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Solid-phase extraction approach for phospholipids profiling by titania-coated silica microspheres prior to reversed-phase liquid chromatography–evaporative light scattering detection and tandem mass spectrometry analysis

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ABSTRACT

A novel strategy for selectively adsorbing phospholipids (PLs) on titania-coated silica core–shell microspheres (TiO_2/SiO_2) was developed. The TiO_2/SiO_2 microspheres were prepared through water-vapor-induced internal hydrolysis and then characterized by SEM, UV–vis spectroscopy, X-ray diffraction, and measurements of Brunauer–Emmett–Teller surface area. Analyses showed that the titania layer was uniformly distributed onto the surface of silica particles. The TiO_2/SiO_2 microspheres were employed as sorbent in solid-phase extraction (SPE), and their absorptive ability was investigated by reversed-phase liquid chromatography–evaporative light scattering detection (RPLC–ELSD). Important factors that affect the extraction, such as loading buffer, eluting buffer, and elution volume, were investigated in detail and optimized by using standard samples. Results reveal that the developed SPE approach had higher recoveries for PLs than that based on pure TiO_2 particles. The proposed SPE method was used for extraction of PLs from serum and showed great potential for identifying more kinds of endogenous PL metabolites by ultra performance liquid chromatography with quadrupole time-of-flight mass spectrometry (UPLC–QTOF MS). The proposed SPE method with the composite sorbent was used to screen PLs from a biological matrix with high selectivity and efficiency. This approach is a promising method for selective extraction of PLs in lipidomics or phospholipidomics.

1. Introduction

Phospholipids (PLs), the main constituents of biological membranes, have structural and functional properties. In particular, some PLs participate in many biological processes such as cell proliferation, differentiation, apoptosis, and oxidative stress [1]. In recent years, an increasing amount of research has demonstrated that metabolic disorders involving PLs and related metabolites and enzymes play an important role in many malignant diseases, including cancer of the breast, endometrium, colon, and kidney, as well as acute leukemia, malignant lymphomas, and multiple myeloma [2].

PLs comprise a polar head group with a phosphate moiety and various fatty acids that are attached to the glycerol backbone. According to differences in their polar head groups, PLs may be divided into classes such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), and lysophosphatidylcholine [3]. Each class of PLs is composed of a mixture of molecular species. This structural diversity and complexity poses challenges in the analysis of PLs.

Selective enrichment of PLs from complex biological samples greatly helps in studying the change of PLs, especially some low-abundance species. It is critical to discover more PL signaling molecules, which have profound significance in lipidomics and phospholipidomics. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) protocols are commonly used to isolate PLs from total lipid extracts. The former is mainly based on the Bligh and Dyer extraction method [4,5], which often uses two organic solvents (methanol and chloroform) and involves phase separation [6]. The latter is a technique designed for rapid, selective sample preparation and purification prior to chromatographic analysis. It is used in sample cleanup, recovery, and concentration necessary for accurate quantitative analysis.

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Commonly used solid materials for SPE include porous silica or silica modified with octadecyl, cyanopropyl, aminopropyl, and 2,3-dihydroxypropoxypropyl groups [7]. However, the selectivity and efficiency of these materials for PL extraction is not very high. Metal oxides (e.g., ZrO_2, TiO_2) have higher selectivity and binding affinity for phosphate groups [8,9]. They have, therefore, been used in the enrichment of phosphopeptides for proteomics studies or of PLs from eggs and dairy products for quality control [10–13].

However, the small surface area, small pore volume, and lack of porous structure restrict the application of pure TiO_2. On the other hand, the interfacial electronic structure and interactions between the metal oxide and silica support lead to the porous structure and physicochemical properties of metal oxide layer. Thus, metal oxide-coated mesoporous silica may be an alternative to metal oxides for highly efficient and selective extraction of phosphates [14]. This material can be used as separation, adsorption, and host material [15,16]. Because of the strong affinity of coordinated Ti^{IV} for the phosphate group, highly dispersed Ti^{IV} preferentially enriches typical phosphate-containing substances such as phosphopeptides on the silica surface [17].

We want to establish a novel strategy for phospholipids pretreatment in order to discover more low-abundance phospholipids and improve their response intensity. In the present study, silica core–shell microspheres coated with a titania layer were synthesized through water-vapor-induced internal hydrolysis [18] and then characterized systematically. The material was employed as a sorbent in a SPE column to enrich PL standard compounds by reversed-phase liquid chromatography–evaporative light scattering detection (RPLC–ELSD). It was also used for comprehensive PL metabolic profiling of human serum followed by ultra performance liquid chromatography with quadrupole time-of-flight mass spectrometry (UPLC–QTOF MS). Such an approach featured the high selectivity of silica microspheres coated with metal oxide in adsorbing PLs, and showed promise in applications for pretreatment of biological samples in lipidomics.

2. Experiments

2.1. Chemicals and reagents

HPLC-grade acetonitrile (ACN), methanol (MeOH), isopropanol (IPA) and *n*-hexane were obtained from CNW Technologies GmbH (Germany). Ammonium formate and ammonia solution (7 mol/L in MeOH) were purchased from Sigma-Aldrich (St. Louis, USA). Chloroform ($CHCl_3$), ammonium acetate (AA), and formic acid (FA) were obtained from Sinopharm Chemical Reagent Company (Shanghai, China). Tetrabutyl titanate ($Ti(OC_4H_9)_4$), NH_3/H_2O (28%), titanium dioxide, and silica microspheres (20 μm–120) were purchased from Aladdin (Shanghai, China), Pinghu Chemical Reagent Company (Jiaxin, China), ZhongLan DingHui Material Company (Guangzhou, China), and Suzhou Global Chromatography Company (Suzhou, China), respectively. The water used was MilliQ grade (Millipore, Bedford, MA, USA). All chemicals used in preparing buffer solutions were analytical-grade reagents.

