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ORIGINAL CONTRIBUTION

Fate of dietary phytosteryl/-stanyl esters: analysis of individual intact esters in human feces

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Abstract
Purpose The objective was to investigate the metabolic fate of phytosteryl/-stanyl fatty acid and ferulic acid esters upon consumption by healthy humans.
Methods A capillary gas chromatographic methodology was employed to follow a randomized, single-blind three group crossover clinical trial and to quantify simultaneously individual intact esters, liberated phytosterols/-stanols and their metabolites in feces. Skimmed milk drinking yogurts enriched with complex mixtures of phytosteryl/-stanyl fatty acid esters and ferulates, respectively, were employed as food carriers.
Results On average, 73 % of total plant stanyl fatty acid esters and 80 % of total plant steryl fatty acid esters were hydrolyzed. Among the individuals, the hydrolysis rates ranged from 40 to 96 %. In addition, there were subject-dependent discrepancies between the amounts of phytosterols/-stanols actually determined in the feces and the calculated hydrolysis rates. On average, 69 % of the amounts of sterols/stanols expected from the amounts of remaining intact esters were found.
Conclusions The study revealed large interindividual variability regarding the recoveries of dietary phytosteryl/-stanyl esters upon gastrointestinal passage in healthy humans. Nevertheless, there was a significant impact of the acid moiety (oleate = linoleate = linolenate > eicosanoate > palmitate > ferulate) on the hydrolysis rates; the influence of the phytosterol/-stanol moiety was less pronounced.

Keywords Phytosteryl/-stanyl fatty acid esters · γ-Oryzanol · Phytosterols · Hydrolysis · Metabolization · Functional food · Randomized clinical trial

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Introduction

Increased plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels are established risk factors for coronary heart diseases. The cholesterol-lowering properties of phytosterols/–stanols and their fatty acid esters in humans have been described in several studies [1–6]. Similar effects on serum cholesterol have been reported for phytosteryl/-stanyl ferulic acid esters in animals [7, 8] and humans [9, 10] using γ-oryzanol, a ferulate mixture obtained from rice bran. Several functional foods (e.g., skimmed milk drinking yogurts, margarines) enriched with fatty acid esters of phytosterols and -stanols are currently available on the EU market [11]. The cholesterol-lowering action of these products is closely associated with the intestinal and hepatic metabolism shared by cholesterol and phytosterols/-stanols [12–14]. The inhibition of intestinal cholesterol absorption by competitive incorporation into dietary mixed micelles appears to play a major role [15–17]. Recently published in vitro data demonstrated that micellar cholesterol solubilization is not affected by intact phytosteryl fatty acid esters and that intact esters are not

solubilized into micelles in a model bile, mixed-micelle system [15]. Therefore, intestinal hydrolysis of these esters by digestive enzymes seems to be a crucial step for the cholesterol-lowering effects of dietary phytosteryl/-stanyl esters. In vitro data indicate that the rate of intestinal hydrolysis, for example, by pancreatic cholesterol esterase, depends on both the phytosterol/-stanol structure and the acid moiety [18]. Feeding stearates of sitosterol, sitostanol and stigmasterol to hamsters confirmed differences in hydrolysis rates depending on the sterol/stanol moiety [19]; the results obtained in rats fed either phytosteryl/stanyl oleates or stearates indicate an impact of the acyl residue [20].

In vitro studies focussing on the enzymatic hydrolysis of phytosteryl/-stanyl ferulic acid esters by different digestive enzymes consistently demonstrate that solely desmethylsteryl ferulates (e.g., campesteryl ferulate) are accepted as substrates; 4,4-dimethylsteryl ferulic acid esters such as cycloartenyl ferulate were not hydrolyzed by the enzymes employed [21–23].

In human trials involving duodenal infusion [24–26] or oral administration of phytosteryl/-stanyl fatty acid esters [27–29], the total fatty acid ester hydrolysis has been calculated either indirectly by determination of the intact ester fraction based on the quantification of free phytosterol/-stanol contents before and after saponification [24–28] or the data reported referred to total esters [29]. Data on the hydrolysis of individual phytosteryl/-stanyl esters and a consideration of the impact of their molecular structures are lacking. In addition, oral administration trials have been confined to ileostomy patients [27–29].

Therefore, the objective of the present study was to investigate the metabolic fate of individual phytosteryl/-stanyl fatty acid and ferulic acid esters upon consumption and digestion by healthy human subjects. For this purpose, a capillary gas chromatography (GC)-based methodology developed for the quantification of individual intact phytosteryl/-stanyl esters in enriched foods [30] was adapted to the analysis of feces. In combination with an efficient isolation procedure, this approach was used to follow a randomized human trial and thus to allow the simultaneous quantification of intact esters, liberated phytosterols/-stanols and their metabolites. As an example for commercially available functional food products, skimmed milk drinking yogurts enriched with complex mixtures of phytosteryl/-stanyl esters were employed as food carriers of the target substrates.

Experimental

Study population

Fifteen male subjects (age: 28 ± 3 years (range: 22–33 years); BMI (kg/m^2): 23.6 ± 1.6 (range: 21.6–25.7)) volunteered to participate in the study; fourteen of them completed the trial. All candidates gave written informed consent to participate; the consent form and the study design were approved by the ethics committee of the Faculty of Medicine of the Technische Universität München (#2943/10). According to personal history, all participants were non-smokers, non-vegetarians and free of chronic metabolic diseases. They reported not to have used antibiotics or statins for at least 6 months before the beginning of the study.

Experimental protocol

A randomized, single-blind three group crossover clinical study lasting for 7 weeks was performed. Healthy volunteers were given three skimmed milk drinking yogurts enriched with different phytosteryl/-stanyl ester mixtures. The participants were asked to consume the yogurts once a day for a period of 3 days besides their habitual diet; they were also advised not to consume other phytosterol-enriched products. Samples were collected for 5 days; wash out periods of 15 days were used between the interventions (Fig. 1).

Administered food products

The following skimmed milk drinking yogurts were administered: (I) Emmi Benecol® enriched with phytostanyl fatty acid esters (produced by Emmi, Switzerland; distributed in Germany by Emmi Deutschland GmbH, Essen; purchased in local supermarkets); the following information was included in the list of ingredients per daily dose (65 mL): plant stanyl ester, corresponding to 2 g plant stanols; total fat content (without stanol) was declared as 1.4 g, thereof 0.1 g saturated, 0.9 g monounsaturated and 0.4 g polyunsaturated fatty acids. (II) Becel pro.activ® enriched with phytosteryl fatty acid esters (produced by Unilever, Germany; purchased in local supermarkets); the following information was given in the list of ingredients per daily dose (100 g): 3.4 % phytosteryl esters, corresponding to 2 g phytosterols; total fat (without 2 g sterols) content of 1.5 g, thereof 0.2 g saturated, 0.4 g monounsaturated and 0.9 g polyunsaturated fatty acids. (III) A skimmed milk drinking yogurt enriched with phytosteryl/-stanyl ferulic acid esters; the product was prepared in-house using γ-oryzanol (purity: 99.8 %), a ferulate mixture obtained from rice bran (Henry Lamotte Oils GmbH, Germany) and a commercial skimmed milk drinking yogurt (0.1 % fat). γ-Oryzanol (58 g) and yogurt (1,042 g) were homogenized using an ULTRA TURRAX®. The daily dose (65 g) of the obtained preparation contained 0.07 g fat (without γ-oryzanol) and 3.4 g γ-oryzanol, that is, the same amount of steryl/stanyl esters as employed in trials I and II.

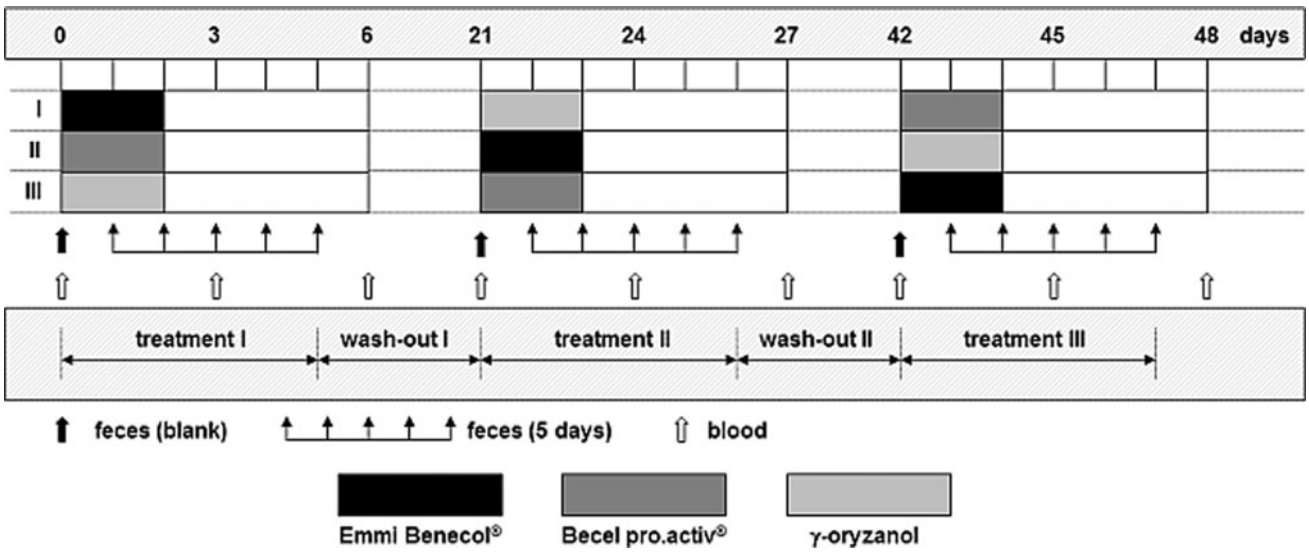

Fig. 1 Study protocol

Chemicals and reference substances

Chemicals

Cholesteryl palmitate (≥98 %), 5α-cholestane (≥97 %), cholesteryl cinnamate (Aldrich^{CPR}), cholesterol (95 %), (+)-4-cholesten-3-one (98 %), coprostan-3-ol, (≥98 %), coprostan-3-one, pyridine (≥99.8 %), *N,O*-bis(trimethylsilyl)trifluoro acetamide (BSTFA) + trimethylchlorosilane (TMCS) (99:1) were obtained from Sigma-Aldrich (Taufkirchen, Germany); 24-Ethylcoprostanol (97.7 %) was obtained from Chiron AS (Trondheim, Norway). Potassium hydroxide (KOH) (pure), hydrochloric acid (37 %), *n*-hexane (SupraSolv®), ethyl acetate (≥99 %) and ethanol (EtOH) (AnalR NORMAPUR®) were purchased from VWR International (Darmstadt, Germany). Methyl *tert*-butyl ether (MTBE, DiveronS) was purchased from Evonik Oxeno GmbH (Marl, Germany) and was distilled prior to use.

