Determination of Ethanol in Fermented Broth by Headspace Gas Chromatography using Capillary Column

ASEM HASSAN MOHAMMED [1], ALAA KAREEM MOHAMMED[2], FIRAS HASHIM KAMAR[1], ASEEL ABDULQADER ABBAS[2], GHEORGHE NECHIFOR[3]*

[1] Institute of Technology- Baghdad, Middle Technical University, Iraq

[2] Department of Biochemical Engineering, AL-Khwarizmi Engineering College, University of Baghdad, Iraq

[3] Department of Analytical Chemistry and Environmental Engineering, University Politehnica of Bucharest, 1-7 Gheorghe Polizu Str., 011061, Bucharest, Romania

The gas chromatography (GC) method in analytical chemistry is a quick and accurate method to detect volatile components like ethanol. A method for determining volatile components known as Headspace chromatography (HS-GC) was developed along with an internal standard method (ISM) to identify ethanol in fermented broth in the laboratory. The aim of this research is determining the concentration of ethanol in fermented broth using capillary column (ZB-1). This method can analyze ethanol concentrations in the fermented medium broth ranging from 10 to 200 g/L. The validation of this method was done in order to obtain the results to be of high precision and the significant, precision was represented as the relative standard deviation (RSD) which was less than 5%, accuracy was less than 4 % and significance level was $p < 0.05$. It was found that this method exhibited good reproducibility.

Keywords: Fermentation, volatile components, internal standard method, headspace sampling-gas chromatography

Bio-analysis knows a continuous and rapid development including electrochemical and, optical methods, but was making possible with advanced chromatography techniques [1-4]. Thus, static headspace gas chromatography (SH-GC) analysis is a procedure applied to the analysis and concentration of volatile organic compounds. This procedure is rather simple and to be able to get sensitivity like dynamic purge and trap operation [5, 6].

Laboratory use of this method has increased and has worldwide validated to analyze alcohol and blood alcohol in biological samples and solvents used in pharmaceutical industries. Other known applications of this method include laboratory analysis of monomers in polymers, plastics and flavor enhancers in beverages, products of food and aroma in perfumes and cosmetics [1, 6, 7].

Complicated samples such as biological solution, blood, cosmetics and plastics contain high-molecular weight materials, some of which are non-volatile that can be deposited into the GC injection port and lead to poor analytical result therefore, lots of analysts use several methods in laboratories for intensive sample preparation to concentrate and extract interest compounds from this undesired material. These evaporation and concentration process can take a long time and more cost. Using analysis of static headspace will reduce time and cost by sampling the volatile component of headspace directly into the GC injection port [8, 9]. A headspace sample is usually prepared in a vial which divide in two phases, liquid phase contain the volatile component, the dilution solvent, a matrix modifier, and vapor phase (headspace) at the top of the vial, see figure 1. The volatile components of the mixture of complex samples (L) will be separated from the non-volatile sample components and collected in the head vacuum or steam part of the sample vial (G). An appropriate volume of the vapor in the headspace is withdrawn to deliver to a GC system for partition and detection of volatile components [10, 11].

The analysis by using of gas chromatography can determine qualitative analysis according to retention time and quantitative analysis using the area under the curve of a chromatographic peak which gives it the best performance compare with among old methods. In this method the sample will be stored in a shut vial, the volatile analytes evaporate toward the vial headspace, in this method of analysis the sample is put in a locked vial. The volatile components evaporate into the vial headspace was shown in figure 1. When equilibrium state between the interesting volatile analytes concentration in the liquid phase (sample) and in vapor phase (headspace) is reached, an appropriate volume is taken from the headspace and injected into the gas chromatography; this injection can be carried out manual or with an auto sampler at higher pressure and temperature than ambient conditions [12]. Nowadays, the headspace gas chromatography method is desired because the contaminants that will reach to the injection port and the separation column of the gas chromatography will be as low as possible [13, 14].

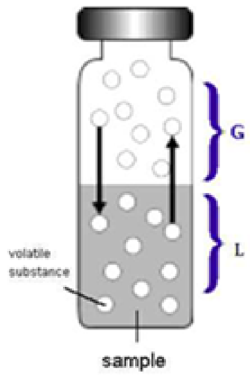

Fig. 1. Phases of sample in the headspace container, G- the vapor phase (headspace), L- the liquid phase [10]

Experimental part

Materials and methods

Chemicals used in this research are: absolute ethanol (HAYMAN, UK); n-butanol (sigma Aldrich), baker's yeast (local market), Glucose and other chemicals used in the fermentation were from Sigma Aldrich.

