

Combining unbiased metabolic profiling with targeted analysis of specific metabolites using high resolution mass spectrometry, a step forward in metabolomics

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Overview

Using high-resolution/high accuracy, mass-stable orbitrap mass spectrometry it is possible to combine unbiased profiling of biological samples and targeted analysis of specific compounds at the same time without loss of sensitivity. In this study, we demonstrate how targeted analysis of phospholipids as well as unbiased profiling is achievable using a benchtop orbitrap mass spectrometer after high-speed reversed phase HPLC. The ability to apply both strategies at the same time on the same data set is an important step forward in comprehensive analysis of the metabolome

Introduction

Metabolomics and lipidomics are relatively new scientific disciplines, currently driven by the performance of the analytical instrumentation used. Advancements are often the result of new developments in mass spectrometry or new tools to interrogate the experimental data. Metabolomics and lipidomics research usually follows one of two possible strategies. If metabolic profiling or fingerprinting is essential, data acquisition techniques focus on capturing as many metabolites as possible without knowledge of the identity of these metabolites. If the aim of the analysis is to identify specific metabolites, it is useful to determine the analyses of interest and these are subsequently classified or identified. This strategy is particularly useful in the search for new compounds, biomarkers, or mechanisms. Some scientists argue that the method is of limited use when underlaid analyses constrains the formation and integration of biochemical networks. For these researchers, it is useful to only collect data on known compounds as they can be interpreted in a biochemical series. As the specific, targeted analysis of known compounds demands a different method (e.g., tandem mass spectrometry, MS/MS) than global metabolite profiling (usually full scan MS spectra), the merger of the two strategies would usually result in compromised quality for one or the other. The ability to obtain mass spectra with a very high degree of mass accuracy at sufficient mass resolutions and scan rates opens the possibility for combining both strategies without any such compromises. Analytical instruments capable of high-resolution mass measurements are time-of-flight (TOF), Fourier transform ion cyclotron resonance (FT-ICR) and orbitrap mass spectrometers. The acquisition of experimental data of sufficient mass resolving power and accuracy has until a few years ago only been possible using FT-ICR-MS, with several limitations with respect to scan speeds, however. Consequently, FT-ICR instruments have not been routinely utilized in high-throughput metabolomics applications. Higher scan speeds are achievable with a TOF analyser, but these do not reach the same mass accuracy and resolution. For very complex samples, both high resolving power and mass accuracy are required, as available from FT-ICR or orbitrap instruments. High resolution orbitrap mass spectrometry is particularly interesting for hybridised LC/MS applications (LC/MS) instruments, where sub-2 micron particle column generated chromatography peaks with peak widths of only a few seconds, requiring at least 2 scans per second to obtain a sufficient number of data points across the peak for quantitation. Importantly, if high mass accuracy can be maintained in metabolomics applications throughout the duration of the sometimes lengthy chromatographic experiments, it will be possible to extract ion chromatograms with a sufficiently high degree of accuracy, so that overlapping isobaric signals from salt adducts and lipids containing longer unsaturated fatty acids can be readily separated. Such applications typically require 5 ppm or less mass measurement accuracy over many hours or days of acquisition time. One of the present studies to evaluate an accurate, benchtop orbitrap mass spectrometry system, the EXACTIVE, for application to high-throughput metabolic profiling. Specifically, we are describing the analysis of human plasma samples, with the goal of achieving simultaneous unbiased fingerprinting as well as the targeted analysis of large numbers of metabolites within a single run, without compromising the analytical quality of the two strategies used.

Methods

Samples Plasma from 10 healthy volunteers was used. Each plasma sample was divided into six different sub-samples, each with different standard additions (including, for example, N-octacosylphosphatidylcholine). A volume of 250 µL of each sample was added to 1 mL of cold acetonitrile. This mixture was centrifuged for 10 min at 13,000 rpm and the supernatant diluted (1:1) with formic acid (0.1%) and transferred to a 96-well plate ready for analysis. The resulting 60 samples were analysed 4 times by LC-MS.

Chromatography Chromatographic separations were performed on a 5.0x2.1mm Hypersil Gold 1.9 µm C18 column (Thermo Scientific, Runcorn, UK) using an Accela UH-PLC system (Thermo Scientific, Hemel Hempstead, UK). The column was maintained at 45°C. A binary mobile phase system was used where A=formic acid (0.1%) and B=acetonitrile/isopropyl alcohol (1:1) containing formic acid (0.1%). The mobile phase program at an initial hold (0.0-4.0 min) at 9% followed by a linear gradient 5-50% B (0.5-5.0 min), then 50-95% B (5.0-5.5 min), the conditions were then held at 95% B (5.5-6.5 min) and returned to the initial conditions (6.5-10.0 min). The total analysis duration was 10 min at a flow rate of 0.25 mL/min. The column eluent was directed to the Exactive mass spectrometer.

