Analytica Chimica Acta 1278 (2023) 341673

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Bifunctional Ti^{4+}-modified paper for selective extraction or removal of phospholipids and paper spray mass spectrometry for bioanalysis in urine and plasma

Wei Luo [a,b], Teris A. van Beek [b], Bo Chen [a,**], Han Zuilhof [a,b,***], Gert IJ. Salentijn [b,c,*]

[a] Key Laboratory of Phytochemical R&D of Hunan Province and Key Laboratory of Chemical Biology & Traditional Chinese Medicine Research of Ministry of Education, Hunan Normal University, Changsha, 410081, China
[b] Laboratory of Organic Chemistry, Wageningen University, 6708, WE Wageningen, the Netherlands
[c] Wageningen Food Safety Research (WFSR), Wageningen University & Research, 6700, AE Wageningen, the Netherlands

HIGHLIGHTS

- Ti^{4+}-modified paper spray tips for selective phospholipids enrichment/removal.
- Ti^{4+} modified paper spray mass spectrometry (TiPS-MS/MS) was developed for analysis of phospholipids.
- Fast extraction/analysis of phospholipids in urine demonstrated at physiological levels.
- TiPS-MS/MS was developed for reducing matrix effects in plasma.

ARTICLE INFO

Keywords:
Paper modification
Paper spray mass spectrometry
Selective enrichment
Phospholipids
Matrix effect

ABSTRACT

Background: Phospholipids (PLs) are major constituents of cell membranes, play important roles in cell proliferation and death, as well as in signal transduction, and therefore are relevant biomarkers for different pathologies. On the other hand, when the analysis of small compounds, such as therapeutics in blood is desired, then phospholipids are part of the matrix and cause serious interference during analysis. Currently, both the analysis and removal of PLs from biological samples are limited by extensive sample preparation and instrumental separation.

Results: A fast and simple quantitative Ti^{4+}-modified paper spray tandem mass spectrometric (TiPS-MS/MS) method was established in urine, where the enrichment of phospholipids was achieved, as well as reduction of matrix effects (primarily caused by high salt content) that ultimately led to improved sensitivity and selectivity. The method could achieve a physiologically relevant limit of detection (0.01–0.03 μg mL^{-1}). Also, the usefulness of the Ti^{4+}-modified paper was investigated in the opposite mode, namely for the selective removal of phospholipids from matrices such as plasma. Clonidine is used as model compound, as the detection of this compound is known to suffer from ion suppression by phospholipids. Compared with blank paper spray tandem mass spectrometry, the limit of detection could be improved from 0.3 μg mL^{-1} to 0.03 μg mL^{-1} by employing a Ti^{4+}-modified paper on top of the spray tip to capture phospholipids from the sample.

Significance and novelty: A novel Ti^{4+}-modified paper was developed to allow for rapid solid-phase extraction of phospholipids from urine or selective removal from plasma, followed by direct paper spray mass spectrometric detection as a fast and convenient sample preparation and analysis combination. The paper properties are based on the Ti^{4+} metal ion, which can selectively bind phosphate-containing compounds under acidic conditions, and its applicability was demonstrated in relevant biological matrices.

* Corresponding author. Laboratory of Organic Chemistry, Wageningen University, 6708, WE, Wageningen, the Netherlands.
** Corresponding author. Key Laboratory of Phytochemical R&D of Hunan Province, Key Laboratory of Chemical Biology & Traditional Chinese Medicine Research of Ministry of Education, Hunan Normal University, Changsha, 410081, China.
*** Corresponding author. Laboratory of Organic Chemistry, Wageningen University, 6708, WE, Wageningen, the Netherlands.
E-mail addresses: dr-chenpo@vip.sina.com (B. Chen), Han.Zuilhof@wur.nl (H. Zuilhof), gert.salentijn@wur.nl (G.IJ. Salentijn).

https://doi.org/10.1016/j.aca.2023.341673
Received 6 June 2023; Received in revised form 28 July 2023; Accepted 29 July 2023
Available online 31 July 2023

1. Introduction

Phospholipids (PLs) are major constituents of cell membranes, and they play important roles in cell proliferation and death, as well as in signal transduction [1]. The PL composition of cell membranes is complex, due to diverse structural combinations of polar head groups and acyl chain lengths with variations in the degree of unsaturation. Moreover, this composition depends on the (patho-)physiological state of cells and the organism, and as such can be used as indicator thereof [2]. Due to this potential of PLs as biomarkers for diseases, lipidomics research is of interest to determine the relative changes in composition and concentration of PLs in cells and biological fluids [3]. Especially urinary phospholipids have attracted increasing attention in current biomedical research, as urine is an easily available and noninvasive source for the discovery of biomarkers. Urinary phospholipids have been shown to be sensitive biomarkers for kidney injury, prostate cancer, and nephropathy [4]. As such, especially the (relative) concentrations of two common PLs, phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) are of great importance, with their concentration in urine from kidney disease patients exceeding 0.1 μg mL^{-1}, compared to less than 0.04 μg mL^{-1} in healthy individuals [5–9].

The analysis of urinary PLs has been described in literature with high-end analytical equipment, and the availability of such instruments is restricted to well-equipped laboratories [10,11]. Tipthara et al. used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), which required a lipid extraction prior to analysis. Zhang et al. used electrospray ionization quadrupole time-of-flight mass spectrometry coupled with ultra-high pressure liquid chromatography (UHPLC-ESI-Q-TOF), relying on the chromatography for achieving selectivity. These methods all require certain pretreatment or column-based separation. To overcome the limitations of such approaches, ambient ionization mass spectrometry (AIMS) has been increasingly applied in clinical and point-of-care settings to decrease time-to-result, improve throughput, and allow more widespread applicability [12–14]. In medical diagnostics, for example, highly specific molecular information in real time for clinical and even point-of-care analysis has been achieved. In our recent work, boronate affinity paper spray mass spectrometry was developed for the detection of catecholamines in urine [15]. The boronate affinity paper contains boronic acid groups that bind catecholamines under alkaline conditions and release them under acidic conditions, thereby demonstrating the potential of combining selective sample pretreatment for enrichment, and direct analysis using the same paper substrate.

