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Application News

High Performance Liquid Chromatograph Mass Spectrometer

Untargeted Metabolomics Analysis of Pancreatic Cancer Using LC/QTOF

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User Benefits

- LC/QTOF method for untargeted metabolomics with flexible workflows for Data Independent Acquisition (DIA) or Data Dependent Acquisition (DDA).
- Data are compatible with all major third-party tools for data processing and statistical analysis of metabolomics data.
- The workflow delivers an end-to-end solution for untargeted metabolomics research.

■ Introduction

Metabolomics is the study of a metabolic phenotype and its impact on a biological system. It involves the measurement of small molecules and can be applied to disease aetiology, discovering potential disease markers in human medicine, but also to other disciplines such as translational and precision medicine, untargeted analyses, nutritional assessment, fermentative optimisations, and agricultural productivity. Metabolomics studies are designed to detect and identify metabolites that are considered to be drivers of a biological process and understand their role in phenotype modulation.

In this application, an LC/QTOF data acquisition method was developed for routine, robust metabolomics research requiring minimal MS expertise which can be applied to the untargeted metabolomics studies of biological tissues and biofluids. Data were processed using commonly used open-source software applications cited in the literature. This study used MS-DIAL (principally for raw data access to detect and align features in each sample) and MetaboAnalyst (for advanced statistical analysis to find features that were differentially expressed in the study).

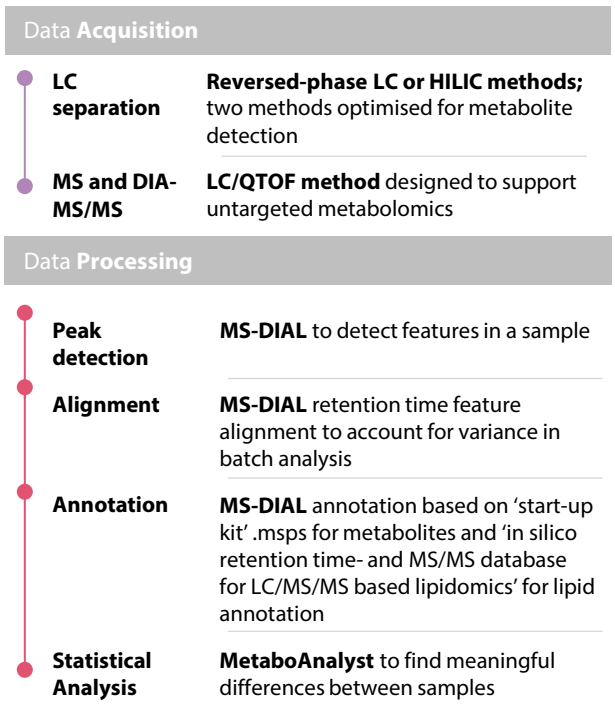


Fig. 1 A typical metabolomics workflow using LC/QTOF data acquisition methods specifically developed for metabolomics and data processing workflows highlighting the flexibility of working with open-source tools such as MS-DIAL and MetaboAnalyst. (Commonly used open-source data processing applications also include MZmine, GNPS and XCMS which can be used in the same data processing workflow).

■ Study design

The end-to-end untargeted metabolomics workflow has been applied to characterise the metabolic profiles of serum from patients with pancreatic ductal adenocarcinoma (PDAC) compared to healthy controls to reveal the key metabolic differences in these phenotypes. The research project was approved by the Pancreatic Cancer Research Fund Tissue Bank (PCRFTB) Tissue Access Committee.

■ Sample preparation and extraction

Serum samples were extracted in methanol (1:3 v/v) for UHPLC-MS/MS analysis. For untargeted metabolomics analysis in positive ion mode, samples were diluted 1 in 10.

■ Data acquisition

High resolution UHPLC-MS/MS data were acquired using TOF MS and DIA-MS/MS in positive ESI mode to exemplify the workflow (Table 1).

Table 1. Analytical conditions

UHPLC:	Nexera X2
Column	C18 ((2.1x100 mm 1.7 µm); column 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
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* 60-1000)
MS/MS	27 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

■ Applying Quality Controls

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]. Pooled QCs were prepared by combining equal aliquots of each sample.

Samples were analysed in a randomised order in positive ESI mode. QCs were used to equilibrate and stabilise the system. Following system stabilisation, pooled QCs were injected after 6 samples to monitor system performance and filter out any component feature that resulted in high variance in signal intensity, mass accuracy, retention time.

