

Lipid Profiling of Complex Biological Mixtures by Liquid Chromatography Mass Spectrometry Using a Novel Scanning Quadrupole Data Independent Acquisition Strategy

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Abstract:	A scanning quadrupole data independent acquisition (DIA) method is described and applied to targeted and untargeted lipidomic identification strategies. The fundamental principle of the scanning quadrupole DIA method is illustrated using lipid applications and the benefits of the fast quadrupole scanning technique are highlighted from both a qualitative and quantitative perspective. The high specificity afforded by the scanning quadrupole is shown to provide identifications of high confidence, whilst the benefits for the analysis of more complex samples, namely improved identification and quantitative accuracy, can be for the most part attributed to the improved specificity of the method. The use of both open and closed source informatics tools is described to emphasise the analytical performance characteristics of the methods and to demonstrate benefits for lipid identification and quantitation.

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Lipid Profiling of Complex Biological Mixtures by Liquid Chromatography Mass Spectrometry Using a Novel Scanning Quadrupole Data Independent Acquisition Strategy

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Abstract

A novel data independent acquisition method is detailed that incorporates a scanning quadrupole in front of an orthogonal acceleration TOF mass analyser. This approach is described and the attributes are compared and contrasted to other DIA approaches. Specific application of the method to both targeted and untargeted lipidomic identification strategies is discussed, with data from both shotgun and LC separated lipidomics experiments presented. The benefits of the fast quadrupole scanning technique are highlighted, and include improvements in speed and specificity for complex mixtures providing high quality qualitative and quantitative data. In particular the high specificity afforded by the scanning quadrupole improves qualitative information for lipid identification.

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Introduction

Significant technology advances have enabled the analysis of complex biological samples by liquid chromatography (LC) coupled with mass spectrometry (MS). The ability to determine not just the presence of specific molecules, but the abundance and response of these molecules to perturbations has been a significant driver in furthering the understanding of biochemical processes and disease onset and progression [1].

The analysis of lipids has become of increasing interest owing to their important function as key constituents of cellular membranes, as mediators of many biological functions and their role in many disease processes such as cardiovascular disease [2], diabetes [3] and cancer [4]. Mass spectrometry based approaches have dominated the developed and applied analytical strategies for the analysis of complex biological lipid mixtures. The approaches used for lipid profiling can be categorised as either targeted or untargeted. In targeted workflows, a common strategy is to focus on known lipid molecules, specifically selected for MS analysis, by coupling LC separation with multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer. These targeted methods have benefits with regard to quantitative performance, providing good linearity, accuracy and precision, but do not provide a comprehensive qualitative and quantitative overview of the lipid and lipid related molecules present within a sample.

In the case of untargeted lipidomics, approaches include both shotgun analysis by infusion of complex lipid mixtures [5], analysis by HPLC and UPLC separation combined with MS analysis [6,7] and the use of both data dependent [8] and data independent [9] MS acquisition strategies, (DDA and DIA, respectively). However, the comprehensive analysis of complex lipids, otherwise known as lipidomics [10], is still challenged due to

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the complexity, and isomeric molecules, of the endogenous lipids present in biological matrices such as human plasma. Previously, several untargeted lipidomics approaches have been described, including the use of data independent LC-MS^E [11] and the generation and use of collisional cross section values as an additional discriminating property for lipidomics profiling [12]. Despite these methodological advances the need for new analytical approaches to profile complex biological mixtures still exists. The implementation and application of a new scanning quadrupole data independent approach (SONAR) is introduced and described, and the specific application to lipid analysis is discussed.

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Experimental

Reagents and Materials

LipidoMIX, a synthetic mixture of various lipid classes and bovine heart extract standards were purchased from Avanti (Alabaster, Alabama, USA). LC-MS grade solvents were used to prepare mobile phases. Water, acetonitrile (ACN) and isopropanol (IPA) were purchased from Fisher Scientific (Loughborough, UK). Additional reagents including formic acid, Leucine Enkephalin, sodium formate (NaF) and ammonium formate were obtained from Sigma-Aldrich (Poole, UK).

Sample preparation

LipidoMIX standard was added to bovine heart tissue extract in a 1:1 ratio, providing a stock solution of LipidoMIX at a concentration of 5 µg/mL. A dilution series was constructed by subsequent dilution of the stock solution to provide working solutions of 3 and 1 µg/mL. Lipid extracts were also prepared from mouse testis tissue as previously described [13]. Briefly, approximately 50 mg of testis tissue was transferred to bead-beater tubes containing 1.5 mL of prechilled methanol/water (1:1) and homogenized using a Precellys bead beater. The resulting mixture was centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant (aqueous extract) was removed and the remaining tissue pellet treated with 1.6 mL prechilled dichloromethane/methanol (3:1) prior to further homogenization. The mixture was additionally centrifuged at 10,000g for 10 min and the supernatant collected in glass vials. Aliquots of 50 µL were then transferred to total recovery vials for LC-MS analysis.

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LC-MS configuration

LC separations were performed using a ACQUITY I-class UPLC system equipped with a CSH C18 1.7 µm, 2.1 x 100 mm analytical column (Waters Corporation, Milford, USA). Mobile phase A consisted of ACN/water (60:40, v:v) containing 10 mM ammonium formate with 0.1% (v/v) formic acid, whilst mobile phase B was IPA/acetonitrile (90:10, v:v) containing 10 mM ammonium formate with 0.1% (v/v) formic acid. Lipids were separated with a gradient of 40 to 99% mobile phase B over 18 min at a flow rate of 400 µl/min. The analytical column temperature was maintained at 55ºC. The lock mass compound, Leucine Enkephalin (200 pg/µl) was delivered at 5 µl/min to the reference sprayer source of the mass spectrometer.

Mass spectrometric analysis was performed using a Xevo G2-XS QTOF mass spectrometer (Waters Corporation, Wilmslow, UK). The mass spectrometer was operated with a resolution of 35,000 FWHM with analyses performed in both positive and negative mode ESI. The ion source block temperature was set to 120ºC and capillary voltage to 3.0 and 1.2 kV for positive and negative ionisation mode respectively. The time-of-flight (TOF) mass analyzer was externally calibrated with sodium formate from *m/z* 50 to 1200. LC-MS data were collected in a novel data independent mode of acquisition (SONAR) over the same mass range used for calibration. In this acquisition mode the quadrupole is continuously scanned between *m/z* 400 to 1100, with a constant quadrupole transmission width of approximately 10 Da. The oa-TOF records mass spectra as the quadrupole scans and stores the MS data into two hundred discrete bins. Two data functions (modes) are acquired in an alternating fashion, differing only in the collision energy applied to the gas cell. In the low energy MS1 mode, data are collected at constant gas cell collision energy of 6 eV. In

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the elevated energy MS2 mode, the gas cell collision energy is ramped from 14 eV to 45 eV (per unit charge). As such, the resulting data set contains both precursor ions and all associated fragment ions. The spectral acquisition time in each mode was 0.2 s with a 0.02 s interscan delay. The reference sprayer was sampled every 60 s and the data post-acquisition lock mass corrected.

Data processing, database searching, and library queries

SONAR quadrupole scanning DIA data were processed using either SimLipid (PREMIER Biosoft, Palo Alto, CA) for structural elucidation and identification purposes or Skyline (University of Washington, Seattle) [14] for targeted quantitative analyses. SimLipid processes raw data and matches experimental fragment ions with *in-silico* generated product ion spectra from LipidMaps [15]. A mass tolerance of 5 ppm was applied for both precursor and product ions. The elemental compositions of matched fragment ions derived from the SimLipid MS/MS libraries were used to direct targeted quantitative analysis with Skyline open source informatics. Additional manual annotation was also conducted using LipidMaps. Relative quantitative and statistical analyses of the data were conducted with Progenesis QI (Nonlinear Dynamics, Newcastle upon Tyne, UK) and EZinfo (MKS Data Analytics Solutions, Umeå, Sweden), respectively. 2D peak detection analysis was conducted with DriftScope (Waters Corporation).

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Results

SONAR is a novel scanning quadrupole DIA method that has a significantly improved selectivity/sensitivity characteristic compared with other DIA approaches. Supplemental Table 1 and Supplemental Figure 1 provide a qualitative comparison of SONAR and the other DIA methodologies available. It can, for example, be configured to give selectivity similar to a traditional data directed approach using a 3 Da isolation window at roughly an order of magnitude higher sensitivity than other DIA strategies (at the same selectivity). Moreover, this method is sufficiently fast to be compatible with rapid ultra performance liquid chromatography (UPLC) separations. To explain how these advantages arise, it is appropriate to first review the scanning single quadrupole mass spectrometer. To generate a spectrum, the isolation window produced by a resolving quadrupole is scanned over a specified *m/z* range containing all species of interest. It is conventionally arranged for the width (W) of the isolation window to remain constant over the mass range. An example spectrum containing a single species generated by a scanning quadrupole with a window of 100 Th is shown in Figure 1A. It is known [16] that, in the absence of interference and with a sufficient number of ions, the *m/z* of this species can be determined with an uncertainty Δm that is significantly smaller than the width of the isolation window. For the usual Poisson ion arrival (counting) statistics, Δm is proportional to $W/\sqrt{N}$ where N is the number of detected ion arrivals for the species in question. This can be rewritten as $\Delta m \propto \sqrt{(W/S)}$, where S is the strength of the ion signal from the instrument source (since for a scanning quadrupole peak, $N \propto W.S$). For a given isolation window, the uncertainty can therefore, in principle, be made arbitrarily small given a sufficiently strong signal (large S). In order for a scanning quadrupole instrument to be compatible with, and accurately

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define, a narrow chromatographic peak, it is necessary for the quadrupole to scan over the *m/z* range of interest with a period that is much smaller than the width of the peak. Modern quadrupole analysers are capable of scanning at over 10,000 *m/z* units per second, resulting in an *m/z* range of 500 Th being profiled at a rate of at least 20 Hz, which is sufficiently fast for the majority of LC applications.

