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FULL ARTICLE

Complementary approach for analysis of phospholipids by liquid chromatography hyphenated to elemental and molecular mass spectrometry

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Abstract

Phospholipids are one of the most important lipid categories with multiple functions in biological systems. Their analysis can contribute to a better understanding of metabolomic and kinetic processes in living cells. Comprehensive methods based on liquid chromatography coupled to mass spectrometry are available for phospholipid identification and quantification. However, quantification of phospholipids using electrospray ionization-mass spectrometry with internal standards is still challenging due to several reasons. In particular, the detector response of phospholipid species differs with variation of the head group as well as the fatty acid chain length and double bond number. Inductively coupled plasma-tandem mass spectrometry (ICP-MS/MS) provides an alternative approach for their absolute quantification with universal detector response for phosphorus independent of its chemical form and proportional to its quantity. Therefore, a quantification method based on compound-independent calibration using hydrophilic interaction liquid chromatography (HILIC) coupled to ICP-MS/MS was developed. An inverse gradient system was implemented for constant mobile phase composition after HILIC separation, which provides steady plasma ionization conditions. Isobaric phosphorus interferences were decreased by using the oxygen reaction mode of the triple quadrupole based ICP-MS/MS instrument. Complementary molecular information was obtained by ESI-high-resolution MS and MS/MS. The applicability of this approach was demonstrated in a proof of concept by complementary analysis of a total lipid extract of baker's yeast.

KEYWORDS

ESI-MS, HILIC, ICP-MS, phospholipids, quantification

Abbreviations: CRC, collision/reaction cell; HRMS, high-resolution mass spectrometry; IPA, Isopropyl alcohol; PL, phospholipid(s); TQ, triple quadrupole.

1 | INTRODUCTION

Phospholipids (PL) are one of the most important cellular lipid categories. These lipids are involved in many biological functions in various organisms. They are the main structural components of cell membranes and of the respiratory chain in mitochondria.[1] Additionally, PL are important for the functionality of membrane proteins such as ion channels[2] and serve as precursor molecules for second messengers.[3] In addition, the importance of PL alterations in various diseases like cancer was described.[3,4] Therefore, the determination of alterations of PL species under different conditions is crucial for the understanding of their biological functions, their involvement in cell processes, and their role in diseases. Relative quantity changes can provide initial insights of lipid involvement. However, absolute quantitative data are required for a comprehensive assessment of cellular relationships and pathways as well as a complete description of most dynamic cell processes.[5] This information requires not only the amount, but also the fluxes of relevant PL classes and species within different cell status. Thus, absolute quantification leads to a higher accuracy of predictions about the metabolic and kinetic lipid response of living cells to environmental changes.[5]

The coupling of mass spectrometry (MS) and tandem-MS (MS/MS) equipped with electrospray ionization (ESI) is the state-of-the-art technique for identification, structural characterization, and relative quantification of lipids. Different techniques for structural elucidation have been developed in recent years. The hyphenation with high-performance liquid chromatography (HPLC) is often used for separation of lipid classes and species, as it exhibits various advantages compared to direct infusion-MS. The HPLC-MS approach allows the identification of isobaric and isomeric lipid species. In addition, PL class separation by hydrophilic interaction liquid chromatography (HILIC) provides benefits for lipid quantification compared to other separation mechanisms due to similar retention times of the lipid species within a lipid class and a class-specific internal standard.

Although lipid identification by ESI-MS often is the method of choice, quantification of PL classes and species is still challenging due to several reasons. The ionization efficiencies depend on varying matrix effects, and a different response of individual lipid species is affected by the head group and the chain length as well as the double bond number of the FA moieties.[6–9] A sufficient coverage with commercial PL standards is not possible due to high structural diversity of lipid species and high complexity of samples.[7] In addition, the chromatographic separation results in changing ionization conditions due to varying mobile phase composition.[7] These effects hamper the quantification of PL with external calibration using ESI-MS.

Inductively coupled plasma-MS (ICP-MS) provides an alternative method for absolute PL quantification. A universal detector response for phosphorus independent of the analytes' chemical binding form and direct proportionality to the elemental concentration as well as wide linear dynamic range and low detection limits are just a few advantages of this approach.[10–12] Hyphenation with prior chromatographic separation is also possible.[10,12,13] Here, the combination of a *MicroFlow* nebulizer and Peltier-cooled cyclonic spray chambers reduces the introduction of organic solvent during aerosol generation. In addition, high rf power and a small inner diameter of the injection tube (1 mm) serve to maintain stable plasma and ionization conditions.[10] Furthermore, oxygen has to be added to prevent elemental carbon deposition. Consequently, sampler and skimmer cones composed of platinum instead of nickel should be used to prevent formation of nickel tetracarbonyl.[10] An ICP-MS instrument equipped with a collision/reaction cell (CRC) in reaction mode is utilized to decrease isobaric polyatomic interferences of phosphorus such as $^{12}C^{1}H_{3}{}^{16}O^{+}$, $^{15}N^{16}O^{+}$, or $^{14}N^{16}O^{1}H^{+}$.[14,15] Reaction with oxygen shifts the mass-to-charge ratio (*m/z*) to a higher value (*m/z* 47 for $^{31}P^{16}O^{+}$), which reduces interferences. The selectivity of the CRC approach has been substantially enhanced by using a triple quadrupole (TQ) system with Q1 and Q3 mass filtering.[14–16] HR-MS instruments such as double-focusing sector-field-MS[17,18] or time-of-flight-MS are alternative approaches to reduce interferences for phosphorus determination.[19] However, these techniques differ significantly in the sensitivity provided. While the sector-field-MS provides a sensitivity roughly one order of magnitude better than the quadrupole-based CRC instrument,[18] the actual TOF-MS reaches approximately one-third of the sensitivity of the quadrupole instrument.[19]

