***In vitro* colonic fermentation of plant sterols standard.** By M. Cuevas-Tena[1], Y. Sanz[2], A. Alegría[1] & M.J. Lagarda[1] (m.j.lagarda@uv.es). [1]*Nutrition & Food Science, Faculty of Pharmacy, University of Valencia, 46100 - Valencia, Spain.* [2]*Microbial Ecology, Nutrition & Health Research Group. Institute of Agrochemistry and Food Technology. Spanish National Research Council (IATA-CSIC). PO Box 73, 46100 – Burjassot. (Valencia), Spain*

Introduction:

Foods enriched with plant sterols (PS) have been used due to their cholesterol-lowering effect [1]. However, PS have low intestinal absorption (1-2%) [2] and can be transformed by the microbiota of the colon [3]. No studies on PS absorption at colon level have been published to date [3]. The present study evaluates the influence of colonic fermentation upon PS using *in vitro* batch culture assays.

Material and methods:

- *In vitro* colonic fermentation assay:

The microbiota were obtained from the stools of 5 healthy donors (pool of stools), who had not taken antibiotics during the three months prior to the assay. The stools were diluted in phosphate buffered saline (PBS). Controls and samples were prepared. Sample: pool stools (375 mg) inoculated in Brain Heart Infusion culture medium (15 ml) with PS (0.6 mg) from a standard of β-sitosterol ≥ 70% (Sigma-Aldrich®, purity ≥ 70%) previously characterized by GC-FID. Controls: A (15 ml culture medium); B (15 ml culture medium + 0.6 mg PS); C (15 ml culture medium + 375 mg pool of stools).Fermentation involved the "batch culture" method, described by Saulnier et al. (2008). The system was maintained at controlled temperature and pH (37ºC and pH entre 6.7-6.9), under anaerobic conditions, for 24 or 48 hours.

- Characterization of sterols in liquid fermentation:

Twenty µg of 5α-cholestane (Sigma-Aldrich®, purity ≥97%) as internal standard were added to 1 ml of liquid resulting from the fermentation, followed by alkaline hydrolysis (KOH 0.71M) and unsaponifiable extraction with hexane. Derivatization was carried out with 40 µl of silanizing reagent (BSTFA + 1% TMCS-pyridine). Identification and quantification were carried out by GC-MS with ion trap ITQ, GC Thermo Science Trace® GC-Ultra; data processor Xcalibur, equipped with capillary column CP-Sil8 CB (50m x 25mm x 0.25µm film thickness) (Agilent Technologies, CA, USA). One µl of the derivatized samples was injected in PTV splitless mode at 80-280ºC. The oven temperature was 150ºC and after 3 min the temperature was raised to 280ºC at 30ºC/min and then to 295ºC at 10ºC/min for 10 min. Hydrogen was used as carrier gas. The standards used for quantification, cholestanol (purity ≥95%), cholesterol (≥99%), coprostanol (≥98%), stigmastanol (≥95%) and sitosterol (≥95%) were purchased from Sigma-Aldrich® (Munich, Germany). Campesterol (≥98.6%) and coprostanone (98%) were obtained from Steraloids® (Newport, RI, USA).

Results

Table 1 shows the data obtained for linearity. The concentrations of sterols and other metabolites quantified expressed as µg/fermentation liquid are summarized in Table 2. We also identified coprostigmastanol.

Table 1: Sterols: linear regression analysis and correlation coefficients.

Sterol	Range (µg)	Linear regression	r
Coprostanol	20 - 100	0.0894x - 0.6792	0.99
Coprostanone	10 - 40	0.00234x - 0.127	0.96
Cholesterol	2 - 60	0.0549x – 0.1078	0.99
Cholestanol	2.5 - 10	0.0518x – 0.035	0.99
Sitosterol	1 - 40	0.058x – 0.0488	0.99
Sitostanol	0.6 - 12	0.034x – 0.0213	0.99
Campesterol	0,4 - 2	0.0177x – 0.0033	0.99

In control A we identified sitosterol traces. The coprostanol was the main metabolite quantified in control C and sample after fermentation at 24 and 48h and pool stools; according to other authors, coprostanol constitutes 60% of the total fecal sterols [5, 6]. The concentration of cholesterol and coprostanol was similar; however, some authors in quantifying these sterols have found coprostanone to be less abundant than cholesterol, since coprostanone is considered an intermediate in the microbial metabolic pathway for cholesterol [3, 7]. Another intermediate metabolite of this pathway is cholestanol [7], which we have identified and quantified. In control C and sample, the coprostanol and cholestanol concentrations after 48 h were slightly higher than at 24 h. For coprostanone this effect was only observed in sample. No differences were found in the metabolite concentrations between sample and stools, except for cholestanol, which showed different concentration in stools versus sample incubated for 48 h.

Table nº1: Concentration of sterols and their metabolites (µg/ml fermentation liquid)

Sterols	Control B		Control C		Sample		Pool Stools
	24h	48h	24h	48h	24h	48h	
Coprostanol	-	-	57.1 ± 1	62.7 ± 2.7	52.3 ± 0,7	69.8 ± 12,2	55.5 ± 8.7
Ethylcoprostanol	-	-	8.5 ± 0.02	8.7 ± 0.06	8.4 ± 0.02	8.7 ± 0.2	8.3 ± 0.4
Methylcoprostanol	-	-	8.1 ± 0.02	8.2 ± 0.05	8.2 ± 0.07	8.4 ± 0.18	8 ± 0.3
Coprostanone	-	-	19.1 ± 1.5	16.9 ± 1.1	18.6 ± 0.5	24 ± 3.9	18.8 ± 3.1
Ethylcoprostanone	-	-	7.2 ± 0.1	8.6 ± 0.8	7.1 ± 0.2	7.8 ± 0.8	7.5 ± 0.3
Methylcoprostanone	-	-	6.2 ± 0.03	6.9 ± 0.06	6.1 ± 0.09	6.2 ± 0.2	6.5 ± 0.2
Cholesterol	-	-	18.1 ± 1	18.1 ± 1.4	16.3 ± 1.3	19.1 ± 5.5	14.4 ± 1.9
Cholestanol	-	-	4.8 ± 0.1	6.1 ± 0.7	5 ± 0.4	7.1 ± 1.4	4.6 ± 0.7
Sitosterol	11.4 ± 2.3	14 ± 4.1	3.2 ± 0.4	7.3 ± 6.1	17.8 ± 1.5	20 ± 5.7	3.4 ± 0.1
Sitostanol	3.01 ± 0.9	3.9 ± 1.8	2.1 ± 0.6	1.9 ± 0.2	5 ± 0.6	5.9 ± 1.7	1.8 ± 0.5
Campesterol	1.6 ± 0.4	3.1 ± 0.1	-	-	-	-	-

Conclusions:

In these preliminary studies we observed that the generation of metabolic products related to PS were detected in control C and sample (24 and 48h), suggesting that the gut microbiota is involved in their metabolism. However, the supplementation of PS did not result in remarkable differences between the two samples. These findings indicate that it would be interesting and useful to evaluate the influence of the microbiota in the stools upon the food matrix enriched with PS, because the low activity of the microbiota upon the added PS and sterols in stools could be due to a lack of substrates.

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