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ORIGINAL ARTICLE

Changes in the color of white chocolate during storage: potential roles of lipid oxidation and non-enzymatic browning reactions

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Abstract Three different samples of white chocolate were prepared: a sample with a synthetic antioxidant, another with casein peptides as natural antioxidant, and a third sample without any kind of antioxidant. Parameters associated with lipid oxidation and non-enzymatic browning were evaluated in the different samples of white chocolate during 10 months storage at 20 and 28°C. Acidity, thiobarbituric acid reactive substances and peroxide values increased with the incubation time. Samples stored at 20°C often showed lower values for these parameters than those stored at 28°C, although the differences were not always significant. The values for water activity increased from 0.4 to 0.53–0.57 during the period of 10 months. The color parameter a* was increased in samples stored at 28°C from month 5, and the parameter b* was lower in samples containing antioxidants from month 2. The addition of antioxidants did not significantly influence most the parameters studied, suggesting that the main parameters governing the alterations of white chocolate during its shelf-life was the storage temperature and increase in water activity.

Keywords Casein · White chocolate · Lipid oxidation · Maillard reaction · Synthetic antioxidant · Natural antioxidant

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Introduction

Chocolates are complex multiphase systems of particulate (sugar, cocoa, certain milk components) and continuous phases (cocoa butter, milk fat and emulsifiers) and its visual characteristics, including gloss, color, shape, roughness, surface texture, shininess and translucency, is summarized into appearance attributes (Briones et al. 2005; Afoakwa et al. 2008). Primary chocolate categories are dark, milk and white that differs in content of cocoa solids, milk fat and cocoa butter (Afoakwa et al. 2007). In manufactured confectionery products such as white chocolate the development of brown colors is one of the main problems that limit the shelf-life. The brown color is more relevant in white chocolates that are often sold in transparent packaging because the consumer can compare them with a white product. Naturally, consumers expect that white chocolate should have a white to pale yellow color and the presence of a dark yellow color, or even light brown color, is undesirable. The white chocolate ingredients reacts each other during processing and storage resulting in the development of brown color (Vercet 2003).

Milk solids have undoubtedly been responsible for the great increase in the consumption of white chocolate (Muresan et al. 2000). These solids have commonly been used in the enhancement of nutritional and technological qualities of another wide range of different foods as well as in adding the desired flavor to them (Tunçturk and Zorba 2006). However, the chemical interaction between milk proteins and the aldehyde groups of the reducing sugar lactose results in the Maillard reaction (Muresan et al. 2000). The flavor and color development due to Maillard reaction are related to temperature, time and presence of water during the processing and/or storage of foods (Moreno et al. 2006). Due to the high percentage of fat

from cocoa butter and milk, lipid oxidation could be also involved in color evolution of white chocolate.

Lipid oxidation is a serious problem because it not only produces off-flavors but also decreases the nutritional quality, safety and shelf-life of the food (Shih and Daigle 2003). Therefore, the control of lipid oxidation in food products is desirable, and the benefits of antioxidants in food storage have been studied by many researchers (Sakanaka et al. 2005). Synthetic antioxidants, such as butylated hydroxylanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ), have been commonly used to suppress the formation of free radicals, preventing lipid oxidation and food spoilage. Although these synthetic reagents are efficient and relatively cheap, special attention has been given to natural antioxidants because of a worldwide trend to avoid or minimize the use of synthetic food additives (Shih and Daigle 2003).

The biological and physiological activities of milk proteins are partially attributed to several peptides encoded in the native protein molecules (Kim et al. 2007). Among the biologically active peptide molecules, casein phosphopeptides (CPPs) have been proposed as potential dietary antioxidants on designing new functional products (Laparra et al. 2008). They indicated that the CPPs contain phosphoseryl residues with the capacity to sequester potential prooxidant metals such as iron. Kitts (2005) suggested that CPPs could inhibit lipid oxidation by chelation with non-phosphorylated groups such as Glu and Asp, and/or via non-chelating mechanisms such as free radical scavenging.

