

Selective enrichment of phosphatidylcholines from food and biological matrices using metal oxides as solid-phase extraction materials prior to analysis by HPLC–ESI-MS/MS

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Abstract A zirconia (ZrO_2)-modified solid-phase extraction sorbent has been evaluated for selective extraction of phosphatidylcholines from biological samples, followed by analysis of the isolated solutes by reversed-phase liquid chromatography–electrospray ionization–tandem mass spectrometry. The clean-up process was optimized using seven standard phosphatidylcholines including two lyso derivatives. Different acidic conditions were tested for the bonding and washing steps; for elution, various aqueous or methanolic bases were studied. Experiments were conducted hydrodynamically using extraction cartridges, and statically in batch mode; the performance of the sorbent was significantly better when used in the flow-through mode. The developed clean-up procedure was used to selectively enrich phosphatidylcholines from whole milk, human plasma, and mouse plasma, to show the wide applicability of the method. For the preceding extraction of total lipids from the matrix, different solvent mixtures (methanol–chloroform, methanol–methyl *tert*-butyl ether, and ethanol–ethyl acetate) were compared. Accuracy and reproducibility of the proposed sample-preparation procedure were evaluated. Matrix effects possibly affecting mass spectrometric analysis were studied before and after the

solid-phase extraction. They were found to be significant for several analytes, stressing the importance of a sample clean-up procedure. Under identical experimental conditions, recovery of bound phosphatidylcholines by zirconia was superior to that by other metal oxides, for example titania (TiO_2) and stannia (SnO_2).

Keywords Phosphatidylcholines · Zirconium dioxide · Titanium dioxide · Tin dioxide · Tandem mass spectrometry · Electrospray ionization

Abbreviations

16:0 lysoPC	1-Palmitoyl-2-hydroxy- <i>sn</i> -glycero-3-phosphocholine
18:0 lysoPC	1-Stearoyl-2-hydroxy- <i>sn</i> -glycero-3-phosphocholine
AcOH	Acetic acid
DAPC	1,2-Diarachidonoyl- <i>sn</i> -glycero-3-phosphocholine
DMPC	1,2-Dimyristoyl- <i>sn</i> -glycero-3-phosphocholine
DPPC	1,2-Dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine
ESI	Electrospray ionization
EtOAc	Acetic acid ethyl ester
EtOH	Ethanol
FA	Formic acid
GPL	Glycerophospholipid
HPLC	High-performance liquid chromatography
iPrOH	2-Propanol
LC	Liquid chromatography
MeOH	Methanol
MS	Mass spectrometry
MTBE	Methyl <i>tert</i> -butyl ether
PC	Phosphatidylcholine

Dedicated to Dipl.-Ing. Dr Dr h.c. Professor Manfred Grasserbauer on the occasion of his 65th birthday

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PE	Phosphatidylethanolamine
PL	Phospholipid
PLPC	1-Palmitoyl-2-linoleoyl- <i>sn</i> -glycero-3-phosphocholine
POPC	1-Palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
TFA	Trifluoroacetic acid
QqQ	Triple quadrupole

Introduction

Phospholipids (PLs) structurally consist of a polar head group, i.e. a phosphonate moiety, and a large hydrophobic part made up of one or two long-chain acyl or alkyl residues [1, 2]. In the group of glycerophospholipids (GPLs), besides mono and diacyl derivatives ether-linked subspecies, namely acyl-alkyl and acyl-alkenyl derivatives (plasmalogens) are also of biological importance [3].

PLs are present in all organisms where they constitute important biochemical intermediates in the growth and functioning of cells, for instance as major components of cell membrane bilayers. Recent studies have also provided evidence that PLs have positive nutritional effects on human health, for instance by minimization of the risk of cardiovascular diseases [4]. Because of their amphiphilic character, bearing lipophilic and hydrophilic properties, PLs are commonly used by food industry as emulsion stabilizers [5].

Analysis of PLs typically involves several steps, namely extraction of total lipids from the matrix followed by separation of the PL fraction from non-phosphorylated lipid classes and separation of the different PL subspecies. The most commonly applied procedures for lipid extraction still use chloroform and methanol as proposed by Folch [6] or Bligh and Dyer [7]. After lipid extraction, separation of PL subspecies is typically done chromatographically using thin-layer chromatography (TLC) [8, 9], gas chromatography, or liquid chromatography (GC, LC) [1]. HPLC is commonly performed in the normal-phase (NP) mode using silica columns as stationary phases in combination with UV or evaporative light-scattering detection (ELSD) [10, 11]. Alternatively, RP-HPLC using C₈ or C₁₈ columns has been used in combination with mass spectrometry (MS) or tandem mass spectrometry (MS/MS approach) which enables highly selective and sensitive detection not only of PL subclasses but also of individual PLs [2, 12–14].

Several studies have described the isolation of the PL fraction from other lipids by solid-phase extraction (SPE) procedures prior to further analysis; this enables simplification of matrix complexity and enrichment of PLs.

Typically, NP materials, for example silica and amino or diol-modified silica have been employed [8, 15, 16]. More selective extraction materials based on metal oxides such as titania (TiO₂), zirconia (ZrO₂), and others which rely on specific Lewis acid-base interactions have particularly been used in the analysis of phosphorylated proteins and peptides [17–26]. Recently, Pucci et al. used zirconia for removal of phospholipids in order to minimize PL-based matrix effects in electrospray ionization (ESI)-MS-based bioanalysis [27]. Up to now, however, these materials have rarely been used for selective enrichment of PLs. Ikeguchi and Nakamura used titania for extraction of PLs from egg yolk prior to their analysis by HPLC with fluorescence detection [28], and Calvano et al. recently reported an SPE procedure based on TiO₂ microcolumns for extraction of PLs from dairy products, followed by qualitative analysis by matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) [29].

