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Effect of cooking methods and intestinal conditions on lipolysis, proteolysis and xanthophylls bioaccessibility of eggs

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Digestibility of macro and micronutrients depends on the ingested food as well as on gastrointestinal conditions, being those suboptimal in exocrine pancreatic insufficiency (EPI) patients. Under this scenario, oral enzyme supplementation improves enzymatic hydrolysis of nutrients. In this study, a static in vitro model was used to assess lipids and protein digestibility as well as lutein and zeaxanthin bioaccessibility of eggs cooked differently and submitted to different intestinal conditions. Boiled, poached and omelette eggs were digested under different intestinal conditions of pH (6 or 7), bile concentration (1 or 10 mM) and doses of the enzyme supplement (1000 to 4000 LU/ g fat). Results showed that poaching resulted in higher digestibility of lipids and proteins, compared to boiling or omelette preparations, under gastrointestinal conditions of EPI (pH 6, bile 1 mM). Concerning xanthophylls bioaccessibility, boiling and poaching led to higher bioaccessibility of lutein and zeaxanthin than omelette under EPI conditions.

Keywords: pancreatic insufficiency; egg; cooking; lipolysis; proteolysis; xanthophylls

1. Introduction

Egg has lately gained attention as a food to be considered into a healthy diet mainly due to its high protein content together with egg yolks antioxidant composition. Concretely, egg white contains around 10 % of high quality protein with a Protein Digestibility Corrected Amino Acid Score (PDCAAS) value of 1. It includes albumins, mucoproteins and globulins, being ovalbumin (OVA) the main protein of egg white which represents 54 % of egg white protein (Sponton, Perez, Carrara, & Santiago, 2015; Weijers, Sagis, Veerman, Sperber, & van der Linden, 2002). Egg yolk, on the other hand, is considered among many food types, one of the most important sources of xanthophylls with higher bioavailability than other common sources such as dark-green leafy vegetables (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2012; Nimalaratne & Wu, 2015; Seuss-baum, 2007; Sunwoo & Gujral, 2015). Xanthophylls, the yellow pigments of egg yolk, are oxygenated carotenoids which all-E-isomeric form predominates in nature. However, processing conditions such as stirring, heating, light, and oxygen exposure may lead to some different changes in protein and lipid digestibility, which may result in changes of the functionality and bioavailability of egg nutrients (Dugave & Demange, 2003; Nimalaratne et al., 2012; Schieber & Carle, 2005). Although the main xanthophylls present in egg yolk are lutein and zeaxanthin, other bioactive compounds such as vitamin E and omega-6/3 polyunsaturated fatty acids are also present (Sunwoo & Gujral, 2015). Due to the lipophilic nature of these compounds, their absorption is closely related to the digestion of lipids. Thus, egg yolk can be an ideal food matrix to deliver highly bioavailable xanthophylls; indeed, bioavailability of lutein from lutein-enriched egg yolk was found to be greater than from lutein supplements or spinach (Chung, Rasmussen, & Johnson, 2004; Handelman, Nightingale, Lichtenstein, Schaefer, & Blumberg, 1999; Nimalaratne, Savard, Gauthier, Schieber, & Wu, 2015).

In order to be bioavailable, lipophilic compounds will have to be released from their food matrix and micellarized, becoming then absorbable (bioaccessible), which means they can be absorbed by intestinal cells and be metabolized (Faulks & Southon, 2005; Nimalaratne et al., 2015). Bioaccessibility of nutrients will depend on different factors related to the food itself such as food matrix, its composition, type of nutrients, processing and cooking methods (Granado-Lorencio et al., 2007; Nimalaratne et al., 2015; Pineda-Vadillo et al., 2017; Ryan, O'Connell, O'Sullivan, Aherne, & O'Brien, 2008). Thus, analyzing the extent to which food matrix and processing can modify the stability, and the bioaccessibility of bioactive compounds is an essential first step for better understanding the actual biological activity of food constituents (Rodríguez-Roque et al., 2015). Furthermore, absorption of this lipophilic bioactive compounds will also depend on individual factors such as gastrointestinal conditions (pH, secretion and composition of the digestive fluids, transit time…) (Ryan et al., 2008; Whitcomb et al.,

2010). All this might modify the extent of digestion and micellarization in the small intestine, and therefore, to absorb liposoluble compounds (Panozzo et al., 2013; Pineda-Vadillo et al., 2017).

Gastrointestinal environment will vary within different individuals depending on their age, gender, diet, etc. (Shani-Levi et al., 2017); these differences however, can become even more relevant under specific digestive disorders. **It** is the case of Exocrine Pancreatic Insufficiency (EPI), which is a disorder associated to several diseases such as pancreatic cancer, chronic pancreatitis (CP) or cystic fibrosis (CF). The obstruction of the pancreatic duct in EPI, produces an insufficient secretion of sodium bicarbonate and pancreatic juice, containing digestive enzymes. Besides this lack of digestive enzymes, the decrease of pancreatic juice may also decrease the intestinal pH, leading to nutrients mal-digestion and mal-absorption (Layer & Keller, 2003; Naikwade, Meshram, & Bajaj, 2009; Whitcomb et al., 2010). Due to pancreatic lipase is the main responsible of lipolysis (Carrière et al., 2000; Sikkens, Cahen, Kuipers, & Bruno, 2010), this scenario compromises lipids' hydrolysis and absorption, leading therefore to a deficit in fat-soluble vitamins (A, D, E and K) as well as other bioactive compounds, causing malnutrition. The current treatment for EPI involves oral administration of an enzymatic supplement in order to improve nutrients digestion and absorption (Armand, Fieker, & Philpott, 2011). Nowadays, the current guidelines for EPI recommend an enzyme dose of 2000-4000 Lipase Units (LU)/ g fat intake, being the only available parameters to guide health professionals on adjusting the prescribed doses, based on the overall fat content of the meals or on patients body weight (Turck et al., 2016). However, the optimal doses are still uncertain since satisfactory levels of fat absorption are not often achieved as they depend on food factors as well as on gastrointestinal (GI) conditions.

