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Original article

Evolution of proteolysis during the ripening of traditional Feta cheese

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Abstract – Four cheesemaking trials were conducted in three different traditional Feta cheese plants. The characteristics of the cheesemaking process had to do with: the heat treatment of the cheese milk, the use of yoghourt as a starter, the use of traditional rennet from kids' and lambs' abomasa, and the packaging in wooden barrels without brine addition. At 60 d (minimum ripening period according to Greek Codex Alimentarius), the percentage of water soluble nitrogen (WSN) varied from 15.88% to 19.58% of total nitrogen (TN) and 73.4%–79.9% of the WSN was nitrogen soluble in 12% TCA. At the same age, 13.3–23.6% of WSN was nitrogen soluble in 5% PTA. The quantitative and qualitative changes of the middle area of the RP-HPLC profiles (A_{220}) indicated the evolution of nitrogenous fractions during the ripening and the effect of each cheese plant technology on them. Most of the qualitative and quantitative changes occured during the pre-ripening period (18 d). In general, the rate of proteolysis was similar to that of the industrially made Feta. However, the percentages of low molecular weight fractions were higher.

Feta cheese / proteolysis

Résumé – **Évolution de la protéolyse au cours de l' affinage de fromage Feta fabriqué par les fromageries traditionnelles.** Quatre fabrications expérimentales de fromage Feta ont été réalisées dans trois différentes fromageries traditionnelles. La technologie adoptée par ces fromageries se caractérise par : une pasteurisation en cuve, l'utilisation de yaourt comme levain, l'emprésurage par la présure traditionnelle obtenue à partir de caillettes d'agneaux ou de chevreaux ainsi que l'emballage et le stockage dans des barils en bois sans ajout de saumure complémentaire. À 60 j (temps minimum d'affinage, selon le Code Alimentaire grec), le pourcentage d'azote soluble variait de 15,88 % à 19,58 %. Par ailleurs 73,4–79,9 % et 13,3–23,6 % de l'azote soluble correspondait à l'azote soluble dans l'acide trichloroacétique (TCA) 12 % et dans l'acide phosphotungstique (PTA) 5 % respectivement. Les variations qualitatives et quantitatives du profil des chromatogrammes obtenus par chromatographie liquide haute performance HPLC (A_{220}) présentent l' évolution des fractions azotées au cours

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de l'affinage ainsi que l'effet de la technologie des différentes fromageries. La plus grande partie des changements qualitatifs et quantitatifs a été observée pendant les 18 premiers jours d'affinage. La vitesse de la protéolyse a été égale à celle de fromage Feta fabriqué industriellement. Cependant les valeurs des fractions azotées de faible poids moléculaire étaient plus élevées.

Feta / fromage / protéolyse

1. INTRODUCTION

Feta is a white cheese in brine, produced in Greece since the Homeric years from ewes' milk or from its mixtures with goat's milk. It is a Controlled Denomination of Origin in Greece [5]. During the last few years, its production has been industrialized and some convenient modifications have been applied to the cheesemaking procedure, with respect to the basic traditional technology. The traditional technology and the new trends in Feta cheese manufacture are described by Anifantakis [3]. According to the traditional technology, the heat treatment of milk is milder than the HTST pasteurisation, which is the common practice for the industrial cheese plants, resulting in different microbiological characteristics of cheese milk. The traditional cheesemakers use rennet produced by themselves from lambs' and kids' abomasa slaughtered before weaning, which has been partially or fully substituted by the classical rennet in the industrial scale of Feta cheese production. Traditional yoghourt is used as a starter instead of mixtures of commercial starters, including mesophilic starters, which are used in industrial production. Also, the draining and pre-ripening conditions vary according to the weather conditions, since usually there is no temperature control in the ripening rooms. Traditional Feta pieces are packaged in wooden barrels, tightly layered one on top of another with dry salt between them and no brine is added, unlike industrially made Feta, which is packaged in rectangular tins filled with brine.

It is well known nowadays that the traditionally made Feta has a "richer" taste and flavor and that it is preferred by the majority of the consumers to the industrially made cheese. Considering the above-mentioned information, the present study was undertaken in order to evaluate the ripening process of Feta made in three different small cheese plants of the Argos region of Peloponnese, in which varied practices of traditional technology were applied.

