Application News

Liquid Chromatography Mass Spectrometry

Fast Profiling of 39 Bile Acids in Plasma, Urine and Feces, by Automated Extraction and LCMS-8060NX.

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User Benefits
◆ Ready to use method for the quantification of a high number of Bile Acids in a short time,
◆ Automated sample preparation protocol for plasma, urine and feces.

■ Background

Bile acids (BA) play an important role in the absorption of fat in the small intestine and are involved in the regulation of cholesterol metabolism through the conversion of cholesterol to BA. Primary bile acids are produced in the liver by cholesterol catabolism, most of which combine with taurine or glycine to form conjugated bile acids. Some primary bile acids are later modified by intestinal bacteria to produce secondary bile acids.

Total bile acid concentration in human peripheral blood is known as a marker of liver dysfunction, and is widely measured together with blood enzyme activity (ALT, AST, etc.). On the other hand, profiling of many types of bile acids is attracting attention because it may be possible to distinguish various liver disorders by measuring multiple bile acids individually.

However, it is difficult to measure fragment ions specific to the difference in structure because the colanic acid structure, which is the basic skeleton of bile acids, is not easily broken by MS/MS. In order to accurately quantify various chemically similar bile acids by simultaneous LC-MS/MS analysis, the isomers must be sufficiently separated by HPLC.

In this report 39 bile acids in bio-samples (human plasma, human urine and mouse feces) were quantitatively analyzed using 10 internal standards. *LC/MS/MS method package Bile Acids Ver.2* [1] was used for the analysis. It contains optimized conditions for LC-MS/MS analysis and an automated sample preparation protocol. By rigorously optimizing the HPLC conditions, we were able to achieve both bile acid separation, high throughput and high sensitivity.

■ Pretreatment

Method Package sample preparation protocol was used (*Figure 1*). It consists of an automated extraction using Biotage® Extrahera™. 96 biological samples are prepared simultaneously in a total of 45 min (so 0.5 min per sample). This is 4 times faster than manual extraction, which takes about 3h for the same number of samples. It has also the advantages of a higher reproducibility and a reduced cost, by using less manpower.

An additional initial hydrolysis step is needed for mouse feces samples. 5 to 10 mg of feces are used. Potassium hydroxide is added, followed by a 20 min incubation at 80℃. Then, potassium phosphate buffer is used to lower down the pH. Afterwards, all samples are processed by automated extraction. For all matrices, plasma, urine and hydrolyzed feces, 250µL aliquots are
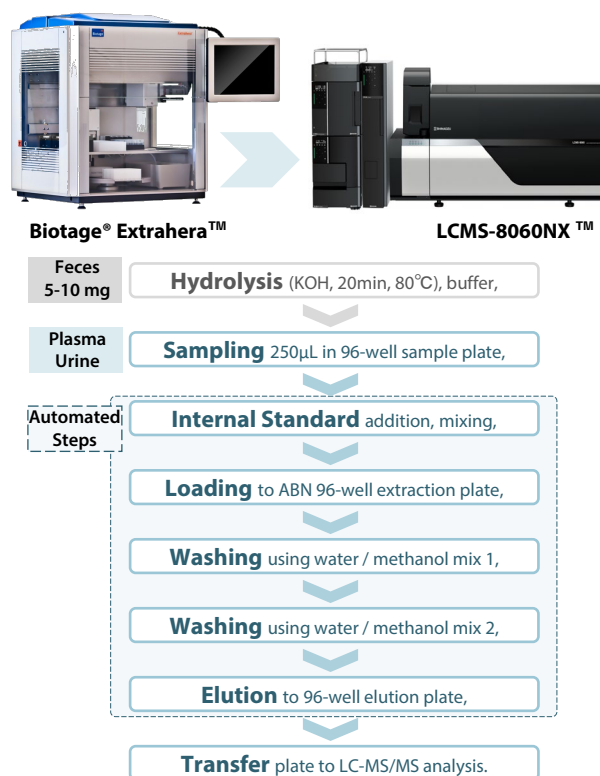

Biotage® Extrahera™   **LCMS-8060NX ™**

Feces 5-10 mg	**Hydrolysis** (KOH, 20min, 80℃), buffer,
Plasma Urine	**Sampling** 250µL in 96-well sample plate,
Automated Steps	**Internal Standard** addition, mixing,
	Loading to ABN 96-well extraction plate,
	Washing using water / methanol mix 1,
	Washing using water / methanol mix 2,
	Elution to 96-well elution plate,
	Transfer plate to LC-MS/MS analysis.

