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Encapsulation of Lipophilic Polyphenols in Plant-based Nanoemulsions: Impact of Carrier Oil on Lipid Digestion and Curcumin, Resveratrol and Quercetin Bioaccessibility

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Abstract

Lipophilic polyphenol compounds (LPCs) are claimed to exhibit a broad spectrum of biological activities that may improve human health and wellbeing, including antioxidant, anti-inflammatory, and anti-cancer properties. Nanoemulsion-based delivery systems have been developed to encapsulate LPCs so as to increase their food matrix compatibility, physicochemical stability, and bioavailability. LPCs vary in their structural features, including the number and position of phenolic hydroxyl, ketone, and aliphatic groups, which results in different molecular, physicochemical, and gastrointestinal properties. In this study, we examined the impact of plant-based carrier oils (coconut, sunflower, and flaxseed oils) and LPC type (curcumin, resveratrol, and quercetin) on the *in vitro* gastrointestinal fate of polyphenols loaded into quillaja saponin-stabilized nanoemulsions. Coconut oil contains high levels of medium-chain saturated fatty acids (MC-SFAs), sunflower oil contains high levels of long-chain monounsaturated fatty acids (LC-MUFAs), and flaxseed oil contains high levels of long-chain polyunsaturated fatty acids (LC-PUFAs). The encapsulation efficiency and gastrointestinal stability of the LPCs were slightly lower in the MC than the LC oils. Differences in the gastrointestinal stability of the three LPCs were linked to differences in their oil-water partition coefficients. Some of the LPCs inhibited lipid digestion for certain oil types. In particular, resveratrol retarded the digestion of all three oils, but it still had the highest GIT stability and bioaccessibility. This study provides valuable information about the gastrointestinal fate of LPC-loaded nanoemulsions and highlights important differences in the behavior of LPCs with different characteristics. This knowledge may facilitate the design of more effective plant-based delivery systems for bioactive lipophilic polyphenols.

Keywords: polyphenols, plant-based carrier oils; lipid digestion; bioaccessibility.

1. Introduction

Polyphenols are found in many commonly consumed foods and beverages, including fruits, vegetables, tea, and coffee [1]. Epidemiological evidence suggests that polyphenol-rich diets are linked to lower incidences of various human diseases, including heart diseases, diabetes, cancer, inflammation, and neurodegenerative diseases [1-3]. There may therefore be benefits to public health in fortifying foods with polyphenols so as to reduce the levels of these chronic diseases in the general population. There are, however, several challenges to incorporating polyphenols into foods and beverages, especially lipophilic polyphenol compounds (LPCs). LPCs have poor compatibility with most food matrices, they are prone to chemical degradation during storage, and they have exhibit poor bioavailability after ingestion [4, 5].

One of the most effective means of overcoming these challenges is to use modern encapsulation technologies [6, 7]. In particular, nanoemulsion-based delivery systems have been shown to be particularly effective at improving the matrix compatibility, stability, and bioavailability of polyphenols [8, 9]. Oil-in-water nanoemulsions are able to encapsulate LPCs within the hydrophobic interior of the emulsifier-coated oil droplets, thereby facilitating their introduction into aqueous food matrices, as well as protecting them from components in the aqueous phase that might promote their degradation (such as acids, bases, transition metals, or enzymes). Moreover, the digestion of the oil phase within the gastrointestinal tract produces free fatty acids (FFAs) and monoacylglycerols (MAGs) that are incorporated into mixed micelles, thereby increasing the solubilization of the LPCs in the intestinal fluids. Researchers are therefore examining the impact of the composition and structure of edible nanoemulsions on their ability to facilitate the utilization of LPCs within functional foods and beverages.

A particularly promising approach of introducing LPCs into nanoemulsions is the pH-driven method, which is based on the change in the water-solubility of certain polyphenols with pH [10, 11]. In particular, many polyphenols are hydrophilic under strongly alkaline conditions because they are negatively charged but are hydrophobic under neutral or acidic conditions because they lose their charge. As a result, the LPCs can be dissolved in an alkaline solution that is then added to an acidified nanoemulsion, causing the polyphenols to move from the hydrophilic aqueous-phase into the hydrophobic droplet interiors [12]. Numerous researchers have investigated the formation and behavior of polyphenol-loaded nanoemulsion- and emulsion-based delivery systems, which have shown that they can be designed to have a high encapsulation efficiency,

stability and bioaccessibility [13-19]. Many factors have been identified as playing a critical role in determining the bioaccessibility or bioavailability of hydrophobic bioactives, including oil-to-water ratio, droplet size, oil type, and interfacial properties [9, 20-22].

The nature of the carrier oil used to formulate the oil droplets in nanoemulsions has been shown to be particularly important for strongly hydrophobic bioactives with relatively large molecular dimensions. For example, β-carotene has a very low bioaccessibility when the oil droplets are comprised of medium chain triglycerides (MCT), because this long hydrophobic molecule is too large to be incorporated into the hydrophobic domains in the mixed micelles formed after lipid digestion [23, 24]. Similar observations have been reported for other relatively large hydrophobic bioactives, including vitamin D_3 [25] and vitamin E [26]. Conversely, relatively small hydrophobic bioactives can be incorporated into the mixed micelles formed after digestion of MCT because they can fit into the hydrophobic domains. For example, the bioaccessibility of 5-demethylnobiletin, a polyphenol, was recently reported to be relatively high when delivered in emulsified MCT oils [27]. Another important factor to consider is that some polyphenols can interact with digestive enzymes *via* noncovalent interactions (*e.g.*, hydrogen bonding or hydrophobic interactions), thereby interfering with the activity [28, 29]. In the case of lipases, this may decrease lipid digestion, and therefore alter the release of the polyphenols from the oil droplets and their solubilization within mixed micelles. LPCs may therefore behave differently in the gastrointestinal tract (GIT) than lipophilic bioactives that do not interact with lipases.

In this work, we focused on the impact of carrier oil type on the gastrointestinal fate of several LPCs encapsulated within plant-based nanoemulsions. These plant-based delivery systems were developed because of the growing interest amongst consumers for more ethical, healthy, and sustainable food products, such as meat, fish, egg and milk analogs [30, 31]. The nanoemulsions were therefore fabricated from plant-based oils (coconut, sunflower, or flaxseed oil) and surfactants (quillaja saponin). The impact of oil and polyphenol type on the gastrointestinal fate of the polyphenol-loaded nanoemulsions was examined, including lipid digestion, LPC stability, and LPC bioaccessibility. Based on previous studies, we hypothesized that: (1) oil type would have little impact on the gastrointestinal fate of the LPCs because they are relatively small molecules that could easily be solubilized within mixed micelles formed from different fatty acids; (2) some of the polyphenols (curcumin, resveratrol and quercetin in **Figure 1**) may inhibit lipid digestion by binding to lipase, which could then impact their

bioaccessibility. This study may therefore have important implications for designing effective LPC-enriched nanoemulsions for encapsulating, protecting, and delivering polyphenols. In particular, these delivery systems may be useful for application within plant-based food products, such as meat, fish, egg, or milk analogs.

