Application Note

Life Science Research

Agilent

Trusted Answers

Deciphering the Microbiome: Targeted LC/MS/MS Analysis of Bile Acids in Biological Samples

Authors

Pietro Morlacchi
Agilent Technologies, Inc.

Justin Cross

Ruben J. F. Ramos
Cell Metabolism Core Facility
Memorial Sloan Kettering
Cancer Center New York
NY, USA

Christophe Deckers
Agilent Technologies, Inc.

Mark Sartain
Agilent Technologies, Inc.

Limian Zhao
Agilent Technologies, Inc.

Daniel J. Cuthbertson
Agilent Technologies, Inc.

Abstract

Bile acids (BAs) are crucial molecules involved in the digestion and absorption of dietary lipids and lipid-soluble vitamins. These amphipathic compounds facilitate the emulsification of fats in the small intestine, aiding in the efficient absorption of nutrients. Primary bile acids are synthesized in the liver from cholesterol through a complex process, tightly regulated to maintain lipid homeostasis. Once produced, these primary bile acids are stored in the gallbladder and released into the duodenum in response to food intake, where they perform their digestive functions. Approximately 95% of these bile acids are reabsorbed in the terminal ileum and recycled back to the liver through enterohepatic circulation, underscoring the efficiency of this system. The remaining 5% escapes reabsorption and undergoes extensive structural modifications by gut microbiota. This process leads to a series of related metabolites, termed secondary bile acids, which have distinct biological activities, many of which remain unknown.

Recent studies suggest that secondary bile acids have broader implications in health and disease. Emerging research points to their involvement in immune regulation, where they may influence the activity of immune cells and inflammatory responses. Additionally, there is growing evidence linking secondary bile acids to carcinogenesis and tumor progression, highlighting their potential roles in the development and progression of certain cancers. These discoveries suggest that bile acids, particularly secondary bile acids, are more than simply digestive aids; they are active participants in complex physiological processes. Understanding their diverse roles could provide new insights into the pathogenesis of various diseases and potentially lead to novel therapeutic strategies. This application note reports a novel standardized LC/MS/MS method for the targeted analysis of a panel of 68 unique BAs in plasma, serum, and fecal samples using an Agilent 6495D triple quadrupole LC/MS (LC/TQ) platform.

Introduction

BA analysis using liquid chromatography-mass spectrometry (LC/MS) is both critical and challenging due to the inherent complexity of bile acids. These compounds encompass a wide range of structurally similar and isomeric species that often exhibit nearly identical mass-to-charge ratios (*m/z*) and retention times. This similarity makes achieving baseline separation and accurate identification particularly difficult.

Additionally, bile acids are relatively stable molecules that do not typically undergo extensive fragmentation in a mass spectrometer. Additionally, the more challenging fragmentation complicates the differentiation of isomeric bile acids emphasizing the need for a standardized method also leveraging retention times.

Compounding these issues, many of the microbiota-produced bile acid species are typically present at low concentrations in biological samples, which often contain matrix components that can lead to ion suppression or enhancement. These matrix effects can interfere with accurate quantification, further complicating the analysis. Moreover, the need for high sensitivity and specificity to distinguish closely related bile acid species adds another layer of complexity.

To address these challenges, standardized and highly optimized chromatographic methods, advanced mass spectrometric techniques, and robust sample preparation protocols are essential. Together, these strategies enhance the reliability of BA analysis, enabling accurate profiling for both research and clinical research applications.

A previously described LC/TQ method was used to analyze 26 bile acids.[1] This present study introduces a new method using a 6495D LC/TQ with fourth-generation ion funnel technology, which improves sensitivity and expands the scope for the analysis of 68 unique bile acids in complex matrix. This bile acid method is compatible and consistent with the Agilent Standardized LC/MS platform for omics analysis and is easily transferable in the laboratory and therefore suitable for rapid delivery of new insights.

Experimental

All chemicals used for the LC mobile phase and LC/MS sample preparation were HPLC or LC/MS grade.

Standards and preparation

Bile acid standards used for method development were sourced from various suppliers, as detailed in Table 1. Powdered standards were dissolved in methanol to prepare stock solutions with concentrations ranging from 1.0 to 10 µg/mL. These stock solutions were used to optimize the Agilent Jet Stream (AJS) ESI source conditions (Table 2). The multiple reaction monitoring (MRM) acquisition parameters were optimized using the source and compound optimizer tools in the Agilent MassHunter acquisition software 12.1.

Briefly, individual BA stock solutions were sequentially injected (0.5 to 1 µL) into the mass spectrometer by way of an automated iterative injection program. The $[M+HCOO]^-$, $[M+H]^+$, and $[M-H]^-$ ion species were selected as precursor ions to identify optimal MRM transitions in both positive and negative modes. These transitions, along with the optimized AJS source parameters (Table 2), were incorporated with their corresponding retention times into the compound dynamic MRM (dMRM) acquisition list of the final acquisition method. All bile acids were analyzed using standard iFunnel settings. The full list of transitions and method details are available upon request in supporting documentation from Agilent.

The guiding principles emerging from this extensive optimization included: (a) amino acid conjugated bile acids had the strongest response in positive ionization mode and a prominent MRM transition due to loss of the conjugated amino acid, (b) unconjugated bile acids produced relatively poor fragmentation spectra, but many formed a stable and intense formate adduct in negative mode, (c) loss of the formate adduct could be included in the MRM schedule to add specificity and sensitivity. Thus, corresponding selected ion monitoring (SIM) transitions were also included to enhance sensitivity and improve signal-to-noise ratios (S/N) in complex matrix. A similar strategy with BA negative-ion mode adducts was described in more detail in the referenced study.[1]

Table 1. List of bile acid standards used for method development (continued on next page).

