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Hard cheese structure after a high hydrostatic pressure treatment at 50 MPa for 72 h applied to cheese after brining

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Abstract — High hydrostatic pressure (HHP) treatment (50 MPa for 72 h) on cheese in the early stages of ripening produced changes in primary proteolysis during the treatment. From the analysis of protein solubilised using bond breaking agents, it was concluded that the incidence of hydrophobic and hydrogen bonds in treated cheeses was reduced after high pressure treatment. Solute diffusion was improved by pressure as salt distribution was enhanced in HHP cheeses. Water was bound more strongly in pressurised cheese compared to the untreated control cheese. The curves of the stress relaxation test were evaluated after modelling of experimental records according to two different models. The mechanical Maxwell model has the drawback of being non-linear and gave no more information than the alternative model evaluated. Cheese texture became more fluid-like.

high pressure / cheese / texture / microstructure / composition

Résumé — Structure d'un fromage à pâte dure après traitement par haute pression hydrostatique de 50 MPa durant 72 h appliquée après salage. L'application de la haute pression hydrostatique (50 MPa durant 72 h) sur les fromages, provoque des changements de la protéolyse primaire durant ce traitement. À partir de l'analyse des protéines solubilisées par des agents de rupture de liaisons, il a été conclu que l'incidence des liaisons hydrophobes et des ponts hydrogènes du fromage traité a été réduite après le traitement par la haute pression. La diffusion des solutés a été facilitée par la pression puisque la distribution saline a été augmentée dans les fromages traités. L'eau s'est liée plus fortement au fromage pressurisé. Les courbes de relaxation de l'effort ont été évaluées après la modélisation des données expérimentales selon deux modèles différents. Le modèle mécanique de Maxwell a l'inconvénient de ne pas être linéaire et ne donne pas plus d'information que le modèle alternatif évalué. La texture du fromage devient plus molle.

haute pression / fromage / texture / microstructure / composition

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1. INTRODUCTION

High hydrostatic pressure (HHP) is transmitted instantly and uniformly to all the volume treated (*Isostatic principle*). The induced volume decrease causes a response to the introduced perturbation leading to a new state where the disturbance is compensated (*Le Châtellier principle*). All the reactions accompanied by a decrease in volume – like chemical reactions, phase transition or change in molecular configuration – are enhanced by pressure.

Low molecular weight food components, which are responsible for nutritional and sensory characteristics, are less affected by pressure, whereas high molecular weight components are sensitive [30]. Tertiary structure of these components is important for functionality determination. This makes this technology an attractive alternative to thermal treatment, due to the possibility of microorganism inactivation and development of new functionality in foods while retaining their freshness and nutritional value.

Most applications of HHP lead to enzyme inhibition, but there are exceptions, like enhanced proteolysis of β-lactoglobulin (e.g. by trypsin) under pressure. This has been suggested for use to reduce the allergenicity of dairy products [30]. Inactivation of enzymes depends upon the composition of the medium, pH, temperature, pressure and processing time, and real foods provide a baroprotective layer, preventing the inactivation of enzymes [29]. 50 MPa is much lower than pressure values described as inactivating enzymes, and it is likely that pressurisation at this intensity increases the reaction rates of enzymes involved in proteolysis due to volume reduction accompanying hydrolysis of peptide bonds.

Changes in protein structure by pressure are based on volume changes of denaturation and the delicate balances of stabilising-destabilising interactions. Free water has a higher compressibility than protein bound water. Ionic bonds are formed under pressure, decreasing the number of free hydrophilic groups and favouring the interactions between proteins [30]. Changes in tertiary structure of proteins may be reversible when pressure is released and hydrophobic interactions return to basal values [8], but association between caseins in milk after pressure exposition is different from the original organisation observed in untreated milk [13, 16].

HHP was investigated on cheese to accelerate ripening [14, 24, 35], accelerate brining [22, 26] and for fresh cheese preservation [3]. Cheese microstructure was not dealt with in previous studies while texture was only briefly looked at. The aim of our work is to relate changes in texture with modifications in microstructure and proteolysis on cheese pressurised at an early stage of ripening. Changes in cheese texture are of great concern for consumer acceptance. The knowledge of the details of those modifications and on possible mechanisms would be of great importance to keep those changes under control. While HHP treatment to accelerate cheese ripening or enhance preservation is in process, texture could also be controlled. Keeping original texture or developing a new one would be possible from the knowledge of mechanisms of HHP effects.

