

Poster Reprint

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

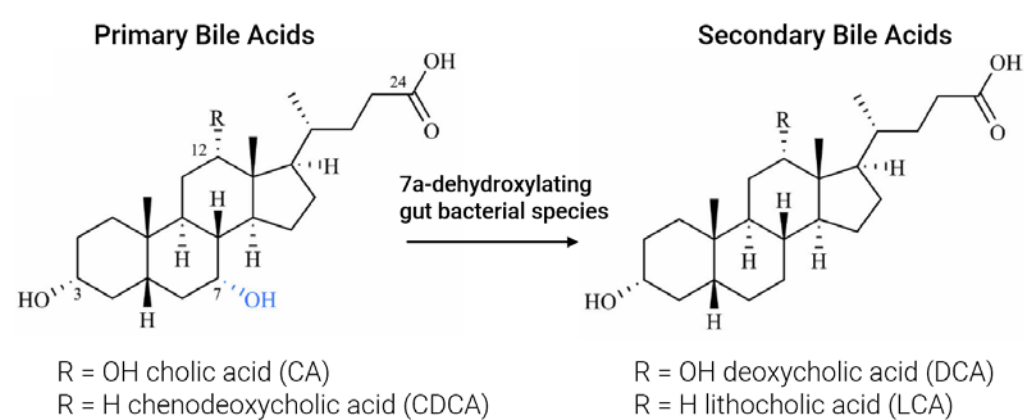
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Introduction

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.



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. 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, enhancing 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 Sample Preparation

Twenty-six authentic chemical standards were purchased from commercial vendors. To establish lower limits of quantification (LLOQs), dilutions of analytes were prepared in neat solvent. Fecal pellets and cecal contents from gnotobiotic and conventionally-raised mice were homogenized and extracted in the presence of methanol, and particulate was removed after addition of an equal volume of water. NIST SRM 1950 plasma was protein-precipitated with methanol.

Experimental

Method Design

MassHunter Optimizer software was used to optimize MRM transitions for 26 bile acids. Major UHPLC and MS parameters are as follows:

Agilent UHPLC 1290 Infinity II System	
Column	Agilent Poroshell EC-C18, 2.1x150mm, 2.7 μ m
Guard column	Agilent Poroshell EC-C18, 2.1x5mm, 2.7 μ m
Column temperature	45 $^{\circ}$ C
Injection volume	2-5 μ L
Mobile phase	A = 0.1% Formic Acid + 20mM Ammonium Acetate in Water B = 0.1% Formic Acid in Acetone
Analytical gradient	0.20 ml/min, 32% B hold for 6 min, 0.4 ml/min to 65% B at 25 min, to 98% B at 25.1 min, followed by further steps for column cleaning and equilibration. Total time = 32.1 min

Agilent 6470 Triple Quadrupole Mass Spectrometer	
Ion source	Agilent Jet Stream ESI
Polarity	Positive and Negative Switching
Drying gas (nitrogen), Temp	12 L/min, 200 $^{\circ}$ C
Sheath gas (nitrogen), Temp	10 L/min, 200 $^{\circ}$ C
Nebulizer gas (nitrogen)	40 psi
Capillary voltage	4500 V (+), 3000 V (-)
Nozzle voltage	2000 V (+), 0 V (-)
Scan type	Dynamic MRM (dMRM)
Cycle time	750 ms
Total number of MRMs	110
Min/max dwell time	17 / 748 ms

