Journal of Chromatography B, 911 (2012) 170–179

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Validation of an LC–MS/MS method for the quantification of choline-related compounds and phospholipids in foods and tissues

Yeping Xiong, Yuan-Yuan Zhao, Sue Goruk, Kirsten Oilund, Catherine J. Field, René L. Jacobs, Jonathan M. Curtis*

Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Edmonton, Canada T6G 2P5

ARTICLE INFO

Article history:
Received 8 June 2012
Accepted 29 October 2012
Available online 3 November 2012

Keywords:
Hydrophilic interaction chromatography (HILIC)
LC–MS/MS
Method validation
Choline
Phospholipids
Egg yolk

ABSTRACT

A hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC LC–MS/MS) method was developed and validated to simultaneously quantify six aqueous choline-related compounds and eight major phospholipids classes in a single run. HILIC chromatography was coupled to positive ion electrospray mass spectrometry. A combination of multiple scan modes including precursor ion scan, neutral loss scan and multiple reaction monitoring was optimized for the determination of each compound or class in a single LC/MS run. This work developed a simplified extraction scheme in which both free choline and related compounds along with phospholipids were extracted into a homogenized phase using chloroform/methanol/water (1:2:0.8) and diluted into methanol for the analysis of target compounds in a variety of sample matrices. The analyte recoveries were evaluated by spiking tissues and food samples with two isotope-labeled internal standards, PC-d_3 and Cho-d_3. Recoveries of between 90% and 115% were obtained by spiking a range of sample matrices with authentic standards containing all 14 of the target analytes. The precision of the analysis ranged from 1.6% to 13%. Accuracy and precision was comparable to that obtained by quantification of selected phospholipid classes using ^{31}P NMR. A variety of sample matrices including egg yolks, human diets and animal tissues were analyzed using the validated method. The measurements of total choline in selected foods were found to be in good agreement with values obtained from the USDA choline database.

1. Introduction

Choline (Cho), a dietary component in many foods, is essential for the normal function of all cells [1]. Adequate choline intake is very important for fetal development, memory function, bone formation, normal liver and kidney functions and the prevention of various diseases [2,3]. The U.S. National Academy of Sciences recommended dietary choline intake level of 550 mg/day for men and 425 mg for women [4]. In many biological pathways, choline is metabolically inter-related to other choline metabolites including betaine (Bet), acetylcholine (AcCho), phosphocholine (PCho), glycerophosphocholine (GPC), and cytidine diphosphocholine (CDP-Cho). Choline is also important for the biosynthesis of cellular phospholipids [5,6], which are essential to membrane structure and functions including signal transduction, efficient storage of energy as with triglycerides, and transport of fat from liver and intestinal cells. The major phospholipids in foods and biological tissues include phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylglycerol (PG).

The measurement of choline-related compounds and phospholipids are therefore important for better understanding their forms, distributions, and biological functions. Many analytical methods have been developed for the analysis of some of these moieties. These involve ^{31}P nuclear magnetic resonance (^{31}P NMR) [7–9], gas chromatography coupled with mass spectrometry (GC/MS) [10], liquid chromatography coupled with evaporative light scattering, fluorescence, or mass spectrometry [11–14]. In reported methods [15], extraction of the choline-related compounds and phospholipids is carried out using both aqueous and organic extraction solvents, or using a single phase extraction method followed by further fractionation. In these methods, the aqueous phase, containing choline-related compounds, and the organic phase, containing phospholipids, then require separate HPLC analyses. The requirement for multiple sample extraction steps results in a greater potential for recovery losses. To our knowledge, there have been no reports of a universal and simple analytical method to simultaneously quantify all choline-related compounds and phospholipids. Such a method would be required to measure these compounds

* Corresponding author.
E-mail address: jcurtis1@ualberta.ca (J.M. Curtis).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.10.038

over a wide range of concentrations and sample matrices, such as exist in food and tissues.

Due to both the diversity of polar head groups found in phospholipid classes and to the polarity of choline-related compounds, a separation scheme based on hydrophilic interaction chromatography (HILIC) would be expected to be a suitable. To date, HILIC has been used to determine either a limited number of water soluble choline compounds or phospholipids classes such as PC and SM [16–18]. Previously, we have reported on the development of a HILIC LC–MS/MS method for the determination of 11 choline containing or closely related compounds in egg yolks [19]. The objective of the present work is to develop the reported method into a more comprehensive method to quantify all of the 14 major choline-related compounds and phospholipids including Cho, Bet, AcCho, PCho, GPC, CDP-Cho, PC, LPC, PE, LPE, SM, PI, PS and PG in a single run. Also, we demonstrate the use of a simple method for the extraction of most phospholipid classes and choline compounds into a single homogeneous phase, which can be directly diluted for LC/MS analysis. This method is applicable to virtually any biological sample matrix, including food samples and animal tissues. Finally, the validation of the combined extraction method and single stage of HILIC LC–MS/MS analysis is described. In our laboratory, this validated method has been successfully applied to the quantification of the target analytes in a wide variety of foods and tissues.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine (from egg yolk, ≥99%) (PC), sphingomyelin (SM) (from egg yolk, >95%), L-α-phosphatidylglycerol (PG) ammonium salt (from egg yolk, ≥99%), choline (Cho) chloride (>98%), choline-trimethyl-d_9 (Cho-d_9) chloride, acetylcholine (AcCho) chloride (>99%), phosphocholine (PCho) chloride calcium salt tetrahydrate (Sigma grade), cytidine diphosphocholine (CDP-Cho) sodium salt dehydrate, and betaine (Bet) hydrochloride (>99%) were purchased from Sigma (St. Louis, MO); 1,2-distearoyl-sn-glycero-3-phosphocholine-N,N,N-trimethyl-d_9 (PC-d_9), L-α-phosphatidylethanolamine (PE) (egg, chicken), L-α-lysophosphatidylethanolamine (LPE) (egg, chicken), L-α-lysophosphatidylcholine (LPC) (egg, chicken), L-α-phosphatidic acid (PA) sodium salt (egg, chicken), L-α-phosphatidylserine (PS) soldium salt (soy, >99%), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-methyl (16:0 monomethyl-PE, MMPE), and L-α-phosphatidylinositol (PI) (Soy, sodium salt) were obtained from Avanti polar lipids, Inc. (700 Industrial Park Drive, Alabaster, Alabama). Glycerolphosphocholine (GPC) was supplied by Bachem Americas Inc. (Torrance, CA). Phosphocholine-N,N,N-trimethyl-d_9 (Pcho-d_9) chloride calcium salt was purchased from C/D/N Isotopes Inc. (Quebec, Canada). 1,2-Distearoyl-sn-glycero-3-phosphocholine-N-methyl-d_3 (PC-d_3) and choline-N-methyl-d_3 (Cho-d_3) iodide were synthesized as described below. HPLC-grade ammonium formate (≥99%) and formic acid were supplied by Sigma (St. Louis, MO). Acetonitrile and water were of LC/MS grade from Fisher Scientific Company (Ottawa, ON, Canada). All other solvents were of HPLC grade.

