Complexes of lutein with bovine and caprine caseins and their impact on lutein chemical stability in emulsion systems: Effect of arabinogalactan

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ABSTRACT

Lutein is an important xanthophyll carotenoid with many benefits to human health. Factors affecting the application of lutein as a functional ingredient in low-fat dairy-like beverages (pH 6.0–7.0) are not well understood. The interactions of bovine and caprine caseins with hydrophobic lutein were studied using UV/visible spectroscopy as well as fluorescence. Our studies confirmed that the aqueous solubility of lutein is improved after binding with bovine and caprine caseins. The rates of lutein solubilization by the binding to bovine and caprine caseins were as follows: caprine α_{S1} -II-casein 34%, caprine α_{S1} -I-casein 10%, and bovine casein 7% at 100 μ M lutein. Fluorescence of the protein was quenched on binding supporting complex formation. The fluorescence experiments showed that the binding involves tryptophan residues and some nonspecific interactions. Scatchard plots of lutein binding to the caseins demonstrated competitive binding between the caseins and their sites of interaction with lutein. Competition experiments suggest that caprine α_{S1} -II casein will bind a larger number of lutein molecules with higher affinity than other caseins. The chemical stability of lutein was largely dependent on casein type and significant increases occurred in the chemical stability of lutein with the following pattern: caprine α_{S1} -II-casein > caprine α_{S1} -I-casein > bovine casein. Addition of arabinogalactan to lutein-enriched emulsions increases the chemical stability of lutein-casein complexes during storage under accelerated photo-oxidation conditions at 25°C. Therefore, caprine α_{S1} -II-casein alone and in combination with arabinogalactan can have important applications in the beverage industry as carrier of this xanthophyll carotenoid (lutein).

Key words: casein, bovine, caprine, lutein, arabinogalactan

INTRODUCTION

Carotenoids are primarily symmetrical, C-40, polyisoprenoid structures with an extensive conjugated double-bond system. The C-40 carotenoids can be divided into carotenes, which are hydrocarbons (e.g., β -carotene), and their oxygenated derivatives, the xanthophylls (e.g., lutein, astaxanthin, zeaxanthin; Sajilata et al., 2008). Lutein, which is mainly extracted from Marigold flowers (*Tagetes erecta*), has recently come into the limelight because of animal and epidemiological studies that support its eye health benefits (Sasaki et al., 2012). The eye is susceptible to photosensitized damage and adequate intake of lutein can provide protection against photo-injury (Krinsky et al., 2003). The role of lutein is filtration of blue light and functions as an antioxidant (Eisenhauer et al., 2017). As the lutein levels increase in the macula of the eye, a significant decrease will occur in the amount of harmful light rays that reach the retinal cells that produce vision. Therefore, lutein reduces the risk of macular degeneration, an age-related disease of the retina that is a common cause of loss of vision in the elderly. Lutein acts as antioxidant by scavenging free radicals or quenching singlet oxygen, thereby decreasing oxidative stress in the retina (Eisenhauer et al., 2017).

Since humans cannot synthesize carotenoids, it is essential that they eat green leafy vegetables such as kale and spinach as part of their daily diet (Eisenhauer et al., 2017). To achieve a lifetime of excellent vision, a dosage of 10 mg of lutein per day is required (Frede et al., 2014). Although green leafy foods are good sources of the carotenoid lutein, enzymatic degradation of this carotenoid occurs immediately when the leaves are cut. Another source of lutein, and one that is the most bioavailable of all, is the egg (Eisenhauer et al., 2017). However, the segment of the population that has been recommended to control dietary cholesterol intake avoids consumption of eggs. Generally, older consumers are more interested in functional foods than the younger generation to improve health and extend life expectancy. Most functional foods that have been developed are beverages, but several challenges in formulating lutein-rich beverages still exist.

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Stabilizing beverages poses a unique challenge to formulators because of their very dilute nature. Formulators have to find a way of stabilizing sensitive ingredients such as colors, flavors, and micronutrients, many of which may not even be soluble in water, in microgram levels in liters of liquid. A liquid dietary food rich in lutein is difficult to design due to its low oral bioavailability. The poor oral bioavailability of lutein has been a challenge and was ascribed to its low water solubility and chemical stability (Mitri et al., 2011). Emulsion delivery systems have enabled the incorporation of lutein at a high dose with minimal formulation issues (Davidov-Pardo et al., 2016). The use of bovine casein assisted in the emulsion delivery process (Davidov-Pardo et al., 2016). However, caprine casein, due to its different composition (i.e., lower α_{S1} -CN and higher β -CN), has been shown to protect algae oil with added carotenoids against oxidation at oil-in-water interfaces (Mora-Gutierrez et al., 2010). The β -CN fraction of caprine whole casein absorbed to the oil-water interface of emulsions formed a dense interfacial layer surrounding oil droplets, thereby participating in the protective effect against lipid oxidation (Mora-Gutierrez et al., 2010). In the dispersion systems of carotenoids, which are lipophilic molecules with near zero inherent aqueous solubility, there is little understanding about the interactions between caseins from different commercially important ruminant species and the lipid-water interfaces.

