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ORIGINAL PAPER

Influence of brine immersion and vacuum packaging on the chemistry, biochemistry, and microstructure of Mihalic cheese made using sheep's milk during ripening

A. A. Hayaloglu · N. Bansal · P. L. H. McSweeney

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Abstract Due to practical and hygienic uses in storage and marketing, cheese maker preference for the use of vacuum packaging instead of the brining method has increased significantly in recent years. The objective of the paper was to study and compare the chemistry, biochemistry, and microstructure of Mihalic cheeses ripened in vacuum packaging or under brine over 360 days. Proteolysis in Mihalic cheese was quite low (e.g., pH 4.6 soluble nitrogen was lower than 15%, as a percentage of the total nitrogen, and α_{s1}-casein was slower in comparison to other brined cheeses) due to its high salt-in-moisture and low moisture contents. Urea-PAGE patterns of the pH 4.6 insoluble fractions of the cheeses showed different degradation patterns of α_{s1}-casein in cheeses of different ages. However, β-casein was relatively resistant to hydrolysis. Packaging the cheese in polyethylene bags significantly increased the levels of pH 4.6 soluble nitrogen, 12% trichloroacetic acid-soluble nitrogen, and free amino acids compared to the cheeses ripened in brine. The total concentrations of free amino acids (FAA) in cheeses increased after 120 days of ripening, and Glu, Val, Leu, Lys, and Phe were the most abundant FAA in the cheeses. Higher levels of these AAs were found in the cheeses ripened in polyethylene bags. The peptide profiles by reversed-phase high-performance liquid chromatography of 70% (*v*/*v*) ethanol-soluble and ethanol-insoluble fractions of the pH 4.6 soluble fraction of the cheeses revealed that slight differences were found in the levels of some peptides between cheeses. Majority of the volatile compounds were present at higher concentrations in vacuum-packaged cheeses than in those ripened in brines. The images obtained by scanning electron microscopy of the cheeses ripened in polyethylene bags were clearly different (the protein matrix was more compact) from those of brine-ripened cheeses. The cheese ripened in vacuum packaging was completely different in terms of

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chemical composition, proteolysis, volatile composition, and microstructure. It was concluded that the vacuum packaging may be useful to maintain the chemical and biochemical qualities of Mihalic cheese, and it is also an easy practice during ripening.

盐水浸渍和真空包装对Mihalic干酪成熟过程中化学、生物化学和微观结构的影响

摘要：考虑到干酪在储藏和销售过程中既要实用又要卫生，近几年来，在干酪成熟过程中和干酪加工采用真空包装代替盐水浸渍。本文究和比较了Mihalic干酪在真空包装袋以及盐水浸渍360多天后，两种干酪成熟过程的化学和生物化学组成以及微观结构的变化。由于盐水浓度高，水分含量低的缘故，Mihalic干酪蛋白酶解程度较低（如，pH 4.6可溶性氮低于总氮的15%, αs1-酪蛋白与其他盐水干酪相比也要低）。尿素-聚丙烯酰胺凝胶电泳 (UREA-PAGE) pH 4.6不溶性组分在干酪的不同成熟阶段αs1-酪蛋白都有不同程度的降解。然而，α-酪蛋白对水解有一定的抵制作用。包装于聚乙烯塑料袋中的干酪与盐水浸渍成熟干酪相比，前者的pH 4.6可溶性氮、12%三氯乙酸可溶性氮以及游离氨基酸的含量增加显著。干酪成熟时间达到120天以后，游离氨基酸 (FFA) 的总含量增加，其中谷氨酸 (Glu), 缬氨酸 (Val), 亮氨酸 (Leu), 赖氨酸 (Lys) 和苯丙氨酸 (Phe) 是干酪中最丰富的氨基酸。在聚乙烯塑料袋中成熟的干酪，这些氨基酸的含量更高。干酪中pH 4.6可溶性组分中的醇溶性和醇难溶性肽经RP-HPLC分析表明：包装于聚乙烯塑料袋中的干酪与盐水浸渍的干酪一些肽的含量略有差异。真空包装干酪的主要挥发性化合物的浓度要比盐水浸渍的干酪要高。真空包装干酪通过扫描电子显微镜所得到的图像与盐水浸渍的干酪相比明显不同。真空包装干酪在化学成分、蛋白酶解、挥发性化合物组成以及微观结构方面完全不同于盐水浸渍的成熟干酪。通过以上实验结果可以认为真空包装更有利于保持Mihalic干酪的化学和生物化学品质，在干酪成熟过程中更实用。

Keywords Mihalic cheese · Proteolysis · Microstructure · Volatile composition · Packaging

关键词 Mihalic 干酪 · 蛋白酶解 · 微观结构 · 挥发性化合物组成 · 包装

1 Introduction

Cheese quality is influenced by several factors such as raw milk flora, type of starters, and the manufacturing and ripening conditions. The ripening of cheese includes complex textural, biochemical, and microbiological events including proteolysis, lipolysis, glycolysis, and further degradation of free amino acids or free fatty acids (McSweeney and Sousa 2000; Hayaloglu et al. 2005). These reactions change the appearance, texture, microstructure, and flavor of the final product (Bosset et al. 1993). Depending on the variety, consumer acceptance of the cheese is mainly dependant on its flavor and textural characteristics.

Volatile compounds result from the degradation of amino acids and fatty acids, with the resulting cheese flavor due to the correct balance and concentration of numerous sapid and aromatic compounds (McSweeney and Sousa 2000). During the cheese manufacture and/or ripening period, many factors including coagulation, acidification, drainage,

starter concentration, rennet type and concentration, salt content, and the ripening period have an effect on the microstructure of cheese (Rovira et al. 2010).

Mihalic cheese is a hard brined cheese with a slightly acidic and very salty taste, regular openings (2–4 mm), and a 3- to 4-mm rind. It has been mainly produced in the cities of Bursa and Balikesir, Turkey, for more than 250 years, and it is consumed throughout Turkey. Raw or heat-treated sheep's milk is coagulated at 32–35 °C using homemade calf or commercial rennet in wooden barrels. Milk is thermized at 65 °C for about 10 min, and the pre-fermentation of milk (about 30 min) is achieved without starter culture. Therefore, the native microflora from milk or the environment is very important on the biochemistry of Mihalic cheese. A high level of NaCl (18% or more) in its brine may result in a salty taste, affecting biochemical reactions and microstructure. Nowadays, Mihalic cheese is sold, after 3 months of ripening, as vacuum-packed pieces weighing about 250–300 g. In comparison to the cheeses in Turkey, no study appears in the literature on the biochemical, textural, or volatile aroma characteristics of Mihalic cheese. Few studies are available on the gross compositional or microbiological status of the cheese sold in retail shops (Hayaloglu et al. 2008). Due to practical and hygienic uses in storage and marketing, cheese makers prefer using the vacuum packaging method instead of brining in recent years. The aim of the study was, thus, to investigate the effect of vacuum packaging or brine immersion on the chemistry, biochemistry, and microstructure of the cheese during extended ripening to test what changes are present after 3 months, especially in proteolysis and flavor. In traditional production, the cheese is ripened more than 3 months in practice; however, no studies are present in the literature. Also, the vacuum packaging and brining methods will be compared and recommended to cheese makers based on the results obtained during ripening (over 360 days) to test what changes occur in proteolysis and flavor compound formation.

