

Rapid Determination of Ethanol in Non-Alcoholic Malt Beverage by ATR-FT-IR Spectroscopy and Headspace Gas Chromatography Confirmation

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ABSTRACT

Malt beverage is one of the most popular drinks in the world. Recently, consumption of non-alcoholic beverages has expanded significantly in many countries. The permitted level of alcohol in malt beverage is less than 0.5% by Iranian National Standards. In this study, a method was developed to determine ethanol content in malt beverages by FTIR, equipped with Horizontal Attenuated Total Reflectance (H-ATR). Here, the Limit Of Detection (LOD) and Limit Of Quantification (LOQ) were 0.07% and 0.23%, respectively. The correlation coefficient of calibration curve was higher than 0.999. Fifty commercial malt beverages from six brands (five Iranian and one imported brand) and three types of flavored malt beverage (classic, equatorial, and lemon) were assessed. The average detected ethanol amount in samples was 0.19%, varying between 0.00-1.47percent. The results showed that the amount of ethanol in lemon malt drinks was more than the maximum permitted limit; that could be attributed to the usage of ethylene glycol for extraction of lemon flavor.

Keywords: Attenuated total reflectance, Fourier transform infrared spectroscopy, Iranian National Standards.

INTRODUCTION

Malt beverage is one of the most popular drinks in the world due to healthy and exquisite tasting that can be appropriate alternatives to sweet and alcoholic drinks. As to the nutritional aspects, malt consists of different vitamins, minerals, starch, protein and trace elements that are vital for better body performances. The non-alcoholic beverages consumption has expanded significantly in many countries; hence, they are considered and monitored by European Union for complying with safety and security in food chains. The amount of alcohol in these kinds of drinks has some

restrictions and classifications. According to regulation (EU: European Union) no. 1169/2011, the beverages that contain more than 1.2% alcohol should be labeled (European Union, 2017; Stupak *et al.*, 2017). The products containing less than 1.2% are classified in two groups: low alcoholic (0.5-1.2%) and non-alcoholic (less than 0.5%) (Hosseini *et al.*, 2011). Due to its importance, determination of alcoholic content in beverages was focused widely on samples claimed to be non-alcoholic. Ethanol is the main type of alcohol found in alcoholic beverages. According to the production way, there are two types of ethanol: fermented ethanol (bioethanol) and synthetic ethanol (Alzeer and Hadeed,

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2016). Bioethanol is made from corn or other biomass materials. Fermentation is a complex series of reactions, in which yeast cells convert sugar into ethanol and carbon dioxide as the major products. Moreover, a series of minor metabolites such as esters, higher alcohols, and acids, which contribute positively to flavor, are the by-products (Erdei *et al.*, 2013; Vijayalaxmi *et al.*, 2013). Synthetic ethanol is produced from ethane by the oil cracking. This reaction also produced toxic by-products, so, synthetic ethanol is never consumed by humans (Chu *et al.*, 2004) and it is the main source of the alcohol consumed in the beverage industry for industrial application (Yue *et al.*, 2014).

Alcohol is widely used for food, pharmaceutical, cosmetic, and other industrial applications. Some studies showed that alcohol consumption has various adverse effects on health. Even small amount of ethanol is absorbed by the tissues rapidly. A higher concentration has an impact on central nervous system as a psychoactive drug increases the risk of organ damage and mental disease. Moreover, long-term ethanol exposure has an effect on behavior and dopaminergic responsivity (Brill and Wagner, 2012; Maldonado-Devincci *et al.*, 2010; Meyer and Quenzer, 2013).

Among Islamic communities, this is noticeable issue for their consumer certifying Halal products, however, it doesn't mean that all forms of alcohol are banned, only because of some mental and physical dangerous situation. Therefore, determination of ethanol content is a principal point in the production of Halal products (Anis Najiha *et al.*, 2010; El-Naggar *et al.*, 2012; Riaz and Chaudry, 2003; van der Spiegel *et al.*, 2012).

