



Multi-target methodology for the screening of blood specimens in drug-facilitated sexual assault cases



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ABSTRACT

The forensic investigation of drug-facilitated sexual assaults (DFSA) requires the toxicological examination of biological matrices to detect possible substances in victims. Whole blood is an important sample since it is the only one available for analysis in a considerable percentage of cases. A new screening methodology was developed for the detection of 96 compounds related to DFSA in blood. Sample treatment was based on a procedure combining protein precipitation and phospholipid removal before HPLC-MS/MS analysis. The new method operates in scheduled multiple reaction monitoring (sMRM) mode and uses the hybrid operating of the third quadrupole as an ion trap. The blood samples preparation was studied comparing four different protein precipitation treatments and two widely used extraction techniques as solid phase extraction and liquid-liquid extraction. The analytical performance of the screening method was evaluated in terms of selectivity and limits of detection. Finally, successful results were obtained applying the new method to the analysis of fifteen samples from real cases of the National Institute of Toxicology and Forensic Sciences.

1. Introduction

Over the last decades, several publications about drug facilitated crimes (DFC) have highlighted the seriousness of this criminal modus operandi in Western societies [1]. In Spain, this phenomenon is frequently referred to as “*sumisión química*”, deriving from the older French term “*soumission chimique*” [2,3]. This involves the cover administration of psychoactive substances to other persons to override their will and subject them to different criminal purposes [4]. However, drug facilitated sexual assaults (DFSA) have received special attention within DFC. In this way, a classification of DFSA offenses based on the type of drugs use distinguishes between proactive and opportunistic assaults. Proactivity relates to the popular expression “drink spiking” and involves the covert or forced administration of incapacitating substances to victims. On the other hand, opportunism concerns intoxications because of the voluntary use of psychoactive substances by the victim [5].

Numerous substances are related to DFSA with a very variable typology differing between countries. International listings enumerate

compounds most frequently associated with this type of sexual assault, including illicit, prescription and over-the-counter drugs. The Society of Forensic Toxicologists (SOFT) published a list including 50 drugs [6,7]. In the same way, the United Nation Office on Drugs and Crime (UNODC) made public a guide for the forensic analysis of DFSA with a list consisting of 90 compounds frequently related to this offense [8]. Despite of any psychoactive substance can be involved, popular denominations as “knock out drugs” and “date-rape drugs” are used to refer some substances typically associated with DFSA in mass media [9], such as *burundanga* (scopolamine), ketamine, flunitrazepam (*Rohypnol*®) and gamma-hydroxybutyric acid (GHB). Specifically, this last compound has received significant attention because of its frequent implication in DFSA cases [10]. GHB is a precursor of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), causing both euphoric and sedation effects [10] and being used as recreational drug of abuse and to enhance sexuality [11].

The early collection of biological samples is a key factor to carry out suitable analytical procedures [12]. Studies agree the potentially underestimated implication of fast-metabolising substances in DFSA

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casuistry. Because of high elimination rates of some compounds from the body of victims, samples collected > 12 h after alleged assaults may lead to negative analytical results, underrating prevalence about implication of certain compounds [13–15]. On the other hand, moreover a proper collection time, types of biological samples collected are another significant feature to carry out toxicological tests. Urine and blood are preferred options [16]. Urine has a detection window up to 7 days, while detection probability in blood samples significantly decrease after 48 h [8]. In this way, the interest of analysing blood samples decreases when collection delay overcomes 24 h from the alleged assault [17]. However, according to the largest case study conducted in Spain, blood was the only available biological sample in 28.9% of alleged DFSA cases, urine was available in 15.8%, and both blood and urine samples were forward to forensic laboratories in 53.9% [18]. Therefore, availability of blood samples is significant in a sizeable percentage of cases, and thus require a specific analytical approach. Regarding blood, proteins and phospholipids are important analytical interferences. The later come mainly from erythrocyte walls and cause an important matrix effect.

Concerning to the analytical technique, nowadays immunoassays remain as a frequently used tool for the toxicological screening of drugs in biological matrices. However, immunochemical methods are not a good option for the screening in alleged DFSA cases. These tests do not cover all drug classes and comprise only a small number of the psychoactive substances frequently related to DFSA, so cannot detect all compounds potentially present in a sample. Moreover, these techniques have too high detection limits of and they are little selective. Furthermore, false positives occur more easily using immunochemical methods, due to cross-reactivity with compounds presenting similar structures to those included in the immunoassay. In addition, most kits are for urine analyses, not for blood. Therefore, confirmatory analyses must be realized after a positive result, using more specific and selective techniques, such as gas or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

During the last years, several publications focused the search of effective analytical alternatives based in LC-MS/MS for the general screening of toxicants in biological samples. Recently, a new LC-MS/MS screening method was developed and validated for the simultaneous analysis of 100 analytes related to DFSA in urine samples [19]. Another methodology used a multi-target screening for the detection and identification of 700 drugs and metabolites in biological fluids, employing a hybrid triple-quadrupole linear ion trap mass spectrometer in a single analytical run [20]. Similarly, a screening LC-MS/MS methodology for the analysis of ante- and post- mortem urine and whole blood samples was lately published [21]. In the same way, other studies using UHPLC-MS/MS addressed the analytical research of specific compounds frequently related DFSA, such as GHB [22,23]. These analytical strategies, based on multi-target screening, needs mass-spectral libraries for the identification of analytes in biological samples, being detectable only a selected group of compounds.

Furthermore, a general unknown screening is possible by analytical strategies using high-resolution mass spectrometers, like time-of-flight (TOF) instruments. Recently, an analytical method employing ultra-performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOF-MS) was developed for the screening of > 150 drugs and metabolites, including pharmaceuticals and different drugs of abuse. This method was successfully tested on real samples [24]. Similarly, another study developed a UPLC-TOF-MS method for the screening of 46 analytes in whole blood from cases of alleged sexual assault [25]. Whereas the use of high-resolution mass systems is not extended, LC-MS/MS remains one of the most specific and selective widely used analytical techniques in toxicology laboratories. The proposed methodology in this work employs the LC-MS/MS technique making use of the Q-Trap potential of the analytical equipment. This particularity enables the hybrid functioning of the third quadrupole as an ion trap, allowing obtaining much more sensitive analytical results. Despite the

fact that Q-Trap is a useful tool, actually its availability is not very extended in LC-MS/MS equipment, probably because of the increased cost. On the other hand, the pre-treatment of complex matrices such as blood needs selective sample preparation procedures. In this sense, solid phase extraction and liquid-liquid extraction are among the processes generally used. However, other less selective procedures such as protein precipitation may also be of interest when preparing complex samples, especially when screening analysis run at low concentrations. In these cases, less selective procedures allow a best passage of both polar and nonpolar compounds in approximately equivalent proportions. Taking this idea in mind and the potential of LC-MS/MS systems in forensic laboratories, this work focused the development of a sensitive and selective analytical LC-MS/MS methodology for the multi-target screening of 96 compounds related to DFSA in whole blood samples, in order to achieve a useful approach for the forensic investigation of alleged DFSA cases.

2. Experimental

2.1. Chemicals

All drug standards and deuterated analogues (used as internal standards) were from Cerilliant® (Round Rock, USA). All solvents were of HPLC grade. Methanol for LC-MS, water for gradient elution (HPLC), ammonium hydroxide solution ca. 25% (v/v) NH_3 and dichloromethane for HPLC, were from Sigma-Aldrich (St. Louis, USA). Acetonitrile (LC-MS grade), 2-propanol for LC-MS and solutions of formic acid 0.1% (v/v) in water and 0.1% (v/v) in acetonitrile were purchased from Thermofisher (Waltham, USA). Panreac Quimica S.A. (Barcelona, Spain) provided 1-chlorobutane and n-hexane 95% (v/v) for analysis. Orto-phosphoric acid 85% (v/v) pure was from Lab-Scan analytical science (Sowinskięo, Poland). Formic acid and ammonium acetate, eluent additives for LC-MS, were obtained from Scharlau (Barcelona, Spain). Deionized water was prepared using a Millipore system (Q-POD®) with a 0.22 μm deionizer cartridge from Millipak® (Darmstadt, Deutschland). Sørensen buffer (0.133 M Na_2HPO_4 and 0.133 M KH_2PO_4) was prepared by technical staff of the National Institute of Toxicology and Forensic Sciences (INTCF). TRIS buffer was prepared with tris(hydroxymethyl)aminomethane at 0.61% (m/v) in water. A solution of 5% (m/v) ZnSO_4 was also prepared.

