GC Determination of Acetone, Acetaldehyde, Ethanol, and Methanol in Biological Matrices and Cell Culture

Helena Pontes[1,*], Paula Guedes de Pinho[1,*], Susana Casal[2], Helena Carmo[1], Agostinho Santos[3], Teresa Magalhães[3], Fernando Remião[1], Félix Carvalho[1], and **Maria Lourdes Bastos[1]**

[1]REQUIMTE, Toxicology Department, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha 164, 4099-030 Porto, Portugal; [2]REQUIMTE, Bromatology Department, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha 164, 4099-030 Porto, Portugal; [3]National Institute of Legal Medicine I.P., Faculty of Medicine, University of Porto, Alameda Prof. Hernâni Monteiro, 4200 - 319 Porto, Portugal

Abstract

A gas chromatography with flame ionization detection method (GC–FID) with direct injection, using a capillary column, was validated to determine ethanol, acetaldehyde, methanol, and acetone in different human matrices, such as whole blood, vitreous humour, and urine, with clinical and forensic interest. This method was also employed to quantify these compounds in cell culture medium, thus being useful in basic research. A good peak resolution was achieved, with linear correlation between concentration and peak areas for all the compounds in all the matrices. The inter- and intra-day precisions of the method were always under 15% and 10%, respectively. The accuracy of the method, calculated as the percentage of the target concentration, was within the acceptable limits. The obtained limits of detection were below 0.85 mg/L for acetaldehyde and below 0.75 mg/L for the other considered compounds. The small injection volume and the high split ratios applied, allied to the high performance of the GC column, resulted in very good peak resolution and high sensitivities. This method is easy to perform, making it suitable for the routine of clinical biochemistry and forensic laboratories.

Introduction

Gas chromatographic (GC) methodologies have been reported, being the volatile fraction analysed by diversified techniques (1–5). Chronologically, the direct sample injection in packed columns (1,3) became obsolete and was gradually substituted by headspace techniques in capillary columns (6) and, more recently, by the selective headspace injection using solid-phase microextraction (SPME) fibers (5,7,8). However, both headspace methodologies require an accurate time- and temperature-controlled sample heating. When the automatic injectors are not available, reproducibility problems are difficult to overcome. Moreover, the influence of the biological specimen on the partitioning of the volatile compounds between liquid and headspace vapour and the selectivity of the SPME fibres increase both the complexity of method development and the total analysis time (9). In addition, the headspace techniques usually require larger sample volumes and have higher detection limits.

Ethanol, acetaldehyde, methanol, and acetone are volatile compounds whose detection and quantification in biological matrices can be used as biomarkers of several diseases and/or intoxications.

Ethanol consumption is under strict regulations in many circumstances, and legal blood alcohol concentration limits for driving are well established. Over-consumption of alcoholic beverages and drunkenness are almost always closely related with fatal accidents, trauma deaths, drowning, suicide, and violent crimes (10). These considerations justify the importance of the determination of ethanol levels in ante-mortem and post-mortem specimens with great importance in the forensic domain (4).

Humans are frequently exposed to naturally occurring acetaldehyde that can exist in the air and that can be ingested as a contaminant of food and alcoholic drinks because it is the main compound formed during ethanol metabolism. Additionally, humans are also exposed to acetaldehyde coming from automobile exhaust, cigarette smoke, fireplaces, and occupational settings. This compound has been associated with cancer development in animal experiments, and has been classified as a possible carcinogenic agent to humans by the International Agency for Research on Cancer of the World Health Organization (11). Thus, from a clinical point of view, its quantification in biological samples is of paramount importance.

The quantification of methanol in body fluids is very important for confirmation of methanol intoxication-related deaths (12). The quantification of methanol in biological fluids can also be a biomarker of intentional (13), accidental (for example, by ingestion in adulterated drinks) (14), or occupational exposure (15).

Acetone can be a biomarker of ketoacidosis, which can help to diagnose ante-mortem diabetes mellitus, though it can also be a likely cause of death in binge drinkers and alcoholics (16), and can be very useful in the diagnosis of death from hypothermia (17). Ketoacidosis is diagnosed by analysis of high levels of ketone bodies in body fluids, namely acetone, acetoacetate, and particularly β-hydroxybutyrate (18). Additionally, the quantification of acetone in biological fluids can be a biomarker of occupational exposure (15).

* Authors to whom correspondence should be addressed: Helena de Oliveira Pontes and Paula Guedes de Pinho; REQUIMTE, Toxicology Department, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha 164, 4099-030 Porto, Portugal; e-mail pguedes@ff.up.pt.

Given the importance of the quantitation of these compounds in blood and urine from ante-mortem and post-mortem specimens, we validated the present method in these matrices. However, as post-mortem compound levels in blood do not necessarily reflect the concentration at the time of death, due to drug instability and post-mortem redistribution phenomena (19), measurements of vitreous humour (VH) concentrations could be of interest for predicting the blood concentration at the time of death in humans (20). VH is a very useful matrix because the vitreous fluid is less influenced by autolytic processes, is simple to collect, and is not affected by hemolysis (10). Additionally, it is a clean fluid that contains less protein than urine and exhibits high stability (21). Thus, VH has been recently demonstrated to be a suitable alternative specimen to post-mortem blood and urine, not only for the analysis of ethanol intoxications, but also for other drugs as well as endogenous biochemical constituents of the body to detect ante-mortem diseases (10). VH is, additionally, more resistant to putrefactive changes than other specimens, such as blood (8,22). Due to these advantages, VH determinations have formerly been performed in order to detect various drugs, in particular ethanol (10) as well as morphine (23), cocaine (24), and amitriptyline (25).

