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ORIGINAL PAPER

Physicochemical, proteolysis and texture changes during the storage of a mature soft cheese treated by high-pressure hydrostatic

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Abstract Physicochemical, proteolysis and textural changes during the refrigerated storage of mature Torta del Casar cheese (a raw ewe milk cheese) subjected to high-pressure treatment (200 or 600 MPa for 5 or 20 min) were studied. Cheeses were analysed after processing at day 60 and after 60, 120 and 180 days of refrigerated storage (days 120, 180 and 240, respectively). High-pressure processing (HPP) had a negligible effect on physicochemical parameters analysed. Nevertheless, nitrogen fractions were significantly affected by HPP. Soluble nitrogen/total nitrogen and non-protein nitrogen/total nitrogen ratios decreased during storage in cheeses treated at 600 MPa compared with control ones. In the same way, proteolysis of casein fractions (para-κ-casein; αs1-CN I, II, III, αs2-CN: αs-caseins; and β1-CN, β2-CN: β-caseins) at day 240 was significantly reduced by HPP at 600 MPa. The levels of αs-caseins and β-caseins found in cheeses treated at 600 MPa and analysed at the end of storage (day 240) were similar to those obtained in control cheeses at day 120 and 180, respectively. Firmness (N) and consistency (Ns) were affected by pressurisation just after HP treatment (day 60), but these changes were reduced during the storage. Therefore, HP treatments at 600 MPa could be a useful tool to delay the Torta del Casar over-ripening associated with an excess of proteolysis during long storage periods.

Keywords High pressure · Raw milk · Cheese proteolysis · Refrigerated storage

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Introduction

High-pressure (HP) treatment or high-pressure processing (HPP) is a "cold pasteurisation" method of food preservation used for a wide range of products to ensure their microbiological safety and extend their shelf life. HPP also maintains the sensorial and nutritional properties of food [1, 2]. Food products are introduced into a vessel and subjected to a high level of isostatic pressure (ranging from 200 to 600 MPa). The HP is instantaneously and uniformly transmitted independently of the size, geometry, shape and composition of food.

Proteolysis has a direct influence on flavour through the production of short peptides and amino acids, some of which can cause bitterness (e.g. from β-casein hydrolysis), by favouring the release of sapid compounds from the cheese matrix and by providing free amino acids (FAA) that are substrates for a series of catabolic reactions that generate important flavour compounds [3]. Unpleasant aromas or tastes could appear due to an excessive proteolysis. In many cheese varieties, the initial hydrolysis of caseins is caused by the coagulant and to a lesser extent by plasmin, which results in the formation of large (water-insoluble) and intermediate-sized (water-soluble) peptides, which are degraded subsequently by the coagulant and enzymes from the starter and non-starter microorganisms of the cheese [4]. However, cheeses manufactured with vegetable rennet from *Cynara cardunculus*, like Torta del Casar cheese, develop an intense proteolysis, giving rise to a more homogeneous structure, thus increasing creaminess and softening of the cheese. Proteolysis by rennet is responsible for the softening of cheese texture early during ripening via the hydrolysis of αs1-caseins, which is sufficient to break the continuous protein matrix [5].

Proteolysis is the most important maturation route for the formation of the characteristic aroma and soft texture of Torta del Casar cheese [6]. The application of HPP could retard the maturation routes of formation of these tastes and thus enhance the shelf life of this cheese. HP treatments are able to modify cheese ripening, in particular, proteolysis by causing alterations in enzyme structure, conformational changes in the casein matrix and/or bacterial lysis [7, 8]. The pattern and extent of proteolysis have been widely studied and vary considerably between cheese varieties because of differences in manufacturing practices and ripening protocols. Furthermore, the effect of HPP on the ripening process has produced a wide range of results in different cheeses, resulting sometimes in ripening acceleration and others in deceleration. Previous studies to evaluate changes in proteolysis of ewe milk cheese observed an increase in the peptidolytic activity and FAA in cheese treated at 300 MPa on day 1 due to bacterial lysis and the release of intracellular aminopeptidases [9]. Similar results have been reported in Hispánico cheese, manufactured with a mixture of cows and ewes milk [10], and La Serena cheese, made from raw ewe milk and vegetable rennet, which showed the highest levels of proteolysis in cheese treated at 400 MPa on day 2 after 60 days of ripening [7]. On the contrary, Juan et al. [11] reported that casein degradation in cheeses treated at 300 or 400 MPa on day 2 was lower than control cheeses after 60 days of ripening. Despite all, studies about ripening changes during long-term refrigerated storage are scarce.

Torta del Casar cheese is one of the traditional Spanish cheeses having the protected denomination of origin (PDO) designation. The region of production is in the south-west of Spain, in which its production plays an important role in the local economy. It has a light and thin semi-hard rind, and the pasta has a very soft texture with relatively high spreadability. Raw milk from Merino and Entrefino ewe breeds is used, and no starter cultures are added. Vegetable rennet, previously obtained from maceration of thistle flowers (*Cynara cardunculus* L.) in water, is used to coagulate the milk. The use of raw ewe milk and vegetable rennet provides a peculiar slightly bitter taste and a spreadable texture. Torta del Casar cheese must be ripened for at least 60 days because it is made from raw milk.

One of the problems of the producers of Torta del Casar is that after a long time of storage, this cheese develops undesirable tastes (bitter, pungent, sharp) and texture changes that are rejected for the consumers. They are associated with a long maturation or commercialisation extent. This fact reduces the shelf life of the Torta del Casar and shortens the commercialisation period of the cheese, producing significant economical losses. Commercials, distributors and/or industrials have great interest on maintaining the quality of cheese during refrigerated storage (e.g. in the supermarket or at home), because it is very important that the cheese retains its characteristics and original properties for as long as possible.

