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CHAPTER TEN

Shotgun Lipidomics by Tandem Mass Spectrometry under Data-Dependent Acquisition Control

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Contents

Abstract

Data-dependent acquisition of full MS/MS spectra from all detectable (or, alternatively, preselected) lipid precursors produces a rich data set, whose subsequent interpretation by the dedicated software LipidInspector emulates the simultaneous acquisition of an unlimited number of precursor and neutral loss scans in a single analysis. Using logical operations, emulated scans can be combined into highly specific data interpretation routines (termed Boolean

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scans) enabling in-depth structural characterization of fragmented precursors. Alternatively, a small number of preselected precursors can be fragmented regardless of their relative intensities in survey spectra, hence emulating selected reaction monitoring (SRM) analysis that attains both high detection specificity and sensitivity. Although the data-dependent acquisition approach is, in principle, cross-platform, it benefits from the high mass resolution capacity of hybrid tandem mass spectrometers with time-of-flight and, especially, Fourier transform or Orbitrap analyzers.

1. Introduction

In shotgun lipidomics (Ekroos *et al.*, 2003; Han and Gross, 2005), total lipid extracts are infused directly into a mass spectrometer via the electrospray ion source and lipid species are subsequently identified and quantified by tandem mass spectrometry using lipid class–specific and (or) lipid species–specific precursor ion scans (PIS) and neutral loss scans (NLS), reviewed in Han and Gross, 2003, 2005; Pulfer and Murphy, 2003; and Wenk, 2005. By varying the solvent composition and spraying conditions, it is possible to specifically enhance the ionization of certain lipid classes and, hence, to improve the dynamic range of lipid detection (Han *et al.*, 2006). PIS and NLS are typical features of triple quadrupole mass spectrometers, the workhorses of the lipidomics field. However, these instruments can only acquire one PIS or NLS spectrum at a time. When quantitative profiling of multiple lipid classes requires several PIS and NLS, the analysis is successively repeated several times (Brugger *et al.*, 1997). In contrast, quadrupole time-of-flight (QqTOF) mass spectrometers can acquire multiple precursor ion spectra in parallel, which, together with the accurate selection of m/z of fragment ions, opens up interesting analytical opportunities (Ejsing *et al.*, 2006a; Ekroos *et al.*, 2002). It is known that collision-induced dissociation (CID) of molecular anions of glycerophospholipid species produces abundant acyl anion fragments of their fatty acid moieties. Therefore, the interpretation of precursor ion spectra, simultaneously acquired for a multitude of plausible acyl anion fragments, could identify and quantify individual molecular species (Ejsing *et al.*, 2006a).

However, QqTOF machines can only acquire multiple precursor ion spectra, but not neutral loss spectra. The absence of NLS hampers profiling of several glycerophospholipid classes, especially in positive ion mode. Once acquired, the data set of precursor ion spectra is "frozen," and is not amenable for further reprocessing or manual interpretation by concidering other fragment ions produced in alternative CID pathways. We note, however, that the mass resolution and accuracy of emerging hybrid

instruments with Fourier transform (Syka *et al.*, 2004) or Orbitrap (Makarov *et al.*, 2006) mass analyzers enable unequivocal determination of the elemental composition of fragment ions solely by their accurately measured masses, hence facilitating the in-depth structural characterization of lipid molecules (Ejsing *et al.*, 2006b). Therefore, the acquisition and interpretation of complete high-resolution MS/MS spectra (rather than their subsets, effectively used in PIS) offers important analytical advantages. Because of the elemental composition constraints, masses of plausible lipid precursors occupy well-spaced m/z slots, rather than populating continuously the entire m/z range. Hence, in many instances, spectra acquisition in scanning mode would be, in fact, impractical.

Here we demonstrate that data-dependent acquisition of full MS/MS spectra from all detectable (or, alternatively, preselected) precursors provides a rich lipidomics data set amenable to the versatile interpretation in a user-defined manner (Schwudke *et al.*, 2006). Rapid extraction of intensities of relevant fragment ions out of each spectrum from the complete MS/MS data set effectively emulates the unlimited number of precursor and neutral loss scans, without compromising the accuracy of identification and quantification of lipid species. Furthermore, in the course of postacquisition MS/MS spectra processing, logical operations could combine emulated scans into lipid structure–specific data interpretation routines, known as Boolean scans.

Alternatively, the inclusion list of plausible precursors could be collapsed down to a very few masses of interest. Hence, MS/MS experiments followed by the extraction of intensities of specific fragment ions from corresponding *dta* files, would effectively emulate the method of selected reaction monitoring (SRM) that dramatically enhances the detection sensitivity and specificity (Liebisch *et al.*, 2006). Rapid and flexible adjustment of data acquisition and processing routines that are tailored to the specific goals of a particular experiment would expand the gamut of generic analytical tools in lipidomics.

