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Determination of underivatised sterols and bile acid trimethyl silyl ether methyl esters by gas chromatography–mass spectrometry–single ion monitoring in faeces

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Abstract

A method for quantification of total faecal sterols and bile acids (BAs) in human stool by using gas chromatography–mass spectrometry–single ion monitoring (GC–MS–SIM) is described. Cholesterol, coprostanol, coprostanone, cholestanol, *iso*-lithocholic acid (*iso*-LCA), lithocholic acid (LCA), *iso*-deoxycholic acid (*iso*-DCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), cholic acid (CA), and 12-oxo-deoxycholic acid (12-oxo-DCA) in faeces of 86 healthy subjects were determined. The sample preparation for sterol analysis requires hydrolysis and liquid extraction from matrix, but no derivatisation. The GC-flame ionisation detection (FID) and total ion current (TIC) in GC–MS were not sufficient for sterol and BA determination, whereas selectivity and specificity of the GC–MS–SIM ensured the analysis of sterols and BAs in faeces.

Keywords: Sterols; Bile acids

1. Introduction

Cholesterol (cholest-5-ene-3β-ol) is mainly converted by oxidation and hydrogenation into coprostanol (5β-cholestane-3β-ol) via coprostanone (5β-cholestane-3-one) by colonic bacteria [1]. Minor products of cholesterol transformation are cholestanol (5α-cholestane-3β-ol) and cholestanone (5α-cholestane-3-one). Furthermore, phytosterols such as sitosterol (24-ethyl-cholest-5-ene-3β-ol) and campesterol (24-methyl-cholest-5-ene-3β-ol) are predominantly converted to the analogous 5β-cholestanes by colonic bacteria [2] (Fig. 1). A variety of different methods of sample preparation for gas chromatographical analysis of faecal sterols have been published [3–8]. Most procedures include derivatisation, often silylation, of the sterols. Formation of artefacts (e.g. enol silyl ether) may occur during silylation of organic compounds with oxo-groups in the molecule [9–11]. However, underivatised sterols like the pairs cholesterol/coprostanone or cholesterol/cholestanol co-elute in GC-analysis [12,13]. Baseline separation of free faecal sterols is achievable by using modified packed columns in the gas chromatographical process [13]. Therefore, a separation into hydroxy-sterol and oxo-sterol fractions by solid phase extraction before estimation with capillary column-GC is necessary [14]. A suitable method of separating co-eluting sterol pairs is using two-dimensional gas chromatography [15].

Primary bile acids (BAs), synthesised in the hepatocytes, and secondary BAs, formed by bacteria in the colon, are identified in faeces of healthy human subjects [16] (Fig. 1). BA extraction from homogenised faeces is the first step in most procedures [16–19]. On the other hand, sample preparation directly from faeces samples is established [5,20]. Furthermore, a method for simultaneous analysis of sterols and BAs using GC-FID has been described [4]. Within this method, methylation and silylation are directly carried out in pul-

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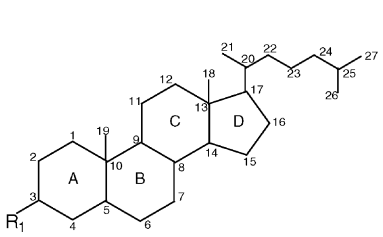

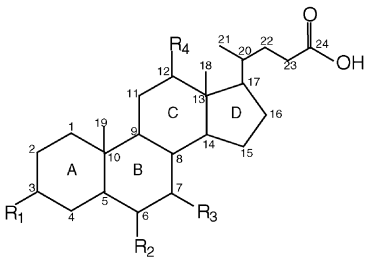

Sterols **Bile acids**

Name	R_1	Configuration A+B/ position of H-atom at C5	Δ5	Substituent at C24	Name	R_1	R_2	R_3	R_4
5α-cholestane	H	*trans*/5α			5β-cholanic acid	H	H	H	H
cholesterol	β-OH		+		lithocholic acid (LCA)	α-OH	H	H	H
sitosterol	β-OH		+	$-C_2H_5$	iso-lithocholic acid (iso-LCA)	β-OH	H	H	H
campesterol	β-OH		+	$-CH_3$	deoxycholic acid (DCA)	α-OH	H	H	α-OH
coprostanol	β-OH	*cis*/5β			iso-deoxycholic acid (iso-DCA)	β-OH	H	H	α-OH
cholestanol	β-OH	*trans*/5α			hyodeoxycholic acid (HDCA)	α-OH	α-OH	H	H
coprostanone	=O	*cis*/5β			chenodeoxycholic acid (CDCA)	α-OH	H	α-OH	H
cholestanone	=O	*trans*/5α			cholic acid (CA)	α-OH	H	α-OH	α-OH

Fig. 1. Structure of main faecal sterols and BAs.

verised faeces and the extraction of the steroids occurs as the last step. Unrecognised co-elution of components of manifold faecal matrix is possible when applying GC-FID. Contrary to the procedure with minimal sample preparation, a further method including three-step lipid extraction, chromatography on solid phases and gels, and derivatisation to trimethyl silyl ether methyl esters has been established [16]. The chromatographic separation and cleaning of faeces samples in this method ensure peak purity, but increase the risk of substance loss during preparation. In further established chromatographic methods for analysis of free and conjugated BAs, high performance liquid chromatographic (HPLC) with MS, fluorescense, ultraviolet, or pulsed amperometric detection is used [21]. However, we preferred faecal steroid analysis by GC with electron impact ionisation (EI) and MS detection, because of the high robustness and reproducity of this technique.