While the analysis of PLs is certainly important, PLs can equally be cause for concern in cases that it is not the analyte, but rather simply part of the matrix. Given the fact that PLs represent a major class of endogenous compounds, substantial matrix effects can occur in biological samples, such as plasma (total PL concentration of 750–1540 μg mL^{-1}) [16]. In particular, the highly ionic nature of PLs makes them responsible for influencing the ionization in the electrospray process, often leading to ion suppression, and decreased sensitivities [17]. Therefore, one of the major challenges in ESI-based bioanalysis is addressing matrix effects caused by endogenous phospholipids, not only because of their pronounced impact on analyte response, but also due to the fact that they are not easily removed from samples due to their amphiphilic nature [18,19]. Currently, a number of strategies are commonly used for phospholipid removal from biological samples [20,21], such as solid phase extraction (SPE) based on cation exchange materials, and liquid-liquid extraction (LLE). Still, such methods require additional steps, consumables and equipment, and it would be more convenient to have such sample preparation integrated with the direct analysis. Again, ambient ionization is an attractive candidate for the development of such a solution, such as recently been demonstrated by the application of a restricted access material combined with paper spray ionization to allow for the selective removal of proteins [22]. Other examples includes work by Liu et al., in which paper was modified with 1-[3-(trimethoxysilyl)propyl]urea to improve the sensitivity of PS-MS analysis in negative mode for salicylic acid in urine, by the selective removal of anions and highly polar compounds, thus decreasing competitive ionization [23]. The aim of the current work has been to develop a paper-based solid-phase extraction material that could be used for both the selective enrichment, and the selective removal of PLs from samples. Given the fact that immobilized metal affinity chromatography (IMAC) is widely used in the separation and purification of compounds containing phosphate groups in biomacromolecules [24,25], we envisioned that metal-ion-modified paper would display a high affinity for phospholipids, and as such could be used for both these purposes. Ti^{4+}-modified materials have been widely used as adsorbents for selective separation and molecular recognition of substances containing phosphate groups, especially for HPLC pretreatment [26,27]. Such compounds can be combined with metal ions under acidic conditions and desorbed under alkaline conditions. This affinity is based on the fact that transition metals (e.g. Ti^{4+}) can coordinate with electron-rich atoms such as phosphorus. The phosphate group is a strong Lewis base that can interact with Ti^{4+} as Lewis acid under acidic conditions [28]. Several studies have shown that this metal coordination is highly selective [29,30]. However, this specific affinity material has not been applied for paper modification in PS-MS, nor to improve the selectivity and sensitivity.

In this work, we thus aimed to develop a Ti^{4+}-modified paper substrate for paper spray. These were then evaluated in two modes, and benchmarked against HPLC-MS/MS: (i) the extraction of phosphatidylcholine and lysophosphatidylcholine from urine, followed by a wash and direct analyte ionization and analysis with PS-MS/MS; (ii) the selective removal of phosphatidylcholine from plasma to reduce ion suppression and improve the sensitivity in the analysis of pharmaceuticals.

2. Experimental

2.1. Chemicals and materials

Epichlorohydrin (EPI) was purchased from Alfa Aesar Chemical Reagent Co., Ltd (Shanghai, China). Iminodiacetic acid (IDA) and methanol (MeOH, HPLC-grade) were purchased from Innochem Scientific Co., Ltd (Beijing, China). Titanium (IV) sulfate was purchased from Rhawn Chemical Reagent Co., Ltd (Shanghai, China). Dimethyl sulfoxide (DMSO), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), concentrated sulfuric acid, ammonium hydroxide (wt%, 25.0–28.0%) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Phosphatidylcholine (PC), and piperine were purchased from Alfa Biotechnology Co., Ltd. (Chengdu, China). Lysophosphatidylcholine (LPC) was purchased from Sigma Aldrich Trading Co., Ltd. (Shanghai, China). Synthetic (phospho)peptides named RIHRA-(pS)-DPGLPA and RIHRA-S-DPGLPA were purchased from All Peptide Biotechnology Co. Ltd. (Hangzhou, China). Citric Acid was purchased from Shanpu Chemical Co. Ltd. (Shanghai, China). Trisodium citrate dihydrate was purchased from Damao Chemical Reagent Factory (Tianjin, China). Clonidine hydrochloride was purchased from National Institute for Food and Drug Control (Beijing, China). Apraclonidine was purchased from MedChemExpress Co., Ltd (Shanghai, China). Formic acid (HPLC-grade, wt%, ≥88.0%) was purchased from Kermel Chemical Regent Co., Ltd (Tianjin, China). Human urine samples were obtained from healthy volunteers in the laboratory. Human plasma samples were obtained from Hunan Provincial Center for Disease Control and Prevention (Changsha, China). Chromatography paper was purchased from J&K Scientific Co., Ltd (Beijing, China). pH paper was purchased from SSS Regent Co., Ltd (Shanghai, China). Filters (13 mm * 0.22 μm, Nylon6) were purchased from BKMAM Biotechnology Co., Ltd (Changde, China). Water was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA).

2.2. Solutions

Fifty μL of formic acid was added to 100 mL of water; the resulting solution is referred to as acidic water. Fifty μL of formic acid was added to 100 mL of MeOH; the resulting solution is referred to as acidic MeOH. Two mL of ammonium hydroxide was added to 100 mL of MeOH, the resulting solution is referred to as alkaline MeOH.

2.3. Preparation of blank paper spray tips

The preparation of blank paper spray tips is described in previous work [15]. In short, paper triangles were cut with a CUTOK DC craft cutting plotter (Hefei CNC Equipment Co. Hefei, China). Then, the cut tips were washed with methanol, dried overnight on a glass plate in a fume hood, and stored in a clean plastic bottle.

2.4. Preparation of Ti^{4+}-modified paper

Based on the protocol reported by Li et al. [31] (see supplementary information (SI), Fig. S1), blank paper tips (2.0 g) were added into a beaker with 50 mL of 2 M NaOH in water. After 30 min, EPI (10 mL), and DMSO (10 mL) were added to the beaker and mixed for 3 h in a water-bath oscillator at 30 °C, in which DMSO is used to improve the solubility of EPI. Afterwards, the solution in the beaker was decanted, and the papers were washed with approximately 1 L of water until the washing solution remained clear. Then, 2.0 g IDA dissolved in 30 mL of 1.5 M Na_2CO_3 in water was added in the beaker and the mixture was allowed to react for 16 h in the water-bath oscillator at 50 °C. Afterwards, the solution in the beaker was decanted, and papers were washed with approximately 1 L of water until the washing solution remained clear. Then, the papers were placed on a clean glass plate and dried overnight in a fume hood.

Forty mL of 0.1 M $Ti(SO_4)_2$ in water with sulfuric acid (1.2%) was prepared in a beaker. Then, the previously modified papers were immersed into the obtained solution to react for 3 h in a water-bath oscillator at 20 °C. Afterwards, the solution in the beaker was decanted, and papers were washed with approximately 1 L of water until the washing solution remained clear. Then, the obtained Ti^{4+}-modified papers were placed on a clean glass plate, dried overnight in a fume hood and stored in a clean plastic bottle until use.

2.5. Instrumental setups

Paper Spray-Tandem Mass Spectrometry (PS-MS/MS) PS-MS/MS analyses were performed by placing a custom-made paper spray setup (SI, Fig. S2, as also described in previous work [15]) in front of an LCMS 8040 triple quadrupole mass spectrometer (Shimadzu Corp., Japan), and positioning paper tips approximately 5 mm in front of the orifice. Samples were either directly deposited on the tips centrally, or the tips were submerged in sample solution. Once dried, spray solvent and a spray voltage (3.5–4.0 kV) were applied to generate spray for ~1 min. Full scan mass spectra were acquired (*m/z* 100–800 for PLs and *m/z* 100–300 for clonidine) with the following conditions: 250 °C desolvation temperature; 300 °C heating block temperature; 4 kV capillary voltage, positive mode. Multiple reaction monitoring (MRM) was used for the analysis of the phospholipids and clonidine (see SI, Tables S1 and S2 for respective MRM settings, and Figs. S3 and S4 for respective chemical structures). Ti^{4+}-modified paper tips were either used directly to generate spray, or placed on top of an unmodified paper tip.

