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Shotgun lipidomics on a LTQ Orbitrap mass spectrometer by successive switching between acquisition polarity modes

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Top–down shotgun lipidomics relies on direct infusion of total lipid extracts into a high-resolution tandem mass spectrometer and implies that individual lipids are recognized by their accurately determined *m/z*. Lipid ionization efficiency and detection specificity strongly depend on the acquisition polarity, and therefore it is beneficial to analyze lipid mixtures in both positive and negative modes. Hybrid LTQ Orbitrap mass spectrometers are widely applied in top–down lipidomics; however, rapid polarity switching was previously unfeasible because of the severe and immediate degradation of mass accuracy. Here, we report on a method to rapidly acquire high-resolution spectra in both polarity modes with sub-ppm mass accuracy and demonstrate that it not only simplifies and accelerates shotgun lipidomics analyses but also improves the lipidome coverage because more lipid classes and more individual species within each class are recognized. In this way, shotgun analysis of total lipid extracts of human blood plasma enabled to quantify 222 species from 15 major lipid classes within 7 min acquisition cycle.

Supporting information may be found in the online version of this article.

Keywords: shotgun lipidomics; LTQ Orbitrap; mass accuracy; high-throughput screening; polarity switching

INTRODUCTION

Shotgun analyses provide rapid quantitative snapshots of the molecular composition of complex lipidomes of eukaryotic and prokaryotic organisms (reviewed in[1–3]). Typically, lipids are extracted from cells, body fluids or tissues, and total extracts are directly infused into a mass spectrometer. Individual molecular species of multiple lipid classes are recognized by a palette of methods relying on tandem mass spectrometry (MS/MS), such as precursor and neutral loss scanning, multiple precursor ion scanning[4–8] or data-dependent acquisition of full MS/MS spectra.[9–11] A combination of over 100 000 (full width at half maximum) mass resolution and sub-ppm mass accuracy of hybrid Orbitrap instruments[12–14] enables to identify and quantify lipid species directly in Fourier transform (FT) MS survey spectra detouring to MS/MS only when peak assignment remains, for any reason, ambiguous. This approach is often termed top–down lipidomics, in contrast to the bottom–up lipidomics where lipid species are quantified via specific fragment ions in their MS^n spectra. With no constraints applied on the composition of hydrocarbon moieties, lipid classes fall into several groups, depending on the unique number of N, O and P atoms in their molecular cations or anions.[15,16] Lipid species from different groups may not have the same exact masses. For example, exact masses of molecular cations of phosphatidylcholines (PC) and sphingomyelins (SM) species (despite sharing the same head group) may not overlap because of different heteroatom compositions, ($N_1O_8P_1$) and ($N_2O_6P_1$), respectively. At the same time, masses of molecular cations of PC or phosphatidylethanolamines (PE) may overlap because their heteroatom composition is the same ($N_1O_8P_1$). Note that these constraints apply to the composition of molecular cations or anions, rather than native zwitterions. For example, in negative ion mode, PC are readily detectable as acetate or formate adducts ($N_1O_{10}P_1$), while PE produce abundant molecular anions ($N_1O_8P_1$); hence, the analysis in negative ion mode circumvents potential overlap of their exact masses.

Because of their remarkable structural diversity, lipid classes differ by their ionization capacity and produce polarity-dependent forms of molecular cations and anions. It is therefore advantageous to acquire both positive and negative ion mode spectra from each analyzed sample provided that mass resolution, mass accuracy and the analysis throughput remain uncompromised. However, rapid switching between the polarities on LTQ Orbitrap instruments – hybrid tandem mass spectrometers comprising a linear ion trap and Orbitrap analyzers – was previously unfeasible because of the severe and immediate degradation of mass accuracy. Although it was possible to acquire spectra seconds after the

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polarity switch, it took more than 60 min to stabilize sensitive electronics and restore the ppm mass accuracy. Therefore, shotgun lipidomics screens were typically performed in one (most often, positive) mode only as a best compromise between the analysis sensitivity and lipidome coverage, despite some lipid classes (such as cardiolipins, phosphatidylinositols, phosphatidylglycerols and phosphatidic acids to mention just a few) remained undetected or nonquantified. Rapid polarity switching could be performed on Exactive instruments of the Orbitrap family[17,18] because of their different design (two power supplies independently supporting positive and negative modes). However, no individual precursor ions could be isolated for MS/MS, which is occasionally required for independent validation of ambiguous peak assignments.[15]

Here, we report on a method to rapidly acquire high-resolution spectra on LTQ Orbitrap instruments in both polarity modes at the sub-ppm mass accuracy and address its implications for high-throughput shotgun lipidomics screening.

EXPERIMENTAL

Chemicals and lipid standards

Synthetic lipid standards and a polar lipid extract of bovine heart were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) or Sigma–Aldrich Chemie (Munich, Germany), common chemicals and solvents of ACS or LC–MS grade from Sigma–Aldrich Chemie (Munich, Germany) or Fluka (Buchs St. Gallen, Switzerland) and methanol (LiChrosolv grade) from Merck (Darmstadt, Germany).

