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Biochemical changes in Picón Bejes-Tresviso cheese, a Spanish blue-veined variety, during ripening

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Abstract

The changes in the gross chemical composition, physico-chemical parameters, nitrogen fractions, caseins and their degradation products, and some fat characteristics were studied during the ripening process of 10 batches of Picón Bejes-Tresviso cheese, a traditional blue-veined variety made in the north of Spain. The values of the different compositional and physico-chemical parameters at the end of ripening did not differ very much from those found in other Spanish and European blue-veined cheeses. The total soluble nitrogen and the non-protein nitrogen increased by factors of 5.4 and 8, respectively, at the end of ripening compared to the values found in cheese curd after salting. The final values of all the nitrogen fractions showed that Picón Bejes-Tresviso cheese undergoes extensive and in depth proteolysis. The intense degradation of the caseins during ripening was confirmed when the caseins and their degradation products were quantified using PAGE techniques. The autooxidation of the fat does not seem very important during the ripening of this cheese. Nevertheless, lipolysis was very intense; the acidity index of the fat values (free fatty acid contents) increased by a factor of about 20 during ripening.

1. Introduction

At the end of the 1980s cheese production in Spain underwent a large increase and this tendency has continued over the last few years, although at low levels, and has currently risen to 284,000 metric tonnes per year, which means an average consumption of 8 kg per person and year (Anon., 1997). This increase in cheese production over the last few years has given rise to an increase in the number of cheese factories, allowing for an increase in the production of the different traditional Spanish cheeses.

The Picón Bejes-Tresviso variety is one of these traditional cheses. It is a blue-veined variety manufactured in the north of Spain, in the Cantabria region and has "Designation of Origin" since 1993. It is cylindrical in shape, between 7 and 15 cm high, and weighs between 1 and 5 kg (2 to 3 kg being the most common), made from cow's milk or from cow's milk mixed with small quantities of ewe's or goat's milk and has a minimum ripening time of 2 months. The mass is white in colour with blue veins due to the growth of moulds of the *Penicillium* genus and its flavour is slightly spicy. It is usually brought to market covered in *Acer pseudoplatanus* leaves.

To date, no information exists on the characteristics, either chemical or microbiological, of this cheese variety which hinders the improvement of its quality and uniformity. The aim of this study is to investigate the biochemical changes which take place during the ripening of Picón Bejes-Tresviso cheese, especially those which affect the protein fractions.

2. Materials and methods

2.1. Cheeses

Ten batches of cheese were produced by ten different industrial cheesemakers following the traditional method. A suspension of *Penicillium roqueforti* spores was added to whole raw cow's milk mixed with small quantities (about 10%) of whole raw ewe's milk which

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was coagulated at 25–28°C by adding about 35 ml of commercial calf rennet (1/10,000 strength) per 100 l of milk. Approximately 1.5 h after the addition of the rennet, the coagulum was cut to the size of a hazelnut and was left standing for about 2 h. Then, the whey was drained and the curd placed in moulds and held for two days at 22°C. The cheeses were salted on both ends and sides by placing an excess of coarse salt on the rind and letting it penetrate. Then, the cheeses were transferred to a drying-room where they remained at 12°C and 80% relative humidity for approximately 15 days. After this time, the cheeses were placed in natural caves at around 6°C and about 95% relative humidities where the rest of the ripening took place.

From each batch, samples of cheese were taken immediately after salting (3-day-old cheese) and after ripening for 3.5 months, when the cheeses were of optimum quality. Each sample consisted of one whole cheese which was taken under refrigeration (2–4°C) to the laboratory where the rind of the cheeses was removed following the Federation Internationale de Laiterie-International Dairy Federation (FIL-IDF) 50B (IDF, 1985) standard method, and the cheeses were ground and held in air-tight containers at −40°C until they were analysed.

2.2. Compositional and physico-chemical parameter analysis

Total solids, protein, salt, ash and lactose were determined according to the FIL-IDF standards 4A (IDF, 1982), 20B (IDF, 1993), 88A (IDF, 1988), 27 (IDF, 1964), and 43 (IDF, 1967), respectively. D- and L-lactic acids were determined by the spectrophotometric method recommended by Boehringer Mannheim (1995). pH and titratable acidity were measured using AOAC methods, 14.022 (AOAC, 1980a) and 16.247 (AOAC, 1980b), respectively. Water activity was measured in a DECAGON CX-1 Water Activity System apparatus (Decagon Devices Inc., Pullman, USA). All the determinations were carried out in duplicate.

