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Research Article

Chromatographic Determination of Denatonium Benzoate in Colognes

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ABSTRACT

Denatonium benzoate, commercially known as Bitrex, is an ionic compound containing a quaternary ammonium cation (denatonium) and negatively charged benzoate. It is commonly used as a bittering agent in various alcohol-based products to deter ingestion. The national regulations regarding the production and domestic-foreign trade of ethyl alcohol and methanol states that ethyl alcohols for domestic use should contain 0.8 g denatonium benzoate per 100 L of absolute alcohol. This leads to the necessity of quantitative analysis of denatonium benzoate in alcoholic products. Cologne samples of different brands were analyzed using reversed-phase high performance liquid chromatography (RP-HPLC) coupled with UV/vis detector. Isocratic elution was performed on an ODS column with a mobile phase containing 75% MeOH:25% H_2O. Since the proposed method does not require any pre-treatment step, the procedure is simple and rapid. The limit of quantitation was found to be 0.17 mg/L. The linearity of the method was tested in the range 0.26 – 15.75 mg/L with a limit of detection of 0.050 mg/L (signal-to-noise ratio of 3).

Keywords: *Denatonium benzoate, Bitrex, Denaturant, Alcohol-based hand sanitizers, Cologne, HPLC*

Kolonyalarda Denatonyum Benzoatın Kromatografik Tespiti

ÖZ

Ticari olarak Bitrex adıyla bilinen denatonyum benzoat, bir kuaterner amonyum katyonu (denatonyum) ve negatif yüklü benzoat içeren iyonik bir bileşiktir. Genellikle çeşitli alkol bazlı ürünlerde kazara yutmayı önlemek amacıyla kullanılır. Etil alkol ve metanol üretimi ve iç-dış ticaretine ilişkin ulusal düzenlemeler, evsel kullanım için etil alkollerin 100 L mutlak alkol başına 0,8 g denatonyum benzoat içermesi gerektiğini belirtmektedir. Bu durum, alkollü ürünlerdeki denatonyum benzoatın nicel analizinin gerekliliğini ortaya koymaktadır. Farklı markalara ait kolonya numuneleri UV/vis dedektörü ile ters-faz yüksek performanslı sıvı kromatografisi (RP-HPLC) kullanılarak analiz edilmiştir. %75 MeOH:%25 H_2O içeren mobil faz ile ODS kolonda izokratik elüsyon gerçekleştirilmiştir. Önerilen yöntem herhangi bir ön işlem adımı gerektirmediğinden, işlem basit ve hızlıdır. Alt tayin sınırı 0,17 mg/L olarak bulunmuştur. Yöntemin doğrusallığı 0,26 – 15,75 mg/L aralığında test edilmiş ve gözlenebilme sınırı 0,050 mg/L olarak bulunmuştur (sinyal-gürültü oranı 3).

Anahtar Kelimeler: *Denatonyum benzoat, Bitrex, Denatüran, Alkol bazlı el dezenfektanı, Kolonya, HPLC*

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I. INTRODUCTION

Ethanol is an organic solvent widely used for scientific research and industrial purposes. It is also used in the production of adhesives, inks, chemicals, plastics, paints, thinners, nail polish remover, cleaning agents and personal care products [1].

Alcohol can be rendered unfit for human consumption by adding a chemical marker that has a very bad taste and/or odor. The process of rendering alcohol unfit for human consumption is called (complete or partial) denaturation [2].

Since alcohol is used in many different industries, from personal care to household cleaning, it is key that the intrinsic properties of alcohol are not altered by the addition of additives; therefore, additives should be selected so that they do not affect the chemical composition [3]. Methanol, benzene, pyridine, castor oil, gasoline, isopropanol, acetone, tertiary butanol, denatonium benzoate are used as additives (i.e. denaturants) in denaturation [4]. Denatonium benzoate (DB) is added to alcohol to make it taste bitter. It can also be found in different types of products:

