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Additional Information

Influence of high pressure homogenization (HPH) on the structural stability of an egg/dairy emulsion

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A B S T R A C T

High pressure homogenization (HPH) is a novel non-thermal preservation technology, which can improve the microbiological quality of products without affecting their stability. The main objective of this paper is to study the influence of different HPH treatments on the structure of a sauce (an egg/dairy emulsion), in order to obtain the higher physicochemical stability. The oil-in-water emulsion was stable up to 100 MPa with the oil droplets surrounded by several layers of natural emulsifiers. Critical pressures, between 150 and 250 MPa, produced a destabilization of the emulsion thus causing a separation of phases. A coalescence phenomenon progressively occurred when pressure increased. This phenomenon was due to the loss of the natural emulsifier barrier. Changes on the electrophoretic pattern were also observed at high pressure levels, showing an insolubilization of proteins. Lipid fraction was observed to be chemically stable after the HPH treatment.

1. Introduction

High pressure homogenization (HPH) is one of the most promising alternatives to traditional thermal treatment of food preservation and diversification (Burns, 2008). This technology is based on the same principle as conventional homogenization, but working at significantly higher pressures (Serra et al., 2009a,b). In HPH the fluid is forced to pass through a narrow gap of the valve, after which it is subjected to an ultrarapid depression (Guerzoni et al., 1999a,b). Then, fluids are subjected to a wide range of forces, such as turbulence, shear, cavitation and large temperature increases (Hayes and Kelly, 2003; Floury et al., 2000).

Homogenizers were developed for the stabilization of food and dairy emulsions. Over the years, the homogenization technology has evolved; the demand for longer shelf-life and products with better stability has led to new developments, based on a very high pressure capacity as well as on a new reaction chamber design. The development of this high pressure technology has influenced research work on emulsions stability, but it has also been observed that such high pressures can affect not only fat globules but also other food constituents such as macromolecules or colloids, and then their functional properties (Paquin, 1999).

The effects of high pressure on the gelation of proteins (Rastogi et al., 1994) and on the creation or modification of functional properties of proteins (Rastogi et al., 1994; San Martin et al., 2002) have been investigated in the last years. In this context, the functional properties of proteins from different origin treated by high pressure homogenization (HPH) were analyzed resulting in an increased foam ability of soybean and egg white proteins and an increased water binding capacity of faba bean protein (Heinzelmann et al., 1994).

Regarding the structure of high pressure treated foods, emulsions and dairy products are the most studied ones. Floury et al. (2002) found that HPH caused denaturation of proteins and reduced droplet sizes in emulsions; they suggested that the gel-like network structure of some emulsions was caused by hydrophobic interactions between proteins. Significant modifications in the structure of emulsions regarding droplet size distribution were observed as the pressure was modified (Desrumaux and Marcand, 2002). Rosenberg and Lee (1993) observed by scanning electron microscopy the microstructure of emulsions consisting of whey protein and anhydrous milk fat. The emulsions prepared under HPH exhibited a bimodal particle-size distribution with clustering of the protein coated droplets. The effects of HPH on the microstructure of oil-in-water emulsions were evaluated by Roesch and Corredig (2003); heating before homogenization had no significant impact on microstructure. SEM analysis of goat cheeses revealed that cheeses made from HPH-treated milk had a more homogeneous microstructure than those made from untreated milk or pasteurized milk (Guerzoni et al., 1999a,b).

However research on the effect of HPH on the structure of emulsions is needed in order to optimize the conditions of the treatments preserving their physicochemical stability.

The main objective of this study was to investigate the influence of different HPH treatments on the structure and physicochemical

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stability of a sauce (an egg/dairy emulsion). The distribution of the main chemical components of the egg/dairy emulsion affected by the HPH treatments was studied by microscopic techniques (TEM and CLSM). Moreover, the effect of these treatments on the soluble protein and lipid fractions was also analyzed. The results of this study could contribute to understand how the HPH treatment affects the functionality of the chemical components of the sauce, some of them acting as possible stabilizers in the system.

2. Materials and methods

2.1. Sauce-making process

In this study, sauce was prepared with sunflower oil (59%), full-fat yoghurt (13%), whole eggs (10%), water (17%) and salt (1%). The whole eggs and the salt were mixed at 700 rpm; then the sunflower oil was added slowly during mixing, and finally the yoghurt and the water were added. pH was adjusted to pH 5 with citric acid (5%).