The aim of the research was to compile methods for capillary column-GC–MS–SIM analysis allowing the determination of all classes (Δ5-sterols, 5α-sterols, 5β-sterols, oxosterols) of sterols and the optimising of BA analysis in faeces with moderate sample preparation and substance-specific detection.

2. Experimental conditions

2.1. Sample collection

Faeces samples were obtained from 86 healthy subjects (34 male, 52 female; age: 30 ± 8 [19–56] a; body mass index: 23.1 ± 3.0 (18.0–29.4 kg/m^2) to acquire a wide inter-individual range. All individual stools were collected in entirety during a period of 5 days in plastic containers, weighed, and, at the end, homogenised and pooled. Aliquots of stools were lyophilised and then stored frozen at $-20\,^{\circ}C$ until analysis.

2.2. Chemicals and reference standards

5α-Cholestane (internal standard, purity: >95%), cholesterol (>99%), coprostanol (>98%), coprostanone (>95%), cholestanol (>95%), cholestanone (>95%), cholest-4-ene-3-one (>95%), sitosterol (>97%), sitostanol (>95%), campesterol (>95%), cholesteryl stearate (>99%), cholesteryl acetate (>99%), lithocholic acid (LCA) (>97%), deoxycholic acid (DCA) (>99%), chenodeoxycholic acid (CDCA) (>98%), cholic acid (CA) (>98%), hyodeoxycholic acid (HDCA, internal standard) (>98%), hyocholic acid (HCA) (>97%), ursodeoxycholic acid (UDCA) (>99%), 3,7,12-trioxo-5β-cholanoic acid (3,7,12-trioxo-CA) (>95%), 3,7-dioxo-5β-cholanoic acid (3,7-dioxo-CDCA) (>98%), 3-oxo-5β-cholanoic acid (3-oxo-LCA) (>95%), and DCA acetate methyl ester (>99%) were purchased from Sigma (Munich, Germany). 24-Methyl coprostanol, 24-ethyl coprostanol, 24-methyl coprostanone, 24-ethyl coprostanone were isolated from human faeces by thin layer chromatography (TLC) [13]. The standard substances of *iso*-LCA, *iso*-DCA, and 12-oxo-DCA were obtained from Steraloids (Newport, Rhode Island, USA). Methanolic hydrochloric acid (3 mol/L) and Sylon HTP (hexamethyl disilazane/chlorotrimethyl silane/pyridine = 3:1:9) were used for

methylation and silylation of the BAs (Supelco, Munich, Germany).

2.3. Sample preparation

For duplicate analysis, two aliquots of 50 mg were weighed into vessels with 250 μg 5α-cholestane as internal standard for sterol determination. Fifty microliters distilled water was added for soaking. After mild hydrolysis with freshly prepared 1 mL ethanolic sodium hydroxide (1 mol/L, 90% ethanol) for 60 min at 70 °C and the addition of 0.5 mL distilled water, neutral sterols were extracted exhaustively with four 1 mL portions of cyclohexane. The solvent of the combined extracts was evaporated under a nitrogen stream. The residues were reconstituted in 500 μL decane and, without further derivatisation, injected in GC in duplicate.

The procedure of sample preparation for BA analysis is adapted to the method of Czubayko et al. [6]. The internal standard (125 μg HDCA) was added to the aqueous phase of sterol extraction. The sample was saponified with 200 μL 10 mol/L sodium hydroxide at 120 °C for 120 min and then acidified to pH 1 with hydrochloric acid. After extraction of BAs with diethyl ether (4 × 1 mL), the solvent-phases were pooled and evaporated under a stream of nitrogen. The residue was methylated with 650 μL dimethoxy propane, 950 μL methanol and 50 μL methanolic hydrochloric acid (3 mol/L) for 45 min at 50 °C. The solution was evaporated to dryness and the residue was dissolved in 150 μL Sylon HTP. The silylation was carried out at 90 °C for 60 min. After evaporation under a nitrogen stream, the residue was dissolved in 250 μL decane. The solution was shaken for 10 min and then centrifuged for 10 min at 1500 × *g*. The clear solution was transferred into a vial for GC-analysis.

