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.

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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™-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

extraction was repeated twice and the combined extract was filtered using sodium sulfate. Ether extracts were evaporated and resuspended with 1 mL of hexane.

2.3.4.1. Chemical oxidation markers. Hydroperoxide content of the extracted fat was determined according to Shantha and Decker (1994). PV was determined in 50 μL of lipid extract at 510 nm, by using a UV–VIS mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan), and it was calculated from the absorbance. The hydroperoxide content was determined using a standard curve prepared with known concentrations of cumene hydroperoxide. Concentrations were expressed as mmol/kg of fat.

2.3.4.2. Determination of fatty acids methyl esters. The chocolates fatty acid profile was determined according to AOCS Ce 1b-89 (AOCS, 2001). The chromatographic analysis was carried out using a gas chromatograph GC (Agilent 7890 A GC System, Agilent Technologies Inc., Santa Clara,USA). A fused silica capillary column (J&W DB-23 Agilent 122-236; 60m × 0.25 mm i.d., 0.15 μm film thickness) was used for injection. High-purity helium was used as carrier gas at a flow rate of 1 mL/min. One μL was injected by a split injector (50:1) at an inlet temperature of 250 °C. The oven temperature was programmed as follows: started at 80 °C, heating rate 5 °C/min up to 175 °C, followed by another gradient of 3 °C/min to 230 °C, and hold at this temperature for 5 min. Detection was carried out by an FID set to 280 °C. The fatty acids were identified by comparing the retention times with those of four purified standard mixtures of fatty acid methyl esters (4-7801; 47085-U; 49453-U and 47885-U from Sigma Chemical Co.). Peak areas were calculated as area % of total fatty acids.

2.3.4.3. Determination of phytosterols content. PS content was determined according to Laakso (2005). Lipids extracts containing about 1–2 mg of PS were mixed with 2 mg of internal standard (5β-cholestan-3α-ol; epicoprostanol) and evaporated to dryness under a nitrogen stream. A hot saponification was carried out by adding 2.5 mL of KOH 2.0 M in methanol followed by extraction with heptane. Sterols were derivatized with 200 μL of bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1 g/100 g trimethylchlorosilane (TMCS) (99:1) and 100 μL of pyridine, at 70 °C for 15 min. An aliquot of 1.0 μL of derivatized sample solution was injected into the column at 250 °C with a split injector (split ratio 1:50). Sterols were separated at 300 °C and detected with flame ionization detector (FID) at 280 °C. The carrier gas was helium at flow rate of 1.0 mL/min. Reference standards were used to identify the peaks. Quantification was calculated based on standard curve prepared with β-sitosterol/IS area ratio, as a linear function ($r = 0.9983$) of sterol concentration (0.5–5.0 mg).

2.3.4.4. Determination of phytosterol oxides. About 20 mg of the chocolate were placed in a tube glass with 19-hydroxycholesterol (0.58 mg in *n*-hexane:isopropanol (3:2, mL/mL)) used as internal standard for the quantification of POPs. The solvent was evaporated under nitrogen and 30 mL of 2 mol equiv/L KOH solution in methanol were added to perform a cold saponification at room temperature for 18 h in darkness and under continuous agitation (Sander, Addis, Park, & Smith, 1989). The unsaponifiable material was extracted with diethyl ether. For determination of POPs, 70 g/100 g of the unsaponifiable matter was purified by silica solid-phase extraction (SPE) according to Guardiola, Codony, Rafecas, and Boatella (1995).

After cartridge activation with hexane (5 mL), PS and impurities were removed with hexane (5 mL) and diverse solvent mixtures of

Table 1
Chemical composition, fatty acids profile, color, hardness, pH, and sensory acceptability score of the three chocolate bars.