The validation of this method for its application in cell culture medium (CCM) samples is also important due to the need of controlling the levels of these compounds when a cell culture is used to study their effects on cell physiology and toxicity (26).

Thus, there is an obvious need to develop an inexpensive, sensitive, rapid, and reliable alternative GC method. In this study, a GC direct injection in capillary columns method is proposed to quantify ethanol, acetaldehyde, methanol, and acetone, and it is applied to different matrices, such as whole blood (B), VH, urine (U), and CCM.

Based on the previously mentioned rationale, the aim of this work was to develop an easy direct injection GC methodology to determine, simultaneously, some of the most important volatiles found in biological samples with clinical, forensic, and research interest and to apply it to several matrices.

Materials and Methods

Reagents

All the chemicals used were of analytical grade: ethanol (> 99.9%, Panreac, Barcelona, Spain), methanol (> 99.9%, Merck, Darmstadt, Germany), 1-propanol (> 99%, Sigma-Aldrich Co., St. Louis, MO), acetone (> 99.9%, Merck), acetaldehyde (> 99.9%, Fluka, Milwaukee, WI), Triton X-100 (Sigma-Aldrich Co.), and acetonitrile (> 99.9%, Merck). William's E culture medium was obtained from Lonza (Brussels, Belgium) and supplemented with 2mM L-glutamine (Lonza), 5 µg/mL insulin (Sigma-Aldrich Co.), 5nM dexamethasone (Sigma-Aldrich Co.), 10mM Hepes (Lonza), 100 units/mL penicillin G, 100 µg/mL streptomycin sulphate, and 250 ng/mL amphotericin B (Sigma-Aldrich Co.).

Biological matrices characterization and preparation

Human B and U samples were collected from healthy volunteers. In the experiments with B, EDTA-blood was used.

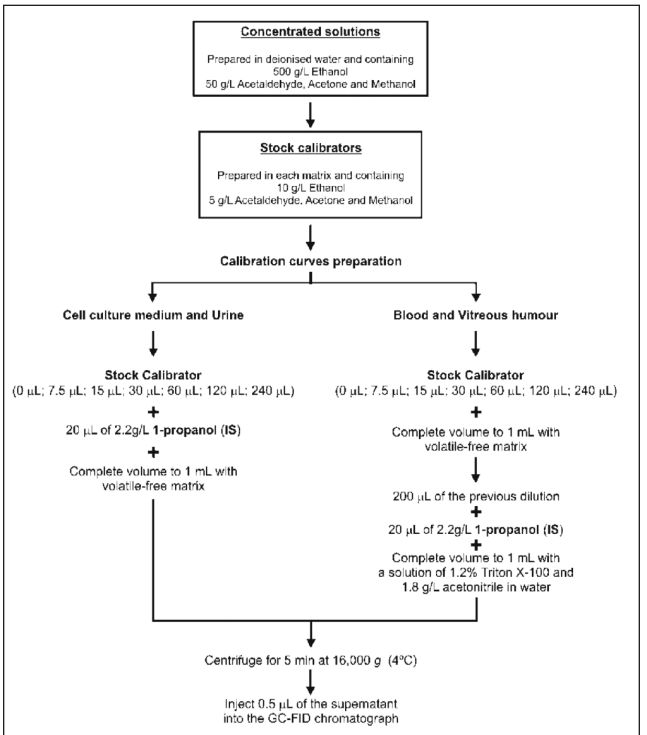

Figure 1. Flowchart for preparation of the calibration curves.

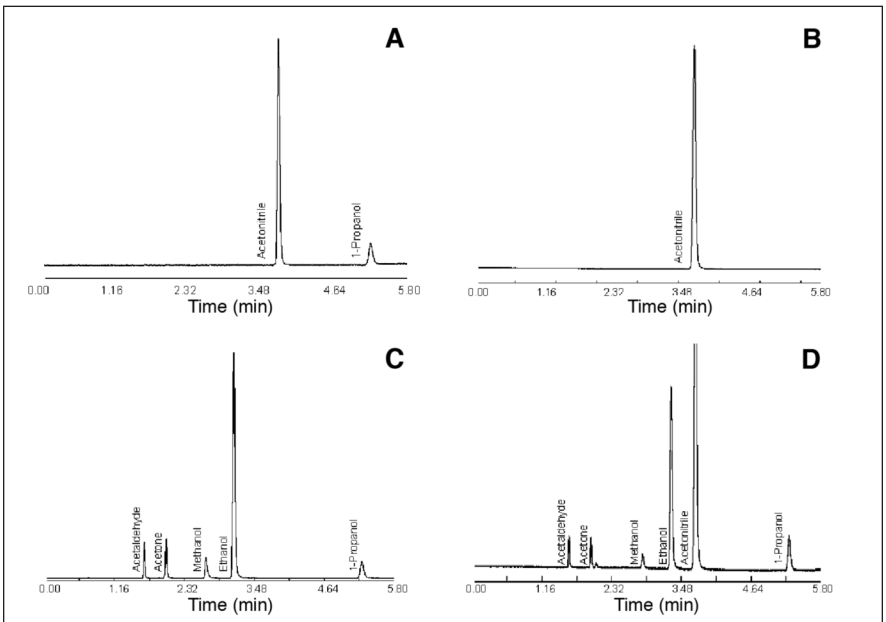

Figure 2. Representative chromatograms obtained from blood sample with IS (A), blood sample with IS (B), calibrator solutions prepared in CCM or U (C), and in B or VH (D) with peak identification and retention times of the quantified compounds.

