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A VALIDATED PROCEDURE FOR DETECTION AND QUANTITATION OF SALVINORIN A IN PERICARDIAL FLUID, VITREOUS HUMOR, WHOLE BLOOD AND PLASMA USING SOLID PHASE EXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

The use of vitreous humor and pericardial fluid as alternative matrices to blood and plasma in the field of forensic toxicology is described to quantitate low levels of Salvinorin A using ethion as internal standard. The method was optimized and fully validated using international accepted guidelines. The developed methodology utilizes a solid phase extraction procedure coupled to gas chromatography mass spectrometry operated in the selected ion monitoring mode. The method was linear in the range of 5.0 to 100 ng/mL with determination coefficients higher than 0.99 in 100 µL of vitreous humor and in 250 µL of each matrix pericardial fluid, whole blood and plasma. The limits of detection and quantitation were experimentally determined as 5.0 ng/mL, intra-day precision, intermediate precision and accuracy were in conformity with the criteria normally accepted in bioanalytical method validation. The sample cleanup step presented mean efficiencies between 80 and 106% in the different biological specimens analysed. According to the low volumes of samples used, and the low limits achieved using a single quadrupole mass spectrometer, which is available in most laboratories, we can conclude that the validated methodology is sensitive and simple and is suitable for the application in forensic toxicology laboratories for the routine analysis of Salvinorin A in both conventional and unconventional biological samples.

Keywords: Salvinorin A; Biological specimens; SPE; GC-MS-EI

1. Introduction

In recent years, the number of stores called *"smartshops"* has increased, especially in major urban centres. In these shops products with psychoactive effects, commonly named *"legal highs"*, are freely sold. *Salvia divinorum* is a plant with hallucinogenic effects that is among the substances sold in those stores. However, it should be kept in mind that "natural" and "legal" do not mean free from danger to health. Another problem related to this type of products refers to is fact that there are no sufficient data or studies on the long-term effects of these substances on the human body, which might expose its consumers to physical and psychological risks. According to the 2012 annual report of the European Monitoring Centre for Drugs and Drug Addiction [1], *Salvia divinorum* is among the three natural "legal highs" most frequently available at online shops.

Salvia divinorum is a plant member of the *Lamiaceae* mint family that has been used for centuries by the Mazatec in Oaxaca, Mexico, in traditional religious practices. Its main active metabolite is the neoclerodane diterpene Salvinorin A and the only known psychoactive terpenoid of *Salvia divinorum* [1, 2]. Salvinorin A is a potent and selective k-opioid receptor agonist with no affinity for the 5-HT_{2A}, the principal molecular target responsible for the action of classical hallucinogens (DOB, LSD, psilocybin, N,N-dimethyltryptamine, mescaline and ketamine) [3-6]. This plant is growing in popularity in Portugal and many other countries as a powerful hallucinogenic recreational drug. Its acquisition is legal in most states of USA and several European countries, and its main consumers are adolescents and young adults. Its availability has been rapidly increasing, due to the spreading of the *"smartshops"* and also to its easy purchase in Internet websites. Since the beginning of this year, several Portuguese hospitals have notified the hospitalization of patients with symptoms including complete loss of contact with reality, uncontrollable laughter, short-term loss of consciousness, headaches, panic crisis, depression, tremor,

nausea, hearing voices, unrealistic visions, sense of death, excitement, increased heart rhythm, potential self injuries without feeling pain, and, sometimes, coma, after the consumption of *Salvia divinorum* [7]. However, no published data is available concerning reported cases of salvinorin-related deaths. Nevertheless, postmortem analysis may be relevant, for instance in those cases where an individual died under the influence of the drug, but whose death was not directly caused by it.

This drug can be taken by smoking, chewing or drinking in a tea [8]. The active ingredient, Salvinorin A, has been reported to induce intense hallucinations in humans, with a typical duration of action between several minutes to an hour [3-11]. Mouth absorption is reduced and it is also poorly absorbed in the gastrointestinal tract. When smoked, the effects of salvinorin A are much more pronounced, inhaled doses of 200-500 µg produce profound hallucinations [3-9, 12-13]. The complete metabolism of Salvinorin A is not well known [14]. Pharmacokinetic studies showed a relatively fast elimination of Salvinorin A, with a half-life (t1/2) of 75 min and a clearance (Cl/F) of 26 L/h/kg [15]. Concerning metabolism, studies using rhesus monkey blood have shown that Salvinorin A is deacetylated to Salvinorin B, a compound with no significant affinity to k-opioid receptors [16-17].

In 2005, two studies were published, in which Salvinorin A was determined in biological fluids: the first one was in human plasma, urine, saliva and sweat using liquid-liquid extraction coupled with gas chromatography-mass spectrometry [13]; and the second was in human and rhesus monkey plasma, human urine and in rhesus monkey cerebrospinal fluid, utilizing solid-phase extraction and high performance liquid chromatography – atmospheric pressure chemical ionization mass spectrometry [10]. Later, in 2008, Salvinorin A was studied in human urine and blood samples by solid-phase extraction and liquid chromatography - electrospray ionization mass spectrometry [18]. Finally, in 2012 and 2013, this compound was analysed in human urine using either liquid-liquid extraction or solid-phase microextraction with comprehensive two – dimensional gas chromatography-time of flight mass spectrometry [19] and microextraction in packed syringe with gas chromatography-mass spectrometry [20]. As we can see, there are limited data and a few analytical methods available in the scientific literature for the determination of this compound in biological fluids, and data related to concentrations obtained in authentic samples are scarce.

