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Food Chemistry 96 (2006) 297–303
Food Chemistry
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Secondary proteolysis of Fynbo cheese salted with NaCl/KCl brine and ripened at various temperatures

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Received 25 October 2004; received in revised form 15 February 2005; accepted 15 February 2005

Abstract

Effects of temperature and salt substitution on secondary proteolysis of Fynbo cheese were studied and different peaks of the chromatographic profiles were examined. Cheeses, salted in solutions of NaCl (190 g l^{-1}) and NaCl/KCl (100 g l^{-1}/100 g l^{-1}) and ripened at 5, 12, and 16 °C, were sampled during 90 days at two different zones. Samples were analysed by RP-HPLC of the trichloroacetic acid-soluble fraction. The information was successfully summarized in 2 dimensions, accounting for 86.5% of data variation using principal component analysis. The source of variation explained by PC1 (77.1% VAR) was related to the ripening time. Two groups of chromatographic peaks were distinguished according to the sign of PC2 loading. Total salt concentration and ripening temperature affected secondary proteolysis significantly, while NaCl replacement by KCl had no affect. An important peptide produced during cheese ripening (α_{s1}-casein (f1-23)) was tentatively identified, taking into account the chromatographic profile and the amino acid composition of the peak isolated.

Keywords: Fynbo cheese; NaCl/KCl; Ripening temperature; TCA-soluble fraction; RP-HPLC

1. Introduction

Proteolysis is the most complex and important event that occurs during ripening of a great number of cheese varieties (Fox & McSweeney, 1996). A part of the casein is converted by proteolysis into water-soluble nitrogenous compounds, such as peptides and amino acids, which contribute to the flavour and texture. Enzymes from coagulant, milk, starter bacteria, and non-starter microflora, catalyse proteolysis in the cheese (Fox, 1987). The activity of these agents varies due to medium conditions, such as salt type, salt content and temperature.

Among the principal effects of sodium chloride (NaCl) in cheese are: direct contribution to flavour, control of microbial growth and activity, control of the enzyme activities, syneresis of the curd, and physical changes in cheese proteins (Guinee & Fox, 1987). Sodium intake has become a key nutrition issue in recent years, due to the association of sodium with undesirable physiological effects such as hypertension. As a result, the food industry has responded to human dietary needs by providing processed foods, such as cheeses, with reduced amounts of sodium (Reddy & Marth, 1991). Potassium chloride (KCl) has been widely and successfully used to partially replace NaCl in cheese (Katsiari, Voutsinas, Alichanidis, & Roussis, 1998; Laborda, 2000; Reddy & Marth, 1993; Zorrilla, 1993). Ripening temperature influences the rate of proteolysis, composition of the cheese microflora, texture, and quality of cheese (Aston, Fedrick, Durward, & Dulley, 1983; Folkertsma, Fox, & McSweeney, 1996; Shakeel-Ur-Rehman, Banks, McSweeney, & Fox, 2000). Although NaCl

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doi:10.1016/j.foodchem.2005.02.026

replacement and temperature are important at the different cheese proteolysis stages, very little information about both factors together is available.

Fynbo is a semi-hard cheese, manufactured from cow's milk, of either regular or low fat content, commonly salted for 10 h at 12 °C in a 20% NaCl brine solution, and ripened for 30 days. The effect of the partial replacement of NaCl by KCl in Fynbo cheese was studied during ripening (Laborda & Rubiolo, 1999; Sihufe, Zorrilla, & Rubiolo, 2003; Zorrilla & Rubiolo, 1997, 1999). Proteolysis is essential in the ripening of Fynbo cheese. Zorrilla and Rubiolo (1997) did not find differences in the kinetics of α_{s1}-casein degradation of cheeses salted in a NaCl or NaCl/KCl brine and ripened at 12 °C during 30 days. Sihufe et al. (2003) determined the characteristic kinetic parameters for α_{s1}-casein degradation in Fynbo cheeses salted with NaCl and with a mixture of NaCl/KCl and ripened at 5, 12 and 16 °C by urea–polyacrylamide gel electrophoresis. Kinetic constants of the α_{s1}-casein degradation were in the range of 0.002–0.016 day^{-1}. As a result, a better understanding of the primary proteolysis was achieved.

