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Safe intake of a plant sterol-enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage

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ABSTRACT

Sterols in foods are susceptible to oxidation to form oxysterols. It is interesting for consumer health to know real intake and the possible associated adverse effects associated to oxysterols. This study measured oxysterol formation and bioaccessibility (BA) in a plant sterol (PS)-enriched milk-based fruit beverage with milk fat globule membrane (MFGM) added at 0, 3 and 6 months of storage at room temperature. The same cholesterol (COPs) and phytosterol oxidation products (POPs) (exclusively from β-sitosterol) (7α/β-hydroxy, α/β-epoxy, triol and 7-keto) were detected in the beverage and its bioaccessible fraction. Total COPs and POPs contents were maintained during storage, and their BA ranged between 58 and 80% and 45–49%, respectively, without significant differences throughout storage. β-Sitosterol showed a lower mean oxidation percentage (0.028%) than cholesterol (1.24%), but the estimated POPs intake (0.5 mg/day) was two-fold higher than that of COPs (0.25 mg/day) from 250 g of beverage. These results show that the presence of milk fat and MFGM in the formulation of this beverage did not imply an increase in the contents of oxysterols and their BA. Thus, the beverage is suitable as a PS-enriched food matrix for the length of its shelf-life, and its consumption appears to be safe for consumers.

1. Introduction

Plant sterols (PS) are well known for their ability to reduce low-density lipoprotein (LDL)-cholesterol concentrations. A recent meta-analysis (Ras et al., 2014) has shown that an average intake of 2.1 g PS/day (3 g PS/day being the maximum allowed dose) gradually reduces LDL-cholesterol by an average of 6–12%. In addition, antiinflammatory and anticarcinogenic properties (against cancer of the breast, prostate, lung, stomach and ovary) have also been proposed as further beneficial effects of the consumption of PS (Othman and Moghadasian, 2011; Bin Sayeed and Ameen, 2015; Ramprasath and Awad, 2015; Shahzad et al., 2017).

In this regard, several foods currently can be enriched with PS due to their cholesterol-lowering effect, since the estimated daily intake of PS from the Western diet usually does not exceed 440 mg PS. Only in the case of vegans can an intake of 1 g PS/day be reached (García-Llatas and Rodríguez-Estrada, 2011; Klingberg et al., 2012; Ras et al., 2015). Such levels fall short of the effective PS doses.

Sterols present in food (cholesterol and PS) are susceptible to oxidation. The oxidized products formed are respectively known as cholesterol oxidation products (COPs) and plant sterol oxidation products (POPs). Overall, COPs and POPs are referred to as sterol oxidation products (SOPs) or oxysterols (García-Llatas and Rodríguez-Estrada, 2011; Brzeska et al., 2016). Milk-based fruit beverages, where the addition of PS has been approved (Commission Decision, 2004), are a good option for obtaining the recommended daily amount of PS in subjects with moderate hypercholesterolemia, and are postulated as a good vehicle for preventing the possible formation of SOPs, thanks to the presence of fruits (natural sources of antioxidants) (González-Larena et al., 2015).

The effects upon the body of consuming COPs have been intensively studied, though less information is available in the case of POPs (Hovenkamp et al., 2008; Otaegui-Arrazola et al., 2010; García-Llatas and Rodríguez-Estrada, 2011; Olkkonen et al., 2015; Brzeska et al., 2016). Recently, Kulig et al. (2016) have reviewed the biological importance of COPs in the human organism and their association to chronic diseases such as atherosclerosis, neurodegenerative disorders or cancer. Given the structural similarity between PS and cholesterol, it

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can be assumed that their oxidation products have similar effects in the human body, though unclear and contradictory information has been published in this regard. The biological actions associated to the consumption of POPs include effects on cholesterol metabolism, atherosclerosis and inflammation processes and cytotoxicity (García-Llatas and Rodríguez-Estrada, 2011; Vanmierlo et al., 2013; O'Callaghan et al., 2014).

Regarding oxysterol occurrence in foods, it has been found that the intensity and time of heat treatment employed are key factors affecting the oxidation of sterols, as well as food composition.

