methods

Charting molecular composition of phosphatidylcholines by fatty acid scanning and ion trap MS^3 fragmentation

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Abstract The molecular composition of phosphatidylcholines (PCs) in total lipid extracts was characterized by a combination of multiple precursor ion scanning on a hybrid quadrupole time-of-flight mass spectrometer and MS^3 fragmentation on an ion trap mass spectrometer. Precursor ion spectra for 50 acyl anion fragments of fatty acids (fatty acid scanning) acquired in parallel increased the specificity and the dynamic range of the detection of PCs and identified the fatty acid moieties in individual PC species. Subsequent analysis of detected PC peaks by MS^3 fragmentation on an ion trap mass spectrometer quantified the relative amount of their positional isomers, thus providing the most detailed and comprehensive characterization of the molecular composition of the pool of PCs at the low-picomole level. The method is vastly simplified, compared with conventional approaches, and does not require preliminary separation of lipid classes or of individual molecular species, enzymatic digestion, or chemical derivatization. The approach was validated by the comparative analysis of the molecular composition of PCs from human red blood cells. In the total lipid extract of Madin-Darby canine kidney II cells, we detected 46 PC species with unique fatty acid composition and demonstrated that the presence of positional isomers almost doubled the total number of individual molecular species.—Ekroos, K., C. S. Ejsing, U. Bahr, M. Karas, K. Simons, and A. Shevchenko. **Charting molecular composition of phosphatidylcholines by fatty acid scanning and ion trap MS^3 fragmentation.** ***J. Lipid Res.*** **2003.** 44: **2181–2192.**

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Phosphatidylcholines (PCs) are the most abundant glycerophospholipids in mammalian cells, accounting for more than 30% of the total lipid content (1). PCs comprise a glycerol phosphate backbone with a choline head group attached at the *sn*-3 position and two fatty acid moieties at positions *sn*-1 and *sn*-2. PCs are rather heterogeneous and have diverse physical properties, because they engage a variety of fatty acids having different numbers of carbon atoms and double bonds.

In mammalian cells, PCs are synthesized via the CDP-choline pathway or via the phosphatidylethanolamine methylation pathway (2–4). Further metabolism involves remodeling processes, which are controlled by the coordinated action of acyltransferases, transacylases, and lipases (5). Inhibiting the CDP-choline pathway is lethal (6, 7) and is not compensated by the alternative phosphatidylethanolamine methylation pathway, presumably because different PC species are produced (8, 9). The majority of de novo synthesized PCs are believed to comprise saturated fatty acids at the position *sn*-1 and unsaturated fatty acids at the position *sn*-2 (10, 11), although they might be altered by subsequent remodeling (12–14).

Certain cellular compartments and membrane microdomains are distinguished by a characteristic composition of PCs, in which very long chain, or saturated, or highly unsaturated fatty acid moieties prevail (15–19). To understand how the specific lipid composition of cellular compartments or microdomains might impinge on a variety of biological processes, it is important to characterize PCs as individual molecular species, i.e., to identify the fatty acid moieties and their exact location at the glycerol backbone.

Electrospray ionization-mass spectrometry is a sensitive and specific tool for the characterization of PCs in total

Abbreviations: FAS, fatty acid scanning; lysoPC, lyso-phosphatidylcholine; M, mass of a zwitterionic PC; PC, phosphatidylcholine; PIS, precursor ion scanning; PIS *m/z* 184.1, scanning for precursor ions that produce a fragment ion with *m/z* 184.1 upon collision-induced dissociation; QqTOF, quadrupole time-of-flight; $[R'CH_2COO]^-$, acyl anion of a fatty acid with hydrocarbon moiety R′; X/Y-PC-PC, PC molecule with X fatty acid moiety at *sn*-1 position of the glycerol backbone, and Y fatty acid moiety at *sn*-2 position; {X;Y}-PC, PC molecule (or a mixture of isomeric molecules) comprising fatty acids X and Y at unidentified position of the backbone.

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lipid extracts (20–23). Because of the positively charged quaternary amine of the choline head group, PCs are readily detected in positive ion mode with the femtomole sensitivity (23). Collision-induced dissociation of cations of PCs yields a characteristic fragment of the phosphorylcholine moiety having *m/z* 184.1, which enables their quantitative profiling in unprocessed lipid extracts by precursor ion scanning (23). Because the fragmentation of sphingomyelins yields the same fragment, PIS *m/z* 184.1 simultaneously detects both PCs and sphingomyelins. Monoprotonated molecular ions of PCs have even nominal masses, whereas ions of sphingomyelins have odd nominal masses and, if not detected in partly overlapping peak clusters, could easily be distinguished. Being very sensitive and specific, PIS *m/z* 184.1 does not determine the fatty acid composition of analyzed PCs directly. Although the total number of carbon atoms and double bonds in both fatty acid chains can be calculated from the intact masses of PC precursors, it is not possible to tell which fatty acids were comprised in isobaric PCs and to estimate the relative amount of their molecular forms.

Hybrid quadrupole time-of-flight (QqTOF) mass spectrometers can simultaneously acquire precursor ion scanning (PIS) spectra for a virtually unlimited number of fragment ions (24). The sensitivity of PIS on QqTOF mass spectrometers is inherently limited by the duty cycle (25), but was significantly improved by trapping fragment ions in the collision cell and pulse-releasing them into the TOF analyzer (26). This sensitivity became comparable with the sensitivity of triple quadrupole machines (24, 27), which currently are the most widely used instruments in mass spectrometric analysis of lipids (28).

Collision-induced dissociation of anions of glycerophospholipids yields abundant acyl anions of fatty acid moieties (20, 22, 29–32). Selecting masses of acyl anion fragments for multiple precursor ion scanning on a QqTOF instrument enables the profiling of the fatty acid composition of individual glycerophospholipids in lipid extracts, a method termed fatty acid scanning (FAS) (27).

However, FAS only covers glycerophospholipids that are detectable in negative ion mode with high sensitivity and therefore is not directly applicable to profiling PCs. FAS accurately determines the fatty acid composition of a particular lipid, and the position of fatty acid moieties (*sn*-1 or *sn*-2) could, in principle, be inferred from the ratio of intensities of peaks of acyl anions. However, the relative quantification becomes ambiguous if a fragmented precursor is a mixture of isobaric species, which might belong to the same class or to different classes of lipids.

In this paper, we present an analytical strategy for quantitative profiling of PCs by a combination of FAS on a QqTOF mass spectrometer and MS^3 fragmentation on an ion trap mass spectrometer. Millimolar concentrations of ammonium salts in the electrosprayed analyte enabled sensitive detection of PCs as negatively charged adducts, which can be directly analyzed by FAS. MS^3 fragmentation of adducts on an ion trap mass spectrometer allowed us to quantify the relative abundance of positional isomers. The analysis of lipid extracts from Madin-Darby canine kidney II (MDCK II) cells and human red blood cells characterized the pool of PCs as a complex mixture of isobaric and isomeric species.

