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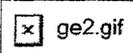


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45.4.10 - Vitamins and Other Nutrients / Nutritionally Related Components

AOAC Official Method 994.10 Cholesterol in Foods

Direct Saponification–Gas Chromatographic Method First Action 1994

(Applicable to determination of  1 mg cholesterol/100 g of foods and food products.)

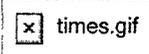
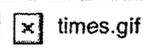
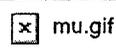
See Table 994.10 for the results of the interlaboratory study supporting the acceptance of the method.

A. Principle

Lipid in test portion is saponified at high temperature with ethanolic KOH solution. Unsaponifiable fraction containing cholesterol and other sterols is extracted with toluene. Sterols are derivatized to trimethylsilyl (TMS) ethers and then quantified by gas chromatography.

B. Apparatus

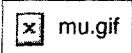
(a) *Centrifuge tubes.*—15 mL, Pyrex No. 13. Silanize tubes as follows: Fill tubes with 10% hydrofluoric acid and let stand 10 min. Rinse tubes thoroughly with H₂O, and then with anhydrous methanol. Dry tubes under stream of nitrogen. Fill tubes with 10% hexamethyldisilane (HMDS) in toluene and let stand 1 h. Rinse tubes thoroughly with toluene, and then with anhydrous methanol. Dry tubes in 100°C oven before use. Alternatively, commercial silinizing reagent may be used. Before each reuse, clean tubes with H₂O, ethanol, hexane, and acetone, and dry in 100°C oven. Tubes can be reused without resilylation if strong alkali wash is avoided. Resilanize tubes at least every 6 months.

(b) *Gas chromatograph (GC).*—With hydrogen flame ionization detector, capillary column, split-mode, 25 m  0.32 mm  0.17  m film thickness, cross-linked 5% phenyl–methyl silicone or methyl silicone gum (e.g., Hewlett Packard No. HP-5, Ultra 2, or HP-1), split inlet liner filled with 10% SP 2100 on 80–100 mesh Supelco packing, and 2 ramp oven temperature programming (Hewlett Packard Model 5890A, is suitable). Operating conditions: temperatures—injector 250°C, detector 300°C, column 190°C, hold 2 min; increase 20°/min to 230°C, hold 3 min; increase 40°/min to 255°C, hold 25 min. Flow rates: helium—column ca 2 mL/min, split vent ca 30 mL/min, purge vent ca 3 mL/min, auxiliary make-up gas ca 20 mL/min; hydrogen—ca 35 mL/min;

air—ca 280 mL/min.

(c) *Rotary evaporator*.—With glass condenser flask between concentration flask and metal shaft.

(d) *Magnetic stirrer-hot plate*.—With variable speed and heat controls.

(e) *Micropipets*.—Capable of delivering 100 and 200  L; metal body.

(f) *Test tube mixer*.

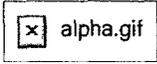
(g) *Balance*.—Analytical, capable of weighing to 0.0001 g.

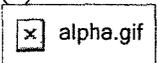
(h) *Glassware*.—Erlenmeyer flasks, 125 and 250 mL; volumetric flasks and pipets; graduated cylinders; separatory funnels, 500 mL.

C. Reagents

(a) *Dimethylformamide (DMF)*.—Distilled in glass.

(b) *Hexamethyldisilane (HMDS)*.

(c) 5 —*Cholestane internal standard solution*.—0.1 mg/mL in *n*-heptane. Standard 5

—cholestane available from Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178, USA, is suitable.

(d) *Cholesterol standard*.—(1) *Stock solution*.—2.0 mg/mL dimethylformamide (DMF). (2) *Working solutions*.—Dilute stock solution with DMF to obtain 6 solutions at concentrations 0.0025–0.2 mg/mL (i.e., 0.0025, 0.005, 0.01, 0.05, 0.1, and 0.2 mg/mL). Eastman Kodak Co., is suitable.

(e) *Potassium hydroxide solutions*.—(1) *50% KOH (w/w)*.—Dissolve 500 g KOH in 500 g H₂O. (2) *1M KOH*.—Dissolve 56 g KOH in ca 800 mL H₂O with cooling and dilute to mark in 1 L volumetric flask. (3) *0.5M KOH*.—Dilute one part 1M KOH solution with one part H₂O.

(f) *Trimethylchlorosilane (TMCS)*.—No. 88531, Pierce Chemical Co., or equivalent.

(g) *Toluene*.—Distilled in glass.

(h) *Sodium sulfate*.—Anhydrous.

(i) *Glass wool*.

D. Saponification

Accurately weigh (usually 2–3 g) test portion (W_1) to nearest 0.001 g into 250 mL Erlenmeyer flask.

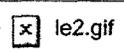
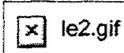
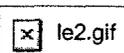
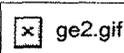
Amount of test portion should contain  1 g fat or  5 g H₂O (i.e., weigh 1 g pure oils, 1.5 g salad dressings, and  5 g substances with high moisture content). Place magnetic stir bar into flask. Add to flask 40 mL 95% ethanol and 8 mL 50% KOH solution, C(e)(1). (Note: Portion of ethanol may be retained and used as rinse after KOH addition. This will help prevent ground-glass joints of flask and condenser from freezing together.)