A previous study on the effect of HP treatment on proteolysis of Torta del Casar cheese has been carried out [12]. Researchers found that HP treatment (400 or 600 MPa, for 5 min) at the midterm of the Torta del Casar ripening (21 or 35 days old) reduced the proteolysis and the cheese over-ripening. However, pressurised cheeses were unpackaged after HPP because they were not yet mature. This fact could be problematic because the rules of the PDO may not allow the cheese processing during their ripening. Therefore, the application of HPP at the end of cheese ripening (day 60) could be a more convenient procedure. In addition, cheese could be placed on the market vacuum-packaged and just after HP treatment ("ready to be sold"). For these reasons, the main objective of the present paper was to study the proteolysis and texture changes after the application of HP treatments at low or HP intensities (200 or 600 MPa) for a short or long periods of time (5 or 20 min) in mature "Torta del Casar" cheeses (60 days old) and evaluate the effect during the refrigerated storage (vacuum-packed cheeses) in order to know whether HPP could extend the commercialisation period of this cheese.

Materials and methods

Cheese-making

Torta del Casar cheese was produced in a PDO dairy from Merino and Entrefino raw ewe milk. The milk was heated to 30 °C. No starter cultures were added, and vegetable rennet obtained from the thistle *C. cardunculus* L. flowers was used. The vegetable coagulant for cheese-making was prepared using 500 g of dry flowers from *C. cardunculus*, ground in a mortar and soaked in tap water (10 l) at room temperature for 24 h and filtering through a cheese cloth. The coagulation took place after ~50 min. The curd was cut, agitated for 15–20 min, transferred to perforate moulds to facilitate the removal of whey, and pressed for 3–4 h at pressure 1–2.5 kg cm^2. After that, cheeses were submerged in brine (sodium chloride 14–16 % w/v; pH 5.5–5.7) for ~4 h. Cheeses were ripened for 60 days at 4–10 °C and 80–90 % relative humidity. The composition of the cheese after 60 days of ripening was 59 % dry matter, 52.5 % fat in dry matter and 1.5 % salt. These values were in line with those included in the regulation of PDO [13].

High-pressure processing

A total of 80 mature cheeses (60 days old) from the same production batch were studied. Cheeses (4 cheeses

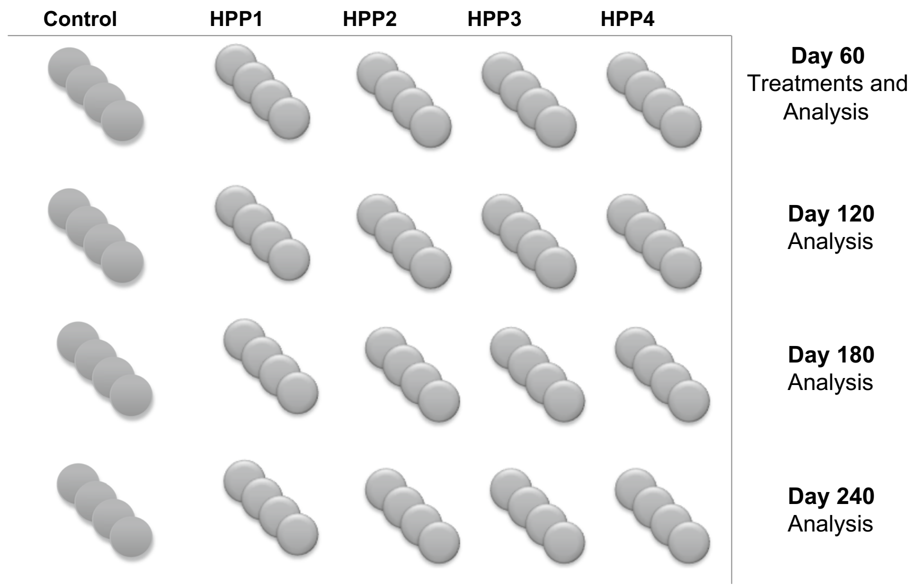

Fig. 1 A schematic illustration of the experimental design. HPP: high-pressure processing. HPP1: 200 MPa, 5 min; HPP2: 200 MPa, 20 min; HPP3: 600 MPa, 5 min; HPP4: 600 MPa, 20 min. Cheeses are symbolised by *circles*

per batch) were HP-treated at 200 or 600 MPa for 5 or 20 min at the end of ripening (day 60) to study the effect of pressure and time of treatment. Cheeses were vacuum-packed in 20/70 polyamide/polyethylene bags with a size of 30 × 40 cm and 90 µm of thickness, O_2 permeability of 50 cm^3 per m^2 per 24 h at 1 atm, 24 °C and 75 % relative humidity (Eurobag & film, Málaga, Spain). HP treatment was carried out in a semi-continuous high-pressure unit Hiperbaric Wave 6000/55 (55–L, Hiperbaric Burgos, Spain). Water was used as the pressure-transmitting medium, and water temperature was 10 °C. Times to reach 600 and 200 MPa were 3 min 50 s and 1 min 5 s, respectively. Cheeses were analysed after processing at day 60 and after 60, 120 and 180 days of refrigerated storage at 6 °C (days 120, 180 and 240, respectively). Cheeses were stored in the same package used for HP treatment. Vacuum-packaged cheeses were stored for 180 days to simulate the conditions of a supermarket. A total of 20 cheeses were analysed in each sampling: 4 control, 4 HP-treated at 200 MPa for 5 min, 4 HP-treated at 200 MPa for 20 min, 4 HP-treated at 600 MPa for 5 min and 4 HP-treated at 600 MPa for 20 min cheeses (Fig. 1). After sampling, cheeses were stored at −40 °C until analysis.

