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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

Introduction: 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.

Methodology: The validation of the method was carried out according to the Guidelines of Scientific Working Group for Forensic Toxicology [13]. The validated method follows: Clarus® 580 GC and Headspace Perkin Elmer® Turbomatrix 16, Rtx®-BAC Plus 1 (30 m, 0.32 mm ID, 1.8 µm df), acquisition software TotalChrom® Navigator version 6.3 with isopropanol internal standard. For method validation, blood's healthy volunteers' samples collected in vivo were obtained from Blood Transfusion Centre 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 and Discussion: A group of 06 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, LOD and 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.

Conclusion: 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: Blood alcohol concentration; forensic toxicology; validation; HS-GCFID; volatile compound

Introduction

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 can be debated [5]. Three possible sources exist 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, 9].

The determination of ethanol metabolites (ethylglucuronide and phosphatidylethanol), considered direct and specific biomarkers of alcohol consumption, is a valuable approach recognized by the international scientific community [10, 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 at Oran University Hospital, ethyl alcohol and cannabis are the most frequently detected psychoactive substances in cases received for post-mortem 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 analytical reagent grade: acetone> 99 % GC quality (SIGMA- ALDRICH), ethanol > 99.8% GC quality (SIGMA- ALDRICH), isobutanol > 99.5 % GC quality (FLUKA CHEMICA), isopropanol 99,9% HPLC quality (SIGMA- ALDRICH), methanol > 99.9% HPLC quality (CHROMASOLV), n-Butanol > 99.5% GC quality (FLUKA CHEMICA), n-Propanol 99.5% HPLC quality (SIGMA- ALDRICH), deionized water for preparation of all the solutions was purified (18.2 MW) using a PURELAB Option ELGA.

Biological Samples

For method validation, 3 blood bags of healthy volunteers collected in vivo were obtained from the blood transfusion center of the university hospital center 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 10 000 mg/L, and the internal standard isopropanol with a concentration of 10 000 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 HS GC-FID PERKIN ELMER Clarus 580 equipped with a flame ionization detector and coupled to a headspace automatic injector PERKEN ELMER Turbomatrix 16. The column used to identify and quantify all the volatile substances was a Rtx®-BAC Plus 1, with dimensions of (30 m x 0.32 mm ID x 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 minute. The carrier gas was nitrogen at 16 psi.

Relative to the headspace, the oven temperature was maintained at 70°C, the syringe 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 programmed to 4,5 minutes with pression time of 1 minute.

Analytical data was processed using the TotalChrom Navigator version 6.3 software.

Validation Procedure

The method was validated according to the Scientific Working Group for Forensic Toxicology guidelines. 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 [13].

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 Figure. Good chromatographic separation between all the compounds was obtained, including the internal standard.

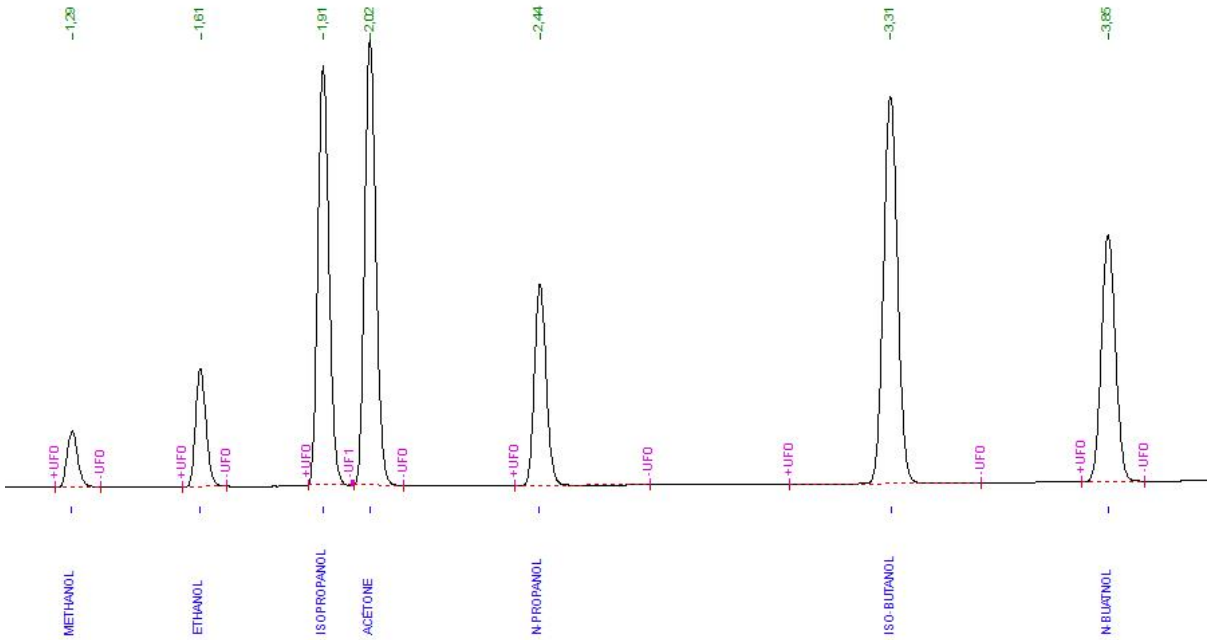

Figure 1: Chromatograms obtained for a mixture of volatile substances by HS-GC\FID

Matrix Effect

The mixture of constituents was successively assayed in 3 different mediums: water, blood and urine. Then the Student's t-test was used to check for significant differences between two regression slopes, thus revealing the absence of a matrix effect between water, blood and urine [14]. The results are presented in the following table. All values obtained with the student's t-test are below the theoretical value, implying the absence of the matrix effect.

