J. Dairy Sci. 90:2103–2112
doi:10.3168/jds.2006-795

Proteolysis in Mozzarella Cheeses Manufactured by Different Industrial Processes

L. Costabel,* M. S. Pauletti,† and E. Hynes‡[1]
*Instituto Nacional de Tecnología Agropecuaria (INTA), Rafaela, Argentina
†Instituto de Tecnología de Alimentos, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe, Argentina
‡Instituto de Lactología Industrial, Facultad de Ingeniería Química, Universidad Nacional del Litoral. Santiago del Estero 2829, Santa Fe, Argentina

ABSTRACT

The objective of the present study was to investigate the influence of stretching temperature, fat content, and time of brining on proteolysis during ripening of Mozzarella cheeses. Seventeen cheese-making experiments (batches) were carried out on an industrial scale on successive days, following the standard procedure with some modifications. Fat content of cheese milk, temperature at the stretching step, and time of brining varied from one batch to another as required by the experimental design, outlined by a surface response model. Proteolysis was assessed during ripening of samples, which was prolonged for at least 3 mo, by means of electrophoresis, nitrogen fractions, and soluble peptide mapping. The amount of soluble nitrogen at pH 4.6 was not significantly different in cheeses obtained by diverse procedures, but it increased during ripening of all samples. This result was coincident with the breakdown of α_{s1}- and β-caseins evidenced by electrophoresis, which reached similar extents at late stages of ripening, regardless of the cheese-making process. Multivariate analysis on soluble peptide profiles obtained by liquid chromatography also detected sample grouping according to ripening time, but did not evidence any separation caused by the cheese-making technology. We concluded that the changes in the cheese-making process assayed in this work were insufficient to produce significant differences in proteolysis of the cheeses. Ripening time had more influence on proteolysis of Mozzarella cheeses than any other assayed variable.
Key words: Mozzarella cheese, proteolysis, cheese making, cheese ripening

INTRODUCTION

The chemical composition and biochemical events that occur during ripening of Mozzarella cheese determine its final quality and acceptance, because they have an effect on the functional properties of this cheese variety, which is consumed worldwide. Dry matter, fat content, Ca content, pH evolution during cheese making, and residual levels of lactose and galactose, among others, have been identified as factors affecting Mozzarella cheese texture and functional properties (Barbano et al., 1993; Rudan et al., 1999; Gunasekaran and Ak, 2003; Joshi et al., 2003a,b; Everett et al., 2004; Sheenan and Guinee, 2004; McMahon et al., 2005). Proteolysis of the caseins that constitute the cheese matrix has been signaled as a source of changes in Mozzarella cheese properties, especially when it is used as a food ingredient (Rudan et al., 1999; Feeney et al., 2002; Kindstedt, 2003).

In this context, most cheese makers would like to know how to combine milk and technology to obtain a customized product for food manufacturers that use Mozzarella cheese as an ingredient. Whereas one customer may need an easy-to-chip, nonbrowning Mozzarella cheese for pizzas, other may want a high-browning, strong-flavored Mozzarella cheese for snacks. Many features of Mozzarella cheese making and ripening processes have been recently elucidated (Bertola et al., 1996; Feeney et al., 2001; Guinee et al., 2001; Kuo et al., 2001), which allows targeting toward a desired final product. However, the effect of other factors has not been well established yet. Proteolysis during cheese ripening, for example, is a well-known group of biochemical reactions for several cheese varieties, most notably Cheddar, but it remains less known in pasta filata cheeses, such as Mozzarella (Johnson and Law, 1999; Feeney et al., 2002).

There are different points of view about the impact of proteolysis on the textural and functional properties of Mozzarella cheese. In cheese, casein molecules form a net that holds fat and water, and it is generally accepted that proteolysis produces the softening of cheese body and changes its functional properties (Fox et al., 2000a; Sousa et al., 2001; Guinee, 2003). However, recent evidence showed that chemical composition and pH are at

Received November 29, 2006.
Accepted January 29, 2007.
[1]Corresponding author: ehynes@fiqus.unl.edu.ar

least as important for cheese texture and functionality as proteolysis (McMahon et al., 2005). In addition, pasta filata cheeses represent a special case, because casein molecules are arranged distinctly after the stretching step that takes place during cheese making (Kindstedt et al., 1999, 2003; Fox et al., 2000b).

On the other hand, the release of amino groups as a consequence of proteolysis has been reported as a requisite to ensure browning via the Maillard reaction when the cheese is heated, regardless of the amount of residual sugars present in the cheese (Oberg, 1991; Barbano et al., 1993; Kindstedt et al., 1999). However, browning has also been related to relatively high contents of residual lactose or galactose (Hutkins, 1993; Mukherjee and Hutkins, 1994; Kindstedt et al., 1999).

Furthermore, the relative contribution of each proteolytic agent involved in Mozzarella cheese ripening is still discussed. Although some authors believe that residual milk-clotting enzyme makes little or no contribution to casein breakdown in cooked cheeses (Kindstedt et al., 1995; Gaiaschi et al., 2000; Sousa et al., 2001), others report that its role may be significant (Hayes et al., 2002; Hynes et al., 2004a,b). Starter bacteria proteolytic and peptidolytic activities have been identified as key factors in proteolysis during Mozzarella cheese ripening, as well as indigenous enzymes such as plasmin (Barbano et al., 1993; Hutkins, 1993; Feeney et al., 2002). In fact, the cheese-making process, both directly and indirectly, determines the relative importance of each proteolytic agent during ripening: it can inactivate enzymes or their inhibitors, and provide a more or less favorable environment for their action (Hynes et al., 2004a).

The objective of the present work was to investigate the effect of different Mozzarella cheese-making processes on proteolysis during ripening. For that purpose, Mozzarella cheeses were manufactured in a dairy plant by the standard process, and by modifying milk fat content, temperature during the stretching step, and time of brining.

MATERIALS AND METHODS

Experimental Design and Mozzarella Cheese Making

Cheeses were manufactured on an industrial scale. After cheese making, samples for this study were taken and the rest of each batch was ripened and sold. Consequently, each studied parameter varied within a relatively narrow rank, compatible with technological possibilities in the factory and reasonable quality standards for the final product. The studied variables were content of fat in the cheese-making milk, temperature at the stretching step during manufacture, and time of brining. These parameters were selected by the decision makers of the dairy industry where the study was conducted, as a first approach in the identification of technological strategies for managing Mozzarella cheese final characteristics. Other variables such as pH, which are more difficult to control during an industrial manufacture, will be approached in further studies.

