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DETERMINATION OF *n*-3 AND *n*-6 UNSATURATED FATTY ACIDS IN VEGETABLE OILS AND FATS BY CAPILLARY GAS LIQUID CHROMATOGRAPHY

Results of a collaborative study and the standardised method

Prepared for publication by

J. L. BEARE-ROGERS[1] and A. DIEFFENBACHER[2]

[1]Food Directorate, Banting Research Centre, Ottawa, Ontario, K1A 0L2, Canada

[2]Nestec Ltd., Central Quality Assurance Laboratory, Avenue Nestlé 55, CH-1800 Vevey, Switzerland

*Membership of the Commission during the period of the collaborative study (1981–83) was as follows:

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†Until 1982 ‡Deceased 1982

Determination of n-3 and n-6 unsaturated fatty acids in vegetable oils and fats by capillary gas liquid chromatography: Results of a collaborative study and standardised method

Abstract - The development, through collaborative study, of a standardised method for the determination of the content of (n-3) and (n-6) unsaturated fatty acids in vegetable oils and fats is described. The procedure involves the preparation of methyl esters of fatty acids and then the analysis of the fatty acid methyl esters by gas-liquid chromatography on capillary columns. Identification of the unsaturated fatty acids has to be carried out using available standards or (gas chromatography—mass spectrometry (GC-MS) methods.

INTRODUCTION

The (n-3) and (n-6) unsaturated fatty acids† have important biological effects and are essential fatty acids (1). Nutritionists are interested in knowing the content of these fatty acids in different foods.

CODEX ALIMENTARIUS has a proposal for the declaration of polyunsaturated fatty acids in edible oils and fats. The existing method for gas liquid chromatography in packed columns proved to be inadequate. To obtain a better resolution of the fatty acids than can be achieved using a packed column, capillary column chromatography was chosen.

The fatty acid methyl esters were prepared according to method 2.301 (2).

PRELIMINARY COLLABORATIVE STUDY AND RESULTS (1ST STUDY)

In a preliminary study organised in 1983/84, (n-3) and (n-6) unsaturated fatty acids were determined in three single samples : A, lightly hydrogenated soybean oil; B, a blend of soybean and evening primrose oil; C, a blend of linseed and evening primrose oil.

Participants used their equipment under usual operating conditions and reported values for linoleic acid and total (n-3) and (n-6) fatty acids.

In the statistical evaluation of the results, importance was given especially to the reproducibility of results - see Table 1. As this was found to be acceptable, it was decided to organise a more comprehensive study.

† n-3 and n-6 designate the position of the double bond at the 3rd and 6th carbon atoms respectively, counted from the end of the chain of n carbon atoms(3).

TABLE 1. Statistical results of the 1st collaborative study (1983/84) (results expressed in g / 100 g)

	(n-3) unsaturated fatty acids			(n-6) unsaturated fatty acids		
Sample	A	B	C	A	B	C
Number of laboratories	17	17	17	17	17	17
Number of accepted results	12	12	12	12	12	12
Mean	2.9	3.3	21.5	29.7	65.4	51.7
Reproducibility standard deviation	0.66	0.18	1.02	0.71	1.13	1.11
Reproducibility coefficient of variation (%)	23.0	5.5	4.7	2.4	1.7	2.1
Reproducibility (R)	1.9	2.3	2.9	2.0	4.8	5.9

2ND COLLABORATIVE STUDY AND RESULTS

For the 1984-85 study, four samples of vegetable oils, including one blind pair, were distributed. Sample A was a blend of linseed oil and soybean oil; sample B was low-erucic rapeseed oil; sample C was identical to sample A of the previous year and had been stored at - 70 °C for one year. Each sample was required to be analysed in duplicate.

The procedure proposed for GC analysis was isothermic at 180 °C, but some participants also used temperature programming. Conditions used by participants are collated in Table 2. The statistical evaluation of the collaborative study (according to ISO 5725) is given in Table 3.

TABLE 2. Capillary GC : operating conditions of the 2nd collaborative study 1984 / 85

Lab.	Column type	column dimensions		operating conditions	
		length m	I.D.† mm	°C	carrier gas
1	Scientific Glass BP 20	25	0.33	210	He
2	Supelcowax 10	30	0.32	172	H_2
3	Durabond 225	30	0.15	180	H_2
4	CP Sil 88	50	0.31	180	He
5	CP Sil 88	50	0.22	195	He
6	FFAP	53	0.2	210	He
7	Carbowax 20M	40		180	H_2
8	Carbowax 20M	57		190	He
9	Grindel 300	40		180	N_2
10	DBS	30	0.35	150-190	He
11	Carbowax 20M	20	0.30	175	He
12	CP Sil 88	50	0.28	180-210	He
13	Silar 5 CP	50	0.22	180	N_2
14	FFAP	50	0.25	208	He
15	Reoplex 400 WCOT	50		175	
16	Silar 5 CP	50		180	H_2
17	Supelcowax 10	30	0.25	200	He
18	CP Sil 88	50	0.32	80-220	H_2

