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Evaluation of Ethanol Formation in Fruit Juices During Refrigerated Storage Time and Its Halal Status

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ABSTRACT

Halal status of ethanol resulting from natural fermentation in food is controversial. According to the GSO standard (2538:2017), the maximum residue level (MRL) of ethanol in fruit juices is 0.1% (v/v). This study examined the effect of storage duration on the formation of ethanol and sugar content in fruit juices. A total of 49 different fruit juice samples (orange, apple, berry, pineapple, and a mixture of grapes and berries) were purchased from local supermarkets and analyzed for ethanol by HS-GC-FID and sugar content (glucose, fructose, and sucrose) by HPLC-RID. The samples were stored in the refrigerator (2-5°C) throughout the experiment and analyzed every week for one month during validity period. Results showed that all juices' ethanol levels were below the MRL 0.1% (v/v) and ranged from < (LOQ: 0.02%) (orange, apple, and berry juices) up to 0.076% *(v/v)* (in mixed grape and berry juice), while total sugar content ranged from 6.5% (v/v) in orange juice up to 12.6% (v/v) in grape and berry mix juice. Ethanol levels and sugar content in all juices remained constant throughout the experiment, indicating that the fermentation process is inactive during storage and that of ethanol MRL in GSO standard is appropriate.

1. Introduction

Fruit Juice is defined as the unfermented but fermentable liquid product obtained from the edible part of sound and ripe fruit of one or more kinds mixed together having the characteristic color, flavor, and taste typical of the juice of the fruit from which it come. It may be fresh or preserved in sound condition by suitable means such as chilling [1], [2]. Fruit juice is fermentable; hence, it is prone to yield alcohol (ethanol) under specific conditions. Ethanol is an organic alcohol compound in which a hydroxyl functional group is bound to a saturated carbon atom (CH3CH2OH) (Figure 1) [3], [4].

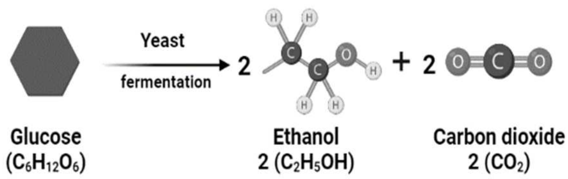

Fig. 1. Glucose fermentation by yeast for ethanol production.

From a biochemical perspective, fermentation is a process of central metabolism that occurs, in the absence of oxygen, when yeast organism converts carbohydrates, like sugar, by an enzyme called *Zymase* into ethanol and carbon dioxide [5]. During fermentation, yeast hydrolyzes starch in the fruit, producing monosaccharide-reducing sugars (such as fructose and glucose) and disaccharides (sucrose). The simple sugars are transformed into pyruvic acid by microbes using the Embden-Meyerhof-Parnas (EMP) route, which is decarboxylated to produce acetaldehyde then subsequently dehydrogenated to produce ethanol [6]. This process is time and temperature dependent.

There are two main types of ethanol, naturally fermented ethanol produced through the natural fermentation process and industrial synthetic ethanol made from ethylene as a petroleum by-product [4], [5]. Ethanol is so ubiquitous in almost all biological systems that even fresh fruits may contain trace amounts of it [7]. Previous studies have reported that ethanol levels in sterilized fruit juices were found to range from 0.01 to 0.35 % (*v/v)* [8]. Ethanol content varies depending on the food products and storage condition. A study by Yilmaz and Goren (2013) found that when fresh pineapple was stored at 4°C for ten days, its ethanol content rose from 0.48% to 1% (*v/v)* [9]. Ethanol is not permissible is Islam (not halal), therefore some of these fruit juices would be viewed as not halal (exceeds the maximum ethanol residues) in some countries, and halal in others.

Halal is synonymous with the manufacturing of food and products that have been certified as being legally lawful under Islamic Law [4]. Islamic sharia has forbidden the consumption of alcoholic products based on the two primary sources of Islamic sharia laws, the Quran, the holy book, and Sunnah, the practice of Prophet Muhammad as recorded in Hadith. There is, however, a considerable controversy surrounding the halal status of ethanol [10]. Islamic laws principles from the Quran and Hadith remain definite and unchanged, but Muslim scholars over time have interpreted it differently. When the two primary sources do not cover the situation, jurisprudence is used to determine the permissibility of food. Muslim scholars' interpretations and applications of Islamic laws might change depending on the surrounding circumstances [11]. The halal status of ethanol and Muslim scholars' interpretations mainly depends on its source and concentration [10]. In terms of halal food laws, when the impurity of food is not detectable by taste, smell, or sight, it does not nullify its halal status. Although ethanol occurs naturally and is unavoidable, that does not void the halal quality of food products it presents [7].