2. MATERIALS AND METHODS

2.1. Cheesemaking and sampling

Four cheesemaking trials were conducted in three different small Feta cheese plants located in the Argos region of Peloponnese within a two-months period. The cheese milk was a mixture of ewes' and goats' milk in a 4:1 ratio. About 20% of the total amount of the cheese milk was defatted and the skim milk was mixed with the rest of the full fat milk in the cheese vat. Yoghourt made in each cheese plant was added as a starter culture. The manufacturing conditions practiced in each cheese plant are shown in Table I. The cheeses were referred to as A, B and C, according to the respective cheese plant. Samples were taken at 3 d (before packaging), at 18 d (before storing at 5 ^{o}C) and at 40, 60 and 120 d (Tab. I).

2.2. Evaluation of proteolysis

Total Solids content was determined in triplicate according to IDF [7].

Water soluble nitrogen (WSN): 20 g of cheese were homogenized with 100 mL distilled water by a Stomacher apparatus

Table I. Manufacture of traditional Feta cheese in three cheese plants.

Cheese plants	A	B	C
Heat treatment of cheese milk	plate heat exchanger, 67 °C·15 s^{-1}	by steam injection, 66 °C·13 min^{-1}	in open vat, 66 °C·6 min^{-1}
kg of yoghourt in 1000 L cheese milk	1.2	0.6	4.0
g of $CaCl_2$ in 1000 L cheese milk	–	–	90
Coagulation temperature (°C)	36–38	37–38	35–37
Cutting into cubes (2 × 2 cm) after (min)	35	60	45–50
Rest (min)	10		
Molding	In stainless molds, open only at the upper side, with oblong (0.2 × 2 cm) and round (0.2 cm) openings and lids of the shape and the size of the bottom of the mold in order to turn them upside down.		
Draining	The filled molds were placed with a certain inclination on a cheese table and were turned around from time to time in order to enhance whey removing. After 3 h, cheese curd was cross cut, salted with salt of rice grain size and the molds with their lids were turned upside down. Then, the molds were removed, the upper surface of curd pieces was salted and the Feta pieces were again put in the molds for 12–14 h.		
Temporary packaging and salting	The next morning, the Feta pieces were placed into plastic barrels in layers with dry salt between them. Dry salt was also added on the bottom of the barrels and on the upper surface of the cheese layers.		
Packaging	3 d after milk coagulation[1].	3 d after milk coagulation[1].	3 d after milk coagulation[1].
	Feta pieces were taken out of the plastic barrels, washed carefully and placed in wooden barrels in layers with no empty space between them. A plastic film with openings was placed on the bottom of the barrel and between the layers. A very thin layer of salted defatted whey cheese (mizithra) was added on the bottom, between and on the upper surface of the Feta pieces. On the top of the cheese layers, there was an empty space of about 2 cm height, due to the small increase in cheese volume during ripening.		
Pre-ripening conditions	In the wooden barrels until 18 d after cheese manufacture, at 15 °C.	In the wooden barrels until 18 d after cheese manufacture, at 17–18 °C.	In the wooden barrels until 18 d after cheese manufacture, at 18 °C.
Ripening conditions	After the pre-ripening period, the barrels were put in cold stores at 5 °C, until 120 d after cheese manufacture[1].		

[1] Sampling points.

for 5 min and left at 40 °C for 1 h, centrifuged (3 000 *g* × 30 min, 4 °C) and filtered through Whatman (Whatman International Ltd, Maidstone, England) filter paper No 40.

Nitrogen soluble in 12% trichloroacetic acid (TCA-SN): an equal volume of 24% TCA solution was added to WSN filtrate. After 2 h the mixture was filtered through Whatman No 40 filter paper.

Nitrogen soluble in 5% PTA (PTA-SN): 70 mL of a 3.95 mol·L^{-1} H_2SO_4 and 30 mL of 33.3% (w/v) phosphotungstic acid (PTA) aqueous solutions were added to 100 mL of WSN filtrate. The mixture was held overnight at 4 °C and then it was filtered through Whatman No 40 filter paper.

