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Quantitative Profiling of Phospholipids by Multiple Precursor Ion Scanning on a Hybrid Quadrupole Time-of-Flight Mass Spectrometer

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A hybrid quadrupole time-of-flight mass spectrometer featured with ion trapping capabilities was employed for quantitative profiling of total extracts of endogenous phospholipids. Simultaneous acquisition of precursor ion spectra of multiple fragment ions allowed detection of major classes of phospholipids in a single experiment. Relative changes in their concentration were monitored using a mixture of isotopically labeled endogenous lipids as a comprehensive internal standard. Precursor ion scanning spectra were acquired simultaneously for acyl anions of major fatty acids in negative ion mode and identified the fatty acid moieties and their relative position at the glycerol backbone in individual lipid species. Taken together, a combination of multiple precursor ion scans allowed quantitative monitoring of major perturbation in phospholipid composition and elucidating of molecular heterogeneity of individual lipid species.

Glycerophospholipids, the main constituents of cellular membranes, consist of a polar headgroup (HG) with a phosphate moiety and two fatty acids (FA) that are attached to the glycerol backbone. Dozens of structural variants are therefore possible within each class of lipids since the headgroup can be combined with a large pool of fatty acids that vary in both chain length and degree of saturation. This yields a multitude of similarly built molecules having a large diversity of physical properties, which allows the living cell to regulate the intrinsic heterogeneity of membranes in a dynamic fashion.

The microheterogeneity of cellular membranes plays an important physiological role. Many biological phenomena are accompanied by continuous changes in qualitative, quantitative, and spatial distribution of individual lipids within membrane microdomains—membrane rafts. Rafts are composed from lipids with saturated fatty acids, are enriched in cholesterol and sphingolipids,[1] and are known to mediate pathogen entry,[2] transmembrane signal transduction,[1,3] and sorting and trafficking through secretory and endocytotic pathways.[4−6] Rafts have also been implicated in prion diseases, Alzheimer's disease, and cancer.[7] To gain insight into these processes, the microcomposition of lipid domains needs to be monitored via comprehensive and quantitative lipid assays and subsequently correlated with dynamic behavior of the relevant protein machines.

Quantitative characterization of individual lipids as well as of lipid classes is conventionally performed by a combination of various analytical technologies,[8] including high-performance liquid chromatography, thin-layer chromatography, gas chromatography, and mass spectrometry.[9,10] Such strategy is time-consuming, requires relatively large amounts of samples, and is prone to biased losses of lipid material that are almost impossible to control. Therefore, it is mostly applied for the characterization of global perturbation of the lipid composition of the whole cell rather than for subtle changes in lipid microheterogeneity. Precursor ion scanning (PIS) has improved significantly sensitivity and reliability of the mass spectrometric analysis of unseparated mixtures.[11,12] The method is based on independent operation of two mass analyzers positioned inside a tandem mass spectrometer at both sides of the collision cell. The second analyzer (quadrupole Q3), placed after the collision cell Q2, is usually set to transmit ions with a certain constant m/z, while the first mass analyzer (quadrupole Q1), positioned in front of the collision cell Q2, is scanning. Ions passing through the first mass analyzer Q1 fragment in the collision cell but are recorded by the detector only if they produce a fragment with the specific m/z at which the second analyzer has been set. Therefore, PIS selectively detects ions (precursors) that produce the same characteristic fragment ion upon their collision-induced dissociation even if a very complex mixture is injected into the mass spectrometer.

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Because of low sample consumption (less than 1 μL in 30 min), nanoelectrospray mass spectrometry (nanoES MS)[13] increased the sensitivity of PIS, which is often limited by ion statistics and therefore benefits from long acquisition time.[14] A combination of nanoES MS and PIS has made possible quantitative analysis of phospholipids in unfractionated mixtures at the low-picomole level.[15]

Until recently, PIS was performed exclusively on triple quadrupole mass spectrometers, mainly due to relatively small losses of ions in quadrupoles in a single-ion monitoring mode. However, on a triple quadrupole mass spectrometer, only a single precursor ion scan can be acquired at a time. If multiple lipid classes have to be profiled, the analysis has to be repeated several times, making it time-consuming and prone to errors in quantification. To achieve the best sensitivity of a triple quadrupole instrument, both analytical quadrupoles should be operated under lower than the unit resolution settings. Usually the second analytical quadrupole Q3 allows transmitting ions in a window of 2−3 Da to maximize the yield of fragment ions from the collision cell. Since the m/z of these ions are typically in 100−300-Da range, fragments originating from other species and from background are often coselected, resulting in "ghost peaks" in precursor ion scan spectra or even in false positive identification.

Hybrid quadrupole time-of-flight (QqTOF) mass spectrometers were introduced a few years ago.[16,17] These instruments revolutionized the field of structural characterization of biomolecules by acquiring tandem mass spectra with high resolution, mass accuracy, and sensitivity provided by parallel detection of all ions (no scanning). However, no efficient precursor ion scanning was possible on QqTOF machines.[18] In PIS mode, the analysis does not benefit from a nonscanning acquisition of spectra by the TOF analyzer. On the contrary, the sensitivity of detecting a selected fragment ion (the very essence of precursor ion scanning) is limited by the duty cycle that can be as low as 5% for low molecular weight species.

The breakthrough emerged when the ion trapping and bunching technology was introduced in QSTAR Pulsar mass spectrometers.[19] Fragment ions of a given m/z can be trapped temporarily in the collision cell and then released as a short ion packet into the TOF analyzer. In this way, 100% duty cycle can be achieved for any fragment ion, and it is improved significantly for ions with m/z close to the specified one.[20] This makes QSTAR Pulsar instruments attractive for lipid analysis because inherent features of the TOF analyzer, such as high mass accuracy, resolution, and nonscanning acquisition of spectra, can help to overcome the limitations imposed by conventional quadrupole mass filters.

We therefore set out to explore potentialities of the quadrupole time-of-flight instrument featured with trapping of fragment ions capabilities for the analysis of complex mixtures of endogenous lipids. We demonstrate here that multiple classes of phospholipids can be profiled simultaneously and quantitatively. Furthermore, QSTAR Pulsar can decipher lipid mixtures via direct monitoring of fatty acid radicals in individual lipid molecules and can deliver much higher confidence in the identification of lipid species.