	Treatments[a]			
	CONT	PHYT	PHAN	P[b]
Chemical composition (g/100 g)				
Moisture	0.70 ± 0.02a	0.52 ± 0.06b	0.48 ± 0.05b	0.015
Protein	11.42 ± 0.98	11.46 ± 0.40	10.62 ± 0.54	0.317
Lipids	33.59 ± 0.34	34.35 ± 0.57	34.66 ± 0.03	0.136
Ash	1.33 ± 0.02	1.38 ± 0.02	1.38 ± 0.05	0.301
Carbohydrate	52.82 ± 1.01	52.51 ± 0.760	52.98 ± 0.79	0.853
Major fatty acids (g/100 g)				
C16:0 – palmitic	27.97 ± 0.01a	23.08 ± 0.35b	22.74 ± 0.12b	0.006
C18:0 – stearic	24.84 ± 0.01	25.28 ± 0.63	26.44 ± 0.06	0.154
C18:1 - oleic (n-9, *cis*)	39.80 ± 0.00	40.49 ± 0.71	39.67 ± 0.12	0.409
C18:2 – linoleic (n-6) - LNA	6.47 ± 0.64	7.15 ± 0.41	7.03 ± 0.02	0.383
C18:3 – α-linolenic (n-3) - ALA	0.22 ± 0.02a	0.74±0.14b	0.77 ± 0.01b	0.011
Color				
L*	23.99 ± 1.26	24.07 ± 0.93	23.96 ± 1.34	0.986
a*	6.13 ± 0.53	5.74 ± 0.21	6.07 ± 0.35	0.189
b*	4.84 ± 0.59	4.38 ± 0.63	4.73 ± 0.37	0.289
Hardness (N)	7.10 ± 2.65	7.39 ± 1.74	7.50 ± 1.91	0.946
pH	5.90 ± 0.01a	5.92 ± 0.01b	5.94 ± 0.01b	0.008
Sensory Acceptability	6.73 ± 1.89	6.33 ± 1.83	6.13 ± 1.81	0.442

[a] CONT – Chocolate without PS; PHYT – Chocolate with PS; PHAN – Chocolate with PS + antioxidants.
[b] Probability value obtained by one-way ANOVA; values are mean ± SD ($n = 3$). Different letters in the same line indicate significant differences ($P < 0.05$).

n-hexane:diethyl ether (10, 30 and 10 mL of 95:5, 90:10, 80:20 (v/v), respectively). POPs were finally eluted with acetone (10 mL), then subjected to silylation, dried under nitrogen stream and dissolved in 40 μL of *n*-hexane. One μL of the TMSE derivatives was analyzed by GC–MS (GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan)), using a Fast GC–MS method suggested by Cardenia, Rodriguez-Estrada, Baldacci, Savioli, and Lercker (2012), with minor modifications. The system was fitted with a capillary RTX-5 Restek column (10 m × 0.10 mm i.d. × 0.10 μm film thickness; Supelco, Bellefonte, PA, USA) and helium was used as carrier gas (0.5 mL/min). The oven temperature was programmed from 220 °C to 325 °C at the rate of 5 °C/min. The injector and transfer line temperatures were set at 310 °C and 280 °C, respectively. Manual injection of 1 μL of the solution containing POPs and the solutions obtained from samples, was performed in the split mode at 1:50 ratio. The Ion source and interface temperatures were set at 230 °C and 210 °C, respectively. The filament emission current was 70 eV. A mass range from 40 to 650 *m/z* was scanned at a rate of 1500 amu/s. The acquisition and integration modes were Full Scan (TIC) and Single Ion Monitoring (SIM), respectively.

