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Evaluation by high-performance liquid chromatography of the hydrolysis of human milk triacylglycerides during storage at low temperatures

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Abstract

The effect of various storage methods on the stability of the triacylglyceride fraction of human milk was evaluated. Samples were treated as follows: Group I – stored at −20°C for 4 months, group II – heated for 1.5 min at 80°C and stored at −20°C for 4 months, group III – stored at −80°C for 4 months and thawed rapidly at room temperature (25°C) just before analysis and group IV – stored at −80°C for 2 months, thawed rapidly at room temperature (25°C), then stored at −80°C for a further 2 months and finally thawed rapidly at 25°C just before analysis. The absence of hydrolysis products in group II and group III indicated that these storage procedures were satisfactory even when samples were rapidly thawed for a short time (group IV). Only storage at −20°C without previous heat treatment led to the hydrolysis of triacylglycerides and the appearance of free fatty acids (group I). On the other hand, the effect that freezing and thawing had over the lipolysis grade was studied. Samples were treated as follows: group V – stored at −20°C for 2 months, thawed slowly at refrigerator temperature (5°C), held at this temperature for one week and stored for a further month at −20°C. Freezing and thawing activated lipolysis and increased the production of free fatty acids, monoacylglycerides and diacylglycerides. Milk samples were analyzed by reversed-phase HPLC with a ternary gradient of acetonitrile–dichloromethane–acetone and an evaporative light-scattering detector. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Recognition of the superiority of breast milk has led to an increase in breast feeding in recent years. The high bioavailability of its nutrients coupled with its immunoprotective factors make human milk unique [1].

Because breast milk is the natural source of fat for the neonate, the structure of its triacylglycerides (TAGs) may be used as a biological reference [2]. Milk TAGs constitute about 50% of dietary energy substrates in breast-fed infants [3]. Nevertheless, the analysis of the composition of human milk has been difficult because of variations during nursing, the effect of diet and other factors due to collection, storage and analysis [4–8]. In addition human milk contains two lipases [9]. One, referred to as bile salt-stimulated lipase (BSSL) or bile salt-dependent lipase is responsible for milk fat degradation in the newborn's intestinal lumen [9,10]. In the breast-fed infant the digestion of milk triacylglycerides is catalyzed by gastric lipase, pancreatic lipase and

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BSSL [8,11]. In contrast to BSSL, only traces of the second milk digestive enzyme in human milk have been reported [9]. Referred to as the serum stimulated lipase (SSL) or lipoprotein lipase, this enzyme has little effect on triacylglyceride molecules.

BSSL does not have positional or fatty-acid specificity and can hydrolyze all three ester bonds of triacylglycerides [3,8,12,13]. This is an important aspect because neither gastric nor pancreatic lipase produces the complete hydrolysis of TAGs [8]. In addition, it has been suggested that BSSL has a role in the use of long chain polyunsaturated fatty acids (LCPUFAs). Pancreatic lipase has been shown to have restricted activity against the ester bond with LCPUFAs in vitro, in contrast BSSL does not discriminate between the fatty acids in TAGs [3].

The BSSL activity does not appear to vary with the period of lactation (colostrum, transitional and mature human milk) [14]. The characteristics of this enzyme are identical in milk from mothers of preterm and full-term infants, and its activity in milk is constant and does not change diurnally or during feeding [8].

The action of BSSL depends on the presence of bile salts. This explains why milk is not hydrolyzed in the mammary gland and why the action of BSSL is restricted to the intestine of the neonate [8,9,15]. However, the appearance of free fatty acids in milk samples stored at −20°C suggests that BSSL loses its dependence upon bile salts during storage at low temperatures and can thus hydrolyze the TAGs in milk. On the other hand, this bile salt dependence appears to be retained during storage at very low temperatures (e.g., −70 to −80°C) [16].

