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The influence of elevated initial ripening temperature on the proteolysis in Reggianito cheese

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ABSTRACT

The effects of elevated initial ripening temperature on proteolysis in Reggianito cheese were evaluated considering different temperature–time combinations. Control cheeses stored at 12 °C for 6 months and experimental cheeses stored at 20 °C for 2 or 4 weeks then at 12 °C up to 6 months, were analysed at 61, 124, and 180 days of ripening by physicochemical analysis, urea-PAGE analysis of the urea-soluble fraction, RP-HPLC analysis on the water-soluble fraction at pH 4.6, and free amino acid analysis. In general, increasing ripening temperature and time resulted in increases of proteolysis products, notably higher levels being observed in experimental cheeses initially stored at 20 °C for 4 weeks. Principal component analysis showed that those cheeses at 124 days of ripening had similar levels of proteolysis products to the control cheeses at 180 days of ripening. In conclusion, promising results related to the proteolysis event in Reggianito cheese were obtained, which may help in the selection of a convenient elevated temperature–time combination for accelerating its ripening.

1. Introduction

Proteolysis is considered the most complex, and for many varieties of cheese, the most important event among all chemical and biochemical pathways occurring in cheese during ripening (Forde & Fitzgerald, 2000). The process is characterised by a sequential breakdown of the caseins into smaller fragments. In a primary proteolysis, caseins are broken into large and medium sized peptides, mainly as a consequence of the action of residual chymosin and plasmin. In a secondary proteolysis, a subsequent degradation of those peptides, mainly due to the action of proteases and peptidases coming from different microorganisms occurring in cheese, releases small peptides and free amino acids, which in turn can be degraded to catabolic products (McSweeney & Sousa, 2000).

Several attempts have been made in order to accelerate cheese ripening, since this is a long and thus expensive step of cheese manufacturing, particularly in low moisture, slow-ripening varieties (Folkertsma, Fox, & McSweeney, 1996). Increasing storage temperature is the simplest method available for the acceleration of cheese ripening period, and it has the additional benefit of potential savings resulting from lower refrigeration costs (O'Mahony, Sheehan, Delahunty, & McSweeney, 2006). Main drawbacks mentioned for this technology are an increased risk of development of undesirable microorganisms and non-specific increases in ripening reactions that can lead to the presence of unbalanced flavours or off-flavours, together with potential body defects such as softening or crumbliness (Law, 2001).

Reggianito cheese is the most important hard cheese variety mainly produced in the central and east-central regions of Argentina (known as the Pampas region), being extensively consumed locally and also exported to several countries worldwide. Its origin is an adaptation of cheesemaking technologies of hard Italian cheeses Grana Padano and Parmigiano Reggiano, brought to the country by Italian immigrants in the late 19th and early 20th centuries. It is manufactured with pasteurised cow milk and natural whey starter is used, mainly composed of *Lactobacillus helveticus* (66%) and *Lactobacillus delbrueckii* subsp. *lactis* (33%) (Reinheimer, Quiberoni, Tailliez, Binetti, & Suárez, 1996), and generally is ripened at 11–13 °C and 82–85% relative humidity. According to CAA (2006), Reggianito must have a cylindrical shape, with 5–10 kg weight, low moisture content (<35.9 g/100 g cheese) and a minimum ripening time of 6 months. In relation to ripening acceleration of Reggianito cheese, different studies have been carried out to assess the impact of an elevated storage temperature of 18 °C for 6 months on the lipolysis (Sihufe et al., 2007), proteolysis (Sihufe, Zorrilla, & Rubiolo, 2010) and sensory characteristics (Sihufe, Zorrilla, Sabbag, Costa, & Rubiolo, 2010), as well as a statistical analysis taking into account all that information (Sihufe, Zorrilla, Perotti, et al., 2010). In addition to characterise main transformations occurring during ripening, those studies allowed establishing an optimal ripening period between 2 and 3 months when cheeses are stored at 18 °C.

As claimed in previous works (Aston, Fedrick, Durward, & Dulley, 1983; Ferrazza, Fresno, Ribeiro, Tornadijo, & Mansur Furtado, 2004; Hannon et al., 2005; O'Mahony et al., 2006), application of high

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storage temperatures during the initial stages of ripening appears to cause a moderate and equilibrated increase in the activity of enzymatic systems, whereas a reduction in the risk of microbiologic contamination or unbalance in biochemical reactions could be achieved. Consequently, application of higher initial storage temperatures emerges as an interesting alternative instead of treatments based on ripening at high temperatures during the whole ripening period. In the present study, our objective was to evaluate the effect of different temperature–time combinations during ripening on the proteolysis in Reggianito cheese.

2. Materials and methods

2.1. Cheese sampling and ripening conditions

Twenty cheeses (7.8 ± 0.1 kg weight, 23.7 ± 0.2 cm diameter, 15.3 ± 0.2 cm height) manufactured with milk from the same cheese vat and by a standard cheese making procedure were brought from a local factory to our laboratory. Two cheeses were used to determine their initial composition, while the other 18 cheeses were stored at 3 different temperature–time combinations for 6 months. As listed in Table 1, 6 cheeses were ripened at 12 °C and 85% relative humidity, while the other 12 cheeses were ripened at two different temperature–time combinations, also at 85% relative humidity. Cheeses were analysed in duplicate at 61, 124 and 180 days of ripening.

2.2. Physicochemical analysis

Samples were completely grated and analysed to determine the moisture and chloride content (Zorrilla & Rubiolo, 1994), total nitrogen (TN), water-soluble nitrogen at pH 4.6 (WSN) (Sihufe, Zorrilla, & Rubiolo, 2003). For pH determination, a pH electrode for solid foods was used (pH Spear, Oakton Instruments, Vernon Hills, IL, USA). Fat content was determined for initial composition (International Dairy Federation, 1969). Maturation index (MI) was expressed as a percentage of WSN of the cheese TN (WSN × 100/TN). Determinations were carried out in duplicate, except for chloride content that was determined in triplicate.

