

Memorial Sloan Kettering Cancer Center

### 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).

	Unconjugated	CA and CDCA
Primary	Glycine	GCA and GCDCA
bile acids	Taurine	TCA and TCDCA
(n=12)	Sulfate	CA3SO, CDCA3SO, GCA3SO, GCDCA3SO, TCA3SO, and TCDCA3SO
Secondary	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
bile acids	Glycine	GDCA, GLCA, GUDCA*, GγMCA, GDHCA, and GHDCA
(n=55)	Taurine	TDCA, TLCA, TUDCA*, ΤαMCA*, ΤβMCA*, ΤγMCA, ΤωMCA, 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**.

Taurine-conjugated

Unconjugated

Glvcine-conjugated • Sulfate-conjugated • ISTD

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

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### Methods

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). Human Human/Mice Human Mice Sulfate Taurine (ACN:1%FA:MeOH with ISTDs 70:20:10) Source Unconjugate Cecal taurocholic acid Fecal tauro-alpha-muricholic acid ursodeoxycholic acid Plasma chenodeoxycholic acid-3-su glycocholic acid Figure 2. Extraction of fecal and cecal samples. Fecal and cecal samples are Figure 3. Extraction of plasma and serum samples. Plasma and serum samples Serum weighed and resuspended in extraction solvent (80% MeOH with ISTDs) to 100 mg/mL, cochenodeoxycholic acid-3-sulfat are extracted with 7:2:1 extraction solvent (Acetonitrile: 1% Formic acid: Methanol with olvcocholic acid-3-sulfate homogenized using a bead ruptor, diluted and filtered before analysis coursodeoxycholic acid-ISTDs), homogenized, centrifuged, dried down, and reconstituted with 50% methanol before MS analysis. Control alpha-muricholic acid beta-muricholic acid ursodeoxycholic acid Treated-ABX enodeoxycholic acid-3-sulfate cholic acid-3-sulfate ursodeoxycholic acid-3-sulfate taurodeoxycholic acid taurolithocholic acid Method performance tauro-omega-muricholic acid tauro-gamma-muricholic acid taurohyodeoxycholic acid taurodeoxycholic acid-3-sulfat taurolithocholic acid-3-sulfate glycodeoxycholic acid alvcolithocholic acid alvcohvocholic acid glycodeoxycholic acid-3-sulfat glycolithocholic acid-3-sulfate deoxycholic acid 3-oxo-deoxycholic acid sodeoxycholic acid lithocholic acid 3-oxo-lithocholic acid isolithocholic acid 3-oxo-cholic acid isocholic acid isochenodeoxycholic ac hyodeoxycholic acid gamma-muricholic aci omega-muricholic acid 7-oxo-deoxycholic Acid 7-oxo-lithocholic acid 12-oxo-lithocholic acid 6-beta-THBA ursocholic acid nordeoxycholic acid deoxycholic acid-3-sulfate lithocholic acid-3-sulfate Figure 7. Heatmap of bile acids profile found in healthy human (fecal, serum, and plasma) samples, and control and antibiotic-**GDCA GLCA GUDCA TCDCA TCA TDCA TLCA TUDCA β-MCA** treated BALB/c mice. Human samples from healthy-control individuals show abundant secondary bile acids and unconjugated primary bile acids. -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 BALB/c mice exposed to ampicillin and enrofloxacin show an overall accumulation of tauro-primary bile acids (TCA/T- $\alpha$ MCA/T- $\beta$ MCA) and low 1.5E+05 2.2E+04 unconjugated-primary and secondary bile acids. The observed profile is due to the loss of commensal bacteria bearing the bile salt hydrolase (BSH) gene 1.3 1.0 1.9 1.2 4.4 1.8 2.5 and the bai operon (primary to secondary bile acids transformation). GDCA GLCA GUDCA TCDCA TCA TDCA TLCA TUDCA β-MC 5 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 **Discovery of new amino acid conjugated-BAs** 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 4.6 3.8 1.9 2.2 4.4 4.1 Leveraging the Q-TOF accurate mass and MS/MS workflow, we were able to assign preliminary IDs to multiple additional ma and fecal samples: peaks, some of which were previously shown to be novel amino acid conjugates (tyrosine, phenylalanine, and leucine), by GDCA GLCA GUDCA TCDCA TCA TDCA TLCA TUDCA β-Μ using GNPS and MS/MS spectral interpretation. [2] 0.1 0.1 0.1 0.1 1.5 0.7 0.6 マ ++ ‡ | Q 団 約 | ピ | 午 🖪 • O O 7 🗉 H 🏽 📥 Δ 🖉 🧏 % % 🖄 | 🖷 Minutes 🖃 🗃 **Figure 8. Extracted ion chromatogram (EIC)** C33H49NO7 Example: Tyr-CA C26H43NO Gly-CA of new amino acid-conjugated bile acids in a GCA human fecal sample. Inserts show the negative HO, O, OH | 101 2000 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 C30H51NO5 C33H49NO6 Example: Leu-CDCA/DCA/UDCA **D)** Leu- $C_{24}H_{40}O_4$  (Leuco-CDCA/DCA/UDCA). Example: Phe-C/ GUDCA LCA TCDCA TCA TDCA TLCA HO TOH 1504 но страни 4.4E+06 5.0E+06 3.9E+06 3.7E+05 7.1E+06 5.1E+06 7.5E+06 7.8E+06 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 3.5 2.7 2.9 2.3 4.4 10.0 3.9 2.8 12.8 2.6 0.0 200 400 600 0.0 200 400 600 m/z TyrCA CA GLCA GUDCA LCA TCDCA TCA TDCA TLCA TUDCA UDCA β-MCA Leu-CDCA DCA UDCA 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 Courts vs. Acquisition Time (nin) **Retention Time precision between plasma and fecal samples** Conclusions CDCA CA DCA GCDCA GCA GDCA GLCA GUDCA LCA TCDCA TCA TDCA TLCA TUDCA UDCA β-Μ( Average STD 0.1 0.0 0.0 0.1 0.0 0.0 CV% 0.4 0.2 0.9 ◆ 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.





