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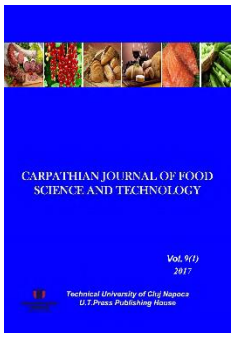

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EVALUATION OF NATURALLY OCCURRING BENZOIC ACID LEVEL IN FETA AND CREAM CHEESE DURING FERMENTATION, PRODUCTION PROCESSING AND STORAGE IN REFRIGERATOR

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ABSTRACT

Benzoic acid and its salts are widely used as preservatives in the food industry, but their addition into cheese and other dairy products has been forbidden. However, it was assumed that it was also naturally produced in fermented dairy products and could mistakenly be considered to be adulteration; therefore, it was difficult to interpret the obtained levels during the examination of suspicious samples. In this study, benzoic acid levels naturally occurring in Feta and cream cheese after fermentation, production and storage in refrigerator were assessed by HPLC. After the fermentation stage, benzoic acid was found in all samples. In Feta cheese, benzoic acid content of samples assessed on various days was significantly different ($P < 0.05$) and showed a positive correlation with lactic acid bacteria (LAB) count (r = 0827). By increasing the storage time from 1 to 30 days, the amount of benzoic acid was significantly increased from 24.45 to 41.10 µg/ kg, although its levels on days 40, 50 and 60 did not significantly change. After production, the benzoic acid level of the samples of cream cheese was 8.52 µg/kg, and its concentration did not significantly change during storage. In general, benzoic acid concentration naturally occurring in cheese depends on the type of lactic starter culture used in fermentation stage, LAB growth and storage time. By considering the measurement of analysis method uncertainty, concentrations of naturally occurring benzoic acid was lower than 46.50 mg/kg in Feta cheese and 9.23 µg/ kg in cream cheese.

1. Introduction

One of the most important methods of controlling food product spoilage caused by microorganisms is the use of food preservatives (Panneerselvam *et al.*, 2015). Benzoic acid and sodium benzoate are commonly used as preservatives in the food industry under code numbers E210 and E211. Their undissociated form, created in media with pH values of 2.5–4, shows an inhibiting effect against the growth of yeasts, moulds and a wide range of bacteria (Chipley, 2005). The Joint FAO/WHO Expert Committee on Food established an acceptable daily intake (ADI) for benzoic acid and sodium benzoate of 0–5 mg/kg body weight. Different countries monitor benzoic acid levels in food products in order to ensure the safety of those products, although the intake of benzoic acid differs in various countries. Benzoic acid has no teratogenic or carcinogenic activity (JECFA, 1996). In doses consumed by a person weighing 60 kg (300 mg), it has no toxic effect, although sensitization reactions such as rhinitis, hives and dermatitis have been observed in some people (Iammarino *et al.*, 2011). Up to now, several methods have been applied to determine benzoic

acid levels, including spectrophotometry, gas chromatography with different detectors (FID, MS) or high performance liquid chromatography (HPLC) with a UV detector. Among these methods, liquid/liquid extraction coupled with HPLC-UV is favoured (Yildiz, 2012).

Some foods and commodities contain benzoic acid naturally, and it has been determined that benzoic acid forms in fermented dairy products (Sieber *et al.*, 1995; Iammarino *et al.*, 2011; Amirpour *et al.*, 2015). The addition of benzoic acid into cheese and other dairy products has been forbidden but in some countries acidified milk, yogurt, kefir and buttermilk are except. However, its natural formation in dairy products might also be considered adulteration; therefore, it is difficult to interpret results obtained in examining suspicious samples. In fact, fermented dairy products such as cheese might contain benzoic acid and be considered "non-compliant" even when no benzoic acid has been added as a preservative (Iammarino *et al.*, 2011). Therefore, the measurement of benzoic acid has considerable importance both to determine adulteration and to estimate humans' daily exposure.

