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DETERMINATION OF TRIGLYCERIDES IN FATS AND OILS

Results of a collaborative study and the standardised method

Prepared for publication by

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Determination of triglycerides in fats and oils: results of a collaborative study and the standardised method

Abstract - The development, by collaborative study, of a standardised method for the determination of the component triglycerides of animal and vegetable oils and fats is described. The procedure involves the separation of the triglycerides into groups containing the same number of carbon atoms, the separation being achieved by direct gas-liquid chromatography of solutions of the lipids on packed columns under temperature programmed conditions.

INTRODUCTION

The determination of the triglyceride composition of animal and vegetable fats and oils provides information which complements that of the fatty acid composition for the identification of these lipids; it also provides limited information concerning the distribution of the fatty acids within the glyceride molecules. For example, as an aid to the identification of genuine palm oil, the Palm Oil Research Institute of Malaysia (PORIM) publishes typical ranges for the levels of C46 through C54 triglycerides in crude Malaysian palm oil (ref. 1), and the draft CAOBISCO* method for the estimation of cocoa butter equivalents (CBE's) and cocoa butter replacers (CBR's) in chocolate and confectionery products requires the accurate assessment of the fat triglycerides (ref. 2).

1st COLLABORATIVE STUDY AND RESULTS

Three approaches to the determination of triglycerides were originally considered, viz.:

1) by gas-liquid chromatography (glc) using a) conventional packed columns and b) capillary columns

2) by high performance liquid chromatography (HPLC)

At the time of the initiation of the first collaborative study (1981) it was considered that the application of HPLC to triglyceride analysis was not sufficiently advanced to ensure the successful development of a standard method based on this technique. For the same reason it was decided that the method to be developed should be based on packed-column GLC rather than GLC using capillary columns - even though the latter offers the possibility of separating isomers of triglycerides, containing the same number of carbon atoms, according to their differing degrees of unsaturation.

By 1981 a significant number of laboratories were already experienced in the analysis of triglycerides by GLC; accordingly for the first collaborative study (1981-82) participants were invited to follow the procedure with which they were familiar, but to keep as far as possible within certain guidelines as respects the GLC working parameters. For the first collaborative study three samples were provided - a cocoa butter, a palm oil and a coconut oil. A summary of the results of the triglyceride analysis of these samples, submitted by nine laboratories, with the values for repeatability and reproducibility calculated according to ISO 5725, are given in Table 1.

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*CAOBISCO: Association of the Chocolate, Biscuit and Confectionery Industries of the EEC

TABLE 1. RESULTS FOR COMPONENT TRIGLYCERIDES (expressed as % by mass of total)

Sample 1. Palm oil

TG No.	C48	C50	C52	C54	C56
min	6.6	36.0	38.4	9.2	0.4
max	8.6	40.6	41.5	11.4	0.7
mean	7.8	38.7	39.4	10.5	0.5
SD (r)	0.19	0.58	1.17	0.48	0.03
SD (R)	0.69	2.04	1.28	0.70	0.11
r	0.54	1.64	3.32	1.37	0.08
R	1.96	5.77	3.63	1.99	0.31

Sample 3. Cocoa butter

TG No.	C48	C50	C52	C54	C56
min	0.1	16.1	41.8	31.1	0.6
max	1.8	18.9	46.0	35.3	2.2
mean	0.7	17.8	45.1	33.7	1.7
SD (r)	0.06	0.13	0.57	0.46	0.11
SD (R)	0.66	1.27	1.32	1.29	0.43
r	0.17	0.38	1.61	1.31	0.32
R	1.88	3.59	3.73	3.65	1.22

