



Ultrasound assisted membrane-assisted solvent extraction for the simultaneous assessment of some drugs involved in drug-facilitated sexual assaults by liquid chromatography-tandem mass spectrometry

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ABSTRACT

A simple and highly efficient ultrasound assisted membrane-assisted solvent extraction (MASE) pre-treatment method for urine has been developed and validated for the simultaneous determination of twenty-two drugs involved in drug-facilitated sexual assaults (DFSAs) by liquid chromatography-tandem mass spectrometry. MASE was performed with 4.0 mL of urine (pH adjusted at 12), 400 μ L of hexane as an organic solvent inside the polypropylene membrane, and ultrasonication (45 kHz, 120 W) for 10 min. A pre-concentration factor of 40 was achieved after evaporation (N_2 stream) and re-dissolution in 100 μ L of methanol. Analytes were separated using a Zorbax Eclipse Plus C18 column under gradient elution with aqueous 10 mM NH_4HCO_3 (pH 8.0) and methanol as mobile phases. Matrix-matched calibrations allowed the assessment of DFSA drugs of quite different octanol-water partition coefficients ($K_{o/w}$), from $1.32 \cdot 10^1$ for pregabalin to $2.45 \cdot 10^5$ for clomipramine (Log P values from 1.12 (pregabalin) to 5.39 (clomipramine)). The limit of detection (LOD) was between 0.0075 to 0.37 μ g L⁻¹, with analytical recoveries ranging from 73 to 103%, and relative standard deviations (RSDs) within the 2–20% range. The applicability of the method was demonstrated after analysing urine samples under forensic investigation.

1. Introduction

Chemical Submission (CS) refers to ‘substance administration to a person without his/her knowledge to cause in him/her a change in alertness, state of consciousness and judgment’ [1]. The term derives from the French term “soumission chimique” (also known as “soumission médicamente use” [2]), and it was first used in 1982 by Poyen et al. [3]. Although chemical submission includes all crimes related to this activity, a new term has arisen due to the high incidence of sexual assaults with drug use: Drug-Facilitated Sexual Assault (DFSA). Previous literature has also used the terms Drug-Enabled Sexual Assault [4] and Incapacitated Sexual Assault [5] to refer to DFSA. Gee et al. have made a distinction between two different categories of DFSA: (i) *Proactive* DFSA, “the covert or forcible administration of a disinhibiting or incapacitating substance to an individual by an assailant for the purpose of sexual assault”; and (ii) *Opportunistic* DFSA, “the act of taking advantage of an

individual who is profoundly intoxicated to the point of near or actual unconsciousness by voluntary ingestion of sufficient amounts of drugs or alcohol” [6]. In 2007, The Advisory Council on the Misuse of Drugs (ACMD) adopted a definition of DFSA that encompasses both premeditated and opportunistic intention [7].

The drugs involved in DFSA cases are known as “date rape drugs”, “predatory drugs” or “club drugs” [8]. However, the use of these terms may be misleading, as is the focus of the mainstream media that has led the public to believe that *proactive* DFSA has a greater incidence than it has. One of the biggest problems of DFSA is the high number of drugs, contrary to the popular opinion, that are suspected to have been used for committing this type of crime. The Society of Forensic Toxicologists (SOFT) has created a list of substances that should be targeted in analysis which includes over fifty common DFSA drugs [9]. amongst these substances we find ethanol, street drugs and traditional drugs of abuse, as well as medicinal drugs, mostly benzodiazepines [10–12]. Most of the

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selected drugs are potent fast-acting central nervous system (CNS) depressants [13], which helps the assailant render the victim passive, unable to fight off the attacker, and powerless to resist sexual assaults [8]. In addition to these effects, the victims usually present total or partial anterograde amnesia (inability to create new memories after having consumed the substance), temporary and spatial disorientation, and sedation and disinhibition [1,2]. The victim may maintain an awoken state and seemingly normal behaviour but with inconsistencies, including a state of suggestion [2].

The same factors that make DFSA substances desirable to sexual predators, also pose challenges for toxicological analysts [8]. The drugs involved in DFSA diagnosis are usually tasteless, odourless, and colourless, orally administered, activated at low doses and may give rise to clinical symptoms like alcohol intoxication [1]. Most of the drugs are rapidly absorbed, metabolized, and eliminated from the body within a few hours after ingestion [8]. Urine and blood are the most usual matrices to document drug exposure [14]. In 1999, SOFT's committee proposed urine as sample of choice in DFSA alleged cases [15]. Drugs and metabolites are generally concentrated in urine [14], which provides a longer window of detection than those in blood. However, other biological samples such as head hair, vomit, and sweat may be considered for complementary analysis [8,10,15]. Regardless of the matrix analysed, highly sensitive and selective analytical methods should be used. Liquid chromatography – tandem mass spectrometry (LC-MS/MS) and gas chromatography – tandem mass spectrometry (GC-MS/MS) are currently considered as a prerequisite in drug-facilitated crime (DFC) cases investigation [14]. However, reports using gas chromatography – mass spectrometry (GC-MS) can be found in the literature for assessing DFSA drugs in biological matrices such as urine and blood, and human placenta and hair [16–18]. In addition, LC-MS/MS using high-resolution mass detectors such as time of flight (TOF) spectrometers has also been used [12].

An adequate sample pre-treatment is quite important for an accurate quantification of ultra-trace levels of DFSA drugs, and urine sample analysis usually requires a pre-concentration stage before DFSA drugs determination. Several approaches such as solid-phase extraction (SPE) [19], solid-phase micro-extraction (SPME) [20], micro-extraction in packed syringe (MEPS) [21] and stir bar sorptive extraction [22] have been proposed for pre-concentrating DFSA drugs and psychoactive substances. Furthermore, hollow-fibre liquid-phase micro-extraction (HF-LPME) [23], dispersive liquid/liquid microextraction (DLLME) [24], and electromembrane extraction (EME), mainly the novel gel-electromembrane extraction (G-EME) mode (also referred as “inside” gel-EME (IG-EME)) [25] have been reported as fast techniques with high pre-concentration factors. The latter extraction technique has been recently used for drugs isolation from complex matrices such as wastewaters and clinical/forensic materials [26–28]. Membrane-assisted solvent extraction (MASE) is a useful option that enables the reduction of organic solvent and can therefore be considered a green technique. MASE methods are becoming very popular due to several advantages, including a high degree of selectivity, proper clean-up from complex matrices, and the possibility for automation and online coupling to analytical instruments [29]. Several applications of this sample pre-treatment have been developed for polycyclic aromatic hydrocarbons in tea infusion [30] and wastewater [31], polychlorinated biphenyls and organochlorine pesticides in seawater [32], alkylphenols in sediments [33], and pharmaceuticals in surface water [34]. As a non-exhaustive technique, MASE requires long extractive times (mechanical shaking) for targets diffusion through the membrane microporous because of concentration gradient (high concentration in the donor phase, usually an aqueous sample, low concentration in the acceptor phase into the membrane). The aim of this study has been to explore the possibilities of ultrasound for speeding-up the MASE process when isolating DFSA drugs from urine, and to validate the LC-MS/MS method for the simultaneous determination of twenty-two DFSA drugs. A previous attempt for speeding-up LPME procedure has been

described by Fashi et al. [35] by using a lab-made device [a polytetrafluoroethylene (PTFE) cylindrical tube which allows a polypropylene (PP) membrane to be housed] for the isolation of malondialdehyde.

