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ABSTRACT

This study compared the bioaccessibility of docosahexaenoic acid (DHA) provided encapsulated or unencapsulated within a food matrix. DHA oil was composed of DHA-enriched triacylglycerols prepared as Pickering emulsion by encapsulation with heat-denatured whey protein isolate particles and then incorporated into homogenized liquid egg to get omelets. The effect of encapsulation was analyzed by using a static *in vitro* digestion model of the adult, which digestive fluid enzymes have also been characterized by proteomics. First, the size of lipid droplets was shown to be smaller and uniformly dispersed in omelets with encapsulated-DHA oil compared to non-encapsulated-DHA oil. Distribution of droplets was more regular with encapsulated-DHA oil as well. As a consequence, we showed that encapsulating DHA oil promoted the hydrolysis by pancreatic lipase during the intestinal phase. A larger proportion of DHA enriched-triacylglycerols was hydrolyzed after two hours of digestion, leading to a greater release in free DHA. Thus, only 32% of DHA remained esterified in the triacylglycerols with encapsulated-DHA oil, compared to 43% with non-encapsulated-DHA oil. The DHA in free form ultimately represented 52% of the total DHA with encapsulated-DHA oil, compared to 40% with non-encapsulated-DHA oil. Finally, our results showed that as much DHA was released after one hour of intestinal digestion when the DHA oil was encapsulated as after two hours when the DHA oil was not encapsulated. Therefore, DHA bioaccessibility was significantly improved by encapsulation of DHA oil in omelets.

1. Introduction

DHA is one of the most important n-3 polyunsaturated fatty acids (PUFAs), mainly known for its health benefits on cognitive development and cardiovascular functions (Bercea et al., 2021; Joffre et al., 2020; O'Connell et al., 2020). Although the recommended dietary intake is consensually estimated at 250 mg per day of DHA, only 14.6 % of the adult population in France appeared to meet the required level (Tressou et al., 2016). DHA may be either *de novo* synthesized from its essential precursor, the α-linolenic acid, or brought as such by food. In the first case, the rate of synthesis is limited in human (Burns-Whitmore et al., 2019; Calder, 2016; Ruxton et al., 2005) so that the food intake of DHA is necessary. While marine fish represents the richest dietary source of DHA, a large part of the population consumes little or no fish. Therefore, fortification of food with DHA is considered as an efficient strategy to reach the recommended intake threshold. However, many challenges are associated with the use of DHA oil in food products such as undesirable fishy flavors and short product shelf-life, a low water solubility reducing the bioaccessibility of DHA in the gastrointestinal tract, and a high risk of oxidation which further leads to the development of rancid odor and taste (Liu et al., 2021; Ma et al., 2020; Singh et al., 2018). As each side effect may finally reduce the potential of DHA on the body functions, integrating DHA oil into food products requires appropriate delivery systems to encapsulate, protect, and incorporate DHA oil into the diet. Numerous emulsion-based delivery systems were reported for encapsulating fish oil (DHA rich oil) to improve its water solubility, its physicochemical stability and its bioaccessibility as well (Haug et al., 2011; Lin & Wright, 2018; Solomando et al., 2020; Viciano et al., 2017; Y. Wang et al., 2016). By bioaccessibility is meant the fraction of a compound which is released from its food matrix in the gastrointestinal

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tract and which thus becomes available for intestinal absorption. However, traditional emulsions are easily affected by environmental conditions such as pH, temperature and ionic strengths. Compared with traditional emulsion, Pickering emulsification of dietary oils has received significant interest as it relies on irreversible adsorption of particles rather than on synthetic surfactants to the oil–water interface, which confers resistance to droplet coalescence and improves the stability of emulsion (Chevalier & Bolzinger, 2013; Li et al., 2019; Low et al., 2020; Wu & Ma, 2016). Based on our previous research, Pickering emulsions were here prepared by encapsulation of DHA oil with heat-denatured whey protein isolate (WPI) as particles (Wang et al., 2020). This emulsion showed a better stability against oxidation than the unencapsulated DHA oil, the characteristic of which is very important during the food process. The encapsulation of the DHA oil in the present study used natural encapsulant materials, allowing a food-grade preparation for further analyses. In this regard, egg products were selected as the target food, as an inexpensive source of high-quality proteins, essential vitamins and minerals. Our previous research showed moreover that the highest bioavailability of DHA was reached with omelet, as compared to various other egg recipes (hard-boiled egg and mousse) (Pineda-Vadillo et al., 2021). Bioavailability means the fraction of component absorbed and available in the body for metabolic use of target tissues. Consequently, omelet was selected as the food model to study the impact of encapsulation of DHA oil on the digestion process.

Several studies have reviewed the importance of a dietary supply of DHA as a strategy to improve DHA accretion in tissues, and especially in brain. Some authors suggested that dietary interventions may be effective in targeting serum lipid pools important for brain DHA uptake (Lacombe et al., 2018). Thereby different works have shown a higher bioavailability of DHA esterified to phospholipids (PLs) as compared to DHA esterified to triacylglycerols (TAGs) (Maki et al., 2009; Ulven et al., 2011; Ramprasath et al., 2013). Other studies contradicted however this finding in showing an equivalent bioavailability between DHA from PLs and DHA from TAGs (Carnielli et al., 1998; Schuchardt et al., 2011; Yurko-Mauro et al., 2015). And others were even more controversial (Ghasemifard et al., 2014; Gázquez & Larqué, 2021), showing that a higher concentration of circulating DHA would not predict brain accretion of DHA (Gázquez et al., 2017; Adkins et al., 2019; Destaillats et al., 2018). In parallel, similar digestion and intestinal absorption were observed *in vivo* between DHA-PLs and DHA-TAGs (Sehl et al., 2019). All these data underline the importance of several factors necessary for a good accretion of DHA in the tissues: the lipid structure, the characteristics of the food vector guaranteeing in particular the stability against oxidation, and the efficiency of digestion. Based on this knowledge, the DHA oil used in this study was enzymatically prepared from fish oil as DHA-enriched TAGs. The DHA oil was then encapsulated with heat-denatured WPI particles as Pickering emulsion, and finally incorporated into homogenized egg liquid and cooked to get omelets. Bioaccessibility of encapsulated DHA oil as compared to non-encapsulated DHA oil was then assessed by using a static *in vitro* digestion model for adults according to the INFOGEST protocol.

2. Material and methods

2.1. Ingredients and reagents

WPI (Lactalis Ingredients, Bourgbarré, France) contained 90 % protein, 5.1 % moisture, 3.0 % lactose and 1.97 % ash (Madadlou et al., 2018). DHA oil was prepared from fish oil by Polaris (Quimper, France). Eggs were from Moisan aviculture (Plestan, France). All reagents were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France) and solvents were purchased from Fisher Scientific (Elancourt, France).

2.2. Preparation of Pickering emulsion and omelets

DHA oil was encapsulated with heat-denatured WPI as described previously (J. Wang et al., 2020). Omelet was prepared with a whole egg homogenized by ultraturrax (10000 rpm, 30 *sec*). Then encapsulated-DHA oil (EN-DHA-O), heat-denatured whey protein dispersion alone (Control) or completed with the DHA oil (UN-DHA-O) were added to liquid eggs and mixed by stirring (500 rpm, 5 min). Eggs were finally molded and cooked in a water bath (80 °C, 10 min).

