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# Oxidative stability of functional phytosterol-enriched dark chocolate

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ABSTRACT

A dark chocolate containing phytosterols (PS) esters was developed to reduce cholesterol in individuals. However, oxidative instability during chocolate processing and storage could reduce the PS bioactivity. Chocolate bars were prepared containing palm oil (CONT) or 2.2 g of PS (PHYT). All samples were stored at 20 °C and 30 °C during 5 months. A peak of hydroperoxides formation was observed after 60 days at 20 °C and after 30 days at 30 °C. PS-enriched samples presented higher values of hydroperoxides than control samples, which could be attributed to the higher level of alpha-linolenic acid present in the PHYT samples. All chocolate bars became lighter and softer after 90 days of storage. However, these physical changes did not reduce their sensory acceptability. In addition, PS bioactivity was kept during the storage, since no significant alterations in the PS esters were observed up to 5 months. However, some PS oxidation occurred in the PHYT bars, being sitostanetriol, 6-ketositosterol, 6β-hydroxycampesterol and 7-ketocampesterol the major phytosterol oxidation products (POPs). The POPs/PS ratio was low (0.001). Therefore, the dark chocolate bars developed in this study kept their potential functionality after 5 months of storage at room temperature, representing an option as a functional food.



## 1. Introduction

Phytosterols or plant sterols (PS) are found in seeds, vegetable oils and cereals with a molecular structure very similar to that of cholesterol. The most frequently found PS in nature are β-phytosterol, campesterol and stigmasterol (Lengyel et al., 2012). These molecules are able to displace cholesterol during micelle formation in the intestine due to their higher hydrophobicity, thus reducing cholesterol absorption (Calpe-Berdiel, Escola-Gil, & Blanco-Vaca, 2009). Additionally, PS increase the expression of ABCG5 and ABCG8 carriers, involved in the reverse transport of cholesterol from enterocyte to intestinal lumen, and also reduce the activity of acetyl-coenzyme A acetyltransferase (ACAT), an enzyme that re-esterifies cholesterol, a necessary step for its incorporation into chylomicrons (Chen, Ma, Liang, Peng, & Zuo, 2011; Garcia-Llatas & Rodriguez-Estrada, 2011). PS are natural compounds that can be taken as drugs or added to some food formulations. Recently, the use of health claim for foods containing PS was revised by the Food and Drugs Administration (FDA) (FDA, 2010). According to the FDA (2010), functional foods should provide at least 0.65 g of vegetable oil sterol esters, eaten twice a day with meals for a daily total intake of at least 1.3 g. According to the epidemiological and clinical studies, the diary intake of 2 g of PS could result in average 8.8% of LDL-cholesterol reduction (Demonty et al., 2009). Based on these studies, several functional food formulations have been developed in order to exploit the PS health claim as dairy products, snack bars, sausages, bakery products, spreads, cereals, salad dressings, breads, orange juice and chocolate (Garcia-Llatas & Rodriguez-Estrada, 2011; Gonzalez-Larena et al., 2011; de Graaf et al., 2002; Micallef & Garg, 2009) at doses that range from 2 to 3 g (Kmiecik et al., 2011). However, some technological limitations should be evaluated when a functional food containing PS is being developed.

Like unsaturated fatty acids and cholesterol, PS are susceptible to oxidation and can generate several types of hydroxy, epoxy, keto, and triol derivatives, known as phytosterols oxidation products (POPs), especially when subjected to heat or long-term storage. The amount of POPs will depend on the sterols structure, water content,

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lipid matrix composition, and presence of light, metal ions, pigments and some oxidant enzymes (Derewiaka & Obiedzinski, 2012; Gonzalez-Larena et al., 2011; Kmiecik et al., 2011; Tabee, Azadmard-Damirchi, Jagerstad, & Dutta, 2008; Yang, Liu, Huang, Zheng, & Zhou, 2011). POPs do not present the health effects of the PS (Liang et al., 2011). In fact, POPs can annul the hypocholesterolemic action of the PS and also show some toxic effects on humans and animals (Garcia-Llatas & Rodriguez-Estrada, 2011; Hovenkamp et al., 2008; Liang et al., 2011). Thus, even though the oxidation range is usually low (<2% of the original PS content), it is still not known the physiological effect of these oxides intake. This fact deserves attention, considering the increase of PS-enriched foods in the market, and the daily and continuous intake of these functional products by individuals with cardiovascular diseases.

Due to its lipophylic aspect and elevated acceptability, chocolate has represented an interesting alternative to be a vehicle for PS supplementation. Although the fatty acid composition and the phenolic compounds present in the dark chocolate matrix exert a natural protection against the PS oxidation (Steinberg, Bearden, & Keen, 2003), oxidative reactions can occur in function of a number of other factors, including the interaction between the ingredients, the processing conditions, storage temperature and packaging type (Nattress, Ziegler, Hollender, & Peterson, 2004). Based on these facts, it becomes essential to evaluate the concentration of PS and their POPs in the chocolate matrix, before offering a functional product for human consumption. Thus, the objective of this study was to develop functional dark chocolate containing PS esters and evaluate its oxidative stability during 5 months of storage.

