# Vegetable protein isolate-stabilized emulsions for enhanced delivery of conjugated linoleic acid in Caco-2 cells

C. Fernandez-Avila[a,b], E. Arranz[a], A. Guri[a], AJ Trujillo[b] and M. Corredig[a]

[a] Department of Food Science, University of Guelph, Guelph, Ontario, N1G 2W1, Canada.

[b] Centre Especial de Recerca Planta de Tecnologia dels Aliments (CERPTA), XaRTA, TECNIO, MALTA-Consolider, Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, Universitat Autònoma de Barcelona, Spain.

**Abstract**

Developing edible delivery systems which offer higher protection and release of bioactive constituents is a current challenge in the food industry. The ability of oil-in-water emulsions (20% oil) stabilized by soy or pea protein isolates (4%) to deliver conjugated linoleic acid (CLA, 6%), was studied. The emulsions were prepared by conventional homogenization (550 bar) using one or five homogenization passes. The physicochemical properties of the emulsions were determined, as well as loading capacity and oxidative stability. The emulsions were subjected to *in vitro* digestion, and tested on absorptive Caco-2 cells. The presence of CLA isomers was followed throughout the process. When comparing similar treatments, soy protein isolate emulsions showed smaller particle size distributions than emulsions prepared with pea protein isolates. Emulsions containing soy proteins showed preferential adsorption of the α' subunit of conglycinin and the $A_1$, $A_2$, $A_4$ subunits of glycinin on the oil droplets. All emulsions protected the encapsulated CLA better than the non-emulsified control in which CLA was oxidized during storage, as well as after *in vitro* digestion and delivery in Caco-2 cells. Similar percentages of bioaccessibility and bioavailability of CLA were found for all the emulsion treatments. The results obtained here open new prospects for using oil-in-water emulsions as structured emulsion-based delivery systems to be used in functional foods containing CLA with health enhancing properties.

**Keywords:** conjugated linoleic acid, emulsion, soy protein, pea protein, Caco-2 cells

## 1. Introduction

The enrichment of foods with lipophilic bioactives, such as omega-3 fatty acids, carotenoids and phytosterols, is an increasingly important area in the food industry, especially in light of the increased tendency for consumption of low-fat good, which in turn decreases the intake of lipophilic bioactives (Gao et al., 2014). A number of emulsion-based technologies could be used as edible delivery systems of these compounds by the food, medical, and pharmaceutical industries, including conventional emulsions, multiple emulsions, multilayer emulsions, solid lipid particles, and filled hydrogel particles. Emulsion technology is particularly suited for the design and fabrication of delivery systems for encapsulating bioactive lipids (McClements, Decker, & Weiss, 2007; McClements & Li, 2010). To create emulsions kinetically stable for a reasonable period of time, stabilizers such as emulsifiers or texture modifiers need to be added to prevent gravitational separation, flocculation, coalescence and Oswald ripening (McClements, 2005). In this sense, commercial protein isolates are commonly used as emulsifiers. However, they exhibit poor solubility compared to other protein fractions or high-soluble isolates obtained in the laboratory (Keerati-u-rai & Corredig, 2010; Wang et al., 2012).

Within bioactive compounds of lipophilic nature, conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid with conjugated double bonds. The CLAs are found naturally in foods derived from ruminant animals, meat, or dairy products. The most representative CLA isomers are c9,t11-18:2 and t10,c12*c*-18:2. CLA has been shown to exert various potent physiological functions such as anticarcinogenic, antiobese, antidiabetic and antihypertensive properties (Koba & Yanagita, 2013; Moon, Lee, Chung, Choi, & Cho, 2008). In recent years, there has been an increasing interest to incorporate CLA into various food products, the most common

being dairy products ~~the most common~~ (Campbell, Drake, & Larick, 2003; Jimenez, Garcia, & Beristain, 2008). However, CLA exhibits very poor chemical stability presenting autoxidation and isomerization during thermal processing, such as UHT, which decrease its nutritional value. To overcome these problems, it is necessary to protect/encapsulate CLA in an efficient delivery system, like an oil-in-water emulsion. Gum arabic was found to be a suitable emulsifier for CLA-in-water emulsions (Yao et al., 2013). However, to date the research made with this bioactive compound has focused mainly on microencapsulation by drying processes rather than oil-in-water-emulsions (Choi, Ryu, Kwak, & Ko, 2010; Costa et al., 2015; Lalush, Bar, Zakaria, Eichler, & Shimoni, 2004; Park et al., 2002; Yang, Gu, Xu, Li, & Zhang, 2010; Yang, Gu, & Zhang, 2009).

There is still significant debate on what are the best strategies for CLA protection since it has been demonstrated that the molecules can decompose to furan fatty acids in the presence of air. Furan fatty acids may have toxicological properties so that spray-drying encapsulation procedure might not be an adequate processing (Buhrke, Merkel, Lengler, & Lampen, 2012). Gao et al. (2014) have succesfully developed soy lipophilic protein nanoparticles (LPP) as a novel delivery vehicle for conjugated linoleic acids. Vegetable proteins, such as soy protein, are rarely used to deliver hydrophobic compounds, in spite of the gained interest for sourcing more sustainable protein isolates for food product development.