A complementary approach for PL analysis with species information, relative ratios and absolute PL class quantification will be presented in this work. The development of the quantification method based on an ICP-MS/MS system equipped with CRC is presented. Hyphenation with HILIC separation represents the basis of the quantification method. Isobaric polyatomic interferences on the phosphorus signal are minimized by reaction with oxygen in the CRC. An inverse HPLC gradient system is implemented by a dual-gradient pump to obtain a constant mobile phase composition reaching the plasma and to ensure steady ionization properties over the complete elution time. Similar approaches have been successfully applied in the HPLC-ICP-MS quantification of phosphorylated peptides[20] and of the thyroid gland hormone thyroxine (3,5,3′,5′-tetraiodothyronine).[21] Besides quantification of phospholipid classes by HILIC-ICP-MS using gradient compensation, the phospholipid species have been identified by complementary ESI-MS detection. The applicability of this complementary approach is demonstrated in this proof of concept study, that is, speciation analysis of PL in a total lipid extract of the baker's yeast (*Saccharomyces cerevisiae*).

2 | MATERIALS AND METHODS

2.1 | Chemicals

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (PC 16:0/16:0), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (*lyso*-PC 16:0), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (PE 16:0/16:0), 1,2-dipalmitoyl-*sn*-glycero3-phospho-(1′-*myo*-inositol) (PI 16:0/16:0), 1,2-dipalmitoyl-*sn*-

glycero-3-phospho-L-serine (PS 16:0/16:0), and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (PG 16:0/16:0) were purchased from Biomol GmbH (Hamburg, Germany). Bis(monooleylglycero)phosphate (S,R isomer) (BMP 18:1/18:1) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Total lipid extract of *S. cerevisiae* (190000C, Avanti Polar Lipids) and dichlorodimethylsilane (>99.5%) were delivered by Sigma Aldrich (Steinheim, Germany).

Acetonitrile (ACN), methanol (MeOH), isopropanol (IPA), 2-butanol (BuOH), acetone, chloroform ($CHCl_3$) (HPLC gradient grade), and toluene (>99.5%) were purchased from VWR International GmbH (Darmstadt, Germany). Nitric acid (HNO_3) (69%) and standard solutions of phosphorus and arsenic (1 g L^{-1}) were acquired from Merck (Darmstadt, Germany). Ammonium acetate (≥99.99%) and acetic acid (≥99.99%) were obtained from Fluka (Darmstadt, Germany). Tune B iCAP Q solution (1 g L^{-1} of the elements Ba, Bi, Ce, Co, In, Li, and U) was delivered by Thermo Scientific (Bremen, Germany). Water was purified by a Milli-Q Academic-System (18.2 MΩ cm; 0.2 μm filter; Millipore, Molsheim, France). All chemicals were used as received.

2.2 | Standard and sample preparation

Glass vials were silanized by dichlorodimethylsilane (1:10 dilution in toluene) to prevent lipid adsorption. Glass vials were soaked with silanization solution for 30 min and rinsed with toluene. Subsequently, vials were soaked with MeOH for 30 min, rinsed with MeOH, and dried with nitrogen.

Note that 1 mM stock solutions of each PL class were prepared. PC, PE, and PS were dissolved in $CHCl_3$/MeOH (95:5, v/v), while solvent mixtures of $CHCl_3$/MeOH (1:1, v/v) and $CHCl_3$/MeOH/Milli-Q H_2O (3:2:1, v/v/v) were used to dissolve PG and *lyso*-PC.

The injection solvent was optimized by evaluation of PL mixtures with each lipid class concentration of 10 μM dissolved in various organic solvent mixtures. Therefore, IPA/ACN/ammonium acetate buffer (35 mM, 48.5:48.5:3, v/v/v), IPA/ACN/ammonium acetate buffer (72.75:24.25:3, v/v/v), BuOH/ACN/ammonium acetate buffer (48.5:48.5:3, v/v/v), BuOH/ACN/ammonium acetate buffer (72.75:24.25:3, v/v/v), $CHCl_3$/IPA/ammonium acetate buffer (48.5:48.5:3, v/v/v), and $CHCl_3$/IPA/ammonium acetate buffer (24.25:72.75:3, v/v/v) as dilution solvents were evaluated.

A calibration series with five PL classes and concentrations of 0.5, 1, 5, 10, 50, and 100 were prepared in IPA/ACN/ammonium acetate buffer (72.75:24.25:3, v/v/v). All samples contained BMP (10 μmol L^{-1}) as an internal standard and were analyzed in triplicate.

A stock solution of *S. cerevisiae* extract was prepared with a concentration of 5 g L^{-1}. For analysis, PL extract stock solution was dissolved 1:10 in IPA/ACN/ammonium acetate buffer (72.75:24.25:3, v/v/v). This *S. cerevisiae* sample also contained BMP (10 μmol L^{-1}) as an IS and was analyzed five times.