2. Procedure

2.1. Sample preparation for mass spectrometric analysis

Stock solutions of lipid standards were prepared in the specified concentrations in $CHCl_3$/MeOH/2-propanol 1:2:4 (v/v/v) containing 7.5-m*M* ammonium acetate. Before the analysis, samples were vortexed thoroughly and centrifuged for 5 minutes at 14,000×*g* (14,000 rpm) on a Minispin centrifuge (Eppendorf, Hamburg, Germany). Samples were loaded onto 96-well plates and sealed with aluminum foil.

2.2. Lipid extraction

Lipids were extracted according to Bligh and Dyer (Bligh and Dyer, 1959). Briefly, three volumes of methanol:chloroform = 2:1 (v/v) were added to 0.8 (v/v) volume aqueous samples. To achieve phase separation, 1 volume of water and 1 volume of chloroform were added, and the chloroform phase separated upon centrifugation was collected and dried. For quantitative analysis, non-naturally occurring lipid species were added as internal standards before extraction.

Where specified, total extracts were separated by TLC, and scrapped bands were extracted and analyzed as described (Schwudke *et al.*, 2006).

2.3. Cholesterol recovery and derivatization

Free cholesterol was converted into cholesteryl acetate as described previously (Liebisch *et al.*, 2006). Briefly, a 1:5 (v/v) mixture of acetyl chloride: chloroform was added to dried lipid extracts, and the solutions were incubated for 60 min at room temperature. Under these conditions, no transesterification of naturally occurring cholesteryl ester species occurred. For the quantification of cholesterol, internal standards of [25,26,26,26,27,27,27-D_7]-cholesterol and two non-naturally occurring cholesteryl ester species—heptadecanoate (CE 17:0) and behenate (CE 22:0)—were added to analyzed samples before lipid extraction.

2.4. Mass spectrometers

Lipid extracts were analyzed on a modified QSTAR Pulsar *i* quadrupole time-of-flight mass spectrometer (MDS Sciex, Concord, Canada) equipped with a robotic nanoflow ion source NanoMate HD (Advion BioSciences, Ithaca, NY). The instrument was calibrated in MS/MS mode using a synthetic lipid standard 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine as previously described (Ekroos and Shevchenko, 2002). Analytical quadrupole Q1 was calibrated according to the instructions from the manufacturer. Its resolution was adjusted such that, from selected precursors, either entire isotopic cluster (low-resolution settings) or only the monoisotopic ions (unit resolution settings), were transmitted. Mass resolution offsets were saved as separate settings tables. MS/MS experiments were performed at the collision energy offset of 40 eV. Ionization voltage at the NanoMate source was set to 950 V, gas pressure to 1.25 psi, and the source was controlled by Chipsoft (v. 6.3.2; Advion BioSciences). Lipid extracts were infused at the flow rate of *ca* 250 nl/min. A typical sample volume of 10 μl allowed more than 40 min of stable electro-spray time. The spraying stability was monitored by the total ion count (TIC) signal reported from survey TOF MS spectra in each DDA cycle.

Acetylated cholesterol and cholesteryl esters were quantified on a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Manchester, UK) by direct-flow injection analysis using a HTS PAL autosampler (Zwingen, Switzerland) and an Agilent 1100 binary pump (Waldbronn, Germany) with a solvent mixture of methanol containing 10 mM ammonium acetate and chloroform (3:1, v/v). A flow gradient was performed starting at 55 μl/min for 6 s followed by 30 μl/min for 1.0 min and then increased to 250 μl/min for another 12 s. The triple quadrupole mass spectrometer was equipped with an electro-spray ion source operated in positive ion mode under the following settings: capillary voltage 3.5 kV, cone voltage 50 V, collision energy 13 eV, collision gas argon, and collision gas pressure 1 mTorr. Both Q1 and Q3 quadrupoles were operated under better than unit mass resolution.

2.5. Data-dependent acquisition setup

In our experiments, data-dependent acquisition (DDA; also called information-dependent acquisition [IDA]) on a QSTAR mass spectrometer was controlled by Analyst QS (v. 1.1; Applied Biosystems). Depending on the targeted lipid classes and, respectively, required methods of post-acquisition processing of MS/MS spectra, DDA cycles could be set up in many different ways. A generic DDA cycle that provides MS/MS data for emulating both precursor and neutral loss scans typically includes one TOF MS survey scan for the time period of 2 s followed by two successive MS/MS experiments (each of 10 to 30 s) that target the same precursor ion. In the first MS/MS experiment, the analytical quadrupole Q1 is operated under the unit resolution settings and only transmits monoisotopic peaks of the fragmented precursors. At the same time, the TOF analyzer is set to detect low-molecular-weight fragments. Note that narrowing TOF m/z range improves the duty cycle (Chernushevich *et al.*, 2001) and, consequently, sensitivity. Subsequently, this spectrum will be taken for emulating precursor ion scans. In the next MS/MS experiment, Q1 is operated under the low resolution settings and the entire isotopic cluster of the fragmented precursor is transmitted. The TOF analyzer acquires spectra in higher m/z range, usually up to the precursor m/z (assuming that precursor ions are singly charged). Fragments detected in the second cycle are mostly used for emulating neutral loss scans. Note that in neutral loss scans, partial co-isolation of neighboring precursors of the same lipid class (e.g., of the two species that differ by one double bond in a fatty acid moiety and, hence, are spaced by 2 Da) does not affect quantification accuracy (Schwudke *et al.*, 2006). However, low mass resolution of Q1 improves ion transmittance, and, hence, enhances the analysis sensitivity. Upon completing the acquisition cycle, the instrument starts a new cycle, in which either the next precursor candidate is selected by considering the intensities of peaks in

