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A Simplified Method of Quantitating Selected Bile Acids in Human Stool by Gas Chromatography-Mass Spectrometry with a Single Extraction and One-Step Derivatization. Supplement: The Unusual Mass Spectral Behavior of Silylated Chenodeoxycholic Acid

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- Gas Chromatography
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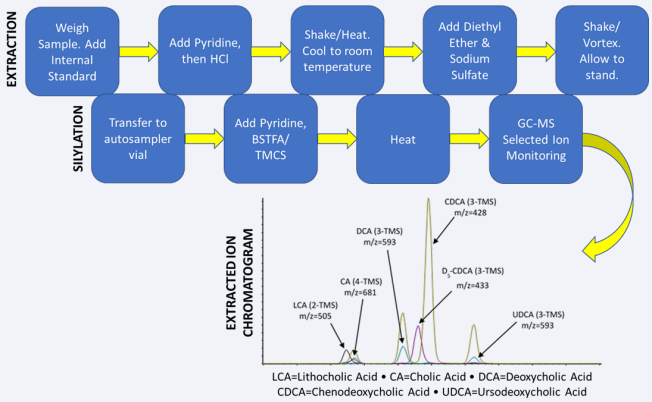
Abstract

A method for quantitating selected bile acids in human stool was developed for lithocholic acid (LCA), cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), and ursodeoxycholic acid (UDCA), using D5-chenodeoxycholic acid (D5-CDCA) as an internal standard. The method is a three-step process: extraction with hot pyridine and hydrochloric acid, extraction into diethyl ether, and derivatization (silylation) with BSTFA/TMCS. The extracts were analyzed by GC-MS operating in selected ion monitoring (SIM) mode. Separation was achieved with an Rtx-50 column followed by an Rtx-5MS column. The LDR (linear dynamic range) was 0.25 to 5.00 µmol/g. The lower limit of quantitation (LOQ) and the detection limit was 0.25 µmol/g. Interday and intraday precision were good, with most CVs less than 5%; Interday and intraday relative recoveries were also good, with most relative recoveries being between 90 and 110%. Interday precision and accuracy were similar without an internal standard.

Graphical Abstract



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INTRODUCTION

Importance of Bile Acids to Gut Health

Bile acids are major components of bile, synthesized in the liver from dietary fats. Intake of a high-fat diet stimulates hepatic bile acid synthesis resulting in production of greater quantities of primary bile acids that escape the enterohepatic circulation and enter the colon where they are converted to secondary bile acids by the 7α-dehydroxylating enzyme of the gut bacteria (mostly *Clostridia* species) [1,3,4]. The 7α-dehydroxylation products of the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA) are the secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. DCA and LCA have been strongly associated with colonic carcinogenesis in experimental studies [5], and high risk of colon cancer in human studies [1,2,6]. Thus, the bile acids are indicators of fat intake, and in turn, may be indicators of gut health.

The method described in this paper is a targeted analysis of the primary bile acids, CA and CDCA, and secondary bile acids, LCA and DCA in fecal samples using gas chromatography-mass spectrometry.

Previous Analytical Methods

Methods which use gas chromatographs for separation require derivatization of the bile acid molecules. Some analytical methods do silylate all the -OH groups with various silylation reagents, but not on actual samples [7,8]. One method uses silylation only, but on fecal sterols, not bile acids. [9] Many of these methods esterify, often by methylation, the carboxylic -OH prior to silylation of the hydroxyl groups on the molecule; often the methylating reagent used is an acid combined with methanol and a saponification step is typically used [10-19]. Still others use an enzymatic reaction to ultimately activate a fluorescing or UV agent to determine total bile acids [19,20]. An enzymatic agent is used to deconjugate conjugated bile acids followed by esterification [21]; however, Street (nee Lillington) was able to use GC-MS on conjugated bile acids by methylation/ silylation without deconjugating them [15-17,21] Many of these methods require multiple extraction and concentration steps, saponification, lyophilization, and pulverization of samples. Methods which use liquid chromatographs for separation do not always require derivatization; however, some still do if the detector is not a mass spectrometer [22-25,31]. An Agilent technical note claims separation of bile acids with no derivatization at all [26]. However, this seems unlikely, as the bile acids would most likely thermolyze before elution.