Information is scarce regarding changes in concentration of benzoic acid in cheeses during fermentation, production processing and storage. The goal of this study was to discover the change trend of benzoic acid concentration during the production and storage of Feta and cream cheese.

2. Materials and methods

2.1. Chemicals and reagents

Benzoic acid (>99%), sodium salicylate (>99.5%), ammonium acetate (98%), acetic acid glacial (100%) and HPLC-grade acetonitrile, MRS agar and other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Manufacturing of cheese

Samples of ultra-filtrated Feta cheese and cream cheese with milk fat were taken at the Pelareh Dairy Factory (Hamadan, Iran) according to processing system set by Tetra Pak. The initial raw milk samples were clarified and bactofugated to remove microorganism spores, and the fat content of the samples was standardized to 3.3%, followed by pasteurization at 72 °C for 15 s and cooling to 50 °C. The milk-solid-non-fat (MSNF) concentration was obtained at 34% using an ultrafiltration system. To produce the Feta cheese, the retentate was homogenized and pasteurized at 78 °C for 1 min and was fermented by adding a starter culture consisting of *Lactococcus lactis* ssp. *lactis*. The *fermentation time* continued until the pH reached 4.8. The fermented retentate was poured into a cheese container and went through a coagulation tunnel (30 min at 37 °C). In a sealing machine, parchment paper containing edible salt (4% w/w) was placed on top of the cheese curd, and each pack was sealed using aluminium foil. The samples were first stored at 27 °C for 24 h and were then transferred into a refrigerator with a temperature of 4 °C for 60 days. The benzoic acid levels, lactic acid bacteria (LAB) count, acidity and pH of the samples were determined after fermentation, after production and on days 1, 10, 20, 30, 40, 50 and 60 of storage in the refrigerator.

The production process for the samples of cream cheese with milk fat was largely similar to that of the Feta cheese. Briefly, milk fat was added to the pasteurized retentate. After homogenization and cooling to 30 °C, a mesophile starter containing *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* was added, and the mixture was transferred into a tank for fermentation until the pH reached 4.9. After the fermentation stage, salt and stabilizer were quickly added, and the mixture was pasteurized at 80 °C for 15 s, cooled to 60 °C, poured into cups in

amounts of 100 g each, and was stored in a refrigerator for 60 days.

2.3. Analysis of benzoic acid and method validation

2.3.1. Sample preparation

Five grams of the homogenized Feta or cream cheese sample was diluted with deionized water to 50 ml in a volumetric flask and was shaken for 2 min. Then 2 ml of the diluted sample was mixed with 200 µl sodium salicylate (1000 mg/kg) as the internal standard, further diluted by mobile phase to 10 ml and shaken vigorously for 2 min. The mixtures were then filtered through a 0.45 µm cellulose acetate syringe filter, and 20 µl was injected into the HPLC.

2.3.2. Preparation of standard solution of sodium benzoate

Stock solutions of benzoic acid were prepared in distilled water (1000 mg/kg). The working standard solutions in a concentration range from 31.25 to 500 mg/kg were obtained by diluting the stock.

2.3.3.Linearity assay

The linearity of the procedure was determined by injecting the standard solution with concentration ranges from 3.125 to 500 mg/kg. Calibration curves were plotted by least squares linear regression analysis of the ratio of the analyte/sodium salicylate peak areas versus the analyte concentration. The regression equation was Y=0.16086X – 0.03590, and the R^2 was calculated as 0.9996. The limit of detection (LOD) and the limit of qualification (LOQ) were determined as follows: LOD = 3.3σ/S, and LOQ = 10σ/S, where σ is the standard deviation of the response, and S is the slope of the calibration curve.