In a Q-TOF mass spectrometer, the detector in the single quadrupole instrument is replaced by a collision cell followed by an orthogonal acceleration time-of flight mass analyser (oa-TOF). TOF mass analysers typically have native acquisition rates exceeding 10,000 spectra/second (although this is usually reduced by grouping spectra before they are stored). This means that it is possible to acquire several hundred TOF spectra within each quadrupole scan (Figure 1B), emulating the detection system in a scanning single quadrupole. However, each point in the quadrupole spectrum is now replaced by a high resolution oa-TOF mass spectrum. At elevated collision energies, ions mass measured by the oa-TOF are predominantly fragments ions that have been produced from precursor ions transmitted through the quadrupole. From the resulting TOF mass spectrum, the *m/z* of a fragment ion may be determined with an uncertainty proportional to $1/(R.\sqrt{(S)})$, where R is the resolution of the oa-TOF and S is the source signal strength as before. On the other hand, given a fragment *m/z*, the scanning quadrupole information preserved by the fast oa-TOF acquisition system allows the *m/z* ratio of the corresponding precursor to be determined with an uncertainty that is proportional to $\sqrt{(W/S)}$. With this method of acquisition, each fragment *m/z* is therefore automatically accompanied by a precursor *m/z*. Moreover, the precursor *m/z* can be measured with an uncertainty that is considerably smaller than the width of the transmission window, typically 1/20th. To illustrate this, extracted quadrupole spectra

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for seven fragments of the parent shown in Figure 1A are shown in Figure 1C. The similarity of the extracted quadrupole profiles can clearly be used to associate fragments with precursors and with each other. In contrast, in an acquisition strategy in which the quadrupole is stepped across the *m/z* range with only small overlaps, the precursor *m/z* is known with an uncertainty of +/- W/2 where W is the width of the quadrupole transmission window. The advantage of the scanning method over the stepped approach therefore scales in proportion to $\sqrt{(W.S)}$, increasing both with the isolation width and the source signal strength. Experiments have shown that the improvement in precursor *m/z* specificity is very often an order of magnitude, or more, for the same isolation width W. In addition, the time taken to step the quadrupole across the chosen *m/z* range is frequently comparable to the peak width in ultra performance LC experiments (>1second), limiting the application of the stepped approach for quantitative analyses.

In the acquisition mode utilised in this paper, termed SONAR, alternate datasets are taken in low (MS1) and elevated (MS2) collision energy mode (Figure 1C). During each low and elevated energy segment, the quadrupole isolation window is scanned linearly between two user-selected positions and 200 TOF spectra are acquired. In the work presented here, the scan duration was 0.2 s. In the elevated energy mode, the collision energy can be ramped between two values, which are chosen to optimise fragmentation efficiency at each quadrupole position.

Initial evaluation of the scanning quadrupole DIA acquisition technique and its applicability to lipidomic applications was performed on a mixture of lipid species spiked into a bovine heart extract. Resolving lipids chromatographically on the basis of class has shown to reduce complexity and thereby aid identification [17]. However,

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even when combining this method of chromatography with a broadband DIA method, where no quadrupole isolation occurs, co-eluting species still prove problematic. Figure 2(A) represents a chromatographic separation of a complex mixture spiked with the LipioMIX standard where the presence of low-abundance lipids and contaminants can be seen. Shown clock-wise is the presence of three co-eluting lipids within a low abundant chromatographic peak (marked with an asterix) eluting at 6.1 min within an area of extreme lipid co-elution. Data acquisition using DIA approaches that employ wider or no mass isolation window would generate a mixed fragmentation spectrum, whereby multiple fragment ions are assigned to the same precursor ions present. However, analysis via the rapid scanning quadruople DIA approach provides an additional degree of specificity. This is illustrated for three of the co-eluting components with different precursor *m/z* that are clearly separated in the scanning quadrupole *m/z* domain, illustrated by the 2D map of quadrupole *m/z vs.* TOF *m/z* and the quadrupole separation, (Figure 2(B)). Detection in the quadrupole *m/z* domain of the quadrupole resolved lipids eluting at 6.1 min was conducted using the 2D peak algorithm of DriftScope (Figure 2(C)) from product ion spectra can be extracted. The elevated collision energy data for these precursors can consist of similar product ion spectra, particularly for the lipids with precursor ions *m/z* 634.4, 674.5 and 745.6, as shown by the resolved MS2 spectra in Figure 2(D). In summary, the selective nature of the SONAR DIA method allows a lipid of interest (*m/z* 634.4) to be clearly distinguished from the potential phosphocholine (PC)/sphingomyelin (SM) co-eluting lipids and background species. The increased specificity in this example is provided by the scanning quadrupole, allowing specific precursor (MS1) and associated product ion (MS2) information to be aligned on the basis of the quadrupole *m/z* position during the scan.

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The non-resolved spectral equivalent of the peak eluting at 6.1 min, representing broadband MS2 DIA detection, including contaminant matrix ions, is shown in Supplemental Figure 2, illustrating that scanning quadrupole isolation of the DIA data, as shown in figure 2(D), significantly reduced spectrum complexity.

In addition to discovery or qualitative identification based strategies, SONAR data can be utilised for targeted quantification studies. Figure 3 illustrates the quantitative performance of SONAR based on a dilution series of the same LipidoMIX standard sample over two orders of dynamic range. It should be noted that the dilution series data presented is not intended to show the total linear dynamic range of the technique, but simply to illustrate the relationship between intensity and concentration. Analysis of the acquired data for a representative lysophosphatidylcholine (LPC) 18:1 is presented within Skyline open source informatics. The results highlight a number of interesting points about the acquisition method and nature of the acquired data. Firstly, despite the speed of the chromatographic separation and the concomitant peak widths (6 seconds at 10% peak height), rapid scanning of the quadrupole and high data acquisition rates of the oa-TOF allow for a significant number of data points across the peak. In this example, acquiring data at rates of 10 Hz provides greater than 24 points across the chromatographic peak, affording sufficient sampling to ensure good quantitative accuracy and precision. This is demonstrated with the linearity and precision obtained from the lipid dilution series. Next to MS1 isolation, accurate mass MS2 read out of precursor and product ions contributes to the overall specificity of the system, increasing the overall peak capacity of the mass spectrometer and therefore the analytical system. An example is shown in Supplemental Figure 3 where lipid product ion *m/z* values were extracted from the data with nominal FWHM resolution settings of

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5000 and 25,000, respectively. It can be seen that a cleaner product ion spectrum, as shown by the reduced number of ion detections within the quadrupole *m/z vs*. TOF *m/z* plot, is obtained at the higher resolution, i.e. the narrower extraction window. Shown inset is the detection of the ^{12}C isotope of the lipids of interest. The importance of sampling speed is further illustrated using a metabolite based example (Supplemental – Figure 4) utilizing a very rapid 3.5 minute UPLC separation [18]. Loss of quantitative precision caused by under sampling occurs in cases where fast data acquisition rates are not employed [19]. In this example, the chromatographic peak width was a mere 1.7 s at half height and the acquisition rates were varied between 0.1 to 0.5 s. The inset bar graph shows that quantitative precision for the three precursor/product ion transitions is retained at an acquisition rate of 0.1 s, but that quantitative inaccuracy and variation in measurement is introduced for the slower acquisition rates of 0.3 s and 0.5 s, respectively.

Data can be processed and visualised using a number of bioinformatic tools. Here we show the structural elucidation of PE (28:2) (*m/z* 632.4), which is based on matching experimentally derived fragments with those generated *in-silico* using SimLipid in combination with a contrived shotgun style experimental design and data analysis approach. All MS1 and MS2 spectra from the LC-MS experiment shown in Figure 2 were combined and stored as single mass spectra in two individual functions whereby the scanning quadrupole information on individual scan/TOF integration basis was retained. This allows the creation of a TOF *m/z* vs. quadrupole *m/z* distribution as shown in Figure 4 where each data point comprises MS1 and MS2 information. In the instance of a shotgun lipidomics experiment, the sample would be infused and the quadrupole slowly scanned to collect collision induced dissociation (CID) MS2 spectra,

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providing a similar distribution as provided in Figure 4. Shown inset are the MS1 and MS2 spectra associated with one of the lipids present within the mixture and the annotation of the data with theoretically predicted fragment ions and its subsequent identification.

