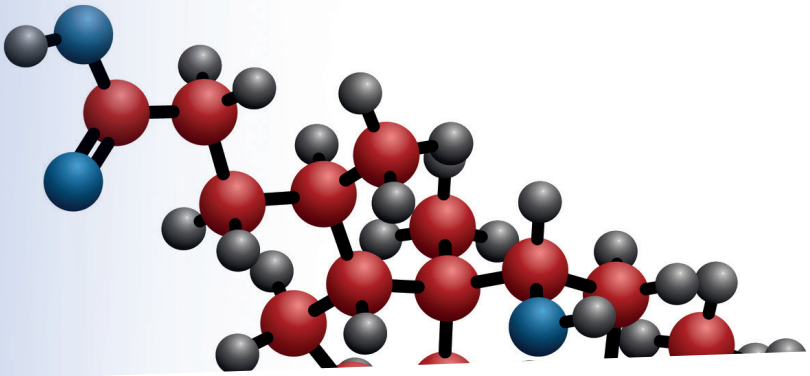

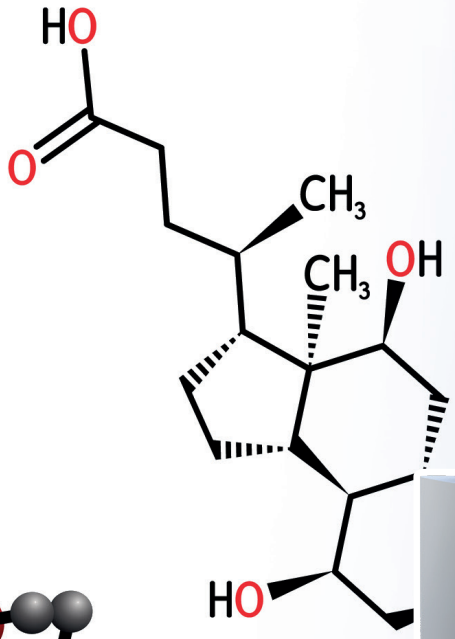

CCS-enhanced annotation confidence in LC-TIMS-HRMS-based bile acid profiling

An optimized LC-TIMS-MS method is presented for the analysis of bile acids in human biofluids (plasma) that leverages trapped ion mobility spectrometry (TIMS) to enhance the measurement specificity and annotation confidence in quantitative and profiling workflows.

Abstract

Seventy-one bile acid standards were used to characterize the approach, and its application was demonstrated using the human plasma standard reference material SRM 1950. The approach shows that confidence in bile acid annotation is increased by the addition of CCS to the commonly used characteristics of accurate mass, isotopic fidelity and retention time. This benefit is particularly valuable for annotation of unconjugated bile acids which lack a characteristic fragmentation pattern, providing a valuable independent parameter to augment conventional UHPLC-MS-based assignment.

Keywords:
LC-TIMS-MS, VIP-HESI, Bile acids, CCS, Annotation confidence

Introduction

Bile acids are synthesized in the liver of mammals as a major component of bile [1] and undergo enterohepatic recirculation and further metabolism in a complex relationship between human host and gut microbiome. They are well known for their role to regulate cholesterol homeostasis, in the absorption of lipids in the gut, and to work as signaling molecules influencing glucose and lipid metabolism [2]. Changes in bile acid metabolism are implicated in a variety of disease states including those involving the liver, gastrointestinal infection, and cognitive dysfunction [3, 4]. Increasing interest in understanding the effects of the microbiome on human health resulted in the recent discovery of novel bile acid conjugates, namely phenylalanocholic acid, tyrosocholic acid and leucocholic acid, which were shown to be enriched in relation to inflammatory bowel disease and cystic fibrosis [5].

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Future research in bile acids is expected to provide further insights into general disease related processes as well as the discovery of novel markers for disease onset or progression. This demands methods that provide rapid and specific profiling and quantitation capabilities that tackle the complex chemical nature of bile acids.

Bile acids are derived from cholesterol and they are classified into two main groups, namely unconjugated and conjugated bile acids. Conjugated bile acids are bound to glycine and taurine or, as described recently, also other amino acids [5]. The complexity of bile acid chemistry arises from the occurrence of hydroxyl or carbonyl groups connected in different stereochemistries. This renders bile acid analysis challenging as they are structurally similar and several isobaric and isomeric forms exists that cannot be separated by common LC methods in all cases.

