Bernat Pérez, N.; Cháfer Nácher, MT.; Rodriguez Garcia, J.; Chiralt A.; González Martínez, MC. (2015). Effect of high pressure homogenization and heat treatment on physical properties and stability of almond and hazelnut milks. Food Science and Technology. 62:488-496. doi:10.1016/j.lwt.2014.10.045.

Effect of high pressure homogenisation and heat treatment on physical properties and stability of almond and hazelnut *milks*

Bernat N[1], Chafer M[1], Rodríguez-García, J[2], Chiralt A[1] and Gonzalez-Martínez, C[1*].

(1) Institute of Food Engineering for the Development.

(2) Food Technology Department**.**

Universitat Politècnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain.

(*cgonza@tal.upv.es*)

ABSTRACT

The effect of high pressure homogenisation (HPH) and heat treatments on physicochemical properties and physical stability of almond and hazelnut milks was studied. Vegetable milks were obtained and homogenised by applying 62, 103 and 172 MPa (MF1, MF2 and MF3, respectively). Untreated and MF3 samples were also submitted to two different heat treatments (85ºC/30 min (LH) or 121ºC/15 min (HH)). Physical and structural properties of the products were greatly affected by heat treatments and HPH. In almond milk, homogenised samples showed a significant reduction in particle size, which turned from bimodal and polydisperse to monodisperse distributions. Particle surface charge, clarity and Whiteness Index were increased and physical stability of samples was improved, without affecting either viscosity or protein stability. Hazelnut beverages showed similar trends, but HPH notably increased their viscosity while change their rheological behaviour, which suggested changes in protein conformation. HH treatments caused an increment of particle size due to the formation oil droplet-protein body clusters, associated with protein denaturation. Samples submitted to the combined treatment MF3 and LH showed the greatest stability.

Key words: Particle size, DSC, viscosity, ζ-potential, confocal.

Abbreviations:

CLSM, confocal laser scanning microscopy; DSC, differential scanning calorimetry ; HH, high heat; HPH, high pressure homogenisation; PI, isoelectric point; LH, low heat; MF1, homogenisation at 62 MPa; MF2, homogenisation at 103 MPa; MF3, homogenisation at 172 MPa; WI, whiteness index.

1. INTRODUCTION

In the last few years, the population ratio demanding vegetable-based products is growing, either because of the increasing problems related with the intolerances to Cow's milk (Fiocchi *et al.*, 2010) or because of changes in the food preferences. As a consequence of new consumer tendencies, food industries are currently producing new nutritionally improved products with added value. Vegetable-based beverages are included in these new products, which are available at any supermarket as an alternative to dairy products, with an increasing consumer acceptance.

There is a wide variety of vegetable-based beverages, although most of the research activity has been focused on those obtained from soy. For soy "milk", studies into the physicochemical characterization, the effects of processing, the application of new technologies, such as electric pulses and ultra-high homogenisation pressures have been carried out (Cruz, Capellas, Hernández, Trujillo, Guamis, & Ferragut, 2007; Li, Chen, Liu, & Chen, 2008).

Research dealing with the use of non-soy vegetable milk is still scarce and most of it is related with the nutritional quality of such products. In this sense, almond and hazelnut beverages have been used as an alternative to milk in lacto-intolerant people, pregnant women and celiacs, due to their high levels of calcium, phosphorous and potassium (Eroski Foundation, 2007; Luengo, 2009). These nuts have low sodium content and an equilibrated mono-unsaturated fatty acid-polyunsaturated fatty acids ratio, which define the products which are healthy for people with heart disease (Mateos, 2007). They are also considered helpful for maintaining cholesterol at healthy levels due to their high content of antioxidant compounds which contributes to heart disease prevention (Fraser, Bennett, Jaceldo & Sabaté, 2002; Jenkins, Kendell, Marchie, Josse,

Nguyen & Faulkner, 2008; Kris-Etherton, Hu, Rose & Sabaté, 2008; Tey, Brown, Chislholm, Delahunty, Gray & Williams, 2011).

Vegetable based beverages are emulsified products where the nut fat is dispersed in an aqueous phase and where the rest of the components play different roles in the product stability. The different process steps, such as homogenisation and heat treatments usually produce changes in the arrangement of components, thus leading to modifications in the particle size, colour, viscosity and physical stability of the product. These physicochemical modifications have to be known to efficiently control the process and to implement the necessary improvements in the production lines. The most commonly used homogenisation pressures in the food industry range between 20 and 50 MPa, although much higher pressures are used in high pressure homogenisation (HPH) processes with some advantages: the deflocculation of clusters of primary fat globules (Floury, Desrumaux, & Lardières, 2000) and uniform dispersion of agglomerates, the changes in protein conformation (Pereda, Ferragut, Quevedo, Guamis & Trujillo, 2009), the increase in emulsion viscosity (Desrumaux & Marcand, 2002) and stability and the microbial inactivation (Diels, Callewaert, Wuytack, Masschalk & Michiels, 2005; Pereda, Ferragut, Guamis & Trujillo, 2006; Smiddy, Martin, Huppertz & Kelly, 2013; Cruz, Capellas, Hernández, Trujillo, Guamis & Ferragut, 2007).

The objective of the present study is to analyze the effect of heat treatments and high homogenisation pressures on the physical properties and stability of almond and hazelnut beverages (nut milks) in order to define processing conditions which ensure the product quality and stability.

2. MATERIALS & METHODS

2.1 Preparation of almond and hazelnut milks

Nut beverages were produced by soaking and grinding *Prunus amygdalus L.dulcis* almonds and *Corylus avellana* hazelnuts, supplied by Frutos Secos 3G S.L. (Valencia, Spain). The extraction was carried out in Sojamatic 1.5 (Sojamatic®; Barcelona, Spain), equipment specifically designed

for the production of vegetable beverages, with a nut-water ratio of 8:100. This equipment carries out both the nut grinding and the solid particles' retention throughout filtration. The manufacturing process takes 30 minutes at room temperature, in which both grinding and filtering are performed discontinuously every two minutes. The milky liquid obtained was used as control sample (untreated).

2.2 High pressure homogenisation and heat treatments

High pressure homogenisation (HPH) treatments were carried out in a high pressure homogeniser M-110P model (Microfluidics International Corporation, Newton, MA, USA) by applying 62, 103 and 172 MPa (samples MF1, MF2 and MF3 respectively). Some samples were submitted to a low temperature heat treatment (LH) at 85 ºC for 30 min by using a temperature-controlled water bath (Precisdig, JP-Selecta; Barcelona, Spain) and to a high temperature heat treatment (HH), 121 ºC for 15 min in an autoclave (Precisdig, JP-Selecta; Barcelona, Spain). The heat treatment conditions chosen were those in which the destruction of all vegetative cells and enzymes are ensured (Walstra, Wouters & Geurts, 2006). Samples submitted to heat treatment were the control samples (LH and HH samples) and those homogenised at 172 MPa (MF3LH and MF3HH samples).

2.3 Characterization of chemical composition.

The quantification of moisture, ash, fat content, proteins and sugars was carried out in the nut milks. Fibre content was estimated by means of the difference in terms of component percentages. Almond beverages were freeze-dried (ioalfa-6 freeze-dryer; TELSTAR, Terrassa, Spain) prior to the analysis. AOAC Official Methods were chosen to determine water, total fats and total nitrogen contents (AOAC 16.006, AOAC 945.16 and AOAC 958.48, respectively) (Horwitz, 2000). Total sugars and ashes were obtained following the protocols suggested by Matissek, Schnepel & Steiner (1998). All the determinations were performed in triplicate.