2. MATERIALS AND METHODS

2.1. Cheese making and high pressure treatment

This study was carried out on Garrotxa cheese. This is a mixed curd, uncooked, pressed, goat milk cheese. The cheese making technology was described in Saldo et al. [28]. Nine cheeses from three different industrial batches (three from each) were obtained from a local dairy farm the day after salting in brine. Each cheese weighed about 1.5 kg and was divided into two parts. Each

half was vacuum-packed and one of them was treated at 50 MPa and 25 ºC for 72 hours while the other halves served as controls (0.1 MPa, 25 ºC, 72 h). Cheeses were analysed to characterise their physical and chemical properties immediately after treatment.

2.2. Cheese composition analyses

Total solids were determined by drying in an oven at 102 ± 2 ºC, until a constant weight was reached [10]. Ash content was determined via gravimetric analysis once the sample had been calcinated in an oven at 550 ºC [9]. The Gerber method with the Van Gulik modification for cheeses was applied for fat content determination [11]. The digestion block method involving a modification of the Kjeldahl method was used for quantitative analysis of total nitrogen [12]. The extraction of soluble nitrogen at pH 4.6 and soluble nitrogen in 12% trichloroacetic acid (TCA) were performed according to McSweeney and Fox [21]. The soluble fraction at pH 4.6 contains whey proteins, proteose-peptone, low molecular weight peptides and amino acids, while the 12% TCA soluble fraction contains only small peptides and free amino acids. A colorimetric method by means of Cd-Ninhydrin reagent [6] was used to measure free amino acids in the pH 4.6 soluble fraction. The pH 4.6-insoluble fraction was analysed by urea-PAGE as described by Pripp et al. [27] and quantified using the software ImageMaster TotalLab v1.00 (Amerham Pharmacia Biotech, Uppsala, Sweden). Cheese compositional analysis was performed in duplicate (dry matter in triplicate).

2.3. Dissolution experiments

From each of the cheeses studied, aliquots of 0.1 g were extracted using 4 solvents in order to solubilise the protein released by bond breaking agents (salt, SDS, urea, DTT) [33]. Salts will break electrostatic interactions, urea and sodium dodecyl sulphate (SDS) will break hydrogen bonds and hydrophobic interactions, and ditiotheriol (DTT) will break disulphide bonds. Protein content of the supernatant was determined by the Lowry method after removing the interfering agents by precipitation of protein by sodium deoxycholate and trichloroacetic acid and resolubilisation of proteins in milliQ ultrapure water (Millipore, Bedford MA, USA).

Conversion of the absorbance values to protein content was achieved by simultaneously analysing a series of diluted cow skim milk by Lowry and Kjeldahl methods. Each cheese was independently extracted six times with each of the four solutions, and colorimetric determinations of solubilised proteins were made in triplicate for each extraction.

2.4. Thermogravimetric analysis

About 20 mg of grated cheese was placed in a silica pan and heated at a rate of 5 ºC min^{-1} in a TGA/SDTA 851^e (Mettler-Toledo GmdH Analytical, Schwerzenbach, Switzerland). The procedure followed the method described by De Angelis Curtis et al. [4], with minor modifications. Weight loss was recorded and the limit between free and bound water was established at 92 ºC according to an inflection point found from the first derivative. Weight loss between 25 ºC and 92 ºC was considered to be free water, and the weight loss step between 92 ºC and 220 ºC was considered to be bound water. The analysis was performed in duplicate for each cheese sample.

2.5. Instrumental texture determination

Cheese texture was studied by using large deformation assay with a TA.XT2 Texture Analyzer (Stable Micro Systems, Haslemere, UK). Samples were maintained at 19 ºC for at least two hours to allow

temperature equilibration. The tests were performed on the inner part of the cheese, after cutting along the equatorial plane. Eight replicates of each test were run for each cheese sample.

A penetration test at 70% of the initial height was applied to the cheese. A test probe of 5 mm diameter breaks through cheese samples at a crosshead speed of 100 mm min^{-1}. The force and penetration distance until fracture were calculated.

