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# Quantitative profiling of bile acids in biofluids and tissues based on accurate mass high resolution LC-FT-MS: Compound class targeting in a metabolomics workflow☆

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ABSTRACT

We report a sensitive, generic method for quantitative profiling of bile acids and other endogenous metabolites in small quantities of various biological fluids and tissues. The method is based on a straightforward sample preparation, separation by reversed-phase high performance liquid-chromatography mass spectrometry (HPLC-MS) and electrospray ionisation in the negative ionisation mode (ESI−). Detection is performed in full scan using the linear ion trap Fourier transform mass spectrometer (LTQ-FTMS) generating data for many (endogenous) metabolites, not only bile acids. A validation of the method in urine, plasma and liver was performed for 17 bile acids including their taurine, sulfate and glycine conjugates. The method is linear in the 0.01–1 μM range. The accuracy in human plasma ranges from 74 to 113%, in human urine 77 to 104% and in mouse liver 79 to 140%. The precision ranges from 2 to 20% for pooled samples even in studies with large number of samples ($n > 250$). The method was successfully applied to a multi-compartmental APOE*3-Leiden mouse study, the main goal of which was to analyze the effect of increasing dietary cholesterol concentrations on hepatic cholesterol homeostasis and bile acid synthesis. Serum and liver samples from different treatment groups were profiled with the new method. Statistically significant differences between the diet groups were observed regarding total as well as individual bile acid concentrations.



## 1. Introduction

Bile acids are steroid acids found predominantly in bile of mammals. The distinction between different bile acids is minute and depends only on the presence or absence of hydroxyl groups at positions 3, 7, or 12 (Fig. 1). Bile acids are synthesized in the liver from cholesterol through the action of specific hepatic enzymes and are excreted into the small intestine via the bile duct as free acids or as glycine or taurine conjugates. Besides being essential for the digestion and intestinal absorption of hydrophobic nutrients such as dietary fats and vitamins [1], bile acids have been implicated in the regulation of key enzymes involved in cholesterol homeostasis [2].

In healthy subjects, only small quantities of bile acids are found in the circulation and urine. In hepatobiliary and intestinal diseases, disturbances in hepatic bile acid synthesis, metabolism and clearance and intestinal absorption will affect the concentration and expression profile of bile acids in serum, liver, gallbladder, urine and faeces. Elevated concentrations of bile acids in peripheral fluids can therefore be used as an indicator of various diseases, and as a marker of liver toxicity.

In humans, there are two primary bile acids, *viz* cholic acid (CA) and chenodeoxycholic acid (CDCA), three secondary bile acids, *viz* deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA), and their glycine and taurine conjugates [3]. In urine, glucuronides, sulfates [4] and acyl galactosides [5] have been detected and characterized. The primary and secondary bile acids are also present in rodents (rats and mice). Furthermore, in

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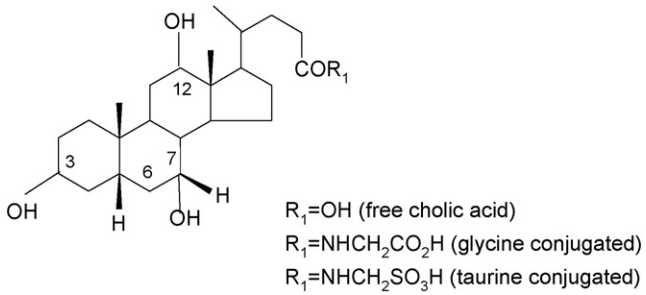


**Fig. 1.** Example of the structures of bile acids: cholic acid and its conjugates.

rodents, some of these bile acids can be converted to muricholic acid (MCA) and hyodeoxycholic acid (HDCA). Accurate measurement and profiling of bile acids and their conjugates is of relevance in many clinical and animal studies.

Thus far, a variety of methods have been described for the analysis of bile acids in various biological matrices, ranging from nuclear magnetic resonance (NMR) [6], gas chromatography mass spectrometry (GC-MS) [7,8] and high performance liquid chromatography (HPLC) methods with ultraviolet (UV) [9,10] or MS [11–13] detection. GC-MS sample preparation procedure involves fractionation of the bile acids according to the conjugation, followed by concentration and derivatisation steps, and is therefore very time consuming. NMR methods are relatively insensitive and only applicable in specific areas where the concentration of bile acids is very high, for example, the analysis of bile composition. HPLC-MS is used frequently and is suitable for the analysis of bile acids and their conjugates without prior fractionation. It is also easily applicable to different matrices provided that an appropriate sample preparation procedure is used, such as solid phase extraction (SPE). In order to achieve sufficient sensitivity and specificity, tandem MS is used [14,15]. A main drawback of the HPLC-MS methods described above is the relatively large volume of plasma (250 μL or more) required. Also, the target approach only provides information on a number of (pre) selected bile acids.

In biomarker discovery and system biology studies quantitative information is required for as many endogenous metabolites as possible and is acquired by applying comprehensive metabolomics approaches [16–18]. However, the above approaches have the drawback of providing only relative concentrations, and the performance, in particular the accuracy, of the methods for most of the detected metabolites is often unknown.

Therefore, there is a clear need for high throughput methods that combine the best of two worlds allowing quantitative determination and identification of a broad spectrum of bile acids in small sample sizes (cf. small mouse plasma samples) and providing relative concentrations (with respect to an internal standard, further referred to as semi-quantitative results) of any other (endogenous) metabolites detected.

