ELSEVIER

Journal of Chromatography A, 823 (1998) 475–482

JOURNAL OF
CHROMATOGRAPHY A

Analysis of human milk triacylglycerols by high-performance liquid chromatography with light-scattering detection

S. Morera Pons*, A.I. Castellote Bargalló, M.C. López Sabater

Unitat de Nutrició i Bromatologia – CERTA, Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII s/n, 08028 Barcelona, Spain

Abstract

A high-performance liquid chromatography (HPLC) method for the separation of human milk triacylglycerols using a C_{18} Spherisorb ODS column and ternary gradient elution with dichloromethane, acetone and acetonitrile is described. The triacylglycerols are detected by light scattering. Several chromatographic conditions were assayed in order to optimize the method: sample solubility, mobile phase, column temperature and the mass detector oven temperature. The linearity, precision and relative response of the method were examined. A total of 34 peaks were separated and quantified based on the percentage peak area in the HPLC chromatogram. Mature human milk analyzed by this method contained six predominant triacylglyceride structures: POO, POL, LOO, POP, OOO and SOP, where P=palmitin, O=olein, L=linolein and S=stearin.
© 1998 Elsevier Science B.V. All rights reserved.

Keywords: Milk; Fats; Food analysis; Triacylglycerols; Lipids

1. Introduction

Human milk is recommended as the main source of food for a healthy infant during the first 4–6 months of life [1]. Better analyses of the composition of human milk are likely to improve our understanding of the infant's nutritional requirements and, thus, feeding practices [2].

The triacylglycerols (TAGs) account for 98% of lipids in human milk [3]. The molecular profile of TAGs is a key to the understanding hydrolysis and absorption of human milk fat. Progress in the knowledge of fat biosynthesis in the human mammary gland can be made from comparison between the random and experimental of triacylglycerol species [4].

While the fatty acids (FAs) of human milk are well documented, only a few studies report the composition of TAGs. Several analytical methods are used for the determination and quantification of TAGs in human milk fat: thin-layer chromatography (TLC) [5–7], reversed-phase high-performance liquid chromatography (RP-HPLC) [8–11], RP-HPLC combined with silver chromatography (Ag-RP-HPLC) [12–15] and tandem mass spectrometry (MS–MS) [16,17]. HPLC has been used extensively in TAG analysis. Although silver HPLC affords high resolving power for TAGs depending on the degree of unsaturation, it is cannot separate TAGs that differ only in the chain length of their constituent FAs. Nevertheless, RP-HPLC can be regarded as providing better separation of individual TAG molecules, because it operates on the principle of both chain length and degree of unsaturation of the FAs [18]. In RP-HPLC elution progresses from the component with the lowest partition number (PN) to

*Corresponding author.

0021-9673/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(98)00584-6

the highest and, within the PN, from the highest degree of unsaturation to the lowest. On the other hand, RP-HPLC offers significant advantages over TLC. Ag-TLC and RP-TLC are techniques not easy to control and the results obtained are mainly qualitative [19].

Various detection methods have been used in the analysis of TAGs separated by HPLC. Evaporative light-scattering detection (ELSD) affords advantages over refractive index detection (RI) and ultraviolet detection (UV), in that no baseline drift occurs and there no limitations on the use of mobile phase solvents. RI is not suitable for analysis of such complex mixtures, as it is applicable only under isocratic conditions. ELSD is stable and sensitive when elution gradients are used, as is required to resolve TAG mixtures as complex as those found in human milk fat. The use of gradient elution is an alternative approach for reducing retention times for higher-molecular-mass saturated TAGs and for improving chromatographic resolution. [20]

In this paper we develop a method for the determination of TAGs in human milk by RP-HPLC with ELSD. This aims to improve the design of new infant formulas to incorporate TAG sources resembling those in human milk structures.