The intention of this study was to evaluate a zirconia-modified SPE sorbent with regard to its selectivity and efficiency for enrichment of selected GPLs from various matrices prior to their quantitative analysis by RP-HPLC–ESI-MS/MS using a triple-quadrupole (QqQ) instrument in the selected reaction monitoring (SRM) mode. Retention of PLs on the solid metal oxide sorbent is presumably based on Lewis acid–base interactions in which the phosphate moiety of the PL, being a strong Lewis base, interacts with the empty d-orbitals of the transition metal which acts as a Lewis acid under acidic conditions [19] (Fig. 1). Accordingly, binding of the GPL analytes should occur at low pH and disruption of these interactions should be achieved by a pH shift towards basic conditions under which the transition metal oxides no longer exhibit Lewis acid characteristics but behave as Lewis bases.

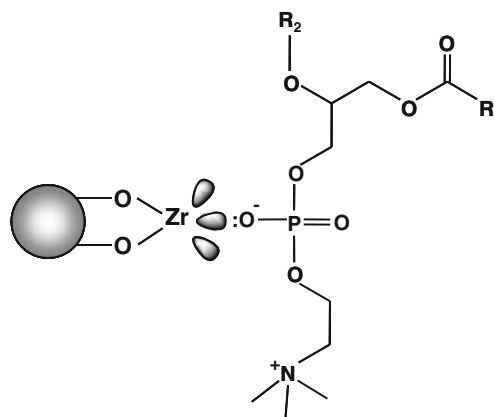


Fig. 1 Proposed binding mechanism underlying the selective retention of phosphatidylcholines on metal oxides exemplified by zirconia (ZrO₂). R₁ denotes an alkyl residue, R₂ stands for an acyl residue (PCs) or for H (lysoPCs)

Phosphatidylcholines (PCs) were chosen as target solutes in this preliminary study to show the applicability of the metal oxide as SPE sorbent. These analytes can be determined by ESI-MS/MS with high sensitivity in the positive-ionization mode, because of the presence of a permanently charged quaternary amine group. In addition, a specific fragment ion of m/z 184 is generated, corresponding to the cleaved phosphocholine residue, which enables highly selective detection of PCs in the SRM mode [2, 30]. Seven PC standards comprising two lysoPCs were used for the development and optimization of the SPE procedure. Additionally, for the preceding extraction of the lipids from the matrix three different solvent systems were compared, namely methanol–chloroform, methanol–MTBE, and ethanol–ethyl acetate mixtures. The developed SPE procedure was used to determine concentrations of the targeted analytes in whole milk and in human plasma and mouse plasma. Matrix effects possibly impairing ionization yield in the ESI source and MS detection were evaluated for the milk matrix before and after SPE clean-up. The accuracy of the proposed method was evaluated by means of spiking experiments. Finally, the performance of the zirconia material was compared with that of two other metal oxides, titania and stannia.

Experimental

Materials and reagents

Titania (Sachtopore NP titanium dioxide, particle size 10 μm , mean pore size 300 \AA , surface area 15 m^2g^{-1}) was obtained from ZirChrom Separations (Anoka, MN, USA). SPE tubes (HybridSPE-PPT) containing 30 mg zirconium dioxide-coated silica (surface coverage 3.5% ZrO_2 (w/w), particle size 20 μm , mean pore size 120 \AA) were from Supelco (Bellefonte, PA, USA). Stannia (tin dioxide) microspheres (particle size 10 μm , mean pore size 50 \AA , surface coverage 50–60 m^2g^{-1}) were prepared as research samples from silica particles according to a nanocasting method described previously [31].

PC standards, including 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine (DAPC), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (18:0 lysoPC) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (16:0 lysoPC), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). A standard mixture containing all seven analytes at a concentration of 10 $\mu\text{g mL}^{-1}$ in methanol, which, after appropriate dilution, was used for development and optimization of

the SPE procedure and for quantitation purposes was prepared freshly every ten days and stored at $-18\text{ }^\circ\text{C}$. Myristic acid (99%), palmitic acid (99%), linoleic acid (99%), and oleic acid (99%) were obtained from Sigma–Aldrich (Steinheim, Germany), stearic acid (99.5%), linolenic acid (98.5%) and arachidonic acid (98.5%) were from Fluka (Buchs, Switzerland).

All solvents used throughout the study were of HPLC gradient grade. 2-Propanol (isopropanol, iPrOH), heptane, chloroform, and acetic acid ethyl ester (ethyl acetate, EtOAc) were obtained from CarlRoth (Karlsruhe, Germany), and ethanol and methanol were purchased from VWR International (Leuven, Belgium). Ammonium hydroxide solution (25%), trifluoroacetic acid (TFA, 99%), formic acid (FA, 98–100%), acetic acid (AcOH, >99.8%), methyl *tert*-butyl ether (MTBE, 99%), and ammonium acetate (99%) were obtained from Fluka. Ammonia solution (7 mol L^{-1} in methanol) and ultra-pure water were purchased from Sigma–Aldrich.

Whole milk (ultra-high temperature-treated and homogenized long-life milk, 3.5% fat) was obtained from a local market in Vienna, extracts from human plasma and mouse plasma were kindly provided by the Medical University of Vienna.

Extraction of total lipids from whole milk

Extraction with MeOH–CHCl₃

Methanol (1.5 mL) was added to 200 μL milk in a glass tube and the mixture was briefly shaken. Then, 3 mL chloroform were added and lipid extraction was performed by vortex mixing at room temperature for 60 min. Phase separation was induced by adding 1.25 mL water. The organic layer was collected, dried in a nitrogen stream, and redissolved in 200 μL iPrOH–heptane (80:20, v/v) containing 1% (v/v) FA for further SPE clean-up.

Extraction with MeOH–MTBE

Methanol (1.5 mL) was added to 200 μL milk followed by brief shaking. MTBE (5 mL) was then added and the mixture was vortex mixed at room temperature for 60 min. The organic layer was collected, dried in a nitrogen stream, and redissolved in 200 μL iPrOH–heptane–FA (80:20:1, v/v).

Extraction with EtOH–EtOAc

Ethanol (1.5 mL) was added to 200 μL milk and the mixture was briefly shaken. Then, 1.5 mL EtOAc were added and the mixture was vortex mixed for 60 min. The organic layer was collected, dried in a nitrogen stream, and redissolved in 200 μL iPrOH–heptane–FA (80:20:1, v/v).