i. Household Cleaning Products such as dishwashing liquids, and window cleaners. Its bitter taste helps prevent accidental ingestion, especially by children and pets.
ii. Automotive Products such as automotive fluids, such as antifreeze, windshield washer fluid, and radiator coolant. These products often contain denatonium benzoate to discourage ingestion due to their potentially toxic nature.
iii. Garden and Pest Control Products such as herbicides, insecticides, and rodenticides, may contain denatonium benzoate. It serves as a deterrent to prevent accidental ingestion or unauthorized use.
iv. Personal Care Products such as nail polish removers, nail-biting deterrents, face mist, and colognes. It is also added to discourage thumb-sucking habits.
v. Paints and Solvents such as inks, varnishes, and solvents. They may incorporate denatonium benzoate to prevent accidental ingestion.
It is important to note that the presence of denatonium benzoate in a product may vary depending on the brand, formulation, and country-specific regulations.

In studies conducted as a result of the use of products containing DB, the effect on the human body has also been examined. It is reported that toxicity data were found to be low [5]. DB has also been reported to affect gastrointestinal functions. In humans, intragastric administration of DB has been reported to impair relaxation of the proximal stomach after infusion of a liquid food and increase satiety during oral food tolerance testing. In studies in men and women, they were given DB before a meal and asked to rinse their mouths with tap water. DB was found to increase satiety compared to normal. In addition, based on the information that women perceive bitter linguistic stimuli more intensely than men, it was observed that women stayed full longer than men [6]. Asthma and respiratory diseases occurred due to high exposure to products such as cologne and disinfectants used during the COVID-19 (the coronavirus disease in 2019) [7].

COVID-19 has become a pandemic, leading to a massive increase in the use of alcohol-based hand sanitizers worldwide. Governments and public health agencies around the world have advocated for the importance of hand hygiene as one of the preventive measures against the COVID-19 pandemic. The ethanol used to produce sanitizers is of industrial quality and is usually denatured to prevent human consumption.

According to the regulations and principles regarding the production and domestic-foreign trade of ethanol and methanol published in the Legal Gazette dated 30/10/2011 and numbered 28100, denaturation of ethanol is carried out by adding 0.8 grams of denatonium benzoate to 100 liters of absolute alcohol [8]. Both companies adding denaturant agents to the products and government agencies monitoring regulatory compliance need an easy analytical technique for the reliable and rapid analysis of products containing denatured alcohol [9].

DB can be analyzed by capillary electrophoresis, liquid chromatography, colorimetry, ion selective potentiometry, UV-Vis spectrophotometry and Raman spectroscopy. Older analysis methods such as colorimetry and thin layer chromatography are time-consuming techniques with low sensitivity and precision. Fast, sensitive, selective and accurate methods are needed to determine whether denaturants have been added at the values allowed by regulations. High performance liquid chromatography (HPLC) has been validated as a qualified method for this purpose [10].

The reasons for denaturing alcohol include protecting the health of individuals, and denaturation is particularly important to prevent people in treatment for alcoholism from secretly drinking alcohol-based industrial products. In this study, the denatonium benzoate content of colognes sold in markets were examined and evaluated their compliance with the legislation. The proposed chromatographic method with a lower limit of detection has been confirmed to be qualified for the analysis of denatonium benzoate as HPLC methods offer significant utility by delivering the necessary separation to attain optimal sensitivity for detecting analytes present at low concentrations.

II. MATERIALS and METHODS

A. REAGENTS and SOLUTIONS

HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Denatonium benzoate (99.5%) (Figure 1) was obtained from The Lakshmiji Organics (Sitapur, India). Samples of five different brands of cologne were purchased from local markets in Turkey.

Stock solution of denatonium benzoate (0.0105 g/100 mL) was prepared using methanol. Working solutions were prepared by diluting the stock solution with the mobile phase at ratios of 1:400, 1:200, 1:130, 1:100, 1:40, 1:20, 1:13, 1:10, 1:8, 1:6.5. An aliquot of 20 µL of each diluted solution was injected to HPLC in three times. Methanol and double distillled water were filtered through a 0.45 µm nylon membrane filter (Supelco, USA). Samples were diluted 1:2 with the mobile phase.

