Cholesterol

Colorimetric method
for the determination of cholesterol in foodstuffs and other materials

Simplified procedure: see "Determination of cholesterol in liquid egg, etc." (see pt.12)

Cat. No. 10 139 050 035

Test-Combination for 31 determinations

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

For use in foodstuff hygiene only.

Store at 2-8°C

For recommendations for methods and standardized procedures see references (2)

Principle (Ref. 1)

Cholesterol is oxidized by cholesterol oxidase to cholestenone (1).

(1) Cholesterol + O_2 $\xrightarrow{\text{cholesterol oxidase}}$ Δ^4-cholestenone + H_2O_2

In the presence of catalase, the hydrogen peroxide produced in this reaction oxidizes methanol to formaldehyde (2).

(2) Methanol + H_2O_2 $\xrightarrow{\text{catalase}}$ formaldehyde + 2 H_2O

The latter reacts with acetylacetone forming a yellow lutidine-dye in the presence of NH_4^+ ions (3).

(3) Formaldehyde + NH_4^+ + 2 acetylacetone $\longrightarrow$ lutidine-dye + 3 H_2O

The concentration of the lutidine-dye (3,5-diacetyl-1,4-dihydrolutidine) formed is stoichiometric to the amount of cholesterol and is measured by the increase of light absorbance in the visible range at 405 nm.

The Test-Combination contains

1. Bottle 1 with approx. 95 ml solution consisting of:
   ammonium phosphate buffer, pH approx. 7.0; methanol, 2.6 M; catalase, approx. 220 000 U
2. Bottle 2 with approx. 60 ml solution, consisting of:
   acetylacetone, 0.05 M; methanol, 0.3 M
3. Bottle 3 with approx 0.8 ml suspension cholesterol oxidase, approx. 12 U
4. Bottle 5 with Cholesterol assay control solution for assay control purposes (measurement of the assay control solution is not necessary for calculating the results.). Use the assay control solution undiluted. (Expiry date: see pack label)

Preparation of solutions

Cholesterol reagent mixture (**"solution 4"** in the assay):
Mix 3 parts of the solution from bottle 1 with 2 parts of the solution from bottle 2 adjusted to 20-25°C (use brown bottle!). Allow the mixture to stand at 20-25°C for 1 h before use.

Use the contents of bottle 3 undiluted (**"solution 3"** in the assay).

Stability of reagents

The contents of bottles 1, 2 and 3 are stable at 2-8°C (see pack label).
Solution 4 is stable for 3 months in a brown bottle at 2-8°C.
Development of a slight yellow color does not interfere with the assay.
Bring solution 4 to 20-25°C and let it stand for 1 h before use.

Procedure

Wavelength: (Hg) 405 nm
Glass cuvette[1]: 1.00 cm light path
Incubation temperature: 37-40°C
Measuring temperature: 20-25°C
Incubation volume: 5.400 ml
Measuring volume: approx. 2.5 ml
Measurement of sample blank and sample one after another in the same cuvette against air (without cuvette in the light path)
Sample solution: 8-160 µg cholesterol/assay[2] (in 0.400 ml sample volume)

Pipette into the tubes made of **glass**	Sample blank	Sample
solution 4	5.000 ml	-
sample solution*	0.400 ml	-
Mix contents of the test tube thoroughly.		
Pipette from the test tube containing the sample blank[3] into another test tube made of **glass**	-	
solution 3	-	2.500 ml
		0.020 ml
Mix thoroughly, cover test tubes containing the sample blank and the sample, and incubate in a water-bath at 37-40°C for 60 min. Allow to cool to 20-25°C. Read absorbances of the sample blank and the sample one after another in the same cuvette against air (see pt. 2.8). Subtract absorbance of the blank from the absorbance of the sample (= ΔA).		

* Rinse the enzyme pipette or the pipette tip of the piston pipette with the sample solution before dispensing the sample solution.

The measured abosrbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt.5).