2. Materials and methods

2.1. Chemicals and reagents

Purified water was obtained from Milli-Q-IQ 7003 water purification system (Millipore Bedford, MA, USA). Methanolic solutions of amitriptyline, atropine, buprenorphine, clomipramine, clozapine escitalopram, haloperidol, ketamine, 3,4-methylenedioxy-n-ethylamphetamine (MDEA), methamphetamine, mirtazapine, norketamine, olanzapine, para-methoxyamphetamine (PMA), pregabalin, sertraline, and proadifen (SKF-525A) at 1.0 mg mL⁻¹; and fluoxetine, imipramine, methadone, quetiapine, trazodone, and venlafaxine (0.1 mg mL⁻¹) were from Cerilliant Corporation (Round Rock, TX, USA). Methanolic solutions of deuterated analogues (internal standards) of 0.1 mg mL⁻¹ (citalopram-d₆, clomipramine-d₃, ketamine-d₄, MDEA-d₅, and norketamine-d₄) were also from Cerilliant. The stock standards, as well as diluted methanolic solutions, were stored at -20 °C. Methanol (Gradient LC) was from Merck (Darmstadt, Germany); whereas methanol (LC-MS Chromasolv) was from Honeywell Riedel-de Hën (Seelze, Germany). Other chemicals/solvents were ACS hydrochloric acid (37%), ACS potassium chloride, ACS ammonium hydrogen-carbonate, and ACS 1-butanol (PanReac, Barcelona, Spain); sodium hydroxide (pellets for analysis), GR for analysis diethyl ether, ACS chloromethane, Chromasolv dichloromethane, and ACS trichloromethane (Merck); ACS sodium fluoride, and ACS hexane (Sigma-Aldrich, San Luis, MO, USA).

2.2. Instrumentation

Analysis was performed using a 3200 QTRAP LC-MS/MS system from ABSciex (Concord, Canada) equipped with an electrospray ionization source, a Flexar FX-15 LC binary pump with integrated vacuum degasser (Perkin Elmer, Waltham, MA, USA), a Flexar LC autosampler (Perkin Elmer), and a GECKO 2000 column heater from Amchro GmbH (Hattersheim, Germany). The chromatographic separation was performed with a Zorbax Eclipse Plus C18 (4.6 × 100 mm, 3.5 µm) from Agilent Technologies (Santa Clara, CA, USA), connected with a C-18 pre-column (4.0 × 3.0 mm) from Phenomenex (Torrance, CA, USA). The LC-MS/MS data handling was done with MultiQuant 2.1 software (ABSciex).

MASE devices consisted of 10 mL glass vial equipped with a polypropylene (PP) membrane (4 cm × 6 mm i.d., wall thickness of 0.03 mm), a stainless-steel funnel with PTFE ring, and a Viton ring and metallic ring cap, from Gerstel (Mülheim, Germany). MASE extraction was assisted with a USC60TH ultrasound cleaner bath (45 kHz, 120 W) from VWR (Leuven, Belgium). Other devices were a Boxcult temperature-controlled incubation chamber (Stuart Scientific, Surrey, UK) with a Rotabit orbital-rocking platform shaker (J.P. Selecta, Barcelona, Spain), a basic 20 pH metre (Crison, Barcelona, Spain), a VLM EC1 metal block thermostat and N₂ sample concentrator from VLM (Leopoldshöhe-Grethe, Germany), a Reax 2000 mechanical stirrer (Heidolph, Kelheim, Germany), and a Classic ML204/01 analytical balance (Mettler Toledo, Columbus, OH, USA).

2.3. Urine samples

Urine samples (drug-free urine) used for optimising and validating the ultrasound assisted MASE and LC-MS/MS procedure were collected daily from laboratory staff volunteers. The optimised method was applied to forensic urine samples from individuals who claimed to have been sexually assaulted. These samples were supplied by the Forensic Sciences Institute “Luís Concheiro” (INCIFOR) at the University of Santiago de Compostela. Collected samples were stored at -20 °C until

further analysis.

2.4. Optimised ultrasound assisted MASE procedure

Vials (10 mL) were filled with 4.0 mL of urine sample spiked with 100 $\mu\text{g L}^{-1}$ of several internal standards (citalopram-D₅, clomipramine-D₃, ketamine-D₄, MDEA-D₅, norketamine-D₄, and SKF). The pH was adjusted to 12 by dropwise addition of a 0.1 M sodium hydroxide solution. The PP bag was fitted into the funnel by Viton ring, dipped into the sample vial, and filled with 400 μL of n-hexane. Finally, the vial was capped and subjected to ultrasound (45 kHz, 120 W) for 10 min. After extraction, the hexane extract was transferred to a glass tube, and the empty PP bag was rinsed with 200 μL of hexane. The rinsing hexane was mixed with the hexane extract before evaporation to dryness by a gentle N₂ stream. The residue was finally re-dissolved with 100 μL of methanol.

2.5. Liquid chromatography-tandem mass spectrometry measurements

Analytes were separated by applying a gradient elution with an aqueous 10 mM NH₄(HCO₃) solution (pH 8.0) and methanol as mobile phases A and B, respectively (Table S1, Electronic Supplementary Information, ESI). MRM transitions (at least two precursor ion→product ion transitions for each analyte), as well as optimized ion source potentials and collision energies for the MRM transitions used for quantification purposes (first MRM transition for each target), are listed in Table 1. Source potentials and collision energies for the remaining MRM transitions (qualitative/confirmation purposes) can be found in Table S2 (ESI). The electrospray source operation conditions were fixed at 5500 kV (ion spray voltage), 300 °C (ion source temperature), 40 psi for nebulizer gas (N₂) and curtain gas (N₂), and high mode for collision gas

(N₂).

For ultrasound assisted MASE optimization, calibrations based on methanolic solutions were prepared daily for each batch of analysis. The standards covered concentrations from 1.0 to 200 $\mu\text{g L}^{-1}$ for escitalopram, fluoxetine, imipramine, ketamine, MDEA, methadone, norketamine, trazodone, and venlafaxine; whereas calibrations were within the 5.0–200 $\mu\text{g L}^{-1}$ range for amitriptyline, atropine, haloperidol, methamphetamine, mirtazapine, and olanzapine. Clozapine determinations involved the use of standards from 10 to 200 $\mu\text{g L}^{-1}$, and the assessment of buprenorphine, clomipramine, PMA, pregabalin, quetiapine, and sertraline was performed with standards within the 50–200 $\mu\text{g L}^{-1}$ range. Internal standards (Table 2) were used at 100 $\mu\text{g L}^{-1}$.

