U.P.B. Sci. Bull., Series B, Vol. 71, Iss. 4, 2009 ISSN 1454-2331

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF BENZOIC ACID IN BEVERAGES

Cornelia Petronela ENE[1], Elena DIACU[2]

Obiectivul prezentei lucrări îl constituie dezvoltarea unei metode HPLC simple şi rapide pentru identificarea şi determinarea conservantului antimicraobial-acid benzoic din diferite matrici alimentare. Metoda constă în injectarea directă a probei, după înlăturarea prealabilă prin filtrare a materialului matricei. Determinarea conservantului s-a realizat cu ajutorul unui sistem HPLC echipat cu un detector UV. Parametrii de lucru au fost optimizaţi şi metoda a fost validată prin stabilirea parametrilor de performanţa analitică. Rezultatele obţinute pentru determinarea acidului benzoic din probe reale de băuturi fabricate de diferite companii romaneşti au arătat valori ale conţinutului de conservant cuprinse intre 0 si 110 mg/L, încadrându-se în limitele legale admise. Metoda HPLC prezentată s-a dovedit corespunzătoare pentru procesul de monitorizare al prezentei legale a acestor conservanţi în băuturi.

The objective of the present contribution was the development of a fast and simple HPLC method for the identification and determination of the antimicrobial preservative benzoic acid in different food matrix. The method consists in the direct injection of the sample, after a prior filtration to remove the particulate matter. The determination of the preservative was performed employing a HPLC system equipped with UV diode array detection. The HPLC working parameters were optimized and the method was validated by establishing the analytical criteria of performance. The results obtained for the determination of benzoic acid on real soft drink samples from different Romanian companies showed values of preservative content between 0 and 110 mg/L, meeting the national regulated limit. The presented HPLC method proved that this method is suitable for the monitoring process of the legal presence of these preservative in beverages.

Keywords: preservatives, benzoic acid, HPLC, soft drinks

1. Introduction

Benzoic acid- benzene carboxylic acid and its salts-benzoates are known as E-numbers E210, E211, E212, and E213 and they are used as food preservatives against yeasts, fungi and common bacteria in acidic products [1-4].

---

[1] PhD Student, Department of Analytical Chemistry, Faculty of Applied Chemistry and Materials Science, University POLITEHNICA of Bucharest, Romania, e-mail: corneliaene2007@yahoo.com

[2] Professor, Department of Analytical Chemistry, Faculty of Applied Chemistry and Materials Science, University POLITEHNICA of Bucharest, Romania, e-mail: e_diacu@chim.upb.ro

The preservation mechanism starts with the absorption of benzoic acid into the cell. If the intracellular pH changes to 5 or lower, the anaerobic fermentation of glucose through phosphofructokinase is decreased by 95%. Therefore, the efficiency of benzoic acid and benzoates is dependent on the pH of the food [5]. In food products with a pH above 5 (slightly acidic or neutral) they are ineffective and not very effective against some bacteria. Acidic food and beverage like fruit juice (citric acid), sparkling drinks (carbon dioxide), soft drinks (phosphoric acid), pickles (vinegar) or other acidified food are well preserved with benzoic acid and benzoates, especially, cloudberries containing so much benzoic acid that they can be stored for long periods of time without bacterial or fungal spoilage.

Benzoic acid can either be added directly or created from reactions as its sodium, potassium, or calcium salt. Benzoic acid is slightly irritant to the skin and irritant to the eye, whereas sodium benzoate is not irritant to the skin and is only a slight eye irritant. High concentrations of benzoic acid have as result a sour taste of beverages and other food matrices, which limits its content of application [1,2]. Due to these reasons and also due to a better solubility, the benzoates are often preferred [1-3].

Benzoic acid (C_6H_5 –COOH) is the simplest aromatic carboxylic acid, being slightly soluble in water and also very soluble in most organic solvents.

First obtained by sublimation from gum benzoin (described by Nostradamus in 1556), benzoic acid is present naturally either free or bound as benzoic acid esters and can be found in many natural products such as berries (cranberries, prunes, cloves) and in animals as the end product of phenylalanine metabolism [2, 4].

