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Research Article

Development and validation of a direct headspace GC-FID method for the determination of sevoflurane, desflurane and other volatile compounds of forensic interest in biological fluids: Application on clinical and post-mortem samples

A simple and reliable headspace GC-flame ionization detection (HS-GC-FID) method has been developed and validated for the simultaneous determination of seven volatile compounds of forensic interest: sevoflurane, desflurane, ethanol, methanol, 1-propanol, acetone and acetaldehyde. All seven compounds including acetonitrile (internal standard) eluted within 10 min and were well resolved with no endogenous interference. Good linearity was observed in the range of 1–12 mg/dL for both anesthetics and 2.5–40 mg/dL for the other five analytes. The method showed good precision, sensitivity and repeatability. Most of the analytes remained stable during the storage of samples at 4°C. Desflurane and acetone degraded (>10%), when the samples remained on the autosampler for more than 2 and 3 h, respectively. The method was finally applied on clinical and post-mortem blood and urine samples. The clinical samples were collected both from patients who underwent surgery, as well as from the occupationally exposed medical and nursing staff of the university hospital, working in the operating rooms. The hospital staff samples were found negative for all compounds, while the patients' samples were found positive for the anesthetic administered to the patient. The post-mortem blood samples were found positive for ethanol and acetaldehyde.

Keywords: Anesthetics / Desflurane / Forensic / Headspace GC-flame ionization detection / Sevoflurane
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1 Introduction

Inhalants represent a group of substances that includes volatile solvents, fuels, nitrous oxide, volatile nitrites and anesthetics. Sevoflurane and desflurane are fluorinated inhalation anesthetics and represent the newest volatile anesthetics in clinical use. They are clear, colorless, nonflammable, volatile lipophilic liquids. Inhalant abuse is a serious public health problem and although the ''flurane'' family of anesthetics is widely used therapeutically, it is also abused [1], sometimes with a fatal outcome [2–5].

Ethanol is one of the most commonly abused substances. It is consumed worldwide in tremendous amounts and is an effective inducer of hepatic drug metabolism, especially of those pathways mediated by the CYP2E1 isoform of the cytochrome P-450 (CYP) family. Sevoflurane is also a substrate of CYP2E1, and therefore its abuse by an individual who is chronically consuming ethanol would lead to its accelerated metabolism and the development of cross-tolerance [6] and would have to be considered in the toxicological setting [7, 8].

Methanol is another volatile substance of forensic interest. Acute methanol intoxication, which can be lethal [9–11], can occur either secondary to the consumption of alcoholic beverages, which have been adulterated with methanol [12], or after accidental (because of their clear and colorless appearance) or intentional (in a suicide attempt) ingestion of cleaning products, antifreeze or industrial solvents [13, 14]. Last but not least, methanol is a congener, a substance contained legally in trace amounts in alcoholic beverages and its determination in forensic congener analysis can help evaluate the allegation of a driver of drinking after the accident [15].

Another volatile substance of medico-legal interest is 1-propanol. It is produced in the body during putrefaction,

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Abbreviations: FID, flame ionization detector; **HS**, headspace

either through lactate fermentation by propionic acid bacteria or through the catabolism of amino acids, glycerol or fatty acids [16]. In an interesting study, it was shown that when examining the postmortem production of ethanol and 1-propanol in the brain of drowned persons, according to the ratio of ethanol/1-propanol, the toxicologist could suspect or exclude ethanol consumption [17] and therefore the determination of both compounds is necessary in such autopsy cases where putrefaction is already in a progressed stage. It is also known that 1-propanol interferes with ethanol analysis by the breath analyzer [18] and therefore its determination is necessary for the correct interpretation of breath analysis results. Furthermore, 1-propanol is an important parameter in forensic congener analysis, which, as mentioned earlier, is useful in order to evaluate claims of drinking alcohol after driving [15].

Acetone is also implicated in medico-legal practice and its presence in post-mortem samples can be used as a criterion for the determination of the cause of death [19]. Its elevated levels can be attributed either to endogenous factors such as hypothermia, diabetic ketoacidosis, starvation or alcoholic ketoacidosis following withdrawal, or to intoxication with acetone containing solvents [20], isopropanol [21] or alcoholic beverages adulterated with acetone.

Furthermore, acetone is produced in a corpse during putrefaction [16] and its determination is of crucial importance in many autopsy cases.