Fragmenting lipids by means of CID generates a range of class-specific fragment ions and associated neutral losses, which can be used for structural elucidation. Typically, experiments involving neutral loss scanning are conducted using triple quadrupole [20] or DDA based [21] methods in conjunction with high resolution platforms. SONAR allows for class specific information to be readily extracted from the data. Figure 5 represents an MS1/MS2 combined multidimensional ion map of precursor and product ions for a mouse testis lipid extract. Precursor MS1 data are displayed on the diagonal trend lines, illustrating the scanning nature of the quadrupole. MS2 fragment ions resulting from neutral losses are also presented as diagonal trend lines residing at lower *m/z*. A number of class-specific neutral losses are readily identifiable that can be extracted on the basis of the detection of an accurate mass difference between the MS1 and MS2 data streams of ions eluting at the same chromatographic time and that are within the same set of average scanning quadrupole bins/windows, including those related to phosphatidylethanolamine (PE) (*m/z* 141.0), phosphatidylserine (PS) (*m/z* 185.0) and triglyceride (TG) (*m/z* 273.3). In addition to the combined loss of $CH_3(CH_2)_{14}COOH$ and NH_3 (*m/z* 273.3) to provide diacyl product ions, we also observe secondary neutral losses. Complimentary information can also be obtained using class specific product ions such as *m/z* 184.1, corresponding to phosphocholine (PC)/sphingomyelin (SM). These product ions are represented on the ion map as horizontal trend lines. Fragment ion data, originating from the asterisk annotated

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precursor ions in Figure 5, representing tentatively identified PC (36:3) and PE (38:5), serving as examples to aid the detection of lipid species that produce either a product ion or a neutral loss when undergoing CID fragmentation, representing given classes, respectively (Supplemental - Figure 5). The dashed lines represent the precursor to product ion relationships, 141.0 (PE) neutral loss and a 184.1 (PC or SM), respectively, to detect the classes of interest based on the correlated quadrupole positions in MS1 and MS2 modes during acquisition.

The same set of data, *i.e.* knockout (KO) from regulated pathways vs. control (CTRL) mouse testis tissue, was used to examine the use of the SONAR scanning quadrupole DIA data for untargeted discovery lipidomics studies. The data were aligned, peak detected and quantitative differences identified via combined use of unsupervised PCA and supervised OPLS-DA. Compounds showing significant abundance differences were compound database searched leading to the identification of study relevant lipids. Part of the identification process is summarised (Supplemental - Figure 6), highlighting a lipid confidently detected with change in abundance within a so-called 'S-plot' distribution, which typically depicts covariance *vs.* correlation, and the search result annotated MS2 DIA spectrum shown inset.

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Conclusions

The mass spectrometry acquisition strategy described in this communication is a novel DIA method using a rapidly scanning quadrupole in front of a gas cell and orthogonal acceleration time-of-flight mass analyser. The results presented show the principle and applicability of this technique to the analysis of lipids mixtures. The technique has the potential to deal with both LC separated lipids as well as the infusion of unseparated lipid mixtures, so called shotgun lipidomics. The key analytical attribute of this mode of data acquisition over previously described DIA approaches is speed in combination with enhanced selectivity. The rapid scanning nature of the quadrupole is compatible with fast LC and could also be potentially applicable for SFC based separations. Quadrupole isolation of molecular species prior to fragmentation provides specificity allowing fragment ions that are commonly produced by multiple lipid species to be easily assigned to the relevant molecular ion. This is a valuable advantage over techniques without quadrupole, or alternative, MS1 isolation. Scanning this in a rapid manner makes best use of modern electronics and enables more precise correlation of precursor product ion relationships, even with relatively wide quadrupole transmission windows. Finally, the unbiased data independent approach allows class based identification of lipids via either diagnostic fragment ions or the constant neutral loss that are indicative of specific lipid classes. This SONAR scanning quadrupole DIA data can be repeatedly probed to determine specific alterations in lipid biochemistry.

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Figures

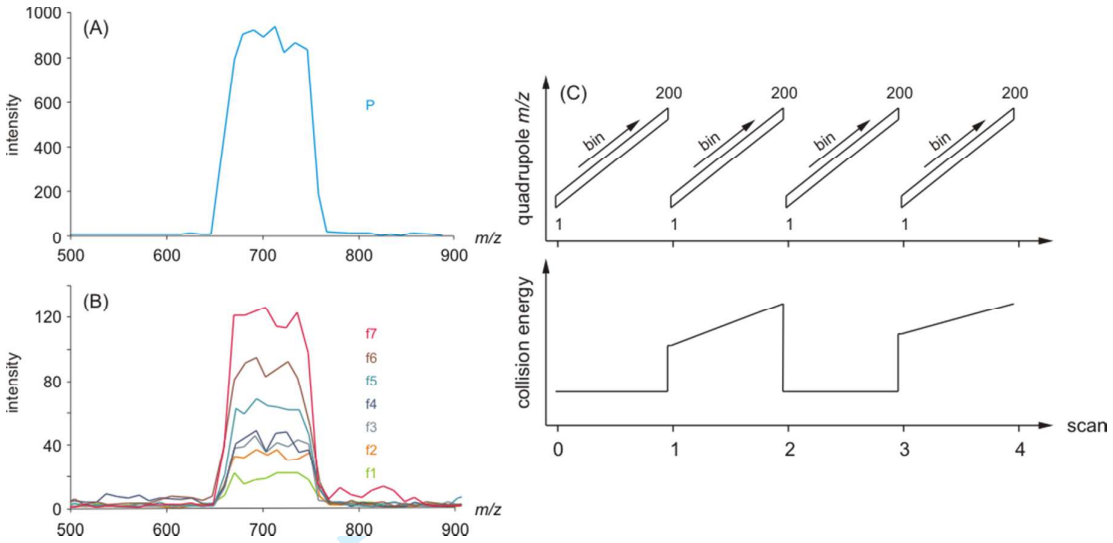


Figure 1 – (A) The quadrupole spectrum for a precursor ion *(P)* obtained using a scanning quadrupole with width 100 Th. (B) A schematic of the SONAR acquisition method. The behavior of collision energy alternates between a constant (low) value and a linear ramp chosen to generate informative fragmentation for as wide a range of precursors as possible. The quadrupole ramps with the same parameters in both low and high energy scans. (C) The extracted quadrupole profiles for fragments *(f1-f7)* of the precursors shown in (A) demonstrating the high degree of correlation between fragment and precursor profiles.

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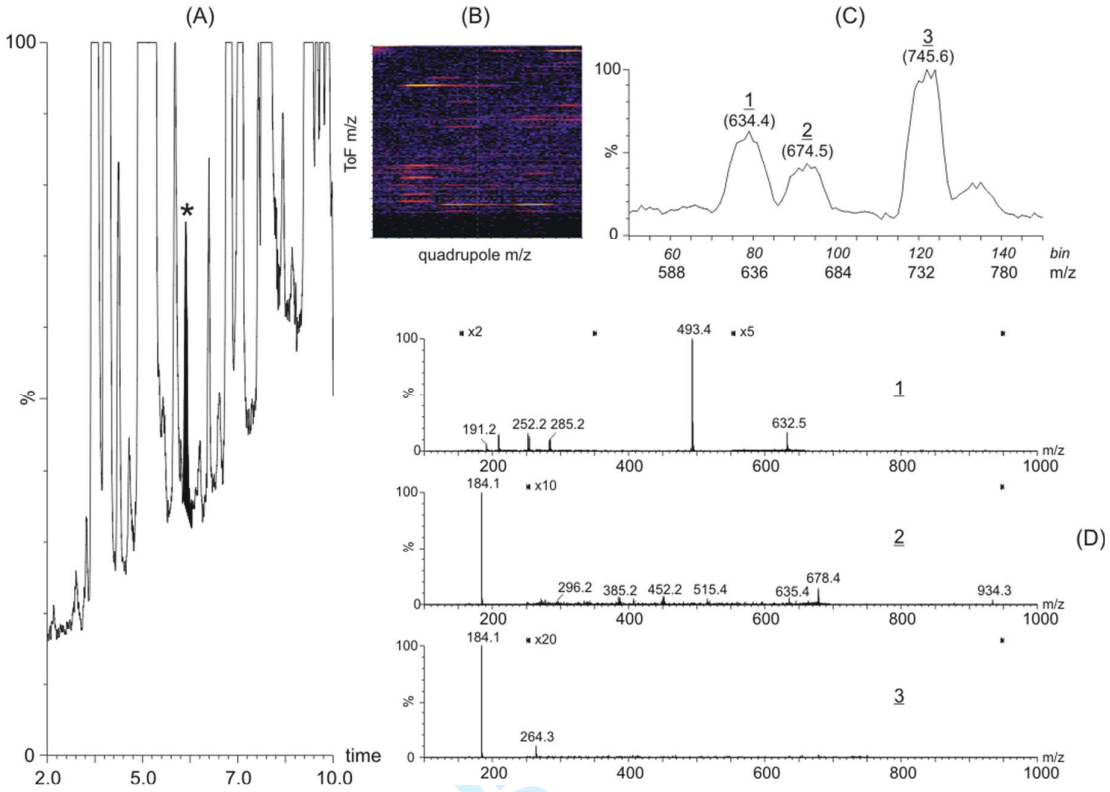


Figure 2 – Chromatographic separation (A) of LipidoMIX standard spiked into bovine heart extract. Based on the chromatographic conditions described in the methods section, the chromatographic peak at retention time 6.0 min (*) shows at least three co-eluting lipids (*m/z* 634.4 (1), 674.5 (2) and 745.6 (C)). Corresponding fragment ion spectra (D) are shown for each lipid, highlighting the specificity of scanning quadrupole DIA to provide accurate identification. This data is also represented as a two dimensional ion map (TOF *m/z* vs. quadrupole *m/z*) (B) were precursor and fragment ions are aligned in vertical trends.

