

# Validation of a Method for Characterization of Ethanol in Water by HS-GC-FID to Serve the Traceability of Halal Measurements

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## Abstract

The determination of the ethanol content in food products is of fundamental importance for HALAL certification. In this work, an analytical method for the determination of ethanol in water by headspace gas chromatography with flame ionization detector (HS-GC-FID) has been developed and validated for the use in characterization of ethanol reference materials. The validation study was carried out in the linear calibration range 100 - 1500 mg/kg using the NIST SRM 2900, nominal 95.6%. The studied performance characteristics of the method were the limit of detection, LOD, the limit of quantification LOQ, selectivity, linearity, precision, recovery and bias. The validation results showed that the method is selective, precise, accurate and free from any significant bias. The LOD and LOQ were 1.27 and 3.86 mg/kg respectively and the estimated expanded uncertainty was 2% indicating that the method is fit for the purpose of certification of ethanol in water reference materials.

## Keywords

Ethanol, HS-GC-FID, Validation, Linearity, Precision, Bias

## 1. Introduction

The global Halal market encompassing both food and beverage products is growing significantly due to the anticipated rise in the global Muslim population [1]. While alcoholic and intoxicating ingredients are strictly forbidden, Halal regulations and standards allow permissible types of ethanol in food and beverage products [2] [3] [4]. These include ethanol naturally occurring in foods/

beverages like fruits, grains and juices as well as ethanol naturally formed through the fermentation process in foods/beverages like soy sauce and vinegar [5]. Additionally, unfermented industrial ethanol used in food processing, preservation, or even the extraction of valuable natural products from plant and animal sources can be permissible for Halal food and beverage production, provided proper regulations are followed [1] [6] [7] [8] [9] [10]. Notably, effective Halal quality control necessitates adequate analytical capabilities particularly for the quantitative analysis of haram ingredients at very low concentrations, especially in products intended for export [11]. There are several methods for determining ethanol concentration. One common technique is gravimetric analysis, which uses a calibrated hydrometer to measure the density of a solution. A reference table then correlates this density to the ethanol content [12]. Distillation is another method, where ethanol is separated from the sample matrix and the collected distillate is weighed. The ethanol content is then calculated based on the initial sample weight and the weight of the isolated ethanol fraction [13]. Spectrophotometric methods can also be used. These methods involve reactions that convert ethanol to a colored compound. The intensity of this color is measured using a spectrophotometer and correlated to the ethanol concentration in the sample [14]. Enzymatic assays utilize enzymes specific to ethanol (alcohol dehydrogenase) to convert it into another product. This conversion can be monitored by measuring changes in absorbance, fluorescence, or other parameters. The measured change is then correlated to the initial ethanol concentration [15]. Finally, Gas chromatography-mass spectrometry (GC-MS) offers both confirmation of ethanol identity and quantification. It can detect ethanol at very low levels, making it suitable for forensic applications like blood alcohol content (BAC) analysis [16]. Headspace Gas Chromatography with Flame Ionization Detection (HS-GC-FID) provides significant advantages compared to traditional methods. It requires minimal sample preparation, typically involving headspace equilibration followed by injection of a small aliquot. This minimizes matrix interferences and potential analyte losses. HS-GC-FID also boasts high sensitivity thanks to FID detectors, allowing detection of very low ethanol concentrations, crucial for applications like BAC analysis. Additionally, HS-GC-FID offers excellent specificity for ethanol determination, differentiating it from other volatile compounds in complex matrices due to the separation power of gas chromatography [17] [18] [19] [20] [21]. However, accurate measurements require calibration with a certified reference material (CRM). CRM is a well-characterized material with certified values for specific analytes, accompanied by a statement of traceability to the SI units [22]. According to ISO Guide 35, reference materials for non-operationally defined measures need to be characterized using at least two techniques, either within a single laboratory or across multiple laboratories [23]. A careful method validation process is essential to ensure the quality and reliability of analytical data. The incorporation of CRMs in method validation serves several critical purposes. Firstly, CRMs enable the assessment of a method trueness which is also known as bias. By comparing the measured concentration of the analyte in the CRM

with its certified value, we can evaluate if the method consistently yields accurate results [24]. This paper presents a comprehensive validation of an HS-GC-FID method for the measurement of ethanol in water utilizing a NIST standard reference material. The validation study has covered the limit of detection (LOD), limit of quantification (LOQ), selectivity, linearity, precision, recovery (accuracy) and bias in accordance with ICH and the EURACHEM guides for method validation [25] [26]. The validated method will provide a reliable analytical tool for applications in precise ethanol determination for reference material characterizations. It will also contribute to improved analytical practices and enhance consumer confidence in Halal product certification.

