Available online at www.sciencedirect.com
SCIENCE DIRECT
International Dairy Journal 14 (2004) 445–454
INTERNATIONAL DAIRY JOURNAL
www.elsevier.com/locate/idairyj

Characterisation of soft cheese proteolysis by RP-HPLC analysis of its nitrogenous fractions. Effect of ripening time and sampling zone

R.A. Verdini[a,b], S.E. Zorrilla[a], A.C. Rubiolo[a,*]

[a] *Instituto de Desarrollo Tecnológico para la Industria Química (INTEC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional del Litoral (UNL), Güemes 3450, Santa Fe 3000, República Argentina*

[b] *Departamento de Química Analítica. Facultad de Ciencias Bioquímicas y Farmacéuticas. Universidad Nacional de Rosario (UNR), Suipacha 531, Rosario 2000, República Argentina*

Received 14 April 2003; accepted 13 September 2003

Abstract

Port Salut Argentino cheeses were studied at different ripening times (1, 6, 13, 27 and 56 days) and two sampling zones (central and external). Moisture content, salt concentration, water-insoluble fraction, water-soluble fraction, and free amino acids in the sulphosalicylic acid-soluble fraction were analysed. Moisture and salt contents at the beginning of ripening were higher in the external zone than in the central zone (51 and 46% w/w moisture, 0.95 and 0.25% w/w NaCl, respectively). Cheeses reached a uniform salt content but not a uniform moisture content during the 56 days of ripening. The hydrolysis of α_{s1}-casein during cheese ripening was modelled using first-order kinetics. The kinetics constant of α_{s1}-casein degradation was 0.017 day^{-1}. Most of the water-soluble peptides increased during cheese ripening, particularly from day 13 of ripening. Free amino acids also increased from day 13 of ripening. Amino acids that characterised the ripening of Port Salut Argentino cheese were leucine, lysine, asparagine, phenylalanine, threonine, tyrosine, glutamine and valine. Proteolysis increased in both zones during ripening and there was no appreciable difference between zones using any of the methods used for investigating proteolysis.
© 2003 Elsevier Ltd. All rights reserved.

Keywords: Proteolysis of soft cheese; HPLC; Port Salut Argentino cheese

1. Introduction

The major production of cheese in Argentina is based on soft cheeses, with Port Salut Argentino being one of the most popular varieties (Zalazar, Meinardi, & Hynes, 1999). Port Salut Argentino is a semi-cooked cheese, produced from pasteurised milk, acidified by lactic acid bacteria, coagulated by rennet and/or other specific enzymes and ripened for a short period (Código Alimentario Argentino, 1981).

During cheese ripening, the caseins are broken down by proteolysis. Several proteolytic agents are involved: casein is hydrolysed to large peptides mainly by the coagulant and some indigenous milk enzymes e.g. plasmin and Cathepsin D, large peptides are hydrolysed to small peptides by microbial proteinases and small peptides are hydrolysed to amino acids by microbial peptidases. The extent of this degradation process plays an important role in determining cheese flavour and texture and depends on the activities of rennet and microbial enzymes (Law, 1987). Proteolysis can vary substantially according to cheese variety. Therefore, every type of cheese has its own characteristic proteolytic pattern, resulting from the enzymatic degradation of peptides by various enzymes, and also from amino acid catabolism (Polo, Ramos, & Sanchez, 1985).

Proteolysis has been widely used as a basis for classification of cheese. An obvious difficulty is that cheese is a dynamic system, and therefore results obtained depend mainly on the age of the cheese (Sousa, Ardö, & McSweeney, 2001). However, several studies have been useful to understand the proteolytic patterns for different cheese varieties. The most commonly used method to monitor cheese proteolysis has been fractional precipitation with acids or solvents (Fox, 1989). Thereafter nitrogen compounds were analysed by

*Corresponding author. Tel.: +54-342-455-91-75/6; fax: +54-342-455-09-44.
E-mail address: arubiolo@intec.unl.edu.ar (A.C. Rubiolo).

0958-6946/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.idairyj.2003.09.002

reverse-phase high-performance liquid chromatography (RP-HPLC) to follow proteolysis during cheese maturation and to evaluate different technological treatments (Christensen, Kristiansen, & Madsen, 1989; Gonzalez de Llano, Polo, & Ramos, 1995; Laborda & Rubiolo, 1999). Furthermore, chemometrical analysis was applied to those profiles to simplify data analysis (Furtula, Nakai, Amantea, & Laleye, 1994a,b; Pripp, McSweeney, Sørhaug, & Fox, 2000; Pripp, Stepaniak, & Sørhaug, 2000; Verdini & Rubiolo, 2002a; Verdini, Zorrilla, & Rubiolo, 2002).

Protein breakdown during the ripening of Port Salut Argentino cheese was studied. Rheological parameters were correlated to the formation of water-soluble nitrogen and trichloroacetic-soluble nitrogen, and to the degradation of α_{s1}-casein (Bertola, Bevilacqua, & Zaritzky, 1991; Verdini & Rubiolo, 2002b). However, chromatographic profiles of the different nitrogenous fractions of Port Salut Argentino cheese were not studied.

Initial concentrations of salt and moisture in cheeses salted by immersion are different depending on the zone of the cheese. A redistribution of salt and moisture occurs during cheese ripening due to diffusion until a uniform concentration is reached (Zorrilla & Rubiolo, 1994). As a result, different salt and moisture contents can be observed at different zones during ripening, which may affect protein breakdown (Zorrilla & Rubiolo, 1997). Consequently, chromatographic profiles of the nitrogenous fractions for cheese zones with different moisture and salt contents may be of important significance when uniform attributes are required to satisfy consumer acceptability.

The objective of our work was to evaluate the proteolysis of Port Salut Argentino cheese by analysing the chromatographic profiles of the nitrogenous fractions at different ripening times and sampling zones.

2. Materials and methods

2.1. Cheese samples

Commercial Port Salut Argentino cheeses (3.55 ± 0.11 kg weight, 23.2 ± 0.3 cm diameter, 7.7 ± 0.3 cm height, $28.7 \pm 0.7\%$ w/w fat, $20.4 \pm 0.9\%$ w/w total protein, $48.8 \pm 2.6\%$ w/w moisture, and 5.2 ± 0.1 pH) were manufactured at a local factory. Cheeses were salted in a brine solution for 3 h at 3°C, stored for 20 h, and packed in heat-shrinkable plastic.

