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DETERMINATION OF TRIGLYCERIDES IN VEGETABLE OILS IN TERMS OF THEIR PARTITION NUMBERS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Results of a collaborative study and the standardized method

Prepared for publication by

J. P. WOLFF[1], F. X. MORDRET[1] and A. DIEFFENBACHER[2]

[1]Institut des Corps Gras, Rue Monge, Parc Industriel, F-33600 Pessac, France
[2]Nestec Ltd, Central Quality Assurance Laboratory, 55 Avenue Nestlé, CH-1800 Vevey, Switzerland

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Determination of triglycerides in vegetable oils in terms of their partition numbers by high performance liquid chromatography: results of a collaborative study and the standardized method

ABSTRACT

The development by collaborative study of a standardized method for the determination of composition of triglycerides in vegetable oils in terms of their partition numbers (or equivalent carbon numbers : ECN) by high performance liquid chromatography is described. The procedure involves the preparation of an oil sample solution, the injection in the HPLC chromatograph , the separation of triglycerides using a reversed phase polarity column and detection with an appropriate system (e.g. refractive index detector). The method allows separation triglycerides into groups of identical partition numbers and the the calculation of their relative contents.

INTRODUCTION

Natural fats and oils are complex triglycerides mixtures. Their physical and chemical properties are closely related to the nature and the content of each individual triglyceride. Certain tirglycerides can represent rather specific compounds of the fat or oil and so offers possibilities for their characterisation. The extensive research in the field of triglycerides analysis (1) and chromatographic methods offer a wide range of possibilities. A GLC method has already been standardized (2) and allows the separation of triglycerides as a function of increasing molecular weight. More detailed separations were obtained with HPLC in reversed-phase mode (3). Identification of triglycerides can be achieved by calculating the "equivalent carbon number" (4) or by using retention time values with respect to triolein (5).

In view of these developments, the Commission decided to evaluate a proposed HPLC method by collaborative study.

1ST COLLABORATIVE STUDY

For the 1st interlaboratory study organized in 1985, the following samples were provided : samples 1 and 2 : pure olive oils, samples 3 and 4 : blends of olive oil with sunflower oil (respectively 90:10 and 95:5 w/w). Results were submitted by 15 laboratories and can be divided in two classes of data depending on the type of detector used (UV or RI). The values for repeatability (r) and reproducibility (R) were considered as good for one or the other detector, but with a too small number of results (mainly for RI detector) for a valuable statistical treatment. The RI detector was considered to be the detector of choice and before continuing on some changes had to be made in the method originally developed for UV detector.

2ND COLLABORATIVE STUDY AND RESULTS

For the 2nd collaborative study organized in 1986, the following six samples were distributed: sample A : soybean oil, sample B : almond oil, sample C : sunflower oil, sample D : blend of almond (B) and sunflower (C) oil (95:5 w/w), sample E : blend of almond (B) and sunflower (C) oil (85:15 w/w). Eighteen laboratories from eleven different countries participated. As an example, we have reported the statistical evaluation for one of these samples (table 1).

For main components the CVR values were fairly good, independently of the type samples and reasonably comparable.

When concentration decreases there is evidently a rapid coefficient increase. For the triglycerides ECN 50, it seems that this increase is stronger than for ECN 40. This increase has to be attributed to some integration problems with these triglycerides which are the last eluted from the column.

In respect with these results, the Commission decided to organize a new ring test to confirm the possibilities of the method.

3RD COLLABORATIVE STUDY AND RESULTS

For the 3rd collaborative study organized in 1987, four samples of vegetable oils were provided : sample A : a blend of palm and sunflower oils, sample B : olive oil, sample C : rapeseed oil, sample D : palm oil. Sixteen laboratories from twelve different countries took part.

The results of the statistical evaluation for sample B are reported (table 2). We can observe that the CVR values are of a similar order to those obtained in the previous study.

