

Determination of *Isomeric di-Acylglycerols* in Olive Oil

GC method

1 Scope and field of application

The method describes the determination 1,2- and 1,3- isomeric *di-acylglycerols* in olive oil.

2 Definitions

The degree of isomerisation is defined as the share of the peak areas of all 1,2-*di-acylglycerols* in refer to the sum of the peaks of all *di-acylglycerols*

3 Principle of the method

A miniaturized column chromatography on a silica gel column from is used to separate the isomeric *di-acylglycerols* as the more polar fraction from the major part of other lipids. The peak areas of 1,3- and 1,2-isomers are determined after silylation by gaschromatography.

4 Reagents

Warning: Attention is drawn to the regulations which specify the handling of dangerous matter. Technical, organizational and personal safety measurements shall be followed. Unless otherwise stated analytical grade reagents must be used;

4.1 Silica gel 60, for column chromatography, (63-100 μm);

4.1.1 Silica gel 60, 5% moisture:

Active the silica gel by heating overnight at 160 °C. After heating, place the silica gel in a desiccator while the gel is cooling and then transfer the silica gel to a stoppered flask. Add 5 % of water and shake until no lumps can be seen and the powder flows freely (1 h in an automatic shake machine). Store the conditioned silica gel overnight before use.

4.2 Cotton wool, defatted;

- 4.3 Dipalmitin (Dipalmitoylglycerol) mixed isomers, approx. 99%;
- 4.4 Distearin (Distearoylglycerol) mixed isomers, approx. 99%;
- 4.5 For each of the following reference substances a reference in toluene ($\rho = 10$ mg/10 ml) is prepared:
 - Distearin;
 - Dipalmitin.
- 4.6 Isooctane;
- 4.7 Acetone;
- 4.8 Diisopropyl ether;
- 4.8.1 Eluent: isooctane + diisopropyl ether (85+15,v/v)
- 4.9 1-Methyl imidazole;
- 4.10 N-methyl-N-(trimethyl-silyl)-hepta-fluorobutyramide (MSHFBA);
- 4.10.1 Silylating reagent: add 50 μ l 1-methyl imidazole in 1 ml of MSHFBA.

5 Apparatus

- 5.1 Analytical balance, capable of weighing to the nearest 0.001 g and displaying 0.0001 g;
- 5.2 Desiccator for storing adsorbents after heating;
- 5.3 Pipette tip: 5 ml, ca 15 cm long (e.g. VWR/Merck: 612-1484 oder 613-2623);
- 5.4 Round bottom flask, of 25 ml capacity, with ground neck;
- 5.5 Beaker, of 10 ml capacity;
- 5.6 Rotary evaporator;
- 5.7 Gas chromatograph for capillary columns, split injector, flame ionisation detector (FID) and suitable integration system.
- 5.8 Fused silica capillary column, for gas chromatography (0.25 mm or 0.32 mm i.d. by 15, 30 or 60 m length) coated with 5 % diphenyl-95 % dimethylpoysiloxane, 0.1 μ m film thickness; .

6 Sampling

Sampling is not part of the method specified in this part. Recommended sampling methods are given in C-I 1 to 5.

7 Procedure

7.1 Preparation of the silica gel chromatography column:

- 7.1.1 A cotton wool ball is compressed 5 mm high in the 5-ml pipette tip. Fill 1 g silica gel 60 in the pipette tip. The silica layer should be covered with a 5 mm high cotton wool ball. Condense filling by slight stamping and slight pushing with a flat ended rod.

7.2 Separation of the fraction containing non polar lipids:

- 7.2.1 Weigh, to the nearest 0.1 mg, about 100,0 mg of the test sample into a 10 ml beaker;
- 7.2.2 Add 1.00 ml toluene to the sample and mix carefully;
- 7.2.3 Transfer the sample onto the column purging carefully the flask with 1 ml eluent. Wash the column with two 3.5 ml portions of isooctane/diisopropylether mixture.
- 7.2.4 Eluate the diacylglycerols with 6 –8 ml diethyl ether and collect the eluate in a 25-ml round bottom flask.
- 7.2.5 Remove the solvent from the eluate to about 1 ml in the rotary evaporator at 20 °C. Transfer the remaining solution into a reaction vial. Blow off the solvent in the reaction vial with a stream of nitrogen.

