Memorial Sloan Kettering Cancer Center

Refined LC/Q-TOF Methodology for Identification and Profiling of Bile Acids Produced from the Gut Microbiome

ThP 328

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Introduction

Bile acids (BAs) play essential roles in the absorption of dietary lipids but have also gained recent attention due to their emerging roles in immune regulation. [1] Here we describe a high-resolution LC/Q-TOF based strategy to resolve and profile 67 bile acids with two stationary phase chemistries. The method incorporates 35 internal standards to allow single-point quantitation and has been applied to human and mouse fecal, cecal, serum, and plasma samples. The method also leverages accurate mass detection, MS/MS fragmentation, and a software workflow to identify and track novel amino acid conjugates and other candidate bile acids.

Bile acid panel

Our bile acid panel consists of 12 human primary bile acids (conjugated and unconjugated) and 55 secondary bile acids (some of which are primary in mice).

Primary bile acids (n=12)	Unconjugated	CA and CDCA
	Glycine	GCA and GCDCA
	Taurine	TCA and TCDCA
	Sulfate	CA3SO, CDCA3SO, GCA3SO, GCDCA3SO, TCA3SO, and TCDCA3SO
Secondary bile acids (n=55)	Unconjugated	DCA, LCA, UDCA*, αMCA*, βMCA*, γMCA, ωMCA, HDCA, 3-oxo-DCA, isoDCA, 3-oxo-CA, isoCA, 7-oxo-CA, 3-oxoLCA, isoLCA, alloLCA, isoalloLCA, 7-oxoLCA, 12-oxoLCA, MDCA, 3-oxoCDCA, isoCDCA, 7-oxo-cholenic, cholenic, DHCA, 6β-THBA, 6α-THBA, UCA, 6,7-dioxo-LCA, 7,12-dioxo-LCA, and norDCA
	Glycine	GDCA, GLCA, GUDCA*, GγMCA, GDHCA, and GHDCA
	Taurine	TDCA, TLCA, TUDCA*, TαMCA*, TβMCA*, TγMCA, TωMCA, THDCA, and TDHCA
	Sulfate	DCA3SO, LCA3SO, UDCA3SO*, GDCA3SO, GLCA3SO, GUDCA3SO*, TDCA3SO, TLCA3SO, and TUDCA3SO*

*mice primary bile acids

Separation of the 67 bile acids

Complete separation of the 67 bile acids is accomplished by leveraging two chromatographic methods with different stationary phases. **Method 1** (BEH Shield RP18, 2.1 x 50 mm, 1.7 µm) can separate 51/67 bile acids. **Method 2** (Cortecs T3, 2.1 x 50 mm, 1.6 µm) completely resolves the problematic (isobaric) compounds.

