
**Animal and vegetable fats and oils —
Determination of monoacylglycerols,
diacylglycerols, triacylglycerols and
glycerol by high-performance size-
exclusion chromatography (HPSEC)**

*Corps gras d'origines animale et végétale — Détermination de la teneur
en monoacylglycérides, en diacylglycérides, en triacylglycérides et en
glycérol par chromatographie liquide d'exclusion (CLHP d'exclusion)*



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Foreword

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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.

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ISO 18395 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

Animal and vegetable fats and oils — Determination of monoacylglycerols, diacylglycerols, triacylglycerols and glycerol by high-performance size-exclusion chromatography (HPSEC)

1 Scope

This International Standard specifies a method for the determination of monoacylglycerols, diacylglycerols and triacylglycerols and also free glycerol by high-performance size-exclusion chromatography. It is applicable to products (e.g. emulsifiers) comprising monoacylglycerols and diacylglycerols as main constituents in concentrations $>10\%$, and to triacylglycerols in a proportion of $<20\%$.

The method is not applicable to dairy fats or fats and oils having a wide range of fatty acid chain lengths, since diacylglycerols of short fatty acids have a lower molecular mass than monoacylglycerols of long-chain fatty acids.

The method has restricted applicability to acylglycerol mixtures based on caprylic and capric acids. Here, only the monoacylglycerol content and the free glycerol content can be determined.

NOTE References [1] to [4] give background information.

2 Normative references

The following referenced document is 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

contents of monoacylglycerols, diacylglycerols, triacylglycerols and glycerol

proportion of monoacylglycerols, diacylglycerols, triacylglycerols and glycerol determined under the conditions of this International Standard

NOTE The contents are expressed as a mass fraction (grams per 100 g) or as a percentage of all peaks.

4 Principle

The sample is dissolved in tetrahydrofuran (THF). The solution obtained is analysed by gel permeation chromatography (GPC) using THF as the mobile phase. The acylglycerols and glycerol are separated according to their molecular size. Detection is by means of a refractive index detector.

5 Reagents

WARNING — Attention is drawn to regulations concerning the handling of hazardous substances. Observe all technical, organizational and personal protective measures.

Use only reagents of recognized analytical grade, unless otherwise specified.

5.1 Tetrahydrofuran (THF), stabilized with 250 µl/l of BHT.

5.2 Standard substances

5.2.1 Glycerol ($w \geq 99,5 \%$).

5.2.2 Monoacylglycerols, diacylglycerols, triacylglycerols¹⁾.

Standards used should be monoacylglycerols, diacylglycerols and triacylglycerols having a fatty acid distribution as similar as possible to those present in the sample. However, the acylglycerol composition does not have to be reproduced exactly. For most samples, monopalmitate and/or monostearate and/or monooleate, dipalmitate and/or distearate and/or dioleate, tripalmitate and/or tristearate and/or trioleate are sufficient.

The response factors for glycerol, monoacylglycerols, diacylglycerols and triacylglycerols are generally the same under the conditions indicated in this International Standard, so that in most cases the use of quantitative reference solutions is unnecessary and percentages by area may be used. However, it is necessary to determine a response factor for glycerol when the concentration in the sample is $> 3 \%$. No response factors at all are employed in the European Pharmacopoeia for acylglycerols. This shall be indicated in the test report.

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 Analytical balance, with a readability of 0,1 mg.

6.2 Volumetric flask, of 10 ml capacity.

6.3 Pipette, of 5 ml capacity.

6.4 Ultrasonic bath.

6.5 HPLC/GPC pump.

6.6 Injector, equipped with a sample loop of 20 µl capacity.

6.7 Detector, differential refractometer (RI detector).

6.8 GPC column combination, with an effective molecular mass up to 4 000 Da (e.g. three columns of 300 mm $\times$ 7,5 mm Plgel²⁾, 5 µm, 100 Å).

It is also possible to use other columns (or column combinations) provided that the separation of the monoacylglycerols and diacylglycerols and also of the diacylglycerols and triacylglycerols is ensured. The use of only two columns is also possible to achieve the required separation.