The aim of this study is to evaluate the lipolytic activity in human milk during storage at −20°C and to examine the effect of freezing and thawing on samples stored at this temperature. We also aim to demonstrate the utility of two methods to preserve the structure and composition of human milk TAGs by inactivation of this enzyme by heating or by storage at very low temperature (−80°C). Furthermore, we describe a high-performance liquid chromatography (HPLC) method that allows the direct, simultaneous separation of monoacylglycerides, diacylglycerides, triacylglycerides and free fatty acids in biological samples. Only one method based on mixtures of reference standards has been reported to date [17].

2. Experimental

2.1. Sample collection

Thirty individual mature milk samples were collected by manual expression from six healthy women who delivered after 38–40 weeks of pregnancy. Samples were divided into series of five groups as follows: Group I – ten samples from two women (five of each one), were divided into two aliquots of approximately 5 ml at the moment of collection, one of them was immediatly stored at −20°C for 4 months. Group II – the second aliquots were rapidly heated at 80°C and held at this temperature for 1.5 min to inactivate BSSL. Samples were then stored at −20°C for 4 months. Group III – ten samples from two other women (five of each one) were divided into two aliquots of ca. 5 ml at the moment of collection, one of which was stored at −80°C for 4 months. Before analyzing the samples, they were thawed in a 25°C thermostatized bath and rapidly extracted. Group IV – the second aliquots were immediately stored at −80°C for 2 months. Samples were then thawed for an hour in a 25°C thermostated bath and stored at −80°C for a further 2 months. At the moment of analysis, samples were thawed in the same way and rapidly extracted.

Group V – ten samples from the last two women were immediately stored at −20°C for 2 months. Then samples were placed directly into a refrigerator (slow thawed) and held at 5°C for one week. Finally, samples were stored at −20°C for another month before lipid analysis.

2.2. 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) and HPLC-grade 1,2-dichloroethane (Merck, Darmstadt, Germany).

Triundecanoin (C33:0) (T-5534) was used as reference standard (99% pure, Sigma, St. Louis, MO, USA). Several glicerides, 1,2-dimyristin (32-1402), 1,3-dimyristin (32-1403), 1,3-dipalmitin (32-1603), trimyristin (Gly Kitt 32), from Larodan (Malmö, Sweden) and 2-monopalmitoyl glycerol (M-8385), palmitic acid (P-0500), stearic acid (S-4751), oleic

acid (O-1008) and linoleic acid (L-1376) (Sigma) were used as standards, and olive oil from Supelco (Bellefonte, PA, USA) as a reference oil.

2.3. Instrumentation

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

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

2.4. Chromatographic conditions and detection

The chromatographic separation was carried out using a linear ternary 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 dichloromethane–acetonitrile (95:5, v/v), 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.

Identification of free fatty acids and monoglycerides, diglycerides and triacylglycerides was carried out by comparing with the relative retention times of the standards [18].

2.5. Sample preparation

A lipid extract was obtained according to a modification of the method described by Folch et al. in 1957 [19].

Twenty five ml of dichloromethane–methanol (2:1, v/v) 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 (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) immediatlely before HPLC analysis. Finally, an aliquot of 200 μl was transferred to a conical flask containing 0.5 mg of triundecanoin (C33:0) as a reference standard (R.S.).

3. Results and discusion

Considerable qualitative diferences were found between the five groups of milk samples analyzed in the presence of free fatty acids, monoacylglycerides, diacylglycerides and triacylglycerides.

3.1. Group I and group II

The chromatograms obtained from milk samples stored at −20°C and from milk samples with previous heating to 80°C for 1.5 min are shown in Figs. 1 and 2, respectively. The presence of free fatty acids and monoglycerides in the first group suggests that BSSL remains stable and active at these temperatures. In contrast, the absence of these hydrolysis products in heated samples, indicates that such treatment may preserve the TAG structure of human milk during storage at low temperatures.

