

# Influence of Proteolysis and Soluble Calcium Levels on Textural Changes in the Interior and Exterior of Iranian UF White Cheese during Ripening

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**Abstract**—The relationships between Proteolysis and soluble calcium levels with hardness of cheese texture were investigated in Iranian UF white cheese during 90 d ripening. Cheeses were sampled in interior and exterior. Results showed that levels of proteolysis, soluble calcium and hardness of cheese texture changed significantly ( $p < 0.05$ ) over ripening. Levels of proteolysis and hardness were significantly ( $p < 0.05$ ) different in interior and exterior zones of cheeses. External zones of cheeses became softer and had higher levels of proteolysis compared to internal zones during ripening. The highest correlation coefficient ( $r^2 = 0.979$ ;  $p < 0.01$ ) was observed between hardness and levels of pH 4.6-soluble nitrogen in exterior zones of cheese. These result showed that proteolysis can contribute to textural softening during ripening of Iranian UF white cheese.

**Keywords**—Calcium, Proteolysis, Softening, Ultrafiltration, White cheese.

## I. INTRODUCTION

**P**RODUCTION of dairy products with high quality needs to control all factors that affect appearance, flavor and texture of the product. The chemical and physical changes occurring during ripening cause the body of the freshly made cheese to lose its hardness and curdy texture. Cheese ripening is a complex process involving many physicochemical changes such as a change in pH, a progressive breakdown of the proteins to smaller peptides and the gradual accumulation of amino acids [10]. Proteolysis in cheese during ripening plays a vital role in the development of texture as well as flavor [11]. Proteolysis affects the level of intact casein, which is a major determinant of the hardness and fracture properties of cheese [6]. The development of texture and proteolysis in cheese has been studied with the aim of elucidating relationships between hydrolysis of the proteins and changes

in. The mineral composition, especially the Ca concentration, is a well-known parameter that influences the textural and functional properties of cheese including hardness/firmness and melt/stretch [22]. Colloidal calcium phosphate (CCP) is one of the primary structural elements of the casein micelles. Recently, it has been proposed that the solubilization of CCP also plays an important role in the development of the texture of mature Cheddar cheese, especially during the early stages of ripening [23]. In recent years, several strategies have been used to study the importance of total Ca concentration, distribution of Ca between insoluble and soluble forms, or both in determining cheese structure, texture, and functionality. Studies have been confined largely to Mozzarella, and to a less extent, Cheddar cheese [10]- [32]. O'Mahony, Lucey & McSweeney [28] showed that the early (d 1 to 21) softening of texture was strongly influenced by CCP solubilisation in Cheddar cheese in which chymosin-mediated proteolysis was inhibited using pepstatin. Joshi, Muthukumarappan, & Dave [27] reported that calcium bound to the casein micelles has a significant influence on functional properties of cheese but addition of soluble calcium through fortification of cheesemilk with calcium chloride did not. The long-ripened cheese may soften drastically due to extended proteolysis that decreases the surface area occupied by the protein fraction in cheese microstructure, leading to a decrease of the force-bearing component in cheese texture [19], and a gradual breakage of the network calcium bonds [8]. The latter is because of the slow solubilization of remaining colloidal calcium phosphate during aging [23].

Application of the membrane processing technologies to the cheese industry has been developed, however, it has been observed that there are some problems, i.e. textural and compositional defects for semi-hard and hard cheeses manufactured from ultrafiltered milk [12]. Softening of texture, after production and during ripening period is one of the main shortcomings of UF white cheeses produced in Iran, which besides reducing of desirability, involves high financial damage for producers. This phenomenon is usually observed in outside parts of the cheeses. The purpose of the present investigation was to study the changes of proteolysis and soluble calcium levels in external and internal zones of Iranian

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UF white cheese to determine which of these factors can be contributed in texture softening.

## II. MATERIALS AND METHODS

### 2.1. Materials

Amixture of mesophilic (G3 mix, composed of *Lc. cremoris* and *Lc. lactis*) and thermophilic (yoghurt 709, composed of *Str. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*) cultures (both prepared commercially by Danisco Deutschland GmbH Alemanha, Germany) in the ratio of 7:1, respectively, was used as starter. Microbial rennet (Fromase® 2200 TL granulate, P2200 IMCU g-1) from Rhizomucor miehei supplied by DSM Food Specialities (Seclin, France) was used for cheesemaking.

