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Specific effect of high-pressure treatment of milk on cheese proteolysis

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The extent of primary and secondary proteolysis of cheeses made from raw (RA), pasteurized (PA, 72 °C, 15 s) or pressure-treated (PR, 500 MPa, 15 min, 20 °C) goats' milk was assessed. Modifications in cheese-making technology were introduced to obtain cheeses with the same moisture content, and thus studied *per se* the effect of milk treatment on cheese proteolysis.

The PR milk cheese samples were differentiated from RA and PA milk cheeses by their elevated β-lg content, and by the faster degradation of α_{s1}-, α_{s2}- and β-CN throughout ripening. Non-significant differences were found in either pH 4·6 soluble-nitrogen or trichloracetic acid soluble-nitrogen contents of cheeses. However, the pasteurization of milk decreased the free amino acid production in cheese. The RA milk cheeses had the highest amount of proline and the lowest concentrations of serine, tyrosine, arginine and α-aminobutyric acid, whereas PR milk cheese showed higher levels of arginine.

Keywords: Proteolysis, ripening, goat cheese, high-pressure treatment.

Europe has a significant tradition in goats' milk production, and most of this milk is currently used for cheese making. Although many goats' milk cheeses have been traditionally made from raw (RA) milk, aspects related to microbial safety have increased the use of pasteurization treatments. The destruction of some desirable indigenous milk microbiota by thermal treatment has been linked to reduced aroma or typical flavour development of cheeses, in relation to those made from RA milk (Grappin & Beuvier, 1997; McSweeney et al. 1993). Likewise, pasteurization attenuates or activates the activity of many indigenous milk enzymes, such as the plasmin/plasminogen complex, lipases or alkaline phosphatase, and it also produces slight denaturation of serum proteins and slight modifications in milk rennetability (Grappin & Beuvier, 1997).

The interest in non-thermal technologies, such as high-hydrostatic pressure, for treatment of milk has increased mainly due to the possibility of reducing the microbial number without significant effects on flavour or nutritional components. In addition to microbial destruction, high-pressure treatments induce numerous effects on milk components: fragmentation of casein micelles, aggregation of whey proteins, modifications to the mineral equilibrium, etc (Felipe et al. 1997; Law et al. 1998; López-Fandiño et al. 1998; Needs et al. 2000), which affect the technological properties of milk (Huppertz et al. 2002; Trujillo et al. 2002b).

Most researchers have found that pasteurization of milk results in higher cheese moisture content compared with cheese made from RA milk (Grappin & Beuvier, 1997). In the same way, Drake et al. (1997) reported that Cheddar cheese made from pressure-treated (PR) milk showed a higher moisture content than RA or pasteurized (PA) milk cheeses. Utilizing PR goats' milk for cheese making, Trujillo et al. (1999b) increased cheese yield basically due to incorporation of pressure-denatured whey proteins into the cheese curd, resulting in higher moisture retention. This property of PR milk has been successfully used by Molina et al. (2000) to make reduced-fat cheeses, where high moisture retention is usually pursued to improve quality.

Nevertheless, moisture in non-fat dry matter (M/NFM) markedly influences the ripening of cheese, affecting the degradation of cheese components (Lawrence et al. 1987). Trujillo et al. (2002a) studied primary and secondary proteolysis in goat cheese made from RA, PA (72 °C, 15 s) and PR (500 MPa, 15 min, 20 °C) milks. During ripening, the hydrolysis of α_{s1}-CN and β-CN in cheeses were in the order PA > PR > RA and PA > PR = RA, respectively. Analysis of secondary proteolysis showed differences between cheese types, especially PR milk cheeses, which contained

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considerably higher concentrations of total free amino acids than either PA or RA milk cheeses throughout ripening. Significant differences in moisture content were also found. According to the authors, the high moisture content of PR and PA milk cheeses, relative to RA milk cheeses, and the effects of technological treatments on milk compounds (native enzymes i.e. plasmin, casein micelles, whey proteins, etc.), could explain the differences in proteolysis between cheeses.

In this work, with the aim of assessing, *per se*, the effect of milk treatment on primary and secondary proteolysis, cheeses with standardized moisture content from RA, PA (72 °C, 15 s) or PR (500 MPa, 15 min, 20 °C) goats' milk were made. However, it is difficult to produce these cheeses with the same M/NFM without modifying the technological parameters of cheese making (Grappin & Beuvier, 1997; Trujillo et al. 1999b). Thus, the necessary modifications in cheese making technology were introduced.

Materials and Methods

Cheese manufacture

Milk was obtained from a herd of Murciano-Granadina goats (Muntanyola, Barcelona, Spain). Goat's cheese was manufactured from RA, PA (72 °C, 15 s) and PR milk in two independent experiments, within an interval of one week. In each experiment, 50 kg RA, 50 kg PA and 50 kg PR milk from the same milk batch were used for cheese making.

Pasteurized milk was produced using a heat-exchanger (Garvía S.A., Barcelona, Spain) at 72 °C at a flow rate of 200 l/h, with a holding time of 15 s.

High pressure-treated milk was obtained using a semi-continuous hyperbar equipment (GEC Alsthom ACB, Nantes, France) by direct compression of the liquid with a piston. Batches of 4 l milk were pressurized at 500 MPa at 20±1 °C with a holding time of 15 min. The PR milk was kept at 4 °C for approximately 4 h until cheese making.

Milk was heated to 31 °C and a starter culture (AM Larbus, Barcelona) containing *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*, was added to final concentration of 20 g/kg. Directly afterwards, a solution containing 350 g $CaCl_2$/kg (food quality grade) was added to cheese milk to final concentrations of 0·20 ml/kg. Ten minutes later, 0·2 ml of calf rennet/kg (Reniflor-15/E, Lamirsa, Barcelona), containing 780 mg chymosin/l was added. After 30 min, the coagulum was cut, and the curds drained and moulded (13·6 × 13·2 cm).

