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The Effect of Different Packaging Methods on The
Formation of Biogenic Amines and Organic Acids
in Kashar Cheese

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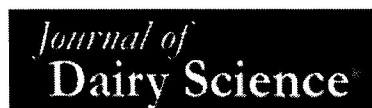
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The effect of different packaging methods on the formation of biogenic amines and organic acids in Kashar cheese

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ABSTRACT

The effects of packaging methods (nonvacuum and vacuum) on biogenic amines (cadaverine, putrescine, tyramine, tryptamine, phenylethylamine, and histamine) and organic acids (citric, lactic, formic, acetic, propionic, and butyric) during storage for 180 d at 4°C were investigated in Kashar cheese. Dry matter, titratable acidity, total nitrogen, water-soluble nitrogen, trichloroacetic acid-soluble nitrogen, phosphotungstic acid-soluble nitrogen, free amino group (proteolysis), pH, fat, and acid degree value were also determined. Storage period had a significant effect on all of the biogenic amines. When compared with vacuum packaging, nonvacuum packaging resulted in no large differences among the amounts of biogenic amines. Vacuum-packaged cheeses had more lactic, formic, acetic, and butyric acids than did cheeses packaged without vacuum. Water-soluble nitrogen, trichloroacetic acid-soluble nitrogen, phosphotungstic acid-soluble nitrogen, proteolysis, pH, and acid degree values of the cheese samples increased continuously until the end of the ripening in all the samples. No significant change was observed in total nitrogen, dry matter, or fat content within the ripening period, whereas titratable acidity values changed significantly in vacuum-packaged cheese and decreased slightly in the non-vacuum-packaged cheeses. The results of this study showed that storage period and packaging method had significant effects on the quality of Kashar cheese.

Key words: Kashar cheese, proteolysis, lipolysis, chemical property

INTRODUCTION

Kashar cheese, a semi-hard cheese, is one of the most important cheese varieties manufactured in Turkey. The reasons for this popularity are long shelf-life and flavor. Kashar cheese is traditionally produced from raw or pasteurized cow milk by local dairy herds or the dairy industry. In Turkey, production of Kashar cheese totals

45,730 t/yr (Özdemir and Demirci, 2006). After the ripening period, the cheese has a unique flavor, taste, and aroma. Cheeses similar to Kashar; for example, Kashkaval and Kasserli, are extensively manufactured in Balkan countries (Keçeli et al., 2004).

Cheese is an ideal substrate for the production of biogenic amines by microbial decarboxylation of the corresponding amino acids. The formation and presence of amines depend on a variety of factors, including the presence of substrate and microbial enzymes, temperature, pH, salt and water content, presence of enhancing substances, and catabolism of amines (Joosten and Northolt, 1987; Joosten, 1988; Roig-Sagues et al., 2002). The increase in the amine content of cheese can be attributed to various microorganisms. These microorganisms may come into existence because of the flora associated with the milk used to make the cheese, or by contamination during cheese making or storage, or may be added to the cheese deliberately in starter cultures (Joosten and Northolt, 1987; Stratton et al., 1992). Tyramine can be consumed without noticeable effects up to 800 mg/kg (Joosten, 1988; Nout, 1994). Histamine production draws the attention of many researchers because the most frequent foodborne intoxications caused by biogenic amines involve this amine. Symptoms of clinical illness have been associated with consumption of 100 to 180 mg of histamine (Joosten and Van Boekel, 1988).

Organic acids are the major products of carbohydrate catabolism created by lactic acid bacteria. Homofermentative bacteria species produce lactic acid as the main end product. Heterofermentative bacteria ferment glucose to multiple end products, such as acetic, formic, lactic, and propionic acids and acetaldehyde, ethanol, and CO₂ (Erkmen and Bozoğlu, 2008). Their ability to produce acidic substances with the concomitant pH decrease is a major factor in milk fermentation. In dairy products, organic acids resulting from the hydrolysis of fatty acids, normal bovine metabolism processes, or direct addition as acidulants are also present. In particular, organic acids contribute to the flavor of most aged cheeses (Hough et al., 1996).

The aim of this study was to determine the effects of different packaging methods on the formation of

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biogenic amines, organic acids, and some chemical and biochemical properties of Kashar cheese during the storage period.

MATERIALS AND METHODS

Cheese Making

Two hundred liters of cow milk was supplied by a commercial dairy plant in Van, Turkey, and Kashar cheeses were produced in the same place. The milk used for cheese making had 12.3% total solids, 3.2% fat, 3.3% protein, and pH 6.54 (mean values). Raw cow milk was pasteurized at 65°C for 30 min, cooled to 32°C, and inoculated with starter culture (*Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*). After 30 min, milk was coagulated with 20 mL of microbial rennet (Mayasan Company, Istanbul, Turkey) for 45 min. After 30 min of additional waiting, the curd was cut. Following this, approximately 15 min elapsed for whey expelling, and the curd was pressed to drain off the whey. The cheese curds were fermented at $20 \pm 2^\circ\text{C}$ about 12 h (overnight). When the pH of curd decreased to 5.4, the curd was cut into thin slices (about 1 cm thick) and transferred into the cooking basket which was placed in hot water. The curd was cooked at 75°C for 50 to 60 s with stirring, and then shaped and put into cylindrical plastic molds. After 1 h, the cheeses in molds were removed and salted with dry salting (dry salt at 5% of cheese weight). The cheeses were kept for 5 d at $15 \pm 2^\circ\text{C}$. Each of the Kashar cheese blocks was approximately 500 g. Afterward, cheeses were packed into plastic bags in vacuum (V) and non-vacuum (NV) forms and ripened at $4 \pm 1^\circ\text{C}$ for 180 d. Kashar cheese was produced 2 times using 2 different milk batches.

Sampling

Samples for the determination of biogenic amines and organic acids were taken at 0, 30, 60, 120, and 180 d of storage and stored at -86°C to prevent further change until the time of analysis. The cheeses were grated, homogenized thoroughly, and analyzed immediately. Each analysis was performed in duplicate. Samples were taken for determination of biogenic amine contents (cadaverine, putrescine, tyramine, tryptamine, histamine, and phenylethylamine), organic acids (citric, lactic, formic, acetic, propionic, and butyric), titratable acidity, DM, total nitrogen (TN), pH, fat, acid degree value (ADV), water soluble-nitrogen (WSN), TCA-soluble nitrogen (TCA-SN), phosphotungstic acid-soluble nitrogen (PTA-SN), and urea-PAGE profile.

