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Changes in soluble nitrogenous compounds, caseins and free amino acids during ripening of artisanal prato cheese; a Brazilian semi-hard cows variety

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Abstract

Proteolytic changes, during ripening (1–60 days) of 12 batches of artisanal Prato cheese, a Brazilian semihard cows'milk variety, were evaluated by determining the soluble nitrogenous compounds, caseins and their degradation products and free amino acids. Levels of pH 4.4-soluble nitrogen (pH 4.4-SN) and 12% TCA-soluble N, expressed as% TN, at the end of ripening were 21.4 and 12.5, respectively.% Aminoacid nitrogen/TN increased significantly ($P<0.05$) during ripening to final values of 4.32. The degradation patterns of α_{s1}- and β-caseins were similar in all cheese batches and α_{s1}-casein was hydrolysed more extensively than β-casein during ripening. The total content of free amino acids increased aproximately 8 times throughout ripening with average final values of 2454 mg/100 g total solids. γ-Amino butyric acid, leucine, lysine, glutamic acid, tryptophan and phenylalanine were the most abundant free amino acids in all batches studied, representing of 62% of total free amino acids.

Keywords: Prato cheese; Proteolysis; Nitrogen compounds; Caseins; Free amino acids; Ripening

1. Introduction

Of all the dairy products made in Brazil, cheese is the most traditional. Cheese production in Brazil is currently running at approximately 470,000 metric tonnes a year (Leite Brazil, 2001). Some 78% of the production of cheese is done under Federal inspection, while the remaining 22% is carried out by small craft producers working outside the legal framework. In Brazil two groups of cheeses are distinguished: common cheeses (95% of the total produced), covering widely-consumed varieties such as Mozzarella, Prato, Requeijao, Minas Frescal, Parmesan and Ricota; and fine or special cheeses (5% of the total) which include types such as Tilsit, Gouda, Gruyère, Gorgonzola and Camembert. Fine cheeses are those the better prospects in the Brazilian market. Nonetheless, common cheeses are those in greater demand by consumers, basically through being eaten indirectly as an ingredient, e.g. in pizzas (Mozzarella), sandwiches (Prato) and pasta dishes (grated Parmesan).

The Southern Region in Brazil (Rio Grande do Sul, Santa Catarina and Paraná states) is the second largest producer of milk in Brazil with approximately 4841 million litres output yearly (Milkbizz, 2002). Rio Grande do Sul state is the third largest milk producer in Brazil and the first by size in the Southern Region.

The dairy trade in Rio Grande do Sul is made up, for the most part, of small businesses, using family members as staff. Around 80% of these producers have associated to form co-operatives that make cheeses of the highest quality. However, the remaining 20% make and sell raw-milk cheeses of poor and inconsistent quality that can be a health risk for consumers. Hence, improvements in the quality of artisanal cheeses entail investments in hygiene, technology and production control with the aim of making consistent, safe cheeses with a traditional aspect. Achievement of this would

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yield, not merely health and technology improvements, but also financial advantages, as it would allow viable sales routes for small artisanal producers.

Among the craft cheeses most widely present in Rio Grande do Sul are the Minas Frescal and Prato varieties. The second of these is the subject of this characterization study.

Prato variety is a ripened cheese made by enzymatic curdling with a smooth, thin rind and an elastic, compact consistency. It is rectangular in shape and has a normal weight of 1.6–1.8 kilogrammes.

Characterization of artisanal Prato cheese includes, as one part, a study of the technological procedures for its production and of the chemical and physico-chemical parameters during its maturation (Cichoscki, Valduga, Valdaga, Tornadijo, & Fresno, 2002). A second part involves the biochemical changes in the principal components during maturation, with proteolysis the most important feature in bacterially-ripened varieties.

Proteolysis in cheese during ripening plays an important part in determining texture and flavour and has been the subject of several reviews (Fox & McSweeney, 1996). Hydrolysis of caseins by rennet and alkaline protease, in the initial stages of maturation releases a considerable quantity of peptides of large or intermediate molecular size. These peptides are substrates of peptidases of the starter and contaminating flora, giving rise to smaller peptides and free amino acids. These contribute directly to the flavour or in some cases indirectly by acting as precursors of aromatic substances (amines, acids, thiols, thioesters, and the like).