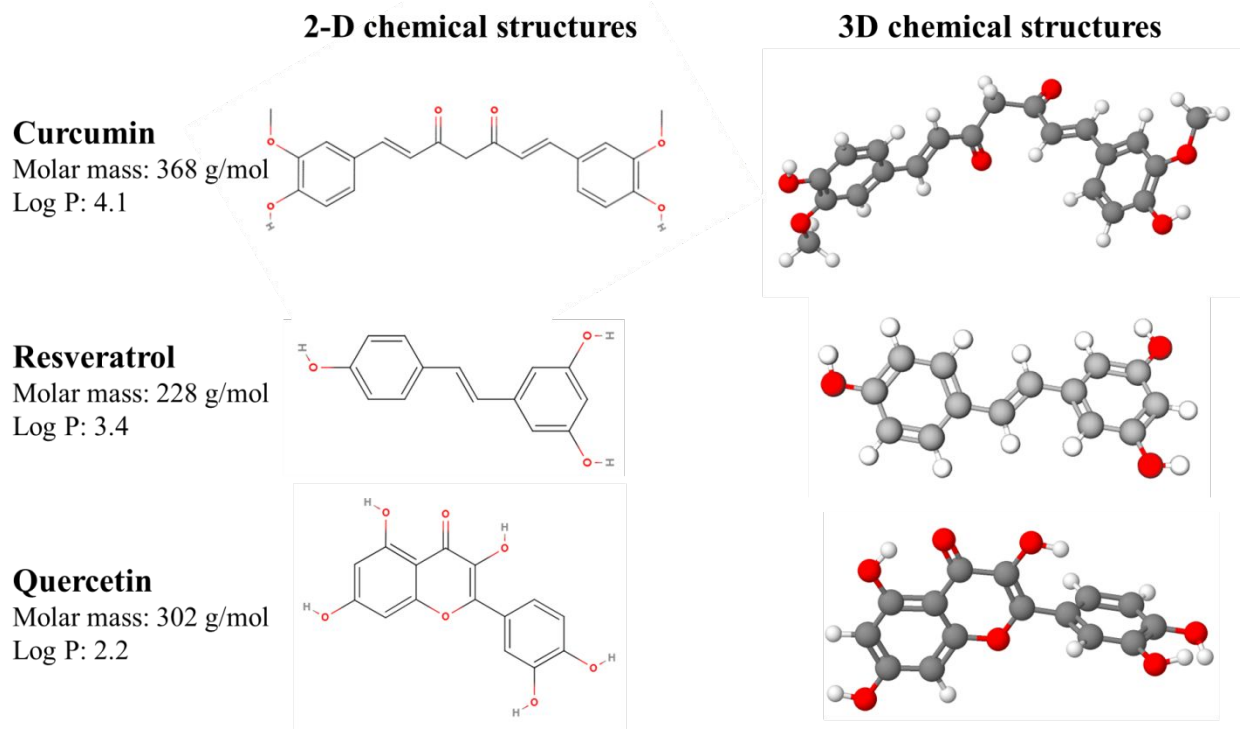


Figure 1. The 2-D and 3-D structures of curcumin, resveratrol and quercetin predicted by an online program (MolView: molview.org). All logP values were calculated from an online database (chemicalize.com).

2. Materials and Methods

2.1. Materials

Resveratrol, curcumin, and quercetin were purchased form Tokyo Chemical Industry (America Division, Portland, USA), who reported their purity to be > 99%, 97%, and 96 %, respectively. Coconut oil (Nature's Way, Pure oil, 10 oz), sunflower seed oil (365, Whole Foods Market, 16.9 oz), and flaxseed oil (365, Whole Foods Market, 8 oz) were obtained from a local supermarket. Based on their nutrition labels, the sunflower seed oil was reported to contain around 7% saturated fat, 79% monounsaturated fat, and 14% polyunsaturated fat. The flaxseed oil was reported to contain around 7% saturated fat, 18% monounsaturated fat, and 64% polyunsaturated fat. The coconut oil was reported to contain around 93% saturated fat, <3.5%

monounsaturated fat, and <3.5% polyunsaturated fat. Quillaja saponin was kindly provided by Ingredion Inc. (Westchester, IL, USA). The reagents used in the *in vitro* digestion model included: potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), hydrogen chloride (HCl), mucin (M2378), pepsin (P7000), pancreatin (P7545), lipase (L3126) and bile extract (B8631), which were also obtained from the Sigma-Aldrich Co. (St. Louis, MO, USA). Double distilled (DD) water was used to prepare the various aqueous solutions used in this study.

2.2. LPC-loaded nanoemulsions

Nanoemulsions formulated using different carrier oils (coconut, sunflower, or flaxseed oil) were fabricated by blending an aqueous phase (90 wt%) and oil phase (10 wt%) together and then passing the resulting emulsion pre-mix through a microfluidizer (M110P, Newton, MA) for 3 times at 12,000 psi. Quillaja saponin was dissolved in the aqueous phase prior to homogenization and was used at a concentration of 1 wt% in the final nanoemulsions. The aqueous phase of the nanoemulsions prepared was measured as pH 5.

The pH-driven method was used to load the LPCs into the nanoemulsions, which has been described in detail in our previous studies [13]. Briefly, polyphenols were dissolved in an alkaline solution (0.1 N NaOH) and then quickly added to the nanoemulsions at room temperature. After that, one drop of HCl (1 N) was used to adjust the pH of the nanoemulsions back to their initial values (pH 5). The final concentrations of curcumin, resveratrol, and quercetin were 450, 300 and 300 μg/g, respectively.

2.3. In vitro gastrointestinal digestion

After preparation, LPC-loaded nanoemulsions were all diluted so they contained the same oil concentration (5 wt%) before passing them through the *in vitro* digestion model, which followed the protocols described in the standardized INFOGEST method [32]. Briefly, the following steps were used to monitor the gastrointestinal fate of the nanoemulsions:

Mouth phase: 5 mL of samples were poured into 50 mL test tubes and then incubated with 5 mL of simulated saliva fluid (SSF), which contained 15 mg of mucin. The samples were maintained in the mouth phase for 2 mins at 37°C with constant swirling to mimic oral processing.

Stomach phase: After the mouth phase was completed, the oral fluids (10 mL) were collected and then an equal volume (10 mL) of simulated gastric fluid (SSF) containing HCl, pepsin (activity 2000 U/mL) and DD water were added. The pH was then adjusted to 3.0 and the samples were maintained in the stomach phase for 2 h at 37°C with constant swirling to mimic gastric processing.

Small intestine phase: After the stomach phase was completed, the resulting gastric fluids (20 mL) were collected and then poured into a 100 mL beaker. An equal volume (20 mL) of small intestinal fluids (SIF) containing DD water, bile extract (10 mM), $CaCl_2$ and pancreatin (lipase activity 2000 U/mL and trypsin activity 100 U/mL) were added. The pH was then adjusted to 7.0 and the samples were kept for 2 h at 37°C to simulate small intestine conditions. The samples were maintained at pH 7.0 throughout the entire small intestine phase by titrating in small aliquots of NaOH solutions using an automatic pH stat method (Metrohm, FL, USA). At the completion of the small intestine phase, 10 mL of the resulting sample was collected for analysis, which was referred to as the "digesta". Another 10 mL of the sample was collected and then centrifuged (18,000 rpm) for 50 mins at 4 °C, and the middle clear layer was collected, which was referred to as the "mixed micelle" phase.

Lipid digestion: The percentage of free fatty acids (FFAs) released from the nanoemulsions in the small intestine phase was monitored using the pH-state method. Briefly, the volume of NaOH solution required to maintain the sample at pH 7.0 throughout the small intestine phase was measured using the pH-stat method. However, this method is only sensitive to FFAs whose carboxyl groups are fully ionized ($-COO^-$). Many long chain FFAs are only partially ionized under neutral pH conditions. For this reason, a back-titration was performed at the end of the small intestine phase, which involved measuring the volume of NaOH solution required to reach pH 9.0. This back-titration therefore measures the remainder of the FFAs in the sample. This approach enabled us to calculate the total FFAs that were released by the end of the small intestine phase. A correction factor was then established: X = FFA(pH 7 + pH 9)/FFA(pH 7), where the two terms in this equation are the FFAs measured at the end of the small intestine phase with and without the back titration, respectively. This correction factor was then applied to the FFA measurements made at pH 7 to compensate for their partial ionization under neutral conditions. The volume of NaOH solution consumed (V_{NaOH}) for blank samples was subtracted from all the measurements made on the test samples. In this case, the blanks were the same as

the test samples, but they did not contain lipids. The molarity of the NaOH solution (C_{NaOH}) used to carry out the titrations was 0.25 M. The molar masses of the oil phases (M_{lipid}) were 554.8, 876.2 and 868 g/mol for coconut, sunflower and flaxseed oils, respectively. The total weight of the oil phase (W_{lipid}) in the small intestine phase was 0.25 g for all samples. The FFAs released from the samples was then estimated using the following expression [33]:

$$\text{FFA}\ (\%) = \frac{V_{NaOH}\, C_{NaOH}\, M_{lipid}}{2\, W_{lipid}} \times 100 \qquad (1)$$

This equation assumes that 2 FFAs are released per triacylglycerol (TAG) molecule. As will be shown later, this assumption may not be true for all systems.

