Influence of particle size and intestinal conditions on *in vitro* lipid and protein digestibility of walnuts and peanuts.

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ABSTRACT

A static *in vitro* model was used to assess walnuts and peanuts macronutrient digestion with two different particle size. Nuts were digested under different intestinal conditions of pH (6 or 7), bile concentration (1-10mM) and pancreatic concentration (1000 to 4000 LU/g fat) the matrix degradation index (MDI), proteolysis and lipolysis were analysed. Results showed that nuts particle size affects proteolysis and MDI the most; intestinal pH was more relevant in free fatty acids release.

Lipolysis extent was lower under suboptimal intestinal conditions of pH 6 and bile salts 1 mM, and in peanuts it was lower than walnuts (567, 585, 134 and 398 mg FFA/ g fat in large and small walnuts, and large and small peanuts, respectively). The higher the pancreatic concentration the higher the proteolysis extent in walnuts; in peanuts, protein digestibility was limited even at high pancreatic concentration at pH 6 and bile concentration 1 mM.

Keywords: *in vitro* digestion; pancreatic insufficiency; nuts; particle size; lipolysis; proteolysis; free fatty acids

1. Introduction

In 2003, the Food and Drug Administration (FDA) of the United States issued a health claim for nuts and cardiovascular disease, which read, “Scientific evidence suggests but does not prove that eating 1.5 ounces (42 g) per day of most nuts, as part of a diet low in saturated fat cholesterol, may reduce the risk of heart disease”. Numerous epidemiological studies establish the relationship between the regular intake of nuts and a reduced prevalence of coronary heart diseases (Kris-Etherton, Hu, Ros, & Sabaté, 2008) and cancers such as those of the prostate (Jain, Hislop, Howe, & Ghadirian, 1999) or colorectum (Yeh, You, Chen, & Sung, 2006). The latest scientific studies on the beneficial effects of nuts proved the relationship between the consumption of nuts and better cognitive function in elderly men (O´Brien et al., 2014).

There are different types of nuts; walnuts (*Juglans regia*) are considered among the most popular edible tree nuts, together with almonds (*Prunus amigdalis*), hazelnuts (*Corylus avellana*), and pistachios (*Pistachia vera*). Peanuts (*Arachis hypogaea*), are botanically legumes, but are widely identified as part of the nuts food group because of their comparable nutritional profile (Griel, Eissenstat, Kris-Etherton, Hsieh, & Juturu, 2004).

The nuts food group is energy dense because of their high protein and lipid content. Their fatty acids profile is characterized by a predominance of unsaturated acids. The major fatty acids found in walnut oil are oleic (18:1 n-9), linoleic (18:2 n-6) and linolenic (18:3 n-3) acids (Zwarts, Savage, & McNeil, 1999). Oleic (18:1v 9) and linoleic (C18:2v6) acids represented 80% of the fatty acid profiles of peanuts; palmitic (16:0) acid account for another 5 to 10% of the total content of fatty acids. Stearic (18:0), arachidic (20:0), eicosenoic (20:1v9), behenic (22:0), and lignoceric (24:0) acids each represent between 1 and 3% of the total profile. (Andersen, Hill, Gorbet, & Brodbeck, 1998; Ozcan & Seven, 2003) At the same time, nuts are considered a nutrient-dense food as they provide dietary fibre, vitamins (e.g. folic acid, niacin and vitamins E and B6), minerals (e.g. copper, magnesium, potassium, zinc) and many other bioactive compounds such as antioxidants, phytosterols and phytochemicals (Dreher, Maher, & Kearney, 1996). Despite the lipid content in nuts (between 50-55%), a meta-analysis of randomized controlled trials indicated that their consumption does not result in an increase of the body weight or body mass index (Flores-Mateo, Rojas-Rueda, Basora, Ros, & Salas-Salvadó, 2013).

This has been attributed to undigested lipid remaining after digestion (Hollis & Mattes, 2007). Of note, Novotny, Gebauer, & Baer (2012) indicated that only 76% of the energy within almonds is metabolized. This can be explained by the well-known fact that intact cell walls protect encapsulated lipids during their passage through the gastrointestinal tract (Ellis et al., 2004; Mandalari et al., 2008), and limit digestibility. That is why chewing is a key