HPLC-MS/MS HPLC-MS/MS analyses were performed using an LCMS 8050 triple quadrupole mass spectrometer (Shimadzu Corp., Japan). The MS/MS conditions were the same as used in Ti^{4+}-modified paper spray mass spectrometry (TiPS-MS/MS), except for: 3.0 L min^{-1} nebulizing gas; 10.0 L min^{-1} drying gas; 10.0 L min^{-1} heating gas; 250 °C interface temperature. An XAmide column (5 μm, 250 × 4.6 mm, Acchrom Technologies Inc., China) was employed for the separation of phospholipids. The mobile phase consisted of (A) water with 10 mM ammonium acetate and (B) acetonitrile with 0.1% formic acid. The gradient elution conditions (with a constant flow of 0.5 mL min^{-1} with high pressure gradient) were as follows: linear decrease from 95% to 85% B in 20 min; then linear decrease of B to 50% in 1 min; 50% B until 26 min; linear increase to 95% B in 2 min; 95% until 35 min. A Diamonsil C18 column (3 μm, 150 × 4.6 mm, Dikma Technologies Inc., China) was used for clonidine analysis [32]. The mobile phase consisted of (A) water containing 0.2% formic acid and (B) methanol. The gradient elution conditions (with a constant flow of 0.5 mL min^{-1} with high pressure gradient) were as follows: linear increase from 10% to 30% B in 3 min; linear increase of B to 80% until 6 min; 80% B until 6.5 min; linear decrease B to 10% in 30s; 10% B until 12 min.

2.6. Characterization of Ti^{4+}-modified paper

X-ray photoelectron spectroscopy (XPS) XPS was performed with a K-Alpha instrument (Thermo Fisher Scientific, USA). Blank papers and Ti^{4+}-modified papers were cut to 5 × 5 mm. The instrument conditions were as follows: Al Kα ray ($h\nu = 1486.6$ eV, excitation source); 400 μm beam spot; 12 kV voltage; 6 mA heater current.

PS-MS/MS 5 μg mL^{-1} phosphatidylcholine was dissolved separately in acidic MeOH and alkaline MeOH. These two solutions were analyzed with blank paper and Ti^{4+}-modified paper by PS-MS. Next, blank papers and Ti^{4+}-modified papers were immersed in fresh 5 μg mL^{-1} phosphatidylcholine in acidic water separately, and after washing with acidic water, acidic MeOH, and drying, the papers were immersed in alkaline MeOH for desorption. These alkaline MeOH desorption solutions were analyzed directly by blank PS-MS. The same experiments were performed for commercial synthetic phosphopeptides and non-phosphopeptides.

2.7. Optimization of Ti^{4+}-modified paper

Addition of internal standard Internal standard (IS, piperine) was added to perform quantitative analysis with the developed Ti^{4+}-modified papers. In order to confirm that the IS does not bind to Ti^{4+}-modified paper under alkaline conditions, it was dissolved in alkaline MeOH as spray solvent in a concentration of 0.5 μg mL^{-1} and analyzed directly with blank PS-MS and TiPS-MS.

Desorption process Ti^{4+}-modified paper tips were first immersed in 5 mL of 0.5 μg mL^{-1} PC in acidic water. After washing with acidic water and acidic MeOH, and drying, the treated papers were analyzed by PS-MS/MS; 40 μL of alkaline MeOH with IS (0.2 μg mL^{-1}) as spray solvent was applied for one paper tip, after which spray was initiated. This was then repeated 2, 3, 4 or 5 times with the same tip. The analyte/IS ratio was used to calculate the quantity of desorbed phosphatidylcholine after each fresh 40 μL spray solvent that was applied. The same experiments were performed for LPC.

Next, Ti^{4+}-modified paper tips were first immersed in 5 mL of 0.5 μg mL^{-1} PC in acidic water. After washing with acidic water and acidic MeOH, and drying, the treated papers were analyzed by PS-MS/MS; alkaline MeOH (40 μL) without IS was added to one paper without applying voltage 0, 1, 2, 3, or 4 times (drying after each addition) for desorption without spraying, and then 40 μL alkaline MeOH with IS (0.2 μg mL^{-1}) as spray solvent was applied on this same paper, after which spray was initiated. The same experiments were performed for LPC.

Extraction time Ti^{4+}-modified paper tips were immersed in 5 mL of 0.5 μg mL^{-1} PC in acidic water for different durations (5, 10, 20, 30, 40, 60 min). After washing with acidic water and acidic MeOH, and drying, the treated papers were analyzed by TiPS-MS/MS using 40 μL of alkaline MeOH with IS (0.05 μg mL^{-1}) as spray solvent. The analyte/IS ratio was used to calculate the quantity of PC. The same experiments were performed for LPC.

Extraction pH Ti^{4+}-modified paper tips were immersed in 5 mL of 0.5 μg mL^{-1} PC solution in acidic water with different formic acid

concentrations (0%, 0.05%, 0.1%, 0.5%, 1%, 5%) for 20 min. After extraction, washing with acidic water and acidic MeOH, and drying, the treated papers were analyzed by TiPS-MS/MS using 40 μL of alkaline MeOH with IS (0.05 μg mL^{-1}) as spray solvent. The analyte/IS ratio was used to calculate the quantity of PC. The same experiments were performed for LPC.

Citrate buffer solution (pH 4.5) was loaded and dried on Ti^{4+}-modified paper tips, to achieve a constant and optimal pH for extraction. The buffer solution was prepared with 0.8 M concentration and consisted of (A) citric acid and (B) sodium citrate in a 7:3 ratio (A:B). Then, 40 μL buffer solution was added to each Ti^{4+}-modified paper, and dried. In order to confirm the effect of the buffer, test solutions were prepared with human urine at different pH values (from 4 to 7, adjusted by formic acid and ammonium hydroxide). Then, the pH values of these solutions were determined before and after the addition of the buffer-loaded Ti^{4+}-modified paper. In addition, these buffer-loaded Ti^{4+}-modified paper tips were afterwards analyzed by TiPS-MS/MS, with 0.05 μg mL^{-1} IS in the spray solvent.

Desorption pH Next, fresh Ti^{4+}-modified papers were immersed in 5 mL of 0.5 μg mL^{-1} PC solution in acidic water with 0.05% formic acid for 20 min. After washing with acidic water and acidic MeOH, and drying, the treated papers were analyzed by PS-MS/MS using 40 μL of MeOH with different ammonium hydroxide concentrations (0%, 0.5%, 1%, 1.5%, 2%, 2.5%) and IS (0.05 μg mL^{-1}) as spray solvent. The analyte/IS ratio was used to calculate the quantity of PC. The same experiments were performed for LPC.

2.8. Analysis of PLs in urine with Ti^{4+}-modified paper

Six standard solutions of PC (0, 0.1, 0.2, 0.5, 1, 2 μg mL^{-1}) in acidic water were prepared to construct a calibration curve. The Ti^{4+}-modified papers were immersed in the standard solution for 20 min, and after washing with acidic water and acidic MeOH, and drying, 40 μL of alkaline MeOH with IS (0.05 μg mL^{-1}) was used as spray solvent. The ratio between analyte and IS signals was used to construct a calibration curve. Calibration curves were similarly constructed for LPC, using the same IS in spray solvent.

Next, calibration curves were constructed from mixtures of the PLs in human urine (after determining the concentration in 'blank' urine via standard addition by HPLC-MS/MS), with different spiked PLs concentrations (0, 0.1, 0.2, 0.5, 1, 2 μg mL^{-1}), following the same TiPS-MS/MS protocol.