2.3. Static in vitro digestion model

In vitro digestion was carried out according to the standardized INFOGEST protocol for adults (Minekus et al., 2014). Briefly, the oral phase was prepared by mixing 5 g of mortar-destroyed omelet with 4 mL simulated salivary fluid (SSF) and 1 mL of 7.5 mmol/L $CaCl_2$. The bolus was incubated for 2 min at 37 °C in a water bath under constant stirring at 170 rpm before the beginning of the digestion process. Then, the gastric phase was prepared by mixing the oral bolus with 8 mL simulated gastric fluid (SGF), 5 μL of 0.3 mol/L $CaCl_2$, 290 μL of 3 mol/L HCl to adjust the pH to 3, and 705 μL of distilled water. After the addition of 0.5 mL porcine pepsin solution (2000 U/mL, P6887, Sigma-Aldrich) and 0.5 mL rabbit gastric lipase (RGE, 70 U/mL, Lipolytech®, Marseille, France), the gastric phase was incubated for 2 h at 37 °C under stirring at 170 rpm. Finally, the intestinal phase started thereafter by adding 8.5 mL simulated intestinal fluid (SIF), 40 μL of 0.3 mol/L $CaCl_2$, 960 μL of 1 mol/L NaOH to adjust the pH to 7, 3 mL of distilled water and 2.5 mL bovine bile extract solution (B3883, Sigma-Aldrich) to obtain 10 mmol/L of bile salts in the final digestion volume. After the addition of 5 mL porcine pancreatin solution made up in SIF (1625 U/mL of lipase activity and 100 U/mL of trypsin activity, P7545, Sigma-Aldrich), the solution was incubated for 2 h at 37 °C under stirring at 170 rpm. For each digestion point, two 500 μL samplings, one for microscopy observation and another one for lipid analyses, were collected and blocked by adding enzyme inhibitors as described elsewhere (Brodkorb et al., 2019).

2.4. Confocal microscopy

Distribution of the DHA oil in omelets during the digestion process was followed by confocal laser scanning microscopy by using a ZEISS LSM 880 microscope (Zeiss, Germany) with a 63X magnification (Plan Apochromat objective, oil immersion, NA1.4). The DHA oil was stained by Nile Red (120 mg/g oil) before the preparation of Pickering emulsion. Proteins were then dyed with Fast Green (50 μL of a 1 % aqueous solution, 10 min at 20 °C) on omelet slices and digested samples. Fluorescence of the DHA oil was obtained by a 488 nm excitation wavelength coupled with a GaasP detector between 550 and 590 nm. Detection of egg proteins was performed by excitation at 633 nm coupled with a PMT detector between 635 and 735 nm. The ZEN lite black software (Zeiss, Germany) was used to process the images produced. Each digestion point was analyzed twice with at least 8 repetitions.

2.5. Lipid analyses

Lipids were extracted according to the Folch's method (Folch et al., 1957) and lipid classes were separated by thin layer chromatography on silica gel-60 plate (Merck, Darmstadt, Germany). Neutral lipids were primarily separated from polar lipids by using a mixture of diethyl ether: acetone 60: 20 (v:v) and were subsequently separated by species with the mixture hexane: diethyl ether: acetic acid 60: 40: 1 (v:v:v). Each lipid species was identified by authentic standards after spraying with primulin and then scrapped off to perform the FA profile. Briefly, collected silica gel was saponified with 0.5 mol/L NaOH in methanol at 70℃ for 20 min and methylated with BF_3 (14 % in methanol) at 70 ℃ for 15 min (Wang et al., 2022). The FA methyl esters were extracted with pentane and then separated by a QP 2010-SE gas chromatograph coupled to a mass spectrometer (MS, Shimadzu, Marne-La-Vallée, France) equipped with a BPX70 capillary column (120 m, 0.25 mm i.d., 0.25 μm film) from

SGE Trajan (Chromoptic, Paris, France). Helium was used as carrier gas at a constant velocity of 27,5 cm/*sec*. The injector temperature was adjusted to 250 °C. The column temperature ramped from 50 °C to 175 °C at 20 °C/min and then from 175 °C to 240 °C at 2 °C/min. The mass spectrometer was operated under electron ionization at 0.2 keV and 200 °C source temperature. Analyses were performed in scan mode over the *m/z* range of 30–450 amu. Components were identified according to the retention time of authentic standards and by using the National Institute of Standards and Technology mass spectral library (version 2.01). Concentrations were determined by using two internal standards (Larodan, Coger SA Paris, France), the first one as TAG-17:0 added before the lipid extraction and the second one as 19:0 in the free FA form added after silica collection. Calibration was performed by standard curves of FA methyl esters. The results were expressed in mass percentages of the total FAs where 100 % designates the sum of the proportions of all the FAs identified, or in mass percentages of the FA considered where 100 % designates the sum of the proportions of the FA considered esterified to each lipid species.

2.6. Proteome of fluids

An exhaustive identification of all the enzymes present in the gastric fluid (SGF + pepsin + RGE) and the intestinal fluid (SIF + pancreatin) was performed by proteomic analysis. Briefly, sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed to concentrate proteins on gel by applying a migration of one centimeter with a Tris-glycine saline buffer. Carrots of gels containing ~ 10 μg of proteins were then digested by porcine sequencing grade trypsin (Promega, Charbonnières-les-Bains, France). Peptide profiles were finally determined by nano liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) as previously described (Huang et al., 2018). Briefly, experiments were performed using a nano RSLC Dionex U3000 system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, CA, United States) equipped with a nano-electrospray ion source. A preliminary sample concentration step was performed on a C18 pepMap100 reverse-phase column (C18 column, 300-μm inner diameter by 5 mm length, 5 μm particle size, 100 Å pore size; Dionex, Amsterdam, Netherlands). Peptide separation was performed on a reversed-phase column (PepMap RSLC C18, 75 μm inner diameter by 250 mm length, 3 μm particle size, 100 Å pore size; Dionex) with a column temperature of 35 °C, using solvent A (2 % acetonitrile, 0.08 % formic acid and 0.01 % trifluoroacetic acid in deionized water) and solvent B (95 % acetonitrile, 0.08 % formic acid and 0.01 % trifluoroacetic acid in deionized water). Peptides were separated using a gradient of 5–35 % solvent B over 80 min followed by 35 to 85 % solvent B over 5 min at a flow rate of 0.3 μL/min. Eluted peptides were directly electro sprayed into the mass spectrometer operating in positive ion mode with a voltage of 2 kV. Spectra were recorded in full MS mode and selected in a mass range 250–2000 *m/z* for MS spectra with a resolution of 70,000 at *m/z* 200. For each scan, the ten most intense ions were selected for fragmentation. MS/MS spectra were recorded with a resolution of 17,500 at *m/z* 200 and the parent ion was subsequently excluded from MS/MS fragmentation for 20 s. The instrument was externally calibrated according to the supplier's instructions. Peptides were identified from the MS/MS spectra using X!Tandem pipeline software (Langella et al., 2017). The search was performed against a database composed of proteomes of bovine (*Bos Taurus*), rabbit (*Oryctolagus cuniculus*) and pig (*Sus scrofa*) downloaded from Uniprot.org (on the April 12, 2022) to which was added the common Repository of Adventitious Protein (https://thegpm.org/crap). Database search parameters were specified as follow: trypsin cleavage was used and the peptide mass tolerance was set to 10 ppm for MS and 0.05 Da for MS/MS. Oxidation of methionine and phosphorylation on threonine, serine and tryptophan were selected as a variable modification. For each peptide identified, a minimum score corresponding to an e-value below 0.05 was considered as a prerequisite for peptide validation.

