

Original Research Article**RP-HPLC Determination of Benzoic Acid in Samples of Children Food Distributed in Aden-Yemen****Abstract**

Revers phase high performance liquid chromatographic method that allows the determination of the preservative benzoic acid is described. The separations were affected by using an initial mobile phase of methanol-acetate buffer (pH 4.4) (35:65). separation of benzoic acid is achieved in 7.10 min. Analytical characteristics of the separation such as limit of detection, limit of quantification, linear range and reproducibility were evaluated. The method was applied to determine the quantities of benzoic acid in 32 local and imported children food products distributed in Aden-Yemen, comprising, dry milk and chesses, food for infants and young children, cakes, juices and jam .Results shows that the ranges of benzoic acid in the studies samples varies greatly from 1.778 mg/L to 849.295 mg/L.

Keywords: RP-HPLC; Children Food analysis ; Benzoic acid ; Aden -Yemen

Introduction

Food preservatives are added to stop or delay nutritional losses due to microbiological, enzymatic or chemical changes of foods during its shelf life. They also prevent consumer hazards due to the presence of microbial toxins or pathogenic microorganisms and economic losses due to spoilage .The role of preservatives has become more prominent with the increase in production of processed and convenience foods. They have been the subject of interest among consumers, health professionals, commercial and industrial agencies, alike because they are widely consumed in the diet by most segments of the population and can exert adverse health effects, especially for children and pregnant women. In fact, such food additives are subject to regulation, since excessive or inappropriate use of them may present food safety problems and can introduce a risk factor [1].

The European scientific committee on food has recommended that special attention should be given to intake by children ,since there is evidence suggesting that the intake of some additives expressed on a body weight basis may be markedly higher than that of adults. The use of benzoic acid (E210) as well their salt sodium benzoate (E211);potassium benzoate (E212); calcium benzoate (E213) is allowed by European legislation and its presence must be declared on the label [2].WHO's International Programme on Chemical Safety (IPCS) suggests that a provisional tolerable intake would be 5 mg/kg body weight per day [3].For these reason the use of food additives in different countries is limited by specific regulations. These preservatives are allowed by legislation but their use demands special care .

Efficient analytical techniques able to detect and to analyze the content of this preservative are available such as spectrophotometric methods [4,5], chromatographic methods [6,7] and capillary electrophoresis methods [8] The spectrophotometric methods in the analysis of food preservative presents major inconvenient, science the interferences required almost an additional extractive step.HPLC methods are more attractive for this purpose, offering the possibility to analyze food additives without a prior step, with high accuracy and precision, and with a very good detection limit, as well.

The objective of present work deals with the development and characterization of a rapid and simple RP-HPLC method ,and to determine the quantities of benzoic acid in 32 local and imported children food products distributed in Aden-Yemen, comprising, dry milk and chesses, food for infants and young children, cakes, juices and jam.

Experimental part**1. Sampling**

Samples were collected in august 2010, from different supermarkets in the governorate .The analyzed food products comprise cakes (14 samples), Juices and drinks (8 samples), jams (3 samples) , Dry Milk and cheese (3 samples), and Food for infants and young children mainly manufactures of grain (4 samples) .

2. Chemicals and reagents

Chemicals and reagents used were obtained from the (BDH): acetic acid (99.8%), Benzoic acid extra pure, ammonium acetate (Analar); methanol (HPLC grade).

3. Chromatographic conditions

Analytical separation was carried out on HPLC from Perkin-Elmer, Germany, model (Series – 200) using (EXCGL 120 ODS-A) C18 column, (15 cm×0.46 mm, 6µm) at room temperature. The detector used was a Perkin-Elmer, Germany UV-VIS model (Series – 200), set at 233 nm. The volume of sample injected was 20 µL. The aqueous phase was prepared by adding 3.8 g ammonium acetate in 1L water and its pH was adjusted to 4.4 using acetic acid. The mobile phase used was methanol – ammonium acetate buffer (pH 4.4) (35:65).

