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Secondary proteolysis in Serra cheese during ripening and throughout the cheese-making season

Abstract Experimental Serra cheeses were manufactured from raw ewe's milk and thistle flowers following a two-way factorial design. The content of nitrogen soluble in water (WSN), in 2% trichloroacetic acid (2% TCA-N), in 12% TCA (TCA-N) and in 5% phosphotungstic acid (5% PTA-N), and the pH and salt-in-moisture concentration were measured throughout the ripening period (sampling at 0, 7, 21 and 35 days) and the cheese-making season (sampling in November, February and May). Proteolysis in 35-day-old Serra cheese was quantitatively high [average values of 34.6% and 11.9% for WSN/TN (total nitrogen) and 2% TCA-N/TN, respectively], but qualitatively low (average values of 5.8% and 1.2% for 12% TCA-N/TN and 5% PTA-N/TN, respectively). The ratios WSN/TN and 2% TCA-N/TN were lowest for cheeses ripened in February, whereas the ratio 12% TCA-N/TN was highest for cheeses ripened in November. By 35 days of ripening, the average pH and salt-in-moisture concentration values were 5.2 and 4.8%, respectively. No correlation was found to occur between the measured pH or salt-in-moisture concentration and the values of soluble nitrogen fractions throughout the cheese-making season.

Key words Proteolysis · Soluble nitrogen fractions · Ripening · Raw milk · Sheep

Introduction

Proteolysis is probably the most complex process that occurs during cheese ripening and is likely to be that which is most important for the development of flavour and texture [1, 2]. Coagulated caseins, which form the cheese matrix, are progressively hydrolysed by enzymes contributed by the milk, the rennet and the adventitious (or deliberately added) microflora leading to generation of nitrogen compounds which are soluble in water (e.g. proteose-peptones, peptides and amino acids). Quite a number of research works have focused on the description of extents and types of proteolysis that occur during the ripening of major cheeses; in order to monitor and quantitate proteolysis, a comprehensive scheme for the fractionation of cheese nitrogen is thus essential. Typically, the initial step of one such scheme is the preparation of a cheese extract containing a very wide mixture of peptides with respect to size, solubility and conformation. Methods for monitoring and quantifying proteolysis have been comprehensively reviewed [2–4].

Serra cheese is the most famous Portuguese cheese manufactured from raw ewe's milk at the farm level. It is shaped like a flat cylinder ($\approx$17 cm diameter and $\approx$5 cm height) and has a mass of $\approx$1.5 kg. It has a buttery texture (which leads to spontaneous and relatively rapid loss of shape upon slicing at room temperature) and possesses a strong aroma and a slightly acid taste. The moisture content ranges from 61% to 69% (fat-free basis) whereas the fat content ranges from 45% to 60% (moisture-free basis). In the traditional manufacturing process of Serra cheese, coagulation of fresh raw ewe's milk occurs at 27–32 °C after addition of a crude preparation of thistle dried flowers (*Cynara cardunculus*, L.), without addition of any starter culture [5]. The gel is cut into irregularly shaped pieces which are then slightly pressed manually to help in the syneresis of whey. The cheeses are salted by adding kitchen salt to milk prior to coagulation and/or by rubbing salt on the surface of the cheese when moulding. Maturation of cheese takes place usually for 40 days in rooms without temperature or relative humidity control. The cheese-making season for Serra cheese typically starts in October and ends in June. During this period the temperature can range

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from 4° C to 30°C and the relative humidity from 42% to 98%. Considering that the ripening process depends chiefly on environmental conditions, proteolysis will be affected by the period within the cheese-making season.

Knowledge pertaining to the biochemical changes that occur during ripening of Serra cheese is scarce and that available has been critically reviewed elsewhere [5]. The aim of the present research was to characterize the secondary proteolysis in Serra cheese during ripening and throughout the cheese-making season via quantitation of the nitrogen fractions that are soluble in water, in trichloroacetic acid (TCA), or in phosphotungstic acid (PTA). Such characterization is important in attempts not only to rationalize the roles of coagulant and microflora in the development of the flavour and texture of Serra cheese, but also in the design of measures leading to technological standardization and improvement of this long-celebrated dairy speciality.

