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A semi-automated workflow for targeted LC/MS analysis of circulating bile acids in plasma samples

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Introduction

Bile acids play important roles in physiology where they are essential for lipid absorption in the small intestine, in addition to regulating and maintaining energy homeostasis. Abnormal levels of circulating bile acids in plasma are associated with liver disfunctions and other metabolic disorders. Bile acids can be efficiently analyzed by mass spectrometry, and various LC/MS methods have been reported over the past few years. In the presence of an underlying conditions, levels of bile acids can fall below instrument detection limits making analysis challenging. In this study, we present a semi-automated workflow for the analysis of bile acids in plasma samples where a novel triple quadrupole mass spectrometer with exceptionally high sensitivity was implemented. The Agilent 6495 triple quadrupole LC/MS system features next generation iFunnel technology with improved ion optics (Figure 1). The instrument is operated by MassHunter 12.0 Acquisition software employing AI autotune, particle swarm optimization, intelligent reflex and optimizer, which overall allow for easier and faster LC-MS/MS method development and implementation.

Figure 1. Agilent 6495 LC/TQ with 4th generation iFunnel technologies.

Experimental

LC-MS/MS Method Development

A panel of 20 biologically relevant bile acids and 15 deuterated-labelled internal standards were acquired from Avanti Polar Lipids Inc. (Birmingham, AL). Methanol stock solutions of individual bile acids were utilized to develop a dynamic-MRM (dMRM) LC-MS/MS method. Analytes were introduced to the 6495 LC/TQ with an Agilent 1290 Infinity II with standard configuration for omics workflows and consisting of a temperature controlled multisampler, multi-column thermostat, and high-pressure binary pump.

Experimental

The built-in MassHunter compound Optimizer and Source Optimization tools were used to determine optimal MS parameters and MRM transitions, respectively. The bile acid mixture was separated by reverse-phase chromatography on an Agilent Poroshell C-18 column with a 13-minute method (Table 1).

Table 1. LC-MS/MS Conditions

LC Conditions	
Column	Agilent Poroshell 120 EC-C18, 2.1 x 50 mm; 1.9 µm (p/n 699675-902)
Column Temperature	50 °C
Injection Volume	2 µL
Autosampler Temperature	4 °C
Needle Wash	Standard Wash, 10 sec, acetonitrile/water (75:25)
Mobile Phase	A: water + 0.1 % formic acid B: acetonitrile + 0.1 % formic acid
Flow Rate	0.50 mL/min
Gradient	Time %B 0.00 25 0.50 25 1.00 25 5.00 36 6.50 37 7.00 45 9.00 47 10.00 98 11.60 98 11.70 25 13.00 25

