In vivo postprandial bioavailability of interesterified-lipids in sodium-caseinate or chitosan based O/W emulsions

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ABSTRACT

Recent studies have shown that it should be possible to control lipid bioavailability through food structural approaches. Nevertheless, the gastrointestinal-tract physiological conditions must also be considered. To get a better understanding of this phenomenon, we evaluated the effect of emulsification, as well as the use of sodium caseinate or chitosan, on the postprandial bioavailability of interesterified-lipids in O/W emulsions after oral gastric feeding Sprague–Dawley rats. We verified that emulsification may increase lipid absorption, as determined after feeding sodium-caseinate emulsions. However, this result could not be generalised. Interesterified-lipids that were emulsified with chitosan were equally absorbed as those contained in non-emulsified interesterified-lipids/distilled-water blends.

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1. Introduction

The importance of lipids in human diet goes beyond their structural and regulatory functions or energy supply; they confer specific sensory attributes to foods, which are critical for consumers' acceptance (O'Brien, 2004). Their intake may be either related to the development or to the prevention of several diseases (Wahrburg, 2004). Thus, it is of interest to understand and control the amount of lipids that are released from a food matrix and become available to be absorbed (a concept referred as bioaccessibility), as well as their bioavailability (the amount that is actually absorbed and becomes available for any metabolic or structural function, or as a source of energy) (McClements, Decker, & Park, 2009).

The fatty acid profile is certainly a critical factor in fat absorption, but the stereochemistry of triacylglycerols also plays a fundamental role. Free fatty acids and sn2-monoacylglycerols are produced by successive hydrolysis and are absorbed in different modes. Monoacylglycerols are readily absorbed and transported through the lymphatic system, whereas free fatty acid absorption and transport depends on their chain length and degree of unsaturation. Shorter fatty acids are absorbed faster than longer ones,

whereas unsaturated fatty acids are absorbed faster than saturated ones (when comparing the same chain length). Thus, fats with a higher melting point are poorly absorbed, as in long-chain saturated free fatty acids absorption (McClements et al., 2009; Ramírez, Amate, & Gil, 2001). Changes of the solid fat content, without altering the free fatty acids profile of fats, may also affect lipids absorption. In fact, Farfán, Villalón, Ortíz, Nieto, and Bouchon (2013) showed that modification of the melting profile through interesterification may delay lipids absorption kinetics, compared to a non-interesterified mix, without altering total lipid absorption.

Lipids bioavailability may be also affected by the diet. Nutrients can interact with partially-digested lipids or with any other molecule involved in lipid digestion, reducing its bioavailability. For instance, divalent cations, such as calcium and magnesium, may form insoluble soaps with long-chain saturated free fatty acids, increasing their excretion (Mu & Høy, 2004; Ramírez et al., 2001). On the other hand, polyphenols from berries or tea may inhibit lipase activity (Juhel et al., 2000; McDougall, Kulkarni, & Stewart, 2009; Nakai et al., 2005).

Certainly, it should be possible to reduce or maximise lipid bioavailability through food structuring. However, the gastrointestinal-tract physiological conditions must also be considered (Lundin & Golding, 2009; Parada & Aguilera, 2007). Accordingly, emulsification has shown to be an appropriate technique to control lipid bioavailability. *In vitro* studies have reported that emulsions produced with different interfacial compositions have large

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differences in lipid digestion (Lundin & Golding, 2009) and that the rate of hydrolysis increases when decreasing droplet size (Borel et al., 1994). This has been confirmed using *in vivo* studies, which in turn have also reported that apparently fat assimilation would not be affected by differences in the initial droplet size (Armand et al., 1999).

