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Phytosterols Esterified with Conjugated Linoleic Acid. In Vitro Intestinal Digestion and Interaction on Cholesterol Bioaccessibility

Maria I. Moran-Valero,[†,§] Diana Martin,*[,†,§] Guzman Torrelo,[†,§] Guillermo Reglero,[†,§,#] and Carlos F. Torres[†,§]

[†]Departamento de Producción y Caracterización de Nuevos Alimentos, Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM), 28049 Madrid, Spain

[§]Sección Departamental de Ciencias de la Alimentación, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain

[#]Imdea-Food Institute, CEI UAM+CSIC, 28049 Madrid, Spain

ABSTRACT: Intestinal in vitro digestion of phytosterols esterified with conjugated linoleic acid (PS-CLA) was performed to study (1) the potential bioaccessibility of the released bioactive-lipid products and (2) the interference with cholesterol bioaccessibility. Commercial food-grade PS ester (PS-C) was assayed as reference. Hydrolysis of PS-CLA by digestive enzymes was similar to that of PS-C (51 and 47%, respectively), most lipids products being mainly included in the bioaccessible fraction, namely, the micellar phase (MP). Control assays in the absence of PS esters showed most cholesterol solubilized within the MP, whereas a displacement of total cholesterol was caused from MP after digestion of PS esters (14 and 36% displacement for PS-CLA and PS-C, respectively), cholesterol being partially precipitated. Precipitated cholesterol was linearly related to a parallel precipitation of saturated-chain PS, mainly determined by sitosterol (R^2 = 0.936). The higher composition in sitosteryl esters of PS-C with respect to PS-CLA might explain their different effects on cholesterol. Therefore, besides being a lipid delivery form of PS similar to other commercial esterified PS, the PS-CLA might have the additional advantage of being a lipid delivery form of CLA. Moreover, PS-CLA might hinder the bioaccessibility of cholesterol. Furthermore, the qualitative/quantitative profile in esterified PS forms might determine the magnitude of cholesterol interaction.

KEYWORDS: *phytosterol, conjugated linoleic acid, bioaccessibility, hypocholesterolemic, lipid delivery systems*

INTRODUCTION

Plant sterols or phytosterols (PS) are currently known as popular hypocholesterolemic ingredients included in food matrices, which reduce serum LDL-cholesterol levels by interfering with dietary and biliary cholesterol for intestinal absorption.[1] PS are only synthesized in plants and are structurally similar to cholesterol, but with the inclusion of an extra carbon chain at the C-24 position, leading to less solubility and higher hydrophobicity compared with cholesterol.[2] Both these similarities and differences between PS and cholesterol would explain the hypocholesterolemic action of PS at the intestinal level. Nevertheless, the exact mechanism by which PS inhibit cholesterol absorption is not fully understood. Several mechanisms have been proposed, including (1) competition with cholesterol for solubilization in micelles within the intestinal lumen, (2) cocrystallization with cholesterol to form insoluble crystals, (3) interaction with digestive enzymes, and (4) regulation of intestinal transporters of cholesterol.[3]

The first studies about the inclusion of PS in foods were performed by using free forms of these molecules.[4] However, the poor solubility of free sterols made necessary the use of high doses of free sterol intake to achieve significant cholesterol reduction. This drawback was solved by improving their solubilization in the provided preparations by diverse strategies. In this respect, PS esterification to fatty acid has been the most common solubilization method in food preparations and dietary supplements. Besides improving the solubilization, lipophilic/hydrophilic balance, and management of PS for inclusion in foods, the esterification of PS is currently of great interest due to the potential of producing lipid delivery systems for both PS and the esterified compound.[4] This is because the esterified molecule might be a bioactive compound by itself regardless of the attached PS; even more attractive might be that synergistic bioactivities between both molecules may result from the process of esterification.[4] In this respect, there are several examples of PS esterified with bioactive compounds, such as n-3 polyunsaturated fatty acids[5] or short- and medium-chain fatty acids[6] and phenolic compounds[7] as well as vitamins such as ascorbic acid.[8]

Recently, PS esterified with conjugated linoleic acid (CLA) (PS-CLA) were synthesized by Torres et al.[9] and by Li et al.,[10] suggesting a combined beneficial effect of PS and CLA within the same molecule. On the one hand, the solubility of PS might be improved by the esterification with a long-chain unsaturated fatty acid such as CLA. On the other hand, the esterified CLA is a well-known bioactive fatty acid of current interest by itself. CLA is the collective name given to positional and stereoisomers of octadecadienoic acid, the *cis*-9, *trans*-11, and *trans*-

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10,*cis*-12 being the most popular isomers, due to their abundance and the huge amount of scientific information on their biological activities.[11] In general, CLA have been shown to have antiadipogenic, anticarcinogenic, antiatherogenic, antidiabetogenic, and anti-inflammatory properties. This lipid is frequently used in its free fatty acid (FFA) form. However, palatability problems of free CLA led to the recommendation of CLA esterified as triacylglycerol (TAG), a more expensive alternative, as the preferred form.[12] Ethyl esters of CLA have been also proposed, but they seem to be worse vehicles of CLA than FFA or TAG.[12,13] PS might also be proposed as an alternative vehicle of CLA, because it might be a molecule with the triple advantages of (1) a lipid delivery form of PS with improved solubility, (2) a lipid delivery form of CLA, and (3) the combination of each individual compound, namely, PS and CLA, within the same molecule.

