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# Determination of benzoic acid content of dairy products consumed in Turkey

*Benzoesäurengehalt von in der Türkei konsumierten Milchprodukten*

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**Summary**

This paper describes quantitative data concerning the natural occurrence of benzoate/benzoic acid in a range of yoghurt, ayran/yogurt drink, strained yoghurt, cheese types (White Pickled, Kashar, Tulum) and butter. During the fermentation or production of dairy products, benzoic acid is produced from hippuric acid. A second metabolic pathway has been described during ripening and additional quantity of benzoic acid originates from phenylalanine degradation with ß phenyl propionic acid and cinnamic acid as intermediate products. Also, a third way could be the autooxidation of benzaldehyde, produced by certain strains of lactic acid bacteria. Samples were purchased from Izmir Region in Turkey in the period of 2011–2013. All dairy products were produced from cow, goat, sheep milk or blends. Benzoic acid levels in cheese, yoghurt, strained yoghurt, ayran and butter varied between 2.3–160, 6.4–83, 7.8–40.70, 0.6–12.8 and 0.0–7.3 µg/l., respectively. The results showed that benzoic acid widely forms in milk products as a result of the metabolic activity of lactic cultures or natural microbial flora employed.

**Keywords:** benzoic acid, benzoate, hippuric acid, traditional dairy products

**Zusammenfassung**

Dieser Beitrag befasst sich mit quantitativen Daten zum Vorkommen natürlicher Benzoate/Benzoesäure in Joghurt, Ayran/Joghurtgetränken, abgetropftem Joghurt, Käse (eingelegter Weißkäse, Kashar, Tulum) und Butter. Während der Fermentation oder der Herstellung von Milchprodukten wird Benzoesäure aus Hippursäure hergestellt. Ein zweiter Stoffwechselweg zur Benzoesäure-Bildung während der Reifung findet durch den Abbau von Phenylalanin mit ß-Phenylpropionsäure und Zimtsäure als Zwischenprodukte statt. Ein dritter Weg könnte die Autooxidation von Benzaldehyd durch bestimmte Milchsäurebakterien Stämme sein. Die Proben wurden aus der Region Izmir (Türkei) in der Zeit von 2011-2013 erworben. Alle Milchprodukte wurden aus Kuh-, Ziegen-, Schafsmilch oder aus deren Mischungen hergestellt. Der Benzoesäurengehalt in Käse, Joghurt, abgetropftem Joghurt, Ayran und Butter variierte jeweils zwischen 2,3–160 µg/l, 6,4–83 µg/l, 7,8–40,70 µg/l, 0,6–12,8 µg/l und 0,0–7,3 µg/l. Die Ergebnisse zeigten, dass die Benzoesäure-Bildung in Milchprodukten häufig als Folge der metabolischen Aktivität von Milchkulturen oder der natürlichen mikrobiellen Flora vorkommt.

**Schlüsselwörter:** enzoesäure, Benzoate, Hippursäure, traditionelle Milchprodukte

## Introduction

In order to inhibit various microorganisms, food preservatives have been widely used in the food industry (Sieber et al., 1995; Qi et al., 1999; Trouni and Toledo, 2002; Lee et al., 2008). The increase in the production of processed and convenience foods have caused developments in the chemical preservation techniques in food technology (Mroueh et al., 2008). Thus, benzoic acid and their salts are extensively used in many countries and in a variety of food products. These additives are effective in inhibiting yeast, mould growth and also exhibit inhibitory effects against a wide range of bacteria (Trouni and Toledo, 2002; Mroueh et al., 2008). Due to its low cost, easy incorporation into products, colorless form, and taste and relatively low toxicity, benzoic acid is a widely used preservative in the world (Qi et al., 2009; Saidi and Raid, 2001; Yildiz et al., 2012). The use of food additives in different countries is allowed by the specific regulations. Although benzoic acid is generally recognized as safe (GRAS), short term exposure to benzoic acid can irritate the eyes, the skin and respiratory tract. Long term exposure or related exposure may cause skin sensitization. Also benzoic acid and its salts can cause non immunological contact reactions at low doses in sensitive people. Adverse effects include asthma, urticarial, metabolic acidosis, convulsions (Filho et al., 2004; Mroueh et al., 2008; Qi et al., 2009; Yildiz et al., 2012). An acceptable daily intake of benzoic acid and benzoate salts, benzaldehyde, benzyl acetate and benzyl alcohol is determined as 0–5 mg/kg body weight by Joint FAO/WHO Expert Committee on Food Additives (Yildiz et al., 2012). This additive doesn't accumulate in the body, and it is transformed into water soluble derivatives through conjugation with glycine and glucuronic acid and it is eliminated in the urine (Mroueh et al. 2008; Qi et al. 2009).