2. Experimental

2.1. Reagents and standards

All chemicals used were of analytical-reagent grade; HPLC-grade acetone (Scharlau, Barcelona, Spain), HPLC-grade acetonitrile (SDS, Peypin, France), HPLC-grade dichloromethane (Merck, Darmstadt, Germany).

Triundecanoin (C33:0) and trinonadecanoin (C57:0) were used as internal standards (99% pure, Sigma, St. Louis, MO, USA). Glyc Kit 32 (99%) tricaproin (CoCoCo), tricaprylin (ClClCl), tricaprin (CaCaCa), trilaurin (LaLaLa), trimyristin (MMM), tripalmitin (PPP), tristearin (SSS), triolein (OOO), trilinolein (LLL), triarachidin (AAA) and tribehenin (BBB) (Larodan, Malmo, Sweden) were used as reference standards and olive oil, soybean oil, palm oil, sunflower oil, and lard oil, purchased from Supelco (Bellefonte, PA, USA) as a reference oils.

A human milk sample was collected by manual expression, heated rapidly to inactivate the lipases [21] and stored at −20°C until analysis.

2.2. Instrumentation

The chromatographic equipment consisted of a Hewlett-Packard (Waldbronn, Germany) Model 1050 pump system, a Rheodyne (Cotati, CA, USA) Model 7125 injector with a 20-μl sample loop, a mass detector (Model 750/14, ACS, Macclesfield, UK), and a HP 3365 series II Chemstation which acquired data from the mass detector.

The analytical column used was a Spherisorb ODS-2 (250×4.6 mm I.D., 5 μm particle size) from Tracer Analitica (Barcelona, Spain).

2.3. Chromatographic conditions and detection

The chromatographic separation was carried out using a linear gradient of acetonitrile–dichloromethane–acetone from (80:15:5, v/v/v) to (10:80:10, v/v/v) in 60 min and after 2 min of isocratic elution with 95% dichloromethane, the initial conditions were reached in 5 min. The flow-rate of the eluent was 1 ml/min and the column temperature was 30°C. The volume of the sample injected was 10 μl. The mass detector oven was at 55°C and the gas flow (from an air compressor) was 10 l/min.

TAGs were identified as described previously [22–24].

TAGs were quantified by normalization assuming that the detector response was the same for all molecules. TAGs were further quantified by grouping them by their PN [22].

2.4. Sample preparation

A lipid extract was obtained according to a modification of the method described by Chen et al. [25].

Twenty-five ml of dichloromethane–methanol (2:1) was added to 1.5 ml of mature human milk contained in a centrifuge tube. The mixture was shaken mechanically for 15 min and centrifuged at 3000 *g* for 8 min. Approximately 8 ml of distilled water was pipetted into a tube and after shaking for a further 15 min the sample was centrifuged (8 min,

3000 *g*). As much of the upper aqueous fraction as possible was removed. The organic layer was washed in a saturated solution of NaCl (Panreac, Barcelona, Spain) and finally mixed (15 min) and centrifuged (8 min, 3000 *g*). The organic fraction was carefully transferred to a separating funnel and filtered through 1PS paper (Whatman, Maidstone, UK) containing anhydrous sodium sulfate (Panreac).

The extract was concentrated by removing solvent in a rotary evaporator and dried under a gentle stream of nitrogen. The residue was stored at −20°C and redissolved in HPLC-grade dichloromethane (5%, w/v) immediately before HPLC analysis. Finally an aliquot of 200 μl was transferred to a conical flask containing 0.5 mg of triundecanoin (C33:0) and trinonadecanoin (57:0) as internal standards (I.S.s).

3. Results and discussion

3.1. Dissolution of sample and standards

Several experiments were carried out in order to optimize the dissolution of sample and standards for HPLC analysis. Good chromatographic resolution of animal fats containing high-molecular-mass saturated TAGs can only be obtained with chlorinated solvents. In addition triundecanoin was used as an internal standard since it is not found naturally in human milk is only soluble in chloroform or dichloromethane. However these solvents are evaporated rapidly which significantly increased the concentration of the analytes. The relative standard deviation (R.S.D.) of the most representative peaks of TAGs and the standards after injecting two aliquots of the same sample dissolved in dichloromethane at different temperatures (4°C, 25°C and 30°C) were investigated. The best results were obtained at 25°C.

