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Effect of ripening time and type of rennet (farmhouse rennet from kid or commercial calf) on proteolysis during the ripening of León cow milk cheese

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Abstract

Classical nitrogen fractions, caseins and their degradation products, and free amino acids were determined during ripening of six batches of León raw cow milk cheese. Three batches were made using commercial calf rennet (average 1/8342 strength and 76% of chymosin) and three were made using farmhouse rennet obtained from kid abomasum (average 1/857 strength and 34% of chymosin). The pH 4.6-soluble nitrogen and the 12% TCA-soluble nitrogen contents increased moderately during ripening, reaching significantly ($P<0.05$) higher average final values in the cheeses made using commercial rennet (18.0 and 11.0% of TN, respectively) than in the cheeses made using farmhouse rennet (8.3 and 5.1% of TN, respectively). The greater extent and intensity of proteolysis registered in the cheeses made using commercial rennet, especially after 15 days of ripening, became clear again on quantifying the caseins and their degradation products. β-Casein did not undergo any appreciable degradation during ripening. In the cheeses made using commercial rennet, 20% of the αs-caseins were degraded during ripening, whilst in those made using farmhouse rennet only 10% of the αs-caseins were degraded. These results show that this cheese undergoes a slight proteolysis and that the rennet plays a preponderant role in protein degradation. The content of total free amino acids increases progressively during ripening. In the batches made using commercial rennet, the final values were double those found in the batches made using farmhouse rennet. In the mature cheeses, the major free amino acid was lysine, followed by leucine, glutamic acid, tryptophan, valine, and phenylalanine. The type of rennet used during manufacture made no difference to the free amino acid profile at maturity.

Keywords: León cheese; Cow milk cheese; Proteolysis; Commercial rennet; Farmhouse rennet

1. Introduction

The changes in the proteins are, without doubt, the most important of the phenomena that occur during ripening of cheese. The proteins retained in the curd (fundamentally caseins) are initially degraded to large peptides, which in turn are degraded to small peptides and, finally, to free amino acids. Lastly, the amino acids can be catabolised by deamination or decarboxylation. The deamination causes the formation of ammonium ions and carboxylic acids, which in turn can be catabolised following different metabolic routes. The decarboxylation generates CO_2 and amines that, in turn, can be degraded by some of the microorganisms that are present.

Proteolysis contributes directly to the texture and flavour of the ripened cheese. It conditions the cheese texture through hydrolysis of the proteins, increase of pH and an increase in the retention of water by the amino and carboxyl groups that are formed during degradation of the protein. The contribution to the flavour is caused by the increase in peptides, free amino acids, amines, acids, thiols and thioesters formed during the previously mentioned degradation processes.

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According to Fox (1989), five different agents are involved in protein degradation in cheese: rennet or rennet substitutes, indigenous proteolytic milk enzymes, starter bacteria and their enzymes, enzymes from secondary starters, and non-starter bacteria. Other authors (Fox, Law, McSweeney, & Wallace, 1993; Grappin, Rank, & Olson, 1985) coincide in marking these same agents.

The rennet or replacement enzymes of vegetal or microbial origin initially act on the k-casein causing coagulation of the milk. After the whey is removed, a small part of this enzyme, generally not more than 10% (Creamer, Lawrence, & Gilles, 1985; Stadhouders, Hup, & Van der Waals, 1977), is retained in the curd and plays a very important role through a well-known mechanism (Fox, 1989; Grappin et al., 1985; Guinee & Wilkinson, 1992; Van den Berg & Exterkate, 1993)

León cheese is a traditional, ripened variety, made in the northwest of Spain (in the province of León) from raw cow milk by a predominantly lactic curdling. Small (<1 kg), it has an irregular cylindrical shape and a slightly moulded and rough rind. Studies on this cheese are limited. In its manufacture, commercial calf rennet or farmhouse rennet, obtained by macerating abomasa from young goats in salted whey, are used without discrimination.

The aim of this study, which forms part of a wider investigation of the biochemical and microbiological characterisation of León cow milk cheese, is to study the proteolysis that takes place during the ripening of this cheese, as well as the effect on the changes produced in nitrogen substances during the ripening process, of using one or the other type of coagulant.

2. Materials and methods

2.1. Rennets and rennet analyses

Commercial calf rennets were supplied from two different laboratories: Productos Nievi (Bilbao, Spain) and Tormol (Santander, Spain). Farmhouse rennet was prepared in the farmhouses that made the cheese by macerating previously minced dehydrated abomasa from kids in salted whey from previous cheese manufactures (3% of NaCl), at a proportion of 100 g of abomasum / l of whey. After 3 days of maceration, the mixture was filtered to eliminate the remains of abomasum. This extract was maintained at room temperature (20 °C) for no longer than 7 days before use.

The strength of the rennets, both commercial and farmhouse, was assessed following the Berridge (1952) method, modified according to Annexe A of the FIL-IDF 110A: 1987 standard (IDF, 1987). The enzymatic composition of the rennets was assessed following the method described by Mulvihill and Fox (1977a), based on selective denaturising of the chymosin using 5 M urea buffers.