2.4 Characterization of physical and structural properties

112

113 **2.4.1 pH and density.**

114 Measurements were carried out at 25ºC using a pH-meter GLP 21+ (Crison Instruments S.A.,
115 Barcelona, Spain) and a digital densitometer DA-110 M (Mettler Toledo, Barcelona, Spain),
116 respectively. These determinations and those described below were carried out in triplicate.

117

118 **2.4.2 Particle size distribution and ζ-potential.**

119 Analysis of the particle size distribution was carried out using a laser diffractometer Mastersizer
120 2000 (Malvern Instruments Ltd, Worcestershire, UK). The Mie theory was applied by considering
121 a refractive index of 1.33 and absorption of 0.1. Samples were diluted in de-ionised water at 2,000
122 rpm until an obscuration rate of 10% was obtained. D_{32} (surface weighted mean diameter) and
123 D_{43} (volume weighted mean diameter) were obtained. The volume-weighted average diameter is
124 sensitive to the presence of large particles, whereas the surface-weighted average diameter is more
125 sensitive to the presence of small particles.
126 ζ-potential was determined at 20 ºC by using a Zetasizer nano-Z (Malvern Instruments Ltd,
127 Worcestershire, UK). Samples were diluted to a fat droplet concentration of 0.4 g/100 mL using
128 a phosphate buffer 0.02 mol/L solution. The Smoluchowsky mathematical model was used to
129 convert the electrophoretic mobility measurements into ζ-potential values.

130

131 **2.4.3 Rheological behaviour**

132 The rheological behaviour of nut milks were characterized by using a rotational rheometer
133 (HAAKE Rheostress 1, Thermo Electric Corporation, Karlsruhe, Germany) with a sensor system
134 of coaxial cylinders, type Z34DIN Ti. The shear stress (σ) was measured as a function of shear
135 rate ($\dot{\gamma}$) from 0 to 112 s^{-1}. The up and down curves were obtained, taking 1 minute to rise and 1
136 minute to fall. The Herschel-Bulkey model (Eq. (1)) was fitted to the experimental points to
137 determine the flow behaviour index (n), consistency index (K) and yield stress (σ_y) by using a
138 non-linear procedure. Apparent viscosities were calculated at 100 s^{-1}.

$$\sigma = \sigma_y + K\,\gamma^{n} \qquad (1)$$

2.4.4. Optical properties

Colour coordinates were measured from the infinite reflection spectrum in a Spectrum-colorimeter CM-3600 d (MINOLTA Co, Osaka, Japan). CIE $L^*a^*b^*$ coordinates were obtained using illuminant D65/10º observer. Colour of samples was characterized as to Lightness (L^*), Chrome ($C_{ab}{}^*$), hue ($h_{ab}{}^*$) and Whiteness Index (WI) as defined in Eq. (3) to (5). Colour difference (ΔE) between treated and untreated samples was also calculated by using Eq. (6).

$$C^* = \sqrt{a^{*2} + b^{*2}} \qquad (3)$$

$$h_{ab*} = \arctan(b^*/a^*) \qquad (4)$$

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \qquad (5)$$

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (6)$$

2.4.5. Protein denaturation

The protein denaturation degree in each sample was analyzed by Differential Scanning Calorimetry in DSC SSC5200-220 calorimeter (Seiko Instruments, Torrance, CA, USA). Prior to the analyses, samples were freeze-dried in an ioalfa-6 free-dryer (TELSTAR, Terrassa, Spain) and afterwards rehydrated with 70 g/100 mL of water. 25 mg of rehydrated samples were introduced in hermetic aluminum capsules (P/N SSC000C008) (Seiko Instruments, Torrance, CA, USA). An empty capsule was used as reference. Sample heating was carried out from 25 ºC to 120 ºC at 5 ºC/min. From the obtained thermograms (heat flux vs. temperature), the peak temperature and enthalpy for protein denaturation were obtained.

2.4.6 Confocal laser scanning microscopy (CLSM)

A Nikon confocal microscope C1 unit, which was fitted on a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan), was used. An Ar laser line (488 nm) was employed as light source to excite fluorescent dyes Rhodamine B and Nile Red. Rhodamine B (Sigma-Alrdrich, Seelze,

Germany) with λex max 488 nm and λem max 580 nm was dissolved in distilled water at 0.2 g/100 mL.This dye was used to stain proteins and carbohydrates. Nile Red (Sigma-Alrdrich, Seelze, Germany) with λex max 488 nm and λem max 515 nm was dissolved in PEG 200 at 0.1 g/L. This dye was used to stain fat. An oil immersion objective lens (60x/1.40NA/Oil/ Plan Apo VC Nikon) was used.

For sample visualization a microscopy slide was elaborated with two razor blades (platinum coated double edge blades with 0.1 mm thickness) stuck to a glass. 20 μL of the sample were placed on the microscope slide, within the central gap of the blades; 10 μL of Rhodamine B solution and 10 μL of Nile Red solution were added and the cover slide was carefully positioned. Observations were performed 10 min after diffusion of the dyes into the sample. Images were observed and stored with 1,024 x 1,024 pixel resolution, using the microscope software (EZ-C1 v.3.40, Nikon, Tokyo, Japan).

2.4.7 Colloidal stability of milks

Colloidal stability of the obtained products was determined through the phase separation analysis throughout storage time (28 days) at 4ºC, in all samples. To this end, about 15 g of almond and hazelnut milks were poured into glass tubes of 16 mm diameter and the height of the separate phases was quantified. 0.04 g/ 100 mL of sodium azide was added to samples, thus assuming no microbial growth took place during storage.

2.5 Statistical Analysis

Results were analyzed by multifactor analysis of variance with 95% significance level using Statgraphics® Centurion XV (Warrenton, Virginia, USA). Multiple comparisons were performed through 95% LSD intervals.

3. RESULTS & DISCUSSION

3.1 Chemical composition

194 Chemical composition of both types of nuts and their beverages has been summarized in Table
195 1.The obtained composition of both nuts was similar to those found by other authors for the same
196 varieties of these products (Luengo, 2009, Saura, Cañellas & Soler, 1988). Composition of both
197 beverages was quite similar, although the protein content, and so the protein-fat ratio, were greater
198 in almond, coherent with the higher content of this component in almonds. In comparison with
199 cow milk (3.2 and 3.4 g/100 mL of fat and protein, respectively), these vegetable milks have a
200 slightly higher fat content and a lower protein content. Nevertheless, they contain vegetable fibre
201 and according to Gallier, Gordon & Singh (2012), 68 and 23 g/100 g of monounsaturated and
202 polyunsaturated fatty acids respectively are present in the lipid fraction of almond milk.
203
204 **3.2 pH and density.**
205 In almond milk, no significant differences were found in pH and density values between the
206 samples submitted to the different treatments ($p > 0.05$), the average values being 6.66 ± 0.08 and
207 1001.1 ± 0.1 kg/m^3, respectively. Regarding to hazelnut milk, non-treated samples showed a pH
208 value of 6.66 ± 0.02 and a density of 1001.2 ± 0.4 kg/m^3, whereas in treated samples (regardless
209 of the treatment) a slight increase in pH (6.81 ± 0.08) and a decrease in density (mean value for
210 treated samples: 995.4 ± 0.6 kg/m^3) was observed. This might be explained by the fact that thermal
211 or pressure effects could cause conformational changes in components (especially biopolymers)
212 which may inhibit the ionization of some acidic groups and induce small changes in density.
213
214 **3.3 Particle size distribution and ζ-potential**.
215 Figure 1 shows the typical particle size distribution obtained for one of the milks (almond) as
216 affected by the homogenisation and thermal treatments. Similar behaviour was found for hazelnut
217 milks.
218 As could be observed, both non-homogenised samples presented bimodal and polydisperse
219 distributions in terms of volume percentage (Figure 1 a, c) but monomodal in terms of the number
220 of particles (Figure 1 b, d), which indicates that there is a very small number of big particles. The
221 finest particle fraction is probably mainly constituted by proteins, whereas fat droplets and