The aim of this study was to investigate the effect of antioxidants in the color alteration of white chocolate during its shelf-life. Two different antioxidants, synthetic Grindox 562 and casein peptides resulting from hydrolysis with Flavourzyme, were tested during storage at two temperatures. Parameters associated with lipid oxidation and non-enzymatic browning reactions were evaluated for the period of 10 months.

Materials and methods

Reagents

The sample of white chocolate was from Florestal Alimentos S/A (Porto Alegre, Brazil). Glucose and 2-thiobarbituric acid were obtained from Sigma (St. Louis, MO, USA). Chloroform, acetic acid, and trichloroacetic acid were from Nuclear (Rio de Janeiro, Brazil). Commercial antioxidant Grindox 562 (in a form of blends, which contains mainly 10% ascorbyl palmitate, 90% propylene glycol, and a food grade emulsifier) was from Danisco (Copenhagen, Denmark), casein was from Farmaquimica (São Paulo, Brazil). Flavourzyme™ (endoprotease and exopeptidase from *Aspergillus oryzae*) was from Novozymes Latin America (Araucaria, Brazil).

Casein hydrolysis

Casein peptides were prepared by the hydrolysis of casein using the proteolytic enzyme Flavourzyme, essentially as described by Rossini et al. (2008). Casein was dissolved in distilled water pH 8.0 at a concentration of 130 gL^{-1} and hydrolyzed by protease (0.4/100 enzyme/substrate ratio) for 4 h at 50°C in a stirred batch. The hydrolysis was stopped by adding trichloroacetic acid (TCA) to reach a final concentration of 10% (w/v). The material was centrifuged at 10,000 x *g* for 20 min to remove insoluble materials, and the supernatant containing the casein peptides was then lyophilized and kept at -18°C until used. Proteolysis was monitored by release free amino groups (Rossini et al. 2008) and antioxidant activity by 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical method (Re et al. 1998).

Confectionery products

Three different formulations of white chocolate samples were produced: a white chocolate with 0.2% (w/w) Grindox (on cocoa butter basis), a white chocolate with natural peptide antioxidants from hydrolysis of casein (0.2%, on cocoa butter) and a white chocolate without antioxidant. Composition of white chocolate was (g/kg): sugar (445), cocoa butter (296), whole milk powder (171), skimmed milk powder (85), soy lecithin (3). The chocolate production procedure follows the traditional process, consisting of mixing the ingredients (powder milk, soy lecithin**,** cocoa butter, sugar and/or no antioxidant), refining and conching (Beckett 1994). Then the white chocolate was tempered and molded in tablets. The tablets had a weight of 25 g and were packed with a polypropylene plastic film. Samples were stored at two temperatures, 20°C±2°C and 28°C±2°C, and at a relative humidity of 65%.

Sample preparation

The technique described by Vercet (2003) was used for preparation of the samples for analyses. For each determination, all the analyses were performed in triplicate. Then, several batches of 4 g were defatted with 25 ml of a mixture of chloroform/methanol (95:5, v/v) and centrifuged at 3,000 x *g* during 30 min. The solvent fraction was decanted and solvent was evaporated. The fat obtained was analyzed for the peroxide value, acidity index and the UV absorbance spectrum (Shimadzu UV-mini 1,240 spectrophotometer, Tokyo, Japan).

The defatted pellet was suspended in water at 50°C in a 50-ml flask under vigorous stirring. Then 0.5 ml of Carrez I reagent was added and afterwards 0.5 ml of Carrez II reagent. The solution was left to rest for 10 min and the volume was adjusted to 50 ml with distilled water. The solution was filtered and the filtrate was used for the analysis of reducing sugars (Vercet 2003).

Peroxide value

The peroxide value was performed by the AOAC method (AOAC 1995). Extracted fat (5 g) was placed in a 100-ml flask and dissolved in 30 ml of an acetic acid-chloroform solution (3:1). Then, 0.5 ml of a saturated solution of KI was added. It was left to stand in the dark for 2 min with a gentle stirring and then 30 ml of water were added. The liberated iodine was titrated with 0.01 N $Na_2S_2O_3$. When the brown color tended to disappear, 1 ml of a 1% soluble starch solution was added to give a better control of the end point.