Since targets must be isolated and pre-concentrated from the urine matrix by the ultrasound assisted MASE, samples analysis and method validation were performed using matrix-matched calibrations (MMA). Aliquots of drug-free urines (4.0 mL) were spiked in duplicate with increasing targets concentrations: 0.025, 0.10, 0.50, 1.0, 2.5, and 5.0 $\mu\text{g L}^{-1}$ for escitalopram, fluoxetine, imipramine, ketamine, MDEA, methadone, norketamine, trazodone, and venlafaxine; 0.125, 0.50, 0.75, 1.0, 2.5, and 5.0 $\mu\text{g L}^{-1}$ for amitriptyline, atropine, haloperidol, methamphetamine, mirtazapine, and olanzapine; 0.25, 0.50, 0.75, 1.0, 2.5, and 5.0 $\mu\text{g L}^{-1}$ for clozapine; and 1.25, 2.0, 2.5, 3.0, 4.0, and 5.0 $\mu\text{g L}^{-1}$ for buprenorphine, clomipramine, PMA, pregabalin, quetiapine, and sertraline. For all cases, deuterated analogues (2.5 $\mu\text{g L}^{-1}$ of internal standards) were also added. The spiked urines were then subjected to the ultrasound assisted MASE procedure (Section 2.4) before LC-MS/MS.

Urine samples from forensic cases were subjected to the ultrasound assisted MASE procedure in triplicate. MRM chromatograms for two suspected urine samples are shown in Fig. 1(a,b), whereas a total ion

Table 1
Multiple reaction monitoring (MRM) transitions and conditions for analytes and internal standards.

Compound	Mass (Da)	Rt ^a (min)	MRM transition ^b	Area ratio ^c	IS ^d	DP ^e (V)	EP ^e (V)	CE ^e (V)	CXP ^e (V)
Amitriptyline	277	29.95	278→233, 191, 105	1.91	SKF-525A	39.5	2.1	22.8	2.5
Atropine	289	18.86	290→124, 93, 103	1.74	SKF-525A	63.1	3.3	31.2	3.7
Buprenorphine	468	32.94	469→55, 396, 109	3.50	SKF-525A	94.1	3.8	64.0	2.3
Clomipramine	315	30.58	316→86, 242, 227	0.68	Clomipramine-D ₃	45.6	3.8	23.0	2.8
Clozapine	327	26.65	328→270, 192, 84	0.95	Citalopram-D ₆	56.1	6.1	21.1	1.3
Escitalopram	324	25.19	325→109, 262, 234	3.33	Citalopram-D ₆	38.2	8.0	32.1	2.3
Fluoxetine	309	27.89	310→91, 117, 148	3.96	SKF-525A	28.9	5.9	84.1	3.8
Haloperidol	376	26.08	377→123, 165, 95	1.02	SKF-525A	55.2	5.0	57.1	2.4
Imipramine	280	29.18	281→86, 208, 193	0.48	Clomipramine-D ₃	43.5	4.3	22.1	2.5
Ketamine	238	22.35	239→125, 220, 179	1.32	Ketamine-D ₄	35.7	6.0	33.3	2.4
MDEA ^f	207	17.41	208→163, 135	4.11	MDEA-D ₅	47.7	4.1	14.4	2.4
Methamphetamine ^f	149	16.85	150→91, 119	0.29	MDEA-D ₅	31.8	4.9	27.9	3.6
Methadone ^f	309	27.89	310→265, 105	2.00	MDEA-D ₅	27.2	4.1	16.3	4.4
Mirtazapine	265	25.45	266→195, 209, 72	0.08	SKF-525A	50.7	7.0	18.1	2.3
Norketamine ^f	224	20.35	225→207, 125	1.44	Norketamine-D ₄	40.3	4.7	13.1	2.8
Olanzapine	313	25.45	314→257, 214, 84	5.96	Clomipramine-D ₃	56.0	8.0	39.0	2.5
PMA- Para-methoxyamphetamine	165	15.92	166→149, 121, 91	2.91	MDEA-D ₅	22.6	5.7	8.2	2.4
Pregabalin	159	7.73	160→142, 55, 97	1.91	MDEA-D ₅	31.4	3.9	14.0	2.3
Quetiapine	383	25.75	384→253, 279, 221	0.10	SKF-525A	56.3	2.8	29.0	2.6
Sertraline ^f	305	29.38	306→159, 129	4.44	SKF-525A	34.4	3.5	27.4	2.9
Trazodone	372	25.45	373→176, 148, 78	1.23	SKF-525A	60.1	5.1	32.4	2.4
Venlafaxine	277	25.19	278→58, 121, 91	1.25	SKF-525A	41.6	4.4	40.1	2.4
Internal standards									
Citalopram-D ₆	330	24.87	331→109, 262, 234	—	—	56.0	5.8	36.9	2.4
Clomipramine-D ₃	318	30.58	319→89, 242, 227	—	—	49.0	3.8	26.1	2.8
Ketamine-D ₄	242	22.35	243→224, 183, 129	—	—	48.0	3.2	17.8	2.5
MDEA-D ₅ ^f	212	17.41	213→163, 135	—	—	43.5	5.3	18.9	2.5
Norketamine-D ₄ ^f	228	20.35	229→211, 129	—	—	41.0	4.5	12.6	2.5
SKF-525A	353	30.84	354→91, 167, 105	—	—	50.9	5.2	49.3	2.7

^a Rt, Retention time.

^b First transition for quantitative purposes.

^c Area ratio of the first and the second MRM.

^d IS, internal standard.

^e DP Declustering potential (first MRM transition), EP Entrance potential (first MRM transition), CE Collision energy (first MRM transition), CXP Collision cell exit potential (first MRM transition).

^f Only one MRM transition (second transition) for confirmative purposes.

Table 2
Limits of detection (LOD), limit of quantification (LOQ) and matrix effect.

