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Original article

In vitro bioaccessibility of ergocalciferol in nanoemulsion-based delivery system: the influence of food-grade emulsifiers with different stabilising mechanisms

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Summary The effect of emulsifier type on the *in vitro* bioaccessibility of ergocalciferol-loaded nanoemulsions was examined (mouth, stomach and small intestinal phases). Oil-in-water nanoemulsions were prepared using emulsifiers with different stabilising mechanisms: decaglycerol monooleate (MO7S; steric), modified lecithin (ML; electrostatic), sodium caseinate (SC; electrosteric) and ML-MO7S (combined electrostatic and steric). The droplet size, size distribution, ζ-potential and microstructure of nanoemulsions during digestion depended on the emulsifier type. The fate of lipid in the small intestinal phase also relied on the emulsifier type, with the free fatty acids release rate decreasing in the following order: MO7S > ML-MO7S > ML > SC. The ergocalciferol bioaccessibilities in nanoemulsions prepared using MO7S (62%), ML (64%) and ML-MO7S (65%) were similar and significantly higher than that stabilised by SC (12%). No significant loss of ergocalciferol was observed in all nanoemulsions after full digestion; they were chemically stable against digestion conditions, regardless of the emulsifier type.

Keywords Bioaccessibility, digestion, emulsifier, ergocalciferol, nanoemulsions, stabilising mechanism.

Introduction

Vitamin D is widely known as a lipid-soluble compound that is essential to humans because this substance plays a crucial role in the absorption of calcium and maintenance of skeletal health (Holick, 2004; Khalid *et al*., 2015). This bioactive compound may also provide a number of other health benefits, such as reducing the risk of certain cancers, inhibiting cardiovascular diseases and improving immunity (Guttoff *et al*., 2015). Vitamin D exists in two major chemical forms: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). In order to exert the functional effects, both these forms should be converted into their biologically active form, which is 25-dihydroxyvitamin D, via hydroxylation in the liver and kidneys (Christakos *et al*., 2012). Cholecalciferol can be produced simply by exposing the human skin to sunlight, whereas ergocalciferol cannot be synthesised by the human body. In other words, ergocalciferol has to be supplied through food, such as mushroom, which is one of the major natural sources of ergocalciferol (Eitenmiller *et al*., 2016). There are huge populations worldwide which are found to be vitamin D deficient due to a number of reasons: (i) insufficient sunlight exposure, (ii) extensive use of UV-protective cream and (iii) poor intake of vitamin D-rich food (Guttoff *et al*., 2015). In view of this problem, there has been an increasing interest in incorporating this lipid-soluble vitamin in food and beverage products. However, vitamin D incorporation poses certain problems related to its limited solubility in water and chemical instability against light, oxygen and high temperature (Luo *et al*., 2012). Additionally, the relatively low absorption of vitamin D from food intake due to its poor water-solubility

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gives rise to another challenge in terms of designing functional foods with increased bioaccessibility.

Emulsion-based delivery system, especially nanoemulsions, is one of the more promising solutions to overcome these challenges and has duly received a considerable amount of attention within the food industry. Oil-in-water (O/W) nanoemulsions are thermodynamically unstable systems that contain small oil droplets ($d < 200$ nm) dispersed in aqueous phase (Walker *et al.*, 2017). Nanoemulsions have better physical stability than conventional emulsions ($d > 200$ nm), which is due to Brownian movement that keep the oil droplets from creaming and eventually forcing coalescence (Tadros *et al.*, 2004). In addition, small oil droplets are more effective at promoting the bioaccessibility of encapsulated lipophilic compounds than large emulsified droplets or nonemulsified oil (Liang *et al.*, 2013; Salvia-Trujillo *et al.*, 2013). Vitamin D-enriched nanoemulsions can be produced simply by dissolving this lipophilic substance in a carrier oil and then homogenising it with the aqueous phase containing a water-soluble emulsifier. As reported previously, there are various factors that influence the formation and stability of O/W emulsions loaded with vitamin D, including homogenisation technique, carrier oil, emulsifier type and so on (Guttoff *et al.*, 2015; Khalid *et al.*, 2015, 2016; Shu *et al.*, 2016). Besides affecting the formation and stability, some researchers have found that the initial carrier oil type and droplet size have significant impacts on the bioaccessibility of this functional ingredient delivered via nanoemulsion-based system (Ozturk *et al.*, 2015; Salvia-Trujillo *et al.*, 2017). The bioaccessibility of encapsulated bioactive lipophilic component in a nanoemulsion is defined as the released fraction that is solubilised within the mixed micelles formed by phospholipids, bile salts and lipid digestion products (such as monoacylglycerols and free fatty acids) (Carbonell-Capella *et al.*, 2014).

Emulsifier is one of the most crucial parameters to consider when producing a stable nanoemulsion. Emulsifier is able to adsorb onto the surface of droplets and act as a barrier to protect the droplets from aggregation and coalescence during emulsification and storage (Shariffa *et al.*, 2016). In general, there are four different mechanisms that are responsible for the way an emulsifier stabilises nanoemulsions: steric, electrostatic, electrosteric and electrostatic-steric mechanisms (Tan *et al.*, 2016). Each category of emulsifier has its own advantages and disadvantages. In recent years, extensive studies on the effects of emulsifiers on the preparation, characteristics and stability of nanoemulsions have been widely reported. Since these aspects have been well-established, the next logical step would be the understanding of the biological fate of these nanoemulsions. However, to the best of our knowledge, there is still limited fundamental information on the bioaccessibility of vitamin D delivered via a nanoemulsion-based system, especially in the context of nanoemulsions fabricated using emulsifiers with different stabilising mechanisms. Therefore, it would be interesting to investigate the influence of these emulsifiers on the release profile of encapsulated compounds, as well as to understand the changes in the gastrointestinal fate of the nanoemulsions during the digestion process.

Thus, in our study, ergocalciferol-loaded O/W nanoemulsions were prepared using either decaglycerol monooleate (MO7S), modified lecithin (ML), sodium caseinate (SC) or combined ML-MO7S, as emulsifier. MO7S (nonionic) and ML (ionic) are small-molecule emulsifiers that possess steric and electrostatic stabilising mechanisms, respectively. Meanwhile, SC is a surface-active protein with flexible structure that stabilises the nanoemulsions by a combination of electrostatic and steric repulsion (electrosteric stabilising mechanism) (Liu *et al.*, 2016; Tan *et al.*, 2016). Finally, the combination of ML and MO7S (electrostatic-steric) was used to mimic the stabilising mechanism of SC. Emulsifiers are critical components of a nanoemulsion, and we believe that the emulsifiers, each with its own stabilising mechanism, will play an important role in maintaining the stability of the emulsified droplets and protecting them from degradation prior to their entry in gastrointestinal tract. Therefore, the aim of this study was to study the impact of emulsifiers with different stabilising mechanisms on the *in vitro* digestion and bioaccessibility of nanoemulsions loaded with ergocalciferol.

