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DETERMINATION OF POLYMERIZED TRIGLYCERIDES IN OILS AND FATS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Results of a collaborative study and the standardized method

Prepared for publication by

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Determination of polymerized triglycerides in oils and fats by high performance liquid chromatography: results of a collaborative study and the standardized method

ABSTRACT

A description is given of the development by collaborative study of a standardized method for the determination of polymerized triglycerides in oils and fats by gel permeation HPLC. This rapid procedure allows the determination of polymer contents equal to or higher than 3 per cent (w/w) of the total amounts of fats.

INTRODUCTION

During the frying process a wide variety of chemical reactions results in the formation of compounds with high molecular weights and a large range of polarities. The presence of air in the frying system and the heating of fats initiate a cycle of thermal and thermo-oxidative decomposition with, as consequence, the formation of polymeric compounds. These mainly contain dimeric triglycerides (*) and higher polymers. The formation of such compounds is closely related to the initial structure of the component triglycerides (unsaturation) and the conditions adopted for the heating of fats.

The appropriate analytical procedure for the determination of such compounds in oils is based on molecular exclusion chromatography. References of some significant research in this field are given [1-3].

A method resulting of this research was previously tested by national bodies [4]. The encouraging results obtained in France (Association Française de Normalisation - Centre National de Coordination des Etudes et Recherches sur la Nutrition et l'Alimentation) with a first ring test led the Commission to organize an international collaborative study.

1ST COLLABORATIVE STUDY AND RESULTS

For the 1st interlaboratory study organized in 1985 the following samples of fats were provided :

Sample 1 :	animal fat
Sample 2 :	exhausted frying oil greatly damaged
Sample 3 :	blend of samples 1 and 2 in the proportions 74 : 26 (w/w)
Sample 4 :	reference material (oil with a known content of polymers)

The method was studied by thirteen laboratories but three of them were unable to obtain any separation with all the four samples. A summary of the results submitted by ten laboratories

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(*) The International Union of Nutritional Sciences (IUNS) proposes to replace triglyceride by triacylglycerol. As the former term has been well introduced in oil and fat chemistry we continue to use it being well aware that the correct expression for monomeric triglycerides would be triacylglycerols.

is given in table 1 and the statistical evaluation in table 2. Except for the sample 3, the variation of CVr in relation with the content of polymers seems to be normal.

However, the fact that a number of the participants were not able to apply the method and the poor results obtained for one of the four samples indicated that more precise directions as column quality and integration requirements had to be provided. The organization of a second ring test was therefore considered necessary.

2ND COLLABORATIVE STUDY AND RESULTS

For the second collaborative study organized in 1986, the following four samples were provided :

Sample 1 : frying oil with an average content of polymers
Sample 2 : industrial animal fat not contaminated with frying oils
Sample 3 : frying oil with a low content of polymers
Sample 4 : frying oil with an average content of polymers and presenting difficulties of resolution between monomers and polymerized triglycerides.

TABLE 1 DETERMINATION OF POLYMERIZED TRIGLYCERIDES FIRST INTERLABORATORY STUDY

LAB CODE	SAMPLE 1		SAMPLE 2		SAMPLE 3		SAMPLE 4	
1	3,1	3,1	23,2	23,3	9,2	9,2	4,1	4,2
2	2,8	2,7	22,4	22,5	8,7	8,6	3,6	3,6
3	1,5	1,5	20,0	19,5	3,8	4,8	3,0	3,2
4	3,1	3,1	22,1	22,3	10,0	10,1	4,2	4,3
5	2,0	2,1	21,0	21,0	7,7	7,4	3,4	3,4
6	2,3	2,2	22,1	22,2	6,0	6,0	3,5	3,5
7	2,6	2,7	22,7	23,0	8,3	7,5	3,6	3,3
8	1,5	1,5	24,1	24,7	3,8	2,8	3,0	2,7
9	2,3	2,1	19,5	19,7	8,0	8,0	4,0	3,9
10	2,3 *	1,8 *	22,1	22,3	7,6	9,0	3,6	3,4

(*) Results eliminated on basis of the Cochran or Dixon tests.