2.3 | Chromatography and mass spectrometry

Detection and quantification of phosphorus was performed on an iCAP TQ ICP-MS (Thermo Scientific) instrument. The interface was equipped with a platinum sampler and skimmer with 2.8 mm insert. Total chromatographic flow was introduced into the ICP by using the combination of a PFA-ST MicroFlow nebulizer (100-1000 μL min^{-1} flow rate range, Elemental Scientific, Omaha, NE, USA), a Peltier-cooled cyclonic spray chamber (Thermo Scientific), and a quartz injector pipe with an inner diameter of 1 mm. The following instrumental settings were used: RF power 1400 W, spray chamber temperature −5 °C, cool gas flow 14 L min^{-1}, auxiliary gas flow 0.7 L min^{-1}, nebulizer gas flow 0.55 L min^{-1}, and oxygen flow 20%. Further parameters were adjusted by automatic instrument tune with an organic solvent matched tune solution and 1 μg L^{-1} of the elements Ba, Bi, Ce, Co, In, Li, and U (Tune B iCAP Q solution [2 mL, 100 μg L^{-1} element stock in 2% HNO_3], Milli-Q H_2O [11.8 mL], HNO_3 [6.2 mL, 69%], ACN [180 mL]). CRC conditions was set to 0.2 mL min^{-1} oxygen (99.999%) and 0.3 mL min^{-1} helium (99.999%). Phosphorus was monitored as $^{31}P^{16}O^+$ with a dwell time of 1 s. Verification of all pre-set system parameters and optimization of CRC conditions were performed by adding a BMP standard solution (20 μg L^{-1}, 5 μL min^{-1}) to the mobile phase flow (411 μL min^{-1}). Their influence on the signal-to-noise ratio of *m/z* of $^{31}P^{16}O^+$ was examined and optimized for maximum signal-to-noise ratio according to the manufacturer recommendations.[22] This system was operated with Qtegra 2.4 software (Thermo Scientific). Data evaluation was carried out using Origin 8.5.0 (OriginLab Corporation, Northampton, MA, USA) and Excel 2016 (Microsoft Corporation, Albuquerque, NM, USA).

PL class separation was carried out on a Thermo Scientific Ultimate 3000 HPLC system equipped with the dual-gradient pump DGP-3600RS providing a left and a right pump head according to a previously described HILIC method.[23] An iHILIC Fusion (250 mm × 2.1 mm, 3.5 μm, 100 Å) (HILICON AB, Umeå, Sweden) was used as stationary phase. The mobile phase gradient was provided by the right pump and consisted of ammonium acetate buffer (35 mM, pH 5.75, 5% ACN) (A) and ACN (B). The gradient started with 97% B from 0 to 0.5 min and decreased to 75% B within 26 min. Subsequently, the mobile phase decreased to 60% B within 0.5 min, followed by a washing step at 60% B for 6 min, prior to an increase to 97% B in 2 min and subsequent equilibration with the initial solvent composition over 10 min. The total run time was 45 min. Additionally, an inverse gradient system was implemented by the left pump to maintain constant mobile phase composition after column separation with the goal to maintain constant ICP ionization conditions. The inverse gradient flow was added to the analytical gradient flow by using a nanocapillary (0.1 mm × 1100 mm) to generate a required working back pressure for a stable pump function and a T-piece (for details, see Figure S1). A flow delay of 604.2 μL for the inverse gradient flow was required to consider the column volume and was determined by injection of acetone to

both inverse gradient and analytical gradient flow paths and ultraviolet detection (265 nm). Thus, the different dwell times of the mobile phase of the two flow paths were compensated. The inverse gradient calculation mode for a steady composition of the mobile phase after column separation was based on the analytical gradient and was set to minimize flow for reduced introduction of organic mobile phase. Analytical gradient and inverse gradient flow rates were 0.300 mL min^{-1} and 0.111 mL min^{-1} (for details, see results and discussion as well as Supporting Information). Injection volume and column temperature were set to 5 μL and 40°C, respectively. The chromatographic system was controlled by the software Chromeleon 7.2 sr3.

Hyphenation of this chromatographic system with the inverse gradient to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) was carried out for lipid species identification. Structural PL annotation was performed in ESI(-) mode using full-scan MS and data-dependent MS/MS acquisition. ESI conditions were optimized with a PL standard solution consisting of BMP and PG. Instrumental settings were used as follows: Source voltage −3200 V, capillary temperature 380°C, probe heater temperature 325°C, sheath gas flow rate 35 AU, auxiliary gas flow rate 15 AU, spare gas flow rate 1 AU, s-lens rf level 90, mass range *m/z* 200-2000, resolution 280 000 (full width at half maximum [FWHM], at *m/z* 200). For MS/MS experiments, resolution was set to 17 500 and normalized collision energy of the HCD cell was set to 35 eV. Accumulation times of the C-trap were set to 100 ms and 50 ms for full-scan and data-dependent scan, respectively. One full scan and 10 data-dependent scans resulted in an average cycle time of 1.8 s. At least, 10 full-scan data points were obtained per chromatographic peak. Both systems were controlled by Xcalibur 4.1 software (Thermo Scientific). Data evaluation was carried out by MZmine 2.32 software.[24,25]

2.4 | Total phosphorus determination by ICP-MS

PL were digested to inorganic phosphate for determination of total phosphorus. Approximately 1 mg total lipid extract of *S. cerevisiae* was weighed into each of the five centrifuge tubes (15 mL, Thermo Scientific) for fivefold determination. Arsenic was used as IS (100 μg L^{-1}) and its solution (0.05 mL, 1 g L^{-1}) was added at the beginning of the digestion. Samples were digested by adding HNO_3 (1 mL, 69%) at 40 °C for 20 h and subsequently filled up with Milli-Q water to 10 mL. Finally, samples were diluted with HNO_3 (2%) by a factor of 50.

Calibration series with element concentrations of 10, 25, 50, 75, and 100 μg L^{-1}were prepared in centrifuge tubes from phosphorus solution (1 g L^{-1}) with arsenic as an IS at a concentration of 100 μg L^{-1}.

The ICP-MS/MS was coupled with the ASX-560 autosampler (Teledyne CETAC Technologies, Omaha, NE, USA) for direct infusion. System parameters were optimized by instrument auto tune with aqueous diluted Tune B iCAP Q solution (1 mg L^{-1} element concentrations) and were set as follows: RF power 1550 W, spray chamber temperature 2.7°C, cool gas flow 14 L min^{-1}, auxiliary gas flow 0.8 L min^{-1}, and nebulizer gas flow 1.19 L min^{-1}. Same CRC gas flows for oxygen and helium were used as for the HILIC-ICP-MS method. Wash time and uptake time of the autosampler were set to 30 s and 60 s, respectively. Wash solution consisted of HNO_3 solution (2%). Each sample was measured five times. The instrument was controlled by Qtegra 2.4 software (Thermo Scientific). Calculations were carried out with Excel 2016 software (Microsoft Corporation).