Biogenic Amine Analysis

Six aqueous standard solutions containing cadaverine dihydrochloride, putrescine dihydrochloride, tyramine hydrochloride, tryptamine hydrochloride, phenylethylamine hydrochloride, histamine dihydrochloride, and 1,7-diaminoheptane as internal standard from Sigma (St. Louis, MO) were derivatized as described for the cheese samples. Biogenic amine contents of the samples were determined according to the method of Eerola et al. (1993). Ammonium acetate (0.1 M) and acetonitrile were used as mobile phases. Ammonium acetate solution was prepared by dissolving 7.7 g of ammonium acetate in 1,000 mL of triple distilled water and filtering through a 0.45- μm Millipore filter (Millipore, Bedford, MA). Acetonitrile was filtered through a 0.45- μm Millipore filter.

Sample Preparation and Extraction. Two grams of cheese containing 0.1 mL of 1,7-diaminoheptane as internal standard (1 mg/mL) was homogenized in 10 mL of 0.4 M perchloric acid using a Waring blender (Torrington, CT). The sample was centrifuged for 10 min at $1,100 \times g$ and filtered. Extraction was repeated with a further centrifugation at 2500 rpm in 10 mL of 0.4 M perchloric acid solution, and the supernatants were combined and made up to 25 mL with 0.4 M perchloric acid. One milliliter of the extracted sample was alkalized by adding 200 mL of 2 N NaOH solution, and 300 mL of saturated sodium bicarbonate was added for buffering. Two milliliters of dansyl chloride solution was added to each sample and incubated for 45 min at 40°C. Residual dansyl chloride was removed by adding 100 mL of 25% ammonia. After 30 min, the solution was adjusted to 5 mL with acetonitrile and centrifuged for 5 min at 2500 rpm; the supernatant was filtered (0.45 μm) and 20 mL was then injected into the chromatograph.

Biogenic amines were extracted from 2.0 g samples with 0.4 M perchloric acid and detected as their dansyl derivatives by HPLC. The gradient-elution system was 0.1 M, with ammonium acetate as solvent A and acetonitrile as solvent B. The graduated elution program started at 50% solvent B and ended at 90% solvent B in 25 min. The system was equilibrated for 10 min before the next analysis. The flow rate was 1.0 mL/min and the column temperature was 40°C. A 20- μL sample was injected onto the column. Peaks were detected at 254 nm using the HPLC system with a column Spherisorb ODS2 150A, 150 \times 4.60 mm (Waters, Milford, MA) and a gradient pump, which included an Agilent HPLC (1100 series, G1311A Quaternary Pump, G1315A Diode Array Detector, G1313A Auto sampler, G1322A Vacuum Degasser; Agilent Technologies, Santa Clara,

CA), and a computer including Agilent package program. The quantitative determinations were carried out by internal standard (1,7-diaminoheptane; Sigma) method, using peak heights. Biogenic amine contents were expressed as milligrams per kilogram.

Standard Curve. The standard biogenic amine solutions of the dansylated derivatives were diluted to 1 mL with 0.4 M perchloric acid to give concentrations from 1 to 10 mg/mL.

Organic Acids Analysis

Citric, formic, acetic, propionic, lactic, and butyric acids were purchased from Sigma, and gradient HPLC-grade H_2SO_4 was purchased from Merck (Darmstadt, Germany). Milli-Q water (Millipore) was used to prepare buffers, stock solutions of each standard compound, and the samples.

Organic acids of cheese samples were extracted by the method of Bevilacqua and Califano (1989) with some modifications. About 100 g of a representative cheese sample was ground (A-10 Analytical Mill, Tekmar, Mason, OH) and 7 g of ground cheese was transferred into centrifuge tube and then homogenized (Heidolph Silent Cruster M, Schwabach, Germany) in 50 mL of 0.009 N H_2SO_4 (mobile phase). Then, organic acids were extracted for 1 h with agitation on a shaker (Heidolph Unimax 1010) and centrifuged (Hettich Zentrifugen Universal 32 R, Tuttlingen, Germany) at $7,000 \times g$ for 5 min. The supernatant was filtered once through filter paper and twice through a 0.45- μm membrane filter (Millex-HV Hydrophilic PVDF, Millipore); 10 μL of sample was injected. Duplicate analyses were performed on all the samples.

Organic acid contents of the samples were also determined according to the method of Bevilacqua and Califano (1989). Samples for organic acid determination were stored at -86°C until the analysis. The HPLC system included a column (Aminex HPX - 87 H, 300 mm \times 7.8 mm; Bio-Rad Laboratories, Richmond, CA) and a gradient pump, an Agilent HPLC (1100 series G 1322 A, Agilent Technologies), vacuum degasser, DAD detector, and a computer including Agilent software package. The UV detector was set at 214 and 280 nm. Operation conditions were as follows: mobile phase, 0.009 N H_2SO_4 , filtered through 0.45- μm membrane filters twice. The quantitative determinations were made by external standard method, using peak area. Organic acid contents were expressed as milligrams per kilogram.