Annotation of lipid species

Lipid classes are as follows: PA, phosphatidic acids; PC, phosphatidylcholines; PC-*O*, 1-*O*-alkyl–2-acyl-glycerophosphatidylcholines; PC-*O*/-*O*, 1,2-di-*O*-alkyl-glycerophosphatidylcholines; LPC, lysophosphatidylcholines; LPC-*O*, 1-*O*-alkyl-2-hydroxy glycerophosphatidylcholines; PE, phosphatidylethanolamines; PE-*O*, 1-*O*-alkyl-2-acylglycerophosphatidylethanolamines; PE-*O*/-*O*, 1,2-di-*O*-alkyl-*sn*-glycerophosphatidylethanolamines; LPE; lysophosphatidylethanolamines; PS, phosphatidylserines; PG, phosphatidylglycerols; PI, phosphatidylinositols; CL, cardiolipins; dLCL, dilysocardiolipins; mLCL, monolysocardiolipins; Cer, ceramides; Chol, cholesterol; CholE, cholesterol esters; SM, sphingomyelins; TAG, triacylglycerols; and DAG, diacylglycerols. Glycero- and glycerophospholipid species were annotated by the total number of carbon atoms and double bonds in their hydrocarbon moieties. Sphingolipid species were annotated by the total number of carbon atoms, double bonds and hydroxyl groups in N-amidated fatty acid and long chain base moieties.

Mass spectrometric analyses

Mass spectrometric analyses were performed on a LTQ Orbitrap XL instrument (Thermo Fisher Scientific, Bremen, Germany) equipped with a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca NY, USA) using nanoelectrospray chips with the diameter of spraying nozzles of 4.1 μm. The ion source was controlled by Chipsoft 8.3.1 software (Advion BioSciences). Ionization voltage was +1.25 kV in positive and −1.25 kV in negative mode; backpressure was set at 0.95 psi in both modes. The temperature of ion transfer capillary was 125 °C; tube voltages were 90 V (MS+) and −150 V (MS−). Under these settings, 10 μl of the analyte could be electrosprayed for more than 50 min.

For the analysis, 10 μl of samples were loaded onto 96-well plate (Eppendorf, Hamburg, Germany) of the TriVersa NanoMate ion source, sealed with aluminum foil and centrifuged for 5 min at 4000 rpm on a Multifuge 3S-R centrifuge from Heraeus DJB Labcare Ltd (Newport Pagnell, UK).

Each sample was analyzed for 7 min. FT MS spectra were acquired within the range of *m/z* 300–1500 from 1.5 min to 3.49 min in negative and from 5.0 min to 7.0 min in positive mode from the start of analysis. Time gaps of 1.5 min were allowed for stabilizing the spray after polarity switching and prior to acquiring spectra.

Polarity switch of the TriVersa NanoMate ion source was triggered by the mass spectrometer via contact closure signal. Timing and polarity of spectra acquisition were controlled by XCalibur 2.0.7 software (Thermo Fisher Scientific).

To improve the mass accuracy, lock mass function of the XCalibur 2.0.7 was engaged. Reference peaks were octadecyl(ditertbutyl-hydroxyphenyl)propionate ($[M-H]^-$, *m/z* 529.462621) and tris (ditert-butylphenyl) phosphate ($[M+H]^+$, *m/z* 647. 458757); tris (ditert-butylphenyl) phosphate ($[M+H]^+$, *m/z* 663.453672) and $[M+NH_4]^+$, *m/z* 680.480221). Reference peaks were selected using LipidXplorer software developed in house as described beneath.

Selection of reference background peaks

FT MS spectra were acquired from five different lipid extracts and one blank at the target mass resolution of $R_{m/z\ 400} = 100\,000$. Spectra were imported by LipidXplorer software into a MasterScan database under the following settings: range of *m/z* 365–1200; minimal peak intensity cutoff $I_{min} = 1 \times 10^5$; mass tolerance 5 ppm. Occupation threshold (the frequency at which peaks should occur in all imported spectra at the intensity above I_{min}) was set to 1.0 (or 100%). Effectively, this implied that peak candidates were detected in each sample and in the blank with the intensities exceeding $I_{min}/\sqrt{n}$, where *n* is the number of acquired scans, and their *m/z* were not affected by overlapping peaks.

MSn of background peaks

MS^n of background peaks was performed by collision-induced dissociation at the LTQ Orbitrap XL instrument. Precursor ions were isolated within the width of 1.8 Th, and upon collision-induced dissociation, fragment ions were detected at the linear ion trap at the unit mass resolution or at the Orbitrap at $R_{m/z\ 400} = 100\,000$. Spectra were acquired under operator control, and normalized collision energy was individually optimized for each analyte by automated ramping.

