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Deciphering the Microbiome: A Targeted LC/MSMS Method for the Analysis of Bile Acids in Biological Samples

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Introduction

Bile acids (BAs) play pivotal roles in the digestion and absorption of lipids and lipid-soluble vitamins. Primary BAs are synthesized in the liver, stored in the gallbladder and secreted into the duodenum. 95% of BAs are reabsorbed in the terminal ileum, and the remaining 5% is subjected to a series of structural modifications by the gut microbes leading to a series of related metabolites termed secondary bile acids. Although the functions of secondary BAs remain elusive, emerging research shows they make important contribution to immune regulation, carcinogenesis, and tumor progression. LC-MS and sample preparation methodologies that are highly curated and robust offer opportunities to expand our comprehension of bile acid dynamics and their implications in both health and disease.

We have developed and standardized an LC/MS/MS method for the targeted analysis of 68 unique BAs in rodent plasma, serum, and fecal samples. The LC/MS system consisted of an Agilent 6495D triple quadrupole mass spectrometer with 4th generation iFunnel technology coupled with an Agilent 1290 Infinity II bioinert UHPLC (BioLC) with Agilent standard configuration for omics applications.

Figure 1. Agilent 6495D LC/MS triple quadrupole, Agilent 1290 Infinity II bio-inert UPLC (BioLC), and Agilent Poroshell EC C-18 column.

Experimental

LC/MS/MS Method Development

Optimal mass spectrometer source conditions (Table 1) and compound MRM acquisition parameters were obtained from authentic bile acid standards and implementing the built-in Method Optimizer tool of Agilent MassHunter 12.1 acquisition software. Data was acquired in Dynamic-MRM (dMRM) mode in both positive and negative ion mode to ensure optimal compound detection.

Experimental

MS Conditions	
Source	Agilent Jet Stream Dual ESI
Sheath Gas Temp, Flow	250 °C; 11 L/min
Gas Temp, Flow	180 °C; 20 L/min
Nebulizer	25 psi
Capillary	2500 V (+)/5000 V (-)
Nozzle	1000 V (+/-)
MS Mode	Positive/negative
Acquisition	Dynamic-MRM
iFunnel Setting	Standard

Table 1. Optimized ESI source conditions and acquisition parameters

An Agilent Poroshell EC C-18 UPLC column (Figure 1) was used for the separation of bile acids by reverse phase chromatography. Optimized LC conditions are summarized in Table 2. The Agilent standard configuration for omics applications of the BioLC system ensures easy method interchange when the platform is also used for targeted metabolomics, lipidomics, and proteomics applications.

LC Conditions			
Column	Agilent Poroshell 120 EC-C18, 2.1 x 100 mm; 1.9 µm (p/n 695675-902)		
Column Temperature	50 °C		
Injection Volume	2 µL, plasma/serum; 1 µL, fecal		
Autosampler Temperature	5 °C		
Needle Wash	Standard Wash, 10 sec, 70% acetonitrile		
Mobile Phase	A: water + 0.1 % formic acid B: acetonitrile + 0.1 % formic acid		
Gradient	Time	%B	Flow Rate (mL/min)
	0.00	20	0.5
	1.50	20	0.5
	17.00	65	0.5
	17.10	98	0.5
	17.15	98	1.0
	19.50	98	1.0
	19.60	20	0.5
	22.00	20	0.5
Total Run Time	23 minutes		

