UNIVERSIDAD
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CÓRDOBA

TESIS DOCTORAL

Programa de Doctorado en Biomedicina

Análisis toxicológico de drogas farmacéuticas e ilícitas en agresiones sexuales facilitadas por drogas por LC-MS/MS usando biodisolventes supramoleculares

Toxicological analysis of pharmaceutical and illicit drugs in drug-facilitated sexual assault by LC-MS/MS using supramolecular biosolvents

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Córdoba, septiembre 2023

TITULO: *Toxicological analysis of pharmaceutical and illicit drugs in drug-facilitated sexual assault by LC-MS/MS using supramolecular biosolvents*

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Tesis Doctora

Análisis toxicológico de drogas farmacéuticas e ilícitas en agresiones sexuales facilitadas por drogas por LC-MS/MS usando biodisolventes supramoleculares

Toxicological analysis of pharmaceutical and illicit drugs in drug-facilitated sexual assault by LC-MS/MS using supramolecular biosolvents

Trabajo presentado, para optar al grado de Doctor, por

Nouman Monzer Almofti

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Que la citada Tesis Doctoral, titulada Análisis toxicológico de drogas farmacéuticas e ilícitas en agresiones sexuales facilitadas por drogas por LC-MS/MS usando biodisolventes supramoleculares, se ha realizado en los laboratorios del Departamento de Química Analítica de la Universidad de Córdoba y que, a su juicio, reúne todos los requisitos exigidos a este tipo de trabajos.

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TÍTULO DE LA TESIS: Análisis toxicológico de drogas farmacéuticas e ilícitas en agresiones sexuales facilitadas por drogas por LC-MS/MS usando biodisolventes supramoleculares.

DOCTORANDO: Nouman Monzer Almofti.

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

La Tesis presentada por el doctorando Nouman Almofti se inició a mediados del año 2020, con dificultades al comienzo debido a un retraso en su incorporación por las restricciones a los viajes internacionales y entrada de viajeros a España a causa del confinamiento durante la pandemia de COVID-19. Desde entonces, realizó una revisión crítica de los métodos analíticos previamente publicados para la determinación de drogas implicadas en delitos de sumisión química, y con posterioridad ha trabajado arduamente en el laboratorio durante la fase experimental, poniendo a punto la técnica para optimizar del proceso de extracción y análisis de los tóxicos más comúnmente utilizados en las agresiones sexuales facilitadas por drogas utilizando distintos disolventes supramoleculares y LC-MS/MS. Nouman Almofti ha demostrado un gran interés por el trabajo de investigación y una completa dedicación al mismo, habiendo obtenido resultados muy satisfactorios, que se corresponden fielmente con los que se presentan en esta memoria de Tesis, y que han podido publicarse en revistas de alto impacto. La Tesis se presenta en la modalidad de compendio de artículos, siendo estos los siguientes:

- Almofti N, Ballesteros-Gómez A, Rubio S, Girela-López E. Analysis of conventional and nonconventional forensic specimens in drug-facilitated sexual assault by liquid chromatography and tandem mass spectrometry. Talanta 250 (2022) 123713 https://doi.org/10.1016/j.talanta.2022.123713, Indexada en JCR (JIF 6,1; D1).

- Almofti N, González-Rubio S, Ballesteros-Gómez A, Girela E, Rubio S. Green nanostructured liquids for the analysis of urine in drug-facilitated sexual assault cases. Analytical and Bioanalytical Chemistry (2023) 415:2025–2035 https://doi.org/10.1007/s00216-022-04358-z, Indexada en JCR (JIF 4,3; Q1).
- Almofti N, Ballesteros-Gómez A, Girela E, Rubio S. Hair analysis of selected drug-facilitated sexual assault substances using green supramolecular solvent extraction and LC-MS/MS analysis. Microchemical Journal (2023) Microchemical J, p. 109144, 2023, https://doi.org/10.1016/j.microc.2023.109144, Indexada en JCR (JIF 4,8; Q1).

Por todo lo anterior, consideramos al doctorando merecedor para aspirar a la obtención del grado de doctor y consecuentemente emitimos informe favorable.

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REPORT ON THE QUALITY INDICATORS OF THE THESIS

Below are the publications that are provided as evidence of the quality of this Doctoral Thesis.

1. **Analysis of conventional and nonconventional forensic specimens in drug facilitated sexual assault by liquid chromatography and tandem mass spectrometry.**
   - Authors: Nouman Almofti, Ana Ballesteros-Gómez, Soledad Rubio, Eloy Girela López.
   - Journal: Talanta 250 (2022) 123713.
   - Impact factor: 6.1 (JCR 2022).
   - Subject area: Analytical Chemistry.
   - Journal category: 9/86 (Q1).

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2. **Green nanostructured liquids for the analysis of urine in drug-facilitated sexual assault cases.**
   - Authors: Nouman Almofti, Soledad González-Rubio, Ana Ballesteros-Gómez, Eloy Girela López, Soledad Rubio.
   - Journal: Analytical and Bioanalytical Chemistry (2022).
   - Impact factor: 4.3 (JCR 2022).
   - Subject area: Analytical Chemistry.
   - Journal category: 20/86 (Q1).

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3. **Hair analysis of selected drug-facilitated sexual assault substances using green supramolecular solvent extraction and LC-MS/MS analysis.**
   - Authors: Nouman Almofti, Ana Ballesteros-Gómez, Eloy Girela López, Soledad Rubio.
   - Journal: Microchemical Journal (2023).
   - Impact factor: 4.8 (JCR 2022).
   - Subject area: Analytical Chemistry.
   - Journal category: 17/86 (Q1).

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I want to dedicate my entire work to my family, who raised me with all the love, wisdom, and knowledge and who have always encouraged me to pursue advanced degrees. My father, Monzer, may his soul rest in peace, who supported my every move and achievement in life, and without him, I would have achieved nothing. My dear Mom, Souha, whose care and love are the source of my strength and persistence in life. My dear sister, Lama, and brother, Mohammed, and his wife, Roula, for the unlimited love and support they have showered upon me during this period.

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ABBREVIATIONS

- ✧ 4-MEC — 4-methylethcathinone.
- ✧ ACN — Acetonitrile.
- ✧ ATS — Amphetamine-type-stimulants.
- ✧ BAC — Blood alcohol concentration.
- ✧ BMIm-PF_6 — 1-Butyl-3-methylimidazolium hexafluorophosphate.
- ✧ BrAC — Breath alcohol concentration.
- ✧ CE — Collision energy.
- ✧ CNS — Central nervous system.
- ✧ CXP — Collision cell exit potential.
- ✧ DFC — Drug-facilitated crime.
- ✧ DFSA — Drug-facilitated sexual assault.
- ✧ DLLME — Dispersive liquid-liquid microextraction.
- ✧ DP — Declustering potential.
- ✧ EME — Electromembrane extraction.
- ✧ GAC — Green analytical chemistry.
- ✧ GC-MS — Gas chromatography-mass spectrometry.
- ✧ GHB — Gamma-hydroxybutyric acid.
- ✧ IL-DLLME — Ionic liquid-dispersive liquid-liquid microextraction.
- ✧ IS — Internal standard.
- ✧ LC-MS/MS — Liquid chromatography coupled to tandem mass spectrometry.
- ✧ LLE — Liquid-liquid extraction.
- ✧ LOD — Limit of detection.
- ✧ LOQ — Limit of quantification.
- ✧ LYS — Lysergic acid diethylamide.
- ✧ *m/z* — Mass-to-charge ratio.
- ✧ MDA — 3,4-Methylenedioxy-amphetamine
- ✧ MDEA — 3,4-Methylenedioxy-N-ethylamphetamine.
- ✧ MDMA — 3,4-Methylenedioxy-methamphetamine (ecstasy).
- ✧ MDPV — Methylenedioxypyrovalerone.
- ✧ MeOH — Methanol.
- ✧ MIPs — Molecularly imprinted polymers.
- ✧ MLOD — Method limit of detection.

- ✧ MLOQ — Method limit of quantification.
- ✧ MPA — Methiopropamine.
- ✧ MRPL — Minimum required performance limits.
- ✧ NIDA — US National Institute on Drug Abuse.
- ✧ OTC — Over-the-counter.
- ✧ PMA — *Para*-methoxyamphetamine.
- ✧ PMMA — *Para*-methoxymethylamphetamine.
- ✧ *q.d.* — once a day.
- ✧ QuEChERS — Quick, Easy, Cheap, Effective, Rugged and Safe.
- ✧ Rpm — Revolutions per minute.
- ✧ RSD — Relative standard deviation.
- ✧ RT — Retention time.
- ✧ SD — Standard deviation.
- ✧ SLM — Supported liquid membrane.
- ✧ SoHT — Society of Hair Testing.
- ✧ SPE — Solid-phase extraction.
- ✧ SSRI — Selective serotonin reuptake inhibitor.
- ✧ SUPRAS — Supramolecular solvent.
- ✧ SXC — Strong cation exchange.
- ✧ THC — Tetrahydrocannabinol
- ✧ THC-COOH — 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol.
- ✧ THF — Tetrahydrofuran.
- ✧ UNODC — United Nations Office on Drugs and Crime.
- ✧ WCX — Weak cation exchange.

ABSTRACT

The research carried out in this doctoral thesis focused on the development of an innovative and eco-friendly extraction method for the qualification and quantification of multi-class drugs involved in drug-facilitated sexual assaults (DFSA) from several biological matrices using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The extraction method was based on the use of supramolecular solvents (SUPRASs) and the target compounds included benzodiazepines, z-hypnotic drugs, amphetamine derivatives, cocaine and its metabolites and other miscellaneous compounds that are frequently used in DFSA. The developed green extraction method was applied to two biological matrices: liquid biological sample (urine) in chapter II and a solid biological sample (hair) in chapter III.

In the Introduction, a general overview on the DFSA cases and involved compounds was reported, then the principle of green chemistry and the available extraction techniques used in forensic toxicology filed, and finally, the need of developing extraction method that allows the green analysis using SUPRASs was discussed.

The results and discussion section starts with a review article (***Chapter I***) discussing the analysis of conventional and nonconventional forensic specimens in DFSA by LC-MS/MS. This review article provides a critical illustration about the DFSA in the last 10 years including the most common drugs reported in literature from different countries around the world and their prevalence. The review concluded that alcohol alone or in combination with a variety of pharmaceutical and illegal drugs (e.g., benzodiazepines, z-hypnotics drugs, cannabinoids, or cocaine) is still on top in the DFSA cases reported around the world. Moreover, the article reviewed the selection requirement of the appropriate biological samples and the increased use of the non-conventional samples (hair, vitreous humor, etc.) in such investigations. It is pointed out in the review that although liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are the most often used extraction procedures, there is a growing interest in the development of microextraction formats and dilute and shoot tactics. Although GC-MS is still employed in forensic routine analysis, LC-MS/MS (both low and high-resolution MS) has been established as a more ideal alternative for multiple drug

analysis due to its high sensitivity, specificity, and lack of the necessity for derivatization.

Chapter II was performed to develop an innovative, green, and effective technique for extracting specific DFSA compounds from human urine using SUPRASs. The method has been optimized and validated for the extraction of 23 targeted-DFSA substances. The SUPRAS extraction process, which involves stirring, centrifugation, and dilution, is simple, quick, and environmentally friendly. The extracted SUPRAS samples are then analyzed using LC-MS/MS. The study demonstrates that the proposed extraction method has a high extraction efficiency ranging from 79% to 119% for many target compounds. Matrix effects are found to be tolerable for most compounds. Additionally, the method achieves low detection and quantification limits, surpassing the minimal performance requirements for these compounds. These findings indicate that the SUPRAS-based extraction technique holds great promise for monitoring illicit substances in DFSA cases within forensic laboratories.

After investigating the SUPRAS extraction technique to liquid biological samples (urine), the efficiency of SUPRAS method was planned to be studied on solid biological samples (hair). For this reason, the third section (***Chapter III***) discusses the development and validation of a single-step extraction method for analysing DFSA compounds in hair samples. Overall, the SUPRAS-based method offers several advantages, including its green and eco-friendly nature, simplified extraction process, increased sensitivity, broad compound coverage, high extraction efficiency, and practical applicability in real forensic sexual assault cases.

At the end of the thesis, the most relevant conclusions of the chapters are pointed out in the Conclusion section. Finally, in the Annexes, the informed consent for extraction, use and storage of biological samples, as well as the communications presented at scientific conferences, are included.

RESUMEN

La investigación llevada a cabo en esta tesis doctoral se centró en el desarrollo de un método de extracción innovador y respetuoso con el medio ambiente para la identificación y cuantificación de fármacos de múltiples clases involucrados en agresiones sexuales facilitadas por drogas (DFSA) a partir de diversas matrices biológicas, utilizando cromatografía líquida-espectrometría de masas en tándem (LC-MS/MS). El método de extracción se basó en el uso de disolventes supramoleculares (*supramolecular solvents, SUPRASs*) y las drogas seleccionadas incluyeron benzodiacepinas, fármacos hipnóticos tipo Z, derivados de anfetaminas, cocaína y sus metabolitos, y otros compuestos diversos que se utilizan con frecuencia en casos de DFSA. El método de extracción desarrollado se aplicó a dos matrices biológicas: muestra biológica líquida (orina) en el capítulo II y muestra biológica sólida (pelo) en el capítulo III.

En la Introducción se presenta una visión general de los casos de DFSA y los compuestos involucrados, a continuación, se aborda el principio de la química verde y las técnicas de extracción disponibles utilizadas en el campo de la toxicología forense, y finalmente se discute la necesidad de desarrollar métodos de extracción más sostenibles.

La sección de resultados y discusión comienza con un artículo de revisión (***Capítulo I***), que trata sobre el análisis de muestras forenses convencionales y no convencionales en casos de DFSA mediante LC-MS/MS. Este artículo de revisión proporciona una revisión crítica sobre los casos de DFSA en los últimos 10 años, incluyendo los fármacos más comunes reportados en la literatura de diferentes países de todo el mundo y su prevalencia. La revisión concluyó que el alcohol solo o en combinación con una variedad de fármacos farmacéuticos e ilegales (por ejemplo, benzodiacepinas, fármacos hipnóticos tipo Z, cannabinoides o cocaína) sigue siendo el más común en los casos de DFSA reportados en todo el mundo. Además, el artículo revisó los requisitos de selección de la muestra biológica apropiada en DFSA y el aumento en el uso de muestras no convencionales (pelo, humor vítreo, etc.) en tales investigaciones. Se señala en la revisión que, aunque la extracción líquido-líquido (LLE) y la extracción en fase sólida (SPE) son los procedimientos de extracción más utilizados, existe un creciente interés en el desarrollo de formatos de microextracción y tácticas

de dilución y análisis directo. Aunque la GC-MS todavía se utiliza en el análisis forense de rutina, la LC-MS/MS (tanto en MS de baja como de alta resolución) se ha establecido como una alternativa más ideal para el análisis de múltiples fármacos debido a su alta sensibilidad, especificidad y la falta de necesidad de derivatización.

En el ***Capítulo II*** se desarrolló una técnica innovadora, ecológica y efectiva para extraer compuestos específicos de DFSA de la orina humana utilizando SUPRASs. El método ha sido optimizado y validado para la extracción de 23 sustancias específicas de DFSA. El proceso de extracción con SUPRASs, que incluye agitación, centrifugación y dilución, es simple, rápido y respetuoso con el medio ambiente. Las muestras de SUPRAS extraídas se analizan directamente utilizando LC-MS/MS. El estudio demuestra que el método de extracción propuesto tiene una alta eficiencia de extracción que oscila entre el 79% y el 119% para muchos compuestos objetivo. Los efectos de matriz se consideran tolerables para la mayoría de los compuestos. Además, el método alcanza límites de detección y cuantificación bajos, superando los requisitos mínimos de rendimiento para estos compuestos. Estos hallazgos indican que la técnica de extracción basada en SUPRAS es eficaz y respetuosa con el medio ambiente y tiene un gran potencial para detectar y cuantificar sustancias ilícitas en casos de DFSA en laboratorios forenses.

Después de investigar el uso de la técnica de extracción SUPRAS en muestras biológicas líquidas (orina), se planeó estudiar la eficiencia del método SUPRAS en muestras biológicas sólidas (pelo). Por esta razón, la tercera sección (***Capítulo III***) trata sobre el desarrollo y la validación de un método de extracción para analizar compuestos de DFSA en muestras de pelo que consta de una única etapa. En general, el método basado en SUPRAS ofrece varias ventajas, incluyendo su naturaleza ecológica, proceso de extracción simplificado, mayor sensibilidad, amplia cobertura de compuestos, alta eficiencia de extracción y aplicabilidad en casos forenses reales de agresión sexual.

Al final de la tesis, se destacan las conclusiones más relevantes de las investigaciones presentadas en los diferentes capítulos en la sección de Conclusiones. Finalmente, en los anexos, se incluyen el consentimiento informado para la extracción,

uso y almacenamiento de muestras biológicas, así como las comunicaciones presentadas en conferencias científicas.

OBJECTIVE

The use of different chemical substances in drug-facilitated crimes DFC has gained greater prominence in recent years due to its association with sexual attacks, robbery, and other criminal actions. The term drug-facilitated sexual assaults (DFSA) or chemical submission consists of the surreptitious administration of psychoactive substances for criminal purposes in which the victim being able to accept situations that he would have considered intolerable in a normal state of consciousness. The substances commonly used have a series of characteristics that make them suitable for the purpose targeted by the attacker because they are easy to obtain, active at low doses, fast-acting, and have relatively short half-lives in the victim's body. The administration to the victim is discreet, usually orally, and added to alcoholic beverages, which are the ideal vehicle because they mask the flavour and colour, while enhancing the effects.

The chemical substances are difficult to detect in the body of the victim and produce unclear symptoms, which can lead to confusing the symptom with alcohol poisoning or some organic disorder that confuses the clinician and delays the diagnosis while the substance is cleared from the body. All these factors, together with the delay in reporting the incident to the responsible authorities because of several factors (social, religious, and fear of guilt), the delay in collecting biological samples, the absence of a complaint in some cases, the inadequate selection of the sample to be taken, or the lack of routine protocols in the laboratory for their toxicological screening means that the result of the analysis is often negative and there is an underestimation of the sexual attack phenomenon.

The traditional available extraction methods that are being used in forensic laboratories worldwide to extract the targeted DFSA from different biological samples are highly effective methods that provide accurate and reliable results; however, the high consumption of organic solvent and the multi-step extraction processes restrain their uses. Nowadays, the appearance of the orientation towards green chemistry in the scientific research field is increasing day by day to keep pace with the requirements of sustainability and green chemistry aspects. One main aspect is in the field of daily scientific research, which is related to the reduction in organic solvent consumption in the daily and routine analysis.

For all these reasons, the **main objective** of this thesis was to develop a generic sample treatment platform, based on the use of supramolecular solvents, for the simultaneous and efficient extraction of substances habitually involved in the crimes of chemical submission that can be applied to some of the biological matrices of interest (urine and hair). This sample treatment platform was combined with chromatography coupled to tandem mass spectrometry (LC-MS-MS) analysis, understanding that with the current methodology these toxicology analyses are not satisfactorily resolved.

The **specific objectives** of this thesis were:

1. To critically review the bibliographic antecedents previously reported for the determination of DFSA drugs in the matrices of interest.

2. To develop a green sample methodology for the extraction of DFSA substances in human urine and hair based on supramolecular solvents made up of water soluble and insoluble alkanediols.

3. To optimize and validate a SUPRAS-LC-MS-MS analytical approach for the determination of DFSA substances in human urine and hair according to the guidelines applied in forensic analysis.

4. To prove the reliability of the developed methodologies in DFSA investigation crimes by the analysis of authentic urine and hair samples from human volunteers.

The **final aim** of the research here presented was to study the potential of green supramolecular solvents to both simplify different biological samples treatment steps and to remove organic solvents consumption (based on green chemistry recommendations) in the determination of substances involved in drug-facilitated sexual assault cases.

INTRODUCTION

1. Drug-Facilitated sexual assault (DFSA)

1.1. General overview.

Sexual assault is a serious and widespread issue that affects countless individuals worldwide. Drug-facilitated crime (DFC) is a broad term that encompasses a variety of acts and violences such as sexual assault, robbery, kidnapped, done while the victim is under the influence of different psychotropic chemicals, and purposeful abuse of vulnerable persons such as the elderly or children [1]. In recent years, there has been growing concern about a specific form of sexual assault known as drug-facilitated sexual assault (DFSA). DFSA entail the use of drugs to impair a person's behaviour, perception, or decision-making abilities to commit illegal activities. It also includes taking advantage of those who have willingly absorbed incapacitating drugs without their knowledge. This state of incapacitation renders the victims unable to resist or provide consent to such assaults. Sexual assault victims are often teen adolescents and young adults and locations are related to leisure places such as nightclubs, social parties, rave clubs, and bars [2]. While the use of drugs for criminal purposes has persisted throughout history, there has been a significant increase in global reports of DFC in recent years [3].

The United Nations Office on Drugs and Crime (UNODC) categorizes drug-facilitated sexual assault (DFSA) crimes as a subset of drug-facilitated crimes [4]. DFSA refers to any crime in which the individual, regardless of gender, experiences a sexual activity while the person is incapacitated or unconscious due to the consumption of alcohol and/or other intoxicating agents (pharmaceutical substances and/or illegal drugs). In certain European countries like, Spain and France, the term DFSA is sometimes used interchangeably with chemical submission [5]. The term “date-rape drugs” was first mentioned in scientific community back in 1982, in an article entitled “Date rape: A campus epidemic” and since then it started to widespread in the media and the scientific community [6]. Although the phrase "date rape" is commonly used, it is erroneous because DFSA may occur at any time and in a variety of contexts or locations.

The United State Department of Justice concluded that approximately 44% of all sexual assaults are perceived to happen under the influence of various drugs and/or alcohol [7]. Furthermore, a recent interesting article discussing the occurrence of alleged drug-facilitated sexual assault in the province of Cordoba, Spain, has drawn the conclusion that among all the documented instances of sexual aggression investigated within the city from 2016 to 2021, only 28% (69 out of 247 cases) were categorized as real DFSA cases. The calculated prevalence was determined to be 1 in 10,000 inhabitants, which is lower than the actual prevalence due to delays in accessing emergency care, collecting samples, and underreporting. Additionally, the research revealed that only 87% of DFSA victims had blood or urine samples taken, with 42% of them yielding positive toxicological results [8]. However, several studies suggested that knowing the exact number of DFSA cases that occur in a country is nearly impossible due to the low reporting rate by the victims due to the difficulties in remembering what happened with them. More reasons for the low reporting rate may be summarized in the social shame, cultural and religious incorrect beliefs, guilty sentiments, and lack of trust in criminal and judicial authorities [9,10].

1.2. Compounds involved in DFSA.

Central nervous system (CNS) depressants, and to less extent CNS stimulants, are the most frequently implicated drugs in sexual assaults. According to various studies, ethanol remains at the forefront of substances involved in drug-facilitated sexual assault, being detected either alone or in combination with other compounds in the victim's body [11–14]. The presence of alcohol in most DFSA cases is not surprising, considering that many of the reported alleged sexual incidents occurred in social contexts such as public houses, pubs, bars, nightclubs, or parties, where the use of alcohol is easy and expected by everyone [2,15]. However, reports have shown that various prescription pharmaceuticals and some over-the counter-drugs (OTC) (see **Table 1**), anxiety medications, muscle relaxers, tranquilizers, and various illicit narcotics are usually associated with DFSA [9]. While numerous drugs, including ethanol, can be utilized in DFSA, the US National Institute on Drug Abuse (NIDA) in its “Community Alert on Club Drugs,” defined “club drugs” as γ-hydroxybutyric acid

(GHB), ketamine, the popular amphetamine derivative ecstasy (3,4-methylenedioxymethamphetamine, or MDMA), Rohypnol® (flunitrazepam), methamphetamine, and lysergic acid diethylamide (LSD) [16].