### 2.2. Cheesemaking

Experimental UF white cheeses were made in four separate cheesemaking days (every day 10 container). The retentate was prepared by Iran Dairy Industry Inc., Pegah Co (Tabriz, Iran) and used for production of Iranian UF white cheese. Raw milk of high microbial quality was standardized to 3.5% fat, and after bactofugation in two stages, pasteurized at 72 °C for 15 s and then ultrafiltered at 50 °C. The membrane cartridges were of the spiral wound type (no UFPH20 Invensys APV, Silkeborg, Denmark) and the membrane had a nominal molecular weight cut-off of approximately 20 kg•mol<sup>-1</sup> with a surface area of 16.9 m<sup>2</sup>. The ultrafiltration unit was operated at an inlet pressure of 5.3 bar and an outlet pressure of 1.7 bar. The retentate was pasteurized at 78 °C for 60 s and then cooled to 35 °C. The starter culture was added and the retentate was adjusted with permeate to 0.34 kg•kg<sup>-1</sup> dry matter as described by Wium, Kristiansen & Qvist [38] and then immediately filled (450 g) into containers and left to coagulate at 30 °C for 60 min. A parchment paper was placed on top of the coagulum and dry salt (3%) was added. The containers were sealed with aluminum foil. Salt gradually adsorbed moisture from curd and a layer of brine formed around the cheeses in the containers. Cheese packs were held at 26–28 °C for 24 h and then transferred to a cool room (8 °C); the next day was considered as the first day of ripening and the samples were ripened for three months. During ripening (d 1 to 90) four cheeses with every 10 day interval were sampled in external and internal zones (sampling zones).

### 2.3. Analytical methods

#### 2.3.1. Compositional analysis

Cheeses were analysed for moisture by the oven drying method at 102 ± 2 °C according to IDF [15], total protein and pH 4.6-soluble nitrogen by the macro-Kjeldahl [27], salt by a potentiometric method [9], and the pH of the cheese was measured by direct insertion of an electrode (Model No. 209, Hanna, Portugal) into grated cheese. Results reported as mean ± standard deviation.

#### 2.3.2. Assessment of proteolysis

The pH-4.6 soluble Nitrogen was prepared by the method

of Kuchroo & Fox [20] and Levels of nitrogen soluble in the prepared extract were determined using a Micro-Kjeldahl Apparatus (Vap 76, Gerhardt, Germany). Urea-polyacrylamide gel electrophoresis (PAGE) of the pH-4.6 insoluble fraction of the cheese was performed using a Protean II XI vertical slabgel unit (Bio-Rad Laboratories Ltd., Watford, UK) according to the method of Shalabi & Fox [31]. The gels were stained directly with Coomassie Brilliant Blue G250, as described by Blakesley & Boezi [4].

#### 2.3.3. Calcium assessment

##### 2.3.3.1. Total calcium

Total calcium of cheese was measured as described by Macedo & Malcata [24].

##### 2.3.3.2. Soluble calcium

Soluble calcium was determined by the method of Pastorino, Hansen & McMahon [30] with some modifications. Cheese samples (5 g) were blended with 50 g of water using Ultra-Turraxs TP 18110 (IKAs Werke, Janke & Kunkel GmbH & Co KG, Staufen, Germany) high-speed homogenizer, and transferred to a beaker. The blending container was then rinsed with water (150 g) and the transferred to the beaker. After standing for 20 min, the solution was filtered through Whatman No. 42 filter paper. The filtrate was then analysed for calcium content.

The concentrations of total and soluble calcium were determined by atomic absorption spectrophotometer (AA 6300, Shimadzu, Japan) with an air/acetylene flame. Standard solutions of 1, 2, 5 and 10 ppm were provided from 1000 ppm calcium solution and used to generate calibration curves. Strontium chloride (SrCl<sub>2</sub>, 6H<sub>2</sub>O) (Merck, Darmstadt, Germany) was added to the final diluted solutions of Ca at a concentration of 1000 ppm in order to avoid interference by phosphate.