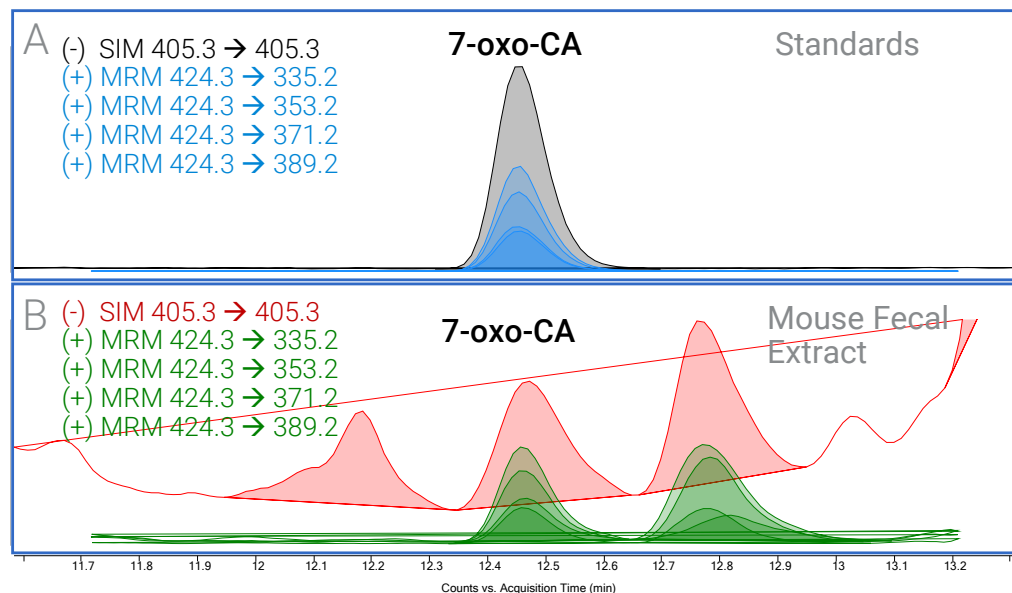


The Agilent 1290 Infinity II UHPLC System with the Agilent 6470 Triple Quadrupole Mass Spectrometer



Compound MS/MS Optimization

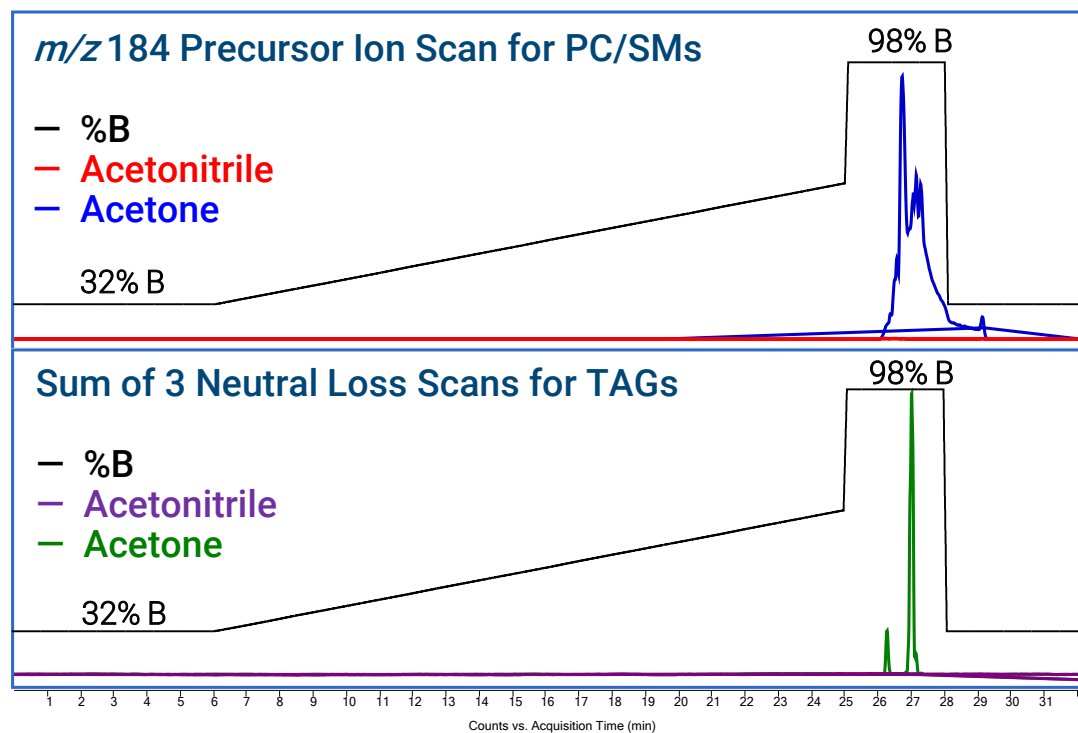
Similar to Wegner et al.², we found that, for many bile acids, MRM-based scans of $(M+NH_4)^+$ precursors were superior to SIM-based scans of $(M-H)^-$ ions. Although detection limits were comparable in neat solvent, the additional analytical selectivity of MRM helped to reduce interference from matrix in biological samples.



Comparison of 7-oxo-CA MRM vs SIM scans for 100 ppb standard in neat solvent (A) and endogenous 7-oxo-CA in mouse fecal extract (B). The same LC/MS method was used for both samples.