Compound ^a	LOD ($\mu\text{g L}^{-1}$)	LOQ ($\mu\text{g L}^{-1}$)	Standard Calibration		Matrix-Matched calibration		Matrix effect (%)
			Slope ($A_{\text{ratio}} \mu\text{g}^{-1} \text{L}$)	R^2	Slope ($A_{\text{ratio}} \mu\text{g}^{-1} \text{L}$)	R^2	
Amitriptyline	0.037	0.12	$6.05 \cdot 10^{-4} \pm 1.20 \cdot 10^{-4}$	< 0.998	$3.90 \cdot 10^{-3} \pm 1.90 \cdot 10^{-3}$	< 0.996	98
Atropine	0.037	0.12	$9.10 \cdot 10^{-3} \pm 1.22 \cdot 10^{-3}$	< 0.997	$1.60 \cdot 10^{-1} \pm 2.89 \cdot 10^{-2}$	< 0.992	95
Buprenorphine	0.37	1.2	$1.38 \cdot 10^{-3} \pm 7.66 \cdot 10^{-4}$	< 0.996	$4.20 \cdot 10^{-3} \pm 2.27 \cdot 10^{-3}$	< 0.995	91
Clomipramine	0.37	1.2	$6.29 \cdot 10^{-3} \pm 1.11 \cdot 10^{-3}$	< 0.997	$3.00 \cdot 10^{-3} \pm 1.11 \cdot 10^{-3}$	< 0.996	100
Clozapine	0.075	0.25	$2.80 \cdot 10^{-3} \pm 1.17 \cdot 10^{-3}$	< 0.997	$2.80 \cdot 10^{-3} \pm 1.40 \cdot 10^{-3}$	< 0.994	100
Escitalopram	0.0075	0.025	$3.41 \cdot 10^{-3} \pm 8.00 \cdot 10^{-5}$	< 0.994	$4.60 \cdot 10^{-3} \pm 9.60 \cdot 10^{-5}$	< 0.995	93
Fluoxetine	0.0075	0.025	$1.52 \cdot 10^{-3} \pm 1.00 \cdot 10^{-2}$	< 1.000	$1.87 \cdot 10^{-1} \pm 5.60 \cdot 10^{-2}$	< 0.997	89
Haloperidol	0.037	0.12	$7.02 \cdot 10^{-3} \pm 1.03 \cdot 10^{-3}$	< 1.000	$7.02 \cdot 10^{-3} \pm 1.03 \cdot 10^{-3}$	< 0.995	100
Imipramine	0.0075	0.025	$5.61 \cdot 10^{-2} \pm 3.44 \cdot 10^{-3}$	< 0.996	$1.29 \pm 2.16 \cdot 10^{-1}$	< 0.998	97
Ketamine	0.0075	0.025	$5.72 \cdot 10^{-2} \pm 3.27 \cdot 10^{-3}$	< 0.997	$7.16 \cdot 10^{-2} \pm 6.67 \cdot 10^{-3}$	< 0.994	81
MDEA	0.0075	0.025	$5.60 \cdot 10^{-3} \pm 7.23 \cdot 10^{-4}$	< 0.998	$6.00 \cdot 10^{-3} \pm 9.10 \cdot 10^{-4}$	< 0.995	94
Methamphetamine	0.037	0.12	$1.81 \cdot 10^{-2} \pm 3.12 \cdot 10^{-3}$	< 0.995	$5.66 \cdot 10^{-2} \pm 7.40 \cdot 10^{-3}$	< 0.996	98
Methadone	0.0075	0.025	$2.52 \cdot 10^{-2} \pm 1.10 \cdot 10^{-3}$	< 0.994	$5.74 \cdot 10^{-3} \pm 7.11 \cdot 10^{-3}$	< 0.995	87
Mirtazapine	0.037	0.12	$5.68 \cdot 10^{-3} \pm 1.29 \cdot 10^{-4}$	< 0.998	$2.96 \cdot 10^{-1} \pm 9.61 \cdot 10^{-2}$	< 0.994	94
Norketamine	0.0075	0.025	$5.30 \cdot 10^{-3} \pm 1.65 \cdot 10^{-3}$	< 0.997	$1.47 \cdot 10^{-2} \pm 2.00 \cdot 10^{-3}$	< 0.992	84
Olanzapine	0.037	0.12	$8.00 \cdot 10^{-4} \pm 1.02 \cdot 10^{-4}$	< 0.998	$1.40 \cdot 10^{-2} \pm 7.52 \cdot 10^{-3}$	< 0.994	94
PMA- Para-methoxyamphetamine	0.37	1.2	$9.23 \cdot 10^{-3} \pm 1.16 \cdot 10^{-3}$	< 0.998	$3.65 \cdot 10^{-2} \pm 1.44 \cdot 10^{-3}$	< 0.997	99
Pregabalin	0.37	1.2	$3.78 \cdot 10^{-3} \pm 1.07 \cdot 10^{-3}$	< 0.996	$7.29 \cdot 10^{-3} \pm 2.05 \cdot 10^{-3}$	< 0.999	100
Quetiapine	0.37	1.2	$1.37 \cdot 10^{-3} \pm 7.72 \cdot 10^{-4}$	< 0.994	$2.00 \cdot 10^{-3} \pm 1.40 \cdot 10^{-3}$	< 0.998	99
Sertraline	0.37	1.2	$5.05 \cdot 10^{-3} \pm 1.12 \cdot 10^{-3}$	< 0.995	$2.45 \cdot 10^{-3} \pm 1.60 \cdot 10^{-3}$	< 0.9997	100
Trazodone	0.0075	0.025	$1.63 \cdot 10^{-2} \pm 4.03 \cdot 10^{-3}$	< 0.996	$9.39 \cdot 10^{-2} \pm 2.32 \cdot 10^{-2}$	< 0.993	99
Venlafaxine	0.0075	0.025	$2.87 \cdot 10^{-2} \pm 6.50 \cdot 10^{-3}$	< 0.997	$8.09 \cdot 10^{-2} \pm 1.86 \cdot 10^{-2}$	< 0.996	89

chromatogram for a $100 \mu\text{g L}^{-1}$ standard solution is given as Figure S1 (ESI). Finally, Figure S2 (ESI) shows the drugs' structure involved in the research together with their predicted ACD LogP (ACD/Labs Percepta Platform - PhysChem Module) [36].

3. Results and discussion

3.1. Optimization of the ultrasound assisted MASE procedure

Optimisation of the variables affecting the ultrasound (45 kHz, 120 W) assisted MASE process has implied the use of 4.0 mL of drug-free urines spiked with targets and internal standards at $2.5 \mu\text{g L}^{-1}$ and 400 μL of n-hexane, 1-butanol, diethyl ether, chloromethane, dichloromethane, and trichloromethane as extraction solvents. Each condition was tested in triplicate using daily prepared standard calibrations (methanolic standards) for measurements and for getting the analytical recoveries under each set of conditions. Two reagent blanks were prepared for each set of conditions. The extracts were evaporated to dryness under N_2 stream, and the residues were finally re-dissolved with 100 μL of methanol.

The optimisation strategy consisted of selecting initial values for the variables and optimising each of them consecutively. After the optimisation of a variable, the optimal value of this variable was used in the study of the next variable (remaining the un-optimised variables at the fixed initial values).

Finally, the MASE pieces (PP membrane, Teflon rings and conic metallic components) were cleaned by soaking in n-hexane and subjecting to ultrasounds (45 kHz and 120 W) for 10 min. The pieces were then allowed to dry at room temperature before being used.

3.1.1. Preliminary experiments

Preliminary experiments by adjusting urine samples at pHs within the 6.0–10 range led to negligible recoveries for all targets, independently of the extractant solvent (hexane, 1-butanol, diethyl ether, chloromethane, dichloromethane, and trichloromethane) used. The selection of the most suitable extraction solvent and the convenience of mechanical or ultrasound assistance was carried out by using 400 μL of the selected solvent, spiked drug-free urines at pH 12 and mechanical (orbital – horizontal) stirring (200 rpm, 15 min, non-optimized operating conditions), and ultrasound assistance (45 kHz, 120 W, 5.0 min, non-optimized operating conditions).