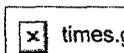
Table 994.10: Interlaboratory study results for determination of cholesterol in foods by direct saponification methods

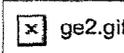
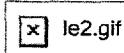
Place flask on magnetic stirrer-hot plate, attach condenser, turn on stirrer-hot plate, and reflux 70 ± 10 min. To ensure complete saponification, occasionally check test portion and disperse any clumps with glass rod or by adding KOH solution to test portion while stirring.

Turn off heat and add 60 mL 95% ethanol through top of condenser while stirring solution. (Caution: Add carefully to avoid spurting of alcohol from top of condenser.) After ca 15 min remove flask from condenser, close with stopper, and cool solution to room temperature. Test solution is stable 24 h.

E. Extraction

Add 100 mL toluene (V_1) to saponified test portion while stirring. Stopper flask and stir  30 s. Pour solution into 500 mL separatory funnel without rinsing. Add 110 mL 1M KOH solution, C(e)(2), and shake funnel vigorously 10 s. Let layers separate and discard aqueous (lower) layer (will be turbid). Add 40 mL 0.5M KOH solution, C(e)(3) to separatory funnel, invert funnel, and gently swirl contents 10 s. Discard aqueous (lower) layer.

Wash toluene layer with 40 mL H₂O by gently rotating separatory funnel. Allow layers to separate and discard aqueous phase. Repeat H₂O wash at least 3  times, shaking more vigorously each time. If emulsification occurs, add small amount 95% ethanol, swirl contents of funnel, let layers separate, and continue with H₂O washes. After final wash, toluene layer should be crystal clear.

Pour toluene layer from top of separatory funnel through glass funnel containing plug of glass wool and ca 20 g Na₂SO₄ into 125 mL Erlenmeyer flask containing ca 2 g Na₂SO₄. Stopper flask and swirl contents. Let mixture stand  15 min. Test solutions may be held  24 h if tightly sealed.

Pipet 25 mL extract (V_2) into 125 g flat-bottom boiling flask and evaporate contents to dryness on rotary evaporator at $40 \pm 3^\circ\text{C}$. Add ca 3 mL acetone and evaporate contents to dryness again. Dissolve residue in 3.0 mL DMF (V_3), C(a). Final concentration of cholesterol in DMF should be within range of working standard solutions, C(d)(2). (Note: If, after quantitation by gas chromatography, test portion concentration falls outside standard curve, change amount of toluene extract evaporated or volume of DMF used to dissolve the residue, or both, so final concentration of cholesterol in DMF falls within range of standards. If test portion contains little or no cholesterol, 75 mL toluene extract dried and redissolved in 2 mL DMF is adequate to detect 1 mg cholesterol/100 g in 1 g test portion.)

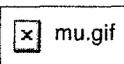
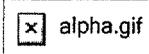
F. Derivatization

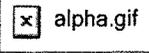
Pipet 1.0 mL aliquots of working standard solutions, **C(d)(2)**, and test solution into separate 15 mL centrifuge tubes, **B(a)**. Add to each tube 0.2 mL HMDS, **C(b)**, and 0.1 mL TMCS, **C(f)**. Stopper tubes and shake vigorously on test tube mixer or by hand for 30 s. Let solution stand undisturbed 15 min. Add to each tube 1.0 mL 5 -cholestane internal standard solution, **C(c)**, and 10 mL H₂O. Stopper tubes, shake vigorously 30 s, and centrifuge ca 2 min.

Transfer sufficient portion of heptane (upper) layer to injection vial. Make sure no aqueous layer is transferred.

Derivatized standards and test solutions must be analyzed within 24 h.

G. GC Analysis

Inject 1  L or other appropriate volume into gas chromatograph. Determine area of 5 -cholestane and cholesterol peaks using height-width measurement or digital integrator.

(Note: 5 -Cholestane and cholesterol should elute in 11–13 and 16–18 min, respectively. If these retention times are not met, adjust carrier flow and temperature.)

Divide cholesterol peak area by internal standard peak area to obtain standard response ratio. Plot response ratios of 4 high standards (0.01–0.20 mg/mL) against cholesterol concentrations. Standard response ratio plot should bracket test solution response ratio. If necessary, plot low standard curve (0.0025–0.05 mg/mL) for low level test solutions. Dilute high-level test solution to fall within standard range.

Calculate g of test portion/mL derivatized as follows:

$$\text{g Test portion/mL derivatized} = (W_1/V_1) \times (V_2/V_3)$$

where W_1 = weight of test portion, g; V_1 = volume of toluene used in extraction, 100 mL; V_2 = aliquot of extract taken to dryness, 25 mL; V_3 = volume of DMF used to dissolve residue, 3 mL.

Calculate cholesterol content in test portion as follows:

$$\text{mg Cholesterol/100 g test test portion} = \text{eqn_0.gif}$$

Reference:

J. AOAC Int. **78**, 75(1995).

Revised: June 2000