Acidity index

Acidity index was determined by titration (method 922.28; AOAC 1995). Extracted fat (1 g) was dissolved in 50 ml of diethyl ether and titrated with KOH (0.1 M) dissolved in methanol. Three drops of a phenolphtalein solution of were added to give a better control of the end point.

Thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid reactive substances (TBARS) were determined as described elsewhere (Bird and Draper 1984). White chocolate samples (5 g) were placed in a 50-ml flask, dissolved in chloroform and transferred to 100 ml volumetric flask and the volume was completed with chloroform. Then, 5 ml solution was transferred to a centrifuge tube of 20 ml, to which 5 ml of trichloroacetic acid (TCA) 10% was added before centrifugation at 3,000 g for 10 min. After centrifuged, 4 ml of supernatant was mixed with 1.25 ml of 30 mM thiobarbituric acid. The sample was then heated in a boiling water bath for 10 min and cooled to room temperature. The absorbance was measured at 530 nm using a Hitachi U-1100 spectrophotometer (Hitachi, Tokyo, Japan). Thiobarbituric acid reactive substances were calculated from a standard curve of malonaldehyde (MDA), a breakdown product of tetraethoxypropane (TEP).

Color determination

The surface color of the chocolate tablets was measured using a CM spectrophotometer model 500-d Series (Minolta, Japan). The parameters determined were L* (luminosity or brightness: L*=0 black and L*=100 white), a* (red-green component: -a*=greenness and+a*=redness) and b* (yellow-blue component: -b*=blueness and+b*=yellowness). Where L*, a* and b* values at the considered storage time were considered with respect to those obtained in just manufactured samples (time zero).

Water activity

Water activity (at 25°C) was determined measuring directly in a water activity instrument (Aqualab 3TE-Decagon, Pullman, WA, USA).

Reducing sugars

The amount of reducing sugars was determined according to Miller (1959). Briefly, 100 µl of an aliquot of samples were reacted with 1,000 µl of 3,5-dinitrosalicylic acid (DNS) reagent. The sample was then heated in a boiling water bath for 5 min and cooled to room temperature. The developed color was measured at 550 nm using a Hitachi U1100 spectrophotometer. A standard curve was developed using glucose (0–5 mg/ml).

Statistical analysis

Results were expressed as means±S.D. of three independent determinations. Data were evaluated statistically by two-way analysis of variance (ANOVA) and Tukey's test. Values were considered different each other when $P<0.05$.

Results

The evolution of several parameters associated to non-enzymatic browning reactions was monitored during storage of white chocolate for 10 months. As a general trend, peroxide values increased in all samples during incubation, most markedly at 28°C (Table 1). At the end of 10 months, the sample without antioxidant and submitted to the temperature of 28°C had the highest increase of peroxide value, significantly different from the other samples ($P<0.05$). The samples with antioxidants stored at 28°C showed difference from the sample without antioxidant stored at this temperature, and also from all samples submitted to the temperature of 20°C. The samples stored at 20°C showed the smaller formation of peroxides.

The values for the acidity index and TBARS showed a significant increase throughout storage time (Table 1). A difference in TBARS values was observed between samples at 20°C and 28°C, at the end of the storage period. However, no significant differences could be seen in all the values at time 0 and among samples with or without

Table 1 Peroxide value, TBARS, acidity and water activity of white chocolate samples stored at 20 and 28°C for up to 10 months

