Improved physicochemical stability of emulsions enriched in lutein by a combination of chlorogenic acid–whey protein isolate–dextran and vitamin E

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Abstract: Lutein, as a bioactive substance, has the ability to decrease the risk of some chronic diseases, but the poor water solubility, chemical instability, and low bioaccessibility limit its wide application in foods. In this study, an emulsion-based delivery system stabilized by chlorogenic acid (CA)–whey protein isolate (WPI)–dextran (DEX) ternary conjugates was prepared and vitamin E (VE) was added to increase the chemical stability of lutein. Molecular weight and conformational information of ternary conjugates were obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, fluorescence spectroscopy, and Fourier transform infrared spectroscopy. o-Phthalaldehyde results suggested that the extent of glycation was 16.4% and 19.5% for (CA–WPI)–DEX and WPI–DEX conjugates, respectively. The physicochemical stability of lutein-enriched emulsions was evaluated under different environmental stresses and long-term storage. The obtained results showed that compared with emulsions stabilized by WPI alone or binary conjugates, ternary conjugates imparted emulsions high stability under different environmental stress conditions (ionic strength, freeze–thaw, and heat) and long-term storage (within 3 weeks). VE can effectively decrease the degradation rate of lutein without changing the physical stability of emulsions. Additionally, the lutein-enriched emulsions prepared by ternary conjugates and VE exhibited a relatively high bioaccessibility.

Keywords: bioaccessibility, emulsion, lutein, physicochemical stability, vitamin E, whey protein isolate

Practical Application: The ternary conjugates constructed in this paper has excellent physicochemical characteristics to stabilize emulsion, and can increase the water solubility of functional factors and reduce their degradation rate. Additionally, this conjugate was prepared by food-grade materials. Therefore, it can be used as emulsion-based delivery systems in food industrials.

1. INTRODUCTION

Lutein, which is found in marigold flowers, has long been used as a natural, lipid-soluble pigment. It was reported to have the potential benefits to human health for its antioxidant and antitumor capacity, and it can protect the DNA of photoreceptive cells from strong light and alleviate the UV damage to the skin (Mora-Gutierrez, Attaie, & González, 2018; Rafi, Kanakasabai, Gokarn, Krueger, & Bright, 2015). Especially, lutein usually accumulates in the pigmented region and may reduce the morbidity of age-related macular degeneration and cataracts (Gumus, Davidov-Pardo, & McClements, 2016). However, due to its conjugated double-bond structure, lutein is highly sensitive to light, heat, oxygen, and low pH (Wang et al., 2011). In addition, the poor water solubility and storage stability resulted in their limited use in food products.

Recently, developing oil-in-water (O/W) emulsion delivery systems to encapsulate bioactive compounds and overcome their limitation in food applications has attracted much attention (Tapal & Tiku, 2012). Particularly, proteins are generally used as emulsifiers to prepare food-grade O/W emulsions as they are safe, biodegradable, and biocompatible, such as milk protein, soy protein, and other cereal proteins (Jiao et al., 2018; Yi, Fan, Zhang, & Zhao, 2016; Yi, Liu, Zhang, & Gao, 2018). However, protein-based emulsions are prone to destabilize when suffered from thermal treatment, pH, ionic strength, and freeze–thaw conditions, due to coalescence, and phase separation (Lam & Nickerson, 2013).

In recent years, fabrication and use of protein–polysaccharide and protein–polyphenol conjugates or complex as emulsifiers to prepare O/W emulsions are considered to solve the problem of protein sensitivity to environmental stress. Protein–polyphenol conjugates were usually prepared via free-radical grafting procedure or alkaline treatment. However, the preparation of protein–polyphenol conjugates by free-radical grafting method involves ascorbic acid (AA)/H_2O_2 redox repair, which may increase the risk of covalent reaction between protein and ascorbic acid (Liu, Sun, Yang, Yuan, & Gao, 2015). The alkaline treatment is that polyphenol is easily oxidized to quinone in an alkaline solution, and quinone further attracts nucleophilic groups of protein (lysine, tryptophan, and tyrosine, etc.) to form conjugates (Kroll, Rawel, & Rohn, 2007). Protein–polysaccharide conjugates are usually prepared by Maillard reaction, and they exhibited superior emulsion stability over protein alone. However, binary conjugates could not simultaneously guarantee the physical and chemical stability of the emulsions during storage. For example, emulsions stabilized

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by protein–polyphenol are not effective in preventing coalescence and phase separation around the isoelectric point (IEP) of protein, whereas emulsions prepared by protein–polysaccharide could not effectively reduce encapsulated material degradation under UV or heat treatment conditions (Gumus et al., 2016).

Emulsions stabilized by protein may be destroyed by the pepsin in the gastrointestinal (GI) tract, leading to the release of lutein into acidic gastral fluid. Acidic conditions could further cause the protonation of carbon and accelerate lutein degradation (Mortensen & Skibsted, 2000). Results have demonstrated that coated polysaccharides could improve the stability of oil droplets by providing a thicker layer than those emulsions stabilized by protein alone (Fan, Yi, Zhang, & Yokoyama, 2018). Polyphenol-modified protein has increased antioxidant capacity compared with natural protein, which may improve the chemical stability of lutein in the GI tract. However, the fate of emulsions stabilized by polyphenol–protein–polysaccharide ternary conjugates in the GI tract is rarely studied.

The carrier oil types also have a significant effect on the physicochemical stability and bioaccessibility of emulsions, and the oxidation of the oil phase may lead to the degradation of fat-soluble ingredients (Qian, Decker, Xiao, & McClements, 2012; Wang et al., 2018). VE, as a fat-soluble antioxidant, has been shown to protect unsaturated fatty acid from oxidant (Wang et al., 2018). Therefore, in this study, VE was considered to be added in emulsion systems to evaluate the physicochemical stability of emulsions prepared by polyphenol (chlorogenic acid, CA)–whey protein isolate (WPI)–polysaccharide (dextran, DEX) conjugates under heat treatment, ionic strength, freeze–thaw and long-term storage conditions and their fate in simulated GI tract.

2. MATERIALS AND METHODS

2.1 Materials

WPI (purity ≥ 90%), VE oil (purity ≥ 98%), and lutein (HPLC ≥ 90%) were obtained from Shanghai Yuan Ye Biotech Co., Ltd. (Shanghai, China). Corn oil was obtained from a local market. DEX (MW 35–45 kDa), Pepsin (porcine, P6887), bile extract (porcine, B8361), and pancreatin (porcine pancreas P7545) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CA (purity ≥ 98%), o-Phthalaldehyde (OPA), and L-leucine were bought from Macklin (Shanghai, China). All other reagents were of analytical grade.