Here we report a new LC-MS method for (semi) quantitative profiling of endogenous metabolites in biofluids and tissues. In

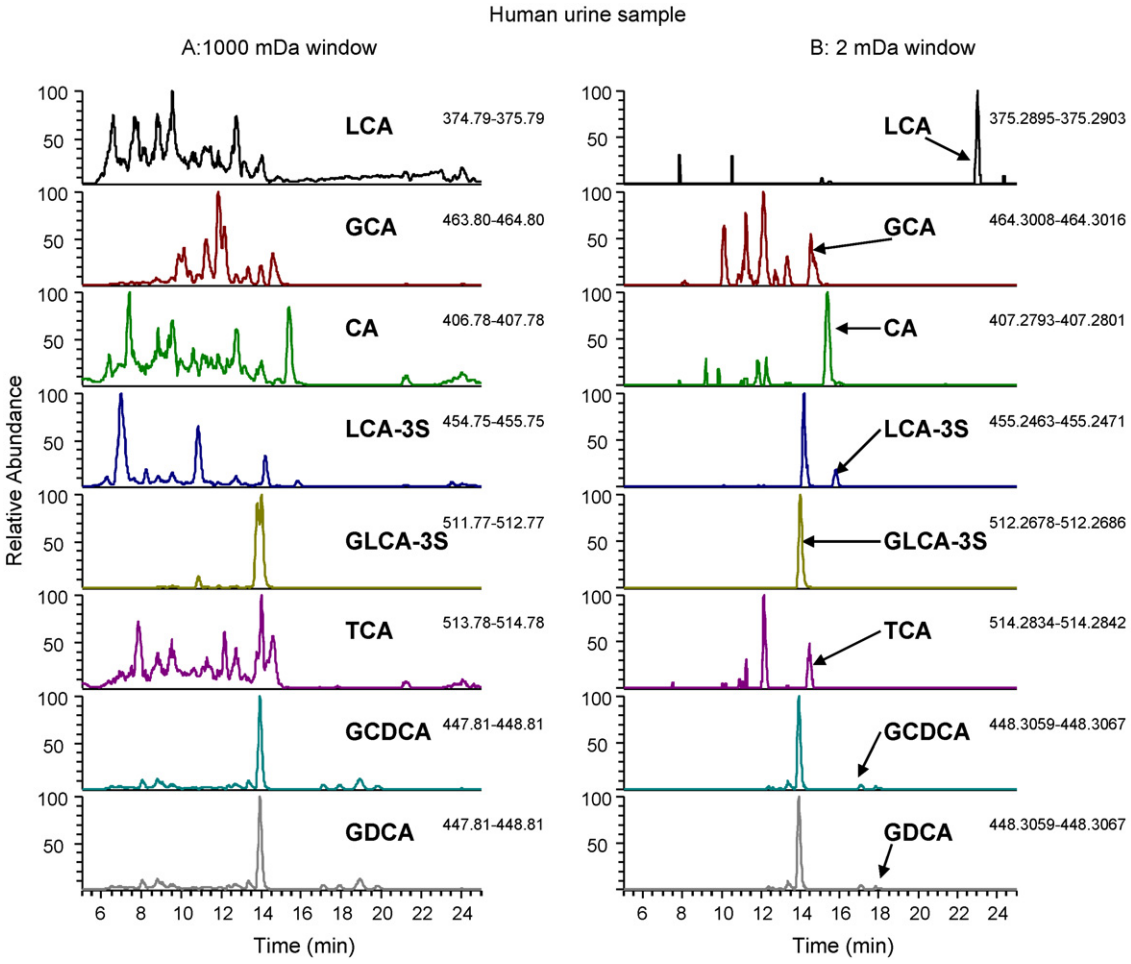


**Fig. 2.** Full scan chromatogram of a human urine sample displayed with (A) 1000 mDa window and (B) 2 mDa window.

this paper the validation of this method for determination of bile acids is described. The high resolution MS reduces chemical background thus providing selectivity more or less equivalent to MS/MS approaches, even in full scan mode. After validation of the method for the main bile acids in urine, plasma and liver, we applied it to a mouse study in which the effect of dietary cholesterol on the hepatic bile acid concentrations was studied. Validation and performance of the method for other detected endogenous metabolites will be described elsewhere.

## 2. Experimental

### 2.1. Materials and reagents

Methanol (Chromasolv) was purchased from Riedel de Haën (Seelze, Germany) and formic acid (p.a. grade) was from Merck (Darmstadt, Germany).

Bile acid standards were obtained from Sigma Chemical (Steinheim, Germany): lithocholic acid (LCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), cholic acid (CA), glycochenodeoxycholic acid (GCDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), taurochenodeoxycholic acid (TCDCA), tauroursodeoxycholic acid (TUDCA), glycolithocholic acid-3-sulfate (GLCA-3S), hyodeoxycholic acid (HDCA), taurolithocholic acid (TLCA), lithocholic acid-3-sulfate (LCA-3S) and taurocholic acid (TCA).

MCA standard was purchased from Steraloids (Quebec, Canada) and TDCA standard was from ACROS (Geel, Belgium).

Deuterated internal standards CA-d4, DCA-d4, GDCA-d4, LCA-d4 were obtained from CDN isotopes (Quebec, Canada).

### 2.2. Calibration and internal standard preparation

The stock solutions of the individual (deuterated) bile acids at a concentration of 1 mg/mL were prepared separately in methanol. All the stock solutions were stored at −20 °C.

Calibration standards were prepared by combining appropriate volumes of each bile acid stock solution and methanol. The calibration range was from 5 to 500 ng/mL of each bile acid in the final solution (this corresponds to approximately 0.1–1 μM). The calibration standards were treated in the same way as samples of biofluids.

### 2.3. Sample preparation

For calibrants, plasma, serum and urine, sample aliquots (a minimum of 50 μL) were deproteinized with 4 aliquots of methanol containing internal standards. After centrifugation, 90% of the sample in the form of the clear supernatant was transferred to a new vial, snap frozen and lyophilized. The sample was then re-dissolved in methanol–water (2:1, v/v), centrifuged and transferred into an auto-sampler vial. A typical dilution factor of sample to extract was 1.5–2, depending on the origin of the sample.