Sihufe, Zorrilla, and Rubiolo (2004) analysed the water-soluble fraction (WSF) and 4% trichloroacetic acid-soluble sub-fraction (TCA–WSF) of Fynbo cheeses (regularly salted) by RP-HPLC. The authors found that peaks eluting after 30 min, in the chromatographic run, were more affected by the 4% TCA treatment. Although, it is not possible to determine a precipitation threshold relating the peptide size to the solubility in TCA, Yvon, Chabanet, and Pélissier (1989), working with different digests of α_{s1}-, β- and κ-casein, determined that all the peptides containing fewer than seven residues were soluble, even in 12% TCA. Thus, the TCA treatment helps in the reduction of the number of peptides and ensures the presence of small peptides in solution. Therefore, the RP-HPLC analysis of the TCA–WSF may be a suitable technique for studying the secondary proteolysis of Fynbo cheese. Moreover, an analysis of this fraction may help to more thoroughly examine different peptides and to give a better understanding of the effects of NaCl replacement and temperature on cheese proteolysis.

Our objectives in this work were to evaluate the effects of temperature and partial replacement of NaCl by KCl on the RP-HPLC chromatograms of the 4% TCA-soluble fraction obtained from the water-soluble fraction and to examine different peaks of the chromatographic profiles during ripening of Fynbo cheeses.

2. Materials and methods

2.1. Cheese sampling

Unsalted low-fat Fynbo cheeses (782.9 ± 27.4 g weight, 11.5 ± 0.3 cm diameter, 6.1 ± 0.2 cm height), manufactured on the same date from the same cheese milk, were brought from a local factory to our laboratory. Their initial composition was: 49.34 ± 0.29% (w/w) moisture, 29.7 ± 1.75% (w/w) protein, 12.56 ± 0.15% (w/w) fat, and the pH was 5.15–5.35. Zorrilla (1993) and Zorrilla and Rubiolo (1997) did not find significant differences between cheeses made from different cheese vats. Therefore, cheeses manufactured on the same date from the same cheese vat were used in this study to reduce the number of cheeses assayed. Twenty-one cheeses were salted for 10 h at 12 °C in a solution of 190 g NaCl l^{-1} (cheese S) and 21 cheeses in a solution of 100 g NaCl l^{-1} and 100 g KCl l^{-1} (cheese K). Both brines also contained 0.55% Ca^{2+} to prevent softening of the cheese rind (Geurts, Walstra, & Mulder, 1972). After brining, each cheese was wiped and packed under vacuum in a heat-shrinkable plastic bag. During ripening, batches of 7 cheeses S and 7 cheeses K were stored at 5, 12, or 16 °C. Although Fynbo cheese is traditionally ripened at the factory for 30 days, this study was extended for a further 60 days to consider changes that may occur during the shelf life of the product. Different cheeses were sampled at 1, 5, 10, 20, 30, 60 and 90 days. Slices (1 cm thickness) were cut parallel to the flat surface from the surface. Concentric rings of 9.9 cm minor diameter and 12 cm major diameter were cut from those slices (external zone, E). Slices (1.5 cm thickness) were cut parallel to the flat surface from the centre. Cylindrical cores of 4.8 cm diameter were cut from those slices (central zone, C). Samples were used to obtain the water-soluble fraction.

2.2. Aqueous extraction and fractionation with TCA

The WSF and the sub-fraction of WSF soluble in TCA 4% (TCA–WSF) were obtained as described by Sihufe et al. (2004).

2.3. RP-HPLC analysis of TCA–WSF

The TCA–WSF was filtered through 0.2 μm disposable filters (Alltech Associates, Inc., Deerfield, IL, USA) and 100 μl were analysed by RP-HPLC, as described by Sihufe et al. (2004).