Since human studies might give very precise information on the bioaccessibility of nutrients, due to its high cost, technical difficulty and ethical reasons, alternative methods are generally used. *In vitro* digestion methodologies represent therefore, a good approach to mimic *in vivo* luminal digestion and to assess the bioaccessibility of bioactive compounds (Faulks & Southon, 2005; Hur, Lim, Decker, & McClements, 2011; Minekus et al., 2014; Pineda-Vadillo et al., 2017).

To the authors knowledge, there are already some studies focusing on lipids absorption and antioxidants bioaccessibility using egg or egg based food matrices (Chung et al., 2004; Handelman et al., 1999; Nimalaratne et al., 2015; Pineda-Vadillo et al., 2017). However, in all of them, gastrointestinal conditions were simulated according to a standard healthy adult. Therefore, the aim of the present study was to in vitro evaluate the influence of some intestinal factors associated to EPI (intestinal pH, bile concentration and the amount of enzyme supplement), as well as the effect of cooking procedure on lipids digestibility and xanthophylls bioaccessibility in eggs.

2. Materials and Methods

2.1. Materials

Pancreatic enzymes supplements (Kreon 10,000 lipase units (LU)) were used to simulate in vitro digestion of an individual with EPI. Each capsule contains 150 mg of gastro-resistant microspheres containing porcine pancreatic enzyme equivalent to 10**,**000 lipase U., 8**,**000 amylase U., and 600 protease U. The specific lipase activity of the Kreon was usually measured before the experiments (Carrière et al., 2000) and the amount of supplement added to the gastric stage was adjusted always to have the corresponding LU/ g fat according to the experimental design.

For the preparation of the simulated digestive fluids (**Table 1**), the following chemicals were needed: pepsin from porcine gastric mucosa (≥2500 U / g protein), bovine bile extract, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$ $(H2O)_6$, $(NH_4)_2CO_3$ and $CaCl_2$ all of them from Sigma-Aldrich Chemical Company (St Louis, MO, USA). NaOH (1 N) and HCl (1 N), were acquired from AppliChem Panreac. For the analytical determinations, Triton-X 100 %, petroleum ether, trichloroacetic acid (TCA), hexane, methanol, acetone, bovine serum albumin (BSA), methyl tert-butyl ether (MTBE), crystalline urea as well as the analytical standards of palmitic acid, lutein and zeaxanthin were all acquired from Sigma-Aldrich.

2.2. Sample preparation

Eggs were purchased from a local supermarket and divided into four equal sets before their use for the experiments that were performed at least 2 weeks prior to the expiry date. One set was used to characterize the raw product and the other three sets were used to analyse the influence of different cooking ways (boiled, poached and omelette). For the boiling, whole shell eggs were placed in a cooking pan, with boiling water covering the eggs, and they were boiled for 10 min (99 ± 1º C) (Nimalaratne et al., 2012). After boiling, the whole eggs were placed under running tap water for 5 min, and they were peeled right after. For poaching, eggs were broken into parafilm and then wrapped before boiling them into a pan filled with boiling water for 4 minutes (99 ± 1º C). After that, the parafilm wraps were placed under running tap water for 5 min. For omelette, eggs whites and yolks were mixed by stirring for 60 seconds, placed in a microwavable plate and cooked in a household microwave oven (model GW72N, Samsung) for 80 s at 750 W, 2450 MHz). After cooking, the samples were in vitro digested by using a static system.

2.3. *In vitro* digestion

Cooked yolks and whites of poached and boiled eggs were separated and sampling was made by weighing both parts in the same proportion as they would appear in a whole cooked egg; in the case of the omelette, raw yolk and white were weighted and added to keep the same proportion of both parts as in the whole egg prior to preparation. The amount of cooked samples to be digested was weighted in order to have 0.35 g fat in each tube (50 mL falcon tubes). Fat content in fresh and cooked eggs was determined previously at the digestion by the official Soxhlet method (AOAO, 2000). The digestion procedure used was based on the standardized static in vitro digestion method for food published by Minekus et al., (2014) with some modifications in order to allow analysing EPI conditions. **Table 1** illustrates the amounts and composition of the fluids required in each of the stages of the digestion process. The digestion fluids were prepared fresh daily from stock solutions, salival (SSS), gastric (SGS) and intestinal (SIS) prepared according to Minekus et al., (2014). The enzymatic activity was tested before each experiment following the protocol proposed by Carrière et al., (2000). Each experimental condition was performed in triplicate. The *in vitro* digestion process was performed as follows:

Oral stage: Simulated salival fluid (5 mL) (SSF; pH 8) at 37 ºC, was added to the egg sample in a ratio 1:1 (v/w) and properly homogenized with a kitchen blender for 3 minutes (Vario Mixer, Ufesa 600 W).

Gastric stage: After the oral stage, simulated gastric fluid (SGF; pH 3) was added to each tube containing the oral bolus (1:1 v/w). Pepsin was added into the SGF to reach a concentration in the gastric mixture of (2000 U/mL). The pH of the mixtures was adjusted with HCl (1N) to pH 2.8 ± 0.1 and samples were flipped from top to bottom at 55 rpm for 2 h at 37 ºC using an Intell-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia) and incubated in a chamber Selecta (JP Selecta SA, Barcelona). These mixing conditions provided constant mechanical energy to induce the breakdown of the food matrix during digestion. The pancreatine supplement was added in the gastric stage in order to simulate swallowing the pill in case of EPI situations.

