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Updating the INFOGEST Digestion Method for Sterol Bioaccessibility by the Simultaneous Addition of Gastric Lipase and Cholesterol Esterase †

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Abstract: Gastric lipase (GL) and cholesterol esterase (CE) are simultaneous added to the INFOGEST digestion method. A bioaccessibility reduction of cholesterol (by 50–60%) present in a plant sterols (PS)-enriched beverage is observed, similar to the individual addition of GL. Furthermore, the bioaccessibility of individual and total PS is reduced (by 9–63% and 42–50%), also similar to individual addition of GL or CE. The bioaccessibility reduction is correlated with an increase in cholesterol content in blanks of digestion. Addition of GL and CE to INFOGEST method for determine sterol bioaccessibility reproduces more faithfully the physiological gastrointestinal conditions.

Keywords: beverage; bioaccessibility; cholesterol esterase; in vitro digestion; phytosterols; rabbit gastric extract

1. Introduction

Gastrointestinal digestion methods are useful to evaluate the soluble fraction theoretically available for intestinal absorption (bioaccessibility) [1]. The great diversity of factors that affect gastrointestinal digestion have promoted the development of a harmonized method by the COST Action INFOGEST network [2,3]. This method was initially adapted for the evaluation of sterol bioaccessibility in a plant sterols (PS)-enriched milk-based fruit beverage (1%, *w*/*v*) [4]. Recently, in order to have an approximation to the *in vivo* physiological gastrointestinal condition, lipid digestion enzymes, such as gastric lipase (GL) or cholesterol esterase (CE), have been incorporated to the simulated digestion method, resulting in an increase of the solubility of cholesterol provided by the digestion reagents [5]. The GL incorporation reduced the cholesterol bioaccessibility by 47% and, in the case of CE, did not allow cholesterol quantification in the bioaccessible fraction (BF). Furthermore, the addition of GL or CE reduces the PS bioaccessibility by 18–62%. However, since both enzymes are physiologically relevant in lipid digestion, their combined incorporation into the harmonized INFOGEST gastrointestinal digestion method is necessary.

Due to the aforementioned facts, the present study aims to evaluate the influence of GL and CE combined addition to the INFOGEST simulated gastrointestinal digestion, on the sterol bioaccessibility in a PS-enriched milk-based fruit beverage.

2. Materials and Methods

2.1. Beverage

A PS-enriched milk-based fruit beverage is used in the present study. The beverage contained (*w*/*v*) skimmed milk, milk fat, whey protein concentrate enriched with milk fat globule membrane (49%), mandarin, juice from concentrate (45%), banana pure (4%) and microencapsulated free microcrystalline PS (1%) from tall oil in a powder form (Lypophytol® 146 ME Dispersible, Lipofoods, Barcelona, Spain) [5].

2.3. Enzymatic and Bile Salts Assay

The enzyme activities and bile salts content of the bovine bile extract were determined according to the guidelines provided by the INFOGEST protocol [2,3]. Regarding CE, the activity of the batch provided by the manufacturer is used since the INFOGEST method does not contemplate its use or a standardized methodology for determining its activity.

2.4. Simulated Gastrointestinal Digestion

A harmonized in vitro gastrointestinal digestion method INFOGEST adapted for sterol bioaccessibility estimation [4] with GL and CE was applied. Briefly, 5 g beverage or deionized water (blank of digestion) was taken and mixed with 3.5 mL simulated salivary fluid, shaking for one minute. Then, 0.5 mL α-amylase solution (1500 U/mL), 25 µL of 0.3 M calcium chloride and 975 µL ultrapure water were added to produce a final volume of 10 mL. The mixture was placed in a shaker bath for 2 min at 37 °C and 95 rpm. Upon completion of the oral phase, 7.5 mL of simulated gastric fluid, 0.98 mL of rabbit gastric extract (1225 U/mL, lipase activity), 0.62 mL pepsin solution (25,000 U/mL) and 5 µL of 0.3 M calcium chloride were added and mixed for one minute. The pH of the mixture was adjusted to 3 and ultrapure water was added up to a volume of 20 mL. The gastric mixture was placed again in a shaker bath for 2 h under the same conditions. To simulate the intestinal conditions, 11 mL of simulated intestinal fluid, 5 mL pancreatin solution (800 U/mL based on trypsin activity), 0.1 or 1 mL of CE solution (30 or 80 U/mL, respectively (for an activity of 0.075 or 2 U/mL, respectively), 40 µL of 0.3 M calcium chloride, and 2.5 mL bovine bile extract solution (166 mM bile salts) were added. The final mixture was stirred for one minute, adjusted to pH 7, and ultrapure water was added to a final volume of 40 mL. Finally, they were incubated in a shaking bath for 2 h at 95 rpm. The digesta obtained was centrifuged (90 min, 4 °C, 3100× *g*) and the supernatant corresponding to the BF was collected.