2.2. Synthesis of PC-d_3

1,2-Distearoyl-sn-glycero-3-phosphocholine-N-methyl-d_3 (PC-d_3) was synthesized from 1,2-distearoyl-sn-glycero-3-phosphocholine (18:0 PC) according to a similar procedure to that described by Wang et al. [20]. Briefly, to a solution of 690 mg of 18:0 PC in 12 ml of dry dimethylformamide was added 440 mg of 1,4,-diazabicyclo[2.2.2]octane (DABCO). The solution was refluxed overnight and evaporated under reduced pressure. The reaction residue was purified on silica gel with $CHCl_3$/MeOH/H_2O (65:25:2, v/v/v) to give 360 mg of the demethylated product phosphatidyl-N,N-dimethylethanolamine. This product was dissolved in 24 ml of dry CH_2Cl_2/MeOH (1:1, v/v) to which 108 μl of cyclohexylamine and 147 μl of iodomethane-d_3 was added in order to methylate it to PC-d_3. The reaction solution was kept in the dark for 16 h and then evaporated under reduced pressure. The reaction residue was then fractionated using a silica gel column with $CHCl_3$/MeOH/H_2O from 65:25:2 to 55:35:4 (v/v/v). The product fraction was evaporated under reduced pressure and lyophilized for 72 h to yield 310 mg of PC-d_3.

2.3. Synthesis of Cho-d_3

Cho-d_3 was synthesized similarly by the above procedure. To the solution of 23 mg of N,N-dimethylethanolamine in 3 ml of dry methanol was added 82 μl of iodomethane-d_3. The reaction solution was kept in dark for 16 h, evaporated under reduced pressure, and recrystalized in ethyl acetate/methanol to give 40 mg clear crystals of Cho-d_3 iodide.

2.4. Preparation of stock and working standard solutions

A 1 mg/ml stock solution of PG, PI, PS, PC, PE, SM and LPC was prepared in chloroform and LPE was 1 mg/ml in chloroform/methanol (9:1, v/v). Solutions were prepared of PC-d_9, PC-d_3, and MMPE (0.5 mg/ml in 1:2 (v/v) chloroform/methanol). Stock solutions of Cho, Cho-d_9, Cho-d_3, AcCho, GPC and Bet were made in methanol at 0.5 mg/ml. Stock solutions of PCho, PCho-d_9, and CDP-Cho were prepared in methanol/water (2:1, v/v). All stock solutions were stored at −20 °C before use.

Calibration solutions were made from the stock solutions of analytes and the internal standards. All calibration curves consisted of eight calibration points with PC-d_9 as internal calibration standard for PC, Cho-d_9 for Cho, Bet, AcCho, and GPC; PCho-d_9 as internal calibration standard for PCho and CDP-Cho and MMPE as the internal calibration standard for all other phospholipids. The calibrated ranges were: PG, PI, PE, LPE and PS between 0.25 and 25 μg/ml; PC, PCho and CDP-Cho from 0.5 to 50 μg/ml; LPC, SM, Cho, GPC and Bet from 0.05 to 5 μg/ml; AcCho from 0.02 to 2 μg/ml; PC-d_3 from 0.02 to 2.5 μg/ml, and Cho-d_3 from 0.012 to 1.2 μg/ml. Triplicate low, medium, and high quality control (QC) standards were prepared separately in methanol at 1 μg/ml (QC, L), 10 μg/ml (QC, M), 25 μg/ml (QC, H) for PG, PI, PE, LPE and PS; 2 μg/ml (QC, L), 10 μg/ml (QC, M), 35 μg/ml (QC, H) for PC, PCho, and CDP-Cho; 0.2 μg/ml (QC, L), 1 μg/ml (QC, M), 3.5 μg/ml (QC, H) for LPC, SM, Cho, GPC, and Bet; 0.08 μg/ml (QC, L), 0.4 μg/ml (QC, M), 1.4 μg/ml (QC) for AcCho.

2.5. Extraction of phospholipids and choline-containing compounds from foods and tissues

Rat livers and stomach contents from suckled rats were collected at necropsy, ground in liquid nitrogen in mortars and stored at −80 °C until extraction. Food samples (homogenized meals) were wet-ground then lyophilized to dryness. Fresh eggs purchased from local markets were carefully broken. The egg yolks were separated from the whites by first decanting the whites then rolling the yolks on filter papers to completely remove the whites. Six egg yolks were pooled together and mixed well. The ground tissues, stomach content, dry foods, and fresh egg yolks were subjected to extraction based on a modified Bligh and Dyer method [21]. In brief, 100 mg of sample was spiked with recovery standards of PC-d_3 and Cho-d_3, homogenized in 2 ml of extraction solvent (chloroform/methanol/water, 1:2:0.8) at 10,000 rpm for 5 min on a Polytron PT1300 D homogenizer (Kinematica AG, Switzerland)

Table 1
MS scan type, scan mass range or transition ions (MRM), scan time and optimized instrumental compound-dependent parameters.

Scan type	Scan mass range or MRM transition ions (amu)	Scan time (s)	Compound	DP[a] (V)	EP[a] (V)	CEP[a] (V)	CE[a] (V)	CXP[a] (V)
Period 1, 0–6.5 min								
NL of 172	600–900	1.2	PG	45	8	30	25	4.5
NL of 260	700–900	1.0	PI	45	8	35	40	4.5
Period 2, 6.5–8.8 min								
NL of 141	600–800	1.2	PE	50	8	20	29.8	27
Period 3, 8.8–12.7 min								
Prec of 185	700–900	1.0	PS	40	8	30	40	5
Prec of 184	720–860	0.5	PC	55	8	20	22	3
NL of 141	400–600	1.0	LPE	50	8	20	29.8	27
MRM	706.3 → 551.5	0.05	IS monomethyl-PE	45	3	20	30	5
	793.7 → 187.0	0.05	PC-d_3	55	8	20	45	3
	799.8 → 192.8	0.05	IS PC-d_9	55	8	20	45	3
Period 4, 12.7–14.8 min								
Prec of 184	400–900	0.8	SM, LPC	55	8	20	45	3
MRM	146.4 → 87.2	0.05	AcCho	25	5	10	20	2.5
Period 5, 14.8–30 min								
MRM	104.2 → 60.1	0.05	Cho	30	9	10	20	2.5
	107.3 → 63.1	0.05	Cho-d_3	30	9	10	20	2.5
	113.2 → 69.1	0.05	IS Cho-d_9	30	9	10	25	2
	118.1 → 58.2	0.05	Bet	30	9	15	40	2.5
	258.0 → 104.0	0.05	GPC	35	5	15	20	2.5
	184.0 → 125.1	0.05	PCho	35	5	10	25	3
	193.1 → 125.1	0.05	IS PCho-d_9	45	3	10	30	3
	488.9 → 184.1	0.05	CDP-Cho	45	5	15	55	3

[a] DP, EP, CEP, CE and CXP are declustering potential, entrance potential, collision cell entrance potential, collision energy and collision cell exit potential.

then centrifuged at 3000 rpm for 5 min. The resultant supernatant was collected and the extraction procedure repeated a further two times. The combined extract solution was centrifuged and the supernatant was diluted with methanol to a final volume of 10 ml using a volumetric flask. The extract was then stored at −20 °C prior to analysis. The extract was further diluted with methanol to ensure that all of the analyte concentrations fell within their respective standard curve ranges and spiked with the internal standard working solution (containing PC-d_9, Cho-d_9, PCho-d_9, and MMPE) for subsequent LC–MS/MS analysis.

