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ORIGINAL PAPER

Using High-Pressure Processing for Reduction of Proteolysis and Prevention of Over-ripening of Raw Milk Cheese

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Abstract High-pressure-processing (HPP) at 400 or 600 MPa was applied to cheeses made from ewe raw milk, on days 21 or 35 after manufacturing, to reduce proteolysis and prevent over-ripening. The characteristics of HPP and non-pressurized (control) cheeses were compared during ripening at 8 °C until day 60 and further storage at 4 °C until day 240. HPP and control cheeses showed similar pH values throughout ripening, but on day 240 pH values remained 0.4–0.6 units lower for HPP cheeses than for the control cheeses. Casein degradation was significantly retarded in the 600 MPa cheeses. Their α-casein concentration was 48–52 % higher on day 60 and 30–33 % higher on day 240 than in the control cheeses while β-casein concentration was 25–26 % higher on day 60 and 100–103 % higher on day 240. No significant differences in para-κ-casein concentration between cheeses were found on day 60, but on day 240, it was 22–35 % higher in the 600 MPa cheeses than in the control cheese. Hydrophilic peptides, hydrophobic peptides and total free amino acids evolved similarly in HPP and control cheeses during the 60-day ripening period. However, on day 240 hydrophilic peptides were at 34–39 % lower levels in the 600 MPa cheeses than in the control cheeses, hydrophobic peptides at 7–16 % lower levels and total free amino acids at 25–29 % lower levels. Flavour intensity scores increased at a slower rate in HPP cheeses than in the control cheese. Flavour quality declined markedly in the control cheeses during refrigerated storage while it did not vary significantly in 600 MPa cheeses.

Keywords High-pressure processing · Proteolysis · Over-ripening · Cheese · Flavour

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Introduction

The breakdown of proteins and lipids, and the metabolism of lactose and citrate, are primary biochemical events which take place during cheese manufacturing and early ripening. Afterwards, secondary biochemical events such as the further hydrolysis of peptides and the catabolism of amino acids, fatty acids and lactate, give rise to the formation of the compounds responsible for the characteristic flavour and aroma of each particular cheese variety (McSweeney and Sousa 2000; Collins et al. 2003; Yvon and Rijnen 2001). Simultaneously, the typical texture and the microstructure of the product develop (O'Reilly et al. 2003; Picon et al. 2013b). Secondary biochemical events occurring during mid- and late ripening pursue during refrigerated storage of cheese at the dairy and during its shelf life at retailers and homes. Consequently, over-ripening defects may surge before consumption, particularly in strongly proteolyzed cheese varieties.

When exceeding a certain threshold, ammonia, amines, alcohols, aldehydes, carboxylic acids and thiol compounds formed in cheese through amino acid catabolism (Yvon and Rijnen 2001) are among the main causative agents for the flavor and aroma defects associated to over-ripening. Freezing of fully ripened cheeses has been assayed to prevent over-ripening and prolong their shelf life, by stopping or retarding enzyme activity and chemical reactions. Even though flavour characteristics of cheeses remained unchanged during frozen storage, texture defects at thawing were common (Tejada et al. 2000; Van Hekken et al. 2005).

High-pressure processing (HPP) is a technology that can achieve the food safety level of heat pasteurization whilst meeting consumer demand for fresher-tasting minimally processed foods (Norton and Sun 2008). The inactivation of pathogenic and spoilage microorganisms has been the main objective when applying HPP to milk and cheese (O'Reilly et al. 2000; Trujillo et al. 2000; Shao and Ramaswamy 2011).

In addition, HPP could be useful to stop or retard some of the chemical reactions taking place during cheese ripening, since cheese-related enzymes, including the proteinases and peptidases responsible for the formation of peptides and free amino acids (FAA), are affected by HPP (García-Risco et al. 2003; Malone et al. 2003; Juan et al. 2007). Pressure level is more crucial to enzyme inactivation than temperature or time of exposure, such as shown for chymosin which loses less than 5 % of its activity in Cheddar cheese at 400 MPa and 93–96 % at 600 MPa (Huppertz et al. 2004b). HPP has been reported to hinder the formation of FAA by some authors. Thus, lower FAA concentrations were found in 1-month-old Cheddar cheese pressurized at 400 MPa on day 1 than in the respective control cheese (O'Reilly et al. 2002) and in 7-month-old Cheddar cheeses pressurized on day 30 at 400, 500 or 800 MPa than in the respective control cheese (Wick et al. 2004). Within the current applications of emerging technologies as alternatives to milk pasteurization in cheese manufacture, pulsed electric fields have also been assayed, with satisfactory results (Yu et al. 2012).

In the present work, we applied HPP to Casar cheese, a variety made from ewe raw milk coagulated with an aqueous extract of *Cynara cardunculus* L. (cardoon) flowers. This extract contains the aspartic proteinases named cyprosins or cynarases, with optimum activity around pH 5.1 (Heimgartner et al.1990). The strong proteolytic activity of cyprosins, the microbial load of raw milk and the high pH value of the cheese thus produced make it prone to over-ripening. Because of the seasonality in ewe milk production, ripe cheeses made during the first half of the year may be held at refrigeration temperatures for several months before marketed. This is also valid for similar varieties such as La Serena and Los Pedroches cheeses in Spain, and Serra da Estrela and Azeitao cheeses in Portugal. With the objective of preventing the undesirable consequences of a prolonged storage period, we applied HPP to cheeses 3 or 5 weeks after manufacture. The breakdown of caseins, the formation of peptides and FAA, the texture and the sensory characteristics of HPP cheeses during a 60-day ripening period and a further 180-day refrigerated storage period were investigated and compared with the characteristics of control cheese.