Name	Abbreviation	Molecular Weight	CAS Number	Vendor	Catalog Number
12-Oxolithocholic Acid	12-oxo-LCA	390.3	5130-29-0	Steraloids Inc.	C1650-000
3-Dehydrocholic Acid (3-Oxocholic Acid)	3-oxo-CA	406.3	2304-89-4	Steraloids Inc.	C1272-000
3-Oxochenodeoxycholic Acid	3-oxo-CDCA	390.3	4185-00-6	Avanti	700255
3-Oxo-Deoxycholic Acid	3-oxo-DCA	390.3	4185-01-7	Avanti	700255
3-Oxolithocholic Acid (Dehydrolithocholic Acid)	3-oxo-LCA	374.3	1553-56-6	Cayman	29544
6,7-Dioxolithocholic Acid	6,7-diketo-LCA	404.3	1643669-23-1	Avanti	700234
3α,6α,7α,12α-Tetrahydroxy Bile Acid	6α-THBA	424.3	80875-92-9	Avanti	700228
3α,6β,7α,12α-Tetrahydroxy Bile Acid	6β-THBA	424.3	80875-93-0	Avanti	700189
7,12-Dioxolithocholic Acid	7,12-diketo-LCA	404.3	517-33-9	Avanti	700230
7-Oxocholic Acid (7-Dehydrocholic Acid)	7-oxo-CA	406.3	911-40-0	Steraloids Inc.	C1250-000
3β-Oh-7-Oxocholenic Acid	7-oxo-cholenic	388.3	25218-38-6	Cayman	29541
7-Oxolithocholic Acid (Nutriacholic Acid)	7-keto-LCA	390.3	4651-67-6	Steraloids Inc.	C1600-000
Allocholic Acid	Allo-CA	408.3	474-25-9	Cayman	30415
Allolithocholic Acid	Allo-LCA	376.3	2276-93-9	Steraloids Inc.	C0680-000
Cholic Acid	CA	408.3	81-25-4	Avanti	700212
Cholic Acid Sulfate	CAS	488.2		Isosciences	13098UNL3SO
Chenodeoxycholic Acid	CDCA	392.3	474-25-9	Avanti	700198
Chenodeoxycholic Acid Sulfate	CDCAS	472.3	59132-32-0	Isosciences	13101UNL3SO
Cholenic Acid	Cholenic	374.3	5255-17-4	Cayman	29543
Deoxycholic Acid	DCA	392.3	83-44-3	Avanti	700197
Deoxycholic Acid Sulfate	DCAS	472.3	67030-48-2	Isosciences	13100UNL3SO
Dehydrocholic Acid	DHCA	402.2	81-23-2	Avanti	700215
Glycocholic Acid	GCA	465.3	475-31-0	Avanti	700265
Glycocholic Acid Sulfate	GCAS	545.3		Isosciences	13443UNL3SO
Glycochenodeoxycholic Acid	GCDCA	449.3	640-79-9	Avanti	700266
Glycochenodeoxycholic Acid Sulfate	GCDCAS	529.3	66874-09-7	Isosciences	13104UNL3SO
Glycodeoxycholic Acid	GDCA	449.3	360-65-6	Avanti	700267
Glycodeoxycholic Acid Sulfate	GDCAS	529.3	67030-54-0	Isosciences	13226UNL3SO
Glycodehydrocholic Acid	GDHCA	459.3	3415-45-0	Steraloids Inc.	C2022-000
Glycohyodeoxycholic Acid	GHDCA	449.3	13042-33-6	Cayman	22643
Glycolithocholic Acid	GLCA	433.3	474-74-8	Avanti	700268
Glycolithocholic Acid Sulfate	GLCAS	513.3	15324-64-8	Isosciences	13231UNL3SO
Glycoursodeoxycholic Acid	GUDCA	449.3	64480-66-6	Avanti	700263
Glycoursodeoxycholic Acid Sulfate	GUDCAS	529.3	133429-88-6	Isosciences	13224UNL3SO
Glyco-Γ-Muricholic Acid (Glycohyocholic Acid)	G-γ-MCA	465.3	32747-08-3	Steraloids Inc.	C1860-000
Hyodeoxycholic Acid	HDCA	392.3	83-49-8	Avanti	700214
Isoallolithocholic Acid	Isoallo-LCA	376.6	2276-93-9	Steraloids Inc.	C0700-000
Isocholic Acid (3-Epicholic Acid)	Iso-CA	408.3	3338-16-7	Cambridge	ULM-10566
Isochenodeoxycholic Acid	Iso-CDCA	392.6	566-24-5	TRC	I789600
Isodeoxycholic Acid	Iso-DCA	392.3	566.17-6	Steraloids Inc	C1165-000
Isolithocholic Acid	Iso-LCA	376.3	1534-35-6	Steraloids Inc.	C1475-000
Lithocholic Acid	LCA	376.3	434-13-9	Avanti	700218
Lithocholic Acid Sulfate	LCAS	456.3	34669-57-3	Isosciences	13099UNL3SO
Murideoxycholic Acid (Murocholic Acid)	MDCA	392.3	668-49-5	Steraloids Inc.	C0910-000
Nordeoxycholic Acid	norDCA	378.3	53608-86-9	Avanti	700240
Taurocholic Acid	TCA	515.3	81-24-3	Avanti	700251

Name	Abbreviation	Molecular Weight	CAS Number	Vendor	Catalog Number
Taurocholic Acid Sulfate	TCAS	595.3	67030-62-0	Isosciences	13232UNL3SO
Taurochenodeoxycholic Acid	TCDCA	499.3	516-35-8	Avanti	700249
Taurochenodeoxycholic Acid Sulfate	TCDCAS	579.3		Isosciences	13105UNL3SO
Taurodeoxycholic Acid	TDCA	499.3	516-50-7	Avanti	700250
Taurodeoxycholic Acid Sulfate	TDCAS	579.3		Isosciences	13225UNL3SO
Taurodehydrocholic Acid	TDHCA	509.2	517-37-3	Avanti	700242
Taurohyodeoxycholic Acid	THDCA	499.3	2958-04-5	Steraloids Inc.	C0892-000
Taurolithocholic Acid	TLCA	483.3	516-90-5	Avanti	700252
Taurolithocholic Acid Sulfate	TLCAS	563.3	15324-65-9	Isosciences	13230UNL3SO
Tauroursodeoxycholic Acid	TUDCA	499.3	14605-22-2	Avanti	700247
Tauroursodeoxycholic Acid Sulfate	TUDCAS	579.3		Isosciences	13106UNL3SO
Tauro-α-Muricholic Acid	T-α-MCA	515.3	25613-05-2	Steraloids Inc	C1893-000
Tauro-β-Muricholic Acid	T-β-MCA	515.3	25696-60-0	Steraloids Inc	C1899-000
Tauro-γ-Muricholic Acid	T-γ-MCA	515.3		Cayman	22669
Tauro-ω-Muricholic Acid	T-ω-MCA	515.3	2456348-84-6	Steraloids Inc.	C1889-000
Ursocholic Acid	UCA	408.3	2955-27-3	Avanti	700229
Ursodeoxycholic Acid	UDCA	392.3	128-13-2	Avanti	700199
Ursodeoxycholic Acid Sulfate	UDCAS	472.3	68780-73-4	Isosciences	13102UNL3SO
α-Muricholic Acid	α-MCA	408.3	2393-58-0	Avanti	700232
β-Muricholic Acid	β-MCA	408.3	2393-59-1	Avanti	700233
γ-Muricholic Acid (Hyocholic Acid)	γ-MCA	408.3	547-75-1	Steraloids Inc.	C1850-000
ω-Muricholic Acid	ω-MCA	408.3		Avanti	700231

