WAGENINGEN
UNIVERSITY & RESEARCH

The effect of cell wall encapsulation on digestion of macronutrients derived from nuts: *lipid bioaccessibility*

MSc Thesis

Eleni Ntone

911015610050

Supervisors: Edoardo Capuano, Nicoletta Pellegrini, Costas Nikiforidis

CHAIR GROUP FOOD QUALITY AND DESIGN

September 2016 - March 2017

Acknowledgments

First of all I would like to express my gratitude to my supervisors, *Edoardo Capuano, Nicoletta Pellegrini and Costas Nikiforidis*, for their valuable help, guidance, support and general contribution to this thesis project. But above all, I would like to thank them for being always there to listen to my concerns, discuss with me and advise me any time needed. Their inputs and their enthusiasm motivated me to try harder for my research.

I would also like to thank my friends for all the priceless moments inside and outside Axis, for all the laughs, for all the discussions. Without them all these months would not be as unique as they were.

I want to thank my family that always supports me in any decision I take and they gave me the opportunity to come to Wageningen and gain all these invaluable knowledge and experience. I am sure that all this effort from both sides will be worthy. Last but not least, I want to thank my friends back home who always care about me and support me no matter how far we are.

Abstract

Food matrix composition, structure and properties are important factors that influence the bioaccessibility of nutrients during food digestion. It has been suggested that plant cell walls remain intact during human digestion, acting as a physical barrier and thus controlling the rate and extent of nutrient release. Nuts are nutrient dense foods and their consumption is associated to health promoting effects, principally due to their fatty acid composition. The aim of this study was to investigate the effect of the cell wall on the rate and extent of lipid hydrolysis during *in vitro* digestion using raw and roasted hazelnut materials with potentially different degrees of bioaccessibility. The results revealed that extracted oil bodies exhibited a significantly higher lipid hydrolysis compared to hazelnut particles, implying that lipid bioaccessibility was directly related to the presence of cell wall. Moreover, roasting had an impact on the structure properties of hazelnut cells as well as on the interfacial properties of oil bodies, facilitating lipid release.Microscopic examination of particles before and after digestion demonstrated a different swelling behaviour in roasting nuts, implying a more efficient diffusion of fluids and enzymes into the cells due to heat treatment. Heat treatment also provoked destabilization of the interfacial proteins of oil bodies, facilitating their proteolysis under gastric conditions, altering the emulsion properties and enhancing FFA release during intestinal digestion. This study underlined the inhibiting role played by the plant cell wall on nutrient release during digestion as well as the beneficial impact of heat processing on nutrient bioaccessibility and hence on the health promoting effects provided by nuts.

Key Words: *cell wall, hazelnuts, oil bodies, roasting, lipid bioaccessibility, FFA release, in vitro digestion*

Abbreviations

CHD: Coronary heart disease
CLSM: Confocal laser scanning microscopy
Cryo-SEM: *Cryo*-Scanning electron microscope technique
FFA: Free fatty acids
GI tract: Gastrointestinal tract
LM: Light microscopy
MGA: Monoglycerides
OBs: Oil bodies
SDS-PAGE: tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM: Scanning electron microscope
SGF: Simulated Gastric Fluid
SIF: Simulated Intestinal Fluid
SSF: Simulated Salivary Fluid
TGA: Triacylglycerides

Table of Contents

1. Introduction

Many beneficial and detrimental health effects of specific nutrients present in foods are well documented. However, although composition tables provide information regarding the total amount of a nutrient that can be obtained, its availability for absorption in the gut is in many cases quite uncertain, depending on processing conditions and presence of other components. The fraction of an ingested nutrient that can be used by the organism is of major importance and several factors like the chemical state of the nutrient, its release from the food matrix and possible interactions with other food components influence its availability[1].

Bioaccessibility is defined as the proportion of nutrients released from a food matrix and therefore potentially available for absorption in GI tract [2-4]. Food matrix composition, structure and properties are important factors that influence the bioaccessibility of nutrients and food components during digestion [5, 6]. It is indicated that for certain nutrients the matrix of natural foods or the microstructure of processed foods may promote or impede their nutritional response in *vivo*[1].

In vitro digestion models are widely used in order to study nutrient bioaccessibility in the GI tract. The *in vitro* gastrointestinal models have been developed (including the oral, gastric and intestinal phase) in order to simulate the real conditions that occur during digestion in human GI tract [7]. They allow to monitor the behaviour of food nutrients or bioactive compounds during digestion, in order to determine whether they are affected by digestion conditions and consequently impact the efficiency of digestion [8].

1.1. Plant cell wall and nutrient digestion

Plant cell wall, consisting of complex networks of polysaccharides mainly cellulose, hemicellulose and pectic components [9](Figure 1), is of high importance concerning the release of nutrients during digestion. These compounds are resistant to degradation by mammalian endogenous digestive enzymes and their breakdown within the gut is partially achieved by the gut microbiota that lives in symbiotic or mutualistic association with the host[10]. Consequently, the cell wall remain intact during human digestion, acting as a physical barrier, encapsulating nutrients and controlling the rate and extent of their release in gastrointestinal tract[11]. Many studies have shown that the relative impermeable cell wall inhibit the release of nutrients during digestion as well as the diffusion of enzymes, restricting their bioaccessibility[4, 11, 12]. This structural integrity and extent of permeability of cell walls, which depend on cell wall strength and inter-cell adhesion, seems to be of high importance regarding nutrient bioavailability[2]. Processing of plant foods may have an impact on these properties , and therefore on nutrient release [13]. It is stated that processing may improve bioavailability most likely as a result of facile disruption of the cell walls of plant tissues during oral processing [1].

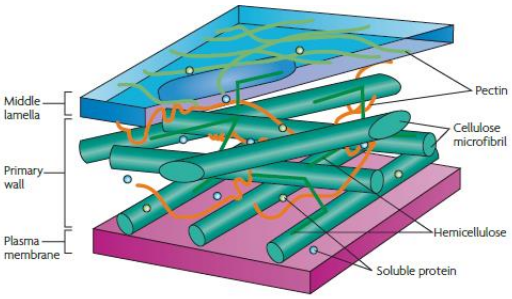

FIGURE 1.Plant cell wall structure. Adopted by Sticklen (2008)

1.2. Nuts and human health

Nuts (tree nuts and peanuts) are nutrient dense foods characterized by complex matrices. Due to their unique composition, nuts are likely to have beneficial impact on health as their consumption is associated with a reduced CHD, obesity, diabetes, as well as with cholesterol-lowering effects [14]. Many of the health benefits provided by nuts are attributed to their fatty acid composition which is low in saturated fatty acid (SFA) content (4–16%) and rich in unsaturated fat such as monounsaturated fatty acids (MUFA) (about half of the total lipid content) and polyunsaturated fatty acids (PUFA). Lipid digestion and absorption is considered to have an impact on postprandial lipaemia which is related to CHD [11]. Moreover, other health-promoting nutrients such as high-quality vegetable protein, minerals as well as bioactive compounds like tocopherols, phytosterols, and phenolic compounds are present in nuts [14].

1.2.1. Nuts and lipid bioaccessibility

As the main health promoting effects of nut consumption are associated with their fatty acid composition, lipid availability during digestion is of great interest. Human studies have shown that nut consumption is inversely associated with weight gain [15, 16] despite their high nutrient content. It has been reported that the energy content of almonds[5, 16], walnuts[15] and pistachios [17] has been overestimated by the Atwater factors due to lower bioavailability of lipids in the GI tract.

A crucial factor that affects lipid digestibility of nuts is their physical behaviour in the GI tract. Specifically, the way of dissemblance as well as the extent to which lipids are released are crucial factors that influence lipid digestion in nuts and consequently their health-promoting effects. The size as well as the microstructure of the particles following oral processing has a significant effect on nutrient release, digestion kinetics and other physiological processes in the GI tract [12]. The disruption of the matrix during the oral processing has an important role on lipid digestion as it determines the facility of enzymes and other components to access the droplet surface. The extent of disruption of the matrix as well as the rate of enzyme diffusion influence the rate and extent of lipid digestion [18]. It is reported that dietary fibres present in the cell wall may alter lipid digestion through different physicochemical mechanisms; they can form a protective coating that prevents lipolytic enzymes to come in contact with the lipids or they can directly interact with the enzymes or bile salts, resulting in reduced activity. Another factor that has an impact on lipid bioaccessibility is the structure of lipids within the nuts. Nut lipids [composed predominantly of triacylglycerols (TGA)][12]are stored in oleosomes or OBs, which are intracellular spherical organelles surrounded by a monolayer of phospholipids (PL) embedded with some proteins, mostly oleosin[9, 19-23] and some minor proteins called caleosin and steroleosin[21] (Figure 2). The size of the OBs is 0.5-2μm [24] in diameter and is determined by environmental factors and differences between seeds of different origin [25]. Oleosins are alkaline proteins of 15-30kDa [23], representing 1-4% of

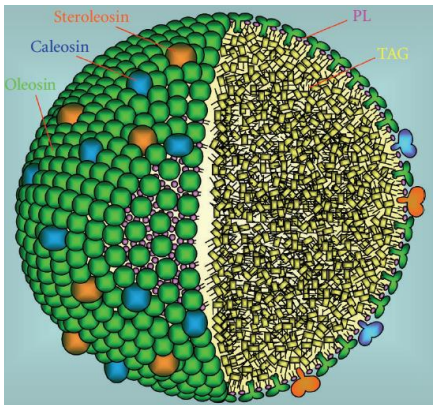

FIGURE 2.Structural model of an oil body. Taken by Tzen(2012)

the oil body mass [22] and they are considered to play role in the stability, synthesis and metabolism of OBs [23]. OBs exhibit a negative charge at neutral pH [21, 22, 26], which prevents their aggregation and coalescence by electrical repulsions [22].

The interfacial microstructure of OBs affects the way that lipids are released in order to be digested and absorbed in the gastrointestinal tract of human body[19]. As lipid digestion is an interfacial process[20], the composition and properties of the surrounding layer can affect the stability of lipid droplets towards disruption and coalescence in the GI tract. This structure may also impact the ability of gastric and pancreatic lipase to adsorb to the surface and act on the substrate for further lipolysis [6].

Gastric lipase is most active on sn-3 ester linkages of TAGs, but hydrolyses sn-1 positions as well. However, only 10-30% of lipid digestion occurs under gastric conditions [27]. Pancreatic lipase hydrolyses the Sn1 and Sn3 positions of TGA, resulting into the production of FFA and 2-monoacylglycerol. The 2-monoacylglycerol molecule is further hydrolysed to release the last fatty acid from the sn-2 position, leaving a glycerol (Figure 3). The optimal activity of the human pancreatic lipase is around pH 6.5 combined with the presence of colipase and in some cases with the presence of surface active bile salts [28].

The study of the OBs behaviour during digestion has gained interest in order to evaluate the effect of oleosins on lipid digestion while excluding the cell wall barrier effect. Moreover, these systems, have been recommended for the encapsulation, protection and delivery of bioactive food components within human body [29][30].

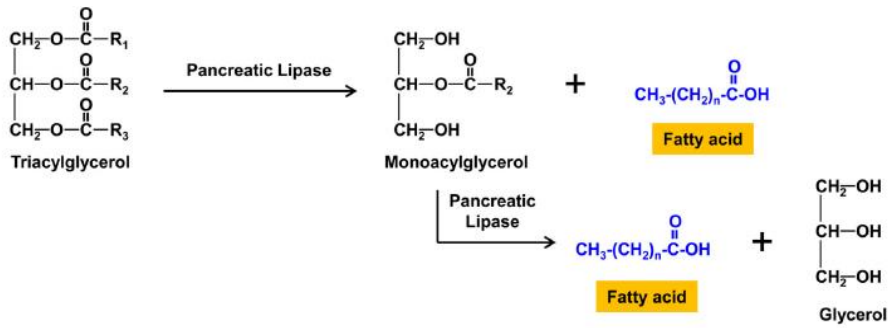

FIGURE 3. Hydrolysis of triacylglycerol by pancreatic lipase during intestinal digestion. Adopted by Wang et al. (2013).

In addition, the chain length of FFA present in the TGA can also influence the digestion of lipids. Nut lipids are composed mainly of long-chain fatty acids, mostly oleic (18:1) and linoleic (18:2) fatty acid [31]. According to studies, long-chain fatty acids are digested slower than medium-chain fatty acids [32]. This effect is attributed mainly to the capability of medium-chain fatty acids to disperse into the water phase during digestion, whereas long-chain fatty acids accumulate to the oil-water interphase for longer times. This behaviour inhibits lipase activity on the lipid surface, and consequently affects the rate and extent of lipid digestion [29].

1.3. Effect of processing on lipid digestion in nuts

Lipid bioaccessibility depends on many factors, such as the size of nut particles which determines the number of ruptured cells [13]. Cooking and/or mechanical processing (including chewing) disrupts cell walls and oil bodies, promoting lipid release[20]. Lipids from ruptured cells are more available for emulsification and digestion by gastric and pancreatic lipase in the GI tract [4, 11]. Particles of smaller size have more fractured cells, hence a greater nutrient release compared to larger particles [12]. The effect of particle size on lipolysis kinetics is underlined in many studies [2-5, 12] emphasizing that lipid bioaccessibility is highly dependent on particle size.

Roasting of nuts is a thermal process that involves dehydration as well as Mallard reactions, which are complex reactions responsible for the brown colour[12]. Different studies indicate that roasting can greatly affect the cells, the cell walls as well as the intra-cellular oil bodies. Moreover, it is reported that heating can promote cellular swelling and rupture and subsequently facilitate mechanical processing [20]. *Mangalore et al.* (2008) [11] mentioned that there is evidence of considerable swelling of cell wall and middle lamella and a further release of lipids from the intact cell wall during digestion which may indicate that diffusion of enzymes, bile salts and lipid products may be facilitated by roasting[11]. At the same time, roasting appears to contribute to the rupture of the oleosome membrane, resulting in coalescence of oil bodies during digestion [3, 20]. The disruption of oleosin layer can be attributed to the fact that oleosins denature in lower temperatures than of these that roasting process requires[12], indicating that a "pre-digest" effect of oil bodies may take place[20] that can alter lipid digestibility.

2. Objective

The purpose of this study was to assess the role played by cell walls and oil body membranes on the bioaccessibility of hazelnut lipids. The effect of processing on cell wall and oleosome integrity was also investigated.

3. Research questions

- How does physical integrity of cell walls affect lipid bioaccessibility in hazelnuts?
- How does roasting affect the cellular integrity of nuts and thus lipid release?
- Does roasting process affect the rupture of the oleosin layer around oleosomes and consequently the action of lipase enzyme and thus lipid release?

4. Materials and methods

4.1. Materials

Raw hazelnuts with the kernel were purchased from Aladdin's notenhoek.
Hazelnut oil was purchased by Albert Heijn.

4.2. Sample preparation

4.2.1. Raw and roasted hazelnut particles

Digestibility experiments were performed on raw and roasted hazelnut particles. Hazelnut particles were created in order to estimate the effect of cell wall encapsulation on lipid bioaccessibility during digestion.