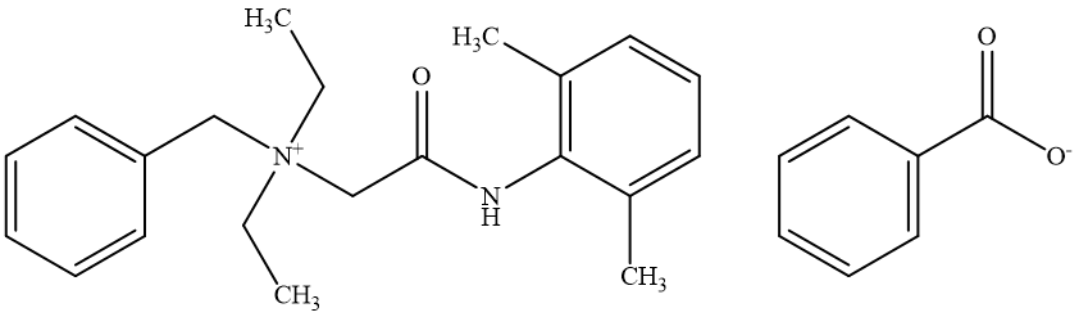


Figure 1. *The structural formula of denatonium benzoate = Bitrex*

B. INSTRUMENTATION

The high-performance liquid chromatography (HPLC) instrument consisted of Shimadzu Prominence LC-20AT pump (Shimadzu, Japan) with DGU-20A5 degasser, CTO-20A column oven, 7725i Rheodyne manual injector and SPD-20A UV/vis detector with data processor LC Solution was used. Double-distilled water was supplied from the mpMINIpure dest model device.

C. HPLC CONDITIONS

The mobile phase consisted of methanol/water (75:25) and was filtered through a 0.45 µm membrane filter and degassed for 10 min. Inertsil ODS-3 (150 × 4.6 mm, 5 µm) (GL Sciences Inc., Japan) column was used for the chromatographic separation. Isocratic elution was performed at a flow rate of 1.0 mL/min. Before injection, all solutions were filtered through a 0.45 µm membrane filter. The column temperature was set to 35 °C, and denatonium benzoate was monitored at 210 nm.

D. VALIDATION

Once the experimental conditions were optimized, the method was validated with respect to the following parameters: linearity, limit of detection (LOD and limit of quantitation (LOQ). The linearity of the method was tested by preparing a calibration curve. The calibration curve was prepared in the range of 0.2 – 15 mg/L standard DB solutions. LOD and LOQ are calculated according to equations 1-2.

$$LOD = 3 \times \frac{s}{m} \quad (1)$$

$$LOQ = 10 \times \frac{s}{m} \quad (2)$$

where, *s* is the standard deviation of 10 different peak areas recorded for the lowest standard DB solution and *m* is the slope of calibration curve. The applicability of the method was evaluated by recovery test through the analysis of samples after the addition of known amounts of standard DB solution.

III. RESULTS and DISCUSSION

At the beginning of the work, the experimental conditions applied in the literature (Table 1) were tested and it was concluded that the two important parameters affecting the separation were organic solvent content and pH. Methanol and acetonitrile were tested as a mobile phase. The effect of phosphate buffered saline (PBS) was also evaluated. The plots of retention time, relative standard deviation (RSD) of retention time, peak area, relative standard deviation (RSD) of peak area, resolution, retention factor and number of plates versus different compositions of mobile phase are presented in Figure 2. In general, the retention time increases as the organic solvent content decreases. Decreasing the amount of organic solvent in reversed-phase HPLC increases the polarity of the mobile phase, weakens hydrophobic interactions with the stationary phase, and can lead to increased retention times for denatonium benzoate molecules. Both methanol and acetonitrile can be suitable solvents for chromatographic analysis of denatonium benzoate, the choice between methanol and acetonitrile as a solvent, particularly for compounds like denatonium benzoate, depends on several factors including the nature of the compound. The solubility of DB in the chosen solvent is crucial for achieving good peak shape and resolution. DB is generally considered to be more soluble in methanol compared to acetonitrile. When the effects of acetonitrile and methanol were examined, it was observed that the retention time has lower values in the case of using methanol as an organic solvent. It was decided to use methanol providing short elution time and cost. In reversed-phase chromatography, a buffer is often used as part of the mobile phase to maintain pH stability and provide suitable ionic strength. However DB may be ionizable, and the presence of a buffer can affect its ionization state. Buffer components can compete with DB for ionization, leading to changes in retention times and peak shapes. This can result in peak broadening or splitting, which can degrade the chromatogram quality. As can be seen in Figure 2, the high RSD for retention time and peak area with PBS buffer is the result of the inability to obtain reproducible chromatograms, therefore no buffer was used. In conclusion, methanol/water ratio of 75:25 (v/v) was selected without any buffer for further separations because it provided the shortest separation time with acceptable resolution. The chromatogram obtained under optimized experimental conditions for stock DB solution (105 mg/L) is shown in Figure 3. The retention time of DB was recorded as 1.50 min. Retention factor, number of plates and resolution were taken from LC Solution data processor and calculated according to equations 3-6.