2.4. Instrumental conditions

The gas chromatographical procedure for sterol analysis using GC17-QP5000, equipped with a split/splitless injector (Shimadzu, Kyoto, Japan), followed an optimised temperature programme, starting at 150 °C for 5 min. The temperature was raised to 240 °C at 40 °C/min. Then the oven was heated to 280 °C at 1 °C/min and remained constant for 10 min. As fused-silica capillary column, optima1 (50 m, 0.2 mm, 0.2 μm; Macherey-Nagel, Düren, Germany) was used. The sample (1 μL) was injected in split mode (1:50) at 280 °C. The carrier gas was helium with a constant linear velocity of 32 cm/s. The temperature of the interface was turned up to 330 °C to guarantee an ion source temperature of 300 °C.

Analysis of BAs was performed with GC17-QP5000 (Shimadzu, Kyoto, Japan), equipped with a capillary column (ZB5; 30 m; 0.25 mm; 0.25 μm; Phenomenex, Torrance, CA, USA). The injection of 1 μL sample solution was carried out in split mode (1:50) at 280 °C. Helium was used as mobile phase with a constant linear velocity of 32 cm/s and the interface temperature was kept at 300 °C. The oven temperature was raised from 150 to 290 °C (5 min at 150 °C; 240 °C (40 °C/min); 255 °C (1 °C/min); 270 °C (4 °C/min); 278 °C (1 °C/min); 9 min at 278 °C; 290 °C (40 °C/min); 4.7 min at 290 °C). The mass spectrometric detection was realised in TIC, multi ion current (MIC), and SIM mode for sterol and BA analysis with an electron beam energy of 70 eV. The sampling rate was 5 s^{-1} and the detector gain was turned to 1.5 kV. The sensitivity adjustment was performed in SIM as part of automatic tuning (perfluoro tributyl amine; m/z = 264.00 amu). Quantification was carried out in SIM mode by using internal standard method and peak areas were obtained from the chromatograms generated by data-handling software Class 5000 (Shimadzu, Kyoto, Japan). Component identification was based on fragmentation and comparison of the retention times with those of standards.

3. Results

3.1. Isolation of the 5β-sterols

The daily phytosterol uptake of humans consuming a western type diet amounts to 0.1–0.5 g [22]. Therefore, the concentrations of phytosterols and their bacterial 5α- and 5β-degradation products are low in faeces. The faeces of one subject having a very high phytosterol intake (1.5 g/d), were used to separate the 5β-sterols to obtain standard substances for the qualitative determination. The faeces were prepared by TLC according to Arca et al. [13] and the isolated 5β-sterols were analysed by GC–MS receiving mass spectral data (Table 1).

3.2. Accuracy and precision of sterol determination

Determining within run-precision, 10 faeces samples of the same origin were prepared and analysed. The within-run precision showed relative standard deviations below 5% for the main sterol components. The between-run precision was estimated by weekly analysis of five faeces samples over 1 month (Table 2).

A solution made up of a mixture of cholesteryl stearate (0.5 mg/mL) and cholesteryl acetate (0.5 mg/mL) was prepared and added to 10 faecal samples of the same origin used for studies of within-run precision. The expected, calculated result was a cholesterol concentration of 0.820 mg/mL. The mean of concentrations amounted to 0.791 mg/mL, which corresponds to a recovery of 96.5%.

The comparability of sterol analysis with and without derivatisation is given based on the results of the 7th Proficiency Test, 2001 [23]. This was an international interlaboratory investigation for analysis of phytosterols and cholesterol in oils, according to the method DGF-F-III1 (98). This validated method contained silylation of sterols as an established method of derivatisation and was performed by most participants in this way. Of interest for present purposes are the cholesterol results without derivatisation, which showed a satisfactory comparability to other laboratories with

Table 1
Fragments with higher mass range of coprostanol and coprostanone standard substances compared to other 5β-sterols isolated from human faeces by TLC

			M-100/M-70	M-(15 + 18)	M-15	M^+
Coprostanol	233 (53)	248 (4.2)	288 (5.5)	355 (20)	373 (29)	388 (14)
24-Methyl coprostanol	233 (53)	248 (4.2)	302 (5.4)	369 (20)	387 (28)	402 (14)
24-Ethyl coprostanol	233 (53)	248 (4.1)	316 (5.4)	383 (19)	401 (28)	416 (13)
Coprostanone	231 (56)	246 (4.1)	316 (34)[a]	353 (12)	371 (4.0)	386 (23)
24-Methyl coprostanone	231 (56)	246 (4.4)	330 (32)[a]	367 (10)	385 (3.7)	400 (22)
24-Ethyl coprostanone	231 (56)	246 (4.0)	344 (32)[a]	381 (10)	399 (3.6)	414 (20)

Data in parenthesis give size as percentage of base peak. M^+, molecular ion; M-15, loss of a methyl group; M-(15+18), loss of a methyl group and water; M-70, loss of ring A inclusive oxo group, M-100, fragments unknown.
[a] M-100/M-70.

a *z*-score of 0.0 and 0.4, whereby $|z| \leq 1$ was assessed as a good finding (Fig. 2).

