Oxidative stability of yogurt with added lutein dye

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ABSTRACT

This study evaluated the effect of adding lutein dye on the oxidative stability of yogurt during 35 d of refrigerated storage, in the presence and absence of light. Yogurts manufactured without and with the equivalent of 1.5 mg of lutein in 120 g of the final product were characterized for their total carotenoid and riboflavin contents, and the behaviors of both riboflavin and lutein were monitored during storage. A decrease in riboflavin content occurred, with concurrent appearance of its derived-oxidation products in the yogurts without added lutein and exposed to light during storage. The yogurts with added lutein dye showed constant lutein and riboflavin contents throughout storage both for the samples stored under light and for those stored in the dark. Yogurts (120 g) with the addition of 0.5, 1.5, and 2.5 mg of lutein dye were evaluated for their sensory acceptance, and the statistical analysis showed no differences between the samples for the attributes of aroma and flavor. These results indicate that the added lutein remained stable throughout the storage period and conferred protection for the riboflavin against photooxidation, preserving the quality of the yogurts.

Key words: fermented milk, carotenoid, riboflavin, photooxidation

INTRODUCTION

Carotenoids are natural pigments known for their biological functions and dyeing properties. They are synthesized by higher plants, algae, bacteria, and certain fungi, and their presence in animals is attributed to the ingestion of foods. Lutein is the second-most-prevalent carotenoid in the human body (Khachik et al., 1997; Calvo, 2005), and accumulates mainly in the macula, in the central region of the human retina, which is responsible for visual acuity and where the greatest number of the photoreceptors is concentrated (Landrum et al., 1999). The richest sources of lutein

include green vegetables such as spinach (40 µg/g) and kale (50 µg/g), and lutein also can be found in yellow vegetables such as corn (5 µg/g), and egg yolk (8 µg/g) (Rodriguez-Amaya et al., 2008; Perry et al., 2009).

It is believed that lutein exerts 2 main protective functions in the eyes: (1) as a filter of blue light and (2) as scavenger of reactive oxygen species (ROS). Blue light is the form of visible light with the greatest energy (wavelength of approximately 450 nm), and is known to induce photooxidative damage by generating ROS. Lutein shows a maximum absorption peak at a wavelength of 446 nm and, for this reason, is capable of absorbing blue light, decreasing the intensity of the light that reaches the retina, and probably reducing the formation of ROS (Krinsky, 1989; Kijlstra et al., 2012). The consumption of lutein has been associated with a decrease and prevention of the occurrence of cataracts and age-related macular degeneration, and hence research on the properties of this carotenoid has been increasing in recent decades (Landrum et al., 1997; Bhosale et al., 2009). Age-related macular degeneration is the main cause of irreversible blindness in the elderly and affects about 50 million people over 75 yr of age throughout the world, including more than 10 million in the United States and more than 190,000 in the United Kingdom (Evans et al., 2004; Klein et al., 2004). The ingestion of 6 mg of lutein per day has been related to a decrease of more than 43% in the risk of age-related macular degeneration (Seddon et al., 1994). Lutein can also be an important ally in reducing oxidative stress and damage to DNA, which may contribute to the development of cancerous cells in the organism (Serpeloni et al., 2010, 2012).

The application of lutein as a functional ingredient in dairy products is a convenient option, considering that, in general, the population is searching more and more for foods that provide health benefits. Nevertheless, it is important that the carotenoid does not degrade during storage, being available at the moment of consumption. Few studies concerning the addition of lutein to dairy products were found up to the present. Jones et al. (2005) evaluated the addition of different concentrations of lutein to Cheddar cheese, and showed that the carotenoid did not degrade during the 24 wk of cheese

Received April 29, 2013.

Accepted August 26, 2013.

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ripening. On the other hand, different concentrations of lutein added to yogurt showed an approximately 10% reduction after 5 wk of refrigerated storage (Aryana et al., 2006).