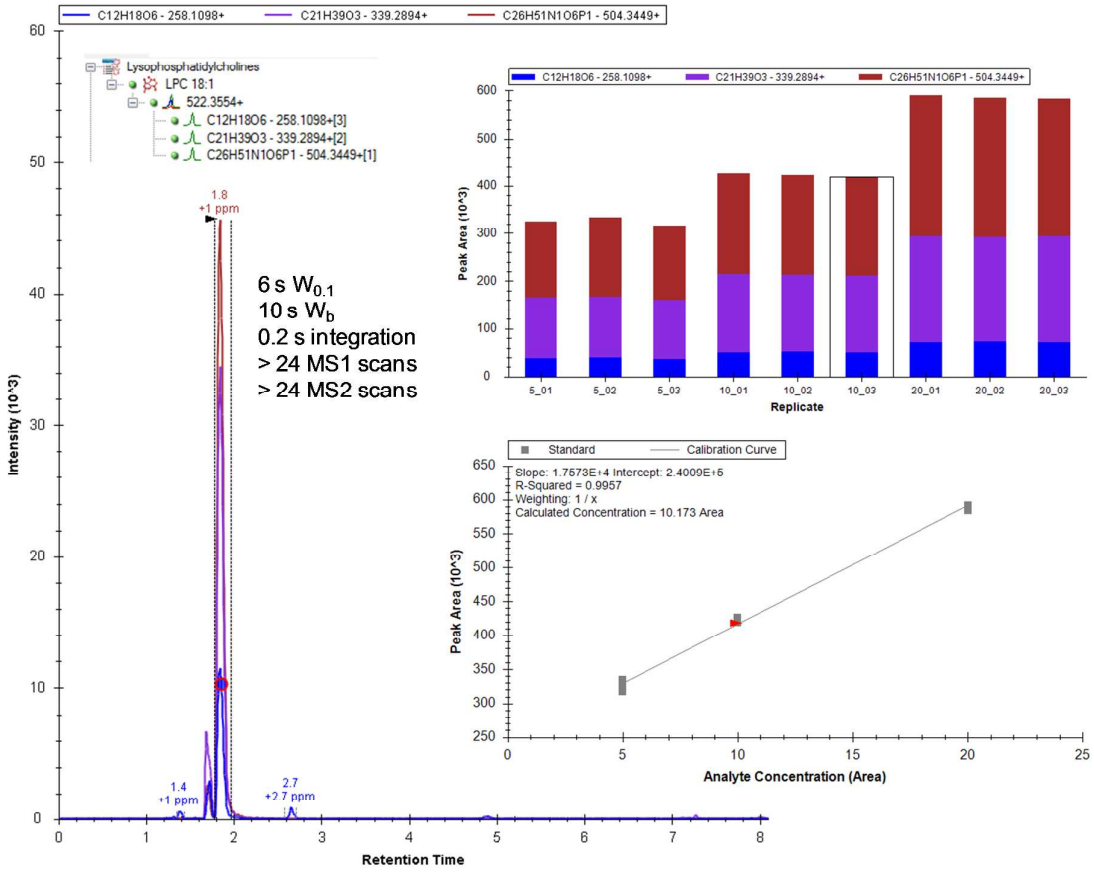


Figure 3 – Multiple dimension ion extraction using retention time, quadrupole (precursor) *m/z* and TOF (product ion) *m/z* for a representative LPC 18:1 in the presence of matrix (bovine heart extract). Three fragment ions (*m/z* 258.1, 339.3 and 504.3) are extracted, with relative abundance shown in the top right hand pane, generating more than 24 MS1 and 24 MS2 data points across a peak of 6 sec at 10% peak height when acquiring at a 0.2 s scan rate. Quantitative precision is demonstrated with an r^2 linearity of 0.9957 as illustrated by the quantitation curve in the lower right corner.

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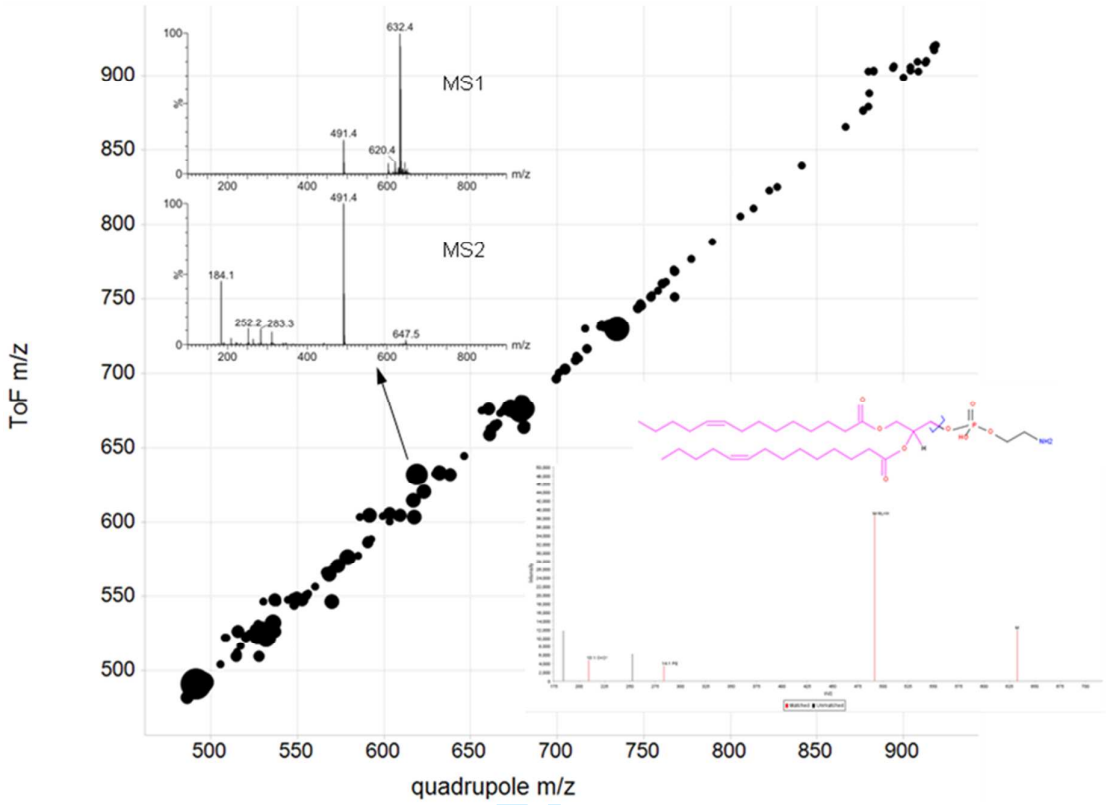


Figure 4 – TOF *m/z vs.* quadrupole *m/z* distribution imitating a shotgun lipidomics experiment. MS1 and MS2 spectra (upper inset) are shown for an example phosphatidylethanolamine (PE 28:2, *m/z* 632.4) with annotation of the MS2 spectrum (lower inset). Data were acquired using a scanning quadrupole window of 10 Da operating over *m/z* 400 to 1100 and TOF range from 50 to 1200 with a 0.2 sec scan rate.

