Increasing reporting confidence in metabolomics; RP and HILIC LC-MS/MS analysis in multiple tissue studies

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Overview

- The chemical and physical diversity of the metabolome creates challenges in developing methods that generate high metabolite coverage and reporting confidence with a sufficiently high analytical throughput.
- Applying a single reverse phase LC-MS/MS method primarily designed to differentiate lipid distributions in tissues may be challenged in identifying highly polar metabolite signals with high certainty.
- HILIC LC-MS/MS has been used to increase reporting confidence and reduce false discovery rate with highly polar metabolites.

1. Introduction

Reverse-phase high resolution LC-MS/MS methods are core analytical tools in tissue metabolomic studies. Their strengths include a high metabolite coverage, particularly with lipid distributions (for example; LPCs, LPEs MGs, fatty acids, fatty acid conjugates and fatty amides) and high reporting confidence for a broad range of polar metabolites (nucleotides, amino acids, purines, pyrimidines and associated derivatives). However, dependent on the study design, tissue type and metabolite class, reporting confidence may be challenged for highly polar metabolite signals. In such cases HILIC can be a powerful complimentary technique to help identify metabolites with greater certainty.

2. Materials and Methods

Multiple tissue types from different metabolomic studies were analysed by a standardized reverse-phase LC-MS/MS method and by HILIC. All tissue extracted samples were reconstituted in 90% acetonitrile. Tissues included brain, liver, pancreas, gut, plasma, caecal and faecal from several murine metabolomics studies. The HILIC method was developed to selectivity analyse highly polar metabolites.

- **Reverse phase LC Separation**.
  - Acquity C18 BEH (2.1x100mm 1.7µm); 50ºC, flow rate 0.4 mL/min
  - Binary gradient; water and 0.1% formic acid, and acetonitrile + 0.1% formic acid
  - Cycle time 35 minutes.
- **HILIC LC Separation**.
  - Shim-pack Velox HILIC (2.1x100mm 2.7µm); 40ºC, flow rate 0.3 mL/min
  - Binary gradient; water + 10mM ammonium formate 0.1% formic acid, and acetonitrile:water+ 10mM ammonium formate 0.1% formic acid [92:8]
  - Cycle time 18 minutes.
- **Mass Spectrometry Detection**. QTOF LCMS-9030 using external mass calibration for positive and negative mode ESI (for the examples shown all m/z values are for positive ion detection)
  - MS mass scan m/z 60-1000; 100 msecs
  - DIA-MS/MS mass scans m/z 40-1000; 33 msecs for each precursor isolation window; isolation width 35 Da; collision energy spread 5-55V; 27 mass scan events. Scan cycle time 0.99 second (28 mass scan in total).
- **Data processing**. A target list of 225 metabolite targets including lipid distributions (LPCs, LPEs, MGs, fatty acids, fatty acid conjugates and fatty amides) and polar metabolites (nucleotides, amino acids, purines, pyrimidines) was processed using LabSolutions Insight software and compared against an in-house metabolomics library (acquired using authentic reference material) for high reporting confidence.

3. Results

3.1 Increasing reporting confidence in metabolomics

Core analytical reverse-phase LC-MS/MS metabolomic analysis; developed for a high metabolome coverage. As one example, feature detection in a mouse brain extract.

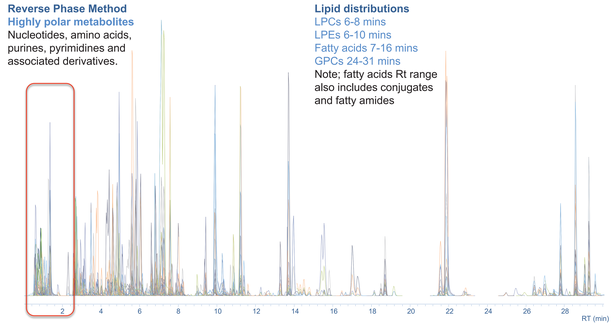

Figure 1 Metabolite features detected by reverse-phase LC-MS/MS for a mouse brain tissue sample as part of a study to determine the effect of ethanol on the metabolome (Insight Analyze was used to detect components using a pre-set threshold). Highly polar metabolites were further qualified using HILIC to increase reporting confidence in isobaric feature identification and further resolving isobaric features in different tissue types.

HILIC LC-MS/MS analysis of highly polar metabolites in mouse brain tissue

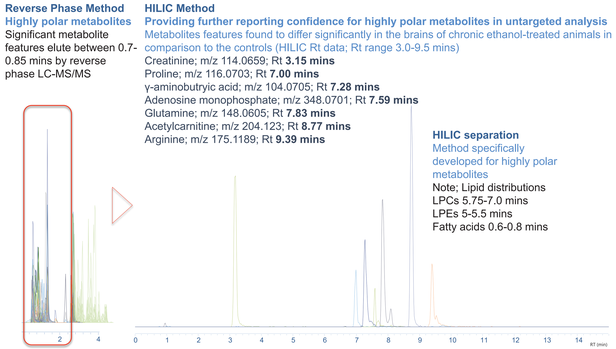

Figure 2 The chronic and acute effect of ethanol administration on the metabolic phenotype of mouse brain was studied in a C57BL/6 mouse model of ethanol abuse using both untargeted and targeted LC-MS/MS. Several highly polar metabolites showed altered brain metabolome perturbations which could be further confirmed using HILIC analysis.