On the other hand, usually the use of benzoic acid and its salt as food additives in dairy products are not permitted. Benzoic acid forms naturally in different foods, such as fruits, vegetables, spices, nuts and also in milk and dairy products at low concentrations (Sieber et al., 1995). The formation and distribution of benzoic acid during the manufacture of fermented dairy products and cheese were explained by several researchers (Overstrom et al., 1972; Chandan et al., 1977; Sieber et al., 1995; Saidi and Raid, 2001; Trouni and Toledo, 2002; Urbienen and Leskauskaite, 2006; Qi et al., 2009; Yildiz et al., 2012; Esfandiari et al., 2013; Lim et al., 2013).

It is known that benzoic acid forms with different biochemical pathways as explained below;

a) Formation of benzoic acid from hippuric acid during cheese manufacture and yoghurt fermentation.
b) Breakdown of phenylalanine as an alternative pathway for the biogenesis of benzoic acid in cheese types (Sieber et al., 1995; Urbienen and Leskauskaite, 2006).

Milk has only low amounts of benzoates but some fermented dairy products can contain higher levels of benzoates (Sieber et al., 1995; Saidi and Raid, 2001; Guler and Park, 2011; Yildiz et al., 2012). Several researchers reported that the presence of benzoic acid in dairy products is not sourced from deliberate addition, but as a result of contamination from rennet, veterinary drugs, teat dips and especially flavouring fruit pulps which contain benzoic acid. It is also known that during the fermentation of milk, lactic acid bacteria convert hippuric acid (naturally present in milk) into benzoic acid (Sieber et al., 1995; Saidi and Raid, 2001; Urbienen and Leskauskaite, 2006).

On the other hand, another metabolic pathway can explain the biogenesis of benzoic acid from phenylalanine and fermentation of aromatic amino acids as well as the simultaneous production of acetophenone (Sieber et al., 1995).

Taking all this information into account, the aim of this study was to measure the concentration of benzoic acid in certain dairy product types consumed in Izmir, Turkey by using high performance liquid chromatography techniques.

## Materials and Methods

### Sampling

A total number of n = 495 commercial products were retained from different local producers in Izmir region. Total n = 345 different kinds of cheese samples (White pickled cheese n = 80, Tulum Cheese n = 62, Kashar Cheese n = 149, Dil Cheese n = 12, Mozzarella Cheese n = 12, Processed Cheese n = 22, Hellim Cheese n = 8) as well as total n = 150 samples other dairy products such as yoghurt n = 52, strained yoghurt n = 67, ayran n = 19, and butter n = 12 were collected and analyzed between December 2010 and July 2012. Samples were taken to the Izmir Food Control Laboratory Directorate at 4 ± 1 °C ensuring cold chain. All the samples were freshly analyzed.

### Chemicals and standards

Benzoic acid standard (1µg/mL) was purchased from Alfa-Aesar (Cas No: 65-85-0, 99 %). The standards were diluted in purified water. All the water used was purified by millipore milli-Q a10 system. HPLC grade acetonitrile was purchased from Carlo Erba (Cas No: 75-05-8, 99.9%). Other reagents were also HPLC grade and were obtained from Panreac (Cas No: 231-633-2), Sigma-Aldrich (Cas No: 778-77-00 98-100%).

### Preparation of samples

Approximately 100 g sample was homogenized with 25 ml purified water by stirring. Homogenized sample was kept for 10 minutes in ultrasonic bath and then in water bath at 25 °C. Then 5 ml of Carrez I and 5 ml Carrez II were added into the tube and stirred in a rotator for 1 minute. The solution was diluted to 100 ml with purified water. After the solution was agitated manually for 1 minute, all the solution and samples were filtered through a 0.45 µm filter (isolab PTFE) before being injected into the liquid chromatograph.