Extraction of total lipids from plasma samples

Fifty microlitres of plasma samples obtained by centrifugation of EDTA-anticoagulated blood were mixed with 1 mL ice-cooled chloroform–methanol (2:1, *v/v*) and shaken for 2 h at -20°C . After centrifugation at 6000 rpm for 15 min at 4°C , the supernatant was carefully removed from the settled protein pellet by use of a micropipette. The solvent was evaporated under a gentle stream of nitrogen and the lipid extracts were stored at -80°C until further analysis. For SPE clean-up, they were redissolved in 200 μL *iPrOH*–heptane (80:20, *v/v*) containing 1% (*v/v*) FA.

Solid phase extraction clean-up

Lipid extracts from 200 μL milk and 50 μL plasma samples, respectively, redissolved in 200 μL *iPrOH*–heptane (80:20, *v/v*) containing 1% (*v/v*) FA were added to the zirconia SPE cartridges and the effluent was collected. After washing with 200 μL *iPrOH*–heptane (80:20, *v/v*) and collection of the effluent, the PLs were eluted by threefold application of 200 μL 7 mol L^{-1} ammonia in methanol and subsequently the three elution fractions were combined. In the case of titania and stannia, the lipid extracts were applied to 1.5 mL microcentrifuge tubes containing approximately 6 mg of the respective sorbent. Binding, washing, and elution were done as outlined above by shaking the beads with 200 μL of the respective solution for 4 min, followed by centrifugation for 2 min at 13,800 rpm and removal of the supernatant by use of a micropipette, avoiding aspiration of the metal oxide particles. All collected effluents/supernatants (binding, washing, and the combined elution fractions) were dried in a nitrogen stream and redissolved in 200 μL methanol–water (80:20, *v/v*) prior to HPLC-ESI-MS/MS analysis.

Spiking experiments

Milk samples (200 μL) were spiked with a standard mixture containing the seven PC analytes to end up with a spiking concentration of 1 $\mu\text{g mL}^{-1}$ milk of each analyte. Then, lipid extraction followed by SPE clean-up on the zirconia cartridges were carried out as outlined above. Simultaneously, non-spiked milk samples were treated under identical conditions. Concentrations of the PC analytes were determined in spiked and non-spiked samples by HPLC–ESI-MS/MS analysis using external calibration, and recovery of the spiked analytes was calculated after correction by the intrinsic PC concentrations found in the non-spiked samples.

HPLC–ESI-MS/MS analysis

PCs were analyzed using an Agilent 1200 HPLC system coupled to an Applied Biosystems 4000 QTrap (triple-

quadrupole linear ion trap hybrid) mass spectrometer equipped with a Turbo V electrospray ion source which was operated in QqQ mode (Applied Biosystems, Foster City, CA, USA). Data acquisition was performed with Analyst software, version 1.5 (Applied Biosystems). In the mass range of the analytes (m/z between ~ 500 and $800 m/z$), mass accuracy of the MS instrument is about $0.2\text{--}0.3 m/z$, i.e. resolution is ~ 3000 (10% valley definition). Analyte detection was performed in positive SRM mode by monitoring of the specific product ion at m/z 184.1 which corresponds to cleaved phosphocholine. The following general settings were used: ESI voltage was 4300 V, temperature of the ion source was set to 500°C , entrance potential (EP) was 10 V. Nitrogen was used as nebulizer, heater, and curtain gas, with the pressure set to 414, 345, and 69 kPa, respectively. Scan time for each SRM transition (dwell time) was 10 ms, pause between two consecutive SRM transitions was set to 5 ms, resulting in a cycling time of 105 ms for the seven SRM transitions. Peak width (at 50% height) for the seven analytes was between 0.17 and 0.25 min under the chromatographic conditions used, leading to 100–140 data points characterizing one peak. Compound-specific MS conditions, i.e. declustering potential (DP), collision energy (CE), and cell exit potential (CXP) were optimized for every analyte individually, using the instrument's automated fragmentation optimization tool. For that purpose, standard solutions of the target compounds at concentrations in the range $0.5\text{--}2.5 \mu\text{g mL}^{-1}$ were infused using a 500 μL Hamilton syringe and a syringe pump at a flow rate of $30 \mu\text{L min}^{-1}$.

Chromatographic separation of the PCs was carried out on a Luna 3u C8(2) column from Phenomenex (Aschaffenburg, Germany) (150 mm \times 2.0 mm, particle size 3.0 μm). Mobile phase consisted of (A) 10 mmol L^{-1} ammonium acetate in methanol–water (80:20, *v/v*) and (B) 10 mmol L^{-1} ammonium acetate in methanol. Gradient elution was performed starting from 55% B increasing to 100% B in 5 min, which were held for 8 min. Afterwards, starting conditions (55% B) were reconstituted within 2 min and held for re-equilibration for 10 min prior to the next analysis. The mobile phase flow rate was $200 \mu\text{L min}^{-1}$. During chromatographic runs, the autosampler temperature was set to 5°C , the temperature of the column compartment was 25°C , and the injection volume was 5 μL . Quantitation of the PC analytes in milk and plasma extracts was done by external calibration using a standard mixture containing each analyte in the concentration range $0.1\text{--}2 \mu\text{g mL}^{-1}$ in methanol–water (80:20, *v/v*) (five calibration levels, performed at the beginning of each analysis sequence). Standard mixtures that were obtained from individual stock solutions (each at $10 \mu\text{g mL}^{-1}$ in methanol, stored at -18°C) were prepared freshly every 10 days to avoid changes in the concentrations because of evaporation of

the solvent during handling at room temperature. In order to ensure analysis in the linear dynamic range of the method, the elution fractions were diluted 1:10 in the case of milk and 1:100 in the case of plasma, with methanol–water (80:20, v/v).

