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Determination of Ethanol in Vinegar and Beverage by Gas Chromatography: A Validated Method for Halal Verification

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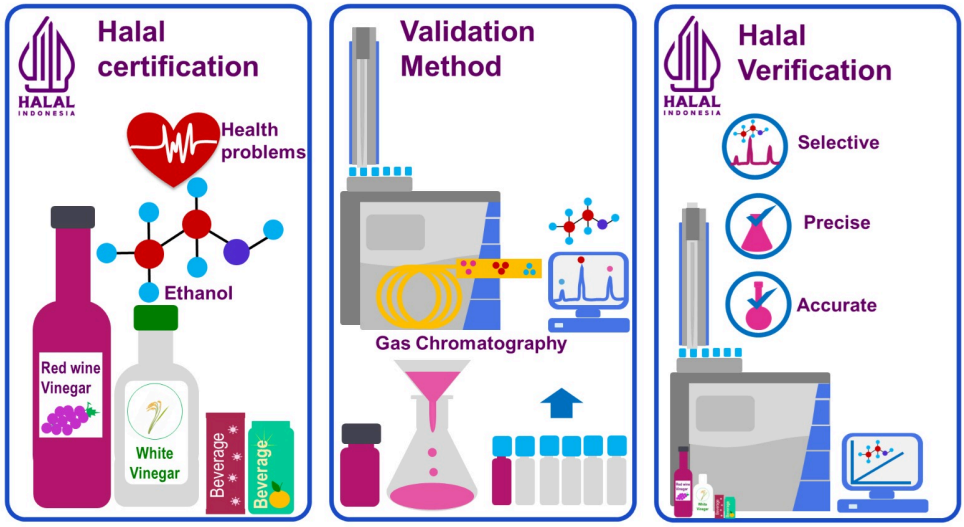
GRAPHICAL ABSTRACT



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ABSTRACT

The method for determining ethanol in vinegar and beverages has been validated in the linearity range of 0.05–1% using GC-FID. This research was conducted using and without an internal standard solution. This study validated the linearity of ethanol determination at low concentrations. This validation helps ensure that the ethanol test method in the sample can be detected at low concentrations. The maximum ethanol content is 5% in the halal authentication process. The method validation results confirm that in the concentration range 0.05-1%, it has good linearity with a correlation coefficient (R) without the addition of internal standards and with the addition of standard solutions being 0.9972 (R^2=0.9943) and 0.9952 (R^2=0.9905). The results of the analysis of variance (ANOVA) test with a 95% confidence interval and 9 degrees of freedom F-count (0.0141) < F-critical (0.3146) show that the range 0.05-1% corresponds to the limit of linearity. The results of the ANOVA test with a 95% confidence interval with df=6 show the F-test value (0.0879) < F-critical (0.2334), indicating that the two proposed

methods have high precision and no significant differences. Both methods also have high accuracy and no significant differences in accuracy, as shown by the results of the ANOVA test with a 95% confidence interval and degrees of freedom = 6, showing the F-test value (1.5204) < F-critical (4.2839). It is recommended that the results of this validation be helpful in the halal verification process for vinegar and beverage products.

1. INTRODUCTION

Halal verification is essential for guaranteeing the halal of food and beverage products. Testing for ethanol content is one of the critical parameters used to test food ingredients or products in the laboratory. Alcohol is an intoxicating and dangerous drink. Excessive alcohol consumption can cause liver injury [1]. At a concentration of 15-50%, ethanol has chronic effects on test animals, and consumption for 160 days can cause death due to damage to the liver, lymph, and kidneys [2].

According to Islamic jurisprudence, the term alcoholic food or drink comes from the word khamr, namely, food or drink that is an intoxicating drink such as beer, wine, and wine that contains ethanol as the main ingredient of alcohol [3]. Alcohol can cause nervous system disorders and poisoning. Khamr is unclean and haram for consumption by Muslims [3]. Alcohol consumption can cause the risk of health problems [4]. Ethanol derived from the khamr manufacturing process intentionally added to food or beverage products, will be haram for consumption [3].