Cheese pieces were compressed with a plunger of 10 mm in diameter a distance of 1 mm. Force attenuation was adjusted to two different models. One was the generalised Maxwell model, and the other a linealisation of the model proposed by Nussinovitch [23].

The Maxwell generalised model needs non-linear regression techniques to be fitted. We used a 5 element Maxwell model with 1 spring F_∞ in series with a parallel arrangement of 2 springs F_1 and F_2 each in series with dashpots τ_1 and τ_2. It was expressed by equation (1), where F_∞ is the equilibrium modulus of the elastic element, F_1 and F_2 are the elasticity modulus, and τ_1 and τ_2 are the relaxation times for both Maxwell elements in parallel [1].

$$F(t) = F_\infty + F_1 \cdot e^{\left(-t/\tau_1\right)} + F_2 \cdot e^{\left(-t/\tau_2\right)}. \quad (1)$$

Stress relaxation curves can be adjusted to the model proposed by Peleg (Eq. (2)), linealised in equation (3), with parameters rearranged according to Pavia et al. [25] (Eq. (4)). The two parameters in this model, *s* and *r*, have values between 0 and 1. A liquid is described in this model by $s = 1$, and $r = 0$ means an ideal elastic body. $1 - s$ is the asymptotic residual modulus and the reciprocal of *r* is the time of semi-relaxation.

$$\frac{F(t)}{F_0} = 1 - \frac{t}{k_1 + k_2 \cdot t} \quad (2)$$

$$\frac{F_0}{F_0 - F(t)} = \frac{k_1 + k_2 \cdot t}{t} \quad (3)$$

$$\frac{F_0 \cdot t}{F_0 - F(t)} = k_1 + k_2 \cdot t = \frac{1}{s \cdot r} + \frac{t}{s}. \quad (4)$$

2.6. Instrumental colour determination

Changes in colour were evaluated using a Miniscan™ XE colorimeter (Hunter Associates Laboratory, Reston, VA, USA) with a Fcw illuminant and observer at 10º. The hunterlab scale (*L, a, b*) was used where "*L*" is the luminosity of the sample, with values from 0 to 100, parameter "*a*" varies from green to red, while "*b*" varies from blue to yellow. Each measurement of colour was repeated six times on freshly cut surfaces of the interior of the cheeses.

The cartesian coordinates of *L, a, b* were converted into polar coordinates by calculation of the longitude of the vector in the $a - b$ plane (namely Chroma) and the angle of the vector (Hue angle) according with the guidelines of Little [18].

2.7. Confocal Scanning Laser Microscopy (CSLM)

Cheese samples were cut into thin slices and stained with Nile Blue for CSLM observation. This technique does not need fixatives and does not alter cheese microstructure as long as it is compatible with the water phase. Protein was observed by fluorescence. Slices of cheese samples were introduced for 5 min into a 0.02% aqueous Nile Blue A solution. After rinsing and draining, sections were mounted in non-fluorescent observation media between two glass slides [34]. Images were taken using a Leica TCS4D (Heidelberg, Germany) confocal microscope. Exciting with a 568 nm Kr/Ar laser, the protein phase can be observed, the remaining field appears as dark holes. The high definition of the observation plane given by the confocal microscopy allows a serial observation of different planes of the same sample, and

the software accompanying the microscope rends a stereoscopic image. The microphotographs presented were obtained with an oil-immersion 60X lens with aperture set to 1.3, and are the three-dimensional reconstrution of 16 individual planes along a 20 μm slice of cheese.

2.8. Statistical analysis

Differences between treatments were estimated using the ANOVA test to discriminate between the means using Fisher's least significant difference (LSD) procedure. The whole experiment was independently repeated three times.

3. RESULTS AND DISCUSSION

After removing packaging material no fat formation on the surface was observed.

Overall composition was typical for fresh-made Garrotxa cheese (42.2% moisture, 2.8% ash, 37.1% fat, and 18.2% protein). The cheese composition does not add up to 100%, maybe due to lack of accuracy in analysis methods for each component [5].