Due to the different milk treatments, and in order to obtain cheese with comparable moisture content, pressing time was prolonged for 12 h (1 h at 1·3 kPa and 11 h at 2·6 kPa). The pressing conditions used in this study were based on the experience gained from previous experiments. After that, cheeses were immersed in brine solution (190 g NaCl/l) for 4 h at 14 °C. Finally, cheeses, approximately 1·31 ± 0·03 kg each, were ripened in a room at 14 °C and 85% relative humidity for 60 d.

Compositional analysis

Analyses of RA, PA and PR milk cheeses from the two experiments were performed in duplicate for dry matter (DM; International Dairy Federation, 1982), fat (F; van Gulik method, ISO, 1975) and total nitrogen (TN; International Dairy Federation, 1993). Moisture in non-fat dry matter (M/NFM) contents of cheeses were calculated as (100-DM)/(DM-F). The pH was measured in a cheese/distilled water (1 : 1) slurry.

Preparation of cheese extracts

Water-soluble fractions (WSF) of cheeses were prepared following the method of Kuchroo & Fox (1982). The pH 4·6 soluble-nitrogen (WSN) and the 12% trichloracetic acid soluble-nitrogen (TCA) were determined from WSF by the Kjeldhal method (International Dairy Federation, 1993).

Capillary electrophoresis

The water-insoluble fractions recovered during the water-soluble extraction were washed three times with 1 M-sodium acetate buffer (pH 4·6) and the remaining fat was eliminated by washing with dichloromethane-sodium acetate buffer (1 : 1, v/v). The final protein precipitate was then lyophilized.

Capillary electrophoresis (CE) analysis were performed following the method of Recio & Olieman (1996). Sample buffer (pH 8·6) contained 167 mM-Tris(hydroxymethyl)-aminomethane (Amresco, Solon, Ohio, USA), 42 mM-3-morpholino-propanesulfonic acid (Biohemika MicroSelect, Fluka, Buchs, Switzerland), 67 mM-ethylenedinitrilotetra-acetic acid disodium salt dihydrate (Tritiplex III; Merck, Darmestadt, Germany), 17 mM-DL-dithiothreitol (Sigma, St. Louis, MI 63178, USA), 6 M-urea (BioChemika Micro-Select, Fluka) and methylhydroxypropyl cellulose (0·5 g/l; Sigma).

The separation buffer (pH 3·0) contained 0·32 M-citric acid, 20 mM-sodium citrate, 6 M-urea and 0·5 g methyl-hydropropyl cellulose/l.

Lyophilized water-insoluble fractions from cheese (18 mg) were dissolved in 1 ml diluted sample buffer (sample buffer/water 1·5 : 1). After incubation for 1 h at room temperature, 20 µl lactic acid (180 ml/l) were added (final pH 7·0) and injected in to the CE apparatus.

Separations were carried out using a BioFocus 2000 capillary electrophoresis instrument (Bio-Rad Laboratories Ltd., Hercules, CA 94547, USA) controlled by BioFocus System operating software. The separations were performed using a hydrophilic-coated fused-silica capillary column (CElect P1, Supelco, Bellefonte, PA 16823, USA) of 0·47 m × 50 µm i.d. (effective length 0·40 m).

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Electrophoresis was run at 40 °C and a final voltage of 20 kV and UV-detection was performed at 214 nm.

The area of each peak was integrated using BioFocus System Integration Software (version 5.0).

Free amino acids analysis

The concentrations of free amino acids (FAA) were analysed by ion exchange chromatography with postcolumn ninhydrin derivatisation and detection at 570 and 440 nm for primary and secondary amino acids, according to the procedure of Spackman et al. (1958).

The cheese WSFs were centrifuged at 10 400 ***g*** for 15 min. Then 250 µl of the supernatant was combined with 25 µl 1 mM-L-norleucine (Sigma) as the internal standard. Samples were filtered through a 10 kDa membrane filter (Ultra Free, Millipore, France), and then 40 µl were injected into the amino acid analyser (Alpha Plus series II, Pharmacia LKB Biotechnology, Sweden). Separation was carried out on a sulphonated divinylbenzene polystyrene column (200 × 4 mm, Biochrom Ltd., Cambridge, UK) using a lithium citrate buffer system (Biochrom) as mobile phase. Individual FAA were identified and quantified, using a mixture of amino acids standards (Sigma).

Statistical analysis

Data were processed by analysis of variance (ANOVA) using the general linear models procedure of SAS® System for WIN™ (8 version). The Student-Newman-Keuls test was used for comparison of sample data. Evaluations were based on a significant level of $P < 0{\cdot}05$.

Principal component analysis (PCA) was performed using Statistica® software (5.0 version; StatSoft Inc., Tulsa, OK, USA). Kaiser's criterion (an eigenvalue greater than 1) was employed to establish the number of final factors from the general parameters considered. Orthogonal rotation of the factors using the Varimax method was used to interpret these results.

Results

Physico-chemical analyses

The composition of RA, PA and PR milk cheeses is shown in Table 1. The pH of PA and PR milk cheeses was lower than in cheeses made from RA milk. No differences ($P > 0{\cdot}05$) in M/NFM content and fat in dry matter were observed between cheeses, but at the end of ripening the PA milk cheese presented the lowest M/NFM.

Primary proteolysis

The water-insoluble fractions at pH 4·6 of RA, PA and PR milk cheeses were analysed by capillary electrophoresis (CE). Designation of CE peaks of intact caseins (α_{s1}-CN, α_{s2}-CN and β-CN) was carried out by comparing the electrophoregrams of skimmed milk and 1-d-old cheeses with those of isolated pure proteins (Trujillo et al. 2000). The relative migration time of peaks was also consistent with previous reports (Recio et al. 1997).