2.4. Isolation and identification of peptides

An attempt at isolation and identification of some significant peaks was carried out by acid hydrolysis of collected peaks. Peaks were collected manually from successive chromatographic runs. Before hydrolysis, acetonitrile and water were removed by evaporation under vacuum and lyophilisation, respectively. Samples were hydrolysed in a Knauer Protein Hydrolyzer with Knauer Air Oven (Knauer-Vertretung Schweiz, Berlin, Germany) with 6 N HCl (sequanal grade) with constant

boiling (Pierce, Rockford, IL, USA) at 110 °C for 24 h. After hydrolysis, each sample was dissolved in water and the amino acid composition was determined by RP-HPLC, as described by Verdini, Zorrilla, and Rubiolo (2002).

2.5. Statistical analysis

Principal component analysis (PCA) was used to reduce the dimensionality of the data obtained from the RP-HPLC chromatograms. Data for PCA were obtained by visually recognizing peaks in the chromatograms and using the peak areas as variables.

Data were analysed by ANOVA. When differences between treatment effects were significant ($p < 0.05$), a multiple comparison of means was performed using Tukey's test. Statistical analysis was carried out using Minitab (Minitab Inc., State College, PA, USA).

3. Results and discussion

RP-HPLC chromatograms of the TCA–WSF from central and external zones of cheeses S and K, ripened at different temperatures and for different ripening times, were obtained. Typical chromatograms of the TCA–WSF, corresponding to 90 days of ripening for external zone of cheese K are shown in Fig. 1. Twenty-one peaks with significant chromatographic area were analysed, as suggested by Sihufe et al. (2004). The effect of ripening temperature was noticeable, mainly when the chromatographic profile at 5 °C was compared with those at 12 or 16 °C.

The peptide chromatographic profiles generally lead to a large amount of data. PCA is a useful tool for simplifying the analysis of the results. In this case, the information obtained from the chromatograms was successfully summarized in 2 dimensions (2D), accounting for 86.5% of the data variation using PCA. Fig. 2 shows the plot of samples in the plane defined by the two principal components. Cheeses ripened for 60 and 90 days are grouped in the left half of the Figure. A compact group in the right of the Figure includes most of the cheeses ripened for less than 60 days. The source of variation explained by PC1 (77.1% VAR) can be related to the ripening time, because the scattering of samples clearly increases as ripening time increases. Moreover, samples of cheeses ripened at 5 °C were less scattered, which may be related to a less pronounced proteolysis.

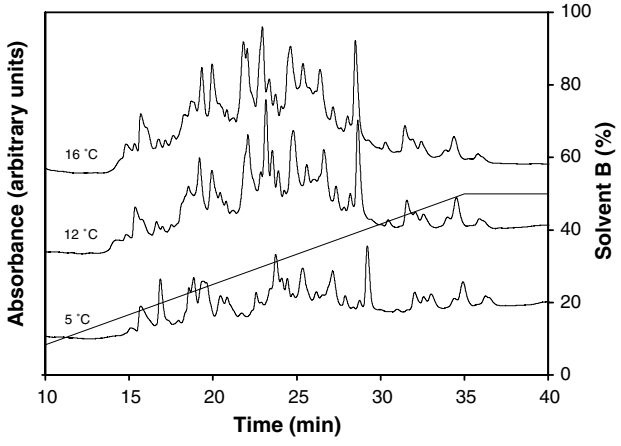

Fig. 1. RP-HPLC chromatograms of TCA–WSF from Fynbo cheeses stored at different temperatures (5, 12 and 16 °C). Samples correspond to cheese K for external zone and ripened during 90 days.

The plot of PC loadings shows the distribution of the 21 studied peaks (Fig. 3). The source of variation explained by PC2 (9.4% VAR) was analysed considering the behaviour of peak areas during ripening according to the sign of PC2 loading. Peaks 8 and 13 are shown to analyse the behaviour of peaks with positive and negative values of PC2 loading, respectively (Figs. 4 and 5). Peaks with positive values of PC2 loading showed a significant increase of the chromatographic area in samples corresponding to the central zone of cheese from the first day of ripening to approximately 30 days (peak 8, Fig. 4). Then, the chromatographic area remained almost constant. Similar behaviour was observed at the external zone but it was not as definite as at the central zone.

