

## Validated analytical method for characterization of alcohols marketed in Tehran, Iran

Anita Chalipour<sup>1,2</sup>, Fatemeh Zamani Mazdeh<sup>2</sup>, Mohammad Reza Fazeli Farsani<sup>3</sup>, Fatemeh Salami<sup>3</sup>,  
Mohsen Amini<sup>2,4</sup>, Yalda H. Ardakani<sup>5</sup>, Mannan Hajimahmoodi<sup>1,2,3\*</sup>

1- Institute of Pharmaceutical Sciences, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

2- Food and Drug Administration, Tehran University of Medical Sciences, Tehran, Iran.

3- Drug and Food Control Department, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

4- Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

5- Pharmaceutical Department, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

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### Abstract

**Background and objective:** Various types of alcohol for several applications are available worldwide, of which, disinfection is one of the most important. In Muslim nations, consumption of alcoholic beverages is prohibited even at low quantities in accordance to halal status. Therefore, denatonium benzoate (commercially known as Bitrex) that has sharp bitterness is added to alcohol to avoid its edible usage. In this regard, at least 10 mg l<sup>-1</sup> of denatonium benzoate is added to industrial alcohol according to Iranian Ministry of Health regulation. In our study, we examined the concentration of denatonium benzoate and also purity of alcohol samples collected from capital city of Iran (Tehran).

**Materials and methods:** In total, 62 samples of alcohol were collected and analyzed by HPLC for Bitrex and alcoholmeter for purity. For HPLC, C<sub>18</sub> column (150×4.6 mm, 5 μm) as stationary phase and phosphate buffer/acetonitrile solution containing sodium lauryl sulfate (50:50 v v<sup>-1</sup>) as mobile phase with flow rate of 1.2 ml min<sup>-1</sup> were used.

**Results and conclusion:** The results revealed that some companies (41 samples out of 62) did not use denatonium benzoate in their products and used fruit essences instead to improve the taste and smell of alcohol. These results were against the force of Ministry of Health in mandatory addition of denatonium benzoate to prevent the samples' further abuse. In addition, purity of most alcohols was not compatible to the information provided by the labels. We concluded that more restriction and supervision is required to prevent adulteration.

**Keywords:** Alcohol, Bitrex, Denatonium benzoate, HPLC

\* Correspondance to: Mannan Hajimahmoodi, e-mail: [hajimah@sina.tums.ac.ir](mailto:hajimah@sina.tums.ac.ir), Tel.: +98-21-66954714, Fax: +98-21-66954714

## 1. Introduction

Alcohol is a popular liquid that produced in various forms by different applications [1]. World ethanol production is growing dramatically. For a long period of time it was just used as ingredient of alcoholic beverages, while its application has spread worldwide during the twentieth century [2]. There are two methods of ethanol production that are fermentation and synthesis. The first process includes fermentation of carbohydrates in some fruits such as grapes and dates or grains such as rye, wheat, barley and corn. In later process, ethanol is synthesized from ethylene. Fermented and synthetic ethanol are not distinguishable chemically or physically. Ethanol is used as main organic solvent for research and industrial purposes. Furthermore, it is applied for production of adhesives, toiletries, detergents, explosives, inks, chemicals, hand creams, plastics, paints, thinners, textiles, vinegar, flavors, candy and personal care products [3]. Ethanol concentrations more than 15% are toxic for drinkers and may cause depression, sensory and motor function impairment, nausea, delayed cognitive abilities, reducing blood flow to brain, unconsciousness and even death [1,4]. Therefore, it cannot be used for beverages but may be useful for industrial and medical uses [3]. In order to reduce ethanol side effects, its acceptable level has been determined and some regulations are followed by industries. With regard, the producers are obliged to control the dose of ethanol in their products [1]. As mentioned earlier, alcoholic beverages are generally prohibited in Islam and addition of alcohol to foods is not allowed even at little quantities [5]. Although, production of fermented products free of alcohol is impossible in some cases, acceptable level of ethanol (Halal status) is controversial in Islamic countries [3]. Therefore, restrictive strategies on ethanol concentration is more important in non-Muslim countries, where there is high level of unregistered alcohol that is not available in official statistics despite their large drinking consumption [6]. Smuggling products and domestic wine or vodka are examples of unregistered alcohol [7]. They are relatively

popular because have no tax that led to their lower price [8].