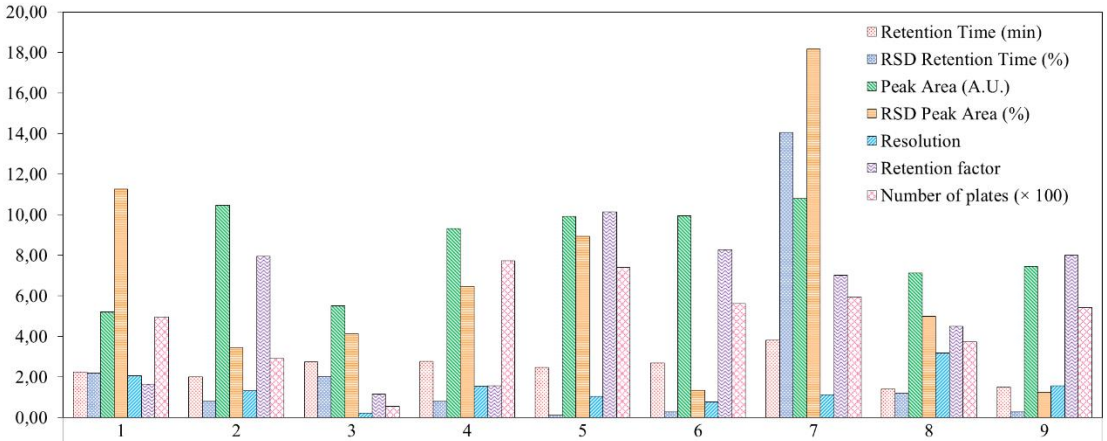
$$k = \frac{t_R - t_M}{t_M} \quad (3)$$

$$\alpha = \frac{k_1}{k_2} \quad (4)$$

$$N = 16 \left(\frac{t_R}{W}\right)^2 \quad (5)$$

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_1}{1+k_1}\right) \quad (6)$$

where, t_R is retention time, t_M is dead time, k is retention factor that is used to define the migration rate of an analyte through a column, , α is selectivity factor that is defined as the ratio of retention factor of more retained component (1) to retention factor of less retained component (2), N is number of plates, W is the width of the peak and R_s is the resolution and defined as a quantitative measure of column ability to separate components.



	1	2	3	4	5	6	7	8	9
Retention Time (min)	2,26	2,00	2,73	2,76	2,48	2,68	3,80	1,39	1,50
RSD Retention Time (%)	2,19	0,81	2,02	0,79	0,11	0,29	14,06	1,21	0,28
Peak Area (A.U.)	5,20	10,48	5,52	9,33	9,91	9,94	10,79	7,13	7,46
RSD Peak Area (%)	11,27	3,46	4,13	6,48	8,93	1,34	18,18	5,00	1,23
Resolution	2,05	1,32	0,21	1,54	1,04	0,76	1,13	3,17	1,56
Retention factor	1,64	7,94	1,16	1,57	10,14	8,27	7,01	4,5	8,01
Number of plates (× 100)	4,97	2,90	0,54	7,73	7,39	5,63	5,94	3,73	5,44