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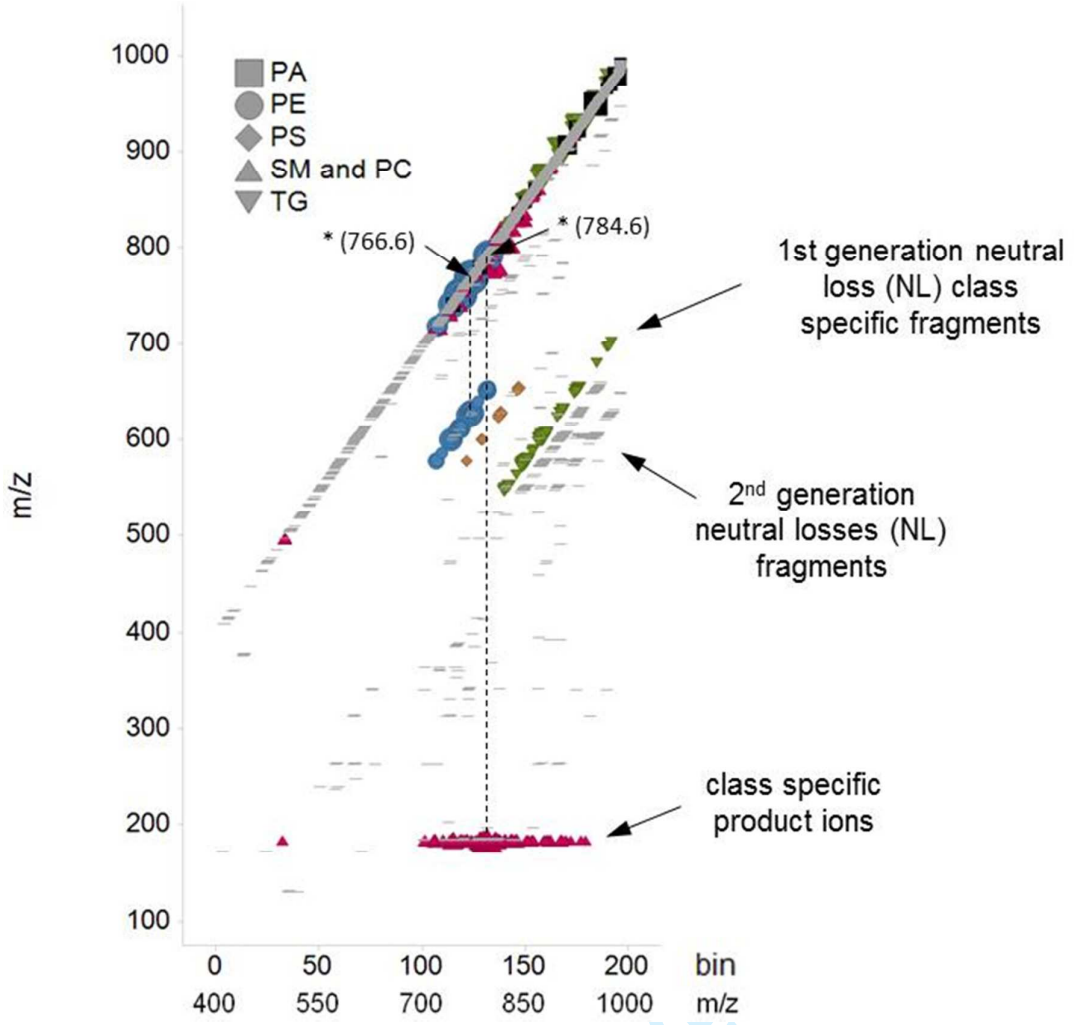
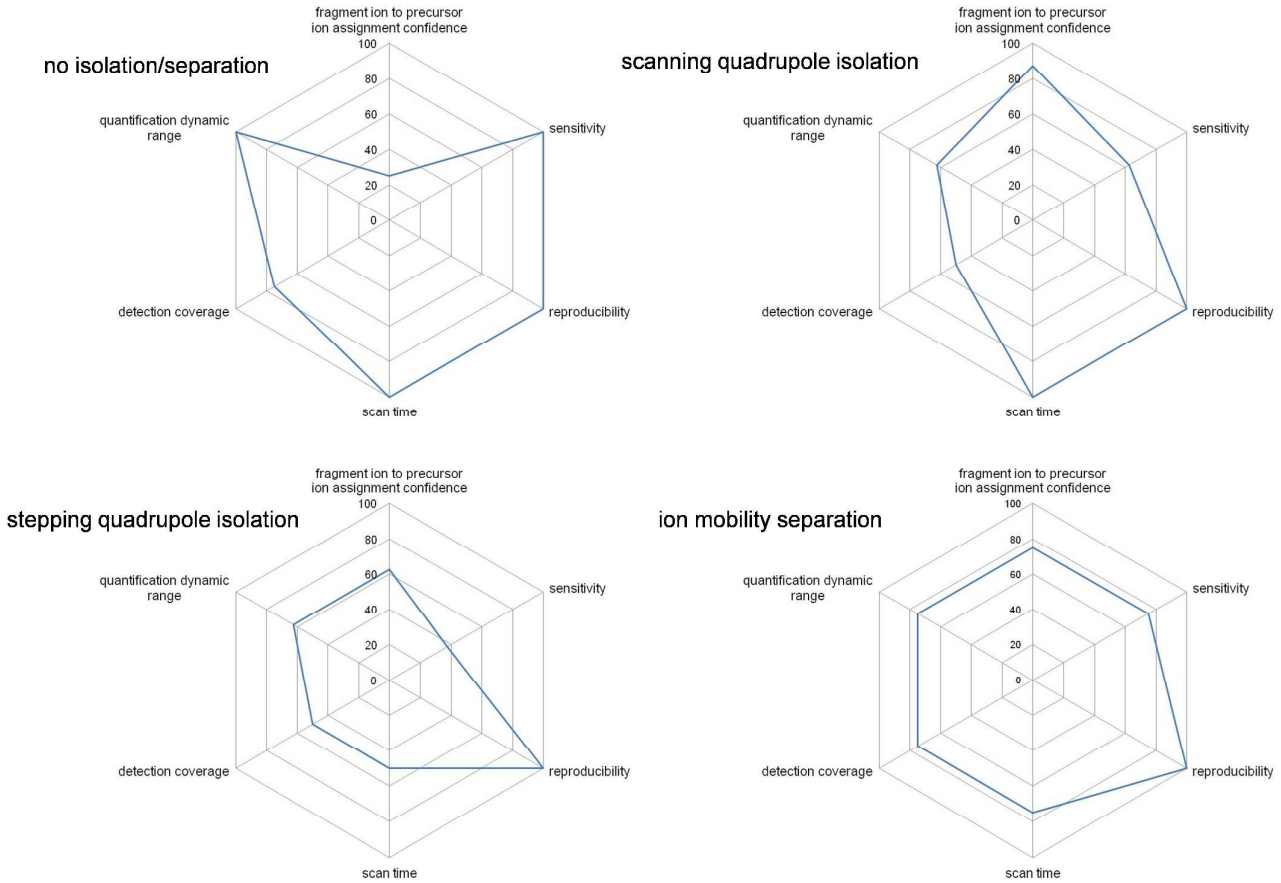
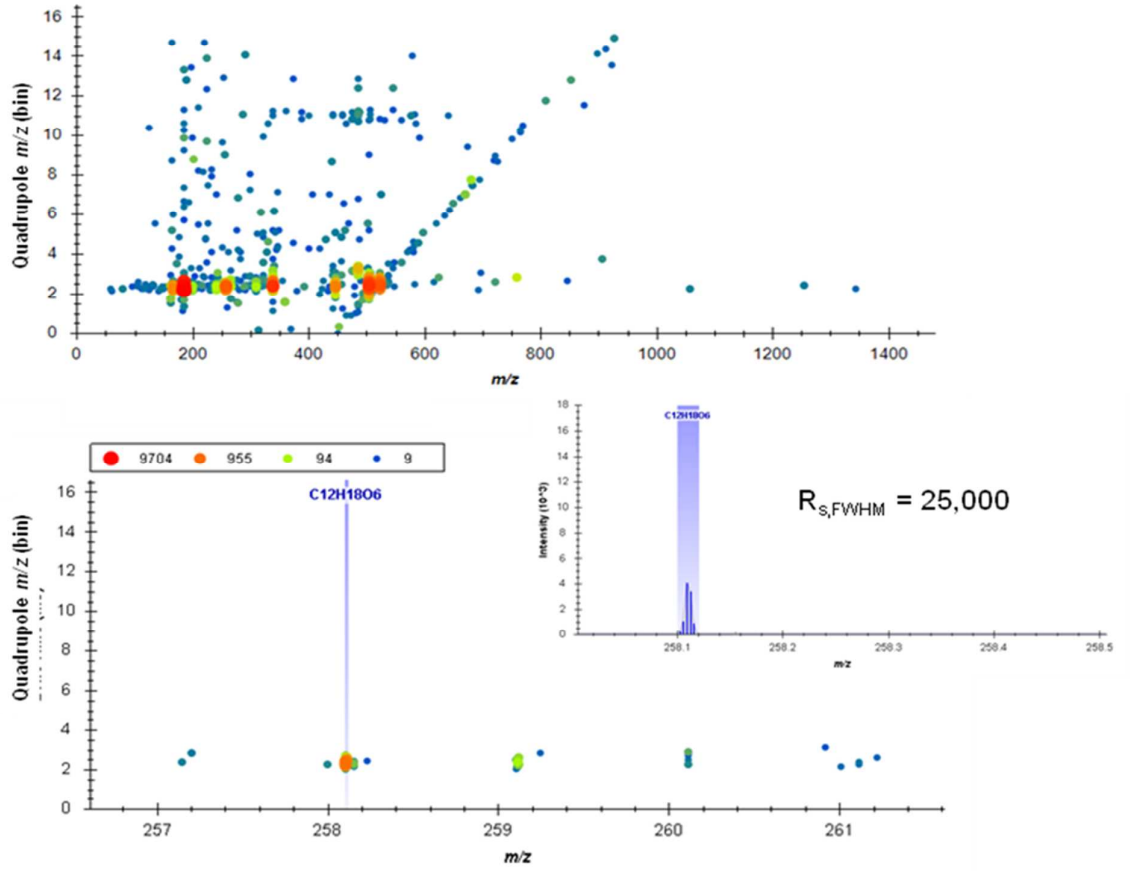


Figure 5 – Multidimensional ion map generated from a mouse testis extract, illustrating class specific information. PA, PE, PS and TG examples show product ions resulting from neutral loss (diagonal trend lines). Lipids generating class specific product ions such as SM/PC are presented as a horizontal trend line. Vertically correlated product ions are MS2 product ions originating from the same quadrupole isolated precursor. Example MS2 spectra for the (*) annotated precursor ions are shown in Supplemental Figure 2. The dashed lines represent the precursor to product ion relationships afforded by the average quadrupole position (bin or *m/z*) in MS1 and MS2 acquisitions modes to detect the classes of interest.