1) Available for example from Sigma-Aldrich (<http://www.sigmaaldrich.com/>).

2) Available for example from Polymer Laboratories (<http://www.polymerlabs.com/>).

This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

6.9 Column oven (column thermostat).

6.10 Data integration and evaluation 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 [5].

8 Preparation of test sample

Prepare the test sample in accordance with ISO 661.

Before taking the test portion from the sample, mix the sample thoroughly to ensure homogeneity. For the same reason, melt solid samples completely for proper mixing.

9 Procedure

9.1 Preparation of the sample solution

Weigh, to the nearest 0,1 mg, about 100 mg of the sample into a 10 ml volumetric flask. The concentration is reported in milligrams of sample per 10 ml. After diluting to the mark with THF, place the flask in the ultrasonic bath for 5 min to 10 min.

The clear solution obtained is directly injected into the GPC system.

9.2 Preparation of reference solutions

Weigh, to the nearest 0,1 mg, amounts of glycerol, monoacylglycerols, diacylglycerols and triacylglycerols above and below the expected concentration in the sample into 10 ml volumetric flasks. After diluting to the mark with THF, place the flasks in the ultrasonic bath for 5 min to 10 min.

The clear solutions obtained in this way are directly injected into the GPC system.

The concentration is reported in milligrams of component per 10 ml for each reference solution. If the expected content is not known, it is possible to make up a number of calibration solutions that cover a wider range.

NOTE Refer to 5.2.2.

9.3 Gel permeation chromatography (GPC)

Set up the GPC system as follows:

Injection volume:	20 μ l
Separation columns:	Range: minimum 0 to 4 000 Da (6.8)
Oven temperature:	35 °C or 40 °C $\pm$ 0,1 °C
Flow rate:	0,8 ml/min to 1,0 ml/min
RI-detector:	Temperature: 35 °C or 40 °C.

Typical chromatograms obtained under these conditions are given in Annex A.

Qualitative testing for the presence of monoacylglycerols, diacylglycerols and triacylglycerols and of glycerol is carried out by comparing the retention times with comparative substances (e.g. C18-monoacylglycerols/-C18-diacylglycerols/C18-triacylglycerols) or acylglycerol groups.

For the quantitative determination of the contents, prepare at least two calibration solutions. Choose the concentrations of the solutions so that they bracket the contents in the sample above and below. These solutions are injected in succession, twice in each case. The calibration curves produced make it possible to calculate the glycerol and acylglycerol concentrations in the sample.

NOTE Refer to 5.2.2.

10 Calculation

The glycerol content in the sample, w_G , expressed as a mass fraction in percent, is calculated as follows:

$$w_G = \frac{m_G \times F_G}{m_S} \times 100 \%$$

where

w_G is the glycerol content of the sample;

m_G is the mass of the glycerol in the sample, in milligrams, calculated from the calibration curve;

m_S is the mass, in milligrams, of the test portion;

F_G is the response factor for glycerol.

The mono-, di- and tri-acylglycerol contents are calculated in the same way.

In the evaluation of the peak areas, a horizontal baseline is used as the integration boundary.

The response factors for glycerol, monoacylglycerols, diacylglycerols and triacylglycerols are generally the same under the conditions indicated here, so that in most cases the use of quantitative reference solutions is unnecessary and the response factors are assumed to be $F = 1$. However it is necessary to determine a response factor for glycerol when the concentration in the sample is $> 3 \%$.

In most cases, free fatty acids are not separated from the monoacylglycerol peak group. Therefore in samples with acid values > 1 the amount of fatty acids, calculated from the acid value, may be subtracted from the monoacylglycerol content. This shall be indicated in the test report.

Report the result of the determination, expressed as a mass fraction in percent, to one decimal place.

11 Precision

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 absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed the value of r given in Tables B.1 to B.5.

11.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5 % of cases exceed the value of R given in Tables B.1 to B.5.

