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Quantifying Phospholipids in Organic Samples Using a Hydrophilic Interaction Liquid Chromatography - Inductively Coupled Plasma High Resolution Mass Spectrometry (HILIC-ICP-HRMS) Method

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ABSTRACT: In this study, a novel method using hydrophilic interaction liquid chromatography (HILIC) coupled with inductively coupled plasma high resolution mass spectrometry (ICP-HRMS) was introduced for the quantification of phospholipids in oil samples. The method employed a bridged ethyl hybrid (BEH) stationary phase HILIC column with a tetrahydrofuran (THF)/water mobile phase, enhancing the solubility and detection of phospholipids. During the study, a gradient/matrix effect on ICP-HRMS sensitivity was observed and successfully compensated for experimentally, ensuring reliable quantification results. This approach has proven effective for a wide range of different oil samples, including vegetable oils, animal fats, and phospholipid supplements. Notably, this method allowed the direct quantification of phospholipids in oil samples, bypassing the need for prior sample preparation methods such as solid phase extraction (SPE), thereby streamlining the analytical process. The precision, accuracy, and reduced need for extensive sample preparation offered by this method mark a significant advancement in lipids analysis. Its robustness and broad applicability have substantial implications for industries such as food, and renewable energy production, where both efficient and accurate lipid identification and quantification are crucial.

~~1.~~ INTRODUCTION.

Phospholipids, with their unique amphiphilic structure, are vital components of cell membranes.[1] Particularly in fish, the need for a dietary supply of phospholipids for good survival, development and growth during the critical early feeding stages has been highlighted.[2,3] These molecules play a crucial role not only in biological systems but also in a range of industrial applications, including pharmaceuticals [4] and commercial product formulation,[5] particularly in drug delivery systems.[5] However, their presence is not always advantageous. In the production of edible oils and fats, for instance, phospholipids can adversely affect product stability, making their removal a critical step in refining processes like degumming and bleaching.[6] Additionally, the issue of phospholipid removal has become increasingly relevant in the energy sector, especially in the context of producing advanced biofuels such as biodiesel (methyl fatty acid ester) and renewable diesel (alkane chains) from non-edible and waste feedstocks like used cooking oil (UCO) and animal fat, through alkali catalyst transesterification,[7] and hydrodeoxygenation (HDO), respectively.[8] In HDO, phospholipids can hinder essential catalytic hydrodeoxygenation processes, which are crucial for converting triglycerides into diesel-range hydrocarbons, by poisoning the active sites of hydrotreating catalysts.[8] This underscores the need for efficient phospholipid removal and corresponding analysis methods in various industrial applications. The need for phospholipid speciation arises from the fact that certain phospholipids exhibit greater resistance to treatment processes such as bleaching and degumming than others, making total phosphorus content an inadequate metric; identifying and quantifying these resistant phospholipids is key to improving the treatment methods.

The chemical structure of phospholipids comprises a hydrophilic head with a phosphate group and two hydrophobic fatty acid tails. This amphiphilic nature enables them to form the structural basis of cell membranes, providing essential barrier and functional properties. Various families of phospholipids exist

depending on a specific molecule attached to the phosphate group, such as phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS), refer to Figure 1. Additionally, within each phospholipid family, there exists a range of compounds characterized by the variability in the carbon chain length and degree of saturation of the fatty acid tails.[5] Phospholipid analysis has significantly evolved from traditional to advanced techniques for their qualitative and quantitative assessment. Techniques such as thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) are relevant approaches in phospholipid analysis, as observed in the IUPAC's Standard Methods for the Analysis of Oils, Fats, and Derivatives.[9] Phospholipid analytes in biological samples, such as food, blood, and tissues, typically require extraction steps for solubilization prior to analysis. Standardized classical methods like Folch and Bligh-Dyer's methods are used for effectively separating lipids from proteins, salts, and non-lipid substances, enabling a more precise analysis.[10,11] In lipid samples, phospholipids are commonly found mixed with other lipids like triglycerides and fatty acids. These additional lipids can interfere in chromatographic systems, necessitating the isolation of phospholipids. This is achieved using solubility separation techniques[12] or solid phase extraction (SPE), a method that effectively separates various phospholipid classes based on their polarity.[13]

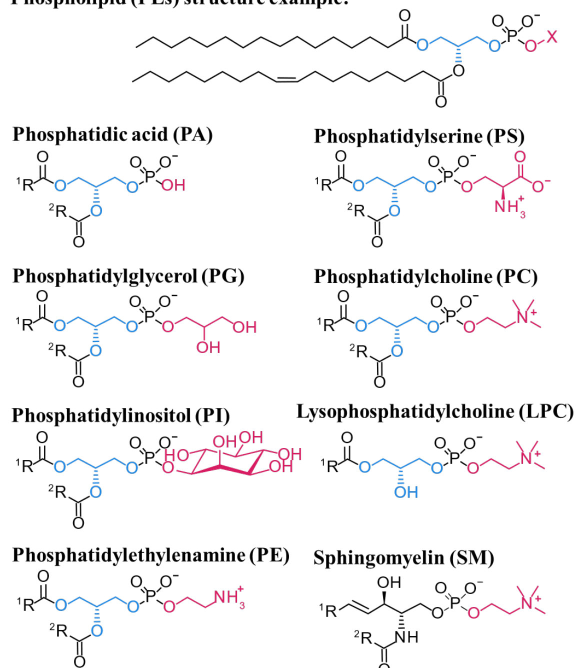


Figure 1. Chemical Structures of Main Phospholipid Families.

TLC can separate phospholipids based on their polar head groups allowing the easy visualization and quantification of phospholipids using various detection methods, i.e. UV visualization (using selective staining agents like molybdate reagent),[9] flame ionization detection (FID),[14] and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). [15] While TLC remains a practical method for phospholipid analysis, HPLC offers greater advantages in the automated analysis of large batches of samples. The initial HPLC approaches relied on normal phase liquid chromatography (NPLC) to separate different phospholipid families. This was performed using silica and diol-modified silica columns, paired with UV-visible (206 nm) detection,[9] evaporative light scattering detector (ELSD),[16] charged aerosol detector (CAD),[12] and phosphorous targeted inductively coupled plasma mass spectrometry (ICP-MS).[17,18] However, as HPLC and mass spectrometry evolved, chromatographic systems such as reversed-phase liquid chromatography (RPLC) using water, and water miscible solvents as a mobile phase that are compatible with MS detection, gained popularity in research. However, RPLC on its own is not effective for phospholipids analysis as it separates them based on their apolar tail lengths.[19] To overcome this limitation, hydrophilic interaction liquid chromatography (HILIC) was developed. HILIC combines a polar stationary phase like NPLC and a polar mobile phase similar to RPLC, commonly using acetonitrile (ACN) as the solvent. The main advantage of HILIC over NPLC is its compatibility with ionization sources more suitable for the analysis of polar compounds, such as electrospray ionization that can be hyphenated to different mass analyzers such as ion trap mass spectrometry (ESI-it-MS),[20,21] ESI-MS/MS,[22,23] quadrupole time-of-flight mass spectrometry (Q-TOF-MS),[24,25] ESI-IT-TOF-MS,[26] and ESI-high-resolution MS,[23,27] as well as traditional light scattering detectors (LSD),[28] and charged aerosol detectors (CAD)[25]. While RPLC alone is inadequate for studying phospholipid families, two-dimensional approaches combining HILIC and RPLC, coupled with MS detectors, have been reported in the literature.[29–31] This innovative fusion of advance analytical techniques marks a significant advancement in the comprehensive analysis of phospholipids, paving the way for deeper insights in lipidomics.