factor that determines the nature and degree of cellular fracture. Some studies reported that oral breakdown of whole nuts leads to a release of 8-11% of oil droplets, making them more available for lipolysis (Mandalari et al., 2014). In addition, proteins in human saliva (mucins) are responsible for the depletion flocculation of some emulsions, depending on the residence time in the mouth and the type of emulsifiers used in stabilizing the emulsion droplets (Gallier & Singh, 2012). In the stomach, proteolysis is the main enzymatic process taking place (38%) of gastric proteolysis that has been reported in almonds (Mandalari et al., 2008), while lipolysis occurs mainly in the duodenum and is highly dependent on intestinal conditions (pH, pancreatin and biliary secretions). The abrupt pH change when the chyme passes through the pylorus causes a rapid change in the physical-chemical properties of lipids. Therefore lipids become partially ionized, and contribute to an improved emulsification (Hernell, Staggers, & Carey, 1990). Simultaneously, biliary lipids ejected from the gallbladder in the form of biliary mixed micelles become rapidly diluted . Bile production is necessary for the efficient intestinal absorption of the dietary lipids and fat soluble vitamins. Moreover, bile salts have a high capacity to solubilize phospholipids and the products of pancreatic lipolysis (Reis, Holmberg, Watzke, Leser, & Miller, 2009). Consequently, the suboptimal intestinal conditions found in some individuals could drastically diminish the intestinal hydrolysis of proteins, and especially of fats. This is the case of individuals suffering from exocrine pancreatic insufficiency (EPI).

EPI is a physiological disorder characterized by a decrease of secretion of Cl^-, water and HCO^{-3} with the consequent reduction in the volume of pancreatic and biliary secretions causing dilation and obstruction of the pancreatic and bile ducts (Li & Somerset, 2014)..The clinical therapy for EPI consists of an enzymatic substitution therapy (EST), which itself consists in the administration of gastro-resistant enzymatic supplements of swine pancreatin.

In this context, the aim of the present study was to evaluate the impact of particle size after oral digestion, intestinal pH, biliary concentration and pancreatic enzyme concentration on proteolysis and lipolysis of walnuts and peanuts by using an *in vitro* digestion model.

2. Materials and Methods

2.1. Raw material

Raw peeled walnuts (*Juglans regia*) and roasted peanuts (*Arachis hypogaea)* were purchased from a local supermarket. They both were available in packets of 200 g each.

2.2. Chemicals

α-amylase from human saliva (1000-3000 U/ mg protein) and pepsin from porcine gastric mucosa (≥ 2500 U / g protein), were obtained from Sigma-Aldrich.

Pancreatin from swine pancreas (Kreon® 10,000 lipase units (LU), Abbot), was kindly donated by "Hospital Universitari Politècnic La Fe" (Valencia, Spain). Each capsule contains 150 mg of porcine pancreatic enzyme equivalent to 10,000 lipase U., 8,000 amylase U. and 600 protease U.

The following chemicals were needed for preparation of the simulated digestive fluids: 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, all solvents were analytical grade; Triton-X 100%, hexane, trichloroacetic acid, glycine, petroleum ether 40:60, BF_3 methanol, H_2SO_4, as well as the analytical standards oleic and linoleic acid, and FAMEs (Supelco®37 Component) were all from Sigma-Aldrich Chemical Company (St Louis, MO, USA).

2.3. In vitro simulation of gastrointestinal digestion

2.3.1. In vitro digestion process

In vitro digestion was performed following the static method proposed by Minekus et al. (2014) with some modifications in order to simulate EPI conditions as detailed in previous studies (Asensio-Grau, Peinado, Heredia, & Andrés, 2018). The digestion fluids (salivary (SSS), gastric (SGS) and intestinal (SIS)) were prepared fresh daily from stock solutions according to Minekus et al. (2014). The enzymatic activity of the enzyme solutions was tested before each experiment following the protocol proposed by Carrière et al. (2000).

The *in vitro* digestion was performed as follows:

Oral stage: walnuts and peanuts were ground in order to simulate chewing and to evaluate the effect of oral breakdown. For this purpose, samples were crushed using a mechanical grinder (Taurus Aromatic SP-7407 50Hz, grinding disc of Ø 80mm, at 1480 rpm) applying grinding pulse of 3 seconds during 1 minute and then passed through a metallic sieve of 1.2 mm to separate two fractions of nuts, peanuts or walnuts, of two particles sizes (large > 1.2 mm and small < 1.2 mm). Simulated salivary fluid (5 mL) with α-amylase from human saliva 1000 - 3000 U / mg protein (SSF; pH 8) was added to the falcon tube containing the ground nut sample; it was properly mixed and incubated for 3 min at 37 ºC without agitation in an incubator chamber Selecta (JP Selecta SA,

Barcelona). The amount of ground sample (walnuts or peanuts) used in each experiment was estimated to always have 0.35 g of fat in the tube.