Solvents such as dichloromethane and diethyl ether were discarded

because they were found to be lost through the membrane. In addition, analytical recoveries lower than 15% were found for all tested solvents when using mechanical stirring, which can be a consequence of the small extraction time (15 min) used. Published methods based on MASE (mechanical stirring) have revealed the need for long shaking (extraction) times for quantitative target pre-concentration/isolation [30–34]. Assistance by ultrasound has led to moderately high analytical recoveries, mainly when using hexane as an extractant (a higher number of resolved chromatographic peaks, and hence, a higher number of extracted analytes, were obtained). Therefore, ultrasound assistance and hexane as a solvent were selected for further experiments.

3.1.2. Effect of the sample pH

The sample (urine) pH is an important factor for achieving a quantitative targets extraction because non-ionised analyte forms migrate easily from the biological matrix to the acceptor phase (organic solvent). The difficulty in choosing the pH derives from the significant differences in functional groups present in the structures of the drugs (and metabolites) under study (analytes with acidic functional groups and other structures with basic functionalities). As previously mentioned, attempts when using pHs close to neutrality (6.0, 7.0, and 8.0), and even pHs of 9.0 and 10, did not allow for the extraction of most targets. Extremely basic pHs such as 11, 12 and 13 were therefore evaluated.

The optimisation of the urine pH (11, 12, and 13) was performed by using an un-optimised extraction time of 5.0 min (other variables have been fixed as previously commented at the beginning of Section 3.1). Fig. 2(a) shows that moderate recoveries were obtained for some targets when using the highest pHs (12 and 13). Therefore, a pH of 12 was finally selected.

3.1.3. Effect of the ultrasonication (extraction) time

After fixing the urine pH at 12, the influence of the ultrasonication time (5.0, 10, 20, and 30 min) was tested. After each set of ultrasonication, the water of the tank was renewed to avoid temperature increases in the water bath. As it can be seen in Fig. 2(b), target pre-concentration was found to increase when sonicating for 10 min (higher recoveries than those observed for 5.0 min sonication). However, recovered concentrations were lower when using long extraction times, contrasting with the equilibrium mechanism of MASE. The back-diffusion phenomenon, documented in other micro-extraction techniques when using long extraction times [37,38], could be responsible of these findings. Therefore, an ultrasonication time of 10 min was finally

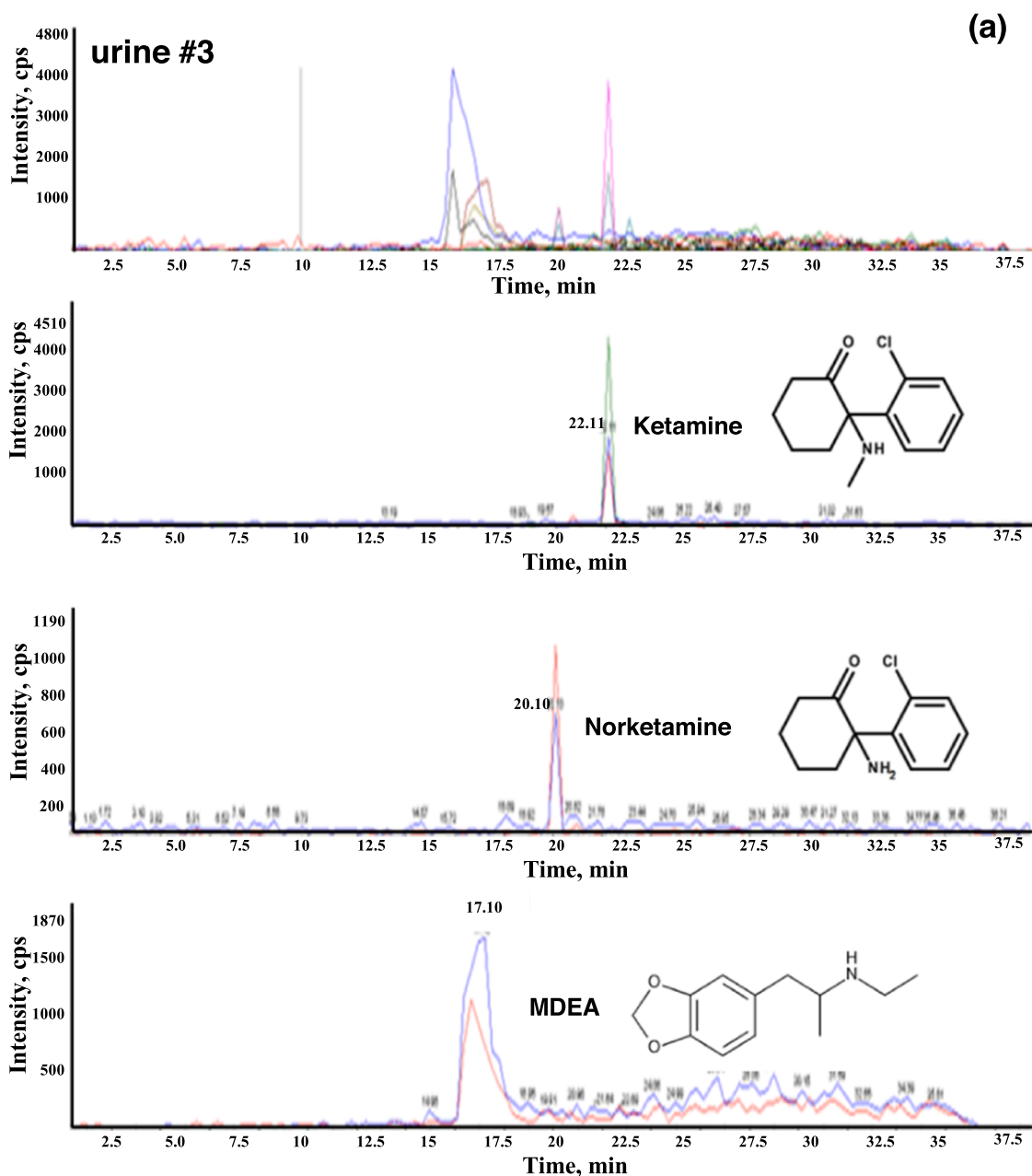


Fig. 1. MRM chromatogram for two authentic urine samples from forensic cases: sample 3 (a) and sample 7 (b).

selected.

A final experiment consisting of adding sodium fluoride at 1.0% (m/v) to urine samples before applying the optimised ultrasound assisted MASE conditions (sample pH 12, 400 μ L of hexane, and sonication at 45 kHz for 10 min) was performed. Similar analytical recoveries for the selected analytes were obtained for urine samples modified with 1.0% (m/v) and for un-modified urine samples, thus implying that the salting out effect does not change the PP membrane permeability towards the analytes.

3.2. Analytical performances

Method validation was performed in accordance with the Guidance for Industry: Bioanalytical Method Validation by the United States Food and Drug Administration (USFDA) [39].