A recent study suggests that the amount of exopolysaccharide (EPS), which has beneficial effects on human health produced by cocultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, is positively influenced by the presence of caprine α_{S1} -I-CN peptides and caprine α_{S1} -II-CN peptides in the culture medium (Mora-Gutierrez, 2016). This report of increased production of EPS by dairy strains of lactic acid bacteria grown in lactobacilli de Man, Rogosa, and Sharpe broth supplemented with caprine α_{S1} -I-CN peptides and caprine α_{S1} -II-CN peptides made these peptides and their parent proteins appealing as possible food ingredients with a potential prebiotic character. Bioactive peptides can be released from the parent protein during gastrointestinal digestion and may interact with *Bifidobacterium longum*, which is a species of bacteria commonly found in the human intestine. Levels of *Bifidobacterium* decline with age, therefore, a food ingredient that on ingestion has the ability to increase levels of bifidobacteria (Fiedorowicz et al., 2016) and production of EPS (Salazar et al., 2009) is a potential food ingredient in the prevention of digestive problems such as constipation and abdominal discomfort.

In this work, the interactions between lutein and the caseins isolated from bovine and caprine milks were

studied by turbidity measurements and fluorescence spectroscopy. Furthermore, the effects of lutein-casein complexes on lutein's chemical stability in lutein-enriched emulsions during storage under conditions for accelerated photo-oxidation were also compared. Moreover, due to the importance of enhancing lutein water solubility and chemical stability in low-fat dairy-like beverages, we used a water-soluble polysaccharide (i.e., arabinogalactan). Arabinogalactan, a highly branched polysaccharide polymer composed of galactose and arabinose in a 6:1 ratio, has been shown to enhance the solubility and photo-stability of lutein in aqueous solutions (Polyakov and Kispert, 2015). Additionally, the effects of caprine α_{S1} -I-CN and caprine α_{S1} -II-CN on the chemical stability of lutein in lutein-enriched emulsions were also examined.

MATERIALS AND METHODS

Materials

A commercial preparation of lutein consisting of 20% (wt/wt) lutein dissolved in corn oil was a gift from Hoffman La Roche (Pleasanton, CA). Mazola corn oil was purchased from a local supermarket. A lutein standard for chromatography analysis was purchased from Extrasynthèse SA (Genay, France). Arabinogalactan, L-tryptophan, ethanol, thimerosal, phenylmethanesulfonyl fluoride, and monobasic potassium phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). Deionized water, prepared by passing distilled water over a mixed bed of cation-anion exchanger, was used throughout this study.

Preparation of Bovine and Caprine Caseins

Caseins were prepared from the milk of a Jersey cow and French-Alpine goats. The samples of caprine milk were collected from individual French-Alpine animals that were raised at the International Goat Research Center in Prairie View A&M University, Texas. The caprine milk caseins were selected based on yielding high levels of α_{S1} -CN as determined by reversed-phase HPLC (Mora-Gutierrez et al., 1991). The equipment for HPLC consisted of a Waters 600 multi-solvent delivery system, a 481 variable wavelength LC spectrophotometer and a 740 data module (Waters Corporation, Milford, MA).

Caseins were isolated from 2 L of fresh, uncooled milk to which phenylmethanesulfonyl fluoride (0.1 g/L) was added immediately to retard proteolysis. The milk was centrifuged at $4,000 \times g$ for 10 min at room tem-

perature to remove the cream fraction using a Beckman Model J2-21 (Beckman Instruments Inc., Palo Alto, CA) centrifuge. Skim milk (500 mL) was diluted with an equal volume of distilled water and warmed to 37°C. Casein was precipitated by gradual addition of 1 *N* HCl to pH 4.5. The precipitate was homogenized with a handheld homogenizer (Biospec Products Inc., Bartlesville, OK) at low speed and dissolved by addition of 1 *M* KOH to yield a solution of pH 7.0. The casein was precipitated again, washed, and then re-suspended. The casein was subsequently cooled to 4°C and centrifuged at $100,000 \times g$ for 30 min to remove residual fat using a Beckman Optima XL-A (Beckman Instruments Inc., Palo Alto, CA) analytical ultracentrifuge. The integrity of the samples was confirmed by SDS-PAGE and densitometry was used to assess the relative concentration of casein components (Basch et al., 1989). The SDS-PAGE of skim milk proteins was carried out on a PhastSystem (Pharmacia, Piscataway, NJ). Densitometry of the gels was carried out on a Bio-Rad Model 222 gel scanner (Bio-Rad Laboratories, Hercules, CA). Data were analyzed using software programs supplied by the manufacturer.

