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Proteolysis during ripening of Manchego cheese made from raw or pasteurized ewes' milk. Seasonal variation

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Changes in nitrogen compounds during ripening of 40 batches of Manchego cheese made from raw milk (24 batches) or pasteurized milk (16 batches) at five different dairies throughout the year were investigated. After ripening for six months, degradation of *p*-κ- and β-caseins was more intense in raw milk cheese and degradation of α_{s2}-casein in pasteurized milk cheese. Milk pasteurization had no significant effect on breakdown of α_{s1}-casein. Hydrophobic peptide content did not differ between raw and pasteurized milk cheese, whereas hydrophilic peptide content was higher in raw milk cheese. There were no significant differences between seasons for residual caseins, but hydrophobic peptides were at a higher level in cheese made in autumn and winter and hydrophilic peptides in cheese made in winter and spring. Raw milk cheese had a higher content of total free amino acids and of most individual free amino acids than pasteurized milk cheese. The relative percentages of the individual free amino acids were significantly different for raw milk and pasteurized milk cheeses. The relative percentages of Lys and Ile increased, while those of Val, Leu and Phe decreased during ripening. There were also seasonal variations within the relative percentages of free amino acids. In raw milk cheeses, Asp and Cys were relatively more abundant in those made in autumn, Glu and Arg in cheeses made in winter, and Lys and Ile in cheeses made in spring and summer. Biogenic amines were detected only in raw milk cheese, with the highest levels of histamine, tryptamine and tyramine in cheeses made in spring, winter and spring, respectively.

Keywords: Caseins, peptides, amino acids, biogenic amines, Manchego cheese.

Proteolysis is the most complex and perhaps the most important biochemical event during the maturation of most cheese varieties (Fox et al. 1996). Caseins retained in the curd are hydrolysed by rennet and indigenous milk enzymes to large and medium peptides during cheese manufacture and ripening. Some peptides derived from the hydrophobic fragments of caseins are bitter and, if present at high levels, may cause a pronounced bitterness in cheese (Visser, 1977). Bitter and non-bitter peptides are further degraded to small peptides and free amino acids (FAA) by peptidases of starter lactic acid bacteria and other microorganisms at a variable rate (Tan et al. 1993). FAA are directly involved in cheese flavour or serve as precursors for flavour development (Fox & Wallace, 1997). On the other hand, the decarboxylation of some amino acids in cheese leads to the formation of biogenic amines, non-volatile amines with important physiological effects in humans (Stratton et al. 1991).

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Manchego cheese, the best known variety among Spanish cheeses possessing a Protected Designation of Origin (PDO), is a hard cheese made in La Mancha region (Central Spain) from Manchega breed ewes' milk and usually consumed after 4–8 months (Nuñez et al. 1989). Some aspects of the changes in nitrogen compounds taking place during ripening of commercial Manchego cheese (Ramos et al. 1976), and of Manchego cheese ripened in olive oil (Ordoñez et al. 1978; Ordoñez & Burgos, 1980) have been investigated. Also, the effects of some technological parameters on the proteolysis of experimental Manchego cheeses have been reported (Gaya et al. 1990; Nuñez et al. 1992; Picón et al. 1994; Uceda et al. 1994; Mohedano et al. 1998; Gomez et al. 1999; Poveda et al. 2003).

Ewes management practices have evolved since early works on Manchego cheese proteolysis were carried out 25–30 years ago. Permanent indoor housing of animals, mechanical milking and milk refrigeration at the farm, nowadays common practices, were practically unknown

in the 70s. Those practices may influence milk chemical composition and bacteriological quality and subsequently those of cheese. An updated and wide-scope study, using more precise analytical methods, of the primary and secondary proteolysis taking place during ripening of Manchego cheese is lacking. Such a study would be useful for the characterization of this PDO cheese variety as currently manufactured by the dairy industry.

Changes in the volatile fraction and the sensory characteristics during ripening of commercial raw and pasteurized milk Manchego cheeses were described in two previous papers (Fernández-García et al. 2002a, b). The objective of the present work was to investigate primary and secondary proteolysis during ripening of Manchego cheese, studying the effects of milk pasteurization and season of manufacture.

Materials and Methods

Cheeses

The cheeses for this study were part of the normal production of five dairies, all located within an area of 110 km diameter in La Mancha region. Three artisan dairy farms transforming raw milk (RM) and two large industries transforming pasteurized milk (PM) were selected among the PDO manufacturers. Cheese making procedures were described in a previous paper (Fernández-García et al. 2002a). Briefly mesophilic starter cultures were added and milk was coagulated with bovine rennet in 30–40 min at 31–32 °C. The curd was cut to rice grain size and scalded at 36–37 °C for 20–30 min. Cheeses, of cylindrical shape, each weighing 2·0–3·5 kg, were pressed for 6–10 h, brine-salted for 24 h, ripened at 10–12 °C for 2 months and held afterwards at 6 °C.

Duplicate batches, 2 weeks apart, were made per dairy in each season (i.e. total of eight batches per dairy). Cheeses were sampled 24 h after manufacture, and at 3 and 6 months of ripening. A different cheese from each batch was analysed at each sampling time. Cheeses were transported to the laboratory at 4 °C. Microbiological analyses were carried out on arrival of samples, after aseptic sampling of the interior of cheeses. For chemical determinations, the rind was removed and sectors of cheese were wrapped in aluminium foil, vacuum packed and stored at −40 °C until analysis. Microbiological and chemical analyses were carried out on duplicate samples.