2.7. Statistical analysis

All experiments were performed three times on 3 independent samples. Results are expressed in mean ± SD. The DHA content in omelets was firstly analyzed by using a one-way analysis of variance followed by a post-hoc test depending on the normality of the data distribution. Lipid analyses on digested omelets were then assessed by a two-way analysis of variance followed by a Tukey's test. Statistical analyses were completed by the lipid species representation and the individual factor map for each repetition of omelets. Significant differences were mentioned by an asterisk or different letters when $p < 0.05$. Analysis was performed with the R software (version 4.0.5) and figures were made with Graph Pad Prism 7.

3. Results

3.1. Characterization of the digestion model

3.1.1. Digestion fluids

The fluids used during the digestion process were analyzed in order to specify the catalytic nature of the enzymes present. First, concerning the gastric phase, the results showed that the pepsin samples contained

Table 1
Digestive enzymes identified by LC-MS-MS in simulated digestion fluids. Proteins were separated by gel electrophoresis and carrots of gels were then trypsinated. Peptides were further analysed by LC-MS-MS to be identified.

Enzymes	Gene number	Protein coverage	Unique peptides	PAI *
Pepsin (Sus scrofa)				
Pepsin B	PGB	20.2 %	5	2.75
Chitinase	CHIA	49.2 %	14	2.50
RGE (Oryctolagus cuniculus)				
Lipase	LIPF	52.4 %	19	5.50
Peptidase A_1 domain-containing protein	LOC100328620	25.3 %	6	2.50
Pepsin II-1	P28712**	20.4 %	6	1.71
Ceramidase (*N*-acylsphingosine amidohydrolase)	ASAH1	13.0 %	3	0.21
Pancreatin (Sus scrofa)				
Carboxypeptidase A_1	CPA1	73.1 %	20	2.82
α-Amylase	AMY2	72.2 %	29	2.60
Trypsin	P00761**	74.0 %	13	1.91
Chymotrypsin-like elastase family member 2A	CELA2A	32.7 %	7	1.80
Peptidase S1 domain-containing protein	LOC100621820	24.7 %	6	1.60
	LOC110261434	41.5 %	9	1.33
	CTRB2	21.4 %	5	1.00
Chymotrypsin-C	CTRC	46.6 %	8	1.50
Trypsinogen isoform X1	LOC100302368	36.2 %	6	1.18
Carboxypeptidase B	CPB1	50.0 %	14	1.18
Pancreatic TAG lipase	PNLIP (PNLIPRP1)	47.3 %	12	1.22
Chymotrypsin-like	CTRL	37.3 %	6	0.91
Chitinase	CHIA	29.5 %	7	0.67
Phospholipase A_2 minor isoenzyme	PLA2G1B	11.3 %	2	0.50
Pancreatic lipase-related protein 2	PNLIPRP2	21.4 %	8	0.48
Phospholipase A_2 major isoenzyme	PLA2G1B	9.6 %	2	0.40
Dipeptidase	DPEP1	25.2 %	7	0.39
Cysteinylglycine-S-conjugate dipeptidase	LAP3	14.6 %	7	0.28
Bile (Bos taurus)				
α-amylase	AMY2B	11.9 %	5	0.39

* Proteins were classified according to their protein abundance index (PAI).
** Accession number.

pepsin B but also chitinase (Table 1 and supplemental Table 1). RGE was composed of lipase but also non-negligible amount of pepsin II-1 and peptidase A1 domain-containing protein. Interestingly, RGE contained ceramidase although probably in low concentration. Then concerning the intestinal phase, classical enzymes involved in protein digestion have been identified. Moreover, lipid digesting enzymes like pancreatic TAG lipase and phospholipase A_2 have been identified. The bile was almost protein-free, although α-amylase was identified. In conclusion, in the fluids analyzed, three enzymes responsible for lipid digestion, namely lipase, phospholipase and ceramidase were identified.

3.1.2. DHA content in omelets

The DHA oil used in this study was prepared from fish oil and was further characterized (Table 2). It was mainly composed of DHA esterified on TAGs (96.7 %), but 3.3 % of the total DHA was found in minor lipid species as well, such as diacylglycerols (DAGs). DHA was the major FA while eicosapentaenoic acid (EPA) and docosapenaenoic acid n-3 (DPAn-3) were also measured at 36 μg/mg and 31 μg/mg respectively (not shown). When the DHA oil was encapsulated as Pickering emulsion, DHA represented 344 μg/mg without modification of the lipid species profile. Then omelet was used as a food matrix, the composition of which was composed of a very small amount of DHA (0.6 % of total FAs) esterified in PLs. When omelets were cooked with DHA oil or with encapsulated DHA oil, DHA reached 9.0 % and 10.3 % of total FAs respectively. It was mainly supplied as TAGs representing 86 % of the total DHA in DHA-enriched omelets. However, heating eggs to get omelets had an effect on the lipid species, as TAGs were partially hydrolyzed into 1,2-DAGs during the food processing. Therefore, we found an increased proportion of DHA esterified in 1,2-DAGs in DHA enriched-omelets as compared to the original DHA oil, whereas the other lipid species were not impacted by the cooking.

3.1.3. Distribution of encapsulated DHA oil in omelets

The DHA oil was incorporated in omelets either in bulk or encapsulated with heat-denatured WPI. The microstructure of Pickering emulsion emphasized the protein layer on the surface of the DHA oil droplets (Fig. 1). However, protein particles were not clearly visible by confocal microscopy since the particle size of heat-denatured WPI was too small, around 42 nm as measured in a previous work (Wang et al., 2020). Then when incorporated in omelets, the DHA oil was differently distributed according to the delivery form (Fig. 1). Droplets of encapsulated DHA oil were uniformly dispersed in omelets with an equivalent repartition of DHA oil in terms of size of droplets. On the contrary, non-encapsulated DHA oil exhibited a more random dispersion in omelets as bigger droplets, but with a wide range of droplet sizes.

3.2. Distribution of DHA oil droplets during the digestion of omelets

Distribution of the DHA oil was followed during digestion of omelets. Firstly, the control omelets deprived of DHA oil showed no red staining (Fig. 2). Protein digestion started during the gastric phase and was particularly emphasized with the reduced size of protein-stained particles in the intestinal phase. Secondly, in the DHA-enriched omelets, the size and the dispersion of DHA oil droplets with UN-DHA-O were less uniform in the beginning of the gastric phase than with EN-DHA-O. Moreover, DHA oil repartition seemed stable during the gastric digestion, whereas the hydrolysis of DHA oil occurred in the intestinal phase as visualized by the reduction of the red-stained droplets. However, using this method of analysis, differences between UN-DHA-O and EN-DHA-O were difficult to quantify. We further analyzed the digestion process by lipochemistry.

3.3. Hydrolysis of lipids and release of fatty acids during the digestion of omelets

Omelets contained initially 92 μg FAs per mg, esterified as TAGs (73 %), PLs (24 %) and other minor species. Digestion of lipids was followed during the gastric and the intestinal phases by the FA profile of the different lipid species present in omelets (Fig. 3). At first, the control omelet showed a higher proportion of FAs in PLs to the detriment of TAGs, as compared to the global FA profile from DHA-enriched omelets. The difference averaged 10 % of total FAs due to the addition of the DHA oil as TAGs and was noticeable during the entire digestion process. Then, concerning more specifically DHA-enriched omelets, the main lipolysis occurred on TAGs during the gastric phase and was completed thereafter during the intestinal phase together with the phospholipid hydrolysis.