Despite having the same moisture content, water in both cheeses was retained in a different manner (Tab. I). Cheeses treated by HHP had more bound water as shown by TGA analysis. The sum of the two weight loss processes (25 ºC to 92 ºC and 92 ºC to 220 ºC) was not equal to the moisture content assessed by the oven drying method probably due to the overestimation produced when long drying times are used. The stronger binding of water could explain the higher moisture content in HHP treated cheese during ripening, as described by Saldo et al. [28].

Table I. Water assigned to the bound or free fraction according with the TGA curves. Values for both treatments differ at $P < 0.05$.

	Free water (%)	Bound water (%)
Control	18.9	21.4
HHP	12.7	27.6

HHP treatment influences proteolysis, but only differences in non-casein fraction (pH 4.6 soluble) were significantly different (Tab. II). This increase could be caused by an increase of proteolysis of caseins produced by enzymes present in the curd (rennet and plasmin, mainly), due to a change in their activity itself or to changes in their substrate structure caused by disruption of hydrophobic bonds under pressure. From the urea-PAGE study of pH 4.6 insoluble fraction (Fig. 1) was observed a decrease in α_{s1}-casein in HHP treated cheese (peaks 8–9), accompanied by an increase of their degradation products (peaks 10–11). This observation could point out an increase of rennet activity under this relatively mild pressure, as long as this enzyme has a special affinity for α_{s1}-casein [7].

Secondary proteolysis was not changed due to the HHP treatment, as is evident from the same values in TCA 12% soluble nitrogen or free amino acids nitrogen (Tab. II). Starter enzymes produce further proteolysis from the products of cleavage of caseins by proteases. Those enzymes are

Table II. Proteolysis indexes as a fraction of total nitrogen corresponding to different proteolysis products. Values within a column with a different subscript differ significantly at $P < 0.05$.

	pH 4.6 soluble N (%)	TCA 12 % soluble N (%)	Free Amino Acids N (%)
Control	13.4_a	7.3_a	1.1_a
HHP	16.5_b	7.3_a	1.2_a

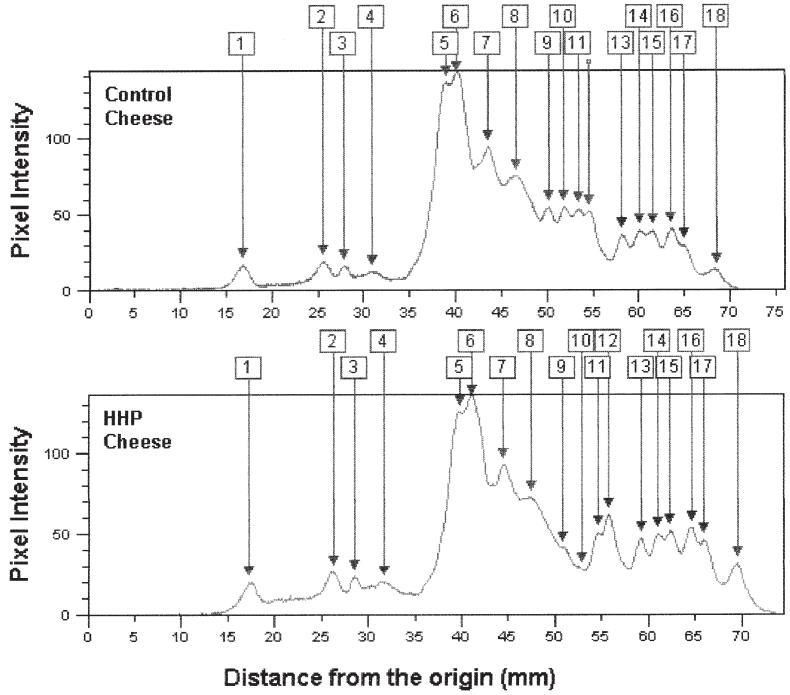

Figure 1. Densitometric representation of urea-PAGE electrophoregram for the pH 4.6-insoluble fraction of cheeses.

not released until a later stage of cheese ripening, when the autolysis of lactic acid bacteria occurs.