Hazelnuts were roasted in the incubator at 140°C for 20 min. The roasting step was performed in the laboratory under known conditions in order to be able to determine the differences between raw and roasted samples, as some variability in lipid content as well as in matrix structure is expected after processing.

Hazelnut particles of 1-2mm (raw, roasted) were obtained by mechanical process with the use of a food processor (blender) followed by sieving using sieves of 1and 2mm.

4.2.2. Hazelnut OBs

Figure 4 presents an overview of the OBs extraction process in aqueous solution. Fifty grams of hazelnuts (50 g) (raw, roasted) were soaked in deionised water at room temperature (ratio of 1:5 w/v) for two hours while stirring with a magnetic stirrer at 200rpm. The pH was adjusted to 8.0 every 15 minutes to induce charge unfoldment and solubilisation of extraneous proteins

present in the nut. The solubilisation of extraneous proteins is important as it facilitates the transfer of lipids and fibres in the water phase. Due to the buffering capacity of proteins the pH had to be adjusted continuously as more protons were phasing the continuous phase and pH was decreasing as more proteins were solubilised. The mixture was stored in fridge overnight. The day after, each milky aqueous suspension was blended for 40 seconds in medium speed after firstly adjusting the pH again to 8.0. The blended mixture was filtered with the use of double layer cheesecloth. The solid residue was mixed again with deionised water (1:4 w/v), the pH was adjusted to 8.0 and the mixture was blended and filtered again. The two permeates were mixed and centrifuged at 3000rpm for 15min at 4^{o}C to remove solid fibres. A second centrifugation (10000 xg/ 30min) followed, after pH adjustment to 8.0toremovethe extraneous proteins. The obtained cream was carefully collected and dispersed again in deionised water (1:5 v/v), the pH was adjusted to 8.0 and a last centrifugation step at 10000xg for 30 minutes followed. This process was important to eliminate any interaction between these proteins and the intrinsic proteins of OBs that may impact their digestion. At the end of the centrifugation the supernatant cream was collected carefully and smoothly to obtain homogeneous OBs creams.

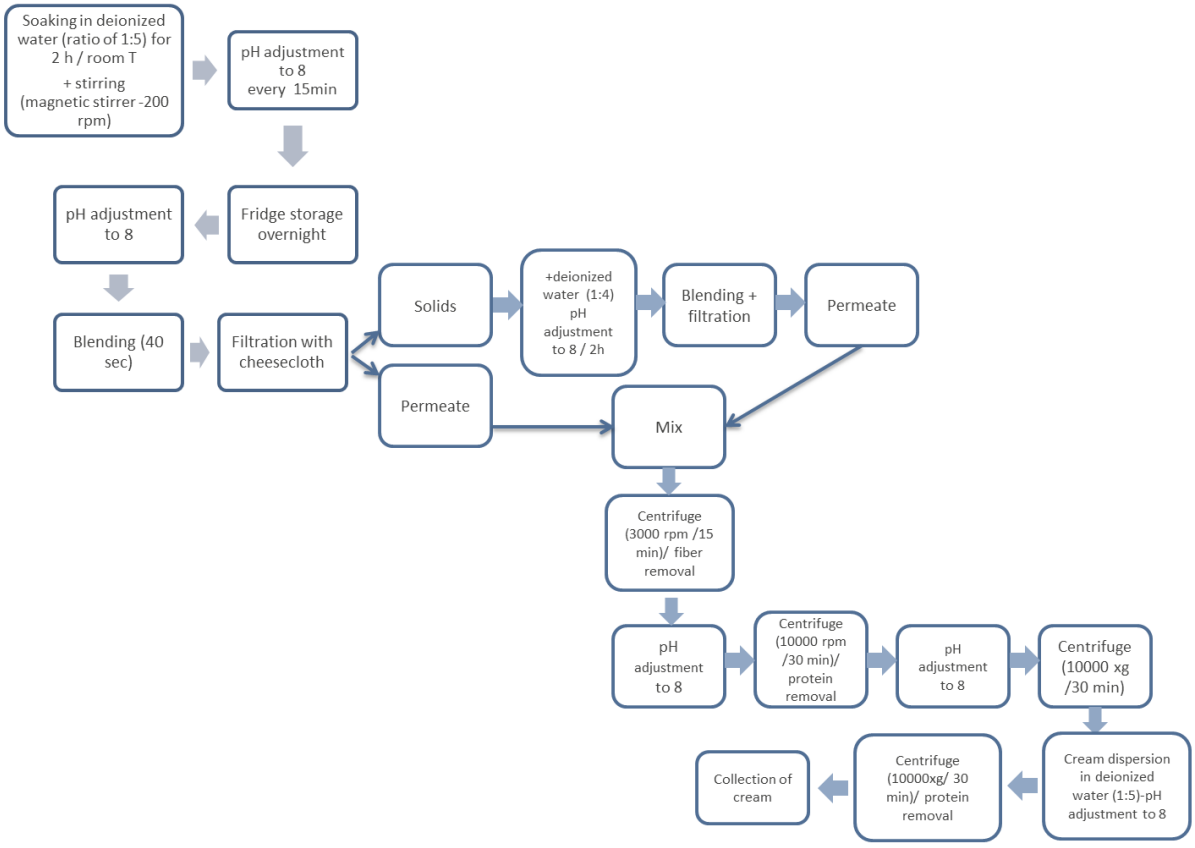

FIGURE 4.Overview flow diagram of OBs extraction process

4.3. *In vitro* digestion

In order to assess the bioaccessibility of lipids derived from hazelnuts and address the effect of cell wall, the *in vitro* digestion method according to protocol [7] was followed. The simulated digestion method includes oral, gastric and intestinal phase. This method tries to mimic the *in vivo* conditions, considering all the factors, including the appropriate enzymes, pH, time, bile salts etc. In Figure 5 an overview of the *in vitro* digestion protocol followed is presented.

Raw and roasted hazelnuts particles, extracted OBs as well as hazelnut oil were digested *in vitro*. Defatted hazelnut particles obtained from Soxhlet lipid extraction were also subjected to *in vitro* digestion to assess the contribution of AA release on pH-stat measurements. Raw and roasted hazelnut particles were also digested *in vitro* without the presence of enzymes to examine if potential endogenous enzymes present in nuts could influence the pH during intestinal digestion. The lipid hydrolysis over intestinal phase was monitoring using pH-stat method, with Titrando 902 (Metrohm). The samples were analysed in duplicates.

A separate digestion experiment was performed on OBs from raw and roasted hazelnuts in order to study their destabilization behaviour during digestion. The digestion was performed according to the protocol in shaking water bath for both gastric and intestinal conditions. The experiments were performed in triplicates.

4.3.1. *In vitro* digestion protocol

Before starting the experiment, the activity of the enzymes used was assessed according to the protocol of supplementary materials mentioned by Minkeus et al. (2014) [7] with some modifications. Porcine Pepsin (3,200-4,500 units/mg protein), Pancreatin extract from porcine pancreas (4 × USP specifications), and Lipase from porcine pancreas Type II, (100-500 units/mg protein) were purchased by Sigma Aldrich Co.

According to this protocol, pancreatic lipase was suggested to be prepared in a concentration of 1mg/ml in order to assess the activity of the enzyme. However, porcine pancreatin extract contains around 1.1% pancreatic lipase [33]. Thus, it was decided to test pancreatin extract in a concentration of 50 and 100 mg/ ml to achieve higher lipase concentration. Furthermore, another experiment using a combination of pancreatin extract and pancreatic lipase (100-400 U/mg) was performedto determine if colipase present in pancreatin mixture can support the action of additional lipase. Different ratios (pancreatin: lipase) were tested in order to achieve 500 U/mg, which is the activity of lipase found in 50 mg of pancreatin. Pancreatic lipase was assumed to exert the lowest activity (100 U/mg) according to the specifications on the label.

Moreover, the influence of the composition of digestive fluids on the pH-stat method was assessed by performing a pH-stat analysis using only the electrolyte solution of the intestinal phase over two hours incubation.

Oral phase

Two and half grams (2.5 g) of raw hazelnut particles were used, which corresponds to half of the amount of sample and fluids suggested by Minekus et al (2014) protocol [7]. As the hazelnut samples had different oil content the sample size was adjusted in order to achieve the same oil

content for each hazelnut sample category and have comparable results. Raw hazelnut particles were considered as reference material with a lipid content of 1.52g. Table 1 presents the amounts used for each hazelnut material.

TABLE 1. Amounts of hazelnut materials used in the *in vitro* digestion experiments

Sample	Amount (g)
Raw hazelnut particles	2.5
Roasted hazelnut particles	2.65
OBs extracted from raw hazelnuts	1.97
OBs extracted from roasted hazelnuts	2.85
Hazelnut oil	1.52

The samples were mixed with SSF electrolyte stock solution to obtain a final ration of 50:50 (v/v). The mixture was incubated for 2 minutes at 37°C. The addition of salivary α-amylase was not included at this step. 12.5 ml of $CaCl_2$(0.3M) were added to achieve 0.75 mM in the final mixture, followed by the addition of 487.5 µl water.

Gastric phase

The sample obtained from the oral phase (5mL) was mixed with SGF electrolyte solution to obtain a final ration of 50:50 (v/v) after the addition of other recipients and water. $CaCl_2$(0.3M) was added to achieve 0.075 mM in the final digestion mixture. The pH was adjusted to 3.0 with 1 M HCl and porcine pepsin was added to achieve 2000 U/ml in the final digestion mixture. Water was also added into the reaction vessel until 10 ml. The digestion time was 2h at 37°C.Sufficient mixing was needed using shaking water bath at 37°C.

Intestinal phase

In this step, the gastric chime (the semi-fluid mass of partly digested food derived from the gastric phase) was mixed with SIF electrolyte stock solution to obtain a final ration of 50:50 (v/v) after the addition of other recipients and water. Bile salts were dissolved in SIFand 1.25 ml was added in the reaction vessel. 20 µl of 0.3M $CaCl_2$ were also added to reach 0.3mM final digestive mixture. The pH was set to 7.0 and 4 ml of pancreatin extract enhanced with pancreatin lipase in a ratio of 10:1 were added to achieve 2000 U/ml in the final digestion mixture. The lipid content in the final mixture was 7.2 wt.%.

The rate of lipolysis was continuously measured over 120 minutes, with 1 minute time interval, using the pH-stat method. The standing tube was placed in water bath at 37°C while stirring at 200 rpm with a magnetic stirrer. The mixture was titrating using 1N NaOH over 120 min with an end point of pH 7.0.

The FFA release as a percentage of the initial total lipid content was calculated by applying the following formula:

$$\%FFA = 100 * \frac{(VNaOH * mNaOH * Mlipid)}{wlipid * 2}$$

where V_{NaOH} corresponds to the volume of NaOH required to neutralise the FFA produced, m_{NaOH} is the concentration of the NaOH solution used (in M), w_{lipid} is the total mass of TAG initially present in the reaction vessel (in g), and $M_{lipid}$is the molecular weight of oil (in g/mol) [18, 34].

The molecular weight of hazelnut oil was estimated to be 853.2 g/mol. This value was calculated from the TAG composition of the oil and the occurrence of the FFA within these TAGs (Oleic :77%, Stearic: 12%, Palmitic: 8% and Linoleic: 8%[35].

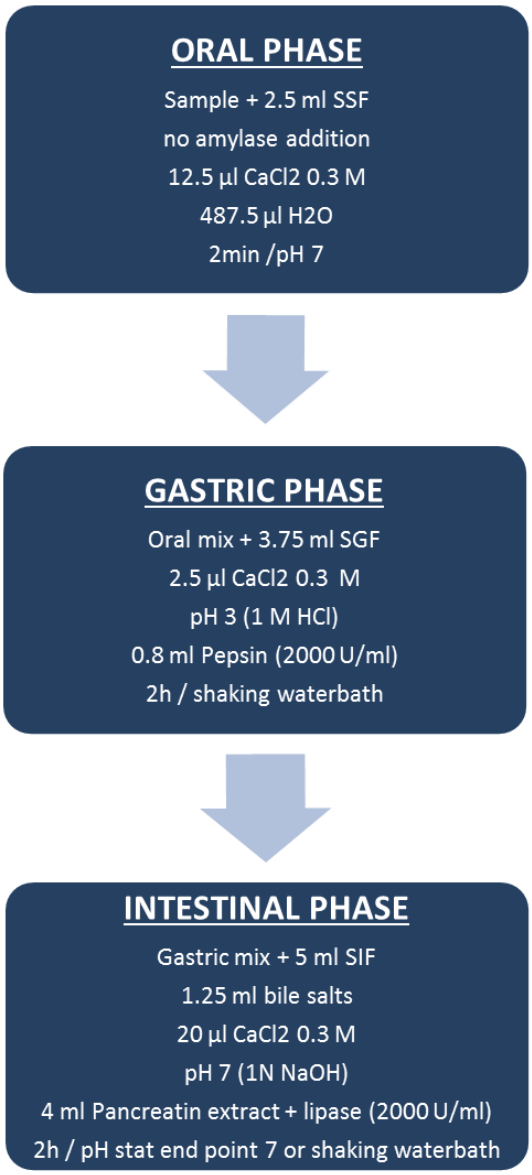

FIGURE 5. Overview flow diagram of simulated *in vitro* digestion method

4.4. Sample characterization

4.4.1. Lipid content

The percentage of lipid present in hazelnut particles and hazelnut OBs (raw and roasted) was determined using Soxhlet extraction with petroleum ether as solvent for ten hours. The digesta residues recovered from in *vitro* digestion were also analysed for total lipid content with Soxhlet extraction. The particles after intestinal digestion were recovered by filtration with Watman folded filters of 185mm and were frozen with liquid nitrogen before the analysis.

4.4.2. Moisture content

One gram (1 g) of hazelnut particles and hazelnut OBs (raw and roasted) was dried at 50°C, in the incubator, until stable weight.

4.4.3. Protein content and composition

Dumas method was used to define the protein content of hazelnuts particles and OBs. Regarding the OBs, the protein composition as well as the size of the surrounding proteins before and after digestion was determined using SDS-PAGE. The protein profile of raw and roasted particles before and after digestion was also examined with SDS-PAGE.

The samples were prepared according to the FQD department protocol with some modifications. Each sample was mixed with sample buffer (NuPAGE® LDS), Sample Reducing Agent (NuPAGE® Sample Reducing Agent) and water in the amounts suggested by the protocol. 17μl of the prepared sample and 8μl of protein marker (BlueRay Prestained Protein Marker 10-180 kDa) were loaded to the gel (NuPAGE® Novex® 12% Bis-Tris Gel). Running buffer (NuPAGE® MES SDS Running Buffer) antioxidant agent (NuPAGE® Antioxidant) were added to the buffer chamber. The gel was washed with water and stained (Coomassie Brilliant Blue R-250 Staining Solution, Bio-Rad Laboratories B.V) overnight under slow shaking.