2.2 Preparation of conjugates

2.2.1 CA–WPI conjugates. CA–WPI conjugates were prepared according to our previous method (Yan et al., 2019). Briefly, 1% (w/w) protein solution was prepared by dissolving WPI in 100 mL ultra-pure water and stirring for 12 hr. Then, 0.2 g of CA was added into the protein solution, and the pH value was adjusted to 9.0. The solution was free to contact air for 24 hr under 125 rpm and freeze-dried under −80 °C to obtain porous solids. The same conditions without CA solution was used to prepare control WPI.

2.2.2 WPI–DEX and (CA–WPI)–DEX conjugates. WPI–DEX conjugates were prepared by Maillard reaction according to a previous method with some modifications (Fan et al., 2018). Briefly, 1 g of WPI and 1 g of dextran were dissolved in water together and stirred for 12 hr to ensure complete hydration. The pH was adjusted to 7.0 using HCl / NaOH and then freeze-dried. The resultant powders were incubated in a desiccator containing saturated KBr solution at 60 °C for 24 hr. (CA–WPI)–DEX conjugates were prepared by replacing WPI with CA–WPI conjugates through the above process.

2.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Changes in relative molecular weight of proteins before and after covalency were analyzed using SDS-PAGE according to an established method with slight modifications (Yi et al., 2018). Briefly, the concentrations of 10% and 5% were used for separating and stacking gel, respectively. After electrophoresis, gel sheets stained with Coomassie Brilliant Blue R-250 were scanned by the imaging system.

2.4 Spectrum analysis

2.4.1 Fluorescence spectroscopy. One mg/mL protein solution was used to obtain the fluorescence spectrum (310 to 500 nm) under room temperature. The parameters of spectrophotometer (F4600; Hitachi, Ltd., Tokyo, Japan) were set (i) 5 nm split width for excitation and emission, (ii) 295 nm for excitation wavelength, and (iii) 670 V for voltage.

2.4.2 Fourier transform infrared spectroscopy (FTIR) spectroscopy. A FTIR spectrophotometer (Nicolet IS50; Thermo Scientific; Waltham, MA, USA) was used to obtain the spectra of different samples (control WPI, CA, CA–WPI conjugate, (CA–WPI)–DEX conjugate, WPI–DEX conjugate and (CA–WPI)–DEX mixture (ternary mixture)). Briefly, 1 mg of samples and 150 mg of KBr were mixed and ground into powders, then compressed to form transparent tablets. The range of 4,000 to 400 cm^{-1} was recorded.

2.5 Extent of glycation (EG)

EG of WPI–DEX and (CA–WPI)–DEX conjugates was measured using OPA assay according to an established method with some modifications (Davidov-Pardo, Perez-Ciordia, Marin-Arroyo, & McClements, 2015). Samples (4 mg/mL protein) solution and OPA reagents were mixed (20:1 [v/v]), then incubated at 35 °C for 2 min. The absorbance was detected at 340 nm. The standard curve was made by L-leucine. The EG of (CA–WPI)–DEX / WPI–DEX conjugate was obtained by calculating the reduction of free amino group of CA-WPI / WPI after the Maillard reaction. Experiments were repeated three times.

2.6 2,2-Diphenyl-1-picryl-hydrazil (DPPH) scavenging activity

DPPH scavenging activity of samples was evaluated according to an established method (Yi et al., 2016). Briefly, WPI or conjugates (0.1 mg/mL protein) were mixed with DPPH solution, and then incubated at room temperature for 1 hr. The absorbance was determined at 517 nm using a spectrophotometer.

2.7 Lutein-enriched emulsions

Lutein-enriched emulsions were prepared according to our previous method (Yan et al., 2019). Briefly, the water phase (1% protein solution) and the oil phase was mixed at 9:1 (v/v), following high-speed shear to obtain pre-emulsions. Then, they were immediately homogenized at 880 bar for three times (AH100B; ATS Engineering Inc., Canada). The final concentration of lutein was 250 mg/mL, and the VE group was prepared by replacing 2% corn oil (w/w) using VE oil in the oil phase. Sodium azide (0.02% w/w) was added to prevent microorganisms.

2.8 Characterization of the emulsions

2.8.1 Diameter, polydispersity index (PDI), and ζ-potential analysis. A dynamic light scattering (DLS) analyzer

(Brookhaven Corporation, Holtsville, NY, USA) was used to obtain the average diameters and PDI at room temperature. The refractive indices of oil droplets and water phase were set 1.45 and 1.33, respectively. To avoid multiple scattering effects, emulsions were diluted using phosphate buffer (10 mM, pH 7.0) prior to the measurements. The ζ-potential values were measured by a Zeta Sizer Nano ZS90 (Malvern, UK) at 25 °C, pH 7.0. All measurements were repeated in triplicate.

2.8.2 Viscosity. The viscosity of emulsions was obtained according to an established method with minor modifications (Zhu, Zhao, Zhang, Saito, & Yin, 2017). Briefly, 1 mL emulsion was deposited on the plateau of the dynamic rheometer (Thermo Fisher HAAKE^{TM} MARS 60, Karlsruhe, Germany), and a steady-state flow method was performed over a shear rate of 10^{-2} to 10^{3} s^{-1}.

2.8.3 Morphological observations. A conventional negatively-staining method was used to observe the morphology of freshly prepared emulsions. One droplet of the emulsion was placed on a copper grid and negatively stained with 2 g per 100 g phosphotungstic acid for 1 minute at room temperature. After drying at ambient temperature, the sample was analyzed using a JEOL JEM-1200 EX transmission electron microscope (TEM; Japan Electronics Company, Tokyo, Japan).

2.9 Environmental stresses

The physical stability of different emulsions was evaluated under different conditions using a Turbiscan Lab analyzer (Formulaction, Co., France). (a) Freeze–thaw: different emulsions (15 mL) were diluted with the same volumes of ultra-pure water, then stored at −18 °C for 20 hr and thawed at 30 °C (water bath) for 2 hr; (b) ionic strength: different emulsions were diluted with the same volumes of 0.4 M NaCl solution, then stirred at 125 rpm for 2 hr and stored at 25 °C for 20 hr; (c) heat treatment: different emulsions were diluted with the same volumes of ultra-pure water, then incubated in a water bath (90 °C) for 10 min and placed at 25 °C for 20 hr. Freshly prepared emulsions (15 mL) diluted with the same volumes of ultra-pure water and stored at 25 °C for 24 hr was used as control. The physical stability was indicated by Turbiscan stability index (TSI).

2.10 Storage stability

Thirty milliliters of each freshly prepared emulsions were sealed in vials and stored at 4, 25, 37, and 50 °C. The physicochemical stability of lutein-enriched emulsions was estimated by the fluctuation of oil droplet size and the retention of lutein. The diameter was measured through above method and the lutein content in emulsions was measured every 3 days according to Liu, Ma, McClements, and Gao (2016). Briefly, ethanol and *n*-hexane were used to extract lutein from emulsions and UV-visible spectrophotometer was sued to measure lutein content at 460 nm.