The HPLC-MS/MS method was used as benchmark. Prior to injection, urine samples were diluted in MeOH (1:3 v/v), and after approximately 3 min of mixing, centrifuged for 10 min at 12,000 rpm, and the supernatant was taken and filtered (13 mm * 0.22 μm, nylon6 filter).

The limit of detection (LOD) and quantification (LOQ) were estimated from the standard deviation of background signal/IS signals in unspiked urine (Sa) and the slope of the calibration curve (b): LOD = 3Sa/b and LOQ = 10Sa/b. Precision was calculated as the relative standard deviation (RSD %). Accuracy was calculated as the relative deviation (%) of the calculated mean value from the actual concentration.

2.9. Reduction of matrix effect in plasma by Ti^{4+}-modified paper

PLs absorption Six standard solutions of PC (10, 50, 100, 200, 500, 1000 μg mL^{-1}) in acidic MeOH were prepared, and analyzed directly by PS-MS/MS and TiPS-MS/MS, without immersing and washing. The peak area was obtained from the PC signal by blank PS-MS/MS and TiPS-MS/MS.

Seven standard solutions of 1 μg mL^{-1} clonidine with different PC concentrations (0, 10, 50, 100, 200, 500, 1000 μg mL^{-1}) in acidic MeOH were prepared, and analyzed by PS-MS/MS and TiPS-MS/MS directly. The signal suppression of clonidine by PCs was calculated from the peak area of clonidine signal in the presence of PC. This was done for the PS-MS/MS method and TiPS-MS/MS separately.

Clonidine analysis Six standard solutions of clonidine (0, 0.1, 0.2, 0.5, 1, 2 μg mL^{-1}) in plasma were prepared to construct a calibration curve with TiPS-MS/MS directly, without immersing and washing. 300 μL of plasma samples were diluted in ACN (1:3 v/v), and after approximately 3 min of mixing, centrifuged for 10 min at 12,000 rpm. Next, the supernatant was taken and filtered (13 mm * 0.22 μm, nylon6 filter). 20 μL of filtered sample was dropped on the paper each time, and 20 μL of acidic MeOH was added. After drying, 40 μL of acidic MeOH with aproclonidine (IS, 0.1 μg mL^{-1}) was used as spray solvent. The signal ratio between clonidine and IS was used to construct a calibration curve. The HPLC-MS/MS method was used as benchmark. LOD, LOQ, accuracy and precision were calculated as described above.

3. Results and discussion

3.1. Characterization of modified paper

According to reported procedures (SI, Fig. S1) [31], paper spray tips were functionalized with IDA and $Ti(SO_4)_2$, which allows their use to extract PLs from complex mixtures. Ti^{4+}-modified paper tips were expected to selectively bind PLs based on Lewis acid-base interaction [28], and thus, PLs would be retained on the paper while other matrix constituents could be washed away, after which the determination of PLs would be achieved. Moreover, such modification would allow the retention of PLs on the paper under specific conditions, so that targeted analytes can be analyzed by MS without or with limited interference from said PLs. Based on the modification procedures, N and Ti should be present on modified paper due to the introduction of IDA and $Ti(SO_4)_2$. The presence of the introduced groups on the paper was confirmed by XPS (SI, Fig. S5). Compared with blank paper (Fig. S5a), the Ti and N element peaks of Ti^{4+}-modified paper (Fig. S5b) are clearly visible in the full scan spectrum. Corresponding XPS spectra of N and Ti are also shown (Figs. S5c–f).

In order to verify that PLs can be bound to Ti^{4+}-modified paper under acidic conditions but cannot be bound or remain bound under alkaline conditions, PC in alkaline MeOH and acidic MeOH were analyzed separately by TiPS-MS to observe any difference (Fig. 1A & 1B). When PC in alkaline MeOH and acidic MeOH were dropped on Ti^{4+}-modified paper directly and analyzed by PS, there was a clear in-source fragment signal (*m/z* 183.87) for PC in alkaline MeOH, but none in acidic MeOH, suggesting that indeed under acidic conditions, PC was captured by the Ti^{4+}-modified paper and could not reach the MS. As control, the same solutions were analyzed on blank paper (Fig. S6), and in both cases a signal for a characteristic PC fragment was obtained.

To further compare the blank papers and Ti^{4+}-modified papers, both were immersed in 5 μg mL^{-1} PC in acidic water separately, and after washing and drying, the same papers were immersed in separate alkaline MeOH solutions for desorption. These desorption solutions were analyzed directly by blank PS-MS (Fig. 1C & 1D). Indeed, PC was captured and desorbed when using Ti^{4+}-modified papers, whereas no signal for PC was obtained by extracting with blank papers. This result could be reproduced with other phosphate-containing compounds, namely phosphopeptides (SI, Fig. S7). Contrastingly, non-phosphopeptides were not recovered from either paper, due to a lack of interaction with the Ti^{4+}.

3.2. Optimization of TiPS-MS/MS

Several steps in the analytical procedure for the phospholipids extraction and desorption were then optimized. Below, the optimization procedures, including extraction processes, desorption procedures, and IS addition are described.

An internal standard (IS, piperine) was chosen for the quantitative analysis of the phospholipids, as it does not have interaction with Ti^{4+}-modified paper, and has good response in mass spectrometry.

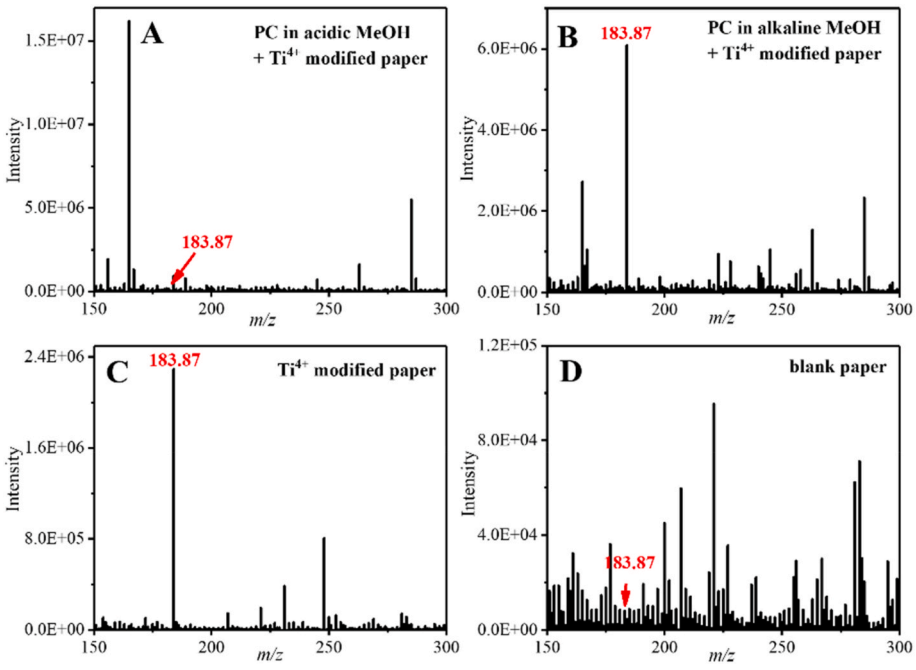

Fig. 1. TiPS-MS spectra in positive mode with Ti^{4+}-modified paper for phosphatidylcholine (5 μg mL^{-1}; *m/z* 183.87, in source fragmentation signal) in acidic MeOH (A) or alkaline MeOH (B). Ti^{4+}-modified papers or blank papers were immersed in 5 μg mL^{-1} acidic phosphatidylcholine solution and, after washing and drying, the papers were immersed in alkaline MeOH for desorption, and analyzed with Ti^{4+}-modified paper (C) or blank paper (D).

