Method development and comprehensive analysis for untargeted metabolomics of mouse stool using TIMS-QToF-MS/MS and NMR

Denis Andrei Ispan [1,2], **Jannik Sprengel** [1,2], **Gyuntae Bae** [3], **Daniele Bucci** [3], **Melek Canan Arkan**[4], **Christoph Trautwein** [1,2,3]

[1]Core Facility Metabolomics, Medical Faculty University of Tübingen, Germany
[2]The M3 Research Center, University of Tübingen, Germany
[3]Werner Siemens Imaging Center, University Hospital Tübingen, Germany
[4]Institute for Tumor Biology and Experimental Therapy, Georg-Speyer-Haus, Frankfurt am Main, Germany

Introduction

The gut microbiota plays a crucial role in regulating immune function, neurological communication, and metabolism, linking it to diseases such as cancer, diabetes, and neurodevelopmental disorders. Studying the fecal metabolome is emerging as a key approach to understanding these metabolic interactions and their implications for health and disease. (1)

Mouse models are valuable in metabolomics due to their genetic similarity to humans and ease of fecal sample collection. However, the complexity of fecal samples poses challenges for consistent and reproducible metabolite extraction, requiring refined methodologies. (2) Additionally, no single technique can detect all metabolites, highlighting the need for multiple approaches.

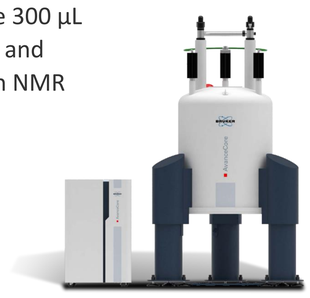
The sample extraction methodology for fecal metabolome analysis was optimized for TIMS-QToF-MS/MS, incorporating Hydrophilic Interaction Liquid Chromatography (HILIC) and Reverse Phase (RP) chromatography to enhance metabolite separation and coverage. Additionally, an established ^{1}H-NMR spectroscopy method was used. As a proof of concept, this approach was applied to stool samples from 6 mice, collected at 4 different time points during treatment.

Sample preparation

1. Add 200 µL 80% methanol to vial containing stool pellet
2. Add 800 µL MTBE
3. Smash the pellet using a plastic spatula
4. Use adaptive focused acoustics® (AFA) technology with CovarisLE220-plus to aid extraction
5. Add 500 µL of water to induce phase separation
6. Centrifuge
7. Transfer polar and organic phases into Eppendorf tubes and LC-vials

Source: www.covaris.com

8. Evaporate 300 µL polar phase and analyze with NMR


Source: www.bruker.com

9. Mix 50 µL of each phase and evaporate
   - reconstitute in 50 µL (50/50 ACN/water)
   - Centrifuge, transfer supernatant into HPLC vial for LC-MS analysis
   - Pool 2 µL of each sample for QC

Extraction solvents proportions, focused ultrasound extraction (Covaris Inc.) parameters, reconstitution solvent, LC gradients and MS settings were all optimized separately

Data Acquisition

- LC system: Elute plus
- Columns: Intensity Solo C18 100 mm * 2 mm * 2 µm (RP)
  BEH Amide 150 mm * 2.1 mm * 1.7 µm (HILIC)
- Mobile phase: RP: A – water + 0.1% formic acid (FA); B – ACN + 0.1% FA
  HILIC: A – 10mM ammonium formate + 0.1% FA;
  B - 10mM ammonium formate + 0.1% FA in 10/90 water/ACN
- LC gradient:

RP	Time (min)	0.0	2.0	10.0	11.0	13.0	13.1	15.5	
0.6 mL/min	%B	5	5	60	98	98	5	5	
HILIC	Time (min)	0.0	1.0	6.0	10.0	11.0	12.0	12.1	21.0
0.5 mL/min	%B	100	100	90	75	60	60	100	100

- Inj. volume: RP: 3 µLH; HILIC: 5 µL
- Source: VIP-HESI
- MS mode: PASEF (data dependent MS/MS) + tims Stepping; HILIC in (+) and RP in (-) mode
- Annotation: Metaboscape 2024b: Target Lists + Spectral Libraries
  match for at least 2 of the following:
  m/z (<2.0 ppm), mSigma (<20), MS/MS score (<900), CCS (<1%)
- MS system/software: timsTOF Pro 2 & HyStar and TimsControl acquisition software
- NMR system/software: ^{1}H-NMR at 600 MHz on a Bruker Avance III HD IVDr system on a TXI room temperature probe. CMPG spectra were used for annotation and quantification using Chenomx V 10.2

