



Digestive fate of emulsions formulated with pea and lupin protein ingredients: Modulation of lipid digestibility and bioaccessibility by the interfacial composition?[☆]

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ABSTRACT

Pea and lupin protein ingredients are suitable sources to promote the transition towards more plant proteins in foods, as they display promising nutritional and emulsifying properties. Oil-in-water emulsions (10 wt% oil, 2.5 g proteins/L) were prepared with pea and lupin protein isolates and concentrates. The homogenization pressure was adapted to obtain droplets with an average diameter around 2.2 μm . The digestive fate of emulsions was examined using the INFOGEST *in vitro* static protocol to elucidate how the microstructure and interfacial composition influenced lipid digestibility and bioaccessibility. Lipolysis was evaluated through lipid class analysis via HPLC. During the oral phase, the microstructure of the emulsions prepared with protein isolates was altered, as the introduction of α -amylase led to the loss of the proteinaceous network bridging the droplets, without inducing coalescence. Comparable lipolysis extent was reached for all four emulsions at the end of the gastric phase (from 11 to 17 % mol/total mol), and after the intestinal phase (from 72 to 81 % mol/total mol). However, lipid bioaccessibility varied depending on the protein source, with approximately 85 wt% of bio-accessible lipids measured for pea protein-based emulsions, against 49 to 63 wt% for lupin ingredients-based ones. These results suggest a marked role of the components present in plant-based ingredients on the digestive fate of the emulsions. As food systems increasingly focus on plant-based innovations, understanding how the composition of these ingredients—particularly their often-overlooked endogenous components—affects their processing and nutritional properties can help the development of more sustainable and nutritious foods.

1. Introduction

In recent years, the use of plant-based protein ingredients in food emulsions has markedly increased, driven by incentives for sustainability, naturality and ethics in food products. This shift raises investigations regarding the nutritional consequences, as the structure of food emulsions, in particular regarding interfacial composition, tailors lipid digestibility and bioaccessibility (Mun, Decker and McClements, 2007; Singh, Ye and Horne, 2009; Couédelo et al., 2015; McClements, 2018).

Our previous research enlightened the inherent complexity of interfaces in emulsions made with pea and lupin protein isolates and concentrates (abbreviated PPI, PPC, LPI and LPC). An intrinsic

competition between their endogenous components, namely polar lipids, native proteins and protein aggregates, to adsorb at the oil–water interface in emulsions was highlighted (Keuleyan, 2024). Consequently, well-controlled emulsions in terms of droplet size (d_{32} around 2.2 μm) displayed different microstructure and interfacial compositions as a consequence of the competitive adsorption of endogenous surface-active phospholipids and proteins. The presence of protein aggregates in PPI and LPI-based emulsions induced extensive droplet–droplet bridging, leading to flocculation. In addition, in PPI-based emulsions, both phospholipids and proteins were found to co-exist at the interface. As a consequence, understanding how such different interfacial compositions and architectures could influence the digestion of dispersed lipids and their bioaccessibility would enhance the knowledge and

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implementation of such ingredients in food formulations, thus promoting the use of complex and moderately processed plant ingredients for targeted nutritional applications.

Lipid digestion is an interfacial phenomenon, as their low solubility in aqueous media requires the digestive system to convert them into dispersed assemblies to be bioaccessible and then bioavailable. Key chemical reactions occur at the surface of the dispersed droplets, where digestive enzymes simultaneously disentangle molecules stabilizing the droplets (i.e., emulsifiers), and perform their catalytic activity (Wilde and Chu, 2011). Triglycerides (TGs) constituting the oil phase are converted into different species, namely diglycerides (DGs), monoglycerides (MGs) and free fatty acids (FFAs), by gastric lipase (which is activated once adsorbed at the interface) and pancreatic lipase (which is activated once interacting with its cofactor named colipase). Phospholipids coating the interface are converted into lysophospholipids and FFAs by phospholipases in the intestine (Meynier and Genot, 2017). The relative proportions of TG hydrolysis into FFAs, MGs and DGs corresponds to the amount of “digestible lipids”. Being surface-active, these lipolysis products accumulate at the surface of oil droplets, hindering further enzyme adsorption. Nevertheless, in addition to helping pancreatic lipase and colipase proceeding further with lipolysis in the intestine, bile salts desorb these lipolysis products and incorporate them into phospholipid vesicles and mixed micelles. Such assemblies comprise MGs, FFAs, bile salts, cholesterol, phospholipids, and correspond to the bio-accessible fraction of lipids, more precisely defined as those “released from foods and present in digestive intestinal fluids in a form suitable for absorption” (Staggers et al., 1990; McClements, 2018). After diffusion through the mucus layer surrounding the intestinal brush border, lipophilic molecules such as FFAs and MGs are able to go through enterocytes to be further metabolized and to form chylomicrons: this fraction corresponds to bioavailable lipids.

When plant protein ingredients are used as emulsifiers, their inherent complexity exacerbates challenges in understanding the digestive fate of emulsified lipids. This complexity is first evidenced with the multiplicity of protein colloidal states (from native to aggregated) in such ingredients (Schmitt et al., 2021). When used as emulsifiers, the presence of protein aggregates increases surface coverage and thickness of interfacial layers. This could hamper the accessibility of digestive enzymes for interfacial adsorption, therefore reducing the efficiency of bile salts to adsorb at the oil–water interface (Qiu et al., 2015; Guo et al., 2017). In addition, plant protein ingredients are rich in non-proteinaceous compounds that might interfere with digestion processes, such as trypsin inhibitors or dietary fibres (Liu et al., 2021). An additional level of complexity is reached with the unexpected presence of phospholipids at the interface. In some dispersed systems comprising mixtures of phospholipids and proteins (mainly dairy-based), such as infant formulas or essential fatty acids delivery systems, phospholipids appeared to modulate (positively or negatively) lipid digestion of droplets coated with both emulsifiers (Wang et al., 2022; Yu et al., 2023). Some authors suggested that the high digestibility of lipids may be due to the ability of certain phospholipids to prevent oil droplet flocculation during digestion. This effect is driven by electrostatic repulsion forces from the polar heads of adsorbed phospholipids (according to the nature of phosphate group) and environmental conditions (salt concentration for instance) (Zhu et al., 2021). Moreover, it was suggested that phospholipids enhance the bioaccessibility of lipophilic molecules by co-forming mixed micelles (Verrijssen et al., 2015). In contrary, the high affinity of phospholipids for the interfacial region makes them less prone for interfacial displacement by bile salts, which may reduce TG hydrolysis (McClements, 2018). In some cases, a single factor can cause both lipid digestibility enhancement and lipid bio-accessibility reduction, as observed with calcium ions. In the former case, their action is explained by their capacity to bind and activate pancreatic lipase and by their ability to desorb FFAs from the oil droplets interface (Hu et al., 2010). In the second case, calcium and FFAs (saturated ones, in particular) form insoluble “calcium soaps”. These

structures are unable to be absorbed through enterocytes, inducing a reduction of lipid bioavailability (Mulet-Cabero and Wilde, 2023).

This brief literature overview highlights the complexity of lipid digestibility and bioaccessibility in plant protein-based emulsions, with many concomitant factors at play, including emulsion microstructure, interfacial properties, and potential presence of enhancers or inhibitors. However, only limited studies have addressed lipid digestibility and bioaccessibility in relation to the interfacial architecture generated with plant protein ingredients. The aim of this work was therefore to assess these parameters using an *in vitro* static model of digestion of emulsions that were highly controlled and characterized prior to digestion (Keuleyan, 2024). Because of the low protein content used (proteins were used for their emulsifying and interfacial role only), proteolysis could not be measured whereas lipolysis was the main targeted output. Results are discussed in light of emulsion microstructure, lipid digestibility, lipid bioaccessibility and lipolysis kinetics.

