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Proteolysis of Livanjski cheese during ripening

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Abstract

Livanjski cheese belongs to the group of hard cheeses which is traditionally produced in Livno (Bosnia and Herzegovina). Proteolytic changes during the ripening of Livanjski cheese have not been investigated extensively. The aim of this paper was to determine its proteolytic changes during the different stages of ripening. Five Livanjski cheeses (from raw cow's or a mixture of sheep's and cow's milk) were observed during the ripening to evaluate its typical proteolytic profile. An electophoretic profile of Livanjski cheese was determined by Urea-polyacrylamide gel electrophoresis (urea-PAGE) and a densitometric evaluation of the urea-PAGE gels was performed using a densitometer. The water-soluble nitrogen fraction in the total nitrogen (WSN %TN) and the 12%-TCA-soluble nitrogen fraction in the total nitrogen (TCA-SN %TN) of the cheese were determined using the Kjeldahl method. Degradation of α_{s1}-casein by chymosin caused a significant decrease ($P < 0.05$) of relative content of this protein in Livanjski cheese at the sixth week point of ripening. Due to the activity of chymosin on αs1-casein, αs1-I-casein and αs1-II-casein developed, which caused a significant increase ($P < 0.05$) of Index alpha. The relative ratio of β-casein significantly decreased ($P < 0.05$) during ripening leading to a significant accumulation ($P < 0.05$) of degraded product (sum γ_1-casein, γ_2-casein and γ_3-casein). These proteolytic changes caused a significant increase ($P < 0.05$) of Index betta. Accumulation of medium, small peptides and amino acids caused a significant ($P < 0.05$) increase of the relative content of WSN %TN and TCA-SN %TN. In general, proteolysis of Livanjski cheese during ripening was moderate probably due to the low moisture content and low water activity, although it was produced from raw milk. Taking into account that the ratio β-casein : α_{s1}-casein at the end of ripening was 1.46, it could be concluded that degradation of α_{s1}-casein could be the indicator of the maturity of Livanjski cheese. Due to that Livanjski cheese could be classified as "α-type of ripening" cheese.

Keywords: Livanjski cheese, proteolysis, ripening

Introduction

Proteolysis is very complexed and one of the most important biochemical process that occurs during ripening of many kinds of cheeses. It has a key role in the forming of taste, flavour and texture of the cheese (Azarnia et al., 2010). The small peptides and free amino acids, which contribute to the development of the taste and flavour of the cheese, are accumulated in the cheese during ripening. As well as, due to the activity of proteolytic enzymes the texture of the cheese becomes softer (Engels and Visser, 1994).

Primary proteolysis in cheese includes degradation of casein due to the activity of enzymes cymosine and plasmine. As a consequence of casein degradation large peptides are accumulated in the cheese (Lynch et al., 1997). Primary proteolysis mainly depends on the type and concentration of the coagulant used, as well as on the activity and concentration of plasmine (McSweeney and Fox, 1999; Kalit et al., 2002). Evaluation of the biochemical changes during ripening of different cheeses starts with the determination of the primary proteolysis. The results of the primary proteolysis do not give enough information for distinguishing of different types of cheeses. Therefore, for distinguishing of different types of cheeses, secondary proteolysis results are also used (Coda et al., 2006).

Livanjski cheese belongs to the group of full fat hard cheeses which is produced from cow's milk and from the mixture of sheep's and cow's milk, in both, industrial plants and on small scale family farms. This cheese obtained the name from the town Livno in Bosnia and Herzegovina where it has been produced since 1888 (Kutle, 1996; Bijeljac and Sarić, 2005) during the Austro-Hungarian Monarchy. The knowledge of this cheese production was spread to surrounding areas. The ripening time of Livanjski cheese varies between 18 to 112 days (on average 50-60 days; Krišto, 1998). Livanjski cheese has a yellowish coloured body, well cared yellowish rind, medium sized eyes on the cut surface and hard and elastic texture (Matić et al., 2014).