These treatments were chosen because literature usually applies treatments between 400–600 MPa and holding times between 5–10 min. Normally, the effect of holding time is not very marked in that range. In addition, some parameters are not affected by the application of pressure intensities between 400 and 600 MPa. For these reasons, it was decided to apply HP treatments with more extreme conditions to produce a more marked effect on cheeses parameters.

Physicochemical analyses

pH was measured directly in the centre of cheeses using a portable puncture pH metre Crison mod. 507 (Crison Instruments, Barcelona, Spain). Dry matter was assessed by a gravimetric method, drying cheese samples at 102 °C [14]. Colour parameters were determined with a Minolta CR–200 colorimeter (Minolta Camera Co., Osaka, Japan) with illuminant D65, an 8 mm port/viewing area and a 0° viewing angle. Before use, the colorimeter was standardised with a calibration plate ($Y = 93.2$; $x = 0.3159$; $y = 0.3324$ to D65). The following colour parameters were determined: lightness (CIE L*), redness (CIE a*: +red, −green) and yellowness (CIE b*: +yellow, −blue). The measurements were repeated at five randomly selected locations on each sample, and average data were reported.

Nitrogen fractions and free amino acid (FAA) measurements

A 0.5 mol L^{-1} tri-sodium citrate dispersion with cheese was used for Kjeldahl analysis of total nitrogen (TN) and soluble nitrogen compounds at pH 4.4 (SN). Soluble non-protein nitrogen (NPN) in 12 % trichloroacetic acid was analysed from the SN fraction [15]. For FAA determination, water-soluble extract was prepared according to Cd-ninhydrin method described by Folkertsma and Fox [16].

Capillary zone electrophoresis (CZE)

The application of CE to the assessment of proteolysis in cheese has acquired an enormous importance in the last years [17, 18] including ewes milk cheese [19]. Caseins were obtained according to Delgado et al. [6] and freeze-dried until analysis. Sample buffer and electrophoresis buffer were prepared according to Feligini et al. [20]. Freeze-dried casein samples for CZE were obtained as described by Clément et al. [21]. Corrected peak areas (CPA) (value obtained dividing the integrated peak area by migration time) were reported. In addition, levels of casein fractions (para-κ-CN, αs2, αs1-I, αs1-II, αs1-III, β2 and β1) in cheese were determined on triplicate samples. Results were expressed as CPA (value obtained dividing the integrated peak area by migration time).

Texture analysis

Texture was measured using a TA-XT2i texture analyser Stable Micro Systems Ltd (Aname, Spain). Texture was measured at 15 ± 1 °C. Cheeses were opened cutting the whole upper rind to evaluate the texture of the soft paste of the cheese. Both cylinder (Aluminium cylinder probe P⁄25, 25 mm diameter; Aname) probes were used and penetrated into the sample at 1 mm s^{-1} to a depth of 8 mm from the surface and then withdrawn at 5 mm s^{-1} through a two-cycle sequence. In the force–deformation curves, the following parameters were calculated: hardness (N); maximum force required for compressing the sample (peak force during the first compression cycle); consistency (Ns) or the area below the first compression curve; and the adhesiveness (Ns) as the work necessary to pull the compressing plunger away from the sample was reported.

Statistical analysis

An analysis of variance (ANOVA) was performed to establish the effect of time of storage and HP treatment. Therefore, this analysis was applied twice. Highest significant difference Tukey's test was applied to compare the mean values of parameters. Mean values and standard error of mean were reported. SPSS software version 14.0 was used for the statistical analysis.

Results and discussion

Physicochemical composition in HP-treated Torta del Casar cheese

Table 1 shows the physicochemical composition of Torta del Casar cheese after HPP at day 60 and after the refrigerated storage (days 120, 180 and 240). pH values were within the range required by the PDO (5.2–5.9) in both control and HP-treated cheeses [13]. Therefore, pH values were as expected for this type of cheese. There were no differences in pH between control and HP-treated cheeses. In addition, pH values did not significantly change during the refrigerated storage. In line with these results, in a previous study, we reported that pH value of a raw goat milk cheese was not modified after HP treatment (400 or 600 MPa, for 7 min) on day 60 and after 30 days of refrigerated storage [22]. In agreement with our results, Garde et al. [7] reported that HP treatments (300 or 400 MPa) at day 50 had no effect on pH values of La Serena cheese analysed at day 60. La Serena cheese is made from raw milk and vegetable rennet, like Torta del Casar cheese; this fact could explain the similar effect of HPP on pH found in both cheeses. On the contrary, according Voigt et al. [8], HP treatments (400 or 600 MPa) of mature blue-veined cheeses caused changes in pH values. Therefore, the effect of HP treatments on pH depends on the type of cheese, among other factors. Changes in pH during cheese ripening may be caused by bacterial inactivation after HP treatment. However, pH differences between HP-treated and control samples become less significant during the cheese ripening [23].

There were no significant differences in moisture content between control and HP-treated cheeses (Table 1), but moisture content was modified during the storage time in HP-treated cheeses (except in cheeses treated at 200 MPa 20 min). Moisture content of these cheeses significantly decreased during storage. In our opinion, moisture decrease (in mass) is due to water condensation on rind cheese caused by both the plastic and low temperature. By contrast, control cheeses showed similar moisture values during that time. According the literature, HP treatments do not change moisture content in cheese [23]. However, the pressurisation of cheese can increase the relation bound water/free water in cheese matrix [24]. In agreement with our results, Garde et al. [7] found that HP treatment did not significantly affect the dry matter content of La Serena cheese (a soft cheese, similar to Torta del Casar cheese); however, the treatment could affect the water retained in the matrix during the storage. On the contrary, Calzada et al. [12] found that pressurisation at 600 MPa at day 21 of Casar cheese increased the dry matter content, but the treatment was carried out during the cheese ripening.