*(Liver) tissue*: About 20 mg of tissue was accurately weighed, lyophilized and the percentage dry weight was calculated. From the powdered sample, 5 mg (±0.5 mg) was accurately weighed and the protein precipitation and extraction was performed by 1000 μL of methanol containing internal standards assisted by ultrasonification. After centrifugation, 900 μL of the clear supernatant was transferred to a new vial, lyophilized and re-dissolved in methanol–water (2:1, v/v). The usual dilution factor of the sample was 1–2 mg of dry liver tissue per 100 μL extract, depending on the origin of the sample. The extract was centrifuged and transferred into an auto-sampler vial.

In the application described in this paper, 5 mg of lyophilized mouse liver and 60 μL of mouse serum were used for sample preparation.

### 2.4. LC-MS analysis

LC-MS analysis was carried out using a Thermo Electron LTQ-FT, equipped with a Thermo Electron Surveyor HPLC with autosampler and column-oven (ThermoFinnigan, Breda, The Netherlands) equipped with an ESI source in negative mode. 20 μL of the clear extract (maintained at 20 °C in the autosampler) was injected onto a C18 column (Waters XBridge C18 125 Å 2.5 μm particle size, 2.1 mm × 50 mm equipped with a Phenomenex guard column Widepore C18 5 μm (4 mm × 2.0 mm). Compounds were separated at a flow rate of 200 μL/min using a methanol–aqueous ammonium acetate ($NH_4OAc$) gradient. Mobile phase A (A) was 5% methanol in water containing 2 mM $NH_4OAc$ at pH 7, mobile phase B (B) was methanol, containing $NH_4OAc$ at 2 mM. Mobile phase C (C) was 2 mM $NH_4OAc$ in IPA-methanol (3:2, v/v). The gradient started at A–B, 9:1, v/v for 2 min and increased to only B in 18 min and was kept for 5 min. The column was then washed with C for 5 min. The column was stabilized at the initial gradient for 10 min before injecting the next sample. The total separation time was 40 min including stabilization of the column, this was sufficient to achieve baseline separation of several isomeric bile acids. The column temperature was maintained at 40 °C. After separation, eluting compounds were ionised in ESI in the negative ion mode and detected in full scan. Operating conditions of the MS were optimized using the initial setting as followed: spray voltage of 4 kV, and the heated capillary temperature of 320 °C. Nitrogen was used for the sheath and auxiliary gas, set at 40 and 20 psi (in arbitrary units) respectively. The resolution was 100,000 at *m/z* 400. The data was processed using the LC Quan software (ThermoFinnigan, Breda, The Netherlands).

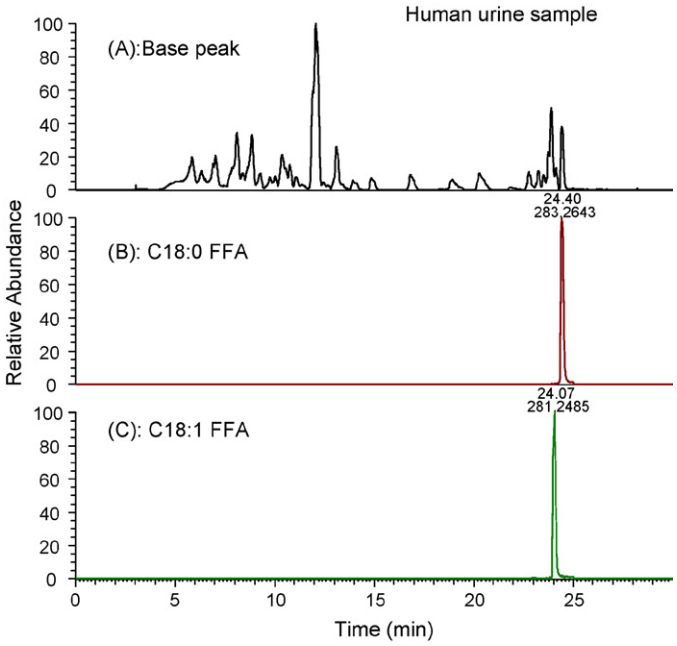


**Fig. 3.** LC–LTQ-FTMS full scan ion chromatogram of a human urine: (A) base peak, extracted ion chromatograms of (B) 283.264 (C18:0 free fatty acid), and (C) 281.248 (C18:1 free fatty acid).

**Table 1**
Calibration range, linearity and instrumental LOD of the developed method

| | [M−H]⁻ | Molecular Formula | $r^2$ range[a] | LOD[b] (pg) |
|---|---|---|---|---|
| CA | 407.2803 | $C_{24}H_{40}O_5$ | 0.994 | 50 |
| CDCA | 391.2854 | $C_{24}H_{40}O_4$ | 0.995 | 50 |
| DCA | 391.2854 | $C_{24}H_{40}O_4$ | 0.999 | 50 |
| GCA | 464.3018 | $C_{26}H_{43}NO_6$ | 0.998 | 10 |
| GCDCA | 448.3068 | $C_{26}H_{43}NO_5$ | 0.998 | 10 |
| GDCA | 448.3068 | $C_{26}H_{43}NO_5$ | 0.998 | 10 |
| GLCA-3S | 512.2687 | $C_{26}H_{43}NO_7S$ | 0.998 | 10 |
| HDCA | 391.2854 | $C_{24}H_{40}O_4$ | 0.997 | 50 |
| LCA | 375.2905 | $C_{24}H_{40}O_3$ | 0.998 | 50 |
| LCA-3S | 455.2473 | $C_{24}H_{40}O6S$ | 0.997 | 10 |
| MCA | 407.2803 | $C_{24}H_{40}O_5$ | 0.997 | 50 |
| TCA | 514.2844 | $C_{26}H_{45}NO_7S$ | 0.998 | 10 |
| TCDCA | 498.2895 | $C_{26}H_{45}NO_6S$ | 0.998 | 10 |
| TDCA | 498.2895 | $C_{26}H_{45}NO_6S$ | 0.998 | 10 |
| TLCA | 482.2946 | $C_{26}H_{45}NO_5S$ | 0.995 | 10 |
| TUDCA | 498.2895 | $C_{26}H_{45}NO_6S$ | 0.997 | 10 |
| UDCA | 391.2854 | $C_{24}H_{40}O_4$ | 0.997 | 50 |