3.2 Identifying isobaric metabolite features

HILIC applied to isobaric metabolite features to increase confidence in metabolite identification in a faecal gut content matrix.

Metabolite features were identified using an in-house library.

- MS/MS data acquired using authentic standards with a fragmentation energy of 5-55V.
- All product ion spectra corrected to theoretical accurate mass values using Insight Assign application.
- MS/MS spectra acquired using targeted MS/MS and a precursor ion isolation width of unit mass. Spectra were acquired positive and negative ion modes
- Retention time mapped to a Shim-pack Velox HILIC LC separation.

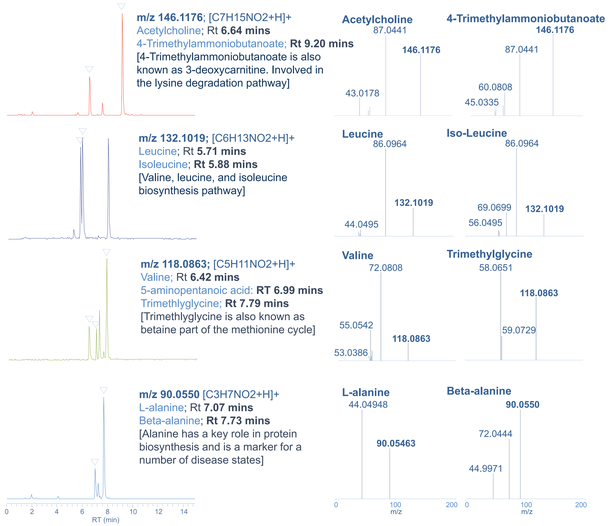

Figure 3 HILIC was applied to the separation of several isobaric metabolite features which elute between 0.7-0.85 mins on a reverse phase LC method. Using HILIC the isobaric metabolite features could be selectivity separated despite the complexity of the faecal gut content matrix. The sample was part of a gut microbiome disruption model following treatment with the antibiotic metronidazole.

Positive identification of highly polar metabolites such as nucleotides, amino acids, purines, pyrimidines is highly dependent on the selectivity of the precursor ion and the chimeric nature of the MS/MS spectra. As the HILIC method was specifically optimized for highly polar metabolites, ion signals at m/z 146.1176 successfully separated acetylcholine and 4-trimethylammoniobutanoate, and at m/z 118.0863 resolved valine, 5-aminopentanoic acid and trimethlyglycine. The isomers leucine/isoleucine and alanine/beta-alanine were also resolved.

3.3 Resolving isobaric features in different tissue types

Applying HILIC to reduce the false discovery rate in highly polar metabolite analysis.

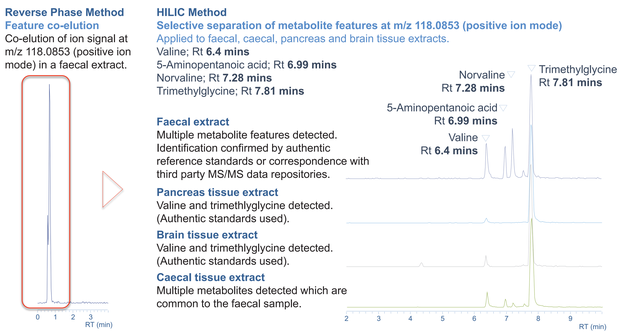

Figure 4 Precursor and data-independent MS/MS acquisition (DIA) methods coupled with reverse phase LC separations are powerful analytical tools in untargeted metabolomics. In this example, the identification of components eluting at 0.67 minutes at m/z 118.0853 (± 5 ppm) by reverse-phase LC was compromised by co-elution. HILIC separation was used to confirm metabolite identification with authentic reference materials. In the case of faecal and caecal samples valine, 5-aminopentanoic acid, norvaline, and trimethylglycine (also known as betaine) were detected. In other tissues such as pancreas and brain, only valine and trimethylglycine were detected.

3.4 Applying HILIC to routine analysis

HILIC LC separations resulted in Rt variance typically less than 0.25% within a batch of tissue sample analysis (pooled QC and tissue extracts; n=55).

Metabolite	Rt variance %RSD	HILIC Rt and m/z (positive ion)
Adenine	0.81%	1.875 mins; m/z 136.0618
Creatinine	1.18%	3.206 mins; m/z 114.0662
Tryptophan	0.58%	5.259 mins; m/z 205.0972
Phenylalanine	0.52%	5.460 mins; m/z 166.0863
Trimethylglycine	0.20%	7.766 mins; m/z 118.0863
Arginine	0.12%	9.401 mins; m/z 175.1190

4. Conclusions

- To increase the metabolome coverage in untargeted tissue metabolomic profiling studies and to ensure high reporting confidence requires more than one analytical approach. Reverse-phase based DIA-MS/MS methods are powerful, robust tools for highly positive metabolite identification. However, identifying highly polar metabolites are challenging.
- Applying HILIC methods to highly polar metabolite analysis resulted in further confidence in metabolite identification and enabled resolution of isobaric features.
- The HILIC metabolite identification was further verified using an in-house metabolomics library acquired on a HILIC LC method.