† I.D. = internal diameter

TABLE 3. Statistical results of the 2nd collaborative study (1984/85)

	(n-3) unsaturated fatty acids				(n-6) unsaturated fatty acids			
Sample	A	B	C	D	A	B	C	D
Number of laboratories	18	18	18	18	18	18	18	18
Number of accepted results	16	16	16	15	16	16	17	16
Mean	24.9	8.1	2.7	8.3	35.0	22.4	29.7	22.4
Repeatability standard deviation	0.32	0.13	0.09	0.10	0.9	0.35	0.61	0.21
Repeatability coefficient of variation (%)	1.3	1.6	3.2	1.2	1.1	1.6	2.1	0.9
Repeatability (r)	0.91	0.37	0.25	0.28	1.10	0.99	1.75	0.59
Reproducibility standard deviation	1.33	1.16	0.50	1.67	0.97	0.96	2.06	1.01
Reproducibility coefficient of variation (%)	5.3	14.4	18.1	20.1	2.8	4.3	6.9	4.5
Reproducibility (R)	3.76	3.28	1.42	4.73	2.75	2.72	5.83	2.86

The conclusions drawn from the results in Table 3 were that for (n-3) unsaturated fatty acids, the reproducibility coefficient of variation was rather high, but overall the results were satisfactory. It was therefore decided to carry out another study to cover also the determination of polyunsaturated fatty acids in partially hydrogenated vegetable oils.

3RD COLLABORATIVE STUDY

Four vegetable oil samples with blind duplicates were distributed, together with a revised draft of the chromatographic method using a capillary column. Sample A was hydrogenated low-erucic rapeseed oil of a IV of 79, sample B was hydrogenated soybean oil of a IV 121, sample C was hydrogenated low-erucic rapeseed oil of a IV 70 and sample D hydrogenated soybean oil of a IV 95.

Results of the participating laboratories are tabulated in Table 4. The results for the sum (n-6) unsaturated fatty acids are given. The statistical evaluation of (n-3) and (n-6) fatty acids is shown in Table in the appendix of the method.

In the statistical evaluation of results we have omitted the data for (n-3) unsaturated fatty acids in samples A and C. Due to the low amounts of these acids, repeatability and reproducibility were close to the detection limits of the apparatus available. The main problems encountered with partially hydrogenated oil samples originated with the difficulty of separating octadecadienoic isomers. This was highlighted by the fact that laboratory repeatability (r) values were good compared with reproducibility values (R). Problems of separation of the isomers are inherent to the working conditions applied, but so are problems of identification of the different trans-unsaturated isomers.

The Commission is of the opinion that the standardized method as described in the following pages is suitable for the determination of (n-3) and (n-6) polyunsaturated fatty acids in vegetable oils and fats.

TABLE 4. Summary of total (n-6) unsaturated fatty acid results of the 3rd collaborative study (1985/86)

Sample	A		B		C		D	
Sample code	1 = 7		2 = 9		3 = 5		6 = 8	
Laboratory								
1	4.1	4.1	43.0	43.9	1.6	1.4	18.1	17.9
2	1.5	1.5	42.9	41.9	0.4[c]	1.6[c]	12.1[c]	15.8[c]
3	1.1	1.6	40.8	37.8	1.2	0.9	12.3	12.2
4	3.4	3.3	44.9	43.0	2.3	2.7	15.1	18.6
5	0.9	1.6	38.8	39.0	1.2	1.1	12.0	12.0
6	2.3	2.3	40.6	40.5	1.8	2.1	12.8	13.2
7	7.3	7.2	44.8	44.9	3.9[d]	3.9[d]	20.7	20.8
8	3.0	2.8	41.9	41.0	1.1	1.4	14.6	13.8
9	1.8	1.8	41.0	39.7	1.0	1.2	12.1	12.1
10	2.8	2.8	41.9	41.8	1.4	1.4	14.0	14.0
11	3.0	3.1	42.2	42.5	1.5	1.5	16.8	16.8
12	4.9	5.2	42.2	42.0	2.8	2.7	17.1	17.2
13	3.3	3.4	43.8	44.2	2.2	2.3	14.7	14.8
14	3.3	3.3	41.3	41.4	2.1	2.1	14.8	14.8
15	3.4	3.4	40.3	40.3	2.2	2.2	14.9	14.8
16	1.1[c]	3.0[c]	39.9	39.8	1.5	1.6	11.1	11.7
17	1.9	1.9	38.9	36.7	1.4	1.4	12.6	12.7
18	1.7	1.8	----	----	1.1	1.1	----	----
19	5.3	5.0	44.3	44.4	1.4	1.5	16.1	17.0
20	1.9[c]	3.1[c]	40.4	41.8	0.9	1.0	14.6	11.7
21	2.2	2.4	41.3	42.4	1.5	1.5	14.2	15.5