2. Materials and methods

2.1. Samples and reagents

2.1.1. Reagents

Sodium phosphate dibasic heptahydrate (Na_2HPO_4 , CAS number: 7782-85-6), sodium phosphate monobasic (NaH_2PO_4 , 13472-35-0), heptadecanoic acid (C17:0) (506-12-7), boron trifluoride methanol (375-57-9), α -amylase from porcine pancreas (9000-90-2), pancreatin from porcine pancreas (8049-47-6), bovine bile (8008-63-7), potassium chloride (7447-40-7), potassium phosphate monobasic (7778-77-0), magnesium chloride hexahydrate (7791-18-6), ammonium carbonate (506-87-6) and calcium chloride (10035-04-8) were from Sigma-Aldrich (St Louis, USA). Cyclohexane, *n*-hexane, chloroform, methanol and isopropanol were from Biosolve Chemicals (Dieuze, France). Sulfuric acid (95–98 %) was from ITW Reagents Panreac (Barcelona, Spain). Sodium chloride (NaCl , 7647-14-5) was from VWR International (Radnor, USA). Sodium hydroxide solution (NaOH 0.1N, 1310-73-2) for titration was from Merck (Darmstadt, Germany). Rabbit gastric extract (RGE) was from Lipolytech (Marseille, France). Rapeseed oil was purchased from a local supermarket.

2.1.2. Plant protein ingredients

Pea and lupin protein ingredients were kindly donated by suppliers. Pea protein isolate (PPI, ref. Nutralys S85F) was from Roquette (Lestrem, France), pea protein concentrate (PPC) was from Elementa (Saint-Nolff, France), lupin protein isolate (LPI) was from Prolupin GmbH (Grimmen, Germany) and lupin protein concentrate (LPC, ref. Fralucon) was from Inveja (Haute-Goulaine, France).

The composition of the powders was thoroughly characterized in previous work (Keuleyan et al., 2023), and is provided in Table 1. The additional determination of trypsin inhibitor activity (TIA) of the powders was performed using a recently published methodology (Locali-Pereira et al., 2024) based on (Page, Quillien and Duc, 2000; Liu, 2021). The results are provided in Supplementary Info 1.

Table 1

Proximate composition of the samples, expressed in g/100 g d.m. (adapted from Keuleyan et al., 2023). The protein content was calculated with nitrogen-to-protein conversion factors specific for each sample: N factors – PPI: 5.66; PPC: .557; LPI: .546; LPC: 5.,9.

g/100 g d.m.	PPI	PPC	LPI	LPC
Proteins	74.6 ± 0.5	47.9 ± 0.7	79.2 ± 0.8	48.0 ± 0.2
Lipids	11.7 ± 0.4	6.0 ± 0.3	3.8 ± 0.4	11 ± 0.4
Fibres	2.6 ± 0.4	15.3 ± 0.6	3.7 ± 0.2	14.9 ± 0.3
Starch	–	5.2 ± 0.1	–	–
Ashes	3.70 ± 0.02	5.91 ± 0.03	5.15 ± 0.13	3.38 ± 0.02

2.2. Emulsion preparation

Aqueous suspensions of the protein ingredients were prepared at 10 g proteins/L in phosphate buffer (10 mM, 90 mM NaCl, pH = 7.0). After 2 h hydration at room temperature under magnetic stirring, the suspensions were pre-treated by high-pressure homogenization (HPH) (Panda Plus 1000, GEA Niro Soavi, Italy) at 300 bars for 3 min (7 cycles). Then, 250 mL of oil-in-water (O/W) emulsions were prepared as previously described (Keuleyan, 2024), and as illustrated in Fig. 1 with 10 wt% rapeseed oil and 2.5 g/L of protein suspension, obtained by diluting the pre-treated aqueous suspension (10 g/L protein) in phosphate buffer. A coarse emulsion was made with a rotor–stator homogenizer (14,000 rpm; 3 min) (Silent Crusher M, Heidolph, Schwabach, Germany) prior to high-pressure homogenization for 3 min (4 cycles). The emulsions were standardized in droplet size (d_{32} centred around 2.2 μm), which required to adapt the pressure to apply according to the protein ingredient used. Therefore, PPI- and LPI-based emulsions were treated at 100 bars, PPC-based ones at 300 bars and LPC-based ones at 600 bars. Then, to ensure the microbial safety of the emulsions for the digestion studies and to inactivate trypsin inhibitors, a heat treatment was applied to the emulsions for 13 min at 90 °C in a water bath under magnetic stirring (Ter 2 Temperierbad, IKA, Germany). For each protein ingredient, three independent emulsions were prepared on the same day, and 2 bottles containing 110 mL of each emulsion were heat-treated: one for the gastric digestion, and the other for the gastro-intestinal digestion. They were all kept at + 4 °C until opening for the digestion assay, that were conducted randomly over two weeks (their physical stability was monitored beforehand, without modification of the particle size distribution over storage (Keuleyan, 2024)).

2.3. In vitro digestion protocol

In vitro static digestions were conducted according to the INFOGEST network protocol (Brodkorb et al., 2019). In a preliminary work, lipolytic activities (using tributyrin) of digestive enzymes were measured (Minekus et al., 2014; Brodkorb et al., 2019; Grundy et al., 2021). Pepsin activities (using haemoglobin) of RGE were measured by the suppliers. A pH-Stat (Metrohm, 905 Titrando) coupled with a dosing system (Metrohm, dosino 20 mL) was used to undertake the digestion assays. The software Tiamo 2.5 was set with the adequate sequence for this digestion assay. An aliquot of heat-treated emulsion (5 mL, pH = 7.0) was poured into the thermostated vessel (conical shape) at 37 °C under stirring using a propeller (speed 5). The sample of this emulsion prior digestion is designated as t_0 . Then, the oral phase was conducted for 2 min, by combining the emulsion with simulated salivary fluid (SSF, 1:1 v/v emulsion) and α -amylase (75 U/mL). A sample of 740 μL was taken to measure the particle size distribution of the emulsion and observe its microstructure after the oral phase (t_2). Then, simulated gastric fluid (SGF, 1:1 v/v digest) was added along with RGE to provide with 60 lipase U/mL of digestive medium (corresponding to 2,000 pepsin U/mL). The gastric phase was conducted for 2 h, after which the digestion was either stopped in the case of an oral-gastric digestion (by increasing the pH to 8.0 using NaOH (1 M)), or pursued in the case of an oral-gastric-intestinal digestion. In the former case, the digest was collected for further analysis (t_{120}) and then stored at –20 °C. In the second case, no digest was sampled at the end of the gastric phase, and the intestinal phase was conducted with bile extract (10 mM/mL, pH = 7.0) and pancreatin (lipase activity: 2,000 U/mL, pH = 7.0). Both were dispersed in simulated intestinal fluid (SIF), and added to reach a dilution of 1:1 (v/v) for this intestinal phase lasting 2 h. During this period, protonated products released in the digest were titrated continuously at pH = 7.0

