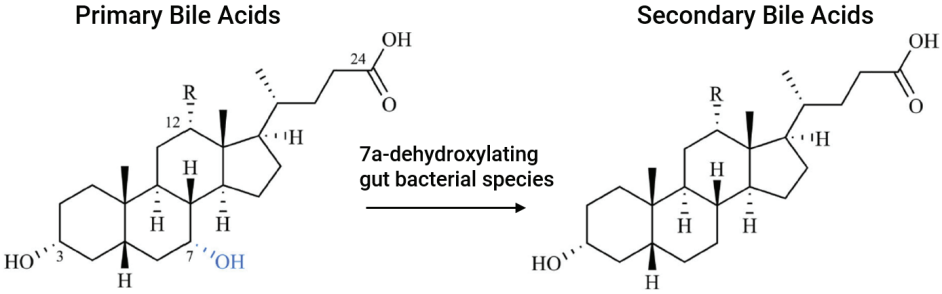
Application Note

Metabolomics

Agilent

Trusted Answers

A Refined LC/MS/MS Method Targeting Bile Acids from the Gut Microbiome



R = OH cholic acid (CA)
R = H chenodeoxycholic acid (CDCA)

R = OH deoxycholic acid (DCA)
R = H lithocholic acid (LCA)

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Introduction

Secondary bile acids are the most abundant and variable microbiota-derived metabolites shown to affect host physiology. Yet, much remains to be discovered regarding their compositional differences across individuals, their biological activities, and the bacterial strains and molecular pathways that produce them.[1]

Recently, there has been increased interest in targeted LC/MS methodologies to profile bile acids, in lieu of established GC/MS approaches that require extensive sample preparation. In this application note, a chromatographic method was developed with a unique mobile phase system to efficiently resolve close structural bile acid isomers, while also clearing problematic lipid accumulation on the column, thereby improving method robustness. In conjunction with the LC method, a triple quadrupole mass spectrometer operated with optimized MRM parameters enabled sensitive and specific detection of bile acids.

Experimental

Reagents and chemicals

All reagents and solvents were HPLC or LC/MS grade. Acetone, acetonitrile, and methanol were purchased from Honeywell (Morristown, NJ, USA). Ultrapure water was produced with a Milli-Q Integral system equipped with a LC-Pak Polisher and a 0.22 µm point-of-use membrane filter cartridge (EMD Millipore, Billerica, MA, USA). Formic acid (Optima LC/MS grade, Fisher, Fair Lawn, NJ), and MS-grade ammonium acetate (Sigma-Aldrich Corp., St. Louis, MO, USA) were used for mobile phase preparation.

Twenty-six authentic chemical standards, representing primary, secondary, and conjugated bile acids were analyzed for curation of an optimized MRM acquisition database. To establish lower limits of quantification (LLOQs), 16 calibration levels with concentrations ranging from 100 pM to 10 µM were prepared in 50/50 methanol/water.

Sample preparation

Fecal pellets (20 to 154 mg) were collected from three germ-free and three conventionally raised mice. The pellets were weighed and added to homogenization tubes with six ceramic beads and 200 µL methanol. The feces were homogenized for 20 minutes in a Qiagen TissueLyser II (Qiagen, Hilden, Germany) and then centrifuged for 10 minutes (16,000 × g, 23 °C). 100 µL of methanol was removed and added to a fresh tube with 100 µL water. The tubes were spun a second time and 100 µL of the methanol/water mixtures were added to MS vials.

NIST SRM 1950 plasma was extracted with four volumes of methanol, incubated on ice for 20 minutes, and centrifuged at 16,000 × g, for 15 minutes at 4 °C to pellet proteins. The supernatant was dried with a vacuum concentrator, and the extract was reconstituted in 50/50 methanol/water for LC/MS analysis.

Instrumentation

Agilent 1290 Infinity II LC including:

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler with thermostat (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 6470 Triple Quadrupole LC/MS system with Agilent Jet Stream Technology source

Method

The LC/MS conditions and parameters are provided in the following tables.

Parameter	Agilent 1290 Infinity II LC
Analytical Column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 150 mm, 2.7 µm (p/n 693775-902)
Guard Column	Agilent InfinityLab Poroshell 120 EC-C18 Fast Guard, 2.1 × 5 mm, 2.7 µm (p/n 821725-911)
Column Temperature	45 °C
Injection Volume	5 µL
Autosampler Temperature	4 °C
Needle Wash	10 seconds in flush port (50/50 methanol/H_2O)
Mobile Phase	A) 20 mM ammonium acetate + 0.1% formic acid B) 0.1% formic acid in acetone
Gradient Program	Time %B Flow Rate (µL/min) 0.00 32 200 6.00 32 200 6.10 32 400 25.00 65 400 25.10 100 400 26.30 100 500 26.40 100 1,000 28.00 100 1,000 28.10 32 400 32.10 32 400
Stop Time	32.10 min
Observed Column Pressure	260 to 540 bar

Parameter	Agilent 6470 LC/TQ
Polarity	Positive and Negative Switching
Time Segments	1) 0.0 to 4.0 minutes, divert valve → waste 2) 4.0 to 25.5 minutes, divert valve → MS (analysis) 3) 25.5 to 32.0 minutes, divert valve → waste
Gas Temperature	200 °C
Drying Gas (Nitrogen)	12 L/min
Nebulizer Gas	40 psi
Sheath Gas	200 °C
Sheath Gas Flow	10 L/min
Capillary Voltage	4,500 V (+), −3,000 V (−)
Nozzle Voltage	2,000 V (+), 0 V (−)
Scan Type	Dynamic MRM (dMRM)
Q1/Q2 Resolution	Unit (0.7 amu)
Delta EMV	200 V
Cell Acceleration Voltage	4 V
Cycle Time	750 ms
Total Number of MRMs	84 (positive 78, negative: 6)
Min/Max Dwell Time	24.94/748.01 ms

Data analysis

MRM data were quantitated using Agilent MassHunter Quantitative Analysis software, version 10.2. For calibration curves, a quadratic fit with weighting 1/x or $1/x^2$ was used. In a few cases, the matching quantitative standard was not available, and a surrogate standard was chosen to approximate endogenous concentrations. In this manner, the LCA calibration curve was used for Allo-LCA and Iso-LCA, UDCA was used for Muro-CA and TUDCA, and GCDCA was used for TCDCA and TDCA. The recorded weights of the fecal pellets were used to calculate the nmol/gram final concentrations. A Quantitative Analysis Report was generated and used as input for differential analysis using Agilent MassHunter Mass Profiler Professional software (MPP) version 15.1, which included the Pathway Architect Module.

