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DIVISION OF CHEMISTRY AND THE ENVIRONMENT
COMMISSION ON OILS, FATS AND DERIVATIVES

DETERMINATION OF MONO- AND DIACYLGLYCEROLS IN EDIBLE OILS AND FATS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND EVAPORATIVE LIGHT SCATTERING DETECTOR: RESULTS OF COLLABORATIVE STUDIES AND THE STANDARDIZED METHOD

(Technical Report)

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Membership of the Commission during the period of the collaborative studies (1992–1995) was as follows:

Chairman: A. Dieffenbacher (Switzerland) until 1993, P. Dysseler; *Vice-Chairman:* P. Dysseler (Belgium) until 1993, F. Mordret, Secretary: P. W. Hendrikse (Netherlands); *Titular Members:* Gunhild Hølmer (Denmark), M. Pike (UK), S. Watanabe (Japan). *Associate Members:* K. Stig-Andersen. *National Representatives (various periods):* R. Brenner (Argentina), J. R. Sabine (Australia), A. Woidich (Austria), R Lago (Brazil), P. P. Kolodziejcyk (Canada) Y. Yang (China), J. Pokorny (Czech Republic), I. Wester (Finland), H. A. Bhakare (India), B. Connolly (Ireland), D. Willard (New Zealand), J. M. Empis (Portugal), C. Dobarganes Garcia (Spain), T. Tuglular (Turkey).

Determination of mono- and diacylglycerols in edible oils and fats by high performance liquid chromatography and evaporative light scattering detector (ELSD): Results of collaborative studies and the standardized method (Technical Report)

Abstract: The development, by collaborative study, of standardized method for the determination of mono- and diacylglycerols in vegetable oils and fats is described. The method involves separation of mono- and diacylglycerols by normal phase high-performance liquid-liquid chromatography (HPLC) and evaporative light scattering detection of a solution of oil, fat or a commercial mono- and diacylglycerol preparation in a organic solvent.

INTRODUCTION

Mono- and diacylglycerols, commonly also designated as mono and diglycerides are partial fatty acid glycerol esters naturally accompanying edible oils and fats being basically triacylglycerols. They are formed by hydrolysis of oils and fats during storage of oil and fat bearing materials. Due to their chemical nature they act as emulsifiers. Their presence in a oil or fat influence its ease of application in food preparation. Therefore, there is a keen interest to relay on a standardized method for the detection and determination of mono- and diacylglycerols in edible oils and fats. Further, commercial preparations of partial acylglycerols are used as authorised food grade emulsifiers.

The determination according to the IUPAC method 2.326 [1] of mono- and diacylglycerols by gas chromatography involves derivatisation of the free hydroxy groups into the more volatile trimethylsilyl ether derivatives prior to gas-chromatographic separation.

Liu *et al*. [2] described an easy to carry out method for determination of partial glycerides based on HPLC separation and determination using an Evaporative Light Scattering Detector (ELSD) [3].

The ELSD is an universal detector whose response is a function of the mass of the analyte. Liu could obtain an approximate linear relationship between the detector response and the mass of the analyte by plotting log (ELSD response) vs. log (mass of the analyte) with a correlation coefficient $r^2 = 0.996$ (Fig. 1)

The ESLD system shows no solvent peak and therefore a solvent gradient system can be applied to increase separation of acylglycerols. As further advantage, no sample pretreatment is necessary,

These facts had emphasised the desirability of standardising a procedure based on HPLC and ELSD.

First collaborative study 1992/93

Four samples with blind duplicates were sent to 12 laboratories for analysis. Samples were based on sunflower oil spiked with known amounts of mono- and diacylglycerols. A sample called ‘practice sample’ was sent for analysis too.

Participants were asked to set up a calibration of their HPLC-detector system with additionally supplied mono and diacylglycerols and to apply a linear and quadratic regression between log detector response and log mass of the test portion. The analytical results should then have been evaluated according to these two ways of correlation. Each participant was further asked to determine twice each coded sample and report these results.

Only seven laboratories sent results in due form and one sent results obtained by linear regression only. One laboratory reported far too high results and was therefore eliminated since the beginning. Among accepted results outliers were eliminated by Cochran’s and Dixon’s outlier tests.