2.11 Bioaccessibility

A simulated GI tract was adopted to obtain the bioaccessibility of lutein following the method of Jiang Yi, Li, Zhong, and Yokoyama (2014). Briefly, 7.5 mL of emulsions and 10 mL of simulated gastric fluids were mixed then incubated at 250 rpm for 1 hr. At the end of gastric digestion, the mixture was adjusted to pH 7.0 and added to 15 mL of intestinal fluids, then incubated at 250 rpm for 2 hr. The solution was kept at pH 7.0 using 0.25 M NaOH.

The values of lutein bioaccessibility were obtained according to previous methods with slight modifications (Liu et al., 2016). Briefly, at the end of the GI tract, aliquots of samples were centrifuged (11,000 × *g* 40 min at 4 °C; 3K15, Sigma, Germany). Syringe filters (0.22 μm; Thermo Fisher Scientific, Waltham, MA, USA) were used to collect purified lutein, and the content of lutein was measured as described above. The bioaccessibility was obtained by the ratio of C/C_0, where C represents residual lutein concentration after digestion and C_0 represents the initial lutein concentration.

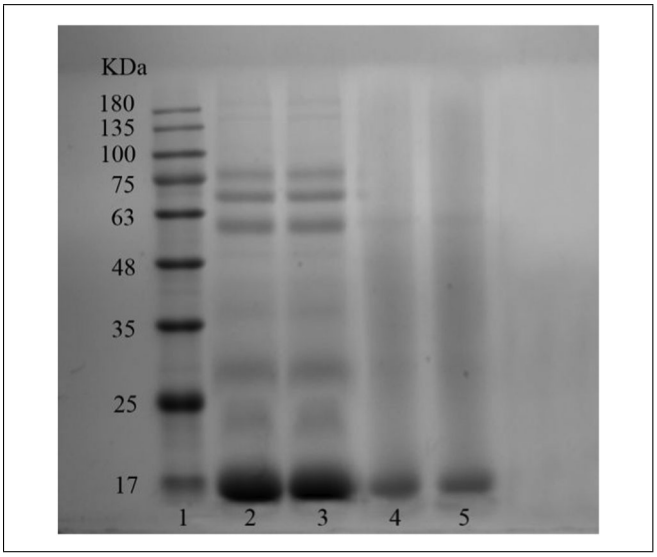

Figure 1--SDS-PAGE results of WPI and conjugates: Lane 1, Markers; Lane 2, WPI; Lane 3, CA-WPI con; Lane 4, WPI-DEX con; Lane 5, (CA-WPI)-DEX con; con, conjugate.

2.12 Statistical analysis

All measurements were performed in at least triplicate. The values are expressed as mean ± standard deviation (SD). Statistical analysis was analyzed by SPSS software and the differences between means were calculated by Duncan's test at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Characterization of conjugations

3.1.1 SDS-PAGE. WPI, which mainly contains β-lactoglobulin (BLG), is widely used in the food industry because of its high nutritional quality and functional properties, such as emulsibility and foaming ability (Yi et al., 2018). SDS can break noncovalent bonds between molecules, and previous studies have shown that the main band in protein will be changed after covalent modification with polyphenol or polysaccharide (Fan et al., 2018; Yi et al., 2016). Figure 1 shows that the main band of WPI is BLG (18.2 kDa). However, this main band moves up slightly in Lane 3, which indicates that some CAs covalently bonded to BLG. Further, the main band become weaker and the weak smeared zones appeared in Lane 4 and 5, which suggests that there was successful glycation between WPI / CA–WPI and dextran.

3.1.2 Fluorescence spectroscopy. Polyphenol contains many hydroxy groups that are easily oxidized to form quinones when exposed to air under alkaline conditions. Quinone, as a reactive electrophilic intermediate, is prone to reactions with nucleophiles such as tryptophan, lysine, and cysteine to form conjugates (Kroll et al., 2007). Tryptophan, as an intrinsic fluorescence group in proteins, is usually used to gain the dynamic changes and local information of WPI. As shown in Figure 2a, the maximum emission wavelength of CA–WPI conjugates was 342 nm, which was red-shifted about 10 nm compared to