Results and summary

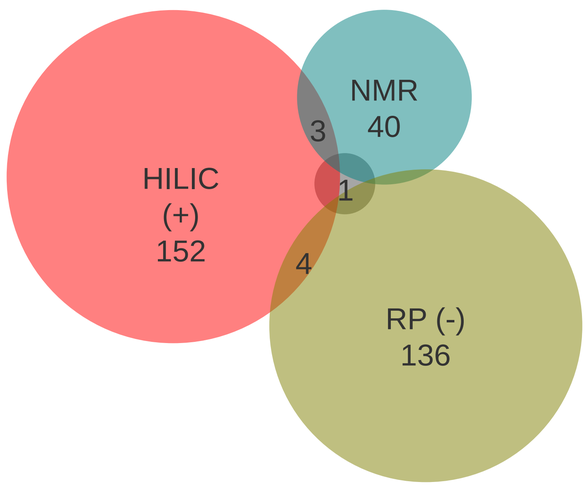


Figure 1. Venn diagrams (created with DeepVenn [3]) display the annotated metabolites across the three analysis types. Metabolite annotations were influenced by the number of samples used for feature extraction and annotation in Metaboscape. Features not present in at least 8 of 48 samples mice (4 time points x 6 mice x 2 analytical replicates) for the 6 mice were excluded. When analyzing only mouse nr. 5 (8 samples) and excluding features not found in at least 2 samples, the annotated metabolites were: 273 in RP, 208 in HILIC, with 21 metabolites overlapping.

Table 1. Counts of metabolite classes classified by analytical technique using ClassyFire [4]. HILIC is particularly effective for detecting more polar organic acids and derivatives, while RP chromatography excels in identifying fatty acids and derivatives and more hydrophobic metabolites. NMR complements these LC-MS techniques by detecting smaller and very polar metabolites.

	HILIC (+)	RP (-)	NMR
Lipids and lipid-like molecules	36	62	6
Organic acids and derivatives	72	30	24
Organoheterocyclic compounds	26	12	1

SUMMARY:

- The integration of NMR with HILIC (+) and RP (-) LC-MS techniques enabled the successful annotation of 345 metabolites, providing a more complete and detailed coverage of the metabolome.
- Distinct trends could be detected in metabolite levels across all six mice at various time points, highlighting dynamic changes throughout the study.
- The methodology proved to be stable across all samples, yielding consistent and reliable results. It is well-suited for further exploration and detailed study of the fecal metabolome in mice.