Thirty cheeses were transported in insulated boxes with ice from the factory to our laboratory and randomly separated into two groups. Fifteen cheeses were held at 5°C for ripening. Three cheeses were sampled at different ripening times: 1, 6, 13, 27 and 56 days. Cubic pieces of 25 mm were cut as described by Creamer and Olson (1982) from two different cheese zones, the central zone (C) and external zone (E), as described by Verdini and Rubiolo (2002a).

2.2. Moisture and chloride content

Moisture contents were measured using a microwave oven CEM AVC 80 (CEM, Mattheus, NC, USA). Chloride concentration was determined with an Automatic Titrator model DL40RC (Mettler Instrumente AG, Greifensee, Switzerland) as described by Fox (1963).

2.3. Fractionation of nitrogenous compounds

Fig. 1 shows the fractionation process. Grated cheese (10 g) mixed with three times the sample weight of water was homogenised using an Ultra-Turrax® T25 (IKA® Werke, Janke & Kunkel GmbH & Co KG, Staufen, Germany) homogeniser for 2 min (Kuchroo & Fox, 1982). The homogenate was held at 40°C for 1 h, pH was adjusted to 4.4–4.6, and the suspension was centrifuged for 30 min at 5°C and 4800 rpm (Biofuge 28RS; Heraeus Sepatech, Osterode, Germany). After centrifugation, three layers were obtained and the upper layer of fat was removed.

The precipitate was dissolved in 7 M urea. The resulting solution was dialysed against water for 48 h using Cellu Sep 5000 MWCO membranes (Membrane Filtration, Inc., San Antonio, TX, USA), lyophilised (Heto Lab Equipment, Allerød, Denmark) and stored in a freezer at −22°C for RP-HPLC analysis, constituting the water-insoluble fraction.

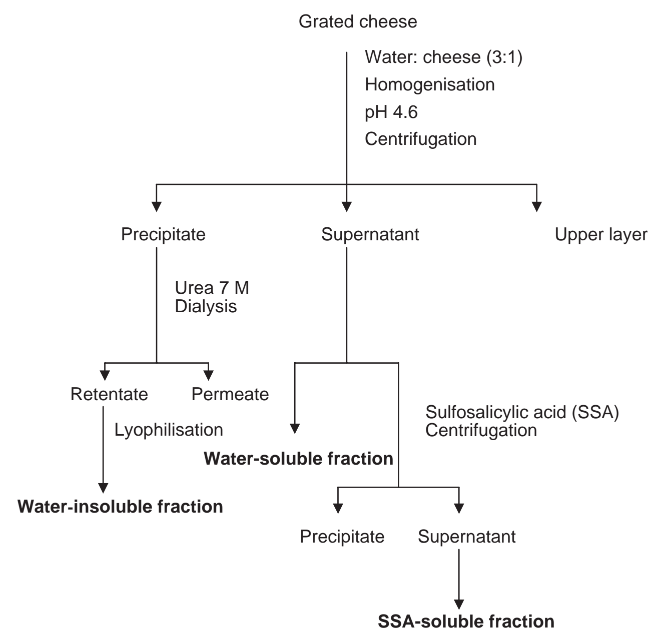

Fig. 1. Scheme of the separation of the nitrogenous fractions.

The supernatant was filtered through Whatman Number 42 paper and diluted to 100 mL. An aliquot of that solution was stored in a freezer at −22°C for RP-HPLC analysis, constituting the water-soluble fraction.

Ten mL of the water-soluble fraction were added to 2 mL of 15% w/v sulphosalicylic acid (SSA) to reach a final concentration of 2.5% w/v SSA (Reiter, Sorokin, Pickering, & Hall, 1969). The suspension was centrifuged for 30 min at 20°C and 4800 rpm (Biofuge 28RS; Heraeus Sepatech). The supernatant solution was adjusted to pH 4.0 and stored in a freezer at −22°C for RP-HPLC analysis, constituting the SSA-soluble fraction.

2.4. Analysis of the water-insoluble fraction

Approximately 20 mg of the lyophilised powder was dissolved in 0.01 M imidazole (pH 7), 0.01 M dithioerytritol and 6.6 M urea (Christensen et al., 1989). The solution was mixed and filtered through a disposable 0.2 μm filter (Alltech Associates, Inc., Deerfield, IL, USA) before 100 μL was injected into the HPLC system. An HPLC system with a gradient programmer model 2360 (Isco, Inc., Lincoln, NE, USA), a V$^{4®}$ variable wavelength absorbency detector and a SynChropak RPP (250 × 4.6 mm) C_{18}, 300 Å column (SynChrom, Inc., Lafayette, IN, USA) at 30°C were used for chromatographic separations. Detection was at 220 nm.

Gradient elution was used with solvent A: 0.1% trifluoroacetic acid (TFA) in water and solvent B: 0.1% TFA in acetonitrile. The gradient programme was: initial composition 0% B, isocratic step at 0% B for 5 min, linear step to 25% B in 5 min, linear step to 35% B for 30 min, linear step to 50% B in 10 min, isocratic step at 50% B for 10 min. The flow rate was 1.0 mL min^{-1}. All the reactants used were of HPLC grade. A chromatogram of a cheese casein extract is shown in Fig. 2a. The α_{s1}-casein peak was identified using a standard of α_s-casein (Sigma-Aldrich, St. Louis, MO, USA).

2.5. Analysis of the water-soluble fraction

The water-soluble fraction was filtered through a disposable 0.2 μm filter before 100 μL was injected into the HPLC system. A SynChropak RPP (250 × 4.6 mm) C_{18}, 300 Å column (SynChrom, Inc., Lafayette, IN, USA) at 30°C was used for chromatographic separations. Detection was at 220 nm.

Gradient elution was used with solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. The gradient programme was: initial composition 0% B, isocratic step at 0% B for 5 min, linear step to 50% B in 30 min, isocratic step at 50% B for 5 min. The flow rate was 1.0 mL min^{-1}. All the reactants used were of HPLC grade. A chromatogram of a water-soluble fraction is shown in Fig. 2b.

