Effect of Frozen Storage Time on the Proteolysis of Soft Cheeses Studied by Principal Component Analysis of Proteolytic Profiles

R.A. VERDINI, A.C. RUBIOLO

ABSTRACT: Port Salut Argentino cheeses were frozen, stored in a freezer at –22 °C for different periods, and slowly thawed. After thawing, some cheeses were immediately sampled while others were sampled after different refrigerated storage times. Reversed Phase High Performance Liquid chromatograms were compared applying principal component analysis. The information obtained from the chromatograms was successfully summarized in 2 dimensions accounting for 93.4% of the data variation. According to sample grouping, there were differences between Port Salut Argentino cheeses due to the ripening during refrigerated storage for up to 60 days at 4 °C. However, for cheeses that were sampled immediately after slow thawing, there was no significant effect of the frozen storage time in cheese proteolysis, from 1 to 60 d.

Keywords: frozen storage time, cheese, proteolysis, RP-HPLC, principal component analysis

Introduction

THERE IS A LARGE VARIETY OF CHEESES ACCORDING TO MANUfacturing conditions. Still more variety is contributed by the regional diversification of traditional cheeses. The major production of cheese in Argentina is based on soft cheeses, with Port Salut Argentino one of the most popular varieties (Zalazar and others 1999). The CAA (1981) describes Port Salut Argentino as a semi-cooked cheese which is produced from pasteurized milk, acidified by lactic bacteria, coagulated by rennet and/or other specific enzymes and ripened for a short period. While most of the rennet added to the milk is lost in the whey, the small amount retaining in the curd is mostly responsible for soft cheese proteolysis, which occurs not only during the ripening period but also continues during refrigerated storage (Fox 1989). Expanding commercialization of Port Salut Argentino cheese has led to increased interest in preserving its characteristics during the marketing period.

Research all over the world has proved that freezing is one of the most effective treatments to ensure high-quality food products. Chemical and physical reactions continue even at very low temperatures and the lower the temperature, the slower the speed of the deteriorative reactions, and the better the quality after defrosting. Rapid freezing and slow defrosting are advantageous, although the changes which are produced during the freeze-thaw cycle may lead to proteins and fat destabilization (Lück 1977).

After evaluating flavor and texture consistency, Lück (1977) concluded that frozen storage was suitable for soft cheeses (cream cheese, unripened Camembert) but not for semi-hard or hard cheeses. Many investigations have concerned the freeze-thaw cycle conditions of Mozzarella cheeses: temperature of the chamber, rate of the freeze-thaw cycle, and time of tempering before freezing or after thawing. Mozzarella cheeses which underwent rapid freeze-thaw cycles and were stored at –15 °C for a wk showed no significant changes in textural characteristics (Cervantes and others 1983). Tempered Mozzarella cheeses, which were frozen at different rates, exhibited the same quality as refrigerated cheeses no matter what the freezing speed was (Bevilacqua 1997). Studies of the effects of slow freezing on Mozzarella cheese showed that there was no difference in the extent of proteolysis after thawing and tempering (Chaves and others 1999).

During cheese ripening, the casein is converted by proteolysis into breakdown products and the extent of this degradation process plays an important role in determining cheese flavor and texture and depends on the activities of enzymes and microorganisms (Law 1987). The most commonly used methods for separating proteins from their breakdown products are fractional precipitation with acids or solvents; the pH 4.6 water-soluble fraction is the most frequently used (Kuchroo and Fox 1982).

The soluble nitrogen compounds have been analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) to follow proteolysis during cheese maturation and to evaluate different technological treatments. Furtula and others (1994a) used RP-HPLC profiles of the water-soluble fraction to analyze Cheddar cheese samples aged by a fast-ripening process. Laborda and Rubiolo (1999) used RP-HPLC profiles of the water-soluble fraction to determine and quantify soluble peptides in Fynbo cheeses salted with a mixture of NaCl/KCl and to evaluate the effects of maturation temperature.

