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# STUDY OF ETHANOL ANALYSIS IN BLOOD BY HEADSPACE/GAS CHROMATOGRAPHY / FLAME IONIZATION DETECTOR FOR ENSURING VALIDATION.

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**ABSTRACT:**

Ensuring the validity of laboratory results is one of the general requirements for the competence to carry out tests. The results provided by the laboratory should be satisfied as far as possible/where relevant with the quality of the measurements, statement of conformity with standard requirements or specifications. A laboratory performing testing should have a procedure for monitoring the validity of its results.

To ensure the validity of laboratory results as required or deemed appropriate for monitoring laboratory performance, a procedure for monitoring the validity of results was established. The activities that are required to fulfil validity of results based on analytical method performance characteristics were identified, analyzed, evaluated, monitored and reviewed. To assure the validity of the results, statistical techniques were applied to the monitoring and reviewing of the performance characteristics resulting data. Specificity, selectivity, linearity, acceptance limits, intermediate checks on measuring equipment, precision and accuracy, of the developed method (Ethanol analysis in blood by Headspace/GC/FID) were monitored using reference materials and positive blood control sample. The present study, assist the analyst to maintain a high level of test performance, reduces variation in test results, improves the confidence of testing results, able to translate the data into answers, which directly relate to solving the customer's problem.

**Keywords:** Reference material; Internal standard; Quality Control; EtOH, Ethanol.

**مستخلص:**

تأكيد صحة نتائج المعمل يعتبر من أهم متطلبات كفاءة إجراء الفحص. النتائج التي يصدرها المعمل يجب أن تفي بقدر ما كان ذلك ممكناً جودة القياسات، حالة من التأكيد مقابلة متطلبات المواصفة. لذا يجب على مختبرات الفحص أن تنشئ إجراء يعنى بمراقبة وتأكيد صحة نتائجة.

تم وضع إجراء لتأكيد صحة النتائج التي يقدمها المختبر كما هو مطلوب أو ما يعتبرمناسباً لمراقبة صحة الإختبارات التي يجريها. تم تعريف الأنشطة المطلوبة للوفاء بمتطلبات التحقق من صحة النتائج بناءً على طريقة التحليل، تحليل، تقييم، مراقبة ومراجعة.

لتأكيد صحة نتائج البيانات الناتجة عن اختبار تحليل الكحول الإيثيلى في الدم باستخدام جهاز – Headspace/GC/FIDتم استخدام الأساليب الإحصائية، النوعية، إختيار الطريقة، العلاقة الخطية، المدى المطلوب، المرجعات البينية لأجهزة القياس، دقة البيانات ودقة النتائج مستخدمين للمواد العيارية والمرجعية. أهمية الدراسة في كونها تساعد المحلل لتثبيت أعلي مستويات أداء، تقلل من إختلاف نتائج الفحص، تحسين مصداقية نتائج الفحص وإمكانية تحويل البيانات إلي أجوبة لها علاقة مباشرة بمشكلة الذبون.