Table 2. Mass spectrometer acquisition parameters.

Parameter	Value
Source	Agilent Jet Stream Dual ESI
Sheath Gas Temperature, Flow	250 °C, 11 L/min
Gas Temp, Flow	180 °C, 20 L/min
Nebulizer	25 psi
Capillary	4,500 V (+)/5,000 V (–)
Nozzle	1,000 V (+/–)
MS Mode	Positive/Negative
iFunnel Settings	Standard

Sample preparation

Certified plasma samples from male and female mice and rats were acquired from BioIVT (Elevated Science, Westbury, NY), while fecal material from various human donors was purchased from Medix Biochemica (Maryland Heights, MO). Optimal BA extraction conditions from biological samples were established. BAs were extracted from plasma/serum samples (50 µL) with four sample volumes of acetonitrile containing 1% formic acid and d-BA internal standards. After incubating at room temperature with shaking for 2 hours, samples were centrifuged (20 minutes, 13,000 rpm, 5 °C) to pellet the proteins, and supernatants were dried down by centrifugation under vacuum at 30 °C.

Fecal samples were extracted at 100 mg/mL with 80% methanol containing d-BA internal standards. Samples were homogenized by bead beating for six 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 minutes, then centrifuged (20 minutes, 13,000 rpm, 5 °C); the supernatants were collected, and the solvent was evaporated as described previously. Dry samples were reconstituted with 50% methanol (100 µL), vortexed for 1 minute, and centrifuged (2 minutes, 13,000 rpm, 5 °C) to pellet insoluble material. Supernatants were then transferred to glass analytical vials for LC/MS analysis.

Alternatively, Agilent Captiva Enhanced Matrix Removal Lipid (Captiva EMR–Lipid) cartridges or plates can be incorporated into the extraction protocol to remove proteins and lipids from samples simultaneously before dry-down, reconstitution, and LC/MS analysis. Bile acid extraction recovery using Captiva EMR–Lipid was evaluated through pre- and post-spike experiments with a series of d-BA standards, achieving recovery rates exceeding 85% (data not shown). These results align with previously reported recovery rates for metabolites.[3]

The inclusion of Captiva EMR–Lipid in this workflow may be particularly relevant when samples are subjected to parallel lipidomics analysis. It is also useful for general sample cleanup to enhance method robustness and to minimize matrix effects on MS signals. Furthermore, the bile acid extraction protocol for plasma samples is compatible with automation using the Agilent Bravo Metabolomics Sample Prep Platform, as described in prior studies.[2,3]

Instrumentation

The LC/MS platform consisted of a 6495D triple quadrupole mass spectrometer with fourth-generation iFunnel technology, coupled with an Agilent 1290 Infinity II Bio LC system with the Agilent standardized configuration for omics applications. The acquisition method is fully compatible with an Agilent 6495C model and a regular stainless-steel Agilent 1290 Infinity II LC with the same standard omics configuration. All methods using the Infinity II LC systems are fully compatible with Infinity III LC systems and give identical method performance.

Software

MS data were acquired using MassHunter acquisition software 12.1, which includes built-in source and compound optimizer tools for method development. Agilent MassHunter Qualitative Analysis 12.0 software was used to visualize the acquired dataset and assess data quality before quantification.

Bile acid levels from the LC/MS/MS datasets were quantified using MassHunter Quantitative Analysis 12.0 software. Quantitation methods for both relative and absolute measurements were developed using one of the quality control (QC) injections. Statistical analysis was performed with Agilent Mass Profiler Professional (MPP) 15.1.

Bile acid separation

BAs were separated using reversed-phase chromatography on an Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, 1.9 µm column (part number 695675-902) with an 18-minute nonlinear gradient. This process was followed by column wash and re-equilibration, resulting in a total run time of 23 minutes. The optimized LC conditions are summarized in Table 3.

Table 3. Chromatographic conditions.

Liquid Chromatography Conditions	
Column	Agilent Poroshell 120 EC-C18, 2.1 × 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 (min) %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

Under these chromatographic conditions, nearly all isomeric and isobaric BAs in the panel were resolved at the base peak level (Figures 1 and 2). The Agilent standard configuration for omics applications using the 1290 Infinity II Bio LC system facilitates straightforward method interchange for targeted metabolomics, lipidomics, and proteomics applications.[4]

