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Selective extraction of phospholipids from dairy products by micro-solid phase extraction based on titanium dioxide microcolumns followed by MALDI-TOF-MS analysis

Cosima D. Calvano · Ole N. Jensen · Carlo G. Zambonin

Abstract A new micro-solid phase extraction (μ-SPE) procedure based on titanium dioxide microcolumns was developed for the selective extraction of phospholipids (PLs) from dairy products before matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis. All the extraction steps (loading, washing, and elution) have been optimized using a synthetic mixture of PLs standard and the procedure was subsequently applied to food samples such as milk, chocolate milk and butter. The whole method demonstrated to be simpler than traditional approaches and it appears very promising for a rapid PLs screening and characterization also in biological matrices.

Keywords Phospholipid · TiO$_2$ · MALDI · SPE · Milk

Introduction

Phospholipids (PLs) are divided into two main groups: glycerophospholipids and sphingolipids. Glycerophospho-

lipids include principally phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) [1]. Sphingolipids are derived from sphingosine and, among them, sphingomyelin (SM) is the most representative [2].

There is an increasing attention toward these compounds since, as main cellular membrane components, they are involved in different biological pathways. In the cell membrane, they play a key role to maintain its physical structure and in the various phenomena linked to its functioning, such as active transportation, selective permeability and electrical conductivity. Recent studies provided also significant evidence that PLs can have a positive nutritional effect on human health, such as reduction of the risk of cardiovascular diseases [3]. Phospholipids are also widely used by the food industry as emulsion stabilizers [4], since they have both lipophilic and hydrophilic properties, and therefore, significantly contribute to the emulsification role of the membrane [5, 6].

Dairy products are among the best sources of PLs; for instance, five major classes of phospholipids are found in milk fat: phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyelin [7–10]. Total polar lipids can be extracted from dairy products by means of different procedures; the most used are those proposed by Folch and Bligh-Dyer [11, 12]. Then, PLs must be isolated from total polar lipids and, for a complete characterization a separation of each PL class is also required. The conventional method for the separation of PL classes requires a preliminary separation by thin-layer chromatography (TLC) [13–15] or by normal-phase liquid chromatography (LC) [16, 17], whereas the separation of molecular species within each class may be performed recurring to gas chromatography (GC) [18, 19] or to

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reversed-phase HPLC [20, 21]. The conversion of PL species to GC suitable compounds is laborious and time consuming, and often results in analyte losses. Moreover, LC does not satisfy always to the required resolving power and sensitivity [22]. Mass spectrometry has also been used for the analysis of PLs using several ionization sources such as electron impact [23], chemical ionization [24] and plasma desorption [25]. However, since PLs are non-volatile and thermally unstable species [26, 27], structural information obtained with these techniques is limited. Afterwards, softer ionization techniques such as fast-atom bombardment (FAB) [28, 29], electrospray ionization (ESI) [30–32] and matrix-assisted laser desorption ionization (MALDI) [33–36] were proposed for the analysis of intact PLs.

In view of the above considerations, it is evident that a fast, simple, cost-effective procedure for the extraction of PLs from dairy products is highly advisable. At this aim, a good alternative to existing methods for PLs extraction from dairy products could be represented by the use of homemade titanium dioxide (TiO$_2$) microcolumns, using the selective phosphate groups–titanium interaction. In fact, the selective affinity of a titanium suspension for PLs was used by Ikeguchi and Nakamura [37] to fractionate hydrophobic phospholipids from total lipids prior to LC analysis. Furthermore, it has been recently demonstrated [38] that an efficient and selective phosphopeptide enrichment can be achieved from complex biological samples using miniaturized solid-phase extraction, by using cheap, simple and fast to prepare homemade microcolumns packed with (TiO$_2$) micro particles.

Thus, in the present work, a simple procedure based on homemade TiO$_2$ microcolumns for the selective enrichment of PLs from complex lipids mixtures, followed by MALDI-TOF MS, was successfully developed for the first time. The method has been optimized using lipids standard mixtures and then applied to the analysis of phospholipids in milk, chocolate milk, and butter samples.

Experimental

Chemicals

Chloroform (CHCl$_3$), methanol (MeOH), trifluoroacetic acid (TFA), and ammonium hydroxide were obtained from Sigma (Sigma Aldrich, St. Louis, MO, USA). Triethyl-amine and formic acid were obtained from Supelco. Lipid and phospholipid standards were obtained from Sigma and stored following the relevant guidelines. 2,5-Dihydroxy-benzoic acid (DHB) was from Fluka (Seelze, Germany); 3 M Empore C8 disk was from 3 M Bioanalytical Technologies (St. Paul, MN, USA). Syringes for HPLC loading were from SGE (Victoria, Australia). Water was

obtained from a Milli-Q system (Millipore, Bedford, MA). Titanium dioxide beads were obtained from a disassembled TiO$_2$ cartridge (4.0 mm ID—5020-08520-5u-TiO$_2$) purchased from GL sciences Inc, Japan. All chemicals and reagents were of the highest grade commercially available. Skimmed milk, butter, and chocolate milk were obtained from a local supermarket. All samples were stored at <4 °C before the analysis.