the survey scan, or another precursor m/z is taken from the inclusion list. The m/zs of already fragmented precursors are excluded until the end of the analysis. It is always worth to include TOF MS survey scan in the DDA cycle, even if it is not required for the subsequent acquisition of MS/MS spectra. Recording TIC from the survey scan at each cycle helps to monitor ESI spray stability throughout the entire DDA experiment, which is essential for robust quantitative determinations. Once the experiment is completed, the acquired MS/MS spectra are exported as individual files in *dta* format. All *dta*s produced in the same analysis are collected into one data set folder for subsequent interpretation.

Because of much slower data acquisition speed, DDA on triple quadrupole mass spectrometers is usually navigated by inclusion lists, in which precursor m/z are either precalculated or deduced from rapid, low-resolution, lipid class–specific PIS or NLS. If samples of limited volumes are infused into a mass spectrometer at 10- to 50-μl/min flow rate, TIC does not reach a plateau, but rather is shaped as a peak. All scans above the half-height of the TIC are then averaged, and the centroided spectrum is produced from the combined continuum spectra. The centroided peak list is exported as a spreadsheet for correcting the peak intensities within partially overlapping isotopic clusters. The peaks detected at the signal-to-noise ratio above the value of 3, and matching the expected masses of species of the targeted lipid class are then compiled into inclusion list for selected reaction monitoring (SRM). The analysis in SRM mode is particularly useful in the quantification of low abundant lipid species. SRM spectra are processed as described above without conversion of continuum to centroided data. Quantification is performed using calibration plots produced by spiking analytes with non-natural synthetic lipid species used as internal standards (Liebisch *et al.*, 2004).

2.5.1. Inclusion lists for DDA-driven lipid profiling

Inclusion lists are important elements of DDA-driven lipidomic routines. Despite structural divergence of glycerophospholipids, species of different classes often have overlapping nominal masses, although their exact masses might differ. Because in MS/MS experiments on a QqTOF mass spectrometer Q1 quadrupole mass analyzer selects precursors with the unit or lower mass resolution, a single inclusion list serving all plausible glycerophospholipid precursors could be compiled. Inclusion lists target the analysis on interesting precursors, irrespectively of other peaks observed in survey scan spectra. This, effectively, balances the exploratory and focused strategies in lipidomics profiling.

Quantification of cholesterol and its esters serves as a good example of the focused lipidomics scenario. In this case, the selected m/zs are analyzed in SRM mode regardless of their intensity ratios to other peaks in the survey spectrum. Alternatively, the exploratory analysis could target all common

glycerophospholipid precursors, or triacylglycerol (TAG) precursors, or ceramide precursors, among others. Once a survey scan spectrum is acquired, the listed precursors are ranked by the abundance of their detectable peaks and analyzed accordingly. Generic inclusion lists used by Schwudke *et al.* (2006) for DDA on a QSTAR Pulsar *i* quadrupole time-of-flight mass spectrometer comprised 100 precursor entries within the range of m/z 690 to 890 for glycerophospholipids and m/z 790 to 990 for TAGs.

3. Automated Interpretation of MS/MS Spectra Acquired in DDA Experiments

A typical DDA experiment produces the pool of *dta* files, each of which represents a single MS/MS spectrum acquired from the selected lipid precursor. The first line of each *dta* file contains the precursor mass and charge, followed by a peak list of centroided m/z and intensities of detected fragments. All *dta* files acquired in the same experiment are processed by LipidInspector (available free of charge from Scionics Computer Innovations, at www.scionics.de). Depending on acquisition mode polarity, collision-induced dissociation of molecular ions of glycerophospholipids yields fragments of head groups and/or fatty acid moieties, either as ions or neutrals. LipidInspector uses the predefined list of m/z of these specific fragments and neutral losses. In each MS/MS spectrum, LipidInspector identifies fragments matching the masses of list entries with defined tolerance and, if requested, checks if they also meet other predefined selection criteria (Fig. 10.1).

Subsequently, LipidInspector evokes a naming routine that annotates the corresponding precursors considering their intact masses and matched fragments. We note that m/z of precursors are either determined in full TOF MS survey scans, or within 0.2-Da window around m/z taken from the inclusion list, Importantly, no residual precursor peaks detectable in the acquired MS/MS spectra are considered. Typically, glycerophospholipid precursors are designated with their lipid class (PE, PC, PG, and so on) and sum composition (the total number of carbon atoms and double bonds in both fatty acid moieties). LipidInspector reports the matched peaks along with their intensities as a tab-delimited text file, which could be directly opened in Microsoft Excel. For the quantitative analysis, isotopic correction of peak intensities is performed as described by Han and Gross (2005).