Calculation

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = final volume [ml]
v = sample volume [ml]
MW = molecular weight of the substance to be assayed [g/mol]
d = light path [cm]
ε = extinction coefficient of the lutidine-dye at 405 nm
= 7.4 [l × mmol^{-1} × cm^{-1}]

Considering the dilution carried out in the test mixture (dilution factor $\frac{2.52}{2.5} = 1.008$), it follows that the sterol content, calculated as cholesterol, is:

$$c = \frac{5.400 \times 386.64 \times 1.008}{7.4 \times 1.00 \times 0.400 \times 1000} \times \Delta A = 0.711 \times \Delta A \text{ [g cholesterol/l sample solution]}$$

If the sample has been diluted further during preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$\text{Content}_{\text{cholesterol}} = \frac{c_{\text{cholesterol}} \text{ [g/l sample solution]}}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \times 100 \text{ [g/100 g]}$$

1. Instructions for performance of assay

The amount of cholesterol present in the assay has to be between 8 µg and 160 µg. In order to get a sufficient absorbance difference, the sample solution is diluted with isopropanol to yield a cholesterol concentration between 0.07 and 0.4 g/l.

1 If desired, disposable cuvettes may be used instead of glass cuvettes.
2 See instructions for performance of assay.
3 The residue left in the pipette is returned into the test tube containing the sample blank.
4 For example, by Parafilm (trademark of American Can Company, Greenwich, Ct., USA), or **better with a stopper made of glass**

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Dilution table

Estimated amount of cholesterol per liter	Dilution with isopropanol	Dilution factor F
< 0.4 g	-	1
0.4- 4.0 g	1 + 9	10
4.0-40.0 g	1 + 99	100

2. Technical information

2.1 Crush solid samples (e.g. noodles) and pass quantitatively through a sieve with < 0.2 mm mesh.

2.2 **From the different possibilities of sample preparation (see pt. 11) the simplified procedure (= boiling under reflux in a 50 ml volumetric flask) can generally be recommended (see pt. 11 c). Volume errors by heating the volumetric flask and the volume displacement by insoluble parts of the sample can be neglected.**

2.3 The volumetric flask which is used for boiling under reflux should posses a flat bottom. The length of the magnetic stirrer should be the same as the diameter of the volumetric flask's bottom, and the speed of mixing should be so high that neither the sample material nor the sea-sand lies on the bottom in order to avoid a delay in boiling.

2.4 Some very small pumice stones may be used instead of sea-sand (volume displacement approx. 0.4 ml). In this case the volume displacement can be neglected.

2.5 Boiling under reflux can be done either with a heatable stirrer or with a magnetic stirrer together with an electric heater. It is recommended to heat slowly in order to avoid boiling over.

2.6 A recovery experiment should be done for test control. For details see "cholesterol assay control solution".

2.7 The incubation with cholesterol oxidase should not be done in plastic tubes because plastic may absorb cholesterol (and the results will be too low); in addition plastic may release formaldehyde (and the results will be too high). It is recommended to incubate in glass tubes which are closed with a glass stopper. It cannot be recommended to cover the test tubes with Parafilm (trademark of American Can Company, Greenwich, Ct., USA) because a hole may be torn in the Parafilm and the solvent could evaporate.

2.8 When doing the photometric readings, A_2 of the sample is measured against A_2 of the sample blank. (The reading of A_1 is omitted.) The following procedure can be recommended:

- pour the sample blank assay mixture into a dry cuvette and measure the absorbance against air;
- pour the solution back into the sample blank tube;
- hit the cuvette with the open side on filter paper;
- pour the sample assay mixture into the same cuvette, pour the contents back into the reaction tube and back into the cuvette (in order to avoid inhomogeneities = schlieren in the solution to be measured);
- measure the absorbance of the sample assay mixture against air.

Use a separate cuvette for each blank and sample assay. Do not measure the blank after the sample: the results would be too low because of contamination - transmission. When measuring a series of analyses measure first all the sample blanks followed by the sample assays (if only one cuvette has to be used).