2.2. Biological material

The Department of Chemistry of the INTCF-Madrid provided whole blood for the development of the new analytical methodology. Blood samples were from healthy voluntary donors with no history of drugs consumption. Moreover, fifteen blood samples from real DFSA cases provided by the same institution were analyzed in order to test the applicability of the developed methodology.

2.3. Preparation of stock and work solutions

Stock solutions of the analytes included in the new methodology were prepared by dilution in acetonitrile of the commercial solutions, reaching the concentration level at 1 $\mu\text{g}/\text{mL}$. In order to facilitate the experimental work, five stock solutions were prepared to divide all the compounds by groups. From each of these stock solutions, other three work solutions were obtained at 1, 10 and 100 ng/mL . By other hand, another solution of internal standards (ISs) in acetonitrile was prepared at 500 ng/mL .

2.4. Blood sample preparation

Three different procedures for the sample preparation were tested, based in solid phase extraction, liquid-liquid extraction and protein precipitation. The blood volume used was 200 μL for the three tested procedures. Following are the characteristics of each procedure:

2.4.1. Solid phase extraction

The blood sample was preprocessed before the extraction, by cell lysis and protein precipitation. For that, 3 mL of Sørensen buffer were added to 200 μ L of blood sample and vortexing for 5 s. Then, 200 μ L of acetonitrile 0.1% (v/v) acidified with formic acid was added and vortexing for 15 s for protein precipitation. After this preprocessing, the extraction was performed using Oasis[®] MCX plates of 60 mg, from Waters[®] (Milford, USA). For a good conditioning and equilibrating, 1.7 mL of MeOH and 1.7 mL of water were passed through the plates, respectively. Sample loading needed two consecutive steps adding 1.5 mL in each one of them. For the first wash 1.7 mL of methanol in water at 5% (v/v) with 2% (v/v) of formic acid was used. Second wash was with 1.7 mL of hexane. After washing, for the drying a nitrogen at 3–6 psi for 5 min was passed. The elution step used a solution of acetonitrile and isopropanol (60:40) with 5% (m/v) of ammonium hydroxide. For that purpose, 800 μ L of this solution was passed twice, consecutively. For drying extracts, a stream of nitrogen (lower than 5 psi) was applied to avoid the evaporation of the more volatile compounds, as amphetamines. To reconstitute the extracts, 200 μ L of the mobile phase were added to them and solution was vortex for 5 s. The final extract solutions showed a clean appearance.

2.4.2. Liquid-liquid extraction

In 10 mL pirex glass tubes, 200 μ L of blood and 400 μ L of TRIS buffer were introduced. After a brief vortexing, 3.2 mL of 1-chlorobutane were introduced and the tubes were vortexed again for 5 s. The tubes were rotated for 30 min and then centrifuged for 5 min at 4000 rpm. For the evaporation of the recovered supernatants to dryness, a stream of nitrogen (lower than 5 psi) was used until total evaporation. The extracts were reconstituted with 200 μ L of the mobile phase and vortexing for 5 s [26].

2.4.3. Protein precipitation with and without phospholipid removal

Four extraction procedures based in protein precipitation were compared. Three combined both protein precipitation and phospholipid removal stages using Phree[®] or Ostro Plates[®], meanwhile one used only protein precipitation. Referred procedures are described following:

- i. Protein precipitation and phospholipid removal using Phree[®] Phospholipid Removal Plates, from Phenomenex[®] (Torrance, USA). First, cell lysis of 200 μ L blood in an Eppendorf tube by vortexing for 5 s with 50 μ L of 5% (m/v) ZnSO₄ was made. Then, protein precipitation by vortexing for 5 s (14,000 rpm; 10 min) with a -20° C chilled mixture of acetonitrile and methanol (95:5) was prepared. Following, the addition of the recovered supernatant to the well containing 25 μ L of 1.0% (v/v) formic acid in acetonitrile, and application of positive pressure for 5 min at 60 psi was carried out. Evaporation to dryness of the recovered volume using a stream of nitrogen (lower than 5 psi) was made previously the reconstitution of the extract by adding 200 μ L of the mobile phase and vortexing for 5 s [27].
- ii. Sonication cell lysis and phospholipid removal using Phree[®] Phospholipid Removal Plates, from Phenomenex[®] (Torrance, USA). First, cell lysis of 100 μ L blood in Eppendorf tube by sonication for 10 min and water addition (100 μ L) and vortexing for 5 s was made. Then, protein precipitation into the well through pipette mixing by aspiration/dispensing with 1.0% (v/v) formic acid in acetonitrile (200 μ L and 5 repetitions) was performed. Following, the application of positive pressure for 5 min at 60 psi was made. Evaporation to dryness of the recovered volume using a stream of nitrogen (lower than 5 psi) was made prior reconstitution of the extract by adding 200 μ L of the mobile phase and vortexing for 5 s.
- iii. Protein precipitation and phospholipid removal using Ostro[®] Sample Preparation Plates, from Waters[®] (Milford, USA). First, cell lysis into the well, using 150 μ L of 0.1 M aqueous ammonium acetate was made. Then, protein precipitation into the well through

pipette mixing by aspiration/dispensing with acetonitrile (200 μ L and 5 repetitions), and vortexing for 3 min was performed. Following, the application of positive pressure for 5 min at 60 psi was carried out. Evaporation to dryness of the recovered volume using a stream of nitrogen (lower than 5 psi) was made prior the reconstitution of the extract by adding 200 μ L of the mobile phase and vortexing for 5 s.

- iv. Simple protein precipitation. First, vortexing 50 μ L blood for 5 s with 40 mM ZnSO₄ in 66% (v/v) aqueous methanol was performed. Following refrigeration for 20 min at -18° C and centrifugation (14,000 rpm, 10 min). Then, evaporation to dryness of the recovered volume using a stream of nitrogen (lower than 5 psi) was made prior the reconstitution of the extract by adding 200 μ L of the mobile phase and vortexing for 5 s.

2.5. Instrumentation and analytical method

2.5.1. High performance liquid chromatography method

The samples were analyzed using a LC-MS/MS system consisted of a AB Sciex 4000 Q TRAP[®] triple-quadrupole ion trap mass spectrometer coupled to a Turbo spray interface used as ion source. This was fitted with an Eksper UltraLC 110-XL instrument. The liquid chromatography system includes a degasser, autosampler, a column oven and two binary pumps. The Software Analyst 1.6.2. was used for the control of the system and data processing. Ionization was performed using an ion source Turbo spray and an Integrated Valve Method. Nitrogen was used as collision gas, and dried and cleaned air was used as curtain gas. The MS conditions were as follows: curtain gas, 25.0 psi; collision gas, high; ion spray voltage, 5500.0 V; source temperature, 600° C; ion source gas 50 psi; ion source gas 2. 6 psi and interface heater, on. The method works for the detection of all the analytes by a positive ionization mode.

Separation was performed with a Kinetex[®] 2.6 μ m Biphenyl 100 \AA (100 \times 2.1 mm) column, provided by Phenomenex (Torrance, USA). The mobile phase was a mixture of 2 mM ammonium formate in water with 0.2% (v/v) formic acid (eluent A) and 2 mM ammonium formate in acetonitrile with 0.2% (v/v) formic acid (eluent B). Gradient conditions started with 10% (v/v) of eluent B during the first 0.5 min at a flow rate of 0.3 mL/min. The level of eluent B increased linearly for 13.5 min to 90% (v/v) and was kept at this concentration for 2 min. Initial conditions were restored at 16.5 min. The injection volume was 5 μ L. The column oven was set to 30° C and the autosampler temperature was 12° C. Column equilibration was performed with starting conditions for 1 min. The flow rate keep constant at 0.3 mL/min throughout the run. For column washing, a mixture of methanol, acetonitrile and water (40:40:20) was used.