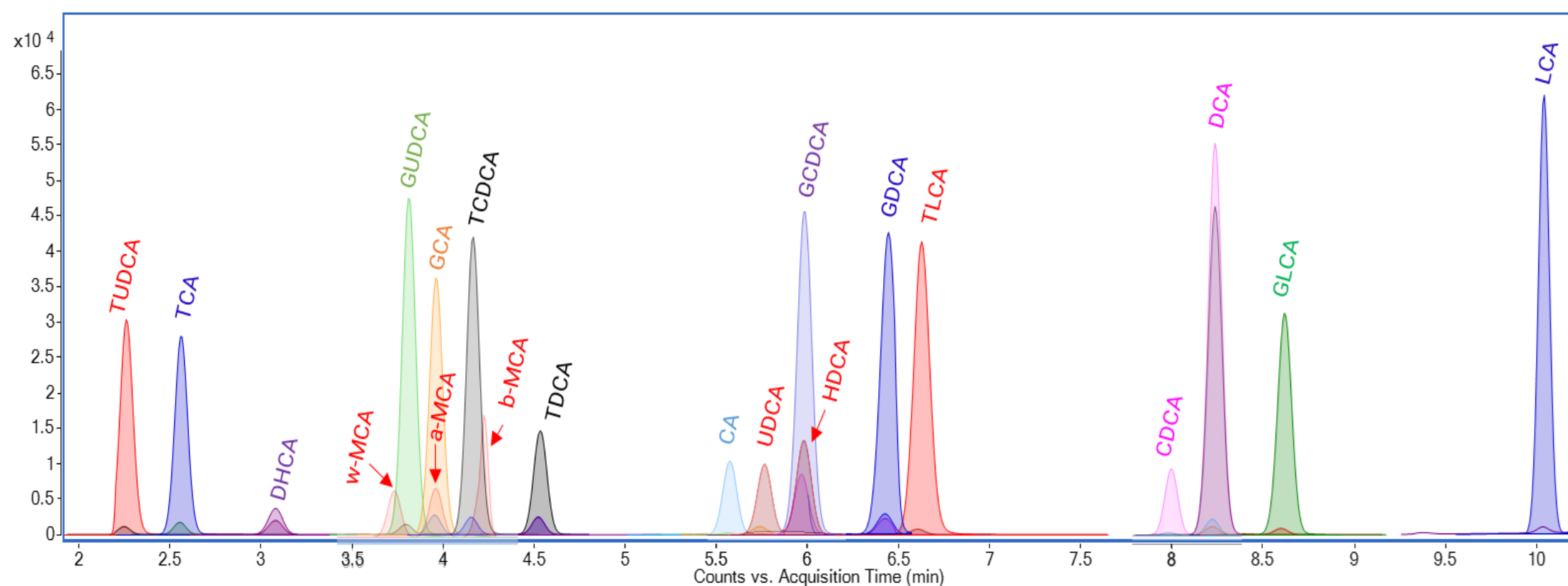| MS Conditions	
Source	Agilent Jet Stream Dual ESI
Sheath Gas Temp, Flow	250 °C; 11 L/min
Gas Temp, Flow	180 °C; 20 L/min
Nebulizer	25 psi
Capillary	5000 V
Nozzle	1000 V
MS Mode	Negative (-ESI)
Acquisition	Dynamic-MRM
iFunnel Setting	Standard

Chromatographic Method Overview and Instrument Response

Figure 2. Chromatographic separation of the 20 bile acids by reverse phase chromatography on an Agilent Poroshell EC-C18 column.
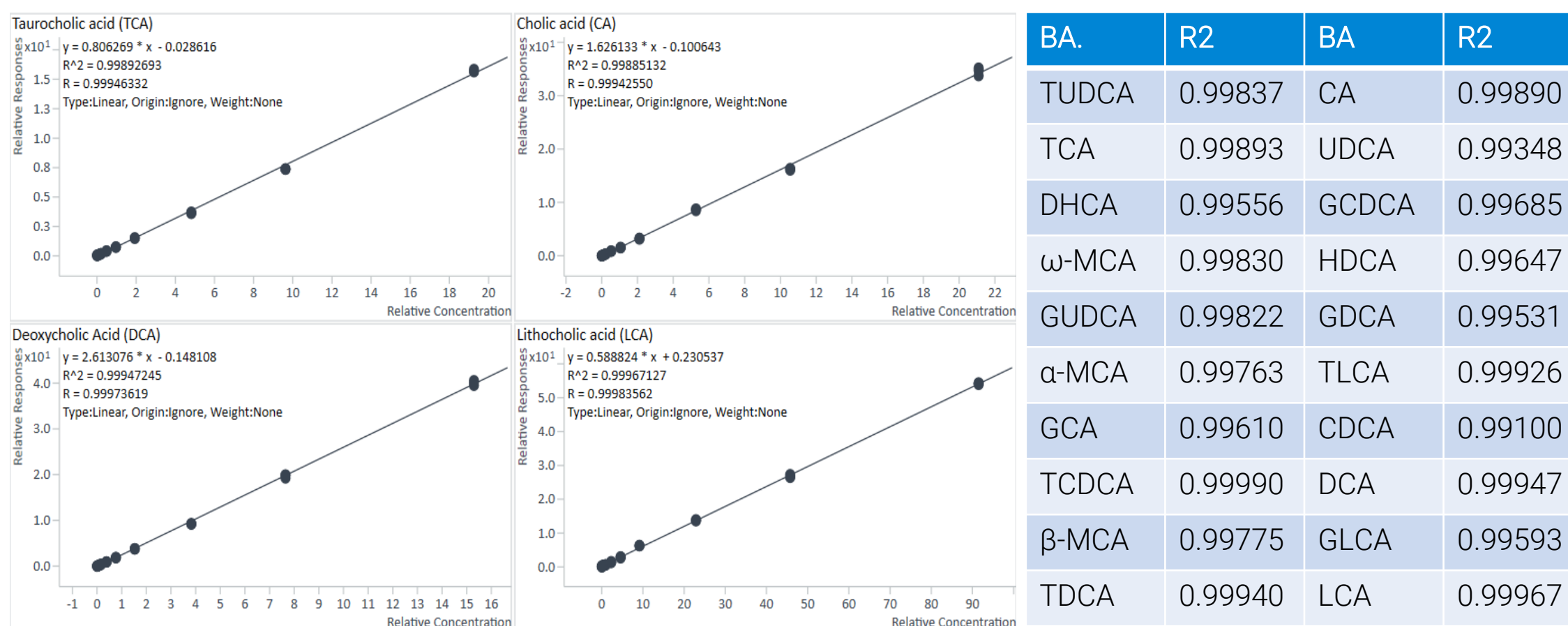