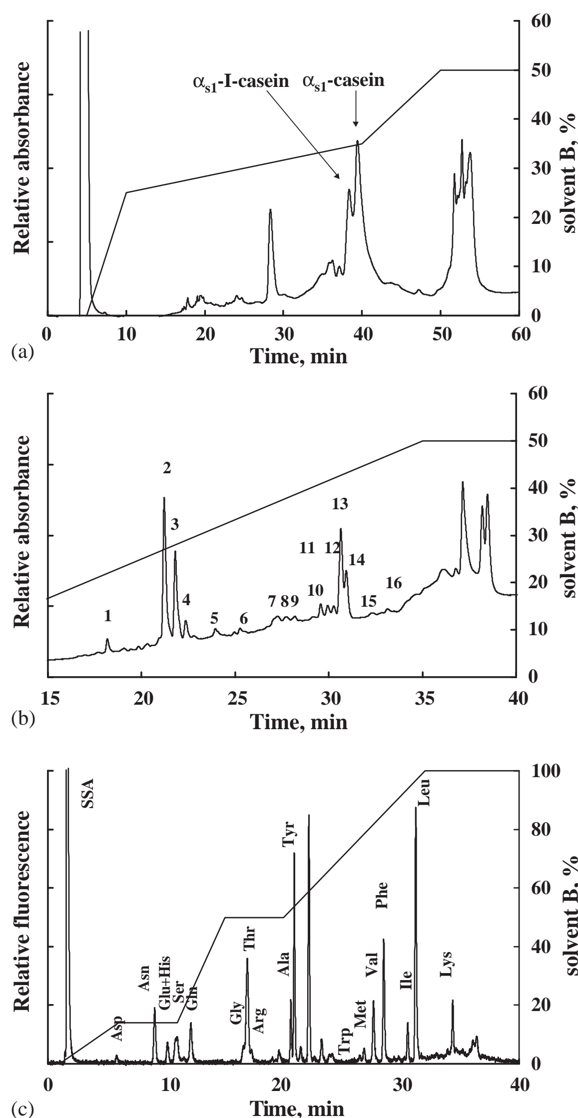

Fig. 2. Chromatograms of the nitrogenous fractions of a cheese sample: (a) water-insoluble fraction, (b) water-soluble fraction (peaks which were recognised and used as variables in the multivariate statistical analysis are indicated by numbers), (c) free amino acids in the SSA-soluble fraction.

2.6. Analysis of the SSA-soluble fraction

Free amino acids were determined in the SSA-soluble fraction using the derivatising procedure with *o*-phthaldialdehyde (OPA) as described by Verdini et al. (2002). The resulting solution was filtered through a disposable 0.2 μm filter before 10 μL was injected into the HPLC system. An Ultrasphere ODS (250 × 4.6 mm, particle size 5 μm) C_{18} column (Beckman Instruments, Inc., Fullerton, CA, USA) at 30°C was used for chromatographic separations. A fluorescence detector model FL-2

(Isco, Inc.) with the following fluorometer settings was used: excitation filter in the 305–395 nm range, emission filter in the 430–470 nm range, time constant of 0.5 s, and a sensitivity of 0.02 units.

Gradient elution was used for OPA derivative separation with solvent A: tetrahydrofuran: methanol: 0.05 M sodium acetate, pH 5.9 (1:19:80), and solvent B: methanol: 0.05 M sodium acetate, pH 5.9 (80:20) (Jones, Pääbo, & Stein, 1981). The gradient programme was: initial composition 0% B, isocratic step at 0% B for 1 min, linear step to 14% B in 5 min, isocratic step at 14% B for 5 min, linear step to 50% B in 5 min, isocratic step at 50% B for 4 min, linear step to 100% B in 12 min, isocratic step at 100% B for 8 min. The flow rate was 1.7 mL min^{-1}. All the reactants used were of HPLC grade. A typical chromatogram of SSA-soluble fraction is shown in Fig. 2c. Amino acids were identified according to their retention times by comparison with a standard solution chromatogram, while amino acid concentrations were obtained through validation parameters as described by Verdini et al. (2002).

2.7. Statistical analysis

Data were analysed using ANOVA with Minitab 13.20 (Minitab Inc., State College, PA, USA). When differences between treatment effects were significant ($p<0.05$), a multiple comparison of means was performed using the Tukey's test. Slopes of the fitted curves were compared using the method proposed by Green and Margerison (1978). Principal component analysis (PCA) was used to reduce the dimensionality of the data obtained from water-soluble and free amino acid profiles. Principal component analysis was applied to the mean centred data matrix as described by Verdini and Rubiolo (2002a).

3. Results and discussion

3.1. Moisture and salt contents

Moisture content during cheese ripening is shown in Fig. 3a. The moisture content at the beginning of the ripening was higher in the external zone (E) than in the central zone (C) (51% and 46%, respectively). The different moisture content between zones E and C at the beginning of ripening (1 day) could be attributed to the fact that cheeses did not reach the moisture equilibrium before packaging (Verdini & Rubiolo, 2002b). Despite the moisture gradient, cheeses did not reach uniform moisture content during the 56 days of ripening. In agreement, Bertola, Bevilacqua, Califano, and Zaritzky (1999) reported that moisture content did not change during ripening for Port Salut, Tybo and Mozzarella cheeses packaged in plastic films.

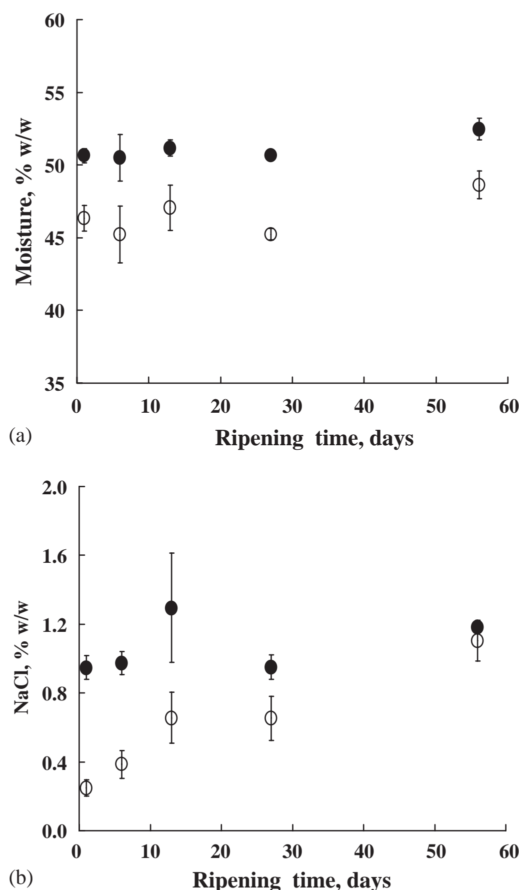

Fig. 3. Moisture and NaCl contents during Port Salut Argentino cheese ripening: (○) central zone, (●) external zone; (a) moisture content, (b) NaCl content. Bars are based on standard deviations.