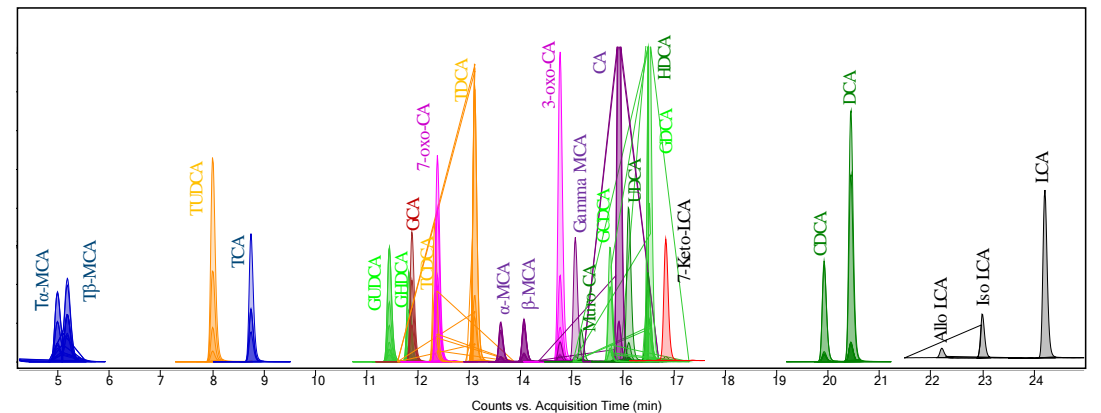
Chromatography

Multiple stationary phases and mobile phase compositions were evaluated. Acetone was found to possess superior lipid elutropic properties in comparison to acetonitrile, fully eluting problematic phospholipids and triacylglycerols (TAGs) at the end of each analytical run.



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

The water-acetone based mobile phase maintained excellent separation of bile acid isomers. The gradient was optimized to achieve baseline separation of 24 of the 26 compounds and partial separation of early-eluting Tauro α - and β -MCA.

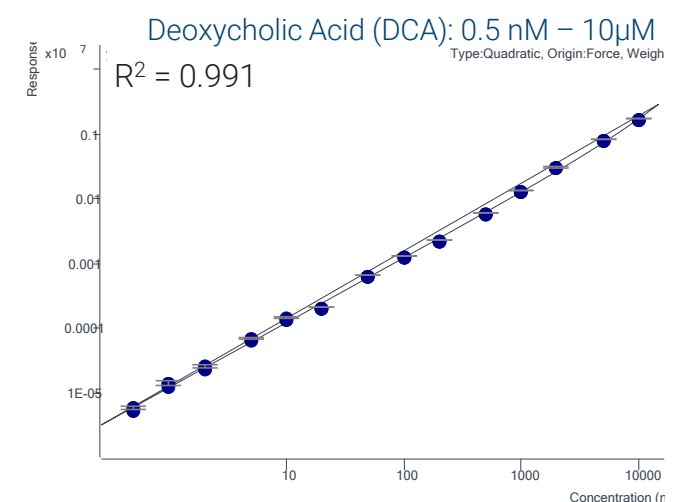
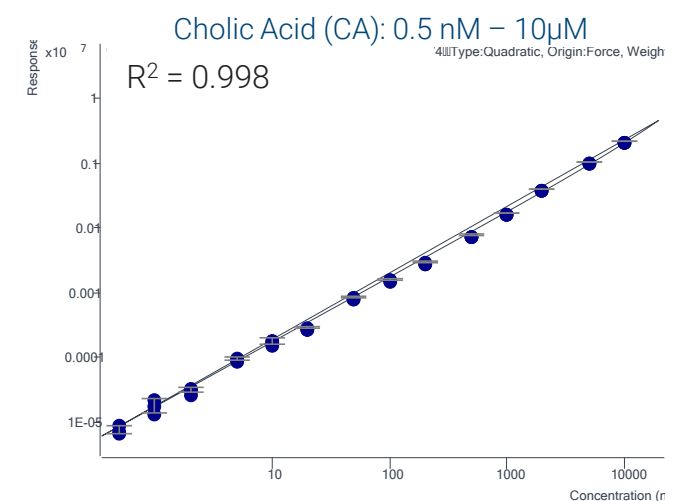


Overlaid MRM and SIM chromatograms from a mixture of bile acid standards acquired with the final dMRM LC/MS method. Bile acids sharing the same precursor m/z are indicated by color.