The measurement of blank and sample assays in two separate cuvettes is only possible when the light path of both cuvettes is absolutely identical at the wavelength used for the measurements.

3. Specificity (Ref. 1.4)

Cholesterol oxidase oxidizes cholesterol and other sterols in which the hydroxyl group at the carbon atom 3 is in the β-position (except lanosterol). Therefore, phytosterols, such as stigmasterol and sitosterol also react in the assay. This must be taken into account when calculating the egg content in "foodstuffs containing eggs".

The International Union of Pure and Applied Chemistry (IUPAC), Commission on Oils, Fats and Derivatives recommends to express the total sterol content arbitrarily either as cholesterol (molecular weight 386.64 g/mol) for animal fats or as β-sitosterol (molecular weight 414.69 g/mol) for vegetable fats (Ref. 2.1).

Table: Average sterol content of plant oils (Ref. 3.11)

Compound	sun-flower	peanut	soybean	cotton seed	corn	olive	palm
Cholesterol	0.5	6.2	0.5	0.5	0.5	0.5	0.5
Brassicasterol	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Campesterol	242	278	563	276	2655	19	88
Stigmasterol	236	145	564	17	499	0.5	42
β-Sitosterol	1961	1145	1317	3348	9187	732	252
Δ^5-Avenasterol	163	253	46	85	682	78	0,5
Δ^7-Stigmasterol	298	0.5	92	0.5	96	0.5	51
Δ^7-Avenasterol	99	34	63	18	102	30	0.5
24-Methylene-cycloartenol	204	0.5	53	0.5	425	580	0.5

data in mg/kg

4. Accuracy of measurement

In the analysis of commercial cholesterol, values of e.g. 92 ± 1.8% have been obtained. The measurement of the Standard Reference Material 911 of the National Bureau of Standards, Washington D.C., USA, resulted in 99 ± 0.5% (Ref. 1.3).

The specificity of the enzymatic method (see pt. 3) has to be considered in the evaluation of data: in the analysis of animal samples, the enzymatic method is relatively specific because the substrates of the nonspecific reactions are practically not contained in the samples. In the analysis of plant samples or of mixtures of animal and plant samples, one has to bear in mind that a number of plant sterols also react (Ref. 1.4).

In the comparison of data for the cholesterol content from different analytical procedures, the different selectivities have to be taken into consideration.

The performance of a recovery experiment (i.e. the analysis of the sample with and without added standard material) is recommended for test control purposes. For details see pt. 9.2 and information about "cholesterol assay control solution".

5. Sensitivity and detection limit

The smallest differentiating absorbance for the procedure is 0.010 absorbance units. This corresponds to a sample volume v = 0.400 ml a cholesterol concentration of 7 mg/l sample solution.

The detection limit of 20 mg/l is derived from the absorbance difference of 0.030 and a sample volume v = 0.400 ml.

6. Linearity (Ref. 1.3)

Linearity of the determination exists from 8 µg cholesterol/assay (20 mg cholesterol/l sample solution; sample volume v = 0.400 ml) to 160 µg cholesterol/assay (0.4 cholesterol/l sample solution; sample volume v = 0.400 ml).

7. Precision

In a double determination using one sample solution, a difference of 0.010 to 0.020 absorbance units may occur. With a sample volume of v = 0.400 ml this corresponds to a cholesterol concentration of approx. 7-15 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.07-0.15 g/100 g can be expected.)

The following data have been published in the literature:

x = 0.05 µmol/assay	CV = 1.05 %	n = 18 in series	
x = 0.2 µmol/assay	CV = 0.80 %	n = 18in series	
x = 0.5 µmol/assay	CV = 1.46 %	n = 18 in series	(Ref. 1.3)
Whole egg powder:			
x = 1665 mg/100 g	r = 111 mg/100 g	$s_{(r)}$ = 39 mg/100 g	
	R = 172 mg/100 g	$s_{(R)}$ = 61 mg/100 g	(Ref 2.2)

8. Interference/sources of error

Commercially available methanolic potassium hydroxide solution usually contains stabilizers which may inhibit cholesterol oxidase. The methanolic potassium hydroxide solution should therefore be *prepared freshly* by the individual user.