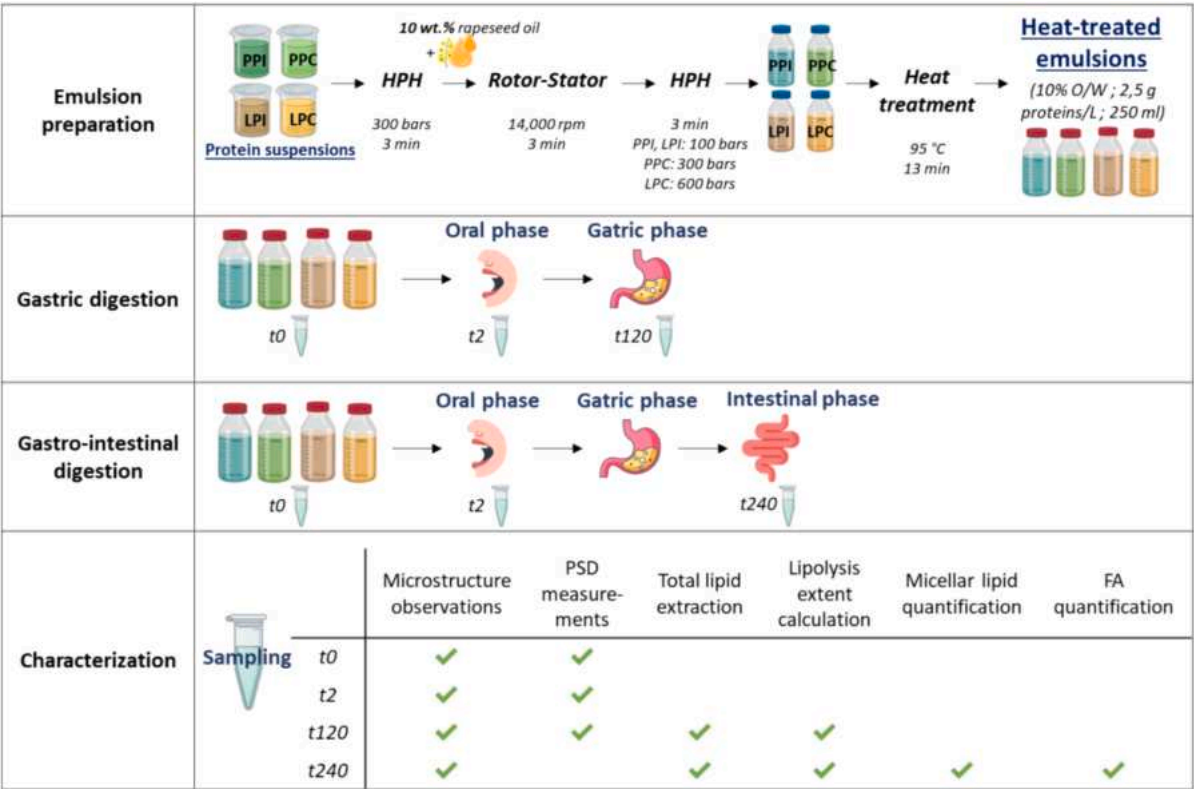


Fig. 1. Schematic view of the experimental set up. First row: emulsion preparation (10 wt% rapeseed oil, 2.5 g protein/L). Second row: gastric digestion, with sampling before digestion, of oral digest, and gastric digest. Third row: gastro-intestinal digestions, with sampling before digestion, of oral digest, and intestinal digest. Fourth row: characterizations of the digest samplings regarding microscopy observations, particle size distribution (PSD) measurements, total lipid and micellar lipid quantifications, lipolysis extent calculation and FA quantification. The indicated times (t_0 , t_2 , etc.) are in minutes.

with NaOH (0.1 M). At the end of the intestinal phase, the digestion was stopped by adding 5 μ L boronic acid (1 M, in methanol) for lipase inhibition, and 50 μ L Pefabloc (0.1 M in water) for protease inhibition. The digest was collected for characterization (t_{240}), and stored at -20°C .

Thereafter, the microstructure of the emulsion was assessed through digestion, as well as the fate of the dispersed lipids. Due to the low protein content in the emulsion formulations, the extent of proteolysis could not be measured (results were below the limit of detection with the o-phthalaldehyde (OPA) assay, results not shown).

2.4. Microstructure analysis of the digesta

2.4.1. Particle size analysis

The particle size distribution of emulsions was measured by static light scattering experiments using a LA-960 (Horiba Scientific, Jobin Yvon, France). The analysis is based on the Mie theory. Few droplets of samples (emulsions at t_0 , t_2 and t_{120}) were poured into the unit filled with ultrapure water under stirring. The samples were both analyzed as such, or after dilution in a SDS solution at 1 % (w/v) (240 μ L of sample in 3 mL of SDS solution). This allows for measuring individual droplets, whereas without SDS, the size of the assemblies of droplets (flocs), if any, is measured. The refractive indices were 1.330 for water and 1.473 for rapeseed oil. The average particle size (μm) was expressed as surface mean diameter (d_{32}). Three independent measurements were performed on each sample for each digestion time, and a representative particle size distribution curve is provided in the results section.

2.4.2. Microstructure observations

Heat-treated emulsions prior digestion and aliquots of digesta were observed by optical microscopy immediately after sampling. All the observations were performed at the same dilution (in phosphate buffer), i.e., the ultimate dilution at the end of the intestinal phase which corresponds to an 8-fold dilution of the starting emulsion. The samples were observed between slide and cover-glass with a Zeiss Axioscope2 (Göttigen, Germany), in differential interference contrast mode (DIC).

2.5. Lipolysis extent

2.5.1. Lipolysis kinetics

The lipolysis extent was determined using two methods. First, it was expressed as the percentage of FFAs released after the hydrolysis of TGs by pancreatic lipases, as titrated with NaOH (0.1 N). According to the action of the lipase/colipase complex, the full hydrolysis of one TG molecule generates two FFAs and one MG. Based on this, we can calculate the number of moles of NaOH required to neutralize the FFAs, and divide it by the amount of molecules of FFAs that would be formed if lipolysis were complete (Li and McClements, 2010). This ratio corresponds to the amount of FFAs released (%) (Equation (1)). It is important to note that the NaOH volume poured into the reactor was corrected by considering the beginning of the titration once pH 7.0 was reached, as previously explained (Okuro et al., 2023). Briefly, as titration begins when both bile salts and pancreatin are poured into the vessel, the initial pH at the beginning of the intestinal phase is systematically lower than 7.0, and therefore requires a fast addition of NaOH. This normalization of data by deducing this added volume allows to account only for the NaOH added for FFA titration. However, it also means that a small fraction of titrated FFAs during this lag-time is not considered.

$$\text{FFAs released}(\%) = \frac{V_{\text{NaOH}} * M_{\text{NaOH}} * MW_{\text{lipid}}}{2 * m_{\text{lipid}}} \quad (1)$$

V_{NaOH} is the total volume of NaOH added at the end of the intestinal phase (L), to which the volume of NaOH added to reach pH 7.0 at the beginning of the titration was deduced. M_{NaOH} is the molarity of the NaOH solution (mol/L), MW_{lipid} is the average molecular weight of one mol of triglyceride (834 g/mol for the used rapeseed oil, as determined

by GC analysis of rapeseed oil fatty acid methyl esters), and m_{lipid} is the initial mass of lipids (g) in the aliquot of the emulsion taken for the digestion assay.

The percentage of FFAs released is plotted against time along with the kinetics of added volume of NaOH. Digestion blanks (both gastric and intestinal, in triplicates) were performed with digestive enzymes and by replacing 5 mL emulsion with 5 mL phosphate buffer. The volume of NaOH added during these blank experiments was subtracted from the calculation of % FFAs released with the emulsions. However, the extent of FFAs released calculated is not used to express the final lipolysis degree, as it was previously shown to be highly underestimated using this methodology (Okuro et al., 2023), which we confirmed here (Supplementary Info 2).

2.5.2. Lipolysis kinetic fitting

Raw data extracted from the titration of protonated free fatty acids by NaOH can be further explored by fitting them with a mathematical model, allowing to deepen some aspects of the time-dependence mechanisms of lipolysis. For this fitting, corrected raw data from pH 7.0 were used. Experimental data were fitted with several mathematical models, and the most appropriate one (based on non-linear regression values) was a first order kinetic model (Equation (2)), which was applied as in a previous work (Okuro et al., 2023). According to this model, digestion kinetics display two rate constants, k_1 and k_2 , witnessing that lipolysis is controlled by two distinct components. This model was chosen after several tests of fittings (data available upon request), and appeared to be the most relevant one.

$$\text{FFA}(t) = \text{FFA}_{\text{final}} - \text{FFA}_{\text{final}} * [f e^{-k_1 t} + (1 - f) e^{-k_2 t}] \quad (2)$$

$\text{FFA}(t)$ corresponds to the concentration of FFAs at time t ; $\text{FFA}_{\text{final}}$ corresponds to the concentration of FFAs in the digest at the end of the intestinal phase, and was restrained to the maximum value of 100 %, and f is the fraction associated with k_1 . The data analysis software Kaleidagraph was employed for this modelling (Synergy Software, Reading, USA).