2.2. High pressure homogenization (HPH)

The sauce underwent the following HPH treatments with industrial equipment: 0 MPa (control sauce), 70, 100, 150 and 250 MPa. All the samples were analyzed just after treatment and after storing them for a week at 4 °C.

A continuous high pressure homogeniser PANDA (Niro Soavi, Parma, Italy) was used for all the treatments. The homogenizer was supplied with a homogenizing PS type valve; the valve assembly included a ball type impact head made of ceramics, a stainless steel large inner diameter impact ring and a tungsten carbide passage head. The inlet temperature of the samples was 2–4 °C and the increase rate of temperature was 3 °C/10 MPa. The increase in temperature was controlled by a heat exchanger installed after the valve. Treatments of the samples were carried out in duplicate.

2.3. Confocal laser scanning microscopy (CLSM)

A drop of the sample was put on a slide and stained with Rhodamine B solution (2 g/L) for protein observation. Then, a solution Nile Red (1 g/L) was used to stain lipids. After that, the mixture was covered with a cover glass. The samples were observed in a CSLM (Nikon confocal microscope C1 fitted to a Nikon Eclipse E800 microscope, Nikon Co. Ltd., Tokyo, Japan) in single photon mode equipped with an Ar-Kr laser. The excitation wavelength and emission maxima of the applied fluorescent dyes were 568/625 nm for Rhodamine B and 647/675 nm for Nile Red. Images were stored using EZ-C1 software (Nikon Co. Ltd., Tokyo, Japan).

2.4. Transmission electron microscopy (TEM)

The ultrastructure of the samples was characterized by means of TEM.

The samples were stabilized by mixing them with a low gelling temperature agarose solution (3%) at 30 °C, which facilitates fixation and embedding prior to TEM observation (Sharma et al., 1996). Next, samples were cut into cubes (1 mm³), fixed (primary fixation with 2.5% glutaldehyde and secondary fixation with 2% osmium tetroxide), dehydrated with 30%, 50% and 70% ethanol, contrasted with uranyl acetate (2%) and embedded in epoxy resin (Durcupan ACM, FLUKA). The blocks thus obtained were cut using a Reichter-Jung ULTRACUT ultramicrotome (Leila, Barcelona, Spain). The ultrathin sections obtained (100 Å) were collected in copper grids and stained with 4% lead citrate to be observed in

the Philips EM 400 Transmission Electronic Microscope (Eindhoven, Holland) at 80 kV.

2.5. Analysis of water soluble protein fraction

2.5.1. SDS polyacrylamide gel electrophoresis (SDS–PAGE)

The SDS–PAGE study of the water-soluble protein fraction was carried out using an aliquot of the sample. The water-soluble protein fraction was extracted, prior to SDS–PAGE, as follows. First, samples were freeze-dried (TELSTAR Lioalfa-6, Terrassa, Spain) for 24 h at 10^3 Pa and –45 °C. After freeze-drying, they were defatted in a continuous extraction method (Soxhlet) for 16 h with *n*-hexane–isopropanol (77:23) as solvent. 2.5 g of defatted samples were mixed with 15 mL of distilled water and centrifuged at 3500 rpm for 20 min in a Sorvall Super T_{21} centrifuge (KENDRO Laboratory Products, Newtown, CT). Protein concentration of these water-soluble extracts was determined by the Bradford method (1976) using standard BSA for the preparation of the standard curve.

The protein concentration of the samples was adjusted to 1.25 mg/mL with Laemmli buffer. Electrophoresis was performed on a Multiphor II Electrophoresis System (Pharmacia Biotech, Piscataway, New Jersey, USA), using 12.5% polyacrylamide gels Excel-Gel SDS Homogeneous (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 600 V, 38 mA, 23 W and 15 °C for 1 h 30 min. Eight micro liter of each sample were loaded in the gel in duplicate.

The standard was an Amersham low molecular weight calibration kit (GE Healthcare, UK) consisting of: phosphorylase b (97,000 kDa), albumin (66,000 kDa), ovalbumin (45,000 kDa), carbonic anhydrase (30,000 kDa), trypsin inhibitor (20,100 kDa) and α-lactalbumin (14,400 kDa).