Results and discussion

Chromatography and MRM optimization

Bile acids are commonly extracted from complex biological samples, with methanol-based extraction procedures. However, with these simple extraction procedures, numerous reports have found that accumulating lipids on the analytical column interfere with LC/MS-based analyses of bile acids. These effects include increasing column backpressure, and observed retention time drift over multiple injections. Following sample protein precipitation, and in lieu of offline solid-phase extraction (SPE) cleanup, several LC mobile phase combinations were investigated for their ability to remove interfering lipid molecules, while maintaining bile acid isomer resolution. Acetone as the eluotropic solvent was found to provide the unique ability to elute phosphatidylcholine (PC) lipids at the end of each analytical run, enhancing method robustness with complex samples. This observation was confirmed in plasma, and it was demonstrated that acetone eluted problematic phospholipids and triacylglycerols (TAGs), when holding the gradient at high percentage B for 3 minutes (Figure 1). This quick lipid elution step was in part accomplished by increasing the flow rate to 1 mL/min, during which time the LC flow was diverted to waste in the final dMRM method. The low viscosity of acetone enabled the increased flow rate, while maintaining acceptable column backpressures.

Further refinements were made to the mobile phase composition that were designed to increase analyte specificity with the tandem-quadrupole (TQ)-based method. Unconjugated bile acids produce very weak fragment ions in negative ion mode, due to the resistance of the steroid backbone to CID processes. Therefore, in lieu of MRM scans, numerous LC/MS methods have used selected-ion monitoring (SIM) for their quantification, where the same deprotonated precursor *m/z* is selected in each quadrupole without fragmentation. However, the unit-mass resolution of TQ instruments in SIM mode often results in reduced specificity and signal-to-noise ratio, especially in complex matrices. Wegner *et al.*[2] found that MRM-based scans of $(M+NH_4)^+$ bile acid precursors were superior to SIM-based scans of $(M-H)^-$ ions. The addition of ammonium acetate to a final concentration of 20 mM in mobile phase A promoted the formation of bile acid $(M+NH_4)^+$ ions, and CID fragmentation resulted in intense product ions, corresponding to losses of NH_3 and multiple H_2O molecules. This study confirmed these observations and demonstrated that multiple MRM transitions built with these product ions increased the analytical selectivity by reducing interference from matrix in biological samples compared to neat solvent (Figure 2). In this manner, MRM selectivity was leveraged for the

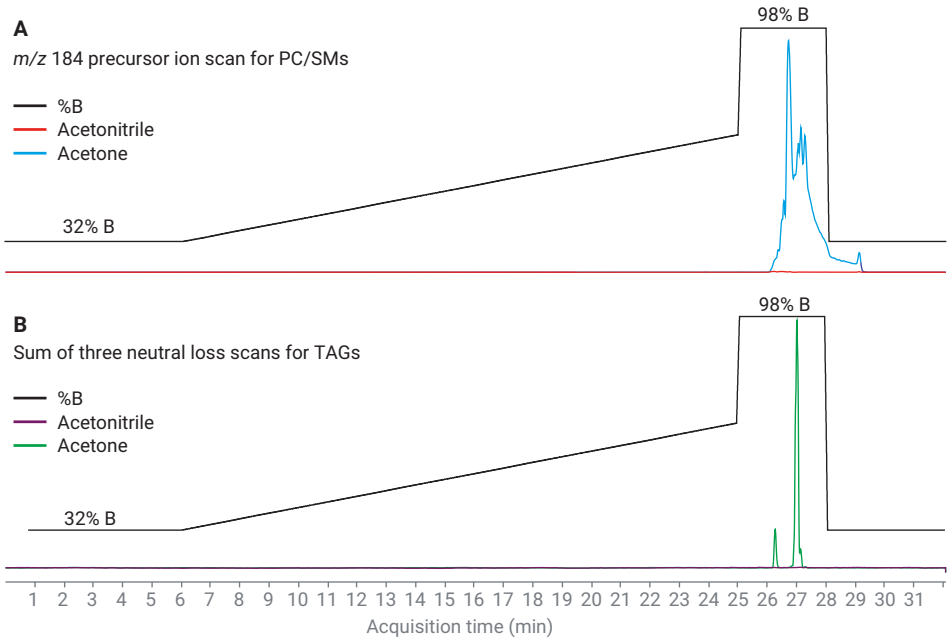


Figure 1. Comparison of phosphatidylcholine/sphingomyelin (PC/SM) elution (A) and triacylglycerol (TAG) elution (B) from protein-precipitated plasma with acetonitrile or acetone as the eluotropic solvent in mobile phase B. Ammonium formate was infused post-column to enable ionization of TAGs.

majority of bile acids targeted in this method. However, it was found that six of the bile acids in this study were still poorly ionized in positive ion mode, and therefore detection of their deprotonated precursors remained in negative SIM mode. Table 1 provides the optimized MRM transition information, along with the observed retention times of standards. The optimized parameters are directly transferable to other Agilent LC/TQ models.

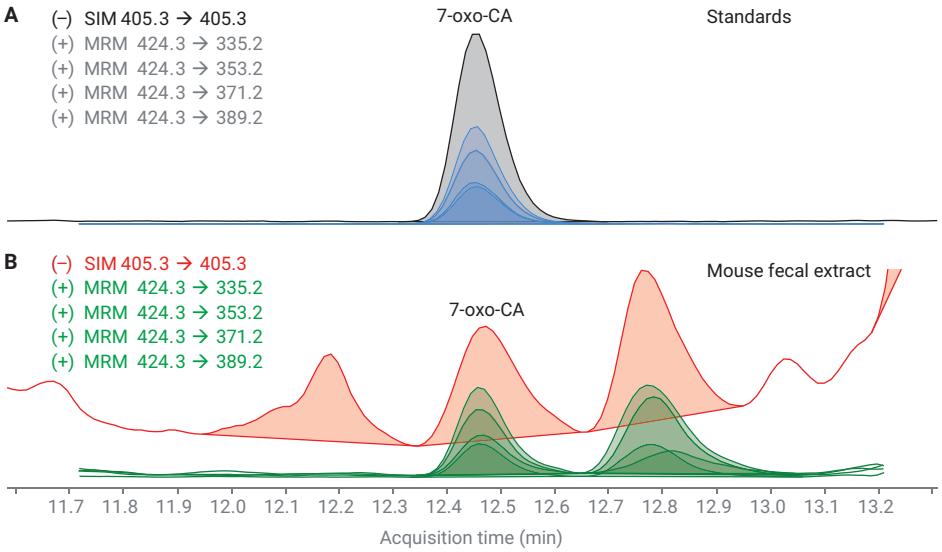


Figure 2. Comparison of 7-oxo-CA MRM versus SIM scans for 100 ppb standard in neat solvent (A) and endogenous 7-oxo-CA in mouse fecal extract (B).