One of the questions that arise is the elucidation of whether esterified forms of PS-CLA would effectively maintain the respective bioactivities of both fractions, would be effectively recognized during their intestinal digestion, and whether their bioaccessibility might be affected. In general, most esterified PS need to be previously hydrolyzed during intestinal digestion by cholesterol esterase to exert the interaction on cholesterol absorption. Subsequently, the main lipid products released from PS esters and dietary fat, namely, FFA, monoglycerides, and free sterols, including PS and cholesterol, are solubilized within bile salt micelles for its transport to the brush border of the intestine.[14] This solubilization of sterols within micellar structures would be one of the competition levels between cholesterol and PS, where the preferred inclusion of PS would hinder the solubilization of cholesterol for its absorption.

Despite this general process of lipid digestion of PS esters, their rate and extent of hydrolysis, as well as the magnitude of cholesterol displacement, can be determined by factors such as the different PS isomers and the different nature of fatty acid.[15,16] With regard to CLA, it seems that its bioaccessibility and bioavailability might be also determined by the lipid-vehicle form, as shown for TAG, ethyl esters, or FFA.[12,13] Taking into account all of this evidence, both the extent of hydrolysis of PS-CLA and the bioaccessibility of the released lipid products, as well as the behavior of this product on cholesterol competition during intestinal hydrolysis, would need to be elucidated to validate this esterified-bioactive form of PS.

In vitro intestinal models of lipid digestion are an interesting approach for obtaining preliminary and valuable information concerning digestion of lipid species. A huge diversity of in vitro intestinal models of lipid digestion can be found in the scientific literature trying to simulate pseudophysiological conditions, the complexity of the composition of the media being diverse.[17] Because there is no standardized method, the selection of a model that closely simulates in vivo conditions is especially essential when in vitro lipid digestion of novel or unknown lipids is performed, to accurately understand obtained results and to avoid misinterpretations due to the used methodology.[17]

The aim of the present research was to evaluate the intestinal digestion of PS-CLA under in vitro conditions, to show the potential bioaccessibility of the released lipid products and their interaction with the bioaccessibility of cholesterol. First, the in vitro intestinal digestion model was tested against a commercial PS ester as reference, to be certain that the model reflected physiological intestinal lipid digestion of these lipid forms and to reject that any artifact or conditions of the digestion method would not interfere with the later results obtained for PS-CLA. Moreover, a comparative study on hydrolysis and cholesterol displacement between PS-CLA and the reference PS ester was established.

MATERIALS AND METHODS

Reagents and Materials. Phytosterol esterified with CLA (PS-CLA) was synthesized according to a methodology previously described by our group.[9] A commercial mixture of phytosteryl esters (PS-C) for food applications (Vegapure 95E) was a gift from Cognis GmbH (Illertissen, Germany). The composition of both products in sterols and esterified fatty acids is shown in Table 1. Trizma, maleic acid, pancreatin from porcine pancreas, cholesterol esterase (35 U/mg), bile salts, triolein, phosphatidylcholine from egg yolk, and cholesterol were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Sodium sulfate anhydrous, sodium chloride, calcium chloride, and ethanol absolute were purchased from Panreac (Barcelona, Spain). *n*-Dodecane for synthesis was purchased from Merck (Darmstadt, Germany). All solvents used were of HPLC grade from Lab-Scan (Dublin, Ireland).

Table 1. Chemical Composition of PS-C and PS-CLA

	PS-C (%)	PS-CLA (%)
sterols and sterol esters composition		
free sterols	2.4	7.5
esterified sterols	97.6	92.5
sterols		
brassicasterol	2.9	1.2
campesterol	15.0	28.7
campestanol	0.9	0.0
stigmasterol	0.8	25.5
sitosterol	69.5	44.8
sitostanol	8.1	0.0
others	2.9	0.0
total saturated-chain PS[a]	93.5	73.5
total unsaturated-chain PS[b]	3.7	26.7
esterified fatty acids		
palmitic and stearic acid	0.0	6.2
oleic acid	6.6	10.3
linoleic acid	92.2	83.4
linolenic acid	0.7	0.0
behenic acid	0.5	0.0

[a]Estimated as the sum of the content of campesterol plus campestanol plus sitosterol plus sitostanol. [b]Estimated as the sum of the content of brassicasterol plus stigmasterol.