Materials and methods

Materials

Sodium caseinate (SC), refined soybean oil, HPLC grade ethanol and α-amylase (015-26372) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Decaglycerol monooleate (MO7S) was provided by Sakamoto Yakuhin Kogyo Co., Ltd. (Osaka, Japan). SC used in our study has a maximum loss of 10% after drying, and its total nitrogen is 12.6–15.8%. MO7S (polymerisation degree 10), has hydrophilic-lipophilic balance (HLB) value of 12.9, and modified lecithin (ML; SLP WhiteLyso), containing phosphatidic acid (0–5%), phosphatidylethanolamine (1–7%), phosphatidylcholine (2–8%), phosphatidylinositol (10–20%) and lysophosphatidylcholine (18–30%), was purchased from Tsuji Oil Mills Co., Ltd. (Tokyo, Japan), and has HLB value of around 12. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): HPLC grade methanol and acetonitrile, mucin from porcine stomach, type II (M2378), pepsin from porcine gastric mucosa (P7000), pancreatin from porcine pancreas (P7545) and bile extract porcine

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(B8631). All other chemicals used in the present work were of analytical grade. Milli-Q water having a resistivity of 18 MΩ cm was used to prepare all the solutions and nanoemulsions.

Formulation of ergocalciferol-loaded O/W nanoemulsions

The O/W nanoemulsions loaded with ergocalciferol were produced using a two-step homogenisation method. Briefly, the oil phase containing ergocalciferol was prepared by dispersing 0.2% (w/w) of crystalline ergocalciferol in refined soybean oil with heating at 80 °C for 20 min, ultra-sonicating at ambient temperature for 60 min, and finally, filtering the oil phase using a membrane filter with pore size of 0.45 μm to remove any undissolved powdered ergocalciferol. The water phase was prepared by dissolving 1% (w/w) emulsifier in Milli-Q water. The emulsifiers used were ML, MO7S, SC or ML-MO7S (ML: MO7S at a 1:1 ratio). A coarse emulsion was obtained in the first step by homogenising 5% of oil phase with 95% of water phase (w/w) using a rotor-stator homogeniser (Polytron, PT-3000 Kinematica-AG, Littace, Switzerland) at 5000 r.p.m. for 5 min. The coarse emulsion was then immediately treated using a high-pressure homogeniser (NanoVater, NV200, Yoshida Kikai, Nagoya, Japan) at 100 MPa for four cycles to produce the final nanoemulsion. The freshly produced nanoemulsions were then placed in a refrigerator (5 °C) for further analyses.

In vitro digestion

Ergocalciferol-loaded nanoemulsions prepared using different emulsifiers were subjected to an *in vitro* digestion model that simulated the mouth, stomach and small intestinal phases. The *in vitro* digestion model applied in our current study was based on those reported previously, with minor modifications (Qian *et al.*, 2012; Xia *et al.*, 2017).

Mouth phase

A simulated saliva fluid (SSF) containing 2.1 g L^{-1} $NaHCO_3$, 0.117 g L^{-1} NaCl, 0.14 g L^{-1} KCl, 2.0 g L^{-1} α-amylase and 1 g L^{-1} mucin was prepared as described previously (Kong & Singh, 2008). Initial nanoemulsion sample (20 g) was mixed with an equivalent weight of SSF (20 g), and the mixture was adjusted to pH 7, if needed. The mixture was then shaken continuously at 100 strokes min^{-1} for 10 min using a temperature-controlled water bath at 37 °C (Personal-11, Taitec, Saitama, Japan).

Stomach phase

The simulated gastric fluid (SGF) was prepared by dissolving NaCl (2 g) and HCl (7 mL, 35–37%) in 1 L of Milli-Q water and then adding 3.2 g of pepsin. Sample collected from mouth phase (20 g) was added into 20 g of SGF, and the pH of the mixture was adjusted to 2.5 using 2.5 mol L^{-1} NaOH solution. Simulations of digestion in stomach phase were performed by shaking the resulting mixture at 100 strokes min^{-1} for 2 h at 37 °C.

Small intestinal phase

Thirty grams of sample collected from the stomach phase was adjusted to pH 7 using NaOH solution (2.5 M). Then, an aliquot (4 mL) of bile extract solution (46.8 mg mL^{-1} in phosphate buffer, pH 7) and 1 mL of calcium solution containing 110 mg of $CaCl_2$ were then added into the 30 g of digesta under magnetic stirring (100 r.p.m. min^{-1}) at 37 °C. The pH of the resulting mixture was adjusted to 7, if needed. Next, 2.5 mL of freshly prepared pancreatin suspension (24 mg mL^{-1} in phosphate buffer, pH 7) was added to the mixture. From this point, the pH of the samples was controlled manually to maintain it at pH 7 by adding NaOH solution (0.5 M) to neutralise the free fatty acids (FFA) released from the lipid during digestion. The volume of the added NaOH against time was recorded during 2 h of digestion in the small intestinal phase and was then used to calculate the FFA release using the eqn (1) (Ozturk *et al.*, 2015).

$$\%\mathrm{FFA}\,(t) = \frac{V_{\mathrm{NaOH}}(t) \times M_{\mathrm{NaOH}} \times m_{\mathrm{Lipid}}}{2 \times W_{\mathrm{Lipid}}} \times 100, \quad (1)$$

where V_{NaOH} (t) is the volume (L) of NaOH solution titrated at digestion time (min), M_{NaOH} is the molarity of NaOH used (M), m_{Lipid} is the molecular weight of soybean oil (g mol^{-1}), and W_{Lipid} is the total mass (g) of soybean oil initially present in the samples during digestion in the small intestinal phase.

Droplet size and size distribution measurement

The droplet size and size distribution in the initial or digested samples were measured using a light-scattering instrument (LS 13 320, Beckman Coulter, Brea, CA, USA). This instrument is able to determine droplet size in the range of 0.04–2000 μm. The refractive indexes were set at 1.471 and 1.333 for the soybean oil and water, respectively. The mean droplet size for every tested sample was reported as the volume mean diameter ($d_{4,3}$). The volume mean diameter ($d_{4,3}$) was chosen instead of Sauter mean diameter ($d_{3,2}$) since it is more sensitive towards the presence of small amount of large droplets (Kaci *et al.*, 2014). All measurements were conducted in triplicates.

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ζ-Potential measurement

The determination of droplet charge (ζ-potential) of the samples was performed using a ζ-potential analyser (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). Prior to analysis, the samples from stomach phase were diluted 100 times with water (pH 2.5), while all the other samples were diluted 100 times with phosphate buffer (5 mM, pH 7). The diluted samples were placed into a folded capillary cell and then automatically measured using the instrument. The refractive indexes were set at 1.471 for the oil and 1.333 for the water, respectively. All measurements were conducted in triplicates.