To determine analyte recoveries as part of method validation, 100 mg of each sample was spiked with a mixture of all 14 of the analytes plus PC-d_3 and Cho-d_3. Analytes were spiked at both low and high concentration levels (see Table 4) prior to extraction using the above procedure. Accuracy and precision of egg yolk measurements was assessed by spiking egg yolks with standards at 2 levels. These were 50 and 100 mg/100 g yolk for PG, PI, LPE, CDP-Cho, PS and PCho; 750 and 1500 mg/100 g yolk for PE; 1500 and 3000 mg/100 g yolk for PC; 100 and 200 mg/100 g yolk for LPC and SM; 4 and 8 mg/100 g yolk for AcCho; and 20 and 40 mg/100 g yolk for Cho, Bet and GPC. Table 5 shows accuracy and precision measured for the high level spike in each case.

2.6. HILIC LC–MS/MS analysis

Standards and sample solutions were analyzed using an Agilent 1200 series HPLC system coupled to a 3200 QTRAP mass spectrometer (AB SCIEX; Concord, ON, Canada) and using Analyst 1.4.2 software for data acquisition and analysis. An Ascentis Express 150 mm × 2.1 mm HILIC column, 2.7 μm particle size (Sigma, St. Louis, MO) was used for LC separations. The column temperature was controlled at 25 °C. The mobile phase A was acetonitrile and B was 10 mM ammonium formate in water at pH 3.0, adjusted using formic acid. The gradient was as follows: 0–0.1 min, 8% B; 0.1–10 min, from 8% to 30% B; 10–17 min, 95% B; and then back to 8% B at 17.1 min for column re-equilibrium prior to the next injection. The flow rate of mobile phase was 400 μl/min for the period from 20 min to 27 min and 200 μl/min for all other periods. The injection volume was 2 μl and the cycle time was 30 min/injection. A turboionspray source was employed in positive ion mode. Nitrogen was used as curtain gas, nebulizing gas and drying gas. Several scan modes, including precursor ion scan (Pre), neutral loss scan (NL) and multiple reaction monitoring (MRM) were used in order to quantify the various choline-containing compounds and other important phospholipids. A valve was programmed by the data system to divert the LC effluent to waste before and after the selected retention time window from 2.5 min to 19 min. Table 1 lists the detailed experimental conditions.

2.7. ^{31}P NMR measurements

Lipid extracts obtained as above were evaporated to dryness by a stream of nitrogen. The residue was re-dissolved in a detergent solution in D_2O, containing 200 mM sodium cholate and 5 mM EDTA with pH adjusted to 7.0 using D_2O solution of sodium hydroxide. The clear solution was spiked with trimethylphosphate (TMP) as a quantification internal standard and subsequently transferred into a NMR tube for analysis [22–24]. The quantitative phosphorus NMR spectra were recorded on Agilent/Varian VNMRS two-channel 500 MHz spectrometer operating at 201.643 MHz for ^{31}P with other parameters as follows: acquisition time 1 s, temperature 27 °C, 30° pulse, pulse delay 10 s. The nuclear overhauser effect (NOE) was removed by using inverse gated decoupling technique [9].

3. Results and discussion

3.1. LC–MS/MS method optimization

In our previous report [19] we successfully separated 11 choline-related compounds or classes that were extracted from egg yolks and quantified 8 of these using a HILIC LC–MS/MS method. Here, we aim to develop and validate a more universal method, suitable for a variety of sample matrices. Therefore, in addition to 11 choline related compounds measured in the previous work, measurement of the two phospholipid classes PG and PS, the choline-containing compound CDP-Cho, and four internal standards MMPE, PC-d_3, Cho-d_3, and PCho-d_9 were added to the method prior to optimization.

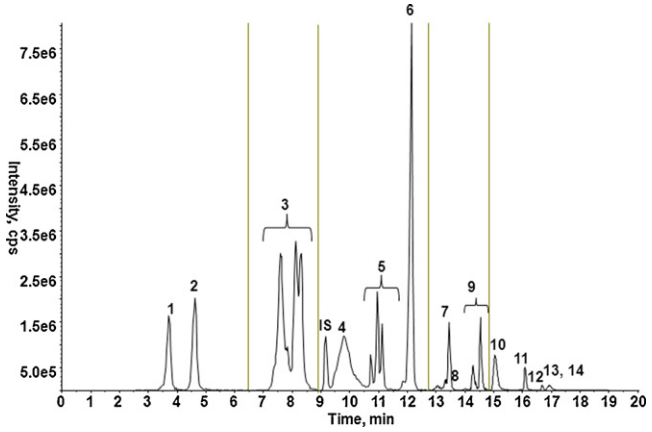


Fig. 1. LC–MS/MS chromatogram of a mixture of standards. Conditions are as described in Experimental sections. Peak: 1, PG; 2, PI; 3, PE; IS, MMPE; 4, PS; 5, LPE; 6, PC, PC-d_3 and PC-d_9; 7, SM; 8, Ace-Cho; 9, LPC;10, Cho – Cho-d_3 and Cho-d_9; 11, Bet; 12, GPC; 13, CDP-Cho; and 14, PCho.

Initially, chromatographic conditions, including the choice of LC column, mobile phase composition and pH, were investigated in order to achieve reproducible separation of all of the 14 choline-related compounds and phospholipid classes (Cho, Bet, AcCho, PCho, GPC, CDP-Cho, PC, LPC, PE, LPE, SM, PI, PS and PG). In the case of phospholipids, the primary goal of this method was to quantify the total amount present for each phospholipid class rather than to quantify an individual molecular species within each class containing one more particular fatty acid(s). It was therefore important to develop an LC/MS method that could separate into phospholipid classes that include the entire fatty acid distribution that is present.

This can be achieved by using a HILIC column where phospholipids were found to be separated into classes based on their head group polarities while species in the same class were eluted over a very narrow retention time window. Hence, most phospholipid classes can be resolved as a single peak by HILIC chromatography. Previously, species within the same class could be very well resolved using a reverse-phase column since the separation was based on the number of carbon atoms and of unsaturated bonds of the fatty acyl chains (or as a function of equivalent carbon number, ECN) on the phospholipid backbone [25,26]. Hence, HILIC separation was identified as the best choice not only because HILIC provides superior retention of polar analytes and enhancement of ESI-MS sensitivity, but also because each phospholipid class elutes as a resolved peak or group of peaks based on head group polarities. During the development of the HILIC separation method, numerous combinations of organic phases and aqueous buffers were explored to separate this mixture of 6 choline related compounds, 8 phospholipids classes plus 6 internal standards (MMPE, PC-d_3, PC-d_9, Cho-d_3, Cho-d_9 and PCho-d_9). For example, different concentrations of ammonium formate buffer in both mobile phase A (acetonitrile) and B (water) were tested but a high level of ion suppression was observed for PG, PI, and PE especially when using the buffer in a mobile phase A of acetonitrile. The pH of the mobile phase also had a significant impact on the retention, selectivity and sensitivity of certain phospholipid classes, especially PI, and PS. Optimal sensitivity and resolution was achieved using acetonitrile (mobile phase A) and ammonium formate buffer (mobile phase B). The final LC conditions used in this study resolved all major phospholipid classes, including PG, PI, PE, PS, LPE, PC, SM, and LPC, and the structurally analogous internal standard MMPE. Fig. 1 shows the LC–MS/MS chromatogram of a mixture of the standards of interest. Because of the excellent resolution of all major phospholipid and polar choline compounds, we concluded that this LC–MS/MS method could be a universal method for the measurement of these compounds and could be therefore be used for a wide range of applications and sample matrices.