In Vitro Lipid Digestion. The in vitro lipid digestion model was based on that of Martin et al.[17] with slight modifications. A sample of 0.5 g of PS ester (PT-C or PT-CLA) was mixed with 0.5 g of triolein, 0.5 g of bile salts, 0.2 g of lecithin, 5 mM $CaCl_2$, 150 mM NaCl, and 52 mL of Trizma-maleate buffer (0.1 M), pH 7.5. In the case of the assays to study the effect of PS on cholesterol bioaccessibility, this was also included within the digestion medium at a molar ratio 3:1 of PS ester to cholesterol. This ratio was previously estimated by Brown et al.[18] as the ratio of the recommended daily consumption of PS as esters by the U.S. Food and Drug Administration (1.3 g/day) to the average cholesterol consumed (257 mg/day).

The mixture was heated at 50 °C for 5 min and homogenized at the same temperature (Ultra Turrax IKA T18) for 25 min at 7000 rpm. The homogenized mixture was placed in a thermostatically controlled vessel (37 °C) under continuous stirring by magnetic stir bar at 1000 rpm. When the mixture reached 37 °C, simulation of intestinal digestion was started by the addition of fresh pancreatin extract (1000 mg of pancreatin in 6 mL of Trizma-maleate buffer, pH 7.5, stirred for 10 min and centrifuged at 1600*g* for 15 min) and 2 mL of cholesterol

esterase solution (2 mg/mL in Trizma-maleate buffer). Reaction was continued during 60 min. Each assay was performed in triplicate.

Separation of Phases after in Vitro Lipid Digestion. At the end of digestion, the medium was submitted to centrifugation at 4000 rpm for 40 min at 37 °C (5810R Eppendorf Iberica, Madrid, Spain) according to the method of Soler-Rivas et al.[19] After centrifugation, an upper oily phase (OP), a lower aqueous or micellar phase (MP), and a minor precipitated pellet (PP) were obtained. Their respective compositions on lipid products were analyzed.

Lipid Extraction. The total lipids from samples were extracted by hexane/methyl *tert*-butyl ether (50:50, v/v) at a ratio of 3:1 (v/v) of solvent to sample. The mixture was vortexed for 1 min and centrifuged for 10 min at 13500 rpm (ScanSpeed mini, Micro Centrifuge). A second extraction was performed by hexane/petroleum ether (50:50, v/v) and a third one by petroleum ether/ethanol (1:0.6, v/v). The three organic phases obtained were mixed, and anhydrous sulfate was added before further analysis.

Analysis of Lipid Products. Hydrolysis products were determined according to the method of Torres et al.[20] by gas chromatography (Agilent Technologies, Santa Clara, CA, USA) with on-column injection using a 7 m 5% phenyl methyl silicone capillary column (Quadrex Corp., New Haven, CT, USA), 0.25 μm i.d. A deactivated column of 12 cm × 530 μm i.d. was used as precolumn. Injector and detector temperatures were 43 and 360 °C, respectively. The temperature program was as follows: starting at 40 °C and then heating to 250 °C at 42 °C min^{-1} with a 10 min hold, followed by heating from 250 to 325 °C at 7.5 °C min^{-1} with a 30 min hold. Helium was used as the carrier gas at a pressure of 5.2 psi. The peaks were computed using gas chromatography Chemstation software (Agilent Technologies) and quantified according to the internal standard of *n*-dodecane.

Statistical Analysis. Statistical analyses were performed by means of the general linear model procedure of the SPSS 19.0 statistical package (SPSS Inc., Chicago, IL, USA) by one-way analysis of variance to compare the extent of hydrolysis of PS-C and PS-CLA, as well as the magnitude of displacement of cholesterol caused by both treatments. Pearson's correlation tests were used to study the bioaccessibility of cholesterol as related to specific PS. Differences were considered to be significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Validation of the in Vitro Intestinal Digestion Model. Before in vitro digestion of the experimental substrate PS-CLA, the lipid digestion model was first performed on the hydrolysis of the reference PS ester (PS-C). This was done to test whether the model was able to reflect physiological in vivo results and to reject that any artifact or the conditions of the digestion method would interfere with the results obtained for the experimental PS-CLA.

A model of lipid digestion previously developed for a standard lipid (olive oil) was used.[17] Such a model was based on the use of pancreatin as the source of main digestive enzymes and properly reflected in vivo physiological results concerning the degree of hydrolysis and the proportion of lipid products from the standard lipid.[17] However, the application of the in vitro digestion model to PS-C in the current study required some brief modifications for the adaptation to a substrate under the form of PS ester. On the one hand, cholesterol esterase was included together with pancreatin, because a minor nonphysiological hydrolysis was observed for PS-C digestion only by pancreatin (data not shown). It was an expected result because diverse studies have suggested that commercial pancreatin lacks an adequate level of cholesterol esterase or that other components of pancreatin may alter the specificity of the cholesterol esterase, which is the enzyme mainly responsible for the hydrolysis of esterified sterols.[18,21,22] On the other hand, an additional lipid was included as a standard triglyceride (triolein), because it would be closer to a real situation in which PS esters would be taken as part of a meal containing diverse lipids, mainly under the form of TAG. Moreover, it has been demonstrated that PS esters need the coexistence of other lipid products in the intestinal lumen that form sufficient surface of mixed micelles of lipid products (FFA and glycerides), where sterols would be included.[23−25]