For data analysis in food research, classification of samples is frequently the main purpose. Chromatographic analysis of elaborate samples generates a large amount of data, which has to be evaluated efficiently. A wide spectrum of multivariate methods is available in order to extract information from the data set, including supervised and unsupervised methods. In supervised methods, samples are grouped into categories according to certain defined criteria of similarity. However, in food technology there are many situations in which for the application of supervised methods is restricted (Vodovotz and others 1993). Therefore, principal component analysis has become one of the most useful unsupervised methods for food researchers, which allows summarizing multivariate data for sample classification (González de Llano and others 1991; Armanino and Festa 1996; Guerrero and others 1997). Pripp and others (2000) considered that multivariate analysis of proteolytic profiles showed to be a powerful method to discriminate cheese varieties, cheese quality and starter strains. Indeed, they suggested that further research

with this method could be a very useful approach to better understand proteolysis during cheese ripening and how it relates to the technology and quality of cheese.

The objective of our work was to evaluate the effect of frozen storage time in Port Salut Argentino cheeses applying principal component analysis to RP-HPLC profiles of the pH 4.6 water-soluble fraction. In addition, chromatograms of cheeses analyzed immediately after thawing were compared with those of cheeses that, after thawing, were stored at 4 °C for different periods.

Materials and Methods

Cheese samples

Commercial Port Salut Argentino cheeses were produced (SanCor United Cooperatives Ltd., Gálvez, Santa Fe, Argentina) by rennet coagulation with a starter mixture of *Streptococcus thermophilus, Lactococcus lactis* subsp. *cremoris* and subsp. *lactis*. Cheeses were salted in a brine for 3 h at 3 ± 1 °C and packed after 20 h. They were cylindrical soft cheeses weighing 3.450 to 3.650 kg, 23 ± 1 cm in diameter and 7.5 ± 0.5 cm in height.

Two batches of cheeses (A and B) were frozen in a TABAI Comstar PR 4GM chamber at –30 °C until the center reached –18 °C. Cheeses were stored at the laboratory freezer at –22 °C for 60 d, then slowly thawed in the TABAI chamber at 4 °C. The temperature of the samples during the freeze-thaw cycle was monitored using a TABAI Comstar THP–18 temperature recorder with T thermocouples.

Six cheeses (A and B), with different frozen storage periods (1, 20, and 60 d), were sampled immediately after thawing (F1, F20, and F60).

Six cheeses (A and B), with 60 d of frozen storage, after thawing were stored in the TABAI chamber at 4 °C for different periods (15, 30, and 60 d), and sampled after refrigerated storage (FR15, FR30, and FR60).

A piece of 60 × 60 mm, with the height of the cheese, was cut in the center perpendicular to the main surface. Then, upper and bottom equal portions were removed from the parallelepiped to obtain a central slice of 30-mm height that was grated for further analysis.

Physicochemical analysis

Moisture and total protein determinations were performed as described by Zorrilla and Rubiolo (1991). Moisture content was determined with a CEM AVC 80 (CEM, Mattheus, N.C., U.S.A.) microwave oven. Total protein content was determined with an automatic digestor model 430, a distillation unit model 322 (Büchi, Flawil, Switzerland), and an automatic titrator DL40RC (Mettler Instrumente AG, Greifensee, Switzerland). Fat content was assessed using the Standard Intenational Dairy Federation method (IDF 1969).

Water-soluble fraction extraction and analysis

The pH 4.6 water-soluble fraction was prepared as reported by Laborda and Rubiolo (1999). Cheese samples were homogenized with a blender SB30 (Black & Decker, Australasia, Australia). The homogenate was centrifuged for 30 min at 5 °C (Biofuge 28RS, Heraeus Sepatech, Osterode, Germany) and filtered through Whatman No. 42 paper. Water-soluble nitrogen was determined using the procedure cited by Zorrilla and Rubiolo (1991) with the same equipment as used for total protein content. Indexes of maturation were expressed as a percentage of the water-soluble nitrogen of the total cheese nitrogen (IM = WSN × 100 / TN). In order to assay all samples simultaneously, water-soluble extracts were stored in a freezer at –22 °C for further chromatographic analysis.