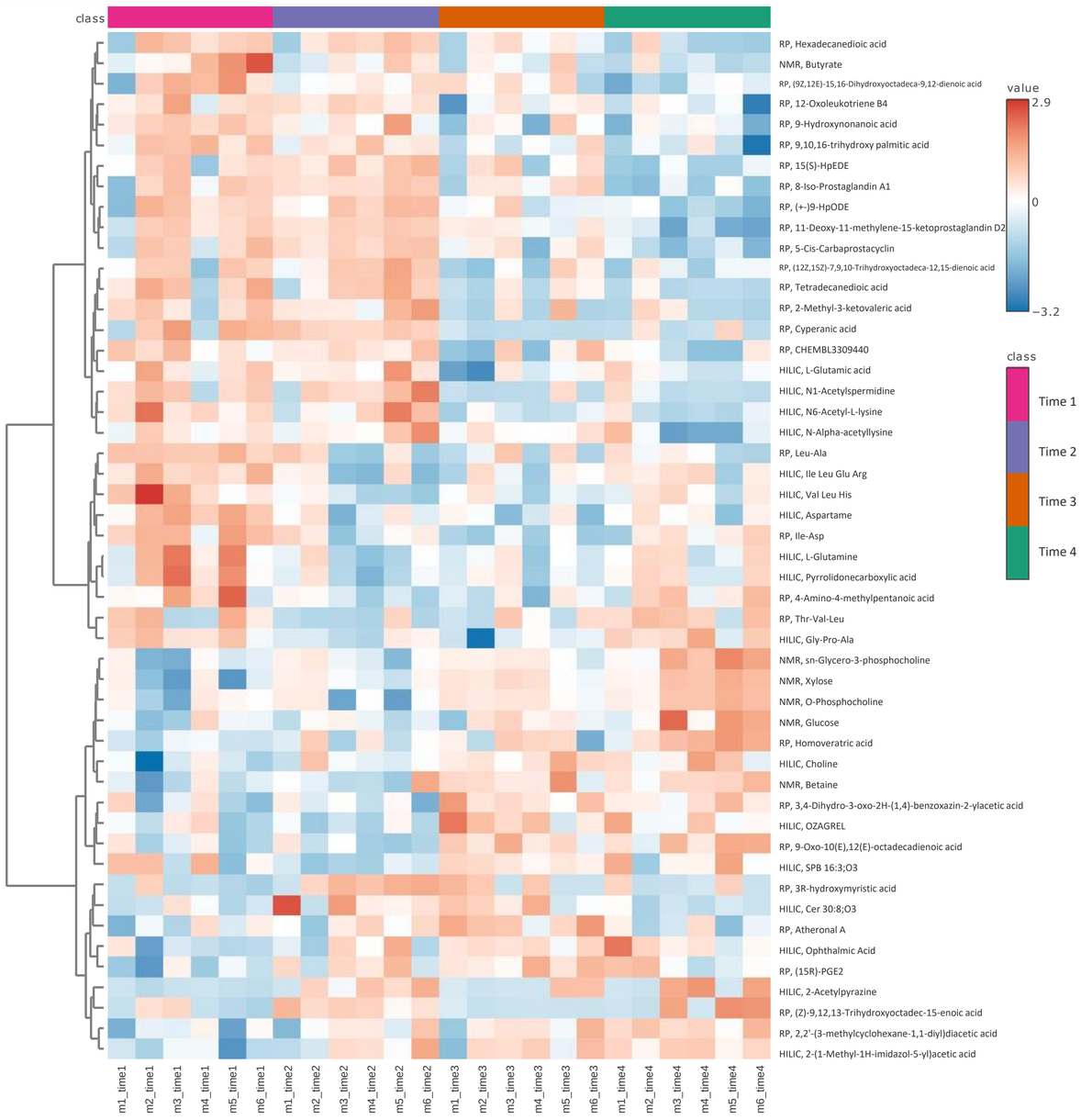


Figure 2. Heat map showing the top 50 most significant metabolites, created with http://www.metaboanalyst.ca. The first half of the heat map illustrates a decline in metabolite concentrations over time, while the second half reveals an increasing trend in metabolite levels.

Universität Tübingen · M3 Forschungszentrum
Core Facility Metabolomics
Otfried-Müller-Str. 37 · 72076 Tübingen· Germany
Telefon +49 7071 29-82546
denis.ispan@med.uni-tuebingen.de

Acknowledgements
The authors would like to thank Bruker Daltonics for the cooperation and their continuous support.

References
(1) Valdes AM, Walter J, Segal E, Spector TD. Role of the gut microbiota in nutrition and health. BMJ. 2018;361
(2) Yu S, Fan J, Zhang L, Qin X, Li Z. Assessment of Biphasic Extraction Methods of Mouse Fecal Metabolites for Liquid Chromatography-Mass Spectrometry-Based Metabolomic Studies. J Proteome Res. 2021 Sep 3;20(9):4487-4494. doi: 10.1021/acs.jproteome.1c00450. Epub 2021 Aug 26. PMID: 34435490.
(3) Hulsen, T., *arXiv,* 2022. 2210.04597. DOI: 10.48550/arXiv.2210.04597
(4) Djoumbou Feunang Y, Eisner R, Knox C, Chepelev L, Hastings J, Owen G, Fahy E, Steinbeck C, Subramanian S, Bolton E, Greiner R, and Wishart DS. ClassyFire: Automated Chemical Classification With A Comprehensive, Computable Taxonomy. Journal of Cheminformatics, 2016, 8:61.DOI: 10.1186/s13321-016-0174-y