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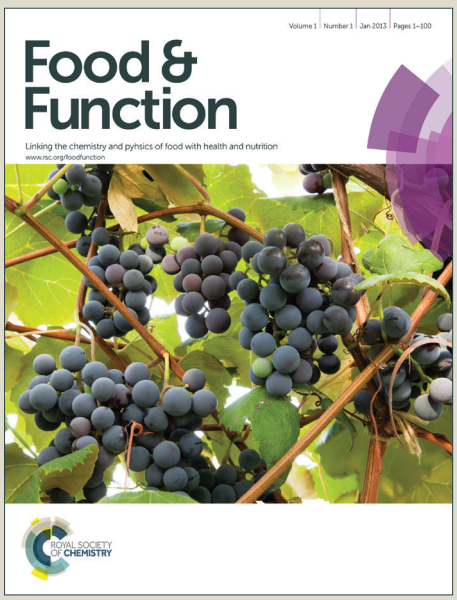

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Designing excipient emulsions to increase nutraceutical bioavailability: Emulsifier type influences curcumin stability and bioaccessibility by altering gastrointestinal fate

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Abstract

The influence of emulsifier type on the ability of *excipient emulsions* to improve the solubility, stability, and bioaccessibility of powdered curcumin was examined. Oil-in-water emulsions prepared using three different emulsifiers (whey protein isolate, caseinate, or Tween 80) were mixed with curcumin powder and then incubated at either 30 ºC (to simulate applications of salad dressings) or 100 ºC (to simulate applications of cooking sauces). The transfer of curcumin into the excipient emulsions was appreciably higher for excipient emulsions held at 100 ºC than those held at 30 ºC, and was appreciably higher for surfactant-stabilized emulsions than protein-stabilized emulsions. For example, the amounts of curcumin transferred into emulsions held at 30 and 100 ºC were 66 and 280 µg/mL for Tween 80, but only 17 and 208 µg/mL for caseinate. The total curcumin concentration in the digesta and mixed micelle phases collected after excipient emulsions were exposed to a simulated gastrointestinal tract (mouth, stomach, and small intestine) depended on emulsifier type. The total amount of curcumin within the digesta was higher for protein-stabilized emulsions than surfactant-stabilized ones, which was attributed to the ability of the proteins to protect curcumin from chemical degradation. For example, the digesta contained 204 µg/mL curcumin for caseinate emulsions, but only 111 µg/mL for Tween 80 emulsions. This study shows the potential of designing excipient emulsions to increase the oral bioavailability of curcumin for food and pharmaceutical applications.

Keywords: Nutraceutical; Curcumin; Nanoemulsions; Excipient Emulsions; Bioavailability

1. Introduction

Turmeric is widely used in food preparations as a spice, pigment, and nutraceutical because of its characteristic flavor profile, yellow color, and biological activity [1, 2]. Curcumin is one of the most biologically active components within turmeric, and has been used for the treatment of numerous ailments, including coughs, fevers, jaundice, wounds, eczema, inflammatory joints, parasitic skin diseases, colds, liver diseases, urinary diseases, anemia, bacterial infections, and viral infections [1]. The claimed health benefits of curcumin are attributed to a broad spectrum of biological effects, including anti-inflammatory, antioxidant, antiviral, antibacterial, antifungal, and antitumor activities [3]. Furthermore, curcumin has a low toxicity, even when ingested at relatively high doses (as high as 12 g per day) as indicated by clinical trials [4].

The potential health benefits of curcumin, as well as its good safety profile, has led to interest in incorporating it into functional food products as a nutraceutical ingredient. However, the high melting point and poor water solubility of curcumin make it difficult to incorporate into many functional foods and beverages, and its poor stability (metabolism and chemical transformation) and low bioaccessibility within the gastrointestinal tract reduce its biological activity [4, 5]. The use of curcumin as a nutraceutical ingredient can be facilitated by developing strategies to improve its solubility, stability, and bioaccessibility. One strategy for improving the functional performance of curcumin is to encapsulate it within food-grade delivery systems such as nanoparticles [6-9], hydrogel beads [10], biopolymer complexes [11], emulsions [12-15], colloidosomes [16] and amorphous solid suspensions [17]. These delivery systems are

designed to facilitate the incorporation of curcumin into commercial food and beverage products, as well as to improve its overall bioavailability characteristics.

An alternative strategy for increasing curcumin bioavailability is to develop *excipient foods* that are consumed with curcumin-rich foods. In general, the composition and microstructure of excipient foods are designed to increase the bioavailability of nutraceuticals (or pharmaceuticals) located in foods (or drugs) they are co-ingested with it [18]. Previous studies have already demonstrated the potential of excipient foods for enhancing the bioavailability of hydrophobic nutraceuticals. For example, the bioavailability of carotenoids from raw fruits and vegetables can be increased by co-ingesting them with certain lipids [19-21]. Oil-in-water emulsions are particularly suitable candidates for creating excipient foods because they can simply be prepared, their composition and structure can easily be manipulated, and they are already widely used in food and beverage products [15, 22-26]. Indeed, in previous studies, we have shown that surfactant-stabilized oil-in-water emulsions containing long chain triglycerides (corn oil) can increase the bioaccessibility of powdered curcumin [27, 28]. This effect was mainly attributed to the ability of the lipid digestion products (free fatty acids and monoglycerides) to form mixed micelles in the intestinal fluids that could solubilize and transport the curcumin molecules.

In the present study, we examined the influence of emulsifier type on the ability of excipient emulsions to solubilize curcumin, and to increase its bioaccessibility in a simulated gastrointestinal tract model. Previous studies have shown that emulsifier type has an important impact on the digestion of lipid droplets, as well as on the release of encapsulated lipophilic bioactive agents [29-32]. We therefore hypothesized

that the type of emulsifier used to coat the lipid droplets in an excipient emulsion would impact its ability to increase curcumin bioaccessibility. Three different food-grade emulsifiers (Tween 80, caseinate, and whey protein isolate) were used to prepare the excipient emulsions. Tween 80 is a small molecule non-ionic surfactant, whey protein isolate (WPI) consists primarily of globular proteins, and caseinate consists primarily of flexible random coil proteins [33]. The excipient emulsions were mixed with powdered curcumin under conditions chosen to simulate practical applications in the food industry: 30 ºC for 30 min to simulate the utilization of curcumin in a salad dressing; 100 ºC for 10 min to simulate the utilization of curcumin in a cooking sauce. The effect of emulsifier type and incubation conditions on the transfer of curcumin from the powder into the excipient emulsions was studied, as well as their influence on curcumin bioaccessibility after exposure of the excipient emulsions to a simulated gastrointestinal tract (GIT). The information obtained from this study would provide important information for the rational design of excipient emulsions to increase the bioavailability and bioactivity of lipophilic nutraceuticals. To our knowledge, this is the first study of the influence of emulsifier type on the potential efficacy of excipient emulsions.

2. Experimental

2.1. Materials

Corn oil was used as an example of a digestible long chain triglyceride, which was purchased from a local supermarket. The following chemicals were purchased from the Sigma Chemical Company (St. Louis, MO): curcumin (C1386-10G); mucin from porcine stomach (M2378-100G); pepsin from porcine gastric mucosa

115 (P7000-25G); lipase from porcine pancreas pancreatin (L3126-100G); porcine bile
116 extract (B8631-100G); Tween 80 (P1754-1L); and Nile Red (N3013-100MG). The
117 purity of the curcumin was ≥ 65%. Sodium caseinate was purchased from the
118 American Casein Company (Burlington, NJ). Whey protein isolate (WPI) was
119 supplied by Davisco Foods International Inc. (Le Sueur, MN, USA). All other
120 chemicals were of analytical grade. Double distilled water was used to prepare all
121 solutions and emulsions.

122 **2.2. Excipient emulsion preparation**

123 Initially, aqueous phase was prepared by mixing 1% (w/w) Tween 80, sodium
124 caseinate, or WPI with an aqueous buffer solution (10.0 mM phosphate buffer saline
125 (PBS), pH 6.5) and stirring for at least 2 h. The emulsifier solution were then stored
126 overnighted at 4 °C to ensure complete hydration. The sodium caseinate or WPI
127 solution were filtered using filter paper (Fisher Scientific, PA) to remove any
128 insoluble particles before further use.

129 Excipient emulsions stabilized by different emulsifiers were prepared by
130 homogenizing 10% (w/w) corn oil with 90% (w/w) aqueous emulsifier solution using
131 a high-speed blender for 2 min (M133/1281-0, Biospec Products, Inc., ESGC,
132 Switzerland). The resulting coarse emulsions were then passed through a high
133 pressure homogenizer (Microfluidizer, M110Y, Microfluidics, Newton, MA) with a
134 75 μm interaction chamber (F20Y) at an operational pressure of 12,000 psi for 3
135 passes. The resulting excipient emulsions were stored in a refrigerator at 4 ºC before
136 use.

2.3. Preparation of curcumin-emulsions mixtures

Curcumin (9 mg) was weighed into a beaker and then excipient emulsions (30 mL) was added. These amounts were selected to ensure that the curcumin could be fully dissolved within the oil phase of the emulsions at equilibrium, since the solubility of curcumin within corn oil has previously been reported to be ≈ 3.2 ± 0.1 mg/mL at ambient temperature [34]. The resulting mixtures were then incubated at either 30 ºC for 30 min or 100 ºC for 10 min to simulate a salad dressing or a cooking sauce, respectively. After incubation, the samples were immediately placed in an ice water bath to cool them down, and then used for the following experiments.