Physical, Chemical, and Electrophoretic Analysis

Cheese samples were analyzed for titratable acidity, also known as lactic acid (LA%), according to the

method described by Case et al. (1985). pH was measured using a pH meter (pH 211, microprocessor pH meter, Hanna Instrument, Cluj, Romania). The cheese samples were analyzed for TN using micro-Kjeldahl digestion and distillation units (Şimşek, Ankara, Turkey; AOAC, 1990). Total solids contents were determined by the weight differences between the samples using a drying oven (Nüve, Ankara, Turkey) according to the methods described by Case et al. (1985). Salt content was determined by the Mohr method (AOAC, 1990). The fat content of the cheese was measured by the Gerber method described by Case et al. (1985); WSN, TCA-SN, and PTA-SN compounds were extracted as described by Butikofer et al. (1993) and their nitrogen contents were determined by micro-Kjeldahl method. The free amino group concentrations of samples were estimated by the trinitrobenzene sulfonic acid method of Polychroniadou (1988). Lipolysis was done by using the Bureau of Dairy Industries (BDI) method and measured as ADV (IDF, 1991). For this test, 10 g of finely ground sample was placed in a lipolysis butyrometer. Twenty milliliters of BDI reagent (30 g of Triton X-100, 70 g of sodium tetrphosphate in 1 L of distilled water) was added and the butyrometers were placed in a boiling water bath for 20 min to extract the fat. The mixture was centrifuged for 1 min and sufficient aqueous methanol was added to bring the fat into the neck of butyrometer and centrifuged at $310 \times g$ for 1 min. Then, the fraction of liquid fat was transferred into a 50-mL glass tube and weighed. Five milliliters of fat solvent (4:1 petroleum ether and n-propanal) was added to the flask and titrated with 0.01 N tetra n-butyl ammonium hydroxide; total free fatty acids were calculated. Casein fractions of cheeses were analyzed using urea-PAGE method of Tarakçı et al. (2004). All determinations were in duplicate.

Statistical Analysis

The results which come into existence with differences in packaging methods and storage periods were analyzed according to a complete randomized design with 2 replicates. All data were subjected to variance analyses and differences between means were evaluated by Tukey multiple range test (significance $P < 0.05$) using the SPSS statistic program (SPSS, version 8.00 software, 1997; SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Biogenic Amines

Statistical analysis results of biogenic amines of the Kashar cheese samples are shown in Table 1. Putrescine,

Table 1. Effect of packaging method (under vacuum or no vacuum) and storage period on the biogenic amines of Kashar cheese (values are means \pm SD)

Biogenic amine (mg/kg)	Packaging method	Storage period (d)				
		0	30	60	120	180
Tyramine	Non-vacuum	0.00	0.93 \pm 0.11 ^{b,C}	23.48 \pm 3.53 ^{a,AB}	31.17 \pm 3.82 ^{b,A}	19.80 \pm 2.57 ^{a,B}
	Vacuum	0.00	12.28 \pm 1.97 ^{a,C}	26.06 \pm 4.18 ^{a,B}	47.88 \pm 3.01 ^{a,A}	11.40 \pm 1.92 ^{a,C}
Histamine	Non-vacuum	0.00	0.00	0.00	3.30 \pm 0.48 ^{a,A}	4.48 \pm 0.76 ^{a,A}
	Vacuum	0.00	0.00	0.00	2.91 \pm 1.19 ^{a,A}	2.40 \pm 0.59 ^{a,A}
Cadaverine	Non-vacuum	0.00	0.00	0.00	0.00	0.00
	Vacuum	0.00	0.00	1.07 \pm 0.13 ^{a,B}	0.69 \pm 0.06 ^{a,C}	1.33 \pm 0.48 ^{a,A}
Putrescine	Non-vacuum	0.10 \pm 0.14 ^{a,C}	1.02 \pm 0.04 ^{b,C}	15.67 \pm 4.82 ^{b,C}	35.07 \pm 5.78 ^{b,B}	174.26 \pm 6.05 ^{a,A}
	Vacuum	0.18 \pm 0.04 ^{a,D}	15.74 \pm 3.24 ^{a,CD}	127.98 \pm 2.87 ^{a,A}	74.30 \pm 7.25 ^{a,B}	28.52 \pm 2.16 ^{b,C}
Phenylethylamine	Non-vacuum	0.00	0.00	0.00	0.00	0.00
	Vacuum	0.00	0.00	0.00	0.00	0.00
Tryptamine	Non-vacuum	89.51 \pm 3.18 ^{a,B}	100.33 \pm 16.52 ^{a,AB}	79.71 \pm 14.17 ^{a,B}	86.87 \pm 10.01 ^{b,B}	141.44 \pm 8.34 ^{a,A}
	Vacuum	92.52 \pm 1.39 ^{a,B}	90.55 \pm 9.14 ^{a,B}	101.39 \pm 5.42 ^{a,B}	143.27 \pm 10.95 ^{a,A}	144.39 \pm 7.62 ^{a,A}

^{a,b}Different lowercase letters within a column and biogenic amine indicate significant differences between cheese samples ($P < 0.05$).

^{A-D}Different uppercase letters within a row (sample) and biogenic amine indicate significant differences between ripening periods ($P < 0.05$).

tryptamine, and tyramine were the major amines in Kashar cheese, whereas phenylethylamine was not found in the samples.

The results show that the levels of each biogenic amines changed differently. Tyramine concentration increased from 0 to 120 d and then decreased in both packaging types. Putrescine content of V cheese increased until 60 d, and then showed a decreasing trend, and the putrescine content of NV cheese reached its highest level at 180 d (Table 1).

Storage period had a significant effect ($P < 0.01$) on all of the biogenic amines except for cadaverine in NV cheese and for phenylethylamine, which was not at detectable level in any samples. The highest tryptamine concentration was determined at the end of the storage period for both packaging methods. Histamine first was determined at 120 d to be at an almost equal level in both samples, then the content of the NV sample slightly increased and the content of the V sample slightly decreased. This may be explained by the appropriate temperature for growth of microorganism and plenty of substrate (free amino acid) formed during storage period. Lactic acid bacteria are still viable under refrigeration conditions and may produce biogenic amines. In addition, nonstarter lactobacilli and enterococci have been implicated in the production of high levels of biogenic amines in most cheese types (Joosten and Northolt, 1987; Roig-Sagues et al., 2002; McSweeney, 2004).

Packaging method had a significant effect ($P < 0.01$) on biogenic amines except that histamine and phenylethylamine were not found during storage. Casein degradation and free amino acid formation remained lower in both cheeses. The reason for this can be attributed to the Kashar cheese processing method (high cook-

ing temperature) and low storage temperature (4°C). Increased concentration of free amino acid in cheese results in the formation of carbon dioxide and amines (Darwish, 1993).