	Peroxide value (mEq O_2/kg)		TBARS (μg/g)		Acidity (mg KOH/g)		Water activity	
Months	0	10	0	10	0	10	0	10
Control								
20°C	2.1±0.19	5.4±0.20[a]	5.4±1.20	14.4±0.60[a]	3.1±0.32	7.4±0.58[a,b]	0.399±0.005 [a]	0.523±0.010 [a]
28°C	2.1±0.21	6.9±0.11[b]	5.6±1.26	18.0±0.78[b]	3.1±0.32	8.4±0.55[a,b]	0.399±0.005 [a]	0.573±0.016 [b]
Grindox								
20°C	2.1±0.18	5.3±0.40[a]	5.2±0.33	14.6±0.47[a]	3.7±0.88	7.4±0.60[a,b]	0.399±0.005 [a]	0.523±0.008 [a]
28°C	2.3±0.29	6.5±0.18[c]	5.9±0.79	17.6±0.13[b]	3.8±0.02	8.1±0.52[a,b]	0.401±0.005 [a]	0.569±0.009 [b]
CH								
20°C	2.3±0.83	5.2±0.14[a]	5.8±0.39	14.7±0.97[a]	3.6±0.28	7.1±0.52[a]	0.408±0.004 [a]	0.533±0.003 [a]
28°C	2.4±0.73	6.0±0.13[d]	5.8±0.34	17.4±0.22[b]	3.6±0.28	8.5±0.42[b]	0.408±0.004 [a]	0.573±0.007 [b]

[a] Different superscript letters indicate significant differences within the same column at $P<0.05$

Differences between time 0 and time 10 were always significant at $P<0.05$

Values are the means of three experiments; *CH* Casein Hydrolysate

antioxidant. Increase in peroxide values, TBARS and acidity was observed under the different experimental conditions. The UV spectra of the samples at time 0 and 10 months were very similar (data not shown).

The values for water activity of the samples increased during the 10 months of shelf life (Table 1). There were no significant differences among the samples submitted to the same temperature. The samples submitted to 20°C had less increase in their water activity values.

In relation to the color, it was possible to observe the increase of the parameter a*, evidencing the rise of intensity of the red color (Fig. 1a). Color a* of the samples stored at 28°C showed a significant increase from 5 months storage. At the end of the storage period, significant differences among the samples stored at different temperatures were observed. However, there were no significant differences when samples submitted to the same temperature were compared. In the color expressed by the b* parameter, there was a rise of intensity of the yellow color for all samples at the end of 10 months of shelf life (Fig. 1b). Despite the large deviations, color b* increased significantly in samples without antioxidants, which had the most accentuated development of the yellow color. These samples showed significantly higher values when compared with samples containing antioxidants, for both temperature of 20°C and 28°C. On the other hand, the analysis of the L* parameter indicated a decrease of brightness at the end of 10 months (Fig. 1c). However, there was no significant difference among all samples.

The results for the amount of reducing sugars showed the reduction of this parameter throughout time (Fig. 1d). Significant differences were verified among the samples stored at different temperatures at the end of storage period. However, there was no difference when comparing different samples stored at the same temperature. The samples submitted to the temperature of 28°C presented the highest losses in the quantity of reducing sugars. Lower values were observed from 8 months at 28°C.

Discussion

In this work, samples of white chocolate were produced with and without the addition of antioxidants and stored at two different temperatures, 20±2°C and 28±2°C. The ideal temperature for the storage of white chocolate has been described as around 20°C (Subramaniam 2000).

The utilization of natural antioxidant derived from the enzymatic hydrolysis of casein is justified since these casein peptides (CPP) have the capability to sequester Fe^{2+} (Kitts 2005). In addition, these peptides were effective to reduce Fe^{2+}-induced liposomal peroxidation and showed direct scavenging affinity for the hydrophilic ABTS radical (Kitts 2005). It can be concluded that peptides derived from bovine casein have both primary and secondary antioxidant properties that specifically involve direct free radical scavenging and sequestering of potential metal prooxidants.

Lipid degradation is related as one of the main deteriorative problems and one of the first mechanisms of quality loss of food products (Haak et al. 2006). In this regard, peroxide values increased for all chocolate samples. The highest value was observed for the sample without antioxidant stored at 28°C, after 10 months of storage (around 7.0 mEq O_2/kg fat). Similarly, Vercet (2003) obtained values of 9.5 mEq O_2/kg fat, after 15 months of storage at 20°C. Although these values increased throughout time, they are still lower than 10 mEq O_2/kg fat indicating that the alterations related to the lipid degradation are still in the initial stage (Mattisek et al. 1998).