MATERIALS AND METHODS

Materials. 1,2-Dilauroyl-*sn*-glycero-3-phosphocholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). ^{13}C-Glucose was purchased from Martek Biosciences Corp. (Columbia, MD) and ^{13}C-methanol (99% atom % ^{13}C) from Cambridge Isotope Laboratories (Andover, MA). *Escherichia coli* strain BL-21 was kindly provided by G. Stier (EMBL, Heidelberg, Germany). *Pichia pastoris* strain SmdII 684 was obtained from Stephen Weeks (EMBL). YNB medium was purchased from Difco Laboratories, Inc. (Detroit, MI). Other components of cell media were obtained from Gibco BRL (Rockville MD). Fetal calf serum (FCS) was obtained from PAA Laboratories GmbH (Cölbe, Germany). Delipidated FCS was obtained from Dr. C. Thiele, (MPI CBG, Dresden, Germany). Oleic acid supplement was purchased from Sigma-Aldrich (St. Louis MO).

Metabolic Labeling of Lipids with Stable Isotopes. *E. coli* strain BL-21 was grown for 24 h at 37 °C in 50 mL of M9 medium supplemented with 1% trace elements, 1 mM $MgSO_4$, 0.3 mM $CaCl_2$, 50 μg each of biotin and thiamine (G. Stier, EMBL), and 0.4% ^{13}C-glucose. Cells were spun down, the supernatant was discarded, and the pellet was frozen in liquid nitrogen. *P. pastoris* cells were continuously grown at 30 °C in YNB medium, without amino acids and ammonium sulfate that were supplemented by 0.4 mg/L biotin and 1% ^{13}C-methanol. At $A_{600} = 1.0$, new culture was made by diluting an aliquot with the fresh media 1:100 (v/v), and the procedure was repeated four times. The final culture (P4) was centrifuged for 5 min at 2000 rpm and 4 °C. The supernatant was discarded, and the pellet was frozen in liquid nitrogen.

Extraction of lipids was performed according to Folch.[21] Briefly, 500 μL of methanol was added to 75 μL of pelleted cells and the resultant mixture was vortexed for 10 min, followed by addition of 1 mL of chloroform and vortexing for another 10 min. The sample was centrifuged for 5 min at 14 000 rpm at room temperature. The supernatant was transferred to a new tube, and 300 μL of H_2O was added, followed by stirring the mixture for another 10 min. Samples were centrifuged at 500 rpm for 5 min at 20 °C. The lower phase (organic) was transferred to a new tube and washed with $CHCl_3$/MeOH/H_2O 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.

Harvesting of MDCK Cells in the Media Enriched with Oleic Acid. MDCK II cells were maintained in supplemented MEM (including 5% FCS, 2 mM glutamine, 100 units/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 ∼30% confluence. MDCK II cells were washed three times with

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Table 1. Detection of Phospholipids by Precursor Ion Scanning

lipid class	mode	cation	selected fragment ions, m/z[a] unlabeled	^{13}C-labeled
phosphatidylcholines (PCs)	+	H^+	184.1	189.1
sphingomyelins (SMs)	+	H^+	184.1	*b*
phosphatidylethanolamines (PEs)	+	Li^+	148.0	150.0
	−		196.0	201.1
phosphatidylserines (PSs)	+	Li^+	192.0	195.0
phosphatidylinositols (PIs)	−		241.0	247.0
phosphatidylglycerols (PGs)	−		153.0[c]	156.0

[a] Fragment ions were selected within mass window of ±0.1 Da centered at the m/z specified in the table. [b] Model organisms used for generating ^{13}C-labeled internal standards (*E. coli*, *P. pastoris*) lack SMs. Instead, peaks of ^{13}C-labeled PCs were used as internal standards for relative quantification of SMs. [c] Since this fragment ion may also be yielded from PIs and PSs, the identification should be confirmed by fatty acid scanning.

PBS buffer. A 5-mL sample of medium, containing 3% delipidated FCS instead of the normal 5% FCS, was added. The concentration of oleic acid was maintained at 100 μM, and the cells were incubated for 24 h. The cells were scraped and pelleted in a 1.5-mL Eppendorf tube for 5 min at 14 000 rpm at room temperature. The pellet was extracted as described above.

Sample Preparation for Mass Spectrometry. Dried lipid extracts were redissolved in 50−500 μL of $CHCl_3$/MeOH 1:2 (v/v) and vortexed for 10 min at room temperature. After brief centrifuging (5 min at 14 000 rpm), a 19-μL aliquot of the sample was mixed with 1 μL of 100 mM ammonium acetate in MeOH, vortexed, and centrifuged for another 5 min followed by a mass spectrometric analysis.

Mass spectrometry was performed on a modified QSTAR Pulsar quadrupole time-of-flight mass spectrometer (MDS Sciex, Concord, Canada) equipped with a nanoelectrospray ion source (Protana, Odense, Denmark). Precursor ion scanning experiments were performed in both positive and negative ion modes typically using a dwell time of 100 ms (5 scans × 20 ms) at a step size of 0.2 Da at unit resolution of the Q1 quadrupole. Peak enhancement (trapping of fragment ions in the collision cell) was applied according to the instructions of the manufacturer and was controlled via either TOF Tune (on Macintosh) or Analyst QS (on PC) software. For multiple precursor ion scanning, trapping conditions were determined experimentally to achieve best transmission of all monitored fragment ions (e.g., in the m/z range 153−283 in negative ion mode).