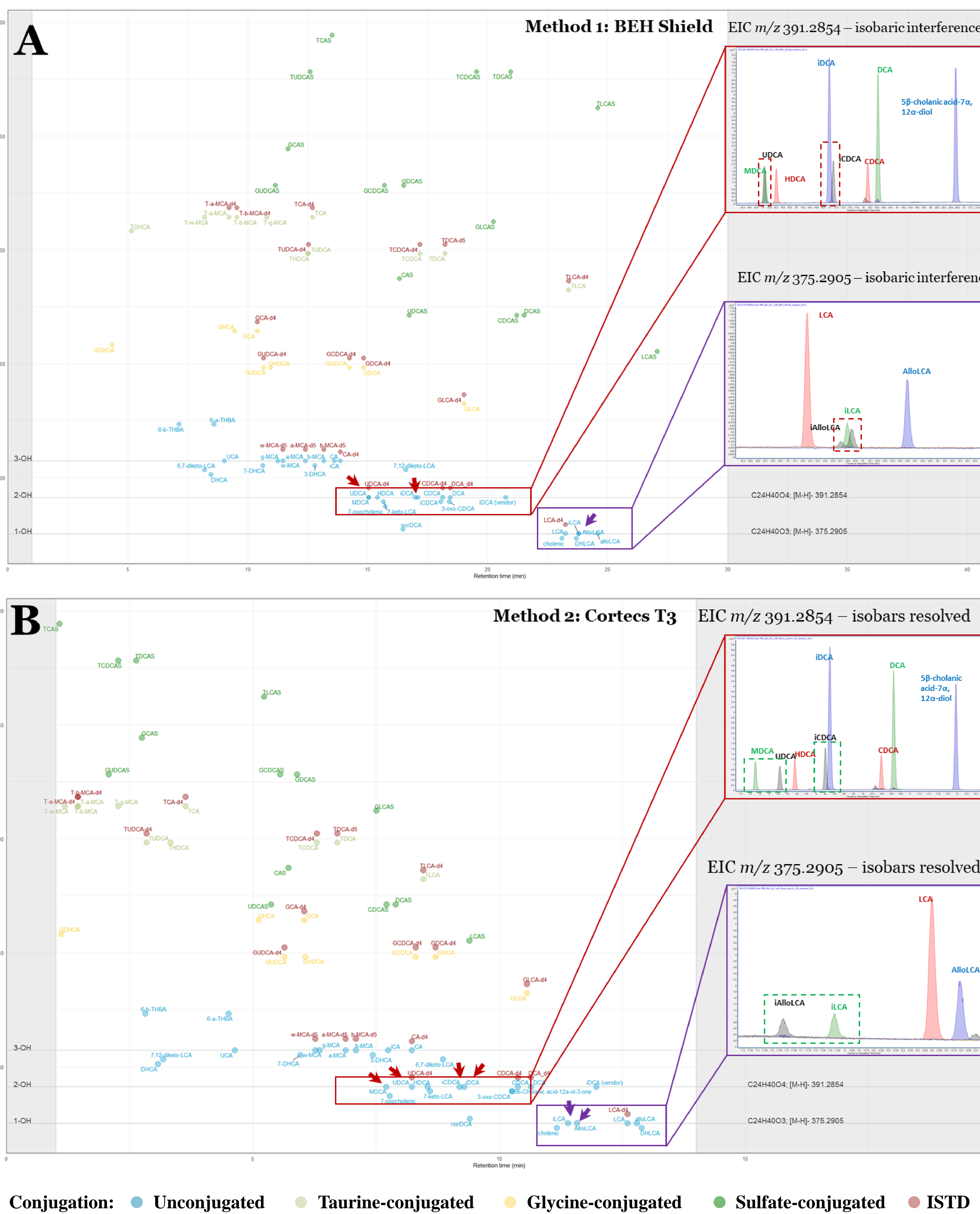


Figure 1. Mass v Retention Time plots of the two separation methods. A. Chromatographic method 1 allows the separation of 51/67 bile acids. Examples of problematic isobars (C24H40O4 and C24H40O3) are shown in inserts. **B.** The chromatographic **Method 2** allows the resolution of problematic compounds on **Method 1.**

Methods

Stool samples were extracted with a modified methanol-based protein precipitation procedure, and plasma samples were extracted with a 7:2:1 Acetonitrile:1% Formic acid:Methanol. The extraction solvent contained 35 labeled internal standards at known concentrations. Extracts were separated with two different 21-minute acetone-based C18 RP-LC methods on an Agilent 1290 Infinity II LC system. Eluents were analyzed with an Agilent 6546 LC/Q-TOF operated in negative-ion mode with both MS and AutoMSMS acquisition parameters. Datasets were analyzed with Agilent MassHunter Quantitative Analysis and MPP software. The metabolite identification workflow leveraged Sirius/CSI:FingerID and GNPS software.

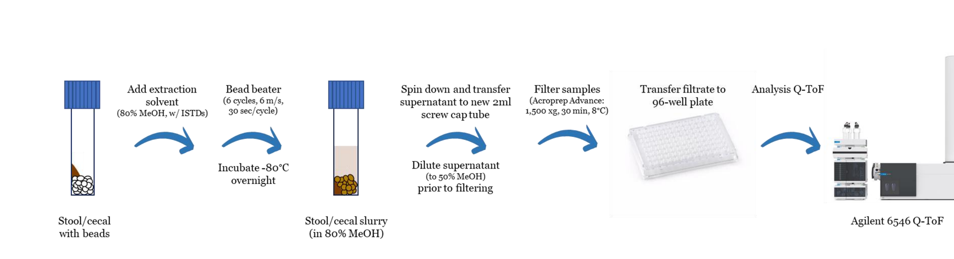


Figure 2. Extraction of fecal and cecal samples. Fecal and cecal samples are weighed and resuspended in extraction solvent (80% MeOH with ISTDs) to 100 mg/mL, homogenized using a bead ruptor, diluted and filtered before analysis.