2.3. Nitrogen fraction analysis

The total nitrogen content (TN) was determined by the Kjeldahl method, as described by the FIL-IDF 20B (IDF, 1993) standard. The procedure of Vakaleris and Price (1959) was followed for the extraction of the total soluble nitrogen (TSN) and non-protein nitrogen (NPN), and that of Johnson (1941) was used for their determination. In the case of NPN, previous precipitation of proteins with trichloroacetic acid at 12% was necessary. The method described by Ordóñez (1974) was used for the determination of ammonia nitrogen (NH_3–N) and amino nitrogen (NH_2–N). Protein, casein, oligopeptides and proteose–peptone nitrogen were calculated as described by Prieto, Fresno, Carballo, Bernado, and Martín-Sarmento (1994). All the nitrogen fractions were analyzed in quadruplicate.

2.4. Electrophoretic analysis

The casein degradation was studied using PAGE techniques following the procedure of Andrews (1983). For the identification and quantification of the casein fractions, the software package Diversity One 1.0 (pdi, New York, USA) was employed after having duly scanned the electrophoresis gels. All electrophoresis analysis were performed in duplicate. The optical density of each region was expressed as percentage of the total optical density.

2.5. Fat characteristics

Fat content was determined according to the FIL-IDF 5B (IDF, 1986) standard. The TBA number was determined as described by Tarladgis, Watts, Younathan and Dugan (1960). The acidity index of the fat was determined as described by the FIL-IDF 6B (IDF, 1989).

3. Results and discussion

3.1. Compositional and physico-chemical parameters

Tables 1 and 2 show, respectively, the values of the main compositional parameters and of the physico-chemical parameters of the cheeses after salting (3-day-old cheeses) and of the ripened cheeses (3.5-month-old cheese) in the 10 cheese batches studied.

The average content of total solids increased during the ripening, being 57.25 ± 2.38 g/100 g at the end. These values are slightly lower than those reported by other authors for other varieties of Spanish blue-veined cheese such as Cabrales (Alonso, Juárez, Ramos & Martin-Alvarez, 1987), Gamonedo (Hernández-Cabría & Abella-García, 1994; Moro, Alvarez Bartolmé, Díez et al., 1992) and Valdeón (López-Díaz, Alonso Calleja, Sanz, Santos & García, 1994) and other blue cheeses such as Stilton (Madkor, Fox, Shalabi & Metwalli, 1987; Muir, Hunter & Watson, 1995) and Roquefort-type cheese (El-Dairouty, El-Sayed, El-Senaity, Tawfek & Sharaf, 1990), but higher than those found in Danish Blue and Gorgonzola (Muir et al.) or Chetwynd (Zarmpoutis, McSweeney, Beechinor & Fox, 1996) cheeses. Changes in the total solids content are strongly influenced by the prevailing conditions in the ripening room/cave. The greatest loss of humidity happens in the first few weeks of ripening coinciding with the highest temperatures (12°C) and the lowest relative humidities (less than 80%). Thereafter, a slower loss of water occurred which was a consequence of transferring the

Table 1
Changes in chemical composition during the ripening of Picón Bejes-Tresviso cheese[a]

	Cheese after salting	Ripened cheese
Total solids (%)	52.76 ± 2.11	57.25 ± 2.38
Protein (%T.S.)	37.2 ± 3.12	38.9 ± 2.61
Fat (%T.S.)	54.08 ± 3.17	56.37 ± 2.96
Ash (%T.S.)	7.84 ± 1.46	9.95 ± 1.27
NaCl (%T.S.)	3.78 ± 1.06	6.67 ± 1.09
Lactose (%T.S.)	1.50 ± 0.41	N.D.[b]
D-lactic acid (%T.S.)	0.17 ± 0.08	0.05 ± 0.04
L-lactic acid (% T.S.)	2.09 ± 0.30	N.D.[b]

[a] Means of 10 batches ± standard deviations.
[b] N.D. = not detected.