Figure 2. *The change in the retention time, relative standard deviation of retention time* ***(%)****, peak area* ***(A.U.)****, relative standard deviation of peak area* ***(%)****, resolution, retention factor and number of plates* ($\times$ $\mathbf{10^2}$) *versus different compositions of mobile phase (1: 85:15 MeCN/H_2O; 2: 75:25 MeCN/H_2O; 3: 60:40 MeCN/H_2O; 4: 50:50 MeCN/H_2O; 5: 85:15 MeCN/PBS buffer; 6: 75:25 MeCN/PBS buffer; 7: 60:40 MeCN/PBS buffer; 8: 85:15 MeOH/H_2O; 9: 75:25 MeOH/H_2O; N=3)*

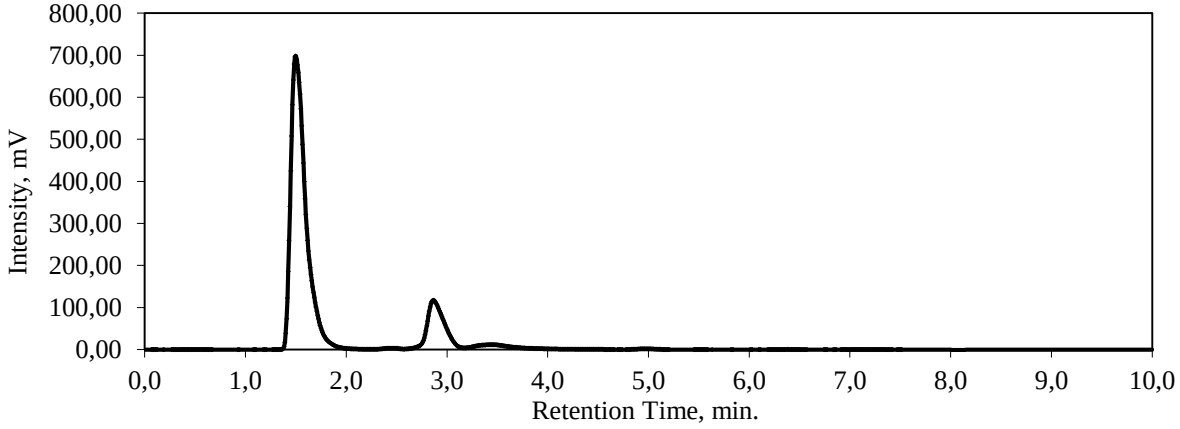


Figure 3. *The chromatogram of stock solution (105 mg/L) of denatonium benzoate (Mobile phase: MeOH/H_2O 75:25; Wavelength: 210 nm; Flow rate: 1.0 mL/min.; Temperature: 35 °C)*

After the optimum conditions were determined, calibration curves were established for the quantitative determination of denatonium benzoate. The linearity of the method was tested by preparing a calibration curve ranging from 0.26 to 15.75 mg/L standard DB solutions with a correlation coefficient of 0.9979

(Figure S1). According to the equations 1-2, LOD and LOQ were found to be 0.050, 0.17 mg/L, respectively. RSD of the peak areas was calculated as 1.25%.