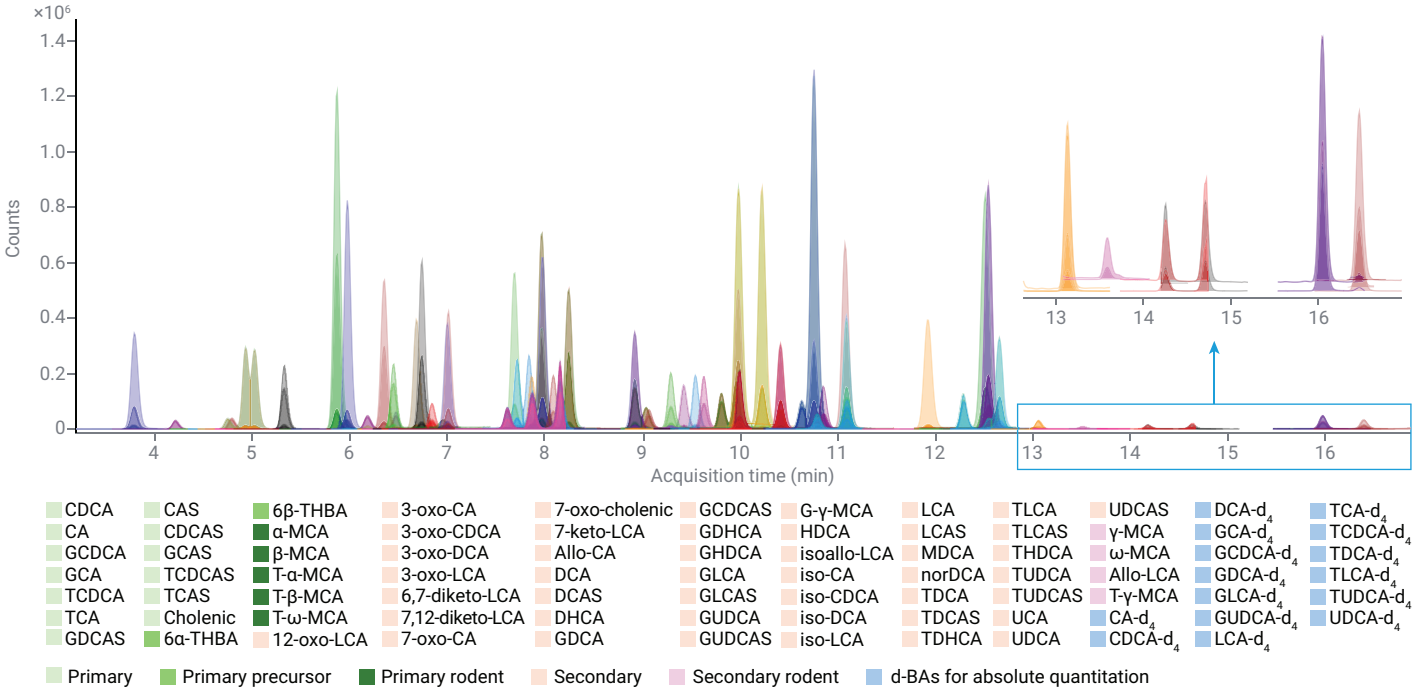


Figure 1. Chromatographic separation of a bile acid standard mixture by C-18 reversed-phase chromatography.

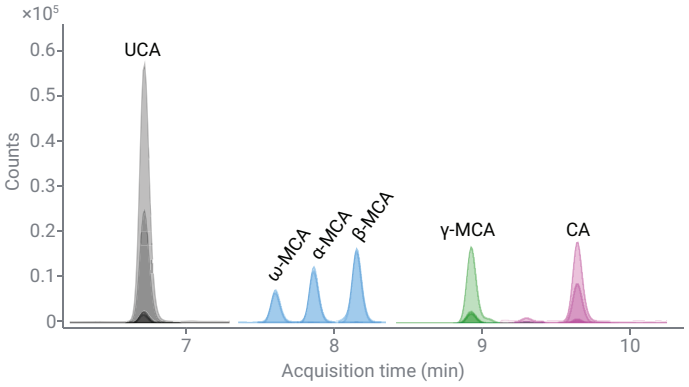


Figure 2. Chromatographic separation of relevant isobaric bile acids. Standardized methodologies with retention times are essential in separating isobaric species that sometimes share MRM transitions.

Absolute quantitation of relevant bile acids

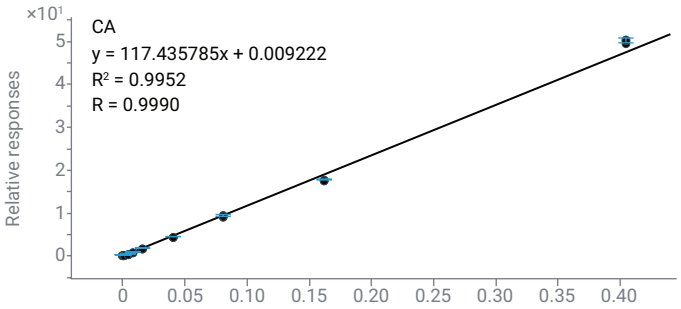
In addition to acquisition parameters for the measurement of 68 endogenous BAs in plasma and fecal samples, the LC/MS/MS acquisition method includes MRM transition and retention times corresponding to 15 deuterated bile acids (d-BAs) from the Bile Acid Splash Lipidomix (Avanti Research). These are designated as internal standards in the LC/MS/MS acquisition method and can be used for the absolute quantification of corresponding primary and secondary plasma BAs.

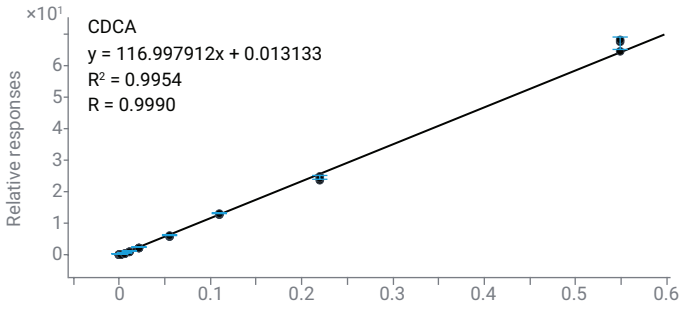
The Bile Acid Splash Lipidomix was diluted 100-fold in acetonitrile and 0.1% formic acid to create the plasma extraction solvent. The d-BA concentrations are reported in Table 4. Quantitation was performed with external calibration curves obtained from neat authentic standards. Twelve calibration standards were employed, each spiked with the d-BA mixture to obtain the same concentrations as those in the plasma samples.