Sample Preparation for Turbidity Measurements

A 2 *mM* stock solution of lutein in ethanol was prepared and stored at 4°C. To prepare the lutein samples (control) in the concentration range of 5 to 100 μ M lutein, stock 2 *mM* lutein solution was added dropwise to 5 *mM* phosphate buffer (pH 7.0) under constant stirring. To prepare the lutein-casein samples, stock 2 *mM* lutein solution was added dropwise to the casein solutions in 5 *mM* phosphate buffer (pH 7.0) under magnetic stirring to get different concentration of lutein (5, 10, 20, 40, 60, 80, and 100 μ M), whereas casein remained constant at 0.5 mg/mL. All samples were mixed by magnetic stirring and then incubated at $25 \pm 1^\circ\text{C}$ for 1 h before analysis.

Turbidity Measurements

Turbidity was determined from the transmission at 500 nm using a Beckman model DU-530 UV/visible spectrophotometer (Beckman Instruments Inc., Fullerton, CA) and expressed as 100% transmission. All experiments were performed in triplicate at $25 \pm 1^\circ\text{C}$.

Fluorescence Studies

The fluorescence studies were performed on a Shimadzu spectrofluorophotometer RF-5000 (Shimadzu Corporation, Columbia, MD) equipped with a calcula-

tor and plotter. The AA L-tryptophan and the bovine casein or caprine casein were excited at 280 nm and their emission spectra were recorded between 300 and 400 nm. Emission spectra were recorded in the concentration range mentioned in the legend of Figure 2.

Fluorescence Quench Titration and Determination of Binding of Lutein to Caseins

The fluorescence titration and calculations of the association constant of lutein with bovine and caprine caseins were performed exactly as described by Levine (1977). The titration consisted of adding aliquots of lutein to the L-tryptophan/casein solutions and measuring the decrease in tryptophan fluorescence after each addition. In almost all cases, the region of interest was the lutein/(tryptophan/casein) ratio of 0 to 5.0. At least 40 points were obtained in the region of interest. The fluorescence quench curves were plotted with the molar ratio of lutein to L-tryptophan/casein on the abscissa and the fluorescence on the ordinate. Fractional quench (*Q*) was calculated from these curves by this equation $\Delta Q = (F_0 - F)/m$, where *F* is the fluorescence at the molar ratio (lutein:tryptophan/protein) of 1, *F*₀ is the fluorescence at zero ratio, and *m* is the maximal quench of the fluorescence. The fluorescence quench curves were used to generate Scatchard plots and analysis of the Scatchard plots was performed, as described by Steiner et al. (1966), to determine the association constant and the number of moles of lutein bound per mole of L-tryptophan or casein.

Emulsion Preparation

A low-fat dairy-like beverage (Davidov-Pardo et al., 2016) was prepared as follows: an organic phase was prepared by diluting 2.5% (wt/wt) of the commercial lutein in corn oil. An aqueous phase was prepared by dispersing 1% (wt/wt) of lyophilized bovine casein or caprine casein into the aqueous buffer solution (5 *mM* phosphate, pH 7.0). A coarse emulsion of oil-in-water was prepared by mixing the organic phase (10%, wt/wt) and the aqueous phase (90%, wt/wt) using a handheld homogenizer (Biospec Products Inc., Bartlesville, OK) at low speed. In emulsions with added arabinogalactan, 2.5% (wt/wt) arabinogalactan was added to the aqueous phase before emulsification. The coarse emulsion was then homogenized 5 times at 82.74 MPa (12,000 psi) through a high-pressure TC5 homogenizer (Stansted Fluid Power, Harlow, UK). The fine emulsion produced was then diluted (1:1, vol/vol) with buffer solution containing an antimicrobial agent [5 *mM* phosphate buffer, pH 7.0, 1 *mM* (wt/vol) thimerosal]. The final diluted emulsions that were used for the stability

studies contained 5% (wt/wt) oil phase and 250 mg/L of lutein.

Conditions for Accelerated Photo-Oxidation

The lutein-enriched emulsions prepared with bovine and caprine caseins were exposed to lighted conditions (fluorescent, daylight type; approximated 2,550 Lx) at 25°C for 96 h (Faraji and Lindsay, 2005). Samples (100 g) were layered in aluminum pans, covered with transparent polymer wrap, and placed under the lighting.

Physical Characterization of the Emulsion

The particle size of the oil droplets in the lutein-enriched emulsions was measured at 1, 48, and 96 h after homogenization with a SALD-2101 laser diffraction particle analyzer (Shimadzu Corporation, Columbia, MD). The charge of the oil droplets in the lutein-enriched emulsion (zeta potential, ζ ; mV) was measured with a Zetasizer nano ZS (Malvern Instruments, Worcestershire, UK). Samples were diluted 100 times in 5 mM phosphate buffer at pH 7.0. All measurements were carried out in triplicate at $25 \pm 1^\circ\text{C}$.

