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ORIGINAL ARTICLE

Volatile compounds screening method in human blood by HS-GC-FID: Application in forensic toxicology to discriminate an antemortem consumption from postmortem formation of ethanol

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ABSTRACT

Background. Determining whether a positive blood alcohol concentration (BAC) originates from antemortem consumption or postmortem formation is a frequent issue in forensic toxicology, especially when markers of alcohol ingestion cannot be analyzed. This study presents a validated HS-GC-FID method for detecting and quantifying volatile compounds in human blood to assess the risk of postmortem ethanol formation in cadaveric samples.

Methods. The validation of the method was carried out in accordance with the Guidelines of the Scientific Working Group for Forensic Toxicology (SWGTOX, 2013). A Clarus 580 gas chromatographic system equipped with a Headspace Perkin Elmer Turbomatrix 16 and Rtx-BAC Plus 1 column (30 m, 0.32 mm ID, 1.8 µm df) were used for method development and method validation, the acquisition software was TotalChrom Navigator version 6.3. Isopropanol was used as internal standard. For method validation, blood's healthy volunteers' samples collected *in vivo* were obtained from the blood transfusion center of the University Hospital of Oran. Seven cadaveric samples, received in order to carry out postmortem toxicological investigations, were analyzed by the validated method.

Results. A group of six volatile substances (acetone, butanol, ethanol, isobutanol, methanol and propanol) well correlated with putrefaction and microbial activity, were qualitatively and quantitatively analyzed by a selective method validated by HS-GC-FID in biological samples. All volatile solvents were studied in the range up to 4000 mg/L in terms of selectivity/specificity, limit of detection (LOD) and limit of quantification (LOQ), linearity, precision, accuracy and bias. The LOD was 1 mg/L for all solvents with a LOQ between 50 mg/L and 100 mg/L. Bias, repeatability, reproducibility and accuracy studies have shown good results. The developed method was applied to real cases to estimate the relevance of the method.

Conclusions. The present method is suitable for the identification and quantification of volatile compounds and can be a reliable tool in forensic toxicology. However, further studies should be carried out to establish the modelling of the relationship between the ethanol produced and the concentration of volatile solvents produced.

Keywords: *HS-GC-FID; Blood alcohol concentration; Volatile compounds; Validation; Forensic toxicology.*

Ethanol (ethyl alcohol) is the most commonly used psychoactive substance worldwide. In Algeria, alcohol consumption is a significant concern. According to the World Health Organization (WHO), Algeria is the second-highest consumer of alcoholic beverages in the Maghreb region.[1] As a result, it is heavily involved in arrests for problem drug use. Blood alcohol concentration (BAC) testing is the most frequently requested and performed analysis in toxicological investigations involving suspected exposure to psychoactive substances, with a positive BAC considered indisputable proof of intoxication. However, relying solely on this parameter can be problematic due to interpretation difficulties encountered in routine forensic cases.[2,3] BAC analysis in autopsy

specimens and its interpretation pose a major challenge in forensic toxicology.[4] The origin of the detected ethanol often becomes a matter of debate,[5] with three possible sources for ethanol detected in postmortem specimens: antemortem ingestion, antemortem endogenous production, and postmortem microbial fermentation in either cadavers or samples after collection.[6,7]

As BAC is routinely used as evidence in criminal and civil litigation, definitively determining its origin, whether exogenous (external) or endogenous (internal), is crucial.[4] Several factors need to be considered when assessing the origin of measured ethanol, such as the state of putrefaction of the cadaver at autopsy, the deceased's medical history, the circumstances of death, the condition of the test sample, and corroboration of results obtained from multiple matrices (e.g., urine and vitreous humor).[4,6,8]

The determination of ethanol metabolites (ethyl glucuronide and phosphatidylethanol), considered direct and specific biomarkers of alcohol consumption, is a valuable approach recognized by the international scientific community.[9-11] Furthermore, detecting volatile solvents produced during putrefaction, which do not occur naturally in the human body and can be identified in cadaveric samples, offers an effective means of better interpreting BAC results. This approach is particularly valuable when routine diagnostic methods for alcohol consumption are not used.[6,8,12]

At the Department of Pharmacology and Toxicology of the University Hospital of Oran, ethyl alcohol and cannabis are the most frequently detected psychoactive substances in cases received for postmortem forensic toxicological expertise. BAC testing is practically the only approach used to document alcohol consumption. Other methods for diagnosing alcohol consumption are not routinely employed. Therefore, this study aims to optimize and validate an HS-GC-FID method for detecting and quantifying volatile compounds in human blood to assess the risk of postmortem ethanol formation in cadaveric samples.

