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Direct determination by high-performance liquid chromatography of *sn*-2 monopalmitin after enzymatic lipase hydrolysis☆

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Abstract

An alternative method to determine the *sn*-2 monopalmitin in infant formulas was developed and validated. This method offers many advantages over the traditional methods. It follows the official method in the first steps, purification of the fat or oil through an alumina column, and subsequently the triglycerides are incubated with pancreatic lipase in order to obtain the *sn*-2 monoglycerides. In traditional methods the *sn*-2 monoglycerides are separated by preparative thin-layer chromatography and then, the 2-monoglycerides are converted into the corresponding fatty acid methyl esters and analysed by gas chromatography. In our method, separation, quantification and identification of the *sn*-2 monoglycerides were achieved by high-performance liquid chromatography with evaporative light-scattering detection. The detection limit (0.19 μg), quantification limit (0.38 μg), linearity range ($r=0.999$, range 1–200 μg) and precision (SD=1.10) show the suitability of the proposed method. This method is faster, cheaper and simple and does not consume large quantities of reagents and materials.

Keywords: *sn*-2 Monopalmitin; Pancreatic lipase

1. Introduction

In human milk, and in most infant formulas, more than 98% of milk fat is in the form of triglycerides (TGs), which contain saturated and unsaturated fatty acids esterified to glycerol [1]. The main saturated fatty acid in human milk is palmitic acid ($C_{16:0}$), which represents about 20–25% of the total milk fatty acids [2]. In human milk, palmitic acid is esterified over 60% in the *sn*-2 position of the triglycerides [3,4].

Vegetable oils and butterfat are the major constituents of the infant formula fat. In those fats palmitic acid is predominately esterified at the *sn*-1 and *sn*-3 positions of the triglycerides [5,6]. In this way, although those formulas resemble the fatty acid profile of human milk, the percentage of palmitic acid in *sn*-2 position is lower than human milk. The specific distribution of the fatty acids in the tri-

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glyceride plays a key role in digestion and absorption of lipids. The absorption of fat from infant formulas is reported to be lower than from human milk [6–9].

Palmitic acid in the *sn*-1 and *sn*-3 positions is hydrolysed by pancreatic lipase and the resulting free palmitic acid may form calcium soaps, which are poorly absorbed. In contrast, palmitic acid in the *sn*-2 position is generally not hydrolysed by pancreatic lipase, and the remaining 2-monoacylglycerol is well absorbed, palmitic acid as 2-monoacylglycerol is unavailable to form calcium soaps [6,10].

The official method [11] of determination of *sn*-2 palmitic acid isolate the *sn*-2 monoglycerides by preparative thin-layer chromatography (TLC), and then 2-monoglycerides are converted into the corresponding fatty acid methyl esters (FAMEs). Those are subsequently analysed by gas chromatography (GC). Tao and Kinderlerer [12] developed a method to determine monoglycerides by GC–mass spectrometry (MS), where, as in the official method, there is a previous separation of the monoglycerides by TLC and then monoglycerides are silylised and analysed by GC–MS.

Several analytical methods are used for the determination and quantification of TGs, diglycerides (DGs) and monoglycerides (MGs) in fats by reversed-phase high-performance liquid chromatography (RP-HPLC) [3,13,14], RP-HPLC combined with silver chromatography (Ag–RP-HPLC) [15–18] and tandem mass spectrometry [19,20]. RP-HPLC can be regarded as providing better separation of individual TGs, DGs and MGs with UV detection [21].

Various detection methods have been used in analysis of monoglycerides separated by HPLC. Evaporative light-scattering detection (ELSD) offers advantages over Refractive Index (RI) and UV detection. When ELSD is used, there is no baseline drift and there are 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; moreover ELSD is more stable and sensitive.

The aim of this work is to develop and validate a new method by HPLC–ELSD as alternative to the official method [11] to determine the *sn*-2 monopalmitin in infant formulas.

2. Material and methods

2.1. Reagents

Aluminium oxide neutral (Brockman activity I), analytical-grade tris(hydroxymethyl)aminomethane (Tris), sodium cholate for biochemistry, TLC plates 20×20 cm Silica gel 60 HPLC-grade dichloromethane, HPLC-grade acetic acid and chemicals of analytical-reagent grade: boron trifluoride in methanol (20% w/v), sodium chloride, *n*-hexane and anhydrous sodium sulfate were purchased from Merck (Darmstadt, Germany).