Results and discussion

Analysis of PCs by HPLC-ESI-MS/MS

Compound-specific MS conditions of the seven herein studied individual PC standards, for example the declustering potential (DP, cone voltage) which is crucial for the ionization efficiency in the ESI source [32, 33] or the collision energy (CE) which affects the fragmentation behaviour of the analytes, were optimized to yield the highest signal intensity for the characteristic product ion of m/z 184.1. The optimized MS conditions are summarized in Table 1 together with the retention times of the analytes under the LC conditions used. In Fig. 2, a standard chromatogram is shown.

Performance of the developed LC–MS/MS method was evaluated with regard to limits of determination (LODs), linear dynamic range, and injection precision using standard mixtures of the PCs in MeOH–H₂O 80:20 (v/v); the results are also summarized in Table 1. LODs, which were determined at a signal-to-noise (S/N) ratio of 10 [34] were in the range between 0.12 and 1.10 ng mL⁻¹ and linearity was found to be good in the studied concentration range (tested between 0.025 µg mL⁻¹ and the ULOQ of the

analytes). Injection repeatability, which was evaluated by means of the peak area at low concentration (0.25 µg mL⁻¹), was below 4% RSD for all analytes ($n=3$) which was satisfactory for the intended use. The effect of the sample matrix on the performance of the developed LC–MS/MS method was evaluated by means of analysis of whole milk and results will be discussed later (vide infra).

Optimization of the SPE clean-up procedure with standard PCs

The optimization experiments were performed using commercially available zirconia SPE tubes and a standard solution containing the seven PCs at a concentration of 1.5 µg mL⁻¹ each in iPrOH–heptane (80:20, v/v). To study the effect of pH on binding of the PC analytes to zirconia, different acidic conditions were tested in the loading step using formic acid (FA), acetic acid (AcOH), and trifluoroacetic acid (TFA) at concentrations in the range between 0.1% and 5% (v/v). Under all tested conditions, 99.4% to 100% of the analytes were bound to the ZrO₂ material. For further experiments, a concentration of 1% (v/v) FA was selected for the loading step.

Subsequent to binding of the analytes to the SPE sorbent, a washing step was included in which matrix components possibly adhering to the zirconia material after the loading step should be removed. Solutions of iPrOH–heptane (80:20, v/v) containing FA, AcOH, or TFA at different concentrations (0–5%, v/v) were studied. At the highest concentration, for all three acids, and especially for TFA, a slight loss of bound PCs (below 1%) was observed

Table 1 HPLC–ESI-MS/MS conditions used for the analytes, and method characteristics regarding linear regression, linear dynamic range, and injection precision

Analyte	MS/MS conditions				t_R (min)	Linear regression ^a				LOD ^c (ng mL ⁻¹)	ULOQ ^d (ng mL ⁻¹)	Precision ^e , as RSD (%)
	Parent ion m/z	Product ion m/z	DP (V)	CE (V)		Slope	Intercept	Weight	r^b			
16:0 lysoPC	496.3	184.1	116	35	4.3	1.5×10^7	-2.6×10^4	1/x	0.999	0.43	3000	4.0
18:0 lysoPC	524.4	184.1	101	39	5.5	1.2×10^7	1.7×10^5	–	0.997	0.48	3000	1.4
DMPC	678.6	184.1	61	39	11.0	3.1×10^7	2.7×10^6	1/x	0.991	0.49	2000	1.7
DPPC	734.6	184.1	96	36	12.7	1.8×10^7	9.3×10^6	–	0.999	0.27	3000	4.2
PLPC	758.6	184.1	105	45	12.3	1.1×10^7	1.2×10^6	1/x	0.993	0.73	3000	2.2
POPC	760.6	184.1	116	45	12.9	4.9×10^7	4.8×10^6	1/x	0.991	0.12	2000	2.5
DAPC	830.6	184.1	116	43	11.9	6.6×10^6	3.2×10^5	1/x	0.991	1.10	3000	0.7

^a Evaluated in the concentration range between 0.025 and 5 µg mL⁻¹

^b Correlation coefficient

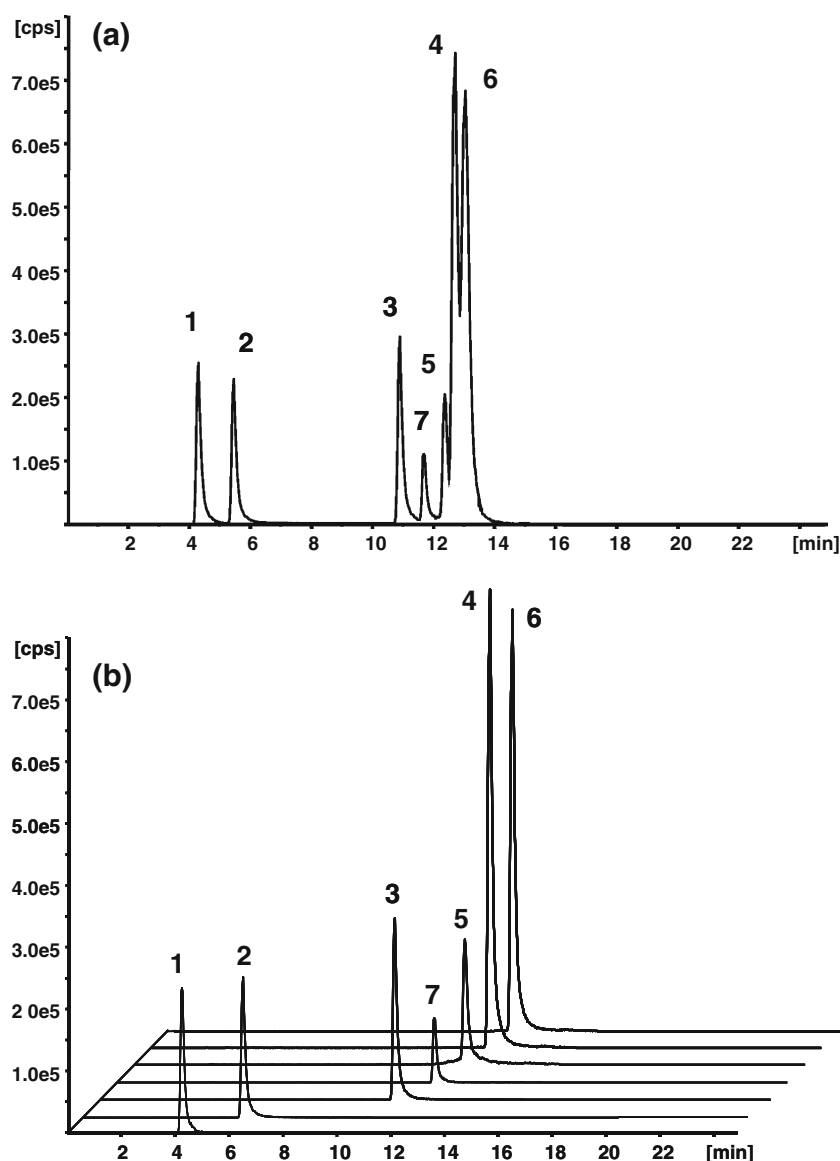
^c Limit of determination at a signal-to-noise (S/N) ratio of 10