Table 1. Composition of cheeses made from raw (RA), pasteurized (PA) and pressure-treated (PR) goat milk

Values are means ± SD for $n = 4$

	Milk	1 d	30 d	60 d
pH				
	RA	5·05 ± 0·03[a]	4·80 ± 0·03	4·89 ± 0·02[a]
	PA	4·99 ± 0·02[b]	4·80 ± 0·01	4·84 ± 0·04[b]
	PR	5·01 ± 0·06[b]	4·79 ± 0·05	4·76 ± 0·02[c]
M/NFM†, %				
	RA	2·45 ± 0·05	1·33 ± 0·05	1·01 ± 0·02[a]
	PA	2·47 ± 0·08	1·29 ± 0·05	0·92 ± 0·01[b]
	PR	2·43 ± 0·05	1·36 ± 0·04	0·98 ± 0·02[a]
F/DM, %				
	RA	54·28 ± 0·32	56·08 ± 0·43	57·96 ± 0·90
	PA	54·30 ± 0·74	55·95 ± 0·44	57·59 ± 0·64
	PR	53·97 ± 0·35	56·07 ± 0·72	57·14 ± 0·87

† M/NFM, moisture in non-fat dry matter; F/DM, fat in dry matter
[a,b,c] Means within a column with a different superscript were significantly different ($P < 0{\cdot}05$)

The main difference observed between cheeses corresponded to the presence of β-lg in PA and PR milk cheeses which, as we expected, was much higher in the later. The β-lg was resistant to proteolysis in PA and PR milk cheese after 60 d ripening.

Except for the β-lg peak, no qualitative differences were observed between the electrophoretic profiles of cheeses at day 1. Nevertheless, significant quantitative differences were evident during ripening (Table 2). An intense decrease in the area of peaks corresponding to α_{s1}-CN was observed at 30 d ripening. The α_{s1}-CN represented the main target for proteolysis, showing at the end of ripening less than 50% of their initial values. The PR milk cheeses exhibited higher ($P < 0{\cdot}05$) α_{s1}-CN degradation than both RA and PA milk cheeses throughout ripening. The hydrolysis of α_{s1}-CN was coincident with the appearance of new peaks with a high electrophoretic mobility, which corresponded to the breakdown product α_{s1}-I. These peaks increased as cheeses aged, and after 30 d ripening cheeses made from PR milk showed elevate values for α_{s1}-I, fact that agrees with the fast degradation of α_{s1}-CN in these cheeses (Table 2).

In all the cheeses analysed, α_{s1}-CN was degraded to a greater extent than β-CN, which in turn was slowly hydrolysed, especially in RA and PA milk cheeses (Table 2). At the end of ripening, cheeses made from PR milk exhibited higher degradation of β-CN (approximately 19%) than both RA and PA milk cheeses.

Plasmin, the main endogenous proteinase of milk, acts mainly on β-CN, forming the γ-CNs (Fox et al. 1993). The area of β-CN peaks increased by approximately 50%

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Table 2. Relative percentages of a_{s1}-CN, a_{s2}-CN, b-CN and a_{s1}-I-CN, determined by CE analyses of the caseins of raw (RA) pasteurized (PA) and pressure-treated (PR) goat milk cheeses during ripening

Values are means ± SD for $n=4$

	1 d	30 d	60 d
α_{S1}-CN†			
RA	100	$43{\cdot}42 \pm 2{\cdot}02^{A}$	$44{\cdot}76 \pm 3{\cdot}54^{A}$
PA	100	$42{\cdot}20 \pm 2{\cdot}76^{A}$	$39{\cdot}85 \pm 1{\cdot}33^{A}$
PR	100	$37{\cdot}88 \pm 0{\cdot}48^{B}$	$34{\cdot}75 \pm 2{\cdot}48^{B}$
α_{S2}-CN			
RA	100	$84{\cdot}10 \pm 3{\cdot}77^{A}$	$71{\cdot}28 \pm 9{\cdot}32^{A}$
PA	100	$60{\cdot}50 \pm 3{\cdot}92^{B}$	$45{\cdot}60 \pm 3{\cdot}13^{B}$
PR	100	$42{\cdot}81 \pm 6{\cdot}18^{C}$	$36{\cdot}94 \pm 9{\cdot}31^{C}$
β-CN			
RA	100	$98{\cdot}42 \pm 0{\cdot}57^{A}$	$93{\cdot}70 \pm 1{\cdot}06^{A}$
PA	100	$97{\cdot}14 \pm 2{\cdot}86^{A}$	$93{\cdot}00 \pm 0{\cdot}59^{A}$
PR	100	$86{\cdot}41 \pm 1{\cdot}11^{B}$	$81{\cdot}51 \pm 0{\cdot}15^{B}$
α_{S1}-I-CN			
RA	100	$342{\cdot}80 \pm 9{\cdot}50^{A}$	$325{\cdot}79 \pm 5{\cdot}63^{B}$
PA	100	$281{\cdot}96 \pm 11{\cdot}81^{B}$	$319{\cdot}22 \pm 1{\cdot}43^{B}$
PR	100	$350{\cdot}90 \pm 6{\cdot}70^{A}$	$423{\cdot}34 \pm 9{\cdot}53^{A}$

† expressed as percentage of area in relation to day 1
^{A,B,C} Means within a column with a different superscript were significantly different ($P < 0{\cdot}05$)

during ripening, but no differences ($P > 0{\cdot}05$) in their values were observed between cheeses.