2.3. Electrophoretic analysis

Electrophoretic analysis was performed as described by Sihufe, Zorrilla, and Rubiolo (2010). Cheese fractions were obtained dissolving grated cheese (3 g) in 25 mL of 8.66 mol/L urea, in a procedure including fat remotion by cold filtration and centrifugation. Electrophoretic runs of the fractions were made on vertical discontinuous polyacrylamide gels using anodic buffers. The current was set at a constant value of 50 mA and Coomasie blue R250 was used to stain the gels. Stained gels were scanned, and using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA) images were processed to obtain relatives areas for each band of interest. Standards of α_{s1}-casein and β-casein (Sigma Chemical Co., St Louis, MO, USA) were run in 2 lanes of each gel.

2.4. Peptide analysis

The extraction of the water-soluble fraction at pH 4.6 (WSF) was performed as suggested by Sihufe et al. (2003). The WSF (100 μL) filtered through a disposable 0.2-μm filter were injected in the chromatograph. Equipment and chromatographic conditions for peptide analysis by RP-HPLC were the same as described by Sihufe, Zorrilla, and Rubiolo (2010), but separation was performed on a Microsorb-MV (250 × 4.6 mm) C18, 300 Å column (Varian Inc., Palo Alto, CA, USA).

Table 1
Summary of the ripening conditions used.

Cheese code	Ripening conditions
C	12 °C for 6 months
E_1	20 °C for 2 weeks followed by 12 °C up to 6 months
E_2	20 °C for 4 weeks followed by 12 °C up to 6 months

2.5. Free amino acid analysis

Soluble fractions in 2.5 g/100 mL sulfosalicylic acid (SSA-SF) were obtained from the WSF. Free amino acids were determined in the SSA-SF using the derivatizing procedure with o-phtaldialdehyde (OPA) as described by Verdini, Zorrilla, and Rubiolo (2002). The resulting solution was filtered through a disposable 0.2-μm filter and 10 μL of the extract were injected. A Waters chromatography system (Waters Corporation, Milford, MA, USA) was used, which consisted of: Waters 1525 Binary HPLC Pump, Waters 717plus autosampler, FL-2 fluorescence detector (Isco, Inc., Lincoln, NE, USA) and Waters Breeze System software. A Microsorb-MV (250 × 4.6 mm) C18, 100 Å column (Varian Inc., Palo Alto, CA, USA) at 40 °C was used for chromatographic separations. Separations were carried out at a flow rate of 1.3 mL/min using solvent A: tetrahydrofuran:methanol: 0.05 mol/L sodium acetate pH 5.9 (1:19:80), and solvent B: methanol: 0.05 mol/L sodium acetate pH 5.9 (80:20) (Jones, Pääbo, & Stein, 1981). The gradient program 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 75% B in 6 min, isocratic step at 75% B for 4 min, linear step to 100% B in 6 min, and isocratic step at 100% B for 4 min. Amino acids were identified according to their retention times by comparison with a standard solution chromatogram.

2.6. Statistical analysis

For statistical analysis, ripening conditions and ripening time were selected as main factors for ANOVA, performed using Statgraphics (Statgraphics Inc., Rockville, MD, USA). When differences between treatment effects were significant ($P<0.05$), a multiple comparison of means was performed using the Least Significant Differences (LSD) test. Principal component analysis (PCA) was applied to reduce the dimensionality of data obtained from chromatograms. Essentially, PCA provides the means to reduce the often large number of interdependent (correlated) variables (e.g. peak areas) represented in the original data set to a few independent (uncorrelated) variables or principal components that are linear combinations of the original variables and explain most of the variation in the original data set (Coker, Crawford, Johnston, Singh, & Creamer, 2005). This analysis was carried out using Minitab (Minitab Inc., State College, PA, USA). Moreover, PCA with the correlation matrix was used. In this form of PCA, data set is scaled before the analysis (each variable has a mean of zero and a standard deviation of one). In this way, the variables are given equal weighting but capturing the effect of all the variables rather the few variables with a comparatively large internal variance (Coker et al., 2005).

3. Results and discussion

3.1. Physicochemical characteristics

The initial composition of cheeses was 40.1 ± 0.2% (w/w) moisture, 20.8 ± 1.5% (w/w) fat, 33.1 ± 0.3% (w/w) protein, nondetectable chloride, and the pH was 5.24 ± 0.02. Table 2 shows the average values for pH, moisture content, chloride content and maturation index obtained during ripening of Reggianito cheese for the treatments studied. There have been only slight differences in pH values during ripening. Final pH values were similar to those previously referred by

Table 2
Average values and standard deviation corresponding to physicochemical parameters determined during Reggianito cheese ripening.

Cheese	Time (days)	pH	Moisture (g/100 g cheese)	Chloride (g/100 g cheese)	MI (%)
C	61	5.54 ± 0.01^{cd}	37.9 ± 0.0^{a}	0.55 ± 0.04^{a}	12.8 ± 0.1^{a}
	124	5.36 ± 0.03^{a}	36.9 ± 0.1^{c}	0.88 ± 0.01^{c}	17.5 ± 1.0^{c}
	180	5.70 ± 0.02^{ef}	36.1 ± 0.4^{e}	1.04 ± 0.01^{d}	20.7 ± 1.2^{d}
E_1	61	5.56 ± 0.04^{d}	37.7 ± 0.1^{ab}	0.59 ± 0.02^{a}	14.8 ± 0.1^{b}
	124	5.41 ± 0.01^{ab}	36.7 ± 0.5^{cd}	0.92 ± 0.05^{c}	19.8 ± 0.8^{d}
	180	5.76 ± 0.02^{fg}	35.8 ± 0.1^{e}	1.08 ± 0.01^{d}	23.0 ± 0.5^{e}
E_2	61	5.60 ± 0.10^{de}	37.2 ± 0.1^{bc}	0.70 ± 0.02^{b}	17.2 ± 0.8^{c}
	124	5.45 ± 0.01^{bc}	36.3 ± 0.2^{de}	0.92 ± 0.01^{c}	20.9 ± 0.2^{d}
	180	5.83 ± 0.02^{g}	35.9 ± 0.0^{e}	1.06 ± 0.00^{d}	25.0 ± 1.5^{f}
Ripening condition		*	*	*	*
Time		*	*	*	*
Interaction		NS	NS	*	NS