Table 1. Optimized MRM transitions.

Compound Name	Trivial name	Steraloids Naming Convention	Class	CAS ID	Polarity/ Precursor Species	Scan Type	Prec. Ion	Product Ion	RT (min)	ΔRT	Frag V	CE
T-Alpha-MCA	Tauro-alpha-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7α, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Muricholic Acid	25613-05-2	Pos/ $(M+NH_4)^+$	MRM	533.3	516.3	5.1	1.5	102	4
T-Alpha-MCA	Tauro-alpha-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7α, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Muricholic Acid	25613-05-2	Pos/ $(M+NH_4)^+$	MRM	533.3	480.2	5.1	1.5	102	12
T-Alpha-MCA	Tauro-alpha-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7α, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Muricholic Acid	25613-05-2	Pos/ $(M+NH_4)^+$	MRM	533.3	462.2	5.1	1.5	102	24
T-Alpha-MCA	Tauro-alpha-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7α, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Muricholic Acid	25613-05-2	Pos/ $(M+NH_4)^+$	MRM	533.3	126	5.1	1.5	102	32
T-Beta-MCA	Tauro-beta-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7β, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Muricholic Acid	25696-60-0	Pos/ $(M+NH_4)^+$	MRM	533.3	516.3	5.33	1.5	112	4
T-Beta-MCA	Tauro-beta-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7β, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Muricholic Acid	25696-60-0	Pos/ $(M+NH_4)^+$	MRM	533.3	480.2	5.33	1.5	112	16
T-Beta-MCA	Tauro-beta-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7β, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Muricholic Acid	25696-60-0	Pos/ $(M+NH_4)^+$	MRM	533.3	462.2	5.33	1.5	112	20
T-Beta-MCA	Tauro-beta-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7β, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Muricholic Acid	25696-60-0	Pos/ $(M+NH_4)^+$	MRM	533.3	126	5.33	1.5	112	32

Compound Name	Trivial Name	Steraloids Naming Convention	Class	CAS ID	Polarity/ Precursor Species	Scan Type	Prec. Ion	Product Ion	RT (min)	ΔRT	Frag V	CE
TUDCA	Tauroursodeoxycholic acid	5β-CHOLANIC ACID-3α, 6β, 7β, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Bile Acid (rodent), Conjugated Secondary Bile Acid (human)	14605-22-2	Pos/ $(M+NH_4)^+$	MRM	517.3	500.3	8.15	1.5	102	4
TUDCA	Tauroursodeoxycholic acid	5β-CHOLANIC ACID-3α, 6β, 7β, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Bile Acid (rodent), Conjugated Secondary Bile Acid (human)	14605-22-2	Pos/ $(M+NH_4)^+$	MRM	517.3	464.2	8.15	1.5	102	16
TUDCA	Tauroursodeoxycholic acid	5β-CHOLANIC ACID-3α, 6β, 7β, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Bile Acid (rodent), Conjugated Secondary Bile Acid (human)	14605-22-2	Pos/ $(M+NH_4)^+$	MRM	517.3	126	8.15	1.5	102	40
TUDCA	Tauroursodeoxycholic acid	5β-CHOLANIC ACID-3α, 6β, 7β, -TRIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Bile Acid (rodent), Conjugated Secondary Bile Acid (human)	14605-22-2	Pos/ $(M+NH_4)^+$	MRM	517.3	81.1	8.15	1.5	102	68
TCA	Taurocholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL N-(2-SULPHOETHYL)	Conjugated Primary Bile Acid	81-24-3	Pos/ $(M+NH_4)^+$	MRM	533.3	480.2	8.88	1.5	114	16
TCA	Taurocholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL N-(2-SULPHOETHYL)	Conjugated Primary Bile Acid	81-24-3	Pos/ $(M+NH_4)^+$	MRM	533.3	462.2	8.88	1.5	114	24
TCA	Taurocholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL N-(2-SULPHOETHYL)	Conjugated Primary Bile Acid	81-24-3	Pos/ $(M+NH_4)^+$	MRM	533.3	337.2	8.88	1.5	114	32
TCA	Taurocholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL N-(2-SULPHOETHYL)	Conjugated Primary Bile Acid	81-24-3	Pos/ $(M+NH_4)^+$	MRM	533.3	126	8.88	1.5	114	52
GUDCA	Glycoursodeoxycholic acid	5β-CHOLANIC ACID-3α, 7β-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Bile Acids	64480-66-6	Pos/ $(M+H)^+$	MRM	450.3	432.3	10.95	1.5	112	4
GUDCA	Glycoursodeoxycholic acid	5β-CHOLANIC ACID-3α, 7β-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Bile Acids	64480-66-6	Pos/ $(M+H)^+$	MRM	450.3	414.3	10.95	1.5	112	12
GUDCA	Glycoursodeoxycholic acid	5β-CHOLANIC ACID-3α, 7β-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Bile Acids	64480-66-6	Pos/ $(M+H)^+$	MRM	450.3	339.3	10.95	1.5	112	20
GUDCA	Glycoursodeoxycholic acid	5β-CHOLANIC ACID-3α, 7β-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Bile Acids	64480-66-6	Pos/ $(M+H)^+$	MRM	450.3	76	10.95	1.5	112	28
GHDCA	Glycohyodeoxycholic acid	5β-CHOLANIC ACID-3α, 6α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Muricholic Acid	13042-33-6	Pos/ $(M+H)^+$	MRM	450.3	432.3	11.35	1.5	110	8
GHDCA	Glycohyodeoxycholic acid	5β-CHOLANIC ACID-3α, 6α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Muricholic Acid	13042-33-6	Pos/ $(M+H)^+$	MRM	450.3	414.3	11.35	1.5	110	12

