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An Improved Reverse Phase LC-MS/MS Method for the Measurement of Bile Acids in Biological Samples

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Introduction

Bile acids are a class of steroidal compounds derived from cholesterol catabolism in the liver.1 These molecules play important roles in the absorption and digestion of lipids, maintaining lipids, glucose, and energy homeostasis, and working as activators of cell signaling pathways associated with their biosynthesis.2 In the intestine, the main secreted bile acids produced in the liver (primary bile acids) undergo additional modifications by gut microbes that results in a bouquet of isomeric and isobaric species (secondary bile acids). Many of these secondary bile acid species are difficult to be accurately measured by LC-MS/MS because of lack of chromatographic baseline separation, poor fragmentation, and low detection limit due to their intrinsic low abundance in biological matrixes. Herein we report a UPLC -MS/MS method for the analysis of bile acids that partially overcomes these limitations

Experimental

LC/MS Method Development and Sample Analysis

A total of 13 bile acid standards were individually dissolved in methanol and sequentially injected in an Agilent 6495C triple quadrupole mass spectrometer (Figure 1) by flow injection to enable automated optimization of MRM compound transitions, AJS source, and iFunnel acquisition parameters with MassHunter Optimizer. Single bile acid standards were then pooled together, and the resulting mixture was utilized to develop a UHPLC method (Table 2) on a high-resolution C-18 column. Details of the MS and LC methods are shown in Table 1 and 2, respectively.

Figure 1. Agilent 6495C Triple Quadrupole Mass Spectrometer with 3rd Generation Ion Funnel Technology coupled to an Agilent Infinity II UHPLC

Experimental

Table 1. 6495C QQQ MS conditions

MS Conditions	
Instrument	Agilent 6495C LC/TQ with iFunnel Technology
Ionization Source	Agilent Jet Stream ESI
Polarity	Negative
Drying Gas	240 °C; 13 L/min
Nebulizer	30 psi
Sheath Gas	400 °C; 10 L/min
Capillary	4000 V
Nozzle	1000 V
iFunnel Parameters	High Pressure Funnel RF 90 V Low Pressure Funnel RF 60 V
Scan Type	Dynamic MRM (dMRM)

Table 2. 1290 Infinity II LC conditions

LC Conditions	
Column	Agilent Zorbax RRHD Eclipse Plus C18, 100 x 2.1 mm,1.8 µm (p/n 959758-902)
Column Temperature	50 °C
Mobile Phase	A: water + 0.1% formic acid B: acetonitrile +0.1% formic acid
Flow Rate	500 µL/min
Gradient	Time (min) %A %B 0 75 25 1 75 25 16 40 60 17 40 60 17.30 2 98 20.30 2 98 21.30 75 25 23.00 75 25 Post Time: 1.0 min
Injection Volume	2-5 µL
Multisampler Temperature	4 °C

Optimized LC-MS/MS Method

The bile acid mixture was chromatographically resolved with excellent baseline peak separation (Figure 2). We attempted to optimize the MS acquisition parameters in both positive and negative ion mode and found that negative ion mode (-ESI) favored better ionization thus more intense MRM transitions Three bile acids in the panel, UDCA, CDCA, and LCA did not produce any measurable MRM transition in both polarities. These compounds were measured in SIM mode in the final method. (Figure 3).

The retention time (RT) of each bile acid in the panel was acquired by single standard analysis. The RTs were included in the acquisition method to build the final dynamic MRM (dMRM) method.

We also analyzed the bile acid mixture on an Agilent 6470 QQQ and found that the 6495C with improved iFunnel technology resulted about 3-5 time more sensitive (Figure 4).