Different methods have been developed for determination of ethanol content including the followings:

1. Instrument-based analysis of alcohols (densitometry based Alcoholmeter, HPLC, GC, Near IR, FTIR, and IR)
2. Chemical sensors for alcohol detection (Optical sensors: Colorimetry based

sensors, Fluorescence based sensors, Infrared spectroscopy based sensors, Refractive index and SPR based sensors, Luminescence based sensors/Electrochemical alcohol sensors/ Electrical alcohol sensors/Mass sensitive alcohol sensors)

3. Biosensors for alcohol detection [Enzyme based alcohol biosensors: ADH based alcohol biosensors (ADH based electrochemical alcohol biosensors, ADH based optical alcohol biosensors), AOX based alcohol biosensors (AOx based electrochemical and electrical alcohol biosensors, AOX based optical alcohol biosensors), Other Enzymes/ Microbial and cell based alcohol biosensors]
4. New trends to determine alcohols (Miniaturization techniques, Other recent techniques) (Thungon *et al.*, 2017).

According to the low acceptance criteria of ethanol content in the non-alcoholic drinks in Halal products, the chosen method should provide results with adequate sensitivity, accuracy, and precision. Furthermore, we should consider other parameters in this measurement, like simplicity, availability, and low cost. The Fourier Transform Infrared Spectroscopy (FTIR) is routinely employed in many quantitative and qualitative determinations. This is because it is simple to handle, fast, and sufficiently precise in analytical techniques. The technique of Attenuated Total Reflectance (ATR) is a new innovation for solid and liquid sample analyses as it could cover some of the sample preparation defects in infrared analyses, such as sample preparation and spectral reproducibility. In addition, the ATR is extremely robust and reliable for quantitative studies involving liquids. The whole process can be evaluated within less than one minute and easily cleaned and ready for the next sampling (Elmer, 2005; Hosseini *et al.*, 2011; Nagarajan *et al.*, 2006).

The National Iranian Standard has not measured alcohol content in malt beverage previously. Therefore, our study is the first accurate and efficient method for monitoring malt beverage quality and determining ethanol content in nonalcoholic drinks in Iranian markets.

Our studies were carried out on three different flavors: classic, tropical, and lemon from imported and local brands in Tehran markets. Our goal in this work was to develop a fast, easy, and efficient method for determining ethanol contents in malt beverage by ATR technique, based on alcohol concentration restriction in Islamic religion.

MATERIALS AND METHODS

Sampling and Reagents

Fifty malt beverages from six brands (five Iranian and one imported brand) and three types of flavored malt beverage (classic, equatorial, and lemon) were purchased from a supermarket in Tehran. Ethanol absolute was obtained from Merck (Darmstadt, Germany). De-ionized water was prepared through the Thermo Scientific Branstead Easypure II system.

The pH and Brix of the samples were measured by 827 models of Metrohm pH meter and Atago Automatic Digital Refractometer RX-7000 α and the samples were categorized according to Iranian National Standard (2011).

Determination of Ethanol

Spectrophotometric Conditions

The infrared spectrometer analysis was carried out on spectrum TwoTM FT-IR Spectrometer from PerkinElmer equipped by ATR with a value version of Horizontal Attenuated Total Reflectance (H-ATR). This accessory was fitted with a 25-reflection, 45

degrees, 50 mm ZnSe crystal trough plate (L1600109). The IR-spectra for all the samples were collected with a resolution of 0.5 cm^{-1} and 32 scans in the wavelength region 2,100-2,480 cm^{-1} by absorbance mode. The 3 mL of liquid samples and standards were poured on the crystal until the whole crystal was covered without any bubble. Some pre-processing including baseline correction, normalization, and smooth were applied for improving signal to noise. The spectrum Software was used to obtain better performance by removing the baseline drift.

Preparation of Standards and Samples

The stock standard solution of ethanol (10% v/v) was prepared in blank solution. According to the Iranian National Standard (2011), Brix of malt beverage should not be less than 4.5% at 20 °C. To prepare blank solution, malt extract was diluted till 4.5% brix with de-ionized water and the blank solution spectrum was applied as background.

The amount of sample was transferred to the beaker and, then, degassed by ultrasonic bath for five minutes. After that, the prepared solution was filtered with 0.45 μm PVDF syringe filter and 3 mL of the solution was poured directly on the surface of clean ZnSe crystal plate monotonically. Finally, the spectral data were collected from all the samples.

Method Validation

The calibration curve of ethanol was evaluated over the range of 0.5-4% (v/v). Each concentration was carried out in triplicate. The linearity and the correlation coefficient for standard curve of ethanol were calculated. The Detection Limit (LOD) and Quantification Limit (LOQ) were calculated as:

$$DL = 3.3S_y/S \text{ and } QL = 10S_y/S.$$

Where, S_y was the Standard deviation of the y intercept and S was the Slope of the calibration curve. The estimate of S_y was



calculated by using the information on calibration curve.