Chemical Stability of Lutein

The chemical stability of lutein was assessed by measuring the concentration of lutein in the lutein-enriched emulsions during storage under accelerated photo-oxidation at 25°C for 96 h. The concentration of lutein was determined from absorbance measurements at 460 nm using a Beckman UV/visible model DU-530 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). To prepare the samples for the spectrophotometric measurements, the lutein-enriched emulsions were diluted 100 times in DMSO (50 μL of emulsion was diluted into 4.95 mL of DMSO). The DMSO was used because it dissolves lutein, oil, and protein to form transparent solutions suitable for UV/visible analysis. The emulsion without lutein was used as a blank. A calibration curve was constructed by dissolving lutein standards in DMSO within a range from 0.5 to 5 mg/L.

Statistical Analysis

All experiments were performed in triplicate and the data are expressed as the means and standard error. Turbidity data were analyzed by 2-way ANOVA with Dunnett's post hoc test using the PROC General Linear Model procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC) at a significance level of $\alpha = 0.05\%$. To evaluate the chemical stability of lutein-enriched emulsions prepared with bovine and caprine caseins and the

polysaccharide arabinogalactan under different storage times, data were analyzed as a factorial arrangement, 3 (casein type) $\times$ 2 (with or without arabinogalactan) $\times$ 5 (stored time), using PROC General Linear Model. Casein type (bovine, caprine $\alpha_{\text{S1-I}}$, and caprine $\alpha_{\text{S1-II}}$), additive (with or without arabinogalactan), and storage time (1, 24, 48, 72, and 96 h) were the main effects of the data. Analysis of variance and Tukey multiple comparison test were used to determine statistical differences ($P < 0.05$) among the main effects and their interactions.

RESULTS AND DISCUSSION

Characterization of Bovine and Caprine Caseins

The bovine and caprine caseins used in this study had different composition in terms of $\alpha_{\text{S1-CN}}$, $\alpha_{\text{S2-CN}}$, $\beta\text{-CN}$, and $\kappa\text{-CN}$ (Table 1). The major source of variation in the caprine caseins is the α_{S1} -fraction, followed by that of the α_{S2} fraction (Ambrosoli et al., 1988). The $\beta\text{-CN}$ content of the $\alpha_{\text{S1-I-CN}}$ and $\alpha_{\text{S1-II-CN}}$ isolated from caprine milks was markedly higher than that of bovine casein (Table 1). $\beta\text{-Casein}$ is the most hydrophobic fraction of whole casein (Kumosinski et al., 1993), and this property makes $\beta\text{-CN}$ -rich whole caseins such as those of caprine $\alpha_{\text{S1-I-CN}}$ and $\alpha_{\text{S1-II-CN}}$ (Table 1) suitable candidates as encapsulating agents for lipophilic molecules such as n-3 fatty acids (Mora-Gutierrez et al., 2014).

Turbidity of Lutein and Lutein-Casein Complex

The complex formed between lutein and bovine casein may have protected the carotenoid molecule from light and temperature degradation (Yi et al., 2016). Besides, this type of encapsulation seems to have increased lutein apparent solubility in water significantly (Yi et al., 2016). To test this hypothesis, turbidity measurements were performed to evaluate the potentials of caprine $\alpha_{\text{S1-I-CN}}$ and $\alpha_{\text{S1-II-CN}}$ to form lutein-casein complexes capable of increasing the apparent solubility

Table 1. Casein distribution of caprine caseins compared with a typical bovine casein¹

Sample	Casein fraction (%)				
	α_{S2}	α_{S1}^*	α_{S1}^*	β	κ
Caprine casein high in $\alpha_{\text{S1-I}}$	9.2	4.0	21.1	51.6	13.8
Caprine casein high in $\alpha_{\text{S1-II}}$	5.3	—	25.6	60.6	9.6
Bovine casein	12.1	—	39.5	37.2	11.2

¹Densitometry.

*Altered SDS-PAGE mobility may represent a truncated version of $\alpha_{\text{S1-CN}}$.

of lutein in the aqueous phase. The turbidity data of lutein alone and lutein-casein complexes are presented in Figure 1. Due to the poor solubility of lutein in water, lutein aggregation is expected to occur in the aqueous phase. This lutein aggregation phenomenon leads to relatively higher turbidity measurements in the control. Compared with the control (lutein alone), the turbidity of lutein-casein complexes for all the treatments were significantly lower ($P < 0.05$) at the same concentrations of lutein from 5 to 100 μM . The turbidity of lutein-casein complexes made with the bovine and caprine α_{S1} -I-CN were significantly higher ($P > 0.05$) than those made with caprine α_{S1} -II-CN at the same concentrations (Figure 1). These results indicated that more lutein was bound with caprine α_{S1} -II-CN than any other caseins.