3.2. Mobile phase

Various mobile phases have been used in TAG analysis by RP-HPLC with ELSD for the separation of complex mixtures like those found in animal fat [12,20,26–33]. A ternary linear gradient of acetonitrile–dichloromethane–acetone from (80:15:5, v/v/v) to (10:80:10, v/v/v) in 60 min was studied.

Due to the complexity and variability in TAG composition three standards oils were used in addition to human milk in order to obtain the best chromatographic resolution under the conditions assayed. Solutions of 5% (w/v) of soybean oil, palm oil, lard oil and human milk fat were prepared and 10 μl were injected into a HPLC system.

3.3. Optimization of column temperature

In order to determine the effect of temperature, the analyses were performed with the column thermostatted at a range of temperatures, from 25°C to 40°C at 5°C intervals. At higher temperatures solubility of TAGs in the mobile phase was greater and this effect was more marked for the least soluble TAGs, the higher-molecular-mass, saturated TAGs. In contrast increases in column temperature resulted in lowered selectivity [34].

Solutions of 5% (w/v) of palm oil and human milk fat were injected at each the above temperatures. The best resolution for most of the saturated TAGs was obtained at 30°C.

3.4. Effects of detector temperature

The evaporation temperature was found to be the most critical parameter for the detector. It had an especially marked effect on the response of saturated TAGs. A temperature of 54°C is the minimum air temperature at which these compounds could be analyzed [35]. The saturated TAGs retain their uniform droplet structure above this temperature, while below it they crystallize and alter the light scattering characteristics.

Solutions of 5% (w/v) of palm oil and human milk fat were injected to each of the following drift tube temperatures: 55°C, 60°C and 65°C. The optimum detector temperature in this study was found to be 55°C. Above this temperature resolution was poor especially for more saturated TAGs.

3.5. TAG identification

The parameters PN, equivalent carbon number (ECN) and logarithms of selectivities (log α) are used in RP-HPLC to characterize the TAG molecules. The ECN, for each individual TAG can be

calculated as follows [36]: (ECN=CN−a'ND), where CN is the number of carbon atoms, ND is the number of double bonds in the FAs attached to the glycerol, and a' is a constant that depends on the chromatographic system. The partition number (PN=CN−2ND) is defined in the same way as the ECN when a'=2. TAGs with the same PN can be differentiated by ECN or by log α. However the procedure for predicting the TAGs in RP-HPLC peaks for a natural fat based on the ECN is complicated for large numbers of FA constituents. In our study log α is calculated on the basis of the retention time of TAGs relative to LOO, considering like t_0, the peak corresponding to triundecanoin (I.S.1) [22,23].

In order to obtain reliable PNs for peak identification, a homologous series of saturated TAGs were injected (Fig. 1). The chromatographic profile of a human milk sample is shown in Fig. 2 and the identification of the major peaks is reported in Table 1. These results showed are consistent with those reported by Breckenridge et al. [5] and Winter et al. [12].

3.6. TAG quantification

Several authors have established that the ELSD response is linear for a broad range of concentrations [29,37,38]. Nevertheless, some authors have reported that the response of the mass detector to increasing amounts of solute injected is non-linear [27,28,30,33] i.e., the response (y) is proportional to the amount (x) injected raised to a power ($y=bx^a$). The exponent, a, is closely linked to the nebulizer shape (pressure and evaporator conditions in the evaporator) [30]. Results from plots of certain selected TAG peak areas versus amount are shown in Table 2. Calibration curves were determined for two