The PL standard solution consisted of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LysoPC (16:0)), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LysoPC (18:0)), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (PC (14:0/14:0), DMPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC (16:0/18:1), POPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE (16:0/18:1), POPE) (Avanti Polar Lipids, Alabaster AL, USA). The purity of all PLs standards is greater than 99%. It was stored at −20 °C and further diluted with MeOH before use.

2.2. Preparation of titania-coated silica composite microspheres

Synthesis of TiO_2/SiO_2 was done through a modification of the water-vapor-induced internal hydrolysis method. Tetrabutyl titanate, an equivalent of 10% (w/w) silica-support, was dissolved in ethanol at a ratio of 1:4 (v/v). The mixture was sealed and then vortexed for 5 min. The ethanol solution was wet-impregnated into the pores of silica under stirring at 60 °C until dryness. The silica was stored at 100 °C for 6 h. Afterward, 1 g of titania-precursor-loaded silica was placed in an open glass vial and kept inside an autoclave containing 20 mL water. There was no direct contact between the solid and water. The sample was heated at 60 °C for 5 h. Subsequently, the silica was removed and dried completely at room temperature. After the desiccated sample was calcined under air at 500 °C for 5 h, 10 wt% TiO_2/SiO_2 microspheres were obtained. Through similar procedures, 7.5 and 5 wt% TiO_2/SiO_2 microspheres could be obtained as well. Pure titanium dioxide was used as the reference material.

2.3. Characterization of TiO_2/SiO_2 composite microspheres

Scanning electron microscopy (SEM) images were obtained on an S-4800 field emission scanning electron microscope (Hitachi, Japan) with an accelerating voltage of 25 kV. Powder X-ray diffraction (XRD) was performed on a D/max-2600PC X-ray diffractometer (Rigaku Corporation, Japan) using Cu Kα radiation ($\lambda = 0.1542$ nm) and operating at 20 kV and 50 mA. N_2 adsorption–desorption experiments were undertaken at 77 K on an automatic ASAP 2020M Micromeritics apparatus. Before adsorption, the materials were degassed at 573 K for 10 h. Brunauer–Emmett–Teller (BET) surface areas were calculated from data on adsorption at relative pressure range of 0.05–0.25, and pore size distributions were calculated from the adsorption branches of the isotherms through the Barrett–Joyner–Halenda (BJH) method. Sorption experiments were performed on an ASAP 2020M surface area and pore size analyzer (Micromeritics Inc., USA). Ultraviolet–visible diffuse reflectance spectroscopy (UV–vis DRS) was performed on a Lambda 950 UV–vis spectrophotometer (Perkin-Elmer Inc., USA).

2.4. Serum sample collection and lipid extraction

Human serum was obtained from healthy volunteers at Ruijin Hospital (affiliated to Shanghai Jiao Tong University). The serum samples were prepared by pooling and mixing the same volume of each sample from 15 healthy volunteers. The total lipids fraction was obtained by LLE according to a modification of the Bligh and Dyer method. In this method, 30 μL of water followed by 150 μL of MeOH was added to 30 μL of human serum. The resulting solution was vortexed for 60 s. Afterward, 300 μL of $CHCl_3$ was added, and the solution was vortexed for another 60 s. Subsequently, 150 μL of water was added to the solution and thoroughly mixed to form a two-phase system. After centrifugation at 10,000 rpm for 10 min, the lower organic phase was collected. The remaining solution was then extracted again with $CHCl_3$. The two lipid extracts were combined and dried under vacuum, and the residue was redissolved in 180 μL of IPA/hexane (8/2, v/v) solution with 1% FA.

2.5. PLs extraction with the TiO_2/SiO_2 SPE cartridge

2.5.1. The recovery of PLs standard with TiO_2/SiO_2 cartridge

The synthesized TiO_2/SiO_2 microspheres (30 mg) were packed into 1 mL SPE columns with top and bottom frits. Columns were prewashed with MeOH and equilibrated by IPA/hexane (8/2, v/v) solution. Lipid extract or standard mixture (150 μL) combined with 300 μL of IPA/hexane (8/2, v/v) solution with 1% FA was added to the TiO_2/SiO_2 SPE columns, and effluent (loading solution, SPE–LS)

was collected. After the sample was applied, the columns were washed with 300 μL of IPA/hexane (8/2, v/v) solution and the effluent (washing solution, SPE–WS) was collected. The PLs were then eluted thrice with 300 μL of 7 mol/L ammonia in MeOH, and then the three eluted solutions were combined (the pooled solution is hereafter referred to as eluting solution, SPE–ES). SPE–LS, SPE–WS, and SPE–ES were dried under vacuum and redissolved in 150 μL of MeOH prior to analysis. Three replicates were prepared to investigate the standard deviation of the recovery. For the reference TiO_2, centrifugation steps instead of SPE steps were used to isolate PLs from the total lipids fraction because the particle size of TiO_2 was too small to be allowed as SPE packing material.