Benzoic acid and its salts are permitted food additives by international laws in processing in restrictive amounts, but their content must be declared and must not exceed the established limits by legislation. The maximum accepted level of benzoates in beverages stipulated by national and European legislation is of 150 ppm. [6-7].

Concern has been expressed that benzoic acid and its salts may react with ascorbic acid (vitamin C) in some soft drinks, forming small quantities of benzene [8]. For humans, the WHO's International Programme on Chemical Safety (IPCS) suggests that a provisional tolerable intake would be 5 mg/kg body weight per day [9].

Therefore, efficient analytical techniques able to detect and to analyze the content of this preservative are required. Several analytical methods have been developed: spectrophotometric methods [10-11], chromatographic methods [12-16] and capillary electrophoresis methods [17]. The spectrophotometric methods in the analysis of food preservative present as major inconvenient the interferences and almost always an additional extractive step is required. 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 present work deals with the development and characterization of a rapid and simple HPLC method for determination of benzoic acid from soft drinks without an extensive sample pre-treatment. The proposed method has excellent performance characteristics, with a real potential to become a powerful tool for soft drink safety.

2. Experimental

Chemicals

Benzoic acid was obtained from Merck (Darmstadt, Germany), acetonitrile Chromasolv was from SIGMA-Aldrich Co. Ltd. (Dorset, UK) and ammonium acetate was from Merck (Darmstadt Germany). All aqueous solutions were prepared using bidistilled water.

Stock standard solution (1000 mg/L) was prepared by weighing benzoic acid and it is preserved at 2 – 8 ° C temperature. This solution is discarded after 6 months. Working calibration standards were prepared by measuring the calculated volumes of stock solution and diluting after the equilibration to room temperature.

The chromatographic separation was achieved using a mobile phases which consists from two components, denoted A and B. The component A (organic constituent) is acetonitrile and the component B (aqueous constituent) consists from ammonium acetate solution. (0.4 g dissolved in 1000 mL bidistilled water, and adjusted to the pH at 4.2 with NaOH). In order to allow a suitable mixture of the two constituents the gradient elution system was A:B = 40:60.

Apparata

The measurements of separation and determination of benzoic acid were performed using an HPLC system *Agilent Series 1100* equipped with diode array detection (DAD) and a column *Luna* 5 µm C_{18} 150 x 4.60 mm.

The pH measurements were performed with a Mettler Toledo pH-meter, equipped with a combined glass electrode. The solution desonication was performed with a sonication bath.

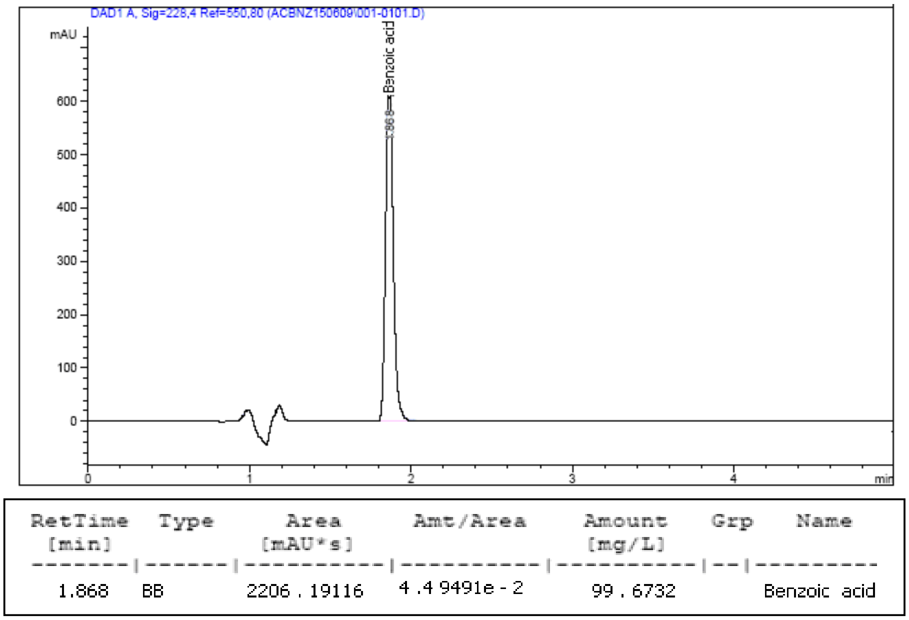
The optimal wavelength for detection was set at 228 nm, being selected from UV-Vis absorption spectra of benzoic acid.