Here we optimized a LC-TIMS-MS method which benefits from trapped ion mobility spectrometry (TIMS) by providing an additional means for bile acid separation in addition to that of LC and mass separation. TIMS can separate bile acid species and measure their specific Collisional Cross Section (CCS) areas which are based on their three-dimensional size and shape. These CCS values are comparable across experiments, among different laboratories (e.g. see the Bruker Application Note LCMS-171), and used to established reference values [6, 7], making them a useful measurement for the enhancement of data collation (i.e. the accurate grouping of bile acid-derived peaks measured across samples in a study) and metabolite annotation confidence. In bile acid analysis, this particularly benefits unconjugated bile acids which lack structurally characteristic MS/MS fragment ions and therefore are often measured and quantified using LC-QQQ systems in low specificity pseudo-MRM mode [1]. TIMS-based CCS measurement is also an effective tool for ensuring data reproducibility within and across laboratories where bile acid LC retention times may differ, limiting confident annotation and replication.

Table 1
MS acquisition parameters

MS	timsTOF Pro 2	
Source	**VIP-HESI source**	
	End Plate Offset	500 V
	Capillary	4500 V
	Nebulizer	2.0 Bar
	Dry Gas	8.0 l/min
	Dry Temp	230°C
	Probe Gas Temp	350°C
	Probe Gas Flow	4.0 l/min
Ionization	Negative ion mode	
Acquisition mode	TIMS-MS	
	Ramp time	300 ms
	Mobility range	0.8 – 1.35 1/K_0
Transfer parameters	Deflection delta	-80 V
	Funnel 1 RF	500 Vpp
	Funnel 2 RF	250 Vpp
	Multipole RF	200 Vpp
	Colission Energy	10 eV
	Collision RF	1100 V
	Quadrupole Low mass	150 *m/z*
	Transfer Time	55 µs
	Pre Pulse Storage Time	5 µs
Transfer parameters	Δt1	20 V
	Δt2	120 V
	Δt3	-80 V
	Δt4	-350 V
	Δt5	0.0 V
	Δt6	-100 V
	Collision Cell In	-220 V
	ICC Target Intensity	7.5 M
Calibration	Automatic internal mass calibration using sodium formate Automatic internal mobility using Agilent Tunemix	

The established LC-TIMS-HRMS method is shown to provide both quantitation and profiling capabilities for bile acids extracted from complex biological matrices, demonstrated here using human plasma but equally applicable to other biological materials including urine, bile, tissue and fecal extracts.

Methods

Here mixtures of pure reference standards were analyzed by an optimized LC-TIMS-HRMS method in negative ionization mode. The chromatographic separation is based on a C8 reversed-phase method published by Sarafian *et al.* 2015 [8] which provides reliable analysis of human biofluids including those additionally containing complex and neutral lipids (e.g. blood plasma).

Bile Acid Standard Mix 1 & 2 were purchased from Cambridge Isotope Labs (Tewksbury, MA, USA). Mixtures were diluted using methanol:water (1:1) in a range between 0.01 nM – 5 µM with 5 µL sample injection volumes.

Table 2
LC parameters

LC	Elute UHPLC		
Column	Waters BEH C8 column (100 x 2.1 mm, 1.7 µm)		
Column oven temp.	60°C		
Mobile phase	A: 100 mL of acetonitrile added to 1 L water, plus 1 mM ammonium acetate; pH adjusted to 4.15 using acetic acid B: Acetonitrile : Isopropanol (1:1)		
15 min Gradient	**Time [min]**	**Flow [ml/min]**	**%B**
	0.0	0.60	10
	0.1	0.60	10
	9.25	0.60	35
	11.50	0.65	85
	11.80	0.80	100
	12.00	0.95	100
	12.10	1.00	100
	12.40	1.00	100
	12.45	0.85	55
	12.50	0.85	10
	12.60	0.80	10
	12.70	0.70	10
	12.80	0.60	10
	15.00	0.60	10
Strong wash	Isopropanol		
Weak Wash	90% Buffer A / 10% Buffer B		

Individual bile acid standards were obtained from Steraloids (Newport, RI, USA) and Medical Isotopes (Pelham, USA) and dissolved in methanol:water (1:1) to a concentration of 0.3 µg/ml with 2 µL sample injection volumes.

NIST SRM 1950 human reference plasma was obtained from Merck / Sigma Aldrich (Taufkirchen, Germany) and extracted based on a protocol adapted from Sarafian *et al.* 2015 [8]. Briefly, 300 µL ice-cold methanol was added to 100 µl plasma in 1.5 ml Eppendorf tubes and vortexed for 15 min at 4°C and 1400 rpm using an Eppendorf MixMate. Samples were centrifuged at 4°C for 15 min at 5900 rpm (Eppendorf 5804 R). The supernatant was transferred to LCMS vials. Five µL of sample was injected.