2.5.2. The recovery of PLs from serum with TiO_2/SiO_2 cartridge

To determine the recovery of PLs from human serum with TiO_2/SiO_2 SPE cartridge, three different kinds of samples were prepared. For sample 1, 30 μL of serum sample was pretreated according to 2.4, and PLs were extracted with TiO_2/SiO_2 SPE followed 2.5.1 protocol. The peak areas of five targeted endogenous PLs, i.e. LysoPC (16:0), LysoPC (18:0), DMPC, POPC and POPE from serum were analyzed with UPLC–MS and labeled as S_1. For sample 2, 18 μL of 100 μg/mL of LysoPC (16:0), LysoPC (18:0), DMPC, POPC and POPE were spiked into 30 μL of serum sample, and the sample was pretreated, extracted and analyzed with the same procedure as sample 1. The peak areas of five PLs were labeled as S_2. For sample 3, 30 μL of serum sample was pretreated and extracted by the same SPE approach as sample 1. Afterwards, the same amount of five PLs standards were respectively spiked prior to UPLC–MS analysis. The areas obtained were labeled as S_3. Each kind of samples was prepared and analyzed in triplicate. The recoveries of PLs were calculated according to the following formula:

$$RE\% = \frac{S_2 - S_1}{S_3 - S_1} \times 100\%$$

2.6. HPLC–ELSD analysis

Optimization of experimental conditions for recovery of PLs from TiO_2/SiO_2 SPE column was performed on a Shimadzu LC-20AB HPLC (Kyoto, Japan) equipped with a UM-3000 ELSD detector (Unimicro Technologies, Shanghai, China). The separation was conducted on a C_8 column with dimensions 4.6 mm i.d. × 250 mm, 5 μm particle size. The mobile phase consisted of (A) 10 mmol/L AA-ammonium acetate in MeOH/water (80/20, v/v) solution and (B) 10 mmol/L AA-ammonium acetate in MeOH. Flow rate was 1 mL/min. The gradient conditions were as follows: 0–15 min, 50–100% B; 15–30 min, 100% B. The column temperature was 35 °C. ELSD evaporating temperature was set at 45 °C. Compressed air (from XWK-3A air pump, Huasheng Analytical Instrument, Tianjin, China) was used as the nebulizing gas for ELSD, and was introduced at 2.5 L/min with a pressure of 3.55 bar.

2.7. UPLC–QTOF MS analysis

An Acquity™ UPLC system (Waters Corp., Milford, MA, USA) was used. Reversed-phase separation was performed on a C_8 column (2.1 × 100 mm 1.7 μm; Thermo Fisher). The mobile phase consisted of (A) 10 mmol/L ammonium formate in ACN/water (3/2, v/v) solution and (B) 10 mmol/L ammonium formate in MeOH. Gradient elution without splitting was carried out for 25 min at a flow rate of 0.4 mL/min. Gradient conditions were as follows: 0–4 min, 5–50% B; 4–6 min, 50–80% B; 6–9 min, 80–85% B; 9–15 min, 85–90% B; 15–16.5 min, 90–100% B; 16.5–22.5 min, 100% B; 22.5–23 min, 100–5% B; 23–25 min, 5% B. A 5 μL aliquot of each sample was injected into the column. The column temperature was kept at 45 °C. All samples were incubated at 4 °C during analysis.

Mass spectrometry was performed on a Waters Q-TOF Micromass (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) source operating in positive ion mode. The capillary voltage was set at 3.0 kV and the cone voltage was set at 35 kV. Nitrogen was used as desolvation and cone gas. The flow rate and temperature of the desolvation gas was set at 700 L/h and 350 °C, respectively, and the flow rate of the cone gas was set at 50 L/h. Source temperature was set at 105 °C. Argon was employed as collision gas and the collision energy was set at 4.0 eV. The data acquisition rate was set at 0.3 s with 0.02 s interscan time. Data between *m*/*z* 250 and 1500 were recorded in continuum mode. Lock mass calibration was applied by using a solution of leucine enkephalin (200 ng/mL, *m*/*z* 556.2707 for positive ion mode) introduced at 0.1 mL/min.

2.8. Data pre-processing and data analyses

UPLC–QTOF MS electrospray ionization (ESI) raw data were analyzed by MarkerLynx Applications Manager version 4.1 (Waters Corp., Milford, MA, USA). Parameters used were 0.5–24 min retention time (RT), 250–1500 Da mass range, and 0.02 Da mass tolerance. Minimum intensity was set to 15% of base peak intensity (BPI). Maximum mass per RT was set at 6 and RT tolerance was set at 0.01 min. A list of the ion intensity of each peak detected was generated by using the RTs, and *m*/*z* data pairs were used as identifiers for each ion. The resulting three-dimensional matrix contains the peak index (RT-*m*/*z* pairs), sample names (observations), and ion-intensity information (variables). Compounds were identified by using the online metabonomics METLIN database (http://metlin.scripps.edu/)

3. Results and discussion

3.1. Characterization of TiO_2/SiO_2 microspheres

Direct observation of TiO_2/SiO_2 particles was done by SEM, which revealed that the TiO_2-coated SiO_2 microspheres had the similar morphology to that of mesoporous silica particles. This similarity also proves that the porous structure of the microspheres was not damaged by the preparation process and that the TiO_2/SiO_2 microspheres were ordered. At least one TiO_2 layer was coated on the surface of the SiO_2 microspheres.

Wide-angle XRD patterns of the synthesized TiO_2/SiO_2 microspheres (Fig. 1) display the broad diffraction peak at 22°, which is the characteristic reflection of amorphous SiO_2. No peaks corresponding to crystalline TiO_2 in all three samples of TiO_2/SiO_2 microspheres appeared, suggesting that the TiO_2 layer coated on the pore wall was amorphous or consisted of particles that were too small to allow detection of Bragg diffraction. Intensities of all diffraction peaks of all TiO_2/SiO_2 microsphere samples increased with increasing titanium content. This variation might be caused by the change in electron density distribution of the core–shell structure following the formation of at least a monolayer of titania. That is, on transition from the center of the pore to the SiO_2 wall, the electron density function reaches a maximum in the titania monolayer [19].