#### 2.3.4. Texture assessment

The simplest fundamental test, uniaxial compression, was performed during ripening using an Instron (1140 Universal Testing Machine, UK) machine with a 5 Kg load cell, a crosshead speed of 10 mm/ min and probe with 35 mm diameter following the method of Madadlou, Khosroshahi & Mousavi [25] except that cylindrical cheese samples (25 mm diameter, 25 mm height) from two different cheese zones, the internal zone and external zone were slowly punched out vertically by a lubricated borer at 5 °C as described by Creamer & Olson [6] and were put into plastic bags to prevent dehydration and left in the test room for 4 hours to reach test temperature of 22 ± 1 °C. Samples were compressed uniaxially with 50% deformation (test end-point: 12.5 mm) from the initial height of the sample in one bite and the maximum stress (for compression to 50% of original height) was expressed as hardness of the cheese.

#### 2.3.5. Statistical analysis

All analysis were performed in four replications. Statistical calculation was performed using SAS Statistical Software version 9.1, and the resulted values are presented as means ± standard deviations. Evaluation of significance was performed

by Duncan's multiple range tests in significance levels of  $P < 0.05$ .

### III. RESULTS AND DISCUSSION

#### 3.1. Composition

Table 1 shows the gross composition of experimental Iranian UF white cheese in two sampling zones at day 1 of ripening. The % SCa/TCa (percent of soluble Ca to total Ca ratio), percent of pH-4.6-soluble nitrogen to total nitrogen ratio (% oh 4.6-SN/TN), pH, hardness and moisture of two sampling zones were not significantly ( $p < 0.05$ ) different, but the NaCl content of these sampling zones were significantly ( $p < 0.05$ ) different. Since powdered salt is added on the surface of cheese curd after cheesemaking process, therefore, absorption of salt in the external zone was higher than in the internal zone at the first day of ripening. No significant ( $p < 0.05$ ) differences were observed in composition among replications.

Changes of pH, salt and moisture content in two sampling zones during ripening are shown in fig. 1, 2 and 3, respectively. No significant ( $p < 0.05$ ) differences were observed in pH values in two sampling zones but pH values were significantly ( $p < 0.05$ ) different during ripening time. The levels of pH decreased UP up to day 60 of ripening and from day 60 to the end of ripening increased in both external and internal zones. As cheese is a fermented dairy product, the metabolism of lactose to lactate by selected cultures of lactic acid bacteria (LAB) decrease the pH value [26]. Increasing pH value from day 60 up to end of ripening may be due to that during cheese ripening, released amino acids raise pH value to a somewhat higher level. The pH of cheese affects the texture of curd directly by influencing the solubility of the caseins; all else being equal, high pH cheeses are softer than more acid cheeses. PH also affects texture and flavour indirectly by affecting the activity of enzymes important to ripening and, in the case of the coagulant, the retention of enzyme in the curd during manufacture [5].

Significant ( $p < 0.05$ ) differences were observed in salt content in two sampling zones and ripening time from day 1 up to day 60. The rate of salt absorption was high from day 1 up to day 60 due to a movement of NaCl molecules as a result of the osmotic pressure and difference moisture contents of cheeses [14]. Salt concentration was significantly lower in internal zone than in external zone until day 60 of ripening. It can be considered that a uniform salt concentration was reached in both zones at 70 days of ripening because there were no significant differences in salt concentrations between 70 and 90 days of ripening. Salting is an essential process in the cheese, since salt has a major effect on the control of microbial growth, the removal of whey from the cheese matrix, and the development of the characteristic flavour and texture of the cheese through the control of the biochemical pathways, i.e., proteolysis, lipolysis and glycolysis [13].

The moisture content at the beginning of the ripening was higher in the internal zone than in the external zone. The different moisture content between external and internal

zones at the beginning of ripening (1 day) could be attributed to the fact that cheeses did not reach the moisture equilibrium before packaging [35]. Despite the moisture gradient, cheeses reach uniform moisture content during the 90 days of ripening.

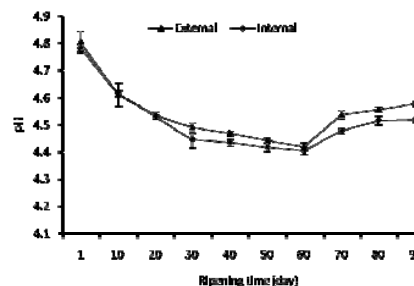


Fig. 1. Change of pH in UF white cheese in external and internal zones during ripening (results were the mean of four replications).