To validate the proper hydrolysis of PS esters by the in vitro model, in vivo results previously published in the scientific literature were taken as reference. However, variable data of hydrolysis of PS esters have been reported, both closer to 40% and even up to 90%.[26−29] One reason that might explain such variability in the hydrolysis of PS esters is the specific section of the intestinal tract where the studies have been performed. Thus, those studies showing lower degrees of PS ester hydrolysis might be related to early events at the first sections of the duodenum, whereas a higher degree of hydrolysis has been found as PS esters advance through the intestinal tract. For example, Miettinen et al.[26] showed around 50% hydrolysis of stanol esters in the intestinal content of the lower duodenum after consumption of margarine containing these PS, and similarly Nissinen et al.[28,30] reported 39−44% hydrolysis of sterol esters during the passage of an infusate through the first 60 cm of the upper small intestine. On the contrary, by the analysis of fecal excretion after the intake of phytosteryl or phytostanyl ester, Miettinen et al.[27] and Normen et al.[29] found that close to 90% of fecal PS were in the unesterified form. Taking into account that the conditions and composition of the in vitro model of lipid digestion of the current study mainly tried to mimic those of the initial sections of the intestinal lumen, we chose those in vivo studies performed at this intestinal level as reference.[26,28]

The application of the improved model of lipid digestion hydrolyzed PS-C up to 47%, leading to the release of the equivalent hydrolysis products, namely 28% of free PS and 19% of FFA (Figure 1a). Compared to the in vivo data,[26,28] the obtained results suggested that the intestinal model of lipid digestion reflected a proper hydrolysis of commercial PS esters

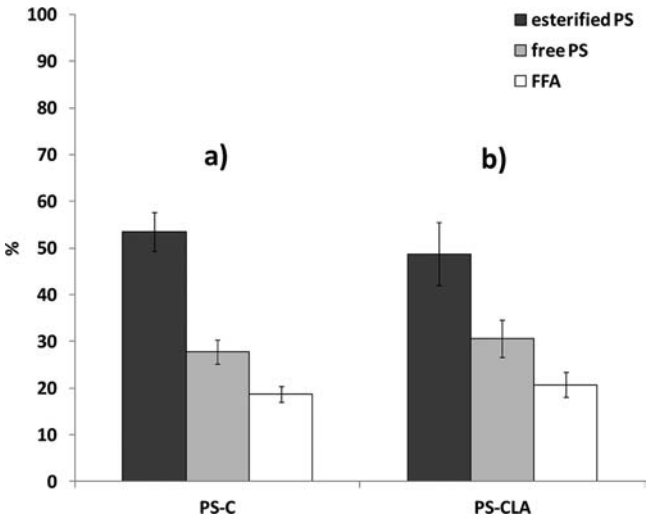

Figure 1. Hydrolysis products (%) after in vitro intestinal digestion of PS-C and PS-CLA (n = 3 for each treatment). Free PS and FFA were calculated by knowing the disappearance of esterified PS and estimating the equivalent appearance of products. The data are referred to 100%. The hydrolysis products from triolein are not shown.

similar to in vivo assays, so this model was used to simulate lipid digestion of the experimental product PS-CLA.

In Vitro Intestinal Digestion of Phytosteryl Esters of Conjugated Linoleic Acid. Digestive enzymes of the in vitro model effectively recognized PS-CLA, leading to around 51% hydrolysis of this substrate (Figure 1b). Such a value was slightly higher but not significantly different from the hydrolysis reached by PS-C (p = 0.355). The hydrolysis of PS-CLA corresponded to the release of the equivalent hydrolysis products, around 30% of free PS and 21% of CLA (Figure 1b), similar to PS-C. Therefore, the different molecular compositions of both PS-C and PS-CLA, concerning either sterols or fatty acid fractions (Table 1), did not determine different magnitudes of their intestinal digestion. This might be considered an interesting result because it would suggest that esterified forms of PS with CLA might be a potential source of free CLA and free PS during lipid digestion at degrees comparable to other commercial esterified PS.

To evaluate the relevance of the level of released CLA, some estimations were performed considering the recommended intake of CLA for obtaining bioactive effects of this fatty acid. Variable daily dose intakes of CLA have been advised. For example, 3 g/day is the most popular amount suggested to obtain the beneficial effects of CLA.[31,32] For comparative reasons, we assumed 3 g/day as the effective bioactive dose of total ingested CLA, which means that this amount of CLA should be bioavailable and bioaccessible. Taking milk as a reference of a natural source of CLA (around 0.6 g CLA/100 g fat),[33] a serving of milk (around 250 g) would contribute approximately 1.8% of the bioactive dose of CLA. According to the results obtained in the current study, the digestion of the recommended intake of PS esters (1.3 g/day; U.S. Food and Drug Administration) for hypocholesterolemic effect would lead at the same time to the release after intestinal digestion of around 9% of the bioactive dose of CLA. This value would be 5 times the CLA contribution from a milk serving. Nevertheless, further in vivo studies to validate these results and estimations would be necessary, because a direct extrapolation of in vitro results to in vivo situations cannot be stated.