2.3.4. Determination of accuracy and recovery

The accuracy of the analysis method was identified by using the recovery method. The recovery method was carried out by adding 50, 125, 250, 500 and 1000 µL of benzoic acid solution (1000 mg/kg) into approximately 5 g blank Feta cheese to obtain samples containing 10, 25, 50, 100 and 200 mg/kg benzoic acid, and their recovery percentage and standard deviation were calculated.

2.3.5. Determination of precision

Intra-day precision was determined by injecting the benzoic acid solution at a concentration of 25 mg/kg six times in the same day. The measurement was continued for three days a week to obtain the intra-day precision.

2.4. Measurement of uncertainty

The measurement uncertainty for the benzoic acid was determined according to previous procedures, with some modifies (Iammarino *et al.*, 2011; Golge *et al.*, 2015). We calculated the uncertainty for the benzoic acid solutions and the cheese sample. Mass (m), volume (v), precision (pre), purity of standard (pur) and the standard calibration curve (S) were used for the uncertainty calculation.

By multiplying the combined uncertainty ($U_{combined}$) by 2, based on a confidence level of 95%, an expanded measurement uncertainty of 8.4% was obtained.

2.5. HPLC analysis

A Waters HPLC system (Milford, MA, USA) equipped with a UV detector and C18 analytical column (250 mm × 4.6 mm, i.d., 5 µm) was applied for the benzoate sodium measurement. The mobile phase contained an aqueous ammonium acetate buffer (pH = 4.2) and acetonitrile (80:20 v/v) with a flow rate of 1 ml/min. The detection of benzoate sodium was carried out using the UV detector at a wavelength of 225 nm.

2.6. Assay of lactic acid bacteria

Lactic acid bacteria (LAB) count of the cheese samples was determined both after the fermentation and the production stages and at 1, 10, 20, 30, 40, 50 and 60 days of storage. Twenty-five grams of the cheese samples was diluted in a stomacher (Seward Ltd., London, UK) with 225 ml quarter-strength Ringer's solution at 45 °C. Subsequently, serial dilutions were

performed in quarter-strength Ringer's solution. LAB counts were performed on MRS agar in order to obtain isolated LAB colonies. Incubation was done in an anaerobic jar at 30 °C for 72 hours until growth was evident.

2.7. Physicochemical properties analysis

The fat and protein content were also measured using the Gerber's and Kjeldahl methods, respectively (AOAC 2000). Moisture content was identified using the gravimetric method, acidity was measured by titration and expressed as lactic acid percentage, and a pH was determined by pH meter.

2.8. Statistical analysis

The obtained data were analysed using version 16 of the SPSS statistical software package (SPSS Inc., Chicago, IL, USA). All the experiments were repeated five times, and the means and standard deviations were reported for each experiment.

The one-way ANOVA and post-hoc Tukey's test were used to compare the mean concentration of benzoic acid, LAB count, pH, and acidity among the samples. The statistical correlation between the LAB count and benzoic acid concentration was assessed using Pearson's correlation coefficient. $P < 0.05$ was considered a statistically significant difference.

3. Results and discussions

The LOD and LOQ values for benzoic acid in Feta cheese were 0.51 and 1.6 mg/kg, respectively. The recovery data ranged from 96.88% to 105.26%, as shown in Table 1. LOD, LOQ and recovery value of benzoic acid in cream cheese was similar to that of Feta cheese (data not shown). The intra-day and inter-day precision data are exhibited in Table 2. The moisture, fat, and protein contents of Feta and cream cheese are showed in Table 3.