MATERIALS AND METHODS

Chemicals and reagents

All reagents used throughout the assay were of analytical grade: acetone > 99% GC quality (Sigma-Aldrich); ethanol > 99.8% GC quality (Sigma-Aldrich); isobutanol > 99.5% GC quality (Fluka Chemika); isopropanol 99.9% HPLC quality (Sigma-Aldrich); methanol > 99.9% HPLC quality (Chromasolv); n-butanol > 99.5% GC quality (Fluka Chemika); n-propanol 99.5% HPLC quality (Sigma-Aldrich). Deionized water used for the preparation of all solutions was purified to 18.2 MΩ using a PURELAB Option system (ELGA).

Biological samples

For method validation, three blood units of healthy volunteers collected *in vivo* were obtained from the blood transfusion center of the University Hospital of Oran. For urine, samples were obtained from five healthy volunteers. For application of the method, seven cadaveric samples, received in order to carry out postmortem toxicological investigations, were analyzed by the validated method. All samples were stored at -80°C until analysis.

Preparation of calibrators, controls and internal standard solutions

Since all the substances under study were soluble in water, only one mixture was prepared and then studied according to the selected working range, 50-4000 mg/L. Stock solutions of mixture acetone, ethanol, isobutanol, n-propanol, methanol, and n-butanol, with concentrations of 10000 mg/L, and the internal standard isopropanol with a concentration of 10000 mg/L, were freshly prepared daily by diluting the commercial solutions in deionized water.

Working solutions of calibrators and controls were prepared by dilution of the stock solutions directly in a 20 mL glass crimped headspace vial. The concentrations of calibrators and controls were, respectively: 4000, 2000, 1000, 500, 200, 100, 50 mg/L and 3000, 1500, 750, 150 mg/L. The concentration of the working solution of internal standard was 900 mg/L. The vials were crimped immediately after addition of the internal standard and the final solution was vortex-mixed. Because of the instability of the mixture of volatiles, the solutions should be immediately used after being prepared and quality controls have to be prepared every day. The same protocol was used to prepare the biologic matrices range in whole blood and urine.

Sample preparation

Regarding the biological samples, these were thawed and thoroughly mixed before analysis. Each vial contained 1 mL of sample + 100 µL of 10 000 mg/L internal standard solution. The prepared solution was lightly mixed manually and placed in the headspace autosampler.

HS-GC–FID conditions

All experiments were carried out using a PerkinElmer HS-GC-FID Clarus 580 system equipped with a flame ionization detector and coupled to a PerkinElmer Turbomatrix 16 automated headspace sampler. The column used to identify

and quantify volatile substances was an Rtx-BAC Plus 1 column (30 m × 0.32 mm ID × 1.8 µm df). The injector temperature was held at 200 °C while the detector temperature was set at 240 °C. The GC oven (column temperature) was held constant at 35°C during 0 minutes, followed by an increase of temperature at a rate of 10°C/min to 80°C, and maintained for 0 minutes. The carrier gas was nitrogen at 16 psi.

For the headspace conditions, the oven temperature was maintained at 70 °C and the syringe temperature at 75 °C. The sample injection volume was 0.06 mL. Before injection, the vials were incubated for 5 min. The GC cycle time was set to 4.5 min, with a pressurization time of 1 min. Analytical data were processed using TotalChrom Navigator version 6.3.

Validation procedure

The validation of the method was carried out in accordance with the Guidelines of the Scientific Working Group for Forensic Toxicology (SWGTOX, 2013).[13] The required validation parameters were: selectivity, linearity, accuracy, precision, reproducibility, repeatability and carryover. The limit of quantification (LOQ) and limit of detection (LOD) were also determined.

