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Influence of rennet milk-clotting activity on the proteolytic and sensory characteristics of an ovine cheese

A. Irigoyen *, J.M. Izco, F.C. Ibáñez, P. Torre

Area de Nutrición y Bromatología, Departamento de Ciencias del Medio Natural, Universidad Pública de Navarra, Campus Arrosadía s/n, Pamplona 31006, Navarra, Spain

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Abstract

Roncal cheese (regulated by an Apellation of Origin) is a traditional hard cheese manufactured from raw ewe's milk in the region of Navarre in Spain. Roncal cheeses, manufactured using two lamb rennets with different milk-clotting activity levels, were evaluated to compare their chemical, proteolytic, and sensory characteristics. A preliminary study of samples of lamb rennets indicated that a large proportion of such rennets did not fulfil current microbiological requirements and likewise revealed considerable variation in the milk-clotting activity of the samples examined. Trends in the overall physicochemical parameter values (pH, dry matter, fat, and protein) were similar in both cheese batches. Proteolysis of the nitrogen fractions was observed to take place at a faster rate in the cheeses made using the rennet with the higher milk-clotting activity (soluble nitrogen, non-protein nitrogen, and amino acid nitrogen values around 13–20% higher than in the cheeses made using the rennet with the lower milk-clotting activity after 180 days of ripening). Urea-PAGE electrophoretic analysis of the caseins from the cheeses manufactured using both types of rennet showed that the β-caseins were less susceptible to proteolysis than the α_s-caseins. The effect of the different milk-clotting activity levels was most pronounced on the α_s-caseins, in which the rennet with the higher milk-clotting activity gave higher breakdown. Nevertheless, the differences in the proteolysis rates did not yield any appreciable sensory differences. © 2001 Published by Elsevier Science Ltd.

Keywords: Milk-clotting activity; Lamb rennet; Ovine cheese; Proteolysis; Sensory analysis

1. Introduction

The flavour and final texture of cheese are the outcome of a series of chemical, biochemical, and microbiological events that occur during ripening. Proteolysis is the major contributor to the changes taking place and occurs in most pressed-curd cheeses, e.g. Roncal cheese. The main proteolytic agents involved are: the natural proteases in the milk itself; the rennet or milk-clotting enzymes retained in the curd; the proteases and peptidases from the microorganisms in the starter; and the enzymes from non-starter bacteria.

All rennets and milk-clotting enzyme complexes capable of coagulating milk at normal physiological pH levels at a temperature of 30°C contain one or more enzymes belonging to the acid proteinase group, such as chymosin and pepsin. Gastric proteinases from young calves, kids, and lambs have traditionally been used in the manufacture of most cheese varieties, with calf chymosin being the type most commonly employed. Lamb and kid chymosins are still used in the production of hard cheeses in parts of Italy.

The milk-clotting activity of the rennet employed is one factor that affects the extent of casein degradation. The level of milk-clotting activity of rennet depends on the chymosin content of the abomasum. The proportion decreases as the animal ages, accompanied by a concomitant increase in the pepsin content. Pepsin is a highly active proteolytic, though relatively non-specific, enzyme that has been held responsible for the production of bitter flavours in cheese (Guinee & Wilkinson, 1992). The proportions normally present in commercial rennets are 70% chymosin and 30% pepsin. The use of rennets with different proportions of these enzymes brings about different proteolytic changes in the cheese.

Roncal cheese, made from raw ewe's milk, was the first Spanish cheese to be granted an Appellation of

* Corresponding author. Tel.: +34-948-169141; fax: +34-948-169187.

E-mail address: lablac@unavarra.es (A. Irigoyen).

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Origin (AO), in 1981. It is an uncooked, pressed-curd cheese, with a minimum ripening time of four months. The regulations of the AO permit the addition of artisanal lamb or commercial calf rennet. Because of the great variety in lamb rennets, the AO regulating authorities are interested in studying the effect of milk-clotting activity on the final product characteristics.

In consequence, the primary object of the present study was to examine variability in the milk-clotting properties and microbiological characteristics of different lamb rennets and the effect of using rennets, with differing milk-clotting activity levels, on the protein fractions, on the casein fractions, and on the organoleptic characteristics of AO Roncal ovine cheese.