MATERIALS AND METHODS

Materials and lipid standards

Synthetic PC standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Chloroform and methanol were liquid chromatography grade from Merck (Darmstadt, Germany). Ammonium acetate and ammonium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Cell media were from Gibco BRL (Rockville, MD). Fetal calf serum (FCS) was from PAA Laboratories GmbH, (Cölbe, Germany), and phospholipase A_2 (PLA_2) was from *Crotalus atrox* venom from Sigma. Nanoelectrospray capillaries (brand "short") were purchased from Proxeon Biosystems A/S (Odense, Denmark).

Preparation of MDCK II cells and human red blood cells

MDCK II cells were maintained in supplemented MEM (including 5% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) in 5% CO_2 at 37°C in a humidified incubator. Cells were grown on 10 cm plastic dishes to ~90% confluence. MDCK II cells were washed three times with PBS buffer before being scraped and pelleted in a 2 ml Eppendorf tube for 5 min at 14,000 *g* at room temperature. Cell pellets were snap frozen in liquid nitrogen and stored at −20°C.

Human red blood cells were pooled by centrifugation for 10 min at 200 *g* at 4°C and washed 5 times with cold 150 mM NaCl and 10 mM Tris-Cl, pH 7.5. Cells were aliquoted into 2 ml Eppendorf tubes, snap frozen, and stored at −80°C.

Lipid extraction was performed according to Folch, Lees, and Sloane-Stanley (33). Briefly, 500 μl methanol was added to a 75 μl cell suspension and vortexed for 10 min, followed by addition of 1 ml chloroform and vortexing for further 10 min. The sample was centrifuged for 5 min at 14,000 *g* at room temperature. The supernatant was transferred to a new tube, and 300 μl water was added, followed by stirring the mixture for 10 min. Samples were centrifuged at 500 *g* for 5 min at 20°C. The lower phase (organic) was transferred to a new tube and washed with chloroform-methanol-water (3:48:47; v/v/v). The phases were separated, and the organic phase was transferred to a new tube. The sample was dried in a vacuum concentrator and stored at −20°C.

Hydrolysis by PLA_2

Synthetic PC standards and their mixtures were hydrolysed by PLA_2 as described by Kates (34). Briefly, 500 nmol of dried PC standard was dissolved in 800 μl diethyl ether-methanol (99:1; v/v) and mixed with 450 μl of aqueous solution containing 40 mM calcium chloride, 20 mM Tris-HCl (pH 8.0) and 7 μg PLA_2. Mixtures were vigorously vortexed for 5 h and then dried in a vacuum concentrator. Dried samples were extracted and prepared for mass spectrometric analysis as described below.

Sample preparation for mass spectrometric analysis

The concentration of PCs in stock solutions was determined by the phosphorous assay described by Rouser, Fkeischer, and Yamamoto (35). Standards were prepared in different concentrations and molar ratios in chloroform-methanol (1:2; v/v) containing 5 mM ammonium acetate or ammonium chloride. Dried total lipid extracts from MDCK II cells and from red blood cells were redissolved in 50–100 μl chloroform-methanol (1:2; v/v). Prior to mass spectrometric analysis, sample aliquots were diluted 10-fold in chloroform-methanol (1:2; v/v) containing an ammonium salt at a final concentration of 5 mM.

QqTOF mass spectrometry

Precursor ion scanning was performed on a modified QSTAR Pulsar *i* QqTOF mass spectrometer (MDS Sciex, Concord, ON, Canada) equipped with a nanoelectrospray ion source (Proxeon Biosystems A/S, Odense, Denmark) as previously described (27). In both positive and negative ion modes, the analytical quadrupole Q1 was operated at unit resolution and 20 msec dwell time with a step size of 0.1 Th. Peak enhancement (trapping of fragment ions of the selected *m/z* in the collision cell) was applied according to the instructions of the manufacturer and was controlled via Analyst QS software.

The characteristic fragment ion of phosphorylcholine, *m/z* 184.1, was selected for detection of PCs and lyso-phosphatidylcholines (lysoPCs) in positive ion mode. For FAS of PC standards, precursor ion spectra of the two acyl anion fragments of the fatty acid moieties were acquired. For FAS analysis of lipid extracts, precursor ion spectra were simultaneously acquired for 30–50 acyl anion fragments of fatty acid moieties, containing 12 to 22 carbon atoms and 0 to 6 double bonds. Collision energy was set at 40 eV in positive and negative ion mode, unless specified otherwise. Fragment ions were selected within an *m/z* window of 0.15 Th. Peak intensities were maintained below 750 counts per scan to avoid saturation of the detector. FAS spectra were interpreted using a beta-version of LipidProfiler 1.0 software (MDX Sciex).

Ion trap mass spectrometry

MS^n fragmentation was performed on a quadrupole ion trap mass spectrometer LCQ (Finnigan ThermoQuest, San Jose, CA) equipped with a nanoelectrospray ion source (Proxeon Biosystems A/S). Approximately 3 μl of the analyte was loaded into laboratory-made, gold-coated glass capillaries and sprayed at −900 to −1,000 V. The temperature of the heated transfer-capillary was 180°C, the capillary voltage was −15 V, and the tube lens was −15 V. Spectra were averaged over 10–50 scans, each scan consisting of three microscans. For MS^2 and MS^3 fragmentation, precursor ions were selected using an isolation width of 5 Th and 2 Th, respectively. The relative collision energy was set between 16% and 22%.

RESULTS

Quantification of positional isomers of synthetic PC standards

To quantify positional isomers of endogenous PCs, we first established the isomeric purity of available synthetic standards. PLA_2 hydrolyses the *sn*-2 ester bond in PCs, yielding 2-lysoPCs. We tested PLA_2 specificity by hydrolyzing 15 mg of synthetic 16:0/18:1-PC standard and analyzing the reaction mixture by ^{1}H-NMR. We detected only the signal from hydrogen atoms of the secondary alcohol, not that from the primary alcohol, which indicated that the *sn*-1 ester bond was not hydrolyzed to any noticeable extent (data not shown). We therefore concluded that under the applied reaction conditions, the hydrolysis of the *sn*-2 ester bond by PLA_2 is specific.

Mass spectrometric analysis of PLA_2-treated PC standards on a QqTOF mass spectrometer by TOF MS and by PIS *m/z* 184.1 suggested that they contained a noticeable amount of positional isomers as presented in corresponding columns in **Table 1** (mol% determined by FAS and ion trap MS^3 fragmentation of the same standard PCs is also presented in Table 1 and discussed below).