Supplemental Figures

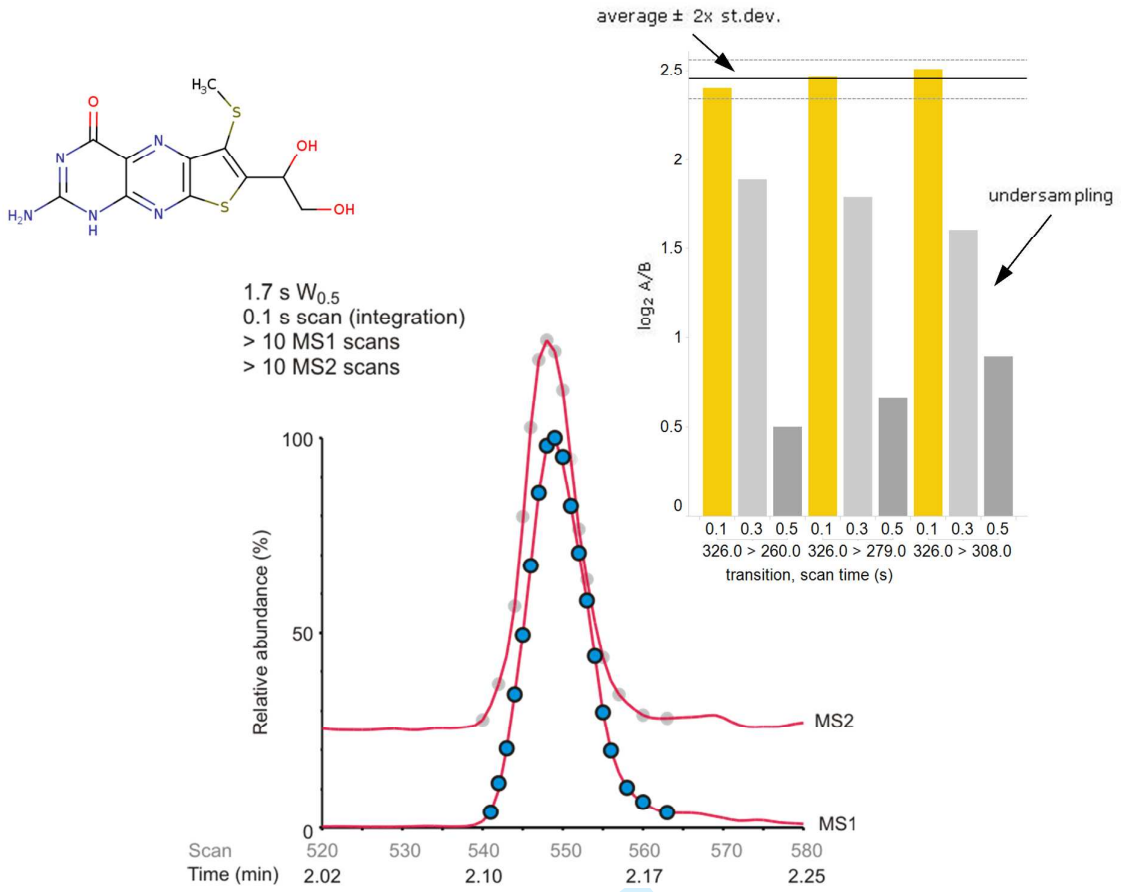


Supplemental Figure 1 – Qualitative comparison analytical performance characteristics DIA methods.



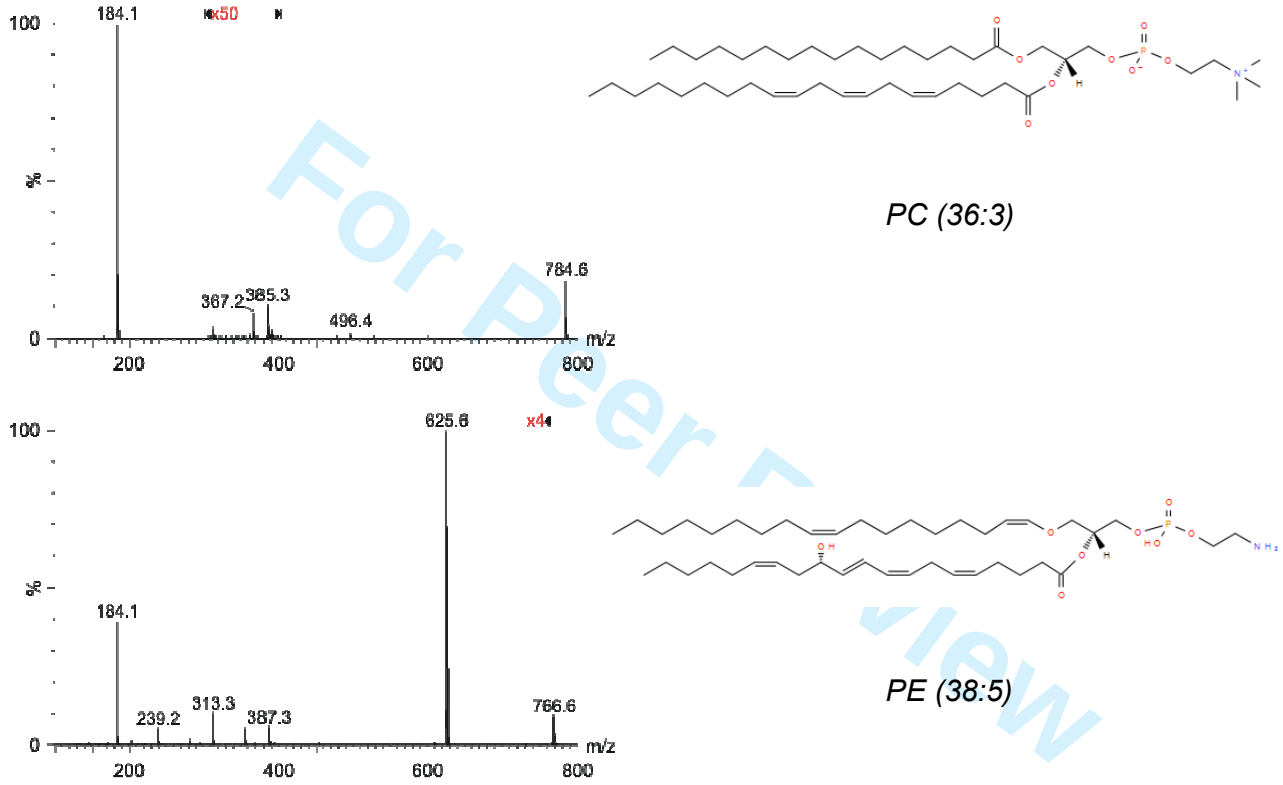
Supplemental Figure 2 – Multiple dimension ion extraction using retention time, quadrupole (precursor) *m/z* and TOF (product ion) *m/z*. Low resolution (5,000 FWHM) data extraction is shown in the top panel and high resolution (25,000) in the bottom pane. Shown inset is the detection of the ^{12}C isotope of protonated $C_{12}H_{18}O_6$ (LPC 18:1).

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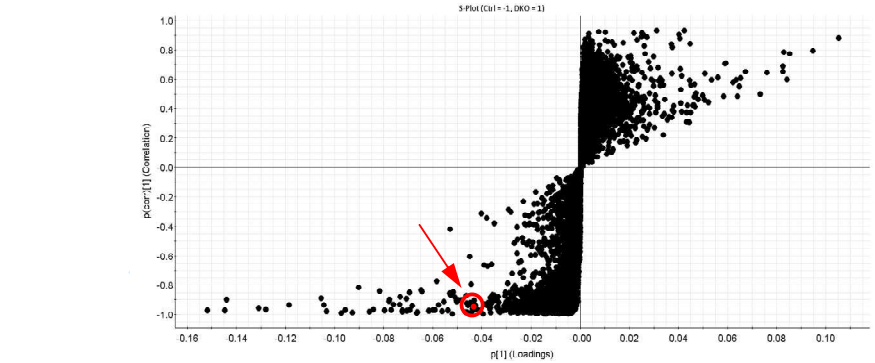


Supplemental Figure 3 – Metabolomic example with an expected fold change of urithion (2.5-fold) across two sample sets highlighting the need for fast data acquisition rates in order to maintain quantitative precision. For three transitions (yellow, light grey, and dark grey bars) acquired at three different scan rates (0.1, 0.3, and 0.5 s, respectively) distortion caused by undersampling is encountered at scan times greater than 0.1 s. Based on a scan rate of 0.1 s it is shown that more than 10 points across the peak (MS1 and MS2) is achievable for UPLC based chromatography.

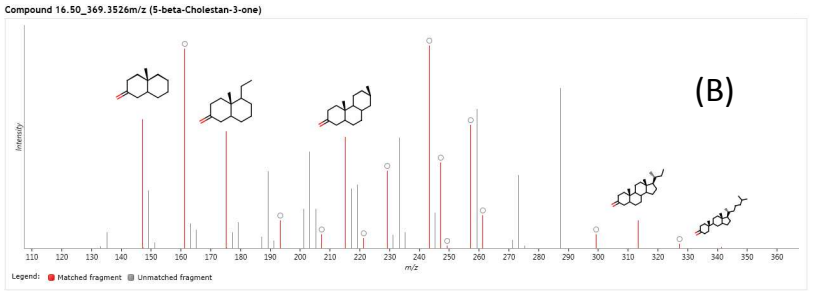
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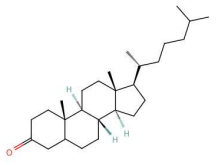


Supplemental Figure 4 – Tentative example lipid identifications based on class specific product ions (upper spectrum) and neutral loss (lower spectrum) highlighted in Figure 5.



(A)





5-beta-cholestan-3-one

Supplemental Figure 5 – Scanning quadrupole DIA untargeted discovery lipidomic workflow. The 'S-plot' (A) distribution depicts features on the basis of covariance and correlation between control and KO mice. One particular feature showing a high probability of differential regulation corresponds to the tentative identification of 5-beta-cholestan-3-one which is highlighted within the distribution along with an accompanying MS2 annotated spectrum (B).