3 | RESULTS AND DISCUSSION

3.1 | Development of a HILIC-ICP-MS/MS method for quantification of PL classes

ICP-MS/MS provides a universal detector response for phosphorus determination and is a complementary method to ESI-MS for the absolute quantification of PL. For method development, the parameters of the ICP-MS system were optimized by an instrument auto tune with a manufacturer recommended tune solution. Utilization of an organic solvent-matched tune solution resulted in a better system performance compared to aqueous tune solution. All parameters were subsequently evaluated by introduction of a BMP standard solution (5 μL min^{-1}, 20 μM) into a mobile phase flow (0.411 mL/min, 90% ACN) and maximized the signal-to-noise ratio of $^{31}P^{16}O^{+}$. Suitable conditions for CRC were determined in the same way. A maximum signal-to-noise ratio was observed at an oxygen flow rate of 0.2 mL min^{-1}, which could be slightly further increased by an additional helium flow rate of 0.3 mL min^{-1}. The enhanced reaction yield may be caused by the effect of collisional focusing due to increased pressure in the CRC and thus a higher number of collisions with gas molecules.[26–29]

For constant ionization conditions and efficiency, an inverse gradient system using a dual-gradient pump with two pump heads was implemented and generated a steady composition of the introduced mobile phase. The right pump head was utilized for analytical gradient flow, whereas the left pump head generated the inverse gradient flow. A small inner diameter capillary was used to create a sufficient back pressure for a constant flow of the inverse gradient pump. Both gradient flows were combined by means of a T-piece after column separation (for details, see Figure S1). The inverse gradient depends on the analytical gradient. By adaptation and merging of both flow paths, a constant composition of the mobile phase after column separation was generated. For this purpose, the used chromatography software provides different modes for calculation of the inverse gradient, resulting in different mobile phase compositions and flow rates for potentially improved analytical properties of the introduced mobile phase instead of simply reflecting the analytical flow: Keep solvent composition, minimize flow rate, and maximize one component of the mobile phase composition (for details, see Table S1). Minimize flow (Table S1, third row, flow rate 0.411 mL min^{-1}, 70.8% ACN proportion of the introduced

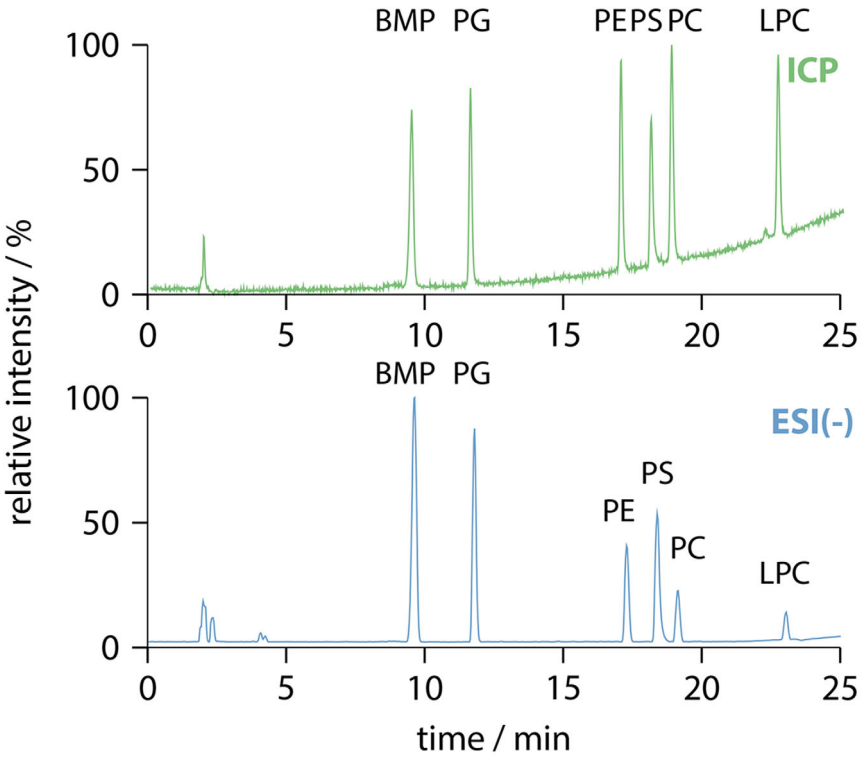

FIGURE 1 HILIC-ICP-MS chromatogram (top) and HILIC-ESI-MS base peak chromatogram (*m/z* 200-2000) (bottom) of PL mixture with a lipid class concentration of 10 μM dissolved in IPA/ACN/ammonium acetate buffer (72.75:24.25:3, v/v/v). Indicated are the following standards: bis(monooleylglycero)phosphate (BMP 18:1/18:1), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (PG 16:0/16:0), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (PE 16:0/16:0), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (PS 16:0/16:0), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC 16:0/16:0), and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (*lyso*-PC 16:0)

mobile phase) showed higher intensities compared to maximize ACN (Table S1, bottom row, flow rate 0.600 mL min^{-1}, 80.0% ACN) due to a lower dilution by the inverse gradient. Maximize buffer was also evaluated in previous measurements but did not show higher intensities than minimize flow. Therefore, reducing the total mobile phase flow rate seemed to have a stronger influence than changing its composition and was used as calculation mode of the inverse gradient for further measurements.

ICP-MS exhibits the advantage of a compound-independent response compared to ESI-MS. While ESI-MS has different response depending on structural differences of PL classes and species, this effect is not observed during atomization and ionization in ICP-MS. A comparison of PL response with standard mixtures (10 μM) is displayed in Figure 1. While the HILIC-ESI-MS chromatogram (bottom) showed different peak areas for all PL class standards with exception of the constitutional isomers PG and BMP, the HILIC-ICP-MS chromatogram presented similar peak areas for the majority of PL classes, although PS appeared with lower peak height due to stronger chromatographic peak tailing. Peak broadening by sample introduction with ICP-MS compared to ESI-MS could not be observed. This comparison showed the compound-independent response for phosphorus in different PL classes and its suitability for quantification.