Post-mortem human VH was collected from autopsy samples at the North Delegation of the National Institute of Legal Medicine I.P., after accomplishment of all legal procedures to post-mortem samples collection. Prior to analysis, each sample was injected into the GC–flame ionization detector (FID) to confirm the absence of each studied compound. Additionally, the interference between the tested compounds and some other volatile biomarkers of some diseases and with great forensic interest was excluded, such as formaldehyde, methyl and ethyl formate, ethylene glycol, propylene glycol, glycerol, 1,4-butanediol, and 2,3-butanediol.

Volatile-free B, U, VH, and CCM were used to prepare the control samples and calibration curves.

Calibration procedures

Concentrated solutions of 500 g/L ethanol, 50 g/L methanol, 50 g/L acetaldehyde, and 50 g/L acetone were prepared daily by dilution of the commercial solutions in deionized water. Daily prepared 2.2 g/L 1-propanol in deionized water was used as internal standard (IS).

A stock calibrator containing 10 g/L ethanol, 1 g/L methanol, 1 g/L acetaldehyde, and 1 g/L acetone was prepared daily in each tested matrix, from the concentrated solutions. The calibration curves were prepared as shown in Figure 1. These procedures resulted in final concentration of 7.5, 15, 30, 60, 120, and 240 mg/L of methanol, acetone, and acetaldehyde, and with 75, 150, 300, 600, 1200, and 2400 mg/L of ethanol. These calibration standards, in B and VH, underwent a 5 times dilution with a solution containing 1.2% of Triton X-100 and 1.8 g/L of acetonitrile in water, to decrease sample viscosity and therefore facilitate volume measurements and sample injection, according to the method described by Dubowski (27).

Each tube was vortex-mixed and centrifuged at 16,000 *g* for 5 min at 4°C. A fixed volume of supernatant (0.5 µL) was injected into the chromatographic system.

Sample preparation

One hundred microliters of B or VH samples were mixed with 10 µL of IS. The samples were diluted to 500 µL with the Triton X-100 and acetonitrile solution, centrifuged, and 0.5 µL of the supernatant were directly injected into the GC as described later. Concerning U and CCM, after adding the IS, samples were centrifuged and 0.5 µL of the supernatant was directly injected into the GC.