Legal regulations relating to the trade in products suspected of containing alcohol apply strict standards, including determining the ethanol content on a product's label [5]. In the Fatwa of the Indonesian Ulema Council (MUI) number 10 of 2018, fermented beverage products have halal criteria if they contain no more than 0.5% alcohol or ethanol. Following halal regulations and standards, the permitted ethanol comes from non-khamr, naturally found in food and drinks, such as fruit, grains, juice, soy sauce, kimchi, and vinegar. One product that needs attention is vinegar and beverages. Vinegar from fermentation, naturally or through engineering, is halal and holy. The ethanol content in vinegar should not exceed 0.5%. Likewise, whether fermented naturally or through engineering, beverage products containing fruit extracts must not contain more than 0.5% alcohol. The analysis results of samples of vinegar and tea, milk, fruit, coffee, flavored, and honey had an ethanol content below 0.5% [6, 7]. The test results verify that the sample is halal for consumption. However, not all vinegar and beverage products have received halal product certification. The presence of ethanol in vinegar and beverage products must still be confirmed to ensure the ethanol content is below 0.5%.

An analytical method with high sensitivity is required to ensure the ethanol content in this concentration range. Therefore, verification steps are needed to detect ethanol content in beverage products that can detect up to a concentration of 0.5%. The gas chromatography flame ionization detector (GC-FID) method is an analytical method developed in the halal verification process to ensure the alcohol content of food and drinks. This method is regularly used in forensic toxicology [5]. Gas chromatography is a qualitative and quantitative analysis method that can provide information regarding retention time, verify the identity of alcohol, and simultaneously measure its concentration [8]. The GC-FID method has high sensitivity, accuracy, validity, and percentage recovery [9-11].

The GC method is sensitive and precise with a sufficiently wide range and has a linear measurement range at intervals of 1-20% [12], 0.01–20% [5], and 1-10% [9]. This concentration range is suitable for alcohol testing in beer, wine, and other high-concentration alcoholic beverages [5]. The ethanol content at low concentrations in samples that have quite complex matrices may not be read by GC-FID [10]. Testing of low-concentration alcoholic beverages, vinegar, fermented products, and low-concentration alcohol requires methods with good sensitivity and detection limits in the lower linearity range. Therefore, various studies were conducted to ensure the linearity range at lower concentration levels. This method has been used to determine the ethanol content in drug samples using a linearity range of 0 – 10,000 µg/mL [13].

This research was conducted to validate the ethanol analysis method with GC-FID in the low concentration range, whether using or without internal standards. This method was developed for vinegar and beverage samples with various sample matrices. The GC-FID method is commonly used for alcohol analysis in halal verification. The development of the GC-FID method has been widely carried out in the halal verification process. However, in low concentration limits and sample matrix conditions, method validation is required to evaluate linearity parameters, detection limits, precision, and accuracy [6, 12]. This method was validated to increase the reliability of the ethanol analysis method with GC-FID for the halal verification process for vinegar and beverage products. Validation of the method for determining ethanol in vinegar with GC-FID in the range of 0.02–1.5 % v/v gave a linearity of 0.9995 [13]. This method provides a limit of detection of 4.89 x 10^{-4} % and a limit of quantification of 1.48x10^{-3} % [13]. Validation of the method for determining ethanol in beverages in the range of 1–5% obtained a correlation coefficient of 0.9999 with a limit of detection of 0.067 % and a limit of quantification of 0.188% [14]. The results of this validation have been tested to determine the ethanol content in vinegar containing 1.17x10^{-2}–2.28x10^{-2} % v/v ethanol [13]. Validation of the method with a concentration range of 12.5–75 µg/mL obtained a correlation coefficient of 0.9995 [15]. Validation of the method with a concentration range of 0.2–3.5 mg/mL resulted in a linearity of 0.993 and a limit detection of 0.13 mg/mL [16]. In this study, method validation will be carried out using internal standards or without internal standards for determining ethanol in vinegar and beverages with a concentration range of 0.05-1%. The validation results of this method can be used as a consideration in determining a routine test method for ethanol content in vinegar and beverage samples that are in the low concentration range.

