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Original Article

Simultaneous determination of ethanol and methanol in alcohol free malt beverages, energy drinks and fruit juices by gas chromatography

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Abstract

A simple, sensitive, and direct method to decide if "alcohol-free" beverages, energy drinks and fruit juices could result in positive "alcohol alerts" based on the use of the gas chromatography with a flame ionization detector (GC– FID) has been developed. The chromatographic conditions such as injection volume and split ratios were optimized in order to increase the sample throughput and sensitivity. Unlike other conventional methods which also employ laborious sample preparations; this method analyzes samples directly without any prior treatment and thus cutting down the sample treatment time, as well as reducing the analysis cost per sample. Additionally, low ethanol concentrations as low as 6 mg/L were detected and quantified; making this method an appropriate technique for routine alcohols analysis in beverages. Optimization of chromatographic conditions gave recoveries in the range of 83.00% to 112.8% with relative standard deviations lower than 9%. The calibration curves were linear over the range from 6.250-200.0 mg/L for methanol and ethanol. The correlation coefficients (r^2) were higher than 0.9997. The limits of quantifications (LOQ) are 4.48 mg/L for ethanol and 5.74 mg/L for methanol. A total of 100 domestic and imported labeled "alcohol-free" in Jordan were analyzed for their alcohol content. Concentrations ranged from non-detectable to 14.9 mg/L for ethanol and from non-detectable to 9.38 mg/L for methanol were found in energy drinks. On the other hand, non- quantifiable amounts of alcohols were found in malt beverage as well as fruit juices.

Keywords: Alcohol-free beverage, Energy drink, Flame ionization detector, Gas chromatography

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Introduction

The popularity of "alcohol-free" beverages and fruit juices have been increased on world markets as an alternative to alcoholic beverages that may cause health problems such as fetal alcohol syndrome (FAS), developmental delay, common cognitive disorders, and social disabilities (Parés and Caballería, 2002; Cabarcos et al., 2015). Drinking too much alcohol over a long period of time or too much alcohol at one occasion (binge drinking) leads to interference in the brain's communication pathways, damage to the heart, liver (hepatitis) and pancreas (pancreatitis); increase risk of infection and weaken the immune system (NIH, 2015). Nevertheless, alcohol has negative social and economic impacts on individual drinker and on the society as a whole. The popularity of "alcohol-free" beverages has increased in Islamic countries that prohibit drinking alcoholic beverages. The consumer understands, or assumes, that these products are completely alcohol-free due to the labeling of these products.

The term "non-alcoholic" may be used for malt beverages containing less than 0.5% (v/ v) (5000 mg/ L) showing alcohol in a clearly legible publication while the term "alcohol free" is used only on malt beverages which does not contain alcohol 0.0% (v/v) (Ayala et al., 2009) .

A literature survey of the manufacturing procedures utilized as a part of making these products showed that the alcohol-free beers and juices have low alcohol content (Morad et al., 1980). Alcohol-free beer is manufactured either by brewing a blend of water, malt and hops, a process in which fermentation stops prematurely, or by decreasing the alcohol content of typically produced beer. On other hand, energy drinks represent beverages that, in addition to calories, contain caffeine in a combination with other energy-boosting components such as taurine, herbal extracts, and B vitamins. First appeared in Europe and Asia in the 1960s due to consumer demand for dietary supplementation that would lead to increased energy (Reissig et al., 2009). A Japanese company called Taisho Pharmaceuticals Lipovitan D was founded in 1962, which controls the Japanese market so far. Lipovitan D contains B vitamins, taurine, and ginseng, all of which are frequent components of energy drinks (Taisho Pharmaceutical Co. Ltd. 2009; Heckman et al., 2010). A large variety of energy drinks claim to provide the consumer with sustainable energy, reducing mental and physical weakness. These products are commonly targeting young adult and very popular among undergraduates worldwide.