Concerning the study of the protein profile of hazelnut particles, small (3-4mg) and big (8-9mg) hazelnut particles after digestion were analysed. Regarding the OBs after digestion, the samples were too diluted, thus a heat treatment at 100°C for 20 minutes was applied after the sample preparation in order to concentrate the solution.

4.4.4. Interfacial changes

The average droplet size of hazelnut OBs before and during digestion was determined using dynamic light scattering using Malvern Mastersizer 3000. The obtained OBs were diluted in deionised water in 1:10 (w/v) ratio and analysed for size distribution. The measurements were done in triplicates. The surface charge of the OBs was determined before and during digestion by measuring the ζ-potential using Malvern Zeta Nano sizer. The samples were diluted in 1:1000 (w/v) in deionised water and analysed in triplicates.

4.4.5. Microstructural analysis

Cryo-SEM analysis concerning the changes in structural integrity of the cell wall as a result of processing and digestion was performed using FEI Magellan 400 scanning electron microscope equipped with a Leica cold stage for cryo-microscopy. The hazelnut particles (raw and roasted) before digestion were soaked in deionised water for two days before microscopy analysis.

Light microscopy (LM) was used during the digestion to observe the behaviour of the OBs regarding their size and structure, using Axioscope 2 Plus with Zen 2011 software.

4.5. Statistical analysis

The data were analysed, using SPSS version 23.0. For all tests, the significance level was set at $p<0.05$ (2 tailed). NaOH release during digestion was assessed by repeated-measures analysis of variance (ANOVA) with time (60 and 120 min) and materials (i.e. raw, roasted particles, raw, roasted OBs and oil) as 'within sample' factors.

5. Results and discussion

5.1. Nut selection

In this study, hazelnut particles and hazelnut OBs were chosen to be studied under *in vitro* digestion in order to assess lipid bioaccessibility in relation to cell wall integrity and to roasting effect.

Most of the studies regarding the cell wall lipid encapsulation phenomenon concern mainly almonds[36, 37] which have been already investigated extensively. Other nuts like walnuts [15, 38], pistachios [17] and peanuts [20] have been also studied but in smaller extent. In addition, all of the studies regarding the digestion of OBs extracted from nuts concern crude but not pure OBs[12, 19, 38]. Furthermore, there are only few studies regarding the impact of roasting of nuts on lipid bioaccessibility during digestion [20, 34].

Human studies regarding the metabolizable energy from nuts including almonds [16], walnuts [15]and pistachios [17], demonstrate that the digestibility of nuts might be nut-dependent. This observation is probably attributed to the physicochemical structure of each nut, mainly in differences in fat and fibre content [15]. Indeed, according to Borges & Peleg [39]the presence of oil appears to enhancenut brittleness.Therefore, this study aimed to investigate 1) the cell wall encapsulation phenomenon for other nuts, 2) the impact of roasting on lipid bioaccessibility, 3) the behaviour of pure OBs extracted from hazelnuts under *in vitro* digestion.

It was assumed that higher lipid content may reflect different failure behaviour and consequently varying lipid-digestibility behaviour. Moreover, hazelnuts are used to be consumed roasted, thereby studying their lipid bioaccessibility related to the roasting process may alter their consumption recommendations.

5.2. Sample characterization before digestion

5.2.1. Hazelnut particles

TABLE 2. Composition of hazelnut seeds and particles before and after roasting

	% MOISTURE	%LIPID	% PROTEIN
Raw hazelnut seeds	3.32 *±0.075*	69.00 ± *3.00*	16.34 ± *0.09*
Roasted hazelnut seeds	3.18 ± 0.01	72.1 ± 3.13	17.07 ± 0.09
Raw particles	3.17 ± *0.03*	60.85 ± *0.85*	14.70 ± *0.17*
Roasted particles	1.90 ± *0.05*	57.31 ± *0.08*	15.66 ±*0.11*

Table 2 outlines the composition of the initial raw and roasted hazelnut seeds and ground particles. The hazelnut seeds after roasting had a loss of 4.3% of initial moisture. The hazelnut particles had a size range of 1-2 mm. This particle size range was chosen as according to literature the threshold particle size that allows swallowing to occur is considered to be <2mm [40]. At the same time, mastication studies on peanuts showed that the median particle size (d_{50})

in peanut boluses was shown to be 0.82mm [40].Thus an intermediate range was chosen in order to standardize the particle size and the homogeneity of the food bolus.

The different composition among whole seeds and particles, both raw and roasted was assigned to the blending and sieving process that was required to obtain the particles which resulted in some losses. Roasted particles had a lower moisture and lipid content presumably due to unlike breaking behaviour. Mastication studies on almonds have shown that roasting leads to smaller particle size in boluses [3, 5] which can probably be attributed to the reduced water content of the nut tissue, including the cell walls. Water acts as a plasticizer and its reduction leads to more brittle cells after roasting [5].

The effect of roasting on hazelnut cell structure was also investigatedusing microscopy analysis. *Cryo*-SEM analysis (Figure6) showed that roasting might had an impact on the structure of hazelnut cells. The cells of the raw hazelnuts appeared to be still packed after two days soaking in deionised water, indicating that water diffusion within the cells was not extensive within these days (Figure 6A). In higher magnification (Figure 6B) protein bodies were more visible but OBs appeared as a unified structure, as they remained packed due to inadequate water penetration. In contrast, the cells of roasted hazelnuts had a more discriminative round shape after two days soaking (Figure 6C) and the intercellular structure was found to be less tight (Figure 6C and 6D). Possibly the permeability of the cell wall had increased upon roasting, allowing a more effective diffusion of the water molecules into the seed and thus permitted a better overview of the inner structure of the cell. This behaviour might be indicative of the one that the cells could exhibit during digestion process. It is possible that roasting might have an effect on the porosity of the cell wall [34, 41] which also seemed to be determinant during digestion, regarding the diffusion of enzymes and digestive fluids.

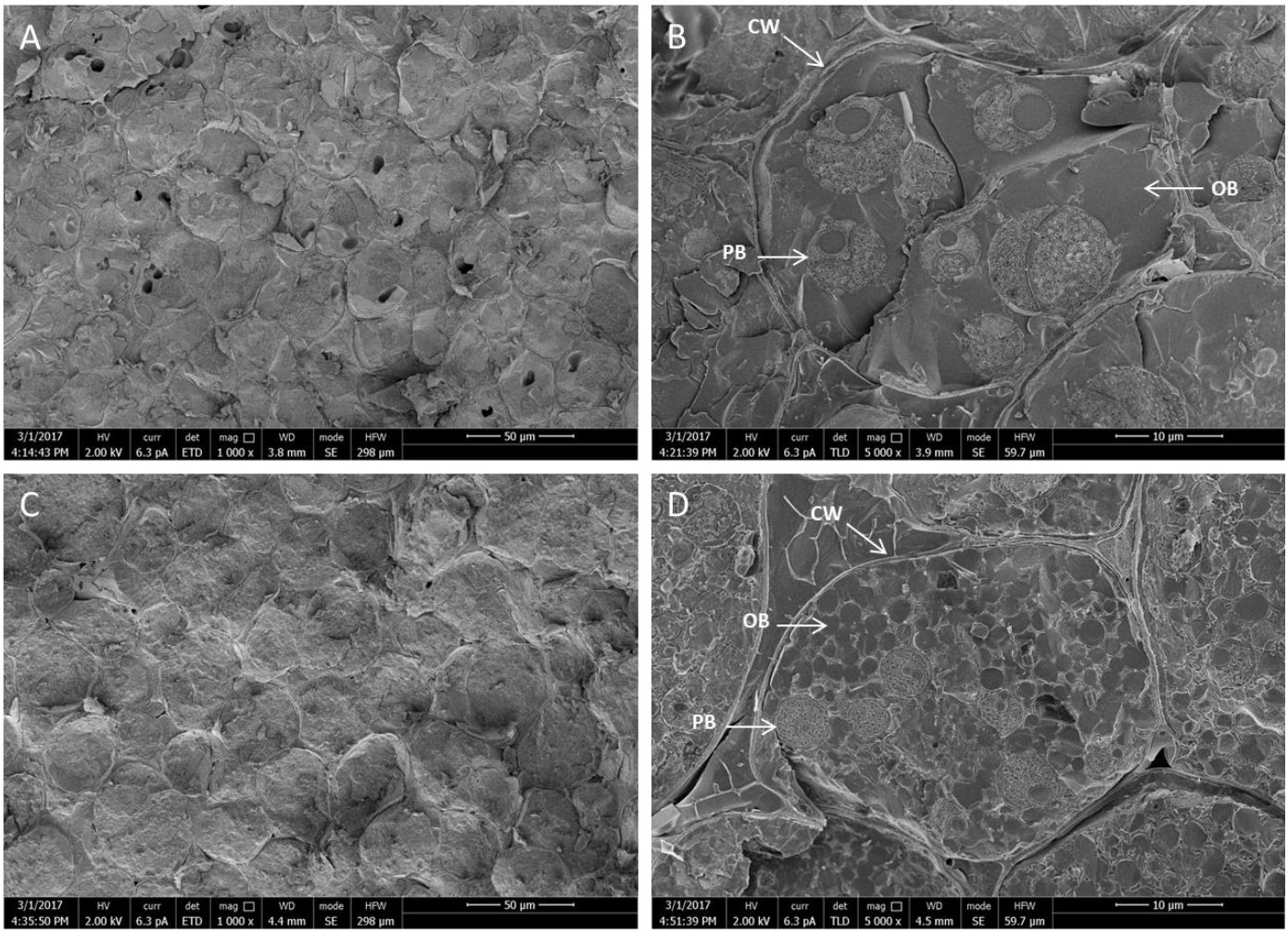

FIGURE 6. *Cryo*-SEM analysis of raw (A,B) and roasted (C,D) hazelnuts before digestion. A,C=50 µm, B,D=10 µm. PB= Protein body, OB=Oil body, CW= Cell wall

5.2.2. Hazelnut OBs

Table 3 summarizes the composition of extracted OB cream from raw and roasted hazelnuts. The extraction yield for raw and roasted OBs was 41.02% and 29.46% respectively of the total lipid present in the seeds.

TABLE 3. Lipid, moisture and protein composition of OBs emulsion creams obtained from raw and roasted hazelnuts after aqueous extraction

	% MOISTURE	% LIPID	% PROTEIN	Protein/oil
OBs cream from raw hazelnuts	17.49 ± *0.12*	77.50 ± *0.50*	1.22 ± *0.13*	0.015
OBs cream from roasted hazelnuts	33.43 ± 1.46	53.27 ± *0.16*	0.96 ± 0.14	0.018

During the aqueous extraction of OBs, simultaneous extraction of oil droplets and proteins occurs. In general, OBs are extracted into the aqueous medium after the diffusion of proteins[42]. The solubility of proteins present in cell is a key factor regarding the effectiveness in OBs extraction. Temperature is a crucial agent that determines the solubility of proteins and subsequently the yield of extraction[42]. Roasting might have resulted to increased protein dissociation, subunit interactions and re-association to larger aggregates [43]. Moreover, Maillard reactions induced by the high roasting temperatures might also have contributed to conformational changes in structure leading to the formation of covalently cross-linked

aggregates [44, 45].Protein analysis on hazelnut particles confirmed the presence of higher molecular weight bands in roasted hazelnuts (Figure 21). Therefore, it is possible that these structural changes of the proteins subsequently lowered their solubility [26, 45].As a result, during the extraction process their removal to the aqueous environment was impeded. Consequently the extraction yield of the OBs from roasted hazelnuts was affected.

Lipid analysis of the solid residue from the cheesecloth during the extraction process showed that 54.29% of the initial lipid content of raw hazelnuts remained in the solid residue while in roasted hazelnuts 68.55% of the initial lipid content was not extracted.

5.2.2.1. Particle size distribution andζ-potential

The particle size distribution of OBs was also assessed before digestion. At time point zero (Figure7) the OBs obtained from raw hazelnuts had a Sauter mean diameter *d*32 of 0.539 ± 0.03 μm and a volume mean diameter, *d*43, of 26.56 ± 2.14 μm. The hazelnut OBs from roasted hazelnuts had a Sauter mean diameter,*d*32, of 0.510 ± 0.06 μm and a volume mean diameter, *d*43, of 4.304 ± 0.16 μm. The small *d*32 value in both creams indicated that intact OBs were obtained during the extraction process. The high *d*43 value of OBs extracted from raw hazelnuts suggested the presence of aggregates, as *d*43 values are associated with the presence of aggregates while *d*32 is related to smaller oil droplets [26]. The trimodal droplet size distribution curve observed in OBs extracted from raw hazelnuts confirmed our observations regarding the presence of aggregates (Figure7, right panel).

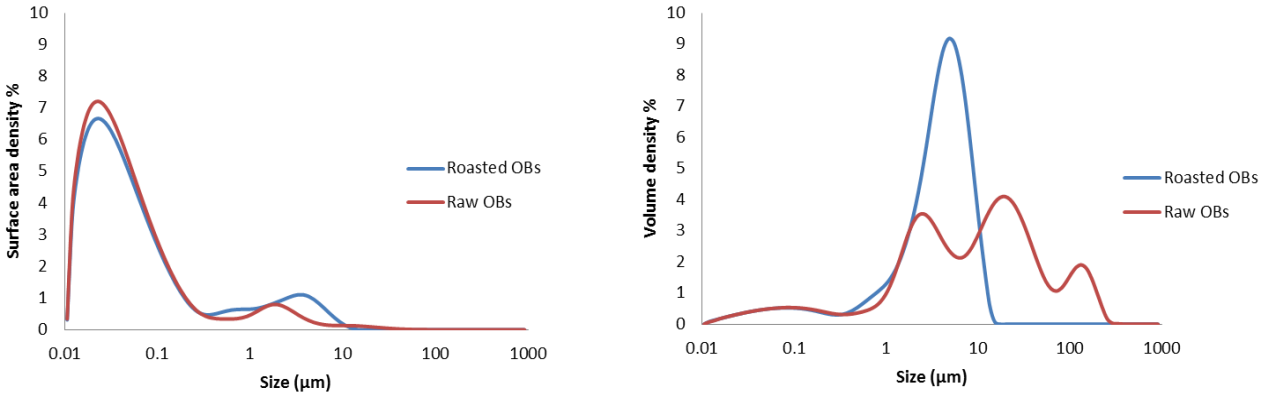

FIGURE 7. Droplet size distribution as a percentage of surface area density (left panel) and volume density (right panel) of oil bodies extracted from raw and roasted hazelnut particles

In contrast, a smaller *d*43 value of OBs extracted from roasted hazelnuts was observed. A monomodal droplet distribution curve was obtained in the volume density measurements (Figure7, right panel), validated our remarks about less aggregates present. This difference can be attributed to changes on the OBs' surface regarding the structural changes in oleosins upon heat treatment [26]. Possible introduction of covalent (disulfide) and physical (hydrophobic) bonds between the surface proteins [6] presumably led to less interactions between the OBs. Thus, more individual OBs than aggregates were present in the cream after the extraction.