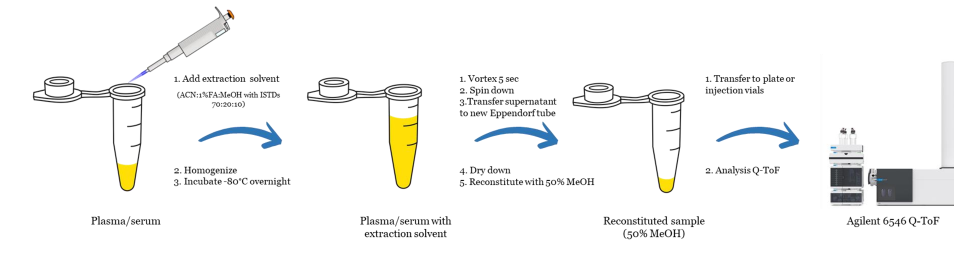
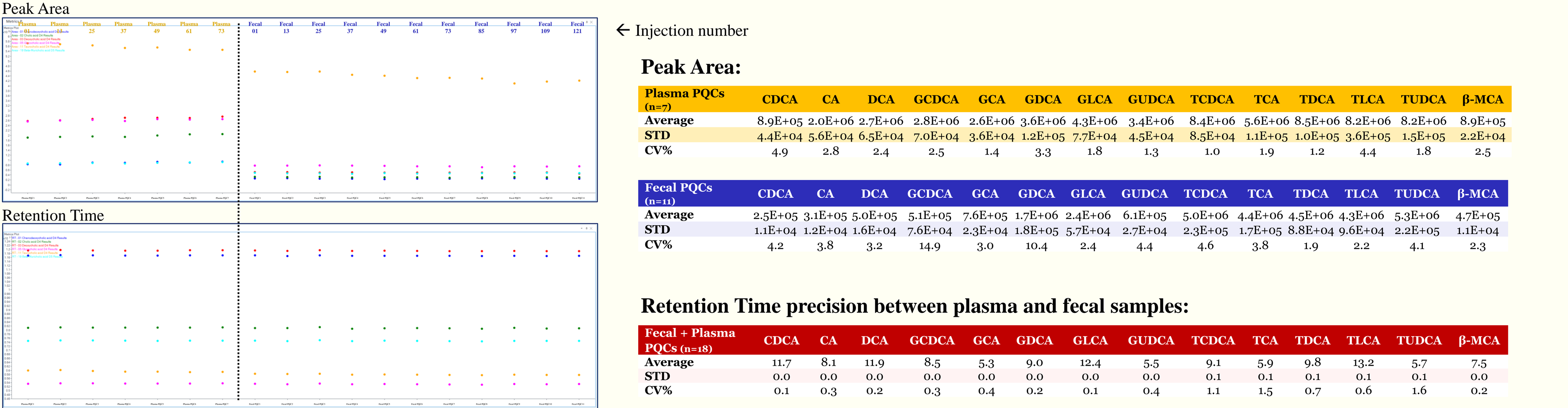


Figure 3. Extraction of plasma and serum samples. Plasma and serum samples are extracted with 7:2:1 extraction solvent (Acetonitrile: 1% Formic acid: Methanol with ISTDs), homogenized, centrifuged, dried down, and reconstituted with 50% methanol before MS analysis.

Method performance

The acetone-based chromatography methods showed great stability over multiple 96-well injection plates and allowed bile acid profiles to be correlated between matched stool and plasma samples. Pooled quality control (PQC) samples were injected interspaced with 12 study samples. For a batch of 2 x 96-well plates containing paired fecal and plasma samples, plus 1 extra fecal plate, injected during an overall 6-day run (per method), 7 plasma PQCs and 11 fecal PQCs were analyzed. Both methods show low CV% (the highest was < 15%, but for the majority of the compounds were <5%). The minimal RT drift between the 2 matrices leads to an easy and fast data analysis and enabled our untargeted and MSMS strategies to be applied to both matrices.

