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In vitro gastrointestinal digestion of marine oil emulsions and liposomal solutions: fate of LC-PUFAs upon lipolysis

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The bioaccessibility and bioavailability of dietary fatty acids depend on the lipid to which they are esterified, the organisation of theses lipids in water and their recognition by lipolytic enzymes. In this work, we studied the release of marine long-chain polyunsaturated fatty acids (LC-PUFA), depending on their presentation either in the form of phospholipids (PL) or triacylglycerol (TAG). Two formulations based on marine PL or TAG extracted from salmon heads (*Salmo salar*) were prepared. Lipolysis was first tested *in vitro* by using individual gastrointestinal lipases and phospholipases to identify the enzymes involved in the digestion. Second, the lipolysis of the prepared formulations by a combination of enzymes was tested under *in vitro* conditions mimicking the physiological conditions found in the GI tract, both in the stomach and in the upper small intestine, in order to evaluate digestibility of TAG and LC-PUFA-containing liposomes. The *in vitro* results showed that TAG emulsion was hydrolyzed by porcine pancreatic extracts (PPE) and pure pancreatic lipase (PPL) with its cofactor, colipase, and to a lesser extent by pancreatic-lipase-related protein 2 (PLRP2) and a gastric extract (RGE) containing gastric lipase while no hydrolysis was observed with purified pancreatic phospholipase A2 (PLA2) and carboxyl ester hydrolase (CEH). The PL substrate was found to be hydrolysed by PLA2, PPE and PLRP2. Their phospholipase activities on liposomes formulation was dependent on the presence of bile salts. Using a two-step *in vitro* digestion model, we measured the kinetics of fatty acid release from TAG and PL during the gastric and intestinal phases of digestion. The highest overall lipolysis level was obtained with liposomes (around 75%) during the intestinal phase while they were preserved during the gastric phase. The overall lipolysis level of TAG emulsion was lower (around 33%), while it started already in the gastric phase. In conclusion, liposomes appear as a better delivery system for intestinal absorption of LC-PUFA than TAG. In addition, their resistance to lipolysis under gastric condition can protect LC-PUFA and provide a gastric stable delivery system for other molecules.

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1. Introduction

The global production of seafood and aquaculture, as reported by the FAO (2024),[1] exceeded 223 million tons in 2022. With seafood consumption continuing to grow (from 9.9 kg in the sixties to 20.6 kg in 2022, with an estimated consumption of 21.3 kg per capita in 2032), it is necessary to maximize the valorization of by-products, which represent 40 to 60% w/w of the resource,[2] in a circular economy approach.[3] For marine oils by-products, classical extraction methods with conventional or green solvents can be used[4,5] complemented by unconventional processes such as microwave-assisted,[6] ultra-high pressure,[7] electric field,[8,9] or supercritical carbon dioxide[10,11] extractions. Moreover, the enzymatic processing of lipid extraction from fish tissues by proteases has long been utilized in the valorization of by-products such as fish and squid eggs, krill, heads, skin, backbones, and cartilages.[12–15] These lipids represent a reservoir of long-chain polyunsaturated fatty acids (LC-PUFA), including docosahexaenoic acid (DHA; C22:6n-3), eicosapentaenoic acid (EPA; C20:5n-3), and docosapentaenoic acid (DPA; C22:5n-3). Global health authorities widely recommend consuming fish 2 times per week or 500 mg LC-PUFAs per day.[16] Indeed, they play important roles in promoting health and are recognized for their ability to mitigate risk factors linked to the onset of diverse disorders, including

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cardiovascular and neurological diseases, inflammation, hypertension and various types of cancer.[17] Studies comparing the compositions of oil fish by-products (head, skin) highlight that the neutral lipid fraction is rich in saturated and monounsaturated fatty acids, while the polar lipids are rich in LC-PUFAs.[3] These are generally esterified predominantly at the *sn*-2 position in marine triacylglycerols (TAG) and PL and are mainly found in phospholipids.[17,18] These amphipathic molecules have been widely used to prepare liposomal formulations of topical, oral, and parenteral drug delivery systems, enhancing membrane permeability and the bioavailability of active compounds.[5] They found various *in vivo* and *in vitro* applications[19] and have the dual functionality of delivering LC-PUFAs present in the liposome bilayer and lipophilic or hydrophilic molecules of interest, or even both simultaneously.[19] Although liposomes are classical delivery systems,[19,20] their use as oral delivery systems is limited by their instability in the gastrointestinal (GI) tract.[21] Their physical stability can be affected by GI factors like the low pH of the gastric juice, the presence of bile salts that might dissolve the liposome phospholipids and the enzymatic hydrolysis resulting in their breakage and release of the loaded molecules in the GI.[22] Recently, more concern was given to the liposome behaviour through the gastrointestinal digestion and its stability towards the digestive fluids. In these studies, the stability of liposomes was investigated at the physical–chemical level, using technics that focus on the liposome integrity with the analysis of microstructure by atomic force microscopy (AFM), size and ζ-potential by dynamic light scattering (DLS) and microelectrophoresis, and the release of bioactive molecules.[23,24] However, no study on the fate of marine PL upon digestion in the GI tract have been reported.

The main goal of the present study was to explore the bioaccessibility of marine LC-PUFA depending on their presentation either in the form of phospholipids (PL) or triacylglycerols (TAG) extracted from salmon heads. A TAG emulsion and a liposomal solution both rich in LC-PUFAs were submitted to digestion by various lipases either tested individually or present in an *in vitro* digestion model including gastric and intestinal steps and relevant enzymes.[25] This study provides information on the fate of LC-PUFAs, such as DHA and EPA, depending on the formulation, as well as on the preservation of vector integrity during digestion.

2. Materials and methods

2.1 Material

Fresh salmon (*Salmo salar*) heads were obtained from a local plant and stored at −20 °C. They were thawed at 4 °C overnight before use.

A controlled enzymatic hydrolysis of salmon heads was performed using Alcalase® 2.4 L and the pH-stat technique as previously described by Gbogouri *et al.*[12] The different hydrolysates, oil, and the pellet containing the heaviest protein fraction were separated by centrifugation at 9000 rpm for 20 min at 4 °C using a Beckman Coulter® Rotor J-10 centrifuge (Beckman Coulter, Brea, CA, USA). Salmon phospholipids were purified through acetone precipitation with the purpose to remove proteins and residual TAGs as described by Lu *et al.*[26]

Sodium taurodeoxycholate (NaTDC; T0557), MES hydrate, chloroform, methanol and acetone were obtained from Sigma-Aldrich. Butylated hydroxytoluene (BHT), copper acetate, PLC Silica gel 60 (20 × 20 cm, 2 mm) and TLC Silica gel 60 (10 × 20 cm, 0.2 mm) plates, *n*-heptane, hexane, diethyl ether, formic acid, *ortho*-phosphoric acid, sodium chloride (NaCl), calcium chloride ($CaCl_2$), boron trifluoride-methanol solution (BF_3) and ammoniac were purchased from Merck.

Lipid standards including triolein (TO), diolein (DO), monoolein (MO), oleic acid (OA), dioleylphosphatidylcholine (PC), dioleylphosphatidylethanolamine (PE), dioleylphosphatidylinositol (PI) and dioleylphosphatidylserine (PS) were obtained from Avanti Polar Lipids INC (Alabaster, Alabama, USA). Individual fatty acids were identified using Standard mixtures (PUFA1 from a marine source and PUFA2 from a vegetable source; Supelco, Sigma-Aldrich, Bellefonte, PA, USA).

2.2 Enzyme sources

Alcalase® 2.4 L (EC.3.4.21.14a) was supllied by Novozymes A/S (Bagsvaerd, Danemark). Porcine pancreatic extract (PPE, 8× USP – P7545) and phospholipase A2 (PLA2) from bovine pancreas (≥20 units per mg – P8913) were supplied from Merck-Sigma-Aldrich. Rabbit gastric extract (RGE15, 15 U mg^{-1}), purified porcine pancreatic lipase (PPL, 10 200 U mg^{-1}), Guinea pig pancreatic lipase related protein 2 (GPLRP2, 2700 U mg^{-1}) and human carboxyl ester hydrolase (CEH, 300 U mg^{-1}) were from Lipolytech (Marseille, France).