Oil-in-water emulsions containing CLA could be used as ingredients in functional foods; however, efficient delivery of the biological compound and its bioefficacy in the emulsion system needs to be demonstrated~~. *In vitro* experiments, such as the uptake by cell cultures (*e.g., Caco-2 cells*) that mimic the human intestinal epithelium, provide useful insights into possible physicochemical mechanisms that occur during digestion~~

~~and absorption of lipids (David Julian McClements & Li, 2010).~~ Previous research demostrated that LPP can protect CLA from oxidation and also show a sustained release profile (Gao et al., 2014). However, so far, no studies evaluated CLA bioavailability in oil in water emulsion systems using human intestinal cell models such as Caco-2 cells. . *In vitro* experiments, such as the uptake by cell cultures (*e.g., Caco-2 cells*) that mimic the human intestinal epithelium, provide useful insights into possible physicochemical mechanisms that occur during digestion and absorption of lipids (David Julian McClements & Li, 2010).

The encapsulation, release and bioefficacy of CLA encapsulated in oil-in-water emulsions stabilized with vegetable proteins have yet to be reported. The aim of this study was to investigate the effect of different oil in water emulsions stabilized by soy and pea protein isolates on the delivery of CLA. Commercially available soy and pea protein isolates were used, to increase the relevance of this research to the food industry (Nishinari, Fang, Guo, & Phillips, 2014). The CLA bioefficacy was studied using *in vitro* digestion and a Caco-2 intestinal cell absorption model.

## 2. Materials and Methods

### *2.1 Materials*

A commercial soy protein isolate (SPI) (PRO-FAM 974) was purchased from Lactotecnia (Barcelona, Spain). The composition of this commercial SPI according to manufacturer was: 90% protein, 4% fat, 6% moisture, and less than 5% ash (dry basis, w/w). Pea protein isolate (PPI) (Nutralys®F85M) was purchased from Roquette Frères S.A. (Lestrem, France). The composition of this commercial PPI according to manufacturer was: 85% protein, 7% moisture, and less than 5% ash (dry basis, w/w). Solubility of both protein isolates at neutral pH was < 40%. Soybean oil was purchased

from Sigma-Aldrich Corporation (Oakville, ON, Canada). A free fatty acid mixture high in isomers of CLA (Neobee®CLA80) was purchased from Stepan Specialty Products LLC (Maywood, USA), containing 80.4% of total CLA. The two main isomers present were c9,t11-CLA and t10,c12-CLA, in a 50:50 ratio. Other CLA isomers (*cis,cis* and *trans,trans*) were present in minor concentrations (<1.2%). Neobee®CLA80 was obtained from natural safflower oil by a gentle, proprietary process without antioxidants added. All other chemicals used were of analytical or better grade. Pepsin (P7000), pancreatin (P1750), phospholipase A2 (P6534) and bile salts (B8631) were obtained from Sigma-Aldrich.

### *2.2 Preparation of oil-in-water emulsions*

Oil-in-water emulsions (20% oil, v/v) containing conjugated linoleic acid (CLA, 6%) and soybean oil were emulsified with a fixed protein content of SPI or PPI (4%, w/v). Firstly, the stock protein dispersion (4%, w/v) was prepared by dispersing the protein in ultrapure water and stirring for 1 h at 40 ºC. Protein dispersions were stored overnight at 4ºC to allow complete hydration. A coarse emulsion was prepared by mixing the protein dispersion with the oil using a hand-held homogenizer (Polytron PT 1200, Kinematica, Fisher Sci., Mississagua, ON, Canada) at 15000 rpm for 1 min. Conventional homogenization was then carried out at 550 bar for one and five passes using Emulsiflex C5, Avestin (Ottawa, ON, Canada). Emulsions were separated using ultracentrifugation (45 min at 50,000 g and at 25 ˚C) (OptimaTM LE-80K, with a Ti-45 rotor, Beckman–Coulter, Mississauga, Ontario, Canada). Cream was carefully removed from the top layer and dried on a filter paper, then resuspended in ultrapure water to the initial volume fraction. The serum phase was withdrawn using a syringe and filtered through 0.45 μm filter (Millipore, Billerica, MA, USA). Fresh emulsions were

immediately used for further analysis and stored at 4 ˚C with 0.02% (w/v) of sodium azide to determine physico-chemical stability.

### *2.3 Determination of oil droplet size distribution*

The particle size distribution of the emulsions was measured using static light scattering (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) using water as the dispersing agent. The refractive indexes of soy oil and water were taken as 1.47 and 1.33, respectively. Emulsion samples were diluted in distilled water until an appropriate obscuration was obtained. Emulsion samples were also diluted in 2 g/L sodium dodecyl sulphate (SDS) at least 30 min before light scattering analyses to check for the presence of aggregated or coalesced droplets that could be dissociated by SDS (Pearce & Kinsella, 1978). The surface-weighted mean diameter ($d_{3.2}$, μm) and the volume-weighted mean diameter ($d_{4.3}$, μm) were determined on the fresh emulsions, the emulsions were kept at 4 ˚C during 10 days and the digestates of the fresh emulsions.