Table 1. *Summary of some studies in the literature and the proposed method*

Technique	**GC-FID**	**LC-APCI-MS**	**HPLC-UV**	**HPLC-UV**	**LC-MS**	**UPLC**	**HPLC-UV**
Analyte	Isopropanol, ethanol, methyl ethyl ketone	Denatonium benzoate	Denatonium benzoate	Denatonium benzoate	Denatonium benzoate	Denatonium benzoate	Denatonium benzoate
Matrix	Denaturated ethanol	Alcoholic beverages	Cleaning products	Ethanol	Liquid detergents	FFP1 and FFP2 masks	Colognes
Column	AT-1 column (30 m × 0.53 mm)	Purospher RP-18e (125 mm × 3 mm × 5 µm); Syringe filter (13 mm/0,2 µm)	Separon TM SGX CN (150 mm × 3 mm × 10 µm)	Chromolith Performance RP-18e (100 mm × 46 mm)	TXB-C10 column (50 mm × 2.1 mm × 3.6 µm)	BEH C18 (50 mm × 2 mm × 1.7 µm)	Inertsil ODS-3 (150 × 4.6 mm, 5 µm)
Experimental Conditions	Equilibration temperature: 170 °C; Equilibration time:35 min; Needle temperature: 180 °C; Transfer line temperature: 190 °C; Pressure time: 1.0 min; Injection time: 0.04 min; Injection temperature: 200 °C; Carrier gas pressure: 130 kPa; Split ratio: 1:5; Injection volume: 10 µL	Gradient elution with solvent A (acetonitrile) and solvent B (25 mM ammonium formic buffer, pH 4.5); flow rate: 0.4 mL/min; Evaporation temperature: 480 °C; Gas flow rate: 65 L/min; Current: 4.5 µA; Capillary temperature: 220 °C; Capillary voltage: 3 V	Mobile phase: 55% CH_3OH and 45% 0.05 mol/L Na_2HPO_4 pH 6.5; Flow rate: 0.5 mL/min; Wavelength: 214 nm	Gradient elution with solvent A (aqueous solution containing 0.1 mol/L H_3PO_4) ve solvent B (acetonitrile)	Mobile phase: Water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B); Injection volume: 5 µL; Flow rate: 0.1 mL/min; Mass range: 50-1600 m/z; Nitrogen gas flow rate: 4.0 L/min; Temperature: 200 °C; Potential: 4.2 kV	Temperature: 50°C; Injection volume: 2.0 µL; Gradient elution with solvent A (water + 0.2% formic acid) and solvent B (acetonitrile + 0.2% formic acid); Flow rate: 0.6 mL/min, Capillary voltage: 2.5 kV; Source temperature: 150 °C; Desolvation temperature: 600 °C; Desolvation gas flow rate: 600 L/h (nitrogen)	Mobile phase: 75% CH_3OH and 25% water; Flow rate: 1.0 mL/min; Wavelength: 210 nm
Reference	[11]	[12]	[13]	[14]	[15]	[16]	Proposed method

To determine the amount of denatonium benzoate, cologne samples were spiked with DB at three different concentrations (2.625, 4.200, 5.250 mg/L) and calibration graphs were generated according to the standard addition method (Figure S2). As given in Table 2, DB amount in the samples ranged from 3.39 mg/L to 7.39 mg/L. The results obtained are summarized in Table 2. As can be seen in the Table 2, the concentrations found were in good agreement with the added concentration (2.100 mg/L) of DB, with recoveries between 98.7 and 104.3 %. These results show that the interferences due to other matrix components are negligible and the proposed method is suitable to achieve sufficient accuracy.

Table 2. *The results of denatonium benzoate in 5 different brands of cologne obtained by standard addition method and their % average recovery (N=3)*

Sample	**Amount of DB (standard addition)** *(mg/L)*	**Calculated DB after spike** *(mg/L)*	**Measured DB after spike** *(mg/L)*	**Recovery** *(%)*
1	7.30 ± 0.14	9.40	9.35	99.46 ± 2.30
2	6.47 ± 0.05	8.57	8.71	101.58 ± 2.51
3	7.39 ± 0.31	9.49	9.40	98.96 ± 0.23
4	3.39 ± 0.08	5.49	5.41	98.68 ± 1.60
5	7.29 ± 0.17	9.39	9.80	104.31 ± 1.00

IV. CONCLUSION

The fact that alcohol is used in many different areas from personal care to household cleaning makes it necessary to analyze the agents used in the denaturation process accurately and quickly. According to the current regulations in our country, ethyl alcohol to be used in cologne production should contain 0.8 g denatonium benzoate per 100 L.

In this study, a reversed-phase HPLC method was developed for determination of denatonium benzoate in colognes and was used to analyze commercially-available consumer products. Optimum HPLC conditions were set methanol/water of 75:25 (v/v) as mobile phase after testing different solvent compositions and ODS column as stationary phase, providing short analysis time with acceptable resolution. The limit of quantitation was found to be 0.17 mg/L which is about fifty times lower than the amount of denatonium benzoate that denaturated alcohol should contain according to the national regulations. The limit of detection of the proposed method was found to be 0.050 mg/L and is comparable to the studies in Table 1 with values of 0.007 mg/L, 0.025 mg/L, 0.45 mg/L, 0.10 mg/L and 0.015 mg/L. With the procedure, the sample preparation was straightforward without significant interference.

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