Table 2. LC method parameters

Results and Discussion

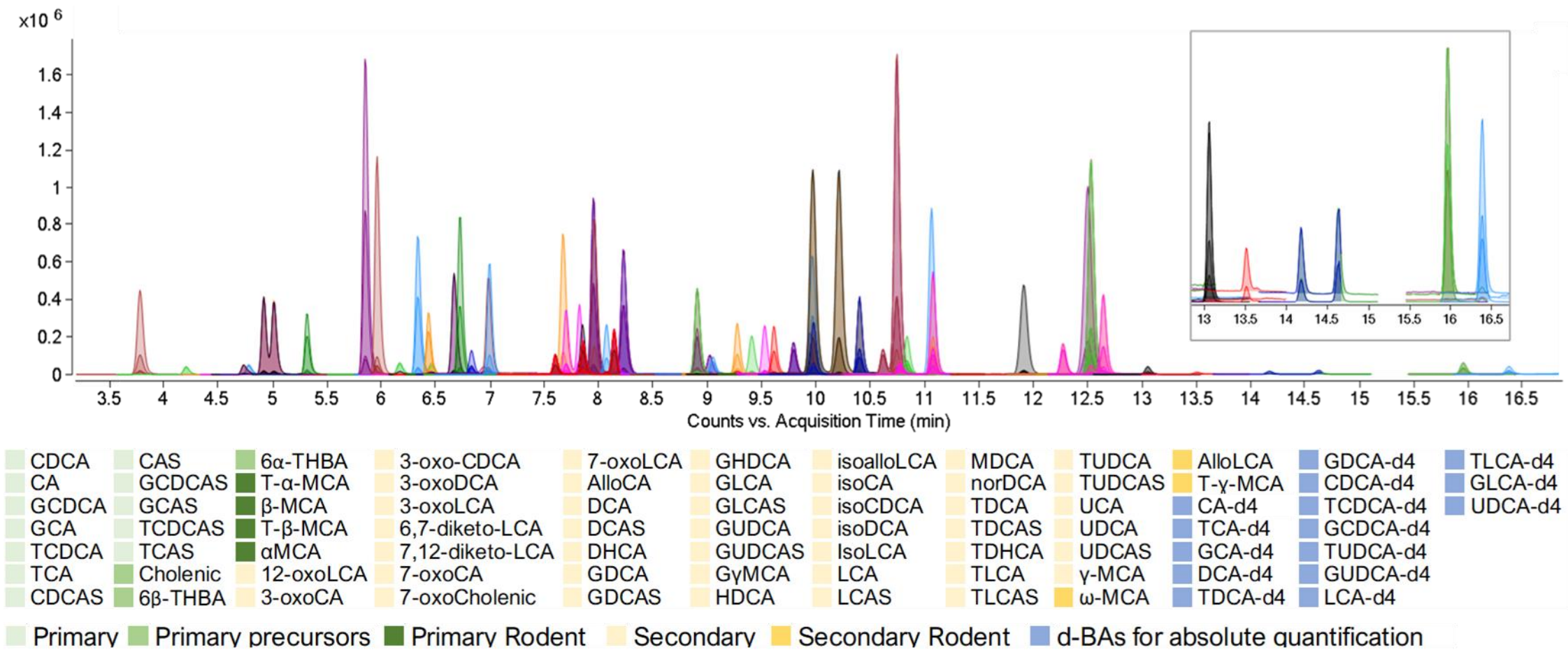

Figure 2. Chromatographic separation of a bile acid standard mixture by C-18 reverse phase chromatography.

