

3-15-2021

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[10.1016/j.aca.2021.338224](https://doi.org/10.1016/j.aca.2021.338224)

This is an author's accepted manuscript of: Shafaei, A., Rees, J., Christophersen, C. T., Devine, A., Broadhurst, D., & Boyce, M. C. (2021). Extraction and quantitative determination of bile acids in feces. *Analytica Chimica Acta*, 1150, article 338224. <https://doi.org/10.1016/j.aca.2021.338224>

This Journal Article is posted at Research Online.
<https://ro.ecu.edu.au/ecuworkspost2013/9566>

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Extraction and quantitative determination of bile acids in feces

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ABSTRACT

With rapid advances in gut microbiome research, fecal bile acids are increasingly being monitored as potential biomarkers of diet related disease susceptibility. As such, rapid, robust and reliable methods for their analysis are of increasing importance. Herein is described a simple extraction method for the analysis of bile acids in feces suitable for subsequent quantification by liquid chromatography and tandem mass spectrometry. A C18 column separated the analytes with excellent peak shape and retention time repeatability maintained across 800 injections. The intra-day and inter-day precision and accuracy was greater than 80%. Recoveries ranged from 83.58 to 122.41%. The limit of detection and limit of quantification were in the range 2.5 to 15 nM, respectively. The optimised method involved extracting bile acids from wet feces with minimal clean up. A second aliquot of fecal material was dried and weighed to correct for water content. Extracting from dried feces showed reduced recovery that could be corrected for by spiking the feces with deuterated standards prior to drying. Storage of the extracts and standards in a refrigerated autosampler prior to analysis on the LC-MS is necessary. Multiple freeze-thaws of both standards and standards lead to poor recoveries for some bile acids. The method was successfully applied to 100 human fecal samples.

Keywords: Bile acids; Fecal; LC-MS/MS; Extraction; Stability

1. Introduction

Bile acids (BA) are water-soluble, amphipathic primary end products of cholesterol metabolism that play an essential role in cholesterol homeostasis as well as the digestion and absorption of dietary lipids [1,2]. Furthermore, they are involved in multiple physiological processes such as the regulation of lipid, glucose and energy metabolism, prevention of bacterial overgrowth, facilitation of intestinal calcium absorption and have recently been described as important signaling molecules central to maintenance of human health [3,4]. During their passage through the intestinal tract BA undergo a series of transformations and the majority (95%) are reabsorbed and recycled by the enterohepatic circulation. The ~5% that reach the colon are modified due to their interactions with the gut microbiota and are excreted in the feces. The complex interplay between BA and the gut microbiota has gained increasing attention as it is now understood to have significant influence on the pathophysiology of many diseases [3]. Fecal BA profiles have been purported to be biomarkers of disease susceptibility, therefore, robust and accurate analysis of BA in feces is important. However, the multiple isometric variations of BA structure and their chemical properties have presented challenges in their separation and detection [3].

A range of techniques have been reported for the analysis of BA including HPLC with UV detection, gas chromatography coupled with flame ionisation (GC-FID) and more recently gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) [3,5–8]. The latter is the technique of choice as it allows for separation of configurational isomers; the BA form stable negatively charged ions that can be sensitively detected by MS and, unlike for GC, requires no derivatisation step [3,5,6].

For LC-MS analysis, fecal samples are typically spiked with one or more isotopically labelled bile acid standard as part of the extraction process. Separation is generally achieved using a

C18 column and gradient elution using a buffered acetonitrile water mobile phase. MS detection is possible for conjugated BA, as glycine and taurine moieties give stable $[M - H]^-$ fragments [3,6]. Selected Ion Recording (SIR) is necessary for unconjugated BA as no stable product ions are usually found and therefore the same parent and product ions are employed [3,6].

An earlier, comprehensive study by Setchell et al (1987) highlighted the lack of homogeneity of human fecal samples as well as observing significant day to day BA fluctuations [7]. Therefore, for best practice it is recommended that multiple samples be collected over a specific timeframe, pooled together and thoroughly homogenised before extraction [2,7]. As feces is a complex matrix some effort has been spent developing methods for sample preparation. These have been comprehensively reviewed and reported elsewhere [5]. In brief, some studies involve preparation of wet fecal samples [9], but a more popular choice appears to be lyophilisation and re-constitution [8,10,11]. Most sample preparation methods involve a solvent extraction step, followed by solid phase extraction to further purify samples and eliminate contamination of the column and the mass spectrometer [5]. More recently, Franco et al (2019) extracted oxobile acids in wet fecal samples with isopropanol and omitted any sample clean-up step, and did not report any detriment to results or instrumentation.

For large studies the stability of the BA post extraction is also of interest, but interestingly does not feature in the literature. The complexity and variability of the sample matrix, the large concentration range reported for BA and the lack of a standardised protocol make comparisons between studies very difficult. Our aim was to develop a robust, simple method suitable for studies involving large cohorts. A secondary aim was to understand the stability of the extracts and standards post extraction and prior to analysis.

2. Materials and methods

2.1. Chemicals

LC-MS grade water, methanol and acetonitrile were purchased from Thermo Fisher Scientific (Sydney, Australia). Chenodeoxycholic acid (2,2,4,4-d4) (CDCA-d4), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), glycodeoxycholic acid (GDCA), glycocholic acid (GCA), glycolithocholic acid (GLCA), taurochenodeoxycholic acid (TCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), tauroursodeoxycholic acid (TUDCA) were purchased from Toronto Research Chemicals (Toronto, Canada). Lithocholic acid (2,2,4,4-d4) (LCA-d4) and taurodeoxycholic acid (2,2,4,4-d4) (TDCA-d4) were obtained from Cambridge Isotope Laboratories, Inc. (Massachusetts, USA). Deoxycholic acid (2,2,4,4-d4) (DCA-d4) was purchased from Sigma- Aldrich (NSW, Australia).

2.2. Preparation of standards

Individual stock solutions (2.5 mM) of 12 target bile acids (DCA, CDCA, UDCA, CA, GCA, GLCA, GDCA, LCA, TUDCA, TCDCA, TDCA, and TCA) and 4 labelled internal standards (DCA-d4, LC-d4, CDCA-d4 and TDCA-d4) were prepared in methanol and stored until use at -80 °C. Mixed calibration standard solutions (nine) containing all 12 target BA at the concentration range of 97.66 – 25,000 nM for UDCA, CA, GCA, GLCA, GDCA, TUDCA, TCDCA, and TDCA; 585.94 – 300,000 nM for LCA and DCA; and 195.31 – 100,000 nM for CDCA and labelled internal standards (5000 nM of TDCA-d4 and CDCA-d4, and 10000 nM of LCA-d4 and DCA-d4) were prepared in 0.1 % aqueous formic acid.