The α_{s2}-CN, like β-CN, is susceptible to plasmin attack. In this work, α_{s2}-CN showed a marked decrease ($P < 0{\cdot}05$) throughout ripening, especially in PR milk cheeses (Table 2). Cheeses made from PR and RA milk showed the highest and lowest degradation of α_{s2}-CN respectively, whereas PA milk cheeses exhibited an intermediate behaviour.

Para-κ-CN peaks showed a decrease (approximately 20%) in all cheeses over ripening time, but no differences were observed at 60 d of ripening.

Secondary proteolysis

The degree of secondary proteolysis was evaluated by the quantification of nitrogen fractions, WSN and TCA, and by the determination of individual FAA. In all cheeses, both WSN and TCA fractions increased ($P < 0{\cdot}05$) by 60 d, indicating the significant effect of the ripening time on their concentration (Table 3). However, no differences ($P > 0{\cdot}05$) in either WSN or TCA values of RA, PA or PR milk cheeses were found at any stage of ripening. In the same way, total FAA content of cheeses also increased ($P < 0{\cdot}05$) over ripening time (Table 3); however, both RA and PR milk cheeses showed higher levels of total FAA than that found in cheeses made from PA milk at 30 and 60 d ripening.

The release of individual FAAs is shown in Table 4. Twenty-three amino acids were identified and quantified from the cheese samples. The main FAA observed throughout ripening in the three types of cheeses were leucine, glutamic acid, proline and phenylalanine, which

Table 3. Values of pH 4·6 water-soluble nitrogen (WSN), 12% trichloracetic soluble nitrogen (TCA) and total free amino acid (FAA) of cheeses made from raw (RA) pasteurized (PA) and pressure-treated (PR) goat milk

Values are means ± SD for $n=4$

	1 d	30 d	60 d
WSN/TN, %			
RA	10·63 ± 1·63	13·61 ± 2·10	17·93 ± 1·74
PA	10·46 ± 2·25	14·31 ± 2·81	18·10 ± 1·09
PR	9·06 ± 0·96	13·59 ± 0·46	17·76 ± 1·27
TCA/TN, %			
RA	5·42 ± 0·48	8·79 ± 2·99	9·15 ± 2·17
PA	5·44 ± 0·73	10·10 ± 2·81	11·26 ± 2·51
PR	4·70 ± 0·76	10·31 ± 3·65	11·20 ± 3·65
Total FAA†			
RA	2·30 ± 0·12	$5{\cdot}59 \pm 0{\cdot}50^{A}$	$6{\cdot}95 \pm 0{\cdot}76^{A}$
PA	2·40 ± 0·37	$3{\cdot}63 \pm 0{\cdot}35^{B}$	$4{\cdot}99 \pm 0{\cdot}51^{B}$
PR	2·01 ± 0·10	$4{\cdot}96 \pm 0{\cdot}17^{A}$	$6{\cdot}41 \pm 0{\cdot}61^{A}$

† Expressed as g/kg dry matter
^{A,B,C} Means within a column with a different superscript were significantly different ($P < 0{\cdot}05$)

together represented approximately 41% of the total FAA content.

Visualisation of the complex data matrix was performed by PCA, with RA, PA and PR milk cheeses as objects and individual FAA data as variables. Results from the PCA showed three interpretable factors that together described about 85·1% of the total variation of sample, 61·3, 13·8 and 9·9% for factors 1, 2 and 3, respectively. Factor 1 was loaded on aspartic acid, threonine, asparagine, glutamic acid, glutamine, proline, glycine, alanine, valine, methionine, isoleucine, leucine, phenylalanine, ornithine, lysine and histidine. The first principal component expressed cheese age, reflecting the fact that the amount of most amino acids increased in all cheeses during ripening. Factor 2 was loaded on serine, α-aminobutyric acid (AABA), tyrosine and arginine, and seems to be linked to cheese milk treatment. The third factor explains the behaviour of cysteine and tryptophan, which both increased up to 30 d ripening and then decreased at the end of ripening.

As shown in Fig. 1, the PCA analysis showed three partially overlapped clusters, which corresponded to the different treatments applied to milk. The clustered PA and PR milk cheese samples differed from RA milk cheese at the end of ripening. The PA and PR milk cheeses were differentiated by their low level of proline, and by their elevate levels of serine, AABA, tyrosine and arginine. The PR milk cheeses showed higher levels of arginine than those found in either RA or PA milk cheeses.

Discussion

Previous studies reported that cheeses made from RA, PA or PR milk showed different moisture content (Drake et al.,

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Table 4. Free amino acids content (mg/kg DM) of cheeses made from raw (RA) pasteurized (PA) and pressure-treated (PR) goat milk during ripening