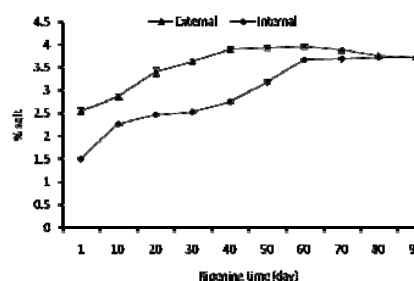


Fig. 2. Change of salt in UF white cheese in external and internal zones during ripening (results were the mean of four replications).

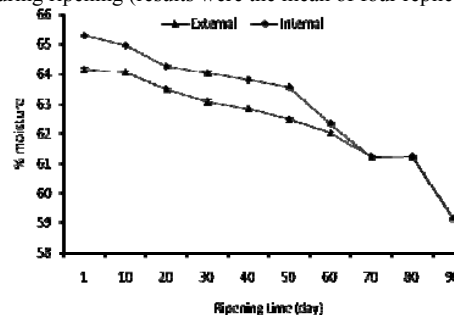


Fig. 3. Change of % moisture in UF white cheese in external and internal zones during ripening (results were the mean of four replications).

#### 3.2. Level of pH-4.6 SN as a percentage of total nitrogen (% pH-4.6SN/TN)

values of % pH-4.6 SN/TN of Iranian UF white cheese in exterior and interior zones during ripening are shown in Fig. 4. The pH-4.6 SN values of the cheeses were changed significantly ( $P < 0.01$ ) during ripening and there was significant ( $p < 0.01$ ) difference between external and internal zones, but interaction of ripening time with sampling zone was not significantly different (data not shown).

The % pH-4.6 SN/TN values increased in both internal and external zones during ripening, but means of % pH-4.6 SN/TN of external zone were significantly ( $p < 0.01$ ) higher than those in internal zone during ripening. The formation of pH-4.6 soluble nitrogen compounds during ripening is an index of the rate and extent of proteolysis, in that it is an indicator of casein hydrolysis brought about by the action of the rennet and the milk proteases present at the start of ripening [16].

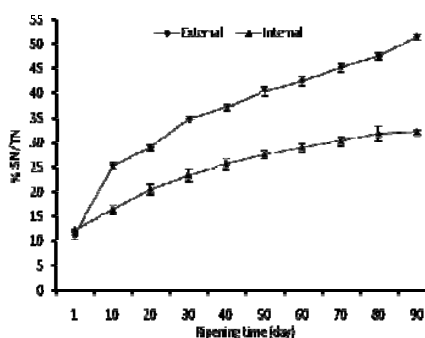


Fig. 4. Change of % pH-4.6 SN/TN in UF white cheese in external and internal zones during ripening (results were the mean of four replications).

Proteolysis contributes to the softening of cheese texture during ripening due to hydrolysis of the casein matrix of the curd and through a decrease in the water activity ( $a_w$ ) of the curd due to changes in water binding by the new carboxylic acid and amino groups formed on hydrolysis and changes in pH (which in turn may cause other changes such as the migration and precipitation of calcium phosphate) [26]. The rate and pattern of proteolysis may be influenced by location within the cheese (e.g., surface-ripened, smear-ripened or young brined-salted cheeses) and a suitable sampling scheme should consider this [33]. In UF Feta cheese,  $\epsilon_f$  (fracture strain) had been shown to be highly correlated with soluble nitrogen (SN) and with non-coagulable nitrogen [37].

Verdini, Zorrilla & Rubioloa [36] reported that maturation indices of Port Salut Argentino cheeses (determined as water-soluble nitrogen as a percentage of total nitrogen) increased during ripening but there was no significant ( $p < 0.05$ ) difference between zones (internal and external). Tarakci & Kucukoner [34] reported that water-soluble nitrogen of Turkish Kashar cheese during 90 days ripening were affected significantly ( $p < 0.05$ ) from the ripening period. The proteolysis values increased both internal and medium zones of cheese throughout ripening, but level of internal cheese was significantly ( $p < 0.05$ ) higher than medium cheese end of ripening.

In a young Feta cheese, this firming effect is more dominant than the softening effect of the proteolysis; however, as storage continues, the proteolytic effect increases to an extent that the cheese becomes fully soft [38].