TABLE 1 STATISTICAL ANALYSIS OF RESULTS FOR SAMPLE C (Sunflower oil)
(2 nd interlaboratory study)

Triglyceride groups	ECN 40	ECN 42	ECN 44	ECN 46	ECN 48	ECN 50
Number of participating laboratories	18	18	18	18	18	18
Number of values	36	36	36	36	36	34
Number of accepted laboratories *	16	17	16	14	17	16
Number of values	32	34	32	28	34	32
Mean value (% m/m)	0,3	29,4	38,4	22,5	8,2	1,3
Repeatability						
Standard deviation Sr	0,1	0,5	0,4	0,4	0,9	0,5
Coefficient of variation CVr %	21,4	1,8	1,2	1,6	10,7	37,0
Repeatability value r	0,2	1,5	1,3	1,0	2,5	1,4
Reproducibility						
Standard deviation SR	0,2	0,95	1,3	0,55	1,0	1,2
Coefficient of variation CVR %	70,4	3,2	3,4	2,4	11,7	92,6
Reproducibility value R	0,6	2,7	3,65	1,55	2,7	3,4

* Elimination on basis of the Cochran and Dixon tests

TABLE 2 STATISTICAL ANALYSIS OF RESULTS FOR SAMPLE B (Olive oil)
(3 rd interlaboratory study)

Triglyceride groups	ECN 42	ECN 44	ECN 46	ECN 48	ECN 50
Number of participating laboratories	16	16	16	16	16
Number of values	31	31	31	31	31
Number of accepted laboratories *	12	13	14	14	14
Number of values	24	26	28	28	28
Mean value (% m/m)	0,6	5,85	21,2	64,7	7,15
Repeatability					
Standard deviation Sr	0,09	0,09	0,28	0,52	0,26
Coefficient of variation CVr %	16,2	1,5	1,3	0,8	3,6
Repeatability value r	0,26	0,25	0,78	1,49	0,74
Reproducibility					
Standard deviation SR	0,12	0,18	0,34	1,01	0,61
Coefficient of variation CVR %	21,1	3,0	1,6	1,6	8,5
Reproducibility value R	0,34	0,50	0,95	2,85	1,73

* Elimination on basis of the Cochran and Dixon tests

TABLE 3 Comparison of the reproducibility coefficients of variation obtained with the 2 nd and the 3 rd collaborative study of triglycerides as a function of their concentration

Triglycerides concentration in L/D m/m	ECN 40		ECN 42		ECN 44		ECN 46		ECN 48		ECN 50	
	2 nd	3 rd	2 nd	3 rd	2 nd	3 rd	2 nd	3 rd	2 nd	3 rd	2 nd	3 rd
0 to 1	21,4			21,1							92,6	
1 to 2											71,7	
2 to 3			27,8								40,3	40,8
3 to 5		8,5	10,8			16,2					16,6 9,1 7,9	
5 to 10	6,7		4,8	4,9		3,0 6,1			11,7			8,5 7,1 9,6
10 to 15			3,5	2,3	6,9				9,5			
15 to 20					4,1							
20 to 25			3,5			2,8	4,3 2,4	1,6 4,0 2,8 2,4				
25 to 30			3,2		3,4		2,4 2,5 2,4					
30 to 40					3,9 3,4				3,7 2,4	2,5		
up to 40									2,9 2,7	2,6 2,3 1,6		

Results of CVR are the average of each ECN-triglycerides of all samples of the 2nd and 3rd collaborative studies

TABLE 4 Main specifications of HPLC columns (3 rd collaborative study)

N° of laboratory	Length (cm)	Column Packing	Internal diameter (mm)	Supplier
1	25	Lichrosorb RP 18	4,7	5 FCC
2	25	Lichrospher RP 18	4,0	Merck
3	25	Lichrospher RP 18	4,0	Merck
4	30	Novapak C18	3,9	Waters
5	25	Ultron S-C18	4,6	Shinwakakou
6	25	Zorbax ODS	4,6	Du Pont
7	25	Zorbax ODS	4,6	Du Pont
8	25	Lichrosorb RP 18	4,6	Merck
9	10	ODS	6,0	Shodex
10	10	C18 Chromospher	3,0	Chrompack
11	25	Lichrospher RP 18	4,0	Merck
12	2 x 25	Nucleosil 5 C18	4,0	Macherey & Nagel
13	15	Nucleosil 5 C18	4,0	Bio-Separation
14	25	100 CH 18/2	4,0	Merck
15	15	ODS 2	4,6	Phasesep
16	25	Lichrosorb RP 18	4,6	Lichrosorb
17	25	Ultra Carb 20	4,6	Phenomenex
18	25	Silasorb C18	4,0	Tessek
19	22	ODS RP C18	4,6	Brownlee
20	23	Pierce C18	4,5	Pierce Chemical

For the samples analysed in the second and third studies a comparison of the variations of CVR depending on the relative concentration of triglycerides in the different samples is given (table 3). Information concerning the characteristics of the columns characteristics used are presented (table 4). Both the repeatability and reproducibility of the method has been shown to be satisfactory.