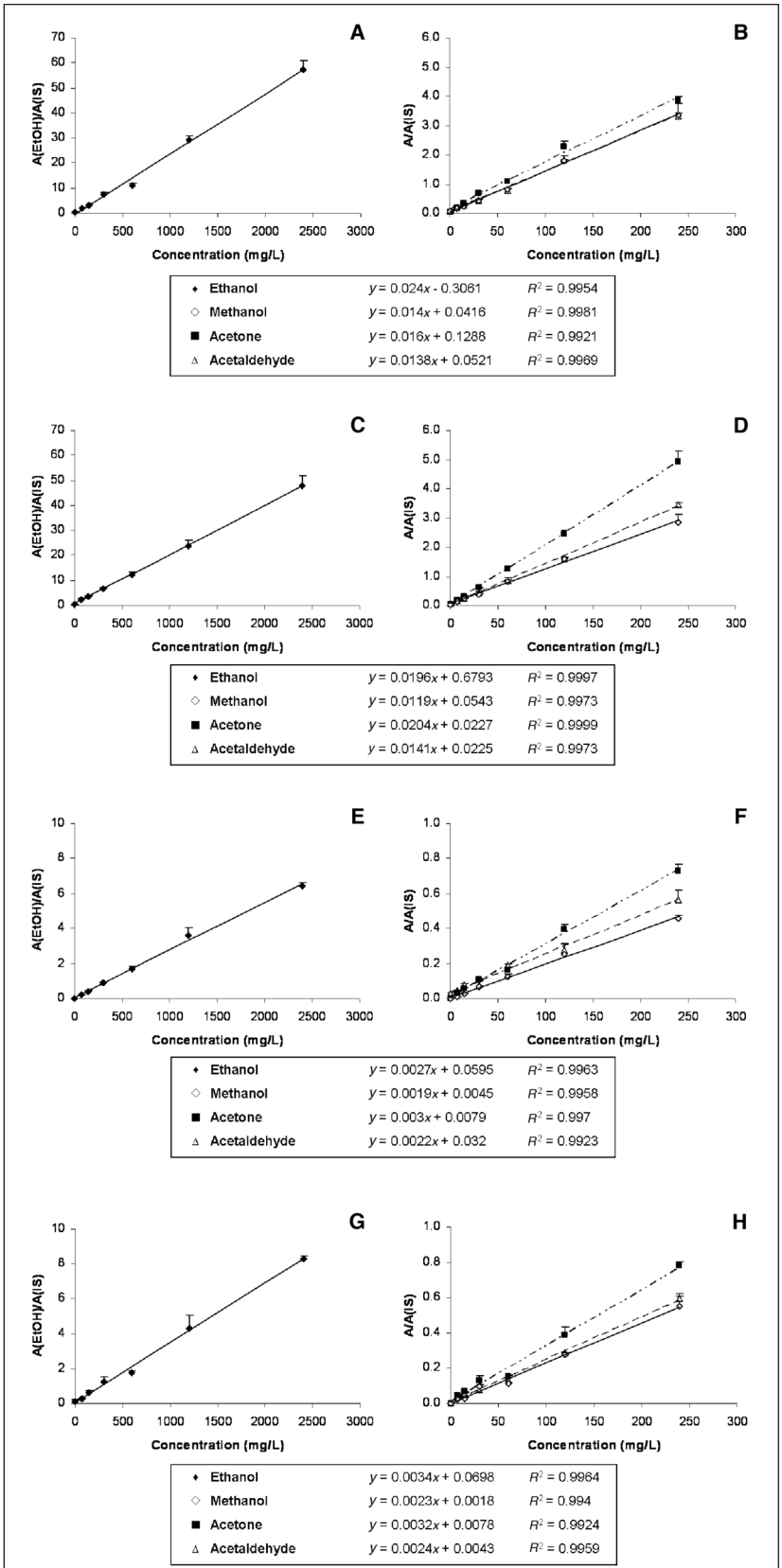

Figure 3. Calibration curves of acetaldehyde, acetone, methanol, and ethanol on the 4 different considered matrices: CCM (A and B), U (C and D), B (E and F), and VH (G and H). Results are presented as mean ± SD of 5 different injections of each calibrator of CCM and U and of 3 different injections of each calibrator of B and VH.

Analytical instrument settings

The GC used was a ThermoFinnigan Model Focus GC equipped with a FID. The injection port of the chromatograph was installed with a glass liner (5-mm i.d.) appropriated for split analysis, to prevent the contamination of the GC column with non-volatile material from the tested matrices. For B and VH, the liner was replaced after 50 injections. For CCM and for U, the liner was replaced after 100 injections.

The analyses were performed under the following chromatographic conditions: Column, CPWax 57 CB (WCOT Fused Silica), 25 m × 0.25 mm i.d., DF = 0.2 µm, from Varian (Palo Alto, CA). The temperature of the FID was 220ºC, and the injector temperature was 220°C. The oven temperature was programmed to 40°C (for 2 min), followed by an increase of 5ºC/min until 200ºC. The carrier gas was helium with a flow of 1.5 mL/min. The injection of B and VH was performed by means of a 10 µL Hamilton syringe (Model 701 RN) with a removable needle (needle gauge 22S), cleaned under vacuum between each injection with the Triton X-100 and acetonitrile solution. On the other hand, the injection of CCM and U was performed by means of a 5-µL SGE syringe (Model 5F-GP) cleaned under vacuum between each injection with deionised water. The volume of injection was 0.5 µL, with a split ratio of 100 and a split flow of 120 mL/min for B and VH; and a split ratio of 60 and a split flow of 90 mL/min for U and CCM.

Table I. LOD and LOQ in mg/L

	Acetaldehyde		Acetone		Methanol		Ethanol	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
B	0.84	2.8	0.75	2.5	0.75	2.5	0.75	2.5
CCM	0.15	0.5	0.38	1.23	0.75	2.5	0.38	1.23
VH	0.15	0.5	0.15	0.5	0.75	2.5	0.25	0.83
U	0.15	0.5	0.75	2.5	0.5	1.67	0.25	0.83

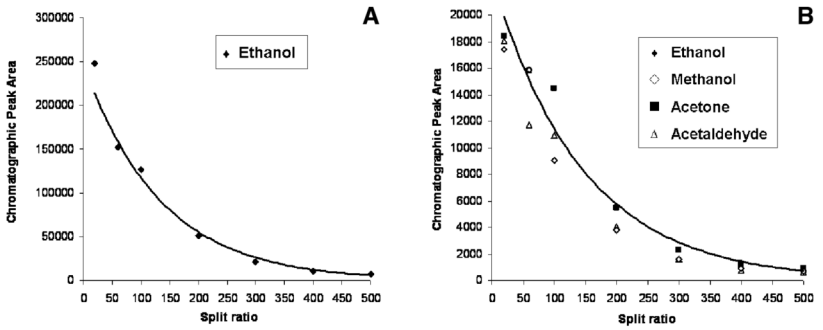

Figure 4. Variation of chromatographic peak area in blood matrix with the injector split ratio.