In one of the most extensive investigations conducted on DFSA cases in the USA, a total of 1000 incidents were examined to analyse the substances involved in sexual assaults. The study findings revealed that ethanol had the highest prevalence, accounting for approximately 30.9% of the cases, closely followed by cannabinoids at 28.8%. Furthermore, stimulant agents (amphetamine, methamphetamine, cocaine, benzoylecgonine) ranked third with 24.1%, just ahead of benzodiazepines (clonazepam and alprazolam), with only 20.9% of the recorded DFSA cases [17].

Table 1. *Drug class, brand name and therapeutic indication for some prescribed pharmaceutical and over-the-counter OTC medication used in DFSA.*

Drug-class	Active compound	Brand name	Therapeutic indication
Benzodiazepines			
	Diazepam	Valium®	
	Alprazolam	Xanax®	Anxiolytic and hypnotic.
	Flunitrazepam	Rohypnol®	
Barbiturates			
	Phenobarbital	Luminal®	Sedative and anti-seizure properties
	Pentobarbital	Nembutal®	short-acting sedative barbiturate
Antidepressants			
	Amitriptyline	Elavil®	Tricyclic antidepressants.
	Sertraline	Zoloft®	Selective serotonin reuptake inhibitor (SSRI).
	Citalopram	Celexa®	Selective serotonin reuptake inhibitor (SSRI).
Muscle relaxants			
	Carisoprodol	Soma	Centrally acting muscle relaxant
	Cyclobenzaprine	Flexeril®	Skeletal muscle relaxant.
Antihistamines			
	Diphenhydramine	Benadryl®	First-generation histamine receptor
	Doxylamine	Unisom®	H1 antagonist.
Opioids			
	Hydrocodone	Vicodin®	Opioid agonist used as an analgesic and antitussive agent.
	Oxycodone	Oxycontin®	Opioid used in the management of moderate to severe pain.

During a recent investigation of 455 cases of Drug-Facilitated Sexual Assault (DFSA) carried out in Peru over a span of three years (2016-2018), a significant prevalence of benzodiazepines was observed, either used alone or in conjunction with

ethanol and/or illegal substances. Ethanol ranked second in frequency, either used alone or combined with psychotropic drugs and/or other illicit substances. In approximately 10% of the cases, the victims had consumed cannabis, whereas cocaine was detected in only 4% of the cases [11]. **Table 2** represent the most common benzodiazepines and z-hypnotic drugs detected in drug-facilitated crimes in various studies [12,17–21]. It is well-noted that diazepam and alprazolam are the most reported benzodiazepines in many studies.

Table 2. *The most common benzodiazepines and z-hypnotic drugs detected in DFSA.*

Biological samples	**Total DFSA cases**	**Total BZD positive cases**	**Most detected (Number, %*)**		**References**
Blood and urine	126	14 in blood 21 in urine	Diazepam	Blood (6/14, 4.7%)	[12]
				Urine (9/21, 7.1%)	
			Lorazepam	Blood (4/14, 3.1%)	
				Urine (5/21, 3.9%)	
			Clonazepam	Blood (3/14, 2.3%)	
				Urine (3/21, 2.3%)	
Blood	145	145	Nordiazepam (87, 60%) Diazepam (81, 55.9%) Temazepam (72, 49.7%) Oxazepam (56, 38.7%) Midazolam (36, 24.8%)		[18]
Blood and urine	1000	209	Clonazepam (76, 7.6%) Alprazolam (72, 7.2%) Lorazepam (66, 6.6%) Diazepam (37, 3.7%) Oxazepam (13, 1.3%)		[17]
Urine	126	55	Flunitrazepam (14. 11.1%) Nimetazepam (10, 7.9%) Clonazepam (6, 4.7%) Zolpidem (5, 4.0%) Diazepam (4, 3.1%) Alprazolam (4, 3.1%)		[19]
Hair	25	12	Zopiclone (6, 24%) Zolpidem (6, 24%) Clonazepam (4, 16%) Oxazepam (3, 12%) Diazepam (2, 8%)		[20]
Urine	178	20	Lorazepam (11, 6.2%) Benzodiazepine's metabolite (5, 2.8%) Clonazepam (2, 1.1%) Diazepam (1, 0.6%) Nitrazepam (1, 0.6%)		[21]

Furthermore, in a recent study concerning the purchase of prescription medicines via social media, the study concluded that among the medications acquired by participants from at least one of the online platforms, narcotics (54.6%) emerged as the most prevalent. Notably, prescription opioids like Vicodin® (Hydrocodone), Xanax® (Alprazolam), Valium® (Diazepam), OxyContin® (Oxycodone), and Percocet® (Oxycodone/paracetamol,) ranked prominently among the drugs frequently abused for nonmedical reasons [22].

It is important to highlight that the guidelines for the forensic analysis of drugs facilitating sexual assault and other criminal acts, issued by the United Nations Office on Drugs and Crime, listed scopolamine as one of the substances involved in both DFSA and DFC [3]. Scopolamine, also known as hyoscine, is a naturally occurring alkaloid in plants and it is used in the treatment of motion sickness and in the prevention of stomach upset [23]. A dose higher than 330 mg can induce delirium, psychosis, and sometime paralysis in addition to its narcotic effects [24].This drug has been illicitly used as hallucinogen. As with GHB, the half-life $t_{1/2}$ (which is defined as the time required for a plasma drug concentration to be reduced by 50%) of scopolamine is relatively short about 9 hours, therefore the drug is rapidly cleared from victims' body, making the confirmation process difficult by scientists [25]. However, scopolamine-facilitated crimes are usually involved in robbery [25,26] and, so far, there are no forensic studies that confirm its use in sexual attacks [27].

It is noteworthy to mention that chloroform; a volatile organic solvent that is widely used in industry, was reported in two different sexual assaults in France [28]. Gaillard *et al.*, 2006 and his colleague reported chloroform in the corpse of sexual assault victim's biological fluids including peripheral blood, bile, and urine at concentrations of 833.9, 148.6 and 9.7 mg/L, respectively. The authors also clarified that the suspect, who committed suicide on the same day, had a high chloroform concentration in many of his biological tissues, such as cardiac blood, urine, and bile at concentrations of 0.25, 0.26 and 0.38 mg/L, respectively. The highest concentration was found in adipose tissue at concentrations of 5.18-5.44 mg/kg. The study concluded that volatile organic solvents may be used in sexual assaults, and it raises the attention to the importance of adipose tissue when suspecting solvent intoxication. Another

DFSA case reported in France by Richeval *et al.*, 2017 was linked to chloroform too [29]. The alleged sexual assault victim was admitted to the hospital and a blood sample was collected in addition to a scarf found next to the victim. The presence of chloroform in the victims' blood was confirmed at a concentration 580 µg/L and traces were also detected on the scarf.

The main classes of DFSA drugs (ethanol, cannabinoids, benzodiazepines, z-hypnotic drugs, amphetamine and amphetamine derivatives, antidepressants, antipsychotic, opioids, ϒ-hydroxybutyric acid (GHB) and ketamine) their prevalence, and their human effects are discussed more into details in the supporting information of the first chapter (Page 94) of this thesis.

2. Green chemistry and extraction techniques.

2.1. Principles of green chemistry.

Green chemistry is best defined as the design of chemical products and processes that aim to reduce or even eliminate the use and generation of hazardous substances, even though the term green chemistry scope extends further [30]. Sustainability and green chemistry in the field of laboratory analysis play critical roles in the development and validation of a novel laboratory extraction techniques, contributing to the development of environmentally friendly and socially responsible practices. It also encompasses goals like reducing waste production, improving energy efficiency, and utilizing renewable raw materials. Green chemistry, with its well-known 12 principles, focuses on the design and implementation of chemical processes that reduce the harmful environmental impact and increase both the safety and efficiency of the proposed developed method [31]. **Figure 1** summarizes the 12 principles of green chemistry.

In 2012, *Chemat et al.* introduced the term "environmentally friendly extraction of natural substances," building upon the principles of "green chemistry" and "green engineering". They defined "Green Extraction" as the exploration and creation of extraction methodologies that minimize energy usage, facilitate the adoption of

substitute solvents and renewable natural materials, and guarantee the production of safe and superior extracts/products [32].

Fig. 1. *The 12 principles of green chemistry.*

The idea of green analytical chemistry (GAC); a term that originates in the year 2000 from the green chemistry, involves the role of analytical scientists in converting analytical laboratory practices (mainly extraction and analysis procedures) to more environmentally friendly in the laboratories around the world [33]. The best approach to implement the various principles of GAC is to utilize a wide range of extraction processes that minimize or eliminate the use of organic solvents [34]. Gałuszka *et al.* set a group of goals to achieve greening analytical methods and can be summarized in then following points:

[1] Eliminate or reduce the use of all types of chemical substances (solvents, reagents, preservatives, pH adjustment additives, and others).

[2] Reduce energy consumption.

[3] Effectively manage analytical waste.

[4] Enhance operator safety.

2.2. Sample preparation techniques in forensic analytical toxicology

Blood serum, blood plasma, whole blood, and urine are often used matrices in analytical toxicology. However, in recent years, there has been a focus on alternate matrices (also known as non-conventional matrices) such as oral fluids [35], hair [36], nails [37], breath [38], and vitreous humor [39]. Some of these samples (for example, saliva, hair, and nails) are easily collected and available in large volume, when compared to blood samples. Furthermore, the collection of both hair and nails, for example, which are keratinized matrices, can give information of history of consumption of several drugs over a lengthy period of time [6,40] .

The demand for forensic toxicologist to detect various pharmaceutical and illegal compounds in human body at low concentrations in complex biological samples requires isolating/enriching the targeted analytes while reducing unwanted matrix effects, before using a highly sensitive detection technique. For this reason, the sample preparation is crucial in analytical procedures, and the implementation of green chemistry principles is considered vital to avoid the use of harmful solvents [33,41].

Both solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are the two most commonly used traditional sample preparation procedures for extracting a wide range of analytes from biological liquid samples (e.g., urine, blood, serum), while the use of organic solvents is mandatory for the extraction of compounds for solid matrices (SLE) (hair, nails, etc.), often followed by purification with SPE. Liquid-liquid extraction is widely used for the extraction of pharmaceutical compounds and illicit drugs from biological matrices [42,43]. LLE can be described as a transfer of analytes between two immiscible liquids phases that do not mix well, often involving water-based solutions and organic solvents (or extraction solvent) [44]. Because most of water-immiscible organic solvents are of non-polar nature, their ability for the extraction of polar drugs is very poor and their capacity for extracting drugs in a wide polarity range is very limited. Although LLE has been used for long time as one of the superior extraction techniques, several disadvantages have been recorded with it in particularly the long-time process and the production of large volume of organic solvent, which contradicts the principles of green chemistry.

Solid-phase extraction is a technique that uses solid sorbents to selectively retain and elute target analytes. It involves passing a liquid sample through a solid phase, where the target compounds are retained while interfering substances are removed. The retained analytes are then eluted with an appropriate solvent. The cartridge is usually comprised of hydrophobic material, such as long chains of carbon (C18) that would specifically bind to non-polar analytes, enabling their retrieval from a polar environment. However, the developing of column materials is continuous and columns are filled upon varying chemical concepts including hydrophobic, hydrophilic, cation-exchange, anion-exchange, and mixed mode (which is a combination of ion exchange and hydrophobic principles) [45]. Over LLE, SPE has advantages such as greater selectivity, improved extraction efficiency, and recovery [46]. On the other hand, SPE is compatible with automation. However, the procedure involves multiple steps, which can increase the chance of errors. A detailed discussion on the extraction techniques used in the extraction of DFSA from biological matrices will be conducted on Chapter I of this thesis.

2.3. Supramolecular solvents

Supramolecular solvents are nanostructured liquids generated as result of the sequential self-assembly of amphiphiles at the molecular and nanoscale levels and coacervation [47]. Molecular self-assembly refers to the spontaneous organization of a group of molecules, leading to a more structured and/or functional arrangement [48]. This process is reversible as the interactions involved are non-covalent, including forces like Van der Waals forces, electrostatic interactions, or hydrogen bonds. On the other hand, the coacervation phenomenon involves the division of a colloidal system into two liquid phases, with one phase containing a significantly higher concentration of the dispersed component than the other phase.

In summary, the production of SUPRASs involves a two-step synthesis process [49]. In the initial stage, an aqueous or organic solution containing amphiphilic molecules is prepared. When the concentration surpasses its critical aggregation point, it initiates the formation of supramolecular structures such as micelles, vesicles, and so forth, resulting in the creation of a colloidal system.

In the second step, some modifications are introduced to the conditions of the colloidal system. This may involve altering factors such as temperature, pH, or even the introduction of salts or other organic solvents. These changes are implemented to encourage the growth of the aggregates and enhance their interaction. As a result, coacervate droplets spontaneously emerge, tending to aggregate together.

The clusters formed have a different density than the medium in which they are dispersed in, so they separate from each other. This separation results in the formation of a new liquid phase in which the coacervate droplets exist as individual entities. This new phase, incorporating most of the amphiphilic molecules, is referred to as SUPRAS. Depending on the system, the SUPRAS may exhibit varying densities in comparison to the equilibrium solution, which could lead it to sink to the bottom (denser) or float on the top (less dense) of the tube. **Figure 2** represents the diagram of the general procedure for the synthesis of supramolecular solvents.

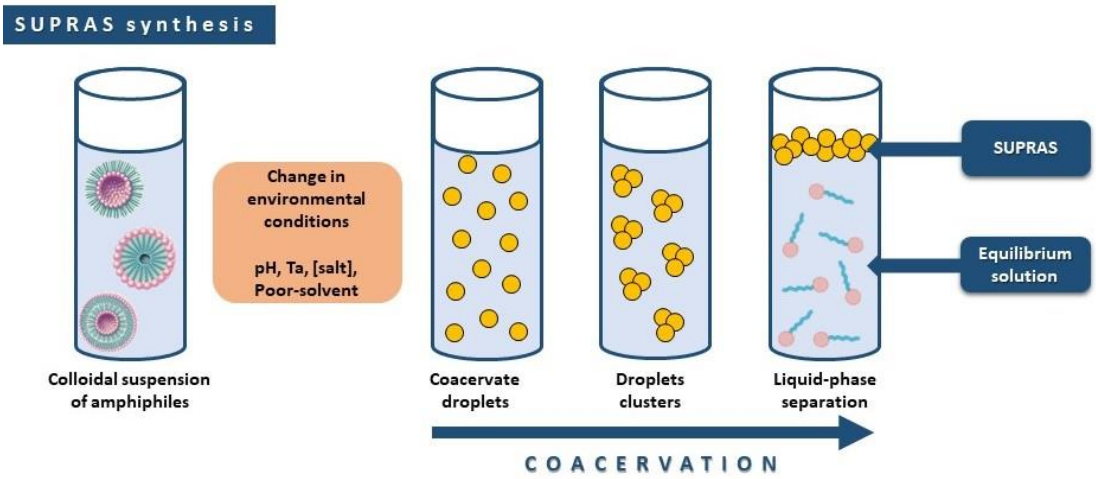

Fig. 2. *Diagram of the general procedure for the synthesis of supramolecular solvents.*

SUPRASs possess the ability to overcome significant drawbacks associated with current extraction methods for detecting DFSA (such as limitations to the extraction of structurally similar drugs, excessive solvent usage, challenging operational conditions, necessitating SPE clean-up, and time-intensive procedures). These capabilities stem from the inherent properties of SUPRASs. In terms of extraction, SUPRASs provide the following advantages [49]:

a) *Diverse polarity microenvironments* allow solutes with a wide range of polarities to be simultaneously solubilized. Unlike traditional solvents, SUPRASs exhibit the capacity to extract substances through various mechanisms (e.g., hydrogen bonding, dipole-dipole interactions, ionic interactions, etc., in the polar region, and dispersion, π-π interactions, etc., in the nonpolar region).
b) *Multiple binding sites* due to the abundant concentration of amphiphiles within the SUPRAS (0.1–1 mg/µL). Therefore, solutes can be extracted using low SUPRAS/sample ratios, enhancing sensitivity, and often eliminating the requirement to evaporate the extracts. This results in reductions in both analysis time and costs.
c) *Large surface area* resulting from the individual coacervate droplets within the SUPRAS, facilitating rapid solute mass transfer during extraction processes.

One of the advantageous features of SUPRAS is their flexibility, as their nanostructures, composition, and properties can be easily tailored by selectively choosing amphiphiles and self-assembly conditions. With regard to sample purification, SUPRASs can be customized to exclude macromolecules using both physical and chemical mechanisms [49]. This integration enables DFSA extraction and sample purification to be accomplished in a single step. Furthermore, SUPRAS fulfils various green chemistry requirements, such as high performance, energy-saving and high-atom economy synthesis, and low toxicity [49].

The self-assembly of amphiphilic compounds opens promising possibilities for creating nanostructured solvents with tunable and functional properties. Advances in supramolecular chemistry have deepened our understanding of self-assembly processes, forming the basis for a bottom-up approach in synthesizing intelligent nanomaterials. However, the application of this knowledge to develop nanostructured liquids has been limited [50].

Figure 3 shows the increased number of publications related to supramolecular solvents from 2002 to 2022, with an increase rate up to 77.2% (Data obtained from Scopus database last access on 27/07/2023).

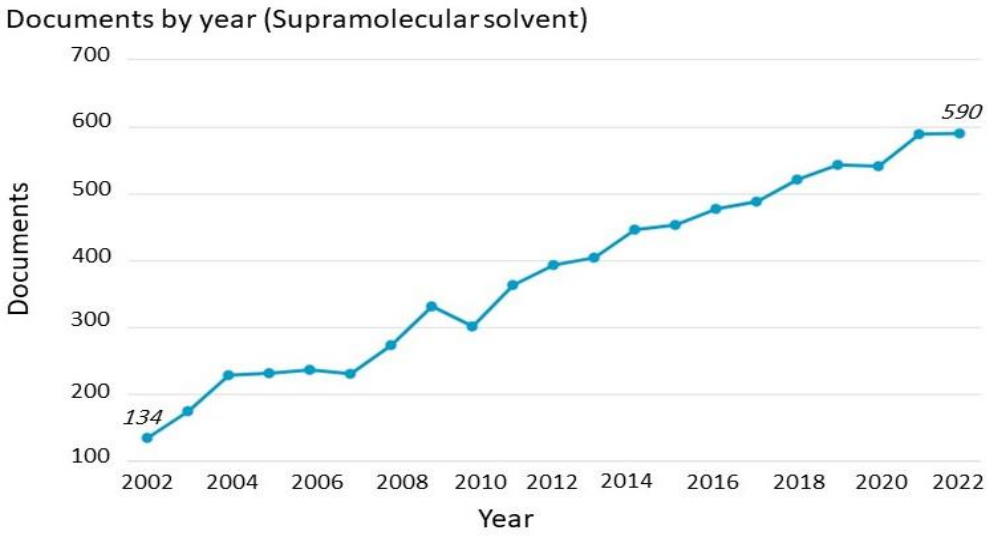

Fig. 3. *Contribution of Supramolecular solvent in the period of 2002-2022. Data extracted from Scopus Database.*

The applications of SUPRASs as an extraction method has been involved into various scientific fields. For instance, it has been applied in food industry to quantify extract organic contaminants in food packaging materials [51], hydroxytyrosol from table olives [52], and in the determination of oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) in meat, seafood and fish tissues [53]. Moreover, several environmental applications have been recorded using SUPRAS to determine several drugs of abuse in tap water from eight European countries [54], and it was efficiently applied in the extraction of carcinogenic polycyclic aromatic hydrocarbons from soils [55].

In general, SUPRASs procedures are simpler, faster, and greener as compared with LLE and SPE for the extraction of a range of analytes from biological matrices. An example is shown in **Figure 4** for the extraction of DFSA substances from urine using LLE (extraction of 3 benzodiazepines [56]), SUPRASs (extraction of 23 DFSA compounds including benzodiazepines, z-hypnotic drugs, amphetamine derivatives, cocaine metabolites, and miscellaneous compounds [57]), and SPE (extraction of 40 benzodiazepines and 3 z-hypnotic drugs [58]).

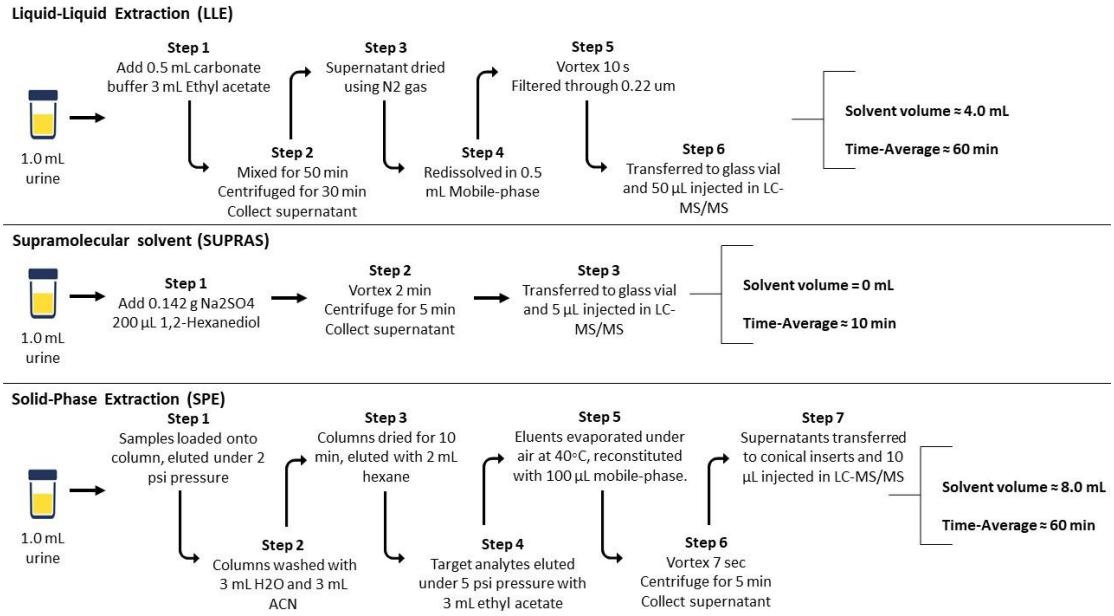

Fig. 4. *Comparison of LLE, SUPRAS, and SPE in the extraction of different drugs from urine sample.*

From the comparison, it is concluded that the SUPRAS used (formed from 1,2-hexanediol and sodium sulphate) offers a distinct advantage, requiring only 3 main extraction steps with a total time of 10 minutes. In contrast, both LLE and SPE involve more extensive steps, up to 6 and 7 steps respectively, resulting in an approximate extraction time of up to 60 minutes for each sample. Moreover, LLE and SPE consume 4 mL and 8 mL of organic solvents per sample respectively, while the SUPRAS method required no consumption of organic solvents.

3. Conclusion and future trends

SUPRAS is a relatively new innovative analytical extraction technique which provides a green and often a single-step method for the possibility of extraction of various toxic compounds or other compounds and can be applied in clinical, forensic toxicological, environmental, and food industries filed. The synthesis of SUPRASs is simple and straightforward, and they are easily transported and stored in the laboratories [47]. However, a main factor to be considered in the development of a new SUPRAS is that its synthesis agrees with the principles of green chemistry.

Green SUPRASs can be easily synthesized from bioamphiphiles, which are biodegradable amphiphiles obtained from natural sources that have not negative impact on both human health and the surrounding environment. Also, of paramount importance is that the coacervation of the amphiphile does not demand extreme conditions related to temperature, pH, or ionic strength. As a continuation of this approach, the exploration of self-assembly and coacervation of additional SUPRAS in aqueous solutions using benign coacervating agents remains a promising avenue for research.

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RESULTS AND DISCUSSION

Chapter I

Analysis of conventional and nonconventional forensic specimens in drug facilitated sexual assault by liquid chromatography and tandem mass spectrometry.