Note that the spectra obtained with low (unit or less) and high (over ~8000 FWHM) mass resolution are interpreted differently by LipidInspector. For example, in high-resolution spectra, it is possible to distinguish, within the same lipid class, species with two ester bonds and species having one

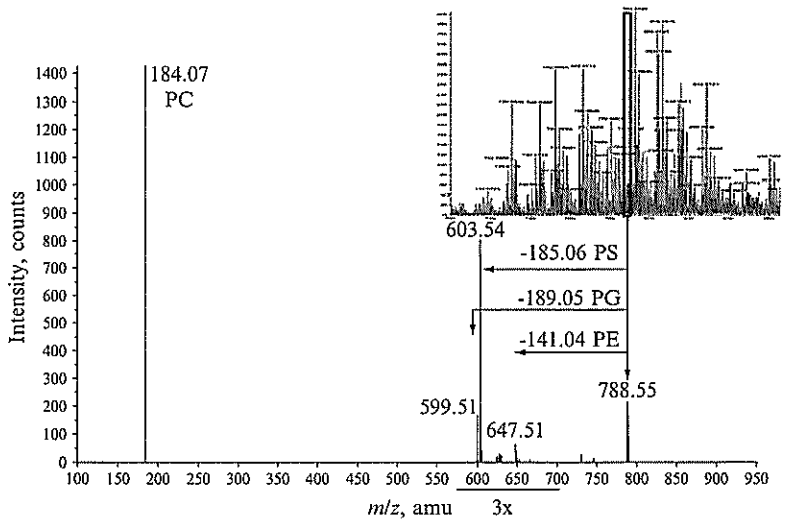

Figure 10.1 Mass spectrum of a total lipid extract acquired at the QSTAR mass spectrometer in TOF MS mode. MS/MS spectrum acquired from the precursor ion with *m/z* 788.55 (framed in the upper pane) contains specific signature ions of several isobaric lipid species co-selected by the quadrupole analyzer. For example, the fragment with *m/z* 184.07 was produced from the phosphocholine group of sphingomyelins and phosphatidylcholines, and the fragment with *m/z* 647.51 was produced by a neutral loss of phosphoethanolamine group from the head group of the PE precursor (other neutral losses are also annotated in the figure). For interpreting each spectrum, the LipidInspector program uses the list of characteristic masses and mass differences relative to m=z of the precursor. Upon opening the spectrum, LipidInspector identifies those ions and, knowing the exact mass of the fragmented precursor, annotates lipid species by their lipid class and sum composition. Here the software concluded that the peak with *m/z* 788.55 isolated by Q1 and fragmented, in fact, stands for PC 36:1 (exact *m/z* 788.6163), PS 36:2 (*m/z* 788.5436), PG 36:4 (*m/z* 788.5436), and PE 39:2 (*m/z* 788.6163). Intensities of corresponding fragment peaks were reported for the subsequent quantification of species. Note that because no chromatographic separation is involved, intensities of fragment peaks are characteristic of the concentrations of corresponding components and do not require integration in the time dimension.

ester and one ether bond, even if they are isobaric. The difference of 0.0364 Da ($m_O - m_{CH4}$) between their exact masses could be confidently determined in MS/MS mode because, compared to survey scans, chemical noise is much reduced, and it is also unlikely that specific fragments (contrary to precursors) overlap with other isobaric ions. Interestingly, hybrid instruments with FT or Orbitrap analyzers could reliably distinguish the corresponding species even in survey scans, if acquired at the target mass resolution exceeding 100,000 (FWHM), with no recourse to MS/MS (Schwudke, 2007).

In experiments on a triple quadrupole mass spectrometer, SRM spectra can be exported as peak lists and analyzed by LipidInspector or by dedicated macros in Microsoft Excel (Liebisch *et al.*, 2004).

4. Boolean Scans: A Novel Scan Type Enabled by Postacquisition Data Processing

Precursor ion scans and neutral loss scans effectively identify molecules that, upon CID, produce either the same fragment, or fragments with the same mass offset relative to the intact precursor mass. In both cases, the characteristic mass (or mass difference) is fixed and is looked upon in all acquired MS/MS spectra, irrespectively of precursor *m/z*. Several CID pathways, however, produce combinations of fragments, whose masses differ in species of the same class, yet they are accountable by the same arithmetical equation. It is hardly possible to use these masses in conventional PIS or NLS, because taken alone, they are not specific for the lipid class. However, these combinations of fragment masses could be recognized in MS/MS spectra, if several selection criteria bundled by Boolean logical operations, are simultaneously applied. These complex data interrogation routines, termed Boolean scans, are performed in parallel with emulating conventional PIS and NLS and reveal important structural details regarding fragmented lipid precursors.