Reference substances

A mixture containing fatty acid esters of sitostanol and campestanol ("Plant stanol ester, STAEST-115") was provided by Raisio Group (Raisio, Finland). The following preparations were gifts from Cognis GmbH (Illertissen, Germany): (I) phytosteryl fatty acid esters ("Vegapure® 95E"; consisting of sitosteryl, campesteryl, stigmasteryl and brassicasteryl esters), (II) plant stanols ("Reducol™ stanol powder"; 91.2 % sitostanol and 8.2 % campestanol) and (III) plant sterols from soy bean oil ("Generol® 122 N"; 46.9 % sitosterol, 28.5 % campesterol, 14.4 % stigmasterol and 3.5 % brassicasterol), respectively.

A dimethylsterol fraction consisting of cycloartenol (90 %) and 24-methylene-cycloartanol (10 %) was isolated from γ-oryzanol by sixfold recrystallization for the enrichment of the dimethylsteryl ferulic acid esters [31], followed by alkaline hydrolysis and extraction of the unsaponifiable sterols.

Sample collection

Feces samples

The entire daily feces was collected by the participants in plastic containers equipped with lockable sterile freezer bags at baseline and during 5 days of each treatment period starting with the second day of application (day 1, 22, 42). The individual daily feces samples were delivered to the laboratory, deep-frozen and stored at −35 °C until sample preparation.

Plasma samples

During each of the three periods, overnight fasting blood samples were taken from subjects at baseline (day 0, 21, 42), at the end of consumption (day 3, 24, 45) and after 6 days (day 6, day 27, day 48). Plasma was separated from blood by centrifugation at 3,000 g for 10 min at 20 °C and stored at −35 °C until further analysis.

Analytical methods

Isolation procedure

For each individual, the feces samples collected over the five consecutive days during the treatment period were pooled, weighed and homogenized with water (1/2, m/m) by magnetic stirring at 500 rpm for 2 h. Three aliquots (25 g each) of the feces homogenate were lyophilized, ground and stored deep-frozen at −35 °C until analysis. Each aliquot was analyzed in triplicate: 100 mg of lyophilized feces were weighed into 15-mL centrifuge tubes containing 375 μg of 5α-cholestane (IS_1) and either 225 μg of cholesteryl palmitate (IS_2) or 750 μg of cholesteryl cinnamate (IS_3) as internal standards. The lipids were extracted three times with 5 mL of *n*-hexane/MTBE (3/2, v/v). After membrane filtration (0.45 μm), 1 mL of the filtrate was evaporated to dryness by a gentle stream of nitrogen (N_2) at room temperature. The residue was derivatized by adding 300 μL of BSTFA + TMCS (99:1) and 200 μL of pyridine followed by incubation at 80 °C for 20 min. After drying under N_2, the resulting trimethylsilyl (TMS) ethers were redissolved in 1 mL *n*-hexane/MTBE (3/2, v/v) and subjected to gas chromatography.

Capillary gas chromatographic separation

Gas chromatographic analysis of individual intact phytosteryl/-stanyl esters and sterols/stanols was performed on an Agilent Technologies instrument 6890 N (Böblingen, Germany) equipped with a flame ionization detector (GC/FID), using a crossbond® trifluoropropylmethyl polysiloxane Rtx®-200MS (Restek, Bad Homburg, Germany) fused silica capillary column (30 m × 0.25 mm ID × 0.1 μm film thickness) [30]. An aliquot of 1 μL was injected by automated injection (7683 Series Injector, Agilent Technologies) at an injection temperature of 280 °C using hydrogen as carrier gas with the constant flow rate of 1.5 mL/min. The detector temperature was set to 360 °C. Split flow was set to 11.2 mL/min, resulting in a split ratio of 1:7.5. Nitrogen was used as a make up gas with a flow of 25 mL/min. The initial oven temperature was kept at 100 °C (2 min), thereafter programmed with 15 °C/min up to 310 (2 min), and 1.5 °C/min up to 340 °C (3 min).

Mass spectrometric analysis (GC/MS)

Identifications of fecal intact esters, liberated phytosterols/-stanols and metabolites were performed on a Finnigan Trace GC ultra (Thermo Fisher Scientific, Austin, TX) equipped with a Finnigan Trace DSQ mass spectrometer (Thermo Fisher Scientific, Austin, TX). An aliquot of 1 μL was injected by automated injection (TriPlus autosampler, Thermo Fisher Scientific, Austin, TX) in a split mode with a ratio of 1:8. Mass spectra were obtained by electron impact ionization (EI) at 70 eV in the full scan mode at unit resolution from 40 to 750 Da (scan time 0.4 s). Helium was used as carrier gas with a constant flow rate of 1.5 mL/min. The interface was heated to 320 °C and the source to 250 °C. The residual conditions were as described for the GC/FID analysis. Identification of the compounds was carried out by comparing their mass fragmentation pattern with those of reference substances, in-house database containing fatty acid esters synthesized at laboratory scale according to a patent application [32] and literature data [30, 33–37].

Quantifications by GC/FID

The concentrations of individual intact esters and liberated phytosterols/-stanols were calculated from calibration functions using the respective internal standard (IS_1, IS_2, IS_3). Linear regression analysis was performed in coordinate ratios of peak areas (component/IS) and amounts (component/IS). Calibrations allowing the selective quantification of individual phytosteryl/-stanyl esters were performed at concentrations ranging from 70 μg to 20 mg STAEST-115/100 mg feces for stanyl fatty acid ester, 70 μg to 4.0 mg Vegapure®95E/100 mg feces for steryl fatty acid esters and 175 μg to 7.0 mg γ-oryzanol/100 mg feces for steryl/stanyl ferulates. The correlation coefficients (R^2) of the calculated calibration functions were in the range of 0.9954–0.9999.

Calibrations allowing the quantification of the individual liberated desmethylsterols/-stanols were performed at concentrations ranging from 34.5 μg to 3.0 mg Reducol™/100 mg feces (campestanol and sitostanol), 3.75 μg to 4.0 mg Generol® 122 N/100 mg feces (campesterol and sitosterol) and 18.75 μg to 2.25 mg dimethylsterol preparation/100 mg feces (cycloartanol, cycloartenol and 24-methylenecycloartanol). The correlation coefficients (R^2) of the calculated calibration functions were in the range of 0.9940–0.9981.

Microbial transformation products (secondary metabolites) of sitosterol and campesterol were calculated using relative response factors (RRF) compared to 5α-cholestane. The RRF were determined using 24-ethylcoprostanol and the corresponding fecal cholesterol metabolites as references. RRF to 5α-cholestane used for the quantification of secondary metabolites were calculated as 0.9690 (24-methylcoprostanol), 1.0427 (24-methylcoprostanone), 1.1278 (24-methylcholest-4-en-3-one) for campesterol metabolites as well as 1.0916 (24-ethylcoprostanol), 1.1746 (24-ethylcoprostanone) and 1.2705 (24-ethylcholest-4-en-3-one) for sitosterol metabolites.

Determination of total fecal phytosterols/-stanols after alkaline hydrolysis by means of GC/FID

Lyophilized feces were hydrolyzed under alkaline conditions according to [38]. Three aliquots of 100 mg were weighed into 4-mL screw-capped vials containing 375 μg of 5α-cholestane as internal standard. After saponification with ethanolic potassium hydroxide (2 mL, 3 M KOH, 90 % EtOH) at 80 °C for 60 min and neutralization with hydrochloric acid (1.0 mL, 6 M HCl), lipids were extracted three times with 5 mL of *n*-hexane/MTBE (3/2, v/v). Sample derivatization was as described for the isolation of intact phytosteryl/-stanyl esters and steroids.

Analysis of plasma lipids

Plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and plasma triacylglycerols (TG) were analyzed with a Roche MODULAR DPE system using established enzymatic methods. (Roche Diagnostics GmbH, Mannheim). The following enzymatic kits were used: (I) TC (Cat.No. 11875523216), (II) LDL-C (Cat.No. 05230438190), (III) HDL-C (Cat.No. 04713265190) and (IV) TG (Cat.No. 1876040216), respectively.