Microbiological analyses

Cheese samples (10 g) were homogenized in 90 ml sterile 20 g sodium citrate/l solution using a Stomacher 400 (Seward Laboratory, London, UK), and decimal dilutions were prepared in sterile 1 g peptone/l solution. Total viable counts were determined on modified plate count agar (Gaya et al. 1999) and lactic acid bacteria on MRS agar (Oxoid, Unipath Ltd, Basingstoke, UK) acidified at pH 5·7, both incubated at 30 °C for 3 d, lactobacilli on Rogosa agar (Oxoid) with anaerobic incubation at 30 °C for 3 d, and enterococci on KF Streptococcus agar (Oxoid) incubated at 37 °C for 2 d. *Micrococcaceae* were determined on mannitol salt agar (Biolife, Milano, Italy) incubated at 37 °C for 2 d, gram negative bacteria on PMK agar (Bio-life) incubated at 37 °C for 24 h, and coliforms on VRBA (Oxoid) incubated at 37 °C for 24 h.

Chemical analyses

Cheese pH was determined using a penetration electrode (Xerolyt 52-32, Crison, Barcelona, Spain), and expressed as the mean value of six measures representative of the cylindrical shape of the cheese.

Caseins were analysed by capillary electrophoresis using a Beckman P/ACE System 2100 (Beckman Instruments España, E-28034 Madrid, Spain), as described by Garde et al. (2002). Residual caseins were calculated as percentage of the total amount of the respective casein initially present in cheese milk using the formula of Picón et al. (1994) for each of the batches.

Hydrophobic and hydrophilic peptides in the water-soluble fraction of cheese were determined by RP-HPLC using a Beckman System Gold chromatograph, as previously described (Gomez et al. 1997). Results were expressed as units of chromatogram area/mg cheese dry matter.

FAA were extracted according to Krause et al. (1995) and analysed by RP-HPLC after derivatization with 6-aminoquinolyl-N-hydroxysuccin-imidyl carbamate (Liu et al. 1995). The concentration of individual FAA in each sample was expressed as mg/kg cheese dry matter. From this concentration, the relative percentage of each individual free amino acid of the total concentration of FAA in the sample was calculated. Biogenic amines were extracted from 20 g cheese with 50 ml 5% trichloroacetic acid and 1 ml 2-amino-3-phenyl-1-propanol (10 mg/ml) as internal standard. Amines were analysed by capillary electrophoresis as described by Fernández-García et al. (1999), in a 57 cm × 50 μm ID hydrophilic coated Celect P150 capillary (Supelco, Bellefonte, PA, USA). Biogenic amines were expressed as mg/kg cheese dry matter.

Statistics

Statistical analysis was performed using the SPSS Win 9.0 program (SPSS, Chicago, IL 60611, USA). Analyses of variance were carried out with type of milk (raw or pasteurized), season of manufacture and cheese age as main effects. Mean comparisons were performed with the Tukey's honestly significant difference test. A two tailed correlation analysis was performed between the proteolysis parameters. A stepwise discriminant analysis was carried out to determine the proteolysis compounds most useful in classifying the samples by dairy and season, using the Wilk's lambda as the statistical selection criterion for the variables.

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Table 1. Values of pH, dry matter and residual caseins in 6-month-old Manchego cheeses made from raw (RM) or pasteurized (PM) ewes' milk throughout the year

Values are means ± SD, for $n = 12$ (RM) or 8 (PM cheese)

	Significance of effects			Season			
	Pasteurization	Season	Milk	Spring	Summer	Autumn	Winter
Cheese pH	***	NS	RM	5·42 ± 0·09^{a}	5·32 ± 0·15^{a}	5·37 ± 0·22^{a}	5·37 ± 0·11^{a}
		*	PM	5·20 ± 0·12^{a}	5·21 ± 0·12^{ab}	5·32 ± 0·13^{b}	5·22 ± 0·07^{ab}
Dry matter	NS	*	RM	69·4 ± 1·79^{ab}	71·8 ± 1·59^{a}	67·9 ± 1·82^{b}	68·8 ± 1·36^{ab}
		*	PM	69·6 ± 1·36^{ab}	69·5 ± 1·67^{ab}	70·9 ± 1·34^{a}	68·8 ± 0·77^{b}
p-κ-casein	*	NS	RM	76·0 ± 10·9^{a}	78·4 ± 29·4^{a}	77·6 ± 17·4^{a}	84·9 ± 25·9^{a}
		NS	PM	85·0 ± 13·8^{a}	89·6 ± 12·5^{a}	98·3 ± 16·9^{a}	95·8 ± 13·7^{a}
α_{s2}-casein	**	NS	RM	37·2 ± 9·1^{a}	32·9 ± 13·7^{a}	40·4 ± 8·0^{a}	32·6 ± 12·8^{a}
		NS	PM	22·6 ± 6·8^{a}	25·4 ± 9·8^{a}	30·1 ± 13·1^{a}	25·3 ± 4·9^{a}
α_{s1}-casein	NS	NS	RM	3·5 ± 3·9^{a}	15·4 ± 21·7^{a}	13·6 ± 14·4^{a}	7·7 ± 5·2^{a}
		NS	PM	3·9 ± 2·3^{a}	6·9 ± 13·8^{a}	14·0 ± 15·5^{a}	7·2 ± 4·1^{a}
β-casein	*	NS	RM	39·1 ± 21·4^{a}	48·9 ± 26·3^{a}	49·2 ± 18·2^{a}	44·8 ± 14·6^{a}
		NS	PM	47·7 ± 10·0^{a}	60·8 ± 22·8^{a}	66·5 ± 16·0^{a}	66·2 ± 10·8^{a}