2. EXPERIMENTAL METHODS

2.1. Materials

Ethanol (≥ 99,9 %; Merck KGaA Darmstadt Germany), 2-propanol (≥ 99,9 %; Merck KGaA Darmstadt Germany), and distilled water (analytical grade; Chemistry Department Laboratory, Universitas Islam Indonesia) were used for analysis. The samples tested were samples of vinegar and beverages sold in supermarkets.

2.2. Determination of Ethanol Using Gas Chromatography

2.2.1 Preparation of alcohol stock and standard solution

Ethanol stock solution 10 % v/v was prepared by diluting 1 mL of ethanol ≥ 99.9 % in a 10 mL volumetric flask and was adjusted to the volume with distilled water. The 2-propanol internal standard solution 10 % v/v was prepared by diluting 1 mL of 2-propanol ≥ 99.9 % in a 10 mL volumetric flask and was adjusted to the volume with distilled water. The standard ethanol solution was made with a concentration of 0.05; 0.1; 0.2; 0.4; 0.6; 0.8; and 1.0% v/v. A total of 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of 10% ethanol stock solution was placed in a 10 mL volumetric flask. The solution was added with 10 µL of 10% v/v 2-propanol internal standard solution [6, 12, 16] and the volume was adjusted with distilled water. The preparation of standard solution without internal standards was carried out as a comparison.

2.2.2 Preparation of sample

The sample was filtered using Wathman 41 filter paper. 500 µL of the sample was added with 10 µL of 2-propanol internal standard solution and homogenized. Accuracy measurements were carried out with 500 µL of sample, 10 µL of 10% v/v ethanol stock solution, and 10 µL of 10% v/v 2-propanol internal standard solution [6, 16]. Sample preparation without internal standards was carried out as a comparison.

2.2.3 Analysis of ethanol using gas chromatography

Quantitative analysis of ethanol in the sample was performed using a GC-FID (Trace 1310 GC System, Thermo Fisher Scientific, United States of America) with a TraceGOLD TG-5MS GC column [30

m (L) × 0.32 mm (ID) × 0.25 µm film thickness; Thermo Scientific, Thermo Fisher Scientific] as a stationary phase. Each sample (0,5 µL) was injected in split mode with a split ratio of 40 (split). The gas flow rates were maintained as follows: carrier gas (helium, 1 mL/min), hydrogen (35 mL/min), and air (300 mL/min) [6], [17]. The injector and detector temperatures were maintained at 250 °C [14]. The oven temperature was initially held at 30 °C for 5 min and increased to 120 °C at a rate of 5 °C/min. The total run time was 23 min.

3. RESULTS AND DISCUSSIONS

3.1. Determination of Linearity and Limit of Detection

In this research, the method for determining ethanol in vinegar and beverages was validated using the GC-FID method, referring to the AOAC 2016.12 method [6], which was modified with the matrix separation method by filtration. The analysis results were quantified using the internal standard 2-propanol in a concentration range of 0.05 - 1% v/v. The results of this validation can be applied in the halal verification process for vinegar and beverage products.