The aim of this work is to study proteolytic changes during the ripening of artisanal Prato cheese by the assessment of soluble nitrogenous compounds, casein and their breakdown products and free amino acids to establish a scientific basis for improvements in the quality of this variety of cheese.

2. Material and methods

2.1. Cheese making procedure and sampling

Twelve batches of Prato cheese were manufactured using traditional methods on 12 different farms in eight municipalities (Aurea, Barao de Cotegipe, Erechim, Gaurama, Jacutinga, Marcelino Ramos, Severiano de Almeida and Viadutos) in the Alto Uruguai region (Rio Grande do Sul, Brazil). They were made from whole unpasteurised cows' milk without commercial starter cultures, as described by Cichoscki et al. (2002).

For each batch of cheese, samples were made up of six rectangular cheeses taken from the ripening places at different periods of time: 1, 7, 15, 30, 45, and 60 days. The samples were taken to the laboratory under refrigeration (4 °C), where the rind of the cheeses was removed and the cheeses were triturated and stored in hermetic containers at −30 °C until they were analysed. Each cheese sample was made up of one whole cheese. The total number of cheeses used for this study was 72. In all cases, the analyses were carried out at least in duplicate.

2.2. Soluble nitrogenous compounds analysis

The total nitrogen content (TN) was determined by the Kjeldahl method as described in the FIL-IDF 20B (1993) standard. The procedure for preparing the cheese extract was adapted from Vakaleris and Price (1959). Ten grammes grated cheese were homogenized with 40 ml of 0.5 M trisodium citrate and 80 ml of deionised water in a Omni-mixer (Sorvall Inc., Newtown, Conn, USA) at highest speed for 3 min. The homogeneous milky solution was quantitatively transferred, using deionised water, into a 200 ml volumetric flask, cooled to ambient temperature, brought up to volume and then thoroughly mixed. This is referred to as the homogenate.

To estimate the fraction of nitrogen soluble at pH 4.4, a 100 ml aliquot of homogenate was transferred to a 150 ml beaker and the pH adjusted to pH 4.4 with 1.41 M hydrochloric acid. The adjusted solution was made up to 125 ml volume with deionised water and held at 30 °C for 1 h. The solution was filtered through a Whatman no. 3 filter and the clear filtrate was used for subsequent measurements.

A 25 ml aliquot of the homogenate and a 25 ml aliquot of 24% (w/v) TCA solution were transferred into a 100 ml beaker and mixed. The mixture was held at room temperature for 1 h and then filtered through Whatman no. 3 filter paper. The clear filtrate was 12% TCA soluble nitrogen (12% TCA-soluble N).

The pH 4.4-SN and 12% TCA-soluble N were determined using the reaction of nitrogen with Nessler reagent (alkaline solution of K_2HgI_4) with the formation of a red-brown colloidal precipitate which was measured spectrophotometrically at 490 nm.

The method of Ordoñez (1974) was used in the extraction of amoniacal nitrogen (NH_3–N) and aminoacid nitrogen (NH_2–N). Amoniacal nitrogen was determined by the same method of pH 4.4-SN. Aminoacid nitrogen was determined by reaction of free amino groups with ninhydrin, as described by Ordoñez (1974). Protein, casein, peptide and polypeptide nitrogen were calculated as described by Prieto, Franco, González Prieto, Bernardo, and Carballo (2002).

2.3. Electrophoretic analysis

The casein degradation was studied by urea-polyacrylamide gel electrophoresis (PAGE) using the Andrews (1983) procedure. Samples were prepared according to Farkey, Kiely, Allshouse, and Kindstedt

(1991) and gels were stained following the method described by Blakesley and Boezi (1977). The identification and quantification of the casein fractions were achieved using the software package Diversity OneTM 1.0 (Pdi, NY, USA), and previously scanned electrophoresis gels. The optical density of each region was expressed as a percentage of the total optical density.