Figure 1. Pretreatment Protocol (Plasma, Urine, Feces).
Details of this pretreatment protocol are provided in
LC/MS/MS method package Bile Acids Ver.2 [1]

used. Extraction media is Evolute Express ABN 30mg 96-well plate (Biotage®). Water and methanol are used as extraction solvents, with formic acid as an additive.

■ Analytical Conditions

Pretreated samples were analyzed on the Nexera ™ X2 UHPLC, LCMS-8060NX system using ready to use method file from *LC/MS/MS method package Bile Acids Ver.2* [1]. The details of the analysis conditions are shown in *Table 1*. We have prepared 39 primary, secondary and conjugated bile acids as standard substances and 10 internal standards (stable isotope-labeled compounds from Alsachim; primary, secondary bile acids, taurine conjugates and glycine conjugates) to achieve high quantification accuracy. 39 bile acid are listed in *Table 2*. In order to reduce the cost of analysis, the number of internal standards has been reduced to 10. High-speed analysis was performed while maintaining good separation between isomers, and the measurement time for mass spectrometry was set to 10 minutes. *Figure 2* shows an example of an MRM chromatogram for each bile acid isomer.

Table 1. Analytical Conditions.

UHPLC (Nexera X2 system)	
Column	ACE Excel C18 Amide (ADVANCED CHROMATOGRAPHY TECHNOLOGIES LTD) [2]
Mobile phase	Aqueous : Formic acid in Water Organic : Acetonitrile / Methanol
LC run time (MS time)	10.95 min (10 min)
Flow rate	0.65 mL/min
Column temperature	45 °C

MS TQ (LCMS-8060NX)	
Ionization	Ion Focus ESI (Negative)
Mode	MRM
Nebulizing gas flow	3.0 L/min
Drying gas flow	5.0 L/min
Heating gas flow	15.0 L/min
DL temperature	250 °C
Heat Block temperature	500 °C
Interface temperature	400 °C

Table 2. 39 Bile Acids can be analyzed by method package

| Compound name	Abbreviation
Tauro-ω-muricholic acid	ToMCA
Tauro-α-muricholic acid	TaMCA
Tauro-β-muricholic acid	TbMCA
7,12-Diketolithocholic acid	7,12-DiketoLCA
Dehydrocholic acid	DHCA
Taurohyocholic acid	THCA
Ursocholic acid	UCA
Glycohyocholic acid	GHCA
Glycocholic acid	GCA
7-Ketodeoxycholic acid	7-KetoDCA
Glycoursodeoxycholic acid	GUDCA
Tauroursodeoxycholic acid	TUDCA
ω-Muricholic acid	oMCA
Norcholic acid	NorCA
Taurohyodeoxycholic acid	THDCA
Taurocholic acid	TCA
Glycohyodeoxycholic acid	GHDCA
α-Muricholic acid	aMCA
Norursodeoxycholic acid	NorUDCA
β-Muricholic acid	bMCA
3-Ketocholic acid	3-KetoCA
Cholic acid	CA
Glycochenodeoxycholic acid	GCDCA
Ursodeoxycholic acid	UDCA
Glycodeoxycholic acid	GDCA
Taurochenodeoxycholic acid	TCDCA
Hyodeoxycholic acid	HDCA
7-Ketolithocholic acid	7-KetoLCA
Taurodeoxycholic acid	TDCA
12-Ketodeoxycholic acid	12-KetoLCA
Allochenodeoxycholic acid	AlloCDCA
Apocholic acid	ApoCA
Chenodeoxycholic acid	CDCA
Deoxycholic acid	DCA
Glycolithocholic acid	GLCA
Taurolithocholic acid	TLCA
Dehydrolithocholic acid	DHLCA
Lithocholic acid	LCA
Allolithocholic acid	AlloLCA
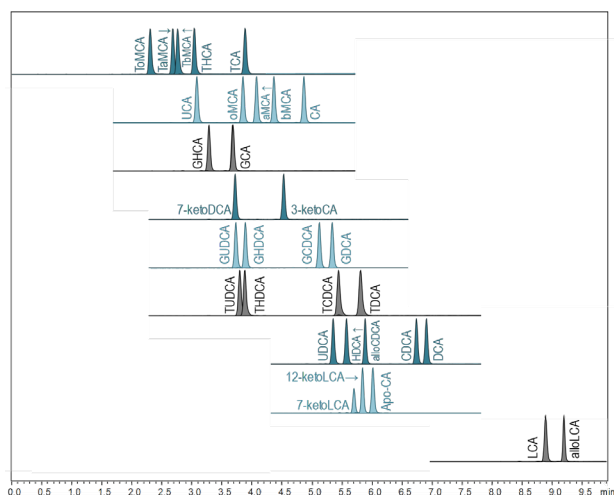
Figure 2. Example Bile Acids Isomers MRM Chromatograms.
Standard solutions at 10 ng/mL. Quantitative MRM transitions.