2.5.3. Lipolysis degree

The lipolysis degree (LD, % mol/mol total) was calculated at the end of both gastric (t_{120}) and intestinal phases (t_{240}) from the quantification of lipid classes in the digesta after separation by ultra high-performance liquid chromatography (U-HPLC, Ultimate 3000 RSLC, Dionex, France). To do so, lipids from the digesta must first be extracted, which was done by mixing aliquots of digesta (1 mL, sampled directly after the digestion assay) with 10 mL hexane and isopropanol mixture (3:2, v/v), 50 μ L sulfuric acid (2.5 M) and 200 μ L NaCl solution (150 mM) (Hara and Radin, 1978; Helbig et al., 2012). The tubes were shaken for 1 min, after which they were centrifuged at 1,800 x g for 5 min at 20°C (Centrifuge 5810-R, Eppendorf, Germany). The upper hexane phase was collected with a glass pipette in a pre-tared glass tube. Then, 6 mL of pure hexane were added to the initial mixture to wash potential remaining lipids, and the procedure of mixing and centrifugation was repeated in the same conditions. Both collected hexane phases were pooled, and the solvent was let for evaporation under nitrogen (N-Evap 111, Organomation, USA) for total evaporation of the solvent. After that, the tubes were weighed, and lipid contents of the digesta were determined by weighing. Lipids were recovered in chloroform and were expressed in mg/mL digest. Lipid extracts were stored at -80°C until lipid classes analysis by HPLC.

For the latter, a calibration curve was prepared between 0.4 μg and 7.5 μg for lipid standards comprising triglycerides (TGs), diglycerides (DGs), monoglycerides (MGs) and free fatty acids (FFAs) (details regarding the standards used are provided in Supplementary Info 3). The HPLC is equipped with an evaporative light scattering detector (ELSD, Sedex 85) and an analytical column packed with a silica normal-phase (Uptisphere CS Evolution SI: 150 mm x 4.6 mm, 2.6 μm , Interchim,

Montluçon, France). A linear gradient of chloroform (eluent A) and a mix of CH₃OH/CHCl₃/NH₄OH (460/5/35; v/v/v) (eluent B) was set for the chromatographic separation of lipid classes (t_0 : 0 % B, $t_{8 \text{ min}}$: 50 % B, $t_{12 \text{ min}}$: 100 % B, and isocratic conditions with 100 % B for 3 min). All lipid classes were quantified, and the LD was expressed using the following equation (Couëdelo et al., 2015) (Equation (3):

$$LD(\% \text{mol/mol total}) = \frac{FFAs}{3TGs + 2DGs + MGs + FFAs} * 100 \quad (3)$$

In this equation, FFAs, TGs, DGs and MGs correspond to the concentrations of recovered corresponding lipids in the digesta as quantified by HPLC ($\mu\text{mol/mL}$).

2.6. Bioaccessible lipid fraction

The fraction of bioaccessible lipids in the digest was assessed at the end of the intestinal phase (t_{240}) as the ratio of fatty acids quantified in the micellar phase to the total fatty acids quantified in the intestinal digest. The procedure to measure fatty acids from one or the other phase is slightly different, and are described in the present section. In both cases, it is based on a methylation of the lipids, either directly in the micellar (aqueous phase) (Berton, Genot and Ropers, 2011; Corstens et al., 2018), or after lipid extraction as described by (Morrison and Smith, 1964).

2.6.1. Micellar phase

To recover the micellar phase containing lipolysis products, a centrifugation of 1.5 mL of intestinal digesta at 21,000 x g for 45 min (4 °C) was conducted in duplicates (Sigma 4 K15, Thermofisher). Then, 200 μL of aqueous supernatant phase were recovered by cautiously crossing the top oil phase with a syringe equipped with a thin needle. An internal standard of heptadecanoic acid (C17:0; 50 μL at 2 mg/mL in hexane) was added, along with 2 mL methanol and 400 μL of pure sulfuric acid (to be added with caution for the latter). Then the tubes were shaken and let in a dry bath at 100 °C for 1 h (Fisher Bioblock Scientific, Ilkirch, France). After cooling, 1 mL ultrapure water and 2 mL cyclohexane were added, and the tubes were shaken. When necessary, the tubes were centrifuged to enhance phase separation (1,800 x g, 5 min, 20 °C). The upper phases were recovered for fatty acid identification and quantification by gas chromatography (GC) (GC Clarus 690, Perkin Elmer).

2.6.2. Lipid extracts

The fatty acid content and composition of total digesta were assessed on lipid extracts from intestinal phase. The appropriate volume of extract to reach 1 mg of lipids was prepared in glass tubes, along with an internal standard of C17:0 (50 μL of a solution at 2 mg/mL). Then the tubes were left under a nitrogen flow until total removal of solvent. After that, 1 mL of toluene and 1 mL of boron trifluoride-methanol solution were added. The tubes were shaken, and let in a dry bath for 45 min at 100 °C. After cooling, 1 mL of cyclohexane with 0.5 mL of ultrapure water were added. The tubes were mixed, and the upper phase was collected for GC analysis. If phase separation was not clear enough, a centrifugation step was performed in the same condition as described above. The percentage of bioaccessible lipids was calculated using Equation (4), where FA_{micellar} is the concentration of FAs from micellar phase (mg/mL digest) and FA_{total} is the concentration of FAs from total digest (mg/mL digest).

$$\text{Bioaccessible lipids (wt. \%)} = \frac{FA_{\text{micellar}}}{FA_{\text{total}}} * 100 \quad (4)$$

2.7. Statistical analysis

Statistical analyses were conducted using XLSTAT software (Version 2024; Addinsoft, Paris, France), with a variance analysis (ANOVA) and a

Tukey post-hoc test. Significant differences were obtained when p -values were below 0.01. Homogenous groups are pointed out using small letters on the graphs. Both the plant source (pea or lupin) and type of ingredient (isolate or concentrate) were used as factors for the variables of FFA_{max} , k_1 , k_2 , m_2 , gastric lipolysis, intestinal lipolysis, and amount of FAs from total or micellar phases.

3. Results and discussion

3.1. Microstructure analysis

The particle size distributions of the emulsions were measured either without SDS, revealing the size of the flocs, or after dilution in SDS solution (1 % w/v), therefore providing with the size of the individual droplets (Fig. 2) (Beaumont and Marze, 2024). As previously mentioned, emulsion preparation parameters had been optimized to obtain a d_{32} around 2.2 μm (droplet size with SDS). The particle size measurement after the heat treatment indicates a slight occurrence of coalescence in the emulsion stabilized with LPC (d_{32} around 3.4 μm). During oral digestion, the individual droplet size remains relatively constant for all samples, ranging between 2 and 3 μm . At the end of the gastric phase, a limited degree of coalescence was observed, with a maximum d_{32} of 10.3 ± 3.1 for the droplet size of LPI. The relative increase in variability observed is attributed to the three independent digestion assays performed).

Without SDS, the size of the droplet flocs was evaluated: when formulated with protein isolates (PPI, LPI), the droplet flocs were about 20 μm large, whereas for the emulsions prepared with protein concentrates (PPC, LPC), the flocs were four times smaller (5 μm). The heat treatment did not modify these particle size distributions, and did not generate additional flocculation. This point slightly differs from some descriptions in the literature, according to which heat treatment either generates thermal destabilization of emulsions because of extensive aggregation, for instance with PPC (Devaki and Ghosh, 2024), or improves the physical stability of emulsions, for instance with soy protein isolate (Q. Li et al., 2020). This most likely depends on the processing history of the protein ingredient, and in particular of the heat treatments possibly applied, which is usual especially for wet-fractionated isolates. For instance, previous studies have shown that heat-treated proteins before emulsification led to emulsions resistant to thermal destabilization (Devaki and Ghosh, 2024). In addition, the protein concentration (and thus the protein ingredient concentration) was low in the present work compared the aforementioned literature examples, which could make the emulsions less susceptible to flocculation.