### Recovery

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Method validation provides documented evidence, and a high degree of assurance, which an analytical method employed for a specific test, is suitable for its intended use (Shabir, 2004). For verifying the accuracy and precision of the analytical methods, recovery studies were carried out. In the study, the known amount of benzoic acid standard aqueous solution was added at two different concentrations in yoghurt, cheese and butter samples (Tab. 1 and Fig. 1). The spiked samples as well as unspiked controls were analyzed in duplicates and the chromatograms indicate of

**TABLE 1:** *Recovery of benzoic acid from different dairy products.*

| Sample | Spiked amount µg/kg | Recovery ± SD (%) | Mean Recovery ± SD (%) |
|---|---|---|---|
| Yoghurt | 10 | 100.3 ± 1.2 | 98.63 ± 2.25 |
| | 50 | 96.96 ± 0.89 | |
| Cheese | 10 | 98.20 ± 0.87 | 99.84 ± 1.80 |
| | 50 | 101.48 ± 1.05 | |
| Strained Yoghurt | 10 | 99.6 ± 0.65 | 95.72 ± 3.80 |
| | 50 | 91.84 ± 0.71 | |
| Butter | 10 | 101.30 ± 1.65 | 104.05 ± 2.34 |
| | 50 | 106.80 ± 2.10 | |

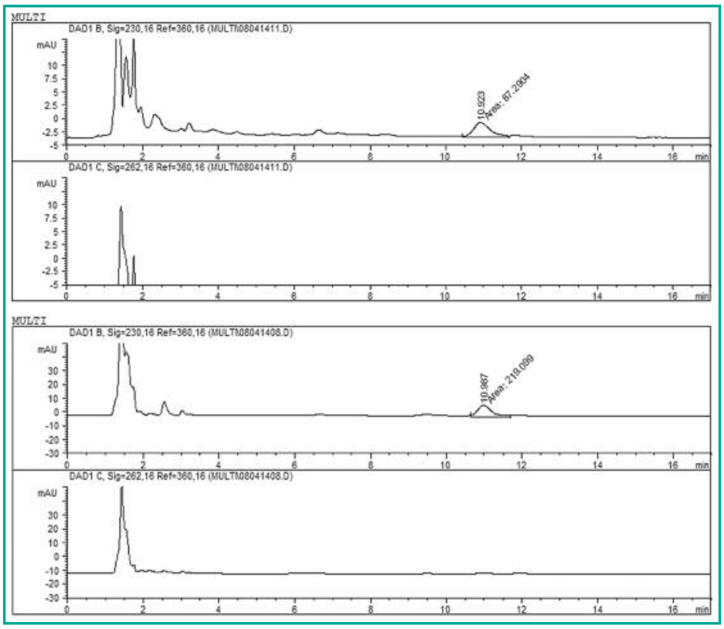

**FIGURE 1:** *Sample containing benzoic acid in recovery studies (unspiked controls of cheese and yogurt).*

benzoic acid in the recovery studies. The average retention time was 7–8 min for benzoic acid. The identification and purification of the peaks were confirmed by comparing their spectra with those of the standard solutions. The average recoveries for yoghurt, cheese and butter were 90 and 110 µg/l.

### Determination of benzoic acid by HPLC

Determination of benzoic acid in dairy products was analyzed according to Prodolliet and Bruelhart (1993). The analysis was carried out by Agilent 1100 series HPLC system equipped with G1316A Column Thermostat, G1315B Diode Array detector, G1379A degasser, G1311A Quatpump. The column used was waters C-18, µ Bondapak, 10 µm, 3.9 x 300 mm. The injection volume was 10 µL. The peak of benzoic acid was measured at 230 nm wavelength. The peak of benzoic acids in samples was identified by comparing retention times and spectra with the aqueous solution of the standard. In quantification, the external standard plot method was used for the best fit standard curve preparing linear regression lines and the concentrations of the benzoic acid varied between 0.5 and 10 µg/l. The analysis was conducted in three repetitions.

### Data analysis

All analyses were performed in three repetitions and the results were expressed after simple descriptive statistical analysis. For the statistical evaluation of results, analysis of variance was used and for the determination of different sample groups, Duncan Test was applied. Accordingly, statistical analysis software SPSS version 19.0 was used.

## Results and Discussion

### High Performance Liquid Chromatography (HPLC) results

Limit of detection (LDD) is defined as the smallest peak detected with a signal height three times that of the baseline while the limit of quantitation (LOQ) refers to the lowest level of analyte which can be determined with an acceptable degree of confidence (Yildiz et al., 2012). In this study, detection and quantitation limits were estimated by decreasing the concentration of prepared standards successfully down to the smallest detectable peak (Yildiz et al., 2012).