Determination of plasma phytosterol/-stanol levels

Saponification, lipid extraction and quantitative determination by means of gas chromatography/mass spectrometry (GC/MS) were performed as combination of two methods [39, 40] with minor modifications. Briefly, 6.25 μg of 5α-cholestane was added as internal standard to 1 mL of plasma. After saponification with ethanolic KOH (3 mL, 3 M KOH, 90 % EtOH) at 100 °C for 60 min and neutralization with HCl (1.5 mL, 6 M), sterols were extracted three times with 2 mL of *n*-hexane/MTBE (3/2, v/v). The combined organic phases were dried completely under a gentle stream of N_2, and the residual sterols/stanols were derivatized to TMS ethers by adding 600 μL of BSTFA + TMCS (99:1) and 400 μL of pyridine followed by incubation at 80 °C for 20 min. GC/MS quantification of phytosterols/-stanols was performed using selected ion monitoring (SIM) mode under identical conditions as described for the identification of fecal compounds. Quantification was carried out by monitoring *m/z* 372 $[M]^+$ for 5α-cholestane, *m/z* 472 $[M]^+$ for campesterol, *m/z* 486 $[M]^+$ for sitosterol, *m/z* 474 $[M]^+$ for campestanol, *m/z* 488 $[M]^+$ for sitostanol, *m/z* 408 $[M\text{-}TMSOH]^+$ for cycloartenol, *m/z* 410 $[M\text{-}TMSOH]^+$ for cycloartanol and *m/z* 422 $[M\text{-}TMSOH]^+$ for 24-methylenecycloartanol. The concentrations of the phytosterols/-stanols were calculated from calibration curves using 5α-cholestane as internal standard. Linear regression analysis was performed in coordinate ratios of peak areas (phytosterol/-stanol / IS) and amounts (phytosterol/-stanol / IS).

Statistical analyses

Statistical analyses were performed with IBM® SPSS® Statistics (version 19.0). Normal distribution and homogeneity of variance were tested with the Kolmogorov–Smirnov–Lilliefors test and Levene's test, respectively. Contents of intact phytosteryl/-stanyl esters in feces were analyzed using ANOVA and post hoc Tukey's HSD test if recoveries were ascertained to be significantly different. Additionally, separate comparisons were performed for the two groups of ferulic acid esters (desmethylsteryl and dimethylsteryl ferulates) by Student's unpaired *t*-test. Plasma samples were compared with either repeated-measures ANOVA including Bonferroni multiple comparisons or pairwise Friedman test depending on data distribution. Differences were considered significant at $P \leq 0.05$, and *P*-values < 0.1 were considered as indicators for a statistical trend. Unless otherwise indicated, data were expressed as mean ± standard error of the mean (SEM).

Results

Methodology

The employed capillary gas chromatographic separation allowed the simultaneous determination of individual intact phytosteryl/-stanyl fatty acid and ferulic acid esters, of phytosterols and -stanols liberated upon hydrolysis and of fecal metabolites in a single GC run. Typical chromatograms obtained from the Benecol®-, the pro.activ®- and the γ-oryzanol-intervention, respectively, are shown in Fig. 2A–C. The two regions of interest—(I) liberated sterols/stanols and microbial transformation products and (II) intact phytosteryl/-stanyl esters—are depicted at enlarged scale. Identifications were based on GC/MS; peak assignments and relative retentions times are given in Table 1.

The limits of detection (LOD), limits of quantification (LOQ) and coefficients of variation (CV) were determined for the individual intact phytosteryl/-stanyl esters (Table 1). The combination of the isolation procedure with the GC/FID analysis across all subjects in the three study periods resulted in high recoveries (92.9 ± 5.6 %, 99.5 ± 5.0 % and 98.1 ± 2.2 %) of the internal standards (5α-cholestane, cholesteryl palmitate and cholesteryl cinnamate), respectively. Based on the analysis of feces of one of the participants in the Benecol®-exposure, data on the repeatability and reproducibility of the employed method

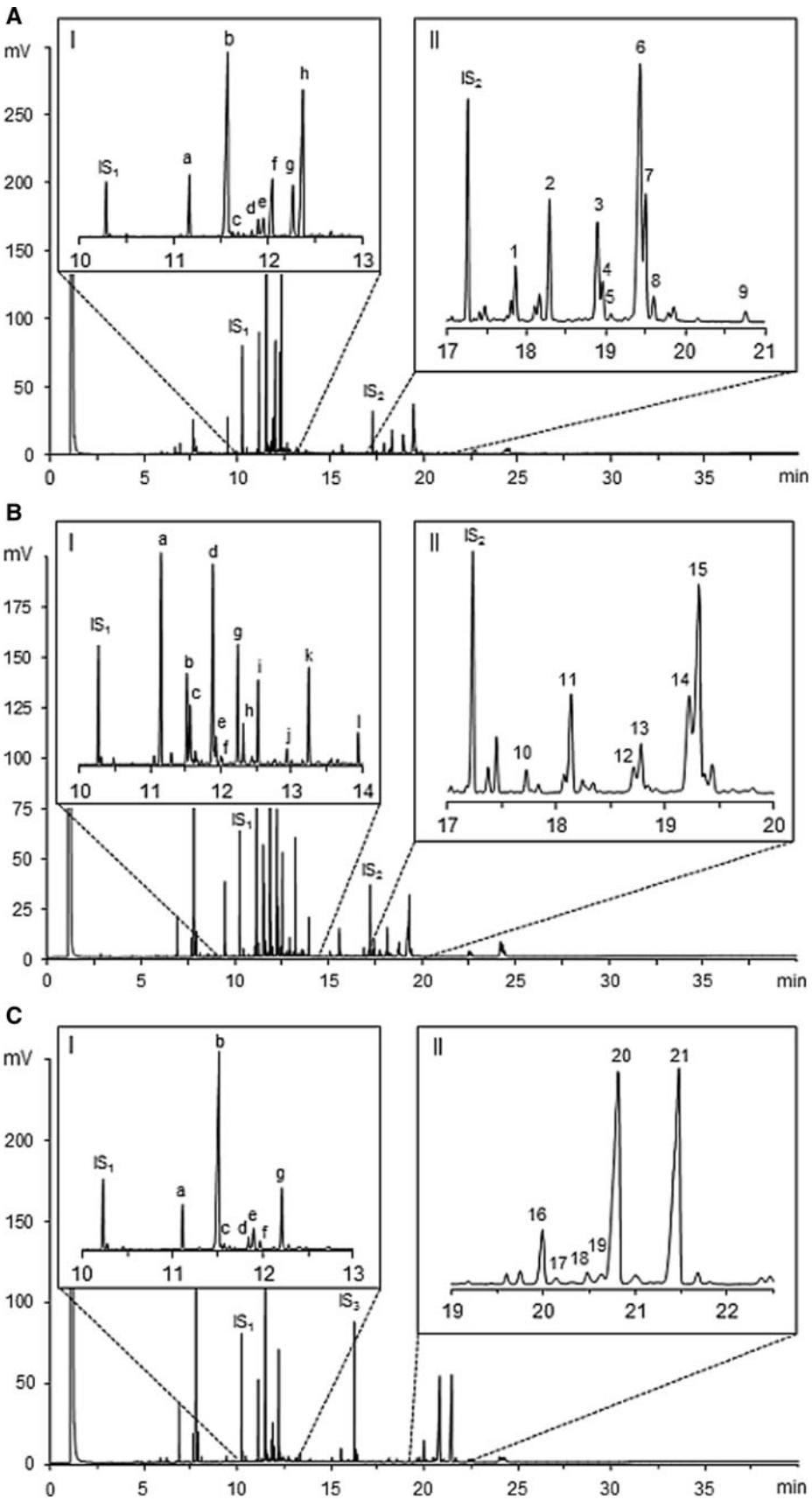

Fig. 2 Capillary gas chromatographic separations of intact phytosteryl/-stanyl esters, upon digestion released sterols/stanols and microbial transformation products in human feces after oral administration of skimmed milk yogurt drinks enriched with phytosteryl/-stanyl esters; **A** Benecol®, **B** pro.activ® and **C** γ-oryzanol; (*I*) = steroids; IS_1 = 5α-cholestane; *a* coprostanol; *b* cholesterol; *c* 24-methyl-coprostanol; *d* 24-ethylcoprostanol; *e* campesterol; *f* campestanol; *g* sitosterol; *h* sitostanol; *i* coprostanone; *j* 24-methylcoprostanone; *k* 24-ethylcoprostanone; *l* 24-ethylcholest-4-en-3-one; (*II*) = intact phytosteryl/-stanyl esters; peak numbering according to Table 1; IS_2 cholesteryl palmitate; IS_3 cholesteryl cinnamate

Table 1 Limits of detection (LOD), limits of quantification (LOQ) and coefficients of variation (CV) determined by analysis of industrial phytosteryl/-stanyl ester mixtures

