A comprehensive method for lipid profiling by liquid chromatography-ion cyclotron resonance mass spectrometry

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Abstract This work aims to combine chromatographic retention, high mass resolution and accuracy, MS/MS spectra, and a package for automated identification and quantification of lipid species in one platform for lipidomic analysis. The instrumental setup elaborated comprises reversed-phase HPLC coupled to a Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT), and Lipid Data Analyzer (LDA) software. Data analysis for lipid species quantification in this platform is based on retention time, mass resolution of 200,000, and mass accuracy below 2 ppm. In addition, automatically generated MS/MS spectra provide structural information at molecular level. This LC/MS technology allows analyzing complex biological samples in a quantitative manner as shown here paradigmatically for murine lipid droplets having a huge surplus of triacylglycerol species. Chromatographic preseparation of the bulk lipid class alleviates the problem of ion suppression of lipid species from other classes. Extension of 1D to 2D chromatography is possible, yet time consuming. The platform affords unambiguous detection of lipid species as low as 0.1‰ within major lipid classes.¶¶ Taken together, a novel lipidomic LC/MS platform based on chromatographic retention, high mass resolution and accuracy, MS/MS analysis, and quantitation software enables analysis of complex samples as demonstrated for lipid droplets.—Fauland, A., H. Köfeler, M. Trötzmüller, A. Knopf, J. Hartler, A. Eberl, C. Chitraju, E. Lankmayr, and F. Spener. A comprehensive method for lipid profiling by liquid chromatography-ion cyclotron resonance mass spectrometry. J. Lipid Res. 2011. 52: 2314–2322.

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Quantitative determination of lipid species provides insights to lipid homeostasis and its dysregulation in diseased states. Thus, lipidomic approaches by mass spectrometry-based technology are attractive for characterization of specific lipids as modulators or disruptors of signaling and metabolic pathways, or as biomarkers in the clinical setting (1–5). Particularly, mass spectrometry and advanced bioinformatics tools enable such approaches today (6, 7).

Direct infusion shotgun mass spectrometry is established for “global” lipidomic analysis (8–13). It is fast and simple, but it suffers from inherent ion suppression effects that may be of advantage in some cases (14). Yet, for certain triacylglycerol-rich samples, a precleaning step is necessary (13). As an alternative approach, liquid chromatography coupled to mass spectrometry (i.e., LC/ESI-MS) can be used. Here, a variety of different reversed-phase and normal-phase liquid chromatographic separation techniques afford higher detection sensitivity in mass spectrometers (15–23). A recent publication describes a HPLC/LTQ-FT system in negative ESI mode for glycerophospholipids from yeast lipid extracts, featuring a duty cycle time of 5 s.

Abbreviations: Cer, ceramide; C1D, collision-induced dissociation; CL, cardiopin; DDA, data-dependent acquisition; DG, diacylglycerol; HILIC, hydrophilic interaction liquid chromatography; IS, internal standard; LDA, Lipid Data Analyzer; LM, LIPID MAPS; LPC, lysophosphatidylcholine; LTQ-FT, hybrid linear ion trap Fourier transform ion cyclotron resonance mass spectrometer; MG, monoacylglycerol; NL, neutral loss; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; Q-Traps, quadrupole linear ion trap mass spectrometer; SM, sphingomyelin; S/N, signal to noise; TG, triacylglycerol; 1D, one dimensional; 2D, two dimensional; 3D, three dimensional. In the article, the notation for lipid species is <lipid class abbreviation> •<number of acyl carbons> •<number of double bonds> (e.g., TG 54:5).

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at a resolution of 50,000, including two data-dependent MS/MS spectra per cycle (19). In addition, hydrophilic interaction chromatography (HILIC), a modified normal-phase chromatography coupled to LC/ESI-MS, allows analyzing cardiolipins (CL) and bis(monoacylglycerol) phosphates (BMP) (22). Increased chromatographic selectivity is obtained by a two-dimensional (2D) separation system of normal-phase and reversed-phase chromatography. Lipid profiles from rat peritoneal surfaces have been reported recently after moving to an online 2D LC/MS noncommercial approach (15).

Taken together, most platforms use MS/MS spectra and exact mass (10), retention time and specific fragments (24), or retention time and exact mass (25). It is our goal to combine all these parameters for the highest possible level of confidence in one platform and to link it to a custom-developed software package for automated identification and quantitation (7).