The identification and quantitation of drugs in biological specimens is one of the most important objectives in forensic toxicology because in some postmortem cases, neither blood nor urine can be collected due to severe exsanguination or advanced putrefaction. In these situations vitreous humor and pericardial fluid can be useful. However, these biological matrices should not be seen as substitutes for blood but as complementary specimens that can provide important information about the intake of toxic substances. Vitreous humor is mainly composed of water (99%) and is anatomically protected from contamination and bacterial degradation due to the protected environment inside the ocular globe. A disadvantage of this matrix is the limited volume that can be collected during autopsy (1-2 mL per eye) [21]. Pericardial fluid, has several advantages as a matrix in forensic toxicology, such as the high volume that can be collected during autopsy (about 10 mL). This specimen is easily obtained from a closed cavity (pericardial cavity), and it is well protected from contamination and by postmortem changes [22-23]. One the other hand, sufficient amounts of this matrix can be obtained even from a completely exsanguinated

body. A study realized with fresh cadavers demonstrated that there is a good correlation between pericardial fluid and blood of the femoral vein, suggesting that drug concentration in pericardial fluid is useful for estimation of intoxication degree [24-28]. However, care should be taken, since pericardial fluid can be contaminated by postmortem diffusion, if a large amount of a drug is present in the stomach [29].

The aim of this study was to develop and validate a sensitive and specific gas chromatography mass spectrometry (GC-MS-EI) method to determine Salvinorin A in pericardial fluid (PF), vitreous humor (VH), whole blood (BL) and plasma (PL) matrices, suitable for the application in forensic toxicology routine analysis.

2. Materials and methods

2.1. Reagents and standards

The analytical standards of Salvinorin A and ethion (internal standard) were purchased from LCG Promochem (Barcelona, Spain) and Sigma-Aldrich (St Louis, USA), respectively.

Acetonitrile (LiChrosolv®), methanol (LiChrosolv®), 2-propanol, dichloromethane, n-hexane and potassium dihydrogen phosphate, all of analytical grade, were obtained from Merck (Darmstadt, Germany).

Oasis® HLB (3mL, 60 mg) extraction cartridges were purchased from Waters (Milford, MA, USA).

A stock solution of Salvinorin A (1 mg/mL) was prepared in acetonitrile. Working solutions at 50, 5, 0.5 and 0.05 µg/mL were prepared by proper dilution of the stock solution with acetonitrile. Additional working solutions, at the same concentrations, were prepared to be used in the quality control samples. A working solution of the internal standard (ethion) at 2 µg/mL was prepared in methanol. All solutions were protected from light and stored at a temperature between 2 and 8ºC.

Potassium dihydrogen phosphate 0.1 M was prepared by dissolving 13.61 g of potassium dihydrogen phosphate in deionized water, obtaining a final volume of 1000 mL of buffer solution.

2.2. Biological samples

For calibration purposes and validation experiments, blank blood and plasma samples were obtained from a local blood bank. Vitreous humor, pericardial fluid and postmortem blank blood were collected during autopsies performed at the Medico-Legal Office of the National Institute of Legal Medicine and Forensic Sciences, Centre Branch, Aveiro, Portugal. These samples were free of drugs of abuse, as they were screened before being used for both calibrators and control samples. All samples were stored at -15 ºC before analysis.

2.3. Gas chromatographic-mass spectrometric conditions

Chromatographic analysis was performed using a HP 6890 gas chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a 5973 mass-selective detector (Hewlett-Packard, Waldbronn, Germany) and a capillary column (30m x 0.32mm I.D., 0.25mm film thickness) with 5% phenylmethylsiloxane (HP-5 MS) supplied by J&W Scientific (Folsom, CA, USA).

The gas chromatograph oven temperature program was as follows: 70 ºC held for 3 min, which was increased by 30 ºC/min to 300 ºC and held for 6 min. The splitless injection mode (2 µL) was used with a constant flow rate (1.2 mL/min) of highly purified helium. The mass spectrometer was operated with a filament current of 300 µA at electron energy of 70 eV in the electron ionization (EI) mode. The temperatures of the injection port and detector were set at 250 and 280 ºC, respectively. Quantitation was done in the selected ion monitoring (SIM) mode, and the ions were monitored at m/z 318, 359, 404 and 432 (quantitation ion) for Salvinorin A, and only one ion was monitored at m/z 231 for the internal standard, ethion. A full-scan mass spectra of Salvinorin A and ethion, as well as their chemical structures are presented in Figure 1.