2.4. Physicochemical and structural properties: Color, size and charge

A colorimeter (ColorFlex EZ 45/0-LAV, Hunter Associates Laboratory Inc., Virginia, USA) was used to quantify the color of the LPC-loaded nanoemulsions. The *L** value of the samples was measured to provide insights into their opacity or lightness, the *a** value was measured to provide information about their redness/greenness (+/-), and the *b** value was measured to provide information about their yellowness/blueness (+/-). A laser diffraction instrument (Mastersizer 2000, Malvern Instruments, Worcestershire, United Kingdom) was used to measure the dimensions of the particles within the LPC-loaded nanoemulsions before and after exposure to the simulated gastrointestinal fluids (*e.g.,* saliva, gastric, intestinal). The average size of the particles in the mixed micelle phase was measured by dynamic light scattering (Nano-ZS, Malvern Instruments). The surface potential of the particles in all the samples was measured by microelectrophoresis (Nano-ZS, Malvern Instruments). Each sample was diluted with DD water (adjusted to the same pH as sample) prior to light scattering or microelectrophoresis measurements to obtain a sufficiently strong signal and avoid multiple scattering.

2.5. LPC concentration determination

High-performance liquid chromatography (HPLC, Agilent 1100 series, Agilent Technologies, CA) with a UV-visible detector was used to measure the LPC concentration in the samples before and after digestion. LPC was extracted from the samples using an acidified ethanol solution (1% acetic acid in ethanol). The samples were then injected into the HPLC instrument. The mobile phase used depended on LPC type and consisted of 30:70, 55:45, and 30:70 of acetonitrile and 1% acetic acid for resveratrol, curcumin, and quercetin, respectively. The concentration of each LPC was determined by measuring the absorbance at an appropriate

wavelength using a UV-visible detector: 307 nm for resveratrol; 420 nm for curcumin; and, 370 nm quercetin. Standard curves were prepared for each LPC, which had R^2 values of > 0.999.

Encapsulation efficiency: The encapsulation efficiency of the LPC-loaded nanoemulsions is a measure of the percentage of the polyphenols added that actually ends up within the final delivery system:

$$EE\ (\%) = 100\ \times \frac{C_{NE}}{C_0} \quad (2)$$

Here, C_{NE} is the concentration of LPC measured in the initial LPC-loaded nanoemulsions soon after they were prepared, while C_0 is the known LPC concentration actually added to the nanoemulsions.

GIT stability: The gastrointestinal tract (GIT) stability of the LPCs is the percentage of polyphenol that remains after they have passed through the entire digestion model:

$$GIT\ stability\ (\%) = 100\ \times \frac{C_D * 8}{C_{NE}} \quad (3)$$

Here, C_D is the concentration of LPC measured in the digesta and the numeral "8" is the dilution factor, which takes into account the fact that the samples were diluted as they passed through the GIT model (5 mL of initial sample was diluted to a final volume of 40 mL in the digesta).

Bioaccessibility: The bioaccessibility of the LPCs is the percentage of polyphenol that is solubilized within the mixed micelle phase is the digesta:

$$Bioaccessibility\ (\%) = 100\ \times \frac{C_M}{C_D} \quad (4)$$

Here, C_M and C_D are the concentrations of a particular type of LPC measured in the mixed micelle and digesta phases after the small intestine phase was completed, respectively.

2.6. Statistical analysis

A full experiment involved preparing a new sample and passing it through the GIT model. This procedure was carried out twice for each system examined. The physicochemical properties, structural attributes, and LPC concentrations were measured at least three times for each procedure. The mean and standard deviation were calculated from these results and ANOVA (post-hoc Tukey HSD Test) was used to ascertain significance differences between results ($p < 0.05$).

3. Results and Discussion

3.1. Physicochemical and structural properties under simulated GIT conditions

3.1.1. Initial properties

Initially, we characterized the optical properties of the LPC-loaded nanoemulsions before they were exposed to the digestion model (**Table 1**)**.** All the original samples had a relatively high L*-value (> 83%), which is a result of strong scattering of light waves by the nanoemulsion droplets. It is sometimes assumed that nanoemulsions are optically clear but this is only true when the droplet diameter is below about 50 nm [34]. In this study, the average diameter of the droplets in the initial nanoemulsions was around 130 to 170 nm. Moreover, the nanoemulsions were polydisperse so an appreciable fraction of the droplets would be larger than these average values, thereby falling in the region where strong light scattering occurs. The lightness of the LPC-loaded nanoemulsions was appreciably lower than that of the equivalent control nanoemulsions (LPC-free). This effect arises because some of the light that was incident upon the surfaces of the nanoemulsions was absorbed by the LPCs, thereby reducing the fraction reflected back to the detector. Carrier oil type did not have a strong impact on the lightness of the nanoemulsions, which can be attributed to the fact that they were not intensely colored, and they were only present at a relatively low concentration (5%).

The addition of the polyphenols had a pronounced impact on the color of the nanoemulsions. In the absence of LPCs, the nanoemulsions appeared white, which is consistent with their high L^* values and low a^* and b^* values. In contrast, the curcumin-loaded nanoemulsions had an intense yellow color ($\Delta b^* = 81\text{-}85$), the quercetin-loaded ones had a slight yellow color ($\Delta b^* = 11\text{-}14$), and the resveratrol-loaded ones were the least yellow ($\Delta b^* = 5\text{-}9$). Indeed, the optical properties of the resveratrol-loaded nanoemulsions were fairly similar to those of the equivalent control nanoemulsions (**Table 1**). The color of the nanoemulsions followed a similar trend to that of the powdered polyphenols they were prepared from: curcumin (orangey-yellow), quercetin (light yellow), and resveratrol (white) [35].

The average diameters (d_{32}) of the emulsifier-coated droplets in the initial nanoemulsions were around 130 to 170 nm (0.13 to 0.17 mm) (**Figure 2**). These measurements indicate that the emulsifier used (quillaja saponin) and homogenization method employed (microfluidization) were efficient at creating small oil droplets in the samples. The surface potential of the saponin-coated lipid droplets in the initial nanoemulsions was strongly anionic, ranging from -60 to -70 mV for the different nanoemulsions (**Figure 3**). Nevertheless, carrier oil and polyphenol type did not have an appreciable effect on the surface potential of the droplets, which suggests that

their electrical characteristics were dominated by the saponin molecules adsorbed to their surfaces. Previous studies have indicated that saponin-coated oil droplets with these small dimensions and high charges have good resistance to aggregation and creaming, thereby leading to nanoemulsions with extended shelf lives, *i.e.,* > 2 months [36, 37].

Table 1. Impact of carrier oil and polyphenol type on the tristimulus color coordinates of pure nanoemulsions and LPC-loaded ones.