2 Materials and methods

2.1 Cheesemaking

The protocol for the manufacture of Mihalic cheese is shown in Fig. 1. Ewe's milk was heat-treated at 65 °C × 12 min and cooled to 35 °C. After pre-ripening of the cheese milk for about 30 min, the cheese milk was coagulated in wooden barrels with a capacity of 200 L using calf rennet. The curd was cut after 90 min and broken into pieces, the slice of a grain of rice (about 2–3 × 2–3 mm in size), using a nailed wooden rod. After 10–15 min, the curd was cooked gradually to 40 °C with stirring and rested for another 10–15 min. Then, the curd was transferred to a cotton cloth for whey drainage. After removing excess whey, the curd was pressed for about 8–10 h and the cheese cloth pierced periodically with a needle to accelerate wheying off. The drained curd was cut into blocks of 3 kg and brined in gradual NaCl solutions (12, 14, 16, and 18% NaCl) for four consecutive days. The blocks were divided into two parts: one was immersed in brine (18% NaCl) and the other was packaged in polyethylene bags under vacuum. Two cheese-making trials were carried out for Mihalic cheese manufacture; the cheeses were ripened for 1 year under vacuum or brine at 6 °C. The samples were taken at 1, 30, 60, 90, 120, 150, 180, and 360 days of ripening.

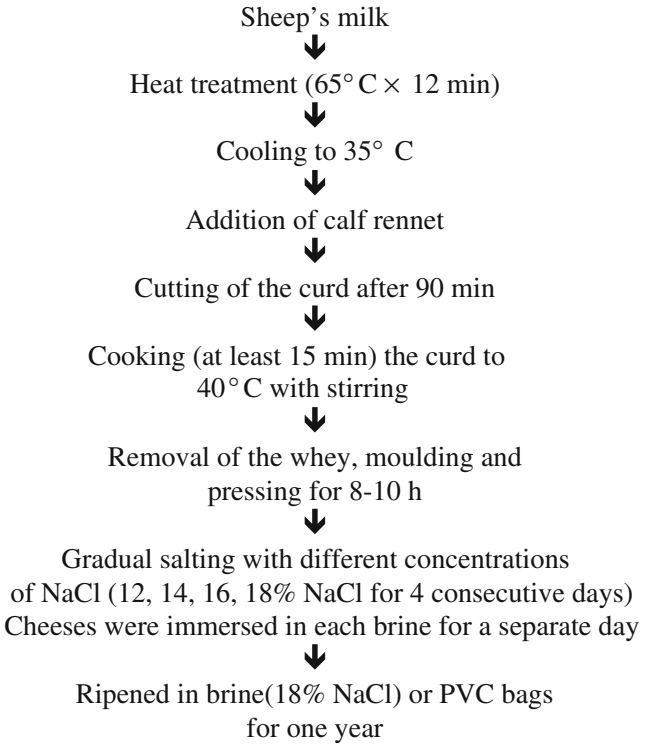

Fig. 1 Protocol for the manufacture of Mihalic cheese using ewe's milk

2.2 Gross composition

Cheeses were analyzed in duplicate for moisture using the gravimetric method of drying at 102 °C, salt by titration with $AgNO_3$, fat using the Van Gulik method, and total nitrogen (TN) using the Kjeldahl method as described in Hayaloglu et al. (2005). For pH measurement, grated cheese (10 g) was macerated with 10 mL of distilled water and the pH of the resultant slurry measured using a digital pH meter (model 420A>>, Orion, Beverly, MA, USA). Titratable acidity was determined as grams of lactic acid per 100 g cheese using the method described in Hayaloglu et al. (2005).

2.3 Proteolysis

Water-soluble nitrogen (WSN), 12% trichloroacetic acid-soluble nitrogen (TCA-SN), and 5% phosphotungstic acid-soluble (PTA-SN) fractions as percentage of the total nitrogen of the cheese were determined using the methods described by Hayaloglu et al. (2005). WSN, TCA-SN, and PTA-SN were expressed as the percentage of TN. Total free amino acid (FAA) levels in the pH 4.6 soluble fraction of the cheeses were determined using the Cd–ninhydrin method described in Hayaloglu (2007). Individual FAA levels were determined in 12% TCA-soluble filtrates of the pH 4.6 soluble extracts using a Beckman 6300 High-Performance Amino Acid Analyzer (Beckman Instruments Ltd., High Wycombe, UK) as described by Hayaloglu et al. (2005). The concentrations were expressed as milligrams per 100 g of cheese. The

pH 4.6 insoluble fractions of the cheeses were freeze-dried and then analyzed using urea polyacrylamide gel electrophoresis (urea-PAGE) using a Protean II XI vertical slab gel unit (Bio-Rad Laboratories Ltd., Watford, UK) according to Andrews (1983). The gels were stained directly using the method of Blakesley and Boezi (1977) with Coomassie Brilliant Blue G-250. The pH 4.6 soluble nitrogen fractions and the fractions resulted after 70% (*v*/*v*) ethanol treatment of the cheeses were also freeze-dried. The 70% ethanol-soluble (EtOH-s) or ethanol-insoluble (EtOH-i) fractions were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) as described by Hayaloglu et al. (2005).

2.4 Volatile composition by SPME

Analysis of the volatiles was performed with the static head space solid phase micro-extraction (SPME) method using a gas chromatography–mass spectrometry system (Shimadzu Corporation, Kyoto, Japan), essentially as described in Hayaloglu et al (2007) and Hayaloglu and Karabulut (2010). The tentative identifications were based on comparing the mass spectra of unknown compounds with those in Wiley 7 (7th edition) and the NIST/EPA/NIH 02 mass spectral library. Identifications were also confirmed by comparing the retention times with the reference standards when available. A total of 33 authentic standard compounds (Sigma Chemical Co., St. Louis, MO, USA) were used to confirm the identities of volatile compounds in the cheese samples. The concentrations were calculated by a comparison of the peak areas of the internal standard and the unknown compounds. Each compound was expressed as micrograms per 100 g of cheese.

2.5 Scanning electron microscopy

The microstructure of the cheese samples was determined at 360 days of ripening as described in Madadlou et al. (2007), with some modifications. Cheeses were cut into specimens with dimensions of 1×1×10 mm using a razor blade and immersed in 2.5% glutaraldehyde (prepared using pH 7.2 phosphate buffer) solution overnight. The fixed specimens were washed six times (1 min each time) using phosphate buffer (pH 7.2) and then dehydrated in a graded ethanol series of 20, 40, 60, 80, 95, and 100% at 30-min intervals. Fat in the specimens was removed using three changes in chloroform (15 min each). The samples were critical point-dried and then the pieces with the heights reduced were mounted on aluminum stubs. The mini cubes were coated with a thin layer of gold–palladium in a sputter coater (BAL-TEC, model SCD 050, Baltec Inc., Liechtenstein) and transferred into high-vacuum scanning electron microscopy (SEM; LEO, EVO 40, Carl Zeiss SMT, Cambridge, UK). Measurements in SEM were carried out at 20 kV and the images were recorded at ×3,000, ×5,000, ×7,000, ×10,000, and ×15,000. Only ×7,000 magnification images were shown for the present paper.

2.6 Statistical analysis

A randomized complete block design which incorporated two treatments (cheeses BR or VP), eight ripening periods (1, 30, 60, 90, 120, 150, 180, or 360 days), and two

blocks (trials) was used to analyze the response variables relating to cheese composition and proteolysis data. ANOVA was performed using a general linear model procedure (SAS 1995), where the effects of treatment and replicates were estimated for the response variables. Duncan's multiple-comparison test was used as a guide for pair comparisons of the treatment means. The level of significance of the differences between treatments was considered at $P<0.05$. Also, data from the RP-HPLC chromatograms of the 70% ethanol-soluble fractions of the cheeses and from the GC-MS data of the volatile components were analyzed using multivariate statistical techniques. Principal component analysis (PCA) was performed using a covariance matrix and varimax rotation between the cheeses. The peak heights (RP-HPLC) or the concentrations of volatile components (GC-MS) were used as variables. PCA was carried out using package programs Unscrambler trial v.9.1 (CAMO Software AS, Oslo, Norway) and SPSS version 9.0 for Windows (SPSS Inc., Chicago, IL, USA).

