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Application News

High Performance Liquid Chromatograph Mass Spectrometer

Untargeted Metabolomics Analysis of Ethanol Exposure in Liver Using LC/QTOF

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User Benefits

- Untargeted metabolomics workflow for High-Resolution LC/QTOF (applicable to LCMS-9030 and 9050).
- Applying an LC/QTOF method with fast scanning MS/MS to an untargeted metabolomics workflow to identify metabolic profiles in biological tissue samples.
- The workflow delivers an end-to-end solution for untargeted metabolomics research requiring minimal prior experience.

■ Introduction

Ethanol is a psychoactive and toxic substance that can cause dependence and is associated with neuropsychiatric disorders, cancers, cardiovascular disease, pancreatitis, and alcoholic liver disease. The underlying pathology of alcohol-induced diseases, particularly liver disease, involves a complex interplay of factors including alcohol metabolism, inflammation and oxidative stress, ultimately leading to liver damage, fibrosis, and cirrhosis. In this paper, a method developed for the untargeted metabolomics analysis of biological tissues and biofluids and was applied to study the metabolic effects of ethanol exposure in the liver using a mouse model.

■ Study design

Metabolic profiles of liver tissue extracts from mice exposed to ethanol were compared to un-dosed controls. C57BL/6 Mice were subjected to a short-term model for exposure to ethanol (8 un-dosed controls and 8 treated over an 11-day duration). Exposure to ethanol was achieved by feeding animals, ad libitum, with the Lieber-DeCarli ethanol diet, containing 5% extra pure ethanol. The study was carried out in accordance with EU and National ethical guidelines and was approved by the Aristotle University of Thessaloniki.

The study was designed to exemplify a high-resolution LC-MS/MS data acquisition method developed for routine, robust metabolomics research requiring minimal MS expertise which can be applied to the untargeted metabolomics studies of biological tissues and biofluids. The method is suitable for the LCMS-9030 and LCMS-9050 (Fig. 1) QTOF instruments and offers flexibility to work with both data independent acquisition (DIA) or data dependent acquisition (DDA). In this application, DIA was applied to an untargeted approach, capturing MS/MS for all detectable features in all samples.

Fig. 1 LCMS-9050 QTOF system

■ LC/QTOF untargeted analysis of tissues

Livers were collected post-mortem and extracted in methanol: isopropanol: water (3:3:1, v/v/v) at a ratio of 1.3mg tissue/µL solvent. Supernatants were evaporated to dryness and reconstituted in methanol for LC/QTOF analysis at a dilution of 1 in 20. For untargeted metabolomics analysis, high resolution UHPLC-MS/MS data were acquired using TOF-MS and DIA-MS/MS in both positive and negative ESI (Table 1) following C18-reversed phase LC separation.

Preparation of quality control (QC) samples was performed for assessing the quality of the acquired data following the metabolomics Quality Assurance and Quality Control Consortium (mQACC) recommendations[1]. QCs were prepared by combining equal aliquots of each sample.

Table 1 Analytical conditions

UHPLC:	Nexera X2
Column	C18 reversed-phase column (2.1x100 mm, 1.7 µm); maintained at 50 °C
Flow Rate	0.4 mL/min
Mobile Phase	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid
Rinse (R0/R1/R2)	IPA/MeOH/MeCN/H_2O (1:1:1:1) + 1% formic acid
Gradient	2% B (0-1min); 40% B (2 min); 90% B (25 min, Curve -3); 100% B (27.5-31 min); 2% B (31.01-35 min)
Injection	0.5 µL; autosampler temperature 4 °C
Mass Spectrometer:	**LCMS-9030**
Interface	300 °C, 1.5 kV (ESI+) / -3.0 kV (ESI-)
Heat block, DL	400 °C, 250 °C
Gas flow rates	Nebulizing gas 3 L/min; Heating gas 10 L/min; Drying gas 15 L/min
CID gas pressure	230 kPa
MS	TOF-MS (*m/z* 100-1000)
MS/MS	45 DIA-MS/MS (*m/z* 40-1000; precursor isolation 20 Da; collision energy spread 5-55 V)
Total cycle time (MS and MS/MS)	1 second

■ Data processing

MS-DIAL, a universal program for untargeted metabolomics, was used to find, align and annotate features across all samples. Default processing parameters were applied to data files without any data conversion (MS-DIAL reads LabSolutions™ raw data files directly). MS-DIAL can be applied to process data from both data dependent or data independent acquisition data types automatically without supplying additional information on data structure. The software includes workflows for metabolomics and lipidomics. In this study, the lipidomics workflow was applied to process data from positive and negative ion mode acquisitions separately. The lipidomics workflow uses an 'in-silico retention time and MS/MS database for LC/MS/MS based lipidomics' to perform lipid annotation. To enable annotation of metabolites in the same dataset, the 'start-up kit' .msp files available from MS-DIAL were added in the identification step. The kit includes external repositories such as MassBank.