Sample 2. Coconut oil

TG No.	C30	C32	C34	C36	C38	C40	C42	C46	C48	C50
min	3.2	14.1	18.1	20.1	17.3	9.7	6.3	3.2	1.4	1.0
max	3.8	15.0	19.1	21.1	18.5	10.7	7.7	3.5	1.7	1.3
mean	3.5	14.3	18.6	20.6	18.0	10.3	6.9	3.4	1.6	1.1
SD (r)	0.12	0.26	0.16	0.12	0.06	0.09	0.09	0.08	0.13	0.07
SD (R)	0.21	0.30	0.25	0.29	0.33	0.37	0.34	0.16	0.13	0.15
r	0.34	0.72	0.45	0.35	0.17	0.24	0.26	0.23	0.37	0.22
R	0.61	0.86	0.71	0.80	0.94	1.06	0.95	0.45	0.37	0.44

The conclusions resulting from this first study can be summarised as follows:

1) A relatively short column of about 0.5 - 0.6 m with a low loading (about 3%) of a stationary phase with a polarity similar to OV-1 gave the most satisfactory chromatographic separations provided:

a) the rate of temperature programming of the column did not exceed 5°C per minute.

b) the injector and detector temperatures were about 25°C above the temperature of the column.

c) the column was temperature-programmed from a minimum of about 220°C to a maximum of 350°C.

2) The amount of sample injected should be limited to the equivalent of not more than 2 µl of a 0.5% (m/v) solution of the fat or oil.

3) Mono- and di-glycerides, if present, must be removed prior to the determination when they are likely to co-elute with triglycerides under the conditions of the analysis.

2nd COLLABORATIVE STUDY AND RESULTS

For the second study samples of the following fats and oils were distributed for analysis: lard, butterfat (2 samples), soyabean oil, hydrogenated soyabean oil, and a groundnut oil to which had been added 1% each of dipalmitin and distearin. Participants were advised to follow the recommendations outlined in the conclusions to the 1st study (see above).

Results were received from 15 participants and a summary of these (except for the hydrogenated soyabean oil) are given in Table 2. The results for the analysis of the hydrogenated soyabean oil have been omitted purely on the grounds of space. The levels of component triglycerides found in this sample (which was the same oil provided as Sample 6 but hydrogenated) were found to be practically identical to those of the non-hydrogenated oil (Sample 6) with mean levels of triglyceride groups C50 at 3.7%, C52 at 27.8%, C54 at 65.0% and C56 1.7%. The values for r and R for the analysis of the hydrogenated soyabean oil (Sample 7) were found to be similar to those for the analysis of the non-hydrogenated oil. In some cases analyses using capillary columns had resulted in the partial separation of triglyceride groups into isomers of triglycerides containing the same number of carbon atoms but the total integrated peak areas were used in the calculations for the results given in the tables.

TABLE 2. RESULTS FOR COMPONENT TRIGLYCERIDES (expressed as % by mass of total)

Sample 4 Lard

TG No.		C50	C52	C54	C56
min	2.2	13.5	54.8	18.9	1.2
max	3.7	16.5	60.7	23.0	2.9
mean	2.8	14.8	58.2	20.6	2.1
SD (r)	0.08	0.25	0.63	0.28	-
SD (R)	0.46	0.85	2.40	1.80	-
r	0.22	0.70	1.78	0.79	-
R	1.30	2.39	6.87	5.12	-

Sample 6. Soyabean oil

C50	C52	C54	C56
2.5	26.8	59.9	0.6
4.9	30.9	69.1	2.7
3.6	27.9	65.5	1.6
0.12	0.77	0.85	0.40
0.78	1.10	2.70	1.00
0.35	2.19	2.39	1.13
2.19	3.34	7.74	3.07

Sample 5. Butterfat

TG No.	C30	C32	C34	C36	C38	C40	C42	C44	C46	C48
min	1.0	2.3	4.9	8.6	11.3	9.6	6.2	5.4	6.2	7.1
max	1.4	3.0	6.2	11.6	13.3	11.3	7.2	6.7	7.5	9.5
mean	1.3	2.7	5.7	10.1	12.5	10.3	6.7	6.1	6.8	8.4
SD (r)	0.07	0.10	0.15	0.26	0.09	0.19	0.10	0.05	0.09	0.08
SD (R)	0.16	0.30	0.52	1.10	0.81	0.56	0.36	0.40	0.43	0.66
r	0.38	0.89	0.46	0.39	0.91	0.78	0.80	0.33	0.49	0.23
R	0.48	1.16	1.87	3.51	2.67	2.98	1.22	1.09	1.35	1.85