An interesting phenomenon is the evolution of the particle size during the oral phase. Within only two minutes, i.e., stirring the emulsion with simulated salivary fluid (SSF) and amylase, the particle size distribution of the individual droplets remained unchanged, however, important modifications were observed in the size of the flocs of both protein isolate-stabilized emulsions: the floc size decreased from around 20 μm down to 9 μm . Blank oral phases, either without SSF but with amylase or with SSF but without amylase, revealed that floc disruption occurred due to the presence of the enzyme, regardless of the addition of SSF (data not shown). This result suggests an interaction between amylase and the interfacial compounds of the emulsions stabilized with PPI and LPI, which partly suppresses the bridging of droplets by protein aggregates. This result was confirmed by optical microscopy observations (Fig. 3).

This observation highlights the importance of the oral phase, which is often overlooked in experimental setups due to the short residence time and typically low starch content in model emulsions (as is the case here, with 32 mg of starch/100 g of PPC-based-emulsion, Supplementary Info 4). Physical destabilization stemming from microstructure modifications of the emulsion in this oral compartment was previously described according to various mechanisms: salt-induced aggregation, depletion flocculation, bridging flocculation or coalescence.

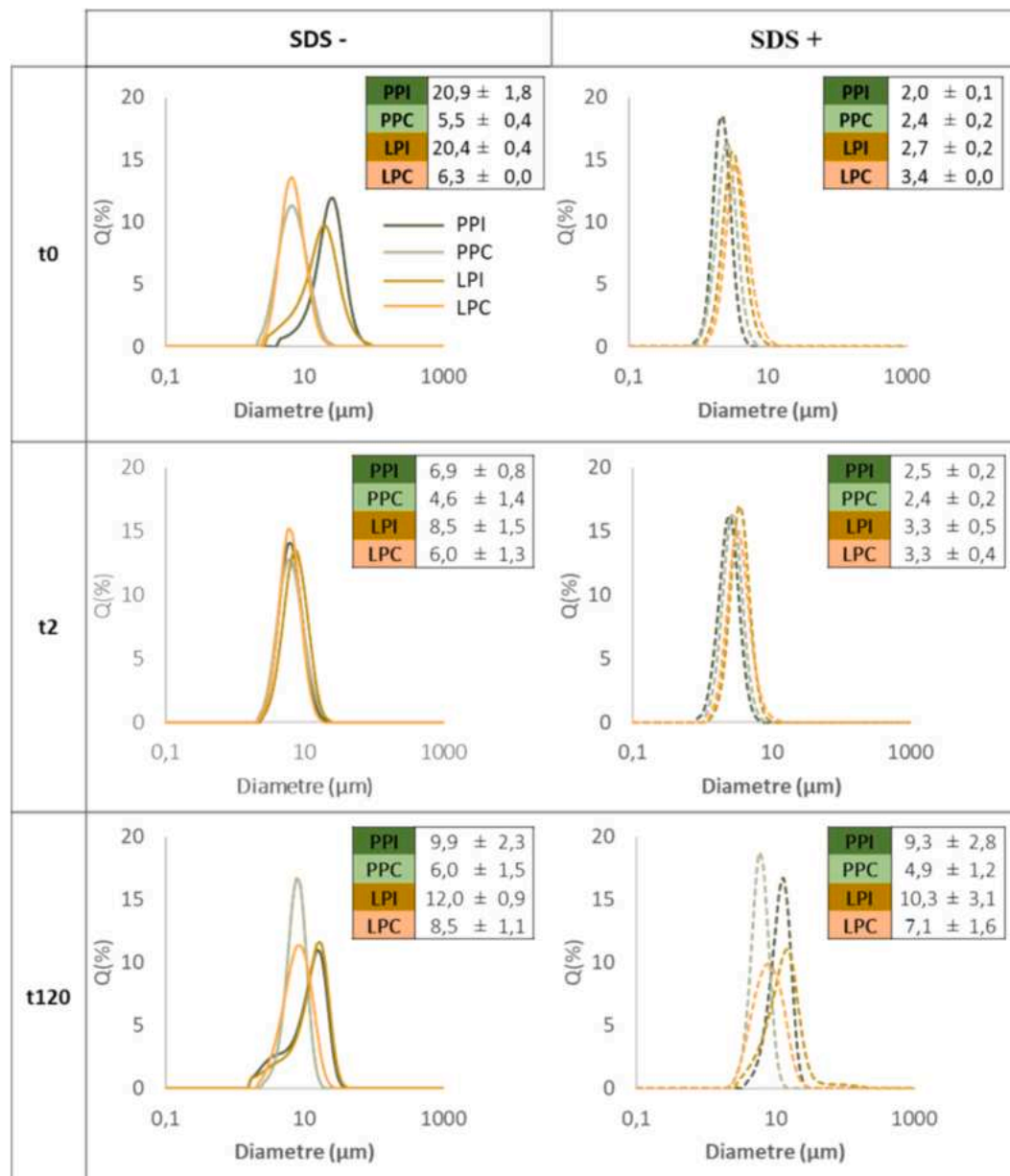


Fig. 2. Particle size distributions measured on heat-treated emulsions prior to digestion (t_0), after the oral phase (t_2), and after the gastric phase (t_{120}), with (left) or without (right) dilution in SDS solution (1 %). Representative curves out of three measurements on independent digestions performed on independent emulsions are shown. The insert tables give the mean $d_{32} \pm$ standard deviations of three independent replicates (μm). The times (t_0 , t_2 , t_{120}) are given in minutes.

Nevertheless, the opposite mechanism of physical destabilization (floculation disruption), as observed here, had not been exemplified before, to the best of our knowledge (Sarkar and Singh, 2012). As discussed in this last review, the physical destabilization of emulsions could impact both subsequent steps of lipid digestion by altering the total interfacial area and thus the accessibility of lipases to the interface, and the oral sensory sensations of food products by modifying the interfacial microstructure, affecting the tribology of food emulsions (Dresselhuys et al., 2008; Sarkar and Singh, 2012).

At the end of the gastric phase (t_{120}), the size of the individual droplets revealed that coalescence occurred to a certain extent. Without SDS, for PPI- and LPI-based emulsions, the signal corresponding to the small droplet size population (below $10 \mu\text{m}$) became slightly more prominent (Fig. 2). This could be due to lipolysis already starting during the gastric phase. At the end of intestinal digestion, some lipid droplets are still visible (Fig. 3), notably with some large ones. The remaining presence of lipid droplets at the end of this phase suggests that lipolysis

was not fully completed, which is further investigated and discussed in the next section.

3.2. Lipolysis extent

3.2.1. Lipolysis kinetics and fittings

Lipolysis kinetics analysis provides valuable insights into the time-dependent mechanisms of lipolysis (Fig. 4). As mentioned earlier, the ultimate percentage of FFAs released is underestimated (Okuro et al., 2023), therefore, these final values will not be discussed. For all emulsions, at the beginning of the intestinal phase, a fast increase in FFAs released in the medium was observed, followed by a more gradual rise after around 80 min (Fig. 4A). The emulsion made with PPC appears to be digested slightly differently compared to the other three samples, which display very similar kinetics.