Protein bands were stained with Coomassie Brilliant Blue tablets (PhastGel Blue R., GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Destaining was performed in an aqueous solution of 25% ethanol and 8% acetic acid. Samples were conserved in a solution of 10% glycerol and 7.2% acetic acid. The molecular weight of each band was determined using a densitometer Intelligent Dark Box II Fujifilm LAS 1000 (Fujifilm, Tokyo, Japan) with the software Image Gauge (Fujifilm USA, Valhalla, NY, USA).

2.5.2. N-Kjeldahl

Water-soluble fraction was quantified by Kjeldahl, using a mixture of K_2SO_4, $CuSO_4$ and Se (10:1:0.1) as catalyser. All the measurements were carried in triplicate and the results were expressed as protein using a conversion factor of 6.25.

2.6. Analysis of lipid fraction

The lipid extracts were obtained by the Folch method (1957).

2.6.1. Acidity index

It was determined in accordance with regulations (AOAC, 1990). The measurements were carried out in triplicate.

2.6.2. Detection of hydroperoxides and secondary oxidation products

The lipid extracts (0.01 g) were dissolved in cyclohexane (100 mL) and their absorbances were taken at 232 and 270 nm to measure hydroperoxides and secondary oxidation products, respectively in a spectrophotometer (Cecil Instruments Limited, Series 1020), using 1-cm quartz cuvettes. The measurements were carried out in triplicate and the results were expressed as:

$$K_\lambda = A_\lambda / ce$$

where A_λ = Absorbance at 232 or 270 nm, c = Concentration (g/100 mL), e = Thickness of the cuvette (cm).

2.7. Statistical analysis

Statistical analysis of the results was performed using analysis of variance (ANOVA); the least significant differences (LSD) were calculated at the $p \leqslant 0.05$ significance level. The Statgraphics 5.1 computer-assisted statistics program was employed.

3. Results and discussion

The sauces were observed by Transmission Electron Microscopy (TEM) and Confocal Laser Scanning Microscopy (CLSM). The use of different microscopic techniques offers complementary information about the changes produced by HPH on the main components of the samples.

3.1. Microstructural analysis by CLSM

Fig. 1A shows a continuous phase stained in red with Rodhamine B, which corresponds to the water phase. This water phase is composed mainly by the soluble components of the sample and water (17%). Another continuous phase, slightly black, probably made up with proteins and fat from the yoghurt and egg (lipoprotein network), can also be observed. Oil droplets (1–15 m) from the sunflower oil were observed closely interacting with the lipoprotein network whereas, they were not observed in the water phase.

The structure of the samples treated at 70 MPa (Fig. 1B) was found to be similar to that of the control sample (0 MPa), although the lipoprotein network appeared to be more extended and the oil droplets were smaller. This was probably due to the homogenization effect during the treatment (Cortés-Muñoz et al., 2009).

When the sample was treated at 100 MPa, the lipoprotein network could be observed denser which indicates aggregation. This could be related to the water-soluble protein fraction study, which showed a progressive decrease in the extraction of soluble proteins as the pressure increased. The oil droplets from the sunflower oil appeared to be deformed and a coalescence phenomenon could

be observed (Fig. 1C). Other authors observed an increase of the apparent rate of coalescence with homogenization pressure, since the concentration of suitable proteins was assumed to be limited. This situation, as expected, got worse with increasing homogenization pressure and the attendant increase in surface areas being created (Agboola et al., 1998).

When higher pressure was applied (150 MPa) very big oil droplets appeared (Fig. 1D). A new fat phase was formed and the other components of the emulsion, mainly proteins, were closely interacting with each other. Similar results were obtained by Floury et al. (2002) when treating soy protein-stabilized emulsions. HPH led to changes in macromolecular structure and interaction of the proteins, enhancing protein–protein interactions and showing emulsions strongly aggregated. They also observed that at pressures above 150 MPa, the droplet size strongly increased, indicating a lack of free protein to cover the newly created interface of the droplets. Moreover, proteins could be much too denatured and aggregated for an efficient adsorption at newly formed oil–water interfaces.

A progressive separation of phases took place as the pressure increased, with a completed disruption of the emulsion at 250 MPa (Fig. 1E). This effect can be also observed in Fig. 2, where samples stained with Nile Red show the fat phase in green. At 250 MPa, two phases were observed: a water phase composed mainly of proteins and lipoproteins from the egg and yogurt, and a fat phase with lipids mainly from the sunflower oil.

Desrumaux and Marcand (2002) described an "overprocessing" phenomenon over 210 MPa, were changes in the conformation of proteins were confirmed, probably because of the combined effects of high pressure treatment and rise in temperature observed. This change probably modified the emulsifying properties of proteins.