Fluorescence Quench Titration and Determination of Binding of Lutein to Bovine and Caprine Caseins

The encapsulation of liposoluble carotenoids (lutein) in milk proteins (i.e., the caseins) may be through the hydrophobic interactions, van der Waals attraction, or hydrogen bonds. Fluorescence spectroscopy is a

technique widely used to study interactions between proteins and small molecules such as resveratrol (Liang et al., 2008; Acharya et al., 2013), folic acid (Liang and Subirade, 2010), and vitamin E (Liang et al., 2011). Tryptophan is one of the major chromophores among the naturally occurring AA and is responsible for the absorbance shown by proteins at 280 nm. When excited at its absorption maximum of 280 nm, tryptophan was found to exhibit a fluorescence emission spectrum with a peak at 375 nm. Milk proteins (caseins and whey proteins) exhibit fluorescence when exposed to this technique. The intrinsic fluorescence of casein is almost solely contributed by tryptophan because the fluorescence of phenylalanine has a very low quantum yield and the fluorescence of tyrosine is almost quenched if it is ionized or located near an amino group, a carboxyl group, or a tryptophan (Lakowicz and Weber, 1973; Lakowicz, 2000). In bovine milk, the major whey protein β -LG contains 2 tryptophan residues, and the minor whey protein α -LA has 4 tryptophan residues (Kelkar et al., 2010), whereas α -CN has 2 tryptophan residues, and β -CN and κ -CN have 1 tryptophan residue each (Kumosinski et al., 1993). However, caprine milk proteins, in particular the caseins, have many sig-

Figure 1. Turbidity of lutein at different concentrations (5, 10, 20, 40, 60, 80, and 100 μM) alone (control) and in the presence of bovine and caprine caseins (0.5 mg/mL) in 5 mM phosphate buffer solutions at pH 7.0. Means within each lutein concentration with different lowercase letters (a–c) are significantly different ($P < 0.05$).

nificant differences in their AA compositions compared with the milk of other mammalian species, especially in relative proportions of the various casein fractions (α_{S1} -CN, α_{S2} -CN, β -CN, and κ -CN) and in their genetic polymorphisms (Jaubert and Martin, 1992; Galliano et al., 2004; Selvaggi et al., 2014). Therefore, AA sequences located in homologous positions in homologous caseins may be different in caprine milk. In this study, the existence of such differences in caprine milk caseins from bovine milk caseins is suggested but not proven.

The changes in the fluorescence emission spectrum of caprine α_{S1} -II-CN in the presence of different concentrations of lutein made with phosphate buffer solutions at pH 7.0 are shown in Figure 2. When a solution of caprine α_{S1} -II-CN was excited at its absorption maximum of 280 nm, a fluorescence emission spectrum with a maxima at 345 nm was observed. Addition of increasing concentrations of lutein to caprine α_{S1} -II-CN resulted in a progressive quenching of the fluorescence emission. The results are suggestive of binding of lutein to caprine α_{S1} -II-CN. The fractional quench was calculated as described in the Materials and Methods. These data were used to construct Scatchard plots for determining the binding of lutein to tryptophan. The Scatchard plot for the binding of lutein to L-tryptophan is shown in Figure 3a. An analysis of this plot revealed that 2.19 mol of lutein are able to bind to 1 mol of L-tryptophan and the association constant of such binding is $4.7 \times 10^7 M^{-1}$ (Table 2). Fluorescence quenching titration of the bovine and caprine caseins was performed by adding increasing concentrations of lutein in exactly the same way as was done with L-tryptophan and the subsequent Scatchard plots are shown in Figures 3b-d. The analysis of Scatchard plots as tabulated in Table 2 revealed that 5.58 mol of lutein binds to 1 mol of caprine α_{S1} -II, whereas 2.89 mol of lutein binds to 1 mol of bovine casein, and 2.82 mol of lutein binds to 1 mol of caprine α_{S1} -I-CN.

