Validation of a method for ethanol analysis in biological and non-biological samples and its toxicological application

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Abstract

A simple, cost-effective and fast headspace gas chromatography method coupled with flame ionization detection (HS-GC/FID) for determination of ethanol was developed and validated for clinical and forensic toxicology purposes. HS-GC/FID is often used for alcohol determination in different biological and non-biological samples. The calibration dependence of the method was linear in the range from 0.15 to 4.00 g dm^{-3} (r^2=0.999) with adequate accuracy (99–106 %) and precision. The limit of detection (LOD) was 0.006 g dm^{-3}. The method was quantitative (LOQ) above 0.020 g dm^{-3}. The new method was successfully used for determination of ethanol in biological samples of intoxicated patients, car accidents participants, participants in criminal acts, and postmortem samples, non-biological samples such as alcoholic beverages, alcohol-based herbal preparations, cosmetic preparations, *etc.* This method is easy to perform, making it suitable not only for the routine applications in clinical biochemistry and forensic laboratories, but also in different fields of industry (*e.g.* for pharmaceutical preparations, cosmetics, dietary supplements, *etc.*). Some of the applications for ethanol determination in different samples related to various clinical-forensic cases are presented.

Keywords: headspace; gas chromatography; alcoholic beverages; cosmetics.

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1. INTRODUCTION

Ethanol is a substance of high toxicological significance. Undoubtedly, it is one of the most commonly found psychoactive substances in forensic and clinical toxicology. Evaluation of ethanol in biological samples is requested for legal purposes such as postmortem alcohol evaluation and driving under the alcohol influence, but also in scientific studies of alcohol metabolism. The most common methods are breath analysis in the field and blood analysis in the laboratory [1]. Also, ethanol analysis is required in quality control of alcohol-based herbal preparations, cosmetic preparations, alcoholic beverages, *etc.* Numerous chemical and enzymatic methods have been described to determine ethanol in biological materials [2-5]. Methods for sample preparation are static and dynamic headspace [6-8], and solid-phase microextraction [3,9], while chemical analytical methods are gas chromatography (GC) [6,7,10,11], infrared spectroscopy (IR) [12] and high-performance liquid chromatography (HPLC) [13,14].

High precision and low limits of detection of methods for ethanol determination are a demand for toxicologists because regulations about the upper limit for permitted blood alcohol concentration are becoming stricter during the time. In Serbia, the allowed blood concentration is 0.20 g dm^{-3}. That is the reason for optimization and validation of new methods for ethanol analysis.

Due to the number of samples received, a toxicological laboratory requires a rapid and accurate analytical method for determining ethanol concentration [15]. Headspace gas chromatography with flame-ionization detection (HS-GC–FID) has become a gold standard for ethanol analysis because of ease of automation, accuracy, sensitivity, and specificity [16]. It allows a relatively large number of samples to be analyzed quickly, with a minimal amount of manual

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handling. Due to the complex matrix of biological samples, static headspace sampling is the sampling method used for ethanol analysis because the column and injector are protected so that contamination will not occur; while GC/FID is employed for analyte separation and detection [11,17,18].

This paper aims to validate a quantitative HS-GC/FID method for ethanol assessment in biological and non-biological samples and to present its application in resolving various clinical-forensic cases.

2. EXPERIMENTAL

2. 1. Reagents

Ethanol standards in distilled water (0.15. 0.3. 0.5. 0.8, 1.0. 1.5. 2.0. 3.0. and 4.0 g dm^{-3}) and ethanol standards in whole blood (0.80 and 1.00 g dm^{-3}) were obtained from Medichem Diagnostica GmbH&Co, Germany, while ethanol standards in whole blood (0.30. 0.50 and 1.1 g dm^{-3}) were purchased from ACQ Science GmbH & Co, Germany.

Standard *n*-propanol, which was used as an internal standard (IS), was purchased from Merck, Germany. IS concentration of 0.5 g dm^{-3} was prepared by diluting *n*-propanol with deionized water prepared in-house.

Standards in whole blood were stored at 5 °C, while standards in water were stored at room temperature.