Considering that Ti^{4+}-modified paper tips extract PLs under acidic conditions and desorb under alkaline conditions, IS should be added into the alkaline desorption solution, which is also used as spray solvent. To prevent possible ionic interaction with the Ti^{4+}-modified paper, a neutral or weakly basic compound should be used. First, a solution of IS in alkaline MeOH was separately analyzed on blank paper and Ti^{4+}-modified paper by PS-MS. The results show that for both papers, there is a clear signal for the IS (SI, Fig. S8).

There are several steps involved in the paper modification process, during which modified papers need to be immersed in different solutions, at different pH values and temperatures. As a consequence, the tip of Ti^{4+}-modified papers can be damaged, and the structure and surface of the papers change (SI, Fig. S9), resulting in spray instability and irreproducibility during paper spray. In order to solve this problem, different desorption and spray processes were studied. As is shown in Fig. 2A, when using the traditional single-tip method, the paper tip is directly held by the copper clip for desorption and spray. In contrast to the traditional method, a new double-tip spray method is applied, in which a blunted Ti^{4+}-modified paper tip is placed on top of a blank paper tip, and the two pieces of paper are held together by the copper clip (Fig. 2B). When the spray solvent is added to the surface of the modified paper tip, phospholipids are desorbed and will elute into the blank paper, where they are sprayed at the tip. The results show that the signal response was strongly enhanced when this new method is applied (see Fig. 2C & 2D).

Next, a Ti^{4+}-modified paper tip was used to extract PC or LPC from a 0.5 μg mL^{-1} solution, and then placed in front of the MS. First 40 μL alkaline MeOH with IS as desorption and spray solution was added and spray was generated. Then, another aliquot was added to the same paper, and this was repeated up to 5 times (Fig. 3A). This experiment indicates that a single addition of 40 μL of spray solvent was insufficient to completely desorb all phospholipids. The absolute signals of PC, LPC and IS are also shown in Fig. S10; more PC or LPC were released each time when fresh spray solvent was added. In addition, one paper with different desorption steps was tested: 40 μL of alkaline MeOH (without IS) was added onto the paper and dried (without application of voltage, i.e. no spray was generated) for 0, 1, 2, 3, 4 times; then 40 μL of alkaline MeOH with IS (0.2 μg mL^{-1}) was applied and spray was generated. (Fig. 3B). This experiment confirms that adding alkaline MeOH multiple times, before applying spray voltage, resulted in increased desorption of PLs. Even though adding increasing aliquots of desorption solutions resulted in higher signal, it also increases the duration of a single measurement. Therefore, three desorption steps (i.e. no spray was generated during the first 2 additions) were employed for all further analyses to optimize detection limits, in a reasonable time. The final desorption protocol is shown in Fig. 2E.

Next, extraction time was optimized. PLs in acidic water were extracted with a Ti^{4+}-modified paper tip and the previously optimized desorption process was used for release (SI, Fig. S11). The extraction of phospholipids did not improve beyond an extraction time of 20 min, which was thus selected for the final protocol.

Then, the influence of the pH during extraction and desorption was assessed. The affinity between Ti^{4+} and phospholipids is based on Lewis acid-base interaction. The phosphate group in phospholipids is a strong Lewis base, which interacts with Ti^{4+} as Lewis acid under acidic conditions [28]. As is shown in Fig. 4A, with an increase in the formic acid concentration in the extraction solution, phosphate groups are more likely to combine with H^+ rather than Ti^{4+}, and the interaction between Ti^{4+} and phospholipids is prevented. Therefore, the optimal extraction solution is acidic water with 0.05% formic acid (pH 4.5). To reach this pH, a paper-based buffer addition step was developed, adapted from the protocol reported in previous work [15]. Buffer solution was directly deposited on Ti^{4+}-modified paper tips and dried. When the buffer-loaded Ti^{4+}-modified tips were placed in acidic human urine samples with different pH, regardless of the initial pH, the final pH was 4.5 (Fig. 4B), and MS signal intensity did not vary substantially when the sample pH initially showed variation, indicating similar extraction efficiency. Next, the alkaline desorption solution was optimized. As shown in Fig. 4C, the signal of the phospholipids increases with the increase of ammonium hydroxide up to 2%. However, when the concentration is higher than 2%, the signal decreases. This is likely caused by ion suppression due to the high ammonia concentration [28]. Therefore, alkaline MeOH with 2% ammonium hydroxide was selected as spray solvent.

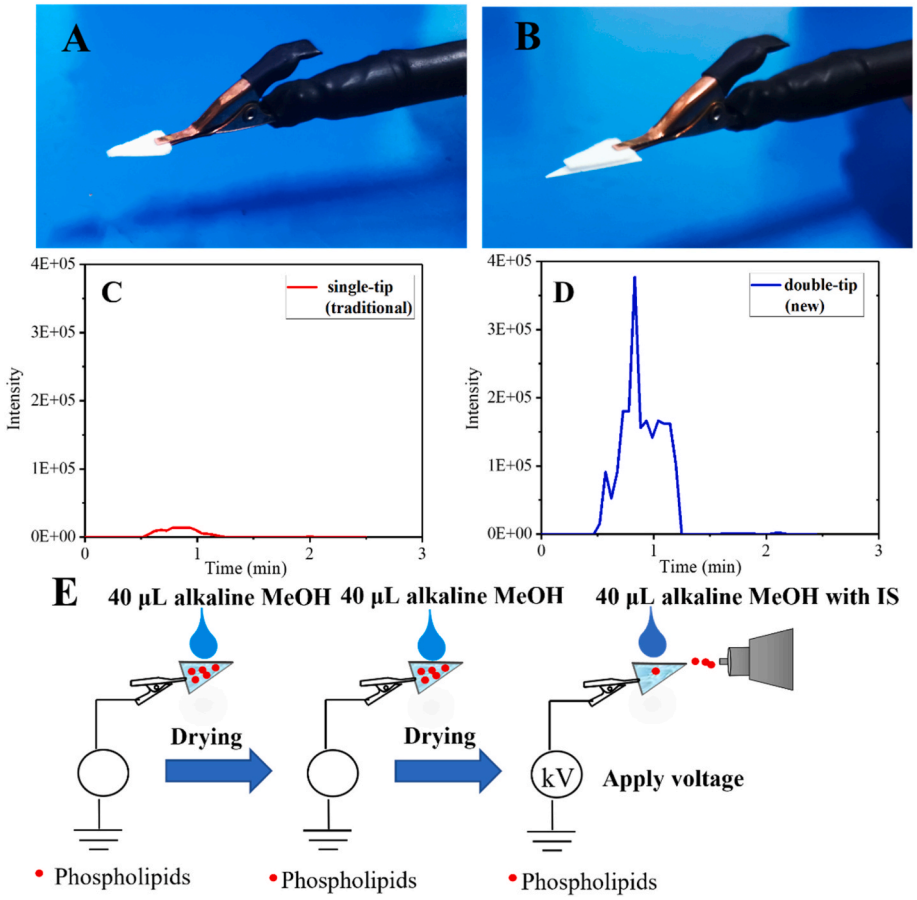

Fig. 2. Photographs of the single-tip (traditional) method (A) and double-tip (new) setups (B), and corresponding chronograms (MRM, 758.50 → 184.00) of phosphatidylcholines obtained viaboth methods (C & D, respectively). Optimized spray process of double-tip (new) method for phospholipids analysis (E). With the double-tip (new) method, 40 μL alkaline MeOH without IS was added twice without applying voltage to desorb the analytes. After evaporation of MeOH, 40 μL alkaline MeOH with IS (0.05 μg ml^{-1}) was used as spray solvent for PS-MS/MS.