Figure 10.2A presents a part of the TOF MS survey scan spectrum of bovine heart polar lipid extract and two representative MS/MS spectra, acquired from precursors with *m/z* 792.56 (Fig. 10.2B) and *m/z* 752.57 (Fig. 10.2C) in the course of DDA-driven profiling. LipidInspector determined that both precursors belong to phosphatidylethanolamine (PE) class, because intense peaks of the neutral loss $\Delta m/z$ 141.02 products were observed at *m/z* 651.540 and *m/z* 611.544. Accurate mass of the latter fragment indicated that it was an ether lipid, although it was not possible to tell whether it belonged to the 1-*O*-alkyl or 1-*O*-alk-1′-enyl (plasmalogen) class.

In Positive ion mode plasmalogen species, contrary to 1-*O*-alkyl PE-species, produce a pair of characteristic fragments that retain moieties of the fatty acid and fatty alcohol, respectively, and the sum of their *m/z* equals M+2, where M stands for the neutral mass of a plasmalogen lipid (Zemski Berry and Murphy, 2004). Therefore, LipidInspector was set to identify in each spectrum, besides other fragment peaks, pairs of fragments $(m/z)_1$ and $(m/z)_2$ with intensities $I(m/z)_1$ and $I(m/z)_2$ that meet the following Boolean-type expression:

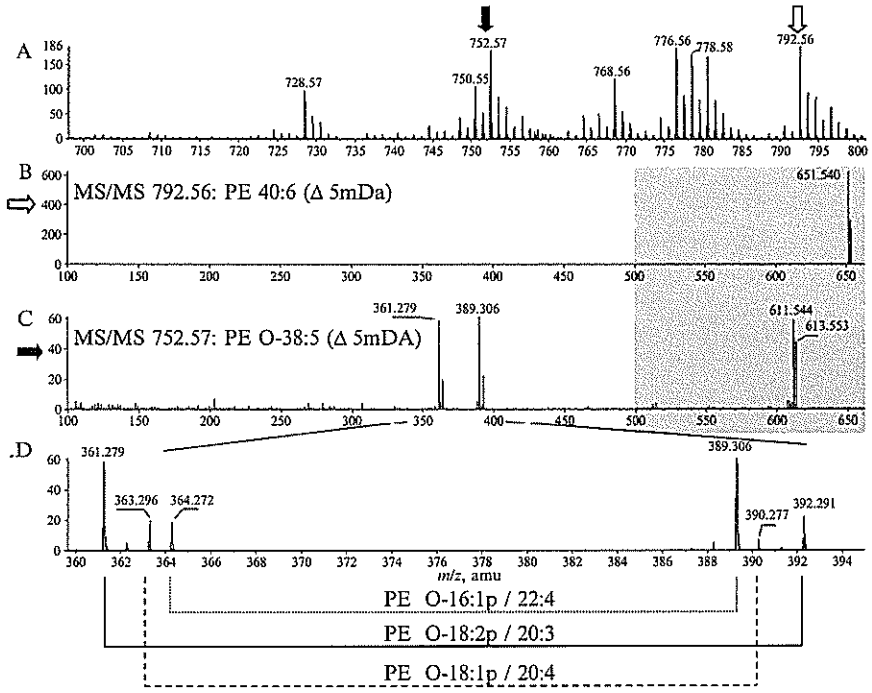

Figure 10.2 Identification of PE 1-O-alk-1′-enyl species by the plasmalogen-specific Boolean scan. Panel A: TOF MS survey spectrum of the polar extract of bovine heart. Although all plausible precursors were fragmented during DDA-driven MS/MS experiments, for the sake of clarity only two representative examples are presented in Panels B and C. The assignment of detectable fragments is explained in the text. Panel D: Zoom of the range of *m/z* 350 to 400 from the spectrum in Panel C. Using the plasmalogen-specific Boolean scan, LipidInspector identified three individual molecular species of plasmalogens, having exactly the same sum composition.

$$\{250 < [(m/z)_1; (m/z)_2] < 450\} \text{ AND } \{(m/z)_1 + (m/z)_2 = \mathrm{M} + 2\}$$
$$\text{AND } \{\mathrm{I}(m/z)_1/\mathrm{I}(m/z)_2 = 0.3 \pm 0.1\} \text{ AND}$$
$$\{\text{peak@M} - 140\} = \text{TRUE}$$

The above expression effectively requested that a pair of candidate fragments should simultaneously meet the following four criteria:

1. Both fragments are within some practical range of *m/z* (in this case, above *m/z* 250 and below *m/z* 450).
2. The sum of their *m/z* equals M + 2.
3. The ratio of their intensities equals approximately 0.3 (with *ca* 30% tolerance).
4. Finally, the same spectrum contains a fragment corresponding to the neutral loss of PE head group.