N_2 adsorption–desorption isotherms (Fig. 2A) provide information on the pore variation. Isotherms of the three samples are typical IV curves with H1 hysteresis loops at relative pressure above 0.7, indicating the presence of mesopores. The BJH plot of pore diameter distribution shows that the average pore size for both SiO_2 and TiO_2/SiO_2 materials was about 12 nm, as shown in Fig. 2B. The BET surface areas for SiO_2, 10 wt% TiO_2/SiO_2, 7.5 wt%

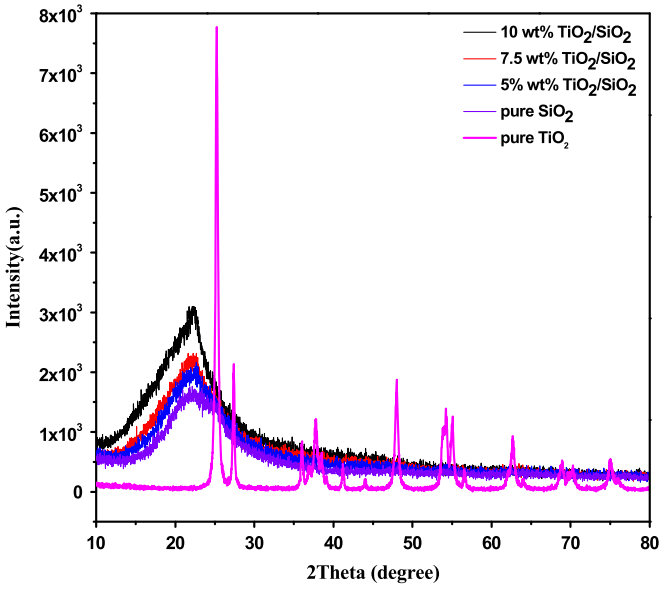

Fig. 1. XRD spectra of the SiO_2, TiO_2, 5 wt% TiO_2/SiO_2, 7.5 wt% TiO_2/SiO_2 and 10 wt% TiO_2/SiO_2.

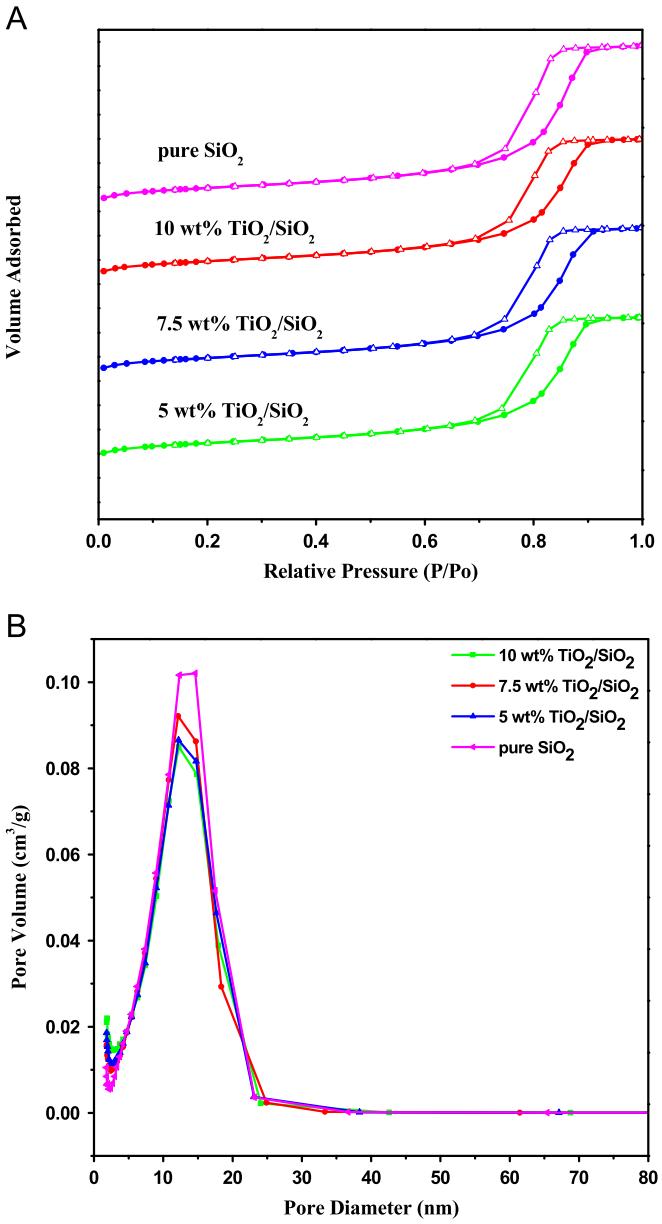

Fig. 2. (A) N_2 adsorption–desorption Isotherm plot of SiO_2, 5 wt% TiO_2/SiO_2, 7.5 wt% TiO_2/SiO_2 and 10 wt% TiO_2/SiO_2 and (B) pore size distribution curve from the BJH Adsorption of SiO_2, 5 wt% TiO_2/SiO_2, 7.5 wt% TiO_2/SiO_2 and 10 wt% TiO_2/SiO_2.

TiO_2/SiO_2 and 5 wt% TiO_2/SiO_2 microspheres were 346.9385, 338.2478, 341.1507, and 335.8712 m^2/g, respectively. The surface areas of the TiO_2/SiO_2 microsphere samples were much larger than that of pure TiO_2, which was only 36.1042 m^2/g, even if they were slightly smaller than the surface area of pure SiO_2. These results suggest that titania was uniformly dispersed on the surface of the mesoporous silica particles without blockage of the mesopores.