The analysis of the association constants of lutein for the bovine and caprine caseins (Table 2) also showed a variation, which appears not to be related to their tryptophan content. The interesting result is that the association constants for tryptophan-containing caprine α_{S1} -I-CN and caprine α_{S1} -II-CN were quite similar ($5.7 \times 10^7 M^{-1}$ and $5.0 \times 10^7 M^{-1}$, respectively). However, the association constant for tryptophan-containing bovine casein was quite low ($1.4 \times 10^7 M^{-1}$) compared with caprine caseins. Reasons for this could be based in structural differences as evidenced by circular dichroism spectroscopy (data not shown). The solubility-related, lutein-binding properties of the potassium forms of the caprine casein submicelles, in the absence of Ca^{2+} , are in part determined by the hydrophobic interactions of

the dominant protein, β -CN. The unique assembly of casein submicelles provides a sufficient nonpolar environment for the bound lutein molecules. Casein submicelles consist of a mixture of proteins, and they are natively unfolded proteins, so it is difficult to predict the exact position of binding of lutein molecules. Taking all this information into account, these results may suggest a complex mode of binding of lutein to bovine casein and the 2 caprine caseins, which may involve, in addition to tryptophan residues, some nonspecific interactions. Moreover, the association constants of lutein-casein complexes of caprine α_{S1} -I-CN and α_{S1} -II-CN were greater than that of lutein-casein complexes of bovine casein, which indicate that caprine α_{S1} -I-CN and α_{S1} -II-CN have higher affinities than bovine ca-

Figure 2. Fluorescence emission spectra of caprine α_{S1} -II-CN in the presence of lutein. Caprine α_{S1} -II-CN (in 5 mM phosphate buffer, pH 7.0) was excited at 280 nm and the emission spectra were recorded between 300 and 400 nm. Traces, from top to bottom, are caprine α_{S1} -II-CN alone (1 μM), α_{S1} -II-CN:lutein molar ratio 1:0.1, 1:0.2, 1:0.4, 1:0.6, 1:0.8, 1:1, 1:1.2, 1:1.5, 1:1.7, 1:2, 1:2.5, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:10, 1:11, 1:12, 1:13, 1:14, and 1:15, respectively.

Figure 3. Scatchard plots for binding of lutein to the AA L-tryptophan and the bovine and caprine caseins. Fluorescence titration of bovine and caprine caseins was performed by adding increasing amounts of lutein to L-tryptophan/casein solutions. These data were used to generate Scatchard plots for binding of lutein to (a) L-tryptophan, (b) caprine α_{S1} -II-CN, (c) bovine casein, and (d) caprine α_{S1} -I-CN. Q is the fractional quench of fluorescence, and A is the molar ratio of total ligand to total protein or total AA.

sein with hydrophobic bioactive molecules (Zhang and Zhong, 2012).

The association constants of lutein for bovine casein and the 2 caprine caseins are of the order of 10^7 L/mol (Table 2). Many drugs (synthetic organic anions) have association constants of approximately 10^4 M^{-1} for physiological proteins, whereas the same antibodies for their antigens are of the order of 10^9 M^{-1} (Meyer and Guttman, 1968). This would suggest that binding of lutein to proteins may not be stereospecific, as in the case of antibodies, but it is still stronger than that observed for nonspecific, electrostatic interactions. This, in part, is accounted by binding of lutein to tryptophan. The results of Table 2 are not very suggestive of tryptophan-specific binding of lutein to milk proteins (the caseins). Instead it appears that lutein, in addition to tryptophan, may bind to other AA and, also, non-specifically to proteins. As mentioned above, structural

changes may make the caprine α_{S1} -II-CN binding site different than those of caprine α_{S1} -I-CN and bovine casein.

Table 2. Calculated association constants (K_a) for the lutein-casein complexes of bovine and caprine caseins and the number of moles of bound lutein per mole of casein molecules¹

AA/casein	Number of Trp residues ²	Association constant (K_a ; L·mol ⁻¹)	Moles of lutein/ moles of casein
L-Trp	1	4.7×10^7	2.19
Bovine	4	1.4×10^7	2.89
Caprine α_{S1} -I	NA	5.7×10^7	2.82
Caprine α_{S1} -II	NA	5.0×10^7	5.58

¹All the values were calculated from the Scatchard plots given in Figure 3.

²The AA sequences of caprine α_{S1} -I and α_{S1} -II-CN are not available (NA).

Physical Stability of Lutein Emulsions Prepared with Bovine and Caprine Caseins

The physical stability of lutein-enriched emulsions prepared with bovine and caprine caseins are needed before comparisons of chemical stability of lutein could be made. The mean particle diameters of oil droplets in the lutein-enriched emulsions stabilized by bovine and caprine caseins ranged from 205.94 to 206.56 nm at pH 7.0 (Table 3). The casein type did not affect ($P > 0.05$) particle size diameters of oil droplets in lutein-enriched emulsions. The mean particle size of oil droplets in lutein-enriched emulsions was significantly lower ($P < 0.05$) with added arabinogalactan. Moreover, we observed no significant differences ($P > 0.05$) in the particle sizes of oil droplets in lutein-enriched emulsions during 1, 48, and 96 h of storage under accelerated photo-oxidation conditions at 25°C. The zeta potentials of the caseinate-coated oil droplets in the lutein-enriched emulsions were negative at pH 7.0 (Table 3). It should be noted here that emulsions containing protein-coated lipid droplets are mainly stabilized by electrostatic repulsion (Dickinson, 2010). The zeta potentials of the oil droplets in the lutein-enriched emulsions prepared with caprine caseins were not different ($P > 0.05$), showing -35.14 mV for caprine α_{S1} -I-CN and -34.80 mV for caprine α_{S1} -II-CN, respectively. The lower zeta potentials of the oil droplets in the lutein-enriched emulsions stabilized by the caprine caseins compared with the lutein-enriched emulsions stabilized by bovine casein can imply a decreased net negative charge (Mora-Gutierrez et al., 1993). Differences in the AA side chains of κ -CN, α_S -CN, and β -CN fractions in caprine whole caseins, where the less charged β quantitatively dominates,