2.5.2. Mass spectrometry method

The mass spectrometry method conducted a survey scan to monitor the ionic fragmentation reactions of the 96 targeted basic compounds, working in positive ionization mode. The QTRAP 4000 Mass Spectrometry System (AB Sciex, Toronto, Canada) operated in scheduled multiple reaction monitoring (sMRM) mode, with a detection window of ± 60 s for the analysis of each MRM transition. The sMRM mode enabled the inclusion of a high number of target analytes in the screening method. This type of approach increases the selectivity of the MRM due to a greater dwell time is available for each transition, as already noted Ferreirós et al. [20]. Dwell time is the time the MRM transitions are monitored in one cycle. In addition, the sMRM mode minimizes the signals originating from matrix components, capable of inhibiting the acquisition of EPI spectra of coeluted analytes. In this sense, interference will not occur if the matrix component elutes out of the retention time window of the analyte [25].

A second scan started when the intensity of the spectral signal reached in the third quadrupole (Q3) exceeded a threshold pre-set at 2000 counts per second (cps). At that moment, the third quadrupole

Table 1

Analytes of the new method, drug class, multi reaction monitoring (MRM) transitions with declustering potential (DP), collision energy (CE), collision cell exit potential (CXP), retention time (tR), and numbering assigned in chromatograms.

Drug class	Analyte (number)	tR (min)	Q1 Mass (Da)	Q3 Mass (Da)	DP (V)	CE (V)	CXP (V)	
BZDs	7-aminoclonazepam (24)	6.08	286.10	121.11	100	41	8	
	alpha-OH-Alprazolam (61)	10.67	325.14	297.2	66	37	16	
	Alprazolam (76)	11.77	309.1	281.1	101	37	16	
	Bromazepam (43)	8.87	315.09	209.1	91	37	16	
	Citalopram (54)	10.24	324.9	109.1	61	41	6	
	Clobazam (90)	13.05	301.07	259.18	13	60	35	
	Clonazepam (83)	12.03	315.9	270	126	35	14	
	Chlordiazepoxide (39)	8.01	300.16	282.1	36	31	4	
	Clotiazepam (94)	13.31	319.1	154.1	60	50	10	
	Diazepam (95)	13.49	284.99	154	106	39	10	
	Flunitrazepam (91)	13.01	314	268.1	111	37	22	
	Loprazolam (50)	9.93	465.2	252.1	68	57	10	
	Flurazepam (51)	9.99	387.99	315.1	80	31	8	
	Lorazepam (65)	11.25	320.95	274.98	66	33	20	
	Lormetazepam (92)	12.93	334.97	288.97	81	33	16	
	Medazepam (58)	10.50	270.98	225	81	41	12	
	Midazolam (48)	9.74	325.98	291.1	106	39	16	
	Nitrazepam (66)	11.26	282.1	236.2	5	35	4	
	Nordiazepam (69)	11.32	270.98	140	106	41	10	
	Oxazepam (63)	11.00	287.02	241.11	46	33	10	
	Prazepam (96)	14.79	325.06	271.13	46	33	10	
	Temazepam (89)	12.64	301.2	255.2	36	33	4	
	Tetraazepam (68)	11.30	288.93	225	61	41	12	
	Triazolam (82)	11.95	343.1	239.2	61	55	10	
	BZDs an.	Zolpidem (41)	8.34	308.1	235.2	98	47	10
		Zopiclone (31)	7.03	389.04	245.05	51	23	20
	Opiates	6-MAM (16)	5.12	328.4	165.3	80	61	10
EDDP (80)		11.9	278.2	234.2	60	35	10	
Buprenorphine (56)		10.28	468.3	414.3	60	50	10	
Codeine (10)		4.34	300.3	215.2	86	35	18	
Oxycodone (15)		5.02	316.15	241.2	60	50	10	
Dextropropoxyphene (73)		11.70	340.2	58	60	35	10	
Dihydrocodeine (6)		4.09	302.2	199.2	60	35	10	
Fentanyl (52)		9.99	337.2	188.2	60	35	10	
Hydrocodone (18)		5.33	300.2	199.1	60	50	10	
Hydromorphone (2)		3.28	286.1	185.1	60	50	10	
Oxymorphone (5)		4.08	302.1	198.1	60	35	10	
Meperidine (34)		7.60	248	220.2	66	31	16	
Methadone (86)		12.38	310.17	265.1	56	21	20	
Morphine (1)		2.67	286.3	201.2	86	33,00	10	
Tilidine (40)		8.19	274	155	36	27	12	
Tramadol (28)		6.63	264.2	57.9	60	31	4	
Tapentadol (29)		6.73	222	107	41	35	10	
AD		Amitriptyline (84)	12.08	278.12	91.14	36	31	6
		Clomipramine (93)	13.16	315.2	86	60	35	10
		Desipramine (77)	11.79	276.18	72.07	60	35	10
	Desmethylvenlafaxine (20)	5.63	264.2	107	60	35	10	
	Doxepine (59)	10.61	280	107	71	27	0	
	Fluoxetine (74)	11.70	310	44	56	39	0	
	Imipramine (71)	11.69	281.2	86.1	60	35	10	
	Mirtazapine (30)	6.92	266.1	195.1	60	35	10	
	Nortriptyline (75)	11.71	264.17	191.16	60	35	10	
	Paroxetine (67)	11.28	330.17	192.1	76	25	10	
	Sertraline (87)	12.51	306	159	60	35	10	
	Trazodone (44)	9.17	372.08	176.13	81	35	14	
	Trimipramine (88)	12.53	295.2	100.1	60	35	10	
	Venlafaxine (37)	7.95	278	260.3	60	35	10	
	AH	Alimemazine (81)	11.90	299.09	100.2	71	25	8
		Cetirizine (72)	11.69	389.16	201	60	35	10
		Chlorpheniramine (33)	7.35	275.1	230.1	60	35	10
Brompheniramine (35)		7.93	319	274.1	60	35	10	
Cyproheptadine (79)		11.82	288.2	191.2	127	53	10	
Diphenhydramine (53)		10.07	256.2	165.1	60	35	10	
Doxylamine (14)		4.77	271.2	167.2	60	35	10	
Hydroxizine (70)		11.52	375.18	201.12	60	35	10	
Promethazine (64)		11.04	285.08	86.3	51	27	6	
AMP		Amphetamine (7)	4.09	135.89	91.11	66	23	14
	MDA (12)	4.67	180.25	105.1	51	30	6	
	MDEA (26)	6.14	208.03	163.13	56	19	10	
	MDMA (17)	5.23	193.74	162.97	51	19	10	
	Metamphetamine (13)	4.71	150.071	119.16	46	17,00	8	

(continued on next page)

Table 1 (continued)

Drug class	Analyxymorpote (number)	tR (min)	Q1 Mass (Da)	Q3 Mass (Da)	DP (V)	CE (V)	CXP (V)
APD	Aripiprazole (85)	12.17	448.1	285.2	100	35	15
	Clozapine (38)	8.00	327.1	270.1	60	30	10
	Haloperidol (62)	10.89	376.14	165.11	46	33	10
	Quetiapine (46)	9.38	384.13	253.06	81	37	30
	Ziprasidone (55)	10.25	413	194	91	41	14
DID	Ketamine (25)	6.08	238	125	71	39	8
	Phencyclidine (49)	9.8	244.2	91.07	60	35	10
	Norketamine (19)	5.47	224.10	125.04	31	35	28
HALL	Mescaline (8)	4.21	212.3	180.3	60	35	10
	LSD (42)	8.34	324.2	223.2	60	35	10
CO	Benzoyllecgonine (22)	5.80	289.9	168.2	76	27	10
	Cocaine (36)	7.93	304	82.1	71	47	4
CAT	Ethylbenzoilecgonine (45)	9.21	318	198.15	71	25	10
	Mephedrone (23)	5.8	78	160.2	60	35	10
AED	4-MEC (27)	6.27	192	144	60	35	10
	Phenitoin (60)	10.62	253.1	104.1	60	50	10
ASD	Pregabalin (3)	3.61	160.1	55.1	46	41	8
	Gabapentin (4)	3.98	172.2	137	60	35	10
MR	Atropine (21)	5.70	290	124.1	111	35	20
	Scopolamine (11)	4.61	304.13	138.1	76	33	8
ANX	Cyclobenzaprine (78)	11.79	276	215	51	57	16
	Carisoprodol (57)	10.48	261	62	51	13	12
AHT	Meprobamate (32)	7.17	219	158	46	13	12
ATU	Clonidine (9)	4.29	230	160	81	41	10
ISs	Dextrometorphan (47)	9.62	272.2	171.2	60	35	10
	Morphine-d ₃ (Is-1)	2.67	289.1	209.1	83	33	10
	Pregabalin-d ₆ (Is-2)	3.30	166.1	148.1	46	17	10
	Metamphetamine-d ₅ (Is-3)	4.71	155.1	121.1	46	17	8
	Ketamine-d ₄ (Is-4)	6.08	241.9	129.1	56	37	8
	Oxazepam-d ₅ (Is-5)	11.00	292.1	246.1	51	31	16
	Zolpidem-d ₆ (Is-6)	8.34	314.3	235.2	61	47	4
	Diazepam-d ₅ (Is-7)	13.49	290.1	198.2	56	43	10

