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Animal and vegetable fats and oils — Determination of sterols and stanols in foods and dietary supplements containing added phytosterols

Corps gras d'origines végétale et animale — Dosage des stérols et des stanols dans les aliments et les compléments alimentaires contenant des phytostérols ajoutés

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Plant sterols and plant stanols, collectively referred to as phytosterols, are increasingly recognized for their role in reducing the risk of coronary heart disease by lowering serum total and low-density lipoprotein cholesterol. Because of these potential health benefits, health claim regulations authorizing the addition of phytosterols and phytosterol esters to foods and dietary supplements now exist in countries throughout Europe, North and South America, Asia, New Zealand and Australia.

This document was developed in response to the worldwide demand for a reference method for quantifying total and individual phytosterols in foods and dietary supplements containing added phytosterols, and in the phytosterol food additive concentrates used to prepare such products. This reference method is based on the single-laboratory validated methods of Clement et al.[1] and Srigley and Haile[2] for the preparation and gas chromatographic separation of phytosterol trimethylsilyl ether derivatives, respectively.

In 2016 to 2017, an international collaborative study co-organized by the United States Food and Drug Administration (FDA), Cargill (USA) and the American Oil Chemists' Society (AOCS) was carried out to evaluate the performance of this method for the determination of total and individual phytosterols in foods, dietary supplements and phytosterol concentrates.[3] A total of 14 laboratories from 6 countries successfully completed the analysis of 18 test materials, upon which the method was approved as AOCS Official Method Ce 12-16[4].

This reference method is appropriate for the determination of the five major phytosterols (i.e. campesterol, campestanol, stigmasterol, β-sitosterol and sitostanol) that are the subject of FDA's health claim regulation for phytosterols and the reduced risk of coronary heart disease[5].

Animal and vegetable fats and oils — Determination of sterols and stanols in foods and dietary supplements containing added phytosterols

1 Scope

This document specifies a reference method for the determination of free sterols/stanols and steryl/stanol esters (0,1 % to 97 % mass fraction) in foods and dietary supplements containing added phytosterols and in phytosterol food additive concentrates.

Milk and milk products (or fat coming from milk and milk products) are excluded from the scope of this document. This method does not apply to matrices that contain rice bran oil at concentrations of more than 20 % due to possible interferences in the gas chromatogram.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

4 Principle

Free sterols/stanols and saponified steryl/stanol esters are derivatized to phytosterol trimethylsilyl (TMS) ethers and separated on a capillary gas chromatography column.

5 Reagents

Use only reagents of recognized analytical grade.

WARNING — Attention is drawn to regulations that specify the handling of hazardous substances. Technical, organizational and personal safety measures shall be followed. A chemical fume hood shall be used for the work.

5.1 Pyridine.

5.2 *N,O*-Bis(trimethylsilyl)trifluoroacetamide +1 % trimethylchlorosilane (BSTFA).

5.3 Internal standard dissolving solvent, toluene (recommended) or ethyl acetate.

5.4 Methanol.

5.5 Sodium hydroxide, pellets, of purity ≥ 97 %.

5.6 Sodium hydroxide solution, 2,3 N.

Weigh 92 g of sodium hydroxide (5.5) into a 1 000 ml volumetric flask (6.14). Add a stir bar (6.20) and 750 ml of methanol (5.4). Place the flask on a magnetic stirrer (6.21) to disperse. Dilute to the mark with methanol, and invert to mix. The sodium hydroxide solution shall be stirred each time before use because it will settle out with time.

5.7 Hydrochloric acid, 3,0 N.

5.8 Epicoprostanol (5β-cholestan-3α-ol), of purity > 95 %.

5.9 Epicoprostanol internal standard (IS) solution, 5 mg/ml.

Accurately weigh 5,0 g of epicoprostanol (5.8) into a glass weighing scoop (6.8). Transfer the epicoprostanol to a 1 000 ml volumetric flask (6.14). Rinse the scoop with internal standard dissolving solvent (5.3) into the flask. Dilute to the mark with toluene or ethyl acetate and invert to mix. The epicoprostanol IS solution of concentration 5 mg/ml is used for sterol/stanol concentrates, steryl/stanol ester concentrates and some dietary supplements.