As shown in Fig. 2, MS-DIAL allows the easy navigation of samples and features. Selecting any peak in the 'alignment spot viewer' shows the extracted ion chromatograms for that feature in all samples. More details about the peak can be found by launching the 'show ion table' feature, which highlights the number of samples the peak was found and aligned in, as well as the scores used to determine the quality of annotation.

The MS spectrum for the feature is displayed in the 'survey scan (MS1) spectrum' for the sample highlighted in the 'file navigator'.

In this case, data were acquired using DIA-MS/MS so MS-DIAL performs deconvolution before annotation. The deconvoluted DIA-MS/MS spectrum is reflected with reference spectrum corresponding to the candidate annotation with the highest match score. In this example, LPC 22:5 provided the best match to the highlighted peak and the MS/MS are presented as the 'Representative vs. Reference' MS/MS spectra. The representative spectrum is the deconvoluted DIA-MS/MS spectrum for the selected sample.

■ Data analysis

Data were processed using MS-DIAL. Aligned peak area data for each ion mode were exported from MS-DIAL for statistical analysis.

The feature arrays from positive and negative ion mode were combined for statistical analysis using MetaboAnalyst. Missing values were automatically detected and the default of replacing missing values by 1/5 of min positive values of their corresponding variable was applied. Features were filtered in MetaboAnalyst based on having relative standard deviation in the QCs < 20%. Interquartile range filtering was applied to the remaining dataset to remove near constant features (default of 40% applied).

Volcano plot analysis was performed using MetaboAnalyst to find the most significant features in the dataset, considering a fold change >4 and p-value <0.05 between treated and control samples (Fig. 3).

■ Metabolite and lipid annotation

The annotations for each of the features found to be significant were reviewed in MS-DIAL. Features of biological relevance were considered for reporting (Fig. 4). Features that were not reported included features that could not be annotated, or considered to be false positives, or previously identified (as an adduct or in a different polarity).

As a result, 15 features were annotated as potential markers of ethanol toxicity in liver (Fig. 4). Most annotations are classified as level 2 annotations (comparison of measured MS/MS to external sources) according to the metabolomics standards initiative (MSI)[2]. Ethyl oleate was annotated by comparison of retention time and MS/MS in the analysis of authentic reference material using the same instrument and analytical conditions used in this study. Ethyl oleate is therefore considered to be identified to MSI level 1.