Despite its antioxidant properties, lutein is sensitive to the action of light due to the presence of conjugated double bonds, as these degrade mainly at wavelengths between 200 and 400 nm and at 463 nm, as observed in a model beverage with added carotenoid filled into transparent packages (Kline et al., 2011).

Lactic products are susceptible to photooxidation due to the presence of riboflavin (**RBF**; vitamin B₂), which is a sensitizing molecule. Riboflavin is capable of absorbing luminous energy and passing from its fundamental singlet state to an excited triplet state (³RBF*), which can follow 2 reaction mechanisms: react directly with a biological molecule, producing free radicals and radical ions, or transfer its energy to oxygen molecules, forming singlet oxygen, which can also degrade biological molecules. These biological molecules can be the proteins, FA, or vitamins present in milk, and hence these reactions can lead to nutritional losses and sensory alterations, representing an indicator of the occurrence of photooxidation in dairy products (Borle et al., 2001).

Riboflavin is characterized by a ring structure with conjugated double bonds and nitrogen bases, with a maximum range of light absorption at 225, 175, 370, and 450 nm (Drössler et al., 2003), the latter being the most critical one with respect to the photooxidation of dairy products, as it matches with the visible light region emitted by the fluorescent light present in the majority of commercial establishments. The distribution of the ROS formed due to the sensitization of RBF depends on the availability of oxygen, RBF concentration, and the presence of other oxidant or antioxidant substances (Choe et al., 2005). Lutein can act as an antioxidant in photooxidation, principally through physical quenching (that is, transference of energy from the singlet oxygen or from the ³RBF* to the carotenoid), resulting in the excited carotenoid plus the oxygen or sensitizer in their fundamental state. The carotenoid rapidly dissipates this energy and returns to its fundamental state, being capable of again scavenging other species (Di Mascio et al., 1989).

Riboflavin is highly fluorescent (with maximum emission around 525 nm) as are also the products generated by its photodegradation, lumichrome and lumiflavin, which show maximum emission in the region from 444 to 479 nm and from 516 to 522 nm, respectively (Fox and Thayer, 1998). Thus, one of the ways of evaluating photooxidation in dairy products is by detecting RBF and its degradation products using fluorescence spectroscopy (Miquel Becker et al., 2003;

Andersen et al., 2005; Wold et al., 2006; Zandomenighi et al., 2007).

The present study evaluated the influence of adding lutein dye on the oxidative stability of yogurt in the presence and absence of light, by monitoring the RBF and lutein contents. At the end of the experiments, a sensory acceptance test was carried out to verify the influence of different concentrations of lutein on consumer preference.

MATERIALS AND METHODS

Materials

Powdered skim milk (Molico; Nestlé, Araçatuba, Brazil) was acquired, all from the same batch and in an amount sufficient for the entire research project. The freeze-dried mixed lactic culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (YO-MIX 505 LYO 200) was provided by Danisco Brasil Ltda. (Cotia, Brazil). The 0.3% lutein formulation used was the water-dispersible Vegex Lutein WS natural lutein dye for food purposes from Chr. Hansen A/S (Hørsholm, Denmark). According to the manufacturer, this formulation contains lutein extracted from marigold flower, water, modified starch, maltodextrin, sunflower oil, ascorbic acid, ascorbyl palmitate, α -tocopherol, and sodium benzoate. In addition, more than 90% of the lutein was found to be esterified with FA (Xavier et al., 2012). The water used in the experiments was initially bidistilled and then deionized. The yogurt was filled into opaque polypropylene cups provided by Dixie Toga SA (Votorantim, Brazil).