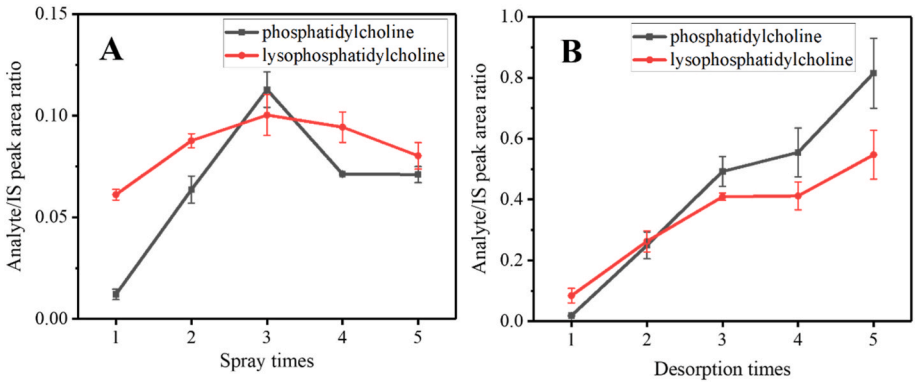

Fig. 3. Signal ratio of analyte/IS by applying spray solvent and spraying multiple, subsequent times from the same paper (A). First 40 μL alkaline MeOH with IS as desorption and spray solution was added and spray was generated. Then, another aliquot was added and this was repeated up to 5 times. Signal ratio of analyte/IS by TiPS-MS/MS with different desorption times (B). Alkaline MeOH (40 μL) without IS was added on one paper without applying voltage 0, 1, 2, 3, or 4 times; then 40 μL alkaline MeOH with IS (0.2 μg mL^{-1}) as spray solvent was applied on this same paper, after which spray was initiated. Error bars represent standard deviation (n = 3).

3.3. *Analysis of PLs in urine with Ti^{4+}-modified paper*

First, a TiPS-MS/MS method was developed for the extraction and determination of PLs in urine, following the workflow demonstrated in Fig. 5A. One Ti^{4+}-modified tip with pre-loaded citrate buffer was immersed in 1000 μL of urine sample for 20 min, washed with acidic water and acidic MeOH, and then the tip was dried. Using the double-tip method, 40 μL of alkaline MeOH without IS was added twice (drying after each addition) without applying voltage; then 40 μL of alkaline MeOH with IS (0.05 μg mL^{-1}) was used as spray solvent for PS-MS/MS.

Calibration curves were constructed for PC and LPC separately in water, and then for the mixture of these two analytes in human urine. HPLC-MS/MS was used as comparison (example of extracted ion chromatogram is shown in Fig. S12). An overview of results of these calibration curves is shown in Table 1. For the TiPS-MS/MS method in standard solutions of the individual PLs, there is good linearity over the range of 0.1–2 μg mL^{-1} (SI, Fig. S13). Then, two PLs were spiked in human urine for constructing a calibration curve. An HPLC-MS/MS method was used as comparison and was used to calculate PC and LPC in blank human urine by standard addition method (0.016 μg mL^{-1} PC and 0.003 μg mL^{-1} LPC, SI, Fig. S14). The results of TiPS-MS/MS method (SI, Fig. S15) show that analysis in this complex matrix affects the performance of the method (see Table 1), but the LOD of 0.01–0.03 μg mL^{-1} can still be achieved. According to Yang et al. [8] and Saulnier-Blache et al. [9], the concentration of the studied PC and LPC in urine from kidney disease patients exceeds 0.1 μg mL^{-1}, which means that the developed TiPS-MS/MS method has an adequate performance to allow fast screening for chronic kidney disease patients by urine analysis. The

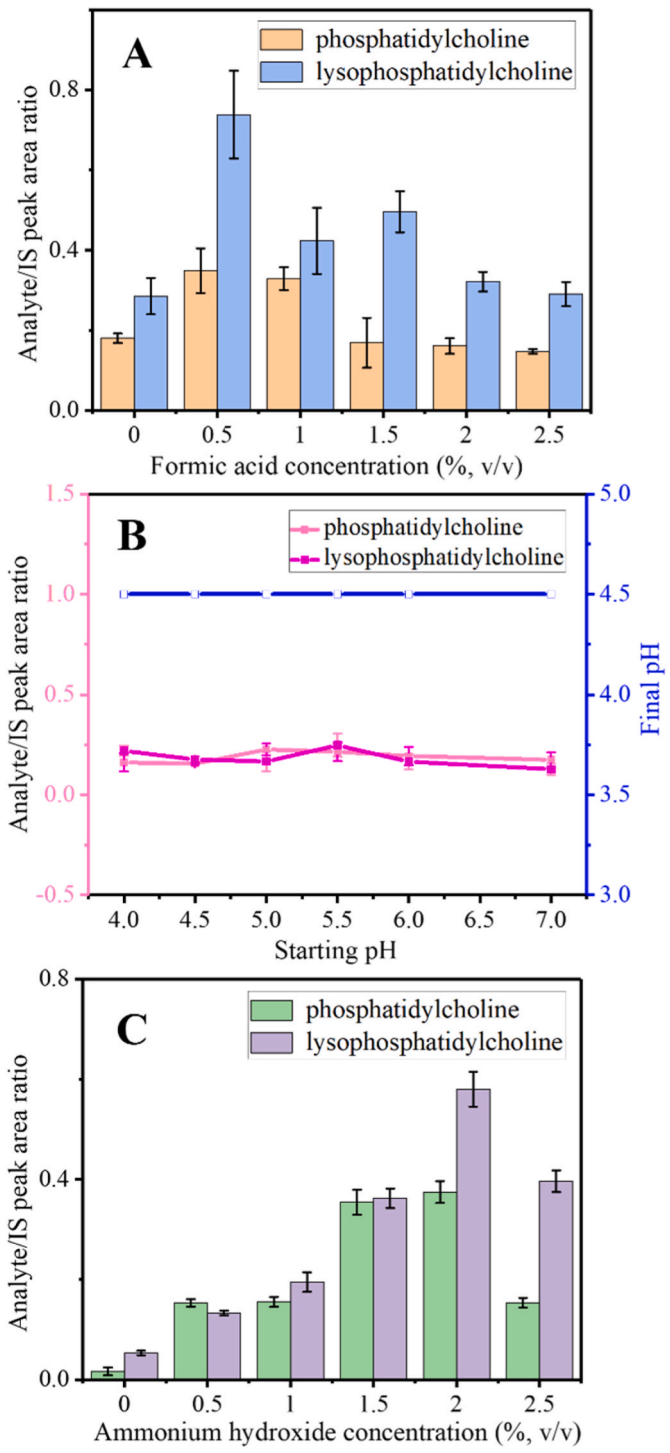

Fig. 4. Signal ratio of analyte/internal standard after extraction of PC or LPC with Ti^{4+}-modified paper from solutions with different acidity, 40 μL alkaline MeOH with IS was used as spray solvent (detailed method is same as Fig. 2E) (A). Signal ratio of analyte/internal standard by TiPS-MS/MS and the final pH of the sample after a pre-loaded buffer paper tip was added to human urine samples with different starting pH values for extraction, and the tips were analyzed using 40 μL alkaline MeOH with IS as spray solvent (detailed method is same as Fig. 2E) (B). Signal ratio of analyte/internal standard using MeOH with different ammonium hydroxide concentrations with IS for desorption and as spray solvent for TiPS-MS/MS, using tips that extracted PC or LPC from acidic PC solution for a duration of 20 min (C). Error bars represent standard deviation (n = 3).