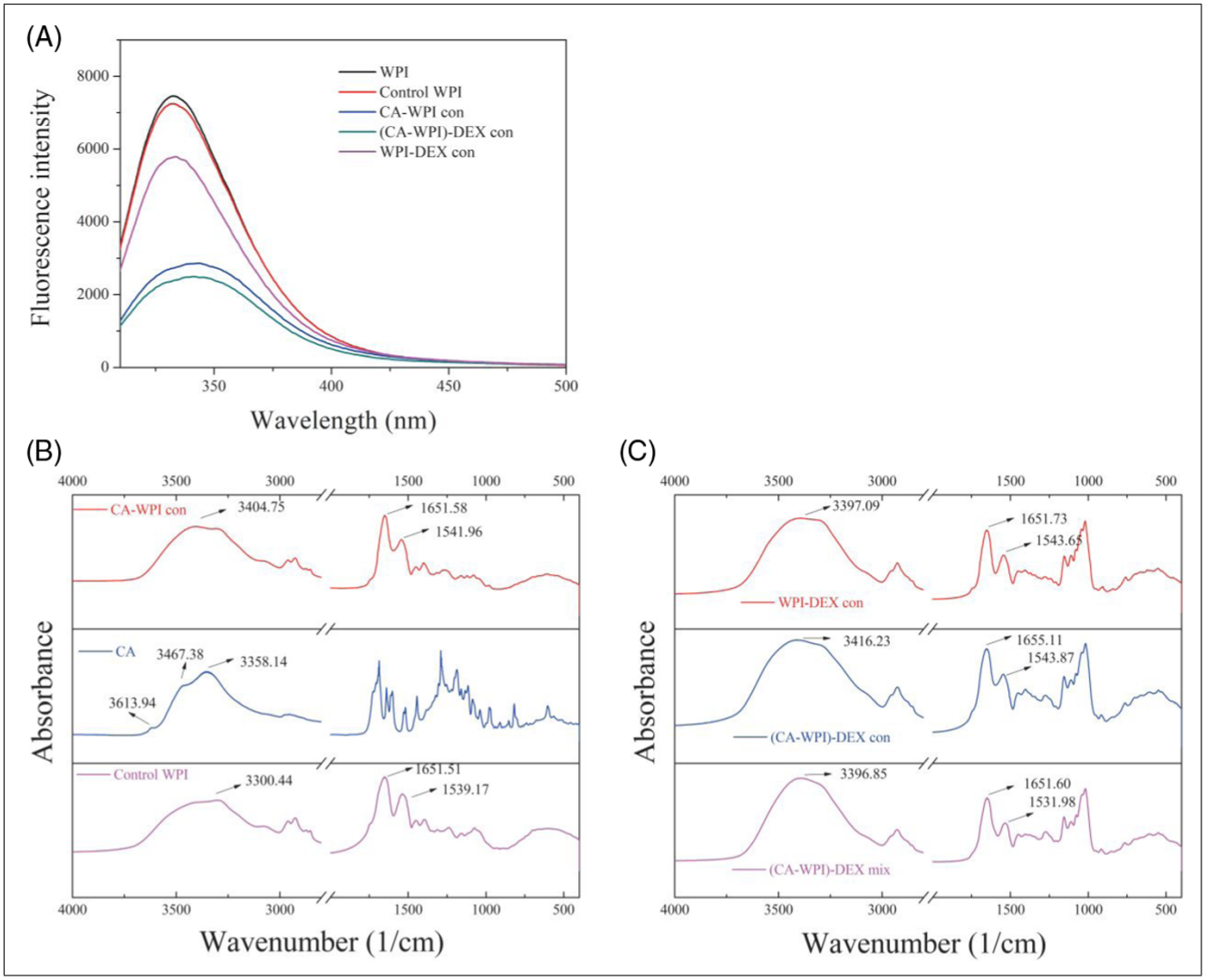

Figure 2--Spectrum analysis: (A) intrinsic fluorescence spectra of native WPI, control WPI, CA-WPI con, (CA–WPI)–DEX con, WPI–DEX con. Protein concentration is 1 mg/mL; (B) and (C) infrared spectra of control WPI; CA; CA–WPI con; (CA–WPI)–DEX con; (CA–WPI)–DEX mix; WPI–DEX con. WPI, whey protein isolate; CA, chlorogenic acid; DEX, dextran; con, conjugates; mix, mixture.

WPI (332 nm). Besides, strong fluorescence intensity quenching happened in CA–WPI conjugates. The similar results have been shown in the previous study (Liu et al., 2015). It may be that the covalent reaction between polyphenol and protein changed the hydrophobic environment of tryptophan and the secondary structure of the protein. However, it is obvious that the effects of dextran on the fluorescence spectrum of tryptophan is less than that of CAs (Figure 2a). In this study, the values of EG were 16.4% and 19.5% for (CA–WPI)–DEX conjugates and WPI–DEX conjugates, respectively. Previous study has suggested that the increase of EG values would increase the quenching degree of fluorescence intensity (Yi et al., 2018).

3.1.3 FTIR spectroscopy. The samples were also characterized by FTIR spectroscopy. The major bands of control WPI are shown in Figure 2b, where amide A band is at 3300.44 cm^{-1} (N–H stretching coupled with hydrogen bonding), amide I band is at 1651.51 cm^{-1} (C = O stretch) and amide II is at 1539.17 cm^{-1} (N–H bend, C–N stretch). Obviously, these band in CA–WPI conjugates were altered in shapes and peak positions compared with the control WPI. Additionally, Maillard reaction is a condensation of carbonyl and amino groups, which generates Amadori compounds (C = O), Schiff base (C = N) and pyrazines (C–N) (Boye, Alli, & Ismail, 1996; Nooshkam & Madadlou, 2016). As shown in Figure 2c, compared with (CA–WPI)–DEX mixture, the Maillard reactions between DEX and WPI/CA–WPI conjugates made the peak strength and positions of amide A, amide I, and amide II bands changed.

The changes in Amide A band suggest that polyphenol and dextran could react with N–H bond in WPI to form conjugates. Amide I and II bands are commonly considered as the sensitive areas of secondary structure. These changed bands in peak positions and shapes indicated that the secondary structure in WPI–CA/WPI–DEX was altered. Yi et al. reported that the conjugation of polyphenol to BLG would cause a decrease in β-sheet and an increase in unordered structure (Yi, Zhang, Liang, Zhong, & Ma, 2015). Rawel et al. also reported that a decrease in α-helix and an increase in random coil when CA was interacted with bovine serum albumin (Rawel, Rohn, Kruse, & Kroll, 2002). Briefly, the changes of structural information obtained by the above spectral analysis indicated that CA was covalently conjugated to WPI.

Table 1—Mean droplet diameter, PDI, and ζ-potential of freshly prepared emulsions stabilized by different emulsifiers at pH 7.0.

Emulsions stabilized by	Mean droplet size (nm)	PDI	ζ-Potential (mV)
WPI	242.16 ± 1.50^{b}	0.131 ± 0.010^{b}	−36.0 ± 0.5^{b}
CA–WPI con	264.63 ± 1.64^{a}	0.178 ± 0.014^{a}	−41.1 ± 0.8^{a}
(CA–WPI)–DEX con	219.40 ± 2.78^{d}	0.117 ± 0.013^{b}	−23.2 ± 0.8^{c}
(CA–WPI)–DEX con +VE	224.73 ± 3.63^{c}	0.124 ± 0.012^{b}	−21.3 ± 0.7^{d}
WPI–DEX con	216.20 ± 3.54^{d}	0.121 ± 0.009^{b}	−17.9 ± 0.8^{e}

Note: Different superscript letters in the same column indicate significant differences ($P < 0.05$).
Abbreviations: WPI, whey protein isolate; CA, chlorogenic acid; DEX, dextran; con, conjugates.

3.2 Characterization of emulsions

3.2.1 Droplet size, PDI, ζ-potential, and morphology. The characterization of freshly prepared emulsions (pH 7.0) was shown in Table 1. The average diameter of emulsions prepared by WPI alone was about 242.16 nm, which was higher than the emulsions coated with DEX. The similar results were also reported previously by Wang, Liu, Xu, Yin, and Yao (2016). The reason could be that covalent dextran increased the layer thickness of oil droplets, causing an increase in steric hindrance between droplets and a decrease in diameter

The PDI values of all emulsions were less than 0.2, which suggested that the droplet size of emulsions showed narrow distribution. The absolute value of ζ-potential reflects the magnitude of electrostatic repulsion, which was related to the physical stability of particles (Zhang, Nie, Martinez-Zaguilan, Sennoune, & Wang, 2016). The ζ-potential value of emulsions stabilized by WPI was about −36.0 mV, which was higher than that of CA-WPI conjugates. It might be due to the negative charge of CAs at pH 7.0 (Liu et al., 2016). However, the ζ-potential absolute values of emulsions stabilized by protein–DEX were significantly smaller than that of WPI and CA–WPI conjugates. This may be that the steric hindrance provided by hydrophilic DEX caused an interference when measuring the ζ-potential (Fan et al., 2018; Wooster & Augustin, 2006){Fan, 2018 #39}.

TEM morphology of freshly prepared emulsions stabilized by (CA–WPI)–DEX conjugates and VE showed that the droplets were spherical and homogeneously dispersed (Figure 2S). However, the average diameter was slightly smaller than that measured by laser light scatting. This could be attributed to the difference in measurement state (one in solution and another in solid-state; Fan et al., 2018).