Elemental detection shows to be an important approach for both total determination of phosphorus and phospholipid speciation in different kinds of lipid samples. The phosphorus-containing polar heads in phospholipid molecules, makes ICP-MS a particularly selective and effective detector for analyzing complex lipidic matrices. The total phosphorus content, which is crucial information for industrial applications, can be measured by techniques such as inductively coupled plasma optical emission spectroscopy (ICP-OES).[32] However, for speciation approaches targeting phospholipid families, a more sensitive technique like ICP-MS is required when coupled with the respective LC system. In ICP-MS, the solubilized sample is nebulized into an aerosol that is then introduced into a plasma heated to thousands of kelvins. This process desolvates the sample, atomizing and ionizing the analyte molecules. ^{31}P is the only stable isotope of phosphorus and is commonly targeted in ICP-MS. The mass spectrometry analyzer is used to detect the positive ion ($^{31}P^+$) with universal and equimolar response to all phosphorus-containing species.[33] Eliminating the need for costly pure phospholipid in chloroform standards, typically used for identification purposes. For routine analysis, a more stable and affordable standard such as Conostan oil can be effectively used for calibration. However, polyatomic interferences such as $^{15}N^{16}O^+$, and $^{14}N^{16}O^1H^+$ require

spectral resolutions ($m/\Delta m$) of at least 1500. These interferences can be avoided using medium resolution ($m/\Delta m$ = 4000) in inductively coupled plasma sector field mass spectrometry (ICP-SFMS), also known as inductively coupled plasma high-resolution mass spectrometry (ICP-HRMS), or by using a mass-shift approach, that is inducing reactions of the analyte of interest in a dynamic reaction cell quadrupole inductively coupled plasma mass spectrometry (DRC-Q-ICP-MS), ~~also known as triple quadrupole inductively coupled plasma mass spectrometry (TQ-ICP-MS),~~ with a reactional or collisional gas, in this case moving from m/z= 31 to m/z= 47 ($^{31}P^{16}O^+$) to avoid the interferences present at m/z 31, this approach has evolved to the triple quadrupole inductively coupled plasma mass spectrometry with Q1 and Q3 mass filtering. Both methods perform well, but ICP-SFMS achieves better results in terms of the limit of detection (LOD) compared to DRC-Q-ICP-MS in phosphorus detection as observed for phosphopeptides analytes.[34] The coupling HPLC ICP-MS for the analysis of complex organic matrices represents an analytical challenge.[35,36] The coupling of HILIC with ICP-MS for phospholipid analysis was pioneered by Vosse *et al.*[23] Utilizing a double pump make-up system compensated for gradient effects by consistently sending the same solvent proportion to the ICP-MS throughout the gradient evolution. This approach maintained stable conditions in the plasma, preserving sensitivity, and addressed the issue through an additional instrumentation solution. This approach is referred to as an inverse gradient, and it is also used in aerosol-based detectors in HPLC that are sensitive to eluent composition, like ELSD, and CAD.[37] Their study evidenced a complementary approach, combining HILIC's elemental detection capabilities with ICP-MS/MS (or DCR-Q-ICP-MS) and molecular analysis using ESI-HRMS.[23]

A common feature of the phospholipid analysis methods previously described is reliance on ACN as the primary mobile phase component because it is compatible with the MS system. However, not all fats and oils fully dissolve in ACN. In this work a novel approach has been introduced with the direct analysis of phospholipids using HILIC-ICP-HRMS with a tetrahydrofuran (THF) based mobile phase that eliminates the need for sample pretreatment for lipid fractionation, and ensuring full solubility of the samples. The high lipid solubility, and water miscibility of THF, and the selective and absolute detection capabilities of ICP-HRMS make this method effective. The method relies on a BEH stationary phase HILIC column, known for its proficiency in phospholipid separations,[38] and resistance to a THF/water mobile phase. To enhance quantification accuracy, the gradient effect on signal sensitivity was assessed and corrected using an experimental measured time-dependent correction factor. This methodology enables direct lipid sample analysis without prior SPE, streamlining phospholipid speciation. The reduction in extensive sample preparation not only saves time but also offers environmental and economic advantages, contributing to sustainable practices. This method holds potential in various field, especially renewable energy production, but future research might expand this method's scalability and application to a broader range of samples, with other THF compatible stationary phases.

~~2.~~ MATERIALS AND METHODS

~~2.1.~~ Reagents, Samples, and Materials

HPLC-grade THF stabilized with 250 ppm BHT (Scharlau, Spain), served both as a solvent in sample preparation and as a constituent of the mobile phase gradient, along with purified water (18.2MΩcm) from a Milli-Q System (Merck Millipore, Molsheim, France). LC MS grade reagents ammonium formate (Fisher Chemical, USA), and formic acid (Sigma-Aldrich, Germany) were used in the buffer system. Asolectin, a soybean extract was used as natural phospholipid standard (Sigma-Aldrich, Germany). A set of isolated phospholipid standards, dissolved at 10 mg/mL in chloroform (Sigma-Aldrich, Germany), was analyzed, among them: L-α-Phosphatidylinositol ammonium salt (bovine liver), L-α-phosphatidylserine sodium salt (porcine brain), L-α-Lysophosphatidylcholine (soybean), 1,2-didecanoyl-sn-glycero-3-phosphoethanolamine (10:0 PE), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphate sodium salt (18:0-18:1 PA), 1,2-dilignoceroyl-sn-glycero-3-phosphocholine (24:0 PC), 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) sodium salt (18:0-18:1 PG), Sphingomyelin (chicken egg). These standards were subsequently diluted to 0.1 mg/mL (100 ppm) in THF for analysis. To quantify phospholipid families in the samples, an external calibration method was employed. The calibration curve was established using a series of solutions with concentrations ranging from 0 to 1 ppm in THF. These solutions were prepared using the Conostan oil-soluble phosphorus standard at 5000 ppm (SCP Science, Canada).

To validate the versatility of the methodology, multiple lipid samples from different matrices were investigated. Among them, crude rapeseed oil from INRAE, and its commercial refined counterpart, and home-recovered UCO produced from commercial rapeseed oil, each diluted five-fold by weight in THF. Analogously, crude pork fat sourced from Haldor Topsoe was compared with commercial refined counterpart (Saindoux), with both samples being 25-fold diluted by weight in THF. Finally, to further demonstrate the method's versatility in both nutritional and pharmaceutical contexts, it was applied to a rainbow trout (*Oncorhynchus mykiss*) egg Folch lipid extract[10] sourced from INRAE, diluted 300-fold by weight in THF, and commercial supplements like soy lecithin and krill oil, each diluted 5,000-fold by weight in THF. More details are indicated in Table 1 for total phosphorus concentrations in samples determined by total area integration of HILIC-ICP-HRMS chromatograms.

Table 1. Phosphorus concentrations in samples determined by total area integration of HILIC ICP-HRMS chromatograms.