RESULTS AND DISCUSSION

A HS-GC-FID method was developed and validated for the qualitative and quantitative analysis of a group of 6 volatile organic substances with different physicochemical properties. The retention times obtained are presented in Fig. 1. Good chromatographic separation between all the compounds was obtained, including the internal standard.

Matrix effect

The mixture of constituents was successively analyzed in three different matrices: water, blood, and urine. The Student's t-test was then applied to assess significant differences between two regression slopes, revealing no matrix effect among water, blood, and urine.[14] The results are summarized in Table 1, with all t-test values below the theoretical threshold, confirming the absence of a matrix effect. Additionally, the liquid–air partition coefficient exhibited a consistent trend in both blood and water. Given that urine contains

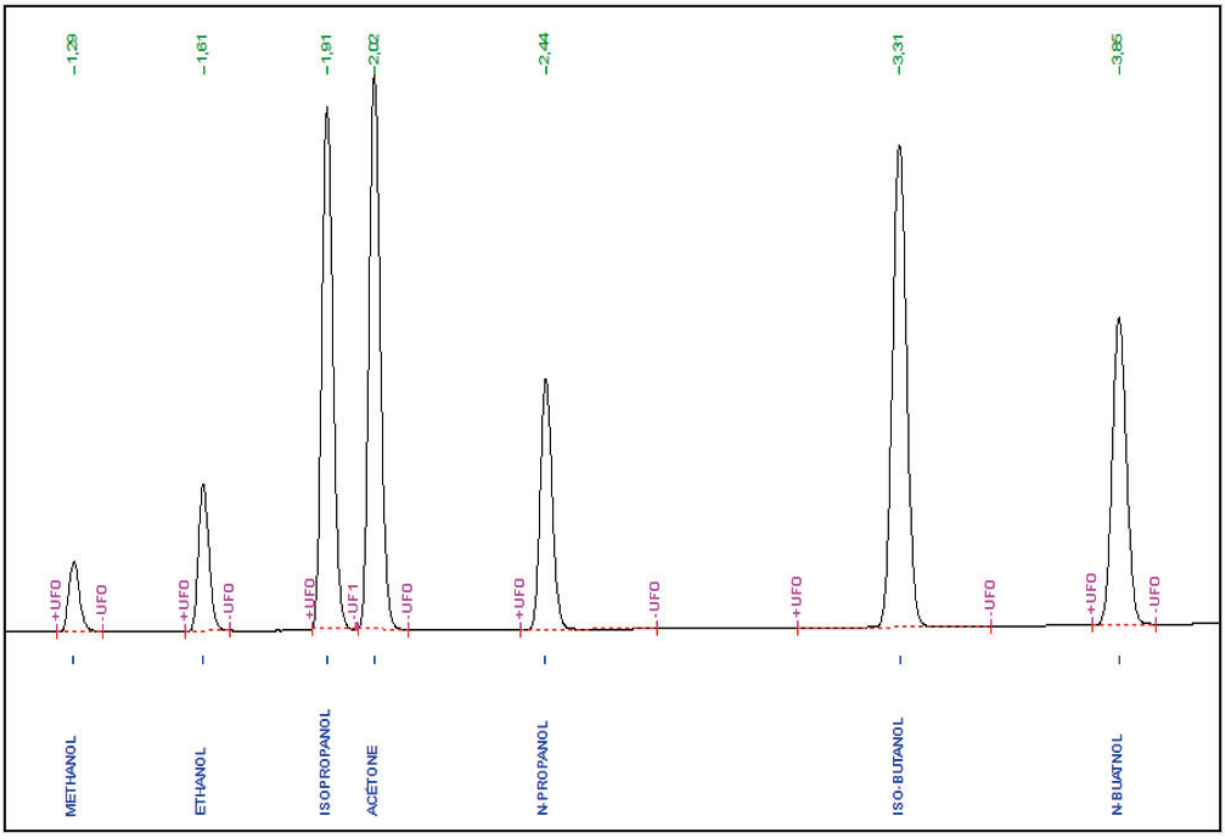

Figure 1. Chromatograms obtained for a mixture of volatile substances by HS-GC-FID (Credits: courtesy of the authors).