Quantitation

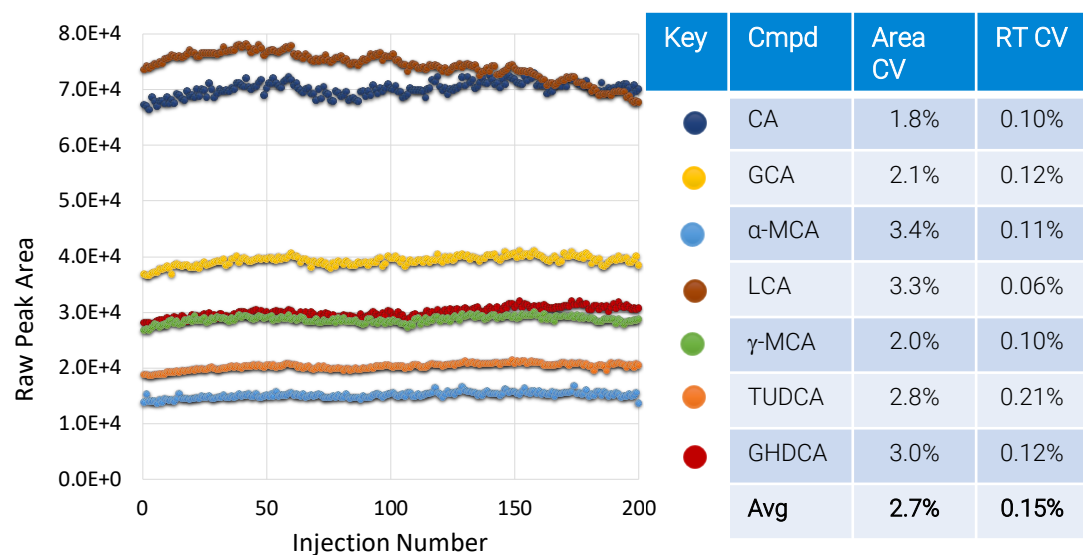
Dilutions of selected standards were prepared in neat solvent and evaluated at 16 standard concentrations ranging from the LOD at 100 pM to 10 μ M. Correlation coefficients (R^2) for calibration curves were higher than 0.99 over up to 4.3 orders of dynamic range.

Compound	LLOQ (nM)
T-Alpha-MCA	0.5
T-Beta-MCA	0.5
TCA	0.5
GUDCA	0.5
GHDA	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



Robustness

To assess robustness, 200 injections of a mouse cecal extract spiked with standards were analyzed over a >4-day period without interruption. The unique LC/MS solvent system enabled excellent retention time reproducibility even with minimal sample preparation.



MRM response over time for 7 representative bile acids. The table provides the corresponding MRM peak area and RT CVs.

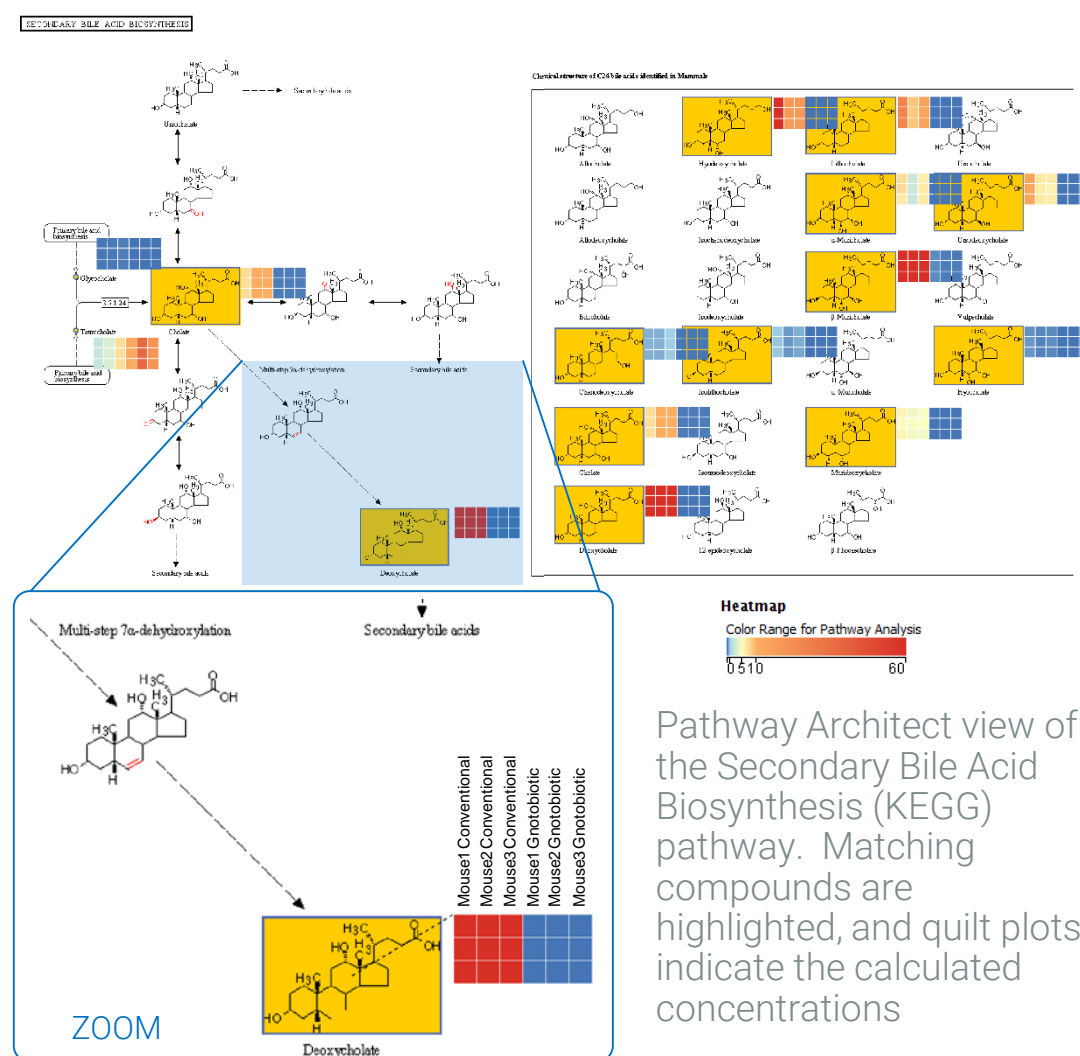
Application: Bacterial Metabolism in the Gut

