Journal of Chromatography A, 1218 (2011) 5470–5479

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Measurement of phospholipids by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry: The determination of choline containing compounds in foods

Yuan-Yuan Zhao, Yeping Xiong, Jonathan M. Curtis*

Department of Agricultural, Food and Nutritional Sciences, University of Alberta, 318F Agriculture/Forestry Centre, Edmonton, Alberta T6G 2P5, Canada

ARTICLE INFO

Article history:
Received 19 December 2010
Received in revised form 14 April 2011
Accepted 8 June 2011
Available online 17 June 2011

Keywords:
Hydrophilic interaction chromatography (HILIC)
Tandem mass spectrometry (MS/MS)
Choline
Egg yolk
Phospholipids

ABSTRACT

A hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC LC–MS/MS) method using multiple scan modes was developed to separate and quantify 11 compounds and lipid classes including acetylcholine (AcCho), betaine (Bet), choline (Cho), glycerophosphocholine (GPC), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphocholine (PCho) and sphingomyelin (SM). This includes all of the major choline-containing compounds found in foods. The method offers advantages over other LC methods since HILIC chromatography is readily compatible with electrospray ionization and results in higher sensitivity and improved peak shapes. The LC–MS/MS method allows quantification of all choline-containing compounds in a single run. Tests of method suitability indicated linear ranges of approximately 0.25–25 μg/ml for PI and PE, 0.5–50 μg/ml for PC, 0.05–5 μg/ml for SM and LPC, 0.5–25 μg/ml for LPE, 0.02–5 μg/ml for Cho, and 0.08–8 μg/ml for Bet, respectively. Accuracies of 83–105% with precisions of 1.6–13.2% RSD were achieved for standards over a wide range of concentrations, demonstrating that this method will be suitable for food analysis. 8 polar lipid classes were found in a lipid extract of egg yolk and different species of the same class were differentiated based on their molecular weights and fragment ion information. PC and PE were found to be the most abundant lipid classes consisting of 71% and 18% of the total phospholipids in egg yolk.

1. Introduction

Choline is a dietary component essential for the normal function of all cells [1]. The major form of choline in the body and in the diet is the phospholipid phosphatidylcholine. Extreme dietary deficiency of choline can result in liver dysfunction, impaired growth, abnormalities in bone formation, kidney failure, and anemia [2]. Choline is critical during fetal development [1], when it affects brain and spinal cord structure and function and thereby the risk for neural tube defects and lifelong memory function. Choline acts as a source of methyl groups for synthetic reactions throughout the body the greatest need for which is during gestation and the early post-natal period. Although choline can be biosynthesized by humans, a large dietary intake is also required. This has been recognized in various countries, e.g. in 1998 the US Institute of Medicine Food and Nutrition Board established dietary reference intake levels of 550 mg/day for men and for women 425 rising to 450 and 550 mg/day during pregnancy and lactation.

Although small amounts of choline are present in many foods, egg yolks are among the richest dietary sources providing ~680 mg choline per 100 g yolk (or 126 mg per 50 g egg) [3]. Considering the newly emerging understanding of the importance of choline in nutrition, there exists an opportunity to promote eggs as an excellent and convenient source.

In addition to phosphatidylcholine (PC), the dominant form of choline in egg yolk, other important and related species present include phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), acetylcholine (AcCho), phosphocholine (PCho), glycerophosphocholine (GPC), phosphatidylinositol (PI), sphingomyelin (SM), choline (Cho), betaine (Bet) and lysophosphatidylcholine (LPC) (Fig. 1). Many analytical methods have been proposed to identify and quantify phospholipids, choline and betaine with various levels of sophistication and specificity. A QC assay for total choline has been developed [4] using combined acid hydrolysis with enzymatic release of choline, derivitisation and colorimetric determination. However, digestion on hydrolysis loses all information on the individual choline-containing compounds. In contrast, phosphorus-31 nuclear magnetic resonance (^{31}P NMR) has been used to quantify phospholipids including PC, PE, and PI in soybean and egg lecithin

* Corresponding author. Tel.: +1 780 492 6364.
E-mail address: jcurtis1@ualberta.ca (J.M. Curtis).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.06.025

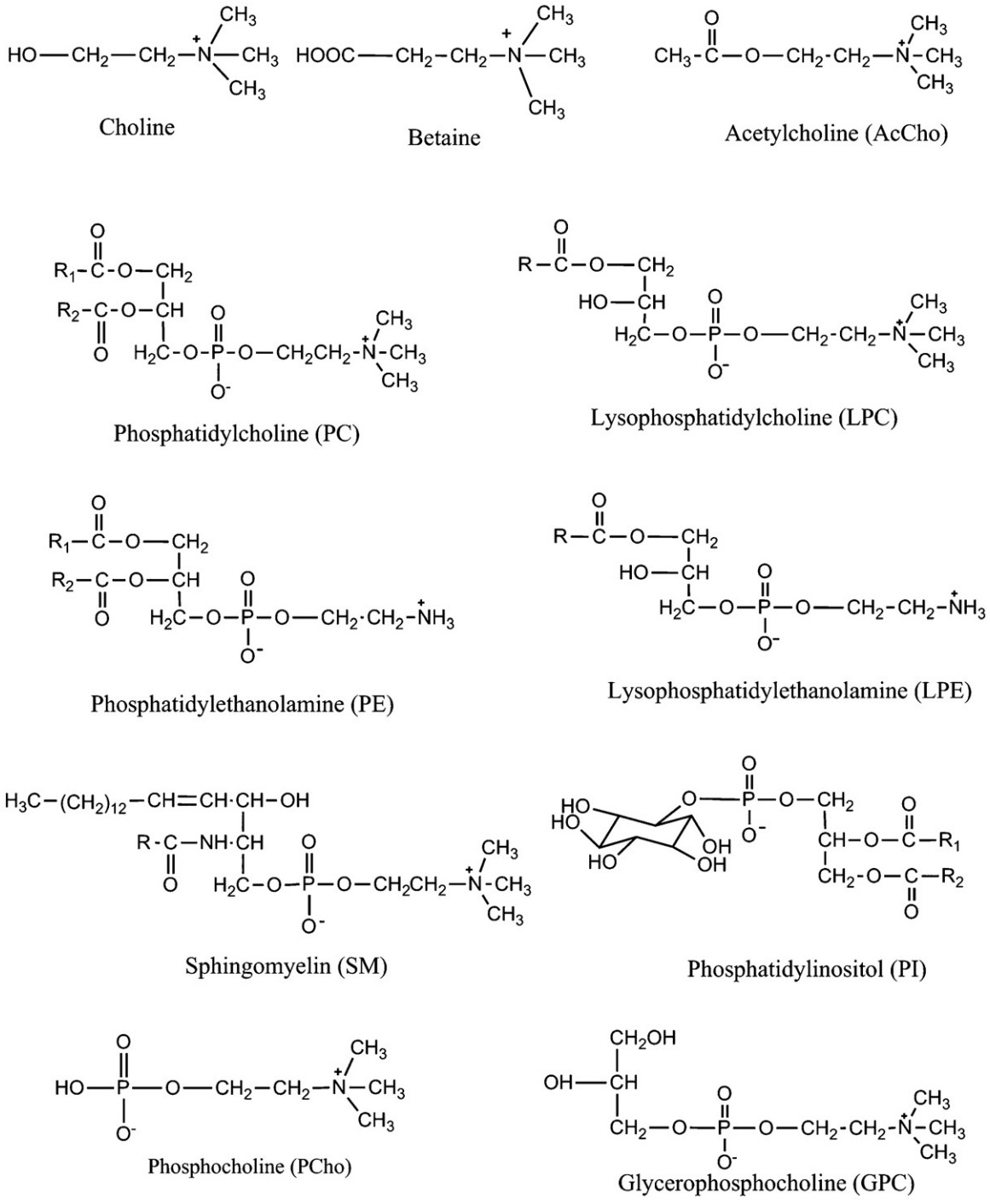

Fig. 1. Structures of the compounds investigated.