In our study a total of 7 cheese types and 345 different samples were analyzed for benzoic acid. The contents of benzoic acid in cheeses at different ages are shown in Table 2 in a chromatogram, and Figure 2 and in Figure 3 in all analyzed samples. The main concentration of benzoic acid observed in cheese samples ranged between 5.46 ± 2.80 and 41.01 ± 48.02 ppm. In processed cheese samples, the maximum concentration of benzoic acid was detected compared to the other cheese varieties. The maximum concentration of benzoic acid can be associated with the processing techniques such as using different type of ripened cheese, lactic acid bacteria used in production of the cheese varieties (types) and non-starter culture flora in cheese.

**TABLE 2:** *The content of benzoic acid in cheeses at different ages (n = 345).*

| Cheeses | N | Positive Sample | Minimum | Maximum | Average | Standard Deviation |
|---|---|---|---|---|---|---|
| Kashar | 149 | 149 (100 %) | 3.0 | 89.30 | 11.88 | 8.26 |
| White | 80 | 80 (100 %) | 2.7 | 66.00 | 14.74 | 11.02 |
| Tulum | 62 | 62 (100 %) | 4.0 | 119.00 | 16.45 | 17.95 |
| Process | 22 | 22 (100 %) | 6.0 | 160.00 | 41.01 | 48.32 |
| Mozarella | 12 | 12 (100 %) | 10.6 | 4.78 | 10.63 | 4.78 |
| Dil | 12 | 12 (100 %) | 6.4 | 38.40 | 14.75 | 8.26 |
| Hellim | 8 | 8 (100 %) | 2.3 | 10.70 | 5.46 | 2.80 |

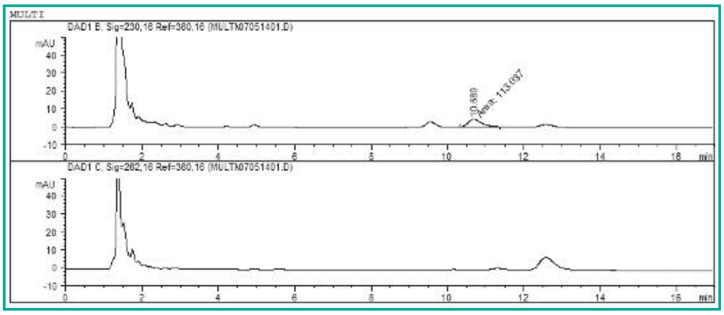

**FIGURE 2:** *Chromatogram of cheese samples containing benzoic acid.*

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**TABLE 3:** *The content of benzoic acid in other dairy products (n = 150).*

| Dairy Products | N | Positive Sample | Minimum | Maximum | Average | Standard Deviation |
|---|---|---|---|---|---|---|
| Strained Yogurt | 67 | 67 (100 %) | 6.4 | 83.00 | 20.32 | 10.62 |
| Yogurt | 52 | 52 (100 %) | 7.8 | 40.70 | 24.48 | 8.50 |
| Ayran | 19 | 19 (100 %) | 0.6 | 12.80 | 5.25 | 3.58 |
| Butter | 12 | 10 ( 80 %) | 0.0 | 7.30 | 3.31 | 2.46 |