2. Materials and methods

2.1. Preparation of lamb rennet samples

The abomasi, or rennet stomachs, removed from suckling lambs, were tied at both ends and stored in a ventilated chamber protected from light for 2 months. When dry, the surface fat was removed, the stomachs were cut lengthwise into strips, and the strips were cleaned and then sliced into small cube-shaped sections. The cubes were mixed with salt (1:2 w/w) and packed in air-tight jars. An amount of 15 g of that mixture was dissolved in 200 ml of water and steeped for 24 h, with stirring. The solution was filtered and the filtrate stored chilled, protected from light until used. A total of 12 different lamb rennets were prepared in that way, and microbiological assays and analyses of coagulating activity were performed. On the basis of the results, two of those rennets were selected for cheese-making, as discussed below in Section 3.

2.2. Microbiological assays

The microorganisms used are listed in the ''General standards for identification and purity of rennet and other milk-clotting enzymes for the domestic market'' (Official Government Gazette of Spain, 1988). A specific culture method and culture medium were used for each microbial genus considered in accordance with the recommendations of the International Commission on Microbiological Specifications for Food (ICMSF, 1982) and Pascual (1992). Total viable counts were determined on plate count agar; *Enterobacteriaceae* on violet red bile glucose agar; yeasts and molds on oxytetracycline glucose yeast extract agar; *Staphylococcus aureus* on Baird-Parker agar base and afterwards tested using the DNSA reagent test; sulfite-reducing Clostridia on sulfite-polymyxin-sulfadiazine agar; *Salmonella* on brilliant green agar following enrichment with selenite-cystine; *Escherichia coli* in brilliant green bile lactose broth.

All determinations were performed in duplicate and have been expressed as colony-forming units per gramme of rennet.

2.3. Milk-clotting activity determination

Milk-clotting activity of the rennet samples prepared as described, was assessed using a modified version of the method of Berridge, with visual determination of the flocculation point of a milk substrate following the addition of coagulating enzyme (IDF, 1987).

2.4. Determination of the chymosin and pepsin contents

This determination was based on separation of the two enzymes on a cellulose column using ion-exchange chromatography, followed by measurement of the milk-clotting activity (IDF, 1987).

2.5. Cheese-making and sampling

Raw ewe's milk was collected from a sheep flock on the morning of cheese-making and transported to the pilot plant. The milk was divided into two equal portions, and cheeses were manufactured simultaneously at the pilot plant in accordance with the conventional methods approved by the Regulatory Board of the Roncal Cheese Apellation of Origin (Official Government Gazette of Spain, 1991). The milk was heated to 22°C, and 1.2 doses of starter (Ezal®, Dangé Saint-Roman, France) per 100 l were added and allowed to work for 30 min. A lamb rennet of the type described in the Results section was added in an amount of 3 ml per 10 l of milk, with coagulation taking 40–50 min at 32°C. The curd thus obtained was sliced, stirred for 30 min, and reheated to 38°C to facilitate drainage of the whey. The curd was packed in cylindrical molds and pressed for 3 h a 2.5 kg/cm^2 at 20°C. The cheeses were brined in a saturated NaCl solution at 13°C for 18 h. The cheeses were then stored for two weeks in an airing chamber at 10°C and a relative humidity of 75%. They were then transferred to a ripening chamber and stored at 10°C and a relative humidity of 85% pending analysis.

Two replications were performed. In each replication, two cheeses manufactured with each type of rennet were selected for analysis at 1, 15, 30, 60, 120, and 180 days of ripening, making a total of 48 cheeses sampled in all. All analyses were performed in duplicate, and hence a total of eight analyses were performed at each sampling date for each cheese type.

2.6. Physicochemical analyses

The pH was measured using the method of Berdague and Grappin (1987). Total dry matter (DM) was determined according to IDF standard no. 4 (IDF, 1986).

ISO standard no. 3433:1975 (ISO, 1975) was used to determine the fat content of the cheeses. The total nitrogen (TN) content was estimated (Kjeldahl method) according to IDF standard no. 25 for cheese (IDF, 1985).

2.7. Proteolysis assays

The soluble nitrogen (SN) fraction was determined by precipitating out the insoluble nitrogen fraction (caseins) in a buffered solution of acetic acid/acetate at pH 4.6 (Basch, Douglas, Procino, Holsinger & Farrel, 1985). The non-protein nitrogen (NPN) fraction was obtained by adding 15% (w/w) trichloroacetic acid to precipitate out the soluble and insoluble proteins (Basch et al.). The sulfosalicylic acid-soluble nitrogen (SSAN) fraction was assayed and quantified as previously described (Izco, Torre & Barcina, 2000).