Materials and Methods

Cheese Manufacture

Two batches of Casar cheese were made on consecutive days, each from 600 L of refrigerated ewe raw milk without added starter cultures, at a Protected Designation of Origin dairy. Milk was coagulated at 30 °C for 60 min in a semi-automated open vat with an aqueous extract obtained by macerating 600 g of dry cardoon flowers overnight in 6 L of tap water and filtering through a cheese cloth. Curds were cut into 10-mm cubes, held at 30 °C for 15 min and distributed into cylindrical moulds. Cheeses, 13 cm in diameter and 6-cm high, were pressed for 3 h in a horizontal press, salted by rubbing dry salt twice on all the surfaces, and ripened at 8 °C and 92 % relative humidity.

High-Pressure Processing

Cheeses coded as 400W3, 600W3, 400W5 and 600W5 were vacuum-packaged in CN300 bags (Cryovac Grace S.A., Barcelona, Spain) and pressurized at 400 or 600 MPa for 5 min, after 3 or 5 weeks of ripening, respectively. HPP was performed in a 120-L capacity isostatic press (Hiperbaric, Burgos, Spain). Times to reach 400 and 600 MPa were 1.85 and 2.83 min, respectively and depressurization times, 7 and 8 s. The temperature of the water used as transmitting fluid remained under 14 °C during the whole process. Pressurized cheeses were unpackaged after HPP. Control cheeses were vacuum-packaged after 3 weeks of ripening and unpackaged simultaneously with cheeses pressurized at that time. Cheeses were ripened at 8 °C and 92 % RH until day 60, and afterwards held at 4 °C until day 240.

Chemical Determinations

Caseins and whey proteins were determined on duplicate samples by capillary gel electrophoresis according to a previously described method (Garde et al. 2002) with some modifications, on an automated P/ACE^{TM} MDQ capillary electrophoresis apparatus controlled by the 32 Karat Software (Beckman Instruments España S.A., Madrid, Spain). Briefly, 5 g of cheese were mixed with 25 mL of 2 % trisodium citrate solution, homogenized for 1 min in an Ultra-Turrax T-10 blender (IKA, Staufen, Germany) at high speed on ice. Twenty microlitres of cheese homogenate was mixed with 170 μL of 100 mM Tris–HCl buffer (pH 9.0) containing 1 % SDS, 10 μL of 2-mercaptoethanol and 4 μL of a 10-kDa internal standard (Beckman), and heated at 95 °C for 10 min before injection at 5 kV. Electrophoretic separation was performed at 15 kV for 30 min after a 4-min ramp, in a bare-fused silica capillary column (Beckman) of 50 μm internal diameter and 30 cm total length, in SDS-buffer gel (Beckman). To calculate the MW of peaks monitored at 214 nm, the coefficient of relative time mobility to the internal standard was compared with those of a mixture of 10, 20, 35, 50, 100, 150 and 225 kDa protein standards (Beckman, SDS-MW protein size standard). Commercial standards (Sigma, Alcobendas, Spain) of bovine α-casein, β-casein, κ-casein, α-lactalbumin, β-lactalbumin, serum albumin and lactoferrin were used for the identification of proteins. Proteins were quantified with respect to the internal standard area and expressed as milligramme of protein per gramme of cheese dry matter (DM).

Hydrophilic and hydrophobic peptides in the water-soluble fraction of cheese were determined on duplicate samples by RP-HPLC using a Beckman System Gold chromatograph (Beckman Instruments España) equipped with a diode array detector module 168, with detection wavelength at 280 nm, as previously described (Lau et al. 1991). Peaks with retention times from 5.5 to 14.6 min were considered to correspond to hydrophilic peptides and those with retention times from 14.6 to 20.5 min to hydrophobic peptides. Peptide levels were expressed in arbitrary units, calculated as units of chromatogram area per milligramme of cheese DM.

Free amino acids were extracted from duplicate samples (Krause et al. 1995) and individual FAA determined by RP-HPLC using a Beckman System Gold chromatograph, after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. They were expressed as milligrammes per gramme of cheese DM.

Cheese pH was measured in triplicate directly with a Crison penetration electrode (model 52–3,2; Crison Instruments, Barcelona, Spain) by means of a Crison GPL 22 pH-meter. Dry matter was determined on triplicate samples after drying to constant weight in an oven at 102 °C.

Cyprosin activity and overall peptidolytic activity in cheese were determined on duplicate samples. A homogenate of 5 g of cheese, 25 mL of 2 % trisodium citrate solution and 2 g of skim milk was acidified with 1 N HCl to pH 5.2, favourable for cyprosin activity, and incubated for 3 h at 37 °C. Then, the pH was adjusted with 1 N NaOH to 6.5, favourable for peptidase activity, and the incubation was prolonged for 3 h. The OPA test (Church et al. 1983), based on the reaction of released α-amino groups with this compound and with β-mercaptoethanol to form an adduct that absorbs strongly at 340 nm, was run on 0-h, 3-h and 6-h samples. Cyprosin activity was estimated as the increase in absorbance (OPA method) from 0 to 3 h during the incubation at pH 5.2 and 37 °C. Overall, peptidolytic activity was estimated as the increase in absorbance (OPA method) from 3 to 6 h during the incubation at pH 6.5, which reduces drastically cyprosin activity, and 37 °C. To discard the possible interference of milk plasmin with cyprosins or enzymes of bacterial origin present in cheese samples, the same assay was run on milk samples (taken from the vat, without added cardoon extract) at pH 6.7, the initial pH value of milk, and at pH values of 6.2, 5.7 and 5.2, after acidification with 1 N HCl.