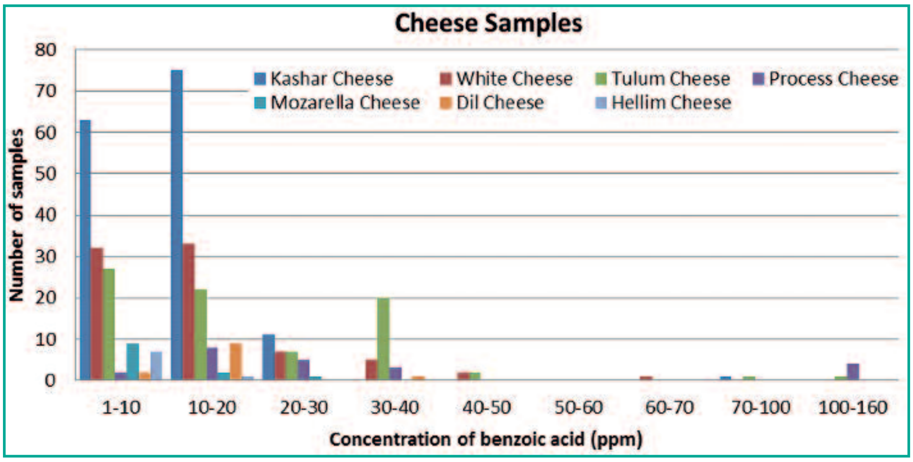


**FIGURE 3:** *Benzoic acid concentration in cheese samples (n = 345).*

The levels of benzoic acid content of yoghurt samples were shown in Table 3, Figure 3 and in chromatogram Figure 4 and in Figure 5. The low level of benzoic acid that was found in samples was probably the indigenous benzoic acid in yoghurt, strained yoghurt, and ayran. Benzoic acid content was detected in 52 yoghurt samples being average 24.42 ppm, in the range of 7.8 and 24.48 ppm. Mean concentrations of benzoic acid in strained yoghurts varied between 6.4 and 83 ppm, with an average 20.32 ppm. A total of 19 ayran samples tested in this study were found to contain benzoic acid between 0.6 and 12.8 ppm. Also the levels of benzoic acid were between 0.0 and 7.3 ppm in butter. The percentage of positive samples for benzoic acid in butter was 80 % (Tab. 3).

It is known that during the fermentation of milk, lactic acid bacteria convert hippuric acid (naturally present in milk) to benzoic acid (Urbienė and Leskauskaitė, 2006). Also an additional quantity of benzoic acid originates from the biogenesis of benzoic acid from phenylalanine, as well as the simultaneous production of acetophenone and ammonia (Sieber et al., 1995). Acetophenone and benzoic acid are formed from phenylvaleric acid with the intermediate product, phenylpropionic acid which can also be generated through the deamination of phenylalanine (Sieber et al., 1995). A third way could be the auto-oxidation of benzaldehyde, produced by certain strains of lactic acid bacteria and some yeast.

Benzoic acid was observed in all yoghurts, strained yoghurts and ayran samples and seems to be a metabolite of lactic acid bacteria, used as a starter in manufacturing and storage. Besides, another possible pathway for the formation of benzoic acid could be the anaerobic metabolism of phenol (Qi et al., 2009; Yildiz et al., 2009). Indigenous benzoic acid levels of 9.36–83.9 mg/kg, 9.79–26 mg/kg, 3.70–13.55 mg/kg have been reported in commercial yoghurt (plain, low fat, fat free, fruit flavored) and butter milk ayran samples (Chandan et al., 1977; Sieber et al., 1995; Mhyar et al., 1995; Mrouch et al., 2008; Yildiz et al., 2012). Also, indigenous benzoic acid level in concentrated yoghurt samples varied between 56.0 and 62.7 mg/kg (Mroueh et al., 2008).

It is shown that the formation of benzoic acid proceeded simultaneously with the logarithmic phase of growing of *Lactobacillus* species (Överström et al., 1972). Besides, a production of benzoic acid up to 16 ppm in growing cheese starters was reported (Överström et al., 1972). Romadur Cheese contained 38 ppm benzoate that was considered to arise from tyrosine metabolism (Chandan et al., 1977). It has been reported that 20, 22, 17, 26 ppm of benzoic acid was found in buttermilk, yoghurt culture, cheddar cheese and mozzarella cheese, respectively. Also, benzoic acid (10–30 mg/100 g dry matter cheese) was detected in Munster and Livarot cheese in free fatty acid fractions (Chandan et al., 1977). Överström et al. (1972) reported between 5–18 ppm of benzoic acid in Swedish, Gouda and Herrgard cheese. The researchers considered that it is a natural metabolite and found that the concentration was higher near the cheese surface. According to Teuber (1987), benzoic acid can be formed at up to 200 µg/kg in Harzer cheese by fermentation of aromatic amino acids. Furthermore, Toppino et al. (1990) detected higher