Tyramine is one of the biogenic amines that can cause some health disorders in sensitive individuals. The tyramine level of the whole Kashar cheese increased until 120 d and decreased thereafter. The tyramine level at 120 d was found to be close to the results found by Schneller et al. (1997) for semi-soft cheeses, but lower than that reported by Valsamaki et al. (2000) (Table 1). Tyramine levels were found to be 212.5 mg/kg by Silvana et al. (1998) in Brazilian cheese. In this study, we found lower tyramine contents than did these authors.

Histamine was not found in Kashar cheeses up to 120 d of storage, and the level of histamine increased to 2.40 and 4.48 mg/kg for V and NV cheeses, respectively, at 180 d (Table 1). Joosten and Van Boekel (1988) investigated histamine in Feta cheese and reported a concentration of 65 mg/kg in the cheese. Silvana et al. (1998) reported a histamine concentration of 196.5 mg/kg in Gouda cheese. These levels are much higher than those found in the current study. Low storage temperature and vacuum packaging were found to be the most important factors in preventing the accumulation of histamine in Kashar cheese in the current study. In our study, histamine formation was first observed after 120 d and a slight increase occurred in NV samples after this period. It is possible that the decarboxylases produced by the microflora, directly in the milk or in the first step of cheese making, could also continue to affect the cheese. Thus, histidine decarboxylase activity increased during the storage period and probably reached its maximum level at the end of the storage

period. From a good manufacturing practice point of view, a level of 100 mg/kg histamine is regarded as acceptable (Nout, 1994). In this study, the maximum histamine concentration determined was 4.48 mg/kg, and it never exceeded a concentration of 100 mg/kg, even at the end of the storage period.

The results show that cadaverine was first determined at 60 d in V cheese and its concentration remained stable until the end of the storage period. Cadaverine was not found in NV samples during the storage period (Table 1). Cadaverine has less pharmacological activity than the aromatic amines but it is probably a potentiator of their toxicity (Joosten, 1988). The cadaverine concentration found in this study was lower than that (1,110.0 mg/kg) reported by Silvana et al. (1998).

The level of putrescine significantly increased ($P < 0.01$) from 0 to 180 d in NV cheese (Table 1). In V cheese, putrescine increased significantly ($P < 0.01$) from 0 to 60 d, and then sharply decreased from 60 to 180 d. In this study, we found that levels of putrescine exceeded 100 mg/kg in NV cheeses at the end of the storage period. Similar levels were found by Joosten and Northolt (1987); however, Schneller et al. (1997) reported lower values. Joosten (1988) reported that putrescine formation ceased after 30 d, and Schneller et al. (1997) observed a decrease in putrescine content after 3 mo of ripening. In contrast, in our experiment, an important increase in putrescine concentration was found in the NV samples throughout the storage period. Silvana et al. (1998) reported a putrescine level of 173.7 mg/kg. In this study, we found slightly higher levels than did those authors.

Phenylethylamine was not found in either cheese packaging type during the storage period (Table 1). Tryptamine was the most abundant biogenic amine at the beginning of storage and its final concentration was found to be higher than its initial level. Tryptamine content of V samples was higher than NV cheeses during storage, except the values in samples stored for 30 d. At the end of storage period, both samples had almost same level of tryptamine (Table 1).

In NV cheeses, tryptamine concentrations were increased up to 30 d and then decreased until 60 d, and then increased again during rest of the storage period. This might be explained by tryptamine being consumed by microorganisms as a nitrogen source. Also, some authors have suggested that the decrease of biogenic amines during ripening could be related to the activity of bacterial amine oxidases (Leuschner et al., 1999).

Organic Acids

The presence of short-chain acids such as acetic, propionic, and butyric acids is mainly related to the

intensity of bacterial fermentation that took place during ripening. Organic acid contents of the Kashar cheese samples are given in Table 2. As seen in the table, significant differences were observed among the contents of organic acids in the samples. Levels of lactic acid were the highest among all organic acids tested, followed by acetic acid (in V cheese samples) and butyric acid. Organic acids in cheese occur due to the hydrolysis of fatty acids, bacterial growth, normal bovine metabolic processes, or direct addition of acidulants (Adda et al., 1982; Akalın et al., 2002; Izco et al., 2002), and the composition of organic acids can differ in different types of cheese. The organic acids produced contribute not only to the flavor and aroma of fermented dairy products but also to their preservation. Packaging method had a significant effect ($P < 0.01$) on all organic acid concentrations at the end of storage period except for butyric acid. The V cheeses under had higher amounts of lactic, formic, and acetic acids ($P < 0.01$) compared with NV cheeses. This could be due to oxidative catabolism of acids by some bacteria and molds grown on cheese surface (McSweeney and Fox, 2004). Coherent with this premise, in this study, mold growth was observed on the surface of NV cheese toward the end of storage period.

Storage period had a significant effect ($P < 0.01$) on all of the organic acids except for lactic acid in V samples and except for butyric acid in NV samples. Citric and butyric acid levels were increased during the storage period. This may be explained by the appropriate temperature for microorganism grown and because plenty of substrate was formed during the storage period. The primary purpose of a dairy starter culture is to produce lactic acid from lactose at a high rate in the early stages (Akalın et al., 2002). As expected, the amount of lactic acid was much greater than the amounts of other organic acids; concentrations of lactic acid ranged from 7,599.2 to 7,888.1 mg/kg for V samples and from 7,828.9 to 2,689.21 mg/kg for NV samples at the beginning and end of the storage period, which is lower than that reported for White cheese (30,000 mg/kg; Akalın et al., 2002).

Citric acid showed a gradual increase in NV samples during ripening (Table 2), similar to findings of Lombardi et al. (1994) for Reggiano cheese. This increase might be explained by citrate metabolism. Citrate is involved in the Krebs or citric acid cycle, where it acts both as a substrate and a product. Citrate can be used as a substrate by the starter to produce pyruvic and acetic acids (Adda et al., 1982).