There are several types of ethanol including 95% ethanol, absolute ethanol (99-100%) and denatured ethanol. Denatured ethanol is produced by addition of one or more chemicals (e.g. isopropanol and methyl ethyl ketone as volatile and denatonium benzoate (DB) as non-volatile agents) to change the taste, smell or appearance of sample without changing alcohol molecule chemically to make it inappropriate for drinking [3,9,10]. DB was first discovered in Scotland as one of the known bitterest substances for human and was produced by the brand named Bitrex [11,12]. Isopropanol and methyl ethyl ketone cause unpleasant odor while Bitrex causes an extremely bitter flavor [10]. They are commonly used to avoid accidental or intended consumption of industrial alcohol [13,14]. Bitrex should be added at maximum concentration of  $3 \text{ mg l}^{-1}$  to ethanol that is significantly lower than the required levels of other denaturant agents. Therefore, a least residual amount of Bitrex would be exist after ethanol evaporation [15]. The structure of Bitrex is shown in Figure 1.

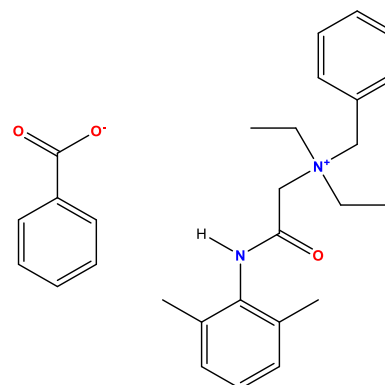


Figure 1- Chemical structure of denatonium benzoate (Bitrex)

The characteristics of alcohol may change in high doses of denaturants. Therefore, an appropriate analytical method should be applied to identify and quantify the concentration of denaturants [10]. Bitrex is analyzed by capillary electrophoresis, liquid chromatography, colorimetry,

ion-selective potentiometry, UV-VIS spectrophotometry and Raman spectroscopy [10,15-17]. The older methods of Bitrex analysis such as colorimetry and thin-layer chromatography had low adequacy and precision and were time-consuming [18]. Therefore, sensitive, selective and accurate methods are needed to determine whether the denaturants are added in the allowed ranges [19]. It has been approved that HPLC-based analysis is a qualified method for this purpose [16]. Although, there is low concern about overconsumption of alcoholic drinks in Iran, there are some reports of ethanol abuse in some regions. However, no national regulation is available for monitoring of alcohol in Iran except for a directive provided by Iran Ministry of Health in which the maximum Bitrex concentration ( $10 \text{ mg l}^{-1}$ ) and purity are determined. In accordance, the objective of present study was to evaluate the concentration of Bitrex and purity degree in alcohol samples available in Tehran markets. The findings of current study can be used to monitor alcohol producing companies.

## 2. Materials and methods

### 2.1. Sampling

In the present study, 62 samples of alcohols from 20 domestic brands were collected from producing plants, stores and pharmacies in Tehran. Purity of purchased samples was mentioned 96% and 70% on the labels.

### 2.2. Chemicals

Sodium dihydrogen phosphate dibasic, hydrochloric acid, acetonitrile (HPLC grade), sodium lauryl sulfate and methanol (HPLC grade) were purchased from Merck (Germany). Denatonium benzoate D5765 (Sigma, USA) was used as standard. Deionized water was prepared through Thermo Scientific Barnstead Easy pure II system.

### 2.3. Preparation of solutions

One molar hydrochloric acid was prepared by addition of 8.33 ml of 12 M hydrochloric acid to

100-ml volumetric flask and then made up to the volume by distilled water.