The fitting of these experimental data with a mathematical model, as in Fig. 4B, provides quantitative information about the kinetic process of

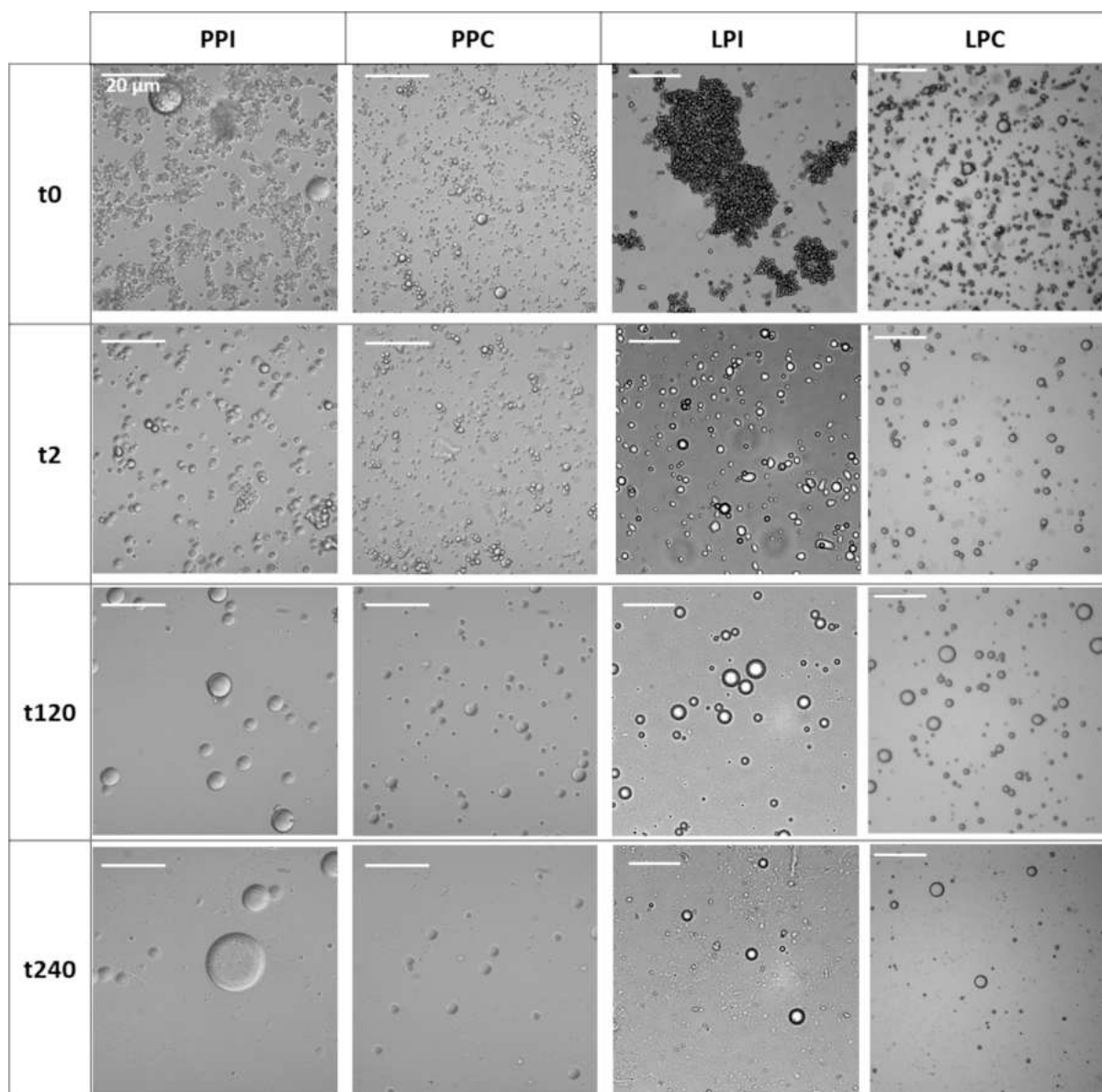


Fig. 3. Optical microscopy images between slides and cover glass of the emulsions prior to digestion (t_0), after the oral phase (t_2), the gastric phase (t_{120}), and the intestinal phase (t_{240}). All the emulsions were observed by differential interference contrast (DIC) microscopy at the same dilution (8-fold in phosphate buffer compared to the starting emulsion with 10 wt% oil). The scale bar is 20 μm . The times (t_0 , t_2 , t_{120}) are given in minutes.

digestion (Verkempinck et al., 2019). Based on the regression values ($R^2 > 0.99$), this model depicts the experimental values very well, much better than a first order kinetic equation with only one rate constant (Supplementary Info 5). The PPC-stabilized emulsion stands apart due to its significantly higher k_1 (i.e., the 'slow' rate constant) compared to the other samples, and lower m_2 value (i.e., the highest fraction of the 'fast' component, compared to the 'slow' one) (Table 2). Despite a slightly lower k_2 (i.e., the 'fast' rate constant) for the PPC-based system, these kinetic features are overall associated with a faster initiation of lipolysis compared to the other samples. Conversely, the lowest FFA_{max} value ($45.7 \pm 1.2\%$) was found for the PPC-based system, against 47.6 to 51.9 % for the other samples. This outcome might be related to the interfacial microstructure of this emulsion, which would enable lipases to reach their substrate quickly in the initial stages of lipid digestion, with a mitigation of this effect later on. The exact underlying mechanisms would deserve more attention, and for instance multi-response modelling could be a valuable tool for further understanding (Infantes-Garcia et al., 2021).

3.2.2. Lipolysis degree

The relative proportions of the different lipid classes (mg/mL digest) are provided alongside final lipolysis values from gastric (A) and intestinal compartments (B) in Fig. 5.

At the end of the gastric phase, the lipolysis degree was overall in the same range (between 11 and 17 wt%) for all four emulsions, though some significant differences were observed. These values are consistent with known maximum ranges of lipolysis previously reported in literature (between 10 and 25 wt%, and up to 30 wt%). This limited extent of lipolysis is associated with the accumulation of lipolysis products (DGs and FFAs) at the surface of oil droplets, preventing further adsorption of gastric lipase (Carey, Small and Bliss, 1983; Meynier and Genot, 2017). As gastric lipase specifically hydrolyses the *sn*-3 bond of TGs, only DGs and FFAs were measured as lipolysis products at the end of this phase, in equivalent ranges. Gastric lipolysis is paramount in the overall physiology of lipid digestion (Golding et al., 2011; Meynier and Genot, 2017), by its action on the disruption of droplets enhancing their emulsification, by triggering the activity of pancreatic lipase (Gargouri et al.,