Sampling

Different soft drink samples were filtered in vacuum on a microfiltration membrane (pore size of 0.45 µm) and were placed on an ultrasonic bath for 15 minutes in order to degas.

3. Results and Discussions

The calibration curve for benzoic acid was plotted on the basis of peak areas of chromatograms obtained for various concentrations of working standard solutions, prepared from the stock solutions.



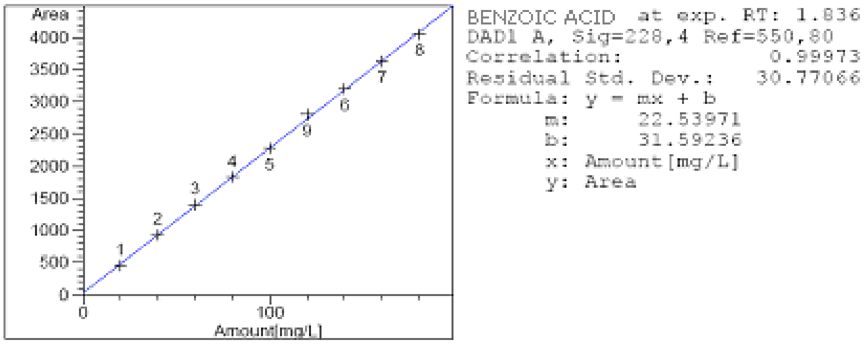
```
RetTime   Type      Area       Amt/Area      Amount    Grp    Name
 [min]            [mAU*s]                    [mg/L]
-------|------|----------|----------|----------|--|---------
 1.868    BB     2206.19116   4.49491e-2     99.6732          Benzoic acid
```

Fig. 1. Chromatogram of 100 mg/L benzoic acid standard solution

The optimum flow-rate of eluent was established at 1.0 mL/min and it was kept to the same value for all determinations. In order to obtain acceptable peaks from the point of view of symmetry, an injection volume of 5 µL has been chosen.

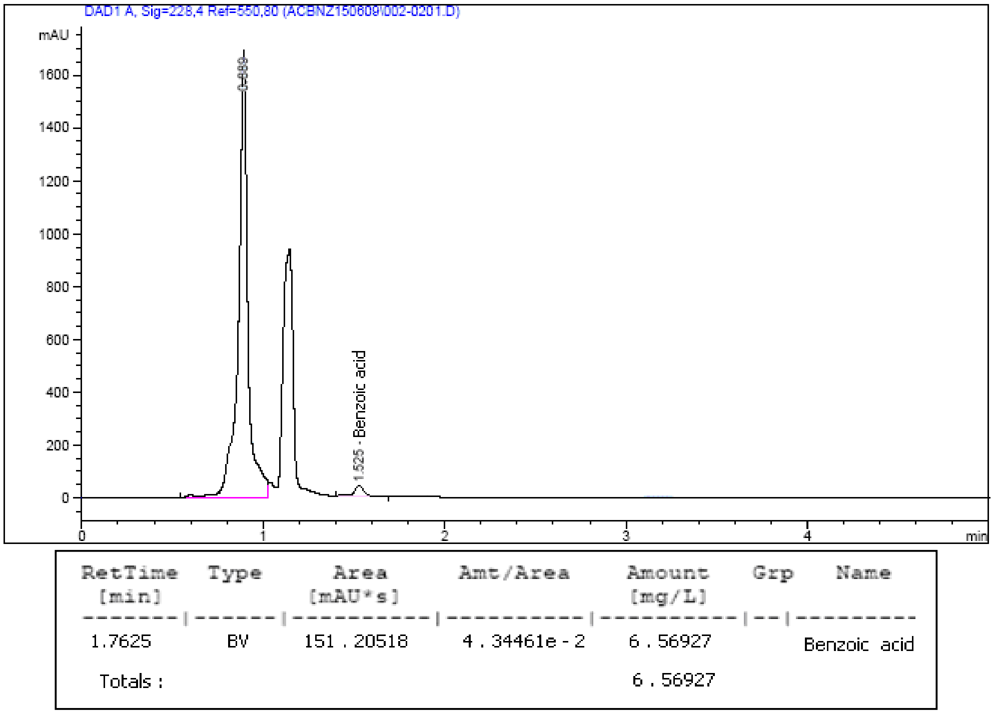
Before the batch injections of the standards, a washing mobile phase (70%water: 30% methanol) was prepared, degassed in an ultrasonic bath and injected through the chromatographic column. From the characteristic HPLC chromatogram of benzoic acid (illustrated in Fig. 1) it can be observed a good resolution of the chromatographic peak. The retention time is 1.7 ± 0.2 min.