Last rows show the ANOVA result for the different factors analysed.
[a–g]Average values in the same column with different letters are significantly different ($P<0.05$).
*Significant effect ($P<0.05$). NS: No significant effect ($P>0.05$).

other authors for this type of cheese (Hynes, Bergamini, Suárez, & Zalazar, 2003; Sihufe et al., 2007; Wolf, Perotti, Bernal, & Zalazar, 2010) and were in a range considered as safe in relation to potential growth of unusual microorganisms such as moulds.

ANOVA showed that both temperature–time combination and ripening time were significant factors affecting moisture and chloride contents. Temperature–time combination modified both salt and moisture distribution at the initial ripening stages, but chloride content evolved with an increasing trend with time while moisture content showed a decreasing one, which is characteristic for cheeses salted by brine immersion and ripened without wrapping (Simal, Sánchez, Bon, Femenia, & Rosselló, 2001). Additionally, moisture and chloride contents were similar for all cheeses towards the end of the ripening period, reaching final levels approximately of 36% and 1%, respectively. Similar values were reported in previous works for this cheese variety (Sihufe et al., 2007; Wolf et al., 2010).

The MI values were significantly affected by temperature–time combination and ripening time. Values corresponding to cheeses stored at 12 °C during the whole period were similar to those reported by other authors for this cheese (Hynes et al., 2003; Sihufe et al., 2007; Wolf et al., 2010). A significant increase in MI was observed with time and temperature–time combination, values at any given storage time being notably higher for experimental (E_1 and E_2) than for control cheeses. Furthermore, MI values for experimental cheeses at 124 days of ripening were found similar to those for control cheeses at 180 days of ripening (Table 2).

3.2. Electrophoretic analysis

Using the urea-PAGE method, identification and quantification of 5 casein fractions with different electrophoretic mobilities were possible (Fig. 1). Casein fractions shown are labelled according to Sihufe, Zorrilla, and Rubiolo (2010). In Table 3, values of integrated optical density (IOD) for all analysed fractions are shown. These values were informed as a relation between the IOD values with respect to the initial IOD value corresponding to each fraction. Hence, values less than 1 indicate a decrease and values greater than 1 indicate an increase on each fraction with respect to initial values.

The principal casein fractions, α_{S1}- and β-casein, decreased with ripening time, especially during the first 124 days of ripening (Table 3). In the case of α_{S1}-casein, the decrease occurring during the first 2 months was accompanied by an increase in the α_{S1}-I and F3 fractions, which are originated as a consequence of α_{S1}-casein hydrolysis. The α_{S1}-casein degradation was significantly affected by the temperature–time combination, occurring a more extensive degradation in cheeses E_2 than in cheeses E_1 at the same ripening time. Furthermore, it was observed a greater (cheeses E_2) or equal (cheeses E_1) degradation in this fraction at 124 days of ripening with respect to that observed towards the end of the ripening period in control cheeses. Even at 61 days of ripening, cheeses E_2 showed a decrease in levels of α_{S1}-casein that was similar to that observed in control cheeses at 180 days of ripening (Table 3).

Levels of α_{S1}-I-casein notably increased at the beginning of the period under study, to decrease later with ripening time. This behaviour can be explained taking into account that α_{S1}-I-casein is the fragment (f24–199) having its origins in α_{S1}-casein initial cleavage, but that is in turn susceptible to suffer proteolytic attack by different enzymes, resulting in smaller fragments (Fox & McSweeney, 1996). Starting from 124 days of ripening, a greater degradation of α_{S1}-I-casein was observed for experimental cheeses, especially in cheeses E_2, suggesting a greater liberation of low-molecular-weight peptides that can act as substrates in subsequent reactions.

The decrease in β-casein resulted in an important increase in γ-caseins, which are produced from this protein by the action of plasmin (Fox & McSweeney, 1996). The levels of β-casein degradation were also affected significantly by the temperature–time combination, being higher in experimental cheeses than in control cheeses, without significant differences in values between cheeses E_1 and E_2. Similarly to α_{S1}-casein behaviour, the level of protein degradation towards 61 or 124 days of ripening in experimental cheeses was similar to the level observed for control cheeses at 124 or 180 days of ripening, respectively. Finally, a significant increase in γ-caseins during the first 61 days of the storage period of cheeses was observed, remaining then practically with no changes until 180 days of ripening, even though β-casein degradation did not stop. The cheeses E_2 showed γ-casein values slightly greater than in control cheeses towards the 61 days of ripening, while there were no significant differences for the treatments studied for the rest of the period.

3.3. Peptide analysis by RP-HPLC

RP-HPLC of water-soluble extracts is among the methods most frequently used for the characterisation of secondary proteolysis in cheese. It is considered to be highly discriminant and it is one of the most valuable techniques for assessing authenticity and quality of cheese (Parente, Patel, Caldeo, Piraino, & McSweeney, 2012). In this study, chromatographic profiles were similar to those reported by another authors for Reggianito cheese (Hynes, Aparo, & Candioti, 2004; Sihufe, Zorrilla, & Rubiolo, 2010). Sixteen peaks totalizing about 90% of area under the curve of chromatograms obtained by RP-HPLC of WSF were selected, eluting between 4 and 96 min (Fig. 2). ANOVA showed that 14 out of the 16 peaks were significantly affected ($P<0.05$) by ripening time, while 12 were significantly affected by the temperature–time combination. Similar chromatographic patterns were obtained for samples corresponding to cheeses C, E1 and E2 (Fig. 2). The differences observed were only related to different height of the peaks, indicating that the ripening conditions studied affect the rate of proteolysis rather than the pathways of proteolysis.