Completely anonymous whole blood samples were used for the validation procedure, which were obtained from ACQ Science GmbH & Co, Germany.

2. 2. Instrumental analysis

The analysis was performed on the GC-2010 Plus (Shimadzu, Japan) gas chromatograph with a flame ionization detector. The system was equipped with an HS-20 Headspace Sampler (Shimadzu, Japan) with 90-sample tray. Zebron BAC1 column (30 m × 0.53 mm × 3.00 µm) was used.

The Headspace sampling system (HS-20) parameters were configured as follows:

- oven temperature = 85 °C,
- sample line temperature = 150 °C,
- transfer line temperature = 150 °C,
- equilibration time = 15 min,
- the time for one cycle = 5 min,
- the sample loop = 1 $cm^{3.}$
- injection time = 0.3 min.

The main advantage of HS-20 sampling system is an overlapping analysis of multiple samples. At the same time, multiple samples are in the equilibration stage, which shortens the overall time for analysis.

The parameters used for GC-2010 Plus are configured as follows:

- hold at 45 °C isothermal for 2.40 min;
- nitrogen (purity 99.9992 %) is employed as a carrier gas, at a constant flow of 30 cm^3 min^{-1};
- the detector gas is a mixture of hydrogen and the air; the flow of hydrogen is 40 cm^3 min^{-1} and the air is 400 cm^3 min^{-1}.
- FID temperature is set to 260 °C.

The analysis of the results and data integration was performed by using the LabSolutions program (Shimadzu, country), while statistical analysis was performed by using Microsoft Excel (Microsoft Corp., USA).

2. 3. Sample preparation

Before starting, all calibration solutions and controls were allowed to equilibrate at room temperature. Whole blood samples were mixed before pipetting.

Standard solutions of ethanol (300 µL) were placed into clean glass headspace vials containing 200 µL of 0.5 g dm^{-3} *n*-propanol as an internal standard (IS). Each vial was sealed with a rubber cap and aluminium crimp seal immediately after addition of the standard.

Depending on the expected concentration, some preparations must be diluted (10, 100, 1000 times) before the analysis.

3. RESULTS AND DISCUSSION

The method for determination of ethanol was validated according to the guidelines established by the International Conference on Harmonization (ICH) and the Scientific Working Group for Forensic Toxicology (SWGTOX) [19].

The selectivity, calibration model (linear), accuracy, precision (interday and intraday), limits of detection and quantification (LOD and LOQ, respectively), carry over are presented.

3. 1. Evaluation of the analytical signal

To improve precision and accuracy, the IS was used so that ethanol and IS peak areas were measured and calculations were carried out considering peak ratios of the analyte to IS.

3. 2. Selectivity

The method demonstrated excellent chromatographic selectivity at the retention times of ethanol and the IS (1.410 and 2.144 min, respectively), which is represented at chromatogram in Figure 1. A selectivity study was conducted analyzing whole blood spiked with possible interferences (methanol, acetone). Interferences with these substances were not observed.