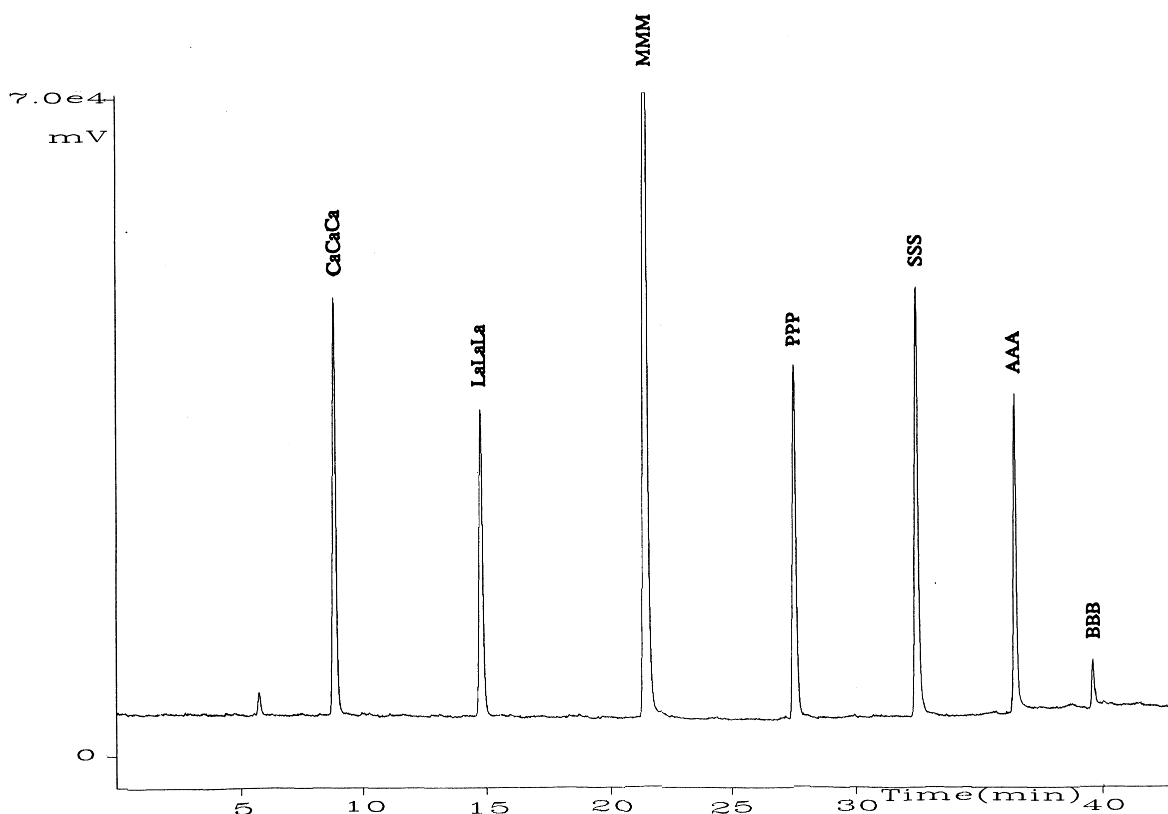

Fig. 1. Chromatogram of mixture of saturated standards. Ca=Caprin; La=laurin; M=myristin; P=palmitin; S=stearin; A=arachidin; B=behenin.