Regarding the effect of HPP on the instrumental colour of Torta del Casar cheese, in general, there were no significant differences in colour between control and HP-treated cheeses (Table 1). The instrumental colour of control cheeses was not modified during storage, while some HP treatments caused colour changes during this time. CIE L* decreased in cheeses treated at 600 MPa for 20 min. In addition, CIE a* decreased during the refrigerated storage in all HP-treated cheeses and CIE b* decreased in some HP treatments (200 MPa/20 min and 600 MPa/5 min).

Table 1 pH, moisture (g 100 g^{-1}) and instrumental colour parameters measured in Torta del Casar cheese after HPP at day 60 and after 60, 120 and 180 days of refrigerated storage (days 120, 180 and 240, respectively)

	Day	Control	200 MPa		600 MPa		SEM	*P* value
			5 min	20 min	5 min	20 min		
pH	60	5.39	5.29	5.39	5.35	5.47	0.03	0.545
	120	5.37	5.39	5.40	5.40	5.40	0.02	0.968
	180	5.59	5.54	5.50	5.45	5.44	0.03	0.630
	240	5.57	5.48	5.46	5.48	5.43	0.02	0.475
P value		**0.048**	0.142	0.609	0.197	0.928		
Moisture	60	41.16	41.65^{A}	40.30	41.45^{A}	43.69^{A}	0.52	0.337
	120	39.14	39.66^{AB}	39.76	40.13^{AB}	39.18^{B}	0.33	0.895
	180	37.94	36.56^{C}	39.45	37.89^{B}	38.33^{B}	0.38	0.195
	240	36.60	37.00^{BC}	36.82	38.74^{AB}	38.95^{B}	0.40	0.219
P value		0.091	**0.001**	0.114	**0.025**	**0.004**		
CIE L*	60	98.67	96.35	98.16^{AB}	99.54	100.42^{A}	0.54	0.135
	120	96.17^{ab}	95.99^{ab}	$95.22^{b,B}$	97.00^{ab}	$98.12^{a,B}$	0.30	**0.012**
	180	97.18	97.04	98.48^{A}	97.97	97.56^{B}	0.22	0.212
	240	97.26	97.54	97.47^{AB}	98.35	97.60^{B}	0.26	0.787
P value		0.175	0.523	**0.023**	0.133	**0.001**		
CIE a*	60	-0.52^{a}	$-1.28^{ab,B}$	$-1.17^{ab,BC}$	$-1.62^{b,B}$	$-0.76^{ab,AB}$	0.13	**0.044**
	120	−0.69	-1.05^{AB}	-1.63^{C}	-1.70^{B}	-1.38^{B}	0.15	0.177
	180	−0.23	-0.40^{AB}	0.12^{AB}	-0.91^{B}	-0.04^{A}	0.15	0.262
	240	−0.12	0.15^{A}	0.19^{A}	0.68^{A}	-0.5^{AB}	0.16	0.178
P value		0.452	**0.042**	**0.003**	**0.002**	**0.029**		
CIE b*	60	2.75	6.34	6.36^{AB}	4.90^{A}	1.90	0.61	**0.019**
	120	3.58	4.32	6.42^{A}	5.04^{A}	4.71	0.48	0.459
	180	3.08	3.71	1.76^{BC}	3.61^{A}	1.95	0.46	0.579
	240	2.54	1.06	0.60^{C}	-2.17^{B}	2.41	0.60	0.058
P value		0.885	0.061	**0.004**	**0.003**	0.121		

P values less than 0.05 are in bold

SEM standard error of mean

a, b: different lower case letters in the same row indicate significant statistical differences (Tukey's test. $P < 0.05$). A, B, C: different capital letters in the same column indicate significant statistical differences (Tukey's test. $P < 0.05$)

Colour of cheese can change after HPP when pressurisation is carried out during the cheese ripening. In this regard, Saldo et al. [25] evaluated colour changes in HP-treated goat cheeses at 400 MPa for 5 min at 14 °C during ripening and 60 days after pressure treatment. CIE L* was significantly lower in HP-treated cheese than in control cheese at the beginning of storage, whereas CIE a* and b* values were higher than control cheeses during all the storage period. In addition, HP treatment at 400 MPa (for 5–15 min) of 1-day-old full fat Cheddar and Turkish white-brined cheeses caused a decrease in CIE a* and an increase in CIE b*, whereas CIE L* remained constant [26, 27]. According to the literature, the treatment conditions (pressure intensity, holding time and processing temperature) have a large influence on the colour of cheese; however, cheese age can be also an important factor. In general, researchers relate the effect of HPP on the colour of cheese to changes in the microstructure of cheese after pressurisation [25, 28]. In this case, the modifications of the moisture content and/or the changes in the conformation of the protein matrix and/or the proteolytic changes produced on it could be associated with these changes.

Nitrogen fractions and free amino acids (FAA) in HP-treated Torta del Casar cheese

The nitrogen fractions (% total nitrogen, TN) and FAA found in Torta del Casar cheese after HPP at day 60 and after refrigerated storage are shown in Table 2. At day 60, SN/TN and NPN/TN values were high in control cheese (42 and 22 %, respectively). Sousa and Malcata [29] reviewed the role of vegetable coagulant in cheese proteolysis and reported that SN/TN was significantly higher for ovine (46 %) than for bovine (33 %) milk cheeses after 68 days of ripening. In addition, La Serena cheese (60 days old), made from ovine milk and vegetable coagulant, showed SN/TN and NPN/TN values of 38 and 15 %, respectively [30]. In a previous study in mature Torta del Casar cheese, SN/TN and NPN/TN values were 45 and 23 %, respectively [31].