2.2. Cheese making and sampling

Six batches of León cheese, three made using farmhouse rennet, and three made using commercial calf rennet and each one composed of five cheeses, were manufactured by six different cheese makers, following traditional methods. Whole, raw cow milk at 35 °C was coagulated by adding 50 ml of farmhouse rennet or 5 ml of commercial rennet per 100 L of milk. No starter cultures were added. Coagulation was carried out at 20 °C (room temperature). Five to seven hours after adding the rennet, the curd was cut into four equal parts and left for approximately 3 h. It was then placed in plastic moulds, 15–20 cm in diameter and 30–40 cm in height, with holes to allow the whey to drain. Twenty-four hours after the curd had been placed in the moulds without being submitted to any pressure, dry salt was added to the surface and was left to penetrate for 12 h. The moulds were then turned over on plates and left for a further 12 h. After this time, the moulds were removed and the cheeses, now consistent and with a defined cylindrical shape, were salted on the other side. Ripening was carried out in well-aerated rooms at a temperature of 5–10 °C and a relative humidity of 70–80%, and the cheeses were turned over periodically.

From each batch, samples of curd (0 days, before placing in the moulds) and of 7, 15, 30, 60 and 90-day-old cheese, were taken. Each cheese sample consisted of one whole cheese. Samples were transported to the laboratory under refrigeration (below 4 °C). At the laboratory, the rind was discarded and the cheeses were grated and kept in airtight containers at −40 °C until analysed.

2.3. Nitrogen fraction analyses

The total nitrogen content (TN) was determined by the Kjeldahl method as described by the FIL-IDF 20B: 1993 standard (IDF, 1993). The Vakaleris and Price (1959) procedure was followed to extract the pH 4.6-soluble nitrogen (pH 4.6-SN) and 12% trichloroacetic acid-soluble nitrogen (12% TCA-SN), and the method of Johnson (1941) was used for their determination. In the case of 12% TCA-SN, prior precipitation of proteins with trichloroacetic acid at 12% was necessary. The method described by Ordóñez (1974) was used to determine ammonia nitrogen (NH_3–N) and amino nitrogen (NH_2–N). All analyses were carried out in quadruplicate. The TN minus the 12% TCA–SN gave the protein nitrogen. The TN minus the pH 4.6-SN gave the casein nitrogen. The pH 4.6-SN minus the 12% TCA–SN gave the proteose–peptone fraction (P–P–N) that is formed by large peptides. Finally, the 12% TCA–SN minus the NH_2–N and minus the NH_3–N gave the

oligopeptide nitrogen (Pept-N) fraction, formed by small sized peptides.

2.4. Electrophoretic analyses

Casein degradation was studied by urea-PAGE techniques according to the procedure of Andrews (1983) using the following polyacrylamide gels: stacking: T = 4.2%, C = 5%, pH = 7.6; resolving: T = 12.5%, C = 4%, pH = 8.9. Samples were prepared according to Farkye, Kiely, Allshouse, and Kindstedt (1991) and gels were stained using the method described by Blakesley and Boezi (1977). For the identification and quantification of the caseins and their degradation products, the software package Diversity One™ 1.0 (pdi, New York, USA), was used after scanning the electrophoresis gels. All electrophoretic analyses were performed in duplicate. The optical density of each region was expressed as a percentage of the total optical density.

2.5. Free amino acids analyses

The extraction of free amino acids was performed as described by Fresno, Tornadijo, Carballo, Bernardo, and González Prieto (1997). Their identification and quantification was carried out by HPLC techniques, using the conditions described by Alonso, Álvarez, and Zapico (1994), with some minor modifications: temperature column, 50 °C; elution time, 47.5 min and variable flow throughout the elution between 0.75 and 1.0 ml min^{-1}. The liquid chromatography equipment consisted of a Spectra Physics Sp 8800 ternary pump (Spectra Physics, San Jose, CA, USA), a 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA), a UV/visible Kontron Uvikon 730 SLC detector (Kontron Uvikon, Middlesex, UK), and a Spectra Physics Sp 4290 integrator (Spectra Physics, San Jose, CA, USA). The column used was a reversed phase Spheri 5-ODS C18, 4.6×250 mm, from Brownlee Labs Columns (Applied Biosystems, Santa Clara, CA, USA). The temperature of the column was controlled to 50 ± 1 °C using a Spectra Physics 8792 column heater (Spectra Physics, San Jose, CA, USA).

2.6. Statistical analyses

Means with a significant difference ($P < 0.05$) were compared by the least squares difference (LSD) test using the programme Statistica© for Windows release 5.1 (StatSoft Inc., 1996, Tulsa, OK, USA).

3. Results and discussion

Table 1 shows the values relating to the coagulant activity and its nature in the rennets used in the manufacture of the batches of León raw cow milk cheese. The commercial calf rennets showed a coagulant activity and a percentage of activity due to the chymosin that were higher than those shown by the farmhouse rennets.