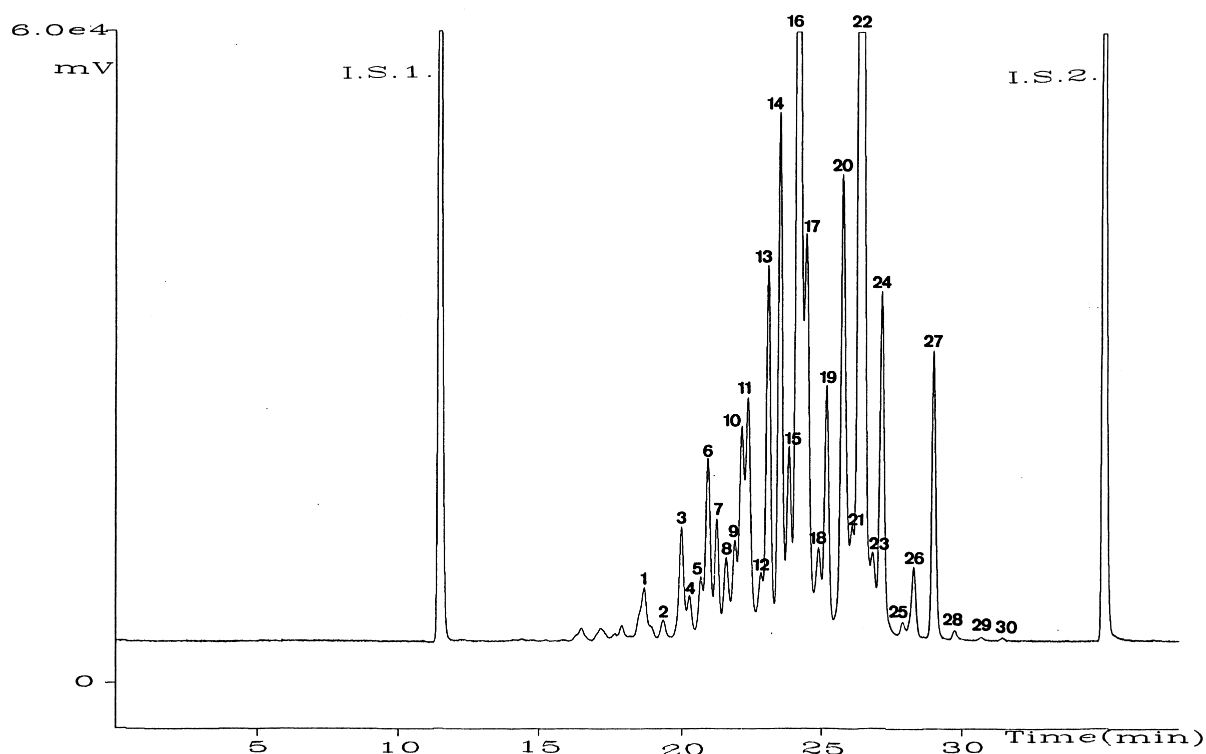

Fig. 2. HPLC profile of human milk triacylglycerols. Peak identification (for abreviations see Table 1): 1=LLL, 2=LnLO, 6=LLO, 8=LnOO, 9=LLP, 10=LnOP, 12=MOL, 14=LOO, 15=SLL/PaOO, 16=POL, 17=PaOP, 18=PPL, 19=MOP, 20=OOO, 21=SLO, 22=POO, 23=SLP, 24=PPO, 25=PPP/PaPS, 26=SOO, 27=SOP, 28=SPP, 29=SOS, 30=SSP.

standard TAGs and for milk samples and a reference oil. Power relationships were detected in all instances with coefficients of determination of 0.999 in the range studied (1–500 μg). Table 2 shows the estimated equations for the log (area) relating (log y) vs. log (amount) injected (log x) for the selected sample TAGs.

Relative response factors (RRFs) calculated for pure homogeneous TAG standards in relation to triolein (OOO) are shown in Table 3. The response factor values close to unity allows quantification on the basis of percentage peak area.

The detection limit (DL) and the quantification limit (QL) were calculated according to the USP criteria [39]. The results obtained for homogeneous standard TAGs were: LD (9.4–11.6) ng and QL (21.2–25.8) ng which show good agreement with the manufacturer's specifications.

The inter-laboratory precision (reproducibility) and intra-laboratory precision (repeatability) were tested by analyzing five replicates of a 5% (w/v) solution of human milk. The R.S.D.s were between (1.71–3.02) for repeatability and between (2.7–4.5) for reproducibility.