Proteins, carbohydrates, and other natural or manufactured substances may be used to stabilise emulsions (McClements, Decker, Park, & Weiss, 2008). Sodium caseinate is a widely used emulsifier (Dickinson & Golding, 1997), which can increase pancreatic-lipase accessibility to lipids, as shown in *in vitro* studies (Mun, Decker, Park, Weiss, & McClements, 2006). On the other hand, the intake of carbohydrates, specifically dietary fibres, may interfere with the digestive process by either changing the viscosity of the bolus, binding to bile salts or by forming a protective coating over emulsified lipids (Beysseriat, Decker, & McClements, 2006). Chitosan, the deacetylated form of chitin, is an abundant and widespread polycationic biopolymer, which has shown good emulsifying properties (Moschakis, Murray, & Biliaderis, 2010) due to its structural heterogeneity, which includes a strongly hydrophilic component (D-glucosamine) and some less deacetylated hydrophobic (N-acetylated) residues (Klinkesorn, 2013). In addition, *in vivo* studies have reported that chitosan intake can reduce plasma cholesterol, total plasma lipids, and total liver lipids, and at the same time, it may increase lipid excretion (Chiang, Yao, & Chen, 2000; Han, Kimura, & Okuda, 1999). Also, *in vitro* studies have reported that lipid hydrolysis may be reduced in chitosan stabilised emulsions when compared to other stabilisers (Beysseriat et al., 2006; Helgason, Gislason, McClements, Kristbergsson, & Weiss, 2009; McClements et al., 2008).

In accordance, the aim of this paper was to study the effect of emulsification, as well as the use of sodium caseinate or chitosan, on the *in vivo* postprandial bioavailability of interesterified-lipids in O/W emulsions.

2. Materials and methods

2.1. Synthesis of dietary fat and test meals

Two oil-in-water emulsions (test meals) were developed to evaluate the effect of emulsification as well as the effect of sodium caseinate or chitosan on lipid bioavailability. To do so, test meals were prepared by mixing an interesterified fat with an emulsifier solution, as explained below.

Dietary fats were synthesised by enzymatic interesterification of linseed oil (LO) and palm stearin (PS), as explained by Farfán et al. (2013). LO was purchased from Fontevita (Nutra Andes Ltd., Santiago, Chile) and PS was supplied by Cox & Co. (Santiago, Chile) as Danfat MIX-4547. Briefly, blends of LO/PS (60/40 mass ratio) were interesterified using 5% (w/w) of lipozyme TL IM (Blumos S.A., Santiago, Chile), a stereospecific catalyst that only hydrolyses the sn-1 and sn-3 positions of triacylglycerols. The reaction was carried out under vacuum (100 Torr) and agitation (150 rpm) at 70 °C for 120 min. After this time, the blend reached thermodynamic equilibrium, as previously reported. The interesterified fat was kept at 4 °C under a nitrogen atmosphere. The most important fatty acids of the raw materials and the interesterified mix are shown in Table 1.

A sodium caseinate solution was prepared by dispersing 10% (w/w) of sodium caseinate (Protevit-HV Ultra Top 2, supplied by Blumos S.A., Chile) in distilled water after stirring for 2 h at 40 °C. On the other hand, a chitosan solution was prepared by dispersing 3% (w/w) of chitosan powder (medium molecular weight with 75–85% deacetylation degree, Sigma–Aldrich, USA) in a 0.15 M acetic acid solution. As reported by Mun et al. (2006), at a relatively low pH (<6.3), chitosan is positively charged and tends to be soluble.

Each emulsion was prepared by homogenising 40% (weight) interesterified fat with 60% (weight) of either the sodium caseinate or the chitosan solution with a high-speed blender (Ultra Turrax®T25 digital, Ika®-Werke, Germany) for 2 min at 20,000 rpm. The emulsions were then stored at 4 °C for 15 h before use.

2.2. Characterisation of sodium caseinate or chitosan emulsions test-meals

Emulsions were characterised in terms of stability, globule size distribution and rheological properties.

2.2.1. Stability

The creaming stability was determined in triplicate by transferring 10 ml of fresh emulsion into a test tube, sealed and stored at 4 °C. The phase separation of a creamy layer at the top and a serum layer at the bottom was monitored daily for 7 days (Khalloufi, Alexander, Goff, & Corredig, 2008; Surh, Ward, & McClements, 2006).