The method was applied to fecal extracts of conventionally-raised and gnotobiotic mice. Shown below are the calculated final concentrations (nmol/g) based on an external calibration curve of standards prepared in solvent. As expected, the results indicate a significant reduction of secondary bile acid metabolism in gnotobiotic mice.

	Conventional			Gnotobiotic			
	1	2	3	4	5	6	
CA	7.022	11.277	10.448	0.072	0.084	0.086	Primary Bile Acids
CDCA	0.608	0.600	0.897	0.029	0.028	0.026	
Alpha-MCA	6.908	2.511	6.199	0.014	ND	ND	
Beta-MCA	84.342	80.800	95.584	0.229	0.090	0.073	
GCA	0.036	0.055	0.096	ND	0.008	ND	
GCDCA	ND	ND	ND	ND	ND	ND	
T-Alpha-MCA	0.354	0.528	1.320	2.235	2.663	1.841	
T-Beta-MCA	7.067	13.795	19.128	71.817	139.076	81.539	
TCA	2.363	3.258	7.071	12.064	37.400	20.216	
TCDCA	0.305	0.293	0.710	1.178	1.094	0.646	
DCA	262.661	97.598	137.053	0.004	0.004	0.006	Secondary Bile Acids
LCA	26.301	8.470	14.261	ND	ND	ND	
3-oxo-CA	0.278	0.316	0.357	0.001	0.002	0.002	
7-ketoLCA	ND	ND	ND	ND	ND	ND	
7-oxo-CA	0.234	0.442	0.408	0.004	0.006	0.006	
UDCA	14.111	6.341	6.066	ND	ND	ND	
IsoLCA	0.841	0.348	0.553	ND	ND	ND	
Gamma-MCA	0.328	0.164	0.234	ND	ND	ND	
HDCA	63.735	19.007	18.582	ND	ND	ND	
Muro-CA	5.442	4.507	4.685	ND	ND	ND	
GUDCA	ND	ND	ND	ND	ND	ND	
GDCA	0.021	0.004	0.007	ND	ND	ND	
GHDCA	ND	ND	ND	ND	ND	ND	
TDCA	0.591	0.371	1.215	0.016	0.014	0.007	
TUDCA	1.219	2.025	4.007	11.603	14.521	6.792	
AlloLCA	0.422	0.132	0.221	ND	ND	ND	

Pathway Mapping

Agilent's 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 pathway for secondary bile acid metabolism.



Conclusions

A targeted LC-MS/MS method for bile acid profiling and quantitation has been newly developed that provides:

- Enhanced selectivity in biological matrices
- Increased robustness due to unique LC mobile phases
- Minimal sample preparation requirements
- Excellent analytical sensitivity

References

1. Devlin, A.S., Fischbach, M.A. Nature Chem Biol. 2015 Sep;11(9):685-90
2. Wegner, K. et al. Anal Bioanal Chem. 2017 Feb;409(5):1231-1245

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