3.3 HARD CHEESE STRUCTURE AFTER A HIGH HYDROSTATIC PRESSURE TREATMENT AT 50 MPA FOR 72 H APPLIED TO CHEESE AFTER BRINING. (2001) J. Saldo, E. Sendra, B. Guamis. *Lait* 81 (5) 625-635

Lait 81 (2001) 625-635

Original article

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

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(Received 5 December 2000; accepted 2 April 2001)

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

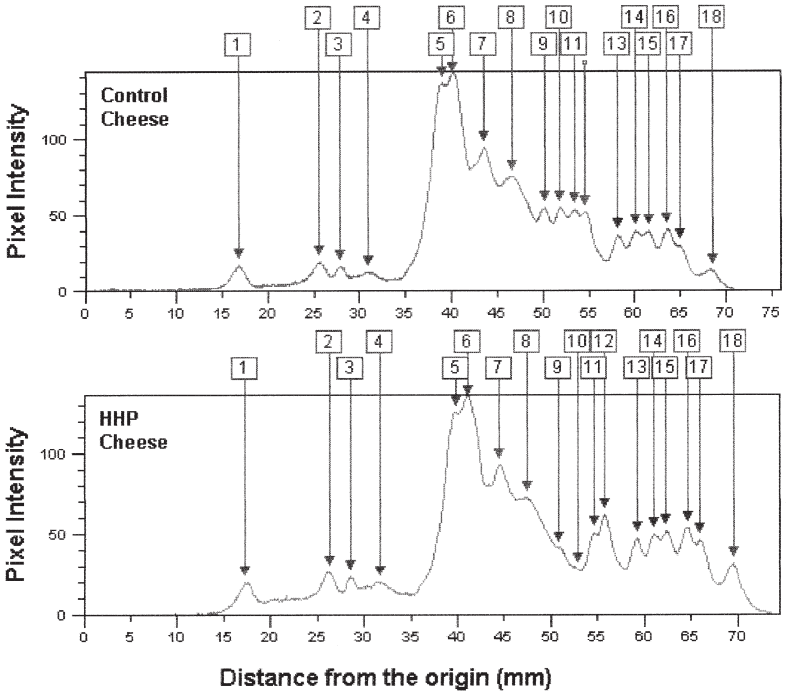

Figure 1. Densitometric representation of urea-PAGE electrophoregram for the pH 4.6-insoluble fraction of cheeses.

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	$93.8^{o}{}_a$

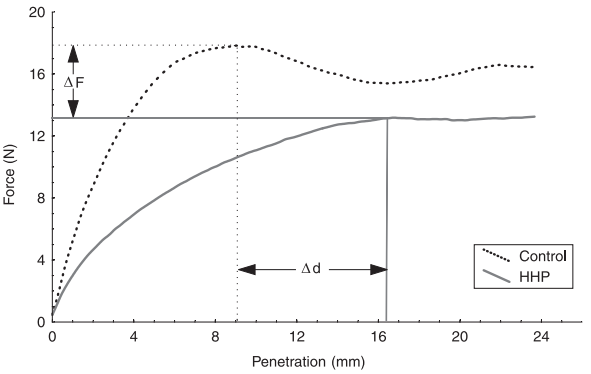

Figure 2. Penetration curves for control and HHP treated cheeses. Results were explained in terms of increase of penetration distance until fracture (Δd) and variation of fracture force (ΔF). The increments were compared with respect to control average.

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

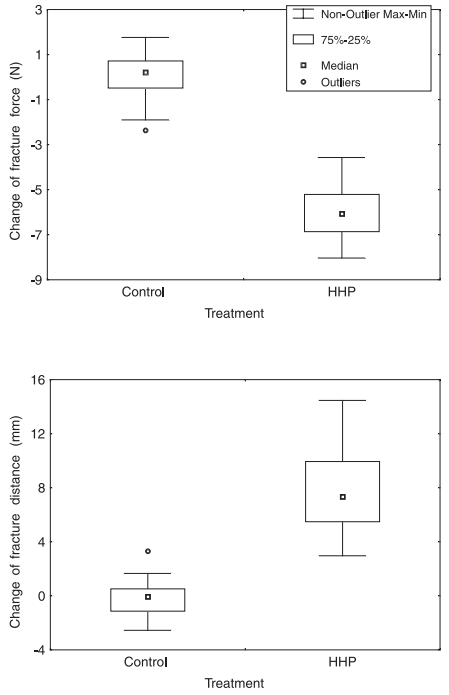

Figure 3. Values for the change of fracture force (ΔF) and distance of fracture (Δd). As distance is measured from control cheese, zero value corresponds to the average for untreated samples.

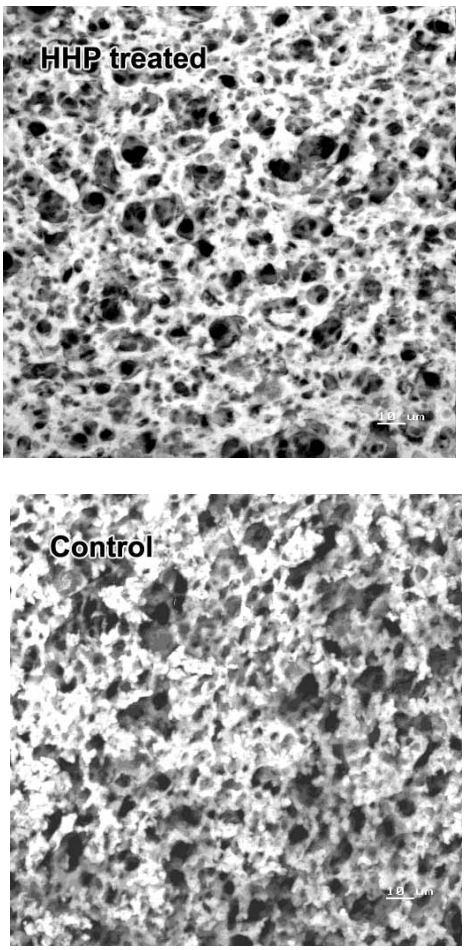

Figure 4. Changes in cheese microstructure due to HHP treatment. Protein network is visible in white, dark holes correspond mainly to globules of fat. Three-dimensional reconstruction of 16 optical sections obtained by laser scanning microscopy along a 20 μm cheese slice.