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Analysis of conventional and nonconventional forensic specimens in drug-facilitated sexual assault by liquid chromatography and tandem mass spectrometry

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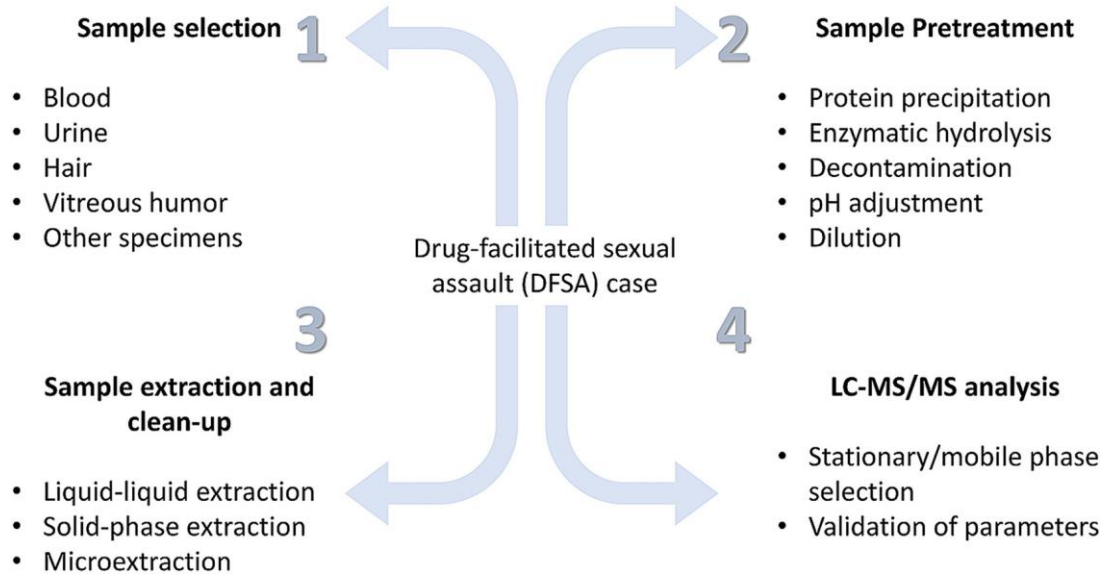
GRAPHICAL ABSTRACT



ABSTRACT

The incidence of drug-facilitated sexual assault (DFSA) has dramatically increased in the last decades. Forensic analytical scientists continuously seek new methods and specimens to prove the incidence of intoxication for the judiciary system. Factors influencing sample selection include the ease of obtaining the samples and the window of detection of the drugs, among others. Both conventional (blood, urine) and non-conventional specimens (hair, nails, fluids) have been proposed as suitable in DFSA cases. Reported sample treatments include a variety of liquid-liquid and solid-phase extraction as well as dilute-and-shoot procedures and microextraction techniques. Regarding analysis, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has emerged as the preferred confirmatory technique, due to its sensitivity, selectivity, and wide-scope applicability. In this review, we critically discuss the most common specimens and sample treatments/analysis procedures (related to LC-MS/MS) that have been reported during the last ten years. As a final goal, we intend to provide a critical overview and suggest analytical recommendations for the establishment of suitable analytical strategies in DFSA cases.

KEYWORDS

Drug-facilitated sexual assault DFSA, Blood, Urine, Hair, LC-MS.

1. INTRODUCTION

Drug-facilitated crimes (DFC) are offenses that include sexual attacks, robbery, kidnapping and other illegal activities under the influence of certain substances [1,2]. According to the United Nations Office on Drugs and Crime (UNODC), drug-facilitated sexual assault (DFSA) is considered a sub-category of drug-facilitated crimes [3]. DFSA is defined as the incidence in which a person; whether a male or female, is subjected to sexual activity while they are incapacitated or unconscious because of the ingestion of ethanol or any other intoxicating substance, resulting in the inability to resist or consent to such acts [4]. The term DFSA is sometimes interchangeably used with chemical submission, which is more common in some European countries, such as Spain and France [5]. Women from different age groups are the major victims [6]. Accordingly, the study by Bosman et al. reported that 120 victims (94%) out of 128 total cases were women [7]. This record is comparable with a recent toxicological assessment in New Zealand on the role of alcohol and other illegal drugs on 162 drug-facilitated sexual assault victims, of which 159 (98%) were women [8].

Forensic evidence of DFSA is particularly difficult to establish [9]. Although most of the drugs used in DFSA induce similar clinical symptoms in the victims (from loss of inhibition to loss of consciousness and sometimes anterograde amnesia), the necessity of analytical confirmation is always required by different health and forensic authorities [10]. Alcohol is still the most used psychoactive substance associated with DFSA, however, the use of other drugs, such as benzodiazepines, anti- depressants, muscle relaxants and antihistamines, is rising fast while drug markets are expanding [11]. The development of suitable analytical methods to detect these other drugs is receiving increasing attention due to the considerable rise in the number of reported cases worldwide [12–14].

The forensic evidence of DFSA in humans requires the toxicological examination of biological matrices to detect the potential substances in the victim's body [15]. This examination is mainly performed in blood and urine. However, sometimes it is difficult or useless to collect them and non-conventional matrices such as oral fluids, hair, nails, and sweat are employed [12,16]. The choice of the biological samples is influenced by many factors, such as whether it is a premortem or postmortem case,

the purpose of the analysis, the target drugs and the time-lapse between the incidence and the collection of the sample [17]. Thus, early sample collection is essential for many DFSA substances that are rapidly metabolized and have high elimination rates. For instance, gamma-hydroxybutyric acid (GHB) is a fast metabolized substance with a short half-life (20–60 min) and a rapid elimination rate from the body [5,18]. It must be also considered that several drugs may be simultaneously administered causing a synergistic effect. This makes the sample selection and analysis even more complex [19].

Immunoassays are still frequently used by forensic laboratories for the initial screening of drugs because they are quick and inexpensive [5, 15]. The use of commercial immunoassays kits is common in large DFSA epidemiological studies for the initial detection of the most used drugs (e.g., cocaine, opiates, cannabinoids, amphetamines, barbiturates, methadone, and benzodiazepines) [20,21]. However, these tests are not sensitive enough for other drugs of interest in DFSA and their applicability is usually limited to urine samples [5]. They also suffer from cross-reactivity, which yields false-positive results. These interferences vary among available kits and they have been reviewed in detail by some authors [22]. For example, dimethylamylamine, which is frequently used as an energy supplement, causes false-positive amphetamine screens [23]. Positive interferences have been also found for THC (cannabinoid) with patients under treatment with niflumic acid (an anti-inflammatory drug) [24]. Due to the lack of specificity of these techniques, positive results must be confirmed by chromatographic techniques coupled to mass spectrometry [25].

Although gas chromatography (GC) coupled to mass spectrometry (MS) is still used in many forensic laboratories as a confirmatory method (mostly based on electron ionization), liquid chromatography (LC-MS) has emerged in the last decade as the preferred choice for the analysis of drugs in biological matrices [26]. The major advantage of LC-MS is its suitability for the separation and ionization of polar substances with minimal sample preparation [27].

This review critically discusses the analytical methods developed in the last decade (2010–2022) for the determination of DFSA drugs. While some reviews and

institutional reports on DFSA have focused on epidemiological and social aspects, less attention has been given to the analytical advances and the challenges to be confronted in this field [10, 18,20,28–31]. In fact, analytical reviews are mostly limited to the analysis of certain groups of drugs, such as benzodiazepines [32], or to the use of certain alternative matrices for forensic purposes, such as hair [33] or nails [34]. To the best of our knowledge, criteria for sample selection, sample preparation and analysis of DFSA drugs as a target group, have not been addressed yet as a whole. With this study, we aim to provide the scientific community working in the field with a critical overview of the key steps involved in DFSA analysis, as a basic guide for future analytical developments.

2. Target compounds in drug-facilitated sexual assault (DFSA)

The term date-rape drugs was first introduced in 1982 in an article entitled “Date rape: A campus epidemic” and since then it began to be used in the media and the scientific community [30]. Since the beginning of the 1990s, many substances have been identified to be involved in rape crimes, being flunitrazepam and GHB among the first recorded. These drugs were reported to alter the consciousness, memory and cognition of the victim [35,36]. These studies already emphasized the importance of the rapid and appropriate identification of the drugs leading to these symptoms in order to provide proper care for the victims [37].

DFSA substances have in common their effect on the central nervous system (CNS), either by stimulating or depressing the mental functions [29]. They consist of pharmaceutical substances that are prescribed for different indications (anxiolytics, hypnotics, antidepressants, etc.) Furthermore, DFSA may include illicit substances like CNS hallucinogens and other illegal drugs, such as cocaine, amphetamine, and cannabis [14]. Moreover, some over-the-counter (OTC) drugs may be employed, such as doxylamine, a first-generation antihistamine [38]. **Table 1** shows the most common DFSA drugs, their molecular formula, therapeutic categories, daily doses, reference toxic concentrations in serum, plasma protein binding and biological half-life.

Table 1. Molecular formulas, therapeutic categories and toxicokinetic data for selected DFSA substances or their metabolites.

Drug class	Drug	Molecular formula	Therapeutic category	Therapeutic daily dose (mg)	[b]Reference therapeutic concentration (mg/L)	[b]Reference toxic concentration (mg/L)	[a]Plasma protein binding	[a]Biological half-life $T_{1/2}$ (hour)
Benzodiazepines	Alprazolam	$C_{17}H_{13}ClN_4$	Anxiolytic and hypnotic	0.25-3.0	0.02-0.04	0.075	80%	11.2 (9-16)
	Bromazepam	$C_{14}H_{10}BrN_3O$	Anxiolytic and hypnotic	6-18	0.08-0.17	0.25-0.5	70%	17 (10-20)
	Clonazepam	$C_{15}H_{10}ClN_3O_3$	Anxiolytic and hypnotic	48	0.03-0.06	0.1-0.12	82-86%	30-40
	Clorazepate	$C_{16}H_{11}ClN_2O_3$	Anxiolytic and hypnotic	3.75-30	-	-	97-98%	30-60
	Diazepam	$C_{16}H_{13}ClN_2O$	Anxiolytic and hypnotic	2-30	0.125-0.75	1.5	89-99%	20-90
	Flunitrazepam	$C_{16}H_{12}FN_3O_3$	Anxiolytic and hypnotic	0.5-2.0	0.005-0.015	0.05	-	18-26
	Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	Anxiolytic and hypnotic	0.5-6.0	0.02-0.25	0.3-0.6	85%	14 (10-20)
	Lormetazepam	$C_{16}H_{12}Cl_2N_2O_2$	Anxiolytic and hypnotic	1.0	0.001-0.02	-	-	11 (10-12)
	Nitrazepam	$C_{15}H_{11}N_3O_3$	Anxiolytic and hypnotic	5-10	0.03-0.12	0.2-0.5	-	26 (15-38)
	Temazepam	$C_{16}H_{13}ClN_2O_2$	Anxiolytic and hypnotic	7.5-30	0.3-0.9	1	96%	9 (3.5-18)
	Zolpidem	$C_{19}H_{21}N_3O$	Anxiolytic and hypnotic	10	0.08-0.3	0.5	92.5%	2-3
	Zopiclone	$C_{17}H_{17}ClN_6O_3$	Anxiolytic and hypnotic	5.0-7.5	0.01-0.05	0.15	45%	5 (3.8-6.5)
Barbiturates	Phenobarbital	$C_{12}H_{12}N_2O_3$	Anticonvulsant and hypnotic	30-120	10-40	60-80	20-45%	53-118
	Amobarbital	$C_{11}H_{18}N_2O_3$	Anticonvulsant and hypnotic	IM 65-100	2-12	>9	-	20-25
	Pentobarbital	$C_{11}H_{18}N_2O_3$	Anticonvulsant and hypnotic	-	1-10	8-10	-	5-50
Antidepressants	Citalopram	$C_{20}H_{21}FN_2O$	Antidepressants	10-40	-	-	80%	90.2
	Amitriptyline	$C_{20}H_{23}N$	Antidepressants	25-100	0.05-0.2	-	95%	25

	Fluoxetine	$C_{17}H_{18}F_3NO$	Antidepressants	20-60	0.1-0.45	-	94%	1-4 *days*
Amphetamines	Amphetamine	$C_9H_{13}N$	CNS stimulant	5-60	0.05-0.15	0.2	20%	1 (9-11)
	Methamphetamine	$C_{10}H_{15}N$	CNS stimulant	20-25	0.01-0.05	0.2-1	-	4-5
	3,4-Methylenedioxymethamphetamine	$C_{11}H_{15}NO_2$	CNS stimulant	-	0.1-0.35	0.35-0.5	-	6-10
Alkaloids	Cocaine	$C_{17}H_{21}NO_4$	CNS stimulant (Local anesthetic)	Nasal solution 4%, 40-160 mg	0.05-0.3	0.25-5	-	1
Opioids	Morphine	$C_{17}H_{19}NO_3$	Opiate (narcotic) analgesic	-	0.08-0.12	0.15-0.5	35%	2-3
	Methadone	$C_{21}H_{27}NO$	Opiate (narcotic) analgesic	-	0.07-0.1	0.2-0.75	85-90%	7-59
	Oxycodone	$C_{18}H_{21}NO_4$	Opiate (narcotic) analgesic	-	0.02-0.05	0.2	45%	3.2-4.5
	Fentanyl	$C_{22}H_{28}N_2O$	Opiate (narcotic) analgesic	50-100 µg	0.001-0.002	0.002-0.02	80-85%	7 (8-10)
	Codeine	$C_{18}H_{21}NO_3$	Opiate (narcotic) analgesic	240	0.01-0.05	0.3-1	7-25%	3
	Dextromethorphan	$C_{18}H_{25}NO$	Antitussives	120	0.01-0.04	0.1	60-70%	3-30
Antihistamine	Doxylamine	$C_{17}H_{22}N_2O$	Antihistamine	25	-	-	-	10
	Diphenhydramine	$C_{17}H_{21}NO$	Antihistamine	-	0.1-1	1	78%	2.4-9.3
Miscellaneous	Ketamine	$C_{13}H_{16}ClNO$	General anesthetic	IV 1-4.5 mg/kg	0.5-6.5	7.0	53.5%	0.45 min (1-3)

			IM 6.5-13 mg/kg				
Scopolamine (hyoscine)	$C_{17}H_{21}NO_4$	Antimuscarinic agent	IV/IM/SC 0.3-0.65	0.0001-0.0003	-	30%	9-10
γ-Hydroxybutyric acid (GHB)	$C_4H_8O_3$	Narcolepsy agents	4.5-9 g	0-1	100-150	-	0.5-1

[a] Values obtained from PubChem, [b] Values obtained from A.C. Moffat, B. Widdop, D.M. Osselton, *Clarke's Analysis of Drugs and Poisons*, third ed., Pharmaceutical Press, London, 2004. IV: intravenous, IM: intramuscular, SC: stratum corneum

The dose ingested by DFSA victims must be well above the daily recommended amounts in the case of pharmaceutical compounds to produce a toxic concentration in serum. The reference toxic concentration is that at which the unwanted and toxic effects appear and it is mainly affected by the half-life (t1/2) of the substance and the patient metabolic status. For instance, the daily pharmaceutical therapeutic dose of amitriptyline, (tricyclic antidepressant) is 50–150 mg and for amphetamine (treatment of narcolepsy) is 20–100 mg, while toxic doses are established at 1 g and 200 mg, respectively [39]. Similarly, medical daily doses of benzodiazepines, which are used to treat sleep and anxiety disorders [40], range between 0.25 mg and 30 mg, and for the pharmacologically related hypnotic Z-drugs, the doses range from 5 mg to 10 mg. The reference toxic concentrations of benzodiazepines in serum range between 0.075 and 1.00 mg/L and for Z-drugs between 0.15 and 0.5 mg/L. For this reason, it is mandatory to ask the victim about any medication or treatment that he/she is using during the sample collection in order to correlate it with the concentration of drugs found in the victim's body.

The biological half-life ($t_{1/2}$) is a key factor related to DFSA since it is the main parameter for the measurement of the metabolization and elimination of drugs and their metabolites from the human body without leaving any trace behind [29]. The $t_{1/2}$ for cocaine and GHB is around 1 h, while other drugs are metabolized more slowly (e.g., $t_{1/2}$ above 10 h for benzodiazepines and amphetamine) (see **Table 1**). Short half-life values are particularly challenging for analysis since this will limit the window of detection in the different biological matrices [41].

Table 2 summarizes some representative epidemiological studies on DFSA in different countries highlighting the top drugs involved in sexual assaults worldwide [7,8,12,14,15,21,28,42–47]. DFSA substances included alcohol, benzodiazepines, amphetamine, and amphetamine metabolites [48] and cocaine and cocaine derivatives [29]. Antidepressant compounds [44], barbiturates [49] and different opioids [42], including the popular powerful synthetic opioid fentanyl, have been also involved in DFSA cases. Other illegal drugs, such as cannabinoids [45], ketamine [50], and scopolamine [51] are also categorized under the DFSA list. Other emerging drugs, not included in DFSA lists, have been reported to be used too, such as 4-methylethcathinone

(4-MEC) and methylenedioxypyrovalerone (MDPV), thus highlighting the need for continuous analytical developments in this field [52].

Most of the substances listed in **Table 2** are prescribed medications, e.g., benzodiazepines, barbiturates and antidepressants, which limit the access to these substances to criminals. Fewer substances are classified as over-the-counter, such as antihistamine medications, which can be easily obtained from any local pharmacy. For other compounds, such as cannabis, the legal status is controversial around the world, since some countries allow its use for medicinal and recreational purposes [53].

3. Specimens' selection

The selection of forensic specimens is a major research issue for forensic scientists nowadays [55]. Once a victim of DFSA is identified, it is crucial to quickly collect the appropriate biological specimens, usually blood and urine. A delay of a few hours in the collection of a blood sample may lead to the administered substance being missed [4]. All collected specimens must be packed separately in suitable containers, properly identified, labeled, tightly sealed, and properly stored [55]. Besides, a unique number or code should be given for each collected sample to maintain a proper chain of custody [56].

Several factors determine the selection of the biological specimen to perform the required chemical analysis. Among them, it is worth noting the circumstances surrounding the case, whether antemortem or post- mortem samples are collected, the availability of specimens and the nature of the target drugs. The postmortem redistribution of drugs may play an important role in the selection of the appropriate matrix [57] since the concentration of the target substances may increase in one organ over other ones [58]. However, this factor is only significant when the sexual assault results in the death of the victim. Two fatal DFSA cases with GHB showed a redistribution of the drug in the corpses, so that it became more concentrated in femoral blood than in cardiac blood, with a femoral/cardiac blood ratio of 1.15–1.76 [59,60].

Table 2. The most common DFSA drugs reported in literature from different countries.

Country	Published year	Sampling period	Total reported DFSA cases N	Drug group/Substances	Positive cases	% Cases per total DFSA	References
Peru	2022	2016-2018	N= 445	Benzodiazepines (alone or in combination with ethanol and/or illicit drugs).	271	58%	[54]
				Ethanol (alone or in combination with psychotropic drugs and/or other illicit drugs)	132	28%	
				Cannabis	49	10%	
				Cocaine	16	4%	
New Zealand	2021	2015-2018	N = 162	Ethanol	76	46.9%	[8]
				Cannabis	46 (31 in blood, 15 in urine)	28.3%	
				Methamphetamine	30**	18.5%	
The United States	2019	March 2015-June 2016	N = 1,000	Ethanol	309	30.9%	[45]
				Cannabinoids (THC/THCCOOH/11-OH-THC)	288	28.8%	
				Stimulants (amphetamine, methamphetamine, cocaine, benzoylecgonine)	241	24.1%	
				Benzodiazepines (clonazepam, Alprazolam)	209	20.9%	
				Antidepressants (citalopram, fluoxetine)	173	17.3%	
Italy	2019	-	N = 120	Ethanol or ethanol biomarker	80	66%	[12]
				Benzodiazepines, z-hypnotic, and their metabolites	22	18%	
				Cocaine and metabolites	15	12.5%	
				Cannabis and THC metabolites	10	8.3%	
				MDMA	8	6.6%	
Italy	2018	2010-July 2018	N = 256	Ethanol	57	22.2%	[20]
				Cannabis	19	7.4%	
				Cocaine	15	5.8%	

				Benzodiazepines	13	5%	
				Opiate/Methadone	12	4.6%	
Taiwan	2018	January 2011-December 2015	N = 126 (Ethanol not measured)	Benzodiazepines	55	43.6%	[21]
				Ketamine and norketamine	10	7.9%	
				New psychoactive substances (methylone, PMA, PMMA)	13	10.3%	
				Stimulants (amphetamines, methamphetamine, MDA, MDMA)	9	7.2%	
				Zolpidem	5	4%	
Spain	2017	January 2010-December 2013	N = 152 total cases (130 positive cases)	Ethanol	100	76.9%	[14]
				Benzodiazepines	35	26.9%	
				Cocaine and derivatives	26	20%	
				Antidepressants	21	16.1%	
				Cannabinoids	17	13%	
The United States	2015	2013	N = 45	Cannabis		58%	[46]
				Ethanol		43%	
				Cocaine		26%	
				Amphetamine		13%	
				Benzodiazepines		11%	
Spain	2015	2011	N = 35 *33 positive cases	Ethanol	15/31	48.4%	[47]
				Stimulants (cocaine and amphetamine derivatives)	7/33	21.2%	
Norway	2013	July 2003-December 2010	N = 264	Ethanol	105	40%	[42]
				Benzodiazepines (alone, with ethanol and with other opioids)	25	9.4%	
				Stimulants (amphetamine, methamphetamine, methylphenidate) with or without other drugs	14	5.3%	
Denmark	2012	June 2007-January 2009	N = 167	Ethanol	59	35.3%	[15]
				Other drugs (benzodiazepines)	35	20.9%	

Netherlands	2011	January 2004-December 2006	N = 135 Only 108 tested for alcohol	Ethanol Illicit drugs (cocaine and its metabolites) Benzodiazepines Stimulants (MDMA and MDA)	51/108 19/134 14/135 14/135	47% 14% 10% 10%	[7]
Canada	2010	June 2005-April 2007	N = 178	Cannabinoids Ethanol Cocaine Antidepressants (Citalopram 12, Venlafaxine 8) Opioids (Codeine 8, Morphine 7)	60 55 38 29 24	33.7% 30.9% 21.4% 16.2% 13.4%	[43]
Sweden	2008	2003-2007	N = 1806 total cases *1247 positive cases	Ethanol (detected in blood or urine) Illicit drugs (alone or in combination with ethanol or other drugs) Benzodiazepines (alone or in combination with ethanol or illicit drugs)	772 150 147	62% 12% 11.7%	[44]
The United Kingdom	2005	January 2000-December 2002	N = 1014	Ethanol (with or without an illicit drug) Cannabinoids (Marijuana) Cocaine Opioids (codeine, dextropropoxyphene) Benzodiazepines (diazepam, temazepam)	470 260 110 103 84	46% 26% 11% 10.2% 8.3%	[28]

*Percentages do not sum up to 100% as more than one drug could be found in a sample. **Number of cases in both blood and/or urine. PMA: Para-methoxyamphetamine, PMMA: Para-methoxymethylamphetamin.

In sexual assault incidents, a critical aspect is the time gap between the incidence and the reporting of the case [61]. According to one of the largest DFSA studies performed in Spain [14], the sample collection time was a key factor. This study found a clear correlation between an early collection of blood samples (not more than 6 h after the incidence) and the positive analytical findings (100% of cases for either drugs or alcohol). Because of the short detection time window of benzodiazepines in human blood (48 h) and urine (96 h) [62], alternative specimens such as hair may be used, in which the detection window may reach up to 6 months after drug exposure [63]. The same happens for GHB and scopolamine.

Blood and urine have been the most widely investigated specimens in DFSA cases [5,62]. Typically, two samples of each type are collected and stored separately so that one of them is analyzed and the other is retained for further confirmatory analysis. A brief description of each specimen is discussed in the following sections.

3.1. Blood

Blood is considered the golden standard forensic sample [5]. Blood should be collected preferably within 48 h of the alleged crime. At least two samples of 5–10 mL should be collected in grey-top tubes containing sodium fluoride and potassium oxalate to prevent degradation and clotting [64,65]. Samples should be refrigerated at 2–8 ◦C and if the analysis is not done within 24 h it is advisable to freeze them after separating the plasma [4].