Values are means ± SD for $n=4$

	1 d			30 d			60 d		
	RA	PA	PR	RA	PA	PR	RA	PA	PR
ASP	24·9±15·6	45·5±9·1	34·4±11·0	128·7±55·6	75·2±10·8	110·6±46·9	236·6±89·0	133·2±56·6	181·0±27·2
THR	21·0±0·3	11·3±8·4	19·1±3·7	78·8±14·7^{AB}	46·0±19·9^{B}	87·6±3·1^{A}	132·1±48·6	74·4±40·6	111·0±27·7
SER	16·8±4·8	17·9±2·3	19·0±1·4	51·0±5·9^{B}	85·4±24·4^{A}	118·7±5·3^{A}	102·0±30·2^{B}	132·7±11·3^{BA}	174·2±40·5^{A}
ASN	90·3±51·6	54·6±13·4	51·2±12·2	371·1±27·2^{A}	178·7±75·4^{B}	243·7±85·3^{AB}	522·9±74·9^{A}	254·9±110·8^{B}	576·2±152·2^{A}
GLU	248·8±39·4	245·3±74·0	254·8±10·6	525·6±65·5^{A}	212·3±108·4^{B}	395·3±59·8^{AB}	775·7±163·6	454·2±270·0	700·6±102·2
GLN	54·5±25·3	37·1±2·5	59·7±1·1	394·6±46·2^{A}	209·0±80·0^{B}	348·0±10·6^{A}	577·4±74·0^{A}	358·1±93·0^{B}	464·8±49·7^{AB}
PRO	335·1±14·5	372·8±60·9	321·3±2·4	363·0±54·2	320·2±50·2	321·4±10·0	517·0±69·6^{A}	444·8±27·5^{AB}	426·2±29·0^{B}
GLY	9·6±1·6	10·2±0·8	7·5±1·6	57·7±12·5	38·0±12·5	52·4±0·1	87·0±35·5	51·9±25·4	64·9±19·5
ALA	49·3±5·4	51·0±10·2	42·3±8·1	167·1±9·1^{A}	109·9±18·7^{B}	152·8±11·7^{A}	238·0±40·8^{A}	149·9±43·5^{B}	194·5±24·1^{AB}
AABA	27·2±2·8	28·2±4·7	26·5±0·6	43·6±28·0^{C}	98·1±3·3^{B}	132·3±9·7^{A}	24·9±16·6^{B}	164·9±33·8^{A}	188·9±42·7^{A}
VAL	211·0±30·7^{A}	146·1±49·8^{AB}	111·8±19·0^{B}	379·8±69·9	204·4±86·1	269·8±4·9	448·8±78·1	277·8±150·1	323·1±69·8
CYST	—	—	—	24·9±18·7^{B}	60·4±7·0^{A}	25·4±16·4^{B}	6·8±9·6	13·5±6·5	4·0±0·8
MET	97·4±18·4	100·7±40·6	88·8±2·5	238·6±37·7	162·4±58·2	191·2±6·9	278·7±55·9	156·8±108·4	210·4±48·9
ILE	38·8±9·7	28·2±5·3	26·6±9·8	184·5±38·1^{A}	87·9±50·7^{B}	159·8±13·8^{A}	325·2±102·3^{A}	175·2±26·4^{B}	204·8±63·7^{A}
LEU	298·7±22·9	280·3±91·2	203·1±37·9	863·5±70·8^{A}	507·7±158·4^{B}	657·5±30·9^{B}	973·3±81·2^{A}	687·1±160·6^{B}	834·7±78·5^{AB}
TYR	109·2±7·9	117·3±2·4	109·5±16·0	42·0±3·4^{C}	123·1±28·5^{B}	242·2±6·6^{A}	68·0±9·5^{B}	191·3±70·7^{A}	290·6±45·7^{A}
PHE	153·7±46·0	153·1±38·4	106·8±6·1	630·7±46·0^{A}	241·3±81·1^{B}	418·4±117·4^{AB}	561·8±62·2^{A}	300·6±96·0^{B}	371·6±42·5^{AB}
GABA	130·9±12·4^{B}	240·6±31·2^{A}	162·9±31·9^{AB}	287·4±29·6^{B}	372·6±15·0^{A}	355·7±23·4^{A}	243·7±14·0	303·4±69·1	298·2±16·9
ORN	54·9±8·7	60·0±4·2	49·4±6·8	303·3±44·3^{A}	164·5±52·4^{B}	186·9±21·0^{B}	219·9±77·4	157·4±38·9	184·3±34·9
LYS	65·1±26·2^{B}	108·6±21·8^{A}	93·7±8·5^{AB}	281·5±15·9	223·6±30·7	318·6±34·9	466·4±122·6	371·4±134·9	435·2±64·1
HIS	54·4±6·2	67·1±15·2	51·1±1·7	68·6±27·2^{AB}	53·8±22·0^{B}	81·9±7·2^{A}	102·9±50·1	85·6±58·0	87·0±40·9
TRP	25·0±2·8	22·7±3·0	21·7±4·9	59·9±26·8	46·8±4·3	37·9±7·4	28·8±7·3	40·6±9·4	28·0±22·5
ARG	8·6±2·7	7·6±0·5	7·4±1·2	30·3±33·9	27·8±7·3	56·8±13·6	18·2±11·7^{C}	33·2±6·2^{B}	53·7±5·2^{A}

^{A,B,C} Means within a row with a different superscript were significantly different ($P<0·05$)