Identification of POPs was performed by comparing the retention time and mass spectra with library of program, as well as with those reported in literature. POPs were recognized and quantified by their corresponding characteristic ions that show a high abundance by SIM mode (*m/z*): IS (19-hydroxycholesterol) (353), 7α-hydroxycampesterol (470), 7α-hydroxystigmasterol (482), 7α-hydroxysitosterol (484), 7β-hydroxystigmasterol (482), α-epoxysitosterol (412), 7-ketocampesterol (486), 6β-hydroxycampesterol (470), stigmastentriol (572), sitostanetriol (431), 6-ketositosterol (473), 7-ketositosterol (500). Considering that POP standards are not commercially available and that POP fragmentation is similar to that of cholesterol oxidation products (COPs), POPs quantification was performed by using the calibration curves obtained for cholesterol oxides in the SIM mode (Cardenia et al., 2012).
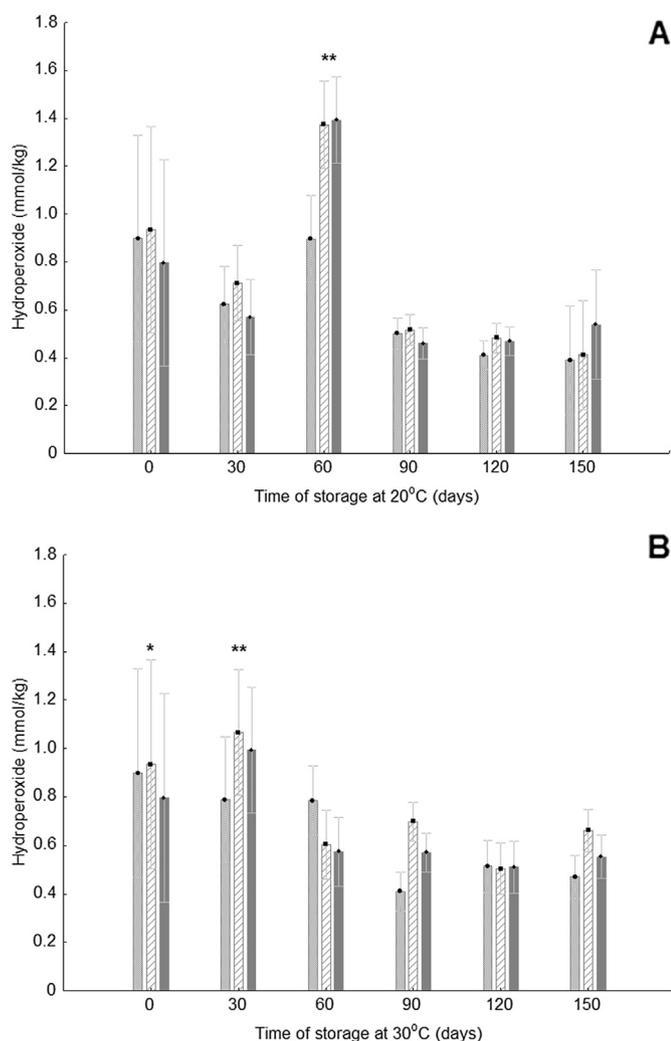
Fig. 1. Hydroperoxides concentration (mmol of hydroperoxide/kg of fat) in the choc-olates containing PS (PHYT), phytosterol added of antioxidants (PHAN) and without PS (CONT), stored for 5 months at 20 °C (A) and at 30 °C (B). Values are means ($n = 5$). Errors bars indicate SEM. *$P < 0.05$, **$P < 0.01$. Conversion rate: 1 mmol/kg = 2 meq O$_2$/kg. ▨ CONT; ▧ PHYT; ▬ PHAN.

2.4. Statistical analysis

The three treatments were compared at the beginning of the study by one-way-ANOVA followed by Tukey HSD test. Previously, all data were submitted to the normality and homogeneity of var-iances tests. Afterward, Repeated Measurements ANOVA was applied to identify the factors that influenced each parameter over time. An α value of 5% was considered to be the risk limit for the type I error. All the calculations and graphics were done using the software Statistica v.9 (Statsoft Inc., Tulsa, USA).

3. Results and discussion

In this study, PS esters were added to bitter Belgium pralines resulting in a functional chocolate. Initially, a dark chocolate formulation was developed using palm oil as filling (CONT). After-ward, the palm oil was replaced by PS esters (PHYT) and PS + antioxidants (PHAN). Table 1 shows the chemical composition, fatty acids profile, color, hardness, pH and sensory score of acceptability of the three chocolate bars evaluated immediately after manufacturing. Except for moisture, no differences were

observed on chemical composition among the three chocolate bars. PS-enriched samples showed lower proportion of palmitic acid and higher proportion of α-linolenic fatty acid when compared with control samples formulated with palm oil. At the beginning of this study, no differences were observed for color, hardness and sensory acceptability among the three treatments. Although pH of the control samples was lower that of the bars containing PS, this dif-ference did not represent a technical relevance.

The objective of this study was to evaluate the oxidative stability of the PS-enriched chocolate bars during 5 months of storage, and its main effects on color, texture, sensory quality and potential bioactivity of the functional food product. As the oxidation of ste-rols reaction can start with the hydroperoxides formation (Lengyel et al., 2012), the primary oxidation of unsaturated lipids was measured by the hydroperoxide concentration (Fig. 1). When stored at 20 °C (Fig. 1A), the hydroperoxide peak (1.39 mmol/kg) occurred after 60 days of storage. Thereafter, the hydroperoxide decomposition rate was greater than its formation. At 30 °C (Fig. 1B), the maximum value (1.06 mmol/kg) was reached after 30 days, thus being earlier but lower than the peak observed at 20 °C. Hamid and Damit (2004) evaluated cocoa butter stability during storage at 15 and 70 °C and observed that the increase of temper-ature anticipated the peroxide peak from 6 to 4 months, even though the maximum values were similar in both storage condi-tions. The peroxide value observed in the chocolate samples during the shelf-life study was lower than 3.0 milli equivalent O$_2$/kg (or 1.5 mmol/kg). This value can be considered low when compared with PV of other fresh vegetable oils, such as coconut (4.9 milli equivalent O$_2$/kg), soybean (2.4 milli equivalent O$_2$/kg) or canola (5.0 milli equivalent O$_2$/kg) (Chaiyasit, Elias, McClements, & Decker, 2007). This low hydroperoxide content observed in chocolates was consequence of the high proportion of saturated (50 g/100 g) and monounsaturated (40 g/100 g) fatty acids present in the cocoa butter. Only less than 10 g/100 g of the fatty acids observed in our samples were polyunsaturated, being the proportion of the most susceptible fatty acid (α-linolenic acid) lower than 1 g/100 g.