### *2.4 SDS-PAGE*

Aliquots (100 μl) of sample (emulsions, serum and cream phases) were treated with 200 μl of extraction buffer, containing 50 mM Tris–HCl, 5 M urea, 1% SDS, 4% 2-mercaptoethanol, pH 8.0. The samples were equilibrated at room temperature for an hour then centrifuged (Eppendorf, Brinkmann Instruments, Westbury, NY, USA) at 10,000 *g* for 10 min, to separate the oil phase before mixing the aqueous extract with 200 μl of the electrophoresis buffer, which was composed by 125 mM Tris–HCl, 5 M Urea, 1% SDS, 20% Glycerol, 4% 2-mercaptoethanol, pH 6.8. Samples were then heated at 95 ˚C for 5 min. After cooling to room temperature, aliquots (5 μl) of all protein samples were loaded onto 12.5% polyacrylamide gel with 4% stacking gel in

Bio-Rad mini-protein electrophoresis (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) for proteins separation. Gels were fixed and stained using Bio-Rad Coomassie blue R-250 stain solution (45% methanol, 10% acetic acid and 0.10% Blue R-250) followed by de-staining using 45% methanol, 45% ultrapure water and 10% acetic acid solution then gel was scanned using a SHARP JX-330 scanner (Amersham Biosciences, Quebec, Canada) and the bands were analyzed using image analysis software (ImageMaster® 1D, Version 2.0, Amersham Biosciences).

### *2.5 Quantification of CLA isomers*

An alkali plus acid-catalyzed methylation method was carried out to quantify the CLA isomers (Moltó-Puigmartí, Castellote, & López-Sabater, 2007). Briefly, saponification with $NaOCH_3$/MeOH (2500 µL) and esterification procedure (2500 µL of $BF_3$) was taken. Fatty acid methyl esters (FAME) were analyzed by gas chromatography, using an automated Agilent 6890 GC system (Agilent, Palo Alto, CA, USA) equipped with a flame ionization detector (FID). FAME was separated on CP-Sil 88 capillary column (100 m × 0.25 mm i.d.× 0.20 µm). Operating conditions were as follows: injector port temperature 250 ˚C; hydrogen as carrier gas at a linear pressure of 17.7 psi; split ratio 1:10 or 1:0.1 and injection volume 1 µL or 2 µL for emulsions and digestates, or Caco-2 cell monolayer samples, respectively. The detector temperature was set to 255 ºC, with $H_2$ flow 40.0 mL/min and air flow 450 mL/min. A programmed temperature gradient was used for the chromatographic separation. The run started at 70 °ºC for 4 min, and then a gradient of 13 °ºC/min was applied to reach 175 °ºC. After 27 min at this temperature, the temperature was risen to 215 °ºC applying a gradient of 4 °ºC/min, and finally held there for 31 min, for a total run time of 80 min (Kramer et al., 2004; Kramer et al., 1997). Gas-chromatographic peaks were identified by comparing the

peaks' retention times to those of a standard FAME mix as well as of a linoleic acid methyl ester isomer mix. CLA peaks were quantified using individual pure standards c9,t11 and t10,c12-CLA. The c9,t11 and t10,c12-CLA isomers were also confirmed by spiking the standards in o/w emulsions containing soybean oil. Calibration curves of t9,c11 and t10,c12-CLA were constructed using pure standards (in a range of 0.05-1 µg/µL for the emulsions and digestates samples and 0.01-0.5 µg/µL for the different Caco-2 cell monolayers phases) containing 50 µL of the internal standard solution (C23:0, 0.2 µg/µL) and were found to be linear, with correlation coefficients >0.985. Other isomers products (*cis,cis* and *trans,trans*-CLA) derived from the main isomers (t9,c11-CLA and t10,c12-CLA) were quantified with t10,c12-CLA due to closer retention time. The ratio percentages between t9,c11; t10,c12; *cis,cis* and *trans,trans*-CLA were also determined. The initial CLA sample, was analysed to confirm its composition, and it was composed by 49% c9,t11; 48% *t10,c12*; 0.45% *cis,cis* and 1.9% *trans,trans*).

### *2.6 CLA encapsulation*

The content of CLA loaded in o/w emulsions was determined by GC-FID technique in the emulsions (initial CLA isomer) and the cream phase resuspended in water (recovered CLA isomer). The amount of loaded CLA per 100 g of initial CLA (in emulsions)—loading capacity (LC) was thus calculated from Eq. (1):

$$\%LC = \frac{C_{recovered\ CLA\ isomer}}{C_{initial\ CLA\ isomer}} \times 100 \quad (1)$$

where *C* is the concentration.