### 3.3. Urea-PAGE

Urea-PAGE electrophoretograms of the pH-4.6 insoluble fraction of experimental UF white cheeses during ripening are shown in Fig. 5. There were some notable differences in electrophoretic patterns between softened and unsoftened cheese types in external. While degradation of caseins was little in exterior and interior of unsoftened cheeses and also in interior zone of cheeses that their external zones became soft, degradation of  $\beta$ -casein and  $\alpha_1$ -casein were apparent in external softened zones of cheeses from day 40 of ripening. Abd El-Salam, Alichanidis & Zerfiridis [1] have reported the resistance of  $\beta$ -casein to hydrolysis during the ripening of many cheese varieties. Alichanidis, Anifantakis, Polychroniadou & Nanou [2] reported that the high NaCl concentration and low pH of Feta cheese markedly reduced the degradation of  $\beta$ -casein by the coagulant and plasmin, but the hydrolysis of  $\alpha_1$ -casein was not inhibited. Primary proteolysis of caseins in cheese is generally due mainly to the activity of chymosin (on  $\alpha_1$ -casein) and of plasmin (on  $\beta$ -casein) but they are not the sole active proteolytic agents. Wium, Kristiansen, & Qvist [38] observed degradation of  $\alpha_1$ -casein in UF Feta cheese made without rennet and ascribed it to cathepsin D activity. Compared to the data reported by Kandarakis et al [18] for traditional Feta cheese, the level of proteolysis for UF Feta cheeses in present study was high in exterior zones. casein network is greatly weakened when only a single bond in about 20% of the  $\alpha_1$ -casein is hydrolyzed by the coagulant to give the peptide  $\alpha_1$ -I- casein [16]. De Jong (1976) reported good correlation between the firmness of a cheese and the quantity of intact  $\alpha_1$ -casein present.

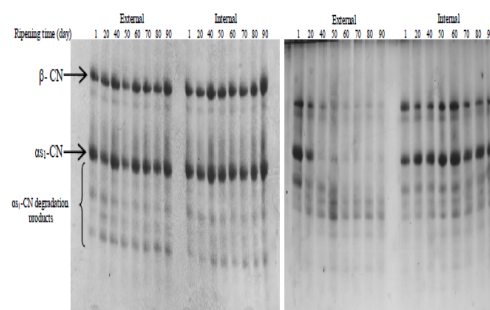


Fig. 5 Urea polyacrylamide gel electrophoretograms of experimental Iranian UF white cheese in softened (left) and unsoftened (right) cheese in two sampling zones during ripening

### 3.4. Calcium assessment

Values of % SCa/TCa of Iranian UF white cheese in exterior and interior zones during ripening are shown in Fig. 6. The % SCa/TCa values of the cheese were significantly ( $p < 0.01$ ) changed during ripening, but the effect of sampling zone and interaction of ripening time with sampling zone were not significant ( $p < 0.05$ ). Calcium phosphate, which is associated with casein, is termed micellar Ca phosphate (MCP) or colloidal Ca phosphate (CCP) plays an important role in maintaining the integrity of casein micelles because

casein micelles are disaggregated into submicelles when MCP is removed. Our results showed changes of % SCa/TCa during ripening had a significant negative correlation ( $r^2 = 0.496$ ,  $p < 0.05$ ) with pH. As pH decreases, ions of  $H^+$  increases and these ions are interchanged with  $Ca^{2+}$  in casein micelles which loads  $Ca^{2+}$  to leave casein micelles. For a similar Ca content, Lawrence & Gilles [21] found that the texture of Cheddar cheese at 35 d varied from curdy ( $pH \geq 5.3$ ) to waxy ( $pH$ , 5.3 to 5.1) and to mealy ( $pH < 5.1$ ). They reported although the total Ca content of cheeses was similar, the proportion of Ca phosphate in an undissolved form likely varied by pH and this variation may have contributed to the observed differences in texture. Lucey & Fox [22] suggested that the proportions of Ca and phosphate in the insoluble form, as opposed to the total Ca and phosphate concentrations are important in modulating cheese structure, texture, and functionality.

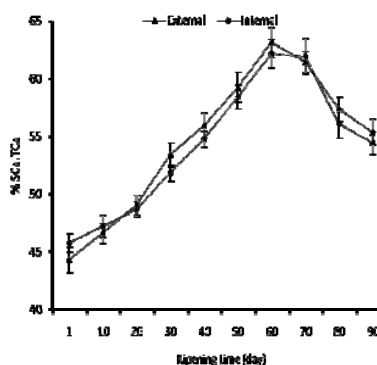


Fig. 6. Change of % SCa/TCa in UF white cheese in external and internal zones during ripening (results were the mean of four replications).