Control	**Coconut**		**Sunflower**		**Flaxseed**	
	Mean	SD	Mean	SD	Mean	SD
ΔL*	89.49	0.12	89.81	0.10	87.73	0.10
Δa*	-0.42	0.03	-0.57	0.02	-0.97	0.01
Δb*	9.15	0.04	5.59	0.07	5.42	0.04
Curcumin	**Coconut**		**Sunflower**		**Flaxseed**	
	Mean	SD	Mean	SD	Mean	SD
ΔL*	83.80	0.13	83.58	0.03	84.42	0.21
Δa*	-10.98	0.22	-5.35	0.05	-6.68	0.07
Δb*	81.49	0.29	85.44	0.34	82.87	0.51
Resveratrol	**Coconut**		**Sunflower**		**Flaxseed**	
	Mean	SD	Mean	SD	Mean	SD
ΔL*	86.57	0.23	88.43	0.01	88.51	0.05
Δa*	-0.15	0.22	0.01	0.03	0.02	0.09
Δb*	5.14	0.12	5.36	0.00	8.58	0.05
Quercetin	**Coconut**		**Sunflower**		**Flaxseed**	
	Mean	SD	Mean	SD	Mean	SD
ΔL*	86.46	0.13	88.23	0.07	88.17	0.18
Δa*	-2.94	0.01	-2.99	0.20	-2.61	0.12
Δb*	11.53	0.49	11.42	0.27	14.35	0.27

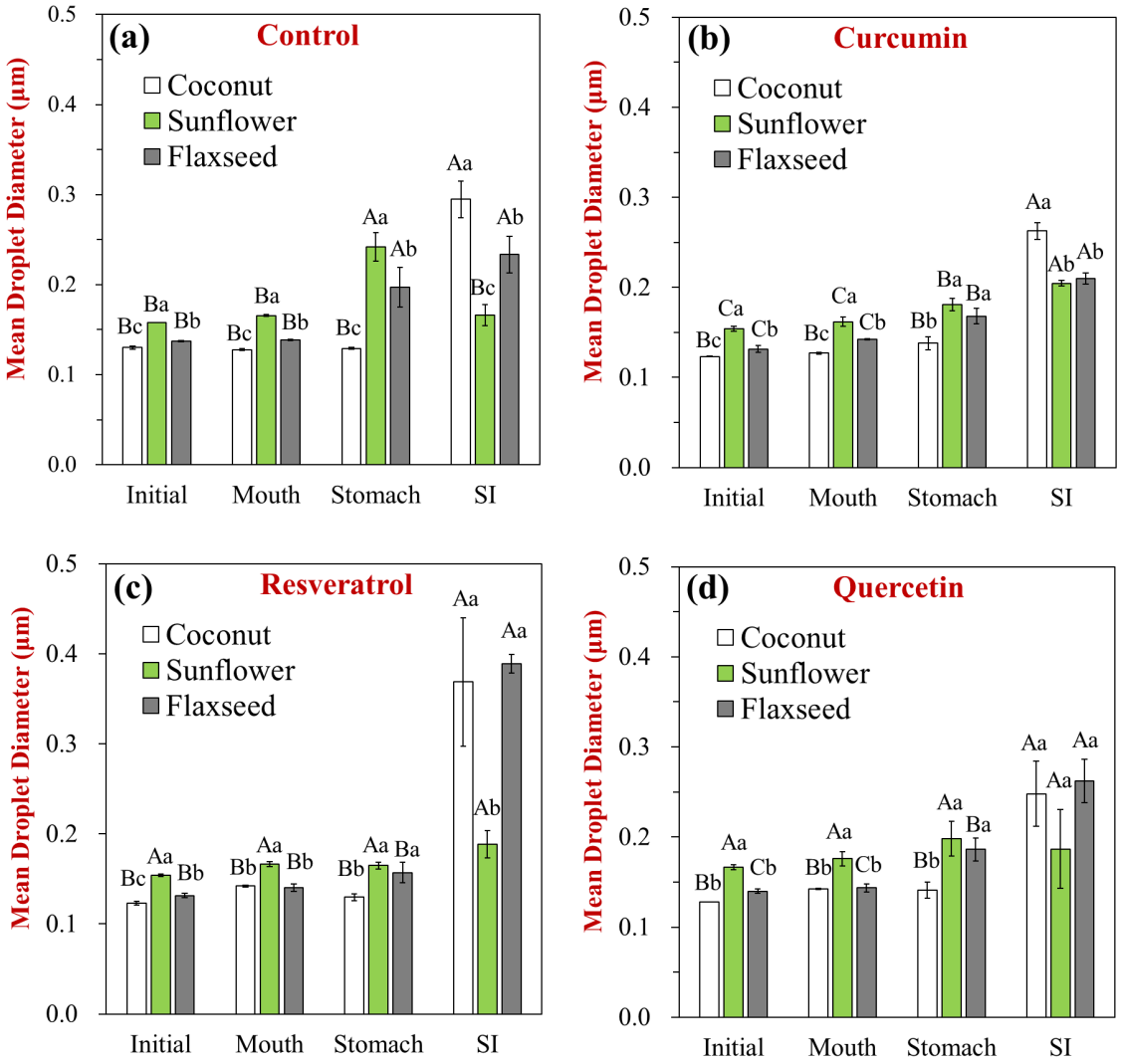
3.1.2. Mouth and stomach phases

The light scattering data indicated that all the nanoemulsions were relatively resistant to droplet aggregation within the mouth and stomach stages, with mean particle diameters ranging from 0.1 to 0.2 mm (**Figure 2**). Moreover, the particle size distributions of all the nanoemulsions remained monomodal within these GIT stages (data not shown). The good aggregation stability of the droplets under these conditions can be attributed to the presence of saponin molecules at their surfaces, which protect the droplets by generating a strong steric repulsion. Indeed, previous studies with nanoemulsions have shown that quillaja saponins can

protect oil droplets from aggregation over a wide range of pH conditions (pH = 2 - 8) and salt concentrations (0 - 500 mM) due to this effect [36].

Changes in the electrical characteristics of the droplets in the nanoemulsions were monitored using particle electrophoresis (**Figure 3**). The magnitude of the surface potential of all nanoemulsions decreased appreciably when they moved from the initial stage (-54 to -65 mV) to the mouth stage (-36 to -49 mV), which was mainly attributed to adsorption of mucin molecules to the oil droplets surfaces, as well as electrostatic screening effects associated with mineral ions dissolved in the simulated saliva. There was a further decrease in the magnitude of the surface potential after the nanoemulsions were incubated in the stomach stage (-8 to -16 mV), which was mainly attributed to partial protonation of the carboxyl groups on the adsorbed saponin molecules.

Overall, our results indicate that carrier oil and polyphenol type did not have a major impact on the size or charge of the oil droplets within the early stages of the GIT model. It should be noted that we did not observe any evidence of crystalized polyphenols in the samples collected from the mouth and stomach phases, *i.e.,* no sediment was visible at the bottom of the test tubes. This result suggests that the polyphenols remained inside the hydrophobic interiors of the oil droplets, rather than moving into the water phase and precipitating.



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310 **Figure 2**. Impact of carrier oil and polyphenol type on the mean droplet diameter (d_{32}) of LPC-
311 loaded nanoemulsions under simulated gastrointestinal conditions. The upper-case letters (A, B,
312 C) and (a, b, c) represent significant differences among different digestion stages and oil types,
313 respectively.