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 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.

ACKNOWLEDGEMENTS

This research has been supported by the European Commission, project FAIR-CT96-1113 (Project title: High pressure treatment of liquid foods and derived products). Author J.S. has benefited from a doctoral grant given by the Catalan Commission for Universities and Research.

We would like to acknowledge the people of Can Bordoi-Formatgeria del Montseny (Cardedeu, Spain) for their kind support in providing cheese samples for this study.

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3.4 PROTEOLYSIS IN CAPRINE MILK CHEESE TREATED BY HIGH PRESSURE TO ACCELERATE CHEESE RIPENING (2002) J. Saldo, P.L.H. McSweeney, E. Sendra, A.L. Kelly, B. Guamis. *International Dairy Journal* 12 (1) 35-44

ELSEVIER

International Dairy Journal 12 (2002) 35–44

INTERNATIONAL DAIRY JOURNAL

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Proteolysis in caprine milk cheese treated by high pressure to accelerate cheese ripening

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Received 9 March 2001; accepted 24 November 2001

Abstract

The application of high-pressure treatment to a hard caprine milk cheese was studied as a method of acceleration of proteolysis during ripening. Levels of proteolysis in the cheese subjected to treatment at 50 MPa for 72 h were only slightly different to those in control cheese, with differences being less apparent by the end of ripening. Treatment at 400 MPa for 5 min caused more significant quantitative and qualitative changes in proteolysis that persisted throughout the ripening. This treatment resulted in increased levels of free amino acids, although cheese treated at 400 MPa had profiles of peptides and caseins, obtained by HPLC and PAGE, similar to younger untreated cheese or cheese treated at 50 MPa. Plasmin activity in cheese was unaffected by pressure treatment, whereas coagulant activity was decreased by treatment at 400 MPa. Overall, application of high pressure at the beginning of ripening significantly increased secondary proteolysis, or conversion of peptides into free amino acids.

Keywords: Multivariate analysis; Goat cheese; High pressure; Proteolysis; Accelerated ripening

1. Introduction

The effect of high hydrostatic pressure (HHP) is transmitted instantaneously and uniformly to all the material treated (isostatic principle). This avoids the possibility of over-treated spots in the foodstuff, as sometimes occurs with other technologies. The volume decrease imposed by HHP enhances reactions having a net decrease in volume, while actions that have a net increase in volume are retarded (Le Chatelier's principle). In the complex environment of a bacterial cell, many processes are influenced by high pressure, resulting in the inability of the bacteria to survive (Tewari, Jayas, & Holley, 1999). These processes range from functions of proteins and action of enzymes, to functions of cellular membranes. Small macromolecules that are responsible for flavour, odour and nutrition are typically not affected by pressure.

Biochemical reactions may be influenced by HHP by means of the effect on volume changes involved in the reaction or by the direct effect of pressure on enzymes. HHP produces conformational changes in proteins, and some changes may affect enzyme modulation sites or active sites directly (Rovere, 1995). Depending on conformation and enzyme function, those changes could activate or inhibit enzyme activity. By Le Chatelier's principle, reactions involving a reduction in volume, such as many proteolytic reactions, are favoured under pressure.

Acceleration of the ripening step of cheese production is an area of scientific and commercial interest. Accelerating ripening allows producers to reduce ripening space requirements and the financial cost of maintaining a large stock of cheese for long periods. This topic has been reviewed periodically (El Soda & Pandian, 1991; Wilkinson & Fox, 1993; Fox et al., 1996). Approaches to accelerating ripening have included elevated ripening temperatures, use of exogenous enzymes, or use of modified microorganisms. In this work, the possible application of HHP to cheese curds to accelerate cheese ripening was investigated.

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0958-6946/02/$ - see front matter
PII: S0958-6946(01)00169-8

HHP has previously been applied to cheese in order to destroy spoilage and pathogenic bacteria (Capellas, Mor-Mur, Sendra, Pla, & Guamis, 1996; O'Reilly, O'Connor, Kelly, Beresford, & Murphy, 2000) and to reduce the salting time required (Messens, Dewettinck, & Huyghebaert, 1999b; Pavia, Trujillo, Guamis, & Ferragut, 2000), with limited success in this last area. Much work involving HHP treatment of cheese has also focussed on acceleration of cheese ripening. Changes in enzymatic activity during HHP treatment (Yokoyama, Sawamura, & Motobayashi, 1992; Saldo, Sendra, & Guamis, 1999), or release of starter enzymes by pressure treatment (Kołakowski, Reps, & Babuchowski, 1998; Saldo et al., 1999; Messens, Foubert, Dewettinck, & Huyghebaert, 2000) may reduce ripening time. The effects of HHP on cheese texture and microstructure have also been studied (Messens, Arevalo, Dewettinck, & Huyghebaert, 1999a; Johnston & Darcy, 2000), with treated cheese generally exhibiting a softer and more plastic texture.