After assessing derivatization with BSTFA/TMCS, in various solvents—even in pure BSTFA/TMCS—and various times and temperatures, it was found that the presence of pyridine was necessary to drive the reaction to completion. Additionally, if pyridine was not used, it was found that the same molecule would yield up to several chromatographic peaks, each at different degrees of silylation. This is consistent with the Supelco® technical note [27,28], which considers pyridine to be the best solvent for the reaction. It is perhaps why some silylation reagents used for bile acids analysis are premixed with pyridine such as Sylon™ HTP (Supelco®). In this method, pyridine and BSTFA/TMCS (1:1 v/v) are used for complete silylation. This new method was developed after reviewing the noted references, as well as two reviews of bile acids analysis [29,30].

MATERIAL AND METHODS

Safety: Sample and standard preparations were carried out under a fume hood, including weighing the samples or standards. Safety glasses, with side shields, a lab jacket, and nitrile gloves, were worn at all times. After the procedure, surfaces were sanitized with a 10% bleach solution in water.

Analytical Instrumentation

Apparatus: Agilent GC-MS (Palo Alto, CA, USA)— Agilent 6890, 7683 autosampler, 5973 MSD; Carrier gas: Helium at 1.0 mL/min.

Software: Agilent ChemStation (Version G1701 D.01.02.16 15-JUNE-2004)

Instrument Conditions: Injection volume was 2 µL, with a split ratio of 10:1 using a 2.3 mm ID inlet liner (Thermo Scientific, #453A1285). Temperature parameters: Inlet temperature, 270°C; oven, 280°C; isothermal; transfer line, 320°C; Ion Source, 250°C; quadrupole temperature, 200°C. Mass Selective Detector was operated in selected ion monitoring (SIM) mode with a 100 ms dwell time. Electron multiplier voltage was about 2100 keV; tuning compound, PFTBA with m/z 502 tuned to 20% of m/z 69. Ions monitored: LCA, m/z M-15=505 (loss of $-CH_3$); DCA m/z M-15=593 (loss of $-CH_3$); UDCA, m/z M-15=593 (loss of $-CH_3$); CDCA, m/z M-180=428 (loss of two TMS-OH); CA, m/z M-15=681 (loss of $-CH_3$); D_5-CDCA, m/z M-180 (loss of two TMS-OH); Wash A and B, pre- and post-injection, 2x and 2x, respectively, with acetonitrile; preinjection sample wash,1x.

Other Equipment

(a) Screw Thread Tubes with Rubber-Lined Cap, Fisherbrand®, 16x100 mm, #1495925B (Fairlawn, NJ, USA)

(b) Teflon Liners, Supelco®, 15 mm, #27157 (Bellefonte, PA, USA)

(d) Vortex-Genie Mixer (Model #21515), (Bohemia, NY, USA)

(e) Micropipettes capable of delivering 1-5000 µL, Eppendorf, (Enfield, CT, USA)

(f) Transfer Pipettes, 3-mL, Samco, #225, (San Diego, CA, USA)

(g) 100-mL volumetric flasks, 250-mL beakers

(h) Autosampler vials with Teflon/Silicone Septa (Thermo Scientific, #CERT5000-78), (Langerwehe, Germany)

(i) Ohaus Explorer Analytical Balance (Model #E10640), (Parsippany, NJ, USA)

(j) Microliter Syringe, 50-µL, fixed needle (Hamilton), (Reno, NV, USA)

(k) Tilt Dispensing Flask, 5-mL (Kimble™Kontes™ #7593000005), (Vineland, NJ, USA)

Reagents

(a) Sodium Sulfate, Sigma-Aldrich, 239313, (St. Louis, MO, USA)

(b) Hydrochloric Acid, Certified ACS Plus, Fisher, A144 SI-212, (Fairlawn, NJ, USA)

(c) Diethyl Ether, Merck, SupraSolv®, 1.00931.2500, (Billerica, MA, USA)

(d) Pyridine, Sigma-Aldrich, 270970-1L (St. Louis, MO, USA)

(e) Acetonitrile, Fisher, A998-4, HPLC Grade

(f) BSTFA/TMCS, Sigma, 15238-25mL, (St. Louis, MO, USA)

(g) Lithocholic Acid, Sigma, L6250-10G, (St. Louis, MO, USA)

(h) Deoxycholic Acid, Sigma-Aldrich, D2510-10G, (St. Louis, MO, USA)

(i) Chenodeoxycholic Acid, Sigma, C9377-5G, (St. Louis, MO, USA)

(j) Ursodeoxycholic Acid, Sigma-Aldrich, U5127-1G, (St. Louis, MO, USA)

(k) Cholic Acid, Sigma, C1129-25G, (St. Louis, MO, USA)

(l) 2,2,3,4,4-D5-Chenodeoxycholic Acid, Cambridge Isotope Laboratories, DLM-9327-0.1 (Tewksbury, MA, USA)

Preparation of Tubes: The Teflon liners were inserted into the caps of the tubes before use and the caps were screwed onto the tubes to ensure a flush fit against the inside of the cap.