Compound Name	Trivial Name	Steraloids Naming Convention	Class	CAS ID	Polarity/ Precursor Species	Scan Type	Prec. Ion	Product Ion	RT (min)	ΔRT	Frag V	CE
GHDCA	Glycohyodeoxycholic acid	5β-CHOLANIC ACID-3α, 6α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Muricholic Acid	13042-33-6	Pos/$(M+H)^+$	MRM	450.3	81.1	11.35	1.5	110	60
GHDCA	Glycohyodeoxycholic acid	5β-CHOLANIC ACID-3α, 6α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Muricholic Acid	13042-33-6	Pos/$(M+H)^+$	MRM	450.3	76.1	11.35	1.5	110	32
GCA	Glycocholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL N-(CARBOXYMETHYL)	Conjugated Primary Bile Acid	475-31-0	Pos/$(M+NH_4)^+$	MRM	483.3	466.3	11.41	1.5	100	8
GCA	Glycocholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL N-(CARBOXYMETHYL)	Conjugated Primary Bile Acid	475-31-0	Pos/$(M+NH_4)^+$	MRM	483.3	430.3	11.41	1.5	100	16
GCA	Glycocholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL N-(CARBOXYMETHYL)	Conjugated Primary Bile Acid	475-31-0	Pos/$(M+NH_4)^+$	MRM	483.3	412.3	11.41	1.5	100	20
GCA	Glycocholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL N-(CARBOXYMETHYL)	Conjugated Primary Bile Acid	475-31-0	Pos/$(M+NH_4)^+$	MRM	483.3	337.2	11.41	1.5	100	28
7-oxo-CA	7-Oxocholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL-7-ONE	Secondary Bile Acid	911-40-0	Pos/$(M+NH_4)^+$	MRM	424.3	389.2	12.38	1.5	112	8
7-oxo-CA	7-Oxocholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL-7-ONE	Secondary Bile Acid	911-40-0	Pos/$(M+NH_4)^+$	MRM	424.3	371.2	12.38	1.5	112	16
7-oxo-CA	7-Oxocholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL-7-ONE	Secondary Bile Acid	911-40-0	Pos/$(M+NH_4)^+$	MRM	424.3	353.2	12.38	1.5	112	16
7-oxo-CA	7-Oxocholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL-7-ONE	Secondary Bile Acid	911-40-0	Pos/$(M+NH_4)^+$	MRM	424.3	335.2	12.38	1.5	112	24
TCDCA	Taurochenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Bile Acid	516-35-8	Pos/$(M+NH_4)^+$	MRM	517.3	500.3	12.47	1.5	112	4
TCDCA	Taurochenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Bile Acid	516-35-8	Pos/$(M+NH_4)^+$	MRM	517.3	464.2	12.47	1.5	112	20
TCDCA	Taurochenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Bile Acid	516-35-8	Pos/$(M+NH_4)^+$	MRM	517.3	126	12.47	1.5	112	44
TCDCA	Taurochenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Primary Bile Acid	516-35-8	Pos/$(M+NH_4)^+$	MRM	517.3	81.1	12.47	1.5	112	72
TDCA	Taurodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Secondary Bile Acid	516-50-7	Pos/$(M+NH_4)^+$	MRM	517.3	500.3	13.26	1.5	102	8
TDCA	Taurodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Secondary Bile Acid	516-50-7	Pos/$(M+NH_4)^+$	MRM	517.3	482.3	13.26	1.5	102	12
TDCA	Taurodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Secondary Bile Acid	516-50-7	Pos/$(M+NH_4)^+$	MRM	517.3	464.3	13.26	1.5	102	20
TDCA	Taurodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(2-SULPHOETHYL)-AMIDE	Conjugated Secondary Bile Acid	516-50-7	Pos/$(M+NH_4)^+$	MRM	517.3	126	13.26	1.5	102	48
Alpha-MCA	Alpha-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7α-TRIOL	Primary Muricholic Acid	2393-58-0	Pos/$(M+NH_4)^+$	MRM	426.3	373.2	13.61	1.5	112	8
Alpha-MCA	Alpha-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7α-TRIOL	Primary Muricholic Acid	2393-58-0	Pos/$(M+NH_4)^+$	MRM	426.3	355.2	13.61	1.5	112	16
Alpha-MCA	Alpha-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7α-TRIOL	Primary Muricholic Acid	2393-58-0	Pos/$(M+NH_4)^+$	MRM	426.3	159.1	13.61	1.5	112	28