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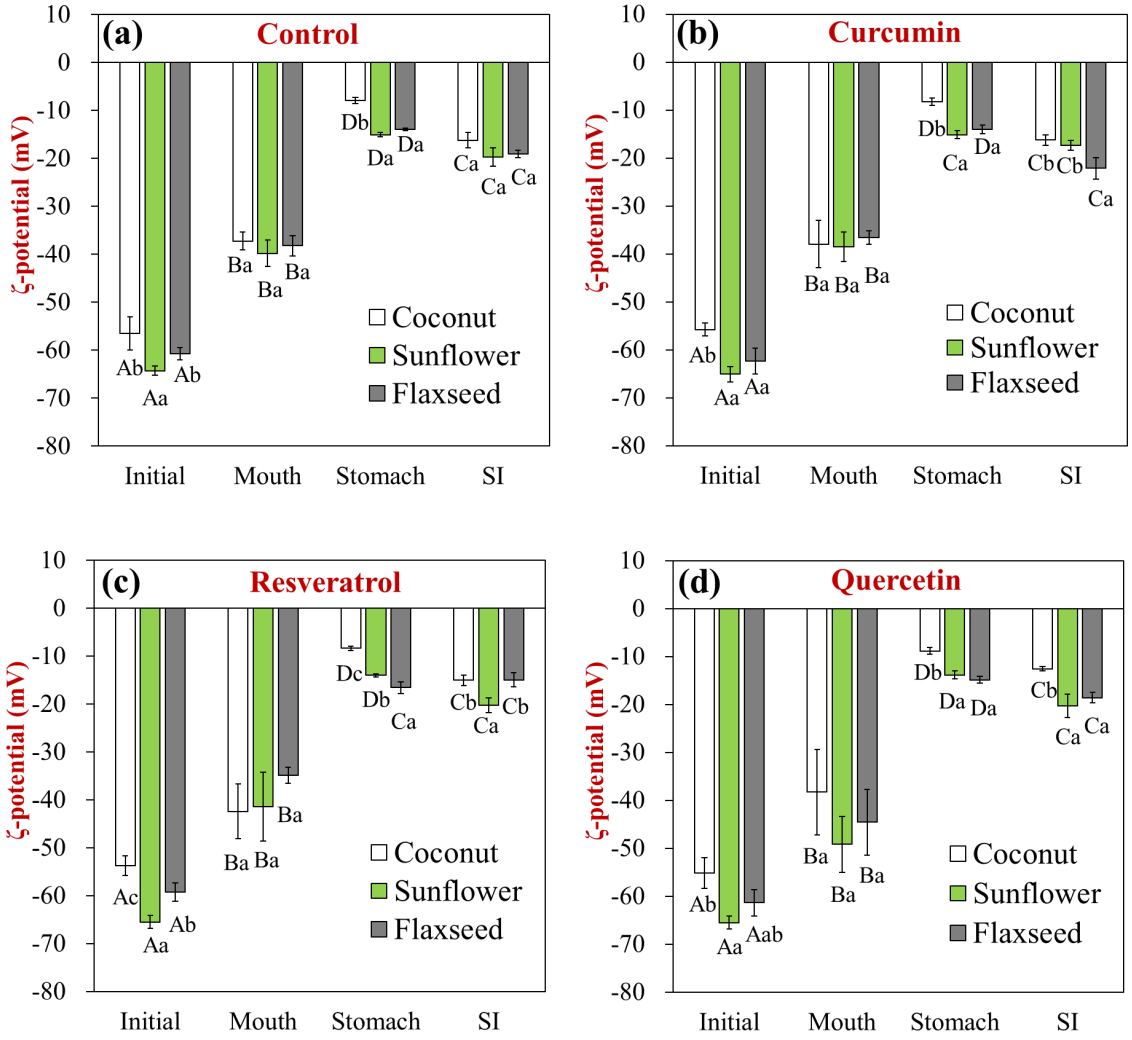

Figure 3. Impact of carrier oil and polyphenol type on the surface potential of LPC-loaded nanoemulsions under simulated gastrointestinal conditions. The upper-case letters (A, B, C) and (a, b, c) represent significant differences among different digestion stages and oil types, respectively.

3.1.3. Small intestine phase

There was an increase in mean particle diameter after the nanoemulsions were exposed to the small intestine phase for most of the samples (**Figure 2**), which can be attributed to digestion of the oil droplets and the formation of new types of colloidal structures [38]. However, the increase in mean particle size in the small intestine phase observed in this study was considerably smaller than that observed in some other *in vitro* studies on related systems [25, 26]. For example, more than 1.0 μm value was found in particle size of previous systems with

medium- or long-chain triacylglycerols [26]. This effect can mainly be attributed to differences in the *in vitro* digestion models used. These other studies used a simulated GIT that had relatively high calcium levels in the small intestine phase, whereas the INFOGEST model used in the current study contains much lower calcium levels [39]. Calcium ions are known to promote aggregation of long-chain ionized free fatty acids, thereby forming insoluble calcium soaps that contribute to the light scattering signal [38].

After lipid digestion, all the digested solution (pH = 7) contained colloidal particles with high negative surface potentials (~ -20 mV), which is due to the presence of anionic species at their surfaces, such as free fatty acids, bile acids, and saponins. Polyphenol type did not significantly affect the measured mean particle diameters or surface potentials, but carrier oil type did. In particular, the average particle diameters in the digested coconut oil nanoemulsions were higher than those in the sunflower or flaxseed oil nanoemulsions, while their surface potentials were less negative. These results suggest that the medium chain FFAs and MAGs released after digestion of the coconut oil impacted the nature of the colloidal particles formed in the mixed micelle phase.

3.1.4 Mixed micelle phase

There were appreciable differences in the properties of the mixed micelle phase collected from the different nanoemulsions (**Table 2**). Visually, the mixed micelle phases appeared optically transparent for coconut oil but cloudy for sunflower and flaxseed oil, which suggested they contained different kinds of colloidal particles. Moreover, three peaks were observed in the particle size distributions of the digested coconut oil samples, but only one broad peak was observed in the digested sunflower and flaxseed oil samples (**Figure 4**). The three peaks in the coconut oil samples probably represent micelles (4-10 nm), liposomes (20-90 nm), and calcium soaps (300-800 nm), respectively. In contrast, the single broad peaks observed in the other two samples probably represented a mixture of liposomes, non-digested fat droplets, and calcium soaps. Despite being optically clear, the mean particle diameters of the coconut oil mixed micelles were appreciably greater than those of the sunflower and flaxseed oil ones (**Table 2**), which suggests that they contain a few large particles (probably calcium soaps) that dominated the light scattering signal. Indeed, a sediment layer was observed at the bottom of the coconut oil samples (**Figure 4**), which may have been formed because the saturated fatty acids had a greater tendency to precipitate in the presence of calcium ions.

The impact of oil type on the appearance and structure of the mixed micelle samples was largely independent of LPC type, which suggested that the polyphenols did not strongly influence the nature of the colloidal particles formed after digestion. The only exception was for the coconut oil, where the presence of the polyphenols appeared to decrease the size of the particles present in the mixed micelle phase (**Table 2**). This may have been because they interfered with the formation of calcium salts from medium chain FFAs.

Table 2. Average particle sizes and surface potentials of the colloidal particles in the mixed micelle phase collected after digestion of nanoemulsions formulated using different carrier oil and polyphenol types.

Samples	Coconut			Sunflower			Flaxseed		
	Z-average (nm)	PDI	ζ-potential (mV)	Z-average (nm)	PDI	ζ-potential (mV)	Z-average (nm)	PDI	ζ-potential (mV)
Control	649.1 ± 19.9	0.81 ± 0.02	-14.7 ± 1.2	142.2 ± 9.9	0.25 ± 0	-29.0 ± 1.9	132.9 ± 5.6	0.24 ± 0	-27.5 ± 2.6
Curcumin	287.3 ± 13.0	0.65 ± 0.2	-15.6 ± 1.8	129.1 ± 1.8	0.26 ± 0.02	-28.1 ± 0.9	129.4 ± 3.5	0.24 ± 0.01	-30.4 ± 1.9
Resveratrol	224.9 ± 39.0	0.49 ± 0.08	-12.5 ± 1.3	124.1 ± 19.7	0.26 ± 0	-30.1 ± 1.2	122.3 ± 6.5	0.25 ± 0.01	-23.4 ± 1.3
Quercetin	161.6 ± 22.5	0.48 ± 0.08	-14.7 ± 0.9	126.0 ± 20.9	0.29 ± 0.07	-29.9 ± 1.2	132.3 ± 6.5	0.24 ± 0	-26.6 ± 0.9