The reproducibility of retention time for every compound was also investigated. The flow rate during the equilibration period was doubled in order to minimize the run time. The retention times for the method were found to be very stable throughout the validation period of several weeks with shifts of less than 0.2 min. Excellent retention time reproducibility and stability is critical for this method since the MS detection was divided into 5 periods (Table 1) each with a different set of scan functions in order to detect and quantify the target analytes. Hence, if a retention time for any analyte were to shift outside of the specified time window to detect that compound, the analyte would not be detected leading to a corresponding error in the quantification.

In order to optimize the mass spectrometer parameters, each analyte in methanol was infused into the Turbospray ion source of the QTRAP mass spectrometer using both positive and negative ionization modes. MS/MS experiments were performed to elucidate the fragmentation patterns in both ionization modes. Following this, the mass spectrometer parameters, such as collision energy and declustering potential, and the most appropriate MS/MS scan functions were selected for the most intense fragmentations. Under the optimized conditions, all of the target analytes of interest gave higher signal-to-noise ratio in the positive ion mode than in the negative ion mode. Therefore positive ion mode was selected for all of the experiments reported in this work. Table 2 summarizes the fragment ions and transitions selected for the ESI-MS/MS experiments. Note that the free choline and related compounds, having unique molecular masses, are analyzed by multiple reaction monitoring (MRM) scan mode to achieve maximum selectivity and sensitivity. In contrast, the phospholipids, with the exception of the internal standards, were measured using a neutral loss or precursor ion scan that covers the full range of likely fatty acyl substituents (see Table 1) to give the total amount of each phospholipid class. However, the internal standards have a unique fatty acid substitution (see Section 2) and so can be measured by MRM (as indicated in Table 1) which is inherently better for quantification.

It is well established that the electrospray ionization process can be influenced by a variety of parameters including the pK_a value and hydrophobicity of the analyte, the eluent pH and buffer concentration [27–29]. The mobile phase composition at which an analyte elutes from the column and passes into the ESI ion source can have a significant impact on its ionization efficiency and the resulting signal intensity. Therefore, the LC gradient used in this work was a compromise between the HILIC separation efficiency and the detection sensitivity for the range of phospholipids and choline compounds investigated. On the one hand, a high ionization efficiency was achieved for all phospholipid classes at a composition of >70% acetonitrile (mobile phase A). On the other hand, the non-lipid, choline-related compounds had to be delivered into the ESI ion source at a high percentage of water in the mobile phase achieve good signal intensity and peak shape. The best signal intensity and peak shape of PCho and CDP-Cho was achieved at the composition of over 90% ammonium formate buffer (mobile phase B).

3.2. Calibration curves

A stable-isotope labeled internal standard (SIL-IS) co-eluting in chromatography with the analyte would be ideal to use in calibration and could overcome ion suppression/enhancement, matrix effects and variations caused by instrument parameters, sample preparations, and injections. However, a SIL-IS is not always available, or can be very expensive to synthesize. This is especially true for phospholipid classes. An alternative approach is to use a structural analog, eluting in chromatography at a different time, or co-eluting with the analyte [30], even though the analog might

Table 2
Fragmentation patterns for detection of choline-containing compounds and phospholipids in ESI positive ion mode.

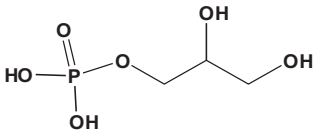
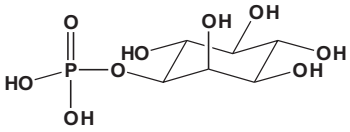
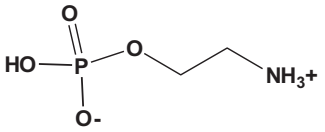
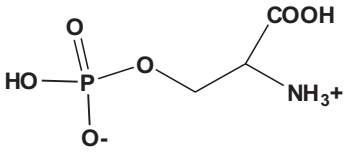
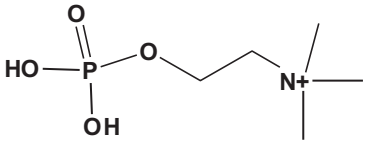
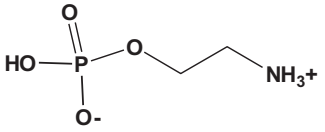
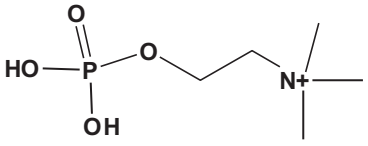
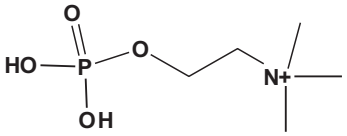
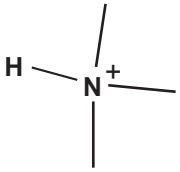
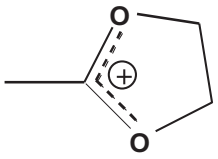
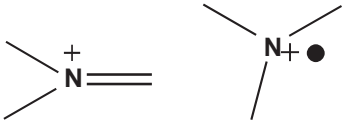
Compound or PL class	Fragment MW or *m*/*z*	Scan mode for detection
PG	Head group, MW 172 	NL of 172
PI	Head group, MW 260 	NL of 260
PE	Head group, MW 141 	NL of 141
PS	Head group, MW 185 	NL of 185
PC	Head group, (+) *m*/*z* 184 	Prec of 184
LPE	Head group, MW 141 	NL of 141
SM	Head group, (+) *m*/*z* 184 	Prec of 184
LPC	Head group, (+) *m*/*z* 184 	Prec of 184
Cho	(+) *m*/*z* 60 	SRM 104 → 60
AcCho	(+) *m*/*z* 87 	SRM 146 → 87

Table 2 (*Continued*)

Compound or PL class	Fragment MW or *m*/*z*	Scan mode for detection
Bet	(+) *m*/*z* 58, 59 	MRM 118 → 58 118 → 59
GPC	(+) *m*/*z* 104: Cho	MRM 258 → 104
PCho	(+) *m*/*z* 125: NL of $N(CH_3)_3$ (+) *m*/*z* 86: NL of H_3O_4P	MRM184 → 125184 → 86
CDP-Cho	(+) *m*/*z* 184: PCho	MRM 488 → 184

experience unequal degree of ion suppression/enhancement and matrix effects. In this work, depending on the availability, both internal standards of stable-isotope labeled standards and a structural analog were spiked into the extract solution. A structural analog of PE with an additional N-methyl group (MMPE) was the internal standard for PG, PI, PE, PS, SM, LPE, and LPC. In addition, PC-d_9 was the internal standard for PC, PCho-d_9 for PCho and CDP-Cho and Cho-d_9 was used for Cho, AcCho, Bet, and GPC.

An eight-point calibration curve of each analyte of interest was constructed based on the peak area ratio of the analyte to the corresponding internal standard versus the analyte concentration. The limits of detection (LODs) and quantitation (LOQs) were measured based on the level of diluted standards giving a minimum signal-to-noise ratio ≥3 and ≥10, respectively. Table 3 shows the results of the calibration curves, the correlation coefficients, the detection limits, and the linear dynamic ranges for all analytes. For most analytes, a two order of linear dynamic range with correlation coefficient greater than 0.993 was obtained. Clearly, lower LOQs and wider dynamic ranges are possible using optimized conditions for a single analyte or for a small range of similar analytes. However the results presented here represent a significant achievement in obtaining a sufficient level of performance over the full range of disparate compound classes.