[c]Results deleted because of large variance
[d]Results deleted as outliers by the Dixon's test

Table 5. Statistical results of the 3rd collaborative study (1985/86)

	(n-3) fatty acids				(n-6) fatty acids			
Sample	A	B	C	D	A	B	C	D
Number of laboratories	21	21	21	21	21	21	21	21
Number of accepted results	18	18	19	20	19	20	19	20
Mean value	0.04	3.10	0.13	0.25	3.05	41.58	1.61	14.70
Repeatability standard deviation (S_r)	0.0	0.10	0.03	0.11	0.17	0.78	0.12	0.98
Repeatability coefficient of variation (%)	0.0	3.36	25.6	43.6	5.57	1.88	7.45	6.67
Repeatability (r) (2.83 x S_r)	----	0.29	----	0.31	0.48	2.21	0.34	2.77
Reproducibility standard deviation (S_R)	----	0.26	----	0.14	1.53	1.96	0.53	2.48
Reproducibility coefficient of variation (%)	----	8.32	----	55.7	50.16	4.71	32.92	16.87
Reproducibility value (R) (2.83 x S_R)	----	0.73	----	0.39	4.33	5.55	1.50	7.02

DETERMINATION OF n-3 AND n-6 UNSATURATED FATTY ACIDS IN VEGETABLE OILS BY CAPILLARY GAS LIQUID CHROMATOGRAPHY

1. SCOPE

This standard describes a method for the determination of (n-3) and (n-6), polyunsaturated fatty acids in vegetable oils and fats by gas-liquid chromatography.

2. FIELD OF APPLICATION

This standard is applicable to the fatty acid methyl esters obtained from vegetable oils and fats according to 2.301.

3. APPARATUS

The instructions given apply to normal equipment used for gas-liquid chromatography employing capillary columns and flame-ionization detection.

3.1. Gas-liquid chromatography

3.1.1. Injection system

The injection system should be specially designed for use with capillary columns. It should be of the split type or on-column (Note 1).

3.1.2. Oven

The oven should be capable of heating the column to at least 220 °C and of maintaining the desired temperature to within 0.1 °C (Note 2).

3.1.3. Capillary column

3.1.3.1. Column

Fused silica or glass. Length : 25 - 60 m. Internal diameter : 0.20 to 0.35 mm.

3.1.3.2.

Stationary phase of moderate polarity, mainly of the type polyglycol (polyethylene glycol 20.000), polyester (butanediol polysuccinate) or polar polysiloxane (cyanosilicones) e.g. Carbowax, Durabond 225, FFAP, Silar 5 CP, Supelcowax, CP Sil 88.

3.1.3.3.

The coating should be 0.1 to 0.2 µm.

3.1.3.4. Assembly and conditioning of the column

Observe the normal precautions for assembling capillary columns : arrangement of the column in the oven (support), choice and assembly of joints (leak tightness), positioning of the ends of the column in the injector and the detector (reduction of dead spaces). Place the column under a carrier gas flow (e.g. 0.3 bar for 25 m of a column of internal diameter 0.3 mm).

Condition the column by temperature programming of the oven at 3 °C / min. from ambient temperature to a temperature 10 °C below the decomposition limit of the stationary phase. Maintain at this temperature for 1 h until stabilization of the baseline. Return to 180 °C to work under isothermal conditions.

3.2. Syringe: Syringe max. capacity 10 µl graduated in 0.1 µl.

3.3. Recorder: According to the method 2.302. It should be compatible with the apparatus used.

3.4. Integrator or calculator

Specifications of the electronic calculator and integrator according to 2.302.

4. REAGENTS

Carrier gas	:	Helium or hydrogen (Note 3).
Auxiliary gas	:	Hydrogen 99.9 per cent min., free from organic impurities air or oxygen, free from organic impurities.
Reference standards	:	a mixture of methyl esters of (n-3) and (n-6) polyunsaturated fatty acids (Note 4).

5. PROCEDURE

5.1 Test conditions

5.1.1. Selection of optimum operating conditions

The properties of efficiency and permeability of capillary columns mean that the separation between constituents and duration of analysis are largely dependent on the flow rate of the carrier gas in the column. It will be necessary to optimize the operating conditions by acting on this parameter (or more simply on the deadspace of the column), according to whether one wishes to improve the separations or to make a rapid analysis.