Determination of the N content: total nitrogen and the N content of the nitrogenous fraction were determined by the Kjeldahl method [8]. Total protein was expressed as total N content × 6.38.

Urea-polyacrylamide gel electrophoresis (PAGE): analysis of cheese samples was done in duplicate, according to Andrews [2]. A suspension of 240 mg cheese in 10 mL stacking buffer containing 6 mol·L^{-1} urea, 0.1 mol·L^{-1} β-mercaptoethanol and 0.4 mL tracking dye solution, was kept at 40 °C for 15 min and then centrifuged at 3 000 *g* for 15 min at 4 °C. The solidified fat layer was discarded and 10 µL of the supernatant were used for electrophoresis. Staining and quantitative determination was done as described by Kandarakis et al. [11]. Residual caseins in each sample were calculated as a percentage of the area of the respective bands at 3 d.

Reverse phase - high performance liquid chromatography (RP-HPLC): 50 µL of WSN were analyzed by a RP C18 Nucleosil wide pore column (5 µm, 30 nm, 250 × 4 mm, Maherey-Nagel, Duren, Germany) as described by Kandarakis et al. [11]. Solvents and samples were filtered through 0.22 µm and 0.45 µm filters, respectively (Millipore Corp., Bedford, MA, USA).

2.3. Statistical analysis

Analysis of variance was used to test the influence of the cheese plant manufacturing conditions, and of the ripening stage, on the Feta nitrogenous fractions. Further testing was carried out by a multiple range tests procedure using the least significant difference (LSD) test ($P < 0.05$). Relationships between different variables were determined by regression analysis. The software STATGRAPHICS Plus for Windows v. 5.2 (1995, Manugistics, Inc., Rockville, MD, USA) was used for data manipulation.

3. RESULTS AND DISCUSSION

3.1. Nitrogenous fractions

The percentage of the total protein over total solids (TP/TS) fraction of cheese B at the packaging date (3 d), was significantly higher ($P < 0.05$) than that of cheese A (Tab. II). This difference could be attributed to the more severe heat treatment of the cheese milk B, resulting in the inclusion of denatured whey proteins in the Feta curd. Furthermore, the significantly lower ($P < 0.05$) pH of cheeses A and C at this age (Tab. II) promoted the expulsion of more whey and therefore of more proteins from the curd.

The change of nitrogenous fractions during the ripening of traditionally made Feta cheese manufactured in three cheese plants is shown in Table II. In general, there were some significant differences in proteolysis between the cheeses of the three plants, resulting from the different manufacturing conditions. The percentage of the WSN/TN fraction increased 1.6–1.7 times in traditional Feta cheese within the ripening period from the packaging date to 120 d. The rate of increase was similar to other reported data concerning Feta manufactured under conditions similar to that of industrial scale production [14, 20]. The

Table II. Biochemical characteristics and nitrogenous fractions during the ripening of traditional Feta cheese manufactured in three cheese plants.

		Cheese plants		
	Days	A	B	C
pH	3	5.08 [b]	5.16 [c]	5.01 [a]
TP/TS[1]	3	33.90 [a]	37.74 [b]	34.47 [a, b]
	18	33.84	35.60	34.86
	40	35.50	36.52	37.16
	60	34.94	37.21	33.57
	120	35.39	35.88	34.89
WSN/TN[2]	3	11.33	12.91	12.17
	18	13.51 [a]	18.01 [b]	15.23 [a, b]
	40	15.26 [a]	19.52 [b]	17.03 [a]
	60	15.88 [a]	19.58 [b]	17.91 [a, b]
	120	18.49 [a]	21.22 [b]	20.51 [a, b]
TCA-SN/WSN[3]	3	42.88	34.44	38.22
	18	72.76 [b]	63.60 [a]	69.17 [a, b]
	40	74.60	70.03	74.21
	60	79.86 [b]	73.37 [a]	75.44 [a, b]
	120	85.19 [b]	77.30 [a]	79.16 [a, b]
PTA-SN/WSN[4]	3	12.70 [a, b]	9.33 [a]	14.69 [b]
	18	21.92	19.13	23.14
	40	18.82 [b]	13.09 [a]	19.07 [b]
	60	23.62 [b]	13.27 [a]	20.71 [b]
	120	22.64 [c]	14.41 [a]	18.50 [b]
Residual α_s-CN[5]	18	70.78	60.98	65.75
	40	63.63	56.10	58.81
	60	67.16 [c]	44.93 [a]	58.78 [b]
	120	56.49 [b]	42.77 [a]	41.58 [a]