A total of 30 peaks (see Fig. 2) were quantified on the basis of the percentage peak area. (Table 4). According to Ruiz Sala et al. [33] the quantitative analysis with an internal standard would not be possible, because the Student t-analysis revealed significant differences between the results obtained for the two I.S.s. This would indicate that TAG response is not dependent on the structure of the component detected, but it is closely related to the large differences in the retention times of the TAGs.

The method proposed is sensitive, rapid and precise, and may be considered suitable for the routine determination of TAGs in animal fats, such as human milk fat by RP-HPLC with ELSD. The complete elution for human milk TAGs was resolved in approximately 35 min. Nevertheless, we advise

Table 1
Identification of the major triacylglycerols in human milk

Peak No.[a]	Log α	Soybean oil	Palm oil	Lard oil	Sunflower oil	Olive oil	PN[b]	CN:ND[b]	Triacylglycerides[c]
1	−0.220	−0.220			−0.219		42	54:6	LLL
2	−0.180				−0.188		42	54:6	LnLO
6	−0.108	−0.098		−0.092	−0.096	−0.090	44	54:5	LLO
8	−0.073	−0.078		−0.079		−0.077	44	54:5	LnOO
9	−0.064		−0.067	−0.064	−0.066	−0.065	44	52:4	LLP
10	−0.050	−0.051		−0.051			44	50:3	LnOP
12	−0.021	−0.021		−0.023			44	52:6	MOL
14	0.000	0.000	0.000	0.000	0.000	0.000	46	54:4	LOO
15	0.014	0.014		0.012	0.014	0.014	46	54:4/52:2	SLL/PaOO
16	0.022	0.023	0.020	0.023	0.024	0.024	46	52:3	POL
17	0.034			0.035		0.036	46	50:2	PaOP
18	0.049	0.046	0.042	0.049	0.048	0.047	46	50:2	PPL
19	0.058			0.060			46	48:1	MOP
20	0.074	0.077	0.074	0.076	0.079	0.074	48	54:3	OOO
21	0.083	0.088			0.089	0.087	48	54:4	SLO
22	0.092	0.097	0.090	0.094	0.098	0.095	48	52:2	POO
23	0.106	0.107	0.102	0.108	0.108		48	52:3	SPL
24	0.115	0.117	0.111	0.118		0.117	48	50:1	PPO
25	0.136				0.132		48	48:0/50:1	PPP/PaPS
26	0.144	0.149	0.146	0.148	0.150	0.148	50	54:2	SOO
27	0.162	0.166	0.162	0.165		0.167	50	52:1	SPO
28	0.180			0.185	0.184	0.182	50	50:0	SPP
29	0.201		0.205	0.208			52	54:1	SOS
30	0.218				0.264		52	52:0	SSP

[a] Peak identification as in Fig. 2.
[b] α=Reduced retention time of TAGs relative to LOO.
Partition number (PN=CN-2ND), where: CN=number of carbon atoms; ND=number of doble bounds.
[c] L=Linolein; Ln=linolenin; O=olein; P=palmitin; M=myristin; S=stearin; Pa=palmitolein.

Table 2
Results from the treatment of experimetal data points of selected TAGs of human milk by linear regression and power curve fitting; estimated equations in the linearity study of ELSD

TAG[a]	Linear regression ($y=ax+b$)			Power curve ($y=bx^a$)			Equation
	a	b	r^2[b]	b	a	r^2[b]	
I.S.1	337 822	−120 583	0.981	502 894	1.79	0.999	Log y=5.70+1.79log x
LOO	743 424	−119 513	0.970	560 184	1.92	0.999	Log y=5.75+1.92log x
PPO	886 301	−130 199	0.975	689 643	1.76	0.999	Log y=5.48+1.76log x
SPO	896 679	−133 618	0.974	692 661	1.76	0.999	Log y=5.84+1.76log x
PPL	479 455	−76 665	0.973	361 778	1.91	0.999	Log y=5.59+1.91log x
SLO	536 645	−85 827	0.973	407 182	1.95	0.999	Log y=5.61+1.95log x
I.S.2	381 284	−132 697	0.976	417 875	1.71	0.999	Log y=5.62+1.71log x

[a] For abreviations see Table 1.
[b] r^2=Coefficient of determination.
I.S.1=Triundecanoin.
I.S.2=Trinonadecanoin.