The list of masses of the characteristic headgroup fragments is provided in Table 1 (see ref 15 for their chemical structures and ref 22 for review of the mechanisms of lipid fragmentation). In negative ion mode multiple precursor ion scanning experiments with lipids from MDCK cells, the following m/z of acyl anions of the fatty acids were selected: 253.2 (16:1); 255.2 (16:0); 269.2 (^{13}C-labeled 16:1); 271.2 (^{13}C-labeled 16:0); 277.2 (18:3); 279.2 (18:2); 281.2 (18:1); 283.2 (18:0); 295.2 (^{13}C-labeled 18:3); 297.2 (^{13}C-labeled 18:2); 299.2 (^{13}C-labeled 18:1); 301.2 (^{13}C-labeled 18:0); 303.2 (20:4); 311.2 (20:0); 323.2 (^{13}C-labeled 20:4); 331.2 (^{13}C-labeled 20:0). Typically, precursor ion spectra for 2 or 4 fragments were simultaneously acquired in positive ion mode and for 19 fragments in negative ion mode. Fragment ions were recorded within the m/z window of 0.2 Da, unless specified otherwise. Peak intensities were kept below 750 counts/scan (cps) to avoid saturation of the detection system.

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Quantitative Monitoring of Changes in Lipid Profile. An equal amount of the total unseparated extract of ^{13}C-labeled lipids from *P. pastoris* was spiked into samples and served as the internal standard. The ^{13}C-labeled lipid mixture was diluted so that intensities of labeled and unlabeled lipid peaks were similar. For quantification, intensities of peaks of interest were normalized to intensities of three internal standards (^{13}C-labeled lipids of the same class) having m/z similar to that of the quantified peak. Relative changes in the concentration of lipid species were calculated as

$$\left[\frac{\left[\frac{(\mathrm{EIp}/\mathrm{EIst1})}{(\mathrm{CIp}/\mathrm{CIst1})}+\frac{(\mathrm{EIp}/\mathrm{EIst2})}{(\mathrm{CIp}/\mathrm{CIst2})}+\frac{(\mathrm{EIp}/\mathrm{EIst3})}{(\mathrm{CIp}/\mathrm{CIst3})}\right]}{3}-1\right]\times 100\%$$

EIp and CIp stand for intensities of experimental and control peaks, respectively; EIst1−EIst3 and CIst1−CIst3 are intensities of peaks of ^{13}C-labeled internal standards in experimental and in control spectra, respectively. The result is presented in percentage along with the mean standard error.

RESULTS AND DISCUSSION

Precursor Ion Scanning on a QSTAR Pulsar Mass Spectrometer. To evaluate the sensitivity of a QSTAR Pulsar mass spectrometer in PIS mode, we analyzed a solution of 1,2-dilauroyl-*sn*-glycero-3-phosphatidylcholine having a concentration of 6 fmol/μL. The isotopically resolved singly charged peak of the lipid was reliably detected (Figure 1), although it could not be distinguished from chemical noise in the TOF MS spectrum (inset). Usually, high resolution of quadrupole time-of-flight machines helps to distinguish multiply charged peptide ions from ions of chemical noise, which are mostly singly charged, by close inspection of the spectra. However, lipids are also detected as singly charged species and high resolution of the instrument alone does not guarantee confident assignment of peaks. Furthermore, intense singly charged ions of chemical background that dominated the TOF MS spectrum (Figure 1) would have provided misleading information regarding the sample identity if not verified by PIS.

The sensitivity of the QSTAR Pulsar was directly compared to the sensitivity of a conventional triple quadrupole instrument (API III from MDS Sciex) and was found to be in the same range (data not shown).

In contrast to triple quadrupole instruments, a TOF analyzer of the QSTAR Pulsar can precisely select masses of fragment ions

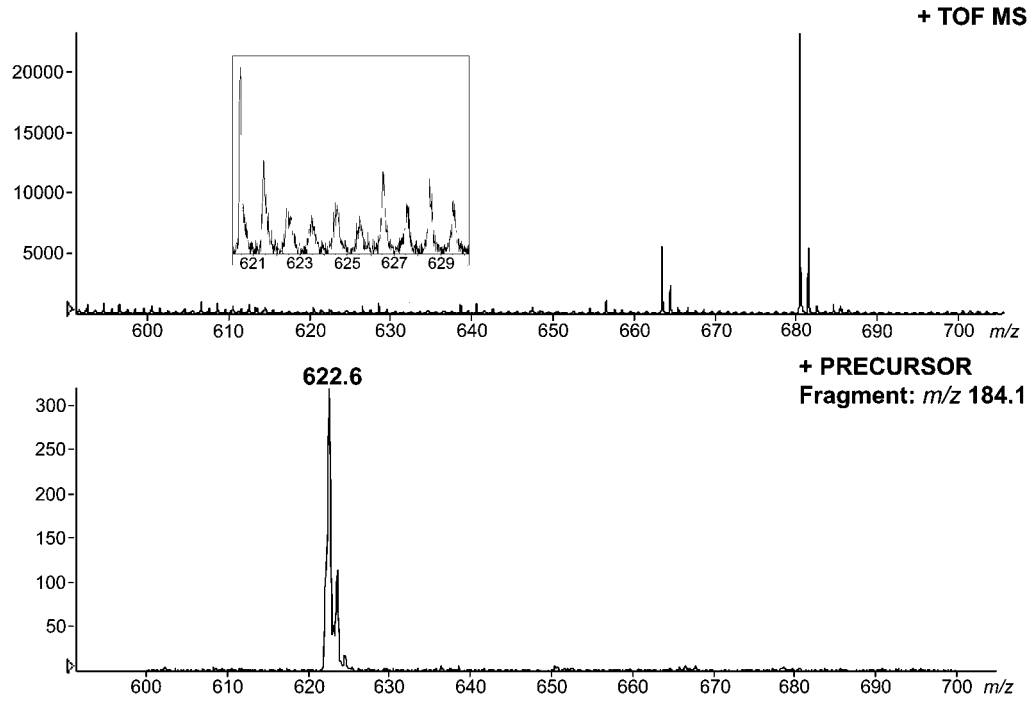

Figure 1. Upper panel: TOF MS spectrum acquired from 6 fmol/μL of the synthetic standard PC 24:0 on a QSTAR Pulsar mass spectrometer. Note that the peak of the PC 24:0 could not be distinguished from chemical noise (inset). Lower panel: precursor ion scan spectrum acquired from the same sample.