Samples observed after 1 week of refrigerated storage (Fig. 3) showed a similar aspect to those observed just after treatment (Fig. 2). Only the fat phase showed greater deformation and appeared unevenly distributed.

The pressure produced microstructural changes in the protein phase, thus allowing a higher aggregation of these components.
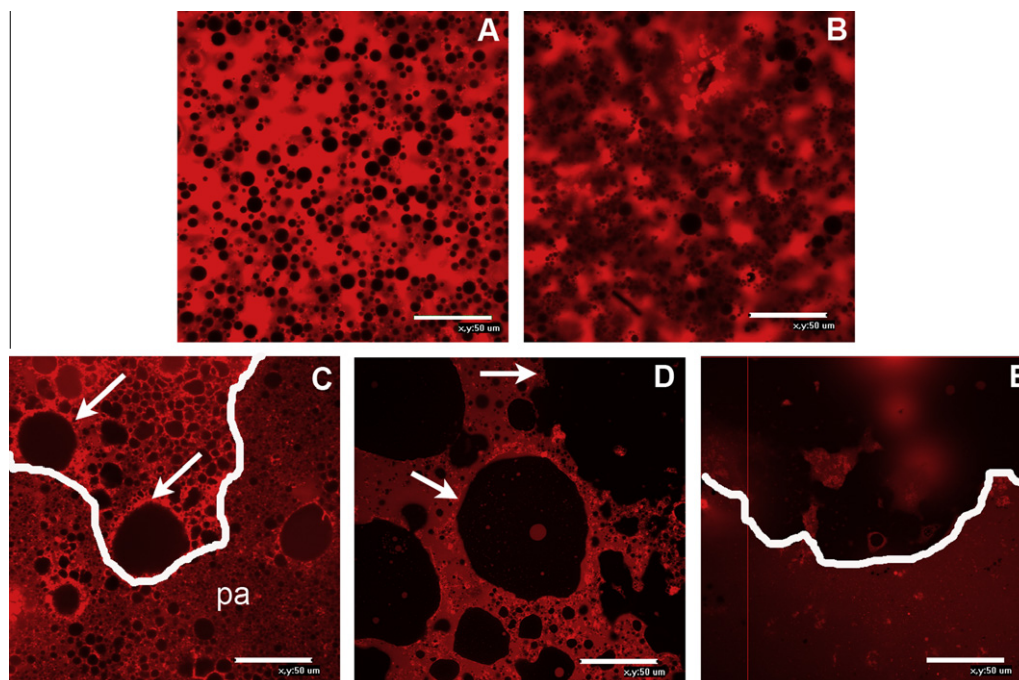

Fig. 1. CLSM (red channel). Sauce treated by HPH at 0 MPa or control sauce (A); 70 MPa (B); 100 MPa (C); 150 MPa (D); and 250 MPa (E) (pa: protein aggregation area, arrows: oil droplets) 60× (bar = 30 m). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
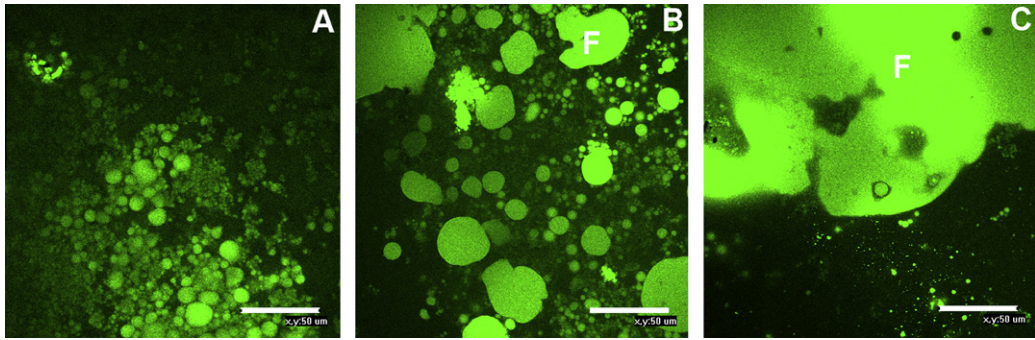
Fig. 2. CLSM (green channel). Sauce treated by HPH at 100 MPa (A); 150 MPa (B); and 250 MPa (C) just treated samples (F: fat) 60× (bar = 30 m). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. CLSM (green channel). Sauce treated by HPH at 70 MPa (A); 150 MPa (B); and 250 MPa (C) after 1 week of refrigerated storage (F: fat) 60× (bar = 30 m). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
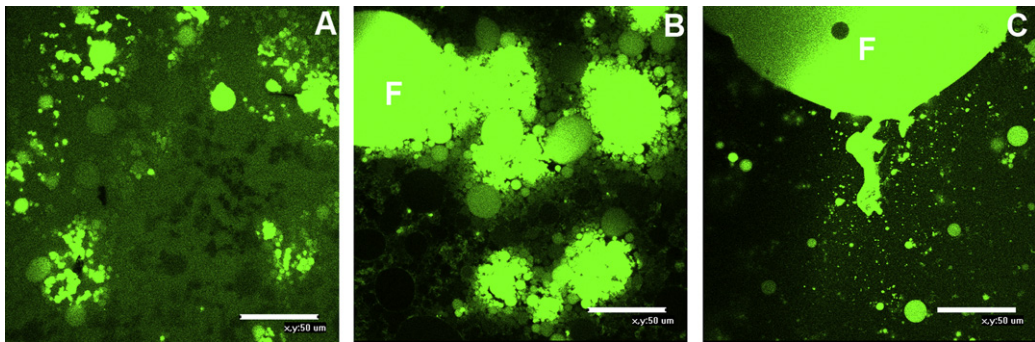
Critical pressures between 150 and 250 MPa produced a destabilization of the emulsion thus causing a separation of phases.