For this, dilute aqueous KOH (10 M; A.R.) with 9 volumes of methanol (A.R.).

9. Recognizing interference during the assay procedure

9.1 Operator error and interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.200 ml and 0.400 ml): the measured differences in absorbance should be proportional to the sample volumes used.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) are used. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

9.2 Insufficient extraction of the sample and possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method (for details see "cholesterol assay control solution").

10. Reagent hazard

Bottle 1 contains approx. 8 g methanol in approx. 100 ml aqueous solution; bottle 2 contains approx. 0.6 g methanol and approx. 0.3 ml acetylactone in approx. 60 ml aqueous solution; bottle 5 contains approx. 32 ml isopropanol.

Methanol in the a.m. concentration is hazardous to health to breathe in or ingest; isopropanol is flammable.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

11. General information on sample preparation

Semi-solid and pasty samples have to be homogenized sufficiently; solid samples have to be crushed and quantitatively passed through a sieve with < 0.2 mm mesh.

Cholesterol must be dissolved out of the sample matrix; its esters have to be hydrolized in alkali. There are the following possibilities for the determination of total cholesterol:

a) Boiling under reflux with methanolic potassium hydroxide, filtration of the supernatant by means of a frit (when a Potterat-Eschmann flask is used) followed by boiling the residue twice under reflux with isopropanol and filtration again, and collecting the filtrates in a volumetric flask.

b) Boiling under reflux in a 50 ml round-bottomed flask with methanolic potassium hydroxide and transfer of the supernatant into a 25 ml volumetric flask, followed by boiling the residue twice under reflux with isopropanol and transfer of the supernatant into the same volumetric flask.

c) **Boiling under reflux in a 50 ml volumetric flask with methanolic potassium hydroxide in the presence of isopropanol ("simplified procedure"). This working technique can generally be recommended, especially if a Potterat-Eschmann flask is not available and if the transfer of a clear supernatant is not possible.**

In the determination of **free cholesterol,** the sample is dissolved in isopropanol or extracted with isopropanol. Undissolved components are then removed by fitration.

Fatty acids can be removed by acidifying the sample in the cold. If this is not sufficient (the sample solution is still turbid), IUPAC recommends the use of a small sample volume (see Ref. 2.1).

Colorants in plant oils like e.g. in raw palm oil can be removed with activated charcoal: 5% of the sample weight (see Ref. 2.1).

In samples containing **low concentrations of cholesterol,** the sample is subjected to boiling under reflux with potassium hydroxide and then extracted with ether/petroleum ether (1:1) at 20-25°C. Afterwards, the organic phase is evaporated and the residue is dissolved in isopropanol (Ref. 3.8).

12. Application examples

Determination of cholesterol in noodles

Accurately weigh approx. 2 g of noodles which have been ground in a powder mill and quantitatively passed through a sieve, (cornsize: max. 0.2 mm) into a 50 ml round-bottomed flask. Add 1 g of sea-sand and heat for 25 min with 10 ml of a *freshly prepared* methanolic potassium hydroxide solution (1.0 M; see pt. 8) under a reflux condenser while stirring (magnetic stirrer).

Transfer the supernatant solution into a 25 ml volumetric flask with a pipette. Boil the residue twice with portions of 6 ml isopropanol each, under a reflux condenser for 5 min.

Collect the solutions in the volumetric flask, allow to cool to 20-25°C, fill up to the mark with isopropanol, and mix. Filter turbid solutions through a fluted filter. The clear solution is used for the assay.

Calculation:

Sterol content, calculated as cholesterol in 100 g of noodles in [mg/100 g]

$$= \text{sterol content of the sample solution [g/l]} \times \frac{100 \times 25}{w}$$

where w = weight of the sample in grams.