HPP did not change the levels of nitrogen fractions when cheeses were analysed immediately after HPP at day 60. However, nitrogen fractions were significantly affected by HP treatment at day 120, 180 and 240 of sampling. SN/TN ratio (a ripening index) showed a significant decrease in cheeses treated at 600 MPa for 20 min compared with

Table 2 Nitrogen fractions (% total nitrogen, TN) and free amino acids (mg Leu g^{-1} cheese) found in Torta del Casar cheese after HPP at day 60 and after 60, 120 and 180 days of refrigerated storage (days 120, 180 and 240, respectively)

	Day	Control	200 MPa		600 MPa		SEM	*P* value
			5 min	20 min	5 min	20 min		
SN/TN	60	42.28^{B}	37.78^{B}	40.55^{C}	41.60^{B}	39.81	0.58	0.107
	120	48.21^{AB}	51.03^{A}	51.35^{B}	45.83^{A}	46.24	0.75	**0.024**
	180	52.39^{ab,A}	52.39^{ab,A}	55.67^{a,A}	46.01^{bc,A}	44.04^{c}	1.23	**0.002**
	240	50.10^{a,AB}	52.93^{a,A}	52.81^{a,AB}	47.29^{ab,A}	42.88^{b}	1.01	**0.001**
P value		**0.025**	**0.001**	**0.001**	**0.009**	0.085		
NPN/TN	60	22.38^{C}	20.90^{B}	21.84^{B}	21.90^{B}	19.39^{B}	0.71	0.735
	120	26.13^{ab,B}	32.43^{a,A}	31.78^{a,A}	25.25^{ab,AB}	20.02^{b,B}	1.25	**0.001**
	180	33.28^{a,A}	33.78^{a,A}	35.77^{a,A}	26.30^{b,AB}	25.69^{b,A}	1.07	**0.001**
	240	33.68^{a,A}	33.52^{a,A}	35.44^{a,A}	28.34^{b,A}	27.40^{b,A}	0.86	**0.001**
P value		**0.001**	**0.003**	**0.001**	0.067	**0.002**		
FAA	60	1.89^{D}	1.78^{C}	1.77^{D}	1.67^{D}	1.64^{D}	0.04	0.301
	120	3.55^{ab,C}	3.17^{b,C}	4.02^{a,C}	3.77^{ab,C}	3.84^{ab,C}	0.09	**0.014**
	180	5.75^{B}	6.04^{B}	6.14^{B}	5.98^{B}	6.01^{B}	0.09	0.783
	240	8.44^{A}	8.47^{A}	8.18^{A}	7.78^{A}	7.99^{A}	0.22	0.872
P value		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		

P values less than 0.05 are in bold

SEM standard error of mean, *SN* soluble nitrogen at pH 4.4, *TN* total nitrogen, *NPN* non-protein nitrogen, *FAA* free amino acids

a, b, c: different lower case letters in the same row indicate significant statistical differences (Tukey's test. $P < 0.05$). A, B, C: different capital letters in the same column indicate significant statistical differences (Tukey's test. $P < 0.05$)

control cheese, during the storage process (Table 2). Cheeses treated at 600 MPa for 20 min and analysed at day 240 showed a SN/TN ratio similar to control cheese at day 60. In addition, cheeses treated at 600 MPa for 5 min and analysed at day 240 had a SN/TN ratio similar to control cheese at day 120. These results indicate that HP treatment at 600 MPa on mature Torta del Casar cheese could reduce the proteolysis development during the storage, and thus, these treatments could avoid the over-ripening during the time of commercialisation of cheese.

NPN/TN ratio significantly decreased in cheeses pressurised at 600 MPa for 5 or 20 min compared with control cheese. This fact suggests that HPP at 600 MPa of Torta del Casar cheese caused a reduction in the production of medium-sized to small peptides or amino acids, which are included in the NPN/TN fraction. Control and HP-treated cheeses had a similar FAA content, which is an index of secondary proteolysis. Therefore, the differences in the proteolytic pattern between control and HP-treated cheeses may be related to a lower content of medium-sized to small peptides in HP-treated cheeses than the control ones. This reduction in the levels of proteolysis in HP-treated cheeses can be caused by the inactivation of proteolytic enzymes from milk (plasmin) and vegetable rennet (cardosin) or polypeptidases from lactic acid bacteria and/or other microorganisms present in cheese. There are few studies about the effect of HPP on these enzymes when treatments are applied directly to cheese. Martínez-Rodríguez et al. [23] evaluated the effect of HPP on *C. cardunculus* aqueous extracts. They indicated that cardosin A and B of the extracts could be more affected by the treatment than chymosin and pepsin. Calzada et al. [12] reported that Torta del Casar cheese pressurised at 400 MPa on day 21 had a significantly ($p < 0.05$) higher level of hydrophilic peptides than the control ones at the end of ripening (day 60). According to those authors, these variations could be due to an enhancement of cyprosin (cardosin) activity by HPP or to changes in the conformation of proteins caused by HPP, which might have favoured the access of cyprosins to their substrates. However, on day 240, hydrophilic peptides showed lower levels in cheeses treated at 600 MPa than in the control ones, which is in line with our results.