Lipids and phospholipids standard mixture

A standard solution, containing D-α-phosphatidylcholine-dipalmitoyl (DPPC, 733.56 Da), D-α-phosphatidylcholine-distearoyl (DSPC, 789.62 Da), L-α-phosphatidylinositol ammonium salt (PI, 850.54 Da), sphingomyelin mixture of stearic (SM18:0, 730.60 Da), lignoceric (SM24:0, 812.68 Da), and nervonic (SM24:1, 814.69 Da) acids, DL-α-phosphatidyl-L-serine-dipalmitoyl (DPPS, 735.97 Da), L-α-phosphatidylethanolamine-β-oleyl-γ-palmitoyl (POPE, 717.53 Da) and lipids, i.e., 1,3-dipalmitin (PP, 569.51 Da) and tristearin (SSS, 891.84 Da), was prepared in chloroform: methanol (1:1, v/v) at a concentration of 1 mg/ml for each PL and neutral lipid. The solution was mixed (1:1, v/v) with DHB (40 mg/ml in 0.1% TFA in MeOH) and 1 μl of the resulting mixture was deposited on the MALDI target plate and analyzed. DHB was selected as MALDI matrix due to its low yield of fragmentation products [33] in PLs analysis.

Polar lipid extraction procedure

The Folch method was slightly modified [39] and used to extract polar lipids from dairy products. Briefly, 1 ml of a chloroform:methanol (2:1, v/v) solution was added to 100 μl of the sample under study in a glass vial. After shaking for 2 min, the mixture was allowed to stand and a clear separation of two phases was observed. The lower chloroform layer was released and the procedure was repeated twice adding 500 μl of the chloroform:methanol (2:1, v/v) mixture to the upper phase. The fractions were collected and washed with sodium chloride (NaCl) 0.1 N (one-fourth its volume). The lower phase was pooled and evaporated under a nitrogen stream. The dried samples were finally dissolved in 100 μl of the chloroform:methanol solution (1:1, v/v).

### Enrichment of phospholipids using TiO$_2$ microcolumns

TiO$_2$ microcolumns (approximately 3 mm length) were packed in tips. A small plug of C8 material was stamped out of a 3 M Empore™ C8 extraction disk using a HPLC syringe needle and placed at the constricted end of a tip. The C8 disk serves only as a frit to retain the titanium dioxide beads within the tip.

The TiO$_2$ beads were suspended in 80% acetonitrile/0.1% TFA and an aliquot of this suspension (depending on the size of the column) was loaded onto the tip. Gentle air pressure created by a plastic syringe was used to pack the column.

Using as starting point the work of Larsen et al [38], the efficacy of different procedures for selective binding of phospholipids was investigated. In these procedures a standard lipids mixture was used. According to the optimized procedure, 3 µl of lipids were dissolved in 20 µl of DHB (10 mg/ml in 0.1% TFA), vortexed for 1 min and loaded onto the TiO$_2$ columns. The column was washed with 5 µl of chloroform, then the bound lipids were eluted using 3 µl of DHB (40 mg/mL) dissolved in a CHCl$_3$:MeOH (54:46, v/v) mixture with 0.1 % formic acid (FA, titrated to pH=5.3 with