Sample Name		[^{31}P] (ppm)
Rapeseed oil	Crude	6.74 ± 0.49
	Commercial	0.31 ± 0.02
	UCO	2.55 ± 0.46
Animal fat	Crude	26.26 ± 0.88
	Commercial	-
Sample Name		[^{31}P] (mg/g)
	Soy Lecithin	13.08 ± 0.12

Supplement phospholipid capsule	Krill oil	17.61 ± 0.09
Rainbow trout egg lipids		1.53 ± 0.03

~~2.2.~~ Instrumentation

For sample analysis, a Dionex high-performance liquid chromatography (HPLC) system was employed, consisting of an UltiMate 3000 microflow pump, an UltiMate 3000 autosampler, and a low port-to-port dead-volume microinjection valve. The separation process was performed using an XBridge BEH HILIC Column (130Å, 3.5 µm, 4.6 mm i.d. x 100 mm length) (Waters Corporation, Milford, MA). The mobile phase gradient was formed by a combination of aqueous ammonium acetate buffer (10 mM, 0.1% formic acid, 5% THF) (A) and THF (B). The gradient initiated at 0 minutes with 95% B, maintaining this composition until 0.5 minutes, then transitioning to 81.8% B by 18.5 minutes, and reverting to 95% B from 19 to 23 minutes with a flowrate of 0.300 mL/min. The overall analysis run-time was 23 min, and the injection volume was 5 µL. The inductively coupled plasma mass spectrometer (ICP-MS) was hyphenated using a custom 1:10 splitter to reduce the flow rate and maintain plasma stability. The experiments were conducted using a Thermo Scientific Element XR double focusing sector field ICP-MS (Thermo Fischer, Germany) at medium resolution (R = 4,000), to accurately detect ^{31}P isotopes while effectively overcoming spectral interferences, notably $^{15}N^{16}O^{+}$, and $^{14}N^{16}O^{1}H^{+}$.[33] A modified DS-5 micro-flow total consumption nebulizer (CETAC, Omaha, NE), was integrated with the system, connected to a custom-made spray chamber, which was heated to 60°C via a custom-made electric heater, pre-heating the solvent before its introduction reduces the energy required from the plasma for vaporization and atomization, thereby allowing more energy to be available for the ionization process.[39] This equipment comprised a quartz injector with a 1.0 mm i.d., a Pt sampling cone with a 1.1 mm i.d., and a Pt skimmer cone with 0.8 mm i.d., all purchased from Thermo Fischer, Germany. To mitigate carbon deposition on the cone surfaces, an O_2 flow rate of 0.08 L/min was incorporated into the argon carrier gas. The mass spectrometer was tuned and calibrated at low (R = 300) and medium (R = 4,000) resolutions, by injecting a standard solution that included elements such as Ag, Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, In, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Sc, Si, Sn, Ti, V, Zn, and Y at 1.0 ng/g in THF. A mass offset was employed to counter the mass drift associated with the sector field magnet.

~~3.~~ RESULTS AND DISCUSSION

~~3.1.~~ Method validation.

The initial phase of the study focused on two primary objectives, with the first one being the identification of the experimental conditions necessary for differentiating various phospholipid families. This was achieved by employing a HILIC-ICP-HRMS system with THF as a substitute for ACN, to ensure sample solubilization and avoid sample pretreatment. Secondly, the study aimed to assess the feasibility of quantifying phosphorus-containing species using this methodology. In this context, the BEH column was selected because it is compatible with a THF mobile phase unlike other HILIC columns.

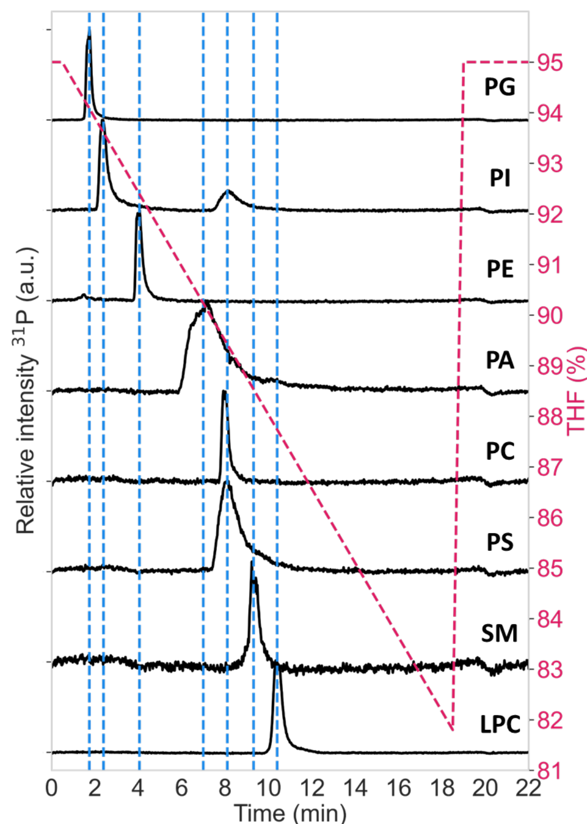


Figure 2. Normalized HILIC-ICP-MS chromatograms for isotope ^{31}P of phospholipid standards: PG, PI, PE, PA, PC, PS, SM, and LPC, and chromatographic gradient in terms of THF percentage.

~~3.1.1. Qualitative analysis~~

Qualitative analysis. During the development of the chromatographic method, the gradient was examined and adjusted to achieve the optimal chromatographic conditions (see Figure S1). The final methodology demonstrated satisfactory separation of key phospholipid families within 12 minutes, by reducing the concentration of phase B (THF), from 95% to 87%, as shown in Figure 2. Non-ionic phospholipids like PG and PI were retained less on the BEH column, with their elution order correlating to the number of oxygen atoms in their glycerol (#O = 2) and inositol (#O = 5) groups. In contrast, phospholipids that consistently remain ionic, such as choline-like (PC, SM, LCP) and amino acid-like (PS) ones, were more strongly retained. Notably, the most retained phospholipid in the standard list was LPC, since it has only one fatty acid group making it more polar than other phospholipids on the list. Two major challenges were encountered: the coelution of PC and PS, and the band broadening of PA and PS. Attempts to overcome these issues by modifying buffer composition and temperature were not successful, largely due to the limitations of the operational conditions of the stationary phase, and the pH dependence of the equilibrium between the protonated and deprotonated species of the phospholipids.[38] Despite these issues, the THF-based HILIC method was effective for separating most lipid standards

and was subsequently applied to assess its quantification capabilities.

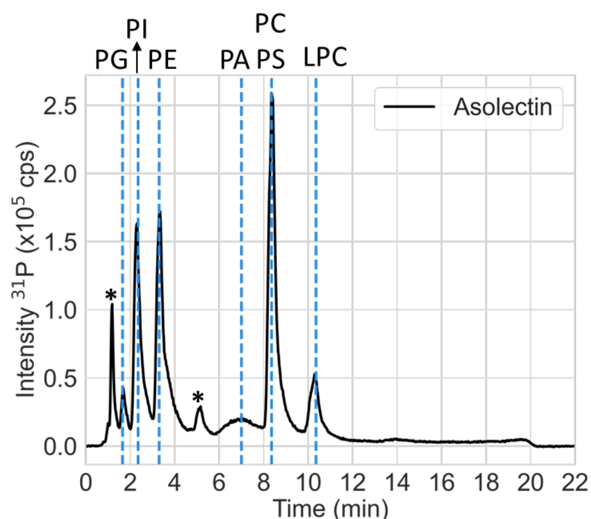


Figure 3. HILIC-ICP-HRMS chromatogram of asolectin soybean extract for isotope ^{31}P.