Besides, during the extraction process, it was noticed that in the case of OBs extracted from raw hazelnuts a thicker layer of cream was obtained with a more transparent subnatant after centrifugation. The thickness of the cream layer might be a sign of aggregates present. Regarding the OBs from roasted nuts the cream layer was thinner while the subnatant more opaque with brownish colour. This observation suggested the presence of indication of individual OBs and colorants formed during roasting process (Figure8).

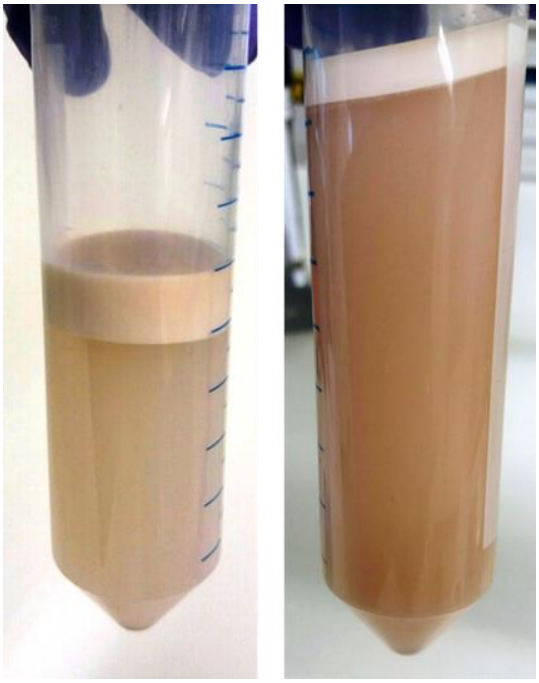

FIGURE 8. Cream obtained by the extraction of OBs from raw (left) and roasted (right) hazelnuts

Protein analysis (Figure 19) of the extracted OBs showed that no extraneous proteins were present in OBs creams confirming that during the extraction process pure OBs were obtained. Oleosin proteins were present in both samples. Nevertheless, the OBs extracted from roasted hazelnuts presented quite more intense bands of high molecular weight peptides (60-100 kDa), which implied the presence of protein aggregates in the extracted cream. These aggregates, as explained before, might have been formed during roasting. As they are more insoluble [45], there might have been extracted in the cream despite the washing steps for their removal. This result is in accordance to the protein/oil ratio in the cream which was slightly higher in the case of cream from roasted hazelnuts. However, these aggregates did not seem to interact with the interfacial proteins of the OBs, as the particle size of the OBs extracted from roasted hazelnuts was comparable to the ones extracted from raw nuts.

Regarding the interfacial charge of the OBs, the ζ-potential values before digestion were -19.31 ± 1.31 mV and -33.23 ± 0.58 mV for OBs extracted from raw and roasted hazelnuts respectively. The measurements showed that slightly stronger repulsive interactions (more negative values) occurred in OBs extracted from roasted hazelnuts, implying he presence of more individual OBs in the cream. The evaluation of the ζ-potential was in accordance to droplet size distribution results regarding the presence of more aggregated OBs (less negative charge) in the cream obtained from raw than roasted hazelnuts.

5.3. *In vitro* lipid hydrolysis

Lipid hydrolysis in hazelnut particles and OBs was investigated under *in vitro* digestion according to Minekus et al. (2014)[7] protocol with some modifications. As enzyme activity is of high importance during digestion, the activity of each enzyme used was calculated. The results are presented in Table 4, expressed in units per mg.

TABLE 4. Enzyme activity measurements

Enzyme tested	Enzyme activity (U/mg)
Porcine Pepsin	3500
Trypsin in Porcine Pancreatin	3.35
Lipase in Porcine Pancreatin	10

According to the digestion protocol when fat digestion is in the centre of the study, pancreatin concentration should be based on lipase activity to achieve 2000 U/ml[7]. In this study, since lipase activity of pancreatin extract was found to be low (10 U/mg) and high amounts of pancreatin extract should have been used to achieve the desired activity. Therefore, another experiment using a combination of pancreatin extract and pancreatic lipase (100-400 U/mg) was carried out, in order to examine if lipase activity in pancreatin extract could be enhanced. Different ratios (pancreatin: lipase) were tested in order to achieve 500 U/mg, which is the activity of lipase found in 50 mg of pancreatin.

The results (Table5) showed that lipase activity in pancreatin extract could be enhanced by the addition of supplementary lipase. The rate of NaOH release (μmol/min) in the combinations tested, which is related to free fatty acid release, was not significantly different from the rate of NaOH released from 50 mg/ml pancreatin ($p>0.05$ in all cases)(Appendices 8.1).Moreover, it was found to be half of the value provided by 100 mg/ml of pancreatin extract (38 ±3.75 μmol/min), as expected. Thus, it was suggested that the same enzyme activity was achieved by using a mixture of pancreatin and lipase. Thereby, lower amounts of pancreatin enhanced by extra lipase could be used, which also facilitated the dissolution of the enzymes in the intestinal fluids. As a result, it was decided to use a ratio of 10:1 pancreatin extract to lipase.

TABLE 5. Rate of NaOH(0.1M) release during lipase activity measurement in porcine pancreatin extract and in porcine pancreatin extract enhanced with additional lipase, using pH-stat method with tributyrin as substrate.

Enzyme combination	NaOH (μmol/min)
50 mg/ml pancreatin extract	23.50 ± 3.53
25 mg/ml pancreatin extract +2.5 mg/ml lipase	19.24 ± 1.21
30 mg/ml pancreatinextract +2 mg/ml lipase	22.40± 0.84
35 mg/ml pancreatin extract +1.5 mg/ml lipase	22.45 ± 9.12

Moreover, when pH-stat analysis was performed using only the electrolyte solution of the intestinal phase, the pH was increasing during two hours incubation (Figure 9) and gas bubbles were present. This observation was attributed to the $NaHCO_3$ present in the solution and specifically to the solubility limit of CO_2 which was low compared to the concentration in which it was added. Therefore, $NaHCO_3$ was substituted with NaCl, in the same molar ratio in all the prepared solutions, which maintained the pH stable. In this way, the effect of fluid composition on pH during digestion was excluded.

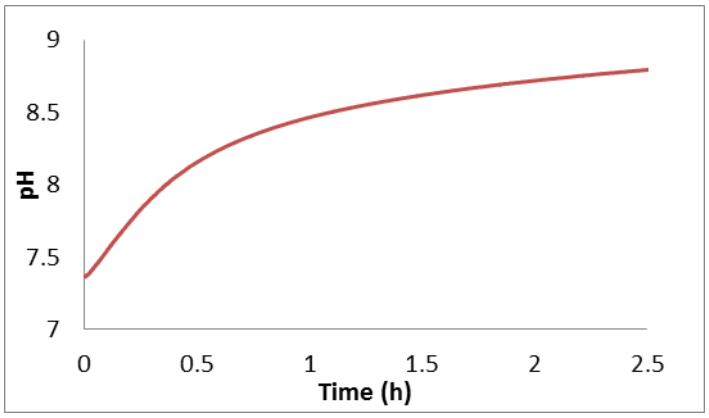

FIGURE 9. pH increase of the electrolyte solution used for intestinal phase, prepared according toMinekus et al. (2014*) in vitro* digestion protocol

5.4. Lipid hydrolysis of hazelnut particles and hazelnut oil bodies during *in vitro* digestion using the pH-stat method

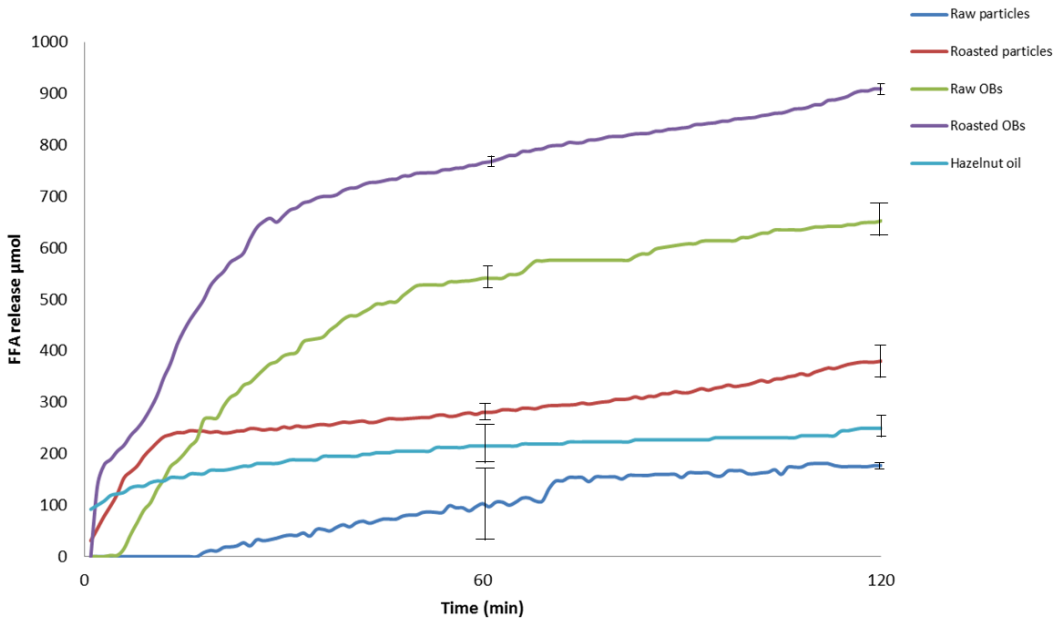

FIGURE 10. FAA release during 120 min of *in vitro* intestinal digestion of hazelnut materials with different degrees of lipid bioaccessibility using pH-stat method

Figure10 presents the FFA release (µmol) from hazelnut samples during 120 min of *in vitro* intestinal digestion. Regarding the raw and roasted hazelnut particles the values were calculated after the subtraction point by point of the values calculated for the defatted hazelnut samples. As hazelnut particles contained also a significant amount of proteins, their hydrolysis could also have contributed to pH changes during digestion. Thus, defatted hazelnut particles (raw and roasted) were also digested *in vitro* order to evaluate and exclude the contribution of AA release to pH reduction and subsequent NaOH addition.

At the first 60 minutes of intestinal digestion the rate of lipolysis was not statistically different between raw and roasted particles (P=0.160). The results were in accordance to the

observations of Grundy et al. (2015)[34] over 60 min of intestinal digestion of raw and roasted almond particles, where no statistically significant differences were observed. In addition, Mandalari et al. (2008) [11]also noted similar amounts of FFA release from almond cubes during the first hour of intestinal digestion. However, after the end of digestion (120 min) the lipolysis rate was statistically different for the raw and roasted hazelnut particles (P=0.041), indicating that thermal processing might have influenced lipolysis kinetics. The microstructural changes of the cells due to heat treatment presumably facilitated the diffusion of the enzymes and bile salts into the cell as well as the diffusion of TGA out of the cell, being more accessible to enzyme degradation and thus promoting lipid hydrolysis. Our results are also in agreement with the study of Groopman et al. (2015) [20], where a decrease in faecal fat excretion was noticed in diets where heat treated peanuts were provided, indicating an increase in fat digestibility by cooking. In addition, higher lipid loss during the digestion of blanched almond cubes compared to the raw ones was also mentioned in other study[11].

Besides, after filtration of the digested particles it was noticed that roasted hazelnut particles showed different damage behaviour compared to the raw ones. Smaller and smoother fragments were observed at the end of digestion (Figure 11), which can be related to the different rupture behaviour of the roasted particles during digestion, as they became more brittle due to heat treatment, leading to an enhanced lipid hydrolysis. Kong and Sigh (2009)[46] also noticed an accelerated disintegration of roasted almond particles during gastric digestion, due to higher porosity of the heat treated cells, allowing better infiltration of the digestive fluids.

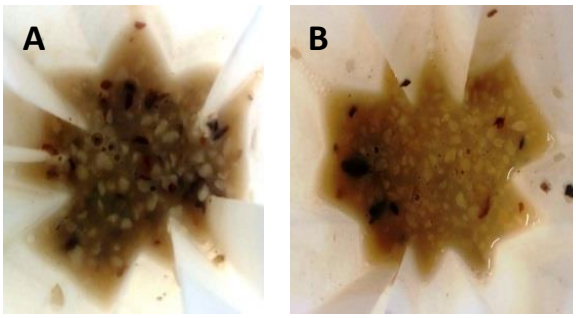

FIGURE 11.Raw (A) and roasted (B) hazelnut particles after 120 min gastric and 120 min intestinal digestion

Both raw and roasted hazelnut particles had statistically significant differences in FFA release with the OBs after two hours of intestinal digestion (P<0.05 in all cases) (Appendices 8.2). OBs (raw and roasted) showed higher lipid release, pointing out that the cell wall can inhibit lipid digestion thus confirming our hypothesis. Other studies have also shown that FFA release over the digestion of almond oil emulsions was significantly higher than the one of almond cells [9, 34], supporting our findings. In addition, Mat et al. (2016) [47] has pointed out that the physical state of the continuous phase (liquid or solid)of lipid emulsions has an important impact on lipid digestion. It was highlighted that in case of solid matrices, lipase has to diffuse into the matrix and oil droplets have to be liberated from the network whereas in liquid emulsion, lipids are already available to be hydrolysed[47].

Regarding the OBs, the rate of lipolysis expressed as μmol of NaOH over 60 and 120 min of *in vitro* intestinal digestion showed that there was a statistically significant difference in both time points (P=0.03 and P=0.036 respectively) between the two different OBs, with the ones extracted by roasted hazelnuts showing higher FFA release. The lipolysis kinetics as well as the surface analysis of OBs extracted from raw and roasted hazelnut particles during digestion, confirmed the differences on their surface destabilization behaviour. These results might be an indication of the "pre-digest" effect of the oleosin [20] as also confirmed by the protein profile analysis and ζ-potential measurements of OBs during digestion (section 5.5.1 and 5.5.2).

The low lipolysis observed during hazelnut oil digestion can be explained by the fact that since oil droplets were not in an emulsified form they tended to coalescein bigger particles before digestion, decreasing the surface area available for lipase to act[47]. According to Singh et al. (2009), the initial size of the droplets governs the digestion behaviour under gastric and intestinal conditions [48].

The fact that lipid hydrolysis was not ended at the end of the experiment was mainly attributed to the long-chain TGA present in hazelnut lipid, as it is known that they are digested in a slower rate than medium-chain fatty acids [29, 47]. Besides, the high lipid content of the digested samples might have influenced the lipolysis rate as they are inversely associated[29]. The ratio of lipase-to-lipid substrate and bile salts to lipid is another factor that possibly have influenced the kinetics. In general, the lipolysis rate increases with a decrease in fat content [29]. However, a slowdown of the lipolysis kinetics was noticed in all cases, probably due to the production and accumulation of lipolytic products that cause a restriction in enzymatic activity [49].