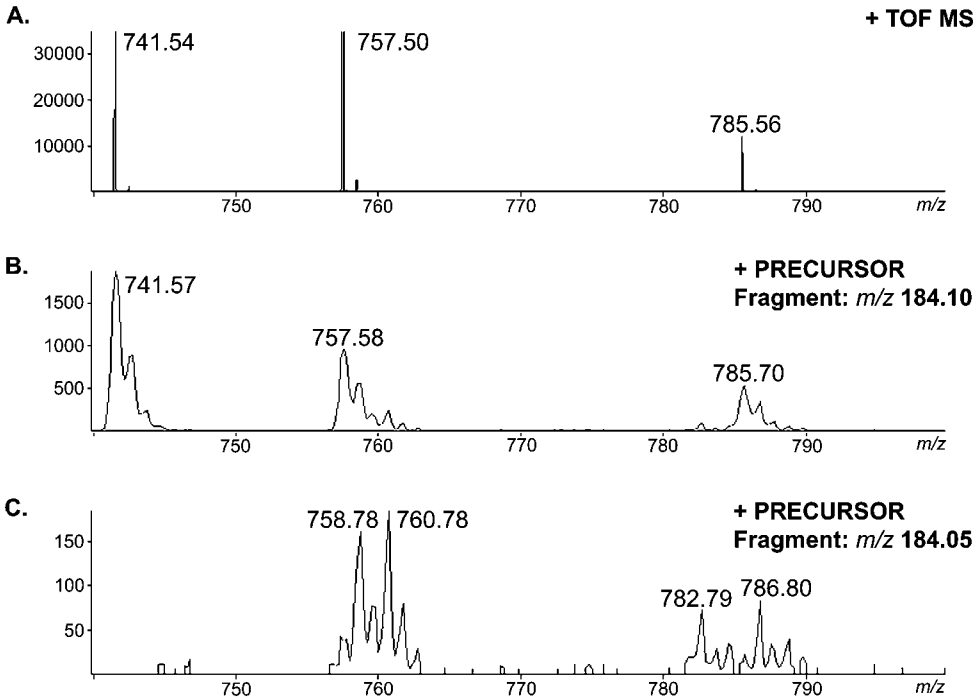

Figure 2. Segments of TOF MS (A) and precursor ion spectra (fragment *m*/*z* 184.1) (B, C) acquired from a fraction of lipids floated in 1% Triton X 100. In panel B, the characteristic fragment ion was recorded within an *m*/*z* window from 184.00 to 184.20, thus including the "parasitic" fragments of the detergent and recording the same peaks as in TOF MS. In (C), the fragment ion was recorded within a narrower window from *m*/*z* 184.00 to 184.10. Only PCs were detected in (C).

without compromising the sensitivity in PIS mode.[23] This feature drastically reduces chemical noise, increases the specificity of PIS, and is therefore important for the analysis of lipids enriched by extraction with nonionic detergents—a common method of isolating membrane rafts.[24] We were able to profile endogenous PCs and SMs in a detergent-insoluble fraction enriched by flotation of a total extract of membrane lipids in cold 1% Triton X 100. No additional cleanup or extensive washing steps were included in the sample preparation routine. TOF MS spectrum acquired from

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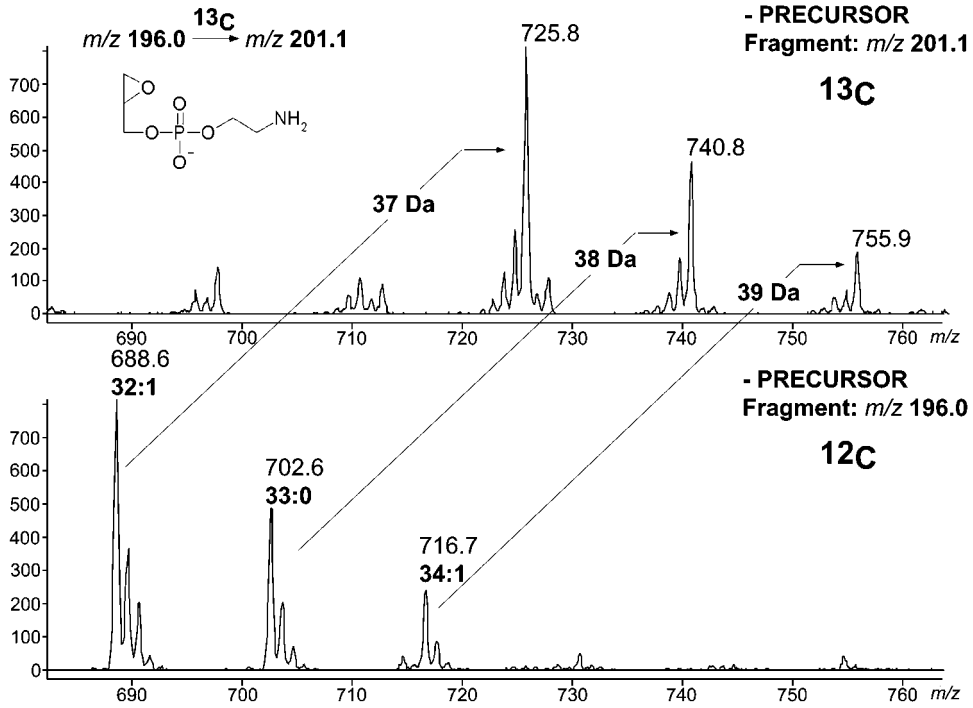

Figure 3. Metabolic labeling of PEs from *E. coli* with ^{13}C isotope and simultaneous detection of labeled and unlabeled forms by multiple precursor ion scanning. Upon labeling, peaks at *m/z* 688.6 (PE 32:1), 702.6 (PE 33:0), and 716.7 (PE 34:1) (lower panel) were shifted according to the total number of carbon atoms (upper panel). *M/z* of the characteristic fragment of the headgroups (upper panel, inset) increased from *m/z* 196.0 to 201.1 and allowed parallel and independent detection of labeled and unlabeled forms from their mixture.

the sample contained only series of clusters of the detergent with sodium and lithium ions that were detected at overwhelming intensity (Figure 2A). The same series of detergent ions appeared even in the precursor ion scan for m/z 184.1 when recorded with a relatively wide m/z window of 0.2 Da (panel B in Figure 2). Lithium adducts of Triton X oligomers produced a fragment ion with m/z 184.129 (its possible structure is $[H(C_2H_4O)_4Li]^+$), which accidentally almost coincide with the mass of the fragment of the headgroup of PCs that is 184.075 Da. Therefore, the m/z window had to be narrowed down to 0.1 Da around m/z 184.05 in order to record lipid ions only (panel C in Figure 2). Note that neither 0.1 nor 0.2 Da isolation of fragment ions is achievable on conventional triple quadrupole mass spectrometers.