Figure 4. ISTD abundance and retention time (RT) plots, and precision (CV%) of PQCs analyzed with **Method 1**



Peak Area:

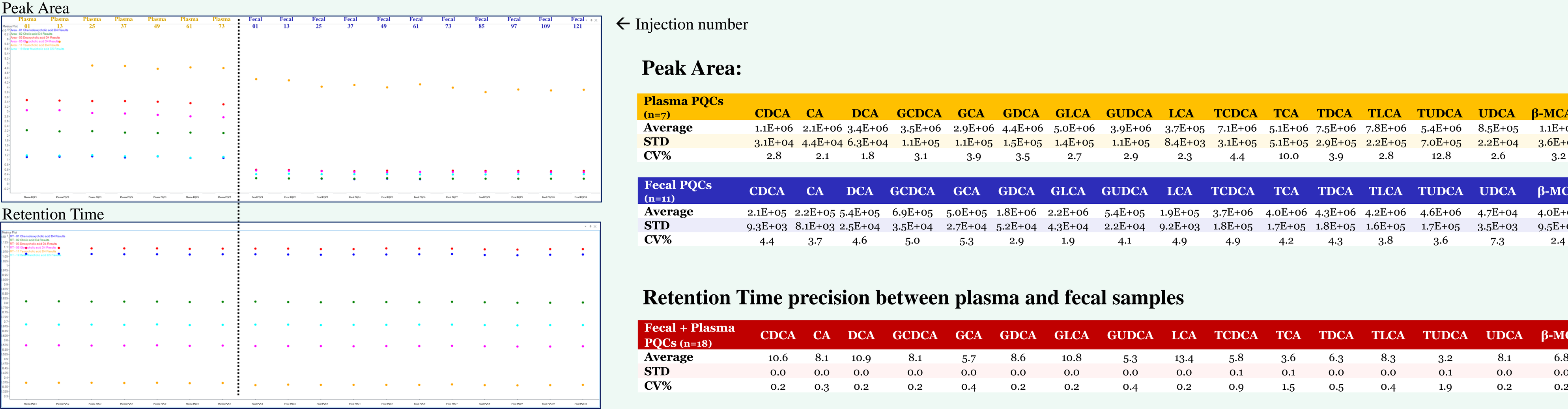
Plasma PQCs (n=7)	CDCA	CA	DCA	GCDCA	GCA	GDCA	GLCA	GUDCA	TCDCA	TCA	TDCA	TLCA	TUDCA	β-MCA
Average	8.9E+05	2.0E+06	2.7E+06	2.8E+06	2.6E+06	3.6E+06	4.3E+06	3.4E+06	8.4E+06	5.6E+06	8.5E+06	8.2E+06	8.2E+06	8.9E+05
STD	4.4E+04	5.6E+04	6.5E+04	7.0E+04	3.6E+04	1.2E+05	7.7E+04	4.5E+04	8.5E+04	1.1E+05	1.0E+05	3.6E+05	1.5E+05	2.2E+04
CV%	4.9	2.8	2.4	2.5	1.4	3.3	1.8	1.3	1.0	1.9	1.2	4.4	1.8	2.5

Fecal PQCs (n=11)	CDCA	CA	DCA	GCDCA	GCA	GDCA	GLCA	GUDCA	TCDCA	TCA	TDCA	TLCA	TUDCA	β-MCA
Average	2.5E+05	3.1E+05	5.0E+05	5.1E+05	7.6E+05	1.7E+06	2.4E+06	6.1E+05	5.0E+06	4.4E+06	4.5E+06	4.3E+06	5.3E+06	4.7E+05
STD	1.1E+04	1.2E+04	1.6E+04	7.6E+04	2.3E+04	1.8E+05	5.7E+04	2.7E+04	2.3E+05	1.7E+05	8.8E+04	9.6E+04	2.2E+05	1.1E+04
CV%	4.2	3.8	3.2	14.9	3.0	10.4	2.4	4.4	4.6	3.8	1.9	2.2	4.1	2.3

Retention Time precision between plasma and fecal samples:

Fecal + Plasma PQCs (n=18)	CDCA	CA	DCA	GCDCA	GCA	GDCA	GLCA	GUDCA	TCDCA	TCA	TDCA	TLCA	TUDCA	β-MCA
Average	11.7	8.1	11.9	8.5	5.3	9.0	12.4	5.5	9.1	5.9	9.8	13.2	5.7	7.5
STD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.0
CV%	0.1	0.3	0.2	0.3	0.4	0.2	0.1	0.4	1.1	1.5	0.7	0.6	1.6	0.2