Lipid droplets are long-neglected cell organelles, composed of a hydrophobic lipid core of triacylglycerols (TG) and sterol esters surrounded by a monolayer of phospholipids, sphingomyelins (SM), and cholesterol. They emerged as a central hub for TG metabolism, among many other metabolic and signaling features (26). The huge concentration difference between TG and the other lipid species (27) makes lipid droplets a highly challenging analytical matrix due to ion suppression effects affected by bulk TG. An efficient chromatographic system and subsequent analysis of lipid species by an ion cyclotron is tested by analyzing this matrix. Moreover, the ultrahigh mass spectrometric resolution delivered by an ion cyclotron should help to reduce the complexity of lipid drop sample, which would be a fundamental advantage over systems with lower resolution.

MATERIALS AND METHODS

Lipid standards

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC 16:0/16:0), 1,2-dibehenoyl-sn-glycero-3-phosphocholine (PC 22:0/22:0), 1,2-dimyrystoyl-sn-glycero-3-phosphocholine (PC 14:0/14:0), 1,2-diaraichidonyl-sn-glycero-3-phosphocholine (PC 12:0/12:0), 1,2-diaraichidonyl-sn-glycero-3-phosphocholine (PC 20:0/20:0), 1,2-disarctearyl-sn-glycero-3-phosphocholine (PC 18:0/18:0), 1-stearoyl-2-araichidonyl-sn-glycero-3-phosphocholine (PC 18:0/22:0), 1,2-dilignoceroyl-sn-glycero-3-phosphocholine (PC 24:0/24:0), 1-palmityl-2-stearoyl-sn-glycero-3-phospho-L-serine (sodium salt) (PG 16:0/18:1), 1,2-diaraichidiny1-sn-glycero-3-phospho-L-serine (sodium salt) (PS 12:0/12:0), 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphoethanolamine (PE 18:0/20:4), 1,2-diaraichidonyl-sn-glycero-3-phosphoethanolamine (PE 12:0/12:0), 1-octadecyl-sn-glycero-3-phosphoethanolamine (LPC 18:0), L-α-lyso phosphatidyicholines (chicken egg), 1,2-diaraichidonyl-sn-glycero-3-phosphoethanolamine (LPE 12:0/12:0), 1-palmityl2-octadecyl-sn-glycero-3-phospho-rac(1-glycerol) (PG 12:0/12:0), 1-palmityl2-octadecyl-sn-glycero-3-phospho-rac(1-glycerol) (PG 16:0/18:1), L-α-lysophosphatidylethanolamines (chicken egg), sphingolipid mix I, Ni-lignocerol-D-erythro-sphingosine ceramide (Cer d18:1/24:0), N-arachidol-D-erythro-sphingosine ceramide (Cer d18:1/20:0), and LIPID MAPS (LM) quantitative lipid standards were supplied by Avanti Polar Lipids (Alabaster, AL). L-α-phosphadidylerines (porcine brain), L-α-phosphatidylethanolamines (corn germ), sphingomyelins (bovine brain), L-α-phosphatidyinositos (bovine heart), 1,2,3-triaraichidonyl-glycerol (TG 16:0/16:0/16:0), 1,2,3-tristeroyl-glycerol (TG 18:0/18:0/18:0), 1,2,3-triarachidonyl-glycerol (TG20:0/20:0/20:0), 1,2,3-heptadecanoyl-glycerol (TG 17:0/17:0/17:0), 1,2-diaraichidonyl-sn-glycero-3-phosphocholine (TG 18:0/18:0/16:0), 1,2-diaraichidonyl-sn-glycerol (DG 12:0/12:0), 1,2-diaraichidonyl-sn-glycerol (DG 16:0/16:0), and 1,2-diaraichidonyl-sn-glycerol (DG 18:0/18:0) were purchased from Laradan (Malmö, Sweden). Standard stock solutions were dissolved in chloroform/methanol 1:1 (v/v) at a concentration of 1 mM and stored at −18°C. Lipid standard mixtures were prepared freshly every day in chloroform/methanol 1:1 (v/v) at a concentration of 3 µM and used immediately.