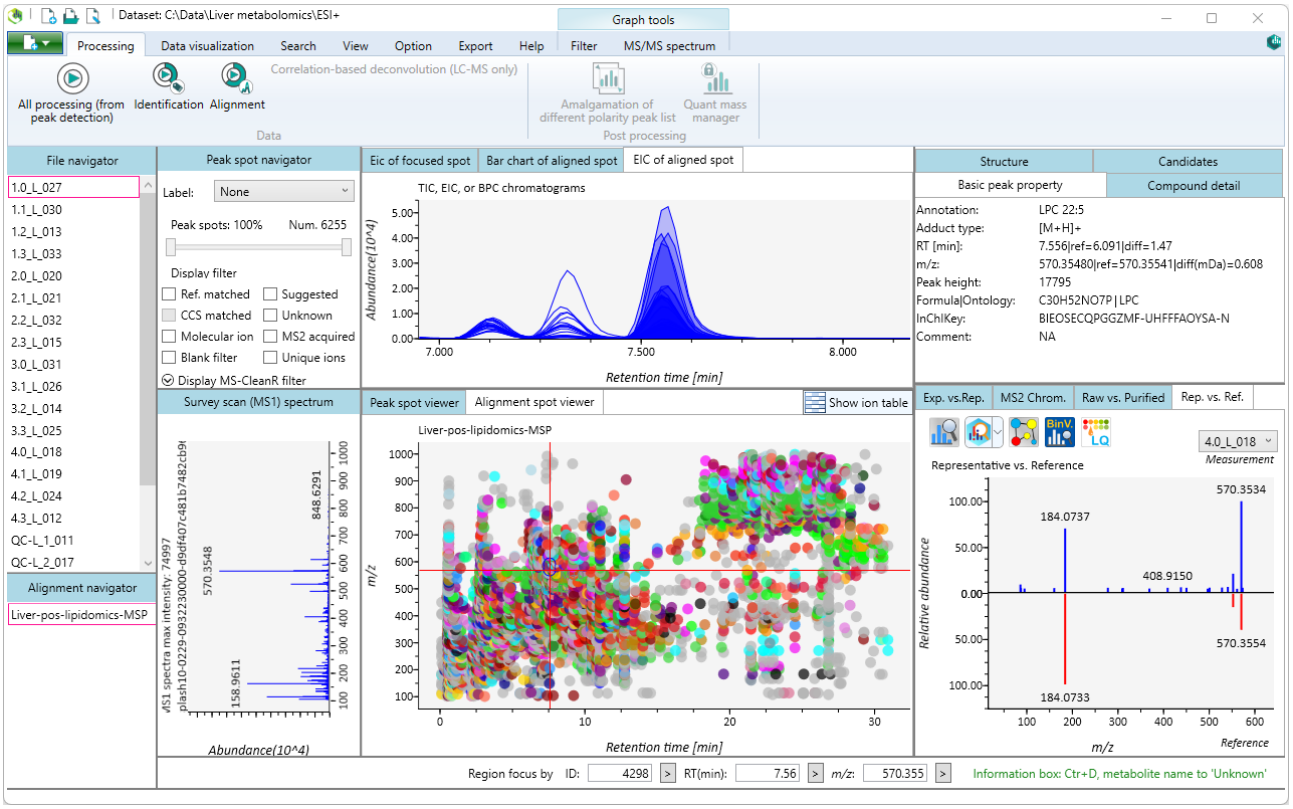

Fig. 2 MS-DIAL was used to process LabSolutions raw data files to find and align features that behaved as chromatographic peaks. The lipidomics workflow was applied in MS-DIAL enabling annotation of lipids to the 'in-silico retention time- and MS/MS database for LC/MS/MS based lipidomics'.

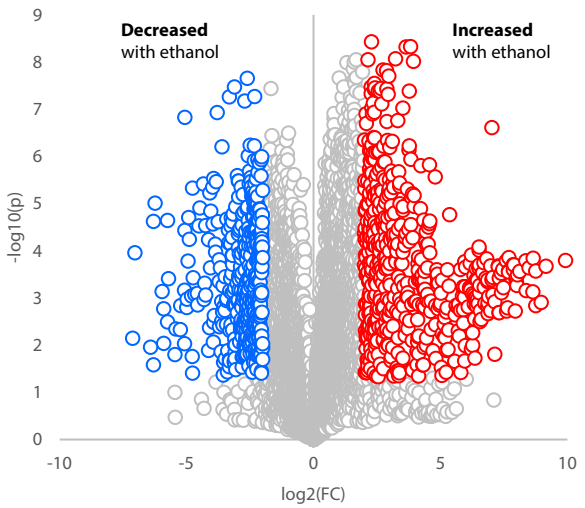

Fig. 3 Volcano plot of features detected using LC/QTOF analysis (positive and negative ESI combined), that were significantly increased (red) or decreased (blue) in liver tissue following ethanol administration (fold change >4, p<0.05).

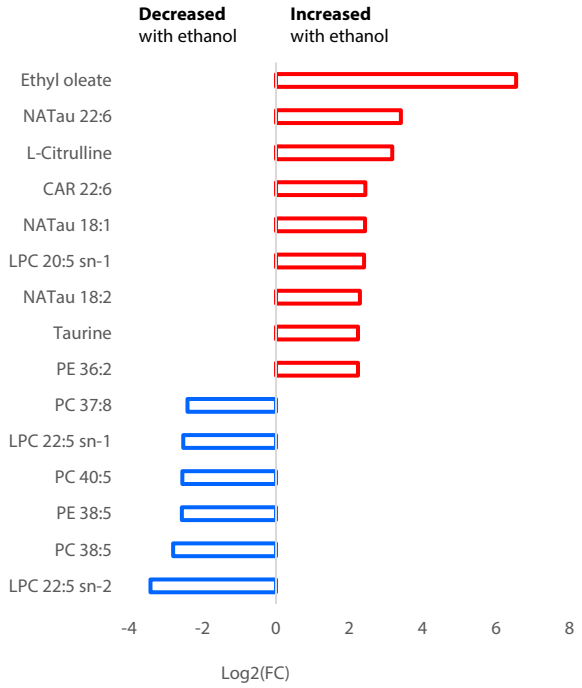

Fig. 4 Bar-chart highlighting annotated metabolites and lipids that exhibited the most significant changes (log2 fold change >2, p<0.05) caused by ethanol administration in liver tissue.

■ Conclusion

An end-to-end workflow developed for untargeted metabolomics on the LCMS-9030 (or LCMS-9050) QTOF has been applied to study ethanol-induced metabolic differences in liver tissue. The high-resolution reversed-phase LC/QTOF method with DIA-MS/MS was applied to analyse profiles from mouse liver tissue extracts following ethanol administration compared to controls.

Data were processed in MS-DIAL and analysed to find significant differences between groups in MetaboAnalyst. Following this workflow, significant alterations were found in the metabolic profiles of the liver tissue of mice exposed to ethanol compared to control animals. Fifteen annotated markers of ethanol toxicity were reported from the most significant differences between the groups (fold change >4; p<0.05). The workflow could be extended to consider the annotations of features with lower fold changes between groups.

■ References

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2. Spicer, R., Salek, R. & Steinbeck, C. (2017) A decade after the metabolomics standards initiative, it's time for a revision. Sci Data 4, 170138.

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