Phospholipids with the same polar head can experience varying retention times during chromatographic analysis due to differences in the lengths of their nonpolar fatty acid tails, which typically range from 14 to 24 carbon atoms.[40] This principle explains the retention time discrepancies observed between pure standards and natural phospholipid mixtures, [26] and is exemplified in Figure 3 for asolectin, a soybean extract used as a natural standard. The extract is composed of roughly equal amounts of PC (phosphatidylcholine), PE (phosphatidylethanolamine), and PI (phosphatidylinositol), with lesser quantities of other phospholipid families, according to Sigma Aldrich's description. Notably, the retention time for the pure standard of PE with a 10:0 fatty acid chain (4.05 min) and PC with a 24:0 fatty acid chain (8.10 min) does not match the times for PE (3.30 min) and PC (8.35 min) found in asolectin, as depicted in Figure 3. The smaller, more polar pure PE standard is retained longer, while the larger, less polar pure PC standard shows less retention, compared to the fatty acid lengths in asolectin. Peaks for PG (phosphatidylglycerol), PA (phosphatidic acid), LPC (lysophosphatidylcholine), and two unidentified compounds marked with asterisks are also observed. Due to these variations, the retention times in asolectin will serve as reference points for analyzing natural samples of the PE and PC families.

~~3.1.2. Quantitative analysis.~~

Quantitative analysis. The Conostan oil standard was used to quantify phosphorous because it has a well-known phosphorus concentration and is not retained in the HILIC column (retention time: 0.8 min). ICP-HRMS is an advanced elemental detector that offers notable advantages for the analysis of inorganic elements such as phosphorus in molecules like phospholipids, effectively overcoming challenges posed by complex matrices with numerous potential interferences. In ICP-MS, the analytes dissolved in liquid solvents must be nebulized to create an aerosol, which is then introduced to the plasma for desolvation, atomization, and ionization. This process's effectiveness can be affected by various parameters, including temperature and the solvent used. In HILIC analysis, where a gradient is applied, the solvent composition shifts over time, potentially impacting the sensitivity of the signal during the gradient. To understand this effect, a calibration curve from triplicate injections of 0 to 1 ppm phosphorus standard solutions was developed under both the initial gradient and specific isocratic conditions that matched the gradient's composition at set times (1.5, 4.0, 9.0, and 11.0 minutes), covering the range where phospholipid families elute.

Figure 4a and Figure 4b demonstrate that modifications in the mobile phase composition impacts signal sensitivity, indicated by the declining slope of the calibration curves as the gradient composition progresses over time in the original data. However, this reduction in sensitivity is not uniform; at the gradient composition corresponding to 11.0 minutes, there is a slight increase in sensitivity compared to that at 9.0 minutes, suggesting a nonlinear decrease. To better understand this non-linear decrease and attempt a correction in the signal, the data was re-plotted, showing time against the integrated area for each concentration (Figure S2a). This visual representation made it clear that the decrease was not linear and that at certain points, the signal intensity began to rise again. When a second-order polynomial regression was applied, this behavior was consistent across all tested concentrations (0.2 – 1.0 ppm). To adjust the signal sensitivity over time, data from various concentrations were normalized. This normalized data was then employed to derive a second-order polynomial regression of relative area versus time (as illustrated in Figure S2b). Following this, the initial time was set to a value of one (t_0=1), and its inverse was used to obtain a time-dependent correction factor.

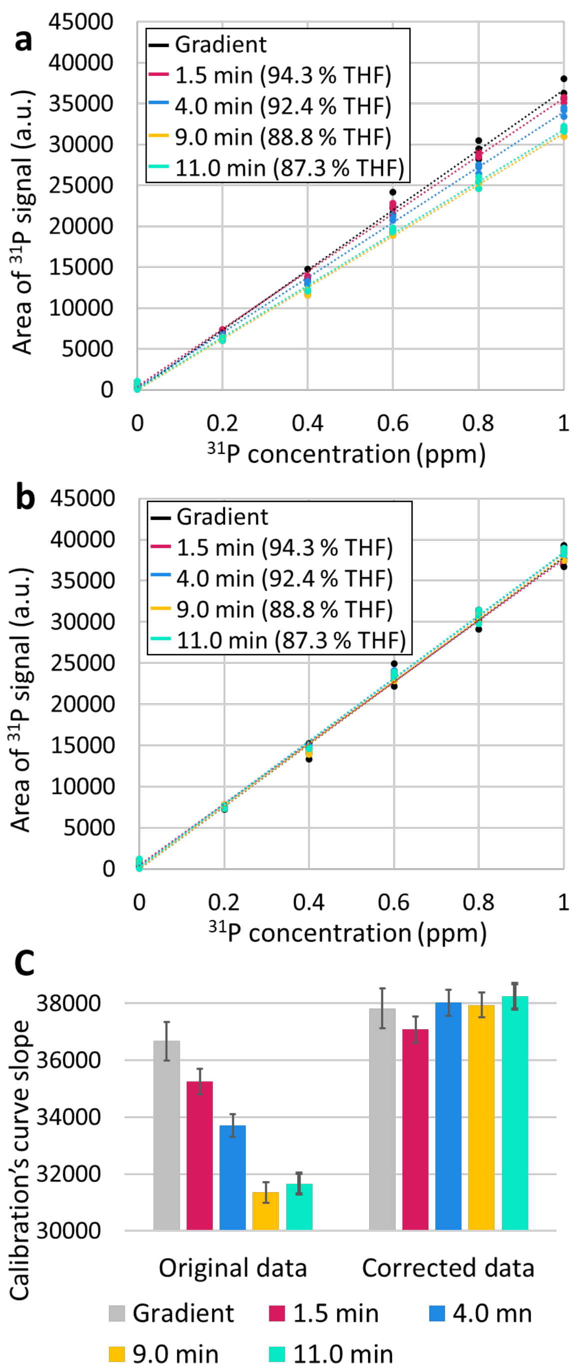
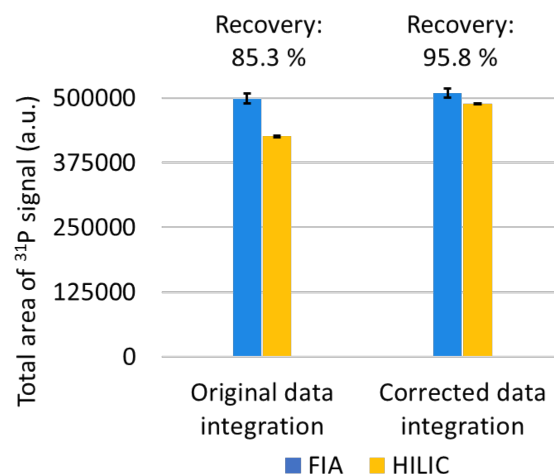


Figure 4. Phosphorus calibration via HILIC ICP-HRMS (triplicate injections 0-1 ppm): (a) original, (b) corrected curves, and (c) slope comparisons under varied isocratic conditions.

This factor, going from 1.000 to a maximum of 1.215 at 9.92 minutes was then applied multiplicatively to the original data (refer to equation 1). The effectiveness of this correction became clear upon comparing the original calibration curves (Figure 4a) with the corrected ones (Figure 4b), as observed in the bar chart of Figure 4c. Notably, this comparison showed that the calibration curves, though based on the same solutions but with varying solvent compositions, exhibited remarkable similarity after the application of the correction factor to the areas. The slopes (A) and determination coefficients (R^2) from the linear regression equation (y = Ax) for all calibration curves are presented in Table S1. The intercept values were disregarded because the associated error was significantly larger than the values themselves. Specifically, the calibration curve employed for sample quantification is the gradient curve of corrected data. Using this calibration curve, the estimated limit of detection (LOD) was 0.09 ppm and the limit of quantification (LOQ) was 0.28 ppm.

$$S_{corr} = S_{origin} \times \frac{1}{1{,}8 \times 10^{-3}\, t^2 - 3.57 \times 10^{-2}\, t + 1} \quad \text{(eq. 1)}$$

Additionally, the interaction between the column and analytes in chromatographic processes may lead to some analytes being permanently retained within the columns. To address this, the mass balance was assessed by comparing the integrated areas of the asolectin standard analyzed with and without the column. Analysis without the column, or flow injection analysis (FIA), results in a single peak rather than a full chromatogram, which is observed with the HILIC column (refer to Figure S3). To ensure an accurate mass balance where intensity varies with the gradient, data integrations were performed in both original data, and corrected data using equation 1. The mass balance results, presented in Figure 5, indicate that prior to signal correction, the recovery stands at 85.3%. After applying the time-dependent correction factor (as outlined in equation 1), the recovery increases to 95.8%. These findings suggest that any potential retention in phospholipid-containing samples is likely to be less than 5%, indicating the method's reliability, particularly when the correction factor is applied.