Calibration

Standard solutions with 6 different concentrations were prepared in the range of 0.5 to 50 ng/mL, and the regression equation was calculated from the measurement results. The peak area was determined automatically using Lab Solutions Insight software (not manually recalculated). Examples of the calibration curve is shown in *Figure 3*. All standard solutions showed an accuracy of 80-120% (*Figure 4*).
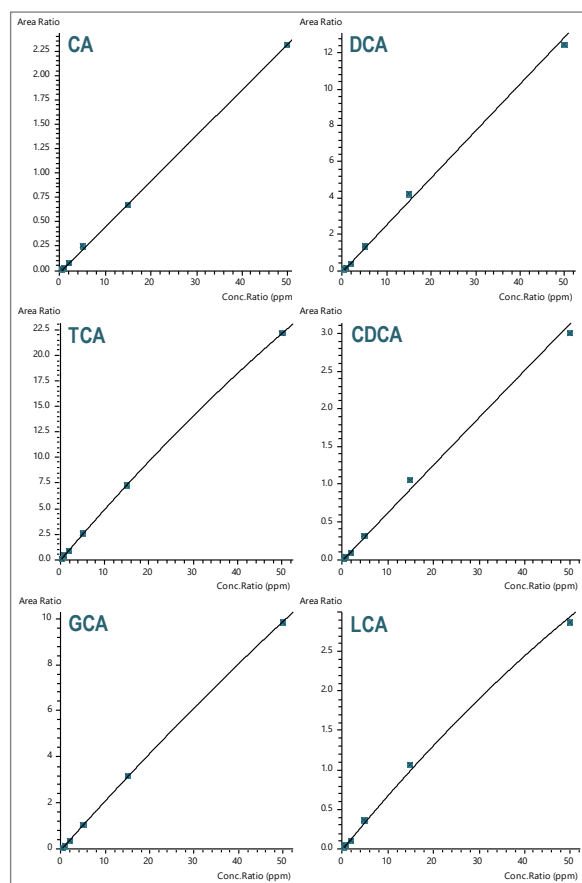

Figure 3. Example Calibration Curves.
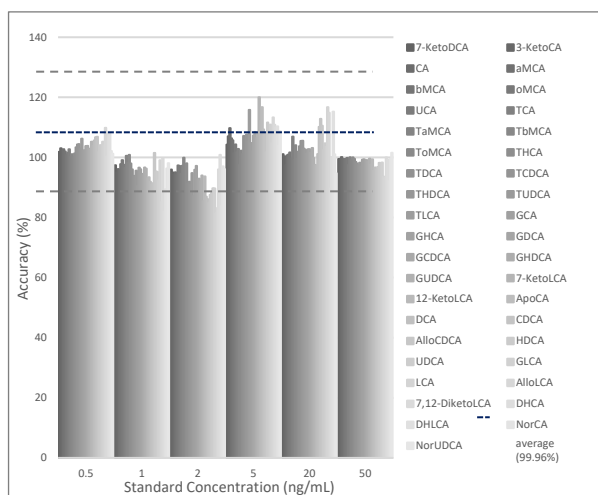Concentrations are spread over 7 standards, in the range 0.5-50 ng/mL.