Sample 5. Butterfat (contd)

TG No.	C50	C52	C54
min	9.6	9.0	4.9
max	11.8	11.2	5.8
mean	10.8	9.9	5.3
SD (r)	0.17	0.19	0.26
SD (R)	0.77	0.86	0.73
r	0.49	0.54	0.73
R	2.16	2.42	2.05

Sample 8. Groundnut oil

C50	C52	C54	C56
2.6	26.8	60.0	0.9
4.8	30.3	69.4	2.7
3.4	27.7	65.5	1.6
0.57	1.00	2.70	0.96
0.18	0.16	0.67	0.07
0.50	0.47	1.88	0.22
1.62	3.03	7.78	2.70

Note: Only the major triglyceride groups reported have been tabulated

Four laboratories submitted results obtained using capillary columns. It was found that there was no significant difference between the levels of triglycerides groups determined by capillary and packed colums and therefore for the statistical calculation of repeatability and reproducibility results obtained using both columns have not been distinguished from each other.

CONCLUSIONS

1) The repeatability and reproducibility values determined from a statistical analysis of the results (Tables 1 and 2) indicate that the determination of triglycerides (according to their carbon number) by packed-column GLC, under temperature-programmed conditions, can be carried out to an acceptable degree of precision for the major triglyceride components of both animal and vegetable fats and oils.

2) It is possible to obtain comparative results using relatively short (i.e. 5 m or less) capillary columns instead of packed columns. By limiting the overall length of the capillary column to 5 m or less the partial resolution, into different isomers, of the peaks representing groups of triglycerides can be reduced or eliminated, thereby diminishing possible peak area integration errors. Details of the capillary columns and the conditions under which they were used in the collaborative study are given in Table 3 which sets out the gas-liquid chromatography operating parameters adopted by the participants.

TABLE 3. GAS-LIQUID CHROMATOGRAPHY OPERATING PARAMETERS

	COLUMN			TEMPERATURE				CARRIER		SOLVENT	
	PHASE		DIMENS. m x mm	INJ. °C	OVEN °C	RATE °C/min	DET. °C	GAS	FLOW ml/min	VOL. µl	TYPE
Lab.											
4	OV1	3%	0.5 x 2.0	350	210-340	5.0	400		50	1.2	$CHCl_3$
5	OV1	3%	0.5 X 3.0	370	220-350	0.6	370	He	50	2.0	$CHCl_3$
7	SP2100		0.6 x 6.3	375	220-350	5.0	380	N	50	2.0	$CHCl_3$
8	OV1		5.0 x 0.3	380	260-340	20.0	380	He		0.5	$CHCl_3$
10	OV1		2.0 x 0.3		180-380	5.0	385	He	3	1.0	Decane
11	OV1	3%	0.6 x 2.0	350	220-350	4.0	400	N	50	1.0	$CHCl_3$
13	OV1	3%	0.5 x 3.0	320	250-350	4.0	370	He	100	1.0	$CHCl_3$
14	SE30		x 0.3	350	240-340	10.0	350	H	6	2.0	Octane
15	OV1		4.0 x 0.3	375	200-350	5.0	375	He	7	1.0	$CHCl_3$
17	OV1	3%	0.6 x 3.0	370	225-355	4.0	370	N	55	1.0	$CHCl_3$
18	OV1	3%	0.6 x 3.0	380	220-350	2.5	380	He	45	1.0	$CHCl_3$
19	OV1	3%	0.6 x 3.0	375	220-350	5.0	375	He	50	2.0	$CHCl_3$

3) The determination of triglyceride groups representing 5% or less of the total triglycerides present in a fat or oil requires particular attention to the accuracy of peak area integration for the triglyceride groups containing 56 or more carbon atoms.