Compared to other lipid vehicles of CLA, Gervais et al.[13] showed that TAG-CLA might be almost totally and readily hydrolyzed, as most TAG. However, with respect to ethyl ester forms of CLA, the bioaccessibility of such a vehicle is limited concerning the rate of hydrolysis and absorption, being worse than TAG-CLA.[12] The current study showed that alternative forms such as PS-CLA might be also potential lipid delivery systems of CLA, with the additional advantage of the vehicle PS as bioactive molecule by itself.

The study of intestinal lipid digestion under in vitro conditions is frequently completed by the subsequent study of the different phases of the digestive media containing the released lipid products. During the intestinal digestion of dietary fat, the intraluminal content has been shown to be structured as an oily phase (OP) dispersed in a micellar bile salt solution (MP).[34] The OP mainly contains undigested lipids, whereas the MP contains bile salt and the end products of enzymatic hydrolysis, namely, fatty acids and monoacylglycerols, as well as cholesterol, all together structured as mixed micelles, vesicles, or emulsion droplets.[35,36] Absorption of lipid products takes place supported by this MP, which enhances the transport of lipid products to enterocites throughout the unstirred water layer close to the microvillous membrane, where they are absorbed.[37] Analysis of the lipid products of these phases contributes to the study of bioaccessibility. Therefore, to study the bioaccessibility of the hydrolysis products, the digestion media after the time of hydrolysis of PS was separated into fractions MP and OP, and their lipid composition was analyzed (Figure 2).

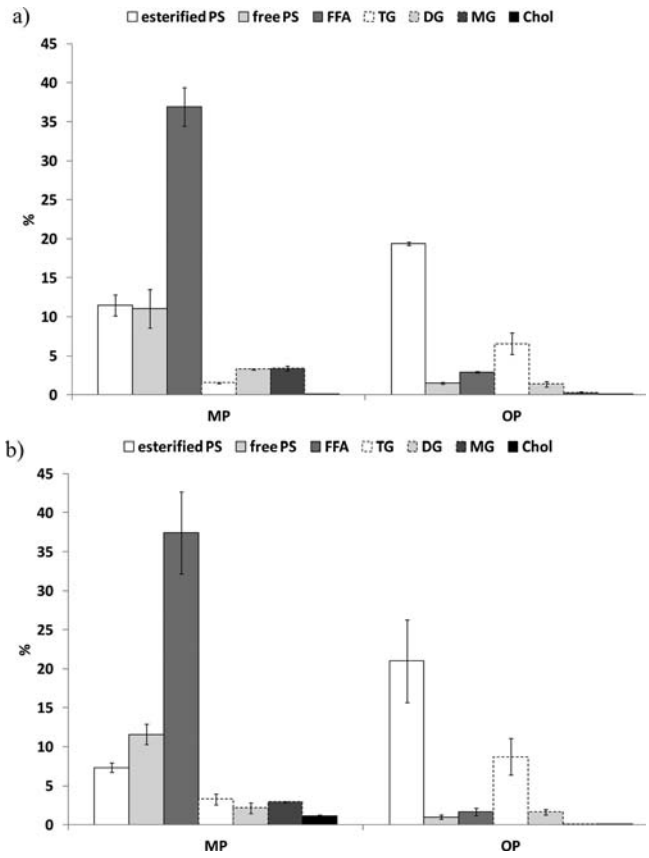

Figure 2. Distribution of total hydrolysis products (%) within MP and OP after in vitro intestinal digestion of (a) PS-C and (b) PS-CLA (n = 3 for each treatment). Minor cholesterol levels detected corresponded to the natural content of the lecithin used to perform the in vitro intestinal model of lipid digestion

The separations of lipid products were quite similar between PS-C and PS-CLA (Figure 2a,b, respectively). Most lipid products were included within the MP (around 68 and 66% of total lipid products for PS-C and PS-CLA, respectively), and the rest of the lipid products were mainly found within the OP (around 32 and 34% of total lipid products for PS-C and PS-CLA, respectively). A minor precipitated pellet (PP) was also separated (around 1% of total lipid products). The MP mainly consisted of the hydrolysis products as FFA and free PS, together with nonhydrolyzed substrates such as PS esters and glycerides from triolein. According to these results, the most interesting bioactive products from PS-CLA were included within the most suitable fraction, namely, MP. On the one hand, the solubilization of free CLA within mixed micelles might allow its bioaccessibility. On the other hand, the solubilization of free PS within mixed micelles would be the desired location to interfere with the bioaccessibility of cholesterol, as will be detailed in the following section. Furthermore, the obtained results would suggest that the distribution of lipid products from the digestion of PS-CLA might be comparable to the distribution of lipid products from

the digestion of other commercial PS. The proper confirmation of these in vitro results under in vivo situations would be a worthwhile study.