Impact of polarity switching on the instrument mass accuracy

FT MS(+) and FT MS(−) spectra of a polar bovine heart extract [2.5 μg/ml in isopropanol/methanol/chloroform 4 : 2 : 1 (v/v/v) containing 7.5 mM ammonium formate] were acquired using polarity switching without engaging the lock mass option. Prior to the experiment, the instrument was calibrated in both modes under stable conditions. Mass resolution was $R_{m/z\ 400} = 100\,000$; target value for automated gain control was 500 000 and maximum injection time was 250 ms. One FT MS scan was acquired within 3.52 s. The total acquisition time for all FT MS spectra was 45 min.

Extracting lipids from human blood plasma

Frozen plasma samples were thawed, and lipids were extracted with methyl-*tert*-butyl ether (MTBE) as described.[19] Briefly, 5 μl

of EDTA plasma was placed in a 1.5 ml vial (Eppendorf, Hamburg, Germany). Then, 700 µl of a mixture of internal standards containing 7.8 µM CholE 12:0, 7.1 µM CholD7, 4.9 µM TAG 36:0, 0.6 µM DAG 24:0, 0.5 µM Cer 30:1:2, 5.4 µM PC-*O*/-*O* 36: 0, 1.1 µM PE-*O*/-*O* 40:0, 0.6 µM PS 24:0, 0.3 µM PI 32:0 and 1.2 µM SM 30:1:1 in MTBE/methanol (10:3; v/v) were added. The sample was vortexed at 4 °C for 1 h. Then, 140 µl of water were added, and tube was thoroughly vortexed for 15 min at 4 °C. After centrifuging for 5 min at 13 400 rpm on a Minispin centrifuge (Eppendorf, Hamburg, Germany), 500 µl of the upper organic phase was transferred into a glass vial and stored at −20 °C until analyzed. 5 µl of total lipid extract were finally diluted in 45 µl isopropanol/ methanol/ chloroform 4:2:1 (v/v/v) containing 7.5 mM ammonium formate and used for MS analysis.

Lipids were identified

Lipids were identified by LipidXplorer software.[20] Molecular Fragmentation Query Language queries were compiled for PA, PC, PC-*O*, LPC, LPC-*O*, PE, PE-*O*, LPE, PG, PI, LPI, PS, SM, TAG, DAG, MAG, Cer, Chol, CholE, CL, mLCL and dLCL lipid classes and are available at LipidXplorer wiki site: https://wiki.mpi-cbg.de/wiki/lipidx/index.php/Main_Page Where specified, lipid identifications were independently validated by FT MS/MS spectra acquired using higher energy collision dissociation.[9]

RESULTS AND DISCUSSION

Automated switching of the acquisition polarity

The controller of TriVersa NanoMate ion source was modified (currently, this function is not a standard option) such that the spray voltage polarity could switch during an infusion experiment by the contact closure signal produced from the LTQ Orbitrap XL. When the status of the relay is set to "close", the TriVersa NanoMate is triggered to positive mode, regardless of the actual polarity set in the acquisition method of the Chipsoft software that controls the ion source. XCalibur MS method file consisted of two segments, one for negative and one for positive mode that use the same tune file: the contact closure was set to "open" status in the first segment and "close" status in the second (Fig. 1). The start of FT MS acquisition by the LTQ Orbitrap XL and sample infusion by the TriVersa NanoMate were time synchronized. The analyte was electrosprayed at −1.25 kV for 3.49 min, while FT MS(−) spectra were acquired from 1.5 min to 3.49 min, i.e. for the time of 2 min. Then, the contact closure signal forced the polarity switch on TriVersa NanoMate, and spray voltage was changed to +1.25 kV, bypassing the polarity settings at Chipsoft software (Fig. 1). The acquisition of FT MS(+) spectra started 1.5 min later, i.e. at the time point of 5.0 min and continued until 7.05 min (also for the time of 2 min), at which point, the sample infusion was stopped and the acquisition cycle was repeated with the next sample. The time gap of 1.5 min between the polarity switch and start of the sample acquisition was allowed for stabilizing electrospray current and achieving better quantification consistency.

Lock mass option preserves sub-ppm mass accuracy upon successive polarity switching

For LTQ Orbitrap machines, switching the acquisition polarity degrades the mass accuracy because of temporarily unstable operation of electronics. It takes more than 60 min to settle it back to the usual low-ppm level, making frequent polarity switching impractical. We reasoned that temporary mass deviations could be compensated by real-time calibration of spectra by using lock masses[12], and therefore spectra could be acquired immediately after the polarity switch. Abundant peaks of chemical background could serve as the lock masses (Fig. 2). Because the actual pattern of background ions is affected by the composition of analyzed extracts, solvents, plasticware and instrument settings, we first established a routine that identified optimal reference peaks in any collection of spectra in a project-specific manner.