3.3. Extraction recovery

A modification of the Bligh and Dyer method [21] was used to extract all phospholipids and aqueous choline compounds from foods and tissues. The modification included sequentially extracting the sample three times using the Bligh and Dyer solvent system (chloroform/methanol/water in the ratio 1:2:0.8), which maintains a monophasic system. Then, instead of causing phase separation by the addition of chloroform and water as described in the original Bligh and Dyer method, in this case the extracts were combined and then all of the diluted with methanol for direct analysis. Thus all analytes including the hydrophilic choline compounds and lipophilic phospholipids could be simultaneously quantified in a single LC–MS/MS run.

Stable-isotope labeled internal standards of PC-d_3 and Cho-d_3 were synthesized in-house and spiked into samples as recovery standards prior to solvent extraction. The concentrations of PC-d_3 and Cho-d_3 were then determined from their own calibration curves, and their recoveries were calculated by comparing the measured and spiked amounts. This ensured that all phospholipids, represented by PC-d_3, and aqueous choline compounds, represented by Cho-d_3, were extracted with high efficiencies, and that the quantitation data for that specific sample was reliable.

To validate the extraction method, the extraction recovery of the 14 analytes investigated plus the two isotopically labeled internal standards PC-d_3 and Cho-d_3 were evaluated. Since a true blank matrix is not available, extraction recovery experiments were carried out by spiking known amounts of authentic standards of all 14 analytes, as well as PC-d_3, and Cho-d_3, at two different concentrations, into a real food matrix. This was a homogenized meal sample containing a variety of foodstuffs. The sample was divided into 9 aliquots of 100 mg; of these 6 were spiked with the authentic standards at low and high levels (see Table 4 for amounts), each in triplicate. The remaining 3 non-spiked (endogenous) aliquots and the 6 spiked aliquots were extracted in the same way and analyzed by the HILIC LC–MS/MS method. The analyte concentrations in all samples were calculated from the calibration curve of each analyte. Extraction recovery for each analyte was calculated as a percentage of the measured spiked amount to the actually spiked amount. Table 4 shows the results of these recovery measurements. Overall, the average recovery of the low level spike for all analytes was 98.0 ± 6.6% and the average recovery of the high level spike was 98.9 ± 7.8%. Thus, the recoveries of most analytes fell between 90% and 110%. These results indicate that excellent recovery of each analyte was obtained throughout the linear dynamic range of the analysis when using the simple extraction method described above. This keeps all of the analytes in one phase, eliminating the need to separate the whole extract into aqueous and organic phases before

Table 3
Calibration curves and linear dynamic ranges.

Compound	Calibration curve	*R* value	LOD (μg/ml)	LOQ (μg/ml)	Linear dynamic range (μg/ml)
PG	$y = 0.382x + 0.0105$	0.9996	0.1	0.25	0.25–10
PI	$y = 0.476x + 0.0245$	0.9997	0.1	0.25	0.25–10
PE	$y = 2.45x + 0.0661$	0.9930	0.1	0.25	0.25–25
PS	$y = 0.914x - 0.0264$	0.9966	0.05	0.1	0.1–10
LPE	$y = 0.496x - 0.00249$	0.9998	0.05	0.1	0.1–17.5
PC	$y = 5.46x - 0.328$	0.9982	0.1	0.5	0.5–50
SM	$y = 1.0x + 0.0279$	0.9998	0.02	0.05	0.05–5
LPC	$y = 1.32x - 0.00274$	0.9999	0.02	0.05	0.05–5
AcCho	$y = 0.436x + 0.00343$	0.9992	0.004	0.01	0.01–1.0
Cho	$y = 0.568x - 0.0136$	0.9996	0.01	0.02	0.02–5
Bet	$y = 0.47x + 0.057$	0.9994	0.02	0.05	0.05–5
GPC	$y = 0.118x - 0.000749$	0.9948	0.005	0.02	0.02–2
PCho	$y = 0.279x - 0.0683$	0.9996	0.1	0.5	0.5–50
CDP-Cho	$y = 0.207x - 0.0352$	0.9999	0.1	0.5	0.5–50

Table 4
Extraction recovery of choline compounds and phospholipids in foods.

Analyte	Added amount (mg/100 g food)		Measured amount (mean ± SD) (mg/100 g food)			Extraction recovery (%)	
	Low-spiked	High-spiked	Non-spiked (endogenous)	Low-spiked	High-spiked	Low-spiked	High-spiked
PG	24	240	12.9 ± 1.3	35.3 ± 0.8	259 ± 15	93.4	102.6
PI	24	240	112.7 ± 2.1	137.6 ± 1.8	354 ± 24	103.7	100.5
PE	24	240	105.1 ± 1.8	128.4 ± 2.4	350 ± 22	97	101.9
PS	50	100	6.3 ± 0.8	56.8 ± 1.7	113.3 ± 4.2	101.2	107.1
LPE	24	240	28.8 ± 0.3	51.9 ± 2.2	225 ± 22	96.2	81.7
PC	48	480	133.2 ± 7.5	180.0 ± 3.2	618 ± 31	97.5	100.9
SM	4.8	48	ND	4.6 ± 0.6	47.9 ± 1.3	96.8	99.8
LPC	100	200	182.8 ± 25	281.6 ± 17	386 ± 82	98.7	101.8
AcCho	1.92	19.2	ND	1.9 ± 0.1	17.5 ± 0.6	98.1	91.3
Cho	4.8	48	30.7 ± 0.5	35.4 ± 0.4	83.0 ± 1.5	99.2	109
Bet	20	40	3.5 ± 0.4	24.0 ± 1.0	43.2 ± 3.2	102.3	99.1
GPC	14.4	144	8.9 ± 0.5	21.2 ± 5.8	8.9 ± 0.5	85.5	87.7
PCho	50	100	9.1 ± 1.3	53.4 ± 4.7	103.0 ± 2.1	88.7	93.9
CDP-Cho	50	100	6.7 ± 0.6	64.0 ± 4.2	114.7 ± 6.5	113.0	107.7

analysis, as previously reported in the literature [15]. In addition, all analytes including choline-related compounds and phospholipids were well resolved on the HILIC column in a single run, thus also eliminating the need to use two separate LC methods.

3.4. Specificity

Since the choline-containing compounds and phospholipids occur in the presence of higher levels of other endogenous components in sample matrices, it was important to confirm assay specificity. This was first demonstrated by comparing retention times and peak shapes for the LC–MS/MS transitions indicated in Table 1 for all of the analytes dissolved in pure solvent with those in the sample matrices. No interfering peaks from the pure solvent samples were found in the MS/MS detection windows specified for each individual analyte and internal standard. More importantly, there were no interfering peaks seen in the specified MS/MS detection windows for analytes in the sample matrices. In addition, there was no retention time shift or peak shape changes caused by interfering biological components in the sample matrices. Those results indicate the specificity of the LC–MS/MS method for each individual analyte in their corresponding MS/MS detection windows.

Specificity was also evaluated for the effect of cross-talk between PC and other analytes. This was done by spiking a standard solution with an excess amount of PC, which is often present in a high abundance in biological matrices. It was found that the presence of high amount of PC did not affect the quantitation results for all other analytes (data not shown), further demonstrating the specificity of this method

3.5. Accuracy and precision

Accuracy and precision of the method were evaluated by replicate analysis of quality control (QC) samples at low, medium and high concentrations (see Section 2.4). The accuracy of the QC samples was evaluated as a percentage of the known spiked concentrations. The precision of the QC samples was expressed as the relative standard deviation for 3 measurements. Accuracy and precision were also investigated in a real sample matrix by spiking egg yolks with authentic standards as described in Section 2.5.