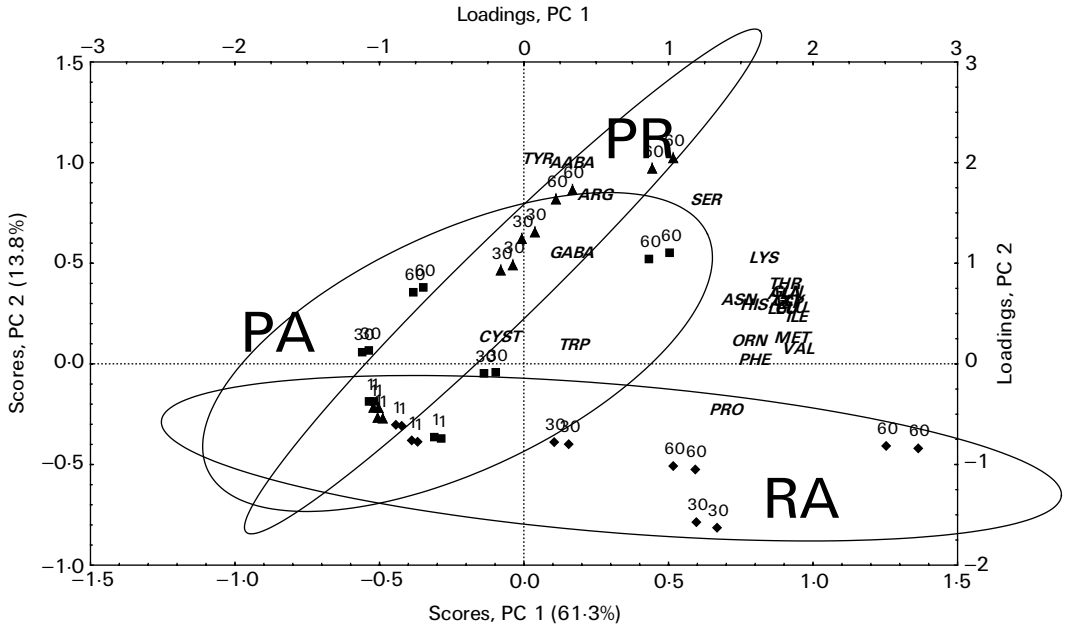

Fig. 1. Score plot and loading vectors obtained by principal component analysis of free amino acid content of cheese made from raw (◆) pasteurized (■) and pressure-treated (▲) goats' milk at 1, 30 and 60 d ripening. Results of each treatment are surrounded by an ellipse, which includes the members of this group ($P < 0{\cdot}05$).

1997; Grappin & Beuvier, 1997; Trujillo et al. 1999a,b; Molina et al. 2000; Trujillo et al. 2002a), with the treated milks yielding higher moisture cheeses. According to Grappin and Beuvier (1997), to obtain cheeses with similar moisture content makes comparisons easier and it is fundamental to have similar microbial and enzymatic activities during ripening. As shown in Table 1, cheeses had similar ($P > 0{\cdot}05$) M/NFM values, but at the end of ripening cheeses made from PA milk showed a lower value. The lower M/NFM level found in PA milk cheeses suggests that they had a greater water evaporation rate than RA or PR milk cheeses. Buffa et al. (2003) studied the water-binding properties of cheeses made from RA, PA and PR goats' milk. In that study, PA milk cheeses showed large decreases in retained water, whereas PR and RA milk cheeses retained more water. These authors suggested that the water loss of cheeses during ripening is also controlled by the internal profiles of water in the cheeses, which in turn is related to the cheese-matrix microstructure, and not only by the external conditions of ripening.

Proteolytic phenomena take place in two steps that are usually called primary and secondary proteolysis. The first of these may be defined as those changes in caseins (α_{s1}-, α_{s2}-, β- and γ-CN) and peptides which can be detected by electrophoretic methods (Fox. 1989). From the CE results, the main qualitative difference between cheeses was the presence of a peak of β-lg in PR milk cheeses, and at a much lower extent in PA milk cheeses. It has been reported that β-lg, in addition to its heat-sensitive behaviour, is the whey protein most easily denatured by the high-pressure treatment of milk (Felipe et al. 1997). Whereas in normal conditions of pasteurization (72 °C, 15 s) about 7% of whey proteins are denatured (Lawrence. 1991), ~80% of β-lg and the 50% of total whey proteins are irreversibly aggregated at 500 MPa (Felipe et al. 1997). These facts could explain the presence, as well as the quantity, of β-lg found in PA and PR cheeses.

Overall, the high-pressure treatment applied to milk produced a faster rate of protein breakdown than that observed in cheeses made from PA of RA milk (Table 2). Peaks corresponding to α_{s1}-CN were strongly degraded throughout ripening of all cheeses, while β-CN was hydrolysed at lesser extend. It has been reported that when animal rennets are used, α_{s1}-CN undergoes considerable proteolysis during ripening of hard and semi-hard bacterially ripened cheeses, but β-CN remains unchanged until an advanced stage of ripening (Fox et al. 1993).

Currently, the effect of high-pressure treatments of milk on cheese proteolysis is not well known. Trujillo et al. (1999a) evaluated the proteolysis in PA and PR goat milk cheeses by SDS-PAGE, reporting no major qualitative differences in the casein degradation pattern of the cheeses. On the other hand, Molina et al. (2000) reported that low-fat cheese made from PR milk showed a faster rate of protein breakdown (α_{s1}- and β-CN) than those made from PA milk. This fact was explained by the higher level of residual rennet retained in PR milk curds, which was attributed in turn to their higher moisture content. According to these authors, the higher moisture content of PR milk cheeses could directly boost proteolysis.

More recently, Trujillo et al. (2002a) found that, even though PR milk cheeses showed the highest moisture

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content compared with PA and RA counterparts, cheeses made from PA milk showed the highest level of hydrolysis of α_{s1}-, α_{s2}- and β-CN. These findings were explained by greater rennet retention and by the activation of plasminogen in plasmin and/or inactivation of plasmin inhibitors following pasteurization. In that work, PR milk cheeses showed an intermediate degradation of α_{s1}-CN (PA>PR>RA). Furthermore, PR and PA milk cheeses displayed similar level of hydrolysis of β-CN at 15 d ripening, but higher than those found in RA milk cheeses.

The application of a high-pressure treatment of 500 MPa at 20 °C for 15 min to milk, led to an increase in the total FAA concentration in cheese in relation to that found in the PA milk cheeses, but similar to that observed in RA milk cheeses (Table 3). Even though PR and RA milk cheeses showed similar amount of total FAA, they differed in the concentration of some amino acids (Table 4).