## 2. Materials and Methods

### 2.1. Chemicals

The NIST SRM 2900 ethanol solution of certified value:  $95.6\% \pm 1.9\%$  was used for calibration. Isopropanol (HPLC Grade > 99% from Merck) served as the internal standard and ultrapure water was obtained from a Millipore Milli-Q RG system (USA).

### 2.2. Calibration Solutions

Six calibration solutions were prepared for the HS-GC-FID by gravimetric dilution from a stock solution of the NIST SRM. These calibration solutions were: 100, 300, 600, 900, 1200, and 1500 mg/kg, each prepared in a 100 mL volumetric flask. An internal standard solution was gravimetrically prepared at a concentration of 1000 mg/kg and added to each calibration solution. The detailed preparation procedure is described elsewhere [27].

### 2.3. The HS-GC-FID Analysis

Analysis of ethanol samples was performed using a Thermo Scientific Trace GC Ultra GC equipped with FID, dual S/SL inlets, and AS2000 autosampler. A WAXMS capillary column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m) was used for the chromatographic separation. The test sample was kept in the headspace oven at 70°C for 10 minutes and 350  $\mu$ L of the sample was injected in a split ratio of 20:1. The oven temperature program began with an initial hold at 50°C for 2 minutes, followed by a ramp to 220°C at a rate of 20 °C/min. The final temperature of 220°C was held for an additional 2.5 minutes. The GC inlet and detector temperatures were 250°C and 300°C, respectively. The carrier gas (helium) flow rate was 35 mL/min, while the hydrogen and air flow rates were 45 mL/min and 450 mL/min, respectively.

## 3. Results and Discussion

### 3.1. The LOD and LOQ

The limit of detection, LOD is defined as the lowest concentration that can be detected with statistical significance by means of a given analytical procedure.

Meanwhile, the LOQ is the smallest concentration of an analyte that can be reliably measured [26]. For the determination of both characteristics, a blank sample was injected, but it did not produce any area values. Therefore, a low concentration of ethanol (100 ppm) was prepared and an IS of 1000 mg/kg was added to it. This sample was injected into the HS-GC-FID using the developed method three times and the obtained area ratio was recorded in **Table 1**.

**Table 1.** Limit of detection (LOD) and limit of quantification (LOQ).

Measurements	A <sub>Eth</sub>	A <sub>IS</sub>	A ratio	$\bar{x}$	SD
n <sub>1</sub>	0.113	2.3602	0.04788		
n <sub>2</sub>	0.124	2.5281	0.04897	0.0483	0.0006
n <sub>3</sub>	0.108	2.2544	0.04795		

The standard deviation (SD) of the average area ratio was found 0.0006. The LOD was calculated as 1.27 mg/kg using equation 1 and the LOQ was calculated as 3.86 mg/kg using equation 2 where  $n$  is the number of measurements.

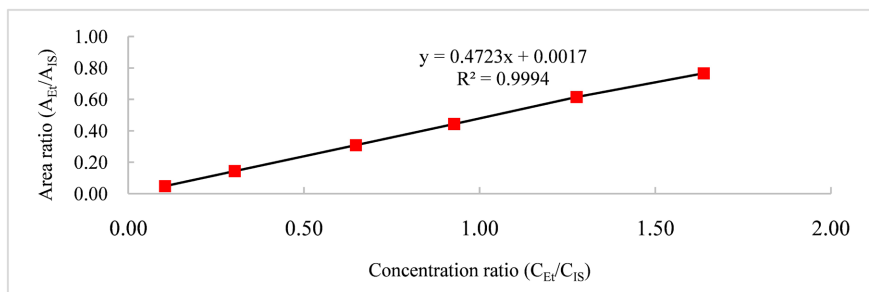
$$LOD = 3.3 \times \frac{SD}{\sqrt{n}} \quad (1)$$