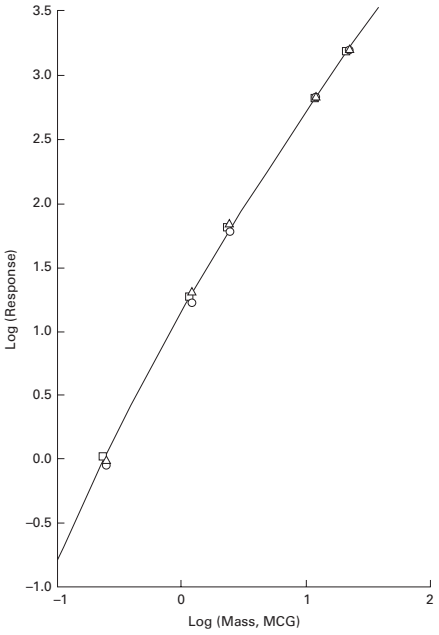

Fig. 1 Response curves of 1-monopalmitin (○), 1-monostearin (□), 1-monoolein (△), with a Varex ELSD using HPLC and ELSD parameters as described in the method.

Reported results were statistically evaluated using both the linear and the quadratic correlation between log detector response and log mass of the test portion for monoacylglycerol and diacylglycerol.

There were considerably more outliers associated with the determination of diacylglycerols than monoacylglycerols; however, the number was less with the quadratic equation than with linear equation. There seemed to be a greater variability associated with the use of the quadratic equation for calculating the partial acylglycerol content, but the average values were closer to the known values than the average values obtained with the linear equation.

The 1-mono and 1,3-dipalmitin reference standards were prepared by dissolving the partial glycerols in corn oil by heating. This formed additional partial acylglycerols so that the theoretical value could not be used as a reference value for the study.

Therefore, the commission agreed to carry out a second collaborative study with more care devoted to the preparation of the reference samples.

Second collaborative study 1994/95

In the following year the study was repeated once again, but with samples prepared more cautiously. Samples similar to the previous study were mailed to 11 laboratories, but only 9 laboratories reported results. Of these, only two laboratories reported results that agreed closely to the estimated values. Results from the other laboratories were off by factors ranging from 200 to 1000. These laboratories were asked to recalculate their results and report them in grams per 100 g of sample. Accepted results from the laboratories are statistically evaluated. Results are given in Tables 1 and 2, for monoacylglycerol (Table 1) and diacylglycerol (Table 2).

In general, the accepted results gave average values that were close to the known values. Values for reproducibility seemed to be reasonable; however, values for reproducibility were rather high, indicating possible problems in the application of the method in the different laboratories although there is consistency within laboratories.

While the method appears to have merits, there do seem to be problems of application of the method although two participant independent laboratories found during each study practically identical results.

Table 1 Second collaborative study 1994/95. Statistical evaluation of monoacylglycerol contents

	Sample (combined duplicates) 1	2	3	4
Evaluation of results using linear equation ($Y = AX + B$)				
Number of laboratories	11	11	11	11
Number of laboratories retained after elimination of outliers	9	8	9	9
Number of accepted results	34	28	34	34
Mean value (g/100 g sample)	0.011	0.025	0.054	0.088
Known value (g/100 g sample)	0.010	0.020	0.0511	0.092
Repeatability standard deviation (S_r)	0.001	0.002	0.003	0.003
Repeatability relative standard deviation (CV_r in %)	10.92	7.43	5.66	3.39
Repeatability limit (r) ($2.83 \times S_r$)	0.003	0.006	0.008	0.008
Reproducibility standard deviation (S_R)	0.001	0.008	0.010	0.017
Reproducibility relative standard deviation (CV_R in %)	12.95	32.01	18.60	19.75
Reproducibility limit (R) ($2.83 \times S_R$)	0.003	0.023	0.028	0.049

	Sample (combined duplicates) A	B	C	D
Evaluation of results using quadratic equation ($Y = A'X^2 + B'X + C$)				
Number of laboratories	11	11	11	11
Number of laboratories retained after elimination of outliers	7	7	8	8
Number of accepted results	28	26	32	32
Mean value (g/100 g sample)	0.011	0.021	0.056	0.092
Known value (g/100 g sample)	0.010	0.020	0.051	0.092
Repeatability standard deviation (S_r)	0.001	0001	0.003	0.003
Repeatability relative standard deviation (CV_r in %)	8.95	2.31	5.16	3.16
Repeatability limit (r) ($2.83 \times S_r$)	0.003	0.001	0.008	0.007
Reproducibility standard deviation (S_R)	0.001	0.002	0.008	0.016
Reproducibility relative standard deviation (CV_R in %)	10.41	8.99	13.28	17.23
Reproducibility limit (R) ($2.83 \times S_R$)	0.003	0.005	0.0211	0.045

The small number of participants could be due to the unfamiliarity of laboratories involved with the ELSD system. The American Oil Chemist's Society adopted this method as a preliminary one as Cd 11d-96 in their methods for the analysis of oils and fats.