BDZs: benzodiazepines; BDZs an.: benzodiazepines analogous; AD: antidepressive drugs; AH: antihistaminic drugs; AMP: amphetamines; APD: antipsychotic drugs; DID: dissociative drugs; HALL: hallucinogens; CO: cocaine; CAT: cathinone; AED: antiepileptic drugs; ASD: antispasmodic drugs; MR: muscle relaxant; AHT: anti-hypertensive drugs; ANX: anxiolytic; AN: antinausea; ATU: antitussive; ISs: Internal Standards. 6-MAM: 6-monoacetylmorphine; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; MDA: 3,4-methylenedioxyamphetamine; MDEA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxyamphetamine; LSD: lysergic acid diethylamide; 4-MEC: 4-methylthcathinone.

started working as an ion trap. This triggered an information dependent acquisition (IDA) scan.

The target scan time was 1.29 s, which supposes 402 cycles per run. The compounds included in the mass spectrometry method comprised several groups of substances. All MRM transitions, analyte-specific settings and retention times for the 96 analytes included in the new methodology are summarized in the Table 1. The MRM mode includes a pause time between the MRM transitions of 2 ms. Q1 and Q3 were used at unit resolution. The monitored transitions and their particular settings, declustering potential, entrance potential, collision energy, and collision exit potential were established for each analyte from pre-existing methodologies in the INTCF laboratory.

The two most intense MRM transitions per cycle exceeding the threshold of 2000 cps, were considered for the generation of the dependent enhanced product ion (EPI) scan. For further improvement of the identification of co-eluted compounds, the MRM transitions triggering the dependent scan were excluded for EPI scans for 20 s after two consecutive occurrences. The EPI scans were performed at a scan range of 50 to 700 amu after a dynamic fill time with a scan rate of 30 amu/s applying a declustering potential (DP) of 60 eV, a collision energy (CE) of 35 eV and a collision energy spread (CES) of 15 eV.

Data processing was necessary for the comparison of the acquired EPI spectra from the sample with those present in the library of the analysis software. Authors built their own library for the 96 compounds. The Analyst® Software 1.6.2. (AB Sciex, Toronto, Canada) was used for data analysis. This comparison provided reports indicating the possible identified compounds, as well as their respective inter-spectral coincidence values, known as purity and expressed as a percentage to characterize the spectral similarity. The purity is a combination of fit

and reverse fit values. The first indicates the similarity between the library spectrum and the unknown spectrum, meanwhile the second provides information about the coincidence of an unknown spectrum with a library spectrum.

2.5.3. Criteria for positive identification

To establish a positive identification of the analytes, different analytical requirements based on the positive identification criteria were selected [28]. Specifically, the monitored transition was required to be present with a signal-to-noise ratio (S/N) ≥ 3 . The acquired spectra matched with the library spectra with a purity $> 70\%$. The peak height ≥ 2000 cps was required, and no compound had to be present in the blank on the same run. At the same time, the internal standard had to be present.

2.6. Assay performance

The proposed methodology was tested with regard to matrix effect (ME), process efficiency (PE) and recovery (RE). Furthermore, the identification of all analytes was tested for spiked blank samples at three different levels of concentration (1, 10 and 100 ng/mL) and a mixture of seven deuterated substances (morphine-d₃, pregabalin-d₆, metamphetamine-d₅, ketamine-d₄, zolpidem-d₆, oxazepam-d₅ and diazepam-d₅) was used to include internal standard along the chromatogram.

First, the influence of the matrix on the detection and identification of the selected analytes in whole blood samples was studied. For that, the parameters of matrix effect, process efficiency and recovery were addressed according to the simplified approach described by

Matuszewski [29]. This procedure implied the preparation of three sets samples and six replications for each set.

For the preparation of the set sample 1, 50 μL of each working solution at 100 ng/mL were evaporated at dryness and reconstituted with 200 μL of mobile phase, reaching a concentration of 250 ng/mL. A stream of nitrogen (lower than 5 psi) was applied to avoid the evaporation of the more volatile compounds.

For set sample 2, blank blood specimens were extracted. Then, prior to evaporation, 50 μL of each working solution at 100 ng/mL were added. After evaporating, the extract was reconstituted with 200 μL of mobile phase.

For set sample 3, blank blood specimens were spiked with 50 μL of the respective spiking solution. Thereafter, the samples were extracted following the steps established for the three tested procedures.

Furthermore, limits of detection (LODs) were also calculated for the analytes included in this study. S/N ratios for each peak of chromatograms registered at 10 ng/mL were used for the calculation of LODs. LOD was calculated as 3S/N.

Finally, the applicability of the proposed methodology was evaluated by the analysis of blood samples from fifteen real DFSA cases previously analyzed in the Department of Chemistry of the INTCF-Madrid. Previous analyses in official forensic laboratories had provided positive results to compounds frequently related to DFSA offenses. These samples were analyzed again using the proposed methodology to compare results. Blood samples from real cases were prepared following the procedure combining protein precipitation and phospholipid removal.

3. Results and discussion

3.1. Screening method for the main DFSA analytes

Compounds frequently related to DFSA cases listed by SOFT [6] and UNODC [7] were included in the proposed methodology. The suitable forensic investigation of alleged DFSA cases highly requires the search of analytes listed by these international authorities in biological samples from victims.

Included compounds were classified into several pharmacologic groups indicated in Table 1. Benzodiazepines are the largest group. Likewise, two analogous of benzodiazepines were also included. Antidepressants are the second largest group, followed by antihistamines. Rest of pharmaceuticals includes antipsychotic, antispasmodic, anti-epileptic, antihypertensive, anti-nausea and antitussive drugs, as well as muscle relaxants. Abused drugs as amphetamines and cocaine were also included, along with cathinones, dissociative and hallucinogenic substances.

New chromatographic conditions were opportunely established to achieve the correct separation of included compounds at their corresponding retention times. The mass spectrometry detector operated in sMRM mode, increasing selectivity and allowing the correct detection and identification of all the included compounds. Therefore, difficulties derived from peaks overlapping between detection windows of different compounds could be overcome. In addition, the third quadrupole operation mode of the mass spectrometer was programmed to work as an ion trap, achieving a greater sensitivity.

All compounds included in the new screening methodology were successfully separated, as Fig. 1 shows. Analyses were carried out grouping all compounds in 5 different groups and at 100 ng/mL. Numbers in Table 1 identify each peak. Morphine was the first detected analyte (1). Detection windows overlapped for some compounds, but this did not prevent the correct observation of their chromatographic peaks. Thus, for chromatogram 1A, retention times of nitrazepam (63), tetrazepam (65) and nordiazepam (66) matched between 11.4 and 11.7 min. In the same way, retention times for triazolam (83) and clonazepam (84) matched between 11.95 and 12.03 min. Similarly, for chromatogram 1B, retention times of citalopram (55) and ziprasidone

(56) matched between 10.24 and 10.25 min, as well as retention times of cetirizine (73), fluoxetine (76) and nortriptyline (78), at 11.69, 11.70 and 11.71 min. Nevertheless, despite of partially overlapped detection windows, all compounds were successfully detected and identified, showing the great selectivity of the proposed screening methodology.

Compounds of the internal standard mixture used to test the proper functioning of the proposed methodology were also correctly separated and identified, such as is showed in the Fig. 2, which illustrates the chromatogram recorded from the internal standard mixture used.