Table 1
Coagulant activities and approximate enzymatic composition of the rennets used in the manufacture of the six batches of cheese studied[a]

	U.R./ml	U.S./ml	Chymosin activity[b]	Residual activity[b]
Farmhouse rennets	3.9 ± 2.0	857.9 ± 430.9	34.5 ± 12.4	65.4 ± 12.4
Commercial rennets	38.1 ± 8.4	8342.7 ± 1848.6	76.9 ± 29.6	23.1 ± 29.6

U.R. = Units of rennet; U.S. = Units of strength.
[a] Means ± standard deviations of three farmhouse rennets and of three commercial rennets.
[b] % of total milk clotting activity.

Tables 2 and 3 show the evolution of the nitrogen fractions in the batches of cheese made using farmhouse and commercial rennet, respectively. The pH 4.6–soluble nitrogen content (about 6-8% of total nitrogen in 0-day cheese) increased slightly during ripening of the cheeses made from farmhouse rennet. The increase was more pronounced in the batches made using commercial rennet. In the three final sampling points, the pH 4.6-soluble nitrogen content in the batches made using commercial rennet were significantly higher ($P < 0.05$) than in the batches made using farmhouse rennets. As the content of chymosin in the commercial rennets are much higher than in the farmhouse rennets used (see Table 1), it would appear that the protagonism of the chymosin in the processes of proteolysis in León cow milk cheese is undisputable.

The amount of chymosin retained in the curd is strongly influenced by the pH at the time the whey is eliminated, increasing as the pH decreases (Creamer et al., 1985; Holmes, Duersch, & Ernstrom, 1977). The low pH values established in the León cow milk cheese curd because of a predominantly acid coagulation (Prieto, 1999; Prieto, Franco, González Prieto, Bernardo, & Carballo, 2002) are, without doubt, favourable to the retention of chymosin. In addition, during the removal of whey, this cheese is not subjected to high temperatures that might cause the enzyme that is retained to become unstable.

The average final values of pH 4.6-soluble nitrogen observed in the batches made using farmhouse rennet (8.3 ± 1.8% of TN) are much lower than those found in the bibliography for the different mature cow milk cheeses (Fritsch, Martens, & Belitz, 1992; Giangiacomo, Iameti, & Bonomi, 1993; Lau, Barbano, & Rasmussen, 1991; Lenoir, 1963; Marcos, Alcalá, León, Fernández-Salguero, & Esteban, 1981; Marcos, Fernández-Salguero, Esteban, León, Alcalá, & Beltrán de Heredia, 1985; Marcos, Millán, Esteban, Alcalá, & Fernández-Salguero, 1983; Paleari, Soncini, Beretta, Dragoni, & Piantoni, 1993; Poznanski & Rymazewski, 1965;

Ramos, Barneto, Suárez, & Íñigo, 1982; Sapru, Barbano, Yun, Klei, Oltenacu, & Bandler, 1997; Thakur, Kirk, & Hedrick, 1975). The batches of cheese made using commercial rennet showed average pH 4.6-SN levels after 90 days of ripening that are notably higher (18.0±10.1% of TN) and which, although lower, are closer to the normal values of cheeses of this type.

The residual action of the rennet on the caseins is translated into the liberation of large peptides that form the proteose–peptone fraction. The values of this nitrogen fraction reflect, once more, the intervention of the chymosin in the proteolysis of this cheese. In the batches made using farmhouse rennet (Table 2), an increase of the nitrogen of the large peptides is hardly noticed, whilst this increase is noticeable during ripening of the cheeses made using commercial rennet (Table 3). The values of the proteose–peptone fraction reflect the result of the balance between the production of large peptides, due fundamentally, to the action of the chymosin, and their degradation produced by the enzymes of microbial origin that are responsible for the formation of the fractions that make up the 12% TCA-soluble nitrogen (small peptides, free amino acids, and ammonia nitrogen). The average levels observed for this nitrogen fraction in León cow milk cheese at the end of the ripening (3.2% of TN in the batches made using farmhouse rennet and 7% of TN in the batches made using commercial rennet) are also below those of most of the matured cow milk cheeses studied up until now. Only Bola (Marcos et al., 1981, 1985) and Gouda cheeses (Marcos et al., 1981) show such low values.

The 12% TCA-soluble nitrogen, which in 0-day cheese was 3.4% of total nitrogen, also showed an increase over the ripening period. This was more

Table 2
Evolution of the nitrogen fractions during the ripening of León cow's milk cheese made using farmhouse kid rennet (average values±standard deviation of three batches)