Ergocalciferol content measurement

The content of ergocalciferol in the nanoemulsions was measured using high-performance liquid chromatography (HPLC) method according to our previous study (Shu *et al.*, 2016). Briefly, demulsification of the nanoemulsions was firstly performed by using ethanol prior to ergocalciferol content determination. A HPLC instrument (JASCO International Co., Tokyo, Japan) equipped with a reverse-phase C_{18} column (4.6 × 250 mm; Shimpack VP-ODS, Japan), an UV-970 UV–Vis spectrophotometric detector, a PU-980 pump system, and an AS-2055 autosampler was used. The column temperature was set at 35 °C. The mobile phase, consisting of 75% acetonitrile and 25% methanol (v/v), was set at a flow rate of 1 mL min^{-1} (isocratic mode). The absorbance was measured at 365 nm to detect the ergocalciferol peak. The ergocalciferol content in the samples was quantified using a calibration curve, and all the measurements were repeated twice.

Bioaccessibility and chemical stability evaluation

Ergocalciferol bioaccessibility in the nanoemulsion-based delivery system was determined by adopting a method described previously (Qian *et al.*, 2012), with some modifications. In brief, an aliquot of the raw digesta (10 mL) was collected after the samples passed through the small intestinal phase and then centrifuged at 9000 ***g*** for 60 min using a MX-307 centrifuge (Tomy Digital Biology Co., Ltd., Tokyo, Japan). Afterwards, an aliquot of the micelles phase was collected by passing the supernatant fraction through a syringe filter (0.45 μm). An aliquot of 0.5 mL raw digesta or micelles phases was mixed with 4.5 mL of ethanol, vortexed, ultra-sonicated for 30 min and then filtered using a membrane filter (0.45 μm) before the ergocalciferol concentration analysis. The concentration of ergocalciferol in the raw digesta or micelles phases was measured using the HPLC method described in Ergocalciferol content measurement Section. The bioaccessibility and retention of ergocalciferol after digestion were calculated from the eqns 2 and 3, respectively:

$$\text{Bioaccessibility}\,(\%) = \frac{C_{\text{Micelles}}}{C_{\text{Initial}}} \times 100, \qquad (2)$$

$$\text{Retention}\,(\%) = \frac{C_{\text{Digesta}}}{C_{\text{Initial}}} \times 100, \qquad (3)$$

where C_{Micelles} and C_{Digesta} are the ergocalciferol contents recovered from micelles phase and raw digesta phase, respectively. C_{Initial} is the ergocalciferol amount calculated in the initial nanoemulsion sample.

Microstructural analysis

Confocal scanning laser microscopy (TCS SP8; Leica Microsystems GmBH, Wetzlar, Germany) was used to monitor the microstructural changes of samples before and after passing through each phase of digestion. Prior to confocal microscopy observation, 1 mL of each sample was mixed with 0.05 mL of Nile Red solution (1 mg mL^{-1} in ethanol) to stain the lipid phase. An oil-immersion objective lens were used to capture all microstructure images.

Statistical analysis

All the experiments were carried out at least two times from the beginning. The results were reported as the average and standard deviation from the obtained data. The data at a confidence level of 95% were analysed using analysis of variance (ANOVA). Least significant difference (LSD) was calculated using Statistix 8.1 software (Tallahassee, FL, USA) according to the method described by Steel *et al.* (1997).

Results and discussion

Effect of emulsifier type on droplet characteristics

Initially, we investigated the influence of emulsifiers with different stabilising mechanisms on the gastrointestinal fate of ergocalciferol-loaded nanoemulsions during digestion. Nanoemulsions were prepared using either MO7S, ML, SC or ML-MO7S as emulsifier. After each digestion phase, an aliquot of sample was taken for measurements of the droplet characteristics, including droplet size, size distribution, microstructure and ζ-potential (Figs 1–4).

Initial nanoemulsions

The droplet size, $d_{4,3}$, was fairly small and similar for all the initial nanoemulsions stabilised using MO7S,

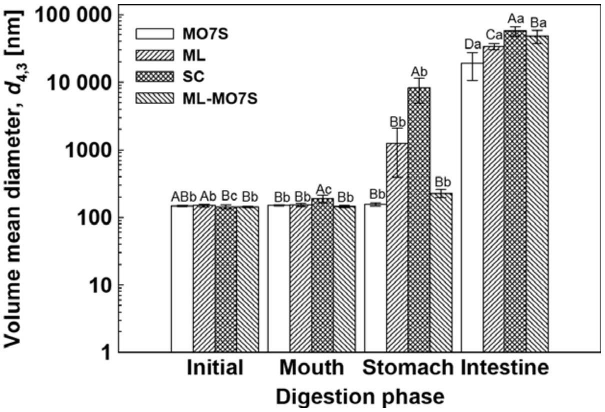

Figure 1 Influence of emulsifier type on volume mean diameter ($d_{4,3}$) of nanoemulsions loaded with ergocalciferol after exposure to different phases of the *in vitro* digestion model. Different lowercase letters indicate significant difference at a 95% probability level between the $d_{4,3}$ of a nanoemulsion at different digestion phases. Different uppercase letters indicate significant difference at a 95% probability level between the $d_{4,3}$ of different nanoemulsions within the same digestion phase.

ML, SC or ML-MO7S: $d_{4,3} = 148$, 149, 142 and 150 nm, respectively (Fig. 1). In addition, all the samples had monomodal size distributions without relatively large droplets in the initial system (Fig. 2). Confocal images showed that the oil droplets in these nanoemulsions were uniformly distributed, without evidence of droplet aggregation (Fig. 3). These results suggested that the homogenisation conditions used in our study were capable of producing stable emulsions containing nano-sized droplets ($d_{4,3} < 200$ nm), regardless of the emulsifier type. For the small-molecule emulsifiers (ML, MO7S and ML-MO7S), their adsorption on the oil droplet surface was fast during the homogenisation process, leading to the formation of nanoemulsions with small droplet sizes (Mao *et al.*, 2009). Protein-based emulsifiers are generally expected to result in larger droplet sizes due to their relatively lower absorption kinetics when compared with small-molecule emulsifiers (Mao *et al.*, 2009; Tan *et al.*, 2016). However, larger droplet size of SC-stabilised nanoemulsions comparing those stabilised by the other three emulsifiers was not observed in our study. For the SC-stabilised nanoemulsions, the mixture of surface-active casein molecule can form a thick layer with a combination of electrostatic and steric repulsion to prevent the newly-formed emulsion droplets from coalescing during the high-pressure homogenisation, which presumably was responsible for the production of droplets with small sizes. The ζ-potential measurements revealed that all the initial electric charge of the nanoemulsions was of negative charge, with the magnitude highly dependent on the emulsifier type (Fig. 4). Relatively high ζ-potential values were obtained for nanoemulsions stabilised using ML, SC and ML-MO7S: −78, −50 and −65 mV, respectively. ML is an ionic emulsifier, and this intrinsic property of ML contributed towards the negative charge shown by ML-stabilised nanoemulsions. As for the case of SC-stabilised nanoemulsions, the ζ-potential will either be negative or positive charge depending on the pH of the nanoemulsions: pH either above or below the isoelectric point of SC (pI = 4.1–4.6) (Liu *et al.*, 2016; Shu *et al.*, 2016). Thus, in this study, the negative