2.3 Lipid formulations preparation

2.3.1. Preparation of TAG emulsion. The oil (TAG) in water emulsion at 3% (w : v) was prepared by mixing 3 g of fish oil in 100 mL of either 10 mM MES buffer (for pH 5.5 and 6.0) or 10 mM Tris-HCl (for pH 8.0), 150 mM NaCl, 10 mM $CaCl_2$ and 4 mM NaTDC at different pH values (pH 5.5 for incubation with gastric enzymes and pH 6.0 or 8.0 for pancreatic enzymes). These solutions contain salts that are classically used for the assay of digestive lipase.[27] 150 mM NaCl is a physiological concentration. 10 mM $CaCl_2$ is a high concentration that allows providing calcium in large excess as a cofactor for some lipolytic enzymes like phospholipases. Finally, 4 mM NaTDC corresponds to a supramicellar concentration of a bile salt well represented in human duodenal contents.[28] It is lower than the concentration (10 mM) recommended in standardized conditions for *in vitro* digestion assays (Brodkorb, *et al.* (2019)),[25] but it still fits with concentrations found in the small intestine.[28] The bile salt NaTDC was used as the emulsifier because we did not want to use compounds that could be substrates for lipolytic enzymes (lecithin, partial acylglycerols) or interfere with lipolysis (surfactant like Twen 80[29] and proteins[30]). NaTDC was selected as bile salt because it can be used in a large pH range and does not precipitate at acidic pH. The mixture was subsequently subjected to ultrasonic treat-

ment for 4 min (Sonicator Vibra-Cell 75115 with the parameters set at 40% intensity; 1 s on and 1 s off).

A characterization of the emulsion was made by DLS analysis for particle size determination and gas chromatography (GC) for fatty acid composition.

2.3.2. Preparation of liposomes. 450 mg of pure fish PL were dispersed in 15 mL of either 10 mM MES buffer (for pH 5.5 and 6.0) or 10 mM Tris-HCl (for pH 8.0), 150 mM NaCl and 10 mM $CaCl_2$ at different pH values (pH 5.5 for gastric enzymes and pH 6.0 and 8.0 for pancreatic enzymes) in the absence or in the presence of bile salts (4 mM NaTDC). The mixture was maintained under low mechanical stirring (Magnetic digital stirrer 444-0623; VWR, USA, 250 rpm) for at least 4 hours, then liposomes were formed by ultrasonic treatment for 4 min (Sonicator Vibra-Cell 75115 with the parameters set at 40% intensity; 1 s on and 1 s off).

A characterization of the liposomes was made by DLS analysis for particle size determination and GC for fatty acid composition. TEM studies on similar liposomes showing their morphology have been reported previously.[31,32]

2.4 Dynamic light scattering analysis of TAG emulsion and PL liposomes

DLS analysis of TAG – NaTDC emulsion, and PL liposome ± NaTDC dispersions were carried out using a Zetasizer Nano S (Malvern Instruments) at 37 °C. Each measurement was performed in triplicate and consisted in 10–15 runs of 10 s at a scattering angle of 173. The determination of the hydrodynamic diameter (D_H) was based on the Einstein-Stokes relation to obtain the intensity-averaged size distribution. A viscosity of 0.736 cP and a refractive index of 1.3335 (at 37 °C) were used for the dispersion medium, while a value of 1.49 was used as an approximation of the refractive index for lipids.[33]

2.5 Hydrolysis of TAG and PL formulations by individual enzymes

1 mL of substrate (TAG emulsion or PL liposome dispersions with or without bile salts), in a 15 mL glass tube with screw-cap, was first incubated in a water bath at 37 °C for 3–5 minutes to stabilize the temperature at 37 °C. Subsequently, 20 μg mL^{-1} enzymes (or enzyme extract) were added, and the mixture incubated for 60 minutes at 37 °C and under low rotative stirring (Stuart rotator SB-3 Cole-Parmer Bibby scientific UK). After 1 hour of incubation, the reaction was stopped by adding 200 μL of a 1 N HCl solution and samples were freeze-dried (CHRIST, Beta 1–8 LD plus, Grosseron – France) for lipid extraction. The experiments were performed in triplicates for each enzyme (PLA2, PPE, PPL + colipase, GPLRP2, CEH and RGE) and pH tested.

For each substrate, a blank without enzyme was also incubated under the same conditions.

2.6 Two-step *in vitro* digestion

A two-step static *in vitro* digestion model including relevant gastric and a duodenal phases was adapted from the standardized INFOGEST 2.0 protocol[25] with some changes regarding the pH values and the use of purified bile salts instead of complete bile. Fifteen mL of substrate dispersion (emulsion in 10 mM MES, 50 mM NaCl, 0.15 mM $CaCl_2$ and, 4 mM NaTDC or liposomes in 10 mM MES, 50 mM NaCl, 0.15 mM $CaCl_2$) were first mechanically stirred in a temperature-controlled (37 °C) reaction vessel of a pH-stat apparatus (Titrando 902, Metrohm, France) and the pH endpoint was adjusted to 5.5. The gastric phase starts by adding 3 mL of a freshly prepared rabbit gastric extract (RGE15; 1.3% w/w GL) solution to the reaction vessel to obtain a final concentration of 60 U ml^{-1} gastric lipase (using tributyrin as substrate[34]) and 2000 U ml^{-1} pepsin. The pH was kept constant at 5.5 during 30 min reproducing the conditions (lipase concentration and pH) found in the stomach at half-gastric emptying of a test meal,[35] *via* the automated titration of free fatty acids (FFAs) with 0.1 M NaOH using the pH-stat device.

At t = 30 min, a PPE/bile salts solution was added to the digestion mixture at a ratio of 1 : 1 v/v and the pH endpoint was shifted to 6.25 and kept constant for 60 min reproducing the conditions (lipase concentration and pH) found in the upper small intestine at half-gastric emptying of a test meal.[35] After adding the PPE/bile salt solution to the reaction vessel, the final pancreatic lipase concentration was 2000 U mL^{-1} (using tributyrin as substrate[34]), the final bile salt concentration was 10 mM, $CaCl_2$ 0.6 mM and the gastric phase was diluted 2-fold. One mL samples were collected at various times (0, 15, 29, 35, 40, 45, 60 and 90 min), immediately acidified with 200 μL 1 N NaOH for the extraction of residual substrate and lipolysis products and subsequent analysis by TLC, TLC-FID and GC-FID.

2.7 Lipid extraction and analysis by thin-layer chromatography

2.7.1. Lipid extraction. Lipid extraction was performed by mixing vigorously the samples (1 mL) with 5 mL chloroform/methanol (2 : 1 v/v), 1 mM BHT in a 15 mL glass tube with a screw-cap. After phase separation, the lower organic phase was collected using a Pasteur pipette, transferred into a 8 mL vial with a screw-cap and the vial was kept at −20 °C until TLC analysis were performed. BHT was added to prevent lipid oxidation upon storage and when the organic solvent was evaporated for further analysis.

2.7.2. Qualitative lipids analysis by thin-layer chromatography (TLC). Qualitative analysis of residual substrate (TAG or PL) and lipolysis products (free fatty acid (FFA); diacylglycerol (DAG), monoacylglycerol (MAG) or lysophospholipids) was performed by thin-layer chromatography (TLC). 5 to 10 μL of lipid extracts or lipid standards (TO, DO, MO and OA, for TAG and PC, PE, PI, PS and OA for PL) were spotted onto thin-layer silica plates. The elution of the lipids was then performed using either heptane/diethyl ether/formic acid (55 : 45 : 1 v/v/v) for the migration of neutral lipids or chloroform/methanol/H_2O (65 : 25 : 4 v/v/v) for the migration of polar lipids. Following chromatography, the plates were dried at room temperature (air-conditioning at 22 °C) for 5 min and then sprayed with a cupric acetate/*ortho*-phosphoric acid solution, prepared by mixing a saturated aqueous solution of cupric

acetate with 85% *ortho*-phosphoric acid in a 1-to-1 volume ratio. The plates were then placed in an oven to ensure heating at 180 °C for 5 min.