CONCLUSIONS

It is submitted that this method of analysis of triglycerides subjected to collaborative study has produced acceptable results **with vegetable oils samples.** One possibility of applications is the study of mixtures of oils and detection of adulterations. On the basis of the results the Commission decided to adopt the method. The text of the standardised procedure is given on the following pages.

Acknowledgements

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2.324 DETERMINATION OF TRIGLYCERIDES IN VEGETABLE OILS IN TERMS OF THEIR PARTITION NUMBER BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. SCOPE

The present standard describes a method of separation and quantitative determination of the triglyceride composition of vegetable oils in terms of their molecular weight and degree of unsaturation as a function of their partition number (or equivalent carbon number. See note 1).

2. FIELDS OF APPLICATION

This standard is applicable to all vegetable oils containing only triglycerides of long-chain fatty acids. The method is especially applicable to the detection of the presence of small quantities of semi-drying oils (rich in linoleic acid) in vegetable oils containing oleic acid as the predominant unsaturated fatty acid.

3. PRINCIPLE

Separation of triglycerides according to their partition number by high-performance liquid chromatography (reversed phase polarity) and interpretation of the chromatograms.

4. APPARATUS

4.1. High-performance liquid chromatograph, allowing thermostatic control of column temperature.

4.2. Injection unit for 10 µl delivery.

4.3. Detector : differential refractometer. The full-scale sensitivity should be at least 10^{-4} unit of refractive index.

4.4. Column : stainless steel tube 250 mm in length and of internal diameter 4.5 mm, packed with 5 µm diameter particles of silica with 22-23 % carbon in the form of octadecylsilane (Note 2).

4.5. Recorder and/or integrator.

5. REAGENTS

The reagents should be of analytical quality. Elution solvents should be de-gassed, and may be recycled several times without effect on the separations.

5.1. : Chloroform

5.2. : Acetone

5.3. : Acetonitrile

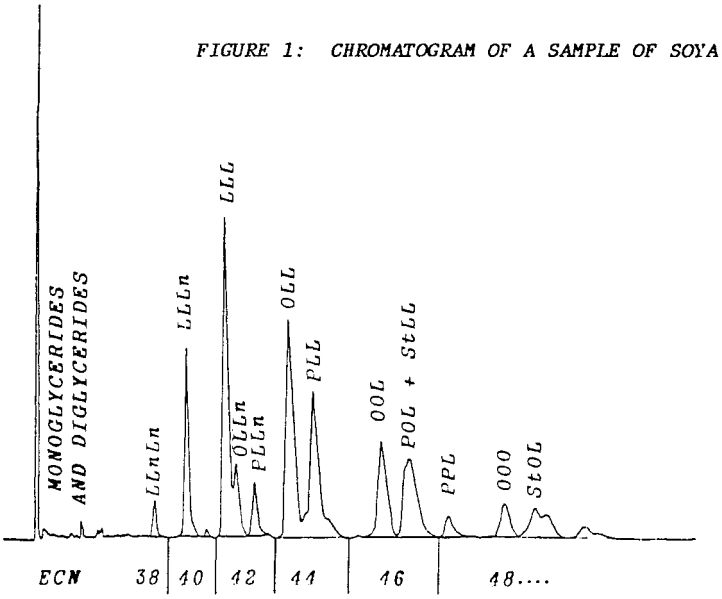
5.4. : Elution solvent : acetonitrile + acetone (proportions to be adjusted to obtain the desired separation ; begin with

50:50 - mixture)

5.5. : Solubilisation solvent : acetone or 1:1 acetone-chloroform mixture.

5,6. : Reference triglycerides : either commercial triglycerides (tripalmitin, triolein, etc.) may be used and the retention times thence plotted in accordance with the equivalent carbon number, or alternatively a reference chromatogram obtained from soya oil (see note 1, 3 and Fig 1 and 2).