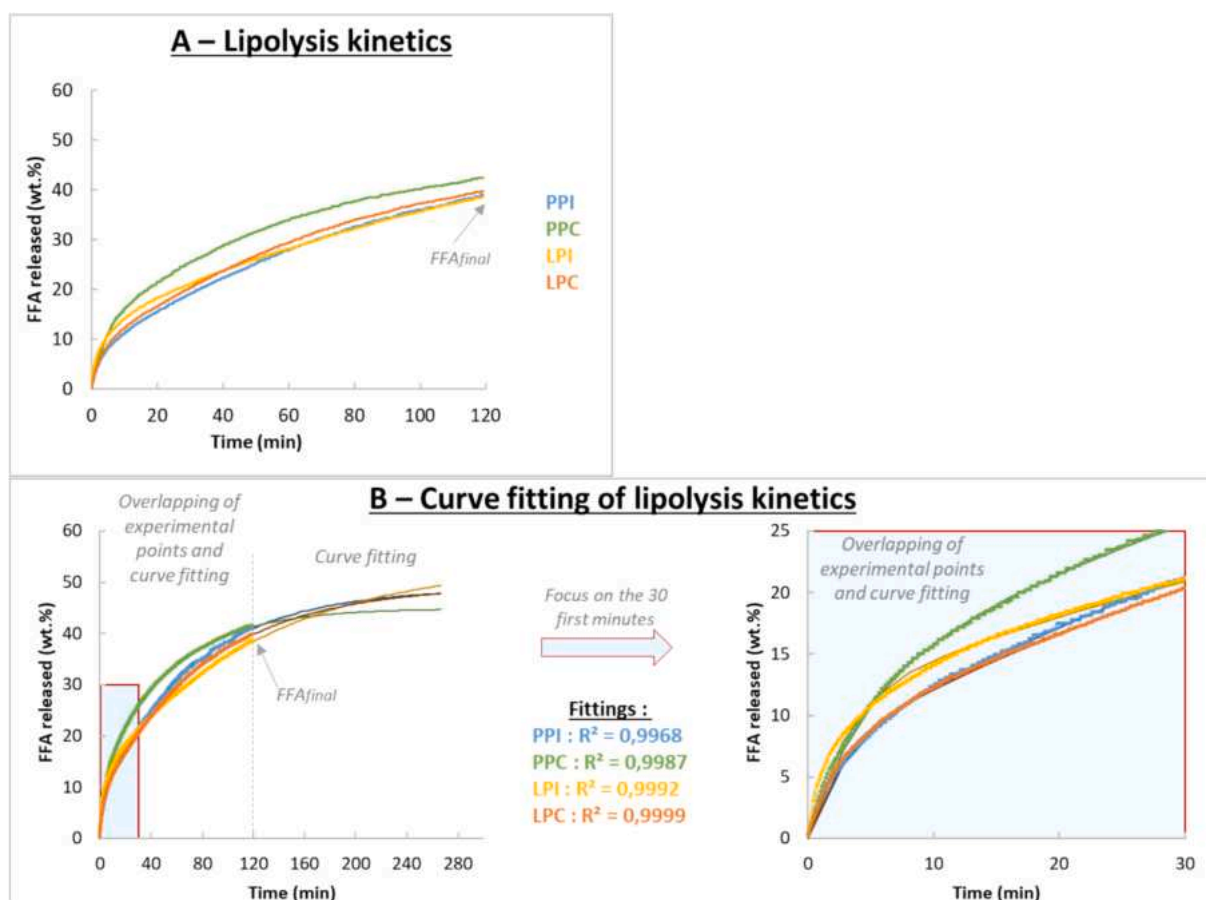


Fig. 4. (A) Percentage of FFAs (%) released through time (min) as quantified with the titration by pH-Stat. (B) Curve fitting of lipolysis kinetics. The FFAs released data were fitted with a mathematical model. Left: experimental values graph (round-shaped and light colour) along with fitted values (thin line and dark colour) for 270 min. Their tight overlap hinders the visibility of fitted values. Right: same graph, but zoomed in until 30 min to enhance the visibility of the first points. Representative curves are provided out of three experimental data sets.

Table 2

Experimental data of FFA_{final} (%) and estimated values of FFA_{max} (%), k_1 (s⁻¹), k_2 (s⁻¹) and m_2 from the independent triplicates of fitting are presented as means \pm standard deviations. ANOVA results with post hoc test Tukey are indicated with small letters (p-values < 0.0001). NS: not significantly different.

Sample	FFA max (%)	k1 (s-1)	K2 (s-1)	m2
PPI	51.9 \pm 3.5 NS	2.1E-04 \pm 2.7E-05 b	5.8E-03 \pm 8.9E-04 ab	0.85 \pm 0.02 ab
PPC	45.7 \pm 1.2 NS	3.0E-04 \pm 2.2E-05 a	4.6E-03 \pm 5.4E-04 b	0.77 \pm 0.03 c
LPI	51.8 \pm 5 NS	1.6E-04 \pm 2.7E-05 b	6.1E-03 \pm 7.7E-04 ab	0.81 \pm 0.02 bc
LPC	47.6 \pm 2.5 NS	2.2E-04 \pm 2.6E-05 b	9.7E-03 \pm 2.7E-03 a	0.88 \pm 0.03 a

1986), stimulating hormone secretions (cholecystokinin) regulating gastric emptying, and by regulating the activity of the colipase/pancreatic lipase activity.

At the end of the intestinal phase, the lipolysis degree ranged between 72 and 81 wt% for all emulsions, without any significant difference between the samples. The main lipid class recovered was FFAs (around 40 mg/mL digest), whereas TGs, DGs and MGs were below 5 mg/mL. Therefore, in the conditions applied in the present work (i.e., all emulsions displaying an equivalent total interfacial area before digestion), no effect of the emulsifier type was observed on the final lipolysis degree. This is worth noticing, since the selected ingredients were complex and contrasted in composition, and led to different interfacial

compositions. For instance, we previously showed the presence of substantial amounts of endogenous phospholipids at the droplet surface in PPI-stabilized emulsions (which was not detected with the other tested ingredients) (Keuleyan, 2024).

The comparison of our results with existing data from literature is challenging, because of the wide variety of emulsion formulation parameters, initial microstructure features (in particular individual droplet size), and characterization techniques of lipolysis. Multiple studies have shown how lipid digestibility can be modulated by food-grade emulsifiers of different nature such as proteins, polar lipids, surfactants (natural, synthetic), or polysaccharides (Hur, Decker and McClements, 2009; Nik, Wright and Corredig, 2011; Tan et al., 2020). Moreover, mixtures of emulsifiers, for instance of polysaccharides and proteins, were suggested to modulate lipolysis as a consequence of competitive adsorption mechanisms (Bellesi, Ruiz-henestrosa and Pilosof, 2020). We suspect that the emulsion conditions used in the present study, albeit generated by a competitive mechanism of adsorption between proteins and phospholipids, do not affect total lipolysis because proteins are prevailing at the interface. Besides, the use of two different plant protein sources (pea and lupin) does not result in any difference in the total lipolysis. Similar observations were obtained by Gumus *et al.*, who analyzed lipid digestibility of emulsions made with pea, faba bean, lentil and whey proteins (2 % protein, pH 7.0, 2 % oil and d_{32} around 0.4 to 0.5 μ m). At the end of intestinal digestion, released FFA percentages were similar between emulsifiers (around 100 wt%, when calculated with the titration methodology of pH-Stat), and no effect of the protein ingredient was noticed (Gumus, Decker and McClements, 2017). Similar lipolysis degrees (around 90 wt%) were measured for pea protein-

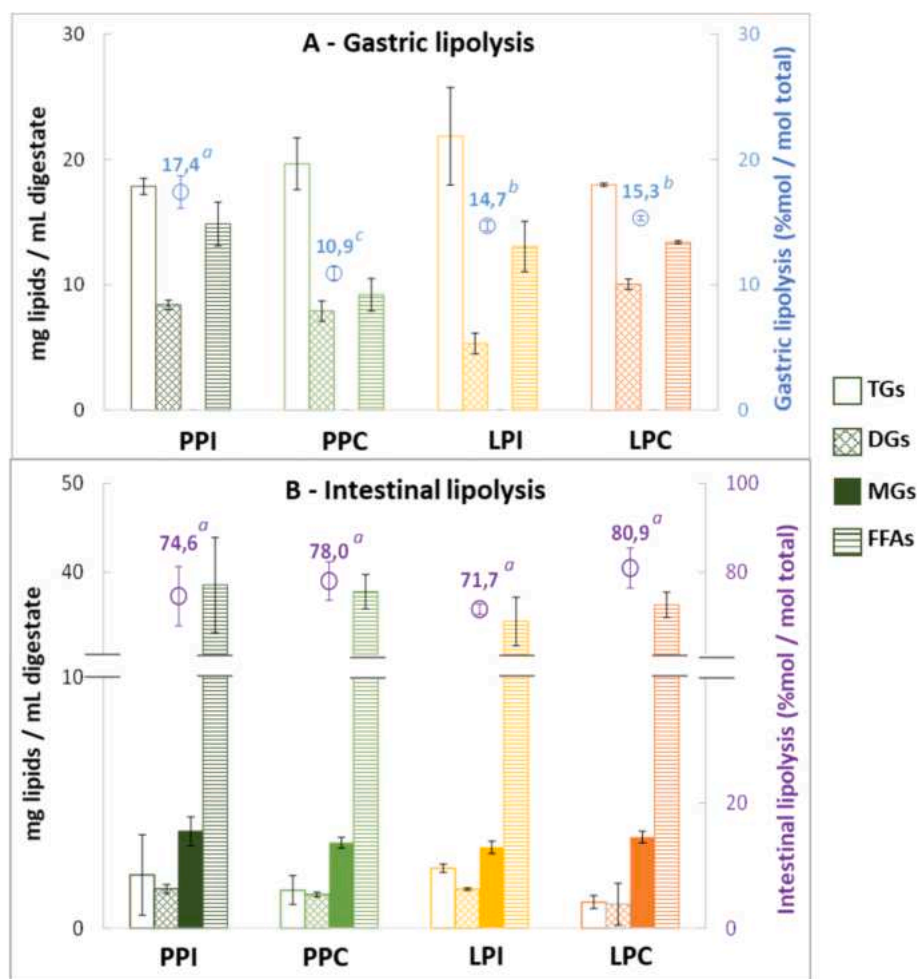


Fig. 5. Lipolysis degree (expressed in % mol/mol total of digest, represented by empty circles) and lipolysis product quantification (expressed in mg lipids/mL of digest, represented by bars) (A) at the end of the gastric digestion and (B) at the end of the intestinal digestion. TGs: triglycerides; DGs: diglycerides (DGs-1,2 and DGs-1,3 were pooled); MGs: monoacylglycerols; FFAs: free fatty acids. Means of three independent digestions are provided, with standard deviations. Significant differences between either gastric or intestinal lipolysis are indicated with different small letters (p-values < 0.0001).