TABLE 2 STATISTICAL ANALYSIS OF RESULTS FIRST INTERLABORATORY STUDY

SAMPLE CODE	1	2	3	4
Number of Laboratories	10	10	10	10
Number of Laboratories retained after elimination of outliers	9	10	10	10
Number of accepted results	18	20	20	20
Mean value (%)	2,35	22,0	7,20	3,60
Repeatibility :				
Standard deviation Sr	0,07	0,18	0,37	0,12
Coefficient of variation CVr	2,8	0,8	5,2	3,3
Repeatability value r	0,19	0,51	1,06	0,34
Reproducibility :				
Standard deviation SR	0,61	1,48	2,14	0,45
Coefficient of variation %	25,9	6,8	29,8	12,5
Reproducibility value R	1,70	4,20	6,05	1,25

TABLE 3 DETERMINATION OF POLYMERIZED TRIGLYCERIDES
SECOND INTERLABORATORY STUDY

LAB CODE	SAMPLE 1		SAMPLE 2		SAMPLE 3		SAMPLE 4		COLUMN PACKING
1	10,0	10,0	2,7	2,6	5,7	5,7	11,6	11,7	Lichrogel PS4 + PS1
2	9,4	9,5	1,5	1,5	4,8	4,9	9,5	9,4	Ultrastyragel
3	9,6	10,0	1,7	1,8	5,1	5,0	10,3	10,8	PL Gel + Styragel
4	9,4	8,8	1,2	1,1	4,2	4,5	12,4	8,9	PL Gel 5 µ
5	8,9	9,6	1,5	1,4	4,9	4,7	10,5	9,6	PL Gel 5 µ
6	9,8	9,7	2,4	2,2	5,0	5,0	10,2	10,3	PL Gel 5 µ
7	8,9	10,2	1,4	1,7	5,7	5,4	4,8*	4,4*	PL Gel 5 µ
8	9,5	9,6	2,0	2,0	5,2	4,8	10,5	10,4	PL Gel 5 µ
9	10,0	10,3	4,8*	5,2*	5,3	5,4	7,4	7,8	?
10	9,5	9,7	1,9	2,0	5,1	5,1	10,5	10,6	2 Ultrastyragel 500 + 100
11	9,7	9,6	2,0	1,9	5,3	5,4	10,2	10,6	Ultrastyragel 500
12	7,3*	8,0*	1,0	1,4	3,5*	3,7*	7,0	7,0	Ultrastyragel
13	10,4	10,0	2,1*	3,3*	5,2	5,2	9,6	9,8	TSK Gel
14	10,7	10,7	2,2	2,3	5,6	5,5	10,6*	10,6*	Shodex PAK
15	9,8	9,8	1,6	1,6	5,4	5,3	10,9	10,8	PL Gel 5 µ
16	9,5	9,4	1,6	1,6	5,3	5,2	10,7	10,8	PL Gel 5 µ
17	9,6	9,8	1,7	1,8	5,1	5,1	10,3	10,4	Waters

(*) Results eliminated on basic of the Cochran or Dixon tests

TABLE 4 STATISTICAL ANALYSIS OF RESULTS
SECOND INTERLABORATORY STUDY

SAMPLE CODE	1	2	3	4
Number of Laboratories	17	17	17	16
Number of Laboratories retained after elimination of outliers	16	15	16	15
Number of accepted results	32	30	32	30
Mean value (%)	9,7	1,8	5,2	10,0
Repeatibility :				
Standard deviation $\underline{S}r$	0,3	0,1	0,1	0,2
Coefficient of variation $\underline{CV}r$	3,2	6,2	2,3	2,2
Repeatability value $\underline{r}$	0,9	0,3	0,3	0,6
Reproducibility :				
Standard deviation $\underline{S}R$	0,4	0,4	0,3	1,2
Coefficient of variation $\underline{CV}R$	4,6	23,7	6,7	12,2
Reproducibility value $\underline{R}$	1,3	1,2	1,0	3,5

The number of participants was higher (17) than for the first study (13). The results submitted to 17 laboratories are given in table 3 and the statistical evaluation in table 4.

In comparison with the results obtained in the first study, the reproducibility is improved but remains rather poor for the low content (2 per cent and less) of polymerized triglycerides.