LC-TIMS-HRMS analyses were conducted as described in Table 1 and 2 using a Elute UHPLC connected to a timsTOF Pro 2 system equipped with a VIP-HESI source (Bruker).

The resulting data (*m/z*, RT, mobility) was processed using TASQ® 2023 and MetaboScape® 2023 (Bruker) for targeted quantitation and non-targeted profiling, respectively. In both software workflow solutions, raw data was automatically recalibrated for mass and mobility.

The dynamic range of quantitation was assessed using TASQ® by applying a linear fit, 1/X weighting and considering <20% calibration residuals. The limit of detection (LOD) was determined with a S/N >3.

The T-ReX® 4D algorithm in MetaboScape® combines common adducts and isotopes belonging to the same compound into features in the feature table. These were annotated using a custom TargetList of 71 bile acids considering accurate mass, isotopic fit, retention time and mobility (CCS) information.

Results and discussion

LC-TIMS-HRMS for profiling and quantitation of bile acids in biological samples

CCS values were obtained for all reference standards analyzed and a target list was built for the annotation of bile acids in plasma. In total 71 bile acids were separated by the LC-TIMS-HRMS method and confidently annotated utilizing the established assay.

Of these, the optimized LC method and HRMS used allowed many bile acid species to be separated by either LC or MS alone (see Figure 1 A). Even in this simplified system of 71 synthetic reference materials, co-elution for several isobaric bile acid pairs was observed. For unconjugated bile acid species, failure to produce characteristic fragment ions when undergoing fragmentation further complicates the unambiguous annotation of these species. In such cases, the additional separation provided by TIMS can enhance the annotation confidence of distinct species, for example in the case of lithocholic acid and allolithocholic acid (see Figure 1 B). Here, TIMS was used to separate their $[M+acetate]^-$ ion species as highlighted in Figure 1 C, clearly demonstrating the additional benefit of TIMS for increasing the measurement specificity and enhancing the confidence in annotation.

Quantitation

To investigate the quantitative capabilities of the established assay we evaluated the linear dynamic range and limit of detection (LOD) for the Cambridge Isotopes Lab (CIL) Bile Acid Standard Mix 1 and 2.

Figure 2 A shows the calibration curve for taurocholic acid with a linear dynamic range of 3.7 orders of magnitude (1 nM – 5000 nM) an R^2 value of 0.9987 and residuals below 20%. The extracted ion chromatogram and extracted ion mobilogram at the lower limit of quantitation (LOQ) are shown for 300 pM and the corresponding blank in Figure 3 A and B, respectively. This demonstrates the quantitative capabilities of the established assay. The LOD was determined by investigating the peaks in the extracted ion chromatogram and mobilogram of the standard compared to a solvent blank. The LOD was determined at 300 pM with a S/N of ~7.

Next, we extracted bile acids from human reference plasma (SRM 1950) and compared the determined concentrations to the MEDM Locations established by an interlaboratory comparison for this reference material [9]. Figure 2 B demonstrates that, for taurocholic acid, the concentrations in the sample are within the linear dynamic range of the established method. Table 3 lists the bile acids that were detected within the dynamic range of the LC-TIMS-HRMS method. All determined concentrations are within the range of the published standard uncertainty locations. This highlights that the LC-TIMS-HRMS method provides reliable quantitative information in addition to the enhanced confidence in bile acid annotation.

Interlaboratory and intertechnology CCS value comparability

To further investigate the advantage of TIMS to increase confidence in target bile acid annotation we evaluated the interlaboratory and intertechnology comparability for bile acids in CIL Bile Acid Mix 1 and 2. We measured the bile acid mixtures using two LC-TIMS-HRMS setups, one in Bremen in Germany and one in Billerica in the US. Six LC-TIMS-HRMS acquisitions were performed in order to assess the standard deviation for CCS value reproducibility. The same MS method was applied, but different LC gradients and column chemistries were used (a 15 min gradient in Bremen and a 12.5 min gradient in Billerica). Table 4 shows the CCS values $[M-H]^-$ for the bile acids generating one major $[M-H]^-$ mobility peak.