Effect of the split ratio on the sensitivity of the method

The effect of the split ratio on the sensitivity of the method was studied for B with 60 mg/L acetaldehyde, acetone, and methanol, and 600 mg/L ethanol. Split ratios between 1:40 and 1:500 were tested: 1:40, 1:60, 1:100, 1:150, 1:200, 1:300, 1:400, and 1:500, which corre-

Table II. Inter-Day CV% and A% of the Method for the 4 Matrices and for 3 Representative Concentrations

	Conc	Acetaldehyde			Acetone			Methanol			Conc	Ethanol		
	(mg/L)	Mean ± SD*	CV%†	A%‡	Mean ± SD	CV%	A%	Mean ± SD	CV%	A%	(mg/L)	Mean ± SD	CV%	A%
CM§	15	0.258 ± 0.021	8.094	–0.3	0.326 ± 0.025	7.588	–9.4	0.249 ± 0.020	7.920	2.8	150	3.359 ± 0.213	6.327	2.0
	60	0.788 ± 0.063	8.060	–10.5	1.099 ± 0.100	9.088	–1.0	0.799 ± 0.038	4.718	–10.7	600	10.951 ± 0.955	8.722	–22.3
	120	1.830 ± 0.148	8.075	7.1	2.288 ± 0.184	8.061	5.1	1.813 ± 0.034	1.878	2.8	1200	29.410 ± 1.562	5.311	3.2
U§	15	0.248 ± 0.009	3.787	6.2	0.319 ± 0.021	6.464	–3.0	0.221 ± 0.008	3.449	–5.2	150	3.738 ± 0.276	7.385	3.3
	60	0.859 ± 0.085	9.869	–1.1	1.262 ± 0.101	8.045	1.2	0.818 ± 0.061	7.413	6.5	600	12.284 ± 1.183	9.629	–1.2
	120	1.579 ± 0.084	5.304	–7.9	2.444 ± 0.131	5.351	–1.1	1.582 ± 0.039	2.445	6.7	1200	23.732 ± 2.179	9.180	–1.9
B**	15	0.077 ± 0.007	9.113	18.0	0.058 ± 0.002	3.483	–9.1	0.026 ± 0.003	10.687	–21.0	150	0.401 ± 0.055	13.720	–13.7
	60	0.193 ± 0.006	3.078	18.0	0.166 ± 0.016	9.648	–11.7	0.122 ± 0.019	15.286	–2.9	600	1.685 ± 0.177	10.483	0.3
	120	0.278 ± 0.041	14.592	–5.9	0.393 ± 0.030	7.511	6.9	0.257 ± 0.056	21.934	10.4	1200	3.622 ± 0.420	11.584	9.8
VH**	15	0.054 ± 0.009	10.886	–21.6	0.066 ± 0.003	5.026	19.0	0.030 ± 0.001	3.498	–16.2	150	0.644 ± 0.078	12.046	11.0
	60	0.128 ± 0.029	22.461	–13.5	0.149 ± 0.012	9.412	–23.4	0.112 ± 0.005	4.210	–20.1	600	1.751 ± 0.114	6.496	–17.0
	120	0.284 ± 0.002	0.818	–2.8	0.387 ± 0.047	12.066	–1.3	0.275 ± 0.006	2.005	–1.2	1200	4.310 ± 0.750	17.413	3.9

* Mean of A/A(IS) ± standard deviation.
† Reproducibility = (standard deviation / mean) × 100.
‡ Accuracy = (mean calculated concentration – nominal concentration) / (nominal concentration) × 100; all CV% are inter-day.
§ Mean of 5 replicates.
** Mean of 3 replicates.

sponds to 60 mL/min, 90 mL/min, 120 mL/min, 150 mL/min, 300 mL/min, 450 mL/min, 600 mL/min, and 750 mL/min, respectively.

Validation experiments and acceptance criteria

Method linearity

Method linearity was determined by evaluating the regression curve and is indicated by the square correlation coefficient (R^2). The line of best fit for the relationship between the ratio of peak area and internal standard area and concentration of analytes in the samples was determined by linear regression performing calibration curves in the considered concentration ranges (7.5–240 mg/L for acetaldehyde, acetone, and methanol, and 75–2400 mg/L for ethanol). For U and CCM, the slopes were calculated taking into account the mean of 5 calibration curves prepared in each matrix on 5 consecutive days. For B and VH, the slopes were based on the mean of 3 curves prepared in these matrices on 3 consecutive days. Linearity was achieved with a minimal R^2 of 0.99.

Precision

For U and CCM, the intra-day precision of the method was determined by injecting, on the same day, 5 different replicate calibrators of each one of the 7 points of the calibration curves; the intra-day precision of the apparatus was determined by analysing 5 times, on the same day, the 7 calibrators of one of the calibration curves; the inter-day precision of the method was determined by analyzing, for 5 consecutive days, daily prepared calibrators of the 7 concentrations considered for the calibration curves.

For B and VH, due to the complexity of these samples, the intra-day precision of the method was determined injecting, on the same day, 6 independent calibrators containing 1200 mg/L of ethanol and 120 mg/L of acetaldehyde, acetone, and methanol; the intra-day precision of the apparatus was determined injecting 6 times, on the same day, one of the concentrations contemplated in the calibration curves (1200 mg/L for ethanol and 120 mg/L for acetaldehyde, acetone, and methanol); the inter-day precision of the method was evaluated by analyzing, for 3 consecutive days, daily prepared calibrators of all the 7 concentrations considered for the calibration curves. Precision was assessed by calculating the mean, standard deviation, and coefficient of variation (CV%) of the observed values.