^{a,b} Means in the same row with the same superscript do not differ significantly ($P > 0·05$). Caseins are expressed as percentages on milk casein content
Significance of effects: NS, $P > 0·05$; * $P \leqslant 0·05$; ** $P \leqslant 0·01$; *** $P \leqslant 0·001$

Results

Microbial counts

Mean counts of all microbial groups were significantly higher ($P < 0·001$) in 1-day-old RM cheeses than in PM cheeses. Total viable counts and lactic acid bacteria in RM cheeses (9·15 and 9·05 log cfu/ml respectively) exceeded by 1 log unit the respective counts in PM cheeses (8·23 and 7·92 log cfu/ml respectively). Differences in mean counts of other microbial groups between RM and PM cheeses were even higher: for *Micrococcaceae*, mean counts were 5·93 log cfu/ml in RM cheeses *v.* 3·34 log cfu/ml in PM cheeses; for enterococci, 6·15 *v.* 3·80 log cfu/ml; for lactobacilli, 6·85 *v.* 4·03 log cfu/ml; for gram negative bacteria 5·92 *v.* 4·01 log cfu/ml and for coliforms 4·36 *v.* 2·44 log cfu/ml.

Cheese pH was influenced by milk pasteurisation, with significantly higher values ($P < 0·01$) for RM than for PM cheeses (Table 1), and by ripening time (data not shown). The season of manufacture had no significant effect on the pH value of RM cheeses; however, significantly higher pH values ($P < 0·05$) were observed for PM cheeses made in autumn. Dry matter of RM and PM cheeses did not differ significantly. The season of manufacture had a significant effect on dry matter of 6-month-old RM and PM cheeses, with higher values for RM cheese made in summer and PM cheese made in autumn (Table 1).

Residual caseins (CN) and peptides

Hydrolysis of β- and *p*-κ-CN were significantly ($P < 0·05$) more intense in RM than in PM cheeses, whereas a higher rate of α_{s2}-CN hydrolysis was observed in PM cheeses (Table 1). Intense proteolysis of α_{s1}-CN was recorded in both types of cheeses, with no significant effect of milk pasteurization. No significant seasonal effect was observed on the levels of residual caseins either in RM or PM Manchego cheeses after ripening for 6 months.

Contents of hydrophobic and hydrophilic peptides are shown in Table 2. RM cheeses showed a significantly ($P < 0·05$) higher content of hydrophilic peptides than PM cheeses, but the level of hydrophobic peptides was not influenced by milk pasteurization ($P > 0·05$). In both RM and PM cheeses, hydrophobic peptides were more abundant in autumn and winter cheeses, while hydrophilic peptides reached the highest levels in winter and spring cheeses. Hydrophobic peptides decreased ($P < 0·001$) with cheese age in all cases, while hydrophilic peptides increased with ripening in PM cheeses and did not vary in RM cheeses. The ratio of hydrophobic peptides to hydrophilic peptides increased with milk pasteurisation ($P < 0·01$), decreased with cheese age ($P < 0·001$) and exhibited significant differences between seasons.

Free amino acids and biogenic amines

Significantly higher ($P < 0·001$) levels of most FAA were recorded for RM than for PM Manchego cheeses, with total contents of 44·64, 33·84, 36·43 and 35·46 g/kg in RM cheeses made in spring, summer, autumn and winter, respectively, and 25·78, 21·10, 18·07 and 31·70 mg/kg in PM cheeses. The exceptions were Arg and Glu, which were not significantly different between RM and PM cheeses, and Tyr, with significantly higher ($P < 0·001$) levels in PM cheeses. In both RM and PM cheeses, the season of manufacture significantly ($P < 0·01$) influenced the levels of all FAA, which increased significantly ($P < 0·001$) with cheese age.

More interesting than the absolute values are the relative percentages of the individual FAA (Table 3). Milk

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Table 2. Hydrophobic and hydrophilic peptides determined at 214 nm and the hydrophobic/hydrophilic ratio in 3- and 6-month-old Manchego cheeses made from raw (RM) or pasteurized (PM) ewes' milk throughout the year

Values are means ± SD, for $n=12$ (RM) or 8 (PM cheese)