CONCLUSIONS

1. Gel permeation HPLC appears to be a suitable method for the determination of polymerized triglycerides in frying fats.

2. With a wide range of molecular weight of polymerized triglycerides formed during the heating of fats, in some cases, poor separations between monomers and polymers were observed with a related increase of CVr mainly for low contents of polymers.

3. Taking in account that :

- sufficient data derived from the Commission's studies are available,
- this method previously studied has been adopted :

a) by Association Française de Normalisation (AFNOR, France) as an experimental method for samples containing at least 3 per cent of polymerized triglycerides (4) ;

b) by the Netherlands Normalization Institute (NNI, Netherlands) with a detection limit of 5 per cent (5),

the Commission decided to adopt the method but with a limitation of application to samples containing 3 per cent or more of polymerized triglycerides.

The text of the standardized method is given on the following pages.

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(5) NNI - Vegetable and animal oils and fats ; Determination of polymerized triglycerides content, Norme NEN 6348 (September 1988).

2.508 DETERMINATION OF POLYMERIZED TRIGLYCERIDES IN OILS AND FATS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. SCOPE

This standard method describes a method to determine the polymerized triglycerides content in oils and fats which contain not less than 3% (m/m) of these polymers.

2. FIELD OF APPLICATION

This method is applicable to any kind of fats and oils, heated or not. It can also be applied to the determination of polymers in animal feed fats.

3. PRINCIPLE

Dissolution of the sample in a suitable solvent and separation of the polymerized triglycerides by gel permeation chromatography according to molecular size.

4. APPARATUS

The recommended equipment must include the following :

4.1. Solvent reservoir of about 250 ml of capacity with a mobile phase liner filter (pore size 1 μ)

4.2. HPLC pump, pulseless, with a flow of 0,7 to 1,5 ml/min.

4.3. Injection valve with a 10 μl loop and a suitable syringe with a volume of 50 to 100 μl (Rheodyne or Valco).

4.4. Stainless steel column length : 300 mm, internal diameter : 7,7 mm packed with a high performance spherical gel made of styrene-divinylbenzene co-polymer diameter of the particles : 5 μ, pore size : 100 Å of the equivalent in term of exclusion power and resolution.
The storage of such column must be achieved in toluene.

4.5. Detector : refractive index detector with a sensivity at full scale at least 1.10^{-4} of refractive index.

4.6. Recorder and/or integrator.
To allow display and accurate quantification of the peak areas.

5. REAGENTS

5.1. Tetrahydrofuran, analytical grade.

5.2. Toluene, analytical grade.

6. PROCEDURE

6.1 Starting an HPLC equipment

It is advisable to follow carefully the manufacturer's recommendations.

Switch on the system and pump tetrahydrofuran at a rate of 1 ml/min. to purge the whole system up to the injection valve. Connect the column to the injection valve and wash it with about 30 ml of tetrahydrofuran. Connect the column to the sample cell of the detector. Fill the reference cell with the tetrahydrofuran. Adjust the mobile phase flow to 0,8 – 1 ml/min. Wait until a convenient stabilization of the system (no appreciable deviation of the base line) is obtained. (Note 1).

6.2 Preparation of the samples and analysis

As the samples may contain suspended particles, resulting in the blockage of the porous fritted filter at the top of the column, it is advisable to filter them before injection. Filtration can be quickly and effectively realized with a 1 μ pore size filter (Note 2).

The samples must be anhydrous. If not, they must first be dried (Note 3).

Weigh about 50 mg of fat and add 1 ml of tetrahydrofuran. Homogenize. Take with the syringe 50 to 100 μl of that solution. Fill the injection loop. Inject and switch on the integrator.

With a mobile phase flow of 1 ml/min., the analysis time is about 10 min.

7. RESULTS

7.1 Qualitative analysis

The chromatographic pattern of the determination may show a main peak representative of monomeric triglycerides (M.W. about 900) and one or several smaller peaks with a shorter pattern retention distance than triglycerides, representative of polymerized triglycerides (dimers and upper oligomers).

