 DOI 10.1002/mnfr.201000572 *Mol. Nutr. Food Res.* 2011, *55*, S278–S289

RESEARCH ARTICLE

Release of lipophilic molecules during in vitro digestion of soy protein-stabilized emulsions

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Received: November 16, 2010
Revised: February 24, 2011
Accepted: March 16, 2011

Scope: Solubilization of lipophilic bioactives in gastrointestinal fluids contributes to their bioavailability, but a better understanding of the transfer processes involved and the impact of molecular structure is required.

Methods and results: The transfer of β-carotene (BC), coenzyme Q10 (CoQ10), vitamin D3 (VitD3), and phytosterols (PSs) from soy protein isolate-stabilized oil-in-water emulsions to the aqueous phase during in vitro digestion was investigated. In the absence of lipolysis, transfer was mainly governed by molecular structure and partitioning within the oil droplets. Less than 3% BC and CoQ10, versus 30.4 ± 0.3% PSs and 24.7 ± 0.4% VitD3, were transferred in this case. However, with lipolysis, PSs and VitD3 rapidly partitioned into the aqueous phase, while lag phases and slower transfer rates were observed for BC and CoQ10. Positive and linear correlations between lipolysis and transfer were observed for all systems. After 2 h exposure to simulated duodenal conditions, there were no differences between percent micellization, except for BC which was proportionally lower. VitD3 and PSs mutually enhanced each other's transfer, while no interactions were observed between VitD3 and BC.

Conclusion: Bioactive molecular structure and co-administration influenced the transfer behaviour, with implications for foods designed to optimize health benefits.

Keywords:
In vitro digestion / Lipolysis / Lipophilic bioactives / Micellization / Oil-in water emulsion

1 Introduction

Lipophilic molecules perform numerous biological roles in human health. However, their poor water solubility means they tend to suffer from low bioavailability. Prior to absorption, oil-soluble molecules must be released from a food or supplement and solubilized within the contents of the small intestine through inclusion in the mixed micellar aqueous phase. The mixed micelles are mainly comprised of bile salts (BSs) and phospholipids (PLs) from the biliary fluids, lysophospholipids produced by phospholipase A2 activity, cholesterol, as well as monoacylglycerols (MGs) and free fatty acids (FFAs), which are produced by digestion of lipids in the gastrointestinal (GI) tract [1].

Various intrinsic and external factors affect lipophilic molecule bioavailability, including chemical structure, food microstructure, the presence of other dietary compounds, the amount of available oil, rate of lipid hydrolysis, and micelle formation [2]. The release in GI fluids is highly variable and determined by whether the molecules is dissolved in the oil phase, in the crystalline form, or bound with other food components [3]. For example, carotenoids are more bioavailable when solubilized in oil droplets, compared to when they are present as crystals in vegetable

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Abbreviations: BC, β-carotene; **BSs,** bile salts; **CoQ10,** coenzyme Q10; **FFAs,** free fatty acids; **GI,** gastrointestinal; **MGs,** monoacylqlycerols; **PSs,** phytosterols; **PTL,** pancreatic triglyceride lipase; **SGF,** simulated gastric fluid; **SO,** soybean oil; **SPI,** soy protein isolate; **VitD3,** vitamin D3

tissues, owing to an easier transfer to the micellar phase [4]. Vitamin D3 (VitD3) solubilized in oil droplets also seems to be more bioavailable compared to VitD3 consumed in powder form [5]. Furthermore, the absorption efficiency of coenzyme Q10 (CoQ10) is significantly higher for oil-solubilized formulations or when complexed with γ-cyclodextrin compared to powder-based and tablet supplements, as demonstrated by in vitro studies with Caco-2 cell cultures [6] as well as human in vivo bioavailability experiments [7].

The chemical and physical properties of molecule, including molecular structure and mass, degree of hydrophobicity and solubility, affect their adsorption behavior through influencing the partitioning into the aqueous phase of the digestate. Therefore, in the present work, a range of lipophilic molecules (i.e. β-carotene (BC), phytosterols (PSs), CoQ10 and VitD3) were studied in terms of their transfer from oil to the aqueous phase during digestion. In terms of the carotenoids, the micellization of BC and lycopene were less efficient than the oxygenated xanthophylls, lutein, and zeaxanthin [8, 9], owing to the differences in hydrophobicity [10]. BC has a melting temperature of 180–182°C and is a highly hydrophobic molecule, only slightly oil soluble at room temperature (0.11–0.14%) [11]. As a point of reference, the BC content of carrots is around 8.14 mg/100 g [12]. BC has pro-vitamin A activity [13] and is sold as a nutraceutical with vision protective, immune enhancement, cancer prevention, and cardiovascular disease risk reduction potential [14–17].

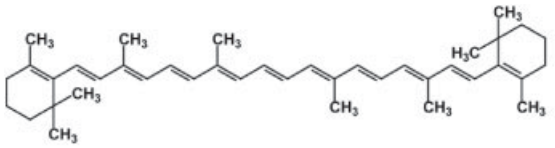
PSs are plant sterols, the main molecule being β-sitosterol followed by campesterol and stigmasterol. They do not occur naturally in the human body, but are found in significant quantities in seeds, nuts, fruits, and vegetables, with vegetable oils being the most concentrated source [18]. For example, soybean oil (SO) contains ~221 mg/100 g PSs [19]. The cholesterol-lowering effects of PS consumption are well-documented [18, 20]. PS consumption reduces serum total cholesterol by up to 15%, and low density lipoprotein cholesterol by up to 22% [18, 21]. One proposed mechanism is that PSs have a higher affinity than cholesterol molecules for mixed micelles and therefore compete during micellization [22]. Another possible explanation is that co-crystallization between PSs and cholesterol during lipolysis reduces cholesterol uptake [22]. Regardless, PS bioavailability has been reported to be very low. It is critically dependent on the structure of both the sterol nucleus and side chains [23]. In one human study, less than 2% of ingested PS was absorbed and the bioavailability of β-sitosterol, the most abundant and particularly hydrophobic PS molecule was only 0.5% [18].

CoQ10 (ubiquinone-10) is lipid soluble and present in all human cells. Particularly high concentrations are found in the heart, kidney, and liver, although tissue levels decrease progressively after the age of 21 [24]. CoQ10 is an essential factor in mitochondrial electron transport and proton translocation during cellular respiration and ATP production. It also protects cellular membranes against oxidation [25]. The major dietary sources of CoQ10 are meat, migratory fish, dairy products, and some vegetables (e.g. broccoli and spinach) [26]. The CoQ10 concentration in SO ranges from 5.4 to 279 mg/100 g CoQ10 [26]. Several formulations have been developed with the goal of increasing GI absorption of CoQ10 [7], as it tends to be slow and limited, because of its high molecular mass (863.34 g/mol) and low aqueous solubility (< 0.1 μg/L) [27].