3 Results and discussion

3.1 Chemical composition and pH

The composition of the cheeses ripened under brine (BR) or vacuum package (VP) was determined at day 30 (Table 1). All compositional parameters were significantly different between the samples, except for pH and fat-in-dry matter values ($P<0.05$). The levels of total solids, fat, and protein were higher in the VP cheeses than the BR cheese. The contents of total solids were higher in VP cheeses than the BR cheeses due to the barrier effect of the polyethylene bag for water. The concentration effect of the total solids caused higher levels of protein and fat in the vacuum-packaged cheeses. No significant changes were noted between the pH values of the two types of cheeses. The pH of the cheese was around 5.0, and the value is in accordance with the same age of Mihalic cheese reported by Oner and Aloglu (2004). Although both VP and BR cheeses were brine-salted during manufacture (see Fig. 1), the salt concentrations were of significantly lower levels in vacuum-packaged cheeses. More salt uptake occurred in cheeses stored in brine. A high concentration of salt is characteristic of Mihalic among brine-ripened cheeses (Hayaloglu et al. 2008).

Table 1 Chemical composition and pH values of Mihalic cheeses at 30 days of ripening

Parameters	BR	VP
Titratable acidity	0.49±0.03a	0.67±0.03b
pH	4.95±0.01a	4.98±0.03a
Total solid (%)	60.40±0.05a	67.08±0.10b
Salt (%)	5.32±0.41a	3.86±0.08b
S/M (%)	13.44±1.04a	11.73±0.22b
Fat (%)	33.00±1.41a	36.75±0.35b
FDM (%)	54.64±2.34a	54.79±0.61a
Total protein (%)	20.44±0.51a	23.20±0.11b

The values presented are the means of duplicate cheese trial and standard deviations. Values within a row not sharing a common letter differ significantly ($P<0.05$) *BR* brine ripened, *VP* vacuum-packaged, *S/M* salt-in-moisture, *FDM* fat-in-dry matter

Similar levels of salt were also reported by Hayaloglu and Karabulut (2010) (4.2–8.4%)) and Oner and Aloglu (2004) (4.5–9.9%). The results indicated that immersing the cheeses under brine during ripening caused an increase in their salt concentrations. The mean values of fat, fat-in-dry matter, and protein concentrations in BR and VP cheeses (respectively) were in agreement with previous work; details are given in Hayaloglu et al. (2008).

3.2 Proteolysis

The level of pH 4.6 soluble nitrogen (SN) in the cheese increased during ripening; however, the rate of increase was not intense at the beginning of the ripening period (until 120 days), as shown in Fig. 2. The levels of pH 4.6 SN in the cheeses ripened under brine or vacuum-packaged did not differ significantly at 1 or 30 days ($P>0.05$). The majority of brine-ripened cheeses have low pH and high moisture values (Hayaloglu et al. 2002, 2005; Abd El-Salam and Alichanidis 2004), conditions that favor the action of chymosin on α_{s1}-casein, resulting in rapid increases of soluble nitrogen. However, low moisture and high salt contents in Mihalic cheese limited the formation of SN during ripening. After 60 days of ripening, VP cheeses had higher values of pH 4.6 SN than BR cheeses; the highest pH 4.6 SN value was recorded at the end of ripening. Differences in the levels of pH 4.6 SN fractions between the BR and VP cheeses increased as ripening proceeded. Karagul-Yuceer et al. (2009) used tinplate packaging (under brine) or vacuum packaging for Ezine cheese (a brine-ripened variety made using mixtures of goat's, ewe's, and cow's milk) and pointed out that no significant differences were noted among the cheeses of this type of soluble nitrogen. The pH 4.6 SN fractions in cheese increased throughout ripening; similar trends were also observed for Ezine (Karagul-Yuceer et al. 2009), Motal (Andic et al. 2010), Kulek (Dervisoglu and Yazici 2001),

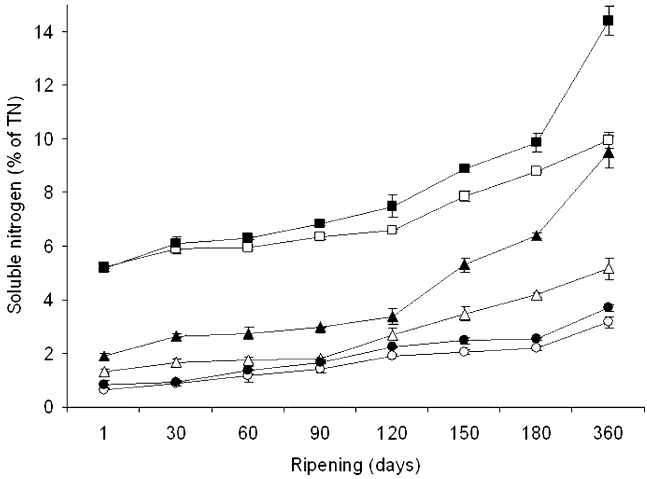

Fig. 2 Levels of pH 4.6 (*square*), trichloroacetic (*triangle*), or phosphotungstic acid (*circle*) soluble nitrogen fraction in Mihalic cheese during 360 days of ripening. *Closed* and *open symbols* represent cheeses ripened in VP and BR, respectively

and Tulum (Hayaloglu et al. 2007) cheeses ripened under different conditions. The fractions soluble in 12% trichloroacetic acid (TCA-SN) showed a similar trend during ripening; the highest differences between the samples were found at the end of ripening (Fig. 2). As with the pH 4.6 SN fraction, the level of TCA-SN was significantly lower in BR than VP cheeses ($P<0.05$). The difference between the first (first days) and the last (360 days) values in the cheeses for the TCA-SN values was about four to fivefold. Ripening under vacuum packaging caused a significant increase in the TCA-SN levels. These increases would be due to the formation of free amino acids and small peptides by the degradation of caseins. Trends for the PTA-SN fractions were the same for the two types of Mihalic cheeses by the end of the ripening period; however, VP cheeses had higher values than BR cheeses at all sampling times (Fig. 2). Increases in PTA-SN were approximately linear during ripening, except for 360 days. The values for all soluble fractions sharply increased at the end of ripening. The sharp increases in all parameters in the cheeses were evaluated as age-related changes in the cheese medium. It was reported that the levels of PTA-SN increased sharply after 9 months of ripening in Ezine cheeses ripened in tinplate containers (under brine) and vacuum packaging (Karagul-Yuceer et al. 2009). Big differences were determined in the contents of amino acids (reported as absorbance at 507 nm, A_{507}) during all sampling times for the cheeses (Fig. 3). The level of A_{507} reached about 3.0 at 360 days for VP cheese; the absorbance value was 2.4 for BR cheese at the same time. The level of total free amino acids in Mihalic cheeses exhibited a similar trend with soluble nitrogen fractions including pH 4.6 SN, TCA-SN, and PTA-SN during ripening, as reported by other workers (Pavia et al. 2000; Hayaloglu 2007; Andic et al. 2010).