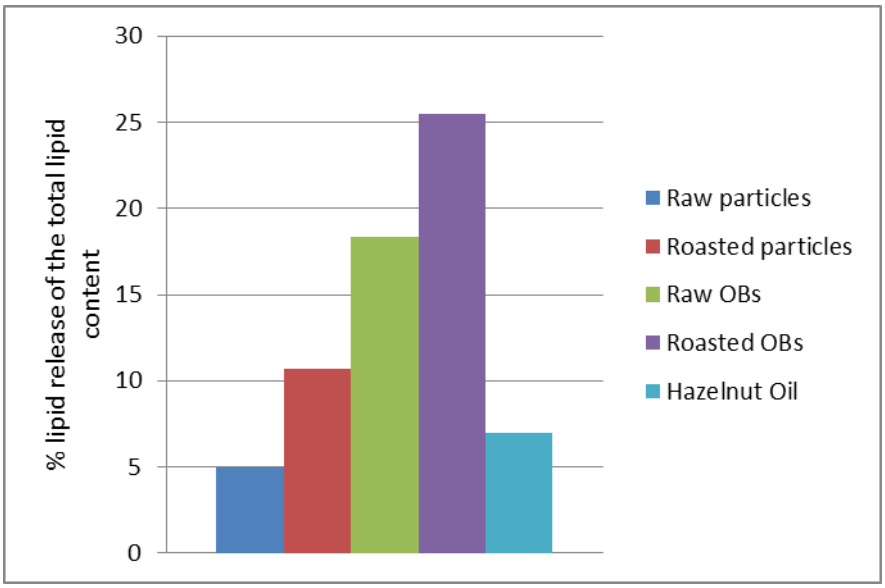

FIGURE 12.Lipid release at the end of 120 min of intestinal digestion as a percentage of the total lipid present in the samples before digestion.

Figure12 illustrates the results of the percentage of FFA release of the total lipid content present in hazelnut samples after the end of the *in vitro* intestinal digestion. The results were in accordance to previous studies, as a limited FFA release during digestion was expected due to the cell wall barrier. Moreover, as explained before, the large amount of lipid present and consequently the low enzyme-substrate ratio and the high accumulation of lipolytic products resulted in slower kinetics in OBs digestion too.

In general, the FFA release (μmol) of hazelnut samples at the end of digestion was observed to be higher than mentioned in other studies[19, 34, 38]. This finding may be related to several factors; Firstly, following a different digestion protocol, higher lipid content was studied (7.6% of the final reaction volume) while in other studies the lipid content was adjusted to 0.8%. Moreover, the duration of intestinal digestion was 60 minutes whereas in the present study was 120 minutes. It has been underlined that bioaccessibility is improved by increasing the residence time under duodenal conditions [11]. In addition, hazelnut samples were subjected also to 120 min of gastric conditions before intestinal digestion, which probably led to

alterations both in the structure of the cells but also in the structure of the OBs emulsions, enhancing FFA release. Moreover, porcine pancreatic extract was used instead of only pancreatic lipase, which also contributed to the higher extent of lipolysis. Grundy et al. (2016) [9]noticed that lipolysis was more effective in the presence of porcine pancreatic extract than porcine pancreatic lipase. Besides, McClements and Li (2010) [18]had also highlighted the synergistic effect of enzymes on lipid hydrolysis. Finally, hazelnuts are nuts with higher lipid content compared to already studied nuts, like almonds [5, 9, 12, 34], pistachios[17] and walnuts[15, 38]. Baer et al. (2016)[15]suggested that physicochemical structure of each nut and mainly the differences in fat and fibre content could influence lipid digestion. Thus, the differences observed in lipid bioaccessibility might be an indication that the digestibility of nutrients is nut-dependent as it is also implied by other studies [15-17].

However, Soxhlet results showed that the remaining lipid inside the cells of raw and roasted hazelnut particles after digestion was 30% and 19% respectively. Nevertheless, these values were lower than expected according to other studies [3, 12, 15, 16, 20]and much lower than the values obtained from the pH-stat results. It is believed that during the sample preparation after the digestion, most of the intracellular lipid leaked out of the cells. It is possible that during the filtration but also during the thawing step after freezing with liquid nitrogen most of the lipid was lost due to softening of the cell structure. Moreover, as the particles were not dried before the lipid extraction, the moisture content could have also limited the extraction process. The leakage was higher for the roasted particles probably due to their different disintegration behaviour as explained before.

Microstructural analysis after digestion showed the presence of large lipid aggregates in both raw and roasted hazelnut particles (Figure 13). Nevertheless, more coalesced lipids were observed in the case of roasted hazelnut particles after digestion (Figure 13B), suggesting that some transformation had occurred leading to a different destabilization of the surface OBs' proteins, that could be attributed to lipase and bile salt penetration [34]. The same observations regarding lipid coalescence in heat treated nuts during digestion were also pointed out in other studies[12, 20, 34, 46]. The presence of more coalesced OBs in roasted cells could be an indication of a more facilitated penetration of the digestive fluids and enzymes as it was highlighted before that could led to higher lipid hydrolysis rates as pH-stat results showed. Moreover, the differences in OBs coalescence could be an indication of alter surface destabilization behaviour in OBs extracted from roasted hazelnuts, as it will be explained in the following section.

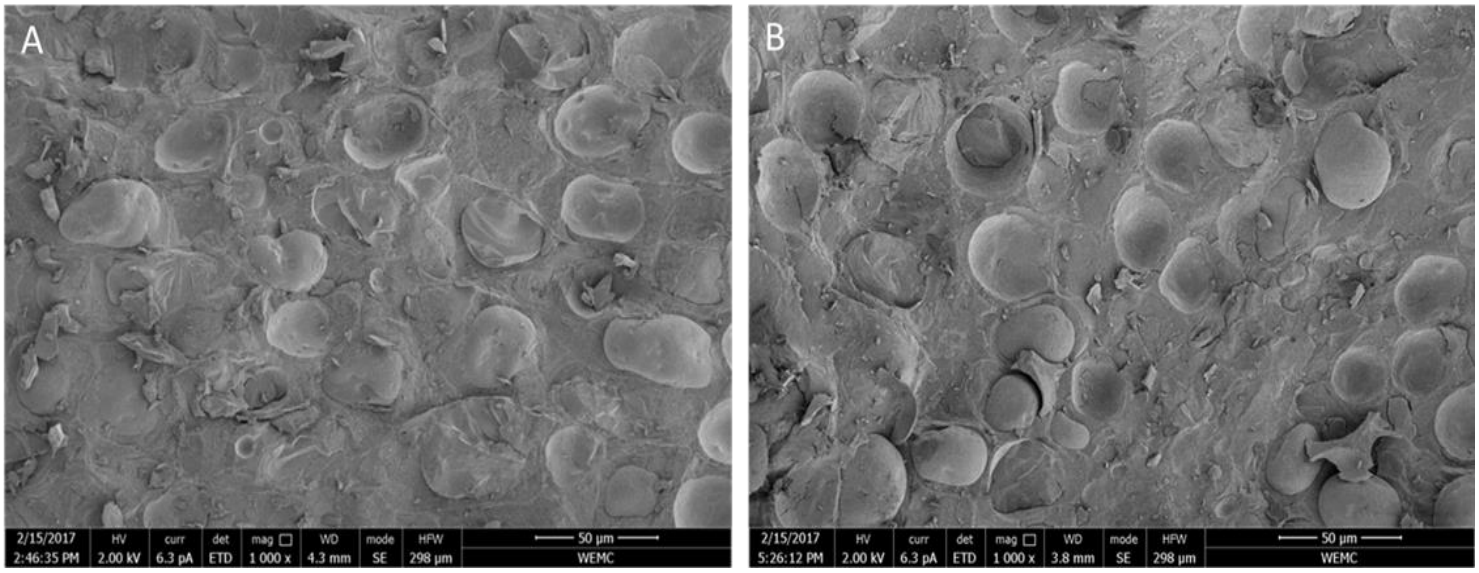

FIGURE 13. *Cryo*-SEM analysis of raw (A) and roasted (B) hazelnut particles after 120 min gastric followed by 120 min intestinal digestion. A,B=50 µm

5.5. Characterization of hazelnut OBs during *in vitro* digestion

5.5.1. Particle size distribution during *in vitro* digestion

In Figure14 an overview of the changes in volume mean and surface mean diameters of the hazelnut OBs under simulated gastric and intestinal conditions is illustrated.

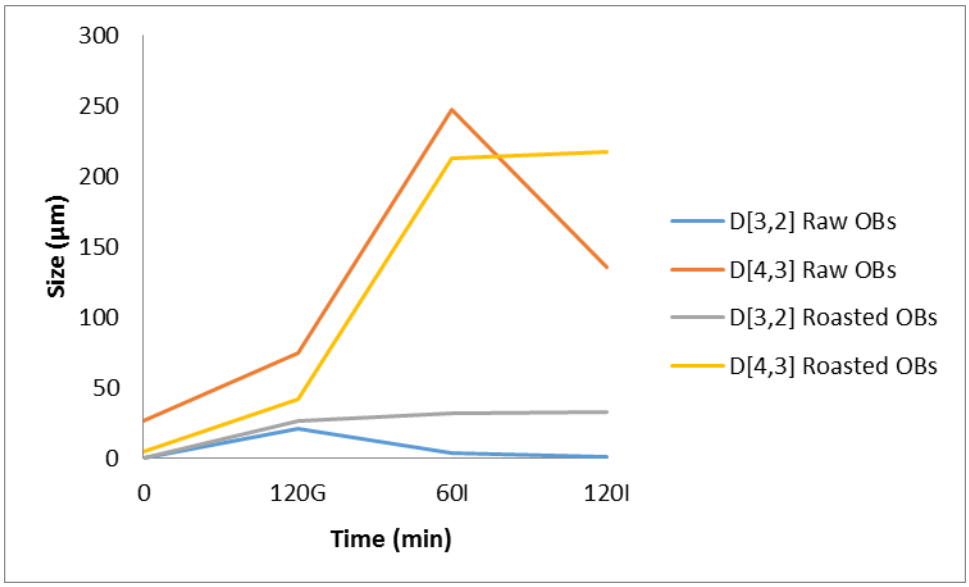

FIGURE 14. Volume mean (*d43*) and surface mean (*d32*) diameters of OBs extracted from raw and roasted hazelnuts after 120 min of gastric digestion followed by 120 min of intestinal digestion. Time 0= 0 min, 120G= 120 min gastric, 60I=60 min intestinal, 120I=120 min intestinal digestion.

After two hours of gastric digestion both the volume and surface mean diameter of the OBs (raw and roasted) were increased (Figure14 and 15), indicating flocculation and/or coalescence of the OBs due to hydrolysis of the interfacial proteins by pepsin. However, a different behaviour between the two different OBs was observed under intestinal conditions.

The OBs derived from roasted nuts had an increase in their size in the first hour of intestinal digestion remaining almost stable until the end (Figure 14 and 15c, d). The increase in droplet size is an implication of droplet coalescence and flocculation as the oil droplets were hydrolysed by the pancreatic enzymes, both on the surface by proteases and subsequently on the TGA surface by lipases. Droplet coalescence is promoted by the formation of FFA and MAG at the droplet surfaces during lipid hydrolysis by pancreatic lipase. These lipophilic surface active substances are ineffective at stabilizing oil-in-water emulsions against coalescence [50]. Moreover, McClements and Li (2010) [18] reported that at high local concentration of FFA and MAG a liquid crystalline or crystalline phase around the lipid droplet can be observed, getting thicker over time thus influencing the *d*43 measurements. The extent of droplet coalescence depends on the ability of lipase to come into contact with the emulsified lipid, as well as on the composition and properties of the interfacial layers surrounding the lipid droplets [50]. The results are also supported by the FFA release measurements, as higher lipid hydrolysis was detected in OBs extracted from roasted hazelnuts.

In contrast, the OBs extracted from raw hazelnuts, displayed a decrease in their *d*32 value after one hour of intestinal digestion (Figure 14 and 15a) with a slight further decrease at the last 60 minutes.As the ζ-potential measurements showed there were high electrostatic repulsions between the OBs extracted from raw hazelnuts during the first hour of intestinal digestion. This behaviourcan be attributed to the disruption of the aggregates formed during the gastric phase by the increase in pH under intestinal conditions and the presence of bile salts[38]. The disruption of aggregates was related to the presence of a pepsin resistant protein domain still present on the OBs surface as explained below (section 5.5.2), that prevented in some extent coalescence of OBs to occur under gastric conditions. A bimodal droplet size distribution curve was observed after the two hours of gastric digestion in OBs extracted from raw hazelnuts (Figure 15a), confirming the presence of both digested (coalesced OBs with bigger droplet size) and individual OBs still undigested due to pepsin resistant protein domains. At the same time period, the *d*43 value was increased showing lipid coalescence by the access of lipase at the oil droplet surface and the production of FFA.

It has been shown that the interfacial proteins have to been hydrolysed before phospholipases can further attack the phospholipid monolayer surrounding the OBs. The oleosins at the surface of the oil bodies protect them from further hydrolysis by lipases and subsequent coalescence [19]. Groopman et al. (2015) [20] reported that as oleosin layer is disrupted, lipase adsorption on the oil surface is facilitated, stimulating gastric fat emulsification. The decrease in *d*32 and *d*43 values at the last hour of intestinal digestion can be assigned to the fact that as lipid droplets were digested by lipolytic enzymes, there might be a decrease in droplet size due to movement of the lipolysis products into the surrounding phase. Moreover, any undigested oil can be expelled as smaller oil droplet out of the crystalline phase[18], influencing the oil droplet distribution measurements. Indeed, pH-stat results (section 5.4) showed that OBs extracted from raw hazelnuts were less digested compared to the OBs derived from roasted hazelnuts.

A clearer overview of the droplet size distribution of the OBs expressed as percentage of volume and surface area density during the gastric and intestinal digestion is presented in Figure 15.