remains of cellular tissue constitute the biggest particles. Nevertheless, particle aggregates could also be present in the biggest particle fraction. Particle size distribution became monomodal when samples were homogenised and the biggest particles of the initial product were greatly reduced in size. However, some finest particles evidenced in the first peak of the distribution of untreated samples seem to aggregate since they are not appear in the tail of the peak of HPH samples. A part of the protein bodies could be unfolded or aggregated by the high pressure effect. The increase in homogenisation pressure progressively reduced the mean particle diameter while distributions became narrower due to the reduction in size of oil droplets and plant cell remains. This can also be deduced from Table 2, where the overall decrease in both the mean particle diameters and the difference between $D_{4,3}$ and $D_{3,2}$ in MF samples can be seen. No significant differences ($p > 0.05$) in these parameters were found when applying 103 (MF2 treatment) or 172 MPa (MF3 treatment) pressures.

The effect of the thermal treatment is shown in Figure 1c. The application of both thermal treatments led to the disappearance of the finest particles probably due to the change in the protein conformation (denaturation) and the promotion of particle aggregations, which increases their hydrodynamic volume, with the subsequent increase in the product viscosity (as observed in the rheological data). Thermal treatments can also promote the increase in the size of fat globules, due to flocculation and coalescence phenomena (Walstra, 2003), this effect being more intense in HH treated samples. When thermal treatments were applied to homogenised samples (treatments MF3LH, MF3HH), the thermal effects seem to be mitigated probably due to the greater stability of the smaller fat globules which are less sensitive to the flocculation and coalescence phenomena than the big ones of non-homogenised samples. Nevertheless, the wider distribution of particles in sample MF3HH is remarkable. This agrees with a greater progress of the aggregation phenomena in this case, in comparison with MF3LH samples, treated a lower temperature.

As far as ζ-potential values are concerned, particles showed negative charge as can be observed in Table 2. This can be explained taking into account the isoelectric point (pI) of the major proteins of almonds and hazelnuts (5 and 4.5, respectively) (Albillos, Menhart & Fu, 2009; Ma, Zhang, Qi & Zheng, 2008). Thus, at the pH of the beverages (above their PI), proteins exhibited negative

charge. Gallier, Gordon & Singh (2012) reported values of ζ-potential of -30 mV for particles of almond milk, which is higher than those found in this work. This can be due to a lower adsorption degree of proteins on the surface of oil droplets in this case, which reduces their effective surface charge.

In almond milks, the homogenisation process led to a higher negative charge of the dispersed particles ($p < 0.05$), which indicates that a re-arrangement of components occurs in the dispersed phase. The interfacial adsorption of proteins with their ionisable groups could be promoted by high pressure, thus increasing the surface charge of the dispersed oil droplets and so, the overall net charge and the ζ-potential value. Changes in the protein conformation could also be promoted, increasing the ratio of the surface ionisable groups and so the water affinity of proteins. In general, this treatment led to smaller particles with a higher electrical charge, in comparison to untreated samples. On the contrary, heat treatments did not significantly affect the ζ-potential of dispersed particles ($p > 0.05$) with respect to the untreated samples.

In hazelnut milks, all treatments led to a slight decrease ($p < 0.05$) in the charge of the particles, especially thermal treatments. The denaturation of proteins and further aggregation processes could explain the lower particle electrical charge in the treated products.

3.4 Rheological behaviour.

Table 3 shows the rheological parameters (K, n and σ_y) of both almond and hazelnut milks submitted to different treatments. Apparent viscosity (η) at a shear rate of 100 s^{-1} and the non-linear correlation coefficient (R^2) of the fitted model are also shown. Rheological parameters of HH samples were anomalous due to the fast phase separation during the rheological measurements and have not been reported.

In almond milks, untreated samples showed a slight shear thickening behaviour (n=1.18) which is typical in dispersions/emulsions when the ortokinetic flocculation occurs associated with the shear rate. Nevertheless, homogenised almond milks exhibited almost Newtonian behaviour (n≈1) probably due to the lowest sensitivity of the smaller particles to shear flocculation.

277 Homogenisation treatment did not cause significant changes ($p > 0.05$) in the consistency index,
278 or apparent viscosity of samples. However, heat treated samples behaved as a Bingham plastic
279 fluid, the MF3HH samples showing the highest yield stress. Moreover, heated samples (submitted
280 or not to homogenisation processes) showed a significant increase ($p < 0.05$) in the apparent
281 viscosity. This behaviour indicates that a weak gelation effect was produced, due to the thermal
282 treatment probably associated with the protein denaturation and subsequent cluster formation.
283 Cluster formations have also been observed in heated and homogenised cow milk (Walstra, 2003).
284 The soluble fibre fraction could also contribute to the increase in the product viscosity by the
285 extension and hydration of the biopolymer chains induced by the temperature.
286 As concerns hazelnut milks, untreated samples showed Newtonian behaviour. Nevertheless, the
287 homogenisation process significantly affected the product rheological behaviour, leading to shear
288 thinning behaviour ($n < 1$). Homogenised samples showed greater values of the consistency index
289 and apparent viscosity than the untreated samples. These results reveal that some changes in the
290 component conformation have been induced by high pressure which makes the system more flow
291 resistant and sensitive to flow orientation. These components could be proteins which can be
292 unfolded by pressure effect. Homogenised samples submitted to thermal treatments also exhibited
293 greater viscosity, as commented on above for almond products, but they showed yield stress only
294 when the highest temperature was applied. However, the LH treatments did not induce significant
295 changes in rheological behaviour as compared to non- treated samples, which indicates that no
296 significant changes in the component arrangement were induced by thermal treatment. This could
297 indicate that hazelnut proteins are more sensitive to pressure than almond proteins and less
298 sensitive to temperature. Their unfolding and denaturation was caused by the high pressure effect
299 but not by the low temperature treatment. Thermal treatments of homogenised samples gave rise
300 to an increase in the sample viscosity which may associate to protein aggregation. Nevertheless,
301 the weak gel formation, reflected in a yield stress value, is only evidenced when the highest
302 temperature was applied. This can be due to the low protein content of hazelnut, as compared to
303 almond. With low protein content, gel formation requires a more intense thermal treatment to
304 induce enough chain aggregation for the network formation. Likewise, it is remarkable that

viscosity of thermally treated almond products was higher than that of hazelnut milks, coherent with their higher protein content and the subsequent greater density of aggregates.

3.5 Protein denaturation.

Figure 2 shows typical thermograms obtained by using DSC for almond milk. As can be observed, homogenisation treatments did not cause protein denaturation, since denaturation endotherms appeared with similar area and temperature peak as in untreated samples. Cruz Capellas, Hernández, Trujillo, Guamis & Ferragut (2007) reported that denaturation of proteins occurs when applying pressures around 200 MPa (partial denaturation) or higher (total denaturation), but it depends on the protein nature. In the case of soy-protein emulsions, protein denaturation phenomena may appear at pressures above 150 MPa (Floury and Desrumaux, 2002). No differences ($p > 0.05$) were found between untreated and homogenised samples which showed endothermic peaks at around 98.0 ± 0.4 ºC, with a total enthalpy of around 10 ± 1 J/g protein. This denaturation temperature is relatively high, in agreement with the reported thermo-stability of the major almond protein (amandin), which represents up to 70 g/100g of the total soluble proteins (Sathe, Wolf, Roux, Teuber, Venkatachalam & Sze-Tao, 2002). On the contrary, both heat treatments provoked total protein denaturation as no endothermic peak was observed in the heated samples.