To assess the changes in benzoic acid levels during fermentation, production processing, and cheese storage in the refrigerator, raw milk was analysed for sodium benzoate levels. No benzoic acid was detected in the milk samples used for cheese manufacturing, but benzoic acid was found in all the samples after fermentation. After fermentation stage, Feta cheese (12.15 mg/kg) showed a higher concentration of sodium benzoate than cream cheese (8.49 mg/kg). In addition, the benzoic acid content of the Feta cheese samples significantly differed after storage in a refrigerator. For example, when the storage time was increased from 1 to 30 days, the benzoic acid concentration increase from 24.45 mg/kg to 41.80 mg/kg. However, the benzoic acid level in Feta cheese that was stored for 40, 50, and 60 days in the refrigerator was not significantly different from the acid level on the 30th day of storage in refrigeration. The benzoic acid concentration in cream cheese stored in refrigerator was similar fermented samples

Table 1. Recovery for the determination of sodium benzoate in spiked cheese sample

N	Spiked levels of sodium benzoate (µg/kg)				
	10	25	50	100	200
1	97.26	99.33	106.79	105.21	106.22
2	98.14	98.25	105.32	106.31	104.23
3	95.24	97.21	103.21	103.45	105.32
Mean (n=3)	96.88	98.26	105.11	104.99	105.26
S.D	1.49	1.06	1.80	1.44	0.99
RSD (%)	1.53	1.09	1.7	1.37	0.94

The LAB count in the Feta cheese samples increased based on fermentation time (3.62×10^8 CFU/g) until 30 days in the refrigerator (3.85×10^{10} CFU/g). Their count in the cream cheese after the fermentation was 1.62×10^8 CFU/g and destroyed after production process completion due to sample pasteurization (Table 4).

Table 2. Intra-day and inter-day precision of the method applied for sodium benzoate analysis

Concentration of sodium benzoate (µg/kg)	Found Concentration		
	Day 1	Day2	Day 3
25	24.45	23.45	24.15
25	24.75	23.75	24.35
25	24.85	23.45	24.25
25	23.95	23.85	23.95
25	23.85	23.95	23.85
25	24.15	23.25	24.55
Mean	24.33	23.62	24.18
SD	0.42	0.27	0.26
RSD (%)	1.71	1.15	1.06

Table 3. Chemical composition of feta and cream cheese

Composition	Cheese type	
	Feta cheese	Cream cheese
Fat (%)	15.21 ± 0.92^b	24.05 ± 1.05^a
Protein (%)	16.12 ± 1.60^a	8.19 ± 0.93^b
Moisture (%)	62.83 ± 1.45^b	65.12 ± 1.85^a

Each value in the table is the mean ± standard deviation of five replications. Mean within the same row followed by different superscripts differ significantly ($P < 0.05$)

In the Feta cheese samples, acidity increment and the pH reduction during storage was significant (Table 4). In cream cheese, acidity and pH showed no significant change.

Cheese is a nutritious dairy product, and different types of cheeses are produced throughout the world (Gheisari *et al.*, 2014; Pakbin *et al.*, 2015). The addition of benzoic acid to cheese and other dairy products has been forbidden (Amirpour *et al.*, 2015). However, this study showed the natural production of benzoic acid in cheese samples and the changes in its concentration during storage.

Table 4. Average values of sodium benzoate, lab count, acidity and pH in the analysed samples