Table 1. Experience plan consisting of 17 cheese-making experiments according to the surface response model, with the option central composite design

	Condition[1]		
Experiment	Fat, %	ST, °C	Time, h
1	2.9	83	18
2	2.4	78	14
3	3.4	78	14
4	2.4	88	14
5	3.4	88	14
6	2.4	78	22
7	3.4	78	22
8	2.4	88	22
9	2.9	83	18
10	3.4	88	22
11	2.06	83	18
12	3.74	83	18
13	2.9	74.6	18
14	2.9	91.4	18
15	2.9	83	11.27
16	2.9	83	24.73
17	2.9	83	18

[1]Fat = content of fat in the cheese-making milk; ST = temperature at the stretching step during manufacture; Time = time of brining.

The experimental design was outlined using a surface response model, with the option central composite design (Gacula and Singh, 1984; Massart et al., 1988), which provided an experience plan consisting of 17 cheese-making experiments, performed on consecutive days (Table 1). Cheese making was carried out following the standard Mozzarella cheese-making technology in the dairy plant of Sucesores de Alfredo Williner S.A. (Bella Italia, Santa Fe, Argentina), introducing changes when appropriate.

Raw milk was HTST-pasteurized, then cooled to 37 to 39°C. Fat content was adjusted to the target values fixed by the experimental design by the addition of pasteurized skim milk. Cheese making was performed in two 10,000-L closed vats provided with automated variable-speed cutting and stirring devices (Bauducco, El Trébol, Santa Fe, Argentina). Starter consisted of selected strains of *Streptococcus thermophilus* (Sacco, Cadorago, Italia), which were inoculated into cheese milk to obtain 10^9 cfu/mL. Milk clotting enzyme was pure chymosin (Chy-Max, Chr. Hansen, Copenhagen, Denmark, 500 International Milk Clotting Units/mL); it was added to the milk immediately after starter at concentration of 0.04 mL/L of milk. The coagulum was cut when it has strengthened enough to withstand cutting, which took 15 to 20 min and was assessed subjec-

tively by the cheese maker. After cutting, the mixture of curd particles and whey was continually stirred and heated up to 42°C, at a rate of 1°C/5 min, then held at this temperature for 30 to 35 min. When the pH of the curd decreased to 6.0 to 6.2, the mixture of curd grains and whey was pumped to finishing vats, where the whey was partially drained. Once pH decreased to 5.5, the remaining whey was drained and curd was cheddared in the finishing vat until a pH of 5.2 was reached. At this point, the curd was milled and stretched in a Mozzarella cooker-stretcher (Equitec, Monte Vera, Santa Fe, Argentina). The temperature of the water in the stretcher varied according to the experimental design. The molten plastic curd was conveyed to a molding unit (Equitec), where it was formed into a parallelepiped-shaped mass, which was then cut into 4-kg blocks. The blocks were placed in molds for 1 h to acquire the proper shape, then taken out and cooled in cold water (10°C) for 1 h. Finally, they were brined in a saturated NaCl solution at 4°C for a time that varied according to the experimental design. After removal from the brine, cheeses were drip dried, vacuum wrapped, and stored at 4°C.

Cheese Composition

Dry matter, salt content, and protein content were analyzed according to International Dairy Federation standards (IDF, 1972, 1982, 1993), and fat and pH by American Public Health Association (APHA) methods (Bradley et al., 1992) on 5-d-old cheeses.

Proteolysis Assessment

Proteolysis was assessed on 3-, 49- and 91-d-old cheeses, by determination of soluble nitrogen at pH 4.6. Electrophoresis and reverse phase (**RP**)-HPLC were also performed. Some of the Mozzarella cheeses, which remained in ripening room up to 131 d, were analyzed as well.

Soluble Nitrogen. Mozzarella cheese samples were treated to obtain crude citrate extract and soluble fraction at pH 4.6, according to Hynes et al. (2001). The crude cheese extract was obtained by adding 20 mL of 0.5 *M* sodium citrate to 10 g of cheese and grinding to homogeneity using a pestle. Deionized water was added to ~90 mL and the pH was adjusted to 4.6. After centrifugation (3,000 × *g* for 15 min), the soluble fraction volume was adjusted to 100 mL. The nitrogen content was determined in duplicate by the macro-Kjeldahl method according to the IDF standard (1993). Soluble nitrogen content was expressed as a percentage of the total nitrogen (**%SN**).

Electrophoresis. The insoluble residue at pH 4.6 was analyzed by urea-PAGE in a Mini-Protean II cube (BioRad Laboratories, Hercules, CA) by the Andrews method (Andrews, 1983), with a concentration of acrylamide of 7.5% (Hynes et al., 1999). Proteins were stained with Coomassie Blue G-250. Samples of cheese casein were prepared by precipitation at pH 4.6 and purified.

RP-HPLC. The HPLC equipment consisted of a quaternary pump, an online degasser, and a UV/visible detector, (all Series 200, Perkin Elmer, Norwalk, CT). An interface module connected to a computer was used for acquisition of chromatographic data with the software Turbochrom (Perkin Elmer). A 250 mm × 4.6 mm Aquapore OD-300 C18, 5 nm–300 Å analytical column was used (Perkin Elmer). Water-soluble extracts of the cheeses were obtained by blending 5 g of cheese and 15 mL of distilled water with a mortar and pestle, then warmed up to 40°C, and maintained for 1 h. The suspension was centrifuged at 3,000 × *g* for 15 min and filtered through fast-flow filter paper. The filtered solution was adjusted to a final volume of 25 mL. Samples were filtered through 0.45-μm membranes (Millex, Millipore, São Paulo, Brazil) and 60 μL was injected into the HPLC chromatograph. Detection was performed at 214 nm and column temperature was 40°C. The gradient, starting from 100% of solvent A (H_2O:trifluoroacetic acid (**TFA**), 1,000:1.1 vol/vol) and 0% of solvent B (acetonitrile:H_2O:TFA 600:400:1 vol/vol/vol), was generated 10 min after injection. The proportion of solvent B was increased at 1%/min (80 min); 20%/min (1 min); 0%/min (4 min) and then returned to starting conditions, which took 1 min. These last conditions were maintained for 10 min.

Statistical Analysis

Cheese-making experimental design was performed with Statgraphics Plus 3.0 (Manugistics Inc., Rockville, MD), applying a surface response model with the option central composite design (Gacula and Singh, 1984; Massart et al., 1988). Soluble nitrogen results were analyzed by ANOVA and multiple ranks test using the same tool.

