A Validated, Fast Method for Quantification of Sterols and Gut Microbiome Derived 5α/β-Stanols in Human Faeces by Isotope Dilution LC–High-Resolution MS

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Chemicals and Reagents

Sodium hydroxide (p.a.), isooctane (2,2,4-trimethylpentane) >99%, cholesterol (cholest-5-en-3β-ol), cholesterol-d_5 (D5-cholest-5-en-3β-ol), campesterol (24(*R*)-methylcholest-5-en-3β-ol), *N*,*N*-dimethylglycine (DMG), *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide (EDC), 5α-sitostanol ((24(*R*)-ethyl-5α-cholestan-3β-ol), cholestanol (5α-cholestan-3β-ol), and cholestanol-d_7 (D7-5α-cholestan-3β-ol) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 2-Propanol HPLC grade was purchased from Karl Roth GmbH (Karlsruhe, Germany). Methanol LiChrosolv gradient grade for liquid chromatography, *N*,*N*-dimethylpyridin-4-amine (DMAP), ammonium acetate, and formic acid were obtained from Merck KGaA (Darmstadt, Germany). Campestanol-d_7 (D7-24(*R*)-methyl-5α-cholestan-3β-ol) and 5α-campestanol (24(*R*)-methyl-5α-cholestan-3β-ol) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Sitostanol-d_5 (D5-5α-stigmastan-3β-ol) 95% D was obtained by Medical Isotopes Inc. (Pelham, NH, U.S.A.). Sitosterol-d_5 (D5-24(*R*)-ethylcholest-5-en-3β-ol) and campesterol-d_5 (D5-24(*R*)-methylcholest-5-en-3β-ol) were purchased from Sugaris (Münster, Germany). 5β-Sitostanol (24(*R*)-ethyl-5β-cholestan-3β-ol) was ordered from Chiron AS (Trondheim, Norway). Sitosterol (24(*R*)-ethyl-cholest-5-en-3β-ol) was purchased from Avanti Polar Lipids (Alabaster, Alabama, U.S.A.). Coprostanol-d_5 (D5-5β-cholestan-3β-ol) was obtained from CDN Isotopes (Point-Claire, Quebec, Canada). All chemicals were of high-purity grade for analysis. Purified water was produced by Millipore Milli Q UF-Plus water purification system (Molsheim, France).

Stock Solutions

All sterol/stanol stock solutions (1.0 mg/mL) were prepared in methanol. The internal standard (ISTD) working solution contained sitosterol-d_5 (8.8 μg/mL), campesterol-d_5 (8.8 μg/mL), sitostanol-d_5 (3.5 μg/mL), campestanol-d_7 (0.4 μg/mL), cholesterol-d_5 (8.8 μg/mL), cholestanol-d_7(1.6 μg/mL), and coprostanol-d_5 (8.8 μg/mL) dissolved in methanol.

Samples

For method development, faeces samples were obtained from 22 healthy volunteers. Samples were collected in 50 mL flat-bottom polypropylene tubes, stored immediately at −20 °C, and transported to the laboratory on ice. Until further processing samples were stored at −80 °C. Sample amounts varied between 5 and 40 g wet weight and were collected from a single defecation. The volunteers were requested to take samples from different faeces locations in order to get a more representative sample.

Preparation of Faecal Homogenates

Raw faeces homogenate was prepared by using up to 2.0 g of faeces, adding 2.5 mL of 70% 2-propanol, and homogenizing in a gentle MACS dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). This homogenate was diluted with 2.5 mL of 70% 2-propanol and again homogenized. Between preparation steps samples were kept on ice. The dry weight (dw) of the raw faeces homogenate was determined by overnight drying of 1.0 mL of this mixture. For further analysis, the raw faeces homogenate was diluted to a final concentration of 2.0 mg dw/mL (diluted faeces homogenate DFH). DFHs were stored at −80 °C until further processing. Sufficient homogenization was evaluated by repeated determination of dry weight from 1 mL of raw faeces homogenate.

The determined dry weight of these portions showed coefficients of variation <8.5% for triplicates of 16 different faeces samples.

For preparation of charcoal-stripped stool matrix, 10 g of charcoal was added to 200 mL of pooled DFH and stirred at 4 °C, overnight. The removal rate for sterols and stanols by charcoal treatment was >97% for all compounds. Aliquots of the charcoal-stripped DFH and pooled DFH, respectively, were supplemented with a combined standard solution to obtain six calibrators in appropriate concentration ranges. The concentration range of the calibrators was estimated based on previous studies and literature values.