A Potterat-Eschmann flask can also be used instead of a 50 ml round-bottomed flask. **When using the simplified procedure (see "Determination of cholesterol in liquid egg, technical yolk and dry egg") weigh out approx. 4 g of the ground sample.**

When calculating the egg content of noodles from the sterol content of the sample, it is necessary to take into account the average phytosterol content of the egg-free material. The results obtained according to the present method correspond well with the results of the method of Acker and Greve (Ref. 3.1) recommended by the Bundesgesundheitsamt (Federal Health Department), Berlin (Ref. 3.2).

In the case of an acid pretreatment (AOAC method no. 14141, Ref. 3.4) a sterol content which is elevated by approx. 10 mg sterol (calculated as cholesterol) per 100 g noodles dry weight is obtained, as had been found by Dresselhaus and Acker, (Ref. 3.5, 3.6). This is caused by hydrolysis of phytosterol glycosides.

It has been reported that the average cholesterol content of eggs, as determined with the digitonid method is lower than formerly found [instead of 2.93% only 2.55% (Ref. 3.5, 3.6) or 2.45%, respectively (Ref. 3.7) in dry egg yolk, corresponding to about 190-200 mg cholesterol/16 g egg yolk].

If the table given in Ref. 3.5 and 3.6 is used for calculation of the egg content of durum noodles, due to the different method of pretreatment, 10 mg sterol/100 g noodles dry weight have to be added to the value found by the enzymatic analysis.

Egg content: eggs/kg flour, semolina	Total sterols in mg/100 g dry matter for noodles made of durum wheat	
	Digitonin procedure after acid hydrolysis (Ref. 3.5, 3.6)	Enzymatic determination after alkaline hydrolysis
0	48.0	38.0
1	71.5	61.5
2	94.5	84.5
3	117.1	107.1
4	139.3	129.3
5	161.1	151.1
6	182.5	172.5

The egg content of noodles can be calculated with high accuracy when the semolina/flour which has been used for production is analyzed enzymatically parallel to the noodle sample. In this case, the phytosterol content of semolina/flour can be taken into account.

Important note:
The procedure (sample preparation by alkaline hydrolysis) cannot be used in the analysis of pre-cooked noodles ("spätzle").

Determination of cholesterol in mayonnaise and remoulade

Weigh approx. 1 g of mayonnaise or remoulade and 1 g of sea-sand accurately into a 50 ml round-bottomed flask. Add 10 ml of a *freshly prepared* methanolic potassium hydroxide solution (1.0 M) and heat under a reflux condenser for 25 min while stirring (magnetic stirrer). Transfer the supernatant solution into a 25 ml volumetric flask with a pipette. Boil the residue twice with portions of 6 ml isopropanol each, under a reflux condenser for 5 min. Collect the solutions in the volumetric flask, allow to cool. Fill the volumetric flask up to the mark with isopropanol and mix. Filter turbid solutions through a fluted filter. The clear solution is used for the assay.

Calculation:

Sterol content of the mayonnaise in [mg/100 g], calculated as cholesterol

$$= \text{sterol content of the sample solution [g/l]} \times \frac{100 \times 25}{w}$$

where w = weight of the sample in grams.

To determine the content of egg yolk in the mayonnaise (remoulade) from the sterol content of the sample, it is necessary to take into account the average phytosterol content of the vegetable fats contained therein (Ref. 3.3)

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	% sterols			% sterols	
Oil or fat	total	free	Oil or fat	total	free
Plant oils					
Castor oil	0.23	0.16	peanut oil	0.24	0.15
Cocoa butter	0.24	0.17	chopped peanut oil	~0.07	0.03
Coconut oil	0.10	0.06	pumpkin kernel oil	0.38	0.22
Cottonseed oil	0.31	0.20	rape-seed oil	0.62	0.27
Linseed oil	0.43	-	safflower oil	0.31	0.22
Corn oil	0.85	0.25	sesame oil	0.50	0.21
Olive oil	0.11	0.06	soybean oil	0.34	0.22
Palm oil	0.4	0.03	chopped soybean oil	0.30	-
Palm kernel oil	0.08	-	sunflower oil	0.35	0.16

When using the simplified procedure (see "Determination of cholesterol in liquid egg, technical yolk and dry egg") accurately weigh approx. 2 g sample.