In order to verify the feasibility of the method, sample recovery was used by analyzing samples before and after addition of known quantities of ethanol (0.5 and 1%). To determine the intra-day precision (RSD_r), each sample was analyzed three times in the same day as described in "Sample Preparation" section.

Statistical Analysis

The obtained data were analyzed with the SPSS statistical package, version 21 (SPSS Inc. Chicago, IL, USA). Analysis Of Variance (ANOVA) was used to evaluate the differences of distribution between different brands and types. For multiple comparisons, Tukey test was used. The results were expressed as mean \pm SD for all the samples in all the tables. Statistical significance was set at ($P < 0.05$).

RESULTS AND DISCUSSION

Analytical Method

There are various analytical methods for determination of ethanol in food, such as pycnometry, gravimetric, electrical gravimetric, conductivity measurement, High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), and Infrared Spectroscopy (IR) (Belghith *et al.*, 1987; Duarte *et al.*, 2004; Somboon *et al.*, 2022; El-Naggar *et al.*, 2012; Esti *et al.*, 2003; Rocchia *et al.*, 2007; Wachełko *et al.*, 2021 ;Mansur *et al.*, 2022). However, IR technique does not have as high resolution as GC or HPLC. Moreover, this equipment is relatively cheaper, simpler, and quicker (Onuki *et al.*, 2008; Nordon *et al.*, 2005). These days, FT-IR is the most common, rapid, simple, and green method for the

quantification of ethanol in beverages (Elmer 2005; Hosseini *et al.*, 2011; Li 2020). ATR method is fast, accurate, and easy to handle for determining ethanol (Nagarajan *et al.*, 2006) Therefore, exploring this technique can be an effective alternative to other methods.

In a similar study for determination of ethanol, two regions suitable for quantitative calibration were found as follows: (1) Near 5,882.35 cm^{-1} (first overtone of C–H stretching) for sample with more than 40% ethanol content and (2) Near 4,166–4,762 cm^{-1} (The O–H bond in ethanol produces a near-infrared band, which is easily distinguished from the O–H band of water that appeared in 6,060 cm^{-1}) for sample with less than 40% ethanol (Davies and Grant, 1988; Stuart, 2005). Figure 1 shows the accuracy of a typical IR spectrum of ethanol in malt beverage, predicted through ATR.

Iranian National Standard method for ethanol determination is based on titration. According to this standard, the amount of ethanol in nonalcoholic malt beverage should be less than 0.5%. However, the limit of detection of ethanol by titration method is more than 0.5% and this method is not accurate and suitable. In this work, we intended to develop a rapid, more sensitive, and simple method for determination of ethanol in malt beverage.

Identification was achieved by comparing the wave number of standard ethanol, and quantification was based on using the calibration curves fitted by linear regression analysis.

The calibration plot is shown in Figure 2.

The results of the calibration data, LOD, LOQ, and the recovery of ethanol are in Table 1.

The average of recoveries was 95.31%. Furthermore, the method had acceptable accuracy and was suitable for determination of ethanol in malt matrix.

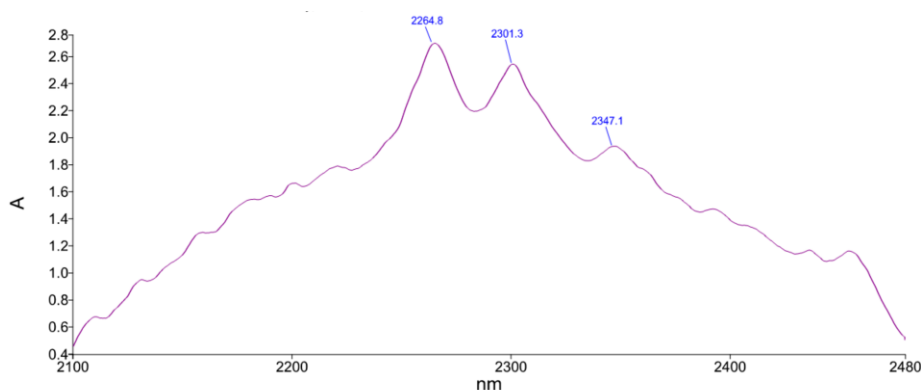


Figure 1. Accuracy of a typical IR spectrum of ethanol in malt beverage, predicted through ATR.