### 3.5. Texture analysis

Fig. 7 shows change of hardness in two sampling zones during ripening of Iranian UF cheeses. The hardness of cheese samples (maximum stress at compression to 50% of original height) decreased during the ripening and there were significant ( $p < 0.01$ ) differences between hardness of sampling zones, but interaction of ripening time with sampling zone was not significantly ( $p < 0.01$ ) different (data not shown). As expected, texture of UF white cheese became softer during ripening but hardness of external zone was significantly ( $p < 0.01$ ) lower than internal zone at each ripening intervals. Al-Otaibi & Wilbey [3] showed that hardness of UF white cheese decreased during ripening. The relationship between proteolysis and texture development was sought by plotting the rheological parameter (hardness) against pH-4.6SN/TN for each of the experimental cheeses. A wide scatter of data was apparent with correlation coefficients of  $r^2 = 0.979$  ( $p < 0.01$ ), which indicated that pH-4.6SN/TN would be a suitable indicator of textural changes in UF white cheeses (Fig. 8). It has been suggested that the hydrolysis of  $\alpha s1$ -casein to  $\alpha s1$ -I-casein is responsible for the early softening of high moisture

cheese [6]. The results from traditional manufacture of Feta cheese would suggest that increased degradation of  $\alpha s1$ -casein during ripening softens the cheese [29]. No significant ( $p < 0.05$ ) correlation was found between % SCa/TCa and texture of Iranian UF white cheese in the present study. However, some other works reported Ca solubilisation as a main factor of some cheese softening. Lucey, Johnson, & Horne [23] reported that various rheological parameters [e.g., storage modulus ( $G'$ ) and maximum loss tangent ( $LT_{max}$ )] of Cheddar cheese were more highly correlated with the level of insoluble Ca than with the extent of primary proteolysis (as monitored by levels of pH-4.6 soluble nitrogen) during ripening. O'Mahony, Lucey, & McSweeney [28] indicated that the hardness of Cheddar cheese during the early stages of ripening (1 to 21 d) was more highly correlated with the concentration of insoluble Ca ( $r^2 = 0.92$ ;  $p \leq 0.001$ ) than were the level of intact  $\alpha s1$ -casein ( $r^2 = 0.63$ ;  $P < 0.05$ ) or level of pH-4.6-soluble nitrogen ( $r^2 = 0.76$ ;  $p \leq 0.05$ ) when residual coagulant was inhibited.

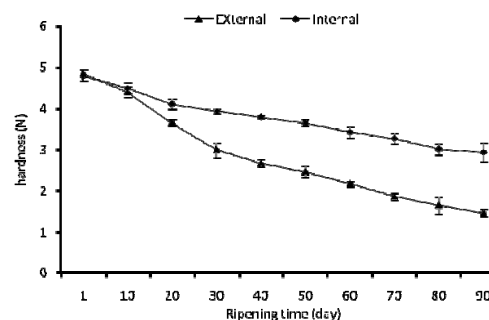


Fig. 7. Change of hardness in two sampling zones during ripening of Iranian UF cheeses

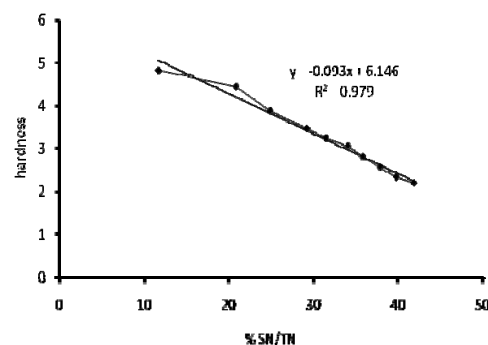


Fig. 8. Correlation coefficients of hardness and % SN/TN in UF white cheese.

### IV. CONCLUSIONS

Although proteolysis and solubilization of calcium are determinant factors on texture of cheeses, but in this study, significant ( $p < 0.05$ ) correlation was found between hardness and % pH 4.6-SN/TN but not between % SCa/TCa and hardness of Iranian UF white cheeses. These results showed that proteolysis and breakdown of  $\beta$ -casein and  $\alpha s1$ -casein is important factor in textural changes of Iranian UF white

cheese during ripening and can be contributed in softening of its texture, especially in external zones.

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