$$LOQ = 10 \times \frac{SD}{\sqrt{n}} \quad (2)$$

### 3.2. Linearity

A key characteristic of an analytical procedure is its linearity. This refers to the method ability to produce results that are directly proportional to the concentration of the analyte within a specific range [25]. In this study, we evaluated linearity within the range of 100 - 1500 mg/kg in which six calibration solutions of 100, 300, 600, 900, 1200, and 1500 mg/kg were used. Each solution was spiked with 1000 mg/kg of internal standard (IS). The area ratio ( $A_{Et}/A_{IS}$ ) of the analyte peak to the IS peak was plotted against the concentration ratio ( $C_{Et}/C_{IS}$ ) to generate the calibration curve shown in **Figure 1**. The linear equation for the calibration curve was determined to be  $y = 0.4723x + 0.0017$ . The near-zero intercept indicates good linearity within the chosen range. Additionally, the  $R^2$  value of 0.9994, which is very close to 1 signifies a strong fit of the calibration points to the line supporting linearity of the method.

Furthermore, we calculated the residuals ( $y_i - \hat{y}$ ) for each calibration point and analyzed their distribution around zero. As recommended by IUPAC guidelines (1998), residuals should be randomly distributed around zero for a linear method [28] [29]. From **Table 2**, it can be seen that the observed residuals are indeed randomly distributed around zero, providing additional evidence for the linearity of the method.



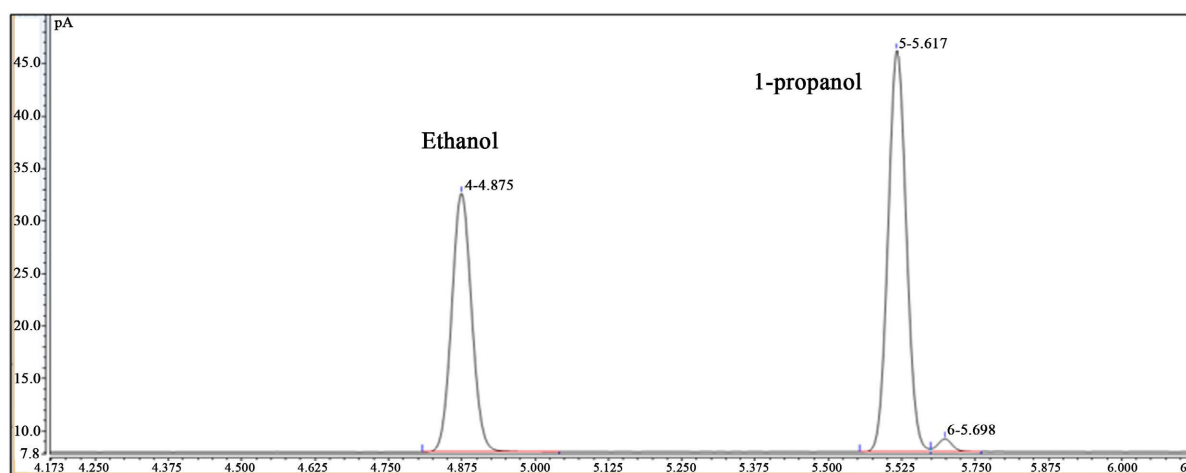
**Figure 1.** The calibration line of the HS-GC-FID by the ethanol CRM.

**Table 2.** Random distribution of the residuals ( $y_i - \hat{y}$ ) of the calibration points around zero axis.

$+(y_i - \hat{y})$	0 Axis	$-(y_i - \hat{y})$
	0	-0.00322
	0	-0.00118
0.00034	0	
0.00355	0	
0.01036	0	
	0	-0.00978

### 3.3. Selectivity

Selectivity refers to the extent to which a particular method can be used to determine analytes under given conditions in the presence of other components of similar behavior [30]. In our study, a blank water sample spiked with 1 mg of ethanol and 1 mg of the internal standard (1-propanol) was analyzed using the HS-GC-FID. The resulting chromatogram shown in **Figure 2** confirms excellent selectivity for ethanol because no interfering peaks from the water matrix were observed near the ethanol peak, which elutes at a retention time (RT) of 4.875 min.