## 2. Material & methods

### 2.1. Materials

Phytosterols (CardioAid$^{TM}$-S) were purchased from ADM Natural Health and Nutrition® (Decatur, IL, USA), derived from vegetal oils esterified with canola oil fatty acids. The phytosterol mixture contained 46 g/100 g β-sitosterol, 26 g/100 g campesterol, 17 g/100 g stigmasterol and 11 g/100 g of others minor PS. Cocoa powder, butter and liquor (Barry Callebaut®, São Paulo, Brazil), palm oil (Agropalma®, Jundiaí, São Paulo), hazelnut paste (La Morela Nuts®, Tarragona, Spain), rice protein (Acerchem International®, Shangai, China), polydextrose (Winway®, São Paulo, Brazil), erythritol (Cargill®, São Paulo, Brazil), maltitol (Huakong®, São Paulo, Brazil), sucralose (Tate Lyle®, São Paulo, Brazil), nut aroma (IFF®, Taubaté, Brazil) and soy lecithin were purchased in a specialized market (São Paulo, Brazil). The antioxidants (ascorbic acid and α-tocopherol) were obtained from Sigma–Aldrich (St. Louis, MO, USA). A chocolate formulation containing 50 g/100 g of cocoa was used to coat the filling and was provided by Chocolife Indústria e Comércio de Alimentos Funcionais Ltda (São Paulo, Brazil). Bis(trimethylsilyl)-trifluoracetamide (BSTFA) containing 1 g/100 g trimethylchlorosilane (TMCS), pyridine, cholesterol, 5β-cholestan-3α-ol (epicoprostanol), (24S)-ethylcholest-5,22-dien-3β-ol (stigmasterol), (24R) –ethylcholest-5-en-3β-ol (β-sitosterol), 24α-ethyl-5α-cholestan-3β-ol(stigmastanol),(24S)-methylcholest-5,22-dien-3β-ol (brassicasterol) and (24R)-methylcholest-5-en-3β-ol (campesterol) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. Experimental design

#### 2.2.1. Chocolate formulation and manufacture

Control chocolates (CONT) were formulated mixing cocoa powder, cocoa liquor, palm oil, polydextrose, rice protein, cocoa butter, xylitol, maltitol, hazelnut paste, erythritol, soy lecithin, polyglycerol polyricinoleate, nut flavor, sucralose and nut flavor. In the PHYT and PHAN formulations, palm oil used to prepare the filling was replaced by PS esters. In the PHAN chocolates, ascorbic acid and α-tocopherol were also added into the filling formulation (0.90 mg/100 g of chocolate).

Belgian pralines were produced in an industry pilot plant as one batch. Firstly, all fats were weighted and placed in the mixer to melt at 45 °C. Afterward, dried ingredients were added to the melted fats and the mixture was conched by a runner mill at 60 °C/6 h, promoting the evaporation of undesirable flavors and water. The mixture was refined at 40–55 °C until an average particle size of 23 μm had been achieved. All samples were manually tempered in a cold marble surface until the temperature reached 29 °C. The chocolate was molded in plastic moulds (14 cm length and 13 mm height) to receive the filling. A thin layer of chocolate was placed in the mould, left to cool and added of 15 g of filling. PS and antioxidants were included in the filling to avoid the negative temperature effect on lipid oxidation during the coaching and tempering process. After cooling the filling at room temperature, another thin layer of chocolate was added to cover the filled chocolate. Thus, each bar (30 g) was composed of 15 g of shell and 15 g of filling. At the end, the filled chocolates were demolded, packaged in metallic BOPP (biaxially oriented polypropylene) commercial pack (60 μm) under normal atmosphere, and stored at 20 ± 2 °C and 30 ± 2 °C for 5 months. Samples kept at 30 °C were only used to evaluate oxidative stability. Samples were taken every month and analyzed for all parameters.

### 2.3. Methods

#### 2.3.1. Chemical analysis

Chemical composition of all six chocolate samples was determined according to the AOAC methods (AOAC, 2005). Carbohydrates were obtained by difference.

#### 2.3.2. Texture and color

Mechanical properties of chocolates (hardness) were measured according to the method proposed by Afoakwa, Paterson, Fowler, and Vieira (2008) using TA-XT2 Texture Analyzer (Stable Micro Systems, Surrey, UK). Maximum penetration and withdrawal forces through a sample were determined (1.0 mm/s, 5 mm of penetration at 20 °C). The color of dark chocolate was measured using a ColourQuest-XE colorimeter (Hunter Assoc. Laboratory, Reston, USA), using the CIE standard illuminant D65 as reference. Ten grams per sample were compressed into an optical cell (vision area 0.37 pol.). Color was expressed as lightness ($L^*$), redness ($a^*$) and yellowness ($b^*$), using CIELab parameters.

#### 2.3.3. Sensory analysis

A hedonic sensory evaluation was carried out by an untrained panel consisting of thirty individuals composed by the students and employees from the Faculty staff, who liked of bitter chocolate. Approximately 10 g of dark chocolate was placed in a small plate coded with 3-digit random numbers. Each panelist received a set of 3 samples (CONT, PHYT and PHAN) in a different order (3!), and they were instructed to rinse their mouth with water between samples evaluation. Acceptability analysis was performed using a 9 point hedonic scale, considering 9 as “extremely like” and 1 as “extremely dislike”.

#### 2.3.4. Fat extraction

The extraction of chocolate lipids was performed according to AOAC official method 920.75 (AOAC, 2002). About 5 g of the chocolate bars was mixed with 10 mL diethyl ether for 1 min. The tubes were centrifuged and the upper phase separated. The