Lutein-enriched emulsion-based delivery systems: Impact of Maillard conjugation on physicochemical stability and gastrointestinal fate

Cansu Ekin Gumus^a, Gabriel Davidov-Pardo^{a,b,*,#}, David Julian McClements^{a,c,#}

^a Department of Food Science, University of Massachusetts, Amherst, MA 01003, USA

^b Department of Human Nutrition and Food Science, California State Polytechnic University,
Pomona, 3801 West Temple Ave, Pomona, CA 91768, USA

^c Department of Biochemistry, Faculty of Science, King Abdulaziz University, P. O. Box 80203,
Jeddah 21589, Saudi Arabia

These authors contributed equally to this manuscript

Corresponding author: **Gabriel Davidov-Pardo**

Department of Human Nutrition and Food Science – California State Polytechnic University
Pomona, 3801 West Temple Ave, Pomona, CA 91768, USA
e-mail: gavidov@cpp.edu Tel.: +1-909-869-5229. Fax: +1-909-869-5078.

Abstract

The utilization of lutein as a natural colorant or nutraceutical in many foods, supplements, and other commercial products is currently limited because of its low water-solubility and chemical instability. The purpose of this study was to evaluate the effect of Maillard conjugates on the physical and chemical stability of lutein-enriched emulsions exposed to different temperatures and pH values, as well as on their potential gastrointestinal fate. Oil-in-water emulsions were prepared using either casein or casein-dextran conjugates as emulsifiers. Both types of emulsions showed a slight increase in particle aggregation at temperatures exceeding 37 °C, and became more prone to color fading (lutein degradation) as the temperature was increased. Casein-coated oil droplets were highly unstable to flocculation near their isoelectric point (pH 4 to 5) due to the reduction in electrostatic repulsion. However, casein-dextran-coated droplets were stable from pH 3 to 7, which was attributed to strong steric repulsion by the dextran moiety. The casein-coated droplets were unstable to aggregation in the gastric phase of the simulated GIT, whereas the casein-dextrin-coated ones were stable, which was again attributed to increased steric repulsion. Emulsifier type did not strongly influence lutein bioaccessibility. This work shows that Maillard conjugates can improve the physical stability of lutein-enriched emulsions without adversely affecting the bioaccessibility of the bioactive agent.

Keywords: Lutein, storage stability, casein, dextran, Maillard conjugates, in vitro digestion.

1. INTRODUCTION

Lutein is a natural colorant found in a variety of biological materials, such as yellow corn, egg yolk and marigold flowers. Lutein belongs to the xanthophyll class of carotenoids, which are oxygenated carotenes (Sajilata, Singhal, & Kamat, 2008). As with other xanthophylls lutein has an intense yellow color when present at low concentrations but a reddish color when present at high concentrations. Its characteristic color is due to selective absorption of electromagnetic radiation in the visible region by conjugated double bonds in its backbone (Sajilata, et al., 2008). Lutein is known to accumulate in the pigmented region of the human eye, which is called the macula. The accumulation of lutein in the macula has been associated with a decrease in the risk of age-related macular degeneration and cataracts (Abdel-Aal el, Akhtar, Zaheer, & Ali, 2013; Boon, McClements, Weiss, & Decker, 2010; Sajilata, et al., 2008). One of the proposed mechanisms for the protection of the macula by carotenoids is the absorbance of damaging light waves (Krinsky, Landrum, & Bone, 2003). Moreover, they can act as antioxidants by scavenging free radicals or quenching singlet oxygen, which protects cells including the ones in the macula from oxidative stress. Lutein cannot be synthesized by the human body and must therefore be ingested through the diet (Khalil, et al., 2012; Nagao, 2014; Sajilata, et al., 2008). The acceptable daily intake for lutein approved by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) is 0 to 2 mg/kg body weight (JECFA, 2005). Moreover, the effective dose of lutein to provide protection against diseases such as age-related macular degeneration and cataracts has been reported to be about 10 mg/day (Frede, et al., 2014).

Due to the beneficial effects of lutein on human health it can be considered to be a nutraceutical ingredient to create functional foods and beverages. Moreover, its yellow-red color and its hydrophobicity make lutein a natural lipid-soluble colorant that can be used to replace

artificial ones. An important trend in the modern food industry is towards products that are manufactured "without artificial additives" such as preservatives, flavorings, and colorings (Sloan, 2015). In addition, consumers are tending to purchase more functional food products that claim to provide additional health benefits beyond their normal nutritional effects (Sloan, 2015). Lutein is a promising ingredient to fulfill these market trends, indeed it has recently been reported that lutein, which is mainly extracted from Marigold flowers (*Tagetes erecta*), has the fastest growing market among the carotenoids with a market value of around US\$233 million in 2010, projected to grow to US\$309 million by 2018 (Berman, et al., 2014).

