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# Determination of phospholipids in dairy products by SPE/HPLC/ELSD

Andrea Avalli, Giovanna Contarini *

*CRA—Istituto Sperimentale Lattiero Caseario, via A. Lombardo 11, 26900 Lodi, Italy*

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**Abstract**

The aim of this work was to evaluate the performance of different methods for both milk lipid extraction and phospholipids separation. As far as the lipid extraction procedure is concerned, the Folch method showed a higher phospholipid recovery with respect to the Rose–Gottlieb method. Different SPE cartridges and solvent phases were tested to carry out the separation of phospholipids from fat. The yield of extraction was evaluated by isolating phospholipids from both milk fat and synthetic fat; Standard Addition Method was applied as well. The isolation of the phospholipids by SPE silica column and subsequent analysis by HPLC/ELSD was shown to be an accurate and reproducible analytical method for the determination of phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine and sphingomyelin in milk fat extracted by Folch method.


*Keywords:* Phospholipids; Milk fat; SPE; HPLC; ELSD

## 1. Introduction

Phospholipids (PLs) are divided into two main groups: glycerolphospholipids and sphingolipids. Glycerolphospholipids are derived from glycerol with a polar headgroup and two fatty acids esterified at the *sn-1* and *sn-2* positions of the glycerol backbone. They include principally phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine [1]. Sphingolipids are derived from sphingosine; sphingomyelin is the dominant species and it is composed of a phosphorylcholine headgroup and a fatty acid linked to the amide nitrogen of the sphingoid long chain base [2].

Recent studies have given considerable evidence that PLs can have a positive nutritional effect on human health, such as reduction of the risk of cardiovascular disease [3–5]. In the food industry, PLs are used as emulsifiers or emulsion stabilisers when they are complexed with proteins [6–9]. Five major classes of phospholipids are found in milk fat, and their approximate percentages are: phosphatidylcholine (PC) 35%, phosphatidylethanolamine (PE) 30%, sphingomyelin (SM) 25%, phosphatidylinositol (PI) 5% and phosphatidylserine (PS) 3% [10–13].

Phospholipids are located on the milk fat globule membrane (MFGM). They have both lipophilic and hydrophilic properties, and therefore, contribute significantly to the emulsification role of the membrane [14,15]. As a result, particular care should be taken during milk fat extraction to recover the whole PL fraction. The analysis of milk PLs involves different steps: fat extraction from milk, isolation of PL fraction from the other lipid classes and separation of the different phospholipid classes. As far as the isolation of PL fraction from the other lipid classes is concerned, thin layer chromatography [16,17], column chromatography [18] and solid phase extraction (SPE) have been applied [19–21]. High performance liquid chromatography with UV or evaporative light scattering detector (ELSD) have been used for the analysis of the different PLs [22–25]. More recently, HPLC/ELSD methods applying on line preconcentration, were developed [26].

The aim of this work was to evaluate the performance of different methods for both the extraction of lipid from milk and the isolation of PLs. Two different fat extraction procedures were tested to evaluate the influence of the solvent polarity on the PL recovery. Moreover, the performances of different SPE cartridges and solvent programs to isolate PLs

* Corresponding author. Fax: +39 037135579.
*E-mail address:* gcontarini@ilclodi.it (G. Contarini).

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from the other lipids were investigated. The quantification of PC, PE, PI, PS and SM was performed by HPLC coupled with ELSD detector.

## 2. Experimental

Cow milk was sampled from the bulk milk tank of the dairy herd of the Institute of Dairy Science of Lodi. Cream, semi-skimmed milk, butter and buttermilk were supplied by a local creamery.

### 2.1. Chemicals and reagents

All the reagents for HPLC analysis were HPLC-grade. Methanol, hexane, diethyl ether and chloroform were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), while the ammonium hydroxide 30% was purchased from Merck (Darmstadt, Germany). Deionized and bidistilled water was used.