[5,6]. However, since this method makes use of the chemical shift observed for phosphorus-containing molecules, it cannot quantify species not containing P such as choline. Furthermore, although a very convenient method requiring little sample preparation, it cannot differentiate fatty acid chains in every class such as PC. Earlier methods using gas chromatography (GC) or GC/mass spectrometry (GC/MS) require collection of peaks by preparative high performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) and derivatization prior to analysis, which is time-consuming and easily causes loss of sensitivity [7,8]. HPLC coupled with evaporative light scattering detector (ELSD) [9,10], fluorometric detection [11], or mass spectrometry [12,13] have also been reported for analysis of choline compounds.

Due to the polar head groups of these compounds, which are very distinctive for identification by mass spectrometry, normal-phase HPLC coupled with electrospray mass spectrometry is often used. This combines the separation power of HPLC with the structural and quantitative information possible by mass spectrometry, thus making it possible to get quantitative information on individual choline-containing compounds or classes [12,14,15]. However, the typical non-polar mobile phases commonly used in normal-phase HPLC are not amenable for direct interfacing to electrospray mass spectrometry; instead either make-up flows containing ionic buffers or else complex solvent/buffer mixtures must be used. An example of the latter was described in the work of Koc et al. [15] who pre-fractionated

extracts of tissues and foods into an aqueous phase containing betaine, choline, glycerophosphocholine, phosphocholine, etc., and chloroformic phase containing phosphatidylcholine and sphingomyelin. The two phases were analyzed in separate injections with different gradients but using the same solvent mixtures of acetonitrile/water/ethanol/1 M ammonium acetate/glacial acetic acid at 800/127/68/3/2 (v/v) as mobile phase A and 500/500/85/27/18 (v/v) as mobile phase B. A further example is the work of Malavolta et al. [14] and Pacetti et al. [12] who used mobile phases of chloroform/methanol/ammonium hydroxide (30%) at 80/19.5/0.5 (v/v) and chloroform/methanol/water/ammonium hydroxide (30%) at 60/34/5.5/0.5 (v/v) in order to separate PE, PC, PI, SM and LPC from egg yolks, with direct coupling to negative ion electrospray mass spectrometry.

Reversed-phase HPLC coupled with tandem mass spectrometry has also been developed for the analysis of phospholipids [16–18]. Based on the difference of acyl chain lengths and the degree of unsaturation, reversed-phase LC can separate different species within one lipid class, such as phosphatidylcholine (PC). However, not all lipid classes, such as PE and PC, can be well separated by reversed-phase LC. Houjou et al. [17] have applied offline 2D LC/ESI-MS/MS of phospholipids, in which each class of phospholipids from the rat liver was separated by a normal phase column prior to analysis of molecular species in each class fraction by reversed-phase LC/ESI-MS-MS. Partial class separation of phospholipids has also been achieved by reversed-phase ion-pair chromatography [19]. However, using ESI with ion-pair reagents in the mobile phase is not desirable because of their strong suppression effect on the analyte response and possible contamination of the ion source.

Hydrophilic interaction chromatography (HILIC) uses a polar stationary phase that can retain polar compounds on the column without the disadvantages of using solvents that are immiscible with water, as used in traditional normal phase HPLC. The high amount polar organic mobile phase (often acetonitrile) used in HILIC is especially compatible with ESI-MS, resulting in high sensitivity. It has been used to determine acetylcholine, choline and butyrobetaine in human liver tissues [20] and acetylcholine, choline and choline-trimethyl-d_9 [21]. However, to our knowledge there has not to date been a report of a comprehensive HILIC LC–MS/MS method for the identification and quantification of the major choline-containing compounds found in egg yolks.

The overall objective of the study reported here is the development of methods both for the qualitative structural identification and the accurate quantification of choline-containing compounds found in egg yolks and other foods. This was achieved by exploiting the advantages of HILIC separation combined with both high resolution ESI mass spectrometry giving accurate molecular weight information and tandem mass spectrometry for species identification. Quantitative methods using the multiple scan modes available on a QTRAP™ mass spectrometer (AB Sciex, Ontario, Canada) including precursor scan, neutral loss scan and multiple reaction monitoring (MRM) were then developed to enable us to determine the abundance of many choline-containing compounds, using a single LC–MS run.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine (from egg yolk, ≥99%) (PC), sphingomyelin (from egg yolk, >95%) (SM), choline chloride (>98%) (Cho), choline chloride-trimethyl-d_9 (Cho-d_9), acetylcholine chloride (>99%) (AcCho), phosphocholine chloride calcium salt tetrahydrate (Sigma grade) (PCho), and betain hydrochloride (>99%) (Bet) were purchased from Sigma (St. Louis, MO); 1,2-distearoyl-*sn*-glycero-3-phosphocholine-N,N,N-trimethyl-d9 (PC-d_9), L-α-phosphatidylethanolamine (Egg, chicken) (PE), L-α-lysophosphatidylcholine (Egg, chicken) (LPC), and L-α-phosphatidylinositol (Soy, sodium salt) (PI) were obtained from Avanti polar lipids, Inc. (700 Industrial Park Drive, Alabaster, Alabama). Glycerophosphocholine (GPC) was supplied by Bachem Americas Inc (Torrance, CA). HPLC-grade chloroform, acetonitrile, and methanol were purchased from Fisher Scientific Company (Ottawa, Ontario). HPLC-grade ammonium formate (≥99%) and formic acid were supplied by Sigma (St. Louis, MO). Water was purified with a Millipore (Bedford, MA) Milli-Q water system. A solution of porcine renin substrate tetetradecapeptide at 10 pmol/μl in acetonitrile/water (50:50) in a chemical standards kit was obtained from Applied Biosystems (Forster City, CA). ESI-L low concentration tuning mix was purchased from Agilent Technologies Canada Inc., Mississauga, Ontario, Canada.

2.2. Preparation of stock and working standard solutions

A 1 mg/ml stock solution of PI, PE, and LPE was prepared in chloroform. Solutions were prepared of PCho (1 mg/ml in 3:1 (v/v) methanol/water) and PC, LPC and SM (0.5 mg/ml in 1:2 (v/v) chloroform/methanol). Stock solutions of Cho, AcCho, GPC and Bet were made in methanol at 0.5 mg/ml. The internal standard, PC-d_9 and Cho-d_9 stock solutions were prepared in methanol at 0.5 mg/ml. All stock solutions were stored at −20 °C before use. Working standard solutions for calibrations were prepared by mixing and diluting stock solutions in methanol to give concentration of PI, PE and LPE from 0.1 to 50 μg/ml; PC from 0.5 to 50 μg/ml; LPC, SM, Cho and Bet from 0.02 to 10 μg/ml; and with internal standards PC-d_9 and Cho-d_9 at 0.2 μg/ml. Solutions were prepared in triplicate for calibrations. Triplicate low, medium and high quality control (QC) standard solutions were made at 1 μg/ml (QC, L), 10 μg/ml (QC, M), 25 μg/ml (QC, H) for PC, PI, PE and LPE; 0.05 μg/ml (QC, L), 0.5 μg/ml (QC, M), 2.0 μg/ml (QC, H) for GPC; 0.08 μg/ml (QC, L), 0.8 μg/ml (QC, M), 2.0 μg/ml (QC, H) for AcCho; 3.0 μg/ml (QC, L), 16 μg/ml (QC, M), 50 μg/ml (QC, H) for PCho, and 0.2 (QC, L), 2 (QC, M), 5 μg/ml (QC, H) for LPC, SM, Cho and Bet.