Supplemental Table 1. Qualitative comparison DIA methods.

	no isolation/separation[1)]	stepping quadrupole isolation[2)]	scanning quadrupole isolation[3)]	ion mobility separation[4)]
fragment ion to precursor ion assignment confidence	+	++/+++ [i)]	+++/++++[i)]	+++
sensitivity	++++	+/++[ii)]	++/+++[ii)]	+++
reproducibility	++++	++++	++++	++++
scan time	0.1 – 0.5 s	1-2 s	0.1 – 0.5 s	~ 0.5 s
search type	targeted/untargeted	targeted/untargeted	targeted/untargeted	targeted/untargeted
detection coverage	+++	++	++	+++
quantification dynamic range	++++	++/+++[iii)]	++/+++[iii)]	+++
primary application	relative quantification	relative quantification	relative quantification	relative quantification

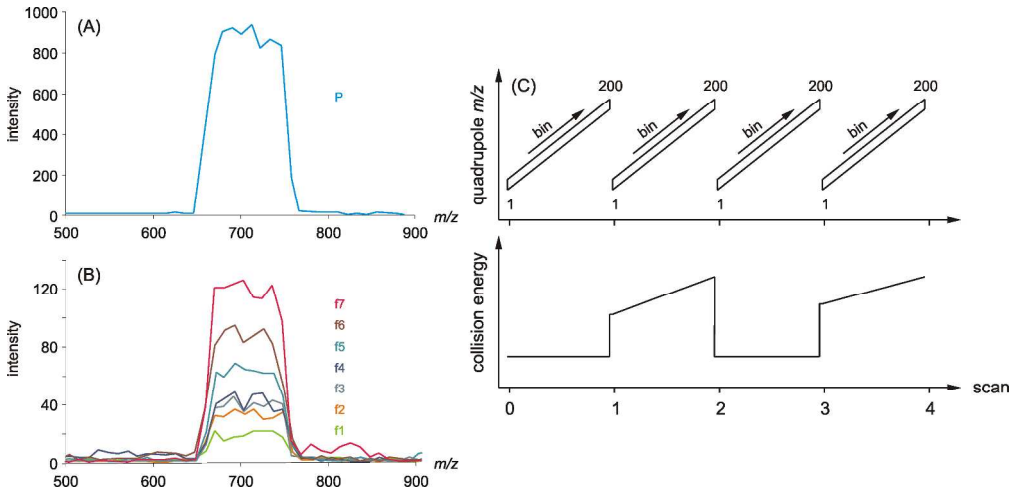
i) Fragment ion to precursor ion assignment confidence is particularly challenging in complex mixtures with multiple co-eluting precursor ions. For quadrupole m/z filters this attribute can be improved by reducing the m/z range transmitted by the quadrupole, making comparisons with other approaches difficult. For the same isolation window scanning quadrupole isolation yields higher fragment to precursor ion confidence than the stepped approach, and both perform better than the no isolation approach. Both ion mobility separation and scanning quadrupole isolation perform better than stepped quadrupole isolation for the for the same precursor ion separation peak capacity but both scanning quadrupole and stepped quadrupole isolation can be improved over ion mobility separation by reducing the quadrupole transmission window at the cost of sensitivity (see ii).

ii) When considering sensitivity it is important to recognise the duty cycle effects of quadrupole m/z filters. For a fixed scanned precursor ion m/z range, increasing the quadrupole transmission window will proportionally increase the duty cycle and thus sensitivity for each precursor ion. This sensitivity increase can reduce the fragment to precursor ion assignment confidence, particularly in complex mixtures.

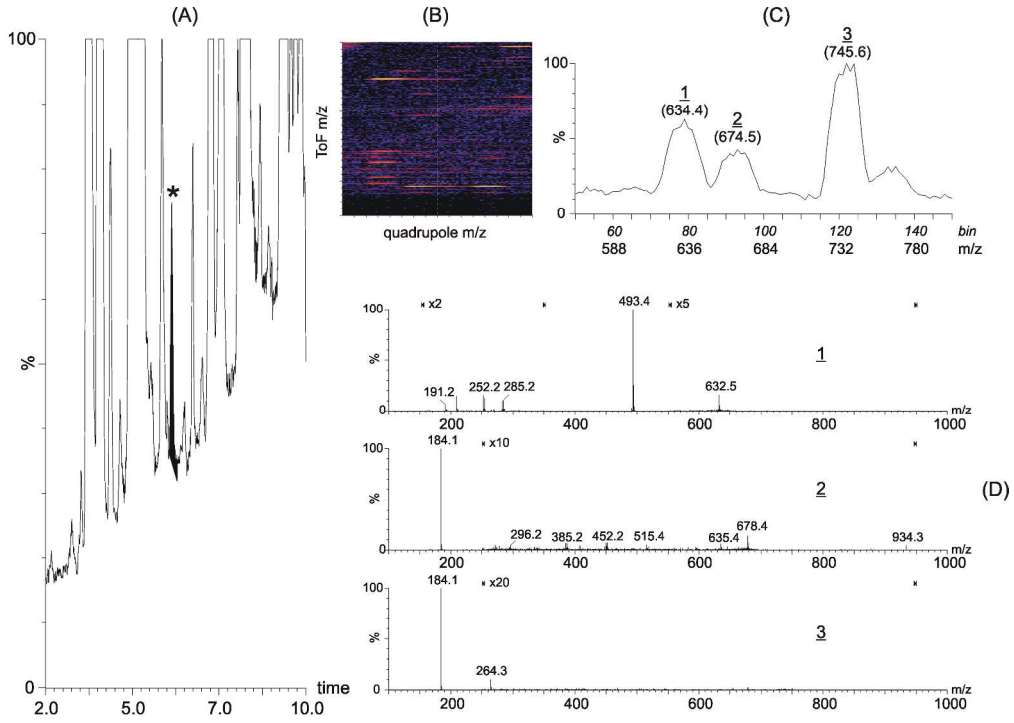
iii) Dynamic range is inherently linked to sensitivity (see ii) for quadrupole m/z filters. The upper limit for quantification remains unchanged when compared with no isolation cases for fast downstream analysers such as time of flight. Typically the lower limit increases with sensitivity and thus with increasing quadrupole transmission windows. This increase will continue until the fragment ion to precursor ion assignment confidence (see i) is reduced such that confidence in the compound identification is lost.

[1)] MSE (W), all ion fragmentation (AIF) (T), all ions MS/MS (A)
[2)] SWATH (S), middle-band DIA (B), wide-band DIA (B)
[3)] SONAR (W)
[4)] HDMSE (W)
A = Agilent Technologies; B = Bruker Corporation; S = Sciex; T = Thermo Scientific; W = Waters Corporation

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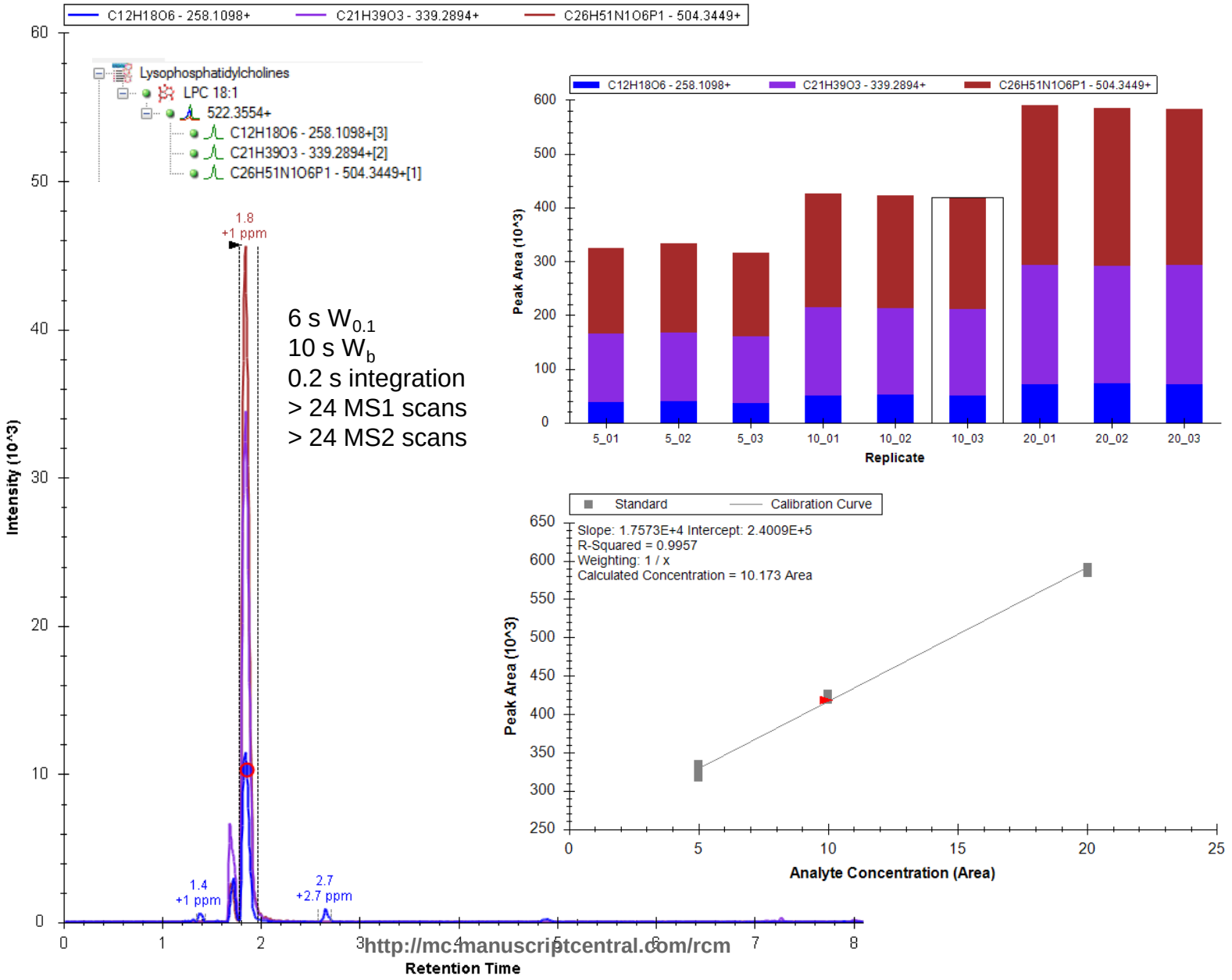