In the early stages of ripening, peptidases and their substrates are in a separate compartment, enzymes are in the cytoplasm and products of primary proteolysis are in the extracellular space. An active transport system, specific for peptides of up to 6 amino acid residues, is necessary to uptake them and put together enzymes and substrates [20]. Pressure enhances plasmatic membrane organisation, causing the separation of peripheral and integral proteins [19]. It is reasonable to suppose that an inhibition of this mechanism occurs under pressure as it depends on a specific active transport [15].

In brine immersion salted cheeses, salt is distributed by diffusion from the rind to the core, reaching an equilibrium in a 1.5 kg cheese in two or three weeks. After obtaining the results by measuring salt content in the core or the rind part of cheese pieces (Tab. III), an increase in salt diffusion is achieved by HHP treatment of salted curd. Salt content is low, because the cheese in this study is in the early stages of ripening, but it will increase later due to moisture loss. In this study salt content decreased in the rind, and increased in the core.

It has been proved that HHP brining of cheese is neither successful for Gouda

Table III. Average salt content (g NaCl / 100 g cheese). Rows with a different subscript differ significantly at $P < 0.05$.

	Core	Rind
Control	0.69_a	1.10_a
HHP	0.80_b	1.00_b

cheese [22] nor for Manchego cheese [26]. The preliminary step of salt intake by capillarity seems necessary to show the increase in solute mobility under HHP.

The pH value was higher in HHP treated cheeses with respect to the control (5.12 in HHP and 5.01 in control). A similar rise in pH due to HHP treatments has been described to be related to pressure-induced dissociation of calcium phosphate in skim milk [17]. This dissociation is irreversible and causes an increase in pH by about 0.05 units when milk is pressurised. In pressurised milk a partial dissociation of the micellar colloidal calcium phosphate occurs, causing a further increase in pH and the disintegration of the casein micelles or casein-whey protein aggregates [16]. This dissociation process is completely reversible in milk but it seems that longer pressure exposure causes a more permanent effect [17]. The pH increase in HHP treated cheese has been previously observed in Gouda [22] and Manchego cheese [26], and was attributed to the same causes. As the cheese was pressurised the day after cheese making, the acidification was not completed. The exposure at 50 MPa for 72 h may stop lactose fermentation. The active intake of lactose is mediated by the multicomponent lactose-specific phosphotransferase system [31], but the organisation in the phospholipid bilayer imposed by the pressure seems to inhibit this transport system as has been discussed previously for peptide transport.

In dissolution experiments (Tab. IV) the total amount of protein solubilised after breaking all weak bonds (solvent 4) was the same for control and HHP treated cheese. The variation in solubilised protein due to the action of DTT was not significant ($P < 0.05$) for any of the treatments, indicating a weak contribution of disulphide bonds to the linkage of proteins in the cheese matrix. The protein solubilised by solvent 1 in Table V corresponds to the fraction tied in the weakest form (just entrapped in the casein gel), and was a little higher in HHP cheese, and the fraction of proteins linked just by electrostatic interactions was also higher. Hydrogen bonds and hydrophobic interactions (solvent 2) were more significant in control cheese, which is demonstrated by the high amount of protein solubilised by solvent 3 in control cheese

Table IV. Protein solubilised ($mg \cdot g^{-1}$ cheese) in different solvents (solvent 1 was milliQ water, solvent 2 was a pH 8 1.6 $mol \cdot L^{-1}$ Tris-Glycine buffer, solvent 3 additionally contains 8 $mol \cdot L^{-1}$ urea and 17 $mmol \cdot L^{-1}$ SDS, and solvent 4 additionally contains 10 $mmol \cdot L^{-1}$ DTT). Rows with a different subscript differ significantly at $P < 0.05$.

	Solvent 1	Solvent 2	Solvent 3	Solvent 4
Control	3_a	81_a	123_b	118_a
HHP	14_b	103_b	115_a	120_a

Table V. Stress relaxation test. Parameters of the Maxwell model (Eq. (1)), obtained by non-linear regression from the instrumental data and parameters of linear model presented in equation (4). All parameters are significantly different between treatments for $P < 0.05$.

	F	F_1	τ_1	F_2	τ_2	s	r
Control	0.65 N	0.66 N	27 s	1.00 N	1.5 s	0.697	0.132
HHP	0.23 N	0.27 N	25 s	0.45 N	1.4 s	0.754	0.142

Table VI. Colour parameters in *L, a, b* scale, and values for Chroma and Hue angle. Rows with a different subscript differ significantly at $P < 0.05$.