Halal related authorities and halal consumers recently started to recognize the value and importance of halal food requirements. Therefore, halal standards and regulations have been introduced to ensure the compliance of imported halal products [12]. The halal market is expanding globally in response to the growing Muslim population worldwide, currently estimated at 1.8 billion people [13]. Approximately 26% of the world's 7 billion population is Muslim, making up a significant portion of the food market today [14]. Globally, halal-relevant sectors, such as food, pharmaceuticals, cosmetics, and tourism etc. are worth around 3.9 trillion USD, and the trend is on the rise [13]. Consequently, the Codex Alimentarius has published in 1997 general guidelines that include requirements for the term halal, such as the scope, definition, criteria, and labeling to prevent and eliminate any unnecessary barrier to global trade [14]. According to the GSO standard (2538:2017), the maximum residue level (MRL) of ethanol in fruit juices should not exceed 0.1% (*v/v)* [15]. The permitted ethanol concentration is listed at 1% in various Association of Southeast Asian Nations (ASEAN) such as Malaysia, Indonesia, and Thailand [16].

Studies in evaluating ethanol content in fruit juices for halal status purposes have mainly focused on analyzing samples when stored at room temperature to stimulate the conditions from the prophet Muhammad (PBUH) times. However, little research has examined the levels of ethanol in fruit juice samples stored in the refrigerator, as practiced nowadays. In addition, previous studies of ethanol contents in fruit juices were short and, in most cases, did not exceed ten days, which does not cover the extended shelf life for preserved fruit juices.

This study focuses on evaluating the effect of storage duration on the formation of ethanol in fruit juices. Juice sugar content was also investigated to investigate the correlation between ethanol formation and sugar content. The proposed study evaluates the influence of storage time on the formation of ethanol in fruit juices. If ethanol levels reach high concentrations, it could be beneficial to suggest an expiry date after opening the bottle and testing the efficiency of ethanol maximum residue level (MRL 0.1% (*v/v)*) in different juices. The key research question of this study was whether ethanol would form in fruit juices during storage in the refrigerator. The experiment was conducted from the production to the expiry date, approximately one-month interval. In line with this, it is essential to study the sugar content of fruit juices since the amount of ethanol formed during fermentation will depend significantly on their initial sugar content [17]. Furthermore, a significant objective of this study is to evaluate ethanol levels found in fruit juices and their compliance with GSO standards (2538:2017) and then discuss the differences of ethanol permissible limits among Islamic halal authorities.

2. Materials and methods

2.1. Sample collection

A total of 49 commercial fruit juice samples were purchased from different local markets in Riyadh, Saudi Arabia. Fruit juice with high sugar content was utilized, such as oranges, apples, berries, pineapple, and a mix of grapes and berries was investigated for the ethanol content and sugar profile across a 28 days interval, the approximate validity period of the fruit juices. The selected fruits were chosen due to their wide availability in the markets and popularity among users.

The sample set consisted of different brands of orange juice (n = 14), apple juice (n = 14), berry juice (n = 7), pineapple juice (n = 7), and a mixture of grapes and berries juice (n = 7). Product specifications were described based on the commercial fruit juice labeling, including fruit type, production type (i.e., organic or conventional), additives (i.e., preservatives, sugars), condition (i.e., filtered, unfiltered with pulp), and expiry date. All samples were kept at (4°C) in the dark.

2.2. Chemicals and Reagents

A 2-butanol (99%) was obtained from Panreac (USA). The ethanol absolute (purity >99.7%) and gradient grade methanol (99.9%) were obtained from VWR chemicals (US). HPLC grade acetonitrile 190 (purity >99.9%) was obtained from Romil (Leicestershire, UK). D-(−)-fructose (≥99% HPLC), D-(+)-glucose (≥99.5% GC), and sucrose (≥99.5% GC), were purchased from Sigma- Aldrich (St. Louis, USA). Deionized water was obtained from a high purity Milli-Q- grade system (Elga Veolia, United Kingdom).