Limits of detection and quantitation

To determine the sensitivity of the method, the calibrators with the lowest concentrations (7.5 mg/L for methanol, acetaldehyde, and acetone and 75 mg/L for ethanol) of each matrix (U, B, VH, and CCM) were progressively diluted to determine the limit of detection (LOD) and quantitation (LOQ). A signal-to-noise ratio of 3 was considered acceptable for estimating the LOD (28). The concentration that originated the peak with a signal-to-noise ratio of 3 was injected 5 times.

The LOQ for each matrix was estimated based on a signal-to-noise ratio of 10 obtained for calibration solutions containing the compounds of interest. The LOQ corresponds to the lowest concentration obtained by successive dilutions of standards that originate a sharp and symmetrical chromatographic peak, required for routine analysis. Peaks that were excessively broad, showing tailing or shoulders, or that did not resolve to within 10% baseline were not considered (29).

Accuracy

Accuracy (A%) was calculated in terms of bias as the percent deviation of the mean calculated concentration at each concentration level from the corresponding theoretical concentration: A% = (mean calculated concentration – nominal concentration) / (nominal concentration) × 100.

Results and Discussion

GC separation

Interferents were ruled out by verifying the absence of peaks in the retention times of the studies analytes (Figures 2A and 2B).

As shown in Figure 2, the retention times for acetaldehyde, acetone, methanol, and ethanol in all tested matrices (Figures 2C and 2D) were 1.66, 2.02, 2.70, and 3.16 min, respectively. The IS (1-propanol) retention time was 5.26 min. The peak at 3.71 min in the chromatogram in Figure 2B (B and VH) corresponds to the peak of acetonitrile, which is part of the triton X-100 and acetonitrile solution used to dilute these complex matrices.

Method validation

Linearity

Regression analysis of calibration data achieved satisfactory linearity over the considered concentration range. Square correlation coefficients (R^2) were always > 0.99, indicating a linear relationship from 7.5 to 240 mg/L for acetaldehyde, methanol, and acetone, and from 75 to 2400 mg/L for ethanol in all the studied matrices.

The slopes and square correlation coefficients are presented in Figures 3A–3H for CCM, U, B, and VH, respectively.

Sensitivity

The LOD and LOQ for each compound in each matrix are shown in Table I.

Effect of the split ratio on the sensitivity of the method. The effect of the split ratio on the sensitivity of the method is illustrated in Figure 4. For the B matrix, higher split ratios were needed to protect liners and the chromatographic column. However, if lower LODs are needed, the split ratio can be decreased until 1:50. For the other matrices, a good peak resolution can still be obtained for ratios up to 1:30 (data not shown).

Inter-day CV% and A% of the method. The results for the 4 matrices and for 3 representative concentrations are presented in Table II. For CCM and U, the simplest matrices, the obtained inter-day CV% were always lower than 10%. For VH and B, due to sample manipulations, the inter-day CV% was less favorable. The average coefficients of variation (ACV%) of all the tested concentrations are presented in Table III and were always lower than 15%.

The A% of the method for the 4 matrices and for 3 representative concentrations of each compound, calculated as the percentage of target concentration, is indicated in Table II.

Intra-day precision of the method and of the apparatus. The intra-day precision of the method and of the apparatus for the 4 tested compounds in the 4 considered matrices are presented in Table III as ACV% that are always lower than 10%.

Concerning the intra-day precision of the apparatus, the ACV% were always between 2%, and 9% was the lowest ACV% obtained for ethanol in U and the highest for acetaldehyde in B. The ACV% values obtained for B and VH were higher than those obtained for CCM and U due to the need of more sample manipulations (Table III).

Concerning the intra-day precision of the method, the ACV% were always between 3%, and 9% was the lowest ACV% obtained for ethanol in B and the highest for methanol in B (Table III).

Discussion

Several methods for the analysis of ethanol that are also concerned with the simultaneous monitoring of acetaldehyde, methanol, or acetone concentrations have been reported (9,30–32). GC–mass spectrometry (MS) methods have also been applied to measure ethanol concentrations in different biological matrices (31,33,34). However, GC–MS is a much more complex technique than GC–FID and requires highly trained personnel. In consonance with these previous methods, the technique described in the present study enables the GC–FID analysis of B, U, VH, and CCM for the presence of ethanol, acetaldehyde, acetone, and methanol without any pretreatment, in the case of U and CCM, and with a simple dilution with triton X-100 and acetonitrile for VH and B.

As shown in Figure 2, the selected chromatographic conditions resulted in a good chromatographic resolution, with good peak separation. In addition, the good chromatographic separation between the studied compounds and some other important volatiles such as formaldehyde, methyl and ethyl formate, ethylene glycol, propylene glycol, glycerol, 1,4-butanediol, and 2,3-butanediol, recognized as biomarkers of some diseases and/or having great forensic interest, evince the interest of this method for a future adaptation to quantify these volatiles. This fact is a great advantage of this method, indicating its possible broad applicability.

A very good linear correlation ($R^2 > 0.99$) between the concentration and the ratio between the compound peak and the IS peak was obtained for all the tested compounds in all the tested matrices.