The total area of the 16 peaks was also significantly affected by both ripening time and temperature–time combination. Total area increased significantly during ripening time. Higher total area values were observed for cheeses E_2 than for cheeses E_1, and for the later with respect to control cheeses, with the exception of 124 days of ripening, where the area for experimental cheeses E_1 and E_2 were similar. That total area level at 124 days of maturation was similar to that of control cheeses at 180 days of ripening. These findings are similar to that corresponding to the MI evolution. It is worth mentioning that MI value is associated with the water soluble fraction at pH 4.6 that consists of proteins, peptides and free amino acids resulting from proteolysis during cheese ripening.

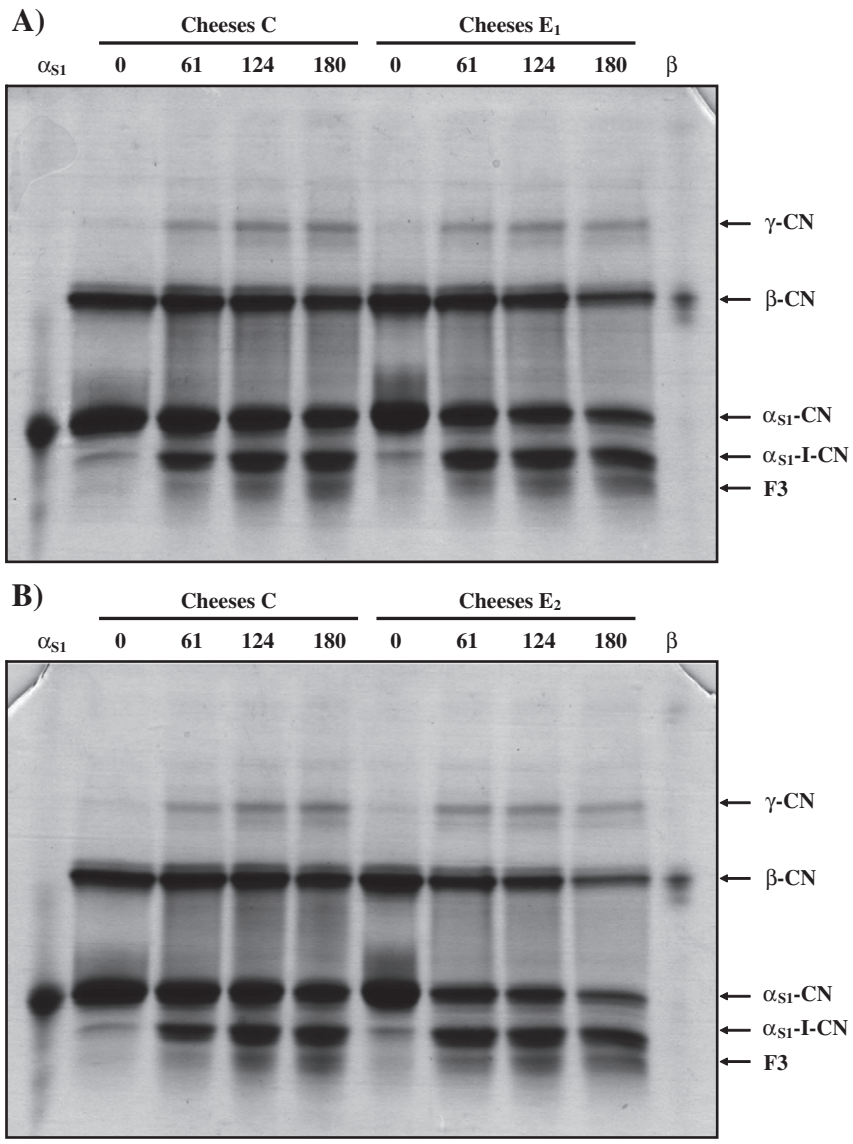

Fig. 1. Urea-PAGE electrophoretograms of Reggianito cheese corresponding to: A) cheeses C and E_1; and B) cheeses C and E_2. First slot: α_{S1}-casein standard; last slot: β-casein standard. Numbers indicate days of ripening.

Table 3
Average values and standard deviation corresponding to integrated optical density referred to its initial values (IOD/IOD_0) for the fractions analysed by urea-PAGE during Reggianito cheese ripening.

Cheese	Time (days)	α_{S1}-casein	α_{S1}-I-casein	β-casein	γ-casein	F3
C	61	0.83 ± 0.03^{e}	10.1 ± 0.3^{bcd}	0.95 ± 0.00^{e}	3.2 ± 0.2^{a}	1.4 ± 0.4^{ab}
	124	0.55 ± 0.05^{d}	13.2 ± 1.1^{e}	0.66 ± 0.04^{d}	5.3 ± 0.0^{cd}	2.5 ± 0.4^{cd}
	180	0.32 ± 0.08^{bc}	9.4 ± 1.7^{bc}	0.39 ± 0.07^{bc}	5.6 ± 0.1^{d}	3.0 ± 0.9^{d}
E_1	61	0.58 ± 0.09^{d}	11.2 ± 1.6^{cde}	0.50 ± 0.08^{c}	3.5 ± 0.1^{a}	0.8 ± 0.1^{a}
	124	0.41 ± 0.02^{c}	10.8 ± 0.5^{cde}	0.32 ± 0.00^{ab}	4.8 ± 0.4^{bc}	1.7 ± 0.1^{abc}
	180	0.35 ± 0.02^{bc}	8.9 ± 2.1^{abc}	0.24 ± 0.03^{a}	5.3 ± 0.3^{cd}	2.3 ± 0.7^{bcd}
E_2	61	0.28 ± 0.04^{b}	13.0 ± 1.1^{de}	0.66 ± 0.07^{d}	4.2 ± 0.0^{b}	1.1 ± 0.0^{a}
	124	0.16 ± 0.02^{a}	7.4 ± 1.9^{ab}	0.38 ± 0.02^{b}	5.1 ± 0.7^{bc}	1.4 ± 0.2^{ab}
	180	0.13 ± 0.01^{a}	6.1 ± 0.6^{a}	0.26 ± 0.02^{a}	5.6 ± 0.3^{d}	1.5 ± 0.1^{abc}
Ripening condition		*	NS	*	NS	*
Time		*	*	*	*	*
Interaction		*	*	*	NS	NS