2.4. Sample preparation and extraction

Samples of vitreous humor (100 µL), pericardial fluid (250 µL), blood (250 µL) and plasma (250 µL) were prepared by the addition of 3 mL of 0.1 M phosphate buffer (pH 4.4) and 25 µL of internal standard solution (ethion) and were homogenised and centrifuged at 3000 rpm for 5 min. The aqueous phases were added to the extraction cartridges, previously conditioned with 2 mL methanol and 2 mL of deionised water. After the samples had passed through, the cartridges were washed sequentially with 2 mL methanol 5% in deionised water and 2 mL of n-hexane. After drying under full vacuum the analytes were eluted with a 2mL of a mixture of dichloromethane: isopropanol (75:25, v/v). The obtained extracts were evaporated to dryness at 30 ºC under a gentle nitrogen stream, reconstituted with 50 µL of ethyl acetate and transferred to autosampler vials to be injected a 2 µL aliquot into the chromatographic system (GC-MS-EI).

2.5. Validation procedure

The described procedure was validated in terms of selectivity, linearity, limits of detection (LOD) and quantitation (LLOQ), precision (intra-day and intermediate) and accuracy, extraction efficiency and stability, according to international guidelines on bioanalytical method validation [30-39]. Validation data were obtained by preparing quality control samples (QC) with drug-free matrices spiked with Salvinorin A at three different concentrations (low, medium and high).

Selectivity was studied by analyzing ten pools from different sources of blank samples of each matrix: vitreous humor, pericardial fluid, blood and plasma. They were checked for interferences at the retention times and monitored ions for the analyte of interest and the internal standard. Also, they were analysed for potential interferences from other substances, namely the most commonly encountered in routine analysis in our laboratory (medical substances, pesticides and drugs of abuse, Table1). From each pool, two sets of samples (n=10) were prepared into 10 mL glass tubes, and they were spiked with the same concentration (100 ng/mL) of all the compounds presented in Table1; in addition, ten of these samples were further spiked with Salvinorin A (10 ng/mL). It was obtained ten positive and ten negative samples which were extracted and analysed by the aforementioned procedure. The criteria for identification the compounds was established according to the recommendations of the World Anti-Doping Agency [39]. For chromatography, the relative retention time of the substance must be within a 1% window, or 0.2 min in absolute terms, from that of the same compound in a quality control sample prepared and analysed

contemporaneously. Mass spectrometric identification in the SIM mode, must include at least three diagnostic ions, and their relative intensities should not differ by more than a tolerated amount from those generated by the same compound in a quality control sample prepared and analysed contemporaneously (if the relative intensity of the ion is within a 25–50 % interval of the base peak in the control sample, a maximum relative tolerance of ±20 % will be allowed for the same ion in the sample; if this intensity is less than 25 % or higher than 50% in the control sample, then absolute tolerances of ±5 and ±10%, respectively, will be allowed for the ion in the sample).

In order to determine calibration curves, a linear range was established between 5 and 100 ng/mL (5, 8, 10, 15, 20, 30, 50, 60, 80, 100 ng/mL) for Salvinorin A, in each biological matrix. The calibration curves were obtained by plotting the peak area ratio between Salvinorin A and ethion (IS) against theoretical concentrations of the compound of interest. The criteria for acceptance included a R^2 value of at least 0.99, and the calibrator´s accuracy [mean relative error (bias) between measured and spiked concentrations] within a ±15 % interval, except at the LLOQ, for which ±20 % was accepted.

The potential for carryover was analyzed by injecting extracted blank, immediately after analysis of the highest calibrator from each calibration curve.

The LLOQ was defined as the minimum concentration of Salvinorin A that could be measured with adequate precision (coefficient of variation <20 %) and accuracy (±20 %). The LOD was defined as the lowest concentration yielding a signal-to-noise ratio of at least three. The intra-day precision was determined by the analysis of five QC samples at each concentration level (low, medium and high) in each matrix on one day. It was characterized in terms of coefficient of variation (CV, %). The intermediate precision and accuracy were assessed by the analysis of three QC samples at each concentration level (low, medium and high) in each matrix on five different days. Accuracy was calculated in terms of mean relative error (RE, %) between the measured and the spiked concentrations for all QC samples; 15% was the limit of the acceptable variability for all concentrations.

The extraction efficiency was evaluated by analysis of six QC samples at each concentration level (low, medium and high) in each matrix, in which the IS was added after extraction. After that, the obtained peak area ratios were compared to those obtained by spiking blank extracts with the same concentrations of Salvinorin A (100 % recovery).

To study the stability of Salvinorin A was used three QC samples at each concentration level (low, medium and high). The stability of the processed samples was evaluated through analysis of the extracts under the conditions of GC-MS analysis during 24h. For bench-top stability, samples of each matrix were spiked and left at room temperature for 3 h, after that they were extracted and compared with freshly spiked samples. To evaluate freeze/thaw cycles, the samples were spiked and stored 24 h at -15 ºC, after this period, they were completely thawed and then frozen once again under the same conditions (a total of three cycles was studied). Storage periods were one day, three days, and seven days, and the samples were analysed after the third cycle. Comparisons between the means concentrations obtained in the control and in the stability samples were made against an acceptance interval of 90-110 %. Furthermore, the 90 % confidence interval has to be within 80-120 % of the control mean , [35-38].