	Significance of effects					Season			
	Pasteurisation	Age	Season	Milk	Months	Spring	Summer	Autumn	Winter
Hydrophobic peptides at 214 nm	NS	***	***	RM	3	$103 \pm 39{\cdot}1^{b}$	$112 \pm 29{\cdot}3^{b}$	$121 \pm 35{\cdot}5^{ab}$	$151 \pm 40{\cdot}2^{a}$
					6	$97{\cdot}8 \pm 30{\cdot}1^{a}$	$92{\cdot}8 \pm 33{\cdot}1^{a}$	$112 \pm 21{\cdot}4^{a}$	$111 \pm 65{\cdot}3^{a}$
		***	***	PM	3	$116 \pm 12{\cdot}5^{b}$	$101 \pm 10{\cdot}2^{b}$	$138 \pm 34{\cdot}9^{a}$	$131 \pm 40{\cdot}8^{a}$
					6	$112 \pm 8{\cdot}0^{b}$	$89{\cdot}3 \pm 22{\cdot}1^{c}$	$124 \pm 29{\cdot}1^{a}$	$106 \pm 38{\cdot}4^{b}$
Hydrophilic peptides at 214 nm	*	NS	***	RM	3	$254 \pm 36{\cdot}1^{a}$	$221 \pm 30{\cdot}2^{ab}$	$203 \pm 51{\cdot}6^{b}$	$241 \pm 53{\cdot}2^{a}$
					6	$239 \pm 67{\cdot}5^{ab}$	$194 \pm 70{\cdot}3^{b}$	$231 \pm 33{\cdot}0^{ab}$	$268 \pm 83{\cdot}5^{a}$
		**	***	PM	3	$205 \pm 29{\cdot}9^{b}$	$196 \pm 18{\cdot}6^{b}$	$172 \pm 44{\cdot}4^{c}$	$236 \pm 50{\cdot}6^{a}$
					6	$226 \pm 33{\cdot}0^{b}$	$175 \pm 39{\cdot}1^{c}$	$195 \pm 48{\cdot}3^{bc}$	$291 \pm 16{\cdot}3^{a}$
Ratio hydrophobic/ hydrophilic	***	***	***	RM	3	$0{\cdot}42 \pm 0{\cdot}17^{b}$	$0{\cdot}50 \pm 0{\cdot}07^{ab}$	$0{\cdot}59 \pm 0{\cdot}05^{a}$	$0{\cdot}63 \pm 0{\cdot}10^{a}$
					6	$0{\cdot}43 \pm 0{\cdot}13^{a}$	$0{\cdot}48 \pm 0{\cdot}04^{a}$	$0{\cdot}49 \pm 0{\cdot}05^{a}$	$0{\cdot}39 \pm 0{\cdot}10^{a}$
		***	***	PM	3	$0{\cdot}57 \pm 0{\cdot}04^{b}$	$0{\cdot}52 \pm 0{\cdot}02^{b}$	$0{\cdot}89 \pm 0{\cdot}42^{a}$	$0{\cdot}54 \pm 0{\cdot}27^{b}$
					6	$0{\cdot}50 \pm 0{\cdot}04^{b}$	$0{\cdot}51 \pm 0{\cdot}03^{b}$	$0{\cdot}72 \pm 0{\cdot}30^{a}$	$0{\cdot}37 \pm 0{\cdot}14^{b}$

Peptides are expressed as units of chromatogram area/mg cheese dry matter

[a,b,c] Means in the same row with the same superscript do not differ significantly ($P>0{\cdot}05$)

Significance of effects: NS, $P>0{\cdot}05$; * $P\leqslant 0{\cdot}05$; ** $P\leqslant 0{\cdot}01$; *** $P\leqslant 0{\cdot}001$

pasteurisation was significant for all relative percentages of FAA except for Gly, His and Pro. Season was significant only for some relative percentages of FAA in RM cheese (Table 3). Asp and Cys were relatively more abundant in autumn cheeses, Glu and Arg in winter cheeses, and Lys and Ile in spring and summer cheeses. The seasonal effect was also significant in PM cheeses, but different from RM cheeses. Winter cheeses had significantly higher relative percentages of the acidic amino acids, Glu and Asp, and lower relative percentages of Tyr, Ala, Gly, Val and Phe. The ripening time was significant for some amino acids, interestingly both in RM and PM cheeses, with the relative percentages of Lys and Ile increasing, and those of Val, Leu and Phe decreasing during ripening. Significant positive correlations ($P<0{\cdot}0001$) between the relative percentages of Lys and Ile were observed both in RM ($r=0{\cdot}63$) and PM cheeses ($r=0{\cdot}78$). Also significant positive correlations ($P<0{\cdot}0001$) were observed between the relative percentages of Val-Leu ($r=0{\cdot}68$ in RM, $r=0{\cdot}77$ in PM cheeses), Leu-Phe ($r=0{\cdot}88$ in RM, $r=0{\cdot}85$ in PM cheeses) and Phe-Val ($r=0{\cdot}68$ in RM, $r=0{\cdot}89$ in PM cheeses).

The capillary electrophoresis method used allowed the detection of the biogenic amines absorbing at 214 nm, tyramine, histamine, tryptamine and phenylethylamine. These amines were only found in RM cheeses, being under the level of detection in all PM cheeses. Concentrations of histamine, tyramine and tryptamine increased significantly ($P<0{\cdot}001$) during ripening (Table 4). Phenylethylamine was found in less than 20% of RM cheeses, at levels always below 20 mg/kg cheese dry matter (data not shown). The season of manufacture had a significant effect on histamine ($P<0{\cdot}05$), with lower levels in winter cheeses, and tryptamine ($P<0{\cdot}01$), which was not detected in summer cheeses. Tyramine levels were not dependent on the season of manufacture.

Discriminant analyses

Discriminant analyses were carried out in order to investigate if Manchego cheeses were susceptible to classification by the dairy and the season of manufacture, with the aim of understanding which proteolysis parameters had a higher influence. Table 5 lists the standardized discriminant function coefficients calculated using dairy as the grouping variable. Hundred percent of the cheeses were separated by the milk type (RM or PM) just applying function 1 (Fig. 1). The FAA determining the position of RM and PM cheeses on the plane were, on one side and with positive coefficient, Leu, Tyr and Arg, with higher relative percentages in PM cheeses, and on the other side with negative coefficient, Ile, Lys, Asp, Thr, Met and Cys, with higher relative percentages in RM cheeses.