Table 2
Changes in physico-chemical parameters during the ripening of Picón Bejes-Tresviso cheese[a]

	Cheese after salting	Ripened cheese
PH	5.13 ± 0.25	6.87 ± 0.54
Titratable acidity (g lactic acid/100 g cheese)	1.82 ± 0.13	2.39 ± 0.92
A_w	0.964 ± 0.004	0.914 ± 0.009

[a] Means of 10 batches ± standard deviations.

cheeses to natural caves where ripening continued and where the temperature fell to $<10°C$ and relative humidity was 95%.

The fat and protein contents, expressed as percentage of the total solids, showed little change between the beginning and end of ripening, with average final values of 38.93 ± 2.61 and 56.4 ± 2.96 g/100 g T.S., respectively. These values did not differ from those reported for other blue-veined cheese varieties, with the exception of the Maldeva variety (Moro, Alvarez-Bartolomé, López et al., 1993) which had values of 26 and 46 g/100 g T.S. for protein and fat, respectively.

The ash content in Picón Bejes-Tresviso cheese showed, from the beginning, high levels which later increased markedly, reaching an average final value of 9.95 ± 1.27 g/100 g T.S., which is higher than that reported for other blue-veined cheese varieties such as Gamonedo (Hernández-Cabría & Abella-García, 1989), Cabrales (Alcalá, Millán & Sanjuán, 1991), Maldeva and La Peral (Moro, Alvarez-Bartolomé, López et al., 1993) and Valdeón (López-Díaz et al., 1994). These values are strongly influenced by the salting method used and by the salt content. At the beginning of ripening the cheeses had high levels of salt, and there were substantial differences between batches, which is a result of a not-well-standardized dry-salting method and of the different rates of salt penetration. At this time, NaCl represents 49% of the ash. However, at the end of ripening the variability observed becomes corrected due to the salt equilibrium which is reached in all the mass, with final NaCl values of 6.67 ± 1.09 g/100 g T.S. which makes up 67% of total ash content. These salt levels are higher than those reported in cheeses such as Valdeón (López-Díaz et al.) and Gorgonzola (Muir et al., 1995), but similar to other blue-veined cheese varieties with the exception of Chetwynd cheese which had values of 8.60–8.70 g salt/100 g T.S. (Zarmpoutis et al., 1996).

The water activity is closely related to water and salt content. The average water activity values in this cheese showed a large decrease throughout ripening from 0.964 ± 0.004 after salting to 0.914 ± 0.009 at the end. Although not many data exist on the behaviour of this physico-chemical parameter in blue-veined cheeses, our values were similar to those reported by Alcalá et al. (1991) and Moro, Alvarez-Bartolomé, Díez et al. (1992) for Cabrales cheese and slightly higher than those reported for Gamonedo (Moro, Alvarez-Bartolomé, Díez et al.) and Valdeón (López-Díaz et al., 1994) cheeses.

The marked decrease in the water activity of Picón Bejes-Tresviso cheese observed throughout ripening seems to be due to the high solute concentration in the cheese mass, due mainly to a high NaCl content, with a molality of 2.67, and low molecular weight nitrogen compounds. The importance of these latter components seems to be greater in blue-veined cheeses than the dehydration per se, since the water loss during ripening in these varieties is very low (Marcos, 1993).

With regard to lactose degradation and its effect on pH, a fairly pronounced drop in the pH of Picón Bejes-Tresviso cheese occurred at the beginning of ripening with average values of 5.13 ± 0.23 after salting, which is a result of the development of the lactic acid flora, which degrades the lactose producing lactic acid through the homofermentative route. This fact determines that, at this time, practically all the lactic acid is in the L form. The L-lactic acid disappears completely at the end of ripening, while D-lactic form is reduced to insignificant levels. These phenomena coincide with a significant increase in pH, the average final values of which were 6.87 ± 0.54. This change of pH was also observed in other varieties of blue-veined cheeses, although our final values were higher than those in the majority of the blue-veined cheese varieties studied and were similar to those found in Cabrales (Alonso et al., 1987) and Gorgonzola (Muir et al., 1995) cheeses. This change in pH is related to the consumption of lactic acid by the moulds and yeasts and to the de-amination and decarboxylation of the free amino acids, producing large quantities of ammonia and amines which also contribute to the neutralization of cheese mass. The importance of this neutralization process in blue-veined cheeses is due to the marked effect which it has on the rheological properties of the cheese, although it occurs to a lesser extent than in mature cheeses by moulds on

the surface such as Camembert or Brie (Karahadian Lindsay, 1987).