Fig. 1 a MALDI-TOF mass spectrum relevant to standard solution dissolved in CHCl$_3$: MeOH (1:1) and mixed with DHB matrix. MALDI-TOF mass spectra relevant to standard solution after TiO2 micropurification after **b** loading, **c** washing, and **d** elution steps. Standard solution content: D-α-phosphatidylcholine-dipalmitoyl (DPPC, 733.56 Da), D-α-phosphatidylcholine-distearoyl (DSPC, 789.62 Da), L-α-phosphatidylinositol ammonium salt (PI, 850.54 Da), sphingomyelin mixture of stearic (SM18:0, 730.60 Da), lignoceric (SM24:0, 812.68 Da) and nervonic (SM24:1, 814.69 Da) acids, DL-α-phosphatidyl-L-serine-dipalmitoyl (DPPS, 735.97 Da), L-α-phosphatidyl-ethanolamine-β-oleyl-γ-palmitoyl (POPE, 717.53 Da) and lipids, i.e., 1,3-dipalmitin (PP, 569.51 Da) and tristearin (SSS, 891.84 Da)
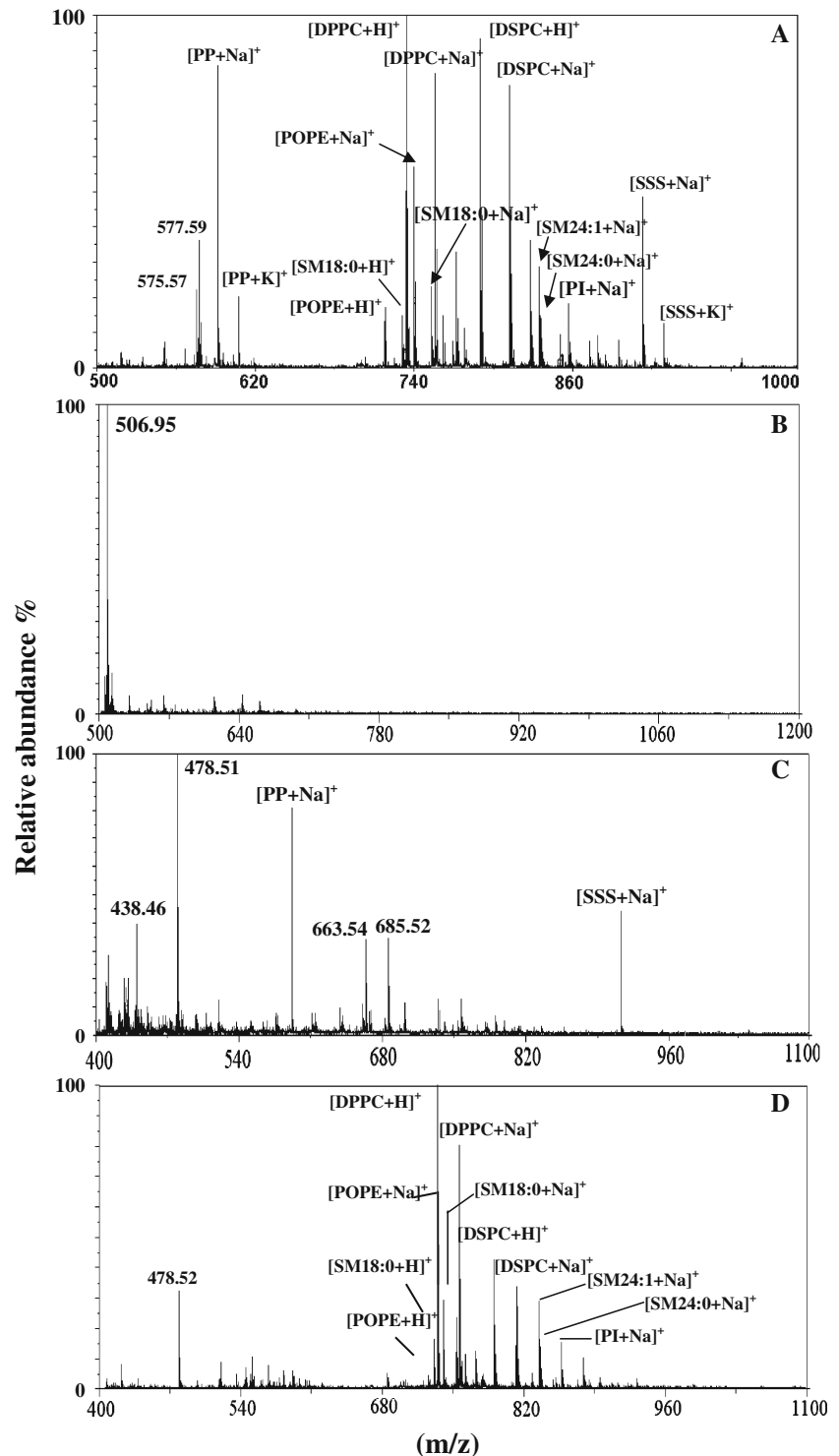
ammonia) and 0.05% of triethylamine (TEA); the eluted fraction was deposited directly on the target plate.

MALDI setup

MALDI-TOF MS spectra were recorded on a Voyager-DE STR instrument (PerSeptive Biosystems, Framingham, MA) operating in the positive ion delayed extraction reflector mode. Ions were generated by irradiation of analyte/matrix deposits by a nitrogen laser at 337 nm and analyzed with an accelerating voltage of 20 kV. Each MALDI-TOF MS spectrum was generated by accumulating data corresponding to 300 laser shots. All lipid mass spectra were acquired with a low-mass gate of 400 Da to prevent the detector from the possible ions saturation arising from DHB matrix degradation. Mass spectrometric data analysis was performed using MoverZ software (www.proteometrics.com).