Preparation of DMG Derivatives

Amounts of 100 µL of ISTD mix, 200 µL of DFH (2 mg dw/mL), 200 µL of an aqueous 5 M NaOH solution, and 500 µL of 70% 2-propanol were combined in a 15.0 mL tube and sealed with a screw cap. Sterol esters were hydrolysed at 60 °C for 60 min in a water bath under constant agitation. After alkaline hydrolysis, samples were neutralized by adding 1.0 mL of 1 M hydrochloric acid and extracted with 3.0 mL of isooctane. An aliquot of 1200 µL of the upper isooctane layer was pipetted into an autosampler vial and evaporated to dryness in a vacuum concentrator. The residue was dissolved in a mixture of 60 µL of DMG (0.5 M) and DMAP (2 M) in chloroform and 60 µL of EDC (1 M) in chloroform at 45 °C for 60 min. The derivatization reaction was stopped by adding 500 µL of methanol. Excess solvent was evaporated, and the residue was dissolved in 300 µL of methanol. The sample was centrifuged, and 100 µL was pipetted into a microinsert for analysis.

Liquid Chromatography–High-Resolution Mass Spectrometry Analysis of Faecal Sterols and Stanols

The analysis of sterols and stanols was performed using an LC–MS/HRMS system consisting of an UltiMate 3000 RS column oven, an UltiMate 3000 XRS quaternary UHPLC, and an UltiMate 3000 isocratic pump (Thermo Fisher Scientific, Waltham, MA U.S.A.) coupled to hybrid quadrupole–orbitrap mass spectrometer QExactive (Thermo Fisher Scientific, Bremen, Germany). The system was equipped with a heated electrospray ionization source. The ion source was operated in positive ion mode using the following settings: ion spray 3500 V, sheath gas 58, aux gas 16, sweep gas 3, and aux gas heater temperature of 463 °C. Capillary temperature was set to 281 °C, and the S-lens rf level to 55. Data analysis was performed with TraceFinder 3.1 Clinical (Thermo Fisher Scientific Waltham, MA U.S.A.) which extracts target ions and generates calibration equations.

Quantification of Faecal Sterols by LC–MS/HRMS

The separation was achieved on a Kinetex 2.6 µm biphenyl, 50 mm × 2.1 mm column (Phenomenex, Aschaffenburg, Germany) at 40 °C. The injection volume was 5 µL. Mobile phase A consisted of methanol/water 5/95 (v/v), and mobile phase B was methanol/acetonitrile 10/90 (v/v), both containing 2 mM ammonium acetate. The gradient elution started at 72% B with a flow rate of 500 µL/min. Solvent B is raised to 84.5% B until 3.5 min followed by a final increase to 100% B in 0.1 min. The flow rate was increased to 800 µL/min at 3.6 min and kept 0.5 min for column cleaning. After 4.1 min solvent B was set back to 72% and held for 0.5 min. Method run time was 4.6 min. The isocratic pump was set to 0.2 mL/min methanol. Data were acquired in parallel reaction monitoring (PRM) mode with the following settings: resolution 17 500, AGC target 1×10^6, maximum IT 50 ms, MSX count 2, isolation window 0.8 *m/z*, and mass extraction window ±5 ppm. PRM monitors full product ion spectra of selected precursor ions, i.e., in our setting, analyte and its internal standard were multiplexed and analysed together (multiplexing of 2). To reduce the contamination of the mass spectrometer, the column flow was directed into the detector from 2.0 to 4.0 min by a divert valve. Collision energy for fragmentation was set to 15 eV.

Quantification of Faecal Stanols by LC–HRMS

The same LC and MS conditions were applied as described above for sterol analysis except the following changes: The solvent B is raised to 88% B until 4.5 min, followed by final increase to 100% B in 0.1 min. The flow rate was increased to 800 µL/min at 4.6 min followed by column cleaning at 800 µL/min kept for 0.6 min. After 5.2 min, solvent B was set back to 72% and held for 0.4 min. The method run time was 5.7 min. Data were acquired in full MS mode with the following settings: resolution 140 000 (at *m/z* 200), AGC target 1×10^6, maximum IT 150 ms, a full scan range from *m/z* 469 to 511, and mass extraction window of ±3 ppm. The sterols and 5α/β-stanols were quantified using the ratio to the corresponding ISTD. Target masses used for quantification are listed in **Table 1**.