Sodium chloride content during cheese ripening is shown in Fig. 3b. Salt concentration was significantly lower in zone C than in zone E until 13 days of ripening. In zone C, salt concentration increased significantly from 1 to 13 days of ripening due to NaCl diffusion. It can be considered that a uniform salt concentration was reached in both zones at 27 days of ripening because there were no significant differences in salt concentrations between 27 and 56 days of ripening.

3.2. Analysis of the water-insoluble fraction

During cheese ripening, the caseins are broken down by proteolysis. In Port Salut Argentino cheese, the α_{s1}-casein degradation by the residual coagulant is expected to be the most important event of casein breakdown. The hydrolysis of α_{s1}-casein by chymosin yields two fragments: a large water-insoluble fragment, α_{s1}-CN (f24–199) known as α_{s1}-I-casein, and a small water-soluble fragment, α_{s1}-CN (f1-23) (Fox & McSweeney, 1996).

The α_{s1}- and α_{s1}-I-casein during ripening of Port Salut Argentino cheese were studied through the water-

insoluble fraction. The α_{s1}-casein peak was identified using a standard of α_s-casein and the α_{s1}-I-casein peak was assigned by comparison of its retention time with those reported in the literature for similar experimental conditions (Hynes, 1998) and according to its behaviour during cheese ripening (Fig. 2a). Peak areas of α_{s1}- and α_{s1}-I-casein per 100 g of cheese are shown in Fig. 4a and b, respectively.

A first-order kinetics model was assumed to represent the primary hydrolysis of α_{s1}-casein as proposed by Zorrilla and Rubiolo (1997) for Fynbo cheese:

$$\alpha_{s1}(\theta) = \alpha_{s1}(0)e^{-K_\alpha \theta}, \quad (1)$$

where $\alpha_{s1}(\theta)$ is the RP-HPLC peak area of α_{s1}-casein $100\,g^{-1}$ cheese changing with ripening time (θ), $\alpha_{s1}(0)$ is the initial value of α_{s1}, and K_α is the kinetics constant. Kinetics constants for α_{s1}-casein hydrolysis during cheese ripening were 0.0179 day^{-1} for zone C and 0.0174 day^{-1} for zone E, showing no significant differences between sampling zones. Additionally, ANOVA showed that there were no significant differences between zones C and E.

The rate of α_{s1}-casein hydrolysis depends on ionic strength, pH, temperature, and enzyme-substrate ratio. Therefore, the rate of α_{s1}-casein hydrolysis may differ from one cheese variety to another depending on the manufacturing and ripening conditions. Moreover, considering the inhibitory effect of a higher salt concentration, differences between sampling zones may be expected in cheeses salted by immersion. Zorrilla and Rubiolo (1997) reported lower values of the kinetics constant for the external zone than the central zone for Fynbo cheese ripened at 12°C. Sihufe, Zorrilla, and Rubiolo (2003) reported lower values of the kinetics constant for the external zone than the central zone for Fynbo cheese ripened at 5°C, 12°C and 16°C. In Port Salut Argentino cheese, although a significant salt concentration difference between sampling zones was found during the first stage of ripening, no significant differences between the kinetics constants for zones C and E were observed.

The fragment of α_{s1}-I-casein undergoes further degradation either by the coagulant or by other proteinases and peptidases of the starter bacteria (McSweeney, Olson, Fox, Healy, & Højrup, 1993). In this study, the content of α_{s1}-I-casein increased significantly between 13 and 56 days of ripening, and no significant differences due to sampling zone were observed during the studied ripening period.

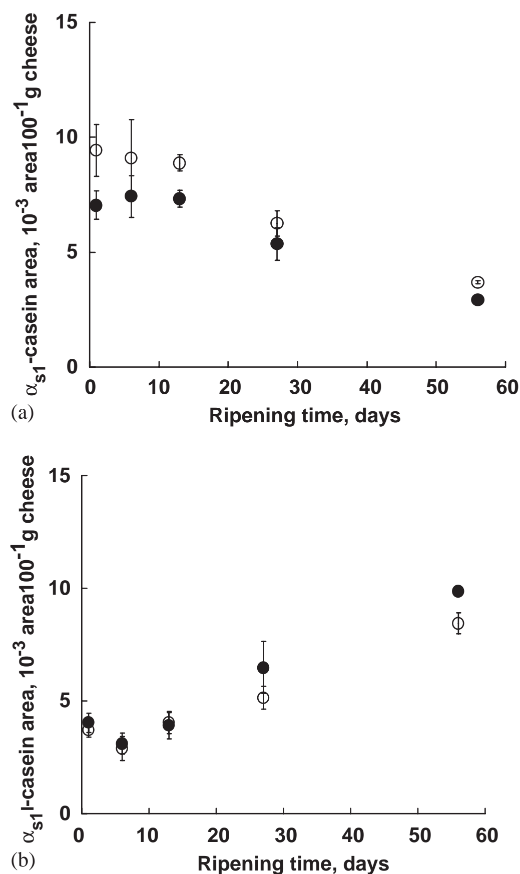

Fig. 4. Areas of α_s-caseins during Port Salut Argentino cheese ripening: (○) central zone, (●) external zone; (a) α_{s1}-casein, (b) α_{s1}-I-casein. Bars are based on standard deviations.

3.3. Analysis of the water-soluble fraction

Maturation indices of Port Salut Argentino cheeses (determined as the percentage of water-soluble nitrogen of the total cheese nitrogen) increased throughout ripening, but there were no significant differences between zones (Verdini & Rubiolo, 2002b). Maturation index measures the water-soluble nitrogen content; however, this index does not consider the hydrolysis of the water-soluble peptides into other water-soluble peptides that still remain in the water-soluble fraction.

To analyse the evolution of the water-soluble peptides, 16 peaks were selected out of the water-soluble fraction chromatograms of Port Salut Argentino cheese samples (Fig. 2b) and peak areas per 100 g of cheese were obtained. Some hydrophobic peaks at the end of the chromatograms coeluted with the serum proteins α-lactalbumin and β-lactoglobulin A and B, consequently they were not included in our analysis. PCA was applied to visualise the distribution of cheese samples according to ripening time and sampling zone, and to identify the peaks that characterised Port Salut Argentino cheese ripening. The 30 samples and 16 mean centred variables yielded two principal components (PC) that explained 96.2% of the data set variation (PC1 91.8% and PC2 4.4%).