Figure 4. Calibration Points Accuracies.

■ Results of Analysis

The evaluation of the method was conducted on biological matrices : plasma (human), urine (human) and feces (mouse). The samples were spiked with a standard solution to a concentration of 10 ng/mL for plasma and urine, or 2.3 ng/mg for feces (in addition to endogenous concentrations). Unspiked samples were also analyzed (blank). Precision was confirmed using spiked plasma sample (retention time and area). Additionally, separations of critical peak pairs were checked, confirming resolution above 1.1 for all BA compounds (data not shown). Lastly, each sample Bile Acids concentrations were measured.

Method Precision

For one experiment, 5 replicate samples were prepared under the same conditions within the same day, using the same plasma sample (standard substance 10 ng / mL spiked), and measured by LC/MS/MS. 4 experiments were performed in 3 days. From these results, Repeatability and Intermediate precision were evaluated using the analysis of variance (ANOVA). Repeatability of the Retention time was RSD <0.4%, and Intermediate precision was RSD <0.5%. Repeatability of Peak area was RSD <12%, and Intermediate precision was RSD <20%. All of them gave good results (*Table 3*).

Table 3. Method Repeatability (n=5) and Intermediate Precision (n=4), at 10 ng/mL in Plasma.

*ANOVA : analysis of variance

Repeatability of each experiment for all Bile Acids

	Experiment 1 RSD (n=5, %)			Experiment 2 RSD (n=5, %)		
	Min.	Max.	Av.	Min.	Max.	Av.
Ret. Time	0.02%	0.33%	**0.07%**	0.05%	0.49%	**0.18%**
Peak Area	1.23%	13.3%	**6.4%**	1.1%	13.5%	**6.2%**

	Experiment 3 RSD (n=5, %)			Experiment 4 RSD (n=5, %)		
	Min.	Max.	Av.	Min.	Max.	Av.
Ret. Time	0.04%	0.29%	**0.15%**	0.02%	0.34%	**0.11%**
Peak Area	1.3%	13.1%	**7.3%**	1.7%	13.1%	**6.4%**

Evaluation results of Repeatability and Intermediate precision by ANOVA*

Repeatability	Intermediate Precision
RT RSD < 0.4% Area RSD < 12%	RT RSD < 0.5% Area RSD < 20%

※Analysis of variance was performed for each bile acid.

Sample Quantification

Bile acids of interest were quantified in plasma, urine and feces. Standard addition (of 10 ng/mL) was used as the reference method for quantification. Two additional methods were evaluated: (i) internal calibration (1-point calibration at 10 ng/mL in water, with ISTD ratio correction) and (ii) direct isotopic dilution (direct calculation by ISTD ratio).

Because 10 ISTD(stable isotope labeled) are used for 39 BA, not all targets are corrected by a chemically equivalent isotope labelled standard. When extraction recoveries of target compounds and internal standards differ, it is necessary to apply a correction factor. It is calculated by the ratio of internal standard recovery and target compound recovery. These factors are matrix-dependent. Typical correction factors are given in the instruction manual of *LC/MS/MS method package Bile Acids Ver.2* [1]. These factors were used in the following calculations.

Quantification by internal calibration was applied both to blank samples (when endogenous bile acid was detected) and to spiked samples. Example unspiked plasma chromatograms are presented in *Figure 5*. The calculated concentrations in all blank matrices are presented in a comparative diagram (*Figure 6*) and their accuracies based on the standard addition are detailed in *Table 3*.