Standards

Internal Standard: 100 mg 2,2,3,4,4-D_5-Chenodeoxycholic Acid (D_5-CDCA; 251.5 µmol) was transferred quantitatively, directly from its original vial, with pyridine, to a 100-mL volumetric flask (final concentration, 251.5 µM). The solution was diluted to volume with pyridine and homogenized (shaken by hand) after stoppering. (On a 0.200 g of stool basis, this amount results in a concentration of 2.514 µmol/g.) (The solution remained capped and refrigerated at 4°C when not in use. Before use, the solution was allowed to equilibrate to room temperature and then rehomogenized (shaken by hand))

Calibration Stock Standard: A 2500 µM solution of analytes was prepared by weighing out the amounts shown in Table 1. Each analyte was transferred quantitatively to one 100-mL volumetric flask with pyridine, diluted to volume with pyridine, and homogenized (shaken by hand) after stoppering (Table 1).

Calibration Standards: The following aliquots were pipetted into five separate tubes, standards 1-5, respectively: 20, 115, 210, 305, and 400 µL. Then, 780, 685, 590, 495, and 400 µL of pyridine were added to each tube, respectively. The amounts of each analyte were 0.0500, 0.2875, 0.5250, 0.7625, 1.0000 µmol, for standards 1-5, respectively. (These amounts correlate to concentrations of 0.2500, 1.438, 2.625, 3.813, 5.000 µmol/g of stool, respectively.)

Preparation was then the same as for the samples, as described in the next section, 2.6, starting with the addition of internal standard and hydrochloric acid. (No additional pyridine was necessary).

A five-point calibration curve was prepared by plotting area ratios (peak area the analyte divided by the peak area of the internal standard) vs. molar ratios (µmol of the analyte divided by µmol of the internal standard) and performing a linear regression.

Sample Preparation and Extraction

University of Pittsburgh Institution Review Board (PRO 08100243); Stellenbosch University Health Research Ethics Committee (REF: N19/02/024); the University of KwaZulu-Natal Medical Ethics and Research Committee (REF: BE006/01); and the Medical Research Council of Zimbabwe (MRCZ/A/1684) provided ethical review and approval for the analysis of human samples in this study. (As soon as possible after collection, stool samples were stored at -80°C. For analysis, they were thawed on water ice.)

A 0.200-0.250 g stool was weighed into a tube, and the weight recorded to four decimal places. To the tubes was added 200µL internal standard solution, 800 µL of pyridine, followed by 200µL hydrochloric acid.

Table 1: Details of selected bile acids in the stock standard solution, internal standard solution, including formula weights, silylated formula weights, and quantitation ions.

Solution	Compound	Abbreviation	FW, g/mol	Amount, g	Concentration, µmol/µL	Nominal Mass	TMS Nominal Mass	TMS Quantitation Ion (m/z)
Calibration Stock Standard	Lithocholic Acid	LCA	376.6	0.0942	0.00250	376	520 (2 TMS)	505 $[M-15]^+$
	Cholic Acid	CA	408.6	0.1022	0.00250	408	696 (4 TMS)	681 $[M-15]^+$
	Deoxycholic Acid	DCA	392.6	0.0982	0.00250	392	608 (3 TMS)	593 $[M-15]^+$
	Chenodeoxycholic Acid	CDCA	392.6	0.0982	0.00250	392	608 (3 TMS)	428 $[M-180]^+$
	Ursodeoxycholic Acid	UDCA	392.6	0.0982	0.00250	392	608 (3 TMS)	593 $[M-15]^+$
Internal Standard	D_5-Chenodeoxycholic Acid	D_5-CDCA	397.6	0.1000	0.00252	397	613 (3 TMS)	433 $[M-180]^+$

The tubes were capped and placed into a 100°C heat block for 10 minutes with occasional shaking. Afterwards, the tubes were removed from the heat block and allowed to cool to room temperature. Then, 5000 µL diethyl ether was added with the dispensing flask followed by about 5 g sodium sulfate. The tubes were then capped, followed by shaking and vortexing for 30 s. More shaking and vortexing were used if the sample did not completely disperse. This mixture was allowed to sit for at least 10 minutes to ensure adsorption of water by the sodium sulfate.

Next, a 500-µL aliquot of the solution was transferred to an autosampler vial. Then, 500 µL pyridine and 500 µL BSTFA/TMCS (or 1000 µL of a 1:1v/v solution) was added and the vial capped tightly. The vials were then heated at 100°C for 10 minutes and allowed to stand for at least five hours prior to analysis by GC-MS. As before, five hours was necessary to allow the analytes to derivatize completely.