Standards for precision and accuracy (PAS) experiments were prepared in water containing 0.1 % formic acid at 4 different concentrations to cover a large concentration range (low to high); PAS1 to PAS4 for CA, GCA, TCA, TCDCA, TDCA, TUDCA and UDCA were 195.31, 390.63, 1562.5, and 6250 nM; for GDCA and GLCA were 390.63, 1562.5, 6250 and 25000

nM; for CDCA were 781.25, 1562.50, 6250, and 25000 nM; and for LCA and DCA were 585.94, 2343.75, 4687.50, and 18750 nM, respectively.

Mixed standards solutions at low (LS), medium (MS) and high (HS) concentration were prepared for the recovery and accuracy experiments. The LS, MS and HS for CDCA, CA, GCA, GDCA, GLCA, TCA, TCDCA, TDCA, TUDCA and UDCA were 200, 2000, and 20000 nM, and for LCA and DCA were 1000, 10000, and 100000 nM, respectively, and were prepared in water containing 0.1 % formic acid.

For the stability study, the following standards were prepared in 0.1 % aqueous formic acid: SS1 to SS5 were 195.31, 781.25, 3125, 12500, and 50000 nM for CA, GCA, GDCA, GLCA, TCA, TCDCA, TDCA, TUDCA and UDCA, 781.25, 3125, 12500, and 50000 nM for CDCA, and 2343.75, 9375, 37500, 150000, and 600000 nM for LCA and DCA, respectively.

2.3. Instrumentation conditions

Chromatographic separation was performed on an Ulti-Mate 3000 Liquid Chromatograph (Thermo Scientific, CA, USA) coupled to a Thermo Scientific TSQ Quantiva Triple Quadrupole Mass Spectrometer equipped with an ESI source. The optimal separation was achieved on ACE C18 (100 mm × 2.1 mm ID; Advanced Chromatography Technologies, Scotland) with 1.7 μm particles and a mobile phase of water containing 0.1% formic acid (A) and acetonitrile (B). The gradient separation was completed in 14 min with the initial conditions of 99.9 % solvent A and 0.1 % solvent B. The initial conditions were held for 1 min, then solvent B was increased to 65 % over 3 min followed by a further rise to 99.9 % over 9 min. Mobile phase B was returned to its initial conditions over 0.5 min before the column was re-equilibrated for 1.5 min. The flow rate was 0.2 mL min⁻¹ and the column temperature was maintained at 40 °C with an injection volume of 2 μL.

The detection was performed in negative mode (2500 V) and the spectra were acquired in multiple reaction monitoring (MRM) mode. The cycle time was set at 0.5 s and the dwell time ranged from 20.8 to 26 ms for all analytes. Argon gas was selected as collision gas and nitrogen as nebulizer and heater gas. The MS conditions were gases (arbitrary units) sheath 35, auxiliary 15, sweep 0; ion transfer temperature 325 °C and vaporizer temperature 275 °C. For optimisation of MS parameters, individual standard solutions were prepared in methanol (5 µM) and introduced into (-)-ESI source by direct infusion at a flow rate of 20 µL min⁻¹. A summary of optimal MS parameters for each standard and internal standard including precursor ion, product ions transitions for BA with stable adequate fragment ions, selected ion recording for BA with unstable fragment ions, and collision energies are presented in Table S-1.

2.4. Fecal sample preparation

Fecal samples were collected from all bowel motions over a 24 h period. If more than one stool sample was collected, they were homogenised as individual samples and then pooled and homogenised again. Aliquots were transferred into 2 mL screw top storage tubes and stored at -80 °C until analysis.

Each accurately weighed (approximately 0.5 g) wet fecal aliquot was thawed and extracted with 1.00 mL ice-cold methanol containing internal standards (5,000 nM of TDCA-d4 and CDCA-d4, and 10,000 nM of LCA-d4 and DCA-d4). The sample was shaken for 30 min at 4 °C and centrifuged at 21,000 rpm for 20 min. The supernatant (100 µL) was transferred to a separate 1.5 mL low-bind microcentrifuge tube with boil-proof cap to ensure maximum sample recovery and diluted v/v (1:5) with 0.1 % aqueous formic acid solution. The extract was then filtered through 0.22 µm polypropylene syringe filter and transferred to 2 mL amber glass short thread LC vial with 0.1 mL clear glass micro insert for analysis.

To obtain the dried weight of each fecal sample analysed, a duplicate 0.5 g of each wet fecal sample was dry-lyophilised using a freeze dryer (Sublimate 2, Esco E.U.) and the content of water in each sample was calculated by comparing the weight of the feces sample before and after drying. The normalised dried weight (in g) was used for calculation of the final BA concentration in fecal samples.

The BA concentration in wet feces was expressed as nmoles L⁻¹ of extract, which was then converted to nmoles g⁻¹ of dry feces by applying the following formula:

$$C = C_0 \times \frac{V}{m} \quad (1)$$

Where C is expressed as concentration in nmoles g⁻¹ of feces, C₀ is the measured concentration as nmoles L⁻¹ of ex-tract, V is the volume of extraction solvent in L, and m is the weight of dried feces in g.

2.5. Optimization of stationary phase

Several columns including a Thermo Scientific Accucore™ 150 Amide HILIC (100 × 2.1 mm; 2.6 μm) column, a Thermo Scientific Synchronis™ HILIC (100 × 2.1 mm; 1.7 μm) column, an ACE C18 PFP (100 × 2.1 mm; 1.7 μm) column, an ACE C18 AR (100 × 2.1 mm; 1.7 μm) column, a Thermo Scientific Hypersil GOLD™ aQ (100 × 2.1 mm; 1.9 μm) column and an ACE C18 (100 × 2.1 mm; 1.7 μm) column were tested to obtain the best separation of the targeted bile acids. All reversed phase stationary phases were tested with water and ACN containing 0.1% formic acid as the mobile phase. HILIC stationary phases were tested with ACN: water containing 15 mM ammonium acetate adjusted to pH 5 with acetic acid as a mobile phase.