5.10 Epicoprostanol internal standard (IS) solution, 2 mg/ml.

Accurately weigh 2,0 g of epicoprostanol (5.8) into a glass weighing scoop (6.8). Transfer the epicoprostanol to a 1 000 ml volumetric flask (6.14). Rinse the scoop with internal standard dissolving solvent (5.3) into the flask. Dilute to the mark with toluene or ethyl acetate and invert to mix. The epicoprostanol IS solution of concentration 2 mg/ml is used for analysis of foods and some dietary supplements.

5.11 Phytosterols, mixture of soya sterols, gas chromatography reference standard.

5.12 Sodium sulfate, anhydrous, granular, of purity ≥ 99 %.

5.13 Sodium chloride, crystalline, of purity ≥ 99 %.

5.14 Sodium chloride solution, saturated.

Weigh approximately 400 g of sodium chloride (5.13) into a storage bottle (6.15). Dilute with 1 000 ml of deionized water.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Gas-liquid chromatograph, equipped with flame ionization detector and capillary split injection system.

6.2 Carrier gas, hydrogen (recommended) or helium, of purity ≥ 99,99 % pure or better, gas chromatography quality, dried, oxygen removed by suitable filters (< 0,1 mg/kg), free from organic impurities.

6.3 Other gases, nitrogen, hydrogen and synthetic air, of gas chromatography quality and purity ≥ 99,99 %.

6.4 Capillary column, with a stationary phase that has been successfully employed to perform the separation of phytosterol TMS ethers.

The use of a bonded (5 %-phenyl)-methylpolysiloxane stationary phase column, such as the HP-5, of length 30 m, internal diameter 0,32 mm and film thickness 0,25 µm, is recommended. The use of a non-bonded (5 %-phenyl)(1 %-vinyl)-methylpolysiloxane stationary phase column, such as the SE-54, of length 30 m, internal diameter 0,32 mm and film thickness 0,25 µm, is also appropriate.

6.5 Injection port split liner, tapered, deactivated, of length 78,5 mm, internal diameter 4 mm and outer diameter 6,3 mm, with glass wool.

6.6 Microsyringe, of capacity 10 µl, with hardened needle for gas chromatography.

6.7 Sample preparation system, suitable for performing saponification and acid hydrolysis procedures, and equipped with the following.

6.7.1 Round bottom flasks, of capacity 50 ml, with 24/40 joints.

6.7.2 Flask neck sleeves, polytetrafluoroethylene (PTFE), size 24/40.

6.7.3 Glass stoppers, full-length, size 24/40.

6.7.4 Hot plate, explosion-proof.

6.7.5 Condensers, water-cooled.

6.8 Glass weighing scoops, suitable for the test portion and insertion into volumetric flasks (6.14).

6.9 Analytical balance, of readability 0,000 1 g and weighing accuracy 0,001 g.

6.10 Spatulas, PFTE-coated.

6.11 Volumetric pipette, of capacities 5 ml, 40 ml and 50 ml, class A, with aspiration bulb.

6.12 Pipette or automatic pipette, of capacities 1 ml and 10 ml, with pipette tips.

6.13 Pasteur pipettes, of length 150 mm, with aspiration bulb.

6.14 Volumetric flasks, of capacities 100 ml and 1 000 ml, class A.

6.15 Storage bottle, of capacity 1 000 ml, glass or polymethylpentene, with screw cap.

6.16 Glass tubes, of length 100 mm and outer diameter 13 mm, with PTFE-lined screw caps.

6.17 Autosampler vials, of capacity 2 ml, amber, with PTFE-lined caps.

6.18 Vortex mixer.

6.19 Boiling chips.

6.20 Stir bar and stir bar remover tool.

6.21 Magnetic stirrer.

7 Sampling

Treat the sample as needed (e.g. cryogrind, homogenize or shake) to obtain a homogeneous composite. Refer to the appropriate International Standard for the matrix concerned.