Fig. 4 shows the macroscopic appearance of the samples treated by HPH at different pressures, just treated and after 1 week at 4 °C. The separation of phases was observed after the HPH treatment at 250 MPa. Furthermore, after 1 week at 4 °C, a slight separation of phases was observed in the control samples but it was not observed in 70, 100 and 150 MPa treated samples. This could indicate that HPH treatment up to 150 MPa helps to stabilize the emulsion during storage.
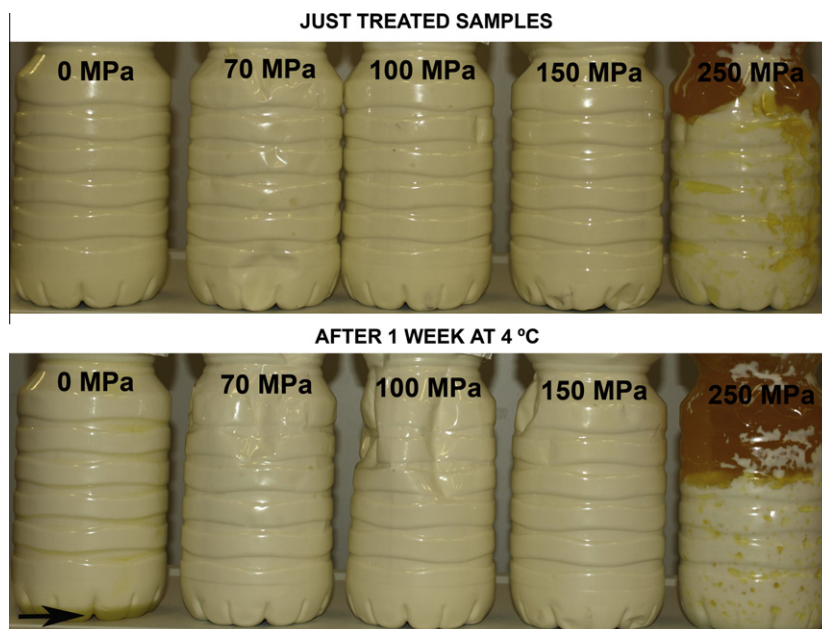