2.6. Optimization of extraction temperature

To determine the optimum extraction temperature a comparison between cold and warm extraction was conducted. Wet fecal samples were extracted in duplicate with ice-cold (4 °C) methanol and with 37 °C methanol containing internal standards as described in section 2.4. Percentage agreement of the target bile acids from cold extraction versus warm extraction were calculated using equation (2), where C_{cold} is the concentration of the given analyte in the cold extraction and C_{warm} is the concentration for the warm extraction.

$$Percentage\ Agreement = \frac{C_{cold}}{C_{warm}} \times 100\% \quad (2)$$

The mean percentage agreement was calculated for each bile acid extracted at two different temperatures.

2.7. Extraction recovery

The extraction recovery or apparent recovery was estimated by spiking 10 µL of LS, MS, HS BA standards (section 2.2) into 3 different fecal samples (0.5 g). The spiked fecal samples were then extracted using 1 mL ice-cold methanol containing internal standards as described in section 2.4. The baseline (unspiked) and spiked feces samples were analysed and percentage absolute recovery was calculated according to equation (3), where $C_{baseline}$ is the calculated unspiked analyte concentration, $C_{recovered}$ is the calculated spiked analyte concentration, and C_{spiked} is the absolute concentration of spiked standard added to the sample.

$$Relative\ Recovery = \frac{C_{recovered} - C_{baseline}}{C_{spiked}} \times 100\% \quad (3)$$

This experiment was repeated in triplicate.

2.8. Clean-up

The optimal pre-analysis clean-up method was investigated: liquid-liquid extraction and a method using a 3kDa molecular weight spin cut-off filter (Amicon Ultra, 0.5 mL Sigma-Aldrich, Cork, Ireland) were tested on methanol extracts from five wet fecal samples.

For liquid-liquid extraction, the BA were isolated from 500 μ L of fecal methanol extract (diluted 5 times with water) with 500 μ L n-hexane (3 times). The n-hexane layers were collected and dried under a nitrogen stream. The hexane residue was reconstituted with 250 μ L 0.1% aqueous formic acid and transferred to the LC vial for analysis. For clean-up using the 3 kDa cut-off filter, 300 μ L of diluted (1 in 5) fecal extracts were filtered and centrifuged for 20 min at 4 $^{\circ}$ C and 21000 rpm. The filtrates were then transferred to the LC vials for analysis.

2.9. Comparison of extraction of wet and dry fecal samples

To compare dried feces versus wet feces extraction methods, wet, dried and spike-dried fecal samples were prepared. A pooled fecal sample was prepared by combining 5g aliquots from 6 individual wet samples. After thorough homogenisation 12 aliquots (0.5 g each) were treated as follows; 4 aliquots (D) were dry-lyophilised using a freeze dryer; a further 4 aliquots (SD) were spiked with four internal standards and then dry-lyophilised; and another 4 aliquots (W) were frozen at -80 $^{\circ}$ C until further analysis. The eight dried fecal extracts were rehydrated to contain water levels consistent with the wet fecal sample.

The four (SD) that were spiked prior to drying were extracted with ice cold methanol while the four (D) un-spiked dried fecal samples were extracted with ice cold methanol containing the internal standards. The four frozen wet fecal samples (W) were thawed and extracted with 1 mL ice-cold methanol containing internal standards. All 12 samples were analysed and the mean percentage agreement for the BA recorded for each extraction method were calculated by applying equation (4), where C_{dried} is the analyte concentration after rehydrating the dried spiked or dried un-spiked sample, and C_{wet} is the analyte concentration in the wet sample.

$$\text{Agreement} = \frac{C_{dried}}{C_{wet}} \times 100\%. \quad (4)$$

2.10. Method validation

The method was validated in accordance with ICH and IUPAC guidelines [12,13].

2.10.1. Linearity, limit of detection and limit of quantification

The linearity of the method was evaluated by injecting the nine calibration standards (see section 2.2) and plotting the peak area ratio of each analyte to the internal standard (y-axis) versus the concentration (x-axis). Limit of detection (LOD) and the limit of quantification (LOQ) of the method were expressed as concentration with S/N equal to 3 and 10, respectively.

2.10.2. Precision, trueness, and accuracy

Method precision and trueness were evaluated by intra-day and inter-day analyses of a set of BA standards (PAS). For intra-day and inter-day analysis, the PASs were injected six times per day and for 6 consecutive days, respectively. The resulting concentrations of the replicate analysis were used to calculate the coefficient of variance (% CV) and thus precision. Subsequently, the mean concentration relative to the nominal concentration was used to calculate % bias and hence the trueness of the method.

Accuracy was evaluated by spiking 10 μ L of LS, MS and HS BA standard solutions (see section 2.2) into 3 separate aliquots of pooled fecal extract (as described in section 2.9). The baseline (un-spiked) and spiked extracts were analysed, and percentage apparent recovery was calculated according to equation (4).

2.10.3. Matrix effect

To evaluate potential matrix effects, and in the absence of a stripped fecal extract (fecal matrix without the BA), the following approach was adopted: a pooled fecal sample was extracted as per the protocol (section 2.4) and the concentration of BA determined. A solution of BA of similar concentration to this fecal sample was then prepared in water with 0.1 % formic acid (no attempt to matrix match). The fecal extract and BA solution were then both spiked with 10 μ L of HS (see section 2.2) and the detector response compared for both and the percentage matrix effect (matrix ion suppression/enhancement) was calculated using equation (5), where R_{matrix} is the response of the given analyte in matrix and $R_{standard}$ is the response in standard solution.

$$ME = \frac{R_{matrix} - R_{standard}}{R_{standard}} \times 100\%. \quad (5)$$

If $ME \sim 0\%$ there is no observed matrix effect, and if $ME > 0\%$ then an ion-enhancement occurred, and if $ME < 0\%$ an ion-suppression occurred.

2.10.4. Short-term storage stability

To investigate the effects of pre-analytical storage on subsequent BA recovery, BA standards (SS1 to SS5) and 4 fecal sample extracts (Ext 1 to Ext 4) were prepared using the optimized method and stored in 2 mL amber glass short thread LC vial with 0.1 mL clear glass micro insert under 2 different storage conditions: at 6 $^{\circ}$ C in the autosampler and at room temperature (22-25 $^{\circ}$ C) for 12 and 24 hours. The baseline ($t=0$) and $t=12$ h and $t=24$ h extracts were analysed, and percentage relative recovery was calculated according to equation (6), where C_0 is the measured analyte concentration at baseline ($t = 0$) and C_t is the concentration at $t = 12$ or 24h.

$$Recovery_t = \frac{C_t}{C_0} \times 100\% \quad (6)$$

Two-way repeated measure analysis of variance (RM-ANOVA) was performed to investigate the interaction of time and sample type (SS vs Ext) and to determine whether storage conditions have a detrimental effect of recovery efficiency.

