HIGH THROUGHPUT PLASMA PROFILING OF HUMAN LIVER DISEASE SAMPLES USING RAPID CHROMATOGRAPHY AND A MULTI-REFLECTING TIME-OF-FLIGHT MASS SPECTROMETER

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INTRODUCTION

Metabolomics is a valuable research strategy in the determination of disease progression, disease stratification, the identification of biological markers for early diagnosis and for the development of new therapeutics. Acute-on-chronic liver failure (ACLF) is a condition characterized by systemic inflammation, multiple organ failure, and high short-term mortality[1]. ACLF develops from the acute decompensation of cirrhosis and currently there is no biomarker identified for the early diagnosis of this disease. Therefore, fully understanding the role metabolites and lipids play in the progression of ACLF and its underlining mechanisms could greatly help determine new diagnostic markers driving development of new therapeutics. Investigating biomarkers for large scale studies requires robust, high-throughput analytical methods.

Rapid microbore metabolic profiling (RAMMP) methods and conventional high-resolution mass spectrometry have previously been shown to considerably reduce analysis time[2-3]. Here we compare these methods with a vacuum jacketed column technology (VJC)[4-5] and ultra high-resolution mass spectrometry[6] for the analysis of plasma from liver disease patients by reversed-phase (RP) for lipid and hydrophilic interaction chromatography (HILIC) for small molecules.

METHODS

Sample preparation

Human plasma samples from four patient groups, healthy control (n=25), cirrhosis (n=25), ACLF (n=25), and acute liver failure (ALF) (n=25), underwent extraction for lipids and small molecules. For lipids, 50 µL of sample underwent protein precipitation with 200 µL of cold isopropanol (IPA) and then incubated for 2 h at 2-8 °C prior to centrifugation and transfer of supernatant to an analytical plate. For the HILIC analysis, 50 µL of plasma was combined with 20 µL of water and 180 µL of acetonitrile . Samples were then incubated for 2 h at 2-8 °C, centrifuged and supernatant transferred to an analytical plate. Quality-control (QC) sample was prepared by combining aliquots of each study sample and phenotypic pools were created by combining aliquots within the study groups. **(Fig1)**

LC-MS method

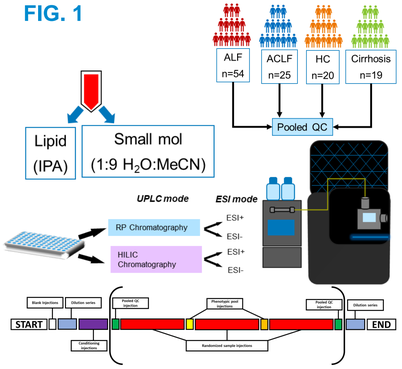
Chromatographic separation was performed using the RAMMP methodology with small molecules analysed using the HILIC[2] ACQUITY™ BEH™ amide (1.0 x 50 mm) and lipids[3] profiled using a ACQUITY BEH C8 (1.0 x 50 mm) column. Mobile phase consisted of (A) water:acetonitrile (95:5) w/ 5 mM ammonium formate and 0.1 % formic acid and (B) water:acetonitrile (5:95) w/ 5 mM ammonium formate and 0.1 % formic acid for RAMMP HILIC and (A) water:isopropanol:acetonitrile (50:25:25) w/ 5 mM ammonium acetate and 0.05% acetic acid and (B) isopropanol:acetonitrile (50:50 v/v) w/ 5 mM ammonium acetate and 0.05% acetic acid for RAMMP lipid. For the VJC assessment, columns of the same dimensions were made in-house using the VJC technology, with all LC conditions maintained from the RAMMP analysis. Mass spectrometry data was acquired on the SELECT SERIES™ MRT mass spectrometer[6] using the data independent acquisition (DIA) mode, MS^E in both positive and negative ESI mode though a low flow ESI probe. Scan speed of 0.033s (30 Hz) was used and leucine enkephalin was used as lockmass (200 pg/ µL).

Data processing

All data were acquired using MassLynx™(v.4.2) and all data processed using MARS and Lipostar2 (Mass Analytica. Spain) software where raw data underwent peak picking, normalization, database searching and statistical analysis.

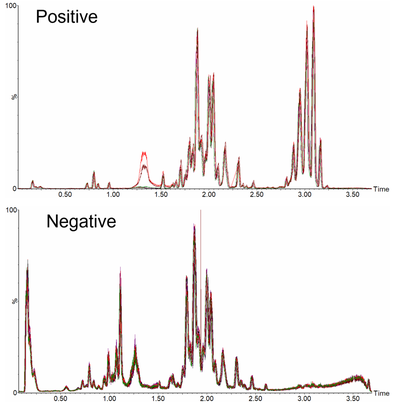
RESULTS

FIG. 1



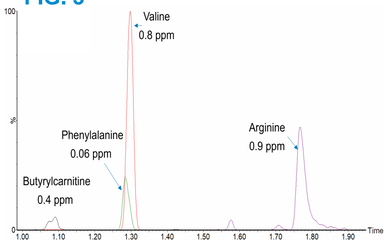
Schematic of sample preparation and LC-MS analysis.

FIG. 2



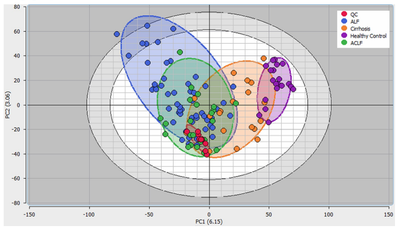
Overlaid TIC chromatograms of pooled QC injections (n=9) from across the batch (168 injections) for both positive and negative ESI for the reversed phase lipid analysis, showing good injection reproducibility.