TABLE 1. Student's t-test results for the matrix effect study.

	Water/Blood		Water/Urine	
	t calculated	t critical value	t calculated	t critical value
Methanol	1,53		2,22	
Ethanol	1,88		2,18	
Acetone	2,27		0,036	
Propanol	1,66	***2,306***	0,035	***2,306***
Isobutanol	2,08		0,035	
Butanol	1,76		0,019	

approximately 98% water, calibration curves prepared in water are considered adequate.[8] Therefore, the calibration curve will be validated using water as the matrix.

Linearity

In order to analyze the linearity of the method under study, five curves (5 replicates per concentration) with seven calibrators (50; 100; 200; 500; 1000; 2000; 4000 mg/L) were performed for acetone, ethanol and methanol; and with six calibrators (100; 200; 500; 1000; 2000; 4000 mg/L) for butanol, isobutanol and propanol. In the forensic toxicology context, the concentrations are properly spaced over the range to assess exposure to different solvents.

The results show that the different points in the range overlap in all five curves. The various volatile solvents included in the study showed a coefficient of determination $R^2 > 0.999$ (Table 2). Generally, in forensic toxicology, an R^2 greater than 0.995 is required for a regression line to be considered sufficiently linear.[15,16] In addition, statistical approaches have been used to analyze the linearity: Student's t-test for comparing the intercept with 0**,** Fischer test for the existence of a significant slope, and analysis of Variance lack-of-fit test (ANOVA-LOF).[13,16]

Concerning the Student's t-test comparing the intercept with 0, the calculated t is below the critical value at 5% risk for methanol and ethanol, so it can be concluded that the intercept is not different from 0 and that the method is specific. On the contrary, it is different from 0 for acetone, propanol, isobutanol and butanol.

Regarding the test for the existence of a significant slope, using Fischer test, from the raw results, F1 exp > F crit with $p < 0.05$, we can conclude the existence of a significant slope for methanol, ethanol, acetone, propanol, butanol and isobutanol. There is therefore a linear dependence between the dependent and independent variables, at the probability threshold considered. Finally, for the Test of validity of the regression line, the results of the F2 test for methanol, ethanol, acetone, propanol, butanol and isobutanol are lower than the critical value, which means that the fit is valid at the probability threshold considered (we accept the null hypothesis).

Another good approach to selecting a calibration model is to study the distribution of standardized residuals between the values obtained and the values predicted by the model. The distribution of these residuals should be in the ± 2 interval and should not be structured.[17] All distributions of standardized residuals obtained for the solvents do not exceed the ± 2 interval.

In conclusion, all the tests used show that the method has acceptable linearity for linear response modeling.

Limits of detection and quantification

Several approaches can be used to determine the LOD and LOQ of an analytical method. The LOD was estimated using statistical tests according to SWGTOX. The blank was analyzed six times, and the LOD was calculated from the mean and standard deviation of the signals obtained for each compound. Subsequently, descending concentrations of 5, 1, 0.75, and 0.5 mg/L were analyzed, and the LOD was determined to be 1 mg/L for all solvents.

The LOQ was determined using the lowest non-zero calibrator. The lowest points of the calibration curves were analyzed nine times to evaluate bias, precision, and accuracy. For acetone, ethanol, and methanol, a concentration of 50 mg/L met the criteria for detection, identification, bias, and precision, and was therefore adopted as the LOQ. For butanol, isobutanol, and propanol, a concentration of 100 mg/L satisfied all criteria and was established as the LOQ for these solvents.

TABLE 2. Validation data of HS-GC-FID assay for the studied analytes.