	Ripening time (days)					
	0	7	15	30	60	90
TN[a]	2.0±0.1a	2.4±0.04b	3.5±0.5c	4.1±0.5d	4.6±0.2e	4.8±0.2e
Protein N[b]	96.6±0.3a	96.4±0.6a	95.5±1.0a	95.5±0.8a	95.1±1.4ab	94.9±0.9b
12%TCA-SN[b]	3.4±0.3a	3.6±0.6a	4.5±1.0a	4.5±0.8a	4.9±1.4ab	5.1±0.9b
Casein-N[b]	93.9±1.2a	92.3±0.3ab	92.6±3.2ab	93.3±1.0a	91.4±3.3b	91.7±1.8b
pH 4.6-SN[b]	6.1±1.2a	7.7±0.3ab	7.4±3.2ab	6.6±1.0a	8.6±3.3b	8.3±1.8b
P–P–N[b]	2.8±1.5a	4.1±0.6b	2.8±2.4ab	2.2±1.1a	3.7±1.9ab	3.2±1.3ab
Pept–N[b]	2.4±0.4a	3.2±0.2a	3.7±0.6b	3.2±0.5ab	2.7±0.5a	3.2±0.4ab
NH_2–N[b]	0.7±0.1a	0.4±0.3a	0.8±0.4a	1.1±0.4b	1.8±0.9c	1.5±0.6bc
NH_3–N[b]	0.2±0.2ab	–[c]	–[c]	0.1±0.2a	0.4±0.2b	0.3±0.1b

Values within the same row, corresponding to the same nitrogen fraction, not followed by the same letter (a–e) differ significantly ($P<0.05$).
[a] Expressed as g/100 g of cheese.
[b] Expressed as g/100 g of TN.
[c] (–) = Not detected.

Table 3
Evolution of the nitrogen fractions during the ripening of León cow's milk cheese made using commercial calf rennet (average values±standard deviation of three batches)

	Ripening time (days)					
	0	7	15	30	60	90
TN[a]	2.0±0.2a	2.7±0.4b	3.5±0.7c	4.2±0.2d	4.7±0.4e	5.0±0.3e
Protein N[b]	96.6±0.9a	95.6±1.0a	94.2±1.7ab	91.9±2.3bc*	91.6±3.1bc*	89.0±5.0c*
12%TCA-SN[b]	3.4±0.9a	4.4±1.0a	5.8±1.7ab	8.1±2.3bc*	8.4±3.1bc*	11.0±5.0c*
Casein-N[b]	92.0±1.5a	89.5±0.7ab	89.0±3.6ab	84.6±3.7bc*	84.7±5.2bc*	82.0±10.1c*
pH 4.6-SN[b]	8.0±1.5a	10.5±0.7ab	11.0±3.6ab	15.4±3.7bc*	15.3±5.3bc*	18.0±10.1c*
P-P-N[b]	4.5±0.6a	6.1±1.7a	5.2±2.0a	7.3±2.1a*	6.9±2.3a*	7.0±6.6a
Pept-N[b]	2.6±0.8a	3.6±0.4ab	4.7±1.4b	4.4±0.4bc	3.5±1.0ab	3.3±0.7ac
NH_2-N[b]	0.7±0.1a	0.7±0.5a	1.1±0.3ab	2.8±1.4bc	3.6±2.1cd	5.0±3.4d*
NH_3-N[b]	0.2±0.2a	0.1±0.2a	–[c]	0.9±0.8ab	1.3±1.2b	2.6±2.2c*

Values within the same row, corresponding to the same nitrogen fraction, not followed by the same letter (a–e) differ significantly ($P<0.05$).
*Sampling point with significant differences ($P<0.05$) associated to the type of rennet used.
[a] Expressed as g/100 g of cheese.
[b] Expressed as g/100 g of TN.
[c] (–) = Not detected.

marked in the cheeses made using commercial rennet, reaching average levels of 5.1% of TN at maturity in those made using farmhouse rennet and 11.0% of TN at maturity in those made using commercial rennet. The values of 12% TCA-SN observed in León cow milk cheese at maturity are at the lowest end of the range of those found in the rest of the ripened cow milk cheeses studied until now. Only in Edam (Poznanski & Rymazewski, 1965), Saint Paulin (Lenoir, 1963), Tetilla (Marcos et al., 1983), Tomme (Sieber, Badertscher, Fuchs, & Nick, 1994) and Vacherin Mont-D'or (Sieber et al., 1994) cheeses, were values of this magnitude observed. If we express the values of 12% TCA-SN as a percentage of pH 4.6-SN, an increase is noted from 43 to 54% in 0-day cheese to 61% in 90-day cheese. This 12% TCA-SN/pH 4.6-SN relation is the most frequent one found in cow milk cheeses.

In the last three sampling points, the contents of 12% TCA-SN in the batches made using commercial rennet were significantly higher ($P < 0.05$) than those observed in the batches made using farmhouse rennet. The formation of 12% TCA-SN (made up of small peptides, free amino acids, and ammonia nitrogen) is due, fundamentally, to the enzymes of microbial origin that act on the large peptides freed by the chymosin as it acts on the α-caseins. The higher quantity of large peptides that are freed in the cheeses made using commercial rennet and, therefore, the greater availability of substrate, could ease the actuation of the agents that convert these peptides into nitrogen substances of lower molecular size.