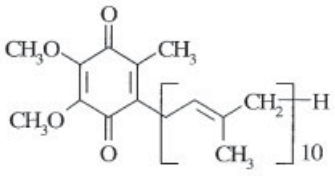
VitD3 (cholecalciferol) is an oil-soluble vitamin with molecular mass of 384.6 g/mol. It is manufactured in response to skin's exposure to sunlight [28], but is also acquired through dietary sources such as fish, meat, egg, and also fortified foods. For example, the VitD3 content of fish is in the range of 0.005–0.084 mg/100 g, depending on the type [29]. VitD3 plays important roles in immune [5] and muscle function [30], but is most established as a vital molecule for bone health, in large part through aiding calcium absorption [31].

There is evidence that the bioavailability of lipophilic bioactive molecules is facilitated during digestion of a food or carrier lipid [2]. For example, Bhagavan et al. [6] reported a positive correlation between the amount of CoQ10 released during in vitro digestion and plasma concentrations of CoQ10 [7]. Other studies have established a relationship between the amount of available oil, rate of lipolysis, and extent of BC micellization under simulated digestive conditions [2]. Therefore, there is rationale for investigating the mechanisms of lipophilic bioactive micellization in relation to lipolysis and oil solubility.

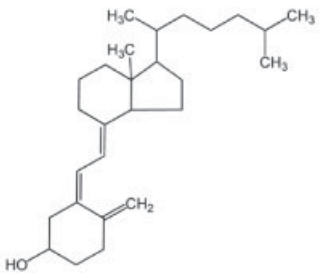
This study aims to elucidate the impact of oil droplet lipolysis on solubilization of lipophilic molecules (BC, CoQ10, VitD3, and PSs) possessing a range of chemical structures, molecular weights, and solubilities from oil-in-water emulsions. Figure 1 depicts the structure of the four model molecules included in this investigation. Emulsion-based delivery systems for the controlled release of lipophilic bioactives, including carotenoids, PSs, vitamins, and CoQ10 from the oil droplet phase, have recently been reviewed [32] and emulsion lipid digestion has been the subject of recent reports [32–35]. Given that nutrients are not typically consumed in isolation, and based on previous reports of competitive interactions between bioactive molecules, combinations of bioactives also need to be investigated in terms of possible interactions. For example in a human trial, consumption of 1.5 g/day of PSs was correlated with reduced blood concentrations of α-carotene, BC, and lycopene by 9, 28, and 7%, respectively [20], although levels of vitamin A, K, and D were not affected [18, 36]. It is reasonable to hypothesize that the co-administration of lipophilic molecules may affect their absorption, at the level of micellization in the GI tract. Therefore, mixed systems containing VitD3-BC and VitD3-PSs were also studied.



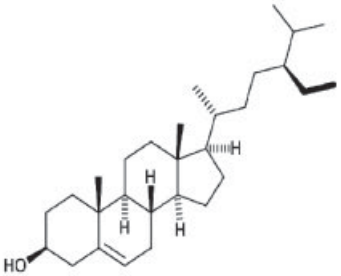
β-carotene: $C_{40}H_{56}$
536.88 g mol^{-1}



Coenzyme Q_{10}: $C_{59}H_{90}O_4$
863.34 g mol^{-1}



Vitamin D_3: $C_{27}H_{44}O$
384.6 g mol^{-1}



Phytosterol (β-sitosterol): $C_{29}H_{52}O$
416.71 g mol^{-1}

Figure 1. Chemical structures of lipophilic bioactive molecules studied.

2 Materials and methods

2.1 Materials

BC (all-*trans*, Type 1 synthetic, >95% purity), VitD3 (cholecalciferol), and CoQ10 (minimum 98% HPLC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Cardio Aid^{TM} Phyto-sterols (containing 40–58% β-sitosterol, 20–30% campesterol, and 14–22% stigmasterol, as determined by the manufacturer) were supplied by ADM Natural Health and Nutrition (IL, USA). Porcine bile extract, porcine colipase (lyophilized, essentially salt free), pyrogallol (99%, A.C.S reagent), porcine pepsin (with activity of 1020 U/mg protein), trypsin (from bovine pancreas), α-chymotrypsin (from bovine pancreas, type II), porcine pancreatin, and porcine phospholipase A2 were also obtained from Sigma Aldrich, as were the analytical grade solvents, hexane (>95%, spectrophotometric grade), acetone (>99.5%, A.C.S reagent), acetonitrile (HPLC grade, 0.2 μm filtered), tetrahydrofuran (HPLC grade), methanol (A.C.S certified), and anhydrous ethanol (A.C.S certified). Bleached and refined SO purchased from Sigma Aldrich was used without further purification. Soybean lecithin was provided by Lipoid (Lipoid GmbH, Ludwigshafen, Germany). The non-esterified fatty acid kit (NEFA-HR2) was purchased from Wako Pure Chemical Industries (Wako diagnostics, VA, USA). Soy protein isolate (SPI) with 86.48±0.23% purity was supplied by the Solae Company (Solae, St. Louis, MO, USA). As a measure of lipophilicity, octanol-water partition coefficients (log *p* at 25°C) reported for VitD3 (provided by BASF chemical company, NJ, USA), β-sitosterol as the main PS [37], CoQ10 [38], and BC [37], are 6.2, 9.6, 14.7, and 17.6, respectively.

2.2 Solubility measurement

The maximum solubility of the selected bioactives (i.e. BC, CoQ10, VitD3, and PS) at room temperature (22±1°C) was determined by gradual addition of each molecule individually to 2 mL of SO until the saturation point, i.e. just before the mixture became turbid. The mixtures of BC, CoQ10, and VitD3 in SO were heated to 30°C to facilitate solubilization and then cooled to room temperature. In the case of PSs, the mixture was heated to 70°C followed by cooling. All mixtures were filtered (0.22 μm nylon filters, Fisher Scientific) to remove any crystalline bioactives and the concentration of bioactive remaining was determined, as per Sections 2.8 and 2.9.