2.3. Sample preparation

Fresh farm eggs purchased from a local supermarket were carefully broken and the egg yolks were separated from the whites. Three egg yolks were pooled together and triplicate samples of 100 mg egg yolks were extracted based on the Bligh and Dyer method [22,23]. In brief, 2 ml of extraction solvent (chloroform/methanol/water, 1:2:0.8) was added to 100 mg of egg yolk. The sample was homogenized at 10,000 rpm for 5 min on a Polytron PT1300 D homogenizer (Kinematica AG, Switzerland) and then centrifuged at 3000 rpm for 5 min. The resultant supernatant was transferred into a separate glass vial. The extraction procedure was repeated twice more, each time transferring the supernatant extract into the same glass vial. The combined extract was dried under a nitrogen stream, re-dissolved into 6 ml of methanol, and further diluted 10-fold with methanol. The diluted extract solution was then filtered through a 0.22 μm membrane and spiked with an internal standard of PC-d_9 and Cho-d_9 for subsequent LC–MS/MS analysis.

2.4. HPLC–ESI-MS/MS method

Standards and sample solutions were analyzed using an Agilent 1200 series UPLC system equipped with binary pump and autosampler (Agilent Technologies, Palo Alto, CA, USA) coupled to a QSTAR Elite mass spectrometer with Turbospray ion source (AB

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 min								
NL of 260	700–900	1	PI	45	8	35	40	4.5
Period 2, 6–7.5 min								
NL of 141	600–800	1.2	PE	50	8	20	29.8	27
Period 3, 7.5–9.5 min								
Prec of 184	700–900	0.6	PC	55	8	20	22	3
NL of 141	400–600	1	LPE	50	8	20	29.8	27
MRM	799.8 > 192.8	0.05	IS PC-d_9	55	8	20	45	3
Period 4, 9.5–11.5 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, 11.5–30 min								
MRM	104.2 > 60.1	0.05	Cho	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

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

SCIEX, Concord, ON, Canada). Analyst QS 2.0 software was used for data acquisition and analyses. The mass spectrometer was tuned by infusing porcine renin substrate tetetradecapeptide (*m/z* 879.9723, doubly charged ion) at a resolution of 13000 full width at half-maximum (FWHM) under positive mode and by taurocholic acid (*m/z* 514.2844) at a resolution of 10,000 FWHM under negative mode, and calibrated using an electrospray ionization (ESI) low concentration tuning mix (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Mass spectrometric analysis was performed using positive ion or negative ion ESI in information dependent acquisition (IDA) mode. The mass range recorded for both full-scan mass spectra and MS/MS product ion spectra was from *m/z* 50 to 1100. Nitrogen was used as curtain gas, nebulizing gas and drying gas. All other instrumental parameters used were as followed: curtain gas at 25 arbitrary units; gas 1 at 40; gas 2 at 60; ionspray voltage at 5500 V for positive mode and −4500 V for negative mode and ion source temperature at 350 °C. The declustering potential (DP), focus potential (FP), and DP2 were 40 V, 150 V, and 10 V for positive mode, respectively and −40 V, −150 V and −10 V for negative mode.

All quantitative experiments were performed on 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. A turbospray ion source was employed under positive ion mode. Various collision induced dissociation CID-MS/MS scan modes including precursor ion scans (Prec), neutral loss scans (NL) and multiple reaction monitoring scans (MRM) were developed in order to quantify the lipid classes containing choline that present in egg yolks. Table 1 lists the detailed parameters for collision induced dissociation CID-MS/MS analyses and is explained in Section 3.2.

HILIC HPLC. An Ascentis Express 150 × 2.1 mm HILIC column, 2.7 μm particle size (Sigma, St. Louis, MO) was used for LC separations. 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; 10–15 min, 70% B; and then back to 8% B at 15.1 min for column re-equilibrium over 20 min prior to the next injection. The injection volume was 2 μl, and the flow rate of mobile phase was 200 μl/min.

Normal phase HPLC. A 150 × 2.1 mm Ascentis silica column with 3 μm particles (Sigma, St. Louis, MO) was employed for normal phase LC. The separation was carried out according to Koc et al. [15]. Briefly, mobile phase A consisted of acetonitrile/water/ethanol/1 M ammonium acetate/glacial acetic acid (800/127/68/3/2, v/v) and B of acetonitril/water/ethanol/1 M ammonium acetate/glacial acetic acid (500/500/85/27/18, v/v). The gradient was as follows: 0–3 min, 0% B; 3–10 min, 40% B; 10–14 min, 45% B; 14–18 min, 60% B, 18–19 min, 100%; 19–27 min, 100% B; 27–28 min, 0% B. Re-equilibrium time was 12 min.

3. Results and discussion

3.1. Mass detection and HILIC separation of choline compounds

Since choline-containing compounds contain charged groups (Fig. 1), they are readily detected using electrospray mass spectrometry. Thus, by infusing into the mass spectrometer a standard solution containing a mixture of the 11 compound classes investigated, $[M]^+$ molecular ions were observed for Cho (*m/z* 104.1) and Bet (*m/z* 118.0) and protonated molecules $[M+H]^+$ and ammonium adducts $[M+NH_4]^+$ were observed for PI, while the protonated molecules $[M+H]^+$ were formed with all other compounds. Although previous work [12] has successfully made use of formate adducts $[M+HCOO]^-$ of PC, LPC and SM under negative mode, in our experiments in positive ion mode, protonated molecules for PI and PE were found to be approximately ten times higher in response than could be achieved in negative ion mode. Therefore, positive ion mode, required to detect M^+ ions for Cho and Bet, was also selected for recording mass spectra of the protonated molecules of molecules for all other choline compounds investigated.

Although high-resolution mass spectra can provide the likely elemental composition of molecular and product ions for compound identification, chromatographic separation giving complementary retention time data is also critical information for compound identification. Furthermore, separation leads to reduced ion suppression for mass spectrometric detection compared to the situation where all species are present at once. As explained above, HILIC appears to be a promising separation method for choline-containing compounds so a comparison was performed between the HILIC method developed in this work and a recently validated normal-phase HPLC method reported in literature [15]. The results for both methods are presented in Fig. 2 for both a high concentration standard solution (20 μg/ml PI, PE, LPE and PCho; 10 μg/ml PC, SM, AcCho, LPC, Cho, Bet, and GPC) and a low concentration standard solution which was a 10× dilution of the high concentration standard solution. With the simpler mobile phase composition used in the HILIC method (**A**:CH_3CN; **B**:H_2O/$HCOONH_4$, pH3) compared to the literature [15] normal phase method (**A**: $CH_3CN/H_2O/C_2H_5OH/CH_3COONH_4/CH_3COOH$;