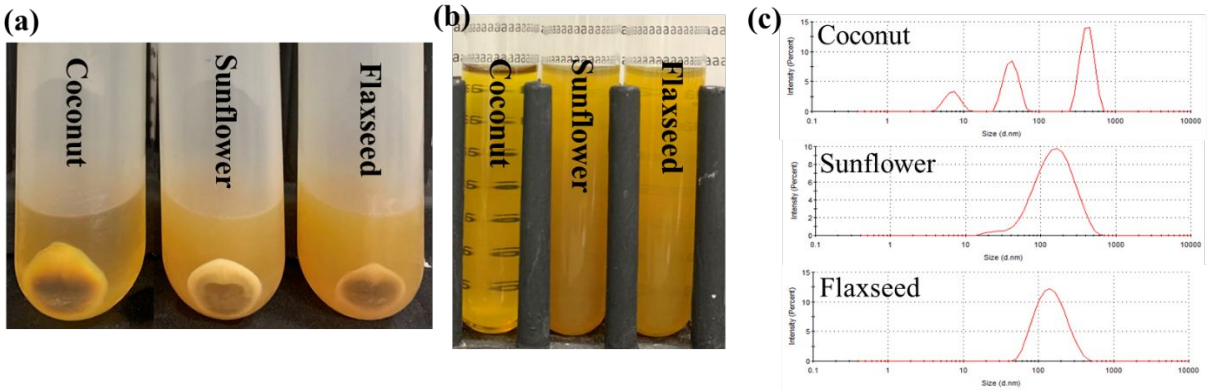

Figure 4. Photographs of quercetin-loaded samples: (a) appearance after centrifugation and (b) appearance of mixed micelle phase. The other LPCs exhibited similar appearances. (c) The particle size distributions of mixed micelles formed from different oil phases. In this case, the curcumin-loaded samples are shown as examples. Again, the other LPCs exhibited similar behaviors.

3.2. Lipid digestion

Impact of carrier oil type: It has been reported that the length of the fatty acid chains within TAGs can significantly affect the overall kinetics and degree of digestion. In particular, TAGs mainly comprised of medium-chain fatty acids (*e.g.*, coconut oil or MCT) are digested more rapidly and extensively than those comprised of long-chain fatty acids (*e.g.*, corn and fish oils) [25, 40]. A similar trend was also observed for the lipase hydrolysis of the LPC-loaded nanoemulsions used in the current study. The corrected percentage of FFAs released from the control and LPC-loaded nanoemulsions during the small intestine phase are shown in **Figure 5**. These values were corrected by calculating a correction factor from the total amount of FFAs released by the end of the digestion process in the absence and presence of a back-titration to pH 9 (**Figure 6**).

3.2.1. Impact of oil type

Initially, we focus on the impact of oil type on the digestion properties of the control nanoemulsions that contained no LPCs (**Figures 5a and 6a**). Rapid hydrolysis of the nanoemulsions occurred during the first 5 to 10 minutes followed by a slower hydrolysis for the remainder of the digestion period. This was to be expected because the oil droplets in the nanoemulsions were relatively small and so have large specific surface areas [22]. As a result, the lipase molecules rapidly adsorb to the oil-water interfaces and break the ester bonds holding the fatty acids to the glycerol backbone. Typically, the ester bonds in the sn-1 and sn-3 positions of TAGs are rapidly hydrolyzed by pancreatic lipase, leading to two FFAs and one MAG [38]. In our study, much more than 100% FFAs were released from the control nanoemulsions formulated using the different plant-based oils, with the coconut oil giving a higher amount (177%) than the sunflower or flaxseed oils (around 150%) by the end of the small intestine phase. As mentioned earlier, it was assumed that only two FFAs were released per TAG in the calculations of the digestion profiles. However, some studies have shown that the ester bond holding the fatty acid molecule to the sn-2 position of MAGs can also be slowly cleaved during digestion, and that isomerization reactions can occur that cause fatty acids to move from the sn-2 position to other positions, thereby making them more available for hydrolysis [41, 42]. Consequently, there may be more than two FFAs released per TAG molecule. Indeed, our results suggest that close to three of the FFAs were released by the end of digestion. This process may not be observed for the digestion of bulk oils or coarse emulsions because the hydrolysis rate is much slower so that the MAGs have less time in the small intestine phase.

In the absence of the back-titration step, the total extent of FFAs released from the control nanoemulsions formulated from long-chain TAGs (sunflower and flaxseed oils) was much less than that from the medium-chain ones (coconut oil) (**Figure 6a**). This effect can be attributed to the fact that the pK_a values of long-chain FFAs are higher than those of medium-chain ones, so only a fraction of them are ionized under neutral conditions [38]. This result highlights the importance of carrying out a back-titration step when using the INFOGEST *in vitro* digestion method to determine the amount of FFAs released.

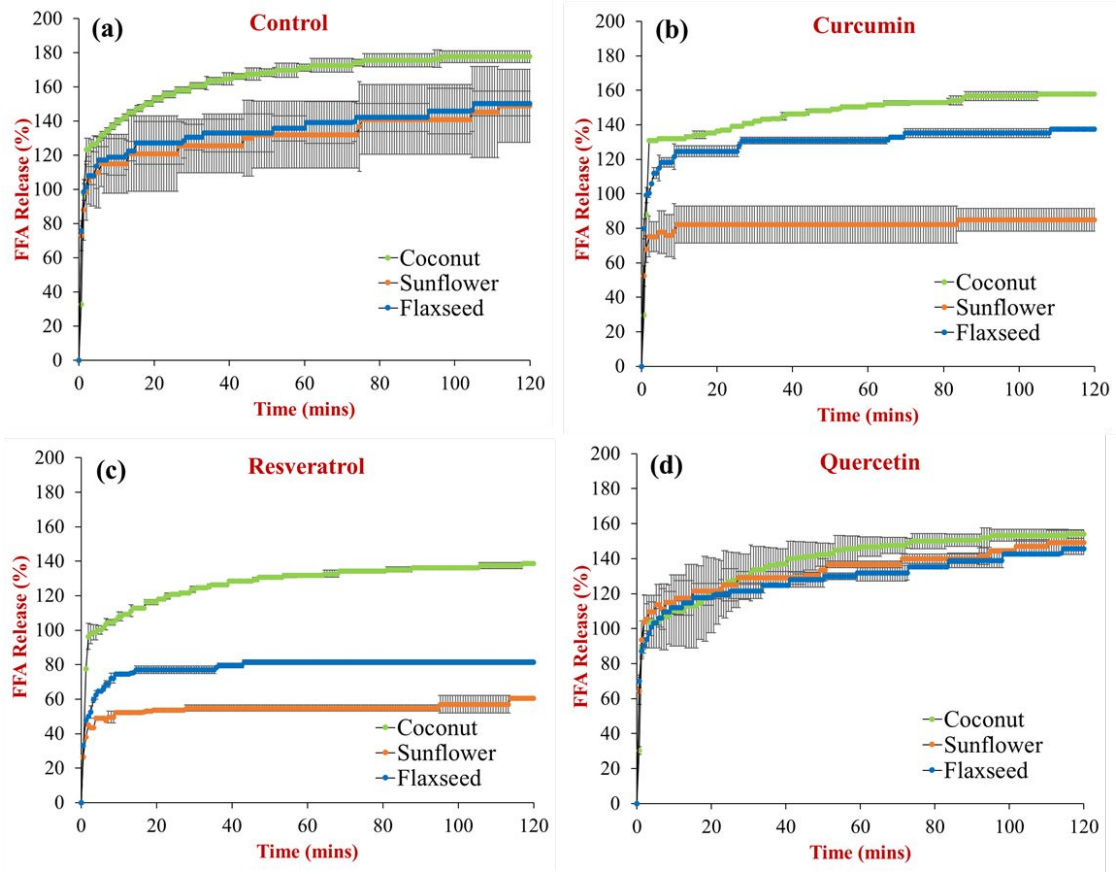

Figure 5. Release of free fatty acids (FFAs) in the control and resveratrol-loaded nanoemulsions under a simulated small intestine condition, the plotted FFA(t) is a scaled FFA(t) curve at pH 7 after multiplying by a ratio of final FFA amount at pH 9 to that at pH 7.