Table 5 shows the results for intraday triplicate measurements. The accuracy ranged from 83% to 116% for QC low (average 100 ± 9%), from 91% to 105% for QC medium (average 98 ± 5%), and from 87% to 104% for QC high (average 95 ± 6%). The precision ranged from 1.6% to 13% for all QC samples. The accuracy for the egg yolk matrix ranged from 94% to 107% for most analytes (average 102 ± 6%), except for GPC and CDP-Cho which were 113% and 115% respectively. The precision for the egg yolk matrix experiment was from 1 to 10% (average 5.6 ± 2.9%). In summary, these results from the egg yolk matrix indicate that the method is sufficiently accurate and precise to quantify all of the analytes in biological matrices using calibrators in the pure solvent rather than in a true "blank" matrix, which is in any case not obtainable.

3.6. Stability

The stability of all analytes was evaluated in both methanol solution and food sample extract solution. The results of the stability studies in methanol solution indicated that all analytes in methanol solution were stable at room temperature for at least 2 days. The stability of all analytes in food extraction solution was tested by re-analyzing the non-spiked and spiked food extract samples, as described in Section 3.3 Extraction Recovery, after storing the samples at −20 °C for 3 months. The results were compared with those from the freshly prepared samples. The long-term stored samples gave the same results with similar accuracy and precision compared to the results in Table 5, indicating the long-term storage stability of the food extract solution for at least 3 months at −20 °C.

3.7. Comparison of quantitation results by LC–MS/MS versus ^{31}P NMR

In order to further validate the accuracy of quantitative HILIC–MS/MS results, NMR was used to give an independent measurement of phospholipid concentrations. Compared to other NMR-active nuclei, phosphorous possesses high magnetogyric ratio and high natural abundance (100%), leading to sensitive detection and simple NMR experimental requirements. ^{31}P NMR is particularly suitable for quantitative analysis of phospholipids classes since most phospholipids classes contain a single phosphorus atom. In the literature [7–9], either a mixture of organic solvents or aqueous detergents have been used as solvents in the ^{31}P NMR analysis of phospholipids. However, the solvent pH and temperature were shown to have a significant impact on the chemical shift and the resolution of phospholipid classes [8,9]. In the present work, a standard mixture of phospholipids was analyzed by ^{31}P NMR both in the deuterated organic solvent ($CDCl_3$/MeOD) system or cholate detergent in D_2O system. Since most of the major phospholipid classes were resolved as sharper peaks in the detergent solvent compared to the organic solvent, this solvent system and associated NMR parameters were then used for absolute quantitation of QC and egg yolk samples. Fig. 2 shows the ^{31}P NMR spectra of a QC sample of phospholipids standards and an egg yolk extract in the cholate detergent. The standard phospholipids were baseline resolved for most phospholipids except for a slighter overlap of the PE and SM peaks (Fig. 2A). In most instances, the ^{31}P NMR in

Table 5
Accuracy and precision of triplicate measurements of QC.

	QC-low		QC-medium		QC-high		Egg yolk	
	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
PI	13.2	90.2	8.5	100.2	8.2	96.5	9.9	96.9
PG	10.7	100.1	6.5	98.7	8.9	96.5	5.2	103.4
PE	9.3	100.2	13.3	104.9	9.1	98.1	2.3	96.6
PS	2.5	116.3	9.7	98.0	7.9	104.3	3.7	106.7
LPE	6.7	97.1	5.8	103.3	1.6	88.4	9.5	96.7
PC	7.9	92.7	11.8	90.0	2.9	93.3	0.7	99.3
SM	4.2	88.7	12.3	101.7	4.9	90.9	8.5	107.4
LPC	9.0	94.3	11.3	100.0	8.3	90.7	6.0	99.5
Cho	5.8	83.0	10.2	90.8	2.0	87.0	4.9	99.5
AcCho	8.6	109.0	10.1	98.2	6.3	89.5	8.6	100.7
Bet	9.2	105.5	10.2	101.2	4.2	104.2	7.5	99.2
GPC	5.0	109.3	7.1	92.4	13.9	93.9	6.6	112.5
PCho	4.7	103.0	6.1	102.2	3.7	99.9	2.0	94.4
CDP-Cho	12.0	110.0	10.5	89.7	2.3	102.0	3.6	115.0

detergent solution would resolve phospholipids by classes, sometimes by fatty acid chain length [9,31]. Fig. 2A shows that both LPE and LPC were resolved as a pair of peaks in the cholate detergent. The ^{31}P NMR spectrum of the egg yolk extract (Fig. 2B) indicates the presence of PC, PE, LPE, LPC, SM, and PI in egg yolk. Due to the high abundances of PC and PE in the egg yolk, SM and PI were partially overlapped with PE and PC, respectively.

To further validate the HILIC LC–MS/MS method, a QC sample containing a mixture of phospholipids standards was analyzed in duplicate by both LC–MS/MS and ^{31}P NMR. However, the ^{31}P NMR samples were diluted 500-fold prior to LC–MS/MS analysis. The average molecular mass reported in the Avanti Polar Lipids Inc. was used to calculate the concentration of each phospholipid class in the ^{31}P NMR experiments. Two different commercial egg yolks were also compared for their phospholipids contents between the LC–MS/MS and ^{31}P NMR measurements. Table 6 shows the results of quantification by the two methods. For the QC standards, both LC–MS/MS and ^{31}P NMR methods provided similarly accurate results (86–110% accuracy measured for LC–MS/MS). For the two egg yolk extracts, the quantitation results are closely matched between the two methods for most of the phospholipids (with difference $\leq$10%). The SM contents were measured higher by the ^{31}P NMR method (with difference $\sim$16%). This is likely an over estimation by the ^{31}P NMR method that can be attributed to the partial overlap of the low abundance SM peak with the high abundance PE peak in the ^{31}P NMR spectra. Similarly, PI was not able to be determined accurately in the egg yolk by the ^{31}P NMR due to the overlap with the peak of the extremely high abundance PC. In addition, the measurement precision (%RSD) for the egg yolk samples by the ^{31}P NMR method was generally, worse than that achieved by the LC–MS/MS method. This is most evident for the measurements of the low abundance phospholipid components of egg yolks, due to the low sensitivity of the ^{31}P NMR method.

In summary, ^{31}P NMR provides accurate measurements of phospholipid classes with the advantages of very simple sample preparation and experimental procedure, lower sensitivity to matrix effects, and feasible choices of internal standards. However, there is a limit to the number of analytes that can be analyzed due to the high limits of quantitation (LOQs), problems with certain overlapping signals and it is only suitable for phosphorus-containing compounds. The HLIC LC–MS/MS method can not only provide accurate measurements of phospholipid classes that are consistent with the ^{31}P NMR method, but can also quantify all analytes of interest in a single run. The close agreement between ^{31}P NMR and HILIC LC–MS/MS data further demonstrate the validity of the LC/MS/MS method.