Acetaldehyde is of great importance in the forensic determination and evaluation of ethanol. It is generated post-mortem in a corpse during the fermentative breakdown of glucose by yeasts, bacteria and fungi [16], but it is also formed by the chemical oxidation of ethanol to acetaldehyde in the presence of red blood cells, during the storage of whole blood samples [22].

To the best of our knowledge, there is currently only one [23] headspace-gas chromatographic (HS-GC) method for the simultaneous determination of sevoflurane and desflurane in biological fluids, while there is no available method combining the above anesthetics with other volatile substances of forensic interest and especially ethanol, which modifies the metabolism of sevoflurane and therefore the simultaneous determination of these two substances is of particular toxicological importance.

The aim of the present study was to develop and validate a new method for the simultaneous determination of seven volatile compounds of forensic interest (sevoflurane, desflurane, ethanol, 1-propanol, methanol, acetone and acetaldehyde) in blood and urine by HS-GC coupled to a flame ionization detector (FID).

The developed method was validated with spiked whole blood and urine samples, using acetonitrile as internal standard. Moreover sample stability was evaluated, both during the storage at 4 and −20°C, as well as during the standing on the autosampler. The method was finally applied on clinical and post-mortem blood and urine samples.

2 Materials and methods

2.1 Chemicals and reagents

Ethanol, 1-propanol, methanol, acetone and acetaldehyde, all of analytical grade, were obtained from Panreac Quimica Srf (Barcelona, Spain). Sevoflurane (1,1,1,3,3,3-hexafluoro-2-(fluoromethoxy)propane), also called fluoromethyl hexafluoroisopropyl ether, was supplied by Abbott Laboratories Hellas A.Q.E.E. (Athens, Greece) and desflurane (2,2,2-trifluoro-1-fluoroethyl-difluoromethyl ether) or 2-(difluoromethoxy)-1,1,1,2-tetrafluoroethane was supplied from Baxter Hellas (Athens, Greece). Dimethyl sulfoxide (DMSO) was purchased from Panreac Quimica Srf and was used for the dilution of the anesthetics. De-ionized water was obtained from an in-house Millipore purification system and was used for the dilution of the standards. Acetonitrile was supplied from MERCK KGaA (Darmstadt, Germany) and was used as internal standard at a concentration of 0.08 mg/mL. A solution of sodium chloride 0.1 M (Mallinckrodt, St. Louis, MO, USA) was used in order to inhibit the oxidation of ethanol.

Drug-free whole blood and urine samples, which were used to prepare the spiked samples, were provided by volunteers, who had not consumed alcohol for 36 h prior to sampling.

2.2 Instrumentation and chromatography

A Focus GC (Thermo Fischer Scientific S.p.A., Rodano, Milan, Italy) equipped with a FID was used for the separation and quantitation of the compounds analyzed. A TriPlus HS autosampler, purchased from the same company, was interfaced with the GC-FID for sample preparation and headspace sample introduction into the GC. The GC-FID instrument was equipped with a SUPELCOWAX 10 (30 m × 0.25 mm id, 0.25 μm film thickness) capillary column purchased from SUPELCO, Sigma-Aldrich (Park Bellefonte, PA, USA). The injector temperature was set at 150°C and the detector was set at 250°C. The injection mode was split, with a split ratio of 14:1. The flow rate of the helium carrier gas was 1.2 mL/min. The temperature program started with the oven at 42°C for 5 min. The temperature was then ramped up at a rate of 4°C/min to 70°C and held for 1 min. The oven temperature was then increased to 100°C at a rate of 10°C/min and held for a further 3 min. The completion of one temperature cycle lasted 19 min.

2.3 Preparation of standards

Stock solutions of the two anesthetics were prepared by adding 0.5 mL of each anesthetic, chilled to 4°C in an ice bath, to a 10-mL volumetric flask, which was then filled to volume with DMSO, chilled to approximately 20°C in a

water bath. A 2-mL aliquot of the stock solution was pipetted into a 25-mL volumetric flask, which was further diluted to volume with chilled DMSO in order to prepare a 3 g/L solution of sevoflurane and desflurane. Shortly before analysis, 2.5 mL of the standard solution were pipetted into a 25-mL volumetric flask and diluted to volume with de-ionized water chilled in an ice bath. Working solutions were prepared at concentrations of 1, 2, 4, 6, 8, 10 and 12 mg/dL. Stock solutions of ethanol, acetaldehyde, acetone, methanol and 1-propanol were prepared by pipetting the appropriate quantity into a 10-mL flask and diluting to volume with de-ionized water. Working solutions were prepared shortly before analysis at concentrations of 2.5, 5, 8, 10, 20, 30 and 40 mg/dL. A 0.8 mg/dL solution of acetonitrile (IS) was prepared into a 100-mL flask by dilution with de-ionized water.