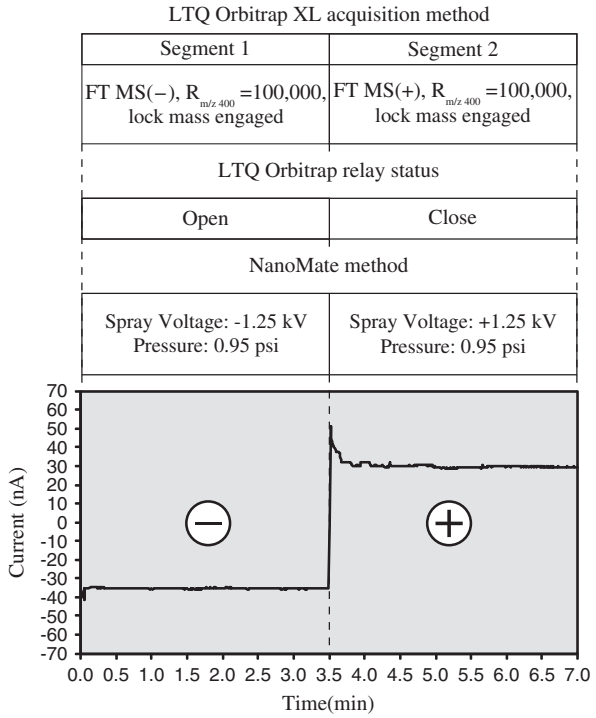

Figure 1. Synchronized acquisition cycles with switching-spraying polarity at the TriVersa NanoMate by the contact closure signal from LTQ Orbitrap XL. In the panels from top to bottom, (1) LTQ Orbitrap method uses 2 segments, separately for negative and positive mode, (2) LTQ Orbitrap relay status is set to "open" in Segment 1 and "close" in Segment 2 to trigger polarity switch on a modified TriVersa NanoMate electronics platform, (3) TriVersa NanoMate method uses negative mode settings for complete acquisition. The contact closure signal only changes the voltage polarity applied to the spraying chip while both its value and gas backpressure remain unchanged, and (4) Profile of the electrospray ionization current typically observed during polarity switching as reported by the Chipsoft software is shown.

We acquired FT MS spectra from a few total lipid extracts in positive and negative modes without changing the polarity between individual samples, i.e. when the instrument calibration was stable. We also acquired spectra from the blank analyte: blanks were processed as experiment samples, but they contained no lipids. All spectra were imported by LipidXplorer software into a flat-file database MasterScan where individual scans were merged for each analyzed sample, and then related peaks were aligned in a mass resolution-dependent manner (refer to [20] for details on the software organization and operation). While processing raw spectra, LipidXplorer applied several user-defined thresholds on mass tolerance, intensities and occupancy of the aligned peaks. The latter controlled the frequency with which each reported peak should be observed in the spectra of individual samples within the entire series of experiments. Under the occupancy threshold of 1.0 (or 100%), LipidXplorer only recognized peaks that were present in all samples and in all blanks, i.e. nonsuppressed ubiquitous background peaks, whose masses were not affected by

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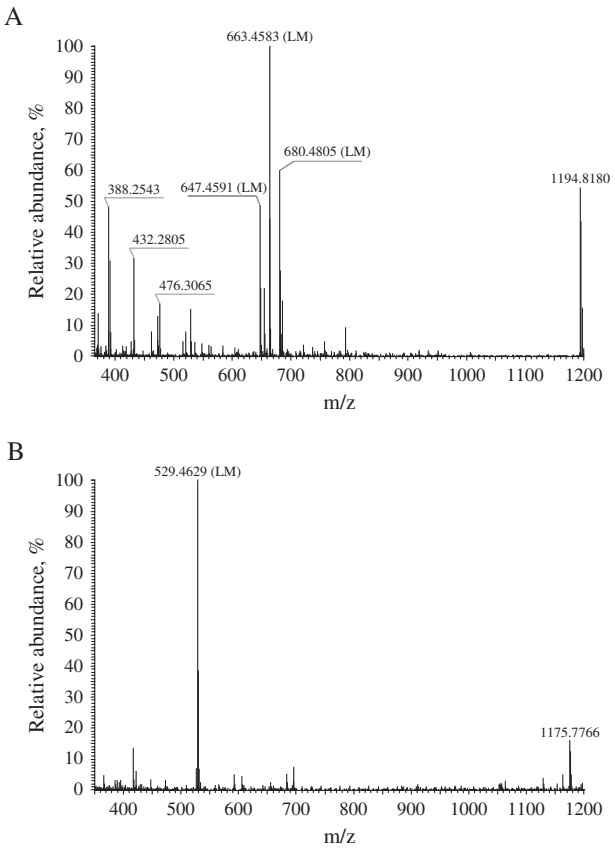

Figure 2. FT MS spectra of the blank analyte acquired in (A) positive and (B) negative modes. Identified background peaks (Table 1) are designated with *m/z* values; LM indicates reference peaks used as lock masses.

