Tools to Understand the Gut Microbiome: A Refined LC-MS/MS Method to Profile Bacteria-derived Bile Acids

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Introduction

Secondary bile acids represent the most abundant and variable microbiota-derived metabolites and have been 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 genes 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. 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.

Method Design

MassHunter Optimizer software was used to optimize MRM transitions for 26 bile acids. Analysis was carried out with positive and negative electrospray ionization in dynamic MRM (dMRM) in a single analytical run. Major UHPLC and MS parameters are as follows:

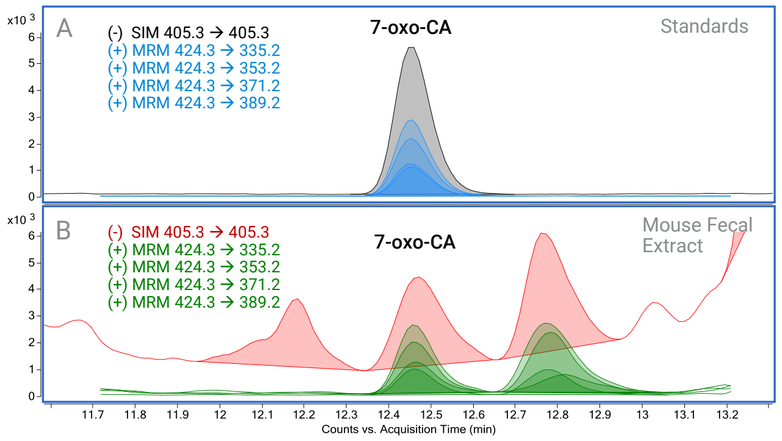
Agilent UHPLC 1290 Infinity II System	
Column	Agilent Poroshell EC-C18, 2.1x150mm, 2.7 µm
Guard column	Agilent Poroshell EC-C18, 2.1x5mm, 2.7 µm
Column temperature	45 ºC
Injection volume	2-5 µL
Mobile phase	A = 0.1% Formic Acid + 20mM Ammonium Acetate in Water B = 0.1% Formic Acid in Acetone
Analytical 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 ºC
Sheath gas (nitrogen), Temp	10 L/min, 200 ºC
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

Experimental

Compound MS/MS Optimization

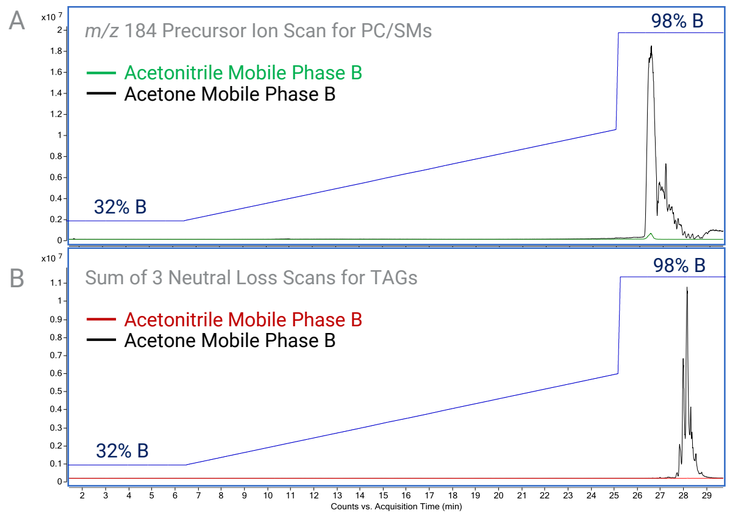
Similar to Wegner et al.[2], we found that for many bile acids MRM-based scans of $(M+NH_4)^+$ precursors were superior to SIM-based analyses of $(M-H)^-$ ions. Although detection limits were comparable in neat solvent, the additional analytical selectivity of MRM helped to reduce interference from matrix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