The commission decided to adopt the method on a preliminary base too. The text of the evaluated method is given on the following pages.

ACKNOWLEDGEMENTS

The commission is thankful to the collaborators in Canada, Denmark, France, the Netherlands, Switzerland, United Kingdom and the United States of America who participated in the study and returned results.

DETERMINATION OF MONO- AND DIACYLGLYCEROLS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND EVAPORATIVE LIGHT SCATTERING DETECTOR: RESULTS OF A COLLABORATIVE STUDY AND THE STANDARDIZED METHOD

1 Scope

This standard describes a method for the determination of mono-and di-acylglycerols (partial glycerides) by high performance liquid chromatography (HPLC) and evaporative light scattering detector (ELSD) in edible oils and fats and commercial food emulsifier preparations based on partial glycerides.

Table 2 Second collaborative study 1994/95. Statistical evaluation of diacylglycerol contents

	Sample (combined duplicates) A	B	C	D
Evaluation of results using linear equation ($Y = AX + B$)				
Number of laboratories	11	11	11	11
Number of laboratories retained after elimination of outliers	6	7	6	7
Number of results	24	24	24	26
Mean value (g/100 g sample)	0.126	0.092	0.055	0.042
Known value (g/100 g sample)	0.127	0.087	0.056	0.046
Repeatability standard deviation (S_r)	0.006	0.003	0.003	0.001
Repeatability relative standard deviation (CV_r in %)	4.87	2.73	5.77	2.18
Repeatability limit (r) ($2.83 \times S_r$)	0.018	0.007	0.008	0.003
Reproducibility standard deviation (S_R)	0.019	0.013	0.006	0.006
Reproducibility relative standard deviation (CV_R in %)	14.82	14.70	10.12	14.36
Reproducibility limit (R) ($2.83 \times S_R$)	0.053	0.038	0.016	0.017

	Sample (combined duplicates) A	B	C	D
Evaluation of results using quadratic equation ($Y = A'X^2 + B'X + C$)				
Number of laboratories	6	7	6	7
Number of accepted results	16	13	14	18
Number of results retained after elimination of outliers	11	5	6	14
Mean value (g/100 g sample)	0.126	0.092	0.055	0.042
Known value (g/100 g sample)	0.127	0.087	0.056	0.046
Repeatability standard deviation (S_r)	0.006	0.003	0.003	0.001
Repeatability relative standard deviation (CV_r in %)	4.87	2.73	5.77	2.18
Repeatability limit (r) ($2.83 \times S_r$)	0.018	0.007	0.008	0.003
Reproducibility standard deviation (S_R)	0.019	0.013	0.006	0.006
Reproducibility relative standard deviation (CV_R in %)	14.82	14.70	10.12	14.36
Reproducibility limit (R) ($2.83 \times S_R$)	0.053	0.038	0.016	0.017

2 Field of application

This standard is applicable to mono-and diacylglycerols in edible oils and fats and mono and diacylglycerol concentrates (see note 1).

3 Principle

Samples are dissolved in a solvent mixture consisting of *n*-hexane and 2-propanol and analysed without derivatisation. Neutral lipid classes are separated with normal phase HPLC and the partial glycerides present are determined with ELSD (evaporative light scattering detector). The ELSD response of the sample components is with a log(response)/log(mass) calibration curve based on ELSD response to mass calibration standards identical to the sample components (see note 2).

4 Apparatus

4.1 HPLC system consisting of a high pressure pump, sample injection device, detector and recording integrator with the following minimum requirements

- column heater;
- programmable dual channel gradient;
- solvent degassing station;

- ELSD detector with drift tube to be heated (see note 3).

4.2 HPLC-Column, 150 mm × 4.6 mm with a stationary phase of silica gel of 10 μm particle size, e.g. Chromegasphere SI-60 stationery phase or similar (see note 4).