Peaks with negative values of PC2 loading showed a plateau during the first stage of ripening and then an increase of the chromatographic area (peak 13, Fig. 5). The increment was observed from 60 days at 5 °C while it was observed approximately from 20 days at 12 and 16 °C. Moreover, the chromatographic area reached a second plateau or started decreasing between 60 and 90 days at 16 °C. Central and external zones showed similar behaviour at 5 °C. However, differences between central and external zones were observed at 90 days at 12 °C, and at 60 and 90 days at 16 °C.

From the 21 peaks studied, 12 (3, 5, 7, 8, 10, 11, 12, 13, 14, 16, 17, and 20) were selected, taking into account their important values of PC1 and PC2 loadings (Fig. 3). ANOVA showed that the partial replacement of NaCl by KCl did not have a significant effect on the profiles of those peaks during ripening. On the other hand, the other variables analysed (temperature, sampling zone and ripening time) had significant effects on those peaks.

The chromatographic areas were lower at 5 °C in most of the cases. The ripening temperature effects on enzyme activities and, consequently, on proteolytic breakdown, were evaluated by different authors (Aston, Giles, Durward, & Dulley, 1985; Gobbetti et al., 1999; Laborda & Rubiolo, 1999). Gobbetti et al. (1999) studied the individual and interactive effects of temperature, pH and NaCl on the peptidase activities of NSLAB by quadratic response surface methodology. The authors

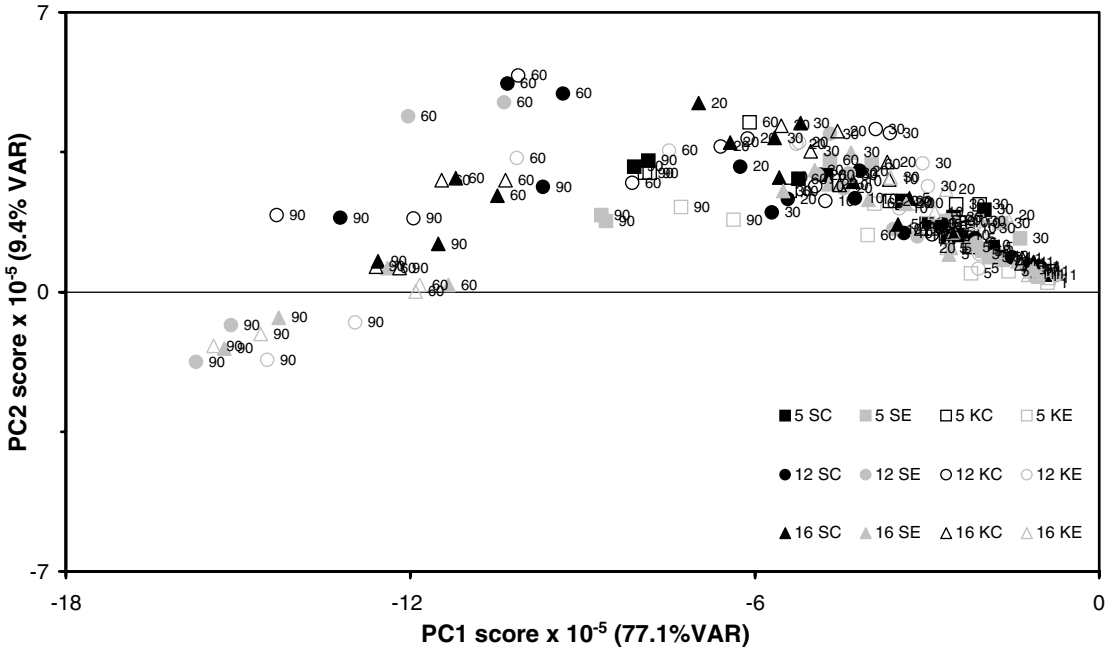

Fig. 2. Score plot from PCA with data from RP-HPLC chromatograms of the TCA–WSF from Fynbo cheese. Numbers indicate the ripening time. Symbols: square, cheeses ripened at 5 °C; circle, cheeses ripened at 12 °C; triangle, cheeses ripened at 16 °C; filled symbol, cheeses S; empty symbol, cheeses K; black symbol, central zone; grey symbol, external zone.