A chromatograph (Isco, Inc., Lincoln, Nebr., U.S.A.) with a gradient programmer model 2360, a V$^{4®}$ variable wavelength absorbance detector and a SynChropak RPP (250 × 4.6 mm) C_{18}, 300 Å column (SynChrom, Inc., Lafayette, Ind., U.S.A.) at 40 °C were used. The method reported by González de Llano and others (1995), modified by Laborda and Rubiolo (1999), was followed. Detection was at 220 nm. Data were processed with the Chem Research Data System Program version 3.0.2. 1994 (Isco, Inc., Lincoln, Nebr., U.S.A.).

Statistical analysis

Principal component analysis (PCA) was applied in order to summarize the large amount of data obtained from the chromatograms with minimal loss of information. PCA is based on the linear combination of the measured variables to produce derived variables called principal components (PCs) which are mutually orthogonal in the principal component space (Gardnier 1997).

For a data set of n samples and p original variables the $n \times p$ data matrix $\mathbf{X} = [\mathbf{x}_1, \mathbf{x}_2,...,\mathbf{x}_i,..., \mathbf{x}_p]$ was defined, where $\mathbf{x}_i$ are the $n \times 1$ variable vectors; and the $n \times p$ mean centered data matrix $\mathbf{X}_C$ was calculated, with $\mathbf{xc}_{ij} = \mathbf{x}_{ij} - \mathbf{x}_j$ where $\mathbf{x}_j$ is the column mean for i = 1, 2,..., n and j = 1, 2,..., p.

Eigenvalues and eigenvectors were obtained from Sw = lw , where S is the $p \times p$ variance-covariance matrix of $\mathbf{X}_C$, l are the eigenvalues and w are the corresponding eigenvectors (providing $w^T w = 1$).

Considering the equivalence between the eigenvalue and the PC variance, eigenvalues were ordered from largest to smallest $\lambda_1 \geq \lambda_2 \geq ...\lambda_i...\geq...\lambda_p$ for i =1, 2,..., p, and the variability that each PC accounted was obtained from PCi variability = $100 \times [l_i / (Sl)]$.

An adequate condensation of the information is achieved when a small number (k) PC explains at least 80 to 90% of the total variability. Thereafter, the k selected PC were calculated from $\mathbf{z}_i = \mathbf{X}_C \mathbf{w}_i$ for i = 1, 2,..., k (Gardnier 1997; Johnson and Wichern 1998; Kellner and others 1998).

The w_i components are called PCi loadings and the z_i components are called PCi scores. The analysis of the PC scores gives evidence of sample grouping in the PC space according to similarities in their characteristics. PC loadings are the coefficients used to construct the corresponding PC scores derived from the originally measured variables. Therefore, the examination of the PC loadings considers the influence of the original variables in the sample arrangement. The higher the absolute value of the loading the more it contributes to what the corresponding PC explains of the data organization (Gardnier 1997).

Distances between samples can be measured after performing PCA. Distances along PC axes are not equally weighted; therefore; Euclidean distances between samples are not equivalent. One of the most useful distance measures is the Mahalanobis distance (MD) between samples calculated in the PC space which is obtained from $MD_{ij} = [(\mathbf{z}_{i:} - \mathbf{z}_{j:}) C^{-1} (\mathbf{z}_{i:} - \mathbf{z}_{j:})^T]^{-1/2}$ for i = 1, 2,..., n and j = 1, 2,..., n, where $\mathbf{z}_{i:}$ and $\mathbf{z}_{j:}$ are the row components of the $n \times k$ matrix $\mathbf{Z} = [\mathbf{z}_1, \mathbf{z}_2,..., \mathbf{z}_i,..., \mathbf{z}_k]$ and C is the $k \times k$ variance-covariance matrix of $\mathbf{Z}$ (Kellner and others 1998; De Maesschalck and others 2000).

PCA and MD between samples in the PC space were calculated with a MATLAB® language subroutine.