Major fatty acids levels observed in the treatments during storage at 30 °C suggested that no significant alterations were detected during the shelf-life. Fatty acids proportion observed in the CONT samples after 150 days at 30 °C were: 27.94 ± 0.06, 18.79 ± 0.51, 41.104 ± 0.06, 7.82 ± 0.29 and 0.26 ± 0.01 g/100 g; for C16:0, C18:0, C18:1, C18:2 n6 and C18:3 n3 respectively; while the mean values obtained to PHYT and PHAN samples were: 22.19 ± 0.12, 24.64 ± 0.21, 40.91 ± 0.15, 7.58 ± 0.10 and 0.90 ± 0.03 g/100 g for C16:0, C18:0, C18:1, C18: 2 n6 and C18:3 n3 respectively. In both storage conditions (20 and 30 °C) it was observed a trend of the PS-enriched bars to oxidize more than the bars formulated with palm oil (Fig. 1). In our chocolate bars, it was expected that C18:3 n3 had been the major responsible for the hydroperoxide formation, since no differences were observed for C18:2 n6 levels between the samples. In fact, the chocolates bars formulated with phytosterols (PHYT and PHAN) presented 236% more C18:3 n3 than those formulated with palm oil (CONT). It was also possible to conclude that the antioxidants applied in this study at the levels here used (0.90 mg/100 g) were not able to reduce oxidation, since no difference was observed between