Yogurt Preparation

The yogurts were made from powdered skim milk, reconstituted to 10% TS, plus a mixed freeze-dried lactic culture. Lutein dye was added to half of the total volume before inoculating with the lactic culture. The lutein dye was added such that the final concentration in the product was equivalent to 1.5 mg of lutein/120 g of yogurt. This amount was added considering that the yogurt with lutein would be a complementary source of lutein in food. The culture was added to the milk and distributed in the polypropylene cups, which were sealed by heat induction. The milk was fermented in a Marconi model MA 415/S incubator (Marconi Equipamentos para Laboratórios Ltda., Piracicaba, Brazil) at 45°C and was interrupted by cooling the cups in an ice bath when the pH value reached 4.8. Yogurt with and without lutein were equally distributed in biochemical oxygen demand (BOD) incubators (Marconi model MA 415; Marconi Equipamentos para Laboratórios Ltda.),

adapted with Osram TL8 15W fluorescent lights (Osram do Brasil Lampadas Elétricas Ltda., Osasco, Brazil) and stored for 35 d at 5°C in the presence and absence of light (light intensity of 1,000 lx). The samples stored under light received radiation on the upper part of the cups, similar to the conditions found in supermarkets. Samples were randomly removed on the specified days and replaced with empty sealed cups.

The yogurt composition was determined 2 d after manufacture. The lutein and RBF contents and the appearance of RBF oxidation products were determined after 0, 7, 14, 21, 28, and 35 d of refrigerated storage. The RBF determinations were carried out in duplicate and the lutein contents in triplicate. Before each analysis, the yogurt was homogenized in the actual cup. The entire procedure of preparing and analyzing the yogurts was then repeated to give duplicate processing results.

Yogurt Characterization

The yogurt composition was determined in triplicate according to the procedures described by AOAC International (2006). The pH was read directly using a Digimed model DM22 pH meter (Digimed Instrumentação Analítica, São Paulo, Brazil). The acidity of the yogurts without added lutein was determined by titration with 0.01 *N* NaOH, whereas that of the yogurts with added lutein was determined by titration with 0.01 *N* NaOH with the aid of a pH meter to pH 8.0 to 8.25, with constant stirring, and the results expressed in Dornic degrees.

The total DM was determined gravimetrically in an incubator with forced-air circulation at 100°C for 24 h. The fat content was determined by the Mojonnier method as described by AOAC International (2006), making 3 consecutive extractions with ethyl alcohol, ethyl ether, and petroleum ether. The organic phase was collected in glass plates, which were heated until complete evaporation of the solvents, and then dried in an oven at 105°C for 50 min. The nitrogen and protein contents were determined by the Kjeldahl method (AOAC International, 2006), obtaining the total protein percentage by multiplying the nitrogen percentage by 6.38.

Analysis During Refrigerated Storage

Lutein Content. The lutein content of the yogurts was determined according to the validated method described by Xavier et al. (2012). For the yogurts with added dye, 1.0 ± 0.1 g of yogurt was extracted 5 times with tetrahydrofuran in a vortex, which was followed by centrifugation at $3,500 \times g$ for 15 min at 20°C to separate the phases. The extracts were combined and

transferred into a mixture of ethyl ether and petroleum ether (2:1, vol/vol) in a separating funnel, followed by washing with distilled water. The solvent was completely evaporated in a rotary evaporator (at $\leq 38^\circ\text{C}$), and the dry extract redissolved in 10 mL of ethanol. The absorbance of the extract was determined at 445 nm in an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies Inc., Santa Clara, CA), and the lutein concentration calculated according to the Lambert-Beer law, using a molar extinction coefficient of 2,550, corresponding to lutein in ethanol. The lutein analyses were carried out in triplicate during the storage period.

The total carotenoid contents of the yogurts without added dye (control) were determined in the same way using a 4.0 ± 0.1 -g sample. The analysis was carried out to evaluate the total carotenoid content of the yogurts coming naturally from the milk. The results showed total carotenoid content in the yogurts below the detection limit of the method (<0.05 $\mu\text{g/g}$). Thus, the results for total carotenoids obtained for the yogurts with added lutein were expressed as micrograms of lutein per gram of yogurt.