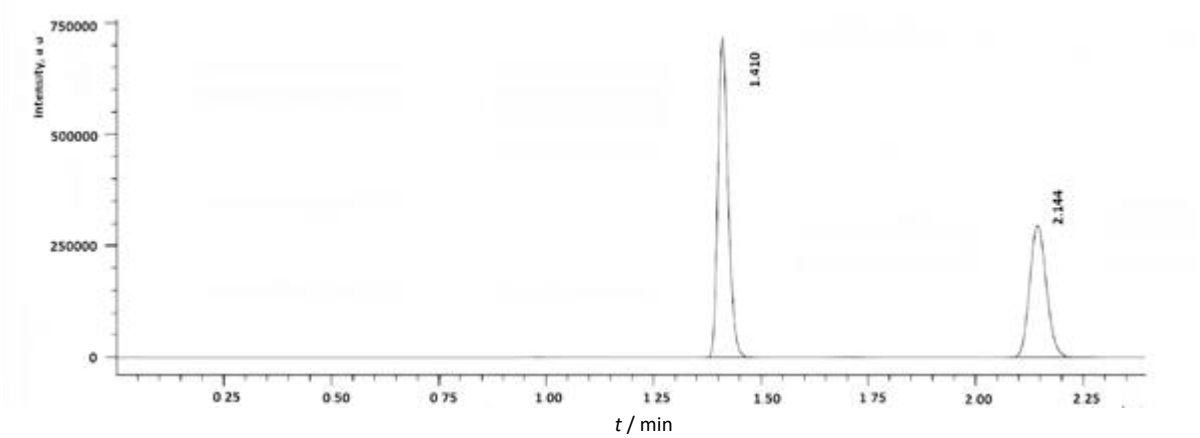

Fig. 1. The representative chromatogram of ethanol (C = 1 g dm^{-3}; R_t = 1.410 min) and n-propanol (C = 0.5 g dm^{-3}; R_t = 2.144 min) standards in whole blood obtained by using Shimadzu HS-GC/FID (HS-20. GC-2010Plus)

3. 3. Matrix-effect

When aqueous ethanol solutions are used for instrument calibration involving a HS-GC technique to quantify this substance, it is necessary to eliminate the matrix effects [20]. The matrix-effect assay was determined using three different whole blood controls, one blank, another containing ethanol, and the last containing both ethanol and IS. Endogenous interference and matrix effect were not observed as peaks were not detected at retention times of analyte and IS in blank whole blood samples. Also, there were no co-eluted peaks with the analyte and IS. All samples were tested in triplicate, in two consecutive days.

Tiscione and coworkers have demonstrated that matrix effects were not occurring between water and whole blood or water and urine standards. They have observed a good correlation for both blood and urine, with r^2 values 0.9999 and 1.0000 respectively, as compared to aqueous standards [21]. Also, selectivity and specificity for all tested compounds in blood, urine and vitreous humor samples was proved in literature [20].

According to the results obtained in the matrix-effect test (matrix effect in whole blood was not observed), as well as results of other authors, it was concluded that aqueous ethanol standards may be used as calibrators and controls when analyzing whole blood, urine and vitreous humour samples [21].

3. 4. Carryover

The sample carryover evaluation was performed by analyzing the ethanol-free whole blood control immediately after the analysis of the standard calibrator with the highest concentration of ethanol in water (4 g dm^{-3}). Carryover was not noticed as peaks were not detected at retention times of the analyte.

3. 5. Linearity

After establishing the chromatographic conditions, the calibration curve was prepared at ethanol concentrations in the range of 0.15–4.00 g dm^{-3}. The calibration model was determined from nine-point calibration curves with calibrators prepared in triplicate as ethanol standards in water. A linear regression of the ratio of the peak area counts of the analyte and IS ($f(C)$) versus the analyte concentration (C) was used to construct the calibration curve. The linear regression equation was:

$$f(C) = 1.94627\ C - 0.0572076 \quad (1)$$

Linearity was obtained with the correlation coefficients r^2=0.9999 and r=0.9999, which proved good linearity (coefficient values above 0.999). The calibration curve is shown in Figure 2.