Table 3
Relative response factors of standard triacylglycerols

Triacylglycerols[a]	RRF[b] (mean±S.D.)	R.S.D. (%)
LLL	1.0938±0.01	1.06
LnLnLn	0.9597±0.01	1.76
MMM	0.9443±0.01	0.81
PPP	1.1702±0.01	1.82

[a] For abreviations see Table 1.
[b] RRF values are given in relation to triolein (OOO) (n=4).

against the use of trinonadecanoin (C57:0) as an internal standard since it increases the time of the analysis. Nonetheless, the analytical method can also be validated using C33:0 as sole internal standard.

Table 4
Composition (%) of the triacylglycerols in human milk

Peak No.[a]	TAGs	Area % (n=4)	S.D.[b]
1	LLL	1.11	0.01
2	LnLO	0.33	0.01
3	NI	1.48	0.02
4	NI	0.54	0.01
5	NI	0.71	0.01
6	LLO	2.32	0.17
7	NI	1.37	0.07
8	LnOO	1.10	0.06
9	LLP	1.13	0.04
10	LnOP	2.59	0.12
11	NI	3.21	0.04
12	MOL	0.77	0.02
13	NI	4.38	0.03
14	LOO	5.88	0.07
15	SLL/PaOO	2.20	0.02
16	POL	15.44	4.40
17	PaOP	5.16	3.21
18	PPL	1.28	0.10
19	MOP	3.08	0.13
20	OOO	6.31	0.03
21	SLO	1.04	0.01
22	POO	28.87	0.72
23	SLP	1.03	0.11
24	PPO	4.31	0.02
25	PPP/PaPS	0.25	0.08
26	SOO	0.98	0.01
27	SOP	2.94	0.03
28	SPP	0.12	0.03
29	SOS	0.04	0.05
30	SSP	0.03	0.03

[a] Peaks numered in Fig. 2 (for abreviations see Table 1).
[b] Standard deviation.
NI=Not identified.

Acknowledgements

The financial support from ‘‘Fundació Mestres Jane’’ is gratefully acknowledged. The authors would like to thank Mr. Robin Rycroft for revising the English of the manuscript.