[a] Calibration range 5–500 ng/ml, approximately 0.01–1 μM.
[b] Instrumental LOD (pg) on column estimated based on the lowest injected standard.

### 2.5. Validation of the method for bile acids

*Quantitative aspects*: Linearity was determined by analysis of calibration curves for all commercially available standards of bile acids in the range 5–500 ng/mL (approximately 0.1–1 μM). Instrumental LOD was estimated from signal in the lowest calibration solution.

Performance characteristics were determined for human plasma, human urine and mouse liver. Accuracy and precision were determined by extracting each matrix 5 times without and with fortification at two concentration levels: 20 and 400 ng/mL. Precision was calculated as relative standard deviation at the lower fortification level for each matrix. Accuracy was calculated based on the recovery for the fortification level closer to the concentrations determined per matrix without fortification. The precision of the method for bile acids was monitored in each study where the method was applied by analyzing a pooled study sample as a QC sample (without fortification with standards).

CA, GDCA, DCA and LCA were corrected for their own deuterated internal standard (CA-d4, GDCA-d4, DCA-d4 and LCA-d4) in all the quantitative determinations. All the other bile acids were corrected for DCA-d4.

Qualitative aspects: stability of the accurate mass was determined in urine. For this purpose, processed urine samples from 5 healthy subjects were injected, totaling over 300 injections. Relative standard deviations of the accurate masses and retention times of bile acids present in the different samples of urine were calculated from the data.

### 2.6. Mice study design

Animal experiments were approved by the Animal Care and Use Committee of TNO and were in compliance with European Community specifications. Serum and liver samples from a published Omics mouse study [19] were used to evaluate the influence of cholesterol feeding on bile acids. A group of E3L mice was treated with a cholesterol-free diet (diet T; Hope Farms, Woerden, The Netherlands) for 10 weeks (control, group T). Two other groups received the same diet but supplemented with either 0.25% (w/w) cholesterol (low cholesterol, group L) or 1.0% (w/w) cholesterol (high cholesterol, group H). After 10 weeks of experimental diet feeding, animals were sacrificed under anesthesia. Serum and tissues were collected, snap-frozen in liquid nitrogen, and stored at −80 °C until use.

### 2.7. Data processing and statistical analysis

Data for the target bile acids was processed using the LC Quan V2.0 software. Target tables were prepared using retention times and accurate masses observed in the calibration solutions. The window for retention time was 0.5 min and the window for accurate mass was 2.5 mDa.

Analysis of variance (one-way ANOVA) was used to compare mean (total) levels of bile acids between the diet groups. In all statistical tests that were performed, the null hypothesis was rejected at the 0.05 level of probability ($\alpha = 0.05$). Statistical analysis of the data was carried out using the SAS statistical software package (SAS/STAT Version 8.2, SAS Institute, Cary, NC).

**Table 2**
Accuracy of the method for bile acids as determined in urine, plasma and liver

| | Average concentration in plasma[a] | Accuracy in plasma (%) | Average concentration in urine[a] | Accuracy in urine (%) | Average concentration in liver[b] | Accuracy in liver (%) |
|---|---|---|---|---|---|---|
| MCA | <5 | 103 | <5 | 80 | 64 | 103** |
| TUDCA | <5 | 94 | <5 | 82 | 44 | 103** |
| GLCA | 553 | 113* | 189 | 102 * | 0 | 170 |
| UDCA | 24 | 80 | <5 | 97 | 3 | 105 |
| TCA | <5 | 88 | <5 | 97 | 681 | 95** |
| GCA | 51 | 109* | 10 | 92 | 1 | 79 |
| HDCA | <5 | 163 | <5 | 77 | 4 | 32 |
| CA | <5 | 94 | <5 | 78 | 4 | 88 |
| LCA-3S | <5 | 109 | <5 | 99 | 0 | 116 |
| TCDCA | 28 | 99 | <5 | 86 | 60 | 97** |
| GCDCA | 320 | 95* | <5 | 97 | 0 | 117 |
| TDCA | 15 | 97 | <5 | 104 | 63 | 100** |
| GDCA | 70 | 74* | <5 | 107 | 0 | 139 |
| CDCA | 26 | 94 | <5 | 107 | 2 | 97 |
| DCA | 134 | 113* | <5 | 94 | 2 | 97 |
| TLCA | <5 | 87 | <5 | 103 | 1 | 100 |
| LCA | <5 | 77 | <5 | 98 | 0 | 127 |

*Default fortification level 20 ng/mL, unless indicated with an *, then fortification level 400 ng/mL; **default fortification level 40 ng/mL, unless indicated with an **, then fortification level 800 ng/mL.
[a] ng/mL without fortification.
[b] ng/mg dry weight without fortification.