Thus, we have demonstrated that the enhanced precursor ion scanning capability of a QSTAR Pulsar mass spectrometer makes it a valuable tool in qualitative analysis of unseparated lipid mixtures since phospholipids can be specifically detected at the level below chemical noise. The sensitivity of PIS on a QSTAR Pulsar mass spectrometer is comparable with the sensitivity of conventional triple quadrupole machines. Furthermore, superior specificity of the fragment ion selection may provide a crucial advantage in analyzing mixtures of lipids, isolated by detergent-assisted extraction or flotation.

Quantitative Analysis of Lipids by Multiple Precursor Ion Scanning (MPIS). To address a variety of biological questions, the lipid analysis methodology should be able to monitor changes in the lipid composition in a quantitative fashion. To this end, a collection of internal standards (typically, synthetic lipids that do not occur in the living cell) is usually spiked into the analyzed sample. However, individual species within the same lipid class are detected with varying sensitivity.[25] Also, conventional lipid extraction procedures, such as Bligh and Dyer's or Folch's, may result in preferential losses of some lipids (K.E. and A.S., unpublished data) or even entire lipid classes, depending on the properties of their fatty acid moieties. It is therefore important that internal standards should be almost identical to endogenous lipids of interest, although for many lipid classes such standards are not readily available. Furthermore, this requirement is impossible to meet in conventional mass spectrometric quantification, because multiple peaks of internal standards would be overlapping with peaks of interest in a complex analyte.

Digests of proteins metabolically enriched in stable isotopes are widely employed as internal standards in quantitative proteomics.[26−28] It is therefore conceivable that metabolic labeling of cells may also produce internal standards useful for lipid quantification. As a test bed, we analyzed total lipid extracts of *E. coli* cells. The pool of *E. coli* lipids consists of three major classes: PEs, PGs, and (in relatively minor amount) cardiolipins.[29] Harvesting cells in the medium that contained ^{13}C-glucose as a single carbon source enriched ^{13}C isotopes in lipid molecules resulted in inverse isotopic distribution of their molecular ions and shifted their m/z according to the total number of carbon atoms (Figure 3). Importantly, ^{13}C atoms were equally incorporated in fatty acids and in headgroups of lipids of all classes.

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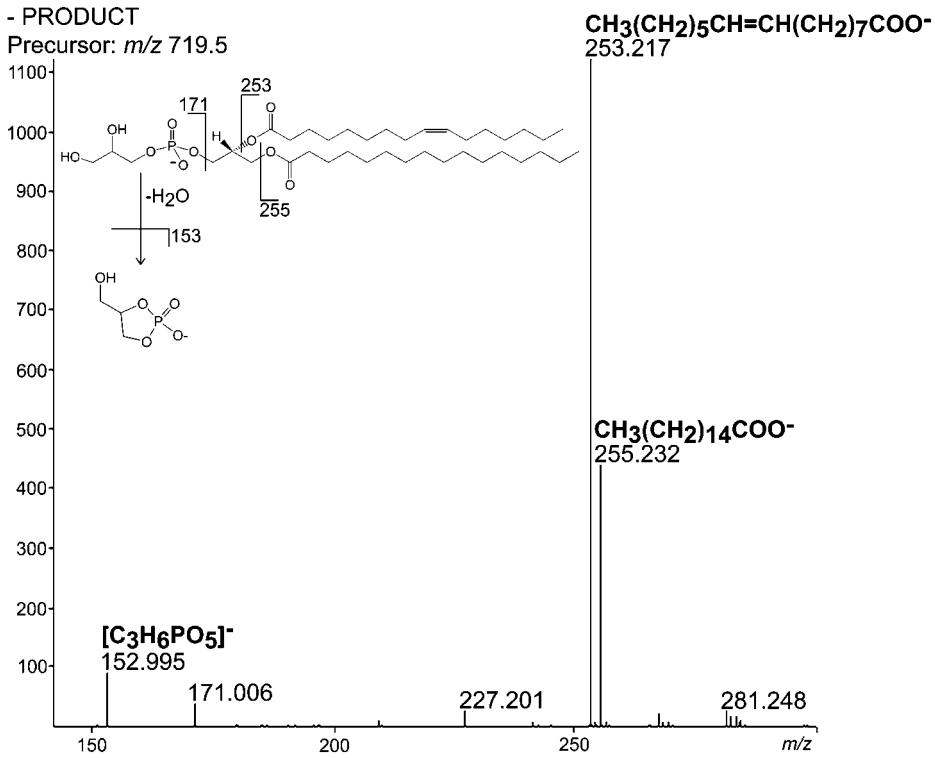

Figure 4. Tandem mass spectrum of PG with *m*/*z* 719.5 acquired in negative ion mode. Peaks of acyl anions of the fatty acid moieties and the peak of a characteristic fragment of the headgroup are labeled.

Therefore, in the mixture, ^{13}C-labeled lipids and normal lipids could be detected simultaneously by PIS using the fragment masses specific for the labeled and for the unlabeled headgroups, respectively (Figure 3). The characteristic fragment of the headgroup of PE contains five carbon atoms, and therefore, its m/z was shifted by 5 Da. Therefore, unlabeled PEs were detected by selecting the fragment with m/z 196.0, ^{13}C-labeled PEs were detected at m/z 201.1, and both precursor ion spectra were acquired in parallel. An equal amount of an unfractionated extract of ^{13}C-labeled lipids was spiked into analyzed samples, thus serving as a comprehensive internal standard. Relative changes in the concentration of lipid species were calculated from the ratio of intensities of their ions and ions of internal standards.