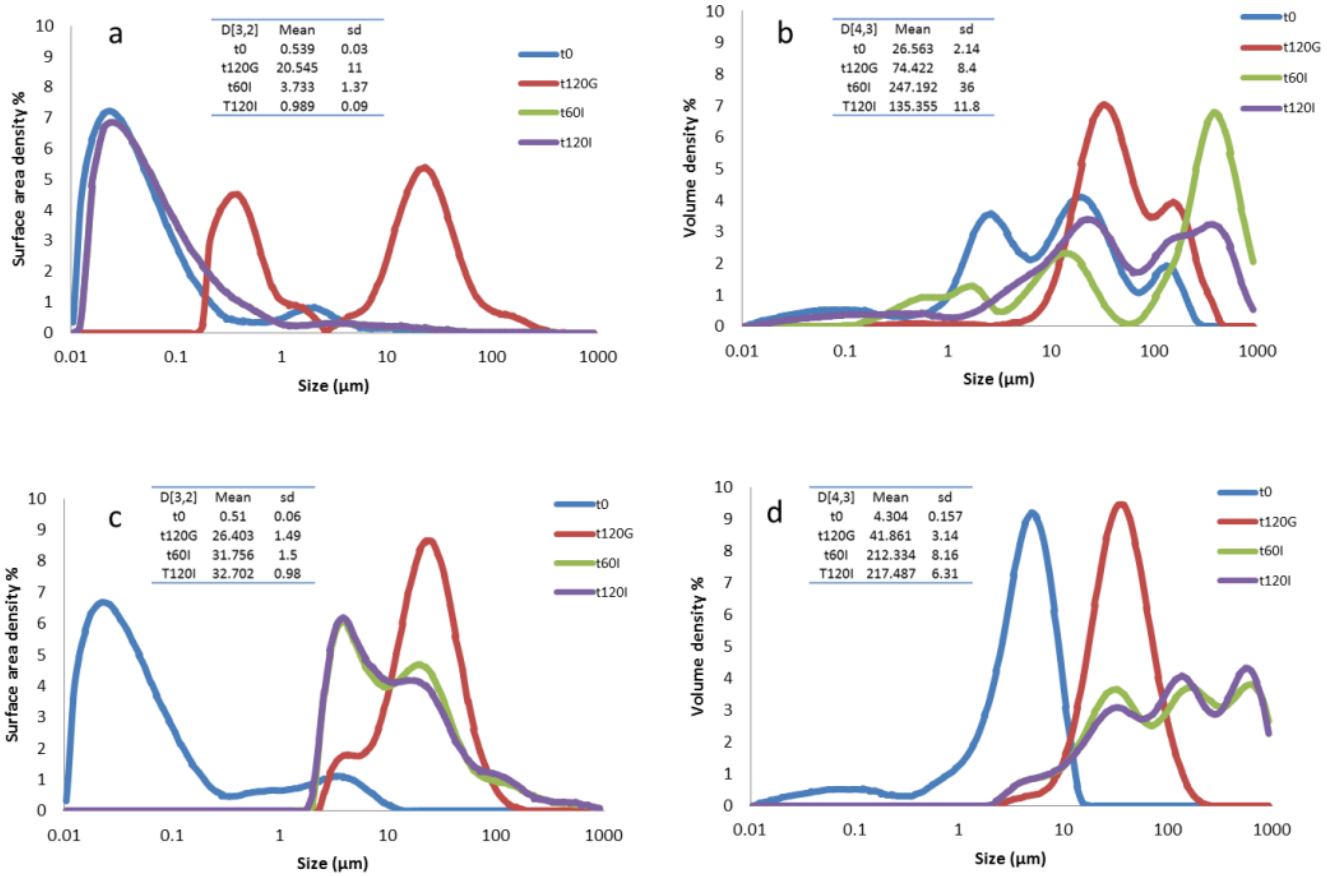

FIGURE 15. Droplet size distribution during in vitro gastric and intestinal digestion of OBs extracted from raw (a-b) and roasted (c-d) hazelnut particles. a,c= represent the particle size distribution as a percentage of surface area density, b,d represent the particle size distribution as a percentage of volume density. Time0= 0 min, 120G= 120 min gastric, 60I=60 min intestinal, 120I=120 min intestinal digestion

5.5.2. Interfacial changes during in vitro digestion of oil bodies

The nature of the adsorbed layer determines the stability behaviour of emulsion droplets under exposure to the gastric environment, its low pH and its proteolytic enzyme activity. The pH reduction during gastric conditions results in changes in the conformation and subsequently in the charge of the adsorbed proteins[48]. Figure 16 illustrates the potential change on emulsion droplets interface under gastric and intestinal digestion.

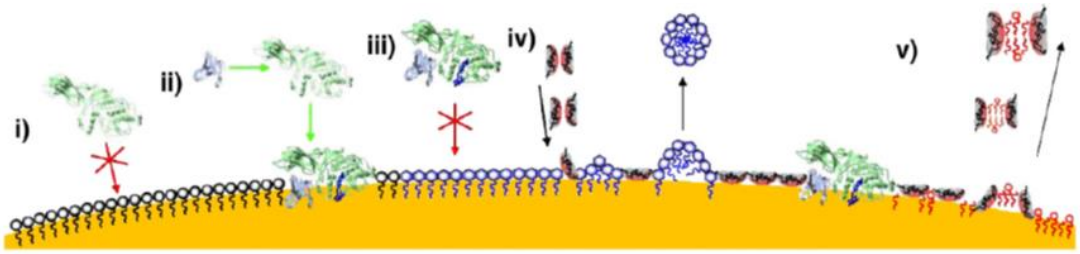

FIGURE 16. Interfacial changes that may occur during OBs digestion by pancreatic lipase: optimal lipase activity occurs in the presence of co-lipase, bound into a complex (i, ii). The interfacial structure of the OBs can sometimes hinder the adsorption of the co-lipase/pancreatic lipase complex (iii). Often, bile salts can remove such inhibitory surfactants from the interface via an orogenic displacement mechanism (iv). As lipolysis proceeds there is a release of 2-monoglycerides and free fatty acids, which can limit lipase adsorption. Normally, these products of lipid digestion are removed from the interface by mixed bile salt and phospholipid micelles (v). Taken from Golding and Wooster, (2010)

Figure 17 presents the interfacial changes of extracted OBs during digestion regarding their surface charge. The OBs extracted from raw hazelnuts had an increase in ζ-potential (more positive) after two hours of gastric digestion.

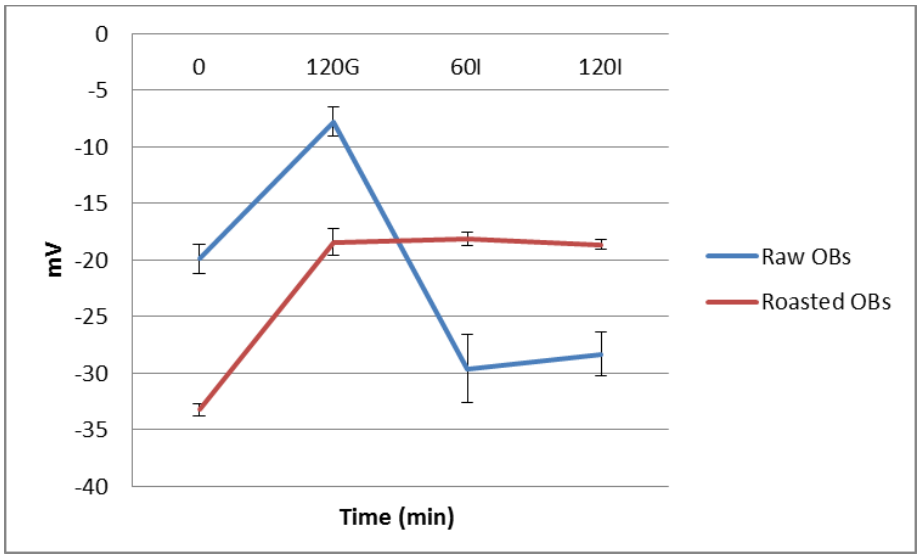

FIGURE 17. ζ-potential changes of OBs extracted from raw and roasted hazelnuts during 120 min of gastric (120G) followed by 120 min (60I and 120I) of intestinal digestion.

As the SDS results confirmed (Figure 19), there was a pepsin resistant oleosin domain of around 8 kDa that was still present after the gastric digestion. Our observations are in accordance with those of Gallier and Singh (2012) [19], and Beisson et al. (2001)[51] who pointed out that the hydrophobic domain of oleosin anchored to the TAG core might be less accessible to pepsin. Therefore, the charge of the interface depended mainly on the protein charge. The oleosins present in hazelnut OBs have an isoelectric point close to pH 3.0 [26] However, after the gastric digestion the pH of the digestion mixture is close to 5.0, in which OBs have a slightly negative charge [26], which is in agreement with our results. As the remaining pepsin resistant proteins pass through their isoelectric point, some reversible aggregation may also had occurred due to less electrostatic repulsion, as confirmed by the increase in droplet size (section 5.5.1). Huang (1992) [22] stated that OBs quickly aggregate when the pH is lowered that 7.0, as the lowering of the pH presumably protonates the histidine residues (pK of about 6) in the oleosins, resulting in a neutralization of the oil body surface. Aggregation occurs, but without coalescence and the

aggregated oil bodies can be dissociated if the pH in the medium is brought back to 7.0. The lack of coalescence is presumably due to the steric hindrance contributed by the shielding oleosins.

Regarding the OBs derived from roasted hazelnuts, more negative ζ-potential values were observed. As Nikiforidis et al. 2016 [26] reported, heat treated OBs exhibited a moderately more negative charge around pH 5.0, which was assigned to possible changes in the interfacial composition as a result of heating. The change of the native structure of proteins by heat has been shown to markedly enhance the accessibility of the specific peptide bonds needed for pepsin action [52]. Indeed, it is possible that a more effective proteolytic action of pepsin on the adsorbed proteins led to a reduction of the droplet charge (less positive) and removal of steric repulsion barriers, leading to further aggregation and possibly some coalescence of emulsion droplets, as confirmed also by other studies (Singh et al. 2009).Microscopy analysis confirmed the presence of flocculated/coalesced lipid droplets after two hours of gastric digestion (Figure18). In addition, due to the hydrolysis of the oleosin layer the emulsion was probably composed mainly of low molecular weight surfactants, such as phospholipids. These negatively charged phospholipids would be acid stable and may not be affected much in the stomach conditions[48].

After one hour of intestinal digestion the ζ-potential of OBs extracted from raw hazelnuts had a steep decrease (more negative). This decrease might have been probably a result of the adsorption of negatively charged bile salts on the surface of the OBs in combination with the charge of the protein domain still present on the OBs' interface. Apparently, the remaining oleosin domain might have shielded the interface of the OBs, preventing to some extent the displacement of the surface by bile salts. Consequently, a hindering effect on lipolysis might have taken place as a lag phase was also noticed in the beginning of the intestinal lipolysis of the OBs (Figure18). Moreover, as light microscopy and size distribution analysis showed, smaller particles were noticed, probably leading to additional electrostatic repulsions.

Contrarily, the OBs from roasted nuts, exhibited a different behaviour, showing a less negative charge. This behaviour may be a combined effect of higher FFA release as presented in section 5.4 and subsequently of their charge, of the displacement of the interfacial surfactants by lipase and of the presence of non-displaced bile salts on the interface. However, it is difficult to assign the values measured to a specific component on the oil droplet interface.

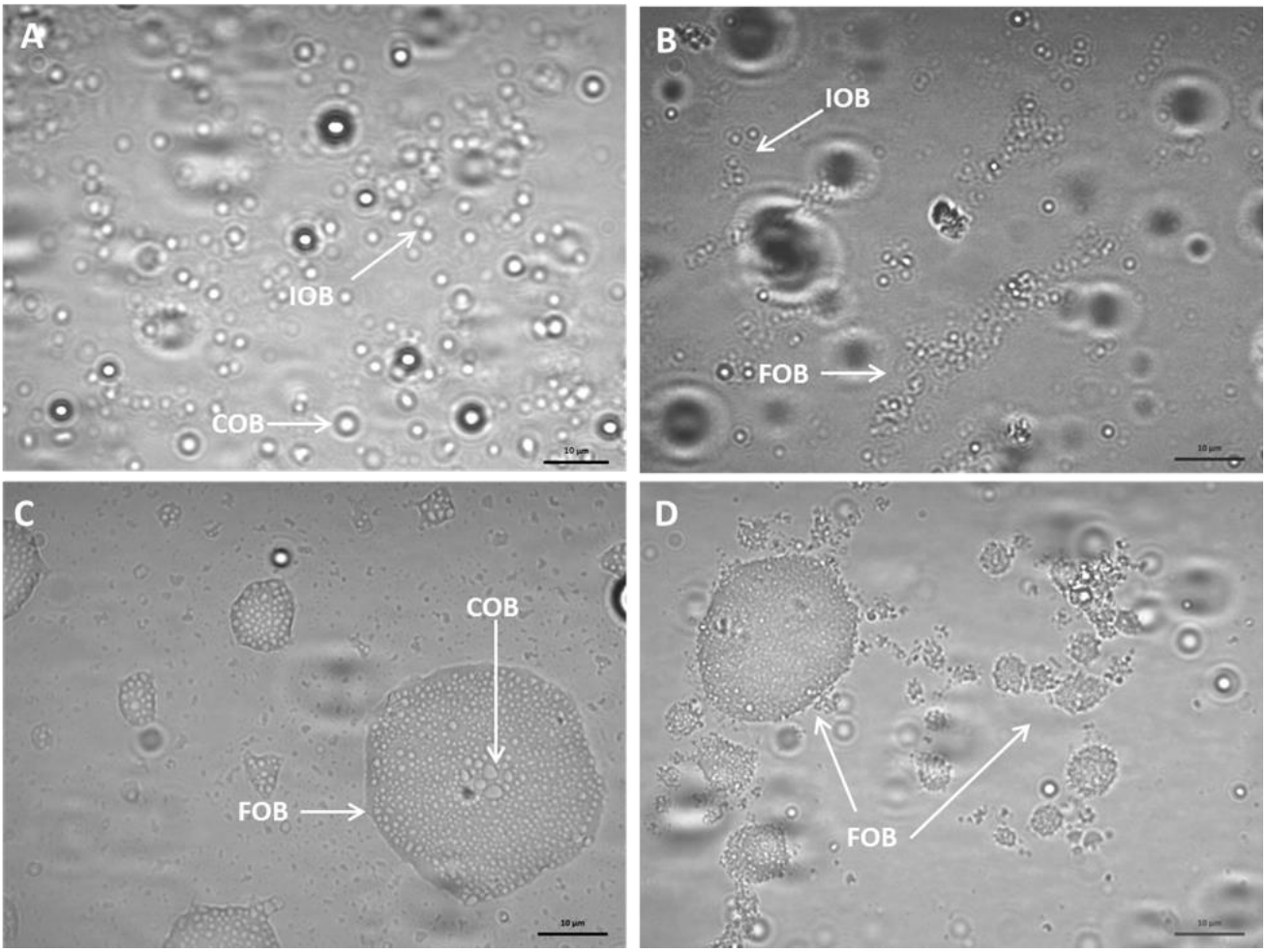

FIGURE 18. Light microscopy images of oil bodies extracted from raw (A,B) and roasted (C,D) hazelnuts during 60 (A,B) and 120 (B,D) minutes of intestinal digestion. IOB=Individual OBs, COB= Coalesced OBs, FOB=Flocculated OBs. A,B,C,D,=10µm

The protein profile of OBs (Figure 19) during the digestion process confirmed our indications regarding the presence of a pepsin resistant oleosin domain in the case of OBs extracted from raw hazelnuts. As explained before, this enzyme resistant domain might have an impact on the digestibility of OBs as it could have an impact on the adsorption of bile salts and enzymes on the OBs surface during digestion. In contrast, oleosins of OBs extracted from roasted nuts were fully hydrolysed after 120 minutes of gastric digestion. The bands of extraneous proteins present in the initial samples of OBs extracted from roasted hazelnuts were still present after gastric digestion pointing out their resistant to proteolytic gastric enzymes as explained below in section 5.6.