3.2. Group III and group IV

All samples stored directly at −80°C maintained the initial TAG profile and neither free fatty acids nor monoacylglycerides or diacylglicerides appear in the chromatograms (Figs. 3 and 4). Hence, in contrast, storage at −20°C, much lower temperatures seem to preserve milk triacylglyceride structure even when rapid freezing and thawing occurs. Our data also suggest that the analysis of samples stored at −80°C may be performed directly without inactivated milk lipases by heat treatments. The method

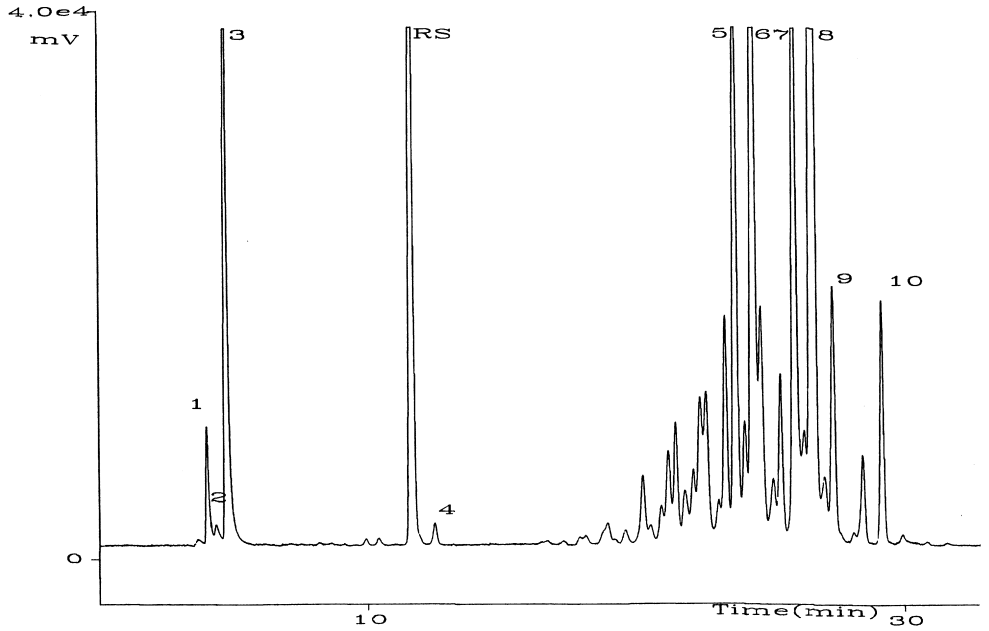

Fig. 1. HPLC of a human milk sample immediately stored at −20°C for 4 months. Peak identifications (for abbreviations see Table 1): (1) linoleic acid, (2) 2-monopalmitoyl glyceride, (3) oleic acid, (4) 1,3-dipalmitoyl diglyceride, (5) LOO, (6) OPL, (7) OOO, (8) OPO, (9) PPO, (10) SPO and (R.S.) reference standard (triundecanoin).