Results and discussion

Moisture, fat and total protein contents of cheeses A and B are shown in Table 1. Indexes of maturation and RP-HPLC peptide profiles of cheese samples were compared; the indexes of

Table 1—Average composition of cheeses

Batch	Moisture (% w/w)	Fat (% w/w)	Protein (% w/w)
A	51 ± 1	22 ± 1	18.8 ± 0.9
B	52 ± 1	22 ± 1	19.3 ± 0.9

Table 2—Indexes of maturation of cheeses (IM = WSN x 100/TN)

Treatment	Batch A[a]	Batch B[a]
F1	9.9	12.8
F20	11.7	13.5
F60	10.5	14.4
FR15	15.0	16.9
FR30	22.7	19.2
FR60	25.7	29.7

[a]Standard deviation ± 0.9

maturation of cheeses A and B are shown in Table 2 and the chromatograms of cheeses A are shown in Figure 1.

Although the chromatograms were complex, peptides with a wide range of polarity were resolved (González de Llano and others 1995; Laborda and Rubiolo 1999).

From the chromatographic data 58 peaks were selected and matched by visual comparison (Furtula and others 1994b). The absolute area of each selected peak was considered a variable and the 12 × 58 data matrix **X** was outlined.

Several researchers applied PCA directly to the original data set (Vodovotz and others 1993; Furtula and others 1994a, 1994b). In some cases, transformation of the data before performing PCA is recommended. Standardization is advisable only when variables are measured on scales with widely differing ranges or with not commensurable measurement units (Gardnier 1997; Johnson and Wichern 1998). However, standardization is not inconsequential because it affects the role that each variable plays in the construction of the PC (Srivastava and Kahtri 1979; Johnson and Wichern 1998). Sometimes mean centering is suggested in order to translate the data set along the coordinate origin (Kellner and others 1998).

In this case, the PCs were calculated from the mean centered data matrix $\mathbf{X}_C$ and the 58 variables yielded 2 principal components (k = 2), which accounted, cumulatively, for 93.4% of the data variation. Most of the variance was accounted for by the first PC according to the bend in the scree-plot in which eigenvalues ordered from largest to smallest were plotted against the number of component (Figure 2).

Grouping of cheese samples according to their characteristics was observed in the plot of PC1 score against PC2 score (Figure 3). Two groups corresponded to cheeses which were analyzed immediately after thawing (F-A and F-B) and were separated from cheeses that after the freeze-thaw cycle were stored at 4 °C (FR). In addition, cheese FR were grouped according to the refrigerated storage time (FR15, FR30, and FR60).

Arrangement of cheeses of the same batch (A or B) sampled immediately after thawing, no matter the frozen storage time, showed that there was no significant effect of the frozen storage time in cheese proteolysis. Separation between cheeses F-A and F-B and the displacement of cheeses F-B towards cheeses FR were in agreement with the differences observed in the indexes of maturation of cheeses F ($\overline{IM}_{F-A} = 11 \pm 1$ *and* $\overline{IM}_{F-B} =$) The latter corresponded with differences within batches. Grouping between cheeses FR was according to their storage time at 4 °C, since ripening continues during refrigerated storage. Indeed, these associations agreed with the variations observed in the in-