In hazelnut samples, in no case were endothermic peaks observed. Since in non-treated samples protein will be in the native state, the non-detection of denaturation endotherm by DSC could be due to the low ratio of proteins of these samples and to the low denaturation enthalpy of these proteins. Therefore, the effect of pressure or temperature on hazelnut protein conformation has not been probed by this technique, although rheological behaviour of the different treated samples suggests changes in the protein conformation due to high pressure.

3.6. Sample microstructure

Figure 3 shows the CLSM images of almond milk untreated and submitted to different treatments. Oil droplets and protein bodies dispersed in the serum phase are clearly distinguished in Figures

3 A and B for the untreated milk. A certain degree of flocculation in protein bodies can be observed, which can be due to their hydrophobic character. Most of the almond proteins belong to the oleosin family with low-molecular-weight and poor water solubility, due to a long highly hydrophobic domain of about 70 amino acid residues (Beisson, Ferté, Voultoury & Arondel, 2001). In some cases, protein bodies appear adsorbed on the oil droplet surface, forming bridges between them. The low affinity of proteins by the aqueous medium contributes to the low stability of the obtained emulsions where steric stability did not occur due to the poor solvent effect (McClements, 2005).

In LH treated samples (Figures 3 C and D), protein aggregates can be observed to be spread over big areas in the sample, whereas isolated protein bodies are not frequently present. In many cases, protein aggregates include oil droplets. This observation is coherent with described rheological behaviour where LH treatment gives rise to a plastic fluid with yield stress and higher apparent viscosity, which may be due to the formation of a weak gel, associated with a three-dimensional network of aggregated particles at relatively low concentration.

The effect of the homogenisation pressure on the product microstructure can be observed in Figures 3 E and F. The great reduction in the particle sizes, detected by the light scattering diffraction, can be observed. Nevertheless, most of the small particles are flocculated through protein bridges, which explain the low stability of the emulsion despite the small particle sizes. The poor stabilizing properties of the protein, associated to its high hydrophobicity and low water affinity, is the cause of the flocculation process and subsequent phase separation, as commented on below.

Combined MF3LH treatment provoked the formation of big oil droplet-protein aggregates which appear embedded in a continuous protein matrix. This new structure is the result of the combined effect of high pressure and temperature. HPH reduces droplet size and promotes partial protein solubilisation and thermal effect provokes soluble protein denaturation and aggregation, as in a gel, thus greatly modifying the product microstructure. Denaturation of the soluble protein gives rise to the formation of a three-dimensional network (evidenced by the yield stress exhibited by

these samples in rheological analyses) which entraps big aggregates of the small protein-lipid particles.

So, microstructural observations of almond milk samples reveal that almond protein did not show good stabilising properties for oil droplets, probably due to their hydrophobic character that negatively affected the steric stabilization effect expected for adsorbed proteins in a good solvent. These proteins were thermal sensitive and denatured during thermal treatments, thus inducing the formation of big aggregates which entrap both oil and protein bodies. In the combined treatments, the big aggregates seemed to be embedded in a continuous protein network (weak gel) which could contribute to stabilise the emulsion.

Although the microstructure of hazelnut milks was not analysed, similar behaviour could be expected, taking into account the similar nature of product.

3.7 Sample colour.

Lightness, hue and chrome values obtained in both milks are shown in Table 4, together with the whiteness index and the colour difference between untreated and treated samples (ΔE).

Almond milks appeared whiter and with greater lightness than hazelnut milk due to the natural brownish colour of hazelnut.

Both milks showed the same trends in the colour parameters when treated, the changes being more intense in the whiter almond milks. Lightness and whiteness index significantly increased ($p < 0.05$) due to the homogenisation process, as the number and size of particles contribute to the light reflection. In heated samples and in samples submitted to the combined treatments, these parameters decreased ($p < 0.05$) in agreement with the observed increase in particle size. On the other hand, hue and chrome significantly decrease ($p < 0.05$) giving rise to a less saturated reddish colour in all treated samples, regardless of the treatment applied. This was more marked when using the highest temperature, to some extent probably due to the occurrence of Maillard reactions.

386 Total colour difference values (ΔE) were low, taking into account that values lower than 3 units
387 cannot be easily detected by the human eye (Francis, 1983). So, only samples submitted to the
388 most intense heat treatment (HH) showed values considered as detectable.
389

390 **3.8 Physical stability over storage time**.

391 All samples, except those MF3 submitted to LH (MF3LH treatment), showed phase separation
392 after 1 storage day and no notable differences in the height of each of the separate phases were
393 observed throughout time. Figure 4 shows the appearance of the samples at 28 storage days where
394 the samples submitted to MF3LH treatments were the only ones which showed colloidal stability,
395 for both almond and hazelnut milks. The combined effect of homogenisation and thermal
396 treatment seems to promote a weak gel formation, mainly associated with the protein
397 solubilisation and subsequent denaturation during thermal treatment, which contributed to
398 stabilise the particle dispersion, thus avoiding phase separation during the product storage.
399 The observed behaviour indicates that nut proteins did not show adequate emulsifying properties
400 to stabilize fat globules by interfacial protein adsorption, as commented on above, even with the
401 particle size reduction induced by HPH. Only when homogenised samples were submitted to
402 thermal treatment and the proteins were denatured, can these contribute to stabilise the emulsions,
403 mainly due to a viscous effect. Capacity of nut proteins to stabilise colloidal systems has not been
404 previously reported.
405 Phase separation occurs in a coherent way with the microstructural observations. A thin cream
406 phase can be seen in almond milks, corresponding to an oil-rich phase, whereas thick sediment
407 corresponding to the contraction of dispersed phase, entrapping protein-oil droplet aggregates,
408 can also be observed. The ratio oil-protein in the clusters determines their mean density. In almond
409 milk, the density of these clusters is higher than that of the serum phase due to the high protein-
410 lipid ratio (0.35) and so, they sediment in the glass tube. In hazelnut milks, the protein-lipid ratio
411 is much lower (0.16) and the proportion of both components in the protein-lipid aggregates is
412 critical to determine the migration direction (up or down) in the tube. In some samples, creaming

was predominantly observed, whereas in others sedimentation occurs. Nevertheless, in all cases, the progressive aggregation of the protein-oil clusters will be responsible for this behaviour, regardless of the lipid-protein ratio present in the clusters. This progressive aggregation process was inhibited in MF3LH samples due to the viscous effect and yield stress induced by combined thermal and homogenisation treatments, probably due to the lower size of the lipid-protein aggregates. In MF3HH samples, with bigger oil-protein clusters, the viscous stabilization is not enough to control the effect of gravitational forces.

4. CONCLUSIONS

Physical properties and stability of almond and hazelnut milks were affected by both homogenisation pressure and heat treatments. The homogenisation process greatly reduced particle size but the resulting emulsions were not stable and phase separation occurred in relatively few hours. Microstructural observations reveal that proteins did not contribute to stabilize the emulsions due to their hydrophobic character which did not favour the steric stabilization in a good solvent. So, flocculation of protein bodies and oil droplets occurred, giving rise to the formation of oil-protein clusters. These clusters suffer progressive aggregation promoting phase separation process. Thermal treatment at the lowest temperature provoked protein denaturation, thus enhancing the aggregation process. Nevertheless, when samples were previously high pressure homogenised, denaturation and aggregation of the serum proteins seem to contribute to the formation of a three-dimensional network (reflected in the sample yield stress), which exerts a stabilising viscous effect that inhibited phase separation during the product storage. So, the combination of low heat treatment with high homogenisation pressure greatly improved the physical stability and appearance of almond and hazelnut milks.