^d Upper limit of quantification

^e Injection repeatability evaluated by means of the peak area at 0.25 µg mL⁻¹ ($n=3$)

Abbreviations: DP, declustering potential; CE, collision energy

Fig. 2 Separation of a standard mixture containing the seven PC analytes at a concentration of $0.25 \mu\text{g mL}^{-1}$ each under the optimized LC-ESI-MS/MS conditions. **(a)** Total ion chromatogram (TIC) obtained in positive ionization mode and **(b)** extracted ion chromatograms (XICs) of the individual SRM transitions. Peak assignment: 1, 16:0 lysoPC; 2, 18:0 lysoPC; 3, DMPC; 4, DPPC; 5, PLPC; 6, POPC; 7, DAPC



for some analytes, whereas under all other conditions tested binding of the PCs was not affected by the washing step. Finally, no acid was added to the washing solvent.

For elution of, e.g., bound phosphopeptides from metal oxides, high-pH buffers have typically been used. Ikeguchi and Nakamura used 0.2 mol L^{-1} ammonia in chloroform-methanol (70:30, *v/v*) to elute bound GPLs from titania with recoveries of PCs and PEs in the range of 70–73% [28]. In comparison, Calvano et al. who extracted PLs from dairy products using TiO_2 -microcolumns, used slightly acidic conditions (pH 5.3) for elution of the analytes. No quantitative information, however, about the extent of recovery was given by the authors [29].

In our study, aqueous solutions of 10–20% (*v/v*) KOH or 25% (*v/v*) ammonia were initially tested for elution of the bound PCs, the former followed by a cation-exchange step to remove excess of KOH prior to the LC-MS/MS

analysis. In any case, however, recovery of the analytes was marginal, probably because of the poor solubility of the PLs in aqueous media, and, when KOH was used, most likely because of hydrolysis of the ester groups. Thereafter, methanolic ammonia was tested at concentrations in the range between 0.1 mol L^{-1} and 7 mol L^{-1} . At concentrations below 5 mol L^{-1} , recovery of the PCs was low, however, under the most concentrated conditions ($5\text{--}7 \text{ mol L}^{-1}$) recoveries above 80% were observed for all of the studied analytes after the three elution steps (Fig. 3a). For all PCs, more than 90% of the total amount of analyte eluted was detected in the first elution fraction when the ammonia concentration was higher than 5 mol L^{-1} , and less than 0.5% was found in the third fraction (Fig. 3b). In comparison, the lysoPCs showed somehow different behaviour, because at an ammonia concentration of 5 mol L^{-1} in the first elution fraction only 55%, in the

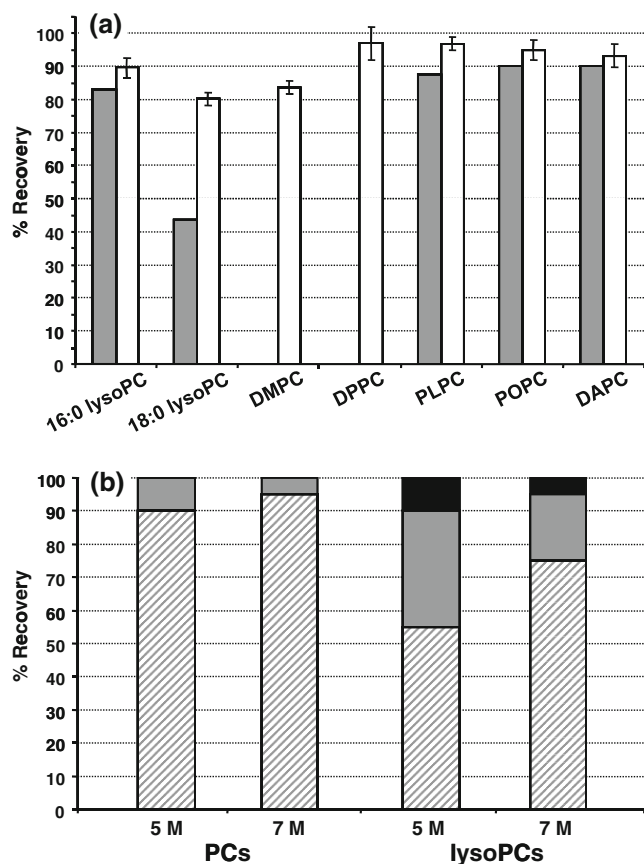


Fig. 3 (a) Recovery of standard PCs from ZrO_2 -containing SPE cartridges after threefold elution with 5 mol L^{-1} ammonia in methanol (grey bars) and 7 mol L^{-1} ammonia in methanol (white bars). (b) Dependence on ammonia concentration of the distribution of the recovered PC and lysoPC analytes in the three elution fractions (dashed bars, 1st fraction; grey bars, 2nd fraction; black bars, 3rd fraction). Error bars in (a) indicate the reproducibility ($n=3$). Note: DMPC and DPPC have not been included in the experiments performed with 5 mol L^{-1} ammonia

second fraction about 35% and in the third fraction still 10% of the recovered analyte were detected (Fig. 3b). Also at the highest ammonia concentration, a minimum of 5% of the total amount of recovered lysoPCs was still found in the third elution fraction, indicating either stronger binding on and/or less effective elution of lysoPCs from the metal oxide under the tested conditions.