Intestinal stage: Following the gastric stage, simulated intestinal fluid (SIF; pH 7) was added in a proportion 1:1 (v/w) to each tube containing the gastric chime. The pH of the mixtures was adjusted to pH 6.0 ± 0.1 or 7.0 ± 0.1, depending on the conditions to be tested, with NaOH (1N). Samples were then being flipped from top to bottom at 55 rpm for another 2 h at 37 ºC. pH was monitored during the digestion process and readjusted if necessary to keep it constant (González-Bacerio, Rodríguez Hernández, & del Monte Martínez, 2010; Prazeres, Garcia, & Cabral, 1994).

2.4. Experimental design

The experimental design for each type of cooked egg (boiled, poached and omelette) consisted on two main sets of experiments. In the first, intestinal conditions were fixed at pH 6 and bile salts concentration 1mM, and different pancreatin supplement doses (0, 1000, 2000, 3000 and 4000 LU/g of lipid) were tested, in order to assess the influence of enzyme concentration. In the second, the dose of enzymes was fixed at 2000 LU/g of fat, and the study variables were different combinations of intestinal pH and bile concentration: pH6/10mM, pH7/1mM and pH7/10mM, in order to analyse the impact of different intestinal scenarios on lipolysis, proteolysis, matrix degradation and lutein and zeaxanthin bioaccessibility. Of note, the combination pH6/1mM would represent the most unfavourable condition in the gastrointestinal tract in EPI (Clarke, Stien, & Walker, 2001; Gelfond, Ma, Semler, & Borowitz, 2013; Norman, 1979; Robinson, Smith, & Sly, 1990; Rovner, Schall, Mondick, Zhuang, & Mascarenhas, 2013; Vu et al., 2000), and the pH7/10mM would approach the standard duodenal conditions of a healthy adult. All the experiments were conducted in triplicate.

2.5. Analytical determinations

2.5.1. Matrix Degradation Index (MDI (%))

Matrix degradation Index was determined in all samples after in vitro digestion. This parameter represents the proportion of solids that were finely dispersed in the digested after the intestinal stage. The total content of a digestion tube was centrifuged (4000 x g-force for 20 minutes, 4 ºC) and filtered on a metallic sieve (1.6 mm x 1.6 mm mesh) to separate out large egg particles. The drained liquid, from now on called micellar phase, was collected and used to determine soluble protein in 12 % trichloroacetic acid (TCA) (5ml), free fatty acids (0.1 ml) and the remaining micellar phase was freeze-dried for xanthophylls determination. The egg particles were rinsed twice with 5 mL of appropriate juice to remove any digested material. Blotting paper was placed around the metallic sieve for 10 min to drain residual digestion juice. The egg particles were then transferred to an aluminum dish and immediately weighed. The aluminum dish was put in a forced air oven at 60º C for 48 h and weighed again to determine the mass of large egg solids. The matrix degradation index (MDI), corresponding to the proportion of egg solids passing the metallic sieve, was calculated according to Lamothe et al, 2012 (Lamothe, Azimy, Bazinet, Couillard, & Britten, 2014; Lamothe, Corbeil, Turgeon, & Britten, 2012).

2.5.2. TCA soluble protein (%)

The extent of proteolysis was determined by measuring the protein soluble in TCA (Lamothe et al., 2014). TCA was added to digested samples to final concentration of 12% (w/w). The mixture was vortexed, incubated for 15 min and filtered using a Whatman no. 40 filter paper. The fraction soluble in 12% TCA was composed of small peptides

and amino acid residues (Rowland, 1938). The filtrate was diluted in buffer (50 mM EDTA, 8 M urea, pH =10) and protein was determined by measuring absorption at 280 nm against a blank prepared with appropriate digestion fluids. A calibration line was determined using bovine serum albumin (BSA) as a standard. The results were reported as the percentage of the total protein concentration initially present in each tube.

2.5.3. Lipolysis extent (%)

Drained juice from digested samples was diluted 100-fold with a solution made of 5.6% Triton X-100 and 6% ethanol in water (Lamothe, Corbeil, Turgeon, & Britten, 2012). This solution was used to solubilize the free fatty acids and stop lipase activity. Fatty acids release during digestion was measured on the diluted samples using a free fatty acid colorimetric assay kit (Roche Diagnostics, Indianapolis, IN, USA) and a spectrophotometer (UV/vis, *Beckman Coulter*) (Lamothe et al., 2014). Palmitic acid standard was used for quantitative determination of free fatty acids (FFA). FFA was expressed as the percentage of total fatty acids that could theoretically be released after complete digestion, assuming the maximum release of 2 fatty acids per triacylglycerol molecule and the average molecular weight of egg triglycerides 860 g mol^{-1} (Hunter, 2001).

2.5.4. Xanthophylls

Initial lutein and zeaxanthin content in egg samples, raw and cooked, were characterized before and after digestion; samples were homogenized, placed immediately at −40 °C and kept at that temperature for at least 48 h before freeze drying (48 h, -45 ºC and 1 mBar). Freeze-dried samples were grounded to obtain a fine powder and they were stored at – 20 ºC before the analysis of lutein and zeaxanthin content.

Extraction of xanthophylls: 6 ml of methanol, acetone, and hexane (1:1:1 (v/v/v)) were added to a glass tube containing 0.15 g of freeze-dried powder. After addition of the solvent mixture, tubes were shaken in a vortex for 30 s and immediately afterwards they continued to be flipped from top to bottom at 55 rpm for 30 min. After this, 2 ml of bidistilled water was added to each tube, and these were shaken for 1 min in the vortex mixer in order to separate the hydrosoluble and liposoluble phases adequately. Next, 1.5 ml of the non-polar phase containing the carotenoid pigments, were filtered with 0.22 mm nylon filters and transferred to amber HPLC glass vials. Hexane was then evaporated under Nitrogen flow and xanthophylls were re-suspended in 400 µL of hexane.