In Vitro Intestinal Bioaccessibility of Cholesterol in the Presence of Phytosterols Esterified with Conjugated Linoleic Acid. In general, the physiological−intestinal absorption of cholesterol is similar to that of most lipids, in the sense that it needs a micellar solubilization prior to absorption, which enhances the transport of cholesterol to enterocites.[37] However, different from most lipids, the absorption of which is very efficient and almost complete, the dietary cholesterol absorption varies from just 40 to 60%, and an even wider range of 20−80% absorption has been reported.[38] In this sense, it is well-known that dietary components can influence the cholesterol availability by either increasing or decreasing its absorption.[38] In this respect, one of the most popular mechanisms proposed for the hypocholesterolemic effect of PS is the limitation of the intestinal absorption of cholesterol, due to its displacement from micelles as a result of PS solubilization instead. As previously detailed, free PS was one of the major hydrolysis products of PS-CLA obtained after in vitro intestinal digestion, and most free PS released (around 92%) was included within the MP (Figure 2). To test the potential bioactive effect of this lipid product as limiting agent on the bioaccessibility of intestinal cholesterol, in vitro intestinal digestion assays of PS-CLA in the presence of cholesterol were performed. After digestion time, the MP, OP, and PP were isolated, their composition in lipid products was analyzed, and the distribution of total cholesterol within the three phases was estimated. Moreover, the distribution of cholesterol within phases in the absence of PS esters was taken as reference. Therefore, knowing the partition of cholesterol within the three phases in the absence and presence of PS allows the potential displacement of cholesterol from the bioaccessible fraction MP to be estimated.

The distribution of all lipid products, including cholesterol, within MP, OP, and PP after digestion of PS-CLA and PS-C is shown in Figure 3a,b, respectively. To show a better display, the specific distribution of total cholesterol within such phases is detailed in Figure 4. The control trial in the absence of PS esters showed that 98.8% of total cholesterol was solubilized within the MP (Figure 4a). However, when the digestion of PS esters was performed, the distribution of total cholesterol changed, and this compound was displaced from MP to OP and PP. Thus, when cholesterol was combined with PS-CLA in the digestion medium, around 14% of cholesterol with respect to the control assay was displaced from MP (Figure 4b).

To compare and evaluate the magnitude of the observed effect on cholesterol for PS-CLA, the in vitro model of lipid digestion was tested against the PS-C product as reference. As shown in Figure 4c, the hydrolyzed PS-C effectively caused a displacement of cholesterol from MP, but the magnitude of such displacement with respect to the control was much higher than the experimental PS-CLA (36% for PS-C and 14% for PS-CLA). Furthermore, such displaced cholesterol by PS-C was mainly found as PP, contrary to the PS-CLA product, where cholesterol was mainly found within OP and partially as PP.

The comparison of the obtained results with in vivo studies is complicated, taking into account that the current results suggest only the displacement of cholesterol from MP under in vitro conditions and do not consider all of the potential mechanisms in the intestinal tract that lead to the total decreased cholesterol absorption by PS. Nevertheless, some examples of in vivo

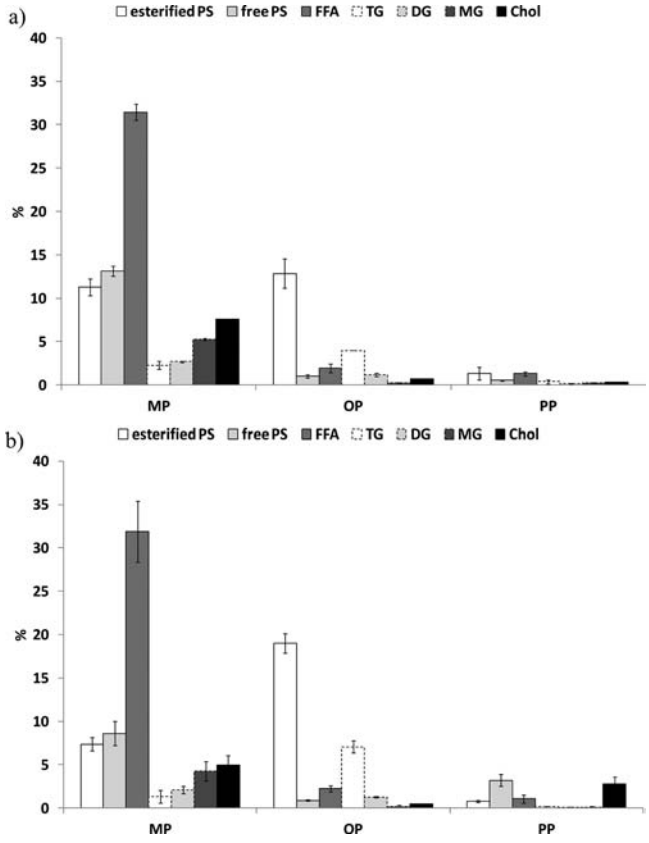

Figure 3. Distribution of total hydrolysis products (%), including cholesterol, within MP, OP, and PP after in vitro intestinal digestion of (a) PS-CLA and (b) PS-C (n = 3 for each treatment).