The ICP-MS chromatogram in Figure 1 showed a raising baseline with increasing buffer percentage of the analytical gradient. An incorrect timing of inverse and analytical gradient could be excluded, which would result in a varying composition of the mobile phase. Residues of PL on column can also be excluded because a new iHILIC Fusion column showed the same signal profile. Thus, the increase in the baseline can likely be traced back to the phosphate group-containing stationary phase of the HILIC column (for details, see Figure S3), that is, column bleeding. In previous experiments, the related iHILIC Fusion(+) with its phosphorus free stationary phase did not show this signal profile. Finally, the rising baseline can be traced back to the phosphorus containing stationary phase of the HILIC column.

Interestingly, the first calibration series revealed nonuniform sensitivity for the selected PL standards (Figure 2, top). Calibration curves of PG, PC, and *lyso*-PC showed almost perfect accordance, and thus, the response of phosphorus was independent of lipid class, whereas PE, and especially PS, had lower sensitivities. Preparation of fresh PL standard stock solutions did not improve their ICP-MS response. Other dissolved PL standard stock solutions were evaluated with the same results. Therefore, PL standards could be excluded as source of errors. Another approach was the silanization of glass vials to avoid lipid adsorption on glass surfaces. However, this had no significant effect in the response of PE and PS. Finally, the injection solvent was investigated, because insufficient solubility was assumed. Therefore, PL standards (10 μM) with different solvent mixtures of ACN, IPA, BuOH, $CHCl_3$, and buffer were examined and the response of PL standards was compared (for details, see Figure S4). A solvent composition containing IPA/ACN/ammonium acetate buffer (72.75/24.25/3, v/v/v) showed similar peak areas for PE, PS, PC, and PG as well as the best

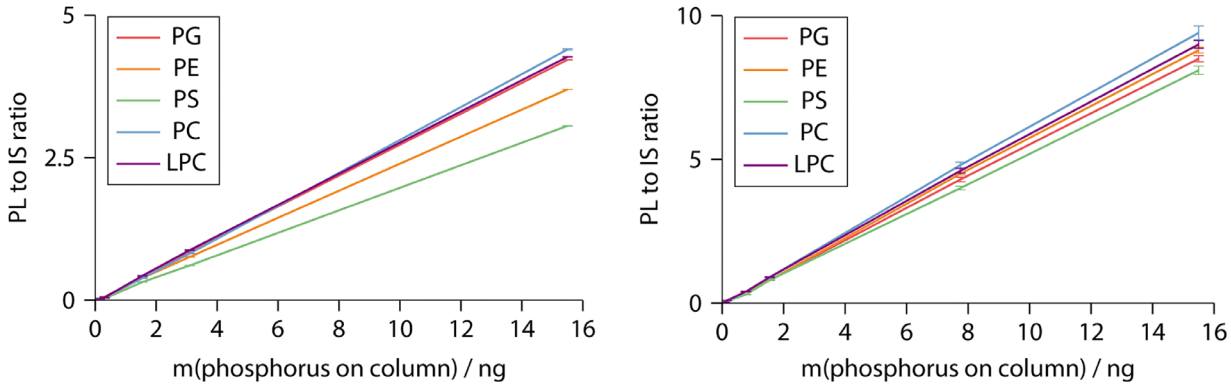

FIGURE 2 HILIC-ICP-MS/MS calibration series of PL classes: First series revealed problems with the universal response, particularly for PS and PE (left), and second series after modification of the injection solvent of the PL samples (right)

results for all PL classes. Finally, this injection solvent was used for further measurements, as it is also compatible with the HILIC mobile phase. It is noteworthy that this solubility problem of PL in the injection solvent would have been hardly discovered with ESI-MS analyses alone.

A new calibration series was recorded and displayed in Figure 2 (bottom). Improvements due to the changed injection solvent are clearly visible and all calibration curves have similar slopes. PS (green) showed a slightly lower, whereas PC (blue) exhibited a corresponding higher sensitivity. The chromatographic separation caused this difference due to peak shape of PS and partial co-elution with PC. This complicated the evaluation of both chromatographic peaks and resulted in decreased areas for PS as well as increased areas for PC. Retention behavior of the different PL classes depending on buffer concentration was already shown in the previously reported method for class-specific PL separation.[23] Consequently, separation of both PL classes could be improved by decrease in the ammonium acetate buffer concentration. Nevertheless, these calibration curves demonstrated the compound-independent response of phosphorus detection with ICP-MS/MS and its suitability for PL class quantification.