4. Preparation of sample

4.1. Liquid samples

10 ml of liquid sample were diluted with 25 ml of methanol into 50 ml volumetric flask and mixed well at 50 °C for about 10 minute on sonicator (vibrating heating plate) (BANDELIN – RK 100H, Germany). The volumetric flask was next subjected to vortex mixing (Model ALC, Germany) for 5 min. The contents were filtered through a 0.45µm syringe filter (Sartorius AG 37070 Goettingen Germany). The content then transferred into another 50-ml volumetric flask and diluted with methanol up to the mark. The clear filtrate was injected into the HPLC column. For concentrated samples, prior dilution with the mobile phase was done.

4.2. Solid samples

Solid samples were finely ground prior to the extraction. About 1.0 g of sample is accurately weighed in a screw capped test tube. 25ml of methanol was added, and placed in a sonicator (BANDELIN – RK 100H, Germany) that was maintained at 50°C for 30 min. The test tube was next subjected to vortex mixing (Model ALC, Germany) for 5 min. The contents were filtered through a 0.45µm syringe filter (Sartorius AG 37070 Goettingen Germany). The content then transferred into 50-ml volumetric flask and diluted with methanol up to the mark. The clear filtrate was injected into the HPLC column. For concentrated samples, prior dilution with the mobile phase was done.

5. Preparation of The Standard Curve

According to the external standard plot method, duplicate injections of 20 µL standard benzoic acid solutions were used to construct linear regression lines (peak area versus concentration). The peaks were identified based on the retention time.

6. Statistical analysis

Results of the RP-HPLC analysis of standard solutions of benzoic acid as well as of studied samples were expressed as mean, Data were analyzed using Origin program.

Results and discussion

The typical chromatogram resulting from injection of standard benzoic acid solution in our work is shown in Fig.1, from the resulting chromatogram the retention time of benzoic

acid was 7.10 minute .

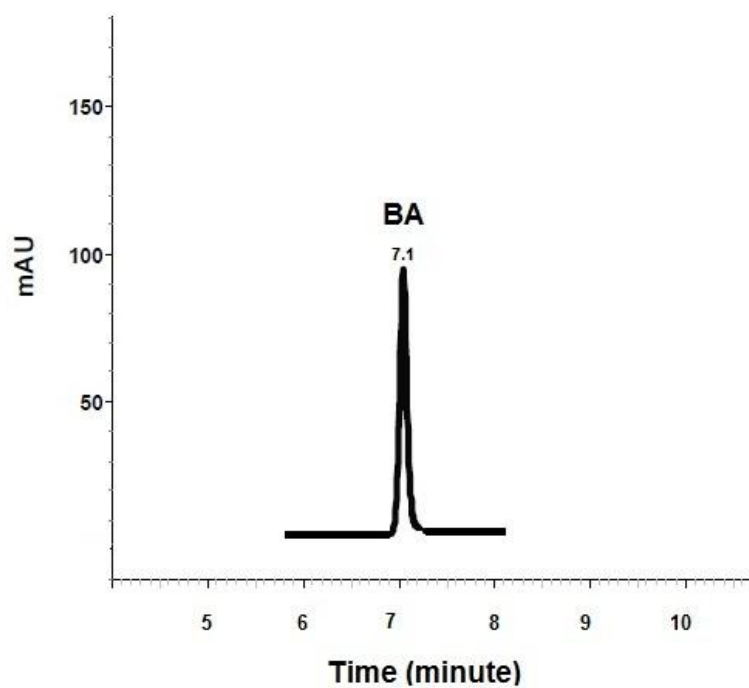


Fig.1. Chromatogram of Benzoic Acid standard solution

Linearity between the concentration of benzoic acid and the UV absorbance at 233 nm was maintained over the concentration range of 5-100 mg/L for benzoic acid is shown in Fig. 2.