## 3. Results and discussion

### 3.1. Method development

We set out to establish a sensitive bile acid profiling method that can be used for high throughput analysis in metabolomic studies (i.e. detection of as many analytes as possible). To that end, we developed a very generic sample preparation procedure suitable for small amounts of biomaterial (preferably <50 μL of body fluids as it is required for rodent studies). For this reason, we limited the sample preparation to protein precipitation with methanol containing internal standards. A concentration step (lyophilization) is then needed to achieve sufficient sensitivity in full scan mode. An advantage is that methanol also extracts many classes of other endogenous metabolites such as (hydroxyl) fatty acids, steroids and phospholipids. Semi-quantitative data can be obtained also for compounds other than bile acids, therefore the method is suitable not only for quantitative profiling of bile acids, but also metabolomics applications.

There are several examples in the literature where bile acids have been separated and detected using HPLC-MS/MS with ESI−. Although methods for baseline separation of bile acid isomers are available with gradients shorter than 30 min [15], we chose a longer gradient in order to better separate also other co-extracted metabolites. A 30 min gradient on a C18 column was found to be sufficient for the baseline separation of the isomeric bile acid, see Fig. 2. However, an additional 10 min rinse and a conditioning step is required to remove other partially co-extracted apolar lipids which are injected, but not eluted with this gradient and not detected in negative ionisation, such as triglycerides and cholesterol esters.

The high mass resolution results in accurate masses with deviations lower than 2 ppm, helping to reduce the chemical background in biological matrices and thereby enhancing specificity compared to unit resolution. This specificity is particularly important for urine and is equivalent to MS/MS methods. In Fig. 2, extracted ion chromatograms of the bile acids detected in extracts of urine are shown, displayed with 1000 mDa (A) and with 2 mDa (B) window. In urine, some bile acids can only be determined if accurate mass is used (LCA, GLCA-3S). Furthermore, at different (shorter) retention times, more peaks are detected with the same accurate mass as some of the target bile acids, see Fig. 2.

Identification of all these peaks was outside the scope of this study, but these peaks are probably bile acid glucuronides, which fragment during ionisation by losing the conjugate to form a fragment with the same elemental composition as the bile acid. Good HPLC separation is essential in order to resolve isomeric bile acids and their conjugates. Bile acids and their polar conjugates are not the only metabolites detected by the method as shown in Fig. 3A. For example, in Fig. 3B and C extracted ion chromatograms are shown corresponding to C18:0 and C18:1 free fatty acids detected in human urine.

### 3.2. Validation

The bile acids are endogenous in mammalian plasma, serum, and liver are always present and blank matrix is not available. The concentrations of the bile acids found in the different biofluids and tissues differ, the standard deviation of replicate measurements of matrix without fortification is therefore not a good indication of the LOQ. In addition, the reported method is based on accurate mass, which is very specific. No background signal is measured in a blank solvent which could be used to estimate the instrumental LOD with the usual methods. LOD is often calculated as: LOD = $3.3\sigma/S$, where $\sigma$ = standard deviation of the area in a blank matrix or a procedural blank and $S$ = the slope of the calibration curve. For these reasons, the instrumental LOD of the method was estimated based on the intensity of the signal observed in the lowest calibration standard and is listed in Table 1. LOQ was not determined

Limited dynamic range can still be a problem, even for the modern high resolution MS instruments. For our method, the linearity of response was determined by analyzing standards in the range of 0.01–1 μM. The $r^2$ ranges from 0.994 to 0.999, see Table 1. Even though this linear range is in some cases lower than reported for bile acids on triple quadrupole instruments [12], it is sufficient for many application(s) (also those described in this paper). Further improvement can be achieved by changing the concentration range of the bile acids standards, depending on the concentration ranges expected in specific studies.

**Table 3**
Precision of the method during validation and during application in large studies

| | Validation[a] (%) | | R.S.D. | Applications[b] (%) | | | R.S.D. |
|---|---|---|---|---|---|---|---|
| | Plasma | Urine | Liver | Chicken plasma | Human plasma | Chicken liver | Human urine |
| total no.of samples | 30 | 30 | 30 | 500 | 50 | 170 | 300 |
| pooled sample replicates | $n=5$ | $n=5$ | $n=5$ | $n=70$ | $n=6$ | $n=22$ | $n=15$ |
| MCA | 4 | 0 | 15 | n.d. | | n.d. | n.d. |
| TUDCA | 4 | 3 | 5 | 11 | 2 | 9 | n.d. |
| GLCA-3S | 2 | 4 | 2 | 7 | 16 | 13 | 4 |
| UDCA | 3 | 4 | 8 | 13 | 4 | n.d. | n.d. |
| TCA | 3 | 2 | 7 | 20 | 2 | 10 | 12 |
| GCA | 1 | 2 | 2 | n.d. | 5 | n.d. | 8 |
| HDCA | 5 | 4 | 7 | n.d. | n.d. | n.d. | n.d. |
| CA | 1 | 1 | 2 | 7 | 3 | 16 | 3 |
| LCA-3S | 1 | 1 | 3 | n.d. | n.d. | n.d. | 5 |
| TCDCA | 1 | 3 | 3 | 16 | 4 | 11 | n.d. |
| GCDCA | 1 | 2 | 2 | n.d. | n.d. | n.d. | 3 |
| TDCA | 2 | 3 | 1 | n.d. | 3 | n.d. | n.d. |
| GDCA | 1 | 1 | 3 | n.d. | n.d. | n.d. | 3 |
| CDCA | 4 | 4 | 4 | 9 | n.d. | n.d. | 3 |
| DCA | 2 | 3 | 3 | 10 | 3 | n.d. | n.d. |
| TLCA | 2 | 2 | 1 | 12 | n.d. | 14 | n.d. |
| LCA | 4 | 5 | 1 | n.d. | n.d. | n.d. | n.d. |

n.d., not detected.

[a] R.S.D. (in %) as determined during the validation of the method in fortified urine, plasma and liver samples. Fortification level 20 ng/ml (urine and plasma) and 40 ng/mg dry weight (liver).