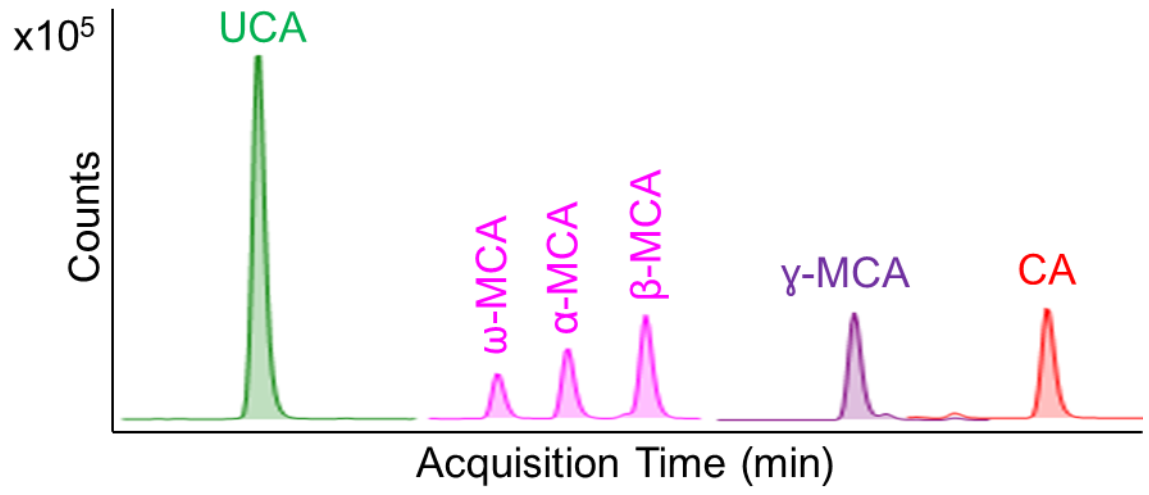

Figure 3. Chromatographic separation of some relevant bile acid isomers

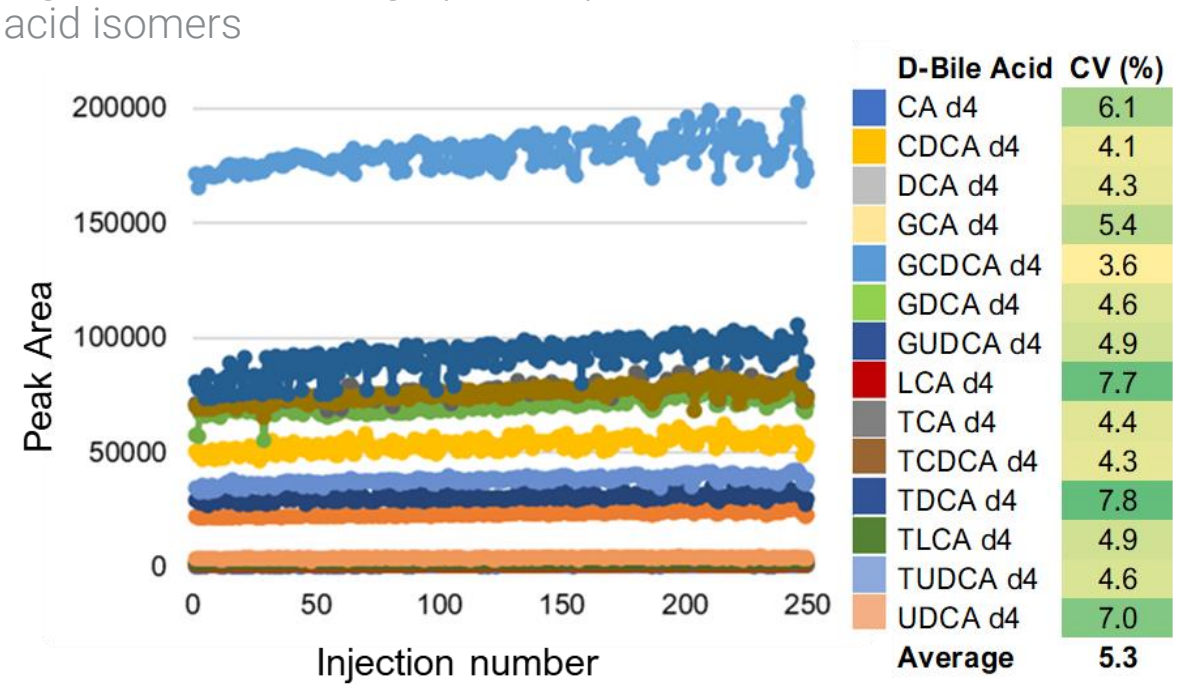

Figure 4. 6495D instrument response signal stability after 250 consecutive injections of extracted mouse plasma spiked with d-BA internal standards.