We additionally requested (although, for presentation clarity, this is not included in the Boolean expression above) that nominal *m/z* of one of the two fragments should be even (this mass corresponds to the loss of the neutral fragment containing a fatty acid ester), whereas nominal *m/z* of another fragment should be odd (it is produced by the loss of the neutral fragment containing the PE head group and a vinyl alcohol; Zemski Berry and Murphy, 2004). If such a fragment pair was identified, LipidInspector annotated the precursor as a plasmalogen and reported the intensities of corresponding fragment peaks for further plasmalogen-specific quantification. We note that the analysis was not affected by co-fragmentation of several plasmalogen species (see Fig. 10.2), or ether PEs or lipids from other classes.

In the current version of LipidInspector, these criteria are bundled under "plasmalogen specific scan," which could be optionally selected by the user, along with other precursor and neutral loss scans, in a check-box menu. However, Boolean scans are generic and, in principle, could take advantage of a variety of CID mechanisms. Therefore, in the future, logical expressions should be programmed such that educated users could link several arithmetic equations and inequalities by various logical operations within a generic meta-language framework.

5. Comprehensive Characterization of Total Lipid Extracts by DDA-Driven Profiling

DDA-driven profiling characterizes the molecular composition of multiple lipid classes in parallel, which makes it a valuable tool for lipidomics screens. To test its performance, we applied the method for the partial characterization of *Caenorhabditis elegans* lipidome. Total lipid extracts were directly infused into a QSTAR Pulsar *i* mass spectrometer using NanoMate HD ion source. Tandem mass spectra were acquired under DDA control and then interpreted by LipidInspector program. While screening MS/MS spectra, LipidInspector was set to emulate three lipid class–specific NLSs, one lipid class–specific PIS, and one Boolean plasmalogen-specific scan (Fig. 10.3). As anticipated, PEs and PCs accounted for the major peaks detected in the survey TOF spectrum (Fig. 10.3A,B, and C). Interestingly, the major plasmalogen species (PE O-18:1p/18:1) was identified at *m/z* 730.58, while at *m/z* 732.58 NLS for $\Delta m/z$ 141.02, together with the accurately determined *m/z* of the corresponding fragment, identified the ether lipid PE O-36:1. With the exception of another two plasmalogen species, other peaks detectable by the same NLS were diacyl PEs. Peaks at *m/z* 820.74 and 822.74 were ammonia adducts of TAG 48:2 and TAG 48:1,