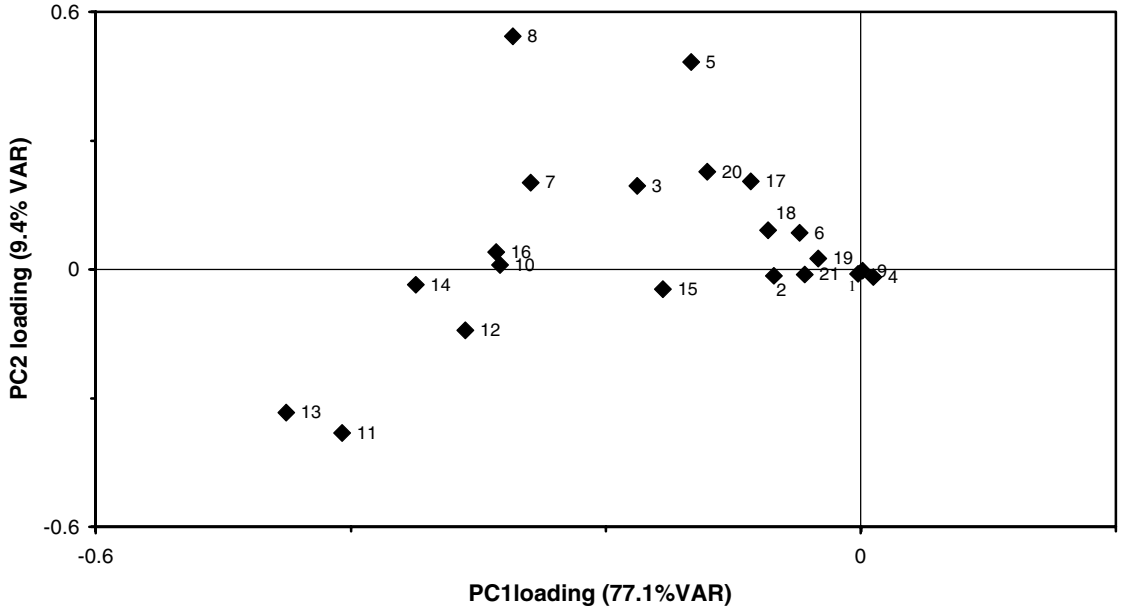

Fig. 3. Loading plot from PCA with data from RP-HPLC chromatograms of the TCA–WSF from Fynbo cheese. Numbers correspond to peaks analysed (as suggested by Sihufe et al., 2004).

reported that, with few exceptions, all the enzyme activities were obviously influenced by the increase of temperature in the range 4–16 °C, working in cheese-like conditions. In general, it is accepted that storing at higher temperature is a tempting option for accelerating cheese ripening because it is technologically so simple to implement. However, it is necessary to ensure that the principal controls have the stability and microbiological safety of the product (Law, 2001). In the case of Fynbo cheese, the values of chromatographic areas of the 12 selected peaks in cheeses ripened at 5 and 12 °C were approximately 45% and 90%, respectively, of the values of chromatographic areas of cheeses stored at 16 °C, after 30 days of ripening. Moreover, the same number