2.8. Electrophoretic analysis of the caseins

Casein separations were carried out according to the method of Ibáñez, Torres, Ordóñez and Barcina (1995). Samples were assayed by urea-polyacrylamide gel electrophoresis (separating gel T = 12.5%, pH 8.8; stacking gel T = 4%, pH 6.8) using a Mini Protean IITM system from Bio-Rad. The gels were stained with Coomassie blue and read using a model GS-700 densitometer (Bio-Rad Laboratories, Richmond, CA, USA) at 600 nm. Ovine milk casein was run in a separate lane for reference purposes.

The residual α_{s1}, α_{s2}, β_1, and β_2-casein fractions were calculated as a percentage of the total amount of the respective casein fraction initially present in the one-day-old cheeses. The α_{s1}-I and γ-casein fractions, which are formed by breakdown of the α_{s1} and β-caseins, respectively, were calculated as percentages of the total casein present on each of the ripening dates considered.

2.9. Sensory analysis

Cheese samples aged for 120 and 180 days underwent sensory analysis by a panel of expert assessors who were members of the Roncal AO tasting board. All samples were evaluated by at least eight trained assessors. The attributes evaluated were characteristic odour, characteristic aroma, the texture components elasticity, firmness, adherence, brittleness, grittiness, moisture, and characteristic texture, the flavour components characteristic flavour, pungency, sweetness, saltiness, bitterness, and aftertaste. Intensity of each attribute was rated on an increasing scale of from 1 to 7. A total score that reflected overall cheese quality was calculated for each cheese sample by multiplying each attribute score by a factor proportional to its contribution to the total sensory characteristics of this type of cheese. The maximum total score was 100, with odour contributing 20, aroma 10, texture 20, flavour 30, and aftertaste 20.

2.10. Statistical analysis

SPSS version 6.1 (SPSS Inc., Chicago, IL, USA) was the statistical package used for statistical processing of the results. Analysis of variance (ANOVA) was employed to establish statistical differences between the physicochemical parameter values, nitrogen fraction values, and sensory analysis scores according to rennet type, ripening time, and the interaction between those two factors.

3. Results

3.1. Lamb rennets

Table 1 sets out the results of the microbiological assays and the assays of the milk-clotting activity of the different rennet samples. *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, and sulfite-reducing clostridia were not present in any of the samples.

Only one-third of the lamb rennet samples prepared (rennets 2, 3, 5, and 6, appearing in boldface) fulfilled the requirements laid down in the Official Government Gazette of Spain (1988) for use in cheese-making (the data items marked with an asterisk exceeded allowable limits). The samples exhibited a broad range of variability with respect to milk-clotting activity. Samples 2 and 3, the former with a high level of milk-clotting activity (batch A, 97.5 RU/ml), the latter with a low level of milk-clotting activity (batch B, 16.3 RU/ml), were selected for use in manufacturing the cheeses. The

Table 1
Results of microorganism counts (expressed as colony forming units per g) and milk-clotting activities of the lamb rennet samples collected[a]

Rennet sample	Mesophiles	Enterobacteriaceae	Molds	Yeasts	Rennet unit
1	3 050	625	20[b]	910[b]	24.5
2	*10 550*	*0*	*0*	*5*	*97.5*
3	*395*	*2*	*1*	*0*	*16.3*
4	455	0	2	44[b]	18.9
5	*4250*	*0*	*1*	*0*	*24.8*
6	*9800*	*0*	*2*	*0*	*16.3*
7	> 300 000[b]	> 30 000[b]	0	> 3000[b]	81.9
8	> 300 000[b]	> 30 000[b]	3	> 3000[b]	77.2
9	820	1050[b]	0	150[b]	14.6
10	885	840[b]	0	102[b]	12.2
11	> 300 000[b]	> 30 000[b]	0	> 3000[b]	45.7
12	> 300 000[b]	> 30 000[b]	0	> 3000[b]	62.6

[a] Lamb rennet samples that fulfilled the requirements laid down in the Official Government Gazette of Spain are in italic.

[b] Data items that exceeded allowable limits.

chymosin and pepsin contents of these two rennets were determined and are given in Table 2.