Materials and methods

Cheese manufacture and sampling

Three batches of 12 0.5-kg cheeses were prepared in a small, certified dairy farm in the Appéllation Serra Controllée region at three sequential periods within the cheese-making season (November, February and May). The three times within the cheese-making season were chosen to be sufficiently apart from each other so as to be representative of varying milk composition (as determined by the local pasture composition and animal physiological state) and ripening condition (as determined by the local weather).

A cheese-making protocol that parallels traditional procedures was used to manufacture the cheeses. Crude kitchen salt (12 g) was dissolved in each litre of fresh raw ewe's milk; the crude rennet coagulant preparation was obtained by grinding dry flowers, mixing them well with tap water, filtering the resulting turbid suspension through a fine, clean cloth, and adding the resulting aqueous extract to the milk so as to give a concentration of 0.4 g/l; and the treated milk was gently stirred. The milk was then allowed to rest at 28 °C until complete coagulation had occurred (for $\approx$1 h); the curd was manually cut in order to obtain small irregularly shaped pieces and was then allowed to rest for $\approx$10 min. The pieces of curd were poured into a fine cloth bag and lightly pressed by hand so as to promote controlled expression of whey; the resulting smaller pieces of curd were then loaded into perforated plastic moulds and slightly pressed to help the development of the correct shape. Drainage of whey was completed by pressing the cheese, while it was in the mould, using a 10-kg metal block for $\approx$12 h (cheeses were turned upside down after $\approx$6 h). Salting was completed by rubbing the surface of the cheese with kitchen salt at a density of 0.04 g per cm^2 of surface area; ripening was started in chambers with temperature and relative humidity maintained at 9 °C and 95%, respectively, for the first week.Thereafter, the cheeses were transferred to a second ripening chamber without control of temperature and relative humidity, where they were turned upside down daily and washed with tap water periodically.

Three cheeses of each batch were randomly taken on the actual day of manufacture and after 7, 21 and 35 days of ripening and transported under refrigeration conditions ($\approx$4 °C) to our premises for further analysis. The four times within the ripening period were chosen to be sufficiently apart from each other so to be representative of varying biochemical parameters in cheese (as determined by the extent of proteolytic breakdown). Each cheese was sampled by adequate homogenization after the rind had been carefully removed, and each sample was frozen at ≈ -30 °C in Whirl-pak vacuum packages (Cole-Parmer, Chicago, Ill., USA) until analysis was in order.

Chemical analysis

The total nitrogen content of cheese (TN) was determined using 0.3-g samples by the micro-Kjeldahl method [6] using a Kjeltec system with a 2012 digester and a 1002 distilling unit (Tecator, Hoganas, Sweden). The pH, moisture and salt contents were determined according to the methods reported elsewhere [7]: the pH of the cheese sample was determined directly using a MicropH 2001 (Crison, Barcelona, Spain); the moisture content was determined by the oven method at 100 °C for 24 h (Ehret, Emmendingen, Germany); and the salt content was determined by the modified Volhard method with reagents supplied by Merck (Darmstadt, Germany). The salt content was expressed as salt-in-moisture concentration (%, w/w).

Biochemical analysis

Fractionation with water. Extracts of cheese soluble in water were prepared as follows: cheese samples (50 g) were homogenized in 100 ml deionized water at room temperature for 10 min using a Stomacher 400 (Colworth, London, UK); the resulting slurry was then held at 40 °C for 1 h with frequent stirring. The material insoluble in water was then separated by centrifugation at 3000 g for 30 min at 4 °C in a refrigerated centrifuge (Sorval RC5C, Wilmington, USA), and the supernatant was filtered through glass wool to remove residual suspended fat. The nitrogen content was determined by the micro-Kjeldhal method using 2-mol aliquots of the filtrate. The analyses were run in duplicate. The content of nitrogen soluble in water (WSN) was expressed as per unit mass of total nitrogen, TN, and will be denoted hereafter as WSN/TN.