Analyte	Retention time (min)	LOD (mg/L)	LOQ (mg/L)	Linear range (mg/L)	Linearity			QC (mg/L)	Bias CV %	Repeatability CV %	Intermediate precision CV %	Accuracy RE %
					Slope	Intercept	R^2					
Methanol	1.29	1	50	50 - 4000	0.2491	0.0048	0.9998	**150**	2.3	6.3	9.9	-2.3
								750	4.4	4	6.5	-4.4
								1500	10	1.1	5.3	-10
								3000	7.2	3	6	-7.2
Ethanol	1.60	1	50	50 - 4000	0.5173	0.0059	0.9999	**150**	4.9	4.5	9	-4.9
								750	2.3	1.9	3.2	-2.3
								1500	10	1.4	2.8	-10
								3000	4	2.1	2.9	-4
Acetone	2.02	1	50	50 - 4000	2.0371	0.0491	0.9999	**150**	-8.9	5	7.7	8.9
								750	-4.2	2.4	5.9	4.2
								1500	-4.6	1.4	2.9	4.6
								3000	-0.5	2.8	5	0.5
Propanol	2.44	1	100	100 - 4000	0.9581	0.0258	1	**750**	-4.6	1.9	2.8	4,6
								1500	-1.1	1.4	3.0	1.1
								3000	-1.9	1.8	2.0	1.9
Isobutanol	3.33	1	100	100 - 4000	2.1132	0.0777	0.9999	**750**	-14.7	2.2	3.8	14.7
								1500	-12.6	2.5	4.1	12.6
								3000	-10.1	2.4	4.1	10.1
Butanol	3.85	1	100	100 - 4000	1.353	0.0575	0.9997	**750**	-12.8	2.3	3.5	12.8
								1500	-8.5	3.3	5.4	8.5
								3000	-8.8	2.8	4.4	8.8

LOD: Limit of detection; LOQ: Limit of quantification; R^2: Coefficient of determination; QC: Quality control; CV: Coefficient of variation; RE: Relative error.

Regarding ethanol, the calculated LOD of 1 mg/L shows that it is possible to detect ethanol below the legal threshold of 200 mg/L. In addition, the LOQ was 50 mg/L, indicating that it will be possible to accurately quantify ethanol concentrations above 50 mg/L, which means that the method can be applied in forensic toxicology for the determination of blood alcohol levels. Similarly, for other solvents, the method is considered sufficiently sensitive to identify and quantify them.

Repeatability, intermediate precision, accuracy and bias

For bias, precision, and accuracy studies, four quality controls at 150, 750, 1500, and 3000 mg/L were used for acetone, ethanol, and methanol, and three quality controls (750, 1500, and 3000 mg/L) for butanol, isobutanol, and propanol. All controls were prepared in blood.

To assess repeatability and reproducibility, the quality controls were analyzed in triplicate over five days. The results were compared with the reference coefficient of variation (CV%). Acceptable CV values were ≤ ± 10% for ethanol and ≤ ± 20% for all other analytes.[13]

Regarding bias, CV values were < ± 10% for acetone, ethanol, methanol, and propanol. For isobutanol, CV exceeded ± 10% at all concentrations, and for butanol, it exceeded ± 10% at 750 mg/L. Nevertheless, all values remained below 20%, which is considered acceptable. In the

repeatability and reproducibility studies, CVs were below 10% for all analytes.

Concerning accuracy, the mean relative error (RE, bias) was within ± 20% of the nominal concentration, meeting the specified criteria.[15] The results of these evaluations are summarized in Table 2.

Carryover

To evaluate carryover several high concentration solutions were tested: 1000, 2000 and 4000 mg/L. A blank was injected after each test. The chromatograms obtained with the blank samples analyzed after the high concentration samples showed no signal, indicating that there was no evidence of carryover. The sequence of injections could therefore be randomized.

Selectivity

To evaluate the selectivity of the method, a random pool of samples (blood and urine) was prepared and divided into two aliquots. The first was directly analyzed with an internal standard only, and the second was spiked with the volatile compound mixture at a concentration of 1000 mg/L. Analysis of the results obtained in the various matrices (blood and urine), and comparison of the blank samples with the spiked samples revealed the absence of false positives and negatives. In addition, the selectivity between the different solvents was studied by calculating the selectivity factor α for the different couples. The selectivity factor α was > 1 for all the solvents tested, confirming that our method is selective.

Application to real cases

To assess the method's effectiveness in a forensic setting, cadaveric blood samples were analyzed using the validated chromatographic method. Cases were selected based on the presence of factors potentially influencing blood alcohol levels, with the additional criteria of compliant (unbroken), non-coagulated, and well-sealed specimens.