2.4. Curcumin solubility in excipient emulsions

The solubility of curcumin in each excipient emulsion was measured spectrophotometrically based on the method of Ahmed, et al. [34] with some modifications. 10 mL of curcumin-excipient emulsion mixture was collected, and then centrifuged at 1750 rpm (≈940 × g) for 10 min at ambient temperature (CL10 centrifuge, Thermo, Scientific, Pittsburgh, PA, USA) to remove non-dissolved (crystalline) curcumin. For the Tween 80 emulsions, 1 mL of the resultant supernatant was mixed with 5 mL of choloroform, vortexed, and then centrifuged at 1750 rpm (≈940 × g) for 10 min at ambient temperature. For caseinate and WPI emulsions, pepsin was used to hydrolyze the protein emulsifier prior to isolation, so as to prevent extensive aggregation of the proteins during extraction. The bottom layer containing the solubilized curcumin was collected, while the top layer was mixed with an additional 5 mL of chloroform and the same procedure was repeated. The two bottom

chloroform layers were combined, and diluted to an appropriate concentration to be analyzed by a UV–VIS spectrophotometer at 419 nm (Ultraspec 3000 pro, GE Health Sciences, USA). A cuvette containing pure chloroform was used as a reference cell. The concentration of curcumin extracted from each mixture was calculated from a calibration curve of absorbance *versus* curcumin concentration in chloroform. The solubility of curcumin in each mixture was then calculated as the concentration of curcumin extracted from each mixture multiplied by the dilution factor.

2.5. Color analysis of mixtures

The color of curcumin-excipient emulsion mixtures after centrifugation was carried out using a colorimeter (ColorFlex EZ 45/0-LAV, Hunter Associates Laboratory Inc., Virginia, USA). Color was expressed in CIE units L^* (lightness/darkness), a^* (redness/greenness), and b^* (yellowness/blueness). An aliquot of sample (15 mL) was placed in a 64-mm path length glass sample cup and then illuminated with D65-artificial daylight (10° standard angle). Three replicate measurements were performed and the results were averaged.

2.6. Particle characterization

The mean particle diameter and particle size distribution of excipient emulsions before and after exposure to gastrointestinal conditions were monitored using static light scattering (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). Samples were diluted with appropriate buffer solutions (same pH as sample) and stirred in the dispersion unit at a speed of 1200 rpm. The particle size is reported as the surface-weighted mean diameter (d_{32}). The electrical charges (ζ -potential) of the

particles in the samples were measured using a micro-electrophoresis instrument (Nano-ZS, Malvern Instruments, Worcestershire, UK). Samples were diluted with appropriate buffer solutions prior to measurements to avoid multiple scattering effects.

The mean particle diameters (Z-average), particle size, and electrical charges (ζ -potential) of micelle centrifuged from raw digesta were measured by a dynamic light scattering instrument and micro-electrophoresis instrument, respectively (Nano-ZS, Malvern Instruments, Worcestershire, UK). Micelles were diluted with buffer solution (5 mM PBS, pH 7.0) prior to measurements to avoid multiple scattering effects.

2.7. Microstructural analysis

The microstructure of samples was characterized using optical or confocal scanning fluorescence microscopy (Nikon D-Eclipse C1 80i, Nikon, Melville, NY). Samples analyzed by confocal microscopy were dyed with Nile Red (0.1%) to highlight the location of the lipid phases. All images were captured with a 10× eyepiece and a 60× objective lens (oil immersion). Changes in the properties of curcumin crystals in the mixtures were observed using a cross-polarized lens (C1 Digital Eclipse, Nikon, Tokyo, Japan).

2.8. Simulated gastrointestinal digestion

Powdered curcumin was mixed with excipient emulsions, and then held at 100 ºC for 10 min. Each sample was passed through a three-step GIT model that consisted of mouth, gastric, and small intestine phases, which was slightly modified from the our previous study [35].

Initial system: The curcumin-excipient emulsion mixtures were placed into a glass beaker in an incubator shaker (Innova Incubator Shaker, Model 4080, New Brunswick Scientific, New Jersey, USA) at 37 °C. The initial concentration of curcumin and corn oil was the same in all curcumin-excipient emulsion mixtures.

Mouth phase: A simulated saliva fluid (SSF) containing 3 mg/mL mucin and various salts was prepared as described previously [36]. SSF was preheated to 37 °C and then mixed with the preheated curcumin-excipient emulsion mixture at a 1:1 mass ratio. The mixture was then adjusted to pH 6.8 and placed in a shaking incubator at 100 rpm and 37ºC for 10 min to mimic oral conditions

Stomach phase: Simulated gastric fluid (SGF) was prepared by placing 2 g NaCl and 7 mL HCl into a container, and then adding double distilled water to 1 L. The bolus sample from the mouth phase was then mixed with SGF containing 0.0032 g/mL pepsin preheated to 37 °C at a 1:1 mass ratio. The mixture was then adjusted to pH 2.5 and placed in a shaker at 100 rpm and 37 ºC for 2 hours to mimic stomach digestion.

Small Intestine phase: 24 mL chyme samples from the stomach phase were diluted with buffer solution (10 mM PBS, 6.5) to obtain a final corn oil concentration of 1.0%. The diluted chyme was then incubated in a water bath (37 ºC) for 10 min and then the solution was adjusted back to pH 7.0. Next, 3 mL of simulated intestinal fluid (containing 0.5 M $CaCl_2$ and 7.5 M NaCl) was added to 60 mL digesta. Then, 7 mL bile extract, containing 375.0 mg bile extract (pH 7.0, PBS), was added with stirring and the pH was adjusted to 7.0. Finally, 5 mL of lipase suspension, containing 120 mg

of lipase (pH 7.0, PBS), was added to the sample and an automatic titration unit (Metrohm, USA Inc.) was used to monitor the pH and control it to a fixed value (pH 7.0) by titrating 0.25 M NaOH solution into the reaction vessel for 2 h at 37 ℃. The percentage of free fatty acids released in the sample was calculated from the number of moles of NaOH required to maintain neutral pH as described previously [37].

2.9. Curcumin concentration and bioaccessibility after digestion

After *in vitro* digestion, 30 mL raw digesta of each mixture was centrifuged (18000 rpm, ≈ 38,465 × g, Thermo Scientific, Waltham, MA, USA) at 25 ºC for 30 min. The clear supernatant was collected and assumed to be the ‘‘micelle’’ fraction in which the curcumin was solubilized. In some samples, a layer of non-digested oil was observed at the top of the test tubes and it was excluded from the micelle fraction. Aliquots of 5 mL of raw digesta or micelle fraction were mixed with 5 mL of chloroform, vortexed and centrifuged at 1750 rpm (≈940 × g) for 10 min at ambient temperature. The bottom layer containing the solubilized curcumin was collected, while the top layer was mixed with an additional 5 mL of chloroform and the same procedure was repeated. The two collected chloroform layers were mixed together, and then diluted to an appropriate concentration to be analyzed by a UV–VIS spectrophotometer at 419 nm. The concentration of curcumin in the overall raw digesta or in the mixed micelle phase were calculated from the absorbance using a standard curve and the known dilution factor.

The bioaccessibility was calculated from this data using the following equation:

$$\text{Bioaccessibility} = 100 \times (C_{Micelle} / C_{Raw\ Digesta})$$

Here, $C_{Micelle}$ and $C_{Raw\ Digest}$ are the concentration of curcumin in the micelle fraction and in the overall raw digesta after the pH-stat experiment, respectively.

2.10. Statistical analysis

All experiments were carried out on at least two freshly prepared samples. The results are expressed as means ± standard deviations (SD). Data were subjected to statistical analysis using SPSS software (version 18.0). Means were subject to Duncan's test and a *P*-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Effect of emulsifier type and incubation conditions on excipient emulsion stability

3.1.1. Influence on particle size

The curcumin-excipient emulsion mixtures were held at two incubation temperatures to simulate different food applications: (i) 30 ºC for 30 min to mimic adding curcumin to a salad dressing; (ii) 100 ºC for 10 min to mimic adding curcumin to a cooking sauce. The mixture of powdered curcumin and excipient emulsion was stirred to ensure homogeneity, and then exposed to one of the incubation conditions.

At 30 ºC, the excipient emulsions were very stable to changes in droplet characteristics (**Table 1** and **Figure 1a**). Indeed, there were no significant changes in mean droplet diameter (d_{32}), particle size distribution, or ζ-potential of the emulsions compared to the initial mixtures (data not shown). There was also little

change in the properties of the protein-coated droplets in the excipient emulsions after being incubated at 100 ºC for 10 min. On the other hand, there was a slight increase in the mean droplet diameter and evidence of a few larger droplets in the particle size distribution for the Tween 80 excipient emulsions after being held at this elevated temperature (**Figure 1a** and **Table 1**). This effect was attributed to some coalescence of the oil droplets coated by the non-ionic surfactant at elevated temperatures due to dehydration of their polar head groups.

3.1.2. Influence on microstructure

The microstructures of the curcumin-excipient emulsion mixtures after incubation at either 30 or 100 ºC were recorded using confocal fluorescence microscopy (**Figure 2a**). There was no observable change in the microstructure of any of the excipient emulsions incubated at 30 ºC, confirming that they had high stability to droplet aggregation at ambient temperature. There was also no observable change in the microstructure of the excipient emulsions containing protein-coated oil droplets after incubation at 100 ºC. However, there was evidence of some larger oil droplets in the Tween 80 emulsions, which suggested that some coalescence had occurred, which again is in agreement with the particle size measurements (**Figure 1a**).

Previous studies have reported that oil droplets stabilized by dairy proteins (caseins or whey) are resistant to heat treatment when heated at neutral pH and low ionic strengths. For example, caseinate-coated oil droplets were shown to be stable to heating at 121°C for 15 min [38], whereas whey protein-coated droplets were stable

to heating at 121 °C for 16 min [39]. The good heat stability of these emulsions can be attributed to the strong electrostatic and steric repulsion between the oil droplets under these conditions.

3.1.3. Influence on particle charge

There was no significant change in the magnitude of the electrical charge on the emulsion droplets for all three emulsifier types when they were incubated at either 30 or 100 ºC (**Table 1**), which suggested that the composition of the interfacial layers did not change appreciably after exposure to either incubation temperature.

3.1.4. Influence on curcumin dissolution and transfer

We also measured the effect of emulsifier type on the amount of curcumin transferred into the excipient emulsions after incubation at 30 or 100 ºC. The amount of curcumin transferred into the excipient emulsions depended on both incubation temperature and emulsifier type (**Table 1**). The level of curcumin within the excipient emulsions was much higher after incubation at 100 ºC than at 30 ºC (**Table 1**). For example, the amount of curcumin solubilized in the caseinate emulsions was around 17 and 208 μg/mL after incubation at 30 and 100 ºC, respectively. This effect can mainly be attributed to the fact that the equilibrium solubility of curcumin in both water and oil phases increases with temperature [40], which would have increased the solubilization rate. In addition, the mass transfer rate of curcumin increases with temperature because of the reduction in aqueous phase viscosity.