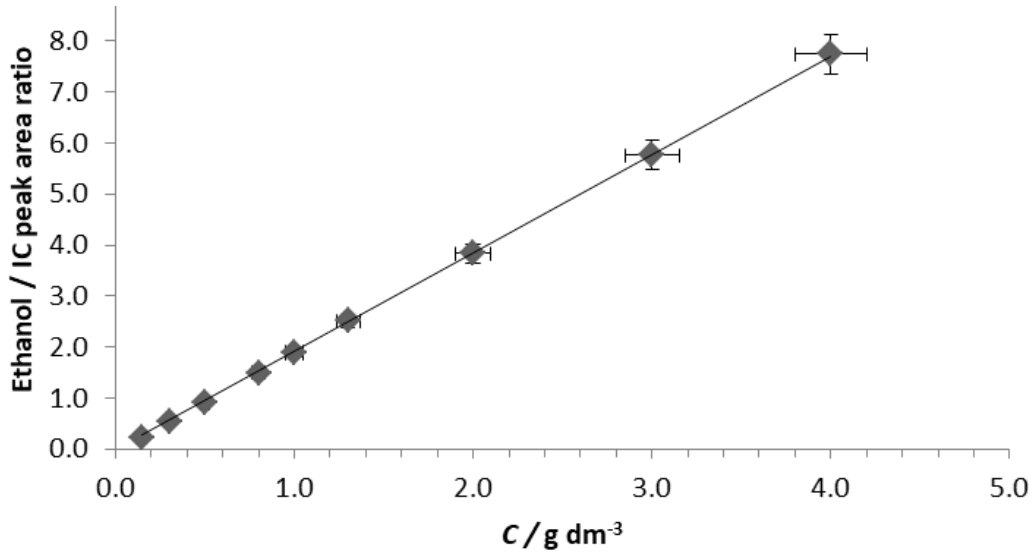

Figure 2. Experimental data of the ratio of peak areas of ethanol and IS (f(C)) vs. ethanol concentration (C) and the best linear fit as the calibration curve for ethanol determination (data present average of n = 3)

Routine laboratory practice requires fast and reliable results. Unknown concentrations of alcohol are present in different samples, so the calibration curve has to be prepared in a wide range of concentrations. The nine-point calibration curve provides the possibility to determine ethanol concentration in all samples, with required precision and accuracy.

3.6. Accuracy

The accuracy of the method was determined by the analysis of whole blood samples. The recovery tests of this analytical procedure were performed by analyzing 5 standard concentrations of ethanol in whole blood in the range 0.30 – 1.10 g dm^{-3}. A recovery rate, bias, standard deviation, and coefficient of variation were obtained, and the results are shown as percentages in Table 1. Accuracy/bias of the assay did not exceed ±10 % over the dynamic range of the assay for the analyte, following SWGTOX guidelines that establish the maximum acceptable bias for ethanol analysis at ±10 % at each concentration level. Coefficients of variation were in the range from 0.85 % calculated for concentration 0.50 g dm^{-3} to 1.59 % for concentration 0.30 g $dm^{-3.}$ while bias was from 0.018 % calculated for concentration 0.50 g dm^{-3} to 5.76 % for concentration 0.30 g dm^{-3}.

Table 1. Recovery test for ethanol in whole blood; bias, standard deviation and coefficient of variation (CV)

Theoretical concentration, g dm^{-3}	Bias, %	Mean measured concentration, g dm^{-3}	Mean recovery, %	SD, g dm^{-3}	CV, %
0.30	5.76	0.3173	105.77	0.0050	1.59
0.50	0.02	0.4991	99.82	0.0042	0.85
0.80	0.93	0.8074	100.93	0.0143	1.77
1.00	0.88	0.9912	99.12	0.0096	0.97
1.10	1.52	1.1167	101.51	0.0112	1.00

3. 7. Precision

Intra-day precision - repeatability, defined as the coefficient of variation, was determined by ten individual replicates of ethanol standard concentration of 1 g dm^{-3}.

The average measured value was 1.0320 ± 0.0059 g dm^{-3}. Values of the obtained standard deviation (0.0059 g dm^{-3}) and coefficient of variation (0.5672 %) indicate good precision for our method, according to ICH [22]. In Table 2 the determined ethanol concentrations and recovery values are presented.

Table 2. Results of ten replicate measurements of the ethanol standard (1 g dm^{-3}) and recovery values

Test number	Measured concentration, g dm^{-3}	Recovery, %
1	1.0437	104.37
2	1.0285	102.85
3	1.0268	102.68
4	1.0311	103.11
5	1.0328	103.28
6	1.0288	102.88
7	1.0276	102.76
8	1.0428	104.28
9	1.0281	102.81
10	1.0301	103.01

Inter-day precision testing using three different concentrations measured three times was performed in two consecutive days and statistical parameters are calculated (Table 3). Coefficients of variation were in the range from 0.03 to 0.32 %. These parameters indicate excellent inter-day precision of this method.