Nevertheless, the use of lutein in the food industry presents challenges related to its poor chemical stability, water-solubility, and bioaccessibility characteristics. In common with other carotenoids, lutein is sensitive to heat and acidic environments, which in the presence of oxygen enhance its degradation through autoxidation (Boon, et al., 2010). The degradation of lutein causes a reduction in its bioactivity, as well as a change in its desirable quality attributes due to color fading and formation of rancid off-flavors (Boon, et al., 2010; Sajilata, et al., 2008). The poor oral bioaccessibility of lutein can be attributed to its low water-solubility, high melting point, and poor chemical stability (Frede, et al., 2014; McClements & Li, 2010). As with other lipophilic compounds, lutein has to be solubilized within the mixed micelle phase formed in the small intestine before it can be absorbed by the epithelial cells, packaged into lipoproteins, and transported to the blood stream (Abdel-Aal el, et al., 2013). The efficacy of solubilization in the mixed micelle phase therefore plays a major role in determining the overall bioavailability of lutein (Nagao, 2014).

Oil in water (O/W) nanoemulsions are a promising platform for creating delivery systems to incorporate lipophilic compounds into food products and increase their bioavailability

(McClements, Decker, & Weiss, 2007). O/W nanoemulsions are thermodynamically unstable colloidal systems in which oil is dispersed in water in the form of small spheres ($r < 100$ nm) (McClements & Rao, 2011; McClements & Xiao, 2012). The functional performance of nanoemulsions can be tailored to specific applications by controlling their compositions or structures. A particularly promising approach to improving nanoemulsion performance is to use novel emulsifiers formed by covalently linking proteins and polysaccharides together using the Maillard reaction (Markman & Livney, 2012; Yang, et al., 2015; Zhou, et al., 2012). The protein part helps the emulsifiers rapidly adsorb to oil droplet surfaces, whereas the polysaccharide part helps prevent the oil droplets from aggregating by generating a strong steric repulsive interaction. For example, studies have shown that protein-polysaccharide emulsifiers formed by the Maillard reaction can improve the physical stability of emulsions, and alter their gastrointestinal fate (Lesmes & McClements, 2012; Yang, et al., 2015).

The Maillard reaction is a non-enzymatic reaction that involves the condensation of the carbonyl group of a reducing carbohydrate with a free amino group of a protein (such as a lysine or a arginine residue or an N-terminal amino group). In the initial stages of the reaction an aldimine (Schiff base) is formed. The Schiff base subsequently undergoes an Amadori rearrangement when aldoses are involved or a Heyns rearrangement in the case of ketoses (Ames, 1990; Oliver, Melton, & Stanley, 2006). It is often important to prevent the later stages of the Maillard reaction from occurring when preparing protein-polysaccharide conjugates since they lead to the degradation of the Amadori products and the formation of a wide variety of undesirable reaction products (Van Lancker, Adams, & De Kimpe, 2011).

The aim of this work was to establish the impact of casein-dextran Maillard conjugates on the physicochemical stability and gastrointestinal fate of lutein-enriched nanoemulsions. In

particular, **this study examined** to determine if these conjugates could improve the stability of the nanoemulsions, without adversely affecting the bioaccessibility of lutein. A source of long chain triacylglycerols (corn oil) was used as the lipid phase since this type of lipid has previously been shown to increase the bioaccessibility of carotenoids (Rao, Decker, Xiao, & McClements, 2013; Salvia-Trujillo, Qian, Martin-Belloso, & McClements, 2013).

2. MATERIALS AND METHODS

2.1 Materials

MariLut (20% lutein in corn oil) was kindly donated by PIVEG (San Diego, CA). Mazola corn oil was purchased from a local store. Spray dried sodium caseinate was purchased from the American Casein Company (Burlington, NJ). A lutein standard for **HPLC** analysis was purchased from Extrasynthese (Genay, France). Sodium azide, calcium chloride, sodium phosphate mono- and dibasic, dextran 37 kDa, porcine bile extract, pepsin and lipase were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO), potassium persulfate, ethanol, and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Fisher Scientific (Waltham, MA).

2.2 Maillard conjugates formation

Sodium caseinate (2.00 w/v%) and dextran (3.5 w/v%) were individually solubilized overnight at 5 °C in water. The completely solubilized and hydrated samples were subsequently mixed in a one-to-one ratio, which led to final sodium caseinate and dextran concentrations of 1.00 and 1.75 w/v%, respectively. The mixture was spray-dried using a mini spray drier (Buchi B-290, Switzerland) with an inlet temperature of 150 °C, a feed rate of 7.5 mL/minute, a compressed air pressure of 600 kPa, and an air flow rate of 35 m³/h (Shah, Davidson, & Zhong,

2012). Maillard conjugation reactions were performed by incubating the spray-dried mixture at 76% relative humidity (using a saturated KBr solution in a desiccator) and 60 °C (in an incubator) for 48 hours (Markman, et al., 2012; Pan, Mu, Hu, Yao, & Jiang, 2006). After conjugation, the samples were allowed to cool to room temperature and ground using a mortar and pestle. The samples were subsequently stored in a desiccator prior to use.