The limit of detection (LOD) and the limit of quantification (LOQ) of phosphorus on column were determined with three and ten times of the signal-to-noise ratio of the representative PE calibration curve at 80 pg and 239 pg, respectively. The developed ICP-MS method exhibited slightly lower LOD compared to Kovacevic et al. (LOD down to 210 pg phosphorus on column).[30] Standard deviation was determined with a maximum of 3%, whereby it ranged from 1% to 8% for the lowest lipid concentrations. Kovacevic et al. analyzed PL in yeast lipid extracts using a normal phase HPLC and an ICP-MS system based on a single quadrupole and a CRC pressurized with helium.[30] A comparison of both ICP-MS methods and their LOD is difficult due to different reasons and could partly explain the only slight improvement of the LOD of the ICP-MS/MS instrument. One of the main differences are the volume of introduced mobile phase. The merged flow paths of the analytical gradient and inverse gradient introduce the mobile phase with a total flow rate of 0.411 mL min^{-1} in the developed method, whereas Kovacevic et al. splitted their mobile phase flow by one-fifth to approximately 0.13 mL min^{-1}. Consequently, the amount of introduced organic solvents was lower and the plasma was less strained. In addition to the sample introduction system, the different organic components of the mobile phase affect the transport efficiency of the analytes into the plasma and thus the signal sensitivity.[31] Especially organic solvents with different physical properties such as volatility, viscosity, density, surface tension, and thermal conductivity as well as their amount and additives such as ammonium acetate as buffer salt have a negative impact on the transport efficiency and the detection limits.[31] However, the contribution of the different organic components of the mobile phase to the transport efficiency of both methods cannot be determined for a comparison so easily. Therefore, ensuring a steady composition of introduced mobile phase using the implemented inverse gradient system is a further important aspect of the developed method for absolute quantification of PL. By changing proportions of hexane and methanol in the method according to Kovacevic et al., the transport efficiency of the sample introduction as well as the ionization properties of the plasma are influenced leading to different response of phosphorus. Further advantage of this complementary analysis approach is the acquisition of absolute quantitative data as well as qualitative data. Besides the determination of individual lipid species and their fatty acid composition, unknown peaks in the ICP-MS chromatogram can be identified using ESI-MS/MS. The ESI-MS-compatible mobile phase of the HILIC separation enables the complementary application of both, element and molecular MS.

3.2 | Complementary analysis of total lipid extract of *Saccharomyces cerevisiae* by HILIC-ICP-MS/MS and HILIC-ESI-MS/MS

Biological samples show higher complexity of PL classes with various species compared to PL standards. Therefore, the developed HILIC-ICP-MS/MS method for PL quantification was applied to the analysis of a total lipid extract of *S. cerevisiae* in this proof of concept study. Complementary, total phosphorus determination was performed by ICP-MS/MS. HILIC-ESI-MS and MS/MS analyses were carried out for identification and structural annotation of PL species as well as determination of their relative abundance within their lipid class.

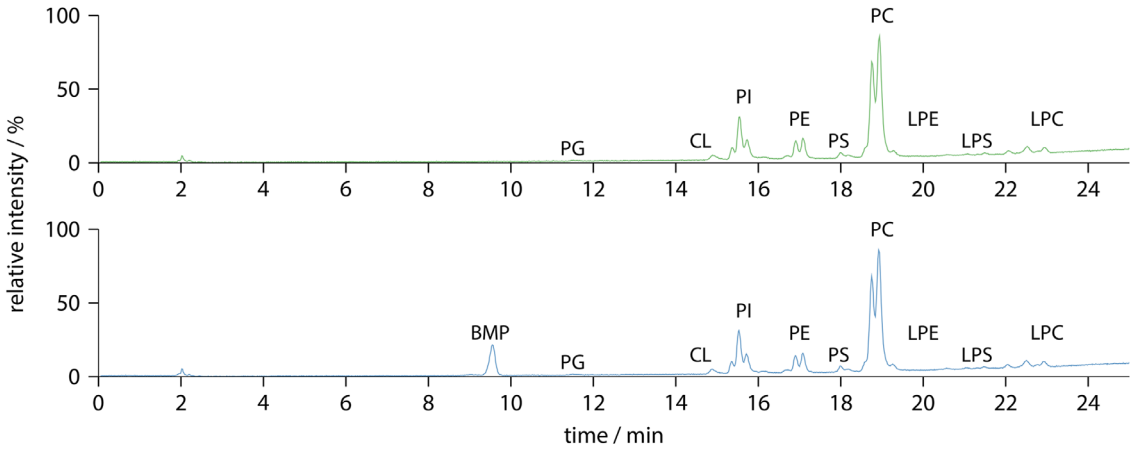

FIGURE 3 HILIC-ICP-MS/MS chromatograms of an *S. cerevisiae* total lipid extract without internal standard (top) and containing BMP (10 µmol L^{-1}) as internal standard (bottom)

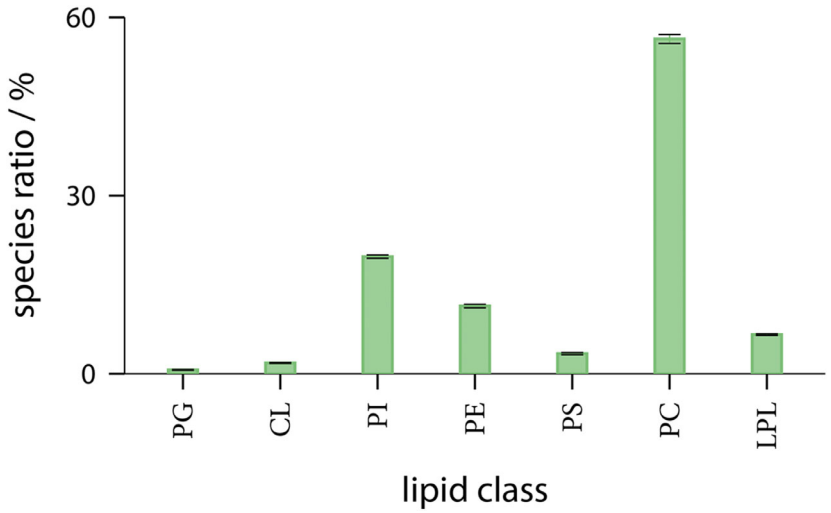

FIGURE 4 PL class distribution of *S. cerevisiae* total lipid extract analyzed with HILIC-ICP-MS/MS. Quantification was carried out with external calibration and internal standard

In order to verify the absence of the used internal standard, the yeast extract was first analyzed without BMP using the HILIC-ICP-MS/MS approach. The chromatograms are displayed in Figure 3 (top). No BMP could be detected. Therefore, BMP was utilized as internal standard containing phosphorus and added to the lipid extract with a total concentration of 10 µM. This chromatogram of *S. cerevisiae* lipid extract containing internal standard is shown in Figure 3 (bottom). Besides BMP, several PL classes were detected including PG, CL, PI, PE, PS, PC, *lyso*-phosphatidylglycerol (*lyso*-PG), *lyso*-phosphatidylethanolamine (*lyso*-PE), *lyso*-phosphatidylserine (*lyso*-PS), and *lyso*-PC.