Analytical-reagent grade: calcium chloride, hydrochloric acid 37%, diethyl ether, sodium, dry methanol and formic acid 98% were purchased from Panreac (Barcelona, Spain).

Pancreatic lipase (Type II, crude, from porcine pancreas, EC 3.1.1.3), tricaprin (CaCaCa), trilaurin (LaLaLa), trimyristin (MMM), tripalmitin (PPP), tristearin (SSS), triolein (OOO), trilinolein (LLL) and trilinolenin (LnLnLn) with purity greater than 99% were purchased from Sigma (St. Louis, MO, USA).

HPLC-grade acetone was purchased from Scharlau (Barcelona, Spain), HPLC-grade acetonitrile was purchased from SDS (Peypin, France) and Milli-Q water (Milli-Q PLUS ultra pure water system, Millipore Iberica, Barcelona, Spain).

Identification of FAMEs was made with Supelco 37 Component FAME Mix and Polyunsaturated Fatty Acid (PUFA) No. 3 (Supelco, Bellefonte, PA, USA).

Sodium methylate (0.5%) was prepared by dissolving 5 g of sodium in 1 l of dry methanol.

2.2. Instrumentation

2.2.1. HPLC–ELSD

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 ACS (Macclesfield, UK) Model 750/14, 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), protected by a guard cartridge system (Tracer, C_{18}, 5 μm).

2.2.2. Gas chromatography instrumentation

FAMEs were analysed by using a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard), equipped with a flame ionisation detection (FID) system. Separations of FAMEs were carried out on a fused-silica column (30 m×0.20 mm I.D.) coated with SP-2330 stationary phase [poly(80% cyanopropylphenyl–20% bis-cyanopropyl)siloxane, 0.20 μm film thickness] from Supelco.

The split–splitless injector was used in the split mode with a split ratio of 1:30. The injection volume of the sample was 1 μl. The injector and detector temperatures were kept at 250°C and 270°C, respectively.

The recording of chromatograms was performed with HP-Chemstation for GC Systems (Software G2070AA, version A.04.02). Chromatographic peaks were identified by comparing the retention times with those of known standards.

2.3. Samples

Two different samples were analysed:

Betapol 45 (Loders Crocklaan, Wormerveer, The Netherlands), synthetic triglycerides, which is used as fat source in some infant formulas. Betapol 45, that contains around 45% of total palmitic in the *sn*-2 position, is produced by interesterifying a tripalmitin-rich palm oil fraction with a mixture of other oils by using the *sn*-1, 3 specific lipase from *Rhizomucor miehei* (code SP-392; Novo Industries, Copenhagen, Denmark) [22].

The other sample was a standard mixture of TGs, prepared by dissolving 500 mg of each commercial triglyceride (MMM, PPP, SSS, OOO, LLL) in 20 ml of *n*-hexane, resulting in a final concentration of 25 μg/μl of each triglyceride in the mixture. The percentage of total palmitic acid in the standard mixture was 20% and 33.33% of total palmitic acid was in the *sn*-2 position.

2.4. Sample preparation

We studied and compared two methods for determining the *sn*-2 monopalmitin composition of the two different samples.

2.4.1. Official method

We followed the official method [11], which consisted in a purification of the fat sample through an aluminium oxide column, followed by an hydrolysis of the triglycerides with pancreatic lipase, and subsequent a separation of the *sn*-2 monoglycerides by TLC. Then fatty acids in the *sn*-2 position of the monoglycerides were methylated and analysed by GC.

2.4.1.1. Purification through alumina. We prepared a suspension of 15 g of activated alumina in 50 ml hexane and poured it, while stirring, onto the chromatographic column. We let the alumina settle evenly, and allowed the solvent level to fall to within 1 to 2 mm above the absorbent. Then, carefully, we poured onto the column a solution of 5 g fat sample in 25 ml of *n*-hexane. After that we collected the whole effluent from the column in a round-bottomed flask. Recovered effluent was evaporated to dryness by distillation under vacuum at 30°C in a rotavapor.

Aliquots of 50 mg of fat sample, exactly weighed, were placed in 125×16 mm test tubes capped with PTFE-lined caps. A small magnetic stirring bar was placed into each sample tube.