the hydro-peroxide content of the PHYT and PHAN bars. Rossini, Norena, and Brandelli (2011) added two types of antioxidants (Grindox and a natural peptide from casein hydrolysis) to white chocolate at 0.25 g/100 g of the fat weight. The authors did not observe differ-ences on PV and TBARs values after 10 months of storage at 20 °C and 28 °C between samples with and without antioxidants. Hashim, Hudiyono, and Chaveron (1997) reported that the pres-ence of 100 ppm of α-tocopherol inhibited the oxidation of cocoa butter while 1000 ppm had accelerated its oxidation. In our
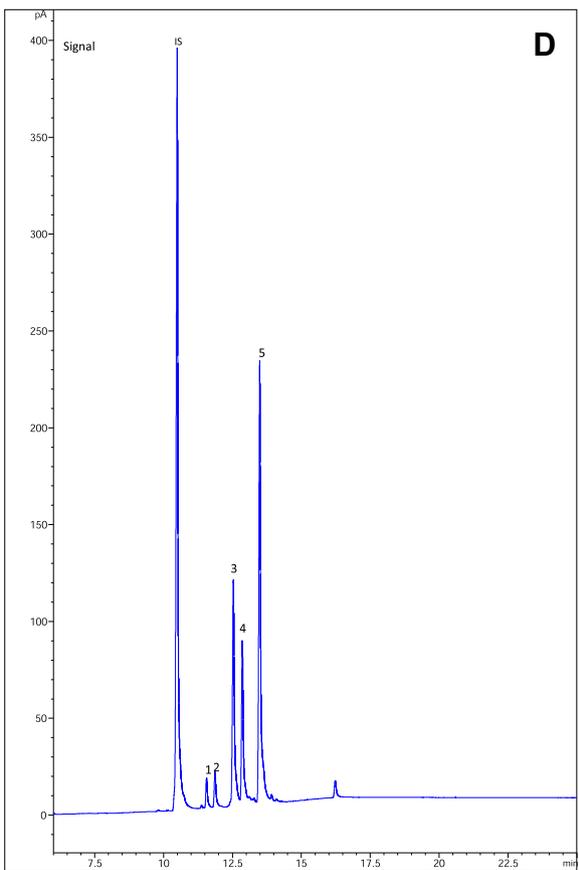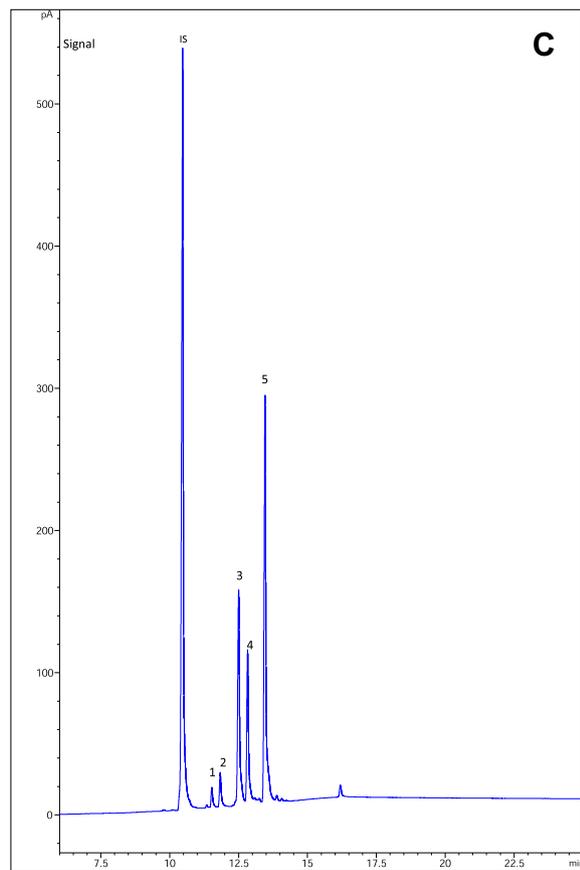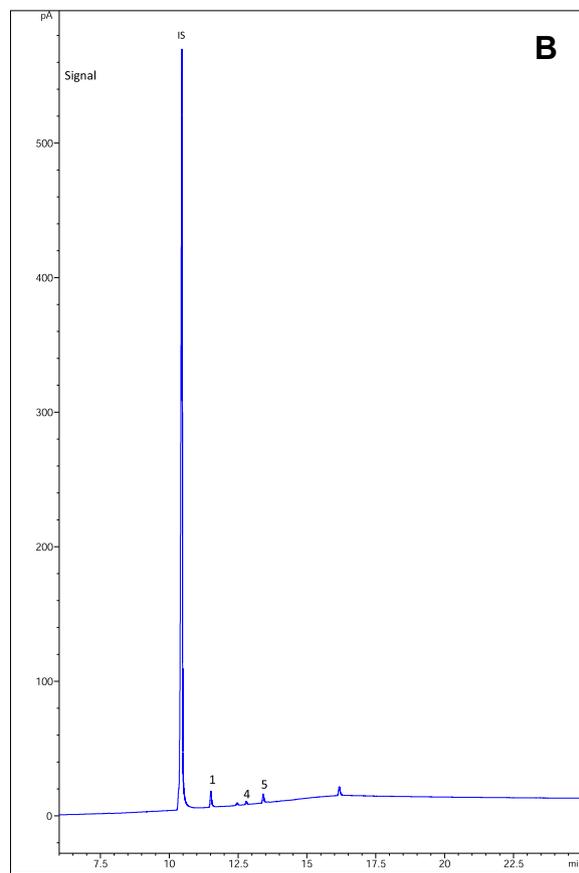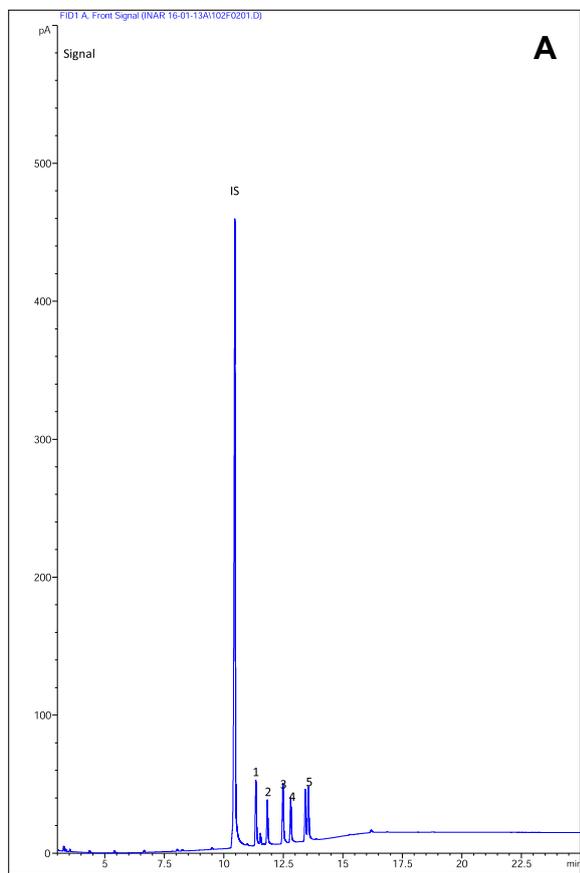
Fig. 2. Trimethythylsilyl (TMS) derivatives of sterols evaluated by GC/FID in the samples: a standard mixture (A), chocolate without PS or CONT (B); chocolate with PS (PHYT) (C) and chocolate with PS and antioxidants (PHAN) (D), after 5 months of storage at 30 °C. Graphs present the signal intensity (from 0 to 500) vs retention time (from 6 to 20 min). Peak identification: IS, internal standard: 5β-cholestan-3α-ol (epicoprostanol), 1, cholesterol; 2, brassicasterol; 3, campesterol; 4, stigmasterol; and 5, β-sitosterol.