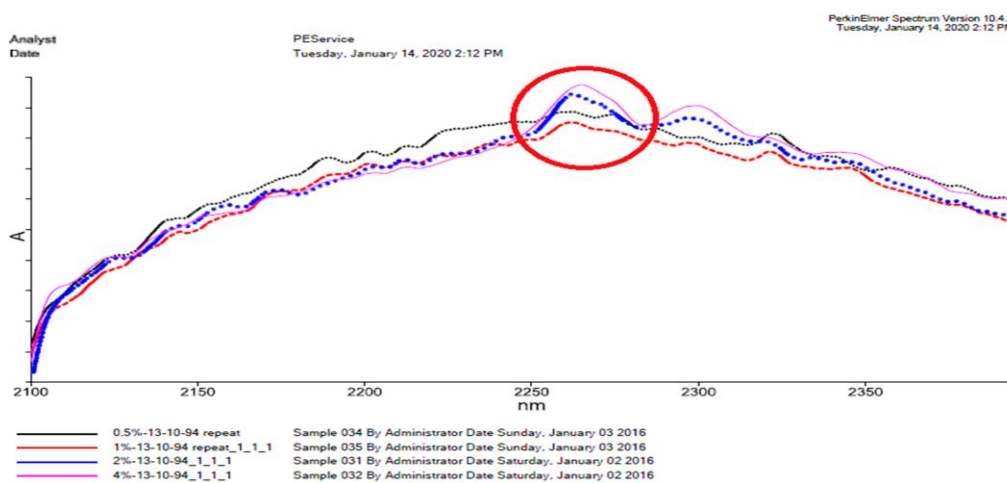


Figure 2. IR-spectra of malt samples containing 4% ethanol.

Table 1. Analytical characteristics of the method validation.

Analytes	LOD (%)	LOQ (%)	Calibration equation	R ²	Recovery (%)	RSDr (%)
Ethanol	0.07	0.23	$y = 1.976x + 2.12$	0.999	95.31	3.91

In this study, an IR method was developed and characterized by minimizing sample preparation for the determination of ethanol in malt beverage, which can reduce the cost of analysis against GC and HPLC. Moreover, this method had some benefits in quantification, LOD, LOQ, and recovery, which was in contrast with titration.

Sample Analysis

The method was applied to 51 samples of different commercial malt beverages. Each

sample was divided according to kinds and brands, as described in sampling. The results are shown in Table 2.

The pH results complied with the relevant standards (pH was between 2.87 and 4.49). The brix content varied between 4.39 and 11.55%. Among the samples, the brix of three classic malts were not accepted as per the Iranian National Standard No.2279 and the brix of these samples were less than 4.5% (Iranian National Standard, 2011).

According to one-way Anova analysis, there was a difference between the pH and the brix amount in various kinds of malt

**Table.2** The range of pH, Brix and ethanol in different kind of malt beverages and relevant limitation in ISIRI.

Kind	pH		Brix%		Ethanol%	
	Range	Limit	Range	Limit	Range	Limit
Classic	3.34 - 4.49	3.6 - 4.5	4.39 - 7.47	≤ 4.5	0.00 - 0.67	≤ 0.5
Equatorial	3.2 - 3.58	2.8 - 3.8	8.13- 9.11	4.5 - 9	0.00- 0.62	≤ 0.5
Lemon	2.87 - 3.47	2.8 - 3.8	8.37 - 11.55	4.5 - 9	0.00 -1.47	≤ 0.5

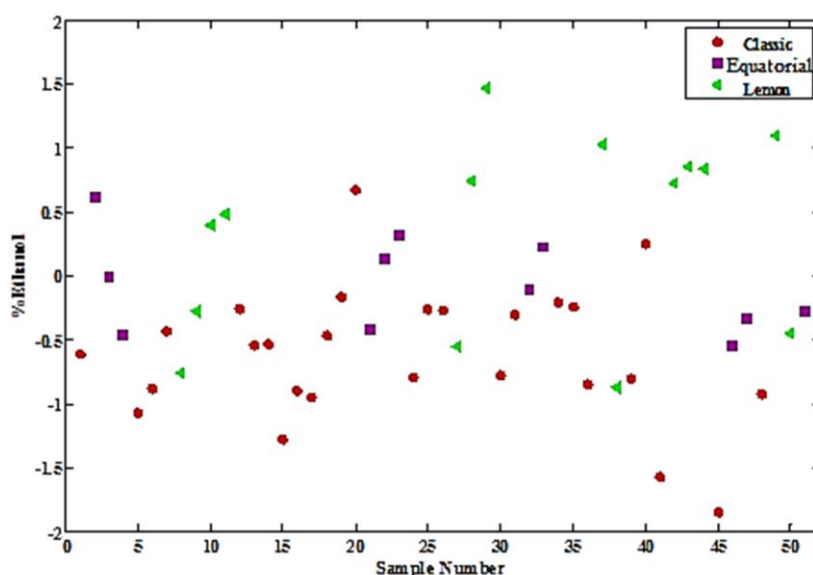
beverages ($P < 0.05$). Tukey analysis showed that the pH in classic malt had significant differences with the other kinds of malt beverages. This result depended on different acceptance criteria in the Iranian National Standard for classic and flavored malt drinks. On the other hand, one-way Anova analysis showed a difference between pH in various brands ($P < 0.05$). However, Tukey analysis indicated that imported brands were different from Iranian brands.