3.2. Physicochemical characteristics

Table 3 presents the differences in the mean values for the batches prepared using the high milk-clotting activity and low milk-clotting activity rennets. The trends in the pH values over the ripening period were similar for both batches. In both cheese batches the pH increased until day 30–60 of ripening, after which it decreased slightly. This same finding has been reported earlier (Oria & Sala, 1992) and can be attributed to the breakdown of lactic acid when all the residual lactose has been metabolized and the production of basic substances is completed (McSweeney & Fox, 1993) and to the metabolization of the non-protein nitrogen components and organic acids (Farkye & Fox, 1990). Percentage DM increased continuously, with values similar to those reported by Oria and Sala (1992) and Millán, Alcalá, Sanjuán, Penedo and Castelo (1992) for this same cheese. The difference in the percentage DM in the two batches was only significant at the end of the ripening period, with the DM value in batch A being 0.5% higher than the DM value in batch B. The fat content remained steady in both cheese batches throughout the ripening period at levels comparable to those reported by Millán et al. (1992) and the values published by those same researchers for Idiazábal cheese (Millán, Alcalá, Sanjuán, Penedo & Castelo, 1991). The percentage total protein decreased slightly over the period considered, with values being comparable to the results previously reported for this same type of cheese (Millán et al., 1992) and for Urbasa cheese (Guindeo, Astasara & Bello 1990).

The ANOVA results (Table 4) showed that milk-clotting activity had no significant effect on the DM, fat, and protein values but that pH was significantly higher in the batch in which the rennet with the higher milk-clotting activity was used.

3.3. Nitrogen fractions

SN/TN values increased throughout ripening in both cheese batches, but levels were significantly higher in the batch made using the rennet with the higher milk-clotting activity (Table 5). The formation of soluble nitrogen compounds during ripening is an index of the rate and extent of proteolysis, in that it is an indicator of casein hydrolysis brought about by the action of the rennet and the milk proteases present at the start of ripening (Visser, 1977). The SN values were similar to the values recorded for this same cheese by Millán et al. (1992) and Oria and Sala (1992). SN values similar to or higher than the values recorded in this study have been published for other ewe's-milk cheeses (Fernández del Pozo, Gaya, Medina, Rodríguez-Maŕin & Núñez, 1989; Gaya, Medina, Rodríguez-Marin & Núñez, 1990; González, Mas & López, 1990; Guindeo et al., 1990; Millán et al., 1992). The different milk-clotting activity levels of the rennets employed yielded significant differences in

Table 2
Percentage chymosin and pepsin in the lamb rennet samples selected

Rennet	Chymosin	Pepsin
A	77.6	22.4
B	54.0	46.0

Table 3
Mean values for pH, dry matter (DM), fat to DM ratio, and total protein to DM ratio in Roncal cheeses made using a lamb rennet with a low level of milk-clotting activity (left-hand column under each column heading) (batch B) and the difference with respect to the values in the cheeses made using a lamb rennet with a high level of milk-clotting activity (right-hand column under each column heading) (batch A)

Day	pH		DM		Fat/DM		Protein/DM	
1	5.00	0.03	57.8	0.2	51.1	0.1	38.5	−0.4
15	5.51	0.06	64.0	−0.2	52.0	0.0	37.0	−0.4
30	5.60	0.07*	66.5	0.4	50.5	−0.2	36.4	0.5
60	5.59	−0.01	66.3	0.5	51.6	0.9	36.9	−0.3
120	5.65	−0.06	72.5	0.3	51.2	−0.6	36.9	−0.7
180	5.44	0.14**	71.2	0.3*	51.2	1.0	35.8	1.3**

*$P < 0.05$.
**$P < 0.01$.