Gastric stage: after the oral stage, the simulated gastric fluid (SGF; pH 3) was added to each tube containing the oral bolus (1:1 v/w). Pepsin was added to the SGF to reach a concentration in the gastric mixture of 2000 U/mL. The pH of the mixture was adjusted with HCl (1N) to a value of pH 2.8 ± 0.1 and samples were flipped from top to bottom at 55 rpm for 120 min at 37 ºC using an Intell-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia) and the incubator chamber Selecta.

Intestinal stage: simulated intestinal fluid (SIF; pH 7) containing bile salt (1 or 10 mM) and pancreatin (0, 1000, 2000, 3000 or 4000 LU/g of fat), was added in a ratio of 1:1 (v/w) to each tube containing the gastric chyme. The pH of the mixture was adjusted with NaOH (1N) to the corresponding pH according to the experimental design (6 ± 0.1 or 7 ± 0.1). Samples were then flipped from top to bottom at 55 rpm for 120 min at 37 ºC and pH was monitored during the digestion process and readjusted if necessary as pH below 5.7 might inactivate lipase activity (González-Bacerio, Rodríguez Hernández, & del Monte Martínez, 2010).

2.3.2. *Experimental design*

The experimental design consisted of two main sets of experiments. In the first set, the dose of pancreatic 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 analyze the impact of different intestinal conditions on the proteolysis and lipolysis. Of note, pH 7 and 10 mM of bile salts would correspond to the standard intestinal conditions of a healthy adult (Minekus et al., 2014), while pH 6 and 1 mM correspond to the most disadvantageous scenario in EPI individuals (Gelfond, Ma, Semler, & Borowitz, 2013). These latter were the conditions fixed for the second set of experiments, and different concentrations of pancreatin (0, 1000, 2000, 3000 and 4000 LU/g of lipid) were tested. All experiments were carried out in triplicate.

2.4. Analytical determination

Digested samples were put in ice for 10 minutes to stop the enzymatic reactions before performing the analytical determinations. In order to separate the solid fraction from the liquid phase (from now on referred as a "micelar phase") resulting from the digestion process, the total content of a digestion tube was centrifuged (4000 x g-force during 20 minutes 10 ºC) and filtered through a metallic sieve (1.6 mm x 1.6 mm mesh) to separate out undigested food particles in order to determine the matrix degradation index. The liquid passing through the sieve (micellar phase) was collected to determine proteolysis and lipolysis; the remaining micellar phase was freeze-dried (-40 °C and 1.25 mbar, Telstar, Terrassa, Spain) and used for free fatty acids profile analysis by GC-MS.

2.4.1. Matrix Degradation Index (MDI %)

Matrix degradation index (MDI) was determined from the proportion of food that was finely dispersed in the digestion juices at the end of the *in vitro* digestion. The undigested nut particles (solid fraction), previously separated after centrifugation as explained before, were transferred onto an aluminum dish previously weighted. Then, the aluminum dishes with the solid residue were placed in a forced air oven at 60 ºC for 48 h and weighed again to determine the dry matter. MDI corresponds to the proportion of nuts solids passing the metallic sieveand was calculated according to Asensio-Grau et al. (2018) ; Lamothe, Corbeil, Turgeon, & Britten (2012).

2.4.2. Protein digestibility

For protein digestibility assessment, digestion tubes were removed at different digestion times (0, 10, 20, 30, 45, 60 and 120 minutes of gastric stage and 0, 10, 20, 30, 45, 60 and 120 minutes of intestinal stage). Samples were immediately placed in ice and after ten minutes, Trichloroacetic acid (TCA) was added to a final concentration of 12 % (w/w), then they were immediately centrifuged (1400 x g-force during 20 minutes). The mixture was vortexed, and incubated for 60 min. Samples were diluted in glycine buffer (33 mM, pH 10.3) and the TCA soluble protein was determined by measuring the optical density (OD) at 280 nm against a blank prepared with appropriate digestion fluids. The protein fraction soluble in 12% TCA is composed of small peptides and amino acid residues. The digestibility of nut protein was estimated according to Bax et al., (2012), with an adaptation of the mathematical model proposed by Gatellier & Santé-Lhoutellier, (2009) . For each digestion trial, an iteration method was applied using Solver of Microsoft® Excel 2011 in order to estimate OD_{max} by minimizing the sum of squares of the differences between the calculated OD values and measured values. **Equation 1** shows the relationship between OD and half-life time. OD_{max} is related to the proteolysis extent achieved at the infinite digestion time; while the half-life time is the time needed to produce half the amount of hydrolysed peptides compared to OD_{max}.