Table 6 lists the standardized discriminant function coefficients calculated using season as the grouping variable for RM and PM cheeses. Seventy five percent of RM cheeses were correctly classified by the season of manufacture (Fig. 2A). Function 1 explained 78·8% of the variance determining the position of spring/summer cheeses, with higher relative percentages of His, and autumn/winter cheeses, with higher relative percentages of Glu. Function 2 explained only 14·7% of the variance. The seasonal classification was precise for PM cheeses, 98·4% of the cheeses being correctly classified (Fig. 2B). Function 1, explaining 74·8% of the variance, determined three groups, winter, summer and spring/autumn cheeses. The higher relative percentage of Arg determined the position of summer cheeses at the left side. The lower Tyr and the

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Table 3. Mean relative percentages of the individual free amino acids found in 6-month-old Manchego cheese made from raw (RM) or pasteurized (PM) ewes milk throughout the year

Values are means ± SD, for $n = 12$ (RM) or 8 (PM cheese)

	Significance of effects				Season			
	Pasteurization	Age	Season	Milk	Spring	Summer	Autumn	Winter
ASP	***	NS	**	RM	5·34 ± 1·42^{b}	7·81 ± 2·30^{ab}	10·25 ± 3·15^{a}	7·51 ± 4·58^{ab}
		**	***	PM	5·16 ± 1·56^{ab}	6·48 ± 2·45^{a}	2·70 ± 2·32^{b}	6·16 ± 1·19^{a}
SER	**	*	NS	RM	1·34 ± 1·10^{a}	1·20 ± 0·29^{a}	1·30 ± 0·70^{a}	1·08 ± 0·58^{a}
		**	NS	PM	0·67 ± 0·35^{a}	0·68 ± 0·29^{a}	0·89 ± 0·79^{a}	0·54 ± 0·04^{a}
GLU	***	NS	*	RM	10·18 ± 3·68^{b}	11·41 ± 2·60^{ab}	13·28 ± 4·57^{ab}	14·10 ± 2·48^{a}
		NS	***	PM	10·15 ± 5·49^{b}	11·04 ± 2·47^{b}	12·72 ± 4·62^{b}	22·38 ± 2·36^{a}
GLY	NS	NS	NS	RM	5·13 ± 2·69^{a}	3·56 ± 0·63^{a}	3·60 ± 0·68^{a}	3·83 ± 1·53^{a}
		NS	**	PM	3·66 ± 0·40^{a}	3·28 ± 0·57^{ab}	3·11 ± 0·26^{b}	2·85 ± 0·22^{b}
HIS	NS	NS	NS	RM	11·90 ± 3·31^{a}	9·45 ± 4·05^{a}	9·48 ± 2·99^{a}	9·26 ± 3·33^{a}
		***	NS	PM	12·31 ± 2·12^{a}	11·59 ± 1·17^{a}	10·63 ± 1·88^{a}	11·52 ± 1·13^{a}
ARG	***	NS	***	RM	0·49 ± 0·36^{c}	1·63 ± 0·57^{b}	1·78 ± 0·66^{b}	2·61 ± 0·94^{a}
		NS	NS	PM	2·43 ± 1·46^{a}	3·61 ± 1·34^{a}	2·94 ± 1·12^{a}	2·44 ± 0·51^{a}
THR	***	NS	NS	RM	2·43 ± 0·37^{a}	2·31 ± 1·36^{a}	2·80 ± 1·75^{a}	3·57 ± 1·11^{a}
		NS	NS	PM	1·61 ± 0·42^{a}	1·47 ± 0·25^{a}	1·24 ± 0·42^{a}	1·44 ± 0·09^{a}
ALA	**	NS	*	RM	4·13 ± 0·19^{a}	3·19 ± 1·40^{a}	3·25 ± 1·03^{a}	3·04 ± 1·04^{a}
		*	**	PM	3·25 ± 0·36^{a}	3·00 ± 0·30^{ab}	3·05 ± 0·37^{ab}	2·65 ± 0·09^{b}
PRO	*	**	NS	RM	4·63 ± 1·82^{a}	3·74 ± 2·00^{a}	3·48 ± 1·35^{a}	5·24 ± 1·42^{a}
		***	NS	PM	4·12 ± 1·42^{a}	4·03 ± 1·04^{a}	4·66 ± 1·48^{a}	3·78 ± 0·76^{a}
CYS	***	NS	*	RM	0·13 ± 0·15^{b}	0·31 ± 0·46^{ab}	0·57 ± 0·57^{a}	0·26 ± 0·12^{ab}
		NS	NS	PM	ND	ND	ND	ND
TYR	***	NS	***	RM	0·88 ± 0·39^{b}	2·75 ± 2·11^{a}	1·08 ± 0·43^{b}	0·85 ± 0·58^{b}
		*	**	PM	4·46 ± 0·30^{a}	4·24 ± 0·72^{a}	4·30 ± 0·72^{a}	3·31 ± 0·54^{b}
VAL	***	***	NS	RM	9·92 ± 0·57^{a}	9·54 ± 0·65^{a}	9·41 ± 0·79^{a}	9·43 ± 0·64^{a}
		***	***	PM	11·20 ± 1·15^{a}	10·44 ± 0·77^{ab}	12·23 ± 2·58^{a}	8·74 ± 0·45^{b}
MET	***	NS	**	RM	3·83 ± 1·03^{ab}	4·64 ± 0·90^{a}	3·73 ± 0·36^{ab}	3·44 ± 0·49^{b}
		NS	NS	PM	2·73 ± 0·60^{a}	3·59 ± 2·04^{a}	2·08 ± 0·88^{a}	2·38 ± 0·43^{a}
LYS	***	***	***	RM	13·05 ± 1·40^{a}	12·60 ± 1·30^{a}	11·36 ± 0·44^{b}	11·17 ± 1·14^{b}
		***	NS	PM	9·38 ± 1·89^{a}	9·52 ± 2·40^{a}	8·79 ± 1·77^{a}	8·68 ± 1·56
ILE	***	***	***	RM	5·41 ± 0·69^{a}	5·02 ± 0·76^{a}	4·70 ± 0·71^{ab}	4·24 ± 0·52^{b}
		***	NS	PM	3·24 ± 0·39^{a}	2·97 ± 0·68^{a}	2·86 ± 0·50	2·91 ± 0·37^{a}
LEU	***	*	NS	RM	13·61 ± 0·70^{a}	13·52 ± 1·39^{a}	12·86 ± 0·89^{a}	13·14 ± 1·29^{a}
		NS	*	PM	17·32 ± 2·16^{a}	16·07 ± 1·18^{ab}	17·88 ± 3·97^{a}	14·01 ± 0·49^{b}
PHE	**	***	NS	RM	7·60 ± 0·37^{a}	7·33 ± 0·71^{a}	7·07 ± 0·57^{a}	7·25 ± 0·43^{a}
		***	**	PM	8·31 ± 1·86^{ab}	7·99 ± 1·07^{ab}	9·87 ± 3·36^{a}	6·19 ± 0·49^{b}