could lead to less repulsive charge-charge interaction and this phenomenon can possibly explain their lower zeta potentials (Table 3). The zeta potentials of the oil droplets in lutein-enriched emulsions prepared with bovine and caprine caseins were not statistically different with the addition of arabinogalactan but still high enough to confer electrostatic repulsive force between the oil droplets (Table 3). Likewise, the zeta potentials of the oil droplets in the lutein-enriched emulsions prepared with the 2 caprine caseins were not affected by the addition of arabinogalactan (Table 3). No significant difference ($P > 0.05$) was observed in the zeta potentials of the oil droplets in lutein-enriched emulsions prepared with bovine and caprine caseins when stored under accelerated photo-oxidation conditions for 1, 48, and 96 h at 25°C. The 2-way and 3-way interactions were not significant ($P > 0.05$) for particle size diameter and zeta potential of the oil droplets in the lutein-enriched emulsion stabilized by the bovine and caprine caseins. On the basis of these findings, we can conclude that the lutein-enriched emulsions prepared with bovine and caprine caseins exhibited relatively good physical stability (McClements, 2005).

Chemical Stability of Lutein Emulsions Prepared with Bovine and Caprine Caseins

The degradation of lutein in lutein-enriched emulsions may be complex, but some possible major causes are related to the increased specific surface area in the emulsions and the possible formation of free radicals during high-pressure homogenization (Boon et al., 2010). In this study, all the main effects (Table 4), and casein type $\times$ storage time and additive $\times$ storage time interactions (Table 5) were significantly different ($P < 0.05$) in lutein concentration. The concentrations of lutein were higher ($P < 0.05$) in the lutein-enriched emulsions prepared with caprine caseins compared with the bovine casein (Table 4). Moreover, lutein-enriched emulsions exhibited higher chemical stability when arabinogalactan was added (Table 4). Overall, higher concentrations ($P < 0.05$) of lutein were observed in the lutein-enriched emulsions prepared with caprine caseins compared with bovine casein during storage under accelerated photo-oxidation conditions at 25°C (Table 5). The data indicated that the degradation of lutein entrapped in the lutein-enriched emulsions prepared with caprine caseins was slower than in those prepared with bovine casein. The concentrations of lutein were higher ($P < 0.05$) with the addition of arabinogalactan in the lutein-enriched emulsions at 72 and 96 h of storage under accelerated photo-oxidation conditions at 25°C (Table 5). Bovine and caprine caseins have the ability to protect liposoluble bioactives

Table 3. Particle size and zeta potential of oil droplets in lutein-enriched emulsions stabilized by bovine and caprine caseins with and without added arabinogalactan in phosphate buffer at pH 7.0 during storage for 1, 48, and 96 h at 25°C

Treatment	Particle size (nm) ¹	Zeta potential (mV) ¹
Casein type		
Bovine	205.94 $\pm$ 2.90	-37.50 $\pm$ 1.86 ^a
Caprine α_{S1} -I	206.44 $\pm$ 2.66	-35.14 $\pm$ 1.77 ^b
Caprine α_{S1} -II	206.56 $\pm$ 2.66	-34.80 $\pm$ 2.46 ^b
Additive		
With arabinogalactan	205.19 $\pm$ 2.88 ^b	-35.41 $\pm$ 2.10
Without arabinogalactan	207.44 $\pm$ 2.04 ^a	-36.22 $\pm$ 2.55
Storage (h)		
1	205.11 $\pm$ 2.30	-36.67 $\pm$ 1.66
48	206.83 $\pm$ 2.48	-35.74 $\pm$ 2.47
96	207.00 $\pm$ 3.07	-35.03 $\pm$ 2.64

^{a,b}Means in the same column within each treatment with a different superscript are significantly different ($P < 0.05$).

¹Mean value $\pm$ SE.