The proposed acceptance limits for the accuracy were 100 ± 20% (28) and, only in the case of VH, and for some concentrations, the obtained accuracy results were out of these limits.

The injection of 0.5 µL of blank matrices showed no interferences with other constituents from B, VH, CCM, and U, allowing the detection of very small amounts of the studied compounds.

The sensitivity depends on the volume injected and on the split ratio. In this work, and in the case of B, to prevent the shelf-life of the chromatographic column, the volume injected was 0.5 µL and the split ratio was 1:100.

For more sensitive determinations, 1 µL or even larger volumes can be injected and the split ratio can also be decreased, easily decreasing the LOD to levels lower than 0.1 mg/L. For the suggested chromatographic conditions, the LOD obtained with B were: 0.84 mg/L for acetaldehyde, and 0.75 mg/L for acetone, methanol, and ethanol. However, by changing the split ratio, the LOD can be easily decreased 10 times, which means: 0.075 mg/L for ethanol, acetone, and methanol, and 0.084 mg/L for acetaldehyde (3).

Table III. Compilation of the ACV% Obtained for the Evaluation of the Intra-Day Precision of the GC–FID Apparatus, the Intra-Day Precision of the Method, and the Inter-Day Precision of the Method

	Acetaldehyde	Acetone	Methanol	Ethanol
Intra-day precision of the GC–FID				
CCM	5.138	5.168	6.292	3.404
U	2.322	2.060	3.839	1.957
B	9.017	4.134	6.342	2.202
VH	4.718	2.410	6.663	4.518
Intra-day precision of the method				
CCM	5.978	5.042	5.697	6.207
U	5.699	4.485	7.003	6.235
B	3.797	5.262	8.829	2.973
VH	8.051	7.871	8.267	5.901
Inter-day precision of the method				
CCM	9.025	7.526	5.179	6.668
U	5.543	5.253	6.802	8.336
B	11.572	8.621	10.728	8.684
VH	12.254	10.759	8.777	12.854

Conclusion

In conclusion, the direct GC–FID injection method using capillary columns presented here is a highly sensitive, rapid, and reliable procedure to determine a plethora of volatile compounds in various biological samples. For the first time, a method was validated for the simultaneous determination of acetaldehyde, acetone, methanol, and ethanol in four different matrices (human B, U, VH, and CCM). The small amounts of sample injected (0.5 µL) and the high split ratios applied (1:60 and 1:100), allied to the high performance of the GC column, result in very good peak resolution and high sensitivities. This method is easy to perform and does not require highly and specifically trained personnel, making it suit-

able to the routine of clinical biochemistry and forensic laboratories.

Acknowledgments

Helena Pontes thanks FCT for financial support (SFRH/BD/18527/2004).