Chromatographic data were analyzed by multivariate methods, including a fuzzy approach for data preprocessing for RP-HPLC chromatograms (Piraino et al., 2004). The objective of this method was to preprocess chromatographic profiles objectively, without visual matching and subjective peak selection on the chromatograms, and to obtain a new reduced data set. Input in this preprocessing technique were peak heights and retention times, and the output were classes of retention time wherein peak heights were cumulated using the distance from the center of class as a weight (Piraino et al., 2004). These data were then analyzed by principal

Table 2. Chemical composition of Mozzarella cheeses

Experiment	Fat, %	pH	Protein, %	NaCl, %
1	23.0[a]	5.48	23.52	1.80[bc]
2	21.5[b]	5.45	24.77	1.58[ab]
3	26.0[c]	5.48	23.69	1.49[ab]
4	23.0[b]	5.48	26.91	1.60[ab]
5	27.0[c]	5.38	22.82	1.73[ab]
6	21.5[b]	5.41	24.44	2.22[d]
7	27.5[c]	5.41	22.98	2.05[d]
8	22.0[b]	5.40	25.85	2.19[d]
9	24.0[a]	5.43	25.38	1.70[bc]
10	28.0[c]	5.42	23.03	2.05[d]
11	21.5[b]	5.43	29.53	1.55[bc]
12	28.5[c]	5.51	22.78	1.98[bc]
13	25.0[a]	5.39	24.29	1.96[bc]
14	24.5[a]	5.51	24.47	2.19[bc]
15	24.0[a]	5.56	24.65	1.31[a]
16	24.0[a]	5.53	24.67	2.31[d]
17	24.5[a]	5.51	24.11	1.58[bc]

[a–d]Means within same column with no common superscripts are different ($\alpha < 0.01$).

Table 3. Soluble N values (%) of experimental cheeses at different stages of ripening

	Days of ripening		
Sample	3	49	91
E1	3.15[a]	5.71[b]	7.60[c]
E2	3.50[a]	5.72[b]	7.25[c]
E3	3.75[a]	6.17[b]	8.27[c]
E4	2.99[a]	3.84[b]	5.16[c]
E5	3.34[a]	4.73[b]	6.04[c]
E6	3.57[a]	4.57[b]	6.09[c]
E7	3.32[a]	4.82[b]	6.24[c]
E8	3.15[a]	4.92[b]	6.67[c]
E9	4.06[a]	4.33[b]	6.69[c]
E10	2.68[a]	4.14[b]	5.83[c]
E11	2.80[a]	4.58[b]	6.08[c]
E12	3.58[a]	5.24[b]	7.29[c]
E13	4.89[a]	5.12[b]	6.35[c]
E14	3.26[a]	4.41[b]	6.05[c]
E15	2.87[a]	4.53[b]	6.41[c]
E16	3.31[a]	4.32[b]	6.44[c]
E17	2.86[a]	5.19[b]	6.66[c]

[a–c]Means within a row with no common superscripts are different ($\alpha < 0.01$).

component analysis (**PCA**) using the covariance matrix (Hair et al., 1999; Pripp et al., 2000). Nonhierarchical cluster analysis (k-means) was performed after PCA, using principal components as variables for the clustering. Multivariate statistical analyses were performed using the SSPS 10.0 software package (SPSS Inc., Chicago, IL).

RESULTS

Chemical composition of Mozzarella cheeses is presented in Table 2. As expected, the fat content of the cheeses showed significant differences ($\alpha < 0.01$). However, although fat content had been adjusted to 5 different values in the cheese milk, only 3 homogeneous groups of cheeses were defined based on fat content. This was probably because small differences in fat content of the milk were evened out during cheese making by the influence of other technological parameters in the fat retention in the curd. The cheese samples showed greater variation in salt concentration. Four homogeneous groups were defined, and notably the cheeses brined for 14 or 18 h were not statistically different ($\alpha > 0.05$). Moisture content of cheese samples, despite the changes in fat matter and the differences in temperature during the cooking step, was not significantly different ($\alpha > 0.05$).

The ANOVA of %SN results did not show significant differences among statistical treatments applied during Mozzarella cheese making. However, %SN of cheeses at different stages of ripening did differ significantly (Table 3), which indicates that ripening time influenced proteolysis more than the other studied variables. Values of %SN for the central points of the experimental design (E1, E9, and E17) are presented in Figure 1. Proteolysis evolved similarly in the 3 samples, showing a moderate increase of %SN.

The %SN values of 4 pairs of cheeses, manufactured at the same stretching temperature and brined for 14 or 22 h are shown in Figure 2. In general, the samples with higher fat content showed slightly greater %SN values, except for E8 and E10. However, the differences were not significant ($\alpha > 0.05$).

Samples E13 and E14 were star points, which represent the extreme values of the rank studied for each variable, of the experimental design, and during the stretching step, were heated at 74.6 and 91.4°C, respectively. They showed very similar %SN values during

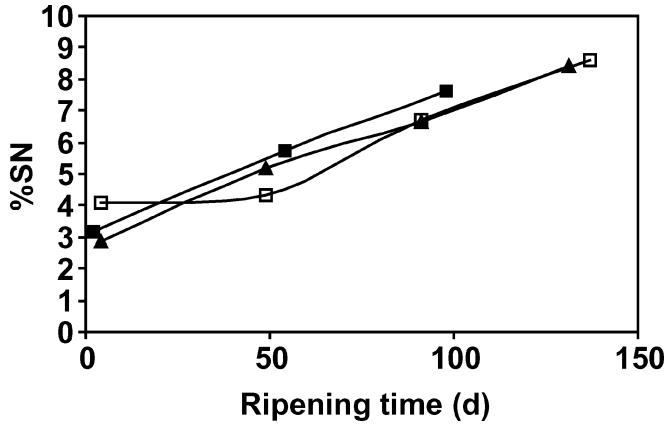

Figure 1. Evolution of soluble nitrogen values (%SN) for the central points (E1, E9, and E17) of the experimental design during ripening time at 4°C. Fat matter in the cheese milk was 2.9%, stretching temperature was 83°C, and brining time was 18 h. ■ = E1, □ = E9; ▲ = E17.