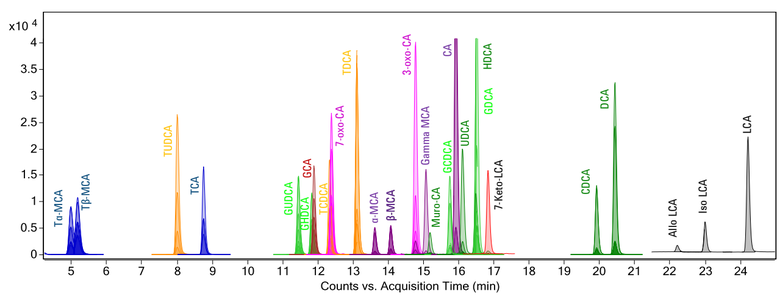
Chromatography

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



Comparison of PC/SM Elution (A) and TAG elution (B) from a liver total lipid extract with acetonitrile or acetone as 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.

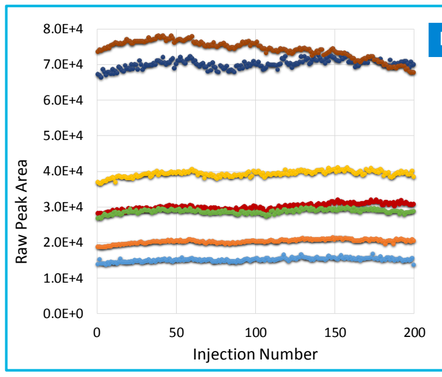


Overlaid MRM and SIM chromatograms from a mixture of bile acid standards acquired with the final dMRM LC/MS method. Bile acids sharing the same precursor *m/z* are indicated by color.

Results and Discussion

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.

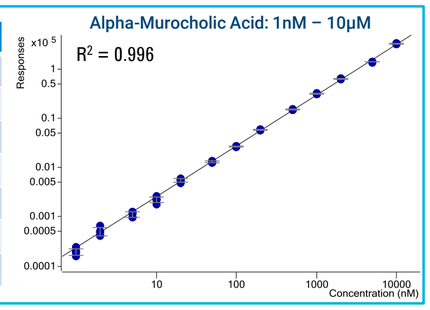


Key	Compound	Area CV	RT CV
	CA	1.8%	0.10%
	GCA	2.1%	0.12%
	α-MCA	3.4%	0.11%
	LCA	3.3%	0.06%
	γ-MCA	2.0%	0.10%
	TUDCA	2.8%	0.21%
	GHDCA	3.0%	0.12%
	Average	2.7%	0.15%

Quantitation

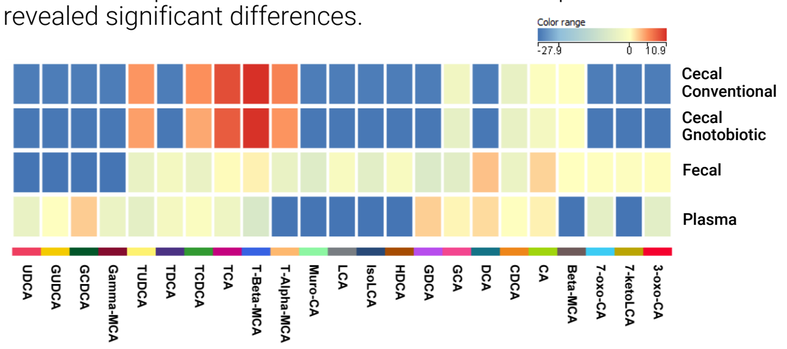
Dilutions of selected standards were prepared in neat solvent and evaluated at 16 standard concentrations ranging from the LOD at 100 pM to the upper limit of quantitation at 10 µM. Correlation coefficients (R2) for calibration curves were higher than 0.99 over up to 4.0 orders of dynamic range.

Compound	LLOD (nM)	LLOQ (nM)
CA	0.2	1
GCA	0.5	1
α-MCA	0.5	1
LCA	2	5
γ-MCA	5	5
GHDCA	0.5	1
3-oxo-CA	0.1	0.2



Profiling

Agilent Mass Profiler Professional software was used to compare bile acid compositions from mouse extracts and plasma and revealed significant differences.



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