chocolate samples, the amount of antioxidants added to the formulation neither inhibit hydroperoxides formation, nor promoted oxidation.

The chocolate bars kept the color stability until 90 days of storage ($L^* = 27.3 \pm 1.5$, $a^* = 5.6 \pm 0.4$ and $b^* = 4.4. \pm 0.7$). After this time, all bars presented a trend to become lighter ($L^* = 31.8 \pm 2.6$) and more red-yellow ($a^* = 6.1 \pm 1.1$ and $b^* = 6.6 \pm 0.8$). This effect may be associated to the formation of white spots on the surface of chocolate, known as *fat bloom*. In our assay, *fat bloom* could have been caused by migration of the lipids from the praline's filling through its shell, since there was no temperature fluctuation during storage, or improper tempering (Cassiday, 2012). According to Depypere, de Clercq, Segers, Lewille, and Dewettinck (2009), filled chocolates are more prone to *fat bloom* due to the characteristics of the fillings and possible incompatibility with the surrounding chocolate shell. The authors suggested that chilling or freezing the chocolates for part of the storage time was found to reduce tri-acylglycerols migration and consequently *fat bloom*. Regarding the bars texture, it was observed a hardness reduction in all bars from the beginning (7.3 ± 2.1 N) until 90 days of storage (4.4 ± 1.6 N). After this time, the treatments showed a hardness elevation (7.7 ± 2.2 N). Mexis, Badeka, Riganakos, and Kontominas (2010) also reported fat bloom and softening of chocolates during storage, followed by a significant decrease of the samples sensory acceptability. However, in our study, the absolute values of the changes in color and texture measurements were much lower than those reported by Mexis et al. (2010). Thus, the color and texture differences in our chocolate bars were not perceived by the tasters, or these differences did not impact sensory acceptability. All samples received scores above 6.0 (mean value 7.0 ± 1.2 after 150 days), suggesting a good acceptability for "dark chocolates". Thus, alterations observed in the oxidative stability including polyunsaturated fatty acids oxidation, color and texture changes were not able to reduce the acceptability of the chocolate bars.

The bioactive compound added to the functional food matrix must maintain its original chemical structure and consequent functionality during the entire shelf-life period. Our hypothesis was that the hydroperoxides formed during the shelf-life could accelerate the PS oxidation, reducing the chocolates functionality, since

hydroperoxides and free radicals catalyze sterol oxidation (Derewiaka & Obiedzinski, 2012; Lengyel et al., 2012). PS content of the chocolates were evaluated in the samples at the beginning and after 150 days of storage at 30 °C (Table 2). Fig. 2 shows the peaks identified in our samples compared with standards. Although PS concentration had changed during the storage time, the values observed after 150 days were 6% higher than the values found at the beginning. This slight difference can have been caused by the sampling variation.

Major products of PS oxidation (POPs) were also measured in the samples at the beginning and after 150 days of storage at 30 °C (Table 2). POPs observed in the chocolates were: 7α-hydroxycampesterol, 7α-hydroxystigmasterol, 7α-hydroxysitosterol, 7β-hydroxystigmasterol, α-epoxysitosterol, 7-ketocampesterol, 6β-hydroxycampesterol, stigmastentriol, sitostanetriol, 6-ketositosterol and 7-ketositosterol (Fig. 3 A–C). Changes observed after 5 months may have been a consequence of increased PS oxidation or even POPs degradation. In the PS-enriched chocolate samples, sitostanetriol, 6-ketositosterol and 6β-hydroxycampesterol were the major POPs, which suggests that PS hydroperoxy derivatives followed two degradation pathways: their conversion, into keto and hydroxy derivatives by dysmutation, and their reaction with sterol that lead to the formation of epoxy derivatives that convert into triol in the presence of water. The higher presence of β-sitosterol oxides is supported by the higher β-sitosterol level present in the PS mixture used in the chocolate formulations, because when the plant sterols individual susceptibility is taken into account, the ranking was campesterol > β-sitosterol > stigmasterol. Although Lengyel et al. (2012) had suggested that the presence of double bonds in the side chain promote an antioxidant potency, this effect was not observed in our study, since both campesterol and β-sitosterol present a saturated side chain. According to Hovenkamp et al. (2008), about 1% sterols are present in oxidized form. In our study, only 0.10% of the initial plant sterols were oxidized in the supplemented samples, and this level did not change until the end of the storage time. The elevated oxidative stability observed in our samples might have been promoted by the use of plant sterol esters instead of free plant sterols, the low effect of the