3.3. Calibration of sterols

For quantification of cholesterol, coprostanol, coprostanone, and cholestanol, a stock solution with substance-specific concentrations (0.010–2.000 mg/mL) was prepared. Each of the six calibration standards was obtained by diluting the stock solution, whereas the concentrations of the analytes were a half, a fourth, an eighth, etc. The internal standard concentration was constant at 0.5 mg/mL 5α-cholestane ($m/z = 357.3$ amu) in all solutions (Table 3).

3.4. Chromatography of BAs

Solutions of single BA trimethyl silyl ether methyl esters and mixed standards were prepared. The chromatogram of a mixed standard (*iso*-LCA, LCA, *iso*-DCA, DCA, CDCA, CA, HDCA, UDCA, HCA, 12-oxo-DCA) by detection in TIC showed separation of all BA trimethyl silyl ether methyl esters (Fig. 3). However, the individual chromatograms of the 3-oxo-BA trimethyl silyl ether methyl esters (3,7,12-trioxo-CA, 3,7-dioxo-CDCA, 3-oxo-LCA) indicated the formation of artefacts. The by-products of 3-oxo-LCA showed a similar fragmentation pattern to *iso*-DCA and DCA, and furthermore, they co-eluted. For this reason, the undisturbed fragments $m/z = 75.1$ amu for *iso*-DCA and $m/z = 255.3$ amu for

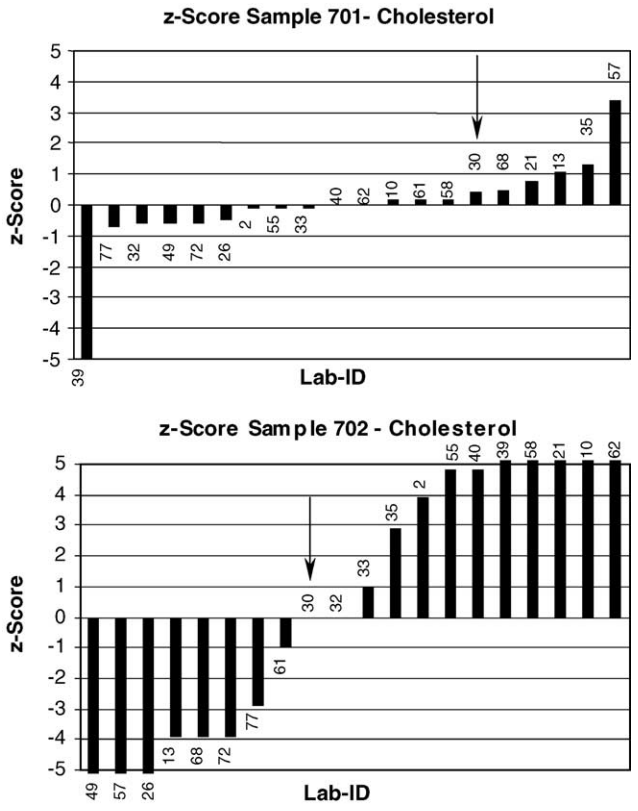

Fig. 2. Comparison of cholesterol results of different laboratories in an international inter-laboratory investigation (our number: 30); samples: different mixtures of fish and vegetable oils.

Table 2
Results of within-run and between-run precision

	Retention time (min)	Fragment (amu)	Within-run precision $n = 10$		Between-run precision $n = 5$	
			Mean ± S.D. (mg/mL)	R.S.D.	Mean ± S.D. (mg/mL)	R.S.D.
Cholesterol	41.2	301.3 (32)	0.073 ± 0.002	2.7	0.072 ± 0.003	4.2
Coprostanol	39.7	373.3 (29)	1.386 ± 0.059	4.3	1.399 ± 0.065	4.6
Coprostanone	40.9	316.3 (34)	0.308 ± 0.010	3.2	0.310 ± 0.015	4.8
Cholestanol	41.5	215.1 (92)	0.025 ± 0.002	8.0	0.025 ± 0.003	11.2
iso-LCA	33.9	215.3 (31)	0.227 ± 0.011	4.9	0.224 ± 0.017	7.6
LCA	34.4	215.3 (58)	0.518 ± 0.027	5.2	0.511 ± 0.035	6.8
iso-DCA	35.8	75.1 (91)	0.080 ± 0.003	3.8	0.081 ± 0.004	4.9
DCA	36.4	255.3 (93)	0.893 ± 0.048	5.4	0.883 ± 0.059	6.7
CDCA	37.4	73.1 (100)	0.076 ± 0.003	3.9	0.077 ± 0.007	9.1
CA	37.8	253.2 (44)	0.184 ± 0.012	6.5	0.179 ± 0.013	7.3
12-oxo-DCA	42.0	231.3 (52)	0.028 ± 0.002	7.1	0.027 ± 0.003	10.7

S.D., Standard deviation; R.S.D., relative standard deviation; data in parenthesis give size as percentage of base peak.