Spike and Recovery and Control Sample: Portions of a composite stool sample, 0.200 ± 0.001 g, were weighed into tubes and spiked at low, medium, and high levels (Tables 2a, 2b, and 3). Five replicates at each of these levels, including unspiked portions, were run prepared and analyzed over four different days.

The "unspiked" sample was used as a control sample, spiked at the mid-range, and was run with each subsequent set of samples.

Analyte amounts were calculated with the following equation. Then, these amounts were divided by the sample weight (g) to determine the concentrations in stool in µmol/g:

$$\mu mol_{analyte} = \left[\left(\frac{Area_{analyte}}{Area_{ISTD}}\right) - b\right]\frac{\mu mol_{ISTD}}{m}$$

Where $\mu mol_{analyte}$ is the amount of analyte in micromoles; $Area_{analyte}$ is the peak area of the analyte; $Area_{ISTD}$ is the peak area of the internal standard; *b* is the y intercept; μmol_{ISTD} is the amount of internal standard in micromoles; and *m* is the slope.

The following equation was used to calculate the recoveries and relative recoveries at the low, medium, and high levels:

$$R = \left(\frac{S-U}{T}\right)\times 100$$

Where *R* is the recovery or relative recovery in percent; *S* is the concentration measured at a given spike level—i.e., L, M, and H (low, medium, and high, respectively)—in µmol/g; *U* is the concentration measured in the unspiked sample—in µmol/g; and *T* is the actual concentration at a given spike level, or theoretical concentration, calculated from the standards—in µmol/g.

Pilot Study (Figure 2): Stool samples were analyzed from African Americans and analyzed using this method.

RESULTS AND DISCUSSION

Sample Preparation: We did not find another method that used acidified pyridine for extraction of bile acids from stool. Initially, it was found that stool did not disperse as well with pure pyridine as when some water was present. In this procedure, it was added as 200 µL concentrated hydrochloric acid (12 M aqueous). It was also found later that the bile acids would not derivatize without the addition of hydrochloric acid. We hypothesize that the carboxylic -OH of the bile acid molecule requires protonation in order to accept a TMS (trimethylsilyl group). With silylation, it was found that all water had to be removed or derivatization would not occur and split phases in the final derivatized extract would sometimes result, with no indication of the analytes. In addition to sodium sulfate, use of diethyl ether facilitated the removal of water from the organic extract, as well as resulted in a massive sample clean up: the pyridine/HCl solutions were dark brown to black before addition of diethyl ether and sodium sulfate. After these additions, and the requisite shaking/vortexing, sample extracts were clear, but not colorless. Standards were both clear and colorless.

Table 2A: Interday accuracy, RR, relative recovery, %; **σ**, population standard deviation; and CV, coefficient of variation , %, using internal standard. Other values are in µmol/g unless otherwise stated. U = unspiked, L = low spike, M= medium spike, H = high spike, T = actual (theoretical) concentration. L-U is the low spike with the unspiked subtracted; M-U is the medium spike with the unspiked subtracted; and H-U is the high spike with the unspiked subtracted.

Interday Accuracy and Precision (Units are µmol/g unless otherwise stated)															
			U	L	M	H	L-U	T	RR	M-U	T	RR	H-U	T	RR
LCA		**Mean**	0.86	1.11	3.51	5.89	0.25	0.25	101	2.65	2.63	101	5.03	5.01	100
		σ	0.03	0.03	0.13	0.27									
		CV	3	2	4	5									
CA		**Mean**	0.70	0.99	3.53	6.13	0.29	0.25	115	2.82	2.63	107	5.43	5.01	108
		σ	0.03	0.03	0.08	0.15									
		CV	4	3	2	2									
DCA		**Mean**	1.69	1.99	4.48	6.95	0.30	0.25	121	2.79	2.63	106	5.26	5.00	105
		σ	0.10	0.05	0.12	0.20									
		CV	6	3	3	3									
CDCA		**Mean**	0.41	0.65	2.98	5.34	0.24	0.25	95	2.57	2.63	98	4.93	5.00	99
		σ	0.02	0.02	0.05	0.07									
		CV	4	3	2	1									
UDCA		**Mean**	0.27	0.45	2.76	5.21	0.18	0.25	74	2.49	2.62	95	4.94	5.00	99
		σ	0.03	0.04	0.09	0.17									
		CV	12	8	3	3									

Table 2B: Intraday accuracy RR, relative recovery; s, standard deviation; and CV, coefficient of variation, %, using internal standard. Other values are in µmol/g unless otherwise stated. U = unspiked,L = low spike, M= medium spike, H = high spike, T = actual (theoretical) concentration. L-U is the low spike with the unspiked subtracted; M-U is the medium spike with the unspiked subtracted; and H-U is the high spike with the unspiked subtracted.