### *2.7 Peroxide value of emulsions*

Emulsions with sodium azide (0.02% w/v) were stored at 37 °C for 20 days. Fresh samples and samples after 10 and 20 days of storage were collected to further analysis. The same mixture of oils (soybean oil and CLA) was used for the control. The formation of lipid peroxides was evaluated according to the AOAC Official Method 965.33 (Hortwitz, 2002). Briefly, 25 mL $CH_3COOH–CHCl_3$ was added into 1.50 ± 0.05 g test portion, and the mixture was stirred to destroy the emulsifier-trapping material. Then 1 mL saturated KI solution was added, followed by vigorously shaking 1 min, and the mixture was held in a dark place for 5 min. Then 75 mL $H_2O$ was added to terminate the reaction. It was slowly titrated with 0.0394 M $Na_2S_2O_3$ with vigorous shaking ~~until yellow disappear~~. Subsequently, drops of 1% starch solution was added, titration and shaking was continued to release all $I_2$ from $CHCl_3$ layer, until blue just disappeared. Peroxide value (Pv) was calculated as follows from Eq. (2):

$$Pv_{(\text{milliequivalent peroxide/kg oil or fat})} = \frac{V \times N \times 1000}{g\ sample} \quad (2)$$

where *V* is the volume (mL) of the $Na_2S_2O_3$ solution used and N is its normality. A blank was titrated to adjust the Pv of samples.

## *2.8 Release of CLA isomers*

### *2.8.1 In vitro gastro-duodenal digestion*

The *in vitro* digestion experiments were performed according to the INFOGEST method (Minekus et al., 2014), with minor modifications. In brief, oil-in-water emulsions and the CLA-control (the CLA without being protected by an emulsification process) were initially dispersed in simulated gastric fluids. The samples were incubated with the gastric fluids containing pepsin (25000 U/mL) at 37 °C, and at pH 2 in a 250 rpm shaking water bath for 1 h. The duodenal digestion was then initiated by the addition of

simulated duodenal fluids containing pancreatin (5 mg/mL), and phospholipase $A_2$ (5 µL of 6.7 mg/mL). Simulated bile fluids containing bile salts (0.4 m M) and phospholipids (1 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 at pH 7. Digestion of the samples was stopped with the same medium that we use to cultivate the cells. We chose the dilution 1:40, prior cytotoxic experiments using sulforhodamine B (SBR) assay demonstrated that this dilution was the minimum with no toxic effect (data not shown). Digestion samples were collected and analysed by GC-FID as described above.

### *2.8.2 Free Fatty Acid (FFA) determination*

The amount of free fatty acids (FFA) liberated after *in vitro* digestion was quantified as described elsewhere (Malaki Nik, Wright, & Corredig, 2011). In brief, the FFAs were extracted under acidic conditions after duodenal digestion. The amount of FFA was determined using a colorimetric method (NEFA kit) by measuring absorbance at $\lambda_{max}$ of 550 nm (UV-VIS microplate spectrophotometer, Spectramax plus, Molecular Devices, CA, USA) and by reference to a standard curve prepared using oleic acid ranging from 0.1 to 2 mM.

### *2.8.3 Transport studies through the Caco-2 cell monolayer*

Caco-2 cell line was provided from the Canadian Research Institute for Food Safety (CRIFS) Culture Collection (Food Science, University of Guelph, ON, Canada). The cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% non-essential aminoacids and 2 mM L-glutamine (Invitrogen, Canada Inc., Burlington, ON, Canada) at 37 ºC in at humidified atmosphere containing 5% $CO_2$.

For transport experiments, Caco-2 cells were seeded in 12-well Transwell® plates (0.4 μm pore size, inserts of 1.2 cm diameter, BD Biosciences, Mississauga, ON, Canada) at a density of $6 \times 10^4$ cells per insert. The cells were maintained for 21 days until they reach full confluency and the complete monolayer was formed. The culture medium was replaced every second day. To ensure the integrity of the monolayer was maintained the transepithelial electrical resistance (TEER) (Evon World Precision Instruments, Sarasota, FL, USA) was measured every other day.

Apical and basolateral compartments were washed with PBS to remove any cell debris and then incubated respectively with 425 μL and 1500 μL of DMEM without FBS. The cells were incubated at 37 ºC for 30 min prior to experiments to equilibrate the monolayers. Digestates were added in the apical compartment at a dilution ratio of 1:40 (sample: medium, v/v) and incubated for 4 h at 37 ºC, 5% $CO_2$. The viability of the monolayers was assessed by measuring the TEER value before and after 1, 2 and 4 h of experiment. Then the cell lysates (collected in PBS), apical and basolateral samples were collected and stored at – 20 ºC till analysed by GC-FID.

The amount of bioaccessible CLA *in vitro* per 100 g of initial CLA (in digestates of emulsions) was thus calculated from Eq. (3):

$$\%\ \text{Bioaccessible isomer} - \text{CLA} = \frac{C_{CLA\ isomer\ in\ cells\ compartment}}{C_{CLA\ isomer\ in\ digestates}} \times 100 \qquad (3)$$

Also, the amount of bioavailable CLA *in vitro* per 100 g of initial CLA (in digestates of emulsions)—was therefore calculated from Eq. (4):

$$\%\ \text{Bioavailable isomer} - \text{CLA} = \frac{C_{CLA\ isomer\ in\ basolateral\ compartment}}{C_{CLA\ isomer\ in\ digestates}} \times 100 \qquad (4)$$

where *C* is the concentration.