Flavour compounds are developed during cheese ripening and confer the characteristics of each type of cheese. Proteolysis is the most complex and the most important of the primary events that occur during the ripening of most cheeses (McSweeney & Sousa, 2000). The breakdown of the protein network plays an essential role in the development of textural properties and in the release of free amino acids. Those amino acids are then available for secondary catabolic reactions, which are of great importance in the production of sapid compounds. Some peptides may impart a bitter flavour to cheese, if proteolysis is not well balanced, leading to the accumulation of an excess of hydrophobic peptides of intermediate size (Habibi Najafi & Lee, 1996). Due to its complexity, proteolysis cannot be described by only one index. Fractionation of cheese and its analysis by electrophoresis, chromatographic analysis of peptides and quantitative determination of free amino acids provide a more complete view of this phenomenon. Multivariate analysis has been proved to be very useful for the study and interpretation of all the data generated from various analytical methods (Dewettinck, Dierckx, Eichwalder, & Huyghebaert, 1996; Pripp, Rehman, McSweeney, & Fox, 1999).

The objective of this study was to explore the changes in proteolysis during ripening of cheese made from goats' milk following HHP treatment.

2. Materials and methods

Garrotxa-type caprine milk cheese was made from pasteurised milk (72°C, 15 s) in the Universitat Autònoma de Barcelona Pilot Plant, as described elsewhere (Saldo et al., 2000). Two independent batches of cheese were manufactured. Each batch of cheese was divided into three groups, and treatments were carried out 1 d after salting. One group was treated at 50 MPa for 72 h, a second one was treated at 400 MPa for 5 min and the last group was kept at 0.1 MPa (control). All treatments were carried out at 14°C, which is the usual ripening temperature of Garrotxa cheese. After treatment, at d 4, the packaging was removed from all cheeses, which were ripened at 14°C and 86% RH until sampling. Samples were taken for all treatments at d 1 (before HHP treatment), d 4 (after the 50 MPa treatment was finished) and at d 14 and 28. Cheese pieces, after sampling, were immediately vacuum packaged and kept at −18°C until analysed.

On sampling, the rind was removed from each cheese and discarded; the remaining cheese was grated to obtain a homogeneous sample for analysis. Overall composition (total nitrogen, fat, moisture and sodium chloride) and pH were determined according to Ardö and Polychroniadou (1999). Residual coagulant activity was evaluated according to the method of Hurley, O'Driscoll, Kelly, and McSweeney (1999), and plasmin activity according to Richardson and Pearce (1981). Proteinaceous products were fractionated at pH 4.4 and using 70% ethanol (McSweeney & Fox, 1997). The concentration of individual free amino acids was determined in the 2% trichloroacetic acid-soluble fraction by RP–HPLC (Rehman, Pripp, McSweeney, & Fox, 1999). pH 4.4—insoluble fractions were analysed by urea-PAGE as described by Pripp, McSweeney, Sørhaug, and Fox (2000) and quantified using ImageMaster TotalLab v1.0 software (Amersham Pharmacia Biotech, Upsala). 70% ethanol-soluble and -insoluble fractions of the pH 4.4—soluble extract were analysed by RP–HPLC (Ardö & Polychroniadou, 1999).

Data from urea-PAGE gels, RP–HPLC chromatograms and free amino acid profiles were subjected to multivariate statistical analysis. Similar bands were recognized visually and matched in the urea-PAGE gels and peak heights of corresponding bands were measured and used as data for the statistical analysis. Peaks in RP–HPLC were identified by their retention time, and their area were used as data for the Principal Component Analysis (PCA) and cluster analysis, which was performed using Statistica v5.0 (StatSoft Inc., Tulsa, OK). A varimax normalized rotational strategy was used to obtain principal components. Complete linkage was chosen as the amalgamation rule in cluster analysis.

PCA allows exploration of the hidden structure of data when there are a large number of influencing variables. The observations in the n-dimensional space of n measured variables are distributed in a manner dependent on the variables. Some variables are dependent upon others, and some directions in the space may be established. The first principal component (PC1) is the direction of maximum variance in the original cloud of points. Coordinates of points over this direction are

called scores and the relationship between this direction and the original variables are the loadings. Subsequent principal components have a direction of maximum variance perpendicular to PC1. Those directions are calculated for all PC with sufficiently high significance (eigenvalues >1). Residual variance is taken as noise.

Cluster analysis is a tool for non-supervised recognition, which allows the study of groups of samples. Distances in *n*-dimensional space are calculated and it is assumed that points that cluster indicate similar samples. Linkage distance was calculated as the Euclidean distance between objects in the 16-dimensions space defined by the concentration (in mg/100 g cheese) of the 16 individual free amino acids quantified.

Significance of differences in compositional results was estimated by using 2-way ANOVA analysis (ripening time × treatment) and Tukey's Honest Significant Difference (HSD) test.

3. Results and discussion

3.1. Overall composition

Garrotxa cheese is ripened without packaging, leading to a decrease in moisture content during ripening. Immediately after HHP-treatment, at d 4, the moisture content of treated cheese was higher than that of control cheese. These differences increased during ripening. This higher moisture content for HHP-treated cheeses was also observed in previous studies (Saldo et al., 2000), and the higher water retention ability seems to be related to a change in the structure of the *para*-caseinate network. Protein content of cheese was 39% and fat content 52%, on a dry matter basis, for all samples.

In brine-salted cheese, salt penetrates by diffusion from the rind, reaching equilibrium in about 2 weeks in cheese of this size (about 300 g). Salt diffusion in HHP-treated cheeses appeared faster than in control cheeses (Table 1), which may be related to the higher moisture content. HHP brining has been studied as a means of promoting salt uptake in Gouda cheese by Messens et al. (1999b) and Pavia et al. (2000), who found that HHP treatment of cheese immersed in brine did not change salt or water profiles but that similar treatment of cheese that had been salted previously by immersion in brine promoted salt movement towards the cheese core.