8 Test portion

Use Table 1 to select the appropriate test portion size and concentration of IS solution based on the estimated content of total phytosterols in the sample.

Table 1 — Guidelines for determining the sample test portion size and concentration of IS solution

Sample type	Phytosterol concentration % (mass fraction)	Test portion mg	IS concentration mg/ml	IS added mg
Sterol/stanol concentrates	> 90	50	5	25
Steryl/stanol ester concentrates, dietary supplements	~50	100	5	25
Foods and dietary supplements	~20	250	5	25
Food and dietary supplements	~10	500	5	25
Food and dietary supplements	< 10	1 000	2	10

9 Procedure

9.1 Selection of analysis protocol

For new formulations (phytosterol type and matrix), determine the optimal protocol by triplicate analysis according to the following protocols: a) 120 min alkaline, b) 15 min alkaline and c) 45 min acid/15 min alkaline. Select the protocol that produces the highest phytosterol recovery and lowest variability. Further refine this protocol, as needed, by decreasing the saponification time from 120 min to 60 min to 30 min, or the acid hydrolysis time from 45 min to 30 min to 15 min, with triplicate analyses for each. The optimal protocol will show a repeatability coefficient of variation ($C_{V,r}$) of less than 5 % for triplicate analyses over a period of five days of testing.

The 120 min alkaline protocol is appropriate for the analysis of most foods and dietary supplements containing added phytosterols. The 45 min acid/15 min alkaline protocol can be required for complete liberation of phytosterol esters from certain matrices, such as some cereals.

9.2 Dilute and shoot protocol

Use this protocol for sterol/stanol concentrates and phytosterol reference standards.

a) Accurately weigh 50 mg of sample into a tared glass tube (6.16). Record the exact weight.

b) Add 5,00 ml of 5 mg/ml epicoprostanol IS solution (5.9). Cap tightly and vortex (6.18) to mix.

c) Add 0,5 ml of pyridine (5.1) and 1 ml of BSTFA (5.2). Cap tightly and vortex (6.18) to mix. Heating can be required because some concentrates are pellets that fail to dissolve at room temperature.

d) Transfer 300 µl of derivatized sample solution, 1 ml of toluene or ethyl acetate (5.3) and 25 mg of sodium sulfate (5.12) to an autosampler vial (6.17). Cap tightly and vortex (6.18) to mix. The sample is now ready for gas chromatographic separation (see Clause 10).

9.3 15 min and 120 min alkaline protocols

Use this protocol for most foods, dietary supplements and steryl/stanol ester concentrates.

a) Accurately weight the test portion into a tarred round bottom flask (6.7.1). Record the exact weight.

b) Add a few boiling chips (6.19) and place a PTFE sleeve (6.7.2) in the neck of the flask.

c) Add 5,00 ml of the appropriate epicoprostanol IS solution (5.9 or 5.10).

d) Add 5 ml of sodium hydroxide solution (5.6).

e) Boil the sample at 100 °C for 15 min or 120 min (see 9.1).

f) Remove the flask from the heat source, insert a stopper (6.7.3) and let cool to room temperature.

g) Add 7 ml of hydrochloric acid (5.7), insert a stopper (6.7.3) and shake to mix.

h) Add 40 ml of sodium chloride solution (5.14), insert a stopper (6.7.3) and shake to mix. Allow the two phases to separate. Wait until the cloudy organic phase is clear before proceeding.

i) Transfer 300 µl of organic phase and 25 mg of sodium sulfate (5.12) to an autosampler vial (6.17).

j) Add 0,5 ml of pyridine (5.1) and 1 ml of BSTFA (5.2). Cap tightly and vortex (6.18) to mix. The sample is now ready for gas chromatographic separation (see Clause 10).