3.2. Blood samples preparation

Blood samples have a significant role in the forensic investigation of alleged DFSA cases. This biological matrix is available in 53.9% of cases, being the only one in > 28.9% [13]. This led to the need of developing sensitive methods for the analysis of blood samples in order to detect any possible substance in the bloodstream of the victim at the time of sample collecting. However, complex composition of blood requires a suitable preparation treatment before the analysis to eliminate possible interferences from the matrix. The previous systematic toxicological analysis at the INTCF-Madrid included solid phase extraction by a procedure using Bond Elut columns, as well as protein precipitation with acetone prior to CEDIA immunoassay. Nevertheless, phospholipids and proteins are the principal interfering substances, so treatments must focus in them or reducing their presence as much as possible. In this way, techniques of protein precipitation, solid phase extraction and liquid-liquid extraction were compared for blood treatment before the analysis. These sample treatment techniques involved six different procedures. Percentages of detected compounds were studied for the six sample preparations procedures at three different concentration levels (100, 10 and 1 ng/mL). Spiked blank blood samples were used. All compounds were analyzed twice (duplicate). Criteria for positivity were checked after analyses in order to verify correct detection and identification. Additionally, results from the six procedures were compared also for matrix effect, extraction recovery and process efficiency using a smaller group formed by 25 benzodiazepines and related Z-drugs.

3.2.1. Detection capacity according to the blood treatment

First, because of proteins and phospholipids are the main interferences from blood, four procedures based in protein precipitation methods were compared. These focused protein precipitation and phospholipid removal as two significant stages in order to remove important analytical interferences. In this way, three procedures combined both stages using Phree® or Ostro Plates® for phospholipid removal, meanwhile one used only protein precipitation. Comparisons were based on detection capacity results shown in Fig. 3. At 100 ng/mL, the procedure combining protein precipitation and phospholipid removal using Ostro® plates reached 85.2%, the highest percentage of detected compounds. However, detection capacity significantly decreased at lower concentrations (29.7% compounds at 10 ng/mL and 15.9% at 1 ng/mL) for this procedure using Ostro® plates. The procedure combining precipitation and phospholipid removal with Phree® plates reached 75.8% at 100 ng/mL, the second higher detection percentage at the greatest concentration level. Interestingly, at lower concentrations it provided the highest results between all procedures based in protein precipitation, thus 60.9% and 31.9% of compounds were detected at 10 ng/mL and 1 ng/mL, respectively. Another alternative combining cell lysis sonication and phospholipid removal by Phree® plates provided poorer detection levels at the three concentration levels tested: 71.9% at 100 ng/mL, 58.8% at 10 ng/mL and 21.4% at 1 ng/mL. Finally, the procedure based only in protein precipitation detected significantly less compounds at 100 ng/mL and 10 ng/mL, reaching 56.6% and 21.9%, respectively. However, this procedure provided a slightly higher result at 1 ng/mL, detecting 26.4%. Therefore, among the four tested precipitation procedures, the combination

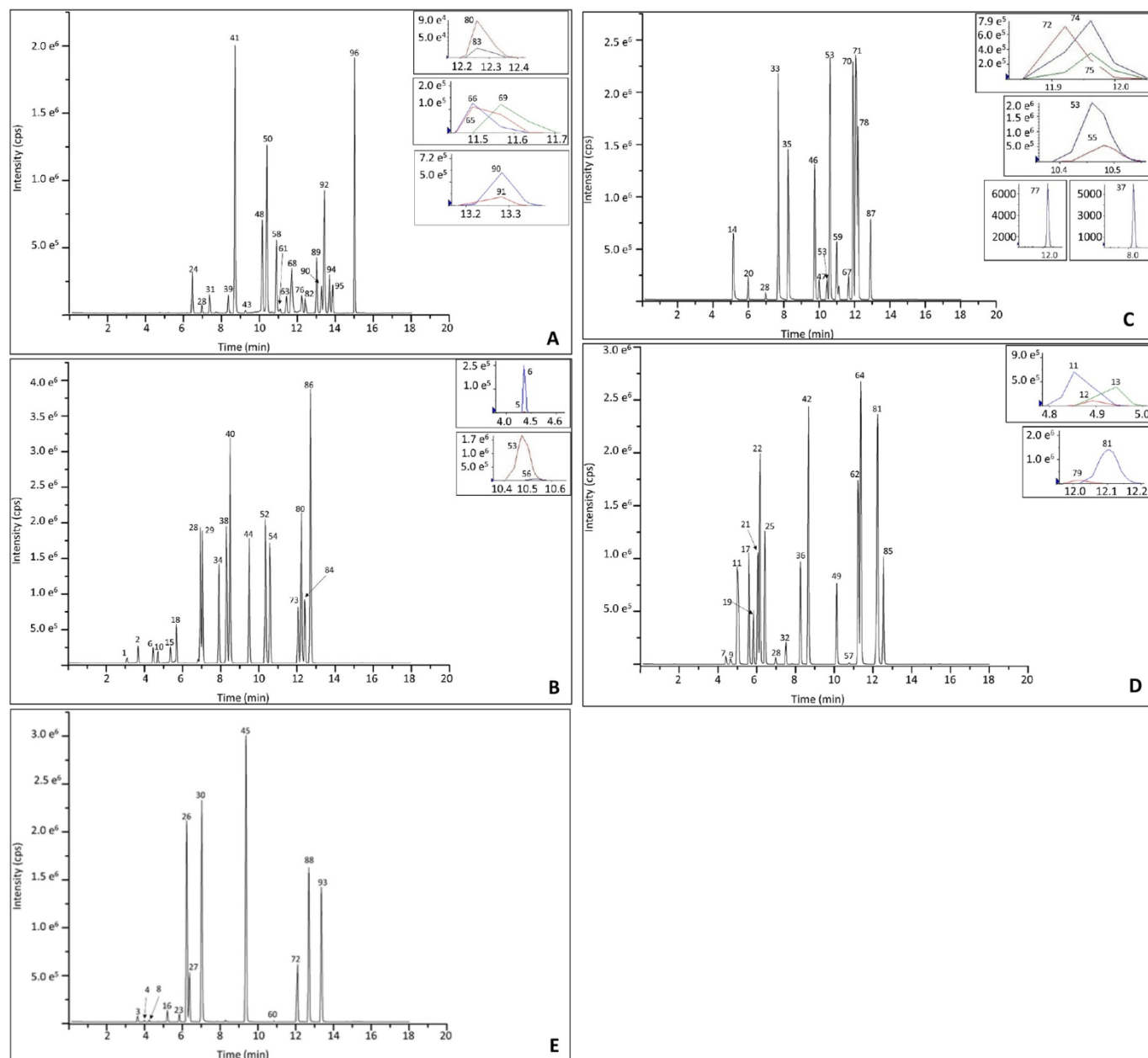


Fig. 1. Chromatograms of the 96 compounds (at 100 ng/mL) studied in the different groups. Table 1 includes some relevant characteristics and the name of the identified peaks.

using Phree® plates was selected because of provided the best results of detection capacity at high and low concentrations, being the most promising alternative for DFSA analytes usually found at low concentrations.

Then, the selected protein precipitation procedure was compared with other two widely used sample preparation techniques, as solid phase extraction and liquid-liquid extraction. Detectability results from both procedures are also included in Fig. 3. At 100 ng/mL, the three treatments detected similar percentages of compounds. Solid phase extraction reached the highest value, 80.2%, meanwhile protein precipitation detected 75.8% and liquid-liquid extraction 74.7%. Once again, protein precipitation and liquid-liquid extraction reported very similar results at 10 ng/mL, detecting 60.4% and 62.1%, respectively. However, detection by solid phase extraction decreased to 47.2%. In this line, solid phase extraction also provided the lowest detection at 1 ng/mL, reaching the 12.1%. At the lowest concentration, the procedure using Phree® plates detected 31.8%; meanwhile liquid-liquid

extraction reached 44.5%.