Mass Spectrometry was performed on an Exactive benchtop, orbitrap mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) operating in positive ion mode. The heated electrospray (HESI-III) source was used. The sheath gas flow was set to 20 (arbitrary units) at a temperature of 200°C, the aux gas set to 10 (arbitrary units) and the capillary temperature set to 250°C. The capillary voltage and spray voltage were set to 5 kV and 4.5 kV, respectively. The instrument was operated in full scan mode from m/z 150-1000 at 50,000 resolving power. The data acquisition rate was 2 Hz. The mass spectrometer was mass calibrated just prior to starting the sequence of 240 injections. All data was acquired using lock mass calibration (m/z 214.0896).

Data analysis

Specific analysis of phosphocholesterols. For the targeted analysis of 50 specific phospholipids (see Table 2), the theoretical exact masses were figured with a significant figure on chromatograms figure with a resolution of 2.5 ppm. The resulting data was integrated and the area-under-the-curve (AUC) was used for relative quantitation. The values were imported into the Dante software, where missing data was imputed using the kNearest Neighbour method. Data was further analysed using analysis of variance (ANOVA), principle component analysis (PCA) and partial least-squares data analysis (PLS-DA).

MZmine The raw files were converted into NetCDF files using the Thermo software package Xcalibur. The converted files were imported into MZmine and peaks were detected using the following settings: noise levels>30,000.0; mass resolution>30,000; peak model function= Savitzky-Golay; min time span>7.0; m/z tolerance>0.0020. The resulting peak lists were aligned using m/z tolerance>0.0025; retention time tolerance>10.0%. The resulting peak list was exported as a CSV file and imported into DANTE where missing data was imputed using the k Nearest Neighbour method, and data was further analysed using ANOVA, PCA and PLS-DA.

Results and Discussion

High mass resolution and accuracy are essential components of the approach described here for targeted analysis with simultaneous, unbiased fingerprinting. However, mass measurement stability is equally critical when analysing large numbers of samples for a given experiment. In table 1 we see that stability of mass measurement is high across the 240 samples analysed in this experiment, which data was acquired in a single LCMS batch.

Table 1. The stability of the mass accuracies across a 240 sample batch. The spread of the measured m/z ratios for five selected ions is shown for every 40th run

Theoretical m/z	Average m/z	Stdev m/z	Min m/z observed	Max m/z observed	m/z spread [ppm]
C ₁₈ H ₃₃ O ₂ N ₂ ⁺	203.07524	203.07524	0.00008	203.07524	1.3
C ₁₈ H ₃₃ O ₂ NH ⁺	274.27349	274.27349	0.00010	274.27346	1.2
C ₁₈ H ₃₃ O ₂ N ₂ ⁺	396.28429	396.28429	0.00012	396.28418	1.0
C ₁₈ H ₃₃ O ₂ N ₂ ⁺	496.37927	496.37927	0.00027	496.37945	1.4
C ₁₈ H ₃₃ O ₂ N ₂ ⁺	606.59641	606.59641	0.00044	606.59630	2.7

The benefit of high resolution coupled with high mass accuracy is shown below in figure 1 for reconstructed ion chromatograms of nominal mass 482, exact masses 482.32404 and 482.36050, a mass difference of 0.03646. With low resolution discrimination of the two peaks is not possible.

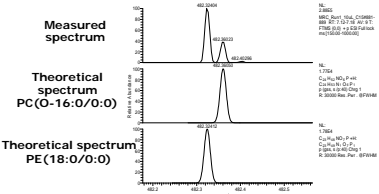


Figure 1 Reconstructed ion chromatograms (m/z 2.5ppm) of m/z 482.32404 and 482.36050 compared with theoretical mass spectra at 30,000 resolution.

Unbiased Approach Peak picking by XCMS or MZmine → Generate peak tables for PCA or PLS-DA → Find differentiating peaks → Annotate using accurate mass

Results are shown below of the unbiased mass spectrometry analysis of samples using principal component and partial-least squares analysis showing the top 7 loadings. All 7 identify ceramide (N-octacosylphosphatidylcholine), which was added to some of the samples but not all.