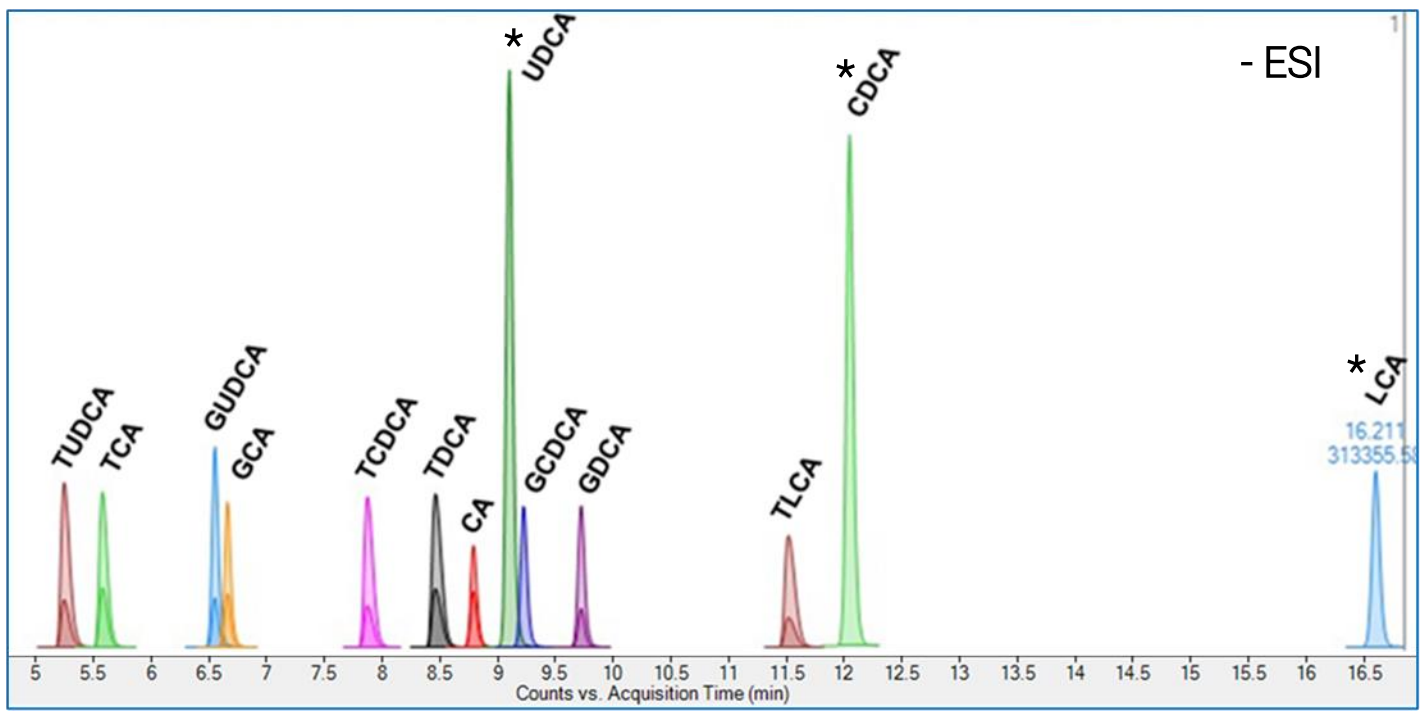

Figure 2. Final LC-MS/MS (-ESI) acquisition method depicting the MRM and SIM chromatograms from the bile acid standard mixture. Asterisks denote bile acids measured in SIM mode.

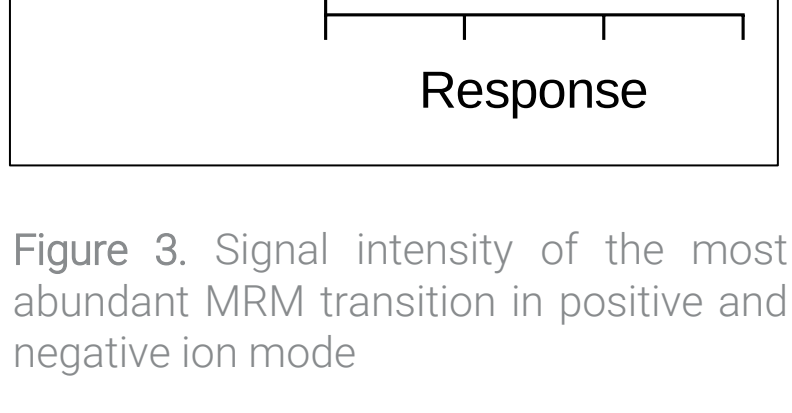

Figure 3. Signal intensity of the most abundant MRM transition in positive and negative ion mode