2.3. Preparation of standard solutions

A 2% of ethanol stock solution was prepared weekly by adding 2 mL of ethanol into a 100 mL volumetric flask, then diluted up to the mark with deionized water. 0.6% of internal standard was prepared by adding 600 µL of 2-butanol into 100 mL volumetric flask, then diluted up to the mark with deionized water. The calibration curve for determination of ethanol was prepared with concentrations of ethanol stock solution as follows (1%, 0.5%, 0.25%, 0.1%, 0.05%, 0.02%, 0.01%, 0.005%) and adding 0.006% (v/v) of internal standard to each working standard solution.

The stock solution of sugars was prepared by weighing 3g of each fructose and glucose and 1g of sucrose into a 100 mL volumetric flask, then dissolved with 50 mL of deionized water. The solution was vortexed for 30 seconds; to dissolve completely then diluted up to the mark with deionized water. The standard solution was diluted to six appropriate concentrations (0.6%, 0.9%, 1.2%, 1.8%, 2.4%, 3%) for fructose and glucose, and (0.2%, 0.3%, 0.4%, 0.6%, 0.8%, 1%) for sucrose. The stock solution of sugars is stable for six months at 4°C. Preparation of stock solution must be done in a sterile volumetric flask; to prevent any growth of fungi and preserve it for a long time.

2.4. Preparation and analysis of samples

Shortly before ethanol analysis, 3.96 mL of respective samples were piped directly into GC-HS 20 mL vial, and then 40 µL of internal standard was added. For sugar content determination, 2 mL of each fruit sample was added into a 50 mL tube, then diluted with deionized water up to 40 mL. Samples were centrifuged for 5 minutes at 5000 rpm, then filtered through a 0.45µm nylon filter and transferred into an HPLC vial. Analyses were performed in triplicates for each type and every 7 days for 28 days. One closed bottle of each juice type was stored as a control and analyzed at each time point (5 phases: day 1, 7, 14, 21, and 28).

The determination of ethanol was conducted on a gas chromatography (GC-2010) (Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector (FID) and a headspace auto-sampler (HS-20) (Shimadzu Corp., Kyoto, Japan). Helium with the flow rate of 30 ml min-1 was used as the carrier gas. A HP Innowax column (30m x 0.25mm, particle size 0.5µm) was used as stationary phase. Phase was operated under programmed temperature conditions: oven was initially set at 50°C and then increased to 85°C at 5°C min-1 in 7 min (detector and injector temperatures of 200 °C), injection volume and mode of 5.0 µL and split ratio of (10:1).

As for the analysis of sugars, a high-performance liquid chromatography (HPLC) system series 1260 (Agilent, USA) was used. Detection was performed with a refractive index detector (RID 1260 Agilent). The HPLC separation was achieved using a carbohydrate column (4.6mm x 150mm, particle size 5µm) (Agilent, USA), carried out by isocratic elution for 8 min using the mixture ACN:H2O (790:210, v/v). The mobile phase was degassed by ultrasonic bath (ASTRA S, Techno-gas, Italy) for 15 min prior to use. The system used a flow rate of 2 ml min-1, a column temperature of 25°C, and an injection volume of 5.0 µL.

3. Results and Discussion

3.1. Ethanol and sugar contents in various fruit juice

In freshly opened commercially available juices, the ethanol and sugar content were reportedly recorded at a 28 days interval to determine a sign of fermentation. All samples of juice flavored showed a detectable amount of ethanol (Figure 2). Apple juice contained a low ethanol content with a 0.006% (v/v) to 0.0058% (v/v) within a 28 days interval. The ethanol content decreased by the 14 days to 0.0048%, which increased to 0.0058% (v/v) by the 28 days. The highest levels of ethanol detected were found in freshly opened mix grape and berry juice which was recorded to be between 0.075% (v/v) and 0.069% (v/v) within a 28 days interval, where the highest detection point was at the 7 days interval with an ethanol content of 0.076% (v/v).