Fractionation with 2% TCA. Extracts of cheese soluble in 2% TCA were prepared as follows: 10 ml of a 4% (w/v) aqueous solution of TCA (Sigma, St. Louis, Mo., USA) was added to 10 ml of the water-soluble nitrogen fraction; the mixture was allowed to stand for 30 min at room temperature and then filtered through Whatman no. 42 filter paper (Whatman, Maidstone, UK). The nitrogen content was determined by the micro-Kjeldhal method using 3-ml aliquots of the filtrate. The analyses were run in duplicate. The quantity of nitrogen soluble in 2% TCA was expressed as per unit mass of total nitrogen, and will be denoted hereafter as 2%TCA-N/TN.

Fractionation with 12% TCA. Extracts of cheese soluble in 12% TCA were prepared as follows: 5 ml of a 48% (w/v) aqueous solution of TCA (Sigma) was added to 15 ml of the water-soluble nitrogen fraction; the mixture was allowed to stand for 30 min at room temperature and then filtered through Whatman no. 42 filter paper. The nitrogen content was determined by the micro-Kjeldhal method using 5-ml aliquots of the filtrate. The analyses were run in duplicate. The quantity of nitrogen soluble in 12% TCA was expressed as per unit mass of total nitrogen, and will be denoted hereafter as 12% TCA-N/TN.

Fractionation with 5% PTA. Extracts of cheese soluble in 5% (w/v) PTA were prepared as follows: 14 ml of a 3.95 M aqueous solution of sulphuric acid (Merck) and 6 ml of a 33.3% (w/w) aqueous solution of PTA (Sigma) were added to 20 ml of the water-soluble nitrogen fraction; the mixture was allowed to stand overnight at 4 °C and subsequently filtered through Whatman no. 542 filter paper. The nitrogen content was determined by the micro-Kjeldhal method

using 5-ml aliquots of the filtrate. The analyses were run in duplicate. The quantity of nitrogen soluble in 5% PTA was expressed as per unit mass of total nitrogen content, and will be denoted hereafter as 5% PTA-N/TN.

Statistical analysis

The Statview 4.0 statistical package [8] was used for statistical treatment of the results by analysis of variance (ANOVA) and Fisher's protected least significance difference test (Fisher's PLSD). Both methodologies are acceptable from a statistical point of view because previous diagnostics (not shown) have indicated that the experimental errors are independent and normally distributed. The two types of tests were employed to determine overall and pairwise, respectively, statistical differences at the 5% level of significance between the pH, salt-in-moisture concentration, WSN/TN, 2%TCA-N/TN, 12% TCA-N/TN and 5% PTA-N/TN throughout the ripening period and the cheese-making season.

Results and discussion

Chemical assays

Changes in pH and salt-in-moisture concentration in Serra cheese with ripening time and time within the cheese-making season are shown in Fig. 1. ANOVA indicates that both the pH and the salt-in-moisture ratio changed significantly with ripening time ($P < 0.0001$ for both, where P is the probability that the value found for the ratio of the mean squares associated with the factor in question to the mean squares arising from experimental variability is due to pure chance) and throughout the cheese-making season ($P = 0.012$ and $P = 0.0003$, respectively). The pH decreased significantly from 6.45 on the actual day of manufacture down to 5.23 by day 21 of ripening, and tended to stabilize thereafter (the pH of the cheese by 35 days was statistically similar to that at day 21, $P = 0.803$). The decrease of pH during ripening is mainly due to conversion of lactose into lactic acid (lactate) by lactic acid bacteria, which reach their maximum numbers by $\approx$21 days of ripening [9, 10]. No statistically significant differences were found to occur between the pH values of cheeses manufactured in November, February or May at day 0 ($P = 0.195$), day 7 ($P = 0.674$) and day 21 ($P = 0.414$) of ripening. However, 35-day-old cheeses ripened in May showed a significantly higher pH value (5.40) than cheeses ripened in November ($P = 0.006$) and February ($P = 0.002$), the pH values of which were statistically similar (5.16 on average, $P = 0.332$). This fact probably can be explained by the lower numbers of lactic acid bacteria in spring than in the other two periods as claimed by Macedo et al. [9, 10]; however, it should be noted that the pH of the cheese is determined not only by the amount of lactic acid produced in situ by the microflora but also by the buffering capacity of the curd, which arises primarily from the quantities of casein and partially soluble inorganic phosphate and citrate [11]. The salt-in-moisture concentration increased significantly throughout ripening ($P < 0.0001$). A significant increase in the salt-in-moisture concentration from 1.82% at day 0 to 3.70% at day 7 was observed ($P < 0.0001$). Cheeses ripened for 21 days showed a salt-in-moisture ratio of 4.12% and these values are significantly lower ($P < 0.0001$) than those measured for 35-day-old cheeses (4.76%). The profile of salt-in-moisture concentration versus ripening time can be explained by both the diffusion of dry salt from the surface into the centre of the cheese, where it gives rise to an increased salt content, and by water evaporation from the surface of the cheese, which accounts for the decreased moisture content. No significant differences were found in the salt-to-moisture ratio for cheeses at day 7 ($P = 0.159$), day 21 ($P = 0.178$) and day 35 ($P = 0.107$) produced at different periods within the cheese-making season. Cheeses manufactured in February showed, at day 0, a significantly higher salt-in-moisture concentration (2.12%) compared with cheeses manufactured in November ($P = 0.041$) and May ($P = 0.015$), values for which were statistically similar to one another (1.67% on average). This variation of the salt-in-moisture ratio between 0-day-old cheeses manufactured at different periods within the cheese-making season may be attributed to the empirical and, to a certain extent, random pattern of salt distribution on the surface of the cheese and to the manual drainage of whey from cheese carried out by the cheesemaker.