The following reference phospholipid standards were supplied by Sigma: L-α phosphatidylethanolamine (PE), L-α phosphatidylcholine (PC), L-α phosphatidyl-L serine (PS), sphingomyelin (SM) from bovine brain and L-α phosphatidylinositol from soybean. Lipid standards for the synthetic milk fat were purchased from Sigma (stigmasterol 96%, 1-mono lauroyl glycerol 99%, triolein 99%, tricaproin 99%) and from Nu-Chek-Prep, Inc. Elysian, MN, USA (1,3-dinonadecanoin 99%).

### 2.2. Fat extraction methods

The methods were tested on 100 ml of milk. Four replicates were performed for each extraction. To assure a complete homogeneization of sample, milk was warmed to about 37 °C with gentle mixing, before extraction.

#### 2.2.1. Rose–Gottlieb modified method (RG)

Milk sample was digested with 15 ml of $NH_3$ (25%, v/v) and mixed with 50 ml of ethanol (96%, v/v). The extraction was performed with a mixture of diethyl–petroleum ether (1:1). The solvent phase was filtered through 30 g of anhydrous $Na_2SO_4$ and evaporated under vacuum. This procedure is based on the IDF method [27].

#### 2.2.2. Folch method

Total lipids were extracted from the samples by homogenisation with chloroform–methanol (2:1, v/v) according to Folch et al. [28]. The extract was shaken and equilibrated with one-fourth its volume of a saline solution (0.05N of NaCl). The solvent phase was filtered and evaporated under vacuum.

### 2.3. Solid phase extraction (SPE)

Lipid sample (400 mg) was dissolved in 1 ml of chloroform–methanol (2:1, v/v). 0.5 ml of the fat solution was applied to different SPE cartridges.

A silica gel bonded column (Supelclean LC-SI, 6-ml volume, 1 g sorbents, Supelco Bellefonte, USA) was used. After conditioning with hexane, the non-polar lipids were eluted with 3 ml of hexane–diethyl-ether (8:2, v/v) and 3 ml of hexane–diethyl-ether (1:1, v/v). The recovery of PLs was performed by using two different conditions: the first with 4 ml of methanol and the second with 2 ml of methanol plus 2 ml of chloroform–methanol–water (3:5:2, v/v/v). The recovered fraction was dried under a gentle stream of nitrogen and it was re-dissolved in 0.2 ml of chloroform–methanol (2:1, v/v) before injecting into HPLC system.

An Octyl (C8) phase bonded column (Accubond, 6 ml volume, 1 g sorbents, Agilent-Technologies Palo Alto, CA, USA) was used. After conditioning with methanol, PLs were recovered according to Caboni et al. [29]. Before injecting into HPLC system, the recovered fraction was treated as described above for silica gel column. The performances of the different SPE columns were tested by using both a synthetic mixture, including the most important costituents of milk fat and milk fat samples spiked with known amounts of PE. The analyses were carried out in duplicate and the results were expressed in mg/g of fat.

### 2.4. Chromatographic system and conditions

HPLC-ELSD analysis was carried out using an HPLC Shimadzu (Kyoto, Japan) instrument equipped with two LC-10 Advp pumps, a SCL-10 Advp gradient system, a DGU-14 Advp module degasser and a Rheodyne manual injector with 10 μl sample loop. The analytical column (250 mm × 4.6 mm I.D., 5 μm) was packed with a silica normal-phase Zorbax Rx-SIL (Agilent-Technologies). The chromatographic separation was carried out using a linear binary gradient according to the following scheme: $t_0$ min: 0%B, $t_{14}$ 100%B, and finally isocratic conditions (100%B) for 9 min. Total chromatographic run time was 40 min per sample, which consisted of a 23 min analysis, 12 min to restore initial conditions and 5 min to re-equilibration. Eluent A consisted of chloroform–methanol–ammonium hydroxide (80:19.5:0.5, v/v/v) and eluent B of chloroform–methanol–ammonium hydroxide–water (60:34:0.5:5.5, v/v/v). The flow rate of the eluent was 1.0 ml/min. A Sedex (S.E.D.E.R.E., Alfortville, France) model 75 ELSD was used; the pressure of nebulizer gas (air) was maintained at 2.2 bar and the drift tube temperature was set at 50 °C.