SD values represent the deviation of the mean concentration measured on the second day from the mean concentration measured on the first day.

Table 3. Results of the test for the inter-day precision: standard deviation and coefficients of variation were calculated for 3 measurements and recovery values for for each concentration measured at the second day compared to mean concentration measured at the first day

Concentration, g dm^{-3}			SD, mg dm^{-3}	CV, %	Recovery, %
Standard	Mean measured, first day	Mean measured, second day			
0.3	0.2999	0.2985	0.969	0.32	99.96
					98.46
					100.20
1.0	0.9968	0.9979	0.777	0.08	100.31
					99.74
					100.27
2.0	1.9972	1.9980	0.565	0.03	100.09
					99.98
					100.04

3. 8. Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the results for the lowest concentration (0.15 g dm^{-3}) according to equations:

$$LOD = 3\ SD \quad (2)$$

$$LOQ = 10\ SD \quad (3)$$

leading to:

$$LOQ = 3.3\ LOD \quad (4)$$

Based on the results of 6 repeated measurements of the standard with the theoretical concentration of 0.15 g $dm^{-3.}$ which yielded the mean value of 0.152 ± 0.002 g dm^{-3} with CV = 1.122 %, the LOD and LOQ values were calculated as 0.006 and 0.020 g $dm^{-3.}$ respectively. The obtained values are suitable for routine analyses.

3. 9. Comparison of the obtained results with the results in literature

Table 4 presents results of different parameters in validation of methods for ethanol determination published by different authors. The obtained coefficient of determination in our study was high *i.e*. 0.999. Our study has shown a narrow range of recovery values, close to 100 %. Only one method exhibited lower limits of detection and quantification [25] than those determined in the present study.

Table 4. Validation parameters obtained in the present study and studies published in literature

Reference	Linear Range	R^2	Recovery, %	LOD, g dm^{-3}	LOQ, g dm^{-3}
This study	0.15-4.00	0.999	99.12-105.77	0.006	0.02
[23]	0.30-3.5	0.993	91.00 – 109.10	0.099	0.13
[20]	0.1-10	0.990	/	0.005	0.01
[24]	0.5-5	0.9992-0.9999	/	0.050	0.05
[25]	0.075-2.4	0.999	89.0–114.4	0.00053	0.002

3. 10. Application of the validated method

This analytical method validated at the toxicological laboratory of the Institute of Forensic Medicine in Nis was accredited by the Accreditation Body of Serbia, according to ISO/IEC 17025/2005 in 2019 and reaccredited according to ISO/IEC 17025/2017 in 2020. This analytical method is precisely set and described in a standard operative procedure (SOP), which is followed by the laboratory personnel. The SOP is available and controlled by experts in charge of the inspection of the laboratory, in the accreditation process. The SOP contains analytical procedures and validation parameters, including information about participation in external proficiency testing. Proficiency testing (PT) is performed four times a year since 2015. and the toxicological laboratory has passed PT schemes every time.

This validated method has found a wide spectrum of applications in resolving many different forensic cases at the Institute of Forensic Medicine in Nis. Daily, it is used for alcohol determination in biological samples:

- antemortem clinical cases (blood, urine of intoxicated patients)
- antemortem legal cases (blood, urine of participants of a road accident, criminal acts, *etc.*)
- postmortem cases (blood, urine, vitreous humour).

Along with biological samples, ethanol content is also routinely analyzed in non-biological samples (alcohol beverages). Also, the method provides the possibility for quantification of potentially present methanol.

Here we present some chosen obtained results of analyses of different samples.

3. 10. 1. Herbal preparations

There have been few cases of people which blood tests for alcohol content showed the presence of alcohol although they claimed that they had not consumed any alcohol beverages, but only herbal preparations containing alcohol. The reason is that ethanol is used as an excipient in various pharmaceutical formulations. Ethanol contents in pharmaceutical products vary in different formulations; higher ethanol concentrations are most commonly used in liquid formulations such as syrups, solutions and suspensions [26]. In one syrup, 1.6 wt.% (16 g dm^{-3}) of ethanol was determined, while even 49 wt.% of ethanol was found in another herbal tincture.