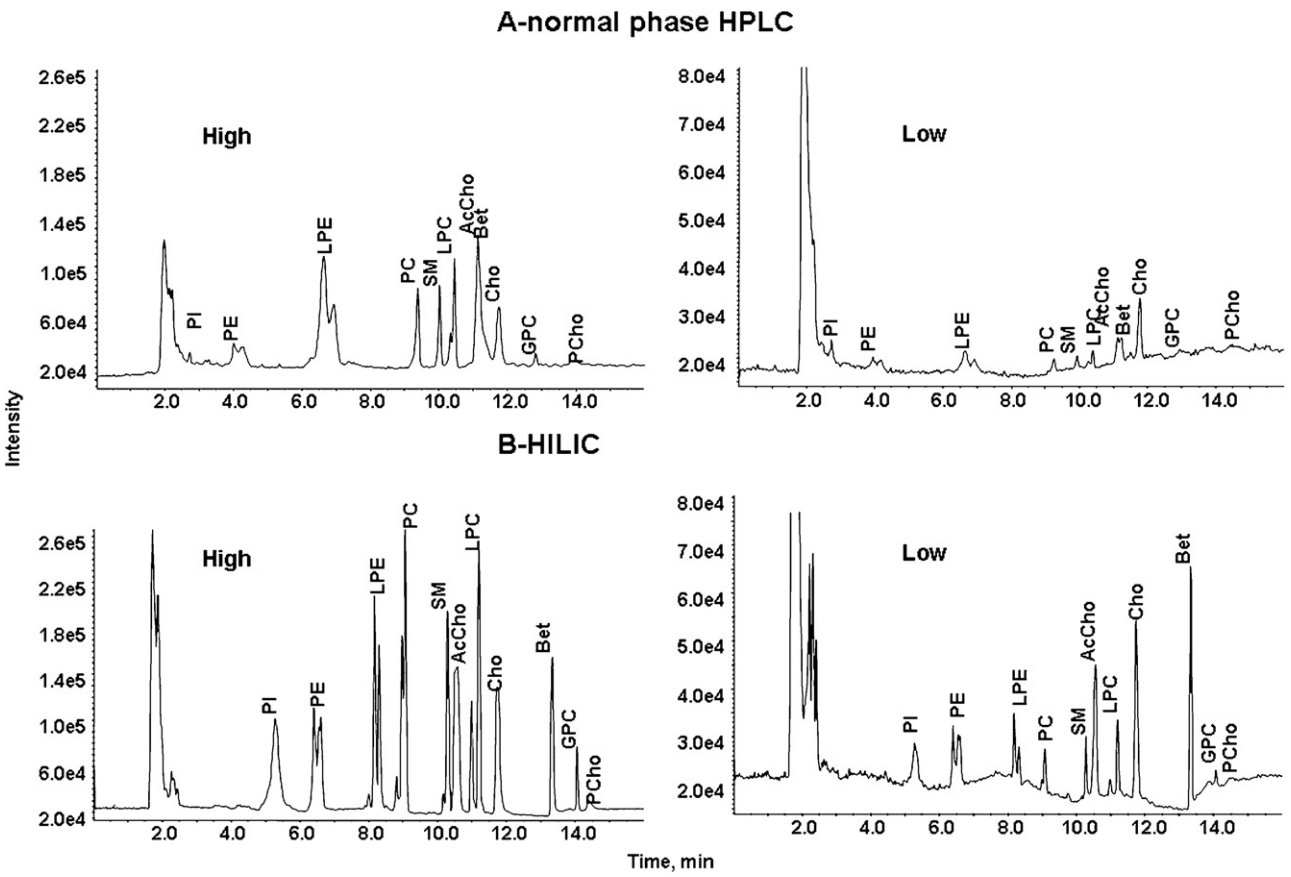

Fig. 2. LC–MS (TIC) chromatograms for the separation of a mixture of PI, PE, LPE, PC, SM, LPC, AcCho, Bet, Cho, GPC and PCho by (A) Normal phase HPLC; and (B) HILIC. The high concentration standard mixture (left panes) was 10 μg/ml of PC, SM, LPC, Bet, Cho, AcCho, GPC and 20 μg/ml of PCho, PE, PI and LPE and the low concentration was a 10 times of dilution factor of this. Other experimental conditions are described in section 2.4 of Materials and Methods.

B: as **A** but at different ratios) a qualitatively comparable separation was achieved but with the advantage that PI is much better retained and well separated from the injection solvent front in the HILIC method. Better peak shape and higher signal-to-noise was also obtained in the HILIC separation, as is evident in Fig. 2 at both concentrations. The chromatograms in Fig. 2 are total ion current (TIC) traces so contain intensity both from the compounds of interest and the background. This also results in the apparently unstable baseline at the lower concentration as the gradient changes, as seen in the figure. However, a low and stable baseline would be obtained in the much more selective MRM or SIR experiments that are employed for quantitation. It is clear From Fig. 2 that limit of detection for the compounds in the mixture would be significantly lower using the HILIC LC/MS method compared to the normal phase-LC/MS method.

The mobile phase initially used for HILIC was acetonitrile and water with 0.1% formic acid at pH 3.0 but the peak for Cho was found to be very broad. The Cho peak shape improved when the mobile phase was modified with ammonium formate (10 mM). However, low sensitivity for PI and PE was observed due to ion suppression from the mobile phase when formic acid and ammonium formate in acetonitrile were used. Therefore, acetonitrile was used as mobile phase A and water with10 mM ammonium formate adjusted to pH 3.0 was used as mobile phase B for the following experiments.

3.2. Characterization of phospholipid species in egg yolk

Using the HILIC LC/MS method, 8 choline containing compounds were observed in egg yolk extracts including PI, PE, LPE, PC, LPC, SM, Cho and Bet (Fig. 3). With the exception of choline and betaine, the phospholipids found in egg yolks all contain a distribution of fatty acyl groups. Due to the similar properties of these species, it is hard to get separation of both lipid class and the fatty acyl substituents in a single LC experiment. However, mass spectrometry provides an additional dimension of separation based on mass-to-charge ratio. Thus, the molecular weight and fragment ion information provided by mass spectrometry makes it possible to differentiate these species to some extent, as shown in the inset of Fig. 3. As an illustration, the selected ion traces for various combinations of

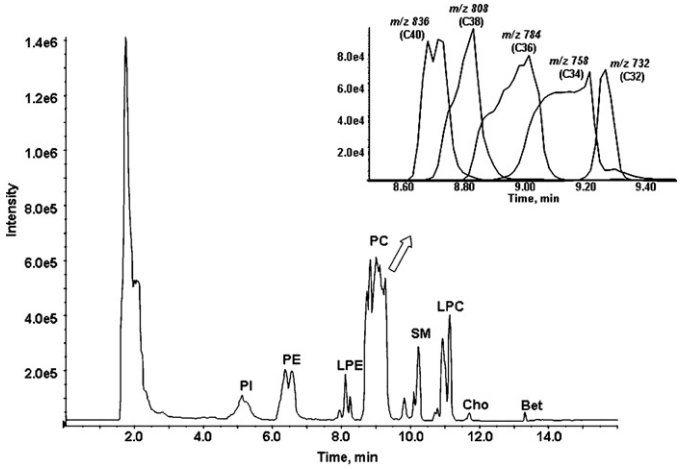

Fig. 3. HILIC chromatogram of egg yolk extracts obtained using the LC–MS/MS method described in Table 1. The inset shows the extracted ion chromatograms (XIC) of PC species. Other experimental conditions are similar to Fig. 2.

Table 2
Compounds identified in egg yolk extracts.