Fig. 4. Macroscopic observations of the samples treated by HPH at different pressures, just treated and after 1 week at 4 °C (arrow: separation of phases).
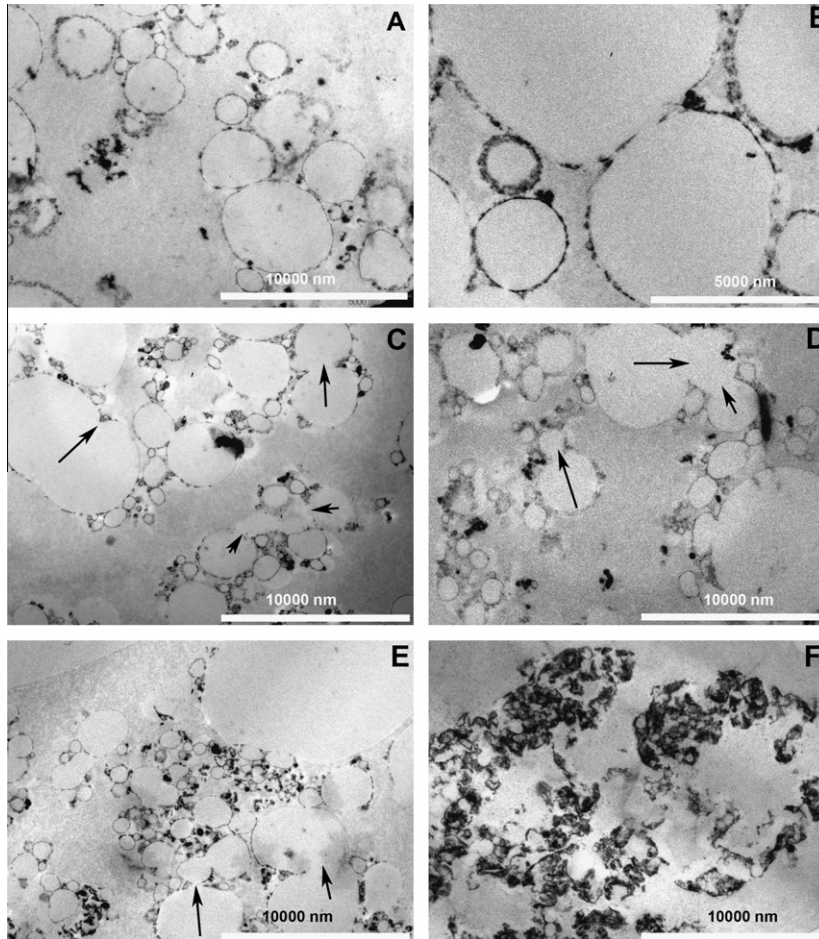
Fig. 5. TEM. Control: sauce treated by HPH at 0 MPa or control sauce (A); detail of control sauce (B) 2200x (bar = 5000 nm); 70 MPa (C); 100 MPa (D); 150 MPa (E); 250 MPa (F) (arrows: coalescence) 1200× (bar = 10,000 nm).

3.2. Ultrastructural analysis by transmission electron microscopy (TEM)

The oil droplets trapped in the protein matrix are observed at an ultrastructural level in Fig. 5A. The oil droplets were surrounded by a barrier of natural emulsifiers (caseins from the yoghurt and phospholipids from the egg) that stabilized the oil-in-water emulsion. Fig. 5B shows a detail of the oil droplets surrounded by several layers of emulsifiers. In the samples treated by HPH (Fig. 5C–F), a coalescence phenomenon progressively occurred as pressure increased. This phenomenon is due to the loss of the emulsifier barrier that was observed surrounding the oil droplets. An accumulation of tensoactives was produced on the interface water–oil, which was separated due to the HPH effect.

After 1 week of refrigerated storage, the tensoactive barrier was still observed in the control sample. Furthermore, the samples treated by HPH showed the same effects due to the treatment: loss of tensoactives, coalescence of the oil droplets and disruption of the oil-in-water emulsion. No significant differences were observed during the storage (images not shown).

3.3. Analysis of protein fraction

The water-soluble protein fraction of the HPH treated samples was studied by SDS–PAGE and quantified by N-Kjeldahl. The results inform about the protein solubility, which can be related to their functionality.

Table 1
Percentages (g/100 g sample w.m) of the water-soluble protein values (Kjeldahl) of the HPH treated samples at different pressures.

Water soluble protein (Kjeldahl)				
HPH treatment (MPa)	Just treated samples		After 1 week at 4 °C	
	Mean	SD	Mean	SD
0	0.77[a]	0.03	0.86[a]	0.09
70	0.77[a]	0.04	0.76[ab]	0.26
100	0.71[ab]	0.01	0.73[ab]	0.15
150	0.57[b]	0.03	0.61[b]	0.02
250	0.25[c]	0.01	0.26[c]	0.01

[a, b, ..., z] Values with different letters within the same columns are significantly different ($p < 0.05$) according to the LSD multiple range test.
The absence of ∗ means that just treated sample does not differ ($p < 0.05$) of the corresponding refrigerated sample.