2.10.5. Long-term storage stability

To investigate the effects of multiple freeze thaw cycles on subsequent BA recovery, standards (SS1 to SS5) and fecal sample extracts (Ext 1 to Ext 4) were again prepared using the optimized method, stored in 2 mL amber glass short thread LC vial with 0.1 mL clear glass micro insert and then exposed to up to three -80 °C freeze-thaw cycles. The baseline (fresh samples) and 3 freeze/thaw extracts were analysed, and percentage relative recovery was calculated according to equation (6), where C_0 is the measured analyte concentration at baseline and C_t is the concentration at $t = 1, 2$ and 3 freeze/thaw cycles. Two-way RM-ANOVA was performed to investigate the interaction of number of freeze/thaw cycles and sample type (SS vs Ext) and to determine whether freeze/thaw cycles have a detrimental effect of recovery efficiency.

2.11. Application

The BA extraction and detection method was employed for quantification of target BA in 100 fecal samples from healthy adult individuals. The measured concentration (nM) was normalised to dried fecal weight (g), thus converted to nmol g⁻¹.

3. Results and Discussion

3.1. Analytical separation method development

Two HILIC and four reversed phase separation columns were trialled for the separation of the target BA. As the analytes were poorly retained on the HILIC columns tested (Thermo Scientific Accucore™ 150 Amide HILIC and Thermo Scientific Synchronis™ HILIC) and there were several instances of coelution, these columns were not explored further (data not

provided). The four reversed phase columns including two C18 columns (ACE C18, Thermo Scientific Hypersil GOLD™ aQ) and two C18 columns with embedded functionalities, specifically aromatic functionality (ACE C18 AR) and pentafluorophenyl rings (ACE C18_PFP) were investigated for separation of the targeted BA and, in particular, the pairs CDCA/DCA and TCDCA/TDCA which form negative molecular ions of the same mass/charge ratio 391 and 498 respectively. A simple mobile phase gradient consisting of acetonitrile and water with 0.1 % formic acid was used for the separations. Yin et al (2017) recommended the use of a weakly acidic mobile phase (pH 3-4) and the absence of ammonium based buffers to maximise separation and formation of negative ions respectively [6]. Also, acidic mobile phases (pH ~ 4) of aqueous acetonitrile and/or methanol with formic acid, where taurine conjugates are deprotonated and glycine and unconjugated bile acids exist predominantly in their unionised form, were recommended by Griffiths and Sjövall (2010) [2]. While close to baseline separation was observed for one of two pairs (CDCA/DCA or TCDCA/TDCA) using the ACE C18 PFP or ACE C18 AR columns, baseline resolution of both BA pairs was achieved using the ACE C18 column. Resolution was calculated as 1.20 and 1.04 for CDCA/DCA and TCDCA/TDCA respectively with excellent symmetrical peaks (Table S-2). Furthermore, the excellent peak shape, and resolution of these pairs was maintained after 800 injections (Fig. 1 and Table S-2).

3.2. Comparison of fecal sample preparation and bile acids extraction methods

A range of methods have been reported for extraction of BA from feces, several of which are not particularly amenable to large scale studies such as refluxing feces sample in alkaline ethanol solution at elevated temperature (80 °C) for 1 h, ultra-sonication for 1h or alkaline hydrolysis using 1 N NaOH followed by long incubation (2.5 h) [8,11]. Franco et al. reported a simple extraction involving wet feces and isopropanol (optimal for oxo-bile acids) at 37 °C [9]. A modified approach was adopted here where wet feces was extracted in methanol, and

the need to use heat to aid extraction as described by Franco et al. was investigated: six fecal samples were extracted at two temperatures (4 °C and 37 °C). The mean recovery of the BA from the cold extraction when compared to the warm extraction ranged from 96.40 to 103.62 % with CV 1.32-5.25 % (Table S-3) indicating that the extraction was not influenced by the temperature. Also, an inspection of the chromatograms generated in full scan MS mode for the 4 °C and 37 °C extracts determined that the cold extraction provided a cleaner spectrum.

The extraction efficiency of the method was then tested. Aliquots of three different fecal samples were spiked with varying concentrations of the BA (zero, low, medium and high concentrations of BA) and then extracted as per the protocol. The apparent recovery of the investigated BA was acceptable and in the range 83.58 to 122.41 % (Table S-4) [12,13].

Two clean-up methods, liquid-liquid extraction with n-hexane and extraction with a 3 kDa cut-off filter were trialled and compared with no clean-up of the extracts other than filtration through a 0.22 µm filter. For all the BA there was loss of analyte when a clean-up method was applied, and the loss of analyte was usually greater when the 3kDa filter applied (Fig. S-1). For example, the signal for LCA, one of the key BA in human feces, reduced by more than 300 times after liquid-liquid extraction and was not detected at all after use of the 3kDa filter compared to no clean-up extracts. The glycine conjugated BA are typically present in relatively low concentrations, and both clean-up methods resulted in a reduction in signal for GDCA and GCA and total loss of signal for GLCA. Similarly, the use of a clean-up method compromised the detection of TDCA and TCA which are typically present in low concentrations in human feces. Therefore, a clean-up method was not adopted, and the possible impact of no clean-up on the column and the MS signal over time was monitored. The loss of resolution of the BA, increase in column pressure and loss of MS signal consequences that can be associated with no sample clean-up was not evident in over 800 injections.