	L	*a*	*b*	Chroma	Hue Angle
Control	93.6_a	-0.73_a	11.0_a	11.0_a	$93{,}7^{o}{}_a$
HHP	91.6_b	-0.85_b	12.9_b	13.0_b	$93.8^{o}{}_a$

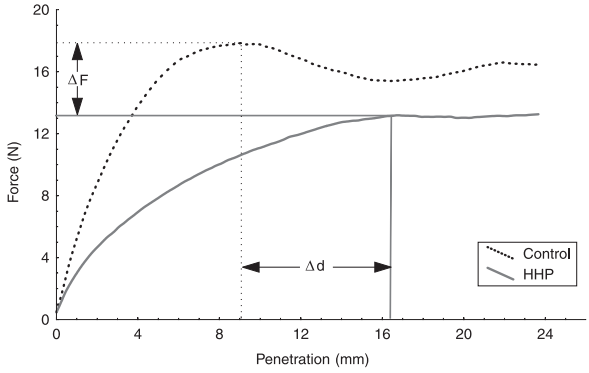

Figure 2. Penetration curves for control and HHP treated cheeses. Results were explained in terms of increase of penetration distance until fracture (Δd) and variation of fracture force (ΔF). The increments were compared with respect to control average.

compared to the protein released in HHP cheese.

The fitting of the Maxwell model for relaxation stress tests was excellent, with an average correlation coefficient of $R^2 = 0.993$ for Eq. (1). The parameters of the model were calculated and are presented in Table VI. The equilibrium modulus (F_∞) was higher for control cheese, and both elasticity modulus F_1 and F_2 were higher too. The force against the deformation decreased in HHP treated cheese. Relaxation times (τ_1 and τ_2) were shorter for HHP cheese.

Textural measurements indicated that 50 MPa-treated cheese was more fluid and less elastic than untreated cheese. HHP treated cheese showed higher values for *s* and *r* parameters, as reported in Table V. The correlation coefficient was $R^2 = 0.998$ for the Eq. (3) model, which is excellent too.

Both models for stress relaxation give the same information about the rheological behaviour of the samples. As R^2 has similar values for both, we recommend the use of the simplified model as used by Pavia et al. [25].

In addition, a less fracturable behaviour in pressurised cheese is evident from the penetration test. In Figure 2 we can see two characteristic plots for force against penetration distance for this test. Some pressurised samples didn't show a fracture point at all. In order to standardise results we refer to all data as variation from control, for each batch. In the penetration test we studied the structural failure of cheese structure. Force and deformation conditions causing such breaking are important because they relate to sensory texture.

The force needed for penetration was lower for 50 MPa-treated cheese, and fracture (if it occurred) needed less force and

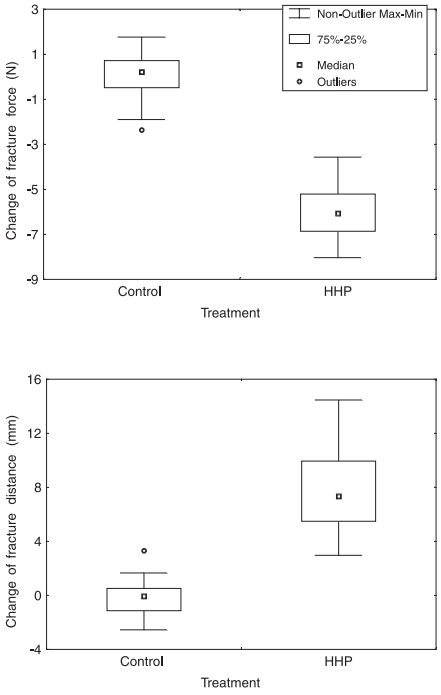

Figure 3. Values for the change of fracture force (ΔF) and distance of fracture (Δd). As distance is measured from control cheese, zero value corresponds to the average for untreated samples.

led to higher deformation, as presented in Figure 3. Hardness and shortness remained higher in control cheese. This would indicate a softening of cheese due to a weakening of the casein matrix. Products from casein degradation have high solubility in water and do not contribute to cheese matrix rigidity [1]. The increase of these products is shown in the increase in non-casein nitrogen (Tab. II).

