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Rapid Microbore Metabolic Profiling (RAMMP) with Ion Mobility for the Lipidomic Investigation of Plasma from Breast Cancer Patients

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APPLICATION BENEFITS

- High throughput
- Efficient instrument usage
- Complete workflow
- High sensitivity

INTRODUCTION

The metabolic and lipidomic profiling of biofluids and tissues has been proven to provide new insights into disease mechanism and progression.[1] Early pilot studies with small sample batches not only demonstrated the capability of the "omics" approach but also revealed the issues arising from small cohort studies, in terms of misleading results due to a lack of population, gender, and age coverage.[2] Large scale metabolic phenotyping studies require the ability to perform accurate and reproducible analysis of large sample cohorts and can often require several batches of analysis to complete.

The analyses of larger cohorts, for example 5000 samples and upwards, require either a large instrument facility or faster methodology.[3] Typical LC-MS profiling methods have sample cycle times of 10–20 minutes, thus a continuous analysis based on conventional UPLC for a study cohort of 1000 samples would require several days of instrumentation time. The benefits of rapid LC analysis combined with microbore LC columns for large batch analysis was demonstrated by Wilson et al. for the analysis of a pre-clinical safety assessment study.[4] The number of overall detected features with RAMMP can be compromised when compared with UPLC. However, combining the RAMMP methodology with data independent acquisition (DIA) strategies involving an ion mobility (IM) schema results in both high peak capacity and ultimately larger numbers of detected features being reported.

WATERS SOLUTIONS

Progenesis™ QI Software

SYNAPT™ G2-S*i* HDMS

ACQUITY™ UPLC™ I-Class System

ACQUITY BEH™ C$_8$ Chemistry

KEYWORDS

Metabolomics, large cohort analysis, lipidomics, LC-MS, ion mobility, HDMS^E

EXPERIMENTAL

Sample description

A pooled sample was prepared by combining 10 µL of all samples (breast cancer patients and healthy controls) which were subsequently stored at -20°C until use. Prior to analysis, each sample was subject to precipitation using the ratio of 1:4 with isopropanol (IPA). The sample was then vortex mixed and centrifuged at 13,000 rpm in a microfuge for five minutes. The resulting supernatant layer was removed and transferred to an autosampler vial for analysis.

Method conditions

LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	ACQUITY BEH C8 1.0 × 50 mm
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	0.2 µL
Flow rate:	250 µL/min
Mobile phase A:	(50:25:25) H_2O:IPA:MeCN w/5 mM ammonium acetate and 0.05% acetic acid
Mobile phase B:	(50:50) IPA:MeCN w/5 mM ammonium acetate and 0.05% acetic acid

Gradient:

Time (min)	Solvent composition (%A)	Solvent composition (%B)
0.05	90	30
2.8	30	90
3.0	0.1	99.9
3.15	0.1	99.9
3.7	90	30

MS conditions

MS system:	Synapt G2-S*i*
Ionization mode:	ESI+
Acquisition range:	50–1200 *m/z*
Capillary voltage:	3.0 kV
Collision energy:	Low energy 5eV, high energy 25eV
Cone voltage:	30 V

Data management

MS software:	MassLynx™
Informatics:	Progenesis QI

RESULTS AND DISCUSSION

The lipidomic analysis of samples arising from large cohorts requires increased analytical throughput. Simply reducing the LC analysis time however, would result in a loss of chromatographic peak capacity. To address these concerns, the separation was geometrically scaled from a 2.1 × 100 mm ACQUITY BEH C$_8$ Column to a narrow bore 1.0 × 50 mm ACQUITY BEH C$_8$ Column, with a mobile phase flow rate of 250 µL/min and an injection volume of 0.2 µL. This approach ensures that the number of column volumes defining the gradient was kept approximately constant between the two separations, resulting in the chromatographic analysis time being reduced from 15 to 3 minutes. The chromatographic performance of the RAMMP lipid methodology was evaluated using the Avanti Lipid differential ion mobility mix. The resulting data displayed in Figure 1, shows that the system delivered a high quality separation that was capable of separating standards in the test mix within the 3.7 minute analysis time.