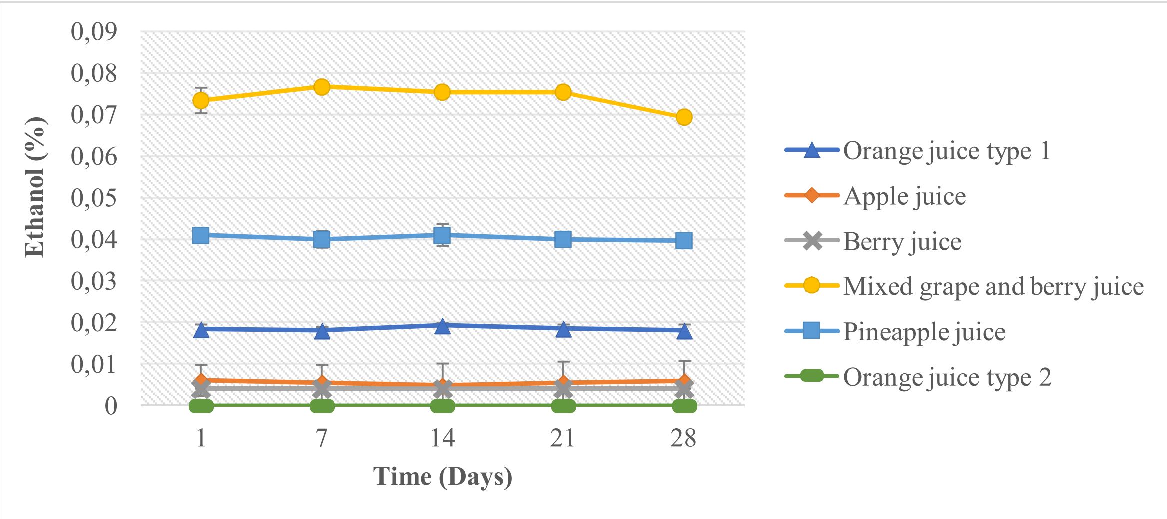

Fig. 2. Percentage of ethanol content in various fruit juices.

In contrast, samples from berry juice differed by more than 10-fold (0.004% (v/v)) in their ethanol content showing a constant ethanol content over a 28-day interval. The fresh pineapple juice showed a somewhat constant fluctuation, with the ethanol content being between 0.041 to 0.040% (v/v) in 28 days intervals. The study has examined two types of orange juice, with and without preservatives, for a spin of 28 days. The freshly opened orange juice without preservatives showed no ethanol content during the tested period. At the same time, the freshly opened orange juice with preservative showed a low ethanol content level between 0.0180% (v/v) to 0.0193% (v/v), with the highest ethanol content detected after 14 days.

A study by Aditya et al. have determined that grape juice has the highest ethanol contents between 0.01% and 0.95% due to the high reducing sugar content. In contrast, apple juice has a low ethanol content between 0% to 0.45% [6]. Gorgus et al. have examined freshly opened apple, orange, and pure grape juice and reported that the ethanol content was between 0.06–0.66 g/L, 0.16–0.73 g/L, and 0.29–0.86 g/L during 7 days in storage at room temperature; respectively [18]. Moreover, Najiha et al. reported a 0.041% to 0.720% increase in ethanol content within 5 days of storage of grape juice [8]. Roswiem et al. have measured the ethanol content for grape juice after 3 days and detected a 0.043 - 0.524% (v/v) [19]. Furthermore, pure grape juice was examined before by several studies. However, mixed grape and berry juice have not been studied before.

A study by Gunduz et al. have analyzed a pineapple juice and detected 0.048 % to 0.99 % of ethanol content during storage for 10 days at 4°C [9]. Storage of pineapple juice at 4°C has caused an increase in the ethanol content to 1%. Our results agree with the reported studies; even though pineapple juice in this study was stored at room temperature, and the effect of storage temperature on the ethanol content was not observed. Based on the previous research, it is recommended that fruits with a high sugar content be ingested fresh.

The percentage of ethanol in mixed grape and berry is much higher than in berry juice; yet the ethanol content is comparable to previous work since the used juice is mixed and not pure grape juice. The detected ethanol content in this study was lower than ethanol content in earlier studies due to the low sugar content in the juice. The findings of this study are consistent literature since the ethanol content of the analyzed fruit juice were found in traces concentrations and does not exceed the set MRL value. The majority of soft drinks, including fruit juices, contained traces of naturally occurring ethanol. The anaerobic fermentation of the fruits' sugars raised the ethanol content of the fruit day by day [9]. The ethanol group, which is typically generated during the fermentation process, is naturally present in fruits. A random examination reveals the existence of ethanol in different meals, including 0 – 0.13% in vinegar, 2 – 3% in organically harvested soy sauce, and 2.2 – 4.9% in fermented rice [4]. This indicates that the presence of ethanol in many food products occurs naturally and not on purpose. The authors do not believe that the detected ethanol content in this study poses an imminent threat to consumers, as most ethanol contents were below the set MRLs. Overall, the levels of ethanol were constant in all juices, and no significant changes in ethanol concentration was noticed. Several publications on the usage of sugar by yeast have been published, and it has been published that the rate of sugar intake essentially restricts the rate of ethanol generation by yeast [8].