During the so far optimized SPE procedure, binding and washing of the PCs was performed with a mixture of *i*PrOH–heptane and elution was with methanol. Because heptane is only poorly miscible with methanol, a probable detrimental effect of the solvent change on the extent of elution of the analytes was examined. Thus, after binding and washing under the above described conditions, a second washing step employing pure *i*PrOH was included in which heptane should be removed from the SPE cartridge before application of the methanolic elution solvent. Recoveries similar to those obtained previously

were observed, i.e. >90% of the PCs and >80% of the lysoPCs were recovered. Interestingly, however, a shift in the distribution of the recovered analytes in the three elution fractions was observed. Thus, after the additional washing step with pure *i*PrOH, only 10% of the total amount of eluted PCs (5% for the lysoPCs) were detected in the first fraction and most, i.e. about 80% of PCs (75% of the lysoPCs) was eluted with the second fraction. Nearly 10% of PCs (20% of lysoPCs) could still be detected in the third fraction.

It is well known that under acidic and, especially, basic conditions fatty acid esters can be hydrolyzed in protic media. Under the relatively harsh basic elution conditions used herein, cleavage of the fatty acid residues from the GPL backbone accompanied by formation of the respective fatty acid methyl ester (FAME) in an alkali-catalyzed transesterification reaction (methanolysis) [35, 36] is conceivable; this could, for instance, explain the lower recovery values observed for the lysoPCs. In order to examine whether such a phenomenon occurs under the developed clean-up conditions a mixture containing the seven PC standards at a concentration of $1.5 \mu\text{g mL}^{-1}$ each was treated with the loading, washing, and elution solvents, but without application to the ZrO_2 cartridge. All the PCs were recovered quantitatively (100% recovery) indicating that no hydrolysis of the fatty acid residues took place and hence, any loss of analyte during the SPE procedure was because of incomplete elution from the metal oxide sorbent. For the two lysoPCs, however, recovery after treatment with the methanolic ammonia solution was somewhat lower (88–96%), which suggests that these analytes at least partly experience cleavage of the palmitic and stearic acid residue, respectively.

Reproducibility of the optimized SPE procedure, i.e. loading in *i*PrOH–heptane (80:20, *v/v*) containing 1% (*v/v*) FA, washing with *i*PrOH–heptane (80:20, *v/v*), and triple elution with 7 mol L^{-1} methanolic ammonia was finally evaluated on the ZrO_2 -cartridges by triplicate repetition of the whole procedure employing the PC standard mixture. RSD values calculated for the recovery of the analytes were between 4% and 10% (Fig. 3a) which was quite acceptable.

Evaluation of non-specific binding of free fatty acids

From the application of metal oxides for enrichment of phosphopeptides it is well known that unspecific binding of non-phosphorylated compounds, for instance via carboxylic or amino groups, may play a crucial role [17]. Larsen et al. introduced aromatic carboxylic acid derivatives, for example 2,5-dihydroxybenzoic acid (DHB), in the loading and washing steps, which turned out to effectively reduce binding of non-phosphorylated peptides [37]. Calvano et al., who adopted the procedure proposed by Larsen et al.

Table 2 Dependence of recovery of PCs from zirconia (ZrO₂) on addition of different amounts of free fatty acids (FFAs)^a

Analyte	ZrO ₂		
	No FFAs	Ratio 1:1	Ratio 1:4
16:0 lysoPC	90±6%	84±3%	88±7%
18:0 lysoPC	80±4%	81±5%	89±2%
DMPC	84±4%	95±4%	93±2%
DPPC	97±10%	94±5%	97±4%
PLPC	97±4%	88±4%	93±6%
POPC	95±6%	94±3%	96±3%
DAPC	93±7%	91±8%	98±2%

^a FFA standards used were myristic, palmitic, stearic, oleic, linoleic, linolenic, and arachidonic acid. Total amount of PCs applied was always 3.5 µg mL⁻¹, total amount of FFAs was 0 µg mL⁻¹ (no FFAs), 3.5 µg mL⁻¹ (ratio 1:1), and 14 µg mL⁻¹ (ratio 1:4)

± Values indicate standard deviations from duplicate ($n=2$ for experiments without FFA addition) and triplicate ($n=3$ for ratios 1:1 and 1:4) experiments

for enrichment of PLs on titania, argued that addition of DHB also reduces binding of neutral lipids such as mono and diglycerides although no data were presented confirming this claim [29].

In our study, we examined the effect of free fatty acids (FFAs) on the binding of PCs to the zirconia material by application of standard mixtures containing the seven PCs and seven FFAs at different PC-to-FFA ratios, namely 1:1 and 1:4. FFA standards included in these experiments were myristic acid (14:0), palmitic acid (16:0), stearic, oleic, linoleic, and linolenic acid (18:0, 18:1, 18:2 and 18:3, respectively), and arachidonic acid (20:4). As can be seen from Table 2, the presence of FFAs had no significant effect

on the binding and recovery of the PCs even when they were added in fourfold excess relative to the PCs. These results, however, may not be applied to other non-phosphorylated matrix components, for example acylglycerides.

Application of the developed clean-up procedure to real samples

To evaluate the usefulness of the developed methodology for selective isolation of GPLs from complex matrices, whole milk was chosen in a first attempt as a “real” sample, because of its known lipid composition and because it is readily available. Prior to the SPE procedure, total lipids were extracted from the milk sample using three different extraction solvents, namely mixtures of methanol–chloroform following the classical procedure proposed by Folch [6], and methanol–MTBE and ethanol–EtOAc which have also been shown to be viable alternatives to chloroform-containing extraction solvents [38, 39]. In order to evaluate possible effects of the milk matrix which could reduce ionization efficiency in the ESI source, for instance via ion suppression and thus may falsify quantitative results [40], aliquots of the different extracts were also analyzed by LC–MS/MS without preceding SPE clean-up.