Excellent performances were observed for both unspiked and spiked samples, for all Bile Acids compounds. Accuracies were within [80-120] % in feces (*Figure 7*), within [82-119] % in urine (*Figure 8*), and within [82-119] % in plasma (*Figure 9*). This quantification method proved its fitness for purpose to assay the selected 39 Bile Acids.
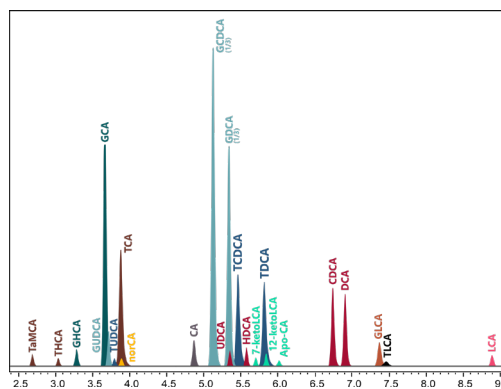

Figure 5. Example Chromatograms in Human Plasma.
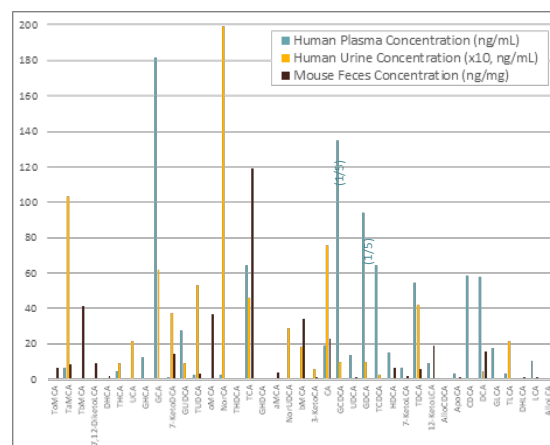Unspiked sample. Calculated concentrations are detailed in *Table 4*.

Figure 6. Calculated Concentrations in Plasma, Urine, Feces.
Unspiked samples. Calculation by internal calibration.

Quantification by direct isotopic dilution was applied both to blank samples (when endogenous bile acid was detected) and to spiked samples. The main advantage of this approach is that no calibration is needed. The concentration is calculated by the area ratio between the internal standard spiked into the sample and the target compound. When the internal standard is not an isotope labelled chemical equivalent of the target, its response may differ from the target one (at same concentration). MS response correction factor is needed (this information can be shared locally by your Shimadzu representative).

Excellent performances were observed for both unspiked and spiked samples, for all Bile Acids. Accuracies were within the range [90-113] % in feces (*Figure 10*), within [84-118] % in urine (*Figure 11*), and within [81-120] % in plasma (*Figure 12*). This quantification method proved its fitness for purpose to assay the selected Bile Acids.

Table 4. Calculated Concentrations and Accuracies.
Unspiked samples (Plasma, Urine, Feces). Internal calibration. Standard addition measured concentration was used for accuracy calculation.

Compound Abbreviation	Plasma Unspiked Conc. (ng/mL)	Accuracy (%)	Urine Unspiked Conc. (ng/mL)	Accuracy (%)	Feces Unspiked Conc. (ng/mg)	Accuracy (%)
ToMCA	ND	N/A	ND	N/A	6.5	84%
TaMCA	6.6	84%	10.3	84%	8.1	80%
TbMCA	ND	N/A	ND	N/A	41.4	N/A*
7,12-DiketoLCA	ND	N/A	ND	N/A	9.4	95%
DHCA	ND	N/A	ND	N/A	1.7	88%
THCA	4.6	89%	0.9	98%	ND	N/A
UCA	ND	N/A	2.1	101%	ND	N/A
GHCA	12.2	97%	ND	N/A	ND	N/A
GCA	181.8	N/A*	6.2	105%	0.2	99%
7-KetoDCA	1.0	104%	3.7	93%	14.0	81%
GUDCA	27.8	116%	0.9	101%	0.0	100%
TUDCA	2.6	106%	5.3	97%	2.8	100%
oMCA	ND	N/A	ND	N/A	36.9	N/A*
NorCA	2.7	99%	19.9	101%	0.1	98%
THDCA	ND	N/A	ND	N/A	0.3	101%
TCA	64.5	82%	4.6	87%	119.0	N/A*
GHDCA	ND	N/A	ND	N/A	ND	N/A
aMCA	ND	N/A	ND	N/A	4.0	83%
NorUDCA	ND	N/A	2.9	97%	ND	N/A
bMCA	ND	N/A	1.8	82%	34.4	N/A*
3-KetoCA	0.2	104%	0.6	105%	0.9	96%
CA	18.7	101%	7.5	110%	23.0	N/A*
GCDCA	673.5	N/A*	1.0	102%	ND	N/A
UDCA	13.4	104%	ND	N/A	1.4	100%
GDCA	470.2	N/A*	1.0	118%	0.1	116%
TCDCA	64.2	99%	0.3	115%	0.8	98%
HDCA	14.7	102%	ND	N/A	6.1	108%
7-KetoLCA	6.4	111%	ND	N/A	1.6	98%
TDCA	54.2	97%	4.2	100%	5.6	95%
12-KetoLCA	8.9	107%	ND	N/A	19.0	103%
AlloCDCA	ND	N/A	ND	N/A	ND	N/A
ApoCA	3.2	106%	ND	N/A	0.9	95%
CDCA	58.6	108%	ND	N/A	0.7	118%
DCA	57.6	116%	0.4	117%	15.5	120%
GLCA	17.9	119%	ND	N/A	ND	N/A
TLCA	3.0	114%	2.2	119%	0.3	120%
DHLCA	ND	N/A	ND	N/A	1.2	97%
LCA	10.0	116%	ND	N/A	1.4	104%
AlloLCA	ND	N/A	ND	N/A	ND	N/A