Table 4. Concentration of lutein in lutein-enriched emulsions stabilized by bovine and caprine caseins with and without added arabinogalactan in phosphate buffer at 25°C and pH 7.0 during storage time

Treatment	Concentration of lutein (mg/L) ¹
Casein type	
Bovine	230.97 ± 10.09 ^c
Caprine α _{S1} -I	238.40 ± 8.59 ^b
Caprine α _{S1} -II	241.73 ± 7.16 ^a
Additive	
With arabinogalactan	239.58 ± 8.18 ^a
Without arabinogalactan	234.49 ± 10.53 ^b
Storage (h)	
1	245.22 ± 3.06 ^a
24	242.61 ± 5.16 ^{ab}
48	239.33 ± 6.64 ^b
72	233.11 ± 7.40 ^c
96	224.89 ± 8.80 ^d

^{a-d}Means in the same column within each treatment with a different superscript are significantly different ($P < 0.05$).

¹Mean value ± SE.

including the carotenoids from oxidation effectively after binding with them (Mora-Gutierrez et al., 2010). The unadsorbed bovine and caprine caseins dissolved in the aqueous phase of the lutein-enriched emulsions may have formed complexes with metal ions (Fe^{3+}), thus preventing their contact with lutein. A reduced reactivity toward metal ions (Fe^{3+}), a lowered amount of reactive oxygen species generated during high-pressure homogenization, and enhanced photo-stability through the formation of lutein complexes with arabinogalactan in the lutein-enriched emulsions stabilized by bovine and caprine caseins may be responsible for enhanced chemical stability. The xanthophyll carotenoids lutein, astaxanthin, and zeaxanthin are sensitive to heat, light, and other oxidative stressors (Martínez-Delgado et al., 2017). Therefore, some loss of lutein in the lutein-enriched emulsions stabilized by bovine and caprine caseins during storage under accelerated photo-oxidation conditions is to be expected (Table 5).

It is important that lutein does not degrade during storage, being available at the time of food consumption. The results of this study clearly indicated that the chemical stability of lutein in lutein-enriched emulsions was improved in the presence of caprine caseins after 48 h of storage under accelerated photo-oxidation conditions at 25°C (Table 5). Added arabinogalactan significantly ($P < 0.05$) enhanced the chemical stability of lutein-casein complexes in lutein-enriched emulsions during storage under accelerated photo-oxidation conditions for 72 and 96 h at 25°C (Table 5).

CONCLUSIONS

The results of this study show that the caseins isolated from caprine milks can contribute to the improvement of lutein's chemical stability in lutein-enriched emulsions from 48 to 96 h of storage under accelerated photo-oxidation conditions at 25°C compared with bovine casein. The improved chemical stability of lutein will be useful in the development of low-fat dairy-like beverages. It is believed that increased binding affinity of caprine caseins, in particular, caprine α_{S1}-II-CN, with lutein leads to improved chemical stability of lutein in lutein-enriched emulsions during storage. These findings stress the importance of choosing the appropriate type of casein for improving the chemical stability of lutein in low-fat dairy-like beverages. The combination of casein with arabinogalactan should also be considered based on the enhanced chemical stability exhibited by lutein in lutein-enriched emulsions during storage under accelerated photo-oxidation conditions at room temperature.

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Table 5. Concentration of lutein as influenced by the interactions of casein type × storage time and additive × storage time

Treatment	Hours ¹				
	1	24	48	72	96
Casein type					
Bovine	242.50 ± 2.88 ^{a,A}	237.83 ± 2.32 ^{ab,B}	232.33 ± 4.72 ^{bc,B}	225.33 ± 4.93 ^{c,B}	216.83 ± 5.85 ^{d,B}
Caprine α _{S1} -I	245.67 ± 2.66 ^{a,A}	244.17 ± 5.00 ^{a,AB}	241.17 ± 3.87 ^{ab,A}	234.83 ± 4.58 ^{b,A}	226.17 ± 7.25 ^{c,A}
Caprine α _{S1} -II	247.50 ± 1.05 ^{a,A}	245.83 ± 4.17 ^{a,A}	244.50 ± 4.23 ^{ab,A}	239.17 ± 4.54 ^{b,A}	231.67 ± 6.44 ^{c,A}
Additive					
With arabinogalactan	246.22 ± 2.49 ^{a,A}	243.67 ± 4.69 ^{a,A}	241.33 ± 6.67 ^{ab,A}	236.67 ± 6.54 ^{b,A}	230.00 ± 8.22 ^{c,A}
Without arabinogalactan	244.22 ± 3.38 ^{a,A}	241.56 ± 5.66 ^{ab,A}	237.33 ± 6.34 ^{b,A}	229.56 ± 6.71 ^{c,B}	219.78 ± 6.18 ^{d,B}

^{a-d}Means in the same row within each treatment with different lowercase superscripts are significantly different ($P < 0.05$).

^{A,B}Means in the same column within each treatment with different uppercase superscripts are significantly different ($P < 0.05$).

¹Mean value ± SE.

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