Figure 2 is an example LC-MS chromatogram representing the extracted lipid QC sample, comparing conventional UPLC and RAMMP lipid methods. Total ion current (TIC) chromatograms demonstrate that the chromatographic profile of the lipid analysis was conserved when transferring from UPLC to RAMMP, maintaining lipid class separation for free fatty acids (FFA), glycerophospholipids and glycerolipids. The reproducibility of RAMMP was evaluated by comparing the chromatographic profile of the QC samples throughout the analysis of the plasma batch. The overlaid chromatograms for four QCs representing the beginning, middle, and end of the analytical batch are displayed in Figure 3. The data demonstrates the reproducibility of the RAMMP Lipid LC-MS methodology and that the quality of the separation is not deleteriously affected by the analysis of multiple plasma samples.
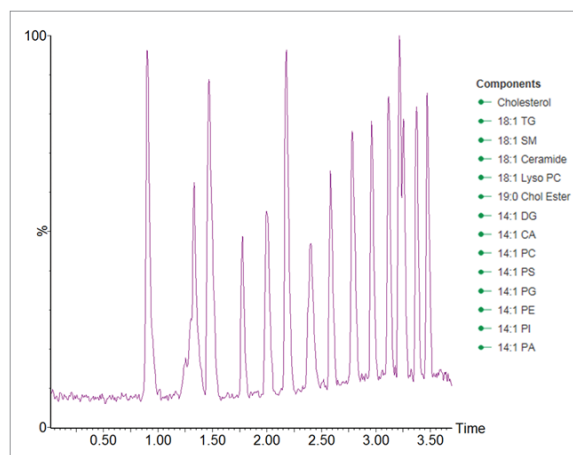

Figure 1. Chromatographic analysis of the Avanti lipid differential ion mobility lipid mix using the RAMMP lipid methodology.
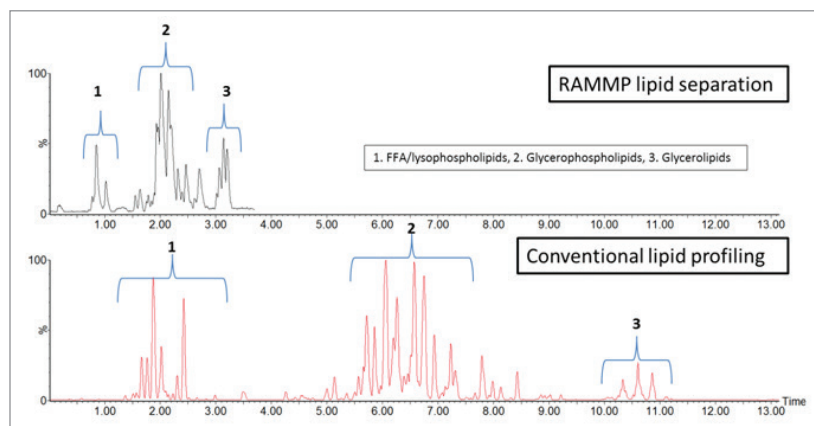

Figure 2. Comparative chromatograms for components of the lipid classes of free fatty acids (FFA), glycerophospholipids and glycerolipids using (a) RAMMP lipid method (b) conventional lipid method and respectively.
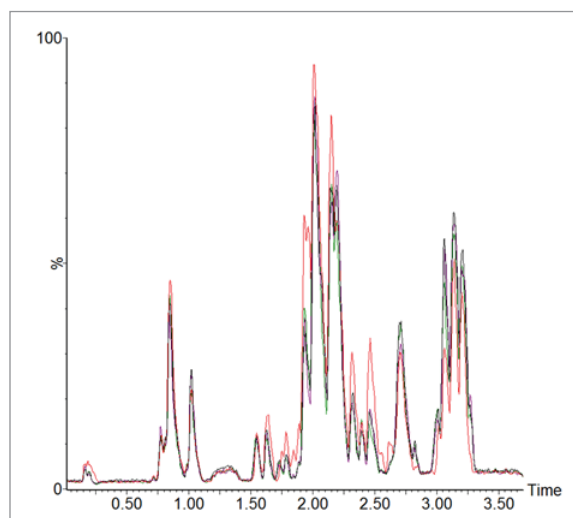

Figure 3. Overlaid extracted ion (BPI) chromatogram of 4 QC from the beginning, middle, and end of the batch.