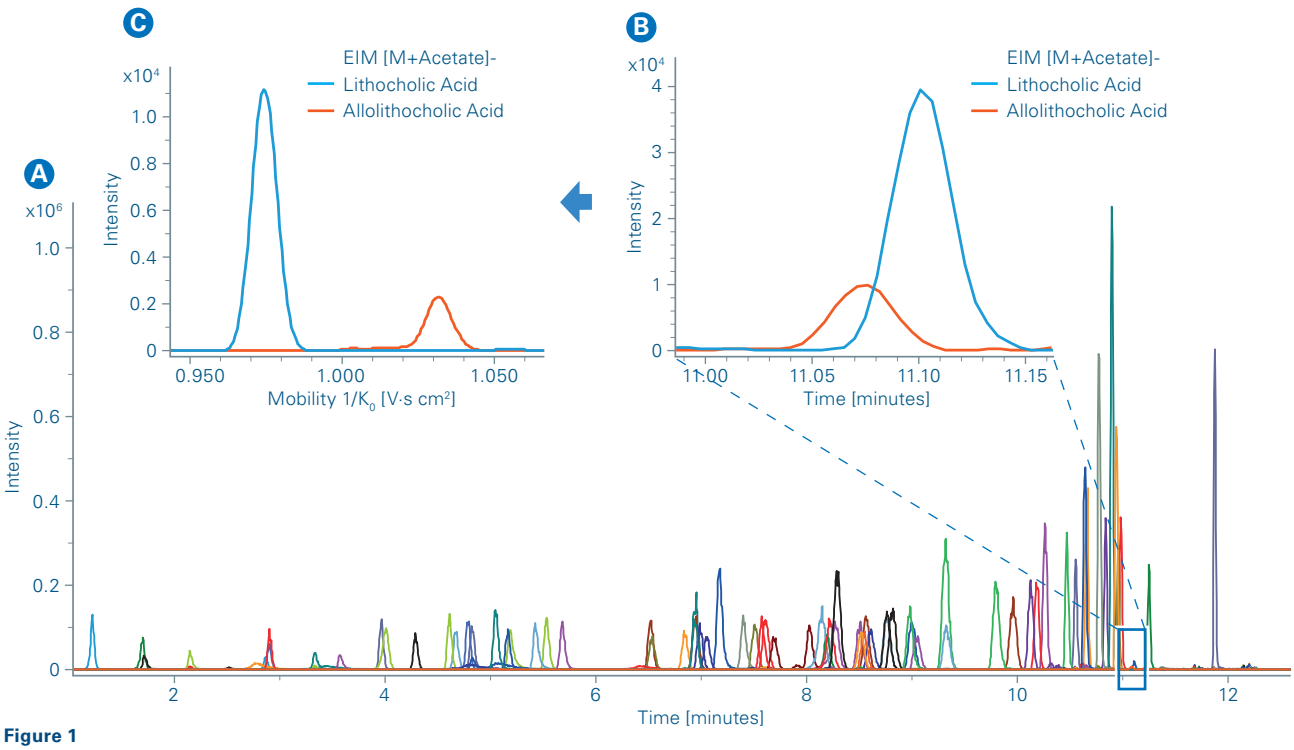


Figure 1
LC-TIMS-HRMS analysis of >70 bile acid standards

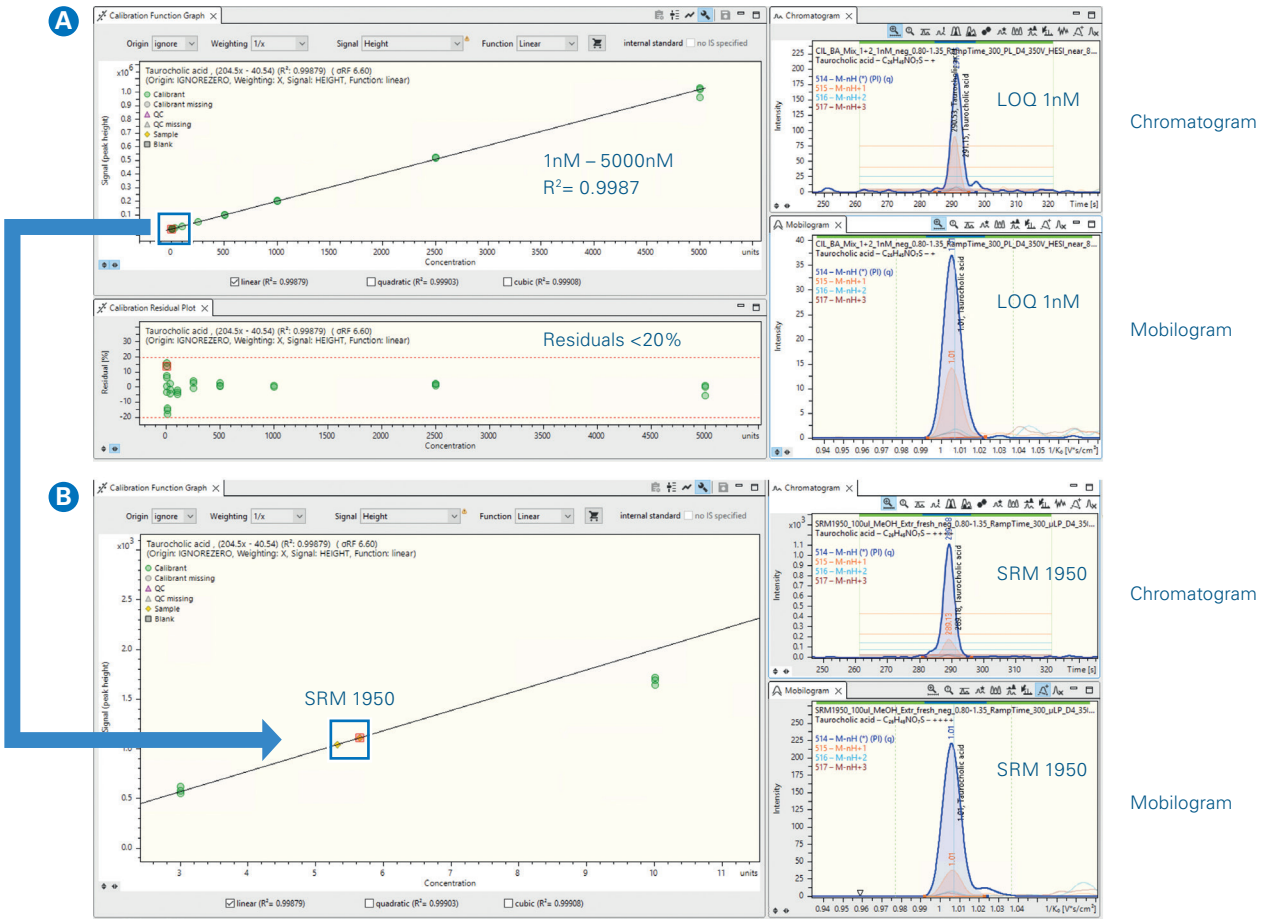


Figure 2
Taurocholic acid quantitation, 3.7 orders of magnitude linear dynamic range