chronograms of PC and LPC in unspiked urine determined by HPLC-MS/MS (SI, Fig. S16a-d), and TiPS-MS/MS (SI, Figs. S16e and S16f) show that there is a substantial difference between the healthy human urine and urine with added 0.1 μg mL^{-1} phospholipids (simulated patient urine). Accuracy and precision were evaluated with 3 samples with different pH and concentrations of the PLs in human urine (SI, Table S3) for HPLC-MS/MS (accuracy, −4.1%-3.8%; precision, 0.9%–3.2%), and TiPS-MS/MS (accuracy, −13.9%-9.5%; precision, 7.9%–13%). Importantly, HPLC takes 35 min for column-based separation and equilibration in each analysis, while TiPS-MS/MS needs less than 5 min after extraction.

3.4. Matrix effect decrease in plasma by Ti^{4+}-modified paper

Phospholipids are endogenous compounds that occur as part of the matrix in plasma. There is a large number of phospholipids (750–1540 μg mL^{-1} total PLs) in plasma, which cause serious matrix effects during analysis. As demonstrated above, Ti^{4+}-modified papers interact with phospholipids under acidic conditions and thus can be used for the selective removal of phospholipids as well. Here, this concept was tested by analyzing plasma spiked with a model agent, clonidine. The same double-tip approach was used, and sample was dropped on the top of the modified paper. Then, 20 μL of acidic water was added on to paper without voltage, resulting in phospholipids from plasma to be retained by the paper. After drying, 40 μL of acidic MeOH with IS (0.1 μg mL^{-1}) as spray solvent was added to elute the analyte clonidine to the normal paper tip for spray, and voltage was applied. The optimized protocol of Ti^{4+}-modified paper spray mass spectrometry for phospholipids removal from plasma is shown in Fig. 5B.

Before assessing the impact of PL removal on the analyte, PC itself was analyzed with blank and Ti^{4+}-modified papers with this approach (Fig. 6A). For PC concentrations below 500 μg mL^{-1}, there is clear reduction in signal with the modified papers, compared to blank paper, indicating removal. When the concentration of PC was 500 μg mL^{-1} (absolute amount was 10 μg with sample volume 20 μL) or higher, the absorption ability of Ti^{4+}-modified papers becomes limiting, and the amount of phospholipids that entered the MS increased.

Next, the impact of different PC concentrations on clonidine was assessed, again with both the Ti^{4+}-modified and blank papers. As shown in Fig. 6B, for the blank PS-MS/MS method, the signal response of clonidine gradually decreases with the increase of phosphatidylcholine concentration, approaching 0 when the phosphatidylcholine concentration is higher than 500 μg mL^{-1}. In contrast, when TiPS-MS/MS was used, with the increase of the phosphatidylcholine concentration, the signal response of clonidine decreased significantly slower than with blank PS-MS/MS. When the phosphatidylcholine concentration reached 1000 μg mL^{-1}, the signal response of clonidine by TiPS-MS/MS is still higher then by PS-MS/MS, which indicates that Ti^{4+}-modified paper can still remove some phosphatidylcholines and improve the clonidine response in a plasma sample. Mass spectra of 1 μg mL^{-1} clonidine with 200 μg mL^{-1} phosphatidylcholines by PS-MS/MS and TiPS-MS/MS method (Fig. 6C & 6D) show that the signal of clonidine is significantly higher when analyzed by TiPS-MS/MS. However, the binding capacity of a single paper is clearly insufficient to remove all PLs from a sample with such a high concentration (>500 μg mL^{-1}).

Finally, the established TiPS-MS/MS method was used for clonidine analysis (0.1–2 μg mL^{-1}) in plasma. The LOQ obtained by TiPS-MS/MS was 0.1 μg mL^{-1} (SI, Fig. S17), which is substantially lower than with blank PS-MS/MS (1.0 μg mL^{-1}). HPLC-MS/MS was used as comparison (extracted ion chromatogram is shown in Fig. S18). Accuracy and precision of the methods were evaluated with 3 samples with different concentrations of clonidine in human plasma (SI, Table S4). The performance of TiPS-MS/MS method (accuracy, −8.9%-13.1%; precision, 6.9%–19.0%) is lower than that of the HPLC-MS/MS method (accuracy, −7.1%-9.7%; precision, 0.5%–3.1%), but considerably faster.