2.3 Preparation of bioactive–SO stock solutions

Stock solutions of BC, CoQ10, VitD3, and PSs, alone or in combination, in SO were prepared as previously described [39]. The solutions containing CoQ10, VitD3, and PSs were each prepared at a final concentration of 0.5 mg bioactive/100 mg of SO; i.e. a concentration well below the solubility threshold of each molecule. In the case of BC, a final concentration of 0.05 mg/100 mg of SO, i.e. close to the

maximum solubility in SO at room temperature, was used. The mixtures were stirred at room temperature for 4–5 h except in the case of PSs where the mixtures were heated at 70°C and stirred until clear (~15 min). Mixtures containing VitD3-BC were prepared as above at the same concentrations used in the experiments for the individual molecules, i.e. 0.05 BC mg/100 mg of SO and 0.5 mg VitD3/100 mg of SO. The VitD3-PSs stock solution was obtained by first solubilizing PSs in SO (0.5 mg/100 mg of SO), as described above, and VitD3 (0.5 mg/100 mg of SO), was added after cooling the mixture to room temperature. Any non-solubilized bioactives were removed by filtration of each stock solution through a 0.22 μm nylon filter (Fisher Scientific) under vacuum. All stock solutions were blanketed with nitrogen, sealed, and kept in amber glass jars at −20°C. The concentration of bioactives in the stock solution was measured (i.e. 0.05 ± 0.00, 0.45 ± 0.01, 0.47 ± 0.01, and 0.55 ± 0.05 mg/100 mg for the BC, CoQ10, VitD3, and PS solutions, respectively), and used in all calculations to minimize the effect of potential losses during processing or storage. The concentrations for the mixed bioactive systems were similar ($p > 0.05$) as with the bioactives in isolation.

2.4 Emulsion preparation

Oil-in-water emulsions were prepared by homogenizing the 10% SO stock solutions containing bioactives into the aqueous protein solution. The aqueous protein solution was prepared by dispersing 1.5 wt% SPI into Milli-Q water followed by stirring at room temperature for at least 5 h prior to homogenization. The mixture pre-homogenized using a handheld mixer (Ultra-Turrax, IKa T18 Basic, Germany) and further emulsified by four passes at 40 MPa through a microfluidizer (110S Microfluidizer Processor, Microfluidics, MA, USA).

2.5 In vitro gastro-duodenal digestion

The in vitro digestion experiments were performed as previously described [40]. In brief, oil-in-water emulsions were initially dispersed in simulated gastric fluids (SGFs) and gastric digestion was carried out at 37°C and pH 2 in a 250 rpm shaking water bath (New Brunswick Scientific, NJ) for 1 h. The duodenal digestion was initiated by the addition of simulated duodenal fluids containing pancreatin (5 mg/mL), colipase (1:1 lipase: colipase, molar ratio), and phospholipase A2 (400 μL in 5 mL of final mixture). Simulated bile fluids containing BSs (8 mM) and PLs (5 mM) were also added. The duodenal phase of digestion was also simulated at 37°C for 2 h in a 250 rpm shaking water bath (New Brunswick Scientific) at pH 6.5. For the in vitro digestions in which pancreatic lipase was omitted, trypsin (1 mg/mL) and chymotrypsin (1 mg/mL) were used instead of pancreatin.

2.6 Isolation of the aqueous micellar phase and bioactive extraction

Partitioning of the bioactive molecules into the aqueous micellar phase was examined by removing and analyzing 1 mL aliquots of digestate at 5, 15, 30, 60, 120 min throughout the duodenal stage. The aqueous phase was separated from any undigested oil and particulate matter by centrifugation at 16 900 *g* for 30 min (5418 laboratory centrifuge, Eppendorf Hamburg, Germany). The supernatant, which represented the aqueous phase, was then filtered (0.22 μm nylon filters, Fisher Scientific). As reported previously [40], the size of mixed micelles was less than 0.22 μm. The aqueous phase was subjected to solvent extraction, as described elsewhere [39]. Briefly, 0.5 mL of aqueous phase was mixed with 0.5, 3.0, and 1.0 mL of ethanol, acetone, and deionized water, respectively, with vortexing for 10 s after the addition of each liquid. 2 mL of hexane was added, the vials inverted ten times, and the organic layer removed after 5 min. The 2 mL hexane extraction was performed in triplicate, and the recovered solvent pooled and evaporated under nitrogen at 35°C (N-Evap, Organomation, Berlin, MA, USA). To determine the proportion of bioactive transferred to the aqueous micellar phase, the concentration of each bioactive in the oil-in-water emulsions was also measured. Aliquots of 3 mL of acetone were added to 0.2 g of each oil-in-water emulsion with vigorous shaking, prior to the addition of 3 mL hexane and 1 mL of Milli-Q water to the mixture. The hexane extraction was repeated three times, with the combined organic layers removed by fine tip transfer pipette and evaporated under nitrogen just to the point of dryness.

2.7 Size and ζ-potential measurements

Size and ζ-potential values of the SPI-stabilized emulsions and mixed micelles were measured by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern Instruments, Southborough, MA). All samples were diluted (1:100) with 5 mM phosphate buffer at a similar pH as during the digestions (pH 6.5) to minimize multiple scattering effects. ζ-Potential was determined from the electrophoretic mobility using laser doppler velocimetry and the Henry equation.

2.8 Determination of BC by UV-VIS spectrophotometry

Following solvent extraction, BC was quantified by adding 2 mL hexane, vortexing for 5 s, and then placing in a sonicator (Branson Ultrasonics Corporation, Danbury, CT) for 10 s to ensure re-dissolution of BC. The contents of each vial were then transferred to a 1 mL microcuvette, the absorbance

at 450 nm (i.e. the maximum wavelength of absorption for all-trans-BC) determined using a spectrophotometer (Hewlett Packard 8451A Diode Array Spectrophotometer), and BC concentration calculated using Beer's law [39].