Last rows show the ANOVA result for the different factors analysed.
[a–e]Average values in the same column with different letters are significantly different ($P<0.05$).
*Significant effect ($P<0.05$). NS: No significant effect ($P>0.05$).

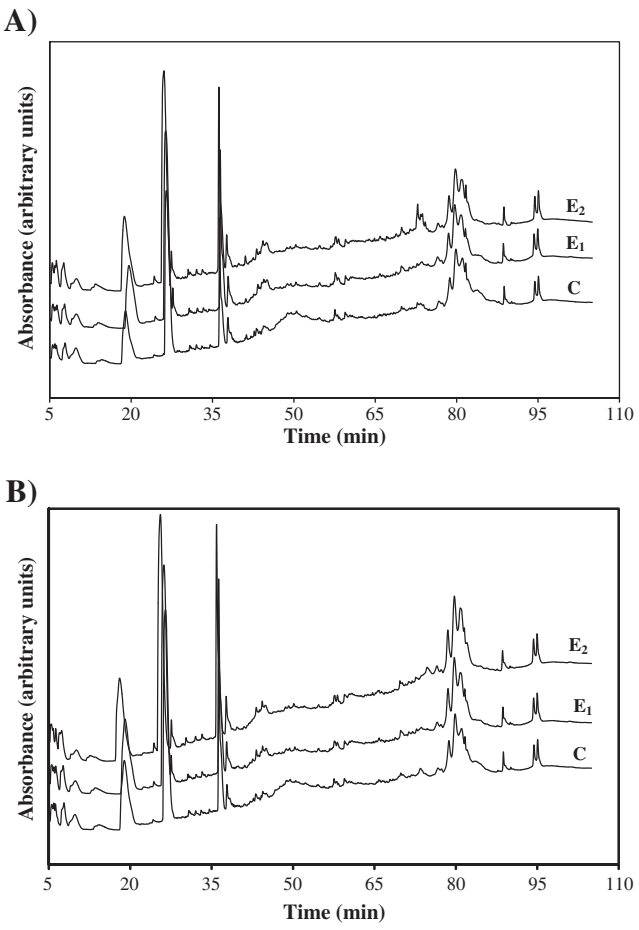

Fig. 2. RP-HPLC chromatograms of the WSF at pH 4.6 for peptide analysis of Reggianito cheese ripened under different temperature–time combinations at: A) 124 days of ripening; and B) 180 days of ripening.

3.4. Free amino acid analysis

Free amino acid concentrations changing during ripening of Reggianito cheese are shown in Table 4. Using calibration curves with standards, 14 amino acids were determined on chromatographic runs. The pairs Ser + His and Thr + Arg coeluted, and they were not quantified.

Higher values of the amino acids Glu, Lys, Leu and Val were observed in all cheese samples, representing altogether more than 50% of total amino acid concentration, individual concentrations being equal or higher than 200 mg/100 g cheese at 6 months of ripening in all cheese samples (Table 4). Similar predominance pattern of these amino acids was observed in previous works dealing with amino acid profiles in Reggianito cheese (Hynes, Zalazar, & McSweeney, 2005; Sihufe, Zorrilla, & Rubiolo, 2010). In several cheese varieties, Glu makes great contribution to umami basic taste, thus acting as a flavour-enhancer (Drake et al., 2007). High levels of Glu have been repeatedly found in Reggianito cheese, therefore suggesting a significant influence of this compound on flavour intensity of this cheese variety.

ANOVA showed that ripening time and temperature–time combination significantly affected the concentrations of all the amino acids studied, except for Gln and Lys that were not affected by temperature–time combination. The concentration of all amino acids increased with ripening time. Concerning temperature–time combination, higher levels of most amino acids analysed were found in cheeses E_2 with respect to cheeses E_1 and C at 180 days of ripening. Total amino acid contents in cheeses E_1 and E_2 at 124 days of ripening were similar to

Table 4
Average concentrations (mg/100 g cheese) and standard deviations of free amino acids determined during Reggianito cheese ripening.