HPLC-DAD Analysis. Xanthophylls (Lutein and Zeaxanthin) were separated using a separation module (Waters, 2695) comprising a pump and DAD detector (2996, Waters, USA), using methanol, tert-methyl-butyl-ether and water as mobile phase (v:v:v), solvent A (83:15:2) and solvent B (8:90:2). Gradient elution was carried out as follows: 90 % A, 0–15 min; 90 % to 5 % A, 15–26 min; 5 % to 90 % A (initial conditions), 26–28 min, at a flow rate

of 1 mL/min, using a Develosil C30 Column 250 mm × 4.6 mm i.d.5 μm (Phenomenex), and UV detection at 450 nm. Each xanthophyll was quantified using a calibration curve of the pure standard. To evaluate the changes undergone by the xanthophylls, results were expressed as % of bioaccessibility, defined as the percentage of lutein and zeaxanthin that are solubilized in the digestion fluids after the intestinal stage. Thus, this index defines the proportion of xanthophylls (lutein or zeaxanthin) that could become available for absorption into the systematic circulation. Samples were prepared in triplicate. Bioaccessibility (%) was calculated according to Eq. (1) (Martínez-Las Heras, Pinazo, Heredia, & Andrés, 2017; Ortega, Reguant, Romero, Macià, & Motilva, 2009):

$$Bioaccessibility\ (\%) = A/B \cdot 100 \qquad \text{(Eq. 1)}$$

Where, A is either lutein or zeaxanthin content (μg/g product (boiled, poached or omelette egg) quantified in the supernatant at the end of gastrointestinal digestion, and B is either lutein or zeaxanthin content in boiled, poached or omelette egg before digestion and expressed in the same units.

Xanthophylls *Quantification*. Six-point standard calibration curves were prepared for quantification purposes. External calibration plots were recorded with sample concentrations ranging from 0.05 to 30.00 μg/mL. Concentrations were calculated using the corresponding all-E standard calibration curves. The limit of detection (LOD) and limit of quantitation (LOQ) were determined by injecting a series of diluted solutions with known concentrations. LOD and LOQ were defined as the signal-to-noise ratio equal to 3 and 10 respectively, according to the International Conference on Harmonization (ICH) Guideline (Dixon, 1999).

2.6. Statistical analyses

In order to study significant differences of the factors (enzyme dosage (LU/ g fat), intestinal conditions of pH-bile concentration (6-1, 6-10, 7-1, 7-10) and cooking method (boiled, poached and omelette egg)), on MDI (%), TCA soluble protein (%), Lipolysis Extent (%), Lutein and Zeaxanthin bioaccessibility (%), a statistical analysis of variance (simple ANOVA) was performed using Statgraphics Centurion, with a confidence interval of 95 % ($p < 0.05$). Additionally, a multi-factor analysis of variance (multivariate ANOVA) was also performed with a confidence interval of 99 % ($p < 0.001$) in order to know out which factor (pH, bile or cooking method) affected the most (*F-ratio*) the studied parameters (MDI (%), TCA soluble protein (%), Lipolysis Extent (%), Lutein and Zeaxanthin bioaccessibility (%)). The multifactor ANOVA was applied only to the data obtained at a fixed dose of 2000 LU/ g fat. All the experiments were performed at least in triplicate.

3. Results and discussion

3.1 Influence of the intestinal conditions and cooking method on macronutrients digestibility

Table 2 shows the results of the different digestibility parameters (MDI (%), TCA soluble protein (%) and lipolysis extent (%)) obtained from varying the intestinal pH and bile concentration using a fixed pancreatic enzyme supplement dose of 2000 LU/g fat (average recommended value for EPI, (Turck et al., 2016)). Complementarily, **Table 3** shows the F-ratio obtained from the multifactor ANOVA considering pH, bile concentration and cooking method as factors and MDI (%), TCA soluble protein (%), Lipolysis Extent (%), Lutein and Zeaxanthin bioaccessibility (%) as response variables. The higher the F-ratio value, the higher the statistical significance of the factors on given response variables. Thus, bile concentration was the factor affecting the most both MDI (%) and lipolysis extent; while cooking method presented a higher impact on proteolysis (TCA soluble protein).

The cooking method has a great impact on the matrix structure of the final egg product. Mixing of egg white and yolk for omelette preparation, as well as the different combinations of cooking temperature-time that were applied in each case (at 100 ° C for 10 minutes in boiled, at 100° C for 4 minutes in poached and 750 W for 80 seconds in omelette), result in different structural changes on egg proteins and lipids; then, different matrices with the same ingredients are obtained (with lower humidity in the case of the omelette, which dehydrates during cooking). Additionally, gastrointestinal digestion is a process that implies food interactions with biological fluids and their exposure to complex flow profiles and mechanical forces (Torcello-Gómez et al., 2011). The overall effect of both processes (cooking and digestion) was evaluated according to their Matrix Degradation Index (MDI (%)). This parameter allows to measure the net result of the changes that take place during gastrointestinal digestion and to analyse the impact of cooking preparation (as food-related factor) and three host-related factors (intestinal pH, bile concentration and pancreatic enzyme activity). In spite of the same composition, different matrices structure degrades in a different way during digestion. Poached egg, which yolk is still fluid showed the highest MDI. The degradation index of boiled egg was lower than poached due to the high coagulation of the white and the solidification of yolk, and even lower for omelette where yolk and white make up a more complex solid structure. The recent available evidence showing that structures of food matrices can modulate bioavailability of lipids, and other macro and micronutrients is based on the accessibility of digestive enzymes to the substrates. This is especially important in some chronic diseases that occur with EPI and require pancreatic enzyme supplementation. Our results reveal that for a certain enzyme dose the MDI and then the digestibility will be higher for poached and boiled egg than for omelette (**Figure 1**).