TABLE 1. Isomeric purity of synthetic lipid standards

	Abundance of Isomeric Species, mol%[a,b]			
	PLA_2 Hydrolysis and QqTOF MS			
Lipid Standard	TOF MS	PIS *m/z* 184.1	FAS	Ion Trap MS^3[c]
16:0/18:1	88/12	87/13	88/12	83/17
18:1/16:0	83/17	82/18	81/19	79/21
16:0/18:0	88/12	85/15	88/12	83/17
18:0/16:0	94/6	95/5	93/7	93/7
18:0/18:1	96/4	96/4	95/5	89/11
18:1/18:0	81/19	81/19	84/16	75/25

FAS, fatty acid scanning; PC, phosphatidylcholine; PLA_2, phospholipase A_2; QqTOF, quadrupole time-of-flight.

[a] Mol% of the indicated lipid standard/mol% of the related isomer, e.g., 16:0/18:1-PC versus 18:1/16:0-PC.

[b] Coefficient of variation for QqTOF measurements was 1.6% and for ion trap measurements 3.6%.

[c] MS^3 fragmentation of chloride adducts in negative ion mode.

MS/MS fragmentation of anion adducts of PCs on a QqTOF mass spectrometer

Because phosphorylcholine moiety comprises a quaternary amino group, high sensitivity detection of PCs in negative ion mode is problematic. Spiking 5 mM ammonium acetate or chloride into an electrosprayed solution of PCs rendered anion adducts $[M + CH_3COO]^-$ and/or $[M + Cl]^-$ (21, 22, 36) (**Fig. 1**).

Upon collision-induced dissociation, anion adducts of PC ions lose both a methyl group from the choline moiety and the counter ion, yielding a fragment ion (designated as $[M - 15]^-$) with *m/z* 15 Th smaller than the mass of the zwitterionic form of the intact PC (Fig. 1). The $[M - 15]^-$ ions undergo further fragmentation via formation of low abundant intermediates by either neutral loss of free fatty acid $[M - 15 - R'CH_2COOH]^-$ or neutral loss of ketene $[M - 15 - R'CH{=}C{=}O]^-$, or by yielding acyl anions of fatty acids directly. A similar pattern of fragment ions was observed in MS/MS analysis of PCs (21) as well as of other glycerophospholipids on a triple quadrupole instrument (29–32). The acyl anion fragment of *sn*-2 fatty acid was more abundant than the fatty acid at *sn*-1 position.

We further examined the way in which the intensity of fragments depends on the applied collision energy (**Fig. 2**). At low collision energy (∼30 eV), MS/MS spectra were dominated by the intact precursor ion and the $[M - 15]^-$ fragment of the demethylated PC. With increasing collision energy, the intensity of both precursor and $[M - 15]^-$ ions decreased, with concomitantly increasing intensity of acyl anion fragments. The intensity of acyl anions peaked at ∼50 eV and then decreased, presumably because high collision energy compromised focusing and steering of the ion beam in the mass spectrometer. In the range of 40–60 eV, altering the collision energy had almost no impact on the yield of acyl anions and, consequently, no tuning of the collision energy was required for optimizing the sensitivity.

Profiling of PCs by FAS

Because acyl anions of fatty acid moieties are efficiently produced by collision-induced dissociation of anion adducts of PCs, they could be directly profiled by FAS, simi-

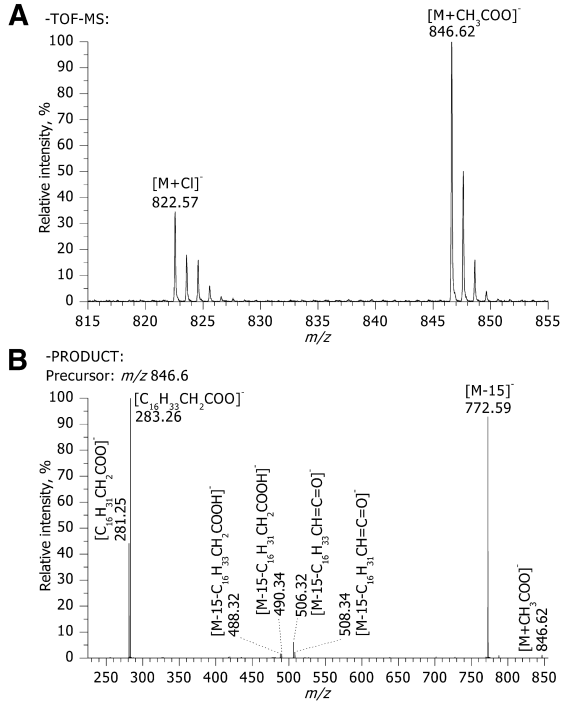

Fig. 1. A: TOF MS spectrum of synthetic 18:1/18:0-phosphatidylcholine (PC) standard in chloroform-methanol (1:2) containing 5 mM ammonium acetate (note that the standard contained ~19% of the isomeric 18:0/18:1-PC, Table 1). PC was detected as anions of acetate and chloride adducts at *m/z* 846.62 and *m/z* 822.57, respectively. B: MS/MS spectrum of the acetate adduct at *m/z* 846.62 acquired at collision energy 40 eV. Peaks at *m/z* 772.59, 283.26, and 281.25 correspond to the fragment ion of demethylated PC ($[M - 15]^-$) and acyl anions of stearic and oleic acid, respectively. Low abundant fragments were produced by the loss of fatty acids as ketenes $[M - 15 - C_{16}H_{31}CH{=}C{=}O]^-$ (*m/z* 508.34) and $[M - 15 - C_{16}H_{33}CH{=}C{=}O]^-$ (*m/z* 506.32) and by neutral loss of free fatty acids $[M - 15 - C_{16}H_{31}CH_2COOH]^-$ (*m/z* 490.34) and $[M - 15 - C_{16}H_{33}CH_2COOH]^-$ (*m/z* 488.32).

lar to other classes of glycerophospholipids (27). FAS analysis of 16:0/18:1-PC and 18:1/16:0-PC standards was performed by selecting *m/z* 255.2 and 281.2, corresponding to acyl anions of palmitic acid (16:0) and oleic acid (18:1), respectively, and precursor ions of acetate and chloride adducts were detected (**Fig. 3**). As expected, in the fragment patterns in MS/MS spectra of adducts (Fig. 1), the peak of the precursor ion was more abundant in FAS for the acyl anion fragment of *sn*-2 fatty acid than of *sn*-1 fatty acid. The ratio of their intensities did not depend on the mode of analysis and pinpointed the major component in the mixture of positional isomers. The plot of areas of precursor peaks in different precursor scans was linear in the 0.4–7 μM concentration range and was not affected when PC standards were spiked into a lipid extract (data not shown).

PLA_2-treated PC standards were subjected to FAS, which demonstrated that lysoPCs were also detectable as acetate and chloride adduct ions (data not shown). Further analysis of precursor ion peak areas demonstrated that FAS produced a quantitatively consistent estimation of the mol% of related positional isomers (Table 1).