7.3 Preparation of trimethylsilyl ethers (Silylation):

- 7.3.1 Add 200 µl of the silylation reagent to the reaction vial containing the diacylglycerols, seal and let react for 20 min at room temperature.
- 7.3.2 After silylation add 1 ml acetone and inject 1-2 µl of the solution directly into the gas chromatography.

7.4 Gas chromatography:

- 7.4.1 Gas chromatograph, with a flame ionization detector, split or

Optimize temperature program and velocity of carrier gas flow so that chromatograms similar to figure 1 are obtained. Test the separation with silylated diacylglycerol fractions.

- 7.4.1.1 Working conditions for split injection

These shall be as follows (Figure 1):

Capillary gas chromatography column: Restek RTX-5, 60 m, 0.25 mm i.d., 0,10 µm film thickness;

(Note: Other columns of similar polarity and selectivity can be used.)

-injector temperature, 340 °C

-detector temperature, 340 °C

-amount of solution injected: 1 µl, injector with 1:50 flow divider;

-carrier gas: hydrogen at a pressure of about ;

-oven programming temperature: initial 240 °C for 1 min, and then rising at 10 °C/min up to 320 °C; 340 °C for 10 min.

8 Result of the determination

8.1 Identification of diacylglycerol isomers:

100 µl dipalmitin (1,2-C32,1,3-C32) and distearin (1,2-C36, 1,3-C36) as references (C24) are collected in a derivatisation vial. Blow off the solvent in the reaction vial with a stream of nitrogen and silylate . . .

8. Result of the determination

8.1 Identification of the diacylglycerols:

8.1.1 To identify the 1,2 and 1,3-diacylglycerols in the test sample, determine the relative retention times (RRT) of the reference standards. The relative retention times of the diacylglycerols containing unsaturated fatty acids may differ a little bit from the RRT of the corresponding saturated fatty acids.

8.2 Determination of the peak areas of 1,2- and 1,3 diacylglycerols in the oil:

8.2.1 Calculation of the percentage of 1,2-diacylglycerols (*w*) in refer to the sum of the areas of the individual 1,2- and 1,3-diacylglycerols (C32,C34,C36):

For the purpose of this method it is assumed that the response factors of all diacylglycerols are equal.

$$W = A_{12} * 100 / A_x$$

Where:

A_x is the sum of the peak areas of the individual 1,2 and 1,3-diacylglycerols (C₃₂,C₃₄,C₃₆);

A_{12} is the peak area of all 1,2-diacylglycerols (C₃₂,C₃₄,C₃₆) present in the sample

8.5 Precision of the method:

The precision of the method is the result of a interlaboratory study organized 2005 by the "Joint Committee for the Analysis of Fats, Oils, Fat Products, Related Products and Raw Materials (GA Fett)" on an

international basis^{*)}. The study was carried out in xxx on five samples. The results are given in table 1.

Table 1: Summary of statistical results

Sample	A	B	C	D	E
Number of participating laboratories (N)	21	21	21	21	21
Number of laboratories retained after eliminating outliers (n)	16	19	18	19	19
Number of individual test results of all laboratories on each sample (z)	32	38	36	38	38
Mean value (m), %	42,18	34,91	60,27	55,44	35,14
Repeatability standard deviation (s_r)	0,507	0,763	1,235	1,626	0,715
Repeatability coefficient of variation (RCV_r), %	1,2	2,2	2	2,9	2
Repeatability limit (r)	1,42	2,14	3,46	4,55	2
Reproducibility standard deviation (s_R)	1,56	1,60	2,15	2,19	2,95
Reproducibility coefficient of variation (RCV_R), %	3,7	4,6	3,6	4	8,4
Reproducibility limit (R)	4,36	4,48	6,02	6,14	8,27

8.6 Repeatability limit (r):

The repeatability limit (r) is the value less than or equal to the absolute difference of two test results which can be expected with a probability of 95 %, under repeatability conditions.

