Enzymatic color test for the determination of cholesterol in foodstuffs and other sample materials
Test combination for 55 determinations

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

This test was validated for the following matrices: whole egg powder, egg yolk, mayonnaise, egg nog, sausage, meat products and butter. For detailed results and further information on validation data, please refer to the validation report.

A recommended sample preparation for pasta and an alternative sample extraction for egg nog were tested and are available on request. Other foodstuffs or sample materials can be tested and must be validated by the user.

1. Test principle

Cholesterol is oxidized to cholest-4-en-3-one by the enzyme cholesterol oxidase (ChOx):

Cholesterol + O_2 $\xrightarrow{ChOx}$ cholest-4-en-3-one + H_2O_2

The enzyme peroxidase (POD) catalyzes the subsequent reaction:

2 H_2O_2 + 4-aminoantipyrine + phenol $\xrightarrow{POD}$ quinoneimine + 4 H_2O

The concentration of the resulting quinoneimine dye is equivalent to the amount of cholesterol and is measured at 492 nm due to its absorption in the visible range. For result calculation, a quinoneimine-specific extinction coefficient is used.

To account for non-specific effects of the sample matrix, a matrix or sample blank is additionally performed by **omitting the cholesterol oxidase in Reagent 1 *Blank***.

2. Reagents

2.1. Content & composition

The test is suitable for manual and automated processing. With manual processing, the reagents are sufficient for 55 determinations. The number of determinations for automated processing is increased by a multiple; however, it depends on the device.

(1) Reagent 1 *Blank*	1 x 116 mL	Buffer
(2) Reagent 1 *Sample*	1 x 116 mL	Buffer, cholesterol oxidase
(3) Reagent 2	4 x 14.5 mL	Buffer, 4-aminoantipyrine, peroxidase

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 indicated shelf life (see labeling) even after opening at 2 - 8 °C if handled properly. Do not freeze reagents.

Caution: Reagent 2 contains the light-sensitive reagent 4-aminoantipyrine and should be protected from light as far as possible during the test and at all times during storage.

The buffer must not be heated above 45 °C, otherwise irreversible turbidity may occur.

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.

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. Additional supplies, reagents and apparatus

The following supplies, reagents and apparatus are **additionally** required for the preparation of samples:

(1) Potassium hydroxide in water for the preparation of 10 M KOH
(2) Methanol for the preparation of a methanolic 1 M KOH
(3) 2-Propanol (Isopropanol)
(4) Hydrochloric acid for the preparation of an 8 M HCl solution
(5) Calcium chloride dihydrate
(6) Glass beads (0.25 - 0.5 mm; e.g. Carl Roth, Art. No. A553.1, Karlsruhe, Germany)
(7) Round bottom flask (50 mL)
(8) Reflux condenser (fitting to 50 mL flasks)
(9) Heating mantle for 50 mL flasks
(10) Fluted or pleated paper filter
(11) Syringe filters Polyethersulfone (PES), 0.22 µm
(12) Graduated flasks (25 mL, 50 mL, 100 mL)
(13) Centrifugal vials
(14) Spectrophotometer for 4 mL cuvettes (set to 492 nm)
(15) Single use acrylic cuvettes (4 mL)

3.2. General sample preparation

- Homogenize **semi-solid and pasty samples** sufficiently; crush them completely and sieve quantitatively (mesh size ≤ 0.2 mm).
- The cholesterol is extracted from the sample matrix by **alkaline hydrolysis**: boiling under reflux with methanolic potassium hydroxide solution and 2-propanol (see **3.1 Additional supplies, reagents and apparatus**).
- For this purpose, a 1 M methanolic potassium hydroxide solution must be **freshly prepared daily**, as commercially available methanolic potassium hydroxide solutions usually contain stabilizers that can inhibit the cholesterol oxidase. For this purpose, an aqueous 10 M KOH is diluted with 9 times the volume of methanol (p.a.).
- **Fatty acids** can be removed by acidifying the sample in the cold.
- The sample preparation for manual and automated testing is identical.