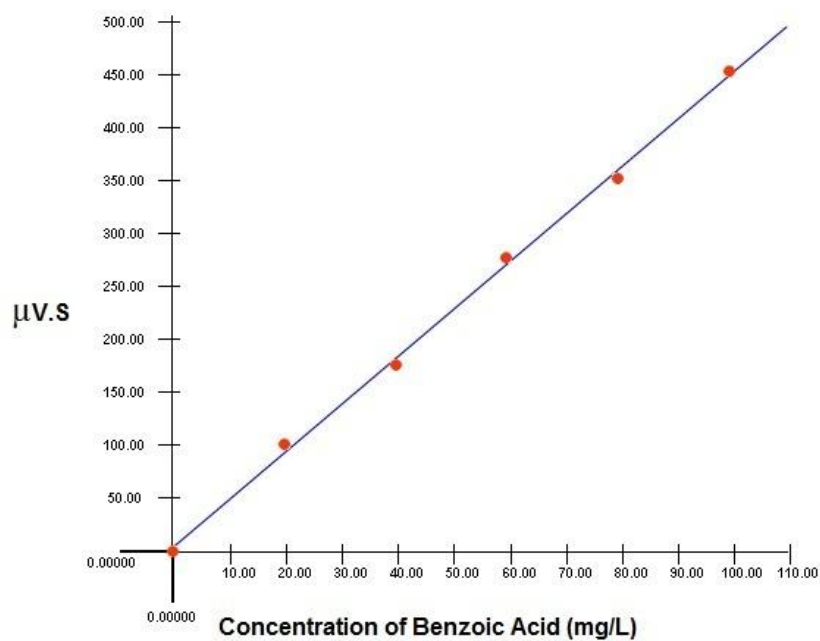


Fig.2. Calibration curve for standard Benzoic Acid

The mean regression equations for concentrations of benzoic versus arbitrary units of peak area were $Y = 48.92 X + 34.51$ (Y represents peak area, X represents concentration in mg/L) and $Y = 134.04 X + 11.30$, respectively. Also the correlation coefficients for standard curves of benzoic were calculated. The limit of detection (LOD) is defined as the smallest peak detected with a signal height three times that of the base line while the limit of quantitation (LOQ) refers to the lowest level of analyte which can be determined with an acceptable degree of confidence. (LOQ) value is often calculated as 10 times the signal height to the baseline. In our work, detection and quantitation limits were estimated by successively decreasing the concentration of the prepared standards, down to the smallest detectable peak. This concentration was multiplied by 3 and 10 to obtain the detection and quantitation limits, respectively. Table 1 shows these parameters and important others.

Table 1 : Characteristic of HPLC method

parameter	Benzoic acid
limit of detection (LOD) (mg/L)	0.404
limit of quantitation (LOQ) (mg/L)	1.348
Linear range (mg/L)	5-120
The correlation coefficient	0.99947

n=3

The selected children food for this study were chosen to full under five categories, the mean concentration of benzoic acid in each sample in the different categories were shown in tables 2,3,4,5 and 6.

Category (1) consist of three samples of dry milk and chesses, the mean concentrations of benzoic acid in these samples ranges from 2.749 to 94.943 mg/L. Sample 1 and 2 had a small mean concentration of benzoic acid 2.749 mg/L, and 11.207 mg/L respectively, while sample 3 had mean concentration of 94.943 mg/L, Table 2.