Results and discussion

Application to standard lipid mixtures

As already stated in the introduction, in order to reach a complete characterization of PLs in dairy products, total polar lipids must be first extracted from the sample, then PLs must be isolated from total polar lipids. Thus, preliminary experiments were conducted on a lipids and phospholipids standard mixture, prepared as described in the experimental section, in order to optimize the home-made TiO$_2$ microcolumns-based PL enrichment procedure. Figure 1a shows the MALDI-TOF MS spectrum of the standard solution without any pre-treatment. Peaks relevant to almost all the target compounds were present in the mass spectrum. Only phosphoserine (PS) was not detected, since the phosphoserine headgroup makes the compound not easily ionizable in positive ion mode (the signal of the deprotonated molecule can be seen in negative ion mode).

As far as neutral lipids are concerned, no protonated molecules were present in the spectrum; sodium (M+23) and, to a minor extent, potassium (M+39) adducts were the main observable species (sodium and potassium were probably present as impurities in the solvent and/or on the target plate). In particular, Na$^+$ adducts of 1,3-dipalmitin and tristearin correspond to the m/z ions 591.43, and 913.81, respectively, while the K$^+$ adduct of 1,3-dipalmitin corresponds to the m/z ion 607.42. In the case of phospholipids, both protonated molecules and sodium adducts were observed. In particular, PCs contain a quaternary nitrogen with a fixed positive charge and readily form positive ions by MALDI, simply after the protonation of a phosphate group, contrary to other phospholipids where negative ions are easily formed. Protonated molecules of D-α-

phosphatidylcholine-dipalmitoyl and D-α-phosphatidylcholine-distearoyl correspond to m/z ions 734.56 and 790.65, while Na$^+$ adducts of the same compounds correspond to the m/z ions 756.55 and 812.64. Protonated molecules of sphingomyelin mixture of stearic (SM18:0), lignoceric (SM24:0), and nervonic (SM24:1) acids correspond to m/z ions 731.60, 813.66, and 815.67, while Na$^+$ adducts of the same compounds correspond to the m/z ions 753.58, 835.64, and 837.65, respectively. Moreover, for L-α-phosphatidylinositol protonated molecules, sodium and di-sodium adducts were observed at m/z 834.55, 856.52 and 878.48, respectively, and for L-α-phosphatidylethanolamine-β-oleyl-γ-palmitoyl both protonated molecules and sodium adducts were found at m/z 718.51 and 740.49. Table 1 lists the main ions observable in the spectrum of Fig. 1a, and their relative attributions.

Different strategies (i.e., loading, washing and elution solutions) were tested on the standard mixture, in order to verify the ability of TiO$_2$ microcolumns to selectively enrich the PL fraction. A MALDI-TOF MS analysis was performed after all the steps of the procedures. Figure 1b, c, and d show the MALDI-TOF MS spectra relevant respectively to the standard mixture subjected to the loading, washing and elution steps of the optimized enrichment procedure described in the "Experimental" section.

As can be seen in Fig. 1b, only one peak at the m/z value 506.96, attributable to the DHB matrix, was observed after the loading step. Figure 1c clearly shows that, after the

Table 1 Attribution of the main ions observable in the spectrum of Fig. 1a

m/z (Th)	Attribution
575.57	[PL]$^+$ (from PI)
577.59	[DPPC+Na-N(CH$_3$)$_3$]$^+$
591.43	[PP+Na]$^+$
607.42	[PP+K]$^+$
718.51	[POPE+H]$^+$
731.60	[SM18:0+H]$^+$
734.57	[DPPC+H]$^+$
740.49	[POPE+Na]$^+$
753.58	[SM18:0+Na]$^+$
756.55	[DPPC+Na]$^+$
790.65	[DSPC+H]$^+$
812.64	[DSPC+Na]$^+$
813.66	[SM24:1+H]$^+$
815.67	[SM24:0+H]$^+$
834.55	[PI+H]$^+$
835.64	[SM24:1+Na]$^+$
837.65	[SM24:0+Na]$^+$
856.52	[PI+Na]$^+$
878.48	[PI-H+2Na]$^+$
913.81	[SSS+Na]$^+$

washing step by CHCl3, only the neutral lipids 1,3-dipalmitin and tristearin were eluted, indicating the selective affinity of the TiO2 microcolumns for PLs. Finally, the target PLs were easily eluted by using 3 μl of DHB (40 mg/mL) dissolved in a CHCl3:MeOH (54:46, v/v) mixture with 0.1 % formic acid (FA, titrated to pH=5.3 with ammonia) and 0.05% of triethylamine (TEA) in the final step, as observable in Fig. 1d, were all the m/z ions relevant to the PLs originally present in the mixture were present. Then, following the optimized procedure, a separation between neutral lipids and phospholipids was reached in a simple and fast way. The procedure was repeated in triplicate, always obtaining the same results.