[1] Total protein expressed as percentage of total solids (TS).
[2] Water soluble nitrogen expressed as percentage of total nitrogen (TN).
[3] Nitrogen soluble in 12% trichloroacetic acid, expressed as percentage of WSN.
[4] Nitrogen soluble in 5% phosphotungstic acid, expressed as percentage of WSN.
[5] Residual α_s-casein, expressed as percentage of the respective area at 3 d.
[a, b, c] Means of the same row with different superscripts were significantly different (LSD test, $P < 0.05$).

percentages of traditional Feta cheese (Tab. II) were in general lower than the reported values [14, 20], but they were similar to the percentages at 60 and 120 d reported by other researchers for Feta made under industrial conditions [1, 11, 16].

The WSN/TN fraction of cheese B was significantly higher ($P < 0.05$) than that of cheese A from the end of the pre-ripening period and thereafter, and the opposite was true for the TCA-SN/WSN and PTA-SN/WSN fractions. This difference could be attributed to the significantly higher ($P < 0.05$) pH of Feta B at the packaging stage that resulted in the retention of more whey proteins in the cheese, as has been shown by Kandarakis et al. [11].

The TCA-SN/WSN fraction was doubled after 120 d of ripening in the cheeses of the three plants. This increase was faster than the respective rates reported for Feta cheese packaged in tin-plated cans filled with brine, in which the increase is 20–60% [1, 16, 20, 21]. Since no brine was added to the containers (wooden barrels) of the traditional Feta, the expulsion of small molecular mass fractions was expected to be limited. It is known that the nitrogenous fractions with molecular mass $< 1\ 000\ g \cdot mol^{-1}$ contribute to cheese flavor [4]. The greatest proportion of this percentage was accumulated during the pre-ripening period, as also happened with the WSN/TN. This finding was in accordance with the conclusions of other studies about Feta [1, 16]. Moreover, as shown in Table II, after the placement of the barrels into the cold stores (at 18 d), the accumulation of amino acids and low molecular mass nitrogenous compounds ($< 600\ g \cdot mol^{-1}$), expressed as PTA-SN/ WSN [9], was retarded.

The significantly ($P < 0.05$) lower PTA-SN/WSN percentage of cheese B was apparently due to the lower amount of yoghourt added as a starter. It has been reported that the starters are the main responsible microbial group for the accumulation of small peptides and amino acids in cheese [12, 15]. In addition, the heat treatment of cheese milk B was expected to cause more extensive inactivation of native milk microflora.

Finally, it seemed that the change in proteolysis in traditional Feta cheese was best described by the changes of TCA-SN/WSN due to the inclusion of various quantities of whey proteins in the WSN fraction. The increase of TCA-SN/WSN during the ripening was logarithmically correlated with cheese age ($r = 0.840$, $P < 0.01$).

The PAGE patterns of the cheeses of the three plants throughout the ripening period were not different regarding the qualitative characteristics of the profiles (Fig. 1). It was shown that plasmin activity resulting in γ-caseins was inhibited while chymosin activity on α_s-casein was high. The hydrolysis of α_s-casein proceeded rapidly during the pre-ripening period (Fig. 1). It was fastest in cheese B, as also happened with the increase of WSN/TN from 3 to 18 d. During the ripening from 3 to 120 d, the area of fractions moving faster than α_s-casein increased 2.79, 2.33 and 2.33 times for the A, B and C cheeses, respectively. It has been shown that these fractions are mainly proteolysis products of α_s-casein [17] due to rennet action, which was expected to be enhanced in our cheeses, bearing in mind their pH and salt in moisture content. According to Michaelidou et al. [18], most of the major water-soluble peptides in 6-month-old Feta cheese originate from α_s-casein. The area of β-casein remained constant and in some cases was increased during the ripening, probably due to the accumulation of hydrolysis products in this area. Moreover, the area of γ-caseins was not remarkably changed, apparently due to the unfavorable cheese pH for plasmin activity [22].