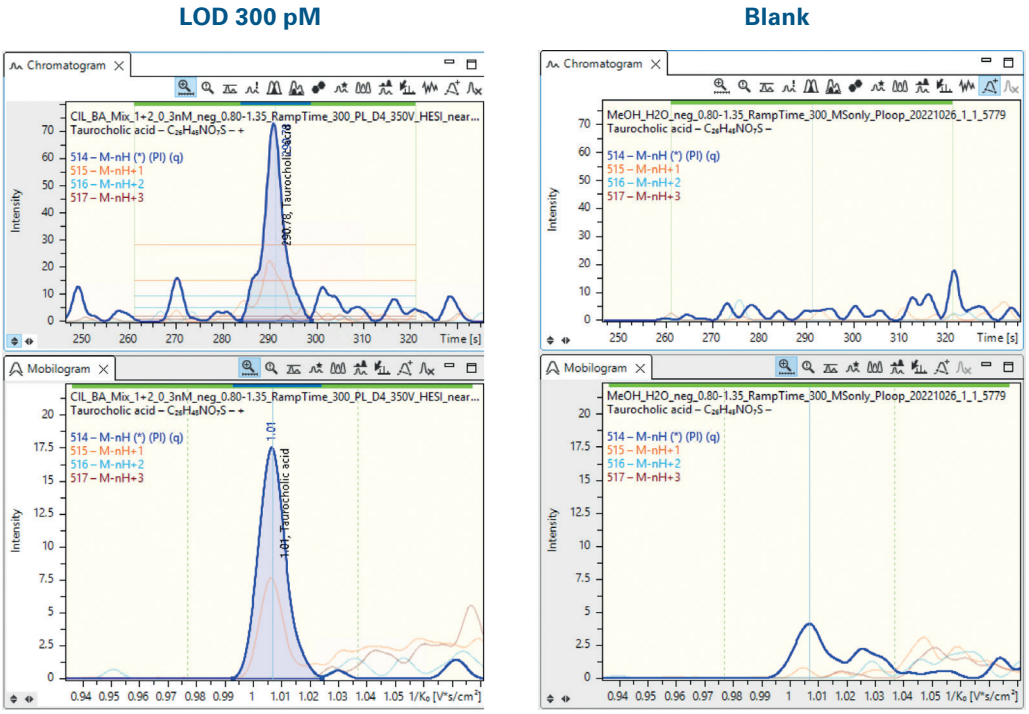


Figure 3
Taurocholic acid – Limit of detection 300 pM

Although the retentions times were different, the CCS values deviated on average by only 0.1% permitting confident interlaboratory annotations.