Textural Determinations

Six cylinder-shaped (17-mm height, 17-mm diameter) samples from each cheese were compressed to 75 % of their original height after 10 min at room temperature (20–22 °C) using an Instron Compression Tester 4301 (Instron, High Wycombe, Bucks, UK), with crosshead and chart speeds of 50 and 500 mm/min, respectively. Fracturability (force at breaking point, expressed in Newtons), elasticity (apparent elastic modulus, expressed in Newtons per square millimetre) and firmness (work done on the cheese, expressed in Joules) were calculated from compression curves (Picon et al. 2013a).

Sensory Evaluation

A trained 15-member panel carried out the evaluation of flavour intensity and quality (preference), scoring on a 10-point scale as previously described (Nuñez et al. 1991). Five additional flavour attributes (acid, bitter, salty, sweet and umami) were also determined by panelists on a 10-point scale (Picon et al. 2013a).

Statistical Analysis

Data obtained were analyzed by a two-way analysis of variance, with HPP treatment and days of ripening as main effects. Means were compared using Tukey's test, with $p=0.05$. The SPSS Win 14.0 software (SPSS, Inc., Chicago, IL) was employed for the statistical analysis of data.

Results and Discussion

Protein Breakdown

Casein concentrations declined significantly ($p<0.001$) with ripening time in control cheese, according to the analysis of variance, from 38.36, 272.55 and 25.76 mg of α-, β- and para-κ-casein per gramme of cheese DM on day 1 (data not shown) to 5.80, 81.93 and 16.09 mg/g DM, respectively, on day 60. These marked decreases in casein levels agree with previous works on cheese varieties made from ewe milk coagulated with cardoon extract (Garde et al. 2007; Delgado et al. 2010). Regarding whey proteins, α-lactalbumin, at a concentration of 1.54 mg/g DM on day 1, could not be detected in control cheese from day 21 onwards (data not shown) while β-lactoglobulin concentration decreased significantly ($p<0.01$) in control cheese with ripening time, from 11.63 mg/g DM on day 1 to 5.13 mg/g DM on day 60.

HPP at 400 or 600 MPa did not significantly influence casein levels immediately after treatment. However, higher α-casein levels were found for the 600 MPa cheeses during ripening and refrigerated storage (Fig. 1), with a concentration of α-casein 48–52 % higher in the 600 MPa cheeses than in control cheeses on day 60 and 30–33 % higher on day 240. The concentration of β-casein in pressurized and control cheeses did not differ significantly during ripening, but it was 100–103 % higher in the 600 MPa cheeses than in the control cheese on day 240. No significant differences in

