Enzymatic UV assay for the determination of D-3-hydroxybutyric acid in foodstuffs and other sample materials
Test combination for 50 determinations

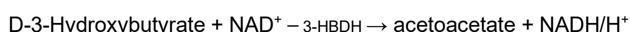
For in vitro use only
Store between 2 - 8 °C

This test was validated for the following matrices: whole liquid egg, whole egg powder and pasta. 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.

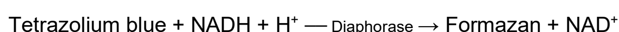
1. Test principle

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. The incubation times for automated processing may deviate and must therefore be verified.

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

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.

Caution: Tetrazolium blue (tetrazoliumchloride) is a light-sensitive reagent and should be protected from light as far as possible during the test and at all times during storage.

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)**.
- 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 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. Assay performance

Wavelength: 492 nm
Temperature: 20 - 37 °C (during the measurement)
Photometer alignment: against air (without cuvette)
Measuring range: 0.5 - 50 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 and read absorbance A_1 at **492 nm**, then add:		
Reagent 2	500 µL	500 µL
Mix, incubate for 15 min at 20 - 37 °C and read absorbance A_2 at **492 nm**.		

- 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 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 D-3-hydroxybutyric acid

The extinction difference ΔA must be calculated for each sample:

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

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

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

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)

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

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 ≙ 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

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 0.05 g/L D-3-hydroxybutyric acid as control for direct use.

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-hydroxy-butyric 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* 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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