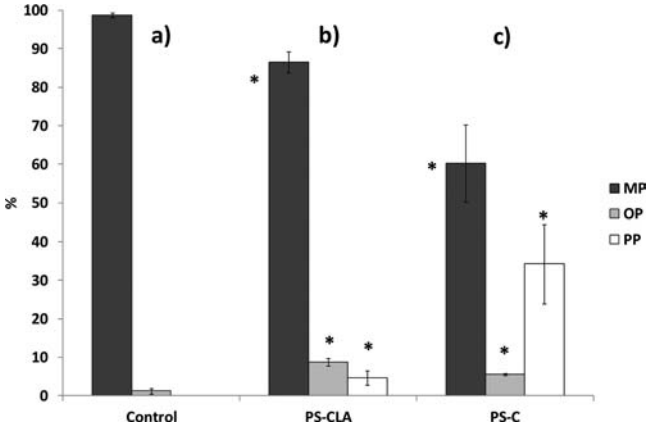

Figure 4. Distribution of total cholesterol (%) within MP, OP, and PP after (a) in vitro intestinal digestion in the absence of PS esters and (b) in vitro intestinal digestion of PS-CLA or (c) PS-C (n = 3 for each treatment). Statistical comparison only between PS-CLA and PS-C was performed. ∗ indicates that values within the same phase were significantly different ($p \leq 0.05$) between treatments.

studies that reported reduced cholesterol absorption are worth mentioning for a comparative attempt. Vanhanen et al.[39] showed values around 17−19% of reduced cholesterol absorption by sitostanol ester, and similarly Varady et al.[40] reported a range of 16−18% in a study of PS combined with exercise in hypercholesterolemic adults. Whether such values were due to micellar intereferences at the intestinal level was not reported in these two studies. One in vivo evaluation of the

effect of PS on cholesterol solubilization at the level of intestinal phases was recently reported by Amiot et al.,[41] who showed reductions of cholesterol in MP at a duodenal level of around 27% witg respect to the control after a PS esters meal. Wider ranges of 5.6−36.7% reductions in cholesterol absorption by sitostanols have been also reported by Ostlund et al.,[42] around 40% by Miettinen et al.[27] in colectomized patients, and between 43 and 49% in ileostomized patients by Normen et al.[26]

Diverse reasons might be hypothesized to explain the observed differences between studies, and especially between PS-C and PS-CLA when digested under the same conditions. On the one hand, the likely different qualitative and/or quantitative compositions of PS-C and PS-CLA in phytosterol/phytostanol (Table 1) might determine different magnitudes on solubilization within micelles and/or competition with cholesterol. One of the major differences was that PS-CLA consisted of only sterols, whereas the PS-C contained stanols; moreover, PS-C almost lacked stigmasterol, whereas such PS was a major PS for PS-CLA. Diverse studies have suggested that free stanols are more effective in reducing cholesterol absorption than free sterols.[43,44] Nevertheless, later studies have not shown different efficiencies between phytosterols and phytostanols or their esters.[45]

Moreover, the side chain that characterizes each individual PS also determines their physicochemical properties. Thus, saturated side chains, such as sitosterol, campesterol, and their stanol analogues, seem to increase hydrophobicity compared to unsaturated side chains of PS, such as stigmasterol.[46] As shown in Table 1, the assayed PS-C contained higher levels of total saturated-chain PS than the experimental PS-CLA. This might lead to a higher hydrophobicity of the PS-C product with respect to PS-CLA. This high level of high hydrophobic PS might be related with another of the popular mechanisms proposed for the hypocholesterolemic action of PS, namely, the cocrystallization of PS plus cholesterol at the intestinal tract and their subsequent precipitation. This has been explained by the existence of an upper limit in the capacity of micelles to solubilize low water-soluble compounds such as sterols, which tend to cocrystallize, and once the micelles are supersaturated with crystallized sterols, they coprecipitate.[47,48] In this respect, a precipitation of cholesterol and PS within PP was observed (Figure 3). In fact, the amount of precipitated cholesterol seemed to follow a positive linear correlation with the amount of total saturated-chain PS precipitated, regardless of the form as PS-C or PS-CLA (Table 2). Furthermore, as shown in Table 2, such precipitation of cholesterol was more clearly explained by saturated-chain PS as sitosterol plus sitostanol forms than by saturated-chain PS as campesterol plus campestanol forms. This last evidence would confirm that the interaction betwenn cholesterol and PS might be different depending on specific isomers of PS, and the current finding suggested that the amount of precipitated sitosterol plus sitostanol might explain the precipitation of cholesterol, in the cases of both PS-C and PS-CLA. This would be in agreement with previous studies, because Mel'nikov et al.[48] showed that sitosterol can form mixed cholesterol/sitosterol crystals. Therefore, the differences between PS-C and PS-CLA on cholesterol precipitation might be just due to the difference in the initial content of sitosterol plus sitostanol of the undigested products, being higher for the PS-C treatment (Table 1). It could be concluded that the precipitation of cholesterol by PS might be mainly evident at specific levels of hydrophobic PS in the media, whereas the major displacement of cholesterol from MP to OP (Figure 4), rather than PP, might be the main finding at lower levels of hydrophobic PS.