### *2.9 Statistical analyses*

Descriptive statistics, mean and standard deviation, were listed for each variable in this study. In order to evaluate each variable of emulsions among type of protein (SPI or PPI) and passes of homogenization (one and five passes), a General Linear Model was performed. The statistical analysis was performed using the SPSS® v17.0 package to a 95% level of significance and Tukey adjustment was performed for multiple comparisons of the means ($P< 0.05$). Also, the Student's *t*-test for paired samples analysis was carried out to compare the changes between day 0, 10 and 20. Significance was defined as $p < 0.05$ for a two-sided test. Experiments were performed in duplicate as separate, independent runs.

## 3. Results and Discussion

### *3.1 Physical characteristics of emulsions*

#### *3.1.1 Particle size distribution*

The diameter distribution of SPI and PPI stabilized emulsions is shown in Figure 1, for samples before and after digestion. While emulsions prepared using only 1 pass through the homogenizer showed a large diameter, between 10 and <100 μm, those prepared with five passes through the homogenizer showed smaller particle size distributions (Fig. 1A,C, for emulsions before digestion). These emulsions had a major peak for diameters of about 14 μm, a value significantly ~~lower~~ higher than for the same emulsions prepared with ~~only one~~five passes. The droplets covered with soy protein isolate after five homogenization passes showed the smallest particle size ($d_{4.3}$ and $d_{3.2}$, $P<0.05$), and they were smaller of the same emulsions prepared with PPI (Table 1). It is widely known that the more an emulsion passes through the homogenization device, the less re- coalescence of droplets will occur (Floury, Desrumaux, & Lardières, 2000;

289 Schultz, Wagner, Urban, & Ulrich, 2004). After one pass through the homogenizer, the
290 emulsions, regardless of the type of protein use to stabilize the system, were extensively
291 flocculated. This is clearly shown by the smaller particle size distribution measured
292 after treatment with SDS (Fig. 1A,C). The residual larger droplets in the emulsions
293 prepared with five passes were also disrupted using SDS, indicating that in this case
294 also some bridging flocculation remained for both SPI and PPI emulsions.
295 The emulsions treated only with one pass through the homogenizer showed a gel-like
296 behaviour, due to the extensive flocculation, and this was not the case for the five passes
297 emulsions, which showed to be shear-thinning (data not shown). With respect to the
298 physical stability of the emulsions after 10 days, only the soy protein isolate emulsion
299 treated at one pass had signs of destabilisation, and this was also shown with an
300 increase in particle size ($P<0.05$).
301 The emulsions were subjected to *in vitro* digestion, and their particle size distribution is
302 shown in Figure 1B,D. In the case of SPI stabilized emulsions, at five passes, there were
303 no significant changes in $d_{4.3}$ and $d_{3.2}$ ($P>0.05$). The particle size of the SPI emulsions
304 prepared with one pass also showed a similar particle size distribution, albeit shifted to
305 smaller diameter, possibly due to the presence of bile salts, disrupting some of the
306 bridging flocs of the emulsions. In the case of PPI emulsions prepared with five passes,
307 ~~while the one pass through the homogenizer did not show significant changes in the~~
308 ~~particle size distribution, the emulsion prepared with five passes showed extensive~~
309 flocculation after digestion was observed. These differences in particle size distribution
310 between the emulsions may cause differences in the fatty acid release and the delivery
311 and absorption of CLA.

312 *3.1.2 Protein composition at the interface*

To determine ~~if homogenization conditions caused~~ differences in the protein adsorption at the interface, SDS-PAGE analysis was carried out on emulsions and cream samples. Differences in the distribution of the proteins in the cream phase would indicate differences in the protein adsorption. The major protein bands were quantified by laser scanning densitometry and the ratios between the main protein fractions present in the emulsions and cream phases of the SPI and PPI emulsions obtained after five passes through the homogenizer are shown in Table ~~2~~ and Figure 2.

In the case of soy protein stabilized emulsions, the major fractions associated with the interface were α' subunit of conglycinin, as well as the A and B subunits of glycinin. Specifically, α' subunit of conglycinin and $A_1$, $A_2$, $A_4$ subunits of glycinin in SPI emulsion showed to be at a higher ratio compared to the emulsions ($P < 0.05$). These results were in contrast with previous reports (Keerati-u-rai & Corredig, 2009) for 1% SPI oil-in-water emulsions. In that case, there was no difference in the subunit distribution between the oil droplets and the serum phase. The higher protein level in the emulsion as well as their different protein history (with a lower solubility index for the proteins used in this study) may be the cause for the discrepancy. ~~There were no statistical differences in the protein composition at the interface between emulsions prepared with one pass or five passes through the homogenizer, as clearly shown by the *P* values in Table 2.~~

For PPI stabilized emulsions, the protein subunits that were present the most at the interface were legumin α and vicilin (Table 2 and Fig. 2). There were no statistically significant differences in the bands intensity of proteins in the cream or emulsion samples (Table 2).