Ion (*m*/*z*)	Relative intensity	Fatty acids	Formula	Theoretical mass	Error (ppm)	Product ions in MS/MS
PE(M+H)+						
716.5231	10.7	16:0/18:2	$C_{39}H_{75}O_8NP$	716.5225	0.8	575, 239, 263, 337, 313
718.5365	56.4	16:0/18:1	$C_{39}H_{77}O_8NP$	718.5381	−2.2	577, 239, 265,
740.519	19.3	16:0/20:4	$C_{41}H_{75}O_8NP$	740.5225	−4.7	599, 361, 313,
744.554	18.6	18:0/18:2, 18:1/18:1	$C_{41}H_{79}O_8NP$	744.5538	0.3	265, 308, 603, 263, 306, 464, 267, 337
746.568	18.6	18:0/18:1	$C_{41}H_{81}O_8NP$	746.5694	−1.9	605, 482, 265, 308,
748.5736	1.9	18:0/18:0	$C_{41}H_{83}O_8NP$	748.5851	−15.3	N/A
764.5227	5.9	16:0/22:6	$C_{43}H_{75}O_8NP$	764.5225	0.3	623, 385, 313
768.5523	62.9	18:0/20:4	$C_{43}H_{79}O_8NP$	768.5538	−1.9	627, 395, 341, 267, 203
792.5542	0.1	18:0/22:6	$C_{45}H_{79}O_8NP$	792.5538	0.5	651, 341, 385
794.5702	0.1	18:0/22:5	$C_{45}H_{81}O_8NP$	794.5694	1	N/A
LPE(M+H)+						
482.32	20.7	C18:0	$C_{23}H_{49}O_7NP$	482.3241	−8.5	341, 310, 155, 89, 71, 62
454.2948	6.4	C16:0	$C_{21}H_{45}O_7NP$	454.2928	4.4	313, 282, 239, 155, 62
PC(M+H)+						
732.5537	68.6	16:0/16:1	$C_{40}H_{79}NO_8P$	732.5537	−0.1	732, 184
734.5667	42.9	16:0/16:0	$C_{40}H_{81}NO_8P$	734.5694	−3.7	734.184
758.5677	71.4	16:0/18:2	$C_{42}H_{81}NO_8P$	758.5694	−2.3	758, 520, 496, 184
760.5814	100	16:0/18:1	$C_{42}H_{83}NO_8P$	760.585	−4.8	760, 577, 496, 478, 184
780.5573	1.1	16:0/20:5,16:1/20:4	$C_{44}H_{79}NO_8P$	780.5537	4.5	780, 184
782.5692	71.4	16:0/20:4	$C_{44}H_{81}NO_8P$	782.5694	−0.3	782, 184
784.5815	63.6	16:0/20:3	$C_{44}H_{83}NO_8P$	784.5851	4.5	784, 184
786.6014	71.4	18:0/18:2, 18:1/18:1	$C_{44}H_{85}NO_8P$	786.6007	0.8	786, 502, 478, 184
788.6106	71.4	18:0/18:1	$C_{44}H_{87}NO_8P$	788.6163	−7.3	788, 184
806.5683	71.4	16:0/22:6,18:1/22:5, 18:2/20:4	$C_{46}H_{81}NO_8P$	806.5694	−1.4	806, 184
808.5808	67.9	18:1/20:4, 18:0/20:5, 16:0/22:5	$C_{46}H_{83}NO_8P$	808.585	−5.3	808, 184
810.5967	71.4	18:0/20:4	$C_{46}H_{85}NO_8P$	810.6007	−5	810, 184
812.6067	28.6	18:0/20:3	$C_{46}H_{87}NO_8P$	812.6163	−11.9	812, 184
832.5772	28.6	18:1/22:6	$C_{48}H_{83}NO_8P$	832.585	−9.5	832, 184
834.5969	54.3	18:0/22:6, 18:1/22:5	$C_{48}H_{85}NO_8P$	834.6007	−4.6	834, 184
836.6099	63.6	18:0/22:5	$C_{48}H_{87}NO_8P$	836.6163	−7.8	836, 184
SM(M+H)+						
703.5738	65.7	C16:0	$C_{39}H_{80}N_2O_6P$	703.5748	−1.5	184, 125
731.6087	2.1	C18:0	$C_{41}H_{84}N_2O_6P$	731.6061	3.5	N/A
LPC(M+H)+						
496.3403	71.4	C16:0	$C_{24}H_{51}NO_7P$	496.3397	1.1	478, 313, 184, 104
518.32	1.3	C18:3	$C_{26}H_{49}NO_7P$	518.3241	−7.9	N/A
520.3392	1.8	C18:2	$C_{26}H_{51}NO_7P$	520.3397	−1.1	184, 104
522.3543	19.3	C18:1	$C_{26}H_{53}NO_7P$	522.3554	−2.1	504, 184, 104
524.37	64.3	C18:0	$C_{26}H_{55}NO_7P$	524.371	−2	506, 341, 184, 104
PI(M+NH4)+						
852.5634	0.1	16:0/18:2	$C_{43}H_{83}NO_{13}P$	852.5597	4.4	852, 575
854.5752	0.1	16:0/18:1	$C_{43}H_{85}NO_{13}P$	854.5753	0.1	854, 577, 339
876.5635	0.05	16:0/20:4	$C_{45}H_{83}NO_{13}P$	876.5597	4.3	876, 599, 577, 313, 265
878.5706	0.04	16:0/20:3	$C_{45}H_{85}NO_{13}P$	878.5753	5.3	878, 601, 313, 239
880.5986	0.6	18:0/18:2	$C_{45}H_{87}NO_{13}P$	880.591	8.6	880, 603, 341
882.6101	0.07	18:0/18:1	$C_{45}H_{89}NO_{13}P$	882.6066	3.9	882, 605
904.5942	1.9	18:0/20:4	$C_{47}H_{87}NO_{13}P$	904.591	3.5	904, 887, 627, 361, 341
906.6096	0.4	18:0/20:3	$C_{47}H_{89}NO_{13}P$	906.6066	3.3	906, 889, 629, 605, 363, 341

fatty acyl substituents from within the PC peak are shown in this inset. The individual mass chromatograms are each combinations of all PC species whose two fatty acyl groups add up to the combined carbon chain length indicated. Although isomeric species are not resolved, an estimation of the abundance of PC species with a particular fatty acyl chain length and degree of unsaturation could be made.

The high resolution mass spectral data for choline containing compounds egg yolk were obtained to aid in identification. Table 2 lists the *m*/*z* for molecular and product ions obtained for the 6 most abundant phospholipids in egg yolk extracts, PE, LPE, PC, SM, LPC and PI. The likely fatty acid composition indicated was assigned based on the accurate mass measured, product ions observed in MS/MS experiments, plus comparisons to standards and the literature [12].

10 PE species (Table 2) were found in egg yolks based on molecular weight information, which is consistent with an earlier report [12]. The base peak in MS/MS for all PE species (except for the ion at *m*/*z* 794, which was at too low an abundance to give measurable product ions) was found to be the fragment ion representing neutral loss of 141 (phosphoethanolamine); the next most intense fragmentation was the loss of a fatty acyl group to leave a structure with the monoacylglyceride backbone. In the latter fragmentation reaction, the charge could reside on either fragment, but a dependence on the degree of unsaturation in the fatty acid chain was observed. If the number of double bonds on one of the fatty acid chains is greater than 3, the ion due to loss of that fatty acid chain was found to predominate. For example, in Table 2, for the PE MH^+ ion at *m*/*z* 764 with a fatty acyl composition of C16:0/C22:6, the fragment ion at *m*/*z* 313 $[M\text{-}141\text{-}C22{:}6+H]^+$ was formed due to the loss of C22:6, while we did not observe the ion at *m*/*z* 311 which would be expected if the charge remained on the C22:6 fatty acyl group. Similarly, for both PE MH^+ ions *m*/*z* 792 (C18:0/C22:6) and *m*/*z* 768 (C18:0/C20:4) the fragment ion observed was *m*/*z* 341 due to loss of C22:6 or C20:4.

