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Improving the annotation of bile acids in fecal samples using a Liquid Chromatography-Ion Mobility-High Resolution Mass Spectrometry method

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Introduction

Bile acids (BAs) are derived from cholesterol and have been linked with a number of disorders, including diabetes, metabolic disruption, and colorectal cancer. Since all BAs have similar structures, their annotation in complex biological matrices can be exceptionally challenging. For their analysis, mass spectrometry (MS)-based methods are frequently used, however identical mass-to-charge ratios and fragmentation patterns are not uncommon[1,2]. Additionally, they share similar polarities and thus their separation by LC-MS methods is also difficult[3]. Trapped Ion Mobility Separation - High Resolution Mass Spectrometry (TIMS-HRMS) is an attractive tool for separation of isomeric and isobaric compounds as the separation is orthogonal to LC.

Here, we established a LC-TIMS-HRMS method to determine BAs in negative electrospray ionization. The proposed method was applied for profiling BAs in fecal and serum samples.

Methods

Standards: Bile Acid/Carnitine/Sterol Metabolite Library of Standards (BACSMLS) was obtained by Merck (Germany).

Samples: Lyophilized (Ly) fecal, aqueous (Aq) fecal, serum

Preparation protocol: Ly fecal: extraction of 10 mg sample with 1 mL H_2O:isopropanol:acetonitrile 2:1:1 v/v/v; Aq fecal: extraction of 30 mg sample with 1 mL H_2O:isopropanol:acetonitrile 2:1:1 v/v/v; serum: extraction with methanol (1:1 v/v)

LC: Elute UHPLC, Waters Acquity BEH C8 column (100 x 2.1 mm, 1.7 µm).

- LC gradient following a modified protocol from Sarafian *et al.*[4]:.

Table 1. Gradient elution program. Solvents: (A) acetonitrile:H_2O 10:90 v/v, 1 mM ammonium formate, pH adjusted to 4.2 with formic acid, (B) acetonitrile:2-propanol, 50:50 v/v.

Time (min)	Flow (mL/min)	%A
0	0.4	90
2	0.4	90
12	0.4	0
15	0.4	0
15.1	0.4	90
18	0.4	90

MS: timsTOF (Bruker Daltonics) equipped with ESI source.

Acquisition: Optimized broad range full scan TIMS-MS acquisition

Software: Compass HyStar Version 6.0, Compass Data Analysis Version 5.3 and MetaboScape® Version 6.0.2 (Bruker Daltonics).

Results

1) Method development

Acquiring four-dimensional data

In total, 32 bile acids were analyzed by established LC-TIMS-HRMS method and an in-house library was built using the resulting data (m/z, RT, ion mobility).

Each compound was prepared in accordance with the manufacturer's instructions, at a concentration of 10 µg mL^{-1}, and was injected into the chromatographic system individually. CCS values were measured in triplicate, under the optimized conditions for BAs analysis, in order to calculate the relative standard deviation (RSD) values. RSD values were below 0.3% in all cases, indicating high system reproducibility.
The established LC method allowed separation of the majority of the studied BAs.
Co-elution for isobaric BAs was still observed. As shown in Table 2, isobaric cholic and allocholic acid, and alpha-muricholic and beta-muricholic acid co-eluted from the LC. TIMS allowed to separate the [M-H]$^-$ ion species. This demonstrated added of TIMS for enhancing the method specificity and increasing confidence in compound annotation compared to conventional LC-MS methods.

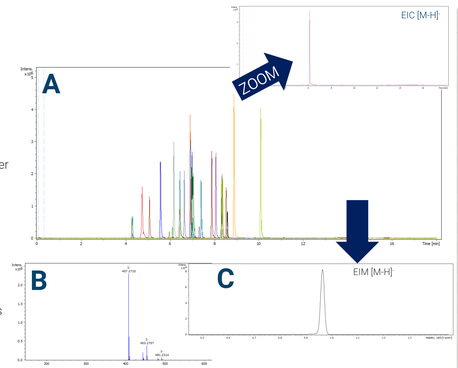


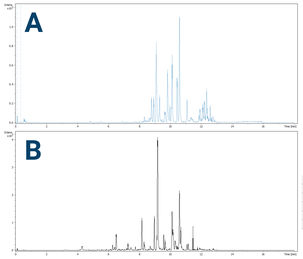
Fig. 1: LC-TIMS-MS analysis of 32 bile acids standards (10 µg mL-1) (A) extracted ion chromatograms (B) MS spectra (C) extracted ion mobilogram.

Table 2. Target List for the 32 bile acids.