Urea-PAGE of the pH 4.6 insoluble fractions was performed; similar patterns of hydrolysis were observed for both cheeses, with minor differences (gel electrophoretograms not shown). The caseins were slowly hydrolyzed during the ripening, and no substantial differences were noted among the cheeses ripened under brine or vacuum-packaged. In aged cheeses, the hydrolysis of α_{s1}-caseins was slightly higher in VP cheeses than in BR cheeses; however, no differences were seen between the extent of β-casein degradation in the two cheeses. The resistance of β-casein to be

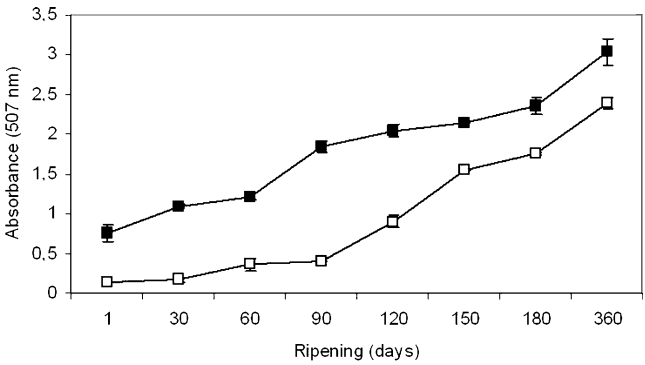

Fig. 3 Absorbance values at 507 nm (A_{507}), indicating free amino acid concentration, of pH 4.6 soluble fractions of Mihalic cheeses during 360 days of ripening. *Dark* and *open symbols* represent cheeses ripened in VP and BR, respectively

hydrolyzed is reported in many cheeses, especially bacterially ripened varieties including Turkish white-brined (Hayaloglu et al. 2005, 2008), Feta (Alichanidis et al. 1984), Tulum (Hayaloglu et al. 2007), and Malatya (a Halloumi-type cheese; Hayaloglu et al. 2010). However, slight degradation of α_{s1}-casein is noticeable in the present study, although the cheeses ripened in quite a long period. A limited degradation of α_{s1}-caseins was also noted for cheeses including Dil, Hellim, and Malatya which are scalded at high temperatures (>80 °C), presumably due to the thermal degradation of proteolytic enzymes and/or microorganisms (Hayaloglu and Karabulut 2010). The limiting factors for casein hydrolysis are the low moisture and the high salt levels. Proteolysis levels were lower in the rind in comparison to the core in some cheeses including Manchego type (Pavia et al. 2000) and Ragudano (Melilli et al. 2004); also, proteolysis was lower in higher concentrations of salt (Guven et al. 2006; Kristiansen et al. 1999). In the above studies, this observation was linked to the higher levels of salt. In the present study, the use of different storage methods did not influence the urea-PAGE patterns of the cheeses due to the high salt and low moisture contents of Mihalic cheese.

The RP-HPLC peptide profiles of the EtOH-s (Fig. 4) or EtOH-i fractions of the pH 4.6 SN extracts were performed during ripening. No major differences were observed during ripening for the EtOH-i fractions (chromatograms not shown); however, some peptides were eluted in VP cheeses at higher concentrations than BR cheeses due to the restricting effects of salt. PCA was performed using peak heights to aid in the interpretation of the results (Fig. 5). A cumulative variation of 78.6% was explained, and PC1 and PC2 accounted for 67.4 and 11.2% of the variation, respectively. PC1 (67.4%) separated the cheeses on the basis of the ripening environment, i.e., vacuum-packaged cheeses positioned on the right side of PC1 and brine-immersed cheese positioned on the negative side of PC1. The ripening period also affected the PCa plot. PC2 (11.2%) separated the cheeses on the basis of the ripening time: the younger cheeses scoring negatively and the older cheeses scoring positively on PC2. However, PC2 has a lower variation. At the beginning of ripening, all samples were closely grouped on the PCA plot. As ripening progressed (after 120 days of ripening), greater differences were recorded between the cheeses, and BR and VP cheeses were completely separated at 360 days. This indicates that the ripening period influenced the peptide profiles of both cheeses in a different manner. Some peaks on the chromatogram at day 30 were specific for Mihalic cheeses, and their concentrations were changed by samples and/or the ripening time. When compared to the other brine-ripened cheeses (Hayaloglu et al. 2005, 2008), the age-related changes were quite limited due to the reasons given above for urea-PAGE or soluble nitrogen fractions.

The extent of proteolysis was also monitored by determining individual FAAs, as shown Fig. 6. Individual FAAs were determined after 1, 120, and 360 days of ripening, and the FAA profiles of the cheeses were quite different at all sampling times. The principal FAAs in the cheese samples were Leu, Glu, Phe, and Lys. The same amino acids were also important for Turkish white-brined (Hayaloglu et al. 2005), Roncal-type ewe's milk cheese (Irigoyen at al. 2007), Teleme (Mallatou et al. 2004; Pappa and Sotirakoglou 2008), and Feta (Alichanidis et al. 1984) cheeses. The levels of individual FFA were similar at 1 day of ripening for both cheeses; however, significant differences were observed between the samples as the ripening period

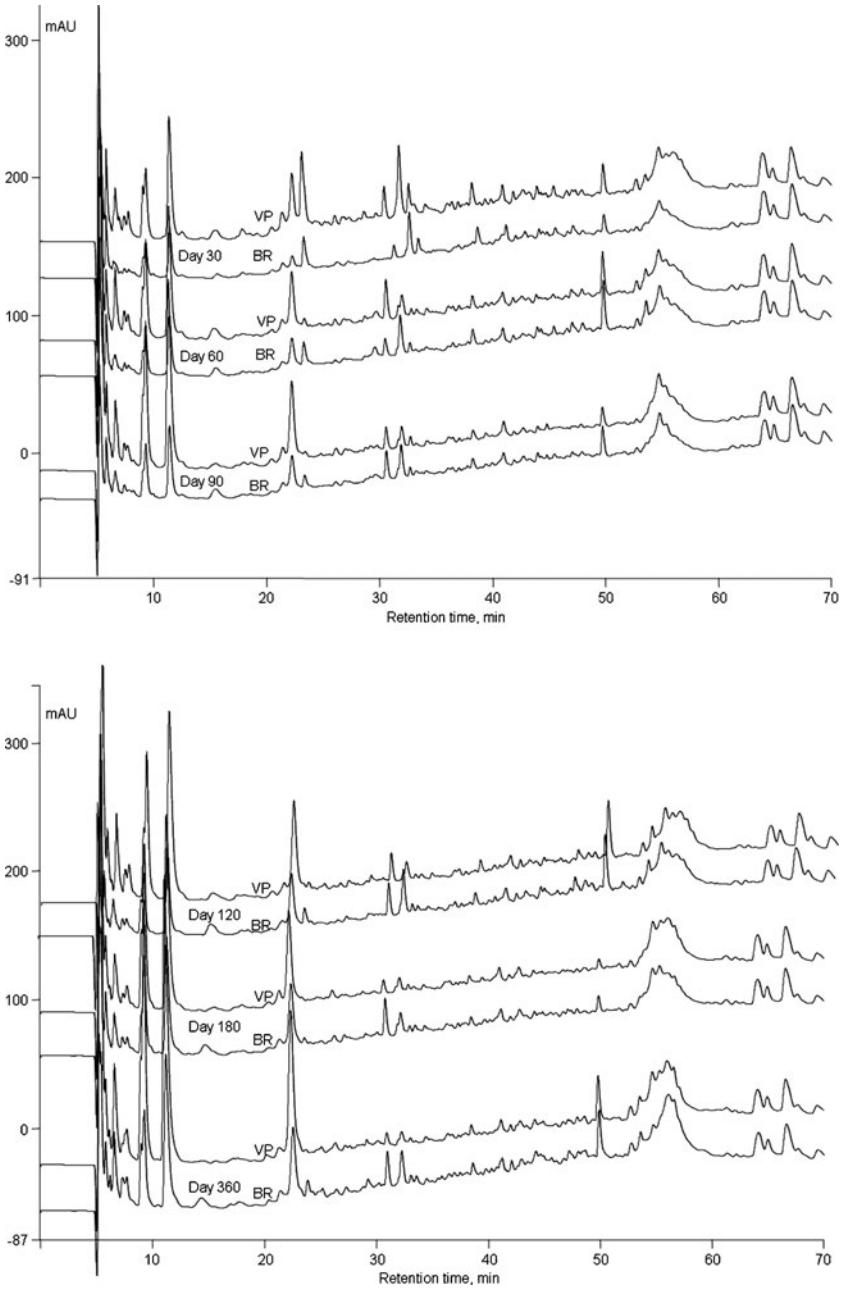

Fig. 4 RP-HPLC profiles of 70% ethanol-soluble fractions of Mihalic cheeses ripened in vacuum packaging (VP) or under brine (BR) at 30, 60, 90, 120, 180 and 360 days of ripening

proceeded ($P<0.05$). Higher concentrations of the principal and other FAAs were found in VP cheeses than in BR cheeses. Age-related changes were observed in the cheeses at 360 days of ripening, and a sharp increase was determined in the FAAs for VP cheese.