With regard to the effect of HPP on the proteolysis of cheese, some HP treatments can increase the levels of proteolysis when the cheese is pressurised at the beginning of the ripening process [32, 33]. Garde et al. [7] reported that La Serena cheese pressurised (300 or 400 MPa for 10 min) on day 2 showed higher proteolysis levels (measured by OPA test) than control cheese when cheeses were analysed at day 60; however, cheeses treated at 300 MPa on day 50 had significantly lower proteolysis levels at day 60 than control cheeses. La Serena cheese has similar characteristics to Torta del Casar: raw ewe milk, vegetable coagulant and soft texture. According to those results and in agreement with our study, the application of HPP at the end of the maturation (day 60) would decrease the proteolysis of cheese during the storage. Therefore, it would be possible to limit proteolysis in soft cheese by HP treatment when cheeses are pressurised at the end of ripening. Similar results have been reported by other authors [8], who also found that mature blue-veined cheeses pressurised at 600 MPa for 10 min on day 42 showed lower levels of SN/TN and NPN/TN than control cheeses after 14 and 28 days of refrigerated storage. However, in a previous study carried out in raw goat milk cheese (60 days old), we noticed that the effect of HP treatment at 600 MPa for 7 min on nitrogen fractions disappeared after 30 days of refrigerated storage [22]. In addition, the same as in the present study, we did not find a significant effect of HPP on FAA levels neither after the pressurisation (day 60) nor after the subsequent storage (day 90) of the goat cheese. We have to highlight that sampling times were longer in the present work than those reported in the literature; therefore, the comparison of results is a difficult matter.

On the other hand, there was a significant increase in nitrogen fractions and FAA during the refrigerated storage of all groups of cheeses (Table 2). The nitrogen fraction levels increased at the beginning of storage, principally at day 120 (60 days of refrigerated storage), and then slight changes were found throughout storage. Therefore, primary proteolysis was limited at the midterm and the end of storage process (day 180 and 240). However, FAA amounts showed a significant increase during the whole time of the refrigerated storage.

Analysis of casein fractions by capillary electrophoresis in HP-treated Torta del Casar cheese

CPA of the casein fractions (para-κ-casein; αs1-CN I, II, III, αs2-CN: αs-caseins; and β1-CN, β2-CN: β-caseins) found in Torta del Casar during refrigerated storage are shown in Table 3. HP treatments of cheese at 200 MPa scarcely affected the proteolysis process. Casein fraction levels in control and cheeses pressurised at 200 MPa did not differ significantly at the end of refrigerated storage (day 240) except for para-k-casein fraction. The ineffectiveness of this treatment could be due to the barostability of cardosin; however, the role of cardosin in pressurised cheeses is difficult to determine because of the absence of studies in this area. In literature, it has been reported that inactivation of enzymes in cheese treated with HP depends on the level of pressure applied [34]. On the other hand, proteolysis of casein fractions at day 240 was significantly reduced by HP treatment at 600 MPa. No significant differences were observed in CPA values at day 240 between HP treatment at 600 MPa for 5 and 20 min.

CPA values reported at day 240 for para-k-casein in cheeses treated at 600 MPa for 20 min (24.5 % of the content at day 60) were significantly higher than control cheeses (14.1 % of the content at day 60). In the same way, CPA values obtained for β2-CN and β1-CN on cheeses treated at 600 MPa were significantly higher than those obtained in the control cheeses at day 240. In cheeses treated at 600 MPa for 5 or 20 min, β1 fraction at day 240 showed higher values (38–43 % of content at day 60, respectively) compared with control cheeses (23 % of content at day 60). The percentage with respect to the initial para-k-casein, β2 and β1 observed at day 240 in cheese pressurised at 600 MPa corresponded to approximately the value obtained at day 180 in control cheeses. Regarding αs1-I, αs1-II, αs1-III casein fractions were also detected significant differences at day 240 in pressurised cheeses at 600 MPa compared with control cheeses. In cheeses treated at 600 MPa, these casein fractions at day 240 showed a similar content to that obtained in control cheeses at day 120.

A recent study [12] has suggested the application of HPP to decelerate proteolytic reactions in similar cheeses as those utilised in this study with the objective of increasing their shelf life. They reported that cheeses treated at 600 MPa for 5 min at day 21 of ripening showed significant differences in the amount of para-k-CN compared with control cheeses at day 180 of storage. In our study, these differences were not observed until day 240 of storage, but we carried out the cheese pressurisation at day 60 (mature cheese), and cheeses were maintained vacuum-packed, which might explain the differences between both studies. Additionally, in cheeses pressurised at day 21 of ripening, they reported significant differences for β-CN at day 120 of storage. Similar results have been obtained for us in β2-CN. However, significant differences in β1-CN fraction were not observed in our study until day 240 of storage. The differences between the two studies may be due to different time in which HP treatments were applied (day 21 or 35 vs. day 60). In our opinion, the application of HPP at day 60 is more appropriate due to the strict regulations to which it is subjected, Torta del Casar cheese which requires a minimum of 60 days of ripening. HPP before this time could not comply the standards required by the PDO, and this product could not be placed on the market as Torta del Casar cheese.

On the other hand, a significant decrease in CPA values was observed for all casein fractions analysed in control and HPP cheeses during the storage time. This decrease over time in the content of casein is in line with the results obtained in previous studies of proteolysis in Torta del Casar cheese [6]. Moreover, the decrease in the residual casein content along the ripening time has been reported in Serpa cheese [35] and La Serena cheese [7], cheese varieties produced by a similar process to Torta del Casar cheese by using ewe milk and vegetable rennet as coagulant. During refrigerated storage of control cheeses was observed higher degradation in αs1-I CN and αs1-II CN showing at day 240 values 3.56 and 2.40 % of the initial content at day 60, respectively, compared with 18.22 and 23.77 % obtained in β2-CN and β1-CN, respectively. These results are in line with those reported previously by Delgado et al. [6] in Torta del Casar cheese, which showed lower levels of proteolysis in β-CN than in αs1 at day 90 of ripening.