The concentration of sugar content in the examined juices has not shown a significant change over time during the 28 days fermentation period (Figure 3). The apple juice sample detected a higher fructose content (5.34%) in comparison to glucose (2.165%) and sucrose (1.32%) (Figure 3C). Berry juice samples showed a higher fructose content (4.33%) in comparison to glucose (3.37%) and sucrose (0.65%) (Figure 3D). The examined mix berry and grape juice sample showed a higher fructose content (6.31%) in comparison to glucose content (6.2%) with no sucrose detected (Figure 3E). In pineapple, the juice sample showed a higher sucrose content (6.31%) in comparison to fructose content (1.95%) and glucose content (1.9%) (Figure 3F). Orange juice with (Figure 3D) or without (Figure 3E) preservative detected a higher sucrose content (4.03% or 3.55%) in comparison to fructose content (1.67% or 1.98%) and glucose content (1.25% or 1.56%); respectively. The total sugar was measured in apple juice (8.82%), berry juice (8.34%), mixed berry and grape juice (12.55%), pineapple juice (10.15%), orange juice with preservative (6.96%), and orange juice without preservative (7.09%) compared to total sugar amount list on the labeled to be 10.8%, 9.2%, 14.5%, 10.5%, 8.90%, and 9.50%; respectively (Table 1).