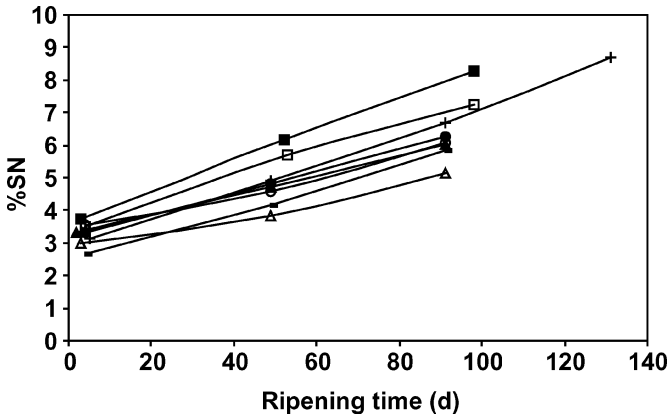

Figure 2. Evolution of soluble nitrogen values (%SN) during ripening at 4°C of 4 pairs of cheeses stretched at 78 or 88°C and brined for 14 or 22 h. E2 (■) and E3 (□): stretching temperature 78°C, 14 h of brining; E4 (△) and E5 (▲): stretching temperature 88°C, 14 h of brining; E6 (○) and E7 (●): stretching temperature 78°C, 22 h of brining; and E8 (+) and E10 (–): stretching temperature 88°C, 22 h of brining.

ripening. The same observation was also valid for the other star point samples: E15 and E16 (brined for 11.27 and 24.73 h, respectively) and E11 and E12 (2.06 and 3.74% of fat matter in the cheese-making milk, respectively; Figure 3).

Electrophoresis showed that all samples were very similar at the beginning of ripening, when only the bands of intact α_{s1}-, β-, and α_{s2}-CN were detected (Figure 4a). After 49 d of ripening, the hydrolysis of α_{s1}-CN was evidenced by the appearance of the band of the peptide α_{s1}(f24-199). The band was more intense in profiles of samples E2 and E3; that is, cheeses in which the stretching step was carried out at 78°C. It was also detected in samples E1, E6, E7, E11, and E13, but was not visible in the electrophoretograms of the cheeses obtained using high stretching temperatures (88 and 91.4°C; Figure 4b). At the end of the ripening (91 d) the peptide α_{s1}(f24-199) was detected in all the samples, but the band was more intense in those Mozzarella cheeses for which the stretching step was performed below 83°C, such as E1, E2, E3, E6, and E13. Samples E2 and E3 showed the highest degradation of α_{s1}-CN; these cheeses had been brined for 14 h. However, the intensity of the bands of α_{s1}-CN and α_{s1}(f24-199), compared visually, was quite similar for all the samples after 91 d of ripening. Samples E13 and E14, in which the stretching step was performed at 74.6 and 91.4°C, respectively, showed little difference, although the band of intact α_{s1}-CN was more intense in the E14 profile (Figure 4c). The bands corresponding to γ-CN also increased during ripening, indicating the hydrolysis of β-CN by plasmin. The other preferential substrate of plasmin, α_{s2}-CN, also disappeared during ripening.

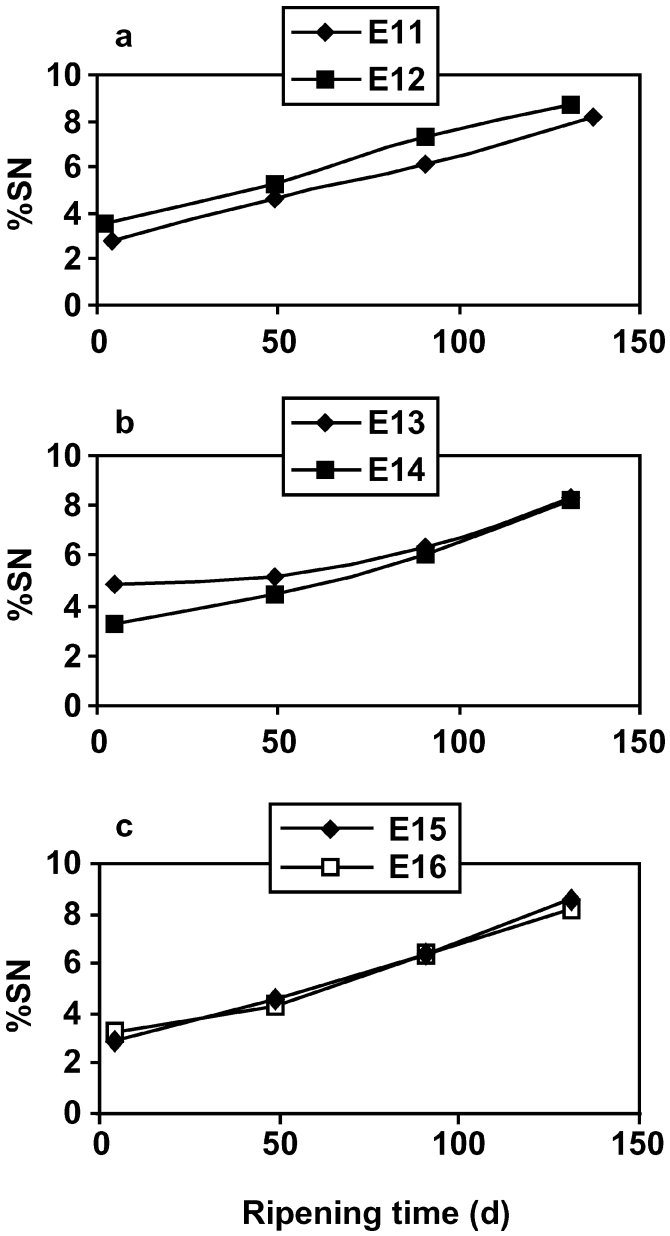

Figure 3. Evolution of soluble nitrogen values (%SN) for the star points of the experimental design during ripening time at 4°C. a) Samples with 2.06 (E11) and 3.74% (E12) of fat content in the cheese-making milk; b) samples stretched at 74.6 (E13) and 91.4°C (E14); c) samples brined for 11.27 (E15) and 24.73 h (E16), respectively.

No differences were detected among the different samples for the hydrolysis of β- and α_{s2}-caseins (Figure 4a, b, and c). Electrophoretic patterns of the 131-d-old samples were very similar (Figure 4d).