Although Livanjski cheese is one of the most popular and valued traditional cheese in Bosnia and Herzegovina, there is a lack of information about its properties. A recent investigation revealed that local consumers prefer Livanjski cheese produced from a mixture of raw ewe's and cow's milk, in contrast to experts who prefer Livanjski cheese which is produced from pasteurized cow's milk (Matić at al., 2014). However, proteolytic changes during the ripening of Livanjski cheese have not been investigated extensively. The only two researches about biochemical changes during ripening of Livanjski cheese were done by Dozet at al. (1975) and Sarić (2002). Therefore, the aim of this paper was to investigate the proteolytic changes of Livanjski cheese throughout ripening.

Materials and methods

Cheese manufacture procedure

The production and ripening of Livanjski cheese were monitored at four family farms and one large scaled dairy plant. Two farms produced Livanjski cheese from mixed sheep's and cow's milk, while other produced Livanjski cheese from cow's milk. Raw milk from the evening and morning milkings was curdled by commercial rennet at the temperature of 32 – 35 °C. When coagulated (setting time was approximately 50 minutes), the curd was cut into cubes of approximately 8 cm and left to rest until the whey begins to separate. Curd grains were heated and stirred, up to a temperature that varied between 46 and 48°C for 30 - 40 minutes. The curd grains (the size of rice or barley) were transferred into moulds lined with cheesecloth. After filling the cloth was firmly wrapped around the curd on which wooden followers are fitted and left for a couple of hours. After that cheese is kept in moulds under pressure for up to 24 hours, and then brining approximately 48 hours in 25% brine. Cheese was ripened 8 weeks at the temperature of 12 - 15 °C and relative humidity of 70 - 85%.

Sampling of cheese

Samples of curd (0 day) and cheese at 2, 4, 6 and 8 weeks of ripening were taken for analysis. A cheese trier was used to take about a 15 g sample from the core to the surface of each cheese loaf. The 1 cm of cheese from the trier that was nearest to the surface of each Livanjski cheese was removed from the core sample and placed back in the hole. The hole was sealed with paraffin before the cheese was again placed in the ripening chamber. Sampling was performed according to the procedure of Licitra et al. (2000).

Analysis of proteolysis of Livanjski cheese

An analysis of proteolysis of Livanjski cheese was performed by densitometric evaluation of the Urea-polyacrylamide electrophoresis (urea-PAGE) gels and by determination of water and trichloro-acetic acid-soluble nitrogen fractions. The analyses were conducted at the Department of Dairy Science, Faculty of Agriculture, University of Zagreb.

Preparation of cheese samples

After the removal of the rind, the cheese samples (0,4 g) were grated and dissolved in five ml of urea buffer [7.5 $g \cdot L^{-1}$ (60 mM) Tri(hydroxymethyl)-aminomethane (Tris-HCl; BIO-RAD, 161-0716), 8 M urea and 20 $mL \cdot L^{-1}$ of 2-mercaptoethanol, adjusted using hydrochloric acid to pH 7.6]. The samples were incubated at 40 °C for 30 min. The dissolved samples were cooled immediately under running water. In order to remove the fat and suspended particles, the samples were centrifuged at 3400 rev/min (acceleration value of 2590 g) and 4 °C for 10 min. The clear supernatant (100 µL) were mixed with 300 µL of sample buffer [7.5 $g \cdot L^{-1}$ (60 mM) Tris-HCl. 8 M urea, 10 $mL \cdot L^{-1}$ of 2-mercaptoethanol and a trace of bromophenol blue, pH 7.6] and 6 µL of the prepared casein sample was applied to a well.

Urea-polyacrylamide gel electrophoresis (urea-PAGE)

Urea-PAGE of the casein samples was undertaken according to the method of Andrews (1983) with some minor modifications. The assay performed on a working unit Mini Protein II (BIO-RAD), using a power supply PAC 300 (BIO-RAD).