2.4. Free amino acids analysis

The extraction of free amino acids was carried out as described Fresno, Tornadijo, Carballo, Bernardo, and Gonzalez (1997). The identification and quantification of amino acids by HPLC techniques was according to Alonso, Álvarez, and Zapico (1994) with some minor modifications. The liquid chromatography equipment consisted of a Waters 2690 quaternary pump (Milford, MA, USA) with autosampler, a Spectra-Physics SP 8792 column heater (San José, CA, USA), a Waters 996 Photodiode array UV/Visible detector, and a software package Millenium 32 Chromatography Manager as integrator. The column used was a Symmetry C18 (5 μm particles; 4.6 × 250 mm; Waters, Mildford, MA, USA) reversed phase type. The temperature was controlled at 50 °C.

2.5. Statistical analysis

One-way analysis of variance was carried out to determine the effect of ripening time on the dependent variables (nitrogenous compounds, caseins and free amino acids). A least squares difference (LSD) test was applied for comparison of means using Statistica software, version 5.1 (Statsoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Changes in soluble nitrogenous compounds during the ripening of artisanal Prato cheese

The changes undergone by the average values for the principal soluble nitrogenous components, expressed as % total nitrogen (TN), during ripening of artisanal Prato cheese are shown in Table 1.

The TN content, expressed as % of the cheese, remained practically constant throughout maturation, with no significant differences observed ($P > 0.05$). The TN values obtained after 2 months, at around 4.62±0.62 g/100 g of cheese, were similar to those noted for other cow cheeses.

The contents of protein nitrogen and casein nitrogen, expressed as% TN, underwent a significant decline ($P < 0.05$) over the process of ripening, with their final values being 87.5±4.9 and 78.6±8.4 g/100 g TN, respectively.

The values for pH 4.4 soluble nitrogen (pH 4.4-SN), shown as % TN, after a period of stabilization during the first two weeks of maturation, rose significantly ($P < 0.01$) in the final phase of ripening, reaching levels of 21.4±8.4 g/100 g TN. These figures are quite similar to those noted for other bovine cheeses, such as Comté (Bütikofer, Rüegg, & Ardö, 1993) or Costa Sur (Suárez-Solís, Cardosa, Iñíguez, & Núñez, 1997) and are higher than those recorded for Fontina (Bütikofer et al., 1993), Fromadzo (Gerbi, Zeppa, Turi, Civera, & Chatel, 1997) and Tetilla (Cámara, 2002) cheeses.

The values for 12% TCA-soluble N, expressed as % TN, showed a significant increase ($P < 0.05$) over the whole ripening process, going from initial figures of 2.8±0.6 to 12.5±4.9 g/100 g TN. When the content of 12% TCA-soluble N is considered with respect to pH 4.4-SN, it is possible to see that, at the start of maturation, it stood at 39%, while, after 1 week, this figure had risen to 59%, and from that point remained unchanged until the end of the ripening process.

The % 12% TCA-soluble N/TN, utilized as an indicator of the depth to which proteolysis of a cheese takes place, was of no great importance in artisanal Prato cheese, being less than that noted for Beaufort or Comté (Chamba, Delacroix-Buchet, Berdagué, & Clement, 1994) and Quesucos de Liébana (Prieto, Urdiales, Franco, Fresno, & Carballo, 2000) cheeses and similar only to the figures recorded for Tybo cheese from Argentina (Bertola, Bevilacqua, & Zaritzky, 1992).

The content of NH_3–N, shown as% TN, remained virtually constant over maturation, with final values of 0.6±0.2 g NH_3–N/100 g TN. The low figures noted in these Prato cheese appear to indicate limited deaminase activity, which is normal for this sort of bacterially-ripened cheese.

As for NH_2–N, expressed as % TN, it proved possible to note that it rose significantly ($P < 0.05$) over the whole maturation process, finally reaching average values of 4.3±1.8 g NH_2-N/100 g TN. Likewise, peptide nitrogen, which is constituted by peptides of low molecular weight, showed a significant increase ($P < 0.05$) during the ripening of Prato cheese, with final average figures of 7.6±3.6 g peptide nitrogen/100 g TN.

The NH_2–N content, shown as the percentage of 12%TCA-soluble N, rose throughout ripening, and by the end of the process, stood at around 35%. Nevertheless,% peptide nitrogen/12%TCA-soluble N grew to 73% during the first fortnight of maturation, then dropped progressively, to reach a percentage of around 60% at the end of two months.