3. 10. 2. Non-alcoholic beverages

There were several cases of symptoms of alcohol intoxication, but the persons in question did not consume any alcoholic beverage on purpose. The suspected drinks were sent for the toxicological analysis. In some cases, the analysis has shown the presence of ethanol, while in the others, along with ethanol there were other psychoactive substances. For example, a liquid resembling water has shown the presence of 1.08 g dm^{-3} of ethanol along with the anxiolytic and sedative drug bromazepam.

3. 10. 3. Cosmetic preparations

It is known that cosmetic preparations contain denatured ethanol (predominantly ethanol with less than 5 % methanol), to prevent abuse by alcoholics. Sometimes, intoxications with cosmetic preparations happen, accidentally or on purpose. In one case, a cosmetic preparation was analyzed for ethanol and methanol contents, showing only methanol presence. The chemical composition of this cosmetic preparation was not in accordance with listed chemical compounds. Also, the concentration of methanol in preparation was above the permitted concentration (35.14 wt.%). According to the methanol toxicological profile, it is extremely life-threatening to consume such cosmetic preparations. In this case it is more dangerous, because the concentration of methanol was above the permitted value.

4. CONCLUSION

A rapid, highly sensitive and reliable headspace-GC–FID method was established for ethanol measurement and was validated in terms of linearity, selectivity, accuracy, precision, and detection and quantification limits. It was verified that this method for ethanol determination is applicable in routine diagnostics and monitoring for forensic–toxicological and analytical purposes.

The results indicated good linearity ranging between ethanol concentrations of 0.15 and 4.00 g dm^{-3} at sufficient accuracy and precision. Since samples can be analyzed directly, without special preparation, results can be obtained rapidly (2.40 min run time).

This method is easy to perform, making it suitable not only for the routine application in clinical biochemistry and forensic laboratories, but also in different fields of industry (pharmaceutical preparations, cosmetics, dietary supplements, *etc.*). We have shown that it can be applied to numerous samples, biological and non-biological. Determination of ethanol content is of high importance for resolving important forensic cases, as presented in this paper.

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SAŽETAK

Validacija metode za analizu etanola u biološkim i nebiološkim uzorcima i njena primena u toksikologiji

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(Stručni rad)

Jednostavna, ekonomična i brza metoda „head-space“ gasne hromatografije sa plamenojonizujućim detektorom (*engl.* headspace gas chromatography coupled with flame ionization detection, HS-GC/FID) za određivanje etanola, razvijena je i validirana radi primene u kliničke i forenzičke toksikološke svrhe.HS-GC/FID se često koristi za određivanje etanola u različitim biološkim i nebiološkim uzorcima. Kalibraciona kriva metode je bila linearna u rasponu od 0,15 do 4,00 g dm^{-3} (r^2 = 0,9999) sa adekvatnom tačnošću (99,12–105,77 %) i preciznošću. Granica detekcije (*engl.* limit of detection, LOD) bila je 0,006 g dm^{-3}. Metoda je bila kvantitativna (*engl.* limit of quantification, LOQ) iznad koncentracije etanola od 0,02 g dm^{-3}. Nova metoda je uspešno korišćena za određivanje etanola u biološkim uzorcima pacijenata, učesnika saobraćajnih nezgoda, izvršioca krivičnih dela, postmortem uzorcima, nebiološkim uzorcima poput alkoholnih pića, biljnih preparata na bazi alkohola, kozmetičkih preparata, itd. Metoda je jednostavna za izvođenje, što je čini pogodnom ne samo za svakodnevnu praksu kliničko-biohemijskim i forenzičkim laboratorijama, već i u različitim poljima industrije (farmaceutski preparati, dijetetski suplementi, kozmetički preparati...). Takođe, prikazana je i primena ove metode za određivanje sadržaja etanola u različitim uzorcima povezanih sa kliničko-forenzičkim slučajevima.

Ključne reči: headspace, gasna hromatografija, alkoholna pića, kozmetika