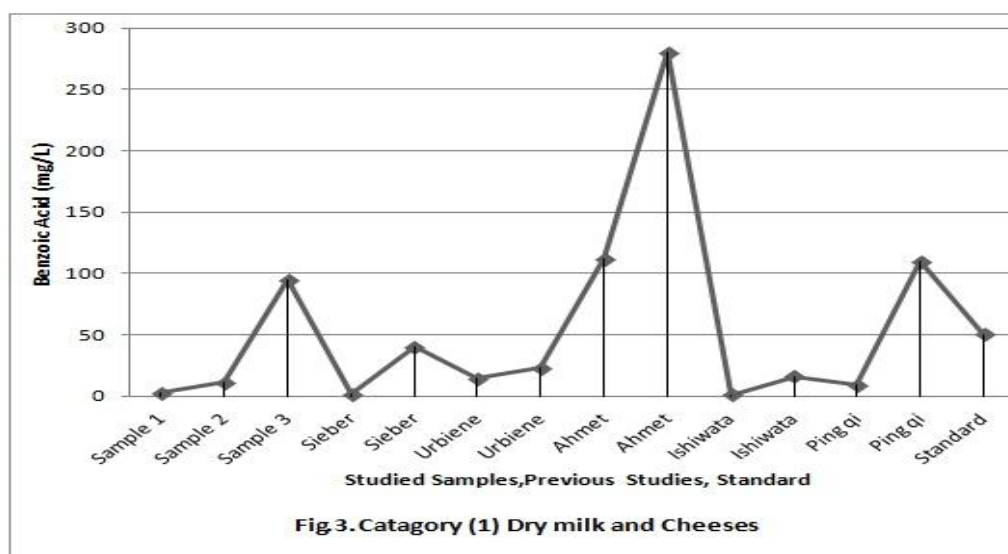
Table 2 : Concentrations (mg/L) of category (1), Benzoic acid in Dry milk and cheese samples

Sample No.	Mean concentration (mg/L)±SD	Mean concentration range of Benzoic acid in the group
1	2.749 ± 0.318	
2	11.207 ± 2.398	2.749 - 94.943
3	94.943 ± 2.826	

n=3

Yemeni standard specifications No. 442 issued 2003, No. 586 issued 2003, No.470 issued 2004, No.1091 issued 2005, No. 373 issued 2008, Codex Alimentarius Commission specification No. 192 issued 1995, and European specification for food additives EU No. 95/2/EC [9-15] all these standards stated that it is not permitted to add benzoic acid to dry milk. Only Yemeni standard specification No. 860 issued 2004 [16] states that the maximum allowable ascorbic acid or benzoic acid or their salts in milk is 50 mg/L (individually or collectively). The content of benzoic acid in the studied samples is due to the nature of the sample, The presence of different amounts of (hippuric acid) in samples of dry milk and cheese, and the culture (thermophilic) in milk and (mesophilic) in cheese, convert (hippuric acid) in milk and cheese to benzoic acid, as well as possibly other factors, such as feeding habits and

conditions of storage and pollution. This explanation is supported by a number of studies which explain the natural presence of benzoic acid in milk such as the European Commission which stated that, most of the benzoic acid is derived from (hydroxycinnamic acids) as components of consumer feed ingested by animals [17]. Wibbertmann and others in 2005 [18] said that, benzoic acid can be seen naturally in many animals and plants, so it is naturally found in many foods, including milk products and did not exceed the average values of 40 mg/L of food. and the maximum benzoic acid added to food for the purpose of conservation were 1000 mg/L. Campell in 2010 [19] found that benzoic acid occurs naturally in milk at low concentration, a few milligrams per kilogram in fermented dairy products, a high concentration lies in the range 20-50 mg/L. During the process of fermentation lactic acid bacteria turning (hippuric acid) which occurs naturally in milk to benzoic acid. When compared our results with previous studies, we found that, Sieber and others in 1995 [20] found that the amount of benzoic acid in samples of Swiss cheese lies in the range 12- 40 mg/L, Urbienė and Leskauskaitė in 2006 [21] found that the amount of benzoic acid in samples of fermented milk in Lithuania lies in the range 14-23 mg/L, Ahmet and others in 2008 [22] found that the amount of sodium benzoate in Turkish samples of dry milk and cheese located in the range 112.1- 280.4 mg/L, Smith in 2008 [23] found that benzoic acid occurs naturally in milk at low levels, also found that the amount of benzoic acid in samples of pasteurized milk and milk brewed located in ranges 4- 6 and 30- 60 mg/L respectively, and that the amount of benzoic acid in samples of white cheese lies in the range 9 - 18 mg/L, Ishiwata and others in 1999 [24] found that the amount of benzoic acid in 399 Japanese sample of milk and cheese, located in range 1.0 -16 mg/L, Ping qi and others in 2009 [25] found that the amount of sodium benzoate in 31 sample of Chinese milk powder located in the range 19-110 mg/L Fig.3.