2.2.2. Oil droplet-size determination

Since droplets were larger than 1 μm, they were directly measured using optical microscopy (Boom, 2008). Emulsions were observed using an Olympus BX-61 TRF (Tokyo, Japan) microscope equipped with a high-resolution CCD colour camera (CoolSnap Pro Colour, Photometrics Roper Division Inc., Tucson, USA), which was used for digital image acquisition. Images were then analysed using Image ProPlus 4.5 (Media Cybernetics, Silver Spring, USA). A circle was fit on each globule and the corresponding diameter was obtained. Thereafter, the Sauter mean diameter (d_{32}), which corresponds to an area-volume mean diameter, was determined according to the following equation (McClements, 2005):

$$d_{32} = \frac{\sum_{i=1} n_i d_i^3}{\sum_{i=1} n_i d_i^2} \tag{1}$$

where n_i is the number of droplets with a diameter d_i. Determinations were carried out at 20 °C in triplicate.

2.2.3. Rheological properties

A stress-controlled rheometer (Physica MCR 301, Anton Paar GMbH, Graz, Austria) with a 25-mm diameter parallel plate

Table 1
Main fatty acid composition of linseed oil, palm stearin and interesterified blend, and amount of fatty acids in oral gastric dose fed through sodium caseinate or chitosan emulsions, or through a non-emulsified interesterified-fat/distilled-water blend.

Fatty acid	Linseed oil (%)	Palm stearin (%)	Interesterified blend (%)	Oral gastric dose (mg)
C14:0	–	1.14 ± 0.006	0.46 ± 0.006	1.84 ± 0.004
C16:0	5.60 ± 0.006	57.52 ± 0.006	26.37 ± 0.006	105.48 ± 0.004
C18:0	4.44 ± 0.010	6.87 ± 0.010	5.41 ± 0.010	21.64 ± 0.006
C18:1n-9	23.97 ± 0.010	27.66 ± 0.012	25.45 ± 0.006	101.80 ± 0.004
C18:2n-6	16.68 ± 0.006	6.81 ± 0.010	12.73 ± 0.006	50.92 ± 0.004
C18:3n-3	49.31 ± 0.006	–	29.59 ± 0.010	118.36 ± 0.006

geometry, separated by a 1-mm gap, was used to carry out all the measurements. Dynamic viscoelastic properties were determined by frequency swept tests in a frequency range of 0.628–628 rad/s. The linear viscoelastic regions of both emulsions were previously determined by amplitude swept tests $\gamma = 0.01$–100% at a fixed frequency ($f = 6.28$ rad/s). Rheological tests were carried out at 20 °C in duplicate, after averaging 3 measurements per replicate.

2.3. Animal tests

Sixty male eight-week old Sprague–Dawley rats weighing $\sim$300 g were given free access to a 4% fat commercial diet and water. They were divided into four groups. One group was given the sodium-caseinate emulsion (Sc), another one the chitosan emulsion (Ch) and a third group was fed with a control test meal (C), which consisted of a non-emulsified interesterified-fat/distilled-water blend using the same mass ratio (40/60). In addition, a blank group (B) was fed with physiological serum.

Overall, after 18 h of fasting with free access to tap water, the rats were anesthetized in an induction chamber with isoflurane/oxygen, and intubated with an oral gastric feeding tube. 1 g of test meal at 36 °C or 1 g of physiological serum was administrated via the feeding tube, and recovery was allowed (Table 1 shows most important fatty acids that were fed in 1-g of test meal). The rats were returned to their cages until blood extraction at 0 (before administration), 1.5, 3, 4.5, and 8 h after test meal or serum administration.

Afterwards, the rats were anesthetized in the induction chamber and arranged in surgery position with an anaesthesia flow through a nosecone. The abdominal cavity was opened and rats were exsanguinated by abdominal aortic blood puncture using a K_2EDTA vacutainer tube. Rats were sacrificed by exsanguination, which was verified by perforating the diaphragm. All procedures were approved by the Bioethical and Biosafety Committee of the Faculty of Biological Sciences of Pontificia Universidad Católica de Chile.