MS^n fragmentation of PC adducts on an ion trap mass spectrometer

Regardless of the applied collision energy, fragmentation of anion adducts of PCs on the QqTOF mass spectrometer did not produce ions that could directly characterize the relative abundance of isomeric PC species (Fig. 1). In contrast with tandem mass spectrometers equipped with a linear collision cell (e.g., triple quadrupole or QqTOF instruments), ion trap mass spectrometers fragment a precursor ion by applying an *m/z*-dependent resonance excitation voltage, which leaves product ions unaffected. If necessary, any product ion can be further trapped and fragmented in another cycle of the tandem mass spectrometric experiment (MS^n experiments) [as reviewed in ref. (37)].

We tested whether fragmenting anion adducts of PCs in an ion trap mass spectrometer would enable direct quantification of positional isomers. MS^2 fragmentation of the chloride adduct of 16:0/18:1-PC standard produced abundant ions of a demethylated fragment of PC $[M - 15]^-$ at *m/z* 744 (**Fig. 4**). Subsequently, MS^3 fragmentation of $[M - 15]^-$ resulted in neutral loss of the fatty acids as ketene $[M - 15 - R'CH{=}C{=}O]^-$, rendering product ions of demethylated lysoPCs at *m/z* 480 and 506, with the peak of the former product as the most abundant. Acyl anion fragments $[R'CH_2COO]^-$ were observed at *m/z* 255 and 281, although they were less abundant than in QqTOF spectra. Minor fragments formed by neutral loss of free fatty acid $[M - 15 - R'CH_2COOH]^-$ from $[M - 15]^-$ were detected at *m/z* 462 and 488. As expected, MS^4 fragmentation of the demethylated lysoPCs produced the acyl anion fragment and corresponding products of ketene and free fatty acid losses at *m/z* 242 and 224, respectively (data not shown). Ion trap MS^n analysis of other PC standards followed the same fragmentation pathways (data not shown).

MS^3 fragmentation of the isomeric standard 18:1/16:0-PC produced a spectrum in which the intensities of fragment ions were reversed, compared with the spectrum of 16:0/18:1-PC standard (Fig. 4), suggesting that fragmentation pathways in ion trap and QqTOF mass spectrometers are similar (**Fig. 5**) and implying that release of fatty acids either as acyl anions or as neutral ketenes is a position-dependent process.

Quantitative analysis of positional isomers by MS^3 fragmentation

MS^3 fragmentation of PC adducts yielded abundant demethylated lysoPC fragment ions, matching the neutral loss of *sn*-2 fatty acid as ketene, that could be employed in direct quantification of isomeric and isobaric species. We therefore evaluated whether the relative intensity of these ions correlated with the estimates of mol% of isomeric species determined by PLA_2 hydrolysis of synthetic standards. The ratio of intensities of demethylated lysoPC fragment ions rendered via neutral loss of fatty acids as ketenes (i.e., $[M - 15 - R'CH{=}C{=}O]^-$) correlated with the estimates of mol% determined by the other three independent methods of detection (Table 1). To further validate the method, we mixed pairs of isomeric standards in various molar ratios, digested aliquots of the mixtures by

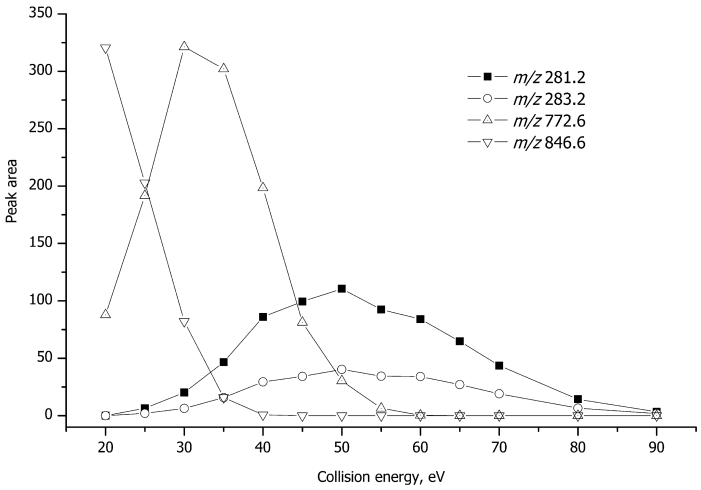

Fig. 2. Fragment ion peak intensities from the acetate adduct of 18:1/18:0-PC are dependent upon collision energy. *m/z* 846.6 ($[M + CH_3COO]^-$); *m/z* 772.6 ($[M - 15]^-$); *m/z* 283.2 and *m/z* 281.2, acyl anions of stearic and oleic acids, respectively.

PLA_2, and determined the content of positional isomers on the QqTOF mass spectrometer. Aliquots of the mixtures of standards were analyzed directly by MS^3 fragmentation, and the results were compared. Linear regression demonstrated a statistically confident correlation between the obtained mol% estimates (**Fig. 6**). The same experiment was performed using another pair of isomeric standards, 16:0/18:0-PC and 18:0/16:0-PC, and produced similar results (data not shown). Taken together, the data indicated that neutral loss of fatty acid as ketene predominantly occurs at the *sn*-2 position.

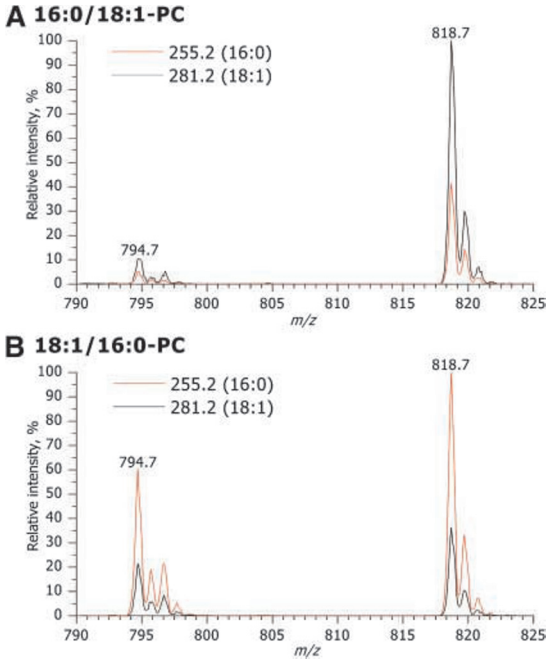

Fig. 3. A: Fatty acid scanning (FAS) spectra of synthetic 16:0/18:1-PC standard. Acyl anions of palmitic and oleic acids (*m/z* 255.2 and *m/z* 281.2) were selected as fragment ions identifying precursor ions of acetate (*m/z* 818.7) and chloride (*m/z* 794.7) adducts. B: FAS spectra of synthetic 18:1/16:0-PC standard, acquired in the same way as the spectrum in panel A. Note reversed intensities of peaks of the precursor, compared with panel A. Isomeric purity of the standards is presented in Table 1.