Free amino acids are expressed as relative percentages on total concentration of free amino acids

^{a,b,c} Means in the same row with the same superscript do not differ significantly ($P > 0·05$)

ND: below detection limit. Significance of effects: NS, $P > 0·05$; * $P \leqslant 0·05$; ** $P \leqslant 0·01$; *** $P \leqslant 0·001$

Table 4. Biogenic amines[1] in 3- and 6-month-old Manchego cheeses made from raw (RM) ewes' milk throughout the year

Values are expressed in mg/kg cheese dry matter means ± SD, for $n = 12$ (RM) or 8 (PM cheese)

	Significance of effects				Season			
	Age	Season	Milk	Months	Spring	Summer	Autumn	Winter
Histamine	**	*	RM	3	24·5 ± 39·9^{a}	50·3 ± 65·0^{a}	40·1 ± 50·8^{a}	ND
				6	229·0 ± 269·4^{a}	110·8 ± 122·4^{ab}	109·2 ± 147·2^{ab}	54·1 ± 108·1^{b}
Tyramine	***	NS	RM	3	304·1 ± 45·2^{a}	275·9 ± 183·9^{a}	289·8 ± 79·3^{a}	233·0 ± 84·3^{a}
				6	533·0 ± 142·6^{a}	402·4 ± 185·2^{a}	443·5 ± 109·0^{a}	486·9 ± 236·8^{a}
Tryptamine	**	**	RM	3	ND	ND	2·3 ± 5·5^{a}	2·8 ± 6·6^{a}
				6	11·1 ± 20·0^{a}	ND	4·0 ± 9·0^{b}	15·3 ± 23·5^{a}

^{a,b} Means in the same row with the same superscript do not differ significantly ($P > 0·05$)

ND, below detection limit. Significance of effects: NS, $P > 0·05$; * $P \leqslant 0·05$; ** $P \leqslant 0·01$; *** $P \leqslant 0·001$

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Table 5. Standardized discriminant function coefficients for the proteolysis indices using dairy as the grouping variable

	Function 1	Function 2
Variance	67·4%	20·8%
Hydrophobic peptides	−0·421	−1·080
Hydrophobic/hydrophilic peptides	0·563	0·420
CYS[1]	−0·451	0·277
TYR	0·700	0·205
VAL	−0·107	1·244
MET	−0·105	0·720
LYS	−0·485	−0·372
ILE	−0·328	0·277
LEU	1·277	0·608
PHE	−1·037	−1·069
GLU	0·475	1·070
GLY	0·585	−0·116
HIS	0·214	0·752
ARG	0·226	−0·313
THR	−0·126	0·568
PRO	0·283	0·349

[1] Values of free amino acids were in relative percentages

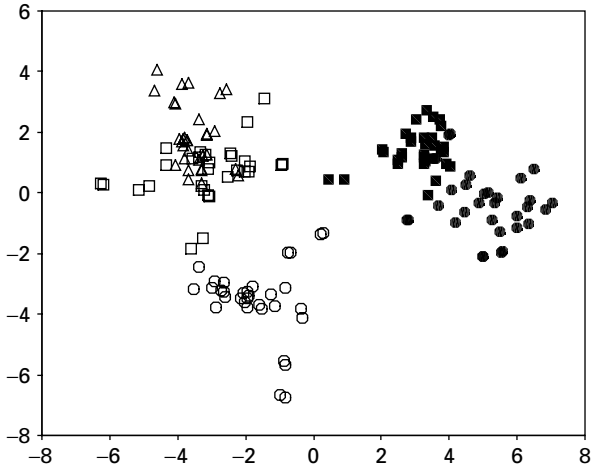

Fig. 1. Plot of sample distribution using the two canonical discriminant functions. Manchego cheeses made from raw milk in artisan dairy A (○), artisan dairy B (□) and artisan dairy C (△). Manchego cheeses made from pasteurized milk in industrial dairy A (●) and industrial dairy B (■).

higher Glu relative percentages and the abundance of hydrophilic peptides determined the position of winter cheeses at the right side. Function 2 explained 19·6% of the variance, determining the separation of spring cheeses from the rest, due to their higher content of hydrophilic peptides and the lower percentage of Arg and Glu.