After we demonstrated the interlaboratory comparability of the CCS values, we compared the $^{TIMS}CCS_{N2}$ values to $^{DT}CCS_{N2}$ reference values published by Poland *et al.* 2020 [10] and submitted to the Unified CCS Compendium [6]. The average deviation of 0.3% in CCS values determined by TIMS vs. Drift Tube (DT) technology underlines the ability to match CCS values acquired by TIMS to reference values in public repositories like the Unified CCS Compendium.

Table 3
Bile acid concentrations determined by LC-TIMS-MS are within the reference location range

Name	MEDM Location ±Standard uncertainty [nmol/ml]; Bowden L, *et al.* 2017 [9]	LC-TIMS-MS determined concentration [nmol/ml] n=2
Chenodeoxycholic acid	0.30 ±0.11	0.28
Cholic acid	0.12 ±0.034	0.14
Deoxycholic acid	0.35 ±0.083	0.3
Glycochenodeoxycholic acid	1.1 ±0.18	1.03
Glycodeoxycholic acid	0.43 ±0.069	0.38
Glycoursodeoxycholic acid	0.15 ±0.024	0.17
Glycocholic acid	0.24 ±0.069	0.18
Taurocholic acid	0.026 ±0.0056	0.022
Ursodeoxycholic acid	0.11 ±0.024	0.11

Bile acid profiling

For qualitative profiling of bile acids, the datafiles of the SRM 1950 plasma extract were evaluated using the MetaboScape® software solution. Four dimensional feature detection technology, namely T-ReX® 4D, extracted and aligned mass peaks belonging to the same metabolite (isotopic peaks, adducts, common fragments) into a Feature Table. This data set was annotated using a custom Target List created based on the measured reference bile acids (see above). The Target List contains, name, molecular formula, retention time and CCS value for 71 bile acids. In total 25 bile acids were annotated in the human plasma extract using this list. These include 14 out of 20 and 13 out of 20 bile acids described by Bowden *et al.* 2017 [9] and Pedersen *et al.* 2021 [11], respectively. Annotation confidence is indicated in the Annotation

Table 4
^{TIMS}CCS values are comparable between laboratories and to reference ^{DT}CCS values

	CCS (²) $[M-H]^-$ Reference			% CCS deviation	
Name	**Poland C., *et al.* 2020 [10]**	**TIMS Bremen [n=6]**	**TIMS Billerica [n=6]**	**TIMS Bremen vs. Poland *et al.* [10]**	**TIMS Bremen vs. Billerica**
Glycolithocholic acid	199.5 ±0.2	198.8 ±0.1	199.1 ±0.2	0.3	-0.1
Glycodeoxycholic acid	199.9 ±0.2	199.2 ±0.1	199.4 ±0.1	0.4	-0.1
Glycoursodeoxycholic acid	201.1 ±0.1	200.5 ±0.1	200.6 ±0.1	0.3	0.0
Glycocholic acid	202.2 ±0.1	200.6 ±0.0	201.8 ±0.1	0.3	-0.1
Taurolithocholic acid	206.4 ±0.1	206.0 ±0.1	206.2 ±0.2	0.2	-0.1
Taurochenodeoxycholic acid	207.2 ±0.2	206.7 ±0.1	206.9 ±0.1	0.2	-0.1
Tauroursodeoxycholic acid	207.6 ±0.1	207.2 ±0.1	207.4 ±0.2	0.2	-0.1
Taurocholic acid	207.6 ±0.2	207.0 ±0.1	207.3 ±0.1	0.3	-0.2
Average				0.3	0.1