The figures obtained for NH_2–N in Prato cheese were higher than those recorded for Quesucos de Liébana (Prieto et al., 2000), Tetilla (Menéndez, Godínez, Centeno, & Rodríguez-Otero, 2001) or Leon cow's milk cheeses (Prieto et al., 2002), but lower than those noted by Suárez-Solís et al. (1997) for Costa Sur cheese.

Table 1
Changes in soluble nitrogenous components during ripening of craft Prato cheese (average values±standard deviation of twelve batches)

	Ripening time (days)					
	1	7	15	30	45	60
TN[a]	3.51±0.38a	3.77±0.22a	4.01±0.36a	4.13±0.36a	4.46±0.45a	4.62±0.62a
pH 4.4-SN[b]	7.23±1.76a	8.78±2.50a	11.5±3.70ab	15.1±5.84bc	17.9±6.43cd	21.4±8.42d
12%TCA-soluble N[b]	2.80±0.59a	5.24±1.21b	6.67±2.41bc	8.36±2.79cd	10.24±3.19de	12.53±4.91e
NH_3-N[b]	0.56±0.16ac	0.45±0.13ab	0.42±0.16b	0.48±0.14ab	0.50±0.16ab	0.64±0.23c
NH_2-N[b]	0.54±0.20a	0.91±0.34ab	1.41±0.47b	2.40±0.93c	3.12±1.17c	4.32±1.75d
Protein N[b]	97.2±0.59a	94.8±1.21b	93.3±2.41bc	91.7±2.79cd	89.8±3.19de	87.6±4.91e
Casein N[b]	92.8±1.76a	91.2±2.50a	88.5±3.70ab	84.9±5.84bc	82.1±6.43cd	78.6±8.42d
Peptide N[b]	1.69±0.67a	3.87±1.32b	4.85±2.35bc	5.48±2.42bcd	6.62±2.68cd	7.78±3.55d
Polypeptide N[b]	4.43±1.47a	3.54±1.72a	4.82±2.99abc	6.73±5.06bcd	7.63±4.51cd	8.89±4.73d

Means within rows without a common letter are significantly different ($P<0.05$).
[a] Expressed as g/100 g of cheese.
[b] Expressed as g/100 g of total nitrogen.

3.2. Changes in casein and breakdown products during the ripening of artisanal Prato cheese

Fig. 1 shows an electrophoretogram characteristic of artisanal Prato cheese over its maturation process and indicates the principal fractions identified. Changes in caseins and their degradation products, expressed as % total optical density, during ripening of Prato cheese, are set out in Table 2.

The breakdown patterns of α_{s1}- and β-caseins were similar in all batches of cheeses and α_{s1}-casein was hydrolysed more extensively (65%) than β-casein (20%) during ripening.

This behaviour was indicative of the fact that breakdown of both fractions in Prato cheese was basically due to the action of the rennet and plasmin. Several authors (Exterkate, Alting, & Slangen, 1995; McSweeney, Olson, Fox, Healy, & Højrup, 1993) have stated that in both "in vitro" studies and in cheese the action of chymosin is directed by preference towards the peptide bond 23-24 of α_{s1}-cn releasing the fragment, $f_{(24\text{-}199)}$, known as α_{s1}-I-cn. This fragment accumulated in noteworthy fashion from the very first moments of ripening of Prato cheese, as did the peptides that arise from it. This behaviour would seem to be related to the pH and salt/moisture (S/M) values in place during maturation of Prato cheese (pH 5.40 and S/M 2–3%) (Cichoscki et al., 2002), facilitating the action of the rennet and of microbial proteases. In Prato cheese, breakdown of α_{s1}-cn and of its degradation products (preα_s-casein) turned out to be much more limited than had been noted in other cow cheeses matured under the same conditions. This fact appears to be related to the limited availability of water in the cheese matrix from the start of ripening as a consequence of the salting of the curd mass, leading to noteworthy changes in the layout of chymosin and/or substrates. Exterkate, Lagerwerf, Haverkamp, and Van Schalkwijk (1997) demonstrated that the effect of solutes was directed fundamentally towards substrates. A small part of the α_{s1}-cn chain has a secondary structure in the β-sheet conformation and with some parts of the spiral random while, in the curd, thanks to environmental conditions, a reorganization occurs of the layout of part of the chain into a folded β-sheet conformation, making certain peptide bonds difficult or inaccesible for the chymosin.