Name	Formula	Experimental m/z	Average experimental CCS*	RSD* (%)	RT
TAURO-BETA-MURICHOLIC ACID (SODIUM SALT)	C26H45NO7S	514.2845	207.9	0.2	4.3
TAURO-ALPHA-MURICHOLIC ACID SODIUM SALT	C26H45NO7S	514.2843	207.9	0.1	4.3
DEHYDROCHOLIC ACID	C24H34O5	401.2332	199.6	0.1	4.8
SODIUM TAUROURSODEOXYCHOLATE	C26H45NO6S	498.2894	206.6	0.3	5.1
GLYCOURSODEOXYCHOLIC ACID	C26H43NO5	448.3067	200.3	0.1	5.3
TAYROCHOLIC ACID SODIUM SALT HYDRATE	C26H45NO7S	514.2842	206.4	0.1	5.3
URSOCHOLIC ACID	C24H40O5	407.2802	202.4	0.1	5.5
GLYCOCHOLIC ACID HYDRATE	C26H43NO6	464.3013	201.0	0.2	5.6
(3ALPHA,5BETA,7ALPHA)-3,7-DIHYDROXY-12-OXOCHOLAN-24-OIC ACID	C24H38O5	405.2647	204.5	0.2	6.1
ALPHA-MURICHOLIC ACID	C24H40O5	407.2801	209.0	0.6	6.1
SODIUM TAUROCHENODEOXYCHOLATE	C26H45NO6S	498.2793	206.0	0.1	6.1
BETA-MURICHOLIC ACID	C24H40O5	407.2803	208.6	0.1	6.2
TAURODEOXYCHOLIC ACID (SODIUM SALT)	C26H45NO6S	498.2898	204.7	0.1	6.3
SODIUM GLYCOCHENODEOXYCHOLATE	C26H43NO5	448.3069	199.3	0.1	6.4
MURIDEOXYCHOLIC ACID	C24H40O4	391.2857	200.0	0.3	6.5
GLYCODEOXYCHOLIC ACID SODIUM SALT	C26H43NO5	448.3072	198.6	0.1	6.6
GLYCOHYODEOXYCHOLIC ACID	C26H43NO5	448.3064	198.7	0.2	6.6
HYOCHOLIC ACID	C24H40O5	407.2807	207.6	0.1	6.7
URSODEOXYCHOLIC ACID	C24H40O4	391.2853	207.1	0.0	6.8
ALLOCHOLIC ACID	C24H40O5	407.2805	203.4	0.3	6.9
CHOLIC ACID	C24H40O5	407.2800	201.9	0.1	6.9
HYODEOXYCHOLIC ACID	C24H40O4	391.2855	209.0	0.3	7.0
NUTRIACHOLIC ACID	C24H38O4	389.2695	206.6	0.1	7.0
SODIUM TAUROLITHOCHOLATE	C26H45NO5S	482.2939	205.3	0.0	7.0
GLYCOLITHOCHOLIC ACID SODIUM SALT	C26H43NO4	432.3118	198.3	0.2	7.3
NOR-DESOXYCHOLIC ACID	C23H38O4	377.2700	197.9	0.1	7.4
CHENODEOXYCHOLIC ACID	C24H40O4	391.2850	207.8	0.1	7.9
DEOXYCHOLIC ACID	C24H40O4	391.2853	200.5	0.1	8.1
7-HYDROXY-3-OXO-CHOLEST-4-EN-26-OIC ACID	C27H42O4	429.3010	203.9	0.1	8.2
3ALPHA,7ALPHA,12ALPHA-TRIHYDROXYCOPROSTANIC ACID	C27H46O5	449.3272	203.4	0.0	8.4
3-OXO-5BETA-CHOLANOIC ACID	C24H38O3	373.2744	204.7	0.1	8.6
LITHOCHOLIC ACID	C24H40O3	375.2911	207.2	0.1	8.9

* Measurements in three different days.

2) Method for profiling BAs in biological samples



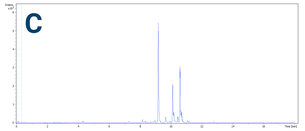


Fig. 2: Total Ion Chromatograms (A) serum (B) Ly fecal (C) Aq fecal.

	RT [min]	m/z meas.	CCS (Å²)	Name	Molecular For...	Annotations	AQ	Flags	ΔRT	Δm/z [ppm]	ΔCCS [%]	Mob. 1/K0
1	8.46	449.3278	204.7	3ALPHA,7ALPHA,12ALPHA-TRIH...	$C_{27}H_{46}O_5$	1	1		0.06	-0.026	0.6	0.992
2	8.96	375.2905	208.0	LITHOCHOLIC ACID	$C_{24}H_{40}O_3$	1	1		0.06	0.169	0.4	1.002
3	8.16	391.2855	202.1	DEOXYCHOLIC ACID	$C_{24}H_{40}O_4$	1	1		0.06	0.384	0.8	0.975
4	7.99	391.2858	208.7	CHENODEOXYCHOLIC ACID	$C_{24}H_{40}O_4$	1	1		0.09	1.041	0.4	1.007
5	8.69	373.2755	201.0	3-OXO-5BETA-CHOLANOIC ACID	$C_{24}H_{38}O_3$	1	1		0.09	1.822	1.8	0.968

Fig. 3: Example of BAs annotation using MetaboScape®.