Method Validation

Method validation was performed according to the European Medicines Agency (EMA) and Food and Drug Administration (FDA) guidelines on bioanalytical method validation (for details see the Supporting Information in the original publication).

Table 1. Analytical Characteristics of the LC–MS/HRMS and LC–HRMS Methods[a]

compd	formula (DMG derivative)	t_R[min]	mass transition	calibration curve[b]	calibration range [nmol/mg dw]	LOD [nmol/mg dw]	LOQ [nmol/mg dw]
cholesterol	$C_{31}H_{53}O_2N$	2.29	472.4 → 369.3508	$y = (0.565 \pm 0.031)x + (-0.299 \pm 0.258)$	0.193–137.68	0.029	0.39
β-sitosterol	$C_{33}H_{57}O_2N$	2.75	500.4 → 397.3831	$y = (1.247 \pm 0.10)x + (-0.025 \pm 0.131)$	0.039–32.28	0.039	0.16
campesterol	$C_{32}H_{55}O_2N$	2.51	486.4 → 383.3670	$y = (1.340 \pm 0.153)x + (-0.003 \pm 0.153)$	0.019–9.06	0.019	0.22
cholesterol-d_5	$C_{31}H_{44}D_5O_2N$	2.28	477.4 → 374.3842				
sitosterol-d_5	$C_{33}H_{52}D_5O_2N$	2.74	505.5 → 402.4144				
campesterol-d_5	$C_{32}H_{50}D_5O_2N$	2.49	491.5 → 388.3984				
compd	**formula (DMG derivative)**	**t_R[min]**	**target mass**	**calibration curve[b]**	**calibration range [nmol/mg dw]**	**LOD [nmol/mg dw]**	**LOQ [nmol/mg dw]**
coprostanol	$C_{31}H_{55}O_2N$	2.34	474.4306	$y = (0.516 \pm 0.023)x + (-0.070 \pm 0.096)$	0.090–138.25	0.09	0.301
cholestanol	$C_{31}H_{55}O_2N$	2.54	474.4306	$y = (1.734 \pm 0.110)x + (-0.011 \pm 0.019)$	0.008–3.78	0.003	0.009
5β-sitostanol	$C_{33}H_{59}O_2N$	2.78	502.4619	$y = (0.349 \pm 0.039)x + (-0.019 \pm 0.047)$	0.033–44.49	0.033	0.111
5α-sitostanol	$C_{33}H_{59}O_2N$	3	502.4619	$y = (2.594 \pm 0.108)x + (-0.012 \pm 0.060)$	0.008–7.09	0.008	0.026
5β-campestanol	$C_{32}H_{57}O_2N$	2.56	488.4462	$y = (3.672 \pm 0.053)x + (-0.026 \pm 0.021)$	0.009–21.36	0.009	0.032
5α-campestanol	$C_{32}H_{57}O_2N$	2.78	488.4462	$y = (3.672 \pm 0.053)x + (-0.026 \pm 0.021)$	0.009–21.36	0.015	0.049
coprostanol-d_5	$C_{31}H_{50}D_5O_2N$	2.33	479.458				
cholestanol-d_7	$C_{31}H_{48}D_7O_2N$	2.53	481.4745				
5α-sitostanol-d_5	$C_{33}H_{54}D_5O_2N$	2.99	507.4932				
5α-campestanol-d_7	$C_{32}H_{50}D_7O_2N$	2.77	495.4901				

[a] Sterols were quantified by PRM analysis. stanols were quantified by full scan HRMS.

[b] Calibration curve (n = 5).

Method Development

The aim of the current study was to develop a comprehensive, robust, and fast LC–MS/HRMS method to quantify FS in human samples. Sterols and stanols were derivatized to DMG esters to permit efficient ionization. For optimized separation of stereoisomeric compounds, different stationary phases with high stereochemical selectivity were tested, i.e., biphenyl and pentafluorophenyl (PFP) columns. With the PFP column the separation of different sterols, i.e., cholesterol, sitosterol, and campesterol, was achieved although their corresponding epimeric 5α/β-stanols have not been separated (Figure S2 in supplementary information provided in original publication). In contrast, the biphenyl stationary phase permits chromatographic separation of isomeric 5α/β-stanols which cannot be differentiated by their mass spectra (**Figure 1**).