2.4.1.2. Hydrolysis. Hydrolysis was carried out adding 40 mg of pancreatic lipase and 4 ml of a Tris solution (1 *M*, pH 8.0). Tubes were shaken and after that, 0.4 ml of a calcium chloride solution (22%, w/v) and 2 ml of a calcium cholate solution (0.1%, w/v) were added. The tubes were then placed in a water bath at 40°C for 15 min with magnetic agitation. They were then removed and cooled in a 25°C water bath, and 2 ml of hydrochloric acid (6 *M*) and 1 ml of diethyl ether were added to each one. The tubes were then shaken for 1 min, followed by centrifugation (3000 rpm, 10 min, 20°C). The clear diethyl ether top layer, containing hydrolysis prod-

ucts, was transferred with a micropipette into 125× 16 mm test tubes.

2.4.1.3. Separation of the sn-2 monoglycerides by TLC. The different products of hydrolysis were separated by TLC. The solution was applied on an activated (105°C, 60 min) plate of silica gel (20×20 cm, 0.2 μm thickness), about 1.5 cm from the bottom edge, in a thin, uniform line, as narrow as possible. Standards of free fatty acid and *sn*-2 monoglyceride were also applied as references. The plate was set in the well-saturated developing tank. Migration was carried out with a mixture of *n*-hexane–diethyl ether–formic acid (70:30:1, v/v/v) up to about 1 cm from the top edge of the plate. Then, the plate was dried at room temperature, sprayed with the (2,7)-dichlorofluorescein solution (0.1%, p/v, in ethanol) and visualised under UV light. The band of *sn*-2 monoglycerides was scraped off and placed in 125×16 mm test tubes capped with PTFE-lined caps, where monoglycerides were converted in their FAMEs as follows.

2.4.1.4. Analysis of the sn-2 monoglycerides by GC. A 200-μl volume of *n*-hexane was added to each sample, which were saponified with 1 ml of sodium methylate (0.5%, w/v) in a water bath at 90°C for 15 min. The tubes were removed from the water bath and cooled to 25°C in a water bath. Then 1 ml of boron trifluoride–methanol (20%, w/v) was added. The tubes were placed in the water bath for another 15 min at 90°C. After that, they were cooled as before, and 400 μl of *n*-hexane was added to each tube. The tubes were then shaken for 1 min. A 1-ml volume of a saturated solution of sodium chloride in distilled water was added, which was followed by centrifugation. The clear *n*-hexane top layer, containing the FAMEs, was transferred with a micropipette to a 1.5-ml Eppendorf tube containing a small quantity of anhydrous sodium sulfate. The Eppendorf was stored at −20°C until injection into the gas chromatograph.

Gas chromatography conditions for fatty acids in *sn*-2 position of the monoglycerides: the oven temperature was programmed as follows; initial temperature 130°C for 3 min, an increase rate of 7°C/min up to 180°C and then an increase rate of 6°C/min up to 240°C, and then left to stand for 5 min at 240°C. Helium was used as carrier gas, with a flow-rate of 0.79 ml/min, and nitrogen was used as make-up gas.

In order to determine total fatty acid composition of Betapol 45, three samples of 50 mg were methylated; the method was the same as described above to determine fatty acids in the *sn*-2 position by GC (Section 2.4.1.4). Analysis was performed in triplicate.

Gas chromatography conditions for total fatty acids: the oven temperature was programmed as follows; initial temperature 70°C for 3 min, an increase rate of 7°C/min up to 180°C and then an increase rate of 4°C/min up to 230°C, and then left to stand for 5 min at 230°C. Helium was used as carrier gas, with a flow-rate of 0.91 ml/min, and nitrogen was used as make-up gas.

2.4.2. HPLC–ELSD method

The proposed direct method follows with official method [11] in the first steps. Purification through alumina and hydrolysis were carried out as in GC method, Sections 2.4.1.1 and 2.4.1.2. After that, diethyl ether was evaporated under a nitrogen stream, and the hydrolysis products were dissolved in 3 ml of HPLC-grade acetone. The samples were then filtered through a 0.45-μm filter and stored at −20°C until injection into the HPLC system.