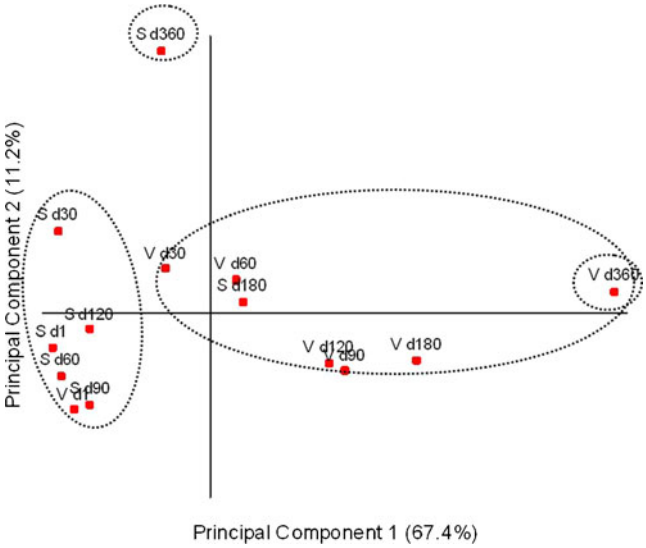

Fig. 5 Principal component analysis of peak (height) data from RP-HPLC of the 70% ethanol-soluble fractions of the cheeses ripened in vacuum packaging (*V*) or under brine (*S*)

3.3 Volatile composition by SPME

A total of 41 volatile compounds belonging to the chemical classes of esters (10), acids (4), ketones (8), aldehydes (3), alcohols (10), and miscellaneous compounds (6) were determined during 360 days of ripening. These compounds were grouped and listed in Table 2 as average values±SD. Esters, alcohols, and ketones were the most abundant compounds isolated in the headspace of Mihalic cheeses. The volatiles in the samples differed based on their ripening environment, so significant differences

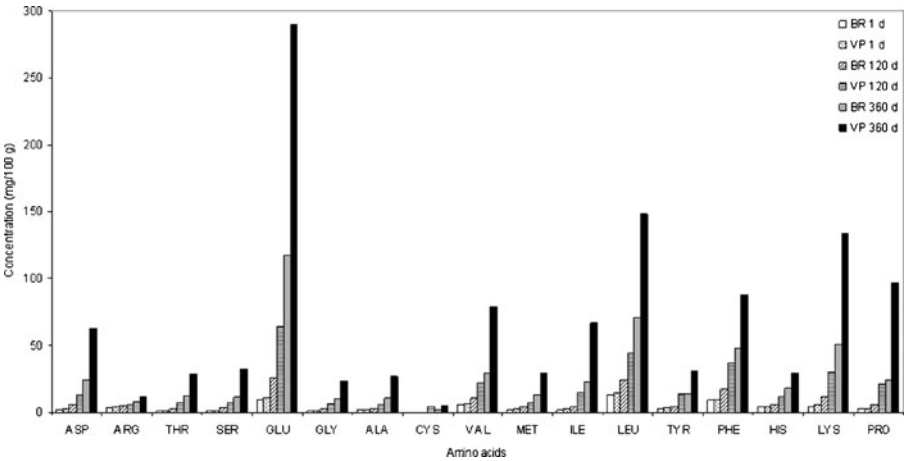

Fig. 6 Levels of individual free amino acids in pH 4.6 soluble fractions of Mihalic cheeses ripened in vacuum packaging (*VP*) or under brine (*BR*) at 1, 120, and 360 days of ripening

were noted between the samples ($P<0.05$). To facilitate the interpretation of the results, multivariate statistical analysis was performed, and the cheeses (BR and VP types) were classified using principal component analysis. In general, the principal volatiles in Mihalic cheese were esters (e.g., ethyl acetate and ethyl and methyl butanoate); ketones (e.g., acetone, 2-butanone, and 2-pentanone); and alcohols (ethanol and 3-methyl-1-butanol).

Ethyl acetate, ethyl butanoate, and methyl butanoate were the principal esters in Mihalic cheese samples during ripening (Table 2). Some compounds in that group, including ethyl acetate, ethyl hexanoate, ethyl butanoate, methyl butanoate, ethyl octanoate, and methyl hexanoate, were determined in VP cheeses at higher concentrations than BR cheeses. In addition, methyl octanoate and ethyl octanoate were found only in VP cheeses, while butyl acetate and hexyl acetate were determined in BR cheeses. These esters were also linked with these types of cheeses by performing multivariate analysis, as shown on the PCA biplot (Fig. 7).

Only four carboxylic acids were isolated from the cheeses, and their concentrations increased during ripening (Table 2). The principal acid was butanoic, and it was determined in all samples at all sampling points. Acetic acid was mainly determined in BR cheese and is also found in other brine-ripened cheeses in Turkey (Hayaloglu and Karabulut 2010), and also Greek Feta (Kondyli et al. 2002) or Spanish Torta del Casar (Delgado et al. 2010) made using ewe's milk. Acetic acid was not determined at the first stage of ripening in VP cheeses; its concentration was about 62 μg/100 g of cheese at 360 days of ripening. Other acids in the cheese were butanoic, hexanoic, and octanoic acids. The concentration of butanoic acid increased linearly during ripening; however, hexanoic and octanoic acids were not isolated in each sampling time (Table 2).

Ketones were one of the major chemical groups in the experimental cheeses (Table 2). The concentrations of ketones increased in VP cheeses during ripening; however, some fluctuations were observed in the levels of ketones in BR cheeses.