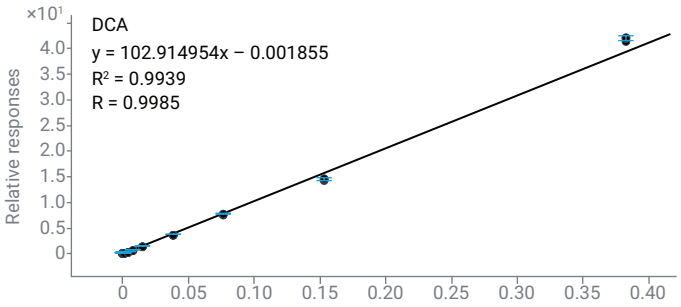
Calibration curves ranged from 0.1 to 2,500 ng/mL. A linear response with an $R^2 > 0.99$ was observed. MassHunter Quantitative Analysis 12.0 generates calibration-at-a-glance figures, allowing quick processing of all calibration curves and customizable visualization to fit any view and subset of compounds (Figure 3).

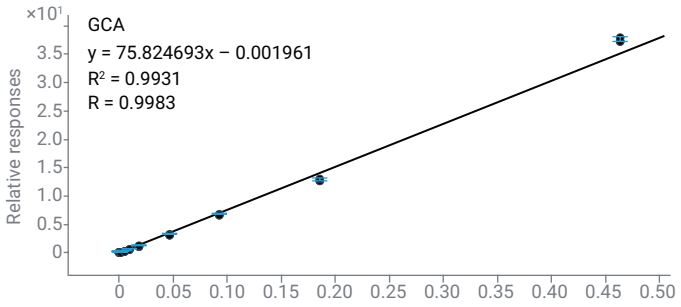
Table 4. Avanti d_4-BA SPLASH composition.

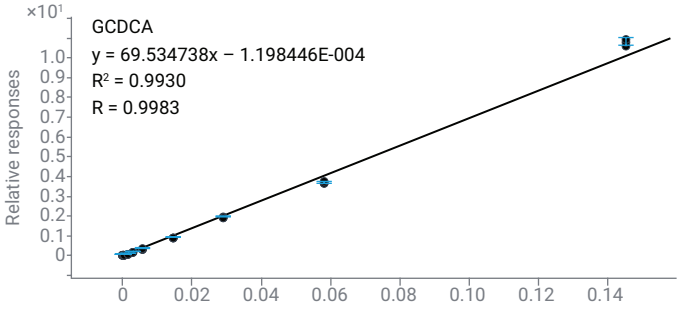
Name	Abbreviation	Concentration in Avanti SPLASH Standard Mix (nM)	Used for Absolute Quantification
Cholic Acid-d_4	CA-d4	1,150	Yes
Taurocholic Acid-d_4, Sodium Salt	TCA-d4	1,000	Yes
Glycocholic Acid-d_4	GCA-d4	1,150	Yes
Deoxycholic Acid-d_4	DCA-d4	1,650	Yes
Taurodeoxycholic Acid-d_4, Sodium Salt	TDCA-d4	1,000	Yes
Glycodeoxycholic Acid-d_4	GDCA-d4	1,275	Yes
Chenodeoxycholic Acid-d_4	CDCA-d4	1,150	Yes
Taurochenodeoxycholic Acid-d_4, Sodium Salt	TCDCA-d4	1,000	Yes
Glycochenodeoxycholic-d_4 Acid	GCDCA-d4	3,800	Yes
Tauroursodeoxycholic Acid-d_4, Sodium Salt	TUDCA-d4	500	Yes
Glycoursodeoxycholic Acid-d_4	GUDCA-d4	600	Yes
3β,5α,6β-trihydroxycholanoic Acid-d_4	THCA-d4	250	No
Lithocholic Acid-d_4	LCA-d4	250	Yes
Taurolithocholic Acid-d_4, Sodium Salt	TLCA-d4	250	Yes
Glycolithocholic Acid-d_4	GLCA-d4	250	Yes
Ursodeoxycholic Acid-d_4	UDCA-d4	300	Yes
Cholenic Acid-d_4	Cholenic-d4	250	No