After the HPH treatments, a progressive decrease in the percentages of water-soluble protein values was observed in just treated samples, as the pressure increased (Table 1), indicating protein insolubilization. Significant differences were observed in the soluble protein values when 150 and 250 MPa were applied if compared to the values of samples treated at lower pressure. The factors involved in the protein insolubilization can be pH and T. With the low pH of the system (pH 5), a relatively low temperature during the process at 150 MPa would explain the denaturation and aggregation of proteins. When high pressure was applied

(250 MPa) the temperature rose to 75–80 °C, which explains the protein denaturation. In these conditions the emulsion was easily destabilized. Moreover, some proteins, as albumins, are especially sensitive to temperature, and it increases during the HPH treatment. Some authors (Sirvente et al., 2007) observed a protein solubility decrease of egg yolk proteins when the HPH treatment was between 100 and 200 MPa. Other type of proteins as soy globulin have been observed to be affected by high pressure treatment above 150 MPa. Increasing the homogenization pressure above 200 MPa led to a quite strong decrease in the globulin solubility due to very strong forced induced phenomena of cavitation, high shear and turbulence, and the rise in temperature (Floury et al., 2002).

After 1 week of refrigerated storage similar effects were observed. However, no significant changes were observed in the treated samples due to the storage. The highest value of the water-soluble protein values was observed in the non-treated sample, probably due to a lower microbiological stability after 1 week at 4 °C.

Fig. 6 shows the electrophoregram obtained for the water-soluble protein fraction of the HPH treated samples immediately after the treatment and after 1 week of refrigerated storage.

Treatment at 150 and 250 MPa produced changes on the electrophoretic pattern of the samples, showing an insolubilization of proteins as albumin (MW = 66 kDa). Furthermore, an intensification of other proteins bands (30–45 kDa) is observed when the sample is treated at 250 MPa. Desrumaux and Marcand (2002) concluded using SDS–PAGE that HPH treatment (from 20 up to 350 MPa) did not show any significant change in the molecular weight of whey proteins from sunflower oil emulsions.

After 1 week of refrigerated storage, similar effects were observed in the samples.

3.4. Analysis of lipid fraction

The fat was analyzed to determine possible chemical changes in this fraction due to the lypolitic activity. The chemical analysis of the fat (Table 2) showed neither significant changes in the acidity

Table 2
Acidity grade of the lipid fraction of the HPH treated samples at different pressures.

Acidity index				
HPH treatment (MPa)	Just treated samples		After 1 week at 4 °C	
	Mean	SD	Mean	SD
0	0.15[a]	0.01	0.17[a]	0.02
70	0.16[a]	0.01	0.19[a]	0.02
100	0.17[a]	0.02	0.16[a]	0.01
150	0.17[a]	0.01	0.18[a]	0.05
250	0.18[a]	0.02	0.17[a]	0.02

[a, b, ..., z] Values with different letters within the same columns are significantly different ($p < 0.05$) according to the LSD multiple range test.
The absence of ∗ means that just treated sample does not differ ($p < 0.05$) of the corresponding refrigerated sample.

Table 3
Oxidation spectrophotometer parameter (K_{232}) of the lipid fraction of the HPH treated samples at different pressures.

K_{232}				
HPH treatment (MPa)	Just treated samples		After 1 week at 4 °C	
	Mean	SD	Mean	SD
0	2.70[a]	0.14	2.71[a]	0.04
70	2.71[a]	0.30	2.73[a]	0.03
100	2.80[a]	0.32	2.84[a]	0.01
150	2.91[a]	0.04	2.88[a]	0.10
250	2.82[a]	0.07	2.81[a]	0.04

[a, b, ..., z] Values with different letters within the same columns are significantly different ($p < 0.05$) according to the LSD multiple range test.
The absence of ∗ means that just treated sample does not differ ($p < 0.05$) of the corresponding refrigerated sample.

index as the pressure increased, nor during storage, which could indicate that lipolysis was not produced.