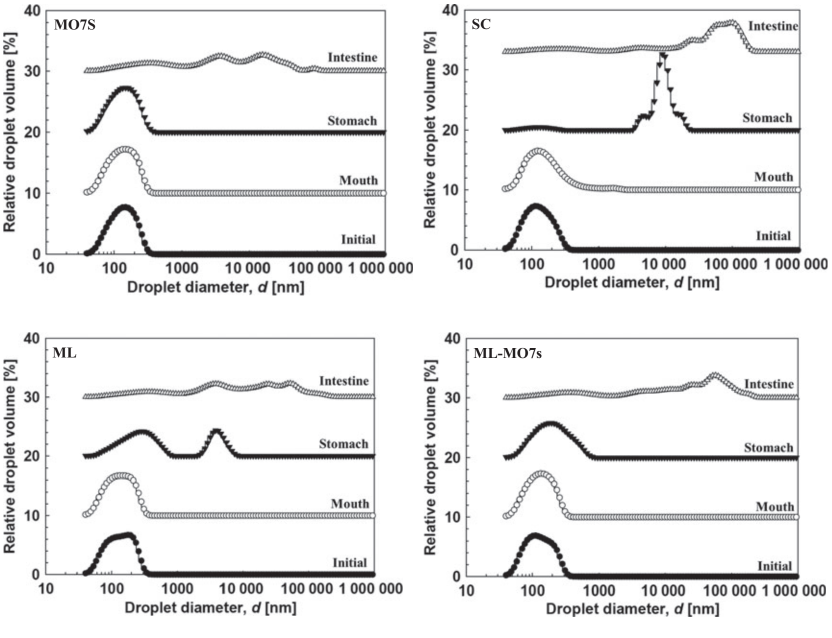

Figure 2 Influence of emulsifier type on droplet size distribution of nanoemulsions loaded with ergocalciferol after exposure to different phases of the *in vitro* digestion model.

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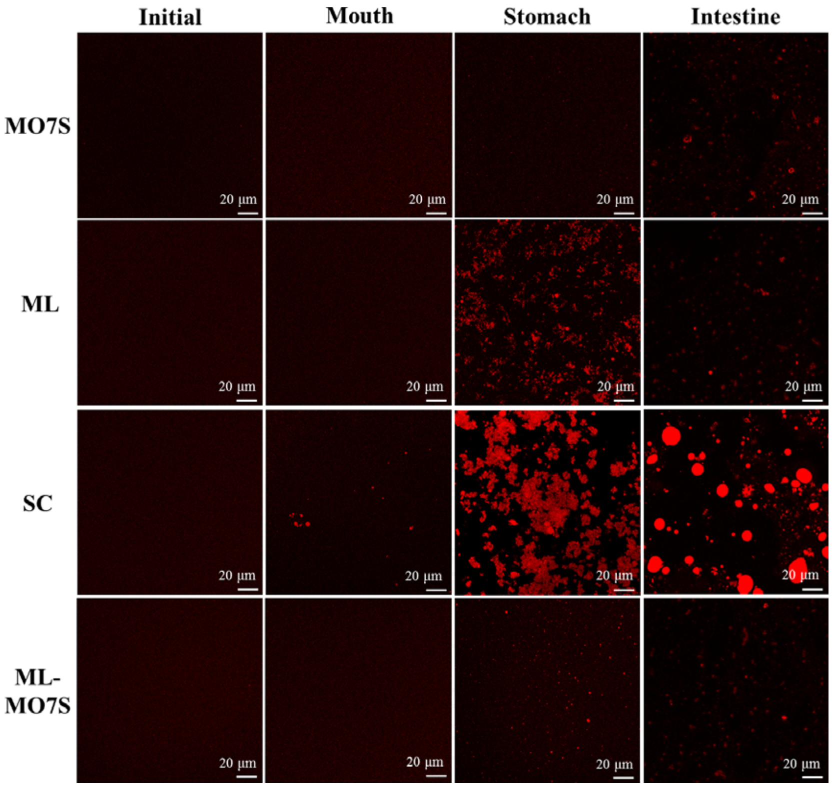

Figure 3 Influence of emulsifier type on the microstructure (confocal microscopy) of nanoemulsions loaded with ergocalciferol after exposure to different phases of the *in vitro* digestion model. (Scale bar = 20 µm). [Colour figure can be viewed at wileyonlinelibrary.com]

charge displayed by the SC-stabilised oil droplets could be attributed to the fact that the pH of primary nanoemulsions (approximately pH 7, data not shown) was much higher than the pI of SC. We anticipated MO7S-coated oil droplets to possess an electric charge which is close to zero because MO7S is a nonionic emulsifier. Unexpectedly, a moderate negative charge (−21 mV) was displayed by the MO7S-stabilised nanoemulsions (Fig. 4). This may be due to the presence of impurities, such as FFA, in the oil phase or surfactant (Qian *et al.*, 2012; Wang *et al.*, 2012).

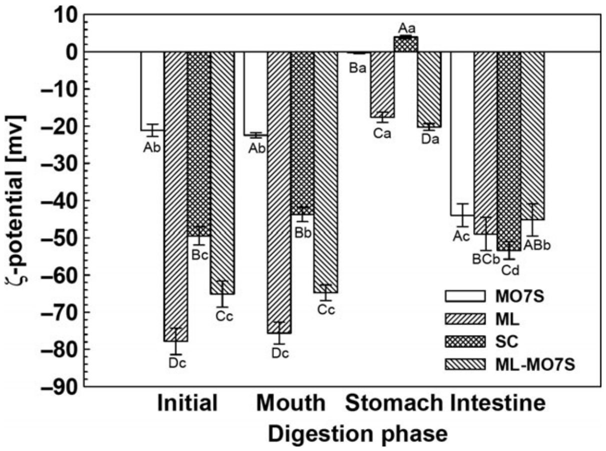

Figure 4 Influence of emulsifier type on the electric charge (ζ-potential) of nanoemulsions loaded with ergocalciferol after exposure to different phases of the *in vitro* digestion model. Different lowercase letters indicate significant difference at a 95% probability level between the ζ-potential of a nanoemulsion at different digestion phases. Different uppercase letters indicate significant difference at a 95% probability level between the ζ-potential of different nanoemulsions within the same digestion phase.