Analysis time (days)		Feta cheese				Cream cheese			
		Benzoic acid concentration (mg/kg)	LAB Count (CFU/ g)	Acidity (% lactic acid)	pH	Benzoic acid concentration(mg/kg)	LAB Count (CFU/g)	Acidity (% lactic acid)	pH
After fermentation		12.15±1.15^{b}	3.62×10^{8f}	0.85±0.01^{c}	4.80±0.01^{a}	8.49±0.74^{a}	1.62×10^{8}	0.79±0.01^{a}	4.91±0.01^{a}
After production		18.38±3.05^{b}	1.25×10^{9e}	0.86±0.03^{c}	4.79±0.01^{a}	8.40±0.21^{a}	ND	0.81±0.01^{a}	4.90±0.01^{a}
After storage in refrigerator (day)	1	24.45±1.42^{b}	1.55×10^{10d}	0.87±0.02^{c}	4.77±0.01^{a}	8.52±0.78^{a}	ND	0.82±0.06^{a}	4.90±0.04^{a}
	10	31.57±4.62^{b}	2.67×10^{10c}	0.89±0.01^{c}	4.75±.01^{a}	8.39±0.93^{a}	ND	0.81±0.02^{a}	4.91±0.10^{a}
	20	35.62±3.26^{b}	3.62×10^{10b}	0.90±0.02^{bc}	4.70±.01^{b}	8.44±0.74^{a}	ND	0.82±0.04^{a}	4.90±0.05^{a}
	30	41.80±2.88^{a}	3.85×10^{10a}	0.92±0.02^{b}	4.62±.02^{b}	7.32±1.24^{a}	ND	0.83±0.08^{a}	4.89±0.08^{a}
	40	42.44±3.58^{a}	7.42×10^{8g}	0.95±0.02^{ab}	4.55±.03^{c}	8.54±0.75^{a}	ND	0.83±0.02^{a}	4.88±0.06^{a}
	50	42.54±4.06^{a}	2.64×10^{7h}	0.96±0.04^{a}	4.48±.02^{c}	8.43±1.13^{a}	ND	0.82±0.06^{a}	4.89±0.04^{a}
	60	43.10±3.9^{a}	7.60×10^{6i}	0.99±0.03^{a}	4.46±.011^{c}	8.66±0.80^{a}	ND	0.84±0.06^{a}	4.86±0.02^{a}

Each value in the table is the mean ± standard deviation of five replications. Mean within the same column followed by different superscripts differ significantly ($P < 0.05$).

Benzoic acid formation in dairy product is due to the transformation of hippuric acid to benzoic acid (Iammarino *et al.,* 2011). Hippuric acid naturally occurs in milk, and is converted to benzoic acid by LAB, such as *streptococci* and *lactobacilli* (Sieber *et al.,* 1995). The hippuric acid content of raw milks from different animals varies. For example, Horníčková *et al.,* (2015) reported sheep's milk (43.3 ±12.3 mg/kg) had a higher hippuric acid level (15.5 ±8.3 mg/kg) than goat's milk (15.5 ±8.3 mg/kg). Therefore, a significant difference between the benzoic acid levels was found in fermented sheep's milk and goat's milk (29.5 ±16.1 mg/kg and 20.3 ±13.9 mg/kg, respectively) (Horníčková *et al.,* 2015). Other bacteria, such as *Lactococcus luctis*, *Escherichia coli,* and *Pseudomonas fluorescens,* also produce benzoic acid in milk (Amirpour *et al.,* 2015*).* However, some bacterial cultures used in the fermentation process did not influence the benzoic acid levels (Horníčková *et al.,* 2015). Our results showed that the benzoic acid level found in Feta and cream cheese was different after the fermentation stage. In the current study, a thermophlic and mesophilic starter cultures were used in the production of Feta and cream cheese, respectively. In previous research, higher levels of benzoic acid were found in hard cheeses (28 mg/kg) produced using a thermophilic LAB starter cultures than in semi-hard cheeses (11 mg/kg) produced using mesophilic LAB starter cultures (Garmiene *et al.,* 2011). The results from the current study indicated that benzoic acid levels depended on cheese LAB growth and type. For example, cream cheese did not show substantial changes in LAB growth during storage because no LAB was found in these samples. In addition, the Pearson correlation test showed a positive and significant correlation (r= 0827) between benzoic acid level and LAB count. The LAB are beneficial, naturally occurring microorganisms found in milk, cheese, meat, beverages, and vegetables. These bacteria play an essential role in cheese production (Al Khalaileh and Ajo, 2013).