Table 2
Phytosterols (g/bar) and their POPs (μg/bar)[a] determined in the three chocolate bars, before and after 5 months of storage at 30 °C.

|	Treatments[b,c] — time (days)					
Phytosterols (PS)	CONT - T_0	CONT - T_{150}	PHYT- T_0	PHYT- T_{150}	PHAN- T_0	PHAN- T_{150}
Brassicasterol	NDa	NDa	0.08 ± 0.00b	0.08 ± 0.00b	0.08 ± 0.00b	0.09 ± 0.00c
Campesterol	NDa	NDa	0.58 ± 0.01b	0.59 ± 0.00bc	0.61 ± 0.00c	0.67 ± 0.01d
Stigmasterol	NDa	0.01 ± 0.00a	0.37 ± 0.00b	0.37 ± 0.00bc	0.39 ± 0.01c	0.42 ± 0.01d
β-Sitosterol	0.01 ± 0.00a	0.02 ± 0.00a	1.04 ± 0.01c	1.08 ± 0.01b	1.12 ± 0.01b	1.21 ± 0.02d
POPs						
7α-Hydroxycampesterol	81.39 ± 3.09a	77.85 ± 1.33a	115.26 ± 4.80b	109.48 ± 3.95b	113.75 ± 2.01b	114.28 ± 2.24b
7α-Hydroxystigmasterol	73.92 ± 0.71a	72.79 ± 2.56a	97.06 ± 5.00b	92.32 ± 2.12b	94.09 ± 3.13b	97.03 ± 0.62b
7α-Hydroxysitosterol	NDc	NDc	188.17 ± 4.00b	173.19 ± 3.02ab	167.33 ± 7.04a	178.32 ± 3.30ab
7β-Hydroxystigmasterol	23.21 ± 2.15a	24.93 ± 0.60a	53.94 ± 5.22b	52.33 ± 3.09b	55.19 ± 4.89b	58.97 ± 2.75b
α-Epoxysitosterol	NDb	NDb	246.78 ± 42.21c	140.36 ± 4.94a	135.71 ± 24.03a	172.77 ± 15.23ac
7-Ketocampesterol	45.73 ± 6.31c	58.83 ± 3.61c	158.14 ± 7.96a	211.57 ± 15.12ab	232.13 ± 30.71b	198.59 ± 8.62ab
6β-Hydroxycampesterol	NDa	NDa	179.50 ± 4.60b	181.08 ± 13.28b	238.00 ± 25.02b	252.94 ± 55.37b
Stigmastentriol	NDa	NDa	35.87 ± 1.65d	45.54 ± 0.31b	51.40 ± 3.86bc	54.35 ± 0.89c
Sitostanetriol	98.91 ± 50.03b	165.84 ± 7.79b	610.90 ± 233.17ab	717.56 ± 108.16b	813.65 ± 6.57b	933.83 ± 109.27b
6-Ketositosterol	39.74 ± 1.90c	67.13 ± 1.27c	186.76 ± 0.99a	228.75 ± 22.70ab	223.84 ± 5.78ab	257.90 ± 35.85b
7-Ketositosterol	55.00 ± 7.89a	66.26 ± 4.46a	193.21 ± 0.62b	179.97 ± 34.37b	207.78 ± 3.11b	160.22 ± 1.57b
Total phytosterols (mg/bar)	10.00	30.00	2070.00	2120.00	2200.00	2390.00
Total POPs (mg/bar)	0.42	0.53	2.06	2.13	2.33	2.48
POPs/sterols ratio	0.042a	0.017a	0.001b	0.001b	0.001b	0.001b