possible overlap with other peaks. By accurately determined *m/z* and subsequently acquired MS^n spectra, they were attributed to common detergents and plasticizers[21] (Table 1). At even lower *m/z* (not included in Table 1), we frequently observed abundant peaks of docosenoamide cation ($[M+H]^+$, calculated *m/z* 338.341740) and anions of palmitic ($[M-H]^-$, calculated *m/z* 255. 232954) and stearic ($[M-H]^-$, calculated *m/z* 283. 264254) fatty acids. Not all peaks from control spectra could be used as references. Out of *ca.* 1400 peaks recognized in blank FT MS(+) spectra, the abundance of 22 peaks exceeded 5% of the base peak intensity, yet only three peaks met the selection criteria and were picked as lock mass candidates (Fig. 2). Note that intensities of peaks selected as lock masses are not reported by XCalibur software. Therefore, peaks of internal standards spiked into total extracts for lipid quantification or any peaks confidently assigned to known lipids should not be used as lock masses.

We then tested if lock masses preserved mass accuracy also when the acquisition polarity was successively changing. First, five FT MS(+) spectra of polar extract from bovine heart and one blank were acquired prior to polarity switching to obtain a reference dataset. Then, FT MS(+) and FT MS(−) spectra of the lipid extract were successively acquired five times with polarity switching, but without using the lock mass option. The *m/z* and intensity of abundant lipid PC-*O* 34:3 ($[M+H]^+$, calculated *m/z* 742.574516 in positive and $[M+HCOO]^-$, calculated *m/z* 786. 565443 in negative modes) were extracted from acquired raw files for each scan. The differences between reported and calculated *m/z* were plotted over the acquisition time (Fig. 3). We observed that, upon switching the polarity, peaks *m/z* were systematically shifted, and it took more than 60 min until the mass accuracy settled back to the ppm level expected for stable operation of the instrument.

FT MS(+) and FT MS(−) spectra were again acquired from the lipid extract, but now with the lock mass option enabled. Tris (ditert-butylphenyl) phosphite and phosphate (*m/z* 647.458757, *m/z* 663.453672 and *m/z* 680.480221) and octadecyl(di-tert-butyl-hydroxyphenyl)propionate (*m/z* 529.462621) were used as reference peaks for positive and negative modes, respectively (Table 1). We observed that the lock mass option/on-line calibration fully compensated mass shifts in both modes starting from the acquisition onset (Fig. 3).

Hence, we demonstrated how lipid identification software LipidXplorer pinpointed suitable common background peaks in the series of FT MS spectra. Using their *m/z* as lock masses helped to maintain the sub-ppm mass accuracy, even when the acquisition started immediately after switching the instrument polarity. Note that the described procedure is generic: it is not bound to any particular type or source of reference masses and can be applied for identifying suitable reference peaks in any series of shotgun spectra acquired at any mass spectrometer; however, the achieved gain in mass accuracy might be instrument dependent.

Benchmarking lipid identification performance

We acquired FT MS spectra of a commercially available polar lipid extract of bovine heart for 3.5 min in negative mode and then

Table 1. Abundant peaks of chemical background in FT MS spectra frequently observed in total lipid extracts

Name	Elemental composition	Precursor ion	Calculated *m/z*	Experimental *m/z*[a]
Octaethylene glycol [b]	$C_{16}H_{34}O_9$	$[M+NH_4]^+$	388.254107	388.2543
Nonaethylene glycol [g]	$C_{18}H_{38}O_{10}$	$[M+NH_4]^+$	432.280322	432.2805
Decaethylene glycol [h]	$C_{20}H_{42}O_{11}$	$[M+NH_4]^+$	476.306536	476.3065
Octadecyl (di-tert-butyl-hydroxyphenyl)propionate [c]	$C_{35}H_{62}O_3$	$[M-H]^-$	529.462621	529.4629
Tris(ditert-butylphenyl) phosphite [d]	$C_{42}H_{63}O_3P$	$[M+H]^+$	647.458757	647.4591
Tris(ditert-butylphenyl) phosphate [e]	$C_{42}H_{63}O_4P$	$[M+H]^+$	663.453672	663.4583
	—	$[M+NH_4]^+$	680.480221	680.4805
Irganox 1010FF [f]	$C_{73}H_{108}O_{12}$	$[M+NH_4]^+$	1194.817903	1194.8180
	—	$[M-H]^-$	1175.776804	1175.7766

[a]Corresponding FT MS(+) and FT MS(−) spectra are presented in Fig. 2
Identification of precursors [b–f] was confirmed by MS^n experiments; peaks of other polyethylenglycols [g,h] were identified by their accurate masses only