Total area integration		Mean (a.u.) (n = 3)	RSD (%) (n = 3)	Recovery (%)
Original data	FIA	498815	1.8	85.3
	HILIC	425590	0.3	
Corrected data	FIA	509457	1.8	95.8
	HILIC	487809	0.4	

Figure 5. Assessment of mass balance in asolectin soybean phospholipid extract with original and adjusted data from HILIC and FIA ICP-HRMS analysis.

In this study, the limit of detection (LOD) using ICP-HRMS as the analyzer/detector is 0.09 ppm, or 0.015 nmol per 5 µL injection volume. This sensitivity is comparable to other

techniques such as ICP-TQMS and CAD, and only ESI-MS shows superior sensitivity, as detailed in Table 2. It is worth noting that refractive index detection is the least sensitive method. Most literature reports LOD in terms of the mass of phosphorus on the column, so the table also presents LOD values in nmol to maintain consistency with these reports.[41]

Furthermore, ICP-HRMS enhances the selectivity and specificity of phosphorus detection, which is crucial for the analysis of phospholipid molecules. This improved selectivity is essential for accurate quantification within complex matrices, where other methods may encounter matrix interferences. Thus, even though the sensitivity of ICP-HRMS may be similar or slightly lower than some techniques like ESI-MS, its significant advantage in minimizing potential errors in complex sample analyses makes it an excellent choice for this research objectives.

Table 2. Comparison of LODs for chromatography methods with various detection systems in phospholipid analysis.

Separation Technique	Analyzer / Detector	LOD (nmol phosphorus)	Standard	Ref. Paper
NPLC	RI	0.6	PC	Grit *et al.* (1991)[42]
		2	LPG	
NPLC	ICP-MS	0.007	PG	Kovačevič *et al.* (2003)[18]
		0.04	PE, PS	
RPLC	(+) ESI-MS	0.0004	PC	Rabagny *et al.* (2011)[43]
		0.004	LPC	
NPLC	CAD	0.007	PC	Kiełbowicz *et al.* (2015)[44]
		0.05	PE	
SFC	CAD	0.0009	LPI	Takeda *et al.* (2019)[45]
		0.04	LPA	
HILIC	ICP-TQMS	0.003	PI	Vosse *et al.* (2020)[23]
HILIC	ICP-HRMS	0.015	Phosphorus Conostan oil	This work

~~3.2.~~ Method application in real samples.

After evaluating the methods for phospholipid speciation in both qualitative and quantitative terms, the selected method was applied to various sample types to assess its robustness and potential as an analytical technique.

~~**3.2.1. Food and energy industry samples (ppm or µg/g level).**~~

Food and energy industry samples (ppm or µg/g level). First, lipid samples with trace amounts of phosphorus from both plant and animal sources, which are used in the food and energy industries, were studied. Rapeseed oil samples were analyzed in different forms: as the original crude vegetable oil, its refined commercial variant for cooking, and a homemade recovered UCO derived from this commercial oil. The chromatograms of these samples are depicted in Figure 6a. Due to the low phosphorus content in these samples, only a 5-fold dilution by weight in THF was applied (refer to Table 1). The analysis revealed that the crude vegetable oil predominantly contained the PA family of phospholipids, along with others such as PG, PI, PE, and PC. Additionally, an unretained unknown peak was also observed~~. (refer to Table 1)~~.

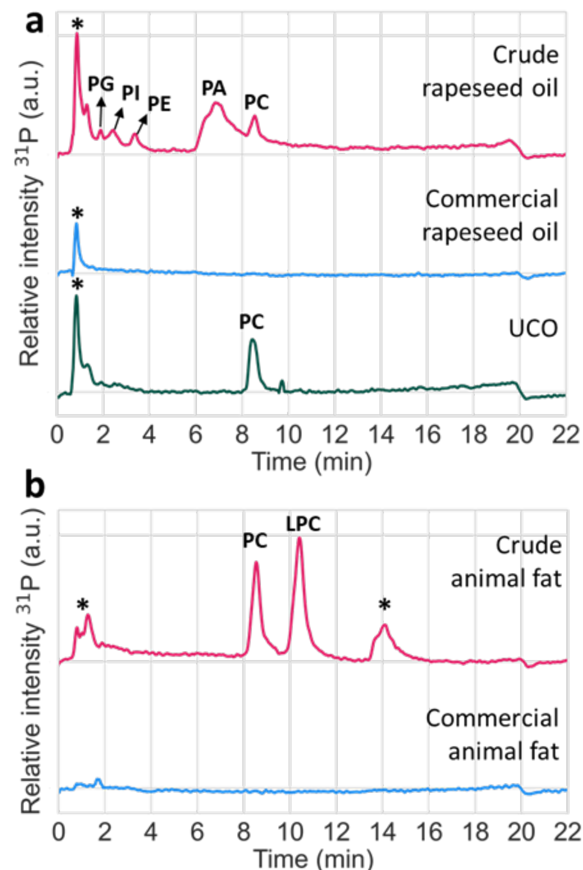


Figure 6. Normalized HILIC-ICP-MS chromatograms for isotope ^{31}P of lipid samples from vegetal and animal origin used in food and energy industry. (a) Rapeseed oil in different versions: crude, commercial (refined) and used cooking oil; and (b) animal fat in crude and commercial (refined) version.

Phospholipids are generally undesirable in cooking oils due to their destabilizing effects, even at ppm levels. The food industry employs various processes to remove these trace phospholipids, which is evident in the commercial rapeseed oil sample, where no phospholipid families were detected except for an unknown unretained peak. In UCO, phospholipids can reappear due to contact with meat and vegetables during cooking, as evidenced by the presence of PC and a significant proportion of the unknown unretained peak (see Figure 6). UCO, often discarded as waste, has gained attention as a potential feedstock for advanced renewable diesel production. Thus, the removal of phospholipids from these oils is crucial to avoid interference with catalytic hydrogenation processes that convert triglycerides into diesel. In this context, phospholipid speciation becomes vital for evaluating the efficacy of pretreatment steps. In the UCO sample examined, the PC concentration was below 1 ppm (see Table 3), though other feedstocks may have higher levels.

Additionally, two animal fat samples from pork were analyzed: a crude sample and a refined commercial sample (illustrated in Figure 6b). A 25-fold dilution by weight in THF was required for the animal samples because they had a higher phosphorous content. The refined sample exhibited no significant peaks, in contrast to the crude sample, which showed an unknown unretained peak and quantifiable amounts of PC and LCP (refer to Table 3). An unidentified band at 14 minutes was also detected, potentially corresponding to phosphocholine, considering that PC and LPC were the only identified phospholipids.[46] The results in Table 3 indicate a higher concentration of phospholipids in animal fat compared to rapeseed oil, attributable to their different biological roles and structures. Animal cells require phospholipids for complex cellular functions, whereas plant seeds predominantly store energy as triglycerides.