Cyclohexane and chloroform were HPLC grade, and ammonium acetate and acetic acid were analytical grade, all obtained from Merck KGaA (Darmstadt, Germany). 2-Propanol was LC/MS grade and supplied by Fluka (Steinheim, Germany). Methanol and acetonitrile were LC/MS grade, methyl tert-butyl ether (MTBE) was HPLC grade, and 28% ammonia p.a. were all purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Nitrogen (purity 5.0) was obtained from Air Liquide (Graz, Austria). Ultrapure water purified by a Milli-Q Gradient system (Millipore, Bedford, MA) was used in all experiments (resistivity > 18 MΩ cm).

Isolation of lipid droplets from murine primary hepatocytes

A pool of lipid droplets isolated from hepatocytes of mice was split into three equal parts and used as representative biological examples for lipid droplets. Primary hepatocytes from C57 black mice (C57BL) were isolated according to literature (28). The hepatocyte pellet obtained was resuspended in 5 ml ice-cold disruption buffer (20 mM potassium phosphate, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM PMSF), and incubated cells were lysed under nitrogen cavitation at 800 psi for 10 min in a nitrogen bomb (Parr Instrument Co., Moline, IL). The resulting homogenate was centrifuged at 1,000 g for 5 min at 4°C to remove cell debris. The supernatant obtained was overlayed with buffer (50 mM potassium phosphate, pH 7.4, 100 mM potassium chloride, 1 mM EDTA, 1 mM PMSF) and centrifuged at 100,000 g for 1 h at 4°C. Lipid droplets concentrated in a white band at the top of the tube and were used for further experiments.

Lipid extraction

Lipid extraction was carried out as described (29). Briefly, 2 ml of the lipid droplet suspension (TG concentration 2.17 mg/ml) was placed in a glass tube with a teflon-lined cap. A volume of 3 ml methanol and then 10 ml MTBE was added, and tubes were shaken for 1 h at room temperature. Upon addition of 2.5 ml deionized water and shaking, phase separation was induced. The upper organic phase was collected, the lower aqueous phase was extracted with MTBE, and the upper phases were combined. The supernatant obtained was overlayed with buffer (50 mM potassium phosphate, pH 7.4, 100 mM potassium chloride, 1 mM EDTA, 1 mM PMSF) and centrifuged at 100,000 g for 1 h at 4°C. Lipid droplets concentrated in a white band at the top of the tube and were used for further experiments.

Identification and quantitation

Dilution factors need to be adapted to expected lipid class concentrations in the sample and to the instrument setup in use. In case of TG class determination lipid droplet samples were diluted 1:57 with chloroform/methanol 1:1 (v/v); in case of determination of all other lipid classes together 1:3 with chloroform/methanol 1:1 (v/v). In both cases, the solvent was evacuated in a SpeedVac (Thermo Fisher Scientific, San Jose, CA), and lipids were redissolved in 4 ml chloroform/methanol 1:1 (v/v).
each lipid class to monitor. Five replicas of extracted lipid samples were prepared for lipid quantification as well as for accuracy and precision analysis. Quantitative IS LM 1000, LM 1002, LM 1003, LM 1004, LM 1601, LM 6002 at 100 pmol/resuspended sample for PC, LPC, and sphingolipid classes; LM 1100, LM 1102, LM 1103, LM 1104, LM 1300, LM 1302, LM 1303, LM 1304, LM 1500, LM 1502, LM 1503, LM 1504 at 250 pmol/resuspended sample for PE, PS, and PI; LM 6001 at 350 pmol/resuspended sample for DG; LM 6002 at 150 pmol/resuspended sample for TG; and LM 4000 at 800 pmol/resuspended sample for cholesterol ester were used. All solutions prepared were stored at −20°C until use.

Accuracy and precision evaluation was performed by spiking five replicas of extracted lipid droplets with 350 pmol TG 17/0:17/0:17/0, 130 pmol PC 12/0:12/0, 270 pmol PE 12/0:12/0, and 220 pmol PS 12/0:12/0. For further determination of the dynamic range, three replicas were spiked with these four standards at nine concentration levels, each ranging from 0.02 to 328 µmol/µl per standard.

**Chromatographic methods**

**High-performance liquid chromatography.** The Accela HPLC system was equipped with a reversed-phase C18 column (reversed-phase C18; 100 x 1 mm i.d., 1.9 µm particle size), both from Thermo Fisher Scientific (San Jose, CA). Mobile phase A was 10 mM ammonium acetate containing 0.1% formic acid. Mobile phase B was acetonitrile/2-propanol 5:2 (v/v) containing 10 mM ammonium acetate and 0.1% formic acid. The binary gradient started with 35 to 70% B for 4 min, then was raised up to 100% B in another 16 min and further held for 10 min. The flow rate was 250 µl/min, the oven temperature was 50°C, and tray temperature 10°C. For analysis, 5 µl samples were injected. After each run, the column was flushed 5 min with 35% B before the next run was started.