3. Results and discussion

3.1. Selectivity

No matrices interferences were observed at the retention times and at m/z values of the monitored ions, by the analysis of the negative blank pools (see Figures 2 and 3). This indicates that neither endogenous matrix constituents nor the substances in Table 1 interfere with the Salvinorin A or the IS. Furthermore, the analytes were successfully identified in spiked samples. These results indicated that the described method is selective for the determination of Salvinorin A in vitreous humor, pericardial fluid, blood, and plasma samples.

3.2. Linearity, limit of detection and limit of quantitation

The linearity, LOD and LLOQ are shown in Table2. Linear calibration curves were obtained for all matrices with $R^2 > 0.99$. The calibrator's accuracy was within the acceptance criteria.

The analysis of extracted blank vitreous humor, pericardial fluid, blood and plasma, immediately after injection of the highest calibrator from each calibration curve did not present any traces of carryover.

The LOD and LLOQ were both determined at 5 ng/mL. These values are comparable to those obtained in previous studies in plasma and blood [10,13,18], but were obtained with lower sample volumes (100 µL of vitreous humor and 250 µL of pericardial fluid, whole blood and plasma), while plasma and blood volumes of 1 and 0.5 mL were used in previous published works. It should be stated that these limits were considered to be good values when compared with previously works, in which were required plasma and blood volumes of 1 [10,13] and the 0.5 mL [18]. Regarding the results in vitreous humor and pericardial fluid, it is not possible to compare limits, since the determination of Salvinorin A in these matrices is not published yet.

3.3. Intra-day precision, intermediate precision, and accuracy

The results of the precision and accuracy are presented in Table 3 and 4. The intra-day and intermediate precision were below 12 % at the studied concentrations for all matrices. The accuracy was ±9 % and thus within the acceptance criteria.

3.4. Extraction efficiency

Extraction efficiencies were between 79.65±4.62 and 99.09±4.68 as shown in Table 5. The reported extraction efficiencies in human plasma [13] and in rhesus monkey plasma [10] ranged between 84.6±4.1 and 99.8 %, respectively. However, an adequate comparison with our results in vitreous humor and pericardial fluid, it is not possible to do, since the determination of Salvinorin A in these matrices is not published yet.

3.5. Stability during bench-top and freeze/thaw cycles

Stability of processed samples in the autosampler was guaranteed for 24h. The study also revealed that Salvinorin A is stable in each matrix for 3 h at room temperature and for the freeze/thaw experiments the acceptance criteria were fulfilled as shown in Table 6.

3.6. Applicability

This study seems to us to be very important especially because the recent cases of people hospitalized with severe psychotic disorders and with serious self-inflicted injuries that occurred during the hallucinogenic episodes. On the other hand, several deaths associated with the consumption of salvia divinorum, were reported in a certain area of Portugal. Despite this worrying situation is occurring, unfortunately the forensic toxicological service did not have any request to analyse Salvinorin A in the routine casework. However, we believe that this situation is due to lack of knowledge and information about this new reality which makes forensic pathologists less prone to make the requests for the analysis of these new substances, as well as to collect the studied matrices. These situations can be an obstacle in the development of new methodologies, so it is important to be prepared for the requests that will be made in the future.

Nevertheless, and even though no request to analyze the substance, the developed methodology is being used routinely, in presumably intoxicated individuals with drugs of abuse. So far, fourteen blood samples and three vitreous humor samples were analyzed for Salvinorin A, but none was positive. Taking into account the low limits of the method, it is expected that the individuals didn't consume *salvia divinorum*, or there was a long period between the consumption and the samples collection.

3.7. Conclusions

According to the low volumes of samples used, and the low limits achieved using a single quadruple mass spectrometer, which is available in most laboratories, the validated methodology proved to be sensitive and specific for the analysis of Salvinorin A in conventional and unconventional biological matrices. Furthermore, the results obtained indicate that the procedure is suitable for application in forensic toxicology laboratories for the routine analysis of Salvinorin A. The small volumes required for the validated procedure are extremely useful in situations when the available volume of the sample isscarce. To the best of our knowledge this is the first procedure developed for the determination of Salvinorin A in vitreous humor and pericardial fluid.

After all, the development of the presented methodology seemed to be very timely, especially due to the increase in the number of cases of intoxication with this type of substances registered in the emergencies of the Portuguese hospitals. So, it is very important that the pathologists are alerted to this new reality that is the consumption of these kinds of drugs, mainly by young people. We believe that this procedure, in the short term will be very useful for national and international application to authentic antemortem and postmortem samples.

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Figure captions

Figure 1. Mass spectra and chemical structures of Salvinorin A and ethion.