The calibration curve was obtained by fortification of a real sample (lemon juice) and it is presented in Fig.2. A very good linearity for benzoic acid in the range of 20-180 mg/L concentrations is obtained, with an excellent regression factor (0.99973).



```
BENZOIC ACID  at exp. RT: 1.836
DAD1 A, Sig=228,4 Ref=550,80
Correlation:              0.99973
Residual Std. Dev.:     30.77066
Formula: y = mx + b
      m:      22.53971
      b:      31.59236
      x: Amount[mg/L]
      y: Area
```

Fig. 2. Calibration curve for benzoic acid



```
RetTime  Type     Area      Amt/Area     Amount    Grp   Name
 [min]          [mAU*s]                  [mg/L]
-------|------|----------|----------|----------|--|---------
 1.7625   BV   151.20518  4.34461e-2   6.56927      Benzoic acid

 Totals :                              6.56927
```

Fig. 3. Typical chromatograms for a sample juice containing benzoic acid

Under the optimized experimental conditions, the HPLC method was then applied to study the benzoic acid content in real samples. The preservative was identified on the chromatograms of the samples by comparison of their retention time with that of standard synthetic solutions.

From Romanian market were purchased randomly forty liquid soft drinks samples of different kind: tomato, orange, peach and apricot juices. A positive in benzoic acid gaseous orange soft drink sample is given in chromatogram of Fig.3.

The results for determination of benzoic acid in fifteen soft drink samples are exhibited in Table 1, where every value represents the mean of two determinations. The expanded measurement uncertainty was $U_{BenzoicAcid}$ = 2.26 mg/L, calculated in agreement with [17,18].

Table 1

Benzoic acid content in different samples

No.	SAMPLE TYPE	BENZOIC ACID (mg/L)
1.	TOMATO SAUCE 1	0
2.	TOMATO SAUCE 2	0
3.	TOMATO SAUCE 3	0
4.	TOMATO SAUCE 4	0
5.	ORANGE JUICE 1	91.14 ± 2.26
6.	ORANGE JUICE 2	96.09 ± 2.26
7.	ORANGE JUICE 3	107.75 ± 2.26
8.	PEACH JUICE 1	60.44 ± 2.26
9.	PEACH JUICE 2	63.25 ± 2.26
10.	PEACH JUICE 3	30.29 ± 2.26
11.	LEMON JUICE 1	67.73 ± 2.26
12.	LEMON JUICE 2	63.25 ± 2.26
13.	LEMON JUICE 3	71.84 ± 2.26
14.	APRICOT JUICE 1	44.46 ± 2.26
15.	APRICOT JUICE 2	43.93 ± 2.26

Analytical performance parameters

Detection limit (LOD) and quantification limit (LOQ) of the applied method have been established taking into account the characteristics of HPLC techniques and the specific literature for analytical performances of analytical methods [17-18]. The found LOD is 7 mg/L and LOQ is 21 mg/L. In order to obtain recovery percentage, fortified soft drink samples were analyzed and this parameter value was ranged between 85- 93 %. More details about the validation parameters of this method will be the subject of a further paper.