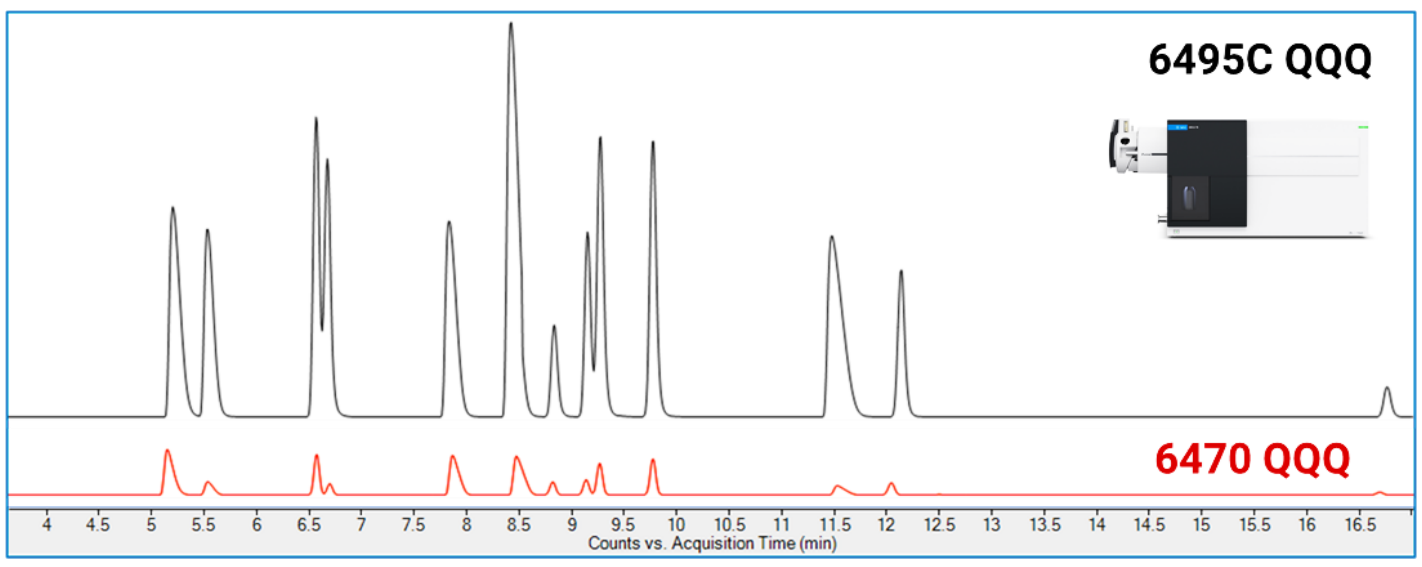

Figure 4. TIC comparison between 6495C and 6470 QQQ models.

Calibration Curve for Quantitation

Calibration standards were prepared from each bile acid stock solution by serial dilution with neat solvent. The concentration of the standards ranged from 1.0 ng/mL to 10 µg/mL. As shown in Table VV, the correlation coefficient (R^2) for most of the bile acid in the mixture was 0.99 over the specified concentration range (Table 3). LLOQ values are also reported in Table 3.