With reference to the evolution of the nitrogen fractions that form the 12% TCA-SN, the oligopeptide nitrogen (small size peptides) increased during the first days of ripening (from 2.4 to 2.6% TN in 0-day cheese to 3.7–4.7% of TN in 15-day cheese) and afterwards slowly decreased until reaching final values of around 3.5% TN. However, the evolution of this nitrogen fraction is more illustrative when expressed as a percentage of 12% TCA-SN. In this case, a small increase is observed during the first week of ripening (from 72 to 88% of 12% TCA-SN in cheeses made using farmhouse rennet and from 75 to 82% of 12% TCA-SN in cheeses made using commercial rennet). This decreased afterwards during the ripening process to average final values of 62.6% of 12% TCA-SN in the batches made using farmhouse rennet and much lower values (30.3% of 12% TCA-SN) in the batches made using commercial rennet. The decrease in the oligopeptide nitrogen was accompanied by an increase in the amino nitrogen (free amino acids). This was much more marked in the batches made using commercial rennet, passing on average from 19.2% of 12% TCA-SN in 0-day cheese to 45.5% of 12% TCA-SN in 90-day cheese in these batches.

The ammonia nitrogen, which reflects the capacity of deamination exercised on the free amino acids by the microbial flora, showed very low values during the first month of ripening and in many cases could not be quantified by the method of analysis used. However, the values of this nitrogen fraction increased in 60 and 90-day cheese, reaching average final values of 0.3% TN in the cheeses made using farmhouse rennet and 2.6% TN in those made using commercial rennet.

The final values of oligopeptide nitrogen found in León cow milk cheese (around 3.5% TN) are below those that have been determined by diverse authors in different varieties of ripened cow milk cheese (Lenoir, 1963; Marcos et al., 1981, 1983, 1985; Poznanski & Rymazewski, 1965). However, the final values of amino nitrogen and ammonia nitrogen are within the range of those found in these varieties of cheese. The quotient $N–NH_2/N–NH_3$ was always very much higher than one (between 1.9 and 4.0) as corresponds to the cheeses in which the microbial flora implicated in the ripening process are the lactic acid bacteria.

The greater extent and intensity of proteolysis in the cheeses made from commercial rennet, above all after 15 days of ripening, with respect to those made using farmhouse rennet, again became clear on quantifying the caseins and their degradation products by electrophoresis techniques (see Tables 4 and 5).

Examining the electrophoretograms from the beginning, various weak bands are found initially, corresponding to the peptides denominated as γ (γ_1, γ_2 and γ_3) which show an electrophoretic mobility similar to the γ-caseins of the milk. Although the intervention of enzymes of microbial origin in their formation cannot be ruled out, their fundamental origin is due to the action of the alkaline protease (plasmin) of the milk on the β-casein (Creamer, 1975). This casein is quite resistant to the degradation activity of the chymosin. Continuing, the area of β-casein with two clearly marked bands can be seen. In most of the cheeses, the band that has the most electrophoretic mobility is the most intensely stained. Following this band is the area of the αs-caseins, where first we find the αs_2-casein formed by two bands and then the αs_1 casein formed by one very thick and intense band. Immediately below this, is the band corresponding to the peptide denominated αs_1-I (Fox & Guiney, 1973). This peptide is the first degradation product of the αs_1-casein of the cow milk, produced by the action of the chymosin on hydrolysing the link $Phe_{23}–Phe_{24}$ (Resmini, Saracchi, Pazzaglia, & De Bernardi, 1976) or the $Phe_{24}–Phe_{25}$ (Creamer & Richardson, 1974). Because of this hydrolysis, two peptides are liberated: a small peptide N-terminal which contains the amino acids of the αs_1-casein from 1 to 23 (Hill, Lahav, & Givol, 1974), and a large peptide C-terminal, denominated αs_1-I, of greater molecular weight, which contains most of the sequence of amino acids of the αs_1-casein (amino acids from 24 to 199). The peptide αs_1-I is very resistant to further degradation and accumulates in large quantities in all the cheeses made from cow milk

Table 4
Evolution of the casein fractions during the ripening of León cow's milk cheese made using farmhouse kid rennet (average values±standard deviation of three batches)

	Ripening time (days)					
	0	7	15	30	60	90
γ-Caseins[a]	3.2±0.7a	3.3±0.9a	2.9±0.9a	3.2±0.8a	3.3±0.7a	3.6±0.5a
β-Caseins[a]	30.1±2.2a	30.8±0.8a	30.7±0.5a	30.3±0.7a	29.5±1.1a	30.8±1.2a
α_s-Caseins[a]	55.3±4.4a	51.9±4.4ab	49.9±3.6b	50.1±5.1b	51.3±5.3ab	50.3±2.9b
α_{s1}-I-Casein[a]	2.5±1.5a	4.6±1.5b	5.8±0.7b	5.4±2.0b	4.3±1.3b	4.1±1.3b
preα_s-Caseins[a]	6.6±1.6a	8.0±0.9ab	8.5±2.1b	8.9±2.6bc	9.8±3.1c	9.0±1.9bc
α_s/β	1.8±0.3a	1.7±0.2ab	1.6±0.1b	1.6±0.1b	1.7±0.2ab	1.6±0.1b

Values within the same row, corresponding to the same casein fraction, not followed by the same letter (a–c) differ significantly ($P<0.05$).
[a] Expressed as percentage of total optical density.