2.4. Postprandial fat absorption

Fat absorption was determined as total plasma lipids during the postprandial period. The collected blood samples were centrifuged at 3000 rpm for 10 min at 4 °C. Plasma was separated and stored at −18 °C until lipid extraction and analysis. The extraction of lipids was carried out using the method described by Bligh and Dyer (1959). Lipids were then methylated (Morrison & Smith, 1964) and analysed by gas chromatography using a GC Hewlett Packard

7890 (column J and W DB-FFAP, 30 m, 0.25 mm ID, 0.25 μm, FID detector).

2.5. Statistical analysis

Animal tests were performed in parallel to reduce the differences in external factors. The results are expressed as the mean ± standard error (SEM) of 3 rats in each group for each individual data point. Statistical significance of differences between groups was determined using two-way ANOVA followed by the Tukey HSD test. Differences were considered significant at $p < 0.05$. Areas under the curve and statistical analysis were determined using GraphPad Prism 5.0 (GraphPad Software, Inc., CA, USA).

3. Results and discussion

3.1. Characterisation of sodium caseinate or chitosan emulsions

Emulsions were characterised in terms of stability, globule size distribution and rheological properties to understand a possible link between these parameters and fat absorption.

3.1.1. Stability

No creaming or phase separation was determined in any emulsion. This was probably due to the high concentrations of sodium caseinate or chitosan that were used, as reported by other authors (Jafari, Assadpoor, He, & Bhandari, 2008; Liang et al., 2014). As we used a high concentration of sodium caseinate, a firm, percolating droplet network must have been formed, which fixed the droplets by a strong force (Dickinson & Golding, 1997; Liang et al., 2014). Similarly, the concentration of medium molecular weight chitosan (with 75–85% deacetylation degree) that was used ensured a good emulsifying capacity (Klinkesorn, 2013).

3.1.2. Oil droplet size

Sodium caseinate and chitosan emulsions had a mono-modal particle size distribution with mean particle diameters of $d_{32} = 36$ μm and $d_{32} = 16$ μm, respectively, as shown in Fig. 1. This indicates that, under these experimental conditions, chitosan was able to form and stabilise smaller droplets than sodium caseinate. This information could indicate that lipids in the chitosan emulsion could be better absorbed. However, other factors than droplet size may play an important role, as will be explained in the next section.
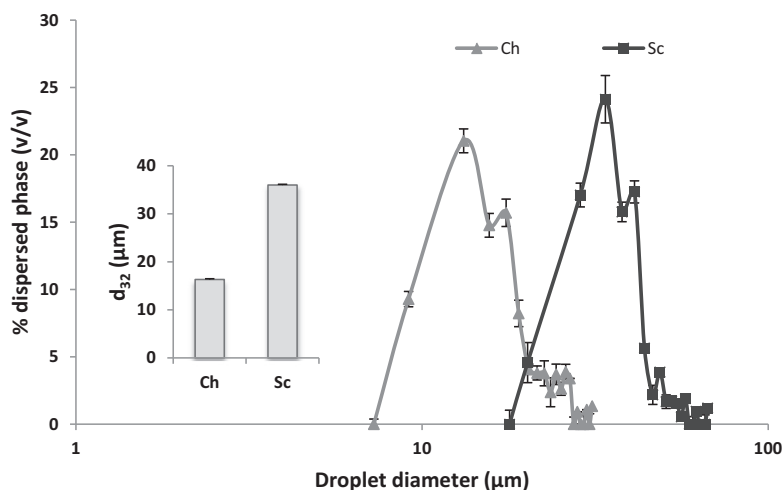

Fig. 1. Droplet size distribution and respective Sautier mean diameter (d_{32}) of O/W emulsions prepared using chitosan (Ch) or sodium caseinate (Sc).