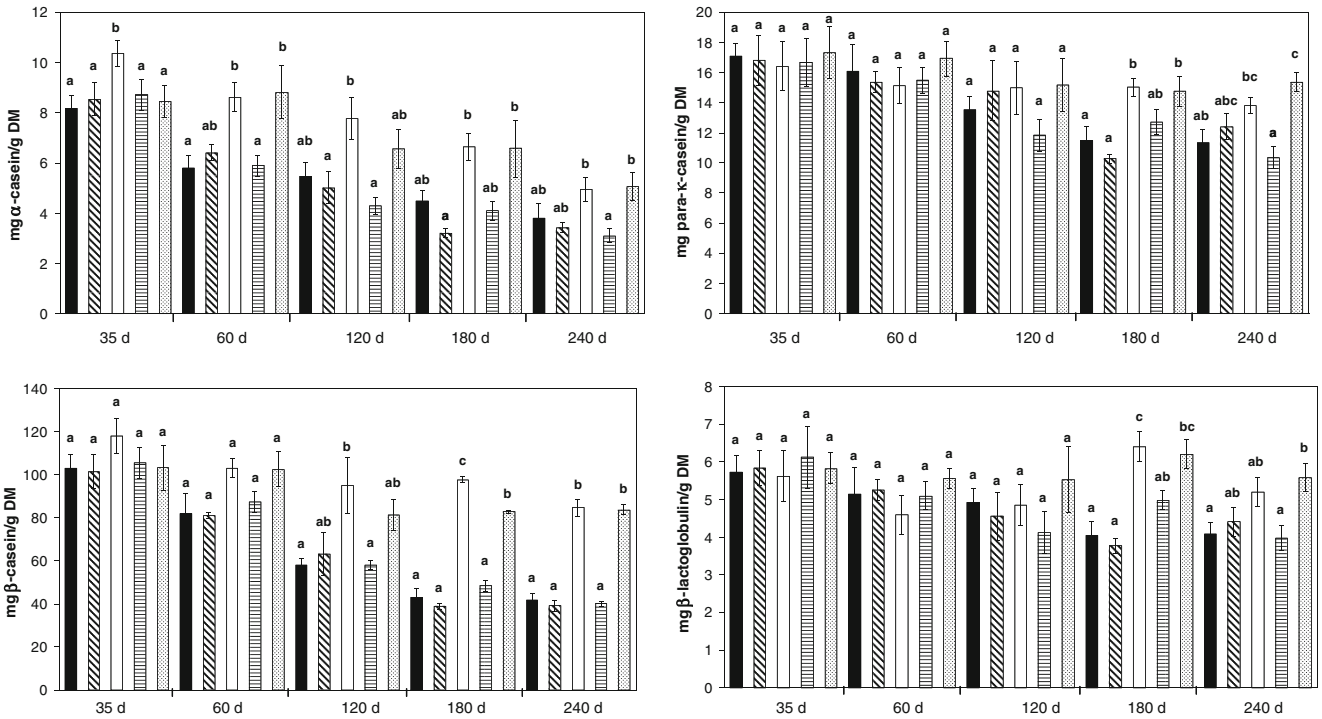

Fig. 1 Concentrations of caseins and β-lactoglobulin during ripening and refrigerated storage of control and HPP cheeses. Control (*black bar*), 400W3 (*obliquely striped bar*), 600W3 (*white bar*), 400W5 (*horizontally striped bar*), 600W5 (*dotted bar*). Means (bars with SEM) at the same sampling date with the same letter do not differ significantly ($p>0.05$)

para-κ-casein concentration between cheeses were found during ripening but on day 240 it was 22–35 % higher in the 600 MPa cheeses than in the control cheese. Similarly, there were no significant differences in β-lactoglobulin concentration between cheeses on day 60, whereas on day 240 it was 27–37 % higher in the 600 MPa cheeses than in the control cheese.

Protein degradation in the control cheese and in the pressurized cheeses until submitted to HPP can be associated to both plasmin and cyprosins. Cheese pressurization at 400 MPa has a negligible effect on plasmin activity, while treatment at 600 MPa reduces its activity by less than 10 % (Huppertz et al. 2004b). Therefore, considerable plasmin activity would remain in pressurized cheeses. The role of cyprosins in the proteolysis of HPP cheeses is difficult to establish since there is no information available on cyprosine barostability. In La Serena cheese, also made from ewe milk coagulated with cardoon extract, treatments at 300 and 400 MPa on day 2 resulted in higher levels of α_s- and β-caseins on days 30 and 60 of ripening than in the control cheese (Garde et al. 2007).

To ascertain the role of cyprosins in protein breakdown, cyprosin activity was estimated in HPP and control cheeses by determining the increase in absorbance by the OPA method after incubation for 3 h at 37 °C and pH 5.2, a pH value favourable for cyprosin activity (Heimgartner et al. 1990) but unfavourable for the activity of plasmin and most proteinases and peptidases from lactic acid bacteria. Apparently, there was a pressure-induced enhancement of cyprosin activity in 400 MPa cheeses, but only the cyprosin activity of 400W5 cheese on day 240 showed a significant ($p<0.05$) difference when compared with the respective control cheese (Table 1). Increases of activity after HPP have been reported for enzymes such as the cell envelope proteinase and the PepC aminopeptidase of *Lactococcus lactis* MG1363 (Malone et al. 2003). Cyprosin activity values in 600 MPa cheeses did not differ significantly from those of the respective control cheese. A high stability of cyprosins under cheese ripening conditions has been reported (Picon et al. 1999). In the present work, considerable cyprosin activity persisted, even in 600 MPa cheeses, until day 240. The increases in absorbance recorded for cheese samples during the 3 h of incubation at pH 5.2 cannot be attributed to the activity of milk plasmin. When the assay was run on milk without added cardoon extract, the increases of absorbance in the OPA test were as low as 0.022, 0.008, 0.004 and 0.003 for milk samples at pH values of 6.7, 6.2, 5.7 and 5.2, respectively. The overall peptidolytic activity was estimated by further incubation of cheese samples at pH 6.5 for 3 h, which resulted in additional increases in absorbance. The peptidolytic activity in 400 MPa cheeses and in control cheese did not differ (Table 1). However, the peptidolytic activity was 74 % lower in 600W3 cheese and 63 % lower in 600W5 cheese than in control cheese on day 60, while it was 90 % and 89 % lower,

Table 1 Cyprosin activity and peptidolytic activity in control and HPP cheeses at the end of ripening (day 60) and after refrigerated storage (day 240)

Days	Cheese	Cyprosin activity[a]	Peptidolytic activity[a]
60	Control	0.148±0.017ab	0.081±0.008b
	400W3	0.172±0.023ab	0.074±0.005b
	600W3	0.123±0.016a	0.021±0.016a
	400W5	0.207±0.025b	0.107±0.010b
	600W5	0.193±0.013ab	0.030±0.007a
240	Control	0.336±0.071a	0.119±0.038a
	400W3	0.439±0.052a	0.125±0.052a
	600W3	0.346±0.041a	0.012±0.015a
	400W5	0.596±0.045b	0.056±0.037a
	600W5	0.424±0.031a	0.013±0.022a

Results are expressed as mean±SEM of duplicate determinations on two cheese making trials. Means in the same column at the same sampling date with the same letters do not differ significantly

[a] Cyprosin and peptidolytic activities are expressed as increases in absorbance (OPA method)

respectively, on day 240 (Table 1). These results point to the inactivation of peptidolytic enzymes of bacterial origin at 600 MPa, in agreement with previous works (Malone et al. 2003; Avila et al. 2006), and may be of practical interest to control over-ripening at the cheese industry.