An increase in pH of cheese after HHP treatment has been described for Gouda (Messens, Dewettinck, Van Camp, & Huyghebaert, 1998), Camembert (Kołakowski et al., 1998) and Père Joseph cheese (Messens et al., 2000). These increases depended on the type of cheese and the high-pressure treatment applied, and ranged from 0.1 to 0.5 pH units. This instantaneous pH increase has been related to the release of colloidal calcium phosphate into the aqueous phase of the cheese (Messens et al., 1998) and considered as reversible. No appreciable changes in pH or ionic calcium activity were found after HHP treatment of bovine (Johnston, Austin, & Murphy, 1992) or caprine milk (de la Fuente, Olano, Casal, & Juarez, 1999). Casein and calcium phosphate are more concentrated in cheese than in fluid milk and thus, the effects of high pressure on those constituents may be more significant in cheese than in fluid milk.

Table 1
Changes in compositional parameters of experimental cheese at different ripening times

	Control	50 MPa	400 MPa
Moisture (%)			
d 1	52.3_{f}	—	—
d 4	52.0_{f}	53.7_{g}	53.2_{g}
d 14	39.9_{b}	47.8_{e}	44.7_{d}
d 28	33.6_{a}	42.7_{c}	39.7_{b}
pH			
d 1	5.7_{c}	—	—
d 4	5.0_{a}	5.3_{b}	$5.6_{b,c}$
d 14	4.9_{a}	5.0_{a}	$5.5_{b,c}$
d 28	5.0_{a}	5.0_{a}	5.4_{b}
Salt content (%)			
d 1	1.3_{a}	—	—
d 4	1.9_{b}	2.2_{c}	2.3_{d}
d 14	2.3_{d}	3.1_{f}	2.8_{e}
d 28	2.7_{e}	3.1_{f}	2.8_{e}

Salt content at the cheese core during ripening was expressed as % NaCl in dry matter. Two-way ANOVA (ripening time and pressure treatment) was applied to data, and values with the same subscripts did not differ significantly ($P<0.05$, HSD test). The experiment was replicated twice, and each value corresponds to the mean of six values (four for pH).

An increase in pH was observed after HHP treatment (Table 1). While cheese treated at 50 MPa reached a final pH equivalent to that of the untreated cheese, the effect on cheese treated at 400 MPa was irreversible and remained throughout ripening. The effect of a treatment at 300 MPa on pH in Gouda cheese was totally reversed after 14 d (Messens et al., 1998).

3.2. Enzyme activity

Residual coagulant and plasmin activities are important for proteolysis in cheese, because they cause primary hydrolysis of caseins into peptides that are available for further degradation by enzymes from lactic acid bacteria. In this study, the activities of coagulant and plasmin were not affected by the age of cheese, in any of the cheese batches (data not shown).

Plasmin, the main indigenous proteolytic enzyme in milk, is formed from an inactive precursor, plasminogen; the system is under the control of a complex system of activators and inhibitors. Its activity in cheese depends on certain factors, including stage of lactation,

somatic cell count, pasteurization conditions, and cheese cook temperature, amongst others. In this study, cheese made in the two trials had different plasmin activities (5.4 and 2.6 arbitrary units), although the milk was obtained from the same herd, and collected within a week. Goats' milk used in the experiment was pooled from a bulk tank containing the whole milking of a herd of more than 400 goats, and the cause of the difference in plasmin activity is not known. As each batch had its own independent control, the differences due to treatment can be interpreted with confidence, reducing the importance of differences between batches.

Plasmin activity in cheese was not significantly affected by HHP treatment at any combination of conditions assayed ($P > 0.05$, HSD test). This barostability of plasmin has been previously reported (Trujillo, Guamis, & Carretero, 1998; Trujillo et al., 1998; Scollard, Beresford, Needs, Murphy, & Kelly, 2000).

In spite of different plasmin activity in the two experimental batches, trends seen suggest that effects of variation in plasmin content do not affect the overall impact of HHP on the cheese. Also, the fact that plasmin activity was not affected by any HHP treatment used suggests that the effects of HHP were not on plasmin anyway and thus, the variation in its activity should not change the effect of HHP treatment.

Residual coagulant activity was reduced in cheese treated at 400 MPa, to about half of the value in control cheese (Fig. 1). Other studies, where coagulants in solution were exposed to pressures of 400 MPa, have also indicated a reduction in activity of calf rennet (Trujillo et al., 2000).

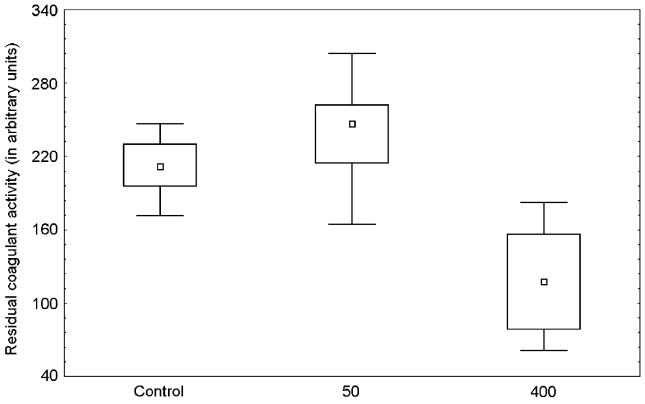

Fig. 1. Residual coagulant activity in cheese exposed to different HHP treatments. Enzyme activity was corrected for nitrogen content, and values from cheese at different ripening stages are presented together. Coagulant activity was significantly lower in cheese treated at 400 MPa for 5 min ($P < 0.01$, HSD test). The box includes the two central quartiles and the whiskers limit the non-outlier distribution.