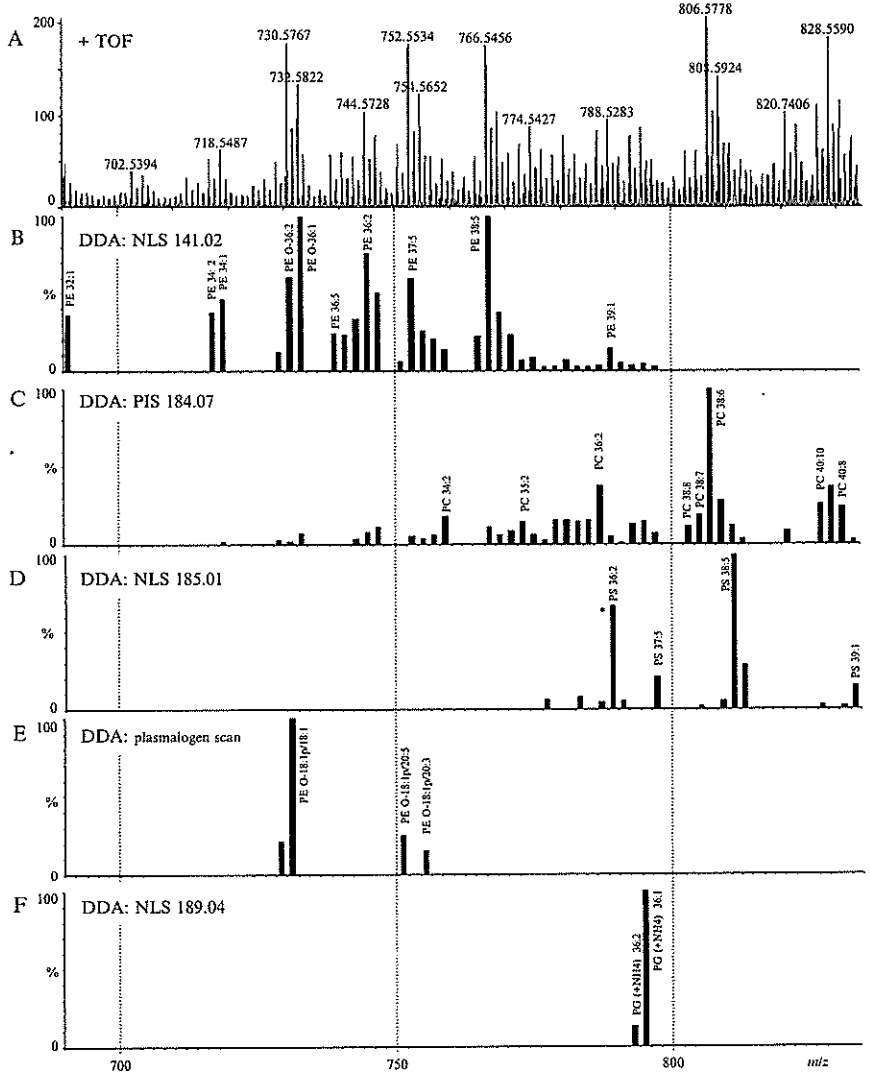

Figure 10.3 DDA-driven profiling of a total lipid extract from *Caenorhabditis elegans*. (A) Survey TOF MS spectrum. (B, C, D) Lipid class–specific profiles obtained by interpreting MS/MS spectra by LipidInspector program, which emulated : (B) neutral loss scan $\Delta m/z$ 141.02, specific for all species having a phosphatidylethanolamine head group (PEs, ether PEs, and plasmalogens); (C) precursor ion scan for the fragment with m/z 184.07, specific for PCs; (D) neutral loss scan for $\Delta m/z$ 185.01, specific for PSs; and (E) Boolean scan specific for plasmalogens. Details are presented in the text and in Fig. 10.2. (D) neutral loss scan $\Delta m/z$ 189.04, specific for PGs in the context of this experiment. The intensities of peaks of identified species were normalized to the total intensity of all species within the class. (With permission, from Schwudke, D., Oegema, J., Burton, L., Entchev, E., Hannich, J.T., Ejsing, C. S., Kurzchalia, T., and Shevchenko, A. (2006). Lipid profiling by multiple precursor and

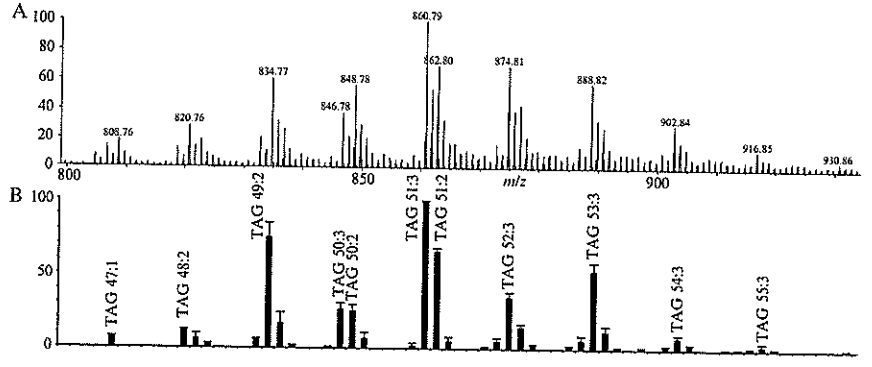

Figure 10.4 The composition of TAGs in *Caenorhabditis elegans*. (A) TOF survey spectrum of TAGs extracted from a TLC band. Note that peaks represent ammonium adducts of TAGs. (B) TAG profile produced by the emulation of 69 parallel neutral loss scans by LipidInspector. TAGs are annotated by their sum compositions and aligned with the spectrum in (A). To calculate the relative abundance of TAG peaks, the intensities of fatty acid loss fragments in each MS/MS spectrum were summed up. (With permission, from Schwudke, D., Oegema, J., Burton, L., Entchev, E., Hannich, J.T., Ejsing, C. S., Kurzchalia, T., and Shevchenko, A. (2006). Lipid profiling by multiple precursor and neutral loss scanning driven by the data-dependent acquisition. *Anal. Chem.* 78, 585–595.)

although the bulk of extractable TAGs were detected at the higher m/z range and are presented separately in Fig. 10.4.

Altogether, DDA-driven identification recognized 90 glycerophospholipids with unique sum compositions and provided their relative (within a given class) quantification in a single experiment. TAGs were analyzed in a separate experiment from the extract of the TLC band. While deciphering MS/MS spectra, LipidInspector emulated 69 scans specific for the neutral loss of fatty acid moieties from molecular adducts of TAGs with ammonium cations. We assumed that fatty acids comprised 9 to 22 carbon atoms and 0 to 6 double bonds. The identified TAGs were annotated with sum formulas and their relative abundance was determined (see Fig. 10.4). However, in all cases the emulated multiple neutral loss scans identified fatty acid moieties that were present in the fragmented precursors. Assuming that the abundance of neutral loss products does not strongly depend on fatty acid moieties and their position at the glycerol backbone, it was also possible to estimate the relative abundance of species with individual fatty acid compositions or, at least, identify major components in the mixture of isobaric precursors (Schwudke *et al.*, 2006).

This and other experiments demonstrated that complex lipid mixtures are fully amenable to DDA-driven profiling, which provides more specific information on the identity of lipid species compared to conventional precursor and neutral loss scans. In perspective, DDA profiling guided by comprehensive

complete lipidomic data set when all fragment ions yielded from all possible precursors become detectable. Once such a data set is acquired, the software can re-process it in several user-specified ways with no recourse to repeating "wet" biology or MS/MS acquisition experiments.