2.4 Headspace procedure

A 1-mL aliquot of a saturated salt solution (0.35 g/mL of NaCl in de-ionized water), 0.25 mL of the IS solution, 0.25 mL of an aquatic standard solution containing a mixture of the different analytes at the different concentrations used (standard mix) and 1 mL of drug-free blood or urine were pooled in a 10-mL headspace vial in order to validate the method on spiked samples. Similarly, for the analysis of clinical and post-mortem samples, 1 mL of the saturated salt solution, 0.25 mL of the IS solution, 0.25 mL of the standard mix and 1 mL of biological fluid were pooled in a 10-mL headspace vial. The vials were sealed immediately with a rubber cap and an aluminum crimp seal, and were then agitated for 30 min in 60°C with an agitator speed of 250 rpm. The gas-tight syringe was heated at 70°C. A 1-mL headspace aliquot was sampled for the analysis and injected directly onto the GC.

2.5 Method validation

The developed analytical method was fully validated in terms of sensitivity, linearity, stability, selectivity, accuracy, within-day and between-day precision.

Selectivity was assessed by the absence of interference in the same chromatographic windows as the examined compounds in biological samples and was demonstrated by the analysis of blank matrices.

Linearity was evaluated by constructing the calibration curves using spiked drug-free whole blood and urine samples. Based on the available literature [23], the selected concentration levels for the two anesthetics were 1, 2, 4, 6, 8, 10 and 12 mg/dL, while the selected concentrations for the other volatile compounds were 2.5, 5, 8, 10, 20, 30 and 40 mg/dL.

Linear regression calibration curves were constructed by plotting the peak area ratios of the analytes to the IS versus nominal drug concentrations.

The LOD and LOQ values were calculated from the calibration curve according to the formulas $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$, where S is the slope and σ is the standard deviation of the intercept.

The assay precision, expressed as RSD, was assessed at three concentration levels, namely 2, 6 and 8 mg/dL for the anesthetics and 5, 10 and 20 mg/dL for the other volatile compounds. Within-day precision was estimated by five replicates, while intermediate (between-day) precision was estimated by triplicate measurements of freshly prepared spiked samples during a period of six consecutive days.

Accuracy, expressed as percentage recovery, was evaluated by comparing the concentrations, as calculated from the calibration lines by linear regression analysis, versus nominal (added) concentrations.

Stability of the analytes in the biological fluids, expressed as percentage recovery, was determined both after several freeze and thaw cycles as well as after long-term storage at 4°C. Stability of the analytes during the standing of the samples on the autosampler was also evaluated.

Aliquots of blood and urine, spiked at 6 mg/dL for the anesthetics and at 10 mg/dL for the other volatile compounds, were stored at 4°C to evaluate their stability.

The stability of the analytes during the standing of the samples on the autosampler was determined for up to 3 h.

The stability during freeze–thaw cycles was examined by deep-freezing (at −20°C) seven aliquots of samples, spiked at 6 mg/dL for the anesthetics and at 10 mg/dL for the other volatile compounds, and then leaving them to thaw at room temperature. One aliquot was analyzed each time while the rest of them were refrozen. The whole procedure was repeated for several cycles until degradation was observed. The 10% degradation criterion was used to evaluate stability.

2.6 Application of the method on clinical and post-mortem samples

Clinical blood and urine samples were supplied by the AHEPA Hospital of Thessaloniki-Greece. They were collected both from patients (10) undergoing neurosurgery, as well as from the occupationally exposed medical and nursing staff (8) working in the operating rooms. Both the patients as well as the hospital staff participated in the study voluntarily and gave their written informed consent.

Clinical blood samples were collected from a venous catheter directly in a vacuum tube, leaving limited residual air, in order to minimize analyte loss. Clinical urine samples were collected from patients undergoing surgery in a similar manner, directly from a urinary catheter in a vacuum tube.

Post-mortem blood samples were provided by the Laboratory of Forensic Medicine and Toxicology, School of Medicine, Aristotle University of Thessaloniki. They were collected from the femoral vein in a vacuum tube during autopsy, taking all the necessary precautions to avoid analyte loss.