Table 3
Calibration statistics of faecal sterols and BAs

Substance	Fragment (amu)	Range of linear calibration (mg/mL)	$y = mx + n$	r^2 ($n = 7$)	Rel. S.D.$_y$ (%)	S.D.$_{xo}$	V_{xo} (%)	Limit of detection (μg/mL)
Cholesterol	301.3(32)	0.015–1.000	*m*: 0.1519 *n*: 0.0065	0.9998	2.6	0.007	2.5	1.19
Coprostanol	373.3(29)	0.030–2.000	*m*: 0.5437 *n*: −0.0060	0.9997	2.9	0.017	3.2	1.35
Coprostanone	316.3(34)	0.015–1.000	*m*: 1.4365 *n*: −0.0110	0.9998	3.4	0.008	3.3	2.40
Cholestanol	215.1(92)	0.010–0.600	*m*: 0.6484 *n*: 0.0070	0.9993	4.5	0.007	4.8	0.90
iso-LCA	215.3(31)	0.005–0.500	*m*: 1.9069 *n*: 0.0001	0.9997	3.0	0.003	3.0	0.45
LCA	215.3(58)	0.010–1.000	*m*: 2.5449 *n*: −0.0007	0.9999	1.7	0.004	1.7	0.31
iso-DCA	75.1(91)	0.005–0.500	*m*: 3.2289 *n*: −0.0095	0.9998	2.1	0.003	2.0	0.86
DCA	255.3(93)	0.020–2.000	*m*: 7.7257 *n*: 0.0079	0.9998	3.2	0.013	3.2	0.16
CDCA	73.1(100)	0.005–0.500	*m*: 3.8610 *n*: −0.0046	0.9999	1.1	0.001	1.1	0.68
CA	253.2(44)	0.010–1.000	*m*: 3.6655 *n*: −0.0023	0.9999	0.3	0.001	0.3	0.64
12-oxo-DCA	231.3(52)	0.005–0.500	*m*: 1.9047 *n*: −0.0028	0.9999	1.0	0.001	1.0	0.61

$y = mx + n$, Linear regression line; r^2, correlation coefficient; Rel. S.D.$_y$, relative residual standard deviation; S.D.$_{xo}$, standard deviation of procedure; V_{xo}, relative standard deviation of procedure; confidence interval, 95%; data in parenthesis give size as percentage of base peak.

DCA were chosen to exclude an overestimation in *iso*-DCA and DCA quantification.

3.5. Accuracy and precision of BA determination

The preparation of the BA trimethyl silyl ether methyl esters was adapted to the established method of Czubayko et al. [6]. The precision of the method was evaluated by repeated analysis of the main human faecal BAs (*iso*-LCA, LCA, *iso*-DCA, DCA, CDCA, CA, 12-oxo-DCA). Within-run precision of 10 samples amounted to 4–7% for all BAs. The between-run precision was approximately 5–10% of five time-shifted measurements over 1 month of faeces samples with different calibrations (Table 2). A standard solution of DCA acetate methyl ester with a DCA concentration of 0.875 mg/mL was added to 10 faecal samples of the same origin also used for within-run precision. Hydrolysis, acidification, extraction, methylation, and silylation were carried out in the same manner described for faeces samples. The mean of concentrations amounted to 1.672 mg/mL corresponding to a recovery of 94.6%.

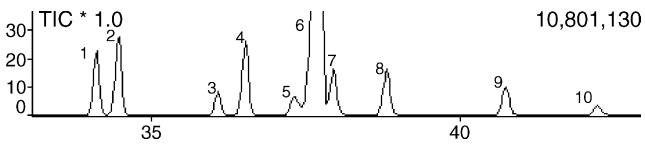

Fig. 3. TIC of BA standards: (1) *iso*-LCA (1 mg/mL), (2) LCA (1 mg/mL), (3) *iso*-DCA (0.25 mg/mL), (4) DCA (1 mg/mL), (5) CDCA (0.25 mg/mL), (6) CA (2 mg/mL); (7) HDCA (0.5 mg/mL), (8) UDCA (0.5 mg/mL), (9) HCA (0.5 mg/mL), (10) 12-oxo-DCA (0.5 mg/mL) (ZB5; 30 m; 0.25 mm; 0.25 μm; analysis parameters are given in instrumental conditions).

3.6. Calibration of BAs

Unconjugated LCA, *iso*-LCA, DCA, *iso*-DCA, CDCA, CA, 12-oxo-DCA, and internal standard (HDCA) were weighed into a vessel and dissolved in ethyl acetate as stock solution. Six dilutions were prepared as described for sterol calibration. Aliquots of each solution were methylated, silylated and resolved in decane as described in sample preparation. The concentration of the internal standard HDCA ($m/z = 81.2$ amu) was 0.5 mg/mL in each of the calibration standards.