2.7.3. Quantitative lipids analysis by thin-layer chromatography coupled to flame ionization detection (TLC-FID). The quantitative analysis of residual substrates (TAG or PL) and their lipolysis products was performed by TLC-FID using a Iatroscan MK5 equipment (Iatron Laboratories, Japan). Each lipid extract (1–2 μL) was spotted onto a quartz rod coated with silica (0.9 mm diameter Chromarod™ S5, LSI Medience Corporation, Japan) and sample migration was performed with either hexane/ether/formic acid (80 : 20 : 0.2 v/v/v) for the migration of neutral lipids or chloroform/methanol/ammoniac (65 : 35 : 5 v/v/v) for the migration of polar lipids. The Chromarods™ were then dried at 100 °C for 1 min (Rod dryer TK8, Iatron Laboratories) and transferred to the Iatroscan MK5 to be scanned using FID for detection and quantification of the compounds separated on the silica. The amounts of each individual lipolysis product were estimated at each time point from the calibration curves established with pure lipid standards. TAG emulsion lipolysis level was estimated from the quantification of residual TAG, FFA, DAG and MAG by TLC-FID. Mass amounts were converted into μmoles using mean molecular masses of 895 g mol^{-1} for TAG, 627.3 g mol^{-1}, for DAG, 359.7 g mol^{-1} for MAG and 285.7 g mol^{-1} for FFA. PL liposomes lipolysis level was estimated from the quantification of residual PC, PE and FFA by TLC-FID. Mass amounts were converted into μmoles using mean molecular masses of 802.4 g mol^{-1} for PC, 759.5 g mol^{-1} for PE and 290.7 g mol^{-1} for FFA.

These molecular masses were estimated from the fatty acid composition of the oil and the PL used for making the TAG emulsions and the liposomes, respectively (Table 1). Lipolysis levels were expressed as FFA% *versus* total fatty acids esterified in the TAG emulsions or PL liposomes. The time-course variations in the amounts of each molecular specie was plotted as a function of time.

2.8 Fatty acid composition determination by gas chromatography (GC)

Fatty acids present in the initial substrate (TAG or PL at 10 mg mL^{-1}) were derivatized into fatty acid methyl esters (FAME) according to the Smith and Morisson method,[36] using 2 mL of BF_3 in methanol. The mixtures were heated at 100 °C for 1 h. After cooling, the FAME were extracted with 2 mL of heptane. Samples were shaken, and the upper heptane layer was removed and stored at −20 °C before GC analysis.

For the characterization of FFAs released upon the hydrolysis of the TAG emulsion or PL liposomes, 500 μL of each total lipid extract were applied as 10 cm bands onto preparative TLC plates, with a 2 mm silica layer. Following elution and lipids separation, the plates were dried at room temperature for 10 min, the bands corresponding to the various lipid fractions were located using a staining with iodine restricted to the edge of the plate/band. Each band was then scraped separately from the TLC plate and transferred into a 15 mL glass tube equipped with a Teflon-lined screw cap and containing 2 mL of a chloroform/methanol mixture (2 : 1, v/v). After mixing vigorously, and centrifugation at 5000 rpm (Thermofisher Scientific laboratory centrifuge 75007214, Germany) for 10 min, the organic phase was transferred into another tube, the extraction of lipids from silica was repeated twice using 2 mL of acetone and the pooled organic phases were taken to dryness under nitrogen stream. All samples were then derivatized into FAME using methanol-BF_3. After extraction with heptane, the volume of each FAME sample was then reduced to 50 μL under a stream of nitrogen for concentration in a vial equipped with 250 μL pulled point conical glass insert (Agilent Technologies, Germany).

The FAME were subsequently analysed by GC on a Shimadzu GC-2010 equipped with a flame-ionization detector (FID) and a SPTM2380 Supelco capillary column (60 m; 0.2 mm internal diameter × 0.25 μm film thickness (Bellefonte, PA, USA)). The oven temperature was set at 200 °C. The detector and injector were at a temperature of 250 °C and nitrogen was used as a carrier gas with a flow rate of 0.79 mL min^{-1}. The oven temperature program used during the run was 120 °C for 2 minutes and then climb to 180 °C for 2 minutes followed by a rise to 220 °C for 25 minutes. The identification of fatty acids was carried out by co-injection of standard compounds (C23 as internal standard and PUFA 1, PUFA2).

Table 1 Fatty acid composition (% w/w) of salmon TAG and PL fractions

	% of total fatty acids	
Fatty acid	TAG	PL
Saturated FA		
C14:0	4.11 ± 0.04	2.45 ± 0.02
C15:0	0.30 ± 0.01	0.81 ± 0.03
C16:0	12.48 ± 0.06	21.22 ± 0.09
C17:0	0.21 ± 0.01	0.67 ± 0.01
C18:0	2.78 ± 0.02	8.64 ± 0.03
C20:0	0.11 ± 0.11	0.17 ± 0.12
C22:0	4.83 ± 0.01	1.83 ± 0.02
Σ Saturated FA	***24.82 ± 0.19***	***35.79 ± 0.33***
Monounsaturated FA		
C16:1n-7	4.28 ± 0.04	2.24 ± 0.03
C18:1n-9	31.88 ± 0.07	17.19 ± 0.14
C18:1n-7	2.95 ± 0.01	2.79 ± 0.02
C20:1n-9	1.16 ± 0.01	0.38 ± 0.27
C22:1n-9	1.17 ± 0.01	0.55 ± 0.01
Polyunsaturated FA		
C18:2n-6	10.94 ± 0.08	3.51 ± 0.03
C18:3n-3 (ALA)[a]	5.71 ± 0.24	2.54 ± 0.02
C18:4n-3	3.57 ± 0.15	1.29 ± 0.01
C20:4n-6 (ARA)[a]	0.14 ± 0.14	2.73 ± 0.02
C22:4n-6	<0.1	2.71 ± 0.06
C20:5n-3 (EPA)[a]	5.03 ± 0.05	6.12 ± 0.02
C22:5n-3 (DPA)[a]	1.89 ± 0.02	2.18 ± 0.01
C22:6n-3 (DHA)[a]	6.46 ± 0.07	19.99 ± 0.01
Σ Unsaturated FA	***75.18 ± 1.47***	***64.21 ± 0.18***
Σ n-3 FA	**22.66 ± 0.53**	**32.03 ± 0.07**

[a] ALA: α-linoleic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid.

3. Results and discussion

3.1. Lipid classes and fatty acid composition determination

3.1.1. Phospholipid classes characterization. The phospholipids were extracted from salmon heads by enzymatic means and purified by precipitation with ice-cold acetone. The composition of the purified PL was determined by TLC FID, showing that PC is predominant at 64.66 ± 0.04% w/w, followed by PE at 14.52 ± 1.41%, and in lower proportions, PS (4.77 ± 1.20%), SM (1.32 ± 0.08%), as well as PI, PA and non-identified polar lipids representing 13.59 ± 2.30%. These results are in agreement with the literature. Indeed, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the predominant phospholipids in marine resources, followed in lesser quantities by phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA) and sphingomyelin (SM).[17] This is precisely the case for salmon by-products, as demonstrated by the work of Haq and Chun (2018),[10] who extracted phospholipids from salmon by-products using supercritical CO_2.

3.1.2. Fatty acid composition of salmon oil TAG and PL

3.1.2.1. Fatty acid composition of TAG. The GC analysis results showed that oleic acid (C18:1) was the main fatty acid found in fish oil TAG in which it represents 34.83% w/w of total FA, followed by palmitic (C16:0) and linoleic (C18:2) acids (12.48% and 10.94% of total FA, respectively) (Table 1). As expected, the fish oil showed a higher level of unsaturated fatty acid (75.18% of total FA) compared to saturated FA (24.82% of total FA) with a high level of omega 3 fatty acids accounting for 22.66% of total fatty acids, with EPA (C20:5n-3) and DHA (C22:6n-3) representing 11.49% of total FA.

3.1.2.2. Fatty acid composition of salmon PL. The GC analysis of the FAME from purified salmon PL showed that palmitic acid (C16:0), DHA (C22:6n-3) and oleic acid (C18:1) were the main fatty acids representing 21.22%, 19.99% and 19.98% of total FA, respectively (Table 1). Similarly, to the oil fish TAG, the PL showed a higher level of unsaturated fatty acids (64.21% of total FA) compared to saturated FA (35.79% of total FA). However, the omega 3 fatty acids level (32.03%) was higher in PL than TAG, with a higher EPA (C20:5n-3) and DHA (C22:6n-3) levels (26.11% of total FAs), *i.e.* more than the double of their proportion in TAG.