3 Results and discussion

3.1 Chromatography

Different retention times for each analyte and resolution ≥ 2 were observed, indicating good selectivity and demonstrating that no interference occurred with elution. Typical chromatograms of blank and spiked blood and urine samples are shown in Figs. 1 and 2, respectively.

3.2 Method validation

The developed method was validated in terms of linearity, sensitivity, precision and accuracy, selectivity and stability.

Linear least-squares regression calibration curves were constructed by plotting the peak area ratios of the analytes to the IS versus the concentration of the working solutions. Linearity and sensitivity data are shown in Table 1.

In blood, the LOD ranged between 0.7 mg/dL for acetone and 1.7 mg/dL for sevoflurane, and the LOQ ranged between 2.2 and 5.2 mg/dL for the same analytes.

In urine, the lowest LOD and LOQ were obtained for sevoflurane (1.3 and 4.1 mg/dL, respectively) and the highest for methanol (2.9 and 8.7 mg/dL, respectively).

The absence of interference in the same chromatographic windows as the examined compounds in blank matrices indicated the selectivity of the method.

Accuracy and within-day precision were determined by analyzing quintuplicates ($n = 5$) of blood and urine samples spiked at three different concentration levels. The between-run precision was evaluated by analyzing the same spiked blood and urine samples on six different days. Precision and accuracy of the method were expressed as RSD and percentage recovery. Within-run and between-run precision in blood ranged from 1.0 to 10.6% and from 1.0 to 14.1%, respectively. Within-run and between-run precision in urine ranged from 1.5 to 11.9% and from 1.3 to 14.6%, respectively. Precision and accuracy data are summarized in Table 2.

Stability of the analytes in the biological fluids was expressed as percentage recovery and was studied both after several freeze and thaw cycles as well as after long-term storage at 4°C. Stability of the analytes during the standing of the samples on the autosampler was also determined.

In blood, the two anesthetics, ethanol and acetaldehyde, lost a large amount of their initial quantity already from the first freeze–thaw cycle. Methanol and 1-propanol required four cycles and acetone needed five cycles to lose 10% of their initial quantity. In urine, all the analytes except for

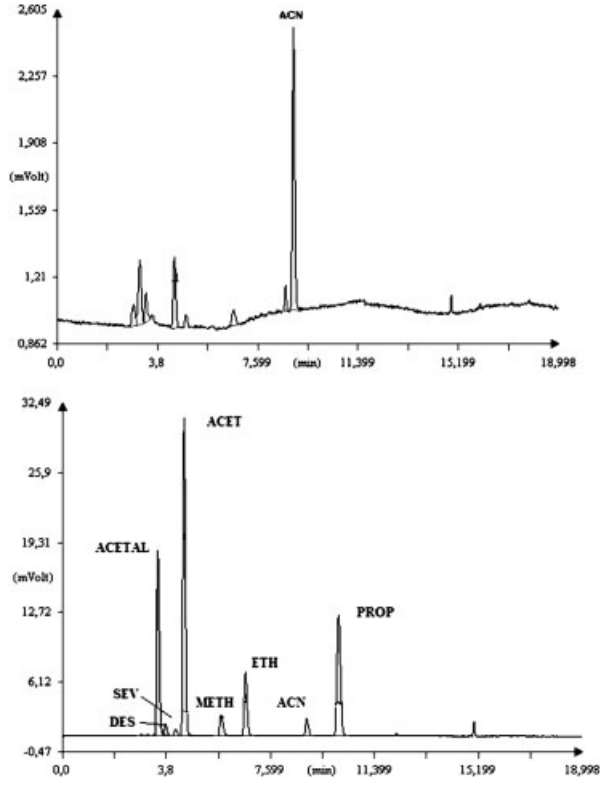

Figure 1. HS-GC chromatograms of whole blood samples: (A) blank; (B) spiked (20 mg/dL). Eluted compounds: acetaldehyde: 3.48 min; desflurane: 3.78 min; sevoflurane: 4.15 min; acetone: 4.45 min; methanol: 5.80 min; ethanol: 6.71 min; IS (0.08 mg/mL): 8.92 min; 1-propanol: 10.08 min. Chromatographic conditions are described in the text.