Table 2. Correlation between Precipitated Cholesterol and Precipitated PS within the Isolated PP after in Vitro Intestinal Digestion of PS-C and PS-CLA

	r (Chol × PS)[c]	coefficient of determination (R^2)	signif
total PS	0.921	0.848	0.009
total unsaturated-chain PS[a]	−0.413	0.171	0.415
total saturated-chain PS[b]	0.956	0.915	0.003
campesterol + campestanol	0.839	0.704	0.037
sitosterol + sitostanol	0.967	0.936	0.002

[a]Total unsaturated-chain PS was estimated as the sum of precipitated brassicasterol plus stigmasterol. [b]Total saturated-chain PS was estimated as the sum of precipitated campesterol plus campestanol plus sitosterol plus sitostanol. [c]Pearson's correlation was performed including the values from PS-C and PS-CLA within the same matrix (n = 6).

On the other hand, the degree of solubilization of either cholesterol or PS in micellar structures depends on the composition in other lipids of the intestinal lumen, being affected by the unsaturation degree or the chain length of fatty acids.[15,16] The PS-C was almost totally esterified by LA, whereas experimental PS-CLA was almost totally esterified by CLA, together with minor fractions of oleic and stearic acid (Table 1). Uehara et al.[49] have studied in detail the physicochemical properties of CLA isomers with respect to similar fatty acids, such as LA. These authors showed a quite different behavior of CLA with respect to LA concerning parameters such as melting point, subcell structures, or polymorphisms, the CLA isomers being closer to saturated and trans-unsaturated fatty acids than LA. Whether these differences between the esterified CLA and LA to PS might influence cholesterol partition would be complicated to explain in the current assay, but previous evidence about the effect of conjugated fatty acids on the bioaccessibility of sterols, in general, or interference in its micellar solubilization have not been found.

It is worth mentioning that during the isolation of the MP, OP, and PP after the digestion of PS-C and cholesterol, a clear separation of the three phases was reached, the MP showing an appreciable and homogeneous turbidity typical of this phase. On the contrary, the separation of the three phases after the digestion of PS-CLA and cholesterol was more unclear, and a heterogeneous turbidity and unclear separation of phases were observed within the MP. In this respect, additional control experiments were performed by isolating the three phases after digestion of PS-C and PS-CLA together with cholesterol, but in the absence of cholesterol esterase. Such study showed that the phases were easily separated for both substrates in the absence of hydrolysis, most lipids being found within MP and OP and the PP being negligible. Therefore, this suggested that the hydrolysis products released during the in vitro digestion of PS-CLA by cholesterol esterase might be likely responsible for the different conformation and composition of phases and likely might determine the different partition of cholesterol from MP compared to PS-C.

In summary, the potential limitation on the bioaccessibility of cholesterol by the synthesized PS esters of CLA by displacement of cholesterol from mixed micelles might be effectively produced, but at lower level than other commercial PS esters. However, further research under in vivo conditions would be necessary to confirm the obtained differences and the proposed explanations. It would be especially interesting to elucidate whether such differences would be just solved by the esterification of CLA with a PS profile similar to that shown by the commercial product PS-C, mainly formed by sitosterol.

It is interesting to point out that the relatively low displacement of cholesterol by PS-CLA compared to PS-C did not undervalue the potential of PS esterified with CLA as bioactive lipid, because additionally such molecules might be a potential vehicle of free CLA after intestinal digestion with expected high bioaccessibility, as previously detailed. Whether released CLA would be effectively bioaccessible and bioavailable under in vivo conditions and whether it would reach sufficient bioactive level would need further evaluation. Especially, the evaluation of the whole hypocholesterolemic action reached by digestion products of PS-CLA, namely, free PS and free CLA, would be of interest. This is because a hypocholesterolemic action of absorbed CLA has been also described by diverse authors.[50,51] In this sense, a recent research work on the esterification of sitosterol with CLA[10] showed that the esterified PS had good cholesterol-lowering properties, could prevent the formation of atherosclerosis, and could moderate the fat pathologic changes of liver in a hyperlipidemic mouse model. Therefore, the production of PS-CLA might be a promising lipid delivery form of both PS and CLA, and the assay performed in the current work would interestingly contribute to the knowledge of the specific events that might take place during the intestinal digestion of these esterified forms. Nevertheless, further in vivo studies would be necessary to validate the findings of the present research.

AUTHOR INFORMATION

Corresponding Author

*Postal address: Instituto de Investigación en Ciencias de la Alimentación (CIAL), Campus de la Universidad Autónoma de Madrid, 28049 Madrid, Spain. Phone: +34 910017930. E-mail: diana.martin@uam.es.

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ABBREVIATIONS USED

CLA, conjugated linoleic acid; FFA, free fatty acid(s); MP, micellar phase; OP, oily phase; PP, precipitated phase; PS, phytosterol(s); TAG, triacylglycerol(s).

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