The levels of lactic acid were significantly decreased from 0 to 180 d in NV samples (Table 1). In V samples, lactic acid was almost stable from 0 to 180 d. Lactate is an important compound for biochemical reactions in

Table 2. Effect of packaging method (under vacuum or no vacuum) and storage period on the organic acids of Kashar cheese (values are means \pm SD)

Organic acid (mg/kg)	Packaging method	Storage period (d)			
		0	30	60	180
Citric acid	Non-vacuum	312.40 \pm 18.96 ^{a,D}	408.60 \pm 13.60 ^{a,CD}	500.80 \pm 19.06 ^{a,BC}	640.20 \pm 38.65 ^{a,A}
	Vacuum	370.40 \pm 15.33 ^{a,BC}	335.50 \pm 49.28 ^{a,C}	419.00 \pm 28.75 ^{a,ABC}	480.20 \pm 25.57 ^{b,AB}
Lactic acid	Non-vacuum	7,828.90 \pm 244.54 ^{a,A}	6,941.30 \pm 1,059.30 ^{a,A}	5,817.00 \pm 260.03 ^{a,AB}	2,689.21 \pm 155.23 ^{b,C}
	Vacuum	7,599.20 \pm 425.56 ^{a,A}	7,538.50 \pm 762.04 ^{a,A}	7,487.00 \pm 356.55 ^{a,A}	7,888.10 \pm 188.48 ^{a,A}
Formic acid	Non-vacuum	0.00	0.00	0.00	0.00
	Vacuum	0.00	0.00	136.40 \pm 21.75 ^{a,C}	272.30 \pm 27.11 ^{a,A}
Acetic acid	Non-vacuum	1,533.10 \pm 192.02 ^{a,A}	899.50 \pm 59.47 ^{a,B}	548.80 \pm 51.59 ^{b,B}	0.00 ^{b,C}
	Vacuum	1,134.10 \pm 238.51 ^{a,B}	1,050.00 \pm 69.61 ^{a,B}	1,219.00 \pm 44.96 ^{a,B}	2,031.30 \pm 62.48 ^{a,A}
Propionic acid	Non-vacuum	95.80 \pm 10.18 ^{a,B}	110.00 \pm 26.31 ^{a,AB}	121.10 \pm 16.32 ^{a,AB}	182.70 \pm 13.32 ^{a,A}
	Vacuum	109.50 \pm 16.09 ^{a,A}	0.00	0.00	0.00
Butyric acid	Non-vacuum	967.00 \pm 33.67 ^{a,A}	1,084.20 \pm 271.57 ^{a,A}	1,191.9 \pm 141.27 ^{a,A}	1,497.20 \pm 175.21 ^{a,A}
	Vacuum	1,041.80 \pm 136.43 ^{a,B}	1,494.2 \pm 86.73 ^{a,AB}	1,635.9 \pm 207.5 ^{a,AB}	1,970.40 \pm 43.40 ^{a,A}

^{a,b}Different lowercase letters within a column and organic acid indicate significant differences between cheese samples ($P < 0.05$).^{A,B}Different uppercase letters within a row (sample) and organic acid indicate significant differences between ripening periods ($P < 0.05$).

cheese ripening. This acid can be catabolized to CO₂ and H₂O by *Penicillium camemberti* during ripening. Additionally, in the presence of oxygen in surroundings, it can be oxidized to formate and acetate by non-starter lactic acid bacteria, particularly by pediococci (McSweeney and Fox, 2004). Lactic acid dominates in aged cheese and its concentration in cheese is reported to range from 1,940 to 17,400 mg/kg (Bevilacqua and Califano, 1989). Lues et al. (1998) reported the values of lactic acid in Cheddar cheese as 23,413.9 mg/kg. The lactic acid concentration in the current study was lower than that reported by these authors. The percentage of lactic acid produced is influenced by the age of the cheese, and the production of lactic acid is essential for consistent ripening. Formation of lactic acid is essential for proper production, flavor development during normal ripening and keeping good quality (Califano and Bevilacqua, 2000).

Acetic acid was the second most abundant acid detected in V samples of Kashar cheese at 180 d (Table 2). Acetic acid content of V samples decreased slightly at 30 d, and then showed a tendency to increase; it reached its highest level at the end of the storage period. On the other hand, the acetic acid concentration in NV samples decreased gradually during storage to an undetectable level at 120 d. Acetic acid is produced by the metabolism of lactose by lactic acid bacteria, by metabolism of citric and lactic acid, or by catabolism of amino acids (McSweeney and Sousa, 2000).

Formic acid was not found during the storage period in NV samples (Table 2). Formic acid was formed after 60 d in V samples. The concentration of formic acid significantly increased from 60 to 180 d in the latter (Table 2). The highest concentration of formic acid was found to be 272.3 mg/kg in Kashar cheese, which was lower compared with brine-pickled White cheese (1,000 mg/kg; Akalın et al., 2002). Heterofermentative metabolism of lactose, by means of a flora different from the starter or secondary flora, may produce formic acid, acetic acid, and ethanol (Law, 1984).

The propionic acid content of NV cheese increased significantly as ripening progressed ($P < 0.01$). Butyric acid concentration slightly increased in NV samples during storage ($P < 0.10$). Propionic acid was not found after 30 d of storage in V samples (Table 2). Butyric and propionic acid fermentations are the result of the lipolytic and especially proteolytic activity of starters and an abundant secondary microflora (Esponda et al., 1983; Akalın et al., 2002). If the milk fat globule membrane is damaged during cheese making, lipolysis and release of fatty acids such as butyric acid would be facilitated (Caboni et al., 1990). Butyric acid production may originate not only from fats but also during proteolysis and subsequent deamination of casein

degradation products (Califano and Bevilacqua, 2000; Akalin et al., 2002).

Hough et al. (1996) stated that propionic acid was a good indicator of flavor development in cheese. Propionic acid is formed from lactic acid by propionic acid bacteria (Califano and Bevilacqua, 2000). Our results were in good agreement with these findings because decreasing the lactic acid in cheese samples without vacuum packaging increased the propionic acid content.

Physical, Chemical, and Electrophoretic Properties

Storage period had a significant effect ($P < 0.01$) on titratable acidity, WSN, TCA-SN, PTA-SN, proteolysis, pH, and ADV. Dry matter and fat did not show any substantial changes during storage. The mean TN content of Kashaar cheese samples was approximately 4.00%; no significant difference was observed between the cheese samples in terms of TN content during ripening ($P > 0.05$; Table 3).