In the gastric phase, TAGs were hydrolyzed to release 1,2-DAGs and free FAs. After 2 h of lipase catalysis, around half of TAGs remained both with UN-DHA-O and EN-DHA-O. Meanwhile, total FAs from 1,2-DAGs increased from 1 % at G0 to 9 % at G120, whereas free FAs raised from 0.7 % at G0 to 30 % at G120, independently of the DHA oil delivery form. No other lipid species evolved otherwise.

In the intestinal phase, less than 10 % of TAGs remained after one hour of digestion for both UN-DHA-O and EN-DHA-O, whereas the hydrolysis was complete for the Control. In parallel, 1,2-DAGs released during the gastric phase decreased to the benefit of the final product 2-monoacylglycerol (2-MAG) during the intestinal phase. PLs were hydrolyzed as well under the action of phospholipase A_2 or other enzymes. Collectively, complete lipolysis led to the release of free FAs, the proportion of which represented 79 %, 73 % and 75 % of the total FAs for Control, UN-DHA-O and EN-DHA-O respectively.

When all data were further considered in the statistical analysis, the representation of lipid species groups was mapped in two dimensions (Fig. 4A). PLs and esters of cholesterol (ECH) were positioned opposite

Table 2
Effect of emulsion and cooking on the concentrations and proportions of DHA in the omelet. Lipids were extracted by the Folch's method and separated by thin layer chromatography. The FA profile from different lipid species was determined by gas chromatography coupled with mass spectrometry. The DHA content was measured in total lipids and in different lipid species. Significant difference between oils or between omelets was indicated by different lowercase and capital letters respectively, when $p < 0.05$.

		Oil		Omelet		
DHA content		**DHA oil**	**EncapsulatedDHA oil**	**Control**	**UN-DHA-O**	**EN-DHA-O**
in total lipids (μg/mg)		615.4 ± 38.8	344.4 ± 27.3	0.5 ± 0.0 ^{A}	7.7 ± 0.7^{B}	9.1 ± 1.1^{B}
in total lipids (% of total FAs)		76.5 ± 0.6	80.8 ± 3.2	0.6 ± 0.1 ^{A}	9.0 ± 0.8^{B}	10.3 ± 0.7^{B}
in lipid species (% of total DHA)						
	TAG	96.7 ± 0.7 ^{a}	97.1 ± 0.2 ^{a}	Nd ^{A}	85.8 ± 1.2^{B}	86.1 ± 2.1^{B}
	1,2-DAG	1.7 ± 0.2 ^{a}	1.9 ± 0.1 ^{a}	Nd ^{A}	7.5 ± 0.8^{B}	8.3 ± 1.5^{B}
	1,3-DAG	1.3 ± 0.3	1.0 ± 0.3	Nd ^{A}	1.4 ± 0.2^{B}	1.1 ± 0.1^{B}
	MAG	Nd	Nd	Nd	Nd	Nd
	FFA	Nd	Nd	Nd	Nd	Nd
	PL	Nd	Nd	100.0 ± 0.0 ^{A}	5.4 ± 0.6^{B}	4.5 ± 0.6^{B}
	ECH	0.3 ± 0.1	0.1 ± 0.1	Nd	Nd	Nd

Nd: Not detected.

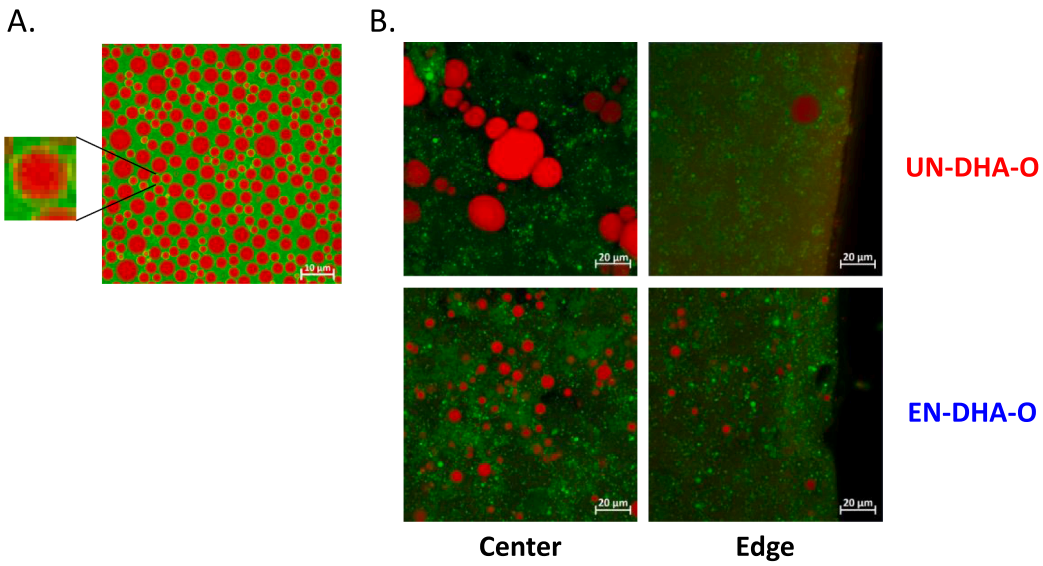

Fig. 1. Encapsulation of DHA oil. A. Encapsulated DHA oil with heat-denatured WPI. B. Distribution of unencapsulated and encapsulated DHA oil in omelets. DHA oil was encapsulated with heat-denatured WPI as Pickering emulsion. Unencapsulated or encapsulated DHA oil was incorporated to the whole liquid egg to get omelets. DHA oil was previously stained by Nile red (red color) whereas WPI was dyed with Fast Green (green color). Emulsion (A) and DHA oil distribution in omelets (B) were visualized by confocal laser scanning microscopy. Pictures were captured in the middle and at the edge of the samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