The intensity of other fragments or a combination of intensities of fragments observed in MS^3 spectra did not correlate well with the mol% of the isomers, most likely because the yield of fragments from other fragmentation pathways is less position-specific than that of ketene loss. Further supporting this notion, we found that lyso-phosphatidic acid fragments formed by loss of ketene from the *sn*-2 position enabled accurate estimates of mol% in mixtures of 16:0/18:1 and 18:1/16:0 phosphatidic acid, although they were much less abundant than fragment ions produced by neutral loss of free fatty acid (C. Ejsing, K. Ekroos, and A. Shevchenko, unpublished observations).

Validation of the mass spectrometric approach

The lipid composition and major molecular species of glycerophospholipids of human red blood cells were previously rigorously characterized (38) by a sophisticated analytical routine that involved several TLC separations, hydrolysis of lipids by phospholipase C, and derivatization of diradylglycerols by benzoic anhydride followed by quantification of diradylglycerolbenzoates by reversed-phase HPLC (39). Diradylglycerolbenzoates were separated into 20 chromatographic peaks (some of which contained a few coeluted species); however, separation of positional isomers was not achieved (39).

To further validate our mass spectrometric approach, we determined the molecular composition of PCs from human red blood cells (**Table 2**) and compared it with the results previously reported by Connor et al. (38). Connor et al. identified 26 molecular species of PCs by HPLC, and 22 species were identified in the present work by a combination of PIS *m/z* 184.1 and FAS. We note that we did not acquire precursor ion spectra for acyl anions of a few minor fatty acids (e.g., 22:6), although Connor et al. found corresponding lipids. Within the datasets obtained independently by Connor et al. and by us (Table 2), 14 molecular species overlapped. All nonoverlapping species were of low abundance, and their relative content was <3 mol%. All eight major PC species with relative content >3 mol% that were reported by Connor et al. were also detected by mass spectrometry, with similar rel-

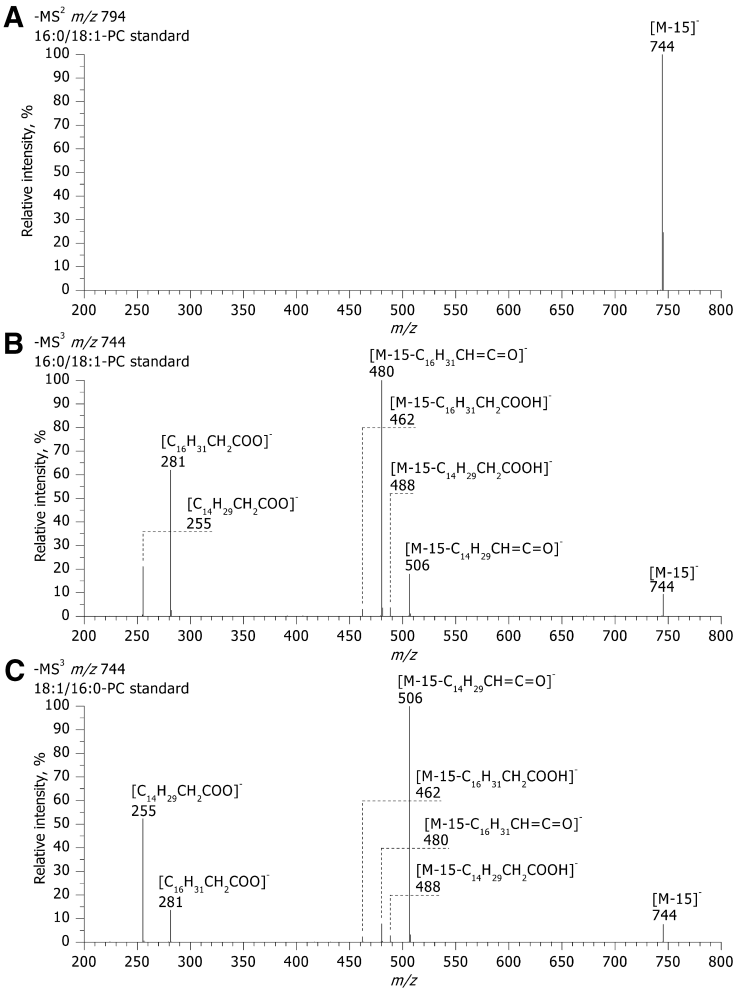

Fig. 4. A: MS^2 spectrum of a chloride adduct of the synthetic 16:0/18:1-PC standard. Abundant fragment at *m/z* 744 corresponds to demethylated PC $[M - 15]^-$. B: MS^3 spectrum of *m/z* 744 ($[M - 15]^-$). The observed fragments are the products of ketene loss {$[M - 15 - C_{14}H_{29}CH{=}C{=}O]^-$ (*m/z* 506) and $[M - 15 - C_{16}H_{31}CH{=}C{=}O]^-$ (*m/z* 480)}, corresponding to ions of demethylated 18:1-lyso-phosphatidylcholine (lysoPC) and 16:0-lysoPC, respectively; acyl anions of oleic (*m/z* 281) and palmatic (*m/z* 255) acids; loss of free fatty acids $[M - 15 - C_{14}H_{29}CH_2COOH]^-$ (*m/z* 488) and $[M - 15 - C_{16}H_{31}CH_2COOH]^-$ (*m/z* 462). C: MS^3 spectrum of $[M - 15]^-$ ion (*m/z* 744) obtained by MS^2 fragmentation of a chloride adduct of synthetic 18:1/16:0-PC standard. The spectrum is similar to that in panel B, but the intensities of fragments at *m/z* 506 and 480, as well as at *m/z* 281 and 255, are reversed.

ative abundance. Furthermore, ion trap MS^3 analysis of major PC species suggested that positional isomers (which remained undetected by Connor et al.) are common in human red blood cells (Table 2). For example, 20:4/16:0-PC, having a highly unsaturated arachidonic acid at the *sn*-1 position, constitutes about 13 mol% of the total 36:4-PC.

Connor et al. detected one PC species comprising a fatty acid with an odd number of carbon atoms, {17:0/18:1}-PC. Mass spectrometry confirmed that 17:0 and 17:1 are major fatty acids with an odd number of carbons that are present in PCs from red blood cells, and we also detected them in another three molecular species (Table 2).

We therefore concluded that qualitative and quantitative concordance of the molecular composition of PCs determined by us and independently by Connor et al. validates the mass spectrometry-based approach.

Molecular composition of PCs from MDCK II cells

The MDCK II cell line is an established model for studying the biogenesis of epithelial surface polarity and the mechanisms of polarized protein and lipid sorting. Here we applied a combination of FAS and MS^3 fragmentation to characterize the molecular composition of PCs in a total lipid extract.