As general rule, RA milk cheeses show higher levels of total FAA than cheeses made from PA milk, a fact usually linked to the amino peptidase activities of indigenous milk micro-organisms (Grappin & Beuvier. 1997). Polo et al. (1985) and Freitas et al. (1999) suggested that the pattern of FAA release depends on the metabolic routes followed by the enzymatic degradation of peptides by micro-organisms, as well as from the interconversion, excresion or degradation of amino acids. In this work, RA milk cheeses showed lower values for arginine, serine and tyrosine and higher for proline in relation to both PA and PR milk cheeses (Table 4 & Fig 1). It has been previously reported that some RA milk cheeses contain lower amount of arginine (Krause et al. 1997; Ordóñez et al. 1999), serine (Ordóñez et al. 1999; Skeie & Ardö. 2000) or tyrosine (Krause et al. 1997; Skeie & Ardö. 2000) than their counterparts made from PA milk. According to Skeie & Ardö (2000) some lactobacilli may oxidize serine under anaerobic conditions (like in ripening cheese), because it contains an –OH group, which could explain the lower amounts of serine found in RA milk cheeses. On the other hand, tyrosine could have been decarboxylated to tyramine by some non-starter lactic acid bacteria (NSLAB; Skeie & Ardö, 2000), and arginine could have been used as energy source for some of these bacteria in ripening cheese (Laht et al. 2002). The high level of proline in RA milk cheese found in this work may also be linked to the NSLAB population, because of some of them have specific peptidases for hydrophobic peptides rich in proline, i.e. prolidases, aminopeptidases P, prolyldipeptidases, etc (Fresno et al. 1997).

It has been reported that, when moisture content of cheese is not controlled, PR milk cheeses contain higher concentrations of total FAA than PA or RA milk cheeses throughout ripening (Trujillo et al. 1999a, 2002a). According to the authors, the accelerated formation of FAA in the PR milk cheese could be explained by the physico-chemical alterations of milk due to high-pressure treatments, which results in different whey protein content incorporated in curd, different moisture content of cheeses, etc. Furthermore, they could also be caused by the residual microbiota after the technological treatment applied to milk. Buffa et al. (2001) compared the microbial groups most relevant during ripening of RA, PA or PR goats' milk cheeses, reporting that both pasteurization and high-pressure treatments reduced the indigenous milk microbiota to a similar extent, yielding cheeses with similar microbiological quality. The main difference between cheeses was the number of lactobacilli (the main microbial group of NSLAB), which were higher (by ~3 logs) in RA milk cheese at all the ripening stages studied. Thus, it is unlikely that the elevated total FAA content observed in PR milk cheeses could be attributed only to the residual microbiota in the cheese, which was basically similar to that of PA milk cheese. In the same way, nor do the differences in moisture content seem to explain by themselves the total FAA content of RA, PA and PR milk cheeses, because when the M/NFS is standardized, cheeses made from PR milk also presented elevated FAA values, similar to the RA milk cheeses (Table 3).

In most cheese varieties, the initial hydrolysis of casein is caused by the coagulant and to a lesser extent by plasmin, which results in the formation of large and medium-sized peptides in cheese. The starter micro-organisms have proteinase activities, which degrade medium peptides produced by plasmin and chymosin into low molecular weight peptides that are subsequently hydrolysed by specific intracellular peptidases to FAA after importation into the cell or following cell lysis (Fox, 1993). High pressures could influence the cheese proteolysis process through action on indigenous milk proteinases (i.e. plasmin) and changes in the cheese matrix, due in turn to the changes in micelle structure induced by high pressure. It has been shown that milk treatments at 400 MPa reduce plasmin activity by 25% (García-Risco et al. 1998; Huppertz et al. 2002). These last authors also examined the proteolysis resulting from the action of plasmin in high pressure-treated skim milk during storage. Milk treatment at 300-400 MPa, despite reduced plasmin activity, enhanced proteolysis of milk in comparison with untreated milk, possibly due to the disruptive effect of high-pressure on the micellar structure, thereby facilitating increasing availability of substrates to plasmin. Similar results were obtained by García-Risco et al. (2003), showing that when exogenous plasmin was added to untreated or high pressure-treated milk, proteolysis was more extensive in the later.

Although cheeses had similar M/NFM contents in this work, the breakdown of cheese proteins (primary proteolysis) was more rapid in PR milk cheeses, and secondary proteolysis was similar in PR and RA milk cheeses. These results suggest that high-pressure treatment made cheese proteins more susceptible to proteolysis. High-pressure treatment of milk (400–600 MPa) causes micelle fragmentation into smaller micelle structures containing denatured β-lg (Needs et al. 2000). These reformed micelles have

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altered structure and/or composition, which may alter the susceptibility of milk proteins to the proteolytic action of cheese enzymes (i.e. plasmin, chymosin, etc.), explaining the extent of primary and secondary proteolysis observed in PR milk cheeses.

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References

Buffa M, Guamis B, Royo C & Trujillo AJ 2001 Microbiological changes throughout ripening of goat cheese made from raw, pasteurized and high-pressure-treated milk. *Food Microbiology* **18** 45–51

Buffa M, Guamis B, Saldo J & Trujillo AJ 2003 Changes in water binding during ripening of cheeses made from raw, pasteurized or high-pressure-treated goat milk. *Lait* **83** 89–96

Drake MA, Harrison SL, Asplund M, Barbosa-Canovas G & Swanson BG 1997 High pressure treatment of milk and effects on microbiological and sensory quality of Cheddar cheese. *Journal of Food Science* **62** 843–845, 860

Felipe X, Capellas M & Law AJR 1997 Comparison of the effects of high-pressure treatments and heat pasteurization on the whey proteins in goat's milk. *Journal of Agricultural and Food Chemistry* **45** 627–631

Fox PF 1989 Proteolysis during cheese manufacture and ripening. *Journal of Dairy Science* **72** 1379–1408

Fox PF, Law J, McSweeney PLH & Wallace JM 1993. Biochemistry of cheese ripening. In *Cheese: Chemistry, Physics and Microbiology* pp. 389–438 (Ed. PF Fox). London, UK: Chapman and Hall

Freitas AC, Fresno JM, Prieto B, Franco I, Malcata FX & Carballo J 1999 How milk type, coagulant, salting procedure and ripening time affect the profile of free amino acids in Picante da Beira Baixa cheese. *Journal of the Science of Food and Agriculture* **79** 611–618