Figure 1 (a) shows the chromatogram profile of a 0.05 - 1% ethanol standard solution without an internal standard (IS), and Figure 1 (b) uses an internal standard. The internal peak of the 2-propanol standard is shown in Figure 1(c). Separation of ethanol produces a chromatogram peak with a retention time of 3.4 minutes, and separation of 2-propanol produces a chromatogram peak with a retention time of 3.52 minutes. Separation of the ethanol standard solution from the 2-propanol internal standard solution provides good resolution (Rs =1.95 ± 0.09). The resolution value indicates the degree of separation of mixture components in gas chromatography analysis with high selectivity at optimum conditions [9]. The selectivity value is categorized as good with Rs ≥ 1.5, shown in the chromatogram, where the mixture is separated [9, 18, 19]. The addition of propanol as an internal standard was modified and used for peak separation and quantitative determination of ethanol [6].

The calibration curve of the ethanol standard solution with the addition of the internal standard solution and without the addition of the internal standard solution is shown in Figure 2. The calibration curve is used to evaluate linearity over the range of the standard series proposed in this study. The linearity of each analysis method shows the ability to obtain test results directly proportional to the variable data in different concentrations [14]. Figure 2 shows that both standard series provide good linearity with a correlation coefficient (R) of 0.9972 (R^2=0.9943) and 0.9952 (R^2=0.9905). This method offers a high linear response between areas peak and concentration [18, 19]. Ethanol measurements with a linearity range between 0.05 and 1 % show satisfactory linearity [6, 12, 16]. Adding an internal standard solution can increase the measurement sensitivity as indicated by the linear regression equation y = 8.6878x + 2.0967. In contrast, in the standard series, without the addition of the internal standard solution, it follows the equation y = 8.2191x + 0,2341. Based on the data in Table VI, the linearity of these two methods can also be compared with the linearity in previous studies.

The ethanol standard solution without adding the internal standard solution provides a lower detection limit than the standard solution with the addition of the internal standard solution. The limit of detection values is 0.09 and 0.13 % v/v, respectively. These two-standard series with test ranges provide detection limits below 0.5%, so this range can be used to verify vinegar and beverage samples with lower concentration levels. Based on the data in Table VI, the detection limits of these two methods provide results at low concentration levels as in the detection limits in previous studies.

The limit of detection is the lowest concentration of analyte in a sample that can be detected and provide a significant response [9]. The research showed a detection limit value of 0.09% for measurements without internal standards. This data indicates that the gas chromatography method has high sensitivity and responds [9, 18] at minimal concentrations up to 0.09% v/v. Determination of ethanol with the addition of internal standards also showed a detection limit of 0.13%. This method provides high sensitivity with a response up to a concentration of 0.13% v/v.