On the other hand, the liquid/air partition coefficient maintains its trend in blood and water. Furthermore, since the water content of urine is 98%, the use of calibration curves in water is sufficient [8]. Based on this information, the calibration curve will be validated in water.

Table 1: Student test results for the matrix effect study

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

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)	Biais	Repeatability cv %	Intermediate precision cv %	Accuracy RE %
					Slope	Intercept	R^2		cv %			
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	1.6	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
Aceton	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	1.1
								3000	-1.9	1.8	2	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

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
Méthanol (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
Acétone (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

Linearity

In order to analyze the linearity of the method under study 5 curves (5 replicates per concentration) with 7 calibrators (50; 100; 200 ;500 ;1000 ;2000 ;4000 mg/L) were performed for acetone, ethanol and methanol, and with 6 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 5 curves. The various volatile solvents included in the study showed a coefficient of determination $R^2 > 0.999$ Table. Generally, in a forensic toxicology, an R^2 greater than 0.995 is required for a regression line to be considered sufficiently linear Tab 2 [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. Whereas it is different from 0 for acetone, propanol, isobutanol and butanol. As for 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 Quantitation

There are various methods for determining the LOD and LOQ of a method. Estimating LOD using statistical tests according to the Scientific Working Group for Forensic Toxicology; the blank was analyzed 6 times and the LOD was calculated from the mean and standard deviation of the signals obtained for each compound, plus descending concentrations were read 5; 1; 0.75;

0.5 mg/L. The LOD was identified at concentration 1 mg/L for all solvents.

Determining LOQ using the lowest non-zero calibrator, the first lowest points of the curves were analyzed 9 times to study bias, precision and accuracy. For acetone, ethanol and methanol the concentration 50 mg/l demonstrate that detection, identification, bias, and precision criteria are met and then it was taken as the LOQ. For butanol, isobutanol and propanol the concentration 100 mg/l demonstrate all criteria and it was identified as LOQ for these solvents.

As regards 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, 4 quality controls were used: 150, 750, 1500 and 3000 mg/L for acetone, ethanol and methanol, and 3 controls (750, 1500 and 3000 mg/L) for butanol, isobutanol and propanol. These controls were prepared in blood. To study repeatability and reproducibility, the controls were analyzed 3 times over 5 days. The various results were compared with the reference CV %. The coefficient of variation (CV (%)) values obtained must be ≤ +/- 10% for ethanol, and ≤ +/- 20% for the other analytes [13].

Concerning bias, CV < +/- 10% was obtained for acetone, ethanol, methanol, and propanol. However, it exceeded +/- 10% in isobutanol at all concentrations and in butanol at 750 mg/l. Nevertheless, the values obtained are below 20%, which is considered acceptable. In the repeatability and reproducibility studies, the CVs obtained were lower than 10% for all substances. As regards accuracy is concerned, the mean RE (bias) was within 20% of the nominal concentration, fulfilling the aforementioned criteria [15]. The results of the various tests are presented in Table.

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 2 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, 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 greater than 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, as well as the presence or not of alcohol breath in the gastric contents, can hinder interpretation. 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), undetermined causes (1 case), and heart failure (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 post--mortem analysis, making it difficult to determine the actual BAC at the time of death [8]. 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 blood alcohol level in a post-mortem scenario of the cases chosen for our application can be influenced by various factors, including storage samples, type of samples and the circumstance of death and the elapsed time between death and the collection of blood samples can impact the accuracy of BAC analysis. As time passes, alcohol may be metabolized or degraded, making it challenging to determine the original BAC accurately.

In the analysis carried out on the blood samples of the selected processes, it was possible to detect and confirm the presence of the volatile substances validated in this study.

The validated method successfully detected and confirmed the presence of the targeted volatile substances in the blood samples. The results are presented in (Tab .3).

- Methanol and butanol: Absent in all samples.
- Acetone: Visually detected in five cases (1, 2, 3, 5, and 6). However, four were below the limit of detection (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 samples.

Our results show that the origin of the ethanol measured for case 5 is debatable and the BAC result may be rejected. Several factors have been incriminated including the delay extended shelf life [4, 19] under undetermined conditions before receiving blood samples taken from tubes not supplemented with NaF [2, 20]. These two critical factors can affect the integrity of samples by promoting the microbial information of ethanol (post-sampling), and by therefore complicate the interpretation of the results [6]. 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 [8].

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.

Conclusion

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 [6].

The HS-CPG/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 (acetone, isobutanol, methanol, n-propanol, n-butanol) within just 4.5 minutes. This method demonstrates excellent precision, bias, and selectivity. Additionally, the sensitivity is exceptional even for low concentrations, as evidenced by the detection and quantification limits that fall well below the Algerian legal limit of 0.2 g/L [20].

The headspace injection technique eliminates the need for prior sample extraction, streamlining the process and enhancing its efficiency. This technique also minimizes column contamination and readily lends itself to full automation with appropriate equipment.

Therefore, this method is particularly suitable for cases involving putrefaction or submersion, where postmortem ethanol production is a possibility. It facilitates a more accurate interpretation of analytically validated BAC results. Furthermore, the applicability of this technique to urine samples (demonstrated absence of matrix effect) allows for a combined approach to BAC determination. This involves comparing alcohol levels between the two matrices and detecting volatile solvents potentially produced during postmortem fermentation processes.

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.

Statement and Declarations

Not applicable

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