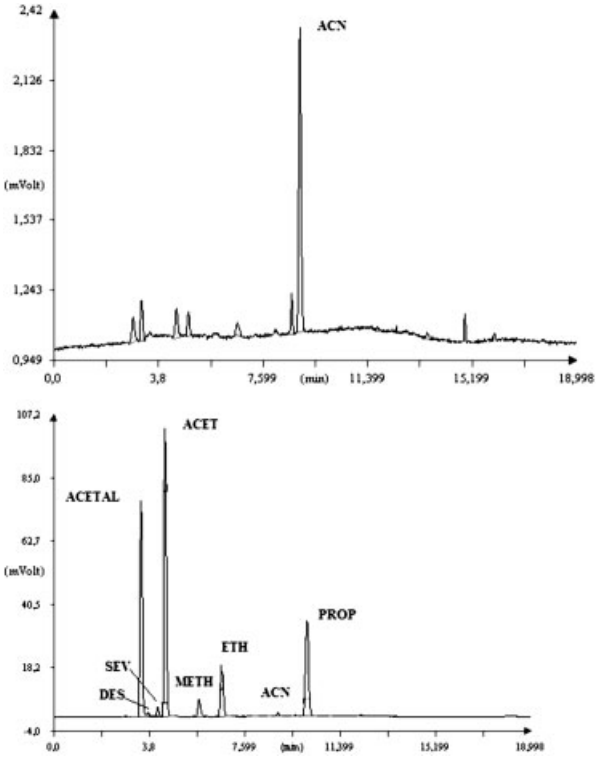

Figure 2. HS-GC chromatograms of urine samples: (A) blank; (B) spiked (20 mg/dL). Eluted compounds: acetaldehyde: 3.48 min; desflurane: 3.78 min; sevoflurane: 4.15 min; acetone: 4.45 min; methanol: 5.80 min; ethanol: 6.71 min; IS (0.08 mg/mL): 8.92 min; 1-propanol: 10.08 min. Chromatographic conditions are described in the text.

Table 1. Linearity and sensitivity data of the analysis of selected compounds in whole blood and urine

Analyte	Blood	Urine
Acetaldehyde	$y = (2.045 \pm 0.045)x + (3.109 \pm 0.952)$ $R = 0.9988$ LOQ = 4.6 mg/dL	$y = (2.43 \pm 0.05)x + (6.52 \pm 1.15)$ $R = 0.9975$ LOQ = 4.7 mg/dL
Desflurane	$y = (0.43 \pm 0.02)x - (0.551 \pm 0.148)$ $R = 0.9944$ LOQ = 3.4 mg/dL	$y = (0.35 \pm 0.02)x - (0.50 \pm 0.14)$ $R = 0.9838$ LOQ = 4.1 mg/dL
Sevoflurane	$y = (0.968 \pm 0.063)x - (2.020 \pm 0.507)$ $R = 0.9938$ LOQ = 5.2 mg/dL	$y = (0.35 \pm 0.02)x - (0.50 \pm 0.14)$ $R = 0.9845$ LOQ = 4.1 mg/dL
Acetone	$y = (3.613 \pm 0.038)x + (9.53 \pm 0.797)$ $R = 0.9997$ LOQ = 2.2 mg/dL	$y = (3.56 \pm 0.12)\ x + (5.74 \pm 2.60)$ $R = 0.994$ LOQ = 7.3 mg/dL
Methanol	$y = (0.342 \pm 0.007)x - (0.437 \pm 0.153)$ $R = 0.9989$ LOQ = 4.5 mg/dL	$y = (0.274 \pm 0.011)x + (0.527 \pm 0.237)$ $R = 0.9916$ LOQ = 8.7 mg/dL
Ethanol	$y = (1.10 \pm 0.03)x - (2.053 \pm 0.519)$ $R = 0.9987$ LOQ = 4.7 mg/dL	$y = (0.73 \pm 0.02)\ x + (1.40 \pm 0.47)$ $R = 0.9952$ LOQ = 6.5 mg/dL
1-Propanol	$y = (1.71 \pm 0.02)x + (2.19 \pm 0.43)$ $R = 0.9996$ LOQ = 2.5 mg/dL	$y = (1.61 \pm 0.06)x + (3.60 \pm 1.26)$ $R = 0.9931$ mg/dL LOQ = 7.8 mg/dL

y = peak area ratio and x = analyte concentration (mg/dL).