Preprocessed peptide profiles were analyzed by PCA, using the covariance matrix. The first 6 PC, which explained 93.84% of the initial variation, were retained based on the criterion of Eigenvalue ≥1 ($\lambda \geq 1$). Nonhier-

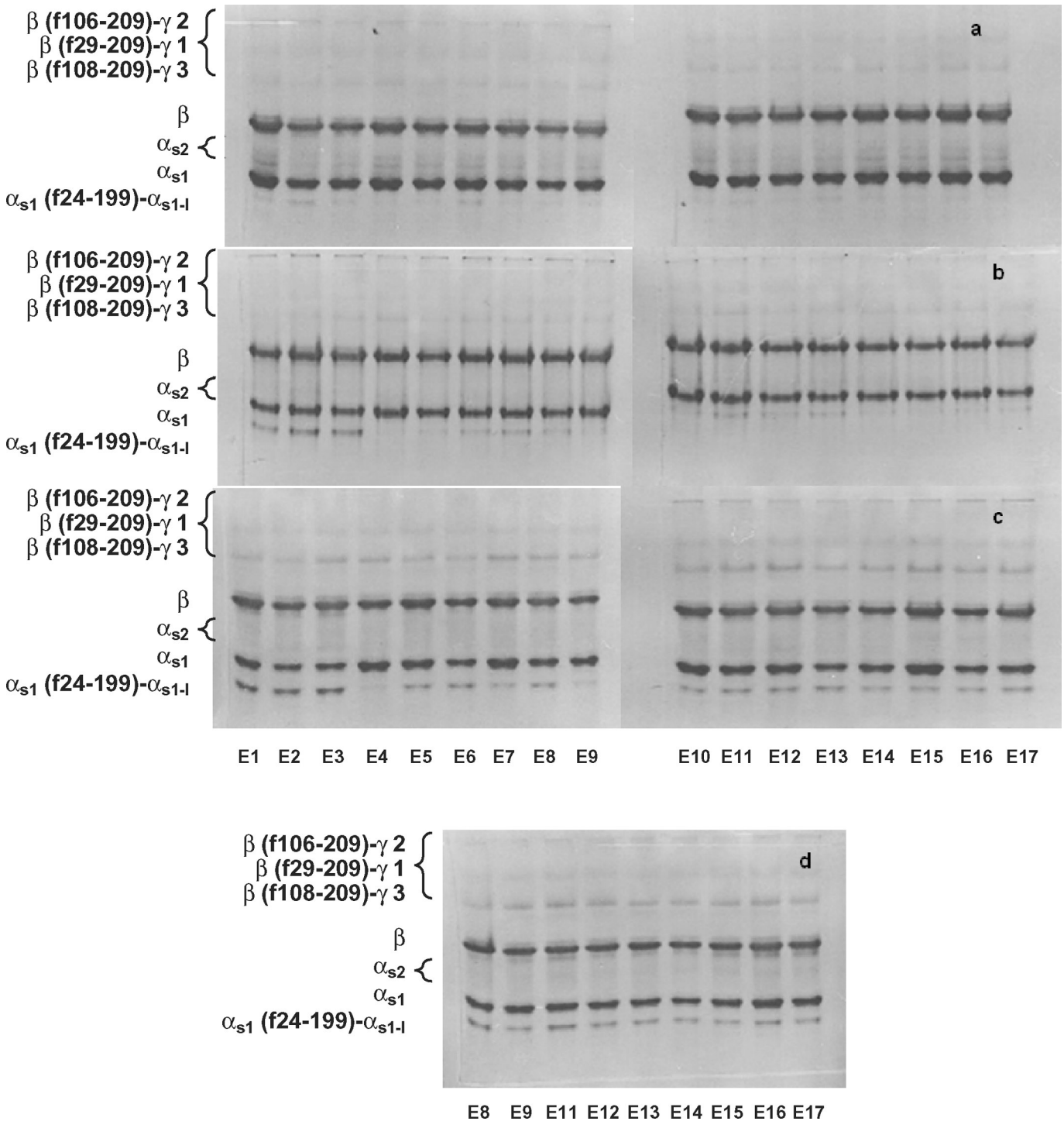

Figure 4. Urea-PAGE of Mozzarella cheese sampled at a) 4, b) 49, c) 91, and d) 131 d of ripening. Fat matter in cheese milk (%), stretching temperature (°C), and brining time (h), respectively, of each cheese was as follows: E1 = 2.9%, 83°C, 18 h; E2 = 2.4%, 78°C, 14 h; E3 = 3.4%, 78°C, 14 h; E = 2.4%, 88°C, 14 h; E5 = 3.4%, 88°C, 14 h; E6 = 2.4%, 78°C, 22 h; E7 = 3.4%, 78°C, 22 h; E8 = 2.4%, 88°C, 22 h; E9 = 2.9%, 83°C, 18 h: E10 = 3.4%, 88°C, 22 h; E11 = 2.06%, 83°C, 18 h; E12 = 3.74%, 83°C, 18 h; E13 = 2.9%, 74.6°C, 18 h; E14 = 2.9%, 91.4°C, 18 h; E15 = 2.9%, 83°C, 11.27 h; E16 = 2.9%, 83°C, 24.73 h; and E17 = 2.9%, 83°C, 18 h. Cheeses E8 to 17 remained in the ripening room and were also sampled at 131 d (panel d).