RBF Content. The RBF content of the yogurts was determined by spectrofluorometric titration of the RBF with a solution of RBF-binding protein (RBPO), based on the method of Zandomenighi et al. (2007). The RBPO (apo form, Sigma R8628) was acquired from the Sigma Chemical Co. (St. Louis, MO). The Cary Eclipse spectrophotometer (Varian Inc., Palo Alto, CA) was equipped with an accessory capable of regulating the inclination of the sample-positioning angle to allow for front-face measurements (Varian Accessory Solid Sample; Varian Australia Pty Ltd., Melbourne, Australia), so as to minimize the radiation that reflects and spreads during incidence of light on the sample. After tests with different angles, an angle of 27° with respect to the incident light was shown to be the best. The measurements were made in quartz cuvettes (1×1 cm) using 0.3 g of yogurt and 400 μL of water to facilitate homogenization of the sample with the added RBPO solution, during titration. The excitation and emission gaps were the same (10 nm), the integration time was 0.5 s, and the increment in wavelength of the scanning spectra was 1 nm. The emission was measured between 480 and 700 nm, with sample excitation at 450 nm. The RBF titration experiments were carried out by adding 10- to 15- μL aliquots of RBPO to the sample to gradually overcome the fluorescence. After each addition, the cuvette was mildly agitated in a vortex for 30 s, before measuring the emission. About 15 to 20 additions were necessary for each experiment. The titrations of the yogurts were carried out in duplicate during the storage period.

Evaluation of Compounds Derived from RBF Degradation. To illustrate RBF photodegradation

and the appearance of degradation products in the yogurts, spectrofluorometric measurements were made using a Cary Eclipse spectrophotometer (Varian Inc.). The conditions used were the same as those described for the determination of RBF, with the exception of the emission, which was measured between 400 and 700 nm, with excitation at 380 nm.

Sensory Analysis. For the sensory acceptance test, yogurts were produced with lutein contents equivalent to 0.5, 1.5, and 2.5 mg per portion (120 g of product), and addition of 1 µg/g per portion of passion fruit artificial aroma (Duas Rodas Industrial Ltda., Jaraguá do Sul, Brazil). One hundred nontrained panelists were recruited among students and staff of the School of Food Engineering of the University of Campinas (UNICAMP, Campinas, SP, Brazil), selected as a function of their yogurt-consuming habits, availability, and interest in taking part in the test. The tests were carried out in individual booths under white light in the Sensory Analysis Laboratory of the Department of Food Technology of UNICAMP (report no. 083/2009 of the Ethics in Research Committee). Twenty-five milliliters of yogurt at 10°C were presented to the panelists in a monadic way and random order. Cream crackers and water were served between evaluations. The attributes of appearance, aroma, flavor, and overall impression were evaluated with the use of a 9-point structured scale, where 1 corresponded to extremely disliked and 9 to extremely liked. A 5-point structured scale was used for buying intention, where 1 corresponded to certainly would buy and 5 to certainly would not buy (Stone and Sidel, 1985).

Experimental Design and Statistical Evaluation of the Results

A split-split-plot design was adopted to evaluate the evolution of the RBF contents during refrigerated storage. The factor studied was the addition of the lutein dye to the yogurts, the storage conditions being the sub-portion of the dye lutein, and the storage time a sub-portion of the storage conditions. The trials were carried out in 2 blocks. The differences between the

sample treatments and the storage times, and the interactions between them, obtained from the RBF and lutein contents, were evaluated by ANOVA. The factors or interactions that presented values of $P \leq 0.05$ were considered to be significantly different. The results obtained in the acceptance test were analyzed by an ANOVA and the Tukey test (5% probability). A bar histogram was prepared with the results obtained for buying intention.

RESULTS AND DISCUSSION

Characterization of the Yogurts

Although the plain yogurts with and without lutein showed significant differences ($P < 0.05$) for pH value and the acidity and fat contents, both presented the typical skimmed yogurt composition (Table 1).