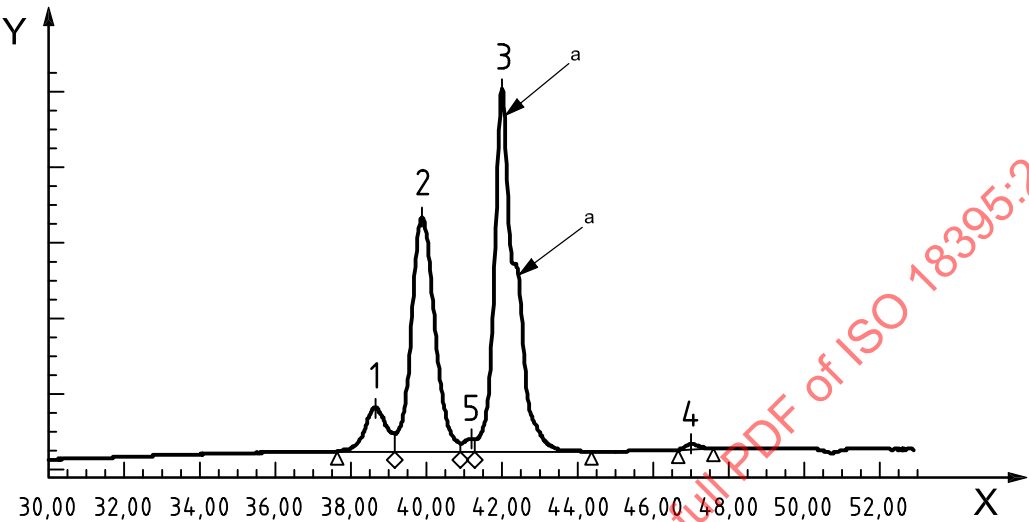
12 Test report

The test report shall specify:

- all information necessary for the complete identification of the sample;
- the sampling method used, if known;
- the test method used, with reference to this International Standard;
- all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);
- the test result obtained;
- if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Examples of chromatograms



Key

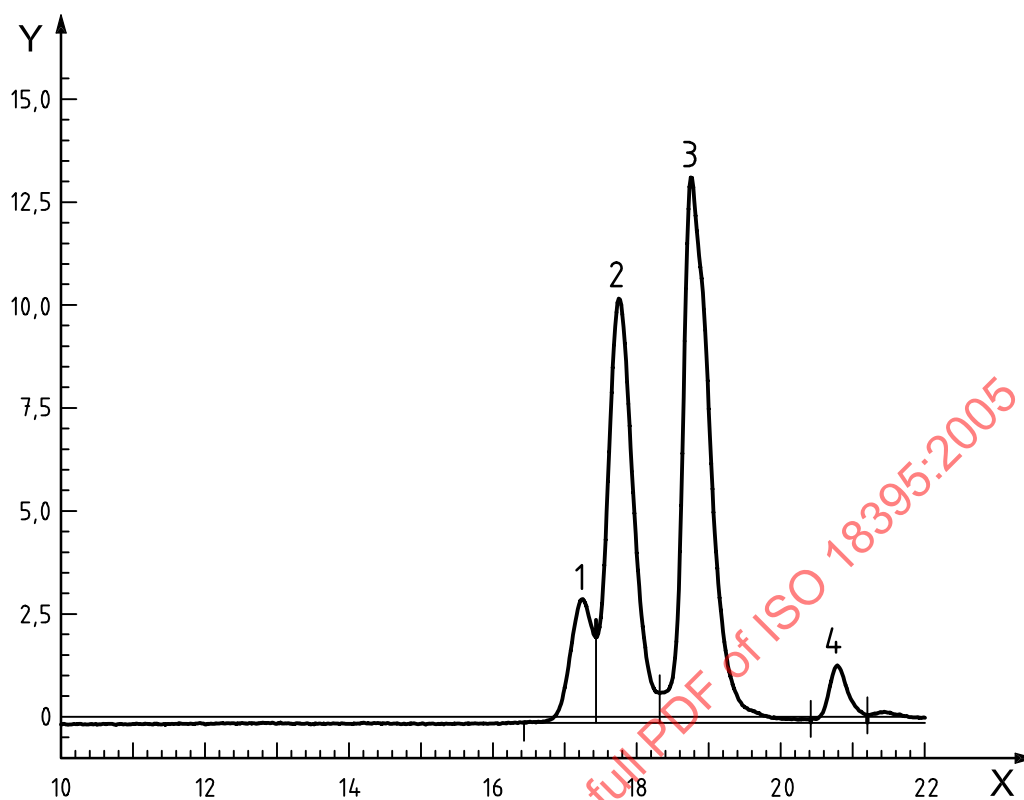
- X retention time, min
Y peak intensity, mV
1 triacylglycerols (39,132)
2 diacylglycerols (40,407)
3 monoacylglycerols (42,552)
4 free glycerol (47,560)
5 diglycerol monostearate (41,719)
a Partial separation of C16-/C18-monoacylglycerols.