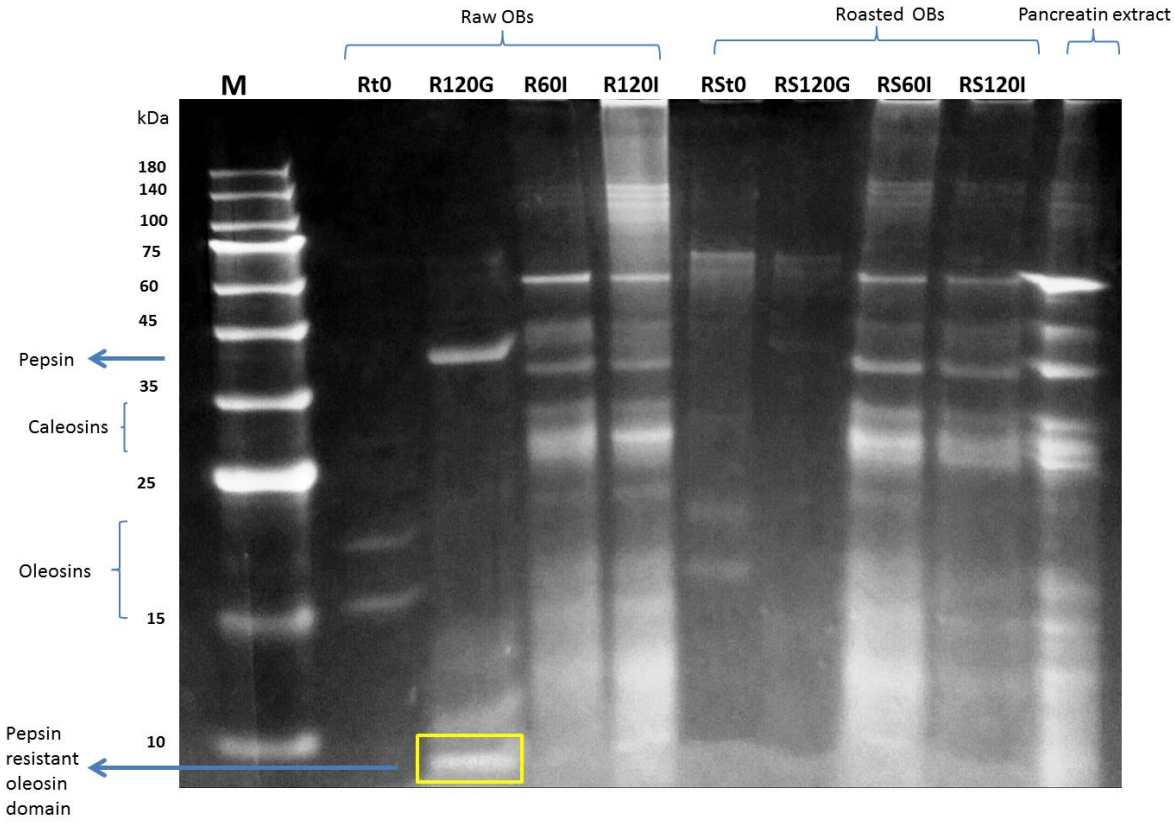

FIGURE 3. Protein profile of OBs extracted from raw (R) and roasted (RS) hazelnuts before (t0) and during 120 min gastric (120G) followed by 120 min intestinal (60I, 120I) digestion. M= Protein molecular weight marker.

5.6. PROTEIN HYDROLYSIS IN HAZELNUT PARTICLES

5.6.1. Determination of protein hydrolysis using the pH-stat method

During the digestion of defatted raw hazelnut particles, higher amount of NaOH release was noticed compared to the one of the defatted roasted hazelnuts (Figure20). The quantity of μmoles released during the study of defatted material reflects the AA release during the hydrolysis of proteins by trypsin and chymotrypsin. The difference in protein hydrolysis between raw and roasted hazelnut particles was also ascribed as it is mentioned before (section 5.2.2) to modifications that occurred in proteins during heat treatment, affecting also their resistance to enzyme degradation. Iwan et al. (2011) [44] noticed a significant decrease in the susceptibility of specific proteins to pepsin hydrolysis after heating. Increased protein aggregation after heat treatment might have reduced the accessibility of pepsin as it has been correlated with stronger protein cross linking [43]. This effect could also have influenced the efficiency of intestinal proteolysis too, as roasting and the induction of Maillard reactions can lead to products that are not degradable by the upper gastrointestinal part.

Concerning the raw particles, it was noticed that protein hydrolysis was dominantat the first minutes of digestion, as a lag phase in lipid hydrolysis was observed. This observation underlined the importance of proteases in the rate of lipid hydrolysis, as lipase might be unable to access the lipids surface until part of the proteins are hydrolysed by proteases [18].

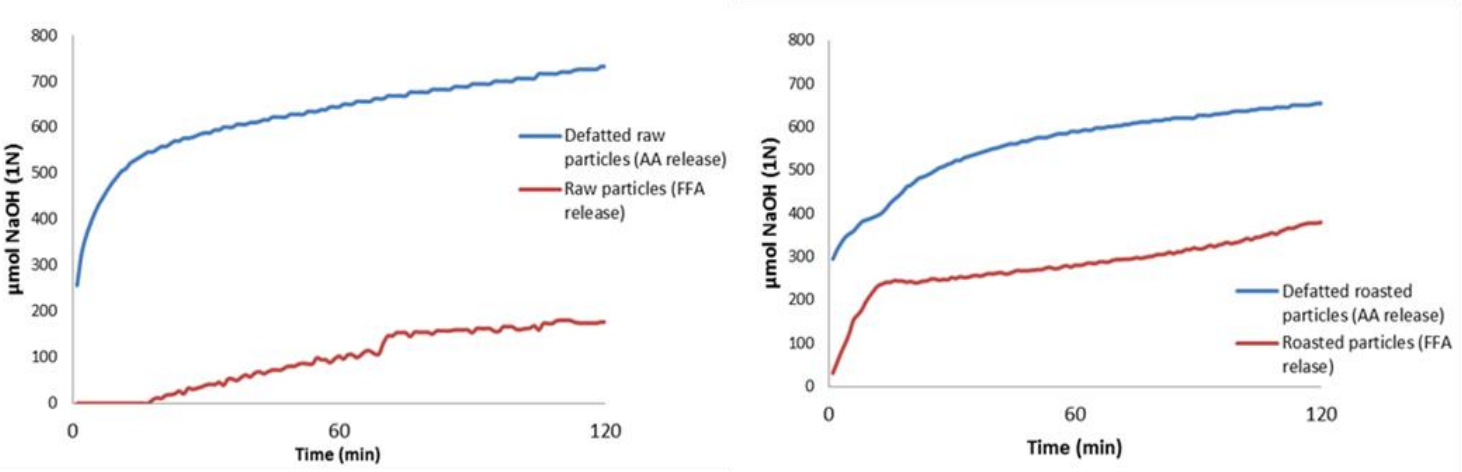

FIGURE 20.NaOH release (µmol) during the intestinal digestion of defatted raw (left panel) and defatted roasted (right panel) hazelnut particles using pH-stat method.

In contrast, in roasted hazelnuts a parallel hydrolysis of proteins and lipid was detected. The conformational changes in the structure of proteins might have hindered their hydrolysis (lower NaOH release over 120 min intestinal digestion) as explained above but subsequent changes in the structure of the cell due to these alterations seemed to facilitate lipid hydrolysis.

As the protein analysis showed (Figure 21), both raw and roasted hazelnuts had a similar protein profile before digestion. However, more intense and slightly thicker protein bands of higher molecular weight (>60 kDa) were observed in roasted hazelnut particles which are indicative of their relative abundance. After the end of the digestion, more bands in low molecular weight range were observed in the roasted hazelnut particles suggesting a limited protein hydrolysis. As explained before, protein aggregates formed during the roasting process, might had been resistant to proteolytic enzymes, due to conformational changes in their structure. Thus, they become less susceptible to the cleavage by proteases. As a result, undigested peptides of low molecular weight appeared during the analysis of the protein profiles of digested particles. Rufián-Henares and Delgado-Andrade (2009) [53] highlighted in their study that although transformations of Maillard reaction products in the intestinal tract can take place, significant amounts of the compounds provide stability and resistance to the digestive process *in vitro*. Erbersdobler and Faistln [54] also mentioned that the metabolism of Maillard reaction products is very low in the upper GI tract(conditions that we mimic during *in vitro* digestion) and only a small amount can be degraded by gut microbiota in colon.

Moreover, small (3-4 mg) and big (8-9 mg) particles were chosen to be examined after digestion to observe potential differences in protein hydrolysis attributed to particle size. It was noticed that smaller hazelnut particles, both raw (R1ad) and roasted (RS1ad), had a higher protein hydrolysis after the end of digestion compared to bigger particles (R2ad, RS2ad respectively). This observation highlighted the importance of particle size in the digestion process [2, 34].

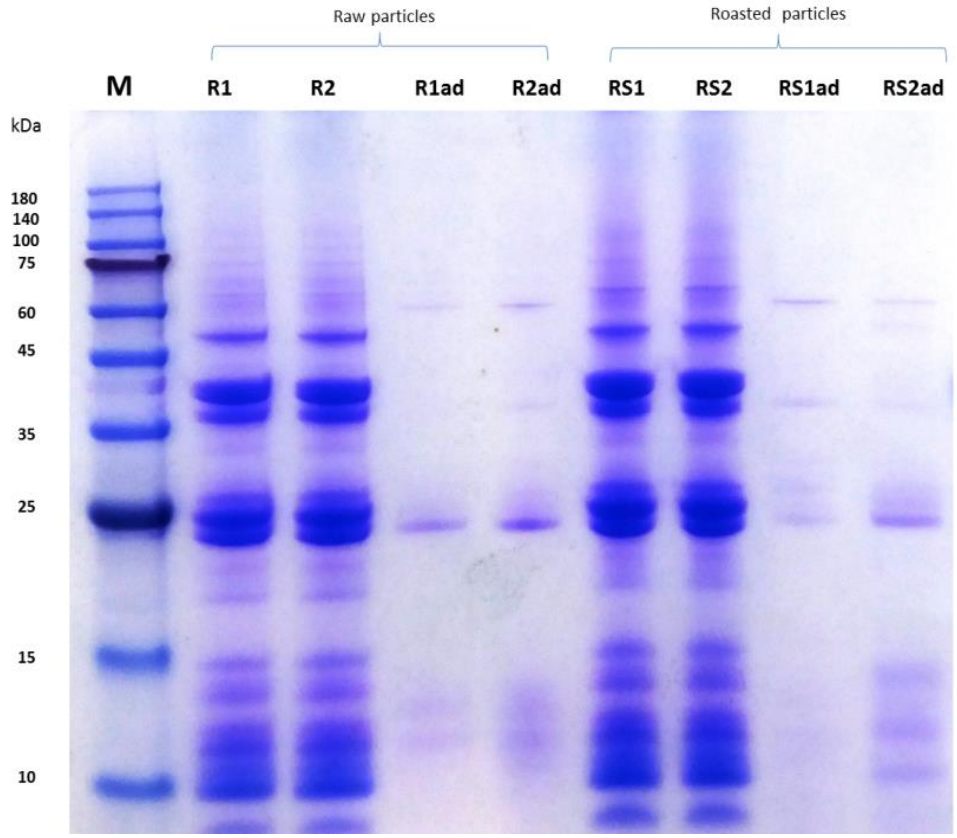

FIGURE 214. SDS-PAGE analysis of raw (R lines) and roasted (RS lines) hazelnut particles before and after digestion. M=protein molecular weight marker. R1,RS1 represent raw and roasted small particles before digestion and R2,RS2 raw and roasted big particles before digestion. Similarly, R1ad, RS1ad represent the protein profile ofraw and roasted small particles after digestion and R2ad, RS2ad the protein profile of bigger particles after digestion.

6. Conclusions and further recommendations

In this research a deeper inside into the potential hindering mechanism of the plant cell wall on lipid hydrolysis in hazelnuts as well as the effect of heat treatment on FFA release was provided. The study showed that lipid bioaccessibility during *in vitro* digestion was greater for OBs compared to hazelnut particles, highlighting the effect of the cell-wall barrier on lipid release. These findings confirmed the important role played by the plant cell wall on nutrient release and digestion.

In addition, it was suggested that roasting impact on the structure of the cell as well of the OBs' surface. Roasted hazelnut particles exhibited significantly higher lipid hydrolysis after the end of the digestion process compared to raw hazelnut particles. As microstructural analysis confirmed, there were apparent differences between raw and roasted hazelnut particles before and after digestion, suggesting a different behaviour upon fluid and enzyme diffusion through the cells. Different disintegration behaviour was noticed in roasted hazelnut particles during digestion, probably attributed to a higher porosity of the heat-treated cells and subsequently to a better enzyme and digestive fluid diffusion into hazelnuts tissues.

In addition, the denaturation of the proteins present in the hazelnut cells under roasting, also contributed to structural changes that might have facilitated lipid digestion but not protein digestion. Besides, protein hydrolysis in raw hazelnuts showed to be dominant at the first minutes of digestion compared to lipid hydrolysis, emphasizing the importance of proteolytic enzyme action on the lipid digestion process.

Furthermore, lipid hydrolysis of OBs extracted from raw and roasted hazelnuts showed to be significantly different, with higher lipolysis rate observed in OBs from roasted hazelnuts. This difference was mainly attributed to the unlike destabilization behaviour during digestion induced by roasting. Roasting was suggested to cause structural changes to the interfacial proteins of OBs leading to more effective oleosin hydrolysis during gastric phase. Subsequently a more efficient displacement of oleosin surface by bile salts and enzymes might have taken place. These findings, confirmed our hypothesis about the existence of a "pre-digest effect" of the oleosins upon heat treatment.

Nevertheless, in order to investigate in a greater extent the effect of cell wall encapsulation of nutrients further studies can be done. In particular, the effect of particle size of nuts on lipid digestion could give a better understanding on the hindering mechanism on lipid release upon digestion. Moreover, a better insight on enzyme diffusion through the cell wall using fluorescence techniques combined with microscopy would be useful to provide information not only about the effect of the particle size but also of the heat treatment on the cell wall barrier mechanism. In addition, CLSM combined with fluorescence technique instead of LM could provide a more detailed image of the structure of OBs before and during digestion. Furthermore, regarding the determination of lipid hydrolysis, another technique like gas chromatography could also be tested as an additional method to assess FFA release during digestion.