Reference shoud be made to the three typical chromatograms presented in figure 1. Under suitable conditions triglycerides and polymerized triglycerides can be separated with good resolution (I and II) even at low levels contents of polymerized triglycerides (I). However in some cases which seem to be connected to the complex degradation phenomena (hydrolysis) the peak pattern preceding the triglycerides peaks may be less clear (III) with consequent difficulties in calculation.

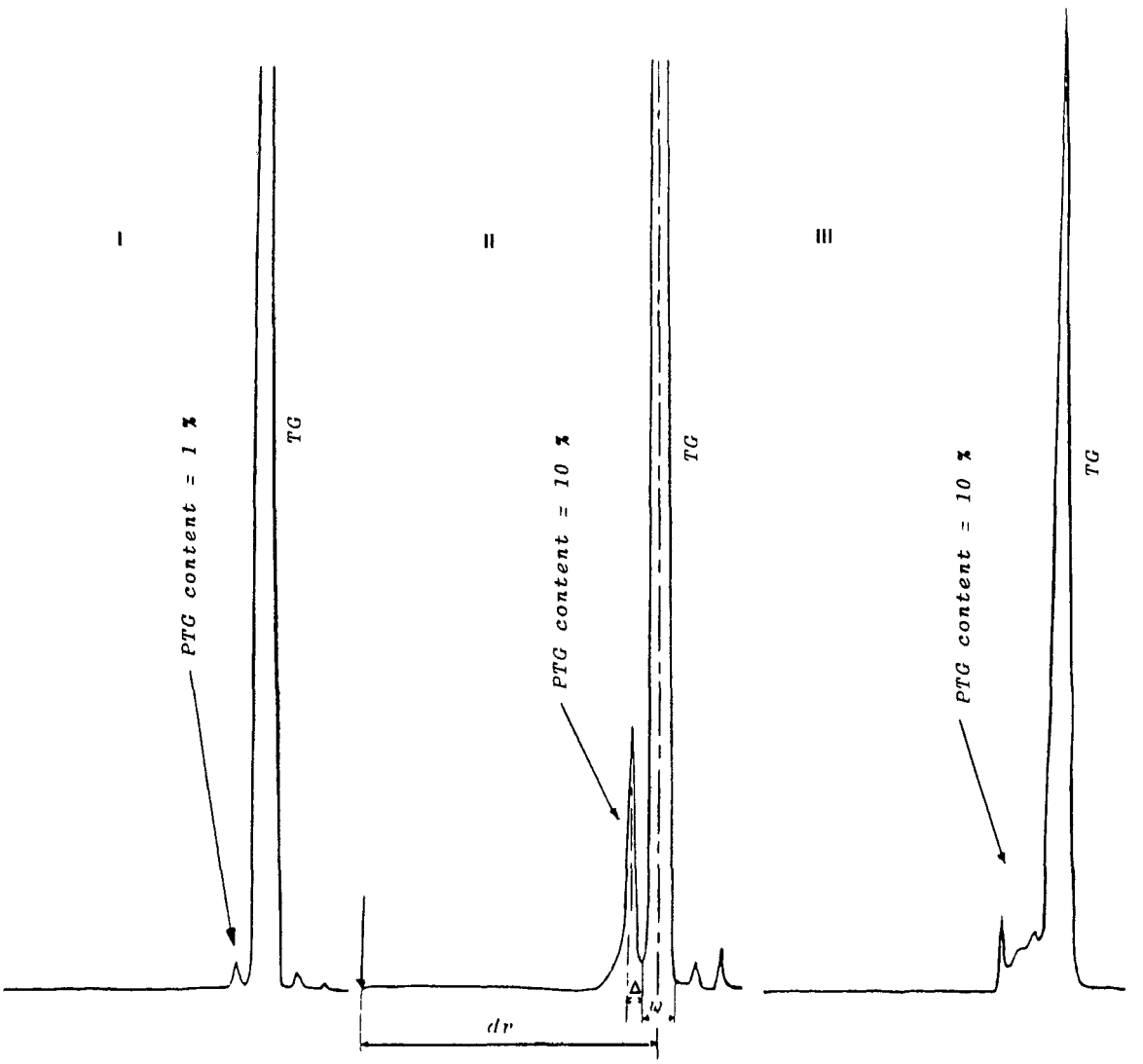

FIG. 1: HPLC OF TRIGLYCERIDES (TG) AND POYLYMERISED TRIGLYCERIDES (PTG)

7.2 Quantitative analysis

The calculation is achieved by the internal standard method assuming that all components of the sample are eluted.