Influence of Light on the Stability of the Dye Lutein Added to the Yogurt

The lutein content remained constant throughout storage for both the yogurts stored in the presence and in the absence of light (Figure 1). Similar behavior was observed by Jones et al. (2005) for lutein added to Cheddar cheese, where no variation was detected in the content of this carotenoid during 24 wk of storage at 4.5°C. In another study evaluating the addition of lutein to skimmed yogurt, the carotenoid showed a reduction of almost 10% of its initial concentration at the end of 5 wk of storage (Aryana et al., 2006). According to Mercadante (2008), the presence of macromolecules could confer photoprotection to the carotenoids in foods, due to complexation between the macromolecules and carotenoids, or by acting as a filter, reducing the incidence of light and, consequently, photodegradation of the pigments.

Influence of Light on the Stability of RBF

Table 2 shows the statistical evaluation of the results (degrees of freedom, sum of mean squares, and prob-

Table 1. Mean ($\pm$ SD) composition of the yogurts with and without the addition of lutein

Composition	Without lutein	With lutein
pH	4.43 $\pm$ 0.08 ^b	4.55 $\pm$ 0.06 ^a
Acidity (% lactic acid)	0.80 $\pm$ 0.04 ^a	0.71 $\pm$ 0.05 ^b
Total protein (%)	3.60 $\pm$ 0.23 ^a	3.60 $\pm$ 0.27 ^a
Total DM (%)	9.40 $\pm$ 0.15 ^a	9.46 $\pm$ 0.07 ^a
Fat (%)	0.06 $\pm$ 0.05 ^b	0.10 $\pm$ 0.01 ^a
Total carotenoids (µg of lutein/g of yogurt)	10.70 $\pm$ 0.18 ^a	0.14 $\pm$ 0.02 ^b
Riboflavin (mg/100 g)	0.22 $\pm$ 0.01 ^a	0.22 $\pm$ 0.004 ^a

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

Figure 1. The behavior of lutein in the yogurts stored under refrigeration in the presence and absence of light. The data presented are the means ($\pm$ SD) of duplicate trials.

abilities) for the addition of lutein, exposure to light, and storage time on the RBF contents of the yogurts. The storage time and light exerted a significant influence on the RBF contents. However, the 3-way interaction of lutein addition $\times$ presence of light $\times$ storage time was also significant. Because the interaction of 3 of the variables exerted an influence on the parameter under analysis, only the effect of the interactions was analyzed and not the independent variables. Figures 2, 3, and 4 show the effects of the interactions of the presence of light with storage time, the addition of lutein with the presence of light, and the addition of lutein with storage time, respectively, on the RBF contents of the yogurts.

The RBF content of the samples stored in the absence of light remained practically constant throughout the storage period (approximately 0.24 mg of RBF/100 g of yogurt; Figure 2). On the other hand, the RBF content of the yogurts exposed to light suffered a loss of about 30% during storage, evidence of the occurrence of photooxidation of the riboflavin and the fact that the photodegradation increased with storage time (Figure 2).

The yogurts with added lutein showed no variation in RBF content, independent of the presence or absence of light (Figure 3), remaining between 0.23 and 0.24 mg of RBF/100 g of yogurt. However, the yogurts without added lutein presented a reasonable reduction in the RBF content (to approximately 0.17 mg of RBF/100 g of yogurt) when exposed to light, compared with those stored in the dark (0.25 mg of RBF/100 g of yogurt). These results suggest that the lutein acted by avoiding photooxidation of the RBF, probably by physical

Figure 2. Behavior of riboflavin in yogurts stored in the presence and absence of light for 35 d. The data presented are the means ($\pm$ SD) of duplicate trials.

quenching of the ROS and by filtering the light (Choe et al., 2005; Montenegro et al., 2007).

The yogurts with added lutein showed little variation in their RBF contents during the storage period, remaining between 0.23 and 0.24 mg of RBF/100 g of yogurt (Figure 4). The yogurts without added lutein showed a reduction of 30% in their RBF contents by the end of storage. These results showed that, in the absence of lutein, RBF degradation became accentuated during the storage period, indicating that it was sensitized by the light and that the degradation process continued with time. When lutein was present in the yogurts, the RBF contents remained practically unaltered during the 35 d of refrigerated storage, which is the approximate shelf life of commercial yogurts.