3.1.3. Rheological properties

Fig. 2 shows the evolution of the shear storage and loss moduli, G′ and G″, of both emulsions during the rheological measurements inside the linear viscoelastic region. G′ was always much higher than G″ in both cases, denoting a predominantly gel-like behaviour (Batista, Raymundo, Sousa, Empis, & Franco, 2006; Romero, Cordobés, Puppo, Guerrero, & Bengochea, 2008). In addition, the higher values of G′ and G″ found in the chitosan emulsion indicate a better developed three-dimensional structure. Overall, in concentrated emulsions as were used here, strong inter-particle interactions are usually observed. The particles are almost touching each other, conferring a high viscoelasticity (Rahalkar, 1992).

3.2. Postprandial lipids absorption after sodium caseinate or chitosan emulsions feeding

The postprandial absorption of selected lipid fractions was analysed over time to understand its relationship to a test-meal composition.
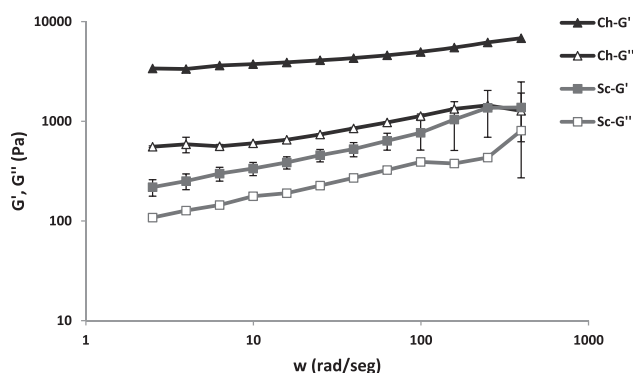

Fig. 2. Evolution of shear storage (G′) and loss moduli (G″) of O/W emulsions prepared using chitosan (Ch) or sodium caseinate (Sc).

3.2.1. Postprandial lipids absorption over time

The absorption curves of linolenic acid (C18:3n3), palmitic acid (C16:0), saturated fatty acids (SFA) and total fatty acids (TFA) in plasma lipids, before and after test meal administration, are shown in Fig. 3. We focused on linolenic acid absorption since this essential fatty acid is present in high amounts in linseed oil and is practically not found on the bloodstream during the fasting state, thus, it is a suitable biomarker. On the other hand, palmitic acid is present in high amounts in palm stearin and was the most abundant saturated fatty acid in the interesterified mix.

As we expected, the test meal intake was quickly reflected in lipemia. All curves showed a similar pattern, that is, an increase after 1.5 or 3 h, which slightly decreased with increasing times. Overall, sodium-caseinate test meals showed a better absorption than chitosan test meals at the beginning ($t = 1.5$ h). These differences were statistically significant ($p < 0.05$) when comparing C16:0, SFA, and TFA concentrations. However, no statistical differences were found in C18:3n3 absorption. In addition, no significant differences were found in postprandial lipid absorption between the chitosan based emulsion and the control mix at any time. The blank group remained at the basal level.

Fig. 3 also shows the plasma level of C16:0, SFA, and TFA, before test meal administration. These blood lipids correspond to those released from the enterocyte or the liver during the fasting state. At the end of the experiment ($t = 8$ h), the amounts of C16:0, SFA, and TFA decreased once again, down to their basal level (no statistical differences were found, $p > 0.05$). Interestingly, an increase in their content was observed in the blank group (only fed with physiological serum) at $t = 8$ h, which was only significant in SFA. This agrees with results obtained in previous work (Farfán et al., 2013), and is attributed to the prolonged fasting and carbohydrate starvation, and the consequent fatty acid mobilisation (Owen, Felig, Morgan, Wahren, & Cahill, 1969).