The extent of proteolysis was evaluated by several methods including WSN. The WSN fraction contains small molecules of proteins (noncasein), peptides, and free amino acids, and it is commonly used as an index of ripening (Guinee and Fox, 1993). A comparison of the WSN data for the cheeses from d 0, 30, 60, 120 and 180 of ripening are given in Table 3. The WSN values of the cheeses were affected significantly ($P < 0.01$) by ripening period and packaging method. It can be seen in Table 3 that the WSN values increased in both V and NV samples throughout ripening, but levels in NV cheese were significantly ($P < 0.01$) higher than those in V cheese at the end of the ripening. The formation of WSN compounds during ripening is an index of the rate and extent of proteolysis, which is an indicator of casein hydrolysis brought about by the action of the residual rennet and indigenous plasmin present at the start of ripening (Irigoyen et al., 2001). The WSN values were lower than values previously reported by Tunçtürk (1996) for non-packaged Kashaar cheese, by Tarakçı and Küçüköner (2006) for vacuum-packaged Kashaar cheese, and by Sert et al. (2007) and Güleç et al. (2004) for Kashaar packed with different materials.

Trichloroacetic acid-soluble nitrogen ratio of NV cheese was slightly higher than that of the V cheese ($P < 0.05$). Trichloroacetic acid-soluble nitrogen is known to be an indication of the amount of small peptides and amino acids present in cheese and its level is regarded as the ripening depth index. These peptides and amino acids are mainly formed by the action of microbial enzymes on the peptides obtained through the action of rennet and plasmin from casein (Tarakçı and Küçüköner, 2006). The environmental conditions

of these cheeses (low acidity, high moisture content) favor the activity of chymosin on α_{s1} -casein, resulting in the rapid production of water-soluble peptides (Romeih et al., 2002). The levels of TCA-SN and PTA-SN increased continuously ($P < 0.01$) in all cheese samples during storage (Table 3). The differences in proteolysis among the cheeses were probably due to differences in the microflora. The TCA-SN values significantly ($P < 0.01$) increased until the end of the ripening. Several authors have found that increasing the ripening time leads to an increase in protein degradation in cheeses (Kurultay et al., 2000; Hayaloğlu et al., 2005; Tarakçı and Küçüköner, 2006). Sert et al. (2007) reported that TCA-SN significantly increased during the ripening period. Tri- and dipeptides and free amino acids are soluble in the PTA-SN fraction. These small peptides and free amino acids are produced mainly by the action of microorganisms (starter culture and non starter organisms) on the caseins and their peptides (Tarakçı, 2004). The PTA-SN contents of V and NV cheeses showed a similar tendency during the storage period ($P > 0.05$).

Titratable acidity of V samples was affected ($P < 0.01$) by storage time (Table 3). Titratable acidity of NV samples decreased during the ripening period and results were generally lower than those reported by Kurultay et al. (2000) for Kashaar cheese produced by addition of different hydrocolloids and by Tarakçı and Küçüköner (2006) for vacuum-packed Kashaar cheese. In this study, we found similar acidity to that reported by Sert et al. (2007) (0.45% LA) in Kashaar cheese. The initial increase in acidity is due to lactic acid and hydrogen formation (Dervişoğlu and Yazıcı, 2001). The following decrease in acidity may be a result of molds and formation of alkaline nitrogenous compounds (Guinee and Fox, 1993). The pH values of cheese samples significantly differed depending on packaging method. The NV samples showed an increasing pH trend during storage; however, the pH values of V samples increased until 60 d and then remained almost stable. This difference may be the result of more proteolysis in NV cheese samples and greater formation of alkaline and buffering compounds than in V samples (Walstra et al., 1999). Moreover, lactate is catabolized to H_2O and CO_2 by *Penicillium camemberti* at the surface of some cheeses. The catabolism of lactic acid causes a large increase in the pH of the surface of these cheeses (McSweeney and Fox, 2004). In our study, we found similar pH levels to that of Sert et al. (2007) (pH 5.30) in Kashaar cheese at the beginning of the storage period (Table 3). Later, pH was increased both in V and NV samples during the storage period, whereas Sert et al. (2007) reported that pH level was decreased. The results show that the level of pH was significantly affected by proteolysis dur-

Table 3. Effect of packaging method (under vacuum or no vacuum) and storage period on some chemical and biochemical characteristics of Kashar cheese (values are means \pm SD)