Despite differences in detection percentages observed at 1 ng/mL between procedures combining precipitation and phospholipid removal and that based on liquid-liquid extraction, the former was selected because of its especially favourable features for the investigation of blood in alleged DFSA cases. In this sense, some features of DFSA casuistry must be considered: toxicological investigation of alleged DFSA cases requires not selective extraction techniques because of both the number of involved substances and their physicochemical properties are unknown, that is why a screening methodology is necessary. Not selective techniques prevent loss of analytes potentially present in the sample. However, victims often delay seeking for help and report to authorities, so biological matrices are collected when a notable time has passed from the alleged attack. Over time, metabolization and excretion processes decrease the detectability of substances in biological matrices. Concentration levels decrease faster in blood, whose detection window is smaller than for other matrices. Less selective extraction

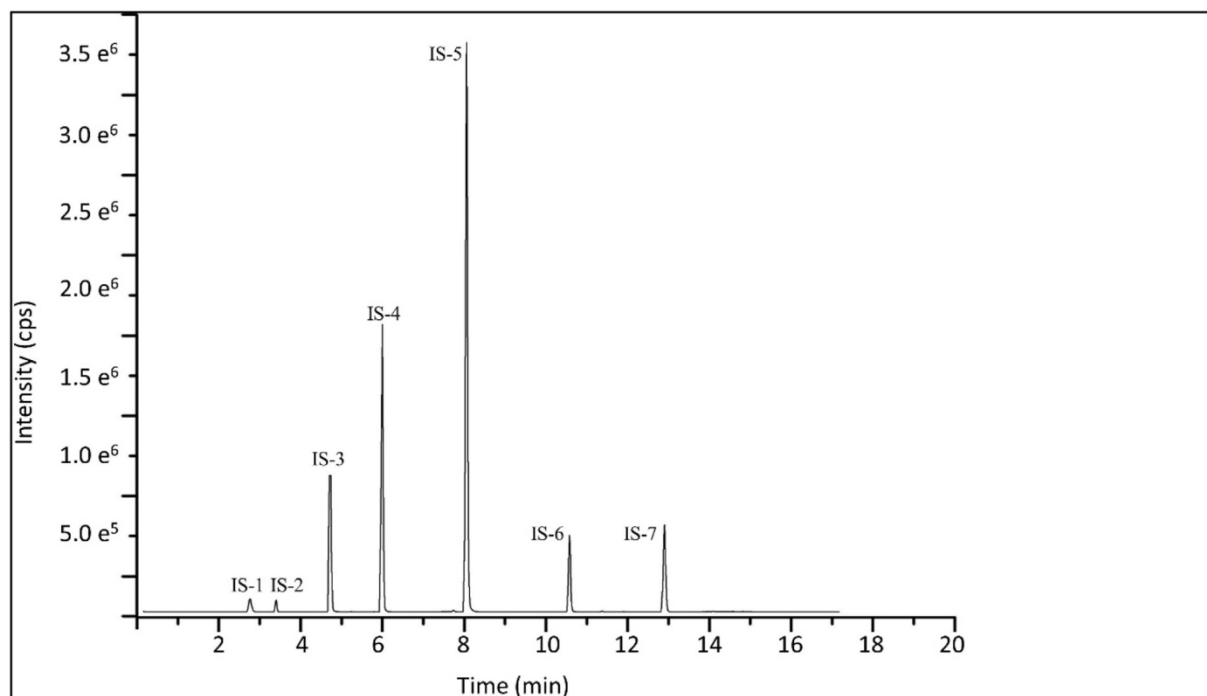


Fig. 2. Chromatogram of the internal standard mixture (at 100 ng/mL). Table 1 includes some relevant characteristics and the name of the identified peaks.

procedures are needed because of the possibility of lower concentration detectability levels. This facilitates the extraction of both polar and non-polar analytes regardless of their concentration in the collected samples from victims. The procedure combining protein precipitation and phospholipid removal is less selective in comparison to solid phase and liquid-liquid extraction techniques. Thus, whereas the first allows the extraction of polar and non-polar molecules, the other two only facilitate the extraction of non-polar compounds. Therefore, the procedure combining protein precipitation and phospholipid removal using Phree® plates was selected for the multi-target screening method developed.

3.2.2. Matrix effect, process efficiency and extraction recovery according to the blood treatment

For this comparison study, a group formed by 23 benzodiazepines and 2 Z-drugs were used. Between procedures based in protein precipitation, the combination of precipitation and phospholipid removal

with Phree® plates reached both highest extraction recovery and process efficiency results, 69% and 103% respectively, as is shown in Fig. 4. Moreover, this procedure provided a properly matrix effect, with a positive average value of 53%. However, extraction recovery gradually decreased for procedures using Ostro® plates (66%), sonication (45%) and only precipitation (36%). Regarding to process efficiency, results from these three procedures were significantly lower than that reached by precipitation combined with phospholipid removal by Phree® plates. In this way, the procedure with sonication reached 27%, meanwhile that using Ostro® plates and only precipitation reached 2% and 6% respectively. Furthermore, these three methods showed significant negative matrix effects: -97% for the method with Ostro® plates, -83% using only precipitation and -39% for the procedure employing sonication cell lysis. Therefore, between tested procedures based in protein precipitation, results indicate again that the combination of protein precipitation and phospholipid removal using Phree® plates was the best alternative.

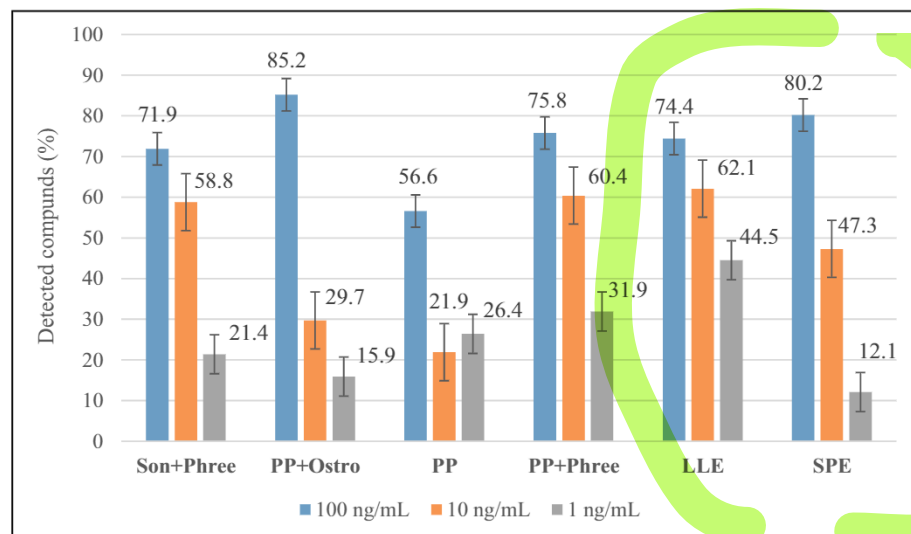


Fig. 3. Bar chart showing percentages of detected compounds by tested sample treatments at three concentration levels (100, 10 and 1 ng/mL). Son: sonication; Phree: Phree® Phospholipid Removal Plates; Ostro: Ostro® Sample Preparation Plates; LLE: liquid-liquid extraction; SPE: solid phase extraction; PP: protein precipitation only.

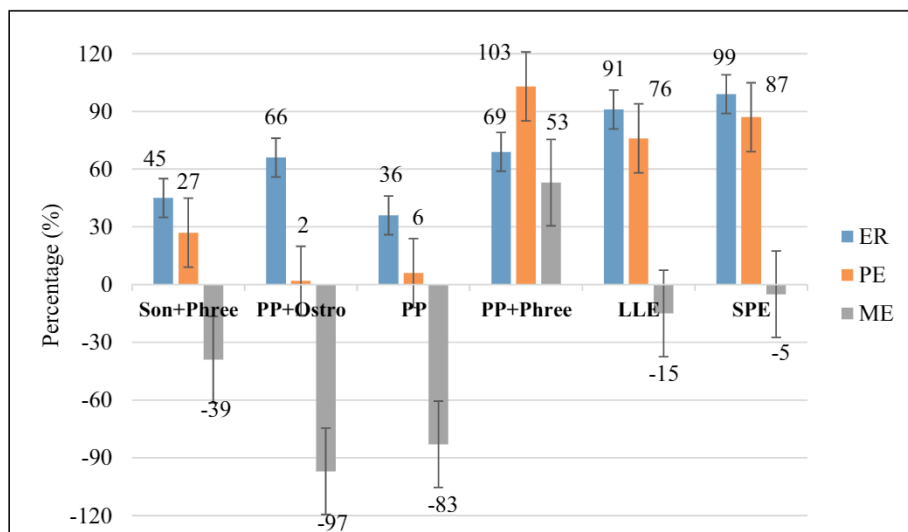


Fig. 4. Bar chart showing percentages of Extraction Recovery (ER), Process Efficiency (EP) and Matrix Effect (ME) tested for 25 compounds at 250 ng/mL. Son: sonication; Phree: Phree® Phospholipid Removal Plates; Ostro: Ostro® Sample Preparation Plates; LLE: liquid-liquid extraction; SPE: solid phase extraction; PP: protein precipitation only.