Table 2 Top seven loadings from the PLS analysis. All 7 identify ceramide. Unbiased approach

Measured (m/z)	R ² (s)	Loading	Mol formulae	Theoretical (m/z)	Identity
408.5834	395.4	0	C ₁₈ H ₃₃ O ₂ N ⁺	408.38361	(Cer-H ₂ O) H ⁺
448.3760	396.6	0.0002	C ₁₈ H ₃₃ O ₂ N ₂ ⁺	448.37667	(Cer) H ⁺
426.3940	396.8	0.0006	426.39405	426.39445	(Cer) H ⁺
873.7631	396.6	0.0008	C ₁₈ H ₃₃ O ₂ N ₂ ⁺ Na ⁺	873.76300	2(Cer) Na ⁺
409.3868	396.9	0.0010	C ₁₈ H ₃₃ O ₂ N ⁺	409.38696	(Cer-H ₂ O) H ⁺ -isotope
874.7664	396.5	0.0012	C ₁₈ H ₃₃ O ₂ N ₂ ⁺ Na ⁺	874.76636	2(Cer) Na ⁺ -isotope
449.3794	396.2	0.0014	C ₁₈ H ₃₃ ClO ₂ N ₂ ⁺ Na ⁺	449.37947	(Cer) Na ⁺ -isotope

A clear difference between ceramide-fortified samples and unfortified samples is observed following PLS analysis (Figure 2).

In figure 3 the benefit of high mass accuracy is observed. At 500 ppm (figure 3A) many background ions are observed and the PE and PC are not obvious. At 2.5ppm however, PE (18:0/O) and PC (16:0/O) are clearly discriminated from each other and from background ions.

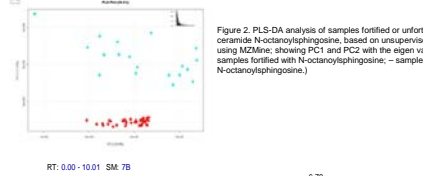


Figure 3A Reconstructed ion chromatogram at 500 ppm resolution.

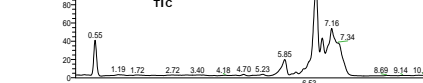


Figure 3B Reconstructed ion chromatogram at 2.5 ppm resolution.

Figure 2. PLS-DA analysis of samples fortified or unfortified with the ceramide N-octacosylphosphatidylcholine. Scaled and unsupervised data extraction using MZmine, showing PC1 and PC2 with the eigen values in brackets. (+ samples fortified with N-octacosylphosphatidylcholine, - samples not fortified with N-octacosylphosphatidylcholine.)

Targeted Approach

Use the same raw data files → Use theoretical mass of known phospholipids + 2.5 ppm window to create reconstructed ion chromatograms → Calculate AUC using the standard quantitation option in the Xcalibur software

The data quality was checked by comparing the results of 4 runs for each sample. SD was <20%.

In table 3 below the results of analysis are shown for 50 phospholipids. Typical average mass error (SD) is of the order of 1ppm.

Table 3. The post-run targeted analysis of 50 phospholipids using their theoretical, exact masses. In 3 runs (#25, #150, #240) across the batch of 240 runs, the measured m/z ratios are reported as well as their deviation from the calculated theoretical m/z. In addition, the average measured m/z is given for 10 runs (#25, #50, #75, #100, #125, #150, #175, #200, #225 and #240).

Lipid name	Run 25			Run 150			Run 240			Average m/z	Lipid mass error (ppm)
	Exp	Meas	Dev	Exp	Meas	Dev	Exp	Meas	Dev		
PC(18:0/O-0)	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	0.0000
PC(16:0/O-0)	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	0.0000
PE(18:0/O-0)	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	0.0000
PE(16:0/O-0)	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	0.0000
PC(18:0/O-0)	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	0.0000
PC(16:0/O-0)	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	0.0000
PE(18:0/O-0)	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	0.0000
PE(16:0/O-0)	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	0.0000
PC(18:0/O-0)	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	0.0000
PC(16:0/O-0)	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	0.0000
PE(18:0/O-0)	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	0.0000
PE(16:0/O-0)	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	0.0000
PC(18:0/O-0)	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	0.0000
PC(16:0/O-0)	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	0.0000
PE(18:0/O-0)	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	0.0000
PE(16:0/O-0)	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	0.0000
PC(18:0/O-0)	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	0.0000
PC(16:0/O-0)	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	0.0000
PE(18:0/O-0)	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	0.0000
PE(16:0/O-0)	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	0.0000
PC(18:0/O-0)	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	0.0000
PC(16:0/O-0)	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	0.0000
PE(18:0/O-0)	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	0.0000
PE(16:0/O-0)	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	0.0000
PC(18:0/O-0)	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	0.0000
PC(16:0/O-0)	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	0.0000
PE(18:0/O-0)	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	0.0000
PE(16:0/O-0)	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	0.0000
PC(18:0/O-0)	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	0.0000
PC(16:0/O-0)	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	0.0000
PE(18:0/O-0)	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	0.0000
PE(16:0/O-0)	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	0.0000
PC(18:0/O-0)	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	608.5835	0.0000	608.5835	0.0000
PC(16:0/O-0)	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	426.3940	0.0000	426.3940	0.0000
PE(18:0/O-0)	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	672.5835	0.0000	672.5835	0.0000
PE(16:0/O-0)	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	482.3605	0.0000	482.3605	0.0000