Acknowledgements

The authors acknowledge the financial support provided by Universitat Politècnica de Valencia (Valencia, Spain) for the project (PAID 05-11-2740). Author N. Bernat appreciates the

441 Consellería de Eduación of Valencia (Spain) for a FPI Grant (Programa VALi+d para
442 investigadores en formación. ACIF/2011).
443

444 **5. REFERENCES**

445

446 Albillos, S.M., Menhart, N. & Fu, T.J. (2009). Structural Stability of Amandin, a Major Allergen from
447 Almond (*Prunus dulcis*) and Its Acidic and Basic Polypeptides. *Journal of Agricultural and Food*
448 *Chemistry*, *57*, 4698-4705.
449 Beisson, F., Ferté, N., Voultoury, R., & Arondel, V. (2001). Large scale purification of an almond oleosin
450 using organic solvent procedure. Plant Physiology and Biochemistry, 39 (7-8), 623-630.
451 Cruz, N., Capellas, M., Hernández, M., Trujillo, A.J., Guamis, B., & Ferragut, V. (2007). Ultra high
452 pressure homogenization of soymilk: Microbiological, physicochemical and microestructural
453 characteristics. *Food Research International, 40 (6)*, 725-732.
454 Desrumaux, A., & Marcand, J. (2002). Formation of sunflower oil emulsion stabilized by whey protein
455 with high-pressure homogenization (up to 350 MPa): effect of pressure on emulsion characteristics.
456 *International Journal of Food Science and Technology*, *37*, 263–269.
457 Diels, A. M. J., Callewaert, L., Wuytack, E. Y., Masschalk, B., & Michiels, W. (2005). Inactivation of
458 Escherichia coli by high-pressure homogenisation is influenced by fluid viscosity but no by water activity
459 and product composition. *International Journal of Food Microbiology*, *101*, 281–291.
460 Eroski Foundation. (2007). "Expertos españoles recomiendan tomar leche de almendras en invierno". In
461 *Eroski consumer* (online). <<http://www.consumer.es/seguridad-alimentaria/2007/01/26/26533.php>>
462 Fiocchi, A., Brozek, J., Schunemann, H., Bahna, S., von Berg, A., Beyer, K., Bozzola, M. Bradsher, J.,
463 Compalati, E., Ebisawa, M., Guzman, M. A., Li, H., Heine, R., Keith, P., Lack, G., Landi, M., Martelli, A.,
464 Rancé, F., Sampson, H., Stein, A., Terracciano, L., & Vieths, S. (2010). World Allergy Organization
465 (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines. A review.
466 *WAO Journal*, 57-161.
467 Floury, J., Desrumaux, A., & Lardières, J. (2000). Effect of high-pressure homogenization on droplet size
468 distributions and rheological properties of model oil-in-water emulsions. *Innovative Food Science and*
469 *Emerging Technologies, 1*, 127-134.

Francis, F.J. (1983). Colorimetry of foods. In Pelef, M., Baglet, E.B. (Eds.), *Physical Properties of Foods (*pp 105-124*)*. AVI Publishing Westport, CT.

Fraser, G. E., Bennett, H. W., Jaceldo, K. B.,& Sabaté, J. (2002). Effect on body weight of a free 76 kilojoules daily supplement of almonds for six months. *Journal American Collection Nutrition*, *21*, 275-283.

Gallier, S., Gordon, K. C., & Singh, H. (2012). Chemical and structural characterisation of almond oil bodies and bovine milk fat globules. *Food Chemistry*, *132 (4)*, 1996-2006.

Horwitz, W. (2000). *Official Methods of Analysis of AOAC International*. 17th edition. Association of Official Analytical Chemists (Eds.). Gaithersburg, MD; USA.

Jenkins, D., Kendell, C., Marchie, A., Josse, A.R., Nguyen, T.H., & Faulkner, D.A. (2008). Almonds Reduce Biomarkers of Lipid Peroxidation in Older Hyperlipidemic Subjects. *Journal of Nutrition*, *138*, 908-913.

Kris-Etherton, P. M., Hu, F. B., Rose, E., & Sabaté, J. (2008). The role of tree nuts and peanuts in the prevention of coronary heart disease: Multiple potential mechanisms. *Journal of Nutrition*, *138*, 1746S-1751S.

Li, Y. Q., Chen Q., Liu X. H., & Chen Z. X. (2008). Inactivation of soybean lipoxigenase in soymilk by pulsed electric fields. *Food Chemistry*, *109 (6)*, 408-414.

Luengo, M. (2009). *La almendra y otros frutos secos: Castaña, pistacho, piñón, nuez*. Oceano AMBAR (Eds). Barcelona, Spain.

Ma, Y., Zhang, L. N., Qi F. Y., & Zheng Y. (2008). Study on extraction of hazelnut protein and its functional properties. *Journal of Food Science*, *29 (8)*, 318-322.

Mateos, M. (2007). *La leche de almendras: complemento alimenticio para el invierno*. Universidad CEU Cardenal Herrera, Valencia [online] <<www.universia.es/html_estatico/portada/actualidad/noticia_actualidad/param/noticia/jbhce.html>>

Matissek, R., Schnepel, F. M., & Steiner, G. (1998). Determinación de azúcares totales: método reductométrico de Luff-Schoorl. In *Análisis de los Alimentos: Fundamentos, Métodos y Aplicaciones* (pp. 123-132). Acribia S.A. publishings, Zaragoza, Spain.

McClements, D. J. (2005). *Food emulsions, principles, practice, and techniques*. LLC: CRC Press, Boca Raton, Florida.

Pereda, J., Ferragut, V., Guamis, B., & Trujillo, A. (2006). Effect of ultrahigh-pressure homogenisation on natural-occurring micro-organisms in bovine milk. *Milchwissenschaft, 61*, 245–248.

Pereda, J., Ferragut, V., Quevedo, J. M., Guamis, B., & Trujillo, A.J. (2009). Heat damage evaluation in ultra-high pressure homogenised milk. *Food Hydrocolloids, 23,* 1974-1979.

Sathe, S. K., Wolf, E. J., Roux K. H., Teuber, S. S., Venkatachalam, M., & Sze-Tao K. W. (2002). Biochemical characterization of amandin, the major storage protein in almond (*Prunus dulcis L.*). *Journal of Agricultural & Food Chemistry, 50 (15),* 4333-4341.

Saura, F., Cañellas, J., & Soler, L. (1988) In. *La Almendra: composición, variedades, desarrollo y maduración*. Instituto Nacional de Investigaciones Agrarias, Madrid.

Smiddy, M. A., Martin, J. E., Huppertz, T., & Kelly, A. L. (2013). Microbial shelf-life of high-pressure-homogenised milk. *International Dairy Journal*. (*in press*).

Tey, S. L., Brown, R. C., Chislholm, A. W., Delahunty, C. M., Gray, A. R., Williams, S. M. (2011). Hazelnuts on blood lipids and α-tocopherol concentrations in midly hypercholesterolemic individuals. *European Journal of Clinical Nutrition, 65*, 117-124.

Walstra, P. (2003). *Physical chemistry of foods*. New York: Marcel Dekker.