The separating gel buffer was prepared by mixing 46 $g \cdot L^{-1}$ (380 mM) of Tris-HCl and 4.5 M urea at pH 8.9. The separating gell (12% T; 3.8% C) was prepared by mixing 3 mL of acrylamide/bisacrylamide (40% acrylamide and 1.6% bisacrylamide) solution, 7 mL of separating gel buffer, 7 µL of TEMED (N,N,N,N-tetramethyl-ethylene diamine) and 0.1 mL of a 100 $g \cdot L^{-1}$ solution of ammonium persulfate (APS).

The stacking gel buffer was made by mixing 7.5 $g \cdot L^{-1}$ (60 mM) of Tris-HCl and 8 M urea at pH 7.5. The stacking gel (5.7% T; 3.8% C) was prepared by mixing 0.5 mL of acrylamide/bisacrylamide (40% acrylamide and 1.6% bisacrylamide) solution, 3 mL of stacking gel buffer, 5µL of TEMED and 70 µof 100 $g \cdot L^{-1}$ solution of APS. The electrophoresis buffer was a solution of 3 g of Tris and 14.6 g of glycine dissolved in a 1000 mL of distilled water (pH adjusted to 8.2). The run was performed at a constant current of 30 mA for 70 min.

The gels were stained with Commassie blue G-250 (0.25% methanol, 500 $mL \cdot L^{-1}$ containing 125 $g \cdot L^{-1}$ of trichloroacetic acid (TCA)) for one hour. Destaining was done overnight in a solution of acetic acid (80 $mL \cdot L^{-1}$).

Densitometry

Densitometric evaluation of the urea-PAGE gels was performed using a Gel Doc (BIO RAD) densitometer. Qualification was based on the measurement of the areas of each peak of β-casein (β-CN) and α_{s1}-casein (α_{s1}-CN) and the degradation products as relative percentage of the total casein in the cheese samples prepared for electrophoresis that was soluble in the urea buffer. Two electrophoretic ripening indexes were used: betta index = sum γ-CN/ β-CN (Mayer, 1997), alpha index = α_{s1}-I-CN + α_{s2}-I-CN + ND /α_{s1}-CN (modified according to Hynes et al., 2001).

Kjeldahl determination of soluble nitrogen fractions

The total nitrogen (TN) content of the cheese samples, the water-soluble nitrogen fraction in the total nitrogen (WSN %TN) and the 12% trichloroacetic acid-soluble nitrogen fraction in the total nitrogen (TCA-SN %TN) were determined according to the Kjeldahl method 8968-2 (ISO 2003).

Statistical analysis

Data's were analysed using the statistical program SAS (SAS version 8.20, 2001). For analysing the influence of ripening time on proteolysis the REG procedure was used.

Results

In general, proteolysis in cheese could be divided into primary and secondary. Primary proteolysis occurs, mainly on α_{s1}-casein and β-casein thanks to the activity

of chymosin and plasmin, respectively (Havranek et al., 2014). Proteolysis of α_{s1}-casein caused a decrease of the relative content of this protein in Livanjski cheese (Figure 1). In the first stage of ripening of Livanjski cheese the relative content of α_{s1}-casein was 24.40% and it significantly ($P < 0.05$) decreased during the ripening of Livanjski cheese reaching 16.27% after 6 week of ripening. However, secondary proteolysis caused a relative increase in the content of α_{s1}-casein from the 6th week of ripening to the end of the ripening period due to the decreasing of proteins and peptides soluble in the urea buffer as a consequence of further primary and secondary proteolysis which will be discussed later. On average the weekly decrease of the relative content of α_{s1}-casein was 1.71%. Due to the activity of chymosin on α_{s1}-casein, α_{s1}-I-casein and α_{s1}-II-casein developed, which caused a significant increase ($P < 0.05$) of Index alpha (Figure 2). Plasmin is the second most important proteolytic enzyme which plays an important role during the primary proteolysis of many types of cheese, especially those in which high cooking temperatures are used in the production (Kalit et al., 2002; Velez at al., 2015). Plasmin cleaves β-casein which leads to the accumulation of degraded products of β-casein such as γ_1-casein, γ_2-casein and γ_3-casein.