The PC1 and PC2 scores plot, representing the cheese samples in the bidimensional PC space is shown in Fig. 5a. The PC1 outlined three groups, a compact group from 1 to 13 days, a group corresponding to 27 days and

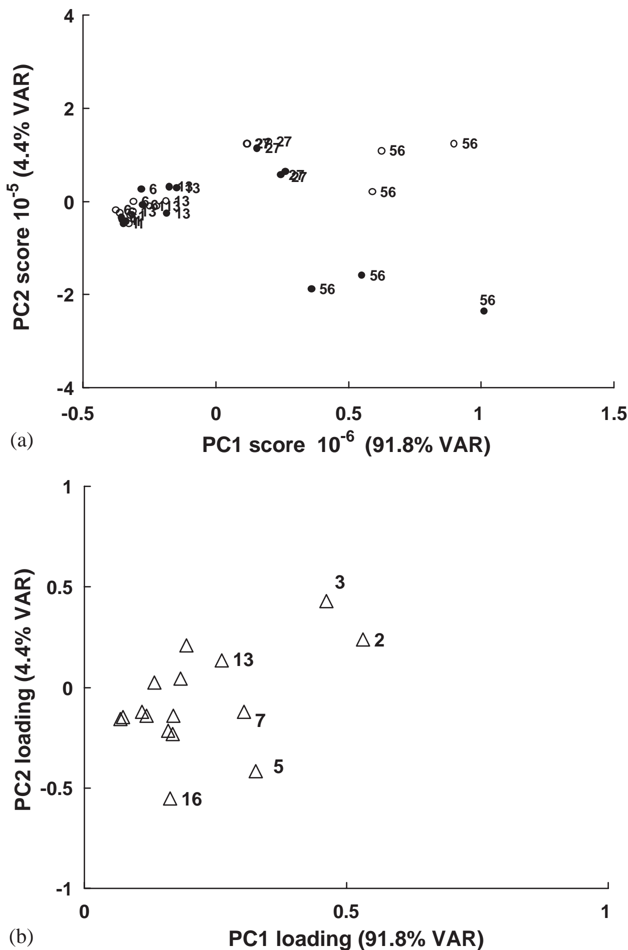

Fig. 5. Plots of the first two principal components when data for the water-soluble fraction of Port Salut Argentino cheeses were analysed: (○) central zone, (●) external zone; (a) scores plot (numbers indicate the ripening time of the samples), (b) loadings plot (numbers correspond to peaks shown in Fig. 2b).

a scattered group corresponding to 56 days. The PC1 and PC2 loadings plot, representing the peptides of the water-soluble fraction in the bidimensional PC space, is shown in Fig. 5b. Peaks with higher PC1 loading were 2, 3, 5, 7 and 13, while those with higher values of PC2 loadings were: 16, 3, and 5 (labelled in Fig. 5b). All PC1 loadings were positive while PC2 loadings were positive and negative.

Fig. 6 shows the area profiles of some selected peaks according to their PC loadings. Peaks with higher PC1 loadings increased their areas during ripening, as occurred with the majority of the peaks (Fig. 6). Consequently, the first PC may be related to the increase in concentration of water-soluble peptides as ripening time increases.

Peaks with higher PC2 loading values showed differences in their rate of formation according to their PC2 loading sign. Areas of peaks with positive values of PC2 loadings (2, 3 and 13) increased with a higher rate between 13 and 27 days (Fig. 6a–c), while areas of peaks with negative values of PC2 loadings (peaks 5, 7 and 16) increased with a higher rate between 27 and 56 days (Fig. 6d–f). Out of the selected peaks, only the area of peak 16 at 56 days of ripening showed differences due to sampling zones. Therefore, the second PC may be related to some differences observed on the rate of peptide formation.

McSweeney et al. (1993) studied the hydrolysis of α_{s1}-casein by chymosin in solution, isolated and identified the produced peptides and proposed cleavage sites and hydrolysis pathways. These authors reported a retention time of 30 min for the fragment α_{s1}-CN (f1–23), and that the area of the corresponding peak increased initially and remained constant in solution at pH 6.5 and increased to a maximum and diminished in solution at pH 5.2. Laborda (2000) studied RP-HPLC profiles of the water-soluble fraction of Fynbo cheese ripened at 12°C and reported that the fragment α_{s1}-CN (f1–23) may be associated to a particular RP-HPLC peak considering its retention time and the behaviour described by McSweeney et al. (1993) in solution at pH 5.2. Therefore, taking into account similar considerations, peak 13 of the RP-HPLC of Port Salut Argentino cheeses was assigned to the fragment α_{s1}-CN (f1–23). The area of peak 13 increased during ripening (Fig. 6f) showing a higher formation rate between 13 and 27 days. The temperature at which Port Salut Argentino cheeses were ripened may explain the absence of a maximum. At 5°C, the rate of the enzymatic reactions is lower than at a higher temperature. On the other hand, no significant differences due to sampling zone were observed in the area of the fragment α_{s1}-CN (f1–23), in agreement with the observations for the water-insoluble fragments (α_{s1}- and α_{s1}-I-casein).

3.4. Analysis of the SSA-soluble fraction

Fourteen amino acids were analysed in the SSA-soluble fraction of Port Salut Argentino cheese samples (Fig. 2c) and concentration was expressed as mg of amino acid per 100 g cheese. Principal component analysis was also applied to visualise the distribution of cheese samples according to ripening time and sampling zone, and to identify the amino acids that characterised Port Salut Argentino cheese ripening. The 30 samples and 14 mean centred variables yielded 2 PC that explained 97.8% of the data set variation (PC1 90.2% and PC2 7.6%).

The PC1 and PC2 scores plot is shown in Fig. 7a. The PC1 outlined two major groups, a compact group corresponding to unripened cheeses (from 1 to 13 days) and a disperse group corresponding to ripened cheeses (27 and 56 days). The PC2 contribute to the dispersion towards the upper corner of Fig. 7a of the samples corresponding to 27 days.