The product ions from MS/MS data helps to identify the species that are present and that differ only by their fatty acid chains. For example, for the PE MH^+ ion at *m*/*z* 740, there are two possible compositions, C16:0/C20:4 and C18:2/C18:2. The MS/MS spectrum of this ion shows product ions at *m*/*z* 313 $[M\text{-}141\text{-}C20{:}4+H]^+$ and *m*/*z*

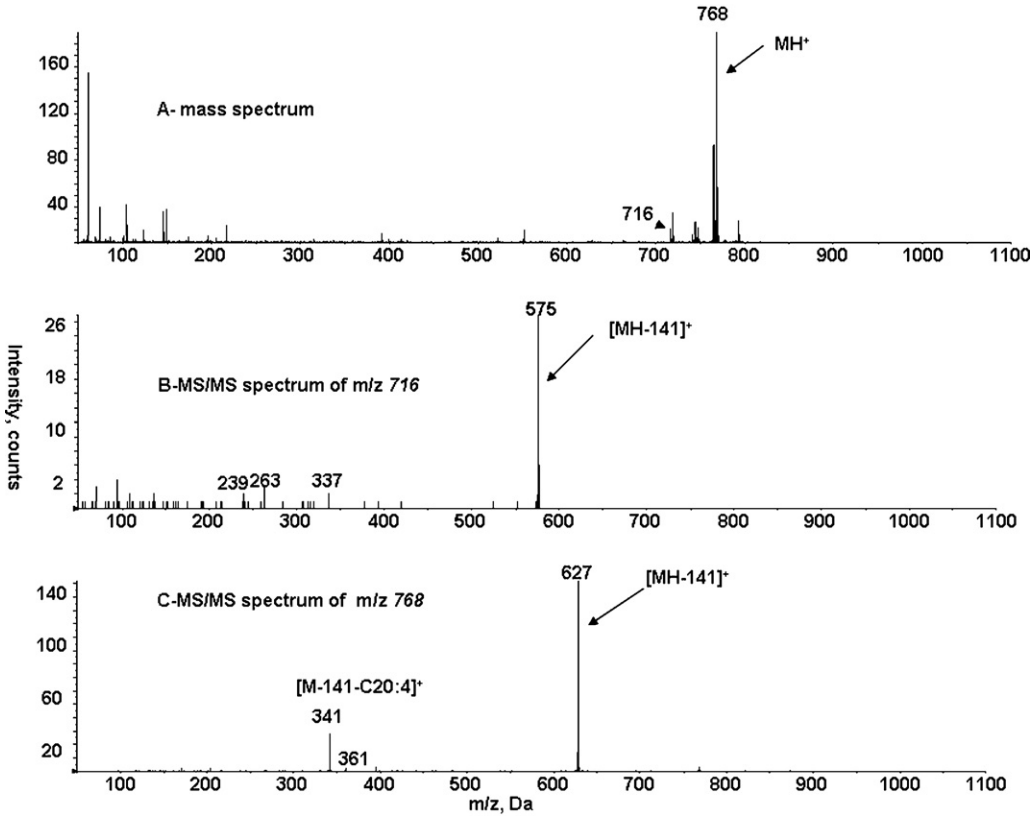

Fig. 4. Examples of full scan and tandem mass spectra of PE found in egg yolk extracts (A) full scan mass spectrum of PE, (B) MS/MS spectrum of *m/z* 716 and (C) MS/MS spectrum of *m/z* 768.

361 [M-141-C16:0+H]$^+$ which confirm this species as C16:0/C20:4. Fig. 4 shows the mass spectrum of PE and the MS/MS spectra of PE MH$^+$ ions at *m/z* 716 (C16:0/C18:2) and *m/z* 768 (C18:0/C20:4). Due to neutral loss of 141, the product ions at *m/z* 575 and *m/z* 627 respectively, were the base peaks in the MS/MS spectra. The ions at *m/z* 239 and *m/z* 263 in Fig. 4B are due to the fatty acyl moieties C16:0 and C18:2 whereas the fragment ion at *m/z* 337 is the complementary fragment to *m/z* 239, i.e. [M-C16:0-141+H]$^+$. In Fig. 4C, the more intense ion at *m/z* 341 (compared to *m/z* 361) was formed with loss of C20:4 (versus loss of C18:0) following the rule described above. Note that despite the signal/noise ratio in the MS/MS spectrum in Fig. 4B being considerably worse than that seen in Fig. 4C, due to the much lower intensity of the precursor ion (*m/z* 716, Fig. 4A), the product ions are still quite reproducible and adequate to confirm the identity of the fatty acid chains present.

PC is the dominant phospholipid class found in egg yolks and 16 PC species were found in this study. Not surprisingly, the main fragment ion for all PC MH$^+$ species was the ion at *m/z* 184, the polar phosphocholine head group. In contrast to PE, little other fragmentation was seen in the MS/MS spectra of PC MH$^+$ ions. In order to investigate this, a 10 μg/ml solution of a PC standard (egg source) was infused into the electrospray source. MS/MS spectra were recorded in multichannel analyzer mode (MCA) in order to accumulate spectra from each scan, to increase sensitivity for detecting low abundance ions. Fig. 5 shows the MS/MS spectrum of the PC MH$^+$ ion at *m/z* 806 (C16:0/C22:6, C18:1/C20:5 or C18:2/C20:4) with 36 scans accumulation. In addition to the dominant ion of phosphocholine at *m/z* 184, product ions at *m/z* 747, due to loss of –N(CH$_3$)$_3$, and at *m/z* 623, due to loss of 183 (phosphocholine-H), were observed. The inset in Fig. 5 is an enlargement of the region showing loss of fatty acyl groups. Four ions at *m/z* 478, 502, 504 and 550 were observed in this region. The ion at *m/z* 478 and *m/z* 550 are the ions [M-C22:6]$^+$ and [M-C16:0]$^+$ indicating the presence of C16:0 and C22:6 (i.e. DHA) in the PC precursor. Similarly, the product ions at *m/z* 502 [M-C20:4]$^+$ and *m/z* 504 [M-C20:5]$^+$ suggested the existence of PCs of composition C18:1/C20:4 and C18:2/C20:5. Therefore, the ion at *m/z* 806 was a mixture of C16:0/C22:6, C18:1/C20:5, and C18:2/C20:4. In order to confirm these results, product ion scan were performed in the negative ion mode. In negative ion mode, chloride adducts of PC were observed resulting in an ion at *m/z* 840 (i.e. [M+^{35}Cl]$^-$) corresponding to the [M+H]$^+$ ion at *m/z* 806 in positive ion mode. In the negative ion tandem mass spectrum of *m/z* 840 (Fig. 5(B)), the product ions at *m/z* 255, 279, 281, 301, 303, and 327 were observed, corresponding to carboxylate anions of C16:0, C18:2, C18: 1, C20:5, C20:4 and C22:6.

Overall, although the MS/MS spectra of PC MH$^+$ ions give mostly low intensity product ions, they are nevertheless diagnostic of fatty acyl substitution. Similar conclusions can be drawn for MS/MS spectra of LPC and SM which also give *m/z* 184 as the dominant fragment ion.