Additionally, results from in vitro digestion revealed that protein and lipid digestion are significantly affected by cooking method. Then, both host and food related factors should be taken into account to optimise egg fat and protein digestion. The highest proteolysis and lipolysis values were observed in poached egg and was the type of cooking that showed to be less dependent on intestinal conditions. According these results, poaching would be the most appropriated way of cooking eggs if the target is to maximize lipolysis, while omelette would be better for contrary purposes.

The higher the intensity of the cooking process (omelette>boiled>poached) the lower the matrix degradation, even at the highest enzyme dose. These changes are related to the thermal denaturation of proteins, which interact to form insoluble aggregates. Concretely, the albumin coagulation depends on temperature as well as on cooking time (Matsudomi, Takahashi, & Miyata, 2001). Moreover, the protein solubility of raw egg can decrease from 98% to 20 % due to thermal treatment (Denmat, Anton, & Gandemer, 1999; Llave, Fukuda, Fukuoka, Shibata-Ishiwatari, & Sakai, 2018; Van Der Plancken, Van Loey, & Hendrickx, 2006). These protein changes are also observed in TCA soluble protein results; no differences between boiled and omelette was observed, and the poached egg samples reached the highest TCA soluble protein values at and above 3000 LU/g fat. Additionally, denatured egg yolk proteins can adsorb at the o/w interface this affecting lipolysis extent at low bile concentrations. This could probably explain the differences observed between lipids digestion in poached and boiled or omelette. In omelette egg, the mixing step previous to the heating process favours the lipids from egg yolk being trapped within the coagulated protein matrix, further reducing the accessibility of the enzymes to lipids. It can be noticed that matrix degradation, and therefore MDI (%), occurs even in absence of pancreatic enzymes. In this context, the simulated gastric stirring together with the previous pepsin action during the gastric stage could be responsible for the MDI (%) value obtained. This is in accordance to the results obtained for TCA soluble protein values for the dose of 0 LU/ g fat, which indicates that proteolysis partially occurred during the gastric stage (about 29% for poached egg, 23% for boiled egg and 28 % for omelette, (**Figure 1b**). After the intestinal stage, however, it ranged from a minimum to a maximum of 40 and 90 %, depending on the type of processing and enzymatic supplement concentration (**Figure 1b**). Increased supplement dose generated a higher proteolysis in all cooking eggs (boiled, poached and omelette), being almost 100% in poached egg. In case of boiled egg and omelette using a 4000 LU/ g fat reached similar TCA soluble protein (%) values to achieved in poached egg using 2000 LU/ g fat. As it has been mentioned above, this fact seems to be related to the structural changes in egg matrix caused by the cooking method. Cooking involves the use of high temperatures that could modified native protein structure resulting in protein aggregates, which might interfere in protein and lipid digestion. Van der Plancken et al. (2006) reported that only 20% of egg proteins

287 remained soluble after 10 min at 80 ºC. Therefore, it is expected a percentage of soluble protein even inferior after
288 cooking treatment at 100 ºC. For this reason, protein and lipid digestibility are affected by cooking conditions and it
289 is important to consider the time and temperature that egg have been subjected.

290 The interactions generated among macronutrients during cooking as well as the chemical modifications undergone
291 by proteins, significantly affected the amount of protein digested ($p < 0.05$). At the end of the digestion, no
292 significant differences were found in the proteolysis achieved between both, omelette and boiled eggs, ($p < 0.05$) at
293 the same enzymatic supplement concentration (**Figure 1b**).

294 Regarding the effect of the dose of enzymatic supplement on proteolysis, an increase from 0 to 2000 LU/ g fat (0 to
295 106.6 protease units'/ g protein) resulted in higher proteolysis values and especially for poached eggs. The softer
296 thermal treatment (in terms of time and/or temperature) of poached egg results in less denaturation and gelation of
297 proteins than in omelette or boiled eggs. The higher temperature applied for boiled and omelette preparations could
298 lead to a coagulation of proteins with an increase of the viscosity, as well as to the formation of aggregates and to a
299 subsequent gelation due to the hydrophobic interactions and the formation of disulphide bonds (Guilmineau &
300 Kulozik, 2006; Kiosseoglou & Parakevopoulou, 2005). The obtained results confirm therefore, that lower
301 denaturation and gelation of proteins allows the matrix for a greater facility to be digested during the intestinal
302 stage. These results are in agreement with previous studies were gelation by heat treatment of protein based
303 products lead to an increase in their mean retention time in the stomach, this leading to lower levels of amino acid
304 absorption (Barbé et al., 2013, 2014). These findings have to be taken into account as this delay and lower amino
305 acid bioavailability might have substantial effects on the protein metabolism. Likewise, a slight increase in TCA
306 soluble protein values could be observed by increasing the pH from 6 to 7 in the case of poached egg and
307 omelette, as well as by increasing the bile concentration when digesting the boiled egg. Comparing the results at
308 pH 6-1 mM bile (exocrine pancreatic insufficiency) with pH 7-10 mM bile (standard healthy conditions), the
309 digestibility of egg lipids only seems to be compromised in the consumption of boiled eggs.

310 According to our results from lipolysis extent (%), it can be concluded that differences in bile concentration, from 10
311 mM to 1 mM, greatly affects the extent of lipolysis in boiled and omelette eggs, while no significant influence of the
312 duodenal pH was found ($p > 0.05$). The role of bile concentration on lipolysis was much higher in boiled eggs in
313 which fat globules are coagulated in yolk, requiring high concentrations of bile to be emulsified and to be more
314 accessible for lipases. Regardless intestinal pH or bile concentration, 2000 LU/g fat seems no to be enough for a
315 complete lipid digestion in egg products. Moreover, an increase on the dose of enzymatic supplement to 4000 LU/

g fat, only promotes a complete lipid digestion (lipolysis close to 100 %) in poached eggs whereas for boiled or omelette the highest extent of lipolysis achieved were ≈ 66 and 42 %, respectively. This could be explained by the capacity of some proteins such ovalbumin to bind hydrophobic compounds (for instance stearic acid) forming complexes that could modify nutrients availability during digestion (Sponton, Perez, Carrara, & Santiago, 2015). In the case of omelette and boiled eggs, where proteins are highly coagulated, these complexes formed within proteins and lipids could lead to the decrease of lipids availability.