Table 5
Evolution of the casein fractions during the ripening of León cow's milk cheese made using commercial calf rennet (average values±standard deviation of three batches)

	Ripening time (days)					
	0	7	15	30	60	90
γ-Caseins[a]	4.9±0.8ab*	4.1±0.9b	4.3±1.2ab*	4.5±1.3ab*	4.9±0.9ab*	5.4±0.8a*
β-Caseins[a]	27.2±2.2a	28.4±1.1a	27.1±0.7 a *	25.8±1.9a*	26.0±2.8a*	26.7±4.4a*
α_s-Caseins[a]	47.7±4.3a	45.2±5.0ab	41.9±5.5ab	39.6±5.2ab*	37.6±8.6b*	37.6±8.8b*
α_{s1}-I-Casein[a]	7.2±5.5a	9.2±4.3a	9.7±3.5a	8.9±3.1a	7.4±2.4a	6.2±1.4a
preα_s-Caseins[a]	9.2±1.4a	9.3±1.2a	13.0±2.4ab	17.4±3.5bc*	19.6±7.2c*	19.9±9.2c*
α_s/β	1.8±0.02a	1.6±0.1ab	1.5±0.2ab	1.5±0.1ab	1.4±0.2b*	1.4±0.1b*

Values within the same row, corresponding to the same casein fraction, not followed by the same letter (a–c) differ significantly ($P<0.05$). *Sampling point with significant differences ($P<0.05$) associated to the type of rennet used.
[a] Expressed as percentage of total optical density.

and chymosin (Marcos, Alcalá, Fernández-Salguero, & Esteban, 1979a; Marcos, Esteban, León, & Fernández-Salguero, 1979b; Resmini et al., 1976). Finally, the bands with the most electrophoretic mobility, denominated, as a whole, as Preαs-caseins, appear to be products generated, fundamentally, from the peptide αs_1-I by the action of the endopeptidases of the lactic acid bacteria.

The β-caseins did not suffer appreciable degradation during ripening. However, the αs-caseins did, although to a moderate degree. Comparing percentages of absorbance due to the αs-caseins in cheeses ripened for 90 days, with those of 0-days (curds), we could see a decrease of around 10% in the cheeses made using farmhouse rennet and of 20% in the cheeses made using commercial rennet. These results again show that the rennet plays a preponderant part in the protein degradation of this cheese, as the degrading action of chymosin is mainly exerted on α_s-caseins (McSweeney, Olson, Fox, Healy, & Hojrup, 1993; Mulvihill & Fox, 1977b) and to a much lesser extent on β-casein, which is much more resistant to its action.

Together with the decrease of the αs-caseins, an increase in the absorbance percentage due to the bands of their degradation products (peptide αs_1-I and Preαs-caseins) was observed. The absorbance percentage due to the αs_1-I band increased in both types of cheese until 15 days of ripening, decreasing afterwards until the end of the process as a consequence of the subsequent degradation of this peptide to small peptides. The percentage of absorbance due to the αs_1-I peptide was, in all points of the sampling, higher in the cheeses made using commercial rennet. This circumstance is normal, taking into account the greater richness in chymosin of the commercial rennets and given that, as previously mentioned, this peptide is generated by the action of the chymosin on the αs_1-casein.

The quantification of the caseins and their degradation products confirm the results obtained when determining the classic nitrogen fractions and allows the conclusion that León cow milk cheese undergoes, throughout ripening, an unimportant proteolysis, as much in extent as in depth.

As mentioned earlier, the rennet appears to be the main agent responsible for the proteolysis in this cheese, but the amounts used in its manufacture are very small. Moreover, the low pH values and the high values of the

salt/moisture quotient observed during ripening (especially after the 15th day of ripening) of León cow milk cheese (Prieto, 1999; Prieto et al., 2002) are not the most favourable for the action of the chymosin and other proteolytic enzymes of different origins. Noomen (1978) proved that the most favourable conditions for the action of the rennet on the αs_1 casein in cheese are found at values of pH of around 5 and values of the quotient salt/humidity of 4%. Similar observations of the optimum pH were made by Mulvihill and Fox (1977b) while studying the degradation *in vitro* of αs_1 casein.

Tables 6 and 7 show the evolution of the free amino acid contents during ripening of the batches of cheese made using farmhouse kid rennet and using commercial rennet, respectively.

The content of total free amino acids increased progressively during ripening, reaching final values in the batches made using commercial rennet of twice those found in batches made using farmhouse rennet. As previously mentioned, the activity of the rennet during ripening is centred mainly on the liberation of medium and large peptides from the caseins (fundamentally the αs_1) and the liberation of the amino acids is due to the endo and exopeptidases of microbial origin. The higher content of free amino acids in the cheeses made using commercial rennet do not appear, a priori, to be very logical. Possibly, in these cheeses, the increased liberation of peptides, because of the higher level of acting chymosin, stimulates the activity of the enzymes of microbial origin that transforms them into free amino acids.