**High-performance liquid chromatography for 2D chromatographic separation.** HILIC-HPLC was carried out in an Agilent 1100 HPLC system (Waldbronn, Germany) consisting of a degasser, a binary pump, a thermostated autosampler, and a thermostated column compartment equipped with a semipreparative column, filled with Nucleosil 100-5 OH, 250 x 10 mm i.d., 5.0 µm particle size (Macherey-Nagel, Düren, Germany). Mobile phase A was cyclohexane, and mobile phase B was 2-propanol/deionized water/acetic acid/28% of ammonia 86:13:1:0.12 (v/v). The HPLC flow rate was 1,000 µl/min at an isotropic composition of 10% A and 90% B, at 35°C oven and 5°C tray temperature. The injection volume was 100 µl. For online monitoring of lipid fractionation, the HPLC system was coupled in positive ESI mode to a 4000 quadrupole linear ion trap mass spectrometer (Q-Trap) (Applied Biosystem/MDS Sciex, Concord, ON, Canada) with a split of 1:21. Two fractions were manually collected, and each was subjected to desalting and concentration. For this, 10 µl deionized water was added to each fraction and the organic phase was collected. After reextraction of the aqueous phase with chloroform/methanol 1:1 (v/v), combined organic phases were dried in a SpeedVac and taken up again in 100 µl chloroform/methanol 1:1 (v/v) for further analysis by reversed-phase-HPLC as described before.

**Mass spectrometry**

**LTQ-FT mass spectrometry.** A 7.0 Tesla hybrid linear ion trap Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT; Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray ion source was used. The instrument was operated in preview mode for parallel MS/MS spectra in the linear ion trap, while running the ion cyclotron in full scan mode at 200,000 resolution (m/z 400) from m/z 400 to 1,050 in positive and from m/z 350 to 1,050 in negative ESI mode. Helium was used as gas for linear ion trap collision-induced dissociation (CID) spectra. From the LTQ-FT preview scan, the four most abundant ions were selected in data-dependent acquisition (DDA), fragmented in the linear ion trap analyzer, and ejected at nominal mass resolution. The following parameters were used for positive and negative ESI-MS/MS experiments: normalized collision energy was 35%, the repeat count was 2, and the exclusion duration 60 s. The activation Q was at 0.2, and the isolation width 2. For positive ESI, spray voltage was set to 5 kV, and the tube lens offset was at 120 V. For negative ESI, spray voltage was −4.8 kV, and the tube lens offset was −87 V. The sheath gas flow was set to 50 arbitrary units, auxiliary gas flow to 20 arbitrary units, sweep gas flow to 2 arbitrary units, and the capillary temperature to 250°C.

**RESULTS**

It is the aim of this study to establish an integrated MS-based analytical platform, relying on chromatography, ultrahigh mass resolution, MS/MS fragmentation, and automated data processing (Fig. 1). We develop a hybrid approach by optimizing both reversed-phase-HPLC and subsequent MS parameters. In addition, we extend reversed-phase chromatography by a HILIC step (2D LC/MS) that may enhance MS sensitivity for lipid classes by minimizing ion suppression. In this process, we test platform performance by analyzing profiles of lipid species present in pooled lipid droplets isolated from mouse hepatocytes.

**Reversed-phase-HPLC development**

The particular challenge is the high excess of TG in the samples. ESI was used because of its extraordinary sensitivity for monitoring lipid species of TG, diacylglycerols (DG), phosphatidylcholines (PC), lysophosphatidylcholines (LPC), phosphatidylethanolamines (PE), phosphatidylserines (PS), and SM in positive mode and phosphatidylinositols (PI) in negative mode. Phosphatidic acids (PA), phosphatidylglycerols (PG), CL, lysophosphatidylethanolamines (LPE), and ceramides (Cer) were searched for, but they are not detectable in positive or negative ionization mode. Cholesterol and cholesterol esters were not determined, as analysis by a separate silica-based, normal-phase chromatography method with postcolumn addition of a polar solvent is the preferred experimental approach (18).