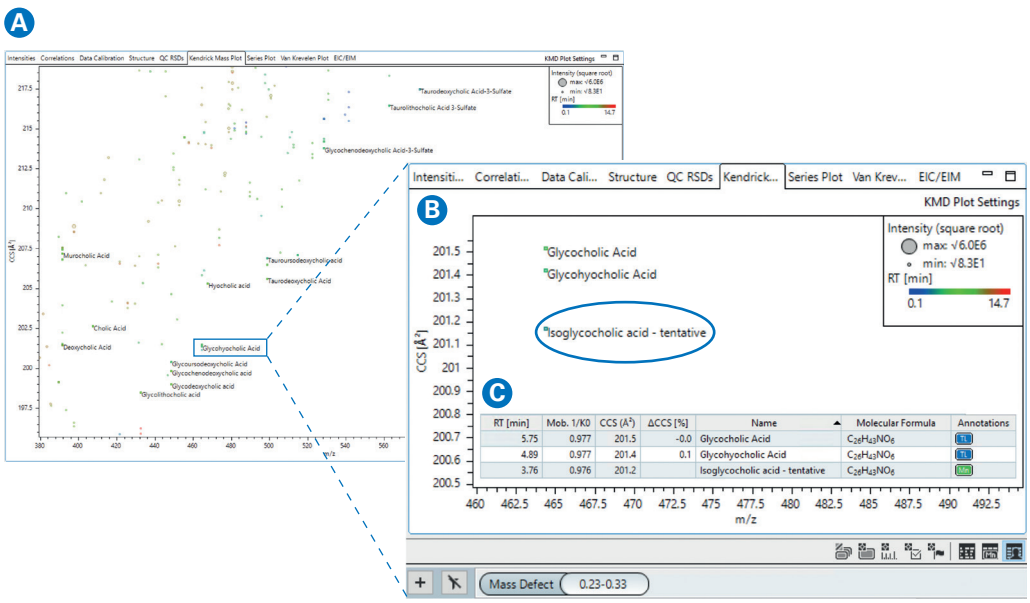
Quality symbol (Figure 4, far right "AQ" column). Each feature in the feature table is categorized by applying criteria to measure the deviation in *m/z*, retention time, isotopic fit (calculated as mSigma) and CCS as compared with known values derived from the Target List. Narrow filters indicate the highest possible fit (two green bars), whilst wider filters can be applied to expand the number of possible annotations (one gray bar). These criteria are user adaptable.

Visualizing annotated and non-annotated features in interactive MetaboScape® plots helped to further mine the data for novel bile acids. Here we used the Kendrick Mass Defect (KMD) plot for visualizing *m/z* vs. CCS values (see Figure 5). Feature intensities are plotted as bubble size and retention times are shown in a color gradient. Additionally, the Feature Table was filtered for the mass defect range 0.23 – 0.33 to show features only in the expected mass defect range for bile acids.

This use of the KMD plot enabled to readily mine the Feature Table for possible bile acids that were not annotated by the Target List. Figure 5 B shows a zoom into the mass and mobility region for glycocholic acid and glycohyocholic acid. A feature was spotted with a similar mass and mobility (in the plot below the two annotated bile acids). The retention time for this potential bile acid at 3.76 minutes is lower compared to the other two glycine conjugated bile acids. A likely annotation (Figure 5 C) for this bile acid is isoglycocholic acid, possessing the same elemental composition, i.e. mass, a similar CCS value and earlier reversed phase elution. Isoglycocholic acid contains a 3 beta-OH orientation (N-(**3beta**,7alpha,12alpha-trihydroxy-5beta-cholan-24-oyl)glycine) compared to glycocholic acid (N-(**3alpha**,7alpha,12alpha-trihydroxy-5beta-cholan-24-oyl)glycine). This difference in orientation renders isoglycocholic acid more polar and can lead to an earlier reversed phase elution [12]. This tentative annotation highlights the potential for discovery of further and potentially clinically relevant bile acids in complex biological extracts.

