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Application News

High Performance Liquid Chromatograph Mass Spectrometer

Increasing Reporting Confidence in Metabolomics; Reversed-Phase and HILIC LC/QTOF Untargeted Analysis in Multiple Tissue Studies

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User Benefits

- Flexible workflows for untargeted metabolomics using LC/QTOF (applicable to LCMS-9030 and 9050).
- A single data independent acquisition (DIA)-MS/MS method for untargeted metabolic profiling of tissues and biofluids.
- Application of reversed-phase and HILIC chromatography increases metabolite coverage and reporting confidence in untargeted metabolomics.

■ Introduction

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. Reversed-phase high-resolution UHPLC-MS/MS methods are core analytical tools in metabolomic studies of tissues and biofluids. Their strengths include high metabolite coverage, particularly with lipid distributions (for example; phospholipids, 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 complementary technique to help identify metabolites with greater certainty. In this application note, a single data independent acquisition (DIA-MS/MS) method is presented for the untargeted metabolomics analysis of different biological tissues with two alternative chromatography solutions: C18 reversed-phase and HILIC. Figure 1 highlights the reversed phase and HILIC analysis of mouse brain, studied in a C57BL/6 mouse model of ethanol abuse. In this example, several metabolites showed altered brain metabolome perturbations that eluted early in the RP gradient which could be further confirmed using HILIC analysis.