* Standard addition concentration is too low compared to endogenous value (1/10 or lower). Quantification by standard addition is not accurate. Accuracy calculation is not possible.
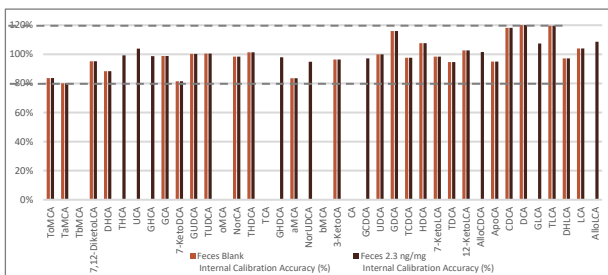

Figure 7. Feces Internal Calibration Accuracies.
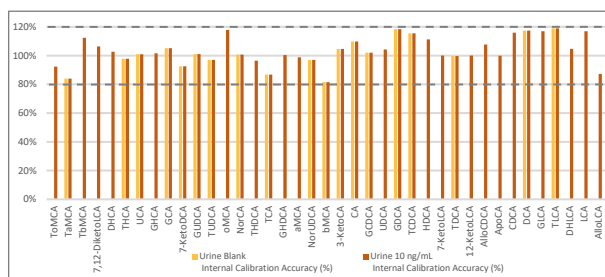Standard addition measured concentration was used as a reference.

Figure 8. Urine Internal Calibration Accuracies.
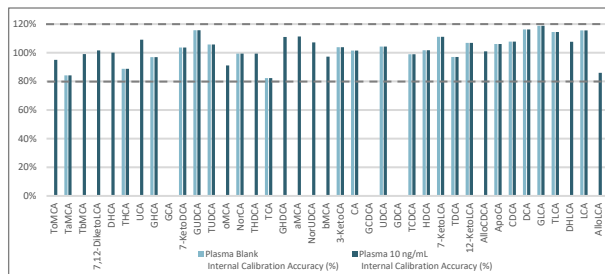Standard addition measured concentration was used as a reference.

Figure 9. Plasma Internal Calibration Accuracies.
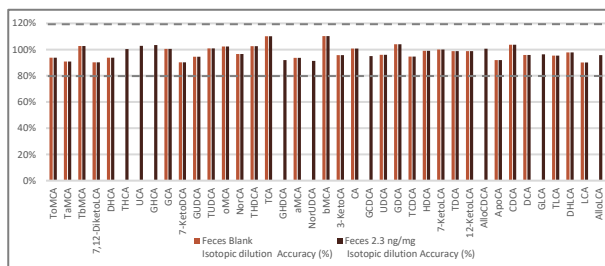Standard addition measured concentration was used as a reference.