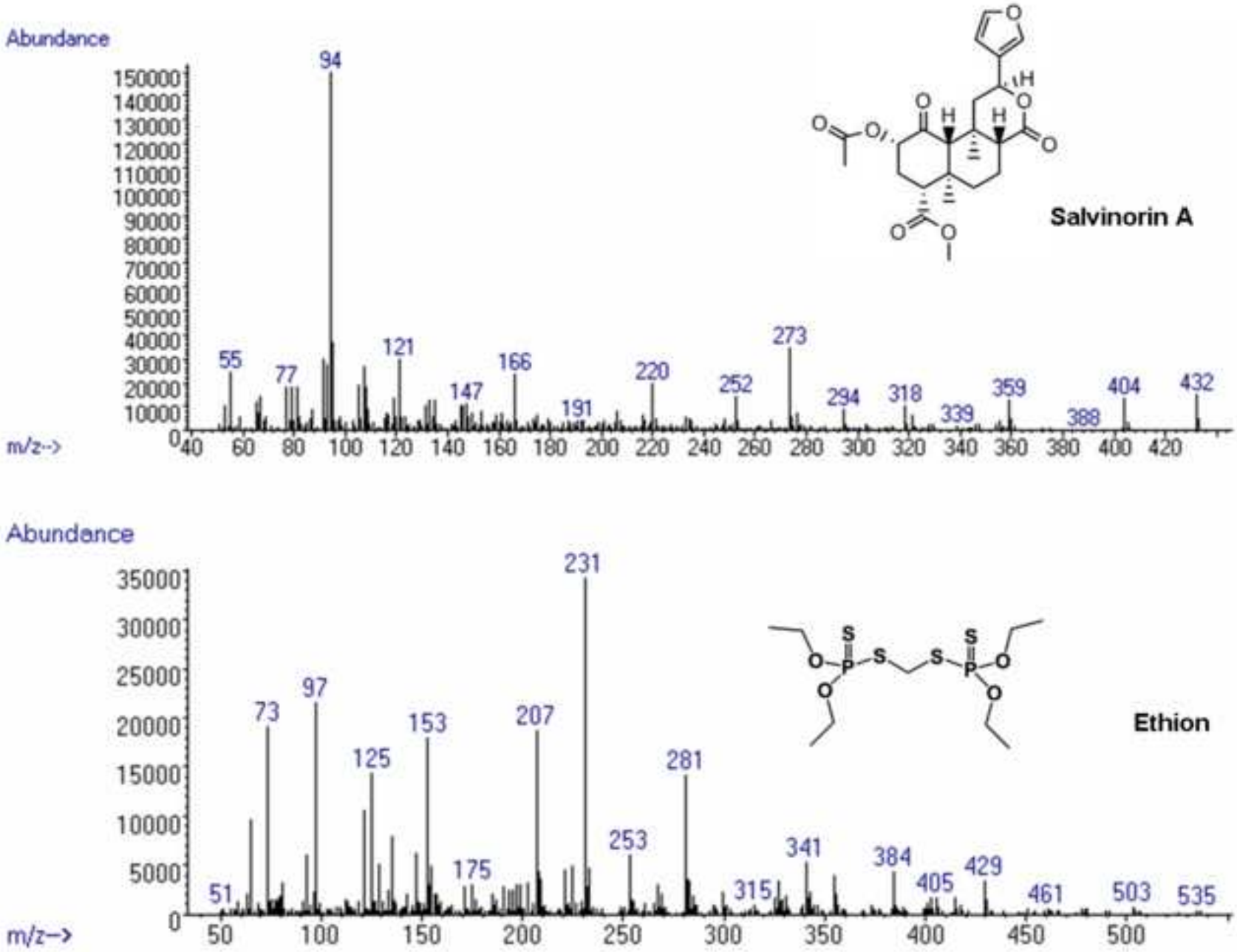
Figure 2. Ion chromatograms of blank and spiked samples (5 ng/mL) of VH (A, B) and PF (C, D) for Salvinorin A at the monitored ions m/z 318, 359, 404 and 432 and at 231 for the internal standard (ethion).

Figure 3. Ion chromatograms of blank and spiked samples (5 ng/mL) of PL (E, F) and BL (G, H) for Salvinorin A at the monitored ions m/z 318, 359, 404, 432 and at 231 for the internal standard (ethion).

HIGHLIGHTS

- First developed methodology to determine Salvinorin A in VH and PF
- VH and PF can be useful in postmortem cases when blood and urine are not available
- *Salvia divinorum* currently has been associated to several cases of intoxication
- Method suitable and quite opportune to clinical and forensic toxicology purposes
- The method has good quantitation limits using low volumes of samples