3.2. Characterization of TAG and PL dispersions by DLS

The TAG emulsion and liposomes were prepared by dispersing the purified marine TAG or PL in buffer followed by the ultrasonic treatment. For the TAG emulsion, the presence of the bile salt NaTDC, was necessary to make the oil in water dispersion, while liposome dispersion was performed in buffer with or without NaTDC.

The particle size distribution of TAG emulsion and liposomes obtained under these conditions were characterized by DLS. For TAG emulsion, average particle sizes of 154.5 ± 1.2 nm, 138.3 ± 1.5 nm and 163.7 ± 1.3 were determined at pH 5.5, 6.0 and 8.0, respectively (Table 2). Liposome dispersions were found to organize in particles of similar size in the absence of bile salt, but with a higher polydispersity than the TAG emulsion. Their average size was found to be slightly affected by the presence of bile salts (Table 2), with a shift from 143–153 nm in absence of bile salts to a smaller average particle size ranging from 108 to 112 nm in presence of bile salts. This suggests that phospholipids organized in smaller size vesicles in the presence of bile salts, or that some phospholipids formed smaller mixed micelles with bile salts, leading to a lower average particle size. The increase in polydispersity (Table 2) supports this hypothesis. The dispersion average size of both TAG emulsion and PL liposomes was found to be slightly affected by the pH (Table 2).

3.3. *In vitro* digestion of marine TAG emulsion and PL liposome by individual gastrointestinal lipases

Preliminary lipolysis experiments with various gastrointestinal lipases were performed at different pH, to determine which lipolytic enzymes could act on each substrate formulation and contribute to the whole digestion process. Lipolysis products were then analysed by TLC.

3.3.1. TAG emulsion lipolysis by individual enzymes. TLC analysis showed that TAG emulsion was mainly hydrolysed by porcine pancreatic extract (PPE) and purified porcine pancreatic lipase (PPL) with its cofactor colipase at both pH 6.0 and 8.0 (Fig. 1, line 4, 5, 9 and 10). PPL was found to be more active on TAG substrate at pH 8.0 (Fig. 1, lane 10) than pH 6.0 (Fig. 1, lane 5) as indicated by the higher intensity of the band corresponding to FFA (oleic acid as standard). Pancreatic lipase related protein 2 at pH 6.0 (GPLRP2; Fig. 1, lane 7) and gastric lipase from RGE at pH 5.5 (Fig. 1, lane 13) were also found to hydrolyse TAG emulsion at a lower extent as indicated by the presence of bands corresponding to the 1,2- and to less extend 1,3-diacylglycerol. No band corresponding to FFA can however be detected on the corresponding lanes, suggesting that the FFA released are at low levels and poorly revealed by the copper acetate-phosphoric acid reagent. Thus, these

Table 2 Determination of the marine TAG emulsion and PL liposome size (*z*-average) and the polydispersity index (Pdi) by DLS

				Liposomes					
Emulsion				0 mM NaTDC			10 mM NaTDC		
pH	5.5	6.0	8.0	5.5	6.0	8.0	5.5	6.0	8.0
Size (nm)	154.5 ± 1.2	138.3 ± 1.5	163.7 ± 1.3	150.8 ± 19.2	153.5 ± 12.8	143.9 ± 6.2	108.4 ± 4.2	104.9 ± 9.6	112.7 ± 12.7
Pdi	0.08	0.07	0.04	0.25	0.25	0.28	0.39	0.39	0.39

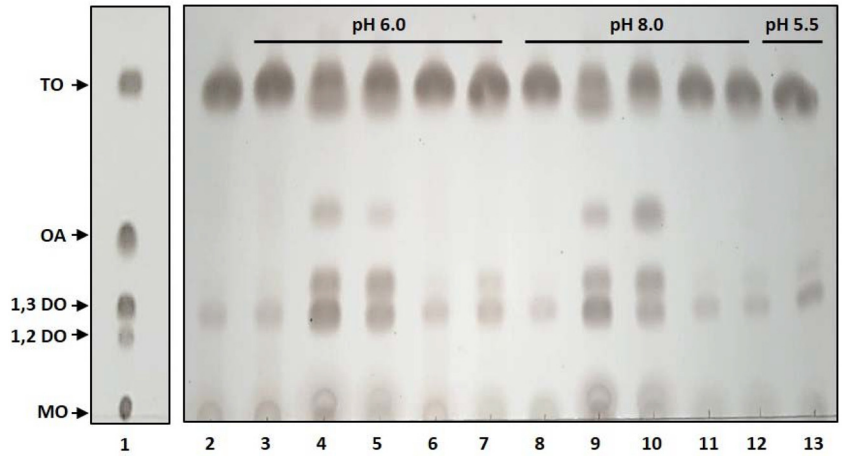

Fig. 1 TAG emulsion hydrolysis by individual enzymes. Lane 1: Neutral lipid standard MO: monoolein, DO: diolein, OA: oleic acid. TO: triolein; Lane 2: blank substrate (20 μL); Lane 3 and 8: bovine phospholipase A2 (PLA2, 20 μg); Lane 4 and 9: porcine pancreatic extract (PPE, 20 μL); Lane 5 and 10: porcine pancreatic lipase (PPL, 20 μg) + colipase (20 μg); Lane 6 and 11: human carboxylester hydrolase (CEH, 20 μg); Lane 7 and 12: guinea pig pancreatic lipase related protein 2 (GPLRP2, 20 μg); Lane 13: rabbit gastric extract (RGE, 20 μL).

lipases might have preferentially released saturated fatty acids under these conditions. GPLRP2 having a stereoselectivity for the hydrolysis of the esters bond at the *sn*-1 position of TAG,[37,38] and gastric lipase showing a stereoselectivity for the *sn*-3 position,[39] these results are in agreement with a preferential location of saturated FA at the external positions in fish oil TAG, while LC-PUFA are preferentially found at the *sn*-2 position. As expected, no activity was observed with bovine pancreatic phospholipase A2 (PLA2, Fig. 1, lane 3), which is consistent with the specificity of this enzyme for phospholipids. No activity was observed with carboxyl ester hydrolase at both pH tested (CEH, Fig. 1, lane 6 and 11) although this enzyme is known to display a wide range of activities including lipase activity. This is not really surprising since this enzyme has a preference for substrates dispersed in solution in the form of mixed micelles (phospholipids, galactolipids, MAG) and poorly interacts with substrates forming oil-in-water emulsions (TAG, DAG),[40] which results in a low TAG lipase activity compared to other lipases.[41]

3.3.2. Liposome lipolysis by individual enzymes. The hydrolysis of liposomes was tested in the absence and in the presence of bile salts (NaTDC) to access the effect of bile salts on the bioavailability of fatty acids from liposomes. In the absence of bile salts, bovine pancreatic phospholipase A2 (PLA2) was able to hydrolyse liposomes phospholipids and generate free fatty acids (FFA) at both pH 6.0 and 8.0 (Fig. 2A and B, lane 7). PLA2 was less active on liposomes phospholipids in the presence of bile salts (Fig. 2C and D, lane 7), which was unexpected because pancreatic PLA2 usually prefers phospholipid substrate in mixed micelles with bile salts rather than in bilayers such as in liposome.[40] Most studies on the biochemical characterization of pancreatic PLA2 have however been performed with PC as substrate. The present findings might therefore result from the particular phospholipid composition of salmon polar lipids (see section 3.1.1. and ref. 5). Indeed, the presence of LC-PUFA in PC can favour the activity of PLA2 on PC bilayers by changing the membrane fluidity and the packing of phospholipid molecules.[42] Moreover, the presence of negatively charged phospholipids (PS and PA) mixed with zwitterionic Phospholipids (PC and PE) might favour the interaction of PLA2 with the liposome membrane by creating an anionic interface.[43,44] Moreover, chain length and unsaturation number of fatty acids can also affect phospholipid digestibility due to the steric hindrance. In both conditions, PLA2 was found to display a higher activity at pH 8.0 then pH 6.0.