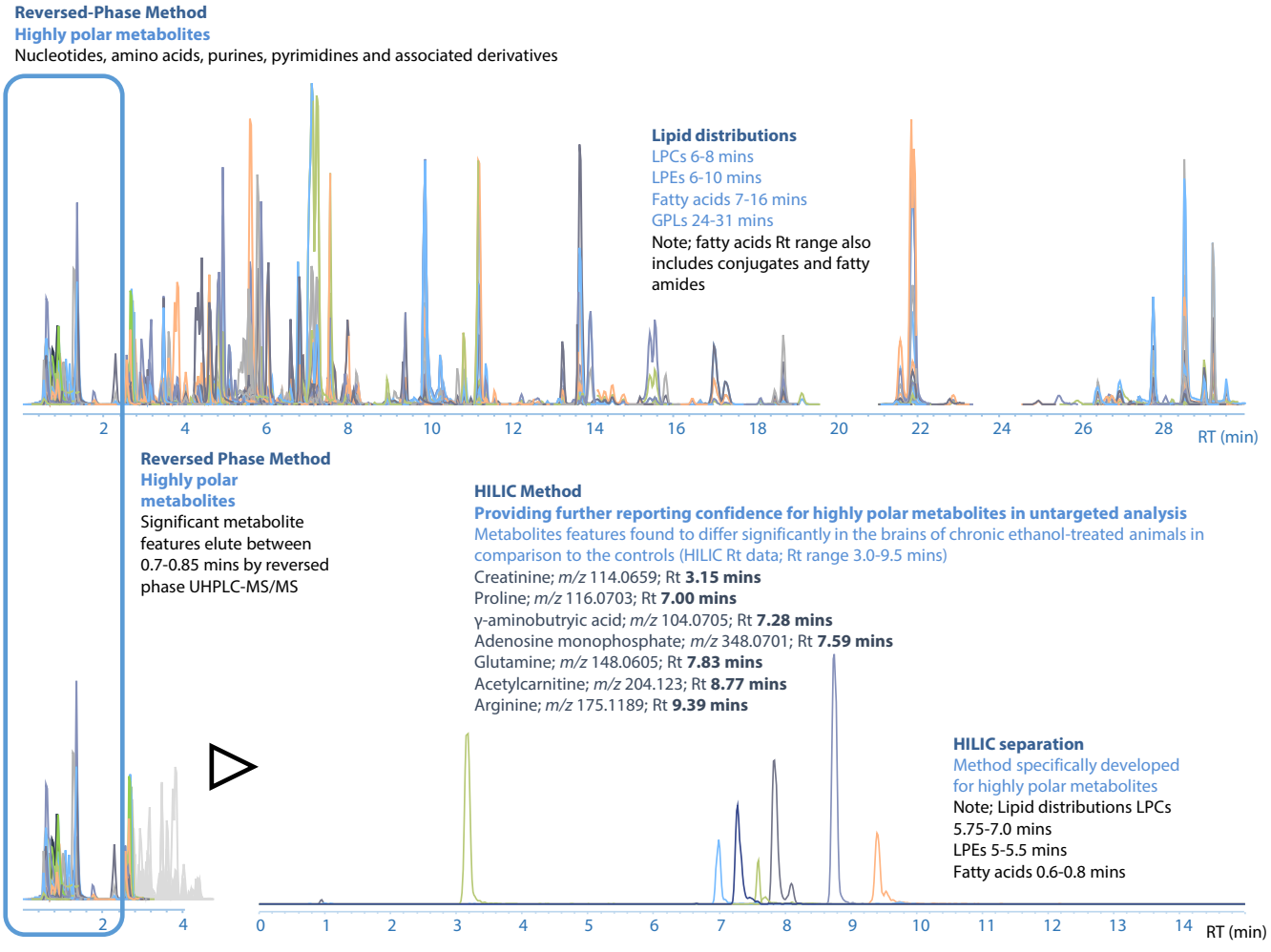


Fig. 1 Metabolite features detected by reversed-phase LC-MS/MS (upper plot) for a mouse brain tissue sample as part of a study to determine the effect of ethanol on the metabolome. Highly polar metabolites were further qualified using HILIC (lower plot) to increase reporting confidence in isobaric feature identification and further resolving isobaric features in different tissue types.

■ Materials and Methods

Multiple tissue types from different metabolomics studies were analysed using a standardised reversed-phase LC/QTOF method and a HILIC LC/QTOF method. Tissue extract samples were reconstituted in 90% acetonitrile. Tissues included brain, liver, pancreas, gut, caecal tissue and faecal gut content from several murine metabolomics studies. The HILIC method was developed to selectively analyse highly polar metabolites. Samples were analysed using the conditions detailed in Table 1. Data were processed using LabSolutions Insight™ software to consider a panel 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). Data were compared against an in-house metabolomics library (acquired using authentic reference material, research use only) for high reporting confidence. Library MS/MS spectra were generated using targeted MS/MS with unit mass resolution and a collision energy spread of 5-55 V. Product ion spectra were corrected to theoretical accurate mass values using the Insight Assign application. Spectra were acquired in positive and negative ion modes, first using the reversed-phase method, then retention time mapped to a Shim-pack Velox HILIC separation.

Table 1. Analytical conditions

UHPLC:	Nexera X2
Reversed phase:	
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, curvi-linear gradient); 100% B (27.5-31 min); 2% B (31.01-35 min)
Injection	0.5 µL; autosampler temperature 4 ˚C
HILIC:	
Column	Shim-pack Velox HILIC (2.1x100 mm 2.7 µm, PN: 227-32025-03); column maintained at 40 ˚C
Flow Rate	0.3 mL/min (0-13 min); 0.4 mL/min (13-17 min); 0.3 mL/min (17-18 min)
Mobile Phase	A: Water + 10 mM ammonium formate + 0.1% formic acid B: Acetonitrile:water [92:8] + 10 mM ammonium formate + 0.1% formic acid
Rinse (R0/R1/R2)	IPA/MeOH/MeCN/H_2O + 1% formic acid
Gradient	98% B (0-1.5 min); 65% B (11 min); 10% B (12.5-14 min); 98% B (14.25-18 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* 60-1000)
MS/MS	27 DIA-MS/MS (*m/z* 40-1000; precursor isolation 35 Da; collision energy spread 5-55 V
Total cycle time (MS and MS/MS)	0.991 seconds

■ Results

To increase the metabolome coverage in untargeted tissue metabolic profiling studies and to ensure high reporting confidence requires more than one analytical approach. Reversed-phase based DIA-MS/MS methods are powerful, robust tools for highly positive metabolite identification. However, identifying highly polar metabolites with high confidence is challenging. As highlighted in Figure 1, the application of a generic analytical reversed-phase LC-MS/MS method results in a high metabolome coverage and has been applied to the untargeted metabolomics analysis of biofluids and tissues.