No[a]	Phytosteryl/-stanyl ester	RRT (min)	Content[g] (g/100 g)	LOD[h] (μg/100 mg dry feces)	LOQ[h] (μg/100 mg dry feces)	CV[h] (%)
Stanyl fatty acid ester[b]						
1	Campestanyl-C16:0	1.045[e]	0.5	0.50	1.37	4.6
3	Campestanyl-C18:1	1.096[e]	5.7	1.50	3.90	2.3
4	Campestanyl-C18:2	1.100[e]	2.1	1.96	5.54	5.4
5	Campestanyl-C18:3	1.106[e]	1.1	2.11	5.27	5.5
2	Sitostanyl-C16:0	1.060[e]	4.1	0.72	2.07	2.8
6	Sitostanyl-C18:1	1.128[e]	54.2	2.75	6.89	2.8
7	Sitostanyl-C18:2	1.131[e]	18.8	4.83	10.26	5.3
8	Sitostanyl-C18:3	1.138[e]	7.5	9.70	24.06	4.8
9	Sitostanyl-C20:0	1.206[e]	2.0	4.41	11.30	5.0
Steryl fatty acid ester[c]						
10	Campesteryl-C16:0	1.029[e]	1.2	0.99	2.70	4.8
12	Campesteryl-C18:1	1.090[e]	5.5	1.00	2.70	5.3
13	Campesteryl-C18:2	1.094[e]	11.6	1.94	5.28	3.2
11	Sitosteryl-C16:0	1.053[e]	4.6	0.44	0.83	5.8
14	Sitosteryl-C18:1	1.117[e]	23.0	1.81	3.54	6.6
15	Sitosteryl-C18:2	1.123[e]	43.5	4.17	8.39	3.5
Steryl/stanyl ferulic acid ester[d]						
16	Campesteryl ferulate	1.235[f]	8.3	15.34	43.66	3.6
17	Campestanyl ferulate	1.244[f]	1.0	2.57	7.08	4.0
19	Sitosteryl ferulate	1.274[f]	2.8	6.93	18.70	4.2
18	Cycloartanyl ferulate	1.266[f]	1.3	2.64	7.48	4.0
20	Cycloartenyl ferulate	1.288[f]	37.7	70.67	199.12	3.4
21	24-Methylenecycloartanyl ferulate	1.326[f]	42.9	83.29	236.50	4.7

[a] Peak numbers correspond to Fig. 2A–C (II)

[b] Phytostanyl fatty acid ester mixture (STAEST-115)

[c] Phytosteryl fatty acid ester mixture (Vegapure®95E)

[d] Phytosteryl/-stanyl ferulic acid ester mixture (γ-orzanol)

[e] Relative retention time [RRT] compared to cholesteryl palmitate (IS_2)

[f] Relative retention time compared to cholesteryl cinnamate (IS_3)

[g] Content in the reference mixtures determined by GC/FID [30]

[h] Determined according to DIN 32645 (2008–2011) [49]

are presented in Table 2. The repeatability is indicated by very low standard deviations of the results obtained by triplicate analysis of the three feces aliquots at each point of analysis. The reproducibility of the results was demonstrated by repeated analysis of the feces in a time-dependent manner.

Simultaneous determination of intact esters and liberated phytosterols/-stanols in feces

The mean weights of daily feces of 145.7 ± 50.6 g (Benecol®), 140.1 ± 49.3 g (pro.activ®) and 139.5 ± 58.9 g (γ-oryzanol) as well as the corresponding feces' dry weights of 25.5 ± 4.6 %, 25.3 ± 5.4 % and 26.0 ± 4.8 % were within the ranges reported for healthy humans [41].

A comparison of the amounts of consumed phytosteryl/-stanyl esters with the amounts of intact esters and liberated sterols/stanols recovered in the feces is shown in Table 3. In the Benecol®-trial, average hydrolysis rates of 73 % were calculated for campestanyl as well as for sitostanyl fatty acid esters on the basis of the intact esters recovered in the feces; the average hydrolysis rates calculated for campesteryl and sitosteryl fatty acid esters in the pro.activ®-trial amounted to approximately 80 %, respectively. Average total recoveries, that is, the sum of intact esters and liberated phytosterols/-stanols in feces, ranged from

Table 2 Reproducibility and repeatability data on the analysis of individual intact stanyl esters in feces upon consumption of enriched skimmed milk drinking yogurts

Stanyl fatty acid ester	Point of analysis		
	Day 1 (mmol/dry weight)[a]	Day 28 (mmol/dry weight)[a]	Day 56 (mmol/dry weight)[a]
Campestanyl-C16:0	0.14 ± 0.00	0.14 ± 0.00	0.14 ± 0.00
Campestanyl-C18:1	0.43 ± 0.01	0.41 ± 0.01	0.43 ± 0.01
Campestanyl-C18:2	0.12 ± 0.00	0.13 ± 0.01	0.13 ± 0.01
Campestanyl-C18:3	0.05 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
Sitostanyl-C16:0	0.38 ± 0.01	0.36 ± 0.01	0.37 ± 0.01
Sitostanyl-C18:1	1.51 ± 0.05	1.40 ± 0.03	1.52 ± 0.04
Sitostanyl-C18:2	0.45 ± 0.01	0.45 ± 0.01	0.47 ± 0.02
Sitostanyl-C18:3	0.15 ± 0.00	0.14 ± 0.00	0.15 ± 0.00
Sitostanyl-C20:0	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.01

[a] Values represent mean ± standard deviation (SD); three aliquots of combined feces of one subject were worked up in triplicate on each time point, respectively

69 % for campesteryl fatty acid esters to 77 % for sitostanyl fatty acid esters. The hydrolysis rates determined for the desmethylsteryl/-stanyl ferulic acid esters (campesteryl ferulate: 26 %, campestanyl ferulate: 12 %, sitosteryl ferulate: 27 %) were significantly lower than those of the fatty acid esters. The average total recovery (intact esters and sterols/stanols) calculated for these substrates amounts to 102 %. For dimethylsteryl ferulates, no hydrolysis products could be detected; the recovery rates of the intact dimethylsteryl ferulates ranged from 82 to 84 %.

Individual data of the fourteen subjects regarding the recoveries of phytosteryl/-stanyl esters and liberated sterols/stanols in the feces are presented in Tables 4 and 5. The data demonstrate large interindividual variability of the hydrolysis rates. For campestanyl esters, the hydrolysis rates ranged from 43 (subject 7) to 88 % (subject 3) and for campesteryl esters from 53 (subject 10) to 95 % (subject 5). It is noteworthy that the same subjects also exhibited the lowest and highest hydrolysis rates, respectively, for the sitostanyl and sitosteryl esters. Surprisingly, there was

Table 3 Amounts of consumed esters and recoveries of intact esters and liberated phytosterols and stanols in human feces upon consumption of enriched skimmed milk yogurt drinks (n = 14)

Substrate	Consumed esters (mmol)	Feces			
		Intact esters (mmol/dry weight)[a]	Free sterol/stanol (mmol/dry weight)[a]	Total (mmol/dry weight)[a]	Recovery (%)[b]
Benecol®					
Campestanyl esters	3.64	0.97 ± 0.12	1.79 ± 0.15	2.76 ± 0.19	75.8
Sitostanyl esters	10.79	2.95 ± 0.79	5.35 ± 0.45	8.30 ± 0.91	76.9
Pro.activ®					
Campesteryl esters	2.22	0.44 ± 0.07	1.08 ± 0.06[c]	1.52 ± 0.09	68.5
Sitosteryl esters	10.03	2.06 ± 0.37	5.59 ± 0.25[c]	7.65 ± 0.45	76.3
γ-Oryzanol					
Campesteryl ferulate	1.46	1.08 ± 0.06	0.27 ± 0.02[c]	1.35 ± 0.05	92.5
Campestanyl ferulate	0.17	0.15 ± 0.01	0.03 ± 0.01	0.18 ± 0.01	105.8
Sitosteryl ferulate	0.49	0.36 ± 0.02	0.15 ± 0.02[d]	0.52 ± 0.03	106.1
Cycloartanyl ferulate	0.22	0.18 ± 0.01	n.d.[e]	0.18 ± 0.01	81.8
Cycloartenyl ferulate	6.39	5.36 ± 0.29	n.d.[e]	5.36 ± 0.29	83.9
24-Methylene-cycloartanyl ferulate	7.10	5.90 ± 0.33	n.d.[e]	5.90 ± 0.33	83.1

[a] Values represent mean ± SEM; content in feces samples related to dry matter; the combined feces samples (5 days) of individual subjects were divided into three aliquots and worked up in triplicate

[b] Recovery: total amount found in feces related to consumed esters

[c] Value represents the sum of campesterol and fecal metabolites (24-methylcoprostanol, 24-methylcoprostanone and 24-methylcholest-4-en-3-one)

[d] Value represents the sum of sitosterol and fecal metabolites (24-ethylcoprostanol, 24-ethylcoprostanone and 24-ethylcholest-4-en-3-one)

[e] Below limits of detection determined according to DIN 32645 (2008–2011) [49]; LOD [μg/100 mg dry feces]: cycloartanol/cycloartenol = 0.15, 24-methylenecycloartanol = 0.24

no correlation between the hydrolysis rates calculated on the basis of the remaining intact esters and the amounts of phytosterols/-stanols actually determined in the feces. On average, only 68.4 % of the amounts of sterols/stanols expected from the amounts of remaining intact esters were found; among the subjects, the levels ranged, for example, from 27.5 to 99.2 % for campestanol and from 32.2 to 100 % for campesterol. As a result of these two non-correlating sources of interindividual variability, the total recoveries, that is, the sum of intact esters and liberated phytosterols/-stanols in feces, also exhibited strong interindividual variability, ranging from 32.7 to 99.9 % in the Benecol®-exposure and from 36.9 to 102.4 % in the pro.activ®-trial.

Total content of fecal phytosterols/-stanols after alkaline hydrolysis

In order to confirm these at first glance conflicting data on the amounts of recovered intact phytosteryl/-stanyl esters and liberated sterols/stanols in the feces and to rule out methodological flaws of the employed quantification of intact esters, the calculated total phytosterol/-stanol contents in feces were compared to those obtained by the classical method based upon saponification. As shown in Table 6 for three randomly selected subjects from the Benecol®- and the γ-oryzanol-trial, the data sets were rather consistent.