Figure 10. Feces Direct Isotopic Dilution Accuracies.
Standard addition measured concentration was used as a reference.
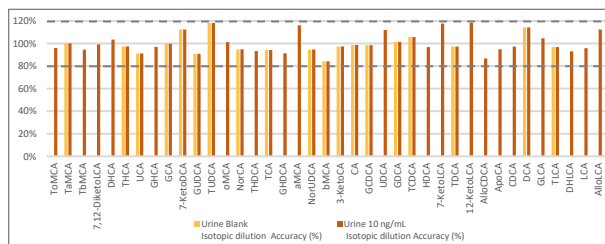

Figure 11. Urine Direct Isotopic Dilution Accuracies.
Standard addition measured concentration was used as a reference.
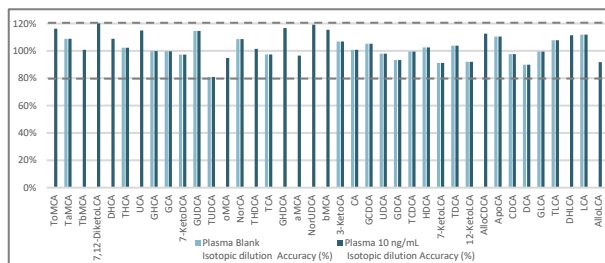

Figure 12. Plasma Direct Isotopic Dilution Accuracies.
Standard addition measured concentration was used as a reference.

Both quantification approaches, internal calibration and direct isotopic dilution, have proved their fitness for purpose to assay the selected 39 BA, with high performances. The correlation with standard addition is also very good for both methods (*Figure 13*).

As previously mentioned, direct isotopic dilution has the main advantage that no calibration is needed. When using this approach, it is however important that the responses of your internal standard and your target are of the same order of magnitude, to guarantee high quantification accuracy.

Still, the tolerance is quite high. In this experiment, for example, the ISTD MS responses were comprised between 1/3 and 80 times of the target responses, and still showing excellent accuracies.

As a conclusion, while using a well designed ISTD mix, direct isotopic dilution becomes a gold standard method for this Bile Acids LC-MS/MS quantification in biological matrices.
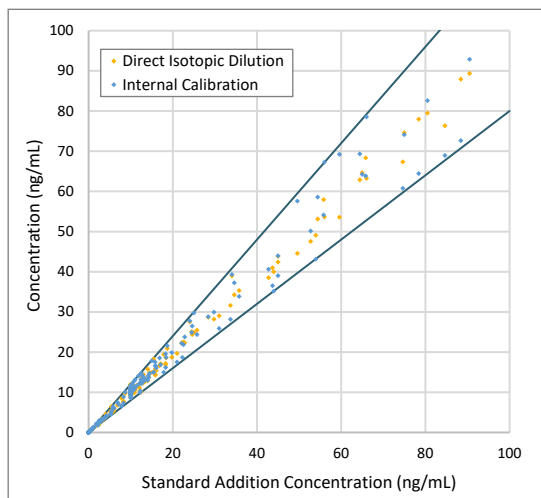

Figure 13. Correlation of Internal Calibration and Direct Isotopic Dilution with Standard Addition method.
Concentration range [0-100]ng/mL, for a clearer data visualization.

■ Conclusion

LC/MS/MS method package Bile Acids Ver.2 proved its fitness for purpose to assay selected 39 BA (primary, secondary and conjugates) in biological matrices of interest such as plasma, urine and feces. Extraction is automatized and can process 96 samples at once, and the MS acquisition is as fast as 10 min, including isomers resolution. Both the fast LC-MS/MS method and the automated sample extraction will enable robust routine analysis of Bile Acids with a high throughput and at a lower cost for the end user.

Novel Aspect

Complete solution for routine analysis of 39 primary, secondary and conjugates Bile Acids, in plasma, urine and feces, with automated sample extraction.

(1) Shimadzu, LC/MS/MS Method Package for Bile Acids version 2 : P/N S225-38610-91.
(2) Advanced Chromatography Technologies, ACE Excel C18 Amide : P/N EXL-1712-7502U.