Lipid extracts of MDCK II cells were first analyzed in positive ion mode by PIS *m/z* 184.1 to detect PCs. The total number of carbon atoms and double bonds in fatty acid moieties was calculated from intact masses of detected precursors (**Table 3**). The same sample was further analyzed by FAS in negative ion mode. Fifty precursor ion scan spectra of acyl anions of fatty acids were matched to the spectrum of PIS *m/z* 184.1 by LipidProfiler 1.0 software, which allowed us to determine the fatty acid composition of all detected PC species (Table 3).

Complementing PIS *m/z* 184.1 by FAS allowed us to resolve ambiguous precursor ion assignments. For example, a minor peak at *m/z* 748.5 was detected by PIS *m/z* 184.1. It was not obvious whether the peak belonged to 33:0-PC, because it overlapped with the second isotopic peak of abundant 33:1-PC at *m/z* 746.5 (**Fig. 7**). Nevertheless, FAS identified 33:0-PC species as {16:0;17:0}-PC and {15:0;18:0}-PC. In total lipid extract of MDCK II cells, we identified 15 PC species (not including positional isomers) comprising fatty acid moieties with an odd number of carbon atoms, although this was rather unexpected, considering the known pathways of de novo lipid biosynthesis in mammalian cells.

We further performed ion trap MS^3 analysis on the most abundant PCs (Table 2). For example, FAS indicated that 32:1-PC (*m/z* 766.8) consists of {16:0;16:1}-PC, {15:0;17:1}-

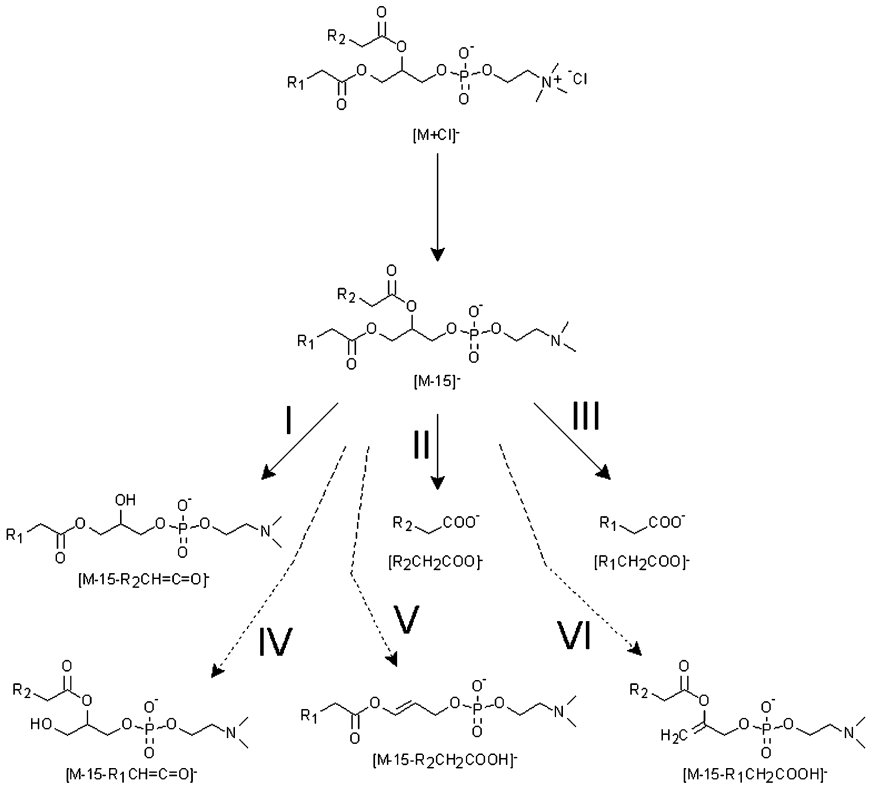

Fig. 5. Fragmentation pathways of the anion adduct of PC under low-energy collision-induced dissociation. In the first step of collision-induced dissociation of the PC adduct anion (e.g., $[M + Cl]^-$), the demethylated fragment ion $[M - 15]^-$ is produced. Fragmentation of the $[M - 15]^-$ ion proceeds along three major pathways: (I), loss of *sn*-2 fatty acid as a ketene yields a demethylated 2-lysoPC; (II) and (III), formation of acyl anions of *sn*-2 and *sn*-1 fatty acids; and three minor pathways: (IV), loss of *sn*-1 fatty acid as a ketene; (V) and (VI), loss of neutral fatty acid from *sn*-2 and *sn*-1 positions.

PC, and {14:0;18:1}-PC. MS^3 analysis confirmed the fatty acid assignment and enabled us to determine that 16:0/16:1-PC and 16:1/16:0-PC constitute 33 and 55 mol% of the total 32:1-PC, respectively, with other species accounting for 3 to 6 mol% (Table 3). We reasoned that the presence of a sizable proportion of positional isomers in PCs is likely to represent a common phenomenon rather than a specific feature of a particular MDCK II cell line.

We note here that *m/z* of PC species with fatty acids having an odd number of carbon atoms might coincide with

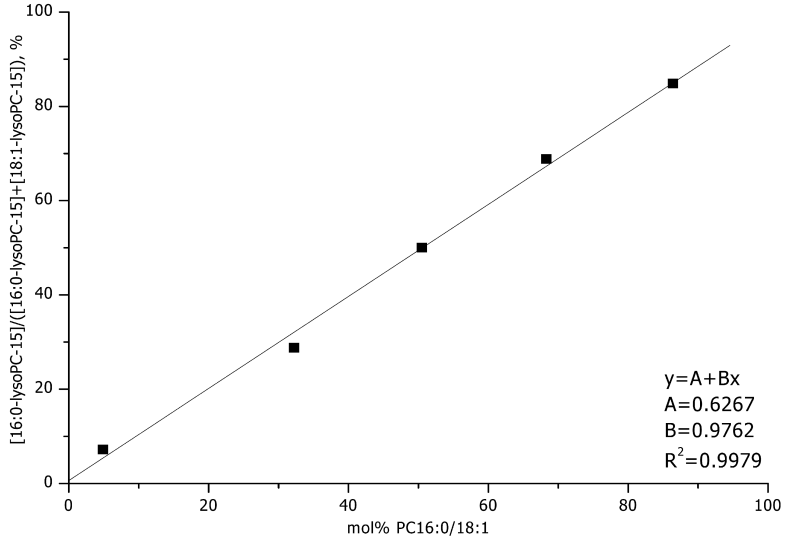

Fig. 6. Quantification of mixtures of 18:1/16:0-PC and 16:0/18:1-PC standards by ion trap MS^3 fragmentation. X-axis: mol% of 18:1/16:0-PC in the mixture determined by phospholipase A_2 hydrolysis and PIS *m/z* 184.1; Y-axis: the peak intensity of demethylated 16:0-lysoPC divided by the sum of peak intensities of demethylated 18:1-lysoPC and 16:0-lysoPC in acquired MS^3 spectra.