Quantification of PL classes was carried out with external calibration and internal standard. The calibration curves in Figure 2 (bottom) served as external calibration and quantification based on calculation of PL class area to internal standard area ratios. This approach offered the possibility for absolute quantification of PL classes with only one internal standard containing phosphorus. The representative calibration curve of PE standard (orange line, Figure 2, bottom) was used for quantification of PL classes, which were not included in the PL standard mixture. The consistent calibration curves and the universal response of phosphorus justified this as a suitable approximation for missing PL standards. In addition, peak area of CL was divided by 2 for correction of the double number of phosphorus atoms in CL molecules. Signal intensity is proportional to the amount of phosphorus, which permitted this simple correction. Furthermore, *lyso*-PE, *lyso*-PS, and *lyso*-PC were summarized as *lyso*-phospholipids (lyso-PL), because their separation for ICP-MS/MS quantification was insufficient due to their isomeric species with bound FA in *sn*-1- or *sn*-2-position and relatively large difference in their retention times.

The PL class distribution using these approximations are presented in Figure 4 (for data, see Table S2). PC was the dominant lipid class with over 56% of the total PL content. PI and PE followed with 19.7% and 11.4%, respectively. *Lyso*-PL and PS were contained in lipid extract with a share of 6.6% and 3.4%. CL and PG exhibited the smallest fractions with 1.8% and 0.7%, respectively. Standard deviation of *S. cerevisiae* total lipid extract analysis varied depending on PL class proportion with 1.0-2.5% for the most abundant four classes, whereas low-concentrated lipid classes

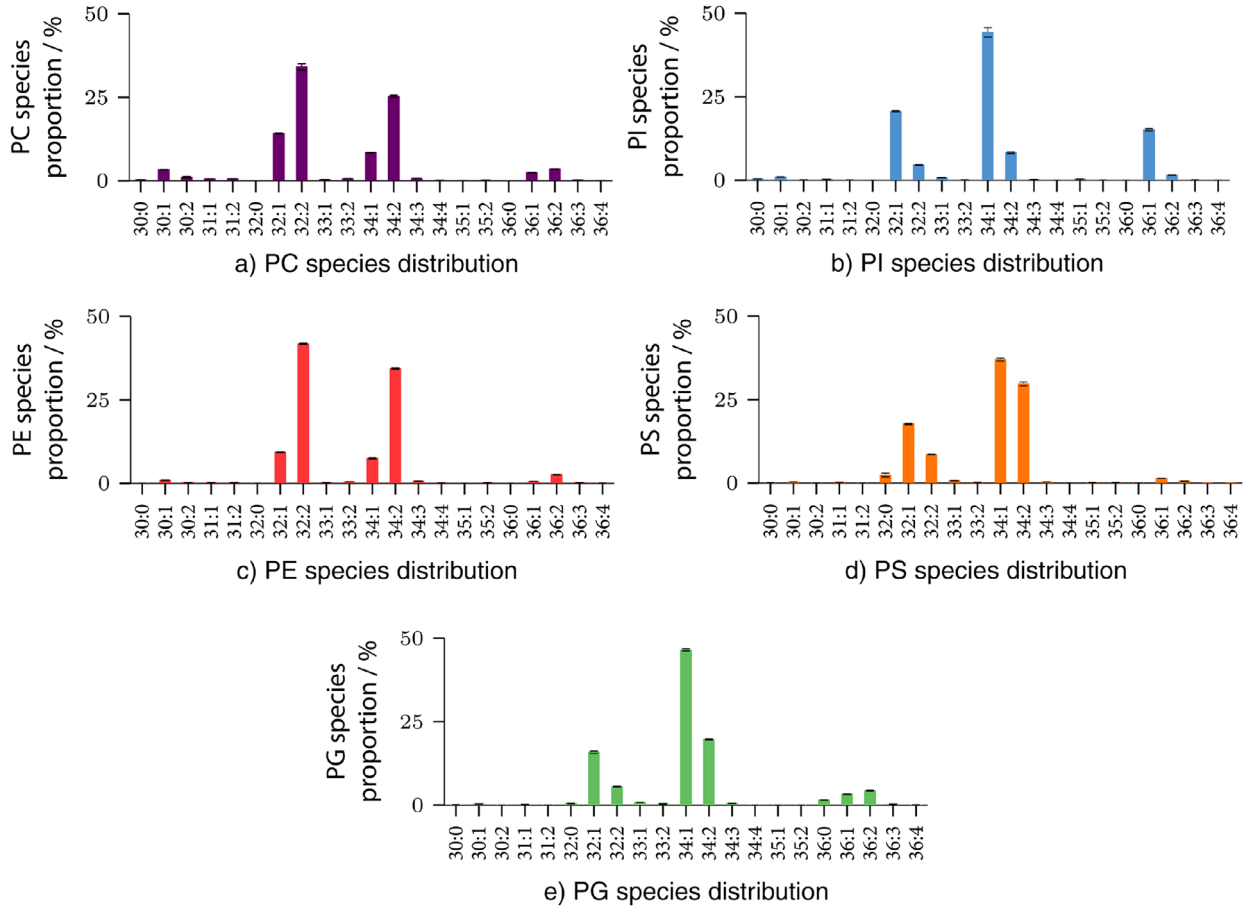

FIGURE 5 Distribution of PG, PI, PE, PS, and PC species of an *S. cerevisiae* total lipid extract analysis with HILIC-ESI-MS

had deviations in the range between 4.0% and 9.5%. A concentration of 7.5 ± 0.1 μg mL^{-1} (five times measured) was calculated for phosphorus in the total lipid extract analyzed with HILIC-ICP-MS/MS. Determination of total phosphorus content in *S. cerevisiae* extract was also performed with ICP-MS/MS. Total phosphorus content was determined with 7.5 ± 2.2 μg mL^{-1} (five replicates). The high standard deviation indicates an inhomogeneous distribution of lipids in the solid extract, which should be improved for future analyses. On the contrary, samples for the HILIC-ICP-MS/MS analyses seemed to be more homogeneous, originating from a prepared sample solution.