The effect of the food lipid matrix, temperature, free or esterified PS (with different degree of unsaturated fatty acids) upon sterol oxidation remains subject to controversy, and has been addressed by a number of recent reviews (Otaegui-Arrazola et al., 2010; Barriuso et al., 2016a). Soupas et al. (2004) indicated that for temperatures above 140 °C, unsaturated lipid matrices result in a lesser PS oxidation rate, since unsaturated lipid matrices are more readily oxidized, thus protecting sterols, while PS in saturated lipid matrices at higher temperatures facilitate sterol reactivity. Similar observations have been made by Soupas et al. (2007) and Barriuso et al. (2016b). However, for temperatures under 140 °C, PS oxidation is highest in unsaturated matrices. Since the mechanism underlying this effect is not clear, sterols could react more rapidly in lipid matrices where oxidation occurs more easily (Soupas et al., 2004; Otaegui-Arrazola et al., 2010). Moreover, the interaction between PS and lipids depends on whether or not PS are esterified: if PS are esterified with fatty acids, the vicinity of the radicals generated is close to the oxidation points of PS, while if the fatty acids are unsaturated, further oxidation of PS may result (Barriuso et al., 2016a). On the other hand, free PS undergo less interaction with the lipid matrix, and the more unsaturated the fatty acids surrounding PS happen to be, the more protected the sterols can be against oxidation. However, highly unsaturated lipids (such as DHA) might not exert this protective effect, due to rapid degradation of the fatty acid and the generation of a high proportion of oxidizing species (Barriuso et al., 2016a). Xu et al. (2011) also reported that polyunsaturated fatty acids possibly may compete for oxygen with sterols – the latter oxidizing first – though the effect of fatty acids upon sterol oxidation is time-dependent and is most unlikely related to their degree of unsaturation. In this regard, Soupas et al. (2007) indicate that at 160 °C, a saturated lipid matrix (butter oil) increases free PS oxidation *versus* an unsaturated lipid matrix (liquid margarine or rapeseed oil) to a greater extent (2- to 3-fold higher) than in the case of esterified PS. Furthermore, sterol oxidation is favored in oil-water emulsions, since they allow more interactions with the aqueous phase and promote the presence of free fatty acids, which accelerate oxidation of the sterols (Cercaci et al., 2007; Pignoli et al., 2009). In designing PS-enriched beverages, it could be of interest to evaluate sterol bioaccessibility (BA), defined as the maximum sterol content available for absorption, as a previous step for *in vivo* studies (Garcia-Llatas et al., 2015). In this sense, we have observed that the presence of milk fat globule membrane (MFGM), a natural emulsifier, and a fat content of 2.4% provided by milk fat, improve sterol BA in milk-based fruit beverages enriched with PS (Alvarez-Sala et al., 2016). Accordingly, the same behavior could be expected referred to the BA of SOPs, which could also be favored in this kind of beverage. To the best of our knowledge, only one study to date has assessed the BA of SOPs in a similar PS-enriched milk-based fruit beverage, though not containing MFGM in its formulation (Alemany et al., 2013). Several beneficial effects from MFGM compounds (phospholipids and gangliosides) present in bovine milk, such as improved blood lipid profiles (Vesper et al., 1999), the lowering of blood cholesterol, and the prevention of coronary heart disease (Rueda, 2014), could counteract the possible atherosclerotic effect of SOPs. Therefore, the aim of the present study was to evaluate the formation of SOPs and their BA during storage in milk-based fruit beverages enriched with PS and containing MFGM.

2. Material and methods

2.1. Chemicals and reagents

The internal standard (IS) used was 5α-cholest-5-en-3β,19-diol (19-hydroxycholesterol) (purity 98%). Other standards of COPs were cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol) (purity 98%), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol) (purity 97%), 5β,6β-epoxycholestan-3β-ol (β-epoxycholesterol) (purity 90%) and cholestane-3β,5α,6β-triol (cholestanetriol) (purity 95%), all acquired from Steraloids (Newport, RI, USA). 5α,6α-Epoxycholestan-3β-ol (α-epoxycholesterol) (purity 80%), and 5-cholesten-3β-ol-7-one (7-ketocholesterol) (purity 90%) were from Sigma Chemical Co. (St. Louis, MO, USA).

Trimethylchlorosilane (TMCS) was purchased from Fluka (Buchs, Switzerland). Ammonium chloride, anhydrous sodium sulfate, chloroform, ethanol, hydrochloric acid (purity 37%), methanol, potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium bicarbonate and urea were supplied by Merck (Whitehouse Station, NJ, USA). Sodium hydroxide was from Panreac (Barcelona, Spain). Uric acid was purchased from Prolabo (Sacramento, CA, USA). Diethyl ether, *n*-hexane, potassium hydroxide and 2-propanol were from Scharlau (Barcelona, Spain). Anhydrous pyridine, α-amylase from human saliva, bovine bile, bovine serum albumin (BSA), butylhydroxytoluene (BHT), calcium chloride dehydrate, cholesterol esterase from bovine pancreas, colipase from porcine pancreas, glucose, glucosamine hydrochloride, glucuronic acid, hexamethyldisilazane (HMDS), lipase from human pancreas, magnesium chloride, mucin from porcine stomach type II, pancreatin from porcine pancreas, pepsin from porcine stomach, phospholipase A2 from porcine pancreas, potassium thiocyanate, sodium dihydrogen phosphate, sodium taurocholate, and tris(hydroxymethyl)aminomethane were from Sigma Chemical Co. (St. Louis, MO, USA). All reagents were of analytical grade. Silica solid-phase extraction (Si-SPE) cartridges (Supelclean LC-Si, 500 mg/3 mL) were purchased from Supelco (Bellefonte, PA, USA). The syringe-driven Millex-FH filters (1 mL, 0.45 μm) were purchased from Millipore, and ultrapure water was obtained by means of a Millipore Q water purification system (Milford, MA, USA).

2.2. Sample

A beverage containing skimmed milk, milk fat, whey protein concentrate enriched with MFGM (Lacprodan® MFGM-10 from Arla Foods Ingredients) (50%), mandarin juice (48%), banana puree (1%) and grape juice (1%) with the addition of microencapsulated free microcrystalline PS (Lipohytol® ME Dispersible from Lipofoods) (2 g PS/250 mL beverage) from tall oil in powder was elaborated. The beverage was prepared by the Hero Global Technology Center (Alcantarilla, Murcia, Spain) specifically for this study (product not commercially available). This sample is one of those used in the study of Alvarez-Sala et al. (2016). Energy and nutritional information per 100 mL of beverage was: energy (kJ/kcal) 263/65.3; protein (g) 3.1; carbohydrates (g) 8.9; fat without considering PS (g) 1.6; fiber (g) 1.5; PS (g) 0.8. The mean sterol contents (mg/100 g of beverage) were: β-sitosterol 704, sitostanol 102, campesterol 34.7, campestanol 9.46, cholesterol 8.15, and stigmasterol 5.19 (Alvarez-Sala et al., 2016).

The beverage was analyzed just after manufacture (time 0) and after 3 and 6 months of storage at room temperature (20–25 °C). The storage time of up to 6 months is the common and usual turnover period for products of this kind at sales points.

2.3. Determination of sterol oxidation products

2.3.1. Beverage

Lipids were extracted according to the procedure described by Alvarez-Sala et al. (2016) To 5 g of beverage (providing approximately