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Vegetable fats and oils — Determination of phospholipids content in lecithins by HPLC using a light-scattering detector

Corps gras d'origine végétale — Détermination de la teneur en phospholipides dans les lécithines par CLHP avec détecteur à diffusion de la lumière

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 11701 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

Vegetable fats and oils — Determination of phospholipids content in lecithins by HPLC using a light-scattering detector

1 Scope

This International Standard specifies a method for the quantitative determination of phospholipids content by high performance liquid chromatography (HPLC) using a diol column and a light-scattering detector.

The method is applicable to crude, oil-containing lecithins, and to oil-free lecithins and lecithin fractions from vegetable fats and oils.

The method is not applicable to animal and ruminant lecithins and enzymatically hydrolysed lecithins as the peak separation of lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI) and lysophosphatidic acid (LPA) is insufficient.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 661, *Animal and vegetable fats and oils — Preparation of test sample*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
content of an individual phospholipid
mass fraction of *N*-acyl-phosphatidylethanolamine (*N*-acyl-PE) or phosphatidylcholine (PC) or phosphatidylethanolamine (PE) or phosphatidylinositol (PI) or phosphatidic acid (PA) or lysophosphatidylcholine (LPC), determined in accordance with the method specified in this International Standard

NOTE The content is expressed in grams per 100 g, numerically equal to a percentage mass fraction.

4 Principle

The individual phospholipids are separated by HPLC using a diol column and a light-scattering detector. For the purpose of quantification, a certified reference mixture is used.

5 Reagents

WARNING — Comply with any local regulations which specify the handling of hazardous substances. Technical, organizational and personal safety measures shall be followed.

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade.

5.1 Water, HPLC grade.

5.2 *n*-Hexane, HPLC grade.

5.3 2-Propanol, HPLC grade.

5.4 Acetic acid, w_{min}, 99,8 % mass fraction.

5.5 Triethylamine.

5.6 Solvent mixture: A mixture of 80 ml *n*-hexane (5.2) and 20 ml 2-propanol (5.3) (volume fraction φ = 80 ml/100 ml for *n*-hexane and φ = 20 ml/100 ml for 2-propanol) is used to dissolve the standards and sample.

5.7 Reference substance (external standard) ILPS-LE01[1)], mixed soy phospholipid reference standard, is a certified reference mixture with defined contents of *N*-acyl-PE, PA, PE, PC, PI, and LPC.

5.8 Mobile phase for the HPLC.

5.8.1 Eluent A. Mix 814,2 ml of *n*-hexane (5.2), 170,0 ml of 2-propanol (5.3), 15 ml of acetic acid (5.4), and 0,8 ml of triethylamine (5.5) (volume fraction φ = 81,42 ml/100 ml for *n*-hexane, φ = 17,00 ml/100 ml for 2-propanol, φ = 1,50 ml/100 ml for acetic acid, and φ = 0,08 ml/100 ml for triethylamine).

In order to obtain a reproducible eluent composition, it is recommended that the solvents be weighed out taking into account their densities. For a batch size of 2,5 l: 1 341,4 g of *n*-hexane, 331,5 g of 2-propanol, 39,4 g of acetic acid, and 1,45 g (2,0 ml) of triethylamine.

5.8.2 Eluent B. Mix 844,2 ml of 2-propanol (5.3), 140 ml of water (5.1), 15,0 ml of acetic acid (5.4) and 0,8 ml of triethylamine (5.5) (volume fraction φ = 84,42 ml/100ml for 2-propanol, φ = 14,00 ml/100 ml for water, φ = 1,50 ml/100 ml for acetic acid and φ = 0,08 ml/100 ml for triethylamine).

In order to obtain a reproducible eluent composition, it is recommended that the solvents be weighed out taking into account their densities. For a batch size of 2,5 l: 1 646,2 g of 2-propanol, 350,0 g of water, 39,4 g of acetic acid and 1,45 g (2,0 ml) of triethylamine.

6 Apparatus

6.1 Analytical balance, capable of being read to the nearest 0,000 1 g.

6.2 HPLC basic equipment with gradient system and light-scattering detector.

6.3 HPLC column oven, adjustable to 55 °C.

6.4 Degasser or similar equipment for degassing the eluent.

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1) ILPS-LE01 is the trade name of a product supplied by the International Lecithin and Phospholipid Society. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to comparable results.

6.5 HPLC column (250 mm × 4,0 mm) with pre-column (20 mm × 4,0 mm) packed with spherical microparticles (5 µm) of diol-bounded silica, e.g. LiChrospher 100 diol (5 µm)[2)]. The age and history of the column, the packaging of the column filling material and the temperature may influence the separation.