The difference in behaviour between SPI and PPI stabilized emulsions was caused by the difference in the amount of protein necessary to obtain a full coverage of the

interface, and the aggregation state of the protein. It has been previously reported that 0.5-2.0% highly soluble soy protein fractions can stabilize emulsions containing 10-20% oil (Floury, Desrumaux, & Legrand, 2002; Keerati-u-rai & Corredig, 2009). However, the commercial SPI used in this study had lower solubility than that reported in the literature (Keerati-u-rai & Corredig, 2010; Wang et al., 2012), and similarly, PPI isolates also did not show good solubility. Hence, in both cases, a higher protein content was needed to stabilize the emulsion. The particle size distribution clearly showed that the emulsions droplets showed some extent of flocculation even after 5 homogenization passes, possibly due to the presence of large protein aggregates bridging between the droplets.

### *3.1.3 CLA loading capacity (%)*

There were no significant differences in the loading capacity of c9t11 and t10c12-CLA isomers within different emulsions (Fig. ~~2~~3). Both CLA isomers showed the same recovery of the CLA initially added into emulsions. However, fresh SPI emulsions prepared with 5 homogenization passes showed a higher recovery of CLA isomers compared to the same emulsion obtained with one pass of homogenization or the emulsions stabilized with PPI. It is important to note that a physically stable emulsion is needed to produce an effective encapsulation of lipids (Hu, McClements, & Decker, 2004; McClements & Li, 2010; Zhang et al., 2014).- Furthermore, evaluating CLA concentration in cream phases, significant differences were seen for all CLA isomers between SPI and PPI emulsions prepared with 5 homogenization passes, in which the emulsion stabilized with PPI had a lower concentration (Fig. 4). The SPI-stabilized emulsion treated with five passes showed the smallest average diameter and the smallest

361 increase in $d_{4.3}$ after 10 days of storage, and this is cause to the higher % loading of
362 c9t11-CLA and t10c12-CLA isomers (both 71±18 %).
363 It was hypothesized that the CLA encapsulated in these emulsions could be protected
364 from oxidation and isomerization of CLA. Very few studies are available on the loading
365 of CLA in oil-in-water emulsions. It has been previously reported (Costa et al., 2015)
366 that CLA could be loaded in PPI inclusions,- and the loading capacity reported was
367 lower than that measured in this work (~~Figure~~ Fig. ~~2~~3).
368 A smaller loading capacity for CLA has been reported also in freeze-dried and spray-
369 dried matrices, compared to the fresh emulsions measured in this study, with values
370 below 15% (w/w) (Choi, Ryu, Kwak, & Ko, 2010; Jimenez, García, & Beristain, 2004;
371 Park et al., 2002). Other authors have studied the complexes formed between CLA and
372 other components such amylose and β-cyclodextrin, such complexes improved the
373 efficiency of the delivery of CLA (Lalush et al., 2004; Yang et al., 2010, 2009).
374 Recently CLA was encapsulated in soy lipophilic protein nanoparticles (LPP) by
375 ultrasonication and coating the particles with sodium caseinate (Gao et al., 2014). The
376 encapsulation efficiency of such systems was about 90%. It is possible to hypothesize
377 that, in the case of SPI and PPI emulsions, complexes may also form between CLA and
378 the protein complexes, precipitating during centrifugation, leading to incomplete
379 recovery of CLA in the cream phase of the emulsions.

380 *3.2 Chemical stability of the emulsions*
381 The double bonds of CLA may be in positions 7,9; 8,10; 9,11; 10,12; or 11,13 along the
382 18 carbon chain. Amongst the isomers, t9t11-CLA and t10c12-CLA isomers have been
383 linked to health benefits (Koba & Yanagita, 2013). The CLA free fatty acid oil used in

this study is rich in those isomers, but further transformation may occur during processing (Martínez-Monteagudo, Saldaña, Torres, & Kennelly, 2012).

The stability of CLA in emulsions is shown in Fig. ~~3~~5, by comparing the concentration of the main isomers in fresh emulsions and in the emulsions after storage at 4 ~~°~~˚C for 10 days. There were no changes in the isomer CLA profiles and their CLA content in emulsions with storage (Fig ~~3A,B~~5). During oxidation, free radicals react with molecular oxygen to form hydroperoxides. It is virtually impossible to have an unsaturated hydrocarbon compound completely free of peroxides (Martínez-Monteagudo et al., 2012; Moon et al., 2008). It is therefore desirable to avoid these compounds or to keep them apart, for example, by controlling the surface charge on emulsion droplets (Hu et al., 2004). Figure ~~4~~ 6 illustrates the peroxide values of the emulsions, as well as of the control (soybean oil with CLA added) as a function of time of storage at 37 ˚~~°~~C. The peroxide value of all the emulsions remained under 10 meq $O_2$/kg fat after 20 days of storage at 37 ˚C, with no significant differences ($P$>0.05) among treatments (Fig. ~~4~~6). In contrast, the peroxide values for the control increased significantly and reached a plateau after 10 days. The relatively low content of peroxides in the emulsions is not surprising, since CLA can act as antioxidant, capturing free radicals responsible for peroxide formation during lipid oxidation (Fagali & Catalá, 2008; Martínez-Monteagudo et al., 2012). The findings suggested that the oxidative stability of the CLA-soybean oil emulsions was preserved in the emulsions compared to free oil control.