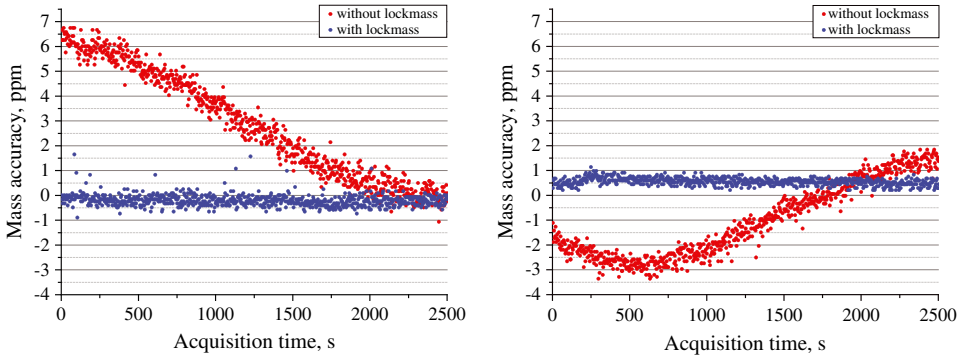

Figure 3. The use of lock masses preserved sub-ppm mass accuracy under successive switching of the spectra acquisition polarity. LTQ Orbitrap XL was switched from negative to positive (panel at the left hand side) and from positive to negative modes (panel at the right hand side). Upon polarity change, masses were shifted by several ppm if lock masses were not engaged; activating lock mass option reduced the average mass shift down to $\Delta\ m/z = -0.21 \pm 0.23$ ppm for negative to positive and $\Delta\ m/z = 0.55 \pm 0.15$ ppm for positive to negative switches, respectively. Mass differences are reported for the precursor of PC-*O* 34:3 ($[M+H]^+$, *m/z* 742.574516 (calc); $[M+HCOO]^-$, *m/z* 786.565443 (calc)). In total, the plots encompass 762 and 712 FT MS(+) and FT MS(−) spectra, respectively.

switched to positive mode, continued the acquisition for another 3.5 min and repeated the same cycle ten times. Within the test run, the first and the last FT MS spectra in the same polarity were acquired in 66.5 min. All spectra were interpreted using the LipidXplorer software.

Table 2 presents the number of species that were only identifiable in FT MS(+), FT MS(−) or in both spectra. By matching accurate masses and compositional constraints, LipidXplorer recognized 191 (146 + 45) and 286 (146 + 140) lipids in positive and negative modes, respectively. CholE were only detected in positive mode; CL, mLCL, dLCL, PG, LPI, MAG and Cer were detected in negative mode. As anticipated, analyzing extracts in only one mode (regardless, positive or negative) would have severely compromised the lipidome coverage, irrespectively of how abundant the missed lipid classes might be and by how many species they might be represented. In total, FT MS analyses in both modes identified 331 lipids, which

Table 2. Number of species per lipid class and acquisition polarity mode identified in the polar bovine heart extract [a]

Lipid class	Species identified in the following spectra: FT MS(+)	FT MS(+) and FT MS(−)	FT MS(−)	Total, per lipid class
CholE	2	—	—	2
TAG	41	37	—	78
DAG	—	6	21	27
MAG	—	—	3	3
Cer	—	—	8	8
SM	—	17	3	20
PE	1	16	11	28
PE-*O*	1	17	18	36
LPE	—	4	6	10
PC	—	17	20	37
PC-*O*	—	17	14	31
LPC	—	6	5	11
PS	—	1	1	2
PI	—	8	10	18
LPI	—	—	1	1
CL	—	—	5	5
mLCL	—	—	3	3
dLCL	—	—	1	1
PG	—	—	10	10
Total, per polarity mode	45	146	140	331

[a]The full list of identified species is in supporting information Table S1. Spectra were acquired in both modes upon polarity switching within the same experiment.

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constituted 73% increase in the number of identified species, compared with FT MS(+) (191 *vs* 331) and 16% (286 *vs* 331) compared with FT MS (−) spectra.

Importantly, the analyses with successive polarity switching practically consumed the same amounts of lipid material: from 10 μl of loaded extract (handling lesser volumes of volatile nonviscous organic solvents is difficult) less than 1.2 μl were consumed for acquiring spectra. Note that running two separate single-polarity analyses (first analyzing all samples in positive mode followed by polarity change, allowing time gap for stabilizing mass calibration, and then analyzing samples in negative mode) would be completely impractical on mass spectrometers that do not support rapid polarity switching. Few microliters of the organic analyte will evaporate instantly once a sealed sample well at the 96-well plate is opened. Therefore, same amounts of samples should be replated, and then the mass spectrometer should be stabilized in another mode prior to reacquiring spectra. Furthermore, TriVersa NanoMate source requires additional time for aspirating samples from individual wells, exchanging sample tips and moving them to the new spraying nozzles. Altogether, two independent acquisitions instead of one might additionally take as long as 3 h per 96-well plate, consume double amount of lipid extracts and use up twice as many single-use nozzles of the expensive nanospray chip. It is therefore not surprising that lipidomics screens were performed only in one (usually, positive) mode, although benefits of switching between the modes were always apparent.