However, several major classes of phospholipids that are present in mammalian cells are missing in *E. coli*. Methylotrophic yeast *P. pastoris*[30] offers a broader spectrum of lipids and may utilize methanol as a single source of carbon. By growing *P. pastoris* cells in the medium that contained ^{13}C-methanol, we generated a mixture of ^{13}C-labeled lipids that was used as a comprehensive internal standard in subsequent experiments with mammalian cells. To check the linearity of the instrument response in MPIS mode, the total lipid extract from unlabeled *P. pastoris* cells was proportionally diluted and the aliquots were spiked with equal amount of the internal standard.

Currently, no neutral loss scanning option, a conventional method of detecting PEs and PSs,[15] is available on QSTAR Pulsar machines. To replace it, samples were spiked with 1 mM LiCl and positively charged lithium adducts of the neutral fragments[31] were selected as characteristic fragment ions in precursor ion scanning (Table 1).

The instrument response was linear in a 10× intensity range in both negative and positive ion modes. However, special precautions had to be taken to avoid saturating of the MCP detector. To this end, the intensities of peaks were carefully monitored during the acquisition and adjusted, if necessary, by varying the spraying voltage or the distance between the spraying capillary and the orifice of the mass spectrometer.[20] Due to the high sensitivity of precursor ion scans for m/z 184.1 and 189.1 (detecting of unlabeled and labeled PCs), the trapping feature had to be turned off to avoid saturation, and therefore, this scan was performed separately from other precursor ion scans in positive ion mode.

We have thus demonstrated that a combination of MPIS and metabolic isotopic labeling of lipids enables quantitative monitoring of changes in any detectable lipid class with no recourse to synthetic standards. Importantly, a comprehensive standard may be spiked into samples before fractionation or extraction, and therefore, the results would be much less affected by biased losses of the analyzed lipids. However, the method only allows monitoring of *relative changes* in the lipid profile rather than determining the *absolute concentration* of individual lipids in the sample. If the latter is required, it will be necessary to once quantify the standard mixture using one of established protocols.

Profiling of Lipids by Fatty Acid Scanning (FAS). Precursor ion scanning for the m/z of the characteristic fragment of the headgroup detects lipid species of the same class and allows calculation of the total number of carbon atoms and the total number of double bonds in fatty acid moieties. However, such a scan does not reveal what fatty acid radicals are present in the particular lipid molecule and the intensity of detected peaks merely

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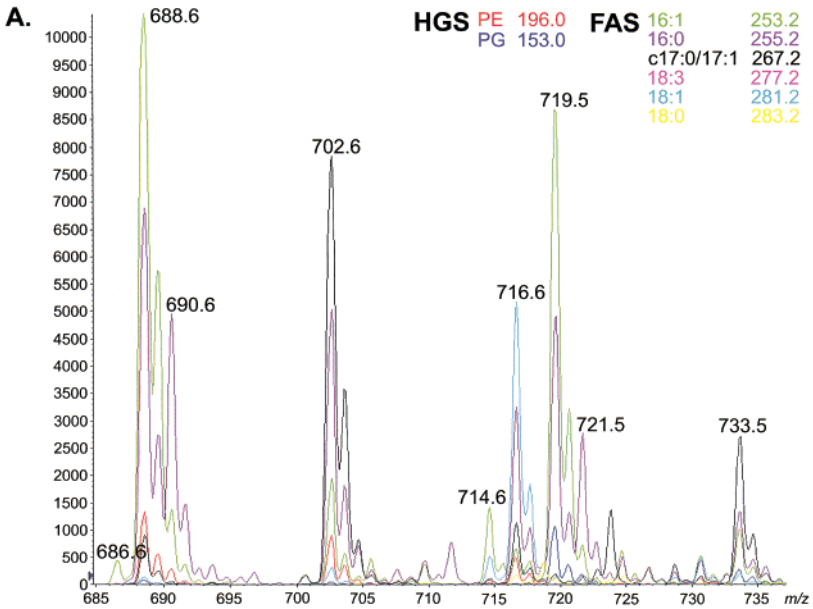

B.

[M-H]- *m/z*		14:0[b]	15:0[b]	16:1	16:0	c17:0[a] /17:1	17:0[b]	18:1	18:0
686.6	PE			*sn-1*					
				sn-2					
688.6	PE			*sn-2*	*sn-1*				
688.6	PE	*sn-1*						*sn-2*	
690.6	PE				*sn-1*				
					sn-2				
702.6	PE				*sn-1*	*sn-2*			
702.6	PE			*sn-2*			*sn-1*		
702.6	PE		*sn-1*					*sn-2*	
714.6	PE			*sn-2*				*sn-1*	
716.6	PE			*sn-2*					*sn-1*
716.6	PE				*sn-1*			*sn-2*	
719.5	PG			*sn-2*	*sn-1*				
721.5	PG				*sn-1*				
					sn-2				
733.5	PG				*sn-1*	*sn-2*			
733.5	PG			*sn-2*			*sn-1*		

[a] Cyclopropane fatty acid c17:0 and unsaturated fatty acid 17:1 are isobaric.
[b] Inferred from *m/z* and another identified fatty acid moiety.

Figure 5. (A) A segment of the overlay of multiple profiles of HGS and FAS of *E. coli* phospholipids. (B) Fatty acid moieties and their localization at the glycerol backbone, as suggested by FAS.

reflects "average" concentration of a pool of isobaric molecules rather than the concentration of the chemically individual lipid.

Hsu and Turk demonstrated previously that in negative ion mode low-energy CID of major glycerophospholipids might produce acyl anions of their fatty acid constituents.[31−34] Indeed, fragmenting of a PG with *m*/*z* 719.5 at moderate (40 eV) collision energy completely destroyed the precursor ion and yielded peaks of acyl anions of both fatty acids moieties, along with the peak of the fragment of the headgroup. Importantly, the intensity of acyl anions was 5−10 times higher than that of the headgroup fragment (Figure 4). Glycerophospholipids are known to comprise ~20 major FAs. Masses of FAs are typically in 200−300-Da range, close to the masses of the headgroup fragments (Table 1) and could be enhanced together under the same trapping settings.[19,20] We therefore reasoned that in a single experiment MPIS could detect lipids by simultaneous monitoring the acyl anions of all major fatty acids, which may (or may not) be present in a particular sample and thus would complement monitoring of characteristic fragments of lipid headgroups. These modes of precursor ion scanning were subsequently termed as FAS and head group scanning (HGS), respectively.