Blood provides a good correlation between the concentration and the administered dose [66]. Both qualitative and quantitative analysis is possible with blood, plasma or serum [67]. In contrast to urine, blood samples usually allow the detection of the parent compounds rather than their metabolites, which facilitates the analysis [68]. With blood testing is often possible to detect substances that have been administered to the human body typically within 2–24 h [69] being the time window of detection shorter than in urine. However, there are exceptions, and a DFSA case reported in Denmark [61] detected the antipsychotic drug quetiapine in blood, urine and hair samples collected after 43 h of the suspected attack. Although the concentration in blood (0.007 mg/L) was

lower than the therapeutic range of quetiapine (0.05–0.06 mg/L), the authors claimed that the detection window in blood was much higher than expected.

3.2. Urine

A sufficient amount of urine, not less than 30–50 mL, and collected within the first 96 h (4 days) of the alleged incidence, must be stored in plastic containers [56]. Samples should be kept at 2–8 ◦C for 24 h or at 18 ◦C for a maximum of 12 months until analysis [4]. Urine may contain both the parent drugs as well as their metabolites and they are usually at higher concentration levels in comparison with other biological samples [38]. This matrix provides longer windows of detection for illicit drugs and their metabolites that may reach up to 96 h [70]. Cannabis is an exception for which the detection window may last for some weeks in urine [71].

3.3. Hair

Although urine and blood are the usual specimens used to prove drug intoxication, the detection window for some of the drugs is only a few days and this makes it very difficult to detect them [72]. Therefore, in many cases, forensic analysts collect hair from the victim, which provides information about the drug ingestion for a longer time (months) [33,73]. Indeed, hair is the most studied non-conventional matrix in DFSA cases. In literature, hair samples have been involved in the detection of many DFSA drugs, such as barbiturates [49], alprazolam [74], bromazepam and clonazepam [75], quetiapine [61], zolpidem [76,77], flunitrazepam and oxazepam [78].

Hair samples should be stored at room temperature in a dry environment [4]. In fact, drugs are very stable in hair, nevertheless, some factors such as weather, pollution, cosmetic treatment and hair-coloring preparations may have a deleterious effect and may reduce the original drug concentration to different extents [79,80]. Hair color plays an important role in drug incorporation. Basic and lipophilic drugs (e.g., amphetamine and cocaine) are more easily incorporated into hair than neutral or acidic drugs (e.g., benzodiazepines and THC), since they possess a higher affinity to melanin, which is more abundant in dark and pigmented hair. Sunlight and weather conditions, heat, and chemical treatments (such as dyeing and bleaching) alter the structure of hair and

generally leads to decreased drug levels. Hydrophilic drugs are probably washed out by regular shampooing [81].

It is advised to take the sample after at least four weeks of the incidence and not to cut the hair during this period of time. The concentration of a drug varies along the length of the hair segment and this can be correlated with the time of administration [33]. The Guidelines for drug testing in hair by the Society of Hair Testing suggest cutting head hair into measured segments between 10 and 30 mm [79]. Cross-sectional hair analysis can also provide information about the medical history of the person and can accurately confirm the administration shift from one drug to another (e.g., from heroin to codeine) [33]. The possibility to perform segmental hair analysis help to differentiate a single exposure from chronic use [82]. Head hair grows at a speed of about 1.0 0.2 cm/month and this is set as a reference for sampling [83]. When only axillary, torso, or leg hair are available, and the growth rate is not established, the analysis must be considered as qualitative since the alleged victim could have consumed the compound at any time (not necessarily at the time of the assault).

Some authors have proposed a micro-segmentation approach [73]. This is based on cutting hair into 0.4 mm segments, considering an approximate daily growth rate of 0.4 mm, and then quantifying the targeted drug in each segment. Kuwayama et al. has successfully used micro-segmental hair analysis technique for the exact determination of specific day of zolpidem ingestion on the day of the incidence [73]. In this way, hair micro-segmentation grants a more specific chronological interpretation of the analytical findings to better understand the DFSA case.

In a case report from Denmark [72], in which a 30-year-old woman was suspected to be a victim of a sexual attack, urine and hair samples were collected 20 h and 34 days, respectively, after the incident. Triazolam was detected in the first inner hair segment at a concentration of 1 pg/mg but not in the other two hair segments. The drug was negative in the aqueous solution coming from the washing of the hair so that external contamination was not expected. The urine sample was positive for α-hydroxytriazolam, the main metabolite of triazolam, at a concentration of 39 μg/L. Other study performed on Risperidone, an atypical antipsychotic; also concluded that the correlation of the concentration of the drug in hair was statistically significant with respect to the serum

concentration [84]. These studies proved that hair was a valuable forensic specimen for corroborating or detecting DFSA incidents.

3.4. Vitreous humour

Vitreous humor is considered a beneficial alternative matrix for postmortem analysis of different substances, but it has been used to a lesser extent in DFSA cases [85]. It is available in a low volume that is not exceeding 0.5–4 mL per eye, nevertheless, it has shown great drug stability, especially for the analysis of some labile drugs, such as heroin metabolites and GHB [86]. Vitreous humor can help in the interpretation of the blood ethanol level; if vitreous humor is positive for ethanol this may suggest an antemortem consumption of ethanol, if negative, the blood ethanol level should be investigated for postmortem putrefaction [56]. A case on a lethal GHB facilitated sexual assault of a 6-year-old girl, reported the use of vitreous humor along with other biological matrices [58]. The concentration of GHB in vitreous humor was 58 mg/L and was in agreement with levels in other biological matrices.

3.5. Other specimens

Recent studies focus on the correlation of the concentration of DFSA drugs in blood and in other non-conventional and non-invasive biological matrices, such as breath, oral fluid and sweat. Oral fluid has been rarely studied in DFSA cases. An early study on this matrix made a comparison of the window of detection of lorazepam in hair, urine, and oral fluid after a single oral intake of 2.5 mg of lorazepam on three volunteers [87]. The peak concentration in oral fluid was detected after 15 min of the ingestion at a level of 18 ng/mL. The concentration peak was detected in urine samples at a concentration between 411 and 880 ng/mL after 24 h. The oral fluid tested positive for the next 8 h with a concentration of 0.3 ng/mL, while urine samples tested positive for 144 h with concentrations between 2 and 4 ng/mL. These results suggested that oral fluid samples would not improve the window of detection of lorazepam compared to urine. A short detection window is very disadvantageous in DFSA cases, which limits the applicability of this matrix. Sweat has been also investigated as a rapid and non-invasive bio- logical sample for the detection of different drugs [88]. In a study per- formed in the United States, three DFSA drugs (flunitrazepam, ketamine, and MDMA) were measured in a

simulated sweat sample. The results highlighted the need for more data regarding the concentration of drugs in this matrix [89].

Other matrices, such as breath and nails have been increasingly used in drug analysis and show potential for their implementation in DFSA cases. Breath analysis has proven suitable as a screening method for the detection of drugs of abuse in many forensic applications [90,91]. Many studies have recently discussed the correlation between the alcohol level in this matrix (breath alcohol concentration, BrAC) and the blood (blood alcohol concentration, BAC) [92] and it has been applied in some DFSA cases [93]. The limitation of breath analysis is the relatively small window of detection after the drug ingestion in comparison with blood and urine. Nevertheless, breath analysis is still considered a promising sample in some situations, such as for testing the drivers who are under the influence of alcohol or any other illegal drugs [94,95]. Unlikely, this is not the case in the determination of DFSA, as there is usually a relatively long-time lapse between the ingestion of the drugs and the reporting of the incidence.

Nails are another promising matrix, which to the best of our knowledge has not been yet reported for the determination of drugs related to DFSA cases. Nevertheless this matrix could play a role as an alternative specimen in DFSA cases in the future [34].

4. Sample pretreatment

Generally, positive samples detected at the preliminary immuno- logical tests are routed for further analysis by GC-MS or LC-MS. GC/MS has long been the gold standard for confirmatory analysis, but LC-MS has emerged as a strong alternative on the basis of its higher sensitivity, versatility and no need for the derivatization of polar compounds [96]. However, LC-MS is particularly prone to matrix effects (suppression or enhancement of the signal due to matrix components) that can lead to unreliable results [97]. In order to prevent this, proper sample preparation is necessary for clean-up and drug enrichment.

Many sample preparation methods have been applied to extract a wide range of DFSA compounds from different biological matrices. **Fig. 1** represents the most often used sample collection and sample treatment steps for the determination of DFSA compounds in blood, urine and hair before LC-MS analysis. **Table 3** gives an overview of

different sample preparation methods along with the sample volume and weight, main sample preparation steps, and average recoveries [16,48,77,80, 98–116]. This table only summarizes the methods where LC-MS was used as the instrumental technique.

As shown in **Table 3**, laboratory sample aliquots of blood are usually in the range 0.1–1 mL, while 1 mL is the preferred volume for urine samples. For hair samples, weights varied from 2.5 mg up to 100 mg.

Regarding sample pretreatment, blood and plasma usually require a deproteinization step that is mostly done by the addition of a certain volume of organic solvent and/or acidic conditions followed by filtration and/or centrifugation [88]. Leal Cunha et al. [111] proposed the addition of 1 mL of cold acetonitrile to 0.25 mL of blood followed by centrifugation for the determination of amphetamine-type stimulants and synthetic cathinones. This simplistic approach relay only on the dilution of the sample (instead of concentrating it), which does not remove other matrix interferents and it requires the sample to be dried and reconstituted in a proper injection solvent for analysis. The method was sensitive enough with LODs in the range 0.5–1.7 ng/mL and acceptable recoveries ranging 60–86%. Similar approaches were pro- posed by Vaiano et al. (deproteinization of 200 µL of blood by adding 600 µL acetonitrile) [118] and Fisichella et al. (deproteinization of 500 µL blood by the addition of 500 µL methanol) [102]. The adjustment of pH is also a common pretreatment step, especially in the liquid-liquid extraction of blood and urine, but also to improve the solvent extractions performed in solid matrices. A suitable pH value is crucial for the favored partition of ionizable analytes from the aqueous matrix to the organic solvent. Banaszkiewicz et al. [112] added carbonate buffer (pH 9.2) to blood samples before the extraction of benzodiazepines and Z-drugs with ethyl acetate. The pKa values for most of these compounds are above 7 so that the neutral form was predominant at alkaline pH and the extraction in the organic layer was ensured. The same approach was performed by Diniz et al. [115] by increasing the pH value to 9.0 using ammonium hydroxide before proceeding with LLE extraction using 1 mL of ethyl acetate.

For urine samples, the enzymatic hydrolysis using β-glucuronidase and arylsulfatase is usually required to convert the conjugated drug to the free form and enable the selective analysis of the parent compounds and prevent false-negative results.

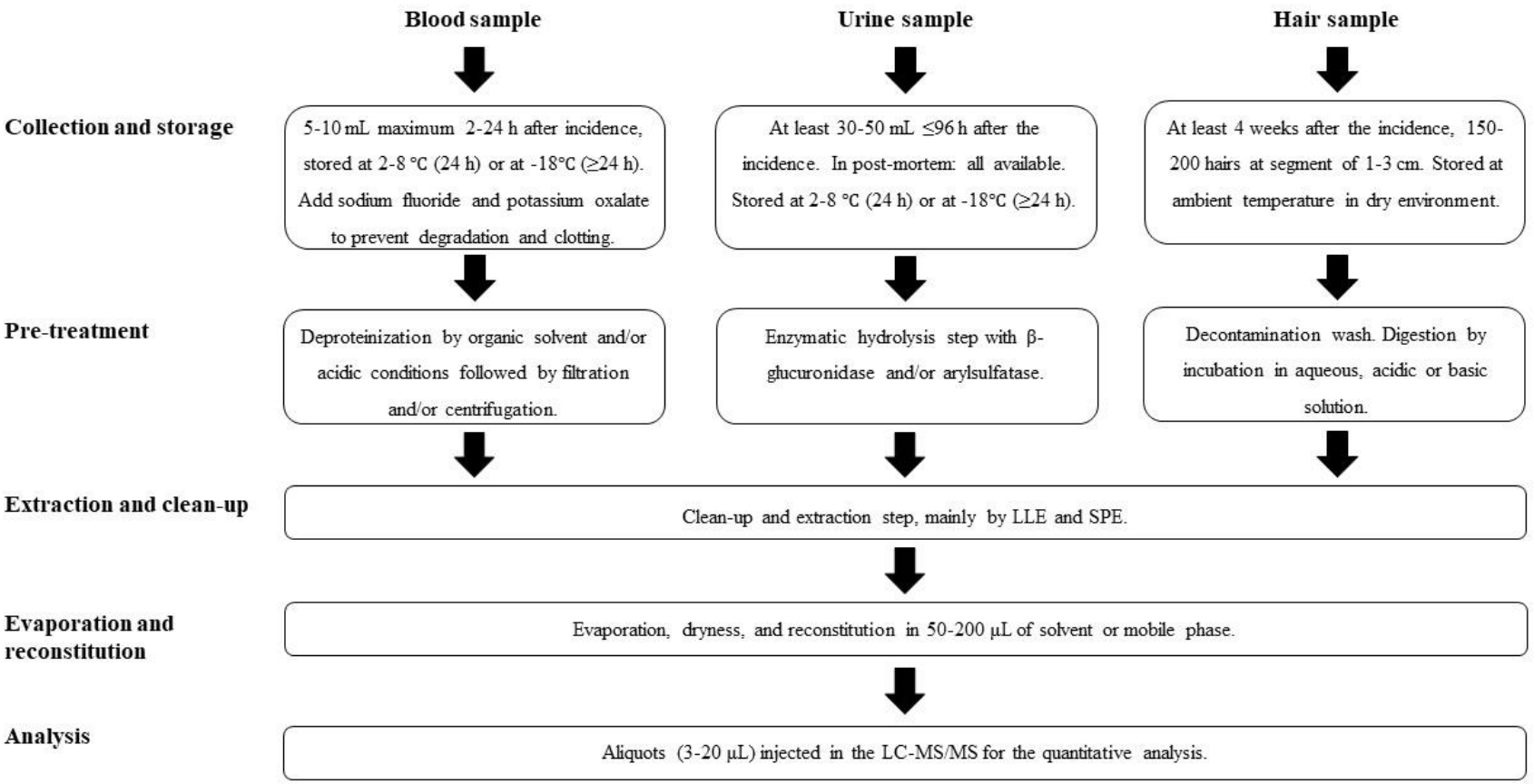


Figure 1. *Steps for sample collection and sample treatment for blood, urine, and hair samples in DFSA cases. LLE: Liquid-liquid extraction, SPE: Solid-phase extraction.*

However, a direct dilute-and-shot approach can be proposed if both parent compounds and metabolites are determined. In this sense, Jeong et al. [105] developed a fast and simple method for the identification and quantification of 18 benzodiazepines, zolpidem and their metabolites in urine. The method proposed the direct injection of 5 µL urine into the LC-MS/MS without any previous sample treatment except for centrifuging. Despite the lack of intensive sample preparation steps, the method was highly sensitive with LOQs ranging from 0.5 to 10 ng/mL and LODs in the range 0.15–1.5 ng/mL.

Hair samples require a decontamination step to avoid false-positive results. This step is usually performed by washing the sample with a small volume of water or organic solvents or both, to get rid of any external contamination. There is no standard procedure for decontamination [33] and different solvents have been proposed. Salomone et al. [77] employed two washing steps with dichloromethane while Wang et al. [82] employed a washing step with isopropanol followed by two steps with water. Leung et al. [108] included an aqueous wash with a surfactant (0.1% sodium dodecyl sulfate, SDS) to remove external contaminants from hair more efficiently. The Society of Hair Testing recommends that the hair washing step include both the use of an organic solvent and an aqueous solution [79]. It is also strongly recommended to study the efficiency of the washing step to remove the surface contamination and to include additional cleanup for hair samples heavily soiled with body fluids [81].

The extraction of hair samples usually involves a digestion step to remove proteins and other organic material so that the drug can be properly solubilized for analysis. There are different approaches for digestion, e.g., the use of aqueous strong basic or acidic conditions or of organic solvents, usually methanol, with or without the addition of a strong acid and enzymatic digestion. Under these procedures, the temperature can be also applied. Special attention must be given to the preservation of chemically unstable drugs and to keep metabolites un- altered. For example, under strongly alkaline conditions, 6-acetylmorphine (6-AM) is hydrolyzed to morphine and cocaine to benzoylecgonine [33].

Table 3. Representative extraction methods for the analysis of DFSA substances in blood, urine, and hair by LC-MS/MS from 2010 to 2021.

Group of drugs	Sample size	Extraction conditions	Evaporation (Yes/No) Reconstitution	Recovery	References
Blood, plasma, and serum					
Amphetamine-type-stimulant (11 drugs)	250 µL	Protein precipitation with 1 mL of cold ACN. Filtration.	Yes 500 µL 1 mM formic acid in MeOH.	60.2-86.2%	[111]
Benzodiazepines and Z-drugs (28 drugs)	500 µL	Pretreatment: pH adjustment by addition of 200 µL of carbonate buffer (pH 9.2). LLE (1 mL ethyl acetate).	Yes 100 µL ACN:H_2O, 2:8 (v/v).	81.0-106.7%	[112]
Benzodiazepines (27 drugs)	85 µL	Pretreatment: redissolution with a buffer for blood dried spots on filter paper. SPE (200 mg Bond Elut Certify I, mixed mode C8-SCX).	Yes 200 µL mobile phase.	30.9-119.2%	[113]
Scopolamine (1 drug).	500 µL	SPE (Oasis MCX cartridges, mixed mode polymeric phase-CX).	Yes 125 µL H_2O:ACN, 90:10 (v/v).	95%	[114]
Benzodiazepines (4 drugs)	200 µL	Pretreatment: pH was adjusted to 9.0 with ammonium hydroxide 5%. LLE (1.0 mL ethyl acetate).	Yes 200 µL ACN/H_2O.	95-109%	[115]
Benzodiazepines and Z-drugs (28 drugs)	1.0 mL	Pretreatment: pH adjustment and dilution to 2 mL with aqueous buffer pH 8.0. IL-DLLME (60 μL IL, BMIm-PF_6).	No Dilution 1:10 v/v with MeOH.	24.7-127.2%	[116]
Cocaine and cocaine metabolites (10 drugs)	-	Pretreatment: pH adjusted to 12 with 100 µL of ammonia solution. SPE (Oasis MCX cartridges, mixed mode polymeric phase-CX).	Yes 74 µL mobile phase.	Above 66.7%	[98]

Opiates, amphetamines, and cocaine metabolites (35 drugs)	100 µL	220 µL ACN and 40 mg of QuEChERS salts ($MgSO_4$, NaCl, sodium citrate).	No Dilution of organic layer 1/3; v/v with 5 mM ammonium formate /0.1 % formic acid buffer.	-	[99]
Methiopropamine (1 drug)	500 µL	SPE (Bond Elut Plexa PCX, CX).	Yes 100 µL 0.1% formic acid.	-	[100]
Amphetamine and psychoactive drugs (69 drugs)	200 µL	Protein precipitation with 600 µL ACN.	Yes 100 µL MeOH.	72-110%	[101]
Drugs of abuse and benzodiazepines (44 drugs)	500 µL	Pretreatment: protein precipitation 500 µL MeOH, dilution with 1 mL water and 100 µL carbonate buffer, pH adjustment to 9. DLLME (100 µL of chloroform and 250 µL of MeOH).	Yes 100 µL mobile phase.	66-120%	[102]
Urine					
γ-hydroxybutyrate GHB (1 drug)	1.0 mL	Pretreatment: dilution with 4 mL ACN and centrifugation.	No Dilution of 10 µL extract with 500 µL 50:50 (ACN/H_2O).	86.2-98.6%	[103]
Date-rape drugs; Z-drugs, ketamine, GHB (13 drugs)	1.0 mL	4-steps LLE Step 1: 6 mL ethyl acetate: dichloromethane, 3:1 at acid pH. Step 2: 2 mL Solvent A at acid pH. Step 3: 3 mL hexane :ethyl acetate : diethyl ether, 1:1:1 at basic pH. Step 4: 3 mL Solvent A at basic pH. *Solvent A =(Hexane:ethyl acetate:dichloromethane:diethyl ether, 1:1:1:1).	Yes 400 µL MeOH.	80.98-99.27%	[104]

Tricyclic antidepressants (5 drugs)	400 µL	Oasis 96-wellplate WCX	No Dilution in 1:1 with H_2O prior to injection.	Above 92%	[117]
Benzodiazepines, zolpidem, and metabolites (18 drugs)	1.0 mL	- Pretreatment: dilution, centrifugation.	No	63-104.6 %	[105]
Benzodiazepines (3 drugs)	1.0 mL	LLE (3 ml ethyl acetate and 0.5 ml 1.5 M carbonate buffer pH 9.5).	Yes. 500 µL ACN with 0.1% formic acid.	70.5-96.7%	[106]
Hair					
Benzodiazepines and Z-drugs (27 drugs)	20 mg	Pretreatment: decontamination wash. Solvent extraction (step 1:1 mL MeOH, step 2: 1 mL MeOH/2 mM ammonium formate buffer pH 3.5).	Yes 500 µL MeOH.	-	[16]
Zolpidem and main metabolites (3 drugs)	10 mg	Pretreatment: decontamination wash (1 mL H_2O then 1 mL acetone), digestion (MeOH, 50 °C, ultrasonication). SPE (HybridSPE-Phospholipid cartridge (30 mg of zirconia-based sorbent).	Yes 200 µL MeOH.	65.2-96.6%	[107]
γ-hydroxybutyrate GHB (1)	10 mg	Pretreatment: washed 5 min with MeOH followed by 2 washing steps with hot H_2O for 5 min. Final decontamination step with dichloromethane for 5 min. Hair samples dried over night at 55 °C. LLE (3 mL ethyl acetate).	Yes 100 µL mobile phase	13-23%	[80]
Amphetamine-type-stimulant (ATS) drugs (14 drugs)	30 mg	Pretreatment: decontamination wash, acid digestion (0.1 % HCl in MeOH). SPE (Strata X-C, mixed mode: reversed phase, normal phase, ion-Exchange).	Yes 75 µL mobile phase	40.5-92.1%	[48]
Ketamine and norketamine (2)	2.5 mg	Pretreatment: decontamination wash acetone, 0.1% sodium dodecyl sulfate (SDS), deionized water and final acetone.	Yes 50 µL mobile phase	Mean 96.7%	[108]

		Solvent extraction (100 µL MeOH: ACN:20 mM ammonium formate in a 25:25:50, v/v/v).			
Benzodiazepines, z-hypnotic, ketamine, and scopolamine (14)	100 mg	2-mL aliquot of methanol was added to the dried hair. The samples were incubated at 55°C for 15 h.	Yes 50 µL mobile phase	-	[77]
γ-hydroxybutyrate GHB (1)	25 mg	Pretreatment: decontamination wash overnight (0.01 N 1.0 mL NaOH, at 56 °C). LLE (600 µL of 1 M H_2SO_4 and 3 mL of ethyl acetate).	Yes. 50 µL mobile phase	89.7-97.9%	[109]
Ketamine and norketamine (2)	10 mg	Pretreatment: decontamination wash and digestion. Molecularly imprinted polymer (MIP cartridges).	Yes 100 µL mobile phase	86% and 88%	[110]

LLE: Liquid-liquid extraction, SPE: Solid-phase extraction, IL-DLLME: Ionic liquid-dispersive liquid-liquid microextraction, BMIm-PF_6: 1-Butyl-3-methylimidazolium hexafluorophosphate, QuEChERS: Quick, Easy, Cheap, Effective, Rugged and Safe; SXC: strong cation exchange, WCX: weak cation exchange

5. Sample extraction/clean-up

Among the different extraction strategies employed in DFSA analysis, the most often used are liquid-liquid extraction (LLE) and solid phase extraction (SPE). LLE does remove proteins and provides some clean-up, but requires the use of hazardous organic solvents, labor-intensive evaporation and reconstitution steps and it can be affected by the formation of emulsions [119]. Due to the polar character of the target drugs, ethyl acetate (1–3 mL, several repetitive steps) has been the most employed organic solvent (as shown in **Table 3**) and applications are usually limited to specific classes of drugs. A low LOD value of 0.01 mg/mL for diazepam, zolpidem and temazepam was obtained after LLE with ethyl acetate of 0.5 mL of blood [112]. Recoveries for diazepam, zolpidem and temazepam were 98.9%, 95.5%, and 99.6% respectively. Alternatively, Anilanmert et al. [104] used a combination of different solvents (hexane, ethyl acetate, dichloromethane, diethyl ether) to perform a four steps LLE under acidic and basic conditions that were able to cover a wide range of DFSA drugs (date-rape drugs; Z-drugs, ketamine, GHB). The LODs ranged from 0.59 to 49.50 ng/mL and the recoveries were between 80.98 and 99.27%.