* email: doru.nechifor@yahoo.com

Sample preparation

Fermentation medium (broth) was prepared by mixing 100 g/L glucose, 8.5 g/L yeast extract, 1.3 g/L NH_4Cl, 0.12 g/L $MgSO_4.7H_2O$ and 0.06 g/L $CaCl_2$, [15]. Broth medium was inoculated via baker's yeast by 100 mL of inoculated broth sample which was prepared in former step, fermentation process was conducted in 1 L sterile shake flasks, metal caps as closures, and sterile pipets, A shaker flask incubate at 35 ± 2°C and 150 rpm for about 3 days, and then it was cooled to 0 °C and stored for another days.

It was taken 5 mL of fermented broth samples at different interval times (6, 12, 24, 48, 72 h) then the samples are taken to centrifuge within one hour after taking the sampling. This can be performed by rotating at 3000 RPM for ten minutes, and then plugged immediately with its ground glass stopper. Filtrated portion of liquid of the sample will pass through a 0.45 mm micro filter after that , sample analyzing was perform as follow: 2 mL of fermented broth was mixed with 3ml of internal standard (n-butanol, 4 g/L) in a 10 mL vial, and then the vial was tightly closed immediately with a suitable caps. Each analysis was undertaken in triplicate with different vials. In order to minimize the loss of volatile compounds, the samples were not analyzed will be saved at 4 °C.

- Internal standard calibration and standardization

Internal standard calibration is a familiar approach to quantitative analysis of static headspace gas chromatography; this method allows overcoming for any deviation caused by matrix effects and injection conditions of gas chromatography. Before the separation by extraction, a known concentration of analyte is added to each standard solutions and sample. That compound is called the internal standard. The choice of an internal standard that is suitable for the analysis of different analyzes is considered the important part of the internal standard calibration. Internal standard compound must be pure form and doesn't exist in the samples, at least at in the analyzed area, according these limitations it was chosen, an appropriate concentration internal standard, using reagent n-butanol. The same percentage of the internal standard solution for each standard and sample analyzed should be added by using this method. For accomplish of calibration curve; 3 mL of (4 g/L) internal standard be added to 2 mL of standard solution into a 10 mL vial .Prepare 4 points of ethanol analytical standards, using 200 proof ethanol, ranging from 10 to 200 g/L, vial is sealed, samples will place in the oven at temperature 70°C for 15 min, more volatile compound (ethanol), which results from heating the sample will rise to the head area until the equilibrium state is reached, 1mL of the headspace volume was taken for injected directly into a GC with tightly syringe, 1-2 intermediate calibration were prepared as verification solutions from the separate ethanol source, then can be account ethanol content by forming calibration curve by plotting the ratio of the analyte signal (area of the peak) to the standard signal as function to analyte concentration of the standards, figure 2 show that.

Instrumentation and GC condition

In this study GC (Shimadzu, Japan, 2014) was used, which was equipped with computer integrator software (GC solution, Shimadzu) and the flame ionization detector (FID). Hydrogen and air flow rate were set at 30 and 300 mL/min, respectively. Temperature of the FID detector and the injection port was set at 250, and 120°C, respectively. The flow rate of nitrogen is 6 mL/min, split ratio is 2, and column flow is 1mL used as the carrier gas. ZB-1 (phenomenx) separation column (L: 30 m, ID: 0.32 mm, HP-5, 5% phenyl methyl siloxane capillary column from Phenomenix with a low-film thickness of 0.5μm) was used to perform in this study. In the beginning: the oven temperature was set at 40°C for 4.5 min, and then increased to the final temperature of 120°C for 1 min at the rate 40°C/ min. The volume of injection was 1mL. Split injection mode was selected. The 10 mL headspace vials and the aluminum crimp caps were obtained from Phenomenix. The 1000 μmL micro pipette were obtained from Dragon (Shanghai, China).

Statistical analysis

All the data used for method validation were analyzed according to SPSS 14.0, and the data used for qualitative analysis. The statistical significance level was set at P < 0.05.