### 2.5. Calibration

Identification of PLs was carried out by comparison with the retention time of pure standards. Calibration curves for each compound were calculated from the area values ob-

tained by injecting 10 μl of chloroform–methanol (2:1, v/v) serially diluted solutions of PE (18–150 μg), PI (10–75 μg), PC (26–200 μg), SM (12–100 μg) and PS (10–75 μg). Each solution was prepared and injected in duplicate. Two different regression equations were applied: linear ($y = a + bx$) and power ($y = ax^b$) [30–33]. The quadratic equation showed good performances for concentration ranges lower than those applied in this research [29,34]; as a consequence it was not calculated.

## 3. Results and discussion

### 3.1. HPLC/ELSD calibration

A chromatogram of a standard PL mixture is shown in Fig. 1. Phospholipids eluted as well-defined peaks. To obtain a quantitative evaluation of PLs, five calibration curves were calculated by applying the equations of both the linear and the power model to the area and concentration values. Results (Table 1) showed that the peak areas fitted the linear model slightly better and demonstrated that, the ELSD response, in the adopted concentration range, was linear. This result was in accordance with other authors [30,31,35] who observed poor linearity for injected masses lower than 5 μg. Therefore, the linear model was applied. As for the sensitivity of ELSD, PS showed a lower value in comparison with the other PLs.

### 3.2. Isolation of PLs from lipids

In order to investigate the effectiveness of different SPE cartridges on the PL isolation, a synthetic matrix, containing the most important lipid classes of milk fat, was prepared and applied to both SPE phases, silica and C8. The same approximate ratio between the milk fat constituents (triglycerides, diglycerides, monoglycerides, sterols and PLs) was maintained in the composition of this matrix (Table 2). Each constituent was weighed separately and dissolved in chloroform–methanol (2:1, v/v). A suitable aliquot of each solution was mixed with the others and the resulting mixture was dried under a gentle stream of nitrogen. The synthetic matrix was redissolved in a suitable amount of chloroform–methanol (2:1, v/v) to obtain the concentration of 200 mg/0.5 ml and applied to silica gel and C8 SPE cartridges.

Table 1
HPLC/ELSD calibration curves

| PLs | Power model | | Linear model | |
|---|---|---|---|---|
| | Equation | $R^2$ | Equation | $R^2$ |
| PE | $y = 17007x^{1.0473}$ | 0.995 | $y = 231075x - 120310$ | 0.997 |
| PI | $y = 11125x^{1.1634}$ | 0.978 | $y = 266330x - 164621$ | 0.986 |
| PS | $y = 39443x^{1.304}$ | 0.984 | $y = 139110x - 498459$ | 0.988 |
| PC | $y = 21317x^{0.9921}$ | 0.999 | $y = 208833x - 272016$ | 0.999 |
| SM | $y = 12734x^{1.085}$ | 0.999 | $y = 212388x - 896712$ | 0.998 |

Table 2
Percentage composition of the synthetic fat sample

| | | | |
|---|---|---|---|
| Triacylglycerol | Triolein + Tricaproin | | 98% |
| Diacylglycerol | 1,3-Dinonadecanoin | | 0.3% |
| Monoacylglycerol | 1-Mono lauroyl glycerol | | 0.2% |
| Sterol | Cholesterol | | 0.5% |
| Phospholipids | Phosphatidylethanolamine | 31.6% | 1% |
| | Phosphatidylinositol | 5.3% | |
| | Phosphatidylcholine | 36.8% | |
| | Sphingomyelin | 26.3% | |