3.3. Products of proteolysis

Urea-PAGE analysis of goat caseins and their degradation products showed some bands appearing as doublets or triplets, due to different phosphorylation levels of β- and α_s-casein. Degradation products of these proteins also appeared as multiple bands (Fig. 2).

From PCA analysis of densitometric data from urea-PAGE gels (Table 2), it can be seen that, for PC1, the highest loading factors corresponded to bands in the α_s-casein region (peaks numbered 10–12 in Fig. 3). Peaks numbered 16–18 had high factor loadings too, but of opposite magnitude. The occurrence of high loadings for both groups of peaks points to a causal correlation. The latter group, with higher electrophoretic mobility, was the degradation products of α_s-caseins. In PC2, among the highest factor loadings were β-caseins (peaks numbered 7–8), while in PC3, high loading values were mainly found for γ-caseins (peaks numbered 1–3).

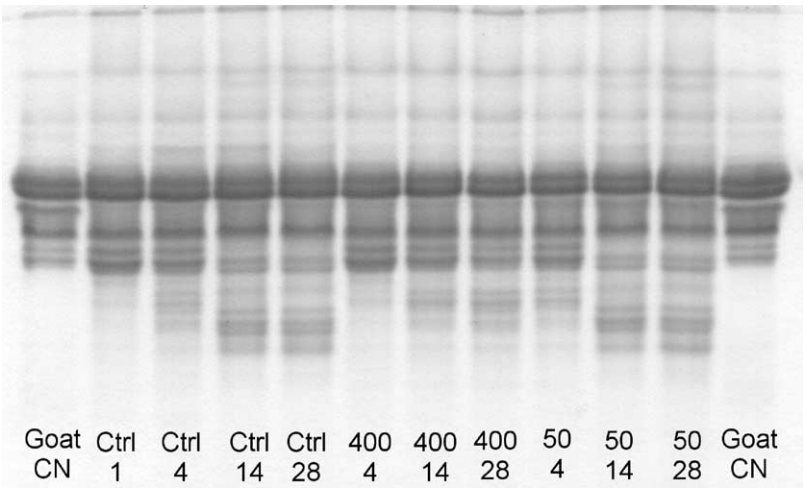

Fig. 2. Urea-PAGE electrophoretograms of caprine sodium caseinate (CN) and pH 4.4-insoluble fractions of cheeses. Cheeses are identified by pressure treatment (Control, 50 MPa/72 h or 400 MPa/5 min) and ripening time (1, 4, 14 or 28 d).

The PCA biplot of the urea-PAGE data (Fig. 4) showed that differences along PC1 between treatments for the early stages of ripening were very small. However, for samples later in ripening, values of PC1 were lower for cheeses treated at 400 MPa. These results are consistent with reduced coagulant activity. Rennet hydrolyses α_s-caseins, and this effect should thus be apparent in PC1 by a reduction of its amount and an increase in degradation products. The PC1 scores were significantly lower ($P<0.05$) for cheese treated at 400 MPa for 5 min than for control cheese.

Table 2
Significant correlations between urea-PAGE bands and principal components

Bands	Principal components			
	1	2	3	4
	Variability explained (%)			
	40.0	24.1	16.0	6.0
1	—	—	0.900^{***}	—
2	—	—	0.820^{***}	—
3	—	—	0.893^{***}	—
4	0.483^{*}	–	0.673^{***}	—
5	—	0.457^{*}	—	—
6	—	0.931^{***}	—	—
7	—	0.943^{***}	—	—
8	—	0.868^{***}	—	—
9	—	0.844^{***}	—	—
10	-0.892^{***}	—	—	—
11	-0.941^{***}	—	—	—
12	-0.920^{***}	—	—	—
13	—	—	0.566^{**}	-0.616^{**}
14	—	—	—	-0.849^{***}
15	—	—	0.474^{*}	0.453^{*}
16	0.905^{***}	—	—	—
17	0.930^{***}	—	—	—
18	0.922^{***}	—	—	—

$^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Band numbers correspond to peak numbers in Fig. 3.

The hydrolysis of β-casein was not well represented on PC2, the results being apparently scattered due to the different responses in the two batches studied. By ANOVA analysis of the PC2 values from urea-PAGE, no significant differences were found between treatments or between times ($P>0.05$). PC3 was selected to be represented in the score plot, as γ-caseins are produced from β-casein. β-Casein is the preferred substrate for plasmin and, in PC2 and PC3, the effect of this enzyme on the cheese ripening is apparent. PC3 scores were significantly higher ($P<0.05$) for cheese treated at 400 MPa for 5 min, in spite of non-significant differences in plasmin activity. This increase in products of plasmin activity may be explained by higher pH in these cheeses (Table 1); the optimum pH for this enzyme being near neutrality (pH 7.5).

