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Validation of a modified alcohol dehydrogenase assay for ethanol determination

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A B S T R A C T

Enzymatic assay, based on oxidation-reduction reaction catalyzed by alcohol dehydrogenase, is one of the methods used to determine ethanol concentration. The present study was directed to determine the exact amount of enzyme required to accomplish oxidation-reduction reaction so that the concentration of ethanol in the sample can be determined precisely and accurately. Results of the present study indicate that the lowest unit activity of the enzyme that can be used for ethanol determination is 4000 units/mL, even though longer incubation time compared to the original method was used to ensure reaction completion. Validation of the method confirmed that the assay have acceptable linearity range within 0.01 - 0.06% (v/v) of ethanol with correlation coefficient of 0.9999. Both accuracy and precision parameters fulfill the Association of Analytical Communities (AOAC) International requirement, and therefore can be accepted as a quantitative analysis method. Limit of detection and limit of quantitation for the modified method were 0.0017% (v/v) and 0.0056% (v/v), respectively.

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1. Introduction

The importance of quantitative analysis of ethanol in foods, medicines, fuel products, and clinical applications require a powerful analysis method. Some of methods that are currently used for ethanol quantitation include high performance liquid chromatography (HPLC)[1], gas chromatography (GC)[2, 3], titration[4], Fourier transform infrared (FTIR)[5-7] and colorimetry[8, 9].

Enzymatic assay for ethanol determination using alcohol dehydrogenase has already proposed by some authors[8, 10, 11]. The assay is based on the oxidation of ethanol to acetaldehyde which followed by conversion of β-Nicotinamide adenine dinucletide in the oxidized form (NAD^+) to the reduced form (NADH) catalyzed by alcohol dehydrogenase (ADH). To force the reaction into completion, semicarbazide is added in the reaction buffer which will bind acetaldehyde. The amount of NADH,

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which equal to the amount of ethanol, can be measured at 340 nm[10]. Addition of phenazine methosulphate-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide (PMS-MTT) to the reaction system also trialled by other authors. The NADH formed from the enzymatic reaction will oxidize the PMS-MTT forming a purple coloured MTT formazan[8].

Previous published methods did not mention the exact amount of enzyme activity required for accomplishing the reaction into completion[10, 11]. According to the proposed method[10], the enzyme required for the assay is ≥ 300 mg which will give enzyme activity about ~8000 - 9000 units/mL. In the present study, we determined the minimal enzyme activity required for the reaction to be accomplished whilst still give acceptable result for ethanol determination. Validation of the modified method is also described.

2. Results and Discussion

2.1 Minimal enzyme required for ethanol determination

Method proposed by Bernt & Gutmann[11] and endorsed by Ough & Amerine[10] did not give the exact value of how much enzyme activity required, while this is very important for the assay. Our study was conducted by varying the unit activity of ADH added to the reaction system so that more precise amount of enzyme activity can be obtained. Utilization of lower enzyme may result in slower reaction rate. Therefore, as an effort to counteract this issue we varied some possible factors that may influence the reaction completion, i.e. temperature and time of incubation.

When 0.05% (v/v) ethanol was measured using various enzyme activity (i.e. 8000, 6000, 4000, 2000 and 800 units/mL), statistical analysis results of the data indicate that there were no significant differences in ethanol measured when 8000, 6000, 4000 and 2000 units/mL of enzyme used in the assay, but 800 units/mL gave inaccurate results by showing significantly lower ethanol concentration than the real value ($P < 0.05$) as can be seen on Table 1. However, individual data of 2000 units/mL enzyme activity showed that the ethanol measured was always ~10% less than the real value (data not shown), and therefore lead us to exclude 2000 units/mL for our proposed method. Low enzyme activity present in the reaction system may lead to incomplete reaction, and therefore gave lower results than expected. Based on the present results, the amount of enzyme that still can be used without sacrificing the efficacy of the assay is 8000, 6000 and 4000 units/mL. As the present study was directed to reduce the amount of enzyme used in the assay, we chose the lowest enzyme activity for the assay. Therefore we suggest that the minimum enzyme activity required for the assay is 4000 units/mL.

Table 1. Comparison of ethanol measurement result using different ADH activities. The concentration of sample measured was 0.05% (v/v). The assay mixture was incubated at 35°C for 40 minutes. Data presented are mean of four independent measurements, except for 2000 and 800 units/mL only from two independent experiments. Means followed by same superscript letter are not statistically different according to Fisher's LSD test.