To prepare buffer solution, pH of sodium dihydrogen phosphate dibasic (1.38 g sodium dihydrogen phosphate dibasic was dissolved in 1000 ml of distilled water) was adjusted to 3.00 with one molar hydrochloric acid.

Phosphate buffer and acetonitrile solution containing sodium lauryl sulfate (50:50) was prepared by adding 250 ml of phosphate buffer to 250 ml of acetonitrile, and then 3.6 g sodium lauryl sulfate was added to the solution. The mixture was used as mobile phase in HPLC.

Preparation of the solution used for initial washing of HPLC column was done by mixing of methanol and water at same volume (50:50). The solution was homogenized and degassed by ultrasonic apparatus.

Ten mg of DB standard was transferred to a 10-ml volumetric flask and made up to the volume with ethanol to prepare DB standard solution ( $1000 \text{ mg l}^{-1}$ ).

### 2.4. HPLC

Chromatographic analysis was carried out by Agilent 1200 series liquid chromatograph equipped with a gradient pump capable of mixing four solvents, a vacuum membrane degasser, a 20- $\mu\text{L}$  loop injector and UV detector. Analysis was performed by  $\text{C}_{18}$  column ( $150 \text{ mm} \times 4.6 \text{ mm} \times 5 \mu\text{m}$ ) and the UV detector was set at 210 nm. Flow rate of  $1.2 \text{ ml min}^{-1}$  was selected for the mobile phase and temperature was set at  $35^\circ\text{C}$ .

### 2.5. Calibration curve

Serial dilutions of standards were prepared by diluting the stock solution. Final concentrations of standard solutions were 2, 4, 8, 10 and  $15 \text{ mg l}^{-1}$ . An aliquot of 20  $\mu\text{l}$  of each dilution was injected to HPLC in three replications.

### 2.6. Method validation

The calibration curve was prepared over the range of  $2\text{-}15 \text{ mg l}^{-1}$  of DB standard solution. The linearity of curve and its correlation coefficient was assessed.

Limit of detection (LOD) and limit of quantitation (LOQ) are calculated as follows:

$$\text{LOD} = 3.3 \times S_y / S \quad \text{Eq. 1}$$

$$\text{LOQ} = 10 \times S_y / S \quad \text{Eq. 2}$$

Where,  $S_y$  and  $S$  are standard deviation of intercept and slope of calibration curve, respectively. Amounts of  $S_y$  and  $S$  are calculated by the data of calibration curve.

Feasibility of method was assessed by recovery test through analysis of samples before and after addition of DB standard solution in known quantities ( $10 \text{ mg l}^{-1}$ ). To estimate the recovery and intra-day precision (as RSDr), each sample was analyzed three times in a same day and in three consecutive days [20].

### 2.7. Determination of DB in alcohol samples

For sample analysis, they were filtered through a  $0.45 \text{ }\mu\text{m}$  PVDF (Polyvinylidene fluoride) membrane and  $20 \text{ }\mu\text{l}$  of filtrate was injected to HPLC without further dilution.

### 2.8. Purity determination of alcohol samples

The purity was evaluated by alcoholmeter. The alcohol concentration of distillate was determined by measuring its density at  $20^\circ\text{C}$  using a special alcohol hydrometer known as alcoholmeter.

### 2.9. Statistical analysis

The obtained results were analyzed using SPSS statistical software version 21 (SPSS Inc. Chicago, IL, USA). Analysis of variance (ANOVA) was conducted to evaluate the differences of samples and Tukey test was used for further multiple comparison. Differences were significant at  $p \leq 0.05$ .

## 3. Results and discussion

### 3.1. Purity of alcohol samples

Based on the results, about half of the samples were not formulated according to the purity mentioned on the labels through which over-dilution was observed in 27 and two samples of those labelled as “70%” and “96%”, respectively). Alcohol kills microbes by denaturation of

their intracellular proteins. However, optimum cellular proteins' denaturation and disinfection is induced by alcohol:water at 70:30 ratio. Over-dilution of pure alcohol by water is of common unconformities that might be intentional due to its cost-effectiveness or unintentional due to uncertainty of process [21,22].