TABLE 2. The molecular composition of PCs from human red blood cells

PIS *m/z* 184.1			FAS		Ion Trap MS^3		
+Precursor Ion	Relative Content	Brutto Composition[a]	−Precursor Ion[b]	Fatty Acid Moieties	−Precursor Ion[c]	Molecular Species	
m/z	%		*m/z*		*m/z*		*mol %*
706.6	1	30:0	764.8	14:0;16:0			
732.6	2	32:1	790.6	16:0;16:1			
734.6	5	32:0	792.6	16:0/16:0			
746.6	1	33:1	804.8	16:0;17:1			
748.6	<1	33:0	806.8	16:0;17:0			
756.6	<1	34:3	814.8	16:1;18:2			
758.6	20	34:2	816.8	16:0;18:2	792	16:0/18:2	94
						18:2/16:0	6
760.6	22	34:1	818.8	16:0;18:1	794	16:0/18:1	85
						18:1/16:0	15
762.6	3	34:0	820.8	16:0;18:0			
772.6	1	35:2	830.8	17:0;18:2			
774.6	<1	35:1	832.8	17:0;18:1			
782.6	7	36:4	840.8	16:0;20:4	816	16:0/20:4	87
						20:4/16:0	13
784.6	4	36:3	842.8	18:1;18:2	818	18:1/18:2	42
				16:0;20:3		18:2/18:1	16
				18:0;18:3		16:0/20:3	31
						20:3/16:0	6
						18:3/18:0	5
786.6	8	36:2	844.8	18:0;18:2	820	18:0/18:2	71
				18:1/18:1		18:2/18:0	7
						18:1/18:1	23
788.6	6	36:1	846.8	18:0;18:1			
				16:0;20:1			
790.6	<1	36:0	848.8	18:0/18:0			
808.6	1	38:5	866.8	18:1;20:4			
810.6	2	38:4	868.8	18:0;20:4			

[a] Total number of carbon atoms:total number of double bonds.
[b] Detected as acetate adduct.
[c] Detected as chloride adduct.

m/z of ether PCs, in which an alkyl chain is linked to the glycerol backbone via an ether rather than an ester bond. However, in the MS/MS spectrum of synthetic 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycerol-3-PC standard, no corresponding alkoxide ion at *m/z* 241.25 was observed (data not shown). Therefore, alkoxide anions of ether lipids will not be detectable by FAS and will not compromise the confidence of peak assignment in FAS spectra. PC peaks in which only a single acyl anion was identified, and in which the complementary fatty acid with an odd number of carbon atoms was not detected, will indicate the presence of ether species. Taken together, PIS *m/z* 184.1, FAS, and ion trap MS^3 fragmentation revealed high complexity of the molecular composition of PCs represented by a variety of isomeric and isobaric species.

DISCUSSION

PCs can be detected at the low μM–nM sensitivity as anion acetate or chloride adducts, which produce structure-specific ions upon tandem mass spectrometric fragmentation. Regardless of the type of instrument, collision-induced dissociation of an anionic adduct first generates abundant $[M - 15]^-$ fragments whose subsequent fragmentation pathways are common for negatively charged glycerophospholipids (29–32, 36) (Fig. 5).

The different fragment patterns observed in QqTOF MS/MS and ion trap MS^2, MS^3, and MS^4 originate in mechanisms by which the collision energy is transmitted to selected precursor ions and affects the yield of unstable intermediates. It is therefore not surprising that the fragmentation of PC adducts in a QqTOF instrument at high collision energy predominantly yielded acyl anion fragments of fatty acids, whereas abundant peaks of demethylated lysoPCs were observed in MS^3 ion trap spectra.

The inspection of fragmentation pathways by ion trap MS^3 revealed that they have different positional specificity (Fig. 5). Neutral loss of fatty acid as ketene accounts for ~58% of the total intensity of fragment ions and occurs almost exclusively via the cleavage of *sn*-2 fatty acid (~99% of ketene fragment ions), thus generating demethylated 2-lysoPC. Its yield does not depend on the fatty acid moieties, as is evident from the accurate estimation of mol% of isomeric species comprising different combinations of fatty acids (Table 1 and Fig. 6). Acyl anion fragments are abundant in MS^3 spectra (~39% of the total fragment ion intensity) and are mostly (although not exclusively!) produced from the *sn*-2 fatty acid (80% of intensity of acyl anion fragments). Neutral loss of free fatty acid appears to be a relatively minor process that accounts for about 2% of the total fragment ion intensity.

Relative (in mol%) quantification of isobaric and isomeric PC species by ion trap MS^3 fragmentation is direct

TABLE 3. The molecular composition of PCs from MDCK II cells

PIS *m/z* 184.1			FAS		Ion trap MS^3		
+Precursor Ion	Relative Content	Brutto Composition[a]	−Precursor Ion[b]	Fatty Acid Moieties	−Precursor Ion[c]	Molecular Species	
m/z	%		*m/z*		*m/z*		*mol %*
692.6	<1	29:0	750.8	14:0;15:0			
704.6	2	30:1	762.8	14:0;16:1			
706.6	1	30:0	764.8	14:0;16:0			
718.6	1	31:1	776.8	14:0;17:1			
				15:0;16:1			
720.6	<1	31:0	778.8	14:0;17:0			
730.6	1	32:2	788.8	16:1/16:1			
732.6	6	32:1	790.8	14:0;18:1	766	14:0/18:1	3
				15:0;17:1		18:1/14:0	6
				16:0;16:1		15:0/17:1	3
						16:0/16:1	55
						16:1/16:0	33
734.6	2	32:0	792.8	16:0;16:0			
744.6	2	33:2	802.8	16:1;17:1			
746.6	8	33:1	804.8	15:0;18:1			
				16:0;17:1			
				17:0;16:1			
748.8	1	33:0	806.8	15:0;18:0			
				16:0;17:0			
756.6	<1	34:3	814.8	16:1;18:2			
758.6	7	34:2	816.8	16:0;18:2			
				16:1;18:1			
760.6	23	34:1	818.8	16:0;18:1	794	16:0/18:1	89
				17:0;17:1		18:1/16:0	10
						17:1/17:0	1
772.6	5	35:2	830.8	15:0;20:2	806	15:0/20:2	6
				16:1;19:1		16:1/19:1	2
				17:1;18:1		19:1/16:1	4
						17:1/18:1	70
						18:1/17:1	18
774.6	2	35:1	832.8	16:0;19:1			
				17:0;18:1			
				18:0;17:1			
782.6	<1	36:4	840.8	16:0;20:4			
784.6	3	36:3	842.8	16:1;20:2			
786.6	17	36:2	844.8	16:0;20:2			
				18:0;18:2			
				16:1;20:1			
				18:1/18:1			
788.6	9	36:1	846.8	18:0;18:1			
				16:0;20:1			
800.6	2.7	37:2	858.8	17:0;20:2			
				18:1;19:1			
				17:1;20:1			
802.6	<1	37:1	860.8	17:0;20:1			
812.6	2	38:3	870.8	18:1;20:2			
814.6	3	38:2	872.8	18:0;20:2			
				18:1;20:1			
816.6	<1	38:1	874.8	18:0;20:1			