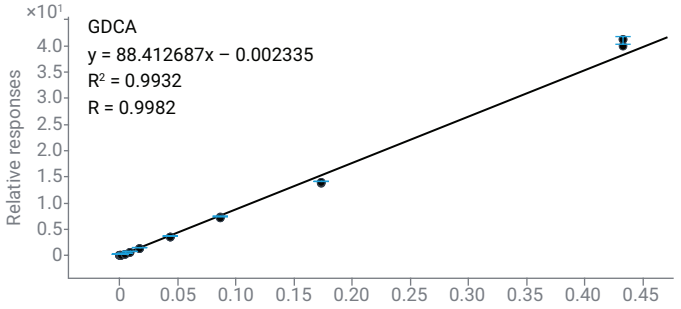

CA
y = 117.435785x + 0.009222
R² = 0.9952
R = 0.9990
Relative responses

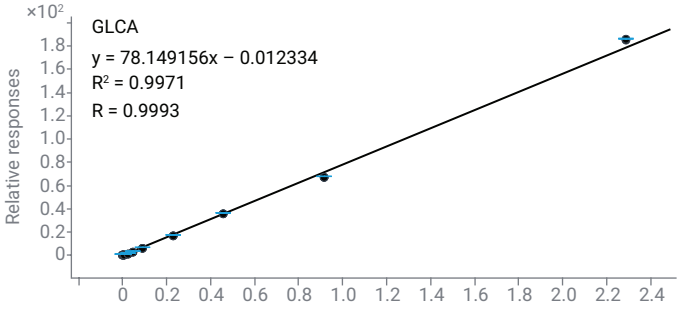

CDCA
y = 116.997912x + 0.013133
R² = 0.9954
R = 0.9990
Relative responses

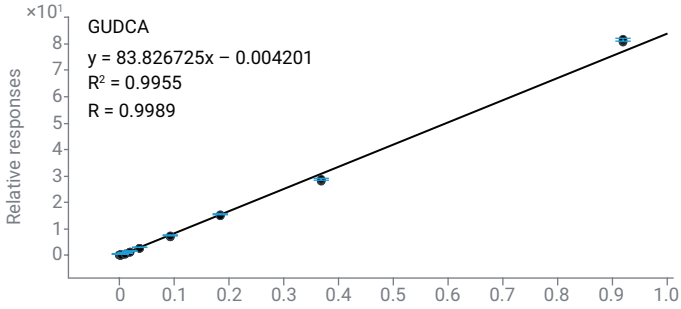

DCA
y = 102.914954x – 0.001855
R² = 0.9939
R = 0.9985
Relative responses

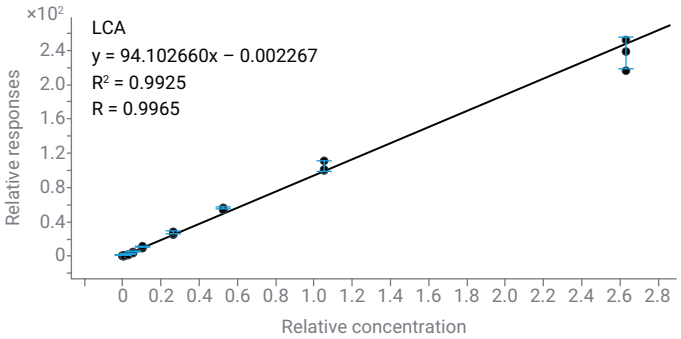

GCA
y = 75.824693x – 0.001961
R² = 0.9931
R = 0.9983
Relative responses

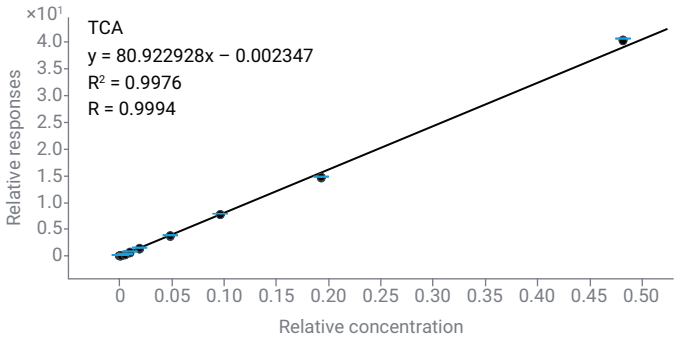

GCDCA
y = 69.534738x – 1.198446E-004
R² = 0.9930
R = 0.9983
Relative responses

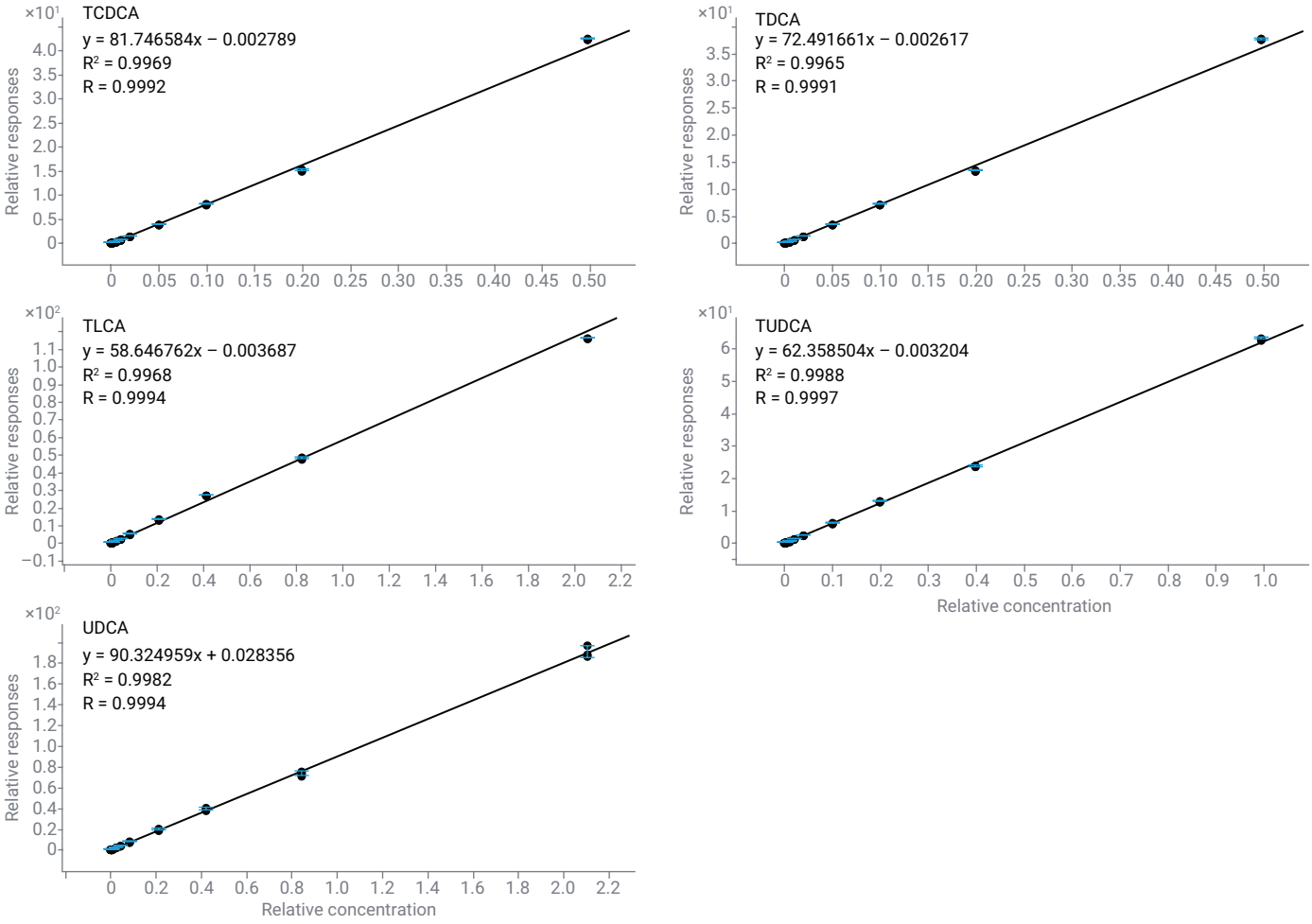

GDCA
y = 88.412687x – 0.002335
R² = 0.9932
R = 0.9982
Relative responses


GLCA
y = 78.149156x – 0.012334
R² = 0.9971
R = 0.9993
Relative responses


GUDCA
y = 83.826725x – 0.004201
R² = 0.9955
R = 0.9989
Relative responses


LCA
y = 94.102660x – 0.002267
R² = 0.9925
R = 0.9965
Relative responses
Relative concentration