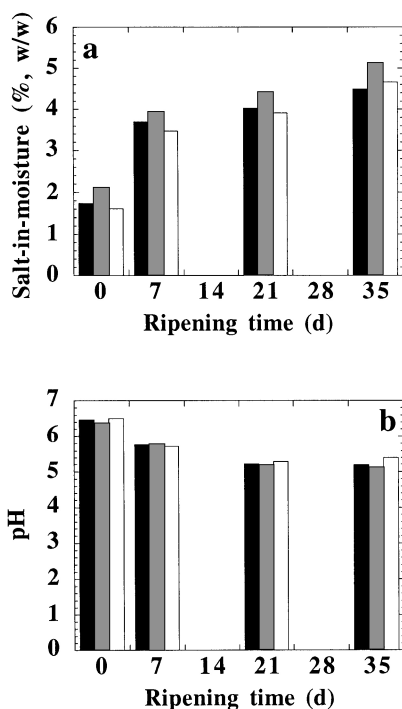

Fig. 1 Changes in **a** salt-in-moisture concentration and **b** pH during ripening of Serra cheese produced in November (*black bars*), February (*stippled bars*), and May (*open bars*). The *height* of each *bar* represents the mean of three replicates (SEM = 0.08 and SEM = 0.27, respectively)

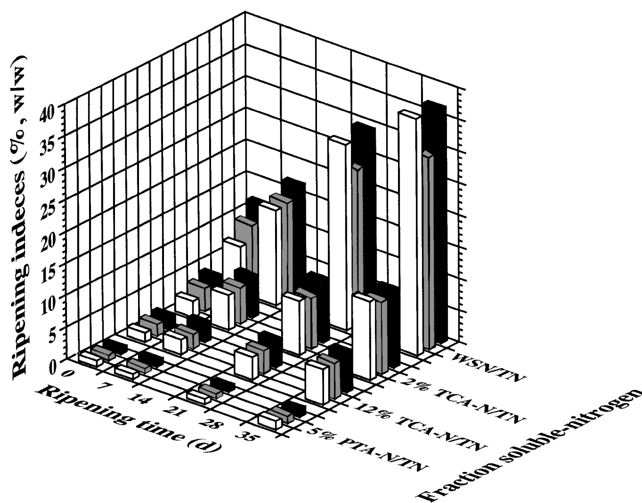

Fig. 2 Changes in water-soluble nitrogen (WSN), trichloroacetic-acid-soluble nitrogen (*2% TCA-N* and *12% TCA-N*), and phosphotungstic-acid-soluble nitrogen (*5% PTA-N*) per unit mass of total nitrogen content (*TN*) during ripening of Serra cheese produced in November (*black bars*), February (*stippled bars*), and May (*open bars*). The *height* of each *bar* represents the mean of three replicates (SEM = 1.22, SEM = 0.46, SEM = 0.19 and SEM = 0.11, respectively)

Biochemical assays

Figure 2 depicts the changes in WSN, 2% TCA-N, 12% TCA-N and 5% PTA-N normalized to total nitrogen in Serra cheese as a function of ripening time and period within the cheese-making season.