2.4.2.1. Separation conditions for monoglycerides and free fatty acids. The chromatographic separation of *sn*-2 monoglycerides and free fatty acids was carried out using an isocratic elution with acetonitrile–acidified water (99:1, v/v), flow-rate of the eluent was 1 ml/min and the column temperature was 30°C. The volume of sample injected was 5 μl. The mass detector oven was at 55°C and the gas flow (from air compressor) was 10 l/min. Quantification was done according to the calibration graph given in the Results and discussion section.

2.4.2.2. Conditions for verification of total hydrolysis. We used the method proposed by Morera et al. [23]. The chromatographic separation of free fatty acids, mono-, di- and triglycerides 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 injected was 5 μl. The mass detector oven was at 55°C and the gas flow (from air compressor) was 10 l/min.

2.5. Validation

The following parameters were determined: linearity, repeatability, detection and quantification limits. The detection and quantification limits were calculated according to the USP criteria [24]. The intra-laboratory precision (repeatability) was tested by analysing six replicates of the sample. In order to check the linearity of the *sn*-2 monopalmitin response, calibration curve was calculated in the range of 1 to 200 μg; the amounts used were 200, 175, 150, 125, 100, 75, 50, 25, 20, 15, 10, 5, 2.5 and 1 μg.

2.6. Statistical analysis

Six samples of Betapol 45 and six samples of the standard mixture were analysed by each method. All data are mean values±standard deviation. Results were evaluated by Statgraphics Plus for Windows 1.4 (Statistical Graphics, USA). The statistical method included Student's *t*-test for differences between groups.

3. Results and discussion

3.1. Hydrolysis conditions

First of all, we assayed different hydrolysis reaction conditions in order to achieve total hydrolysis of the triglycerides to *sn*-2 monoglycerides and free fatty acids. Fig. 1, chromatogram 1, shows a sample of Betapol 45 without hydrolysis treatment. We made eight assays in triplicate (Table 1), in order to verify whether hydrolysis was carried out completely, the hydrolysis products of Betapol 45 were analysed by HPLC–ELSD using the method developed by Morera et al. [23]. In those assays we found that the official method [11] (assay 1) did not lead to total hydrolysis (Fig. 1, chromatogram 2), while assay 8 produced in all cases total hydrolysis of triglycerides to their *sn*-2 monoglycerides and free fatty acids (Fig. 1, chromatogram 3). This assay was repeated 10 times and the results were satisfactory in all cases. Therefore, we used the conditions of assay 8 instead of those of the official method [11].

3.2. HPLC–ELSD method validation

Using the Morera et al. [23] HPLC–ELSD method conditions, we could prove if there were free fatty acids, tri-, di- and monoglycerides. In the chromatogram (Fig. 1, chromatogram 3) the only peaks that appear correspond to *sn*-2 monoglycerides and free fatty acids. And, since under those chromatographic conditions, free fatty acids and monoglycerides could not be separated, we assayed different mobile phases to improve separation.

We found that better separation conditions were reached when acetonitrile–acidified water (99:1) was used as mobile phase under isocratic conditions. In order to know the elution order and times of the monoglycerides and free fatty acids, we hydrolysed triglycerides of trilaurin, trimyristin, tripalmitin, tristearin, triolein, trilinolein and trilinolenin, because there are few *sn*-2 monoglyceride standards available. Table 2 shows elution time of the monoglycerides and free fatty acids obtained from hydrolysis of those triglycerides. Fig. 2 shows an HPLC–ELSD chromatogram obtained from a sample of Betapol 45 completely hydrolysed. Determination of free fatty acids and *sn*-2 monoglycerides by HPLC–ELSD was carried out in 15 min.

We determined the linearity of *sn*-2 monopalmitin. Several authors have established that the ELSD response is linear for a broad range of concentrations [25–27]. Nevertheless, some authors have reported that the response of the mass detector to increasing amounts of solute injected is non-linear [28–30], i.e., the response (y, expressed as peak area) is proportional to the amount injected (x) raised to a power ($y=bx^a$). The exponent, a, is closely linked to the nebulizer shape (pressure and evaporator conditions in the evaporator) [29]. The result, by power curve fitting, from plot of *sn*-2 monopalmitin peak areas versus amounts was $y=1986.79x^{1.78}$ (x are μg), $r=0.999$, range 1–200 μg. *sn*-2 Monopalmitin detection and quantification limits were calculated according to