Figure


Abundance
94
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m/z-->
Salvinorin A
Abundance
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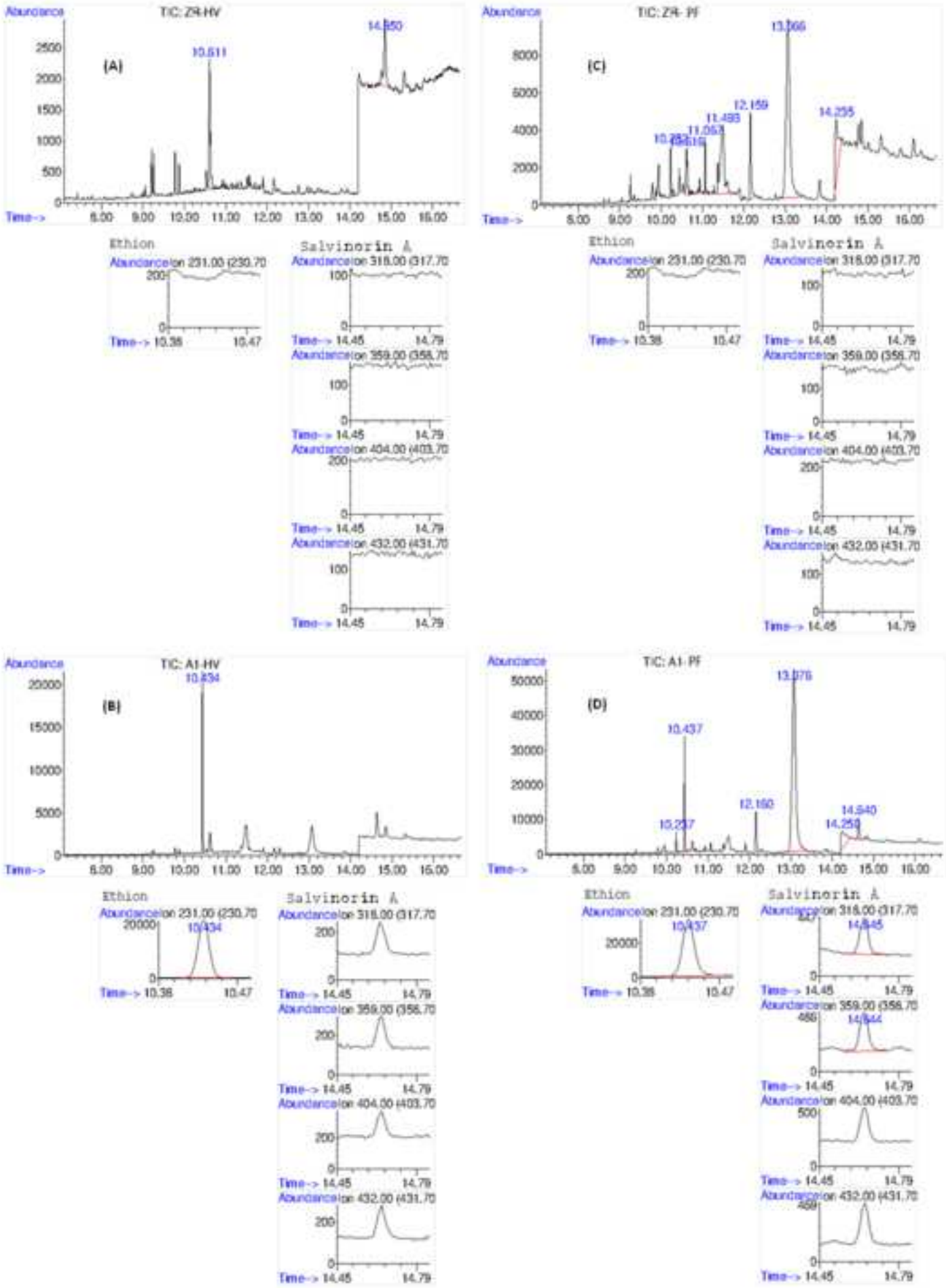