### 3.2. Bitrex concentration

A reversed phase HPLC method was developed for determination of Bitrex in alcohol. Retention time of Bitrex in samples was recorded as 4.6 min by comparing with standard solution. The calibration curve fitted by linear regression analysis, was used for quantification. According to the calibration curve, equation and correlation coefficient were  $Y = 51.84 X + 14.55$  and 0.99, respectively. Furthermore, LOD, LOQ and recovery were determined  $0.31 \text{ mg l}^{-1}$ ,  $0.95 \text{ mg l}^{-1}$  and 98.99%, respectively. Our study had some advantages including use of general and routine UV-Vis detector, isocratic mode in HPLC and one wavelength for determination of Bitrex. In comparison, Zuba et al. used gradient mode in HPLC using diode-array detector and two wavelengths for detection of Bitrex in denatured spirits [23]. Another research was carried out on denatured alcohols for determination of Bitrex, quassia powder and sucrose octa-acetate along with diethyl phthalate and camphor by Kovar and Loyer. In their study, UBondapak-CN column was used while we used  $C_{18}$  column that is more available [24]. Kwiatkowski et al. investigated non-commercial alcoholic beverages for detection of Bitrex using Raman spectroscopy. Their technique was qualitative compared to HPLC results that are qualitative and quantitative [9]. Iran Ministry of Health set minimum concentration of  $10 \text{ mg}$  Bitrex per  $1000 \text{ ml}$  of alcohol that is comparable to  $6$  and  $10 \text{ mg l}^{-1}$  in USA and UK, respectively [11]. In our study, the measured concentrations of Bitrex in the samples were in the range of non-detectable (ND) to  $53.75 \text{ mg l}^{-1}$ . Distribution of Bitrex concentration is illustrated in Figure 2 and average concentration

of Bitrex in different brands are shown in Figure 3. As shown in the figures, Bitrex concentration of most samples was lower than 10 mg l<sup>-1</sup> and significant differences were observed between

various brands (p<0.05). In contrast, Bitrex concentration differences in the samples 70% and 96% were not statistically significant.