3.2. Proteolytic parameters

The average values obtained for the different nitrogen fractions at the beginning and at the end of ripening, expressed as percentage of the total nitrogen, are shown in Table 3.

The values obtained for TSN, NPN and NH_2–N at the end of ripening show that Picón Bejes-Tresviso cheese undergoes extensive and in-depth proteolysis. Relatively high values of TSN were observed in cheese immediately after salting, as a consequence of the proteolytic activity of the rennet, which is favoured by the low pH values (5.13) and by the high moisture content (47.24%). At the end of ripening, the TSN increased markedly reaching average values of 72.7 ± 13.6 % of TN, which were higher than those reported for Stilton (Madkor et al., 1987), Bleu de Bresse, Danablue, Edelpilzkäse and Gorgonzola (Fernández-Salguero, Marcos, Alcalá & Esteban, 1989), Cabrales (Alcalá et al., 1991), Gamonedo (González de Llano, Ramos, Rodríguez, Montilla & Juárez, 1992) and Chetwynd (Zarmpoutis et al., 1996) cheeses.

NPN increased markedly during ripening (8-fold compared to 5.4-fold for TSN) and represented an average of 49.9 ± 13.6% of TN at the end of ripening. This level represents approximately 70% of TSN which was similar to that in the six varieties of blue-veined cheese studied by Fernández-Salguero, Marcos et al. (1989) which had values of NPN between 70 and 85% of TSN.

The intense proteolysis which this cheese variety undergoes is correlated to the changes in Protein N and Casein N, the levels of which decreased by 50 and 70%, respectively. The final values of both nitrogen fractions were lower than those reported by Alcalá et al. (1991) for Cabrales cheese.

Table 3
Changes in nitrogen fractions during the ripening of Picón Bejes-Tresviso cheese[a]

	Cheese after salting	Ripened cheese
Total nitrogen (%)	3.07 ± 0.25	3.49 ± 0.27
Protein nitrogen (%T.N.)	93.7 ± 1.30	50.1 ± 13.56
Non protein nitrogen (%T.N.)	6.25 ± 1.30	49.94 ± 13.56
Casein nitrogen (%T.N.)	86.5 ± 2.78	27.3 ± 13.6
Total soluble nitrogen (%T.N.)	13.5 ± 2.80	72.7 ± 13.6
Proteose-peptone nitrogen (%T.N.)	7.24 ± 2.09	22.7 ± 7.48
Oligopeptides nitrogen (%T.N.)	4.51 ± 1.08	17.6 ± 8.91
Amino nitrogen (%T.N.)	1.23 ± 0.41	24.4 ± 6.97
Ammonia nitrogen (%T.N.)	0.52 ± 0.15	7.93 ± 3.48

[a] Means of 10 batches ± standard deviations.

The Proteose-peptone N fraction consists of polypeptides soluble at pH 4.6 while the Oligopeptides N fraction consists of small- and medium-sized peptides. Table 3 shows that both fractions increased during ripening to 3- or 4-fold. However, when both fractions are expressed as a percentage of TSN, proteose–peptone N decreased by almost 50% and the oligopeptides N by 30%. This change is logical since the main proteolytic agent initially is the rennet, the action of which produces large polypeptides (Fox & McSweeney, 1996). As the ripening process progresses, the liberation to the medium of different enzymes (proteases and peptidases) is produced by the lactic acid flora and moulds, which intervene in the breaking of polypeptides into small-sized peptides and aminoacids. This is shown in a decrease in the proteose–peptone N values and, to a lesser extent, in oligopeptides N values and in a marked increase, around 3-fold, in NH_2–N and NH_3–N when they are expressed as % of TSN.