In Table 3, concentrations determined under the different experimental conditions are compared. For most of the studied analytes, extraction efficiency of the three tested solvent systems was comparable, although concentrations of DPPC, POPC, and DAPC and thus total PC concentration were slightly higher in the ethanolic solvent. This is in line with results described by Lin et al. who also found higher extraction efficiency for phospholipids with ethanolic solvents [38]. Highest concentrations were determined for DPPC, POPC, PLPC and 16:0 lysoPC.

Table 3 Concentration of selected PCs (in µg mL⁻¹ milk) determined in whole milk (3.5% fat) after lipid extraction with different solvents (methanol–chloroform, methanol–methyl *tert*-butyl ether or ethanol–

ethyl acetate) determined by RP-HPLC–ESI-MS/MS with and without preceding SPE clean-up on a zirconia-modified sorbent^a

Analyte	With SPE clean-up			Without SPE clean-up		
	MeOH–CHCl ₃	MeOH–MTBE	EtOH–EtOAc	MeOH–CHCl ₃	MeOH–MTBE	EtOH–EtOAc
16:0 lysoPC	2.16±0.07	2.15±0.001	1.90±0.20	0.33±0.01	0.58±0.15	0.58±0.04
18:0 lysoPC	0.60±0.03	0.65±0.04	0.58±0.04	0.11±0.002	0.17±0.001	0.17±0.03
DMPC	0.78±0.05	0.64±0.04	0.73±0.01	0.75±0.03	0.85±0.11	0.72±0.05
DPPC	4.70±0.20	4.77±0.11	5.51±0.17	3.91±0.50	4.22±0.41	4.80±0.81
PLPC	1.61±0.03	1.26±0.02	1.44±0.005	1.38±0.05	1.62±0.30	1.69±0.03
POPC	2.63±0.12	4.47±0.02	4.73±0.10	2.74±0.03	2.81±0.33	3.06±0.007
DAPC	<LOQ	<LOQ	0.015±0.008	<LOQ	<LOQ	<LOQ
Total	12.48±0.77	13.94±0.23	14.90±0.53	9.22±0.62	10.21±1.26	11.04±0.94

^a Quantitation by external calibration.

± Values indicate standard deviations of duplicate experiments ($n=2$)

Total PC concentrations obtained with the clean-up procedure (about 0.015 mg mL^{-1} milk, corresponding to 0.43 mg g^{-1} fat) agreed well with data available in literature, which are in the range between 0.05 and 0.14 mg mL^{-1} milk [15, 41] (Note, reference data are given for total PC content whereas herein we analyzed seven individual PC analytes). Regarding the occurrence of lysoPCs in milk, Sánchez-Juanes et al. reported concentrations of lysoPCs of about $1.1 \pm 0.3 \text{ } \mu\text{g mL}^{-1}$ in fresh whole milk [42] whereas other studies only found traces of the lyso-derivatives of PLs [41]. Because it was shown in the course of this study that under the clean-up conditions proposed herein no hydrolysis (methanolysis) of the fatty acid residues occurs (vide supra) it can be assumed that the lysoPC contents found in the long-life milk analyzed herein are not a result of careless sample treatment, as has been stated in other studies [43], but can probably be attributed to the ultra-high temperature and homogenization treatment during processing which has been shown to alter phospholipid composition of milk [44] or to enzymatic hydrolysis caused by phospholipases [45].

When lipid extracts were analysed without preceding SPE clean-up, concentrations found were significantly lower for several analytes, indicating the occurrence of matrix effects (Table 3). These effects were especially significant for the lysoPCs for which concentrations in the non-purified extracts were as low as 15%, 27%, and 30% for MeOH-CHCl₃, MeOH-MTBE, and EtOH-EtOAc, respectively, compared with the concentrations determined after clean-up on ZrO₂. Also for DPPC and POPC slight matrix effects were observed when no SPE was carried out prior to LC-MS/MS analysis.

To determine the accuracy of the whole sample treatment, spiking experiments were performed in which

Table 4 Influence of the extraction solvent on the accuracy of the clean-up procedure in sample matrix using zirconia-modified SPE sorbent in sample matrix, determined by spiking of whole milk with individual PC standards^a

Analyte	MeOH-CHCl ₃	MeOH-MTBE	EtOH-EtOAc
16:0 lyso PC	92±10%	87±1%	55±4%
18:0 lyso PC	97±4%	97±3%	93±3%
DMPC	73±1%	102±4%	93±1%
DPPC	97±9%	110±8%	98±3%
PLPC	92±8%	95±2%	67±1%
POPC	108±15%	79±4%	100±2%
DAPC	98±8%	97±2%	96±2%

^a Results expressed as percentage recovery of the spiked standard, concentration of the spike: $1 \text{ } \mu\text{g mL}^{-1}$ milk

Quantitation was by external calibration and correction for the intrinsic PC concentrations

± Values indicate standard deviations of duplicate experiments ($n=2$)

Table 5 Concentrations of selected PCs in human plasma and mouse plasma determined by LC-MS/MS after SPE on ZrO₂ functionalized silica particles^a

Analyte	Human plasma		Mouse plasma	
	$\mu\text{g mL}^{-1}$	$\mu\text{mol L}^{-1}$	$\mu\text{g mL}^{-1}$	$\mu\text{mol L}^{-1}$
16:0 lyso PC	36.48	73.64	42.30	85.39
18:0 lyso PC	14.30	27.32	14.81	28.29
DMPC	11.79	17.40	7.32	10.80
DPPC	14.04	19.14	6.24	8.50
PLPC	281.3	371.2	280.4	370.0
POPC	67.17	88.42	47.88	63.02
DAPC	n.d.	n.d.	n.d.	n.d.
Total	425.1	597.1	398.9	566.0

^a Quantitation by external calibration

n.d., not determined

standards of all the analytes under investigation were added to the milk and recovery of these standards after lipid extraction and SPE clean-up was determined. Accuracy was generally satisfactory—recovery was usually >90% (Table 4). For the ethanolic extracts, however, for two analytes, 16:0 lysoPC and PLPC, recoveries were as low as $55 \pm 4\%$ and $67 \pm 1\%$, respectively.