Methanol is the simplest type of alcohol. It is much related to ethanol which is a kind of alcohol usually found in wine, beer and spirits - but methanol is much more harmful. Methanol is formed in very small quantities during fermentation process by which alcohol is produced from plant products such as grape juice or grain. During distillation process of fermented fruits, methanol is distilled simultaneously with ethanol due to their comparative physicochemical properties. This is why all alcoholic beverages obtained from fermented fruits contain methanol beside ethanol (Croitor et al., 2013). The likelihood of their presence in home-made beverages made from distilled spirits poses a health hazard. The legal limits that established by the European Union (EU) of methanol in alcoholic beverages are 0.4% (v/v) (4000 mg/L) (Paine and Davan, 2001).

Several analytical techniques have been developed for the determination of ethanol in liquor, and pharmaceutical preparations. Sensitive, rapid and relatively inexpensive ethanol quantification is the primary analytical procedure for alcohol analysis in the beverage.

There are several approved methods for ethanol analysis in alcoholic beverages products (AOAC, 1990). The oldest and most commonly used method is semi-quantitative ebullioscopic, based on an identification of boiling beverages (Amerine, 1972). For a long time, pycnometric density was determined by the reference method for analysis of alcoholic content in spirits and wines. But the pycnometric method should be preceded by a distillation step (Debebe et al., 2017). A specific gravity method, which is based on hydrometric measurement, is more accurate but it is time-consuming; also it requires sample extraction prior to laboratory analysis (Ough and Amerine, 2018). The electronic density meter was later introduced to determine alcoholic strength has achieved a similar or better performance based on of accuracy (Strunk et al., 1979; Mark and Vaughn, 1980; Brereton et al., 2003).

These techniques are inexpensive and do not need standards, chemicals and reagents. In addition, they do not require sample preparation omitting the needs of skillful operator. However, it may suffer from high detection limit and therefore the techniques are not suitable to sample with small amount. To overcome the problems associated with the high detection limit mentioned above, the alcohol content in the beverages is now determined today using faster, and simpler spectroscopic methods such as Fourier Transform Mid -Infrared Spectroscopy (FT-MIR) (Debebe et al., 2017), Raman Spectroscopy (Nordon et al., 2005; Pappas et al., 2016), Nuclear Magnetic Resonance Spectroscopy (NMR) (Isaac-Lam, 2016) and non-invasive Near-Infrared (NIR) (Nordon et al., 2005). Besides, no sample preparation is required other than degassing in spectroscopic methods, it permits the direct analysis of alcohol in liquid samples without any sample pre-treatment, and in addition, analytes can be monitored simultaneously within milliseconds. Despite spectroscopic methods advantages, government bodies around the world have relied upon gas chromatography with flame ionization detection (FID) (Tiscione et al., 2011) for routine alcohol analysis in alcoholic beverages and for the testing of "alcohol-free" beverages, whether non-alcoholic and dealcoholized products (Spedding, 2015). This procedure can be used to analyze alcoholic beverages without any other sample preparation. Recently, gas chromatography coupled with mass spectrometry (GC-MS) (Jones et al., 1983) and gas chromatography with tandem mass spectrometry (GC-MS/MS) (Tiscione et al., 2011) have been adopted.

In capillary gas chromatography (GC) there are four main techniques for vaporizing a sample and transmitting it onto the inlet of the analytical column: direct, on-column, split, and splittless injections. The most commonly used techniques are split and splittless injections. In the split injection mode, which has been applied, a small fraction of the vaporized sample is transmitted onto the head of the column and removing the remainder of the vaporized sample from the injection port through the separate ventilation line. Split injections were required when sample concentrations are high to enable a portion of the sample to be discarded through the injection process and maintaining an adequate concentration of analytes for the detector so as to produce a signal (Chasteen, 2000). It should be used to minimize the nonvolatile sample components including sugar, pigments and others and yield suitable chromatography.

This study aims to develop a simple, sensitive, and direct method to decide if “alcohol-free” beverages, energy drinks and fruit juices could result in positive “alcohol alerts” based on the use of the gas chromatograph with a flame ionization detector (GC–FID). The proposed method was in-house validated concerning linearity, accuracy, intra-day precision, inter-day precision, limit of detection (LOD), and limit of quantification (LOQ).