Walstra, P., Wouters, J. T. M., & Geurts, T. J. (2006) In CRC Press (Eds). *Dairy Science and Technology* (pp 207-296). Taylor and Francis Group, Boca Raton, England.

517 **Table 1.** Chemical composition (g/100 g product) of almond)and hazelnut nuts and derivative milks used

518 in the study. Mean values ± standard deviation (n = 3).

Composition (g/100 g)	Almond nut	Almond milk	Hazelnut	Hazelnut milk
Moisture	3.06 ± 0.05	93.4 ± 0.5	3 ± 1	94.1± 0.5
Lipid	55.77 ± 0.29	3.96 ± 0.2	62.4 ± 0.4	4.02 ± 0.00
Ashes	3.86 ± 0.06	0.325 ± 0.012	3.14 ± 0.11	0.20 ± 0.04
Total sugars	4.9 ± 0.4	0.030 ± 0.002	4.13 ± 0.25	0.03 ± 0.00
Protein	25.55 ± 0.12	1.37 ± 0.03	13.43 ± 0.12	0.65 ± 0.05
Fibre	6.82	0.58	14.28	0.40
Dry matter	96.94 ± 0.05	6.64 ±0.5	97 ± 1	5.3 ± 0.4

519

520

521 **Table 2.** Particle size parameters (volume mean diameter ($D_{4,3}$) and surface mean diameter ($D_{3,2}$)) and

522 ζ-Potential values of untreated and treated samples. Mean values ± standard deviation (n = 4).

Treatment	Almond milk		
	$D_{4,3}$ (μm)	**$D_{3,2}$ (μm)**	**ζ-Potential (mV)**
Untreated	92.9 ±1.9 ^{ab}	5.2 ± 0.2 ^{ab}	-17.0 ± 1.4 ^{a}
MF1	35 ± 20 ^{cd}	5.7 ± 0.6 ^{a}	-21.2 ± 1.3 ^{b}
MF2	15.9 ± 1.7 ^{ce}	4.8 ± 0.3 ^{ab}	-19.41 ± 1.06 ^{c}
MF3	14 ± 7 ^{e}	3.91 ± 0.14 ^{b}	-19.16 ± 1.43 ^{c}
LH	78 ± 2 ^{b}	21.4 ± 0.6 ^{e}	-15.99 ± 1.18 ^{a}
HH	158 ± 20 ^{f}	24.5 ± 1.0 ^{c}	-17.01 ± 2.12 ^{a}
MF3LH	23 ± 3 ^{cde}	8.7 ± 0.3 ^{f}	-16.7 ± 1.3 ^{a}
MF3HH	40 ± 4 ^{d}	13.0 ± 1.3 ^{d}	-15.0 ± 1.0 ^{d}
Treatment	**Hazelnut milk**		
	$D_{4,3}$ (μm)	**$D_{3,2}$ (μm)**	**ζ-Potential (mV)**
Untreated	101 ± 13 ^{a}	6.5 ± 0.5 ^{abc}	-23.8 ± 1.2 ^{a}
MF1	39 ± 2 ^{b}	7.94 ± 0.14 ^{b}	-21.6 ± 0.8 ^{bc}
MF2	26 ± 3 ^{c}	6.94 ± 0.09 ^{bc}	-21.2 ± 0.5 ^{c}
MF3	17.7 ± 0.9 ^{c}	5.6 ± 0.5 ^{cd}	-23.6 ± 0.8 ^{a}
LH	113 ± 4 ^{a}	6.0 ± 0.3 ^{cd}	-18.2 ± 1.2 ^{d}
HH	147 ± 15 ^{d}	17.9 ± 0.8 ^{e}	-22 ± 2 ^{bc}
MF3LH	15.7 ± 0.2 ^{c}	5.88 ± 0.06 ^{cd}	-22.4 ± 1.2 ^{bd}
MF3HH	62 ± 15 ^{e}	15 ± 3 ^{f}	-21 ± 2 ^{c}

523 $^{a, b, c, d}$ Different letters in same column indicates significant differences between treatments in 95% of confidence

524 MF = homogenisation at 62 (1), 103 and 172 (3) MPa, HH = high temperature heating; LH = low temperature heating

525

526 **Table 3.** Mean values and standard deviation of consistency index (K), flow behaviour index (n) and yield
527 stress (σ_y) obtained from fitting experimental data to Herschel-Bulkey model (non-linear correlation
528 coefficient R^2 is included). Apparent viscosity (η) was calculated at shear rate of 100 s^{-1}. (n = 3 in duplicate).

Almond milk					
Sample	**K (x10^3) (Pa s^n)**	**n**	**σ_y (Pa)**	**R^2**	**η_{100} (x10^3) (Pa·s)**
Untreated	0.62·± 0.09^a	1.18 ± 0.03^a	0 ^{a}	0.990	1.44· ± 0.01^a
MF1	1.6 ± 0.2^a	1.039± 0.006^{abc}	0 ^{a}	0.999	1.9· ± 0.2^a
MF2	2.25 ± 1.05^a	0.925 ± 0.001^b	0 ^{a}	0.980	1.6· ± 0.7^a
MF3	1.55 ± 0.03^a	1.026 ± 0.006^{bc}	0 ^{a}	0.998	1.75 ± 0.02^a
MF3HH	15 ± 10^b	0.97 ± 0.12^{bc}	0.875±0.007^b	0.990	12 ± 2^b
LH	4 ± 2^a	1.09 ± 0.09^{ac}	0.20 ± 0.04^c	0.997	5.5· ± 0.7^c
MF3LH	4.7 ± 0.5^a	1.084 ± 0.009^{ac}	0.44 ± 0.04^d	0.990	6.9 ± 0.5^c
Hazelnut milk					
Sample	**K (x10^3) (Pa s^n)**	**n**	**σ_y (Pa)**	**R^2**	**η_{100} (x10^3) (Pa·s)**
Untreated	1.1·± 0.2 ^{a}	1.08 ± 0.02 ^{a}	0^a	0.990	1.61· ± 0.03 ^{ab}
MF1	4.7 ± 0.7 ^{ab}	0.84 ± 0.02 ^{b}	0^a	0.999	2.21· ± 0.09 ^{bc}
MF2	8 ± 5 ^{b}	0.79 ± 0.08 ^{b}	0^a	0.980	3.0· ± 0.7 ^{de}
MF3	7.9 ± 0. 3 ^{b}	0.769 ± 0.005 ^{b}	0^a	0.998	2.72· ± 0.05 ^{cd}
MF3HH	2.59 ± 0 ^{ab}	1.08 ± 0.00 ^{a}	0.2 ± 0.0 ^{b}	0.990	3.8·± 0.0 ^{e}
LH	0.91 ± 0.05^a	1.085 ± 0.007 ^{a}	0 ^{a}	0.980	1.35 ± 0.03 ^{a}
MF3LH	8.0 ± 0.2 ^{b}	0.796 ± 0.005 ^{b}	0^a	0.990	3.121· ± 0.002 ^{de}

529 **$^{a, b, c, d}$** Different letters in same column indicates significant differences between treatments in 95% of confidence
530 MF = homogenisation at 62 (1), 103 (2) and 172 (3) MPa, HH = high temperature; LH = low temperature
531

532 **Table 4** Mean values ± and standard deviation of Lightness (L^*), hue (h_{ab*}), chrome (C^*) and whiteness
533 index (WI) of almond and hazelnut milks and colour difference between untreated and treated samples. (n
534 =3 in duplicate).