Nitrogen soluble in water

The ratio WSN/TN increased throughout the whole of ripening ($P < 0.0001$). The water-soluble fraction is a very heterogeneous solution in terms of components, which include whey proteins, high-, medium- and low-molecular-weight peptides and free amino acids [3]. The components of WSN are mainly accounted for by nitrogen-based compounds, which results from reactions brought about by rennet [12, 13] and, to a lesser extent, by milk proteinases [14]; this network of reactions is generally termed secondary proteolysis [15]. Although plasmin is the major constituent of the family of adventitious proteinases in milk, it is not expected to contribute considerably to the WSN in cheeses because γ-caseins (i.e. the major peptides released via the action of plasmin on β-caseins) are not extracted in water [15]. On the other hand, the plant extract utilized as the coagulant in the manufacture of Serra cheese is also highly proteolytic [16]; therefore, most of the WSN of Serra cheese is likely to be the result of the breakdown of ewe's milk caseins (and of their first breakdown products) effected by cardosins. At day 0, cheeses showed WSN/TN levels of 9.5%, on average, which are higher than that of Manchego cheese (manufactured from ewe's milk cheese with animal rennet), which possesses $\approx$7% of WSN/TN by day 2 of ripening [17]; the differences are probably due to the higher proteolytic activity of the extracts of *Cynara cardunculus* than animal chymosin [16].

The WSN/TN ratio changed throughout the cheese-making season ($P < 0.0001$); however, after 7 days of ripening no statistically significant differences between cheeses ripened in either November, February or May could be found ($P = 0.146$). Possible reasons for this observation are that the temperature and relative humidity during this period were similar for all cheeses, because the early stage of ripening takes place in a chamber with controlled temperature and relative humidity (9 °C, 95%), and that the (already mentioned) values of pH and salt-in-moisture concentration are similar (see Fig. 1), which thus account for the similar values of the four intrinsic factors that are most likely to constrain enzyme activity (i.e. temperature, pH, water activity and ionic strength). Cheeses ripened in November and May showed similar WSN/TN values after 21 days (29.2% on average) and after 35 days (36.9% on average), but these values were statistically different to those of the cheeses manufactured in February and ripened for 21 days (24.3% on average, $P = 0.004$) or 35 days (30.1% on average, $P = 0.002$). During the second week of maturation the ripening cheeses were transferred to a second ripening room without control of temperature or relative humidity; the ripening conditions thereafter were thus mainly determined by the local weather. Since, of all three periods studied, the lowest temperatures ($\approx$4 °C) and the highest relative humidities ($\approx$95 %) are reached in February, whereas the highest temperatures ($\approx$18 °C) and the lowest relative humidities ($\approx$70%) are reached in May (with intermediate values prevailing in November), it is expected (and was confirmed by our results) that the secondary proteolysis in Serra cheese will be affected by the period within the cheese-making season after the first week has elapsed, and that cheeses ripened in May will be richer in WSN than those ripened in November, which will, in turn, be richer in WSN than those ripened in February. Nuñez et al. [18] reported that increasing the ripening temperature of Manchego cheese from 10 to 15 °C causes an increase in the WSN content by 17%. Since enzymatic activity is affected by salt-in-moisture concentration and pH, in addition to temperature, it could be argued that such experimentally observed variations could also be due to changes in these physicochemical parameters between the months. However, as already discussed, the salt-in-moisture concentration did not exhibit significant differences throughout the cheese-making season. On the other hand, although our results indicate that the pH of cheeses ripened for 35 days in February or November (5.2 on average) is statistically lower than that of cheeses ripened for 35 days in

May (5.3), lower pH values tend to favour the action of the plant rennet (an assertion that actually contradicts our observations pertaining to the extent of proteolysis). This point if further backed up by the results of Heimgartner et al. [19], who reported that the maximum activity of the enzymes extracted from the thistle flower is obtained at pH 5.1 and is rather insensitive to pH in the vicinity thereof.