$OD = OD_{max} \cdot e^{\left(\frac{-B}{time}\right)}$ Equation. (1)

where B = (half-life time)·ln (2)

Furthermore, initial slope of the curve (ΔOD/h) was calculated in order to better analyze the dynamics of the protein digestibility. The **Equation 2** shows the rate of digestion calculated from the derivative of **Equation 1.**

$\frac{d(OD)}{d(time)} = 60 \cdot OD_{max} \cdot B \cdot \frac{1}{(time)^2} \cdot \exp(-\frac{B}{time})$ Equation (2)

Where B= (half-life time) ·ln (2)

Initial slope was calculated over the first 20 minutes of gastric and intestinal stages (Bax et al., 2012)

2.4.3. FFA analysis

Two types of methods were used to measure the FFA in the micellar phase of the digestion medium; a spectrophotometric method which allows estimating the overall FFA was used for all the digested samples, and a chromatographic method which allows the determination of the FFA profile was additionally used in a selection of samples.

The overall FFA released after digestion was measured by means of a spectrophotometric assay kit for this purpose (Roche Diagnostics, Indianapolis, IN, USA) in a spectrophotometer (UV/vis, Beckman Coulter). The solubilisation of free fatty acids was achieved by mixing 100 μl of micellar phase from digested samples with a solution made of 5.72% (w/w) Triton X-100 and 4.80% (w/w) ethanol in water accounting for a total of 10 mL (Lamothe et al., 2012). Linoleic or oleic acid for walnut or peanut respectively, was used as standard for quantitative determination of free fatty acids (FFA). The results were expressed as mg of FFA per gram of fat, considering the average of molecular weight of oleic or linoleic acid (282.47 and 280.45 g mol^{-1} respectively).

The FFA profile was analyzed by GC-MS after transesterification to methyl esters (FAMEs) with a mixture BF_3 methanol (14% in methanol) at 20 °C according to the IUPAC standard method (IUPAC, 1992; Yaich et al., 2011). The digested lipid fraction from samples was extracted as follows: micellar phase previously freeze-dried (60-100 mg) was mixed with hexane (3 mL) and the mixture flipped from top to bottom at 55 rpm for 90 min using an Intell-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia). After that, 1 mL of the hexane containing the lipid fraction, was pipetted into a reaction vial, the hexane evaporated under nitrogen flow and the residue submitted to methylation. For GC-MS analyses, samples of extracted oil from raw nuts (10 mg) or the lipid fraction after evaporation of hexane (1 mL of the extraction hexane containing the digested lipid fraction) were placed in 2 mL glass reaction vials. Internal standard (50 µL of a solution 1 mg / mL), hexane (40 µL) and BF_3 (100 µL) were added into the vials and then heated at 70 °C for 90 min. After transesterification, saturated salt solution (100 µL, 25 % NaCl), H_2SO_4

(40 µL, 10 %) and hexane (0.7 mL) were added to the reaction medium. Analyses of FAMEs were carried out with an Agilent 5977A GC equipped with an auto sampler, an Agilent 5977A and a HP-5MS UI (30m x 0.25mm, 0.25um film thickness) capillary column. The oven temperature was programmed from 90 °C for 2 min, increased to 222ºC at 5ºC/min for 5 min, and increased to 280ºC at 20ºC/min for 2 min, and the injector and detector temperatures were set at 280 °C. The carrier gas was helium at 1.0 mL/min constant flow (split ratio 10:1). Data analysis identification and quantification of FAMEs was accomplished by comparing the retention times of the peaks with those of pure standards (Supelco®37 Component FAMEs Mix, Sigma), and analyzed under the same conditions. Pentadecanoic acid was used as internal standard. The results were expressed as percentage of individual fatty acid compared to the initial content before digestion.

2.5. Statistical Analysis

Analyses of Variance (Multivariate ANOVA) followed by Fisher LSD post-hoc tests were performed to find out the statistical significance of the particle size and intestinal variables (pH, bile salt concentration and pancreatin) on the matrix degradation index, proteolysis, lipolysis and free fatty acids profile in peanuts and walnuts by means of Statgraphics Centurion, and differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1 Influence of intestinal conditions and particle size of nuts on Matrix Degradation Index.