3.2.1. Study of matrix interferences

Matrix-dependant signal suppression or enhancement characterizes a most important drawback in quantitative analysis with liquid chromatography-mass spectrometry. Therefore, the influence of matrix (matrix effect, ME) was evaluated in accordance with Truffelli et al. [40] by analysing in triplicate 100 μ g L⁻¹ standard solutions in methanol and free-drug urine sample spiked at 100 μ g L⁻¹, and applying the Eq. (1)

$$ME(\%) = \frac{B}{A} \times 100 \quad (1)$$

where A is the MS/MS peak area from quantifier ions in the methanolic standard, and B is the MS/MS peak area of the quantifier ions in spiked drug-free urine after MASE.

A ME value equal to 100% means absence of matrix effect and ME higher or lower than 100% would imply ion suppression or ion enhancement, respectively. As shown in Table 2, matrix effect was found

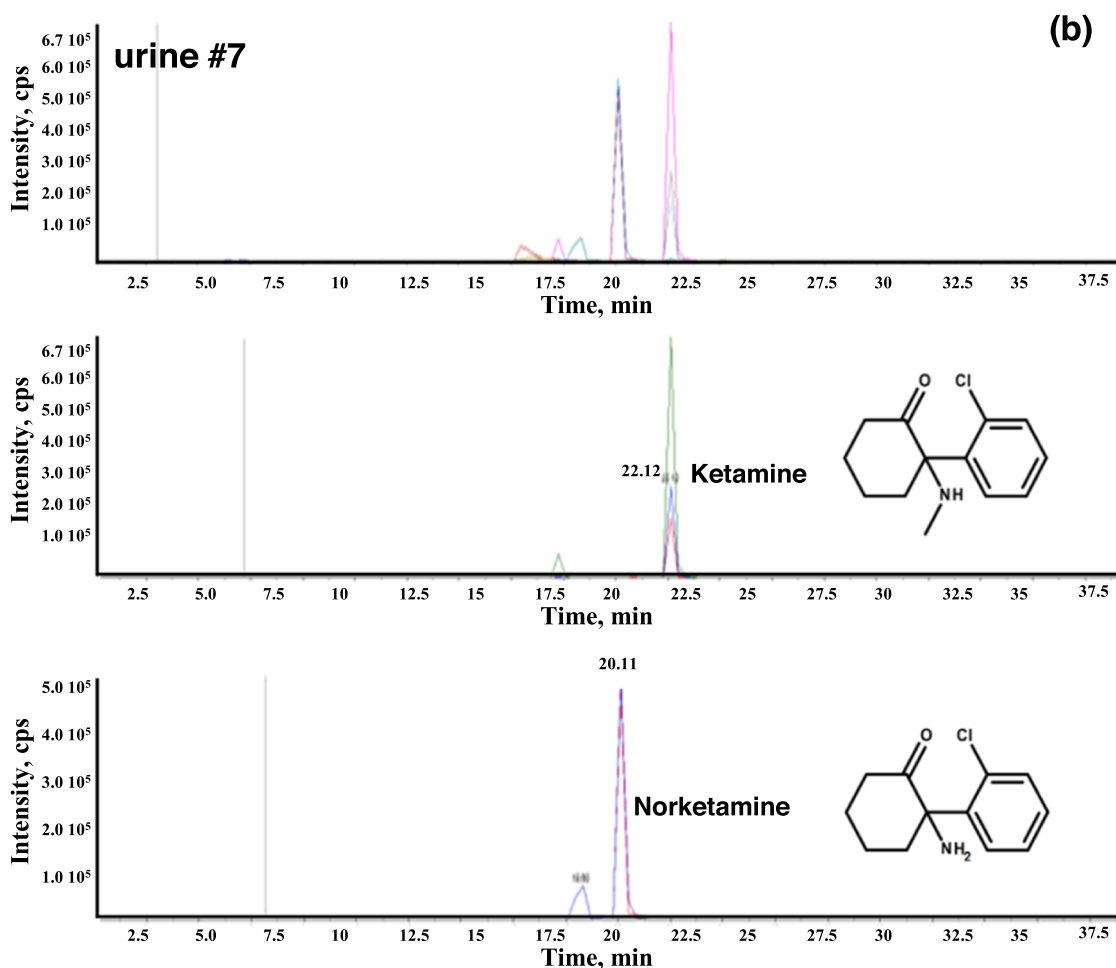


Fig. 1. (continued).

to be low for some targets, whereas atropine, buprenorphine, escitalopram, ketamine, MDEA, methadone, mirtazapine, norketamine, olanzapine and venlafaxine showed low/moderate ion suppression (ME within the 81–94% range, Table 2). Since low/moderate ME was observed in some targets, a MMC has been proposed for analysis. The matrix effect in LC-MS and LC-MS/MS has been reported for several analytes such as ketamine [10,11], synthetic cathinones [10,12,21], synthetic cannabinoids [10], amphetamines [11,12], methadone [11, 21], benzodiazepines [11], cocaine and opioids [21] in biological matrices by using sample pre-treatments such as solid-liquid extraction [10], liquid-liquid extraction [11], solid phase extraction [12], and micro-extraction by packed sorbent [21]. However, the matrix effect was found to be negligible for benzodiazepines and other psychotropic medications when using dispersive liquid-liquid micro-extraction [24]. Regarding MASE, a moderate matrix effect (lower than 50%) has been reported for alkylphenols in sediments (sample pre-treatment based on pressurized hot water extraction followed by MASE) [33].

Finally, the coefficients of determination (R^2) for SC ranged from 0.990 to 1.000; whereas R^2 were within the 0.986–1.000 range for MMC.

3.2.2. Limit of detection and limit of quantification

The assessment of the limit of detection (LOD) and the limit of quantification (LOQ) was established by spiking drug-free urines at low target concentrations and registering the peak area of the m/z (precursor ion) \rightarrow m/z (product ion) transition for each target at each retention time until after applying the ultrasound-assisted MASE procedure. The LODs and LOQs were obtained considering MS/MS signals lower than 3 and 10

times the signal-to-noise ratio, respectively. Considering the pre-concentration factor of 40 (Table 3), quite low LODs/LOQs ($0.0075/0.025 \mu\text{g L}^{-1}$) were obtained for some targets (ketamine and norketamine included), whereas low LODs (within the $0.037\text{--}0.37 \mu\text{g L}^{-1}$ range) and LOQs (within the $0.12\text{--}1.2 \mu\text{g L}^{-1}$ range) were assessed for the remaining drugs.

3.2.3. Selectivity

Selectivity was studied by subjecting in triplicate six different drug-free urine samples (blanks) to the ultrasound assisted MASE procedure and analysing the extracts by LC-MS/MS. No signals from potential interferences were detected at the retention times and selected precursor ion \rightarrow product ion transitions for each analyte. In addition, only negligible contribution of chemicals used in sample preparation and in the chromatographic separation has been found. These results confirm the good selectivity of the method for the targets and that there was no interference from urine constituents.