Amino acid	Cheeses C			Cheeses E_1			Cheeses E_2			Ripening condition	Time	Interaction
	61 days	124 days	180 days	61 days	124 days	180 days	61 days	124 days	180 days			
Asp	43.8 ± 2.4^{a}	69.0 ± 6.0^{b}	108.8 ± 26.2^{cd}	51.6 ± 2.9^{ab}	94.9 ± 2.1^{c}	120.3 ± 5.1^{d}	60.2 ± 11.8^{ab}	70.0 ± 3.8^{b}	150.8 ± 9.3^{e}	*	*	*
Glu	355.5 ± 12.9^{a}	512.9 ± 58.7^{bc}	675.8 ± 143.3^{de}	388.4 ± 12.1^{ab}	611.7 ± 16.3^{cde}	737.5 ± 35.4^{e}	486.8 ± 70.8^{abc}	556.7 ± 7.5^{cd}	963.4 ± 64.1^{f}	*	*	NS
Asn	108.6 ± 3.1^{a}	150.3 ± 17.1^{bcd}	186.5 ± 38.6^{de}	121.4 ± 1.6^{ab}	178.2 ± 5.4^{cde}	205.9 ± 9.7^{e}	144.5 ± 18.9^{abc}	159.3 ± 2.8^{bcd}	258.0 ± 20.8^{f}	*	*	NS
Gln	118.5 ± 8.1^{a}	157.5 ± 24.6^{abc}	178.5 ± 44.6^{c}	128.3 ± 3.1^{ab}	170.0 ± 6.0^{bc}	184.2 ± 1.7^{cd}	147.0 ± 8.8^{abc}	162.7 ± 3.5^{abc}	227.2 ± 31.9^{d}	NS	*	NS
Gly	37.5 ± 1.9^{a}	63.1 ± 6.7^{b}	82.8 ± 14.7^{cde}	42.2 ± 2.2^{a}	71.4 ± 2.4^{bcd}	97.5 ± 12.1^{e}	65.6 ± 4.6^{bc}	85.9 ± 3.6^{de}	143.3 ± 11.8^{f}	*	*	NS
Ala	78.2 ± 0.3^{a}	97.9 ± 9.4^{ab}	116.7 ± 24.1^{bc}	77.1 ± 1.1^{a}	111.1 ± 3.0^{bc}	123.9 ± 7.0^{c}	106.8 ± 14.0^{bc}	109.4 ± 5.5^{bc}	172.8 ± 4.2^{d}	*	*	*
Tyr	45.6 ± 4.8^{a}	60.1 ± 16.4^{ab}	85.6 ± 11.4^{bc}	47.4 ± 5.1^{a}	71.2 ± 2.1^{abc}	89.7 ± 5.7^{c}	70.2 ± 3.0^{abc}	83.8 ± 8.2^{bc}	145.2 ± 25.4^{d}	*	*	NS
Trp	16.6 ± 0.3^{a}	38.1 ± 1.7^{d}	46.7 ± 2.8^{e}	22.0 ± 2.1^{b}	41.0 ± 0.4^{d}	48.4 ± 0.2^{e}	31.6 ± 1.6^{c}	48.8 ± 0.1^{e}	70.2 ± 0.3^{f}	*	*	*
Met	39.3 ± 1.7^{a}	63.2 ± 6.6^{b}	83.2 ± 15.9^{d}	44.5 ± 1.1^{a}	75.2 ± 2.0^{bcd}	90.4 ± 4.4^{d}	65.4 ± 8.5^{bc}	78.9 ± 0.2^{cd}	131.7 ± 4.7^{e}	*	*	*
Val	133.2 ± 5.4^{a}	193.1 ± 21.8^{ab}	249.9 ± 60.5^{bc}	151.3 ± 3.2^{a}	238.3 ± 9.6^{bc}	280.2 ± 14.8^{c}	190.8 ± 29.4^{ab}	216.1 ± 6.4^{b}	385.7 ± 30.5^{d}	*	*	NS
Phe	84.5 ± 4.2^{a}	138.7 ± 15.8^{b}	182.5 ± 39.0^{c}	96.6 ± 4.1^{a}	161.4 ± 5.7^{bc}	192.2 ± 12.2^{c}	161.4 ± 22.6^{bc}	184.3 ± 3.0^{c}	307.0 ± 22.3^{d}	*	*	*
Ile	88.1 ± 2.5^{a}	143.1 ± 16.4^{bc}	205.4 ± 45.6^{d}	101.8 ± 5.9^{ab}	173.8 ± 10.7^{cd}	215.8 ± 7.5^{d}	139.3 ± 19.1^{bc}	171.6 ± 2.5^{cd}	308.2 ± 24.7^{e}	*	*	NS
Leu	174.1 ± 4.8^{a}	237.3 ± 28.6^{ab}	303.1 ± 63.1^{bc}	188.3 ± 6.0^{a}	277.0 ± 10.2^{bc}	316.3 ± 16.9^{c}	294.2 ± 44.0^{bc}	306.3 ± 0.7^{c}	492.8 ± 30.6^{d}	*	*	NS
Lys	274.9 ± 3.6^{a}	404.3 ± 44.8^{ab}	522.0 ± 134.6^{bc}	267.6 ± 15.0^{a}	424.6 ± 13.6^{ab}	517.0 ± 27.9^{bc}	283.6 ± 130.5^{a}	493.8 ± 3.3^{abc}	658.6 ± 233.3^{c}	NS	*	NS
Total	1598.6 ± 52.2^{a}	2328.5 ± 274.5^{abc}	3381.2 ± 745.2^{de}	1949.6 ± 68.5^{a}	3018.2 ± 81.6^{cde}	3585.7 ± 163.5^{e}	2247.3 ± 126.4^{ab}	2727.6 ± 26.7^{bcd}	4414.9 ± 513.9^{f}	*	*	NS

Last columns show the ANOVA result for the different factors analysed.
[a–f]Average values in the same row with different letters are significantly different ($P<0.05$).
*Significant effect ($P<0.05$). NS: No significant effect ($P>0.05$).

that content in control cheeses at 180 days of ripening (Table 4). As can be seen in Fig. 3, similar free amino acid distribution patterns were observed for cheeses C, E_1 and E_2 at the final ripening stages.

3.5. Principal component analysis

Principal component analysis (PCA) was applied to the 16 peaks from the RP-HPLC analysis of the WSF and the 14 free amino acids studied, together with MI values. The first 2 principal components contained the meaningful variance in the data set, representing 87.0% of the total variance. Biplot of the first 2 principal components is shown in Fig. 4.