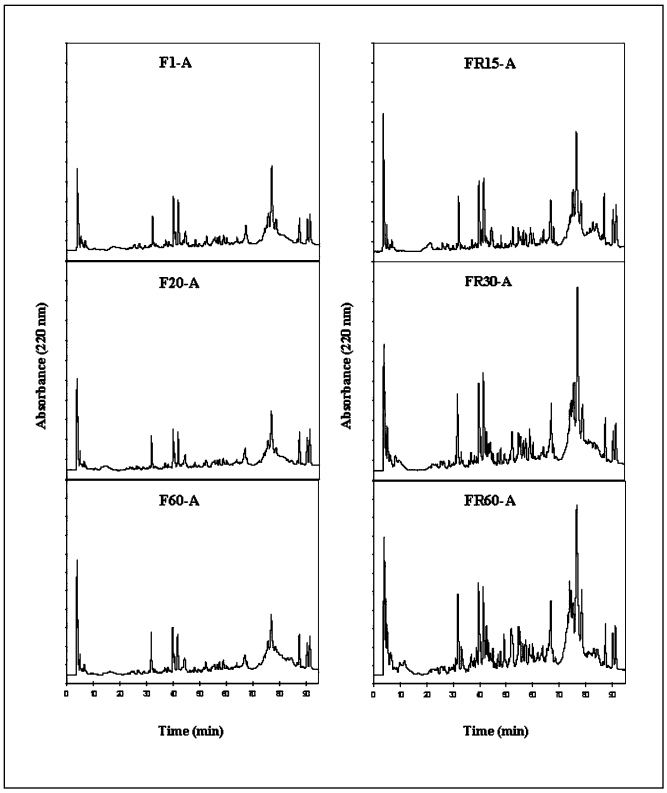

Figure 1—RP-HPLC profiles of the water-soluble fraction of cheeses A. F1-A, F20-A, and F60-A: stored at –22 °C for different periods. FR15-A, FR30-A, and FR60-A: stored at –22 °C, thawed and stored at 4 °C for different periods

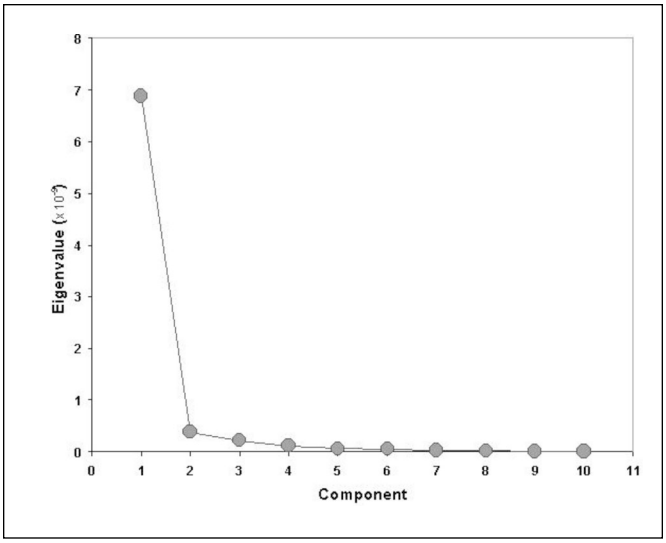

Figure 2—Scree-plot of the largest eigenvalues for the principal component model of selected peak areas from the RP-HPLC peptide profiles of cheese samples

Table 3—Mahalanobis distances between cheese samples in the 2-dimensional principal component space.

Samples	F1-A	F20-A	F60-A	FR15-A	FR30-A	FR60-A	F1-B	F20-B	F60-B	FR15-B	FR30-B	FR60-B
F1-A	0	0.10	0.03	3.50	7.95	7.18	1.21	2.11	0.96	4.39	7.36	9.37
F20-A		0	0.23	4.80	9.72	7.73	1.99	3.12	1.66	5.77	9.12	9.54
F60-A			0	2.93	7.04	6.65	0.87	1.66	0.66	3.72	6.48	8.95
FR15-A				0	1.23	6.46	0.65	0.22	0.87	0.19	0.83	10.50
FR30-A					0	5.56	2.99	1.93	3.39	0.55	0.08	9.65
FR60-A						0	5.10	5.11	5.02	5.02	6.45	0.56
F1-B							0	0.13	0.02	1.00	2.60	8.16
F20-B								0	0.23	0.42	1.59	8.51
F60-B									0	1.24	3.00	7.93
FR15-B										0	0.39	8.80
FR30-B											0	10.80
FR60-B												0

dexes of maturation (Table 2).

Loadings of the original variables in the 2-dimensional PC space are reported in Figure 4. Absolute values of PC1 and PC2 loadings were analyzed; none of the variables presented loadings with absolute values higher than 0.5 and only a few variables presented loadings with absolute values higher than 0.1 (labeled peaks in Figure 4).

Sometimes it is difficult to translate PC loadings information into a chemically interpretable solution (Gardnier 1997). However, it could be considered that variables with higher absolute values of PC loadings explained the separation of cheeses F according to differences between batches and the separation of cheeses FR according to their refrigerated storage time at 4 °C.

Examination of the PC loadings as function of peak number was performed and "spectra" of PC1 and PC2 loadings were obtained (Adams 1995). These "spectra" of PC1 and PC2 loadings, overlapped to peak areas of cheeses F1-A, FR15-A, FR30-A and FR60-A, are shown in Figure 5. The contribution of PC1 and PC2 loadings to cheese samples separation due to their ripening time could be observed in these "spectra".