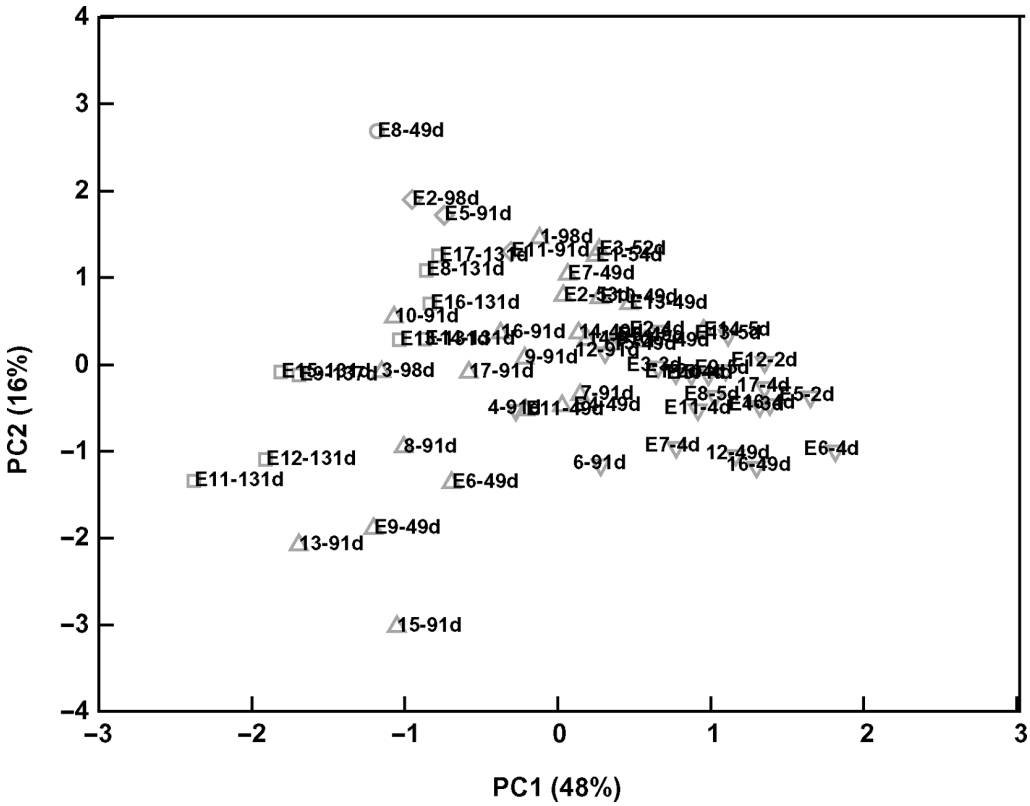

Figure 5. Principal component analysis and sample clustering (k-means) of preprocessed data obtained from reverse phase-HPLC chromatograms of the water-soluble fraction of Mozzarella cheeses. Numbers indicate sample and ripening time; different symbols show different cluster membership.

archical clusters analysis (k-means) was performed using as input variables these first 6 PC. Several analyses were performed, proposing a number of 3, 4, 5, or 6 clusters. The best results were obtained for the assay with 5 clusters, which was verified by analyzing visually the grouping of samples in graphical representation of the first 6 PC scores taken by pairs (i.e., PC1 vs. PC2, PC1 vs. PC3, and so on), and also taking into account the presence of outsiders and relating the results of the k-means analysis to the conceptual aspects of the study. In Figure 5, PC scores of samples in PC1 and PC2 are represented, as well as cluster membership. The 3 samples that represent the central points of the experimental design (E1, E9, and E17) were grouped in the same clusters either at 3, 49, or 91 d of ripening. Sample E8 constituted a one-sample cluster at 49 d of ripening. However, we considered that it was not an outsider because it was not detected as such in the preliminary data analysis, nor did the cluster analysis improve after taking it out (Hair et al., 1999).

The levels of some peaks in the chromatograms of the water-soluble fraction increased with ripening time, and new peaks appeared as well. The retention time classes that corresponded to hydrophobic peaks with high retention times (45 to 85 min) had negative loadings on PC1, which represented most of the data variation (51.5%). On the other hand, the retention time categories corresponding to hydrophilic peptides with short retention times showed positive loadings on PC1. The same categories showed a practically inverse influence on PC2 (representing 15.8% of variation; Table 4). The relation of PC2 to proteolysis progress, however, was more difficult to determine. The development and height increase of the peptides that influenced this PC could not always be related to higher proteolysis, because they are the consequence of an equilibrium between production and degradation processes. Some peaks increased at all ripening times, but others diminished after an initial increase.

Nonhierarchical cluster analysis failed to detect any effect of the experimental treatments on the grouping of the samples, which were mostly clustered by ripening time.

DISCUSSION

Proteolysis during ripening of Mozzarella cheeses depends on the active proteolytic agents and the environ-

Table 4. Loadings of the retention time classes for the first and second principal components (PC1 and PC2)

Retention time (min)	PC1	PC2
2.6	0.336	0.087
2.8	0.486	0.065
3.2	0.854	–0.455
3.5	0.494	–0.109
3.9	0.703	–0.297
4.3	0.597	–0.004
4.8	0.059	0.215
5.4	–0.542	0.298
6.0	0.370	0.004
6.6	0.395	0.026
7.4	0.287	0.049
8.2	–0.312	0.383
9.1	–0.667	0.166
10.2	0.605	–0.055
11.3	0.212	–0.017
12.6	0.156	0.049
14.0	–0.264	–0.026
15.5	0.245	0.153
17.3	0.635	–0.067
19.2	0.233	0.019
21.4	–0.060	0.200
23.8	0.646	–0.032
26.4	0.161	0.128
29.4	0.127	0.118
32.7	0.169	0.038
36.3	–0.734	0.228
40.4	–0.576	0.186
44.9	–0.227	0.521
49.9	–0.623	0.460
55.5	–0.712	0.272
61.8	–0.519	0.502
68.7	–0.616	0.156
76.4	–0.838	–0.520
84.9	0.378	0.307
94.4	0.305	0.275
105.0	0.250	0.148

mental conditions in the food matrix, determined in turn by the cheese-making technology. However, the changes in cheese-making process assayed in this work were insufficient to produce significant differences on proteolysis of the cheeses.

The degree of hydrolysis of caseins in Mozzarella cheeses obtained after stretching at temperatures as different as 74.6 and 91.4°C was similar, although the cheeses treated at the highest temperatures showed a slightly slower evolution of proteolysis. Kindstedt et al. (1995) observed a similar trend for cheeses made with different concentrations of coagulant, and Yun et al. (1993) found that proteolysis rate in Mozzarella was affected by cooking temperature. Kuo et al. (2001) reported that meltability of Mozzarella decreased when the holding time during the stretching step was increased. The delay in proteolysis may be due to a decrease in the residual activity of coagulant enzyme in the cheeses treated at the highest temperatures. However, the residual rennet activity may be significant in cooked, long-ripened cheeses, as it probably reactivates after cheese making (Delacroix-Buchet and Fournier, 1992; Hayes et al., 2002; Hynes et al., 2004a,b). Plasmin global activity has been shown to increase when cooking temperature is increased in Cheddar cheese (Somers and Kelly, 2002), but we found no evidence of higher production of γ-CN in Mozzarella samples obtained after cooking-stretching at high temperatures.

The fact that stretching-cooking temperature (within the assayed rank) did not affect proteolysis represents a main conclusion of this work, because strong heat treatments of the curd have been empirically applied in the dairy industry to prolong the textural stability of Mozzarella cheeses. There is evidence that pasta filata cheeses obtained at high stretching-cooking temperatures (>65°C) take longer to achieve the same rheological characteristics as those stretched at 55°C (Kindstedt, 2003). However, our results suggest that textural changes may not be due to differences in proteolysis, and temperature increases above 74°C in the heating water (~60°C in the curd) may be useless.