2.3 Maillard conjugates characterization

2.3.1 Conjugation efficiency

The conjugation efficiency was determined by measuring the reduction in free amino groups using the OPA assay (Pan et al., 2006). The OPA reagent was prepared according to Pan, et al. (2006). In short, 40 mg OPA (dissolved in 1.0 mL 95% ethanol), 25 mL 0.100 M sodium tetraborate buffer (pH 9.5), 2.5 mL 20% SDS solution, and 0.10 mL 2-mercaptoethanol were mixed together and brought to a final volume of 50 mL. The OPA reagent was prepared freshly before use. After dispersing the conjugates, 0.10 mL of the dispersion was mixed with 2.70 mL of OPA reagent and incubated for 1 minute at room temperature, the absorbance at 340 nm was measured immediately using an UV–visible spectrophotometer Ultrospec 3000 pro (Biochrom Ltd., Cambridge, England). A calibration curve was constructed using L-leucine (0,2-5 mM) as a standard amino group-containing compound (Markman, et al., 2012; Pan, et al., 2006). The conjugation efficiency was defined as follows:

$$\text{Conjugation efficiency (\%)} = \left(1 - \frac{\text{amine groups after conjugation (M)}}{\text{amine groups before conjugation (M)}}\right) \times 100$$

2.3.2 Conjugation yield

The quantification of the non-complexed protein remaining in the system was assessed

based on the methodology described by Markman, et al. (2012). The samples were dissolved in double distilled water at concentrations of 10 mg/mL, and subsequently acidified to pH 4.6 with HCl. The sample suspensions were centrifuged at 1000 g for 10 minutes and then filtrated through P5 filter paper (Fisher Scientific, Pittsburgh, PA). The pH of the supernatant was readjusted to 7.0 with NaOH. The protein content of the suspension (after pH adjustment and before centrifugation) and supernatant was measured using an UV–visible spectrophotometer (Ultrospec 3000 pro, Biochrom Ltd., Cambridge, England) at 278 nm. The amount of protein was subsequently calculated using a calibration curve prepared using sodium caseinate concentrations ranging from 0.2 to 1.0 mg/mL. The conjugation yield was defined as follows:

$$\text{Conjugation yield (\%)} = \left(\frac{\text{protein in supernatant} \left(\frac{\text{mg}}{\text{L}} \right)}{\text{protein in the suspension} \left(\frac{\text{mg}}{\text{L}} \right)} \right) \times 100$$

2.3.3 Antioxidant activity measurement

The antioxidant activity of protein and Maillard conjugate solutions were calculated using an ABTS assay (Re, et al., 1999). For this assay, 7 mM ABTS and 2.45 mM potassium persulfate were allowed to overnight at room temperature in the absence of light to produce the ABTS radical. The ABTS radical solution was then diluted in ethanol to give an absorbance of 0.7 cm⁻¹ at 734 nm. 10 µL aliquots of samples to be analyzed were mixed with 1 mL of the diluted radical solution and absorbances were read at 734 nm after 1 minute. The percent reduction in absorbance at 734 nm and the antioxidant capacities were calculated using a trolox standard curve and the results are reported as Trolox equivalents (TE)

2.4 Emulsion formation

An organic phase was prepared by diluting the lutein in corn oil to a final concentration of 2.5% lutein (w/w). An aqueous phase was prepared by dispersing 1% (w/w) powdered

sodium caseinate or 1% (w/w) **caseinate-dextran complexes** based on the protein weight of the Maillard conjugates into aqueous buffer solution (5 mM phosphate, pH 7.0). A coarse oil-in-water emulsion was prepared by mixing the organic phase (10% w/w) and the aqueous phase (90% w/w) using a high-speed mixer M133/1281-0 (Biospec Products, Inc. Bartlesville, OK, USA) for 2 min at 10,000 rpm. The resulting coarse emulsion was then passed through a high-pressure microfluidizer for three passes at 20,000 psi (Purenano, Microfluidics, Newton, MA). The fine emulsion produced was then diluted (1:1) with buffer solution containing an antimicrobial agent (5 mM phosphate buffer, pH 7.0, 0.04% (w/v) sodium azide). The final emulsion therefore contained 5% (w/w) oil phase and 250 mg/L lutein.

2.5 Stability study

Selected emulsions were stored for 7 days at different temperatures (5, 20, 37, 55 and 70 °C) or at different pH values (pH 3-8). The pH values were adjusted to the desired values using 0.1 and 1.0 N of hydrochloric acid and/or sodium hydroxide solutions. The emulsions with pH values of 3, 4, 5, 6, 7 and 8 were stored at 37 °C to accelerate their destabilization, while samples at pH 7 were stored at 5, 20, 37, 55 and 70 °C.

2.5.1 Chemical stability

The chemical stability of lutein was assessed by measuring the change in color in the emulsions during storage. The color was monitored using a colorimeter (ColorFlex EZ, HunterLab Reston, VA, USA). For the color analysis, 10 mL of emulsion was pipetted onto a plastic petri dish and the readings were performed against a black background.

2.5.2 Physical stability

The physical stability of the emulsions was assessed by measuring changes in particle size and charge after 7 days of storage. The mean droplet diameters, particle size distributions,

and ζ -potentials were measured using a dynamic light scattering/micro-electrophoresis instrument (Zetasizer Nano ZS, Malvern Instruments, Malvern, England) and a laser diffraction particle size analyzer (LS 13 320, Beckman Coulter, Brea, Ca, USA). Samples were diluted in buffer solution (5 mM phosphate) at the proper pH to avoid multiple scattering and measurements were made at 25 °C.

2.6 In vitro digestion model

A dynamic *in vitro* gastrointestinal model was used to study the influence of the emulsion interface on the bioaccessibility of lutein. The gastrointestinal model was based on the work by Salvia-Trujillo, et al. (2013) with some slight modifications. The mouth phase was not included in this work since most liquids do not require an oral phase, mainly due to the very short residence times in the oral cavity (Minekus, et al., 2014).