3.2.4. Precision and accuracy

Intra-day assays (intra-day precision or repeatability, and intra-day analytical recovery) were established by spiking several aliquots of a drug-free urine sample at three or two DFSA drugs concentration levels depending on targets sensitivity (Table 3) which were subjected to the ultrasound assisted MASE procedure (each spiked concentration was replicated seven times in the same day). Similarly, inter-day assays (inter-day precision or reproducibility, and inter-day analytical recovery) were assessed by spiking drug-free urine samples at six, five or three DFSA drugs concentration levels, also depending on targets sensitivity

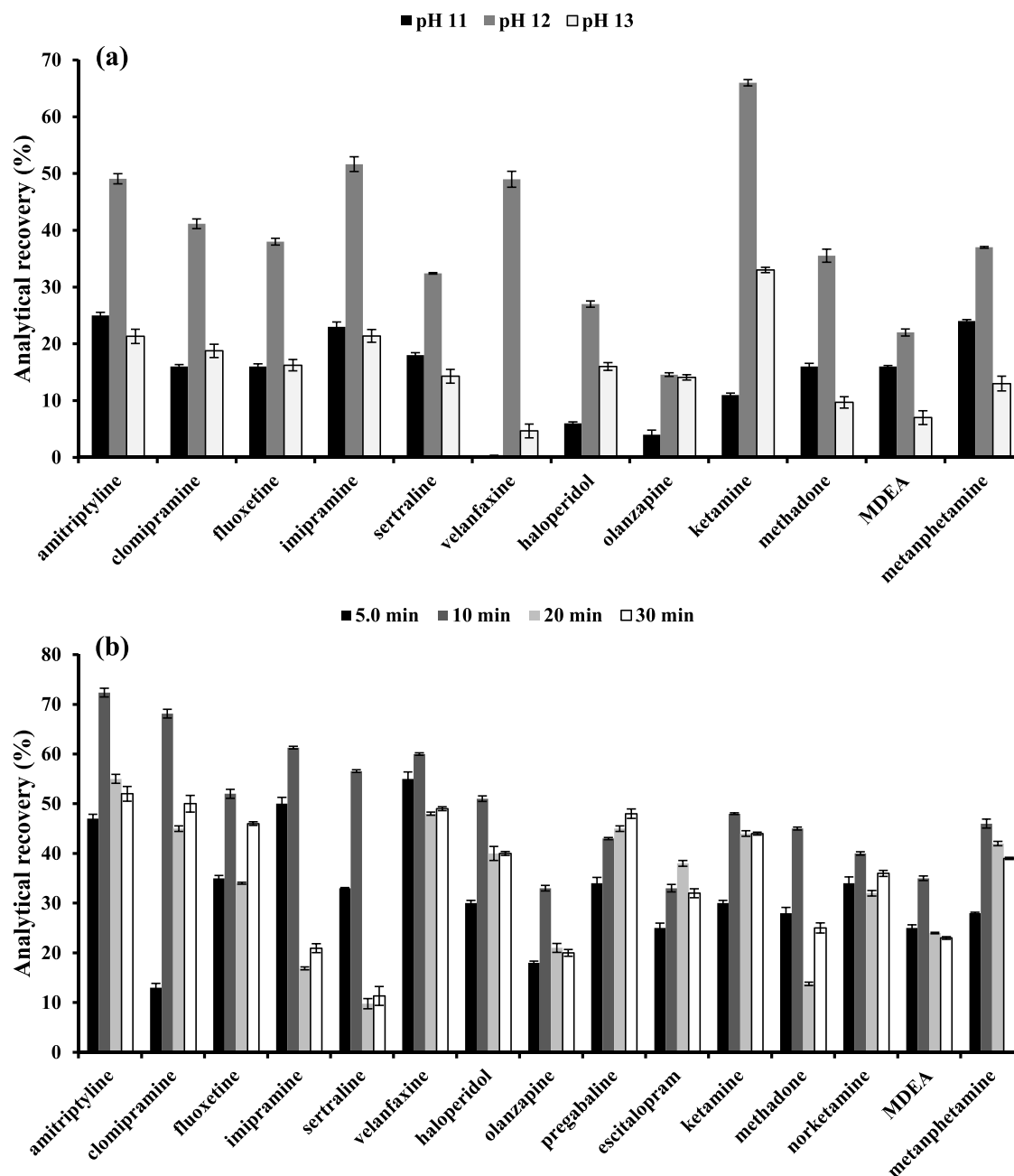


Fig. 2. Effect of the urine sample pH (a) and the ultrasonication (extraction) time (b) on the extraction efficiency (analytical recovery) of DFSA drugs from urine.

(Table 3), and by subjecting each one to the ultrasound assisted MASE procedure in duplicate and repeating the experiment in seven different days.

Results listed in Table 4 show intra-day precision (RSD values) within the 2–15% range, whereas, the inter-day precision varied from 2 to 20%. Intra-day and inter-day analytical recovery were found to be higher than 80% for most of DFSA drugs concentrations, although analytical recoveries lower than 80% were obtained when testing the lowest concentration levels ($0.025 \mu\text{g L}^{-1}$) for escitalopram, imipramine, MDEA, and methadone. In addition, intra-day and inter-day analytical recoveries for olanzapine were 77 ± 10 and $72 \pm 13\%$, respectively, at the lowest concentration tested ($1.25 \mu\text{g L}^{-1}$).

3.3. Application

Ten authentic urine samples from forensic cases were analysed in

triplicate by the proposed ultrasound assisted MASE procedure combined with LC-MS-MS detection. The presence of the drugs/metabolites was assessed by monitoring the most sensitive $m/z \rightarrow m/z$ transition (quantitative purposes) and the less sensitive $m/z \rightarrow m/z$ transitions (two MRM transitions or at least one MRM transition for analytes with few sensitive MRM transitions) for confirmative purposes. In addition, the peak area ratio of the first and second $m/z \rightarrow m/z$ transitions was also recorded and compared to those obtained for standards. Under this assumption, all samples, except for the sample coded as 9, were found to contain some of the drugs (Table 4). Ketamine (and the metabolite norketamine) was present in all positive samples and reached concentrations higher than $1000 \mu\text{g L}^{-1}$ in some cases. In addition to ketamine, two samples were found to contain MDEA, another two samples contained methamphetamine, two other samples contained pregabalin, and one sample contained atropine. Other drugs (amitriptyline, buprenorphine, clomipramine, clozapine, escitalopram, fluoxetine, haloperidol,

Table 3
Intraday and inter-day precision and analytical recovery of the method.