ADH activity (units/mL)	Measured ethanol concentration (% (v/v))
8000	$0.0485 \pm 0.0024^{a,b}$
6000	0.0502 ± 0.0034^{a}
4000	$0.0472 \pm 0.0008^{a,b}$
2000	0.0448 ± 0.0001^{b}
800	0.0365 ± 0.0018^{c}

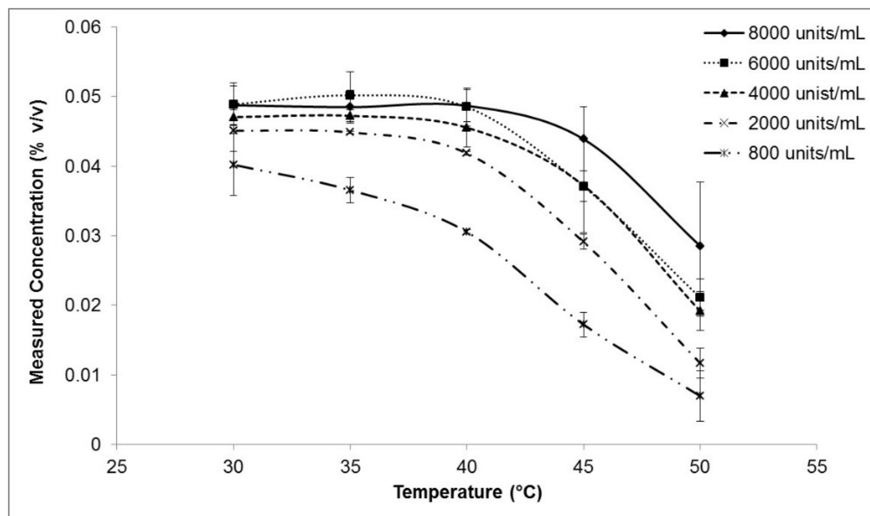

Fig. 1. The effect of incubation temperature on measured ethanol concentration. The assay was performed using activity as described on the legend. The concentration of sample used for the assay was 0.05% (v/v) and incubated for 40 minutes. Data presented are mean of four independent experiments, except for 2000 and 800 units/mL only from two independent experiments. Error bars represent standard deviation.

When we vary incubation temperature in which the reaction conducted, it was observed that temperature above 40°C gave lower ethanol concentration than expected and therefore should be avoided. This observation was more apparent when 800 and 2000 units/mL of enzyme activity was used as presented on Fig. 1. This result is slightly different to what Zenon *et al.*[8] found in which the activity of ADH starts to decline when the temperature reached above 45°C. However, either the present study or the result observed by Zanon *et al*.[8] indicate that the best temperature for ADH assay is between 30-40°C. Inaccurate results due to increasing temperature are consequence of enzyme inactivation due to denaturation. Since the enzyme activity is very crucial factor in this assay, we prefer to incubate the reaction mixture for the assay at 35°C.

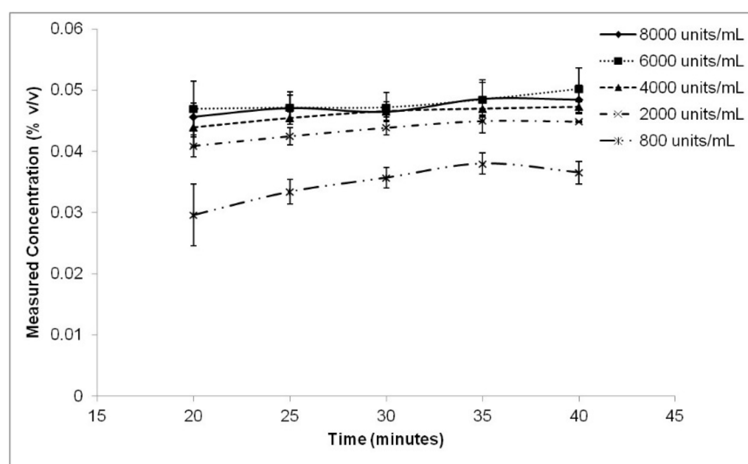

Fig. 2. The effect of incubation time on measured ethanol concentration. The assay was performed using activity as described on the legend. The concentration of sample used for the assay was 0.05% (v/v) and incubated at 35°C. Data presented are mean of four independent experiments, except for 2000 and 800 units/mL only from two independent experiments. Error bars represent standard deviation.