Fresno JM, Tornadijo ME, Carballo J, Bernardo A & González-Prieto J 1997 Proteolytic and lipolytic changes during the ripening of a Spanish craft goat cheese (Armada variety). *Journal of the Science of Food and Agriculture* **75** 148–154

García-Risco M, Cortés E, Carrascosa AV & López-Fandiño R 1998 Microbiological and chemical changes in high-pressure-treated milk during refrigerated storage. *Journal of Food Protection* **61** 735–737

García-Risco M, Recio I, Molina E & López-Fandiño R 2003 Plasmin activity in pressurized milk. *Journal of Dairy Science* **86** 728–734

Grappin R & Beuvier E 1997 Possible implications of milk pasteurization on the manufacture and sensory quality of ripened cheese. *International Dairy Journal* **7** 751–761

Huppertz T, Kelly AL & Fox PF 2002 Effects of high pressure on constituents and properties of milk. *International Dairy Journal* **12** 561–572

International Dairy Federation 1982 *Cheese and processed cheese. Determination of the total solids content*. Brussels: IDF (*FIL–IDF Standard* no. 4A)

International Dairy Federation 1993 *Milk determination of the total nitrogen content*. Brussels: IDF (*FIL–IDF Standard* no. 20B)

ISO 1975 Cheese- Determination of fat content- van Gulic method. International Organization for Standarization. Standard, 3433, International Organization for Standarization, Leusden, Netherlands

Krause I, Bockhardt A & Klostermeyer H 1997 Characterization of cheese ripening by free amino acids and biogenic amines and influence of bactofugation and heat-treatment of milk. *Lait* **77** 101–108

Kuchroo NC & Fox PF 1982 Soluble nitrogen in cheese: comparison of extraction procedures. *Milchwissenschaft* **37** 331–334

Laht TM, Kask S, Elias P, Adamberg K & Paalme T 2002 Role of arginine in the development of secondary microflora in Swiss-type cheese. *International Dairy Journal* **12** 831–840

Law AJR, Leaver J, Felipe X, Ferragut V, Pla R & Guamis B 1998 Comparison of the effects of high pressure and thermal treatments on the casein micelles in goat's milk. *Journal of Agricultural and Food Chemistry* **46** 2523–2530

Lawrence RC 1991 Incorporation of whey proteins in cheese. In *Factors Affecting the Yield of Cheese*, pp. 79–87. Brussels: IDF (*International Dairy Federation. Special Issue* no. 9301)

Lawrence RC, Creamer LK & Gilles J 1987 Texture development durig cheese ripening. *Journal of Dairy Science* **70** 1748–1760

López-Fandiño R, de la Fuente MA, Ramos M & Olano A 1998 Distribution of minerals and proteins between the soluble and colloidal phases of pressurized milks from different species. *Journal of Dairy Research* **65** 69–78

McSweeney PLH, Fox PF, Lucey JA, Jordan KN & Cogan TM 1993 Contribution of the Indigenous Microflora to the Maturation of Cheddar Cheese. *International Dairy Journal* 613–634

Molina E, Álvarez MD, Ramos M, Olano A & López-Fandiño R 2000 Use of high-pressure-treated milk for the production of reduced-fat cheese. *International Dairy Journal* **10** 467–475

Needs EC, Stenning RA, Gill AL, Ferragut V & Rich GT 2000 High-pressure treatment of milk: effects on casein micelle structure and on enzymic coagulation. *Journal of Dairy Research* **67** 31–42

Ordóñez AI, Ibáñez FC, Torre P & Barcina Y 1999 Effect of ewe's-milk pasteurization on the free amino acids in Idiazabal cheese. *International Dairy Journal* **9** 135–141

Polo C, Ramos M & Sánchez R 1985 Free aminoacids by high performance liquid chromatography and peptides by gel electrophoresis in Mahon cheese during ripening. *Food Chemistry* **16** 85–96

Recio I, Amigo L, Ramos M & López-Fandiño R 1997 Application of capillary electrophoresis to the study of proteolysis of caseins. *Journal of Dairy Research* **64** 221–230

Recio I & Olieman C 1996 Determination of denatured serum proteins in the casein fraction of heat-treated milk by capillary zone electrophoresis. *Electrophoresis* **17** 1228–1233

Skeie S & Ardö Y 2000 Influence from Raw Milk Flora on Cheese Ripening Studied by Different Treatments of Milk to Model Cheese. *Lebensmittel Wissenschaft and Technologie* **33** 499–505

Spackman DH, Stein WH & Moore S 1958 Chromatography of amino acids on sulfonated polystirene resins. *Analytical Chemistry* **30** 1185–1190

Trujillo AJ, Buffa M, Casals I, Fernández P & Guamis B 2002a Proteolysis in goat cheese made from raw, pasteurized or pressure-treated milk. *Innovative Food Science and Emerging Technologies* **3** 309–319

Trujillo AJ, Capellas M, Saldo J, Gervilla R & Guamis B 2002b Applications of high-hydrostatic pressure on milk and dairy products: a review. *Innovative Food Science & Emerging Technologies* **3** 295–307

Trujillo AJ, Pavia M, Ferragut V, Casals I & Guamis B 2000 Analysis of proteins in milk and cheese by capillary zone electrophoresis. *Recent Research Developments in Agricultural and Food Chemistry* **4** 239–255

Trujillo AJ, Royo C, Ferragut V & Guamis B 1999a Ripening profiles of goat cheese produced from milk treated with high pressure. *Journal of Food Science* **64** 833–837

Trujillo AJ, Royo C, Guamis B & Ferragut V 1999b Influence of pressurization on goat milk and cheese composition and yield. *Milchwissenschaft* **54** 197–199