Intraday Accuracy and Precision (Units are µmol/g unless otherwise stated)															
			U	L	M	H	L-U	T	RR	M-U	T	RR	H-U	T	RR
LCA	DAY 1	Mean	0.85	1.13	3.60	6.16	0.28	0.25	113	2.75	2.63	105	5.32	5.01	106
		s	0.02	0.03	0.05	0.06									
		CV	2	2	1	1									
	DAY 2	Mean	0.88	1.12	3.59	5.92	0.24	0.25	95	2.72	2.63	103	5.04	5.01	101
		s	0.01	0.01	0.08	0.12									
		CV	2	1	2	2									
	DAY 3	Mean	0.86	1.09	3.31	5.52	0.23	0.25	91	2.45	2.63	93	4.66	5.01	93
		s	0.04	0.02	0.07	0.24									
		CV	4	2	2	4									
	DAY 4	Mean	0.84	1.10	3.54	5.94	0.26	0.25	103	2.70	2.63	103	5.09	5.01	102
		s	0.03	0.03	0.04	0.14									
		CV	3	3	1	2									
CA	DAY 1	Mean	0.71	1.00	3.61	6.28	0.29	0.25	116	2.91	2.63	111	5.57	5.01	111
		s	0.02	0.02	0.03	0.10									
		CV	3	2	1	2									
	DAY 2	Mean	0.73	1.04	3.57	6.18	0.31	0.25	123	2.84	2.63	108	5.45	5.01	109
		s	0.02	0.02	0.03	0.08									
		CV	3	2	1	1									
	DAY 3	Mean	0.69	0.97	3.41	5.93	0.28	0.25	113	2.72	2.63	103	5.24	5.01	105
		s	0.02	0.01	0.05	0.12									
		CV	3	1	1	2									
	DAY 4	Mean	0.68	0.95	3.51	6.12	0.27	0.25	107	2.83	2.63	108	5.43	5.01	108
		s	0.02	0.01	0.03	0.06									
		CV	4	1	1	1									
DCA	DAY 1	Mean	1.66	2.01	4.56	7.12	0.35	0.25	142	2.90	2.63	110	5.46	5.00	109
		s	0.01	0.03	0.04	0.09									
		CV	1	1	1	1									
	DAY 2	Mean	1.76	2.05	4.59	7.08	0.29	0.25	116	2.83	2.63	108	5.32	5.00	106
		s	0.17	0.04	0.05	0.16									
		CV	9	2	1	2									
	DAY 3	Mean	1.67	1.94	4.30	6.67	0.27	0.25	108	2.63	2.63	100	5.00	5.00	100
		s	0.06	0.04	0.04	0.04									
		CV	4	2	1	1									
	DAY 4	Mean	1.66	1.96	4.46	6.92	0.29	0.25	117	2.79	2.63	106	5.26	5.00	105
		s	0.08	0.03	0.02	0.08									
		CV	5	2	1	1									
CDCA	DAY 1	Mean	0.39	0.65	3.04	5.43	0.26	0.25	103	2.65	2.63	101	5.04	5.00	101
		s	0.01	0.01	0.03	0.05									
		CV	1	2	1	1									
	DAY 2	Mean	0.41	0.64	2.96	5.31	0.24	0.25	95	2.56	2.63	97	4.90	5.00	98
		s	0.01	0.01	0.05	0.04									
		CV	3	1	2	1									
	DAY 3	Mean	0.43	0.67	2.95	5.28	0.24	0.25	97	2.52	2.63	96	4.85	5.00	97
		s	0.00	0.01	0.02	0.05									
		CV	1	1	1	1									
	DAY 4	Mean	0.41	0.62	2.98	5.33	0.22	0.25	87	2.57	2.63	98	4.93	5.00	98
		s	0.02	0.01	0.02	0.04									
		CV	4	1	1	1									
UDCA	DAY 1	Mean	0.24	0.43	2.81	5.31	0.19	0.25	76	2.57	2.62	98	5.07	5.00	101
		s	0.00	0.01	0.02	0.06									
		CV	2	3	1	1									
	DAY 2	Mean	0.32	0.50	2.87	5.38	0.18	0.25	74	2.55	2.62	97	5.06	5.00	101
		s	0.00	0.01	0.04	0.04									
		CV	1	2	1	1									
	DAY 3	Mean	0.27	0.46	2.63	4.96	0.19	0.25	77	2.36	2.62	90	4.69	5.00	94
		s	0.00	0.01	0.01	0.05									
		CV	1	2	1	1									
	DAY 4	Mean	0.24	0.41	2.73	5.18	0.17	0.25	69	2.49	2.62	95	4.94	5.00	99
		s	0.00	0.01	0.01	0.04									
		CV	2	2	0	1									