In the RP-HPLC chromatograms of the water-soluble fraction, the group of hydrophilic peptides consisted mainly of peaks with retention times lower than 35 min and the group of hydrophobic peptides consisted mainly of peaks with retention times higher than that value (González de Llano and others 1995).

Peaks with absolute values of PCs loadings ≥ 0.1 were classified as hydrophilic or hydrophobic according to their retention times. Most peaks with absolute values of PC1 loadings ≥ 0.1 were hydrophobic and accounted for 88.4% of the variability that produced the separation of the samples according to their ripening time, while peaks with absolute values of PC2 loadings ≥ 0.1 were homogeneously distributed in both hydrophobic and hydrophilic zones (Figure 4 and Figure 5). González de Llano and others (1995) reported that for artisanal cheeses there was a significant increment of hydrophilic peptides during ripening in particular for cheeses that displayed a high degree of proteolysis. Laborda and Rubiolo (1999) reported that the increment of hydrophilic and hydrophobic peptides was almost constant during the ripening of Fynbo cheeses. These differences are in agreement with variations in the mechanisms of protein degradation, which are in direct relation to the manufacturing conditions. Port Salut Argentino cheese has a low degree of proteolysis, mainly due to residual coagulant activity (Fox 1989). Further hydrolysis is produced by proteinases and peptidases from the starter.

In many cases, PCA is an adequate method for objective and reliable classification of samples, but sometimes PCA serves as

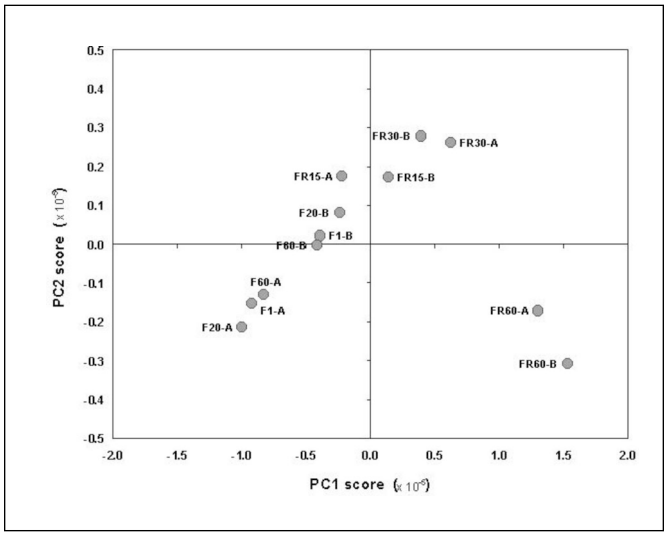

Figure 3—Plot of the samples projected on the plane of the 2 first-principal components

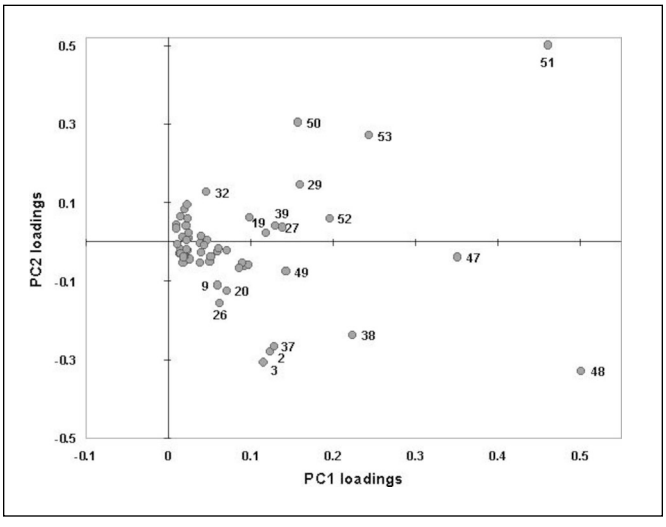

Figure 4—Plot of the loadings of the 2 first-principal components (labeled variables have PC1 loadings and/or PC2 loadings ≥ 0.1)

an intermediate step for the application of other methods. Furtula and others (1994a, 1994b) found that principal component similarity (PCS), which derives from PCA, was more accurate for classifying cheese samples based on age. Vodovotz and others (1993) concluded that, although PCS was more quantitative than PCA, the latter was more efficient when only a few principal components accounted for a major portion of the variation of the original data. In our work, where 2 PCs accounted for 93.4% of the total variation, PCA was more adequate than PCS.