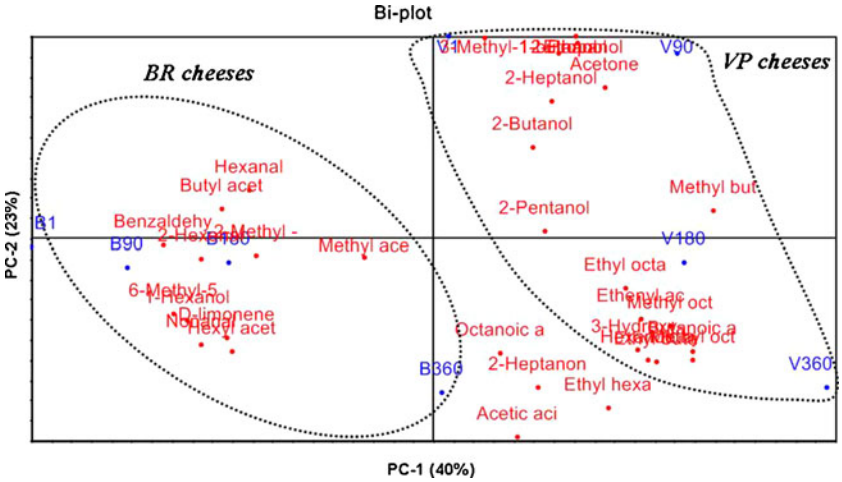

Fig. 7 PCA of the volatile data from SPME/GC-MS analyses of Mihalic cheeses ripened in vacuum package (*VP*) and under brine (*BR*) during ripening

Table 2 Volatiles in Mihalic cheeses during ripening (in micrograms per 100 g cheese)

Volatiles	RI	ID method[a]	Cheeses							
			VP				BR			
			1 day	90 days	180 days	360 days	1 day	90 days	180 days	360 days
Methyl acetate	867	MS, RI	ND	0.8±0.1	0.9±0.2	0.3±0.1	ND	0.2±0.0	9.9±12.7	0.4±0.1
Ethyl acetate	892	MS, RI	57.3±43.6	44.0±13.0	32.3±17.0	28.3±0.7	33.8±5.1	15.8±4.1	20.9±29.6	37.0±7.7
Ethyl hexanoate	1,235	MS,RI	3.5±0.5	14.7±4.5	13.7±5.5	62.6±7.0	2.1±0.1	3.5±1.0	18.2±8.1	50.0±13.3
Methyl octanoate	1,438	MS, RI	ND	1.0±1.43	3.0±1.6	4.9±0.9	ND	ND	ND	ND
Ethyl butanoate	1,033	MS, RI	12.8±2.9	21.6±6.5	17.3±7.9	58.6±2.3	3.4±0.2	3.6±1.1	19.1±10.5	34.1±7.9
Methyl butanoate	984	MS, RI	14.9±9.8	36.5±8.3	37.5±17.0	35.7±1.0	5.2±0.6	5.0±0.7	9.7±3.9	11.1±1.6
Butyl acetate	1,069	MS, RI	1.3±1.8	ND	ND	ND	4.8±0.0	1.4±0.5	ND	ND
Hexyl acetate	1,275	MS, RI	ND	ND	ND	ND	3.2±0.3	1.2±0.5	3.5±0.1	3.8±1.1
Ethyl octanoate	1,393	MS, RI	ND	2.0±0.5	15.3±17.9	6.9±1.5	ND	ND	ND	ND
Methyl hexanoate	1,189	MS, RI	9.7±5.4	21.1±4.7	31.6±14.0	42.3±2.0	9.0±0.9	9.0±3.1	13.2±2.6	24.5±6.7
Acetic acid	1,478	MS, RI, AS	ND	ND	40.7±56.9	61.8±11.7	4.5±6.3	9.5±0.5	53.0±15.3	96.3±33.9
Butanoic acid	1,643	MS, RI, AS	71.8±5.7	320.4±1.2	440.7±622.4	545.9±68.7	15.0±0.2	35.9±5.5	193.3±46.7	409.0±127.2
Hexanoic acid	1,878	MS, RI, AS	66.9±2.3	176.0±21.1	382.0±276.8	337.1±38.2	ND	42.4±10.1	ND	458.8±107.6
Octanoic acid	2,048	MS,RI, AS	ND	ND	93.0±51.2	ND	ND	ND	ND	139.9±30.9
Acetone	858	MS,RI, AS	63.5±15.1	52.9±14.6	43.5±2	40.0±0.8	31.2±4.9	16.2±5.1	36.0±18.7	40.0±8.6
2-Butanone	913	MS, RI, AS	47.8±4.6	43.9±12.9	36.6±19.1	33.6±11.3	23.9±12.3	20.6±10.1	40.8±15.7	51.7±10.7
Diacetyl	972	MS, RI, AS	1.6±0.4	1.8±1.2	1.9±0.1	8.5±0.2	0.9±0.1	0.4±0.2	0.7±0.2	0.8±0.2
2-Pentanone	974	MS, RI, AS	21.8±2.2	21.5±5.5	17.0±8.11	13.6±0.5	11.6±0.5	8.1±2.6	15.3±6.5	24.6±6.2
2-Heptanone	1,186	MS, RI, AS	4.6±2.2	10.1±1.7	11.7±5.7	11.4±0.4	9.1±0.5	4.8±1.2	9.0±3.9	19.0±3.9
2-Nonanone	1,396	MS, RI, AS	2.6±1.0	3.2±0.9	3.1±1.3	2.6±0.1	5.3±2.0	3.2±1.7	3.4±1.3	6.2±1.6

Table 2 (continued)

Volatiles	RI	ID method[a]	Cheeses							
			VP				BR			
			1 day	90 days	180 days	360 days	1 day	90 days	180 days	360 days
3-Hydroxy-2-butanone	1,291	MS, RI, AS	ND	5.3±0.7	14.0±6.9	40.3±1.3	2.6±0.9	0.8±0.8	4.4±1.8	1.1±1.0
6-Methyl-5-hepten-2-one	1,342	MS, RI	ND	ND	ND	ND	1.9±0.3	1.0±0.4	0.7±0.0	0.9±0.4
Hexanal	1,082	MS, RI, AS	16.9±0.9	7.2±0.7	4.7±2.0	3.2±0.1	12.9±1.6	9.3±3.3	24.2±6.2	12.8±2.2
2-Hexenal	1,225	MS, RI	ND	ND	ND	ND	4.0±2.2	3.5±2.1	ND	ND
Benzaldehyde	1,542	MS, RI	0.62	ND	ND	ND	3.1±0.2	2.3±0.6	3.0±1.0	ND
Nonanal	1,399	MS, RI, AS	ND	ND	ND	ND	4.5±3.2	4.5±2.1	7.1±2.5	5.5±1.5
Ethanol	937	MS, RI, AS	200.5±37.2	171.8±57.3	110.6±68.0	106.9±4.2	56.8±6.2	46.1±20.0	98.9±48.1	214.3±29.1
3-Methyl-1-butanol	1,207	MS, RI	23.6±1.5	20.4±2.9	13.1±5.5	7.8±0.1	11.4±0.2	7.9±2.4	14.0±20.2	14.6±1.9
2-Butanol	1,026	MS, RI, AS	ND	1.2±0.2	0.2±0.2	ND	ND	0.2±0.3	ND	0.5±0.6
2-Pentanol	1,119	MS, RI	0.4±0.5	1.4±0.4	0.3±0.4	0.8±0.0	ND	0.2±0.2	1.3±1.1	1.0±0.0
1-Pentanol	1,251	MS, RI, AS	ND	ND	ND	ND	ND	0.5±0.6	ND	ND
2-Heptanol	1,320	MS, RI, AS	2.6±0.1	3.2±0.6	2.2±0.7	1.4±0.3	1.5±0.0	1.1±0.3	2.2±1.1	2.6±0.3
2-Propanol	933	MS, RI	6.8±2.6	8.0±3.9	4.7±2.5	3.1±0.5	3.3±0.7	0.8±0.6	3.4±2.0	3.1±0.8
1-Hexanol	1,355	MS, RI, AS	0.5±0.8	0.4±0.5	0.6±0.4	0.2±0.3	1.0±0.1	1.1±0.4	0.8±0.3	1.1±0.2
1-butanol	1,144	MS, RI	7.1±0.8	24.0±3.7	3.8±3.4	ND	ND	ND	ND	ND
2-Methyl -1-propanol	1,092	MS, RI	ND	ND	ND	ND	4.7±6.7	0.1±0.2	ND	0.4±0.2
D-Limonene	1,205	MS, RI	ND	ND	ND	ND	4.0±0.7	4.6±2.1	9.1±4.5	6.0±2.1
1,3-Dimethylbenzene	1,144	MS, RI	13.8±0.2	4.4±0.1	9.3±5.1	12.6±1.0	7.9±0.9	5.5±2.4	4.7±6.3	14.1±6.3
Chloroform	1,015	MS, RI	55.5±12.2	49.3±11.8	35.5±17.1	22.8±0.9	27.5±3.8	17.6±3.3	29.4±15.3	29.8±5.9
Toluene	1,039	MS, RI	51.8±3.3	43.9±10.8	39.1±20.4	35.7±8.2	43.2±2.1	31.2±10.9	49.7±14.3	57.7±23.0