3.2 Influence of the intestinal conditions and cooking method on carotenoid bioaccessibility

Lutein and zeaxanthin constitute the main xanthophylls in egg yolk (Schlatterer & Breithaupt, 2006), while other xanthophylls, such as 13′-Z-lutein, and 13-Z-zeaxanthin, all-E-canthaxanthin or all-E-β- apo-8′ carotenoic acid ethyl ester, are in much lower amounts. Additionally, no significant *trans−cis* isomerization of xanthophylls previously reported during digestion (Granado-Lorencio et al., 2007, 2010; Nimalaratne et al., 2015). Thus, the impact of the intestinal conditions on xanthophylls bioaccessibility in eggs has been focused on lutein and zeaxanthin compounds, and the bioaccessibility assessed from the chromatographic quantification of *trans* isomer of each respective carotenoid. In this context, the LOD and LOQ were found to be 0.032 and 0.12 (µg/mL) for lutein and 0.033 and 0.103 (µg/mL) for zeaxanthin respectively, and the concentration of both xanthophylls in the extracts was always above the LOQ. According to our results (**Table 4**), lutein and zeaxanthin contents in raw egg (mean value of 0.27 and 0.35 µg/g, respectively) were similar to those reported by other authors (Handelman et al., 1999; Nimalaratne & Wu, 2015), although they could be significantly affected by hens' feed composition. With regard to the influence of the cooking method (boiling, poaching, and microwaving), it could be affirmed that all methods preserved the xanthophylls content (µg/g of dry matter). The carotenoid stability during cooking has been also reported in the study carried out by Nimalaratne et al. (2012) in which a slight decrease of lutein and zeaxanthin contents (6-20 %), was found as a consequence of cooking. It is important to point out, the higher intake of xanthophylls per gram of omelette compared to the ingestion of one gram of raw, boiled or poached egg (values of µg/ g product) as a result of the concentration taking place during cooking by microwaves due to the loss of water.

Table 5 gathers the influence of intestinal conditions of pH and bile concentration as well as enzymatic dosage of Kreon on lutein and zeaxanthin bioaccessibility (%). With regard to the effect of the dosage of the enzymatic supplement under intestinal pH of 6 and bile concentration of 1 mM, bioaccessibility of both xanthophylls seems to follow a similar trend to that observed for lipids digestion as it was expected due to the fat-soluble nature of these antioxidants. Thus, the solubility and extractability of xanthophylls from boiled and poached eggs seems to

increase as it does the dose of the enzyme supplement from 0 to 3000 LU/ g fat; whereas in omelette, the maximum bioaccessibility of xanthophylls was reached at 1000 LU/ g fat. When the digestibility of both xanthophylls is compared, zeaxanthin appears to be slightly more bioaccessible than lutein in poached and omelette eggs at 2000, 3000 and 4000 LU/ g fat. Apparently, lutein and zeaxanthin may exhibit different orientations in the phospholipid bilayer, which might contribute to their different bioaccessibility (Britton, Liaaen-Jensen, & Pfander, 2008; Nimalaratne et al., 2015).

The effect of intestinal pH and bile concentration on xanthophylls bioaccessibility was dependent on the egg matrix. The results obtained from the multifactorial ANOVA showed a significant influence ($p < 0.001$) of cooking method on the bioaccessibility of lutein and zeaxanthin; while only the lutein bioaccessibility was significantly affected ($p < 0.001$) by bile concentration (**Table 3**).

Boiling process resulted in higher bioaccessibility of lutein and zeaxanthin, under standard intestinal conditions of pH 7 and bile concentration of 10 mM when compared to the other intestinal scenarios (pH 6/ 1 mM or 10 mM and pH 7/ 1 mM). In poached egg, neither pH nor bile concentration seems to present a significant influence on xanthophylls bioaccessibility, even if slight higher values were found at pH 7 and bile concentration of 1 mM. Finally, a notable increase of xanthophylls bioaccessibility was found when omelette was digested at pH 7 instead of 6. This fact could be linked to the higher digestibility of macronutrients (**Table 2**) in omelette, both lipids and proteins, at pH 7 than at 6, leading to a higher release of micronutrients at this pH (Peinado, Larrea, Heredia, & Andrés, 2018).

Of notice, chemical and structural changes of proteins and lipoproteins in egg yolk occurring during different cooking conditions will influence the micellarization efficiency (Nimalaratne et al., 2015) giving as a result higher carotenoid bioaccessibility in boiled eggs and poached eggs than in omelette, excepting at pH 7 at 2000 LU/ g fat. Concretely, the homogenization during omelette preparation, together with the thermal treatment, could increase the interaction among ingredients and therefore matrix consistency, generating a network that might entrap the carotenoids (Panozzo et al., 2013), leading to a lower bioaccessibility.

4. Conclusions

From the present study, it could be concluded that both structural changes undergone by egg matrix during cooking and the host intestinal conditions highly affect the digestibility and bioaccessibility of macro and micronutrients. To this regard, omelette cooking and boiling resulted in lower digestibility of lipids and protein compared to poaching after in vitro digestion under exocrine pancreatic insufficiency (EPI) conditions. Thus, the highest lipolysis (100 %)

was registered for poached eggs under the highest dose of enzyme supplementation of pancreatin (4000 LU / g fat).