However, in metabolomics studies which report early eluting features as significant, a HILIC method was specifically developed to further increase reporting confidence using the same precursor and data-independent MS/MS acquisition (DIA) methods as applied with a reversed-phase UHPLC separation. The HILIC method resulted in the separation of highly polar features with minimal variance in retention time throughout a metabolomics batch analysis (retention time variance was typically less than 0.25% within a batch of tissue sample analysis n=55; pooled QC and tissue extracts, Table 2 and graphically represented in Figure 2).

Table 2. Variance of retention time for metabolites detected in tissue matrix extracts using HILIC. Data include extracts from brain, caecal, gut, liver, pancreas, faecal gut content and plasma. The RT stability with this method is high despite the wide variance in biological matrix.

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

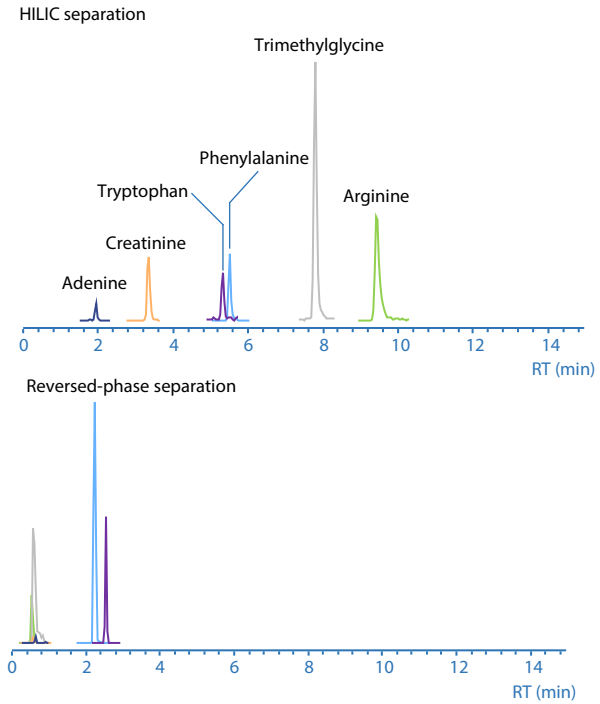


Fig.2 Chromatographic separation of adenine, creatinine, tryptophan, phenylalanine, trimethylglycine and arginine in pancreas tissue extract by HILIC and reversed-phase. Peaks are displayed to a ±5ppm mass tolerance, and the same colours are used for each metabolite in HILIC or reversed-phase.

HILIC is particularly powerful for the separation of isobaric compounds that elute in the first minute of a reversed-phase gradient developed for a high metabolite and lipid coverage.

Figure 3 highlights the application of HILIC to the separation of several isobaric metabolite features which elute between 0.7-0.85 mins on a reversed-phase UHPLC method in faecal gut content. 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.

Applying HILIC can also reduce the false discovery rate in highly polar metabolite analysis. 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 examples of reducing the false discovery rate, ion signals at *m/z* 146.1176 successfully separated acetylcholine and 4-trimethylammoniobutanoate, and at *m/z* 118.0863 resolved valine, 5-aminopentanoic acid, norvaline and trimethylglycine. The isomers leucine/isoleucine and alanine/beta-alanine were also resolved. HILIC separation was used to confirm metabolite identification with authentic reference materials (with the exception of 5-aminopentanoic acid and norvaline). These examples are highlighted in Fig. 3.