Different types of cheese made with different production technologies contain different benzoic acid levels. Sieber et al., (1995) reported that the high concentration of benzoic acid in the outer zone of smear-ripened cheeses is more than the stoichiometric transformation of the total amount of hippuric acid available in the milk to benzoic acid. Therefore, benzoic acid might be formed from other natural pathways including phenylalanine degradation, and the auto-oxidation of benzaldehyde. According to results, benzoic acid wasn't produce in cream cheese during storage in refrigerator. Therefore, it seems role of pathway of phenylalanine degradation, and the auto-oxidation of benzaldehyde in benzoic acid production in cheese is low and insignificant. In the current study, the maximum concentration of benzoic acid found in Feta cheese, by considering the measurement of analysis method uncertainty, was 46.50 mg/kg. Our results were similar to other studies. For example, Amirpour et al., (2015) investigated the occurrence of sodium benzoate in cheeses in Iran. The collected UF-Feta cheese, Lighvan cheese, and lactic cheese samples contained mean benzoate concentrations (range) of 50.6 ± 17.4 (27.6–91.2), 34.6 ± 12.3 (16.7–84.1), 17.3 ± 3.7 (11.9–25.6) $mgkg^{-1}$, respectively. In Turkey, the benzoic acid levels in cheese ranged from 3.17–56.77 mg/kg (Yildiz et al., 2011). Iammarino et al., (2011) analysed various cheeses in Italy and reported natural benzoic acid levels below 40.0 mg/kg. Higher levels of benzoic acid in some cheese studies were comparable to the samples assessed in current study. For example, Garmiene et al., (2011) found that the benzoic acid content in hard and semi-hard cheese increased during the ripening stage, reaching 152 mg/kg at 48 months in hard cheeses. However, the authors found that the level of benzoic acid in provolone cheese did not change during the ripening stage. Sieber et al, (1995) reported higher content and wider range of benzoic acid (from traces to 341 mg/kg) in various types of cheese produced in Switzerland. In comparison with our findings, lower value of benzoic acid has been reported in some cheeses. Camembert cheese and processed cheese had the benzoic acid in range of < 0.18–4.2, and 0.18–20.8 mg/kg, respectively (Lim *et al.*, 2013).

The LAB count in Feta cheese increased from fermentation to 30 days of storage in the refrigerator. Our results were similar to previous research. For example, Kamleh *et al*., (2012) found increased lactic acid bacteria levels in Halloumi cheese during storage. The LAB count of 0.9 log CFU/g in freshly-packed Halloumi reached 3.38, 3.64, and 3.43 log CFU/g at 25°C/20 days, 15°C/54 days, and 5°C/170 days, respectively (Kamleh *et al.*, 2012). The changes in cheese acidity depended on the growth of lactic acid and aerobic bacteria (Hasani *et al.*, 2016). The average LAB counts of the Karin Kaymagi cheese samples were 6.11 log CFU/g, and these levels increased for 60 days after production (Yangılar and Ozdemir, 2010). Souza *et al*., (2003) analysed six Serrano cheeses and found 431–484 LAB isolates in these cheese samples. The *lactobacilli* were the most abundant lactic bacteria, followed by *enterococci* and *lactococci*. The LAB count reduced during a 60-day fermentation process. The growth of *lactococci* and *leuconostoc* was inhibited due to the salt that was added to the cheese (Souza *et al.*, 2003). These discrepancies in the LAB count may also be due to different cheese making and ripening techniques.

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4. Conclusions

In this study, the benzoic acid was produced after the fermentation stage in all

the cheese samples. The benzoic acid concentration was dependent on the type of cheese, storage time, and LAB count. An increase in the storage time from 1 to 30 days significantly raised the Feta cheese benzoic acid level and LAB count from 24.45 to 41.80 mg/kg and 1.55×10^{10} to 3.85×10^{10} CFU/g, respectively. The benzoic acid levels in Feta cheese had a positive correlation to the LAB count (r = 0827). After the fermentation stage, the benzoic acid concentration in cream cheese was 8.52 mg/kg, but no significant changes in this level were observed during the 60 days the cheese was stored in the refrigerator. In general, the maximum benzoic acid level (46.50 mg/kg) in cheese occur naturally after 30 days of storage and this concentration of benzoic acid and lower levels were attributed to LAB activity.

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