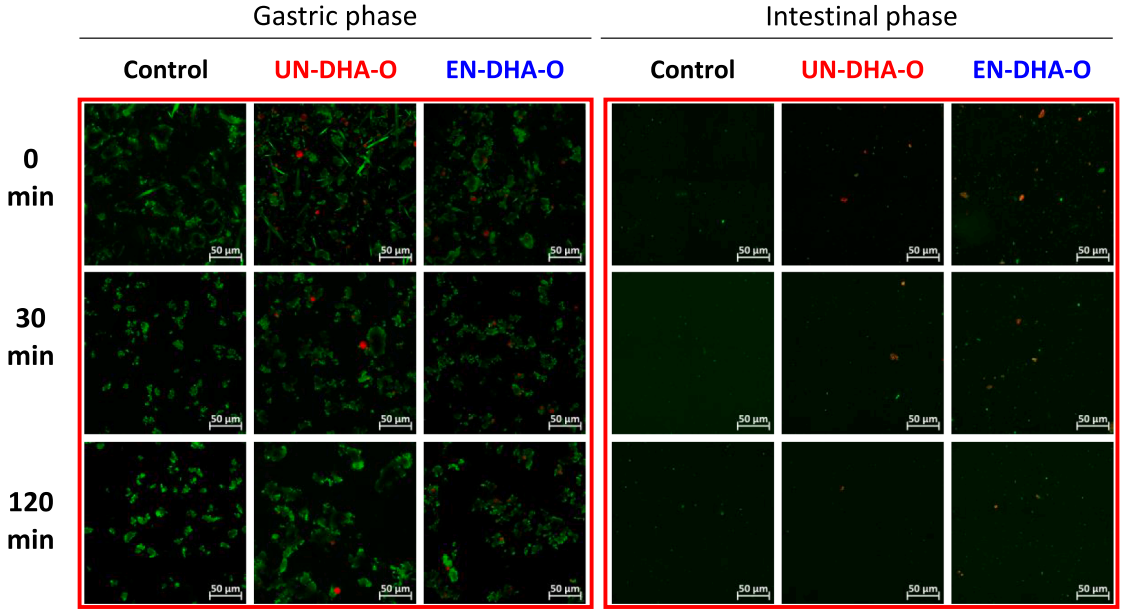

Fig. 2. Distribution of DHA oil in omelets during the gastric and intestinal phases of digestion. Unencapsulated or encapsulated DHA oil was incorporated to omelets. DHA oil was previously stained by Nile red (red color) whereas proteins were dyed with Fast Green (green color). Omelets were then digested by using the static INFOGEST model of adults. Distribution of DHA oil was finally followed by confocal laser scanning microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to neutral lipids and free FAs, in both axis dimensions. TAGs, considered as a discriminant lipid species of omelets, were not highlighted in this representation. The principal component analysis of samples showed however a high discrimination between DHA-enriched omelets and the control omelet, the representation of which was opposed in the first dimension of the individual factor map (Fig. 4B). The discrepancy between control and DHA-enriched omelets was specifically marked with TAGs as visualized on the multifactorial analysis (Fig. 4C). The effect was linked to specific FAs of the DHA oil, mainly EPA and DHA, but also 22:5n-6 and 24:1n-9, DHA and 22:5n-6 being less present in eggs unlike EPA and 24:1n-9 absent from eggs (Wang et al., 2022). Otherwise the second dimension showed no prominent difference between samples of omelets, this axis being represented by monounsaturated FAs as 16:1n-7 and 18:1n-7, and saturates like 16:0 and 18:0 (not shown). In conclusion, overall, the digestion pattern was uniform regardless of the samples, although the statistical analysis underlined a difference inherent in the enrichment of omelets with the DHA oil. We further studied the specific digestion of DHA oil supplied as such or encapsulated with whey protein isolate in omelets.

3.4. Hydrolysis of DHA oil and release of DHA during the digestion of omelets

Release of DHA from DHA oil was followed during the digestion process (Fig. 5). Firstly, DHA was not released during the gastric phase but only during the intestinal phase, whether esterified from TAGs or from PLs. This contrasts with the hydrolysis of total TAGs present in the samples, as the lipase digestion began in the gastric phase and released half of the total FAs esterified in TAGs (Fig. 3). Secondly, digestion of DHA-TAGs was more important with EN-DHA-O than with UN-DHA-O.

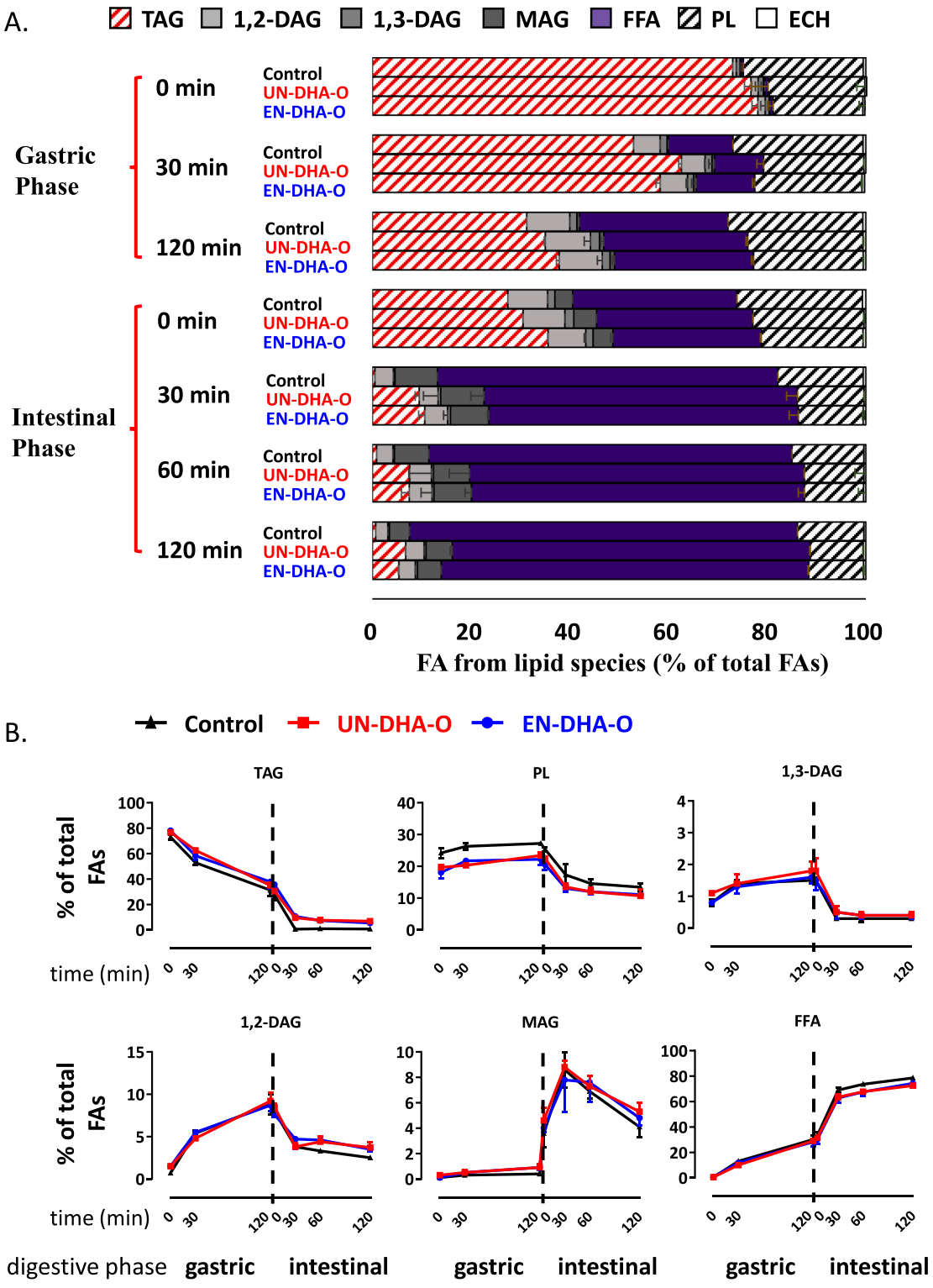

Fig. 3. The fatty acid proportions depend on the lipid species during digestion. Lipids were extracted by Folch's method and separated by thin layer chromatography. The FA profile from different lipid species was determined by gas chromatography coupled with a mass spectrometer. The FA proportion was determined according to the lipid species during the digestion.