Innovative approaches based on the use of alternative solvents and miniaturization have been also proposed to a lesser extent. A method based on the ionic liquid-based microextraction of 28 benzodiazepines in blood was proposed to fulfill the need for faster and more environ- mentally friendly alternatives [116]. The technique consists in adding a small volume of an ionic liquid (60 µL of BMIm PF6) to 1 mL of blood. The extraction was done in just 5 min and the extract was analyzed after simple dilution in methanol. Recoveries and matrix effects (which were compensated by matrix-matched calibration) were generally worse than those reported with conventional procedures (recoveries: 24.7%– 127.2%, matrix effects: 20.0%–92.6%) and the method could only be validated for 19 out of 28 compounds. This method was later applied to the analysis of 24 benzodiazepines in postmortem blood samples [120]. Dispersive liquid-liquid microextraction (DLLME) has been also employed in blood samples for the detection of antidepressants and anticonvulsants [102] and the detection of GHB in both beverage and hair samples [121]. In the DLLME study of Fisichella et al. [102], the volume of the extractant phase (chloroform, 100 µL) and

dispersion phase (methanol, 250 µL) were optimized to improve recoveries (66–100%) and matrix effects (28% suppression or enhancement).

Other promising treatment procedure was presented in 2006 as electromembrane extraction (EME). The method is based on applying an electric field in which charged substances will migrate through a sup- ported liquid membrane (SLM) and into a solution carrying the inverse charged electrode. In comparison with other approaches, EME provides short extraction times, reduction in the consumption of organic solvents and high selectivity [122]. This method has been successfully tested on real forensic samples for the extraction of 3 barbiturates from human blood and urine with recoveries between 51% and 90% [123]. Authors proposed the use of tributyl phosphate with zero hydrogen-bond acidity as supported liquid membrane (SLM) due to its high polarity-polarizability. Nevertheless, it has not been applied in DFSA studies yet.

Regarding hair, ethyl acetate has been the most used solvent for the extraction of both polar and nonpolar drugs [109]. More specific solvent mixtures have been proposed for studies oriented towards specific compounds, e.g., a mixture of methanol:acetonitrile:20 mM ammonium formate (25:25:50, v/v/v) was employed for the determination of ketamine and norketamine in hair [108].

SPE has been used to a wider extent than LLE in DFSA analysis. It enables sample clean-up, preconcentration and the production extracts that are readily amenable for LC-MS analysis. There is a wide variety of commercially available sorbents with different binding mechanisms and hybrid operation modes so that a wide range of drugs can be covered. In general, SPE methods are also regarded as lengthy and labor-intensive, and they can require some sample pretreatment to avoid clogging. However, there are well-established protocols for drug analysis as well as novel formats (e.g., disposable syringe barrel or 96-well plates) intended to increase the sample analysis throughput in forensic sciences. Mixed-mode SPE sorbents, with a combination of reverse phase and ion exchange mechanisms, have been the most employed to extract a wide spectrum of compounds. Benzodiazepines have been extracted from blood with a nonpolar C8 strong cation-exchange (SCX) sorbent (trade name: Bond Elute Certify), which is recommended for the extraction of basic drugs. Recoveries greatly

varied between 31 and 119% [113]. Both blood and dried blood spots (DBS) on filter papers were analyzed, the latter providing recoveries about 10%–15% lower due to incomplete desorption from the paper prior to SPE. DBS allowed stabilizing the analytes at room temperature during a 3-months period facilitating the storing of blood postmortem samples [124]. However, if the method is applied to other compounds, stability issues can arise at room temperature, especially for compounds that carry ester moieties (e.g., cocaine) or sulfur atoms (e.g. phenothiazines or olanzapine) or drugs that are easily oxidized or reduced, such as nitrobenzodiazepines [125]. Lendoiro et al. [48] used a normal phase/reverse phase/ion ex-change multi-mode sorbent (trade name: Strata X–C) to efficiently extract 14 amphetamine-type stimulants from hair (after decontamination and digestion) with recoveries between 41 and 91%.

Alternative sorbents have been also proposed in the extraction of different compounds. Thus, Kim et al. [107] employed a HybridSPE technique in which a porous silica sorbent coated with zirconia was used for clean-up of hair samples. Zr acted as a Lewis acid. The sorbent was used to bind zolpidem and metabolites, which acted as Lewis bases since their carboxylic moieties were supposed to be deprotonated at the pH of the hair extract (~5) [126]. This type of sorbent was proved superior to other tested materials namely, filtration with PTFE filter paper (0.2 µm), dispersive SPE (QuEChERS, MgSO4 and PSA), hydrophilic-lipophilic balanced SPE (Waters Oasis HLB), cation-exchange-based SPE (Waters Oasis MCX), and anion-exchange-based SPE (Waters Oasis MAX). Among the tested techniques, the zircon-based HybridSPE provided the highest efficiency.

On the other hand, Dulaurent et al. [99] proposed the use of QuEChERS (a one-step quick, easy, cheap, effective, rugged, and safe method) to cover the analysis of 35 compounds including opiates, amphetamines, and cocaine metabolites in blood. Highly selective molecularly imprinted polymers (MIPs) have been applied to specific compounds, namely ketamine and norketamine [110]. Recoveries from hair samples were in the range 86–88% with minimal matrix effects (ion suppression —6.8%/ion enhancement +0.2%)

Table 4. Stationary and mobile phases and MS analyzers used in the LC-MS/MS analysis of DFSA substances and the corresponding detection and quantification limits.

Drugs	Stationary phase	Mobile phase	Type of instrument	Injection volume	LOD	LOQ	Ref
Amphetamine-type stimulants and synthetic cathinones	Restek Raptor Biphenyl column	A) H_2O. B) MeOH. Both containing 2 mM ammonium formate and 0.1% (v/v) formic acid.	LC-triple quadrupole MS.	3 µL	0.5-1.7 ng/mL	5 ng/mL	[111]
Benzodiazepines and Z-hypnotic drugs	EC-C18 column	A) H_2O. B) ACN. Both containing 0.05% formic acid.	LC-triple quadrupole MS.	10 µL	0.01-0.33 ng/mL	1 ng/mL	[112]
Benzodiazepines and Z- hypnotic drugs	Kinetex® F5 column	A) H_2O. B) ACN. Both containing 1 mM ammonium formate and 0.1% formic acid.	LC-QTrap MS.	-	-	0.5-10 pg/mg	[16]
Benzodiazepines	Kinetex C18 column	A) H_2O. B) ACN. Both with 0.1% (v/v) formic acid.	LC-QTrap MS.	5 µL	0.1-50 ng/mL	5-100 ng/mL	[113]
γ-hydroxybutyrate GHB	Symmetry C18 column	A) H_2O. B) ACN. Both containing 0.2% ammonium formate and 0.2% formic acid.	LC-QTrap MS.	10 µL	0.1 ng/mg	0.3 ng/mg	[80]
Scopolamine	Zorbax XDB-C18 column	A) H_2O. B) ACN. Both containing 0.1% v/v formic acid.	LC-triple quadrupole MS.	15 µL	-	5 pg/mg *LLOQ*	[114]

Drugs of abuse	1) Kinetex XB C18 2) Kinetex PFP	A) H_2O. B) MeOH/ACN 50:50 Both with 0.1% formic acid.	LC-Q Exactive MS.	6 µL	0.1-5	0.2-50	[134]
Fentanyl and fentanyl analogues	Raptor biphenyl LC column	A) H_2O with 10.0 mM ammonium formate and 0.1% formic acid. B) ACN with 0.1% formic acid.	LC-triple quadrupole MS.	-	0.05 ng/mL	0.1 ng/mL *LLOQ*	[132]
Amphetamine-type-stimulant (ATS) drugs	Atlantis® T3 reversed-phase C18	A) Ammonium formate 2mM with 0.1% formic acid. B) ACN.	HPLC- triple quadrupole-MS	30 µL	0.2-5 pg/mg	2-20 pg/mg	[48]
Benzodiazepines	Symmetry C18 column	Isocratic elution: MeOH: H_2O:ACN (50:30:20, v/v/v) with 0.05% of formic acid.	LC-triple quadrupole MS.	10 µL	0.8-34.8 ng/mL	10-100 ng/mL	[115]
Benzodiazepines and Z-hypnotic drugs	Kinetex Biphenyl LC	A) Aqueous buffer pH 8.0. B) MeOH.	LC-QTrap MS.	10 µL	0.003-4.74 ng/mL	2-50 ng/mL	[116]
GHB	BEH C18 column	A) H_2O 0.1% formic acid of 10 mM ammonium acetate. B) ACN.	LC-triple quadrupole MS	-	0.05 µg/mL	-	[103]
Opiates, amphetamines, and cocaine metabolites	Pinnacle DB Pentafluorophenyl (PFP) column	A) 5 mM ammonium formate/0.1 % formic acid buffer. B) ACN.	LC-triple quadrupole MS.	5 µL	3 ng/mL *LLOD*	5 ng/mL *LLOQ*	[99]
Amphetamines and new psychoactive substances	Zorbax Eclipse Plus C18	A) 5 mM aqueous formic acid. B) ACN.	LC-triple quadrupole MS.	7 µL	0.05-0.3 ng/mL	0.1-0.5 ng/mL	[118]
GHB, ketamine, norketamine, phenobarbital,	Poroshell C18 column	A) 0.1% formic acid. B) ACN.	LC-triple quadrupole MS.	10 µL	0.59-49.5 ng/mL	6-80.8 ng/mL	[104]

thiopental, zolpidem, zopiclone, phenytoin							
Drugs of abuse, benzodiazepines, and other psychotropic medications	Kinetex Biphenyl column	A) H_2O. B) MeOH. Both with 0.1% formic acid.	LC-triple quadrupole MS.	10 µL	0.05-2 ng/mL	0.2-10 ng/mL	[102]
Benzodiazepines, zolpidem, and their metabolites	Zorbax SB-C18	A) H_2O. B) ACN. Both containing 2 mM ammonium trifluoroacetate and 0.2% acetic acid.	LC-QTrap triple-quadrupole MS.	5 µL	0.15-3 ng/mL	0.5-10 ng/mL	[105]
Zolpidem, zopiclone and metabolites	PFP column	A) H_2O 0.05% formic acid and 10mM ammonium formate. B) MeOH 0.05% formic acid.	UHPLC-HR-MS Orbitrap	10 µL	0.5 ng/mL	0.5-2 ng/mL	[133]
Flunitrazepam, nimetazepam and nitrazepam	C18 column	A) 5:95 ACN:H_2O. B) 95:5 ACN:H_2O. Both with 0.1% formic acid.	LC-triple-quadrupole MS.	50 µL	0.125-1 ng/mL	0.25-5 ng/mL	[106]
Benzodiazepines, z-hypnotic, ketamine, and scopolamine	Synergi Fusion-RP column based on C18	A) H_2O 5 mM formic acid. B) ACN.	LC-triple-quadrupole MS.	15 µL	0.2-4 pg/mg	0.7-13.2 pg/mg	[77]
Ketamine and norketamine	Synergi Hydro RP column based on C18	A) H_2O 3 mM ammonium formate +0.001% formic acid. B) ACN.	LC-IonTrap-MS	20 µL	0.10-0.14 ng/mg	0.18-0.23 ng/mg	[110]

6. Instrumental analysis

Immunoassays are mainly used for initial screening purposes since the presence of interfering substances can lead to false-positive results [127], while GC-MS and LC-MS are the preferred confirmatory techniques. Many authors point out that LC-(ESI)MS/MS is the most suitable technique for the determination of both illegal drugs and pharmaceutical substances in the different human matrices [128]. The main advantage is the direct applicability to a wider polarity range of com- pounds and to non-volatile compounds. The fact that GC-MS instruments are cheaper, and the availability of universal GC-MS libraries are benefits that can explain why this technique is still widely implemented in routine laboratories.

Some authors have compared GC-MS and LC-MS methods for the analysis of drugs of abuse and the advantages of the latter were high- lighted. For example, Perez et al. [27] found similar analytical features and compliance criteria for the LC-MS/MS and GC-MS analysis of benzodiazepines in urine. LC-MS/MS offered the advantages of not requiring extensive sample preparation and provided shorter run times while matrix effects were corrected by the use of deuterated internal standards. Vaiano et al. [129] compared the LC-MS/MS and GC-MS analysis of propofol (a hypnotic agent) by using two different derivatization methods (silylation for GC-MS and azo-coupling derivation for LC-MS/MS). The latter provided an easily ionizable compound in ESI that resulted in better detection limits (0.0004 in urine and 0.1 ng/mL in blood) than those obtained by GC-MS (0.3 in urine and 5 ng/mL in blood) and shorter run times.

The use of CE-MS is by far more limited in comparison with LC-MS and GC-MS in forensic drug analysis. Nevertheless, some authors have claimed their potential in terms of low consumption of reagents and of samples and short analysis times with high-resolution separation for the analysis of charged drugs [130,131].

Table 4 provides an overview of representative DFSA studies that employ LC-MS analysis [16,80,99,102,104–106,111–116,118,132,133]. The target compounds, stationary and mobile phases, injection volume, detection (LODs) and quantification limits (LOQs), and the type of MS instrument are indicated.

Liquid chromatographic separation of drugs has been primarily carried out on an alkyl-bonded silica column made up of octadecylsilane (C18) [80,112,113,134]. However, this stationary phase often exhibits peak tailing for basic compounds due to the presence of free silanol groups, even with the use of improved bonded phases [135]. Further- more, polar and charged compounds are poorly retained giving rise to peak asymmetry, low system efficiency and poor reproducibility [136]. In this sense, alternative reverse-phase liquid chromatography phases have been proposed, which offer secondary polar interactions through embedded polar groups or hydrophobic π–π active aromatic moieties. Thus, the use of biphenyl (BP) [102] and pentafluorophenyl (PFP) columns [99] have been proposed to promote retention via π–π interactions in DFSA analysis [132].

Regarding the mobile phase, gradient elution with water (usually buffered and/or acidified with ammonium formate and formic acid) and acetonitrile are preferred. LC with tandem MS based on triple quadrupole or QTRAP (see **Table 4**) has been the most employed technique given its robustness and suitability for quantification at trace levels. The registration of at least two MS/MS transitions per compound is required for drug confirmation. Reported studies with QTRAP employed the instrument in "triple quadrupole" operation mode and did not use the linear ion trap for MS3 measurements. Deuterated standards were widely employed for calibration purposes in order to account for sample treatment losses, matrix effects and instrument signal fluctuations [137]. The use of high-resolution MS instruments (mainly Orbitrap) has been proposed to a lesser extent. Metabolites of zolpidem were properly identified with LC-Orbitrap-MS analysis [133]. The higher identification capacity and possibilities for retrospective analysis of the new generation of high-resolution MS instruments suggest an increasing use for DFSA analysis in the near future.

It is also worth mentioning that both parent compounds and metabolites are determined [7,12,48,72,95,98,99,105,107,133], which is common due to the fast metabolic rate of the drugs. Regarding Phase II metabolites (glucuronide or sulfate conjugates), as we specified in Section 4, urine samples are usually subjected to enzymatic treatment before analysis, so that they are converted into the free form and the parent compound is analyzed. However, some authors propose to analyze the

conjugated forms too in order to get more information. Regarding phase I metabolites, they are frequently analyzed together with parent compounds, for example, for MDMA (ecstasy), we find MDA (3,4-methylenedioxyamphetamine), HMMA (4-hydroxy-methoxymethamphetamine) and HMA (4-hydroxy-3-methoxyamphetamine) in urine. For THC, the major urinary phase I metabolite is 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH). Benzoylecgonine, ecgonine methyl ester, norcocaine and norbenzoylecgonine are major phase I metabolites of cocaine. For heroin and morphine, we find 6-acetylmorphine or 6-monoacetylmorphine (6-MAM) in urine [138]. All these metabolites are more polar than their parent compounds and this can require a suitable adjustment of the chromatographic separation, e.g., by the selection of a column providing secondary polar interactions (e.g. PFP) [99] and a suitable mobile phase gradient. Analysts have to be also careful during sample preparation since polar compounds are more easily missed.

The analysis of metabolites can be also key for preventing false- positives coming from external contamination, which is especially relevant in hair analysis [33]. In this case, only metabolites that are not prescribed as medication can be targeted. For example, diazepam can be metabolized to nordazepam, temazepam, and oxazepam but all these drugs can be ingested as parent compounds too. Wang et al. targeted nordazepam and temazepam in hair since there were not prescribed pharmaceuticals in Denmark, where the study was carried out [82]. The ratio of the parent compound and the targeted metabolites can be used to distinguish a single dose from chronic use which is essential in DFSA cases [81].

According to the guidelines for the forensic analysis of drugs facilitating sexual assault and other criminal acts by the UNODC issued in 2011 [4], the minimum required performance limits (MRPLs) in urine have been set for the analytical methods used in this field. These MRPLs rank from 1 ng/mL for ketamine and norketamine to 10 mg/L for GHB. The MRPLs of benzodiazepines in urine is 5 ng/mL for clonazepam, flunitrazepam, nitrazepam, phenazepam and triazolam while for the rest of benzodiazepines is set at 10 ng/mL. Consequently, the reported LODs and LOQs of LC-MS methods (**Table 4**) are sensitive enough to determine these substances in urine.

For example, method LODs ranged from 0.1 ng/mL to 3 ng/mL for benzodiazepines [105,106] and were 0.05 mg/L for GHB [103].

Finally, for an analytical method to be adopted in DFSA investigations, guidelines recommend at least evaluating the following analytical parameters: selectivity, calibration model (linearity), accuracy and precision, the lower limit of detection (LLOD), the lower limit of quantification (LLOQ) and analytical stability. Furthermore, other analytical parameters that may be required during the validation study are recovery, ruggedness (robustness) and matrix effects. Matrix effects are of particularly major concern for LC-MS analysis with ESI since it is common under this soft ionization technique. Matrix effects should be evaluated in any method development because it is detrimentally affecting other analytical parameters such as accuracy, precision, and sensitivity [139].

7. Conclusion

Despite the widely use of alcohol for intoxicating victims, a wide variety of pharmaceutical and illegal drugs (e.g., benzodiazepines, z-hypnotics drugs, cannabinoids, or cocaine) are being increasingly employed by perpetrators alone or in combination with alcohol to commit DFSA crimes. The analysis of traces of these compounds demands more sensitive and wide-scope analytical approaches and sampling procedures. Studies prove that blood and urine are still the desired matrices in alleged DFSA cases although the use of other nonconventional samples (hair, vitreous humor, etc.) is increasing due to the need for longer windows of detection. LLE and SPE are still the most widely applied extraction methods but there is an increasing interest in the development of microextraction formats and dilute and shoot strategies. Although GC-MS is still used in forensic routine analysis, LC-MS/ MS (both low and high-resolution MS) has been consolidated as a more suitable choice for the analysis of multiple drugs due to its high sensitivity, specificity, and no need for derivatization.

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.

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Appendix A. Supplementary data

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SUPPORTING INFORMATION

Analysis of conventional and nonconventional forensic specimens in drug-facilitated sexual assault by liquid chromatography and tandem mass spectrometry

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Main classes of DFSA drugs: prevalence and human effects

Alcohol

According to **Table 2**, alcohol drinks or ethanol is the most detected substance in alleged sexual assault globally. The study of Hindmarch et al. [1] showed that 67% of all analyzed urine samples from alleged sexual assault victims (2,026 cases) were positive for ethanol, followed by cannabis in 30% of the cases. The Forensic Science Service (London, UK) confirmed that 470 out of 1,014 DFSA cases (46%), were positive for alcohol alone or with other substances [2]. In a recent study carried out in the United States, ethanol was also the most prevalent substance in a total of 1,000 studied cases of alleged sexual assault with a frequency of around 30% followed by cannabinoids with 28% [3]. In another recent study published in Peru on DFSA cases reported between 2016 and 2018 [4], ethanol came in the second place; alone or in combination with psychotropic drugs and other illicit drugs, counting for 28% of the cases, while benzodiazepine group was the first group with 58% of the cases.

In general, the percentage of cases that identified ethanol as an intoxicating substance among those listed in **Table 2**, ranged from 22.2% to 76.9%. The leading role of ethanol in DFSA cases has been frequently reported by previous literature and technical studies, either alone or in combination with other pharmaceutical or illicit drugs [5]. The clear predominance of ethanol could be referred to the wide acceptance of alcohol consumption in the western communities and to the fact that the majority of DFSA cases occurred in leisure settings [6].

Cannabinoids

A Cannabinoid is a group of compounds found in the *Cannabis Sativa* plant. They exert psychoactive effects and they have been used for a long time for medical and recreational purposes. Cannabis derivatives contain a wide variety of chemical compounds being the primary psychoactive constituent of the well-known delta-9-tetrahydrocannabinol (THC) [7]. Other frequently detected compounds are 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) as the main active metabolite of THC and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) as the main secondary metabolite. Many cannabis-based medications have been approved for the treatment

of different symptoms associated with chemotherapy, such as nausea and vomiting and they are also used with multiple sclerosis to reduce pain and spasticity [8].

In a very recent DFSA study in New Zealand [9], cannabis was the most frequently detected drug after alcohol, with 31 positive blood samples and 15 positive urine samples out of 162 DFSA cases. In another study performed in the USA in 2019 [3], 288 out of 1,000 cases (28.8%) were positive for cannabinoids ranking also second after alcohol. The study also stated that cannabinoids were the most abundant drugs detected in combination with ethanol in all the alcohol-drug combination positive cases. Accordingly, in a DFSA study performed in Italy in 2018 [10], cannabis was detected in 19 cases (7.4%) raking also second after alcohol (57 cases, 22.2%). Finally, the Forensic Science Service London Laboratory outlined in 3 years study [2] that out of 1,014 claimed DFSA cases, 260 (26%) were positive for cannabis, being the second most detected drug after alcohol (470 cases, 46%) in this study too. In 2 out of the 14 reported studies in **Table 2**, cannabinoids were the most detected substances in DFSA cases with a detection frequency of 58% and 33.7% in both the USA and Canada, respectively, being even more frequently detected than alcohol (30.9-43%) [11,12].

The frequent use of cannabis in sexual assaults can be referred to as the ease of obtaining it and the emergence of less stringent regulations that allow its use for medical or recreational aspects or both. For example, in the USA cannabis has been legalized for medicinal and/or recreational uses in around 34 states [13].

Benzodiazepines and z-hypnotic drugs

Benzodiazepines are prescribed sedative-hypnotic substances that are indicated for the treatment of several CNS disorders, such as insomnia, anxiety, seizures, convulsions, agitation, muscle spasms, and alcohol withdrawal [14,15]. At higher doses, benzodiazepines can develop loss of consciousness, dissociation, memory loss, and respiratory depression [16].