2.6.1 Gastric phase

Simulated gastric fluid **stock solution (SGFSS)** was prepared by dissolving 2 g of NaCl and 7 mL of HCl (37%) in 1 L of double distilled water. **Simulated gastric fluid work solution (SGFWS)** was prepared by mixing 20 mL of SGFSS and 0.064 g of pepsin (amounts are per sample) 45 minutes before running the gastric phase. The emulsion was diluted to 1 % (w/w) oil and then mixed with SGFWS at a 50:50 volume ratio so that the final mixture contained 0.5% (w/w) oil. The pH of the sample was adjusted to 2.5 using NaOH and incubated at 37 °C for 2 h with continuous agitation at 100 rpm in an incubator shaker (Innova 4080, New Brunswick Scientific, Enfield, CT).

2.6.2 Small intestinal phase

An instrumental automatic titration (pH-stat) device (835 Titrando, Metrohm USA Inc., Riverview, FL) was used to simulate the conditions in the small intestinal phase of the

gastrointestinal tract. An aliquot of 30 mL of the gastric chyme was placed in a water bath at 37 °C and the pH was set to 7.0 using NaOH solution. Then, 1.5 mL of calcium chloride (37 mg/mL) and sodium chloride (219 mg/mL) and 3.5 mL of bile extract (53.5 mg/mL) dissolved in 5 mM phosphate buffer solutions were added to the sample and the pH was re-adjusted to 7.0. Afterwards, 2.5 mL of freshly prepared lipase suspension (24 mg/mL) dissolved in 5 mM phosphate buffer was incorporated into the mixture. The pH of the mixture was monitored and the volume of 0.1 M NaOH (mL) necessary to neutralize the free fatty acids (FFA) released from the lipid digestion (i.e., to keep pH at 7.0) was recorded during 2 h. The amount of free fatty acids released was calculated using the following equation:

$$\%FFA = 100 \times \frac{V_{NaOH} \times m_{NaOH} \times m_{lipid}}{w_{lipid} \times 2}$$

Where, V_{NaOH} is the volume of titrant in liters, m_{NaOH} is the molarity of the sodium hydroxide, m_{lipid} is the molecular weight of corn oil (872 g/mol), w_{lipid} is the weight of oil in the digestion system in grams.

2.7 Bioaccessibility

The bioaccessibility of lutein was evaluated after the samples had passed through the simulated small intestine phase of the gastrointestinal model. Aliquots of 10 mL of the samples were centrifuged (4000 rpm for 40 min at room temperature) using a bench top centrifuge (Sorvall ST8, Thermo Scientific, Tewksbury, MA). After centrifugation, the samples separated into a sediment phase at the bottom and a clear micelle phase at the top. The intestinal phase and the micelle phase were dissolved in dimethyl sulfoxide and read at 460 nm. The bioaccessibility (BA) was determined using the following equation:

$$BA(\%) = \frac{C_M}{C_R} \times 100$$

where, C_M is the lutein concentration in the micelle phase and C_R is the concentration of lutein in the entire intestinal phase, which is considered the raw digesta (Pool, Mendoza, Xiao, & McClements, 2013). The emulsions without lutein were used as blanks. The concentration of lutein was determined from absorbance measurements (460 nm) made using a UV–visible spectrophotometer (Cary 100 UV-VIS, Agilent Technologies, Santa Clara, Ca, USA). A calibration curve was prepared by dissolving lutein (standard) in DMSO in a range from 0.5 to 5 mg/L ($r^2=0.9992$).

2.8 Data analysis

All experiments were performed in triplicate and the results are given as mean values $\pm$ standard deviation. Differences among the treatments were determined using an analysis of variance (ANOVA) and a post-hoc Tukey test with a confidence level of 95 %. The analyses were made using SPSS software (IBM Corporation, Armonk, NY, USA).

3. RESULTS & DISCUSSION

3.1 Maillard conjugates formation and characterization

Sodium caseinate and dextran mixtures were spray dried and stored at 60 °C and 76% relative humidity for 48 h to produce Maillard conjugates. Dry conditions were used to minimize the presence of post-Amadori reactions (Morris, Sims, Robertson, & Furneaux, 2004). After 48 hours, we monitored the extent of conjugation using the OPA test, which measures the unreacted amino groups of the protein, and the conjugation efficiency was found to be $23.8 \pm 0.4\%$. It has been reported that the number of amino groups per caseinate molecule available to react with polysaccharides is 13.6 (G. Davidov-Pardo, Perez-Ciordia, Marin-Arroyo, & McClements, 2015; Markman, et al., 2012; Pan, et al., 2006). This means that the number of

dextran molecules attached to each of the caseinate molecules in our system was around 3.3. The percentage of protein participating in the Maillard reaction was measured by quantifying the protein that did not precipitate at pH 4.6 (isoelectric point of casein). This percentage is referred as the conjugation yield. For this study the conjugation yield was determined to be $93.7 \pm 3.2\%$, which verifies that the majority of the caseinate was conjugated to dextran molecules. Formation of Maillard conjugates was also verified using SDS-PAGE (data not shown).