Instrumentation: All gas chromatography methods we found in the literature used a single column, including methods that use silylation only. We, however, did not achieve the desired separation with a single column. Additionally, cholic acid coeluted with CDCA. Under these conditions, it was difficult to find a quantitation ion of sufficient specificity and abundance for CDCA with which cholic acid did not interfere. (Cholic acid did not have interference from CDCA because the quantitation ion was higher than the formula weight of CDCA.) Eventually, two columns in series (Rtx-50 followed by Rtx-5MS) were tried which did resolve these interference issues and produced excellent resolution and peak shape (Figures 1a and 1b). The oven temperature is isothermal at 280°C for this method, which is unusual; however, various temperature programs were tried and did not result in better separation than the isothermal program. The isothermal oven temperature was advantageous in that the oven did not have to cool down between runs, substantially eliminating oven recovery time. For all analytes, except CDCA, $[M-15]^+$ (loss of $-CH_3$) was chosen as the quantitation peak. Chenodeoxycholic acid did not produce a very abundant $[M-15]^+$, so M-180 (loss of two TMS-OH groups) was chosen instead. The same was true for the deuterated analog, D_5-CDCA.

(The supplement provides more details on this issue.) In many stool samples, a leading hump was present on the LCA peak (Figure 1b). The oven temperature was reduced to resolve this peak from LCA. By comparison with the mass spectrum of LCA, this peak is most likely an isomer of LCA.

Accuracy and Precision (Spike and Recovery)/ Efficacy of the Internal Standard

Interday and Intraday Accuracy and Precision (Tables 2a and 2b): Interday and Intraday precision was good, with most analytes having CVs of less than 5%. Interday and Intraday relative recovery was also good, with most analytes being between 90 and 110%. Intraday precision and relative recovery were slightly better than the interday results. For the lowest spike, both CVs and relative recoveries were more variable, with LCA, CA, DCA, and CDCA being above 100% and UDCA being under 80%.

Efficacy of the Internal Standard (Table 3): Surprisingly, spike and recoveries calculated without use of the internal standard yielded similar results to those with the internal standard. It was expected that the accuracy and precision without an internal standard would be much worse than with an internal standard; however, R^2 values were generally better for calibration curves with the internal standard.

Long-Term Precision (Table 4): A stool sample with sufficient amounts of the analytes was not used. Instead, the control sample was analyzed over different days and months. This had the advantage of not only showing precision over time, but also accuracy. Accuracy, in terms of relative recovery, ranged from 94 to 104%, over 17 different days and five different months with CVs of 5% or lower.

Chromatography/Calibration Curves: Separation and peak shapes were excellent in both standards and samples. Calibration curves yielded R^2 values of 0.99 or better, with slightly negative intercepts. We tried an expanded calibration range, from 0.125 to 10.0 µmol/g which also yielded R^2 values of 0.99 or better.

Pilot Study: The method performed as expected on the samples from African Americans. No unexpected problems were experienced during sample preparation. Results were extrapolated below 0.25 µmol/g before averaging. Even so, the average CDCA and UDCA concentrations fell below the LOQ. Although these results meet our current research needs, they also indicate an opportunity for lowering the LLOQ: Higher sensitivity at lower levels could be achieved by any or all of the following: concentrating the extract; increasing the injection volume on the GC; using a smaller volume of diethyl ether for extraction; and derivatizing a larger volume of the extract.

CONCLUSIONS

The method described in this paper provides a simple, accurate, and precise method for the measurement of selected bile acids in human stool by GC-MS. In addition, we were able to achieve the desired separation. As result, this method has shown the following innovations: First, it uses a single derivatization, silylation. Other methods, which are used to analyze actual samples for bile acids, rather than pure compounds, all use two-step derivatizations: methylation of the carboxylic acid moiety, followed by silylation of the hydroxyl groups on the rings. Second, it employs a dual Column: A mid-polarity Rtx-50, followed by a low-polarity Rtx-5MS. This arrangement essentially functions as continuous two-dimensional gas chromatography. Third, it requires only one tube per sample for preparation. Granted, the derivatization is performed in an autosampler vial, but the sample would have to be transferred to an autosampler vial in any case. Fourth, sample preparation is simple: The sample preparation in this method is simple compared to other methods, many of which require lyophilization, pulverization, followed by saponification and then the two-step derivatization, multiple extractions, and concentration or drying steps. Fifth, it uses a pyridine extraction: No other method found uses a pyridine extraction. Pyridine is an excellent solvent for bile acids, and it will even dissolve the salts of bile acids. Additionally, the presence of pyridine helps drive the derivatization. Sixth, it takes advantage of isothermal GC. Since the oven "program" is isothermal, the oven has no recovery time, meaning that the next sample is injected almost immediately. Seventh, the addition of sodium sulfate not only aids in removal of water, but also aids in the dispersion of the sample, which enhances extraction of target analytes.