Dairy products

The optimized strategy was then applied to dairy products, i.e., cow milk, chocolate milk, and butter. Samples were

first subjected to lipids extraction following the procedure described in the "Experimental" section. The resulting extracts were then loaded onto the TiO2 microcolumns and subjected to the enrichment procedure. Figure 2a shows the MALDI-TOF MS spectrum acquired after total lipid extraction from a cow milk sample while Table 2 provides a summary and structural assignments of the main lipid species observed in the mass spectrum. MALDI-TOF confirmed to be a fast and simple technique for lipid analysis. Whilst the identification of high molecular weight TAGs was reasonably straightforward, the attribution of the lower molecular weight species becomes more difficult as an ion at a particular mass could correspond to losses of $RCOO^-Na^+$ or $RCOO^-K^+$ molecules from TAGs or to shorter TAGs species containing butyric acid (4:0). For example, m/z 577.59 could be attributed to a $[PO]^+$ ion or to Na^+ adducts of shorter TAGs, such as [4:0–10:0–16:0],

Fig. 2 MALDI mass spectra relevant to the lipid extract from milk a without any enrichment, b after the washing, and c the elution steps from TiO2. See Tables 2 and 3 for the attribution relevant to a and c, respectively

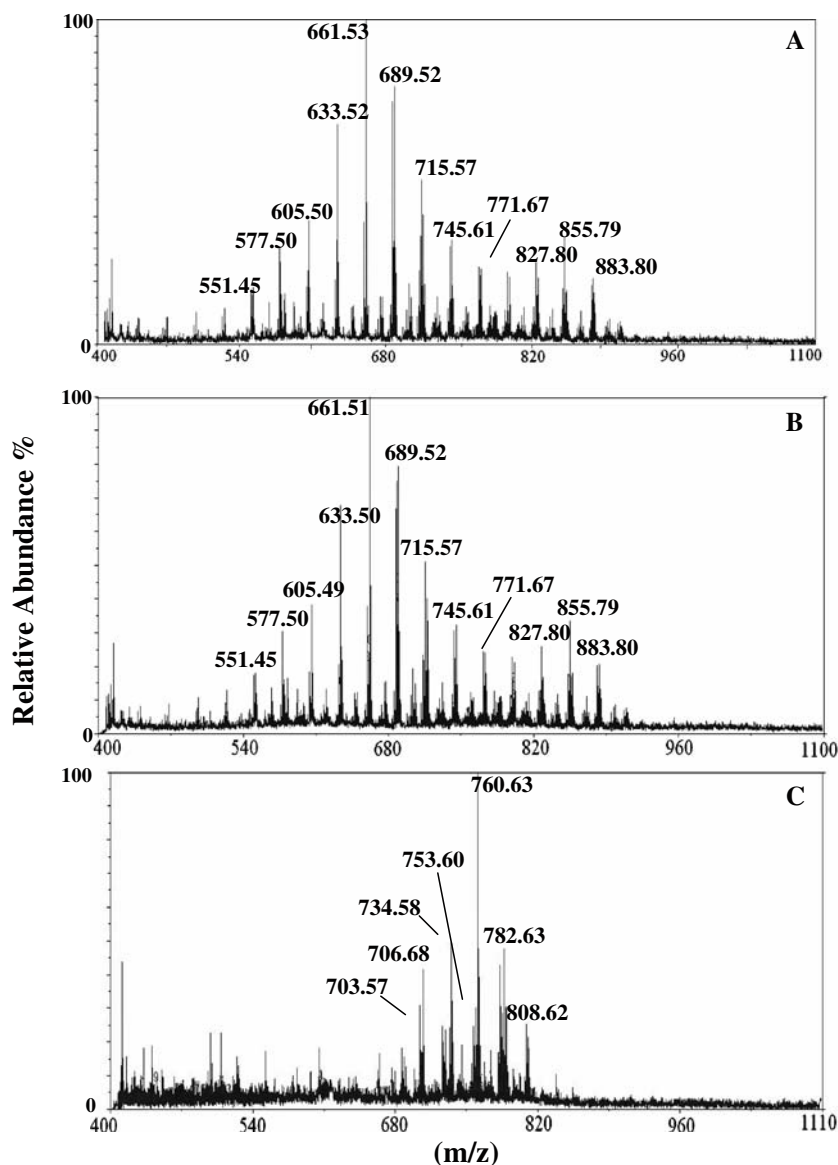**Table 2** Attribution of the main ions observable in the spectrum of Fig. 2a