3.2 Emulsion formation

Oil-in-water emulsions were made using 10% corn oil as the dispersed phase and 90% aqueous solution as the continuous phase (phosphate-buffered saline at pH 7). The continuous phase contained either caseinate or caseinate-dextran conjugates as emulsifiers, with the caseinate concentration being the same (1%) in both cases. Commercial lutein (20 % in corn oil) was dispersed at a concentration of 2.5 % in the oil phase to reach a lutein concentration of 500 mg/L in the final emulsion. The lutein concentration used in this study was based on the amount required to have a biologically significant effect on macular degeneration, i.e., 10 mg of lutein for a 200 mL serving size of a 1% fat beverage. Initially, emulsions containing relatively small droplets ($d < 300$ nm) that were stable to phase separation could be produced using both emulsifiers.

3.3 Influence of pH on emulsion stability and properties

Emulsion-based delivery systems may be utilized in food products that have different pH values, and they are exposed to changes in pH as they pass through the different regions of the gastrointestinal tract. For this reason, the influence of pH on the physical and chemical stability of lutein-enriched emulsions stabilized by either caseinate or caseinate-dextran conjugates was

measured. The pH-stability of the emulsions was compared by adjusting them to different pH values (pH 3 to 8), and then storing them at 37 °C for one week.

The physical stability of the emulsions was determined by measuring their particle size, particle charge, and overall appearance at the end of the storage period. The emulsions stabilized by caseinate alone underwent extensive droplet aggregation at pH 3, 4 and 5, as seen by a large increase in particle size (Figure 1a) and visible phase separation (Figure 1c). This effect can be attributed to a reduction in the magnitude of the electrical charge on the caseinate-coated oil droplets around their isoelectric point (Figure 1b), which reduces the electrostatic repulsion between them (McClements, et al., 2011). Presumably, the casein layer around the droplets was not sufficiently thick to prevent aggregation through steric repulsion (Dickinson, 1999, 2010). Probably, the emulsions exhibited droplet aggregation at pH 3 (Figure 1a), despite the fact that the droplets had a relatively high positive charge at this pH (Figure 1b), because they were initially prepared at neutral pH and then adjusted to the final acidic pH. Consequently, they had to pass through the isoelectric point, which may have resulted in some irreversible droplet flocculation. The change in ζ -potential with pH for this system was typical of that observed for protein-coated droplets, changing from highly negative at pH values well above the isoelectric point to highly positive at pH values well below the isoelectric point. The point of zero charge (between pH 4 and 5) for the protein-coated emulsions was consistent with the published isoelectric point of caseins (around pH 4.6).

The emulsions containing droplets coated with caseinate-dextran conjugates were stable to droplet aggregation across the entire pH range studied, with no evidence of an increase in particle size or visible phase separation (Figures 1a and 1c). The high stability of these systems can be attributed to the ability of the hydrophilic dextran molecules to generate a steric repulsion

that is strong enough to overcome any attractive interactions (such as van der Waals) between the droplets (Jiménez-Castaño, Villamiel, & López-Fandiño, 2007). The ζ -potential *versus* pH profile of the emulsions containing conjugated caseinate followed a similar general trend to those containing non-conjugated caseinate, *i.e.*, the droplet charge went from negative at high pH to positive at low pH, with a point of zero charge between pH 4 and 5 (Figure 1b). However, the magnitude of the ζ -potential values was appreciably lower for the caseinate-dextran coated droplets than the caseinate-coated droplets at the same pH. This effect can be attributed to the influence of the dextran molecules on the distance from the droplet surfaces where the effective electrical properties are measured, *i.e.*, the shear plane (Lesmes, et al., 2012). In the presence of dextran, the electrical properties are measured at a distance that is further from the droplet surfaces, and so there has been a greater decay in the electrical potential. These results show that conjugation of caseinate with dextran leads to an appreciable increase in the pH-stability of emulsions.

Lipid oxidation reactions produce reactive species that can further interact with other oxidizable compounds, such as the lutein in our samples. The interfacial characteristics of the emulsion will affect the susceptibility of lutein to degradation. Proteins located at the oil-water interface can inhibit oxidation at pH levels below their isoelectric points due to electrostatic repulsion of transition metals since they are both positively charged (Donnelly, Decker, & McClements, 1998; Elias, Kellerby, & Decker, 2008; Hu, McClements, & Decker, 2003). On the other hand, proteins in the continuous phase act as pro-oxidants above their isoelectric points due to their iron-binding properties since they are oppositely charged (Faraji, McClements, & Decker, 2004; Waraho, McClements, & Decker, 2011). Previous work has shown that carotenoid oxidation (including lutein) leads to color fading of the emulsion and that the Pearson correlation

values between these two phenomena was close to >0.9 (Gabriel Davidov-Pardo, Gumus, & McClements, 2016). Therefore, the influence of pH on the chemical degradation of lutein in the emulsions was monitored by measuring changes in the L, a, b values and then calculating the total color change (ΔE). The observed total color change (Figures 2a and 2b) was primarily the result of a reduction in positive b-values (decreased yellowness), a reduction in positive a-values (decreased redness), and an increase in lightness (color fading) during storage, which is indicative of lutein degradation (data not shown).