FIGURE 1: CHROMATOGRAM OF A SAMPLE OF SOYA OIL



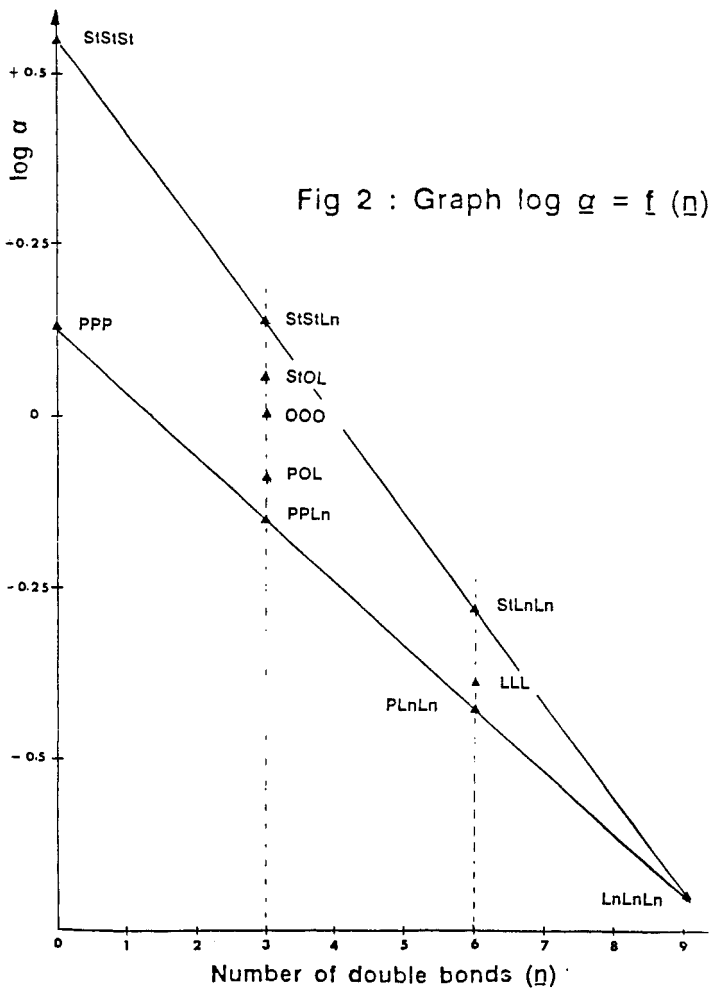


Fig 2 : Graph log $\alpha = f(n)$

6. PREPARATION OF SAMPLES

A 5 % solution of the samples to be analysed is prepared by weighing 0.5 + 0.001 g of the sample into a 10 ml graduated flask and making up to 10 ml with the solubilisation solvent (5.5).

7. PROCEDURE

7.1. Set up the chromatographic system. Pump elution solvent (5.4) at a rate of 1.5 ml/mm to purge the entire system. Wait until a stable base line is obtained. Put on the integrator.

Inject 10 µl of the sample prepared as in (6) and wait until the elution of the last peaks of the chromatogram ($\underline{ECN}$ 48 or $\underline{ECN}$ 50).

8. CALCULATION AND EXPRESSION OF RESULTS

Use the internal standardisation method. It is assumed that the sum of the areas of the peaks corresponding to the various triglycerides is equal to 100 %. Calculate the relative percentage of each triglyceride using the formula :

$$\% \text{ triglyceride} = \frac{\text{area of peak}}{\text{sum of peak areas}} \times 100,$$

the result to be given to one place of decimals.

9. NOTES

Note 1

The elution order can be determined by calculating partitions numbers or the equivalent carbon numbers, often defined by the relation $\underline{ECN} = \underline{CN} - 2\,\underline{n}$, where $\underline{CN}$ is the carbon number and $\underline{n}$ is the number of double bonds. It can be calculated much more precisely by taking into account the origin of the double bond. If $\underline{n}_1$ $\underline{n}_2$ and $\underline{n}_{1n}$ are the numbers of double bonds attributable to oleic, linoleic, linolenic acids respectively, the equivalent carbon number can be calculated by means of a relation of the formula :

$$\underline{ECN} = \underline{CN} - \underline{d}_1\underline{n}_1 - \underline{d}_2\underline{n}_2 - \underline{d}_3\underline{n}_3$$

where the coefficients $\underline{d}_1$ $\underline{d}_2$ and $\underline{d}_3$ can be calculated by means of the reference triglycerides. Under the conditions specified in this method the relation obtained will be close to :

$$\underline{ECN} = \underline{CN} - [2.60\ \underline{n}_1] - [2.35\ \underline{n}_2] - [2.17\ \underline{n}_3]$$

Note 2

Examples : Lichrosorb (Merck) RP18 Art 50333 ;
Lichrosphere (Merck) 100 CH18 Art 50377.