The HILIC-ICP-MS results are in accordance with the ICP-MS/MS analyses, which indicated that no further phosphorus species were contained in the total lipid extract and no loss of phosphorus species occurred in the LC-MS system.

Determination of PL species was performed by HILIC-ESI-MS. MS/MS was used for structural identification. Lipid species distributions were provided. Identification of PL species was carried out based on retention time, accurate mass, and characteristic fragments. MS/MS data were not obtained for all PL species. Possible reasons for this could be low intensities or a too short exclusion time, which is why more intense masses were more preferably selected compared to masses of lower intensity in the data-dependent acquisition mode of the used quadrupole-orbitrap MS. PL species with retention times matched by the homologous series are also listed in Table S3.

The distribution of PL species in *S. cerevisiae* total lipid extract is presented in Figure 5. Lipid classes of PG, PI, PE, PS, and PC were dominated by species with a total FA composition with chain lengths of 32 and 34 carbon atoms as well as zero to two double bonds. However, further species were also identified with total chain lengths of 26-36 carbon atoms and zero to four double bonds, whereby even FA compositions with up to 44 carbon atoms were determined for PI. The individual FA consisted of even- and odd-numbered chain lengths with 10-18 carbon atoms (for PI up to 26 carbon atoms) and zero to three double bonds. *Lyso*-PL species were characterized by FA 16:0, FA 16:1, FA 18:0, and FA 18:1. Interestingly, all *lyso*-PL species exhibited one preferred constitutional isomer, which had a higher retention time compared to the other isomer. A discrimination approach of *sn*-1 and *sn*-2 regioisomers of *lyso*-PL is the determination of the ratio of diagnostic product ions in the MS/MS data in the positive ionization mode.[32,33] These data were not obtained in the present study. Fang et al. used a RP C8 stationary phase to separate *lyso*-PL.[33] Their results indicated a *lyso*-PL regioisomer elution order of *sn*-2 and *sn*-1. Under the HILIC conditions applied in our study, the *sn*-1 regioisomers would be the main isomers of *lyso*-PL.

4 | CONCLUDING REMARKS

The complementary analysis approach with HILIC-ICP-MS/MS and HILIC-ESI-MS/MS is a powerful tool for PL profiling. ICP-MS/MS can provide the absolute quantification of PL classes by using compound-independent calibration, whereas ESI-MS/MS provides structural information and relative distributions of PL species within a lipid class. This approach enables the determination of alterations in PL class concentrations and lipid species distributions. In addition, the obtained complementary data can provide more detailed insights into dynamic processes and cellular relationships with a higher accuracy of predictions about metabolic and kinetic response of living cells to environmental changes.

An ICP-MS/MS method for compound-independent PL quantification was developed by hyphenation to HILIC separation. The unexpected nonuniversal response of PL classes revealed solubility problems of some PL classes necessitating further optimization of the composition of the injection solvent. An inverse gradient system was implemented for constant mobile phase composition after HILIC separation. Thus, constant plasma and ionization conditions were provided. Polyatomic interferences were decreased in the CRC of the ICP-MS/MS instrument by shifting the *m/z* for phosphorus after reaction with oxygen to $^{31}P^{16}O^{+}$ from *m/z* 31 to *m/z* 47. The optimized CRC gas composition consisted of oxygen and helium. Calibration series of PL with BMP as internal standard showed similar calibration curves, which confirmed the universal response for phosphorus when applying ICP-MS/MS detection and exhibited a LOD of 80 pg phosphorus on column.

The applicability of the developed complementary approach of PL classes and species was demonstrated by analysis of a total lipid extract of *S. cerevisiae*. Absolute quantification of PL classes was performed by HILIC-ICP-MS/MS. BMP could be utilized as phosphorus-containing internal standard due to its absence in the extract. PL class distribution was dominated by PC, followed by PI and PE. Standard deviations were in a range of 1.0-2.5% for most abundant and 4.0-9.5% for lower concentrated lipid classes. Determination of total phosphorus by ICP-MS/MS resulted in the same phosphorus amount as determined by HILIC-ICP-MS/MS.

Structural information of lipid species and their distribution within lipid classes were obtained by HILIC-ESI-MS/MS. Their identification was based on retention time, accurate mass, and characteristic fragments. Species distributions were dominated by four similar FA compositions of each PL class. The selection parameters for data-dependent MS/MS data acquisition should be improved to increase confidence of identification in future experiments.

The developed HILIC-ICP-MS/MS method provides potential for improvements. Buffer concentration for PL class separation can be fine-tuned to increase chromatographic resolution, particularly for PS and PC. Reduction of total flow rate resulted in higher intensities for phosphorus. Comparable chromatographic separation can be achieved by using columns with smaller inner diameter and adjusting the flow rate to lower values. Another approach is the optimization of the sample introduction. Improvement of the LOD and LOQ for phosphorus detection could be achieved by using other desolvation systems. A more efficient sample introduction into the plasma as well as the formation of a mostly dry aerosol with decreased organic solvent could lead to a higher transport efficiency into the plasma as well as enhanced phosphorus ionization, and thus to improved detection limits compared to the conventional cyclonic spray chamber introduction system. Birka et al. showed an increase in the intensity by factor 2.7 of the Apex Q desolvation system for the ionization of gadolinium based contrast agents in surface waters also using an HILIC-ICP-MS approach.[34] Thereby, the quantification of minor PL classes such as BMP, for example in tissue samples, could be enabled.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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