The environment of the Ti metal center on the mesoporous silica surface can be elucidated from the UV–vis DRS spectra (Fig. 3). No diffraction peaks related to the TiO_2 crystalline structure were observed [20]. The adsorption band around 240 nm corresponds to the charge-transfer transition between Ti^{4+} and O^{2-} of the lattice, and the adsorption band at 290 nm is due to hexacoordinated titanium, which contains Ti–O–Ti bonds. The absorption band of the TiO_2/SiO_2 materials (corresponding to Ti–O–Ti bonds) remarkably shifted toward shorter wavelengths relative to those for the TiO_2 reference. The blue shift of the band edge indicates an increase in dispersion of the titanium species [21]. Therefore, UV–vis DRS plots reveal that the titania was highly dispersed on the mesoporous silica surface.

3.2. Development and optimization of the TiO_2/SiO_2 SPE method

Sample pretreatment is critical in biochemical analysis because of the complexity of the biological matrix. SPE is the technique of choice for sample cleanup and trace enrichment. In our experiment, the synthesized TiO_2/SiO_2 material was packed into the SPE column for selective extraction of PLs. Several parameters that influenced the efficiency of PL extraction by TiO_2/SiO_2 microspheres packed in the SPE column were investigated and optimized. A PL mixture of the standards LysoPC (16:0), LysoPC (18:0), DMPC, POPC, and POPE was used. RPLC–ELSD was performed for sample quantification and detection.

The effect of the amount of TiO_2 coated on the SiO_2 surface on the extraction recovery of PLs was the first extraction parameter investigated (Table 1). The lowest recovery ratios for PLs were obtained with pure TiO_2 sorbent. This result is due to the relatively low surface area and microporosity of TiO_2. Recoveries of the five PLs increased with increasing amount of TiO_2 coated on the SiO_2 surface, confirming the importance of coating the silica with metal oxide for enrichment of PLs. Larger amounts of tetrabutyl titanate could strongly enhance the speed of tetrabutyl titanate hydrolysis, resulting in a large number of titania nuclei in a short time; thus, titanium dioxide particles without silica core are easily formed. Therefore, 10 wt% TiO_2-coated silica microspheres was the optimum sorbent for SPE cartridges. They were used for further experiments.

Effects of the loading and eluting buffer on the recovery were also investigated. Retention of PLs on the solid metal oxide sorbent is presumably based on Lewis acid–Lewis base interactions. Titanium oxide is known to have amphoteric properties, that is, it can behave as a Lewis acid or Lewis base depending on the pH of the reaction solution. At lower pH, TiO_2 behaves as a Lewis acid with positively charged titanium atoms, displaying high binding affinity for phosphate ions. At higher pH, it exhibits Lewis base characteristics. Thus, the adsorption of PLs on metal oxide proceeds under acidic condition, whereas desorption occurs under alkaline condition.

Effects of the three acidic loading buffers, namely, IPA/hexane (8/2, v/v) with 1% FA, MeOH with 1% FA, and MeOH with 1% AA,

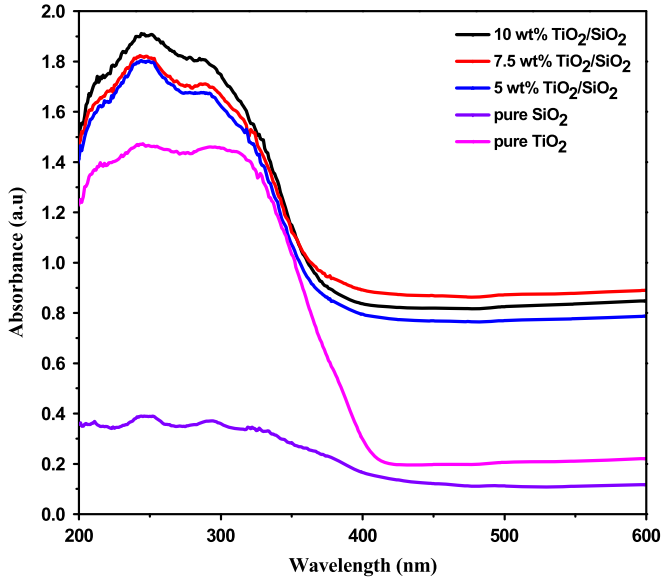

Fig. 3. UV–vis diffuse reflectance spectra of the SiO_2, TiO_2, 5 wt% TiO_2/SiO_2, 7.5 wt% TiO_2/SiO_2 and 10 wt% TiO_2/SiO_2.

Table 1
Recoveries of PLs standards using different amount TiO_2-coated material used as the sorbent ($n=3$).

Recovery ratio	10 wt% TiO_2/SiO_2	7.5 wt% TiO_2/SiO_2	5 wt% TiO_2/SiO_2	Pure TiO_2
LysoPC16:0	93.9 ± 2.8	89.0 ± 2.6	81.0 ± 2.4	53.3 ± 7.6
LysoPC18:0	85.1 ± 2.4	78.7 ± 2.8	76.3 ± 1.0	43.2 ± 4.4
DMPC	85.5 ± 3.7	71.0 ± 1.5	67.9 ± 4.6	36.6 ± 4.2
POPE	89.2 ± 4.1	85.3 ± 4.2	79.7 ± 3.8	34.6 ± 7.8
POPC	90.1 ± 1.3	83.0 ± 1.4	77.7 ± 3.9	28.9 ± 11.6

on the recovery of PLs were compared. The highest recovery for the five PLs was achieved by using IPA/hexane (8/2, v/v) containing 1% FA as loading buffer. The influence of alkaline solutions, namely, MeOH/ammonia (95/5, v/v), chloroform/MeOH/ammonia (75/20/5, v/v/v), and 7 mol/L ammonia in MeOH, on the extraction recovery for PLs was also investigated. Greater amounts of PLs were desorbed by using 7 mol/L ammonia in MeOH as eluting buffer.