Table 2. Within-run ($n = 5$) and between-run ($n = 6$) accuracy and precision of the analysis of selected compounds in blood and urine

Analyte	Spiking level (mg/dL)	Blood				Urine			
		Within-run $n = 5$		Between-run $n = 6$		Within-run $n = 5$		Between-run $n = 6$	
		RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)
Acetaldehyde	5	5.2	95.6	8.2	98.5	11.9	102.9	6.3	100.2
	10	4.8	100.5	5.8	98.8	3.1	146.8	2.1	106.5
	20	2.5	105.6	3.7	105.8	5.3	112.7	5.8	103.4
Desflurane	2	1.0	110.5	3.9	112.4	2.4	102.5	5.4	134.2
	6	1.6	41.7	11.4	46.6	5.6	87.3	8.5	92.9
	8	1.4	32.0	14.1	44.8	11.1	76.4	6.6	89.6
Sevoflurane	2	10.6	146.4	9.6	131.3	3.4	98.9	14.6	129.8
	6	3.9	115.3	4.6	103.1	4.4	107.3	2.8	102.0
	8	6.8	98.0	4.4	87.6	11.5	134.3	3.2	98.7
Acetone	5	7.8	64.5	9.5	78.6	10.2	125.2	1.7	105.8
	10	1.7	111.7	1.5	97.5	2.9	133.5	2.4	81.9
	20	1.7	96.2	2.7	100.0	5.5	105.4	2.2	106.4
Methanol	5	1.5	128.0	11.6	102.1	2.5	85.0	7.6	91.7
	10	4.6	118.0	4.0	103.2	4.4	106.9	6.4	101.2
	20	4.5	90.5	4.9	92.4	5.4	104.2	4.1	114.1
Ethanol	5	1.9	120.3	2.9	101.1	1.5	95.0	2.6	97.1
	10	4.1	111.9	6.8	93.2	3.9	114.7	2.9	106.1
	20	1.3	76.7	2.8	77.5	11.9	102.9	6.9	107.7
1-Propanol	5	6.8	101.1	2.7	99.9	3.1	146.8	8.0	74.3
	10	6.5	113.1	3.1	101.9	5.3	112.7	4.1	105.1
	20	6.8	97.5	1.0	94.0	2.4	102.5	1.3	108.0

Table 3. Concentration of volatile compounds analyzed (mg/dL) in clinical blood and urine samples and post-mortem blood samples

Analytes	Blood samples from patients undergoing surgery	Blood samples from hospital staff	Post-mortem blood samples	Urine samples from patients undergoing surgery
Acetaldehyde	<4.6	<4.6	4.6–6.6	<4.7
Desflurane	3.4–13.3	ND	ND	ND
Sevoflurane	5.2–20.4	ND	ND	4.1–9.9
Acetone	<2.2	<2.2	<2.2	<7.3
Methanol	4.5–5.3	<4.5	<4.5	<8.7
Ethanol	<4.7	<4.7	4.7–109.3	<6.5
1-Propanol	<2.5	<2.5	<2.5	ND

desflurane were stable during seven freeze–thaw cycles. Desflurane required two cycles to lose over 10% of its initial amount.

Ethanol, desflurane and methanol were stable in blood for 15 days while 1-propanol remained stable for 10 days when the samples were stored at 4°C. In urine, all volatile compounds, except the anesthetics remained stable for 24 days, while the two anesthetics lost over 10% of their initial amount in 16 days.

The stability of the compounds analyzed, during the standing of the samples on the autosampler, was determined for 3 h. Acetone lost over 10% of its initial amount after 3 h, while desflurane was unstable and lost a large proportion of its initial quantity after 2 h on the autosampler. The rest of the analytes remained stable during the 3-h standing on the autosampler.

3.3 Analysis of clinical and post-mortem samples

The applicability of the developed and validated method was proved by the successful analysis of clinical and post-mortem blood and urine samples (Table 3).

Blood and urine samples from patients undergoing surgery were found positive for the anesthetic (sevoflurane or desflurane) administered to the patient and negative for most of the other volatile compounds.

Blood samples from the medical and nursing staff working in the operating rooms were found negative for all examined compounds, ruling out the possibility of them being occupationally exposed to the anesthetics administered to the patients.

Finally, the post-mortem blood samples were found positive for ethanol and its metabolite acetaldehyde.

4 Concluding remarks

To the best of our knowledge, this is the first HS-GC-FID method for the simultaneous determination of sevoflurane and desflurane in combination with other volatile compounds of forensic interest in biological fluids.

The developed and validated method herein is characterized by good precision, accuracy, selectivity and high throughput, since it does not require lengthy sample pre-treatment techniques, which are the bottleneck in biological fluid analysis. It can therefore be readily applied to any forensic toxicology laboratory. The applicability of the method was proved by the successful analysis of clinical and post-mortem blood and urine samples.

The authors have declared no conflict of interest.

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