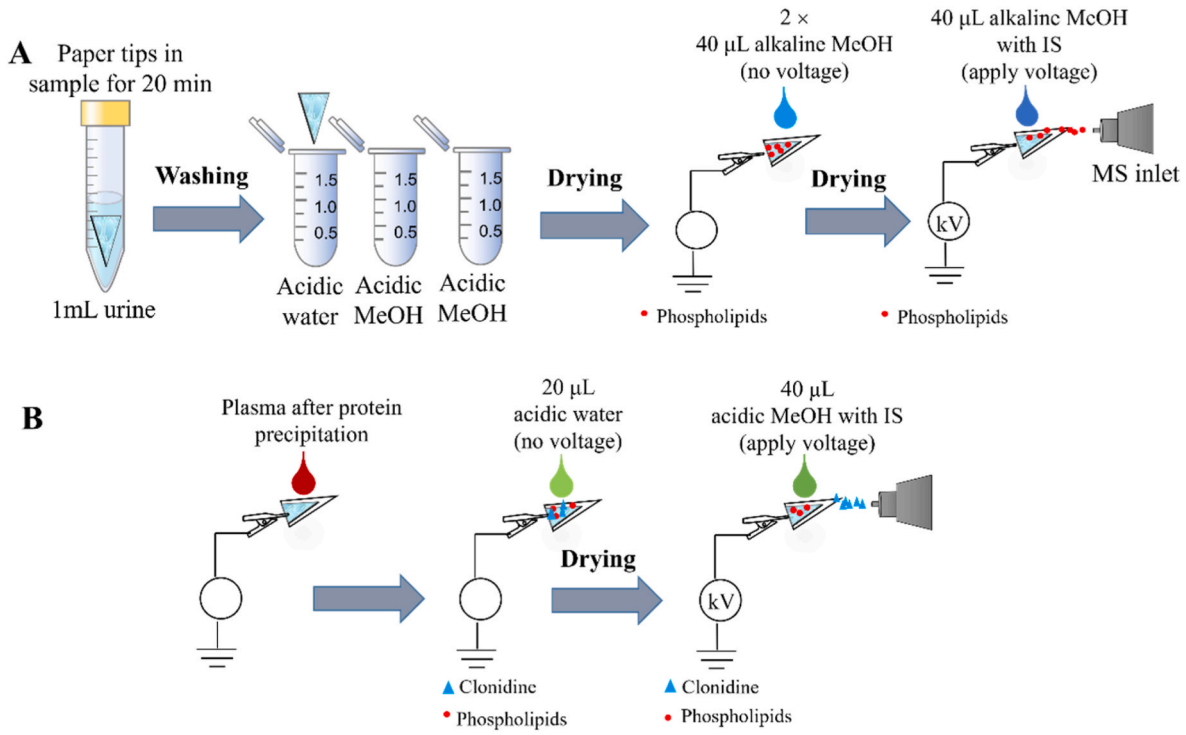

Fig. 5. Optimized protocol of TiPS-MS/MS for phospholipids enrichment and analysis in urine (A). One Ti^{4+}-modified tip with pre-loaded citrate buffer was immersed in 1000 μL of urine sample for 20 min, washed with acidic water (1 ×) and acidic MeOH (2 ×), and then the tip was dried. With the double-tip method, 40 μL of alkaline MeOH without IS was added twice (drying after each addition) without applying voltage, and then 40 μL of alkaline MeOH with IS (0.05 μg mL^{-1}) was used as spray solvent and the voltage was applied. Optimized protocol of TiPS-MS/MS for phospholipids removal and clonidine analysis in plasma (B). After adding of plasma sample, 20 μL of acidic water without IS was added to the paper without applying voltage. After drying, 40 μL of acidic MeOH with IS (0.1 μg mL^{-1}) as spray solvent was added to elute the analyte clonidine and the voltage was applied.

Table 1
Analytical performance of Ti^{4+}-modified paper PS-MS/MS and HPLC-MS/MS for PLs analysis.

Method	Phospholipids	Linear equation	Correlation coefficients (R^2)	Linear range (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
PS-MS/MS of PLs separately in standard solution	PC	$y = 0.571x+0.011$	0.999	0.1–2	0.010	0.040
	LPC	$y = 1.785x+0.081$	0.999	0.1–2	0.020	0.060
PS-MS/MS of PLs together in human urine sample	PC	$y = 0.305x+0.002$	0.994	0.1–2	0.010	0.040
	LPC	$y = 0.263x+0.027$	0.998	0.1–2	0.030	0.100
HPLC-MS/MS of PLs together in human urine sample	PC	$y = 3.7e^5x+620$	0.990	0.05–1	0.002	0.006
	LPC	$y = 1.5e^5x+477$	0.996	0.05–1	0.003	0.010

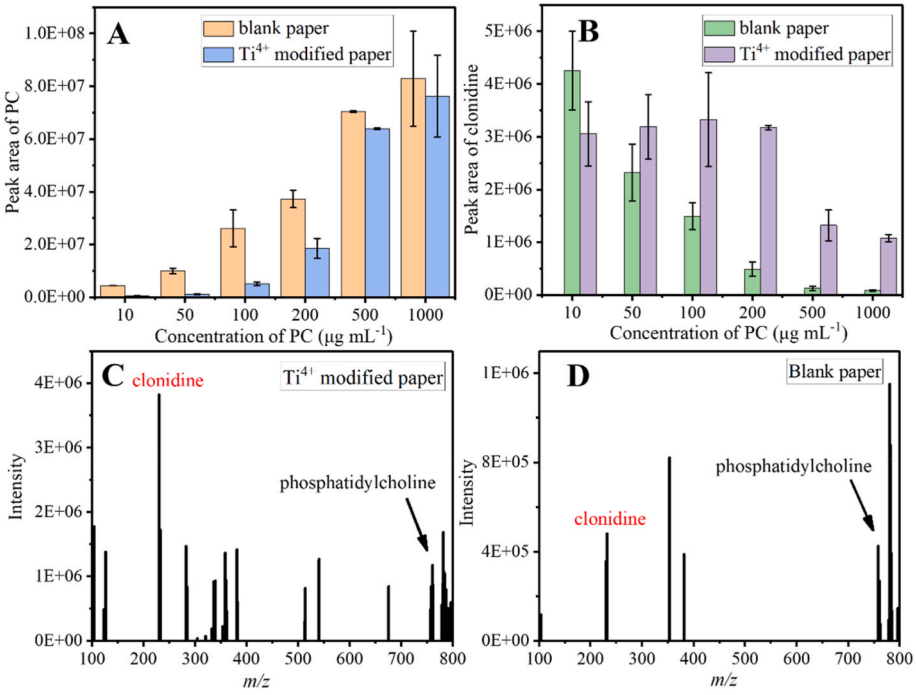

Fig. 6. Peak area of phosphatidylcholines (10–1000 μg mL^{-1}) when TiPS-MS/MS and blank PS-MS/MS were used (A). Peak area of 1 μg mL^{-1} clonidine with different phosphatidylcholine concentration (10–1000 μg mL^{-1}) by blank PS-MS/MS and TiPS-MS/MS (B). Mass spectrum of 1 μg mL^{-1} clonidine with 200 μg mL^{-1} phosphatidylcholine analyzed by TiPS-MS/MS (C) or PS-MS/MS (D).

4. Conclusions

A new Ti^{4+}-modified paper spray method was established for either the enriched detection, or in contrast, removal, of phospholipids. It is based on the selective binding between Ti^{4+} metal ions and phosphate-containing compounds, such as phospholipids, under acidic conditions. The method cannot only extract phospholipids in urine, but also remove phospholipids from plasma. When used in combination with MS/MS (TiPS-MS/MS), the LOD of this method is 0.01–0.03 μg mL^{-1} for phosphatidylcholine and lysophosphatidylcholine, allowing for fast determination of phospholipids and fast analysis of urine samples for chronic kidney disease, such as glomerulonephritis. In addition, the TiPS method can also be used for the removal phospholipids prior to analysis to reduce matrix effects in plasma. The LOD of this new TiPS-MS/MS method improved by a factor of 10 for a model drug in plasma compared to the use of PS-MS/MS without the use of Ti^{4+}-modification. This is highly promising for future applications– even though further gain in sensitivity would be needed for practical implementation. Finally, the established method should be easily adaptable to the analysis of other phosphate group-containing analytes, such as phosphopeptides.

CRediT authorship contribution statement

Wei Luo: Methodology, Investigation, Writing – original draft. **Teris A. van Beek:** Supervision, Validation, manuscript drafting, editing & revision. **Bo Chen:** Funding acquisition, Supervision, editing & revision. **Han Zuilhof:** Funding acquisition, Supervision, editing & revision. **Gert IJ. Salentijn:** Conceptualization, Funding acquisition, Methodology, Formal analysis, Supervision, manuscript drafting, editing & revision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (22276050, 22276049), the China Scholarship Council 2020 International Cooperation Training Program for Innovative Talents, the Aid Program for S&T innovation research team in higher education institutions, the construction program of key disciplines of Hunan Province (2015JC1001), and the Hunan Province 100 experts project. Gert Salentijn acknowledges financial support from the Dutch Research Council (NWO) (Veni grant 17328).

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.341673.

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