Table 1 shows the statistical effect of intestinal pH, bile concentration, particle size, and their interactions, on the Matrix Degradation Index (MDI (%)) of walnuts and peanuts digested at a fixed concentration of pancreatin of 2000 LU/g of fat. As can be observed, particle size was the variable with the greatest effect on MDI. **Table 2** shows the Matrix Degradation Index (MDI (%)) of digested nuts, walnuts and peanuts of two particle sizes, at different conditions of pH-bile concentration and at a fixed pancreatic enzyme concentration of 2000 LU/g fat (average recommended value for EPI (Turck et al., 2016)). The influence of the pancreatin concentration (0-4000 LU/ g fat), at fixed intestinal conditions of pH 6 and 1mM bile concentration, on MDI is also reported. Food matrix absorbs a significant amount of water during digestion, which combined with the action of digestive enzymes, promotes the softening of the food and the reduction of cohesive forces that hold the matrix structure; depending on the food composition it will result in different degradation profiles (Kong & Singh, 2009). The MDI corresponds to the

218 percentage of finely dispersed solid particles and it provides, therefore, information of the overall mechanical
219 disruption undergone by the food matrix during the whole digestion process (oral, gastric and intestinal stages).
220 The relevance of the particle size can be noted since it was the variable affecting MDI the most in both walnuts
221 and peanuts regardless of the intestinal conditions of pancreatin concentration, intestinal pH or bile concentration.
222 In fact, MDI ranged from 19 to 36 % (average values) in the large particle size samples (that simulate a slight
223 mastication) while MDI reached 52-86 % (averages value) in small particle samples (that simulate a more intense
224 mastication). It might be noted that the minimum value of the above-mentioned intervals of MDI was achieved in
225 peanuts, and the maximum value was achieved in walnuts. These results confirmed that mastication is a key factor
226 that determines the matrix degradation during digestion in most food. The first physical transformation of food
227 matrices during eating occurs in the mouth, producing fractured surfaces with some ruptured parenchyma cells
228 and decreasing the particle size. This phenomenon enlarges the surface area of intra-cellular nutrients exposed
229 to the digestive fluids and increase their availability for hydrolysis by digestive enzymes; thus improving the overall
230 digestion efficiency (the higher the MDI the higher the digestibility of proteins and lipids) and the gastrointestinal
231 absorption of nutrients (Mandalari et al., 2008; Parada & Aguilera, 2007). In addition, the degradation of foods
232 under digestion depends on characteristics and composition of the food matrix, the nature of bonds and the
233 permeability of the matrix to small molecules as well as other parameters such as hardness, cohesiveness, and
234 elasticity that have been previously associated with resistance to matrix degradation such as in cheese or pasta
235 (Lamothe et al., 2012).
236
237 ***3.2 Influence of intestinal conditions and particle size of nuts on Protein Digestibility***
238 Walnut protein is highly digestible and has a good balance of essential amino acids the major protein fraction
239 being glutelins (≈70%) followed by globulins (≈18%), albumins (≈7%), and prolamins (≈5%) (Sze-Tao & Sathe,
240 2000). Peanuts are actually a legume and have more protein than any other nut. Peanuts proteins have been
241 customarily classified as albumins or globulins. Globulins make up 87% of the total protein and are made up of
242 two major proteins, arachin and conarachin (Arya, Salve, & Chauhan, 2016). In this work, two parameters were
243 used to characterize protein digestibility: the maximum optical density (OD_{max}), which is an indirect indicator of the
244 maximum proteolysis extent, and the initial slope of OD that changes with time ($\Delta OD/h$) indicating the initial rate
245 of the proteolytic reaction (Bax et al., 2012). **Table 3** gathers the statistical effect of pH, bile concentration, particle
246 size, and their interactions on the protein digestibility parameters (OD_{max}, and $\Delta OD/h$) of walnuts and peanuts