According to **Table 2**, benzodiazepines are in the second or third rank of drugs reported in DFSA cases with roughly 9% to 43% of DFSA cases. In the study performed in Taiwan, where the level of alcohol in blood was not monitored, benzodiazepines constituted the first class of drugs involved in sexual assault in about 43% of the cases

[17]. Clonazepam was detected in 7.6% of cases and ranked first among benzodiazepines in the study of Fiorentin and Logan [3] followed by alprazolam (7.2%). In a three-year study by Banaszkiewicz et al. [18], nordiazepam; one of the diazepam metabolites, was prevalent in the blood samples of 87 victims out of 145 DFSA cases (60%) followed by diazepam (81 victims, 55.9%). Diazepam was also frequently detected in the study by Poulsen et al. [9] in 6 out of 14 blood samples and 9 out of 19 urine samples. Another frequently detected benzodiazepine metabolite, known as oxazepam, was observed in 56 out of 145 blood samples (38.7%) from Poland [18], in 13 out of the 1,000 blood and urine samples (1.3%) from the USA [3] and in 3 out of 25 hair samples (12%) from Denmark [19].

Z-compounds or z-hypnotics, namely, zopiclone and zolpidem, also belong to the benzodiazepine receptor agonists and due to their similar pharmacological effects, they can be also detected in DFSA cases [20]. Lee et al. [17] reported a total of 5 cases (4%) using zolpidem to facilitate sexual assault. Furthermore, Fiorentin and Logan [3] detected zolpidem in 6 out of 1,000 cases (0.6%) and in another 2 cases with zopiclone (0.2%). In a very recent case report by Carfora et al. [20], in which a female was sexually assaulted by a group of men after offering her an alcoholic drink, hair samples were collected 7 months after the incident.

Amphetamine and amphetamine derivatives

Amphetamine, the very well-known CNS stimulant, is a psychostimulant drug that is prescribed to treat attention-deficit hyperactivity disorder (ADHD). Globally, they are the second most widely used group of illicit drugs after cannabis [21]. Synthetic amphetamines are known as amphetamine-type stimulants (ATS) and include a wide range of substances such as 3,4-methylenedioxyamphetamine (MDA), the famous 3,4-methylenedioxymethamphetamine (MDMA), and 3,4-methylenedioxy-N-ethylamphetamine (MDEA).

These drugs rank after alcohol, cannabis, and benzodiazepines with 5.3% to 24.1% of alleged sexual cases (**Table 2**). In a very recent study performed in New Zealand on 162 DFSA victims between 2015 and 2018 [9], methamphetamine was the second most commonly involved drug and it was detected in 46 blood and urine

samples out of 162 cases. MDMA was also detected in this study but in lesser extent in comparison to methamphetamine with only 3 positive cases.

Antidepressants, antipsychotic, and opioids

Although they are not as prevalent as the previously mentioned drugs, other pharmaceutical compounds such as antidepressants, antipsychotics, and opioids have been also widely reported [22]. Antidepressants are a group of pharmaceutical compounds that are used to relieve depression disorder symptoms, treat anxiety disorder, and treat chronic pain [23]. Since antidepressants inhibit the reuptake of neurotransmitters in the brain and lead to produce dizziness, drowsiness, sleepiness, and blurred vision [16], they are attractive to criminals for their use in sexual assault. In the study performed by Poulsen et al. [9] in New Zealand on 162 DFSA cases, citalopram and fluoxetine were the most detected antidepressants being present in 8 blood and 10 urine samples and 6 blood and 8 urine samples, respectively. Citalopram was also the most frequently detected antidepressant (50 cases out of 173 DFSA positive cases) in a study carried out in the USA on 1,000 suspected DFSA cases [3].

Poulsen et al. [9] reported the presence of quetiapine, a well-known antipsychotic drug, which is used to treat schizophrenia, bipolar disorder, and major depressive disorder combined with antidepressants [24]. Quetiapine was the third most frequently detected drug in the category of antidepressants, anticonvulsants, and antipsychotics, being present in 4 blood and 5 urine samples out of 28 blood samples and 41 urine samples. A recent case report from Denmark also confirmed the use of quetiapine in a DFSA case that was committed by spiking a female teenager's drink and sexually attacking her. The report was the first case to detect quetiapine in hair after a single dose and it correlated well with the hair concentration with levels in blood and urine samples collected after 43 hours from the incidence [25].

Opioids are another natural pharmaceutical group used as painkillers [16]. Opioids are the second group of drugs most commonly abused by adults [26]. In the DFSA study in the UK in 2005, opiates, such as codeine, morphine, dextropropoxyphene, and methadone, were detected with a frequency of 10.2%, being codeine and morphine the most frequently detected compounds [2]. In the

Canadian study of 2010 on 178 DFSA cases [12], opioids were detected in 24 cases (13.4%) in which codeine was present in 8 cases and morphine in 7 cases. In a study conducted in 2013 in Norway [27], opioids (mainly codeine) were detected in 3.6% of the DFSA cases.

Ɣ-hydroxybutyric acid (GHB)

Another well-known and one of the first discovered DFSA substances which act as CNS depressant is the γ-hydroxybutyric acid GHB. GHB was prescribed for the treatment of alcohol withdrawal symptoms [28]. GHB produces sedation, drowsiness, and memory lapses [29]. The onset time of GHB is between 20-60 min and the intoxication dose might start from 15 mg/kg [16]. Due to the very short $t_{1/2}$ of GHB of around 1 hour, it has a narrow window of detection that is not exceeding 12 hours. Consequently, the prevalence of GHB in suspected sexual attacks studies is difficult to determine and it is highly dependent on the time of sampling [3]. Another significant challenge with GHB is that it is synthesized endogenously by the neurotransmitter GABA (gamma-aminobutyric acid) [30]. As a result, its concentration increases normally between death and autopsy, and this should be considered in postmortem cases. The overlapping between the normal endogenous increase and due to the exogenous antemortem administration makes the interpretation of the analysis very challenging [31].

In the study performed in the USA on 1,000 DFSA cases, 5.9% were positive for GHB [3]. In an earlier study also performed in the USA on 2,026 cases [1], 100 positive cases (4.9%) were also linked to the use of GHB. Authors stated that these figures could have been underestimated due to the time lapse between the incidence and the analysis. In a Canadian study [12], GHB was only detected in 1.1% of the reported sexual assaults.

Ketamine

Ketamine is a short-acting dissociative anesthetic agent that causes amnesia and difficulty in fighting off an assailant [32]. Ketamine amnesia may last from 1-2 hours after a single dose of 6-13 mg/kg, in which victims will not be able to remember the events while they were under the influence of the drug [33]. Moreover, ketamine

is odorless and tasteless, and for this reason, it can be readily added to drinks without being detected by victims [34]. Recreational use of ketamine is also widely spread in young people at parties and nightclubs. In fact, ketamine is known as club-drug, due to its stimulant and hallucinogenic effects [35]. It is sometimes mixed synergistically with other drugs, mainly with cocaine. Ketamine has been reported in a retrospective study on alleged DFSA cases by the National Institute of Toxicology and Forensic Sciences in Madrid and it was detected in 3 out of 38 positive cases related to the use of illicit-drugs (7.9%) [6]. In Taiwan, the analysis urine samples from 126 DFSA victims' revealed that 10 (7.9%) were positive for ketamine and its main metabolite norketamine, making this drug the second most detected after flunitrazepam (11.1%) [17].

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Chapter II

Green nanostructured liquids for the analysis of urine in drug-facilitated sexual assault cases.

Analytical and Bioanalytical Chemistry
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PAPER IN FOREFRONT

Green nanostructured liquids for the analysis of urine in drug-facilitated sexual assault cases

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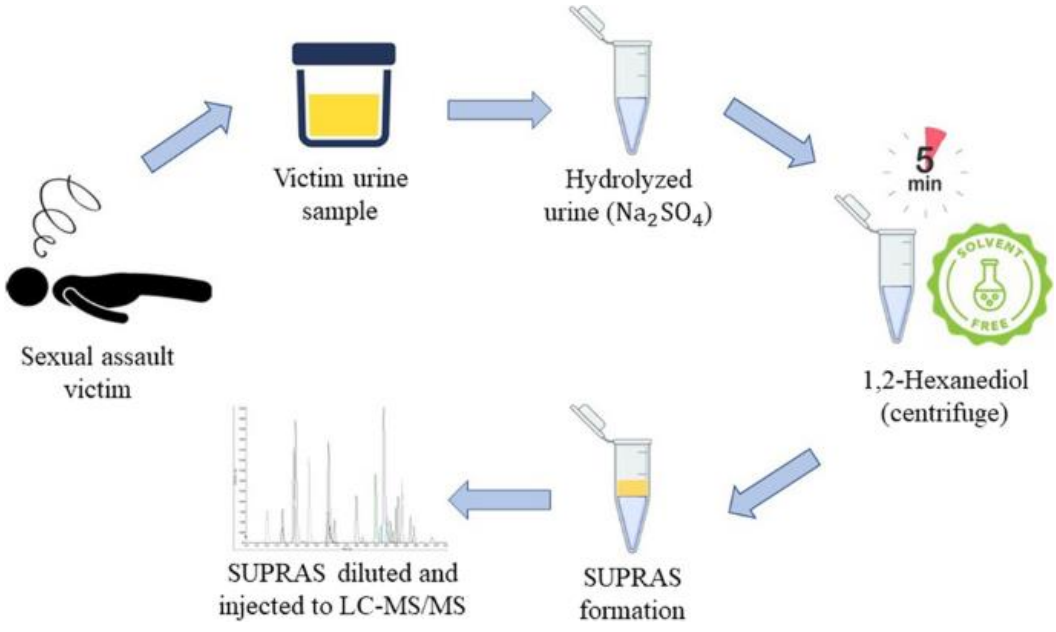
GRAPGICAL ABSTRACT



ABSTRACT

In this work, we optimize and validate a simple, time-saving, and environmentally friendly sample preparation method based on supramolecular solvents (SUPRAS), green nanostructured liquids, for the extraction of selected drug-facilitated sexual assault (DFSA) substances from human urine. The methodology was fast and simple (stirring, centrifugation, and dilution). Cubosomic SUPRAS were formed by the addition of 1,2-hexanediol (200 µL) to 1.0 mL of human urine containing 1 M Na_2SO_4. SUPRAS extracts were analyzed by LC-MS/MS. The method was fully validated for 23 DFSA compounds including 10 benzodiazepines, 1 z-hypnotic drug, 5 amphetamine derivatives, 3 cocaine metabolites, and 4 miscellaneous compounds. Extraction efficiency varied between 79 and 119%, and matrix effects were acceptable (−14.3/+21.5) for 87% of the compounds. Method detection and quantification limits ranged from 0.003 to 0.75 ng/mL and from 0.01 to 2.50 ng/ mL, respectively. These values were low enough for the established minimum required performance limits (MRPL) of these substances. This simple and green method has a great potential to be implemented for the monitoring of illegal drugs involved in DFSA cases by forensic laboratories.

KEYWORDS

Supramolecular solvents · Drug-facilitated sexual assault · Urine · Benzodiazepines · Cocaine · LC-MS/MS

INTRODUCTION

In the last two decades, social concern regarding drug- facilitated sexual assault (DFSA) has rapidly increased, a phenomenon in which a sexual attack is conducted under the influence of a certain pharmaceutical or other illegal substances [1, 2]. These substances incapacitate the victim to resist and bring them to a state of inability and delirium which facilitates any assault or violation [3]. DFSA cases are complicated to solve and require collaboration between the victim, police, medical staff, and scientific experts [4].

The analysis of substances involved in DFSA cases is challenging for forensic laboratories, and it has been an intensive field of research since the 1990s. DFSA compounds, like other illegal drugs, are determined in a variety of biological matrices that include blood, urine, and hair [5–8]. However, in sexual assault incidences, the time lapse between the administration of the drug and the reporting of the sexual attack is usually more than 12 h [9], which limits the use of blood as a suitable sample, since most of the DFSA compounds have short half-lives and a fast metabolism [10]. Therefore, urine is a priority sample that provides longer windows of detection for both parent compounds and their metabolites that can reach up to 96 h after the alleged sexual attack [11].

Sample preparation methods for the determination of DFSA substances in urine mostly include solid-phase extraction (SPE) [12] or liquid-liquid extraction (LLE) [6, 13], which involve multiple time-consuming and costly steps with the use of a considerable volume of organic solvents.

In this study, we propose and validate a simple, rapid, and eco-friendly method based on supramolecular solvent (SUPRAS) extraction. SUPRAS are nanostructured liquids made up of colloidal solutions of amphiphiles by self-assembly and coacervation [14]. They are excellent extractants with unique properties for solute solubilization. SUPRAS present regions of different polarities, multiple binding sites (high concentration of amphiphile, ~ 0.1–1 g/mL), and mixed-mode interactions, which allow the efficient solubilization of a wide polarity range of substances. They also provide a large surface area for fast mass transfer due to their discontinuous character,

since they are formed by coacervate droplets which in turn are made up of tridimensional aggregates. The nanostructures, composition, and proper- ties of SUPRAS are easily tunable by the proper selection of amphiphiles and self-assembly conditions. Furthermore, SUPRAS comply with many green chemistry criteria (e.g., high performance, energy-saving and high-atom economy synthesis, low toxicity and volatility, etc.) [15].

SUPRAS have been successfully employed for the extraction of organic compounds and metals from different samples, including urine [14, 16]. The suitability of SUPRAS made up of alkanols and alkanediols (C6-C10) for the extraction of multiple drugs for anti-doping control procedures in urine has been recently reported [17]. SUPRAS made of alkanediols were superior in terms of extraction efficiency. Optimal results were found with cubosomic SUPRAS made up of 1,2-hexanediol in salty aqueous solutions due to both the higher efficient extraction rates and green properties, since organic co-solvents were not needed. The nanostructures of these SUPRAS provided highly hydrophilic and also apolar regions to solubilize both very polar and nonpolar drugs. Based on these promising results, SUPRAS made up of alkanediols (C6–C10) formed under different conditions were investigated for the extraction of 23 DFSA drugs from urine. Target compounds belong to five different classes, namely benzodiazepines, Z-hypnotic drugs, cocaine and cocaine metabolites, amphetamine derivatives, and miscellaneous compounds (ketamine, fentanyl, scopolamine, and THC- COOH), and they were selected on the basis of their wide use in sexual assaults [18, 19] and because they are listed as targets by the Guidelines for the Forensic analysis of drugs facilitating sexual assault and other criminal acts of the United Nations office on Drugs and Crime [24]. Although methiopropamine (MPA) is not listed as a frequent DFSA substance, it has been included in this study as it is an analog to methamphetamine. Methiopropamine is structurally categorized as a thiophene ring-based meth- amphetamine derivative, and its abusive potential has been recognized in the literature [20]. **Table S1** lists the chemical structures, molecular formulas, and physico-chemical parameters for the selected DFSA. The proposed method was optimized, and it was validated in terms of selectivity, limits of detection, limits of quantification, recoveries, matrix effects, and precision.

The main aim of this study is to develop an analytical method based on the use of a green nanostructured liquid for the monitoring of compounds involved in drug-facilitated sexual assault. The method offers a green, fast, and simple alternative method suitable for routine analysis in forensic laboratories.

Material and methods

Chemicals and solutions

All chemicals were of analytical grade and used as supplied. Methanol (MeOH) and tetrahydrofuran (THF) were purchased from VWR-Prolabo Chemicals (Bois, France). Ultra-high-quality water was generated from a Milli- Q water purification system (Millipore-Sigma, Madrid, Spain). A KH2PO4 (0.4M) /$Na_2HPO_4x2H_2O$ (0.4 M) buffer solution (pH 6.5) was monthly prepared and stored at 2–8 °C until use. 1,2-Hexanediol, 1.2-octanediol, and 1,2-decanediol were all supplied by Sigma-Aldrich (St. Louis, MO, USA). The enzyme β-glucuronidase from E. coli K12 was purchased from Roche Diagnostics GmbH (Mannheim, Germany) with specific activity of at least 140 U/mL. The following compounds were purchased from LGC GmbH (Luckenwalde, Germany): alprazolam, bromazepam, flunitrazepam, diazepam, lorazepam, lormetazepam, nitrazepam, temazepam, clonazepam, zolpidem tartrate, methiopropamine hydrochloride, coca- ethylene (benzoylmethylecgonine), methamphetamine, cocaine, benzoylecgonine, fentanyl, rac-MDEA (rac- 3,4-methylenedioxy-N-ethylamphetamine), and ketamine hydrochloride. Scopolamine hydrobromide trihydrate was supplied by DR.EHRENSTORFER (Augsburg, Germany). Clorazepate dipotassium and (±)-MDMA (3,4-methylenedioxy-methamphetamine) were obtained from Supelco (USA). 3,4-Methylenedioxyamphetamine (±)-MDA was obtained from Cerilliant (Round Rock, TX, USA). 11-nor- Δ9-Tetrahydrocannabinol-9-carboxylic acid was obtained from Cerilliant. The following deuterated internal standards diazepam-d5, nitrazepam-d5, methamphetamine-d14, benzoylecgonine-d3, and scopolamine-d3 were all sup- plied by Sigma-Aldrich (Barcelona, Spain).

Stock solutions of DFSA substances (mix with individual concentrations of 1000 ng/mL and 100 ng/mL) and of the internal standards (IS) (mix with individual concentrations of 1000 ng/mL) were prepared in methanol and stored at –20 °C.

Intermediate and working solutions were made by appropriate dilution and stored at −20 °C until use.

Urine samples

Urine samples from volunteers were collected according to the "Ethics Committee of Andalusian's Biomedical Research" and the Declaration of Helsinki.

For method validation, a pooled sample was prepared from 10 urine samples collected from healthy volunteers and mixed at equal volumes. Prior to SUPRAS treatment, the pooled sample was centrifuged (15,000 rpm, 5 min) and enzymatically hydrolyzed. First, 5 mL of KH_2PO_4 Na_2/HPO_4 $2H_2O$ buffer was added to 100 mL of urine. After that, 2.5 mL of the enzyme β-glucuronidase was added and the solution heated at 52 °C for 1 h. Then, the hydrolyzed urine was stored in a closed glass bottle at −20 °C until use (within 1 week).

The validated method was applied to 10 other urine samples that were individually collected from volunteers, centrifuged (15,000 rpm, 5 min), and enzymatically hydrolyzed as explained before. The volunteers consisted of 6 patients under treatment with benzodiazepines, 2 frequent consumers of cannabis, and 2 volunteers with expected negative results for DFSA substances. All samples were spot urine samples collected in the early morning, and they were immediately treated and analyzed (within the same day).

For SUPRAS extraction optimization, synthetic urine was prepared according to a previously published protocol [21]. **Table S2** shows the composition of the synthetic urine.

SUPRAS extraction optimization

The extraction efficiency of different types of SUPRAS for DFSA compounds was investigated. SUPRAS were directly produced into synthetic urine (1 mL) from three different alkanediols, namely 1,2-hexanediol (200 µL), 1,2-octanediol (190 mg), and 1,2-decanediol (190 mg), with or without the presence of tetrahydrofuran (THF ~ 4–14% v/v) and salt (1 M Na_2SO_4 in urine). The synthetic urine was fortified with the target compounds at 100 ng/mL each. Extractions were performed in triplicate in 2-

mL Eppendorf tubes. The tubes were vortex shaken for 2 min for extraction and then centrifuged for 5 min at 15,000 rpm to accelerate the phase separation. The SUPRAS phase remained on the top of the tube, and the equilibrium solution (salty aqueous solution containing residual amounts of amphiphile and solvent, if any, plus non-extracted urine components) stayed at the bot- tom. The generated SUPRAS volume was calculated from a cylindrical volume equation by measuring its height in the tube using a digital Caliper from Medid Precision, S.A. (Barcelona, Spain). SUPRAS extracts were collected using a micropipette and transferred to a glass LC vial, fortified with the IS at 25 ng/mL (to account for instrumental fluctuations) and diluted to 1 mL with water in the case of SUPRAS of 1,2-hexanediol and with methanol in the case of SUPRAS of 1,2-octanediol and 1,2-decanediol. Finally, the extracts were measured by LC-MS/MS analysis. Extraction recoveries were calculated by using a post-fortified synthetic urine sample as a reference, which underwent SUPRAS treatment.

Recommended procedure for SUPRAS extraction of DFSA

Aliquots of 1.0 mL of hydrolyzed urine containing 0.142 g Na_2SO_4 and 200 µL of 1,2-hexanediol were vortex-stirred in 2-mL Eppendorf tubes for 2 min and then centrifuged at 15,000 rpm for 5 min. The volume of SUPRAS extracts formed on the top of the Eppendorf tube (~ 280–300 µL) was withdrawn using a micropipette and made up to 1.0 mL with distilled water before LC-MS/MS analysis. A schematic of the SUPRAS-based sample treatment is depicted in **Fig. 1**.

LC-MS/MS analysis

Measurements were carried out using an Agilent Technologies 1200 series LC (Palo Alto, CA, USA) coupled to a hybrid triple quadrupole/linear ion trap (Applied Biosystems MSD Sciex, 4000 QTRAP, Foster City, CA, USA) equipped with a Turbo V Ion Source (TIS). Data was processed using the Analyst 1.5.1 software. The stationary phase was a Rap- tor FluoroPhenyl (2.7 µm × 100 × 3.0 mm) provided by RESTEK (Bellefonte, PA, USA), which was operated at 35°C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B), and the gradient conditions are specified in **Table S3**. Stationary and mobile phases were selected on the basis of the results of previous studies [17]. The flow rate was 250 µL/

min, and the injection volume was 5 µL. The source was operated in positive ionization mode with the following parameters: source gas temperature: 400 °C, capillary voltage: 4500 V, nebulizer gas pressure: 50 psig, drying gas pressure: 50 psig, curtain gas pressure: 20 psig; and declustering potential: 30 V. MRM transitions (quantifier and qualifier ions), corresponding internal standard, and detection parameters for each compound are given in **Table S4**.

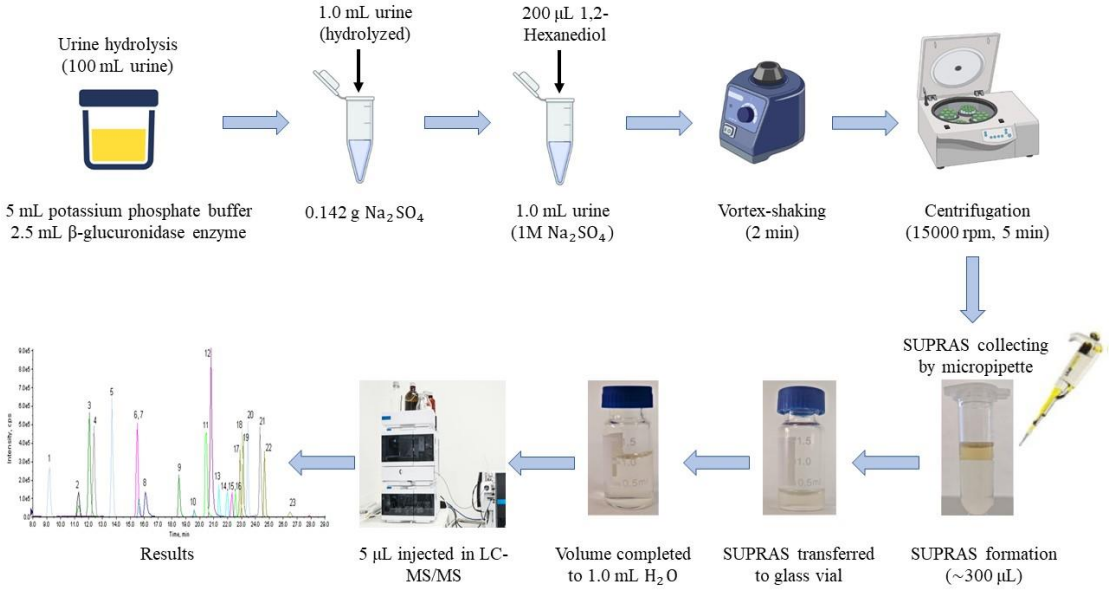


Fig. 1 *Procedural steps of SUPRAS formation and extraction of DFSA substances from a urine sample. Chromatogram peaks: methiopropamine (1), scopolamine (2), methamphetamine (3), MDA (4), MDMA (5), MDEA (6), ketamine (7), benzoylecgonine (8), cocaine (9), bromazepam (10), cocaethylene (11), zolpidem (12), lorazepam (13), nitrazepam (14), clonazepam (15), clorazepate (16), lormetazepam (17), fentanyl (18), temazepam (19), flunitrazepam (20), alprazolam (21), diazepam (22), and THC-COOH (23). Some images were generated from Biorender.com.*

Method validation

The SUPRAS method was validated under optimal extraction conditions (see "Recommended procedure for SUPRAS extraction of DFSA") by using the pooled urine sample (see "Urine samples"). Calibration was prepared in water: SUPRAS 70:30 v/v standards at concentrations in the range 0.1–5.0-fold of the MRPL set for each compound by the United Nations Office on Drugs and Crime [24]. Due to its higher limit of quantification (LOQ), standards for THC-COOH were prepared in the range

0.25–5.0-fold the MRPL. All solutions contained 25 ng/mL of IS. Each calibration standard was run in triplicate in the LC-MS/MS system. The calibration curves were built by plotting the relative peak areas (ADFSA substance/AIS) as a function of the corresponding concentrations. Limits of detection (LOD) and LOQ were determined at S/N of 3 and 10, respectively.