LC/MS sample preparation

Optimal bile acid extraction conditions from biological samples were established. Bile acids were extracted from plasma/serum samples (100 µL) with four-sample volume acetonitrile containing 1% formic acid and d-BA internal standards. After incubation at room temperature with shaking for two hours, samples were centrifuged (20 min, 13000 RPM, 5 °C) to pellet proteins and supernatants were dried down under reduced pressure. Fecal samples were extracted at 100 mg/mL with 80% methanol containing d-BA internal standards. Samples are bead beat for 6 cycles, at 6 m/s, 30 sec/cycle, with a 5-second dwell time between cycles. After overnight incubation at -80 °C to facilitate protein precipitation, samples were allowed to thaw on wet ice for 30 min, centrifuged (20 min,13000 RPM, 5 °C) to collect the supernatants, and the solvent was removed as described above. Dry samples were reconstituted with 50% methanol for LC/MS analysis. Agilent Captiva EMR-lipid cartridges/plates can be implemented in the extraction protocol for simultaneous removal of proteins and lipids from the samples. Bile acid extraction recovery from Captiva EMR-lipids was calculated with a pre- and post-spike experiment using a series of d-BA standards with a recovery >85%. Selective lipid removal might be relevant when samples undergo parallel lipidomics analysis. The bile acids extraction protocol from plasma samples is also amenable for automation on an Agilent Bravo Metabolomics liquid handler as described previously[1,2].

Results and Discussion

Analysis of biological samples and data analysis

The dMRM acquisition method was used to measure levels of bile acids on a series of certified plasma (BIOIVT Elevated Science, Westbury, NY) and fecal (Medix Biochemica, Maryland Heights, MO) samples and test method feasibility on biological samples. After LC/MS analysis, the raw data was processed with MassHunter Quantitative Analysis 12 software and peak areas were imported into Mass Profiler Professional for downstream chemometric analysis.