Figure 4 shows the separation of valine, 5-aminopentanoic acid, norvaline, and trimethylglycine (also known as betaine) at *m/z* 118.0863 in different biological tissues highlighting the need to apply HILIC for increased reporting confidence for highly polar features.

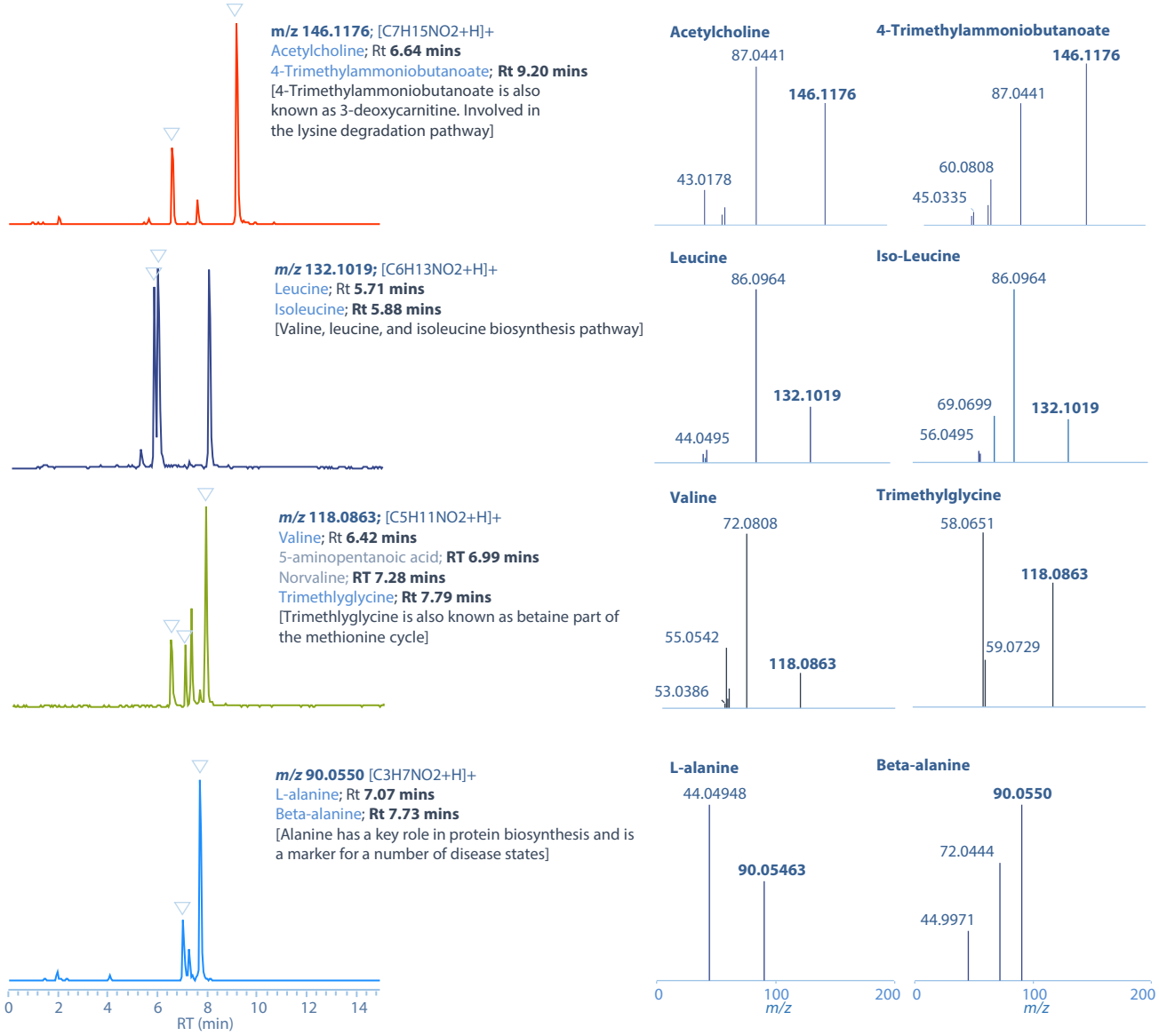
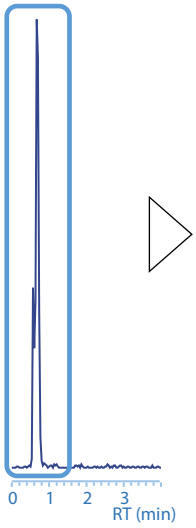