Our goal was to develop a robust, high throughput method for extraction and quantification of BA in human feces that maintained sample integrity, hence our approach to extract from wet feces. The variable water content in feces is well documented, and to be able to report BA concentrations that are independent of water content, a second aliquot of fecal material was dried to determine and correct for water content. The duplicate preparations also allowed presentation of BA data as wet or dried weight, allowing for greater comparisons with the literature. The BA concentrations for 100 wet fecal samples when converted to dry weight (Table 1), were consistently higher than those re-reported in the literature for dried feces [8,10,11,14]. It is well recognised and reported that BA concentrations vary dramatically between individuals and even for a given individual [7]. Furthermore, meaningful comparisons between studies is often difficult with differences in extraction, type of sample (healthy vs diseased etc), population size, reporting format (mean versus median) and unit of BA concentration reported (nmol g^{-1} ; ug mL^{-1} dried or wet). However, the differing concentrations for wet and dried feces did prompt an investigation into the impact of drying the fecal sample prior to extraction on BA recovery. An initial experiment involved lyophilising aliquots of pooled fecal material, re-hydrating with water and then extracting with methanol as per the wet fecal protocol. Aliquots of the same pooled, but wet fecal material, were extracted as per the protocol for comparison purposes. The concentrations of BA were significantly lower in the dried extracts when compared to the wet fecal extracts (data not shown) and this was true for all of the BA, so much so that the experiment was repeated, but with an extra variable. In the follow up experiment a further four aliquots of pooled wet feces were spiked with internal standards prior to drying. The recovery of the two main BA in human feces, LCA and DCA, in the dried ex-traction was 60 and 70 % less (respectively) of that detected in the wet fecal extraction. The recovery for CDCA and CA from dried feces was 50% that recovered from wet feces. The recovery of the more minor BA, the taurine and glycine conjugated bile acids were

even less: the glycine conjugated BA recoveries were between 0.2 and 23 % (Fig. 2). To confirm that the low concentration recorded for the dried extracts was not a detection issue, the LOQ for the BA in fecal samples was determined. The LOQ for the taurine conjugated BA were at least 10 times lower than the concentrations reported for the dried fecal samples (see Table 2 for LOD and LOQ data). The taurine conjugated BA recoveries from the dried fecal extracts were just 2% or less, and a check of LOQ data confirmed that it was not due to a detection issue. The aim of this work was not about developing an extraction method for dried fecal material, so no attempt was made to optimise the extraction. Undoubtedly, a longer extraction time, and/or sonication or heat might improve extraction efficiency. However, these results do highlight the potential for reduced and inconsistent bile acid recovery from dried fecal samples. Fortunately, the inclusion of internal standards in the feces prior to drying was able to largely correct for the poor extraction efficiency of BA. Percentage agreement between dried fecal extraction after correction using the internal standards was between 78 -115 % of the wet fecal extracts for all BA (Table 5-S). These findings highlight how sample preparation can significantly impact extraction efficiency and may explain some of the variation in concentrations reported for the BA in the literature.

3.3. Linearity, limit of detection and limit of quantification

A 10-point calibration curve for each analyte was constructed by plotting analyte response (peak area of the analyte to the peak area of corresponding internal standard) versus concentration in nM. Linearity ($R^2 > 0.995$) was observed for all analytes in the concentration range 97.66-25,000 nM for UDCA, CA, GCA, GLCA, GDCA, TUDCA, TCDCA, and TDCA; 585.94-300,000 nM for LCA and DCA; and 195.31-100,000 nM for CDCA. Linear regression was used to interpolate the concentration of analytes in fecal extracts.

The limit of detection (LOD) is the lowest level of detectable analyte concentration that can be reliably distinguished from the background by an analytical system ($S/N = 3$). Whereas limit of quantification (LOQ) is the lowest quantifiable level of analytes that can be measured with an acceptable level of confidence ($S/N = 10$). The LODs and LOQs for all analytes were in the range 2.5-7.5 nM and 10 - 15 nM, respectively (Table 2), The LOQs recorded for the bile acids here are consistent with those reported by Humbert et al (2012) which ranged from 14.6-18.6 nM [10]. More useful is to relate the LOQ of the method to the concentration of BA in fecal extracts, which interestingly has rarely been reported. This measure of LOQ was an order to several orders of magnitude below the median bile acid concentrations reported for a study involving over 100 fecal samples (Tables 1 and 2).

3.4. Precision, trueness, and accuracy

Precision (%CV) and trueness (%bias) of the method were determined by intra-day and inter-day analyses of four PAS's (different concentration of calibration standards; PAS1, PAS2, PAS3 and PAS4). For intra-day analysis, six replicates of PAS's were injected in one day, whereas for inter-day, one replicate of each PAS were injected for six consecutive days. For both intra-day and inter-day analyses, the CV ranged from 0.62 to 19.41 % and bias ranged from -20.68 to 20.23 %, which complies with the acceptable limit (Table 6-S) [12,13]. These results indicated that the method is precise and accurate. Additionally, recoveries across three concentration ranges from pooled fecal extracts were calculated to evaluate the analytical method accuracy. The % apparent recovery ranged from 86.32 to 116.95 % which confirms the accuracy of the developed method (Table 7-S). These results were in agreement with the acceptance criteria for method recovery (100 ± 20 %) and also demonstrate an excellent alignment between expected and true values with the confidence intervals in the complex fecal matrix [12,13].

3.5. Matrix effect

In quantitative LC–MS analysis matrix effects are major concerns due to their dramatic effect on accuracy, precision and sensitivity of measurement especially when the concentration of target analyte is low such as the taurine conjugated BA in this study. Although, matrix effect cannot be completely avoided during LC-MS analysis, a number of strategies can be employed to correct for matrix effects [15]. In this study, labelled internal standards were used to correct for signal enhancement or suppression due to matrix effects and to correct for loss of analyte during sample preparation. Since isotopically labelled standards were not available for all studied BA, four isotopically labelled standards (TDCA-d4, CDCA-d4, LCA-d4 and DCA-d4) were used that reflected the different classes of BA. To evaluate potential matrix effects and to see if the applied internal standards were able to account for matrix effect in sample extracts, parallelism was assessed. Parallelism was evaluated by comparing the analyte response in post-extract spiked matrix to that of a standard solution at the same concentration as the spike. Ion suppression/enhancement was calculated to be less than $\pm 5\%$ for all target BA ranged -3.40 to 3.66% (Table 7-S).

These results revealed that the matrix effects were not significant and thus the applied internal standards were able to compensate for any loss or enhancement and therefore, matrix effects.

3.6. Stability

The stability of the BA standards and the BA in the fecal extracts was investigated. Five standards (SS1 to SS5) and four fecal extracts (Ext1 to Ext 4) were stored under three different conditions: at 6 °C in the LC auto sampler for 24 h; on the lab bench (23-25 °C; room temperature) for 24 h, and stored at -80 °C freeze but with up to three freeze-thaw cycles.