5.1.2. Determination of efficiency and resolution

Use correspondingly the formula given in 2.302. Number of theoretical plates calculated should be at least 2000 per meter.

5.2. Test portion

Use the syringe 3.2. to take 0.1 to 2 µl of the solution of 1 to 5 % of methyl esters prepared according to 2.301.

5.3. Analysis

Operate the oven isothermally at a temperature of 180-210 °C or operate by linear heating from 80 °C to 220 °C (Note 5).

Set injector and detector temperatures 30 °C to 50 °C above the column temperature.

5.4. Number of determinations

Carry out two determinations (each consisting of duplicate injections of the prepared test solution) in rapid succession, using a fresh test portion for each determination.

6. EXPRESSION AND CALCULATION OF RESULTS

6.1. Calculation of results

Obtain the area for each peak by electronic integration and express it as per cent of the total fatty acid methyl esters. Use formulas given in 2.302. Identify peaks with available standards and material of known composition (Note 4).

Critical pairs or triplets of fatty acid peaks may be formed depending on the stationary phase (3.1.3.2.) used :

- C 20:0 and C 18:3 (n-6)
- C 22:0 and C 20:3 (n-6)
- C 20:3 (n-3); C 22:1 (n-9) and C 20:4 (n-3) (Note 6)

6.2. Report of results

Report as the final result the mean of the values obtained from two determinations, provided that the requirements for repeatability are met.

7. PRECISION

The results of interlaboratory studies organised at the international level gave the statistical results (evaluated in accordance with ISO 5725-1986) which are summarised in the table in the appendix.

7.1. 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 test sample, lies within the range of the mean values cited in the table in the appendix, the difference between the two values obtained should not be greater than the repeatability value (r) for the level of fatty acids comparable to those cited in the annexed table.

7.2. Reproducibility

When the values for the final result, obtained by operators in different laboratories using different apparatus under different conditions, from the analysis of the same laboratory sample, lie within the range of mean values cited in the table in the appendix, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility (R) for the level of fatty acids comparable to those cited in the annexed table.

8. NOTES

1. On-column injection is preferred as it generally gives a better resolution.

2. Apparatus equipped with a temperature programmer is recommended.

3. Either helium or nitrogen may be suitable as a carrier gas but these may increase helium elution times with respect to hydrogen.

4. Standards of most known (n-3) and (n-6) fatty acids are available from suppliers such as Nuspet : Nu-Check-Prep, Inc. Po Box 172, Elysian Minnesota 56028 US, for Europe : Bast of Copenhagen V, Denmark.

Otherwise oils with specific fatty acids should be used as standards such as blackcurrant seed oil containing C 18:3 (n-6) (gamma-linoleic acid)† and C 18:4 (n-3) (stearidonic acid)‡.

5. Operating the oven by linear heating increases the speed of fatty acid elution but does not improve or reduce the resolution of the gas chromatograms. A suitable heating program is : Start at an oven temperature of 80 °C. Hold it for 2 minutes, then heat up at a rate of 20 °C per minute until 125 °C. Hold it there for one minute, then continue to heat up at a rate of 3 °C per minute until 220 °C. Hold at this temperature for at least 5 minutes until all high carbon number fatty acid methylesters are eluted.

6. The abbreviated symbol C 18:2, respectively C 18:3 stands for the number of carbon atoms (C 18) whereas :2 respectively :3 indicates the number of double bond in the fatty acids chain e.g. :0 means saturated fatty acids.

APPENDIX Table - Statistical results of the 3rd collaborative study

	(n-3) fatty acids				(n-6) fatty acids			
Sample	A	B	C	D	A	B	C	D
Number of laboratories	21	21	21	21	21	21	21	21
Number of accepted results	18	18	19	20	19	20	19	20
Mean value	0.04	3.10	0.13	0.25	3.05	41.58	1.61	14.70
Repeatability standard deviation (S_r)	0.0	0.10	0.03	0.11	0.17	0.78	0.12	0.98
Repeatability coefficient of variation (%)	0.0	3.36	25.6	43.6	5.57	1.88	7.45	6.67
Repeatability (r) (2.83 x S_r)	----	0.29	----	0.31	0.48	2.21	0.34	2.77
Reproducibility standard deviation (S_R)	----	0.26	----	0.14	1.53	1.96	0.53	2.48
Reproducibility coefficient of variation (%)	----	8.32	----	55.7	50.16	4.71	32.92	16.87
Reproducibility value (R) (2.83 x S_R)	----	0.73	----	0.39	4.33	5.55	1.50	7.02

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† systematic name : 6,9,12-octadecatrienoic acid

‡ systematic name : 6,9,12,15-octadecatetraenoic acid