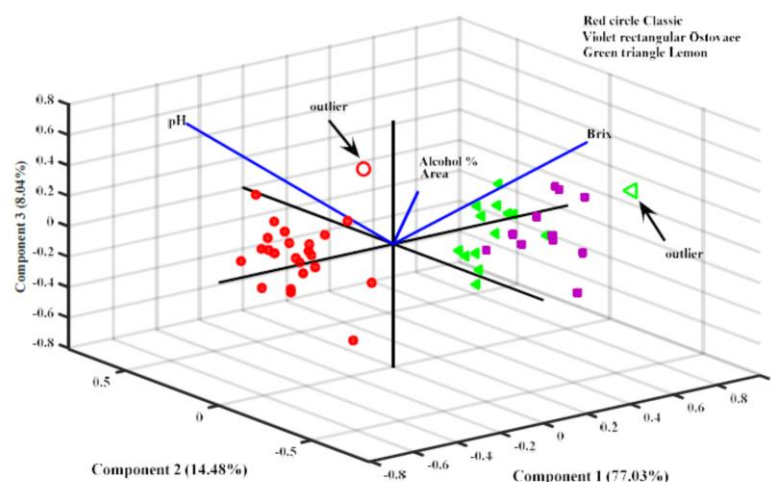
Average ethanol content of samples were $0.19 \pm 0.35\%$. This amount was lower than the acceptance criteria of ethanol for non-alcoholic malt beverage (0.5%). The amount of ethanol varied between 0.00 and 1.47%. More than 0.5% of ethanol was detected in nine samples, of which eight samples were lemon malt beverage (Figure 3).

Tukey analysis approved that ethanol in

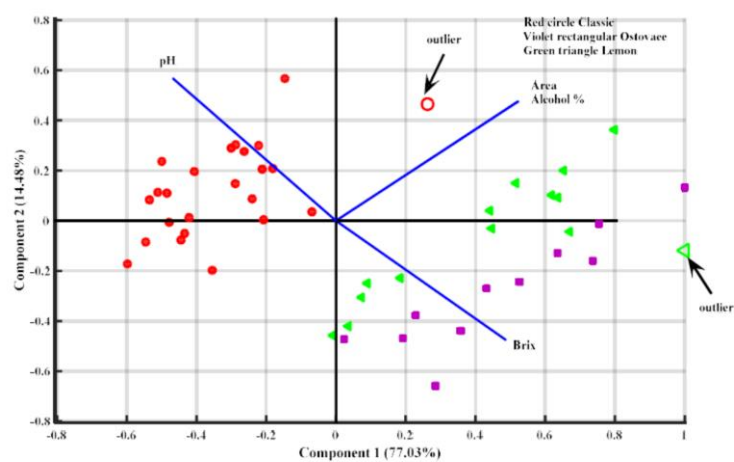
lemon malt was significantly different than the other kinds of malt beverages. One of the reasons for the presence of ethanol in the lemon malt drinks could be lemon essence and this essence was extracted with alcohol ethylic (Moshonas and Shaw, 1972; Yilmaz and Goren, 2013).