The volume of eluent directly influences the recovery of targeted analytes and must be sufficient to elute the compounds of interest from the sorbent. The effects of elution volume (300–1200 μL) were monitored and results show that the recoveries of PLs increased with increasing elution volume from 300 to 900 μL. Above 900 μL, recoveries for the five PLs did not increase further. Thus, to reduce the consumption of solvent, the smallest satisfactory volume for eluting PLs from 10 wt% TiO_2/SiO_2 SPE cartridges was 900 μL, which was selected as the elution volume for further experiments.

3.3. Method of determination of PL standards by using TiO_2/SiO_2 SPE and RPLC–ELSD

3.3.1. Method of investigation of the five PLs standards

Five endogenous PLs were selected as the standards to evaluate the recovery through the TiO_2/SiO_2 SPE approach. The RPLC–ELSD chromatograms of the standard mixture subjected to the loading, washing, and eluting steps are shown in Fig. 4. It is obvious that no peaks corresponding to the five PLs appeared after the loading and washing steps (Fig. 4B,4C), indicating the high affinity of the TiO_2/SiO_2 SPE column for the PL standards. Peaks for the five PLs

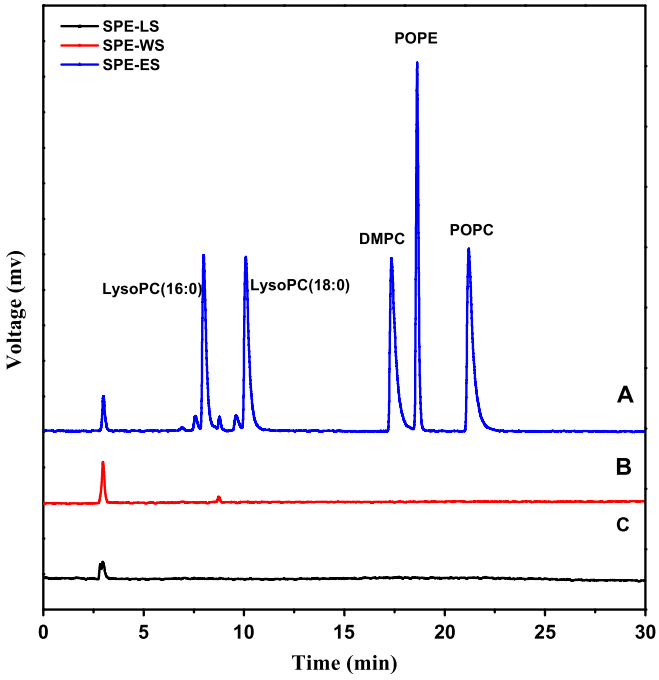

Fig. 4. RPLC–ELSD chromatogram of five phospholipids standard for (A) SPE–ES; (B) SPE–WS; (C) SPE–LS.

appeared only in the chromatogram of the eluting solution (Fig. 4A), demonstrating the effectiveness of the SPE approach.

3.3.2. Method validation

Linear equations, correlation coefficients (R), significance of F-statistics, linear range, limit of detection (LOD), and limit of quantitation (LOQ) were determined under optimized conditions to assess the practical application of the method developed for PL analysis. Results are summarized in Table 2. R value was found to be between 0.996 and 0.999, which showed excellent goodness-of-fit of linearity. The significance of F-statistics ($1.35–9.11 \times 10^{-7}$) was substantially smaller than the significance level ($\alpha=0.05$), which demonstrated that the linear relationship between $Y=\log 10$ (peak area of PLs) and $X=\log 10$ (concentration of PLs) was quite significant. LODs were in the range of 0.5–1.0 μg/mL at signal-to-noise ratio (S/N) of 3, and LOQs were in the range of 2.5–5.0 μg/mL at S/N of 10.

3.4. Extraction of PLs from human serum by TiO_2/SiO_2 SPE followed by UPLC–QTOF MS

PLs in human serum have attracted increasing interest because of their important role in explaining biological and pathological variations and in discovering useful biomarkers for cancers. Because the structures of PLs are very complicated and the complex matrix may obscure signals of some low-abundance subclasses, analysis of PLs in human serum is difficult. By far, applications of metal oxides in PLs extraction are quite rare and mostly focus on the use of pure metal oxide via centrifugation instead of the use of metal oxide-coated silica composites in SPE cartridge.

The above experiments suggest that the TiO_2/SiO_2 SPE cartridges exhibited high efficiency of extraction of the PL standards. In addition, recoveries of five PLs standards from human serum had also been investigated. The results showed that good recoveries (76.7–91.5%, RSD < 8%) were achieved for five PLs standards from serum with the TiO_2/SiO_2 SPE cartridge.

To further demonstrate whether this approach could selectively adsorb PLs from a complex biological sample, the SPE method was applied to adsorb endogenous PLs from human serum prior to analysis by UPLC–QTOF MS.

In our experiment, C_8 instead of C_{18} reversed-phase column was used to elute more compounds from the column. MeOH/ACN/water solution containing ammonium formate was the mobile phase and positive ESI was the ionization mode in LC–MS. PC and SM are more ionizable in positive ion mode than in negative ion mode. Thus, the compounds eluted around 2–6, 9–14, and 20–24 min might be LysoPC, PC and SM, and triacylglycerols (TGs), was compared on the basis of metabolites selected according to their occurrence in human serum. Fig. 5 presents the extracted ion chromatograms of four different PL metabolites and their corresponding tandem mass spectra: LysoPC (16:0) (*m*/*z* 496.33), LysoPC (18:0) (*m*/*z* 524.37), PC (16:0/18:1) (*m*/*z* 760.58), and *N*-(tetracosanoyl)-4*E*, 14*Z*-sphingadienine-1-phosphocholine, SM (d18:2/24:0) (*m*/*z* 813.68). Evidently, the response intensity of the eluting fraction is higher than that obtained by analysis without the SPE approach. This further proves that the SPE cartridge strongly absorbed PLs, thereby allowing enrichment and detection of more PCs.