Table 2. Mean squares and probabilities for the behavior of riboflavin in the yogurts during storage

Factor	Riboflavin		
	df	MS	P-value
Lutein	1	0.00776	0.10
Error I	1	0.00020	
Light	1	0.01672	0.01
Lutein $\times$ light	1	0.02200	0.005
Error II	2	0.00022	
Time	4	0.01067	<0.0001
Lutein $\times$ time	4	0.00812	<0.0001
Light $\times$ time	4	0.00895	<0.0001
Lutein $\times$ light $\times$ time	4	0.00338	0.02
Error III	56	0.01563

Figure 3. Behavior of riboflavin in yogurts with and without addition of the dye lutein, stored in the presence and absence of light. The data presented are the means ($\pm$ SD) of duplicate trials.

Formation of Riboflavin Degradation Products

Figure 5 shows that a reduction in fluorescence at a wavelength close to 525 nm occurred during the period of refrigerated storage in the presence of light, indicating degradation of the RBF. In contrast, an increase in fluorescence in the region between 414 and 490 nm occurred during storage, which could indicate the formation of lumichrome, one of the fluorescent compounds resulting from the degradation of RBF, together with lumiflavin (Fox and Thayer, 1998). Wold et al. (2002, 2006) and Andersen et al. (2005) also found the same profile of reduction in fluorescence of RBF during the evaluation of photooxidation in dairy products.

Sensory Acceptance and Buying Intention

The yogurt samples with the addition of different amounts of the dye lutein showed good sensory acceptance, with mean scores varying between 6 (liked

Table 3. Mean scores attributed by the panelists to the yogurts with the addition of different concentrations of lutein, for the attributes of appearance, aroma, flavor, and overall impression

Attribute	Lutein content (mg/120 g)		
	0.5	1.5	2.5
Appearance	6.55 ^a	6.43 ^a	5.34 ^b
Aroma	6.25 ^a	6.59 ^a	7.2 ^a
Flavor	6.44 ^a	6.42 ^a	6.34 ^a
Overall impression	6.43 ^a	6.43 ^a	6.01 ^b

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

Figure 4. Riboflavin contents in yogurts with and without addition of the dye lutein during 35 d of storage. The data presented are the means ($\pm$ SD) of duplicate trials.

slightly) and 7 (liked moderately) for the attributes of appearance, aroma, flavor, and overall impression. The samples showed no differences for the attributes of aroma and flavor ($P > 0.05$). However, the sample with the addition of 2.5 mg of lutein showed a lower mean ($P < 0.05$) for the attribute of appearance, probably due to the more-intense yellow color in relation to the other samples. This could also be the reason for the difference presented by the same sample for the attribute of overall impression (Table 3). In general, all samples presented an elevated buying intention, with approxi-

Figure 5. Fluorescence spectra of the yogurts without addition of the dye lutein during 35 d of exposure to light.

Figure 6. Histogram of the frequency of buying intention for the yogurts with the addition of different concentrations of lutein, as attributed by the panelists. Score 1 = would certainly buy; score 5 = would certainly not buy.

mately 50% of the panelists affirming that they would certainly or probably buy yogurts with the addition of the dye lutein (Figure 6).

CONCLUSIONS

The addition of lutein conferred oxidative stability on the yogurts. Even when yogurts were exposed to light, no changes were observed in the RBF levels or production of degradation compounds during refrigerated storage. The lutein contents remained practically unaltered during the time exposed to light, signifying that the lutein added to the yogurt would be present in the product throughout the entire shelf life. In addition, the good sensory acceptance and buying intention of the yogurt with added lutein indicated the feasibility of producing the yogurt developed in the present study on a commercial scale.

ACKNOWLEDGMENTS

The authors are grateful to São Paulo Research Foundation (FAPESP) grant no. 05/59552-6 and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their financial aid.

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