In addition to whole milk, plasma samples were also analyzed. For these samples isolation and/or enrichment of the analytes may be crucial, because of limited sample availability. Lipid extracts from human plasma and mouse plasma, obtained by Folch extraction, were subjected to SPE using the ZrO₂ functionalized cartridges. The results summarized in Table 5 agree well with reference data from

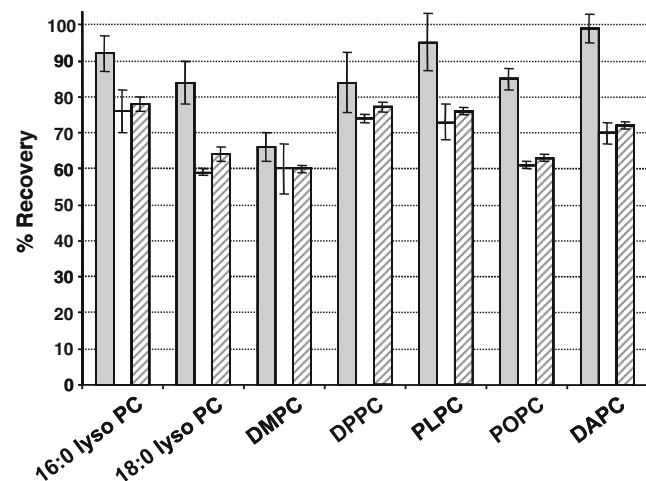


Fig. 4 Recovery of standard PCs from ZrO₂ (grey bars), TiO₂ (white bars), and SnO₂ (dashed bars) materials in batch extraction experiments ($n=3$). Experimental conditions: total PC concentration applied, $0.12 \text{ } \mu\text{g}$ per mg sorbent; volume, $200 \text{ } \mu\text{L}$; amount of sorbent, 6 mg. Binding, washing and elution conditions as outlined in the experimental section

the literature according to which total PC content of both, human and mouse plasma is in the range of 1000–3000 $\mu\text{mol L}^{-1}$ with concentrations of lysoPCs between 200 and 400 $\mu\text{mol L}^{-1}$ [46–48]. Total concentration of the herein analyzed PCs was 597 $\mu\text{mol L}^{-1}$ (425 $\mu\text{g mL}^{-1}$) and 566 $\mu\text{mol L}^{-1}$ (399 $\mu\text{g mL}^{-1}$) in human and mouse plasma, respectively; this is somehow lower compared with reference data, because in this study five individual PCs and two lysoPCs were quantified, not total PC content. Takatera et al., who analyzed lysoPCs in human serum, reported concentrations between 31.3 and 57.9 $\mu\text{g mL}^{-1}$ for 16:0 lysoPC and between 9.5 and 18.6 $\mu\text{g mL}^{-1}$ for 18:0 lysoPC [49]; this is in the same range as the concentrations determined herein (36.5 $\mu\text{g mL}^{-1}$ for 16:0 lysoPC, 14.3 $\mu\text{g mL}^{-1}$ 18:0 lysoPC).

Comparison of ZrO₂, TiO₂, and SnO₂

To compare the selective retention of the PCs on different metal oxide sorbents, the developed SPE procedure was repeated using TiO₂ and SnO₂ materials. A standard mixture containing 0.5 $\mu\text{g mL}^{-1}$ of each analyte in iPrOH–heptane–FA (80:20:1 v/v) was used for these experiments. Because no commercial cartridges comparable with those containing zirconia were available for titania and stannia, experiments with all three metal oxides were simply performed in 1.5 mL centrifuge tubes containing about 6 mg of the loose solid sorbent. ZrO₂ material was removed from commercial SPE cartridges and was weighed into tubes similar to the TiO₂ and SnO₂ materials and clean-up was carried out by shaking of the beads in 200 μL of the respective solvent followed by centrifugation, removal, and analysis of the supernatant.

The first experiments soon revealed that working with the metal oxides in the batch mode was problematic. The zirconia, especially, had a very fine powdery character which hampered centrifugation and thus quantitative removal of the supernatants. Accordingly, recoveries were lower than those obtained when clean-up was performed using the ZrO₂ SPE cartridges. Values as low as 66 \pm 4% were obtained, and reproducibility also was poorer when clean-up was performed statically (RSD up to 18%, $n=3$) (Fig. 4). Interestingly, in these experiments recovery of the lysoPCs from the zirconia material was not markedly lower than that of the PCs.

The performance of the two other metal oxides under identical experimental conditions turned out to be significantly worse than that of the zirconia sorbent. However, recovery of the analytes from titania was comparable with that from stannia (Fig. 4). Recovery of the PC analytes from titania and stannia could probably be improved by fine-tuning of experimental conditions individually for each material; this, however, was not examined in the course of

this study. In addition, performance of the two sorbents in the flow-through mode, for instance using self-packed SPE cartridges remains to be evaluated.

Conclusion

In this study it was shown for the first time that zirconia-modified SPE sorbent is useful for selective enrichment of phosphatidylcholines from complex matrices. Application of the developed SPE procedure to whole milk revealed that clean-up of organic lipid extracts prior to HPLC–ESI–MS/MS analysis is necessary in order to avoid impairment of quantitative results because of ion suppression in the ESI source caused by matrix components. Comparison of different extraction solvents revealed slightly higher extraction efficiency for mixtures of MeOH–MTBE and EtOH–EtOAc compared with classical Folch extraction, indicating that halogenated solvents, for example chloroform, may be easily substituted. In comparison with zirconia, the performance of the other metal oxides tested, titania and stannia, was significantly worse under the same experimental conditions. Evaluation of the metal oxides for enrichment of other PL species, for example phosphatidylethanolamines, phosphatidylserines, or sphingomyelins, and ether-linked GPLs, which are partly characterized by low abundance, remains topic of future work, because these compounds may have different binding and elution characteristics. Oxidized PLs, especially, but also plasmalogens will be of interest because, owing to their base and acid lability, respectively, the proposed binding and elution conditions may turn out to be unsuitable.

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