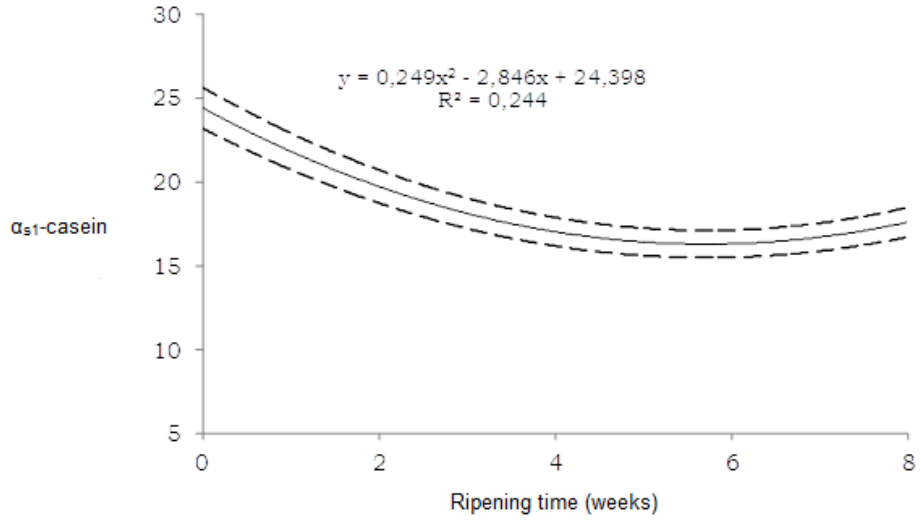

Figure 1. Best fit regression of decreasing ($P < 0.05$) of the relative content of α_{s1}-casein (solid line) and 95% confidence interval (dotted line)

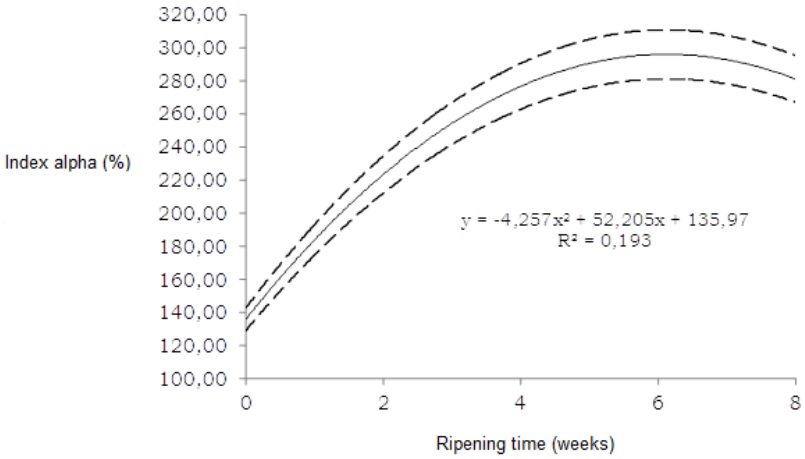

Figure 2. Best fit regression of Index alpha (solid line) and 95% confidence interval (dotted line)

At the beginning of the ripening of the Livanjski cheese, more than 30% of the urea soluble proteins and peptides belonged to β-casein (Figure 3). However, the relative ratio of β-casein significantly decreased ($P < 0.05$) during ripening (Figure 3) leading to a significant ($P < 0.05$) accumulation of degraded product (sum of γ_1-casein, γ_2-casein and γ_3-casein; Figure 4). These proteolytic changes caused a significant increase ($P < 0.05$) of Index betta (Figure 5). Similar results were obtained by other authors who observed that plasmin is a key enzyme in the ripening of some cheeses produced from raw ewe's milk such as Krk or Istrian cheese (Mikulec et al., 2008; Magdić et al., 2013) or from raw cow's milk such as Tounj cheese (Kalit et al., 2005). α_{s1}-casein could be an indicator of the maturity of Livanjski cheese while the ratio β-casein : α_{s1}-casein at the end of ripening was 1.46. Due to that Livanjski cheese could be classified as "α type of ripening" cheese.