Nitrogen soluble in 2% TCA

The values of 2% TCA-N/TN were significantly lower than those of WSN/TN ($P < 0.0001$). This result was anticipated as 2% TCA has been widely used [20–22] to subfractionate nitrogen-based components that are soluble in water, and also because of the assertion by Kuchroo and Fox [23] that 2% TCA precipitates mainly large peptides from the water-soluble fraction, thus leaving medium-sized peptides, small peptides and free amino acids in the supernatant (the length of peptides precipitated varies between 2 and 64 amino acid residues [13]).

The 2% TCA-N/TN ratio increased significantly throughout ripening ($P < 0.0001$). The ripening agents that may be implicated in the generation of nitrogen-based-compounds soluble in 2% TCA are peptidases released from viable (or lysed) lactic acid bacteria and possibly those from the thistle flower. Since lactic acid bacteria are nutritionally fastidious and have complex amino acids requirements, coupled with the fact that the naturally occurring concentration of free amino acids in milk is insufficient to support bacterial growth, there has been selective pressure on adventitious lactic acid bacteria in milk to develop proteolytic systems that involve the concerted action of proteinases and peptidases that will ultimately hydrolyse milk polypeptides down to free amino acids [1]. Furthermore, Gram-negative psychrotrophic bacteria also possess proteinases capable of degradating casein and polypeptides [24]. Finally, it has been reported [9] that the microflora in early ripening Serra cheese is dominated by *Lactococcus lactis* spp. *lactis* and *Hafnia alvei* in terms of lactic acid bacteria and Enterobacteriaceae, respectively. Since the proteinases from the plant rennet employed achieve a relatively fast and efficient degradation of caseins into polypeptides, peptidases from *Lactococcus* spp. and Enterobacteriaceae could easily and promptly digest polypeptides into large peptides, which are thus soluble in 2% TCA.

The 2% TCA-N/TN is statistically different between months within the cheese-making season ($P < 0.0001$), although the 7-day-old cheese ripened in the different periods showed statistically similar values of 2% TCA-N/TN (5.84% on average, $P = 0.147$). This observation was expected in view of previous discussion pertaining to the constancy of ripening conditions prevailing during the first week of ripening. By 21 days of ripening, the 2% TCA-N/TN for cheese manufactured in November (9.7%) was significantly higher than that for cheese manufactured in February (8.0%, $P = 0.006$) and May (8.4%, $P = 0.021$), which were statistically similar to one another ($P = 0.354$); by 35 days of ripening, cheeses manufactured in November and May showed statistically similar 2% TCA-N/TN values to one another (12.2% on average, $P = 0.401$) but these values were higher than those shown by cheeses manufactured in February (11.2%, $P = 0.024$ and $P = 0.008$, respectively). As discussed before, these differences are mainly due to higher average ripening temperatures in May and November than in February, rather than to variations in pH or salt-in-moisture concentration.

Nitrogen soluble in 12% TCA

The values of 12% TCA-N/TN were significantly lower than those of WSN/TN ($P < 0.0001$) and 2% TCA-N/TN ($P < 0.0001$). This was expected in view of the reports by several researchers [13, 20, 22] who have shown that the nitrogen fraction that is soluble in 12% TCA contains only small peptides (with a chain length of between 2 and 20 amino acid residues) and free amino acids; therefore, several of those peptides that are part of the WSN and of the 2% TCA-N will precipitate in the presence of 12% TCA.

The 12% TCA-N/TN increased significantly throughout ripening ($P < 0.0001$). This ratio changed at approximately the same rate between 0 and 21 days (average of 0.10% per day) but increased thereafter to 0.14% per day. O'Keefe et al. [21] have reported that the action of starter enzymes leads primarily to the formation of small peptides and amino acids and is thus the source of most 12% TCA-N; therefore, such observation is probably the result of the activity of peptidases from lactic acid bacteria which reach their maximum numbers by $\approx$21 days of ripening [9, 10].