Repeatability conditions are defined as conditions 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.

8.7 Reproducibility limit (R):

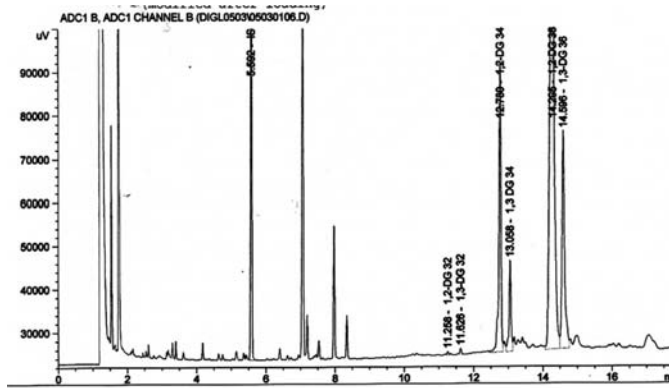
^{*)} Evaluated according to DGF Standard Method A-II 1 "Execution and evaluation of ring tests" and "ISO 5725:1994, Precision of test methods - Determination of repeatability and reproducibility for a standard test method by interlaboratory tests".

The reproducibility limit (R) is the value less than or equal to the absolute difference of two test results which can be expected with a probability of 95 %, under reproducibility conditions.

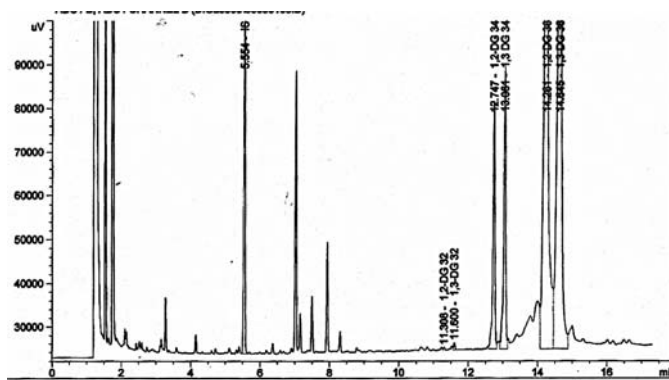
Reproducibility conditions are defined as conditions under which test results are obtained with the same method, on identical test material, in different laboratories, by different operators, using different equipment and reagents, within a short interval of time.

Figure 1: Gas Chromatograms obtained from olive oil samples analyses

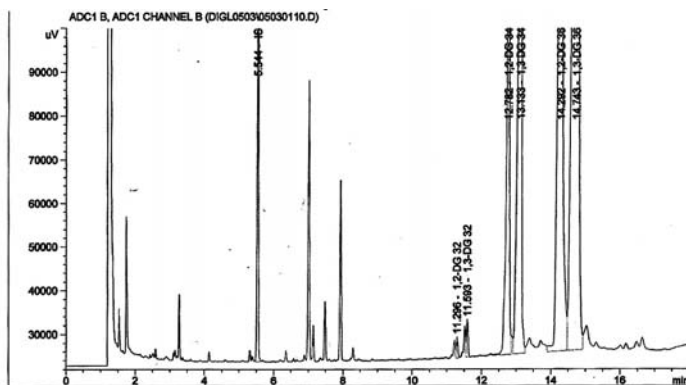
- a) Olive oil, extra virgine
- b) Olive oil, lampante
- c) Olive oil, desodorised (refined)



Olive Oil, extra virgine



Olive Oil, lampante



Olive Oil, refined