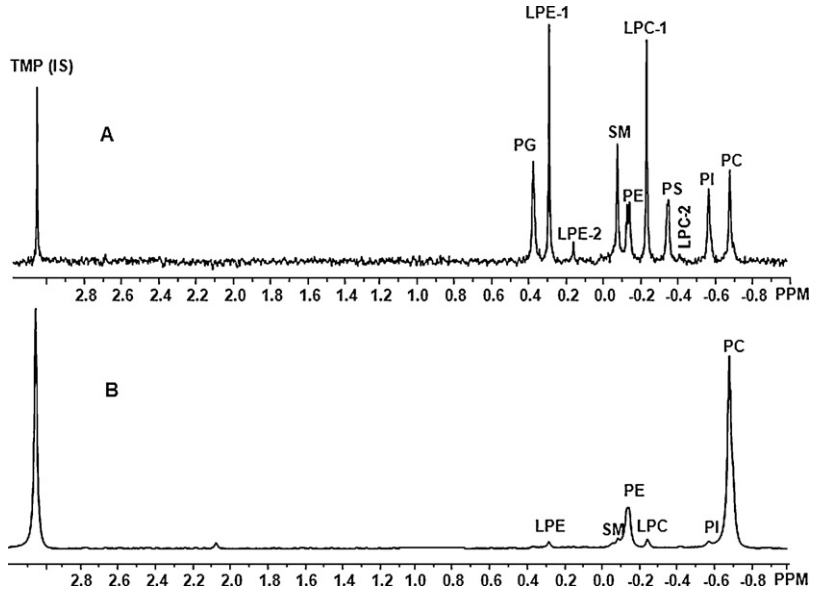


Fig. 2. ^{31}P NMR spectra of a standard mixture of phospholipids (A) and egg yolk extract (B) in the cholate detergent system (pH 7.0).

Table 6
Comparison of quantitation results by LC–MS/MS versus ^{31}P NMR.

Analyte	QC standards (μg/ml)			Egg yolk 1 (mg/100 g)			Egg yolk 2 (mg/100 g)		
	LC–MS/MSmean ± SD(accuracy)	^{31}P NMRmean ± SD(accuracy)		LC–MS/MSmean ± SD	^{31}P NMRmean ± SD	Diff (%)	LC–MS/MSmean ± SD	^{31}P NMRmean ± SD	Diff (%)
PC	2.05 ± 0.11	(103%)	1015 ± 50	(102%)	6300 ± 479	6189 ±876	1.8	6545 ± 31	7313 ± 370	10.5
PE	1.71 ± 0.00	(86%)	994 ± 95	(99%)	1444 ± 158	1504 ±233	4.0	1577 ± 10	1752 ± 102	10.0
LPE	1.76 ± 0.06	(88%)	966 ± 93	(97%)	116.6 ± 2.7	112 ± 16	4.5	100.5 ± 0.8	94.6 ± 6.2	6.2
LPC	1.90 ± 0.13	(95%)	1004 ± 148	(100%)	169 ± 11	175 ± 33	3.1	164.2 ± 4.1	170 ± 21	3.2
SM	2.23 ± 0.02	(112%)	1066 ± 50	(107%)	254 ± 31	237 ± 55	7.3	195.8 ± 6.1	233.1 ± 4.0	16.0
PG	2.07 ± 0.14	(104%)	1159 ± 42	(116%)	ND	ND	–	ND	ND	–
PI	2.07 ± 0.16	(104%)	1048 ± 57	(105%)	145 ± 11	NA	–	146 ± 14	NA	–
PS	2.00 ± 0.08	(100%)	900 ± 85	(90%)	6.0 ± 0.8	ND	–	7.0 ±	ND	–

All data in duplicate; ND, none detectable; NA, not available; QC samples of ^{31}P NMR were diluted 500-fold for LC–MS/MS analysis.
Diff (%) = value difference/NMR value × 100.

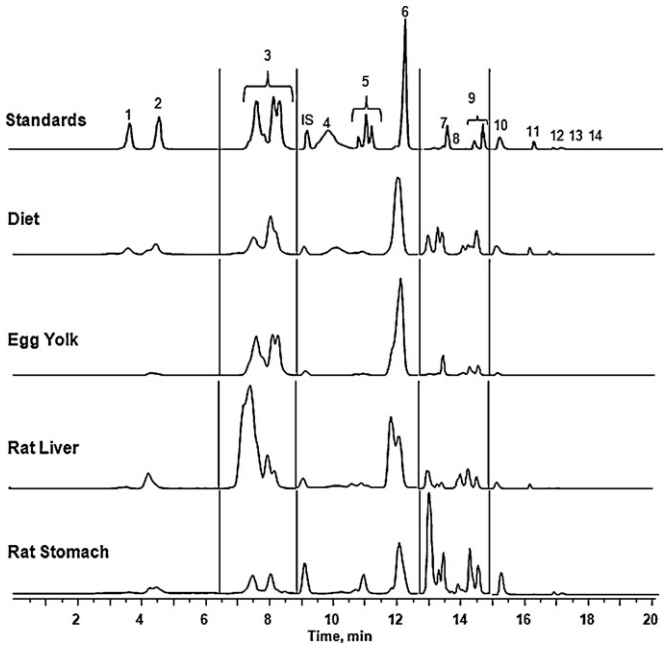


Fig. 3. LC–MS/MS profiles of different sample matrices. Peak: 1, PG; 2, PI; 3, PE; IS, MMPE; 4, PS; 5, LPE; 6, PC – PC-d_3 and PC-d_9; 7, SM; 8, Ace-Cho; 9, LPC; 10, Cho – Cho-d_3 and Cho-d_9; 11, Bet; 12, GPC; 13, CDP-Cho; and 14, PCho.

3.8. Application to a variety of experimental samples

The HILIC LC–MS/MS method established above was used to quantify the total choline present in a variety of biological samples including foods, rat livers and rat stomach content from rodent pups whose dams had been fed choline enriched diets. All sample matrices were extracted using the method as described in the experimental section. Fig. 3 shows the LC–MS/MS profiles of the different sample matrices. As expected, PC and PE were the major phospholipids in chicken egg yolks and rat livers, whereas PC and SM were the major components in the lipid extracts of suckled rats stomach contents. Fig. 3 demonstrates that the method is applicable to the analysis of a wide variety of samples relevant to nutrition and animal studies; quantitative data on these studies will be reported elsewhere.

In our previous publication [19], we found that the PC content of fresh egg yolk measured by the new LC–MS/MS method was different to the value reported in the USDA database for the choline content of common foods [32]. This may be due to a number of factors including yolk moisture content, extraction efficiency and egg type. This difference was also confirmed by the data shown in Table 6, indicating that the PC content of fresh egg yolks measured by both the LC–MS/MS method and ^{31}P NMR of 6190–7300 mg PC/100 g yolk. This corresponds to ~840–990 mg PC choline/100 g egg yolk compared to the USDA database value of 630 mg PC choline/100 g egg yolk. To further investigate the difference, we analyzed a wide range of eggs from local markets for their moisture, phospholipids and choline contents. The moisture measurements were conducted by freeze-drying of pooled egg yolks. The moisture content of egg yolk for all types of eggs were relatively similar, ranging from ~48% to 52%. The measurements of choline compounds and phospholipids in egg yolks were (per 100 g fresh yolk): 6048–7164 mg PC (818–969 mg in terms of choline moiety), 1576–1828 mg PE, 100–118 mg LPE, 194–285 mg SM (28.8–42.3 mg in terms of choline moiety), 140–169 mg PI, 142–191 mg LPC (29.4–39.5 mg in terms of choline moiety), 1.1–1.6 mg Cho, 1.4–2.3 mg GPC (0.6–1.0 mg in terms of choline moiety), and 0.9–3.9 mg Bet. The values from the USDA database all fall in the ranges of those measurements except

for the PC values measured by this method were higher, which was also confirmed by the ^{31}P NMR analysis discussed above. We can only speculate that the lower value in the USDA database might be due to a lower extraction yield of phosphatidylcholine, which is in extremely high abundance in egg yolk.