6. Selected Reaction Monitoring in Quantification of Cholesterol and Cholesteryl Esters

Compared to QqTOF instruments, triple quadrupole machines QqQ lack mass resolution and scan speed of the TOF analyzer, and therefore acquiring full MS/MS spectra from all possible precursor masses is impractical. However, the DDA approach specifically targets precursors of interest and use selected reaction monitoring (SRM) instead of conventional PIS for their quantification. Quantitative analysis of free cholesterol and cholesteryl ester in SRM mode serves as a good example. Cholesteryl esters (CE) are readily ionized as ammonium adducts, which produce a fragment ion of m/z 369.3 upon their CID. To determine free cholesterol content, it was first acetylated and ammonium adduct of the cholesterol acetate subjected to MS/MS analysis. Quantification was achieved in SRM mode using spiked synthetic internal standards—that is, D_7-cholesterol and CE17:0 and CE22:0—as reference compounds (Fig. 10.5) (Liebisch *et al.*, 2006).

SRM outperforms conventional or DDA-driven, lipid class–specific scans in the analysis of a small number of very low abundant precursors, especially if only a single fragment ion should be monitored and there is no need in acquiring full MS/MS spectra. For example, a human LDL fraction was analyzed in six replicates by PIS m/z 369 and by SRM targeting only CE precursors identified by survey PIS (see Fig. 10.5). PIS was performed within the range of m/z 600 to 760 with a scan speed of 200 amu/s. Assuming that peak width at half maximum was close to 0.7 amu, the effective scan time was 3.5 ms per analyzed species. In the direct flow injection analysis performed as described above, 50 scans were averaged to produce the spectrum for reliable quantification. However, in this analysis, each precursor of interested was analyzed for only 175 ms. In contrast, within the same time frame, SRM analysis of CEs, including acetyl ester of free cholesterol (see Fig. 10.5), was performed with a dwell time of 70 ms and 18 SRMs were averaged. Hence, each precursor was analyzed for 1260 ms. This difference in scan time enhanced the quantification accuracy. Major species, such as CE 18:2, 18:1, or 16:0, were quantified with CVs below 5% in PIS mode, compared to ~2% using SRM. However, low abundant species like CE 20:3 or CE 24:1 were quantified with CVs of only ~15% in PIS, whereas less than ~5% was achieved by SRM. In summary,

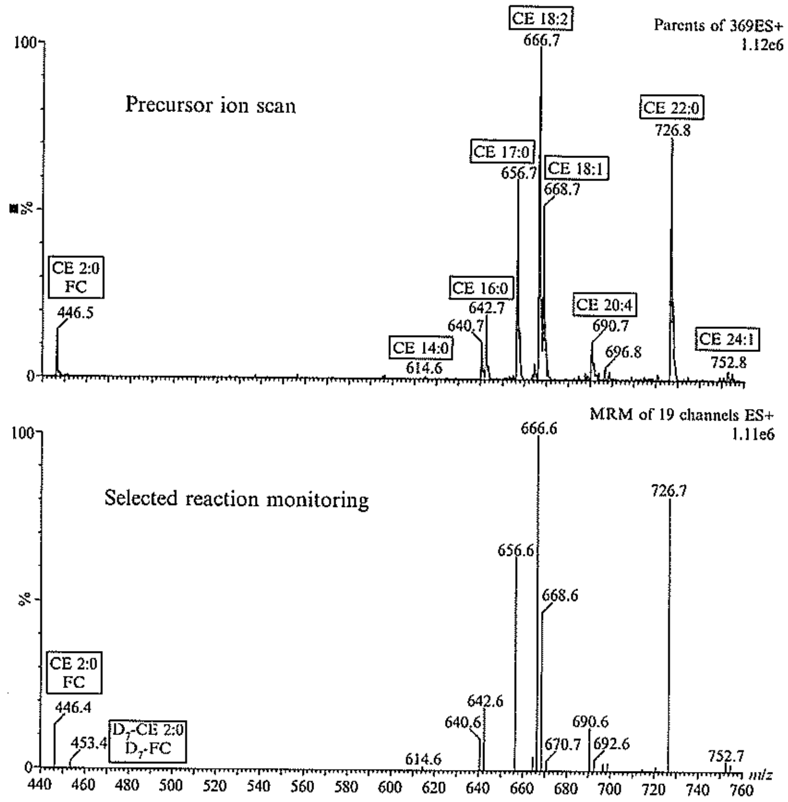

Figure 10.5 PIS m/z 369 and SRM for CE and FC quantification. Free cholesterol was acetylated before analysis. The upper panel shows a PIS m/z 369.3 of a human LDL fraction in positive ion mode specific for CE. The lower panel shows the spectrum of the same sample reconstructed from SRM data. Note the internal standard for quantifying free cholesterol D_7–CE 2:0 was detected using different transition (m/z 453.4→376.3). CE, cholesteryl ester; FC, free cholesterol. (See color insert.)

for a moderate number of lipid precursors, SRM on triple quadrupole instruments produced significantly higher accuracy, compared to experiments in scanning mode. SRM driven by the inclusion list of rapid PIS used as a survey scan is especially beneficial for low abundance species and samples with known lipid species composition.

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