247 digested at a fixed concentration of pancreatin of 2000 LU/ g of fat. In walnuts the three digestion variables (pH,
248 bile concentration and particle size) affect both protein digestibility parameters, while pH does not seem to affect
249 peanuts proteolysis. The interactions between digestion variables especially influence both the kinetics (initial
250 slope) and the expected maximum proteolysis (OD_{max}) of walnuts. In peanuts, pH-bile-particle size interaction
251 presented a remarkable influence on the kinetics of proteolysis (OD_{max}). With regard to the effect of intestinal
252 conditions of pH and bile concentration (**Figure 1)**, a slight increase in the initial slope (ΔOD/h) was observed in
253 large walnuts and peanuts by increasing bile concentration from 1 to 10 mM at pH 6 (**Figure 1 C and D**). Although
254 the effect of intestinal pH and bile concentration on OD_{max} was statistically not significant, the differences between
255 values are not considerable (**Figure 1B**). In contrast, intestinal conditions affected the kinetics and potential
256 maximum extent of proteolysis in small particle size walnuts with a gradual increase of the values from pH 6 to 7,
257 obtaining the maximum value at 10 mM of bile concentration (**Figure 1A**). In general, much higher rate (ΔOD/h)
258 and potential extent (OD_{max}) of proteolysis were found in digested nuts. The particle size is especially relevant in
259 the hydrolysis of walnut and peanuts proteins (**Table 3**) (**Figures 1 and 2**). It evidences the impact on proteolysis
260 of the higher access of proteolytic enzymes to proteins favored by the great surface area in small particle size
261 samples. Smaller particles maximize protein surface exposure to hidrophilic zones thus, promoting the
262 bioaccessibility of enzymes to cleavage sites. Proteolytic enzymes, pepsin in stomach and trypsin in duodenum,
263 cleaves hydrophobic aromatic amino acids such as alanine, leucine, isoleucine, proline and valine which are
264 predominant in nuts composition and specially in walnuts (Sze-Tao & Sathe, 2000).
265 **Figure 2** shows the proteolysis of both nuts digested at intestinal pH of 6, bile concentration of 1 mM and varying
266 the concentrations of pancreatin (0 to 4000 LU/g of fat which are equivalent to 0, 229, 459, 688, 918 PU/g protein
267 in walnuts, and 0,124, 247, 371, 494 PU/g protein in peanuts). As can be observed, supplementation with
268 pancreatin led to an increase of proteolysis in walnuts, with respect to the proteolysis achieved in the previous
269 gastric stage, regardless of the dosage. (**Figure 2 A and C**). Uniquely, a significant increase of both parameters
270 was found at 918 PU/ g protein (4000 LU/ g fat) in large walnuts. In the case of peanuts (**Figure 2 B and D**), the
271 protein hydrolysis was limited even at high doses of pancreatin. Nevertheless, a significant improvement of the
272 proteolysis was found in large and small peanuts, especially in small ones, at 3000 LU/ g fat (equivalent 371 PU/g
273 protein).
274 Proteolysis parameters achieved in digested peanuts and walnuts, and shown in **Figure 1,** are not directly
275 comparable because of different proteases units per g of protein used in digestion studies of walnuts (459 PU/ g

276 protein) and peanuts (247 PU/ g protein). Results show a higher affinity of proteases to walnut proteins than to
277 peanuts ones. This fact can be directly observed by comparing the values of initial slope (ΔOD/h) and potential
278 extent (OD_{max}) for walnuts and peanuts at similar proteases units, shown in **Figure 2** (PU/ g of protein) (1000 LU/g
279 fat equivalent to 229 PU/g protein in walnuts, while 2000 LU/g fat equivalent to 247 PU/g protein in peanuts). The
280 differences in protein digestibility observed between the two types of nuts might be due to differences in their
281 amino acid composition. The lower values of proteolysis in peanuts could also be related to protein structural
282 changes occurring during roasting. Results from previous studies indicate that roasting of peanuts at 160°C for 30
283 min, adversely affect the quality of proteins and oil in peanut kernels (Damame, Chavan, & Kadam, 1990). After
284 air-roasting, significant damages to the epidermis and the tissue are observed, resulting in cell separation, loss of
285 cellular shape, destruction of the endoplasmic network, distortion of protein bodies, and increase in the size of oil
286 bodies (Altan, McCarthy, Tikekar, McCarthy, & Nitin, 2011; Perren & Escher, 2013). It is well known that heat
287 generates structural changes in food proteins. These mechanisms may include the initial deployment of a protein
288 molecule, the loss of secondary and tertiary structure, the formation of covalent and non-covalent intra- and / or
289 intermolecular interactions (Davis & Williams, 1998; Rahaman, Vasiljevic, & Ramchandran, 2016). Heating can
290 also alter the susceptibility of the proteins to gastrointestinal digestion. After roasting, peanut protein such as Ara
291 h1 forms compact polymers by covalent cross-linking and hydrophobic interactions. This aggregation causes the
292 protein to be inaccessible to some extent to gastrointestinal digestion (Rahaman et al., 2016).
293 Additionally, from the comparison of initial slope values in gastric (walnuts >1.2 mm= 0.196ΔOD/h walnuts
294 <1.2mm= 0.521ΔOD/h; peanuts >1.2mm= 0.178ΔOD/h peanuts <1.2mm= 0.169ΔOD/h) and intestinal stages
295 (**Figure 1 and Figure 2**), kinetics of proteolysis occurs faster in the intestine than in the stomach, and especially
296 in walnuts.
297
298 ***3.3. Influence of intestinal conditions and particle size of nuts on lipids digestibility***
299 In plant food tissues, the physicochemical structure and properties of cell walls are critical factors involved in
300 nutrients digestibility and bioaccessibility in the gastro intestinal lumen (Ellis et al., 2004). In nuts, lipid is the main
301 storage component and the largest proportion of available energy, which comprises ≈50% of the total weight of
302 the kernel and is located in intracellular oil bodies in the form of triacylglycerol (TAG) (Ellis et al., 2004). The oil-
303 bodies have an average diameter of 2-3 μm, approximately, and are surrounded by a single layer of phospholipids
304 in which proteins, mainly oleosins, are embedded (Beisson, Ferté, Voultoury, & Arondel, 2001). The mechanism