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. 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.

Metrics Plasma Metrics Plot (10 <sup>6</sup> Area - 01 <b>01</b> rodeox	Plasma	Plasma 25	Plasma 37	Plasma 49	Plasma 61	Plasma 73	Fecal	Fecal 13	Fecal 25	Fecal 37	Fecal 49	Fecal 61	Fecal 73	Fecal 85	Fecal 97	Fecal 109	Fecal * × 121	$\leftarrow$ Injection number	r				
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2.8- 2.6- 2.4-	•	•	:	•	. •	:												Average	8.9E+05	2.0E+06	2.7E+06	2.8E+06	2.6E+
22- 2- 18-	•	•	•	•	•	•												STD	4.4E+04	5.6E+04	6.5E+04	7.0E+04	3.6E+
1.6- 1.4- 1.2-																		CV%	4.9	2.8	2.4	2.5	1.4
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Reten	tion ]	Гime																Average	2.5E+05	3.1E+05	5.0E+05	5.1E+05	7.6E+c
Metrics Plot																	* *×	STD	1.1E+04	1.2E+04	1.6E+04	7.6E+04	2.3E+0
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0.68- 0.66- 0.64- 0.62-																		Average	11.7	8.1	11.9	8.5	5.3
0.6-		:		:	:	:	•	•	:	•	:	•	•	•	•	•		STD	0.0	0.0	0.0	0.0	0.0
0.56-	•			•	•		: ·	•		•	•	•	•	•	•	•	•	CV%	0.1	0.3	0.2	0.0	0.4
0.56- 0.54- 0.52- 0.5- 0.40-																		0170	0.1	0.0	0.2	0.3	0.4

**Figure 4.** ISTD abundance and retention time (RT) plots, and precision (CV%) of PQCs analyzed with **Method 1 Figure 5.** ISTD abundance and retention time (RT) plots, and precision (CV%) of PQCs analyzed with **Method 2**  $\frac{1 \text{ Can Filed}}{\frac{1}{1 \text{ First}} \text{ Field}} = \frac{1}{25} \frac{1}{37} \frac{1}{49} \frac{1}{61} \frac{1}{73} \frac{1}{13} \frac{1}{25} \frac{1}{37} \frac{1}{49} \frac{1}{61} \frac{1}{13} \frac{1}{25} \frac{1}{37} \frac{1}{49} \frac{1}{61} \frac{1}{73} \frac{1}{25} \frac{1}{37} \frac{1}{49} \frac{1}{61} \frac{1}{73} \frac{1}{3} \frac{1}{25} \frac{1}{37} \frac{1}{49} \frac{1}{61} \frac{1}{73} \frac{1}{13} \frac{1}{25} \frac{1}{37} \frac{1}{49} \frac{1}{61} \frac{1}{73} \frac{1}{13} \frac{1}$ 

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Peak Area	:					
Plasma PQCs	CDCA	CA	DCA	CCDCA	CCA	
(n=7) Average	1.1E+06	2.1E+0	6 3.4E+06	3.5E+06	2.9E+06	5
STD	3.1E+04	4.4E+0	4 6.3E+04	1.1E+05	1.1E+05	,
CV%	2.8	2.1	1.8	3.1	3.9	
Fecal PQCs (n=11)	CDCA	CA	DCA	GCDCA	GCA	
Average	2.1E+05	2.2E+05	5.4E+05	6.9E+05	5.0E+05	
STD	9.3E+03	8.1E+03	2.5E+04	3.5E+04	2.7E+04	
CV%	4.4	3.7	4.6	5.0	5.3	

# 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





- antibiotic exposures.

### 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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◆ 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 acidconjugated-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

• 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).