The final values of total free amino acids in León cow milk cheese are lower than those found in other mature cow milk cheeses, such as Taleggio (Resmini, Saracchi, Volonterio, & Bozzolati, 1969) and Cheddar (Laleye, Simard, Gosselin, Lee, & Giroux, 1987; Puchades, Lemieux, & Simard, 1989). However, they are similar to those described in Mahón cheese (Polo, Ramos, & Sánchez, 1984) and only higher than those found in Ulloa cheese (Ordóñez & Burgos, 1977).

Proline was the major free amino acid in the curd (0-day cheese), a fact observed by other authors in other varieties of cheese, such as Taleggio (Resmini et al., 1969), Télémé (Polychroniadou & Vlachos, 1979), Armada (Fresno et al., 1997) and Babia-Laciana (Franco, Prieto, Bernardo, González Prieto, & Carballo, 2003).

The different free amino acids showed an irregular increase during ripening. The highest level found in 90-day cheese was of lysine, followed by leucine, glutamic acid, tryptophan, valine and phenylalanine. These six amino acids accounted for 48% of the total free amino acids in the cheeses made using farmhouse rennet and 59% of total free amino acids in cheeses made using commercial rennet.

Table 6
Evolution of free amino acids during the ripening of León cow's milk cheese made using farmhouse kid rennet (average values ± standard deviation of three batches)[a]

	Ripening time (days)					
	0	7	15	30	60	90
Asp	0.2±0.1a	0.7±0.2 a	2.0±0.4b	2.2±1.5b	11.9±9.3b	7.4±5.5b
Glu	1.7±0.2a	5.5±3.3b	13.3±4.7bc	19.6±6.6c	46.1±22.1c	40.9±23.5c
Gln	0.9±0.1a	1.6±0.7a	3.0±0.3ab	4.7±1.5b	5.9±1.1b	3.8±1.4ab
Ser	1.3±0.6a	6.2±2.1b	12.0±1.6bc	15.4±5.2bc	32.1±15.2c	24.9±9.8bc
Asn	0.6±0.3a	6.4±2.3b	19.8±8.9bc	21.8±11.8bc	40.4±29.6c	25.6±19.4bc
Gly	0.7±0.1a	1.7±0.6a	2.9±1.4a	4.9±2.3ab	11.9±6.0b	12.1±6.2b
His	1.5±0.1a	3.6±1.7a	7.6±2.4ab	10.9±3.8bc	24.6±11.2c	23.1±11.9c
Tau	1.4±0.2a	1.5±0.1a	1.5±0.6a	1.8±0.9a	2.5±0.6ab	5.4±2.4b
GABA	3.8±3.1a	12.5±3.9b	18.0±2.9bc	22.2±5.4bc	52.1±19.8c	36.7±10.8c
Thr	1.7±0.3a	3.7±0.6ab	6.3±1.0b	7.9±2.5b	19.6±6.6c	17.2±5.4c
Ala	2.0±0.5a	9.2±3.3b	15.3±6.9bc	14.6±5.3b	29.3±12.0c	27.2±9.9c
Pro	10.4±1.0a	10.5±5.1a	16.0±10.9a	12.2±7.7a	22.2±9.7a	24.7±10.4a
Tyr	2.3±0.3a	3.9±1.2a	7.0±2.7ab	6.9±3.1ab	13.4±7.3b	12.3±7.3b
Val	3.9±0.6a	6.9±1.7ab	10.2±4.2b	10.8±2.8b	27.9±12.4bc	29.9±12.1c
Met	0.6±0.1a	2.6±1.0ab	5.8±2.6b	7.5±2.4b	15.9±7.4b	15.5±7.1b
Cys	1.0±0.6a	1.9±0.3a	2.4±0.3ab	2.9±0.5ab	2.6±1.2ab	3.0±2.0b
Ile	0.6±0.2a	1.9±0.9b	3.6±2.0bc	4.5±1.2c	12.5±4.3d	15.7±6.0d
Leu	4.3±1.5a	13.3±5.6ab	24.6±10.0b	26.4±8.8b	50.6±22.5b	48.5±21.4b
Phe	2.4±0.9a	9.9±4.4ab	17.5±7.0b	17.5±7.3b	30.6±13.0b	26.8±14.7b
Trp	2.6±1.0a	6.9±3.0a	11.3±3.3a	13.5±5.8ab	24.2±11.9b	23.9±10.3b
Lys	3.0±0.3a	9.1±4.4a	19.9±9.6a	23.4±6.6ab	57.6±26.1ab	64.8±28.6b
Σ faa	46.7±7.9a	119.5±43.7a	220.0±78.5b	251.6±87.0b	534.2±242.2c	489.4±218.3c

Values within the same row, corresponding to the same free amino acid, not followed by the same letter (a–d) differ significantly ($P < 0.05$).
[a] Expressed as mg/100 g of total solids.