After storage in the autosampler at 6 °C for 24 h the mean percentage recoveries for the target BA in both the standards and the extracts ranged from 90.58-107.89 %, which is within ± 20

% and therefore acceptable [12,13] (Table 8-S). Furthermore, there was no statistically significant change in the mean percentage recoveries reported after 12 or 24 h storage at 6 °C (Fig. 3A). In contrast, the storage of the sample extracts and BA standards at room temperature significantly impacted the recovery of some BA. Specifically, the recoveries of CA, UDCA, TCA and GLCA in both the standards and the extracts were significantly and negatively impacted ($p_{condition} = 4.37 \times 10^{-6}$, 0.0004, 0.006, 1.44×10^{-7} , respectively) by storage at room temperature after just 24 h compared to 12 h (see Table 10-S). For example, the mean percentage recovery of CA after 12 h, irrespective of storage conditions and sample type, was around the ~100% mark. However, after 24 h both the extracts and standards stored at room temperature were less than 70 % recovery (see Fig. 3A). For GLCA the mean percentage recovery was ~100% for all samples stored at 6 °C at both the 12 h and 24 h time points. However, all samples stored at room temperature had reduced recovery to < 70%. For the bile acids CDCA, DCA, GCA, LCA, TCDCA and TDCA the mean % recovery after 24 h storage at room temperature ranged from 91 to 100 % and from 89 to 106 % for the standards and extracts respectively and was not statistically different to the recoveries reported for no storage, or storage at 6 °C. Interestingly, the results were different again for TUDCA and GDCA, where their mean percentage recoveries were significantly lower in the standards, but not in the extracts ($p = 0.0006$ and 0.040 , respectively). Comparative plots of all BA are provided in Fig. S-2. Recovery summary data for all BA are provided in Table 8-S. The instability of some BA in the sample extracts stored at room temperature may be due to the presence of bacteria in feces, which can modify the BA composition [7]. However, the use of MeOH as the extraction solvent should minimise bacterial growth in the sample extract. Storage of the sample extracts at low temperature is recommended to avoid possible bacterial degradation of BA structures.

The stability of the BA in standard solutions and in fecal extracts stored at -80 °C and subject to freeze-thaw cycles was also investigated. The percentage recovery of the BA in the extracts

and the standards varied with the specific BA and the number of freeze thaw cycles. After just one freeze-thaw cycle, all the BA, with the exception of GLCA in the extract, in both the extracts and the standard solutions, had mean recoveries of greater than 90 %. After three freeze-thaw cycles, CA, DCA, TCDCA and TUDCA maintained mean percentage recoveries of greater than 90%, while the recoveries for GCA, CDCA, TCA, TDCA, UDCA and GDCA were somewhat compromised with the extra freeze thaw cycles and reported mean percentage recoveries of between 74-105 %, and in some instances this lower recovery was statistically significant when compared to the recovery after just one freeze-thaw cycle ($p=$ 0.60, 0.003, 0.42, 0.08, 0.44, and 0.23, respectively; see Table 10-S). Both CDCA and GLCA had recoveries of less than 80 % for the extracts after three freeze thaw cycles. Comparative plots of all BA are provided in Fig. S-3. Recovery summary data for all BA are provided in Table 9-S.

While it is not clear why some analytes are more susceptible to varying storage conditions, this study does highlight the need for fecal extracts and standards to be queued in a refrigerated autosampler for LC-MS analysis to maintain analyte integrity. When longer term storage of standards and extracts is required prior to analysis, it is important to minimise freeze-thaw cycles. For standard solutions, sub-aliquoting is recommended to avoid multiple freeze thaws.

3.7. Application to fecal samples

The extraction of BA from wet feces and analysis on an ACE C18 column was successfully applied for quantification of the 12 BA in 100 fecal samples obtained from healthy individuals. A second aliquot of fecal material for each sample was dried and weighed to normalise for water content. The concentration range recorded for each BA is reported in Table 1. The literature reports for BA in fecal material tend to focus on just the four key BA (CA, CDCA,

DCA and LCA) and data is presented either as dry weight or wet weight, but not both, making comparisons difficult [8–11]. Therefore, here we have presented mean, median, % BA concentrations for all the BA and total primary and secondary BA concentrations (Table 1). We have included available data from the literature for comparison purposes [8,10,11,14]. In agreement with other studies, DCA and LCA are the two key BA present in feces and they contribute 80% or more of the total BA content. A comparison of our BA data (generated by extracting from wet feces but expressed as dry weight) with the available dry weight literature, highlights the higher concentrations (mean and median) reported for our study for DCA and LCA. This may be due purely to the cohort, given the large natural variation in BA concentrations, but it may also be due in part to the poor extraction recovery of BA from dried feces, as our study has highlighted. Importantly, the higher concentrations reported for our wet fecal extraction method, also ensured that the minor BA were efficiently extracted and quantifiable (Table 1).

4. Conclusions

We report here a robust, sensitive method for the quantitative determination of 12 BA from feces using LC-MS. The minimal sample preparation, extraction into methanol, makes the method applicable to studies with large cohorts. The extraction from wet feces maximises sample integrity and avoids issues of poor recovery experienced with dried fecal material. If the use of dried fecal material is necessary, then extraction efficiency and repeatability need to be tested, and spiking of the fecal material before drying is essential to capture any loss in recovery. The extracts must be stored cold (6 °C) prior to analysis and if analysis is not possible after extraction, the extracts need to be stored at -80 °C and multiple freeze-thaws are to be avoided.

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Table 1. Comparison of fecal bile acid concentrations in this study with other studies