The storage condition, such as temperature, light, etc., could have impact on microorganism performance in biological process. This converts sugars such as glucose, fructose, and sucrose into cellular energy, producing ethanol and carbon dioxide (Abdel-Banat *et al.*, 2010). Hence, the microbiological analysis (total microorganism count, yeasts, molds, acid resistant, and lactic acid bacteria) was carried out on samples that had more than 0.5% ethanol. The result showed compliance with the relevant limitation. Therefore, the

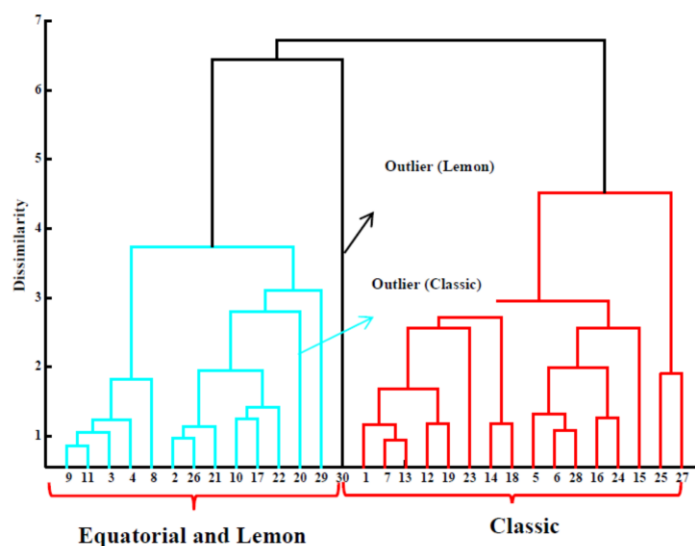
**Figure 3.** Ethanol content in the different types of malt beverage.



(a)



(b)



(c)

Figure 4. Classification the different kinds of malt beverage: (A) 3D biplot, (B) 2D biplot, (C) Dendrogram.