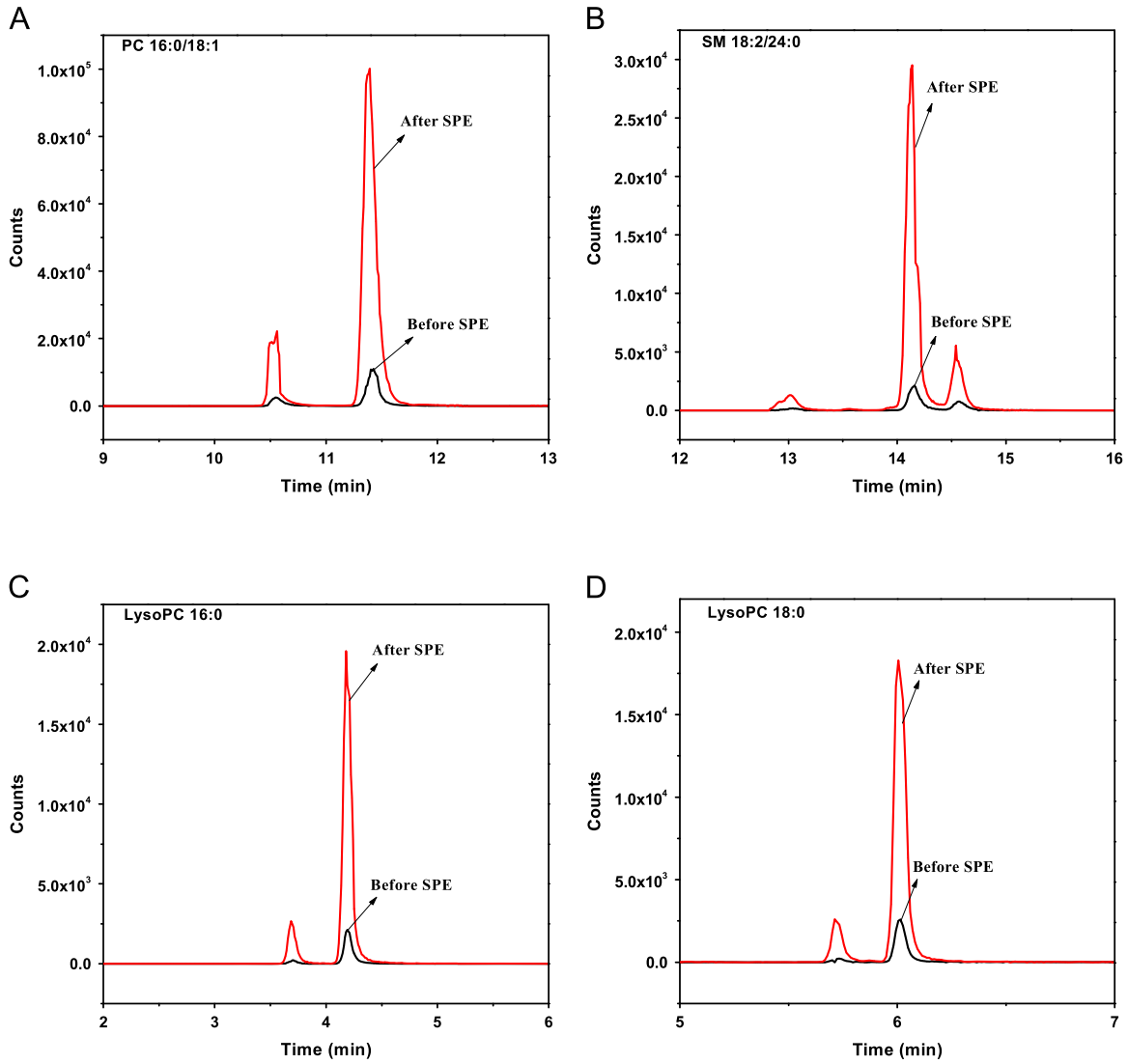

Fig. 5. Extracted ion chromatograms and tandem MS from UPLC–QTOF MS in positive ion mode for (A) PC 16:0/18:1 (*m*/*z* 760.58); (B) SM d18:2/24:0 (*m*/*z* 813.68), (C) LysoPC 16:0 (*m*/*z* 496.33); (D) LysoPC 18:0 (*m*/*z* 524.37).

respectively, as indicated by data from RT, *m*/*z* ratio, and tandem MS. Comparison of samples extracted without using SPE and with SPE procedures was made. The loading and washing fraction were mainly composed of TGs, as can be inferred by the relevant *m*/*z* ions and RT. This result suggests that most of the TGs were removed by the SPE column packed with titania-coated silica microspheres. After the elution step, peaks for more PLs are clearly manifested in the spectrum. These observations indicate that the TiO_2/SiO_2 SPE column strongly adsorbed PLs.