Ions	m/z meas.	Δm/z [mDa]	RT [min]	ΔRT	mSigma	Mob. 1/K0	CCS (Å²)	ΔCCS [%]	Name	Molecular For...	Annotations	AQ
	389.26957	-0.164	10.27	0.02	24.5	0.961	199.2	0.1	5-beta-Cholanic Acid 12-alpha-ol-3-one	$C_{24}H_{38}O_4$	RT	
	391.28503	-0.353	9.35	0.03	2.8	0.972	201.5	-0.0	5-beta-Cholanic Acid-3-beta, 12-alpha-diol	$C_{24}H_{40}O_4$	RT	
	448.30638	-0.467	5.22	0.04	8.2	0.971	200.4	-0.0	Glycoursodeoxycholic Acid	$C_{26}H_{43}NO_5$	RT	
	464.30126	-0.502	5.75	0.06	10.8	0.977	201.5	-0.0	Glycocholic Acid	$C_{26}H_{43}NO_6$	RT	
	498.28943	0.526	6.97	0.03	10.3	0.999	205.6	1.0	Taurodeoxycholic Acid	$C_{26}H_{45}NO_5S$	RT	
	391.28482	-0.559	10.66	0.00	8.0	0.972	201.5	0.1	Deoxycholic Acid	$C_{24}H_{40}O_4$	RT	
	391.28482	-0.562	7.70	0.02	3.3	1.000	207.2	-0.8	Murocholic Acid	$C_{24}H_{40}O_4$	RT	
	391.28477	-0.612	10.55	0.01	17.9	1.001	207.6	-0.2	Chenodeoxycholic Acid	$C_{24}H_{40}O_4$	RT	
	448.30642	-0.244	8.09	0.01	16.4	0.964	199.1	-0.1	Glycodeoxycholic acid	$C_{26}H_{43}NO_5$	RT	
	432.31161	-0.323	9.87	0.04	6.5	0.961	198.5	-0.2	Glycolithocholic acid	$C_{26}H_{43}NO_4$	RT	
	498.28903	0.389	6.55	0.02	8.7	1.004	206.5	-0.1	Taurochenodeoxycholic Acid	$C_{26}H_{45}NO_5S$	RT	
	407.27967	-0.397	8.79	0.03	14.8	0.979	202.7	0.2	Cholic Acid	$C_{24}H_{40}O_5$	RT	
	514.28392	-0.480	4.82	0.01	16.7	1.007	207.1	0.1	Taurocholic Acid	$C_{26}H_{45}NO_7S$	RT	
	528.26340	-0.257	2.83	0.05	33.5	1.049	215.7	0.0	Glycoursodeoxycholic Acid-3-Sulfate	$C_{26}H_{43}NO_8S$	RT	
	578.24657	0.270	3.61	0.02	45.9	1.061	217.5	0.1	Taurodeoxycholic Acid-3-Sulfate	$C_{26}H_{45}NO_8S_2$	RT	
	562.25185	0.471	4.87	0.03	42.7	1.055	216.5	-0.2	Taurolithocholic Acid 3-Sulfate	$C_{26}H_{45}NO_7S_2$	RT	
	391.28473	-0.652	9.21	0.17	12.3	1.001	207.5	0.5	Hyodeoxycholic Acid	$C_{24}H_{40}O_4$	RT	
	391.28426	-1.125	8.55	-0.06	3.2	0.998	206.8	-0.2	Ursodeoxycholic acid	$C_{24}H_{40}O_4$	RT	
	448.30594	-0.905	7.64	-0.01	5.8	0.968	199.9	-0.1	Glycochenodeoxycholic acid	$C_{26}H_{43}NO_5$	RT	
	528.26339	-0.951	5.22	0.14	34.4	1.043	214.2	-0.0	Glycodeoxycholic Acid-3-Sulfate	$C_{26}H_{43}NO_8S$	RT	
	528.26332	-0.341	4.94	0.13	25.8	1.041	213.8	0.0	Glycochenodeoxycholic Acid-3-Sulfate	$C_{26}H_{43}NO_8S$	RT	
	464.30078	-0.977	4.89	0.06	41.7	0.977	201.4	0.1	Glycohyocholic Acid	$C_{26}H_{43}NO_6$	RT	
	467.30254	1.110	8.22	0.02	43.1	0.996	205.3	0.2	Hycholic acid	$C_{24}H_{40}O_5$	RT	
	455.24883	1.546	9.02	0.04	58.4	1.040	214.5	0.0	Lithocholic Acid 3-Sulfate	$C_{24}H_{40}O_6S$	RT	
	498.28848	-1.001	4.33	-0.02	61.4	1.005	206.9	-0.1	Taurursodeoxycholic acid	$C_{26}H_{45}NO_6S$	RT	

Figure 4
Bile acids annotated in SRM 1950 human reference plasma using MetaboScape® software



RT [min]	Mob. 1/K0	CCS (Å²)	ΔCCS [%]	Name	Molecular Formula	Annotations
5.75	0.977	201.5	-0.0	Glycocholic Acid	$C_{26}H_{43}NO_6$	RT
4.89	0.977	201.4	0.1	Glycohyocholic Acid	$C_{26}H_{43}NO_6$	RT
3.76	0.976	201.2		Isoglycocholic acid - tentative	$C_{26}H_{43}NO_6$	Iso

Figure 5
Discovery and assignment of bile acids using interactive vizalizations in MetaboScape®

Conclusion

- The optimized LC-TIMS-HRMS based method for 71 bile acids leverages CCS as a comprehensive criterium for annotation confidence.
- The LC-TIMS-HRMS method provides quantitative results covering up to 3.8 orders linear dynamic range and LODs down to 300 pM.
- Determined bile acid concentrations in SRM 1950 reference plasma are within the abundance range of published reference concentrations.
- Untargeted profiling using the MetaboScape® solution enabled to readily pinpoint and annotate a bile acid not contained in the custom Target List of 71 standards: isoglycocholic acid. Confidence was increased by similarity of the CCS value to bile acids with similar structure.

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