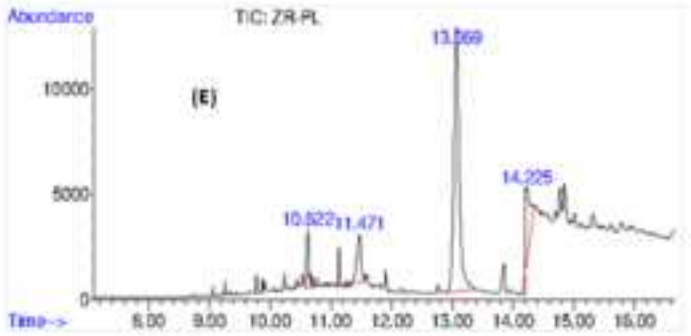

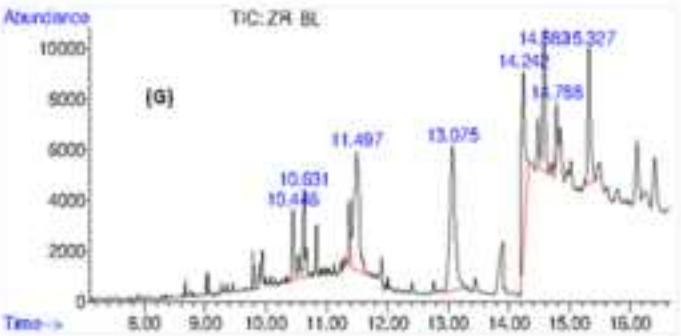

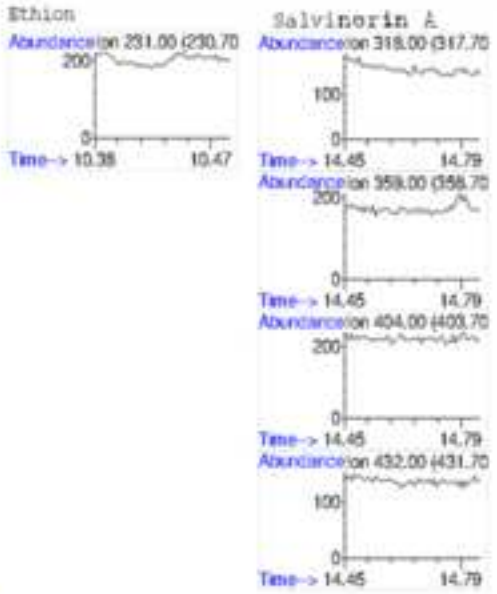

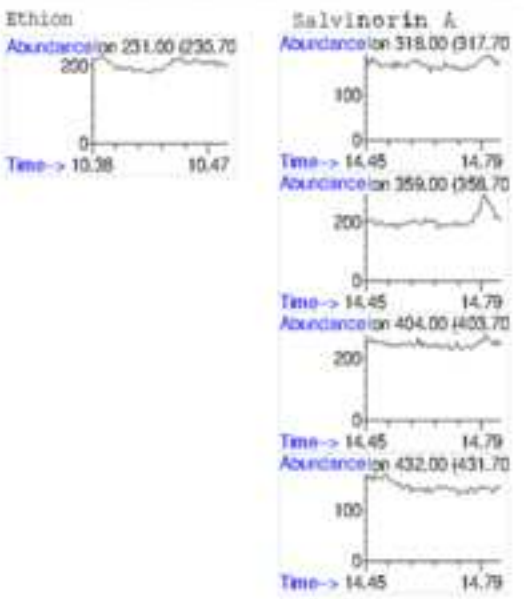

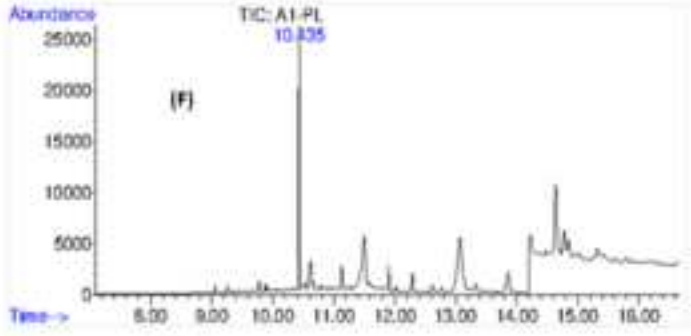

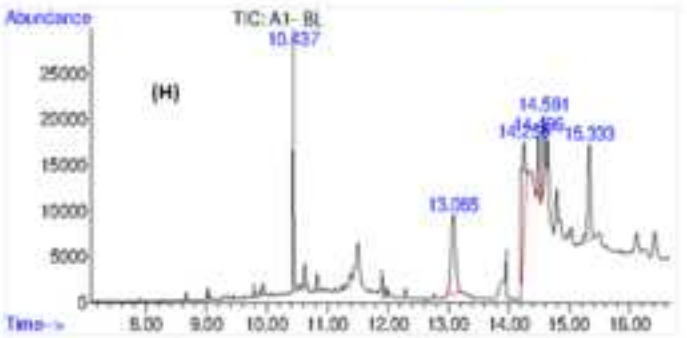

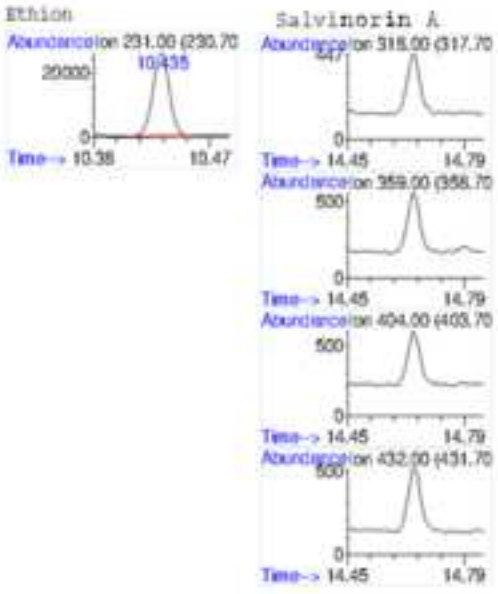

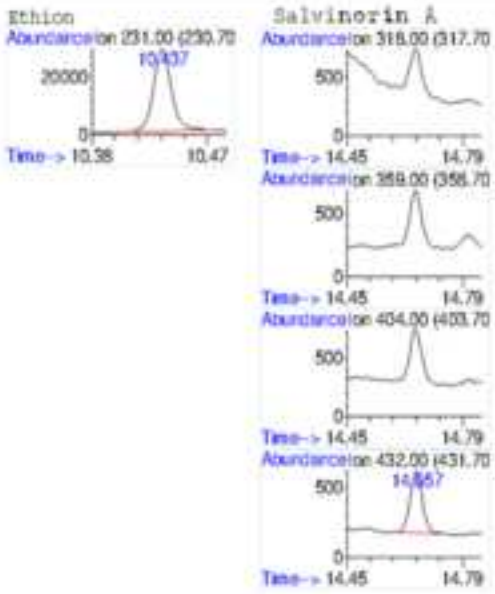

Table 1. Substances tested for interferences.