The peptide with mobility slightly greater than β-cn (fraction β-I-cn) underwent no variation during the ripening of Prato cheese. This might be explicable in terms of the aggregation state of this substrate, which would affect access of chymosin to β-I-cn (Creamer, 1976).

In Prato cheese, although the S/M concentration was not very high over the ripening process (around 0.5 M), its uniform distribution from the start of manufacture brought with it a striking competition with β-cn for hydration water. As a consequence of this, hydrophobic interactions were set up in the β-cn chain that determined its aggregation and the masking of several peptide bonds sensitive to the action of chymosin (Exterkate et al., 1997).

Plasmin (with an optimal pH for acting of 7.5) is associated with the casein micelle, depending on the degree of association of pH and the concentration of salt in the medium. Grufferty and Fox (1988) recorded that with pHs below 4.6 and concentrations of salt in milk of 1 M, there was total dissociation of the plasmin from the casein micelle and it was lost in the whey, while at pHs above 4.8 the degree of dissociation was low to nil. Thus, under the pH conditions for Prato cheese, it is logical to assume that the greater part of the plasmin was retained in the curd.

In Prato cheese it might be expected that the action of plasmin would be as noteworthy as that seen in Gouda cheese by other authors (Venema, Herstel, & Elenbaas, 1987), since the two varieties of cheese are very similar. However, the drop in the content of the β-cn fraction

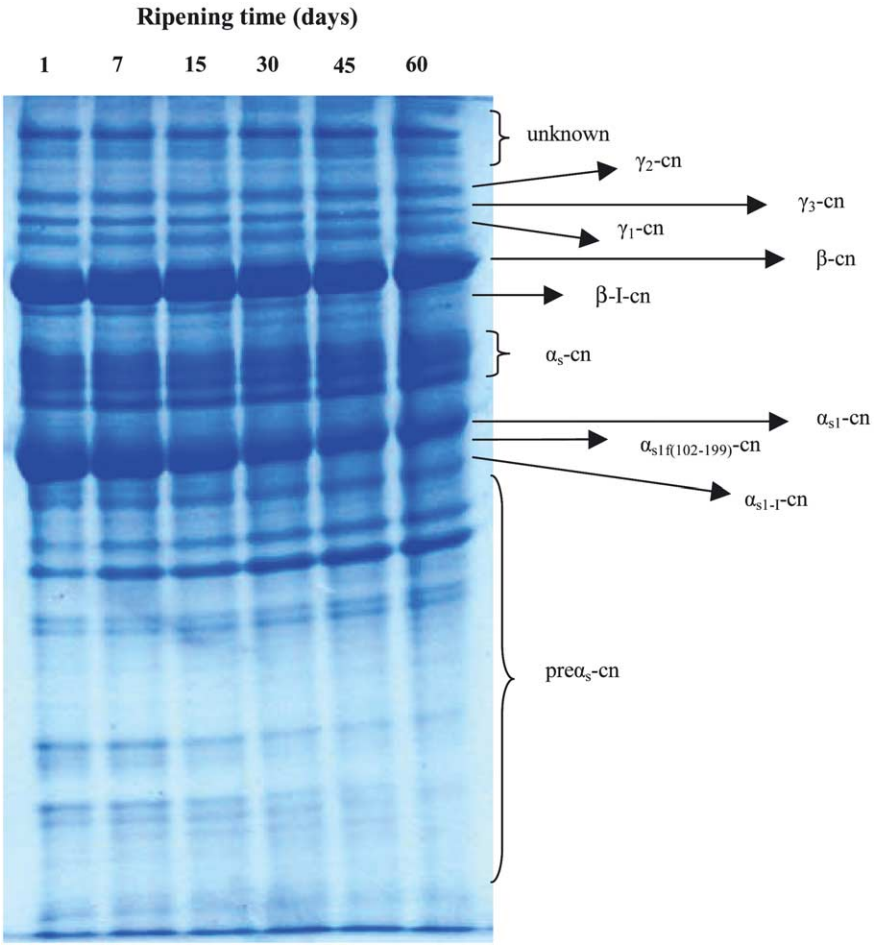

Fig. 1. Electrophoretogram characteristic of craft Prato cheese during ripening.