To determine the best incubation time, we used various enzyme activity with different incubation time at fixed incubation temperature (35°C). A default incubation time for 20 minutes was used based on previously published method[11]. As our initial assumption was that lower enzyme activity will require

longer incubation time to reach reaction completion, longer incubation time than the default time was investigated in the present study. Fig. 2 presents the result of ethanol measurement with different incubation time. Based on statistical analysis results of the data, there were no significant differences of ethanol concentration measured at different incubation times ($P > 0.05$). However, it was observed that better results, as indicated by closer ethanol content to real value, obtained when longer incubation time used. Longer incubation time was chosen to ensure that the reaction reach completion. Therefore, we propose incubation time for 40 minutes to be used and validated in the present study. The ethanol content obtained when 800 units/mL enzyme activities used was significantly lower than the real value and when 2000 units/mL used the results was slightly lower than the real value as presented on Figure 2. These results support our previous suggestion to use 4000 units/mL in the modified assay method.

In summary, we proposed that the assay should be conducted using 4000 units/mL, 35°C and 40 minutes for the unit of enzyme activity, incubation temperature and incubation time, respectively, in the modified method. In the present study, we validate our proposed method and the method proposed by Bernt & Gutmann[11] to compare the validity of our modified method.

2.2 Validation of the modified method

Method validation is performed to verify the performance of a method in order to demonstrate that the method is accurate and powerful to determine a sample in a particular concentration and sample matrix. The validations performed in the present study covered linearity, precision, accuracy, limit of detection and limit of quantitation.

Linearity

To check linearity of the method, a standard curve between absorbance values against theoretical concentration (the concentration of ethanol made from stock, represented by C_t) was plotted so that the linear regression of the curve can be calculated. The concentration of which a method can be accepted as an assay is if the method gives a correlation coefficient of $R > 0.990$[12]. The regression equation was then used to back-calculate the ethanol concentration of the sample to give calculated concentration (C_c) from the absorbance data. Recovery was then calculated as percentage ratio of C_c to C_t. Recovery can be accepted if the value falls within 95-105% range.

Recovery of the method proposed by Bernt & Guttmann[11] for 0.0030, 0.0040 and 0.0050% (v/v) of ethanol was outside of the acceptable range as presented on Table 2. This result indicates that there were high errors within that range of concentrations. This also supported by the result of quantitation limit, as will be described in the next section, which showed that the particular ethanol concentrations range fall below the quantitation limit of the method, and therefore measurement within the range will give high errors and bad precision[13, 14].

High error also indicated when the C_t compared with concentration calculated using Equation (1) (C_f). For example, when the concentration should be 0.0030% (v/v), a result of 0.0078% (v/v) was obtained as C_f as presented on Table 2. Therefore we suggest that the linear range for the assay proposed by Bernt & Guttman[11] is between 0.01 – 0.06% (v/v) with correlation coefficient and linear regression of 0.9997 and $y=18.68x + 0.0751$, respectively.

The modified method gave similar result as the original method. However an anomaly was detected at 0.0050% (v/v) in which the recovery was actually acceptable, with value of 102.9%. However this concentration is still below its quantitation limit (0.0056% (v/v)) and doubtful since the C_f value was 0.0090% (v/v), not 0.0050% (v/v). Therefore we proposed that the linearity range for the modified method is 0.01 – 0.06% (v/v) with correlation coefficient and linear regression of 0.9999 and $y=17.60x + 0.0961$, respectively. Both methods showed good and acceptable linearity, since both of the methods have correlation coefficient $R > 0.990$.

Table 2. Data used to verify linearity of method proposed by Bernt & Gutmann. Assay was conducted using 8000 units/mL of ADH activity. The assay mixture was incubated at 37°C for 25 minutes.

C_t (%(v/v))	A	C_c (%(v/v))	Recovery (%)	C_f (%(v/v))
0.0030	0.161	0.0037	123.6	0.0078
0.0040	0.177	0.0046	115.1	0.0086
0.0050	0.194	0.0055	110.3	0.0094
0.0100	0.271	0.0098	97.5	0.0131
0.0150	0.353	0.0143	95.1	0.0171
0.0200	0.438	0.0189	94.5	0.0212
0.0250	0.539	0.0244	97.8	0.0261
0.0400	0.834	0.0406	101.6	0.0404
0.0500	1.001	0.0497	99.5	0.0484
0.0600	1.198	0.0606	100.9	0.0580

Table 3. Data used to verify linearity of the modified method proposed in this study. Assay was conducted using 4000 units/mL of ADH activity. The assay mixture was incubated at 35°C for 40 minutes.