BA.	R2	BA	R2
TUDCA	0.99837	CA	0.99890
TCA	0.99893	UDCA	0.99348
DHCA	0.99556	GCDCA	0.99685
ω-MCA	0.99830	HDCA	0.99647
GUDCA	0.99822	GDCA	0.99531
α-MCA	0.99763	TLCA	0.99926
GCA	0.99610	CDCA	0.99100
TCDCA	0.99990	DCA	0.99947
β-MCA	0.99775	GLCA	0.99593
TDCA	0.99940	LCA	0.99967

Figure 3. Calibration curves for all bile acids ranged between 0.1 – 1000 ng/mL. A linear response with an R^2 > 0.99 was observed. MassHunter Quantitative Analysis 12.0 generates calibration-at-a-glace figures allowing for quick processing of all calibration curves and customizable visualization to fit any view and subset of compounds. %RSDs were <10% across 50 replicate injections of the ISTD mixture.

Plasma Sample Preparation for LC-MS/MS Analysis

Bile acids were extracted from plasma with methanol containing the heavy-labelled internal standards. After incubation for 30 min at room temperature, samples were centrifuged (10 min, 13,000 rpm) to remove the proteins. Supernatants were transferred to clean tubes and dried out by centrifugation under reduced pressure. Dry samples were reconstituted with 100 µL (MeOH:H_2O, 1:1) and transferred to glass analytical vials for LC-MS/MS analysis.