In total, 12 BAs were annotated in the human aqueous fecal, 14 BAs in the lyophilized fecal and 7 BAs in human serum, using the Target List for 32 bile acids (containing name, molecular formula, retention time and CCS value). Raw data were automatically recalibrated for mass and mobility using MetaboScape®.

Table 3. Bile acids annotated in biological samples using MetaboScape®. Columns Δm/z, ΔRT and ΔCCS show the deviation between theoretical and experimental values.

Name	Δm/z	ΔRT	ΔCCS
(3ALPHA,5BETA,7ALPHA)-3,7-DIHYDROXY-12-OXOCHOLAN-24-OIC ACID	2.117	-0.02	-0.39
3-OXO-5BETA-CHOLANOIC ACID	0.186	0.09	0.23
3ALPHA,7ALPHA,12ALPHA-TRIHYDROXYCOPROSTANIC ACID	1.270	0.07	0.62
7-HYDROXY-3-OXO-CHOLEST-4-EN-26-OIC ACID	1.378	0.15	0.26
ALLOCHOLIC ACID	-0.258	0.14	-0.03
CHENODEOXYCHOLIC ACID	0.491	0.09	0.45
DEOXYCHOLIC ACID	-0.384	0.06	0.90
HYOCHOLIC ACID	0.927	0.07	0.34
HYODEOXYCHOLIC ACID	0.569	-0.09	0.27
LITHOCHOLIC ACID	-0.279	0.06	0.37
MURIDEOXYCHOLIC ACID	0.121	0.02	0.32
NUTRIACHOLIC ACID	1.426	0.11	0.51
TAURODEOXYCHOLIC ACID (SODIUM SALT)	2.800	0.04	-0.40
URSOCHOLIC ACID	0.779	0.09	0.47
BETA-MURICHOLIC ACID	-0.840	0.07	0.08

3) CCS value comparability

CCS values reported here were compared to drift tube (DT) values published by Picache et al.[5] in the unified CCS Compendium database (Table 4). The errors between the ^{TIMS}CCS and ^{DT}CCS Compendium were below -1.7% in all cases. Table 5 shows the CCS deviation using two LC-TIMS-MS setups. Different MS methods and LC gradients were applied in two different laboratories (Bremen, Germany and Thessaloniki, Greece). CCS values were highly reproducible with deviation below 0.3%.

Tables 4 and 5. ^{TIMS}CCS values are comparable between different laboratories (right) and to the CCS Compendium database (below).

Name	CCS (Å²)		%CCS deviation
	Picache et al.[5]	TIMS Thessaloniki	TIMS Thessaloniki vs. Picache et al.[5]
SODIUM GLYCOCHENODEOXYCHOLATE	200.6	199.3	0.6
GLYCOCHOLIC ACID HYDRATE	202.2	201.0	0.6
CHOLIC ACID	203.1	201.9	-0.6
URSOCHOLIC ACID	203.3	202.4	-0.5
HYOCHOLIC ACID	204.2	207.6	-1.7
ALPHA-MURICHOLIC ACID	205.6	209.0	-1.6

Name	CCS (Å²)		%CCS deviation
	TIMS Bremen	TIMS Thessaloniki	TIMS Thessaloniki vs. TIMS Bremen
GLYCOLITHOCHOLIC ACID	198.8	198.3	-0.3
GLYCODEOXYCHOLIC ACID	199.2	198.6	-0.3
GLYCOURSODEOXYCHOLIC ACID	200.5	200.3	-0.1
GLYCOCHOLIC ACID	201.6	201.0	-0.3
TAUROLITHOCHOLIC ACID	206.0	205.3	-0.3
TAUROCHENODEOXYCHOLIC ACID	206.7	206.0	-0.3
TAUROURSODEOXYCHOLIC ACID	207.2	206.6	-0.3
TAUROCHOLIC ACID	207.0	206.4	-0.3

Summary

We developed and optimized an UHPLC-TIMS-HRMS method which provides confident annotation for 32 bile acids in complex human biomatrices. Furthermore, we tested system reproducibility and compared our results with similar methods published before.

References

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Conclusions

- CCS values are used as a annotation criterion in the LC-TIMS-MS method for 32 bile acids. This increased confidence in compound annotations.
- The proposed method was tested for its reproducibility and deviations in CCS values below 0.3% in three different days were observed.
- We extracted bile acids from three different biological matrices (lyophilized fecal, aqueous fecal and serum).
- Untargeted profiling using MetaboScape® software enabled automatic annotation of bile acids or other compounds of interest.
- ^{TIMS}CCS values reported here were comparable between different laboratories, demonstrating the capability for confident interlaboratory annotations.

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