Mouth phase

After passing through the mouth phase, there was nonsignificant ($P > 0.05$) increase in $d_{4,3}$ and little change in droplet size distributions of the nanoemulsions stabilised by MO7S, ML and ML-MO7S (Figs 1 and 2). These results were also confirmed by confocal microscopy, which showed that the oil droplets remained small and no droplet flocculation or coalescence occurred during the 10 min of incubation in the artificial saliva (Fig. 3). The observed high stability of these nanoemulsions was possibly related to the steric hindrance provided by MO7S and/or electrostatic repulsion generated by ML. In comparison, the droplet size of SC-stabilised nanoemulsions slightly increased from 142 to 188 nm when the sample moved from the initial to the mouth phase. In addition, the droplet size distribution of SC-stabilised nanoemulsions showed a small amount of population with slightly larger droplet size after those had similar droplet dimensions to the initial sample (Fig. 2). Minor droplet coalescence was also detected by confocal images as shown in Fig. 3, suggesting that SC-stabilised nanoemulsions became slightly unstable towards droplet growth when exposed to the oral phase. Previous studies have reported that the depletion and/or bridging flocculation caused by the presence of mucin in the artificial saliva could contribute to the growth of oil droplets in protein-stabilised emulsions (Vingerhoeds *et al.*, 2005; Sarkar *et al.*, 2009; Zhang *et al.*, 2015b). Generally, for all nanoemulsions, the ζ-potential remained fairly close to those displayed by the initial samples, even after being exposed to the artificial saliva (Fig. 4). The results indicated that the conditions of the mouth phase used in our study (neutral pH, with the presence of salt, mucin and α-amylase) had little impact on the ζ-potential of the nanoemulsions.

Stomach phase

The droplet characteristics of the nanoemulsions after gastric digestion were highly dependent on the emulsifier type (Figs 1–4). The MO7S-stabilised nanoemulsions did not show changes in the droplet size (Fig. 1), size distribution (Fig. 2) and microstructure (Fig. 3), suggesting that this emulsion system was highly stable against droplet aggregation under stomach conditions. The reason behind this observation could be related to the fact that the steric repulsion conferred by MO-7S (a nonionic emulsifier) is insensitive to the changes in pH, ionic strength and protease activity (Zou *et al.*, 2015). Conversely, there was an appreciable increase in droplet size for the nanoemulsions stabilised by ML and SC (Fig. 1). Measurements of size distributions and confocal images also showed that a fraction of large oil droplets was present in ML- and SC-stabilised nanoemulsions (Figs 2 and 3). This indicated that these two nanoemulsions were highly unstable towards droplet growth after exposure to the stomach phase. A previous study has also found that emulsions stabilised by nonionic emulsifiers were more stable towards flocculation and coalescence under gastric condition than those prepared using protein- and phospholipid-based emulsifiers (Chang & McClements, 2016). The instability of nanoemulsions stabilised by ML and SC under gastric phase was correlated with a series of physicochemical phenomenon: (i) the stomach phase reduced the electrostatic repulsion between ML and SC-coated droplets (as will be discussed later), thereby inducing aggregation; (ii) hydrolysis of the adsorbed protein layer by pepsin could possibly have reduced the ability of the protein layer to protect the droplets from growth; and (iii) the biopolymers might have promoted the depletion or bridging flocculation of oil droplets in the protein-stabilised nanoemulsions under acidic condition (Zou *et al.*, 2015). Meanwhile, the nanoemulsions stabilised by the combined emulsifiers (MO7S and ML) were more resistant towards droplet growth as compared to the ML-stabilised ones, suggesting that the physical stability of nanoemulsions stabilised by ML could be improved by the addition of MO7S. This phenomenon was presumably attributed to the combined effects of steric hindrance and electrostatic interaction could help to protect the oil droplets from aggregating under the simulated conditions of the stomach phase. The magnitude of negative charge in all the nanoemulsions was significantly ($P < 0.05$) decreased after exposure to the stomach phase (Fig. 4). The reduction in ζ-potential between ML- and ML-MO7S-coated droplets could be related to two reasons: (i) the presence of salt produced a screening effect which reduced the electrostatic repulsion between the droplets; and (ii) the anionic phosphate groups on ML have pKa values of approximately 1.5 and thus would lose their negative charge at low pH (Carvalho *et al.*, 2014; Ozturk *et al.*, 2014). As mentioned earlier, the SC-coated droplets would be expected to be highly positively charged at highly acidic condition (pH 2.5), because this particular pH value was well below the pI of SC. As such, the anionic mucin molecules could adsorb onto the cationic SC-emulsified droplets in the stomach phase, as has been reported in recent studies (Zou *et al.*, 2015; Chang & McClements, 2016). Consequently, the negatively charged mucin molecules and the positively charged SC-coated droplets cancelled off each other, leading to the SC-stabilised nanoemulsions having a ζ-potential value of near to zero.

Small intestinal phase

After 2 h of incubation in the small intestinal phase, a drastic increase in droplet size was observed in all samples (Fig. 1). Examination of the droplet size distribution revealed that the raw digesta for all the samples contained droplets with a broad size range (Fig. 2). These measured droplets were presumably made up of a complex mixture of calcium salts sediment, nondigested oil, micelles, vesicles and/or nondigested protein aggregates (Zhang *et al.*, 2015b). The microscopy images also indicated the presence of visibly large particle aggregates in all the samples (Fig. 3). However, there was clearly a difference in the nature of droplets present in the raw digesta for the nanoemulsions prepared using different emulsifiers (Fig. 3). A numerous of irregularly-shaped clumps of small particles were observed in the nanoemulsions stabilised by small-molecule emulsifiers (MO7S, ML and ML-MO7S). On the hand, there were many spherical droplets with large sizes present for the SC-stabilised nanoemulsions. Apparently, a full lipid hydrolysis led to the generation of a mixtures of vesicles and micelles present in the

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small emulsifier-based nanoemulsions. On the other hand, partial digestion of lipid induced the coalescence between undigested oil droplets that was responsible for the large spherical droplets detected in the digesta of SC-based nanoemulsions. All samples exhibited relatively high negative electric charges after incubation in the small intestinal phase, as seen in Fig. 4. The increase in the magnitude of negative charge on all the nanoemulsions could be attributed to several factors. Firstly, emulsifier molecules would be more negatively charged as the pH increased to 7 in the small intestinal phase (Ozturk *et al.*, 2015). Secondly, the adsorption of surface–active anionic substances (bile salt and phospholipids from bile extract) on the droplets would contribute to a relatively high negative charge at neutral pH. Thirdly, the presence of anionic free fatty acids generated from lipid digestion would also lead to a relatively high negative charge in the small intestinal phase. Similar trend in the ζ-potential when the nanoemulsions moved from the stomach phase to the small intestinal phase was also reported by other authors (Zhang *et al.*, 2015b; Xia *et al.*, 2017).