Through the analyses of TBARS, it was possible to reinforce the result described above. The results showed no

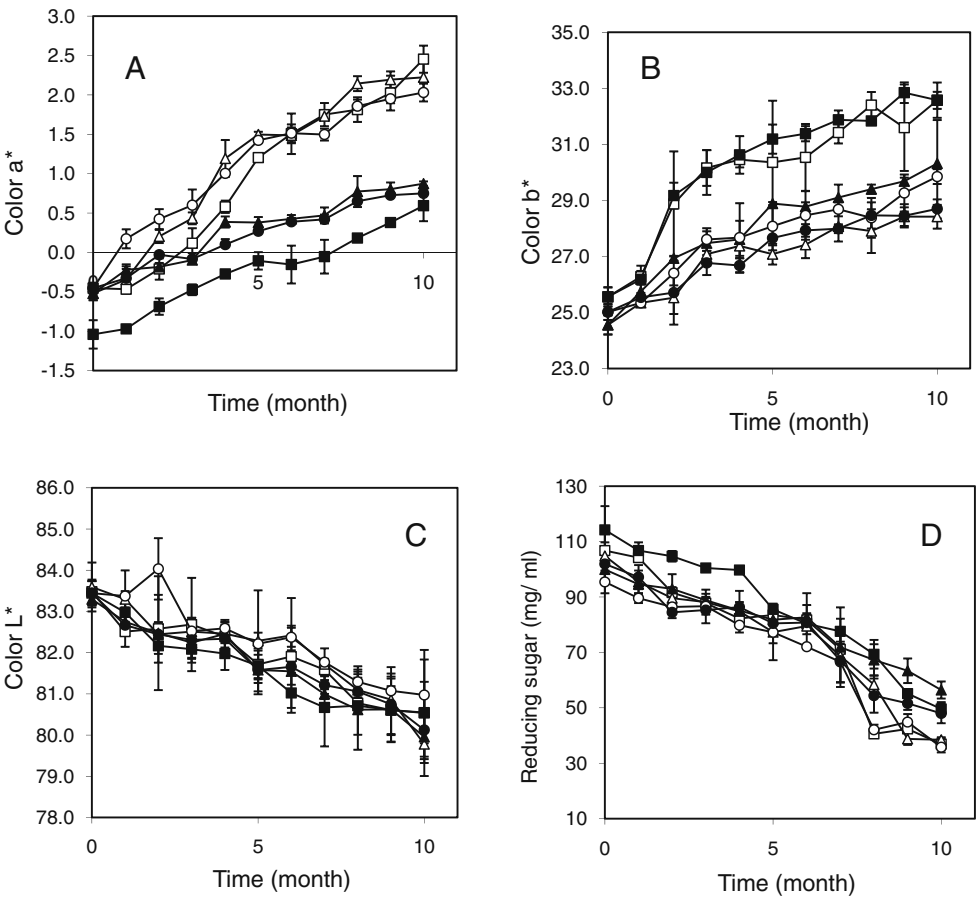

Fig. 1 Color parameters a* **a**, b* **b** and L* **c** and concentration of reducing sugars **d** of white chocolate during storage at 20°C with Grindox (●), 20°C with casein hydrolysate (▲), 20°C without antioxidant (■), 28°C with Grindox (○), 28°C with casein hydrolysate (Δ), 28°C without antioxidant (□). Values are the means of three experiments

significant difference among the samples with and without antioxidants, suggesting that lipid degradation was not main factor in the color alteration of the white chocolate samples in the conditions they were analyzed. Vercet (2003) showed that fatty acid methyl esters profiles of fat extracted from chocolates at the beginning and at the end of 15 months storage at 20°C are very similar. This indicates that there is no significant loss in the unsaturated fatty acids present in the chocolate.