References

[1] ESPGAN Acta Paediat., Scan. 302 (1982) 1–27.
[2] A. Lewis, Am. J. Clin. Nutr. 46 (1987) 168–170.
[3] R.G. Jensen, R.M. Clark, A.M. Ferris, Lipids 15 (1979) 345–355.
[4] J. Gresti, M. Bugaut, C. Maniongui, J. Bezard, J. Dairy Sci. 76 (1993) 1850–1869.
[5] W.C. Breckenridge, L. Marai, A. Kuksis, Can. J. Biochem. 47 (1969) 761–769.
[6] W.W. Christie, J.L. Clapperton, J. Soc. Dairy Technol. 35 (1982) 22–24.
[7] J.Ch. Martin, P. Bougnoux, J.-M. Antoine, M. Lanson, C. Couet, Lipids 28 (1993) 637–643.
[8] K.D. Dotson, J.P. Jerrell, M.F. Picciano, E.G. Perkins, Lipids 27 (1992) 933–939.
[9] B.G. Lyapkov, T.V. Kiseleva, Vopr. Med. Khim. 38 (1992) 8–9.
[10] B.G. Lyapkov, T.V. Kiseleva, Vopr. Pitan. 5–6 (1992) 59–61.
[11] J.H. Fiebig, M. Arens, Fat Sci. Technol. 98 (1992) 283–285.
[12] C.H. Winter, E.B. Hoving, F.A.J. Muskiet, J. Chromatogr. 616 (1993) 9–24.
[13] L. Brühl, E. Schulte, H.P. Thier, Fat Sci. Technol. 95 (1993) 370–376.
[14] L. Brühl, E. Schulte, H.P. Thier, Fat Sci. Technol. 96 (1994) 147–154.
[15] L. Brühl, E. Schulte, H.P. Thier, Fat Sci. Technol. 96 (1994) 223–227.
[16] J. Currie, H. Kallio, Lipids 28 (1993) 217–222.
[17] H. Kallio, P. Rua, J. Am. Oil Chem. Soc. 71 (1994) 985–992.
[18] V. Ruiz-Gutierrez, L.J.R. Barron, J. Chromatogr. B 671 (1995) 133–168.
[19] R. Tarandjiiska, I. Marekov, B. Nikolova-Damyanovand, B. Amidzhin, J. Liq. Chromatogr. 18 (1995) 859–871.
[20] M.T.G. Hierro, A.I. Najera, G. Santa-Maria, Rev. Esp. Cienc. Tecnol. Aliment. 32 (1992) 635–651.
[21] J. Bitman, D.L. Wood, M. Hamosh, P. Hamosh, N.R. Mentha, Am. J. Clin. Nutr. 38 (1983) 300–312.
[22] J.P. Goiffon, C. Reminiac, D. Furon, Rev. Franç. Corps Gras 28 (1981) 199–206.
[23] M. Parreño, A.I. Castellote, R. Codony, J. Chromatogr. A 655 (1993) 89–94.
[24] J. Parcerisa, M. Rafecas, A.I. Castellote, R. Codony, A. Farràn, J. Garcia, A. Lopez, A. Romero, J. Boatella, Food Chem. 50 (1994) 245–249.

[25] C.-S. Chen, J. Shen, A.J. Sheppard, J. Am. Oil Chem. Soc. 58 (1981) 599–601.
[26] A. Stolyhwo, H. Colin, G. Guiochon, Anal. Chem. 57 (1985) 1342–1345.
[27] B. Herslöf, G. Kindmark, Lipids 20 (1985) 783–790.
[28] J.L. Perrin, A. Prevot, Rev. Franç. Corps Gras 33 (1986) 437–445.
[29] W.W. Christie, Rev. Franç. Corps Gras 38 (1991) 155–160.
[30] M.T.G. Hierro, M.C. Tomas, F. Fernandez-Martin, G. Santa Maria, J. Chromatogr. 607 (1992) 329–338.
[31] J.A. Garcia Regueiro, J. Gibert, I. Diaz, J. Chromatogr. A 667 (1994) 225–233.
[32] K. Aitzetmüller, M. Gröheim, Fat Sci. Technol. 95 (1993) 164–168.
[33] P. Ruiz-Sala, M.T.G. Hierro, I. Martinez-Castro, G. Santa Maria, J. Am. Oil Chem. Soc. 73 (1996) 283–293.
[34] L.J.R. Barron, G. Santa Maria, J.C. Díez Masa, J. Liq. Chromatogr. 10 (1987) 3193–3212.
[35] Application Note PL-EMD 950 Evaporative Mass Detector Technical Note 004: Lipid Analysis, Polymer Labs., UK.
[36] B. Herslöf, O. Podlaha, B. Töregard, J. Am. Oil Chem. Soc. 56 (1979) 864–866.
[37] W.S. Letter, J. Liq. Chromatogr. 15 (1992) 253–266.
[38] H.C. Gérard, R.A. Moreau, W.F. Fett, S.F. Osman, J. Am. Oil Chem. Soc. 69 (1992) 301–304.
[39] The United States Pharmacopeia, (U.S.P. XXIII), Mack Printing, Easton, 1989, p. 1711.