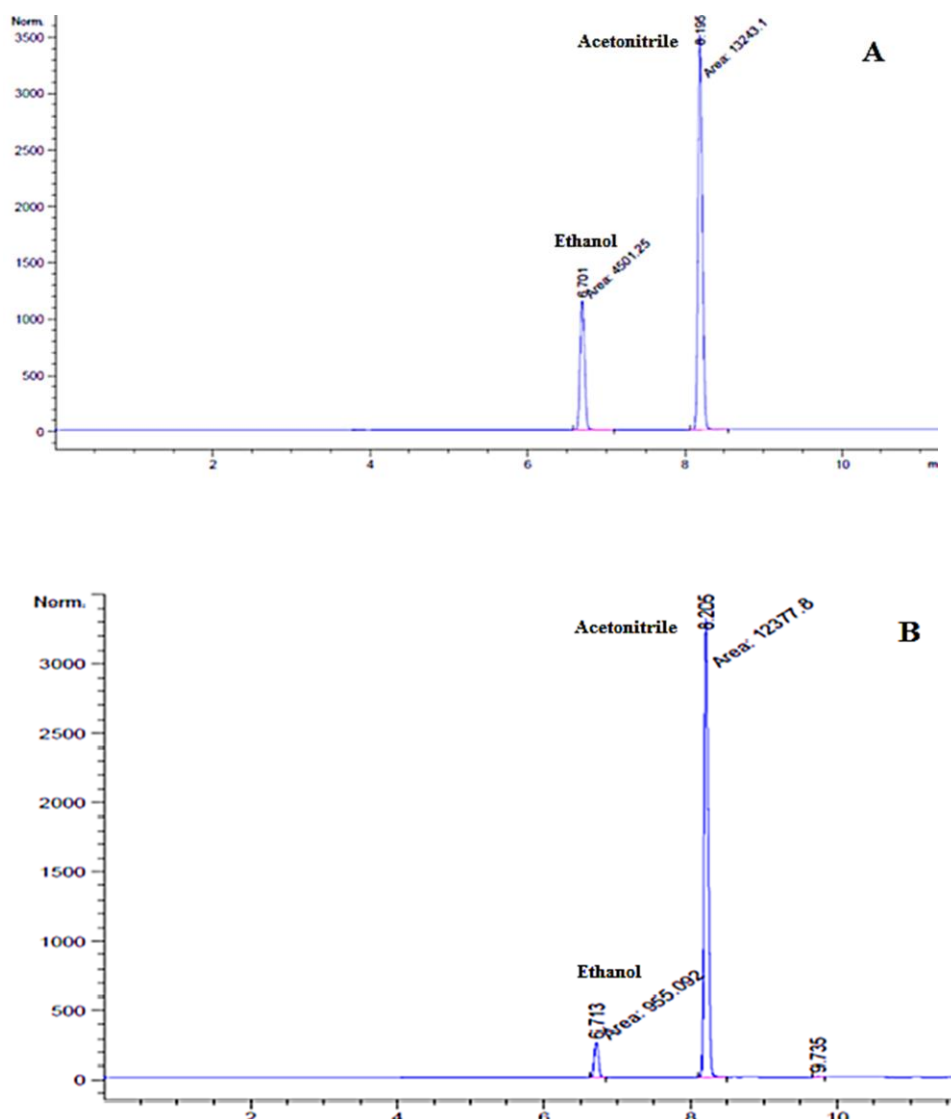


Figure. 5 Chromatogram from headspace gas chromatography confirmation: (A) Standard 0.5%, (B) Sample.

activities of the microorganisms could not produce this amount of ethanol.

In this study, the obtained data from different beverages (pH, brix, and % ethanol content) was analyzed by Principal Component Analysis (PCA). All results that were found using classical methods could be proven by statistical data analysis precisely.

PCA analysis proved that the pH in classic malt had significant differences with other kinds of malt beverages. This was mentioned by Tukey analysis and the percentage of alcohol in lemon malt was found to be more than the other kinds

(Figures 4-a and -b). Figures 4-a, -b (using biplot), and -c (using dendrogram) showed that there were two different classes of beverage malts based on pH, brix, and ethanol content. Equatorial and lemon malts were in the same group and classic malt was in another group.

Headspace Gas Chromatography Confirmation

Notably, confirmation of low ethanol levels (less than 0.5%) requires more

sensitive analytical instrumentation, such as Headspace Gas Chromatography (HS-GC), whose Limit Of Quantification (LOQ) is less than 0.01% (Varlet and Augsburg, 2013; Wachelkoe *et al.*, 2021).

Confirmation testing with the help of Agilent 7890A gas chromatograph, equipped with a 7697A head space provided the definitive chromatogram and results that are presented in Figure 5.

CONCLUSIONS

In this research, we aimed to evaluate the amount of ethanol among non-alcoholic malt beverages. The proposed method was ATR-FT-IR Spectroscopy. This method was chosen due to its simplicity, reliability, sensitivity, and rapidness in detecting ethanol. In addition, the approved method for ethanol evaluation in Iranian National Standard is a titration technique, which is not an accurate quantification. However, according to this study, we could help to codify a new method for the determination of ethanol in malt drinks. The results of this investigation indicated the presence of ethanol more than the maximum permissible limit in lemon malt drinks. Ethanol content of these drinks was more than the level authorized by Iranian National Standards, which could cause the flavor. However, ATR-FT-IR is convenient for routine quality control of ethanol.

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تعیین میزان اتانول در نوشیدنی مالت توسط طیف سنجی ATR-FT-IR و تایید آن توسط روش کروماتوگرافی گاز با تکنیک Headspace

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چکیده

نوشیدنی مالت، یکی از محبوب ترین نوشیدنی ها در جهان است. مصرف نوشیدنی های غیرالکلی به طور چشمگیری در بسیاری از کشورها گسترش یافته است. میزان حداکثر مجاز الکل در نوشیدنی مالت بر طبق استانداردهای ملی ایران کمتر از ۰.۵ درصد ثبت شده است. در این مطالعه، روشی برای تعیین اتانول موجود در نوشیدنی های مالت توسط دستگاه FT-IR (مجهز به H-ATR) ستاپ شده است. نتایج به دست آمده در مقایسه با روش مرجع در استانداردهای ملی ایران، برخی از مزایا را در مقیاس سنجی، LOD، LOQ و Recovery نشان داد. مقدار حد تشخیص (LOD) و حد کمی سازی (LOQ)، مقدار ۰.۰۶ و ۰.۲۳ درصد را به ترتیب نشان داد. ضریب همبستگی منحنی کالیبراسیون بیشتر از ۰.۹۹۹ بود. این روش برای ۵۰ نمونه تجاری مالت از شش برند مختلف (پنج برند ایرانی و یک برند خارجی) و همچنین سه طعم متفاوت (کلاسیک، استوایی و لیمویی) به کار رفت. میزان متوسط اتانول در نمونه ها ۰.۱۹ درصد شناسایی شد که بین ۰.۰۰ تا ۱.۴۷ درصد متغیر بود. نتایج حضور اتانول بیشتر از حداکثر حد مجاز در نوشیدنی های مالت لیمویی نشان داده شد.