Results and discussions

This type of column (ZB-1, 40 meter) was selected because we intend to know the ability and the range of ethanol concentration which can determine by using standard solution of ethanol and real solution that represent

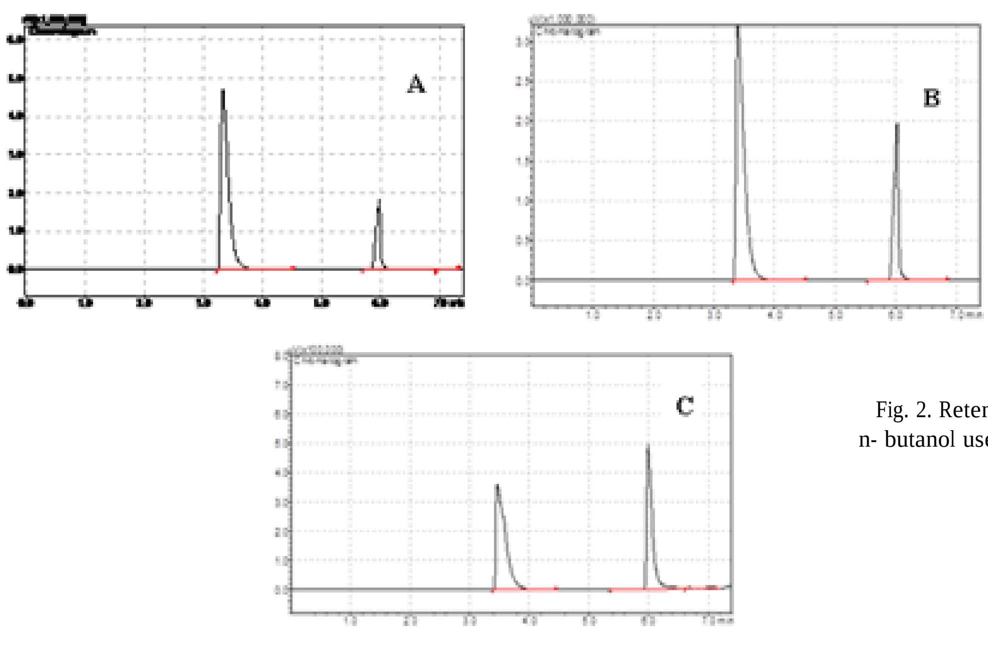

Fig. 2. Retention time of ethanol (2.73 min) : n- butanol used as internal standard solution

of bioethanol in fermented broth when we use static headspace gas chromatography ((SH-GC)) technique instead of direct injection of liquid.ZB-1 (100, 150 meter) column is the most popular column for the determination of ethanol content of denatured fuel ethanol by direct injection of liquid [16, 17]

The GC conditions used which is described in materials and methods are selected after many times of runs for reaching the best resolution, the retention time of ethanol was 2.73 min and n- butanol which used as internal standard solution was 7 min as shown in figure 2.

Validation of the analysis method

A pure ethanol solution (99.9% w/w) was mix with distilled water to get ethanol concentrations (200, 100, 50, 25, 12, and 10 mg/mL). Each diluted solution sample (2 mL) was poured into a 10-mL caped vial. After mixing with (3 mL) of a (4m g/mL) internal standard spiking solution (equivalent to 12 mg), vial is tightly closed, samples will place in the oven at temperature 70 °C for time 15 min, volatile compound (ethanol) in the sample will partition into the headspace until reaches equilibrium state, 1 mL of the head space phase was directly injected into a GC with syringe. All concentrations of were measured in three time (triplicate).

Relative standard deviation (RSD) or called coefficient of variation (CV) was used to evaluate precision which obtained of each internal standard solution have the same concentration at a rate of three times of each sample, which was less than 5% for most samples.

Relative error of the mean (REM) was used to measure the accuracy, This relationship was calculated as difference between measured value and true value of concentrations divided by true value by a gas chromatograph, generally (REM) was less than 4% for all samples.

Relative response factor and quantitative ethanol determination

Taken 2 mL of ethanol at (200, 100, 50, 25, 12.5 and 10 mg/mL) and 3 mL of (4 mg/mL) internal standard solution were poured into an 10 mL vial, after that the sample is putted inside a clean vial and the vial is tightly sealed, samples will place in the oven at temperature 70 °C for time 15 min, volatile compound (ethanol) from the sample will partition into the headspace until a state of equilibrium is reached, 1mL of the head space of samples was injected directly into a GC with tightly syringe. It will be generated a regression line from the GC peak area under curve (A) ratio of ethanol to n-butanol (y-axis) (A_S/A_{IS}), against the concentration ratio of ethanol (x-axis) (C_S), and (fig. 3) show that.