We next checked if successive dual-polarity acquisition could impact the species quantification. To this end, we correlated the relative abundance of 37 peaks of all PE, PE-*O* and LPE species identified in both FT MS(+) and FT MS(−) spectra of bovine heart extract (Table 3) and observed no influence of both polarity switching and using lock masses.

We therefore concluded that top–down analysis with successive switching of the acquisition polarity identified more lipid classes and more species within each class, compared with the analysis in a single-polarity mode. When analyzing a series of samples, switching the polarity for each individual sample saved lipid material, drastically decreased the total analysis time and running costs, while the lipid identification and quantification were not affected.

Switching acquisition polarity in clinical lipidomics screens

We applied a previously established top–down screening method[16,19] to total extracts of human blood plasma. However, this time, FT MS spectra were acquired with successive switching between positive and negative modes (Fig. 4). For each sample FT MS(−) and FT MS(+) spectra were acquired successively within the same experiment and then independently interpreted by LipidXplorer. Table 4 presents lipid species detectable in FT MS(+), FT MS(−) and in both spectra.

In the test series of ten individual plasma samples, the top–down analyses recognized 222 lipid species from 15 classes. From those, 62 lipids were detected only in FT MS(+) spectra (among them, 44 out of the 50 TAG species, as well as Chol and CholE) and 68 lipids only in FT MS(−) spectra (Table 4).

Dual-polarity acquisition had three important implications. First, we detected 44% more species (222 *vs* 154) and encompassed five more lipid classes: DAG, Cer, LPC-*O*, PS and PI, compared with the previously reported positive mode-only top–down shotgun screen.[19] Second, for each plasma extract, both FT MS(+) and FT MS(−) spectra were successively acquired from the same 10 μl sample. Replating samples for repeating the acquisition in a different mode was not required, which drastically reduced the analysis time and cut the sample consumption by twofold. Third, major glycerophospholipid species of PE (PE 36 : 1, PE 36 : 2, PE 38 : 3) and LPE (LPE 18 : 0) were accurately quantified because, in negative mode, they were unequivocally distinguished from isobaric endogenous PC with odd-numbered fatty acid moieties (e.g. PC 33 : 1, PC 33 : 2, PC 35 : 3 and LPC 15 : 0). The total content of major lipid classes calculated from the abundances of individual species agreed well with the previously reported values.[19,22] Within each lipid class, the relative abundance of individual species generally followed the published profiles[23], although in some instances substantial deviations occurred. This, however, should be expected considering remarkable compositional diversity of the blood plasma lipidome confirmed in numerous clinical screens.[19,24–27]

To validate potentially ambiguous assignments, we further acquired HCD FT MS/MS spectra[9] from precursor ions of the lipid classes that (1) were only detectable in one polarity mode and (2) might overlap with precursors from another major lipid class because of the identical composition of heteroatoms.[15,16] In this way, we were able to distinguish molecular anions of PS from formate adducts of PC with odd-numbered fatty acid moieties and identical exact masses (supporting information Fig. 1S).

We underscore that, in top–down lipidomics, compromising the mass accuracy increases the rate of false positive assignments.[20] Under mass tolerance of +/− 10 ppm that corresponds to the mass accuracy achieved without lock mass option (Fig. 3), the interpretation of plasma extracts spectra becomes ambiguous and points to putative polyunsaturated species (such as TAG 55 : 12 (+7.27 ppm),

Table 3. Correlation of relative abundances of PE, PE-*O* and LPE species identified in FT MS spectra of polar lipid extract from bovine heart [a]

Aquisition mode	Polarity switch	Lock mass	Correlation coefficient [(a)]	Slope [(a)]
FT MS(+)	no	no	—	—
FT MS(+)	no	yes [(b)]	0.9997	0.9914
FT MS(+)	yes	yes [(b)]	0.9998	0.9727
FT MS(−)	yes	yes [(c)]	0.9799	1.0157

[a]Relative abundances were calculated by normalization to summed peak intensities of all PE. Correlation coefficients were calculated relative to the spectrum acquired with no polarity switching and no lock mass option engaged.

[b]Lock masses for FT MS(+) were *m/z* 647.458757, 663.453672 and 680.480221.

[c]Lock mass for FT MS(−) was *m/z* 529.462621.