Table 4
P-values from the analysis of variance performed on the pH, dry matter (DM), fat, and protein values

Factor	pH	DM	Fat/DM	Protein/DM
Ripening time (t)	< 0.0001	< 0.0001	0.0762	0.0019
Rennet type (T)	0.0099	0.9359	0.5970	0.9504
Interaction ($t \times$T)	0.0113	0.8591	0.7884	0.2222

Table 5
Mean percentage values for soluble nitrogen (SN), non-protein nitrogen (NPN), and sulfosalicylic acid-soluble nitrogen (SSAN) on total nitrogen in Roncal cheeses made using a lamb rennet with a low level of milk-clotting activity (left-hand column under each column heading) (batch B) and the difference with respect to the values in the cheeses made using a lamb rennet with a high level of milk-clotting activity (right-hand column under each column heading) (batch A)

Day	SN/TN		NPN/TN		SSAN/TN	
1	5.50	0.05	2.80	−0.08	0.19	−0.03
15	9.57	2.84*	4.47	0.79	0.77	0.22
30	10.3	3.12**	7.72	2.28	1.15	0.18
60	14.8	5.45*	10.7	3.19**	2.02	0.35**
120	17.0	5.87**	14.2	5.14**	2.92	0.93**
180	19.5	6.12**	17.5	5.30***	3.77	0.55

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.0001$.

the SN values (Table 5); the batch made with the rennet with the higher chymosin content presented higher SN values. This finding is in accordance with the findings published by other researchers (Santoro & Faccia, 1998).

The percentage NPN/TN values increased continuously over the ripening period in both cheese batches but did present significant differences (Table 5). On the whole, the cheeses made using the rennet with the higher level of milk-clotting activity displayed higher NPN values, with the differences turning significant from 60 days of ripening (Table 5). In other studies performed on Roncal cheese (Millán et al., 1992; Ordóñez, Masso, Marmol & Ramos, 1980; Oria & Sala, 1992) percentage values of between 9.80 and 17.7% were recorded at 90 days of ripening.

The SSAN/TN ratio increased in both batches over the ripening period, with significant differences for the different rennets employed (Table 5). In this case too, the differences between the two cheese batches were significant at 60 and 120 days of ripening, with the cheeses made using the rennet with the higher level of milk-clotting activity exhibiting the higher values. The formation of small peptides and amino acids is caused mainly by the action of the microbial enzymes contributed by the starter culture (Furtado & Partridge, 1988). Consequently, the level of milk-clotting activity of the rennet would not, in principle, be expected to exert an influence on the two indices of proteolysis (NPN and SSAN), in that the rennet does not act directly on the processes concerned. However, the action of the coagulating enzymes on the caseins does give rise to the peptides used by the bacteria as substrates, thereby producing dipeptides and amino acids.

3.4. Electrophoresis of the caseins

The α- and β-casein values underwent a significant decrease with ripening time (Fig. 1). This was a result of the action of the residual rennet in combination with the action of the hydrolytic enzymes of the microorganisms present in the cheeses (Fontecha, Peláez, Juárez, Requena, Gómez & Ramos, 1990). Table 6 presents the analysis of variance results and shows that the differing milk-clotting activities of the two rennets tested did not have a significant influence on the β_1-, γ- , and α_{s1}-I casein fractions.

The first casein fraction to be broken down was the α_{s1}-casein in both cheese batches, although breakdown proceeded at different rates in the two batches. The influence of the milk-clotting activity was most apparent in the case of the α_s-caseins. That finding was in agreement with the results reported by Irigoyen, Izco, Ibáñez and Torre (2000). Their capillary electrophoretic study of the caseins in these same two cheese batches showed differential degradation of the α_s-caseins according to the milk-clotting activity of the rennets used. At the end of the ripening period, the residual α_{s1}-casein values in the batch made using rennet A were significantly ($P < 0.01$) lower than in the batch made using rennet B (18.3 vs. 38.9%). The α_{s1}-casein fraction is the fraction most intensely broken down by rennet proteases during ripening (Farkye & Fox, 1990; Fox & Stepaniak, 1993). The trend recorded for the α_{s2}-casein fraction was similar to the results described above for the α_{s1}-casein fraction, the percentage residual α_{s2}-casein at the end of ripening being significantly lower for the batch made using rennet A (25.3 vs. 39.9%). Both α_s-casein fractions were hydrolyzed in the early stages of ripening, resulting in important alterations in the texture of the cheeses, as reported previously by Creamer and Olson (1982).