It should be noted that autopsy findings are often missing, and the presence or not of alcohol breath in the gastric contents, can make interpretation difficult. Alcohol breath suggests recent intake, potentially during the pre-absorption phase. In addition, the risk of passive diffusion of alcohol from the stomach to the bloodstream after death can occur, leading to artificially high BAC readings that don't reflect levels at the time of death.[18]

A total of seven real cases were analyzed. The subjects included one female and six males, ranging from 22 to 50 years old. Causes of death varied, encompassing drowning (1 case), violent trauma (4 cases), heart failure (1 case), and undetermined causes (1 case). The time between death and autopsy ranged from 1 day to 4 months (unknown in one case) which could be the cause of the post-mortem production of alcohol by a process of putrefaction. This can lead to artificially elevated BAC during postmortem analysis, making it difficult to determine the actual BAC at the time of death. Six samples were peripheral blood, while one was cardiac blood. Notably, none of the samples were supplemented with sodium fluoride (NaF) for preservation, and they were stored for 4 to 15 days under unspecified conditions.

The BAC in our specimen can be influenced by various factors, including type of samples, storage conditions, the circumstance of death and the elapsed time between death and the collection of blood samples. As time passes, alcohol may be metabolized or degraded, making it challenging to determine the original BAC accurately. The validated method had successfully detected and confirmed the presence of the targeted volatile substances in the blood samples (Table 3):

TABLE 3. Results of the solvents analysis by HS-GC-FID.

Patient	01	02	03	04	05	06	07
Blood nature	**PB**	**CB**	**PB**	**PB**	**PB**	**PB**	**PB**
Methanol (g/L)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ethanol (g/L)	2,56	0,155	0,77	1,96	0,29	0,093	0,13
Acetone (g/L)	< LOQ	< LOQ	< LOQ	< LOQ	0,34	< LOQ	< LOQ
Propanol (g/L)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Isobutanol (g/L)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Butanol (g/L)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

PB: Peripheral blood; CB: Cardiac blood; LOQ: Limit of quantification.

- *Methanol and butanol:* absent in all samples
- *Acetone:* visually detected in five cases (1, 2, 3, 5, and 6). However, four were below the LOD of 1 mg/L and considered negative. Only case 5 had a quantifiable amount (340 mg/L)
- *Propanol:* detected in four cases (1, 5, 6, and 7). Considering the LOD, only the propanol peak in case 5 was deemed detectable
- *Isobutanol:* not detected in any of the samples

Our results show that the origin of the ethanol measured for case 5 is debatable and the BAC result may be rejected. According to the literature,[2,4,19] several factors have been incriminated including the delay extended shelf life under undetermined conditions before receiving blood samples taken from tubes not supplemented with NaF. These two critical factors can affect the integrity of samples by promoting the microbial formation of ethanol (post-sampling),[6] and by therefore complicate the interpretation of the results. Moreover, the duration elapsed between death and autopsy is undetermined. This is a very important factor in take into account when interpreting, any extension strongly evokes the putrefaction hypothesis and therefore the neoformation of ethanol postmortem**.** On the other hand, for the other cases it is rather the hypothesis of neoformation of ethanol which can be rejected given the absence of volatile solvents indicating the absence of contamination microbial.

CONCLUSIONS

The analysis of volatile organic compounds offers a promising alternative approach for distinguishing the source of ethanol and confirming antemortem alcohol intake, particularly in settings where routine methods for diagnosing alcohol use are unavailable. The HS-GC-FID method developed in this work allows for the identification and quantification of ethanol alongside five other solvents of interest for assessing microbial ethanol production.

Our future endeavors in this area involve expanding the scope of this method and conducting more in-depth studies to model the relationship between the concentrations of ethanol produced and the concentration of volatile solvents generated by microbial activity. This modeling would enable us to calculate the microbially generated BAC in postmortem blood based on the concentrations of volatile solvents detected, ultimately leading to a more accurate estimate of the actual BAC level. Additionally, the development of techniques for measuring biomarkers of alcoholism remains crucial for a more comprehensive understanding of the challenges associated with BAC interpretation.

Conflicts of interest

The authors declare no conflicts of interest.

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