4) The removal of mono- and di-glycerides prior to analysis is essential for the accurate analysis of lipids such as coconut oil and palm kernel oil which contain a wide range of triglyceride groups. It was found that this removal can be best achieved by column chromatography using method 2.321 (ref. 3).

5) Response factors were determined for each triglyceride group by the gas-liquid chromatography of solutions of high purity triglycerides under the same glc conditions as adopted for the analysis of the samples. Varying or relatively high values for the response factors may indicate inadequate conditioning of the column. For satisfactory quantitative results to be achieved the response factors should not exceed a ca. 1.1, although for triglyceride groups containing 54 or more carbon atoms it may not be possible to obtain response factors which do not exceed 1.1.

6) The column must be properly conditioned before any quantitative analyses are attempted. On the basis of the experience gained in the collaborative study it is recommended that the column should be conditioned for not less than 24 hours at a maximum temperature close to, but not exceeding, 350°C. Details of the conditioning procedures adopted by eight of the laboratories which participated in the 2nd collaborative study are given in Table 4.

7) 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.

TABLE 4. CONDITIONING OF GAS-LIQUID CHROMATOGRAPHY COLUMNS

Lab.	phase	loading %	temperature °C	time hours	carrier gas
DK	SE 30		300	36	He
UK	Dexsil	1	450	24	He
FRG	SE 30	3	50 - 250 @ 1°/m	5	N
			250 - 320 @ 1°/m	24	
B	SP 2100	3	50	30 min	N
			50 - 355 @ 1°/m	24	
N	OV 1	3	350	48	He
F	SE 30	cap	340	3	H
J	OV 1	3	350	36	He
SA	SP 2100	3	40 - 370 @ 5°/m	24	He

Note: For reasons of space it has not been possible to include data provided by all the participating laboratories although their data have been taken into consideration.

2.323 DETERMINATION OF TRIGLYCERIDES BY GAS-LIQUID CHROMATOGRAPHY

1. SCOPE

This Standard describes a method for the determination of the content of triglycerides having the same carbon number (Note 1).

2. FIELD OF APPLICATION

This Standard is applicable to animal and vegetable oils and fats.

3. DEFINITION

The content of a group of triglycerides having the same carbon number is a quantity expressed as a percentage relative to the total triglycerides content of the sample, separated according to the present procedure.

4. PRINCIPLE

Separation of the triglyceride groups having the same carbon number by direct gas-liquid chromatography of a solution of the oil or fat, under temperature programmed conditions. Identification by reference to a standard triglycerides solution. Content determination by peak areas ratio.

5. APPARATUS

5.1 Gas-liquid chromatography with facilities for on-column injection (Note 2), oven temperature programming up to at least 350°C and preferably equipped with an electronic integrator.

5.2 Column, glass, suitable for the chromatograph (5.1) about 0.5 - 0.6 m long, and 2 - 4 mm internal diameter (Note 3), filled with 3% (or less) of a methyl polysiloxane on an acid-washed silanised support (Note 4). The carrier gas flow through the column should be about 50 ml/min (Note 5).

5.3 Microsyringe 2 µl.

6. REAGENTS

6.1 Chloroform, analytical reagent quality. (Note 6)

6.2 Triglycerides, purity 99%, standard solution in chloroform (6.1)
Prepare a standard solution containing about 10 mg/ml of each of tricaprin, tricaprylin, trilaurin, trimyristin, tripalmitin and tristearin according to the type of oil or fat to be analysed.