The amount of curcumin transferred into the excipient emulsions was considerably higher for the surfactant-coated oil droplets (Tween 80) than the protein-coated ones (WPI and caseinate) at both incubation temperatures (**Table 1**). A possible explanation for this phenomenon is that the proteins formed a physical barrier around the oil droplets that was more effective at inhibiting the transfer of the curcumin molecules across the oil-water interface. Alternatively, adsorbed proteins may have bound some of the curcumin molecules at the droplet surfaces, or non-adsorbed proteins may have bound some of the curcumin molecules in the aqueous phase.

Differences in the amount of curcumin transferred into the excipient emulsions could also be clearly observed by visual observation of mixtures after centrifugation (**Figure 1b**). The yellow color of the mixtures incubated at 100 ºC was much deeper than that of the mixtures incubated at 30 ºC. Additionally, there were more curcumin crystals at the bottom of the mixtures incubated at the lower temperature. Emulsifier type also had an appreciable impact on the visual appearance of the excipient emulsions. At similar incubation temperatures, the yellow color of the Tween 80 emulsions was much deeper than that of the caseinate or WPI emulsions, and they had less crystals visible at the bottom of the tubes. These differences in appearance were quantified instrumentally using a colorimeter (**Table 1**). The a^* values of all the mixtures was fairly similar, but there were significant differences in the b^* and L^* values (**Table 1**). This is because the b^* values are sensitive to color changes in the yellow-blue axis, whereas the a^* values are only sensitive to color

changes in the red-green axis. In addition, the L^* values are reduced when there is a decrease in light scattering or an increase in color intensity [41]. The b^* values of all mixtures incubated at 100 ºC were higher than those incubated at 30 ºC, indicating that they had a more intense yellow color due to greater curcumin solubilization. Additionally, the b^* values of the Tween 80 emulsions were higher than those of the WPI and caseinate emulsions at both incubation temperatures, indicating that more curcumin was incorporated into the surfactant-stabilized emulsions than into the protein-stabilized emulsions. These results are therefore in good agreement with the measurements of the amount of curcumin transferred into the excipient emulsions discussed earlier.

The presence of curcumin crystals within some of the mixtures was confirmed using a crossed polarizer lens in the optical microscope (**Figure 2b**). Crystalline material was clearly observed in all the excipient emulsions incubated at 30 ºC, which suggested that the curcumin crystals did not fully dissolve when held at this temperature in the presence of the excipient emulsion. Conversely, no crystals were observed in any of the excipient emulsions incubated at 100 ºC, which indicated that all the curcumin crystals had rapidly dissolved and been incorporated into the oil droplets. The microscopy measurements also indicated that there were some large oil droplets in the Tween 80 emulsions after heating at 100 ºC for 10 min, again suggesting that some droplet coalescence had occurred.

3.2. Influence of emulsifier type on gastrointestinal fate of excipient emulsions

In this section, we examined the influence of initial emulsifier type on the potential fate of the excipient emulsions in the gastrointestinal tract (GIT) using an *in vitro* model that simulated the mouth, stomach, and small intestine. Curcumin-excipient emulsions incubated at 100 ºC for 10 min were selected for this study, since they gave the highest amount of curcumin transfer. The results of these experiments are useful for understanding the influence of excipient emulsions (such as cooking sauces) on the bioaccessibility of powdered curcumin.

3.2.1. Influence on particle size

The mean particle diameters (d_{32}) of the Tween 80, caseinate, and WPI excipient emulsions after incubation at 100 ºC were around 0.24, 0.21 and 0.18 μm, respectively. Consequently, all of the excipient emulsions initially had fairly similar particle sizes prior to exposure to the simulated gastrointestinal tract.

Surfactant-stabilized excipient emulsions: For the Tween 80 emulsions, the mean particle diameter remained fairly constant after exposure to the mouth and stomach phases, but increased appreciably after exposure to the small intestine phase (**Figure 3**). Examination of the particle size distributions of the Tween 80 emulsions after exposure to the mouth and stomach phases indicated that they contained a large fraction of droplets with similar sizes to those in the initial emulsions, which suggested that these droplets were relatively stable to coalescence (**Figure 4a**). This was presumably because the oil droplets were coated by a layer of non-ionic surfactant and were therefore fairly resistant to changes in pH, salt, and protease

activity. The particle size distribution of the Tween 80 emulsions after exposure to the small intestinal phase indicated that there was a wide range of different-sized particles present. Presumably, these particles consisted of non-digested lipid droplets, mixed micelles (micelles and liposomes), and insoluble calcium salts remaining after the lipid digestion step.

Protein-stabilized excipient emulsions: For the caseinate and WPI emulsions, the mean particle diameter remained relatively constant after exposure to the mouth, increased markedly after exposure to the stomach phase, and then decreased after exposure to the small intestine phase (**Figures 3**). The particle size distributions indicated that an appreciable fraction of the particles in the excipient emulsions exposed to the mouth phase had fairly similar dimensions to those in the initial emulsions (**Figure 4b and 4c**), which suggested that the oil droplets were stable to coalescence under simulated oral conditions. However, there was an appreciable increase in the particle size in the excipient emulsions after exposure to the stomach phase, indicating that extensive droplet aggregation had occurred. This effect may be attributed to a number of physicochemical phenomenon occurring in the simulated gastric phase, including reduction of the electrostatic repulsion between the oil droplets due to changes in pH and ionic strength, bridging and depletion flocculation induced by biopolymers, and coalescence resulting from digestion of the adsorbed protein layer by pepsin. After exposure to simulated small intestine conditions, the particle size decreased somewhat, but was still much larger than the initial emulsions. These particles were probably a mixture of non-digested lipid droplets, mixed

micelles, and calcium soaps. Similar behavior has been reported in other studies of the digestion of oil-in-water emulsions containing oil droplets coated by different kinds of emulsifiers [35, 42, 43].

3.2.2. Influence on microstructure

Information about changes in the microstructure of the excipient emulsions in different regions of the GIT was obtained by confocal fluorescence microscopy.

Surfactant-stabilized excipient emulsions: The microscopy images indicated that the droplets in the Tween 80 emulsions underwent extensive flocculation in both the mouth and stomach phases (**Figure 5**), which was not detected by light scattering (**Figures 3 and 4**). These results suggest that the flocs in these emulsions were held together by relatively weak attractive forces that were disrupted when the samples were prepared for the light scattering measurements. This behavior is in agreement with previous studies [26, 35], and may be caused by sample dilution and/or mechanical agitation during sample preparation [33, 44]. After exposure to the small intestine stage, some large lipid particles were clearly visible in the confocal microscopy images (**Figure 5**), which may have been non-digested oil droplets or large colloidal particles formed by lipid digestion products, such as vesicles.

Protein-stabilized excipient emulsions: The microscopy images indicated that the protein-stabilized emulsions contained larger aggregates than the surfactant-stabilized ones after exposure to mouth and stomach conditions (**Figure 5**). Again, the microscopy images showed that appreciable droplet flocculation occurred within the

mouth phase, which was not detected by the light scattering method (**Figures 3 and 4**). This effect can again be attributed to dissociation of the flocs during sample preparation. Previous studies have also indicated that protein-stabilized emulsions are more sensitive to aggregation under gastric conditions than surfactant-stabilized ones [35]. The instability of the protein excipient emulsions may occur for a number of reasons: (i) hydrolysis of adsorbed proteins may reduce droplet stability to coalescence; (ii) changes in pH and ionic strength may reduce droplet stability to aggregation by weakening electrostatic repulsion; (iii) biopolymers (such as mucin) may promote depletion or bridging flocculation [35]. In the small intestine phase, the size of the particles was appreciably less than that observed in the stomach phase. As mentioned earlier, these particles are probably a mixture of non-digested fat, lipid digestion products, and calcium soaps [26].

Overall, the particle size and microstructure measurements indicated that the protein-stabilized emulsions behaved differently than the surfactant-stabilized ones in the simulated gastrointestinal tract. In particular, the protein-stabilized emulsions were much more prone to extensive aggregation in the stomach, which may have an appreciable influence on lipid digestion and curcumin release in the small intestine.

3.2.3. Influence on particle charge

The electrical characteristics (ζ-potential) of the particles in the excipient emulsions was also measured after exposure to different regions of the simulated GIT (**Figure 6**).

436 *Surfactant-stabilized excipient emulsions:* The particles in the initial Tween 80
437 emulsions had relatively low negative charges (≈ −5 mV) due to the fact that the
438 droplets were coated by a non-ionic surfactant. After exposure to the mouth phase the
439 particles became more negative (−11 mV), which can be attributed to adsorption of
440 anionic species (such as mucin) from the simulated saliva to the droplet surfaces [26].
441 After exposure to the stomach phase, there was a decrease in the magnitude of the
442 negative charge on the droplets (≈ −2 mV), which may be due to electrostatic
443 screening effects associated with the low pH and high ionic strength of the gastric
444 fluids. After exposure to the small intestine conditions, the particle charge became
445 highly negative (≈ -40 mV), which is probably due to the presence of colloidal
446 particles containing various types of anionic constituents, such as bile salts,
447 phospholipids, and free fatty acids [26].
448 *Protein-stabilized excipient emulsions:* The initial caseinate- and WPI-stabilized
449 excipient emulsions had relatively high negative charges (−34 and -38 mV) due to the
450 fact that the pH (6.5) used was appreciably higher than their isoelectric point (around
451 pH 4.5 to 5). The charge on these particles became less negative after exposure to
452 the mouth phase (−26 and -32 mV), which may have been due to electrostatic
453 screening due to the presence of mineral ions in the simulated oral fluids, or due to
454 interactions with mucin [35]. After passing through the stomach phase, there was a steep
455 decrease in the magnitude of the negative charge on the droplets in the
456 protein-stabilized emulsions (≈ −2 mV). This effect can be attributed to a number of
457 phenomena: (i) electrostatic screening effects caused by the high ionic strength of the

458 gastric fluids; (ii) alterations in protein charge due to the change in solution pH; (iii)
459 adsorption of anionic biopolymers (mucin) to the droplet surfaces; and, (iv) digestion
460 of the protein layer by proteases in the gastric fluids. One might expect
461 protein-stabilized oil droplets to have positive charges under highly acidic conditions
462 (pH 2.5) because this pH is well below their isoelectric points. However, the
463 adsorption of anionic mucin to the droplet surfaces may have caused the
464 protein-coated droplets to have a net negative charge [35]. After exposure to the small
465 intestine fluids, the protein-stabilized excipient emulsions both became highly
466 negative (≈ -40 mV), which is due to the relatively high levels of anionic constituents
467 in the intestinal fluids after digestion, such as bile salts, phospholipids, and free fatty
468 acids [26].