3.2.2 Viscosity. The viscosity of emulsions was measured at different shear rates in this study. As shown in Figure 3, the emulsions prepared by (CA–WPI)–DEX conjugates and VE exhibited shear thinning behavior at the shear rate below 1 s^{-1}, whereas the viscosity of the emulsions was constant at the shear rate over 1 s^{-1}. Because the low shear rate is rarely used in food processing, it can be considered that the ternary conjugates and VE do not change the Newtonian behavior of emulsions. Those emulsions stabilized by WPI, CA–WPI conjugates, (CA–WPI)–DEX conjugates, and WPI–DEX conjugates exhibited a similar trend. The results showed that this emulsion may be suitable for tube feeding without tube clogging because of its good fluidity.

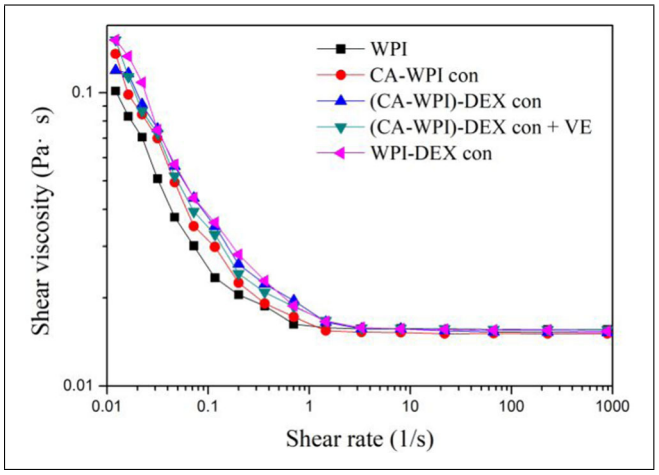

Figure 3--Viscosity of the emulsions stabilized by WPI, CA-WPI con, (CA-WPI)-DEX con, WPI-DEX con, (CA-WPI)-DEX con, and VE. con, conjugates.

3.3 Influence of environmental stresses on emulsion physical stability

It will seriously affect the sensory effects if the emulsion-based delivery system goes through severe aggregation, flocculation, and phase separation under different storage conditions. Therefore, it is very important to evaluate the physical stability of the emulsions stabilized by different emulsifier types under different environmental stresses such as ionic strength, heat treatment and freeze–thaw cycles. In this study, the physical stability was assessed by TSI. As shown in Figure 4a, all the emulsions of the control group have small TSI values (below 0.35), which suggested that there was no obvious change in oil droplets.

As shown in Figure 4b, under the 0.2 M NaCl condition, the TSI values of all emulsions became bigger than control. This indicated that the droplet diameter has changed. Especially, the emulsions stabilized by WPI/CA–WPI conjugates went through more serious aggregation compared with those emulsions stabilized by protein–DEX. This may be that NaCl decreased the amounts of the ζ-potential and thus reduced the electrostatic repulsion between droplets, whereas the emulsions coated with dextran had strong steric hindrance that can effectively prevent droplets aggregation (Fan et al., 2018).

The results of heat treatment exhibited the same trend as that of ionic strength (Figure 4c). Heating may cause the exposure of the hydrophobic group of protein molecules, and further resulted in the aggregation of droplets (Liu et al., 2016). However, the emulsions prepared by protein–DEX conjugates still exhibited better physical stability than that of WPI/CA–WPI. A previous study has indicated that the thermal denaturation temperature of protein may be improved after the glycation with saccharides or polysaccharides (G. Liu & Zhong, 2013; Liu et al., 2016). Therefore, this could be attributed to the increased denaturation temperature and steric hindrance provided by hydrophilic dextran.

As shown in Figure 4d, under freeze–thaw condition, the TSI values of emulsions stabilized by WPI alone were over 29. In addition, there was visible aggregation and phase separation on the emulsions stabilized by WPI and CA–WPI conjugates. These results indicated that the oil droplets were highly unstable under the freeze–thaw condition. Interestingly, the emulsions coated with