C_t (%(v/v))	A	C_c (%(v/v))	Recovery (%)	C_f (%(v/v))
0.0030	0.126	0.0018	58.6	0.0061
0.0040	0.185	0.0051	127.7	0.0090
0.0050	0.186	0.0051	102.9	0.0090
0.0100	0.267	0.0098	97.6	0.0129
0.0150	0.363	0.0152	101.3	0.0175
0.0200	0.451	0.0202	101.1	0.0218
0.0250	0.541	0.0253	101.2	0.0262
0.0400	0.791	0.0395	98.8	0.0383
0.0500	0.978	0.0501	100.2	0.0473
0.0600	1.154	0.0601	100.1	0.0558

As presented on Table 2 and Table 3, C_f tends to have higher error compared to C_c. Therefore, we endorse utilization of standard curve of ethanol to determine ethanol content of the samples. When standard curve used, introduction of error from the particular instrument used for measurement can be reduced, and therefore can give better result than calculating the ethanol using Equation (1).

Precision

A method considered has an acceptable precision if the %RSD value of measurement is lower than %RSD Horwitz[12]. The results of %RSD value of measurement of both methods are presented on Table 4. The results indicate that both methods have %RSD value of measurement lower than %RSD Horwitz. Therefore both methods can be considered has good precision for ethanol determination.

Table 4. Comparison of analytical characteristics of the method proposed by Bernt & Gutmann[11] and the modified method in this study

Parameter	Bernt & Gutmann method[11]	Modified method (present work)
Regression equation[a]	A = 18.68 (C_c) + 0.0751	A = 17.60 (C_c) + 0.0961
R	0.9997	0.9999
Linear Range (% (v/v))	0.01 – 0.06	0.01 – 0.06
RSD (%)[b]	1.99 (%RSD Horwitz = 4.90)	1.49 (%RSD Horwitz = 4.80)
Recovery (%)[c]	91.2 ± 1.7	104.5 ± 1.6
LOD (% (v/v))	0.0019	0.0017
LOQ (% (v/v))	0.0062	0.0056

Note: [a] C_c is calculated concentration of ethanol using linear regression equation
[b] Relative standard deviation of seven replicates for determination of 0.0200% (v/v) ethanol
[c] Recovery of seven replicates

Accuracy

Recovery of an analyte is used to determine accuracy of a method. In the present study, recovery was determined by measuring known concentration of ethanol added to a blank solution. The concentration of the analyte in the mixture was then determined and therefore the recovery can be calculated. According to AOAC International, for 0.02% (v/v) concentration, the acceptable recovery should fall within 85-110% range[15]. The recovery of both methods is presented on Table 4. Both of the methods showed acceptable recovery, and therefore both of the method can be concluded to have good accuracy.

Limit of detection and Limit of quantitation

Limit of detection (LOD) is the lowest concentration in which an analyte can be detected and discriminated from the blank solution. While limit of quantitation (LOQ) is the lowest concentration of analyte that still can be quantified with repeatability, precision and accuracy that are acceptable[13, 16]. The detection and quantitation limit result of the present study is presented on Table 4. The results showed that the methods have slightly different LOD and LOQ. LOQ for both of the methods are about 0.006% (v/v). Therefore we suggest that both of the methods can be used to determine ethanol with minimal concentration of 0.006% (v/v). However, to ensure the best result we suggest that ethanol determination using this methods should be conducted within 0.01 – 0.06% (v/v) range in order to get accurate results

3. Conclusions

Modification of a method for determination of ethanol using ADH assay has been conducted. The proposed modified method uses 4000 units/mL enzyme activity, incubation at 35°C for 40 minutes for the assay. The modified method gave acceptable linearity, precision, accuracy, detection limit and quantitation limit which comparable to the original method. We also suggest that the assay should be used to measure ethanol within 0.01-0.06% (v/v) range to get accurate results. Utilization of standard curve rather than calculation using equation derived from Lambert-Beer equation is endorsed to minimize error in ethanol determination in sample. If the sample predicted to have higher concentration of the suggested range, dilution should be made.