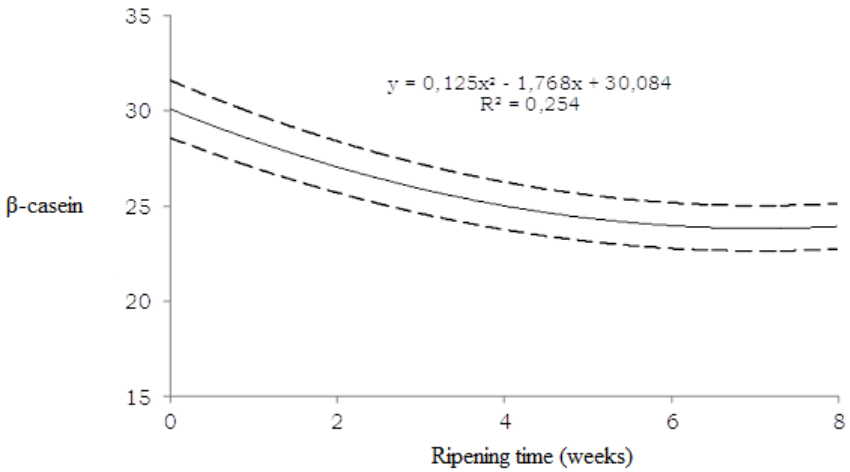

Figure 3. Best fit regression of decreasing ($P < 0.05$) of the relative content of β-casein (solid line) and 95% confidence interval (dotted line)

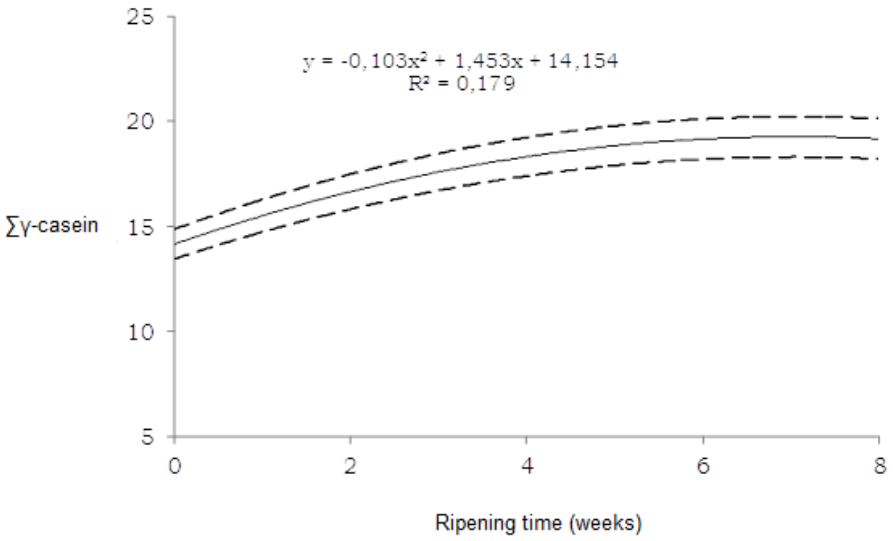

Figure 4. Best fit regression of increasing ($P < 0.05$) of the relative content of sum of γ-casein (∑γ-casein; solid line) and 95% confidence interval (dotted line)