Proportion of DHA from TAGs was reduced from 86 % to 32 % with encapsulated-DHA oil as compared to 43 % with non-encapsulated-DHA oil. Therefore, release of DHA as free FA reached 52 % of the total DHA with EN-DHA-O at the end of the intestinal digestion, against only 40 % of the total DHA with UN-DHA-O. Moreover, DHA from 1,2-DAGs and subsequently from 2-MAGs was equivalently measured in UN-DHA-O and EN-DHA-O during the intestinal phase. Likewise, DHA from egg yolk was shown to be presumably esterified in PLs mainly at the *sn*-2 position but possibly also at the *sn-1* position since 80 % of it was released after two hours of the intestinal hydrolysis. No difference between omelets was observed on the phospholipid catalysis. Altogether, these data showed that encapsulating DHA oil enhanced the lipase

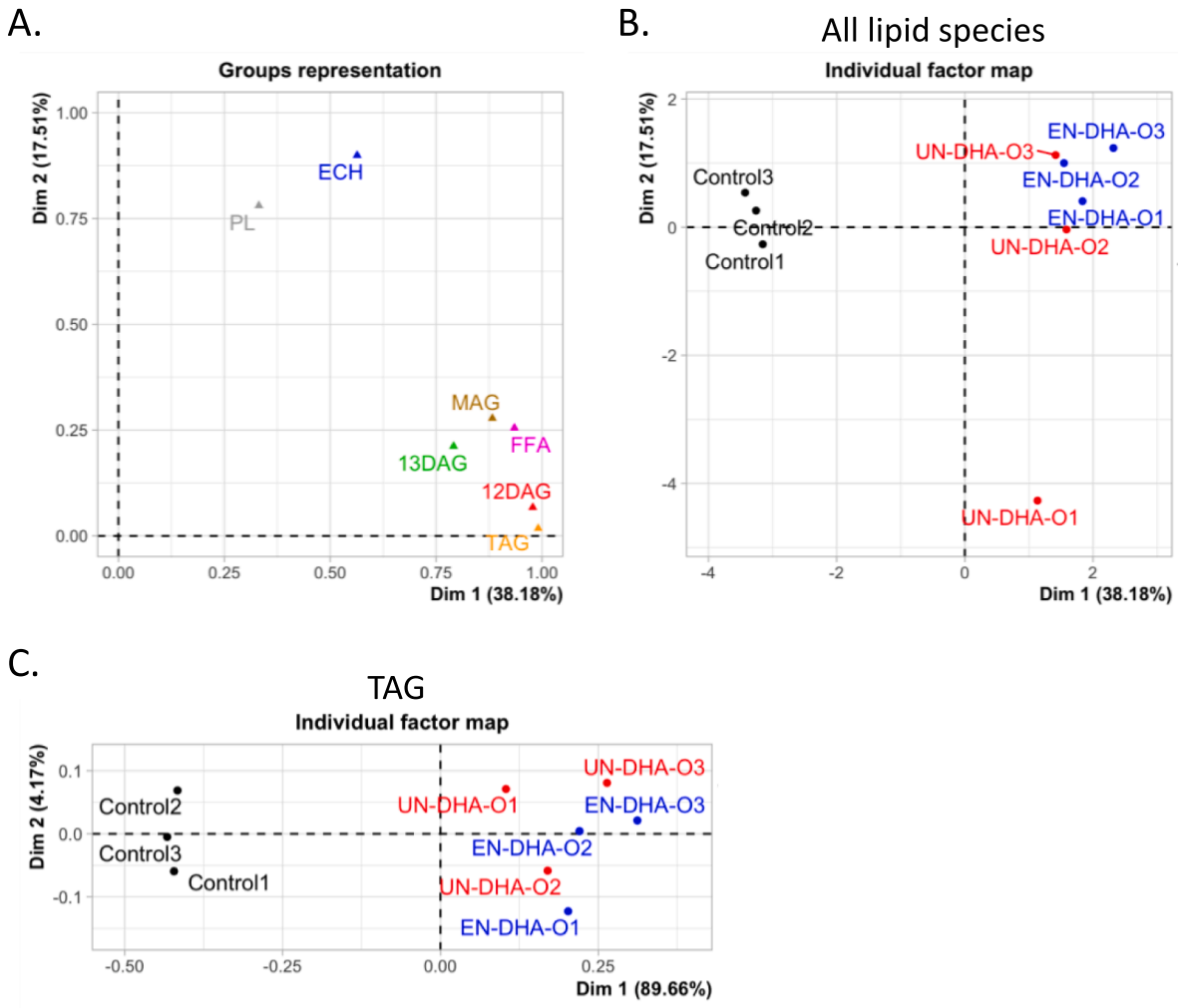

Fig. 4. The digestion process is analyzed by lipid species and by omelets. In A, each point represents a lipid species obtained from the digestion data. In B, each point represents a repetition from the digestion data. In C, each point represents TAGs of each repetition from the digestion data.

hydrolysis during the process of digestion.

3.5. Comparison of digestion profiles according to the nature of the fatty acid and its origin

The DHA oil also contains a very low amount of other very long chain FAs such as DPA (22:6n-3), EPA (20:5n-3) and nervonic acid (24:1n-9). The same profile of digestion as that of DHA was observed, without hydrolysis of TAGs during the gastric phase and a tendency for a greater FA release when the DHA oil was encapsulated (Fig. 6 and supplemental Fig. 1). This contrasts with another long chain PUFA present in egg and DHA oil, esterified mainly in PLs but also in TAGs, the arachidonic acid (ARA, 20:4n-6). Indeed, the ARA of the TAGs was hydrolyzed to almost 50 % during the gastric phase (Fig. 6). Similar results were obtained on other saturated and unsaturated FAs particularly abundant in omelets, but with a shorter chain length (C16, C18) (supplemental Fig. 2 and supplemental Fig. 3). 16:0, 18:1n-9, 18:2n-6 and 18:3n-3 esterified in TAGs were almost 60 % hydrolyzed after the gastric phase. In both cases (ARA and other C16-C18 FAs), no impact of DHA oil encapsulation was observed.

4. Discussion

The present study demonstrates that encapsulating DHA oil into Pickering emulsion allows to increase the bioaccessibility of DHA in the small intestine. Indeed, a 12 % increase of DHA released as free FA was observed in EN-DHA-O compared to UN-DHA-O. This increase was in line with the 11 % decrease of DHA in TAGs in EN-DHA-O compared to UN-DHA-O.

Very often, oil encapsulation is a way of limiting the hydrolysis and oxidation of compounds in the stomach in order to make them available in the small intestine for absorption. The present study showed that DHA was not released in the stomach at all in both EN-DHA-O and UN-DHA-O. This may be related to the origin of the gastric lipase as the enzyme was extracted from rabbit, an animal that never consumes food containing very long chain PUFAs such as DHA. Besides, the triglyceride structure carrying three DHA is, most certainly one of the best factors to explain this non-activity of gastric lipase. The spatial conformation of such a substrate molecule probably strongly modifies the affinity of the enzyme for it and thus the subsequent catalysis.

Therefore, the beneficial effect of encapsulation mainly occurred in the small intestine where encapsulation tended to exacerbate the release of DHA mainly at the end of the intestinal phase. It looks like that the heat-denatured whey protein shell that was surrounding the DHA oil was not an obstacle to the hydrolysis action of the pancreatic lipase. It could be interesting to investigate at which stage this protein shell was hydrolyzed allowing the digestive lipase to anchor at the interface of the lipid droplet. One possible explanation for the difference observed in the kinetics of release of DHA in the small intestine during *in vitro* digestion might be the difference observed in the lipid droplet size between the two omelets. Indeed, in UN-DHA-O, lipids appeared as non-homogenous large droplets that were visible by CLSM throughout the gastric phase. In