From all the peaks observed in HPLC chromatograms of 70% ethanol-soluble fraction of cheese (Fig. 5), only those with loadings higher than 0.6 in the PCA of the correlation matrix were considered for further analysis (Table 3). Samples from cheese treated at 400 MPa were clearly different from the control and samples treated at 50 MPa, mainly at the end of ripening, according to the values of the second principal component (Fig. 6). PC2 had higher loadings mainly for the most hydrophilic peaks (peaks A–J, Fig. 5) while PC1 had higher loadings for hydrophobic peaks (peaks K–X, Fig. 5). The index of hydrophobicity, or area of hydrophobic peaks divided by area of hydrophilic peaks, decreased during ripening for all cheeses (data not shown). HHP-treated cheese had higher index of hydrophobicity than control cheese, and cheese treated at 400 MPa had the highest index of hydrophobicity throughout ripening. High amounts of hydrophobic peptides of high molecular weight have been related to bitterness in cheese (Habibi, Najafi, & Lee, 1996). In another study where HHP was applied to accelerate cheese ripening (Saldo et al., 2000), sensory assessment of cheese bitterness after 28 d of ripening found HHP

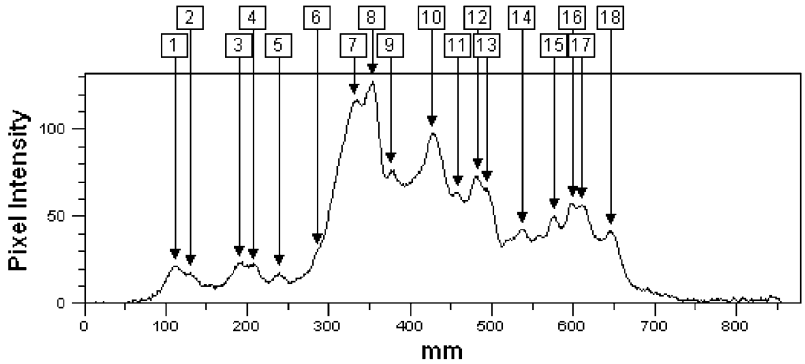

Fig. 3. Densitometric profile of the urea–polyacrylamide gel electrophoretogram of the pH 4.4-insoluble fraction of cheese treated at 400 MPa, after 14 d of ripening. Distances are from the beginning of the stacking gel. Corresponding bands recognized and compared in the gels are indicated by arrows.

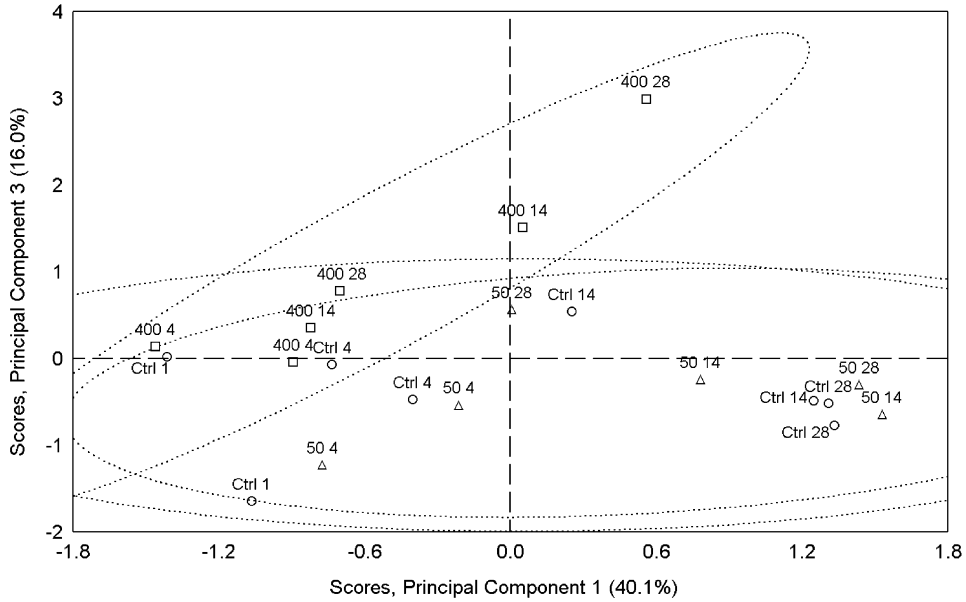

Fig. 4. Score plot obtained by PCA of urea-PAGE analysis of cheeses exposed to different HHP treatments. Cheeses are identified by pressure treatment (○: control, △: 50 MPa/72 h, □: 400 MPa/5 min) and ripening time (1, 4, 14 or 28 d). Results of each treatment are surrounded by an ellipse, which includes the members of this group with a probability of 0.95.

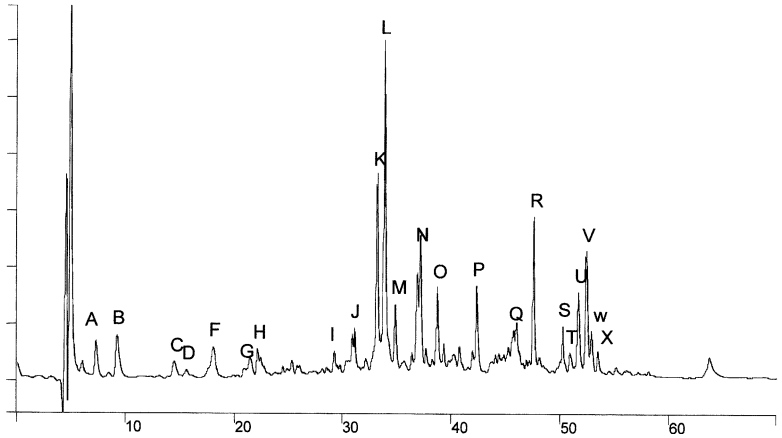

Fig. 5. Reversed-phase HPLC chromatogram of the 70% ethanol-soluble fraction of control cheese after 14 d of ripening. Peaks recognized and used as variables for multivariate statistical analysis are indicated by letters.

cheeses to be slightly more bitter than the control, cheeses treated at 400 MPa having the higher bitterness. These differences in sensory characteristics did not affect the preferences of panelists (Saldo et al., 2000). The reduction in primary proteolysis, and more specifically the lack of debittering activity due to reduced secondary proteolysis, may be linked to reduced residual rennet activity in cheese treated at 400 MPa.