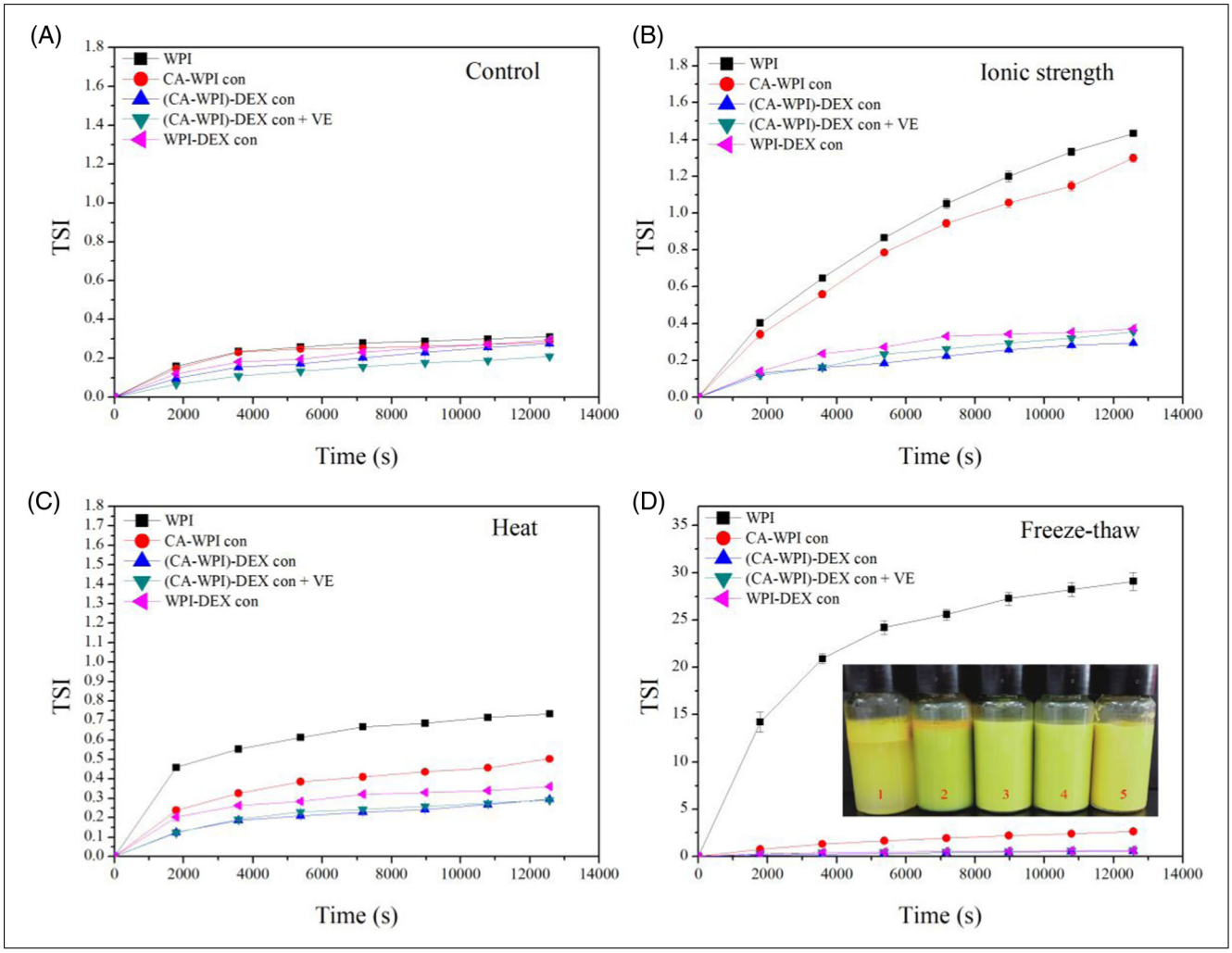

Figure 4--The changes of Turbiscan stability index (TSI) under different environmental stresses. TSI values were recorded at 30 min intervals. (A) Control; (B) ionic strength stability; (C) Heat treatment; (D) freeze-thaw stability.

dextran were still stable and seemed to be more homogeneous. Mun, Cho, Decker, and McClements (2008) indicated that the freeze–thaw stability of oil droplets is related to the properties of the interfacial layer (Mun et al., 2008). The coated dextran might change the interfacial properties of the emulsions prepared by WPI, which makes the emulsions stabilized by protein–DEX more stable under freeze–thaw conditions.

Additionally, we found that the CA-WPI-DEX ternary mixture was not effective in preparing an emulsion with good physical stability under these environmental stresses (shown in Figure 1S). This could be that the mixture of CA and/or DEX does not change the steric hindrance and charge properties of the emulsion stabilized by WPI. The above results suggested that the coated CAs could improve the physical stability of WPI-stabilized emulsions, but the effect was not obvious. Emulsions prepared by WPI–DEX conjugates exhibited better physical stability than that of WPI–CA, and the emulsions stabilized by ternary conjugates were most effective in preventing aggregation, creaming, and phase separation between droplets under different environmental stresses.

3.4 Physicochemical stability under storage

Lutein-enriched emulsions may suffer from different temperatures during processing. Consequently, it is very important that emulsion-based delivery systems can protect core materials from degradation during storage. For this reason, different temperature conditions were set to assess the physicochemical stability of lutein-enriched emulsions.

As shown in Table 2, the droplet size of all emulsions stabilized by different emulsifiers increased after 3 weeks of storage at different temperatures. Under 50 °C, the droplet diameter of emulsions prepared by WPI became 365.80 nm. Hydrophilic dextran can provide a strong steric hindrance that could prevent oil droplets from aggregating. However, long-term storage at high temperatures may trigger lipid oxidation and a chainlike reaction, further promoting a decomposition of Amadori compounds, and increasing droplet diameter (Mastrocola & Munari, 2000). The droplet size of emulsions stabilized by ternary conjugates increased from 219.4 to 246.80 nm, which still exhibited better physical stability than emulsions prepared by WPI alone. Previous study also indicated that the emulsions stabilized by WPI–soy soluble polysaccharides exhibited better physical stability at long-term storage than that of WPI alone (Yang et al., 2015).

In addition, CA, as a water-soluble antioxidant, is dispersed in the aqueous phase. The above results also showed that the unreacted CA did not markedly affect the physical stability of emulsions compared to WPI–DEX. Therefore, in this study, the unreacted CA was retained in the emulsion-based delivery system. Further, the antioxidant capacity of CA–WPI and (CA–WPI)–DEX

Table 2—Mean droplet diameter, PDI, and ζ-potential of emulsions after 21 storage under 4, 25, 37, and 50 °C conditions.

Emulsions stabilized by	4 °C	25 °C	37 °C	50 °C
WPI	254.17 ± 3.67^{b}	291.03 ± 3.98^{b}	326.67 ± 3.72^{b}	365.80 ± 4.56^{b}
CA–WPI con	274.87 ± 3.41^{a}	305.03 ± 3.07^{a}	334.17 ± 4.10^{a}	394.17 ± 4.26^{a}
(CA–WPI)–DEX con	228.07 ± 2.57^{c}	233.30 ± 2.11^{c}	238.10 ± 2.72^{c}	246.80 ± 3.90^{c}
(CA–WPI)–DEX con +VE	227.73 ± 3.25^{c}	233.43 ± 2.85^{c}	234.30 ± 2.62^{c}	253.57 ± 3.20^{c}
WPI–DEX con	226.60 ± 4.56^{c}	231.73 ± 6.05^{c}	234.03 ± 3.26^{c}	249.53 ± 4.83^{c}

Note: Different superscript letters in the same column indicate significant differences ($P < 0.05$).
Abbreviations: WPI, whey protein isolate; CA, chlorogenic acid; DEX, dextran; con, conjugates.