References

1. L.A. Ferrari, M.G. Arado, C.A. Nardo, and L. Giannuzzi. Postmortem analysis of formic acid disposition in acute methanol intoxication. *Forensic Sci. Int.* **133:** 152–158 (2003).
2. T. Fukunaga, P. Sillanaukee, and C.J. Eriksson. Problems involved in the determination of endogenous acetaldehyde in human blood. *Alcohol Alcohol.* **28:** 535–41 (1993).
3. A. Tangerman. Highly sensitive gas chromatographic analysis of ethanol in whole blood, serum, urine, and fecal supernatants by direct injection method. *Clinical Chem.* **43:** 1003–9 (1997).
4. M. Zilly, P. Langmann, U. Lenker, V. Satzinger, D. Schirmer, and H. Klinker. Highly sensitive gas chromatographic determination of ethanol in human urine samples. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **798:** 179–186 (2003).
5. D. Zuba, A. Parczewski, and M. Reichenbacher. Optimization of solid phase microextraction conditions for gas chromatographic determination of ethanol and other volatile compounds in blood. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **773:** 75–82 (2002).
6. I.A. Wasfi, A.H. Al-Awadhi, Z.N. Al-Hatali, F.J. Al-Rayami, and N.A. Al Katheeri. Rapid and sensitive static headspace gas chromatography-mass spectrometry method for the analysis of ethanol and abused inhalants in blood. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **799:** 331–6 (2004).
7. B.S. De Martinis and C.C. Martin. Automated headspace solid-phase microextraction and capillary gas chromatog- raphy analysis of ethanol in postmortem specimens. *Forensic Sci. Int.* **128:** 115–9 (2002).
8. B.S. De Martinis, M.A.M. Ruzzene, and C.C.M. Martin. Determination of ethanol in human blood and urine by automated headspace solid-phase microextraction and capillary gas chromatography. *Anal. Chem. Acta* **522:** 163–168 (2004).
9. M.T. Watts and O.L. McDonald. The effect of biologic specimen type on the gas chromatographic headspace analysis of ethanol and other volatile compounds. *Am. J. Clin. Pathol.* **87:** 79–85 (1987).
10. F.C. Kugelberg and A.W. Jones. Interpreting results of ethanol analysis in postmortem specimens: a review of the literature. *Forensic Sci. Int.* **165:** 10–29 (2007).
11. WHO-IARC. Re-Evaluation of Some Organic Chemicals, Hydrazine and Hydrogen Peroxide. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* **71:** 14–18 (1999).
12. A. Pla, A.F. Hernandez, F. Gil, M. Garcia-Alonso, and E. Villanueva. A fatal case of oral ingestion of methanol. Distribution in postmortem tissues and fluids including pericardial fluid and vitreous humor. *Forensic Sci. Int.* **49:** 193–6 (1991).
13. P.E. Hantson. Acute methanol intoxication: physiopathology, prognosis and treatment. *Bull. Mem. Acad. R. Med. Belg.* **161:** 425–34 (2006).
14. A. Paine and A.D. Davan. Defining a tolerable concentration of methanol in alcoholic drinks. *Hum. Exp. Toxicol.* **20:** 563–68 (2001).
15. M. Imbriani and S. Ghittori. Gases and organic solvents in urine as biomarkers of occupational exposure: A review. *Int. Arch. Occup. Environ. Health* **78:** 1–19 (2005).
16. J.L. Thomsen and B. Frohlich. Drug abuse and intoxication in alcoholics. *Alcohol Alcohol.* **30:** 379–83 (1995).
17. G. Teresinski, G. Buszewicz, and R. Madro. The influence of ethanol on the level of ketone bodies in hypothermia. *Forensic Sci. Int.* **127:** 88–96 (2002).
18. B. Brinkmann, G. Fechner, B. Karger, and A. DuChesne. Ketoacidosis and lactic acidosis—Frequent causes of death in chronic alcoholics? *Int. J. Legal Med.* **111:** 115–9 (1998).
19. D.J. Pounder and G.R. Jones. Post-mortem drug redistribution—A toxicological nightmare. *Forensic Sci. Int.* **45:** 253–63 (1990).
20. E.A. De Letter, P. De Paepe, K.M. Clauwaert, F.M. Belpaire, W.E. Lambert, J.F. Van Bocxlaer, et al. Is vitreous humour useful for the interpretation of 3,4-methylenedioxymethamphetamine (MDMA) blood levels? Experimental approach with rabbits. *Int. J. Legal Med.* **114:** 29–35 (2000).
21. J. Sebag. Age-related differences in the human vitreoretinal interface. *Arch. Ophthalmol.* **109:** 966–71 (1991).
22. W. Miekisch, J.K. Schubert, D.A. Vagts, and K. Geiger. Analysis of volatile disease markers in blood. *Clinical Chem.* **46:** 1053–1060 (2001).
23. J. Wyman and S. Bultman. Postmortem distribution of heroin metabolites in femoral blood, liver, cerebrospinal fluid, and vitreous humor. *J. Anal. Toxicol.* **28:** 260–3 (2004).
24. P. Fernández, M. Aldonza, A. Bouzas, M. Lema, A.M. Bermejo, and M.J. Tabernero. GC-FID determination of cocaine and its metabolites in human bile and vitreous humor. *J. Appl. Toxicol.* **26:** 253–7 (2006).
25. A. Tracqui, P. Kintz, S. Ritter-Lohner, P. Mangin, A. Lugnier, and A. Chaumont. Toxicological findings after fatal amitriptyline self-poisoning. *Hum. Exp. Toxicol.* **9:** 257–61 (1990).
26. C.S. Schaffert, S.L. Toderoa, B.L. McVickera, P.L. Tumac, M.F. Sorrella, and D. Tumaa. WIF-B cells as a model for alcohol-induced hepatocyte injury. *Biochem. Pharmacol.* **67:** 2167–2174 (2004).
27. K.M. Dubowski. Methods for specific substances: Ethanol. In *Methodology for Analytical Toxicology*. I. Sunshine, Ed. CRC Press, Cleveland, 1975, pp. 149–154.
28. EMEA. Note for guindance on validation of analytical procedures: Text and methodology. CPMP/ICH/381/95:1–15 (1995).
29. D.A. Armbruster, M.D. Tillman, and L.M. Hubbs. Limit of detection (LOD)/limit of quantification (LOQ): Comparison of the empirical and the statistical methods exemplified with GC–MS assays of abused drugs. *Clin. Chem.* **40:** 1233–1238 (1994).
30. R. Hernandez-Munoz, X.L. Ma, E. Baraona, and C.S. Lieber. Method of acetaldehyde measurement with minimal artifactual formation in red blood cells and plasma of actively drinking subjects with alcoholism. *J. Lab. Clin. Med.* **120:** 35–41 (1992).
31. H.M. Liebich and J. Wöll. Volatile substances in blood serum: Profile analysis and quantitative determination. *J. Chromatogr.* **142:** 505–16 (1977).
32. M. Varga, G. Somogyi, J. Posta, and L. Buris. Effect of different columns and internal standards on the quality assurance of the gas chromatographic determination of blood ethanol. *Eur. J. Clin. Chem. Clin. Biochem.* **31:** 773–6 (1993).
33. H.M. Liebich, H.J. Buelow, and R. Kallmayer. Quantification of endogenous aliphatic alcohols in serum and urine. *J. Chromatogr.* **239:** 323–9 (1982).
34. B.K. Tang. Detection of ethanol in urine of abstaining alcoholics. *Can. J. Physiol. Pharmacol.* **65:** 1225–7 (1987).

Manuscript received June 5, 2008;
Revision received July 31, 2008.