

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 (36 – 46 °F)

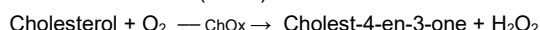
This test was validated using selected samples of the following matrices: whole egg powder, egg yolk, mayonnaise, egg liqueur, sausage, meat products, and butter.

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

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



The enzyme peroxidase (POD) catalyzes the subsequent reaction:



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.

- Reagent 1 *Blank*: 1 x 110 mL (buffer, **without** enzyme)
- Reagent 1 *Sample*: 1 x 110 mL (buffer, ChOx)
- Reagent 2: 4 x 14.5 mL (buffer, 4-Aminoantipyrin, POD)

### 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.

**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 (113 °F), otherwise irreversible turbidity may occur.

### 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. 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 (176 °F, boiling point 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 (68 °F) or the calibration temperature of the volumetric flask.
- Mix and allow to cool for 20 minutes at 2 – 8 °C (36 – 46 °F, 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**.

An alternative sample extraction for egg nog was tested and is available on request.

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

- Weigh  $1 \pm 0.05$  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 (176 °F, boiling point 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 (68 °F) 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!** → see 5.1. Total concentration of cholesterol in sample solutions).

### 3.5. Determination of cholesterol in pasta (not validated)

The following method for sample preparation of pasta has been tested for its basic suitability but has not been fully validated; therefore, it is provided solely as a recommendation.

A separate document is available as a *Recommendation* upon request.

- Weigh 1 g of glass beads and 1 g of the sufficiently homogenized sample into a 50 mL round bottom flask.
- Add 10 mL of freshly prepared methanolic KOH (1 M).
- Heat the sample solution under stirring in a reflux condenser and, once the boiling temperature is reached, incubate for 25 minutes.
- Transfer the supernatant through a funnel into a 50 mL glass bottle with a screw cap and allow it to cool.
- Add 5 mL of 2-propanol to the residue and mix gently by swirling.
- Bring the contents of the flask to a boil while stirring, then boil for another 5 minutes. Transfer the supernatant to the glass bottle as well.
- Repeat the previous step with 5 mL of 2-propanol, transfer the supernatant back into the glass bottle, and allow it to cool.
- Add 4 mL of 2-propanol to the residue, mix by gently swirling, and transfer the 2-propanol together with the residue into the glass bottle.
- Add 1 mL of 8 M HCl and mix carefully (the volume of the extract solution should be 25 mL after all reagents have been added).
- Filter the extract solution through a pleated filter; store the extracts overnight at 4 °C (39 °F). If the extract becomes cloudy again during this time, the filtration must be repeated.
- Use 100 µL of the clear extract solution in the test.

### 3.6. Further advice

- The sample extracts should be as clear as possible and without precipitation. 2-Isopropanol 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 and 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 (39 °F) and measured the following day.

### 4. Manual test procedure

|                            |                               |
|----------------------------|-------------------------------|
| Wavelength:                | 492 nm                        |
| Temperature (measurement): | 37 °C (99 °F)                 |
| Photometer alignment:      | against air (without cuvette) |
| Measuring range:           | 20 – 900 mg/L                 |

|                                                                                                                                                            | Sample blank | Samples / controls |
|------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|--------------------|
| Reagent 1 <i>Blank</i>                                                                                                                                     | 2000 µL      | -                  |
| Reagent 1 <i>Sample</i>                                                                                                                                    | -            | 2000 µL            |
| Sample / control                                                                                                                                           | 100 µL       | 100 µL             |
| Mix, incubate for <b>3 minutes at 37 °C (99 °F)</b> . Read absorbance <b>A<sub>1</sub></b> at <b>492 nm</b> , then addition of:                            |              |                    |
| Reagent 2                                                                                                                                                  | 500 µL       | 500 µL             |
| Mix, incubate for <b>10 minutes</b> (wait for the end of the reaction) at <b>37 °C (99 °F)</b> and read absorbance <b>A<sub>2</sub></b> at <b>492 nm</b> . |              |                    |

### Important notes for assay procedure

- When performing the test, a **sample blank** is used **instead of a reagent blank**, using reagent 1 *Blank* (**without enzyme**). This means that **two** cuvettes are required for each determination, into each of which 100 µL of sample is added.
- The sample blank (SB) must be determined for **each** sample and subtracted from the respective sample result. The sample blank and the sample must be measured **in the same** run and under the same conditions.
- The specified incubation times were validated at an optimal temperature of 37 °C (99 °F). The test should generally **not** be performed at temperatures **below** this value. If the measurement temperature is below 37 °C (99 °F), this can result in approx. a 10 % lower sample recovery for cholesterol concentrations below 100 mg/L.
- The use of a multistep pipette is recommended for the addition of Reagent 1 *Blank*, Reagent 1 *Sample*, and Reagent 2. Use a separate tip for each component.
- The reagents should only be gently swirled and not shaken, as shaking may cause excessive foaming.
- 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.
- Ensure that the light path does not pass through the foam, as this can cause light scattering and may result in an excessively high absorption reading.
- Use separate tips for each sample extract and the control solutions to avoid cross-contamination; rinse the tip before pipetting.
- 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.

### 5. Calculation of results

#### 5.1. Total concentration of cholesterol in sample solutions

The extinction difference  $\Delta A$  must be calculated for each sample:

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

df: Dilution factor  
RB: Reagent blank

$$df = \frac{\text{sample volume [mL]} + \text{volume R1 [mL]}}{\text{reaction volume [mL]}} = 0.808$$

The specified df value of **0.808** applies to a base application of **100 µL**. An increase in sample volume is possible (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 cholesterol in sample solutions is calculated using Lambert-Beer's law:

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

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 cholesterol [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. Content of cholesterol in 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:

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

## 5.3. Controls & acceptance criteria

Control or reference samples should be included in each run for quality control purposes. For this purpose, we recommend certified reference material, for example:

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

The recovery of control solutions from extracted food samples should be within  $100 \pm 10 \%$ .

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 a recommended measuring range of 20 – 900 mg/L for a sample volume of 100 µL.

The limit of detection (LoD) was determined according to the method DIN 32645:2008-11 in 2-propanol. For a sample volume of 100 µL, the calculated LoD is 4 mg/L and 2 mg/L for a sample volume of 500 µL.

The limit of quantification (LoQ) was determined by precision profile. The calculated LoQ is 20 mg/L cholesterol for a sample volume of 100 µL and 5 mg/L for a sample volume of 500 µL.

## 7. Supporting documents

On request, we offer the following documents:

- Enzytec™ Liquid Cholesterol Validation report
- Enzytec™ Liquid Cholesterol Excel template for results
- Enzytec™ Liquid Cholesterol Technical Information
- Enzytec™ Liquid Sample preparation guide
- 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 to 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

On request, we offer the following services:

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

**10. Disclaimer**

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- b. Failure to use trained and qualified personnel;
- c. Failure to apply appropriate industry standard practices, including Good Laboratory Practices;
- d. Failure to otherwise use, and when necessary validate or verify, suitable controls, samples, matrices, or processing procedures;
- e. Improper use;
- f. Product alterations or modifications;
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