A sample diet was prepared based on the foods reported by a woman in a detailed 24 h recall taken from the Alberta Pregnancy Outcomes and Nutrition Study (APrON [33]). Her entire food intake for the day, including breakfast, lunch, dinner, and snacks, was prepared, homogenized and analyzed. Then, the measured average total daily intake of phospholipids and total choline were compared with the values estimated from the USDA database [32]. Good agreement was obtained for the total PC amount and total choline amount between the values measured using the LC–MS/MS method described here and the value estimated from the USDA database. The measured PC amount was 142 ± 87 mg against the estimated value from USDA database 146 ± 56, and the total choline measured was 280 ± 140 mg against the estimated 240 ± 90 mg. Details of this analysis will be discussed in detail elsewhere but the agreement between the present analysis and the USDA database values obtained by an independent method and referring to the range of foods that make up the daily diet, further validates the HILIC LC–MS/MS method and extraction scheme described in this report.

4. Conclusions

The HILIC LC–MS/MS method has been validated for the simultaneous quantification of 6 choline-related hydrophilic compounds and 8 major phospholipids classes in a single run. Method validation was demonstrated through measurements of sensitivity, linear dynamic ranges, extraction recovery, accuracy and precision, specificity and stability. Measurements of phospholipids classes by the HILIC LC–MS/MS method were found to be in good agreement to values obtained from ^{31}P NMR, further confirming the accuracy of the method.

The LC–MS/MS method has been successfully applied for the quantification of the 14 analytes in biological samples including egg yolks, human diets, and rat livers and stomach contents. Both the hydrophilic choline-related compounds and the phospholipids were extracted by the Bligh and Dyer method and directly diluted with methanol for LC–MS/MS analysis without the need for further separation or multiple LC methods. This universal method can be used for the simultaneous quantification of all major phospholipids and choline compounds in any biological sample matrix.

Acknowledgments

This work was financially supported by the Alberta Egg Producers (AEP) and the Alberta Livestock and Meat Agency Ltd. (ALMA). We also thank the Department of Chemistry of the University of Alberta for supporting phosphorous NMR analysis and the APrON study for access to dietary intake data.

References

[1] S.H. Zeisel, Annu. Rev. Nutr. 26 (2006) 229.
[2] S.H. Zeisel, K.A. da Costa, Nutr. Rev. 67 (2009) 615.
[3] S.H. Zeisel, K.A. da Costa, P.D. Franklin, E.A. Alexander, J.T. Lamont, N.F. Sheard, A. Beiser, FASEB J. 5 (1991) 2093.
[4] Institute of Medicine, National Academy of Sciences, U.S.A., Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline, National Academy Press, Washington DC, 2000.
[5] V. Michel, Z. Yuan, S. Ramsubir, M. Bakovic, Exp. Biol. Med. 231 (2006) 490.
[6] A. Ramírez de Molina, D. Gallego-Ortega, J. Sarmentero-Estrada, D. Lagares, T. Gómez del Pulgar, E. Bandrés, J. García-Foncillas, J.C. Lacal, Int. J. Biochem. Cell Biol. 40 (2008) 1753.
[7] T. Glonek, J. Am. Oil Chem. Soc. 75 (1998) 569.
[8] N. Sotirhos, B. Herslöf, L. Kenne, J. Lipid Res. 27 (1986) 386.
[9] J. Schiller, K. Arnold, Med. Sci. Monit. 8 (2002) MT205.
[10] E.A. Pomfret, K.DaCosta, L.L. Schurman, S.H. Zeisel, Anal. Biochem. 180 (1989) 85.
[11] N.U. Ollson, N. Salem, J. Chromatogr. B 692 (1997) 245.
[12] A. Sala Vila, A.I. Castellote-Bargalló, M. Rodríguez-Palmero-Seuma, M.C. López-Sabate, Anal. Chem. 74 (2002) 4734.
[13] P.I. Holm, P.M. Ueland, G. Kvalheim, E.A. Lien, Clin. Chem. 49 (2003) 286.
[14] P. Andrieux, T. Kilinc, C. Perrin, E. Campos-Giménez, J. AOAC Int. 91 (2008) 777.
[15] H. Koc, M.-H. Mar, A. Ranasinghe, J.A. Swenberg, S.H. Zeisel, Anal. Chem. 74 (2002) 4734.
[16] Y. Wang, T. Wang, X. Shi, D. Wan, P. Zhang, X. He, P. Gao, S. Yang, J. Gu, G. Xu, J. Pharm. Biomed. Anal. 47 (2008) 870.
[17] A. Fischbeck, M. Krüger, N. Blaas, H.-U. Humpf, J. Agric. Food Chem. 57 (2009) 9469.
[18] D. Desoubzdanne, C. Claparols, N. Martins-Froment, C. Zedde, S. Balayssac, V. Gilard, F. Tercé, R. Martino, M. Malet-Martino, Anal. Bioanal. Chem. 398 (2010) 2723.
[19] Y.-Y. Zhao, Y. Xiong, J.M. Curtis, J. Chromatogr. A 1218 (2011) 5470.
[20] P. Wang, D.H. Blank, T.A. Spencer, J. Org. Chem. 69 (2004) 2693.
[21] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 37 (1959) 911.
[22] A. MacKenzie, M. Vyssotski, E. Nekrasov, J. Am. Oil Chem. Soc. 86 (2009) 757.
[23] M.H. Nouri-Sorkhabi, N.S. Agar, D.R. Sullivan, C. Gallagher, P.W. Kuchel, Comp. Biochem. Phys. B 113 (1996) 221.
[24] A. Puppato, D.B. DuPré, N. Stolowich, M.C. Yappert, Chem. Phys. Lipids 150 (2007) 176.
[25] Y. Xu, P.-A. Siegenthaler, Lipids 31 (1996) 223.
[26] G. Isaac, D. Bylund, J.-E. Månsson, K.E. Markides, J. Bergquist, J. Neurosci. Methods 128 (2003) 111.
[27] N.B. Cech, C.G. Enke, Mass Spectrom. Rev. 20 (2001) 362.
[28] M. Oss, A. Kruve, K. Herodes, I. Leito, Anal. Chem. 82 (2010) 2865.
[29] R. Kostiainen, T.J. Kauppila, J. Chromatogr. A 1216 (2009) 685.
[30] G. Shi, Rapid Commun. Mass Spectrom. 17 (2003) 202.
[31] J.M. Pearce, R.A. Komoroski, Magn. Reson. Med. 44 (2000) 215.
[32] K.Y. Patterson, S.A. Bhagwat, J.R. Williams, J.C. Howe, J.M. Holden, USDA Database for the Choline Content of Common Foods Release Two, USDA, Agricultural Research Service, Beltsville Maryland, US, 2008, http://www.nal.usda.gov/fnic/foodcomp/Data/Choline/Choln02.pdf
[33] Alberta Pregnancy Outcomes and Nutrition (APrOn) study. http://www.apronstudy.ca