2.9 Determination of CoQ10, VitD3, and PSs by RP-HPLC

Following solvent extraction, the dried samples containing CoQ10 and VitD3 were re-dissolved in 5 and 1 mL of fresh ethanol, respectively. In the case of the PSs, 1 mL of fresh methanol was added to the mixture followed by heating at 70°C for 10 min to ensure solubilization of all crystalline PSs prior to analysis by RP-HPLC with an Agilent Technologies 1200 series HPLC. The unit consists of a quaternary pump, auto degasser, diode array detector, auto-injector, and Chem Station software. A constant column temperature of 25°C and flow rate of 1 mL/min were maintained under isocratic conditions and using a ZORBAX Eclipse XDB-C18 (4.6 × 150 mm, 5 μm) column (Agilent Technologies). Injection volumes of 20, 25, and 50 μL were used for CoQ10, VitD3, and PSs, respectively. The mobile phase of acetonitrile:tetrahydrofuran:Milli-Q water (55:40:5 v/v/v) for CoQ10, methanol:Milli-Q water (99:1 v/v) for VitD3, and acetonitrile:methanol (60:40 v/v) for PSs were utilized and peaks corresponding to CoQ10, VitD3, and PSs were detected at wavelengths of 275, 265, and 208 nm, respectively. Standard curves based on CoQ10, VitD3 and PS molecules were prepared by dilution at concentrations ranging from 3 to 125 μg/mL in ethanol for CoQ10, 12.5–250 μg/mL in methanol for VitD3, and 25–500 μg/mL in methanol for PSs.

All data are expressed as a percentage of transferred bioactive and calculated based on the initial amount of each molecule present in the SO stock solutions. In the case of VitD3-BC the aqueous micellar phase extracts were subjected to analysis by both RP-HPLC and UV-VIS spectroscopy. According to initial HPLC measurements, the PSs powder contained 48% β-sitosterol and 52% campesterol and stigmasterol, which eluted together. The two fractions were found to incorporate in the mixed micellar phase equally during the digestion (data not shown) and so solubilization of individual PS molecules was not investigated.

2.10 Determination of oil droplet lipolysis

The hydrolysis of oil droplets by pancreatic triglyceride lipase (PTL) was monitored by extracting and determining the amount of FFAs liberated during the duodenal stage of in vitro digestion, as described elsewhere [40]. In brief, the FFAs were extracted under acidic conditions after 5, 15, 30, 60, 120 min of duodenal digestion. The amount of FFA was determined using a colorimetric method (NEFA kit) by measuring absorbance at λmax of 550 nm (UV-VIS microplate spectrophotometer, Spectramax plus, Molecular Devices, CA, USA) and using a standard curve based on oleic acid ranging from 0.1 to 2 mM.

2.11 Statistical analysis

Results are reported as mean ± standard deviation based on at least three independent experiments and differences analyzed using ANOVA and Duncan multiple comparisons testing with SAS version 9.1 (SAS Institute, NC, USA). Results were considered statistically significant at $p < 0.05$.

3 Results and discussion

3.1 Solubility of lipophilic molecules in SO

The maximum solubility of BC, CoQ10, VitD3, and PSs in SO at room temperature is presented in Fig. 2. Among the molecules tested, VitD3 and CoQ10 were the most soluble, i.e. 12.1 ± 0.1 and 11.5 ± 0.2%, respectively. A maximum solubility of CoQ10 in SO of 7.4% was previously reported [41]. The solubility of PSs was 2.6 ± 0.1%, which was significantly lower than VitD3 and CoQ10 (Fig. 2, $p < 0.05$) and in the range of previous reports for PSs in corn oil (i.e. 2–3%) [42]. BC was the least soluble molecule tested in SO (i.e. 0.051 ± 0.001%). The solubility was lower than previous reports of 0.11–0.14% solubility in tricaprylin, triolein trilinolein, and fish oil [11].

3.2 The effect of lipophilic molecules on emulsion physical properties

Droplet characteristics, including size and interfacial properties, can affect lipolysis and the release of molecules from the oily phase. Further, micellization behavior depends not only on the chemical structure of bioactive molecules, but

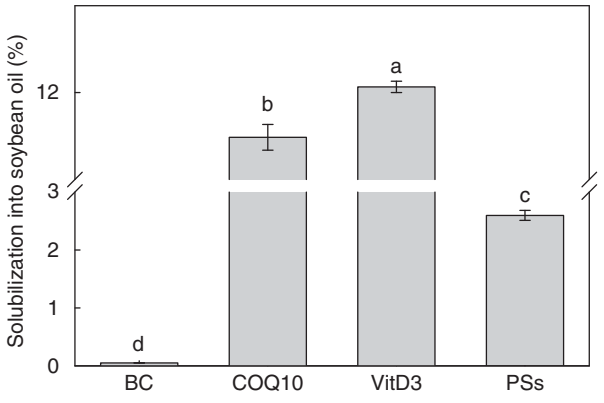


Figure 2. The maximum solubility (%) of selected lipophilic molecules in SO at room temperature (22 ± 1°C).

Table 1. Size and ζ-potential values of SPI-stabilized emulsions containing BC, CoQ_{10}, $VitD_3$, PSs, $VitD_3$-BC, or $VitD_3$-PSs[a),b)]

	Control[c)]	BC	CoQ_{10}	$VitD_3$	PSs	$VitD_3$-BC	$VitD_3$-PSs
Hydrodynamic diameter (nm)	271.5 ± 2.8^a	304.8 ± 3.9^b	300.3 ± 1.4^b	307.6 ± 0.1^b	257.0 ± 0.4^c	301.5 ± 0.4^b	273.4 ± 3.9^a
ζ-Potential (mV)	-39.1 ± 0.2^a	-36.4 ± 0.0^a	-38.3 ± 1.1^a	-37.3 ± 1.2^a	-44.0 ± 1.7^b	-36.4 ± 0.1^a	-42.8 ± 1.8^b

a) Mean ± SD, $n = 3$.
b) Within each row, means with different superscripts are significantly different at $p<0.05$.
c) Control sample contains no bioactive molecule.

also on the properties of the emulsion droplets and the changes occurring to the population of surface-active molecules in the systems. The average diameter and ζ-potential of the oil droplets for all the emulsions studied are shown in Table 1. A control SPI-stabilized emulsion without bioactives incorporated had a hydrodynamic diameter of 271.5 ± 2.8 nm and ζ-potential value of −39.1 ± 0.2 mV. A significant increase in droplet diameter was observed with incorporation of BC, CoQ10, or VitD3 alone and also with the mixture of VitD3-BC ($p<0.05$). The hydrodynamic diameter for these emulsions was about 300 nm (Table 1). In contrast, the presence of PSs led to significantly smaller oil droplets compared to all the other emulsions (257.0 ± 0.4 nm). The mixed VitD3-PSs emulsion droplets were similar in size compare to the control sample. The incorporation of different carotenoids was previously found not to affect the mean droplet diameter of PL-stabilized oil-in-water emulsions [11]. In the present study, incorporation of the bioactive molecules had no effect on droplet ζ-potential, except in the case of the PSs. The emulsion droplets containing PSs had significantly higher charges (Table 1, $p<0.05$) (−44 mV) compared to the control emulsions (−39 mV). These results suggest an interaction between PSs and the proteins adsorbed at the interface, with PSs contributing to changes in the interfacial layer, causing the formation of smaller oil droplets and a higher negative charge.