469 Overall, these results again show that there were appreciable differences in the
470 properties of the excipient emulsions depending on the initial emulsifier type, which
471 may influence their digestion and curcumin release in the small intestine.

472 *3.2.4. Influence on lipid digestion*

473 In this section, we used the pH stat method to evaluate the effect of initial
474 emulsifier type on the rate and extent of lipid digestion of excipient emulsions that
475 had been incubated with curcumin at 100 ºC for 10 min. The volume of NaOH that
476 had to be titrated into the mixtures to maintain a constant pH (7.0) was measured as a
477 function of digestion time, and then the fraction of titrable free fatty acids released
478 was calculated.

Emulsifier type had an appreciable influence on the digestion of the excipient emulsions (**Figure 7**). The behavior of the Tween 80 and WPI excipient emulsions was fairly similar: most of the emulsified lipid was digested within the first 10 min, and then there was a more gradual increase in free fatty acids afterward, until a relatively constant final value was reached. The initial rate of digestion was slightly faster in the Tween 80 emulsions, which might be attributed to their smaller initial droplet size when entering the intestinal phase, which would lead to a higher surface area of lipid exposed to the lipase. The fact that the WPI-coated and Tween 80-coated droplets were digested at a similar rate was somewhat surprising since these two excipient emulsions had very different behaviors in the mouth and stomach phases, as seen in the particle size and microstructure measurements (**Figure 3 and Figure 5**). This was probably due to the fact that the bile salts and phospholipids in the small intestinal fluids were still able to adsorb to the oil droplet surfaces and displace the original emulsifiers, thereby enabling subsequent lipase adsorption and digestion of the triacylglycerols to occur [43]. In addition, any flocs formed in the stomach by the WPI-coated droplets may have been disrupted under small intestine conditions. The excipient emulsion containing caseinate-coated droplets was digested considerably slower than the other two emulsions, and the final amount of free fatty acids produced was also lower. Previous studies have also reported a slower rate and lower extent of lipid digestion for caseinate-stabilized emulsions compared to other emulsions [35, 43]. The inhibition of lipid digestion in caseinate-stabilized emulsions mixture has been attributed to extensive droplet flocculation in the stomach. When the chyme from the

stomach phase is mixed with simulated small intestinal fluids, it is more difficult for lipase molecules to access the undigested triacylglycerol molecules in flocculated emulsions than in non-flocculated ones. Additionally, caseinate forms a relatively thick steric layer around oil droplets [45], which may also significantly hinder the ability of lipase molecules to access undigested triacylglycerols within the droplets.

3.2.5. Influence on curcumin bioaccessibility

As mentioned earlier, the oral bioavailability of crystalline curcumin is normally limited by its chemical instability and low solubility in gastrointestinal fluids. We therefore measured the concentration of curcumin in the mixed micelle phase and in the total digesta to provide a measure of the amount of curcumin that was solubilized and the amount that was not chemically degraded (**Table 2**). These values were also used to calculate the bioaccessibility of the curcumin, *i.e.,* the fraction of curcumin in the small intestine phase that was solubilized within the mixed micelles. The properties of the colloidal particles present in the mixed micelle phase were also measured to assess whether emulsifier type influenced them (**Table 2**).

The mixed micelles obtained from all three different excipient emulsions had a highly negative charge, with the ζ-potential ranging from -46 to -53 mV. The most likely reason for this phenomena is that the micelles consisted primarily of anionic bile salts, phospholipids, and free fatty acids. In addition, the pH was above the isoelectric point of the proteins so that any undigested proteins would also have a negative charge. The mean diameters of the particles in the micelle phase obtained

from the three different excipient emulsions was also fairly similar ($d \approx 160$ nm), which suggested that the initial emulsifier type did not have a major impact on the nature of the final digestion products formed. It should be noted that the mixed micelle phase actually contains a mixture of small micelles (typically < 10 nm), and vesicles (d > 100 nm), which would account for the fairly large size measured in our study [46, 47].

The amount of curcumin present within the raw digesta and the mixed micelle phase provides a good indication of the stability of the curcumin during *in vitro* digestion and the amount available for absorption. The amount of curcumin present in the raw digesta decreased in the following order: caseinate > WPI > Tween 80 (**Table 2**). The amount of curcumin present in the micelle phase was fairly similar for both caseinate and WPI excipient emulsions, but lower for the Tween 80 excipient emulsion. The higher levels of curcumin present in the total digesta of the protein-stabilized emulsions suggest that the proteins protected the curcumin from chemical degradation within the simulated gastrointestinal fluids. Curcumin may have been protected in the presence of the proteins for a number of reasons. First, the proteins may have some antioxidant properties that inhibited the chemical degradation reaction. Second, the proteins formed a relatively thick layer around the oil droplets that may have physically reduced the interactions between the curcumin molecules and the aqueous phase [48, 49]. Third, the proteins may have formed complexes with the curcumin molecules that protected them from degradation. Fourth, the slower digestion rate of the caseinate emulsions may have increased the

544 amount of time the curcumin spent in the hydrophobic core of the lipid droplets
545 (rather than in close contact with the aqueous phase), which may have also enhanced
546 curcumin stability.

547 Interestingly, the bioaccessibility of curcumin was lower in the
548 caseinate-stabilized excipient emulsions than in the WPI- and Tween 80-stabilized
549 ones. There are two possible reasons for this phenomenon: (i) more undigested oil
550 remained in the caseinate emulsions, and some of the curcumin was trapped within
551 this undigested oil; (ii) there were more mixed micelles generated for the WPI and
552 Tween 80 emulsions that could solubilize the curcumin.

553 ## 4. Conclusions

554 The present study focused on the influence of the type of emulsifier used to form
555 excipient emulsions on their ability to increase the bioaccessibility of powdered
556 curcumin. Prior to digestion, the amount of curcumin transferred into the excipient
557 emulsions depended on incubation temperature and emulsifier type. There was a
558 greater amount of curcumin transferred from the powder into the oil droplets for
559 mixtures incubated at 100 ºC than for those incubated at 30 ºC. The transfer of
560 curcumin into the excipient emulsions was higher when the oil droplets were coated
561 with a surfactant (Tween 80) than when they were coated with a protein (caseinate or
562 WPI). The amount of curcumin in the raw digesta phase depended on emulsifier
563 type (caseinate > WPI > Tween 80), which was attributed to the ability of the proteins
564 to protect the curcumin from chemical degradation under simulated gastrointestinal
565 conditions. The bioaccessibility of curcumin also depended on emulsifier type (WPI ≈

Tween 80 > caseinate), which was attributed to the fact that caseinate inhibited lipid digestion. Consequently, there would be less curcumin released from the oil phase, and less mixed micelles available to solubilize the curcumin. Overall, the total amount of curcumin available for adsorption was highest for the caseinate-stabilized emulsions, and therefore they may be most suitable as excipient emulsions. A schematic representation of these effects is shown in **Figure 8**. Our results have important implications for designing excipient emulsions to increase the oral bioavailability of other lipophilic nutraceuticals and vitamins. Future research should focus on testing excipient emulsion bioavailability using cell culture, animal feeding, and human feeding studies.

Acknowledgments

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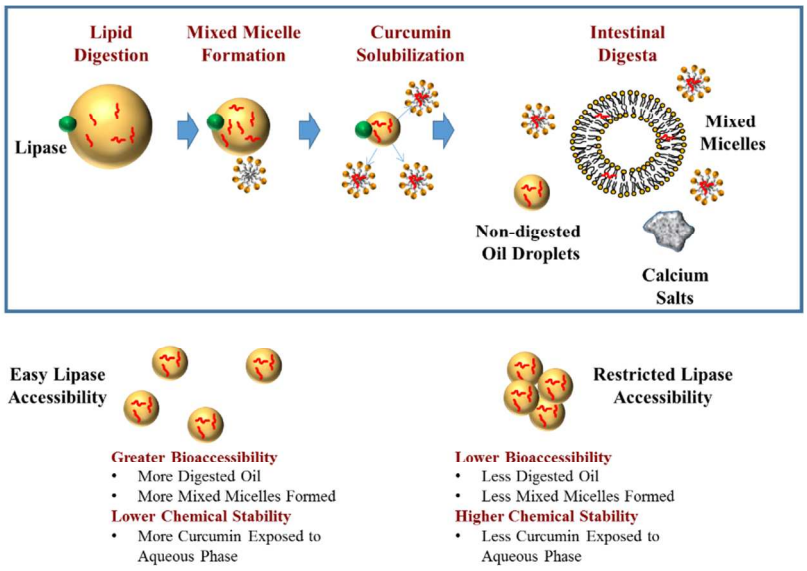
Graphical Abstract



"Designing excipient emulsions to increase nutraceutical bioavailability: Influence of Lipid Droplet Size on Solubility and Bioaccessibility of Powdered Curcumin"

Zhou et al

Excipient emulsions can be specifically designed to increase the bioavailability of curcumin from the powdered form. The bioavailability depends on the nature of the emulsifier used to coat the droplets in the excipient emulsion.

Figure 1 (a).