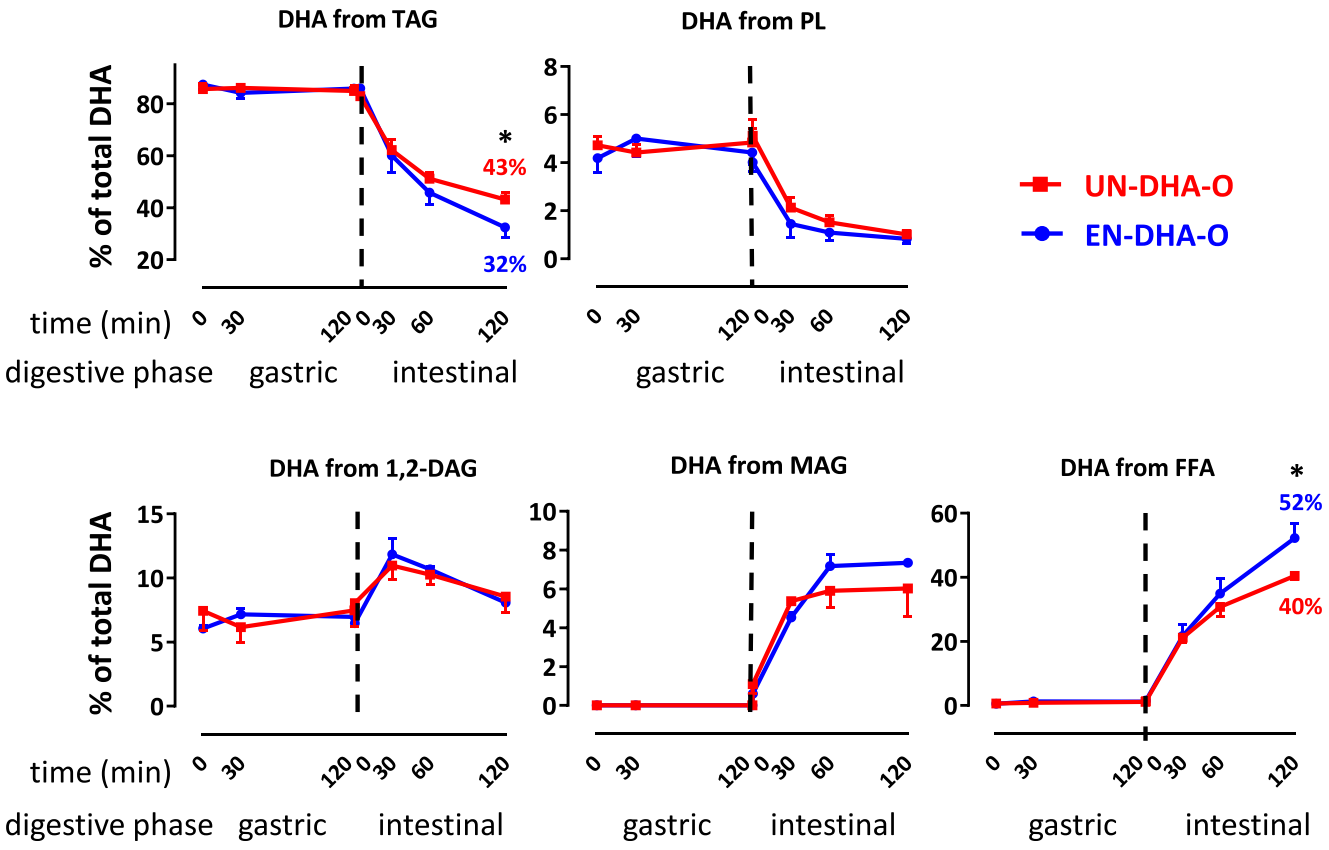

Fig. 5. The evolution of DHA from different lipid species during digestion. Lipids were extracted by Folch's method and separated by thin layer chromatography. The FA profile from different lipid species was determined by gas chromatography coupled with mass spectrometry. The total DHA corresponds to the sum of DHA esterified to each lipid species. * Indicates a significant difference between values at each time point ($p < 0.05$).

contrast, encapsulation of DHA oil led to the formation of a high number of small droplets of around 4.34 μm in diameter as previously described (Wang et al., 2020), that increased the specific surface available for digestive lipases. It has previously been shown that increasing the specific surface of lipids, by homogenization for instance, accelerates the kinetics of lipid digestion *in vitro* (Bourlieu et al., 2015) and *in vivo* (de Oliveira et al., 2017). The effect of oil encapsulation on DHA bioaccessibility has been investigated previously. Indeed, DHA encapsulation in caseinate/alginate microparticles enhanced its bioaccessibility (Ma et al., 2020). It was shown that the digestion of DHA microparticles led to more open loose structures than the large aggregated lipid droplets that were observed with DHA emulsion droplets. Consequently, the rate of lipid digestion was function of the surface area of lipids exposed to the lipase molecules as well as the capability of these enzymes to adhere and further contact the underlying TAG molecules. Similarly, the lipid hydrolysis, oxidative stability and bioaccessibility of algae oil (42 % of DHA) were compared for three lipid delivery systems, i.e bulk oil, soy protein stabilized O/W emulsion and carrageenan gelled emulsion (Gayoso et al., 2019). Emulsification techniques were linked to a slight 3 % increase in DHA released during *in vitro* gastrointestinal digestion. Finally, the addition of pectin to an emulsified algal oil rich in DHA was shown to limit lipid digestion and DHA bioaccessibility (Lin & Wright, 2018). The effect of pectin was attributed to its interaction with lipids and other digestive molecules during simulated gastrointestinal digestion that limited the action of pancreatic lipase.

At the beginning of the digestion process, TAGs and PLs were the main lipid species, accounting for approximately 73 % and 24 % of total FAs, respectively. In addition, there were a small amount of 1,2-DAGs, 1,3-DAGs, MAGs, free FAs and ECH. This result is in concordance with previous egg studies, which have shown that TAGs were about two-thirds and PLs were about one-third of the total lipids in eggs (Wang & Wang, 2009). Nevertheless, our results showed a release of FAs up to 70 % of the total FAs present in the samples, whereas we could only expect 61 % of FAs released from PLs (12 % from *sn*-2) and from TAGs (49 % from at *sn*-1 and *sn*-3). This difference may be provided by ceramide present in eggs (Fujino & Momma, 2008) as ceramides release only partially FAs during saponification used to determine total FAs, whereas the fluids used for the digestion process contained ceramidase as demonstrated in the study. This hypothesis seems, however, to be ruled out since ceramides usually contain very long chain FAs such as 24:0 and 22:0, which were not found in the control omelet. The second hypothesis was the contribution of the enzyme extracts. Other works in the team showed that enzymes used in the fluids were prepared without a complete delipidation. In order to follow the digestion of the samples selectively without the bias of that of the blanks, the digestion of the latter should be carried out in parallel.

Then, when looking at the evolution of the different classes of lipids over digestion, the present study has also shown that while TAGs started to be hydrolysed in the gastric phase, phospholipids were not affected and only slowly digested in the intestinal phase. This is in agreement with the fact that the gastric lipase exhibits a high hydrolytic activity towards TAGs, lower towards DAGs, is only slightly active on MAGs and has no action on PLs (Lengsfeld et al., 2005). In contrast, PLs are the favourite substrate of the pancreatic phospholipase A_2 (Borgström, 1993) and can be hydrolysed by other pancreatic enzymes such as the carboxylester hydrolase and the type-2 (Lombardo et al., 1980). The addition of DHA oil to the omelet did not affect the evolution of different lipid species during digestion. In gastric and intestinal phases, the released free FAs accounted for approximately 29 % and 46 % of the total FAs, respectively. It is consistent with previous *in vitro* studies that the lipolysis degree of lipids in gastric and in intestinal phase is 10–40 % and 40–70 %, respectively (Armand et al., 1999; Carriere et al., 1993).