3.3 Emulsion changes during digestion

SPI-stabilized emulsions were employed in this investigation as the details of their changes during in vitro digestion was recently reported [35]. The protein fractions in both the serum and cream phases were rapidly hydrolyzed in the presence of gastric fluids, and the emulsion droplets had a net positive charge of around +10 mV at pH 2 [35]. Hydrolysis of the interfacial layer, along with the lower electrostatic repulsion under the acidic and ionic conditions, led to substantial droplet flocculation (i.e. mean floc diameter of around 7 μm, data not shown).

As expected, transfer of the lipophilic molecules to the aqueous phase at the end of the gastric digestion was negligible (data not shown), given the absence of gastric lipase, BSs, and PLs in the SGF. Practically, this verifies that a range of lipophilic bioactive molecules solubilized in oil droplets exhibit minimal gastric release, but instead remain protected from the gastric environment, to later be released in the small intestine where they are absorbed [28]. Changes in the emulsion droplets continued in the duodenal phase, with the presence of the bio-surfactants (BSs and PLs). In the presence of BSs-PLs-COL-PLA2, the droplet average diameter decreased and there was an increase in the magnitude of the negative droplet charge from around −40 to −70 mV. Changes in droplet size and interfacial composition during duodenal in vitro digestion directly affect oil droplet lipolysis [35].

3.4 The release of lipophilic molecules during duodenal digestion, but in the absence of PTL

To study the influence of chemical structure on the release of the bioactive molecules, the oil-in-water emulsions were subjected to SGF, and then duodenal digestion, but in the absence of PTL. This provided insights into bioactive distribution in the oil droplets and their affinity to incorporate in the mixed BS micelles. Figure 3 shows the percentage of each molecule transferred to the aqueous

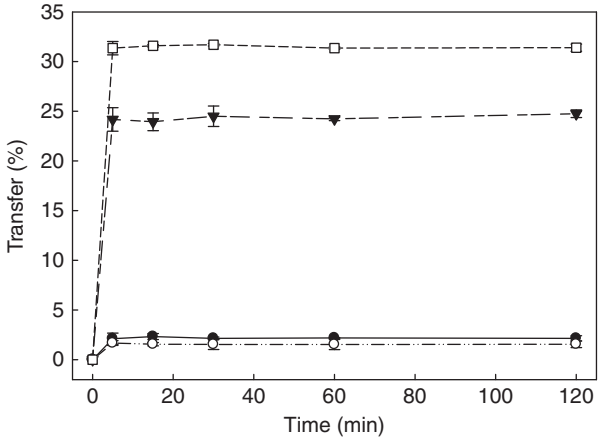


Figure 3. Proportion of BC (●), CoQ10 (○), VitD3 (▼), and PSs (□) transferred to the aqueous phase during the duodenal stage of in vitro digestion, but in the absence of PTL. Time 0 represents the end of the gastric stage. Data represent mean and standard deviation for three independent experiments.

phase as a function of incubation time. Between 5 and 120 min, only ~2 and 1.5% BC and CoQ10, respectively, was transferred ($p<0.05$). In contrast, 24.2 ± 1.2% of the VitD3 and 31.3 ± 0.7% of the PSs were transferred by 5 min, with no further increases observed.

Therefore, even in the absence of lipolysis, rapid and substantial partitioning to the aqueous phase of some bioactives was observed with exposure to the simulated duodenal fluids. CoQ10 and BC have higher molecular weights (Fig. 1) and are more hydrophobic (as per the log *p* values stated above) than VitD3 and PSs. Thus, it is expected that BC and CoQ10 are preferentially distributed towards the core of an oil droplet, i.e. away from the interface. This is consistent with a release mechanism in which their transfer is highly dependent on the digestion of the oil droplet. In contrast, with VitD3 and PSs, the presence of a hydroxyl group (OH) in their chemical structures imparts some hydrophilicity, enabling the molecules to locate closer to the oil–water interface. The highest release was observed in the case of the PSs, most likely because of their interaction with the soy protein at the interface. It was previously shown that the relatively hydrophilic carotenoids (i.e. xanthophylls) preferentially locate at the oil droplet interface, with readier access to the mixed micelles, compared to more highly hydrophobic carotenoids (such as BC and lycopene) [4, 8, 43]. The present results support the conclusion that relatively less hydrophobic molecules locate towards an oil droplet surface and can be solubilized in the aqueous micellar phase without oil droplet hydrolysis.

3.5 Hydrolysis of oil droplets during duodenal digestion

Table 2 summarizes the extent of lipid hydrolysis at 5, 15, 30, 60, and 120 min of simulated duodenal digestion. In general, these results followed the trends reported earlier for SPI-stabilzed emulsions [35]. Within the first 5 min, lipid hydrolysis was already pronounced and ranged from 42.6 to 53.8%. No significant differences were observed among the samples at 5 min, ($p>0.05$) except for the VitD3-PS system, where significantly less lipolysis was observed (42.6%, $p<0.05$). After 120 min, the extent of lipolysis for the CoQ10 and BC emulsions was higher (94.4 ± 2.3%) and lower (79.2 ± 2.2%), respectively ($p<0.05$). The reason for these observed differences in FA release is not known and not previously reported. Of note, the amount of lipid hydrolysis at each time point was highly related to the bioactive release kinetics (see below).

3.6 The release of lipophilic molecules during duodenal digestion

Figure 4 shows the release kinetics of the bioactive molecules present in isolation in the emulsion droplets during in vitro duodenal digestion in the presence of PTL. Overall, and as expected, transfer was markedly increased over when PTL was absent (compare Figs. 3 and 4). For example, 19.2 ± 0.6% BC was transferred within the first 5 min, compared with 2.1 ± 0.6% in the absence of lipolysis. This is consistent with the understanding that lipid digestion products (mainly MGs and FFAs) enhance the solubility of lipophilic drugs [44].

In the presence of PTL, increases in BC and CoQ10 release beyond 5 min were also observed until about 60 min (Fig. 4). By 15 min, proportionally more CoQ10 than BC was transferred to the aqueous phase ($p<0.05$). This difference is consistent with the greater extent of hydrolysis observed for the CoQ10-containing droplets than for the BC-containing droplets (Table 2). It is especially significant given that there was nearly ten times more CoQ10 than BC present in the original emulsions, although both were present within their limits of solubility in the oil. Therefore, the results suggest that the transfer behavior of theses two lipophilic molecules was mainly due to the difference in lipolysis rather than their chemical structures.