Determination of cholesterol in egg liqueur
Accurately weigh approx. 1 g of egg liqueur into a 50 ml round-bottomed flask. Add 1 g of sea-sand and heat for 25 min with 10 ml of a *freshly prepared* methanolic potassium hydroxide solution (1.0 M) under a reflux condenser while stirring (magnetic stirrer). Transfer the supernatant solution into a 25 ml volumetric flask with a pipette. Boil the residue twice with portions of 6 ml isopropanol each, under a reflux condenser for 5 min. Collect the solutions in the volumetric flask, allow to cool, dilute to the mark with isopropanol, and mix. Filter turbid solutions through a fluted filter. The clear solution is used for the assay.
To calculate the egg content from the sterol content of the sample solution, the average cholesterol concentration of approx. 200 mg cholesterol/egg yolk (16 g), is taken as basis (Ref. 3.5-3.7). With this value it follows:

eggs (egg yolks)/l egg liqueur

$$= \text{sterol content of the sample solution [g/l]} \times \frac{1000 \times 25 \times D}{w \times 200}$$

where w = weight of the sample in grams
D = specific gravity of the egg liqueur [g/ml].

When using the simplified procedure (see "Determination of cholesterol in liquid egg, technical yolk and dry egg") accurately weigh approx. 2 g sample.

Determination of cholesterol in lard (total cholesterol)
Accurately weigh a sample of about 2 g into a 50 ml round-bottomed flask. Add 10 ml of a methanolic potassium hydroxide solution (1.0 M) and heat under a reflux condenser for 25 min. Allow to cool, transfer the contents of the round-bottomed flask with a pipette into a 25 ml volumetric flask. Rinse the round-bottomed flask with isopropanol and transfer these rinses also to the volumetric flask.
Add 1 ml HCl (8 M) and fill up to the mark with isopropanol. Place in an ice-bath for 10 min. The free fatty acids precipitate.
Filter the turbid solution quickly through a fluted filter. The filtrate is used immediately for the assay.
After mixing of solution 4 and sample solution a slight turbidity appears. For removing turbidity, place the test tube for 10 min into a water-bath of 37°C or add 0.5 ml KOH (1 M), mix and continue the analysis. The volume alteration must be taken into account in the calculation.

Determination of cholesterol in liver sausage
a) *Total cholesterol:* Accurately weigh a sample of approx. 2.5 g liver sausage into a 50 ml round-bottomed flask. Add 1 g of sea-sand and 10 ml of a *freshly prepared* methanolic potassium hydroxide solution (1.0 M). Heat under a reflux condenser for 25 min while stirring (magnetic stirrer). Transfer the supernatant solution into a 25 ml volumetric flask with a pipette.
Boil the residue twice with portions of 6 ml isopropanol each, under a reflux condenser for 5 min. Collect the solutions in the volumetric flask, allow to cool. Fill volumetric flask up to the mark with isopropanol and mix. Filter through a fluted filter and use the clear solution for the assay.
b) *Free cholesterol:* Homogenize approx. 10 g liver sausage. Accurately weigh approx. 1 g and extract three times at 20-25°C by shaking with 5 ml portions of isopropanol each. Filter through a fluted filter into a 25 ml volumetric flask.
Add 5 ml HCl (8 M), fill up to the mark with isopropanol and place into a refrigerator for 20 min in order to separate the fat. Filter through a fluted filter and use the clear solution for the assay.