On the basis of the following considerations the method was split into two separate runs, an LC–MS/HRMS run for sterols and a second LC–HRMS run for stanols: (i) Due to their double bond at C atom 5 DMG derivatives of sterols form an abundant product ion of the steroid backbone representing a fragment with a high specificity for sterols. A schematic illustration of the fragmentation is shown in Figure S1B. (ii) Stanols do not contain that double bond, and their DMG derivatives generate primarily a DMG-derived fragment at m/z 104.0711 which has low specificity for stanols. (iii) Because of the fast chromatography all compounds elute in a narrow retention time window resulting in an insufficient number of scans per chromatographic signal in MS/MS experiments. (iv) Full scan HRMS enables the separation of the stanol species from the isobaric interference of the second isotope peak of the coeluting sterol (Figure S3 in supplementary information provided in original publication). Coprostanol, cholestanol, 5α-sitostanol, and 5α-campestanol were quantified with the corresponding deuterated standard. 5β-Sitostanol and 5β-campestanol are quantified with the deuterated 5α-stanols since no deuterated 5β-stanols are commercially available. 5β-Campestanol was quantified with the calibration curve of 5α-campestanol since a 5β-campestanol standard is not available. Target compounds, internal standards, and retention times are listed in **Table 1**.

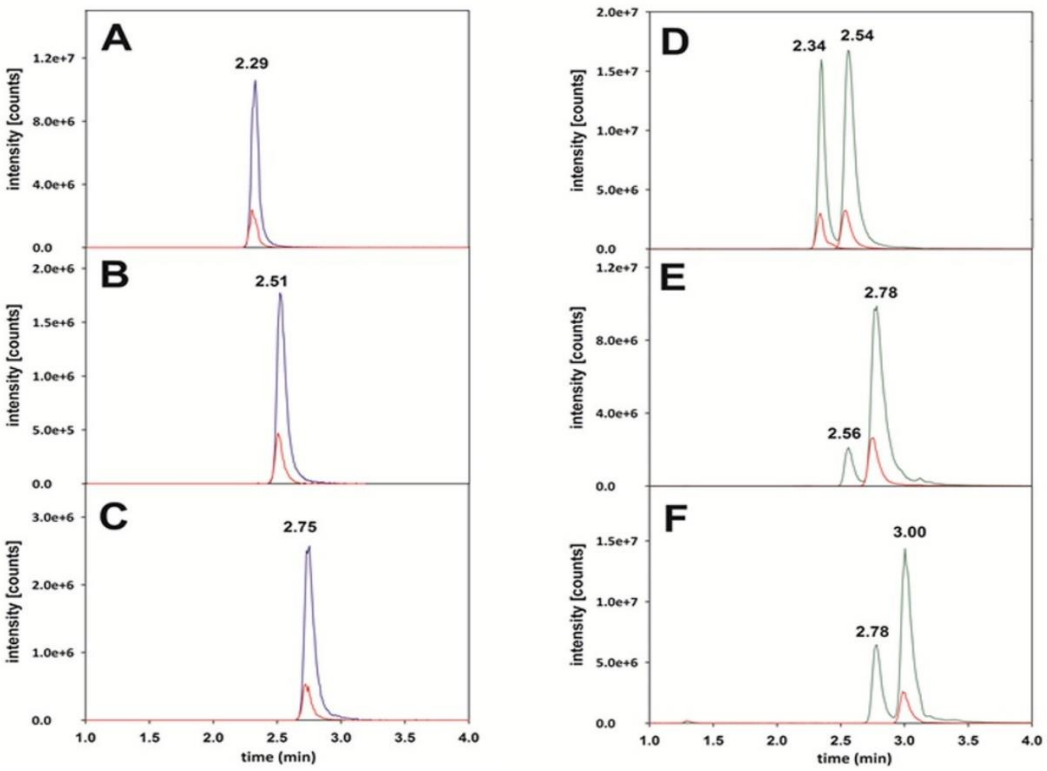

Figure 1. Extracted ion chromatograms of sterols and stanols of a human faeces sample. Panels A–F show the chromatograms of faecal sterols (blue), stanols (green), and deuterated internal standards (red) on a biphenyl column: (A) cholesterol; (B) campesterol; (C) sitosterol; (D) coprostanol and cholestanol; (E) 5α- and 5β-campestanol; (F) 5α- and 5β-sitostanol.