At 5 min, more VitD3 and PSs were solubilized in the aqueous phase (i.e. 46.9 ± 1.8 and 72.1 ± 0.4%, respectively) compared to CoQ10 and BC ($p<0.05$). However, there were no differences in terms of the extent of lipid digestion between the emulsions at this time point. Therefore, these differences can be attributed to differences in molecular hydrophobicity and are consistent with the solubilization

Table 2. Hydrolysis (%) of oil droplets containing BC, CoQ_{10}, $VitD_3$, PSs, $VitD_3$-BC, or $VitD_3$-PSs during exposure to simulated duodenal conditions[a),b)]

Time (min)	Control[c)]	BC	CoQ_{10}	$VitD_3$	PSs	$VitD_3$-BC	$VitD_3$-PSs
5	50.1 ± 1.2^{x}	52.5 ± 0.7^{x}	53.6 ± 2.3^{x}	50.2 ± 1.8^{x}	53.8 ± 3.8^{x}	50.3 ± 1.3^{x}	42.6 ± 0.5^{y}
15	63.1 ± 2.1^{y}	65.3 ± 1.8^{y}	75.5 ± 3.2^{x}	70.1 ± 1.7^{x}	75.5 ± 0.6^{x}	71.8 ± 2.7^{x}	62.1 ± 2.5^{y}
30	77.5 ± 3.3^{y}	76.4 ± 2.5^{y}	87.9 ± 4.2^{x}	78.9 ± 3.0^{y}	83.8 ± 4.0^{x}	79.9 ± 1.2^{y}	70.1 ± 3.9^{z}
60	79.2 ± 1.2^{y}	74.9 ± 1.6^{z}	90.4 ± 3.8^{x}	83.9 ± 3.3^{y}	83.4 ± 4.7^{y}	83.6 ± 3.4^{y}	75.1 ± 3.4^{z}
120	85.2 ± 2.6^{y}	79.2 ± 2.2^{z}	94.4 ± 2.3^{x}	84.8 ± 1.9^{y}	87.2 ± 5.2^{y}	83.6 ± 2.8^{y}	84.7 ± 5.2^{y}

a) Mean ± SD, $n = 3$.
b) Within each row, means with different superscripts are significantly different at $p<0.05$.
c) Control emulsion contains no bioactive molecules.

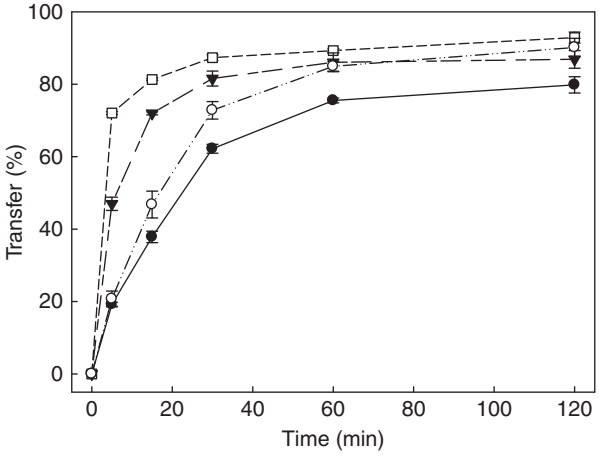


Figure 4. Proportion of BC (●), CoQ10 (○), VitD3 (▼), and PSs (□) transferred to the aqueous phase during the duodenal stage of in vitro digestion in the presence of PTL. Data represent mean and SD for three independent experiments.

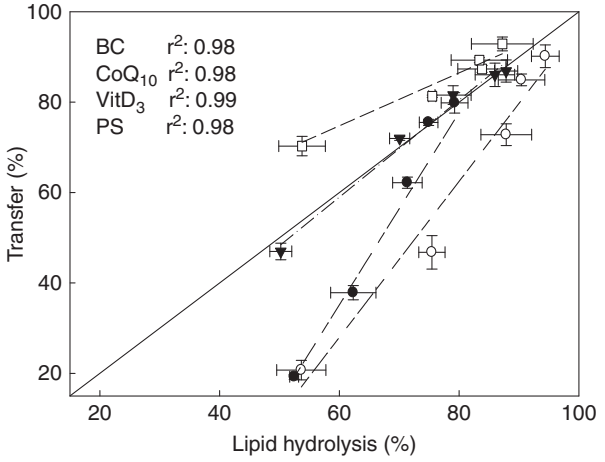


Figure 5. Proportion of BC (●), CoQ10 (○), VitD3 (▼), and PSs (□) from the single bioactive systems transferred to the aqueous phase as a function of lipid hydrolysis. Data represent mean and SD for three independent experiments.

trends observed in the absence of PTL activity. The trends are generally in agreement with the bioactives' octanol-water partition coefficients, which increased in the order of VitD3 (6.2), PSs (9.6 for β-sitosterol), CoQ10 (14.7), and BC (17.6) [37]. Increases in VitD3 and PSs micellization continued until 30 min, when a plateau was reached, i.e. earlier than for CoQ10 and BC (Fig. 4). By 120 min duodenal digestion, relatively more PSs and CoQ10 were transferred, followed by VitD3 and BC. These results are not in line with the extent of lipid hydrolysis, which was most extensive in the CoQ10-containing emulsion and the lowest in the emulsions containing BC. This discrepancy suggests that, while lipolysis plays a significant role in bioactive release and micellization, the degree of hydrophobicity and molecular structure are also important.

3.7 Correlation between lipid digestion and bioactive transfer

The relationship between bioactive release and lipid hydrolysis is shown in Fig. 5. Positive and linear correlations were observed in the case of each bioactive. Although there were differences in terms of partitioning between the oil and micellar phase, more extensive lipid digestion led to an increase in molecular transfer. For BC and CoQ10, a clear lag phase was observed prior to the linear region in Fig. 5, such that approximately 55% lipolysis resulted in approximately 20% bioactive transfer. This lag phase might be explained by a preferential partitioning and hence a concentration of these lipophilic bioactives in the oil phase during digestion. A similar trend was recently reported for the lipophilic drug progesterone, which became more concentrated in the oil phase as the oil was digested [45].