Profiling of a total extract of *E. coli* lipids by FAS and HGS is presented here as an example (Figure 5). Spectral profiles for six major fatty acids and for two headgroups were acquired simultaneously. Overlaying HGS profiles with FAS profiles allowed us to identify fatty acids linked to the glycerol backbone for every individual species of *E. coli* lipids (inset in Figure 5). In tandem mass spectra of glycerophospholipids *sn*-2 carboxylate fragment ions are more abundant than *sn*-1 carboxylate ions.[31−34] Therefore,

(32) Hsu, F. F.; Turk, J. *J. Mass Spectrom.* **2000**, *35*, 595−606.
(33) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 892−899.
(34) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 797−803.

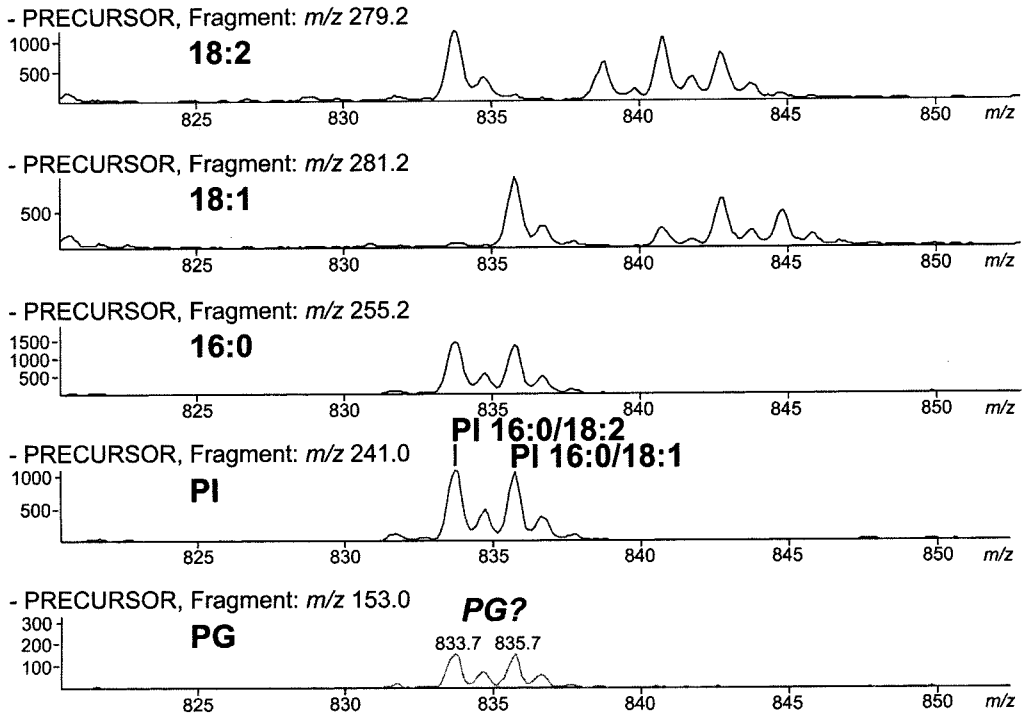

Figure 6. A segment of precursor ion scan spectra in the *m*/*z* 820−850 range, acquired in parallel for PG (*m*/*z* 153.0), PI (*m*/*z* 241.0), 16:0 (*m*/*z* 255.2), 18:1 (*m*/*z* 281.2), and 18:2 (*m*/*z* 279.2). Overlaying of multiple precursor ion scan spectra reveals the identity of individual lipid species.

the position of fatty acid moieties at the glycerol backbone can also be determined from FAS spectra. Taken together, these data elucidated the full complexity of glycerophospholipid composition. For example, the lipid detected at *m*/*z* 702.6 (PE 33:1) is a mixture of at least two isobaric forms: PE 16:0/17:1 and PE 15:0/18:1. Importantly, no tuning of collision energy was required to yield acyl anions from molecules of various classes of lipids (see also the discussion below) and their abundance was always much higher than that of the headgroup fragments, thus increasing the sensitivity of lipid detection.

Acyl anions of fatty acid moieties are produced by collisional fragmentation of anions of glycerophospolipids. Therefore, under conventional settings, FAS does not identify the fatty acid moieties of strongly positively charged lipids, such as PCs or SMs. However, the mass of the fatty acid radical in sphingomyelins could be calculated from their intact masses determined in positive ion mode. In principle, negative ionization of PCs could be achieved via chloride anion clusters.[35] Collisional dissociation of the products of orifice fragmentation of these clusters may also yield acyl anions[15] and subsequently be used for FA profiling.

The reliability of identification and quantification of lipids by precursor ion scanning depends on the accurate selection of fragment masses and may be compromised if other molecules (of lipid and nonlipid origin) yield fragments of similar mass. In ambiguous cases, complementing HGS by FAS provided independent verification of the identity of lipids. Profiling of *P. pastoris* lipids may serve as an example (Figure 6). Peaks at *m*/*z* 833.7 and 835.7 were detected independently at the precursor ions scans specific for PGs and for PIs (Table 1). However, taken together with FAS spectra, these peaks were unambiguously identified as PI 16:0/18:2 and PI 16:0/18:1, respectively. Subsequent inspection of data revealed that the fragment ion with *m*/*z* 153.0 is yielded upon collisional fragmentation of PGs, PIs, and PSs but not of PEs or PCs.

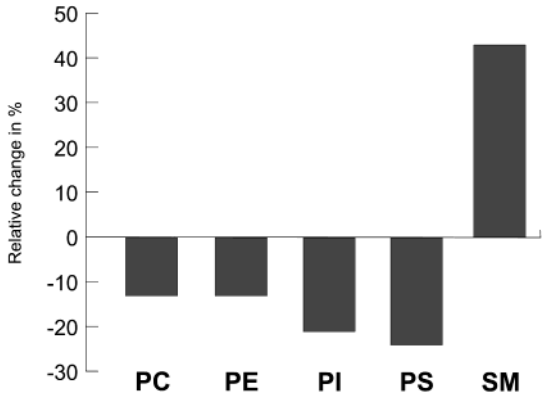

Figure 7. Relative changes in total composition of phospholipid from MDCK II cells treated with oleic acid. MDCK II cells grown in the conventional media served as control.