Table 3 Corrected peak areas of the casein fractions found in Torta del Casar cheese after HPP at day 60 and after 60, 120 and 180 days of refrigerated storage (days 120, 180 and 240, respectively)

	Day	Control	200 MPa		600 MPa		SEM	*P* value
			5 min	20 min	5 min	20 min		
pK	60	7813,0^{ab,A}	7936,3^{ab,A}	6941,0^{b,A}	8036,3^{ab,A}	8452,3^{a,A}	165.62	0.081
	120	4422,1^{a,B}	4568,0^{a,B}	5492,7^{a,B}	2771,1^{b,B}	4353,2^{a,B}	193.00	**0.001**
	180	2827,3^{C}	3272,5^{C}	3058,6^{C}	3050,6^{B}	2351,8^{C}	141.29	0.353
	240	1099,3^{b,D}	2085,3^{a,C}	1766,2^{a,D}	2150,3^{a,B}	2074,1^{a,C}	81.73	**0.001**
P value		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		
αs2	60	1099,9^{b,A}	1205,1^{b,A}	1136,7^{b,A}	1153,5^{b,A}	1607,8^{a,A}	49.29	**0.007**
	120	286,8^{b,B}	505,9^{ab,C}	580,1^{a,B}	298,4^{b,B}	504,7^{ab,B}	34.57	**0.006**
	180	340,1^{b,B}	770,4^{a,B}	585,3^{ab,B}	352,1^{b,B}	618,9^{a,B}	38.64	**0.001**
	240	306,1^{b,B}	536,5^{a,C}	427,4^{ab,B}	435,4^{a,B}	524.2^{a,B}	22.59	**0.035**
P value		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		
αs1I	60	4353,5^{bc,A}	4905,4^{ac,A}	3453,4^{b,A}	4336,4^{bc,A}	5715,2^{a,A}	151.66	**0.001**
	120	494,3^{b,B}	901,6^{a,B}	490,1^{b,B}	715,8^{ab,C}	778,6^{a,B}	39.07	**0.001**
	180	289,1^{b,B}	569,7^{c,BC}	516,7^{c,B}	1658,8^{a,B}	616,5^{c,B}	84.76	**0.001**
	240	155,0^{b,B}	181,5^{b,C}	107,5^{b,C}	431,8^{a,C}	550,5^{a,B}	37.97	**0.001**
P value		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		
αs1II	60	6721,3^{ab,A}	6476,7^{ab,A}	5032,2^{b,A}	5921,1^{b,A}	7743,6^{a,A}	216.45	**0.002**
	120	781,0^{b,B}	1491,2^{ac,B}	923,9^{bc,B}	898,7^{bc,C}	1768,5^{a,B}	87.80	**0.001**
	180	554,4^{b,B}	393,8^{b,BC}	322,9^{b,C}	1891,9^{a,B}	2412,6^{a,B}	152.96	**0.001**
	240	161,5^{b,B}	248,2^{b,C}	216,6^{b,C}	2052,5^{a,B}	1648,4^{a,B}	168.14	**0.001**
P value		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		
αs1III	60	4310,4^{A}	4110,4^{A}	3240,1^{A}	3623,5^{A}	4247,4^{A}	133.54	0.066
	120	1229,3^{b,B}	1482,7^{bc,B}	1272,1^{bc,B}	1930,6^{ac,B}	2532,1^{a,B}	104.49	**0.001**
	180	836,6^{b,B}	1289,1^{c,B}	1142,2^{bc,B}	1260,8^{c,B}	1715,8^{a,C}	58.88	**0.001**
	240	842,2^{c,B}	101,5^{b,C}	95,3^{b,C}	1337,0^{a,B}	1407,4^{a,C}	120.17	**0.001**
P value		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		
β2	60	8265,0^{ab,A}	9965,7^{a,A}	7223,5^{b,A}	8582,6^{ab,A}	8869,9^{ab,A}	242.22	**0.005**
	120	4052,2^{ab,B}	4600,3^{a,B}	4264,7^{a,B}	2680,6^{b,B}	4575,1^{a,B}	186.93	**0.002**
	180	1734,5^{b,C}	2871,2 ^{cd,BC}	2136,6^{bc,C}	2963,0^{ad,B}	3692,4^{a,BC}	136.71	**0.001**
	240	1506,1^{bc,C}	1386,1^{b,C}	1139,6^{b,C}	2231,1^{a,B}	2160,5^{ac,C}	107.74	**0.001**
P value		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		
β1	60	6357,8^{A}	6503,7^{A}	5906,4^{A}	6372,8^{A}	6302,4^{A}	192.25	0.908
	120	4103,7^{ab,AB}	4697,8^{a,B}	5924,0^{a,A}	2492,3^{b,B}	4573,8^{a,B}	276.16	**0.001**
	180	2320,0^{ab,BC}	3135,6^{a,C}	2412,2^{ab,B}	2176,9^{b,B}	3085,8^{a,C}	113.76	**0.006**
	240	1511,2^{b,C}	1530,9^{b,D}	1886,3^{bc,B}	2760,8^{a,B}	2445,2^{ac,C}	125.66	**0.001**
P value		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		

P values less than 0.05 are in bold

SEM standard error of mean, *pK* para-κ-casein

a, b, c: different lower case letters in the same row indicate significant statistical differences (Tukey's test. $P < 0.05$). A, B, C: different capital letters in the same column indicate significant statistical differences (Tukey's test. $P < 0.05$)