Compound Name	Trivial Name	Steraloids Naming Convention	Class	CAS ID	Polarity/ Precursor Species	Scan Type	Prec. Ion	Product Ion	RT (min)	ΔRT	Frag V	CE
Alpha-MCA	Alpha-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7α-TRIOL	Primary Muricholic Acid	2393-58-0	Pos/ $(M+NH_4)^+$	MRM	426.3	105	13.61	1.5	112	68
Beta-MCA	Beta-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7β-TRIOL	Primary Muricholic Acid	2393-59-1	Pos/ $(M+NH_4)^+$	MRM	426.3	373.2	14.08	1.5	102	12
Beta-MCA	Beta-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7β-TRIOL	Primary Muricholic Acid	2393-59-1	Pos/ $(M+NH_4)^+$	MRM	426.3	355.2	14.08	1.5	102	20
Beta-MCA	Beta-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7β-TRIOL	Primary Muricholic Acid	2393-59-1	Pos/ $(M+NH_4)^+$	MRM	426.3	159.1	14.08	1.5	102	32
Beta-MCA	Beta-muricholic acid	5β-CHOLANIC ACID-3α, 6β, 7β-TRIOL	Primary Muricholic Acid	2393-59-1	Pos/ $(M+NH_4)^+$	MRM	426.3	105	14.08	1.5	102	64
3-oxo-CA	3-Oxocholic acid	5β-CHOLANIC ACID-7α, 12α-DIOL-3-ONE	Secondary Bile Acid	2304-89-4	Pos/ $(M+NH_4)^+$	MRM	424.3	353.2	14.78	1.5	142	20
3-oxo-CA	3-Oxocholic acid	5β-CHOLANIC ACID-7α, 12α-DIOL-3-ONE	Secondary Bile Acid	2304-89-4	Pos/ $(M+NH_4)^+$	MRM	424.3	335.2	14.78	1.5	142	28
3-oxo-CA	3-Oxocholic acid	5β-CHOLANIC ACID-7α, 12α-DIOL-3-ONE	Secondary Bile Acid	2304-89-4	Pos/ $(M+NH_4)^+$	MRM	424.3	55.1	14.78	1.5	142	80
3-oxo-CA	3-Oxocholic acid	5β-CHOLANIC ACID-7α, 12α-DIOL-3-ONE	Secondary Bile Acid	2304-89-4	Pos/ $(M+NH_4)^+$	MRM	424.3	371.2	14.78	1.5	142	20
Gamma-MCA	Gamma-muricholic acid	5β-CHOLANIC ACID-3α, 6α, 7α-TRIOL	Secondary Muricholic Acid	547-75-1	Neg/(M-H)-	SIM	407.3	407.3	15.07	1.5	294	0
Muro-CA	Murocholic acid	5β-CHOLANIC ACID-3α, 6β-DIOL	Secondary Muricholic Acid	668-49-5	Pos/ $(M+NH_4)^+$	MRM	410.3	357.3	15.2	1.5	112	8
Muro-CA	Murocholic acid	5β-CHOLANIC ACID-3α, 6β-DIOL	Secondary Muricholic Acid	668-49-5	Pos/ $(M+NH_4)^+$	MRM	410.3	95.1	15.2	1.5	112	44
Muro-CA	Murocholic acid	5β-CHOLANIC ACID-3α, 6β-DIOL	Secondary Muricholic Acid	668-49-5	Pos/ $(M+NH_4)^+$	MRM	410.3	81.1	15.2	1.5	112	48
Muro-CA	Murocholic acid	5β-CHOLANIC ACID-3α, 6β-DIOL	Secondary Muricholic Acid	668-49-5	Pos/ $(M+NH_4)^+$	MRM	410.3	67.1	15.2	1.5	112	72
GCDCA	Glycochenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Primary Bile Acid	640-79-9	Pos/ $(M+H)^+$	MRM	450.3	432.3	15.321	1.5	114	8
GCDCA	Glycochenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Primary Bile Acid	640-79-9	Pos/ $(M+H)^+$	MRM	450.3	414.3	15.321	1.5	114	16
GCDCA	Glycochenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Primary Bile Acid	640-79-9	Pos/ $(M+H)^+$	MRM	450.3	76.1	15.321	1.5	114	36
GCDCA	Glycochenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Primary Bile Acid	640-79-9	Pos/ $(M+H)^+$	MRM	450.3	55.1	15.321	1.5	114	80
CA	Cholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL	Primary Bile Acid	81-25-4	Pos/ $(M+NH_4)^+$	MRM	426.3	373.2	15.91	1.5	112	12
CA	Cholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL	Primary Bile Acid	81-25-4	Pos/ $(M+NH_4)^+$	MRM	426.3	355.2	15.91	1.5	112	20
CA	Cholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL	Primary Bile Acid	81-25-4	Pos/ $(M+NH_4)^+$	MRM	426.3	105	15.91	1.5	112	72
CA	Cholic acid	5β-CHOLANIC ACID-3α, 7α, 12α-TRIOL	Primary Bile Acid	81-25-4	Pos/ $(M+NH_4)^+$	MRM	426.3	55.1	15.91	1.5	112	80
GDCA	Glycodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Bile Acid	360-65-6	Pos/ $(M+H)^+$	MRM	450.3	414.3	16.07	1.5	108	12

Compound Name	Trivial Name	Steraloids Naming Convention	Class	CAS ID	Polarity/ Precursor Species	Scan Type	Prec. Ion	Product Ion	RT (min)	ΔRT	Frag V	CE
GDCA	Glycodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Bile Acid	360-65-6	Pos/$(M+H)^+$	MRM	450.3	339.2	16.07	1.5	108	24
GDCA	Glycodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Bile Acid	360-65-6	Pos/$(M+H)^+$	MRM	450.3	105	16.07	1.5	108	76
GDCA	Glycodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(CARBOXYMETHYL)-AMIDE	Conjugated Secondary Bile Acid	360-65-6	Pos/$(M+H)^+$	MRM	450.3	55.1	16.07	1.5	108	80
UDCA	Ursodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(CARBOXYMETHYL)-AMIDE	Primary Bile Acid (rodent), Secondary Bile Acid (human)	128-13-2	Pos/$(M+NH_4)^+$	MRM	410.3	357.2	16.11	1.5	102	8
UDCA	Ursodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(CARBOXYMETHYL)-AMIDE	Primary Bile Acid (rodent), Secondary Bile Acid (human)	128-13-2	Pos/$(M+NH_4)^+$	MRM	410.3	105	16.11	1.5	102	60
UDCA	Ursodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(CARBOXYMETHYL)-AMIDE	Primary Bile Acid (rodent), Secondary Bile Acid (human)	128-13-2	Pos/$(M+NH_4)^+$	MRM	410.3	81.1	16.11	1.5	102	56
UDCA	Ursodeoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL N-(CARBOXYMETHYL)-AMIDE	Primary Bile Acid (rodent), Secondary Bile Acid (human)	128-13-2	Pos/$(M+NH_4)^+$	MRM	410.3	55.1	16.11	1.5	102	80
HDCA	Hyodeoxycholic acid	5β-CHOLANIC ACID-3α, 6α-DIOL	Secondary Muricholic Acid	83-49-8	Neg/$(M-H)^-$	SIM	391.3	391.3	16.48	1.5	246	0
7-ketoLCA	7-Ketolithocholic acid	5β-CHOLANIC ACID-3α-OL-7-ONE	Secondary Bile Acid	4651-67-6	Neg/$(M-H)^-$	SIM	389.3	389.3	16.82	1.5	248	0
CDCA	Chenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL	Primary Bile Acid	474-25-9	Pos/$(M+NH_4)^+$	MRM	410.3	357.2	19.93	1.5	92	8
CDCA	Chenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL	Primary Bile Acid	474-25-9	Pos/$(M+NH_4)^+$	MRM	410.3	95.1	19.93	1.5	92	44
CDCA	Chenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL	Primary Bile Acid	474-25-9	Pos/$(M+NH_4)^+$	MRM	410.3	81.1	19.93	1.5	92	56
CDCA	Chenodeoxycholic acid	5β-CHOLANIC ACID-3α, 7α-DIOL	Primary Bile Acid	474-25-9	Pos/$(M+NH_4)^+$	MRM	410.3	55.1	19.93	1.5	92	76
DCA	Deoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL	Secondary Bile Acid	83-44-3	Pos/$(M+NH_4)^+$	MRM	410.3	357.2	20.46	1.5	110	12
DCA	Deoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL	Secondary Bile Acid	83-44-3	Pos/$(M+NH_4)^+$	MRM	410.3	95.1	20.46	1.5	110	52
DCA	Deoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL	Secondary Bile Acid	83-44-3	Pos/$(M+NH_4)^+$	MRM	410.3	81.1	20.46	1.5	110	56
DCA	Deoxycholic acid	5β-CHOLANIC ACID-3α, 12α-DIOL	Secondary Bile Acid	83-44-3	Pos/$(M+NH_4)^+$	MRM	410.3	67.1	20.46	1.5	110	68
AlloLCA	Isoallolithocholic acid	5α-CHOLANIC ACID-3β-OL	Allo Secondary Bile Acid	2276-93-9	Neg/$(M-H)^-$	SIM	375.3	375.3	22.23	1.5	218	0
IsoLCA	Isolithocholic acid	5β-CHOLANIC ACID-3β-OL	Secondary Bile Acid	1534-35-6	Neg/$(M-H)^-$	SIM	375.3	375.3	23.02	1.5	238	0
LCA	Lithocholic acid	5β-CHOLANIC ACID-3α-OL	Secondary Bile Acid	434-13-9	Neg/$(M-H)^-$	SIM	375.3	375.3	24.24	1.5	248	0