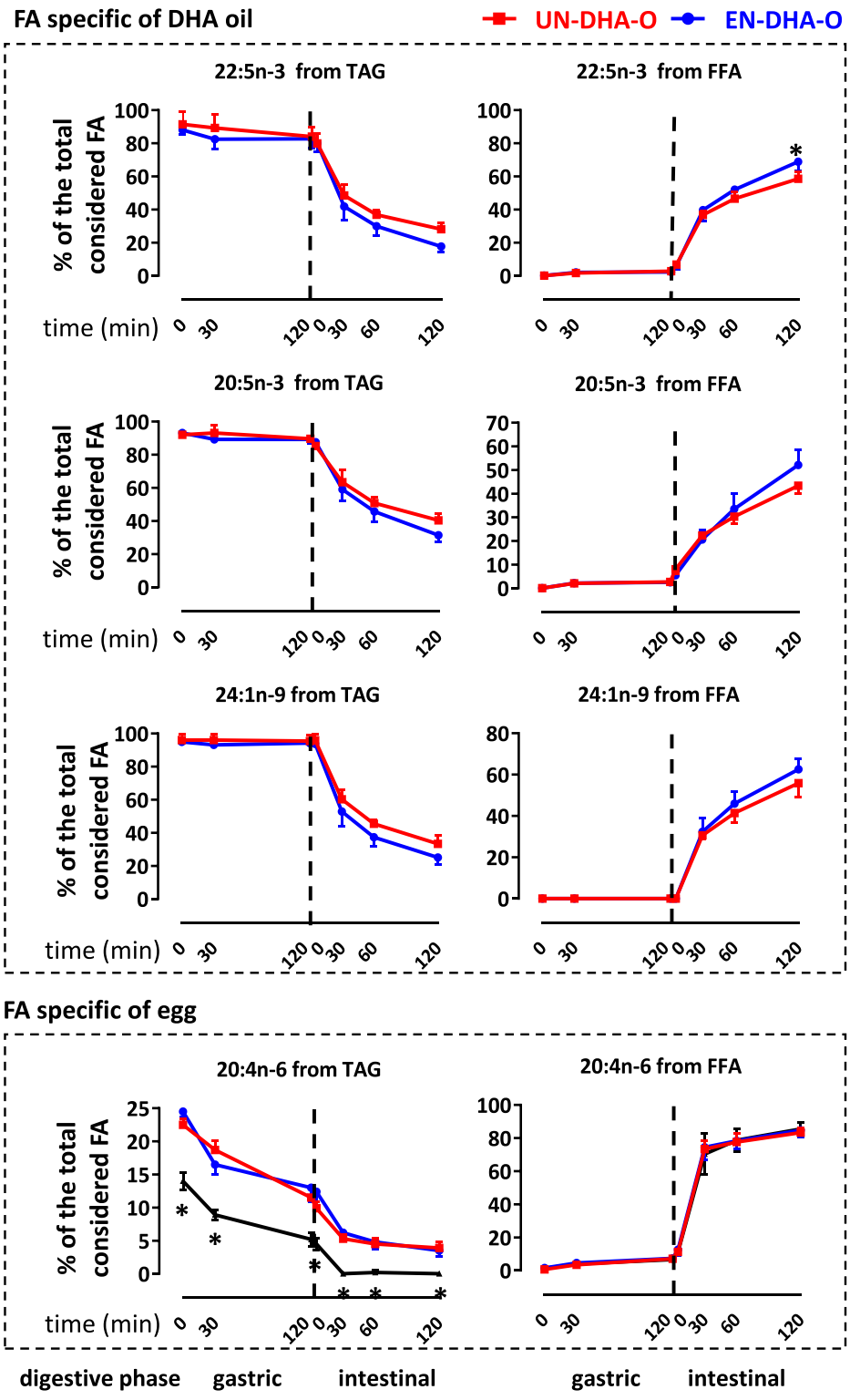

Fig. 6. The evolution of very long chain PUFAs hydrolyzed from TAGs and released as free FAs during digestion. Lipids were extracted by Folch's method and separated by thin layer chromatography. The FA profile from different lipid species was determined by gas chromatography coupled with mass spectrometry. The FA considered total corresponds to the sum of the FA considered esterified to each lipid species. * Indicates a significant difference between values at each time point ($p < 0.05$).

Oil encapsulation did not affect the proportion of DHA in the oil indicating that the quality of the oil was maintained during the encapsulation process and that DHA oxidation was avoided. In the present study, the control omelet contained a low amount of DHA (0.5 μg/mg) exclusively brought by the PLs. In DHA enriched groups, the enrichment of DHA oil greatly increased DHA concentration that reached 7.7 and 9.1 μg/mg in UN-DHA-O and EN-DHA-O, respectively. Besides, the proportion of DHA in different lipid species was similar between DHA-enriched groups. In addition, the theoretical DHA concentration in omelets should be 9.1 μg/mg of sample, which was the same as the DHA concentration in EN-DHA-O and higher than in UN-DHA-O. It can be attributed to the loss of unencapsulated DHA oil that can easily adhere to the containers and castings during the omelet preparation. In both UN-DHA-O and EN-DHA-O, the proportion of DHA in TAGs was around 86 %, which was significantly lower ($p < 0.05$) than in DHA oil (97 %). At the same time, the proportion of DHA in 1,2-DAGs was around 8 %, which was significant higher ($p < 0.05$) than in DHA oil (2 %). Therefore, the preparation of omelets affects the DHA oil quality.

The microstructure of Pickering emulsion exhibited a protein layer on the surface of the DHA oil droplet. However, the heat-denatured WPI particles could not be clearly seen from the microstructure of Pickering emulsion. This was due to the fact that the particle size of the heat-denatured WPI that was too small (42 nm) to be clearly observed by a CLSM. But the lipid droplets obtained by encapsulation were homogenous and small (4.34 μm) leading to an intense lipolysis. In contrast, for UN-DHA-O, the droplet size and distribution of Pickering emulsion were not uniform and large patches of lipids were observed. Finally, this study underlined the great importance of delivery system of food to improve digestibility of meal and the subsequent absorption of nutrients. In our case, we showed in another study carried out *in vivo* that encapsulation of DHA oil did not significantly changed the plasma bioavailability of DHA, but greatly modified its metabolism into derivatives such as oxylipins, measured in the brain and the heart, thus emphasizing the key role of the form of dietary intake of a nutrient (Wang et al., 2022).

5. Conclusion

We have demonstrated that encapsulating DHA oil with whey protein and adding it to an egg-based food structure (omelet) modified the bioaccessibility of the DHA oil, and thus accelerated the kinetics of the digestion process by releasing DHA as free fatty acid earlier during the intestinal phase. This result was obtained by using an internationally agreed digestion model and a complete study of the lipid species of the samples. Note that emulsifying the DHA oil did not affect the structure of oil, although cooking in an omelet triggered a slight hydrolysis of TAGs into DAGs. Thus, this DHA oil, commercially used as a food supplement, is an asset to guarantee nutritional needs, especially if it is introduced in an encapsulated form in food. Encapsulation is presented here as a key factor in the digestion process, and even in nutrient metabolism as demonstrated elsewhere. It opens the field to food possibilities to improve DHA intake through products of quality that are widely consumed.

Abbreviations.

DAG: diacylglycerol; DHA: docosahexaenoic acid; DHA-PL: PL containing DHA; DHA-TAG: TAG containing DHA; EN-DHA-O: omelet with encapsulated DHA oil (Pickering emulsion); ECH: Ester of Cholesterol; EPA: eicosapentaenoic acid; FA: fatty acid; FFA: free fatty acid; MAG: monoacylglycerol; PL: phospholipid; TAG: triacylglycerol; UN-DHA-O: omelet with unencapsulated DHA oil; WPI: whey protein isolate.

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.

Data availability

Data will be made available on request.

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