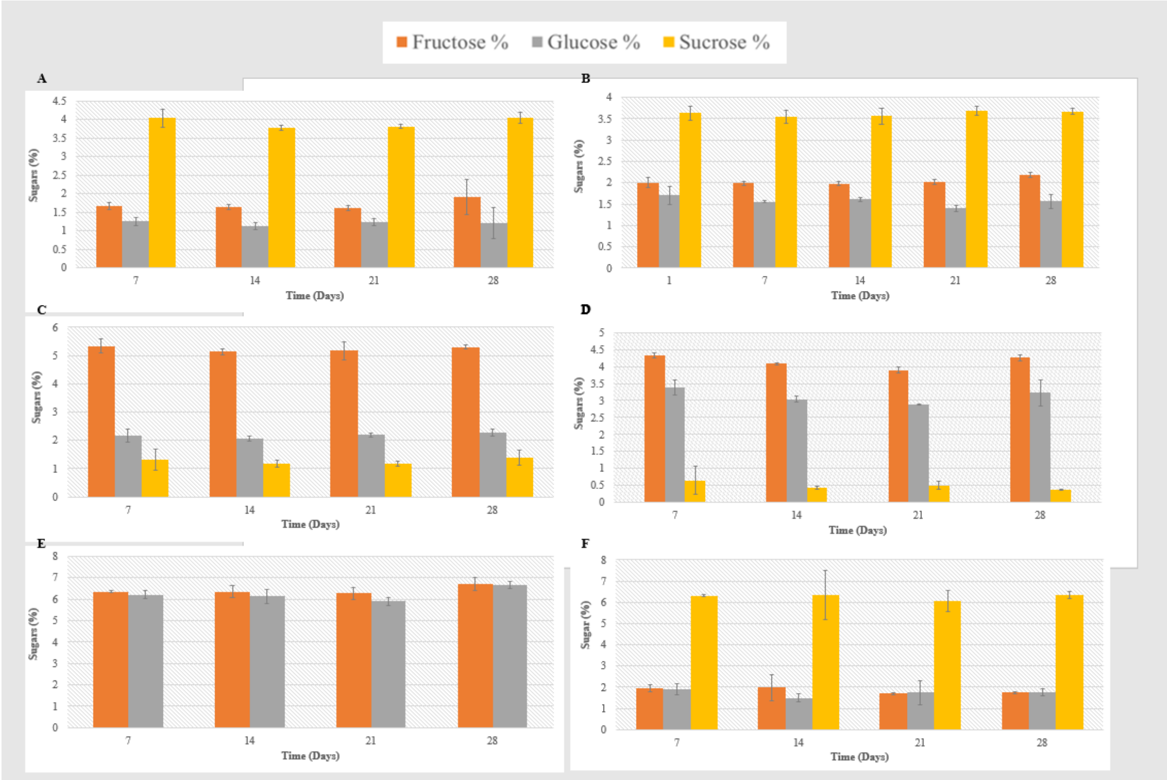

Fig. 3. Percentage of sugar content in various fruit juices. (A) Apple juice. (B) Berry juice. (C) Berry and grape mixed juice. (D) Orange juice with preservative. (E) Orange juice without preservative. (F) Pineapple juice. All plots use orange color for percentage of fructose, grey color for percentage of glucose and yellow color for percentage of sucrose.

Table 1. Ethanol and sugars content in fruit juice samples.

Samples	Alcohol	Sugar Content			Total Sugars (g/100ml)	
	Ethanol %	Fructose %	Glucose %	Sucrose %	Experimental	Labeled
Apple	0.009	5.34	2.165	1.32	8.82	10.8
Berry	<LOQ	4.33	3.37	0.65	8.34	9.2
Grapes and Berries	0.041	6.35	6.2	ND	12.55	14.5
Orange without preservative	ND	1.98	1.56	3.55	7.09	9.5
Orange with preservative	0.018	1.67	1.25	4.03	6.96	8.9
Pineapple	0.073	1.95	1.9	6.31	10.15	10.5

The amount of ethanol and acetic acid produced during fermentation is heavily influenced by the starting sugar concentration. Because different types of fruits have varying sugar levels, it is critical to know the sugar content of the fruit juices. Interestingly, ethanol levels and sugar content in all juices were constant during the experiment, indicating that the fermentation process is inactive during storage. The sugar level of fresh fruit juice is typically 10% for apples, 9% for oranges, and 15% for grapes [20]. At the end of the fermentation process, 55% of the sugars found in grapes were transformed into ethanol [16]. The recorded total sugar content for all test juice was higher than the stated sugar content in the manufacture labeled.

3.2. Limits on ethanol level in juice in comparison to some Islamic countries

In respect to compliance of fruit juice with the GSO standard (2538:2017) for ethanol, all results were below the MRL 0.1% (v/v), ranging from not detected to 0.076% (v/v). The findings match those observed in previous studies of Gorgus et al. found that orange, apple, and grape juice contain substantial amounts of ethanol up to 0.077 % [18]. In view of these findings, the results indicate that ethanol MRL in GSO standard is adequate and appropriate for its purpose in food matrix. The GSO standards include requirements for halal accreditation bodies, halal certification bodies, management systems for halal, animal slaughtering requirements according to Islamic rules, detection of lard in food, and the maximum limits for ethanol residues in food. Regarding ethanol MRL in food, GSO standard (2538:2017) sets limits for ethanol that naturally occur in some food matrices (Table 2). The GSO standard prohibits the addition of ethanol or alcoholic products to food during food manufacturing. In some cases, it is permissible to use additives in which ethanol is used as a solvent when its usage is indispensable in the food manufacturing process, not exceeding the maximum level of ethyl alcohol (ethanol) in the final product [21].

Table 2. Ethanol maximum limits in final food precuts in (GSO: 2538) standard.

Food matrix	Ethanol MRL
Grape vinegar	1%
All vinegar types except grape vinegar	0.5%
Souses and ketchup, drinks/ concentrated juices, concentrates, and food mixtures prepared for manufacture and aromatic herbal oils	0.5%
Juices including nectars, cocktails and drinks on different types and shapes and flavored water ready to drink	0.1%
Fresh or processed foods produced from meat, dairy, grains, pulses, oils, eggs, seafood, spices and candy	0.3%
Raw materials such as protein concentrates, sugars, yeast, essential oils, raw cocoa and other similar raw materials	0.5%
Chocolate	0.02%
Other foods	0.02%

The GSO technical committee established the maximum ethanol limits in food by reviewing related national and international standards, with approval from the official Islamic authorities in Gulf countries and supported by scientific opinion via laboratory analysis (GSO,2017). In comparison to other Islamic halal bodies, the religious authorities in the Southeast Asian region, including Brunei, Malaysia, Indonesia, and Singapore, are among the first to issue certifications and accreditations for halal products. In line with the Association of Southeast Asian Nations (ASEAN), those countries collaborate through a governing body known as MABIMS (The Informal Meeting of Religious Ministries of Brunei, Indonesia, Malaysia, and Singapore). MABIM's primary role is to ensure that there are no disputes among its members regarding the rules and regulations of halal certification. Although ASEAN members are from one region and follow the same school of thought in Islamic law, it can be seen in Table 3 that there are dissimilarities between them regarding halal standards [4].