	Ours (n = 100)					Humbert et al (2012) ⁹ (n = 19)			Kakiyama et al (2014) ¹¹ (n = 10)		Perwaiz et al (2002) ¹⁰ (n = 5)				Mouzaki et al (2016) ¹³ (n = 25)				
Method	LC-MS					LC-MS			LC-MS		GC-MS				LC-MS-MS				
Unit	nmol/g dried feces					nmol g ⁻¹ dried feces			umol g ⁻¹ dried feces		ug 100mg ⁻¹ dried feces				nmoles g ⁻¹ dries feces				
BA*	12					26			9		4				18				
	Concentration range		Mean	±	Median	Total (%)	Mean	±	Total (%)	Median	Total (%)	Mean	±	Total (%)	Mean	±	Total (%)	Median	Total (%)
CA	BLOQ** 2118.48	-	214.3 553	±	20.8	1.1	44.7 48	±	1.4	0.01	22.0	3.5 3.9	±	2.0	3.7 2.5	±	< 1	28	1.3
CDCA	10.98 4495.22	-	431.6 1065	±	81.4	2.1	54.8 72	±	1.7	0.0	0.0	-	-	-	-	-	-	45	2.1
DCA	202.37 32506.10	-	10536. 8491	±	8482.7	51.8	1920.1 1391	±	60.6	1.9	41.8	97.5 2.2	±	41.0	158.5 ± 4.2	±	37.0	1101	51.5
LCA	353.27 16656.03	-	6914.5 4498.7	±	6251.4	34.0	1016.6 647	±	32.1	1.5	33.1	155.5 ± 4.4	±	66.0	199.4 ± 3.0	±	46.0	952	44.5
UDCA	BLOQ** 1933.92	-	187.10 505	±	43.76	0.92	-	-	-	-	-	-	-	-	-	-	-	-	-
GDCA	5.42 167.65	-	43.26 43	±	30.04	0.21	-	-	-	-	-	-	-	-	-	-	-	-	-
GCA	BLOQ 98.13	-	1968.8 5879	±	6.47	9.68	-	-	-	-	-	-	-	-	-	-	-	-	-
GLCA	BLOQ 51.88	-	17.96 29	±	11.55	0.09	-	-	-	-	-	-	-	-	-	-	-	-	-
TCDC	BLOQ 25.00	-	5.16 6.6	±	2.91	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-
TCA	BLOQ 75.18	-	4.48 16	±	0.61	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-
TDCA	BLOQ 49.96	-	9.38 13	±	5.46	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-
TUDCA	BLOQ 486.30	-	8.11 ± 81	±	0.00	0.04	-	-	-	-	-	-	-	-	-	-	-	-	-
PBA***	-	-	2624.2 6055	±	208.4	12.9	137.7	±	4.3	-	-	-	-	-	-	-	-	65	3.0
SBA****	-	-	17716.9 12683	±	16548.6	87.1	2950.9	±	93.1	-	-	-	-	-	-	-	-	2063	96.5
Total BA	-	-	20341.1 15984.6	±	17097.5	-	3170.5	-	-	-	-	-	-	-	-	-	-	2138	-

*Number of detected bile acids, **BLOQ: Below Limit of Quantification, ***Primary bile acids, ****Secondary bile acids

Table 2. LOD and LOQ of the method

Compound	LOD (nM)*	LOQ (nM)*	LOD (nmole g ⁻¹)**	LOQ (nmoles g ⁻¹)**
CDCA	5	15	0.23	0.76
CA	5	15	0.34	1.15
DCA	2.5	10	0.14	0.46
GCA	5	15	0.14	0.48
GDCA	5	15	0.18	0.59
GLCA	5	15	0.25	0.82
LCA	2.5	10	0.45	1.51
TCA	7.5	12.5	0.07	0.23
TCDC	5	15	0.16	0.53
TDCA	5	15	0.28	0.93
TUDCA	5	15	0.08	0.25
UDCA	7.5	12.5	0.47	1.56