The method developed here is a compromise between chromatographic resolution and running time as shown in Fig. 2. The chromatogram reveals that in the first 10 min, the most-polar lipids, like LPC, elute, followed by...
could influence ionization efficiency. However, in a validation experiment with standard LM 6000 (eight deuterated TG species), we found that the HPLC gradient applied and TG structural features have only a minor impact [coefficient of variation (CV) of signal intensities of 8.4% only, data not shown].

### Lipid identification and quantitation

**Accurate mass determination.** The solvent chosen facilitates the formation of ammonia adducts [M+NH$_4]^+$ for TG in positive ESI to yield an elemental fingerprint of C$_x$H$_y$O$_z$N. The exact mass of better 2 ppm is sufficient in most cases for unambiguous identification of the elemental composition. The resolution of 200,000 at m/z 400 results in 90,000 at m/z 950, the upper limit for molecular mass of major lipid species in lipid droplets. This resolution does not provide baseline separation of C$_x$H$_y$O$_z$N and $^{13}$C$_x$H$_y$O$_z$N, but it is still good enough for peak top separation at 80% peak height. The instrument would be able to deliver much higher resolution, but the resolution chosen is a compromise because ion cyclotron sensitivity decreases with increasing resolution on the one hand, and acquisition time increases with increasing resolution, resulting in a slower duty cycle, on the other hand.

**Determination of lipid molecular species by MS/MS fragmentation.** For interpretation of exact mass data in terms of fatty acid composition of lipid species and for confirmation of identity by specific fragments, MS/MS spectra were acquired in DDA. In this setup, the cyclotron and linear ion trap operate as two separate instruments. This method allows acquisition of low-resolution MS/MS spectra in the linear ion trap on the most intense peaks in parallel to high-resolution spectra acquired by the ion cyclotron (30). The MS/MS coverage of lipid species attained in DDA is 66%. The method circumvents the need either to rerun the sample for obtaining MS/MS spectra on precursors identified in a previous high-resolution run or to operate the instrument sequentially in high-resolution MS and subsequent MS/MS mode, which would result in a lower duty cycle. This is illustrated paradigmatically in an extracted ion chromatogram shown in [Fig. 3A](#) for TG 54:5 eluting with a peak maximum at 24.0 min. The respective MS/MS spectrum in positive ionization mode is presented in [Fig. 3B](#). The LDA software identifies TG 54:5 by exact mass at m/z 988.78578 ([M+NH$_4]^+$), and the exact mass trace at an isolation width of 0.015 Da shows a main peak at 24.0 min (Fig. 3A). The structural elucidation of the detected compound is done by manual analysis of targeted MS/MS data. The MS/MS spectrum (Fig. 3B) of m/z 898.78578 (TG 54:5) depicts fragmentation patterns typically encountered with ammonium adducts of TG. The fragment ion of m/z 881.7 derives from the neutral loss (NL) of NH$_3$ (MW 17) originating from the ammonium adduct of TG 54:5. Moreover, Fig. 3B clearly shows abundant fragment ions at m/z 599.5 [M+H$_2$R$_3$COOH]$^+$ and m/z 601.5 [M+H$_2$R$_3$COOH]$^+$ and the less-abundant ion m/z 603.5 [M+H$_2$R$_3$COOH]$^+$, corresponding to the NL of FA 18:1, 18:2, and 18:3, respectively. This information and

![Lipid profile](#)

**Fig. 1.** Profiling of lipid species by 1D and 2D LC/MS. For 2D LC/MS, lipid extracts are first separated in two fractions by normal phase HILIC-HPLC monitored online by the Q-Trap mass spectrometer. After liquid-liquid extraction (concentration and desalting), the two fractions are acquired by reversed-phase-HPLC/LTQ-FT (identical to 1D LC/MS). In the last step, high-resolution mass at m/z 350-1,050.

- **Fig. 2.** Total ion chromatogram of lipids from a representative lipid droplet sample acquired by reversed-phase-HPLC/LTQ-FT. A reversed-phase C18 column is used for separation of lipid species and monitored in positive ESI mode. The scan range of the LTQ-FT is m/z 350-1,050.
combinatorial restrictions arising from elemental composition allow for deducing the molecular species to be TG 18:1/18:2/18:2 and TG 18:1/18:1/18:3.