Item	Packaging method	Storage period (d)				
		0	30	60	120	180
DM (%)	Non-vacuum	60.48 \pm 0.71 ^{a,A}	57.38 \pm 0.68 ^{a,A}	57.98 \pm 0.87 ^{a,A}	57.66 \pm 0.82 ^{a,A}	59.04 \pm 1.32 ^{a,A}
	Vacuum	59.19 \pm 0.76 ^{a,A}	59.44 \pm 0.27 ^{a,A}	59.10 \pm 0.42 ^{a,A}	59.80 \pm 0.26 ^{a,A}	59.46 \pm 0.47 ^{a,A}
Fat (%)	Non-vacuum	28.25 \pm 0.35 ^{a,A}	27.50 \pm 0.71 ^{a,A}	28.25 \pm 1.06 ^{a,A}	28.00 \pm 1.41 ^{a,A}	28.50 \pm 0.71 ^{a,A}
	Vacuum	27.75 \pm 0.35 ^{a,A}	27.50 \pm 0.70 ^{a,A}	28.75 \pm 0.35 ^{a,A}	27.75 \pm 0.35 ^{a,A}	28.25 \pm 0.35 ^{a,A}
Total N (%)	Non-vacuum	4.00 \pm 0.16 ^{a,A}	3.79 \pm 0.16 ^{a,A}	4.01 \pm 0.16 ^{a,A}	4.01 \pm 0.16 ^{a,A}	4.01 \pm 0.16 ^{a,A}
	Vacuum	4.00 \pm 0.13 ^{a,A}	3.83 \pm 0.15 ^{a,A}	3.92 \pm 0.32 ^{a,A}	3.94 \pm 0.24 ^{a,A}	4.02 \pm 0.22 ^{a,A}
Water-soluble N (%)	Non-vacuum	0.20 \pm 0.03 ^{a,D}	0.35 \pm 0.01 ^{a,C}	0.41 \pm 0.02 ^{a,BC}	0.43 \pm 0.01 ^{a,AB}	0.49 \pm 0.00 ^{a,A}
	Vacuum	0.19 \pm 0.01 ^{a,D}	0.28 \pm 0.01 ^{b,C}	0.32 \pm 0.01 ^{b,BC}	0.36 \pm 0.02 ^{b,AB}	0.39 \pm 0.01 ^{b,A}
TCA-soluble N (%)	Non-vacuum	0.08 \pm 0.001 ^{a,D}	0.11 \pm 0.002 ^{a,CD}	0.13 \pm 0.01 ^{a,BC}	0.18 \pm 0.02 ^{a,B}	0.26 \pm 0.02 ^{a,A}
	Vacuum	0.08 \pm 0.001 ^{a,C}	0.09 \pm 0.001 ^{b,C}	0.10 \pm 0.00 ^{b,C}	0.16 \pm 0.00 ^{a,B}	0.19 \pm 0.07 ^{b,A}
Phosphotungstic acid-soluble N (%)	Non-vacuum	0.02 \pm 0.004 ^{a,C}	0.03 \pm 0.00 ^{a,C}	0.04 \pm 0.01 ^{a,BC}	0.06 \pm 0.00 ^{a,AB}	0.07 \pm 0.00 ^{a,A}
	Vacuum	0.02 \pm 0.001 ^{a,C}	0.02 \pm 0.00 ^{a,C}	0.03 \pm 0.00 ^{a,C}	0.05 \pm 0.01 ^{a,B}	0.07 \pm 0.00 ^{a,A}
Titratable acidity (%)	Non-vacuum	0.61 \pm 0.04 ^{a,A}	0.54 \pm 0.01 ^{a,A}	0.56 \pm 0.01 ^{b,A}	0.48 \pm 0.02 ^{b,A}	0.47 \pm 0.09 ^{a,A}
	Vacuum	0.67 \pm 0.01 ^{a,A}	0.56 \pm 0.02 ^{a,B}	0.66 \pm 0.01 ^{a,A}	0.67 \pm 0.01 ^{a,A}	0.60 \pm 0.01 ^{a,B}
pH	Non-vacuum	5.25 \pm 0.00 ^{a,C}	5.48 \pm 0.02 ^{a,BC}	5.72 \pm 0.03 ^{a,AB}	5.89 \pm 0.24 ^{a,AB}	6.18 \pm 0.08 ^{a,A}
	Vacuum	5.24 \pm 0.01 ^{a,C}	5.39 \pm 0.01 ^{b,B}	5.45 \pm 0.01 ^{b,A}	5.46 \pm 0.03 ^{a,A}	5.46 \pm 0.01 ^{b,A}
Proteolysis (free amino groups; mmol)	Non-vacuum	75.49 \pm 5.70 ^{a,D}	108.98 \pm 2.75 ^{a,CD}	147.32 \pm 9.82 ^{a,BC}	170.31 \pm 15.32 ^{a,B}	238.97 \pm 13.75 ^{a,A}
	Vacuum	71.96 \pm 2.65 ^{a,E}	105.93 \pm 1.57 ^{a,D}	140.65 \pm 9.82 ^{a,C}	170.31 \pm 3.53 ^{a,B}	215.36 \pm 2.37 ^{a,A}
Acid degree value (mEq/100 g of fat)	Non-vacuum	1.10 \pm 0.01 ^{a,C}	1.40 \pm 0.26 ^{a,BC}	1.97 \pm 0.06 ^{a,AB}	2.26 \pm 0.09 ^{a,A}	2.53 \pm 0.27 ^{a,A}
	Vacuum	1.12 \pm 0.05 ^{a,C}	1.49 \pm 0.27 ^{a,C}	1.65 \pm 0.35 ^{a,BC}	2.41 \pm 0.12 ^{a,AB}	2.66 \pm 0.15 ^{a,A}

^{a,b}Different lowercase letters within a column and item indicate significant differences between cheese samples ($P < 0.05$).^{A-E}Different uppercase letters within a row (sample) and item indicate significant differences between ripening periods ($P < 0.05$).