The first principal component (80.8% VAR) can be related to ripening time, due to the samples spread from left to right according to ripening time. On the right of the plot, it can be observed a grouping of amino acids and almost all peptides with a positive PC1 loading value, showing an increasing trend of their levels in cheese with time, whereas peak 9, with a decreasing trend along with time, has a negative PC1 loading. On the other hand, PC2 (6.2% VAR) showed, despite some group overlapping, a slight separation of samples according to temperature–time combination, with positive average PC2 scores for cheeses E_2, lower negative PC2 scores for cheeses E_1, and intermediate, close to zero PC2 values for cheeses C.

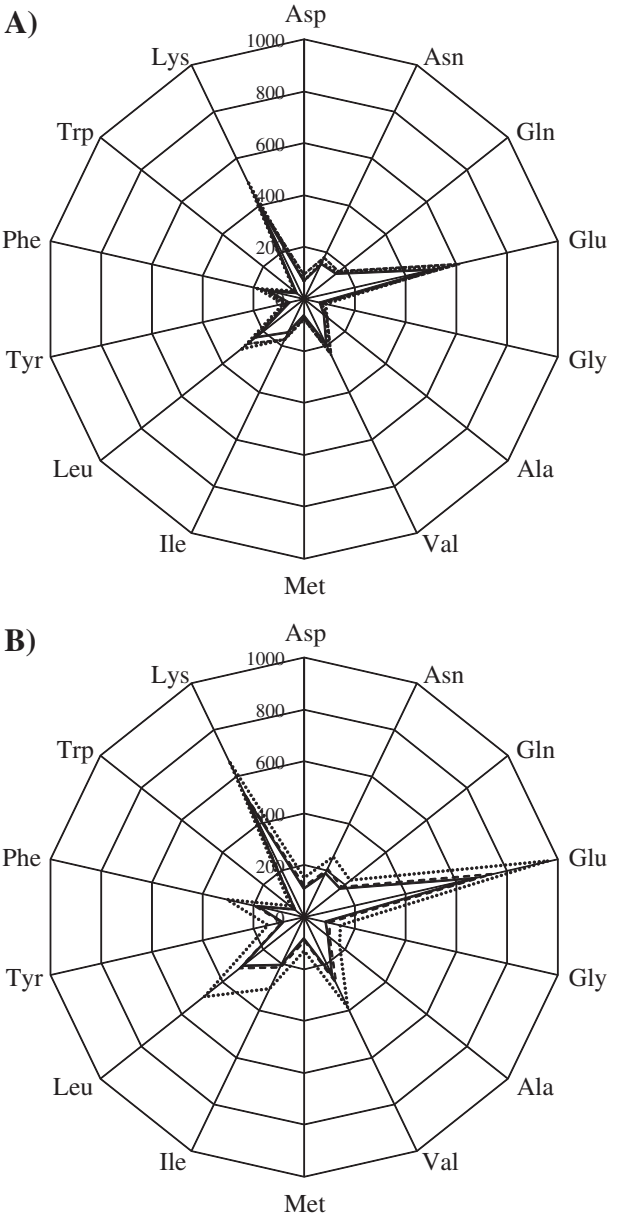

Fig. 3. Levels of individual free amino acids (mg/100 g cheese) determined for Reggianito cheeses at: A) 124 days of ripening; and B) 180 days of ripening. (—) cheeses C; (- - -) cheeses E_1; and (…) cheeses E_2.

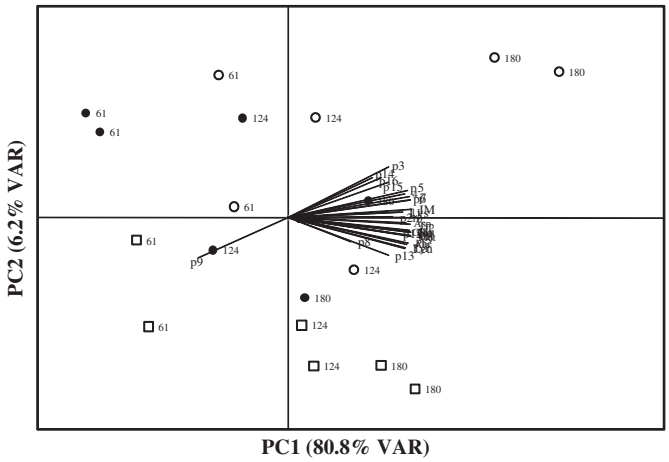

Fig. 4. Biplot of scores and loadings of data corresponding to MI and RP-HPLC chromatograms for peptides and free amino acids in Reggianito cheese samples: (•) cheeses C; (□) cheeses E_1; and (O) cheeses E_2. Numbers close to symbols indicate days of ripening.

Taking into account the relative position of the experimental cheeses on the biplot to the position of the control cheeses, it can be considered that cheeses E_2 at 124 days of ripening had an equivalent ripening time to control cheeses at 180 days of ripening (Fig. 4).

4. Conclusions

Taking into account the levels of proteolysis products studied, the use of an initial elevated ripening temperature resulted in a controlled acceleration of Reggianito cheese maturation. In general, the concentrations of major caseins decreased while the levels of peptides and amino acids increased at higher rates in experimental cheeses but following a similar pattern than in control cheeses, indicating that the initial elevation of ripening temperature may be an adequate strategy to accelerate Reggianito cheese ripening. The results of our study are very encouraging and will complement other important areas such as lipolysis or sensory analysis to determine an appropriate temperature–time combination for accelerating Reggianito cheese ripening.

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References

Aston, J. W., Fedrick, I. A., Durward, I. G., & Dulley, J. R. (1983). The effect of elevated ripening temperatures on proteolysis and flavour development in Cheddar cheese I: Higher initial storage temperatures. *New Zealand Journal of Dairy Science and Technology*, *18*, 143–151.