### *3.3 In vitro digestion*

Figure ~~5~~ 7 illustrates the changes occurring to the CLA isomers after *in vitro* digestion. No significant differences ($P$>0.05) were found in the CLA isomer profile among

emulsions after digestion (Fig. ~~5~~7). However, the CLA-control showed significant ($P$<0.05) increased percentages of isomerization for *trans, trans* isomers compared to emulsions containing CLA, even though *trans,trans* CLA isomers are more stable in air (Moon et al., 2008).

The CLA concentration of different isomers in the digestates was also evaluated, to avoid misleading interpretations by the isomer CLA profile using internal normalisation, and the concentration of the isomers is summarized in Figure ~~5B~~7B. No significant differences were seen for isomerization products from t9c11 and c10t12-CLA bioactive compounds between different treatments. However, slight changes in the two major groups of CLA isomers were observed in the digestates of the CLA-control (free CLA), which had a lower concentration of these isomers than in the emulsions. Thus, these results are in concordance with oxidation or losses of CLA isomers in oil that is not protected in an emulsion delivery system (Fig. ~~5~~7). This indicated that c9t11-CLA and t10c12-CLA isomers are more susceptible to oxidative degradation than isomerization, as other authors have also indicated (Moon et al., 2008)(Moon et al., 2008). ~~It is also interesting to note the decrease in the means in the main CLA isomers (c9t11 and t10c12-CLA) in the digestates of PPI emulsion treated at one homogenization pass, compared to five passes (Fig. 5B). The higher content of lipoxygenase in the cream phase of these emulsions treated at one pass could have affected the oxidation of CLA due to its unsaturation (Table 2).~~ In general, emulsions clearly protected t9c11-CLA and t10c12-CLA isomers from transformation and oxidation.

The release of CLA isomers could have been affected by the interfacial composition of the emulsions after the digest process. Anyway, it has been previously characterized that bile salts have an important role in the duodenal phase for the total displacement of

proteins from the interface even without the presence of phospholipids (Malaki Nik et al., 2011; Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). So that interfacial composition of the digestates was evaluated by SDS-PAGE and empty gels ~~in~~ were found. This indicated a complete hydrolysis of all soy and pea polypeptides (data not shown). The extent of lipolysis of the emulsions was also measured after digestion. Emulsions-stabilized with SPI (4%, w/v) at one and five passes of homogenization presented FFA release percentages of 23.3±0.7 and 22±1.5%, respectively. In parallel, emulsions-stabilized with PPI (4%, w/v) at one and five passes of homogenization showed 25.3 ± 0.3 and 24.8 ± 3.1, respectively. The extent of lipolysis of the oil (soybean oil and CLA) in these emulsions did not vary between treatments suggesting that the *in vitro* digestibility of the oil within the emulsions would not be affected by the type of protein present at the interface (SPI or PPI), and that the particle size of the emulsions did not affect the final lipolysis. This fact is in concordance with complete hydrolysis of all soy and pea polypeptides after digestion of the emulsions. Other authors reported >80% lipid hydrolysis values for emulsions containing 10% of soybean oil and 1,5% (w/v) SPI (Malaki Nik et al., 2011; Nik, Corredig, & Wright, 2011). The smaller FFA values obtained in this study compared to those found by other authors, can be explained by the differences in the composition of the *in vitro* gastric and intestinal fluids, and the high amount of protein used also had an impact on the release of free fatty acids, as residual protein at the interface may act as physical barrier at the lipid droplet surface during digestion, decreasing lipase activity and therefore catalysis of lipid substrate into free fatty acids. (Malaki Nik *et al.*, (2011) showed that the presence of bile salts in the emulsions led to disruption of the flocs formed in the gastric phase and a significant decrease in mean droplet diameter was observed by the end of the duodenal stage. However, as we used lowers~~maller~~

concentration of bile salts (0.4 mM) in the duodenal phase compared to these authors, ~~they~~which could have not been enough to disrupt flocs during digestion process, as particle size measurements showed in most of the emulsions after digestion process (Table 1). Also, larger particle size might be explained by less interfacial activity of mixed bile salts-phospholipids compared to bile salts alone, as previously suggested (Malaki Nik et al., 2011). Then, larger particle size of the emulsions in this study could have led to the reduction of the lipolysis rate. It is well understood that changes in composition of the emulsions can be used to modulate free fatty acid release in the gastrointestinal tract, and affect satiety and release of bioactives (Guri & Corredig, 2014; McClements & Li, 2010).