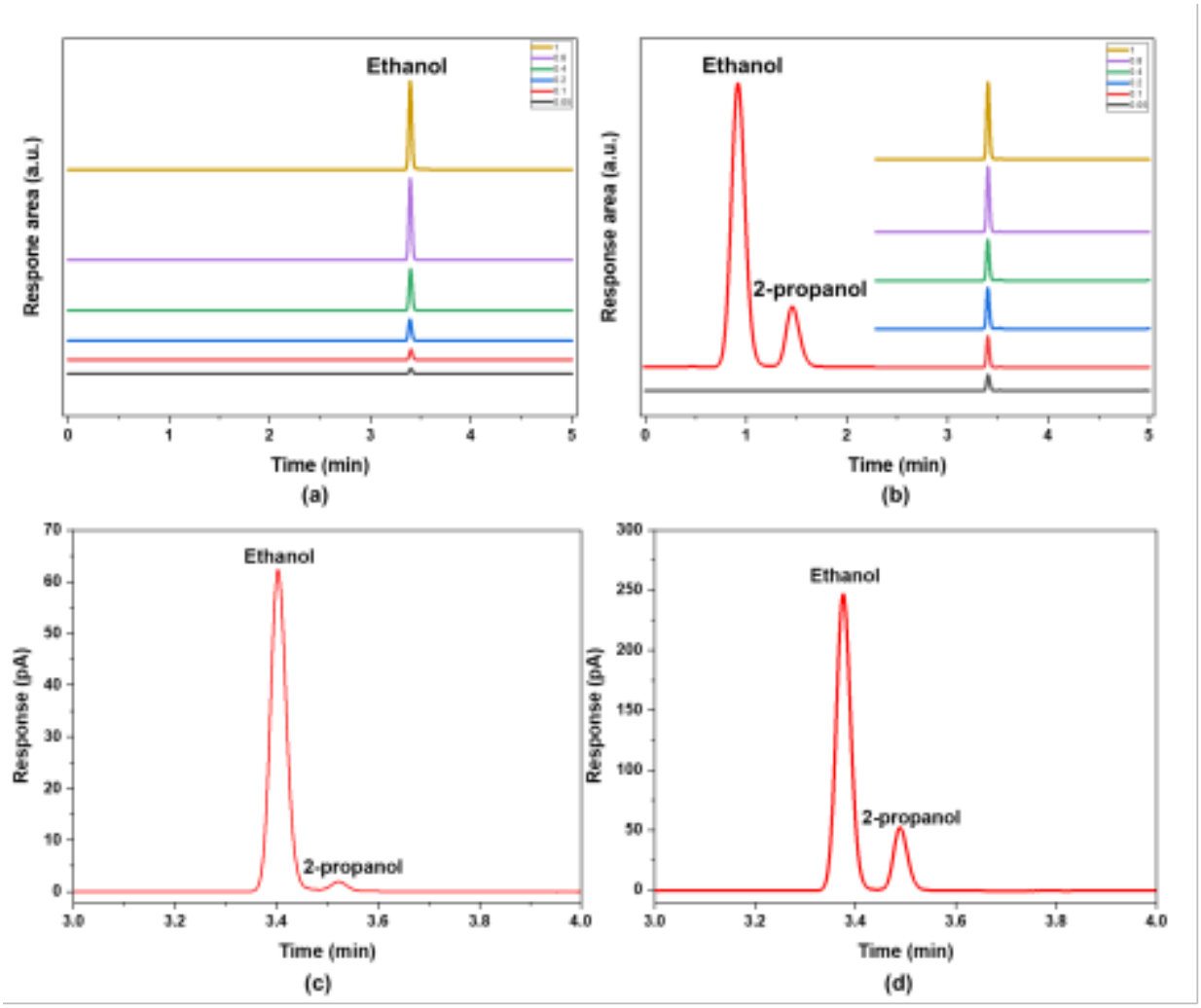

Figure 1. Chromatogram for (a) standard ethanol without internal standard, (b) standard ethanol with internal standard (2-propanol), and (c) ethanol standard (0.05 % v/v) with internal standard (0,001 % v/v 2-propanol), and (d) sample with internal standard (50 : 1 v/v).

The standard series range of 0.05-1% can be recommended as a measurement range for vinegar and beverages. The linearity acceptance criteria can be reviewed by determining the limit of linearity. The evaluation was carried out using the F-test to compare the differences in variance [16] in standard solutions at the lowest and highest concentration limits. The results of the limit of linearity test by measuring concentrations of 0.05 and 1% with ten replications are shown in Table I. The results of the analysis of variance (ANOVA) test with a 95% confidence interval and 9 degrees of freedom in Table II show an F-test (0.0141) < F-critical (0.3146). The results of the ANOVA test show that the results of measuring the chromatogram area with concentrations of 0.05 and 1% have the same variance.