For the emulsions containing caseinate alone, reliable color measurements could not be made during storage at pH 4 and 5 because the samples rapidly phase separated (Figure 1c). For the remainder of the samples it was clear that the rate of color fading increased with decreasing pH (Figure 2). Previous studies have reported that carotenoids undergo chemical degradation under acidic conditions due to protonation of carbon atoms (Boon, et al., 2010; Mortensen & Skibsted, 2000). Color measurements could be made across the entire pH range for the emulsions containing droplets coated by caseinate-dextran conjugates because they were physically stable (Figure 1). In these systems, the rate of color fading clearly increased with decreasing pH (Figures 2a and 2c), which is in agreement with previous studies with other carotenoids (Boon, et al., 2010; Mortensen, et al., 2000). Conjugation did not appear to have a major impact on the rate of color degradation (Figure 2c); however, color fading did appear to occur somewhat more rapidly for the emulsions stabilized by the caseinate-dextran complexes at pH 3, but more slowly for the same systems from pH 6 to 8.

Overall, these results indicate that it may be better to store lutein-enriched emulsions under neutral conditions to inhibit color fading, and that these systems may be susceptible to some degradation under the highly acidic conditions of the stomach fluids.

3.4 Influence of temperature on emulsion stability

Food and beverage products are often exposed to a range of temperatures during their production, storage, transport, and utilization, and therefore it is useful to examine the influence of temperature on the physical and chemical stability of the lutein delivery systems. Emulsion thermal-stability was established by storing them at different temperatures (5 to 70 °C) for 7 days at pH 7, and then the particle size, particle charge, and emulsion appearance were measured.

A significant increase in droplet diameters was only observed at 70°C when the emulsions were stabilized with protein alone. However, significant differences in droplet size were observed at storage temperatures 37, 50 and 70°C when the conjugates were used to stabilize the emulsions ($p<0.05$) (Figure 3a). Figure 3c shows that the polydispersity index values of the treatments follow the same trend as the droplet size values. There was also an appreciable change in the electrical charge at elevated temperatures, particularly of the emulsions stabilized by the non-conjugated protein (Figure 3b).

The temperature-dependence of the chemical stability of lutein was determined by monitoring the changes in emulsion color during storage (Figure 4). For both emulsion systems, higher temperatures led to faster color fading. Previous studies have also reported that exposure of carotenoids to elevated temperatures leads to more rapid chemical degradation and formation of colorless products (Aparicio-Ruiz, Minguez-Mosquera, & Gandul-Rojas, 2011; Lim, Griffin, & Roos, 2014). The use of casein-dextran conjugates did not have a significant effect on the rate of color change in the emulsions. Some studies have reported that the Maillard reaction between proteins and low molecular weight sugars resulted in an increased antioxidant activity of the conjugates (Gu, et al., 2009; Gu, Abbas, & Zhang, 2009; Hiller & Lorenzen, 2010; Yilmaz &

Toledo, 2005). In the case of this study, the antioxidant activity of the Maillard conjugates and the casein alone measured by ABTS (909 ± 29 mM TE and 879 ± 25 mM of TE, respectively) were not significantly different ($p > 0.05$). This lack of difference in antioxidant activity of the emulsifiers may account for the similar trends of color fading observed in both systems (Figure 4). Similar results were found by Drusch, et al. (2009). In their work, the conjugation of caseinate with dextran did not prevent the oxidation of fish oil, while conjugation of casein with glucose showed a protection against lipid oxidation.

After the storage period at temperatures above 37°C the ζ -potential of the enriched emulsions stabilized by the Maillard conjugates presented a significant decrease ($p < 0.05$) and the droplet diameter of the samples stored at 70°C had the same increase as the samples stabilized with sodium caseinate alone. The decrease in the ζ -potential and the increase in droplet diameter could be explained by the possible breakdown/degradation of conjugates between the sodium caseinate and dextran when they are exposed to intermediate products of the carotenoids autooxidation reactions. The degradation of Amadori compounds can be initiated by lipid oxidation products and continue as a chainlike reaction (Mastrocola & Munari, 2000). As mentioned before, the exposure of carotenoids to temperatures of 37°C and above will cause their oxidation and produce highly reactive radicals (Boon, et al., 2010). The degradation of Maillard products due to the continuous heating of emulsions in the presence of casein and long chain carbohydrates has also been attributed to the progression of the Maillard reaction and the presence of post-Amadori products (O'Regan & Mulvihill, 2009). To verify this possibility, a solution of the conjugates was heated using the same conditions as the emulsions and the increase in absorbance was measured at 420 nm (an indicator of the formation of post-Amadori

products). The observed lack of browning in the samples supported the hypothesis that the degradation of the Maillard products was probably initiated by lipid and lutein oxidation.

