measurement** and subtracted from **each** sample result.
- Reagent blank and sample must be measured **in the same run** and under the same conditions.
- Specified incubation times were validated at 25 °C (77 °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 D-3-hydroxybutyric acid

The extinction difference ΔA must be calculated for each sample:

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

df: dilution factor
RB: reagent blank

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

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 D-3-hydroxybutyric acid acid is calculated using Lambert-Beer's law:

$$\mathbf{C}_{\text{D-3-hydroxybutyric acid}}\ \mathbf{[g/L]} = \frac{(V \times MW \times \Delta A)}{(\varepsilon \times d \times v \times 1000)} = \mathbf{0.1512 \times \Delta A} \times F$$

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]	= 2.6
MW:	Molecular weight D-3-hydroxybutyric acid [g/mol]	= 104.1
d:	Optical path [cm]	= 1.0
v:	Sample volume basic application [mL]	= 0.1
ε:	Extinction coefficient formazan [L/mmol x cm]	= 17.9 (at 492 nm)

5.2. Note on calculation for extraction with PEG 8000

Since liquid whole egg contains about 25 % of proteins and fat, a concentrating factor needs to be considered in the calculation:

10 g liquid whole egg contains 2.5 g precipitating solids by adding 1.4 g PEG 8000 which is solubilized in the aqueous phase. After precipitation the theoretical liquid amount is 10 g + 1.4 g – 2.5 g = 8.9 g. The concentrating factor is 10 g/8.9 g = 1.12. The same calculation applies for whole egg powder that is reconstituted by adding water before extraction.

5.3. 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:

$$\text{Content}_{\text{D-3-hydroxybutyric acid}}\ [\text{g/100 g}] = \frac{C_{\text{D-3-hydroxybutyric acid}}\ [\text{g/L sample}]}{\text{weigh-in}_{\text{sample}}\ \text{in g/L sample}} \times 100$$

Example:

$C_{\text{D-3-hydroxybutyric acid}} = 0.454$ g/L Weigh-in = 5.02 g/100 mL $\mathrel{\widehat{=}}$ 50.2 g/L

$$\text{Content}_{\text{D-3-hydroxybutyric acid}} = \frac{0.454\ \text{g/L}}{50.2\ \text{g/L}} \times 100 = 0.904\ \text{g/100 g (or \%)}$$

5.4. 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; 0.050 g/L D-3-hydroxybutyric acid).

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

6. Performance data

6.1. Specificity & side activities

The enzyme 3-hydroxybutyric acid dehydrogenase (3-HBDH) is specific for D-3-hydroxybutyric acid.

L-Ascorbic acid and SO_2 as typical redox-reactive, as well as L-3-hydroxybutyric acid (S-3-hydroxybutyric acid), carnitine and 3-hydroxyglutaric acid as they are structurally related to D-3-hydroxybutyric acid were analyzed. Except for ascorbic acid, none of these showed ΔA values of more than 0.002.

6.2. Interferences

Isocitric acid and L-tartaric acid showed no interferences up to concentrations of 2.5 g/L.

Sodium sulphite leads to slightly elevated signals for D-3-hydroxybutyric acid for concentrations of 1.25 g/L and above.

For relevant sugars and organic acids, no interferences were identified for concentrations at 5 g/L and 10 g/L.

6.3. Linearity, measuring range & sensitivity

For a sample volume of 100 µL, linearity is given up to 50 mg/L D-3-hydroxybutyric acid, leading to a recommended measuring range of 0.5 – 50 mg/L and 0.15 – 2 mg/L for a sample volume of 1000 µL respectively.

The limit of detection (LoD) was determined according to method DIN 32645:2008-11 in stabilized aqueous solution. This results in an LoD of 0.16 mg/L and 0.015 mg/L D-3-hydroxybutyric acid for a sample volume of 100 µL and 1000 µL, respectively.

The limit of quantification (LoQ) was determined by precision profile and confirms a concentration of 0.5 mg/L for 100 µL and 0.05 mg/L for 1000 µL sample volume respectively.

7. Supporting documents

On request, we offer the following documents:

- Enzytec™ *Liquid* D-3-Hydroxybutyric acid Validation report
- Enzytec™ *Liquid* Sample preparation guide
- Enzytec™ *Liquid* D-3-hydroxybutyric acid Excel results template
- Enzytec™ *Liquid* D-3-hydroxybutyric acid 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, 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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