On the other hand, the C18:3n3 concentration was approximately 10 µg/ml, before oral gastric feeding (Fig. 3). This low concentration proved that C18:3n3 was practically not found in the bloodstream during the fasting state, and thus, was an appropriate
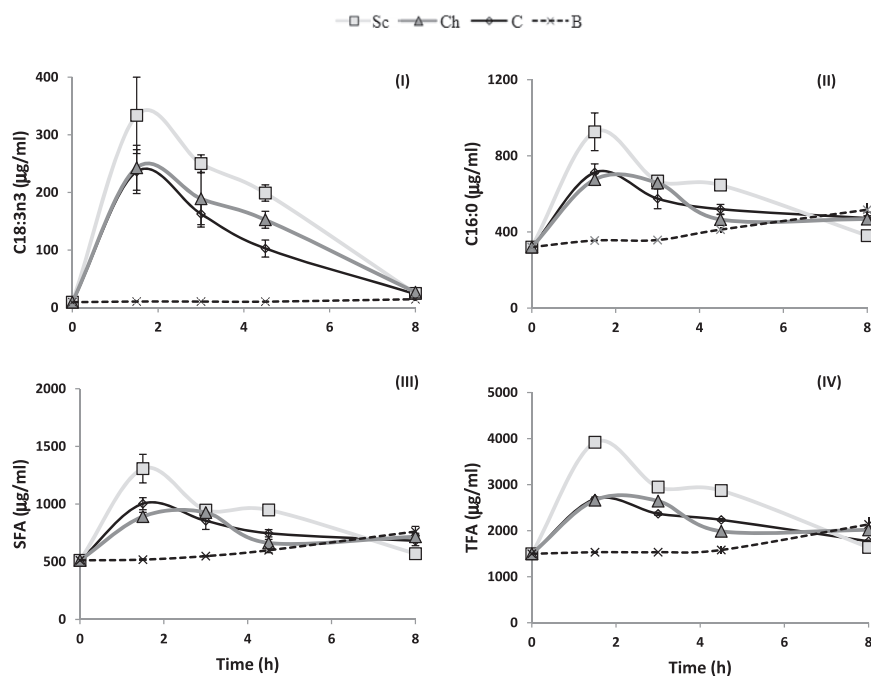

Fig. 3. Postprandial absorption of C18:3n3 (I), C16:0 (II), saturated fatty acids, SFA (III), and total fatty acids, TFA (IV), after oral gastric feeding of sodium caseinate emulsion (Sc), chitosan emulsion (Ch), non-emulsified control (C) or physiological serum blank (B).

biomarker. After feeding any test meal (Sc, Ch or C), the amount of C18:3n3 was quickly reflected. The peaks were obtained in the first measurement ($t = 1.5$ h). They were statistically higher than the ones found in the blank group, which remained at the basal level. All concentrations diminished gradually, down to the basal level at $t = 8$ h.

3.2.2. Total postprandial lipids absorption over time: area under the curve

Fig. 4 shows the areas under the C18:3n3, C16:0, SFA and TFA plasma concentration time curves (AUC), which reflect the total absorbed amounts after oral gastric feeding. As expected, lipid absorption after test-meal feeding was significantly higher at all times compared to lipid absorption after physiological-serum feeding (blank, B). Results were certainly consistent with those reported in Fig. 3. Sodium-caseinate emulsion feeding resulted in a significantly higher absorption of C16:0 (after 4.5 h), SFA (after 4.5 h) and TFA (after 3 h), compared to those obtained after chitosan-emulsion or control feeding. No significant differences were obtained in C18:3n3 total absorption when comparing both emulsions, despite the fact that the mean values were always higher in sodium-caseinate based emulsions. Also, a significant difference between the sodium-caseinate emulsion and the control was observed after 8 h. Overall, no significant differences were found in the total postprandial absorption of any lipid fraction, between the chitosan-based emulsion and the control mix at any time.

The higher absorption observed after sodium-caseinate emulsion feeding, confirms the effectiveness of emulsification in lipid absorption. Results agree with those showed by Garaiova et al. (2007) and Vors et al. (2013) who reported an enhanced absorption of emulsified fat compared to non-emulsified fat, during postprandial absorption in humans.