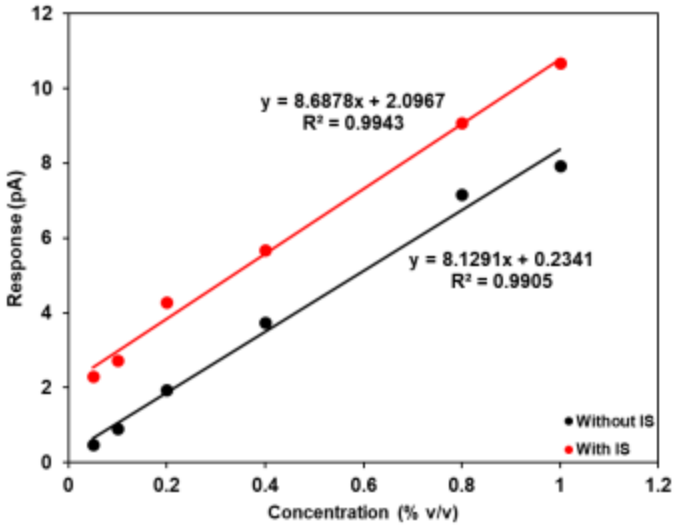

Figure 2. Calibration curve for standard ethanol without internal standard (IS) and standard ethanol with internal standard (2-propanol).

TABLE I. Chromatogram peak area of 0.05 and 1% v/v ethanol standard solutions.

Replication	pA of 0.05 % v/v ethanol standard	pA of 1 % v/v ethanol standard
1	0.353	7.013
2	0.343	6.519
3	0.461	6.96
4	0.343	6.777
5	0.375	7.451
6	0.321	6.271
7	0.305	6.852
8	0.343	7.423
9	0.344	6.902
10	0.294	7.385

TABLE II. F-Test Two-Sample for Variances.

	pA of 0.05 % v/v ethanol standard	pA of 1 % v/v ethanol standard
Mean	0.3482	6.9553
Variance	0.0021	0.1507
Observations	10	10
df	9	9
F	0.0141	
P(F<=f) one-tail	2.79×10^{-7}	
F Critical one-tail	0.3146	

3.2. Determination of Precision and Accuracy

The determination of ethanol in vinegar and beverage samples is presented in Table III. The results of the analysis without internal standards showed that the ethanol content in the table vinegar, red wine vinegar, rice vinegar, and beverage samples was below the detection limit value, so it could not be quantified properly. The ethanol content in the white and red wine vinegar samples was above the limit of detection value.

Different from the results of the analysis with the addition of internal standards. White vinegar and red wine vinegar samples can be observed in the analysis results by adding internal standards. This shows that testing with the addition of internal standards can increase the response of ethanol measurements at low concentration levels. Internal standards are used in analysis based on chromatograms due to fluctuations in instrument parameters, which affect the accuracy of the analysis [9, 18]. However, both tests with and without internal standards provide good precision, respectively, with a % relative standard deviation (%RSD) of 3.31 and 2.12. Test precision satisfies the requirements if it is less than 5%, which indicates that the GC-FID method for measurements at low concentration levels has high validity [5, 11, 18].

TABLE III. Concentration of ethanol, precision, and accuracy.

	GC-FID without IS			GC-FID with IS		
Sample	% Ethanol	% RSD	% Recovery	% Ethanol	% RSD	% Recovery
Table vinegar	-0.02 ± 0.00	1.67	96	0.03 ± 0.00	0	82
White vinegar	0.24 ± 0.01	2.55	99	0.58 ± 0.03	4.99	102
Red wine vinegar	0.08 ± 0.01	2.04	76	1.00 ± 0.03	3.01	83
Rice vinegar	0.05 ± 0.01	3.12	98	0.35 ± 0.02	5.41	101
Plain beverage	-0.01 ± 0.00	2.82	97	0.05 ± 0.00	0	92
Flavored beverage	-0.02 ± 0.00	2.65	75	0.32 ± 0.00	0.34	81
Energy enhancing beverage	-0.02 ± 0.00	0	81	0.25 ± 0.02	9.45	94

TABLE IV. F-Test Two-Sample for Variances of Precision

	Precision value without IS	Precision value with IS
Mean	2.12	3.31
Variance	1.11	12.63
Observations	7	7
df	6	6
F	0.0879	
P(F<=f) one-tail	0.0047	
F Critical one-tail	0.2334	