3.3. Determination of cholesterol in whole egg powder, egg yolk, mayonnaise and egg nog

- Weigh 1 ± 0.05 g glass beads in a 50 mL round bottom flask
- Add 0.25 g ± 0.01 g sample.
- Add 20 mL 1 M methanolic KOH and 10 mL 2-propanol.
- **Heat the mixture to boiling point and then** heat for 30 minutes while stirring on the reflux condenser.
- Transfer the supernatant solution to a 50 mL volumetric flask and allow to cool to below 80 °C (boiling temperature of 2-propanol).
- Add **in the following order**:
  (1) 6 mL 2-propanol
  (2) 2 mL 8 M HCl
  (3) 1 g calcium chloride dihydrate
  Complete cooling can take place after this addition.
- Fill the volumetric flask with 2-propanol up to the mark when the sample has reached 20 °C or the calibration temperature of the volumetric flask.
- Mix and allow to cool for 20 min at 2 - 8 °C (e.g. on ice).
- Filter the solution through a pleated filter and use the clear solution in the test.
  If the sample stands for longer, further precipitation of the fatty acids may occur. The sample may then have to be filtered again. Alternatively, use a syringe filter.

The extraction method described above is also recommended for the characterization of the phytosterol content of **pure vegetable oils**.

3.4. Determination of cholesterol in sausage, meat products and butter

- Weigh 1 ± 0.05 g glass beads in a 50 mL round bottom flask.
- Add 0.25 g of the meat product or 0.125 g of butter.
- Add 10 mL of 1 M methanolic KOH.
- **Heat the mixture to boiling point and then** heat for 25 minutes while stirring on the reflux condenser.
- Transfer the supernatant solution to a 25 mL volumetric flask and allow to cool to below 80 °C (boiling temperature of 2-propanol).
- Heat the residue **twice** with 6 mL 2-propanol each time while stirring at the reflux condenser.
- Transfer the solution to a 25 mL volumetric flask and add 1 mL 8 M HCl.
- Fill the volumetric flask with 2-propanol up to the mark when the sample has reached 20 °C or the calibration temperature of the volumetric flask.
- Mix and filter the solution through a pleated filter and use **200 µL** of the clear solution in the test (**take note of the reagent dilution factor df!** → 5.1.1. Total cholesterol concentration).

3.5. Further advice

- The sample extracts should be as clear as possible and without precipitation. 2-Isoproanol can cloud and/or damage acrylate cuvettes if left to act for a long time. This can lead to turbidity in the lower part of the cuvette. A longer standing time (e.g. in devices) should therefore be avoided.
- The **cuvette turbidity should not be in the beam path during the measurement**, as otherwise the measured absorption will be too high.
- Pay attention to/avoid boiling retardation!
- It has been shown that the recovery of cholesterol in sample solutions can be significantly improved if they are left overnight at 4 °C and measured the following day.

4. Assay procedure

Wavelength: 492 nm
Cuvettes: 1 cm light path
Temperature: 37 °C (during the measurement)
Measuring range: 20 - 900 mg/L

	Sample blank	Samples / controls
Reagent 1 *Blank*	2000 µL	-
Reagent 1 *Sample*	-	2000 µL
Sample / control	100 µL	100 µL
Mix, incubate for 3 min at 37 °C. Read absorbance **A_1** at **492 nm**, then add:		
Reagent 2	500 µL	500 µL
Mix, wait for the end of the reaction (10 min at 37 °C) and measure absorbance **A_2** at **492 nm.**		

The sample blank value (SB) must also be determined **for each sample** and subtracted from the respective sample result.

If the measuring temperature is below 37 °C, this can lead to an approximately 10 % lower sample recovery for cholesterol concentrations below 100 mg/L.

4.1. Important notes on the assay procedure

When performing the test, **no reagent** blank is used, but a **sample** blank using reagent 1 *Blank* (**without** cholesterol oxidase). This means that **two** cuvettes are required for each determination, into each of them 100 µL of sample is added.

- Sample blank and sample must be measured **in the same run** and under the same conditions.
- The use of a multistepper pipette is recommended for the addition of reagent 1 *Blank*, reagent 1 *Sample* and reagent 2. Use a separate tip for each component.
- Use separate tips for each sample extract (and the control solutions) to avoid cross-contamination; rinse the tip before pipetting.
- The reagent may only be swirled and not shaken, as this can lead to increased foam formation. Make sure that the beam does not pass through the foam, otherwise light scattering may occur and too high absorbance may be measured.
- Reagent 2 contains a light-sensitive dye and should only be drawn up immediately before addition and pipetted quickly. The photometer should be closed immediately after the addition of reagent 2.