The base peak in the product ion spectra of all PI species was found to be the ion with neutral loss of inositol phosphate (molecular weight 260, see Table 2). The product ion spectra also show diglyceride (DG) and monoglyceride (MG) ions; the *m/z* difference between DG and MG ions is due to loss of a ketene [18] and so identifies one of the fatty acyl chains. For example (Table 2), the precursor [M+NH$_4$]$^+$ ion at *m/z* 904 fragmented to give 4 product ions at *m/z* 887, 627, 361 and 341. The product ion at *m/z* 887 is formed by loss of ammonia to give the protonated molecule [M+H]$^+$. The neutral loss of inositol phosphate from *m/z* 887 results in the DG ion at *m/z* 627. The MG ion at *m/z* 361 is in turn due to loss of C18:0 ketene from *m/z* 627 whereas the MG ion at *m/z* 341 is due to loss of C20:4 ketene. Thus, the fatty acid composition of the PI species is C18:0/C20:4.

All of the phospholipids classes discussed above, including PE, LPE, PC, LPC, SM and PI, contain some distribution of fatty acyl

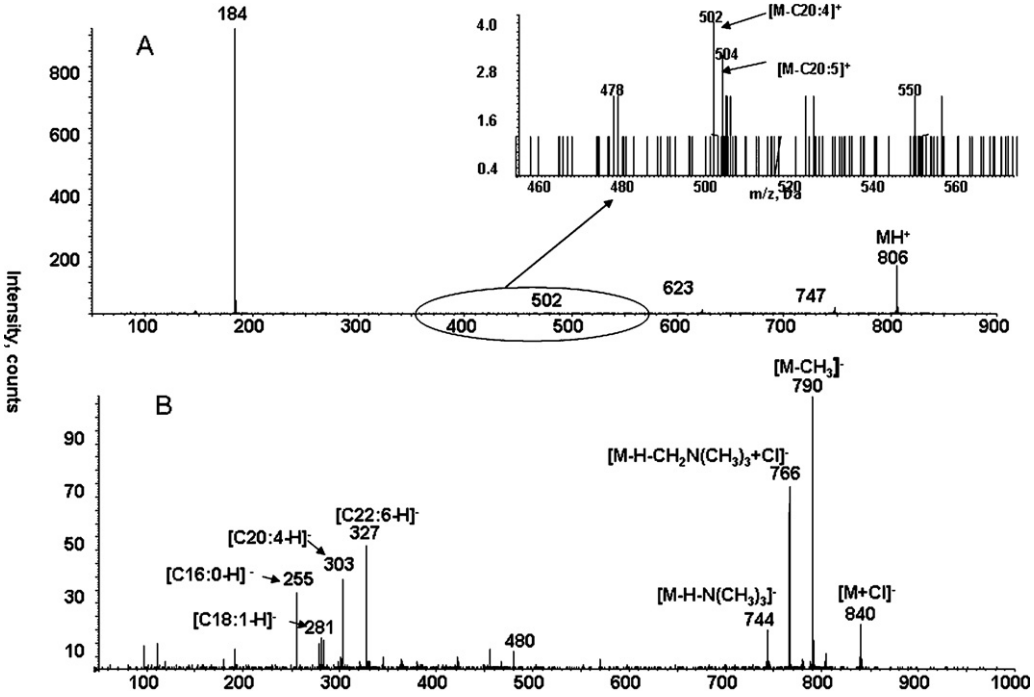

Fig. 5. Tandem mass spectrum of PC species at (A) positive mode *m*/*z* 806 and (B) negative mode *m*/*z* 840.

groups. Thus, multiple molecular species are present in the mass spectra of each of class, such as the 10 MH^+ ions seen for PE (Table 2). However, the existence of common structural features within each class results in characteristic fragmentation patterns seen in their MS/MS spectra. For example, the ion at *m*/*z* 184 is the base peak in product ion spectra for PC, LPC and SM and the fragment ion resulting from the neutral loss of 141 is the base peak of the product ion mass spectra for PE and LPE. Hence, in order to achieve maximum sensitivity, precursor scans of *m*/*z* 184 covering the expected molecular weight range considering likely fatty acid distributions will be used in this study for the quantification of PC, LPC and SM; similarly neutral loss scans of 141 over suitable *m*/*z* ranges will be used for PE and LPE. However, in the case of Cho (*m*/*z* 104) and Bet (*m*/*z* 118) there are unique molecular species since there are no fatty acyl groups. Also, their MS/MS spectra show major product ions at *m*/*z* 60 for Cho and *m*/*z* 58 for Bet. Since selected ion recording of low molecular weight species in electrospray would result poor selectivity and unacceptable background, multiple reaction monitoring (MRM) of the transitions *m*/*z* 104 → 60 and *m*/*z* 118 → 58 will be used for quantification of Cho and Bet respectively. A summary of the optimized parameters for quantification of the major choline and betaine containing compounds by HILIC LC–MS/MS are given in Table 1.

3.3. Quantification and method validation

Using the methods described above, separation of the choline containing compounds found in egg yolk can be achieved in a single HILIC LC–MS/MS run. Multiple scan modes, including precursor scans, neutral loss scans, and multiple reaction monitoring (MRM), were developed as described in Table 1. However, since the method does not separate all species within a class containing different combinations of fatty acyl groups, quantification of lipid classes rather than every individual species is the focus of this work.

In order to determine the amount of all choline compounds in a single HPLC run, we set up 5 periods in the quantification method. Since the abundance of PC is much higher than that of other components, a 5-fold decrease in sensitivity for PC determination was achieved by decreasing the collision energy from 45 eV to 22 eV during the PC precursor scan. The detection sensitivity for SM and LPC were increased by including them in a separate period in which the MS parameters were fully optimized and the scan times were enhanced compared to the period for PC determination.

Calibration curves were constructed using mixtures of standards in solutions at 10 concentrations levels. These ranged from 0.1 μg/ml to 50 μg/ml for PI, PE, PC, PCho and LPE; from 0.02 μg/ml to 10 μg/ml for SM, LPC, GPC, Cho, AcCho, and Bet; and contained internal standards PC-d_9 and Cho-d_9 at 0.2 μg/ml. In the analyses, PC-d_9 was used as the internal standard for PC, PI, PE, SM, LPE and LPC quantification, whereas Cho-d_9 was used as internal standard for GPC, PCho, Cho, AcCho and Bet. Calibration curves were made based on the peak area ratios of analytes to internal standards versus the analyte concentrations. The linear ranges for the 11 analytes, with correlation coefficients (r^2) > 0.993 were: 0.25–25 μg/ml for PI and PE, 0.5–50 μg/ml for PC, 0.05–5 μg/ml for SM and LPC, 0.5–25 μg/ml for LPE, 0.02–2.0 μg/ml for GPC, 0.02–2.0 μg/ml for AcCho, 0.8–50 μg/ml for PCho, 0.02–5 μg/ml for Cho, and 0.08–8 μg/ml for Bet. Subsequently, calibration curves constructed in this manner were used for all choline measurements.

For the 11 analytes defined in Table 1, limits of quantification for greater than 10:1 signal-to-noise ratio were found to lie in the range 0.02–0.25 μg/ml. For the purposes of nutritional measurements, this was considered satisfactory. To investigate the precision and accuracy of the method, triplicates of 3 QC standards (described under Materials and Methods) at low, medium and high concentrations were prepared and analyzed. The accuracy was defined as the percentage of the expected concentration that was actually measured. As shown in Table 3, the accuracy for measuring the 8 classes of compounds ranged between 83 and 105% with an average across compounds of 94%, 99% and 94% for QC low, medium and high respectively. The precision ranged from 1.6 to 13.2% relative standard deviation (RSD); the overall average RSD was 8% with an average RSD of 5% for the higher concentration test sample. These preliminary results indicate that the method, which measures up to 11 compounds or compound classes in a single run, may be

Table 3
Precision and accuracy of triplicate measurement for 8 polar lipid classes found in egg yolk. Accuracy refers to the ratio of the measured to the expected amount of each lipid in a standard mixture.