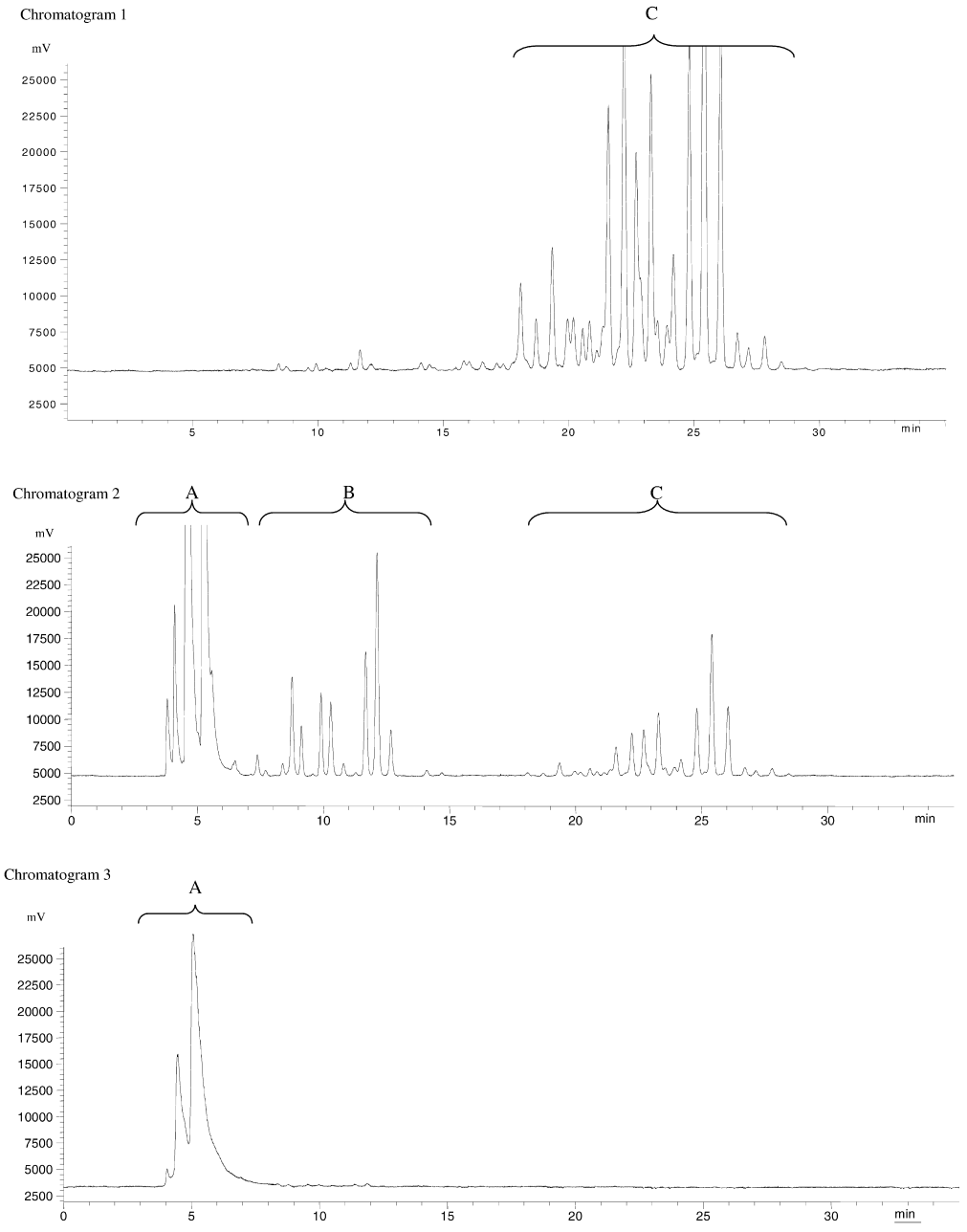

Fig. 1. HPLC–ELSD chromatograms of unhydrolysed, partially hydrolysed and fully hydrolysed Betapol 45. HPLC–ELSD conditions: linear gradient of acetonitrile–dichloromethane–acetone from (80:15:5, v/v/v) to (10:80:10, v/v/v) in 60 min. The flow-rate of the eluent was 1 ml/min and the column temperature was 30°C. The ELSD oven was at 55°C and the gas flow (from air compressor) was 10 l/min. *sn*-2 (A) Monoglycerides and free fatty acids. (B) Diglycerides. (C) Triglycerides. Chromatogram 1: example of HPLC–ELSD chromatogram of unhydrolysed Betapol 45. Chromatogram 2: HPLC–ELSD chromatogram of partially hydrolysed Betapol 45. Hydrolysis conditions: 100 mg Betapol 45, 20 mg lipase, 1 min bath 40°C, 2 min vortex (see Table 1, assay 1). Chromatogram 3: HPLC–ELSD chromatogram of fully hydrolysed Betapol 45. Hydrolysis conditions: 50 mg Betapol 45, 40 mg lipase, 15 min bath 40°C, with magnetic agitation (see Table 1, assay 8).

Table 1
Hydrolysis conditions assayed

Assay	Fat mass (mg)	Lipase mass (mg)	Cholate 0.1% solution (ml)	$CaCl_2$ 22% solution (ml)	Tris[a] (ml)	Hydrolysis conditions	Di- and triglycerides hydrolysis
1	100	20	0.5	0.2	2	1 min bath (40°C), 2 min vortex	Partial
2	100	40	1	0.4	4	1 min bath (40°C), 2 min vortex	Partial
3	100	60	1	0.4	4	1 min bath (40°C), 2 min vortex	Partial
4	50	20	0.5	0.2	2	3 min bath (40°C)	Partial
5	50	40	1	0.4	4	3 min bath (40°C)	Partial
6	50	40	1	0.4	4	5 min bath (40°C), with magnetic agitation	Partial
7	50	40	1	0.4	4	10 min bath (40°C), with magnetic agitation	Partial
8	50	40	1	0.4	4	15 min bath (40°C), with magnetic agitation	Total