Because of a consistent graduation in the analyte concentration within the calibrated range, a single calibration with three replicates per concentration was applied. The limits of detection were calculated from the calibration curve (Table 3).

3.7. Faecal amounts of sterols and BAs

The total sterol content in faeces of 86 subjects amounted to 19.4 ± 9.1 mg/g dry weight (Table 4). Coprostanol, as

Table 4
Comparison of sterol contents in faecal dry matter of healthy subjects

	Dry weight (mg/g)						
			Korpela and Adlercreutz[a] [44]		Perogambros et al. [43]	Reddy et al. [38]	Weststrate et al. [39]
	86 Subjects	81 Subjects[b]	19 Omnivores	20 Vegetarians	10 Subjects	19 Subjects	11 Subjects
Cholesterol	2.7 ± 3.0	2.0 ± 1.2	4.4 ± 0.9	5.1 ± 1.5	1.9 ± 0.4	3.2 ± 3.1	6.1 ± 1.2
Coprostanol	15.0 ± 9.4	16.6 ± 8.6	19.3 ± 3.4	8.3 ± 1.9	19.2 ± 3.9	14.9 ± 5.8	18 ± 3.1
Coprostanone	1.3 ± 0.9	1.4 ± 0.9	2.2 ± 0.5	0.81 ± 0.19	0.70 ± 0.13	2.4 ± 1.3	2.4 ± 0.5
Cholestanol	0.37 ± 0.17	0.39 ± 0.15				1.6 ± 0.4	
Total sterols	19.4 ± 9.1	20.3 ± 9.1			21.8 ± 3.7	23.1 ± 10.9	
Conversion (%)	81 ± 24	89 ± 7	75 ± 6	66 ± 8			

[a] Original data in μmol/g dry weight.
[b] Exclusion of low-converters; Data are presented as mean ± standard deviation of mean.

main bacterial product of cholesterol degradation, appeared among the faecal sterols with the highest concentrations of 15.0 ± 9.4 mg/g dry weight. A wide inter-individual range in conversion rate of cholesterol into degradation products (coprostanol, coprostanone, cholestanol) was observed (conversion rate: 2–99%, $n = 86$). These differences led to high standard deviations of the mean of faecal sterol concentrations and the conversion rate. The majority of subjects were high-converters, so the amount of excreted cholesterol in faeces was low. Five subjects were low-converters (conversion rate: <50%) meaning a higher excretion of cholesterol than of coprostanol occurred.

The faecal total content of BAs amounted to 10.6 ± 3.5 mg/g dry weight in the study population of 86 subjects (Table 5). The secondary BAs (*iso*-LCA, LCA, *iso*-DCA, DCA, 12-oxo-DCA) included the major components with a concentration of 10.1 ± 3.6 mg/g dry weight. The proportion of primary BAs averaged to 5% in regard to the total BA concentration.

4. Discussion

The potential of the modern analysis of faecal sterols and in particular BAs offers manifold possibilities of investigation into the effects of faecal steroids in the development or prevention of diseases like cholelithiasis, familial polyposis, ulcerative colitis, Crohn's disease, hyperlipidemia, and colon cancer [24,25]. Different analytical methods are available for the faecal steroid determination. Particularly enzymatical, spectroscopical, and liquid or gas chromatographical methods have been published [21,26,27]. Combining chromatographical separation and mass spectrometrical detection adds up to a potential system with high specificity and sensitivity, making it useful in analysing complex biological matrices like faeces [28].

Cholesterol and more than 40 known phytosterols may enter the colon via nutrition digestion. All these sterols are hydrolysed, hydrogenated, dehydrogenated, and/or epimerised by the gut bacteria into many degradation products and establish an extensive faecal sterol pattern. Standard substances

Table 5
Comparison of BA contents in faecal dry matter of healthy subjects

	Dry weight (mg/g)						
		Hofstad et al. [40]		Kaibara et al. [41]	Reddy et al. [38]	Spanhaak[a] et al. [42]	Weststrate et al. [39]
	86 Subjects	35 Subjects	12 Subjects[b]	10 Subjects	19 Subjects	10 Subjects	11 Subjects
iso-LCA	1.48 ± 0.74			0.69 ± 0.32			
LCA	2.20 ± 0.92	1.78 ± 0.15	2.55 ± 0.44	1.24 ± 0.48	2.96 ± 2.00	2.14 ± 0.79	2.94 ± 0.20
iso-DCA	1.44 ± 0.96			0.48 ± 0.22			0.83 ± 0.19
DCA	4.60 ± 1.83	1.96 ± 0.33	3.80 ± 0.85	1.61 ± 0.42	3.42 ± 2.50	3.45 ± 1.53	3.83 ± 0.47
12-oxo-DCA	0.37 ± 0.35			0.47 ± 0.29	0.34 ± 0.02		
CDCA	0.27 ± 0.32	0.01 ± 0.01	0.14 ± 0.11	0.24 ± 0.21	0.51 ± 0.08	0.51 ± 0.74	0.14 ± 0.03
CA	0.26 ± 0.48	0 ± 0	0.09 ± 0.09		0.27 ± 0.16	0.61 ± 1.51	0.13 ± 0.02
Total BA	10.6 ± 3.5	4.0 ± 0.5	6.9 ± 1.4	5.2 ± 1.4	9.5 ± 6.4		8.3 ± 0.8
Secondary BA	10.1 ± 3.6				4.9 ± 1.4		8.0 ± 0.8
Primary BA	0.5 ± 0.8			0.2 ± 0.2			0.3 ± 0.1