[b] R.S.D. (in %) as determined in pooled study samples without fortification in studies where the method was applied.

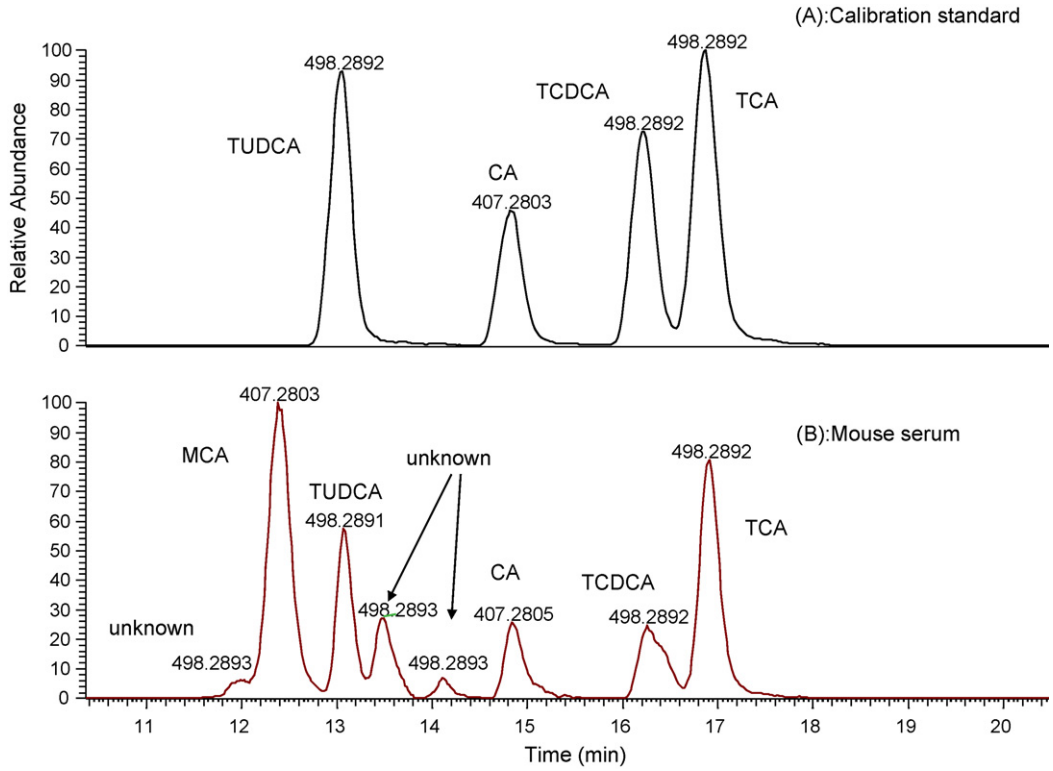


**Fig. 4.** Extracted ion chromatograms of *m/z* 498.289 and 407.280 (display window: 2 mDa) in (A) calibration standard and (B) mouse serum.

Other performance characteristics of the method were determined in the most common matrices: human plasma, human urine and rat liver (all healthy individuals). The concentrations of the individual bile acids differ even within one matrix, from low (0.02 μM) to high (0.5 μM). It is important to know the performance characteristics at these biologically relevant levels. The accuracy was therefore determined by fortifying the samples at two different concentrations, 0.04 and 0.8 μM or 40 and 80 μmol/kg dry weight (liver). Depending on the concentrations determined without fortification, the accuracy was determined using the lower or the higher concentration level whichever was more comparable with the 'normal' concentration. The results are summarized in Table 2. The accuracy (after deuterated internal standard correction) in plasma ranges from 74% (GDCA) to 113 % (DCA) with the exception of HDCA (163% at the low fortification level that could not be explained). In urine, the accuracy ranges between 77% (HDCA) and 104% (TDCA) and in liver 79% to 140%, with the exception of HDCA (32%) and GLCA-3S (170%). It should be noted that despite the lower accuracy (<80 and >120) of the method for the mentioned bile acids, these are relevant concentration levels and not high concentration levels often reported in determinations of accuracy and precision. At the high fortification level the accuracy of the method was ~100% for all the file acids (individual results not shown).

Precision was also determined in all three matrices and is shown in Table 3. The precision is very good and ranges from 1–8% with the exception of MCA in liver, where the R.S.D. was 15%. Reproducibility of the method was not determined. However, the method was applied in several studies with a large number of samples. In every study a pooled study sample with and without fortification is analyzed several times, evenly distributed throughout the series. In this way the reproducibility and accuracy of the method can be determined for each application and monitored over a large number of injections and in various matrices. Several examples are shown in Table 3. The relative standard deviation (R.S.D.) is in all cases below 20%, even in a very long series of samples where 70 replicates of a pooled liver study sample were analyzed (total analysis time for all the samples is approximately 250 h).

For a quantitative method based on full scan and accurate mass, the stability of both retention time and accurate mass is essential and was therefore validated. Over 300 injections, the R.S.D. (in ppm) of the accurate mass of the bile acids detected in human urine ranges from 0.1 ppm (for GLCA-3S) to 0.37 ppm (for GDCA), see Table 4. This was considered to be sufficient for use in a target

**Table 4**
Stability of the retention time and accurate mass of the method

| Bile acid in human urine | $R_t$ (min) mean[a] | R.S.D. $R_t$[a] (%) | Theoretical mass | Average observed mass | R.S.D. mass (ppm)[a] | Average delta (mDa) |
|---|---|---|---|---|---|---|
| Glycocholic acid | 14.5 | 0.1 | 464.3018 | 464.3013 | 0.12 | 0.5 |
| Cholic acid | 15.4 | 0.15 | 407.2803 | 407.2798 | 0.27 | 0.5 |
| Lithocholic acid-3-sulfate | 15.7 | 0.1 | 455.2473 | 455.2469 | 0.19 | 0.4 |
| Glycolithocholic acid-3-sulfate | 13.9 | 0.10 | 512.2687 | 512.2682 | 0.10 | 0.5 |
| Taurocholic acid | 14.4 | 0.05 | 514.2844 | 514.2840 | 0.26 | 0.4 |
| Glycochenodeoxycholic acid | 16.9 | 0.1 | 448.3068 | 448.3065 | 0.30 | 0.3 |
| Glycodeoxycholic acid | 17.7 | 0.1 | 448.3068 | 448.3065 | 0.37 | 0.3 |

[a] Determined over 300 urine injections, calibrated only once.