5. Calculation of results

5.1. Calculation of sample solutions

5.1.1. Total concentration of cholesterol

$$\Delta A_{\text{Cholesterol}} = (A_2 - A_1 \times df)_{\text{Sample or control}} - (A_2 - A_1 \times df)_{\text{SB}}$$

df: (Reagent) dilution factor
SB: Reagent blank

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

Stated df of 0.808 applies for a basic application of 100 µL. **Increasing the sample volume** (refer to validation report) with unchanged reagent volumes **requires conversion of the dilution factor (df)**.
If the volume is increased, the test system may be affected. In general, this must be checked depending on the matrix. It is recommended to adjust the sample blank to the increased sample volume.

The cholesterol concentration is calculated using Lambert-Beer's law:

$$\mathbf{C}_{\text{Cholesterol}}\ \mathbf{[g/L]} = \frac{(V \times MW \times \Delta A)}{(\varepsilon \times d \times v \times 1000)} = \mathbf{1.648 \times \Delta A} \times F$$

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

V:	Test volume (basic application) [mL]	= 2.600
MW:	Molecular weight [g/mol]	= 386.65
d:	Optical path [cm]	= 1.00
v:	Sample volume (basic application) [mL]	= 0.100
ε:	Extinction coefficient quinoneimine [L/mmol x cm]	= 6.1 (at 492 nm)

5.2. Calculation of solid samples

When analyzing solid and semi-solid samples that are weighed in for sample preparation, the analysis result is based on the sample weight:

$$\text{Content}_{\text{Cholesterol}}\,[\text{g}/100\ \text{g}] = \frac{C_{\text{Cholesterol}}\,[\text{g/L sample solution}]}{\text{weight}_{\text{Sample}}\ \text{in g/L sample solution}} \times 100$$

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 %. For this purpose, we recommend the use of reference materials or standard solutions. For example:

- NIST SRM 1845a Whole egg powder
- NIST 1546a Meat homogenate

Due to its insolubility in water, cholesterol is weighed out for the preparation of control or reference samples and dissolved in 2-propanol at the desired concentration.

The properties of the solvent must be taken into account during handling (e.g. good wetting of the pipette tip, use of a positive displacement pipette).

6. Performance data

6.1. Specificity & side activities

The cholesterol oxidase oxidizes cholesterol and other sterols in which the hydroxyl group on carbon atom 3 is in the β-position (except lanosterol). Therefore, phytosterols such as stigmasterol, β-sitosterol, campesterol and delta-5-avenasterol also react to almost 100 %. This must be taken into account when calculating the egg content of "egg-containing foods".

When analyzing animal fats, the International Union of Pure and Applied Chemistry (IUPAC) recommends calculating the results as cholesterol (molecular weight 386.64 g/mol); when analyzing vegetable fats, it recommends calculating the results as β-sitosterol (molecular weight 414.69 g/mol).

6.2. Interferences

Sulphite, ascorbic acid, nitrate, nitrite and oxalic acid do not interfere up to a concentration of 1 g/L.

6.3. Linearity, measuring range & sensitivity

Linearity is given up to 900 mg/L cholesterol, with the recommended measuring range between 20 and 900 mg/L (sample volume of 100 µL).

The limit of detection (LoD) was determined according to method DIN 32645:2008-11 in 2-propanol. This results in an LoD of 4 mg/L and 2 mg/L cholesterol for a sample volume of 100 µL and 500 µL, respectively. The limit of quantification (LoQ) was determined by precision profile and confirms a concentration of 20 mg/L and 5 mg/L for 100 µL and 500 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* Troubleshooting guide

Safety data sheets (SDS) und certificates of analysis (CoA) are available in digital under 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 validation of the sample matrix of interest will need to be performed.

Due to the multitude of sample types, matrix effects cannot be excluded. These can lead to increased results, but also impair or suppress the enzymatic reaction. Such matrix effects are independent of the specificity of the enzymes used in the test and can be made visible by spiking experiments.

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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