DG have similar fragmentation behavior as TG, both having \([M+NH_4]^+\) ions in positive ESI mode. The characteristic MS/MS fragments are the NL of NH\(_3\) and the NL of constituent FA. Similarly, determination of other lipid classes, like PC, PE, PS, PI, SM, and LPC, by MS/MS relies upon specific fragments and neutral losses described in detail in various publications (6, 31). Constituent FA is determined by neutral losses in positive ionization mode and carboxylates in negative ionization mode.

**Identification of lipid species by retention time.** Low abundant compounds sometimes do not generate reliable MS/MS spectra. In this case, accurate retention in addition to accurate mass becomes an important criterion for identification. The method of choice is reversed-phase chromatography, where separation of lipid species within one class, expressed by equivalent carbon numbers (32), are mainly based on interaction between hydrophobic stationary phase and acyl carbon chains of the lipid. In fact, chain length and degree of unsaturation of constituent FA of the lipid matter. Consequently, species having FA of same chain lengths (same number of acyl carbons) can be separated by reversed-phase chromatography by their degree of unsaturation in the molecule; the chromatograms shown in Fig. 4 impressively illustrate such behavior. Elution times of TG with the same number of acyl carbons decrease by approximately one minute per additional double bond in the lipid. Taking the example of TG with 48 acyl carbons (i.e., TG 48:0 to 48:8), MS/MS data are not available for TG 48:0, 48:1, 48:7, and 48:8. Nonetheless, as shown in Fig. 4 and supplementary Tables I–IV, retention times demonstrate the occurrence of these very minor species by decreasing elution order of these compounds according to number of double bonds. Clearly, identification of lipid species becomes possible by retention time even without availability of reliable MS/MS spectra as long as any other species of the same lipid class with the same carbon number has a reliable MS/MS spectrum.

**2D HPLC approach.** The chromatogram in Fig. 2 reveals that in reversed-phase-HPLC/ESI-MS analysis, lipid species from different lipid classes overlap, particularly at retention times between 10 and 20 min. Owing to excellent ionization efficiency in positive electrospray mode, PC species are a case in point. They exert a dominating ion suppression on other species of partially coeluting lipid classes. Thus, a chromatographic selectivity complementary to reversed-phase-HPLC is needed to attain separation of PC species from other polar lipids. Our choice is a diol-based stationary phase allowing for HILIC. The semi-preparative diol column is preconditioned once for 1 h with the isocratic solvent composition as described in Materials and Methods. The advantage of isocratic elution over gradient elution is that it requires no time-consuming preconditioning between individual runs and has better...
Lipid profiling by high-resolution LC/MS/MS

Application of reversed-phase-HPLC/MS to profile lipid species from lipid droplets. As the gain in significant improvements by 2D chromatography is low in relation to considerably more experimental effort and time needed, our method retains time reproducibility. For method development, standards are used as described in Materials and Methods. During HILIC of lipid droplet samples, the eluent of the first 10 min is discarded, then lipids eluting between 10 and 24 min (TG, DG, PS, PE, and PI) are collected in fraction 1. The second fraction includes PC, SM, and LPC and elutes between 24 and 48 min (Fig. 5). The subsequent liquid-liquid extraction after fractionation has two purposes. On the one hand, it is necessary to desalt fractions from exceeding ammonium acetate and transfer them into a solvent compatible with the second chromatographic dimension. On the other hand, concentrations of lipids in fractions could be increased by a factor of 140 by this procedure.

Our findings indicate that the 2D method elaborated results in a higher sensitivity for certain lipid classes. The data shown in Fig. 6A demonstrate that DG species in this sample can be detected by the 2D approach with a higher sensitivity compared with the 1D approach, and the same is found for PE species (Fig. 6B).
Calibration curves were computed for TG 17:0/17:0/17:0, PC 12:0/12:0, PE 12:0/12:0, and PS 12:0/12:0, showing a linear range of four orders of magnitude (supplementary Table IV). The lowest concentration points of the calibration curves are all at a signal-to-noise (S/N) ratio greater than 10:1, a value generally referred to in the literature, thus defining the limit of quantitation (LOQ). Accuracy and precision were determined for five replicas spiked with these compounds. The former is within 20% deviation; the latter is below 10% relative standard deviation (supplementary Table IV). All species detected show an S/N ratio of at least 34:1 or greater, well above the limit of detection (LOD) defined at S/N 3:1. The mean retention time deviation of all acquired lipid species is 0.37% (n = 5).