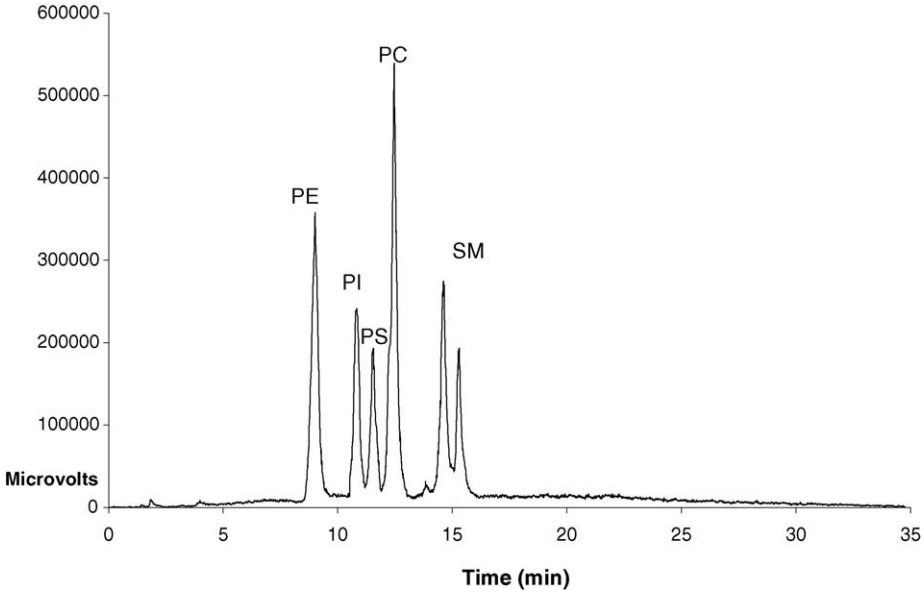


Fig. 1. HPLC/ELSD chromatogram of PL standard mixture (PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin).

Table 3
Recovery of PLs from synthetic fat mixture

| SPE column | PLs yield (%) | Mean (mg) | SD | RSD% |
|---|---|---|---|---|
| Silica A | 61.1 | 1.03 | 7.64 | 12.5 |
| Silica B | 96.0 | 1.91 | 7.49 | 7.8 |
| C8 | 47.5 | 0.83 | 8.60 | 18.1 |

Silica A = PL elution with methanol, Silica B = PL elution with methanol followed by a mixture of chloroform–methanol–water.

Table 4
PL composition of milk fat extracted by Folch method (four replicates)

| | PE | PI | PS | PC | SM | Total |
|---|---|---|---|---|---|---|
| Mean (mg/g fat) | 2.92 | 0.55 | 0.26 | 2.98 | 2.17 | 8.87 |
| SD | 0.16 | 0.01 | 0.02 | 0.21 | 0.11 | 0.39 |
| RSD% | 5.49 | 1.08 | 8.11 | 6.89 | 5.22 | 4.35 |
| Mean (%) | 32.9 | 6.2 | 2.9 | 33.5 | 24.5 | 100 |

Table 3 shows the PL recovery obtained with the two cartridges; silica gel phase was tested by using two different elution step programs as well. The best recovery was obtained by the silica column, eluting the PLs by 2 ml of methanol followed by 2 ml of a mixture of cloroform–methanol–water (3:5:2, v/v/v). The incomplete recovery of PLs from the C8 column was probably due to an interaction between the PL diacylglicerol group and the alkyl chain of the SPE solid phase. In the silica normal-phase cartridge polar lipids are strongly retained by hydrogen bonding and dipolar interactions. The elution of PLs with methanol provided unsatisfactory results, particularly for SM recovery. On the contrary, improved results were obtained by the use of an additional more polar solvent mixture, according to Christie [21]. The performance of this SPE procedure, on the PE evaluation, was also demonstrated by the results obtained from a sample of natural milk fat. Known amounts of PE were added to a milk sample and the Standard Addition Method was applied. The PE values determined in the spiked samples were plotted versus the values for added PE. Calculating the intercept of the regression line allowed the natural PE content of milk fat to be estimated. The value calculated (3.09 mg/g of fat) was included in the range obtained by the PE mean value ± standard deviation (3.11 ± 0.12) found in the unspiked samples. The same procedure was applied to natural milk fat spiked with bovine brain SM as standard and it showed less satisfactory results. The large differences of composition between milk fat and bovine brain SM may be one of the reasons of this result, as previously reported by other authors [26,36].