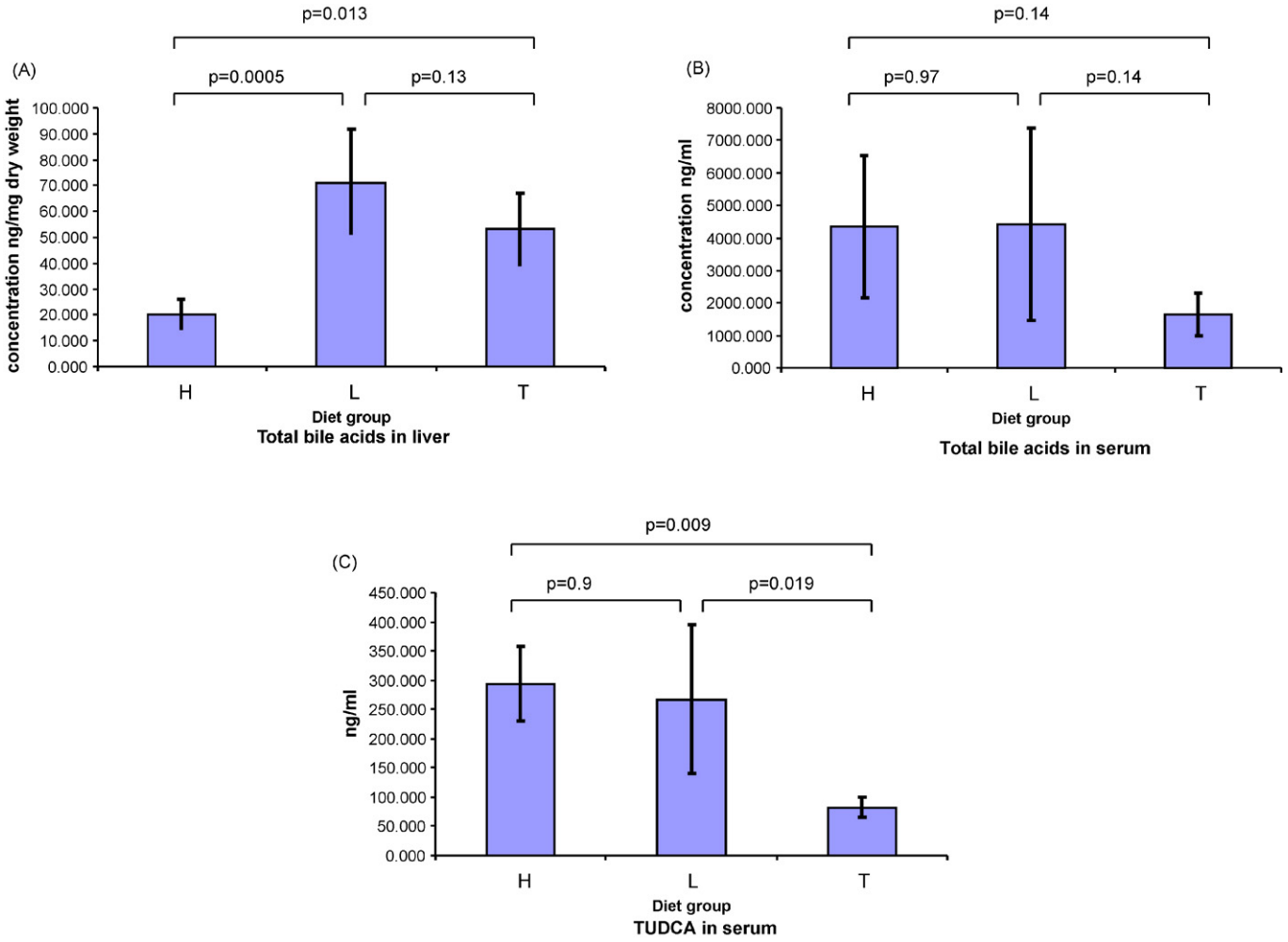


**Fig. 5.** Group averages and standard deviations of the total bile acids (calculated as sum of the individual bile acids) as determined in (A) mouse liver and (B) mouse serum. (C) Group averages and standard deviations of TUDCA as determined in the mouse serum samples.

approach where accurate mass is used with a window of ~2 ppm. We would like to stress that proper calibration of the instrument before the start of the study is very important, but even for studies running for >250 h additional calibration during the study was not required. The R.S.D. of the retention time in urine was <0.2%.