■ Data Processing using MS-DIAL

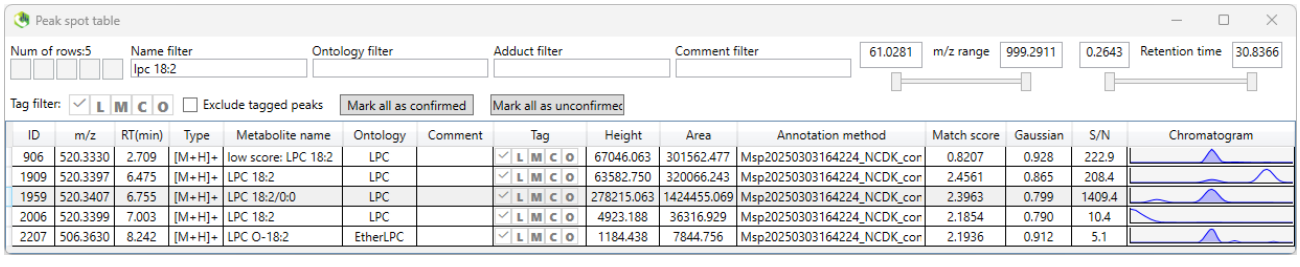
MS-DIAL, a universal program for untargeted metabolomics, was used to find, align and annotate features across all samples applying default processing parameters without any data conversion (MS-DIAL reads LabSolutions™ raw data files directly; Fig.2). MS-DIAL can be applied to process data from both data dependent or data independent acquisition data types automatically without supplying additional information on data structure.

In this study, the lipidomics workflow was applied to detect, align and annotate features in positive ion mode data. 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 'peak spot viewer' shows the extracted ion chromatogram for that feature in the sample highlighted in the 'file navigator' and the MS spectrum for the feature in 'survey scan (MS1) spectrum'. 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 18:2/0:0 provided the best match to the highlighted peak and the MS/MS are presented as the 'Deconvoluted vs. Reference' MS/MS spectra.

The 'show ion table' feature launches the peak spot table for the highlighted sample. This allows easy navigation of all peaks found in a sample (Fig. 3). Applying a name filter enables the user to review all peaks annotated with the same name. The chromatogram and match scores can be useful to indicate the confidence in each annotation.

Fig. 2 MS-DIAL was used to process LabSolutions raw data files to find features that behaved as chromatographic components and align individual components as features across samples. Once all features are aligned across all samples, statistical analysis is applied to identify features that are differentially expressed between the control and treatment groups.



ID	m/z	RT(min)	Type	Metabolite name	Ontology	Comment	Tag	Height	Area	Annotation method	Match score	Gaussian	S/N	Chromatogram
906	520.3330	2.709	[M+H]+	low score: LPC 18:2	LPC			67046.063	301562.477	Msp20250303164224_NCDK_cor	0.8207	0.928	222.9	
1909	520.3397	6.475	[M+H]+	LPC 18:2	LPC			63582.750	320066.243	Msp20250303164224_NCDK_cor	2.4561	0.865	208.4	
1959	520.3407	6.755	[M+H]+	LPC 18:2/0:0	LPC			278215.063	1424455.069	Msp20250303164224_NCDK_cor	2.3963	0.799	1409.4	
2006	520.3399	7.003	[M+H]+	LPC 18:2	LPC			4923.188	36316.929	Msp20250303164224_NCDK_cor	2.1854	0.790	10.4	
2207	506.3630	8.242	[M+H]+	LPC O-18:2	EtherLPC			1184.438	7844.756	Msp20250303164224_NCDK_cor	2.1936	0.912	5.1	

Fig. 3 Peak spot table in MS-DIAL used to navigate all detected peaks in a sample when launched from the peak spot viewer (shown here) or all aligned features when launched from the alignment spot viewer. When launched from the peak spot viewer, the chromatogram for each feature is displayed for the highlighted sample. Applying filters such as 'name filter' applied here, allows the user to review a subset of the data more closely. In this example, all annotations containing 'lpc 18:2' are shown.

■ Statistical analysis

To find features that differed between the PDAC group and controls, a single aligned and filtered feature array for positive ion was analysed using MetaboAnalyst. MetaboAnalyst has the advantage of supporting a wide variety of commonly used statistical methods such as fold change, t-test, volcano plot, ANOVA, correlation analysis.

The aligned data array was filtered (criteria included present in at least 50 % of all pooled QCs (n=11) with RSD <20 % and present in at least 80 % of either the PDAC or healthy control group).

Upon loading the data to MetaboAnalyst, missing values were automatically detected by the software and the default of replacing missing values by 1/5 of min positive values of their corresponding variable was applied. A volcano plot was used to identify significantly different features in the PDAC group compared to controls (Fig. 4). Using volcano plot analysis (scatterplot that shows statistical significance (-log10 p-value) versus magnitude of change (log2 fold change) a total of 229 features were found to be significant (criteria set to fold change >1.5 and p-value <0.1). Features meeting the criteria are coloured pink for fold increases in PDAC samples compared to controls and purple for fold reductions.