[a] Tris(hydroxymethyl)aminomethane (1 *M*, pH 8.0) solution.

the USP criteria [24] and were 0.19 and 0.38 μg, respectively.

We analysed six samples of Betapol 45 and six samples of the standard mixture, in order to prove repeatability. Percentage of *sn*-2 monopalmitin in total *sn*-2 monoglycerides was 30.46±1.10% in Betapol 45 and 19.42±1.51 in the standard mixture.

3.3. Methods comparison

We compared both methods, the percentage of *sn*-2 monopalmitin (*sn*-2 monopalmitin·100/total *sn*-2 monoglycerides) obtained by HPLC–ELSD with the percentage of palmitic acid in the *sn*-2 position (*sn*-2 palmitic acid*100/total *sn*-2 fatty acids) obtained by GC (official method [11]).

Table 2
Elution time of *sn*-2 monoglycerides and free fatty acids

	Retention time (min)
Free fatty acids	
$C_{12:0}$	3.806
$C_{18:3}$ *n*-3	4.654
$C_{18:2}$ *n*-6	5.718
$C_{14:0}$	5.745
$C_{18:1}$ *n*-9	7.391
$C_{16:0}$	8.069
$C_{18:0}$	11.441
sn-2 Monoglycerides	
$C_{12:0}$	3.755
$C_{18:3}$ *n*-3	4.098
$C_{14:0}$	4.892
$C_{18:2}$ *n*-6	5.351
$C_{18:1}$ *n*-9	6.217
$C_{16:0}$	6.638
$C_{18:0}$	9.012

When Betapol 45 was analysed according the official method [11] it was found that 30.60±0.79% of total fatty acids in the *sn*-2 position was palmitic acid, while when it was analysed according our new method, 30.46±1.10% of total *sn*-2 monoglycerides was *sn*-2 monopalmitin. When the standard mixture, was analysed by the official method it was found that the 19.73±0.91% of total fatty acids in the *sn*-2 position was palmitic acid, while according our new method that 19.42±1.51% of total *sn*-2 monoglycerides was *sn*-2 monopalmitin.