Data are presented as mean ± standard deviation of mean.
[a] Original data in μmol/g dry weight.
[b] Subjects with colonic polyps.

are not available for a few sterols, though they are necessary for clarifying the sterol distribution in faeces. Particularly the 5β-sterols of the main phytosterols sitosterol and campesterol were of substantial interest, and therefore, isolated from faeces samples which were phytosterol-enriched by nutrition according to Arca et al. [13]. The fragmentation pattern of the methyl and ethyl substituted 5β-sterols isolated from faeces agreed with those of coprostanol and coprostanone standards in consideration of their different molecular weight. Furthermore, the mass spectral data of substituted coprostanols were similar to the results of Seidel et al. [29].

The hydroxyl groups of steroids are conventionally transformed into alkyl silyl ether derivatives with the objective of enhancement of peak shape, resolution and sensitivity [30]. The evaluation of the TIC of underivatised sterols of a faeces sample showed a symmetric peak form without tailing, which may occur in the presence of active centres in the molecule like hydroxyl groups. Baseline separation of Δ5-sterols and their corresponding 5α-cholestanols (cholesterol/cholestanol, campesterol/campestanol, sitosterol/sitostanol) was achieved with a non-polar capillary column in this investigation. However, no-baseline separation for the Δ5-sterols and their corresponding 5β-cholestanones (cholesterol/coprostanone, campesterol/24-methyl coprostanone, sitosterol/24-ethyl coprostanone) was obtained by using non-polar capillary column (optima1; 50 m; 0.2 mm; 0.2 μm; 100% dimethyl polysiloxane; Macherey-Nagel, Düren, Germany). Increased polarity of stationary phase of the fused-silica capillary column (DB5; 50 m; 0.25 mm; 0.25 μm d.f.; 5% phenyl 95% dimethyl polysiloxane; DB1701; 60 m; 0.25 mm; 0.25 μm d.f.; 14% cyanopropyl phenyl methyl polysiloxane, J&W Scientific, Folsom, CA, USA) had little effect on the resolution of the Δ5-sterol/5β-cholestanone pairs. In agreement with Dutta and Normén [31] and Phillips et al. [32] the resolution of the Δ5-sterol/5α-cholestanol pairs was enhanced using middle-polar stationary phase. However, the substance specificity of detected fragments (Δ5-sterols: M-85; 5β-cholestanones: M-70) allowed quantification of underivatised sterols in SIM mode by using capillary column (Fig. 4).

An alkaline hydrolysis was preferred as the first step in the sterol sample preparation. No artefact formation of the oxo-compound coprostanone occurred during mild alkaline hydrolysis of a pure standard solution. Under more rigorous conditions (increased temperature and sodium hydroxide concentration) artefacts of coprostanone were verified primarily in the faecal matrix after silylation.

The hydrolysis results in saponification of sterol esters, but also of triacylglycerides or phospholipids. As a result of hydrolysis, the sum of free and esterified sterols was quantified. Sterols can be esterified with fatty acids differing in chain length and saturation grade. The determination of the whole faecal sterol spectrum including all the kinds of their esters is not yet practicable in a single analysis run by this method. Since, the aim of our study was just the pattern

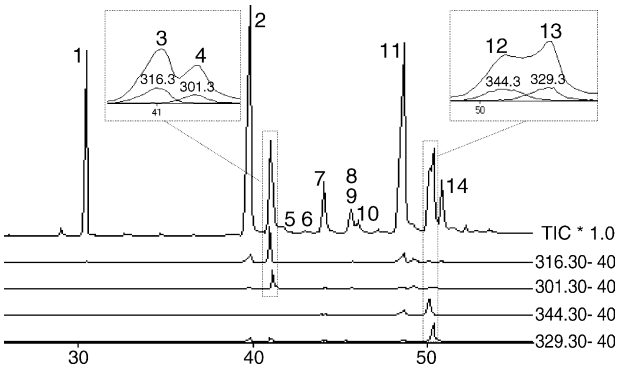

Fig. 4. TIC of the underivatised faecal sterol profile of a healthy subject after phytosterol intake of 1.5 g/d and fragments for cholesterol/coprostanone and sitosterol/24-ethyl coprostanone determination: (1) 5α-cholestane, (2) coprostanol, (3) coprostanone, (4) cholesterol, (5) cholestanol, (6) cholestanone, (7) 24-methyl coprostanol, (8) 24-methyl coprostanone, (9) campesterol, (10) cholest-4-ene-3-one, (11) 24-ethyl coprostanol, (12) 24-ethyl coprostanone, (13) sitosterol, (14) sitostanol, (optima; 1 ms; 50 m; 0.2 mm; 0.2 μm; analysis parameters are given in instrumental conditions).

of sterols instead of sterol fatty acid esters, hydrolysis was applied.