### *3.4 Bioaccessibility and bioavailability of CLA isomers*

The transport of CLA isomers after digestion and absorption was investigated using a human intestinal cell culture model, by quantifying CLA in the cells and basolateral compartments. Figure ~~6~~ 8 summarizes the concentration of CLA recovered in the cells and basolateral compartment after 4 h of incubation, showing the percentages of the bioaccessibility and bioavailability of CLA. These values were calculated dividing the concentration which remained in the compartments by the amount in the digestates of the emulsions (Eq. (3,4)).

All the treatments showed very similar CLA bioaccessibility results from the main CLA isomers (c9t11 and t10c12) in digestates of the emulsions loaded into the human intestinal cell culture model, except for the higher concentration recovered in the CLA-control digestates. The results clearly suggest that when present in the emulsion, there is a delayed absorption of the CLA in the cells with a lower recovery after 4 hours. In concordance with oxidation/isomerization analysis of CLA-control after digestion, there

was an extensive absorption and possibly in cell metabolism of CLA for control samples, where CLA was unentrapped.

After absorption, there were no significant differences in the amount of t9c11-CLA recovered in the basolateral fraction, between the treatments and the CLA-control ($P$>0.05). This may indicate that albeit there was a higher concentration of CLA in the cell for control, the CLA was also metabolized to a higher extent. Only less than 1% of each CLA isomer from digestates of emulsions loaded to the cell media was recovered in the basolateral phase. On the other hand, a higher concentration of t10c12 was found in the basolateral phase for control than for the emulsion treatments. In this case also, the amounts recovered were around 1.5%. There was no significant difference between the emulsion treatments.

Turning now to the experimental evidence on low lipid hydrolysis, CLA bioactive isomers solubilized in oil droplets exhibited minimal duodenal release, but instead remained protected from the gastric and duodenal environment, to later be released after absorption and transport through Caco-2 monolayers.

Recently, nanoemulsified CLA in an obesity rat model had greater antiobesity effect than in the non-emulsified CLA-treated group (Kim, Park, Kweon, & Han, 2013). However, chemical stability of different CLA isomers among non-emulsified CLA diet and nanoemulsified CLA (with lecithin) diet and its possible effect on metabolism was not assessed. In this respect, we have shown that the oil-in-water emulsion formulations protected the encapsulated CLA better than the corresponding CLA-control, and the longer residence times may also be beneficial for controlled delivery. In that manner, further work needs to be done to establish whether CLA chemical stability preservation with emulsifiers widely used in the food industry, as protein isolates, is correlated to health benefits to the human body.

## 4. Conclusions

This research demonstrated the importance of oil in water emulsions as delivery matrices for highly hydrophobic bioactive compounds. This work was undertaken to design different oil-in-water emulsions formulations to evaluate CLA delivery. All emulsions protected CLA from oxidation during storage, and after *in vitro* digestion, absorption and transport through Caco-2 monolayers compared to non-emulsified CLA control. Both SPI and PPI isolates were effective in stabilizing the emulsions encapsulating and delivering CLA. The emulsions showed a similar *in vitro* digestibility, and similar results of CLA absorption and transport through Caco-2 monolayers. ~~After absorption, there were no significant differences in the amount of t9c11-CLA recovered in the basolateral fraction, between the treatments and the CLA-control ($P$>0.05). This may indicate that albeit there was a higher concentration of CLA in the cell for control, the CLA was also metabolized to a higher extent.~~

Over the last few decades, CLA have been intensively investigated in numerous *in vivo* and *in vitro* studies ~~(Koba & Yanagita, 2013)~~. However, the bioactive main isomers of CLA are already available as food supplements in the market, and the risks and benefits associated with supplementation are currently under discussion. Auto-oxidation of CLA in the presence of molecular oxygen leads to ~~formation of toxic compounds as furan fatty acids leading to~~ isomerization of CLA into *cis,cis* and *trans,trans*-isomers through the Caco-2 cells ~~(Buhrke et al., 2012)~~. The results of this study indicate that isomers form during storage and digestion and further investigation is needed to evaluate the risks that these isomers may cause on the human body. Novel-technologies of encapsulation of CLA are necessary to protect the main biological CLA isomers ~~(Costa et al., 2015; Gao et al., 2014; Kim et al., 2013; Yao et al., 2013)~~ and oil-in-water

emulsions seem to be a suitable solution. -In fact, the emulsions presented in this manuscriptwork are suitable to produce a functional food-based product, such as skimmed milk, with CLA enriched emulsion containing 2% of fat to deliver 0.6% CLA. According to literature and food industry demands to label the CLA functional products with health claims, one milk glass would have 1.5 g CLA which is in the range required to obtain health benefits.

To the best of our knowledge Tthis work was the first attempt in which CLA was preserved into oil-in-water emulsions stabilized with vegetable protein isolates. The findings of this study have a number of important implications for future practice in the food industry for development of novel functional foods.

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