### 3.3. Application to liver and serum

The validated LTQ-FTMS method was applied to liver and plasma samples of a mouse study to evaluate whether feeding of increasing concentrations of dietary cholesterol would affect intrahepatic

**Table 5**
Summary of the bile acids detected in mouse serum and plasma

| | (Proposed) name | Retention time (min) | $[M-H]^-$ | Mol formula | Mouse serum | Mouse liver |
|---|---|---|---|---|---|---|
| LCA | Lithocholic acid[a] | 22.7 | 375.2900 | $C_{24}H_{40}O_3$ | n.d. | n.d. |
| CDCA | Chenodeoxycholic acid[a] | 18.4 | 391.2854 | $C_{24}H_{40}O_4$ | n.d. | n.d. |
| DCA | Deoxycholic acid[a] | 18.7 | 391.2854 | $C_{24}H_{40}O_4$ | + | n.d. |
| UDCA | Ursodeoxycholic acid[a] | 13.7 | 391.2854 | $C_{24}H_{40}O_4$ | + | n.d. |
| CA | Cholic acid[a] | 14.9 | 407.2803 | $C_{24}H_{40}O_5$ | + | + |
| GCDCA | Glycochenodeoxycholic acid[a] | 16.6 | 448.3068 | $C_{26}H_{43}NO_5$ | n.d. | n.d. |
| GDCA | Glycodeoxycholic acid[a] | 17.3 | 448.3068 | $C_{26}H_{43}NO_5$ | n.d. | n.d. |
| GCA | Glycocholic acid[a] | 14.2 | 464.3018 | $C_{26}H_{43}NO_6$ | + | n.d. |
| TDCA | Taurodeoxycholic acid[a] | 17.1 | 498.2885 | $C_{26}H_{45}NO_6S$ | + | + |
| TCDCA | Taurochenodeoxycholic acid[a] | 16.6 | 498.2885 | $C_{26}H_{45}NO_6S$ | + | + |
| TUDCA | Tauroursodeoxycholic acid[a] | 13.1 | 498.2885 | $C_{26}H_{45}NO_6S$ | + | + |
| GLCA-3S | Glycolithocholic acid-3-sulfate[a] | 13.6 | 512.2687 | $C_{26}H_{43}NO_7S$ | + | n.d. |
| TCA | Taurocholic acid[a] | 14.1 | 514.2844 | $C_{26}H_{45}NO_7S$ | + | + |
| MCA | Muricholic acid[b] | 12.8 | 407.2803 | $C_{24}H_{40}O_5$ | + | + |
| TMCA | Tauromuricholic acid (proposed) | 11.9 | 514.2844 | $C_{26}H_{45}NO_7S$ | + | + |
| THDCA | Taurohyodeoxycholic acid (proposed) | 12.2 | 498.2895 | $C_{26}H_{45}NO_6S$ | + | + |

n.d., not detected.
[a] Standard available for calibration.
[b] Identified based on literature and confirmed with standard.

and plasma/serum bile acid levels. In liver we observed a statistically significant difference between the % of the dry weight after lyophilization between the control and the low cholesterol diet groups (average dry weight of 38%) and the high cholesterol diet group (average dry matter 56%). For both TvsH and LvsH the $p$ value was <0.0003, showing that a moderate increase of cholesterol in the diet results in a significantly higher dry matter (lipid) content of the liver.

Despite the small volumes and amounts of sample used (60 μL serum and 5 mg dry liver) many bile acids were detected in one or both matrices. In addition to the bile acids available as standards, several unknown peaks were detected (Fig. 4). Several of the unknown peaks have the same accurate mass and elemental composition as the analyzed bile acid standards and differ only in the retention time (Fig. 4) it was therefore assumed that they were bile acids. An overview is given in Table 5. Based on literature, one of the peaks was identified as muricholic acid. The identity was confirmed by analyzing a standard (data not shown) and muricholic acid was added as a calibration standard of this method. The other unknown bile acids could not be confirmed with standards but can either be different isomers of taurodeoxycholic acid or fragments formed in the ion source from other bile acid conjugates.

Large inter-individual differences of the concentrations of bile acids were observed within all the groups, shown as standard deviation of the group averages in Fig. 5.

Differences in intrahepatic bile acid levels between the groups were statistically significant. This holds for most of the individual bile acids and the total bile acids (calculated as a sum of all the measured concentrations), shown in Fig. 5. For total bile acids, $p = 0.013$ for TvsH and $p = 0.0005$ for HvsL groups.

In serum, lower concentrations were found in the control group and the low cholesterol diet, higher concentrations were found in the high cholesterol diet group. However, if the total bile acid concentration is evaluated by ANOVA, the differences are statistically not relevant ($p > 0.1$). After statistical evaluation of the individual bile acids, two taurine conjugated bile acids, TCDCA and TUDCA show statistically significant differences between the H and T groups, as is shown in Fig. 5 for TUDCA.

The observed large inter-individual differences in relative concentrations of the bile acids most probably reflect biological variation between individual mice rather than methodological variation, since comparable variations were seen for other biological parameters [19]. The different cholesterol intake results in differences in the bile acid production in the liver. Further interpretation of the biological relevance of the differences in the individual bile acid profiles is outside the scope of this paper and will be published elsewhere.

## 4. Conclusions

A new quantitative method for determination of bile acids by HPLC-MS with accurate mass is reported. A fully validated target method for 17 free and conjugated bile acids is combined with a comprehensive metabolomic profiling, generating (semi)quantitative data for many other endogenous metabolites in body fluids and tissues. The applied method used much smaller sample volumes and tissue amounts than most of the recently described methods. The straightforward sample preparation procedure can be applied in a high throughput manner. The accurate mass, in combination with retention time provides sufficient specificity for quantitative determination of all the studied bile acids, with the exception of HDCA. The performance of the method is adequate to detect subtle changes in bile acids at endogenous levels in biofluids and tissues. For other endogenous metabolites the method can be used for comparing samples from the same study.

Application of this method in a mouse study with different cholesterol diets revealed statistically significant differences between the intrahepatic and serum concentrations of bile acids in control and low *vs.* high cholesterol diet. The high quality data set generated in this study will be of significant use for further research directed at a better understanding of hepatic cholesterol homeostasis and intracellular cholesterol trafficking.

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