Leu_{190}-Tyr_{191} and Ala_{187}-Phe_{188} are the regions on the β-casein that are most susceptible to the action of chymosin (Whyte, 1995). Breakdown of the β-caseins was less pronounced than that of the α_s-caseins in both the experimental cheese batches. Several other workers have previously reported greater resistance to enzymatic hydrolysis by β-caseins (Choisy, Desmazeaud, Gripon, Lamberet, Lenoir & Torneur, 1990; Fontecha et al., 1990; Fox & Law, 1991). Comparing two rennets that contained differing proportions of chymosin and pepsin in Burgos and Hispànico cheeses, Medina, Gaya, Guillén and Núñez (1992) observed that the rennet with the higher chymosin content resulted in higher breakdown of the β-caseins. In this study, the degree of β_2-casein degradation was the only significant difference between the two rennets tested (Table 6).

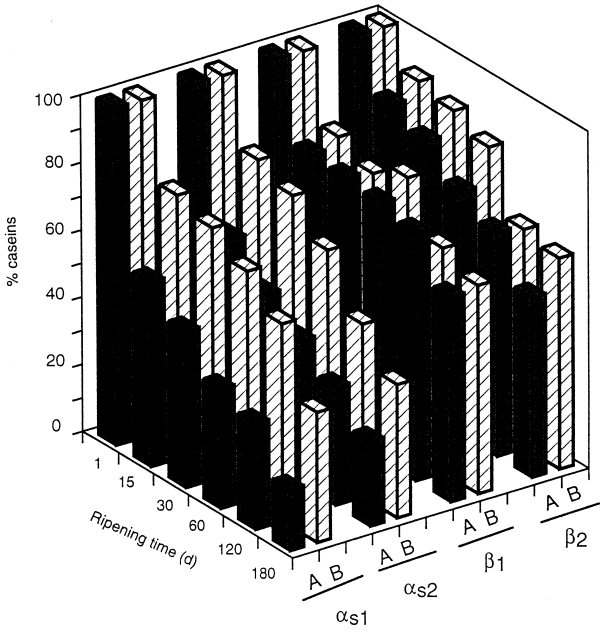

Fig. 1. Fractional breakdown of caseins caused by enzymatic hydrolysis over the ripening period in both cheese batches. Batch A: cheeses made using rennet with a higher level of milk-clotting activity; Batch B: cheeses made using rennet with a lower level of milk-clotting activity.

The α_s-/β-casein ratio decreased by 0.3 over the course of ripening in both trial cheese batches. The ratio was slightly lower in the cheeses made with the rennet with the higher milk-clotting activity due to greater hydrolysis of the α_s-caseins. An α_s-/β-casein ratio of 0.5 has been reported for ovine cheese (Fernández-Salguero & Sanjuán, 1999), similar to the values for batch B and slightly higher than the value for batch A (around 0.4).

The primary breakdown product of α_{s1}-casein degradation, α_{s1}-I-casein, was present in higher quantities in the cheeses made using the lamb rennet with the lower level of milk-clotting activity (batch B), though the differences between the two batches were not significant (Table 6). Cleavage of C-terminal residues from the β-casein gives rise to γ-casein. These breakdown products increased over the ripening period, with no differences observed between the batches made using the two rennets (Table 6). Statistical analysis of the results of the electrophoretic study indicated a highly significant ($P < 0.0001$) correlation between the γ-casein and β-casein fractions in both cheese batches. That same finding has also been reported by other researchers (Fernández-Salguero, Barreto & Marsilla, 1981; Marcos, Esteban, León & Fernández-Salguero, 1979) in different varieties of Spanish and European cheese.

Table 6
P-values from the analysis of variance performed on the casein fractions[a]

Factor	β_2-CN	β_1-CN	α_{s2}-CN	α_{s1}-CN	γ-CN	α_{s1}-I-CN
Ripening time (t)	< 0.0001	< 0.0001	0.0011	< 0.0001	< 0.0001	< 0.0001
Rennet type (T)	0.0102	0.8921	0.0008	< 0.0001	0.2346	0.3842
Interaction($t \times$T)	0.4408	0.2287	0.9468	0.9287	0.0954	0.0030

[a] β_2-CN, β_2-casein; β_1-CN, β_1-casein; α_{s2}-CN, α_{s2}-casein; α_{s1}-CN, α_{s1}-casein; γ-CN, γ-casein; and α_{s1}-I-CN, α_{s1}-I-casein.

3.5. Sensory analysis

Fig. 2 shows the sensory attributes considered for each cheese batch. The results of the sensory analysis plainly showed the appreciable influence of ripening time, with the cheeses becoming less moist and less elastic with higher adherence and characteristic texture scores after 180 days. Nevertheless, the results of the analysis of variance of the total scores revealed that the assessors did not perceive differences between the two cheese batches.