Selectivity was determined by analyzing 10 blank urine samples and checking for any interfering peak in the chromatograms. Peaks in these chromatograms were compared to those obtained for the same urines spiked at the MRPL values. Recoveries and relative matrix effects were evaluated by spiking urine and water samples at the MRPL levels. Three sets of samples were prepared: set A: hydrolyzed urine spiked with the target compounds and the IS at 25 ng/mL prior to SUPRAS extraction, set B: hydrolyzed urine sample spiked with the target analytes and IS after SUPRAS extraction (at the final dilution step), and set C: distilled water sample spiked with the target analytes and IS after SUPRAS extraction (at the final dilution step). The comparison of the relative peak areas ($A_{DFSA\ substance}/A_{IS}$) × 100 for set A with respect to set B was employed for the calculation of the extraction recovery. Relative matrix effects were calculated as [($A_{DFSA\ substances}/A_{IS}$)× 100] − 100 for set B with respect to set C.

Precision was evaluated by spiking hydrolyzed urine (n = 6) blank samples at three concentration levels: high 10 × MRPL levels, medium at MRPL levels, and low at 0.5 × MRPL levels, with 25 ng/mL of IS in the three levels. The samples were analyzed daily (inter-day precision, n = 6) and on three different days (intra-day precision, n = 3). Precision was expressed as relative standard deviation (RSD). The stability of SUPRAS extracts was confirmed during three consecutive days after extraction of the pooled sample fortified at the MRPL, by keeping the samples at 4 °C.

Results and discussion

SUPRAS optimization

Mixtures of 1,2-alkanediols in THF:water mixtures have been reported to give sponge-like structures which are made up of interconnected bilayers separated by abundant channels of water [22, 23]. In this research, SUPRAS consisting of alkanediols (C6-C10), water, THF, and Na_2SO_4 were synthesized by adding the amphiphile dissolved

in THF to the synthetic urine containing the salt. The urine (aqueous solution), which is a poor solvent for alkanediols, was the driver of their spontaneous self-assembly and coacervation. The salt was expected to enhance the extraction rate of the analytes by salting-out. First, the influence of the chain length of the alkanediols on the extraction of DFSA com- pounds was investigated by producing SUPRAS under the same synthetic conditions. **Table S5** shows the results for SUPRAS made up of 1,2-hexanediol, 1,2-octanediol, and 1,2-decanediol.

Absolute recoveries (IS added at the final dilution step) were very similar for the tested SUPRAS and in the ranges 40–96%, 48–100%, and 37–95% for 1,2-hexanediol, 1,2-octanediol, and 1,2-decanediol, respectively (**Table S5**). However, if the number of drugs with recoveries in the range of 70–120% was considered, values varied from 57% for 1,2-decanediol to 69% for 1,2-octanediol and up to 91% for 1,2-hexanediol (see **Fig. 2**). Thus, when the extraction was performed with 1,2-hexanediol-based SUPRAS, only two compounds showed absolute recoveries outside this range, namely scopolamine (49%) and methiopropamine (44%). The SUPRAS water content, and consequently its hydrophilicity, increase as the amphiphile chain length decreases, which favors the extraction of the more polar compounds and explains why sponge-based SUPRAS of 1,2-hexanediol were superior to those obtained from 1,2-octanediol and 1,2-decanediol in the extraction of DFSA compounds [22]. Based on these results, sponge-like SUPRAS of 1,2-hexanediol were selected as optimal.

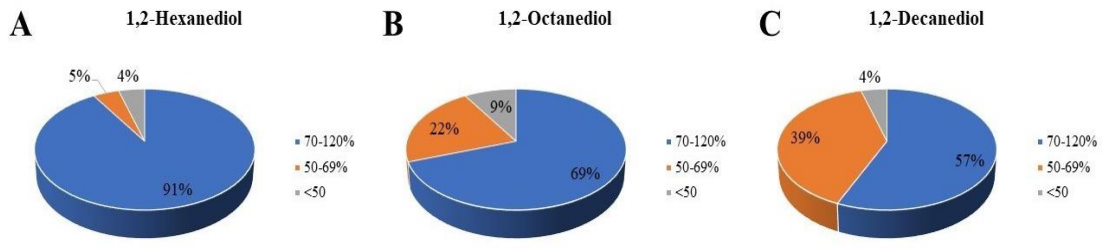


Fig. 2 *Absolute recoveries of DFSA substances [70–120% (blue), 50–69% (orange), and less than 50% (gray)] with SUPRAS synthesized from the following amphiphiles: 1,2-hexanediol (**A**), 1,2-octanediol (**B**), and 1,2-decanediol (**C**).*

Sponge-like SUPRAS of different compositions were prepared from a given amount of 1,2-hexanediol and different percentages of THF in the hydro-organic

colloidal solution, and their extraction efficiency for DFSA compounds was evaluated. **Table S5** shows the results. The number of compounds within the optimal recovery interval (70–120%) hardly changed under the tested conditions (83–91%), indicating that the content of THF in the SUPRAS was not relevant and that any of these SUPRAS was a good extractant for the selected drugs.

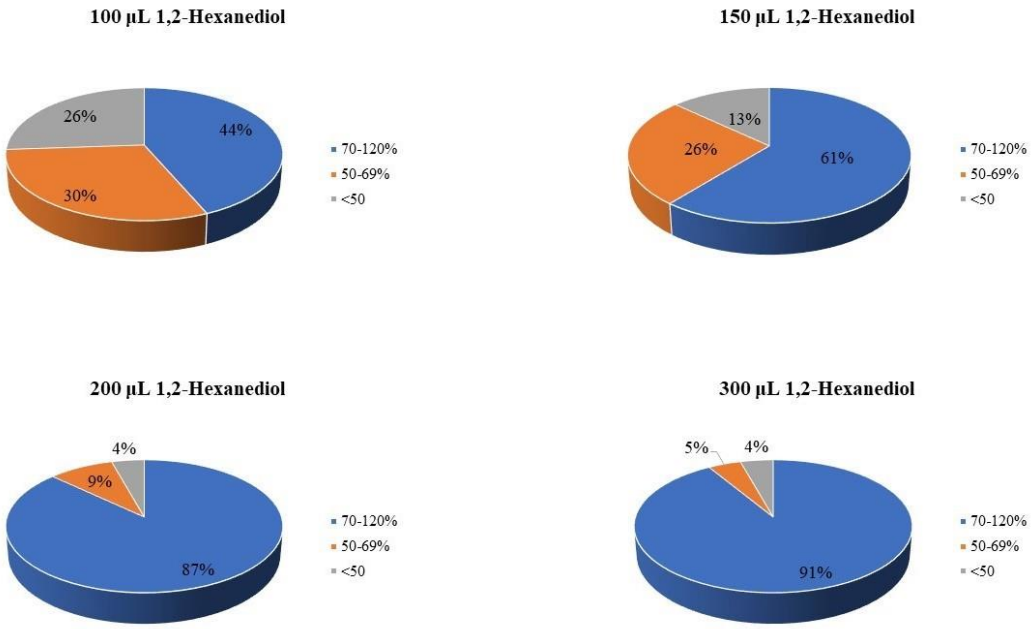


Fig. 3 *Absolute recoveries of DFSA substances [70–120% (blue), 50–69% (orange), and less than 50% (gray)] for SUPRAS synthesized from different amounts of 1,2-hexanediol.*

Considering the negligible influence of THF in the extraction of DFSA drugs and that 1,2-hexanediol gives salt-induced cubosomic SUPRAS in aqueous colloidal solutions without the need of co-solvents [17], several SUPRAS were prepared from a given amount of salt (1 M Na_2SO_4) and variable amounts of 1,2-hexanediol. Results are shown in **Table S5** and **Fig. 3**. Recoveries clearly improved up to 200 µL of 1,2-hexanediol, with 87% of the compounds with absolute recoveries between 70 and 120%, and they only slightly increased at 300 µL. Final optimal conditions consisted in the use of cubosomic SUPRAS, which were directly produced in urine by adding 200 µL of 1,2-hexanediol and 1 M Na_2SO_4. This green methodology was further validated for the extraction of DFSA compounds.

Table 1. Minimum required performance limits**, c**alibration parameters and method detection and quantification limits for the investigated DFSA compounds.

Analytes	[a]**MRPLs** (ng/mL)	[b]**Linear range** (ng/mL)	**Slope ± SD** (mL/ng)	**Determination coefficient (*R²*)**	**MLOD** (ng/mL)	**MLOQ** (ng/mL)
Benzodiazepines						
Alprazolam	10	1-50	0.0399±0.0006	0.999	0.06	0.20
Bromazepam	10	1-50	0.0113±0.0002	0.999	0.30	1.00
Clorazepate	10*	1-50	0.0159±0.0004	0.997	0.30	1.00
Diazepam	10	1-50	0.0200±0.0018	0.961	0.06	0.20
Lorazepam	10	1-50	0.0397±0.0013	0.995	0.10	0.40
Lormetazepam	10	1-50	0.0625±0.0012	0.998	0.09	0.30
Temazepam	10	1-50	0.0651±0.0013	0.998	0.09	0.30
Clonazepam	5	0.5-25	0.0256±0.0006	0.997	0.07	0.25
Flunitrazepam	5	0.5-25	0.0550±0.0009	0.999	0.07	0.25
Nitrazepam	5	0.5-25	0.0428±0.0011	0.997	0.15	0.50
Z-hypnotic drug						
Zolpidem	10	1-50	0.4432±0.0089	0.998	0.003	0.01
Cocaine and cocaine metabolites						
Cocaine	50	5-250	0.0339±0.0004	0.999	0.05	0.15
Benzoylecgonine	50	5-250	0.0381±0.0011	0.996	0.05	0.15
Cocaethylene	50	5-250	0.0672±0.0023	0.994	0.009	0.03
Amphetamine derivatives						
Methamphetamine	10	1-50	0.0527±0.0004	0.999	0.30	1.00

Methiopropamine	n/a	1-50	0.0052±0.0005	0.958	0.30	1.00
MDA	10	1-50	0.0328±0.0006	0.998	0.06	0.20
MDMA	10	1-50	0.0397±0.0012	0.995	0.006	0.02
MDEA	10	1-50	0.0898±0.0018	0.998	0.01	0.04
Miscellaneous compounds						
Fentanyl	10	1-50	0.1081±0.0009	0.999	0.05	0.20
Scopolamine	10	1-50	0.0474±0.0008	0.999	0.07	0.25
Ketamine	1	0.1-5	0.0483±0.0010	0.998	0.05	0.20
THC-COOH	10	2.5-50	0.0102±0.0002	0.998	0.75	2.50

[a]The minimum required performance limits (MRPLs) values obtained from the Guidelines for the Forensic analysis of drugs facilitating sexual assault and other criminal acts of the United Nations office on Drugs and Crime (UNODC). [b]The calibration curve was made within the range 0.1-5-fold the respective MRPL, except for THC-COOH (0.25-5.0-fold the MRPL). *Clorazepate MRPL value was obtained from Drug-Facilitated Crimes Committee (DFC) in the Society of Forensic Toxicologists (SOFT).

SUPRAS method validation

Table 1 lists the calibration parameters (slope ± SD and determination coefficient R^2), method limit of detection and quantification (MLOD and MLOQ), and the minimum required performance limits (MRPL) for each DFSA com- pound. The determination coefficients of the calibration curves were in the interval 0.958–0.999. The MLOD ranged between 0.003 and 0.75 ng/mL, while MLOQ varied in the range 0.01–2.5 ng/mL, values well below the MRPL levels.

Selectivity was confirmed because no peaks were detected in 10 different blank urine samples at the extracted ion chromatograms of each compound and at the retention time (RT) window of each analyte (RT ± 0.25 min). **Figure 4** shows, as an example, the extracted ion chromatograms obtained of three representative DFSA substances in blank urine, fortified at the 1 × MRPL value (A), and unfortified (B) by measuring the quantifier and qualifier transitions.

Table 2 shows the recoveries (calculated according to "Method validation") which were all within the accept- able range of 70–120% and varied between 79% ± 13 for methiopropamine and 119% ± 7 for nitrazepam. Relative matrix effects were evaluated in terms of signal suppression and enhancement, and values were mostly within the acceptable range − 20 to + 20%, except for clorazepate (+ 35%), lorazepam (+ 40%), methiopropamine (+ 27%), and MDMA (+ 35), for which moderate signal enhancement was observed. For these DFSA compounds, the use of isotopically labeled compounds as IS (instead of related compounds) could be beneficial. The intra-day and inter-day precision ranged in the intervals 4.0–8.9% and 4.1–13.7%, respectively.

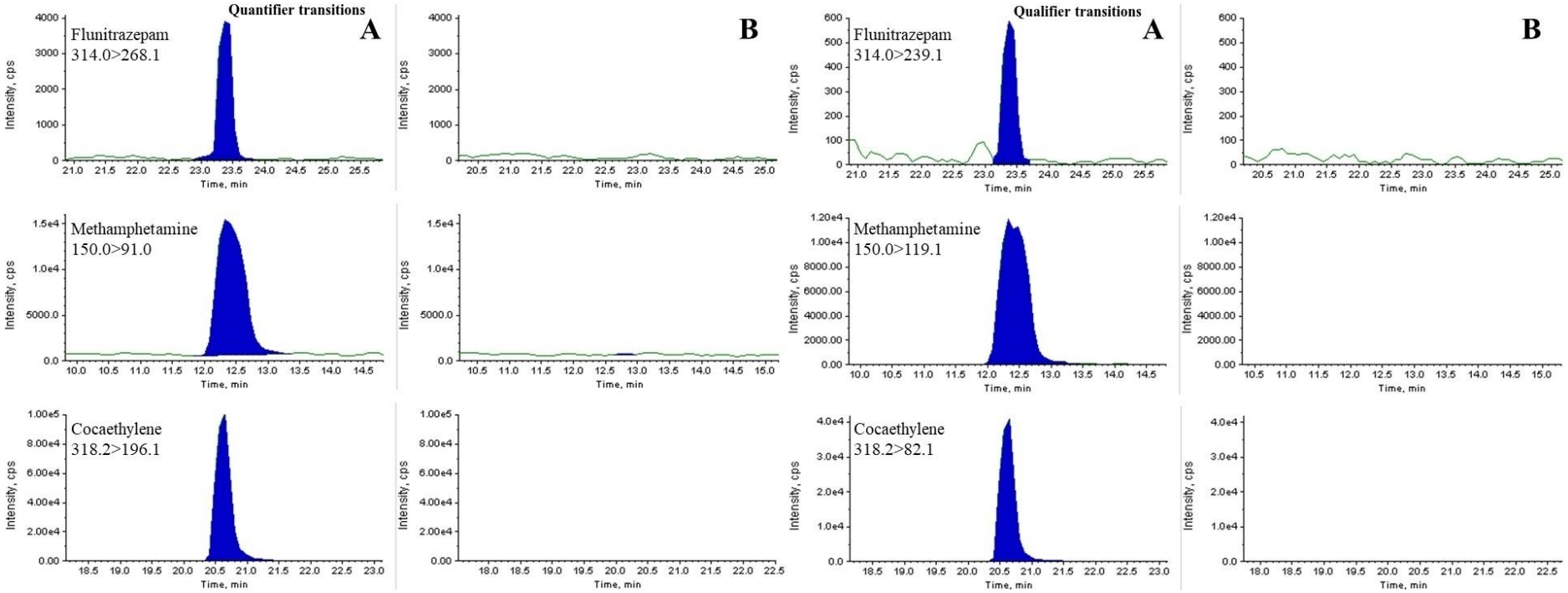


Fig. 4 *Extracted ion chromatograms of the quantifier and qualifier transitions of three representative DFSA substances flunitrazepam (314.0→268.1), methamphetamine (150.0→91.0), and cocaethylene (318.2→196.1) in a blank urine sample (**A**) fortified at the 1 × MRPL level and (**B**) unfortified.*

Table 2. Recoveries, relative matrix effects and intra- and inter-day precision for DFSA compounds in urine at the respective MRPL values and using the proposed cubosomic SUPRAS-LC-MS/MS method.

Analytes	Recovery ± SD (%) at MRPL	Relative matrix effect (%) at MRPL	Intra-day precision (n=6), RSD%			Inter-day precision (n=3), RSD%		
			10 x MRPL	MRPL	0.5 X MRPL	10 x MRPL	MRPL	0.5 X MRPL
Benzodiazepines								
Alprazolam	97 ± 6	8.3	5.9	4.4	2.4	4.3	3.5	4.1
Bromazepam	99 ± 2	9.1	6.9	8.9	5.3	13.7	5.2	5.3
Clonazepam	95 ± 5	8.1	9.4	6.9	2.2	7.6	3.7	6.4
Clorazepate	97 ± 5	34.7	2.5	6.1	3.6	5.9	4.1	5.9
Diazepam	96 ± 6	14.0	1.8	4.7	5.1	5.7	2.2	4.7
Flunitrazepam	89 ± 9	-3.8	3.6	4.5	2.8	6.3	3.8	4.4
Lorazepam	83 ± 5	40.3	6.1	7.2	8.7	7.3	4.6	5.8
Lormetazepam	98 ± 1	1.9	1.4	6.5	3.1	6.6	5.3	6.5
Nitrazepam	119 ± 7	-13.9	4.2	7.1	8.2	7.3	6.5	7.2
Temazepam	100 ± 6	19.1	3.5	6.9	4.6	6.8	6.2	5.7
Z-hypnotic drug								
Zolpidem	94 ± 6	9.8	6.5	4.0	6.5	4.8	5.7	6.3
Cocaine and cocaine metabolites								
Cocaine	115 ± 6	20.2	4.5	6.8	2.8	6.6	4.5	5.6
Benzoylecgonine	100 ± 2	-6.0	3.6	7.5	2.9	8.9	3.7	4.9
Cocaethylene	115 ± 1	6.3	3.3	5.3	6.1	7.2	6.6	6.4
Amphetamine derivatives								
Methamphetamine	104 ± 1	-4.6	0.6	4.8	2.8	5.8	3.7	6.2
Methiopropamine	79 ± 12	27.6	7.2	5.1	4.4	5.7	5.8	5.3
MDA	104 ± 3	9.1	3.8	4.6	3.4	4.1	5.4	4.1
MDEA	112 ± 9	-2.8	6.2	5.6	7.4	6.5	6.2	7.1
MDMA	106 ± 5	35.2	4.8	6.9	3.2	5.9	4.4	6.4

Miscellaneous compounds								
6.Fentanyl	97 ± 8	19.5	5.5	5.8	2.6	5.7	5.6	3.8
Scopolamine	84 ± 9	-14.3	6.5	7.4	7.1	8.3	5.4	6.3
Ketamine	79 ± 13	21.5	4.3	6.7	5.5	7.5	4.9	5.4
THC-COOH	81 ± 7	9.2	7.1	8.1	6.6	8.0	5.1	6.2

Urine sample analysis

The validated method was applied to urine from ten volunteers consisting of six patients under treatment with benzodiazepines, two frequent consumers of cannabis, and two individuals with expected negative results for DFSA substances. **Table 3** shows the results obtained. **Figure 5** shows the extracted ion chromatograms for the detected compounds in the positive urine samples. The half-lives of these are 12–15 h for alprazolam and lorazepam [24]. Half-lives for THC-COOH can reach up to 3 days for frequent users [25].

Levels in urine were normalized according to the urine specific gravity [26]. The presence of THC-COOH was con- firmed in the urine sample of volunteers 1 and 2 at concentrations of 90 ± 4 ng/mL and 79 ± 7 ng/mL, respectively. The two volunteers, who are in their twenties, are mentioned to be occasional cannabis smokers. The concentration of urinary THC-COOH can highly vary, and it is very dependent on the frequency of use, time since the last consumption, and percentage of delta-9-THC in the consumed material. For example, in a previous study performed on 21 heavy cannabis users, the concentration of the THC-COOH in urine exceeded 1000 ng/mL for some volunteers [27]. In another study, where “light cannabis,” which contains less than 0.2% of the main psychoactive component delta-9-THC, was smoked, the detected concentration of THC-COOH in urine was as low as 1.8 ng/mL [28].

The third volunteer, an 84-year-old female who has been taking 1 mg/day of lorazepam for 5 years, tested positive for it with a concentration of 218 ± 12 ng/mL. Lower lorazepam levels between 69 ± 8 and 146 ± 7 were found for volunteers after a single use (volunteers 8–10). Values were all within the range of a previously reported study (2.5–97% range 58–4838 ng/mL, median 516 ng/mL, *n* = 3807). Volunteers 4 and 5 were positive for alprazolam and were both under treatment with 0.25 mg/day for 20 and 3 years, respectively. Detected concentrations were 37 ± 2 and 19 ± 2 ng/mL, respectively. These values were in the low range of a previously reported study (2.5–97% range 22–878 ng/mL, median 96 ng/mL, n =26,479) [29]. None of the studied substances were detected in volunteers 6 and 7, as expected.

Table 3. Concentrations of DFSA drugs found in 10 human urine samples analyzed with the cubosomic SUPRAS-LC-MS/MS method.

Volunteer	**Sex**	**Age**	**Detected substance**	**Urine concentration (ng/mL) (mean ± SD)**	**Condition**
1	Female	24	THC-COOH	90 ± 4	Occasional cannabis smoker.
2	Male	25	THC-COOH	79 ± 7	Occasional cannabis smoker.
3	Female	84	Lorazepam	218 ± 12	Lorazepam 1.0 mg q.d.* for 5 years.
4	Female	55	Alprazolam	37 ± 2	Alprazolam 0.25 mg q.d. for 20 years.
5	Male	38	Alprazolam	19 ± 2	Alprazolam 0.25 mg q.d. for 3 years.
6	Female	39	-	-	-
7	Male	35	-	-	-
8	Female	59	Lorazepam	69 ± 8	Lorazepam 1.0 mg single dose.
9	Male	58	Lorazepam	80 ± 3	Lorazepam 1.0 mg single dose.
10	Female	38	Lorazepam	146 ± 7	Lorazepam 1.0 mg single dose.

*q.d.: once a day.