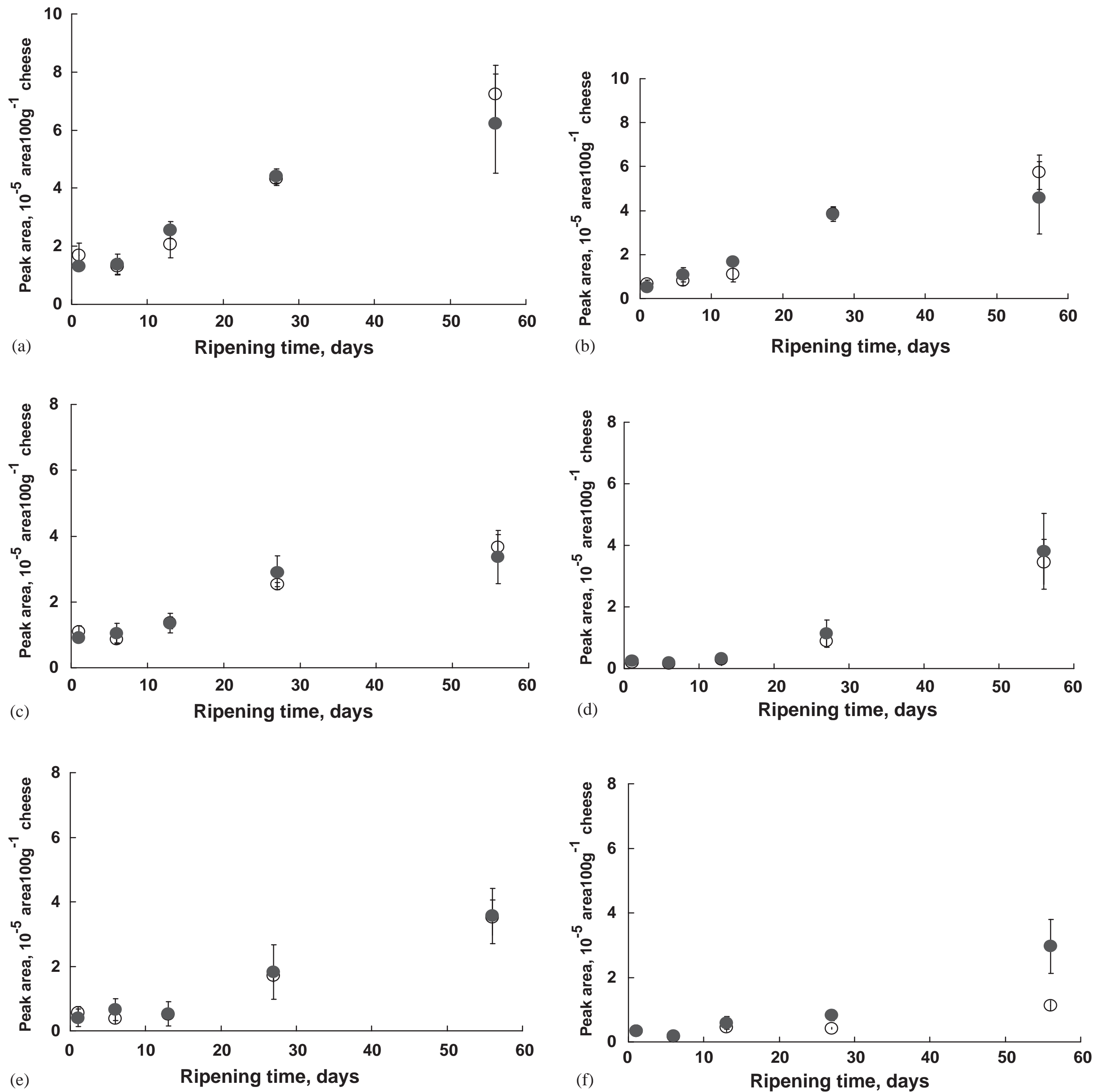

Fig. 6. Areas of peaks of the water-soluble fraction during Port Salut Argentino cheese ripening: (○) central zone, (●) external zone; (a) peak 2, (b) peak 3, (c) peak 13, (d) peak 5, (e) peak 7, (f) peak 16. Bars are based on standard deviations.

The PC1 and PC2 loadings plot is shown in Fig. 7b. Amino acids with higher PC loadings that characterised the ripening of Port Salut Argentino cheese were leucine, lysine, asparagine, phenylalanine, threonine, tyrosine, glutamine and valine (labelled peaks in Fig. 7b). Some authors detected the same amino acids during early ripening of cheeses. Fresh Mahon cheese (10 days of ripening) had high values of leucine, phenylalanine and valine (Frau, Massanet, Roselló, Simal, & Cañelas, 1997). The major free amino acids found in young Feta cheese were leucine and valine (Katsiari, Alinchanidis, Voutsinas, & Roussis, 2000).

All PC1 loadings were positive while most PC2 loadings were negative except for lysine, tyrosine and glutamine. Fig. 8 shows the area profiles of some selected amino acids according to their PC loadings for further discussion.

Amino acids with higher PC1 loadings (leucine, asparagine and lysine) increased their areas during ripening, as occurred with the majority of the amino

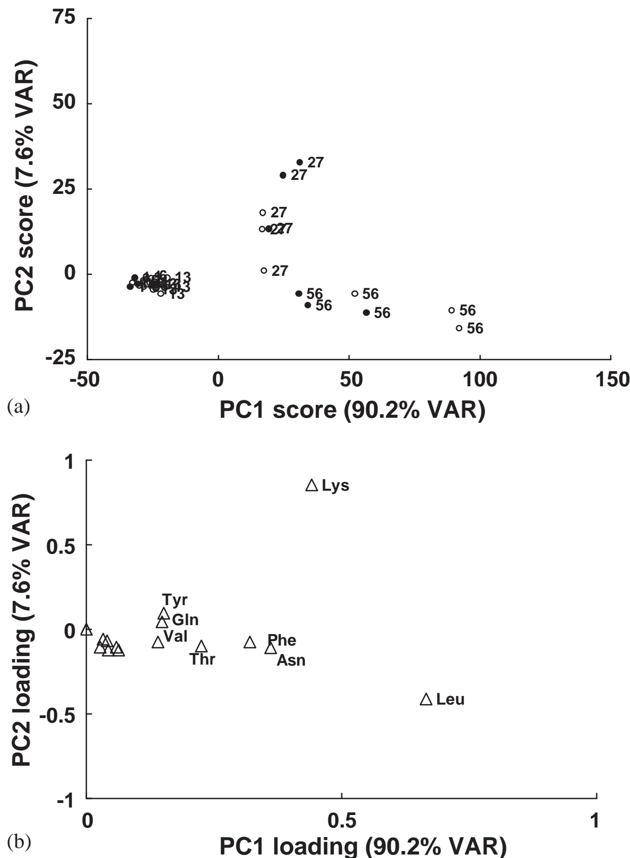

Fig. 7. Plots of the first two principal components when data for the free amino acids in the SSA-soluble fraction of Port Salut Argentino cheeses were analysed: (○) central zone, (●) external zone; (a) scores plot (numbers indicate the ripening time of the samples), (b) loadings plot.