4.3 Volumetric flasks:

4.3.1 50 mL

4.3.2 100 mL

4.4 Injection syringe of 20 μL volume

5 Reagents

5.1 HPLC-grade solvents

5.1.1 *n*-Hexane

5.1.2 2-Propanol

5.1.3 Ethyl acetate,

5.2 Formic acid 88%, analytical grade

5.3 Solvent solutions for HPLC

5.3.1 Formic acid (5.2)/2-propanol(5.1.2) in a volume ratio 10/90.

5.3.2 Mobile phase A: *n*-hexane (5.1.1).

5.3.3 Mobile phase B: *n*-hexane (5.1.1)/2-propanol (5.1.2)/ethyl acetate (5.1.3)/formic acid-2-propanol solution (5.3.1) in a volume ratio 80/10/10/1.

5.3.4 Column rinse (to remove formic acid when the column is not in use): *n*-hexane (5.1.1)/2-propanol (5.1.2)/ethyl acetate (5.1.3) in a volume ratio 80/10/10.

5.3.5 Solvent solution for sample preparation: *n*-hexane (5.1.1)/2-propanol (5.1.2) in a volume ratio 90/10.

6 Procedure

6.1 Sample preparation

6.1.1 Vegetable oils

Weigh to within 0.1 mg, approximately 1 g of vegetable oil into a 50 mL volumetric flask (4.3.1). Dissolve the sample in the solvent mixture (5.3.5) and bring to volume with the same solvent (5.3.5). The sample is ready for analysis.

6.1.2 Monoacylglycerol emulsifiers

Weigh to within 0.1 mg, approximately 100 mg of vegetable oil into a 50 mL volumetric flask (4.3.1)

Dissolve sample in *n*-hexane (5.1.1)/2-propanol (5.1.2) mixture (5.3.5) and bring to 50 mL volume with the solvent mixture (5.3.5).

6.2 Calibration of the detector

6.2.1 Mono- and diacylglycerols

Determine the responses of the ELSD to 1,3-distearin, 1,3-dipalmitin, 1,3-diolein (and other diacylglycerols as needed), and to 1-monostearin, 1-monopalmitin and 1-monoolein (and other monoacylglycerols as needed) by preparing series of solutions from individual standards. Standards should be of at least 99% purity

Approximately 0.1 g of each of 1-monopalmitin and 1,3-dipalmitin are accurately weighted into a 100-mL volumetric flask (4.3.2), dissolved in a portion of the *n*-hexane/2-propanol solvent mixture (5.3.5), diluted to 100 mL with solvent mixture (5.3.5) and mix well to provide a stock solution.

From this stock solution of a concentration of approximately 1 mg/mL, prepare 1/2 and 1/10 dilutions with the *n*-hexane/2-propanol solvent mixture (5.3.5), to obtain approximately 0.5 and 0.1 mg/mL

standard solutions. Use the 1, 0.5 and 0.1 mg/mL standard solutions to establish the calibration curve for mono-and diacylglycerols (see note 5) (see note 6).

Following the analysis of the three standard solutions according to 6.3 the log of the peak area is plotted against the log of the concentration of 1-mono-palmitin and 1,3-dipalmitin to obtain the calibration curves (three-point calibration curve). Use a quadratic equation to calculate results. A typical ELSD response curve for working standards is shown in Figs 1 and 2.

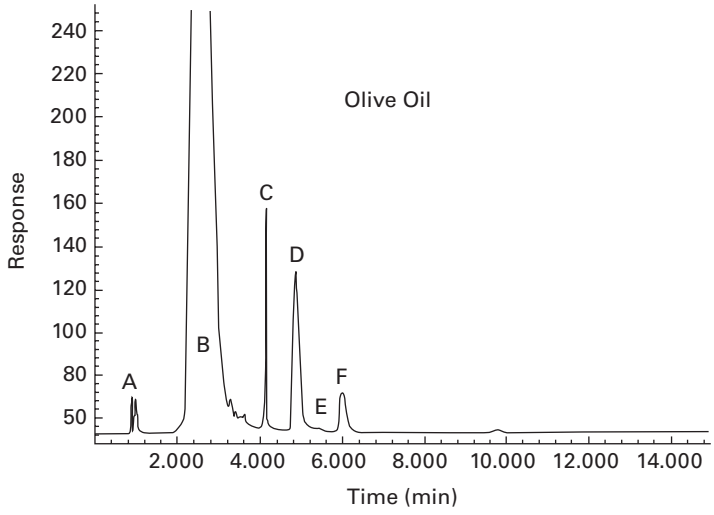

Fig. 2 Chromatogram of an olive oil obtained with a VAREX ELSD using HPLC and ELSD parameters as described in the method. (A) steryl (octadecyl) esters, (B) triacylglycerols, (C) free fatty acids, (D, E) traces of minor 1,3-diacylglycerols and/or phytosterols, and (F) 1,2-diacylglycerols. Peaks identified by retention time of reference standards only.