The values of total free amino acid content at d 1 differed between batches (105.1 and 52.1 mg Leu/100 g cheese for batches 1 and 2, respectively), but the proportional increase in free amino acids during ripening was almost identical. The production of free amino acids in control cheese and cheese treated at 50 MPa was similar at all stages of ripening. Cheese treated at 400 MPa, however, had a slower production of free amino acids, but later in ripening, the rate was enhanced, reaching twice the value found in control cheese after 28 d (Table 4). An initial reduction in peptidolytic activity in cheese immediately after a treatment above 300 MPa has been described elsewhere (Sendra, Saldo, Capellas, & Guamis, 2000). The increased peptidolytic activity after this initial phase inmediately after the HHP treatment was caused by an increase in peptidase activity and not merely by an increase in the amount of peptides that would be substrate for this reaction, as shown by the increase in levels of free amino acids without an increase in peptide

amount. Peptidases in cheese derived from the cytoplasm of lactic acid bacteria and the increase in activity of such enzymes in the cheese matrix should be the result of an enhanced contact between the substrate and enzyme. Peptidases may have been released because of cell lysis caused by the HHP treatment applied. A reduction of about 3 log cycles on starter counts after a 400 MPa treatment was described by Saldo, McSweeney, Sendra, Kelly, and Guamis (2000).

Levels of individual free amino acids in cheese were also determined and subjected to multivariate analysis. Arginine was not included in the analysis because it was not possible to quantify this amino acid in all the samples. PCA simplifies the information about samples, and the profile of free amino acids was explained by only two variables (Table 5). PC1 gave information mainly about the total amount of free amino acids, while PC2 contained most of the information about the profile of free amino acids, allowing the treatments applied to cheese to be distinguished (Fig. 7). Despite there being higher free amino acid levels in cheese treated at 400 MPa at the end of ripening (Table 4), their profile was different from the one in control and cheeses treated at 50 MPa. The amino acids Asp, Cys and His were present in lower concentration in cheese treated at

Table 3
Significant correlations between original HPLC peaks and principal components

Peaks	Principal components			
	1	2	3	4
	Variability explained (%)			
	43.0	26.7	10.2	8.9
A	—	0.859^{***}	—	—
B	—	0.885^{***}	—	—
C	—	—	—	0.890^{***}
E	—	0.904^{***}	—	—
H	0.849^{***}	—	—	—
I	—	0.865^{***}	—	—
J	0.953^{***}	—	—	—
K	—	—	—	0.917^{***}
L	0.830^{***}	—	—	—
M	—	—	—	0.865^{***}
N	—	—	—	0.804^{***}
O	0.684^{***}	—	—	0.672^{**}
P	0.701^{***}	—	0.512^{*}	—
Q	—	0.811^{***}	—	—
R	0.792^{***}	—	—	—
S	0.742^{***}	—	0.517^{*}	—
T	0.846^{***}	—	—	—
U	—	—	0.962^{***}	—
V	0.649^{**}	—	0.573^{**}	—
W	—	—	0.799^{***}	—

$^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Peak names correspond to those on RP–HPLC chromatograms in Fig. 5.

Table 4
Increases in total free amino acid levels in cheeses during ripening, expressed as mg Leu/100 g cheese

	Control	50 MPa	400 MPa
d 4	38.1 $_{a}$	16.2 $_{a}$	0.8 $_{a}$
d 14	60.2 $_{a,b}$	85.4 $_{a,b}$	110.9 $_{b}$
d 28	136.1 $_{b}$	135.2 $_{b}$	307.2 $_{c}$

Two-way ANOVA analysis (ripening time and treatment) was applied to data and values with the same subscripts did not differ significantly ($P<0.05$, HSD test).

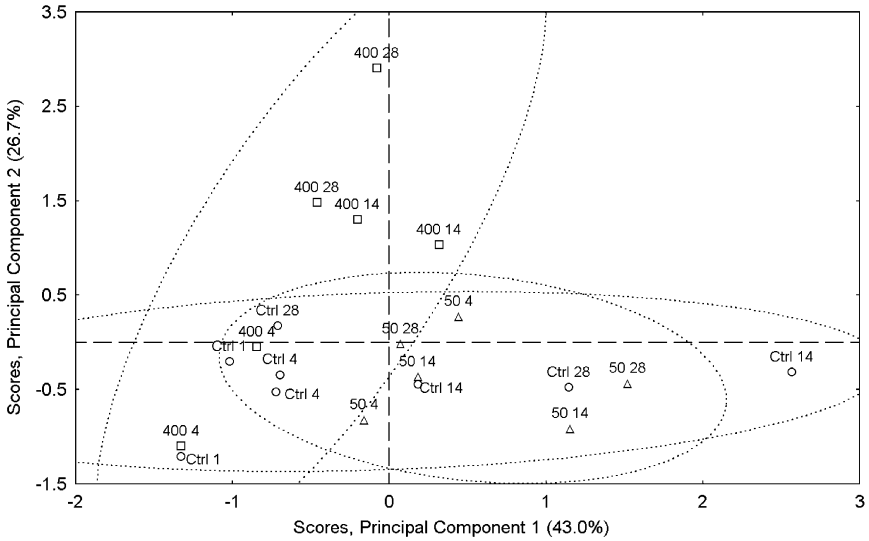

Fig. 6. Score plot obtained by PCA of HPLC chromatograms of the ethanol (70%)-soluble fraction from cheeses exposed to different HHP treatments. Cheeses are identified by pressure treatment (○: control, △: 50 MPa/72 h, □: 400 MPa/5 min) and ripening time (1, 4, 14 or 28 d). Results of each treatment are surrounded by an ellipse, which includes the members of this group with a probability of 0.95.