In order to make the analyses of the PC scores plot more quantitative, MDs between samples in the 2-dimensional PC space were measured. The values of the MDs (Table 3) were in accordance with the sample grouping previously outlined from the PC scores plot (Figure 3).

Therefore, PCA in addition to MD between samples clearly showed sample arrangement according to differences in ripening times and batch characteristics.

Conclusion

ALTHOUGH THE RP-HPLC PEPTIDE PROFILES OF THE WATER-soluble fraction of cheeses were complex, PCA applied to the 58 peak areas selected from the chromatograms of each of the 12 samples provided distinguishable evidence of sample organization in a 2-dimensional space. Therefore, PCA was useful not only because it summarized the large amount of information obtained from the chromatograms in 2 dimensions, but also because it showed sample arrangement in correspondence with differences in ripening times and batch characteristics. MDs between samples in the 2-dimensional PC space provided a useful measure to assess the grouping outlined from the PC scores plot. According to the sample grouping of Port Salut Argentino cheeses stored at –22 °C, there were distinctions due to their refrigerated storage times at 4 °C and in relation to differences within batches. However, for cheeses that were sampled immediately after slow thawing there was no significant effect of the frozen storage time in cheese proteolysis from 1 to 60 d.

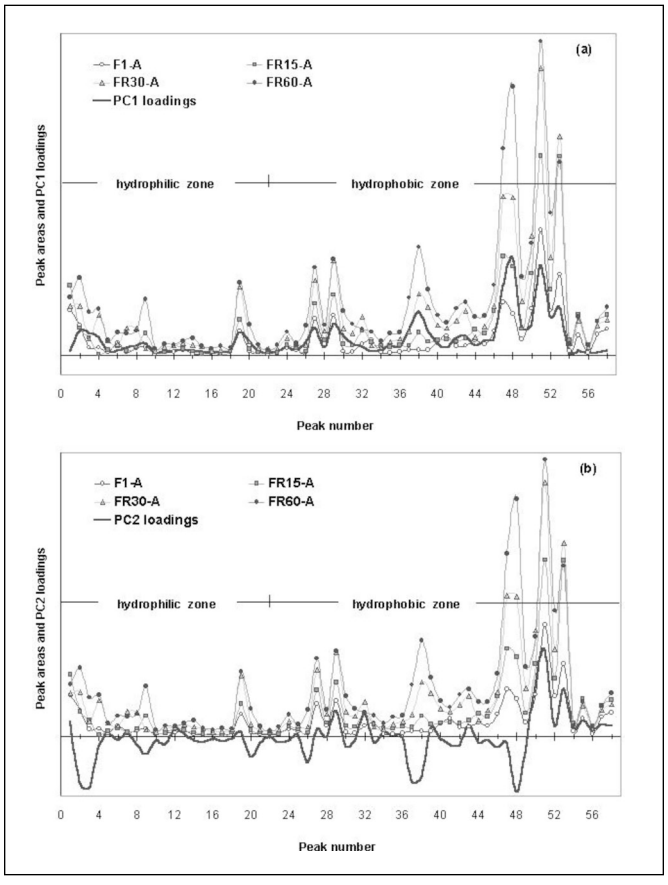

Figure 5—"Spectra" of the variables and the PC loadings (in arbitrary units) as a function of peak number: (a) PC1 loadings and (b) PC2 loadings

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Authors Verdini and Rubiolo are affiliated with Instituto de Desarrollo Tecnológico para la Industria Química (INTEC) - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional del Litoral (UNL) - Güemes 3450, (3000) Santa Fe, República Argentina. Author Verdini is affiliated with Universidad Nacional de Rosario (UNR), Suipacha 531, (2000) Rosario, República Argentina. Direct inquires to author Rubiolo (E-mail: arubiolo@intec.unl.edu).