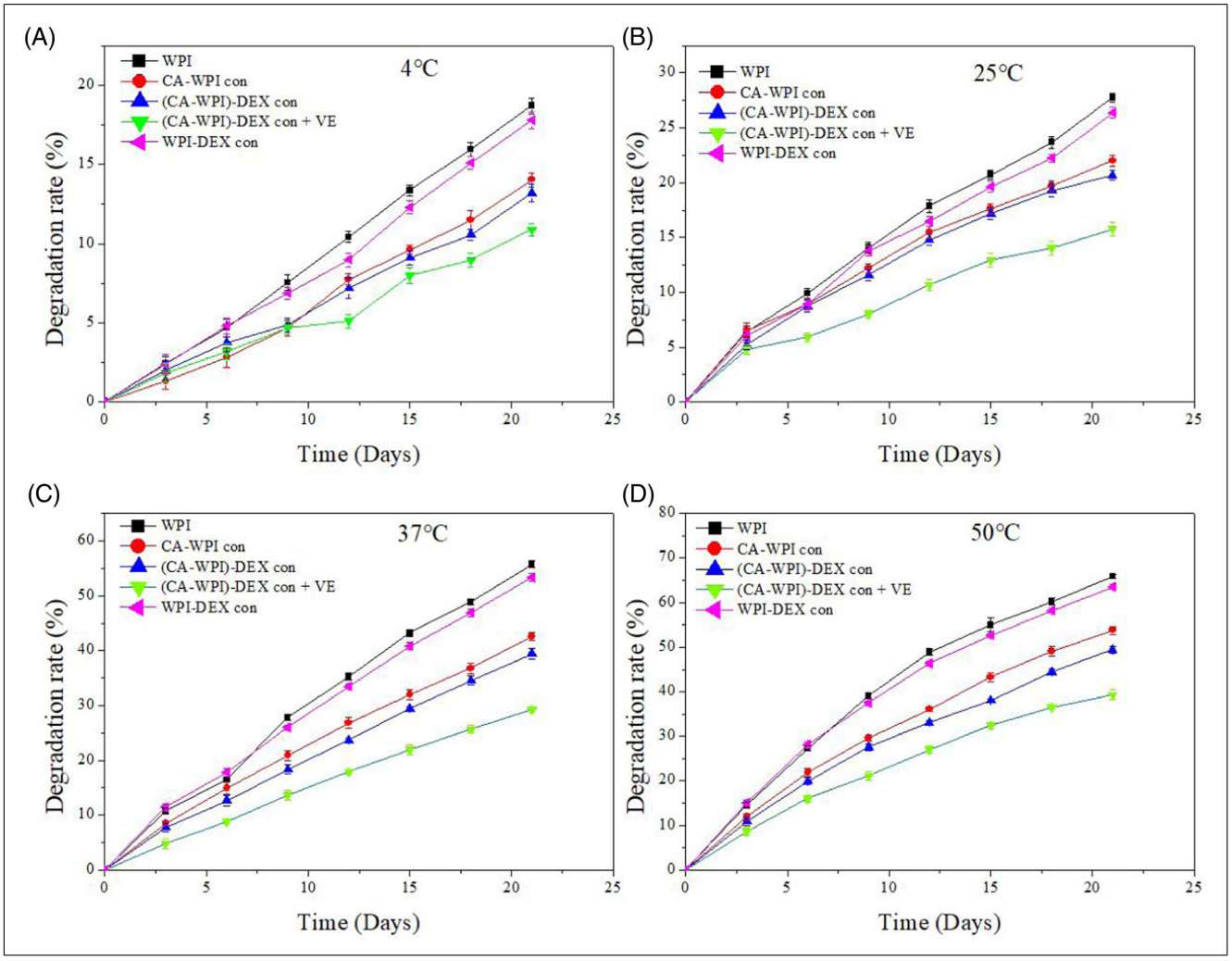

Figure 5--The degradation of emulsions stabilized by different emulsifiers. The values were measured at 3 days intervals. Different letters at the same temperature indicate significant differences ($P < 0.05$).

conjugates was evaluated by DPPH experiment. The results showed that, compared with WPI alone, CA–WPI conjugates exhibited better antioxidant activity. Although the antioxidant activity of CA–WPI conjugates grafted dextran decreased, it was still significantly improved compared with WPI alone (Figure 3S; $P < 0.05$).

The chemical stability of lutein was evaluated by measuring the degradation rate of lutein (Figure 5). As a thermal sensitive substance, the degradation rate of lutein increases with the increase of temperatures (Aparicio-Ruiz, Mínguez-Mosquera, & Gandul-Rojas, 2011). The degradation rate of emulsions prepared by WPI was about 65% after 3 weeks of storage at 50 °C, whereas that was only about 18% at 4 °C (Figure 5a and d). The chemical stability of lutein-enriched emulsions stabilized by WPI–DEX was slightly improved, and was significantly increased by CA–WPI conjugates compared with WPI ($P < 0.05$). According to Yi et al. (2016), the emulsions containing water-soluble antioxidants exhibited better protective effects on β-carotene against isomerization, oxidation, and degradation (Yi et al., 2016). Figure 5d also showed the emulsions stabilized by ternary conjugates and VE had the lowest degradation rate (39%) at 50 °C after storage. The above results showed that the emulsions stabilized by (CA–WPI)–DEX conjugates and VE were more effective at inhibiting the degradation of lutein.

Table 3—Reaction rate constants (k) of lutein degradation in emulsions.

Emulsions stabilized by	$k \times 10^{-2}$/d (R^2)			
	4 °C	25 °C	37 °C	50 °C
WPI	1.03 (0.99)	1.40 (0.99)	3.90 (0.99)	5.07 (0.99)
CA–WPI con	0.78 (0.99)	1.02 (0.99)	2.55 (0.99)	3.58 (0.99)
(CA–WPI)–DEX con	0.66 (0.99)	1.01 (0.98)	2.37 (0.99)	3.09 (0.99)
(CA–WPI)–DEX con +VE	0.53 (0.97)	0.76 (0.99)	1.67 (0.99)	2.32 (0.99)
WPI–DEX con	0.96 (0.99)	1.33 (0.99)	3.60 (0.99)	4.64 (0.99)

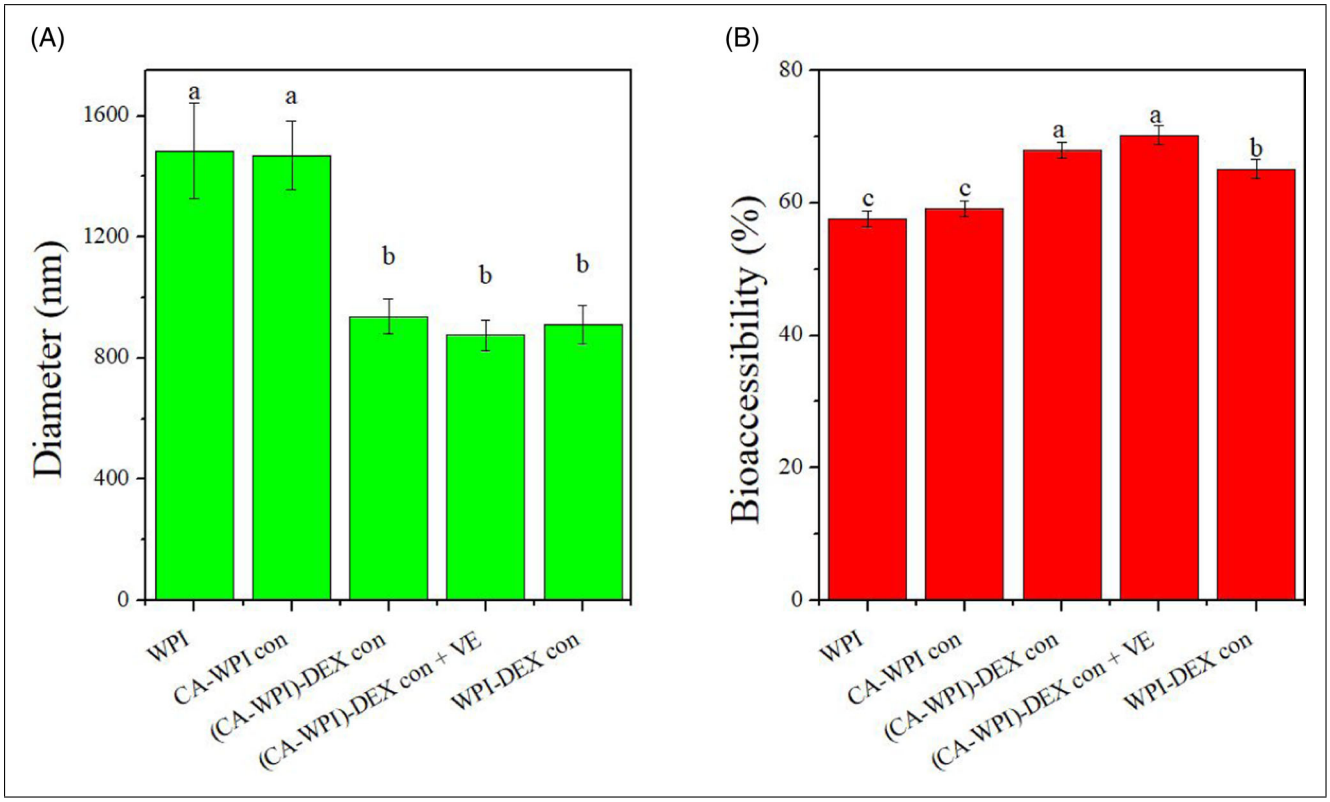

Figure 6--(A) The changes of droplet diameter after intestinal digestion; (B) bioaccessibility (%) after in vitro digestion. Different letters indicate significant differences ($P < 0.05$).