3.2. RP-HPLC profiles

The RP-HPLC profiles of 60-d-old WSN monitored at 220 nm are shown in

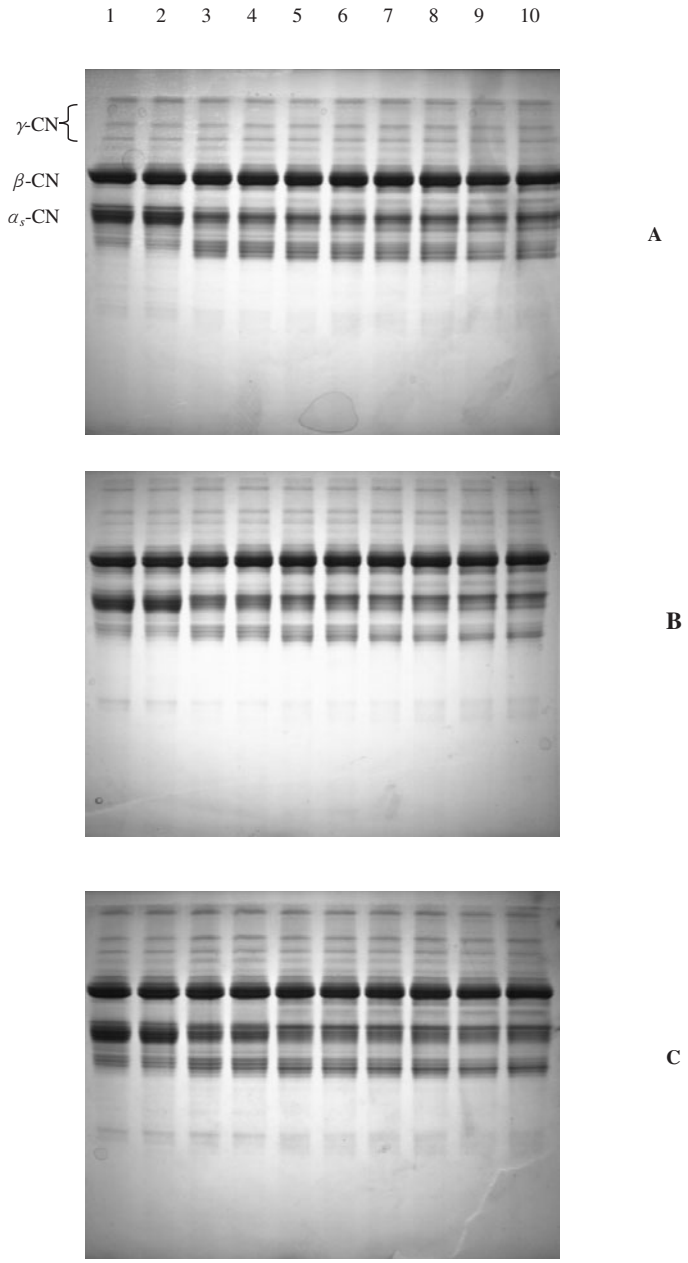

Figure 1. Urea-polyacrylamide gel electrophoresis (PAGE) profiles of traditional Feta manufactured in three cheese plants (A, B, C). Lanes 1-2: 3 d; lanes 3-4: 18 d; lanes 5-6: 40 d; lanes 7-8: 60 d; lanes 9-10: 120-d-old Feta. Sample: 10 μL from cheese suspension in urea buffer (24 mg·mL^{-1}).

Figure 2 and some of their quantitative characteristics are shown in Table III. The comparative profiles of WSN, TCA-SN and PTA-SN are shown in Figure 3.

The total area of the chromatograms monitored at 220 nm was significantly correlated with cheese age (logarithmic $r = 0.834$, $P < 0.01$) with the WSN/TN and TCA/TN fractions and with residual α_s-casein (logarithmic $r = 0.844$, linear $r = 0.911$ and linear $r = –0.861$, respectively).