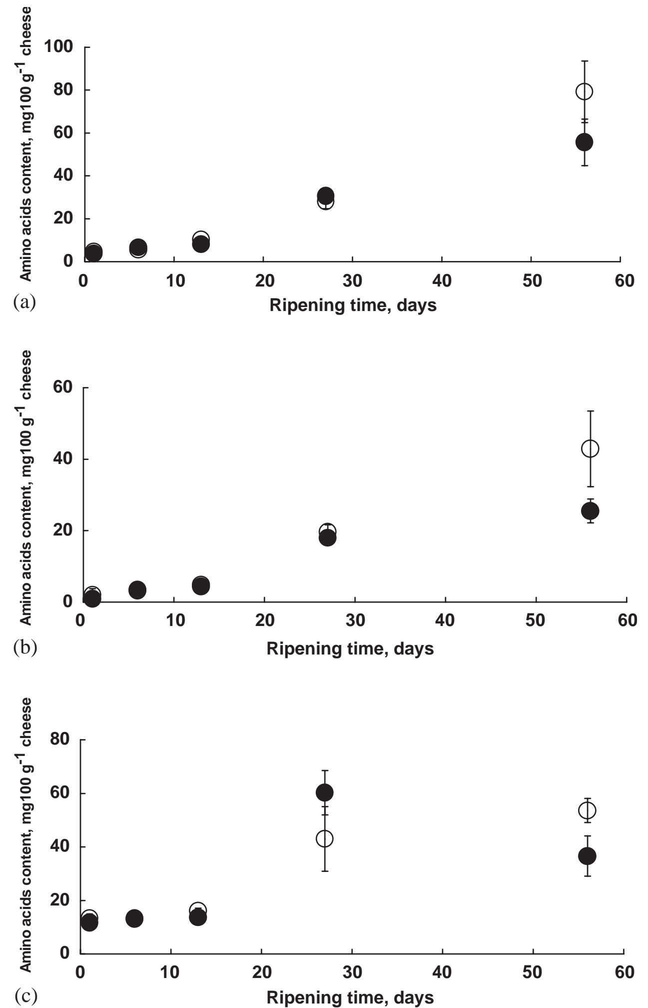

Fig. 8. Free amino acid contents in the SSA-soluble fraction during Port Salut Argentino cheese ripening: (○) central zone, (●) external zone; (a) leucine, (b) asparagine, (c) lysine. Bars are based on standard deviations.

acids (Fig. 8a–c). Consequently, the first PC may be related to the increase in concentration of free amino acids as ripening time increases. Amino acids with higher PC2 loading values showed differences in their rates of formation according to their PC2 loading sign. Leucine and asparagine, which had negative values of PC2 loading, increased their concentration between 13 and 56 days (Fig. 8a and b), while lysine, which had a positive value of PC2 loading, increased its concentration between 13 and 27 days and reached equilibrium between 27 and 56 days (Fig. 8c). Out of the selected amino acids, leucine and asparagine at 56 days of ripening showed differences due to sampling zone. Therefore, the second PC may be related to some differences observed on the rate of amino acid formation.

Casein hydrolysis by rennet occurs during the first stage of cheese ripening. Large peptides are hydrolysed to small peptides by microbial proteinases and small peptides are hydrolysed to amino acids by microbial peptidases (Sousa et al., 2001). Then, amino acids situated in the terminal position or next to the points of hydrolysis are more exposed to bacterial peptidase activity. Results obtained from model cheeses in which the production of amino acids from limited rennet-digested casein showed the production of amino acids: leucine, arginine and phenylalanine followed by glutamic acid, valine, lysine, tyrosine and isoleucine (Exterkate, Lagerwerf, Haverkamp, & Van Schalkwijk, 1997). In this study, some of the amino acids produced are related to the amino acids reported by Exterkate et al. (1997).

4. Conclusions

Cheeses reached a uniform salt content but not a uniform moisture content during the 56 days of ripening. The water-insoluble fraction analysis showed the hydrolysis of α_{s1}-casein and the subsequent formation of α_{s1}-I-casein during cheese ripening. The hydrolysis of α_{s1}-casein was modelled using first-order

kinetics, and showed no significant differences due to sampling zone. Most of the water-soluble peptides increased during cheese ripening, particularly from 13 days of ripening. The chromatographic area of the fragment α_{s1}-CN (f1–23) increased during ripening, and no significant differences due to sampling zone were observed, in agreement with the observations for α_{s1}- and α_{s1}-I-casein. Free amino acid content also increased from 13 days of ripening and no differences due to sampling zone were observed except for 56 days of ripening. Amino acids that characterised the ripening of Port Salut Argentino cheese were leucine, lysine, asparagine, phenylalanine, threonine, tyrosine, glutamine and valine. Proteolysis increased in both zones during ripening and there was no appreciable difference between zones using any of the methods used for investigating proteolysis.

Acknowledgements

The authors acknowledge SanCor Cooperativas Unidas Ltd. for the supply of cheeses. This work was done with the financial support of Universidad Nacional del Litoral (Santa Fe, Argentina), Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina), and Agencia Nacional de Promoción Científica y Tecnológica (Argentina).

References

Bertola, N., Bevilacqua, A., Califano, A., & Zaritzky, N. (1999). *Vinculación entre el comportamiento reológico y los cambios microestructurales en quesos madurados en películas plásticas.* VIII Congreso Argentino de Ciencia y Tecnología de Alimentos, Rafaela, Santa Fe, Argentina.

Bertola, N. C., Bevilacqua, A. E., & Zaritzky, N. E. (1991). Changes in rheological and viscoelastic properties and protein breakdown during the ripening of Port Salut Argentino cheese. *International Journal of Food Science and Technology*, *26*, 467–478.