CAA (2006). Código Alimentario Argentino. [Accessed 2011 Nov 30]. Available from:. http://www.anmat.gov.ar/alimentos/normativas_alimentos_caa.asp

Coker, C. J., Crawford, R. A., Johnston, K. A., Singh, H., & Creamer, L. K. (2005). Towards the classification of cheese variety and maturity on the basis of statistical analysis of proteolysis data — a review. *International Dairy Journal*, *15*, 631–643.

Drake, S. L., Carunchia Whetstine, M. E., Drake, M. A., Courtney, P., Fligner, K., Jenkins, J., et al. (2007). Sources of umami taste in Cheddar and Swiss cheeses. *Journal of Food Science*, *72*(6), S360–S366.

Ferrazza, R. E., Fresno, J. M., Ribeiro, J. I., Tornadijo, M. E., & Mansur Furtado, M. (2004). Changes in the microbial flora of Zamorano cheese (P.D.O.) by accelerated ripening process. *Food Research International*, *37*, 149–155.

Folkertsma, B., Fox, P. F., & McSweeney, P. L. H. (1996). Accelerated ripening of Cheddar cheese at elevated temperatures. *International Dairy Journal*, *6*, 1117–1134.

Forde, A., & Fitzgerald, G. F. (2000). Biotechnological approaches to the understanding and improvement of mature cheese flavour. *Current Opinion in Biotechnology*, *11*, 484–489.

Fox, P. F., & McSweeney, P. L. H. (1996). Proteolysis in cheese during ripening. *Food Review International*, *12*(4), 457–509.

Hannon, J. A., Wilkinson, M. G., Delahunty, C. M., Wallace, J. M., Morrissey, P. A., & Beresford, T. P. (2005). Application of descriptive sensory analysis and key chemical indices to assess the impact of elevated ripening temperatures on the acceleration of Cheddar cheese ripening. *International Dairy Journal*, *15*, 263–273.

Hynes, E., Aparo, L., & Candioti, M. C. (2004). Influence of residual milk-clotting enzyme on α_{s1} casein hydrolysis during ripening of Reggianito Argentino cheese. *Journal of Dairy Science*, *87*, 565–573.

Hynes, E., Bergamini, C. V., Suárez, V. B., & Zalazar, C. A. (2003). Proteolysis on Reggianito Argentino cheeses manufactured with natural whey cultures and selected strains of *Lactobacillus helveticus*. *Journal of Dairy Science*, *86*, 3831–3840.

Hynes, E., Zalazar, C. A., & McSweeney, P. L. H. (2005). Influence of defined and natural 'wild' thermophilic cultures on proteolysis in Reggianito Argentino hard cooked cheese. *The Australian Journal of Dairy Technology*, *60*(3), 55–59.

International Dairy Federation (1969). *Determination of the fat content of cheese and of processed cheese products.* IDF Standard 5A. Brussels, Belgium: International Dairy Federation.

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

Law, B. A. (2001). Controlled and accelerated cheese ripening: The research base for new technologies. *International Dairy Journal*, *11*, 383–398.

McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Le Lait*, *80*, 293–324.

O'Mahony, J. A., Sheehan, E. M., Delahunty, C. M., & McSweeney, P. L. H. (2006). Lipolysis and sensory characteristics of Cheddar cheeses ripened using different temperature–time treatments. *Le Lait*, *86*, 59–72.

Parente, E., Patel, H., Caldeo, V., Piraino, P., & McSweeney, P. H. L. (2012). RP-HPLC peptide profiling of cheese extracts: A study of sources of variation, repeatability and reproducibility. *Food Chemistry*, *131*, 1552–1560.

Reinheimer, J. A., Quiberoni, A., Tailliez, P., Binetti, A. G., & Suárez, V. B. (1996). The lactic acid microflora of natural whey starters used in Argentina for hard cheese production. *International Dairy Journal*, *6*, 869–879.

Sihufe, G. A., Zorrilla, S. E., Mercanti, D. J., Perotti, M. C., Zalazar, C. A., & Rubiolo, A. C. (2007). The influence of ripening temperature and sampling site on the lipolysis in Reggianito Argentino cheese. *Food Research International*, *40*, 1220–1226.

Sihufe, G. A., Zorrilla, S. E., Perotti, M. C., Wolf, I. V., Zalazar, C. A., Sabbag, N. G., et al. (2010). Acceleration of cheese ripening at elevated temperature. An estimation of the optimal ripening time of a traditional Argentinean hard cheese. *Food Chemistry*, *119*, 101–107.

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*(1), 117–123.

Sihufe, G. A., Zorrilla, S. E., & Rubiolo, A. C. (2010). The influence of ripening temperature and sampling site on the proteolysis in Reggianito Argentino cheese. *LWT — Food Science and Technology*, *43*, 247–253.

Sihufe, G. A., Zorrilla, S. E., Sabbag, N. G., Costa, S. C., & Rubiolo, A. C. (2010). The influence of ripening temperature on the sensory characteristics of Reggianito Argentino cheese. *Journal of Sensory Studies*, *25*, 94–107.

Simal, S., Sánchez, E. S., Bon, J., Femenia, A., & Rosselló, C. (2001). Water and salt diffusion during cheese ripening: Effect of the external and internal resistances to mass transfer. *Journal of Food Engineering*, *48*, 269–275.

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*(9), 3264–3270.

Wolf, I. V., Perotti, M. C., Bernal, S. M., & Zalazar, C. A. (2010). Study of the chemical composition, proteolysis, lipolysis and volatile compounds profile of commercial Reggianito Argentino cheese: Characterization of Reggianito Argentino cheese. *Food Research International*, *43*, 1204–1211.

Zorrilla, S. E., & Rubiolo, A. C. (1994). Fynbo cheese NaCl and KCl changes during ripening. *Journal of Food Science*, *59*(5), 972–975 & 985.