TCA
y = 80.922928x – 0.002347
R² = 0.9976
R = 0.9994
Relative responses
Relative concentration



Bile Acid	R^2	Fit	Weight
CA	0.995	linear	1/x
CDCA	0.995	linear	1/x
DCA	0.994	linear	1/x
GCA	0.993	linear	1/x
GCDCA	0.993	linear	1/x
GDCA	0.993	linear	1/x
GLCA	0.997	linear	1/x
GUDCA	0.995	linear	1/x

Bile Acid	R^2	Fit	Weight
LCA	0.993	linear	1/x
TCA	0.998	linear	1/x
TCDCA	0.997	linear	1/x
TDCA	0.997	linear	1/x
TLCA	0.997	linear	1/x
TUDCA	0.999	linear	1/x
UDCA	0.998	linear	1/x

Figure 3. Bile acid calibration curves.

Method robustness and reproducibility

To evaluate the performance and reproducibility of the LC/MS acquisition method, a rigorous stress test was conducted. A pooled plasma sample was prepared by combining equal volumes (50 µL) of plasma from 10 male and 10 female mice (BioIVT). Bile acids were isolated with acidic acetonitrile spiked with the Avanti d-BA Splash mixture following the optimized extraction procedure from plasma described previously. After solvent removal, the sample was reconstituted with 50% methanol, thoroughly mixed, and injected consecutively 250 times onto a 6495D LC/MS system over a total acquisition period of 96 hours (4 days).

This extensive injection series was designed to assess the method's robustness under conditions that simulate long-term, high-throughput analytical workflows. Peak areas corresponding to 15 distinct d-BAs were integrated with MassHunter Quantitative Analysis 12.0 software to evaluate signal response and retention time reproducibility over time. Despite the extended analysis period, the method demonstrated excellent performance. The average coefficients of variation (%CV) for peak area measurements across the 250 injections remained below 5% and the %CV relative to retention time (RT) reproducibility was 0.15% (Figure 4). These results confirmed the method's ability to maintain consistent signal intensity and data quality over prolonged use.

d-Bile acid	Peak area %CV (n = 250)	RT %CV (n = 250)
TUDCA-d4	4.3	0.31
TCA-d4	4.4	0.29
GUDCA-d4	4.7	0.08
GCA-d4	5.4	0.10
TCDCA-d4	4.2	0.27
TDCA-d4	7.7	0.30
CA-d4	6.1	0.11
UDCA-d4	5.8	0.07
GCDCA-d4	3.4	0.08
GDCA-d4	4.0	0.07
TLDCA-d4	4.8	0.33
CDCA-d4	6.1	0.07
DCA-d4	4.1	0.07
GLCA-d4	3.1	0.08
LCA-d4	4.9	0.04
Average	**4.9**	**0.15**

Figure 4. Method reproducibility study after 250 consecutive injections of a pooled plasma sample over a four-day acquisition period.

Sample analysis

To evaluate the applicability of the LC/MS/MS method to biological matrices, bile acid levels were measured in a series of representative plasma and fecal samples. BAs were extracted from aliquots of plasma (100 µL) from 10 distinct male and 10 female mice and rats (BioIVT). Pooled QC samples, prepared by combining extracted mouse and rat plasma, were injected to condition the column to the matrix prior to sample analysis and to monitor instrument response throughout data acquisition. All rodent plasma samples were divided into species-specific blocks and injected in a randomized order within each block. This approach ensured an even distribution of instrument response fluctuations, allowing more accurate comparisons across sample groups.

After LC/MS/MS analysis, raw data were processed using MassHunter Quantitative Analysis 12.0 software. Bile acid signal abundances (peak areas) were used to generate a MassHunter quantitative report, which was used to import the data into MPP for downstream chemometric analysis. Alternatively, integrated peak areas can be exported from the MassHunter Quantitative Analysis 12.0 software as a Microsoft Excel table and loaded into MPP through the generic import option. This flexibility allows the use of MPP for statistical analysis even when raw LC/MS/MS data sets are processed with external software such as Skyline.

BA extracts (1 µL) from fecal material collected from cohorts of human donors, both healthy and with various underlying conditions (Medix Biochemica), were analyzed, processed, and evaluated using MPP. In addition to primary BAs, many relevant secondary BAs were detected in both plasma and fecal samples.

To assess differences across sample groups, the datasets were subjected to multivariate analysis in MPP. Hierarchical clustering analysis (HCA) and principal component analysis (PCA) revealed distinct bile acid compositions in plasma samples. PCA demonstrated proper sample grouping across rodent biological replicates, with a pronounced separation by animal species along principal component 1 (PC1, 49%) and a more modest separation by gender along principal component 2 (PC2, 9%) (Figure 5A). In contrast, no significant variations were observed across fecal sample groups (data not shown).

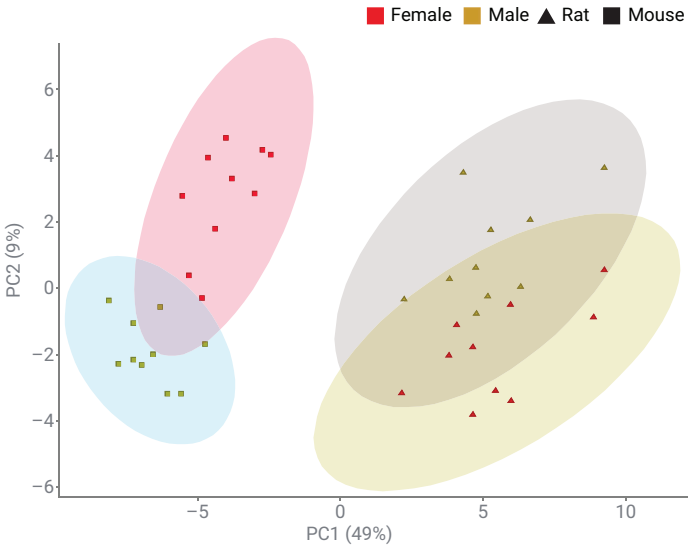


Figure 5A. Principal component analysis (PCA) showed differences in BA distribution across sample groups.