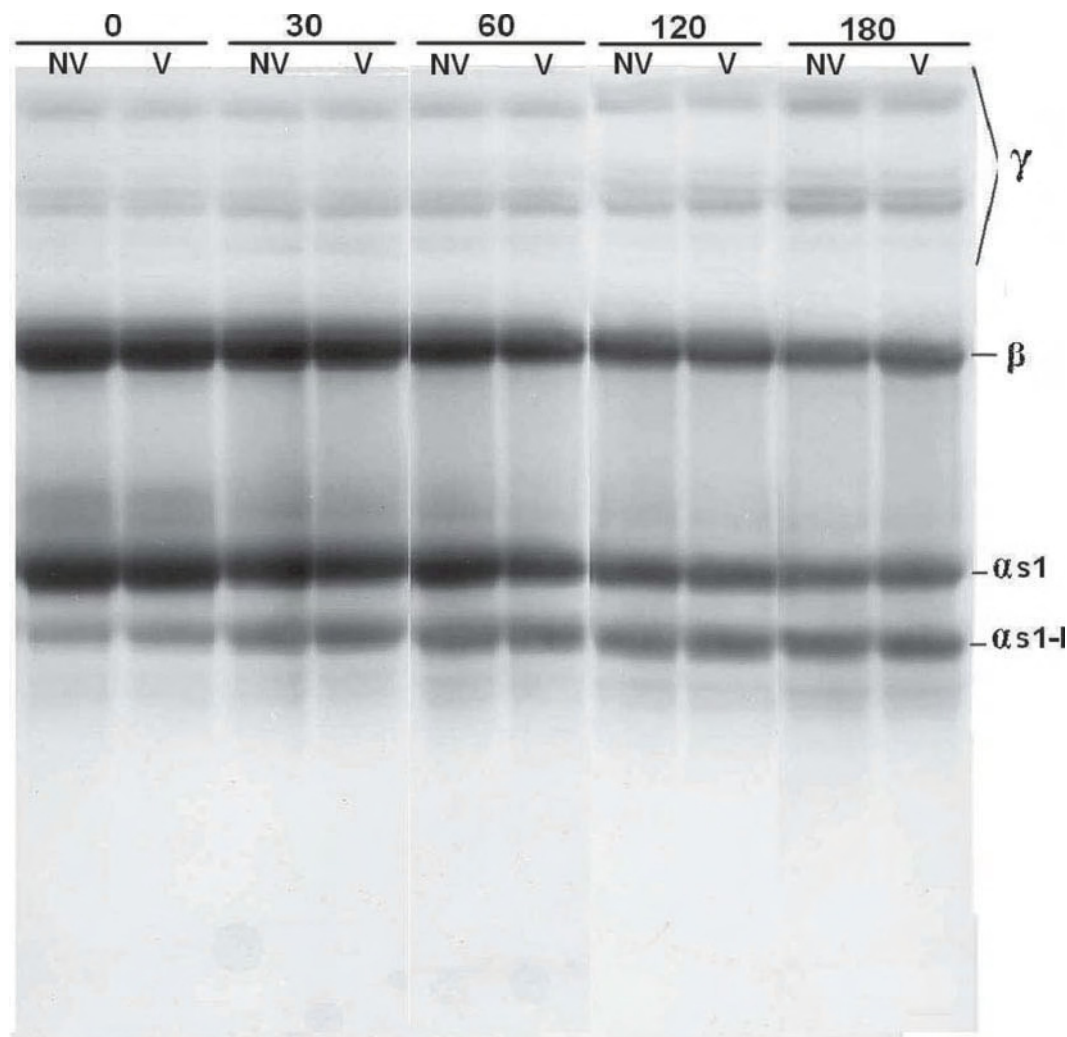


Figure 1. Urea-PAGE profile of Kashar cheese samples. Samples were taken at 0, 30, 60, 120, and 180 d of storage. Packaging method: NV = non-vacuum; V = vacuum.

ing the storage period. In addition, pH level of cheese samples was found to be in good agreement with nitrogen fractions (WSN, TCA-SN, and PTA-SN) for both samples.

Formation of free amino groups (proteolysis) was one of the most important changes during storage period. Concentration of free amino groups was increased during the storage period in all samples. It can be seen in Table 3 that the values of proteolysis level were not significantly different ($P > 0.05$) in the cheeses stored under NV or V conditions. Storage period had significant effects ($P < 0.01$) on proteolysis (Table 3). Contents of tyramine, histamine, and putrescine were in good agreement with proteolysis criteria. Correlation coefficients between tyramine, histamine, and putrescine and proteolysis criteria were as follows: for free amino groups 0.516, 0.832, 0.664; for WSN 0.524, 0.715, 0.541;

for TCA-SN 0.427, 0.914, 0.630; and for PTA-SN 0.401, 0.866, 0.487, respectively.

The lipolysis values of V or NV Kashar cheese samples during ripening are shown in Table 3. Lipolysis (ADV) level increased during the storage period in all samples. Storage periods had significant effects ($P < 0.01$) on ADV (Table 3). In this study, ADV of V samples ranged from 1.12 to 2.66. These values were similar to those reported by Tarakçı and Küçüköner (2006) for vacuum-packed Kashar. In the Kashar cheeses, lipolysis values increased linearly to a significant level during ripening ($P < 0.01$). Lipolysis values were lower than those reported by Güler and Uraz (2004) for commercial Kashar cheeses, but similar to those reported by Dervişoğlu and Yazıcı (2001) on Kulek cheese produced without starter. These differences might be due to packaging conditions, usage of different milks, manufacturing

procedure, or ripening conditions. Lipolytic agents in cheese generally originate from the milk, the coagulant (in the case of rennet paste), and the cheese microflora (starter, nonstarter, and adjunct microorganism). Milk contains a potent indigenous lipase, lipoprotein lipase. Lipoprotein lipase activity is significant in raw milk cheeses because the enzyme is largely inactivated by pasteurization, although $85^{\circ}\text{C} \times 10 \text{ s}$ is required to inactivate the enzyme completely (McSweeney, 2004).

From the appearance of the gel obtained by urea-PAGE, a remarkable decrease was observed in protein bands (α_{S1} -casein and β -casein) for both Kashar cheese samples during ripening (Figure 1). However, β -casein and α_{S1} -casein of NV cheese samples hydrolyzed further than casein fractions of other samples. In particular, α_{S1} -casein of NV cheese was more affected by the ripening process. We determined that the decreases in band intensities were more evident at 120 and 180 d of ripening. Casein can be hydrolyzed by milk plasmin, microbial enzymes, or the clotting enzyme used (Carmona et al., 1999). Kashar cheese is classified as hard or semi-hard cheese type, and proteolytic activity is generally lower than in softer cheeses. Electrophoretic results were in good agreement with other proteolysis parameters such as WSN, TCA-SN, PTA-SN, and free amino groups.

CONCLUSIONS

The results of this study showed that storage period had significant effects on the formation of biogenic amines except phenylethylamine. Tyramine, putrescine, and tryptamine were found to be the most abundant biogenic amines in Kashar cheese. Low storage temperature (4°C) and vacuum packaging in Kashar cheese storage hindered or limited formation of phenylethylamine, cadaverine, and histamine. Histamine content was found to be much less than 100 mg/kg at the end of storage and its formation accelerated slightly, especially after 120 d of storage period. Packaging method had a significant effect ($P < 0.01$) on concentrations of all organic acids at the end of storage period except butyric acid. Vacuum-packaged cheeses had higher amounts of lactic, formic, acetic and butyric acid ($P < 0.01$) compared with non-vacuum-packaged cheeses. Storage period had a significant effect ($P < 0.01$) on WSN, TCA-SN, PTA-SN, proteolysis, pH, and ADV in all of the samples and on titratable acidity in vacuum-packaged samples. To extend the shelf life of Kashar cheese, evaluation of the effects of storage conditions and packaging methods on chemical and biochemical characteristics is of utmost importance. However, we found no evidence of a serious problem in Kashar

cheese in terms of biogenic amine formation for either packaging method.

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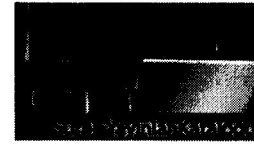
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