Next Steps: For the targeted analysis of short-chain fatty acids we previously published [32], we found that by injecting the underivatized extract, the short-chain fatty acids, starting with acetic acid, do separate from the pyridine and ether solvent peaks on a DB-FFAP column. However, the underivatized extract must be injected, because BSTFA/TMCS will derivatize, and, therefore, damage a DB-FFAP column. Silylated short-chain fatty acids do not produce good chromatography on the column

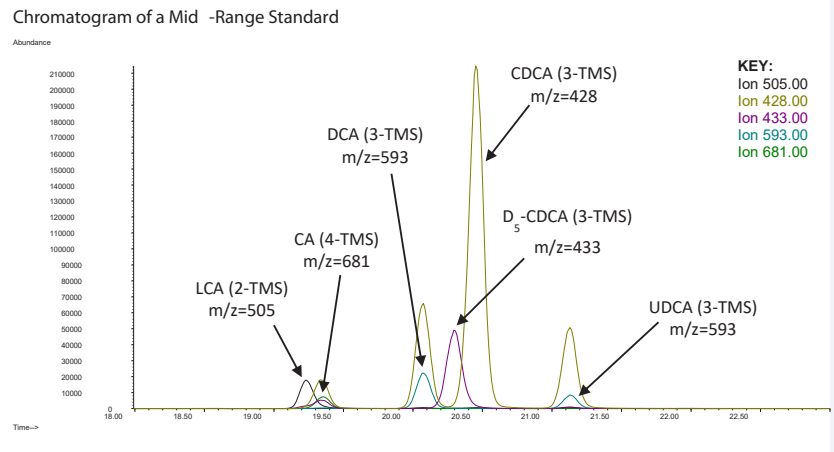

Figure 1A Example Chromatogram (Mid-Range Calibration Standard), with TMS (trimethylsilyl) substitutions designated for the completely derivatized molecules.

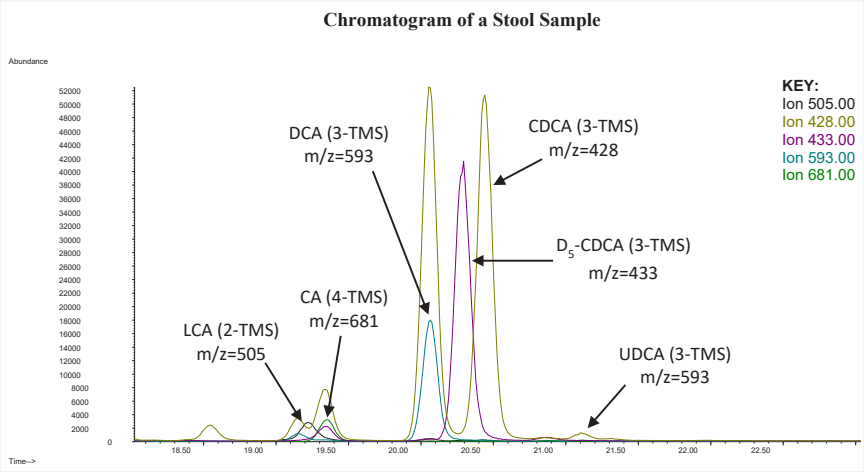

Figure 1B Example Chromatogram (Stool Extract) with TMS (trimethylsilyl) substitutions designated for the completely derivatized molecules.