Microstructure images (Fig. 4) displayed the irregular matrix distribution in control cheese, with plenty of holes of different size and shape. The biggest cavities correspond to mechanical holes, while globules of fat occupy the others. The protein network looked like a sponge cake with few structures connecting over long distances. HHP treatment produced a more

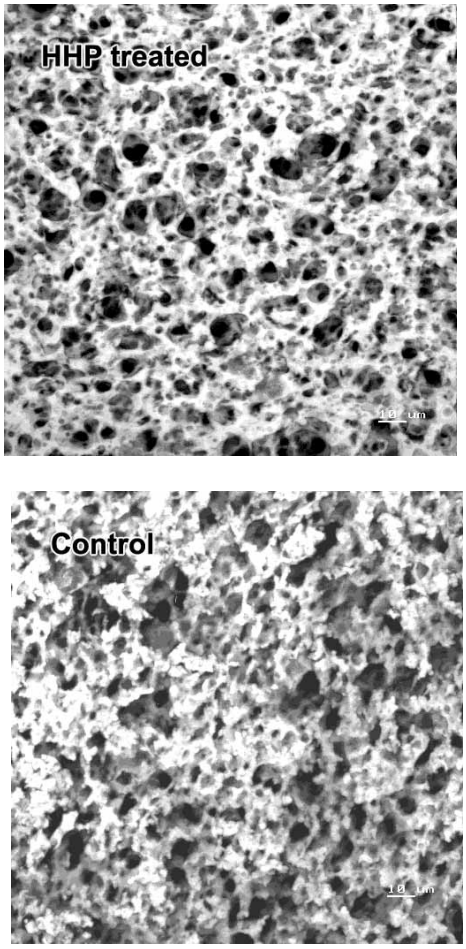

Figure 4. Changes in cheese microstructure due to HHP treatment. Protein network is visible in white, dark holes correspond mainly to globules of fat. Three-dimensional reconstruction of 16 optical sections obtained by laser scanning microscopy along a 20 μm cheese slice.

regular distribution of hole-size, along with an even and more continuous protein network. The matrix in these cheeses was made of filament-like structures, connecting the structure over long distances. Similar changes were found in fresh goat cheese pressurised at 400 MPa [2]. Torres Mora et al. [32] reported the possibility of a new

texture generation in cheese by means of HHP treatments.

These changes in the casein matrix could account for the changes in textural properties. The structure in microphotographs of HHP cheese could explain the texture change found by instrumental analysis. A continuous and evenly distributed network will store or dissipate energy of compression in a more efficient way, and be more resistant to fracture.

Colour in cheese is lightly yellow-greenish. The HHP treatment changed this colour together with a decrease in lightness (Tab. VI). For a better understanding of the evolution of colour, the original cartesian position of each measurement has been converted into polar coordinates. The longitude of the vector (Chroma) is larger for HHP treated cheese, while the angle (Hue) did not change. This means that the shade of colour did not change at all and only an intensifying effect occurred. The denser microstructure of the casein matrix could account for this increase in Chroma, because of the reduction of the gaps.

4. CONCLUSION

Water retention was enhanced by the HHP treatment applied to the cheese, as was shown by the increase in bound water. The pH value was higher in treated cheese, because of the effect on calcium equilibrium and the effect of pressure on the acidifying activity of starter bacteria. Salt distribution in the cheese became more uniform, and diffused quickly from the rind to the core. The microstructure of the cheese developed into a more uniform structure, and the paracasein network had a regular distribution. The proteins turned out to be linked more strongly by electrostatic interactions and less by hydrogen bonds or hydrophobic interactions. Primary proteolysis was enhanced under pressure, while the subsequent peptidolysis remained unaffected. The cheese texture became softer, large deformations were achieved with less force applied, and a more difficult fracture was obtained.

This HHP treatment could lead to the development of new cheese characteristics by means of the modification of microstructure. Those changes produce a more efficient water binding and a softening in the cheese texture. The decrease in the force involved in the interactions between proteins favour the yielding texture development. The modification of salt distribution and of acidification should be monitored closely to avoid defects during ripening.

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