NH_2–N and NH_3–N, expressed as % of TN, markedly increased during ripening representing, at the end, average values of 24.4 ± 6.97% and 7.93 ± 3.48%, respectively. These values coincide with those reported for other blue-cheese varieties described in literature with the exception of Gamonedo cheese (González de Llano et al., 1992) which has higher NH_2–N values and of Cabrales (Alcalá et al., 1991), Bleu de Bresse (Fernández-Salguero, Marcos et al., 1989) and Roquefort (Fernández-Salguero, Marcos et al.) cheeses which have higher NH_3-N values. If we express these nitrogen fractions as a percentage of NPN, of which they are important components together with the oligopeptides N, we can clearly observe what we have previously pointed out; that is, if the content in oligopeptides N represented 73% of NPN at the beginning of ripening, at the end of ripening it is reduced more than 50%, while the content in NH_2–N increases more than 100%, then becoming the most important component of NPN (almost 50% of the same).

Fig. 1 shows the typical electrophoretogram of the caseins and their degradation products in the cheese after salting and in the ripened cheese with values of Rf and optical density of each one of the bands detected. Table 4 shows the changes in the percentage of total optical density of the electrophoretical regions in the stained gels of caseins during the ripening of Picón Bejes-Tresviso cheese.

The αs-CN fraction is initially degraded by the rennet to $\alpha_{s1\text{-}I}$-CN which, later as ripening advances, is a substract of other enzymes, above all the aspartyl protease of *P. roqueforti* or the quimosine (Trieu-Cuot, Archieri-Haze & Gripon, 1982) originating an increase in the bands of greater mobility (fraction integrated by the preα_s-CN).

The β-CN concentration also drops markedly during ripening (see Fig. 1) fundamentally by the action of the

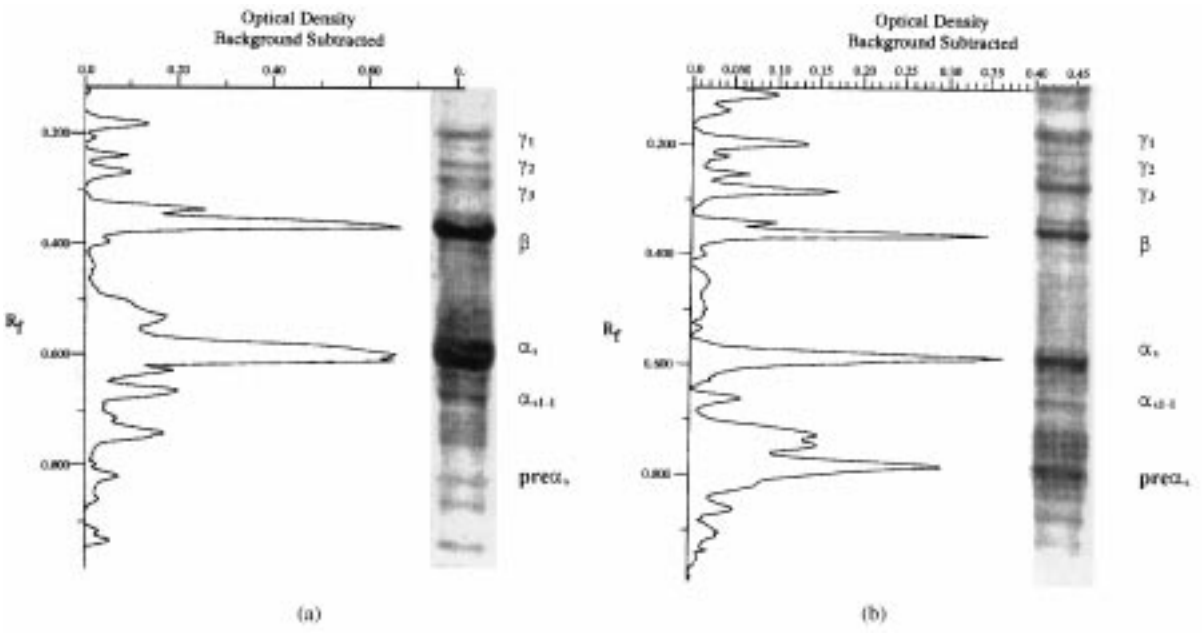

Fig. 1. Typical electrophoretogram of the casein fractions in cheese after salting (a) and in ripened cheese (b) with Rf and Optical Density values of each one of the bands detected.