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Table 3. Concentrations in ppm of phosphorus in phospholipid families and unknown compounds detected by HILIC-ICP-MS in lipid samples from vegetal and animal origin used in food and energy industry. Rapeseed oil in different versions: crude, commercial (refined) and used cooking oil; and animal fat in crude and commercial (refined) version. The hyphen (-) represent, not observed species. The analysis was performed by triplicates (n = 3).

Phospholipids	Phosphorus concentration (ppm)				
	Rapeseed oil			Animal fat	
	Crude	Commercial	UCO	Crude	Commercial
Unknown 1	1.23 ± 0.26	0.31 ± 0.02	0.78 ± 0.08	2.79 ± 0.04	-
PG	~~0.23 ± 0.03~~ <LOQ	-	-	0.59 ± 0.07	-
PI	0.35 ± 0.05	-	-	1.10 ± 0.11	-
PE	0.35 ± 0.10	-	-	1.18 ± 0.07	-
PA	2.84 ± 0.19	-	-	-	-
PC	0.56 ± 0.04	-	0.84 ± 0.11	6.36 ± 0.25	-
LPC	-	-	-	9.23 ± 0.15	-
Unknown 2	-	-	-	3.92 ± 0.03	-
TOTAL	5.57 ± 0.35	0.31 ± 0.02	2.13 ± 0.16	25.17 ± 0.31	-

~~3.2.2. Pharmaceutical and biological samples (mg/g level).~~

Pharmaceutical and biological samples (mg/g level). The HILIC-ICP-MS method was extended to analyze phospholipids in pharmaceutical supplements and biological samples where the phosphorus content is on the order of milligrams per gram (mg/g). This study included two phospholipid supplements, soy lecithin and krill oil, representing vegetal and animal origins, respectively; and a Folch lipid extract from rainbow trout eggs. The high phosphorus concentrations for the phospholipid supplement capsules required substantially higher 5,000-fold dilution by weight. Figure 7 illustrates a comparative analysis of the supplements. The soy lecithin sample contained a more diverse array of phospholipid families compared to the krill oil, which primarily contained PC and LPC, with minimal presence of other lipids (refer to Table 4). Additionally, the profile of the soy lecithin closely resembled that of the asolectin standard from Sigma Aldrich, likely due to their common soybean origin (see Figure 3 and Figure 7).

The lipid extract from rainbow trout eggs was diluted 300-fold by weight in THF and contained mainly PC (see Figure 7). This suggests a predominance of this phospholipid family in the cell membranes of the sample, in accordance with data obtained by Vassallo-Agius *et al.* by separation on Sep-Pak silica gel cartridges followed by Iatroscan MK-5 TLC/FID chromatographic analysis.[47]

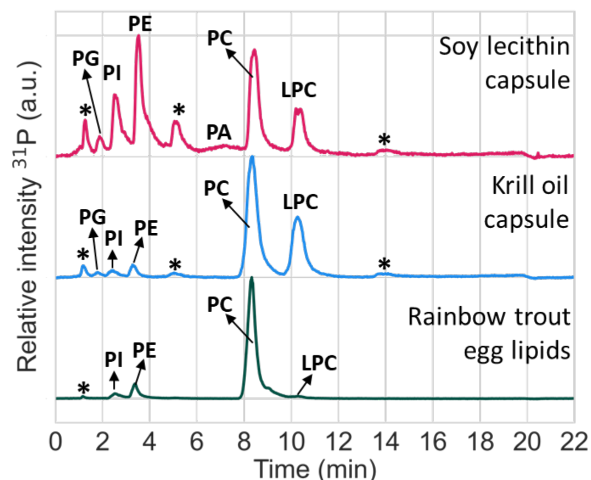

Figure 7. Normalized HILIC-ICP-MS chromatograms for isotope ^{31}P of pharmaceutical and biological samples: Capsule of phospholipid supplements from soy lecithin, and krill oil, respectively; and lipid extract from rainbow trout eggs obtained by Folch's method.

The pure krill oil showed a higher overall phosphorus content (16.572 mg/g) compared to the soy lecithin supplement (12.098 mg/g). According to the manufacturer of the commercial krill oil supplement, ach capsule contained 500 mg of pure krill oil with 200 mg of phospholipids. Based on the atomic mass of phosphorus (^{31}P, 30.973762 g/mol) and a proposed 750 g/mol average

molecular weight for phospholipids, each capsule contained an estimated 16.519 mg/g of phosphorus. This closely aligns with the experimentally obtained value of (16.572 ± 0.069) mg/g, confirming the accuracy of the results and providing insights into the phospholipid content of the supplements.

4. CONCLUSIONS.

The HILIC-ICP-HRMS method introduced in this study effectively facilitated the speciation of phospholipids in a variety of sample types with a wide range of phospholipid contents, without any prior sample processing such as SPE. In the chromatographic evaluation, the method typically achieved satisfactory separation for the majority of primary phospholipid families. Nonetheless, certain challenges were encountered, including the co-elution of PC and PS, and the broadening of the PA peak. Addressing these issues might be possible by experimenting with various mobile phases that are compatible with THF. From a quantitative standpoint, it was possible to elucidate the impact of the gradient on signal sensitivity. This was achieved by measuring sensitivity under isocratic conditions at different stages of the gradient. The effectiveness of this corrective approach was validated by comparing calibration curves before and after the adjustments, and also by confirming the mass balance of asolectin analysis, which showed a recovery rate of nearly 96%. The versatility of the method is demonstrated by application to a wide range of samples with trace and high concentrations of phospholipids, including vegetable oils, animal fats, phospholipid supplements, and a biological sample, highlighting its broad applicability in research. The accuracy of the method was further corroborated by comparing the total phosphorus content of a krill oil phospholipid supplement, which was experimentally determined to be (16.572 ± 0.069) mg/g, with the estimated value based on the supplement's composition, which was 16.519 mg/g. This innovative approach marks a significant advancement in phospholipid analysis, combining efficiency, accuracy, and a commitment to environmental sustainability.

Table 4. Concentrations in mg/g of phosphorus in phospholipid families and unknow compounds detected by HILIC-ICP-MS in pharmaceutical and biological samples: Capsule of phospholipid supplements from soy lecithin, and krill oil, respectively; and lipid extract from rainbow trout eggs obtained by Folch's method. The hyphen (-) represent, not observed species. The analysis was performed by triplicates (n = 3).

Phospholipid	Phosphorus concentration (mg/g)		
	Soy lecithin capsule	Krill oil capsule	Rainbow trout egg lipids
Unknown 1	0.450 ± 0.004	0.321 ± 0.004	0.008 ± 0.001
PG	0.333 ± 0.014	0.203 ± 0.004	-
PI	1.341 ± 0.017	0.384 ± 0.004	0.043 ± 0.005
PE	2.787 ± 0.013	0.496 ± 0.025	0.108 ± 0.004
Unknown 2	1.186 ± 0.023	0.332 ± 0.009	-
PA	0.783 ± 0.010	-	-
PC	3.131 ± 0.032	9.164 ± 0.054	1.290 ± 0.038
LPC	1.767 ± 0.021	5.245 ± 0.030	0.050 ± 0.005
Unknown 3	0.319 ± 0.009	0.428 ± 0.016	-
TOTAL	12.098 ± 0.053	16.572 ± 0.069	1.500 ± 0.039

ASSOCIATED CONTENT

Supporting Information

Gradient effect on phospholipid separation in asolectin standard solution (Figure S1); Effect of Gradient Composition on Phosphorus Calibration Sensitivity and Correction in HILIC-ICP-HRMS (Figure S2); Calibration curve parameters (y = Ax + B) for both original and corrected data (Table S1); Comparative analysis for mass balance of phospholipid species. Chromatograms of asolectin soybean extract by HILIC ICP-HRMS (left) versus FIA ICP-HRMS peak signals (right) (Figure S3). (PDF)

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Notes

The authors declare no competing financial interest.