3.5 Digestion and Bioaccessibility

The digestion rate (FFA release) depends on several factors including droplet size and interfacial structure (McClements, et al., 2010). The effect of using Maillard conjugates as emulsifiers on the fate of the digestion of the enriched emulsions was examined. The pH-stat method was used to compare the rate and extent of lipid digestion among the emulsions stabilized with protein only and protein-polysaccharide conjugates (Salvia-Trujillo, et al., 2013). The digestion model utilized for these samples do not include a mouth phase since the liquids do not spend long enough time in mouth to cause a significant change. Also, it is not necessary for liquid foods that does not contain a significant amount of starch (Minekus, et al., 2014). Figure 5 shows the changes in ζ -potential, particle size distribution and average particle diameter of the emulsions along the *in vitro* digestion model. The ζ -potential of the emulsions at pH 7 prior starting the digestion model was -38.73 ± 0.76 mV for the emulsions with non-conjugated emulsifier. The ζ -potential of the emulsions stabilized by Maillard conjugates was significantly lower ($p < 0.05$) with a value of -7.75 ± 0.67 mV. Both samples presented a negative charge because the pH of the emulsion was higher than the isoelectric point (pI) of sodium caseinate. The lower charge of the droplets in the emulsions stabilized by the Maillard conjugates has been explained earlier in the emulsion formation and stability section. In the stomach phase the pH drops to 2.5 and this affects the electric charge of the droplets which became positively charged 9.15 ± 1.38 mV and 2.52 ± 1.09 mV for samples with non-conjugated and conjugated sodium caseinate, respectively. Positive charge is expected since the pH is lower than the pI . Another reason for the lower charge in the stomach phase can be that the pepsin present in the gastric

juice hydrolyzes sodium caseinate, especially at around pH 2 (Guo, Fox, Flynn, & Kindstedt, 1995) and the resulting peptides yield in a lower ζ -potential. The emulsion stabilized with the Maillard conjugates is still stable at the gastric phase (will be explained further below). That is why the change in the ζ -potential is less drastic than the emulsion stabilized with protein alone, which is not stable at this step anymore (Figure 5a). During the intestinal phase the pH is 7 and this yields to negatively charged droplets in both samples. The average charge became strongly negative and was -40.88 ± 2.30 mV for the emulsions stabilized by sodium caseinate alone and -37.75 ± 0.91 mV for the Maillard conjugates. The ζ -potentials in this phase are similar and high not only because sodium caseinate has strong negative charge in neutral pH but also because the bile salts and phospholipids in the intestinal fluid are negatively charged and may contribute to the charge load. Furthermore, the FFAs produced can also yield to negative charges (Zou, Liu, Liu, Xiao, & McClements, 2015).

All samples showed monomodal particle size distribution (Figure 5c) in the initial stage. The average initial droplet diameter of the emulsion formed by using sodium caseinate was 144 ± 0.82 nm, whereas it was 138.25 ± 0.5 nm for the emulsions stabilized by Maillard conjugates, showing that Maillard conjugates reduced the droplet size significantly ($p < 0.05$) (Figure 5b). Markman and Livney (2012) claim that the Maillard conjugation changes the packing characteristics of the surface-active materials and increases the curvature of the emulsion droplets that stabilize smaller droplet sizes. Also, the microstructures of the emulsions after each stage are presented in Figure 6. It can be seen that the droplets were evenly distributed and no coalescence or flocculation were observed in the initial stage. The emulsions with Maillard conjugates stayed stable during the gastric phase with an average droplet diameter of 136 ± 0.82 nm ($p > 0.05$). On the other hand, the enriched emulsions stabilized by sodium caseinate yielded

to $3.19 \pm 0.48 \mu\text{m}$ in average droplet diameter (Figure 5b). The emulsion stabilized with sodium caseinate flocculated and showed coalescence as shown by the increase in average droplet diameter and the appearance of multimodal particle size distributions. This was further confirmed by microscopic observations (Figure 6). The sodium caseinate emulsions destabilization might have occurred because of the loss of charge due to the pH changes or due to proteolysis by pepsin present in this phase (G. Davidov-Pardo, et al., 2015; Lesmes, et al., 2012; Oliver, Augustin, & Sanguansri, 2009). The stability of the emulsions made with the Maillard conjugates in the gastric phase could be due to the steric repulsion caused by the dextran moieties on the surface of the droplets. The steric hindrance prevents the pepsin from reaching the surface of the droplet and therefore impedes the proteolysis of the caseinate molecules (Corzo-Martinez, Soria, Belloque, Villamiel, & Moreno, 2010; G. Davidov-Pardo, et al., 2015; Markman, et al., 2012). In the intestinal phase both emulsions showed a much higher average droplet size; $11.27 \pm 0.90 \mu\text{m}$ for the emulsions with sodium caseinate alone and $13.6 \pm 1.54 \mu\text{m}$ for the samples with the Maillard conjugates (Figure 5b). Both treatments showed flocculation and coalescence (Figure 6) leading to bigger droplet sizes after the intestinal phase, probably due to the replacement of sodium caseinate or Maillard conjugates from the droplet surface by the bile salts and free fatty acids present in the intestinal fluid (Dang, et al., 2015; G. Davidov-Pardo, et al., 2015; Oliver, et al., 2009; Salvia-Trujillo, et al., 2013). It has been shown that the bile salts in the gastric fluids strongly replace the emulsifiers around the droplets, even in multilayer emulsions stabilize by enzymatic cross-linking (Dang, et al., 2015). The replacement by the bile salts and FFA can be further confirm by the similarity between the samples stabilized with sodium casein alone and Maillard conjugates after the intestinal phase (Figure 5a).