**Figure 2.** Chromatogram of a spiked blank water with ethanol and IS.

### 3.4. Precision

Precision refers to the closeness of repeated measurements of the same sample [31]. In this study, precision of the method was investigated in the range of 100 - 1500 ppm and we investigated how well the method can reproduce results for low, middle, and high mass fraction levels. Thus, three ethanol samples were prepared as 99.72, 867.35 and 1403.67 mg/kg and three IS mass fraction, 953.33, 934.94 and 856.79 mg/kg were added respectively to them. Each sample was measured by HS-DC-FID three times and the results are given in **Table 3**. The precision was evaluated as RSD% by equation 3, which expresses the variability of the measurements as a percentage of the mean value.

$$RSD \% = \frac{SD}{\bar{x}} \times 100 \quad (3)$$

**Table 3.** Precision at low, middle and high ethanol mass fraction (mg/kg).

$C_{Et}$ (mg/kg)	$C_{IS}$ (mg/kg)	Area ratio	Average	SD	RSD %
99.72	953.33	0.048	0.048	0.0006	1.26
		0.049			
		0.048			
867.35	934.94	0.443	0.445	0.0020	0.45
		0.447			
		0.445			
1403.67	856.79	0.766	0.799	0.0038	0.47
		0.801			
		0.796			

A lower RSD% indicates higher precision, meaning that the repeated measurements are more consistent. If the RSD% increases or decreases significantly at a specific mass fraction (low, middle, or high), it might indicate limitations in the method precision at that particular mass fraction. The factors outlined the precision of the method can be the stable carrier gas flow, detector response, and column performance throughout the GC analysis minimize random fluctuations. Also, the use of NIST SRM calibration standards with known ethanol concentrations allows for precise quantification. In addition, employing a consistent integration method for the ethanol peak on the chromatogram reduces errors in measurement

Analysis of the RSD% values in **Table 3** reveals that the middle and high ethanol mass fractions exhibit consistent RSD% values below 0.5%. However, the RSD% for the low mass fraction is about three times larger (1.26%). This indicates that the method precision is lower when determining smaller ethanol mass fractions. Nevertheless, the overall precision across the three ethanol levels remains acceptable well below the 5% threshold. This demonstrates that the method is suitable for its intended purpose of reference material characterization.

### 3.5. Recovery

This analytical method is simpler because it does not involve extraction or cleanup steps that can lead to analyte loss. Consequently, to evaluate how well the method recovers the target analyte, a spike of a blank approach was used. A specific mass fraction (1035.29 mg/kg) from the ethanol NIST SRM was used for spiking and was measured 9 times by HS-GC-FID. The average and standard deviation were calculated and presented in **Table 4**. Equation 4 was then used to calculate the percentage recovery, which was found to be 98.14% [26] [32].

$$R(\%) = \frac{\bar{x}}{C_{CRM}} \times 100 \quad (4)$$

where,

$\bar{x}$  —average mass fraction of ethanol (mg/kg);

$C_{CRM}$ —mass fraction of the CRM sample (mg/kg).

This near 100% recovery indicates that the method captures almost all of the spiked ethanol and is reliable to quantify ethanol in water within an acceptable error range.

**Table 4.** Recovery data of ethanol measurement using a sample of NIST SRM.

$C_{spike}$ (mg/kg)	Measurements, n	$\bar{x}$ (mg/kg)	SD	$SD/\sqrt{n}$	bias	Recovery %
1035.29	1015.15	1016	3.28	1.093	-19.29 (1.86%)	98.14
	1020.63					
	1012.08					
	1014.25					
	1020.14					
	1013.24					
	1015.60					
	1019.63					
	1013.28					

### 3.6. Bias

The method bias was calculated by subtracting the average of measurements from the reference value, as described by equation 5 and was reported in **Table 4** as -19.29 mg/kg.

$$b = \bar{x} - x_{CRM} \quad (5)$$

The calculated bias was tested if significant using the criterion in equation 6. If the bias falls within the range established by this criterion, the method is considered unbiased.

$$-2\sigma \leq b \leq +2\sigma \quad (6)$$

The standard deviation ( $\sigma$ ) was calculated using equation 7, which considers uncertainties from both the repeatability of measurements and the standard uncertainty of the NIST SRM (1.9%/2) [32].

$$\sigma = \sqrt{\left(\frac{SD}{\sqrt{n}}\right)^2 + (u_{CRM})^2} \quad (7)$$

Notably, the reference material uncertainty was expressed as a percentage, while the repeatability uncertainty was given in mg/kg. To address this unit inconsistency, an uncertainty ratio was employed to eliminate units and then the resulting number was multiplied by the average mole fraction of ethanol to obtain  $\sigma$ , which was found to be 10.16. Thus, it can be observed that the bias of  $-19.29$  mg/kg falls within the range of  $-2\sigma$  ( $-20.32$ ) and  $+2\sigma$  ( $+20.32$ ). This confirms that the method exhibits no significant bias.