### 3.3. Evaluation of different fat extraction methods

Compared to other food, milk is a very complex matrix with a high amount of water, and PLs, in this type of matrix, interact with both lipids and proteins. Therefore, particular care should be taken during fat extraction from milk and other dairy products, to recover the whole PL fraction. Two fat extraction procedures with different solvents were compared: Folch (F) and the Rose–Gottlieb (RG) modified procedure. In order to obtain a suitable amount of fat, a proportional increase of the original milk portion and reagents was applied to the RG procedure. Fig. 2 reports the HPLC chromatograms of PLs of milk fat samples extracted by the two procedures. According to Karlsson et al. [36], milk fat SM peak was characterised by three sub-peaks due to the presence of a larger number of molecular species than bovine brain SM used in standard mixture (Fig. 1). As far as the extraction procedure is concerned, it is worth noting that RG was not able to extract PS and PI. The presence of ammonia in the RG reagents, as MFGM dissociating agent, probably increased the water solubility of PS and PI, due to their acidic characteristics. Four replicates of the Folch estraction were performed and the results (mean value and standard deviation) are reported in Table 4. The repeatability (RSD%) of the entire analytical procedure, ranging between 1.1 and 8.1, was comparable with the literature data [20,29]. The PL composition of milk fat, expressed as percentage, were in accordance with the results obtained by other authors [10,18,20].

### 3.4. PL composition of dairy products

The analytical procedure showing the best performance was applied to dairy products having different fat content. The results are reported in Table 5.

The cream samples were obtained by both natural creaming (natural) and centrifugation (centr.). The sample indicated as “milk in vat” was the semi-skimmed milk remaining after the natural creaming and used for cheesemaking of typical Italian hard cheeses, such as Grana Padano and Parmigiano Reggiano.

The PL content of the natural cream was higher than that of cream obtained by centrifugation. This result can be explained by the incorporation, during the natural creaming, of a large number of small fat globules, having a higher ratio between the MFGM and the fat content. As a consequence, a higher concentration of polar lipids, located on the MFGM, can be expected. The two types of cream showed important differences in the percentage composition of PLs as well.

The PL composition of milk in vat was closer to cream obtained by centrifugation than to natural cream.

The churning process, usually applied for the butter production, is responsible for the disruption of MFGM, resulting in an important increase of membranous material in buttermilk. This behaviour was demonstrated by the high PL content of buttermilk and the corresponding low content of but-

Table 5
PL composition of different dairy products

| Sample | Fat (%) | PLs (mg/g fat) | PE | PI | PS (%) | PC | SM |
|---|---|---|---|---|---|---|---|
| Cream (natural) | 22.0 | 8.60 | 42.7 | 6.8 | 7.2 | 14.6 | 28.6 |
| Cream (centr.) | 18.0 | 5.32 | 29.9 | 8.6 | 15.2 | 25.9 | 20.4 |
| Milk in vat | 2.6 | 3.59 | 32.3 | 9.3 | 10.5 | 27.3 | 20.5 |
| Butter | 81.7 | 1.95 | 31.0 | 11.9 | 15.3 | 24.7 | 17.1 |
| Buttermilk | 0.2 | 44.85 | 33.5 | 2.4 | 10.3 | 35.5 | 18.3 |