On the other hand, Fig. 4 also presents results from solid phase and liquid-liquid extractions. In this way, the comparison with these other two extraction techniques showed as the selected procedure based in protein precipitation produced the greatest increase of the analytical signal. Solid phase and liquid-liquid extraction provided negative matrix effects, reaching average values of -5% and -15% , respectively. These two procedures led to decrease of analytical signal intensity for most compounds. This coincides with expected results, since solid phase and liquid-liquid extraction are cleaner techniques.

Chlordiazepoxide signal intensity significantly decreased for the three compared techniques, meanwhile alpha-hydroxy-alprazolam provided the greatest increase by solid phase extraction and protein precipitation. Matrix effect for solid phase extraction was between 132% for alpha-hydroxy-alprazolam and -90% for chlordiazepoxide. Concerning liquid-liquid extraction, matrix effect varied from 40% for bromazepam to 86% for chlordiazepoxide. For the procedure combining protein precipitation and phospholipid removal, matrix effect ranged from 314% for alpha-hydroxy-alprazolam to -88% for chlordiazepoxide.

Regarding extraction recovery, liquid-liquid and solid phase extraction procedures provided the highest average results, reaching 99% and 92% , respectively. Protein precipitation reached an average recovery value of 66% . Extraction recovery for solid phase extraction ranged from 329% for chlordiazepoxide to 60% for 7-aminoclonazepam. For liquid-liquid extraction, it was between 117% for tetrazepam and 56% for alpha-hydroxy-alprazolam. Values for protein precipitation oscillated between 135% for zolpidem and 27% for chlordiazepoxide.

Furthermore, the procedure combining protein precipitation and phospholipid removal showed the highest process efficiency, reaching an average value of 103% . Results for liquid-liquid and solid phase extraction was 76% and 87% , respectively. For solid phase extraction, process efficiency was between 209% for alpha-hydroxy-alprazolam and 32% for chlordiazepoxide. Values for liquid-liquid extraction ranged between 115.1% for nitrazepam and 12% for chlordiazepoxide. Finally, the procedure combining protein precipitation and phospholipid removal showed a process efficiency between 274% for alpha-hydroxy-alprazolam and 3% for chlordiazepoxide.

The matrix effect increased analytical signals when the sample preparation using protein precipitation was applied. Likewise, an important decrease in the analytical signal was observed for most compounds when applying the more selective solid phase and liquid-liquid extractions. However, matrix effect is not a serious problem in this study because of the screening methodology is qualitative. Moreover,

even a slight increase of the analytical signal can facilitate a better qualitative observation of target analytes. Furthermore, the procedure combining protein precipitation and phospholipid removal using Phree® plates reached the highest average value for process efficiency. These results, as well as already referred suitable percentages of detected compounds in the previous section, led to the selection of this procedure in the multi-target screening method developed.

3.3. Analytical performance of the screening method

The new developed methodology focuses to the multi-target screening of analytes related to DFSA casuistry in blood samples. As qualitative method, analytical performance was evaluated studying selectivity and LODs.

3.3.1. Selectivity

Selectivity of the new methodology was confirmed by obtained results from blank blood samples analysis. Any relevant interference from the matrix was observed at the retention time of the 98 compounds included in the proposed methodology. The Fig. 5 shows the chromatogram resulting from the analysis of a blank blood sample. Minor signals from the matrix were observed around minutes 7.86 and 7.58, coinciding with the retention times of cocaine, chlordiazepoxide, clozapine, venlafaxine and brompheniramine. However, these signals had an intensity lower than 2000 cps, so they did not interfere the analytical detection. Another interference from the matrix appeared at 15.08 min and reached 5580 cps, coinciding with the retention time of prazepam at 14.79 min. However, neither problem was observed for detection and identification of this compound. Therefore, results confirmed the good selectivity of the new methodology, as well as the suitability of the extraction procedure combining protein precipitation and phospholipid removal for the analyses of blood samples in alleged DFSA cases.

3.3.2. Limits of detection

LODs were calculated using the S/N obtained for each compound from chromatograms at 10 ng/mL . In this way, as is shown in Fig. 6, most analytes provided LODs under 1 ng/mL , the lowest concentration level analyzed. Thus, for 59 substances LODs were between 0.1 and 0.4 ng/mL , meanwhile values for other 15 compounds ranged 0.5 to 0.9 ng/mL . Beyond 1 ng/mL , LODs for 9 compounds were between 1.0 and 1.4 ng/mL . Obtained values oscillated between 2.1 and 2.5 ng/mL for other 3 compounds, whereas 4 analytes had LODs ranged from 3.3 to 5.0 ng/mL . Finally, LODs for 7 compounds were higher than 5 ng/mL . Likewise, 6 substances were between 5.1 and 10 ng/mL , meanwhile

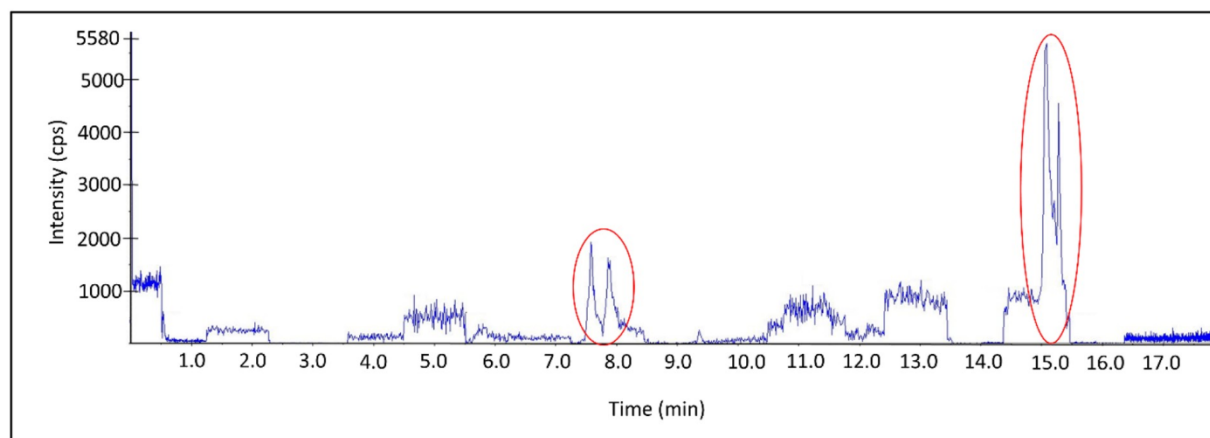


Fig. 5. Chromatogram of a blank blood sample treated by protein precipitation and phospholipid removal.

there was one analyte with a LOD of 10.7 ng/mL. Table 2 shows names of compounds included in each LODs range.

3.4. Application to the analysis of real forensic samples

The proposed methodology was applied to the analysis of fifteen blood samples from DFSA cases, using protein precipitation with phospholipid removal as sample preparation procedure. These samples had been previously analyzed in the INTCF-Madrid by the analytical systematic of the official laboratory. Through this systematic, firstly proteins were removed by precipitation with acetone and shaking. Then, supernatant was mixed with hydrochloric acid, following an assay by CEDIA enzyme-linked immunosorbent. Moreover, the systematic includes solid phase extraction with BondElut and subsequent analysis by HPLC-MS/MS.

The Table 3 shows the obtained analytical results, observing as the proposed methodology confirmed initial results for eight cases. Specifically, those identified in Table 3 as number one, seven, eight, nine, eleven, twelve, fourteen and fifteen. In these cases, both official systematic and the proposed methodology provided exactly the same results. So, none substances were detected in cases number seven, nine, fourteen and fifteen. For case number one, both the two methodologies detected the previous consumption of ecstasy or MDMA. Similarly, in case number eight both match one more time, with the detection of cocaine, benzoilegonine and ethylbenzoilegonine. In the same way,

lorazepam and sertraline were detected in cases number eleven and twelve, respectively. On the other hand, several false negatives were observed for different substances. So, in case number six, the proposed methodology detected the presence on mirtazapine, which had remained hidden to the official procedure. The same happened respectively in samples ten and thirteen, with the detection of nordiazepam and mirtazapine. However, the greatest differences between the both procedures were observed for cases identified as two, three, four and five. In these samples, analytical findings differed for the detection of two or more compounds. Curiously, in case number three, the official procedure identified a compound not detected by the proposed methodology.