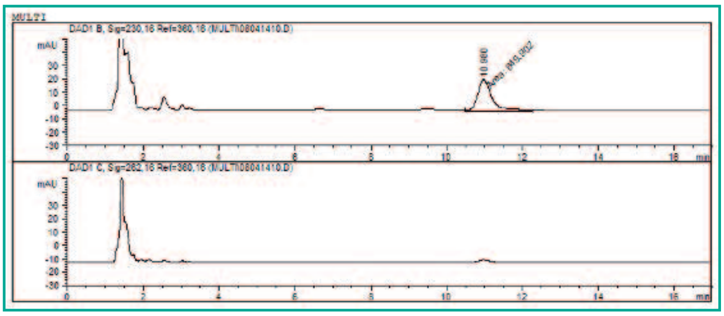

**FIGURE 4:** *Chromatogram of yoghurt samples containing benzoic acid.*

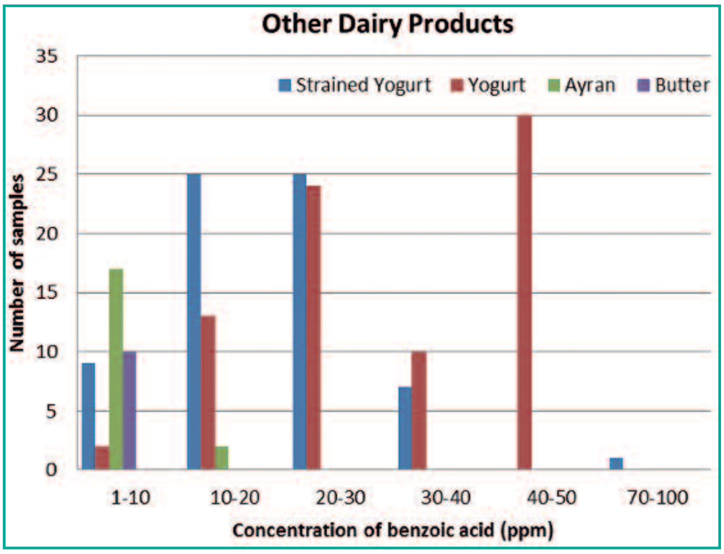


**FIGURE 5:** *Benzoic acid concentration in other dairy products (n = 150).*

concentrations of benzoic acid in Provolone cheeses, in which an additional amount of benzoyl peroxide, equivalent to 60 mg benzoic acid per kg, was permitted.

Sieber et al. (1990b) and Kopp (1992) reported that ripened cheeses and non-starter ripened cheeses contain benzoic acids. Whereas the variable concentration of benzoic acid in the bottom and upper sides as well as the hoop side of smear-ripened cheese, depends on many factors such as the composition of the microbiological flora, the frequency and kind of cheese curing and ripening conditions as temperature, humidity, ripening time etc. Yildiz et al. (2011), observed that benzoic acid was detected in 50 samples of cheese, being 25 white cheese and 25 based on kashar and melted cheese, in the range of 3.17 and 56.77 mg/kg. According to the researchers, in Istanbul, the maximum concentration of benzoic acid in cheese samples was detected compared with other cheese and related with the processing conditions, starter culture used, ripening time and conditions of this cheese. The results obtained in this paper as well as in earlier reports indicate that nearly all the naturally occurring benzoate in cultured/fermented dairy products, cheese varieties and butter is elaborated as a result of activity of lactic cultures used.

Gul and Dervisoglu (2013) analyzed sodium benzoate and potassium sorbate in fresh Kashar cheese by HPLC-DAD and found out that the average level of sodium benzoate of the cheese samples was 68.63 mg/kg. It was also stated that sodium benzoate might occur in fresh cheese at concentrations of up to 50 mg/kg.

Benzoic acid is legally permitted as preservative in several countries including Turkey in foods such as coffee, fruit juices, soft drinks, flavorings, rennet, and syrup. The permitted levels usually range between 160 and 2000 ppm. However, it is not permitted as an additive in dairy products in most countries.

## Conclusions

In the present study, we investigated the concentrations of benzoic acid in commercial yoghurt, strained yoghurt, ayran, cheese types and butter, produced in Izmir region in Turkey. An isocratic HPLC technique was described for the determination of benzoic acid in dairy products. The results showed that benzoic acid forms naturally in fermented dairy products and cheese types by using lactic cultures. The levels of benzoic acid found in the samples varied, but according to our results, it can be stated that the real utilization of benzoates is significantly lower than the maximum authorized levels. The data also indicates that the legislation on additives needs revision in order to accommodate the natural presence of benzoate in dairy and dairy related products.

## Authors contribution

The authors declare that they have no competing interests. O.K., L.G., S. M. collected samples and E.A., M.K., G.K. and H.G.Y. prepared all samples for analysis. O.Y. and O.K. produced data analysis and wrote the manuscript, O.K. contributed to field experiments, O.K. and O.Y. conceived and designed the written manuscript. All authors read and approved the final manuscript.

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