Material and Methods

Certified standard solutions of ethanol (99.8%) and methanol (99.8%) for gas chromatography electron capture detector (ECD) and FID SupraSolv® were obtained from Merck KGaA (Darmstadt, Germany). Water (HPLC-grade) was purchased from VWR International (EC). In the month of February 2018, 100 samples of “Alcohol-free” beverages, energy drinks and fruit juices samples of different brands were randomly obtained from groceries and stores in Amman, Jordan. The samples were stored at 4.0 ± 0.5 °C in a refrigerator.

Sample preparation

The beverage sample solution was dispensed into a 2 mL capped sample vial and injected directly into the GC-FID instrument.

GC analysis

The analysis was performed using gas chromatography, Perkin-Elmer workstation, with model Clarus 600 GC (Llantrisant, UK), which was equipped with a liquid auto sampler and FID detector. The chromatographic separation was performed with RTX-WAX (fused-silica), 30 m x 0.32 mm ID x 0.25 µm film thickness of capillary column, which was purchased from Restek Corporation (USA) under the following condition. The instrument has an oven with an initial temperature of 40°C for 8 min and a ramp program which elevates up to 100°C ramping 5°C/min, followed by further 2 min isothermal hold. Samples with 2 µL volumes were injected using split ratios of 10:1. The temperature of the injection port was set at 100°C. The carrier gas was helium at the flow rate of 1 mL/min with the pressure set at 7.1 psi and average velocity at 22.5 cm/sec. The FID detector temperature was maintained at 200°C with a flow of 45 mL/min H_2 and 450 of mL/min H_2O. Split injection mode was selected.

To optimize the chromatographic conditions such as injection volume and split ratios, different splitting ratios between 1:20 and 1:5 were studied at injections volume between 0.5 µL and 4 µL.

The accuracy was calculated by the determination of the recoveries of the alcohols from Alcohol-free” beverages samples and energy drinks samples at 20, 40 and 80 mg/L of ethanol, and methanol standards; the spiked samples were analyzed in triplicates (Table

3) and calculated according to the following formula (Shabir, 2003):

$$\text{Recovery (\%)} = \frac{\text{Recovered Amount (mg/L)}}{\text{Added Amount (mg/L)}} \times 100$$

The linearity was tested using the mix of alcohol standards in a concentration range from 6.250 to 200.0 mg/L for both ethanol and methanol. The limit of detection (LOD) and limit of quantification (LOQ) were calculated experimentally as the lowest concentration giving a response of three- and six-times, respectively, the base-line noise given by the software, obtained from alcohol-free samples. Intra-day precision was calculated by assaying five replicates of the same sample at a spiked level of 20 mg/L of ethanol and methanol on the same day. For the inter-day precision, five replicates of the same sample at a spiked level of 20 mg/L of ethanol, and methanol were analyzed on three consecutive days.

Statistical analysis

The statistical analysis at an alpha level of 0.05 were performed using Microsoft Excel 2016 for ethanol and methanol determination in beverages.

Results and Discussion

The optimization procedure for the gas chromatography parameters was carried out by modifying the splitting ratio between 1:20 and 1:5 until the maximum sensitivity (peak area counts of ethanol and methanol standards mixture) was obtained. In general, higher split ratios, up to 1:20, were required to protect liners and the chromatographic column. On the other hand, the split ratio were reduced to 1:5 if lower LODs are needed. It can be observed from Table 1 that, there were significant differences in the peak area counts for ethanol and methanol when applying different split ratios. The peak area percent for both ethanol and methanol gradually increased from 43% and 40% to 100%, respectively. The split ration changed from 20:1 to 10:1. In contrast, when the split ration was decreased from 10:1 to 5:1, the peak area percent for ethanol decreased from 100% to 84%, while that for and methanol decreased from 100% to 80%. Hence, the optimum split ratio of 10:1 was employed. Alternatively, as injection volume increased from 0.5 till 2 µL the peak area percent for both ethanol and methanol gradually increased from 76% and 68% to 100%, respectively (Table 1). But further increase in injection volume to 4 µL resulted in dramatic decrease in peak area percent for both ethanol and methanol to 84% and 88%, respectively. Therefore, the optimum 2 µL injection volume at split ratio of 10:1 were adopted.