Cheese pH and DM increased significantly ($p<0.001$) with time, according to the analysis of variance. They modulate enzyme activity and may influence protein breakdown. Control cheese and HPP cheeses had similar pH values until day 60 (Fig. 2) but afterwards the pH rose more rapidly in the control cheese, which showed significantly ($p<0.05$) higher values than HPP cheeses from day 120 onwards. The higher pH values of the control cheese during refrigerated storage would have been less favourable for the activity of cyprosins, with optimum pH values around 5.1 (Heimgartner et al. 1990), but more favourable for plasmin, with maximal activity at slightly alkaline pH (Visser 1981). This fact may contribute to explain the lower concentrations of residual proteins in the control cheese than in the pressurized cheeses. Regarding cheese DM, no significant differences were found between HPP and control cheeses during ripening, until day 60. At the end of the refrigerated storage period, on day 240, higher ($p<0.05$) DM content was recorded for 600W3, 400W5 and 600W5 cheeses than for the 400W3 cheese and control cheese (Fig. 2).

Peptide and FAA Formation

The levels of hydrophilic and hydrophobic peptides and their ratio were significantly ($p<0.001$) influenced by time, according to the analysis of variance. There were no significant changes in peptide levels attributable to HPP immediately after treatment on days 21 (data not shown) and 35. On day 60, at the end of ripening, the 400W3 cheese had a significantly ($p<0.05$) higher level of hydrophilic peptides than the control cheese (Fig. 3) while the rest of the HPP cheeses did not differ from the control cheese. This result may be ascribed either to an enhancement of cyprosin activity by HPP at 400 MPa, a pressure level which apparently increased its activity, or to changes in the conformation of proteins caused by HPP (García-Risco et al. 2000; Huppertz et al. 2004a) which might have favoured the access of cyprosins to their substrates. The level of hydrophobic peptides on day 60 in control and HPP cheeses did not differ, with the only exception of 400W5 cheese which showed a lower level. In 60-day-old La Serena cheese pressurized at 400 MPa on day 2, hydrophilic peptides also attained a higher level than in the control cheese, while hydrophobic peptides were at a lower level (Garde et al. 2007). The hydrophobic peptides/hydrophilic peptides ratio reached on day 60 values of 1.55–1.68 in HPP cheeses and 1.87 in control cheese, markedly higher than the 0.54 mean value recorded for ewe raw milk Manchego cheese made using animal rennet (Gaya et al. 2005) but close to the 1.50 value found for La Serena cheese (Garde et al. 2007).

The level of hydrophilic peptides increased from day 60 to day 240 by factors of 3.19 in the control cheese and 1.78–2.56

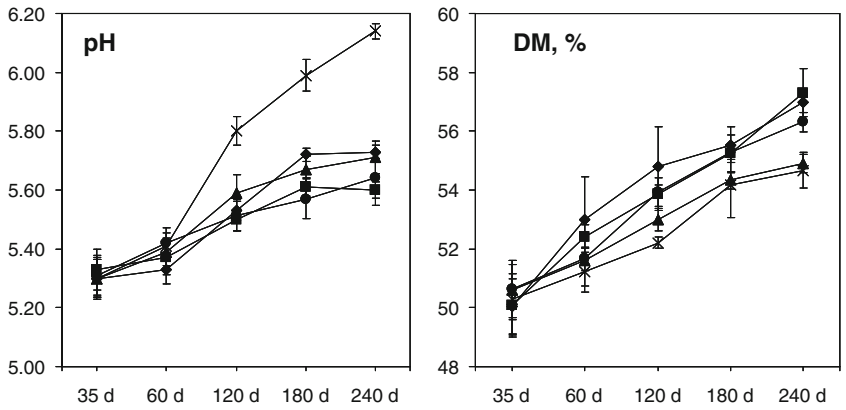

Fig. 2 Values (means with SEM) of pH and dry matter (DM) content during ripening and refrigerated storage of control and HPP cheeses. Control (*X*), 400W3 (*triangle*), 600W3 (*circle*), 400W5 (*diamond*), 600W5 (*square*)

in the HPP cheese. At the end of refrigerated storage, the level of hydrophilic peptides in the control cheese was significantly ($p<0.05$) higher than in the rest (Fig. 3), in agreement with its low concentrations of residual proteins. On the contrary, the levels of hydrophobic peptides hardly varied during refrigerated storage and on day 240 there were no significant differences in the contents of hydrophobic peptides between HPP and control cheeses. The hydrophobic peptides/hydrophilic peptides ratio fell sharply during refrigerated storage, to values of 0.64–0.92 on day 240, with no significant differences between HPP and control cheeses. To our knowledge, no information is available on the post-ripening evolution of peptides in the Casar or La Serena cheeses during the refrigerated storage period, which may span for several months because of the seasonality in milk and cheese production.