Compounds	QC low		QC Medium		QC high	
	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)
PI	13.2	90	8.5	100	8.2	97
PE	9.3	100	13.3	105	9.1	98
PC	7.9	93	11.8	90	2.9	93
LPE	6.7	97	5.8	103	1.6	88
SM	4.2	89	12.3	102	5.0	91
LPC	9.0	94	11.3	100	8.3	91
Cho	5.8	83	10.2	91	2.0	87
Bet	9.2	105	10.3	101	4.2	104

Table 4
Concentration of phospholipids and choline compounds determined for fresh wet egg yolk using the HILIC-LC–MS/MS method.

	PC	PE	SM	LPC	LPE	PI	Cho
Concentration (mg/100 g egg yolk)	5840 ± 250	1500 ± 100	190 ± 9	270 ± 6	90 ± 6	330 ± 23	4.4 ± 0.1
Percentage in lipid extract (%)	71.1	18.3	2.3	3.3	1.1	4.0	
Concentration of choline moiety (mg/100 g egg yolk)	791 ± 34		28 ± 1.5	56 ± 1			4.4 ± 0.1

All samples were analyzed in triplicates ($n = 3$).

sufficiently reproducible and accurate for the purpose of measuring choline levels in foods. A more in-depth method validation for the determination of choline levels in foods will be reported in a later communication. This will include with the use of a PC-d_3 recovery standard that we have recently synthesized.

3.4. Measurement of choline in egg yolk

As an example of the application of the method, we determined the levels of choline containing compounds in lipid extracts from fresh egg yolk. Fig. 3 shows the resulting LC–MS/MS chromatograms of the egg yolk extracts. In this experiment, all lipid classes were identified by comparison of retention times to authentic standards using the HILIC method and by the use of the class-specific scan modes described above. Compound assignments were also confirmed by accurate mass measurements and fragment ion (MS/MS) patterns and are given in Table 2.

In order to quantify the phospholipids and choline compounds in egg yolk, triplicate samples of egg yolk extracts were spiked with the internal standard PC-d_9 and Cho-d_9. Table 4 shows the measured concentration in fresh wet egg yolk using the LC–MS/MS method developed in this work. As expected, PC and PE were found to be the most abundant lipid classes in the extract, consisting of 71% and 18% of the total phospholipids in egg yolk, respectively, which is slightly lower than an earlier report of 76% and 21% for PC and PE [24,25]. The egg yolk extract also contained minor amounts of other compounds including SM, LPC and Cho (Table 4).

From the measured PC concentration in egg yolk we have estimated the choline contribution from PC to be 791 ± 34 mg choline moiety per 100 g yolk compared to the value of 634 mg choline recently published USDA database [3]. Differences between these two values may be due to a number of factors including yolk moisture content, extraction efficiency and egg type. Future work will focus on validation of the method for the measurement of choline in a variety of foods. This includes properly accounting for possible inaccuracies due to the recoveries of each lipid class, which has not been addressed in this report where the focus was on the development of the LC–MS/MS method.

4. Conclusions

A HILIC LC–MS/MS method using multiple scan modes has been developed to separate and quantify 11 lipid classes including all major choline-containing compounds. The method offers some advantages over other LC methods since HILIC chromatography is more readily compatible with electrospray ionization compared to normal phase methods and resulted in higher sensitivity and improved peak shapes.

Accurate mass measurements aids the identification of choline containing compounds, and product ions in MS/MS experiments gives information on the fatty acid composition of compounds within a phospholipid class. The LC–MS/MS method described in this work allow quantification of all choline compounds in a single LC run and accommodates both the high concentration of PC and low concentration of other lipid classes. A preliminary demonstration of accuracy and precision indicates that the method will be suitable for food analysis. Results obtained for fresh egg yolks indicate the presence of 8 polar lipid classes.

References

[1] S.H. Zeisel, Annu. Rev. Nutr. 26 (2006) 229.
[2] S.H. Zeisel, IUBMB Life 59 (2007) 380.
[3] J.C. Howe, J.R. Williams, J.M. Holden, http://www.nal.usda.gov/fnic/foodcomp/Data/Choline/Choline.pdf.
[4] D.C. Woollard, H.E. Indyk, G. Angyal, G. Bancan, M. Benoit, W. Chase, A. Clark, C. Dunne, C. Ellis, E. Evans, M. Gern, P. Gilliland, G. Gross, M. Jagodic, L. Jianhua, L. Kuo, B. Lacheron, T. Lam, P. Manzi, H. Matsuda, L. Oehrl, R. Pelletier, M. Petty, L. Pizzoferrato, C. Rayner, K. Reardon, A. Reid, K. Saito, P. Scheelings, W. Schuep, M.L. Silva, R. Simmie, P. Swartz, E. Tagliaferri, N. Tsalkani, P. Van Niekerk, B.J.C. Van der Vat, W. Verschoor, E. Walker, C. Wo, Y. Zhou, J. AOAC Int. 83 (2000) 131.
[5] N. Sotirhos, B. Hersloef, L. Kenne, J. Lipid Res. 27 (1986) 386.
[6] T. Glonek, J. Am. Oil Chem. Soc. 75 (1998) 569.
[7] E.A. Pomfret, K.A. daCosta, L.L. Schurman, S.H. Zeisel, Anal. Biochem. 180 (1989) 85.
[8] B.F. Dickens, C.S. Ramesha, G.A. Thompson, Anal. Biochem. 127 (1982) 37.
[9] E. Boselli, D. Pacetti, F. Curzi, N.G. Frega, Meat Sci. 78 (2008) 305.
[10] A. Sala Vila, A.I. Castellote-Bargallo, M. Rodriguez-Palmero-Seuma, M.C. Lopez-Sabater, J. Chromatogr. A 1008 (2003) 73.
[11] J. Ricny, S. Tucek, I. Vins, J. Neurosci. Methods 41 (1992) 11.
[12] D. Pacetti, E. Boselli, H.W. Hulan, N.G. Frega, J. Chromatogr. A 1097 (2005) 66.
[13] H. Ishimaru, Y. Ikarashi, Y. Maruyama, Biol. Mass Spectrom. 22 (1993) 681.
[14] M. Malavolta, F. Bocci, E. Boselli, N.G. Frega, J. Chromatogr. B 810 (2004) 173.
[15] H. Koc, M. Mar, A. Ranasinghe, J.A. Swenberg, S.H. Zeisel, Anal. Chem. 74 (2002) 4734.
[16] D.Y. Bang, E.j. Ahn, M.H. Moon, J. Chromatogr. B 852 (2007) 268.
[17] T. Houjou, K. Yamatani, M. Imagawa, T. Shimizu, R. Taguchi, Rapid Commun. Mass Spectrom. 19 (2005) 654.
[18] H. Nakanishi, Y. Iida, T. Shimizu, R. Taguchi, J. Biochem. 147 (2010) 245.

[19] Y.C. Ma, H.Y. Kim, Anal. Biochem. 226 (1995) 293.
[20] 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.
[21] E.S. Grumbach, D.M. Wagrowski-Diehl, J.R. Mazzeo, B. Alden, P.C. Iraneta, LCGC North America (2005) 89.
[22] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 37 (1959) 911.
[23] M. Schreiner, Int. J. Food Prop. 9 (2006) 573.
[24] A. Kuksis, Biochim. Biophys. Acta 1124 (1992) 205.
[25] M. Anton, in: R. Huopalahti, R. López-Fandiño, M. Anton, R. Schade (Eds.), Bioactive Egg Compounds, Springer-Verlag, Berlin, 2007, pp. 1.