Table 2 (continued)

Volatiles	RI	ID method[a]	Cheeses							
			VP				BR			
			1 day	90 days	180 days	360 days	1 day	90 days	180 days	360 days
Ethylbenzene	1,129	MS, RI	5.8±2.8	4.2±0.6	4.5±2.1	3.1±0.9	6.6±0.3	5.4±1.7	8.9±3.4	18.4±5.9
Styrene	1,263	MS, RI	20.7±0.5	18.2±0.8	21.9±14.5	15.4±1.0	46.7±0.5	33.8±8.1	55.8±20.1	58.5±19.7

VP vacuum-packaged, *BR* brine-ripened, *ND* not detected

[a] *MS*—mass spectral identification using NIST 07 and Wiley 7 library; *RI*—identification using alkane series (C8–C20) by calculation of the retention indices at the same chromatographic conditions; *AS*—identification using authentic standard at the same chromatographic conditions

Acetone (propanone), 2-butanone, and 2-pentanone were the most abundant ketones in the two types of cheeses during ripening. Therefore, it can be thought that these three ketones contributed to the aroma of these types of cheeses. Also, the level of 3-hydroxy-2-butanone was higher in VP than in BR cheeses. Similarly, these three compounds were the major ketones in another study of Mihalic cheese (Hayaloglu and Karabulut 2010). Diacetyl was identified at increased levels in VP cheeses, while its concentration did not change substantially in BR cheese. Four aldehydes were identified in the experimental cheeses; only hexanal was unique in VP cheese, and its concentration decreased during ripening. Benzaldehyde was present at a low level (0.62 μg/100 g) in VP cheese at 1 day of ripening, and it disappeared at other sampling times. When compared to the experimental cheeses, the concentration and/or the availability of aldehydes in Mihalic cheese during ripening were significantly influenced by ripening in brine or vacuum packaging.

Alcohols were one of the principal chemical groups in the cheese volatiles, and they may have an effect on the flavor of Mihalic cheese. Ethanol, 3-methyl-1-butanol, 2-heptanol, and 2-propanol were the alcohols with the highest concentrations in Mihalic cheese cheeses during ripening, and their concentrations exhibited different trends in VP and BR cheeses. In general, the concentrations of alcohols increased in BR cheeses, while their levels decreased in VP cheeses during 360 days of ripening. Ethanol was the most abundant volatile in both cheeses throughout ripening. In our previous study (Hayaloglu and Karabulut 2010), the level of ethanol was not high; however, the principal alcohols were 2-butanol, 1-butanol, and 3-methyl-1-butanol. 3-Methyl-1-butanol was also among the main alcohols in the present study, and it was present at about 15 μg/100 g, implying a strong contribution to the flavor characteristics of Mihalic cheese. There are some compositional differences between the two types of cheeses; 1-butanol was identified only in VP cheeses, while 2-methyl-1-butanol was identified in BR cheeses during ripening. The compounds which were found in the cheeses are highlighted on the PCA plot (Fig. 7). It is noticeable that the alcohols including 2-butanol, 2-pentanol, 2-heptanol, 2-propanol, and 1-hexanol have been found in the majority of the samples (Table 2).

Six miscellaneous compounds were identified in Mihalic cheeses. This group of compounds is not related to cheese manufacture or the ripening processes; they originate from animal feed (like D-limonene) or contamination (chloroform, etc.) during manufacture or cheese analysis. D-Limonene was only determined in BR cheeses, and the highest concentration (9.12 μg/100 g) was observed at 180 days of ripening. Toluene and styrene, which are aromatic hydrocarbons, were identified at high levels, and their concentrations did not change significantly during ripening. Toluene has been identified at similar levels in Mihalic cheese (Hayaloglu and Karabulut 2010) and Tulum (Hayaloglu et al. 2007) cheeses.

PCA was used to visualize the SPME/GC-MS results of the cheeses during ripening. A biplot of the sample scores and variable loadings for PC1 and PC2 is shown in Fig. 7 and explained 63% of the total variance of the GC-MS data. PC1 and PC2 explained 40 and 23% of the variation, respectively. Based on the volatile data of the cheeses, a clear grouping or classification was obtained between VP and BR cheeses. Cheeses ripened under BR were located on the negative side of PC1 axis,

while VP cheeses were located on the positive side (Fig. 7). Some esters (methyl acetate, butyl acetate, and hexyl acetate) and aldehydes (hexanal, nonanal, and benzaldehyde) had high negative factor loadings. At 90 and 180 days of ripening, the cheeses ripened under brine had higher contents of these compounds compared to VP cheeses (see also Table 2). Also, D-limonene was only found in BR cheeses and was present at the same locations on the biplot. As ripening proceeded in BR cheeses, the volatiles exhibited some similarities to VP cheeses both qualitatively and quantitatively. On the other hand, the VP cheeses were characterized by higher levels of some alcohols (3-methyl-1-butanol, 2-heptanol, 2-butanol, etc.); acids (acetic acid and octanoic acid); and esters (methyl butanoate, ethyl octanoate, ethyl hexanoate, methyl octanoate, etc.). Age-related distributions were observed; mature cheeses were located on the positive side of PC1. BR and VP cheeses at 360 days were separated from the other cheese samples, and 3-hydroxy-2-butanone and diacetyl, which contributed to the buttery note of the cheeses, were identified at higher concentrations in 360-day-old VP cheese.

3.4 Scanning electron microscopy

The SEM microphotographs of the cheeses are shown in Fig. 8. The treatments did not directly affect the microstructure of the cheeses; however, salt diffused into the cheese matrix for each type of cheese in a different manner. Therefore, SEM images would be a successful means to explore possible differences between each specimen of the cheeses. Slight differences were observed between the images (Fig. 8) in terms of protein structure. More dense and larger protein aggregates were observed in the SEM images of VP cheeses (right) in comparison to the images of BR cheeses (right). The number of openings was higher in VP than BR cheeses, probably due to the impact of NaCl on the hydrophobic interactions of casein. Rowney et al. (2004) found that caseins were homogenously coalesced by NaCl diffusion during ripening and that chlorides promote a hydrophobic interaction among caseins. The stronger brine gave denser a microstructure with large protein aggregates in Iranian white-brined cheese (Madadlou et al. 2007). The BR cheese has a higher NaCl content than VP cheese, and small holes were present in BR cheese, probably with spaces filled by the brine.