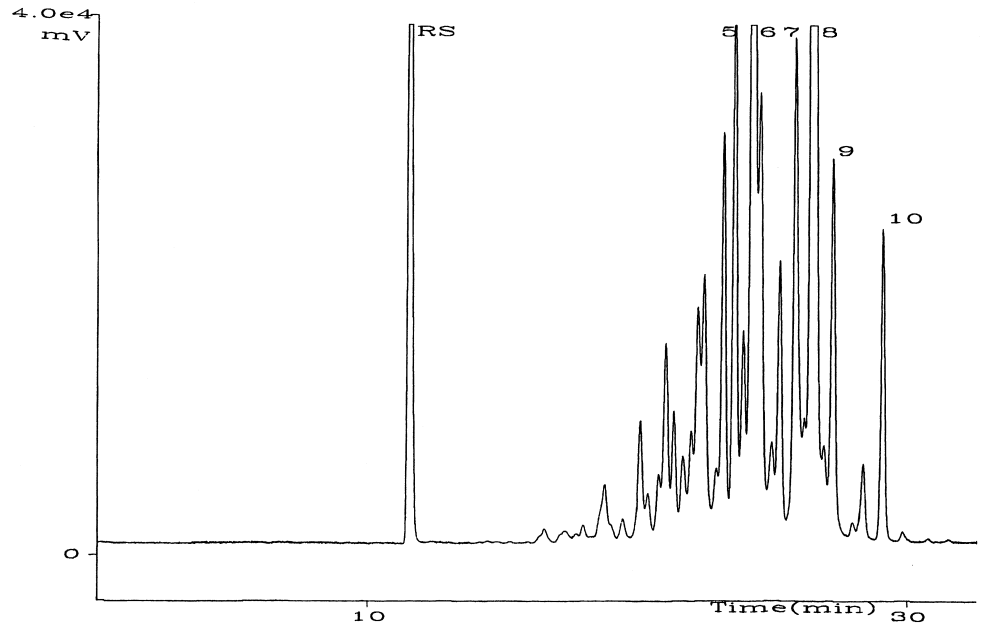

Fig. 2. HPLC of a human milk sample which was rapidly heated at 80°C, held at this temperature for 1.5 min, and then stored at −20°C for 4 months. Peaks identified in Fig. 1.

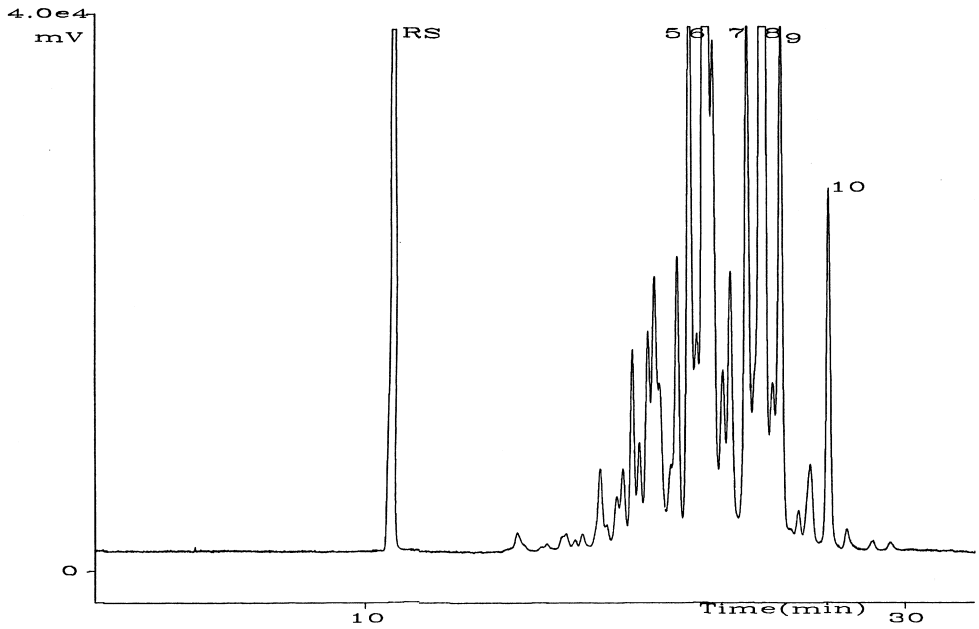

Fig. 3. HPLC of a human milk sample immediately stored at −80°C for 4 months. Peaks identified in Fig. 1.