3.2.2. Impact of polyphenol type on lipid digestion

Some researchers have reported that certain types of LPCs inhibit lipid digestion, which has been attributed to non-covalent interactions between the polyphenols and digestive enzymes that reduced their activity [28]. For this reason, we examined the impact of LPC type on the lipid digestion profiles of the different oils in the nanoemulsions (**Figure 5**). In general, the FFA

release profiles followed a similar trend as the controls: a rapid rate of lipid hydrolysis during the first few minutes, then a slower rate later. However, there was clear evidence of inhibition of lipid digestion in several of the nanoemulsions, with the final amount of FFAs generated being considerably lower than the controls (**Figure 6**). The extent of this effect depended on both oil and LPC type.

For the coconut oil nanoemulsions, there was only a slight reduction in the total amount of FFAs released by the end of lipid digestion, decreasing from 177% for the control to 159, 139, and 154% for the curcumin-, resveratrol-, and quercetin-loaded nanoemulsions, respectively. This result suggests that the LPCs only had a small impact on the activity of the lipase when coconut oil was used as the lipid phase. In contrast, they had a pronounced effect on the total amount of FFAs released from the sunflower and flaxseed nanoemulsions, depending on LPC type: resveratrol caused an appreciable decrease in both nanoemulsions; curcumin only caused an appreciable decrease in the sunflower nanoemulsions; and quercetin did not greatly inhibit digestion in either nanoemulsion (**Figure 6**). A previous study examining the effects of various phenolic compounds on *in vitro* lipid digestion reported that the inhibition of lipolysis followed the following trend: resveratrol > quercetin > curcumin [43]. Thus, these authors also found that resveratrol was the most potent lipase inhibitor, but they found that quercetin was more potential than curcumin, which was opposite to our results. It should be noted that these researchers used triolein as a model lipid compound, which consists of long-chain monounsaturated fatty acids. The triolein was therefore most similar to the sunflower oil used in our study, where we still found that quercetin had the least inhibitory effect. These differences may be a result of the different kinds of delivery systems and in vitro digestion models used. In our study, the lipid phase was converted into a nanoemulsion that was passed through mouth, stomach, and small intestine phases. However, in the other study, the triolein was not emulsified and only a small intestine phase was utilized.

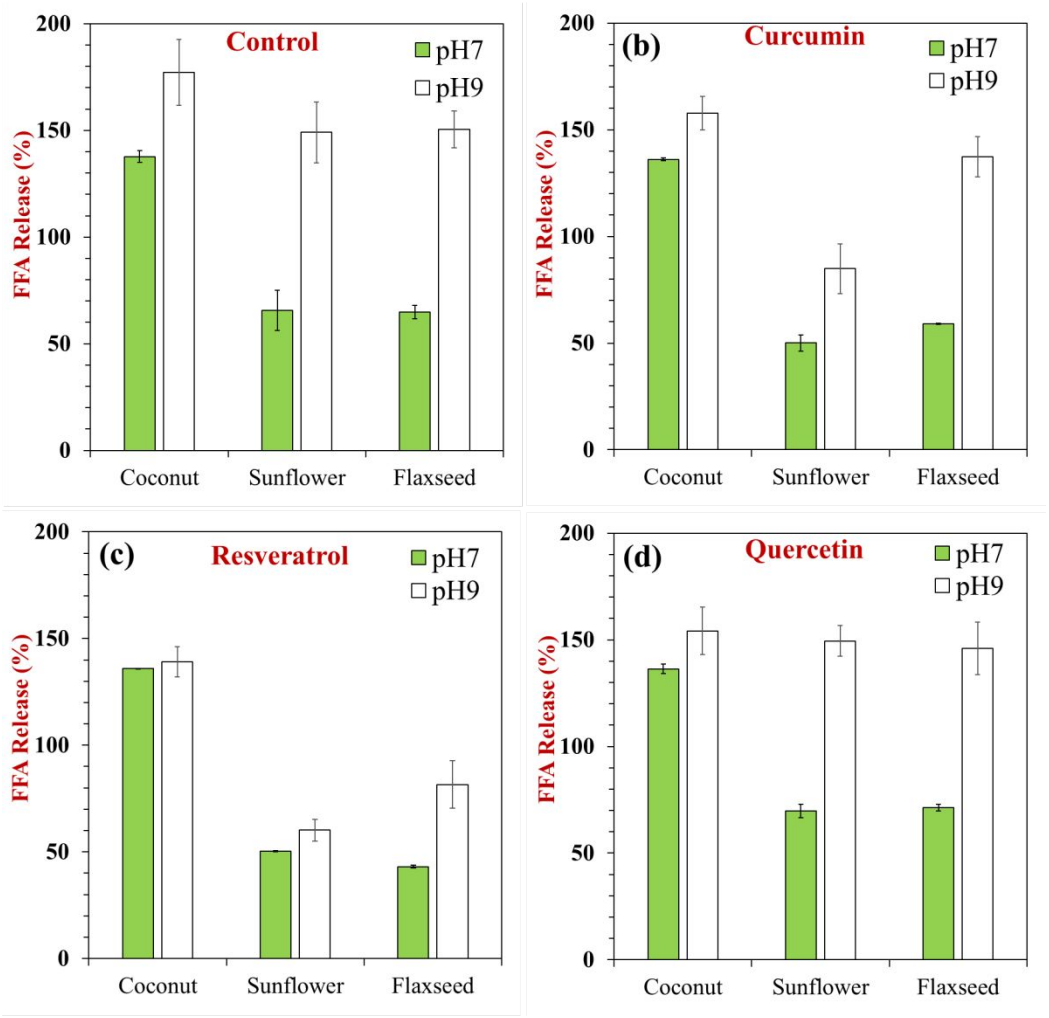

Figure 6: The total fraction of free fatty acids released under simulated small intestine conditions assuming two FFAs released per TAG molecule. Measurements were made at pH 7 after digestion and at pH 9 following a back titration.

3.3. Encapsulation efficiency and gastrointestinal tract stability

The three LPCs used in our study have previously been reported to be susceptible to chemical degradation under conditions they may experience during storage or during passage through the GIT [35]. For this reason, we measured the impact of carrier oil and polyphenol type on the GIT stability of the LPCs after they had been exposed to the full simulated digestion model.

Initially, we measured the encapsulation efficiency of the three polyphenols after they were loaded into the nanoemulsions using the pH driven method. In all cases, the encapsulation efficiency was relatively high (70 - 90 %) but it did depend on oil type. For all LPCs, a higher

461 percentage of the polyphenols was successfully encapsulated for the long-chain TAGs (flaxseed
462 and sunflower oils) than the medium-chain ones (coconut oil). This effect suggests that the LPCs
463 were more readily solubilized inside the oil droplets when the oil phase contained larger TAG
464 molecules.

465 After passing through the *in vitro* digestion model, the GIT stability of the polyphenols in
466 the LPC-loaded nanoemulsions was measured and compared to that of LPC crystals dispersed in
467 water (**Figure 7**). The GIT stability of the curcumin and resveratrol was relatively high (85-
468 100%) in all nanoemulsions and crystals. In contrast, the GIT stability of the quercetin was
469 relatively low in the nanoemulsions (35 to 43%) and in the crystals (73%). These results show
470 that the quercetin is much more susceptible to chemical degradation under GIT conditions than
471 the other two polyphenols used. Oil type did not have a major impact on GIT stability, with the
472 exception of curcumin, which appeared to be more stable in sunflower oil than in coconut oil or
473 flaxseed oil.

474 The differences in the GIT stabilities of the LPCs was attributed to differences in their oil-
475 water partition coefficients and chemical reactivities (**Figure 1**). Curcumin (LogP = 4.1) and
476 resveratrol (LogP = 3.4) are more lipophilic than quercetin (logP = 2.2). Consequently, there
477 may have been a lower fraction of quercetin in the oil phase than for the other two polyphenols
478 [35]. Previous studies have shown that polyphenols tend to be more stable when located in an oil
479 phase than in an aqueous phase [44].