m/z	Probable attribution
551.45	Matrix peak
577.55	Fragment [PO]$^+$
579.54	Fragment [PS]$^+$
591.53	[DAG32:0+Na]$^+$
603.56	Fragment [OO]$^+$
605.50	Fragment [OS]$^+$
617.54	[DAG34:1+Na]$^+$ [**]
619.53	[DAG34:0+Na]$^+$ [**]
633.52	[TAG34:0+Na]$^+$
647.54	[TAG35:0+Na]$^+$
659.54	[TAG36:1+Na]$^+$
661.54	[TAG36:0+Na]$^+$
675.53	[TAG37:0+Na]$^+$
677.51	[TAG36:0+K]$^+$,
687.53	[TAG38:1+Na]$^+$
689.54	[TAG38:0+Na]$^+$
703.54	[SM16:0+H]$^+$, [TAG39:0+Na]$^+$
706.52	[PC30:0+H]$^+$
713.55	[TAG40:2+Na]$^+$
715.57	[TAG40:1+Na]$^+$
717.55	[TAG40:0+Na]$^+$
731.54	[SM18:0+H]$^+$, [TAG42:1+H]$^+$
743.56	[TAG42:1+Na]$^+$
745.61	[TAG42:0+Na]$^+$
759.65	[TAG43:0+Na]$^+$
771.67	[TAG44:1+Na]$^+$
773.68	[TAG44:0+Na]$^+$
787.71	[TAG45:0+Na]$^+$
799.72	[TAG46:1+Na]$^+$
801.75	[TAG45:0+Na]$^+$
825.80	[TAG48:2+Na]$^+$
827.80	[TAG48:1+Na]$^+$
829.79	[TAG48:0+Na]$^+$
841.73	[TAG49:1+Na]$^+$
853.78	[TAG50:2+Na]$^+$
855.79	[TAG50:1+Na]$^+$
857.80	[TAG50:0+Na]$^+$
871.18	[TAG51:0+Na]$^+$
879.77	[TAG52:3+Na]$^+$
881.79	[TAG52:2+Na]$^+$
883.78	[TAG52:1+Na]$^+$
885.80	[TAG52:0+Na]$^+$
895.74	[TAG53:2+Na]$^+$
897.75	[TAG53:1+Na]$^+$
899.74	[TAG53:0+Na]$^+$
905.78	[TAG54:4+Na]$^+$
907.77	[TAG54:3+Na]$^+$
909.78	[TAG54:2+Na]$^+$

P palmitic acid, *O* oleic acid, *S* stearic acid, *SM* sphingomyelin, *PC* phosphatidylcholine, *DAG* diacylglycerol, *TAG* triacylglycerol

[4:0–12:0–14:0], [10:0–10:0–10:0]. In the higher-mass region the combination of different fatty acids can generate a high number of isobaric TAGs. For example, the m/z ion 661.53 could correspond to Na$^+$ adducts of TAGs such as [12:0–12:0–12:0] or [6:0–14:0–16:0] or [4:0–16:0–18:1]; in particular, TAGs with short-chain fatty acids are abundant in bovine milk. Since the complete elucidation of all lipids species composition is outside the aim of this work, for simplicity an attribution corresponding to the total number of carbon in each TAG/DAG/PL has been considered. Lipid assignments were made on the basis of the LIPID MAPS database [40] and by considering the composition of bovine milk lipids [41].

As apparent, few phospholipids were directly observed without any enrichment. The lipid mixture extracted from cow milk was then loaded onto the TiO$_2$ microcolumns and subjected to the enrichment procedure. Figure 2b and c shows the mass spectra relevant to the washing and elution steps. After the washing step, the eluted fraction was mainly composed of a triacylglycerols mixture, as can be inferred by the relevant m/z ions and by other m/z ions

Table 3 Attribution of the main ions observable in the spectrum of Fig. 2c

m/z	Probable attribution
703.57	[SM16:0+H]$^+$
706.58	[PC30:0+H]$^+$
725.55	[SM16:0+Na]$^+$
728.51	[PE36:3+H]$^+$
731.58	[SM18:0+H]$^+$
732.60	[PC32:1+H]$^+$, [PE36:1+H]$^+$
734.58	[PC32:0+H]$^+$
753.60	[SM18:0+Na]$^+$
756.57	[PC32:0+Na]$^+$, [PC34:2+H]$^+$
758.65	[PC34:1+H]$^+$
759.60	[SM20:0+H]$^+$
760.63	[PC34:0+H]$^+$
781.62	[SM22:3+H]$^+$
782.63	[PC34:0+Na]$^+$, [PC36:4+H]$^+$
783.61	[SM22:2+H]$^+$
784.62	[PC36:3+H]$^+$
785.63	[SM22:1+H]$^+$
786.64	[PC36:2+H]$^+$
787.63	[SM22:0+H]$^+$
788.65	[PC36:1+H]$^+$
790.62	[PC36:0+H]$^+$
808.62	[PC36:2+Na]$^+$
809.63	[SM22:0+Na]$^+$
810.67	[PC36:1+Na]$^+$