Alternatively, extraction of bile acids and LC/MS sample preparation can be efficiently achieved with the Agilent Bravo Metabolomics liquid handler following a procedure similar to that described in the Agilent application note 5994-2156EN[1,2].
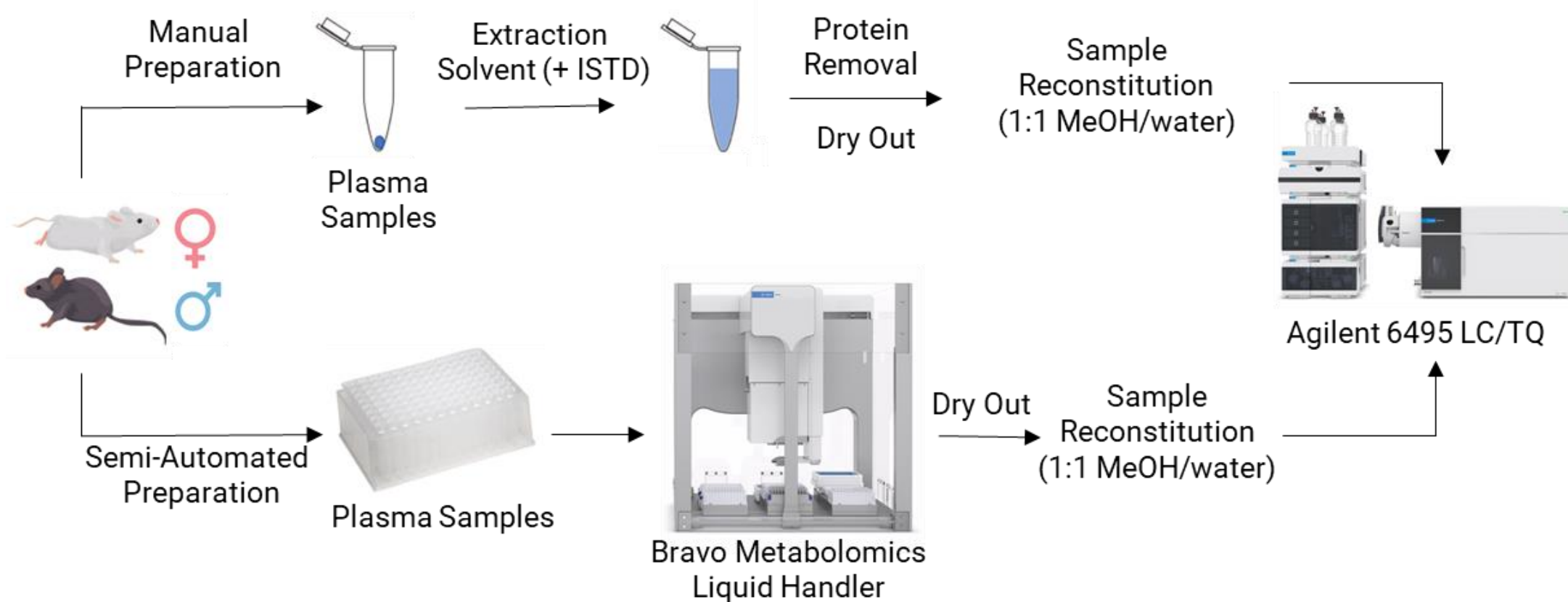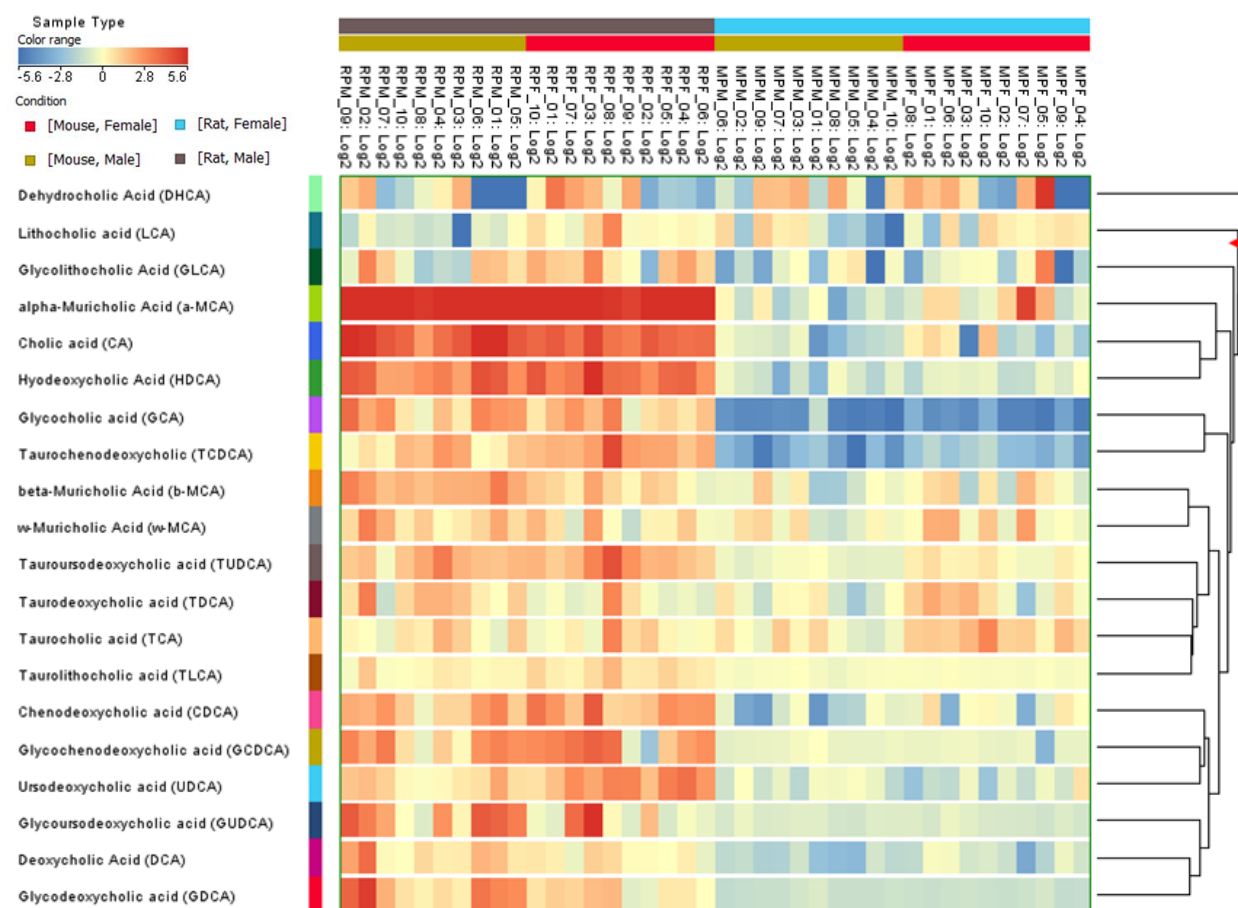
Results and Discussion