The polymerized triglycerides content can be calculated using the formula :

$$\% \text{ PT} = \frac{\underline{A}_{PT}}{\Sigma \underline{A}} \times 100$$

where :

PT : percentage of polymerized triglycerides

$\underline{A}_{PT}$: sum of areas of the polymerized triglycerides peaks

$\Sigma \underline{A}$: sum of areas of all peaks.

For calculating $\underline{A}_{PT}$ two cases are possible :

a) Good resolution between peaks ($\underline{R} \geq 1$) (similar to I and II). The general methods of integration (manual and electronic) can be used to calculate individual and total areas.

b) Poor resolution between peaks ($\underline{R} < 1$) (similar to III).

It is assumed that all components eluted before $\underline{dr}_T$ are polymerised triglycerides.

The resolution is calculated from

$$\underline{R} = \frac{\Delta}{\underline{W}}$$

where

Δ is the width, in mm, of the triglycerides peak at the baseline, measured between the points of intersection between the tangents and baseline.

$\underline{W}$ is the width, in mm, of the triglycerides peak at the baseline, measured between the points of the intersection between tangents and baseline.

$\underline{dr}_T$ is the retention distance, in mm, from the beginning of the chromatogram to the peak maximum for triglycerides.

Using electronic integration, the integrator has to be carefully adjusted (backward horizontal integration) to integrate all the surfaces included between the curve and the baseline. If a manual technique is used, it is necessary to determine the triglyceride peak area by triangulation.

Express the results to one place of decimals.

8. QUALITY ASSURANCE

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

8.2 For specific applications of analytical quality control see the Annexe to this standard method.

9. NOTES

1. If the composition of the column is as indicated, an acceptable stabilization of the system should be obtained in about 15 min. With other column packings, the stabilization of the system may be more difficult: for example changing the mobile phase should be done stepwise from toluene to tetrahydrofuran, with different mixtures, each time 25 per cent higher in tetrahydrofuran. Acceptable stabilization is normally obtained in about 12 hr.

2. The sample can be filtered with a 1 micron pore size filter made of polytetrafluoro-ethylene or cellulose esters. Syringe disposable filter units are available from manufacturers of HPLC equipment.

3. Samples which may contain water must first be dried: dissolve 50 to 100 mg of the sample in 1 ml tetrahydrofuran. Add 50 mg anhydrous sodium sulphate, shake, and leave for about 2 min. Filter upper layer through a 1 micron pore size filter.

APPENDIX

1. 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 table 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 table below.

2. 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 the mean values cited in the table 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 table below.

3. 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 10 and 17 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with ISO 5725-1986) summarised in the following table:

TABLE

Sample		A	B	C	D	E	F	G	H
Number of laboratories	..	17	10	10	10	17	16	10	10
Number of laboratories retained after eliminating outliers	..	15	9	10	16	10	16	15	10
Number of accepted results	..	30	18	20	32	20	32	30	20
Mean value (% of total triglycerides)	..	1.8	2.4	3.6	5.2	7.2	9.7	10.0	22.0
Repeatability standard deviation (S_r)*	..	0.1	0.07	0.12	0.1	0.37	0.3	0.2	0.18
Repeatability relative standard deviation†	..	0.1	2.8	3.3	2.3	5.2	3.2	2.2	0.8
Repeatability limit (r)* [2.8 x S_r]	..	0.3	0.19	0.34	0.3	1.06	0.9	0.6	0.51
Reproducibility standard deviation (S_R)*	..	0.4	0.61	0.45	0.3	2.14	0.4	1.2	1.48
Reproducibility relative standard deviation†	..	23.7	25.9	12.5	6.7	29.8	4.6	12.2	6.8
Reproducibility limit (R)* [2.8 x S_R]	..	1.2	1.7	1.25	1.0	6.05	1.3	3.5	4.2

†(coefficient of variation) *(expressed as % of total triglycerides)