Figure 5. ISTD abundance and retention time (RT) plots, and precision (CV%) of PQCs analyzed with **Method 2**



Peak Area:

Plasma PQCs (n=7)	CDCA	CA	DCA	GCDCA	GCA	GDCA	GLCA	GUDCA	LCA	TCDCA	TCA	TDCA	TLCA	TUDCA	UDCA	β-MCA
Average	1.1E+06	2.1E+06	3.4E+06	3.5E+06	2.9E+06	4.4E+06	5.0E+06	3.9E+06	3.7E+05	7.1E+06	5.1E+06	7.5E+06	7.8E+06	5.4E+06	8.5E+05	1.1E+06
STD	3.1E+04	4.4E+04	6.3E+04	1.1E+05	1.1E+05	1.5E+05	1.4E+05	1.1E+05	8.4E+03	3.1E+05	5.1E+05	2.9E+05	2.2E+05	7.0E+05	2.2E+04	3.6E+04
CV%	2.8	2.1	1.8	3.1	3.9	3.5	2.7	2.9	2.3	4.4	10.0	3.9	2.8	12.8	2.6	3.2

Fecal PQCs (n=11)	CDCA	CA	DCA	GCDCA	GCA	GDCA	GLCA	GUDCA	LCA	TCDCA	TCA	TDCA	TLCA	TUDCA	UDCA	β-MCA
Average	2.1E+05	2.2E+05	5.4E+05	6.9E+05	5.0E+05	1.8E+06	2.2E+06	5.4E+05	1.9E+05	3.7E+06	4.0E+06	4.3E+06	4.2E+06	4.6E+06	4.7E+04	4.0E+05
STD	9.3E+03	8.1E+03	2.5E+04	3.5E+04	2.7E+04	5.2E+04	4.3E+04	2.2E+04	9.2E+03	1.8E+05	1.7E+05	1.8E+05	1.6E+05	1.7E+05	3.5E+03	9.5E+03
CV%	4.4	3.7	4.6	5.0	5.3	2.9	1.9	4.1	4.9	4.9	4.2	4.3	3.8	3.6	7.3	2.4

Retention Time precision between plasma and fecal samples

Fecal + Plasma PQCs (n=18)	CDCA	CA	DCA	GCDCA	GCA	GDCA	GLCA	GUDCA	LCA	TCDCA	TCA	TDCA	TLCA	TUDCA	UDCA	β-MCA
Average	10.6	8.1	10.9	8.1	5.7	8.6	10.8	5.3	13.4	5.8	3.6	6.3	8.3	3.2	8.1	6.8
STD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.0	0.0
CV%	0.2	0.3	0.2	0.2	0.4	0.2	0.2	0.4	0.2	0.9	1.5	0.5	0.4	1.9	0.2	0.2

Method application

Our approach enabled changes in primary and secondary bile acid composition and amino acid conjugation to be monitored in mice samples. Additionally, control human fecal, plasma, and serum samples were used to validate our method.

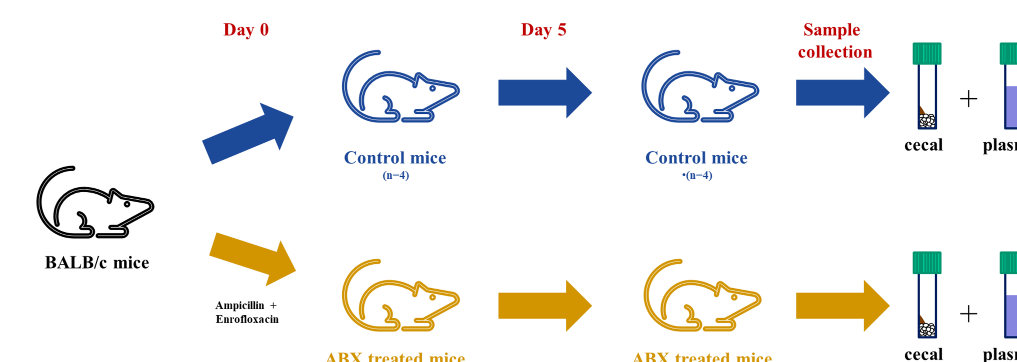


Figure 6. Experimental design for profiling of the bile acid pool in control and antibiotic-treated BALB/c mice. Plasma and cecal of control and antibiotic-treated (ampicillin and enrofloxacin) Balb/c mice were collected 5 days after treatment.