In the same way, xanthophylls bioaccessibility was also affected by cooking method, registering the highest bioaccessibility of lutein and zeaxanthin in boiled and poached egg. Xanthophylls bioaccessibility of boiled and omelette eggs was significantly lower when in vitro digested under EPI. Additionally, pH 7 seems to greatly favour xanthophylls bioaccessibility in omelette eggs compared to pH 6; while lutein bioaccessibility was positively and significantly affected by bile concentration.

To sum up, it is expected that these results help to adjust the dosage of pancreatic supplementation for individuals with exocrine insufficiency taking into account the preparation method. In this context, poached egg would be the most advisable under these intestinal conditions in terms of fat and protein digestibility.

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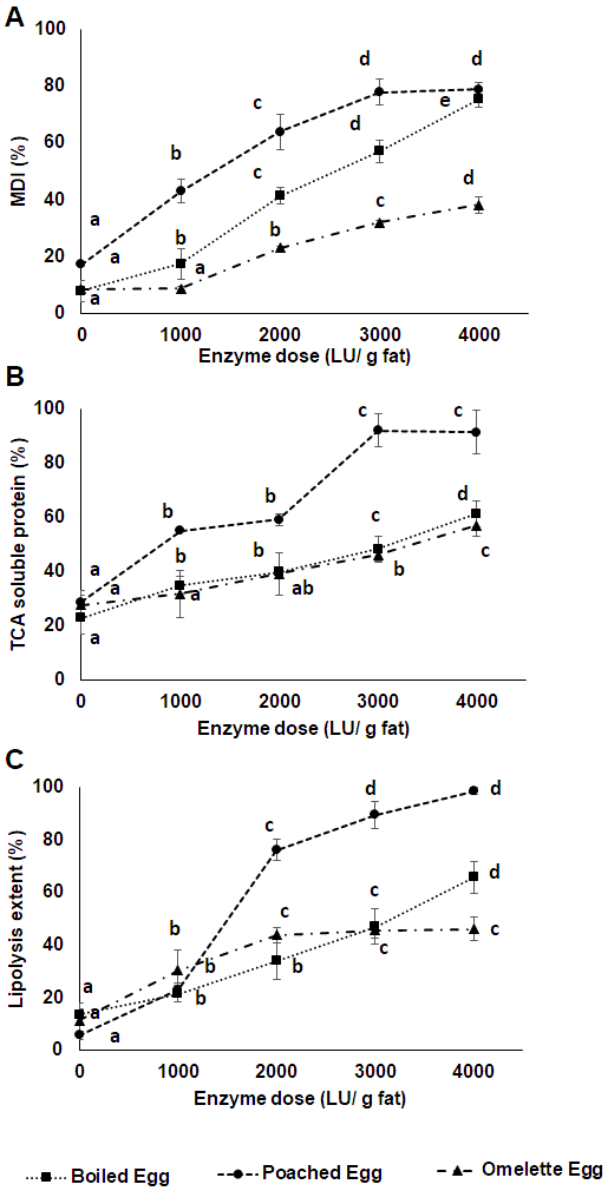

Figure 1. Matrix degradation index (%) (A), TCA Soluble Protein (%) (B) and Lipolysis extent (%) (C) obtained for the different egg matrices after in vitro digestion at fixed duodenal conditions of pH 6 and Bile concentration 1 mM using different doses of Kreon (0-4000 LU/ g fat ≈ 0-57-107-160-213 Protease Units / g of protein).

Letters (a-e) refer to the homogenous groups obtained for different doses (0 – 4000) for the same egg matrix (boiled, poached and omelette) at a statistical significance of 95 % (p-value < 0.0

Table 1

Table 1: Composition of simulated digestion fluids.

Constituent	**SSF** mmol/ L	**SGF** mmol/ L	**SIF** mmol/ L
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25	85
NaCl	-	47.2	38.4
$MgCl_2(H_2O)_6$	0.15	0.1	0.33
$(NH_4)_2CO_3$	0.06	0.5	-
$CaCl_2$	1.5	0.15	0.6

The addition of pepsin, Ca^{2+} solution and water will result in the correct electrolyte concentration in the final digestion mixture. SSF: Simulated Salival Fluid; SGF: Simulated Gastric Fluid; SIF: Simulated Intestinal Fluid.

Table 2

Table 2: Matrix degradation index (%), TCA Soluble Protein (%) and Lipolysis extent (%), obtained for the different egg matrices after the in vitro digestion process using a fixed enzyme dose (2000 LU/ g fat) and different duodenal conditions of pH and Bile concentration.

	Boiled Egg	Poached Egg	Omelette Egg
MDI (%)			
pH 6 - 1 mM	41 ± 2 ^{aB}	64 ± 12 ^{aC}	23.2 ± 0.3 ^{aA}
pH 6 - 10 mM	71.23 ± 0.03 ^{bB}	73 ± 10 ^{aC}	25 ± 4 ^{aA}
pH 7 - 1 mM	46 ± 1 ^{aB}	70 ± 4 ^{aC}	34.77 ± 1.14 ^{aA}
pH 7 - 10 mM	83 ± 7 ^{cC}	70 ± 6 ^{aB}	40 ± 4 ^{aA}
TCA Soluble protein (%)			
pH 6 - 1 mM	40.0 ± 0.4 ^{aA}	58 ± 2 ^{bB}	39 ± 6 ^{aA}
pH 6 - 10 mM	50 ± 2 ^{bB}	49 ± 3 ^{aB}	36 ± 2 ^{aA}
pH 7 - 1 mM	39 ± 5 ^{aA}	68 ±1 ^{cC}	51 ± 5 ^{bB}
pH 7 - 10 mM	57 ± 6 ^{bB}	54 ±4 ^{bAB}	44 ± 6 ^{abA}
Lipolysis extent (%)			
pH 6 - 1 mM	30 ± 7 ^{aA}	76 ± 8 ^{bC}	44 ± 3 ^{aB}
pH 6 - 10 mM	78 ± 14 ^{bB}	63 ± 2 ^{aB}	42 ± 5 ^{aA}
pH 7 - 1 mM	28 ± 4 ^{aA}	55 ± 2 ^{aB}	49 ± 9 ^{abB}
pH 7 - 10 mM	87 ± 12 ^{bB}	75 ± 8 ^{bB}	55 ± 3 ^{bA}

Superscript letters (a-c) refer to the homogenous groups obtained for different duodenal conditions (pH and Bile concentration) for the same egg matrix (boiled, poached and omelette) and at a statistical significance of 95% (p-value <0.05). Superscript letters (A-C) refer to the homogenous groups for different cooking methods at the same intestinal conditions and at a statistical significance of 95% (p-value <0.05).