Plasma lipids and phytosterol/-stanol levels

Concentrations of LDL-C, HDL-C and TG measured across all dietary treatments were in the ranges described for normolipidemic subjects (data not shown). No treatment-related differences were observed for any of the parameters. Plasma phytosterol/-stanol concentrations and their ratios to plasma TC are presented in Table 7. Mean plasma concentrations of campestanol and sitostanol were significantly increased on day 3 ($P < 0.001$ and $P < 0.01$) compared with day 0 after consumption of Benecol® by 275 and 127 %, respectively, but were not significantly different compared with the concentrations on day 6. Without further consumption, plasma concentrations of campestanol and sitostanol on day 6 compared with day 3 decreased by 51.7 and 38.2 %. In the pro.activ®-trial, plasma concentrations of campesterol and sitosterol on day 3 increased ($P < 0.02$ and $P < 0.005$) relative to day 0 by 43.2 and 66.2 %, respectively. On day 6, concentrations of campesterol and sitosterol decreased ($P = 0.01$ and $P < 0.005$) compared with day 3 by 24.6 and 27.5 %, but were not significantly different from day 0. Consumption

Table 4 Individual data on fecal recovered intact esters and liberated phytostanols within the Benecol®-trial

Subject	Benecol®											
	Campestanyl esters						Sitostanyl esters					
	Intact esters		Stanol		Total		Intact esters		Stanol		Total	
	(mmol)	(%)[b]	(mmol)	(%)[c]	(mmol)	(%)[b]	(mmol)	(%)[b]	(mmol)	(%)[c]	(mmol)	(%)[b]
Substrate	3.64						10.79					
Feces[a]												
1	0.69 ± 0.07	19.0	2.08 ± 0.10	70.5	2.77 ± 0.12	76.1	2.51 ± 0.08	23.3	6.55 ± 0.34	79.1	9.05 ± 0.34	83.9
2	0.55 ± 0.02	15.1	1.24 ± 0.05	40.1	1.19 ± 0.06	32.7	1.80 ± 0.06	16.7	3.97 ± 0.18	44.2	5.77 ± 0.19	53.5
3	0.44 ± 0.02	12.1	1.61 ± 0.06	50.3	2.05 ± 0.07	56.3	1.41 ± 0.06	13.1	4.88 ± 0.20	52.0	6.29 ± 0.20	58.3
4	0.74 ± 0.02	20.3	2.29 ± 0.07	79.0	3.03 ± 0.07	83.2	2.61 ± 0.08	24.2	7.30 ± 0.22	89.2	9.91 ± 0.23	91.8
5	0.55 ± 0.01	15.1	0.85 ± 0.02	27.5	1.41 ± 0.02	38.7	1.59 ± 0.03	14.7	2.41 ± 0.05	26.2	4.00 ± 0.06	37.1
6	1.06 ± 0.06	29.1	2.32 ± 0.13	89.9	3.38 ± 0.14	92.9	3.58 ± 0.18	33.2	6.22 ± 0.35	86.3	9.81 ± 0.40	90.9
7	2.06 ± 0.05	56.6	1.20 ± 0.05	75.9	3.26 ± 0.08	89.6	6.42 ± 0.15	59.5	2.95 ± 0.12	67.5	9.37 ± 0.19	86.8
8	1.00 ± 0.07	27.5	2.49 ± 0.09	94.3	3.49 ± 0.11	95.1	2.75 ± 0.17	25.5	7.30 ± 0.30	90.8	10.05 ± 0.34	93.1
9	1.30 ± 0.10	35.7	1.67 ± 0.15	71.4	2.96 ± 0.18	81.3	4.66 ± 0.33	43.2	5.42 ± 0.47	88.4	10.08 ± 0.57	93.4
10	1.75 ± 0.04	48.1	0.99 ± 0.05	52.4	2.74 ± 0.06	75.3	4.57 ± 0.10	42.4	3.60 ± 0.13	57.9	8.17 ± 0.17	75.7
11	1.10 ± 0.02	30.2	2.52 ± 0.04	99.2	3.62 ± 0.04	99.5	3.06 ± 0.09	28.4	7.72 ± 0.14	99.9	10.78 ± 0.16	99.9
12	0.64 ± 0.02	17.6	1.88 ± 0.05	62.7	2.52 ± 0.05	69.2	1.56 ± 0.05	14.5	6.10 ± 0.13	66.1	7.66 ± 0.14	71.0
13	0.72 ± 0.02	19.8	1.48 ± 0.04	50.7	2.20 ± 0.05	60.4	2.09 ± 0.05	19.4	3.95 ± 0.11	45.4	6.05 ± 0.12	56.1
14	1.03 ± 0.03	28.3	2.41 ± 0.11	92.3	3.45 ± 0.12	94.9	2.74 ± 0.09	25.4	6.50 ± 0.24	80.7	9.24 ± 0.26	85.6

[a] Values represent mean ± SD; content in feces samples related to dry matter; the combined feces samples of individual subjects were divided into three aliquots and worked up in triplicate, respectively

[b] Recovery related to consumed esters

[c] Recovery related to amount of hydrolyzed esters

Table 5 Individual data on fecal recovered intact esters and liberated phytosterols within the pro.activ®-trial

Subject	Pro.activ®											
	Campesteryl esters						Sitosteryl esters					
	Intact esters		Sterol[b]		Total		Intact esters		Sterol[c]		Total	
	(mmol)	(%)[d]	(mmol)	(%)[e]	(mmol)	(%)[d]	(mmol)	(%)[d]	(mmol)	(%)[e]	(mmol)	(%)[d]
Substrate	2.22						10.03					
Feces[a]												
1	0.45 ± 0.01	20.3	1.12 ± 0.05	63.3	1.57 ± 0.05	70.7	2.21 ± 0.04	22.0	4.86 ± 0.22	62.1	7.07 ± 0.22	70.5
2	0.72 ± 0.02	32.4	1.50 ± 0.02	100.0	2.21 ± 0.03	99.5	3.51 ± 0.07	35.0	6.74 ± 0.06	103.4	10.26 ± 0.09	102.3
3	0.42 ± 0.01	18.9	0.58 ± 0.05	32.2	0.99 ± 0.06	44.6	0.79 ± 0.02	7.9	2.92 ± 0.07	31.6	3.70 ± 0.08	36.9
4	0.22 ± 0.01	9.9	1.47 ± 0.11	73.5	1.69 ± 0.11	76.1	0.96 ± 0.07	9.6	8.24 ± 0.42	90.8	9.20 ± 0.43	91.7
5	0.10 ± 0.00	4.5	0.85 ± 0.01	40.1	0.95 ± 0.01	42.8	0.38 ± 0.01	3.8	4.79 ± 0.03	49.6	5.17 ± 0.04	51.5
6	0.47 ± 0.02	21.2	1.23 ± 0.06	70.3	1.70 ± 0.06	76.6	2.32 ± 0.08	23.1	5.22 ± 0.24	67.7	7.54 ± 0.25	75.2
7	0.84 ± 0.01	37.8	0.94 ± 0.04	68.1	1.78 ± 0.04	80.2	4.20 ± 0.04	41.9	3.98 ± 0.15	68.3	8.18 ± 0.16	81.6
8	0.13 ± 0.00	5.9	1.09 ± 0.03	52.2	1.22 ± 0.03	55.0	0.93 ± 0.04	9.3	6.03 ± 0.11	66.3	6.96 ± 0.12	69.4
9	0.55 ± 0.02	24.8	1.48 ± 0.04	88.6	2.02 ± 0.05	91.0	2.54 ± 0.07	25.3	7.74 ± 0.21	103.3	10.27 ± 0.22	102.4
10	1.05 ± 0.03	47.3	0.60 ± 0.03	51.3	1.65 ± 0.04	74.3	5.24 ± 0.15	52.2	2.70 ± 0.10	56.4	7.94 ± 0.18	79.2
11	0.10 ± 0.01	4.5	1.09 ± 0.01	51.4	1.19 ± 0.01	53.6	0.55 ± 0.01	5.5	9.39 ± 0.05	99.1	9.94 ± 0.05	99.1
12	0.41 ± 0.02	18.5	1.26 ± 0.05	69.6	1.67 ± 0.05	75.2	1.40 ± 0.04	14.0	7.86 ± 0.25	91.1	9.26 ± 0.26	92.3
13	0.42 ± 0.02	18.9	1.39 ± 0.04	77.2	1.81 ± 0.04	81.5	2.39 ± 0.06	23.8	5.64 ± 0.15	73.8	8.02 ± 0.16	80.0
14	0.28 ± 0.01	12.6	0.87 ± 0.05	44.8	1.16 ± 0.05	52.3	1.45 ± 0.04	14.5	4.83 ± 0.21	56.3	6.28 ± 0.21	62.6

[a] Values represent mean ± SD; content in feces samples related to dry matter; the combined feces samples of individual subjects were divided into three aliquots and worked up in triplicate, respectively

[b] Value represents the sum of campesterol and fecal metabolites (24-methylcoprostanol, 24-methylcoprostanone and 24-methylcholest-4-en-3-one)

[c] Value represents the sum of sitosterol and fecal metabolites (24-ethylcoprostanol, 24-ethylcoprostanone and 24-ethylcholest-4-en-3-one)

[d] Recovery related to consumed esters

[e] Recovery related to amount of hydrolyzed esters

Table 6 Comparison of total phytosterol/-stanol content in feces of three randomized selected subjects before hydrolysis (BH)[a] and after alkaline hydrolysis (AH)[b] (mean ± SD)