400 MPa than in control, and there were high loadings of those amino acids in PC2 (Table 5).

After 28 d of ripening, the free amino acid composition of the cheese treated at 400 MPa was different from all the other cheese samples (Fig. 8). All other cheeses were grouped in a separate cluster, although it was possible to distinguish between ripening stages.

Table 5
Significant correlations between original concentrations of individual free amino acids and principal components

	Principal components	
	1	2
	Variability explained (%)	
	80.6	10.5
ASP	—	0.879***
THR	0.905***	—
SER	0.929***	—
GLU	0.950***	—
PRO	0.852***	—
GLY	0.845***	—
ALA	0.757***	0.636**
CYS	—	0.949***
VAL	0.862***	0.459*
MET	0.922***	—
ILE	0.950***	—
LEU	0.848***	0.523*
TYR	0.693***	—
PHE	0.883***	—
HIS	—	0.800***
LYS	0.701***	0.654**

*$P<0.05$; **$P<0.01$; ***$P<0.001$.

4. Conclusions

HHP treatment of caprine cheese produced a more regular salt distribution and higher moisture content in cheese. Cheese treated at 400 MPa maintained a higher pH than control cheese, presumably due to reduced acidification activity of lactic acid bacteria after HHP treatment. These conditions, high moisture and high pH, favour accelerated ripening of cheese.

None of the HHP treatments studied affected plasmin activity, whereas a decrease in residual coagulant activity was apparent after treatment at 400 MPa. This partial inactivation of some key enzymes produced changes in the profiles of proteolysis, which were studied

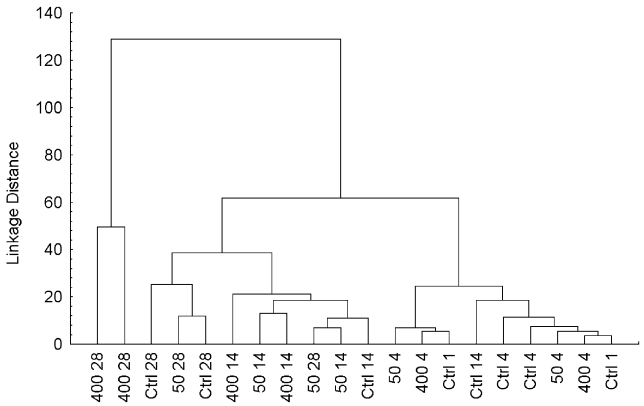

Fig. 8. Dendrogram from hierarchical cluster analysis of individual free amino acids (mg 100 g^{-1} cheese) from cheeses exposed to different HHP treatments. The amalgamation rule was complete linkage using Euclidean distance. Cheeses are identified by pressure treatment (control, 50 MPa/72 h or 400 MPa/5 min) and ripening time (1, 4, 14 or 28 d).

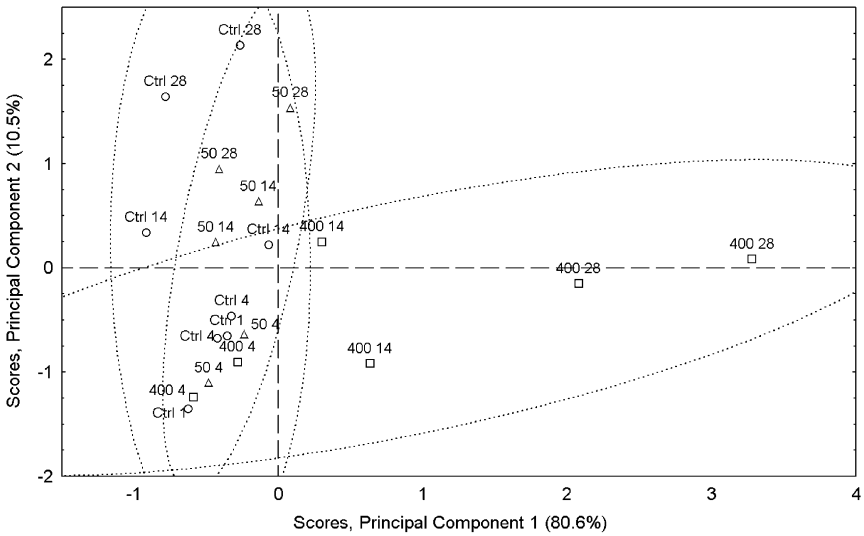

Fig. 7. Score plot obtained by PCA of free amino acids from cheeses exposed to different HHP treatments. Cheeses are identified by pressure treatment (○: control, △: 50 MPa/72 h, □: 400 MPa/5 min) and ripening time (1, 4, 14 or 28 d). Results of each treatment are surrounded by an ellipse, which includes the members of this group with a probability of 0.95.

using multivariate analysis. In terms of primary proteolysis, fully mature cheese (28 d) treated at 400 MPa was similar to less-ripened samples (4 and 14 d) of cheese treated at 50 MPa and control cheese. However, cheese treated at 400 MPa had a higher amount of free amino acids, usually used as indices of cheese maturity, possibly a result of higher peptidase activity. The treatment at 400 MPa reduced the activity of some proteases, reducing the rate of degradation of α_s-caseins and rendering a profile of primary proteolysis products similar to the profile corresponding to the samples of control and 50 MPa-treated cheese earlier in ripening.

The HHP treatments tested in this study are the extremes of likely pressure treatments to produce acceleration of ripening in cheese. The optimum treatment would increase peptidase activity without reducing protease activity. The results of the present work will open the way to deal with the restrictions of developing such a method of accelerated ripening.

Acknowledgements

Author J.S. has benefited from a doctoral grant given by the Catalan Commission for Universities and Research. He would also want to acknowledge the help and the assistance received from all the people of the Department of Food Science, in Cork, especially from Dr Sousa.

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