## INTRODUCTION

Analytical performance should be monitored by operating quality control scheme appropriately to the type and frequency of testing undertaken by a laboratory to ensure the integrity of the test results. The laboratory results must be fit for its intended purpose and provided "accurately and objectively" (ISO/IEC 17025, 2017, 7.8.1.2). The laboratory should confirm that its measurement procedures are fulfils specified requirements, (AOAC, 2007; ISO/IEC 17025, 2017, 7.2.1.1). The quality control activities required to fulfil ensuring the validity of results, "based on analytical method performance characteristics" (AOAC, 2007), accuracy of a measuring instrument ("ability of a measuring instrument to give responses close to a true value" (ISO 3534-1, 1993; Eurachem, 1998), precision (a measure "of how close results are to one another" (Eurachem, 1998) or "aspects of random error" (NATA, 2009)), specificity / selectivity ("the accuracy of its measurement in the presence of interferences" (NATA, 2009)), LOD (the minimum amount of a substance that an analytical process can reliably detect), LOQ (the minimum amount of a substance that can be reported with a specified degree of confidence) and Linearity/Range. So several "statistical techniques" (Miller and Miller, 2010) have been developed through implementing a quality control methods. The laboratory should maintain an "internal quality control" (IUPAC, 1995) program, which is a systematic process that controls the validity of results which are appropriate to the type and frequency of testing undertaken by the laboratory, (ILAC-G19, 2002; Ludwig, 2009). Quality control data should be recorded in a way to be useful whenever use (ISO/IEC 17025, 2017, 7.7.1). When quality control data found to be outside the acceptable criteria, planned action should take to correct the problem and to prevent incorrect results. In order to ensure the production of quality data, the laboratory should participate in "Proficiency testing (PT) studies" (ISO/IEC 17025, 2017, 7.7.2 (a)), or "Inter-laboratory comparisons" (ISO/IEC 17025:2017, 7.7.2 (b)). The laboratory should use the information collected from these sources with the routine QC data to develop plans to prevent the deterioration of data quality.

## MATERIALS AND METHODS

### Materials

### Developed methods

The developed method, Ethanol Analysis in Blood by Headspace /Gas chromatography/Flame Ionization Detector, was chosen as guidelines and techniques for single-laboratory for monitoring the validity of its results.

### ISO/IEC 17025:2017 standard

The ISO/IEC 17025 (2017) standard, general requirements for the competence of testing and calibration laboratories was used as standards for establishing procedure for Ensuring the validity of results of testing laboratory.

### Control Standards

### Reference materials (RM)

These material composed of internal standards solution (IS) (200 mg 2-Propanol/100 ml water). Positive Blood Control Sample (80 mg EtOH/ 100 ml Blood).

### Certified reference materials (CRM)

The material used in certified reference materials were 10g EtOH /dl aqueous ethanol standard solution, 50 mg EtOH /dl aqueous ethanol standard solution, 100 mg EtOH /dl aqueous ethanol standard solution, 200 mg EtOH /dl aqueous ethanol standard solution, 300 mg EtOH /dl aqueous ethanol standard solution and 400 mg EtOH /dl aqueous ethanol standard solution.

**Resolution mixture control sample**

Solution of control sample were 80 ml of distilled water, 100 µl of ethanol, 100 µl of 2 – Propanol, 100 µl of acetone and 100 µl of methanol.

**Preparing controls**

**Internal standards solution (IS)**

About 250 µl of Iso-Propanol were dilute in 1000 ml of distilled water, and dilution existed at room temperature prior directly to use for not less than half hour.

**Positive blood control sample**

One ml of absolute ethanol was diluted in 1000 ml negative whole blood using Calibrated fixed Micropipette 1000 µl in 1000 ml volumetric flask. The Volume of absolute ethanol was determined to prepare blood control sample as follows:

$$V_{EtOH} = \frac{C_{BCS} \times V_{BCS}}{d_{EtOH} \times 10^3}$$

Where: $V_{EtOH}$ is the volume of absolute ethanol (ml); $C_{BCS}$ is the Conc. of blood control sample (mg EtOH /100 ml blood); $V_{BCS}$ is the volume of blood control sample (ml);$d_{EtOH}$ is the density of absolute ethanol (g/ml); i.e. at density = 0.791 (g/ml).

**Method Blank control sample**

Distilled water with Internal Standard were used, by drawing the ratio of 0.9 ml IS solution and 0.1 ml of distilled.

**Method**

The activities that are required for fulfilment validity of results based on analytical method performance characteristics, were identified, analyzed, evaluated, monitoring and reviewed. The method used according to ISO/IEC 17025(2017) standard.

**Quality of test results**

To assure the validity of the proposed method data, the performance characteristic, specificity, selectivity, linearity, acceptance limits, intermediate checks on measuring equipment, precision and accuracy (ISO/IEC 17025, 2017, 7.7.1) using reference materials and positive blood control sample.