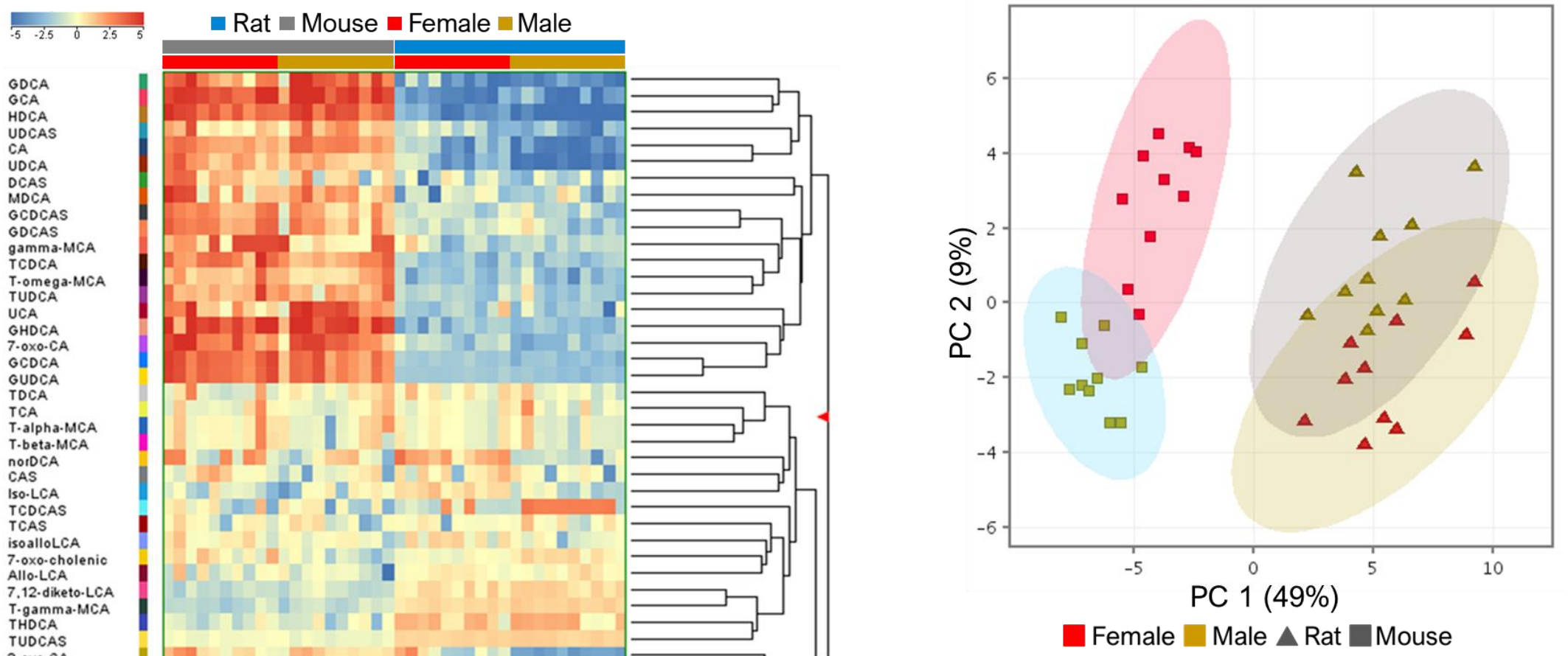

Figure 5. Principal component analysis (PCA) and hierarchical clustering (left) showed differences in BAs distribution across sample groups.

In addition to primary BAs, many relevant secondary BAs were detected in plasma as well as fecal samples. Hierarchical clustering and PCA analysis showed differential bile acid composition across rodent sample groups (Figure 5).

Conclusions

We developed a novel LC/MS/MS method for the targeted analysis of 68 unique bile acids in relevant biological matrices with a single chromatographic run.

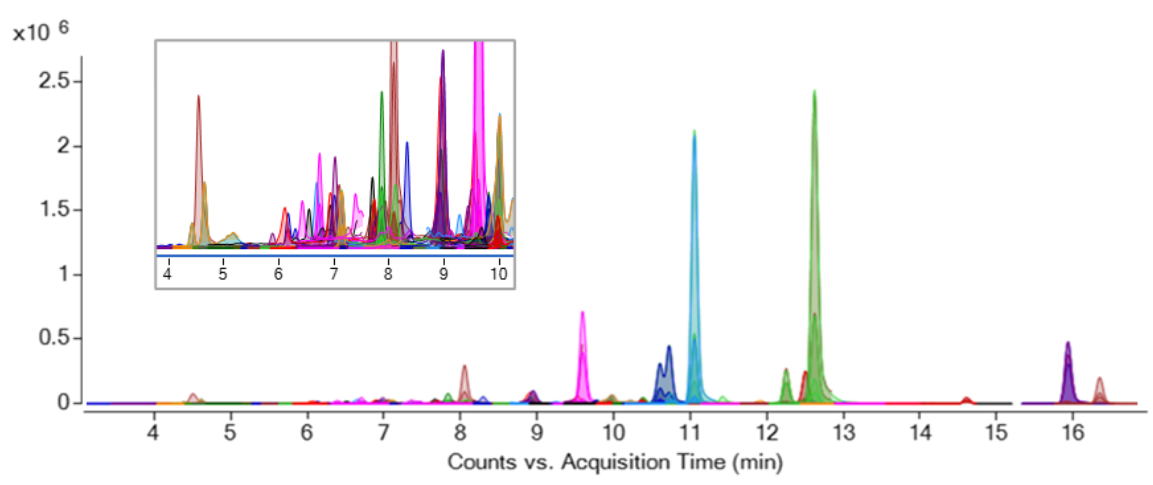

Figure 6. MRM bile acid overlay chromatograms of a representative fecal sample

References

[1]Pietro Morlacchi et al. ASMS 2023 Poster MP 123. A semi-automated workflow for the analysis of circulating bile acids in plasma samples

[2]Sartain, M et al., Enabling Automated, Low Volume Plasma Metabolite Extraction with the Agilent Bravo Platform Agilent Application Note 5994 2156 EN 2020

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