4. Experimental

4.1 Materials

Alcohol dehydrogenase (ADH) used in this study was sourced from *Saccharomyces cerevisiae* (Sigma # A7011) and bought from Sigma Aldrich. All the chemicals and solvent used were analytical grade reagent purchased from Sigma Aldrich, except double distilled water were purchased from local chemical store.

4.2 Preparation of reagent solutions

4.2.1 Semicarbazide buffer

The semicarbazide buffer was made by weighing 3.34 g tetrasodium pyrophosphate, 0.84 g semicarbazide hydrochloride and 0.16 g glycine and dissolved with 90 mL double distilled water. The pH of the solution was adjusted to 8.7 by addition of 8 N NaOH, and the volume is made up to 100 mL.

4.2.2 NAD^+ solution

To make the NAD^+ solution 0.08 g NAD^+ was weighed and dissolved using double distilled water to give total volume of 5 mL.

4.2.3 Alcohol dehydrogenase solution

The stock enzyme solution is made to give final unit activity of 8000 units/mL. The amount of the enzyme weighed is depending on the specific activity of the available batch indicated on the label. The enzyme batch that was used in the present study has specific activity of 415 units/mg, the amount of lyophilized enzyme that has to be weighed for 1 mL of the enzyme solution is: 8000 unit ÷ 415 unit/mg = 19.27 mg. When lower enzyme activity is required, the stock enzyme solution was diluted as necessary to give the required activity.

4.2.4 Standard ethanol solution

Absolute ethanol solution was diluted with double distilled water to give aqueous ethanol with the required concentration as noted on the text.

4.3 Determination of ethanol concentration

To a tube containing 1.25 mL semicarbazide solution, 25 µL of sample and 25 µL NAD^+ solutions was added and mixed thoroughly. After finely mixed, 5 µL alcohol dehydrogenase solutions was added to the solution and mixed thoroughly. The reaction mixture was then incubated at a particular temperature for a designated time as indicated on the text. The absorbance was then read at 340 nm after setting the spectrophotometer to zero with reagent blank.

In case of protein containing samples, a treatment to remove protein content should be made by adding perchloric acid prior to semicarbazide solution addition as described in the original method and dilution factor also should be taken into account. In addition, based on our experience, all reagents should be made freshly since we observe absorbance value decline when the reagents (especially NAD^+ and ADH solutions) were kept for more than two weeks even though they were stored in -20°C.

4.4 Derivation of formula to calculate ethanol based on Lambert-Beer equation

Assuming absorbance value of 1.000, path length is 1 cm and molar extinction coefficient for NADH is 6300 $M^{-1}.cm^{-1}$ [17]:

- Concentration of NADH in the assay tube
  Using Lambert-Beer equation, $A = \varepsilon.b.c$
  where A is absorbance, ε is molar extinction coefficient, b is path length and c is concentration of the analyte in molar concentration, we can construct the following calculation:
  $1.000 = 6300 \times 1 \times c$, so $c = 1.000/6300 = (1.5873 \times 10^{-4})$ M or $(1.5873 \times 10^{-4} \times 10^{6})$ µM = 158.73 µM (µmole/L)
- Amount of NADH in the assay tube (equal to the amount of ethanol, total assay volume 1.305 mL)
  158.73 µmole/L × 1.305 × 10^{-3} L = 0.2071 µmole
- Concentration of ethanol in the sample (25 µL sample was taken to give total volume of 1.305 mL of the assay)
  0.2071 µmole / 25 µL = 0.008284 µmole/ µL or 0.008284 mole/ L = 8.284 × 10^{-3} mole/L
- Concentration of ethanol in the sample (%(w/v))
  8.284 × 10^{-3} mole/L × 46.08 gram/mole × 0.1 L/100 mL = 0.0382 gram/100 mL = 0.0382% (w/v)
- Concentration of ethanol in the sample (%(v/v))
  0.0382 gram/100 mL × (1/0.789 gram/mL) = 0.0382 gram/100 mL × (1/0.789) mL/gram = 0.0484 % ((v/v))

Therefore, the equation to calculate ethanol concentration is:

$$\text{Ethanol Concentration (\%v/v)} = \frac{A}{6300} \times 10^{6} \times (1.305 \times 10^{-3}) \times \frac{1}{25} \times 46.08 \times 0.1 \times \frac{1}{0.789} \quad (1)$$

4.5 Statistical analysis

Raw data were analysed using Minitab® 15 for Windows®. Analyses include one way analysis of variance (one way ANOVA) followed by the Fisher's LSD (least significant difference) test to determine which data differed significantly.

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