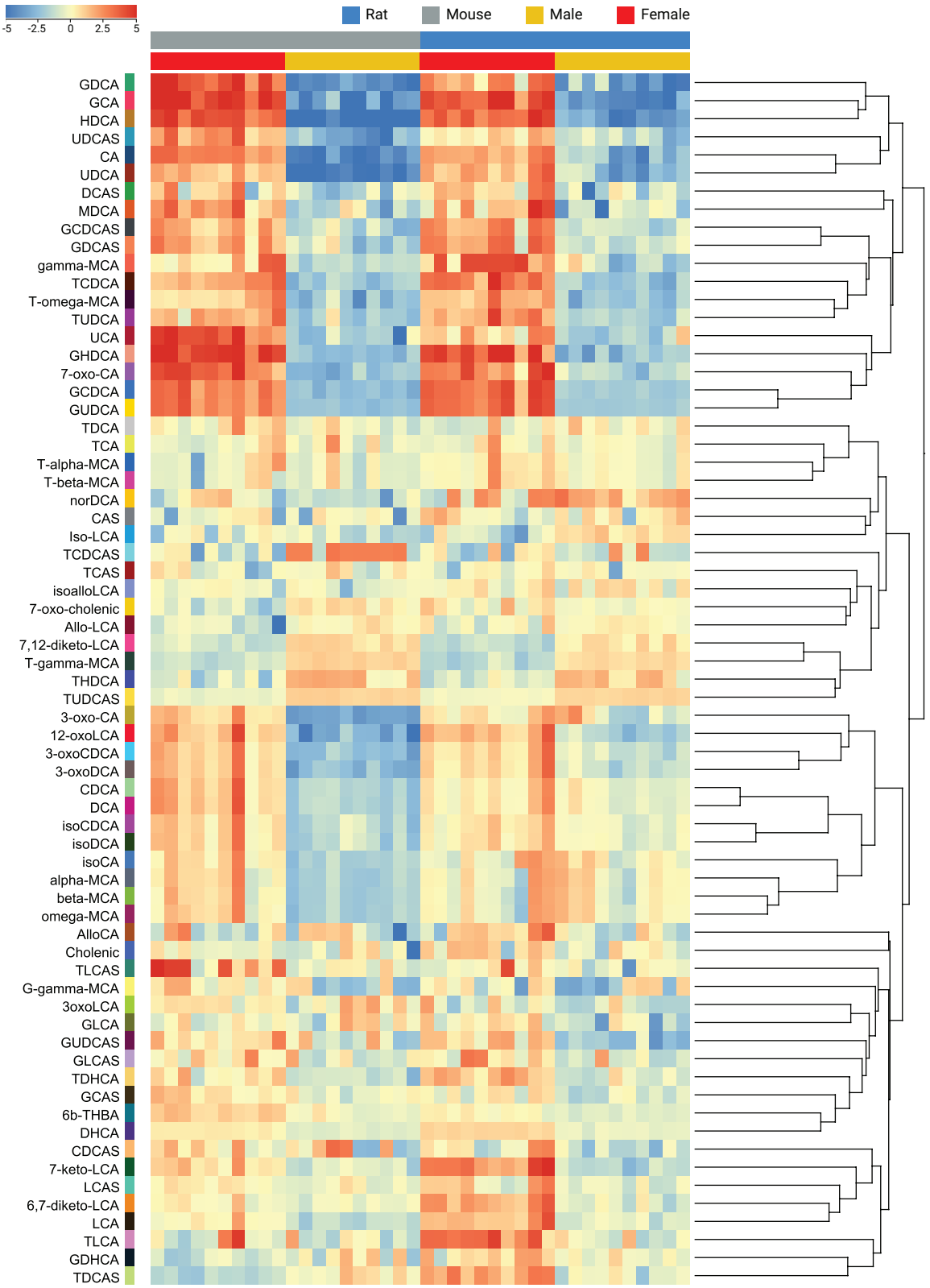


Figure 5B. Hierarchical clustering showed differences in BA distribution across sample groups.

Conclusion

A robust and standardized targeted dynamic-MRM LC/MS/MS method for the analysis of bile acids was developed on an Agilent 6495D triple quadrupole/LC platform. Optimal bile acid extraction conditions from plasma and fecal samples were established along with LC/MS conditions that allowed the measurement of a large panel of bile acids in a single chromatographic run. This analytical method is highly versatile and applicable to both plasma/serum and fecal samples, offering valuable insights into systemic and gut-specific bile acid profiles. In plasma, it provides a reliable means to assess circulating bile acids, which are key indicators of liver function, metabolic health, and disease states. In fecal samples, the method enables detailed profiling of bile acids involved in gut microbiota interactions and intestinal health, shedding light on digestion and enterohepatic circulation dynamics.

References

1. Sartain, M.; *et al.* A Refined LC/MS/MS Method Targeting Bile Acids from the Gut Microbiome, *Agilent Technologies application note*, publication number 5994-4956EN, **2023**.
2. Van de Bittner, G. C.; *et al.* An Automated Dual Metabolite + Lipid Sample Preparation Workflow for Mammalian Cell Samples, *Agilent Technologies technical overview*, publication number 5994 5065EN, **2022**.
3. Sartain, M.; *et al.* Enabling Automated, Low-Volume Plasma Metabolite Extraction with the Agilent Bravo Platform, *Agilent Technologies application note*, publication number 5994-2156EN, **2020**.
4. Highly Curated Workflows for Targeted Omics Using a Standardized LC/TQ Platform, *Agilent Technologies flyer*, publication number 5994-7447EN, **2024**.

www.agilent.com

For Research Use Only. Not for use in diagnostic procedures.

RA250421.245

This information is subject to change without notice.

Printed in the USA, July 8, 2025
5994-8263EN

Agilent
Trusted Answers