Table 1. Peak area percent of alcohol standards mixture at different split ratios as well as different injections volume.

Alcohol standards mixture	**Split Ratio**			
	1:20 (Peak Area %)		1:10 (Peak Area %)	1:5 (Peak Area %)
Ethanol	43%		100%	84%
Methanol	40%		100%	88%
	Injections Volume			
	0.5 µL (Peak Area %)	1 µL (Peak Area %)	2 µL (Peak Area %)	4 µL (Peak Area %)
Ethanol	76%	84%	100%	43%
Methanol	68%	88%	100%	40%

The described method that has been developed has many advantages including speed and accuracy as well as showing non-blank effects or interferences by any endogenous materials with the analyte in beverages that was analyzed directly without any prior treatment. Besides, low ethanol concentrations that reach 6 mg/L are detected and quantified.

Out of total 100 samples of malt beverages, energy drinks and fruit juices that have been analyzed, only energy drinks showed alcohol content at concentrations ranged from non-detectable to 14.9 mg/L for ethanol and from non-detectable to 9.38 mg/L for methanol. On the other hand, non-quantifiable amounts of alcohols were found in malt beverage as well as fruit juices. In spite of the fact that ethanol and methanol were detected at a concentration of 14.9 mg/L and 9.38 mg/L, respectively, in energy drinks (Red-Bull) these concentrations are considered relatively low compared to regular beer or other alcoholic beverages, this is in opposition to the claim that these beverages are "alcohol-free". These results are also considered low compared to study conducted by Ayala et al. (2009) that reported 293 mg/L of ethanol in Red-Bull energy drinks. Also it was reported that ethanol concentrations in soft drinks and flavored beverages were in the range of 0–960 mg/L and 0–840 mg/L, respectively (Ayala et al., 2009). Another study by Morad et al. (1980) also detected ethanol in the “alcohol-free" beverages and fruit juices tested, the reported range were 90–3850 mg/L.

The existence of ethanol as well as methanol in these beverages can possibly be attributed to the inefficiency of the alcohol removing technique. It is additionally conceivable that ethanol may be framed after bottlings and during storage if the pasteurization process were insufficient in the destruction of microorganisms. The addition of sugar was expected to increase the ethanol to methanol ratio because formation of methanol from sugar fermentation has not yet been described (Croitoru et al., 2013). The retention time of methanol and ethanol were 4.1 min and 4.8 min, respectively (Figure 1). Symmetric sharp peaks were obtained, allowing peak height measurements to be used with maximum accuracy as peak areas (Wesselman, 1960).

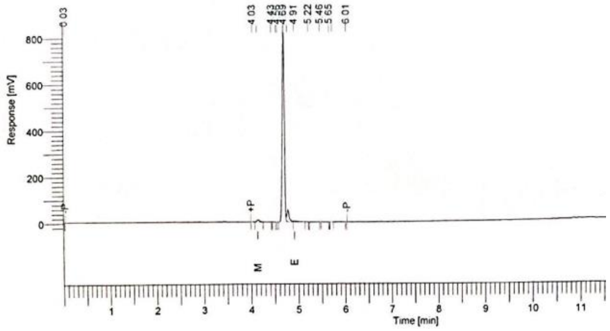

Fig. 1. HPLC chromatogram of alcohol standard solutions containing 50 mg/L of ethanol and with a retention time 4.1 min and 4.8 min of methanol and ethanol, respectively.

The method was validated internally regarding linearity, accuracy, intra-day precision, inter-day precision, LOD and LOQ.