7. References

1. Parada, J. and J.M. Aguilera, *Food microstructure affects the bioavailability of several nutrients.* J Food Sci, 2007. **72**(2): p. R21-32.
2. Grassby, T., et al., *Modelling of nutrient bioaccessibility in almond seeds based on the fracture properties of their cell walls.* Food Funct, 2014. **5**(12): p. 3096-106.
3. Mandalari, G., et al., *The effects of processing and mastication on almond lipid bioaccessibility using novel methods of in vitro digestion modelling and micro-structural analysis.* Br J Nutr, 2014. **112**(9): p. 1521-9.
4. Ellis, P.R., et al., *Role of cell walls in the bioaccessibility of lipids in almond seeds.* American Journal of Clinical Nutrition, 2004. **80**(3): p. 604-613.
5. Grundy, M.M., et al., *Effect of mastication on lipid bioaccessibility of almonds in a randomized human study and its implications for digestion kinetics, metabolizable energy, and postprandial lipemia.* Am J Clin Nutr, 2015. **101**(1): p. 25-33.
6. McClements, D.J., E.A. Decker, and Y. Park, *Controlling lipid bioavailability through physicochemical and structural approaches.* Crit Rev Food Sci Nutr, 2009. **49**(1): p. 48-67.
7. Minekus, M., et al., *A standardised static in vitro digestion method suitable for food - an international consensus.* Food Funct, 2014. **5**(6): p. 1113-24.
8. Fernandez-Garcia, E., I. Carvajal-Lerida, and A. Perez-Galvez, *In vitro bioaccessibility assessment as a prediction tool of nutritional efficiency.* Nutr Res, 2009. **29**(11): p. 751-60.
9. Grundy, M.M., et al., *The role of plant cell wall encapsulation and porosity in regulating lipolysis during the digestion of almond seeds.* Food Funct, 2016. **7**(1): p. 69-78.
10. Flint, H.J. and E.A. Bayer, *Plant cell wall breakdown by anaerobic microorganisms from the Mammalian digestive tract.* Ann N Y Acad Sci, 2008. **1125**: p. 280-8.
11. Mandalari, G., et al., *Release Of Protein, Lipid, And Vitamin E From Almond Seeds During Digestion.* J. Agric. Food Chem, 2008. **56**: p. 3409-3416.
12. Grundy, M.M., K. Lapsley, and P.R. Ellis, *A review of the impact of processing on nutrient bioaccessibility and digestion of almonds.* International Journal of Food Science and Technology, 2016. **51**(9): p. 1937-1946.
13. Berry, S.E.E., et al., *Manipulation of lipid bioaccessibility of almond seeds influences postprandial lipemia in healthy human subjects.* Am J Clin Nutr, 2008. **88**: p. 922–9.
14. Ros, E., *Health benefits of nut consumption.* Nutrients, 2010. **2**(7): p. 652-82.
15. Baer, D.J., S.K. Gebauer, and J.A. Novotny, *Walnuts Consumed by Healthy Adults Provide Less Available Energy than Predicted by the Atwater Factors.* J Nutr, 2016. **146**(1): p. 9-13.
16. Novotny, J.A., S.K. Gebauer, and D.J. Baer, *Discrepancy between the Atwater factor predicted and empirically measured energy values of almonds in human diets.* Am J Clin Nutr, 2012. **96**(2): p. 296-301.
17. Baer, D.J., S.K. Gebauer, and J.A. Novotny, *Measured energy value of pistachios in the human diet.* Br J Nutr, 2012. **107**(1): p. 120-5.
18. McClements, D.J. and Y. Li, *Review of in vitro digestion models for rapid screening of emulsion-based systems.* Food & Function, 2010. **1**(1): p. 32-59.
19. Gallier, S. and H. Singh, *Behavior of almond oil bodies during in vitro gastric and intestinal digestion.* Food Funct, 2012. **3**(5): p. 547-55.
20. Groopman, E.E., R.N. Carmody, and R.W. Wrangham, *Cooking increases net energy gain from a lipid-rich food.* Am J Phys Anthropol, 2015. **156**(1): p. 11-8.
21. Bonsegna, S., et al., *Plant oil bodies: novel carriers to deliver lipophilic molecules.* Appl Biochem Biotechnol, 2011. **163**(6): p. 792-802.
22. Huang, A.H., *Oil bodies and oleosins in seeds.* Annual review of plant biology, 1992. **43**(1): p. 177-200.
23. Frandsen, G.I., J. Mundy, and J.T. Tzen, *Oil bodies and their associated proteins, oleosin and caleosin.* Physiologia plantarum, 2001. **112**(3): p. 301-307.
24. Tzen, J.T., *Integral proteins in plant oil bodies.* ISRN Botany, 2012. **2012**.

25. Nikiforidis, C.V., V. Kiosseoglou, and E. Scholten, *Oil bodies: An insight on their microstructure — maize germ vs sunflower seed.* Food Research International, 2013. **52**(1): p. 136-141.
26. Nikiforidis, C.V., S. Donsouzi, and V. Kiosseoglou, *The interplay between diverse oil body extracts and exogenous biopolymers or surfactants.* Food Research International, 2016. **83**: p. 14-24.
27. Whitcomb, D.C. and M.E. Lowe, *Human pancreatic digestive enzymes.* Dig Dis Sci, 2007. **52**(1): p. 1-17.
28. Golding, M. and T.J. Wooster, *The influence of emulsion structure and stability on lipid digestion.* Current Opinion in Colloid & Interface Science, 2010. **15**(1-2): p. 90-101.
29. Li, Y. and D.J. McClements, *New mathematical model for interpreting pH-stat digestion profiles: impact of lipid droplet characteristics on in vitro digestibility.* J Agric Food Chem, 2010. **58**(13): p. 8085-92.
30. Porter, C.J., K.M. Wasan, and P. Constantinides, *Lipid-based systems for the enhanced delivery of poorly water soluble drugs.* Adv Drug Deliv Rev, 2008. **60**(6): p. 615-6.
31. Ros, E. and J. Mataix, *Fatty acid composition of nuts – implications for cardiovascular health.* British Journal of Nutrition, 2007. **96**(S2): p. S29.
32. Pouton, C.W. and C.J. Porter, *Formulation of lipid-based delivery systems for oral administration: materials, methods and strategies.* Adv Drug Deliv Rev, 2008. **60**(6): p. 625-37.
33. Capolino, P., et al., *In Vitro Gastrointestinal Lipolysis: Replacement of Human Digestive Lipases by a Combination of Rabbit Gastric and Porcine Pancreatic Extracts.* Food Digestion, 2011. **2**(1-3): p. 43-51.
34. Grundy, M.M., et al., *Impact of cell wall encapsulation of almonds on in vitro duodenal lipolysis.* Food Chem, 2015. **185**: p. 405-12.
35. OÈzdemir, M., et al., *Evaluation of new Turkish hybrid hazelnut (Corylus avellana L.) varieties: fatty acid composition, α-tocopherol content, mineral composition and stability.* Food Chemistry, 2001. **73**(4): p. 411-415.
36. https://ndb.nal.usda.gov/ndb/foods/show/3666?fgcd=&manu=&lfacet=&format=&count=&max=50&offset=&sort=default&order=asc&qlookup=hazelnuts&ds=.
37. https://ndb.nal.usda.gov/ndb/foods/show/3635?fgcd=&manu=&lfacet=&format=&count=&max=50&offset=&sort=default&order=asc&qlookup=almonds&ds=.
38. Gallier, S., H. Tate, and H. Singh, *In vitro gastric and intestinal digestion of a walnut oil body dispersion.* J Agric Food Chem, 2013. **61**(2): p. 410-7.
39. Borges, A. and M. Peleg, *Effect of water activity on the mechanical properties of selected legumes and nuts.* Journal Of The Science Of Food And Agriculture, 1997. **75**(4): p. 463-471.
40. Jalabert-Malbos, M.-L., et al., *Particle size distribution in the food bolus after mastication of natural foods.* Food Quality and Preference, 2007. **18**(5): p. 803-812.
41. Yaacoub, R., et al., *Formation of lipid oxidation and isomerization products during processing of nuts and sesame seeds.* Journal of agricultural and food chemistry, 2008. **56**(16): p. 7082-7090.
42. Nikiforidis, C.V., A. Matsakidou, and V. Kiosseoglou, *Composition, properties and potential food applications of natural emulsions and cream materials based on oil bodies.* RSC Adv., 2014. **4**(48): p. 25067-25078.
43. Ruiz, G.A., et al., *Denaturation and in Vitro Gastric Digestion of Heat-Treated Quinoa Protein Isolates Obtained at Various Extraction pH.* Food biophysics, 2016. **11**(2): p. 184-197.
44. Iwan, M., et al., *Impact of Maillard reaction on immunoreactivity and allergenicity of the hazelnut allergen Cor a 11.* J Agric Food Chem, 2011. **59**(13): p. 7163-71.

45. Nagaraj, R.H., I.N. Shipanova, and F.M. Faust, *Protein cross-linking by the Maillard reaction isolation, characterization, and in vivo detection of a lysine-lysine cross-link derived from methylglyoxal.* Journal of Biological Chemistry, 1996. **271**(32): p. 19338-19345.
46. Kong, F. and R.P. Singh, *Digestion of Raw and Roasted Almonds in Simulated Gastric Environment.* Food Biophysics, 2009. **4**(4): p. 365-377.
47. Mat, D.J., et al., *In vitro digestion of foods using pH-stat and the INFOGEST protocol: Impact of matrix structure on digestion kinetics of macronutrients, proteins and lipids.* Food Research International, 2016. **88**: p. 226-233.
48. Singh, H., A. Ye, and D. Horne, *Structuring food emulsions in the gastrointestinal tract to modify lipid digestion.* Prog Lipid Res, 2009. **48**(2): p. 92-100.
49. Giang, T.M., et al., *Dynamic modeling highlights the major impact of droplet coalescence on the in vitro digestion kinetics of a whey protein stabilized submicron emulsion.* Food Hydrocolloids, 2015. **43**: p. 66-72.
50. Mun, S., E.A. Decker, and D.J. McClements, *Influence of emulsifier type on in vitro digestibility of lipid droplets by pancreatic lipase.* Food Research International, 2007. **40**(6): p. 770-781.
51. Beisson, F., et al., *Oil-bodies as substrates for lipolytic enzymes.* Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 2001. **1531**(1–2): p. 47-58.
52. Singh, H. and A. Ye, *Structural and biochemical factors affecting the digestion of protein-stabilized emulsions.* Current Opinion in Colloid & Interface Science, 2013. **18**(4): p. 360-370.
53. Rufián-Henares, J.A. and C. Delgado-Andrade, *Effect of digestive process on Maillard reaction indexes and antioxidant properties of breakfast cereals.* Food Research International, 2009. **42**(3): p. 394-400.
54. Erbersdobler, H. and V. Faist, *Metabolic transit of Amadori products.* Molecular Nutrition & Food Research, 2001. **45**(3): p. 177-181.

8. Appendices

8.1. Statistical analysis of enzyme activity determination

Descriptive Statistics

		Mean	Std. Deviation	N
1	50 mg/ml pancreatin	23.5000	3.53553	2
2	25mg/ml pancreatin +2.5 mg/ml lipase	19.2400	1.21622	2
3	30mg/ml pancreatin +2 mg/ml lipase	22.4000	.84853	2
4	35 mg/ml pancreatin +1.5 mg/ml lipase	22.4500	9.12168	2

Pairwise Comparisons

Measure: MEASURE_1

(I) ENZYMES_COMB	(J) ENZYMES_COMB	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
1	2	4.260	3.360	.425	-38.433	46.953
	3	1.100	1.900	.666	-23.042	25.242
	4	1.050	3.950	.835	-49.140	51.240
2	1	-4.260	3.360	.425	-46.953	38.433
	3	-3.160	1.460	.276	-21.711	15.391
	4	-3.210	7.310	.737	-96.092	89.672
3	1	-1.100	1.900	.666	-25.242	23.042
	2	3.160	1.460	.276	-15.391	21.711
	4	-.050	5.850	.995	-74.381	74.281
4	1	-1.050	3.950	.835	-51.240	49.140
	2	3.210	7.310	.737	-89.672	96.092
	3	.050	5.850	.995	-74.281	74.381

Based on estimated marginal means

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

8.2. Statistical analysis of pH-stat results

Within-Subjects Factors

Measure: MEASURE_1

condition	Time	Dependent Variable
1	60	Raw_particles60
	120	Raw_particles120
2	60	Roasted_Particles60
	120	Roasted_Particles120
3	60	Raw_OBs60
	120	Raw_OBs120
4	60	Roasted_OBs60
	120	Roasted_OBs120
5	60	Oil60
	120	Oil120

Descriptive Statistics

	Mean	Std. Deviation	N
Raw_particles60	103.0000	53.74012	2
Raw_particles120	177.0000	2.82843	2
Roasted_Particles60	280.5000	10.60660	2
Roasted_Particles120	380.0000	21.21320	2
Raw_OBs60	543.5000	16.26346	2
Raw_OBs120	654.5000	21.92031	2
Roasted_OBs60	767.0000	1.41421	2
Roasted_OBs120	909.0000	2.23484	2
Oil60	215.0000	26.87006	2
Oil120	249.5000	16.26346	2

Pairwise Comparisons

Measure: MEASURE_1

Time	(I) condition	(J) condition	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
60min	1	2	-177.500	45.500	.160	-755.632	400.632
		3	-440.500*	26.500	.038	-777.214	-103.786
		4	-664.000*	37.000	.035	-1134.130	-193.870
		5	-112.000	57.000	.300	-836.254	612.254
	2	1	177.500	45.500	.160	-400.632	755.632

		3	-263.000*	19.000	.046	-504.418	-21.582
		4	-486.500*	8.500	.011	-594.503	-378.497
		5	65.500	11.500	.111	-80.621	211.621
	3	1	440.500*	26.500	.038	103.786	777.214
		2	263.000*	19.000	.046	21.582	504.418
		4	-223.500*	10.500	.030	-356.915	-90.085
		5	328.500	30.500	.059	-59.039	716.039
	4	1	664.000*	37.000	.035	193.870	1134.130
		2	486.500*	8.500	.011	378.497	594.503
		3	223.500*	10.500	.030	90.085	356.915
		5	552.000*	20.000	.023	297.876	806.124
	5	1	112.000	57.000	.300	-612.254	836.254
		2	-65.500	11.500	.111	-211.621	80.621
		3	-328.500	30.500	.059	-716.039	59.039
		4	-552.000*	20.000	.023	-806.124	-297.876
120min	1	2	-203.000*	13.000	.041	-368.181	-37.819
		3	-477.500*	17.500	.023	-699.859	-255.141
		4	-732.000*	3.000	.003	-770.119	-693.881
		5	-72.500	13.500	.117	-244.034	99.034
	2	1	203.000*	13.000	.041	37.819	368.181
		3	-274.500	30.500	.070	-662.039	113.039
		4	-529.000*	16.000	.019	-732.299	-325.701
		5	130.500	26.500	.128	-206.214	467.214
	3	1	477.500*	17.500	.023	255.141	699.859
		2	274.500	30.500	.070	-113.039	662.039
		4	-254.500*	14.500	.036	-438.740	-70.260
		5	405.000*	4.000	.006	354.175	455.825
	4	1	732.000*	3.000	.003	693.881	770.119

		2	529.000*	16.000	.019	325.701	732.299
		3	254.500*	14.500	.036	70.260	438.740
		5	659.500*	10.500	.010	526.085	792.915
	5	1	72.500	13.500	.117	-99.034	244.034
		2	-130.500	26.500	.128	-467.214	206.214
		3	-405.000*	4.000	.006	-455.825	-354.175
		4	-659.500*	10.500	.010	-792.915	-526.085

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).