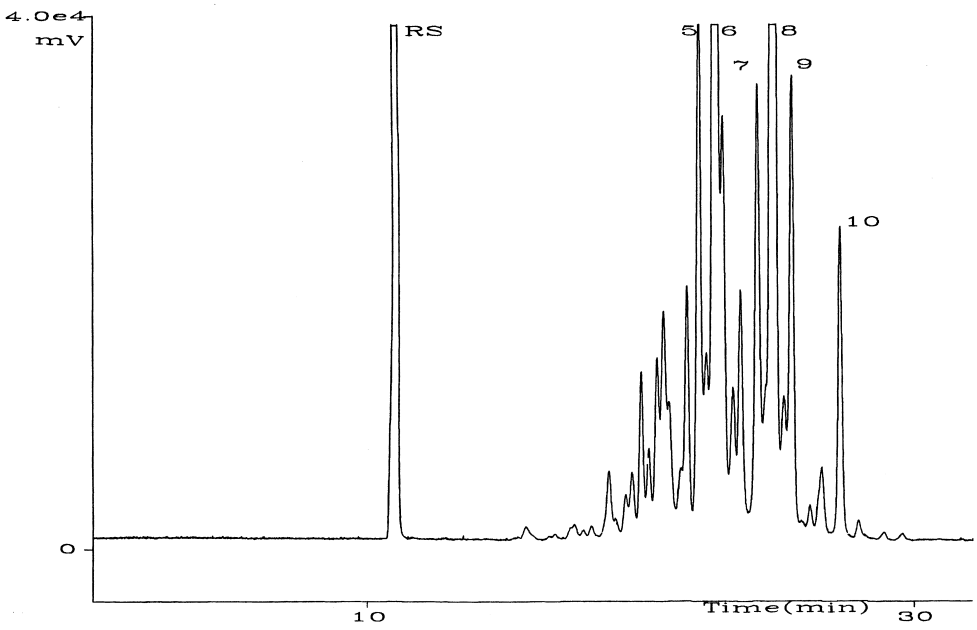

Fig. 4. HPLC of a human milk samle stored at −80°C for 2 months, frozen and thawed once then stored at −80°C for another 2 months, finally thawed rapidly at 25°C just to lipid extraction. Peaks identified in Fig. 1.

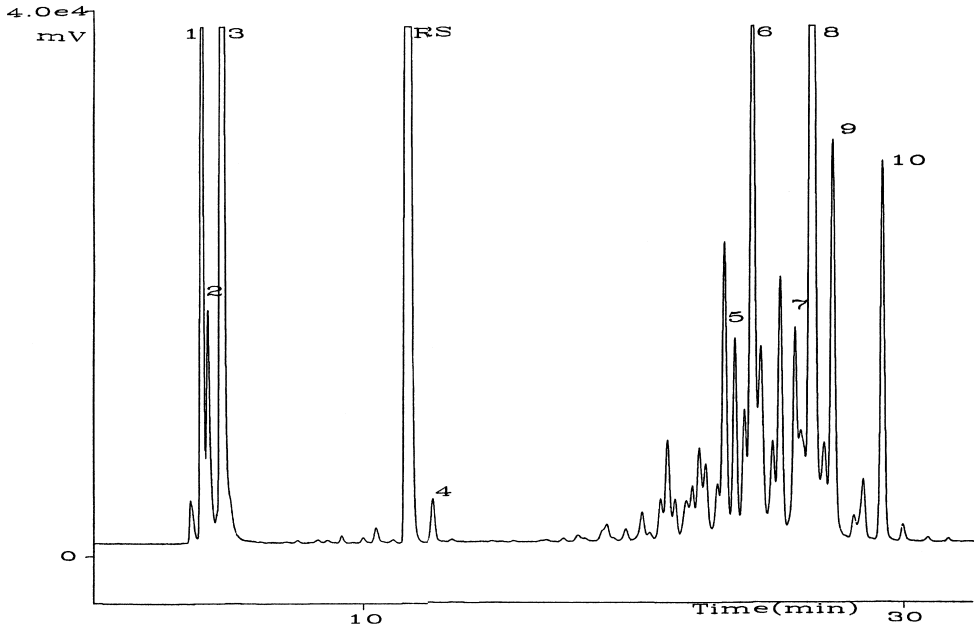

Fig. 5. HPLC of a human milk sample stored at −20°C for 2 months, thawed for a week (5°C) and refrozen prior to lipid extraction. Peaks identified in Fig. 1.

Table 1
Mixture of standard compounds

Standard compound[a]	PN[b]	Retention time (min)	Relative retentionc time[c] (min)
1-Monomyristoyl glyceride	14	3.741	−6.801
Linoleic acid	–	3.994	−6.548
2-Monopalmitoyl glyceride	16	4.018	−6.524
Oleic acid	–	4.753	−5.789
Palmitic acid	–	4.844	−5.698
Stearic acid	–	5.787	−4.755
1,3-Dimyristoyl glyceride	28	7.854	−2.688
1,2-Dimyristoyl glyceride	28	8.311	−2.231
1,3-Dipalmitoyl glyceride	32	11.341	0.799
MMM	42	19.986	9.444
LOO	46	21.627	11.085
OPL	46	22.208	11.666
OOO	48	23.638	13.096
OPO	48	24.293	13.751
POP	48	24.959	14.417
SOO	50	26.092	15.548
SOP	50	26.730	16.188

[a] L=Linolein; M=myristin; O=olein; P=palmitin; S=stearin.
[b] Partition number (PN=CN+2ND), where CN is the number of carbons atoms, ND is the number of double bonds in the fatty acids attached to the glycerol.
[c] Relative retention time to triundecanoin (R.S.).