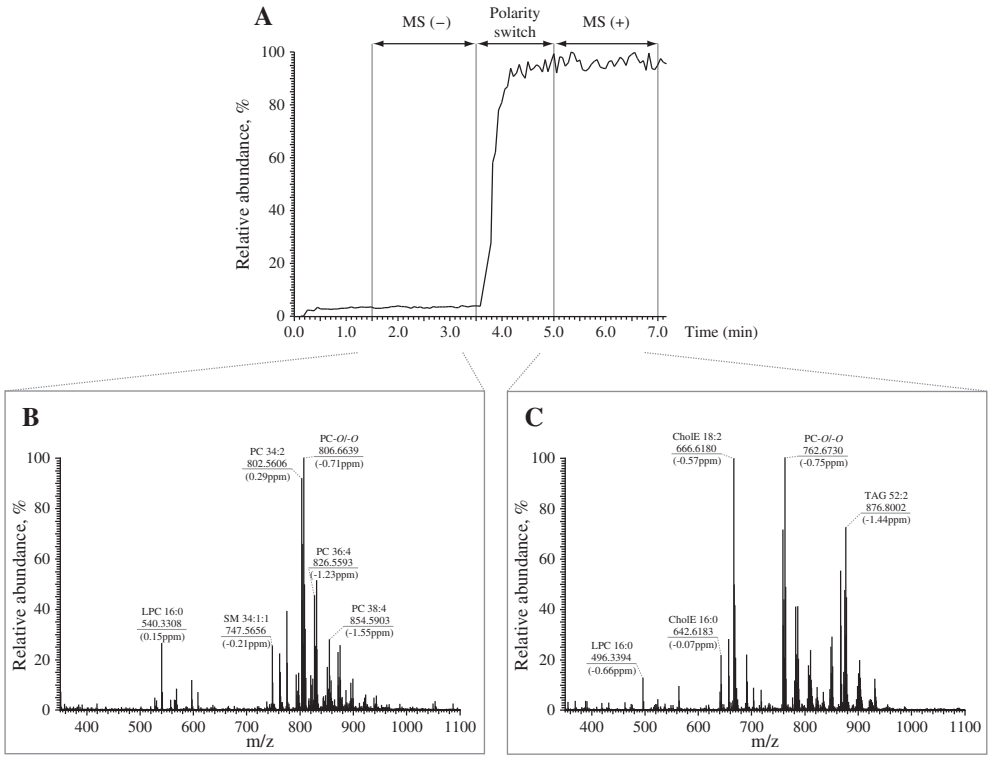

Figure 4. Top–down lipidomics of total extracts of human blood plasma. (A) Total ion current recorded over the full analysis time. (B) Merged FT MS spectra acquired from 1.5 to 3.5 min in negative mode. The polarity was switched to positive after 3.5 min, and another 1.5 min were allowed for stabilizing the spray. (C) Merged FT MS spectra acquired from 5.0 to 7.0 min in positive mode. In both spectra, most abundant peaks are annotated with lipid identification, *m/z* and its difference with calculated *m/z* (in ppm); PC-*O*/-*O* stands for PC-*O*/-*O* 36:0 (one of the internal standards used for the lipid quantification). LipidXplorer software identified 154 and 160 species in positive and negative modes, respectively.

Table 4. Number species per each lipid class and acquisition polarity mode identified in total extracts of human blood plasma [a]

Lipid class	Species identified in the following spectra: FT MS(+)	FT MS(+) and FT MS(−)	FT MS(−)	Total, per lipid class
Chol	1	—	—	1
CholE	17	—	—	17
TAG	44	6	—	50
DAG	—	6	7	13
Cer	—	2	6	8
SM	—	27	—	27
PE	—	—	11	11
PE-*O*	—	3	13	16
LPE	—	—	7	7
PC	—	18	13	31
PC-*O*	—	14	—	14
LPC	—	9	4	13
LPC-*O*	—	3	—	3
PI	—	4	4	8
PS	—	—	3	3
Total, per polarity mode	62	92	68	222

[a]The full list of lipid species and their concentrations in human blood plasma is in supporting information Table S2. Spectra were acquired in both modes upon polarity switching within the same experiment.

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PE 41 : 7 (+6.36 ppm) and PC-*O* 37 : 8(−5.8 ppm), to mention only a few) that could not be independently confirmed by MS/MS (data not shown).

Altogether, the dual-polarity top–down screen covered 222 lipid species from 15 major lipid classes, while the total analysis time was 7 min per sample and could further be reduced because no system re-equilibration and washing are required for preventing carry-over of the lipid material between samples.

CONCLUSIONS AND OUTLOOK

Shotgun lipidomics holds remarkable potential for high-throughput screening and biomarker discovery.[24,28] High-resolution mass spectrometers can unequivocally recognize the majority of lipid species by direct analysis of total extracts. The entire procedure is simple, and both sample preparation and acquisition are easy to automate. The software supporting lipid identification on high-resolution machines has also become available.[20,29]

Here, we demonstrated that top–down screens on LTQ Orbitrap mass spectrometers could use successive switching of the acquisition polarity. For each sample in both positive and negative mode, FT MS (and if required, many MS/MS) spectra could be acquired in rapid succession within the same noninterrupted sequence. Neither throughput nor sensitivity was compromised, while lipid identification specificity and coverage of lipid classes were markedly improved. Because hybrid LTQ Orbitrap mass spectrometers are becoming common instruments in *omics* facilities worldwide, this development paves the way for technically simple, robust and comprehensive lipidomics screening supporting a variety of biological and clinical applications.

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Supporting Information

Supporting information may be found in the online version of this article.

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