Figure 4. Schematic representation of the manual and semi-automated LC/MS sample preparation workflows.

To test its applicability to biological matrices, the final LC-MS/MS method was implemented to measure levels of circulating bile acids in plasma samples from male and female, mice and rats (BIOIVT Elevated Science, Westbury, NY). Absolute concentrations of bile acids in the samples were calculated with MassHunter Quantitative Analysis 12.0 and processed in Mass Profiler Professional (MPP) for chemometric analysis.
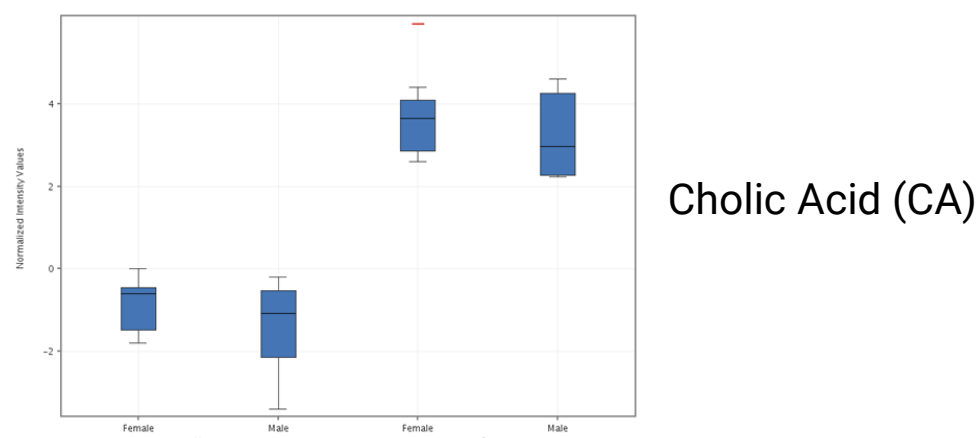
Cholic Acid (CA)

Figure 5. Hierarchical clustering analysis in MPP. Levels of CA, α-MCA and HDCA appeared to be elevated in rat plasma samples. All bile acids were detected at fg levels across sample groups.

Conclusions

- Novel triple quadrupole LC/MS with ion optic improvements for high sensitivity.
- Easy LC-MS/MS reverse-phase method development and smart data acquisition.
- Semi-automated workflow, feasible for high-throughput applications.

References

[1] Van de Bittner, GC et al. An Automated Dual Metabolite + Lipid Sample Preparation Workflow for Mammalian Cell Samples. Agilent Technical Overview 5994-5065EN. 2022.

[2] Sartain, M et al. Enabling Automated, Low-Volume Plasma Metabolite Extraction with the Agilent Bravo Platform. Agilent Application Note 5994-2156EN. 2020.

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