The concentration of FAA increased significantly ($p<0.001$) with time. It did not vary immediately after the HPP of cheeses on days 21 (data not shown) and 35. However, total FAA were on day 35 at significantly ($p<0.05$) higher levels in the 400W3 cheese than in the rest (Fig. 3), as already found for hydrophilic peptides, which served as substrates for the activity of peptidolytic enzymes. Pressurization of cheeses at 400 MPa most probably lyses a fraction of the bacterial cells without a negative effect on the activity of the enzymes which are released into the medium (Picon et al. 2013a), thus increasing the extracellular peptidolytic activity in cheese. Higher FAA concentrations on days 30 and 60 of ripening were also found for La Serena cheese pressurized at 400 MPa on day 2 (Garde et al. 2007). In the present work, the FAA concentration was 53 % higher in the 400W3 cheese than in the control cheese on day 60, but afterwards the differences in FAA between the 400W3 cheese and the control cheese were no longer statistically significant. The higher pH values of the control cheese during refrigerated storage most probably enhanced the activity of the peptidolytic enzymes of microbial origin, increasing the FAA concentration to levels close to those of the 400 MPa cheeses. At the end of the refrigerated storage period, on day 240, the cheeses pressurized at 600 MPa showed significantly ($p<0.05$) lower FAA concentrations than the rest (Fig. 3), a result which can be associated to the observed inactivation of peptidolytic enzymes at the higher pressure level (Table 1).

Texture

Firmness and elasticity declined significantly ($p<0.001$) with time. A marked softening of cheese texture was observed during the first weeks of ripening, resulting in the soft creamy texture desirable for this type of cheese. Firmness, as determined from the compression curves, reached low values in HPP and control cheeses (Fig. 4), consequently with the

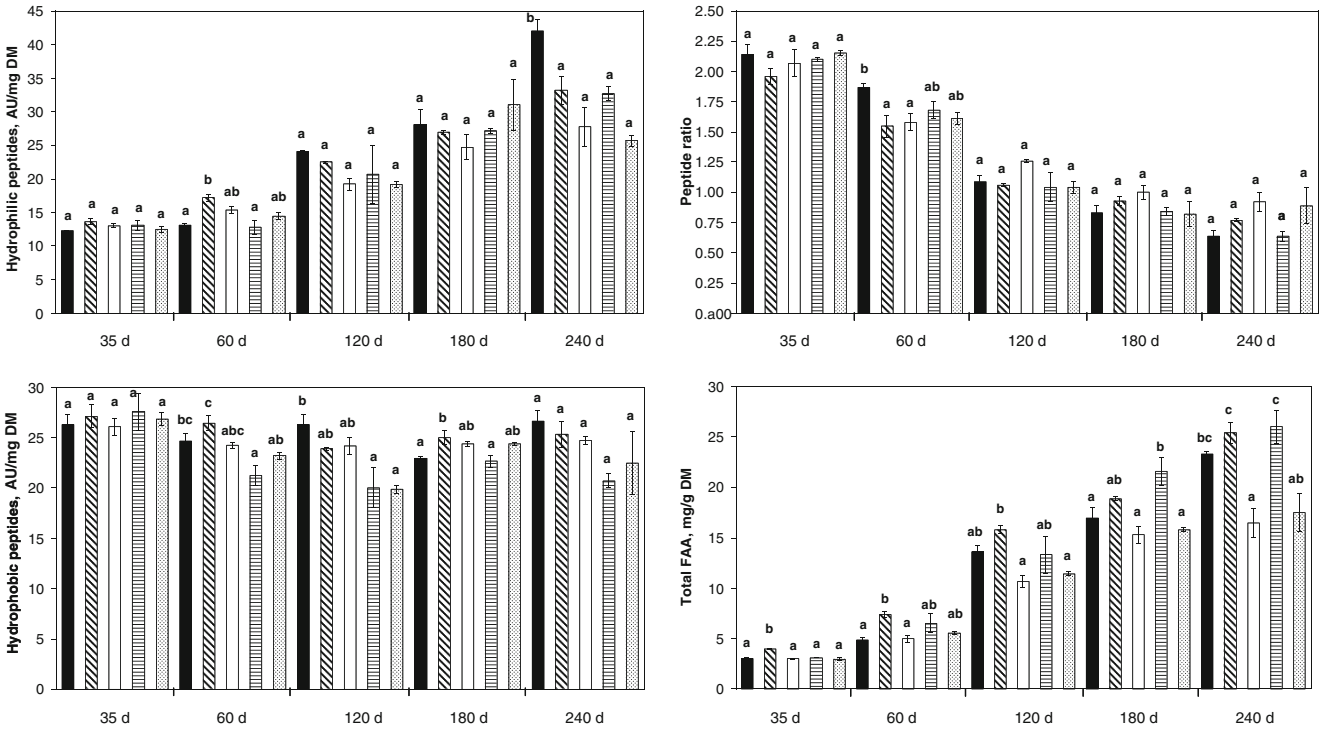

Fig. 3 Levels of hydrophilic peptides, hydrophobic peptides, hydrophobic/hydrophilic ratio and total free amino acids (FAA) during ripening and refrigerated storage of control and HPP cheeses. Control (*black bar*), 400W3 (*obliquely striped bar*), 600W3 (*white bar*), 400W5 (*horizontally striped bar*), 600W5 (*dotted bar*). Means (bars with SEM) at the same sampling date with the same letter do not differ significantly ($p>0.05$)