Table 2
Changes in casein fractions, expressed as% total optical density, throughout ripening of craft Prato cheese (average values ± standard deviation of twelve batches)

	Ripening time (days)					
	1	7	15	30	45	60
Unknown	9.98±3.21a	10.2±3.15a	10.9±2.89a	10.6±2.16a	11.2±3.50a	11.4±4.69a
γ_2-cn	4.27±1.07a	4.69±1.23a	4.94±1.29a	5.12±1.09a	5.24±1.22a	5.18±1.32a
γ_1-cn	2.96±0.68ab	2.80±0.56a	3.07±0.47ab	3.07±0.40ab	3.13±0.43ab	3.30±0.83b
γ_3-cn	2.99±0.59a	3.33±0.65ab	3.60±0.52bc	3.70±0.54bc	3.78±0.69bc	4.10±0.96c
β-cn	18.6±3.20ab	18.4±3.61a	16.8±3.19abc	16.0±2.96abc	15.7±3.50bc	14.9±3.57c
β-I-Cn	1.65±0.60a	1.58±0.47a	1.67±0.63a	1.73±0.43a	1.69±0.31a	1.67±0.41a
α_s-cn	12.6±1.49a	11.9±1.64ab	10.9±1.91bc	11.2±1.34bc	10.8±1.62bc	10.2±1.86c
α_{s1}-cn	18.4±6.02a	16.2±4.81ab	14.2±5.39bc	12.8±4.38bc	12.5±4.76bc	12.1±3.90c
$\alpha_{s1f(102-199)}$-cn	2.06±0.89a	2.29±0.63a	2.16±0.78a	2.31±0.93a	2.00±0.57a	1.85±0.33a
α_{s1}-I-cn	10.5±4.98a	11.1±3.82a	11.7±4.04a	12.0±3.73a	12.3±3.01a	11.5±2.91a
Pre-α_s-cn	16.5±3.84a	17.5±3.71a	20.2±4.50ab	21.6±3.81bc	21.7±4.38bc	23.8±4.51c

Means within rows without a common letter are significantly different ($P < 0.05$).

and the rise in γ-cn fractions were very small when compared to what happens with the α_{s1}-cn fraction, and this shows a behaviour more like that occurring in Cheddar cheese (Farkye & Fox, 1992). Unlike the process for Gouda cheese, curds for Prato cheese are not subjected to washing with hot water during manufacture, so that there is no major loss of plasmin inhibitors or plasminogen activators. Moreover, salting of Prato cheese is not carried out in brine but done in the curd mass, like the procedure for Cheddar cheese, and this implies that, at the start of maturation, there was a value for S/M (2%) close to the optimum for plasmin action. Nevertheless, as ripening progressed, this parameter rose to reach nearly 3.5%, with a consequent reduction in alkaline protease activity on β-cn. Very similar results were described by Kristiansen, Deding, Jensen, Ardö, and Qvist, (1999) when studying the influence of salt content on proteolysis of Danbo-style cheeses.

Finally, the absence of any heat treatment of the milk during the manufacture of Prato cheese would permit plasminogen inhibitors to remain active in the curd and so cause a slowing down in transformation of plasminogen into plasmin (Richardson, 1983).

3.3. Changes in free amino acid content during the ripening of artisanal Prato cheese

The developments in the average values for free amino acids during the maturation of artisanal Prato cheese are shown in Table 3.

The total content of free amino acids grew significantly ($P < 0.05$) during ripening of the Prato cheese, rising from 298 mg/100 g TS at the start of maturation to 2454 mg/100 g TS at the end.