[a] Total number of carbon atoms:total number of double bonds in fatty acid moieties.
[b] Detected as acetate adduct.
[c] Detected as chloride adduct.

and does not require internal standards. The determination is very specific because of the accurate selection of precursor masses at MS^2 and MS^3 stages and therefore is reliable even if applied to total lipid extracts. Furthermore, matching pairs of demethylated 2-lysoPCs and acyl anion fragments of *sn*-2 fatty acid additionally verify the peak assignment. At the same time, MS^3 fragmentation is poorly suited for the detection of PC precursors in lipid mixtures, as well as for the quantification of PC species with different molecular masses. Because no efficient precursor ion scanning is possible on ion trap mass spectrometers, complementary analysis by QqTOF mass spectrometry and by ion trap mass spectrometry should be used in concert.

We applied FAS on a QqTOF mass spectrometer and MS^3 fragmentation on the ion trap mass spectrometer to characterize the molecular composition of PCs. FAS complemented the previously established method of PIS *m/z* 184.1 and not only increased the specificity and the dynamic range of detection but also allowed us to identify the fatty acid moieties and their relative localization on the glycerol backbone of the individual PC molecules. MS^3 fragmentation of precursor ions detected by FAS in-

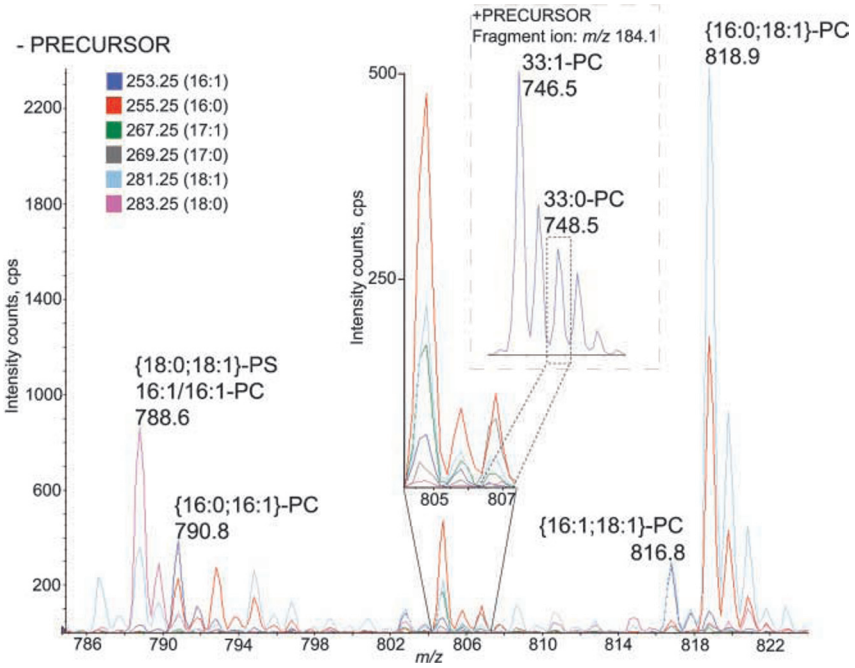

Fig. 7. In PIS *m/z* 184.1, the peak of 33:0-PC (*m/z* 748.5) overlaps with the second isotope peak of abundant 33:1-PC (*m/z* 746.5) (inset). FAS (zoomed) suggested that 33:1-PC is a mixture of {15:0;18:1}-, {16:0;17:1}-, and {17:0;16:1}-PCs (only representative precursor scans are presented in the figure). At the same time, FAS identified 33:0-PC as {16:0;17:0}- and {15:0;18:0}-PCs. Fifty precursor ion scan spectra were simultaneously acquired in the FAS experiment.

dependently enabled quantitative estimation of the relative amounts of their positional isomers. Because a combination of mass spectrometric methods could account for individual molecular species of PCs, we assumed that this is currently the most detailed and comprehensive approach for their characterization.

A combination of FAS and MS^3 was applied to profile PCs in a lipid extract from MDCK II cells. The conventional analysis by PIS *m/z* 184.1 detected 25 peaks of isobaric PCs (Table 3). Further analysis of the same sample by FAS revealed that they represent 46 species with unique fatty acid composition. MS^3 fragmentation of the most abundant peaks suggested that they are mixtures of positional isomers, and therefore the total number of molecular species is close to 100. We note that although we detected no sizeable amount of ether PCs, we could not exclude that they might be present in the extract (40) but were masked by more abundant species.

Although PCs of MDCK II cells and human red blood cells have been extensively characterized, no indication of the possible presence of positional isomers has been reported (41). Biochemical studies have shown that fatty acid remodeling is a common phenomenon in eukaryotic organisms (5), which potentially accounts for the observed presence of isomeric lipid species. Biophysical studies have demonstrated that positional isomers display different types of phase behavior and have differences in phase transition temperatures (42, 43). Importantly, positional isomers differ in their ability to interact with sterols, thus affecting the dynamics of membrane microdomains. With the analytical tools now available, the biological role of the observed complexity can be evaluated in molecular detail.

Charting the molecular composition of PCs (and, conceivably, of other glycerophospolipids) by mass spectrometry has several advantages over conventional methods. A combination of head group scanning (PIS *m/z* 184.1), FAS, and ion trap MS^3 fragmentation provided the most detailed and comprehensive characterization of the molecular composition, including the relative quantification of individual molecular species. The sensitivity of detection was at the low picomole–femtomole level. The sample preparation routine was vastly simplified and did not require preliminary separation of lipid classes and of individual molecular species, enzymatic digestion, or chemical derivatization. In FAS, *m/z* of acyl anions can be selected with high accuracy, thus increasing the dynamic range and the specificity of detection of corresponding lipid precursors. Therefore, glycerophospholipids comprising fatty acid moieties of possible medical diagnostic interest [such as very long chain fatty acids (16), fatty acids having an odd number of carbon atoms (44), etc.] can be reliably detected and quantified in total extracts in the presence of overwhelming amounts of lipids with more-prevalent fatty acid moieties. It is therefore conceivable that upon further development, this technology will pave the way for high throughput "shotgun lipidomics," the approach in which molecular species of multiple lipid classes will be identified and quantified by computer processing of a very large number of simultaneously acquired precursor ion scans for *m/z* of characteristic fragments, in-

cluding (but not limited to) the fragments of head groups and acyl anions of a variety of fatty acids.

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