Compound	Concentration ($\mu\text{g L}^{-1}$)	Intra-day		Inter-day	
		Analytical recovery (%)	RSD (%)	Analytical recovery (%)	RSD (%)
Amitriptyline	0.125	90±10	11	101±15	16
	0.250	-	-	89±6	8
	1.25	93±3	3	93±3	4
	2.5	-	-	82±3	5
	5.0	90±6	7	90±8	9
Atropine	0.125	88±7	6	103±9	15
	0.250	-	-	89±13	9
	1.25	90±8	9	85±7	5
	2.5	-	-	87±2	11
	5.0	92±6	9	94±17	10
Buprenorphine	1.25	81±7	7	79±8	7
	2.5	-	-	85±11	10
	5.0	91±4	8	87±15	7
Clomipramine	1.25	80±13	12	94±12	19
	2.5	-	-	101±11	16
Clozapine	5.0	88±7	7	90±11	17
	0.250	93±11	10	91±19	20
	1.25	88±8	8	91±15	17
Escitalopram	2.5	-	-	88±8	5
	5.0	90±7	6	84±5	9
	0.025	76±5	8	75±4	6
	0.125	-	-	86±8	18
	0.250	-	-	92±10	11
Fluoxetine	1.25	89±1	2	94±17	19
	2.5	-	-	100±5	7
	5.0	82±1	2	89±6	7
	0.025	92±11	13	89±18	6
	0.125	-	-	93±16	12
Haloperidol	0.250	-	-	82±8	2
	1.25	84±15	12	97±14	4
	2.5	-	-	82±15	11
	5.0	90±7	14	86±12	5
	0.125	85±12	11	92±17	11
Imipramine	0.250	-	-	108±11	15
	1.25	81±12	12	90±16	18
	2.5	-	-	80±18	8
	5.0	80±13	4	95±17	10
	0.025	77±7	8	84±10	8
Ketamine	0.125	-	-	95±15	8
	0.250	-	-	93±8	4
	1.25	91±15	8	93±8	10
	2.5	-	-	99±11	11
	5.0	87±7	7	83±12	5
MDEA	0.025	101±13	12	86±9	29
	0.125	-	-	100±16	17
	0.250	-	-	80±8	11
	1.25	88±11	13	116±8	8
	2.5	-	-	99±10	12
Methamphetamine	5.0	85±10	12	91±5	6
	0.025	77±8	10	76±6	7
	0.125	-	-	101±3	3
	0.250	-	-	80±1	2
	1.25	80±9	8	95±9	11
Methadone	2.5	-	-	86±8	11
	5.0	99±7	3	96±10	11
	0.125	93±9	9	98±3	5
	0.250	-	-	95±5	7
	1.25	87±7	8	95±6	7
Mirtazapine	2.5	-	-	94±3	4
	5.0	86±7	9	96±6	6
	0.025	73±16	14	88±11	16
	0.125	-	-	89±11	14
	0.250	-	-	78±12	8
Norketamine	1.25	82±18	12	90±18	15
	2.5	-	-	84±17	9
	5.0	90±8	11	88±9	7
	0.125	86±8	9	82±14	9
	0.250	-	-	80±17	9
Norquetamine	1.25	93±10	10	79±10	3
	2.5	-	-	91±7	10
	5.0	96±8	13	91±10	5
	0.025	102±11	11	88±8	10
	0.125	-	-	95±4	5

(continued on next page)

Table 3 (continued)

Compound	Concentration ($\mu\text{g L}^{-1}$)	Intra-day		Inter-day	
		Analytical recovery (%)	RSD (%)	Analytical recovery (%)	RSD (%)
Olanzapine	0.250	-	-	99±3	4
	1.25	88±4	5	93±6	8
	2.5	-	-	99±7	8
	5.0	94±10	11	92±6	7
	0.125	77±10	10	72±13	13
	0.250	-	-	100±17	8
PMA	1.25	91±9	9	95±12	15
	2.5	-	-	91±15	2
	5.0	93±8	8	103±14	2
	1.25	92±5	7	90±10	12
	2.5	-	-	95±10	8
Pregabalin	5.0	90±11	10	93±7	8
	1.25	89±11	10	93±12	12
	2.5	-	-	89±8	9
Quetiapine	5.0	99±9	5	89±7	8
	1.25	84±13	9	81±16	10
	2.5	-	-	94±15	8
Sertraline	5.0	91±4	5	93±6	5
	1.25	94±8	7	94±10	12
	2.5	-	-	96±7	9
Trazodone	5.0	96±6	7	99±16	5
	0.025	95±12	13	87±18	17
	0.125	-	-	91±15	14
	0.250	-	-	95±6	6
	1.25	86±7	8	95±8	5
Venlafaxine	2.5	-	-	96±4	3
	5.0	104±6	6	96±7	5
	0.025	84±10	11	95±13	14
	0.125	-	-	88±9	11
	0.250	-	-	93±5	8
	1.25	90±7	8	100±7	9
	2.5	-	-	98±6	7
5.0	91±4	6	101±4	7	

Table 4

Found DFSAs concentrations in forensic urine samples (concentrations as mean \pm standard deviation of three replicates).

	Concentration expressed as $\mu\text{g L}^{-1}$					
	Atropine	Ketamine	MDEA	Methamphetamine	Norketamine	Pregabalin
Urine 1	—	1183±380			785±21	
Urine 2		28.2 \pm 9.9	12.2 \pm 0.35			94.9 \pm 4.62
Urine 3		45.0 \pm 7.8	16.9 \pm 0.12		40.7 \pm 4.4	
Urine 4						48.9 \pm 2.86
Urine 5		12.6 \pm 3.6		10.3 \pm 1.2		
Urine 6	12.62±1.34	104±10		8.53±0.45	14.2 \pm 0.98	
Urine 7		10,441±1868			7285±954	
Urine 8		13.8 \pm 1.4				
Urine 10		200±2		81.8 \pm 0.23	80.8 \pm 1.79	

imipramine, methadone, mirtazapine, olanzapine, PMA, quetiapine, sertraline, trazodone, and venlafaxine) were not detected in any urine sample.

4. Conclusions

Ultrasound assistance has been found to be useful for speeding-up the MASE process, allowing for fast and simultaneous isolation/pre-concentration of twenty-two DFSA drugs from urine. The developed sample pre-treatment is simple, highly efficient and environmentally friendly since organic solvent consumption is reduced. DFSA drugs migration towards the acceptor phase has been found to require extreme alkaline pHs (12) and short ultrasonication times (10 min). However, LC-MS/MS measurements have shown important signal suppressions for all targets, and a matrix matched calibration was required for avoiding/minimizing the matrix effect. Ketamine has been found as the most common DFSA drug in the authentic urine samples from forensic cases, followed by amphetamines (MDEA and methamphetamine).

CRedit authorship contribution statement

Ana Justo-Vega: Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Kamal K. Jinadasa:** Formal analysis, Investigation, Software, Writing – original draft. **G.D. Thilini Madurangika Jayasinghe:** Formal analysis, Investigation. **Iván Álvarez-Freire:** Data curation, Supervision, Validation. **Ana María Bermejo-Supervision, Validation, Writing – review & editing.** **Pilar Bermejo-Barrera:** Resources, Project administration, Funding acquisition. **Antonio Moreda-Piñero:** Conceptualization, Methodology, Software, Validation, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.chroma.2023.464284](https://doi.org/10.1016/j.chroma.2023.464284).

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