Results and Discussion

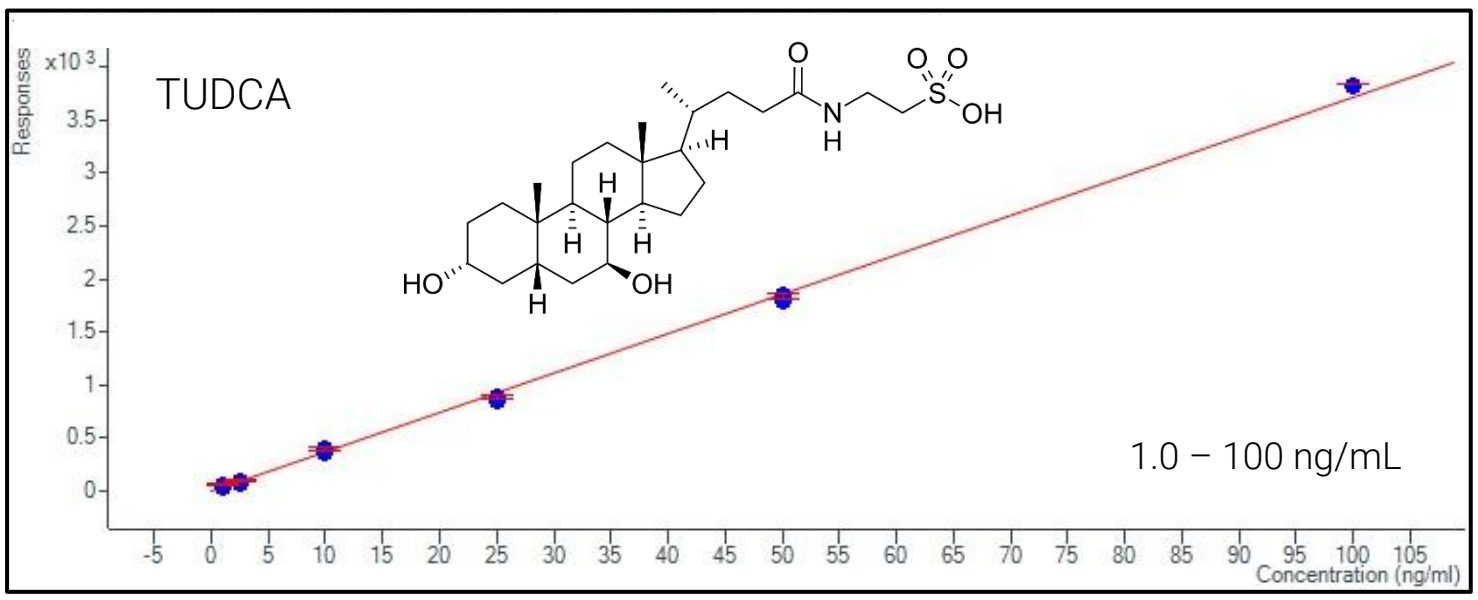

Figure 4. TUDCA calibration curve representative of the bile acids mixture

Bile Acid	R^2	LLOQ (pmol/mL)
TCDCA	0.999	0.5
TUDCA	0.999	0.6
TDCA	0.999	0.6
TCA	0.999	0.6
GUDCA	0.996	0.6
CA	0.995	0.6
TLCA	0.987	1.5
GCA	0.993	1.5
GDCA	0.992	1.6
GCDCA	0.993	1.6
UDCA	0.995	18.2
CDCA	0.985	18.2
LCA	0.958	30.6

Table 4. LLOQ and correlation coefficient relative to the bile acids in the mixture