TABLE V. F-Test Two-Sample for Variances of Accuracy

	Accuracy value without IS	Accuracy value with IS
Mean	88.86	90.71
Variance	120.47	79.24
Observations	7	7
df	6	6

F	1.5204
P(F<=f) one-tail	0.3119
F Critical one-tail	4.2839

The % RSD value of testing without an internal standard is lower than testing with an internal standard. The results of the ANOVA test with a 95% confidence interval with df=6 in Table IV show the F-test value (0.0879) < F-critical (0.2334). The ANOVA test showed no significant difference in variance in the precision of the results of ethanol analysis with or without internal standards. This data indicates that the limit of detection influences ethanol testing in the low concentration range.

The ANOVA test results in Table V with a 95% confidence interval and degrees of freedom = 6 show the F-test value (1.5204) < F-critical (4.2839). The results of the ANOVA test indicated that testing with an internal standard and without an internal standard did not provide a difference in variance in the accuracy values. The average accuracy in ethanol testing was 90.71 and 88.86%, respectively, which indicates high accuracy [6, 11]. The accuracy value is determined from the % recovery.

The chromatography method provides reliable, precise, and accurate results for testing samples with complex matrix characteristics [6, 12, 20]. This GC method can be developed as simple, easy, specific, precise, accurate, selective, and reliable for determining ethanol at low concentration levels [14, 16]. Table VI shows that the two proposed methods provide precision and accuracy based on previous studies' range of precision and accuracy data.

TABLE VI. The comparison of determination of ethanol using GC-FID methods.

Sample	Methods	Linear range	Linearity	LOD	Precision (%)	Accuracy (%)	References
Vinegar and beverages	GC-FID	0.05-1 % (v/v)	0.9952	0.09 % (v/v)	2.12	88.86	Present work
Vinegar and beverages	GC-FID with IS	0.05-1 % (v/v)	0.9972	0.13 % (v/v)	3.31	90.71	Present work
Vinegar	GC-FID with IS	0.025-1.5 % (v/v)	0,9995	$4,89.10^{-4}$ %	5,63	101,25	[21]
Beverages	GC-FID	1-5 % (v/v)	0.9999	0.067 % (v/v)	0.008-0.143	98.453-101.833	[14]
Beverages	GC-FID	6.25-200 mg/L	0.9997	2.24 mg/L	<9%	83.00-112.8%	[11]
Beverages	GC-FID with IS	0.01-20.0% (v/v)	0.999	0.003%	<5%	-	[5]
Foods and beverages	GC-FID with IS	1-10 % (v/v)	0.9984	0,15 ng	≤ 2%	98-102	[9]
Foods and beverages	GC-FID with IS	0.003-0.161 % (v/v)	-	-	-	-	[4]
Foods and beverages	GC-FID with IS	0.0003–0.0012 mg/mL	>0.999	0.0003 mg/g	<5%	96-105	[6]
Bioethanol	GC-FID	0.049-100 g/L	0.99	0.012 g/L	0.943	-	[19]
Drug	GC-FID	12.5-75 µg/mL	0.9995	-	1.09	-	[15]
Drug	GC-FID	0-8000 µg/mL	0.999	2,8 mg/L	-	-	[13]
Blood	GC-FID	20-200 mg/dL	0.999	0.5 mg/dL	4,47	99.8	[17]
Blood	GC-FID with IS	0.1-3.5 mg/mL	0.993	0.099 mg/mL	27	91.0-109.1	[16]

4. CONCLUSIONS

The study results validate the linearity, detection limit, precision, and accuracy in determining ethanol in vinegar and beverage products by considering the condition of the sample matrix. Testing can be recommended in the linearity range of 0.05-1% v/v, either with the addition of an internal standard solution or without an internal standard solution. The research results have been validated with high linearity,

precision, and accuracy for detection limits for low ethanol concentration levels. This method of determining ethanol at low concentration levels can be used in the halal authentication process for vinegar and beverage products.

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