REFERENCES:

(1) Iwasaki, Y.; Ishihara, K. Cell Membrane-Inspired Phospholipid Polymers for Developing Medical Devices with Excellent Biointerfaces. *Sci. Technol. Adv. Mater.* **2012**, *13* (6), 64101.

(2) Tocher, D. R.; Bendiksen Eldar Åand Campbell, P. J.; Bell, J. G. The Role of Phospholipids in Nutrition and Metabolism of Teleost Fish. *Aquaculture* **2008**, *280* (1–4), 21–34.

(3) Daprà, F.; Geurden, I.; Corraze, G.; Bazin, D.; Zambonino-Infante, J.-L.; Fontagné-Dicharry, S. Physiological and Molecular Responses to Dietary Phospholipids Vary between Fry and Early Juvenile Stages of Rainbow Trout (Oncorhynchus Mykiss). *Aquaculture* **2011**, *319*

(3–4), 377–384.

(4) Betz, G.; Aeppli, A.; Menshutina, N.; Leuenberger, H. In Vivo Comparison of Various Liposome Formulations for Cosmetic Application. *Int. J. Pharm.* **2005**, *296* (1–2), 44–54.

(5) Li, J.; Wang, X.; Zhang, T.; Wang, C.; Huang, Z.; Luo, X.; Deng, Y. A Review on Phospholipids and Their Main Applications in Drug Delivery Systems. *Asian J. Pharm. Sci.* **2015**, *10* (2), 81–98.

(6) Čmol\'\ik, J.; Pokorn\`y, J. Physical Refining of Edible Oils. *Eur. J. Lipid Sci. Technol.* **2000**, *102* (7), 472–486.

(7) Meng, X.; Chen, G.; Wang, Y. Biodiesel Production from Waste Cooking Oil via Alkali Catalyst and Its Engine Test. *Fuel Process. Technol.* **2008**, *89* (9), 851–857.

(8) Verdier, S.; Alkilde, O. F.; Chopra, R.; Gabrielsen, J.; Grubb, M. Hydroprocessing of Renewable Feedstocks-Challenges and Solutions. *Haldor Topsoe A/S Copenhagen, Denmark* **2019**.

(9) Dieffenbacher, A.; Pocklington, W. D. *Standard Methods for the Analysis of Oils, Fats and Derivatives: 1st Supplement to the 7th Edition*; Blackwell Scientific Publications: Oxford, UK, 1992.

(10) Folch, J.; Lees, M.; Sloane Stanley, G. H.; others. A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. *J biol Chem* **1957**, *226* (1), 497–509.

(11) Bligh, E. G.; Dyer, W. J. A Rapid Method of Total Lipid Extraction and Purification. *Can. J. Biochem. Physiol.* **1959**, *37* (8), 911–917.

(12) Gładkowski, W.; Chojnacka, A.; Kiełbowicz, G.; Trziszka, T.; Wawrzeńczyk, C. Isolation of Pure Phospholipid Fraction from Egg Yolk. *J. Am. Oil Chem. Soc.* **2012**, *89*, 179–182.

(13) Kaluzny, M. A.; Duncan, L. A.; Merritt, M. V; Epps, D. E. Rapid Separation of Lipid Classes in High Yield and Purity Using Bonded Phase Columns. *J. Lipid Res.* **1985**, *26* (1), 135–140.

(14) De Schrijver, R.; Vermeulen, D. Separation and Quantitation of Phospholipids in Animal Tissues by Latroscan TLC/FID. *Lipids* **1991**, *26* (1), 74–76. https://doi.org/10.1007/BF02544028.

(15) Fuchs, B.; Schiller, J.; Süß, R.; Schürenberg, M.; Suckau, D. A Direct and Simple Method of Coupling Matrix-Assisted Laser Desorption and Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) to Thin-Layer Chromatography (TLC) for the Analysis of Phospholipids from Egg Yolk. *Anal. Bioanal. Chem.* **2007**, *389*, 827–834.

(16) Sas, B.; Peys, E.; Helsen, M. Efficient Method for (Lyso) Phospholipid Class Separation by High-Performance Liquid Chromatography Using an Evaporative Light-Scattering Detector. *J. Chromatogr. A* **1999**, *864* (1), 179–182.

(17) Axelsson, B.-O.; Jörnten-Karlsson, M.; Michelsen, P.; Abou-Shakra, F. The Potential of Inductively Coupled Plasma Mass Spectrometry Detection for High-Performance Liquid Chromatography Combined with Accurate Mass Measurement of Organic Pharmaceutical Compounds. *Rapid Commun. Mass Spectrom.* **2001**, *15* (6), 375–385.

(18) Kovačevič, M.; Leber, R.; Kohlwein, S. D.; Goessler, W. Application of Inductively Coupled Plasma Mass Spectrometry to Phospholipid Analysis. *J. Anal. At. Spectrom.* **2004**, *19* (1), 80–84.

(19) Ritchie, M.; Mal, M.; Wong, S. *UPLC BEH HILIC: The Preferred Separation Chemistry for Targeted Analysis of Phospholipids*; Milford, MA, USA, 2012.

(20) Schwalbe-Herrmann, M.; Willmann, J.; Leibfritz, D. Separation of Phospholipid Classes by Hydrophilic Interaction Chromatography Detected by Electrospray Ionization Mass Spectrometry. *J. Chromatogr. A* **2010**, *1217* (32), 5179–5183.

(21) Zhu, C.; Dane, A.; Spijksma, G.; Wang, M.; der Greef, J.; Luo, G.; Hankemeier, T.; Vreeken, R. J. An Efficient Hydrophilic Interaction Liquid Chromatography Separation of 7 Phospholipid Classes Based on a Diol Column. *J. Chromatogr. A* **2012**, *1220*, 26–34.

(22) Xiong, Y.; Zhao, Y.; Goruk, S.; Oilund, K.; Field, C. J.; Jacobs, R. L.; Curtis, J. M. Validation of an LC – MS / MS Method for the Quantification of Choline-Related Compounds and Phospholipids in Foods and Tissues. *J. Chromatogr. B* **2012**, *911*, 170–179. https://doi.org/10.1016/j.jchromb.2012.10.038.

(23) Vosse, C.; Thyssen, G. M.; Sperling, M.; Karst, U.; Hayen, H. Complementary Approach for Analysis of Phospholipids by Liquid Chromatography Hyphenated to Elemental and Molecular Mass Spectrometry. *Anal. Sci. Adv.* **2020**, *1* (1), 46–55.

(24) Ali, A. H.; Zou, X.; Lu, J.; Abed, S. M.; Yao, Y.; Tao, G.; Jin, Q.; Wang, X. Identification of Phospholipids Classes and Molecular Species in Different Types of Egg Yolk by Using UPLC-Q-TOF-MS. *Food Chem.* **2017**, *221*, 58–66.

(25) Wei, J.; Zhao, X.; Wang, S.; Zhang, M.; Yao, W.; Yuan, Y. Determination of Related Substances in Egg Yolk Lecithin by HPLC-CAD and Characterization of Its Profiling by HPLC-Q-TOF-MS. *J. Pharm. Biomed. Anal.* **2022**, *221*, 115079.