9.4 45 min acid/15 min alkaline protocol

Use this protocol for some foods and dietary supplements containing added phytosterol esters. Acid hydrolysis prior to saponification is needed for the complete liberation of phytosterol esters from certain matrices, such as some cereals (see 9.1).

a) Accurately weight the test portion into a tarred round bottom flask (6.7.1). Record the exact weight.

b) Add a few boiling chips (6.19) and place a PTFE sleeve (6.7.2) in the neck of the flask.

c) Add 5 ml of hydrochloric acid (5.7).

d) Add 5,00 ml of the appropriate epicoprostanol IS solution (5.9 or 5.10).

e) Boil the sample at 100 °C for 45 min.

f) Remove the flask from the heat source, insert a stopper (6.7.3) and let cool to room temperature.

g) Add 40 ml of sodium chloride solution (5.14), insert a stopper (6.7.3) and shake to mix. Allow the two phases to separate.

h) Transfer the organic phase (as much as possible without disrupting the solids on the surface) to a new round bottom flask (6.7.1).

i) Add 5 ml of sodium hydroxide solution (5.6).

j) Boil the sample at 100 °C for 15 min.

k) Remove the flask from the heat source, insert a stopper (6.7.3) and let cool to room temperature.

l) Add 7 ml of hydrochloric acid (5.7), insert a stopper (6.7.3) and shake to mix.

m) Add 40 ml of sodium chloride solution (5.14), insert a stopper (6.7.3) and shake to mix. Allow the two phases to separate. Wait until the cloudy organic phase is clear before proceeding.

n) Transfer 300 µl of organic phase and 25 mg of sodium sulfate (5.12) to an autosampler vial (6.17).

o) Add 0,5 ml of pyridine (5.1) and 1 ml of BSTFA (5.2). Cap tightly and vortex (6.18) to mix. The sample is now ready for gas chromatographic separation (see Clause 10).

10 Gas chromatography

Operating conditions are as follows.

a) Injection port temperature: 290 °C.

b) Detector temperature: 290 °C.

c) Oven temperature: 250 °C for 60 min, ramp at 15 °C/min to 265 °C, hold for 7 min.

d) Post-run temperature: 250 °C for 3 min.

e) Carrier gas (6.2): hydrogen or helium, 1,0 ml/min, constant flow rate.

f) Detector gases (6.3): air, 400 ml/min, hydrogen, 30 ml/min.

g) Makeup gas (6.3): nitrogen, 30 ml/min.

h) Split ratio: 25:1.

i) Injection volume: 1,0 µl.

j) Phytosterol concentration: 10 mg/ml.

11 Peak identification

Identify phytosterol TMS ethers by retention time (see Table 2) and by comparison with reference chromatograms (see Figures A.1 to A.4). Peaks of unknown identity should be omitted from the summation of peak areas, unless they are confirmed to be phytosterols.

Samples containing added plant stanols and stanol esters require special attention during peak identification and integration. Refer to Figure A.4 when analysing samples known to contain high concentrations of plant stanols.

Table 2 — Elution order and theoretical correction factors (TCFs) for sterols and stanols commonly found in foods and dietary supplements containing added phytosterols

Peak #[a]	Compound	Retention time min	Relative retention time min	TCF[b]
1	Epicoprostanol	25,3	1,00	1,000 0
2	Cholesterol	30,5	1,21	0,995 6
3	Brassicasterol	33,7	1,33	0,988 7
4	Ergosterol	37,8	1,49	0,984 5
5	24-Methylene cholesterol	38,3	1,51	0,988 7

[a] Peak numbers are used in Figures A.1 to A.4. Peaks corresponding to cholesterol and ergosterol are not included in the quantitation of total phytosterols.

[b] TCFs were calculated relative to epicoprostanol TMS ether, which had a factor of 1,000 0. Atomic weights were as follows: carbon, 12,011; hydrogen, 1,007 9; oxygen, 15,999 4; silicon, 28,085 5. TCFs account for the proportion of active carbon atoms contributing to the response of the flame ionization detector[6].