Substrate and sterols/stanols	Subject 1		Subject 2		Subject 3	
	BH (mmol/dry feces)	AH (mmol/dry feces)	BH (mmol/dry feces)	AH (mmol/dry feces)	BH (mmol/dry feces)	AH (mmol/dry feces)
Benecol®						
Campestanol	3.03 ± 0.07	3.04 ± 0.05	3.38 ± 0.14	3.27 ± 0.05	2.74 ± 0.06	2.75 ± 0.19
Sitostanol	9.91 ± 0.23	9.79 ± 0.14	9.81 ± 0.40	9.79 ± 0.05	8.17 ± 0.17	7.94 ± 0.12
γ-Oryzanol						
Campesterol	1.54 ± 0.02	1.44 ± 0.04	1.01 ± 0.05	0.93 ± 0.03	1.31 ± 0.06	1.27 ± 0.03
Campestanol	0.19 ± 0.00	0.18 ± 0.01	0.14 ± 0.01	0.13 ± 0.00	0.15 ± 0.01	0.14 ± 0.01
Sitosterol	0.65 ± 0.01	0.64 ± 0.02	0.49 ± 0.02	0.46 ± 0.03	0.41 ± 0.02	0.43 ± 0.04
Cycloartanol	0.22 ± 0.00	0.20 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.19 ± 0.01	0.17 ± 0.00
Cycloartenol	6.39 ± 0.02	6.22 ± 0.20	3.67 ± 0.21	3.81 ± 0.09	5.48 ± 0.30	5.57 ± 0.10
24-Methylenecycloartanol	7.10 ± 0.07	6.89 ± 0.20	4.04 ± 0.32	4.02 ± 0.08	6.06 ± 0.39	5.94 ± 0.08

[a] Values represent the sum of phytosterols/-stanols determined as total intact ester, in their free form and as fecal metabolites (in the case of campesterol and sitosterol); three feces aliquots of each subject were worked up in triplicate, respectively

[b] Values represent the sum of phytosterol/-stanols determined in their free form and as fecal metabolites (in the case of campesterol and sitosterol); onefold analysis of three feces aliquots of each subject

Table 7 Plasma phytosterol/-stanol concentrations determined by GC/MS selected ion monitoring and their ratios to cholesterol during the treatment periods (mean ± SEM; n = 14)

Phytosterol/-stanol	Day 0		Day 3		Day 6	
	(μg/dL)	($\times 10^2$ μg/mg)[a]	(μg/dL)	($\times 10^2$ μg/mg)[a]	(μg/dL)	($\times 10^2$ μg/mg)[a]
Benecol®						
Total cholesterol (mg/dL)	153.3 ± 11.0		162.4 ± 9.3		158.1 ± 9.2	
Campestanol	2.5 ± 0.4	1.6 ± 0.3†	9.3 ± 1.4	6.0 ± 1.0‡	4.7 ± 0.8	2.9 ± 0.5†,‡
Sitostanol	7.2 ± 1.1	4.5 ± 0.8†	15.9 ± 2.1	10.2 ± 1.5‡	9.7 ± 1.3	6.3 ± 1.0†,‡
Pro.activ®						
Total cholesterol (mg/dL)	157.3 ± 12.5		165.7 ± 9.6		172.4 ± 11.8	
Campesterol	343.9 ± 65.6	198.9 ± 26.5†	493.9 ± 79.8	284.8 ± 30.3‡	395.4 ± 70.7	214.8 ± 22.6†
Sitosterol	215.0 ± 42.6	123.6 ± 18.5†	353.6 ± 53.3	205.4 ± 21.5‡	273.4 ± 46.7	149.0 ± 16.9†
γ-Oryzanol						
Total cholesterol (mg/dL)	149.4 ± 8.2		160.0 ± 9.7		157.5 ± 9.5	
Campesterol	303.2 ± 40.3	187.3 ± 19.9	339.3 ± 40.7	213.3 ± 23.0	303.0 ± 41.0	189.8 ± 20.6
Campestanol	1.9 ± 0.2	1.2 ± 0.2	1.9 ± 0.2	1.2 ± 0.1	1.8 ± 0.2	1.2 ± 0.1
Sitosterol	171.4 ± 26.2	105.9 ± 13.5	167.9 ± 25.1	105.6 ± 15.1	153.0 ± 24.4	95.8 ± 13.0
Cycloartanol	n.d.[b]		n.d.[b]		n.d.[b]	
Cycloartenol	5.9 ± 0.5	3.8 ± 0.3†	7.3 ± 0.6	4.7 ± 0.5‡	6.6 ± 0.4	4.3 ± 0.4†,‡
24-Methylenecycloartanol	n.q.[c]		n.q.[c]		n.q.[c]	

[a] Values represent ratio to total cholesterol calculated as mean of the individual ratios of the subjects; different superscript symbols indicate significant differences ($P < 0.05$ or less) between time points within each treatment

[b] Not detectable within limit of detection determined according to DIN 32645 (2008–2011) [49]; LOD (μg/dL): cycloartanol/cycloartenol = 0.57

[c] Not quantifiable within limit of quantification determined according to DIN 32645 (2008–2011) [49]; LOQ (μg/dL): 24-methylenecycloartanol = 2.47

of γ-oryzanol-enriched skimmed milk yogurt drinks solely resulted in a significantly higher plasma level of cycloartenol on day 3 ($P < 0.01$) compared with day 0 by 23.7 %. The plasma cycloartenol concentration decreased on day 6 compared with day 3 by 8.5 % and was neither significantly different compared with day 3 nor with day 0.

Recoveries of individual intact phytosteryl-/stanyl esters in feces

In all three trials, the spectra of esters found in the feces of the subjects corresponded qualitatively to those consumed with the enriched skimmed milk drinking yogurts. However, there were significant quantitative differences between the individual esters (Table 8).

Mean recoveries of individual intact phytosteryl/-stanyl fatty acid esters in feces ranged from 18.8 ± 2.7 % for campestanyl linolenate to 71.4 ± 4.8 % for campestanyl palmitate in the Benecol®-trial and from 17.6 ± 3.8 % for campesteryl linoleate to 50.0 ± 4.5 % for campesteryl palmitate in the pro.activ®-trial. Only for the ferulates administered in the γ-oryzanol-trial, recoveries in a more narrow range (between 73.4 ± 3.5 % and 83.9 ± 3.7 %) were observed. When comparing esters with the same acyl moieties, stanyl esters always exhibited higher recoveries than the respective steryl esters.

Fecal recoveries of individual fatty acid esters also revealed large variability within the study population. The data shown in Fig. 3 demonstrate that the three selected subjects did not only differ in the recoveries of total sitostanyl fatty acid esters (Fig. 3A) and total sitosteryl fatty acid esters (Fig. 3B) but also showed clear differences in the profiles of the recovered esters compared to the consumed substrates. This variability was less pronounced for the ferulates of desmethylsterols/-stanols (Fig. 3C) and dimethylsterols (Fig. 3D).

Despite this interindividual variability, the data summarized in Fig. 4 demonstrate the impact of the acyl moieties (Fig. 4A, B) and of the steryl/stanyl moieties (Fig. 4C, D) on the recoveries of the administered esters. Ferulates were consistently recovered at higher rates than all other esters. Palmitates of both campestanol and sitostanol were recovered to significantly higher degrees than the respective esters of unsaturated C18 fatty acids (Fig. 4A). The recovery rate of sitostanyl eicosanoate was significantly lower than that of sitostanyl palmitate but significantly higher than those of the respective esters of unsaturated C18 fatty acids. The recovery rates of oleic

Table 8 Content of individual phytosteryl/-stanyl esters in the substrates and in collected feces samples (n = 14)

No[a]	Phytosteryl/-stanyl ester	Substrate (mmol)[b]	Recoveries in feces (mmol)[c]	(%)
Benecol® (stanyl fatty acid ester)				
1	Campestanyl-C16:0	0.21	0.15 ± 0.01	71.4 ± 4.8
3	Campestanyl-C18:1	2.31	0.60 ± 0.08	26.0 ± 3.6
4	Campestanyl-C18:2	0.79	0.17 ± 0.03	21.5 ± 3.6
5	Campestanyl-C18:3	0.32	0.06 ± 0.01	18.8 ± 2.7
2	Sitostanyl-C16:0	0.57	0.37 ± 0.02	64.9 ± 4.2
6	Sitostanyl-C18:1	6.93	1.74 ± 0.23	25.1 ± 3.4
7	Sitostanyl-C18:2	2.30	0.57 ± 0.10	24.8 ± 4.3
8	Sitostanyl-C18:3	0.81	0.19 ± 0.03	23.5 ± 3.7
9	Sitostanyl-C20:0	0.19	0.08 ± 0.01	42.1 ± 4.7
Pro.activ® (steryl fatty acid ester)				
10	Campesteryl-C16:0	0.14	0.07 ± 0.01	50.0 ± 4.5
12	Campesteryl-C18:1	0.77	0.14 ± 0.02	18.2 ± 2.9
13	Campesteryl-C18:2	1.31	0.23 ± 0.05	17.6 ± 3.8
11	Sitosteryl-C16:0	0.64	0.31 ± 0.03	48.4 ± 5.4
14	Sitosteryl-C18:1	3.71	0.69 ± 0.11	18.6 ± 3.0
15	Sitosteryl-C18:2	5.67	1.07 ± 0.24	18.9 ± 4.4
γ-Oryzanol (steryl/stanyl ferulic acid ester)				
16	Campesteryl ferulate	1.46	1.08 ± 0.06	74.0 ± 3.4
17	Campestanyl ferulate	0.17	0.15 ± 0.01	88.2 ± 2.8
19	Sitosteryl ferulate	0.49	0.36 ± 0.02	73.4 ± 3.5
18	Cycloartanyl ferulate	0.22	0.18 ± 0.01	81.8 ± 3.7
20	Cycloartenyl ferulate	6.39	5.36 ± 0.29	83.9 ± 3.6
21	24-Methylenecycloartanyl ferulate	7.10	5.90 ± 0.33	83.1 ± 3.7

[a] Peak numbers correspond to Fig. 2A–C (II)

[b] Content in the substrates consumed within 3 days; determined according to [30]

[c] Content in feces samples (mean ± SEM, n = 14) related to dry matter and collection time of 5 days; the combined feces samples (5 days) of individual subjects were divided into three aliquots and worked up in triplicate, respectively

(C18:1), linoleic (C18:2) and linolenic (C18:3) acid esters were in the same range. Differences between campestanyl esters and the corresponding sitostanyl esters could not be observed (Fig. 4A).