Effect of emulsifier type on lipid digestion

The influence of emulsifier type on lipid digestion was determined by measuring the percentage of FFA released from the nanoemulsions in the small intestinal phase. According to Fig. 5a, there was an appreciable difference between the rate and extent of lipid digestion, depending on the emulsifier type used. The behaviour of lipid digestion for the nanoemulsions stabilised by small-molecule emulsifiers (MO7S, ML and ML-MO7S) showed relatively similar trends: most of the emulsified triacylglycerols were digested by lipase within 20 min, after which the digestion rate slowly reduced until a relatively constant plateau was reached. Looking at these nanoemulsions individually, we found that the initial rate of FFA release decreased in the following order: MO7S > ML-MO7S > ML. There are two possible reasons behind the lowest initial lipid digestion detected in ML-stabilised nanoemulsions. Firstly, for the ML-stabilised nanoemulsions, extensive droplet flocs formed under gastric conditions, thus reducing the exposed surface area and making it more difficult for pancreatic lipase to digest them when they move to the small intestinal phase (Zhang *et al.*, 2015a; Zou *et al.*, 2015). Secondly, it is known that calcium ion can bind with phospholipids, which is the main composition of ML (Sjoblom, 2005). This interaction, to a certain extent, reduced the total amount of free Ca^{2+} present in the small intestinal phase, thereby decreasing the FFA release (Li *et al.*, 2011; Zhang *et al.*, 2015b). In the case of nanoemulsions stabilised by SC, the initial rate and extent of FFA release was much lower than in those prepared using the small-molecule emulsifiers (Fig. 5a). This finding was in agreement with previous studies reported that SC-stabilised emulsions revealed slow rate of lipid digestion in the small intestinal phase (Li *et al.*, 2012; Zhang *et al.*, 2015b). We believe the severe aggregation of SC-coated lipid droplets within the stomach phase, and the reduction of free Ca^{2+} due to calcium bridging between the caseins were the main reasons to explain its lowest initial rate of lipid digestion. In addition, the SC forms thick protective layer around the lipid droplets, which might also have inhibited the access of pancreatic lipase to the triacylglycerols. As indicated in Fig. 5b, there was an appreciable amount of undigested oil remaining in the SC-stabilised nanoemulsions after full digestion. This finding was further confirmed through the images

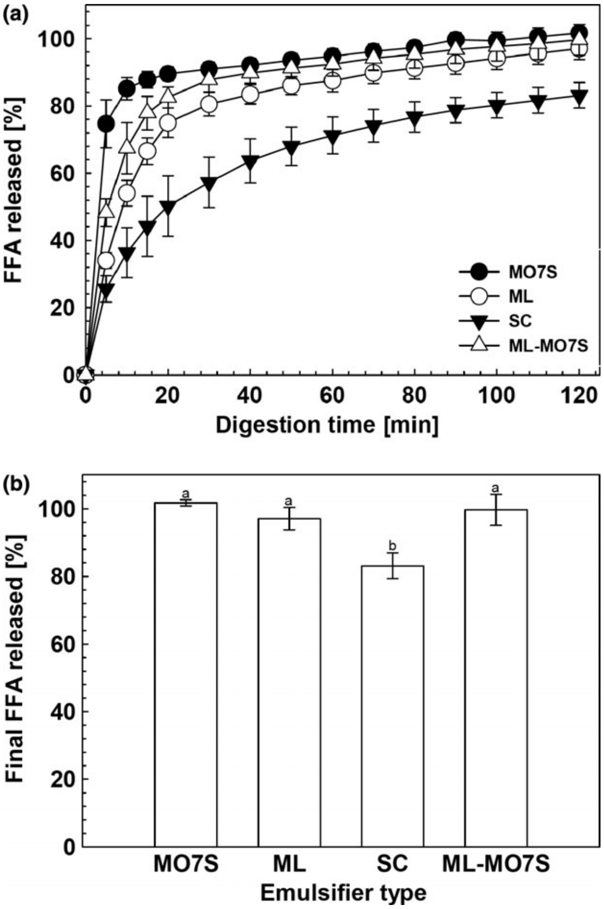

Figure 5 (a) Influence of emulsifier type on the release of free fatty acids (FFA) from nanoemulsions loaded with ergocalciferol during digestion in small intestinal phase. (b) Influence of emulsifier type on the final amount of FFA released after 2 h of small intestinal digestion. The different lowercase letters indicate significant difference at a 95% probability level between the final FFA releases of nanoemulsions prepared using different emulsifiers.

obtained from the confocal microscopy (Fig. 3). We suppose this was because of lipase having insufficient time to fully access the SC-coated lipids due to the severe flocculation of the lipid droplets in the small intestinal phase.

Effect of emulsifier type on bioaccessibility and retention of ergocalciferol after digestion

Ergocalciferol bioaccessibility

The influence of emulsifiers with different stabilising mechanisms on the bioaccessibility of ergocalciferol was evaluated by measuring the ergocalciferol concentration in the micelle fraction. According to Fig. 6a, there were no significant ($P > 0.05$) differences in the ergocalciferol bioaccessibility between nanoemulsions stabilised using different small-molecule emulsifiers. We believe that this finding was possibly due to MO7S, ML and ML-MO7S emulsifiers generating similar percentages of FFA release at the end of the digestion process in the small intestinal phase. Conversely, the bioaccessibility of ergocalciferol in SC-stabilised nanoemulsions was only 12%, which was much lower than those shown by the other three nanoemulsions. Several potential reasons could be used to account for this phenomenon; (i) the lower amount of FFA released from SC-stabilised nanoemulsions resulted in lesser formation of mixed micelles available to solubilise the ergocalciferol, (ii) the oil droplets in the SC-stabilised nanoemulsions were not fully digested; therefore, more ergocalciferol was retained within this undigested oil portion, (iii) the interaction between ergocalciferol and β-casein, one of the major protein fractions of SC, might have promoted the aggregation and precipitation of mixed micelles during digestion (Forrest *et al.*, 2005; Mun *et al.*, 2015), and (iv) MO7S and ML might participate in the formation of mixed micelles, thereby increasing the capacity to solubilise ergocalciferol.