NOTE Five columns of 300 mm x 7,5 mm Plgel, 5 µm, 100 Å were used.

Figure A.1 — Chromatogram of a sample comprising mono-, di- and tri-acylglycerols derived from tallow

Table A.1 — Sample comprising mono-, di- and tri-acylglycerols derived from tallow

Substance	Retention time min	Area	% Area
Triacylglycerols	39,132	1 694 320	6,2
Diacylglycerols	40,407	10 574 930	38,5
Diglycerol- monoester	41,719	311 730	1,2
Monoacylglycerols	42,552	14 719 338	53,5
Free glycerol	47,560	155 732	0,57

**Key**

X retention time, min

Y peak intensity, mAU (absorption units)

1 triacylglycerols

2 diacylglycerols

3 monoacylglycerols

4 glycerol

NOTE Two columns of 300 mm $\times$ 7,5 mm Plgel, 5 μ m, 100 Å were used.

Figure A.2 — Separation of a test mixture

Annex B (informativ)

Results of interlaboratory test

A collaborative study was organized by the *Joint Committee for the Analysis of Fats, Oils, Fat Products, Related Products and Raw Materials* in 2002 on an international basis. Five different samples were investigated. The results, evaluated in accordance with ISO 5725-2 [6] are given in Tables B.1 to B.5.

Table B.1 — Results of interlaboratory test (fatty acids)

Sample identification: Mono-/di-acylglycerols of C8/C10 fatty acids	Monoacyl- glycerols	Di-/triacyl- glycerols	Glycerol
Number of participating laboratories	10	10	10
Number of laboratories retained after eliminating outliers	10	10	8
Number of test results in all laboratories	20	20	16
Mean	45,78	41,76	10,96
Repeatability standard deviation (s_r)	0,24	0,27	0,14
Repeatability relative standard deviation	0,5	0,7	1,20
Repeatability limit (r)	0,67	0,77	0,38
Reproducibility standard deviation (s_R)	1,38	1,08	0,61
Reproducibility relative standard deviation	3,0	2,6	5,5
Reproducibility limit (R)	3,85	3,03	1,70

Table B.2 — Results of interlaboratory test (tallow)

Sample identification: Mono-/di-acylglycerols based on tallow	Monoacyl- glycerols	Diacyl- glycerols	Triacyl- glycerols	Glycerol
Number of participating laboratories	10	10	10	9
Number of laboratories retained after eliminating outliers	10	9	9	8
Number of test results in all laboratories	20	18	18	16
Mean	48,92	43,52	6,09	1,72
Repeatability standard deviation (s_r)	0,15	0,12	0,08	0,04
Repeatability relative standard deviation	0,3	0,3	1,3	2,1
Repeatability limit (r)	0,41	0,33	0,22	0,10
Reproducibility standard deviation (s_R)	0,64	0,49	0,23	0,48
Reproducibility relative standard deviation	1,3	1,1	3,8	27,9
Reproducibility limit (R)	1,78	1,37	0,65	1,35