The 12% TCA-N/TN ratio varies statistically throughout the cheese-making season ($P < 0.0001$). The values of 12% TCA-N/TN at 7 days, 21 days and 35 days for cheeses ripened in November (2.9%, 4.2%, 6.2%, respectively) were significantly higher than those for cheeses ripened in February ($P = 0.006$, $P = 0.006$, and $P = 0.015$, respectively) and in May ($P = 0.010$, $P = 0.004$, and $P = 0.013$, respectively), which were statistically similar to one another (2.3% on average, $P = 0.635$; 3.6% on average, $P = 0.625$; and 5.5% on average, $P = 0.914$, respectively). Although the ripening temperature is considerably higher in May than in November and higher in November than in February, the number of lactic acid bacteria in cheeses is statistically higher in November than in May for the same ripening time [9]; hence, a compromise between temperature and viable counts of lactic acid bacteria probably leads to better results in terms of proteolysis compared with lower temperatures and higher counts

(as happens in February) or higher temperatures and lower counts (as happens in May).

Nitrogen soluble in 5% PTA

The values for 5% PTA-N/TN were significantly lower than the values of WSN/TN ($P < 0.0001$), 2% TCA-N/TN ($P < 0.0001$) and 12% TCA-N/TN ($P < 0.0001$). Since precipitation with 5% PTA is used to subfractionate the water-soluble fraction [3] so as to allow only small peptides (with molecular weight below 600 Da) and free amino acids to remain in solution [25], several peptides soluble either in water, 2% TCA or 12% TCA will precipitate in the presence of 5% PTA. However, the values obtained for 5% PTA-N/TN were particularly low, which is an indication that the plant rennet has little activity against peptides containing a small number of amino acid residues; hence, the microflora are probably the major source of those peptidases responsible for generation of the nitrogen-based compounds that are soluble in 5% PTA [4].

The 5% PTA-N/TN changed significantly as ripening progressed ($P < 0.0001$). However, this ratio decreased consistently from 0.93% at day 0 down to 0.79% at day 21, to increase thereafter up to 1.24% by day 35. It is likely that lactic acid bacteria (which, as already mentioned, possess strict nutritional requirements for amino acids) directly utilize the free amino acids available in the aqueous phase during their stage of exponential growth, which occurs before day 21 [9, 10]; after depletion of free amino acids, a stationary phase is reached at which the microrganisms are forced to utilize less usable forms containing amino acid residues via hydrolysis of small peptides effected by extracellular peptidases from viable cells or endocellular peptidases from lysed cells [26].

Cheeses ripened at different months within the cheese-making season showed no statistical differences in terms of 5% PTA-N/TN ($P = 0.427$); for 35-day-old cheeses, the average values of 5% PTA-N/TN obtained in November, February and May were 1.2%, 1.2%, and 1.3%, respectively.

Overall comparison and conclusions

The values obtained in this work are consistent with those obtained in an independent work [27] where values for WSN/TN, 12% TCA-N/TN and 5% PTA-N/TN were measured to be 42%, 8% and 1%, respectively, for 42-day-old cheeses manufactured from raw ewe's milk using extracts of *Cynara cardunculus* as rennet. In addition, Serena cheese (a Spanish cheese also manufactured from raw ewe's milk using extracts of *Cynara* spp. as coagulant) also suffers extensive proteolysis characterized by 38–39% for WSN/TN, 10–12.5% of TCA-N/TN and 1.6% PTA-N/TN by 60 days of ripening [28, 29]. Conversely, Manchego cheese (a Spanish cheese manufactured from raw ewe's milk using standard animal rennet) exhibited much lower values for WSN/TN, namely 20%, but similar values for 2% TCA-N/TN and 5% PTA-N/TN, i.e. 13% and 1%, respectively [17].

Based on the average values of 34.6% for WSN/TN, 11.9% for 2%TCA-N/TN, 5.8% for 12% TCA-N/TN and 1.2% for 5% PTA-N/TN obtained for 35-day-old Serra cheeses in our work, one concludes that secondary proteolysis proceeds to a high extent but to a low degree. The rationale for this general conclusion is that the soluble nitrogen fractions are preferentially recovered in water than in TCA or PTA, which means that polypeptides and large peptides are preferentially released during ripening relative to medium-sized and small-sized peptides.

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