These figures were higher than those given by other authors for bovine cheeses, such as Samsoe, Maribo, Danbo or Havarti (Ismail & Hansen, 1972), León cow's milk cheese (Prieto et al., 2002), Tetilla or Arzúa-Ulloa (Linares, 2002) and were similar to those for Taleggio (Resmini, Saracchi, Volonterio, & Bozzolati, 1969), Montasio (Innoccente, 1997) and Mahón (García-Palmer, Serra, Palou, & Gianotti, 1997) cheeses.

All the batches of Prato cheese studied showed similar behaviour in respect of the changes in their free amino acid contents, during ripening, although noticeable differences were found in the total content from batch to batch. This could be associated with two features: modifications in the technological process of manufacture of the cheese (hygiene levels of the milk used, temperature of milk storage, quantity of rennet added, temperature during whey drainage (38–47 °C), conditions under which the curd mass was pressed, and so on) and changes in ambient conditions during ripening (type of storage, temperature and relative humidity).

The profile for the principal free amino acids in Prato cheese by the end of maturation turned out to be very similar to the state of affairs when ripening started, being constituted of a mix involving taurine/Gaba, glutamic acid, leucine, lysine, tryptophan and phenylalanine, which represented 62% of the total amount of free

Table 3
Changes in free amino acids, expressed as mg/100 g of total solids, during ripening of Prato cheese (average values ± standard deviation of twelve batches)

	Ripening time (days)					
	1	7	15	30	45	60
Asp	15.4±5.14a	22.1±7.60a	38.6±18.8ab	61.1±39.7b	116±58.3c	126±51.1c
Glu	36.5±22.6a	39.7±18.0a	62.3±27.0ab	141±87.7b	282±168c	368±202c
Asn	9.61±5.31a	13.2±8.56ab	21.7±17.2ab	29.0±19.8b	54.2±31.7c	53.6±35.9c
Ser	8.92±2.62a	11.6±4.22a	17.6±7.45b	17.4±5.5b	25.4±4.20c	25.4±7.98c
Gln	10.9±7.18a	14.6±12.0a	29.7±24.6ab	45.8±42.8b	72.0 ±55.5b	70.9±53.3b
Gly	4.18±1.06a	5.23±1.55a	8.68±3.25ab	13.0±4.80b	25.1±8.53c	31.6±13.8d
His	3.39±1.01a	4.40±1.70a	6.15±1.98a	7.28±3.29a	14.0±7.1b	17.1±10.5b
Arg	8.44±4.78a	12.7±8.22ab	19.3±11.9ab	18.6±5.21b	25.9±5.9ab	24.5±4.43ab
Tau/Gaba	36.7±24.6a	80.1±47.7a	155±118a	206±148a	336±239b	406±294b
Thr	8.13±2.68a	9.98±4.02a	18.5±11.3ab	29.6±20.0b	55.2±26.1c	77.9±39.7d
Ala	12.2±4.34a	15.6±5.58a	25.3±9.48ab	40.3±20.9b	64.1±29.5c	89.0±43.2d
Pro	13.1±6.57a	13.2±8.36a	16.5±11.5a	22.9±15.3a	40.9±25.3b	59.4±31.6c
Tyr	5.81±3.64a	7.23±4.33ab	9.60±6.72ab	14.3±12.8bc	19.0±10.3cd	24.6±17.4d
Val	10.0±5.70a	14.2±7.06a	24.8±11.0ab	44.8±24.6b	70.7±31.7c	101.3±44.7d
Met	3.49±1.64a	4.85±2.49a	9.54±4.40ab	18.5±10.6b	32.2±15.2c	48.4±24.6d
Cys	17.0±0.87a	21.0±1.01a	29.2±2.33a	29.0±2.2a	54.5±3.3b	52.5±2.7b
Ile	4.06±2.53a	5.46±3.14a	11.9±7.80ab	22.3±14.5b	37.7±18.7c	61.2±33.9d
Leu	29.8±26.2a	39.1±23.7a	72.8±46.3ab	133±83.0b	212±109c	286±140d
Phe	21.4±17.7a	25.2±13.4a	42.1±22.4ab	63.7±36.3b	98.2±39.0	128±47.5d
Trp	18.6±13.6a	31.4±25.1a	56.6±46.8ab	99.6±62.1b	151±84.4c	167±97.2c
Lys	20.8±10.4a	23.9±12.1a	40.5±20.6ab	76.5±41.4b	147±68.8c	236±130d
TOTAL	298±140a	415±183a	716±355ab	1134±583b	1933±791c	2454±1063d

Means within rows without a common letter are significantly different ($P < 0.05$).

amino acids. Patterns like this have been noted by Laleye, Simard, Gosselin, Lee, and Giroux (1987) for Cheddar cheese and by García-Palmer et al. (1997) for Mahón cheese made with raw milk.