Comparing Betapol 45 results no significant differences between both methods ($p<0.05$), $p=0.30$ were observed and neither for standard mixture, $p=0.45$

Total fatty acid composition of the sample was determined by GC. Then, palmitic acid in position *sn*-2 was expressed as percentage of total palmitic acid. The amount of palmitic acid present at the *sn*-2 position as percentage of total palmitic acid (*a*) was calculated as follows: $a=[b/(3c)]\cdot 100$, where *b* is the percentage of *sn*-2 monopalmitin of all *sn*-2 monoglycerides, and *c* is the percentage of total palmitic acid, by GC, in the sample. When Betapol 45 and the sample mixture were analysed, it was found that palmitic acid was 23.41±0.2% (percentage of total palmitic acid in Betapol 45 stated by the manufacturer 23.80%) and 19.82±0.4% (expected value of the standards mixture 20.00%) of total fatty acids, respectively. Consequently, the value of *c* for Betapol 45 was 23.41 and for the standard mixture was 19.82. When we change *b*, for the value found

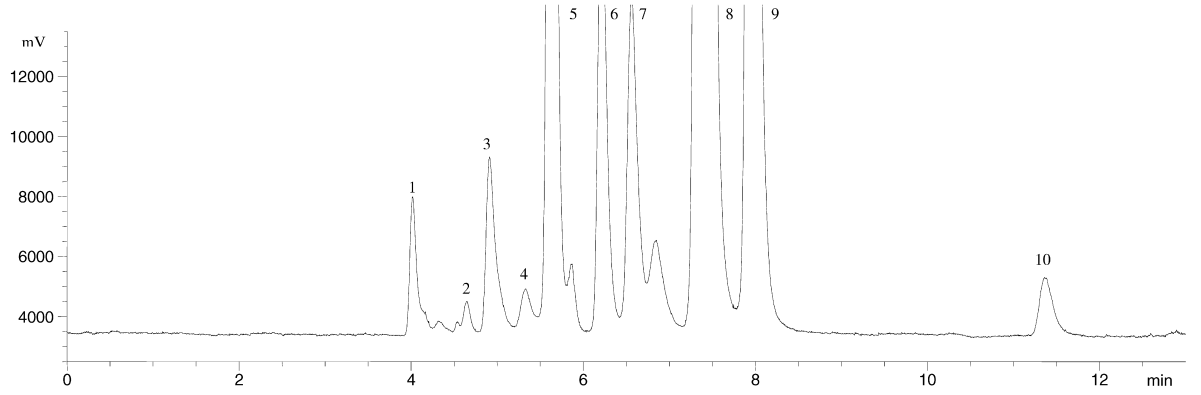

Fig. 2. ELSD–HPLC chromatogram of separated monoglycerides and free fatty acids, after complete hydrolysis of Betapol 45. HPLC–ELSD conditions: isocratic elution with acetonitrile–acidified water (99:1, v/v), 15 min. The flow-rate of the eluent was 1 ml/min and the column temperature was 30°C. The ELSD oven was at 55°C and the gas flow (from air compressor) was 10 l/min. 1=*sn*-2 Monolinolenin, 2=linolenic acid, 3=*sn*-2 monomyristin, 4=*sn*-2 monolinolein, 5=myristic and linoleic acids, 6=*sn*-2 monoolein, 7=*sn*-2 monopalmitin, 8=oleic acid, 9=palmitic acid, 10=stearic acid.

for Betapol 45 by our new method and by the official method (30.60 and 30.46, respectively), we found that 43.20±1.59% and 43.58±1.13% of total palmitic acid was in the *sn*-2 position (manufacturer Betapol 45: around 45%). Acting equally for the standard mixture it was found that according the official method 33.18% of total palmitic acid was in the *sn*-2 position, while according to our new method it was 32.66% (theoretical value of the mixture 33.33%).

Therefore, we concluded that our method is adequate for the determination of palmitic acid in the *sn*-2 position. This method offers a lot of advantages over the official method [11], as it is faster, cheaper, less cumbersome and requires a minimum consumption of reagents and material, because we avoid the TLC, derivatization and GC steps. In this method after hydrolysis we analyse samples by HPLC–ELSD (15 min), whereas in the official method it is necessary to separate the *sn*-2 monoglycerides by TLC (1 h), after that derivatization of the *sn*-2 monoglycerides to its fatty acid methyl esters (45 min) and then, analysis of the samples by GC (25 min). These three steps needs a higher consumption of reagents and material than the HPLC–ELSD step, therefore HPLC–ELSD method is cheaper than the official method.

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