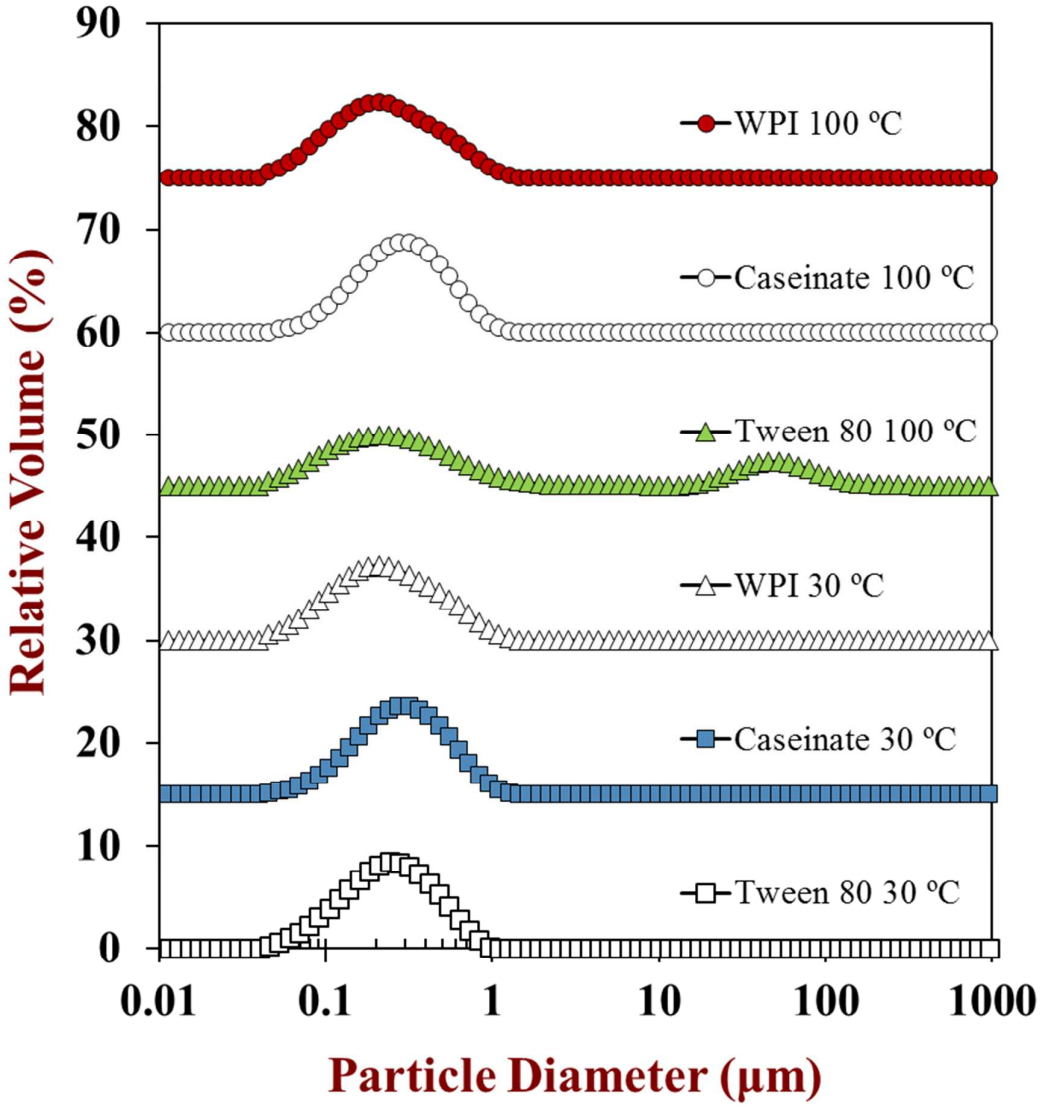


Figure 1 (a). Particle size distributions of mixtures of curcumin and excipient emulsion (Tween 80, caseinate and whey protein isolate (WPI) blank emulsion) measured by static light scattering after incubation at 30 ºC for 30 min and 100 ºC for 10 min;

Figure 1 (b).

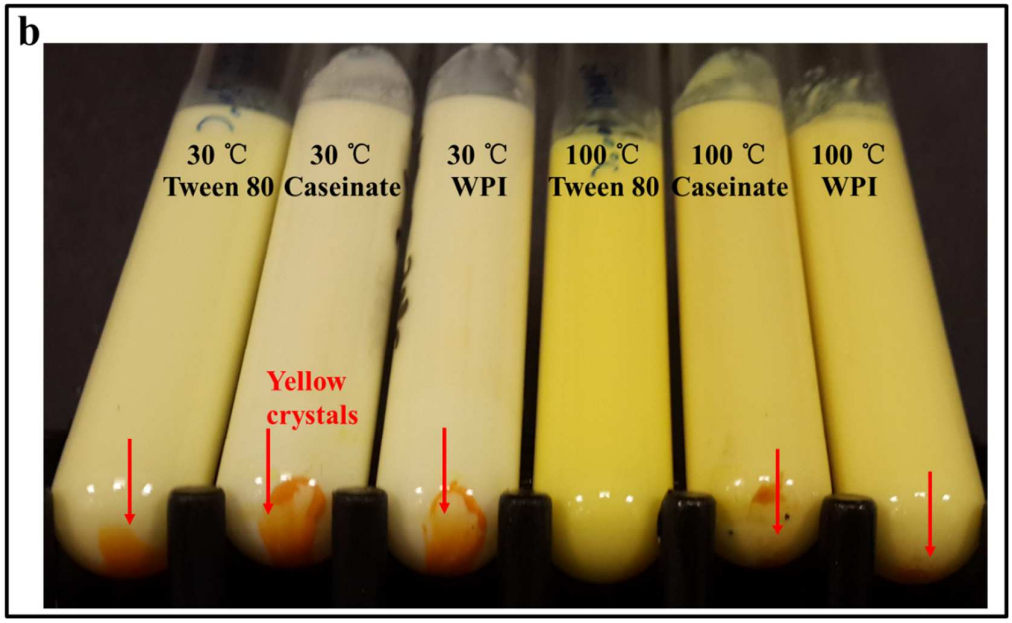


Figure 1 (b). Image of mixtures of curcumin and excipient emulsion (Tween 80, caseinate and whey protein isolate (WPI) blank emulsion) after incubation at 30 and 100 ºC, and the concentration of curcumin in the mixtures is 3 mg/mL.

Figure 2. Effect of temperature and emulsifier type (Tween 80, caseinate and WPI) on: (a) microstructure or (b) polarized light microscopy of mixtures of curcumin and excipient emulsion.

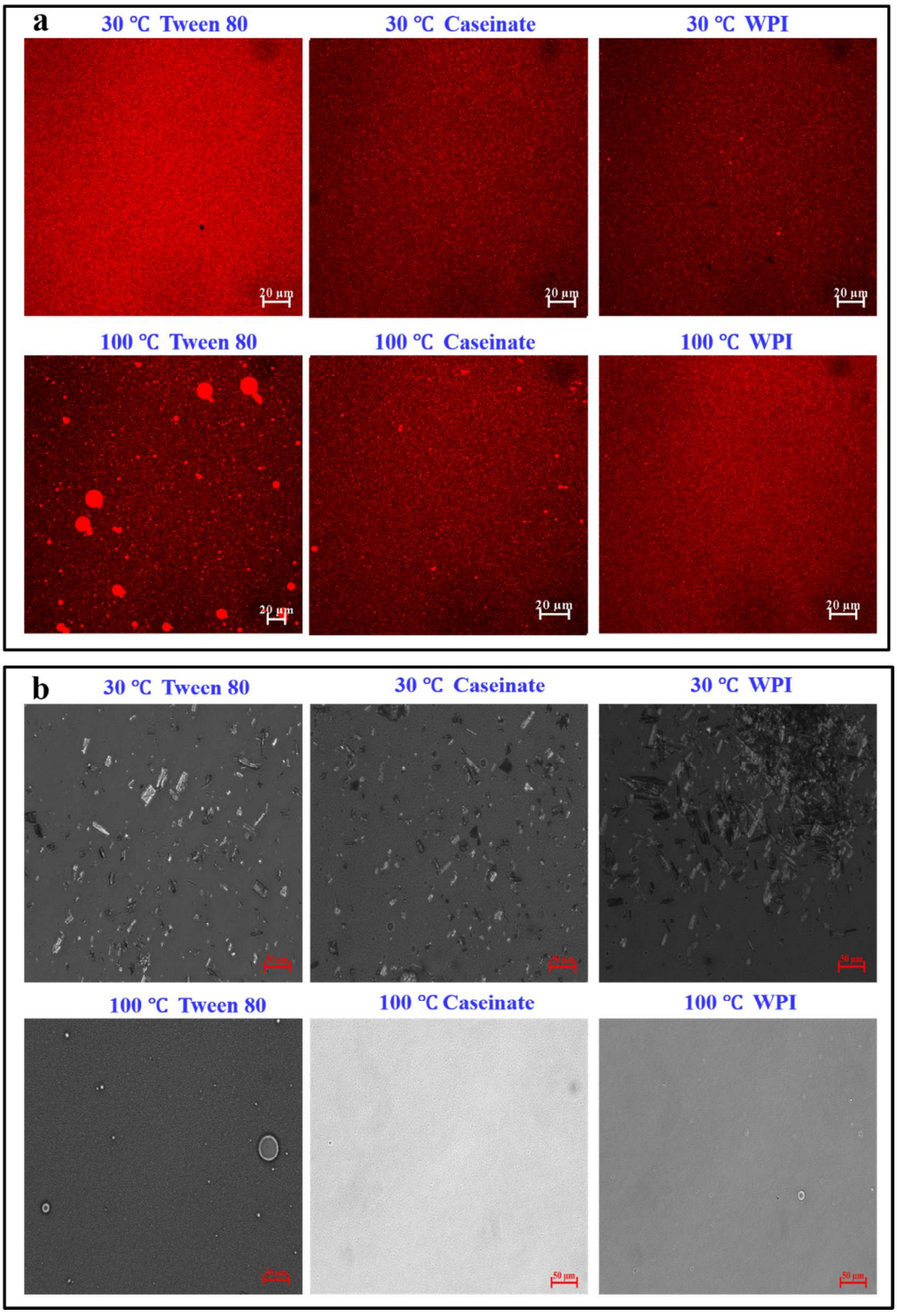


Figure 3. Influence of simulated gastrointestinal conditions on the mean droplet diameter (d_{32}) of curcumin-emulsion with three different emulsifier (Tween 80, caseinate and WPI blank emulsion) mixtures after incubation 100 ºC for 10 min. Different lowercase letters mean significant differences ($p < 0.05$) of the droplet diameter of an emulsion between digestion phases; Different capital letters mean significant differences ($p < 0.05$) of the droplet diameter between three different emulsifier (Tween 80, caseinate and WPI blank emulsion) within the same digestion phase. The experiments were carried out three times.