The question remains why postprandial lipid absorption after oral gastric feeding with a chitosan-based emulsion did not improve? This behaviour could be a consequence of pH variation through the gastro-intestinal tract. In fact, chitosan has shown to have gelling properties when the pH is greater than its pKa (>6.3), due to the lower solubility of its deprotonated form (Klinkesorn, 2013). Thus, when the pH rises (as in the duodenum), chitosan-stabilised droplets may become more rigid, entrapping the emulsion (Beysseriat et al., 2006). This could restrict the access of pancreatic lipase to lipids within the droplets, as explained by Agulló, Rodríguez, Ramos, and Albertengo (2003) and Mun et al. (2006). This behaviour could be intensified by a direct interaction between chitosan and the active site of the pancreatic lipase, reducing its enzymatic activity, as explained by Mun et al. (2006). If this happened, the limited access of pancreatic lipases to lipids could counterbalance the positive effect of size reduction, resulting in a null net effect. Conversely, the lower postprandial lipid absorption found in chitosan-based emulsions could be due to the breakdown of the emulsion in the small intestine, into oil and water phases.

4. Conclusions

Throughout this study, we were able to successfully evaluate the effect of emulsification, as well as the use of sodium caseinate or chitosan, on the postprandial bioavailability of interesterified-lipids in O/W emulsions after oral gastric feeding Sprague–Dawley rats. In addition, we confirmed the suitability of linolenic acid as plasma biomarker.

Overall, we confirmed that through emulsification it is possible to increase lipid absorption, as determined after feeding sodium-caseinate emulsions. However, this result could not be generalised. Interesterified-lipids emulsified with chitosan were not better absorbed than those contained in non-emulsified interesterified-lipids/distilled-water blends. This may be due to a limited access of pancreatic lipases to lipids due to the pH increase in the duodenum, which may counterbalance the positive effect of size reduction, or to the breakdown of the emulsion in the small intestine. Future studies may consider a deep analysis of the physical stability of the chitosan-based emulsions at different pH values, as well as additional *in vitro* studies under simulated gastrointestinal conditions to analyse lipase digestibility.
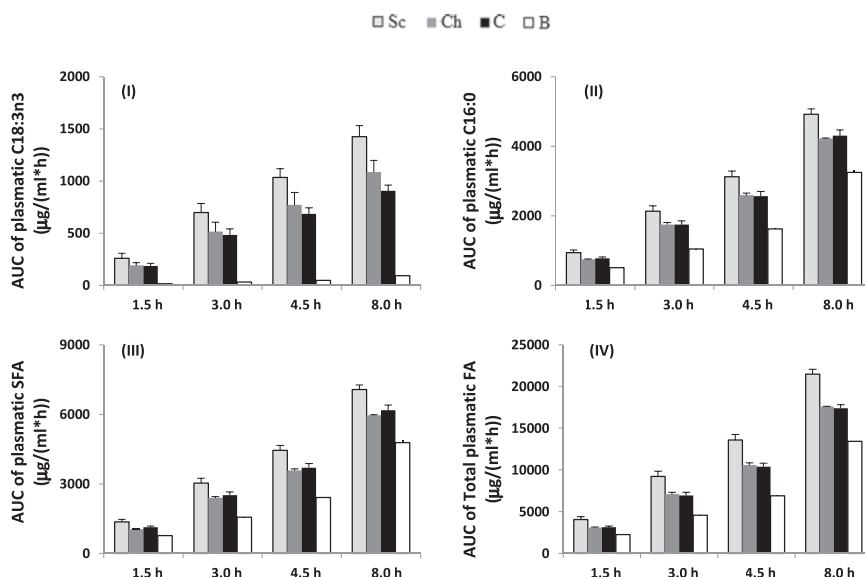
Fig. 4. Areas under the C18:3n3 (I), C16:0 (II), saturated fatty acids, SFA (III) and total fatty acids, TFA (IV) plasma concentration time curves (AUC), after oral gastric feeding of sodium caseinate emulsion (Sc), chitosan emulsion (Ch), non-emulsified control (C) or physiological serum blank (B).

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