With PPE and in absence of bile salts (Fig. 2A and B, lane 8), several bands corresponding to PE and PC totally disappeared but no band corresponding to FFA were detected. As previously proposed regarding the lipolysis of TAG and the absence of a detectable FFA band, these results suggest a preferential release of saturated FA by PPE, which are preferentially esterified in the *sn-1* position of the phospholipids. This hypothesis is further supported by the analysis of the FFA released upon digestion (see section 3.4.2. and Fig. 5) and it is consistent with the presence of the PLA1 activity in the PPE.[45] In the case of PLA2, specifically releasing FA from the *sn*-2 position, FFA should be enriched in LC-PUFA and thus well stained using the copper acetate-phosphoric acid reagent preferentially acting on double bounds. However, with PPE, an intense band that migrates just below the front appears on the TLC plate (Fig. 2A and B, lane 8). Since phospholipids and their lipolysis products are polar and migrate in the lower part of the plate, it seems that some apolar compounds are either generated during the reaction or, more probably, are originating from PPE. This hypothesis remains to be confirmed but it is known that PPE contains some residual fat (TAG and lipolysis products) from the pancreas.[45] Interestingly, GPLRP2 was found to hydrolyse liposomes since bands corresponding to FFA appeared and bands corresponding to PC and PE substrates disappeared (Fig. 2, line 11). This finding is unexpected

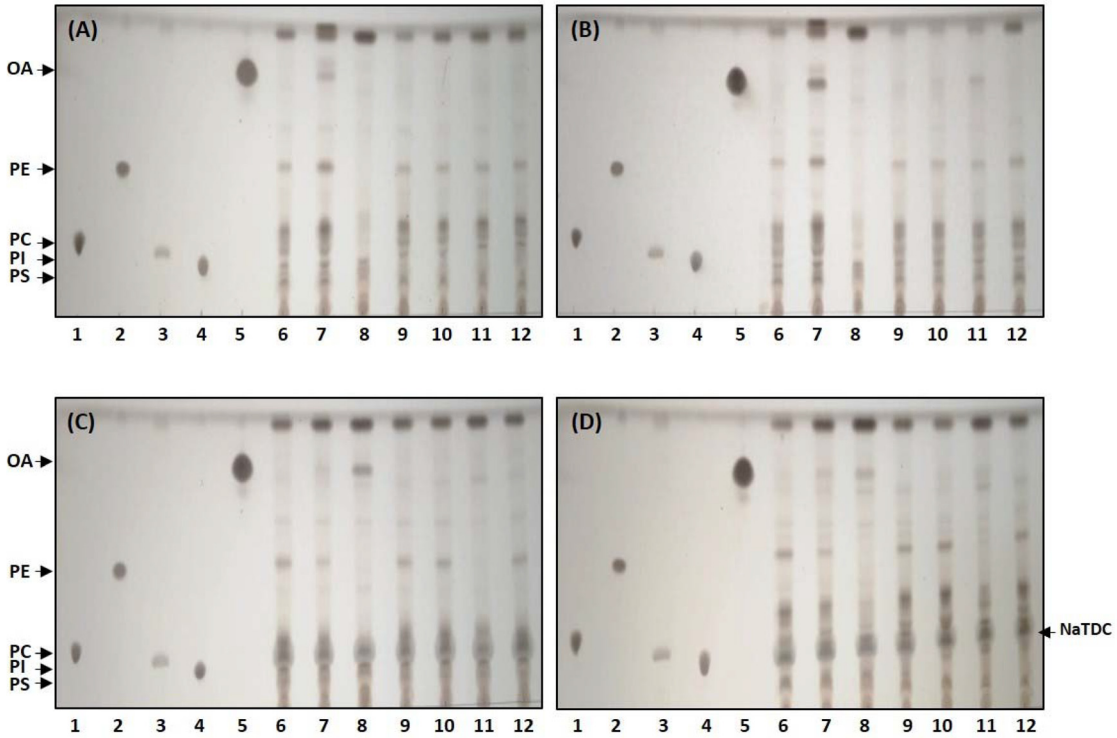

Fig. 2 Liposome phospholipid hydrolysis by individual enzymes in absence (Panel A and B) or in presence (Panel C and D) of bile salts (NaTDC) at pH 6.0 (Panel A and C) and pH 8.0 (Panel B and D). Lane 1: PC (dioleylphosphatidylcholine); Lane 2: PE (dioleylphosphatidylethanolamine); Lane 3: PI (dioleylphosphatidylinositol); Lane 4: PS (dioleylphosphatidylserine); Lane 5: OA (oleic acid); Lane 6: blank, substrate alone (20 μL); Lane 7: bovine phospholipase A2 (PLA2, 20 μg); Lane 8: porcine pancreatic extract (PPE, 20 μL); Lane 9: porcine pancreatic lipase (PPL, 20 μg) + colipase (20 μg); Lane 10: human carboxylester hydrolase (CEH, 20 μg); Lane 11: guinea pig pancreatic lipase related protein 2 (GPLRP2, 20 μg); Lane 12: rabbit gastric extract (RGE, 20 μg).

since GPLRP2 poorly interacts with PC liposomes and preferentially acts on mixed PC-NaTDC micelles.[46,47] Nevertheless, it has been shown that GPLRP2 can act on heterogenous biomimetic membranes[48] and, as indicated before, the presence of negatively charged phospholipids in marine PL might favour the interaction of GPLRP2 with the liposome membrane. However, and as expected, the activity of GPLRP2 was found to be higher in presence of bile salts (Fig. 2C and D, line 11) and at pH 8.0 (Fig. 2B and D, line 11). This finding is consistent with the substrate preference of PLRP2 for micellar substrates.[41,48,49] Interestingly, DLS analysis indicated that the liposomes structure was not drastically disturbed by bile salts, according to their particle size that was only slightly reduced on average. These findings suggest that either PLRP2 is able to interact with these liposomes and hydrolyse their phospholipids or the activity observed results from the action of GPLRP2 on PL-bile salts mixed micelles co-existing with liposomes.

Regarding CEH, no action of this enzyme on liposomes was observed whatever the bile salt and pH conditions tested (Fig. 2, line 10), although it is known to display some phospholipase A1 activity.[49] Similarly, no activity was observed with both PPL and RGE, what is consistent with their lipase activity and high specificity for TAG substrates.

3.4. Two-step *in vitro* digestion of TAG and PL substrates under simulated physiological conditions

The experimental conditions adopted for the *in vitro* digestion[25,50] were based on *in vivo* studies and parameters measured at 50% meal gastric emptying.[34,35,51] The chosen pH values, 5.50 for the gastric phase and 6.25 for the intestinal phase, differ from those recommended by the INFOGEST static *in vitro* digestion model. However, they better fit with the optimum activity of digestive lipases. For the gastric phase, rabbit gastric extract (RGE) was chosen as the source of gastric enzymes because it contains pepsin and gastric lipase and the activity of rabbit gastric lipase has a similar range of activity as the human gastric lipase (HGL).[52] Porcine Pancreatic Extract (PPE) was used as the source of pancreatic enzymes for the intestinal phase of *in vitro* digestion. PPE consists of a mixture of digestive enzymes produced by the exocrine cells of the porcine pancreas and contains trypsin, chymotrypsin, α-amylase, and all the lipolytic enzymes required for lipid digestion including pancreatic lipase and its cofactor colipase, pancreatic phospholipase A2, PLRP2 and CEH/BSSL.[45] PPE is commonly used for *in vitro* digestion studies as a substitute of human pancreatic enzymes.[25,50]

3.4.1. TAG emulsion lipolysis during *in vitro* gastrointestinal digestion. According to the TLC analysis of TAG digestion