Category (2) consist of four food samples for infants and young children manufactures from grains, the mean concentrations of benzoic acid in these samples ranges from 84.753 mg/L to 180.066 mg/L, Table.3.

Table 3 : Concentrations (mg/L) of category (2) Benzoic acid in Food for infants and young children mainly manufactures of grain samples

Sample No.	Mean concentration (mg/L)±SD	Mean concentration range of Benzoic acid in the group
4	84.753±0.591	84.753-180.066
5	91.245±5.818	
6	114.842±5.546	
7	180.066±3.126	
n=3		

Identity card of samples of this group, although not clarify the presence of benzoic acid , no, the presence of milk and grain samples in this group are likely to be one of the reasons for the possibility of a natural concentration of benzoic acid in this group, and this is what was stated by Wibbertmann and others in 2005 [18] where they seen that benzoic acid occurs naturally in many plants and animals .Yemeni standard No.1028 issued in 2004 and No. 22 issued in 2009. Gulf Standard No. 677 issued in 2007, [26-28] which specializes in infant and children food , and the Codex Alimentarius Commission specification No. 73 ,No. 74 issued in 1981,[29-30] and Codex Alimentarius Commission specification No. 192 issued 1995, [14] for canned foods for children mainly manufactures from grain, all these standards does not allow adding benzoic acid in canned baby food manufacturer mainly of grain. There is no possibility to compare the contents of benzoic acid in samples of this group with standards since all these standard did not allow the addition of benzoic acid as preservative for this category of children food, so it is a violation of the specification

Category (3) consist of fourteen samples of cake , the mean concentrations of benzoic acid in these samples ranges from 1.778 mg/L to 849.295 mg/L . It can be divided into three subgroups ,first one which consist of samples 8 to12 with mean concentration range of benzoic acid from 1.778 mg/L to 5.492 mg/L , the second subgroup which consist of samples 13 to 17 with mean concentration of benzoic acid from 27.220 mg/L to 94.670 mg/L , and the third subgroup which consist of samples 18 to 21 with mean concentration of benzoic acid from 108.107 mg/L to 849.295 mg/L,Table 4.

Table 4 : Concentrations (mg/L) of category (3) Benzoic acid in cakes samples

Samples No.	Mean concentration (mg/L)±SD	Mean concentration range of Benzoic acid in the sub group	Mean concentration range of Benzoic acid in the group
8	1.778±0.707	1.778 – 5.492	1.778 - 849.295
9	2.206±0.933		
10	2.587±0.429		
11	4.689±0.411		
12	5.492±0.076		
13	27.220±1.924	27.220 - 94.670	
14	46.417±4.677		
15	66.151±2.185		
16	69.103±5.928		
17	94.670±5.129		
18	108.107±8.484		

19	298.949±5.516	108.107- 849.295
20	632.029±4.065	
21	849.295±5.609	
n=3		

Yemeni Standard No. 586 issued in 2003 [10] and No. 104 issued in 2006 [31] which specializes in all types of cakes not include mixtures of different kinds of cakes or cakes prepared for special purposes, stated the use of sorbic acid up to 0.4% of the weight of flour and did not mention the use of sodium benzoate or benzoic acid. Food Additives of the European Union No. 95/2/EC, [15] did not allow adding benzoic acid in the cake, and it allows adding sorbic acid, and the maximum allowable is 1000 mg/L. As well the specification for the Codex Alimentarius Commission specification No.192 issued in 1995 on food additives [14] has stated that the maximum allowable benzoic acid is 1000 mg/L for products filling, fruit pastry and bakeries goods products and did not mention a cake within the food in this specification. Therefore according to the specifications mentioned, all cake samples studied is a violation of the specification. The presence of different amounts of benzoic acid in the studied samples of cake can be attributed to the quality of materials used in its manufacture.