Characteristics of the Method

Validation confirmed the selectivity of the method. The specificity of the method was investigated using qualifier ions in six different faecal samples. The ion ratios quantifier/qualifier correspond to those of authentic standards with a maximum deviation of ±15% for all compounds. Calibration lines were linear over a wide range (**Table 1**) and verified by lack-of-fit testing. Limits of detection (LOD) and limits of quantitation (LOQ) for stanols were assessed by signal-to-noise ratio and for sterols by functional testing because in PRM analysis almost no noise was present. The limits of detection and quantification were determined in the range of 0.003–0.09 and 0.026–0.301 nmol/mg dw, respectively. Imprecision of the method was tested in four real samples and showed coefficients of variation (CV) below 15% except for 5β-campestanol which reached CVs up to 22% (**Table 2).**

The apparent recovery at low, medium, and high levels was between 88% and 111% (**Table 3**). Internal standard corrected matrix factor (isMF) was determined in seven different samples at high and low spike concentration with a maximum variation of 16% CV. Derivatized samples were stable at least for 10 days at a temperature of 4–8 °C. Carryover of analytes was not detected.

Faecal Sterol and Stanol Concentration Range in Human Faeces

The FS concentrations were determined for 22 healthy volunteers (**Table 4**). In all samples both sterols and their related stanols were detected except in the sample of an infant where neither plant stanols nor coprostanol were detected. Highest concentrations were detected for cholesterol and its related 5β-stanol coprostanol followed by sitosterol and campesterol and their related stanols. Interestingly, cholesterol and sitosterol were mainly converted to their 5β-stanols, whereas campesterol was mainly converted to 5α-campestanol (**Figure 2**).

Table 2. Intraday and Interday Assay Imprecision of Sterols and 5α/β-Stanols in Faeces

	intraday imprecision		interday imprecision	
sample	**mean (*n* = 6) [nmol/mg dw]**	**coefficient of variation [%]**	**mean (*n* = 4) [nmol/mg dw]**	**coefficient of variation [%])**
		Cholesterol		
1	3.24	7	3.3	3.5
2	5.69	2.2	5.42	3.8
3	2.76	4.6	2.66	6.8
4	2.89	1.6	2.82	5.6
		Coprostanol		
1	20.78	1.1	21.38	6.2
2	28.78	1.8	29.52	5.6
3	37.14	1.6	38.52	4.4
4	9.33	1.9	9.71	5.1
		Cholestanol		
1	0.56	3	0.56	3.5
2	0.8	4.6	0.77	3.3
3	1.04	1.8	1.03	2.8
4	0.42	0.9	0.45	6.5
		Sitosterol		
1	1.93	4	1.93	6.8
2	1.89	4	1.7	4.9
3	1.36	0.8	1.25	8.4
4	2.31	1.7	2.3	3.6
		5β-Sitostanol		
1	11.67	3	11.96	8.9
2	10.54	1.1	10.42	10.4
3	18.12	3.3	18.01	11.8
4	6.36	1.6	6.63	13.2
		5α-Sitostanol		
1	0.73	3.6	0.75	1.8
2	0.44	1.4	0.44	1.8
3	0.78	1.9	0.78	1
4	0.45	1.7	0.49	8
		Campesterol		
1	0.54	4.7	0.49	12.9
2	0.73	4.7	0.68	2
3	0.54	2.3	0.49	10.4
4	1.01	3	0.91	9.9
		5β-Campestanol		
1	0.29	1.8	0.34	15.1
2	0.38	2.4	0.45	16
3	0.76	4.3	0.93	21.4
4	0.3	3.2	0.38	21.1
		5α-Campestanol		
1	0.5	2.4	0.5	4.6
2	0.25	2.1	0.24	2.5
3	0.55	1.3	0.55	0.4
4	0.36	2	0.36	3

Table 3. Apparent Recovery Data of Sterols and 5α/β-Stanols in Human Faeces[a]