After the lag phase, the slope of the linear region between BC transfer and lipid digestion was steep, i.e. a 20% increase in lipid hydrolysis resulted in 40% BC micellization. Similar behavior was observed for CoQ10, albeit, at a slower rate than for BC release. This could suggest a higher affinity of CoQ10 for the oil droplet compared to BC. Although both molecules are highly hydrophobic, the higher affinity of CoQ10 for the oil droplet may be attributed to its larger size (Fig. 1). However, because of the extensive lipolysis by the end of the duodenal digestion, more than 90% of CoQ10 was still transferred to the aqueous phase (see Fig. 3).

VitD3 release followed the trend expected for a molecule with an equal affinity for the oil and aqueous phases (i.e. the theoretical, solid line in Fig. 5). VitD3 has been previously reported to concentrate in oil droplets during lipolysis [45], although this was based on experiments without BSs, and hence with substantially less micellization potential. In the present study, BSs, PLs, lysophospholipids, MGs, and FFAs were all present and presumably influenced the affinity of VitD3 for the oil versus aqueous phases. A linear relationship was also found between lipid digestion and PSs micellization (Fig. 5), although the rate of micellization was higher than the rate of lipid digestion.

3.8 The release profile of mixed systems

To investigate whether co-administration of lipophilic molecules alters their micellization behavior, two mixed systems (VitD3-BC and VitD3-PSs) were studied. VitD3, BC, and PSs were selected based on the differences observed in their transfer behaviors. Table 3 summarizes the release kinetic for the mixed systems, in comparison with the bioactives in isolation. Figures 6 and 7 summarize the results as a function of lipid hydrolysis. Inclusion of both

Table 3. Proportion (%) of each bioactive transferred to the aqueous phase during in vitro duodenal digestion[a),b),c)]

Time (min)	BC	CoQ_{10}	$VitD_3$	PSs	BC ($VitD_3$)[d)]	$VitD_3$ (BC)[d)]	$VitD_3$ (PSs)[d)]	PSs ($VitD_3$)[d)]
5	$19.2\pm0.6^{d,z}$	$20.7\pm2.2^{d,z}$	$46.9\pm1.8^{c,y}$	$72.1\pm0.4^{c,x}$	$21.7\pm3.1^{d,z}$	$49.7\pm5.9^{c,y}$	$54.4\pm1.2^{c,y}$	$68.1\pm2.4^{b,x}$
15	$37.8\pm1.5^{c,z}$	$46.8\pm3.7^{c,z}$	$71.9\pm0.4^{b,y}$	$81.3\pm0.3^{b,x}$	$62.7\pm3.3^{c,y}$	$66.2\pm6.2^{b,y}$	$80.3\pm4.3^{b,x}$	$88.1\pm0.5^{a,x}$
30	$62.2\pm1.2^{b,z}$	$72.8\pm2.4^{b,y}$	$86.6\pm2.1^{a,x}$	$87.3\pm0.8^{a,x}$	$70.9\pm3.4^{b,y}$	$76.8\pm2.7^{a,y}$	$90.7\pm1.8^{a,x}$	$90.7\pm1.7^{a,x}$
60	$75.5\pm0.7^{a,z}$	$84.9\pm1.3^{a,y}$	$86.1\pm2.6^{a,y}$	$89.3\pm0.5^{a,x}$	$83.1\pm2.1^{a,y}$	$80.2\pm1.6^{a,y}$	$92.4\pm0.7^{a,x}$	$91.3\pm1.8^{a,x}$
120	$79.8\pm2.3^{a,z}$	$90.1\pm2.5^{a,x}$	$86.9\pm2.4^{a,y}$	$92.9\pm1.5^{a,x}$	$85.2\pm0.9^{a,y}$	$80.3\pm1.3^{a,z}$	$92.6\pm0.5^{a,x}$	$92.7\pm1.6^{a,x}$

a) Mean $\pm$ SD, $n = 3$.
b) Within each row, means with different superscripts a–d are significantly different at $p < 0.05$.
c) Within each column, means with different superscripts x–z are significantly different at $p < 0.05$.
d) Indicates BC, $VitD_3$, $VitD_3$, and PSs transferred from $VitD_3$-BC, $VitD_3$-BC, $VitD_3$-PSs, and $VitD_3$-PSs, respectively.

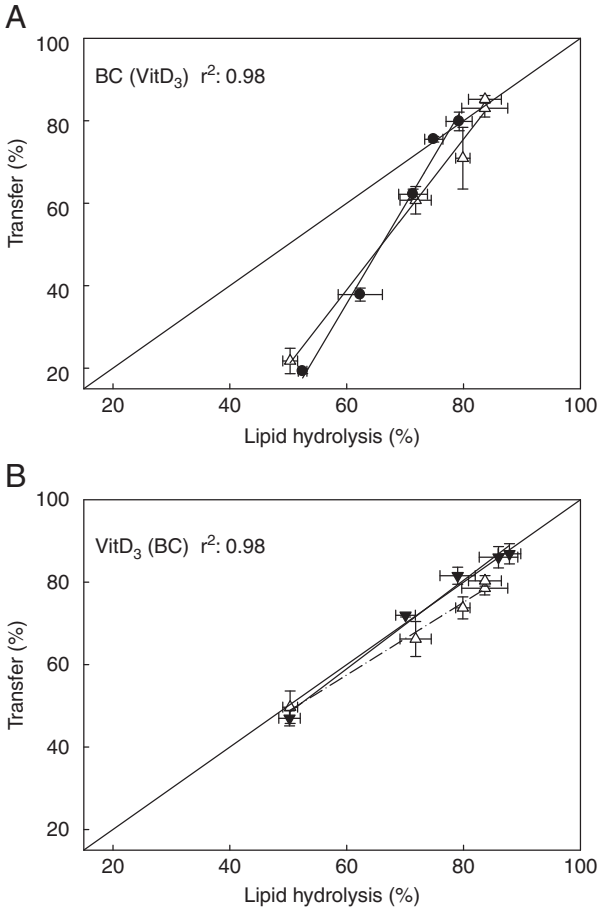


Figure 6. Proportion of BC (A) transferred to the aqueous phase in the absence (●) and presence (Δ) of VitD3 and proportion of VitD3 (B) transferred to the aqueous phase in the absence (▼) and presence (Δ) of BC. Data represent mean $\pm$ SD for three independent experiments.