The different milk-clotting activities of the two lamb rennets tested did not affect the overall physicochemical parameters (pH, DM, fat, and protein). The values for the nitrogen fractions were higher in the cheeses made using the rennet with the higher milk-clotting activity, and

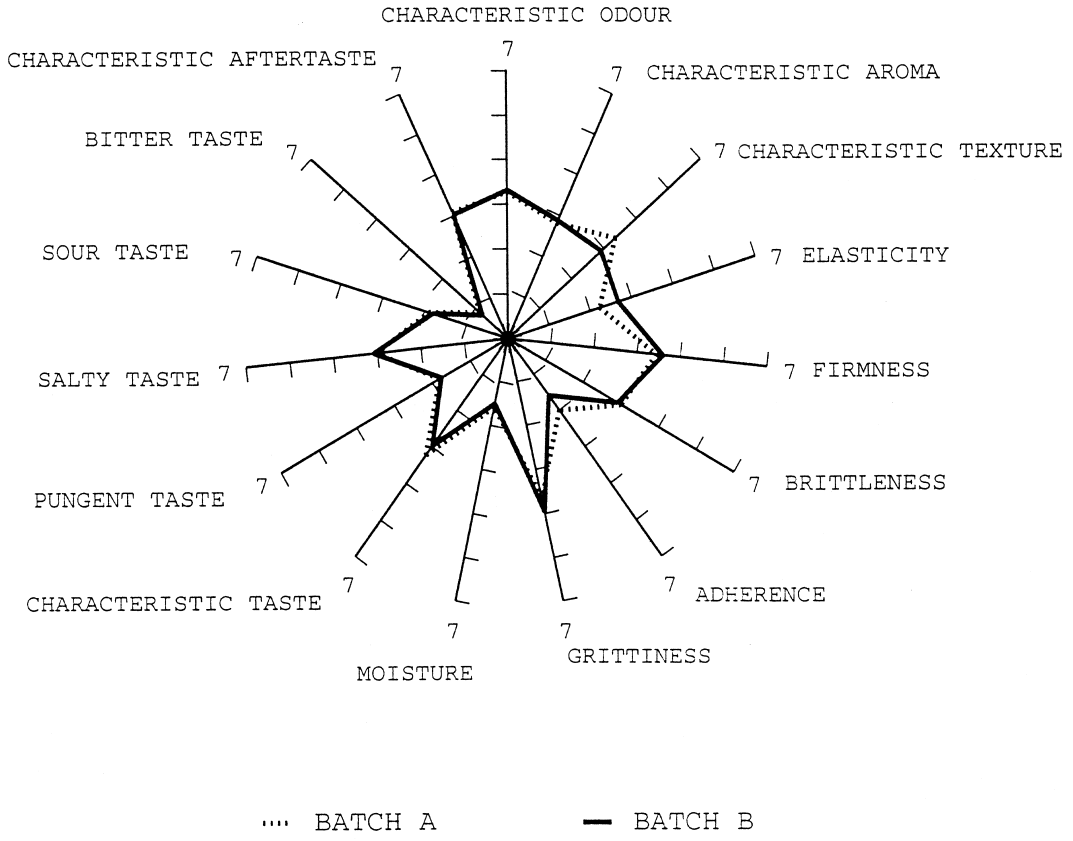

Fig. 2. Sensory profile constructed using the scores, for the sensory attributes of Roncal cheeses made using a rennet with a higher level of milk-clotting activity and a rennet with a lower level of milk-clotting activity after 120 days of ripening, awarded by a taste panel.

proteolytic activity on the β and α_s-caseins, especially on the α_{s1}-caseins, was likewise higher. Thus, both the formation and the subsequent degradation of the α_{s1}-I-casein took place more rapidly in the cheeses made using the rennet with the higher level of milk-clotting activity. The γ-casein values increased with ripening time, but no differences were observed according to the type of rennet employed. Nevertheless, these changes did not result in any appreciable sensory differences in the batches after 120 and 180 days of ripening. According to the results obtained in this work, we could speculate that the use of lamb rennet containing around 50% chymosin does not affect significantly the physicochemical and sensory characteristics of Roncal cheese. Therefore, this would allow the manufacture of Roncal cheese with rennet from older lambs.

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