SM sphingomyelin, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine

arising from their fragmentation. Finally, after the elution step, more phospholipids, that were not observable in Fig. 2a, were clearly present in the spectrum. Table 3 provides a summary and the structural assignments of the main lipid species observed in the mass spectrum of Fig. 2c. As apparent, only PLs were present in the elution fraction, indicating the selectivity of the present approach for PLs and its potential for their isolation and enrichment from other lipid classes. It is worth noting that, as already observed in the case of the standard mixture, the PLs present in the elution fraction are essentially sphingomyelin and phosphatidylcholines. It is likely that the presence of more concentrated PCs lead to a suppression effect toward the other two PL classes.

The procedure was then applied to the analysis of PLs in chocolate milk and butter samples.

Figures 3a and 4a show the MALDI-TOF MS spectra acquired after total lipid extraction from a chocolate milk

sample and from a butter sample, respectively, while Figs. 3b and c, and 4b and c, show the MALDI-TOF MS spectra relevant to the same extracts after the washing and the elution steps, respectively, of the developed enrichment procedure.

As apparent, similar results than those already observed in the case of cow milk were obtained, clearly showing the ability of the present method to isolate and enrich PLs from total lipids in a variety of food samples.

Possible mechanism for the selective enrichment of phospholipids by TiO_2

Larsen et al [38] have shown as the presence of DHB in the loading buffer dramatically enhances the selective retention of phosphopeptides on TiO_2. The effect was attributed to a competition for binding sites on TiO_2 between non-phosphorylated peptides and DHB molecules. The same effect could be invoked also in present case, since the DHB