**Specificity**

To confirm the specificity of the proposed method, each sample was analyzed on the gas chromatograph by the transfer of approximately 0.4 ml of headspace. The vials of the samples are analyzed in the following sequence: One method blank control sample; Resolution mixture control sample; One positive blood control sample; One method blank control sample; One blood samples. To ensure the integrity of the test, verification of validity of reference material was performed, by assessing calibration/ verification intervals, by continuously monitoring the standards, (ISO/IEC 17025, 2017, 7.7.1 (a)).

**Selectivity**

To evaluate the effect of background interferences and/or laboratory contamination on sample results of the proposed method, method blank control sample was used. One ml of blank sample (0.9 ml IS solution and 0.1 ml of distilled water) was injected into GC /HS and 0.4ml of every solvent of the resolution mixture, was injected individually before injecting the resolution mixture into GC /HS. The vials of the samples were analyzed in the following sequence; Method blank control sample, Resolution mixture solvents, Resolution mixture control sample, positive Blood control and the sample.

**Linearity**

The method linearity (Thompson *et al.*, 2002) was determined by evaluation of the regression curve. The calibration curve, using six different ethanol concentrations, 10 mg EtOH /dl, 50 mg EtOH /dl, 100 mg EtOH /dl, 200 mg EtOH /dl, 300 mg EtOH /dl and 400 mg EtOH /dl aqueous ethanol standard solution, was constructed. To monitor the functional of equipment used by the proposed method, "quantitative analysis" (AOAC, 2007), using different ethanol concentrations standards and IS, was performed.

**Acceptance limits**

To determining whether a process of the determination of the concentration of ethanol is in a state of statistical control, a control chart/Shewart-chart was created (Montgomery, 2009; Miller and Miller, 2010; WHO, 2011). A series of 8 reading of the 3 separate reference materials (positive blood control sample) with different level of the concentration were used at any one time, over a 20 days period using GC/MS. Standard deviation (s) calculated as follows:

$$S = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$

where: $S$ is the standard deviation; $x_i$ is the value of particular analysis; $\bar{x}$ is the mean value of all analysis; $n$ is the number of all analysis performed.

The control limits based on method performance was setting. Data, standard deviation and the mean value, obtained from precision of the proposed method using reference materials (RM) was using as the acceptance criteria for the assay method and for drawing up control charts. Data as was setting on a separate control chart for each RM concentration. The control limits setting are:

Warning limits will be $\bar{x}$ +2*s* and –2*s*.

Action limits will be $\bar{x}$ +3*s* and –3*s*.

where: $S$ is the standard deviation; $\bar{x}$ is the mean value.

The control lines of 2S and 3S for all were determined. The control lines "illustrated as below.


Where: Upper control limit = Mean+3STD, Upper warning limit = Mean+2STD, Control line = Mean, Lower warning = Mean-2STD and Lower control limit = Mean-3STD.

**Precision**

A sample duplicate was prepared for monitoring the validity of proposed method results, by homogenizing and splitting the sample into two equal portions before sample preparation process. The sample precision, the closeness of repeated individual measures of analyte, associated with preparation as well as matrix-specific and precision associated with the analytical method was measured. Precision of the proposed method was expressed as the coefficient of variation (%*CV*) and relative standard deviation "RSD" (ISO 3534-1, 1993) for both standard and sample solutions in nominal concentration. "Intraday precision

(repeatability)" (NATA, 2009; Eurachem, 1998) were "analyzed" (Rabeea, 2007; Miller and Miller, 2010; Montgomery, 2009). *CV* and RSD given by:

$$CV = \text{RSD} = 100\ s/x$$

Where: $s$ = standard deviation. $x$ = mean.