Results

Our workflow incorporates a quantitation strategy allowing 35 (the most abundant primary and secondary human and rodent) bile acids to be quantified using a single-point ISTD strategy, although this requires samples to be injected at two dilutions. Additionally, the LC methods completely separate important low-concentration immunoregulatory bile acids (isoDCA, isoLCA, alloLCA, isoalloLCA, and 3-oxoLCA).

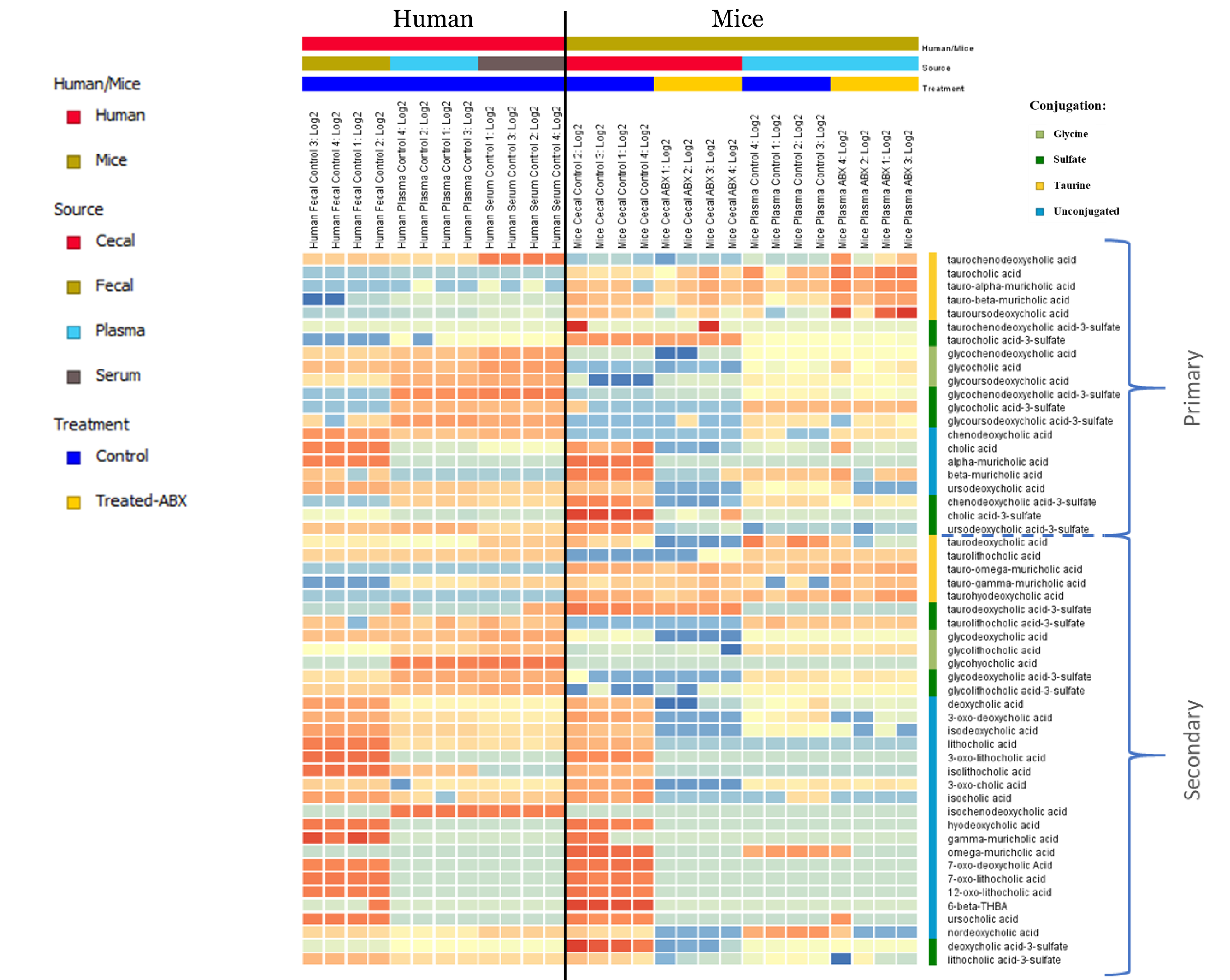


Figure 7. Heatmap of bile acids profile found in healthy human (fecal, serum, and plasma) samples, and control and antibiotic-treated BALB/c mice. Human samples from healthy-control individuals show abundant secondary bile acids and unconjugated primary bile acids. BALB/c mice exposed to ampicillin and enrofloxacin show an overall accumulation of taurine-primary bile acids (TCA/T-αMCA/T-βMCA) and low unconjugated-primary and secondary bile acids. The observed profile is due to the loss of commensal bacteria bearing the *bile salt hydrolase* (*BSH*) gene and the *bai* operon (primary to secondary bile acids transformation).

Discovery of new amino acid conjugated-BAs

Leveraging the Q-TOF accurate mass and MS/MS workflow, we were able to assign preliminary IDs to multiple additional peaks, some of which were previously shown to be novel amino acid conjugates (tyrosine, phenylalanine, and leucine), by using GNPS and MS/MS spectral interpretation. [2]

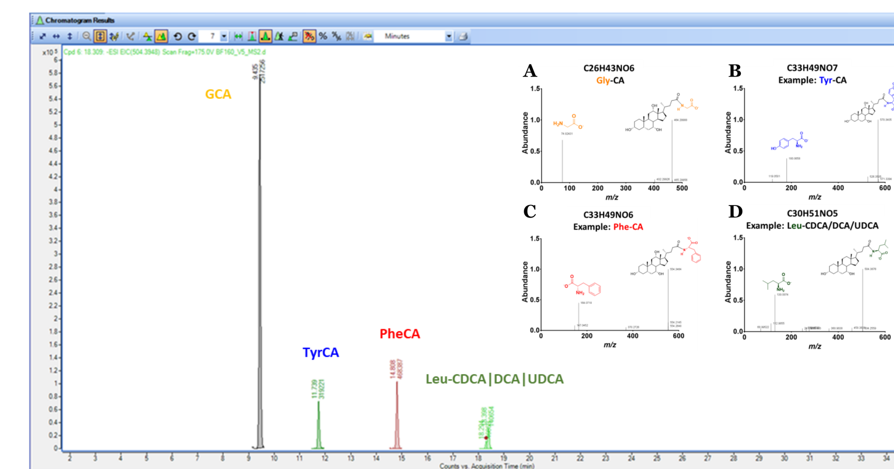


Figure 8. Extracted ion chromatogram (EIC) of new amino acid-conjugated bile acids in a human fecal sample. Inserts show the negative mode fragmentation pattern for: **A)** glycocholate, **B)** Tyr-$C_{24}H_{40}O_5$ (most likely tyrosocholate), **C)** Phe-$C_{24}H_{40}O_5$ (most likely phenylalanocholate), and **D)** Leu-$C_{24}H_{40}O_4$ (Leuco-CDCA/DCA/UDCA).