*Calculated from the standard solutions

**nmoles/g dry weight, calculated from fecal samples (n = 5)

Figure 1

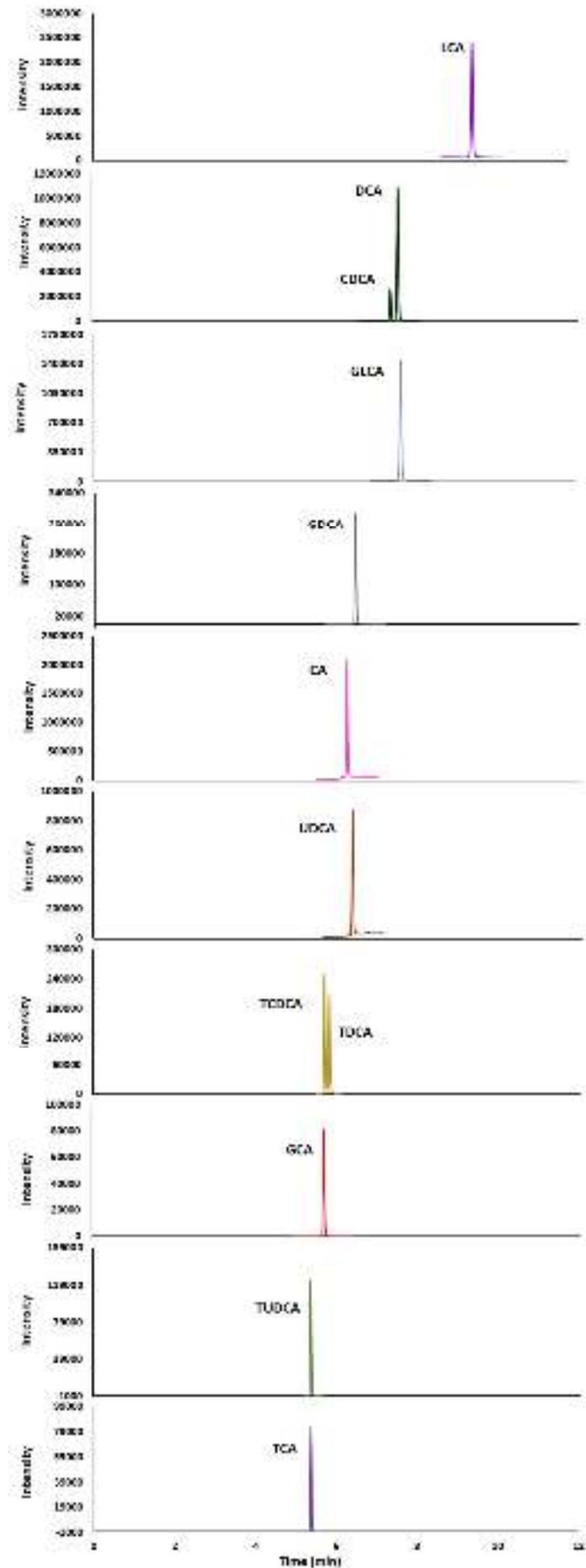


Figure 2

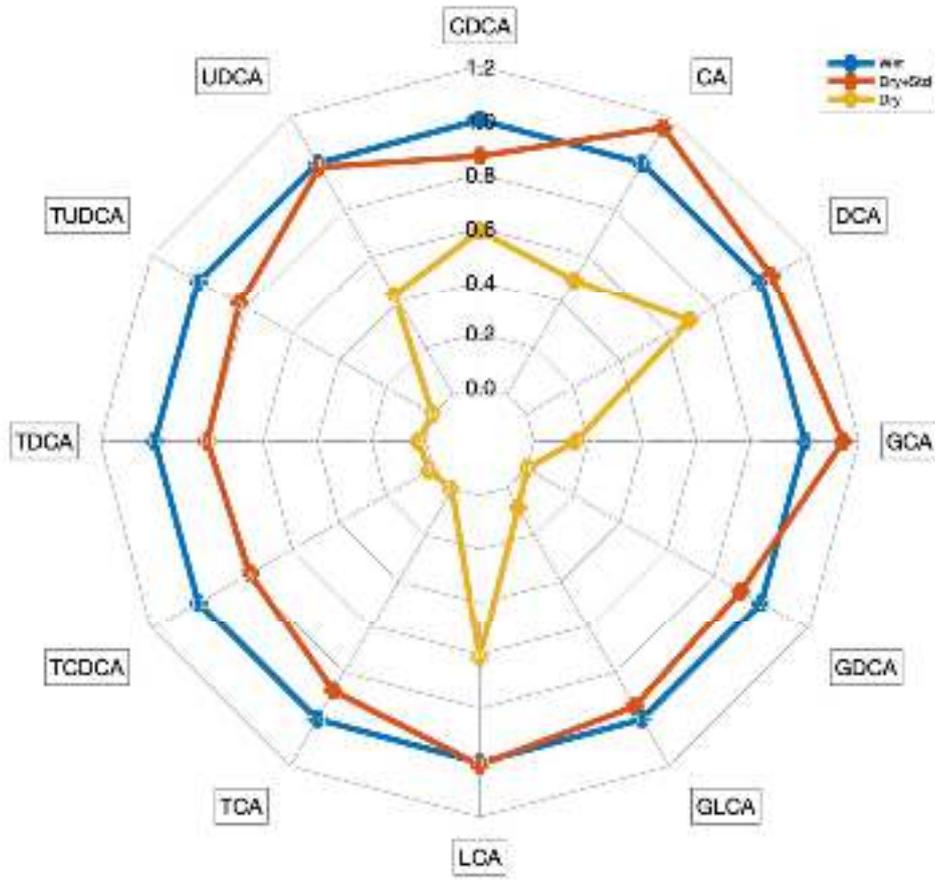


Figure 3

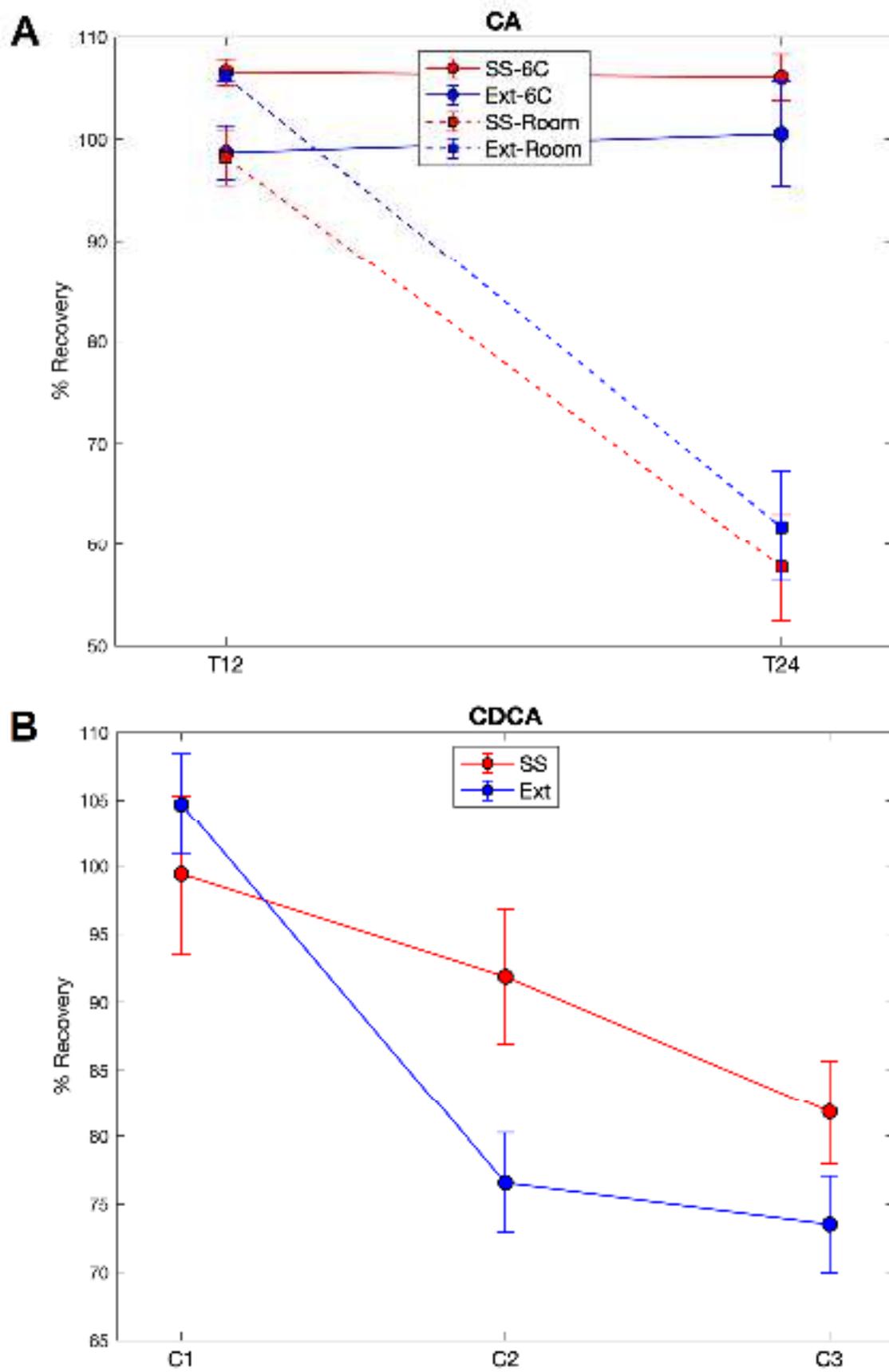


Figure Captions

Fig. 1. Total ion chromatograms of bile acid in fecal extract

Fig. 2. Recoveries of wet, dried and spike-dried extractions

Fig. 3. Stability of cholic acid (CA) in standard solutions and fecal extracts at 6°C and at room temperature over 24 h (A); and stability of chenodeoxycholic acid (CDCA) in standard solutions and fecal ex-tracts after three freeze-thaw cycles (B)