The effect of Maillard conjugates on the digestion rate and extent of the corn oil

triglycerides **was also examined**. The FFA release is calculated by using the amount of sodium hydroxide used during the titration at intestinal phase. Figure 7 shows the FFA release measured during the intestinal phase of the in vitro digestion model. The steep increase in the FFA release in the beginning of the digestion shows that lipase quickly hydrolyzed the triglycerides (Figure 7). However, the increase in the FFA release during the first 5 minutes of digestion is slower for the samples stabilized with sodium caseinate alone. This effect could be due to the lower droplet diameter of the emulsions stabilized with Maillard conjugates after the gastric phase that yielded in a higher surface area at the beginning of the intestinal step. These findings are in accordance with some other studies (Dang, et al., 2015; Ray & Rousseau, 2013; Yang, et al., 2015). Dang, et al. (2015) explained that the interfacial properties do not play an important role in the fate of the digestion, whereas the stability and particle size tend to be more determinative. On the other hand, there is no obvious difference between the extents of FFA releases of the two emulsions. This may be due to exchange of surface-active materials during the digestion.

Finally, the bioaccessibility of the lutein contained in the enriched emulsions **was measured**. The bioaccessibility was monitored by measuring the lutein present in the mixed micelle phase after the digestion was finished (Joye, Davidov-Pardo, & McClements, 2014). There was not a negative effect in the bioaccessibility of lutein when the sodium caseinate was replaced by the Maillard conjugates. The bioaccessibility of lutein was 8.20 ± 0.73 % when the emulsion was stabilized by the sodium caseinate, and 7.55 ± 0.66 % when the emulsion was stabilized by the conjugates and there was no significant difference between the two ($p > 0.05$). The similar size and charge of both samples after the small intestine phase might be a reason for the similar bioaccessibility results. The fact that the bioaccessibility percentages were lower than 10 % could be explained by the high amount of lutein in the original emulsions and the

maximum capacity of the micelles to incorporate it. Further research with higher and lower lutein concentrations should be done to prove this hypothesis.

4. CONCLUSIONS

Lutein can be used as a natural colorant in foods, as well as a functional ingredient in functional foods to improve eye health. Sodium caseinate alone yields to unstable lutein-enriched emulsion at acidic pH values (pH 3-5), whereas the conjugation with dextran through Maillard reaction results in a surface-active complex that provides stability to the emulsions in the same conditions. The oxidation of lutein at high temperatures had an impact on the stability of the Amadori compounds of the Maillard conjugates, which resulted in a slight increase in the particle size of those emulsions stored at high temperatures. Finally, the Maillard conjugates did not affect the fate of digestion and the bioaccessibility of lutein. **The results of this study should facilitate the rational design and fabrication of nanoemulsion-based delivery systems for utilization in functional foods and beverages.**

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FIGURE CAPTIONS

Figure 1: (a) Mean droplet diameter (b) ζ -potential (c) pictures of lutein-enriched emulsions stabilized by non-conjugated sodium caseinate and Maillard conjugates adjusted to pH 3-8 and stored at 37 °C, values were taken at the end of 7 days of storage. Different lower case letters are significantly different for non-conjugated samples ($p < 0.05$). Different upper case letters are significantly different for conjugated samples ($p < 0.05$).

Figure 2: Changes in ΔE values of lutein-enriched emulsions prepared with (a) sodium caseinate and (b) Maillard conjugates adjusted to different pH values (pH 3-8) (c) Effect of storage at different pH values on lutein color degradation rate. All samples were kept at 37 °C for 7 days. Different lower case letters indicate significant difference between non-conjugated and conjugated samples at the same pH ($p < 0.05$).

Figure 3: (a) Mean droplet diameter and (b) ζ -potential (c) polydispersity index of lutein-enriched emulsions stabilized by Maillard conjugates adjusted to pH 7 and stored at different temperatures (5-70 °C), values were taken at the end of 7 days of storage. Different lower case letters are significantly different for non-conjugated samples ($p < 0.05$). Different upper case letters are significantly different for conjugated samples ($p < 0.05$).

Figure 4: Changes in ΔE values of lutein-enriched emulsions prepared with (a) sodium caseinate and (b) Maillard conjugates exposed to different temperatures (5-70°C) (c)

Effect of storage at different temperatures on lutein color degradation rate. All samples were adjusted to pH 7 and kept for 7 days. Different lower case letters indicate significant difference between non-conjugated and conjugated samples at the same temperature ($p < 0.05$).

Figure 5: Changes in (a) ζ -potential (b) average particle diameter and (c) particle size distribution and of lutein-enriched emulsions stabilized by non-conjugated sodium caseinate and Maillard conjugates during the in vitro digestion model. Different lower case letters indicate significant difference between non-conjugated and conjugated samples at the same digestion stage ($p < 0.05$). Different upper case letters indicate significant difference between different digestion stages for each of the samples ($p < 0.05$).

Figure 6: Microscopic images of lutein-enriched emulsions stabilized with (a) sodium caseinate and (b) with Maillard conjugates (scale bar 20 μ m).

Figure 7: Free fatty acids (FFA) release during the intestinal stage of the in vitro digestion model lutein-enriched emulsions stabilized with non-conjugated sodium caseinate and Maillard conjugates. Insert: FFA release during the first five minutes.

Figure 1

(a)

(b)

(c)

Non-conjugated

pH = 3 4 5 6 7 8

Conjugated

pH = 3 4 5 6 7 8

Figure 2

Figure 3

(a)

(b)

(c)

Figure 4

(a)

(b)

(c)

Figure 5

Figure 6

Figure 7

Lutein-enriched corn oil emulsions stabilized by:

Caseinate

Caseinate-dextran conjugates

pH = 3

4

5

6

7

8

pH = 3

4

5

6

7

8