2.5. Determination of Sterols

The methodology used by Blanco-Morales et al. (2018) for sterols determination in beverages and BF was applied. Briefly, 5 g beverage was taken, and the lipid fraction was extracted with dichloromethane:methanol (2:1, *v*/*v*) with 0.05% butylhydroxytoluene at 60 °C. Then, 1 M potassium chloride solution was added and kept at 4 °C overnight. The chloroform phase was concentrated using a rotary evaporator and dried under a nitrogen stream. The lipid fraction extracted was dissolved with hexane:isopropanol (4:1) (*v*/*v*) at final volumen of 10 mL. For saponification, 0.5 mL of lipid fraction of beverage or 5 g of BF were taken, and an internal standard (200 µg of epicoprostanol) was added. The samples were subjected to hot saponification (10 mL of 2 M or 1 mL of 1 M potassium hydroxide in ethanol:water (9:1, *v*/*v*), for beverage or BF, respectively) in the water bath (1 h, 65 °C) and unsaponifiable fraction extraction with diethyl ether was carried out. The unsaponifiable fractions were derivatized with 600 µL of pyridine: hexamethyldisilazane: trimethylchlorosilane (5:2:1, *v*/*v*/*v*) (25 min, 40 °C). After derivatization, the trimethylsilyl ether (TMSE) derivatives were solubilized in n-hexane and filtered. The TMSE derivatives were dissolved in 100 µL of n-hexane and analyzed (1 µL) by GC-FID equipped with a CP-Sil 8 lowbleed/MS (50 m × 0.25 mm × 0.25 µm film thickness) capillary column, with

the chromatographic conditions described in a previous work [6], modifying the carrier gas flow (2 mL/min) and the split ratio (1:20).

2.6. Statistical Analysis

T-test and one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, were applied to determine statistically significant differences ($p < 0.05$) between different conditions of the INFOGEST method assayed for the same sterol in BF or bioaccessibility. Statistical software Statgraphics Plus 5.1 (Statpoint Technologies Inc., Warrenton, VA, USA) was used for statistical analysis.

3. Results and Discussion

After carrying out the simulation of the gastrointestinal digestion on PS-enriched beverage, the following order of sterol bioaccessibility (%) was obtained: cholesterol (20.9–26.1), campestanol (15–16.6), campesterol (9.9–10.9) and sitostanol (8.8–10.1), β-sitosterol (6.3–7.3) and stigmasterol (5.1–5.5) (Table 1). The bioaccessibility values obtained were not affected by the CE activity tested.

Table 1. Sterol content in bioaccessible fractions and bioaccessibility after different condition of INFOGEST gastrointestinal digestion in a PS-enriched beverage.

Sterol \ Beverage	Beverage	GL + CE (0.075 U/mL)		GL + CE (2 U/mL)	
		BF	BA	BF	BA
Cholesterol	12.7 ± 2.3	2.7 ± 0.4	20.9 ± 2.8	3.3 ± 0.3	26.1 ± 2.2
Campesterol	62.0 ± 4.0	6.8 ± 0.3	10.9 ± 0.5	6.1 ± 0.5	9.9 ± 0.8
Campestanol	12.0 ± 0.2	2.0 ± 0.2	16.6 ± 1.5	1.8 ± 0.1	15.0 ± 0.5
Stigmasterol	5.0 ± 0.3	0.33 ± 0.02	5.5 ± 0.3	0.25 ± 0.03	5.1 ± 0.6
β-Sitosterol	742.2 ± 56.0	54.2 ± 1.5	7.3 ± 0.2	46.6 ± 0.6	6.3 ± 0.1
Sitostanol	91.0 ± 1.7	9.2 ± 0.5	10.1 ± 0.6	8.0 ± 0.5	8.8 ± 0.5
Total PS	911.1 ± 59.8	72.6 ± 2.4	8.0 ± 0.3	62.8 ± 0.4	6.9 ± 0.1

Sterol content in BF (mg/100 g beverage) and BA (%) are expressed as mean ± standard deviation (n = 4). The values of sterol content in BF and BA are not significantly different between the two different digestion conditions assayed. BA: bioaccessibility; BF: bioaccesible fraction; CE: cholesterol esterase; GL: gastric lipase; PS: plant sterols.

The bioaccessibility values of cholesterol are significantly lower (by 50–60%) than those reported in previous studies in which the use of GL and CE was not contemplated [4,5]. These results are consistent with a previous study, in which the individual incorporation of GL reduced the cholesterol bioaccessibility by 47% [5]. The authors indicated that GL improve the solubility of the cholesterol provided by the digestion reagents, displacing the sterol of the beverage from the micelle. It should be noted that the combination of these enzymes reduces the cholesterol bioaccessibility similar than individual addition of GL, indicating that the cholesterol solubility of the reagents does not improve with the combined use of GL and CE, compared with the individual addition of GL. In turn, Lopez-García et al. [5] observed that the individual addition of CE did not allow the quantification of cholesterol in the BF. The authors suggested that the formation of emulsifying agents such as free fatty acids and monoacylglycerols by the action of GL would contribute to the micellarization of cholesterol, allowing its quantification, in contrast to CE. In the present study it is confirmed that the use of GL and CE reduces the cholesterol bioaccessibility, and that GL allows its quantification.

On the other hand, the addition of GL and CE reduces the bioaccessibility of individual and total PS by 9–63% and 42–50%, respectively, regardless of the CE activity assayed. These reduction values are similar to those obtained by López-García et al. [5], in which it incorporates GL and CE individually. As observed for cholesterol, the reduction in the PS

bioaccessibility could be explained by an increase in the cholesterol content of the blank of digestion.

5. Conclusions

The INFOGEST method considering the combined use of GL and CE is an adequate protocol for the evaluation of sterol bioaccessibility since reproduces more faithfully the physiological gastrointestinal conditions.

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Abbreviations

The following abbreviations are used in this manuscript:

BA	Bioaccessibility
BF	Bioaccessible fraction
CE	Cholesterol esterase
GC-FID	Gas chromatography-flame ionization detection
GL	Gastric lipase
PS	Plant sterols

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