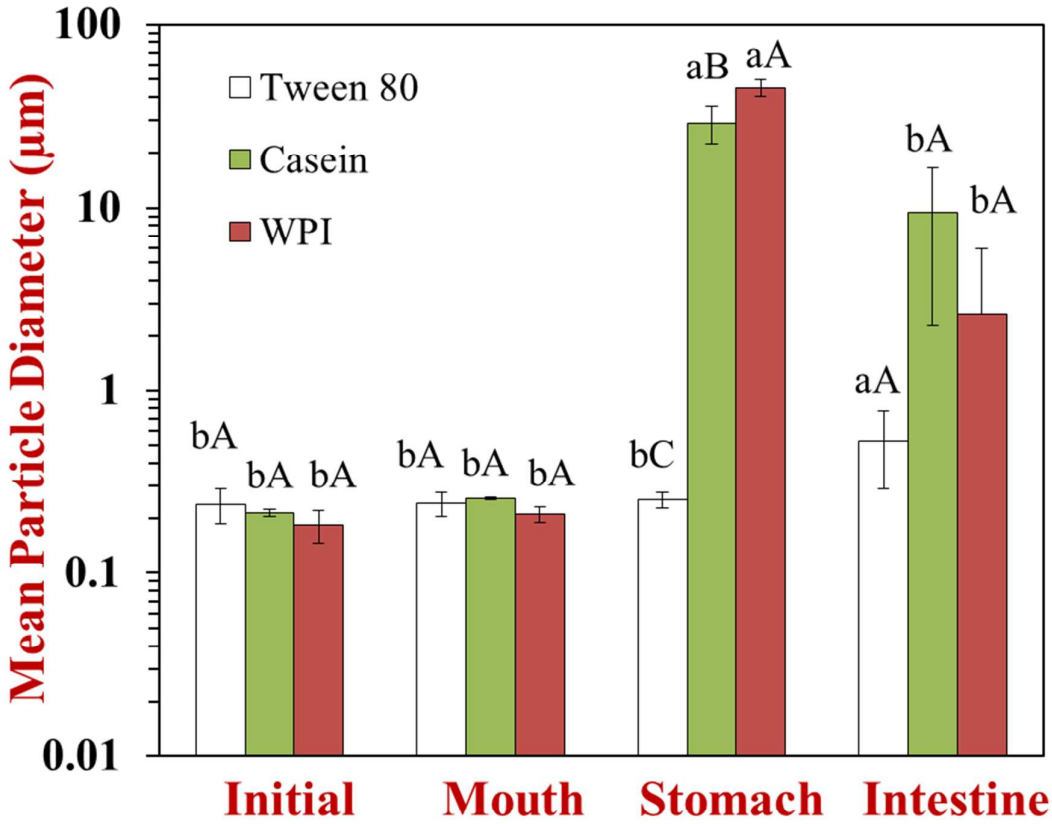


Figure 4 (a).

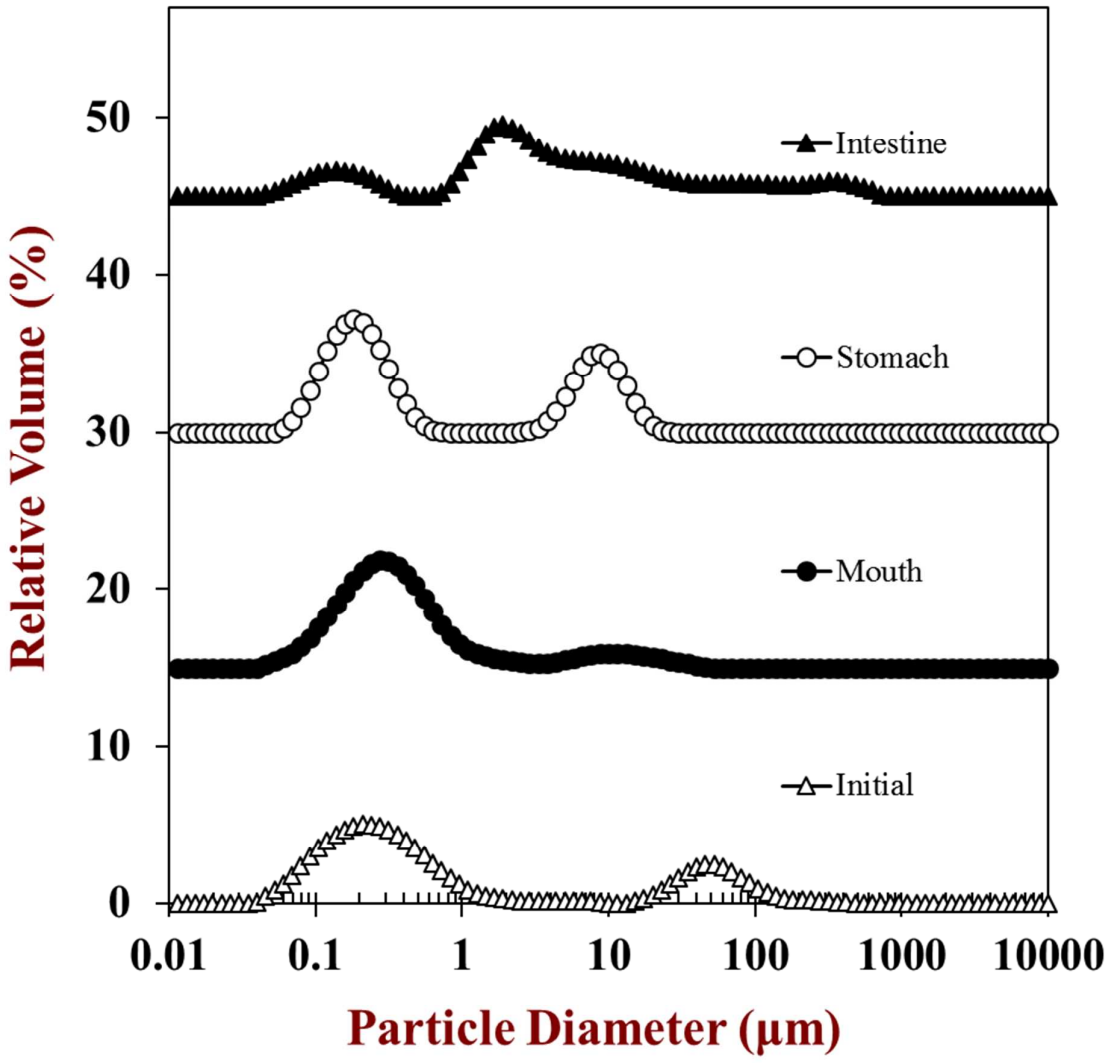


Figure 4a. Influence of simulated gastrointestinal conditions on the particle size distributions of: (a) curcumin- Tween 80 emulsion mixture after 10 min incubation at 100 ºC;

Figure 4 (b).

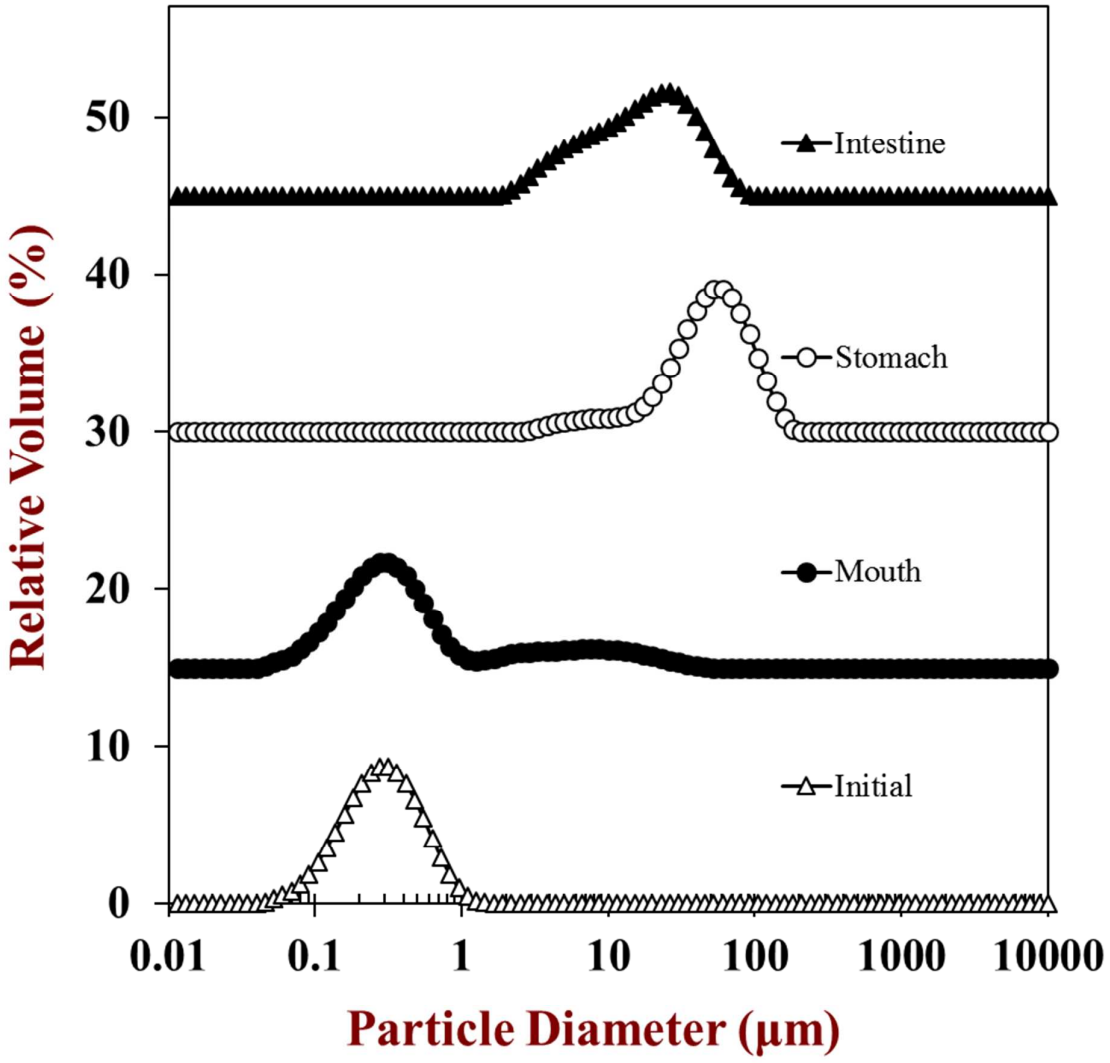


Figure 4b. Influence of simulated gastrointestinal conditions on the particle size distributions of: (b) curcumin- caseinate emulsion mixture after 10 min incubation at 100 ºC;

Figure 4 (c).