**Accuracy**

To determine accuracy of the proposed method, recovery experiments (Thompson *et al.*,2002; NATA, 2009; Miller and Miller, 2010) were performed by spiking a known concentration of the ethanol sample with appropriate concentration of the test sample (fortified/ spiked sample) prior to analysis to produce 80%, 100% and 120% of nominal standard concentration. Samples were prepared in triplicate at each levels. The recovery percentage (%R) was determined (Eurochem, 1998) as follows:

$$\text{Recovery (\%)} = [(Cf - Cu)/Ca] \times 100$$

Where: Cf is the concentration of analyzing measure in fortification sample; Cu is the concentration of analyzing measure in unfortification sample; Ca is the concentration of analyzing measure added in fortification sample.

## RESULTS AND DISCUSSION

### Quality of test results

**Specificity**

The GC/HS chromatogram of the proposed method showed 6 peaks. The signals produced which has been attributed to the resolution mixture solvents (Ethanol, 2-Propanol, Acetone and Methanol), Positive blood control sample and Blood sample only. More over the ethanol peak, purity was 99.99%, which confirm the identity of the proposed method, and indicate that the quantitative analysis of ethanol was under control. Specificity measured assessing the reliability of measurements in the presence of interferences, (Eurachem, 1998).

To demonstrate that the quantitative analysis was under control the laboratory should use "appropriate reference materials" (ILAC-G19, 2002), whose property values are sufficiently "homogeneous" (Eurachem, 1998) for "calibration or identification purposes" (ISO/IEC Guide 30, 1992) and for "estimation bias" (NATA, 2009).

**Selectivity**

The GC/HS chromatogram of the proposed method showed no interference peaks was detected in retention time of ethanol, which indicates that ethanol was analyzed at standard laboratory conditions, verified the absence of carryover following the analysts of high concentration and all standard solutions were prepared using pure solvents and "the capability of instrument to respond to a target substance" (ISO/IEC 17025, 2017, 7.7.1 **(c)),** functional check(s) of equipment). An acceptable Method Blank Control Sample of the proposed method should contain 0.002% w/v ethanol, (Sandra, 2017). To check the suitability of the used distilled water and to verify the absence of any possible contamination, 1ml of distilled water was injected into HS/GC. According to Eurachem (1998) those interferences may inhibit confirmation, for example by distorting the signal arising from the analyte, enhancing the concentration of the analyte by contributing to the signal attributed to the analyte or conversely suppressing the concentration of the analyte if they contribute a negative signal.

**Linearity**

A linear correlation was found between the peak areas and concentrations of Ethanol in the concentrations of 10 to 400 mg EtOH /dl and the correlation coefficient ($R^2$ = 0.9995) was found highly significant. The greater the sensitivity/ slope, the better a method is able to distinguish small changes in analyte concentration (NATA, 2009). Any curved pattern suggests lack of fit is due to a nonlinear (Thompson *et al.*, 2002). Since blood alcohol concentration results <10mg/100ml reported negative and 450 mg/dl is the fatal dose for

most people (Sandra, 2017), 10 to 400 mg EtOH /dl are applicable range of the proposed method.

**Acceptance limits**

The results were shown in table 1 representing of measurements of a quality statistic characteristic, the target mean or target concentration of the Quality Control sample (reference material/ positive blood control sample) and two upper control limits (upper warning and upper action lines) and two lower control limits (lower warning and lower action lines). The results shown in Table 2 and (fig. 1) shown that 7 of the values were fall within upper and lower warning limit of the mean, i.e. 95% of confidence level, and one value fall outside the warning lines. Therefore, 6 of consecutive results were fall inside the range of acceptable values for the control material, which indicate that a process of the determination of the concentration of ethanol was in a state of statistical control.