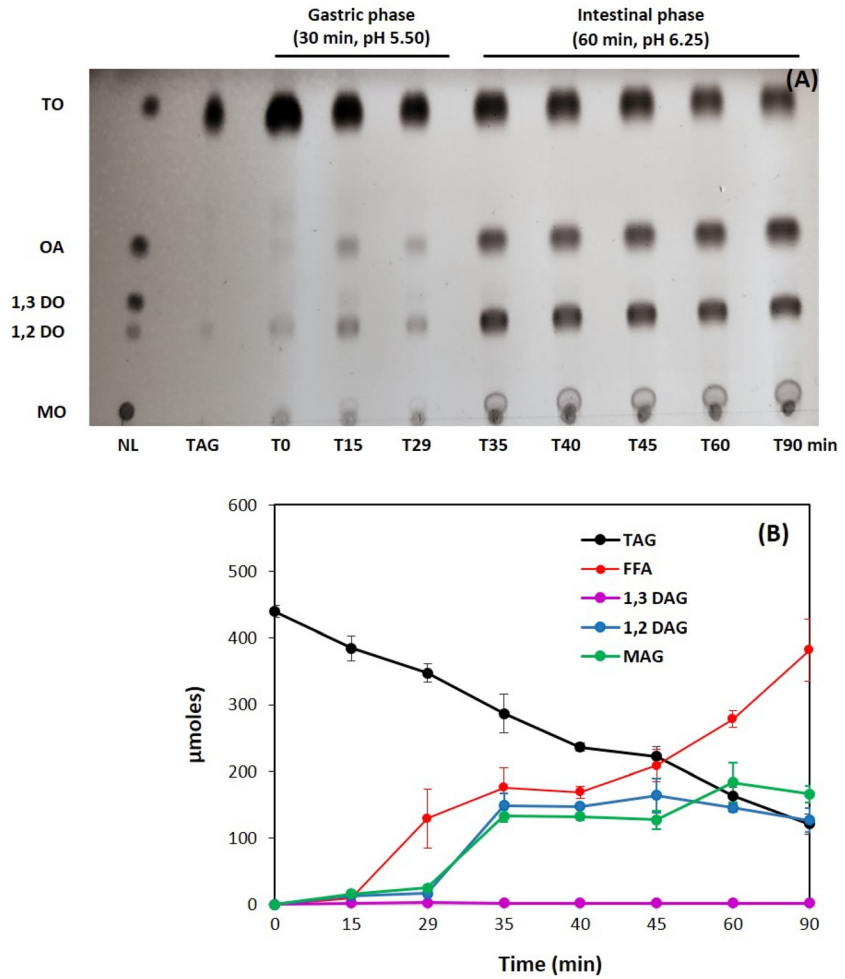

Fig. 3 *In vitro* digestion of TAG emulsion. (A) TLC analysis of TAG emulsion hydrolysis products in the course of the two-step static digestion model. NL: neutral lipid standard; TAG: triglyceride fraction used to make the emulsion; MO: monoolein; DO: diolein; OA: oleic acid; TO: triolein. Lane T0 to T90: the different time point analysis. (B) Variations with time of the level of individual lipolysis products generated during the *in vitro* lipolysis of TAG emulsion using the two-step static digestion model. Individual lipolysis product levels were measured by TLC-FID and expressed in µmoles present in the reaction mixture. TAG, residual triacylglycerols; FFA, free fatty acids; 1,3-DAG, 1,3-diacylglycerides; 1,2-DAG, 1,2-diacylglycerides; MAG, monoacylglycerols. Values are mean ± SD obtained from 3 independent hydrolysis experiments.

(Fig. 3A), TAG emulsion was hydrolysed and converted to diacylglycerol (DAG), monoacylglycerol (MAG) and free fatty acids (FFA) during the whole digestion process. This digestion started during the gastric phase with a release of lipolysis products (FFA, DAG and MAG) and a decrease in the initial TAG by around 20% as determined by TLC-FID quantification (Fig. 3B). The hydrolysis level (FFA% *vs.* total fatty acids) obtained during the gastric phase of TAG emulsion lipolysis (0–29 min) was 8.4 ± 1.4% as determined by TLC-FID quantification (Fig. 4). The FFA generated during that period mainly resulted from the hydrolysis of TAG but also from some intermediate DAG since the production of MAG was observed (Fig. 3B).

When pancreatic enzymes were added at 30 min to initiate the intestinal phase of digestion, a clear jump in the release of fatty acids, 1,2-DAG and MAG was observed while TAG decreased at a higher rate (Fig. 3A and B). During the first 10 min of the intestinal phase, TAG decreased by around 46% as determined by TLC-FID quantification (Fig. 3B). It is worth noticing that the levels of 1,3-DAG did not increase during the lipolysis experiment, suggesting that either they were not formed or were rapidly hydrolyzed and converted in MAG.[53] Both 1,2(2,3)-DAG and MAG were found to accumulate and reached a plateau after 35 min. Since FFA still increased while TAG decreased, especially after 45 min (Fig. 3A and B), these results suggest that some MAG are further converted into glycerol (not measured). At the end of the duodenal phase of digestion (t = 90 min), the hydrolysis level reached 32.8 ± 1.2% (Fig. 4) whereas lipolysis levels of emulsions usually exceed 50% after 90 min under these conditions.[29,54] The level of absorbable fatty acids is around 46% if we consider the sum of FFA and MAG *versus* total fatty acids.

In these experiments NaTDC was used as the unique bile salt source in the intestinal phase of the *in vitro* digestion,

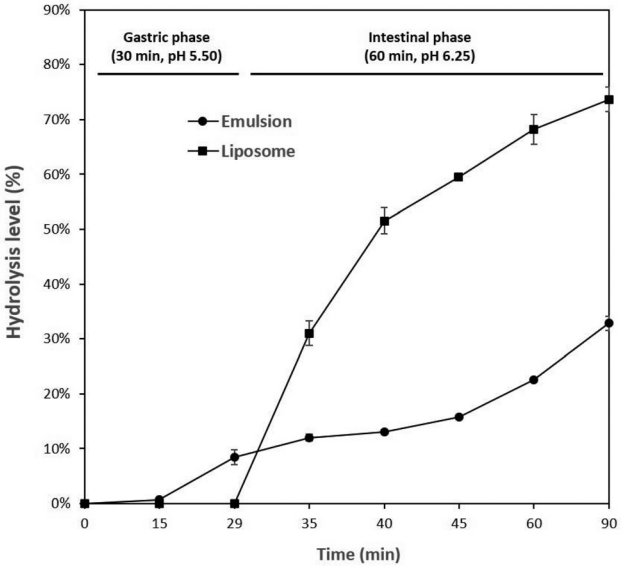

Fig. 4 Time-course evolution of the lipolysis levels of TAG emulsion (circle dots) and PL liposomes (square dots) during two-step *in vitro* digestion. Data are FFA % and are expressed as the percentage of total fatty acids esterified in the TAG or PL initially presents in the emulsion and liposomes, respectively. Values are mean ± SD obtained from 3 independent hydrolysis experiments.

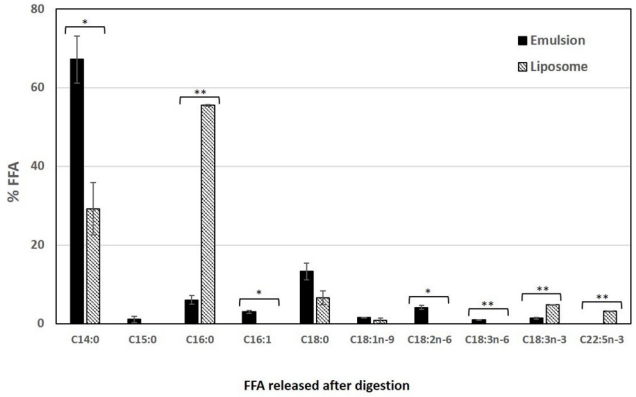

Fig. 5 Fatty acid composition of the FFA released at the end of digestion of TAG emulsion (black column) and PL liposome (hatched column), after purification from silica plate and GC analysis. Values are mean ± SD obtained from 3 independent hydrolysis experiments. Statistical analyses were performed with Origin software 2019 (Massachusetts, USA) using one-way ANOVA multiple comparisons followed by Tukey correction. *p* values were indicated in the legends if considered significant (* $p < 0.05$, ** $p < 0.01$).

whereas bovine bile was recommended in the standardized INFOGEST protocol (Ref Brodkord). Our choice was justified by the presence of endogenous PC and PE in bovine bile which can count up to 22% (w:w).[55] Thus, these phospholipids might have interfered with the digestion of the PC and PE present in the liposome substrate. The aim of this study being a comparative study of liposome digestibility *vs.* emulsion, it was necessary to keep the same experimental conditions during *in vitro* digestion. The use of NaTDC as bile salts (and no other emulsifier) might explain the lower salmon oil TAG lipolysis compared to other oils and emulsions.[29]