stabilized emulsions (5 wt% oil, 1 wt% protein, pH 7.0, d_{32} around 0.5 μm) based on the titration method with pH-Stat (R. Li et al., 2020). However, the corrections applied to calculate the titration values are not explicitly explained, and such high digestibility degrees obtained with pH-Stat titration should be considered carefully. A previous study from our group drew attention to significant underestimation of the lipolysis degree using pH-Stat compared to HPLC, because (i) of a lack of knowledge of the nature of the lipolysis products (lipid classes), or (ii) the chemical environment of the intestinal phase affects the apparent pK_a of FFAs, or (iii) titration does not allow for distinguishing the possible contribution of proteolysis products which are simultaneously titrated with lipolysis products (Okuro et al., 2023).

3.3. Lipid bioaccessibility

The results of FA quantification in one or the other phases are provided in Fig. 6. No differences could be measured in the quantity of FAs from total digest according to the samples, which was expected since the total lipid content is supposed to be the same for all samples, for a given digestion phase. However, significant differences were obtained regarding the amount of FAs quantified in the micellar phase, in particular for lupin-based ingredients. Significantly less FAs in the micellar phase were measured for LPI and LPC, corresponding to a bioaccessibility of 48 and 63 wt%, respectively, against 84 to 86 wt% for PPI and PPC, respectively. This reduced lipid micellization extent for

lupin-based emulsions is worth noticing, since the overall lipolysis degree was, conversely, similar for all four emulsions (Fig. 5). This outcome suggests the occurrence of a phenomenon specific to lupin protein ingredients, hindering partly the solubilization of lipolysis products into mixed micelles.

Several hypotheses can explain this phenomenon. First, lupin protein isolates have been mentioned as cholesterol-lowering agents, due to the high capacity of lupin proteins and peptides to form complexes with bile salts (Yoshie-Stark and Wäsche, 2004; Radtke et al., 2014). This was suggested to hinder bile salts' capacity to desorb lipolysis products from the oil droplet surface, and their incorporation into mixed micelles. Lentil protein ingredients and hydrolysates were reported to largely display this effect too, and it was also mentioned for other plant proteins such as soy, pinto bean or black bean proteins, for instance (Barbana, Boucher and Boye, 2011; Naumann et al., 2020). Herrera et al. recently reported that the less aggregated and denaturated pea proteins were, the more they would bind to bile salts. Such results are not observed with the present results, as no effect of ingredient processing could be distinguished. More work would be required to deepen the interactions between bile salts and proteolysis products from multiple plant sources. Non-proteinaceous components from lupin-based ingredients, such as polysaccharides, could also interact with bile salts, as reviewed by Naumann et al. (2020); however this phenomenon is probably not the predominant explanation for our results, since the LPC-based emulsion, which contains more fibres than the LPI-based emulsion, displayed

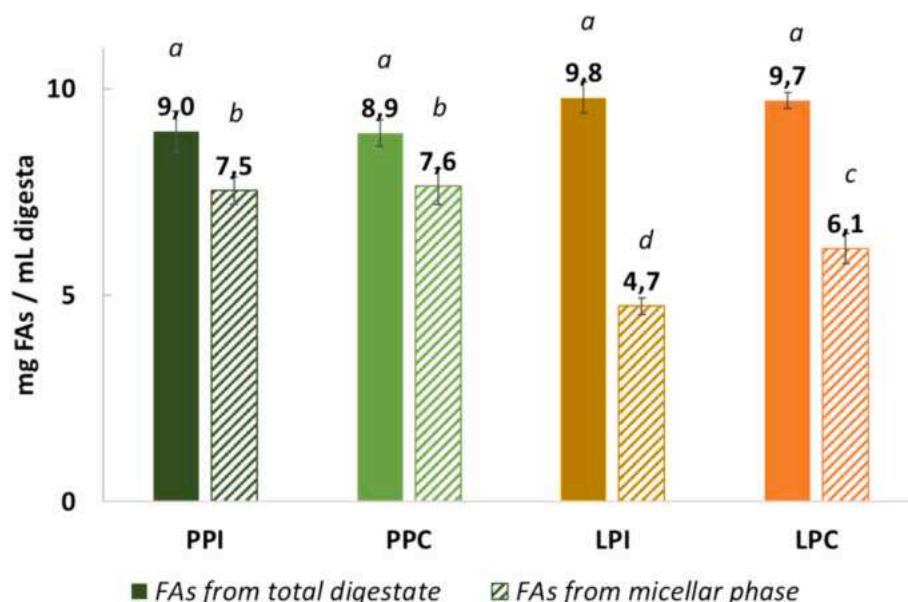


Fig. 6. Fatty acid contents (mg FAs/mL digest) at the end of intestinal digestions in total digest (full bars) and in the micellar phase (striped bars). The results are expressed as means (numerical values on top of the bars) \pm S.D. of three independent digestions. Small letters on top of the values indicate significant differences among the groups (p -value < 0.0001).

slightly yet significantly higher lipid bioaccessibility (Supplementary Info 4). Bile acids can also be bound by minor compounds such as polyphenols (Naumann et al., 2020), which also raises attention to minor co-passengers that could be extracted along with proteins during plant protein ingredient fractionation.

4. Conclusion

This work investigated the digestive fate of emulsions formulated with plant protein ingredients (pea and lupin) of different purities (concentrates, isolates), having a similar droplet size but contrasted compositions and interface structures. Notable modifications in the structure of the emulsions were highlighted, already from the oral phase. For instance, droplet flocs were partly disrupted during the oral phase as a result of amylase interaction with the interfacial components, independently of the presence of residual starch in the protein ingredients. The PPC-stabilized emulsion displayed a slightly different time-dependent digestion of lipids, especially with a faster initiation, whereas the final extent of lipolysis was similar for all samples. However, a significant decrease of lipid bioaccessibility (i.e., the proportion of lipid digestion products incorporated in mixed micelles upon the intestinal phase) was highlighted for the emulsions formulated with the lupin-based ingredients compared to pea-based ingredients. This outcome suggests the inhibition of lipolysis products' micellization when lupin protein ingredients are used. Further studies would be needed to determine which compounds present in lupin-based ingredients could be involved. Although the *in vitro* static digestion protocol employed in this study was relevant for screening the lipid digestive fate, moving to more advanced models, such as semi-dynamic or dynamic systems, would provide deeper insights into the mechanisms at play, in particular in relation with pH variations during the gastric step. This work supports the critical importance of investigating multiple parameters (lipolysis, bioaccessibility) to probe a global complex phenomenon, such as lipid digestion in dispersed systems based on compositionally complex ingredients.

CRedit authorship contribution statement

Eléna Keuleyan: Writing – review & editing, Writing – original

draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sophie Laurent:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alain Riaublanc:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Claire Berton-Carabin:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Anne Meynier:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2025.115749>.

Data availability

Data will be made available on request.

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