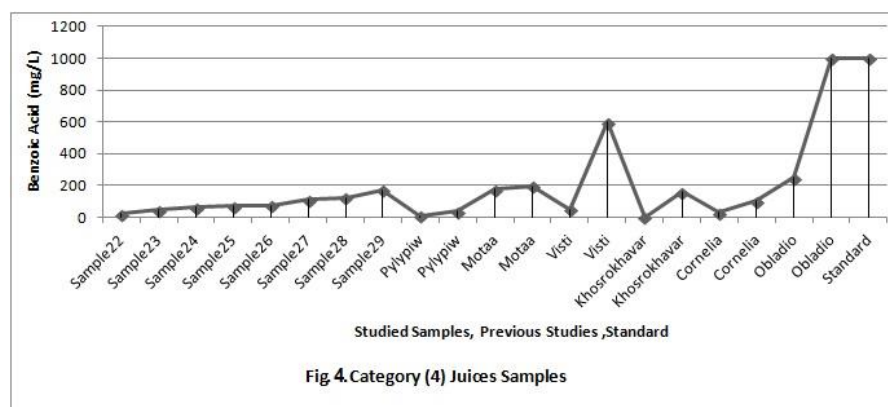
Category (4) consist of eight samples of juices, the mean concentrations of benzoic acid in these samples ranges from 22.942 mg/L to 175.940 mg/L, (Table 5).

Table 5 : Concentrations (mg/L) of category (4) Benzoic acid in Juices samples

Samples No.	Mean concentration (mg/L)±SD	Mean concentration range of Benzoic acid in the group
22	22.942±3.889	22.942 - 175.940
23	46.053±5.974	
24	66.621±0.818	
25	71.001±6.036	
26	77.508±5.230	
27	115.060±2.218	
28	125.003±2.057	
29	175.940±0.617	
n=3		

All above concentration of benzoic acid in samples of juices and drinks were within the permitted national and international limits. Yemeni standard No.1859 issued in 2007, which specializes in drinks and juices [32], the Codex Alimentarius Commission specification No.192 issued in 1995 on food Additives [14], these standard reported that the maximum allowable benzoic acid in juices and beverages is 1000 mg/L. When comparing the content of benzoic acid in samples of this group of juices and drinks with the benzoic acid values of previous studies of samples of juices and beverages, we find that, Pylypiw and Grether in 2000 in the United States [33], found that the concentration of sodium benzoate in different kinds of juices fall in the range 12-38 mg/L. Mota and others in 2003 [34] found that the concentration of benzoic acid in different samples of juices in Portuguese lies in the range 179-198 mg/L. Visti and others in 2003 in Finland [35] found that the concentration of benzoic acid in various kinds of juices fall in the range 52-602 mg/L. Oladipo and others in 2009 in Nigeria [36] found that the concentration of benzoic acid in various kinds of canned fruit juices fall in the range 250 -1000 mg/L. Cornelia and Elena in 2009 [37] found that the concentration of benzoic acid in

different samples of the juice in the city of Bucharest - Romania is located in the range 30.29 - 107.75 mg/L. Khosrokhavar and others in 2010 in Iran [38], found that the concentration of sodium benzoate in different types of fruit drinks lies in the range 3.9 - 164.0 mg/L , Fig. 4.