**DISCUSSION**

Excellent LC/MS methods were developed based on Orbitrap and LTQ-FT mass spectrometry, although to date, they are restricted exclusively to analysis of phospholipids (19, 20, 33). LC/MS analysis of lipid species by low-resolution multiple reaction monitoring (MRM), applicable only to a preset list of expected lipids, was also described (34, 35). In this article, however, a method for comprehensive profiling of lipid species is presented that enables data acquisition in one HPLC run per ionization polarity. Our platform provides full scan data of high-resolution ion cyclotron resonance mass spectrometry. Untargeted MS/MS data, which can be used in an untargeted manner in subsequent bioinformatics analyses, are also provided. This is an important aspect when high-throughput data of choice is reversed-phase HPLC/MS. This method allows identification of lipid species by exact mass, MS/MS spectra, and retention time in one platform (Fig. 1). Exact mass and retention time are then used by LDA for identification and peak integration as described previously (7), whereas MS/MS spectra are inspected manually for confirmation of identity and fatty acid analysis of lipid molecular species. If MS/MS spectra for some species are not available, confirmation of identity can be deduced from retention time shifts (Fig. 4); a practical example of the latter is given in Fig. 7. As a result of these procedures, the platform is able to identify lipid species as low as 0.1‰ of the respective base peak of a given lipid class with a high degree of certainty, even beside bulk amounts of a few lipid species. Thus, we were able to identify 103 minor TG species in addition to 19 major TG species, with each of the minor TG species contributing less than 10‰ of the total amount of TG. The total number of lipids identified accounts for 122 TG, 41 DG, 28 PC, 18 PE, 4 PS, 9 PI (PC compared with the latter three being the dominant phospholipid class), 13 LPC, and finally, 7 SM species. Ether-linked phospholipids (e.g., plasmalogens) were not detected. Fig. 8 shows the molar proportion of each lipid class relative to total lipid amount in the pooled samples. All individual lipid species found in pooled lipid droplets are shown in supplementary Tables I–III, which presents quantitative analysis data, including respective retention times. For example, TG 62:14 contributes as little as 0.066‰ to total amount of TG; in LPC 16:0, 18:2, 18:0, 22:6, and 20:4, fatty acyls predominate; and in minor lipid classes, PE, PS, and PI 38:4 species (18:0/20:4) are the most prominent. Very long-chain fatty acyls 22:0, 24:0, and 24:1 predominate in SM species.

**Validation of method**

Calibration curves were computed for TG 17:0/17:0/17:0, PC 12:0/12:0, PE 12:0/12:0, and PS 12:0/12:0, showing a linear range of four orders of magnitude (supplementary Table IV). The lowest concentration points of the calibration curves are all at a signal-to-noise (S/N) ratio greater than 10:1, a value generally referred to in the literature, thus defining the limit of quantitation (LOQ). Accuracy and precision were determined for five replicas spiked with these compounds. The former is within 20% deviation; the latter is below 10% relative standard deviation (supplementary Table IV). All species detected show an S/N ratio of at least 34:1 or greater, well above the limit of detection (LOD) defined at S/N 3:1. The mean retention time deviation of all acquired lipid species is 0.37% (n = 5).
Lipid profiling by high-resolution LC/MS/MS

In fact, the platform is a valuable tool for detection of changes occurring in very low concentration ranges of a lipid species compared with the total amount of lipid species in a given sample. The limitation of reversed-phase-HPLC is separation of PC from other phospholipid classes. This could be overcome by adding a second chromatographic dimension with complementary selectivity. A separator linking the two chromatographic dimensions for automated analysis was developed recently, but it is not commercially available (15). In the case of lipid droplet analysis reported here, we carried out such an approach by manually linking the two chromatographic dimensions, and we found enhanced sensitivity for detection of PE and DG species (enhancement of PS species was not significant). Obviously, in positive ESI mode, these lipid classes benefit particularly from PC removal by the first chromatographic dimension.

Shotgun lipidomics of chromatographically not separated, overlapping M+2 peaks requires isotopic correction (8). This is not necessary here, due to chromatographic resolution and the extremely high mass resolution delivered by the ion cyclotron. Several combinations of chromatography (retention time), high mass accuracy, and MS/MS information are addressed in the literature (15, 19, 20, 25). But the combination of all these parameters, including bioinformatic tools, integrated in one platform applicable to a wide variety of lipid classes is the novel concept realized in this work. All of this results in high identification certainty, sensitivity, and selectivity, and it contributes significantly to unambiguous detection of minor lipid species.

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