Almond milk	**L^***	**C^***	**$h_{ab}{}^*$**	**ΔE**	**W.I.**
Untreated	86.1 ± 0.2^{a}	7.15 ± 0.15^{a}	96.1 ± 0.6^{a}	-	84.3 ± 0.2^{a}
MF1	87.4 ± 0.1^{c}	6.66 ± 0.21^{b}	95 ± 1^{a}	1.9 ± 0.2^{b}	86.1 ± 0.2^{c}
MF2	90.5 ± 0.2^{e}	5.80 ± 0.05^{c}	96.6 ± 0.5^{b}	4.81 ± 0.12^{c}	89.1 ± 0.1^{e}
MF3	88.5 ± 0.1^{d}	5.22 ± 0.02^{d}	94.7 ± 0.2^{b}	2.93 ± 0.08^{d}	87.2 ± 0.1^{d}
HH	78.8 ± 0.5^{f}	5.48 ± 0.16^{e}	94.6 ± 0.5^{b}	7.2 ± 0.4^{e}	77.5 ± 0.4^{f}
MF3HH	86.8 ± 0.1^{b}	7.67 ± 0.08^{f}	95.2 ± 0.3^{c}	1.54 ± 0.11^{f}	85.5 ± 0.16^{b}
LH	$86.0 \pm 9 \cdot 10^{-3a}$	6.00 ± 0.02^{g}	90.3 ± 0.2^{a}	0.43 ± 0.02^{a}	84.3 ± 0.15^{a}
MF3LH	87.8 ± 0.1^{c}	6.73 ± 0.03^{bf}	96.6 ± 0.3^{a}	2.23 ± 0.01^{b}	86.5 ± 0.1^{c}
Hazelnut milk	**L^***	**C^***	**h_{ab*}**	**ΔE**	**W.I.**
Untreated	83.4 ± 0.4^{a}	9.9 ± 0.5^{a}	90.2 ± 1.2^{a}	-	80.6 ± 0.6^{a}
MF1	83.0 ± 0.2^{ab}	9.33 ± 0.11^{b}	85.9 ± 0.7^{bc}	1.01 ± 0.09^{ab}	80.6 ± 0.2^{ab}
MF2	83.9 ± 0.2^{cd}	9.4 ± 0.4^{b}	86.1 ± 1.4^{bc}	1.11 ± 0.13^{a}	81.4 ± 0.4^{c}
MF3	84.38 ± 0.14^{c}	8.24 ± 0.12^{c}	86.2 ± 0.8^{bc}	2.04 ± 0.02^{a}	82.34 ± 0.07^{d}
HH	77.1 ± 0.3^{e}	11.5 ± 0.3^{d}	89.4 ± 0.5^{a}	6.5 ± 0.4^{bc}	74.3 ± 0.4^{e}
MF3HH	78.7 ± 0.8^{f}	10.0 ± 0.2^{ae}	82.2 ± 0.9^{d}	4.9 ± 0.7^{d}	76.4 ± 0.7^{f}
LH	79.6 ± 0.3^{g}	10.5 ± 0.4^{e}	86.9 ± 0.3^{b}	3.9 ± 0.3^{cd}	77.1 ± 0.4^{g}
MF3LH	83.88 ± 0.07^{d}	7.90 ± 0.03^{b}	85.29 ± 0.03^{c}	2.19 ± 0.02^{d}	82.05 ± 0.05^{c}

535 **[a, b, c, d]** Different letters in same column indicates significant differences between treatments in 95% of
536 confidence
537 MF = homogenisation at 62 (1), 103 (2) and 172 (3) MPa, HH = high temperature; LH = low temperature
538

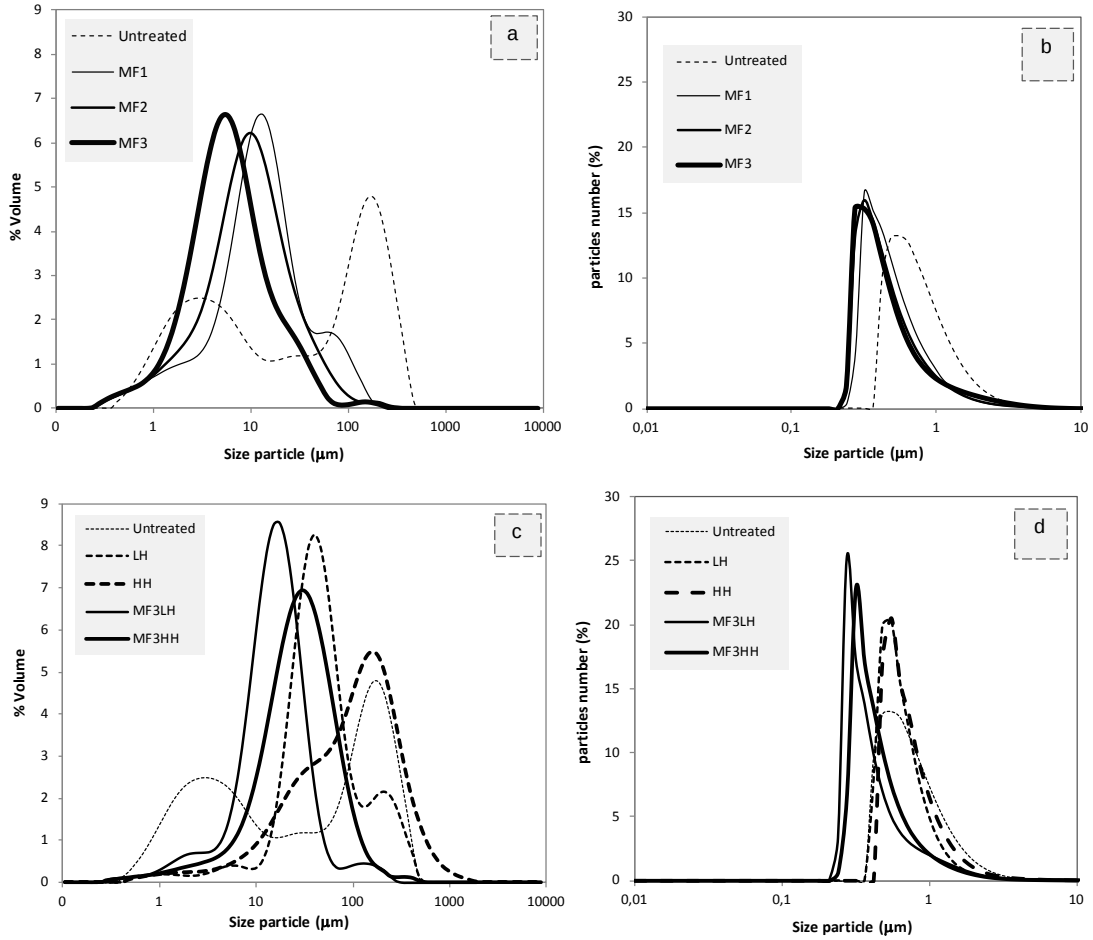


Figure 1. Typical particle size distribution curves for the untreated and treated almond milks in terms of percentage of volume (a,c) and percentage of number of particles (b,d). (MF = homogenised samples; HH = High Heat treated samples; LH = Low Heat treated samples; MF3HH and MF3LH= samples homogenised at 172 MPa and high and low heat treated, respectively).