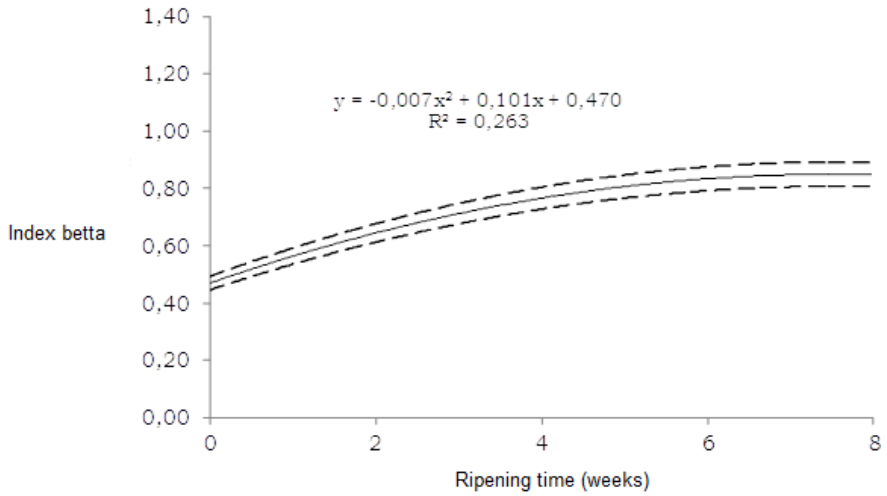

Figure 5. Best fit regression of increasing ($P < 0.05$) of Index betta (solid line) and 95% confidence interval (dotted line)

Accumulation of medium and small peptides and amino acids caused accumulation of water soluble nitrogen fractions and three chlorine acetic acid soluble nitrogen fractions (WSN %TN and TCA-SN %TN, respectively; Figures 6 and 7). In the first stage of ripening of Livanjski cheese content of WSN %TN was less than 10% and significantly ($P < 0.05$) increased towards the end of the ripening to around 21%. A similar occurrence was noted with the content of TCA-SN %TN. In the first stage of

ripening of Livanjski cheese the content of TCA-SN %TN was less than 4% and significantly ($P < 0.05$) increased towards the end of the ripening to around 10%. The fastest increase of WSN %TN and TCA-SN %TN was during first four weeks, after what the growth was moderate by the end of ripening (Figures 6 and 7).

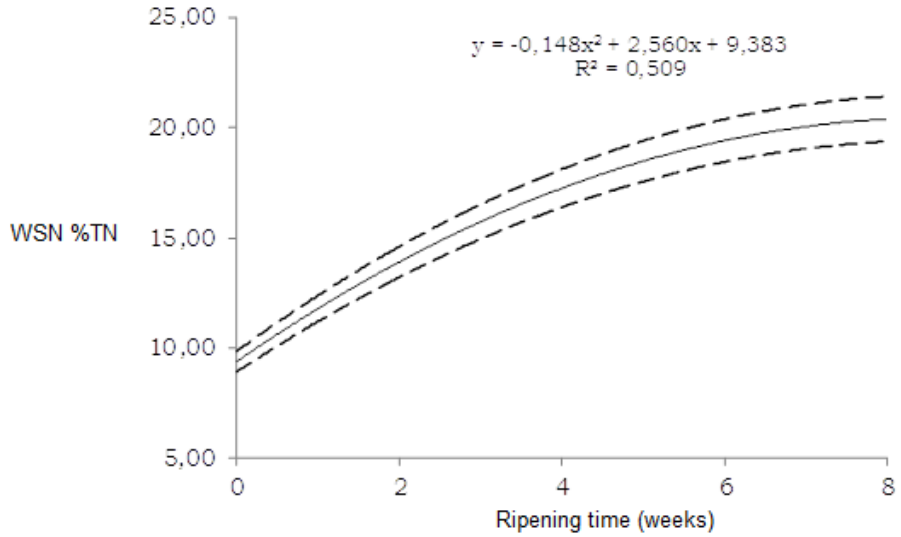

Figure 6. Best fit regression of increasing ($P < 0.05$) of the relative content of water soluble nitrogen of total nitrogen (WSN %TN; solid line) and 95% confidence interval (dotted line)