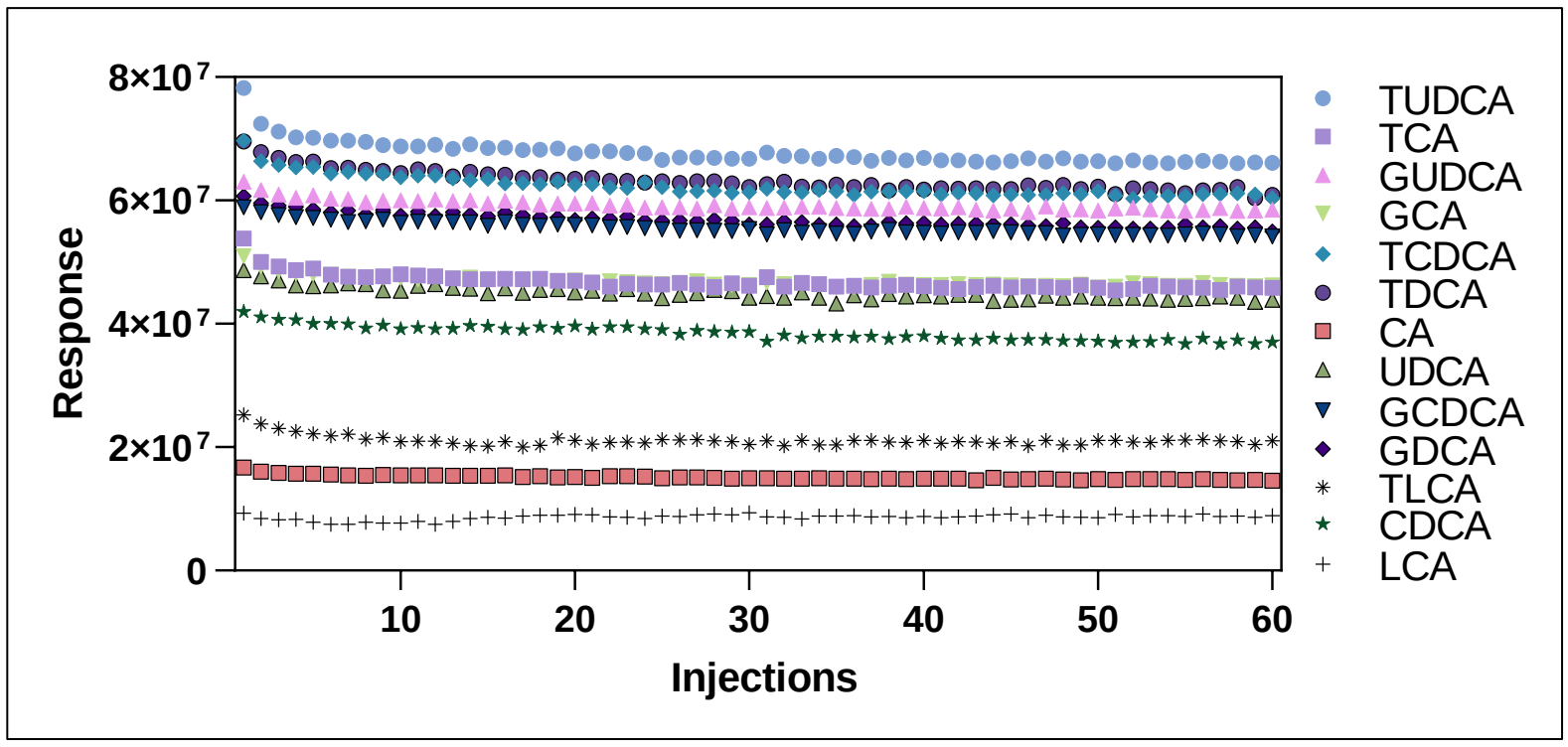

Figure 4. Bile acid instrument response across consecutive injections and corresponding RSD (%)

Bile Acid	RSD (%)
TUDCA	2.9
TCA	2.9
GUDCA	1.5
GCA	1.9
TCDCA	2.8
TDCA	2.8
CA	2.6
UDCA	2.3
GCDCA	1.9
GDCA	2.0
TLCA	4.6
CDCA	3.2
LCA	5.3

Conclusions

The implementation of the Agilent 6495C triple quadrupole mass spectrometer in the development of this analytical method allowed a much higher sensitivity towards bile acid detection compared to previous models. Overall, the method presented good linearity over a wide range of concentrations, and the use of the dynamic multiple reaction monitoring (dMRM) ensured the acquisition of higher quality data. Although this method requires a more rigorous validation in complex matrices, it should greatly simplify bile acid quantification and thereby facilitate research and discovery of novel biomarkers in biological samples.

References

[1]John Y.L. Chiang, J Lipid Res, 2009 Oct; 50(10): 1955-1966

[2]Tiara R. Ahmad & Rebecca A. Haeusler, Nature Reviews Endocrinology 2019, 15: 701-712

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