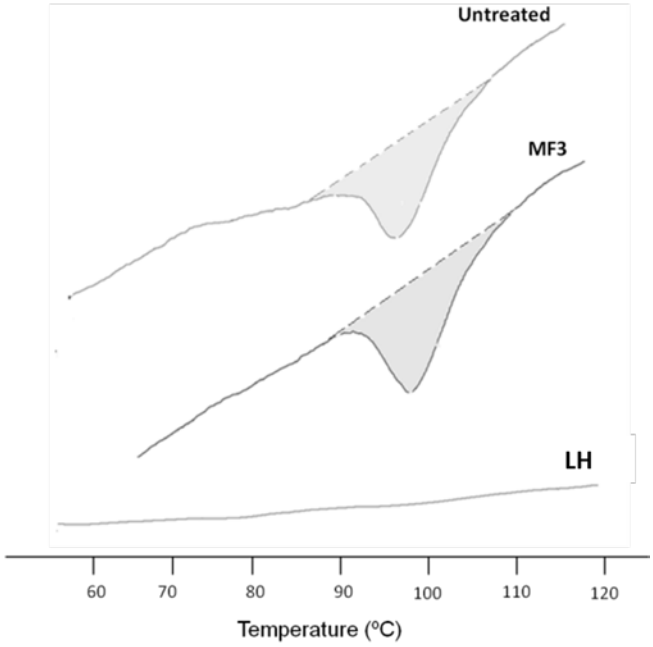


Figure 2. Typical DSC thermograms obtained for almond samples submitted to different treatments. (MF3 = homogenised samples at 172 MPa; LH = Low Heat treated samples).

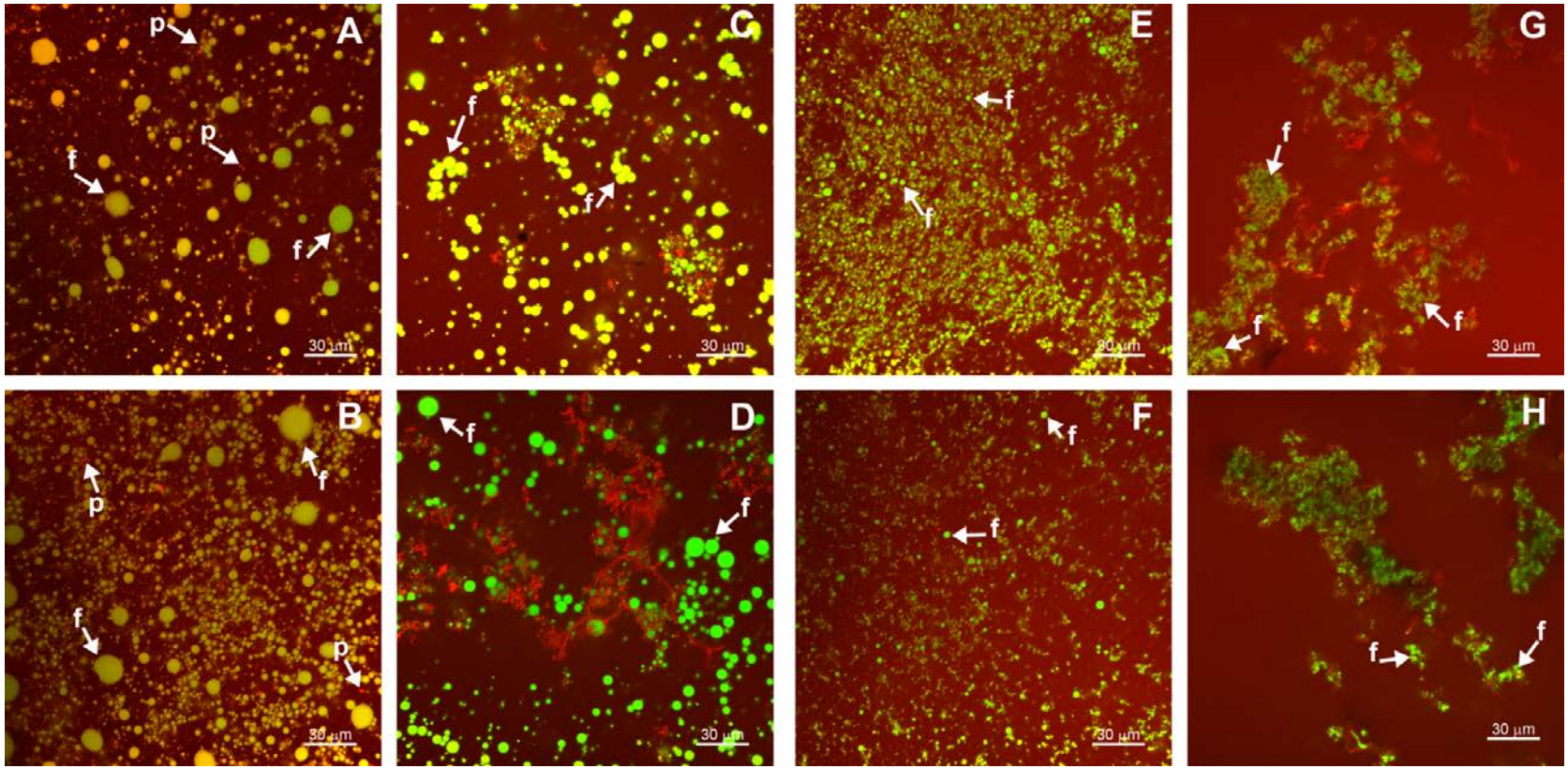
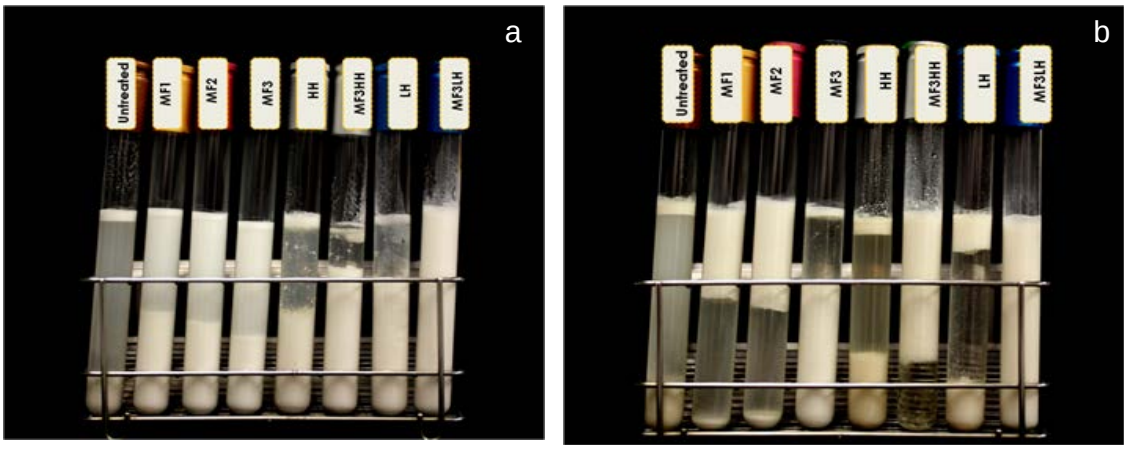


Figure 3. Confocal laser scanning microscopy (CLSM) images of almond milks stained with Rodamine B and Nile Red (proteins and carbohydrates in red, fat in green).A and B: untreated product, C and D: Low Heat treated milks, E and F: MF3 treated milks, and G and H: combined MF3 and Low Heat treated milks. (pa: protein aggregates; o: oil bodies; opc: oil-protein clusters)



553
554 **Figure 4.** Phase separation observed in almond (a) and hazelnut (b) milks submitted to different treatments
555 after 28 storage days at 4ºC. (MF = homogenised samples; HH = High Heat treated samples; LH = Low
556 Heat treated samples; MF3HH and MF3LH= samples homogenised at 172 MPa and high and low heat
557 treated, respectively).

558

559