The response intensity of extracts obtained without the SPE approach (before SPE) and with the SPE eluting fraction (after SPE)

To explore further the role of metal oxide-coated silica SPE column for lipidomics, a Venn diagram technology was used to compare molecular features determined with and without using SPE prior to UPLC–QTOF MS. Molecular features obtained after loading, washing, and elution steps were combined and compared with those obtained without using SPE method. Without SPE extraction, 662 molecular features were observed, whereas 1527 molecular features were detected with the SPE approach. The set of molecular features obtained without SPE extraction shows approximately 76.7% overlap with that obtained with SPE extraction. The SPE method provided 66.7% larger number of unique

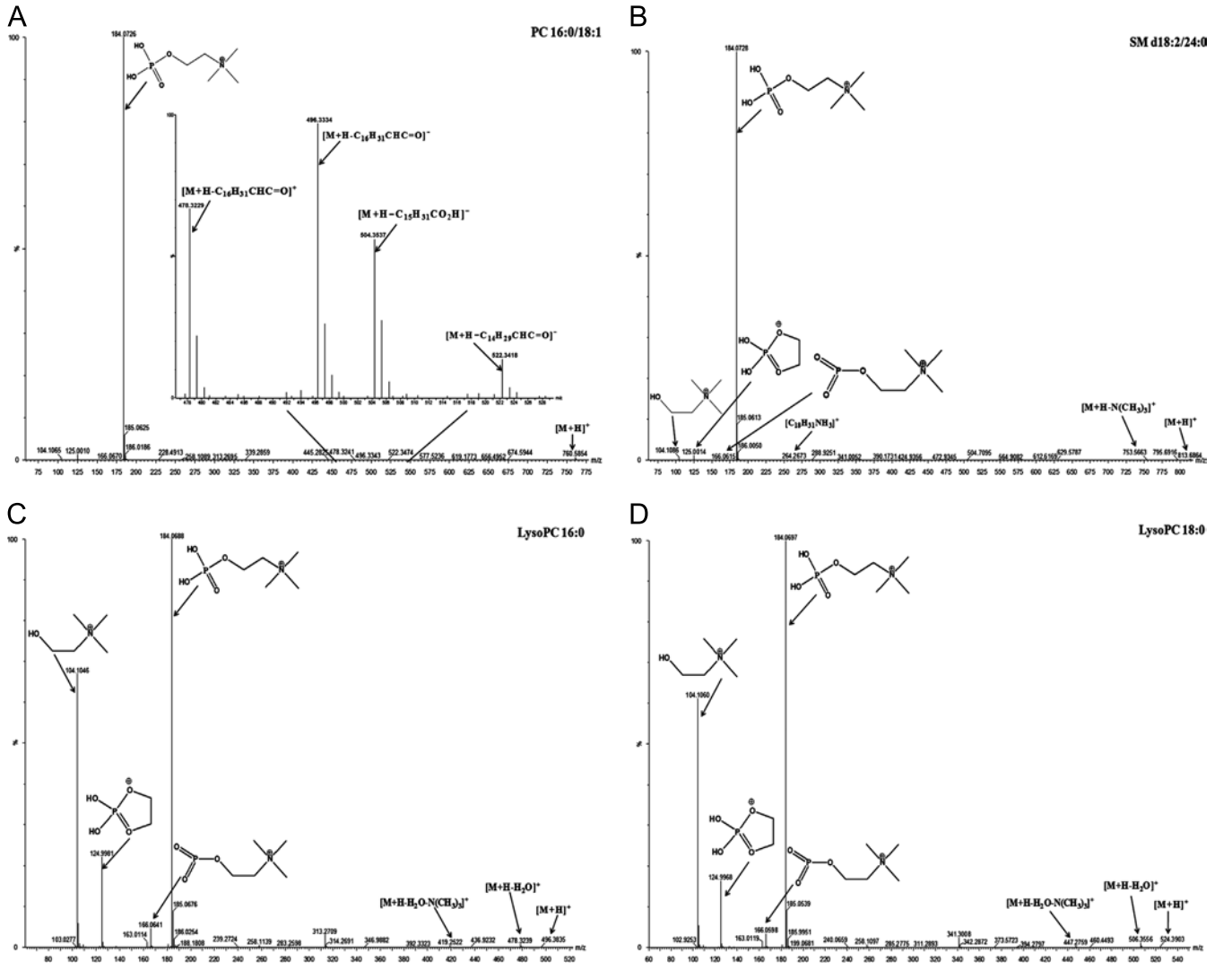

Fig. 5. (*continued*)

Table 2
Analytical parameters of SPE using 10 wt% TiO_2/SiO_2 as sorbent for determination of five PLs.

Analytes	Regression equation[a]	Correlation coefficient (*R*)	Significance *F*	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
LysoPC (16:0)	$Y=1.737X+0.972$	0.992	1.40E−7	30–300	0.6	3.0
LysoPC (18:0)	$Y=1.847X+0.616$	0.998	1.35E−7	40–400	1.0	2.5
DMPC	$Y=1.790X+0.563$	0.998	1.68E−7	50–500	1.0	2.6
POPE	$Y=1.750X+1.579$	0.997	9.11E−7	15–150	1.0	5.0
POPC	$Y=1.719X+0.798$	0.994	3.54E−7	50–500	0.5	2.8

[a] $X=\log_{10}$ (concentration of PLs), $Y=\log_{10}$ (peak area of PLs).

molecular features compared with that observed without using the SPE method. These results show that SPE provides a greater chance of finding more lipid metabolites from serum.

4. Conclusions

Mesoporous silica microspheres coated with well-dispersed titania layer were synthesized and applied to specifically adsorb PLs. Their high binding affinity for PLs could be attributed to the amounts of coordinatively unsaturated titanium species dispersed on the mesoporous silica surface, large surface area, and large pore volume. SPE based on this material was performed in the highly selective and efficient isolation of PLs from a complex biological matrix. It not only increased the response intensity for metabolites such as sphingomyelin and phosphoglyceride, but also enabled discovery of more PL features in human serum, especially those that are low-abundance metabolites. This protocol offers a new means of large-scale extraction of PLs as well as applications for biomarker discovery in phospholipidomics.

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