6.3 HPLC-working parameters

6.3.1 ELSD operating conditions

The working conditions for the ELSD depend from the system available.

For Varex ELSD II apply the following settings:

- Drift tube temperature maintained at 90 °C
- Nitrogen carrier flow rate maintained at 30 mm (approximately 1.2 L/min) on the flow meter of the ELSD.

6.3.2 Elution program

- Column temperature maintained at 40 °C
- Gradient program in Table 3

Table 3 Gradient program

Time (min)	flow (mL/min)	Mobile phase (mixing rate in volume parts) A(5.3.2)	B(5.3.3)
0	2	98	2
8	2	65	35
8.5	2	2	98
15	2	2	98
15.1	2	98	2
19	2	98	2

6.3 Inject 20 μL of solution of sample (6.1) or calibration standard (6.2)

When not in use, the column must be rinsed with the mixture of *n*-hexane/2-propanol/ethyl acetate (5.4.3) to remove formic acid.

7 Calculation and expression of results

7.1 Identify peaks by comparison of retention times of known substances of or the reference standard solutions (6.2).

Figure 2: Eventually delete E and adjust the legend correspondingly.

7.2 Calculate the coefficients A, B and C of the quadratic regression obtained from graph (6.2).

In applying a quadratic equation

$$Y = A + BX + CX^2$$

where: Y is the log detector response; X is the log of mass (mg/mL) of the calibration standards; A is the intercept of the quadratic regression line with the y-axis; B and C are coefficients of the quadratic equation

Calculate X from the following equation:

$$0 = A' + BX + CX^2$$

where: A' is $A - Y$; $X = -B/2A' \pm (B^2 - 4A'C)^{1/2}/2A'$

Discard negative or unrealistic solutions for X

Acylglycerol in g/100 g = 100×10^x/mass of the test portion in mg/mL (6.1.1).

8 Notes

1 Phytosterols may interfere with the determination of 1,3-diacylglycerols in vegetable oils. The 1,3-diacylglycerols are resolved from the 1,2-diacylglycerol positional isomers, although some 1,3-diacylglycerols of low molecular weight interfere with the 1,2-diacylglycerols of high molecular weight. For monoacylglycerols, the separation between 1-and 3-acyl- and 2-acylglycerols is optimised only between those pairs with identical fatty acyl groups.

2 A suitable ELSD is supplied Varex Corp., Rockville, Maryland, USA (Varex ELSD II).

3 A suitable column may be a stationery phase Chromegasphere SI-60.

4 ES Industries, 8 South Maple Ave., Marlton, New Jersey 08053 (USA).

5 Convenient standards may be obtained from Sigma Chemical Co., St. Louis, MO, or Nu-Check-Prep, Elysian Minnesota USA.

6 Reference standard stock solutions (6.2.1) can be kept in the deep freezer at <10 °C. They have to be warmed up to room temperature before use. Prepare calibration standard solutions each time freshly before use.

9 Quality assurance

9.1 For general principles of analytical quality control, see section on Quality Assurance in the introductory part of the *Compendium of Standard Methods*.

9.2 For specific applications of analytical quality control, see the Appendix to this standard method.

REFERENCES

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APPENDIX

Analytical quality control

1 Repeatability

When the mean value of two single test results obtained under *repeatability conditions* (conditions where independent test results are obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time) lies within the range of the values shown in the table below, the absolute difference between the two test results obtained should not be greater than the repeatability limit (r) deduced by linear interpolation from the data in Tables 1 and 2 (quadratic equation sections).

2 Reproducibility

When the values of two single results obtained under reproducibility conditions (conditions where test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment) lie within the range of the values shown in the table below, the absolute difference between the test results obtained should not be greater than the reproducibility limit (R) deduced from the data in Tables 1 and 2 (quadratic equation sections).

3 Results of the inter-laboratory test

An inter-laboratory test carried out at the international level in 1995 by the IUPAC Commission on Oils, Fats and Derivatives, in which seven laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with ISO 5725-1986) summarised in Tables 1 and 2.