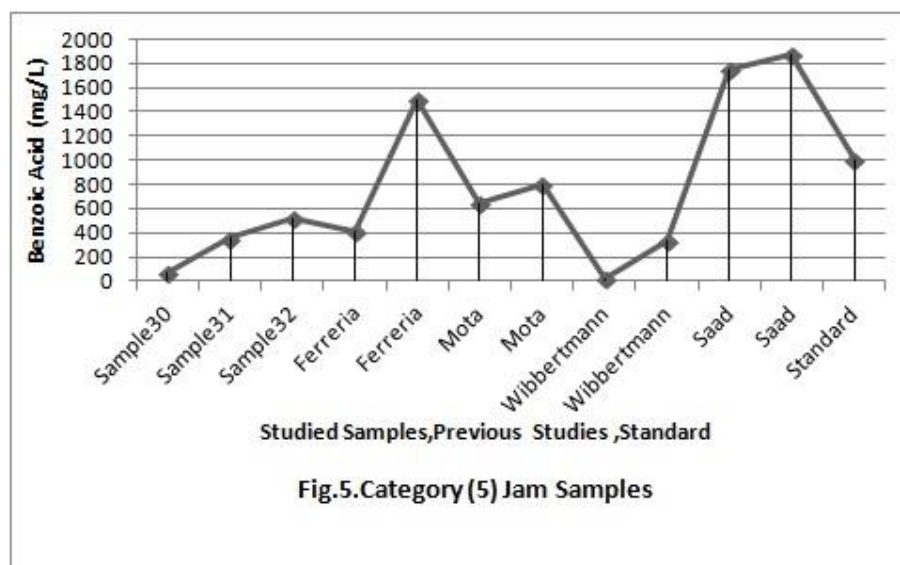


Category (5) consist of three samples of jam , the mean concentrations of benzoic acid in these samples ranges from 74.016 mg/L to 519.688 mg/L Table 6.

Table 6 : Concentrations (mg/L) of category (5) Benzoic acid in Jam samples

Samples No.	Mean concentration (mg/L) ± SD	Mean concentration range of Benzoic acid in the group
30	74.016±0.252	74.016 - 519.688
31	357.019±6.092	
32	519.688±29.368	
n=3		

When comparing the content of benzoic acid in samples of this group of jam with some previous studies , we find that, Ferreira and others in 2000 [39] found that the concentration of benzoic acid in different samples of Portuguese jam lies in the range 413.9 - 1501.0 mg/L ,Motaa and others in 2003 [34] found that the concentration of benzoic acid in different samples of Portuguese jam lies in the range 646-798 mg/L, Wibbertmann and others in 2005 [18] found that the concentration of benzoic acid in different samples of British jam lies in the range 20-333 mg/L and Saad and others in 2005 [40] found that the concentration of benzoic acid in different samples of Malaysian jam lies in the range 1756.3-1872.1 mg/L. When comparing the concentration of benzoic acid in samples of jam of this group with the amount of benzoic acid allowed in jam according to the local, regional and international standard such as Yemeni standard No. 586 issued 2003 [10], Codex Alimentarius Commission specification No.192 issued in1995 [14] and Codex Alimentarius Commission specification No.296 issued in 2009[41] as well as Scientific Committee on Consumer Products (SCCP/0891/05) [42] which permits 1000 mg/L of benzoic acid. we find that samples of jam of this group contain concentrations of benzoic acid within the limits of the above mentioned standards. Fig.5.



Conclusion

The method was found to be suitable for the routine determination of benzoic acid in food samples with acceptable parameters such as limit of detection, limit of quantitation. The levels of the benzoic acid in dry milk and cheese samples were of natural occurrences, small amount, and to some extent similar to those reported in the previous studies. Only Yemeni specifications No. 860 issued 2004 allow addition of benzoic acid as preservatives to this food category. The levels of the benzoic acid in samples for infants and young children manufactures from grains, and samples of cake were of natural occurrences, and is a violation of all mentioned specification. The level of benzoic acid in samples of juices and drinks as well as jam were within the permitted national and international limits, and to some extent similar to those reported in the previous studies.

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