by which intracellular lipid and other nutrients are released will depend on the physicochemical properties of the nut tissue in the gut lumen. Thus, a critical factor will be whether the cell walls are disrupted during oral processing (chewing) and further transit along the gastrointestinal tract (Ellis et al., 2004; Guo, Ye, Bellissimo, Singh, & Rousseau, 2017).

The present study analyzed the effect of chewing (particle size) and intestinal conditions (pH, bile and pancreatin concentration) on lipolysis extent achieved after the *in vitro* digestion of peanuts and walnuts (**Table 5**). **Table 4** shows the statistical effect of pH, bile concentration, particle size, and their interactions on the lipolysis (mg FFA/g Fat) of walnuts and peanuts digested at a fixed concentration of pancreatin of 2000 LU/ g of fat. pH and particle size, and its interaction, were the variables with the greatest effect on peanut lipids digestion, while only the pH seems to influence the hydrolysis of fat walnuts. The particle size only affected fat lipolysis in peanuts and has no significant impact in walnuts. The higher concentration of FFA after digestion of small particles of peanuts, compared with the large ones, can be attributed to the greater number of rupture cells, and therefore an increase in lipid bioaccessibility (Ellis et al., 2004; Grassby et al., 2014; Grundy, Wilde, Butterworth, Gray, & Ellis, 2015; Mandalari et al., 2014). In fact, large particles of peanuts exhibited the lowest values of FFA/g of fat even under standard conditions of pH and bile concentration (7/10). Apparently, remaining intact cell walls after chewing might significantly reduce the rate and extent of lipolysis during the digestion of both starch-rich leguminous seeds and nuts (Edwards, Warren, Milligan, Butterworth, & Ellis, 2014; Tovar, De Francisco, Bjork, & Asp, 1991). In fact, some human studies supplying a peanut-rich diet for 6 days reported that undigested lipid from nuts transported to more distal sites of the gastrointestinal tract led to an increased excretion of faecal energy, and thus to malabsorption (Levine AS, 1980). Similarly, more recent studies indicated that significant amounts of lipid were excreted by subjects under diets rich in either almonds or pecans (Sabaté, 2003). According to our results, an effective disruption of the walnuts matrix seems to be easily achieved during *in vitro* digestion as compared to disruption of peanuts matrix in which lipolysis was limited.

The influence of intestinal pH, bile and pancreatin concentration on lipolysis was also explored (**Table 5**); and in order to analyze if there is any impact of these factors on the digestion of different TAG, the free fatty acids profile was additionally determined only in the released and digested lipids from small particles nuts (**Figures 3).** A significant influence of intestinal pH on overall lipolysis of walnuts was only observed when bile concentration was 10mM (**Table 5**); the same effect was observed in the release of linoleic acid (ω-6 fatty acid) (**Figure 3**) which is the majority FFA (Sze-Tao & Sathe, 2000), while no effect of pH and bile concentration was observed for palmitic

and stearic acids. Similarly, in the case of peanuts only pH showed a significant influence on the overall lipolysis. However the analysis of the released FFA profile revealed a significant influence not only of pH but also of bile concentration, this was observed for almost all the free fatty acids and not only for the predominant fatty acid which in this case is oleic acid. The evidenced influence of bile salts is related to their surfactant properties that play a crucial role in lipid digestion (Maldonado-Valderrama, Wilde, MacIerzanka, & MacKie, 2011) by promoting colipase and subsequently lipase adsorption at the interface of fat globules. Bile salts are also required to remove the products resulting from lipolysis accumulated at the interface, and prevent lipase inhibitions (Grundy et al., 2015). Regarding the effect of pancreatin concentration (0 to 4000 LU/ g fat), the overall lipolysis increase with the pancreatin concentration up to achieving a maximum value from which an increase of enzymes did not result in a significant increase of lipolysis (i.e. peanuts), or even promoted a slight decrease (i.e. walnuts). The obtained results revealed that the concentration of pancreatine that maximizes lipolysis, under *in vitro* digestion with intestinal pH 6 and bile concentration 1mM, is 3000 LU/g fat for walnuts and 2000 LU/g fat for peanuts. The low values of lipolysis in digested roasted peanuts have been also found in roasted almonds compared to raw almonds, where the distribution of lipids is uneven owing to heat-induced partial coalescence of lipid into larger droplets (Mandalari et al., 2014). In parallel, it is possible that roasting process results in an increase of cell wall porosity, thereby allowing greater access of digestive fluids but without a significant intracellular lipolysis because of the presence of coalesced lipids (i.e. lower surface area: volume) (Grundy et al., 2015).