7. PROCEDURE

7.1 Determination of triglycerides correction factors

By means of a microsyringe (5.3) inject onto the column (5.2) about 1 µl of the triglycerides standard solution (6.2) with the injection and detector temperatures of the chromatograph (5.1) set at about 375°C and an initial oven temperature of about 220°C. Immediately, commence programming the oven tempearature to increase at a rate of about 4 - 5°C/min (but not exceeding 5°C) and continue the analysis until the temperature reaches about 350°C. This temperature should be maintained until all triglycerides have eluted from the column.
Assume that trilaurin is completely recovered from the column and calculate the correction factor, f_i, for each of the remaining triglycerides from:

$$f_i = \frac{C_{si}}{C_L} \times \frac{A_L}{A_{si}}$$

where A_L is the peak area for trilaurin

A_{si} is the peak area for the standard triglyceride i

C_L is the concentration - in mg per ml - of trilaurin

C_{si} is the concentration - in mg per mil - of the standard triglyceride i

Determine f from at least two injections of the standard solution (6.2).
Plot a graph of the average values for f for each triglyceride against the corresponding carbon number (Note 7).

7.2 Identification graph

Plot the values of the retention time for each standard triglyceride peak against the corresponding carbon number. Normally a straight line will be obtained from which the expected retention times for other triglycerides can be determined.

7.3 Preparation of test sample solution

Warm the sample as necessary so that it is completely liquefied, following the procedure described in method 2.001. Homogenise the liquid sample by gently shaking the container. Prepare a 50 mg/ml solution of the sample in chloroform (6.1).
For example, transfer about 1.25 g of the liquid sample to a 25 ml graduated flask using a pipette and dissolve the sample (while still liquid) in a few ml of chloroform (6.1) Make up to the mark with the same solvent. Mix. (Note 8).

8. EXPRESSION OF RESULTS

8.1 Determination of the triglycerides groups composition

Identify each peak using the identification graph (7.2).

8.2 Calculation of the triglycerides groups content

Determine the peak areas of each group of triglycerides. Calculate the corrected peak areas using the correction factors determined either by calculation (7.1) or by interpolation from the graph (7.2) of correction factors obtained for the standard triglycerides. The quantity of each group of triglycerides having the same carbon number expressed as a percentage relative to the total triglycerides content is given by the formula:

$$\frac{A_{TGi}}{A_T} \times 100$$

where A_{TGi} is the corrected peak area of triglycerides group i.

A_T is the total corrected peak area of triglycerides groups contained in the sample. ($A_T = \Sigma A_{TGi}$)

8.3 Repeatability

The difference between the results of two determinations carried out on the same day by the same analyst using the same apparatus for the same test material and for triglycerides present in excess of 10% should not exceed a figure of 1% absolute. For triglycerides present at a level of 10% or less, the difference should not exceed 0.5% absolute (Note 9).

9. NOTES

1. The carbon number is the number of carbon atoms in the acyl chains of the triglycerides. For example, tristearin can be designated as C_{54}.

The procedure described does not allow triglycerides having the same carbon number to be determined individually.

Triglycerides having different degrees of unsaturation but an identical carbon number are not separated from each other. Partial separation of unsaturated triglyceride isomers may be achieved by capillary column gas-liquid chromatography. (See note 3)

2. An all-glass system should be used whenever possible.

3. Equivalent results to that using a packed column may be obtained by use of a short capillary column i.e. 6 m or less.

4. OV-1 is suitable.

5. Helium is recommended as the carrier gas but nitrogen may be used. However, some loss in resolution of the peaks may be experienced with nitrogen. The column should be conditioned prior to use by heating it at about 350°C for at least 36 hours with a carrier gas flow-rate of about 5 ml/min.

6. If preferred, di-isopropyl ether may be used in place of chloroform.

7. Correction factors greater than 1.1 should be considered unsatisfactory. A reduction in the stationary phase loading or an increase in the carrier gas flow-rate may achieve acceptable correction factors.

8. If the sample is known to contain significant amounts of mono- or diglycerides or free fatty acids, these should be removed according to method 2.321 before proceeding with the analysis (7.4).

9. Triglycerides present at a level of 5% or less are determined less accurately.

REFERENCES

1. B. K. Tan and C.H. OH Flingoh, **Chemical and Physical Characteristics of Palm Oil** - PORIM technology No 3, May 1983

2. CAOBISCO document 7301/262, September 1984

3. **Standard Methods for the Analysis of Oils, Fats and Derivatives** 6th Edition (Pergamon Press, 1979)

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