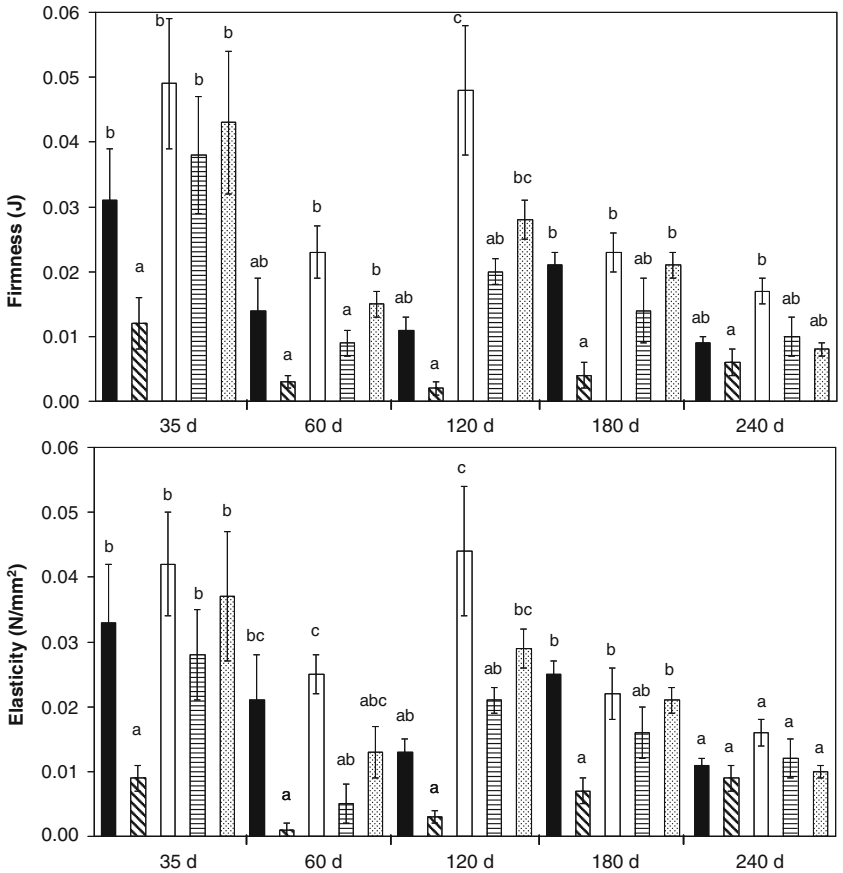

Fig. 4 Texture parameters (firmness and elasticity) during ripening and refrigerated storage of control and HPP cheeses. Control (*black bar*), 400W3 (*obliquely striped bar*), 600W3 (*white bar*), 400W5 (*horizontally striped bar*), 600W5 (*dotted bar*). Means (bars with SEM) at the same sampling date with the same letter do not differ significantly ($p>0.05$)

extensive proteolysis due to the use of cardoon extract as milk coagulant. The level of intact caseins, in particular of α_{s1}-casein, influences the stability of the cheese protein network (Creamer and Olson 1982). In the present work, higher firmness values were generally found for the 600 MPa cheeses, which showed the highest levels of intact caseins. A strong correlation between residual caseins and firmness had been recorded for La Serena cheese (Fernández del Pozo et al. 1988). Also, HPP by itself strengthens the texture of cheeses made from milk coagulated with cardoon extract, with an increase in the values of texture parameters immediately after treatments which is more marked at higher pressure levels (Garde et al. 2007).

Two additional factors influencing cheese texture are pH value and DM content. At high pH values the casein molecules acquire a negative net charge and ionic interactions change from attraction to repulsion, weakening cheese texture (Creamer and Olson 1982). In the present work, the lower pH values of 600 MPa cheeses during refrigerated storage probably contributed to their relatively higher firmness. Negative correlations between pH values and firmness of La Serena cheese had been reported (Fernández del Pozo et al. 1988). A higher DM content is known to strengthen the matrix structure (Nuñez et al. 1991). However, the small differences in DM content between cheeses observed in the present work precluded a significant effect of DM on texture.

Elasticity exhibited a pattern similar to that of firmness, with higher values generally for the 600 MPa cheeses and lower values for the 400 MPa cheeses (Fig. 4). The compression curves showed no breaking point, impeding the determination of the fracturability parameter.

Sensory Evaluation

Both flavour intensity and flavour quality were significantly ($p<0.001$) influenced by time, according to the analysis of variance. HPP at 600 MPa applied on day 21 retarded flavour development during ripening, with lower ($p<0.05$) intensity scores on day 60 for the 600W3 cheese than for the respective control cheese (Fig. 5) and no significant differences between the control cheese and the rest of the HPP cheeses. Afterwards, flavour intensity increased in all cheeses, with significantly ($p<0.05$) lower scores for the 600 MPa cheeses than for the control cheese from day 180 onwards (Fig. 5) and no significant differences between the 400 MPa cheeses and control cheese. A significant ($p<0.01$) correlation was found between flavour intensity scores of cheeses during the whole refrigerated storage period and the respective levels of total FAA. Flavour intensity scores of 60-day-old La Serena cheeses pressurized at 300 or 400 MPa on days 2 and 50 after manufacturing did not differ significantly from that of control cheese (Garde et al. 2007). The results obtained in the present