The most quantitative differences between the cheese profiles were displayed in the portions 40–70 and 10–40 min, especially concerning cheese B. The percentage of the 10–40 min area of cheese B was significantly lower ($P < 0.05$) than that of the other two cheeses, and the opposite was true for the percentage of the 40–70 min portion. In the 10–40 min portion are eluted small peptides and free amino acids [6, 10] that are included in the TCA-SN and PTA-SN, as was also evident from Figure 3. The participation of these fractions in WSN was also significantly lower ($P < 0.05$) in cheese B (Tab. II). In the portion 70–100 min are eluted hydrophobic and mainly high molecular mass peptides along with the whey proteins [13, 18, 19]. This was also apparent from the TCA-SN profiles, in which there was almost no peak in this portion (Fig. 3). At 3 d the mean area ($\times 10^6$) of peaks with a retention time similar to that of ovine and caprine whey proteins was 12.0, 16.5 and 14.1 for the A, B and C cheeses, respectively. These data were in accordance with the higher WSN/TN content of cheese B at the same age, which was attributed to higher retention of whey proteins due to higher pH.

Table III. Quantitative changes of RP-HPLC profiles (A_{220}) of WSN during the ripening of traditional Feta cheese manufactured in three cheese plants.

		Cheese plants		
	Days	A	B	C
Total area ($\times 10^6$)[1]	3	30.1	41.8	37.2
	18	56.9 [a]	73.8 [b]	62.9 [a, b]
	40	67.9 [a]	88.1 [b]	83.4 [a, b]
	60	74.1	83.1	81.3
40–70/10–40 min[2]	3	0.72	1.13	0.85
	18	1.25	1.61	0.88
	40	0.96 [a]	1.64 [b]	0.89 [a]
	60	1.10 [a]	2.21 [b]	1.11 [a]
40–100/10–40 min[2]	3	3.49	4.87	4.02
	18	2.95	3.59	2.40
	40	1.97	3.16	1.92
	60	1.93 [a]	3.81 [b]	1.95 [a]

[1] Chromatogram Area Units (CAU).
[2] Ratio of the respective areas in CAU.
[a, b, c] Means of the same row with different superscripts were significantly different (LSD test, $P < 0.05$).

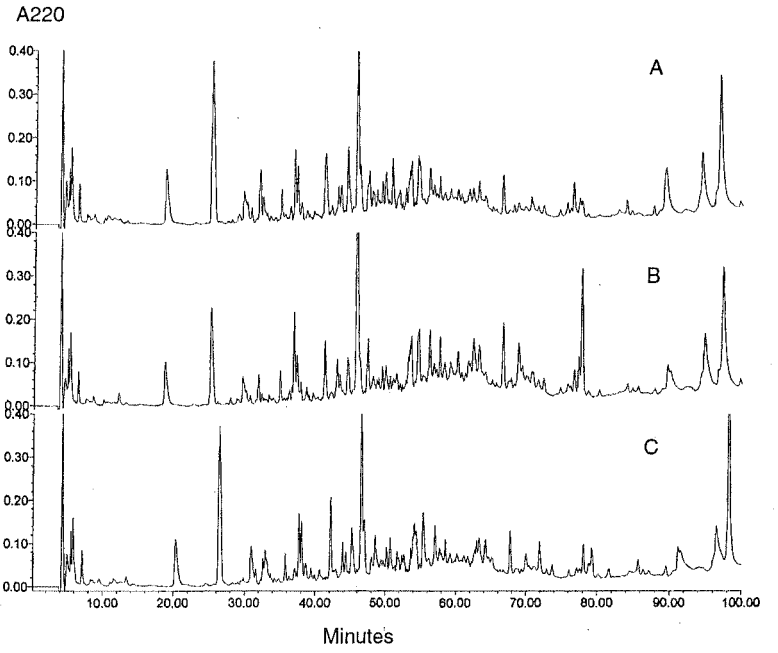

Figure 2. RP-HPLC profiles (A_{220}) of 60-d-old traditional Feta made in three cheese plants (A, B, C). Sample: 50 μL WSN.