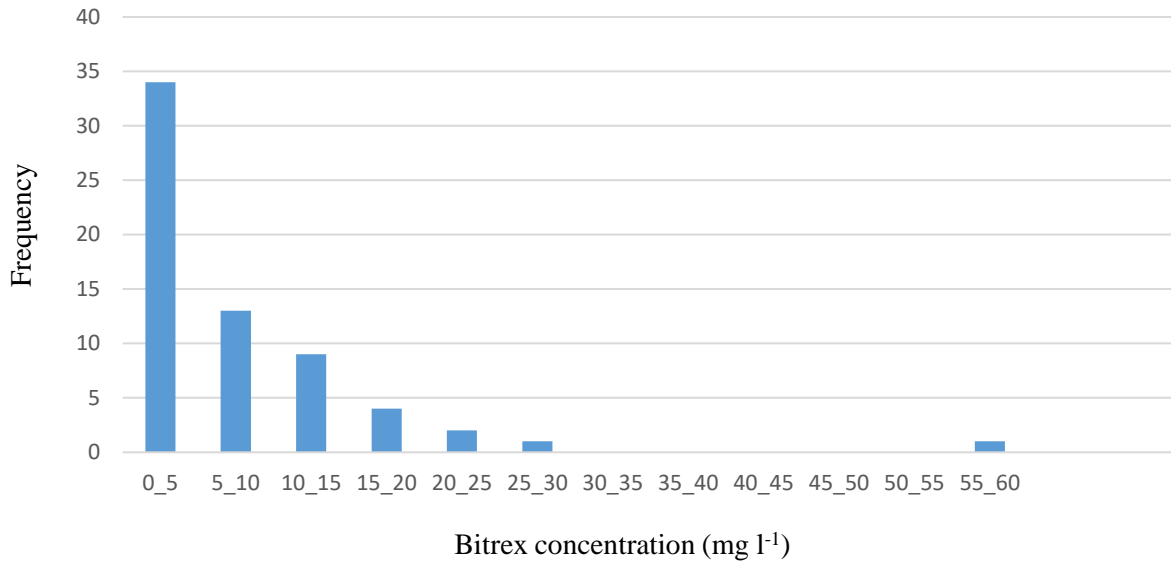


Figure 2- Distribution of Bitrex concentration in alcohol samples

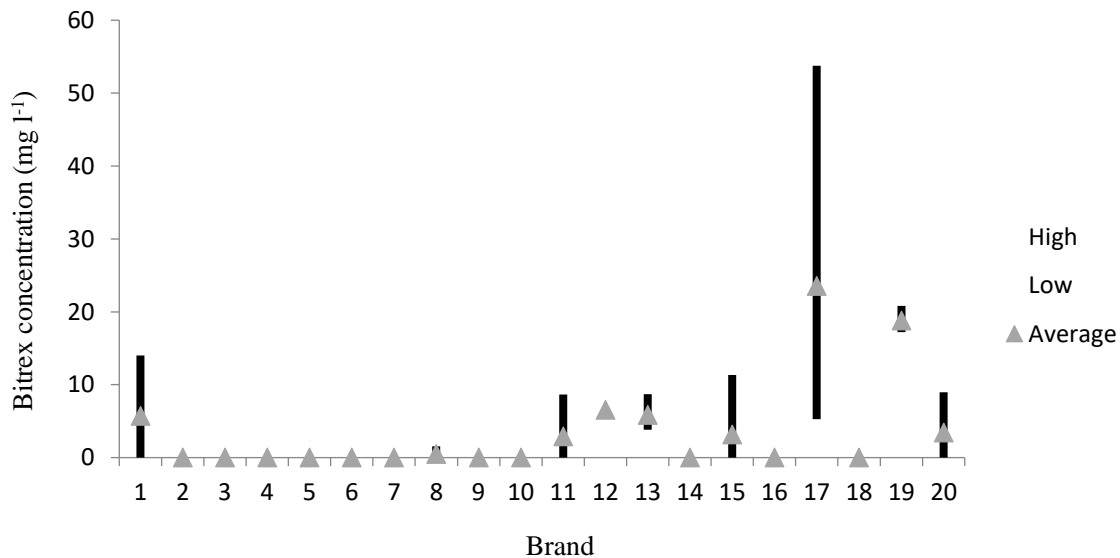


Figure 3- Average concentration of Bitrex in different brands of alcohol

According to our findings, a large number of samples (41 out of 62) were free of Bitrex. This outcome will be a concern for health authorities so that it can lead to alcohol abuse. Therefore,

more restrictions and monitoring protocols are required in the country to improve the conformity of marketed alcohols.

#### 4. Conclusion

Countries may have national limitations for some of domestic products based on their religion and culture. For instance, drinking of alcohol is banned in Islamic countries. In this regard, some chemical agents are added to alcohol to make it inappropriate for edible use. Unfortunately, there is no national regulation about acceptable concentration of Bitrex in alcohol in Iran. Although, there is a directive provided by Iran Ministry of Health that set minimum 10 mg l<sup>-1</sup> of Bitrex in alcohol. Accordingly, a simple and applicable method was developed in the current study to measure Bitrex concentration of alcohol samples. It was observed that most of the samples did not contain Bitrex and fruit essences were detected instead. In contrast, little unconformity was observed in purity of samples. The results highlighted that urgent and restrictive actions are required for alcohol monitoring strategies in Iran.

#### 5. Acknowledgement

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#### 6. Conflict of interest

The authors have no conflict of interest.

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