Discussion

Effect of milk pasteurization on Manchego cheese proteolysis

The more intense breakdown of β- and p-κ-CN, but not of α_{s1}-CN, found in this study for RM Manchego cheeses, are opposite to the results reported by Gaya et al. (1990) and Gomez et al. (1999), who observed a more intense degradation of both α_s- and β-CN in PM cheese compared with RM cheese. In the case of α_s-CN this fact was explained by the greater retention of rennet in PM cheese. Results reported for Cheddar cheese proteolysis also vary: a slower breakdown of β-CN and of the high molecular weight peptides was observed in pasteurized milk cheese by Lau et al. (1991), compared with RM cheese, coinciding with our results for ewes' milk Manchego cheese. However, McSweeney et al. (1993) did not find significant differences in primary proteolysis between RM and PM Cheddar cheeses. Milk microorganisms and the activity of enzymes other than rennet, such as plasmin and cathepsin D may influence casein hydrolysis during cheese ripening (Grappin & Beuvier, 1997). Plasmin activity, with a higher specificity for β-CN, is enhanced by pasteurization, while cathepsin D, acting preferably on α-CN, is only partially thermally deactivated (Kitchen, 1985). These facts would not explain a higher β-CN breakdown in RM cheeses. A more plausible explanation would be the decreased susceptibility to proteolysis of the complex β-CN-β-lactoglobulin in pasteurized milk cheeses as reported by Lau et al. (1991). The variable strain composition of starter cultures used for the manufacture of RM and PM Manchego cheese and the considerably higher populations of lactobacilli, enterococci, *Micrococcaceae*, gram negative bacteria and coliforms in RM cheeses, could be also responsible for the differences in degradation of the various casein fractions, depending on the type and activity of their proteinases.

The hydrophilic peptide content of RM cheese was higher than that of PM cheese, in agreement with data

Table 6. Standardized discriminant function coefficients for the proteolysis indices of RM and PM Manchego cheeses, using season as the grouping variable

		Function 1	Function 2
RM cheeses	Variance	78·8%	14·7%
	TYR[1]	−0·579	0·029
	GLU	0·738	0·148
	HIS	−0·562	−0·108
	ARG	1·062	−0·456
	PRO	−0·052	1·066
PM cheeses	Variance	174·8%	119·6%
	CYS[1]	−0·378	0·914
	TYR	−0·834	0·318
	LEU	1·349	−1·152
	GLU	1·235	0·861
	ARG	−0·495	1·417
	THR	−1·120	0·033
	ALA	0·877	0·129
	PRO	1·833	0·089
	ASP	−0·196	0·624
	Hydrophilic peptides	1·473	−0·730

[1] Values of free amino acids were in relative percentages

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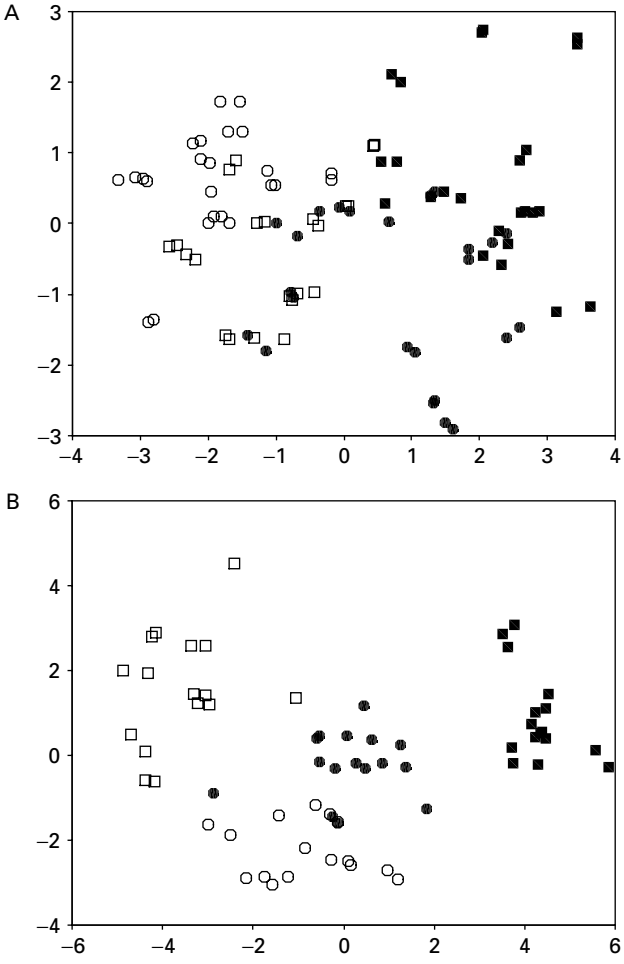

Fig. 2. Plot of raw milk (A) and pasteurized milk (B) Manchego cheeses distribution using the two canonical discriminant functions. Cheeses made in spring (○), summer (□), autumn (●) and winter (■).

reported for Manchego cheese by Gomez et al. (1999) and for Cheddar cheese by Lau et al. (1991). Although the hydrophobic peptide content was not significantly different between RM and PM cheeses, the former cheeses showed lower hydrophobic peptide/hydrophilic peptide ratio, as reported for ripe RM Manchego cheese by Taborda et al. (2003). The water-soluble nitrogen fraction of 3-month-old RM and PM Cheddar cheeses exhibited quite different RP-HPLC profiles, both qualitatively and quantitatively, according to McSweeney et al. (1993).

The most dramatic differences between RM and PM Manchego cheeses were observed in the FAA fraction, in agreement with the results of McSweeney et al. (1993) who reported a reduction by 50% of the total FAA content when Cheddar cheese was produced from PM. Compared with earlier and recent studies on Manchego cheese proteolysis (Ordoñez & Burgos, 1980, Taborda et al. 2003), our results show a more intense secondary proteolysis, although the most abundant individual FAA found were basically the same, i.e. Leu, Glu, Lys, His, Val, Phe and Ile.

The relative amounts of individual FAA found in Manchego cheese were not a reflection of the theoretical concentration of amino acids in intact caseins, they were not constant during ripening, and differed between RM and PM cheeses. For the calculation of the theoretical concentration of amino acids, the composition of the variant A of the ovine α_{s1}-CN and the ovine β-CN and the percentages of these caseins in ovine milk were taken into account. FAA like Ser, Thr and Pro were at much lower values (<50%) than theoretical values in both RM and PM cheeses, while Asp, Phe and specially His were at higher values than theoretical. Zarmpoutis et al. (1996) also reported lower Pro and Thr concentrations in Irish blue cheese than theoretically expected.