11-OH-THC	Clobazam	Fenarimol	Mirtazapine
6-acetylmorphine	Clomipramine	Fentanyl	Morphine
7-aminoclonazepam	Clonazepam	Fenthion	Naproxen
7-aminoflunitrazepam	Clonazepam	Flunitrazepam	Nordazepam
Acetaminophen	Clorpromazine	Fluoxetine	Olanzapine
Acetylsalicylic acid	Clozapine	Flurazepam	Oxacarbamazepine
Alprazolam	Cocaine	Foxyme	Oxazepam
Amitryptiline	Codeine	Imipramine	Papaverine
Amphetamine	Cyalothrine	Ketamine	Paroxetine
Atrazine	Cyamemazine	Ketoprofen	Penconazole
Atrazine	Cyfluthrine	Lamotrigine	Phenacetin
Azinphos-ethyl	Cypermethrine	Levomepromazine	Phenobarbital
Azinphos-methyl	Deltamethrine	Lidocaine	Phenytoin
Bendiocarb	Demeton-S-methyl	Lindane	Propranolol
Bentazone	Demeton-S-methylsulphon	Lorazepam	Quetiapine
Benzoylecgonine	Desalquylflurazepam	Maprotiline	Quinalphos
Bitertanol	Diazepam	MBDB	Sertraline
Bromazepam	Diazinon	MCPA	Strychnine
Bupirimate	Dichlorvos	MDA	Sulphotep
Caffeine	Dimethoate	MDEA	Temazepam
Carbamazepine	Dinocap	MDMA	THC
Chlorenvinphos	DNOC	Methadone	THC-COOH
Chlorpyrifos	Ecgonine methyl esther	Methamphetamine	Topiramate
Chlorpyrifos-methyl	EDDP	Mianserine	Tramadol
Citalopram	Estazolam	Midazolam	Venlafaxine

Table 2. Calibration and limits (LOD/LLOQ) data (n=5).

Biological Specimens	Linear range (ng/mL)	Linearity			LOD/LLOQ (ng/mL)
		Slope(*)	Intercept(*)	$R^{2(*)}$	
VH	5-100	8.8 E-03 ±1.1E-02	11.4 E-03 ±6.6 E-02	0.9972±1.9E-03	5
PF	5-100	1.2 E-03 ±1.1E-03	1.5 E-03 ±5.7E-03	0.9980±0.7E-03	5
BL	5-100	8.7 E-03 ±1.5E-02	26.1 E-03 ±3.3E-02	0.9973±2.3E-03	5
PL	5-100	0.3 E-03 ±0.4E-04	0.7 E-03 ±0.9E-03	0.9973±1.3E-03	5

(*) Mean values ± standard deviation

Table 3. Intra-day precision (n=5).

Biological Specimens	Spiked Concentration (ng/mL)								
	10			25			100		
	Concentration Found (ng/mL)	CV (%)	RE (%)	Concentration Found (ng/mL)	CV (%)	RE (%)	Concentration Found (ng/mL)	CV (%)	RE (%)
VH	10.2	8.8	1.9	25.1	11.7	0.2	93.9	6.3	-6.1
PF	9.1	10.9	-9.3	25.4	5.7	1.7	97.8	5.4	-2.1
BL	10.6	7.5	6.2	26.9	6.8	7.8	94.7	7.1	-5.3
PL	10.0	1.7	-0.3	26.2	1.2	4.8	96.4	4.0	-3.6

CV: coefficient of variation; RE: relative error [(concentration found-spiked concentration)/ spiked concentration x 100]

Table 4. Intermediate precision and trueness data (n=15).

Biological Specimens	Spiked Concentration (ng/mL)								
	10			25			100		
	Concentration Found (ng/mL)	CV (%)	RE (%)	Concentration Found (ng/mL)	CV (%)	RE (%)	Concentration Found (ng/mL)	CV (%)	RE (%)
VH	10.1	3.8	0.5	24.3	6.6	-2.8	98.8	3.0	-1.2
PF	10.4	11.2	3.9	25.7	5.1	2.7	102.3	6.8	2.3
BL	10.0	2.3	0.4	24.9	4.6	-0.3	100.3	3.8	0.3
PL	10.0	1.1	-0.1	25.6	1.9	2.2	100.8	3.5	0.8

CV: coefficient of variation; RE: relative error [(concentration found-spiked concentration)/spiked concentration x 100]

Table 5. Extraction efficiency (%) (n=4).

Biological Specimens	Concentration (ng/mL)		
	5	25	100
VH	97.4±9.9	100.6±7.2	79.6±4.6
PF	100.2±6.4	93.4±0.2	98.0±3.1
BL	98.9±10.8	88.8±9.8	99.1±4.7
PL	91.3±5.9	88.3±3.6	98.0±4.6

Mean values ± standard deviation

Table 6. Freeze/thaw stability (%).

Concentration (ng/mL)	Freeze/thaw stability (7 days)							
	VH		PF		BL		PL	
	% of controls	90% CI	% of controls	90% CI	% of controls	90% CI	% of controls	90% CI
10	105	85-116	103	99-107	103	99-108	100	93-106
25	105	96-114	92	87-98	97	87-108	105	96-113
100	104	95-113	84	81-87	86	85-87	100	95-105

CI: Confidence Interval