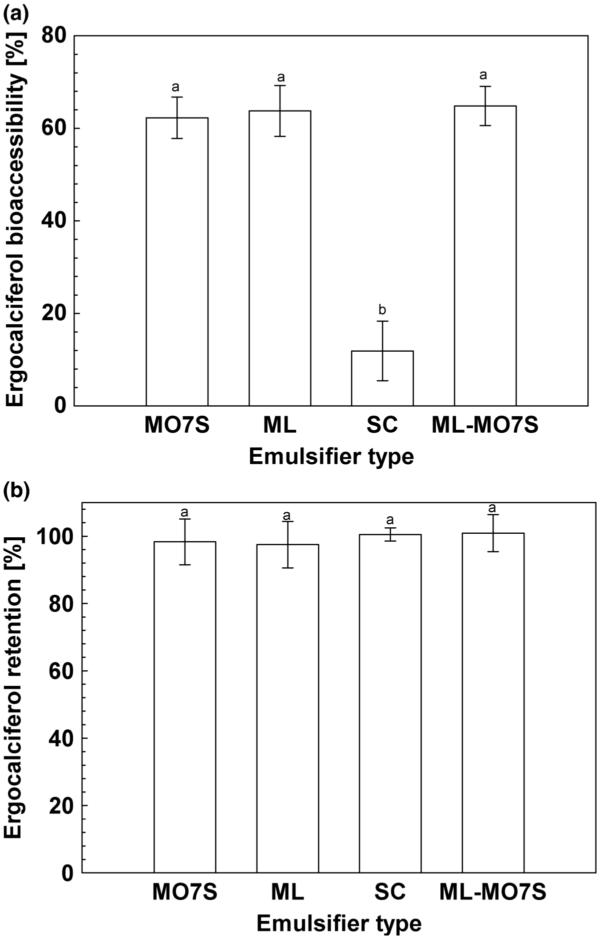

Figure 6 (a) Influence of emulsifier type on the bioaccessibility of ergocalciferol in nanoemulsion-based delivery system. (b) Influence of emulsifier type on the chemical stability of ergocalciferol after full digestion. The different lowercase letters indicate significant difference at a 95% probability level between (a) the ergocalciferol bioaccessibility and (b) retention of nanoemulsions stabilised by different emulsifiers.

Ergocalciferol retention

Finally, the stability of ergocalciferol after full digestion was also examined since it is one of the major factors affecting its bioaccessibility. According to Fig. 6b, almost no loss of ergocalciferol was observed in any of the samples, which demonstrated that this encapsulated oil-soluble vitamin was very stable against degradation under the digestion conditions. Previous studies also showed that β-carotene (a type of carotenoids with poor chemical stability) were chemical stable in nanoemulsion-based system during the digestion process, which was attributed to the short digestion time (Qian *et al.*, 2012; Yi *et al.*, 2014).

Conclusion

The present work has shown the importance of emulsifier type in the ergocalciferol-loaded nanoemulsions on their potential biological fate (e.g., emulsion droplets stability, digestibility, and ergocalciferol bioaccessibility and stability) within an *in vitro* digestion model. The physical stability of the prepared nanoemulsions during different phases of digestion was evaluated by measuring their droplet size, size distribution, ζ-potential and microstructure. The obtained results indicated that the physical stability of the nanoemulsions after passing through the mouth, stomach and small intestinal phases was strongly dependent on the type of emulsifier used. Emulsifier type considerably affected the initial rate of FFA release from nanoemulsions by pancreatic lipases, and the results demonstrated that

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the access of pancreatic lipase to emulsified oil droplets decreased in the following order: MO7S > ML-MO7S > ML > SC. The nanoemulsions stabilised by small molecular emulsifiers (MO7S, ML and ML-MO7S) had similar ergocalciferol bioaccessibilities, which may be related to the similar amount of lipid digestion products present. However, SC-stabilised nanoemulsions displayed much lower ergocalciferol bioaccessibility as compared to the three aforementioned nanoemulsions. This observation could be attributed to the fact that there was more undigested oil and less mixed micelles to solubilise the released ergocalciferol. The ergocalciferol remained stable in all samples, proving that the nanoemulsion-based delivery system was suitable to protect this oil-soluble vitamin from degradation under the *in vitro* digestion conditions. We are positively confident that the findings obtained from this study will be important and useful for future designs of nanoemulsion-based delivery system to encapsulate lipophilic functional compounds, such as vitamin D.

References

Carbonell-Capella, J.M., Buniowska, M., Barba, F.J., Esteve, M.J. & Frígola, A. (2014). Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: a review. *Comprehensive Reviews in Food Science and Food Safety*, **13**, 155–171.

Carvalho, A., Silva, V. & Hubinger, M. (2014). Microencapsulation by spray drying of emulsified green coffee oil with two-layered membranes. *Food Research International*, **61**, 236–245.

Chang, Y. & McClements, D.J. (2016). Influence of emulsifier type on the in vitro digestion of fish oil-in-water emulsions in the presence of an anionic marine polysaccharide (fucoidan): Caseinate, whey protein, lecithin, or Tween 80. *Food Hydrocolloids*, **61**, 92–101.

Christakos, S., Ajibade, D.V., Dhawan, P., Fechner, A.J. & Mady, L.J. (2012). Vitamin D: metabolism. *Rheumatic Disease Clinics of North America*, **38**, 1–11.

Eitenmiller, R.R., Landen, W. Jr & Ye, L. (2016). *Vitamin Analysis for the Health and Food Sciences*. Boca Raton, FL: CRC Press.

Forrest, S.A., Yada, R.Y. & Rousseau, D. (2005). Interactions of vitamin D3 with bovine β-lactoglobulin A and β-casein. *Journal of Agricultural and Food Chemistry*, **53**, 8003–8009.

Guttoff, M., Saberi, A.H. & McClements, D.J. (2015). Formation of vitamin D nanoemulsion-based delivery systems by spontaneous emulsification: factors affecting particle size and stability. *Food Chemistry*, **171**, 117–122.

Holick, M.F. (2004). Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. *The American Journal of Clinical Nutrition*, **79**, 362–371.

Kaci, M., Meziani, S., Arab-Tehrany, E., Gillet, G., Desjardins-Lavisse, I. & Desobry, S. (2014). Emulsification by high frequency ultrasound using piezoelectric transducer: formation and stability of emulsifier free emulsion. *Ultrasonics Sonochemistry*, **21**, 1010–1017.

Khalid, N., Kobayashi, I., Wang, Z. *et al.* (2015). Formulation characteristics of triacylglycerol oil-in-water emulsions loaded with ergocalciferol using microchannel emulsification. *RSC Advances*, **5**, 97151–97162.

Khalid, N., Kobayashi, I., Neves, M.A., Uemura, K., Nakajima, M. & Nabetani, H. (2016). Encapsulation of cholecalciferol and ergocalciferol in oil-in-water emulsions by different homogenization techniques. *European Journal of Lipid Science and Technology*, **119**, 1600247.

Kong, F. & Singh, R.P. (2008). A model stomach system to investigate disintegration kinetics of solid foods during gastric digestion. *Journal of Food Science*, **73**, E202–E210.