The water-acetone based mobile phase maintained excellent separation of bile acid isomers (Figure 3). The gradient was optimized to achieve baseline separation of 24 of the 26 bile acids, and partial separation of early-eluting Tauro α- and β-MCA, key bile acids of interest in the mouse model system.

Robustness

To assess robustness, 200 injections of a mouse cecal extract spiked with standards were analyzed over a more than 4-day period without interruption (Figure 4). The unique LC/MS solvent system enabled excellent retention time reproducibility, even with minimal sample preparation. The overall average MRM peak area CV was 2.7% per bile acid. Excellent retention time reproducibility was also observed with an overall average CV of <0.2% per bile acid. With some samples, chromatographic peak shape began to noticeably worsen over multiple analytical injections (n = 50). It can be speculated that protein precipitation on the head of the guard column was the primary cause of the worsening peak shape. Although the samples were prepared with a methanol-based protein-precipitation step, methanol is known to incompletely remove all proteins from typical samples. For future experiments, alternative protein-precipitation solvents and reagents could be evaluated, or a 3,000 MWCO ultrafiltration step may help to remove residual proteins from the sample prior to analysis. Alternatively, it was found that replacing the guard column or simply washing the guard column with isopropanol completely recovered peak shapes, and the latter procedure can be reasonably implemented between sample batches.

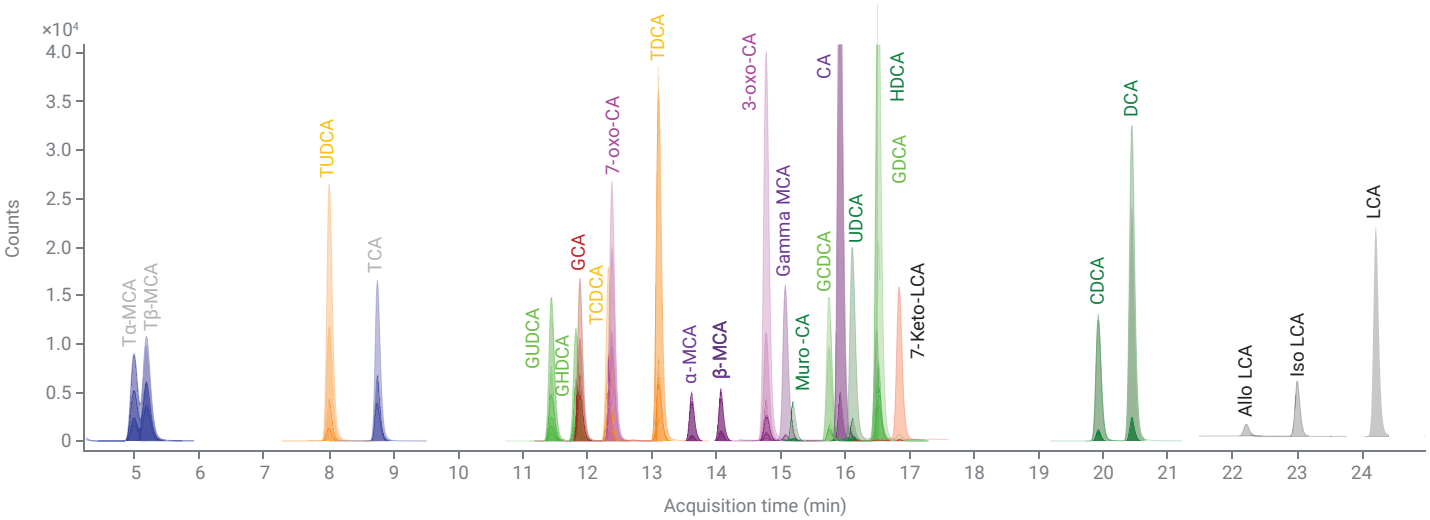
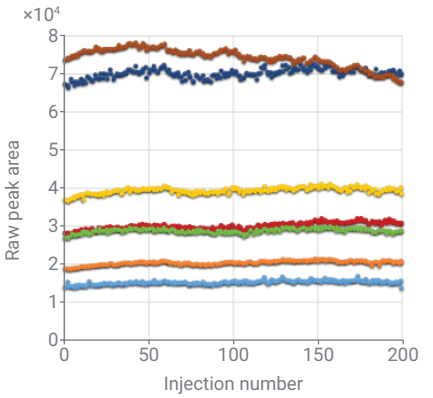


Figure 3. Overlaid MRM and SIM chromatograms from a mixture of bile acid standards acquired with the final dMRM LC/MS method. Isobaric bile acids sharing the same precursor *m/z* are indicated by color.