Table 2 shows good linear relationships between the concentration of the analytes and the peak response with correlation coefficients of 0.9997 for ethanol and 0.9999 for methanol. Calibrations with standard solutions were used for quantitation because moderate signal suppression was noticeable for both analytes. Furthermore, the ANOVA test did not give any significant difference at $p > 0.05$.

Table 2. Linearity range, Equation, r^2 value, LOD and LOQ of ethanol and methanol.

Alcohol standards mixture	**Linearity Range (mg/L)**	**Equation**	**r^2**	**LOD (mg/L)**	**LOQ (mg/L)**
Ethanol	6.250 – 200.0	$Y = (2787.3) + (1797.4) X$	0.9997	2.24	4.48
Methanol	6.250 – 200.0	$Y = (-209.64) + (572.23) X$	0.9999	2.87	5.74

The obtained recovery percentages ranged from 83.00% to 112.8%, with a relative standard deviation (RSD) of less than 9% (Table 3). The recoveries for methanol were slightly better than the ethanol.

Table 3. Mean of recoveries and RSDs (n=5) of ethanol and methanol spiked into clean energy drink and malt beverage samples at three spiking levels using HPLC method

Alcohol standards mixture	**Spiking Level (mg/L)**	**Energy Drink**	**Malt Beverage**
		Mean of Recovery (%) ± RSD (%)	
Ethanol	20	112.8±6.7	83.0±7.7
	40	97.9±6.0	88.6±8.8
	80	97.7±5.3	108.1±4.9
	20	84.9±7.4	86.0±7.4
Methanol	40	85.7±8.3	110.2±5.0
	80	85.4±6.7	96.5±6.3

The sensitivity was determined by estimating the limit of detection (LOD) and limit of quantification (LOQ). The LOD of ethanol and methanol were 2.24, and 2.87 mg/L, respectively and the LOQ were 4.48 and 5.74 mg/L for ethanol and methanol, respectively (Table 2).

The intra-day precision and inter-day precision were calculated and tabulated in Table 4. The intra-day precision (n = 5) values were between 8.4 and 8.5%, while the inter-day variation (n=15) values were between 10.4 and 11.0%. These values determined are lower than 15% (maximum 20%), confirming the good reproducibility and repeatability of this method (FDA 2001).

Considering the data obtained from the method validation, the current GC–FID analysis measured, and sample preparation procedures are considered as a selective, precise, and robust method to determine ethanol, and methanol in Alcohol-free" beverages, energy drinks and fruit juices samples.

Table 4. The intra-day precision and inter-day precision of ethanol and methanol expressed as RSD% values

Alcohol standards mixture	Spiking Level (mg/L)	Intra-Day Precision (n = 5)^a	Inter-Day Precision (n = 15)^a
Ethanol	40	5.3	7.6
Methanol	40	6.7	9.2

Conclusion

A simple, rapid, inexpensive, and effective sample preparation method has been developed for the determination of methanol and ethanol in Alcohol-free" beverages, energy drinks and fruit juices. The sensitivity of the GC–FID instrument could be significantly enhanced by optimizing the chromatographic conditions. Extensive and expensive clean-up procedures could be replaced by analyzing samples directly without any prior treatment followed by GC–FID separation of a mixture of methanol and ethanol. Separation of the methanol and ethanol with higher selectivity and sensitivity, and within reasonable retention time was performed. Excellent linearity, high recoveries, acceptable repeatability, and reproducibility with lower LOQ values were achieved indicating the suitability of the proposed method for the determination of alcohols in Alcohol-free" beverages, energy drinks, and fruit juices.

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Contribution of Authors

Sirhan AY: Conceived idea, conduct the study, collected data, performed the statistical analysis and write up of article
Wong R: Conceived idea and amend the article write up.
Abdulra'uf LB: Assisted in the statistical analysis and amend the article write up.
Aljabar JA: Design the study and assisted in the statistical analysis
Mostafa A: Conceived idea, conduct the study and collected data
Talhouni A: Conceived idea and amend the article write up.

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