### 3.7. The Uncertainty of Measurements

An ethanol sample of concentration 1028.85 mg/kg was analyzed 10 times using HS-GC-FID calibrated by the NIST SRM, and the mole fraction ( $x$ ) was calculated using the calibration function 8.

$$x = \frac{\left( \frac{A_{Un}}{A_{IS}} - b \right) \times C_{IS}}{a} \quad (8)$$

where

$x$ —mass fraction of unknown (mg/kg)

$A_{Un}$ —area of unknown

$A_{IS}$ —area of IS

$b$ —intercept

$C_{IS}$ —concentration of IS added to the sample

$a$ —slope.

From this mathematical model, it is clear that the explicit sources of uncertainty are: area of unknown, area of IS, intercept, slope and concentration of the IS added to the unknown sample. In addition, the NIST SRM calibration solutions and the mass of the ethanol sample are two implicit sources of uncertainty. They were combined into the uncertainty of the concentration of IS ( $C_{IS}$ ) to fit the model equation 8. This means that these two implicit sources of uncertainty are not explicitly accounted for in the model calculations, but their contribution is assumed to be reflected in the overall uncertainty of the IS concentration [27]. The calculation of the uncertainty contributions was carried out according to the ISO GUM [33]. The combined standard uncertainty,  $u_c$  was calculated according to equation 9 in which the  $dx/dy_i$  were the sensitivity coefficients obtained by differentiation of equation 8.

$$u_c = \sqrt{\left( \frac{\partial x}{\partial A_{un}} \cdot u_{A_{un}} \right)^2 + \left( \frac{\partial x}{\partial A_{IS}} \cdot u_{A_{IS}} \right)^2 + \left( \frac{\partial x}{\partial a} \cdot u_a \right)^2 + \left( \frac{\partial x}{\partial b} \cdot u_b \right)^2 + \left( \frac{\partial x}{\partial C_{IS}} \cdot u_{C_{IS}} \right)^2} \quad (9)$$

The expanded uncertainty  $U_{exp}$ , was then calculated at a 95% confidence level using a coverage factor  $k = 2$  as in equation 9.

$$U_{exp} = k \times u_c \quad (10)$$

The resulting uncertainty budget is presented in **Table 5** that shows value ( $x_i$ ) of each source, and the corresponding uncertainty,  $u(x_i)$ , the probability distribution,



**Table 5.** The uncertainty budget of ethanol in water measurements by HS-GC-FID.

Uncertainty source	$x_i$	$u(x_i)$	Probability	$c_i$	$c_i \cdot u(x_i)$
Area of unknown	1.2005	0.004	normal	865.0975	11.974300
Area of IS	2.4586	0.015	normal	-422.4150	40.147751
Slope	0.47	0.0024	normal	-2183.9616	27.473405
Intercept	-0.0025	0.0022	normal	-2126.9288	21.895318
Concentration of IS	1006.25	1.7481	normal	1.0400	3.305287
Combined standard uncertainty, $u_c$				10.24	
Expanded uncertainty, $U_{exp}$ (mg/kg)		$k = 2$		20.47	
Expanded uncertainty, $U_{exp}\%$				1.99	

the sensitivity coefficient,  $c_i$  and the uncertainty contribution,  $c_i \cdot u(x_i)$ . From this table it is clear that the expanded uncertainty,  $U_{exp}\%$ , produced by the method reached 2%. Thus, by identifying and quantifying these uncertainties, we can get a better understanding of the limitations of the ethanol measurements by HS-GC-FID and express the results with more confidence.

#### 4. Conclusion

The HS-GC-FID rapid and low-cost method presented in this study has been successfully validated for the determination of ethanol in water samples. The method showed low limits of detection and quantification and good linearity in range of 100 - 1500 mg/kg with excellent selectivity for ethanol. It also exhibited good precision at low, middle and high mass fractions in the specified range. Recovery studies confirmed minimal matrix effects, minimal bias and 2% associated expanded uncertainty suitable for the intended application. This validated HS-GC-FID method provides a reliable approach for the accurate quantification of ethanol in water for the purpose of characterization of ethanol reference materials.

#### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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