Similarly, samples that significantly differed in salt content did not show significant changes on proteolysis. This was not surprising, as salt content consistently comprised between 1.31 and 2.31%. Inhibition of proteolytic events during cheese ripening by NaCl was reported when much greater concentrations of salt were present (Noomen, 1978a,b; Kristiansen et al., 1999).

Fat matter did not significantly influence proteolysis of the Mozzarella cheeses, although %SN increased faster in those Mozzarella cheeses with the highest fat content. Increased fat content has been found to correlate with degree of proteolysis in cheese (Rudan et al., 1999), although differences were mainly detected between cheeses made with skim (fat content between 4.10 and 9.41%) and full-fat milk (fat content between 16.48 and 26.73%). In our study, fat content varied in a narrower range (21.5 to 28.5%), as all Mozzarella cheeses fulfilled Argentinean regulations (fat content in DM >35%, Código Alimentario Argentino, www.anmat.gov.ar).

Increase in proteolysis during ripening is a well-documented fact for many cheese varieties and has been recently proposed for describing and predicting cheese maturity (Fallico et al., 2004; Piraino et al., 2004; Cocker et al., 2005). In our study, ripening time had a more important influence on proteolysis of Mozzarella cheeses than any other assayed variable. Unlike fat content, stretching temperature, and brining time, ripening time had an effect on the proteolysis of the samples as revealed by multivariate analysis of the data. All the Mozzarella cheeses that were ripened for 131 d, regardless of the manufacture process, were grouped together. This implied that, if ripening time were long

enough, all differences in proteolysis among samples would eventually even out. Similar proteolytic enzymes were probably active in the food matrix, and the differences originated from changes on their relative activities were only perceived at early ripening times.

ACKNOWLEDGMENTS

The authors thank Sucesores de Alfredo Williner S.A (Bella Italia, Santa Fe, Argentina) for cheese-making experiments and partial funding for this research work, and M. C. Candioti for electrophoresis assays. Erica Hynes acknowledges financial support from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

REFERENCES

Andrews, A. T. 1983. Proteinases in normal bovine milk and their action on caseins. J. Dairy Res. 50:45–55.

Barbano, D. M., K. Y. Chu, J. J. Yuri, and P. S. Kindstedt. 1993. Contribution of coagulant, starter and milk enzymes to proteolysis and browning in Mozzarella cheese. Pages 65–80 in Proc. 30th Marschall Italian and Specialty Cheese Seminars. Rhodia-Marschall, Madison, WI.

Bertola, N. N., A. N. Califano, A. E. Bevilacqua, and N. E. Zaritzky. 1996. Textural changes and proteolysis of low-moisture Mozzarella cheese frozen under various conditions. Lebensm. Wiss. Technol. 29:470–474.

Bradley Jr., R. L., E. Arnold Jr., D. M. Badano, R. G. Semerad, D. E. Smith, and B. K. Vines. 1992. Chemical and physical methods. Pages 433–531 in Standard Methods for the Examination of Dairy Products. 16th ed. R. Marshall, ed. American Public Health Association, Washington, DC.

Cocker, C. J., R. A. Crawford, K. A. Johnston, H. Singh, and L. K. Creamer. 2005. Towards the classification of cheese variety and maturity on the basis of statistical analysis of proteolysis data – A review. Int. Dairy J. 15:631–643.

Delacroix-Buchet, A., and S. Fournier. 1992. Protéolyse et texture des fromages à pâte cuite pressée. II. Influence de la chymosine et des conditions de fabrication. Lait 72:53–72.

Everett, D., M. Rowney, M. Hickey, and P. Roupas. 2004. Salt induced changes in Mozzarella cheese and the impact upon free oil formation in ripening time. Lait 84:1–11.

Feeney, E. P., P. F. Fox, and T. P. Guinee. 2001. Effect of ripening temperature on low moisture Mozzarella cheese: 1. Composition and proteolysis. Lait 81:463–474.

Feeney, E. P., T. P. Guinee, and P. F. Fox. 2002. Effect of pH and calcium content on proteolysis in Mozzarella cheese. J. Dairy Sci. 85:1646–1654.

Fallico, V., P. L. H. McSweeney, K. J. Siebert, J. Horne, S. Carpino, and G. Licitra. 2004. Chemometric analysis of proteolysis during ripening of Ragusano cheese. J. Dairy Sci. 87:3138–3152.

Fox, P. F., T. P. Guinee, T. M. Cogan, and P. L. H. McSweeney. 2000a. Biochemistry of cheese ripening. Pages 236–281 in Fundamentals of Cheese Science. Aspen Publishers, Inc., Gaithersburg, MD.

Fox, P. F., T. P. Guinee, T. M. Cogan, and P. L. H. McSweeney. 2000b. Cheese as Food Ingredient. Pages 452–483 in Fundamentals of Cheese Science. Aspen Publishers, Inc., Gaithersburg, MD.

Gacula, M. C., Jr., and J. Singh. 1984. Statistical Methods in Food and Consumer Research. Academic Press, Inc., Orlando, FL.

Gaiaschi, A., B. Beretta, C. Polesi, A. Conti, M. G. Giuffrida, C. L. Galli, and P. Restani. 2000. Proteolysis of α_s-casein as a marker of Grana Padano cheese ripening. J. Dairy Sci. 83:2733–2739.

Guinee, T. P. 2003. Cheese rheology. Pages 341–349 in Encyclopedia of Dairy Science. Vol. 1. H. Roginsky, J. W. Fuquay, and P. F. Fox, ed. Elsevier, London, UK.

Guinee, T. P., E. P. Feeney, and P. F. Fox. 2001. Effect of ripening temperature on low moisture Mozzarella cheese: 2. Texture and functionality. Lait 81:475–485.

Gunasekaran, S., and M. M. Ak. 2003. Factors affecting functional properties of cheese. Pages 399–428 in Cheese Rheology and Texture. CRC Press, Boca Raton, FL.

Hair, J. F., Jr., R. E. Anderson, R. L. Tatham, and W. L. Black. 1999. Análisis multivariante. 5th ed. Prentice Hall Iberia, Madrid, Spain.

Hayes, M. G., J. C. Oliveira, P. L. H. McSweeney, and A. L. Kelly. 2002. Thermal inactivation of chymosin during cheese manufacture. J. Dairy Sci. 69:269–279.