Li, Y., Hu, M. & McClements, D.J. (2011). Factors affecting lipase digestibility of emulsified lipids using an in vitro digestion model: proposal for a standardised pH-stat method. *Food Chemistry*, **126**, 498–505.

Li, J., Ye, A., Lee, S.J. & Singh, H. (2012). Influence of gastric digestive reaction on subsequent in vitro intestinal digestion of sodium caseinate-stabilized emulsions. *Food & Function*, **3**, 320–326.

Liang, R., Shoemaker, C.F., Yang, X., Zhong, F. & Huang, Q. (2013). Stability and bioaccessibility of β-carotene in nanoemulsions stabilized by modified starches. *Journal of Agricultural and Food Chemistry*, **61**, 1249–1257.

Liu, X., McClements, D.J., Cao, Y. & Xiao, H. (2016). Chemical and physical stability of astaxanthin-enriched emulsion-based delivery systems. *Food Biophysics*, **11**, 302–310.

Luo, Y., Teng, Z. & Wang, Q. (2012). Development of zein nanoparticles coated with carboxymethyl chitosan for encapsulation and controlled release of vitamin D3. *Journal of Agricultural and Food Chemistry*, **60**, 836–843.

Mao, L., Xu, D., Yang, J., Yuan, F., Gao, Y. & Zhao, J. (2009). Effects of small and large molecule emulsifiers on the characteristics of β-carotene nanoemulsions prepared by high pressure homogenization. *Food Technology and Biotechnology*, **47**, 336–342.

Mun, S., Kim, Y.-R., Shin, M. & McClements, D.J. (2015). Control of lipid digestion and nutraceutical bioaccessibility using starch-based filled hydrogels: influence of starch and surfactant type. *Food Hydrocolloids*, **44**, 380–389.

Ozturk, B., Argin, S., Ozilgen, M. & McClements, D.J. (2014). Formation and stabilization of nanoemulsion-based vitamin E delivery systems using natural surfactants: Quillaja saponin and lecithin. *Journal of Food Engineering*, **142**, 57–63.

Ozturk, B., Argin, S., Ozilgen, M. & McClements, D.J. (2015). Nanoemulsion delivery systems for oil-soluble vitamins: influence of carrier oil type on lipid digestion and vitamin D3 bioaccessibility. *Food Chemistry*, **187**, 499–506.

Qian, C., Decker, E.A., Xiao, H. & McClements, D.J. (2012). Nanoemulsion delivery systems: influence of carrier oil on β-carotene bioaccessibility. *Food Chemistry*, **135**, 1440–1447.

Salvia-Trujillo, L., Qian, C., Martín-Belloso, O. & McClements, D. (2013). Influence of particle size on lipid digestion and β-carotene bioaccessibility in emulsions and nanoemulsions. *Food Chemistry*, **141**, 1472–1480.

Salvia-Trujillo, L., Fumiaki, B., Park, Y. & McClements, D. (2017). The influence of lipid droplet size on the oral bioavailability of vitamin D2 encapsulated in emulsions: an in vitro and in vivo study. *Food & Function*, **8**, 767–777.

Sarkar, A., Goh, K.K. & Singh, H. (2009). Colloidal stability and interactions of milk-protein-stabilized emulsions in an artificial saliva. *Food Hydrocolloids*, **23**, 1270–1278.

Shariffa, Y., Tan, T., Abas, F., Mirhosseini, H., Nehdi, I. & Tan, C. (2016). Producing a lycopene nanodispersion: the effects of emulsifiers. *Food and Bioproducts Processing*, **98**, 210–216.

Shu, G., Khalid, N., Zhao, Y., Neves, M.A., Kobayashi, I. & Nakajima, M. (2016). Formulation and stability assessment of ergocalciferol loaded oil-in-water nanoemulsions: insights of emulsifiers effect on stabilization mechanism. *Food Research International*, **90**, 320–327.

Sjoblom, J. (2005). *Emulsions and Emulsion Stability: Surfactant Science Series/61*. Boca Raton, FL: CRC Press.

Steel, R.G., Torrie, J.H. & Dickey, D.A. (1997). *Principles and Procedures of Statistics: A Biometrical Approach*. New York, NY: McGraw-Hill.

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Tadros, T., Izquierdo, P., Esquena, J. & Solans, C. (2004). Formation and stability of nano-emulsions. *Advances in Colloid and Interface Science*, **108**, 303–318.

Tan, T.B., Yussof, N.S., Abas, F., Mirhosseini, H., Nehdi, I.A. & Tan, C.P. (2016). Stability evaluation of lutein nanodispersions prepared via solvent displacement method: the effect of emulsifiers with different stabilizing mechanisms. *Food Chemistry*, **205**, 155–162.

Vingerhoeds, M.H., Blijdenstein, T.B., Zoet, F.D. & van Aken, G.A. (2005). Emulsion flocculation induced by saliva and mucin. *Food Hydrocolloids*, **19**, 915–922.

Walker, R.M., Gumus, C.E., Decker, E.A. & McClements, D.J. (2017). Improvements in the formation and stability of fish oil-in-water nanoemulsions using carrier oils: MCT, thyme oil, & lemon oil. *Journal of Food Engineering*, **211**, 60–68.

Wang, Z., Neves, M.A., Yin, L., Kobayashi, I., Uemura, K. & Nakajima, M. (2012). In vitro gastrointestinal digestibility of soybean oil-in-water emulsion droplets stabilized by polyglycerol esters of fatty acid. *Food Science and Technology Research*, **18**, 149–156.

Xia, Z., McClements, D.J. & Xiao, H. (2017). Influence of lipid content in a corn oil preparation on the bioaccessibility of β-carotene: a comparison of low-fat and high-fat samples. *Journal of Food Science*, **82**, 373–379.

Yi, J., Li, Y., Zhong, F. & Yokoyama, W. (2014). The physicochemical stability and in vitro bioaccessibility of beta-carotene in oil-in-water sodium caseinate emulsions. *Food Hydrocolloids*, **35**, 19–27.

Zhang, C., Xu, W., Jin, W., Shah, B.R., Li, Y. & Li, B. (2015a). Influence of anionic alginate and cationic chitosan on physicochemical stability and carotenoids bioaccessibility of soy protein isolate-stabilized emulsions. *Food Research International*, **77**, 419–425.

Zhang, R., Zhang, Z., Zhang, H., Decker, E.A. & McClements, D.J. (2015b). Influence of emulsifier type on gastrointestinal fate of oil-in-water emulsions containing anionic dietary fiber (pectin). *Food Hydrocolloids*, **45**, 175–185.

Zou, L., Liu, W., Liu, C., Xiao, H. & McClements, D.J. (2015). Designing excipient emulsions to increase nutraceutical bioavailability: emulsifier type influences curcumin stability and bioaccessibility by altering gastrointestinal fate. *Food & Function*, **6**, 2475–2486.

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