The annotations for each of the features found to be significant were reviewed in MS-DIAL. Features that were not reported included features that could not be annotated, annotated features that were not considered to be biological markers of PDAC (e.g. medicines), annotations that were not at the expected retention times for this reversed-phase method and annotations for additional adducts that were already reported for the dominant ion. Following this, 45 features could be annotated as potential markers of PDAC. All annotations are classified as level 2 annotations (comparison of measured MS/MS to external sources) according to the metabolomics standards initiative (MSI)[2].

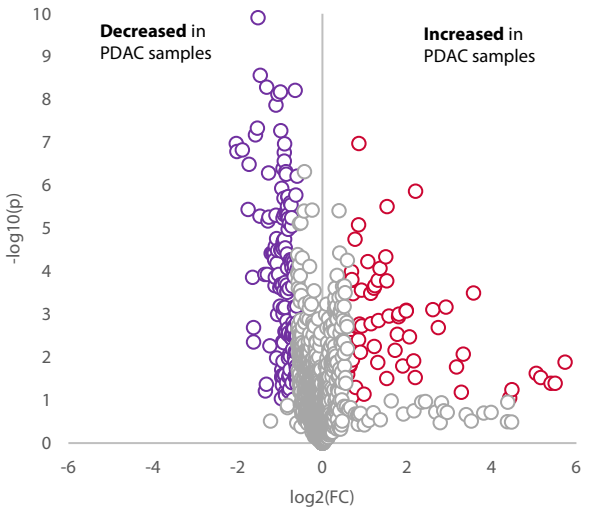


Fig. 4 Volcano plot of features detected using LC/QTOF in positive ion mode. Significantly increased features in the serum profiles of PDAC features are coloured pink and significantly decreased features in PDAC samples compared to healthy controls are coloured purple (criteria for significance defined as fold change >1.5, p<0.1).

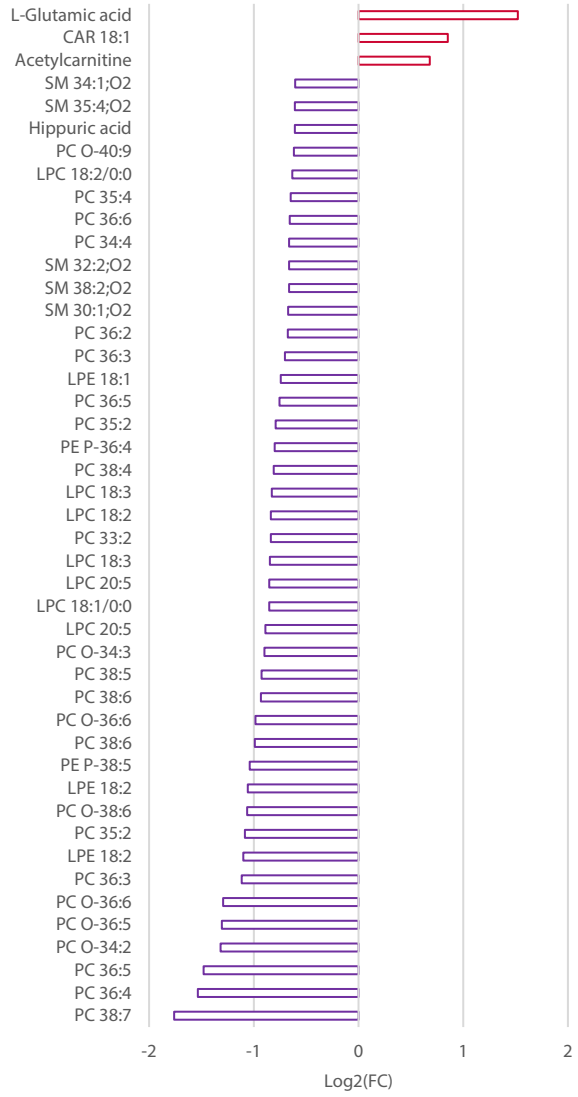


Fig. 5 Box plots of metabolites lipids annotated using MS-DIAL found to be significantly different in PDAC serum profiles versus healthy controls.

■ Conclusion

An end-to-end workflow has been developed for untargeted metabolomics and applied to the study of pancreatic cancer. An LC/QTOF method with reversed-phase separation and DIA-MS/MS was applied to analyse profiles of serum from patients with pancreatic ductal adenocarcinoma (PDAC) and healthy controls. Data were processed in MS-DIAL and analysed to find significant differences between groups in MetaboAnalyst. Following this workflow, 45 potential markers of PDAC were found in positive ion mode. The workflow could be extended to consider negative ion mode markers of PDAC.

■ References

1. Kirwan, J. A., Gika, H., Beger, R. D., et al. (2022). Quality assurance and quality control reporting in untargeted metabolic phenotyping: mQACC recommendations for analytical quality management. Metabolomics. 18 (9).
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.

05-SCA-210-086-EN First Edition: Jul. 2026

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