compd		spiked concn [nmol/mg dw]	concn ± SD [nmol/mg dw]	apparent recovery [%]
cholesterol	unspiked		8.63 ± 0.50	
	spike low	2.73	11.43 ± 0.41	102.5
	spike medium	13.67	22.09 ± 0.64	98.4
	spike high	68.36	73.71 ± 2.52	95.2
coprostanol	unspiked		15.18 ± 0.48	
	spike low	2.42	17.34 ± 0.25	89.2
	spike medium	12.11	26.92 ± 0.56	97.0
	spike high	60.56	72.17 ± 3.60	94.1
cholestanol	unspiked		0.53 ± 0.01	
	spike low	0.09	0.62 ± 0.01	97.5
	spike medium	0.45	0.99 ± 0.02	102.1
	spike high	2.24	2.66 ± 0.13	95.2
sitosterol	unspiked		3.85 ± 0.03	
	spike low	0.77	4.58 ± 0.07	94.8
	spike medium	3.85	7.57 ± 0.18	96.7
	spike high	19.24	21.77 ± 0.82	93.2
5β-sitostanol	unspiked		8.03 ± 0.29	
	spike low	3.59	11.25 ± 0.31	89.6
	spike medium	17.96	25.01 ± 0.44	94.5
	spike high	89.82	95.39 ± 7.64	97.3
5α-sitostanol	unspiked		0.54 ± 0.02	
	spike low	0.16	0.70 ± 0.02	94.0
	spike medium	0.81	1.30 ± 0.03	93.5
	spike high	4.07	4.28 ± 0.22	92.0
campesterol	unspiked		1.45 ± 0.11	
	spike low	0.11	1.56 ± 0.05	95.0
	spike medium	0.57	1.98 ± 0.09	92.6
	spike high	2.86	3.97 ± 0.10	88.1
5α-campestanol	unspiked		0.38 ± 0.01	
	spike low	0.08	0.47 ± 0.00	111.3
	spike medium	0.39	0.79 ± 0.02	103.9
	spike high	1.96	2.27 ± 0.10	96.7

[a] Concentrations determined in five replicates.

Table 4. Concentrations of Sterols and 5α/β-Stanols in Human Faeces

compd	mean ± SD [nmol/mg dry weight][a]	median	min	max
cholesterol	20.24 ± 20.05	11.03	2.62	65.66
coprostanol	20.41 ± 15.97	16.83	0.06	64.49
cholestanol	0.74 ± 0.38	0.62	0.23	1.77
sitosterol	6.01 ± 4.69	4.58	1.16	16.65
5β-sitostanol	7.82 ± 6.95	5.88	0.02	23.60
5α-sitostanol	0.61 ± 0.27	0.60	0.10	1.23
campesterol	2.22 ± 1.69	1.74	0.50	5.66
5β-campestanol	1.17 ± 2.90	0.55	0.00	13.70
5α-campestanol	0.51 ± 0.22	0.48	0.07	1.01

[a] Values of 22 volunteers.

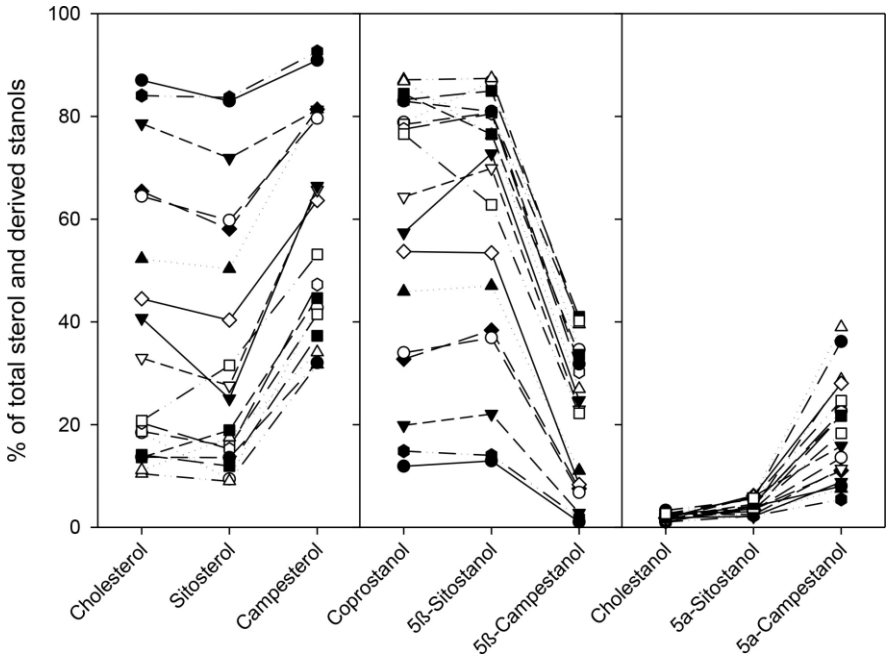

Figure 2. Faecal sterol fractions in healthy volunteers. Displayed are the fractions of cholesterol, sitosterol, campesterols, and their corresponding 5α/β-stanols as percent of their sum (sterol plus 5α- plus 5β-stanols). Each symbol represents an individual subject.