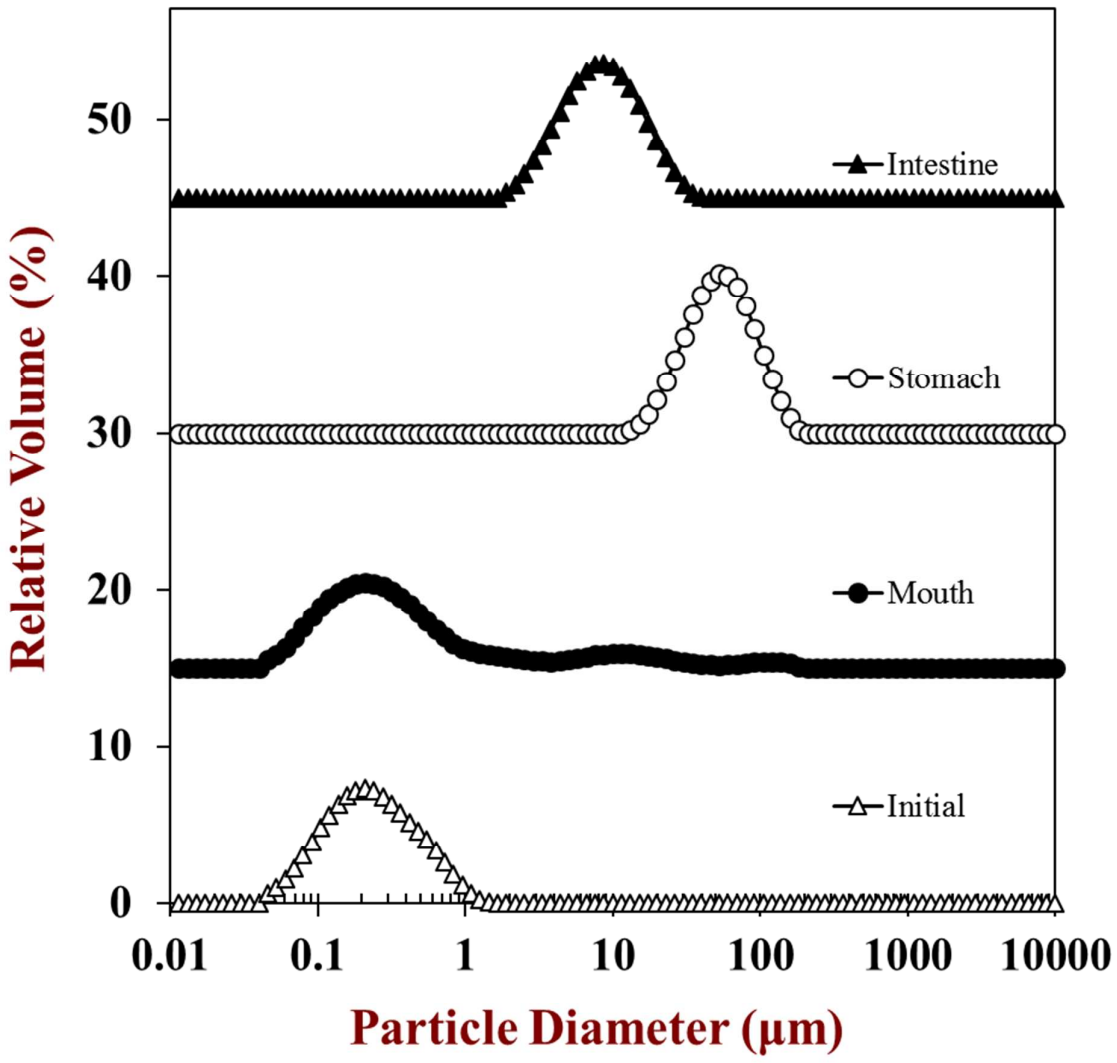


Figure 4c. Influence of simulated gastrointestinal conditions on the particle size distributions of: (c) curcumin- WPI emulsion mixture after 10 min incubation at 100 ºC;

Figure 5. Influence of simulated gastrointestinal conditions on microstructure of curcumin-emulsion with three different emulsifier (Tween 80, caseinate and WPI blank emulsion) after 100 ºC for 10 min incubation determined by confocal fluorescence microscopy. The scale bars represent a length of 20 μm, and the red regions represent lipids.

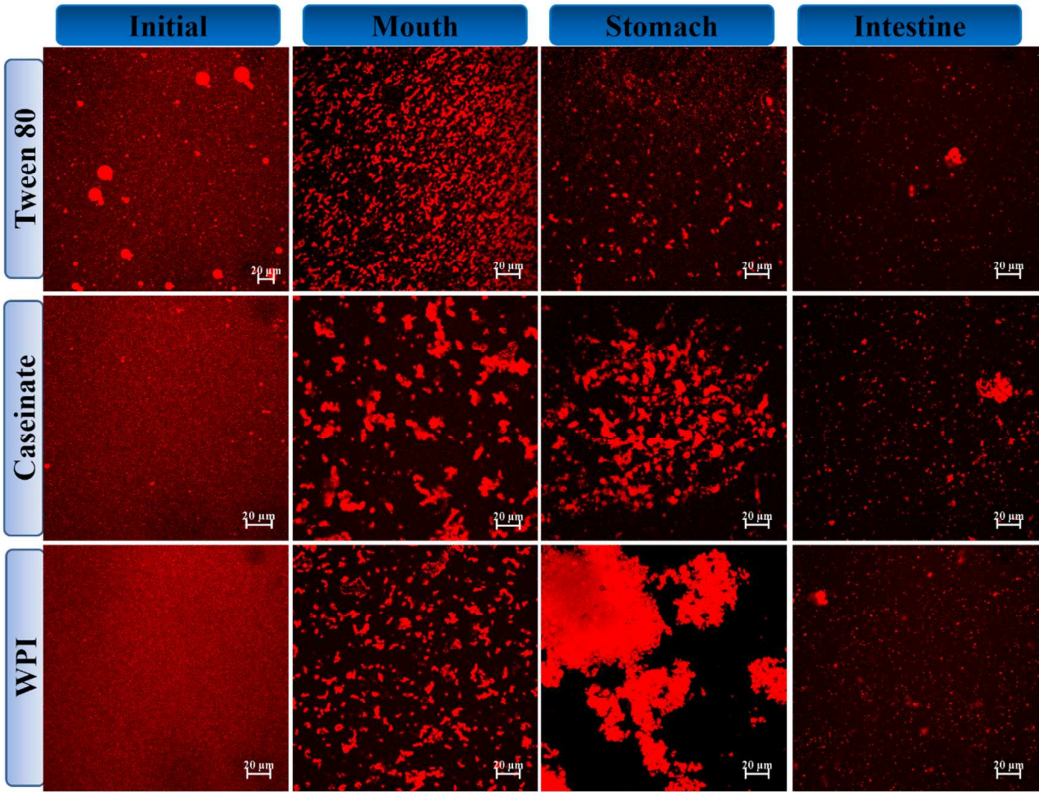


Figure 6. Influence of simulated gastrointestinal conditions on the particle charge of curcumin-emulsion with three different emulsifier (Tween 80, caseinate and WPI blank emulsion) after 100 ºC for 10 min incubation. Different lowercase letters mean significant differences ($p < 0.05$) of the particle charge of an emulsion between digestion phases; Different capital letters mean significant differences ($p < 0.05$) of the particle charge between three different emulsifier (Tween 80, caseinate and WPI blank emulsion) within the same digestion phase. The experiments were carried out three times.