Conclusions

- The combination of two separation methods allows for the complete resolution of 67 (confirmed by standards) bile acids (many of them isobars), plus many other bile acid-like molecules.
- Both methods show excellent precision and robustness. The 18 PQC samples (7 plasma + 11 fecal PQCs) run interspaced by 12 study samples clearly shows that even after injecting 3 x 96-well plates (58 plasma + 119 fecal samples), run back-to-back, the variation in the ISTD signal was below 5% for most compounds.
- Leveraging the Q-TOF accurate mass and MS/MS workflow allowed identification of the newly described amino acid-conjugated-bile acids and additional candidate bile acids (data not shown).
- Here we show the versatility of our workflow by measuring bile acids in fecal, cecal, plasma, and serum samples. Additionally, we show that bile acid profiles are dramatically altered by changes in the microbiome composition and antibiotic exposures.
- Our workflow already allowed us to investigate population cohorts undergoing fecal-microbiota transplantation (FMT), where we observed restoration of bile acid profiles in various disease settings (data not shown).

References

1. Guzior DV and Quinn RA. Review: microbial transformations of human bile acids. Microbiome. 2021 Jun 14;9(1):140. PMID: 34127070.
2. Quinn RA et al. Global chemical effects of the microbiome include new bile-acid conjugations. Nature. 2020 Mar;579(7797):123-129. PMID: 32103176.

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