Degradation kinetics of lutein were also analyzed to better comprehending the correlation of emulsifiers and storage temperature on lutein loss. Reaction rate constants (k-value) were obtained by the formula: $C/C_0 = \exp(-kt)$, in which C represents lutein concentration at storage time t, C_0 represents initial concentration, k-values were listed in Table 3. The results showed that the degradation of lutein in emulsions confirms to the first-order kinetic model (R^2 varied from 0.97 to 0.99). The k-values increased with the increase of storage temperature in the range of between 4 and 50 °C and the highest lutein loss appeared in emulsions stabilized by WPI alone at 50 °C ($k = 5.07$ d^{-1}). Emulsifier has a significant impact on the stability of lutein, and ternary conjugates-stabilized emulsions have lower k-values than protein alone and binary conjugates. In addition, the addition of VE could decrease the degradation rate of lutein.

3.5 Bioaccessibility

Lutein can decrease the risk of some chronic diseases, but it is extensively destroyed in the GI tract and rarely accumulates in the pigmented region of human eyes (Gumus et al., 2016). Therefore, it is important that the emulsion-based delivery systems can release the lutein in an appropriate place, usually at the end of small intestinal, to increase its bioaccessibility. In this study, the bioaccessibility of emulsions stabilized by different emulsifiers was evaluated by a two-step simulated GI tract, and it was obtained by measuring the fragment of lutein content after the intestinal digestion (Qian et al., 2012; Yi et al., 2014).

As shown in Figure 6a, the average diameter of all emulsions increased significantly after the GI digestion compared to the initial measurement (Table 1). This might be due to (i) the presence of other highly surface-active substance in the GI tract, such as phospholipids and bile salts, which can replace the WPI or conjugates; and (ii) the digestion of corn oil into free acids and monoacylglycerols, resulting changes in oil phase materials (Qian et al., 2012).

Markman pointed out that conjugates between proteins and saccharides can alter the packing characteristics of emulsifiers and improve the curvature of oil droplets which stabilize the smaller oil diameter (Markman & Livney, 2012). As shown in Figure 6a, the droplet size was 937.6, 876.3, and 911.7 nm for the emulsions stabilized by (CA–WPI)–DEX conjugates, (CA–WPI)–DEX conjugates plus VE and WPI–DEX conjugates, respectively, which is less than that of WPI/CA–WPI conjugates. The similar results were

also found in the emulsions stabilized by lactoferrin–DEX (Liu et al., 2016).

Finally, we evaluated the bioaccessibility of lutein in emulsions prepared by different materials, and the results showed that the bioaccessibility ranged from 55% to 70% (Figure 6b). On the whole, the bioaccessibility of the emulsions coated with dextran was higher than that of WPI/CA–WPI conjugates. Compared with WPI, the bioaccessibility of emulsions stabilized by WPI–DEX/(CA–WPI)–DEX conjugates improved 13.2% and 18.1%, respectively. The similar results have been reported in the previous study, where decreasing the droplets diameter can improve the bioaccessibility of carotenoids (Salvia-Trujillo, Qian, Martin-Belloso, & McClements, 2013; Yi et al., 2014). The previous study also indicated that the free fatty acids digested from lipid by pancreatic lipase may form mixed micelles with the phospholipids and bile acids, which can transfer the carotenoids to epithelium cells and cause an increase in bioaccessibility of lutein (Tyssandier, Lyan, & Borel, 2001). Therefore, the reason for high bioaccessibility might be that the droplets diameter of emulsions stabilized by protein–DEX conjugates was smaller than that of WPI/CA–WPI conjugates (Table 1), resulting an increase in the surface area and a faster lipid digestion.

In addition, the results indicated that the emulsions with higher bioaccessibility was accompanied by smaller particle size after digestion. The previous study has shown that the emulsions with different interfacial structure have different micellization rate and particle size after digestion (Liu, Hou, Lei, Chang, & Gao, 2012). Therefore, two possible reasons may explain this: (i) a portion of emulsion droplet coated with dextran remain intact and exhibited small diameter due to its strong steric hindrance; and (ii) the others underwent rapid lipolysis, and formed small lutein micelles with phospholipids and bile acids. Additionally, it will further affect the metabolism of lutein in the blood system.

However, compared with the emulsions prepared by (CA–WPI)–DEX conjugates, the VE-based emulsions exhibited high bioaccessibility but no significance ($P > 0.05$). As the emulsifiers used in the both emulsions were the same (ternary conjugates), it is supposed that the VE could not significantly regulate lipid digestion in the GI tract. Further experiments are needed.

4. CONCLUSIONS

Lutein can be used as a functional supplement to reduce the risk of chronic diseases, as well as a colorant in foods. WPI–CA–stabilized lutein emulsions exhibit good chemical stability but are prone to aggregation under different environmental stresses, whereas WPI–DEX–stabilized emulsions have a good physical stability but are not significant to improve the chemical stability. The ternary conjugates could produce a physically and chemically stable emulsion under the same conditions. The addition of VE into emulsions stabilized by ternary conjugates can effectively reduce the degradation rate of lutein without affecting the physical stability of the emulsion. In addition, VE group exhibited the best bioaccessibility. Consequently, the emulsion stabilized by polyphenol–protein–polysaccharide and VE can be an effective delivery system in food products.

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AUTHOR CONTRIBUTIONS

Hao Wang and Qiao-mei Zhu conceived and designed the experiments; Yong Yan, Xiang-ru Feng, Zi-jian Wu, Ya-tu Guo, and He-yu Li performed the experiments and analyzed the data simultaneously; and Hao Wang wrote the paper and had primary responsibility for final content. All authors have read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure 1S–The changes of Turbiscan stability index (TSI) under different environmental stresses.

Figure 2S–TEM image of the emulsions stabilized by (CA–WPI)–DEX con and VE.

Figure 3S–DPPH scavenging activity of WPI, CA–WPI conjugates, and (CA–WPI)–DEX conjugates