4. Conclusion

From the present study, it can be concluded that both the type of nuts and the particle size determine the digestibility and bioaccessibility of proteins and lipids. Results from the study revealed that the MDI was affected by the particle size in both nuts, regardless of the intestinal conditions and the dose of enzyme supplementation, the highest values were observed in small particles 52-86% (average value) versus 19-36% (average value) in large particles. This parameter provides information on the relevance of chewing on the effective access of digestive enzymes to macronutrients.

Results from *in vitro* digestion simulating altered intestinal conditions allowed the quantification of differences in macronutrient digestion from nuts under healthy and EPI conditions. Kinetics and hydrolysis of proteins during intestinal stage occur in a greater extent in small particles nuts than in large ones, and especially in walnuts than peanuts, under similar conditions of protease units per g of protein. Supplementation with pancreatin led to an

increase of proteolysis in walnuts regardless of the dosage. Intestinal conditions of EPI, pH 6 and bile concentration 1 mM, only seemed to limit proteolysis in small particles size walnuts.

Regarding lipolysis, results report the release of FFA in peanuts increased as long as both bile concentration and pH do. In the case of walnuts, only linoleic acid (majority FFA) showed the greatest significant difference in its quantification at intestinal conditions of pH 7 and bile concentration of 10 mM.

Finally, lipolysis in both nuts increases as the dose of pancreatin increases until a maximum extent is reached. According to the obtained results, recommended dose of pancreatin in EPI would be 2000 and 3000 LU / g fat for peanuts and walnuts, respectively.

Conflicts of interest

There are no conflicts of interest to declare.

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Figure Captions:

Figure 1. Protein digestibility parameters (OD_{max} and Initial Slope ΔOD/h) of walnuts and peanuts with large (> 1.2 mm) and small (< 1.2 mm) particle sizes digested at different conditions of intestinal pH (6 or 7), bile concentration (1 or 10 mM) using a fixed pancreatic enzyme dose (2000 LU/ g fat equivalent to 459 PU/g protein in walnuts and 247 PU/g protein in peanuts). Letters a-d refer to the homogenous groups obtained by the ANOVA applied to data of each nut in the same size and it provides information about the effect of intestinal conditions (pH-bile concentration) on the protein digestibility parameters (OD_{max} and Initial Slope) (p-value <0.05). Line indicates the value in the gastric stage for particles size <1.2mm, segmented line indicates values in the gastric stage for particles size >1.2mm, dark bar indicates the values in the intestinal stage for particles size >1.2 mm, light bar indicates values in the intestinal stage for particles size <1.2 mm.

Figure 2. Protein digestibility parameters (OD_{max} and Initial Slope ΔOD/h) of walnuts and peanuts with large (> 1.2 mm) and small (< 1.2 mm) particle sizes digested at intestinal condition pH 6, and bile concentration 1mM, with different pancreatin doses (0-1000-2000-3000-4000 LU/ g fat equivalent to 0-229-459-688-918 PU/g protein in walnuts, and to 0-124-247-371-494 PU/g protein in peanuts).
Letters a-d refer to the homogenous groups obtained by the ANOVA applied to data of each nut in the same size and it provides information about the effect of pancreatin concentration on the protein digestibility parameters (OD_{max} and Initial Slope) (p-value <0. 05). Line indicates the value in the gastric stage for particles size <1.2mm, segmented line indicates values in the gastric stage for particles size >1.2mm, dark bar indicates the values in the intestinal stage for particles size >1.2 mm, light bar indicates values in the intestinal stage for particles size <1.2 mm.

Figure 3. Release of the individual free fatty acids after *in vitro* digestion of small particle sized walnuts and peanuts. **A** and **B** *in vitro* digested with a fixed pancreatin concentration (2000 LU/ g fat) and different combinations of intestinal pH and bile concentration (pH 6 or 7, bile salts concentration 1 or 10 mM); **C** and **D** *in vitro* digested under fixed intestinal conditions (pH 6 and bile concentration 1 mM) and different pancreatin concentration (0-4000 LU/ g fat).
Letters a-e refer to the homogeneous groups obtained by the ANOVA applied to data in each individual free fatty acid release under different pH-bile concentration or pancreatin doses.