The results obtained for steryl fatty acid esters confirmed these findings. The C16:0 esters of both phytosterols were also recovered to a significantly higher degree than the respective C18:1 and C18:2 esters, whereas the recovery rates of the campesteryl and the corresponding sitosteryl esters were in the same range (Fig. 4B).

The comparison of different palmitates, oleates and linoleates demonstrates the impact of the steryl/stanyl moieties on the recoveries (Fig. 4C); the palmitates of campestanol and sitostanol were recovered at significantly higher rates than the corresponding esters of campesterol and sitosterol. There were similar trends for the oleates and linoleates; however, the differences in the recoveries between stanyl and steryl esters were not significant.

ANOVA comparing the ferulates of all containing phytosterols/-stanols within the γ-oryzanol-trial revealed borderline significance ($P = 0.066$). To control this overall trend, the ferulic acid esters of desmethylsterols/-stanols (campestanol, campesterol and sitosterol) as one group and of dimethylsterols (cycloartanol, cycloartenol and 24-methylenecycloartanol) as second group were investigated using Student's unpaired *t*-test. Significant differences could be proven between the two groups ($P \leq 0.05$) and within the desmethylsteryl/-stanyl ferulic acid esters when comparing the recoveries of campesteryl and sitosteryl ferulate with campestanyl ferulate ($P \leq 0.05$) (Fig. 4D).

Discussion

This study presents for the first time the quantitative data on the recoveries of individual phytosteryl/-stanyl esters in feces upon consumption of enriched skimmed milk drinking yogurts by healthy humans. Comparisons of the amounts of intact esters recovered in the feces with those consumed indicated that on average 73 % of total plant stanyl fatty acid esters and 80 % of total plant steryl fatty acid esters were hydrolyzed (Table 3). These hydrolysis rates are only slightly lower than those calculated on the same basis for total phytostanyl fatty acid esters (86 %) and phytosteryl fatty acid esters (88 %) after small intestine transit for seven ileostomy patients [29]. In all other available consumption studies, hydrolysis rates have been derived based on the amounts of excreted phytosterols/-stanols before and after saponification of the esterified

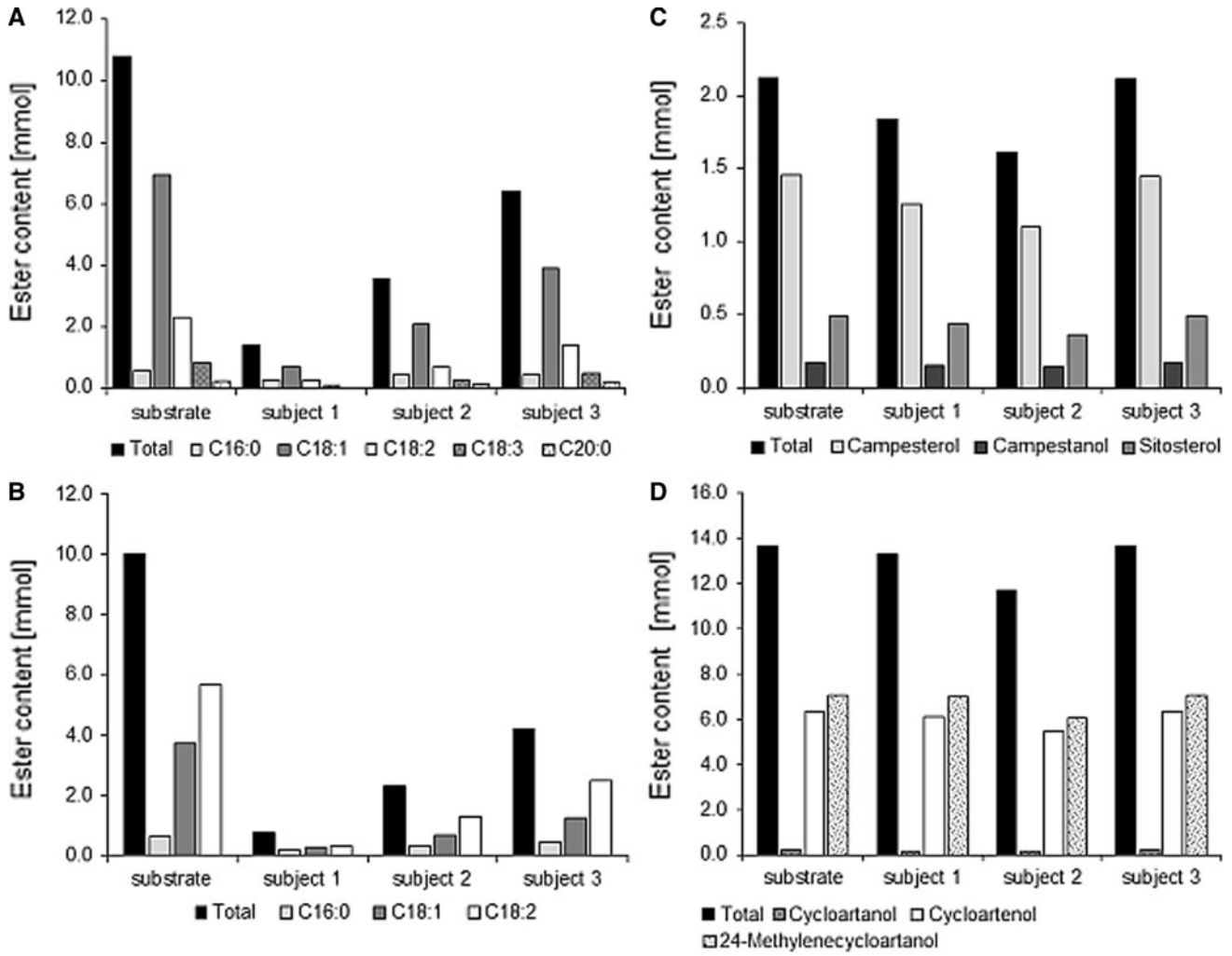

Fig. 3 Contents of sitostanyl fatty acid esters (**A**), sitosteryl fatty acid esters (**B**), desmethylsteryl ferulates (**C**) and dimethylsteryl ferulates (**D**) in feces of 3 subjects after oral administration and in the consumed substrate; **A** Benecol®, **B** pro.activ® and **C**, **D** γ-oryzanol

fraction. Administration of phytostanyl fatty acid ester-enriched margarine in eleven colectomized patients revealed 90 % hydrolysis [27]. Almost 60 % of sitostanyl and campestanyl fatty acid esters supplied in fat-free pastilles were hydrolyzed during gastrointestinal passage in nine colectomized patients when consumed with a normal-fat diet [28]. Intubation studies in humans with a normal intestinal tract described hydrolysis rates of 39–67 % for stanyl esters and 23–42 % for steryl esters during their 50–60 cm duodenojejunal transit [24–26].

The hydrolysis rates calculated in this study exhibit large interindividual variability ranging from 40 to 96 % (Tables 4, 5). This might be explained by both subject-dependent differences in the activities of digestive enzymes as well as by effects of the habitual and non-controlled diets of the volunteers on the bioavailability of the employed steryl/stanyl fatty acid esters. The range of hydrolysis rates reported for ileostomy patients (75–96 %) despite controlled dietary conditions confirms the dependence on the individual digestive system [29]. An impact of the type of administration, in particular the dietary fat content, was indicated by a study performed in eight colectomized patients in which phytostanyl fatty acid esters supplied in fat-free pastilles were hydrolyzed to a greater extent (70 %) when consumed with a normal-fat diet rather than with a low-fat diet (40 %) [28].

In a previous study, hydrolysis rates calculated by comparing the amounts of intact esters recovered in the ileostomy excreta to those consumed were only marginally lower than hydrolysis rates calculated by comparison of the amounts of recovered intact esters to the sum of esterified and free sterols/stanols in the excreta [29]. In contrast, in the present study, the hydrolysis rates calculated on the basis of the latter method were considerably lower than those calculated by comparing the amounts of intact esters recovered in the feces to those consumed (for phytostanyl