Multivariate statistical analysis (MVA) shows clear separation between breast cancer samples (BC) and healthy controls (HC), when applying an orthogonal partial least squares (OPLS-DA) approach (Figure 4). To ensure that the loss in peak capacity was minimized when switching from the conventional more extensive UPLC analysis to RAMMP, IMS was implemented as part of the workflow resulting in increased feature discrimination per unit time. The implementation of IMS improved the spectral clarity and facilitated the measurement of collision cross section (CCS) values. The fragment ion spectra and derived CCS values were used to identify differentially expressed lipids within the breast cancer cohort. Features contributing to the MVA discrimination were subjected to database analysis. The CCS enabled searches resulted in a number of top hit database searches (Figure 5), including PC 36:2, which was shown to be up regulated in healthy control plasma. The same lipid was also identified as a high ranking hit when searched against Lipidblast and METLIN™ databases. The lipids detected and shown to be differentially expressed in the breast cancer samples compared to the healthy controls are listed in Table 1. From this data we can see that the phosphatidylcholines lipids, triglycerides, and diglycerides showed lower expression in the breast cancer samples whereas the phosphatidylserine showed greater expression in the breast cancer samples.
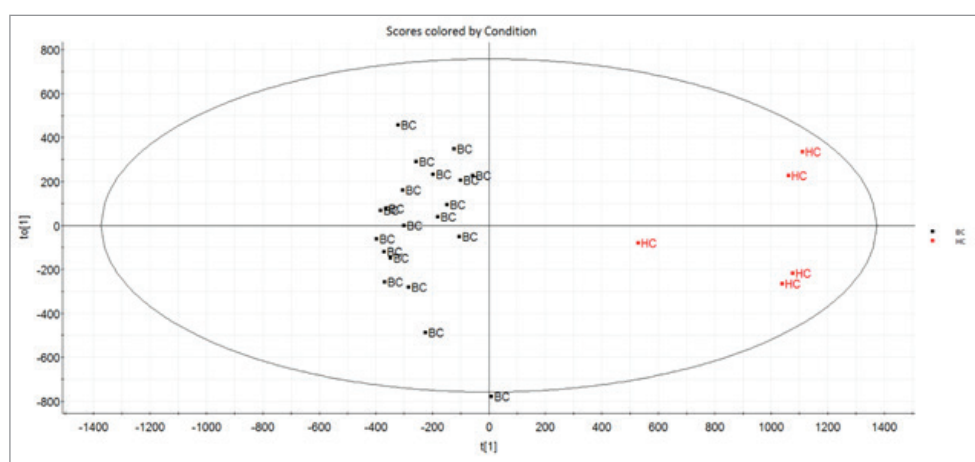

Figure 4. OPLS-DA plot differentiating healthy controls (HC) from breast cancer (BC) subjects.
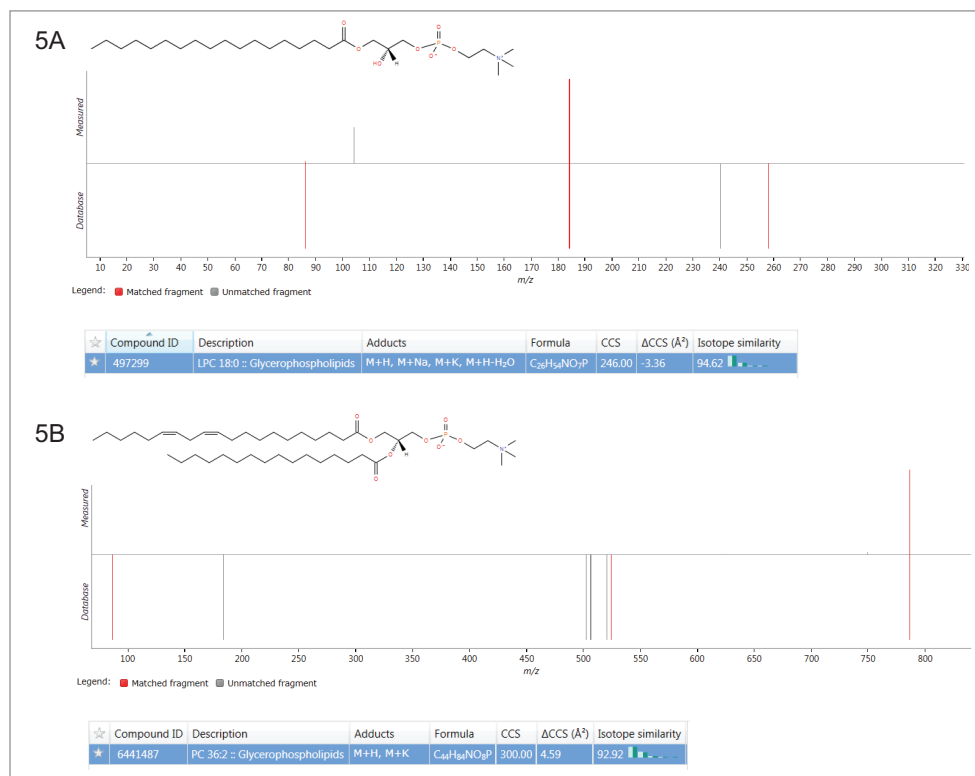