The composition of the fatty acids released upon digestion of emulsion was determined by GC after purification from silica plate. The results showed that C14:0 was released at the end of digestion (67.11 ± 5.93% of total FFA), followed by the long chain FFA C18:0 (13.27 ± 2.08% of total FFA) (Fig. 5). These results suggest that these FAs are present at the *sn-1* or *sn-3* positions of the TAG substrate which are preferentially hydrolyzed by pancreatic lipase. These findings are in accordance with previous studies that have showed that LC-PUFA are mainly esterified at the *sn-2* position of TAG.[56] Knowing the contents of C14:0 (4.11 ± 0.04% w/w) and C18:0 (2.78 ± 0.02% w/w) in TAG (Table 1) and the fact that they are not the main fatty acids, their high levels in FFA is constituent with a low lipolysis level of TAG with a preferential release of saturated FAs at the *sn-1* or *sn-3* positions of the TAG. Polyunsaturated fatty acids C16:1 and C18:2n-6 were also released at the end of digestion (Fig. 5), but at lower levels (2.97 ± 0.34% and 4.07 ± 0.52%, respectively). The total polyunsaturated FFA released at the end of the *in vitro* digestion of TAG emulsion is calculated to be around 11% of total FFAs released (Fig. 5).

3.4.2. Liposomes lipolysis during *in vitro* gastrointestinal digestion. The lipolysis of liposomes was tested under *in vitro* conditions mimicking the physiological conditions found in the GI tract. Some FFA were already observed at T0 (Fig. 6A). These FFA were also found in the lecithin fraction (Fig. 6B, lane 1), used for liposome substrate preparation (Fig. 6B, lane 2), indicating that a slight hydrolysis of phospholipids had occurred in the course of marine lecithin extraction/purification process and was due to endogenous fish enzymes with phospholipase activity. The level of these FFA was 13.0 ± 1.8% of the total FA as determined by TLC-FID quantification (data not shown). In order to take in account only the % FFA released by the added digestive enzymes and for the calculation of the related hydrolysis level, the FFA initially presents in liposome substrate have been subtracted from those released upon digestion in Fig. 4 and 6C. After addition of the gastric extract, the FFA level did not increase (Fig. 6A, lane T0 to T29 and Fig. 6C) which is consistent with the absence of phospholipase activity in the gastric extract, and results obtained with individual enzymes with RGE on liposome (Fig. 2). Nevertheless, liposomes were found to be further hydrolysed and converted into FFA when pancreatic enzymes and bile salts were added (Fig. 6A, lane T35 to T90 and Fig. 6C). For individual species and hydrolysis levels quantification, only FFA release and PC and PE decreases were considered (Fig. 6C and 4). Lipolysis of liposome phospholipids was very efficient with PE and at a less extend with PC since they appeared to be hydrolyzed during the first 10 minutes of the intestinal phase (Fig. 6A and C). At this time point, their residual levels were estimated to be 41.4 μmoles and 74.2 μmoles, which corresponds to around 24% and 40% for

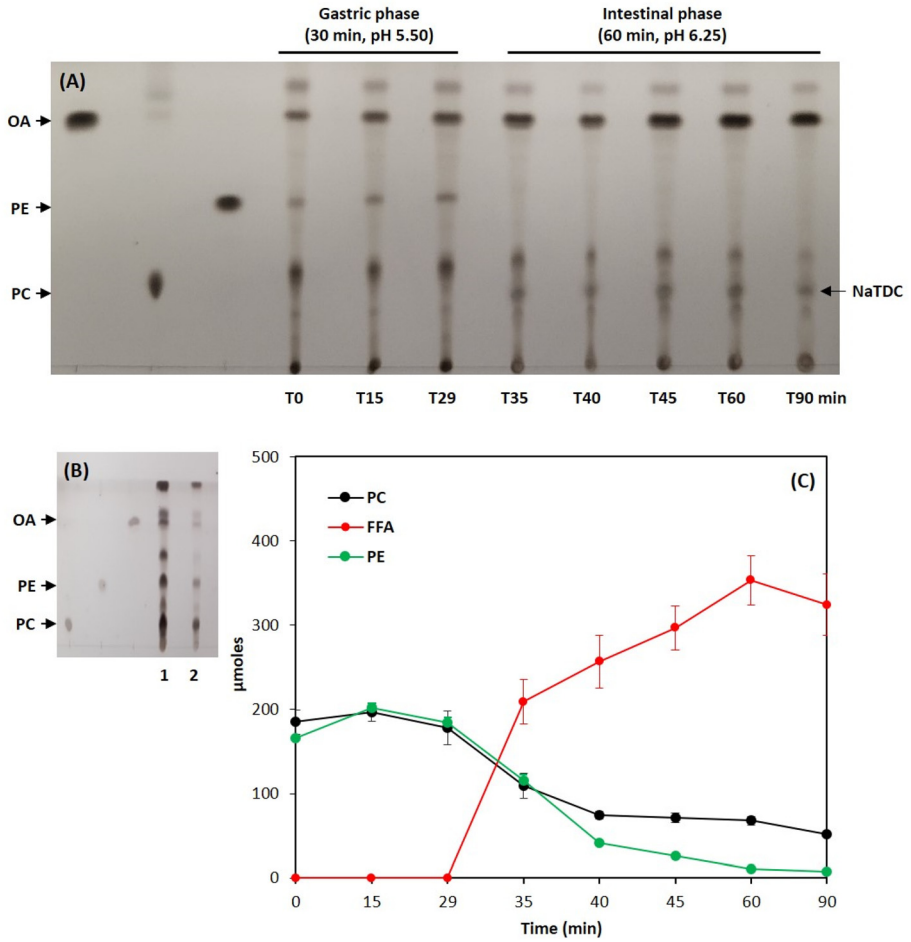

Fig. 6 *In vitro* digestion of liposome phospholipids. (A) TLC analysis of liposome hydrolysis in the course of the two-step static *in vitro* digestion. PC: standard phosphatidylcholine; PE: standard phosphatidylethanolamine; Lane T0 to T90: the different time point analysis. (B) TLC analysis of the initial lecithin (lane 1) used for liposome substrate (lane 2) preparation. (C) Variation with time of the level of the individual lipolysis products generated during the *in vitro* lipolysis of liposomes. Individual lipolysis product levels were measured by TLC-FID and expressed in µmoles present in the reaction mixture. FFA: free fatty acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine. Values are mean ± SD obtained from 3 independent hydrolysis experiments.

PE and PC, respectively (Fig. 6C). The hydrolysis level of liposome phospholipids already reached around 51% at this time point (Fig. 4). The PE and PC disappearance showed a reversed correlation with the increase in FFA level (256.7 µmoles, Fig. 6C) which corresponds to around 50% of total FA during the same period. At the end of the intestinal phase of digestion (t = 90 min), the hydrolysis level reached 73.7 ± 2.2% (Fig. 4), the total FFA liberated (384.2 ± 34.6 µmoles) constitutes around 74% of total FA and the residual PC and PE were 28% and 5%, respectively as determined by TLF-FID quantification (Fig. 6C). This high hydrolysis level might be explained by a combined action of pancreatic phospholipases (PLA2 and PLA1 activities), and the presence of bile salts. Although the initial DLS analysis showed that bile salts alone had a minor effect on the average size of the liposomes, they might gradually organise in smaller size particles in the course of digestion and are better hydrolysed by pancreatic phospholipases. Control experiments revealed that the addition of a solution mimicking the intestinal fluid and containing 10 mM NaTDC with no PPE, to the liposome substrate solution slightly affected the liposome size, after incubation at 37 °C, for 1 h. The DLS analysis showed particle sizes of 125 ± 6.4 and 118 ± 9.9 at t = 0 and t = 60 min of incubation compared to the initial size of the liposome substrate 144.14 ± 7.59 (data not show). One can therefore exclude a drastic destructuration of liposomes and the formation of mixed phospholipid/bile salts micelles that would have a much smaller size (around 5 to 40 nm (ref. 57)).

Thus, we have shown that liposomes are resistant to gastric conditions and are largely digested when they reached the upper small intestine conditions. With such a high level of phospholipid hydrolysis, the probability that liposome structure still exists is very low. Therefore, these liposomes appear as a perfect vehicle for delivering hydrophilic molecules in the small intestine while preserving them from gastric environment (Fig. 7).