The reported pattern of FAA in Manchego cheese would be consistent with our present knowledge about chymosin cleavage sites on caseins and about the activity of lactic acid bacteria peptidases. The high positive correlation between free Leu, Val and Phe found in this study would be compatible with the position of these residues in or close to the major cleavage sites of α_{s1}-CN by chymosin. The first cleavage site would be the Phe_{23}-Val_{24} bond, in the same manner as reported for caprine caseins (Trujillo et al. 1998). The cleavage in this site releases the peptide f(1-23), which is rapidly hydrolysed by the lactococcal cell envelope proteinases. Another sensitive cleavage site would be the Leu_{149}-Phe_{150} bond according to McSweeney et al. (1993). These authors reported that the peptide bonds most susceptible to hydrolysis by chymosin generally had an aromatic or hydrophobic residue at the N-terminal side of the bond in bovine αs_1-CN, result corroborated by Trujillo et al. (1998) in variant A of caprine α_{s1}-CN. A weak potential of chymosin to cleave sites in the phosphorylated region of the α_{s1}-CN was observed by Trujillo et al. (1998), which would agree with the lower relative percentage of free Ser found in Manchego cheese. On the other side, the high positive correlation found between Lys and Ile would be compatible with the proximity of these amino acid residues in the more exposed N-terminal of γ_1-CN and C-terminal of the β-CN f(1-28) corresponding peptide. Many peptidases of lactic acid bacteria do not cleave bonds that contain Pro, a fact which would agree with the low percentages of this free amino acid in Manchego cheese.

Regarding the variation during the ripening time, amino acids Leu and Val seemed to be released at a higher rate at the beginning of ripening, consistent with the early action of chymosin on α_{s1}-CN. However, the subsequent degradation of these amino acids into 3-methyl-1-butanol and 2-methyl-1-propanol, would have caused the decrease of their relative percentages with the ripening time in RM cheeses. Alcohols coming from the metabolism of Val and Leu were found to be significantly more abundant in RM than in PM Manchego cheese (Fernández-García et al. 2002a).

Some FAA showed significantly different relative percentages between RM and PM cheeses, which points to quantitative and qualitative differences between the

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peptidase activity of starter cultures and of the indigenous microbiota. The relative percentage of free Lys was higher than theoretical in RM cheeses and that of Ile was much lower than theoretical in PM cheeses. This would indicate that peptidases liberate Lys and Ile at a higher rate in RM cheeses, which would agree with the higher primary proteolysis of β-CN in these cheeses. On the other hand, the relative percentages of free Arg and Tyr were lower than theoretical in RM cheeses and higher in PM cheeses, pointing to a subsequent metabolism of these amino acids into biogenic amines, which were exclusively detected in RM cheeses.

The FAA determining the separation of RM and PM cheeses in the discriminant analysis were those subjected to further degradation especially in RM cheeses, such as Tyr, decarboxylated to tyramine, Leu, probably metabolised to 3-methyl-1-butanal and 3-methyl-1-butanol, and Phe, probably metabolised to phenyl derivative compounds in RM cheeses.

Seasonal effect on Manchego cheese proteolysis

A seasonal effect was not equally observed on all the proteolysis fractions determined in Manchego cheese. While the degradation rate of caseins was independent of the season of manufacture, a significant seasonal effect was observed in the peptide content although the results, differing between RM and PM cheeses, can not be easily explained. It was clear that the hydrophobic peptides/hydrophilic peptides ratio was significantly higher in PM cheeses made in autumn and that cheeses made in spring exhibited the highest content of total FAA, histamine and tyramine among RM cheeses. Qualitative more than quantitative differences of the milk microbiota would account for the variation in the seasonal proteolysis patterns. For example, different strains of *Lactococcus* have different peptide formation and degradation abilities (Morales et al. 2002), and the same is probably true among strains of *Lactobacillus*, a genus reaching high counts during late maturation stages of Manchego cheese (Nuñez et al. 1989). Although the seasonal differences were not statistically significant, the highest content of total FAA in spring RM Manchego parallels the richer volatile fraction reported for these cheeses (Fernández-García et al. 2002b).

Discriminant analyses can help interpreting the seasonal effect. In RM cheeses only five variables met the conditions to be used in the analysis, and only 75% of the cheeses were classified correctly (Fig. 2A). Cheeses made in spring and summer were closer to each other and so were cheeses made in autumn and winter. Contrary to expected, the seasonal variability of PM cheeses relating to the proteolysis indices seems clearer than that of RM cheeses (Fig. 2B), winter cheeses being completely separated from the rest and especially from the summer cheeses. One variable determining the separation of winter cheeses in both RM and PM cheeses is their higher Glu relative content, but other variables were different for RM or PM cheeses. The separation of summer and winter cheeses would be expected, provided the large variation in climatic conditions between summer and winter in this inland region of Spain. Spring and autumn cheeses seem more alike in PM cheeses, maybe because of similar climate conditions of these two seasons in La Mancha.

As has been reported in previous works (Fernández-García et al. 2002a), Manchego cheeses made from raw or pasteurized milk are significantly and remarkably distinct, even though the same PDO label is protecting both types. This should be taken into account if a homogeneous product is desired. The seasonal variability is not easy to evaluate, but strikingly it has been found more noticeable in PM than in RM cheeses. The high variability between RM cheeses from different dairies might have masked the seasonal effect in this type of cheese.

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