Table 3

Table 3. F-ratio obtained from factorial ANOVA analysis for MDI (%). TCA soluble protein, Lipolysis extent (%) and Zeaxanthin and Lutein bioaccessibility (%). The factors for the analysis were pH, bile concentration and cooking method. The multifactor ANOVA was applied only to the data obtained at a fixed dose of 2000 LU/ g fat.

Principal effects	MDI (%)		TCA soluble protein (%)		Lipolysis extent (%)		Lutein bioaccessibility (%)		Zeaxanthin bioaccessibility (%)	
pH	16.3	*	26.5	*	1.04	n.s.	0.04	ns	3.6	ns
Bile	54.9	*	0.36	ns	61.9	*	57.89	*	0.18	ns
Cooking	151	*	46	*	21.8	*	18.54	*	100.5	*

ns: non-statistical differences ($p > 0.05$). *: $p < 0.001$.

Table 4

Table 4. Lutein and Zeaxanthin content in raw egg, boiled, poached and omelette before digestion. Contents are expressed in µg / g product and µg / g dry matter.

	Lutein		Zeaxanthin	
	(µg/ g product)	**(µg/ g dry matter)**	**(µg/ g product)**	**(µg/ g dry matter)**
Raw egg	0.27± 0.09 [a]	1.2± 0.3 [a]	0.35 ± 0.18 [a]	1.5 ± 0.7 [a]
Boiled	0.25 ± 0.04 [a]	0.89 ± 0.15 [a]	0.38 ± 0.08 [a]	1.3 ± 0.3 [a]
Poached	0.27 ± 0.09 [a]	0.9 ± 0.3 [a]	0.35 ± 0.06 [a]	1.3 ± 0.2 [a]
Omelette	0.67± 0.17 [b]	1.1 ± 0.3 [a]	0.68 ± 0.17 [b]	1.14 ± 0.16 [a]

Superscript letters (a-b) refer to the homogeneous groups obtained by the ANOVA (p-value <0.05).

Table 5

Table 5. Lutein and Zeaxanthin bioaccessibility (%) as a function of different doses of Kreon (0-4000 LU/g fat) and intestinal conditions of pH (6 and 7) and bile concentration (1 and 10 mM).

	Lutein Bioaccesibility (%)		
	Boiled Egg	**Poached Egg**	**Omelette Egg**
Enzyme dose (LU/ g fat)			
0	27 ± 2 ^{aA}	30 ± 2 ^{aA}	35 ± 7 ^{bA}
1000	41 ± 3 ^{bA}	38 ± 11 ^{aA}	37 ± 4 ^{bA}
2000	66 ± 7 ^{cB}	70 ± 11 ^{bB}	19 ± 7 ^{aA}
3000	80 ± 8 ^{dB}	99 ± 5 ^{cC}	28 ± 6 ^{abA}
4000	84 ± 8 ^{dB}	91 ± 14 ^{bcB}	22 ± 2 ^{aA}
Intestinal conditions pH – Bile mM			
6 – 1	66 ± 7 ^{aB}	70 ± 11 ^{aB}	19 ± 7 ^{aA}
6 – 10	59 ± 10 ^{aB}	69 ± 3 ^{aA}	33 ± 9 ^{bB}
7 – 1	65 ± 11 ^{aA}	92 ± 10 ^{bB}	83 ± 3 ^{dAB}
7 – 10	104 ± 21 ^{bB}	57 ± 10 ^{aA}	69 ± 7 ^{cA}
	Zeaxanthin Bioaccesibility (%)		
	Boiled Egg	**Poached Egg**	**Omelette Egg**
Enzyme dose (LU/ g fat)			
0	26 ± 0 ^{aA}	28 ± 8 ^{aAB}	43 ± 11 ^{aB}
1000	48 ± 1 ^{bB}	32 ± 3 ^{aA}	61 ± 7 ^{bC}
2000	63 ± 5 ^{cAB}	65 ± 13 ^{bB}	31 ± 7 ^{aA}
3000	70 ± 2 ^{dB}	103 ± 17 ^{bC}	45 ± 13 ^{abA}
4000	77 ± 5 ^{dB}	98 ± 7 ^{bC}	34 ± 7 ^{aA}
Intestinal conditions pH – Bile mM			
6 – 1	63 ± 5 ^{aB}	65 ± 13 ^{aB}	31 ± 7 ^{aA}
6 – 10	67 ± 7 ^{aB}	70 ± 4 ^{bB}	47 ± 8 ^{bA}
7 – 1	62 ± 6 ^{aA}	86 ± 3 ^{bB}	111 ± 12 ^{aAB}
7 – 10	98 ± 16 ^{bB}	65 ± 6 ^{aA}	106 ± 13 ^{bB}

Superscript letters (a-d) refer to the homogenous groups obtained for different doses (0 – 4000) or duodenal conditions (pH and Bile concentration) for the same egg matrix (boiled, poached and omelette) and at a statistical significance of 95% (p-value <0.05). Superscript letters (A-C) refer to the homogenous groups for different cooking methods at the same dose or intestinal conditions and at a statistical significance of 95% (p-value <0.05).