Figure 5. LPC 18:0 (A) and PC 36:2 (B) are example top scoring, tentative lipid identifications, which incorporate CCS as an identification parameter. PC 36:2 in particular, is shown to be under expressed in breast cancer subjects.

Lipid identification	Neutral mass (Da)	m/z	Retention time (min)	CCS (Å2)	ΔCCS (Å2)	Chromatographic peak width (min)	Anova (p)	q Value	Max Fold Change	Minimum CV%
TG(52:3)	856.75	874.79	3.14	334.1	–	0.20	5.5E-05	0.00166	1.5	6.03
TG(52:4)	–	872.77	3.06	331.4	–	0.23	1.0E-04	0.00201	1.8	4.85
TG(54:5)	880.75	898.78	3.07	337.6	–	0.18	1.5E-04	0.00237	2.6	5.53
PS (40:4)	839.57	822.56	1.62	304.4	–	0.30	2.1E-04	0.00274	2.0	4.38
TG(54:3)	–	902.82	3.22	341.1	–	0.24	3.4E-04	0.00386	2.0	5.54
DG(34:1)	–	577.52	3.2	267.1	–	0.18	3.7E-04	0.00404	1.8	2.97
TG(50:1)	832.75	850.79	3.19	332.5	–	0.16	3.9E-04	0.00408	1.7	5.15
PS(O-36:2)	773.56	774.56	1.55	295.8	–	0.31	5.0E-04	0.00452	1.9	3.16
PS(O-38:3)	799.57	782.57	1.62	299.7	–	0.28	6.5E-04	0.00529	1.8	3.09
PS(36:1)	789.55	790.56	1.54	298.7	–	0.41	2.3E-03	0.01119	2.9	2.21
PC(36:4)	781.56	782.57	2.01	306.6	–	0.28	6.6E-03	0.02300	1.5	5.15
PC (38:4)	809.59	810.60	2.19	312.2	7.2	0.37	8.3E-03	0.02592	1.6	4.06
PC (36:2)	785.60	786.60	2.19	304.6	4.6	0.20	1.9E-02	0.04109	1.3	4.69
PS (38:2)	815.57	816.57	1.63	306.3	–	0.41	2.2E-02	0.04428	1.5	4.56
PC(34:2)	757.57	758.57	2.02	296.9	–	0.24	2.7E-02	0.04926	1.3	4.84

Table 1. Summary of lipids which show differential expression in breast cancer samples. Over and under expressed lipids are shown as light blue and blue rows respectively.

CONCLUSIONS

- A rapid lipid profiling method has been successfully developed and applied for the investigation of lipids originating from human plasma derived from breast cancer and control samples.

- Reducing the analysis time from 15 to 3.7 minutes using a RAMMP approach has shown that retention and peak shape of lipids is maintained and the same statistically relevant features are identified, while increasing throughput and improving batch to batch robustness.

- Acquiring LC-MS data using a DIA-IMS workflow has shown increased peak capacity, specificity, and spectral clarity, which ultimately provided improved confidence for the identified compounds.

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