(26) Song, S.; Cheong, L.-Z.; Wang, H.; Man, Q.-Q.; Pang, S.-J.; Li, Y.-Q.; Ren, B.; Wang, Z.; Zhang, J. Characterization of Phospholipid Profiles in Six Kinds of Nut Using HILIC-ESI-IT-TOF-MS

System. *Food Chem.* **2018**, *240*, 1171–1178.

(27) Vosse, C.; Wienken, C.; Cadenas, C.; Hayen, H. Separation and Identification of Phospholipids by Hydrophilic Interaction Liquid Chromatography Coupled to Tandem High Resolution Mass Spectrometry with Focus on Isomeric Phosphatidylglycerol and Bis (Monoacylglycero) Phosphate. *J. Chromatogr. A* **2018**, *1565*, 105–113.

(28) Ferraris, Q.; Hale, J.; Teigland, E.; Rao, A.; Qian, M. C. Phospholipid Analysis in Whey Protein Products Using Hydrophilic Interaction High-Performance Liquid Chromatography-Evaporative Light-Scattering Detection in an Industry Setting. *J. Dairy Sci.* **2020**, *103* (12), 11079–11085.

(29) Dugo, P.; Fawzy, N.; Cichello, F.; Cacciola, F.; Donato, P.; Mondello, L. Stop-Flow Comprehensive Two-Dimensional Liquid Chromatography Combined with Mass Spectrometric Detection for Phospholipid Analysis. *J. Chromatogr. A* **2013**, *1278*, 46–53.

(30) Sun, C.; Zhao, Y.-Y.; Curtis, J. M. Characterization of Phospholipids by Two-Dimensional Liquid Chromatography Coupled to in-Line Ozonolysis--Mass Spectrometry. *J. Agric. Food Chem.* **2015**, *63* (5), 1442–1451.

(31) Granafei, S.; Azzone, P.; Spinelli, V. A.; Losito, I.; Palmisano, F.; Cataldi, T. R. I. Hydrophilic Interaction and Reversed Phase Mixed-Mode Liquid Chromatography Coupled to High Resolution Tandem Mass Spectrometry for Polar Lipids Analysis. *J. Chromatogr. A* **2016**, *1477*, 47–55.

(32) Cassap, M.; Schmelzel, J. *Elemental Analysis of Canola Oil Using the Thermo Scientific ICAP 7400 ICP-OES*; Bremen, Germany; California, US, 2017.

(33) Bandura, D. R.; Baranov, V. I.; Tanner, S. D. Detection of Ultratrace Phosphorus and Sulfur by Quadrupole ICPMS with Dynamic Reaction Cell. *Anal. Chem.* **2002**, *74* (7), 1497–1502.

(34) Maes, E.; Brusten, W.; Beutels, F.; Baggerman, G.; Mertens, I.; Valkenborg, D.; Landuyt, B.; Schoofs, L.; Tirez, K. The Benefits and Limitations of Reaction Cell and Sector Field Inductively Coupled Plasma Mass Spectrometry in the Detection and Quantification of Phosphopeptides. *Rapid Commun. Mass Spectrom.* **2015**, *29* (1), 35–44.

(35) Bernardin, M.; Bessueille-Barbier, F.; Le Masle, A.; Lienemann, C.-P.; Heinisch, S. Suitable Interface for Coupling Liquid Chromatography to Inductively Coupled Plasma-Mass Spectrometry for the Analysis of Organic Matrices. 1 Theoretical and Experimental Considerations on Solute Dispersion. *J. Chromatogr. A* **2018**, *1565*, 68–80.

(36) Bernardin, M.; Bessueille-Barbier, F.; Le Masle, A.; Lienemann, C.-P.; Heinisch, S. Suitable Interface for Coupling Liquid Chromatography to Inductively Coupled Plasma-Mass Spectrometry for the Analysis of Organic Matrices. 2 Comparison of Sample Introduction Systems. *J. Chromatogr. A* **2019**, *1603*, 380–387.

(37) Magnusson, L.-E.; Risley, D. S.; Koropchak, J. A. Aerosol-Based Detectors for Liquid Chromatography. *J. Chromatogr. A* **2015**, *1421*, 68–81.

(38) Cífková, E.; Hájek, R.; Lísa, M.; Holˇ, M. Hydrophilic Interaction Liquid Chromatography Mass Spectrometry of (Lyso) Phosphatidic Acids,(Lyso) Phosphatidylserines and Other Lipid Classes. *J. Chromatogr. A* **2016**, *1439*, 65–73.

(39) Caumette, G.; Lienemann, C.-P.; Merdrignac, I.; Paucot, H.; Bouyssiere, B.; Lobinski, R. Sensitivity Improvement in ICP MS Analysis of Fuels and Light Petroleum Matrices Using a Microflow Nebulizer and Heated Spray Chamber Sample Introduction. *Talanta* **2009**, *80* (2), 1039–1043.

(40) Paula, S.; Volkov, A. G.; Van Hoek, A. N.; Haines, T. H.; Deamer, D. W. Permeation of Protons, Potassium Ions, and Small Polar Molecules through Phospholipid Bilayers as a Function of Membrane Thickness. *Biophys. J.* **1996**, *70* (1), 339–348.

(41) Schoeny, H.; Rampler, E.; Binh Chu, D.; Schoeberl, A.; Galvez, L.; Blaukopf, M.; Kosma, P.; Koellensperger, G. Achieving Absolute Molar Lipid Concentrations a Phospholipidomics Cross-Validation Study. *Anal. Chem.* **2022**, *94* (3), 1618–1625.

(42) Grit, M.; Crommelin, D. J. A.; Lang, J. Determination of Phosphatidylcholine, Phosphatidylglycerol and Their Lyso Forms from Liposome Dispersions by High-Performance Liquid Chromatography Using High-Sensitivity Refractive Index Detection. *J. Chromatogr. A* **1991**, *585* (2), 239–246.

(43) Rabagny, Y.; Herrmann, W.; Geisel, J.; Kirsch, S. H.; Obeid, R. Quantification of Plasma Phospholipids by Ultra Performance Liquid Chromatography Tandem Mass Spectrometry. *Anal. Bioanal. Chem.* **2011**, *401*, 891–899.

(44) Kiełbowicz, G.; Trziszka, T.; Wawrzeńczyk, C. Separation and Quantification of Phospholipid and Neutral Lipid Classes by HPLC--CAD: Application to Egg Yolk Lipids. *J. Liq. Chromatogr. \& Relat. Technol.* **2015**, *38* (8), 898–903.

(45) Takeda, H.; Takahashi, M.; Hara, T.; Izumi, Y.; Bamba, T. Improved Quantitation of Lipid Classes Using Supercritical Fluid

Chromatography with a Charged Aerosol Detector. *J. Lipid Res.* **2019**, *60* (8), 1465–1474.

(46) Lewis, E. D.; Zhao, Y.-Y.; Richard, C.; Bruce, H. L.; Jacobs, R. L.; Field, C. J.; Curtis, J. M. Measurement of the Abundance of Choline and the Distribution of Choline-Containing Moieties in Meat. *Int. J. Food Sci. Nutr.* **2015**, *66* (7), 743–748.

(47) Vassallo-Agius, R.; Watanabe, T.; Yoshizaki, G.; Satoh, S.; Takeuchi, Y. Quality of Eggs and Spermatozoa of Rainbow Trout Fed an N-3 Essential Fatty Aciddeficient Diet and Its Effects on the Lipid and Fatty Acid Components of Eggs, Semen and Livers. *Fish. Sci.* **2001**, *67* (5), 818–827.