Key	Compound	Area CV	RT CV
	CA	1.8%	0.10%
	GCA	2.1%	0.12%
	α-MCA	3.4%	0.11%
	LCA	3.3%	0.06%
	γ-MCA	2.0%	0.10%
	TUDCA	2.8%	0.21%
	GHDCA	3.0%	0.12%
	Average	2.7%	0.15%

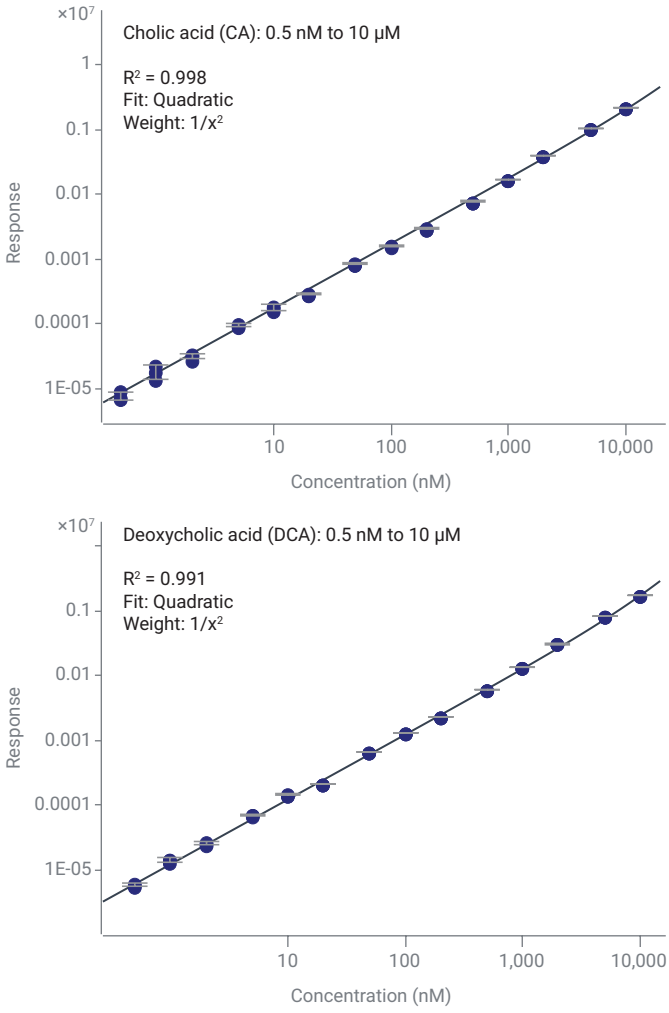
Figure 4. MRM response over time for seven representative bile acids (left). Corresponding MRM peak area and RT CVs (right).

Quantitation

Twenty of the 26 standards were diluted in neat solvent and evaluated at 16 standard concentrations ranging from the LOD at 100 pM to 10 µM. Excellent assay precision (RSD% <20% at the LLOQ and <15% at the rest of the levels) as well as average accuracy (80 to 125% at the LLOQ and 85 to 115% at the rest of the levels) were obtained. Correlation coefficients (R^2) for calibration curves were higher than 0.99 over up to 4.3 orders of dynamic range (Figure 5).

Evaluating the effect of the gut microbiome on secondary bile acid production

The principal actions of gut bacteria on bile acids are C_{24} amide hydrolysis and 7α-dehydroxylation, transforming the glycine and taurine conjugates of CA and CDCA into the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. An additional ~50 different secondary bile acids have been detected in human feces.[1] With respect to bile acid research, germ-free mice are devoid of gut bacteria, and thus lack secondary bile acids, providing an invaluable experimental model to study interactions between the host and its microbiota. As a proof-of-principle, the LC/MS/MS method was applied



Compound	LLOQ (nM)
T-Alpha-MCA	0.5
T-Beta-MCA	0.5
TCA	0.5
GUDCA	0.5
GHDCA	1.0
GCA	1.0
7-oxo-CA	1.0
Alpha-MCA	2.0
Beta-MCA	5.0
3-oxo-CA	0.5
Gamma-MCA	10.0
GCDCA	2.0
CA	0.5
GDCA	0.5
UDCA	10.0
HDCA	20.0
7-ketoLCA	10.0
CDCA	1.0
DCA	0.5
LCA	5.0

Figure 5. Examples of the dynamic range and linearity achieved with the final dMRM method. The table provides LLOQs for the 20 bile acid standards.

to fecal extracts of three biological replicates each, of conventionally raised and germ-free mice. Fecal bile acid concentrations were approximated based on an external calibration curve of standards prepared in solvent. A quantitative standard was not available for six of the 26 compounds, and in these cases, the calibration curve of a related standard with an analogous fragmentation pattern was applied. It should be emphasized that without the use of labeled internal standards, the concentrations provided are rough estimations, especially due to known significant ionization suppression effects observed in fecal matrices. However, the MRM could easily be modified to enable absolute quantitation through the incorporation of stable-isotope-labeled standards.

While there were variations in bile acid concentrations across the individual mice, the overall results demonstrated a universal absence of secondary bile acid metabolism in germ-free mice (Figure 6).