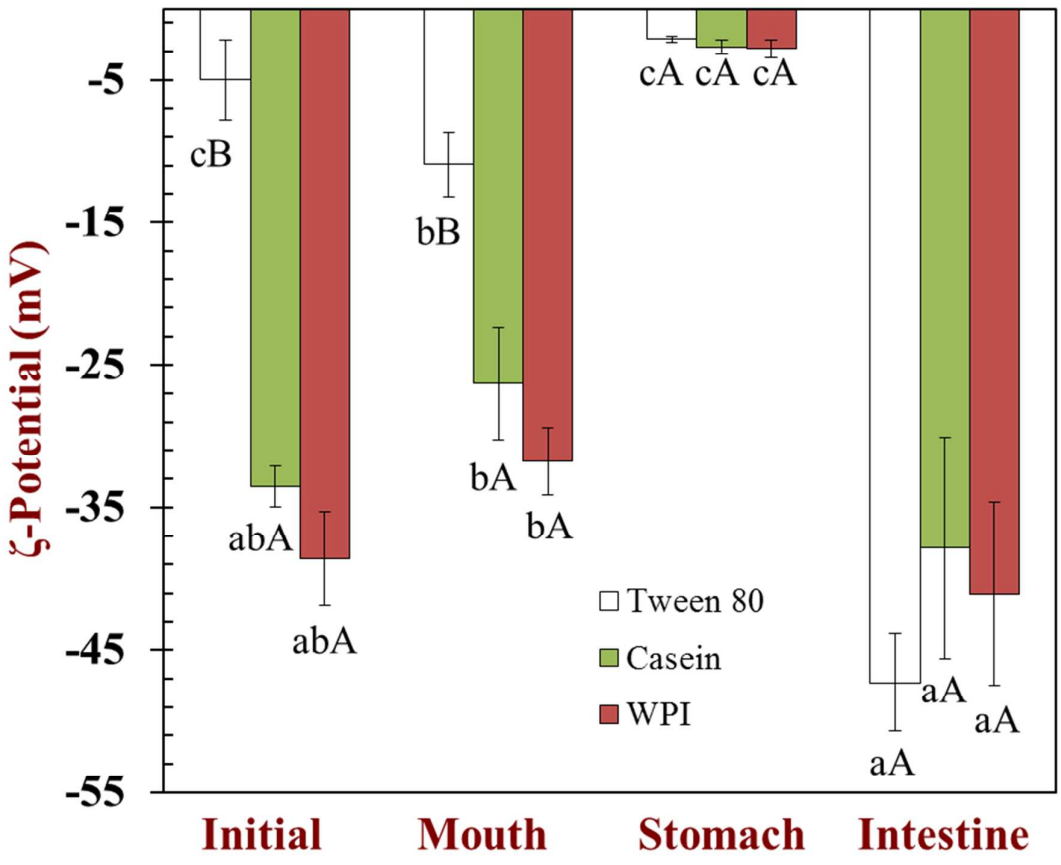


Figure 7. Influence of incubation temperature on the free fatty acids release profile at small intestine phase for curcumin-emulsion with three different emulsifier (Tween 80, caseinate and WPI blank emulsion) mixtures after 100 ºC for 10 min incubation

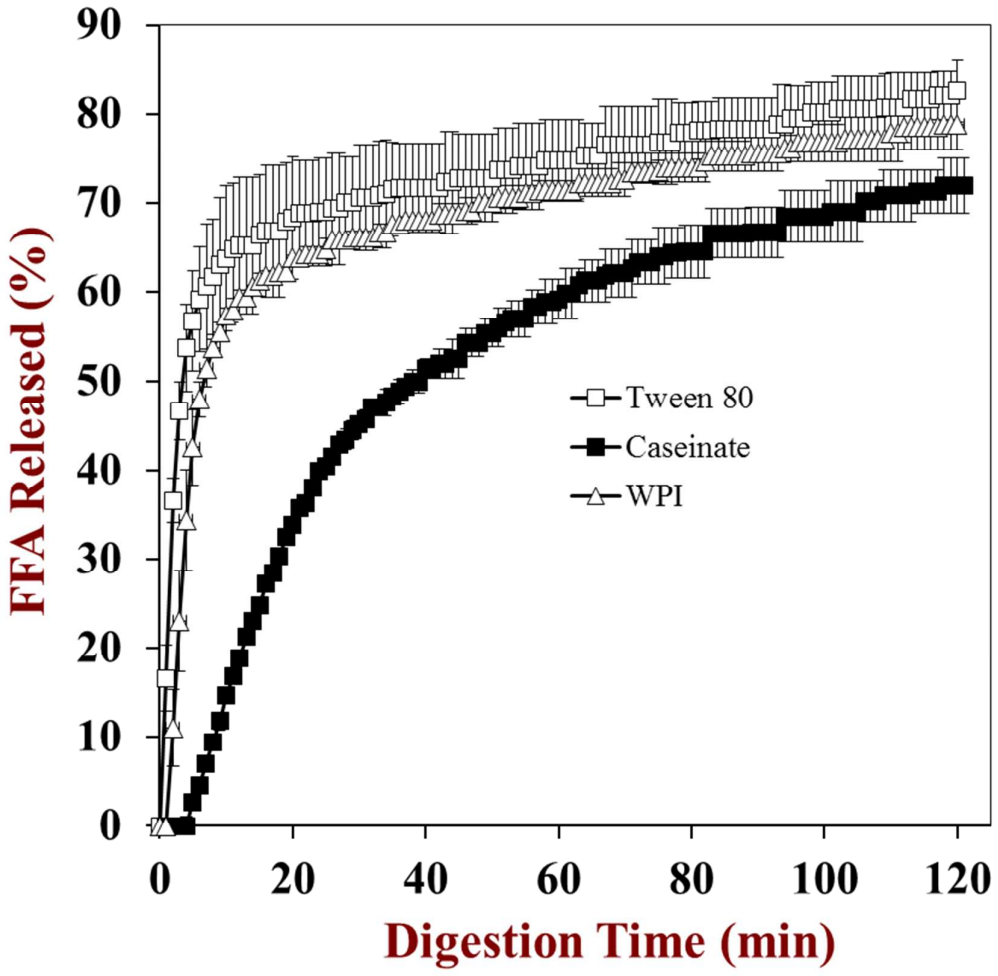
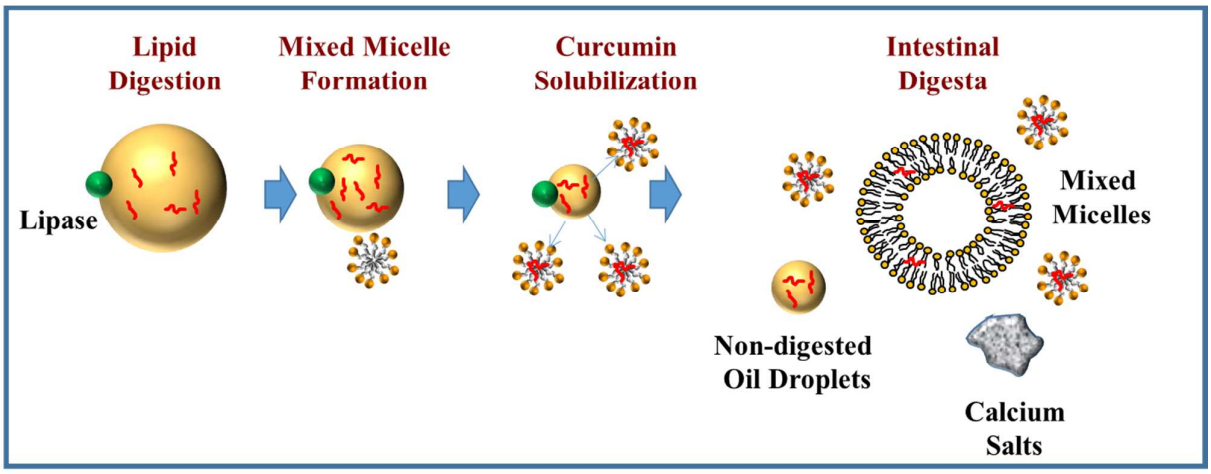
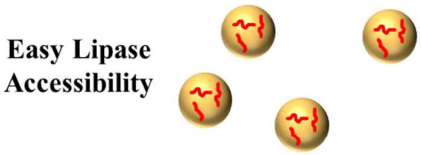


Figure 8. The curcumin trapped within the excipient emulsions may be released and solubilized in the mixed micelles after digestion of the lipid droplets by lipase.





Easy Lipase Accessibility

Greater Bioaccessibility
- More Digested Oil
- More Mixed Micelles Formed

Lower Chemical Stability
- More Curcumin Exposed to Aqueous Phase

Restricted Lipase Accessibility

Lower Bioaccessibility
- Less Digested Oil
- Less Mixed Micelles Formed

Higher Chemical Stability
- Less Curcumin Exposed to Aqueous Phase

Table 1. Effect of temperature on the mean droplet diameter (d_{32}), ζ-potential, curcumin solubility, and tristimulus color coordinates of mixtures of curcumin and excipient emulsion prepared using three different emulsifiers. Samples designated with different letters (a, b, c) were significantly different (Duncan, $p < 0.05$).

	30 °C			100 °C		
	Tween 80	Caseinate	WPI	Tween 80	Caseinate	WPI
d_{32} (μm)	0.178±0.001 [b]	0.211±0.004 [ab]	0.162±0.003 [b]	0.237±0.051 [a]	0.212±0.009 [ab]	0.183±0.037 [b]
ζ-potential (mV)	-5.8±0.4 [c]	-34.1±1.0 [b]	-39.8±0.8 [a]	-5±2.8 [c]	-33.5±1.5 [b]	-38.6±3.3 [a]
Solubility (μg/mL)	66 ± 1 [c]	17 ± 1 [d]	22±1 [d]	280±38 [a]	208±14 [b]	213±15 [b]
L	93.67 ± 0.05 [b]	94.64±0.16 [a]	94.53±0.00 [a]	90.49±0.00 [c]	88.33±0.01 [d]	90.36±0.23 [c]
a	-12.72 ±0.05 [a]	-9.82±0.09 [d]	-10.11±0.02 [c]	-11.0±0.01 [b]	-8.49±0.01 [e]	-10.92±0.21 [b]
b	47.52 ±0.04 [d]	29.57±0.04 [f]	31.90±0.01 [e]	80.80±0.01 [a]	65.02±0.01 [c]	69.54±0.45 [b]

Table 2. Influence of emulsifier on curcumin bioaccessibility (BA), curcumin concentration in the raw digesta ($C_{\text{Raw digesta}}$), and curcumin concentration (C_{Micelle}), mean particle diameter, polydispersity index (PDI), and ζ-potential of the micelle phase isolated from raw digesta. Samples designated with different letters (a, b, c) were significantly different (Duncan, $p < 0.05$).

	Tween 80	Caseinate	WPI
BA (%)	68.1±11.6 [ab]	55.2 ±7.6 [b]	76.2±1.5 [a]
$C_{\text{Raw digesta}}$ (μg/mL)	111.1±11.1 [c]	203.5 ±21.9 [a]	140.5±4.9 [b]
C_{Micelle} (μg/mL)	74.9±7.1 [b]	110.7±3.4 [a]	107.1±5.9 [a]
Mean diameter (nm)	167±19 [a]	161±37 [a]	160±24 [a]
PDI	0.23±0.09 [a]	0.28±0.10 [a]	0.40±0.20 [a]
ζ-potential (mV)	-52.2±6.9 [a]	-46.9±9.6 [a]	-45.7±10.6 [a]