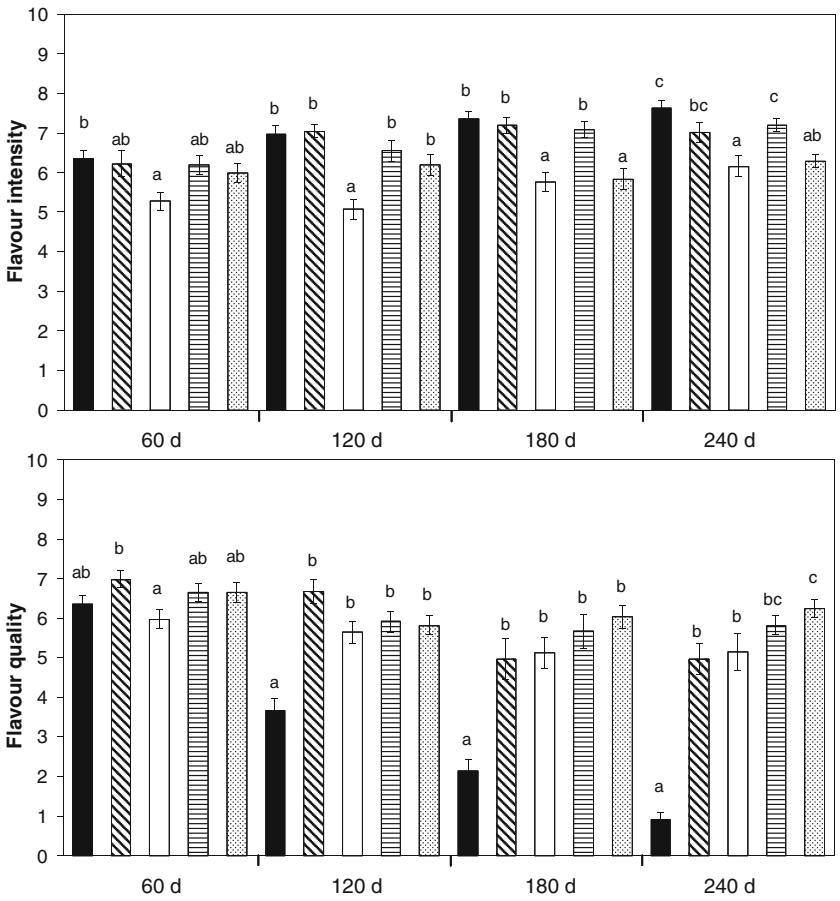

Fig. 5 Sensory characteristics (flavour intensity and flavour quality) during ripening and refrigerated storage of control and HPP cheeses. Control (*black bar*), 400W3 (*obliquely striped bar*), 600W3 (*white bar*), 400W5 (*horizontally striped bar*), 600W5 (*dotted bar*). Means (bars with SEM) at the same sampling date with the same letter do not differ significantly ($p>0.05$)

work for 400 MPa cheeses are in agreement with those obtained for La Serena cheese treated at 400 MPa in spite of the different days of ripening at the time of pressurization.

Flavour quality scores at the end of ripening, on day 60, showed few significant differences between cheeses. However, the flavour quality of the control cheese declined dramatically throughout refrigerated storage reaching significantly ($p<0.05$) lower scores than all the HPP cheeses from day 120 onwards (Fig. 5). The flavour quality score of 400W3 cheese also declined with time, attaining significantly lower values on days 180 and 240 than on day 60. The highest flavour quality score on day 240 was that of 600W5 cheese, which did not vary during refrigerated storage. La Serena cheese pressurized at 400 MPa on day 2 after manufacture showed a significantly lower flavour quality score on day 60 than control cheese, but there was no difference in quality if the cheese was pressurized on day 50 (Garde et al. 2007). As the study on La Serena cheese ceased on day 60, the results of the present work during refrigerated storage cannot be compared.

Flavour descriptors "acid", "salty", "sweet" and "umami" were not influenced by HPP. Differences were occasionally found during refrigerated storage for "bitter" scores, which attained higher ($p<0.05$) values on day 120 for control cheese and 400W3 cheese in comparison with 600W3 cheese, on day 180 for control cheese in comparison with 600W3 and 600W5 cheeses, and on day 240 for control cheese in comparison with 600W3 cheese (data not shown). These differences could not be associated to the levels of hydrophobic peptides. An opposite effect of HPP was reported for La Serena cheese, which showed increased bitterness when pressurized on day 2 at 300 or 400 MPa and on day 50 at 300 MPa in comparison with the control cheese (Garde et al. 2007). The different ripening time of cheeses at HPP and the higher pressure level (600 MPa) applied in the present work may be responsible for the variation in results.

Conclusions

Research on HPP application to cheese has been mainly focused on its effects during the ripening period. The objective of the present work was to preserve cheese flavour quality during post-ripening refrigerated storage, mostly of cheese varieties with seasonal variations in production. According to the results obtained, HPP appears as a useful tool to prevent over-ripening of raw milk cheeses, in particular of those

varieties which suffer extensive proteolysis because of manufacturing procedures, e.g. if cardoon extract is used for milk coagulation. HPP at 600 MPa was particularly effective in retarding the breakdown of proteins and the formation of peptides and FAA during prolonged refrigerated storage. Flavour development during the 60-day ripening period was similar in pressurized and control cheeses. Afterwards, HPP prevented the dramatic decline in flavour quality recorded for the control cheese throughout the refrigerated storage. HPP cheeses, with the only exception of 400W3 cheese, retained until day 240 flavour quality scores not differing from those of the respective 60-day-old cheeses. The HPP of cheeses at 600 MPa may thus be recommended to prevent over-ripening and maintain flavour quality during prolonged refrigerated storage.

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