(A)
intensity
m/z
P
(B)
intensity
m/z
f7
f6
f5
f4
f3
f2
f1
(C)
quadrupole m/z
bin
collision energy
scan

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(A)
(B)
(C)
100
%
0
time
2.0
5.0
7.0
10.0
*
ToF m/z
quadrupole m/z
1
(634.4)
2
(674.5)
3
(745.6)
60
588
80
636
100
684
120
732
140
780
bin
m/z
x2
493.4
x5
191.2
252.2
285.2
632.5
200
400
600
800
1000
m/z
184.1
x10
296.2
385.2
452.2
515.4
635.4
678.4
934.3
x20
264.3
(D)

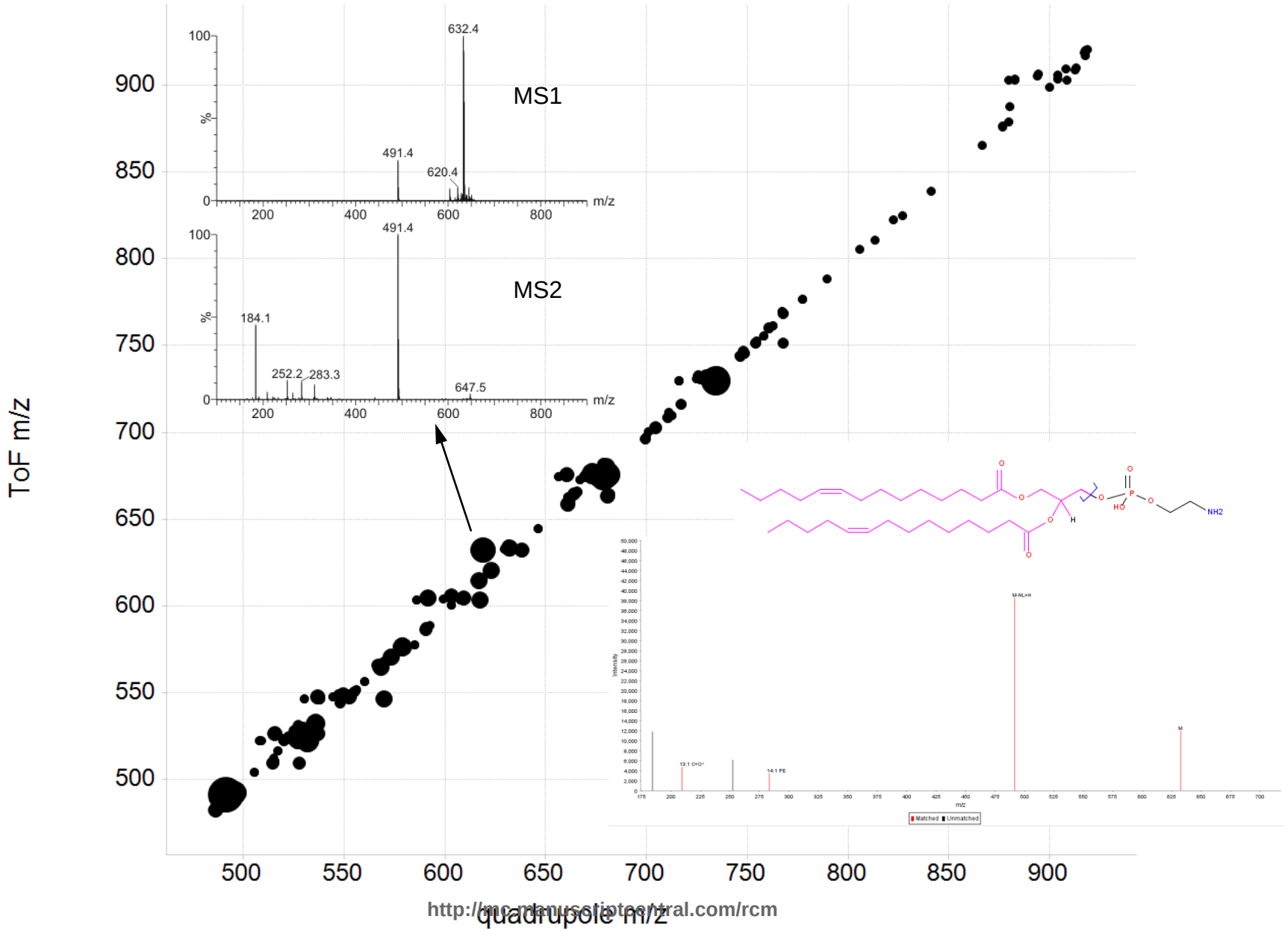
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Figure 3



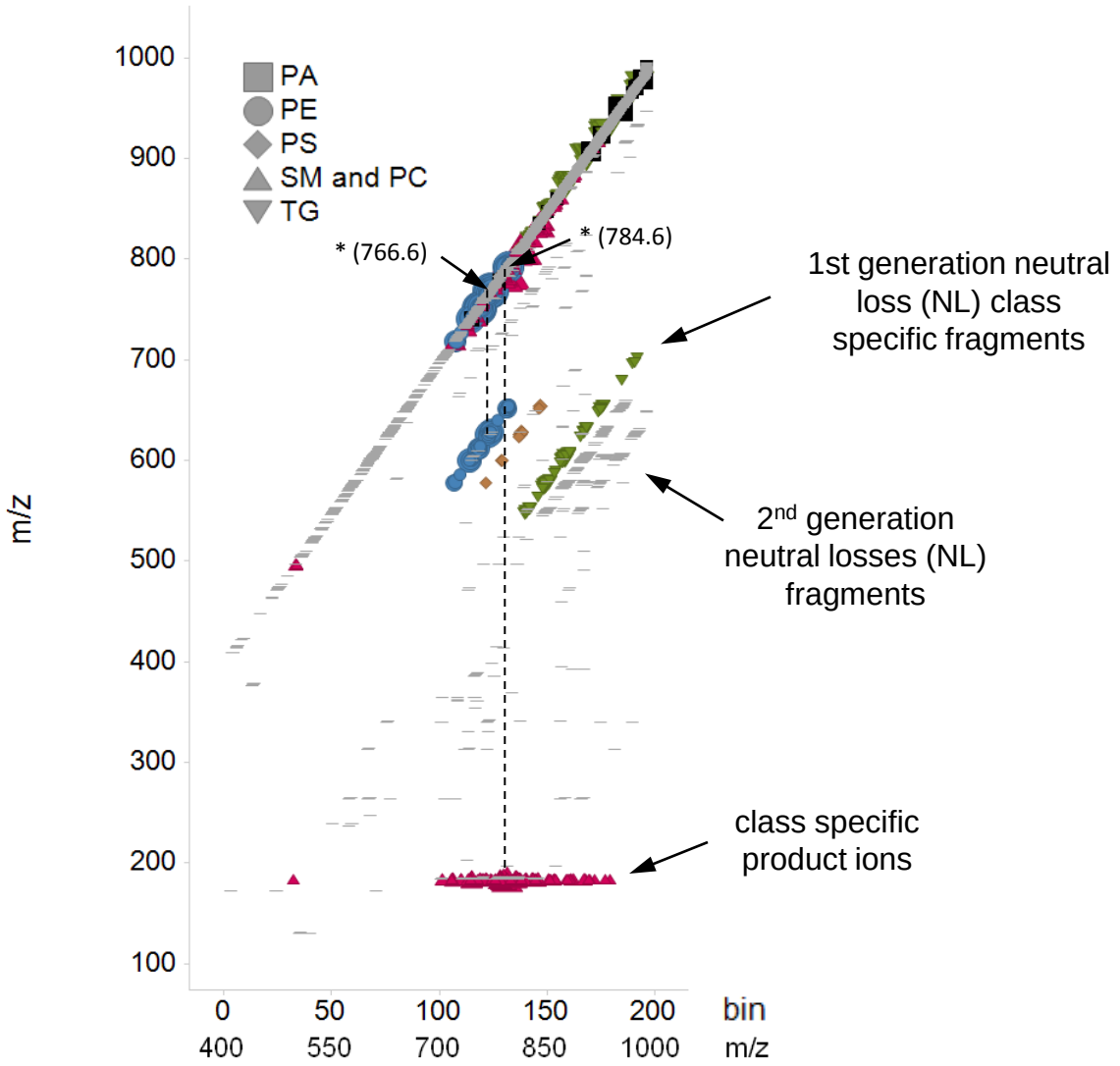
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Figure 4



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Figure 5


PA
PE
PS
SM and PC
TG
* (766.6)
* (784.6)
1st generation neutral loss (NL) class specific fragments
2nd generation neutral losses (NL) fragments
class specific product ions
m/z
1000
900
800
700
600
500
400
300
200
100
0
50
100
150
200
bin
400
550
700
850
1000
m/z