Hutkins, R. W. 1993. How starter culture metabolism affects properties of Mozzarella cheese. Pages 1–8 in Proc. 30th Marschall Italian and Specialty Cheese Seminars. Rhodia-Marschall, Madison, WI.

Hynes, E., L. Aparo, and M. C. Candioti. 2004a. Influence of residual milk-clotting enzyme on α_{s1} casein hydrolysis during ripening of Reggianito Argentino cheese. J. Dairy Sci. 87:565–573.

Hynes, E., M. C. Candioti, C. A. Zalazar, and P. L. H. McSweeney. 2004b. Rennet activity and proteolysis in Reggianito Argentino hard cooked cheese. Aust. J. Dairy Technol. 59:209–213.

Hynes, E., A. Delacroix-Buchet, C. A. Meinardi, and C. A. Zalazar. 1999. Relation between pH, degree of proteolysis and consistency in soft cheese. Aust. J. Dairy Technol. 54:24–27.

Hynes, E., C. A. Meinardi, N. Sabbag, T. Cattaneo, M. C. Candioti, and C. A. Zalazar. 2001. Influence of milk-clotting enzyme concentration on the α_{s1} casein. J. Dairy Sci. 84:1334–1340.

International Dairy Federation (IDF). 1972. Standard 17A. Determination of the salt content of cheese. IDF, Brussels, Belgium.

International Dairy Federation (IDF). 1982. Standard 4A. Cheese and processed cheese. Determination of the total solids content. IDF, Brussels, Belgium.

International Dairy Federation (IDF). 1993. Standard 20B. Milk. Determination of nitrogen content. IDF, Brussels, Belgium.

Johnson, M., and B. A. Law. 1999. The origins, development and basic operations of cheesemaking technology. Pages 1–32 in Technology of Cheesemaking. B. A. Law, ed. Sheffield Academic Press, Sheffield, UK.

Joshi, N. S., K. Muthukumarappan, and R. I. Dave. 2003a. Understanding the role of calcium in functionality of part skim Mozzarella cheese. J. Dairy Sci. 86:1918–1926.

Joshi, N. S., K. Muthukumarappan, and R. I. Dave. 2003b. Effect of calcium on physicochemical properties of fat-free Mozzarella cheese. J. Food Sci. 68:2289–2294.

Kindstedt, P. S. 2003. Pasta-filata cheeses. Pages 386–391 in Encyclopedia of Dairy Science. Vol. 1. H. Roginsky, J. W. Fuquay, and P. F. Fox, ed. Elsevier, London, UK.

Kindstedt, P. S., M. Rowney, and P. Roupas. 1999. Technology, biochemistry and functionality of pasta filata/pizza cheese. Pages 193–221 in Technology of Cheesemaking. B. A. Law, ed. Sheffield Academic Press, Sheffield, UK.

Kindstedt, P. S., J. J. Yun, D. M. Barbano, and K. L. Larose. 1995. Mozzarella cheese: Impact of coagulant concentration on chemical composition, proteolysis, and functional properties. J. Dairy Sci. 78:2591–2592.

Kristiansen, K. R., A. S. Deding, D. F. Jensen, Y. Ardö, and K. B. Qvist. 1999. Influence of salt content on ripening of semi-hard round eyed cheese of Danbo type. Milchwissenschaft 54:20–23.

Kuo, M. I., Y. C. Wang, S. Gunasekaran, and N. F. Olson. 2001. Effect of heat treatments on the meltability of cheeses. J. Dairy Sci. 84:1937–1943.

Massart, D. L., B. G. M. Vandeginste, S. N. Deming, Y. Michotte, and L. Kaufman. 1988. Chemometrics: A textbook. Data Handling in Science and Technology. Vol. 2. Elsevier, London, UK.

McMahon, D. J., B. Paulson, and C. J. Oberg. 2005. Influence of calcium, pH and moisture on protein matrix structure and functionality in direct-acidified nonfat Mozzarella cheese. J. Dairy Sci. 88:3754–3763.

Mukherjee, K. K., and R. W. Hutkins. 1994. Isolation of galactose-fermenting thermophilic cultures and their use in the manufac-

ture of low browning Mozzarella cheese. J. Dairy Sci. 77:2839–2849.
Noomen, A. 1978a. Activity of proteolytic enzymes in simulated soft cheese (Meshanger type). I. Activity of milk protease. Neth. Milk Dairy J. 32:26–48.
Noomen, A. 1978b. Activity of proteolytic enzymes in simulated soft cheese (Meshanger type). II. Activity of calf rennet. Neth. Milk Dairy J. 32:49–61.
Oberg, C. J. 1991. Factors affecting stretch, melt and cook color in Mozzarella cheese. Pages 1–8 in Proc. 28th Marschall Italian and Specialty Cheese Seminars. Rhodia-Marschall, Madison, WI.
Piraino, P., E. Parente, and P. L. H. McSweeney. 2004. Processing of chromatographic data for chemometric analysis of peptides from cheese extracts: A novel approach. J. Agric. Food Chem. 52:6904–6911.
Pripp, A. H., Shakeel Ur Rehman, P. L. H. McSweeney, T. Sørhaug, and P. F. Fox. 2000. Comparative study by multivariate statistical analysis of proteolysis in a sodium caseinate solution under cheese-like conditions caused by strains of *Lactococcus*. Int. Dairy J. 10:25–31.
Rudan, M. A., D. M. Barbano, J. J. Yun, and P. S. Kindstedt. 1999. Effect of fat reduction on chemical composition, proteolysis functionality, and yield of Mozzarella cheese. J. Dairy Sci. 82:661–672.
Sheenan, J., and T. P. Guinee. 2004. Effect of pH and calcium level on the biochemical, textural and functional properties of reduced-fat Mozzarella cheese. Int. Dairy J. 14:161–172.
Somers, J. M., and A. L. Kelly. 2002. Contribution of plasmin to primary proteolysis during ripening of cheese: Effect of milk heat treatment and cheese cooking temperature. Lait 82:181–191.
Sousa, M. J., Y. Ardö, and P. L. H. McSweeney. 2001. Advances in the study of proteolysis during cheese ripening. Int. Dairy J. 11:327–345.
Yun, J. J., L. J. Kiely, D. M. Barbano, and P. S. Kindstedt. 1993. Mozzarella cheese: Impact of cooking temperature on chemical composition, proteolysis, and functional properties. J. Dairy Sci. 76:3664–3673.