Exploring Perturbations in a Global Lipid Profile. As demonstrated above, MPIS on a QSTAR Pulsar mass spectrometer enables fast, quantitative, and comprehensive profiling of major glycerophospholipids in unfractionated total lipid extracts via HGS. At the same time, FAS helps to elucidate the heterogeneity of isobaric lipid species and verifies identification of lipids achieved via HGS. Therefore, a combination of FAS and HGS may shed light on complex changes in composition of lipids that accompany a variety of cellular phenomena.

(35) Munster, H.; Stein, J.; Budzikiewicz, H. *Biomed. Environ. Mass Spectrom.* **1986**, *13*, 423−427.

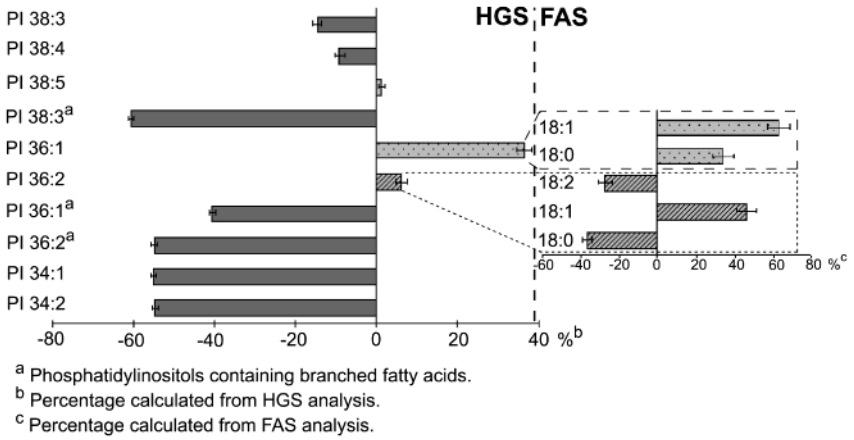

Figure 8. Quantitative profiling of PI in MDCK II cells after treatment with oleic acid. The change in quantity (%), monitored in HGS mode of PI species compared to the PIs of the control cells. Individual species of PI 36:1 and PI 36:2, as revealed by FAS (inset). Values are means ± SEM.

To further evaluate MPIS-based lipid profiling, we set out to chart perturbations in the lipid composition of membranes of MDCK cells that were triggered by a high concentration of oleic acid spiked into the cell medium.[36] First, we determined how the increased concentration of oleic acid affected the balance between various lipid classes in cellular membranes. Second, we elucidated changes in fatty acid composition of individual lipid species within a given class of phospholipids. To achieve these objectives, we profiled total lipid extracts from MDCK cells treated with oleic acid and from the control cells by a combination of FAS and HGS. The analysis revealed a complex pattern of changes in the lipid composition (Figure 7). The amount of major glycerophospholipids was decreased, and their fatty acid composition was significantly altered. Changes in fatty acid composition of PIs are discussed below in detail (Figure 8). Although treatment with oleic acid decreased the total amount of PIs (Figure 7), the amount of two PI species (PI 36:1 and PI 36:2) substantially increased. The content of major fatty acid in PI 36:1 stearic (18:0) and oleic (18:1) acids increased by 35 and 60%, respectively, and was probably accompanied by their redistribution at *sn*-1/*sn*-2 positions at the glycerol backbone, e.g., 18:0/18:1 and 18:1/18:0 (*sn*-1/*sn*-2). At the same time, in PI 36:2, the content of oleic acid (18:1) increased by 40%, while the content of its "complementary" fatty acids linoleic (18:2) and stearic (18:0) was down by 25 and 40%.

Similarly, the relative content of oleic acid was increased in PE and PS species, although their total amount, as estimated from HGS, decreased (Figure 7).

Treatment with oleic acid (an unsaturated fatty acid) increased its presence in glycerophospholipids of all classes, as was concluded from FAS spectra. We speculate that decline in the total amount of glycerophospholipids and increased concentration of SMs (SM 16:0; SM 24:0, and SM 24:1), the major raft sphingolipids in mammalian cells, might constitute a part of the compensating mechanism that preserves the physical properties of cellular membranes. Treatment with oleic acid did not affect the SM profile and the sphingomyelin containing oleic acid moiety was neither detected in the experiment sample nor in the control.

Thus, a combination of HGS and FAS allowed us to monitor the balance between major phospholipid classes. At the same time, the analysis revealed details on how those changes occurred at the most upstream level of individual lipid molecules.

CONCLUSION AND PERSPECTIVES

MPIS on a QSTAR Pulsar mass spectrometer has significantly improved the lipid analysis by making it more rapid, accurate, unaffected by chemical noise, and much less dependent on internal standards of synthetic origin. FAS, a variation of MPIS, has brought new analytical dimension to the field. Compelling information on fatty acid composition of individual lipid molecules can now be obtained in parallel with conventional profiling of lipid classes. Thus, it has become possible to identify individual lipid species, rather than charting an intrinsically heterogeneous isobaric subpopulation of molecules, sharing the same number of carbon atoms and double bonds. We also envision that FAS could become a powerful method for discovering novel lipid classes since detection of lipid species does not rely on preliminary knowledge of their headgroups or other radicals.

Currently, multiple scanning has been set up only in precursor ion scanning mode. Future technical development will include the implementation of multiple neutral loss scanning that will increase the specificity and sensitivity of detection of various lipid classes. A combination of various methods of scanning may help to profile glycolipids and signaling lipids, as phosphatidylinositol phosphates, which do not yield a single distinct fragment ion and therefore are not reliably detectable by any specific scan. Powered by the appropriate software, comprehensive lipid profiling may eventually match the sensitivity and throughput of the characterization of proteomes and thus provide a more comprehensive view of molecular machines embedded into cellular membranes.

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