Table 4
Changes in the percentage of total optical density of the electrophoretical regions in the stained gels of caseins during the ripening of Picón Bejes-Tresviso cheese[a]

	Cheese after salting	Ripened cheese
#[b]	1.74 ± 0.48	18.3 ± 4.47
γ_1	2.85 ± 0.73	7.88 ± 0.63
γ_2	1.32 ± 0.53	1.97 ± 1.45
γ_3	2.40 ± 0.44	5.99 ± 1.60
β	23.7 ± 1.24	4.83 ± 3.12
#[b]	1.82 ± 0.51	0.37 ± 0.64
α_s	45.8 ± 2.21	8.54 ± 5.97
#[b]	3.77 ± 0.09	1.79 ± 0.85
α_{s1-I}	7.80 ± 2.03	0.94 ± 0.90
Pre-α_s	8.79 ± 1.49	50.4 ± 7.96
α/β	1.93 ± 0.10	1.70 ± 0.58

[a] Means of 10 batches ± standard deviations.
[#b] Unknown degradation products.

plasmin since the pH values present in this cheese are close to its optimal range of action (Grufferty & Fox, 1988) giving rise to various products of low mobility which are known as γ_1-CN, γ_2-CN and γ_3-CN.

Other bands whose intensity increases during ripening and which we have named as "unknown", were also observed. These bands, which were also described by Fernández-Salguero, Marcos et al. (1989) in other blue-cheese varieties, are derived from β-CN by the action of aspartyl protease and metaloprotease of *P. roqueforti* (Le Bars & Gripon, 1981).

3.3. Fat parameters

The average values of TBA number and acidity index of the fat are shown in Table 5.

Data on the TBA number in blue-veined cheeses could not be found in the literature. Nevertheless, taking into account the data reported for other types of cheese (Carballo et al., 1994; Fresno, Tornadijo, Carballo, Bernardo & González Prieto, 1997; Prieto et al., 1994), it does not seem that autooxidation is very important in Picón Bejes-Tresviso cheese.

During the ripening of Picón Bejes-Tresviso cheese, the lipolytic processes did seem to have great relevance. The average values of the acidity index of the fat increased about 20-fold during ripening, being 40.1 ± 11.5 mmol KOH/100 g of fat at the end of the process, similar to those reported for other blue-veined cheese varieties such as Cabrales (Fernández-Salguero, Florido, Alcalá, Marcos & Esteban, 1986) and Danablue (Vanbelle, Vervack & Foulon, 1978) and higher than those reported by Vanbelle et al. for Roquefort cheese. Alonso et al. (1987) also showed similar values (50 mmol KOH/100 g of fat) for Cabrales cheese at the end of ripening. The values of acidity index of the fat obtained in Picón Bejes-Tresviso ripened cheeses seem to indicate that this cheese undergoes a very intense lipolysis during ripening.

The native lipase of the milk, as well as other enzymes freed by the lactic acid flora are of great importance in lipolysis, although, without doubt, the lipases of fungal origin (*P. roqueforti*) are the main ones responsible for this process. Two types of lipase produced by *P. roqueforti*

Table 5
Changes in acidity index of the fat and T.B.A. number during the ripening of Picón Bejes-Tresviso cheese[a]

	Cheese after salting	Ripened cheese
Acidity index[b]	2.41 ± 0.89	40.1 ± 11.5
T.B.A. number[c]	0.39 ± 0.23	0.92 ± 0.13

[a] Means of 10 batches ± standard deviations.
[b] Expressed as mmol of KOH/100 g of fat.
[c] Expressed as mg of malonaldehyde/Kg of cheese.

were detected: one acid (optimum pH 6.0–6.5) and the other alkaline (optimum pH 9.0–9.5) (Gripon, 1993). It is possible that, at pH values found in Picón Bejes-Tresviso cheese, the acid lipase activity is maximal while the alkaline lipase activity is reduced in an important way, even though it may maintain up to 20% of its activity at pH values of 6.0 (Menassa & Lamberet, 1982). Both enzymes are characterized by showing a special affinity to the liberation of short chain fatty acids whose contribution to the final characteristic aroma of these cheese varieties will be very important, either by themselves or as a source of metilketones, through their oxidation (Kinsella & Hwang, 1976; Law, 1984; Urbach, 1997).

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