Table 1 shows the relationship between ethanol content in the standard solution with (A_S/A_{IS}), (RSD) %, (REM) %. The slope of the regression line will represent relative response factor (RRF) is the, as in the equation (1):

$$RRF = (A_S/ A_{IS}) \div C_S \qquad (1)$$

And then ethanol content was calculated using the following equation:

$$C_S(mg/mL)=(A_S/A_{IS})x_1/(RRF) \qquad (2)$$

where A_S is the ethanol peak area under curve; A_{IS} is n-butanol peak area under curve; and C_S is the ethanol content (mg/mL).

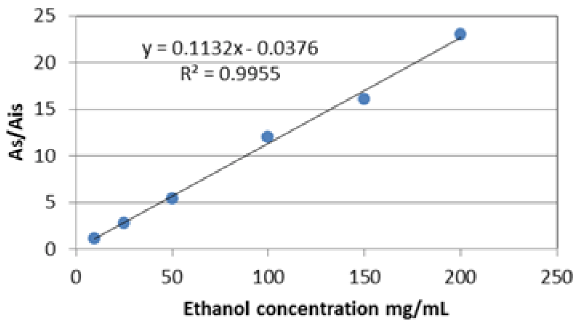

Fig. 3. Linear Regression of Calibration curve of ethanol concentration with (A_S/A_{IS})

GC Conditions

For selection of the GC conditions, it has been selected n-butanol as internal standard because of these reasons; first, n-butanol as chemical structural is similar to the analyte (ethanol), and the it will undergo to same extraction conditions and chromatography, otherwise the compensation will be lost, second n-butanol available in extremely pure form and never appear in the samples third it has retention time (3.5 min) difference about ethanol retention time (7.3 min) in the chromatogram. As shown in figure 2.

For choosing of ethanol concentration it was the highest concertino 200 mg/mL and the lowest concentration was 10 mg/mL. This selection depend on fact that the percent of ethanol in fermented broth by yeast of saccharomyces least than 20% and the concentration of ethanol less than 1% has deform chromatogram. Also for choosing the concentration of internal standard solution we should to match between concentration and the volume of internal standard was added to vial with standard and real solution to get the best resolution without change the volume of internal standard solution for ethanol content range depend on this concept the best concentration of internal standard was 4 g/mL. The rest of other conditions, were initially depended on previous studies [17, 18], until were reached the best conditions. As mentioned before.

Table 1
ETHANOL CONTENT USING GAS CHROMATOGRAPHY BY STATIC HEADSPACE GAS CHROMATOGRAPHY METHOD WITH (RSD) AND (REM)

Ethanol Content (mg/mL)	(A_S/A_{IS})	(RSD)%	(REM)%
10	1.1	5	2
25	2.8	4.5	2.4
50	5.4	4.5	3
100	12	4	3.5
150	16	3	1.5
200	23	2.5	3.5

Table 2
ETHANOL CONTENT DETECTION IN FERMENTED BROTH

Sample No.	Time (hr.)	Ethanol Conc. (mg/mL)
1	6	11
2	12	25
3	24	55
4	48	110
5	72	120

Determination of ethanol concentration in fermented broth

Five samples of fermented broth were taken at different time (6, 12, 24, 48, 72 h) for determination of ethanol content by head space gas chromatography as mentioned before in sample preparation and the results shows in table 2 as follow.

Conclusions

Utilizing a headspace sampling-gas chromatography which is mixed with an internal standard for the determination and quantification of ethanol content in fermented broth medium samples in the laboratory seems to be very promising in the analysis of volatile components. Collected data in this research will help to monitor the fermentation process and determine the concentration of ethanol in biofuel. Although ZB-1 column (40 meter) is smaller than common (ZB-1) columns which used to determine the ethanol in the solutions but this column show that it has a good performance under certain condition for detection of ethanol. It can be useful for the analysis of ethanol in biofuel samples.

References

1.*** Technical Guide of static headspace analysis by using GC. RESTEC, www.restek.com.