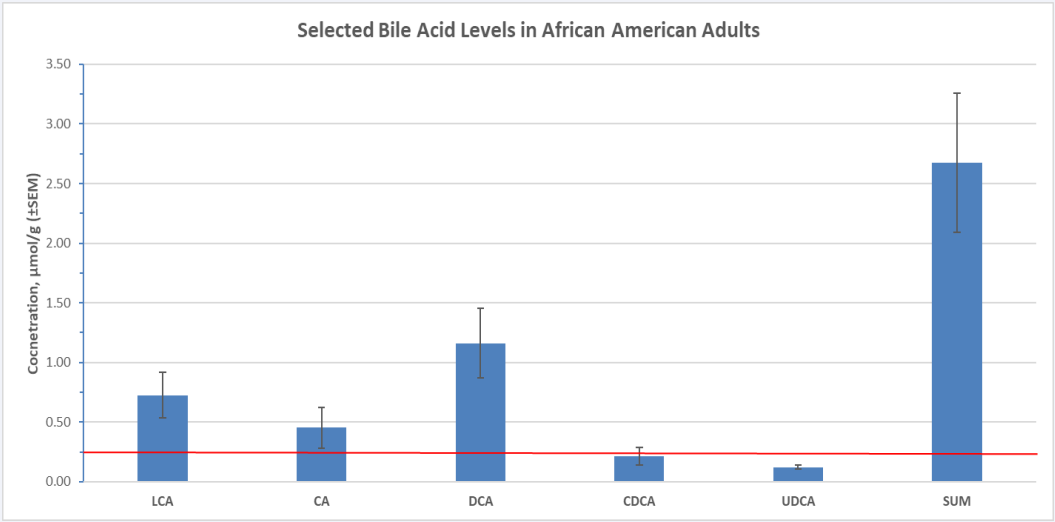

Figure 2 Analysis of targeted bile acids in the stool of healthy African American adults. Results are in µmol/g and the red line indicates the lower limit of quantitation, 0.25 µmol/g. Results below 0.25 µmol/g were extrapolated before averaging.

Table 3: Intraday accuracy R, recovery, %; σ, population standard deviation; and CV, coefficient of variation, %, without using and internal standard. Other values are in μmol/g unless otherwise stated. U = unspiked,L = low spike, M= medium spike, H = high spike, T = actual amount spiked. L-U is the low spike with the unspiked subtracted; M-U is the medium spike with the unspiked subtracted; and H-U is the high spike with the unspiked subtracted.

Efficacy of the Internal Standard (Interday Only) (Units are μmol/g unless otherwise stated)													
		U	L	M	H	L-U	T	R	M-U	T	R	H-U	T	R
LCA	Mean	1.00	1.20	3.65	5.96	0.20	0.25	80	2.66	2.63	101	4.96	5.01	99%
	σ	0.06	0.07	0.31	0.59									
	CV	6	6	8	10									
CA	Mean	0.85	1.09	3.68	6.20	0.24	0.25	95	2.82	2.63	107	5.34	5.01	107%
	σ	0.06	0.07	0.30	0.57									
	CV	7	7	8	9									
DCA	Mean	1.84	2.03	4.63	7.00	0.19	0.25	77	2.79	2.63	106	5.16	5.00	103%
	σ	0.18	0.15	0.40	0.68									
	CV	10	7	9	10									
CDCA	Mean	0.59	0.79	3.14	5.41	0.20	0.25	81	2.56	2.63	97	4.83	5.00	96%
	σ	0.05	0.06	0.24	0.49									
	CV	9	7	8	9									
UDCA	Mean	0.41	0.58	2.91	5.29	0.17	0.25	68	2.49	2.62	95	4.87	5.00	98%
	σ	0.06	0.05	0.24	0.52									
	CV	13	8	8	10									

Table 4: Precision, N=35, spiked composite stool, 18 different days, 6 different months. "CV" and "Relative Recovery" are expressed as percentages. Other values are in μmol/g unless otherwise stated. The population standard deviation is indicated by σ, and the mean±2σ range is the 95% confidence interval.

Long-Term Precision of a Control Sample (Units are μmol/g unless otherwise stated)					
	LCA	CA	DCA	CDCA	UDCA
Mean	3.40	3.40	4.38	2.93	2.68
σ	0.21	0.18	0.20	0.08	0.15
2σ	0.42	0.36	0.40	0.17	0.29
CV	6	5	5	3	5
Mean-2σ	2.98	3.04	3.98	2.77	2.39
Mean+2σ	3.81	3.76	4.79	3.10	2.97
Unspiked (N=20)	0.86	0.70	1.69	0.41	0.27
Spike	2.63	2.63	2.63	2.63	2.62
Relative Recovery	97%	103%	103%	96%	92%

arrangement described in this paper. Nevertheless, two types of analyses, which are important in the analysis of stool, could be accomplished with only one extract.

Some work was done on a few pure conjugated bile acids. The method described in this paper works for unconjugated bile acids, but not for the conjugated bile acids, as the procedure does not deconjugate them.

Further work could be done to determine if lengthening the derivatization time, or perhaps direct derivatization of the stool, could be efficacious.

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