ND: not detected.
[a] Each bar contained 30 g of chocolate.
[b] CONT − Chocolate without PS; PHYT − Chocolate with PS; PHAN − Chocolate with PS + antioxidants.
[c] Values are expressed as means ± SD ($n = 5$). Different letters in the same line indicate significant differences ($P < 0.05$).
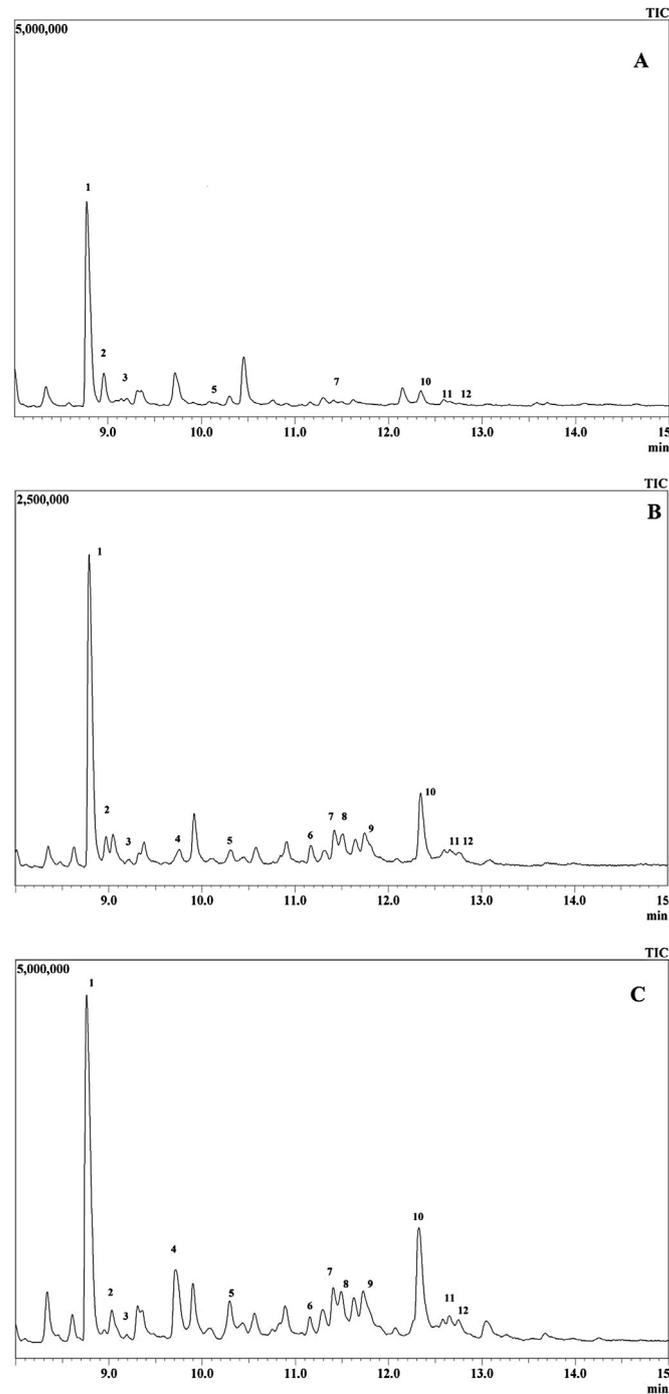
Fig. 3. GC–MS chromatograms of phytosterol oxides identified in the treatments: chocolate without PS or CONT (A); chocolate with PS (PHYT) (B) and chocolate with PS and antioxidants (PHAN) (C). All Compounds were analyzed as TMS derivatives. Peaks: (1) 19-hydroxycholesterol (I. S); (2) 7α-hydroxycampesterol; (3) 7α-hydroxystigmasterol; (4) 7α-hydroxysitosterol; (5) 7β-hydroxystigmasterol; (6) α-epoxysitosterol; (7) 7-ketocampesterol; (8) 6β-hydroxycampesterol; (9) stigmastentriol; (10) sitostanetriol; (11) 6-ketositosterol; (12) 7-ketositosterol.

temperature during the chocolate manufacturing and by the high level of fatty acid saturation and phenolic compounds found in cocoa butter and cocoa, respectively.

Based on the analysis performed in this study, it can be concluded that these PS-enriched chocolate bars attained all relevant aspects for a satisfactory functional food development and can be stored for up to 150 days without significant modifications in their nutritional and sensory profile. The daily intake of 1 bar (30 g) provided about 2.2 g of PS esters, that is higher than the amount required by the FDA (1.3 g). In addition, the chocolate bar developed in this study did not contain sugar and was formulated with 50 g/100 g of cocoa, becoming an interesting option for individuals with dyslipidemia, type 2 diabetes or metabolic syndrome.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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