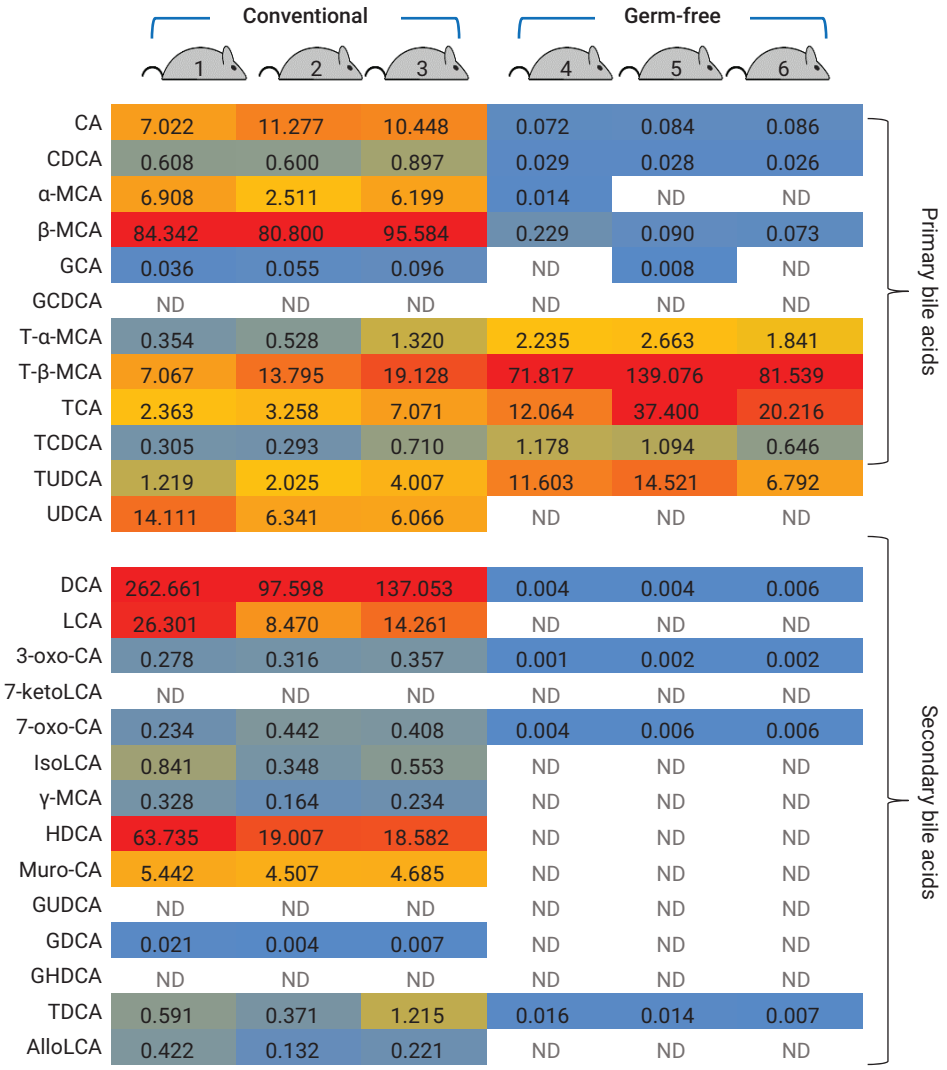


Figure 6. Estimated fecal bile acid concentrations in conventionally raised versus germ-free mice. Values are expressed as nmol/g = nmol bile acid per gram fecal material. ND = Not detected.

Agilent Mass Profiler Professional software (MPP) provides the ability to import, compare, and visualize LC/MS datasets. With the Pathway Architect module, the calculated bile acid concentrations were mapped onto the MetaCyc pathway for "Bile Acid 7Alpha-dehydroxylation" that is common to many taxa of bacteria (Figure 7). This enabled visualization of differences in individual bile acid concentrations across the six mouse samples, in context with the biosynthetic pathway.

Conclusion

A targeted TQ-based LC/MS/MS method for bile acid profiling and quantitation was developed that provides enhanced selectivity in biological matrices, increased robustness due to unique LC mobile phases, minimal sample preparation requirements, and excellent analytical sensitivity. This application note further demonstrates that the method and software workflow is fit-for-purpose to compare bile acid profiles from typical complex samples of microbiome research interest.

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2. Wegner, K. *et al.* Rapid Analysis of Bile Acids in Different Biological Matrices Using LC-ESI-MS/MS for the Investigation of Bile Acid Transformation by Mammalian Gut Bacteria. *Anal. Bioanal. Chem.* **2017**, *409*, 1231–1245.

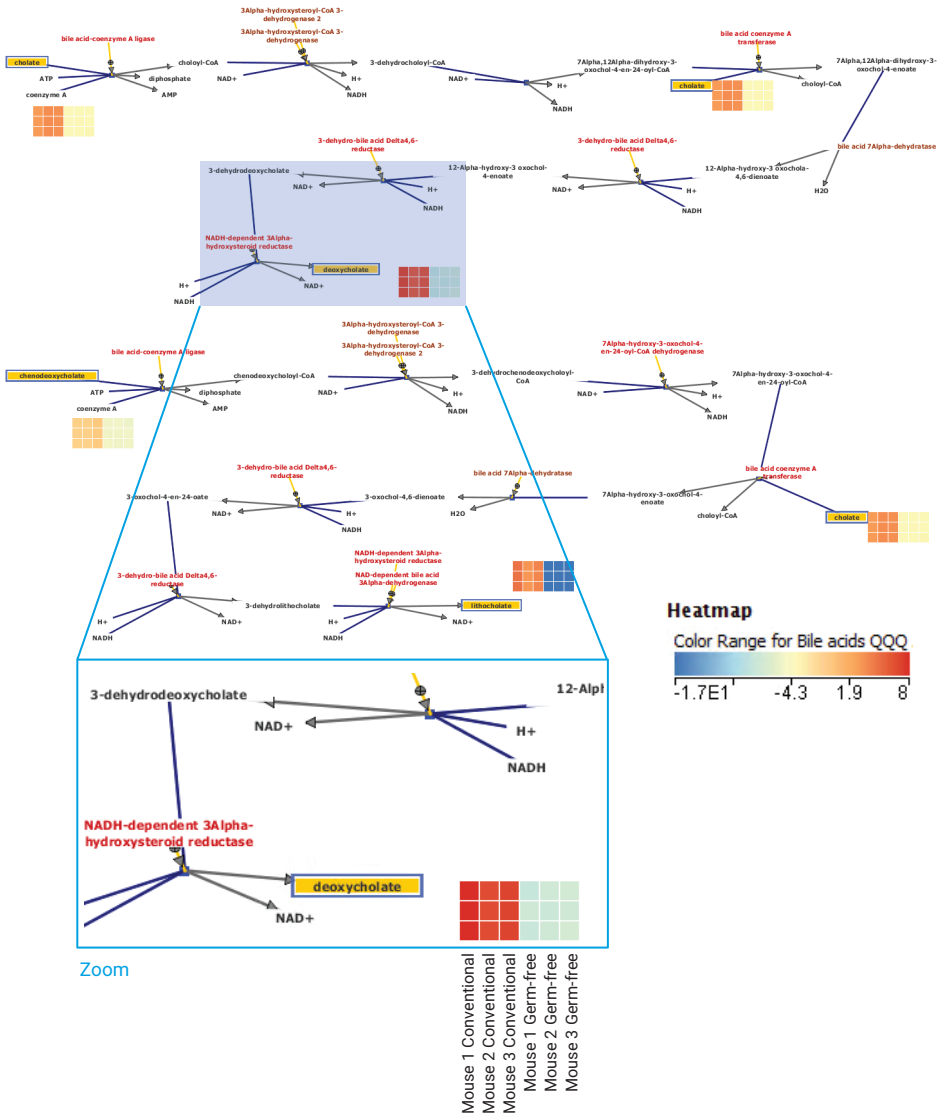

Figure 7. Pathway Architect view of the "Bile Acid 7Alpha-dehydroxylation" pathway from BioCyc (MetaCyc). Matched compounds in the dataset and pathway are highlighted in yellow, and quilt plots indicate the calculated concentrations.

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