An increase in water activity throughout time was observed for all samples. Water activity of chocolate is often between 0.4 and 0.5, but several factors such as the raw materials used, the surface area of the materials, and the temperature and humidity of refining and conching may influence this parameter (Biquet and Labuza 1988). Chocolate is protected from the external water by its fatty surface, which strongly difficult moisture uptake (Richardson 1987). However, the presence of amorphous sugars in chocolates should be considered. Amorphous sugar is a metastable form and tends to crystallize under the influence of a number of factors, mainly temperature and moisture (Gloria and Sievert 2001). There are two important sugars in white chocolate, sucrose and lactose. During the milling of sucrose to reduce particle size and incorporate it into the chocolate, high temperatures are reached and a considerable amount of amorphous sucrose is formed (Niediek 1991). Otherwise, lactose largely occurs in the amorphous state, which is thermodynamically unstable and hygroscopic, absorbing moisture from the surrounding and subsequently plasticizing. When crystallization occurs, water is suddenly liberated and local water activities could increase. The temperature at which this transition from an amorphous solid-state to a viscous rubbery state occurs is known as the glass transition temperature. The glass transition temperature can fall below ambient temperature when the powder absorbs sufficient moisture during storage (Ibach and Kind 2007). In the results presented here, significant differences were observed for the values of water activity among the samples stored at different temperatures, with higher values for the samples stored at 28°C, suggesting that this parameter influenced in the quantity of free water in the samples, through the crystallization of lactose (Fitzpatrick et al. 2007).

Although the values of water activity have increased, the highest values did not reach 0.6, thus prohibiting microbial growth. According to Richter and Lannes (2007), the lowest value of water activity allowing the bacterial growth was 0.75, and 0.65 and 0.61 for xerophilic fungi and osmophilic yeasts, respectively. Nevertheless, although this raise of water activity does not influence the microbial growth, it contributes for the development of non-enzymatic browning

reactions. Although the optimal range of water activity for Maillard reaction is 0.52–0.75, an increase in the development of this reaction during the storage of egg powder is reported for water activity ranging 0.32–0.35 (Caboni et al. 2005). Moreno et al. (2006), observed the occurrence of this reaction for values of water activity of 0.44 and temperature of 30°C in onions. García-Banos et al. (2005) visualized the development of Maillard reaction in powder enteral formulas during storage at 30°C and water activity of 0.44. Thus, the increase in water activity observed during storage of white chocolate may favor the development of non-enzymatic browning reactions.

The increase of a* parameter along the time indicates that the white chocolate is becoming darker. This fact agrees with the decrease in the concentration of reducing sugars, which was higher at 28°C and higher water activity. The main reducing sugar in white chocolate is lactose, a disaccharide formed by glucose and galactose found in milk in concentrations of 4.5–5.0 g/100 ml (Messia et al. 2007). In white chocolate, the initial stage of the Maillard reaction involves interaction between the ε-NH_2 of protein-bound lysine with lactose to form lactuloselysine [ε-(deoxylactose)lysine] (Friedman 1996; Van Boekel 1998). Decrease in reducing sugars (mainly lactose from milk solids) during storage of white chocolate is possibly associated to its involvement in non-enzymatic browning. Miao and Roos (2005) observed that non-enzymatic browning rate in low-moisture food system was accelerated by crystallization of the component sugars.

The result of the b* parameter, which represents the intensity of the yellow color, demonstrated that the presence of antioxidants may act to prevent the accentuated development of yellow color, since the samples without any kind of antioxidants presented the highest values in this analysis, independent from the temperature they were stored. The L* parameter, that is the brightness of white chocolate, has diminished in all samples throughout time, without significant difference among the samples at the final time, indicating that this parameter is not influenced neither by the addition of antioxidants nor by the temperature.

Excepting for the color parameter b*, white chocolate samples stored at the temperature of 20±2°C showed minor alterations during storage independent from the addition of antioxidants. Besides, the development of browning in white chocolate is mainly due to the non-enzymatic browning reactions and the ambient conditions during the storage period. The lipid oxidation was evidenced, but in its initial stage, causing no important alterations among the samples of white chocolate. These results suggest that increase of water activity and storage temperature are the main factors governing color alteration in white chocolate.

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