Fig. 3 MALDI mass spectra relevant to the lipid extract from chocolate milk **a** without any enrichment, **b** after the washing, and **c** the elution steps from TiO_2
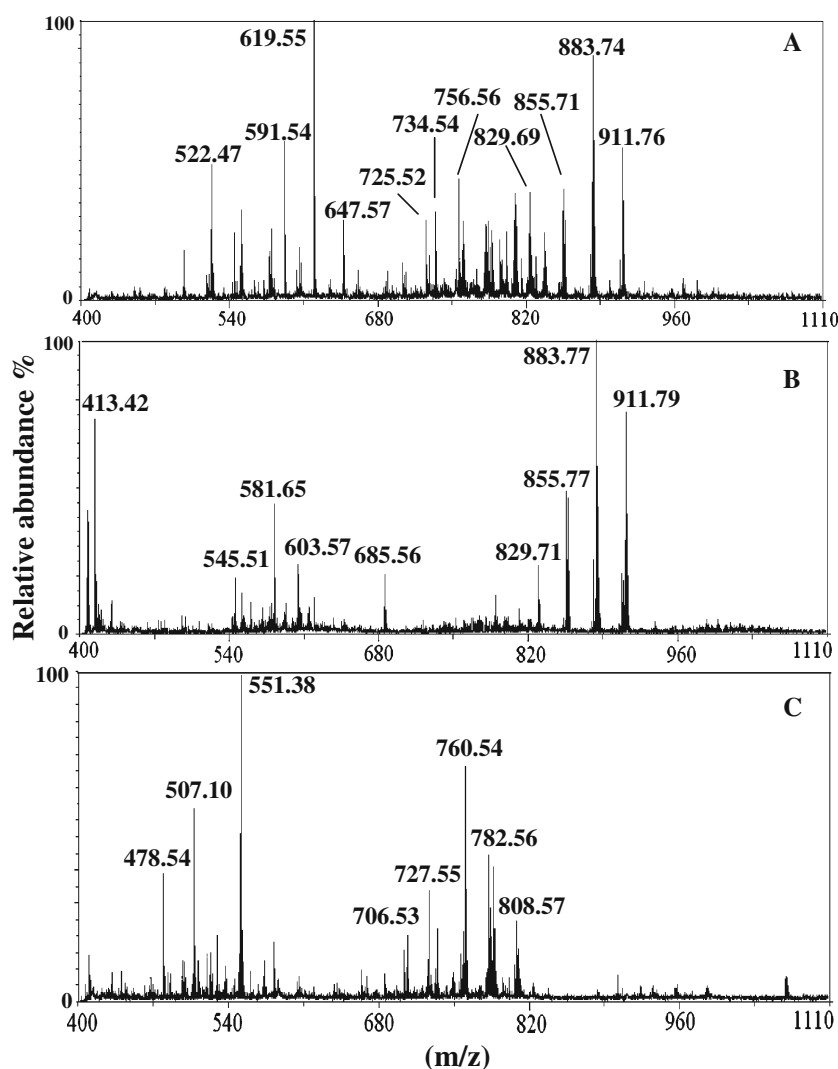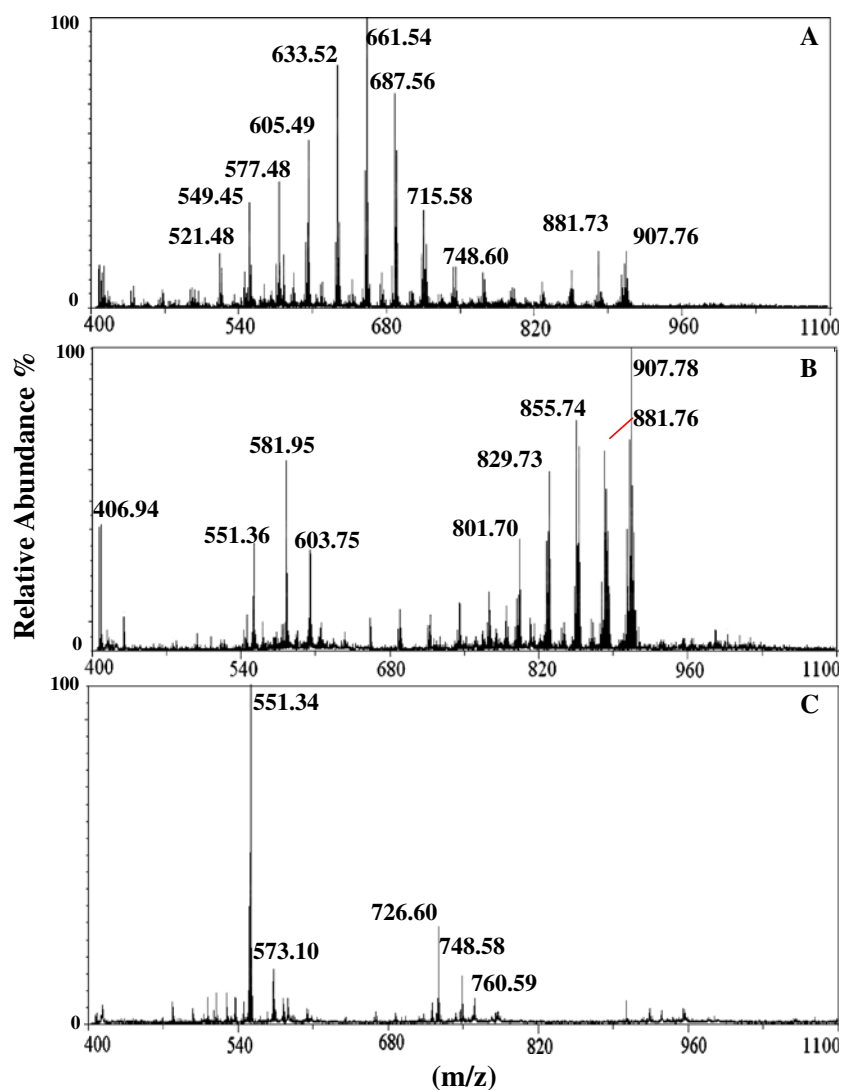
Fig. 4 MALDI mass spectra relevant to lipid extract from butter **a** without any pre-treatment, after **b** washing, and **c** elution steps from TiO$_2$ microcolumns

molecules could compete with neutral lipids such as diglycerides and monoglycerides. The competition of free carboxylic acid eventually present in dairy products should be minimal, since it has been demonstrated by infrared spectroscopy that monofunctional carboxylic acids (including benzoic acid and acetic acid) interact weakly with TiO$_2$ [42]. Then, the presence of substituted aromatic carboxylic acids such as DHB, that strongly coordinate on the surface of TiO$_2$, could improve the enrichment in PLs, since it effectively competes with non-phosphorylated lipids for the adsorption on TiO$_2$ surface, whereas it does not have influence on phosphorylated lipid binding.

In any case, it has to be pointed out that the binding mechanism of the phosphate group to TiO$_2$ differs from that of substituted aromatic carboxylic acids. In fact, in the case of DHB, a chelating bidentate bond could be hypothesized

[42, 43], whereas only monodentate complexes are possible for disubstituted phosphates.

Conclusions

A new simple μ-SPE procedure based on homemade titanium dioxide microcolumns was developed for the selective enrichment of PLs, followed by MALDI-TOF MS. The main advantage of the approach is the possibility to obtain a very fast PLs profiling without using more time-consuming separation technique. All the extraction steps (loading, washing, and elution) have been optimized using a synthetic mixture of PLs standard and the procedure was subsequently applied to food samples such as milk, chocolate milk, and butter.

The whole method appears very promising for a rapid PL screening and characterization also in other matrices.

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