4. Conclusions

This contribution proposes a simple, selective and rather fast HPLC method for the determination of benzoic acid in soft drinks commercial samples. Practically the HPLC analysis is complete within 5 min, after the sample filtration and sonication. Under the optimized values of experimental parameters, the method is adequate for the determination of the benzoic acid, in different beverages with good analytical performances. The method also demonstrated its applicability for the study of a large number of soft drinks samples with complex matrices. The obtained results showed that the benzoic acid concentration varied between different kinds of beverages samples, with levels lower than the maximum values established by national and international legislation. All these data regarding the concentrations of benzoic acid in different beverages will be included in a data base that can help the consumers in the selection of more adequate products for their health.

REFERENCES

[1] *P. M. Davdison*, *John N. Sofos, A.L. Branen*, Antimicrobials in food, Third edition, Taylos & Francis, 2005

[2] *H. M. Pylypiw, M. T. Grether*, *Journal of chromatography A,* , **883**,1-2, 299-304, 2000

[3] *L. P. Loginova1, A.U. Kulikov, E. Y. Yakovleva1, A. P. Boichenko1*, Chromatographia, **67**, 7-8, 615-620, 2008

[4] *A D Warth*, *Appl Environ Microbiol*, **1**, PMID 1785916, 1991

[5] *I. Pastrorova, C.G. de Koster, J.J. Boom*, Phytochem. Anal. **8**: 63–73. GSFA Online Food Additive Group Details: Benzoates 2006

[6] EUROPEAN PARLIAMENT AND COUNCIL DIRECTIVE No **95/2/EC** of 20 February 1995 on food additives other than colours and sweeteners (Consleg - versions do not contain the latest changes in a law), 1995

[7] *M. C. Bennett, D. R. Petrus*, Journal of Food Science, **42,** 5, 1220 – 1221, 2006

[8] Concise International Chemical Assessment Document **26**: BENZOIC ACID AND SODIUM BENZOATE, 2000. http://www.inchem.org/documents

[9] *I. A. Badea , L. Vladescu*, Analele Universitatii din Bucuresti – *Chimie*, **XIV**, I – II, 401 – 406

[10] *M.P. Aquilar – Caballos, A. Gomez – Hens and D. Perez - Bendito*, Analyst, **124**, 1079- 1084, 1999

[11] *C. Galletti and P. Bocchini,* Journal of Chromatography A, **729**, 1-2, 5, 393-398, 1996

[12] *T. Ittipon and S. Ranee, Journal of food composition and analysis,* **20**, 3 – 4, 220-225, 2007

[13] *M. S. Ali, S. Rafiuddin , M. I. Murin , M. Ghori and A. R. Khatri*, Journal of AOAC Internatonal, **91**, 5, 2008

[14] *M. Gonzalez, M. Gallego and M. Valcarcel*, Journal of Chromathography A, **823**, 1-2, 321 – 329, 1998

[15] *Y. A. Onishi, M. Yomota, N. Sugimoto, H. Kubota, K. Tanamoto*, Journal of Chromatography A , **1040**, 209-214, 2004

[16] *K. L. Kuo and Y. Z. Hsieh*, Journal of Chromatography A, **768**, 2, 334 – 341, 1997

[17] ISO/IEC 17025:1999. General Requirements for the Competence of Calibration and Testing Laboratories. ISO, Geneva, 1999, EURACHEM, Quantifying Uncertainty in Analytical Measurement. Laboratory of the Government Chemist, London 1995. ISBN 0- 948926-08-2

[18] ISO GUM, Guide to the expression of uncertainty in measurement, 2nd edition, 1995, with Supplement 1, Evaluation of measurement data, JCGM 101: 2008, Organization for Standardization, Geneva, Switzerland.