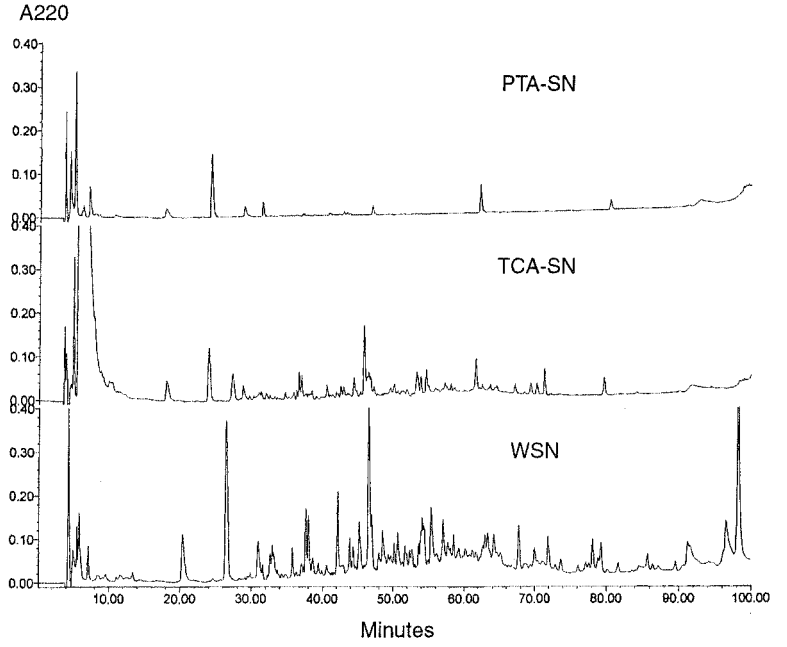

Figure 3. RP-HPLC profiles (A_{220}) of the nitrogenous fractions of 60-d-old traditional Feta C. Sample: 50 μL of the filtrate.

The profiles of the present study were different from the WSN profiles of Feta made with mesophilic starters and packaged in tins filled with brine according to the industrial practice, concerning in particular the 10–40 min portion. The 40–100/10–40 min ratio of those profiles is higher and ranges from 6.1–7.8 [11]. This observation is in accordance with the above-mentioned lower TCA-SN/WSN of Feta made under conditions similar to the industrial ones.

The percentage of the 70–100 min portion after 60 d decreased by half, while the percentage of the 10–70 min portion was increased 81–94% during the ripening. The rate of these changes was similar for the three cheese plants and most of them occurred during the pre-ripening period, as it has been also mentioned for the changes in the nitrogenous fractions.

The ratios 40–100/10–40 and 40–70/10–40 min of 60-d-old cheese B WSN were significantly different ($P < 0.05$) from those of A and C. The decrease in the 40–100/10–40 ratio during the ripening could be attributed to the hydrolysis of hydrophobic medium and large peptides to smaller hydrophilic ones and free amino acids. The increase in the 40–70/10–40 min ratio indicated that during the ripening peptides tended to accumulate in the 40–70 min portion rather than in the 10–40 min portion. This could be attributed to the small amount of starter used, which is mainly responsible for the accumulation of amino acids and small peptides in cheese [12, 15]. In cheese B, the higher values of these ratios were in accordance with the lower values of TCA-SN/WSN and PTA-SN/WSN (Tab. II).

4. CONCLUSIONS

In general, the rate of proteolysis in traditional Feta was similar to that reported for industrially made Feta, but the values and the rate of formation of the low molecular mass nitrogenous fractions were higher. Therefore, the accumulation of these compounds along with lipolysis products and aromatic substances could be responsible for the particular organoleptic characteristics of traditionally made cheese.

The observed differences in qualitative and quantitative characteristics of proteolysis related to the small peptide and amino acid fractions, could be attributed to the different manufacturing conditions applied in each cheese plant, concerning mainly the heating process of the cheese milk and the amount of yoghourt used as a starter. Most of the qualitative and quantitative changes occured during the pre-ripening period (until 18 d), before the placement of the Feta into the cold stores. Plasmin activity was inhibited while chymosin activity was high. The differences in the nitrogenous fractions between the three different cheese plants were satisfactorily represented by the ratios of the partial regions of RP-HPLC profiles.

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