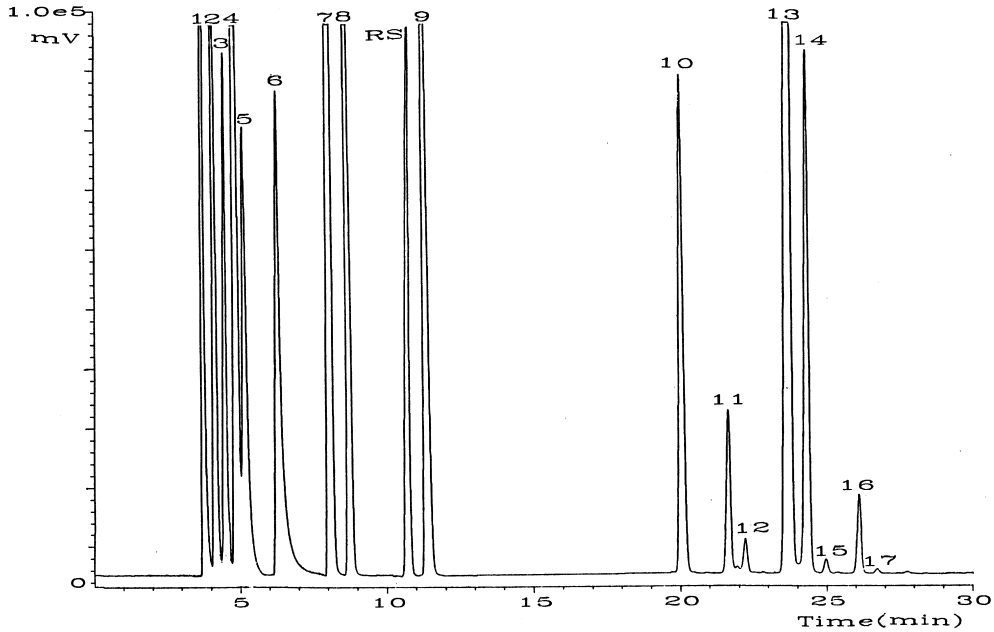

Fig. 6. Chromatogram of standard mixtures. Peak identification (for abbreviations see Table 1): (1) 1-monomyristoyl glyceride, (2) linoleic acid, (3) 2-monopalmitoyl glyceride, (4) oleic acid, (5) palmitic acid, (6) stearic acid, (7) 1,3-dimyristoyl glyceride, (8) 1,2-dimyristoyl glyceride, (9) 1,3-dipalmitoyl glyceride, (10) MMM, (11) LOO, (12) OPL, (13) OOO, (14) OPO, (15) PPO, (16) SOP.

thus offers the same storage stability with much less effort and equipment.

3.3. Group V

The effect of freezing and thawing on the chromatographic TAG profile in milk samples stored at −20°C is represented in Fig. 5. In these samples the monoacylglycerides, diacylglycerides and free-fatty acid fractions showed significant increases. This higher hydrolysis suggests that slow but continuous thawing causes activation of a milk lipase that raises its lipolytic activity.

In order to identify some hydrolysis products a mixture of standards was injected under conditions given in Section 2.3. These compounds are listed in Table 1 together with their retention times and relative retention times to triundecanoin (R.S.). The major identified lipolysis products were: oleic acid (free fatty acid), 2-monopalmitoyl glyceride (monoacylglyceride) and 1,3-dipalmitoyl glyceride (diacylglyceride), which are shown as the standard mixture in Fig. 6. Thus the method allows the simultaneous determination in single run of the various glycerides and also the free fatty acids.

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