Furthermore, as expected, no significant changes in the oxidation parameters (K_{232} and K_{270}) were observed due to the HPH treatment and after 1 week of storage at 4 °C (Tables 3 and 4).
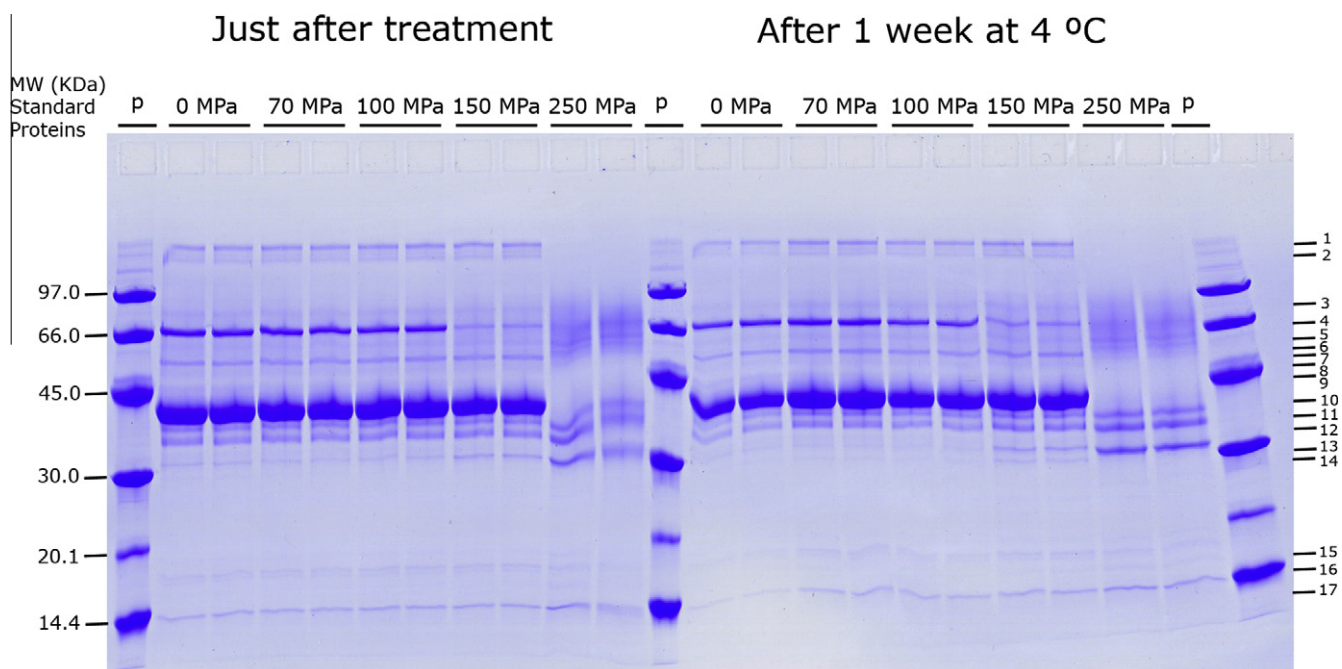

Fig. 6. Water soluble protein electrophoregram of the sauces treated by HPH, just after the treatment and after 1 week of refrigerated storage (p: standard proteins).

Table 4
Oxidation spectrophotometer parameters (K_{270}) of the lipid fraction of the HPH treated samples at different pressures.

HPH treatment (MPa)	K_{270}			
	Just treated samples		After 1 week at 4 °C	
	Mean	SD	Mean	SD
0	0.55[a]	0.03	0.54[a]	0.01
70	0.55[a]	0.05	0.54[a]	0.03
100	0.54[a]	0.06	0.53[a]	0.01
150	0.52[a]	0.02	0.54[a]	0.02
250	0.53[a]	0.01	0.54[a]	0.02

[a, b, ..., z] Values with different letters within the same columns are significantly different ($p < 0.05$) according to the LSD multiple range test.
The absence of ∗ means that just treated sample does not differ ($p < 0.05$) of the corresponding refrigerated sample.

Therefore, according to the analysis, the lipid fraction showed to be chemically stable after HPH treatment and after 1 week of refrigerated storage.

4. Conclusion

The physicochemical stability of this emulsion is directly related to the pressure applied during the process. The oil-in-water emulsion is stable up to 100 MPa with the oil droplets surrounded by several layers of natural emulsifiers. Critical pressures, between 150 and 250 MPa, affect the stability of the emulsion thus causing a separation of phases. A coalescence phenomenon progressively occurs when pressure increases. This phenomenon is due to the loss of the natural emulsifier barrier. Changes on the electrophoretic pattern are observed at 150 and 250 MPa, showing protein insolubilization while lipid fraction is chemically stable during the HPH treatment.

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