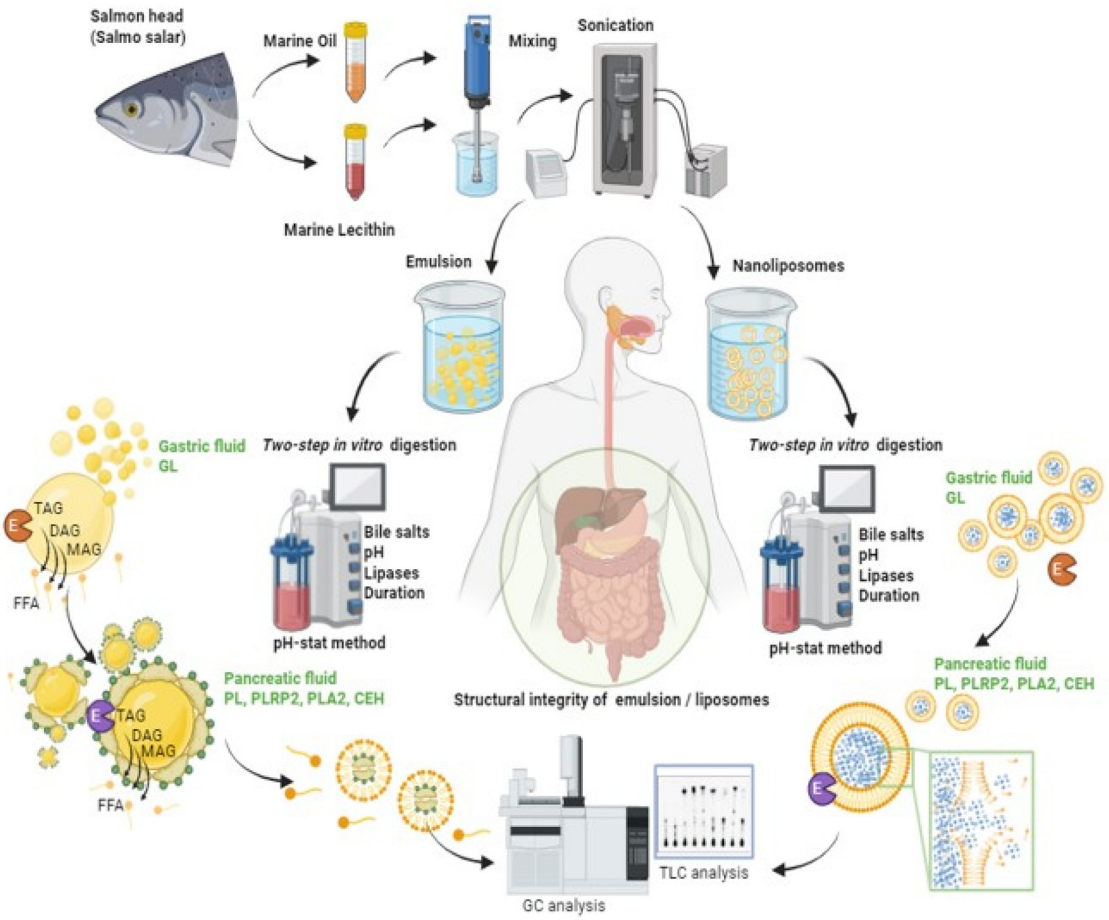

Fig. 7 Schematic presentation of the fate of marine lipids purified from salmon head during the *in vitro* digestion of TAG emulsion and PL liposomal solution. GL, gastric lipase; PL, pancreatic lipase, PLRP2, pancreatic lipase related protein 2; CEH, carboxylester hydrolase.

The composition of the FFA liberated upon digestion of PL liposomes was determined by GC after purification from silica plate. The results showed that C16:0 was the main FFA released at the end of digestion (55.53 ± 0.23% of total FFA released), which is consistent with its high proportion in PL fatty acids (21.22 ± 0.09% w/w; Table 1) followed by C14:0 (29.20 ± 6.62 of total FFA released) and C18:0 (6.58 ± 1.72% of total FFA released) (Fig. 5). Since these FA are mainly present at the *sn-1* position of the fish PL substrate,[58] the results indicate that the phospholipid hydrolysis occurring during the intestinal phase involves at least a phospholipase A1 activity. Therefore, PLRP2 and not pancreatic PLA2 seems to be the most important enzyme from PPE involved. Indeed, PLRP2 is a phospholipase A1[59] and the phospholipase activity of GPLRP2 on PL liposomes was shown here (Fig. 2 line 11).

In contrast to the digestion of TAG, significant levels of C18:3n-3 and C22:5n-3 were released from liposomes phospholipids (Fig. 5), in accordance with the PLA2 activity present in the PPE (Fig. 3, lane 7) and the distribution of these FA at the *sn-2* position of marine PL.[3,18] Nevertheless, the total polyunsaturated FFA released at the end of the *in vitro* digestion of liposomes (around 9% of total FFA released) was similar to that calculated for TAG emulsion (around 11% of total FFA released (Fig. 5)). No EPA and DHA were observed. Since these fatty acids are mainly located at the *sn-2* position,[58] this results suggest the low phospholipase A2 activity level in the PPE. Besides, the low phospholipase A2 activity obtained in these conditions (intestinal phase contains 10 mM NaTDC and performed at pH 6.25) fits with the individual activity of PLA2 on PL liposomes in presence of bile salts and at pH 6.0 compared to same results obtained in absence of bile salts and at pH 8.0 (Fig. 2 line 7).

We have seen that, in the case of TAG, the absence of EPA and DHA may simply results from a low lipolysis level and the preservation of the ester bond at the *sn-2* position of TAG where most LC-PUFA are located. Aursand *et al.*[56] used ^{13}C NMR to study positional distribution of fatty acids in TAG in salmon oil and found the majority of DHA in the *sn-2* position while EPA was more randomly distributed on all three positions. In the case of phospholipids, LC-PUFA can remain esterified at the *sn*-2 position of lysophospholipids after the removal of the fatty acid at the *sn-1* position. Lysophospholipids were however not quantified in this study nor their FA analysed.

Previous studies have shown that EPA and DHA present at the *sn-2* positions are more readily absorbed compared to the structured lipids in which these fatty acids are in one of the other positions in the molecule.[18] It has been also shown that lysophospholipids with DHA at the *sn-2* position were preferentially absorbed and more efficiently transported to the brain by the acetyl, docosahexaenoyl-glycerophosphocholine (AceDoPC).[60]

To conclude, it seems that the digestion of PL liposomes is in part insured by the phospholipase A1 activity contained in pancreatic extracts PPE. One explanation could be that in the presence of several phospholipases provided by pancreatic extracts, phospholipase A1 (PLRP2) acts first on PL and generates 2-lysophospholipids which was slightly hydrolyzed by phospholipase A2. The 2-lysophospholipid would be absorbed as such in the intestine.

4. Conclusion

The objective of this study was to compare the lipid digestion of oil-in-water emulsion and liposomal solutions formulated from neutral lipids and phospholipids, extracted from salmon heads (*Salmo salar*). The lipolytic enzymes involved in this digestion were initially identified by testing individual enzymes and the specificity of their action on each of the formulations was defined. Subsequently, an *in vitro* gastrointestinal digestion of these formulations rich in LC-PUFAs (EPA and DHA) was conducted using a two-step *in vitro* digestion model to study the stability of these carriers. The FFA analysis showed that DHA and EPA were absent among the FA released from both formulations used. This supports the fact that LC-PUFAs are predominantly esterified at the *sn*-2 position of TAG and phospholipids, in accordance with the literature. Fatty acids esterified at the *sn*-2 position are less hydrolyzable by lipases (as is the case for emulsions) or by enzymes with phospholipase A1 activity such as GPLRP2, and to lower extent CEH, in the case of liposomes. Marine-origin nanoliposomes are naturally richer in LC-PUFAs than TAG and they are not submitted to lipolysis in the stomach. This will allow increased protection of vectorized biomolecules, such as EPA and DHA, as well as the much more assimilable 2-lysophospholipid form at the intestinal barrier, as described in the literature.

Conflicts of interest

The authors have declared no conflict of interests.

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