FIG. 3



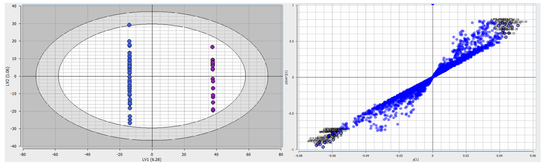
Example overlaid XIC's of 4 small molecule annotated compounds in the pooled QC samples analysed by positive mode HILIC, with their corresponding mass accuracy measurements, all <1ppm.

FIG. 4



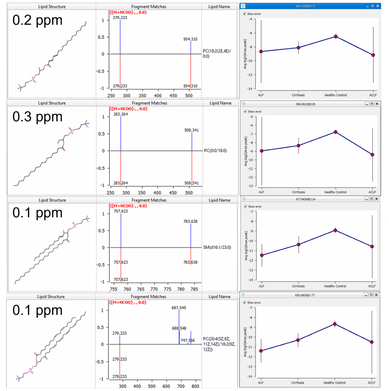
Lipid negative PCA plot of all samples highlighting tight QC clustering (red) with group overlaps between ALF and ACLF samples and significant separation of Healthy controls (purple) when compared to ALF and ACLF patients.

FIG. 5



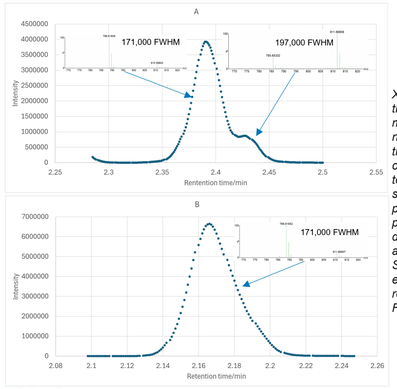
OPLS-DA of ALF and Healthy control patients with the corresponding S-plot with significant features contributing to the variation between these groups highlighted.

FIG. 6



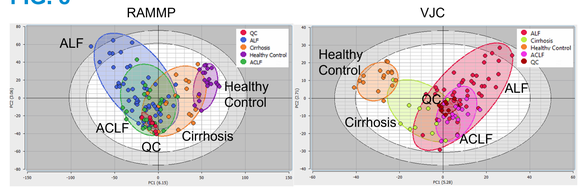
Example database annotations of statistically significant lipids with corresponding mass errors of < 0.3 ppm alongside matched MS/MS fragment ions (red). Adjacent trend plots for each, highlighting the dysregulation seen in the ACLF and ALF patient groups compared to healthy controls and cirrhosis groups.

FIG. 7



XIC of example lipid peaks from the VJC (A) and the RAMMP methodology (B) highlighting the number of scans collected across the analytical peak at 30 Hz combined with the VJC technology's ability to produce sharper peaks allowing better peak fidelity. This enabled the partial separation and determination of two lipid species, annotated as PC36:1 and SM42:3,O2. Both with mass errors of 0.1 ppm at mass resolutions of between 170-200K FWHM.

FIG. 8



Comparison of PCA plots of negative RAMMP lipid and VJC Lipid analysis highlighting similar group distributions and clustering of each study group showing the clear distinction of the healthy control groups from the diseased groups.

CONCLUSION

- **Conventional LC-MS methods used for large cohort studies suffer from batch response variation. The rapid chromatographic methods reduce this impact and demonstrated reproducible response across the entire batch of 168 injections per polarity (Fig. 2).**
- **The SELECT SERIES MRT mass spectrometer generated highly accurate mass measurements (<1 ppm), improving annotation confidence for both lipids and small molecules, including essential amino acids and carnitines from the HILIC analysis (Fig. 3).**
- **PCA of the RAMMP data showed good analytical reproducibility from the clustered QC samples and group separation with some overlap between the different disease groups (Fig. 4).**
- **Group comparisons were performed by OPLS-DA where statistically significant features were highlighted for database annotation (Fig. 5).**
- **A number of lipids classes, particularly lysophosphatidylcholines (LPC), phosphatidylcholines (PC) and sphingomyelins (SM), showed dysregulation in the diseased samples when compared to healthy controls and were annotated through Lipostar2 with mass accuracies between 0.1-0.3 ppm (Fig. 6).**
- **For the HILIC small molecule analysis, differences in amino acid response were detected in comparisons between diseased and healthy controls.**
- **The SELECT SERIES MRT MS achieved mass resolution of 170-200K FWHM at 30 Hz compatible with RAMMP (a) and VJC (b) methodologies for example lipids, PC36:1 (0.18 ppm) and SM42:3, O2 (0.16 ppm), with the VJC providing better peak shape and resolution of adjacent peaks (Fig. 7)**
- **Comparison of the RAMMP and VJC negative lipid analysis showed very similar distribution of disease and healthy control groups demonstrating minimal impact on the overall statistics (Fig. 8).**
- **Both RAMMP and VJC methodologies combined with the SELECT SERIES MRT provided highly accurate reproducible metabolic profiling for a biomarker study, with the VJC providing improved peak fidelity complemented by the fast scanning high resolution capabilities of the SELECT SERIES MRT.**
- **This work demonstrated an accurate, reproducible and high throughput metabolomic method for the rapid screening of a large number of biological samples for biomarker discovery.**

References

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