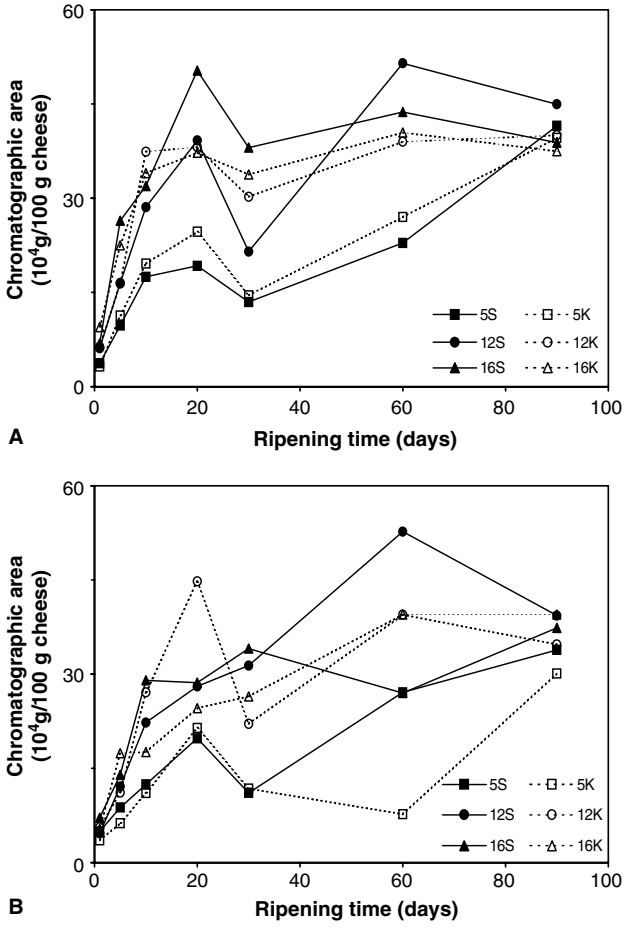

Fig. 4. Chromatographic area profile corresponding to peak 8 from TCA–WSF of Fynbo cheese: (A) central zone; (B) external zone.

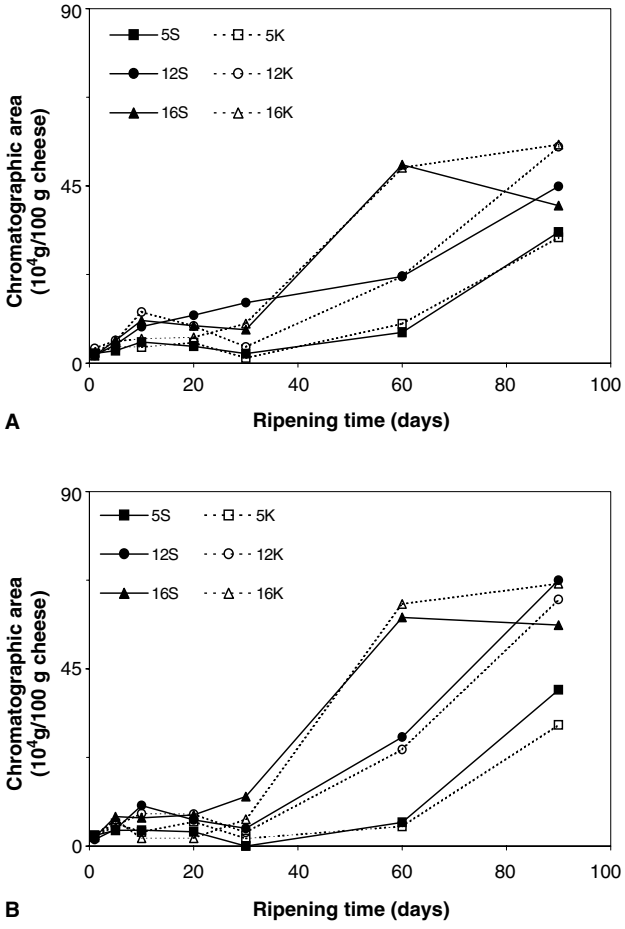

Fig. 5. Chromatographic area profile corresponding to peak 13 from TCA–WSF of Fynbo cheese: (A) central zone; (B) external zone.

of peaks was detected in the chromatograms of cheeses ripened at 5, 12 or 16 °C. This may indicate that the ripening temperature probably affects the rate of proteolysis rather than the pathways of proteolysis.