CAA. (1981). In J. De La Canal (Ed.), *Código Alimentario Argentino* (pp. 107–108). Buenos Aires, Argentina: De La Canal & Asociados SRL.

Christensen, T. M. I. E., Kristiansen, K. R., & Madsen, J. S. (1989). Proteolysis in cheese investigated by high performance liquid chromatography. *Journal of Dairy Research*, *56*, 823–828.

Creamer, L. K., & Olson, N. F. (1982). Rheological evaluation of maturing Cheddar cheese. *Journal of Food Science*, *47*, 631–636, 646.

Exterkate, F. A., Lagerwerf, F. M., Haverkamp, J., & Van Schalkwijk, S. (1997). The selectivity of chymosin action on α_{s1}- and β-caseins in solution is modulated in cheese. *International Dairy Journal*, *7*, 47–54.

Fox, P. F. (1963). Potentiometric determination of salt in cheese. *Journal of Dairy Science*, *46*, 744–745.

Fox, P. F. (1989). Proteolysis during cheese manufacture and ripening. *Journal of Dairy Science*, *72*, 1379–1400.

Fox, P. F., & McSweeney, P. L. H. (1996). Proteolysis in cheese during ripening. *Food Review International*, *12*, 457–509.

Frau, M., Massanet, J., Roselló, C., Simal, S., & Cañelas, J. (1997). Evolution of free amino acid content during ripening of Mahon cheese. *Food Chemistry*, *60*, 651–657.

Furtula, V., Nakai, S., Amantea, G. F., & Laleye, L. (1994a). Reverse-phase HPLC analysis of cheese samples aged by a fast-ripening process. *Journal of Food Science*, 59, 528–532, 567.

Furtula, V., Nakai, S., Amantea, G. F., & Laleye, L. (1994b). Reverse-phase HPLC analysis of reference Cheddar samples for assessing accelerated cheese ripening. *Journal of Food Science*, *59*, 533–538.

González de Llano, D., Polo, M. C., & Ramos, M. (1995). Study of proteolysis in artisanal cheeses: high-performance liquid chromatography of peptides. *Journal of Food Science*, *78*, 1018–1024.

Green, J. R., & Margerison, D. (1978). Statistical treatment of experimental data. Amsterdam, Holland: Elsevier.

Hynes, E. R. (1998). *Estudio de la proteólisis durante la maduración de quesos blandos. Cremoso Argentino*. D.Phil. thesis, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe, Argentina.

Jones, B. N., Pääbo, S., & Stein, S. (1981). Amino acid analysis and enzimatic sequence determination of peptides by an improved o-phtaldialdehyde precolumn labeling procedure. *Journal of Liquid Chromatography*, *4*, 565–586.

Katsiari, M. C., Alinchanidis, E., Voutsinas, L. P., & Roussis, I. G. (2000). Proteolysis in reduced sodium Feta cheese made by partial substitution of NaCl by KCl. *International Dairy Journal*, *10*, 635–646.

Kuchroo, C. N., & Fox, P. F. (1982). Soluble nitrogen in Cheddar cheese: Comparison of extraction procedures. *Milchwissenschaft*, *37*, 331–335.

Laborda, M. A. (2000). *Maduración de quesos con bajo contenido de cloruro de sodio*. D.Phil. thesis, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe, Argentina.

Laborda, M. A., & Rubiolo, A. C. (1999). Proteolysis of Fynbo cheese salted with NaCl/KCl and ripened at two temperatures. *Journal of Food Science*, *64*, 33–36.

Law, B. A. (1987). Proteolysis in relation to normal and accelerated cheese ripening. In P. F. Fox (Ed.), *Cheese: Chemistry, physics and microbiology, Vol. 1, general aspects* (pp. 365–392). London, New York: Elsevier.

McSweeney, P. L. H., Olson, N. F., Fox, P. F., Healy, A., & Højrup, P. (1993). Proteolytic specificity of chymosin on bovine α_{s1}-casein. *Journal Dairy Research*, *60*, 401–412.

Polo, C., Ramos, M., & Sanchez, R. (1985). Free amino acids by high-performance liquid chromatography and peptides by gel electrophoresis in Mahon cheese during ripening. *Food Chemistry*, *16*, 85–96.

Pripp, A. H., McSweeney, P., Sørhaug, T., & Fox, P. F. (2000). Quantitative contribution of rennet and bacterial enzymes to the primary proteolysis in sodium caseinate solution. *Milchwissenschaft*, *55*, 263–266.

Pripp, A. H., Stepaniak, L., & Sørhaug, T. (2000). Chemometrical analysis of proteolytic profiles during cheese ripening. *International Dairy Journal*, *10*, 249–253.

Reiter, B., Sorokin, Y., Pickering, A., & Hall, A. J. (1969). Hydrolysis of fat and protein in small cheeses made under aseptic conditions. *Journal of Dairy Research*, *36*, 65–76.

Sihufe, G. A., Zorrilla, S. E., & Rubiolo, A. C. (2003). Casein degradation of Fynbo cheese salted with NaCl/KCl brine and ripened at various temperatures. *Journal of Food Science*, *68*, 117–123.

Sousa, M. J., Ardö, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, *11*, 327–345.

Verdini, R. A., & Rubiolo, A. C. (2002a). Effect of frozen storage time on the proteolysis of soft cheeses studied by principal component

analysis of proteolytic profiles. *Journal of Food Science*, *67*, 963–967.

Verdini, R. A., & Rubiolo, A. C. (2002b). Texture changes during the ripening of Port Salut Argentino cheese in 2 sampling zones. *Journal of Food Science*, *67*, 1808–1813.

Verdini, R. A., Zorrilla, S. E., & Rubiolo, A. C. (2002). Free amino acid profiles during ripening of Port Salut Argentino cheese after frozen storage. *Journal of Food Science*, *67*, 3264–3270.

Zalazar, C., Meinardi, C., & Hynes, E. (1999). Quesos típicos argentinos. Santa Fe, Argentina: Centro de Publicaciones Universidad Nacional del Litoral.

Zorrilla, S. E., & Rubiolo, A. C. (1994). Fynbo cheese NaCl and KCl changes during ripening. *Journal of Food Science*, *59*, 972–975.

Zorrilla, S. E., & Rubiolo, A. C. (1997). Kinetics of casein degradation during ripening of Fynbo cheese salted with NaCl/KCl brine. *Journal of Food Science*, *62*, 386–389.