Table 1: Measurements of a quality statistic characteristic of positive blood control sample.

| **Mean($\bar{x}$)** | **St. Dev. (*s*)** | **UCL (CL+ 3*s*)** | **LCL (CL - 3*s*)** | **UWL (CL+ 2*s*)** | **LWL (CL - 2*s*)** |
|---|---|---|---|---|---|
| 80,00 | 1.65 | 84.95 | 75.05 | 83.3 | 76.7 |

Table 2: Results of Ethanol concentration in blood.

| Date | Ethanol Conc. (mg/100 ml) |
|---|---|
| 1st Day | 81 |
| 2nd Day | 81 |
| 3rd Day | 80 |
| 4th Day | 81 |
| 5th Day | 79,2 |
| 6th Day | 79 |
| 7th Day | 85 |
| 8th Day | 81 |

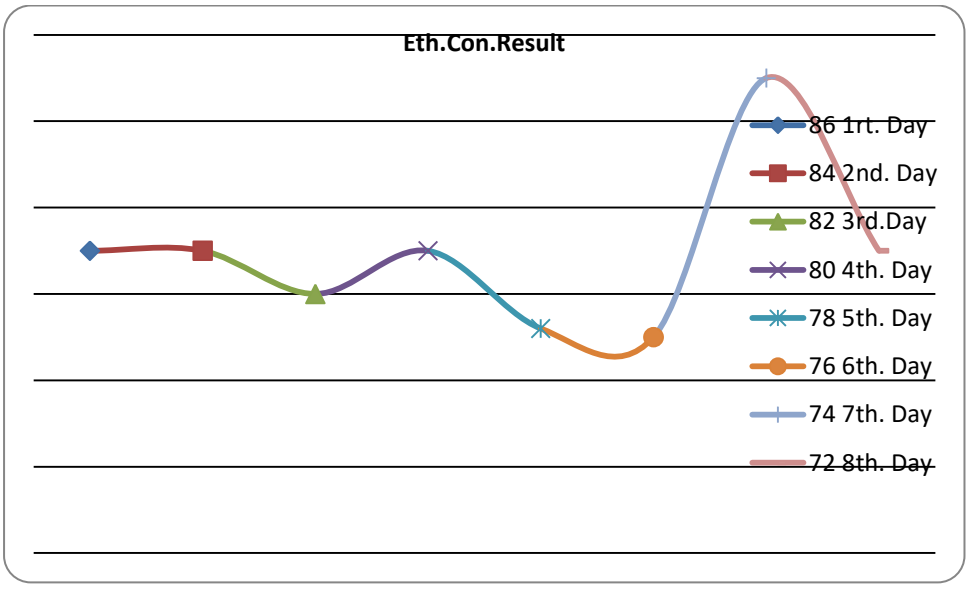


**Figure 1:** Ethanol concentration standard chart.

Variation, as a characteristic of repeated of measurements, may be due to operator, environmental conditions or the performance characteristics of an instrument. Some variation is normal, even when all of the factors listed above are controlled (WHO, 2011). Any control data found to be out of range, the cause should determine and a corrective action should take.

**Precision**

The results of proposed method was presented in terms of variation coefficient *CV* of the measurements for intraday variation shown in table 2. The *CV* of the proposed method was 2.9 indicating good precision of the develop method. Acceptance criteria for (*CV)* of concentration < 5.0. Ideally, the value of the *CV* should be less than 5% (WHO, 2011).

The proposed method used was a chromatographic method for determining the concentration of ethanol in blood sample. The performance of the proposed method was checked, at regular intervals by applying it, with a number of replicate analysts (ISO/IEC 17025, 2017, 7.7.1 (f)), to a standard reference material (SRM). The less varation a set of measurements has, the more precise it is. In more precise measurements the width of the curve is smaller because the measurements are all closer to the mean.

**Accuracy**

Accuracies of positive control sample of the proposed method was found less than 6% the measured concentration of the control sample.

**Conclusions**

A appropriate quality control procedures was established for monitoring the validity of tests undertaken. Statistical techniques was applied to the monitoring and reviewing of the performance characteristics resulting data. In addition, acceptance criteria was created. The performance characteristics established assist the analyst to maintain a high level of test performance reduces variation in test results, improves the confidence in the accuracy of testing results, able to translate the data, generated during analysis of samples, into answers, which directly relate to solving the customer's problem. Quality controls based on the monitoring of results can be use to confirm that the validity of results. The customer will not have the technical skills to appreciate the significance of the data. These facts demonstrated the importance of the proposed method.

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