6.6 One-mark volumetric flasks, of capacities 50 ml, 100 ml and 2 500 ml, ISO 1042[1] class A.

6.7 Microlitre syringe, of capacity 25 µl, graduated in microlitres.

6.8 Filter for filtering the external standard and test sample solutions, e.g. Millex HV[3)].

6.9 Integration system.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 5555[2].

8 Preparation of test sample

See ISO 661.

The sample is heated to a maximum of 60 °C to soften it (overheating shall be avoided) and then homogenized by vigorous stirring.

9 Procedure

9.1 Preparation of standard reference solutions and test portions

9.1.1 Standard reference solutions R_1, R_2, and R_3

Prepare three different standard reference solutions. For this purpose, accurately weigh out in three different 100 ml one-mark volumetric flasks approximately 550 mg, 850 mg and 1 150 mg of the certified reference mixture (5.7), dissolve in the solvent mixture (5.6) and make up to the mark with the same solvent.

Filter (6.8) the standard reference solutions before injection into the HPLC.

9.1.2 Test sample solutions and test portions

Weigh, to the nearest 0,001 g, 425 mg of the sample in the case of crude lecithin or 255 mg of the sample in the case of deoiled or fractionated lecithin into separate 50 ml one-mark volumetric flasks, dissolve in solvent mixture (5.6) and make up to the mark with the same solvent.

Filter (6.8) the test sample solutions before drawing test portions S_{1a} and S_{1b} from them.

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2) LiChrospher 100 diol is the trade name of a product supplied by Merck. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

3) Example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

9.2 HPLC analysis

Adjust the working conditions in the equipment using the test samples and reference samples in order to get a separation according to the chromatogram in Figure A.1. Optimize the separation profile, depending on the type of column and gradient. The following conditions are recommended (see Table 1):

— Temperature of the oven: 55 °C

— Sensitivity of the detector: 5 to 6

— Temperature of the detector: 50 °C

— Pressure of the detector: 0,20 MPa (2,0 bar)

— Flow: 1,0 ml/min

— Flow for column rinsing: 2,0 ml/min

Table 1 — Gradient programme for HPLC

Time min	Eluent A %	Eluent B %	Flow ml/min
0,0	95	5	1,0
5,0	80	20	1,0
8,5	60	40	1,0
15,0	0	100	1,0
17,5	0	100	1,0
17,6	95	5	1,0
21,0	95	5	1,0
22,0	95	5	2,0
27,0	95	5	2,0
29,0	95	5	1,0

9.3 Calibration

Use 20 µl injection volumes for calculation of the linear regression lines and for determination of the test sample. Plot peak area against concentration to obtain a calibration curve.

NOTE Light-scattering detectors are not linear over the whole range (S-shaped calibration curves). The concentrations of the standard reference solutions have been chosen to be in the linear range.

The following analysis sequence is recommended for the quantitative determination of phospholipids: R_1, R_2, R_3 (one injection each), S_{1a}, S_{1b} (test portions drawn from the test sample, two injections each), R_1, R_2, R_3 (one injection each).

9.4 Determination

Inject 20 µl of the test sample solution into the HPLC and register the peak areas. Identify the peaks by comparing the retention times of the substance in the chromatograms of the standard reference solutions and the test portions (see Figure A.1).

10 Calculation and expression of results

Use the calibration curve to calculate the individual content of phospholipids (see 9.3). Three calibration points shall have lower concentrations and three calibration points shall have higher concentrations compared to the sample. Solutions R_1, R_2, and R_3 (9.1.1) are diluted depending on the sample to obtain the six calibration points.

The mass fraction, w_i, in grams per 100 g of the test sample, of the individual phospholipid is given by:

$$w_i = \frac{m_{\mathrm{p}i}}{m} \times 100$$

where

$m_{\mathrm{p}i}$ is the mass, in milligrams, of the individual phospholipid determined from the calibration curve;

m is the mass, in milligrams, of the test sample (9.1.2).

Express the result to one decimal place.

11 Precision of the method

11.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

11.2 Repeatability

The repeatability limit, r, is the value less than or equal to which the absolute difference between two final values, each of them representing a series of test results, obtained under repeatability conditions, is expected to be with a probability of 95 %.

Repeatability conditions are those under which test results are obtained with the same method, on identical test material, in the same laboratory, by the same operator, using the same equipment and reagents, within a short interval of time.

11.3 Reproducibility

The reproducibility limit, R, is the value less than or equal to which the absolute difference between two test final values, each of them representing a series of test results, obtained under reproducibility conditions, is expected to be with a probability of 95 %.

Reproducibility conditions are defined as conditions under which test results are obtained with the same method, on identical test material, in different laboratories, by different operators, using different equipment and reagents, within a short interval of time.