The major faecal BA fraction is present in unconjugated form as a result of bacterial activity. Even though hydrolysis during sample preparation is not necessary in either case [4], hydrolysis was preferred (200 μL sodium hydroxide: 10 mol/L) in order to exclude possible discrepancies in deconjugation behaviour due to the wide inter-individual range in bacterial flora and enzyme activity among a group of 86 subjects. Implementing the hydrolysis step, the measured results represent the sum of free and conjugated BAs. Among the different methods for hydrolysis of conjugated BAs like enzymatical or chemical procedures, a strong alkaline hydrolysis under atmospheric air pressure was selected in sample preparation [6,20,33,34].

Analysing BAs, derivatisation reactions were necessary for GC-analysis in spite of artefact formation of oxo-BAs [11], with the aim of lowering the high boiling points especially of dihydroxy or trihydroxy BAs. Although, the conversion into oximes before further derivatisation prevents enolisation of the oxo-BAs [35,36], we excluded the additional derivatisation step from sample preparation, since a low portion of the oxo-BAs in total faecal BA content exists.

The comparison of the MIC (m/z = 255.3 and 253.3 amu) of CA in a mixed standard solution and in the faeces sample showed different ratios and shifting in peak maxima of these fragments (Fig. 5). The quantification based on TIC-data would lead to overestimation of CA as a result of co-elution of components of complex faecal matrix. Consequently, detection in SIM mode was preferred. In agreement with this, Miyazaki et al. [37] used mass chromatography analysis for quantification of BAs, which have almost identical retention times and fragmentation patterns.

The concentrations of total faecal BAs of the study population (n = 86) were similar to the BAs contents described by Reddy et al. [38] and Weststrate et al. [39], but elevated

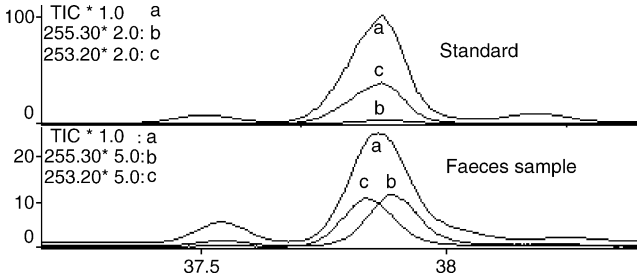

Fig. 5. Comparison of fragments of CA in standard solution and faeces sample (ZB5; 30 m; 0.25 mm; 0.25 μm; analysis parameters are given in instrumental conditions).

compared to the results of Hofstad et al. [40] and Kaibara et al. [41] (Table 5). The consideration of *iso*-BAs in total BAs amounts predominantly caused the differences. A high variation was pronounced in different investigations in primary BAs CA and CDCA concentration [38–42]. The mean of the cited CA and CDCA results is nearly equivalent to our findings of faecal CA and CDCA contents of 86 healthy subjects.

The total sterol concentration found in faeces of 86 subjects was similar to the sterol contents of healthy participants in studies conducted by Reddy et al. [38] and Perogambros et al. [43] (Table 4). The presence of low-converters of cholesterol within our study population confirmed the studies of Wilkins and Hackman [45], who also demonstrated low cholesterol degradation in faeces of healthy subjects. Exclusion of low-converters from statistics resulted in decreased faecal cholesterol concentrations and higher amounts of conversion products along with increased conversion rate.

5. Conclusions

The described sterol analysis is suitable for quantitative determination of free Δ5-sterols and their corresponding 5α- and 5β-degradation products in faeces by capillary-column-GC–MS–SIM. The method for BA analysis includes hydrolysis, acidification, extraction, and derivatisation for sample preparation. The BA trimethyl silyl ether methyl esters are separated by GC and detection in substance-specific MS–SIM mode prevents overestimation in BA analysis due to components of faecal matrix. Determined BAs represent the total of conjugated and unconjugated derivatives in faeces. The association of the chromatographical characteristics to the specific fragments ensures the selectivity and specificity of both methods. A moderate sample preparation, the high efficiency and robustness of the capillary-column-GC also allow faecal steroid analysis in routine use. Our results of faecal sterol and BA concentrations of 86 healthy subjects confirm current findings. There is a wide inter-individual range likely as a result of the individual nutrition associated with individual bacterial microflora activity in the colon.

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