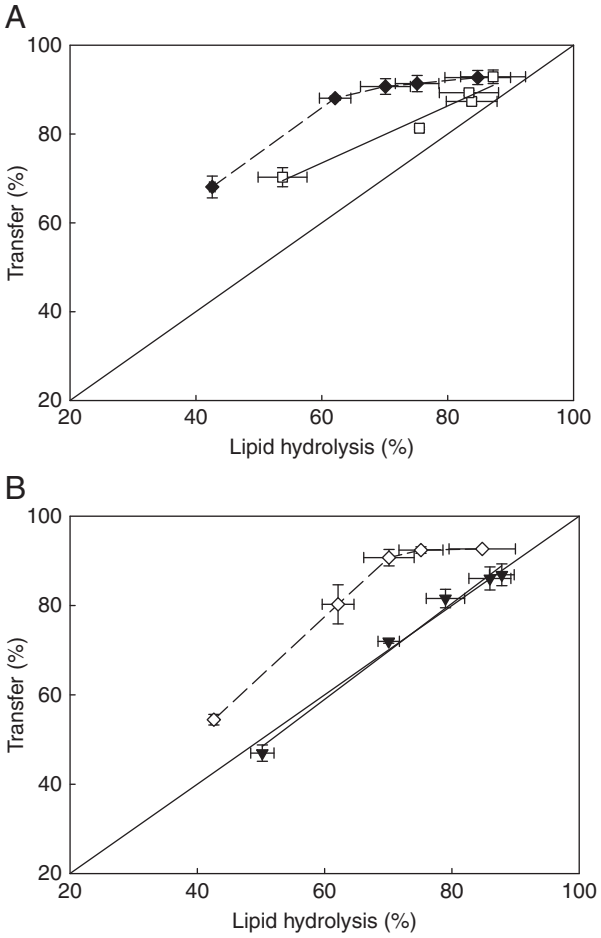


Figure 7. Proportion of PSs (A) transferred to the aqueous phase in the absence (□) and presence (♦) of VitD3 and proportion of VitD3 (B) transferred to the aqueous phase in the absence (▼) and presence (◇) of PSs. Data represent mean $\pm$ SD for three independent experiments.

VitD3 and BC in the emulsion did not change BC's solubilization behavior (Table 3 and Fig. 6A). BC (0.05% in the SO) had similar release profiles in the absence and presence of VitD3 (0.5 mg in 100 mg SO), with an initial gradual increase and plateau reached by roughly 60 min (Table 3). The percentage of BC transfer was linearly correlated with lipid digestion (Fig. 6A).

Incorporation of BC and VitD3 had no effect on the release profile of VitD3, either as a function of incubation time (Table 3) or in relation to lipid hydrolysis (Fig. 6B). A

Table 4. Size and ζ-potential values of aqueous micellar phase isolated after 120 min duodenal in vitro digestion and containing BC, CoQ_{10}, $VitD_3$, PSs, $VitD_3$-BC, or $VitD_3$-PSs [a),b)]

	Control[c)]	BC	CoQ_{10}	$VitD_3$	PSs	$VitD_3$-BC	$VitD_3$-PSs
Hydrodynamic diameter (nm)	74.9 ± 6.6^{a}	$63.3\pm7.3^{b,c}$	77.3 ± 7.4^{a}	$62.1\pm7.1^{b,c}$	66.7 ± 0.9^{b}	60.1 ± 1.3^{c}	66.7 ± 0.5^{b}
ζ-Potential (mV)	-71.3 ± 0.4^{a}	-54.6 ± 1.1^{c}	-57.9 ± 2.4^{c}	-56.4 ± 1.1^{c}	-64.9 ± 0.6^{b}	$-61.0\pm1.2^{b,c}$	-59.7 ± 4.1^{c}

a) Mean ± SD, $n = 3$.
b) Within each row, means with different superscripts are significantly different at $p<0.05$.
c) Control sample contains no bioactive molecules.

significant increase in VitD3 micellization was obtained within the first 30 min, but not after that point. This is similar as for VitD3 alone (Table 3 and Fig. 6B). The differences observed between the single and mixed systems (Table 3) seem to stem from differences in lipid hydrolysis, as opposed to the presence of another bioactive molecule. Although, it cannot be ruled out that the bioactives influence the interfacial environment and therefore digestion. Co-administration of BC and VitD3 did not impact the micellization behavior of either bioactive, possibly because of their relatively low concentrations in the oil-in-water emulsions.

As shown in Table 3, as with VitD3 alone, VitD3 release from the mixed PSs and VitD3 systems reached a plateau around 30 min. In contrast, the release of PSs was more rapid in the presence of VitD3. Surprisingly, the mixed VitD3-PSs system had significantly less lipolysis (Table 2). VitD3 and PSs mutually enhanced each other's solubility into the mixed micelles, as clearly shown in Fig. 7. Therefore, at the concentrations studied, interactions between VitD3 and PSs led to greater micellization.

3.9 The effect of lipophilic molecules on the physical properties of the mixed micelles

The hydrodynamic diameter and ζ-potential of the mixed micelles in each digestate are summarized in Table 4. Differences were observed based on the lipophilic compound present. The largest micelles were observed for the digestate containing CoQ10. Those derived from the emulsions containing PSs and PSs-VitD3 were identical in diameter (66.7 nm, Table 4, $p>0.05$). The smallest diameters for the aqueous micellar phase were observed for the BC, VitD3, and VitD3-BC digested emulsions. In terms of charge, ζ-potential values were also affected by the presence of the lipophilic molecules. According to Table 4, less negative values were observed for all the bioactive-containing samples compared to the control mixed micelles ($p<0.05$). Therefore, incorporation of these bioactives in the mixed micelles affected the micelle physical properties, namely size, but also charge.

4 Concluding remarks

The results of this study confirm that the physical and chemical properties of lipophilic bioactive molecules play important roles in the kinetics of their solubilization into the mixed micellar phase. There was evidence that the bioactives were distributed differently in the emulsion oil droplets, leading to differences in transfer behavior. BC and CoQ10 distributed towards the oil droplet core compared to VitD3 and PSs. In all cases, lipid hydrolysis facilitated the rate of bioactive transfer. Inclusion of two bioactives in an emulsion affected the micellization behavior in some, but not all, cases. The presence of both VitD3 and BC had no impact on either molecule. In contrast, the co-administration of VitD3 and PSs significantly enhanced the transfer of both compounds. The present results contribute to a better understanding of the relationships between lipophilic molecular structure, oil droplet hydrolysis, and solubilization in the aqueous phase during digestion, towards the goal of designing emulsions for optimal bioavailability. While the work provides insights into pre-absorptive events, the model used cannot account for differences in intestinal absorption which also impact bioavailability.

The authors have declared no conflict of interest.

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