Determination of cholesterol in liquid egg, technical yolk and dry egg (simplified procedure)
Accurately weigh approx. 1 g liquid egg, 0.5 g yolk or 0.25 g dry egg, respectively, into a 50 ml volumetric flask and add 1 g sea-sand (the volume displacement of 0.400 ml must be taken into account in the calculation formula); heat under a reflux condenser for 30 min with 20 ml *freshly prepared* methanolic potassium hydroxide solution (1.0 M) and 10 ml isopropanol while stirring (magnetic stirrer). Allow the turbid solution to cool, and fill up to the mark with isopropanol at 20-25°C after removal of the magnetic rod (rinse with isopropanol); mix, filter through a fluted filter and use the clear solution for the assay.

Calculation:
Sterol content of the egg in [mg/100 g], calculated as cholesterol

$$= \text{sterol content of the sample solution [g/l]} \times \frac{100 \times 49.6}{w}$$

where w = weight of the sample in grams.

Determination of cholesterol in milk fat (Ref. 3.8)
Accurately weigh approx. 5 g milk fat into a 250 ml round-bottomed flask; heat for 30 min under a reflux condenser with 50 ml *freshly prepared* methanolic potassium hydroxide solution (2 M) while stirring (magnetic stirrer). Transfer the still warm solution with 100 ml redist. water into a 1000 ml separating funnel. After cooling to 20-25°C shake with 100 ml ether/petroleum ether (1+1). After 20 to 30 min drain off the clearly separated bottom phase into the saponification flask and transfer the organic phase into a 500 ml round-bottomed flask. This extraction has to be repeated twice. The collected ether/petroleum ether phases are to be evaporated under a rotation evaporator at 35°C and the residue is to be transferred with isopropanol into a 50 ml volumetric flask. Fill up to the mark with isopropanol at 20-25°C, mix and filter through a fluted filter. Use the clear solution for the assay.

Calculation:
Sterol content of milk fat in [mg/100 g], calculated as cholesterol

$$= \text{sterol content of the sample solution [g/l]} \times \frac{100 \times 50}{w}$$

where w = weight of the sample in grams.

13. Further applications

The method may also be used in the examination of cosmetics. For the determination of cholesterol in "egg"-shampoos see König, H. & Walldorf, E. (1979) Fresenius Z. *Anal. Chem.* **299,** 1-18; for the determination of cholesterol in cosmetic products, which are prepared with wool wax and wool wax alcohols, see Orlick, B. & Montag, A. (1982) Mitt. Lebensm. Chemie u. gerichtl. Chemie **36**, 30-31.

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Cholesterol assay control solution (Bottle 5)

Concentration: 1.00 mg cholesterol/ml

Cholesterol assay control solution is a solution of cholesterol in isopropanol. It serves as an assay control solution for the enzymatic determination of cholesterol in foodstuff and other materials.

Application:

1. *Addition of cholesterol assay control solution to the assay mixture:*
   Instead of the sample solution as stated in the pipetting scheme of the instruction, pipette into the test tube:

assay control solution:	0.100 ml
isopropanol:	0.300 ml.

   This corresponds to a diluted assay control solution of a concentration of 0.25 mg cholesterol/ml; the assay mixture contains 100 µg cholesterol from the assay control solution.

2. *Addition of cholesterol assay control solution to the sample before saponification of cholesterol esters:*
   Before saponification of the cholesterol esters add 5.0 ml of cholesterol assay control solution to the sample mixed with *freshly prepared* methanolic potassium hydroxide (1 M). Saponify the mixture in the usual manner by heating under a reflux condenser as mentioned under "Instructions for sample preparation". Fill up solution (after saponification) to 25 ml and use 0.400 ml for the assay. This way, the assay mixture contains 80 µg cholesterol from the added assay control solution.

Quality of the standard

The cholesterol used in the assay control solution is checked against the *Standard Reference Material 911b* of the National Bureau of Standards, Washington D.C., USA.

R-BIOPHARM AG
An der neuen Bergstraße 17
64297 Darmstadt/Germany
Phone + 49 61 51 / 81 02-0
Fax + 49 61 51 / 81 02-20
www.r-biopharm.com

Roche