Table 7
Evolution of free amino acids during the ripening of León cow's milk cheese made using commercial calf rennet (average values ± standard deviation of three batches)[a]

	Ripening time (days)					
	0	7	15	30	60	90
Asp	0.5±0.2a	0.9±0.1a	2.1±0.4a	14.5±10.8b*	21.2±15.3b	17.3±18.5b
Glu	5.5±0.5a	7.0±1.6a	15.3±13.9a	30.0±35.3a	27.6±15.3a*	30.4±7.9a*
Gln	1.5±0.2a	1.9±0.8a	3.6±1.6ab	11.2±9.4c	8.8±7.7bc	6.8±4.0ac
Ser	2.7±1.3a	7.4±2.7ab	12.3±4.7ab	14.6±7.5abc	17.7±7.6bc*	28.0±22.3c
Asn	1.0±0.1a	5.5±6.2ab	9.3±7.5ab	29.6±34.5ab	36.6±43.9b	25.7±29.2ab
Gly	1.0±0.1a	1.7±0.3a	4.0±0.9a	16.1±10.0ab*	25.6±17.5bc	40.4±30.5c*
His	3.4±0.3a	4.3±0.7a	8.7±7.0a	17.5±18.2a	16.2±7.9a	18.0±4.1a
Tau	1.4±0.1a	1.4±0.01a	1.4±0.01a	4.4±3.2b*	3.4±1.3b	3.8±0.3b
GABA	7.8±4.4a	17.1±4.3ab	31.7±6.1b*	53.4±15.2c*	54.2±16.4c	73.0±31.9d*
Thr	3.0±0.9a	4.6±1.2a	8.0±1.4ab	21.5±11.7bc*	29.5±18.4cd	41.3±27.7d*
Ala	3.6±1.0a	10.0±4.0a	16.7±4.7ab	29.6±11.0bc	39.8±23.7cd	54.0±33.7d*
Pro	9.8±1.2a	7.2±1.0 a	7.4±4.1 a	17.9±10.3a	24.0±18.3a	48.6±36.6b*
Tyr	3.1±0.8a	5.0±1.1a	7.9±4.3a	9.8±6.5a	9.8±6.5a	11.4±5.5a
Val	5.8±0.9a	10.8±1.9a	15.2±3.4a	43.9±22.0ab*	67.7±44.9b	120.2±83.1c*
Met	0.9±0.3a	3.2±0.6a	8.2±0.9a	24.0±9.3b*	35.4±19.6bc*	44.3±24.4c*
Cys	1.7±1.0a	2.5±1.1ab	2.1±0.8ab	5.8±2.4bc	7.2±3.0c*	13.1±9.1d*
Ile	1.0±0.3a	1.5±0.2a	4.0±1.0a	18.3±11.8ab*	36.6±25.0b*	65.3±46.6c*
Leu	7.0±2.4a	19.2±2.9a	42.1±4.8ab*	87.3±31.9bc*	109.7±49.1c*	161.1±94.1d*
Phe	5.5±2.1a	17.6±0.4a*	31.2±9.2ab*	55.3±21.1bc*	67.9±32.0cd*	96.3±66.2d*
Trp	7.1±1.1a	11.3±1.8a	20.6±9.4ab	71.1±46.0bc*	99.8±70.4cd*	134.3±112.5d*
Lys	6.5±2.2a	10.5±4.5a	20.8±14.2a	85.7±56.1ab*	140.6±101.4b*	224.7±162.2c*
Σ faa	79.8±11.8a	150.7±15.0a	272.5±57.3ab	661.5±305.4bc*	879.5±496.3cd*	1291.2±828.1d*

Values within the same row, corresponding to the same free amino acid, not followed by the same letter (a–d) differ significantly ($P<0.05$). * Sampling point with significant differences ($P<0.05$) associated to the type of rennet used.

[a] Expressed as mg/100 g of total solids.

The high contents of γ-aminobutyric acid (GABA) stand out. As different authors have indicated (Ismail & Hansen, 1972), the GABA possibly comes from the decarboxylation of the glutamic acid. Our results appear to confirm this possibility. The cheeses made using commercial rennet, that show higher contents of GABA, also show much lower contents of glutamic acid than those found in the cheeses made using farmhouse rennet. The high contents of GABA could also partially explain the low contents of glutamic acid observed in the León cow milk cheese when compared with those of other cow milk cheeses (Ismail & Hansen, 1972; Laleye et al., 1987).

It is well known that each type of cheese has its characteristic profile of free amino acids that result from the balance between the degradation of the peptides to free amino acids and the degradation and inter-conversion of the different free amino acids. These are definitively determined by the nature of the microbial flora that are present and implicated in these phenomena of degradation. The profile of free amino acids in León cow milk cheese at the end of ripening is independent of the type of rennet used in cheese making and basically coincides with those of other cow milk cheeses matured by bacteria (Burniana & Zeidan, 1982; Dilanian, 1980; Ismail & Hansen, 1972; Laleye et al., 1987; Ordóñez & Burgos, 1977; Puchades et al., 1989; Resmini et al., 1969).

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