Texture in HP-treated Torta del Casar cheese

Texture analysis data of Torta del Casar cheese are showed in Table 4. According to the ANOVA, HPP significantly affected the firmness and consistency of the pressurised cheeses, except at day 180 of sampling. However, when we carried out the multiple comparison procedure of the means, we found that differences between control and HP-treated cheeses were only significant at day 60 of analysis (just after pressurisation). Treatments at 600 MPa (for 5 or 20 min) caused the most intense changes, by reducing the firmness and consistency of the pressurised cheeses in comparison with the control ones. Typically, there is a relationship between changes in proteolysis and texture in cheese. However, in the present study, changes in texture did not correlate with those changes reported in proteolysis, probably due to the soft (spreadable) texture of mature Torta del Casar. The changes in texture found in this cheese variety at day 60 could be associated with physical changes induced by HPP rather than biochemical changes. Regarding the adhesiveness, this parameter showed significant differences between control and HP-treated cheese at day 120 and 240 of analysis. At day 120, the adhesiveness was higher in control cheese than those pressurised. However, at the end of storage

Table 4 Texture analysis of Torta del Casar cheese after HPP at day 60 and after 60, 120 and 180 days of refrigerated storage (days 120, 180 and 240, respectively)

	Day	Control	200 MPa		600 MPa		SEM	*P* value
			5 min	20 min	5 min	20 min		
Firmness (N)	60	3.41^{b,C}	5.21^{a,C}	2.11^{bc,C}	1.94^{c,C}	1.89^{c,B}	0.33	**0.001**
	120	13.02^{ab,B}	13.42^{ab,AB}	8.18^{b,B}	17.43^{a,A}	15.39^{a,A}	0.96	**0.015**
	180	19.93^{A}	17.60^{A}	14.42^{A}	14.44^{A}	17.09^{A}	0.87	0.197
	240	11.39^{ab,B}	10.80^{ab,BC}	13.05^{a,A}	8.15^{b,B}	7.62^{b,B}	0.58	**0.003**
P value		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		
Consistency (Ns)	60	15.10^{b,C}	22.62^{a,B}	9.47^{c,C}	8.86^{c,C}	8.34^{c,B}	1.36	**0.001**
	120	59.33^{ab,B}	59.76^{ab,A}	36.75^{b,B}	78.21^{a,A}	71.61^{ab,A}	4.54	**0.023**
	180	91.35^{A}	85.38^{A}	66.17^{A}	71.46^{A}	77.34^{A}	4.52	0.460
	240	54.58^{ab,B}	55.60^{ab,A}	66.16^{a,A}	41.11^{b,B}	38.45^{b,AB}	2.91	**0.004**
P value		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		
Adhesiveness (Ns)	60	2.06^{BC}	2.46^{AB}	2.15^{BC}	1.92^{B}	1.98^{B}	0.11	0.614
	120	5.44^{a,A}	3.53^{b,A}	3.76^{b,A}	2.89^{b,A}	2.98^{b,AB}	0.25	**0.001**
	180	2.83^{B}	3.25^{A}	2.92^{AB}	2.71^{AB}	3.26^{A}	0.14	0.633
	240	1.35^{b,C}	1.35^{b,B}	1.85^{ab,C}	2.04^{ab,B}	2.32^{a,AB}	0.11	**0.003**
P value		**0.001**	**0.013**	**0.001**	**0.011**	**0.044**		

P values less than 0.05 are in bold

SEM standard error of mean

a, b, c: different lower case letters in the same row indicate significant statistical differences (Tukey's test. $P < 0.05$). A, B, C: different capital letters in the same column indicate significant statistical differences (Tukey's test. $P < 0.05$)

time (day 240), only cheeses treated at 600 MPa for 20 min showed an adhesiveness value significantly higher than the other ones. Therefore, in general, the changes of texture of HP-treated cheeses were reduced during refrigerated storage.

Similar to the effect observed in the present paper, other authors [7] have noticed that HP treatment applied at La Serena cheese (a soft cheese) at day 50 of ripening had no significant effect on the texture of mature cheese (day 60). However, studies about the effect of HP treatment on the texture of soft cheeses are very limited. Wick et al. [36] assessed the effect of HP treatment (200–800 MPa for 5 min at 25 °C) on the texture of 30- and 120-day-old Cheddar cheese. They found that pressures up to 300 MPa applied to 30-day-old cheeses had no significant effect on the fracture force of the cheese. In a similar manner, HP at 800 MPa did not affect the fracture stress of cheeses throughout 160 days of storage. HP treatment applied to 120-day-old cheeses had no significant effect on texture for the most of the parameters analysed. Therefore, according to the literature, it is difficult to change the texture by pressurisation of mature cheese.

Firmness and consistency increased significantly with cheese age, except at day 240. The increase in these texture parameters could be related to the loss of moisture throughout refrigerated storage (Table 1). However, at day 240, the extensive proteolysis might be responsible for the decrease in firmness and consistency found in Torta del Casar cheese.

Conclusions

HPP had a negligible effect on physicochemical parameters. However, some proteolysis indices were modified. Nitrogen fractions decreased and casein fraction increased in cheeses treated at 600 MPa compared with control ones, while the treatments at 200 MPa had a minor effect. Textural parameters were modified in cheeses pressurised and analysed at day 60, but these changes were reduced during the storage time. These results suggest that HP treatment at 600 MPa on mature Torta del Casar cheese could reduce the proteolysis, thus delaying the over-ripening during the marketing. This effect would also maintain the soft texture of cheese during longer time. This fact would be of great importance to ensure the quality of the product for a longer period of storage.

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