As observed through comparisons, in some cases the official systematic did not detect several substances. However, these analytes were detected by the new multi-target screening methodology. It should be noted that the official systematic also included the search of these substances not detected initially. These differences in results are due to the greater sensitivity of the proposed methodology. In this sense, to harness the capacity of the third quadrupole for operating as an ion trap was a key factor. Enhanced product ion (EPI) scans were generated when the spectral signal reaches a pre-set intensity, which contributed in a very important way to improve sensitivity. In addition, an extraction procedure of blood samples directly focused on the elimination of proteins and phospholipid helped to the improvement of the detected compounds in real DFSA cases.

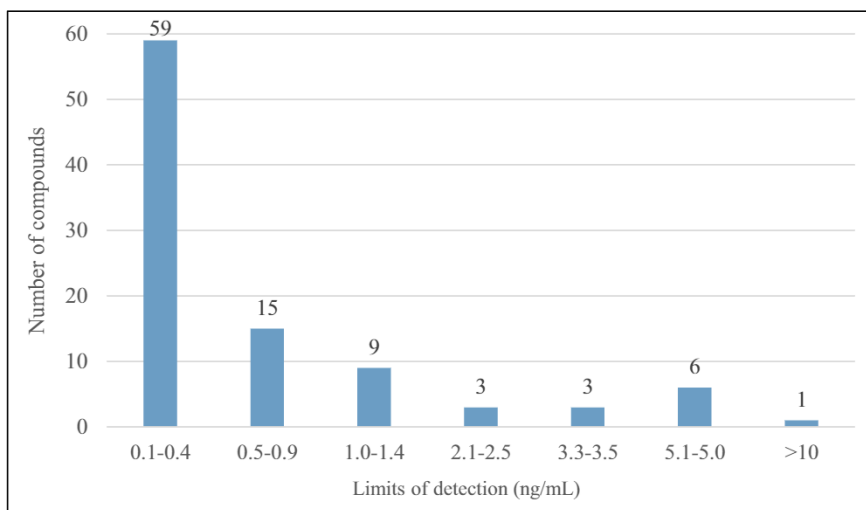


Fig. 6. Limits of detection (LODs) for the compounds included in the screening methodology.

Table 2
Limits of detection (LODs) for the compounds included in the screening methodology.

LODs (ng/mL)	Compounds
0.1–0.4	Scopolamine, MDA, MA, doxylamine, oxycodone, hydrocodone, norketamine, atropine, benzoylecgonine, 7-amineclonazepam, ketamine, MDEA, 4-MEC, tapentadol, tramadol, mistazapine, chlorpheniramine, meperidine, brompheniramine, cocaine, clozapine, tilidine, zolpidem, LSD, trazodone, ethylbenzoilecgonine, quetiapine, midazolam, phencyclidine, loperazolam, flurazepam, fentanyl, citalopram, ziprasidone, buprenorphine, medazepam, haloperidol, promethazine, paroxetine, hydroxyzine, imipramine, cetirizine, dextropropoxyphene, fluoxetine, nortriptyline, cyclobenzaprine, cyproheptadine, alimemazine, amitriptyline, aripiprazole, methadone, trimipramine, temazepam, clobazam, flunitrazepam, clomipramine, clotiazepam, prazepam
0.5–0.9	Hydromorphone, oxymorphone, dihydrocodeine, codeine, MDMA, zopiclone, meprobamate, doxepine, lorazepam, nitrazepam, tetrazepam, nordiazepam, alprazolam, desipramine, lormetazepam, diazepam
1.0–1.4	Morphine, 6-MAM, desmethylvenlafaxine, venlafaxine, chlordiazepoxide, oxazepam, diphenhydramine, triazolam, clonazepam
2.1–2.5	Amphetamine, mephedrone, dextrometorphan
3.3–5.0	Pregabalin, clonidine, sertraline,
5.1–10.0	Gabapentine, mescaline, Bromazepam, phenitoin, alpha-OH-alprazolam, EDDP
> 10	Carisoprodol

Table 3
Comparison of the detected compounds after the analysis of fifteen blood samples from DFSA cases by the proposed methodology and by an analytical systematic previously used in official laboratories.

Detected compounds		
Case	Previous analytical systematic	Proposed methodology
1	MDMA	MDMA
2	Diazepam, codeine	Diazepam, codeine, hydrocodone, nordiazepam, sertraline, temazepam
3	Clonazepam, mirtazapine	Clonazepam, 7-aminoclonazepam, zopiclone, lorazepam
4	Cocaine, benzoilecgonine	Cocaine, benzoilecgonine, MDMA, haloperidol
5	Lorazepam	Lorazepam, benzoilecgonine, 7-amineclonazepam, cocaine, alprazolam clonazepam
6	N.D.	Mirtazapine
7	N.D.	N.D.
8	Cocaine, benzoilecgonine, ethylbenzoilecgonine	Cocaine, benzoilecgonine, ethylbenzoilecgonine
9	N.D.	N.D.
10	Aripiprazole, fluoxetine, clotiapine	Aripiprazole, fluoxetine, clotiapine, nordiazepam
11	Lorazepam	Lorazepam
12	Sertraline	Sertraline
13	Citalopram	Citalopram, mirtazapine
14	N.D.	N.D.
15	N.D.	N.D.

N.D: no detection. Other abbreviations as defined in Table 1. Compounds detected only by the proposed methodology are highlighted in bold.

4. Conclusion

The proposed analytical methodology was developed for the multi-target screening of 96 compounds related DFSA in whole blood samples from alleged victims of this type of sexual violence. Referral listings from recognized organizations compiling substances involved in DFSA were considered for the selection of all compounds. The proposed method uses a HPLC-MS/MS system operating in scheduled multiple reaction monitoring (sMRM) mode. A survey scan monitoring all ionic fragmentation reactions is complemented by the hybrid operating of the third quadrupole as an ion trap and provides enhanced product ion (EPI) spectra. The high sensitivity of the new methodology allowed the correct separation, detection and identification of the 96 targeted compounds included. Furthermore, the whole blood is an important sample for the forensic investigation of alleged DFSA because this is usually submitted to the forensic laboratory in this type of cases and frequently the only one available biological matrix. Consequently, analytical methodologies specifically focused on blood treatment are necessary. However, these must consider the elimination of phospholipids and proteins as principal interfering substances in the analytical

examination of blood samples. The proposed methodology combines a blood sample preparation treatment including these two necessary stages focused on protein precipitation and phospholipid removal. The implemented extraction technique is also advantageous considering the delay frequently characterizing report DFSA cases and subsequent sample collection. Blood concentration levels decrease as time goes by, so bit selective sample preparation techniques are especially required for the toxicological screening of these sexual assaults. Results from the capacity of detection and influence from the matrix were compared for extraction techniques based on protein precipitation, solid-phase extraction, and liquid-liquid extraction. The treatment combining protein precipitation and phospholipid removal increased the analytical signal for most compounds in comparison with liquid-liquid and solid phase extractions. However, no significant interferences from the matrix were observed, so detection and identification were not affected. Although blood treatment based on protein precipitation has shown lower detection capability than liquid-liquid extraction, was the selected sample preparation procedure because of its suitability to analytical particularities of blood analyses during the toxicological screening of alleged DFSA cases. To bear in mind these aspects is necessary to improve the forensic investigation of this form of sexual violence. Obtained results are good, but further investigations in blood preparation procedures based on protein precipitation can be focused to reduce the matrix influence and improve the capacity of detection. Due to the proposed methodology works in positive ionization mode only, so acidic compounds related DFSA are not included, such as GHB. In this line, the inclusion of pending substances must ever be accompanied by a periodical check of compounds to be searched, those progressively appearing related to DFSA. Despite this, the proposed methodology comprises a significant number of compounds included in international listings. Moreover, successful results obtained through the analysis of fifteen blood samples from real cases demonstrated the potential of the proposed multi-target screening method for the forensic investigation of DFSA cases.

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