2.BADEA, M., CHIPEREA, C., BALAN, M., FLOROIAN, L., RESTANI, P., MARTY, J.-L., IOVAN, C., TIT, D. M., BUNGAU, S., TAUS, N., New Approaches for Electrochemical Detection of Ascorbic Acid, Farmacia, **66**, no. 1, 2018, p. 83.

3.BUNGAU, S., TIT, D. M., IOVAN, C., COPOLOVICI, L., BOSCENCU, R., CIOCA G., COPOLOVICI D., New Method for Simultaneous Determination of Ascorbic and Acetylsalicylic Acids in Effervescent Tablets, Rev. Chim.(Bucharest), **68**, no. 11, 2017; p. 2495.

4.GITEA, D., VICAS, S., GITEA, M.A., NEMETH, S., TIT, D.M., PASCA, B., PURZA, L., IOVAN, C., HPLC screening of bioactives compounds and antioxidant capacity of different hypericum species, Rev. Chim. (Bucharest), **69**, no. 2, 2018, p. 305.

5.ERNESTO BERNAL, Determination of Volatile Substances in Forensic Samples by Static Headspace Gas Chromatography, Gas Chromatography in Plant Science, Wine Technology, Toxicology and Some Specific Applications, **2012**, www.intechopen.com.

6.MACCHIA, T., MANCINELLI, R., GENTILI, S., LUGARESI, E. C., RAPONI, A. & TAGGI, F., Ethanol in biological fluids: Headspace GC measurement, Journal of analytical Toxicology, **19**, 1995, p. 241.

7.KUMAZAWA, T., SENO, H., LEE, X. P., ISHII, A., SUZUKI, O., and SATO, K., Detection of ethanol in human body fluids by headspace solid phase micro extraction (SPME) capillary gas chromatography, Chromatographia, **43**, no. 7, 1996, p. 393.

8.GALI McCARVER-MAY, D., LUBICA DURISIN, L., An Accurate, Automated, Simultaneous Gas Chromatographic Headspace Measurement of Whole Blood Ethanol and Acetaldehyde for Human in Vivo Studies, J. Anal. Toxic, **21**, 1997, p. 134.

9.SMITH, R. M., Before the injection-modern methods of sample preparation for separation techniques, Journal of Chromatography A, **1000**, no. 1-2, 2003, p. 3.

10.DE KONING, S., JANSSEN, H-G., BRINKMAN, UDO A. TH., Modern Methods of Sample Preparation for GC Analysis, Chromatographia, **69**, 2009, p. 33.

11.KOIB, B., ETTRE, L.S., Static Headspace-Gas Chromatography: Theory and Practice, John Wiley & Sons, Inc., 2nd Edition, Book, **2006**, p. 349.

12.TAGLIARO, F., LUBLI, G., GHIELMI, S., FRANCHI, D., MARIGO, M., Chromatographic methods for blood alcohol determination, Journal of Chromatography, **580**, no. 1-2, 1992, p. 161.

13.SETO, Y., Determination of volatile substances in biological samples by headspace gas chromatography, Journal of Chromatography A., **764**, no. 1-2, 1994, p. 25.

14.KUGELBERG, F.C., JONES, A.W., Interpreting Results of ethanol analysis in postmortem Specimens: A review of literature, Forensic Science International, **165**, no. 1, 2007, p. 10.

15.MUSSHOFF, F., Chromatographic methods for the determination of markers of chronic and acute alcohol consumption, Journal of Chromatography B., **781**, no. 1-2, 2002, p. 457.

16.HO NAM CHANGE, WOOD GI LEE, BEOM SOO KIM, Cell Retention Culture With An internal Filter Module: continuous Ethanol Fermentation, Bioenergy and Bioengineering, **41**, no. 6, 1993, p. 677.

17.MEI-LING WANG, YOUK-MENG CHOONG, NAN-WEI SU, MIN-HSIUNG LEE, A Rapid Method for Determination of Ethanol in Alcoholic Beverages Using Capillary Gas Chromatography, Journal of Food and Drug Analysis, **11**, no. 2, 2003, p. 133.

18.CSAVDARI, A.A., RACZ, C., SABOU, D.M., TONK, S.A., SPIRIDON, I.D., Economically Efficient Production of Hydro-Alcoholic Extracts from Medicinal Plants, Rev. Chim. (Bucharest), **67**, no. 10, 2016, p. 1906.

Manuscript received: 27.02.2018