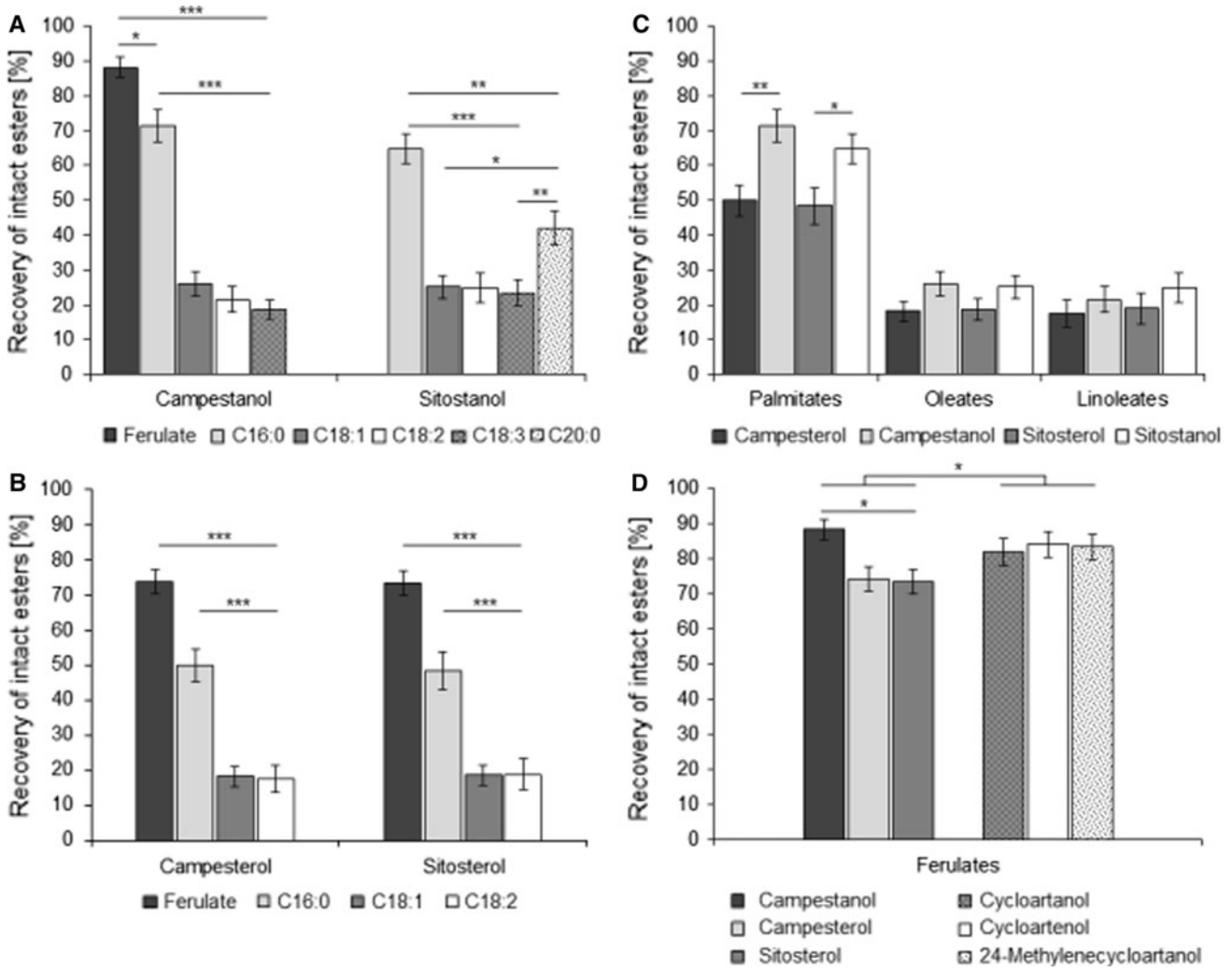

Fig. 4 Structure-dependent recoveries of intact phytosteryl/-stanyl esters in human feces after oral consumption of enriched skimmed milk yogurt drinks; **A**, **B** impact of acid moiety; **C**, **D** impact of phytosterol/-stanol structure; significance levels: $*P \leq 0.05$; $**P < 0.01$; $***P < 0.001$

fatty acid esters: 65 % vs. 86 %; for phytosteryl fatty acid esters: 73 % vs. 80 %). The employed quantification revealed that the amounts of liberated phytosterols/-stanols determined in the feces did not necessarily correlate with the amounts expected from the hydrolysis rates calculated on the basis of recovered intact esters. In consequence, the overall recoveries of fecal intact esters and released phytosterols/-stanols in healthy humans did not reflect the amount of respective esters consumed (Table 3). It is noteworthy that this phenomenon also becomes obvious when calculating total recoveries on the basis of the substrate amounts reported in the study with ileostomy patients [29]; the sum of sterols/stanols and intact esters recovered in the ileostomy excreta amounted only to 82 % of the phytosterol esters and to 88 % of the phytostanyl esters consumed by the patients. In the present study, this discrepancy between the amounts of sterols/stanols actually recovered in the feces and the amounts expected from the remaining intact esters was in addition strongly dependent on the individuals (Tables 4,5).

The lengths of the periods of feces collection during the three interventions were based on an average transit time of the diet described as 55–72 h [42, 43]. Nevertheless, it might be hypothesized that delayed excretion of free phytosterols/-stanols owing to their turnaround time in the intestinal and enterohepatic circulation might be responsible for the differences between experimentally determined and expected contents in the feces. However, it is important to note that this delayed excretion did not result in permanently increased plasma phytosterol/-stanol concentrations (Table 7). Baseline plasma phytosterol/-stanol concentrations that were comparable to published data [44–46] significantly

increased during substrate consumption (day 3), but returned nearly to baseline levels within day 6.

Using γ-oryzanol as substrate, the present study provides for the first time the data on the metabolic fate of phytosteryl/-stanyl ferulic acid esters upon human digestion. Almost 80 % of the consumed ferulates were recovered in intact form in the feces (Table 3). This is comparable to the results obtained after feeding labeled γ-oryzanol to rats as well as to in situ experiments using isolated rat ileum [47]; upon oral administration, about 10 % of the radioactive dose referable to ferulic acid and its metabolites was excreted in urine and almost 85 % was recovered in feces. The data are also in agreement with hydrolysis rates for γ-oryzanol (exhibiting a composition comparable to the substrate employed in the present study) by cholesterol esterases from bovine and porcine between 5 and 10 % [21]. In an earlier in vitro study employing the same bovine cholesterol esterase, hydrolysis rates ranged from 34 to 56 % [23]; however, in this study, no information on the composition of the γ-oryzanol used as substrate was provided.

In the present study, the hydrolysis of dietary phytosteryl/-stanyl esters during gastrointestinal passage in healthy humans was significantly affected by their molecular structures. Large interindividual variations of the hydrolysis rates of the different phytosteryl/-stanyl esters were observed in analogy to the variability seen for the total ester hydrolysis (Fig. 3). Despite this variability, the findings clearly demonstrated the impacts of both the acid moiety and the phytosterol/-stanol structure (Fig. 4). The impact of the acid moiety seems to be more relevant. For the rates of hydrolysis, the following order depending on the acid moiety was determined: oleate = linoleate = linolenate > eicosanoate > palmitate > ferulate. For rats fed oleates and stearates mainly of phytosterols, also a significant impact of the acid moiety has been described; oleic acid esters were virtually absent in feces, whereas almost 80 % of the stearates were recovered in their intact form [20]. A recent in vitro study focussing on the substrate specificity of pancreatic cholesterol esterase further supports the findings of this study; oleates were preferred substrates for hydrolysis by pancreatic cholesterol esterase compared to the respective palmitic and stearic acid esters that were in the same order of magnitude [18].

A significant impact of the desmethylsterol/-stanol structure was proven for the palmitic acid and ferulic acid esters in this order of rate of hydrolysis: desmethylsterol > desmethylstanol. But the consistently lower recoveries of the remaining desmethylsteryl esters compared to the analogous desmethylstanyl esters indicated an additional impact of the sterol/stanol structure (Table 8). Similar results were reported in hamsters fed either sitosteryl stearate or sitostanyl stearate [19]. Although the hydrolysis rates of both esters were very low, almost 5 % for sitosteryl-C18:0 and 1 % for sitostanyl-C18:0, the difference was described as being significant. In contrast, no difference has been reported for the hydrolysis of sitosteryl fatty acid esters compared to the respective sitostanyl esters in an in vitro study investigating the substrate specificity of pancreatic cholesterol esterase [18]. Further indications for an impact of the sterol/stanol moiety can be inferred from the results obtained by in vitro hydrolysis of steryl ferulates compared to sitostanyl ferulate [21]. Steryl ferulate mixtures from rye and wheat composed of 80 % stanyl (campestanol and sitostanol) and 20 % steryl (campesteryl and sitosterol) ferulates were hydrolyzed by cholesterol esterases to a significantly higher degree.

Employing several mammalian digestive enzyme preparations, it has been concluded that dimethylsteryl ferulates are not hydrolyzed [22] or that desmethylsteryl ferulates are preferentially hydrolyzed compared to dimethylsteryl ferulates [21, 23]. The hypothesis arising from the available in vitro data that dimethylsteryl ferulates are not hydrolyzed upon human digestion is confirmed by the results of the present study. The exact mechanism underlying the cholesterol-lowering activity reported for γ-oryzanol and rice bran oil [8, 9] remains unclear.

In conclusion, this is the first human study that demonstrates the structure-dependent hydrolysis of phytosteryl/-stanyl esters upon digestion of complex ester mixtures via enriched skimmed milk drinking yogurts. Despite large interindividual variability, the results clearly indicate that hydrolysis is significantly affected by the acid moiety (order of hydrolysis: oleate = linoleate = linolenate > eicosanoate > palmitate > ferulate) and the phytosterol/-stanol moiety (order of hydrolysis: desmethylsterols > desmethylstanols (= dimethylsterols)); however, the impact of the sterol/stanol moiety is less pronounced than the impact of the acid moiety. Considering the large variability observed in this study, more detailed investigations into the correlations between the molecular structures of phytosteryl/-stanyl fatty acid ester, their hydrolysis rates and the resulting cholesterol-lowering properties for individual subjects seem reasonable. The simultaneous quantitation of individual intact esters and liberated sterols/stanols revealed subject-dependent discrepancies between the amounts of phytosterols/-stanols found in the feces and those expected from the calculated hydrolysis rates. This phenomenon should also be further investigated, in particular in the light of the ongoing discussions regarding effects of plant sterols on vascular function [48].

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