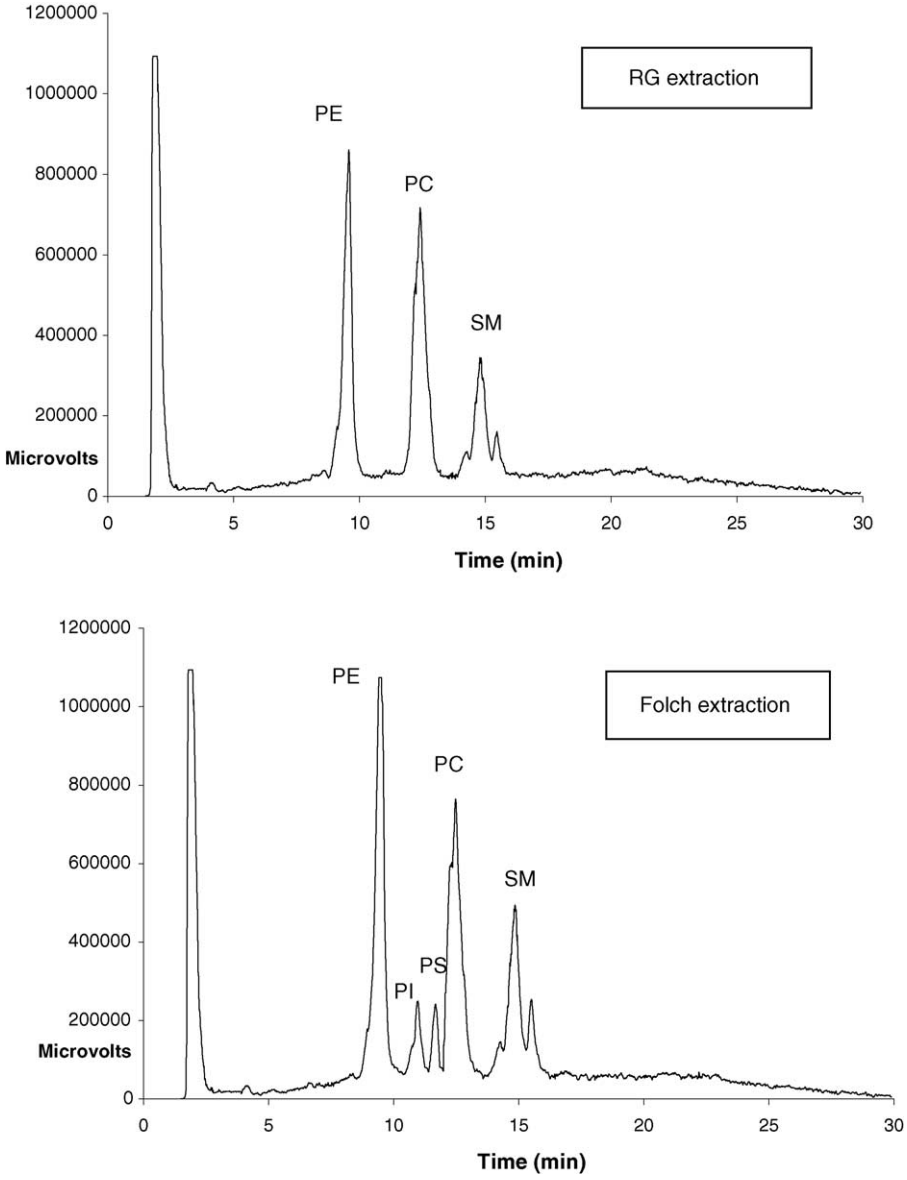


Fig. 2. HPLC/ELSD profiles of milk fat extracted by two different procedures. The peak identities are given in Fig. 1.

ter. Moreover, buttermilk showed a lower PI and a higher PC percentage, in comparison with the PL composition of butter.

## 4. Conclusions

The isolation of the phospholipids by SPE silica column followed by HPLC/ELSD analysis has shown to be a suitable method for the determination of phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine and sphingomyelin in milk fat. Moreover, from this study, Folch has been demonstrated as being the most reliable method to extract PLs from the milk matrix.

The whole analytical procedure, applied to other dairy products, containing different fat percentages, was able to determine quantitative differences of PL composition as affected by the technological processes.

## References

[1] C. Huang, Lipids 36 (10) (2001) 1077.
[2] L. Nyeberg, in: G. Cevc, F. Paltauf (Eds.), Proceedings of the 6th International Colloquium on Phospholipids, Hamburg, Germany, October 25–27, 1993, AOCS Press, Champaign, Illinois, 1995 (Chapter 12).
[3] M. Pfeuffer, J. Schrezenmeir, Bull. IDF 363 (2001) 47.
[4] A.H. Merrill Jr., E.M. Schmelz, M. Cameron Sullards, D.L. Dillehay, Bull. IDF 363 (2001) 27.
[5] E.R. Eckhardt, D.Q. Wang, J.M. Donovan, M.C. Carey, J. Gastroenterol. 122 (4) (2002) 948.
[6] T. Lefèvre, M. Subirade, Int. J. Biol. Macromol. 28 (2000) 59.