Table 3. The percentage of permissible ethanol in halal food and drinks in ASEAN members.

Nation	Percentage of Ethanol
Malaysia	1% of naturally formed ethanol 0.5% of industrial ethanol
Singapore	Naturally formed ethanol is not stated Industrial ethanol is less than 0.5% additives, 0.1% remains in the final product
Indonesia	1% of naturally formed ethanol Industrial ethanol is1 % for additives, but in the final product must have 0.0% presence of ethanol
Brunei	2% of naturally formed ethanol Industrial ethanol is prohibited

The differences between halal standards in Islamic countries, especially in the maximum amount of ethanol in food, are due to the different interpretations of Islamic laws according to the different Islamic schools. It is also attributable to different approaches in setting the maximum limits between halal authorities. To illustrate, the GSO standards set a variety of ethanol maximum residue limits based on the food matrix by evaluating normal ethanol presence levels within a specific matrix. At the same time, ASEAN countries have standardized ethanol maximum limit for all food commodities, though there are different means to define the maximum limit among ASEAN countries.

Differences in halal standards and requirements among Islamic countries is a significant issue causing trade barriers. To address these issues, the Standards and Metrology Institute of Islamic Countries (SMIIC) was established in Turkey under the umbrella of the Organization of Islamic Cooperation (OIC) with 36 members. SMIIC is developing a reliable certification system based on common international halal standards. Although some progress has been made in creating a common halal accreditation system within the Institute, it is not yet operational [13]. Differences between halal governing bodies regarding permissible ethanol levels have raised academic debate among scholars and is considered one of the main areas of halal research [4]. In a study of ethanol and its halal status in food industries, [5] suggested that any ethanol from natural fermentation of less than 1% is considered a preserving agent and is therefore halal, relying on the prophet Mohammad saying, “Anything which intoxicates in large quantity is prohibited even in a small quantity” (Sunan Ibn Majah, Book 30, Hadith no. 3392).

A study by Alzeer and Abou Hadeed assumed that if a female weighing 55 kg consumes 1% ethanol within an hour, intoxication is reached (BAC = 0.09%) after drinking 4000 ml volume [5]. Consumption of 4 liters of water in an hour will result in rapid and severe hyponatremia, a condition that occurs when the sodium level in the blood is abnormally low and leads to the entry of water into the brain cells, causing brain swelling then subsequently comma and death. They argued that ethanol below 1% should be viewed as halal, Since reaching intoxication from less than 1% ethanol is practically impossible. However, these conclusions need to be interpreted with caution since sensational changes can be reached at BAC levels starting from 0.01% [22]. That leads to a question that needs to be asked, what is the level of intoxication meant in Prophet Mohammad saying, “Anything which intoxicates in large quantity is prohibited even in a small quantity”.

The GSO standards set ethanol limits based on the natural presence of ethanol in the dietary products, considering the dietary product's consumption. This method of determining ethanol MRL is in agreement with the ideas of Pauzi et al., 2019 [4], who argued that the consumption manner of dietary products is often ignored in determining the permissible threshold of ethanol in halal dietary products. Indeed, it is not justified to compare the ethanol content of vinegar with that of beer because both products are consumed differently. As a result, there would be a different threshold for intoxication among dietary products.

4. Conclusion

The study examined the ethanol levels and sugar content in fruit juices during a 28 days interval. Our results show that there were no significant changes in ethanol levels during the experiment and that ethanol content ranged from <LOQ (0.005%) up to 0.076%, which is well below GSO standard (2538:2017) for ethanol. The findings of this study support the appropriateness of GSO standards of permissible ethanol limits in fruit juices. However, there are several approaches for determining maximum ethanol levels in food and drinks, which has resulted in the divergence of ethanol MRL's among different authorities. This may lead to the confusion of halal customers and the complication of trade. Therefore, a definite need for the establishment of common and effective halal standards to support halal trade between Islamic countries.

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