Fig. 3 HILIC was applied to the separation of several isobaric metabolite features which elute between 0.7-0.85 mins on a reversed-phase UHPLC method. Using HILIC, the isobaric metabolite features could be selectively separated despite the complexity of the faecal gut content matrix. The sample was part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

Reversed-Phase Method
Feature co-elution
Co-elution of ion signal at *m/z* 118.0853 (positive ion mode) in a faecal extract.



HILIC Method
Selective separation of metabolite features at *m/z* 118.0853 (positive ion mode)
Applied to faecal, caecal, pancreas and brain tissue extracts.
Valine; Rt **6.4 mins**
5-Aminopentanoic acid; Rt **6.99 mins**
Norvaline; Rt **7.28 mins**
Trimethylglycine; Rt **7.81 mins**

Faecal extract
Multiple metabolite features detected. Identification confirmed by authentic reference standards or correspondence with third party MS/MS data repositories.

Pancreas tissue extract
Valine and trimethlyglycine detected. (Authentic standards used).

Brain tissue extract
Valine and trimethlyglycine detected. (Authentic standards used).

Caecal tissue extract
Multiple metabolites detected which are common to the faecal sample.

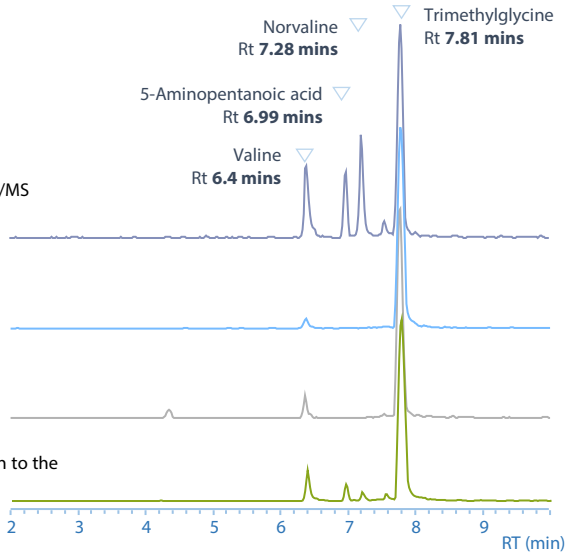


Fig. 4 Identification of components eluting at 0.67 mins at *m/z* 118.0853 (±5 ppm) by reversed-phase UHPLC was compromised by co-elution. HILIC separation was used to confirm metabolite identification of valine and trimethylglycine with authentic reference materials. In the case of faecal and caecal 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.

■ Conclusion

To increase the metabolome coverage in untargeted tissue metabolic profiling studies and to ensure high reporting confidence requires more than one analytical approach. Reversed-phase based DIA-MS/MS methods are powerful, robust tools for highly positive metabolite identification with a high metabolite coverage. However, identifying highly polar metabolites with high reporting confidence is challenging given near zero chromatography resolution and isobaric mass spectra. Applying HILIC methods to highly polar metabolite analysis resulted in increased confidence in metabolite identification and enabled resolution of isobaric features. The metabolite identification was further verified using authentic standards analysed with the HILIC method.

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