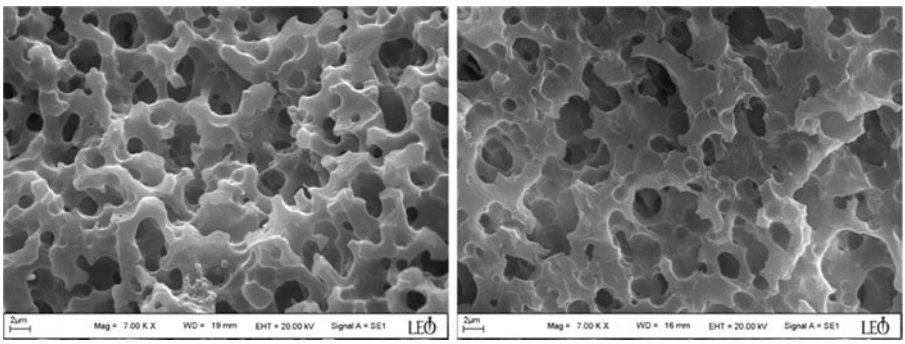

Fig. 8 Scanning electron micrographs of Mihalic cheese ripened under brine (*left*) or vacuum-packaged (*right*) at 360 days of ripening. Magnification is ×7,000

4 Conclusions

The use of brine immersion or vacuum packaging methods in the manufacture of Mihalic cheeses influenced some quality parameters of the cheeses. Total solids, fat, and protein contents were higher in VP than BR cheese; however, a high salt concentration was found in BR cheese. High salt contents in BR cheese caused lower proteolysis in the cheeses; that is, casein breakdown, the levels of pH 4.6 soluble nitrogen, 12% trichloroacetic acid-soluble nitrogen, and total and individual FAA were lower in BR cheese in comparison to VP cheese. The concentrations of FAA in the cheeses increased with age, and Glu, Val, Leu, Lys, and Phe were the most abundant amino acids in both cheeses. The peptide profiles (reversed-phase HPLC) of 70% (*v*/*v*) ethanol-soluble and ethanol-insoluble fractions of the pH 4.6 soluble fraction exhibited some differences among the cheeses, reflecting a cheese environment that affected its peptide profile. Esters, alcohols, and ketones were the most abundant compounds, and the concentrations of volatiles were higher in VP than those of BR cheeses. The images obtained by scanning electron microscopy of the cheeses ripened in polyethylene bags were clearly different (the protein matrix was more compact) from those of brine-salted cheeses. It was concluded that vacuum packaging in the manufacture of Mihalic cheese can be recommended for cheese makers to maintain the overall quality of the cheese.

References

Abd El-Salam MH, Alichanidis E (2004) In: Fox PF, McSweeney PLH, Cogan TM, Guinee TP (eds) Cheeses varieties ripened in brine cheese: chemistry, physics and microbiology, vol. 1. Elsevier Academic, London, pp 227–249

Alichanidis E, Anifantakis EM, Polychroniadou A, Nanou M (1984) Suitability of some microbial coagulants for Feta cheese manufacture. J Dairy Res 51:141–147

Andic S, Genccelep H, Tuncturk Y, Kose S (2010) The effect of storage temperatures and packaging methods on properties of Motal cheese. J Dairy Sci 93:849–859

Andrews AT (1983) Proteinases in normal bovine milk and their action on caseins. J Dairy Res 50:45–55

Blakesley RW, Boezi JA (1977) A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. Anal Biochem 82:580–581

Bosset JO, Collomb M, Sieber R (1993) The aroma composition of Swiss Gruyere cheese. IV. The acidic volatile components and their changes in content during ripening. Lebensm Wiss Technol 26:581–592

Delgado FJ, Gonzalez-Crespo J, Cava R, Garcia-Parra J, Raminez R (2010) Characterization by SPME-GC-MS of the volatile profile of a Spanish soft P.D.O. Torta del Casar during ripening. Food Chem 118:182–189

Dervisoglu M, Yazici F (2001) Ripening changes of Kulek cheese in wooden and plastic containers. J Food Eng 48:243–249

Guven M, Yerlikaya S, Hayaloglu AA (2006) Influence of salt concentration on the characteristics of Beyaz cheese, a Turkish white-brined cheese. Lait 86:73–81

Hayaloglu AA (2007) Comparisons of different single strain starter cultures for their effects on ripening and grading of Beyaz cheese. Int J Food Sci Technol 42:930–938

Hayaloglu AA, Karabulut I (2010) Turkiye'de ekonomik degeri olan onemli bazi peynir cesitlerinin proteoliz, lipoliz ve aroma yonunden karakterizasyonu. Project final report (no: 2007/31). Inonu University, Malatya

Hayaloglu AA, Guven M, Fox PF (2002) Microbiological biochemical and technological properties of Turkish white cheese "Beyaz Peynir". Int Dairy J 12:635–648

Hayaloglu AA, Guven M, Fox PF, McSweeney PLH (2005) Influence of starters on chemical, biochemical, and sensory changes in Turkish white-brined cheese during ripening. J Dairy Sci 88:3460–3474

Hayaloglu AA, Cakmakci S, Brechany EY, Deegan KC, McSweeney PLH (2007) Microbiology, biochemistry and volatile composition of Tulum cheese ripened in goat's skin or plastic bags. J Dairy Sci 90:1102–1121

Hayaloglu AA, Ozer BH, Fox PF (2008) Cheeses of Turkey: 2. Varieties ripened under brine. Dairy Sci Technol 88:225–244

Hayaloglu AA, Deegan KC, McSweeney PLH (2010) Effect of milk pasteurization and curd scalding temperature on proteolysis in Malatya, a Halloumi-type cheese. Dairy Sci Technol 90:99–110

Irigoyen A, Ortigoza M, Juansaras I, Oneca M, Torre P (2007) Influence of an adjunct culture of *Lactobacillus* on the free amino acids and volatile compounds in a Roncal-type ewe's-milk cheese. Food Chem 100:71–80

Karagul-Yuceer Y, Tuncel B, Guneser O, Isleten M, Yasar K, Mendes M (2009) Characterization of aroma-active compounds, sensory properties and proteolysis in Ezine cheese. J Dairy Sci 92:4146–4157

Kondyli E, Katsiari MC, Massouras T, Voutsinas LP (2002) Free fatty acids and volatile compounds of low-fat Feta-type cheese made with a commercial adjunct culture. Food Chem 79:199–205

Kristiansen KR, Deding AS, Jensen DF, Ardo Y, Qvist KB (1999) Influence of salt content on ripening of semi-hard round-eyed cheese of Danbo-type. Milchwissenschaft 54:19–23

Madadlou A, Khosrowshahi A, Mousavi ME, Farmani J (2007) The influence of brine concentration on chemical composition and texture of Iranian white cheese. J Food Eng 81:330–335

Mallatou H, Pappa EC, Boumba VA (2004) Proteolysis in Teleme cheese made from ewes', goats' or a mixture of ewes' and goats' milk. Int Dairy J 14:977–987

McSweeney PLH, Sousa MJ (2000) Biochemical pathways for the production of flavour compounds in cheese during ripening: a review. Lait 80:293–324

Melilli C, Barbano DM, Manenti M, Lynch JM, Carpino S, Licitra G (2004) Lipolysis and proteolysis in Ragusano cheese during brine salting at different temperatures. J Dairy Sci 87:2359–2374

Oner Z, Aloglu H (2004) Some characteristics of Mihalic: a traditional Turkish cheese. Milchwissenschaft 59:628–631

Pappa EC, Sotirakoglou K (2008) Changes of free amino acid content of Teleme cheese made with different types of milk and culture. Food Chem 111:606–615

Pavia M, Trujillo AJ, Guamis B, Ferragut V (2000) Proteolysis in Manchego-type cheese salted by brine vacuum impregnation. J Dairy Sci 83:1441–1447

Rovira S, Lopez MP, Ferrandini E, Laencina J (2010) Hot topic: microstructure quantification by scanning electron microscopy and image analysis of goat cheese curd. J Dairy Sci 94:1091–1097

Rowney MK, Roupas P, Hickey MW, Everett DW (2004) Salt-induced structural changes in 1-day old mozzarella cheese and the impact upon free oil formation. Int Dairy J 14:809–816

SAS (1995) User's guide: statistics. Version 6.12 ed. SAS Institute, Inc., Cary, NC