Complete characterisation of proteolysis in cheese requires isolation and identification of individual peptides. Scarce information about the peptides that characterize Fynbo cheese proteolysis is available. Therefore, an attempt at identifying some important chromatographic peaks was made. The primary site of chymosin action on α_{s1}-casein is Phe_{23}–Phe_{24}, producing α_{s1}-casein (f1-23) peptide (McSweeney, Olson, Fox, Healy, & Højrup, 1993). The quoted authors studied the proteolytic specificity of chymosin on bovine α_{s1}-casein in solution and identified different peptides produced at pH 6.5 and at pH 5.2 in the presence of 5% (w/v) NaCl. In their chromatographic run, the peak corresponding to α_{s1}-casein (f1-23) had a retention time of ≈30 min. In this case, α_{s1}-casein hydrolysis by chymosin is essential in Fynbo cheese ripening (Sihufe et al., 2003; Zorrilla & Rubiolo, 1997) and there were several peaks with important values of chromatographic area in the region of 20–30 min of retention time in the chromatogram (peaks 12, 13, 14, 16, and 17). It must be pointed out that the chromatographic conditions used in this study are very similar to those used by McSweeney et al. (1993). Therefore, peaks 12, 13, 14, 16, and 17 were isolated and analysed by amino acid analysis, preceded by acid hydrolysis, for comparisons with α_{s1}-casein (f1-23).

Hydrochloric acid hydrolysis generally yields over 95% recovery for 10 aminoacids: aspartic and glutamic acids, glycine, alanine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine (Gehrke, Wall, Absheer, Kaiser, & Zumwalt, 1985). Table 1 shows the molar ratio (mol residue/mol peptide) of these amino acids calculated for α_{s1}-casein (f1-23) peptide and for peaks 12, 13, 14, 16, and 17. Due to during acid hydrolysis, asparagine and glutamine are quantitatively converted to aspartic and glutamic acids (Gehrke et al., 1985), the results for these amino acids are showed as Asx and Glx, respectively. A deviation value was calculated as the squared difference between theoretical and experimental molar ratios of amino acids with yields over 95%. Peak 17 showed the lowest deviation value.

The α_{s1}-casein (f1-23) peptide, or the peptides produced by the starter proteinase action on α_{s1}-casein

Table 1
Molar ratio (mol residue/mol peptide) of α_{s1}-casein (f1-23) peptide and the hydrolysed of peaks 12, 13, 14, 16, and 17

Aminoacid	α_{s1}-casein (f1-23)	Peak 12	Peak 13	Peak 14	Peak 16	Peak 17
Asx	2	2.42	2.06	2.02	1.58	1.82
Glx	4	4.78	5.27	5.64	5.90	5.88
His	2	0.00	0.00	0.58	0.56	0.51
Gly	1	3.85	2.57	1.31	1.30	1.34
Ala	0	1.96	1.29	0.88	0.95	0.95
Tyr	0	0.00	0.00	0.61	0.39	0.30
Phe	1	1.51	1.47	0.70	0.90	0.78
Leu	4	1.79	1.80	1.52	1.66	2.02
Lys	2	0.00	0.88	1.23	1.24	1.02
Deviation[a]	0	25.91	16.05	12.75	13.05	11.84

[a] Deviation calculated as the squared difference between theoretical and experimental molar ratio of amino acids with yield over 95%.