Gamma-aminobutyric acid (γ-aminobutyric acid) is a non-caseinous amino acid originating from the decarboxylation of glutamic acid. The changes in the γ-aminobutyric and glutamic acid content showed them to be virtually equal at the start of maturation of artisanal Prato cheese. However, after a fortnight, the glutamic acid content had increased slightly (1.7 times), while γ-aminobutyric had increased nearly fourfold. During the last phase of ripening, there was an inversion in the behaviour of these two substances, since the glutamic acid content rose 2.6 times and γ-aminobutyric acid went up only twofold. This fact confirms that in the first phase of maturation of Prato cheese there was predominant decarboxylase glutamate activity favoured by the low pH values in the curds, very close to the optimum for its action (Nomura, Kimoto, Someya, Furukawa, & Suzuki, 1998) while, at the end of the ripening period, there appeared to be a predominance of aminopeptidase glutamate activity by the flora present. Resmini et al. (1969) and Laleye et al. (1987) established in their studies of Taleggio and Cheddar cheese, respectively, that cheeses with higher content of γ-aminobutyric acid presented deficient sensorial characteristics. The present study was able to demonstrate that those batches of Prato cheese with the highest levels of γ-aminobutyric acid had a strong and poorly defined flavour, and also a fruity taste, which was indicative of the development of abnormal fermentations during ripening.

The remaining majority free amino acids in Prato cheese (leucine, lysine, phenylalanine and valine), are distributed principally in α_s and β caseins, so that their presence in the cheese is related to the hydrolytic activity of certain peptidases in the natural microbial flora of the cheese, fundamentally lactobacilli, on the fragments released by chymosin and plasmin from both caseins. This fact has been noted by several authors (Lynch, Mcsweeney, Fox, Cogan, & Drinan, 1996; Swearingen, O'Sullivan, & Warthesen, 2001) who found high levels (up to four times greater in some cases) in the glutamic acid, leucine, phenylalanine and valine content of cheeses to which lactobacilli were added jointly with the starter culture.

The proline content of Prato cheese did increase significantly ($P < 0.05$) during ripening, but its final values were lower than those recorded by Innoccente (1997) or García-Palmer et al (1997) for other cow cheeses. The low proline concentrations in Prato cheese might be linked to the limited activity of prolin aminopeptidase, X prolyl dipeptidyl aminopeptidase and prolidase on small peptides or dipeptides released from β-cn, of which they constitute the chief amino acids. The importance of proline lies in its contribution to the aroma of cheeses, as it is formed of dipeptides or tripeptides presenting a bitter taste (Shiraisi, Kazuo, Sato, Yamaoka, & Tuzimura, 1973), while when it is in free form it gives the cheese a mild flavour (Biede & Hammond, 1979).

The asparagine and glutamine contents increased over the first 45 days of ripening, and by the end of the process represented respectively 2.1 and 2.8% of the total free amino acids in Prato cheese. These low figures were also observed by García-Palmer et al. (1997) in Mahón cheese made with raw milk. The lower concentration of asparagine and glutamine in cheeses made with milk not subjected to heat treatment would appear to be related to the activity of asparaginase and glutaminase, two enzymes that are broken down during pasteurization of milk. This fact has led some authors (Bullock & Irvine, 1956) to use the asparagine content of milk as an indicator of its having been pasteurized. These same authors also point out that serine contents were higher in cheeses made with pasteurized milk, owing to the denaturation of the enzymes responsible for its release, while the aspartic acid content was greater in cheeses produced from raw milk. These two facts seem to explain the behaviour noted in the evolution of serine and aspartic acid contents during the ripening of Prato cheese.

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