480 **3.4. Bioaccessibility**

481 The bioaccessibility of LPCs provides a useful indication of the fraction that is available in a
482 form suitable for absorption by the epithelium cells. Experimentally, it is defined as the ratio of
483 LPCs solubilized within the mixed micelle phase relative to the total amount in the digesta. In
484 general, the bioaccessibility of lipophilic bioactive agents is known to depend on the type and
485 concentration of carrier oil used, as well as the molecular structure of the bioactives themselves
486 [22]. For this reason, we examined the impact of carrier oil and bioactive type of LPC
487 bioaccessibility.

488 *3.4.1. Impact of carrier oil type*

489 The mean diameters of the colloidal particles remaining in the mixed micelle phase after
490 they were separated by centrifugation were less than 300 nm (**Table 2**), which means that they
491 would be small enough to move pass through the mucus layer covering the epithelium cells. The

bioaccessibility of the polyphenols did not depend strongly on carrier oil type (**Figure 7**), with the exception of quercetin, which was less bioaccessible for coconut oil than for the other two oils. A lower bioaccessibility in mixed micelles comprised of medium chain fatty acids has previously been reported for strongly hydrophobic bioactive substances encapsulated within nanoemulsions, such as carotenoids and oil-soluble vitamins [24-26]. However, the quercetin (302 g/mol) had a molecular weight that was between those of resveratrol (228 g/mol) and curcumin (368 g/mol). It is therefore possible that the molecular geometry and distribution of the polar and non-polar groups on the quercetin molecule make it more difficult for it to easily fit within the hydrophobic domains in smaller mixed micelles. Nevertheless, further research is required to elucidate the relationship between the molecular structure of polyphenols and their incorporation into mixed micelles comprised of different fatty acids. In general, both the length and unsaturation of the fatty acids would be expected to impact lipid digestion and polyphenol bioaccessibility, and so these factors should be the subject of future studies.

3.4.2. Impact of polyphenol type

The bioaccessibility of the LPCs clearly depended on the molecular structure of the polyphenols. For resveratrol, curcumin and quercetin, the measured bioaccessibility values were 85.5, 51.8 and 48.7% for coconut oil, 80.2, 53.2, and 75.2% for sunflower oil, and 77.2, 59.1 and 69.2% for flaxseed oil, respectively. These results suggest that the resveratrol had the highest bioaccessibility in all of the digested nanoemulsions. In the nanoemulsions formulated with long-chain fatty acids, the bioaccessibility of the LPCs decreased in the following order: resveratrol > quercetin > curcumin. But in the nanoemulsions formulated with medium-chain fatty acids it decreased in the following order: resveratrol > quercetin » curcumin. A relatively low bioaccessibility of curcumin has also been reported in other kinds of colloidal systems containing lipid droplets, such as soymilk (59%) and bovine milk (41%) [45, 46]. The molecular origin of the different bioaccessibilities of the different LPCs is still not clearly understood. As discussed earlier, the molar masses of the three LPCs decrease in the following order: curcumin > quercetin > resveratrol, whereas their hydrophobicities (logP) decrease in the following order: curcumin > resveratrol > quercetin. This suggests that some other factors must also influence the concentrations of LPCs incorporated into the mixed micelles, such as binding to proteins or incorporation into calcium soaps. Again, it will be important in the future to identify the molecular origin of these effects.

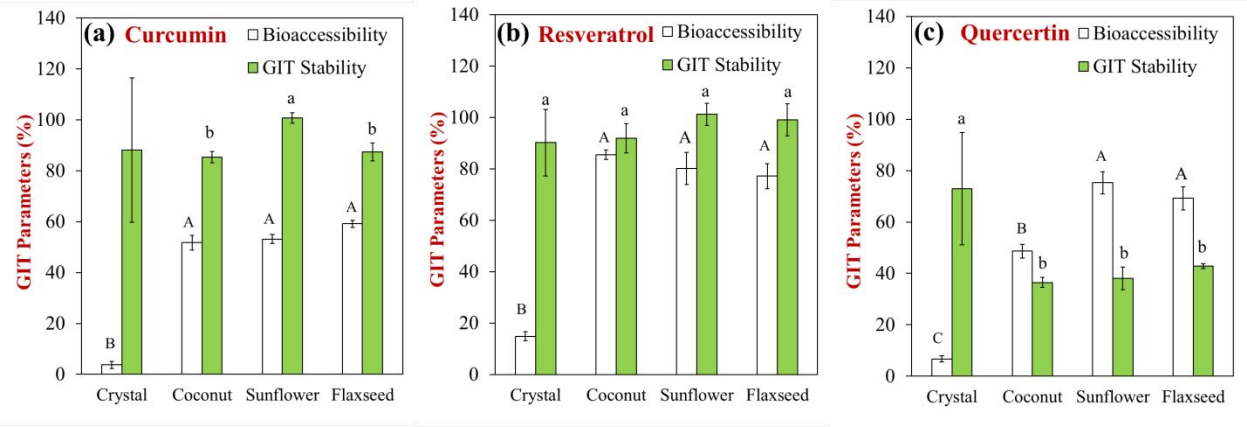

Figure 7. The gastrointestinal stability and bioaccessibility of the polyphenols in LPC-loaded nanoemulsions formulated using different carrier oil types. The upper-case letters (A, B, C) and lower-cased letters (a, b, c) represent significant differences among the bioaccessibility and GIT stability values of the samples, respectively.

3.5. Relationship between digestion inhibition, stability, and bioaccessibility

As discussed in Section 3.2.2., resveratrol was the most effective polyphenol at inhibiting lipid digestion in the nanoemulsions containing long-chain fatty acids (**Figure 5**). However, it also had the highest bioaccessibility and GIT stability (**Figure 7**). One possibility is that the resveratrol was located near the surfaces of the lipid droplets during the digestion process, which inhibited the lipase molecules from interacting with the TAGs inside the droplets. Nevertheless, there was still sufficient digestion for most of the resveratrol to be released from the lipid droplets and solubilized within the mixed micelles formed. All the polyphenols have multiple polar hydroxyl groups, as well as multiple non-polar groups, which means that it may be possible for them to be located at the lipid droplet surfaces. The quercetin did not appear to strongly inhibit lipid digestion (**Figure 5**) but it had the lowest GIT stability and a moderate bioaccessibility. These results may be due to the more polar nature of this polyphenol, which reduced its binding to the lipase and increased its solubility in the aqueous phase, thereby leading to more chemical degradation. Overall, these results suggest that there are many different physicochemical phenomena occurring within the GIT that can influence the LPC bioavailability and lipid digestion and more detailed studies are still required.

4. Conclusion

This study systematically examined the gastrointestinal fate of LPC-loaded nanoemulsions formulated from carrier oils containing fatty acids with different chain lengths and degrees of unsaturation. In contrast to previous studies, we found that the bioaccessibility of LPCs encapsulated in nanoemulsions formulated using medium-chain fatty acids (coconut oil) is not always lower than in those formulated using long-chain fatty acids (sunflower and flaxseed oil). In particular, there was only a modest decrease for quercetin and no difference for resveratrol and curcumin. We also found that resveratrol and curcumin could substantially inhibit lipid digestion in some of the nanoemulsions, which may be because they can interact with lipase and decrease its activity. The bioaccessibility of the three LPCs was different but there was no strong correlation to their molar masses or hydrophobicities. Clearly, further research is required to establish the relationship between the molecular structure of polyphenols and their bioaccessibility, GIT stability, and inhibitory effects on lipid digestion. The information gained from this study will facilitate the development of more effective delivery systems for polyphenols, especially those intended for applications in plant-based foods. In future studies, it would be advantageous to establish whether similar results were found when LPC-loaded nanoemulsions were exposed to real GIT conditions using animal and human studies. In particular, there are a number of metabolic enzymes in real GITs that may promote the metabolism of the polyphenols, which were not accounted for in this study.

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