(f1-23), were isolated and identified in vitro or in cheese extracts, by different authors (Exterkate & Alting, 1993, 1995; Kaminogawa, Yan, Azuma, & Yamauchi, 1986; McSweeney et al., 1993). In some cheese varieties, the α_{s1}-casein (f1-23) peptide is rapidly hydrolysed by starter proteinases (Fox & McSweeney, 1996). Gagnaire, Mollé, Herrouin, and Léonil (2001) reported an increment in the chromatographic area corresponding to the α_{s1}-casein (f1-23) until the end of the period evaluated (50 days), working with Emmental cheese. In our case, the chromatographic area of peak 17 increased during the first 20 days of ripening and then reached a plateau with a trend to decrease at 90 days of ripening (Fig. 6). This profile would indicate a production of α_{s1}-casein (f1-23) up to the end of the period studied. This behaviour is in accordance with Sihufe et al. (2003), who observed an important production of the complementary peptide, α_{s1}-casein (f24-199), during the same ripening period using electrophoretic analysis. Therefore, peak 17 might be related to α_{s1}-casein (f1-23).

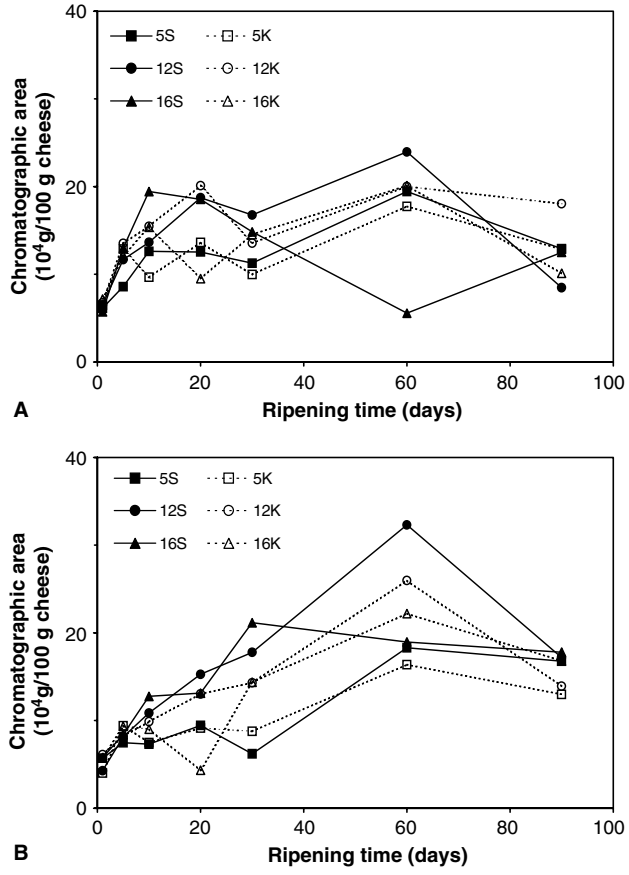

Fig. 6. Chromatographic area profile corresponding to peak 17 from TCA–WSF of Fynbo cheese: (A) central zone; (B) external zone.

This study showed the influence of different technological variables on the second stage of Fynbo cheese proteolysis and the behaviour of different chromatographic peaks during ripening. The results give an interesting prospect for further studies where peptides may be identified and different proteolytic pathways may be proposed. In this way, further studies are necessary to improve the isolation and identification technique.

4. Conclusions

The effects of partial NaCl replacement by KCl and ripening temperature on Fynbo cheese secondary proteolysis were studied. Cheeses salted with the mixture of NaCl/KCl had similar secondary proteolysis as cheeses salted with NaCl. The information obtained from the RP-HPLC chromatograms was successfully summarized in 2D, accounting for 86.5% of the data variation. Ripening time was the main cause of variation between the samples analysed. Secondary proteolysis in Fynbo cheese was significantly affected by total salt concentration and ripening temperature. An important peptide produced during cheese ripening was tentatively identified as α_{s1}-casein (f1-23), taking into account the chromatographic profile and the amino acid composition of the peak isolated. These results may lead to a better understanding of the influences of different technological variables on secondary proteolysis in Fynbo cheese, which may be used to surmount some modifications in the elaboration process.

Acknowledgements

This research was partially supported by Universidad Nacional del Litoral (Santa Fe, Argentina), Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina), and Agencia Nacional de Promoción Científica y Tecnológica (Argentina). We thank SanCor Cooperativas Unidas Ltda. for the supply of cheeses.

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