[7] A. Kristensen, T. Nylander, M. Paulsson, A. Carlsson, Int. Dairy J. 7 (1997) 87.
[8] O. Korver, H. Meder, J. Dairy Res. 41 (1974) 9.
[9] C.H. McCrae, Int. Dairy J. 9 (1999) 227.
[10] H.C. Deeth, Aust. J. Dairy Technol. 52 (4) (1997) 44.
[11] A.V. McPherson, B.J. Kitchen, J. Dairy Res. 50 (1983) 107.
[12] W.W. Christie, in: P.F. Fox (Ed.), Advanced Dairy Chemistry-2: Lipids, Chapman and Hall, London, 1995, p. 1.
[13] J.C. Hawke, M.W. Taylor, in: P.F. Fox (Ed.), Advanced Dairy Chemistry-2: Lipids, Chapman and Hall, London, 1995, p. 37.
[14] T.W. Keenan, D.P. Dylewski, in: P.F. Fox (Ed.), Advanced Dairy Chemistry-2: Lipids, Chapman and Hall, London, 1995, p. 89.
[15] P. Walstra, J. Dairy Res. 52 (1985) 309.
[16] D.M. Kariotoglou, S.K. Mastronicolis, Lipids 36 (11) (2001) 1255.
[17] W.W. Christie, in: W.W. Christie (Ed.), Lipid Analysis-Isolation, Separation, Identification and Structural analysis of lipids, Oily Press, Dundee (UK), 2003, p. 137.
[18] R.J. Maxwell, D. Mondimore, J. Tobias, J. Dairy Sci. 63 (1986) 3.
[19] V. Ruiz-Gutiérrez, M.C. Pérez-Camino, J. Chromatogr. A 885 (2000) 321.
[20] W.W. Christie, R.C. Noble, G. Davies, J. Soc. Dairy Technol. 40 (1987) 10.
[21] W.W. Christie, in: W.W. Christie (Ed.), Advances in Lipid Methodology-One, Oily Press, Dundee (UK), 1992, p. 1.
[22] M. Careri, A. Mangia, M. Masci, J. Chromatogr. A 794 (1998) 263.
[23] L.C. Boyd, N.C. Drye, A.P. Hansen, J. Dairy Sci. 82 (1999) 2550.
[24] K.C. Arnoldsson, N.U. Olsson, in: G. Cevc, F. Paltauf (Eds.), Proceedings of the 6th International Colloquium on Phospholipids, Hamburg. Germany, October 25–27, 1993, AOCS Press, Champaign, Illinois, 1995 (Chapter 6).
[25] F. Mancini, E. Miniati, L. Montanari, Ital. J. Food Sci. 4 (9) (1997) 323.
[26] P. Fagan, C. Wijesundera, J. Chromatogr. A 1054 (2004) 241.
[27] IDF (1996) Standard, Milk-Determination of Fat Content (reference method) Brussel: International Dairy Federation.
[28] L. Folch, M. Lees, G.H.S. Stanley, J. Biol. Chem. 726 (1957) 497.
[29] M.F. Caboni, S. Menotta, G. Lercker, JAOCS 73 (11) (1996) 1561.
[30] A.M. Descalzo, E.M. Insani, N.A. Pensel, Lipids 9 (2003) 999.
[31] S.L. Melton, JAOCS 69 (8) (1992) 784.
[32] J.W. Dolan, C.S. Young, LCGC North America 21 (2) (2003) 120.
[33] T. Gunnarsson, A. Karrlsson, P. Hansson, G. Johnson, C. Alling, G. Odham, J. Chromatogr. B 705 (1998) 243.
[34] R. Homan, M.K. Anderson, J. Chromatogr. B 708 (1998) 21.
[35] M.N. Vaghela, A. Kilara, JAOCS 72 (6) (1995) 729.
[36] A.L. Karlsson, P. Michélsen, G. Odham, J. Mass Spectrom. 33 (1998) 1192.