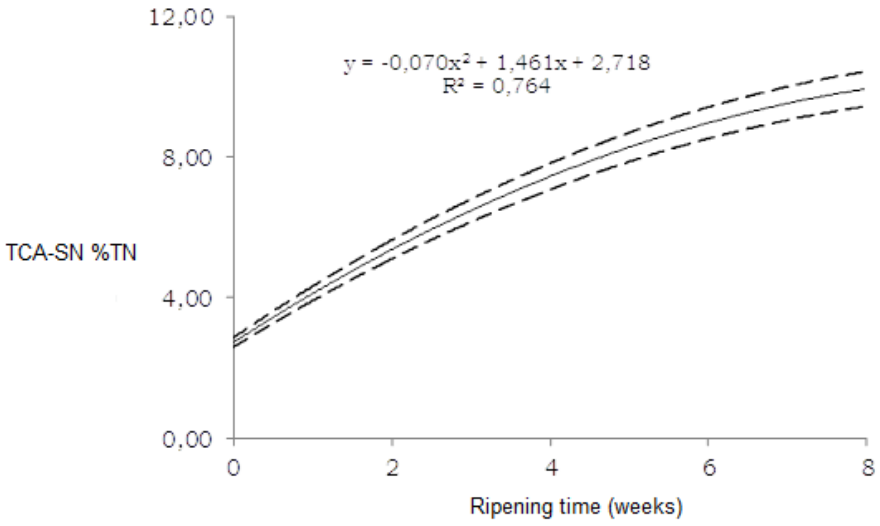

Figure 7. Best fit regression of increasing ($P < 0.05$) of the relative content of three chlorine acetic acid soluble nitrogen of total nitrogen (TCA-SN %TN; solid line) and 95% confidence interval (dotted line)

WSN %TN and TCA-SN %TN fractions increased moderately during ripening of Livanjski cheese in comparison to ewe's milk cheeses such as Istrian cheese (Magdić et al., 2013) and cheese ripened in an lamb skin sack (Tudor Kalit et al., 2014). The intensity of the increment of WSN %TN and TCA-SN %TN fractions in Livanjski cheese was higher than in Tounj cheese (Kalit et al., 2005). The cause can be found in the usage of cow's milk for the production of Tounj cheese, while in this study two cheeses researched were from the mixture of cow's and ewe's milk. The WSN content in Serra da Estrela cheese increased from 1% (on the day of manufacture) up to 43 %TN by 180 days of ripening, thus reflecting the intense proteolytic activity of the enzymes contributed by the plant coagulant utilized. The TCA-SN %TN was also found to be high in this cheese by the end of ripening (16–20%), which suggests a high extent of free amino acids released throughout maturation (Tavaria et al., 2003). The reasons of moderate intensity of proteolysis in Livanjski cheese could be found in the fact that this cheese is produced from cow's and mixed cow's and ewe's milk as well as because of the low moisture content of Livanjski cheese (on average 33.84%; Matić et al., 2014), which is lower than the moisture content of Istrian cheese (on average 37.97%; Magdić et al., 2013) and cheese ripened in a lamb skin sack (on average 35.03%; Tudor Kalit et al., 2014). Water activity together with the pH value of the cheese determines the intensity of proteolysis. Water activity of cheese is mainly determined by two parameters: moisture and salt content (Choisy et al., 2000). In general, lower water content with a higher salt content means less water activity of the cheese which leads to the less intensive proteolytic activity of the cheese. On average, the moisture content in Livanjski cheese is lower and the salt content is higher (on average 33.84 $g \cdot 100g^{-1}$ and 2.67 $g \cdot 100g^{-1}$, respectively; Matić et al., 2014) in comparison to Istrian cheese (38.00 $g \cdot 100g^{-1}$ and 2.34 $g \cdot 100g^{-1}$, respectively; Magdić et al., 2013) and cheese in a lamb skin sack (36.00 $g \cdot 100g^{-1}$ and 2.33 $g \cdot 100g^{-1}$, respectively; Tudor Kalit et al., 2014). The pH value could also influence the proteolytic activity in cheese, but the pH values of all cheeses were similar.

Conclusion

The investigation of proteolysis of different types of cheese during ripening is essential to understand the final sensory characteristics of each type of cheese. Proteolysis of Livanjski cheese during ripening was moderate probably due to the low moisture content and low water activity, although it was produced from raw milk. Taking into account that the ratio β-casein : α_{s1}-casein at the end of ripening was 1.46, it could be concluded that degradation of α_{s1}-casein could be the indicator of the maturity of Livanjski cheese. Due to that Livanjski cheese could be classified as "α-type of ripening" cheese.

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