Note 3

With several reference triglycerides (i) it is also possible to calculate the relative retention with respect to triolein,

$$\underline{\alpha}_i = \underline{RT}_i'/\underline{RT}'_{\text{triolein}}$$

by use of the reduced retention time $\underline{RT}_i' = \underline{RT}_i - \underline{RT}_{\text{solvent}}$

The graph of $\log\underline{\alpha}$ against $\underline{n}$ (number of double bonds) enables the retention values to be determined for all the triglycerides of fatty acids contained in the reference triglycerides - see Fig 2.

10. QUALITY ASSURANCE

10.1. For general principles of analytical quality control see the section on Quality Assurance in the introductory part of the Compendium of the Standard methods.

10.2. **Repeatability**

When the mean of the values obtained from two single determinations carried out in rapid succession by the same operator, using the same apparatus under the same conditions for the analysis of the same laboratory sample, lies within the range of the mean values cited in the tables below, the difference between the two values obtained should not be greater than the repeatability limit (r), which can generally be deduced by linear interpolation from the values in the tables 1 and 2 below.

TABLE 1 STATISTICAL ANALYSIS OF RESULTS FOR SAMPLE C (Sunflower oil)
(2 nd interlaboratory study)

Triglyceride groups	ECN 40	ECN 42	ECN 44	ECN 46	ECN 48	ECN 50
Number of participating laboratories	18	18	18	18	18	18
Number of values	36	36	36	36	36	34
Number of accepted laboratories *	16	17	16	14	17	16
Number of values	32	34	32	28	34	32
Mean value (% m/m)	0,3	29,4	38,4	22,5	8,2	1,3
Repeatability						
Standard deviation Sr	0,1	0,5	0,4	0,4	0,9	0,5
Coefficient of variation CVr %	21,4	1,8	1,2	1,6	10,7	37,0
Repeatability value r	0,2	1,5	1,3	1,0	2,5	1,4
Reproducibility						
Standard deviation SR	0,2	0,95	1,3	0,55	1,0	1,2
Coefficient of variation CVR %	70,4	3,2	3,4	2,4	11,7	92,6
Reproducibility value R	0,6	2,7	3,65	1,55	2,7	3,4

* Elimination on basis of the Cochran and Dixon tests

TABLE 2 STATISTICAL ANALYSIS OF RESULTS FOR SAMPLE B (Olive oil)
(3 rd interlaboratory study)

Triglyceride groups	ECN 42	ECN 44	ECN 46	ECN 48	ECN 50
Number of participating laboratories	16	16	16	16	16
Number of values	31	31	31	31	31
Number of accepted laboratories *	12	13	14	14	14
Number of values	24	26	28	28	28
Mean value (% m/m)	0,6	5,85	21,2	64,7	7,15
Repeatability					
Standard deviation Sr	0,09	0,09	0,28	0,52	0,26
Coefficient of variation CVr %	16,2	1,5	1,3	0,8	3,6
Repeatability value r	0,26	0,25	0,78	1,49	0,74
Reproducibility					
Standard deviation SR	0,12	0,18	0,34	1,01	0,61
Coefficient of variation CVR %	21,1	3,0	1,6	1,6	8,5
Reproducibility value R	0,34	0,50	0,95	2,85	1,73

* Elimination on basis of the Cochran and Dixon tests

10.3. **Reproducibility**

When the values for the final result, obtained by operators in different laboratories using different apparatus under the same conditions, for the analysis of the same laboratory sample lie within the range of mean values cited in the tables below, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility limit (R), which can generally be deduced by linear interpolation from the values in the tables 1 and 2 below.

10.4 **Results of the interlaboratory tests**

Two interlaboratory tests carried out at an international level in 1986-87 by the IUPAC Commission on Oils, Fats and Derivatives, in which 18 and 16 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with ISO-5725) summarised in tables 1 and 2.