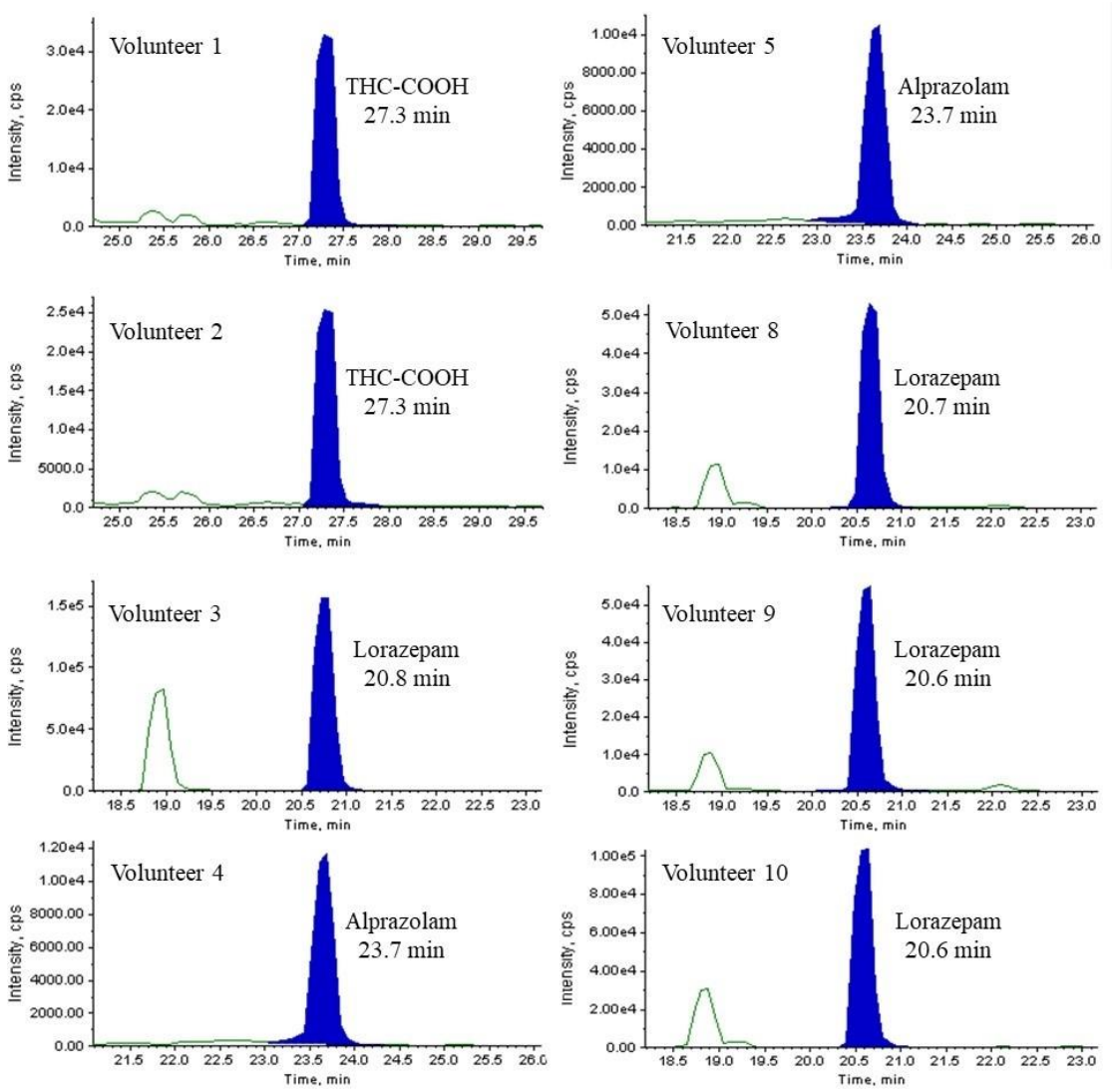


Fig. 5 *Extracted ion chromatograms of the positive samples from the 8 volunteers*

Conclusions

An extraction method based on green SUPRAS of 1,2-hexanediol in salty urine samples has been optimized and validated to extract 23 DFSA compounds. After simple and fast extraction and centrifugation steps, extracts were analyzed by LC-MS/MS. The use of the proposed method provides a precise, fast, green, and cheap approach to the monitoring and observation of DFSA cases. Extraction efficiency was in the range 79–119% with negligible matrix effects for 19 out of 23 compounds and with intra- day and inter-day precision in the ranges 4.0–8.9% and 4.1–13.7%, respectively. The applicability of the proposed method was proven by analyzing 10 human urine samples from volunteers, and it was able to detect various targeted substances at different concentrations.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s00216-022-04358-z.

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Declarations

Informed consent Volunteers' information sheets were given to all the participants, and samples were collected with their consent. The study was approved by the "Ethics Committee of Andalusian's Biomedical Research."

Conflict of interest The authors declare no competing interests.

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SUPPLEMENTARY INFORMATION

Green nanostructured liquids for the analysis of urine in drug-facilitated sexual assault cases

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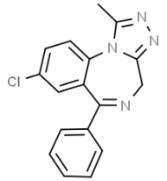
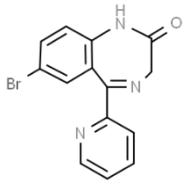
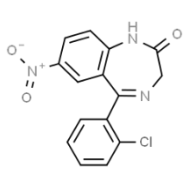
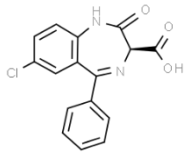
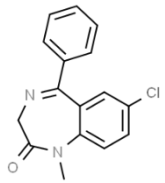
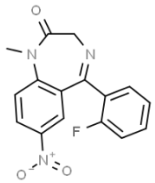
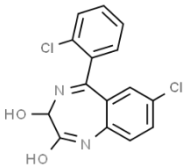
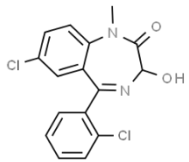
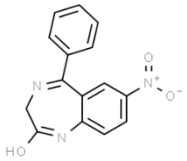
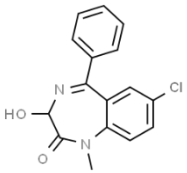
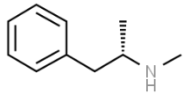
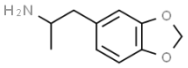
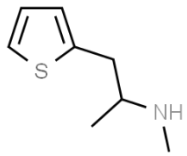
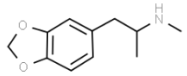
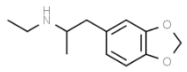
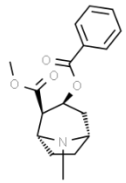
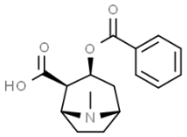
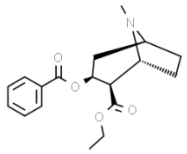
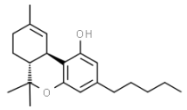
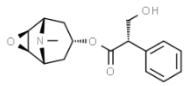
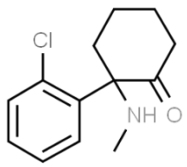
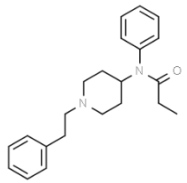
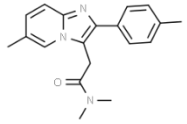
Table S1. Chemical structures, molecular formulas, and different parameters of interest for the retention behavior of the selected DFSA substances or their metabolites.

Main drugs involved in DFSA	**[a]Chemical structure**	**Molecular formula**	**[b]Molecular weight (g/mol)**	**[b]Partition coefficient, log *P***	**[b,c]pK_a acidic**	**[b,c]pK_a basic**	**[b]H-bond donors**	**[b]H-bond acceptors**
Alprazolam		$C_{17}H_{13}ClN_4$	308.8	2.1	18.3	5.08	0	3
Bromazepam		$C_{14}H_{10}BrN_3O$	316.15	1.7	12.24	2.68	1	3
Clonazepam		$C_{15}H_{10}ClN_3O_3$	315.17	2.4	11.89	1.86	1	4
Clorazepate		$C_{16}H_{11}ClN_2O_3$	314.72	3.3	3.32	-0.64	2	4

Diazepam		$C_{16}H_{13}ClN_2O$	284.74	3.0	n/a	2.92	0	2
Flunitrazepam		$C_{16}H_{12}FN_3O_3$	313.28	2.1	n/a	1.7	0	5
Lorazepam		$C_{15}H_{10}Cl_2N_2O_2$	321.2	2.4	10.61	-2.2	2	3
Lormetazepam		$C_{16}H_{12}Cl_2N_2O_2$	335.2	2.4	10.68	-2.2	1	3
Nitrazepam		$C_{15}H_{11}N_3O_3$	281.27	2.2	11.9	2.61	1	4

Temazepam		$C_{16}H_{13}ClN_2O_2$	300.74	2.2	10.68	-1.4	1	3
Methamphetamine		$C_{10}H_{15}N$	149.23	2.1	n/a	10.01	1	3
3,4-Methylenedioxy-amphetamine (MDA), Tenamfetamine		$C_{10}H_{13}NO_2$	179.22	1.6	n/a	10.01	1	3
Methiopropamine		$C_8H_{13}NS$	155.26	1.9	n/a	n/a	1	2
3,4-Methylenedioxy-methamphetamine (MDMA), Ecstasy		$C_{11}H_{15}NO_2$	193.24	2.2	n/a	10.14	1	3

3,4-Methylenedioxy-N-ethylamphetamine (MDEA)		$C_{12}H_{17}NO_2$	207.27	2.5	n/a	10.22	1	3
Cocaine		$C_{17}H_{21}NO_4$	303.35	2.30	n/a	8.85	0	5
Benzoylecgonine (metabolite of Cocaine)		$C_{16}H_{19}NO_4$	289.33	-0.3	3.15	9.54	1	5
Cocaethylene (metabolite of Cocaine)		$C_{18}H_{23}NO_4$	317.4	2.7	n/a	n/a	0	5
11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid		$C_{21}H_{30}O_2$	314.5	7.0	9.34	-4.9	1	2

Scopolamine		$C_{17}H_{21}NO_4$	303.35	0.9	15.15	6.95	1	5
Ketamine		$C_{13}H_{16}ClNO$	237.72	2.2	18.78	7.45	1	2
Fentanyl		$C_{22}H_{28}N_2O$	336.5	4.0	n/a	8.77	0	2
Zolpidem		$C_{19}H_{21}N_3O$	307.4	2.5	n/a	5.65	0	2

[a]Chemical structures obtained from Chemspider, [b]Values obtained from PubChem, [c]Values obtained from DrugBank, n/a: non-available value.

Table S2. Composition of synthetic urine prepared in distilled water.

Component	Formula	Molarity (mM)
Sodium sulfate	Na_2SO_4	11.965
Uric acid	$C_5H_4N_4O_3$	1.487
Sodium citrate tribasic dihydrate	$C_6H_5Na_3O_7.2H_2O$	2.450
Creatinine	$C_4H_7N_3O$	7.791
Urea	CH_4N_2O	249.750
Potassium chloride	KCl	30.953
Sodium chloride	NaCl	30.053
Calcium chloride	$CaCl_2$	1.663
Ammonium chloride	NH_4Cl	23.667
Sodium oxalate	$Na_2C_2O_4$	0.19
Magnesium sulphate anhydrous	$MgSO_4$	4.389
Sodium dihydrogen phosphate monohydrate	$NaH_2PO_4. H_2O$	18.667
Di-sodium hydrogen phosphate	$Na_2HPO_4.2H_2O$	4.667

Table S3. Stationary phase and mobile phase parameters.

Phase	**Specification**			
Stationary phase				
Packing material	Raptor FluoroPhenyl			
Column length	100 mm			
Inside diameter	3.0 mm			
Particle size	2.7 µm			
Mobile phase				
Composition	Solvent A: 0.1% formic acid in H_2O.			
	Solvent B: 0.1% formic acid in MeOH.			
Gradient conditions	**Total time (min)**	**Flow rate (µL/min)**	**A%**	**B%**
	5.0	250	90	10
	0.5	250	90	10
	25.0	250	20	80
	26.0	250	10	90
	36.0	250	10	90

Table S4. MS parameters and internal standard used for analysis of the target drugs.

Drug	Precursor ion	Quantifier Qualifier	DP (volts)	CE (volts)	CXP (volts)	Internal standard
Alprazolam	309.1	281.2	91	39	6	Diazepam-d5
		205.3	91	37	10	
Bromazepam	318.0	182.1	51	37	16	Diazepam-d5
		209.2	51	37	16	
Clonazepam	315.9	270.1	126	35	14	Nitrazepam-d5
		214.4	126	35	14	
Clorazepate	271.0	140.1	65	50	18	Diazepam-d5
		165.0	65	50	18	
Diazepam	285.1	154.3	101	47	10	Diazepam-d5
		193.3	101	39	12	
Flunitrazepam	314.0	268.1	111	37	22	Nitrazepam-d5
		239.1	111	37	22	
Lorazepam	320.95	274.98	66	33	20	Diazepam-d5
		229.00	66	33	20	
Lormetazepam	334.97	288.97	81	33	16	Diazepam-d5
		317.00	81	33	16	
Nitrazepam	282.1	236.2	5	35	4	Nitrazepam-d5
		180.1	5	35	4	
Temazepam	301.2	255.2	91	33	16	Diazepam-d5
		177.3	91	57	8	
Zolpidem	308.1	235.2	98	47	10	Diazepam-d5

		236.1	98	47	10	
Methamphetamine	150.071	91.00	46	17	8	Methamphetamine-d14
		119.16	46	17	8	
Methiopropamine	155.9	97.05	46	23	8	Scopolamine-d3
		58.10	46	13	8	
3,4-Methylenedioxy-amphetamine (MDA)	180	163.2	42	15	9	Methamphetamine-d14
		105.1	42	32	8	
3,4-Methylenedioxy methamphetamine (MDMA)	194.2	163.0	62	19	10	Methamphetamine-d14
		105.2	62	37	8	
3,4-Methylenedioxy-N-ethylamphetamine (MDEA)	208.2	163.20	31	15	2	Methamphetamine-d14
		132.95	31	21	2	
Cocaine	304.2	77.0	36	89	12	Benzoylecgonine-d3
		182.1	36	14	12	
Benzoylecgonine (metabolite of Cocaine)	290.23	168.24	30	29	12	Benzoylecgonine-d3
		105.06	30	55	12	
Cocaethylene (metabolite of Cocaine)	318.2	196.1	10	27	20	Benzoylecgonine-d3
		82.1	10	43	4	
Fentanyl	337.1	105.0	30	36	9	Diazepam-d5
		188.0	30	22	9	
Scopolamine	304.1	156.0	66	31	8	Scopolamine-d3
		138.1	66	23	8	
Ketamine	238.0	125.0	60	20	4	Diazepam-d5
		220.0	60	10	4	

11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid	345.179	327.3	136	23	6	Diazepam-d5
		299.2	136	27	22	
Diazepam-d5	290.0	154.0	96	41	24	-
		198.0	111	47	14	
Nitrazepam-d5	287.1	241.1	16	37	40	-
		185.1	16	34	40	
Methamphetamine-d14	164.0	98.0	45	22	20	-
		130.0	45	10	20	
Benzoylecgonine-d3	293.0	171.0	60	30	8	-
		105.0	91	43	8	
Scopolamine-d3	307.1	141.1	30	26	20	-
		159.0	30	16	20	

Table S5. Optimization of SUPRAS type and composition in terms of amphiphile chain length, and organic solvent and amphiphile concentration.

1,2-alkanediol sponge-like SUPRAS		**SUPRAS volume**	**Absolute recovery range of all compounds**	**Number of compounds with optimal absolute recoveries (70-120%)**
Amphiphile optimization				
190.2 mg 1,2-Decanediol, [a](~13% v/v)	140 µL THF, [a](~10% v/v) Na_2SO_4 (1M)	400 µL	37 – 95%	13 / 23 (57%)
190.2 mg 1,2-Octanediol, [a](~13% v/v)		420 µL	48 – 100%	16 / 23 (69%)
200 µL 1,2- Hexanediol [a](~13% v/v)		460 µL	40 – 96 %	21 / 23 (91%)
1,2-hexanediol sponge-like SUPRAS				
Organic solvent optimization				
200 µL 1,2- Hexanediol	60 µL THF, [a](~5% v/v) Na_2SO_4 (1M)	355 µL	31 – 103 %	20 / 23 (87%)
	100 µL THF, [a](~8% v/v) Na_2SO_4 (1M)	400 µL	35 – 101 %	19 / 23 (83%)
	140 µL THF, [a](~10% v/v) Na_2SO_4 (1M)	460 µL	40 – 96 %	21 / 23 (91%)
	200 µL THF, [a](~14% v/v) Na_2SO_4 (1M)	540 µL	40 – 99 %	20 / 23 (87%)
1,2-hexanediol cubosomic SUPRAS				
Amphiphile concentration optimization				
100 µL 1,2-Hexanediol, [a](~9% v/v)	Na_2SO_4 (1M)	120 µL	33 – 85%	10 / 23 (44%)
150 µL 1,2-Hexanediol, [a](~13% v/v)		220 µL	48 – 85%	14 / 23 (61%)
200 µL 1,2-Hexanediol, [a](~17% v/v)		285 µL	49 – 98 %	20 / 23 (87%)
300 µL 1,2-Hexanediol, [a](~23% v/v)		455 µL	49 – 87%	21 / 23 (91%)

[a]Percentage v/v in the ternary synthesis mix (amphiphile:urine:THF)

Chapter III

Hair analysis of selected drug-facilitated sexual assault substances using green supramolecular solvent extraction and LC-MS/MS analysis.

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Hair analysis of selected drug-facilitated sexual assault substances using green supramolecular solvent extraction and LC-MS/MS analysis

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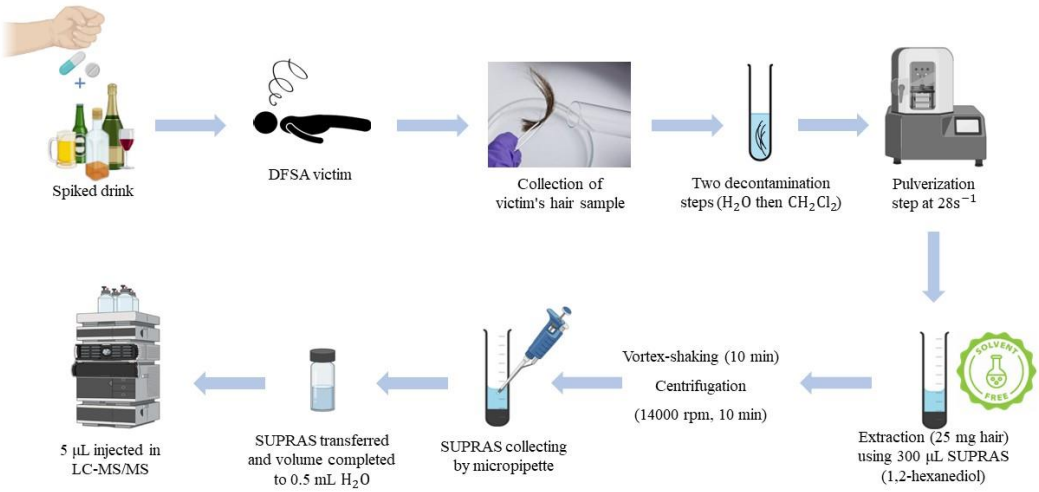
GRAPHICAL ABSTRACT



Abstract

Drug-facilitated sexual assault (DFSA) investigations require the consumption history of certain drugs by the victim. Hair; as a keratinized biological sample, offers the possibility to perform retrospective quantitative analysis due to its large window of detection in comparison to other biological matrices. Many analytical methods have been reported to determine class-specific DFSA substances. However, generic methods able to determine multiclass DFSA substances and which are also more sustainable remain pending. In this paper, we develop an efficient and eco-friendly generic single-step extraction method, based on the use of a supramolecular solvent (SUPRAS) made up of 1,2-hexanediol, to extract benzodiazepines, z-hypnotic compounds, cocaine and metabolites, amphetamine derivatives and other miscellaneous compounds involved in DFSA from hair. The proposed method offers a high extraction recovery (>86%) and acceptable matrix effects for 91% of the 23 tested substances, which cover a wide polarity range (log P from − 0.3 to 7.0). The estimated method detection and quantification limits were in the ranges 0.1–24.2 pg/mg and 0.4–80 pg/mg, respectively, and were lower than the recommended drug cut-off levels for all the studied substances, except for 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid. This fast and environmentally friendly method has been successfully applied to quantify DFSA substances in different hair samples.

Keywords

Hair, green extraction, drug-facilitated sexual assault, supramolecular solvent, LC-MS/MS.

1. Introduction

Drug-facilitated sexual assaults (DFSAs) are violent crimes committed by impairing the state of awareness and degree of consciousness in the victim under the influence of a drug [1] and have dramatically increased worldwide in the past few years [2]. The employed substances are pharmaceutical compounds (such as benzodiazepines and opioid analgesics) and other illegal drugs (such as cocaine, cannabis, and ecstasy). These substances are attractive due to the sedative, hypnotic and amnesic effects that they produce on the victim's central nervous system [3].

Investigators and forensic toxicologists of DFSA incidents are requested to elucidate the substances that are involved in the sexual crime, their concentration, and the approximate time of administration. An important aspect in most DFSA cases is the delay in reporting the incident (several days or even weeks after the administration of the drug [4]) due to the embarrassment from the surrounding society, cultural and religious false beliefs, guilty feelings and lack of confidence in the criminal and judicial authorities [5]. Due to this delay, the drug is metabolized and eliminated from the victim's body before it can be detected in the traditional biological samples (blood and urine) [6].

Despite blood and urine are still the first-line samples to be employed in toxicological analysis and they are requested to be collected in all cases of alleged DFSAs [7], the window of detection for most of the drugs is limited to not more than several days [8]. The considerable long time-lapse between the incidence of the sexual attack and the collection of the biological samples encourages forensic scientists to search for alternative biological matrices [9].

The human keratinized biological matrices, such as hair, which are made up of 65–95% protein, mostly keratin [10], are able to incorporate and accumulate the ingested drugs thus allowing the performance of a retrospective evaluation of the drug consumption history [11]. Drugs can incorporate into hair and be deposited during months [12]. The amount of drugs deposited into the hair is primarily affected by physicochemical properties of the drug or its metabolites (such as its lipophilicity and basicity), hair pigmentation (the amount of melanin in the hair) and the individual variations in the rate of drug metabolism from one victim to another [13], [14]. The United Nation Office on Drugs and Crime (UNODC) guidelines recommend the

collection of hair samples after a minimum of 4 to 6 weeks post-alleged assault. This period is critical to allow the drug or their metabolites to be incorporated into the hair and be present at a minimum distance above the surface of the skin for efficient collection [15].

Hair analysis has a number of advantages over other types of biological specimens. For instance, the process of collecting hair samples is painless and there is no need for needles or any other invasive methods [16]. The hair sample can be easily stored and transported [17]. Furthermore, drugs and their metabolites are considered stable in hair for a long time [18]. Another significant advantage is that hair analysis can point-out a positive result after even a single-dose administration. This has been reported for diazepam [19], for ketamine and norketamine [20] and for other hypnotic agent [2]. Table 1 lists some the detection of drugs in hair from reported cases of DFSA after a single dose intake, indicating the time lapse before sampling, the sample size and the detected concentration [6], [21], [22], [23], [24], [25], [26], [27].

The Society of Hair Testing (SoHT) and The European Workplace Drug Testing Society have set threshold concentrations for each drug and/or metabolite of interest to determine whether the hair sample is positive or negative [28]. This is known as the drug cut-off level and any developed method must show a quantitation limit below this recommended value [29].

The quantification of drugs and their metabolites in hair includes several challenging and time-consuming steps before analysis, usually carried out by liquid or gas chromatography coupled to mass spectrometry (LC-MS, GC–MS). These steps consists of sample treatment, including decontamination and digestion of hair samples, and effective drug extraction and sample cleanup [30].

Several extraction methods have been used for the extraction of DFSA substances from hair samples. Thus, methanol and methanol/ammonium formate buffer was applied to extract benzodiazepines and z-hypnotic drugs, from hair samples, [29] and a mixture of methanol:acetonitrile:20 mM ammonium formate in a ratio of 25:25:50 (v/v/v) was used to extract ketamine and norketamine form hair of ketamine users [31]. In other cases, liquid–liquid extraction (LLE) was applied after the hair sample was washed, decontaminated and digested, as it is the case of the extraction γ-hydroxybutyrate (GHB) with 3 mL of ethyl acetate [32], [33].

Table 1. Some reported cases for detection of DFSA compounds in hair after a single dose intake.

Drug	Victim sex	Victim age (years)	Dose	Time-lapse before sampling	Collected sample length	Sample size	Drug concentration (hair segment analyzed from hair root (proximal) in cm)		Reference
Zolpidem Flunitrazepam Oxazepam	Female	56	Single dose	7 months	28 cm	20 mg	0.7-1.06 pg/mg (4.6-5.5 cm) 55-67 pg/mg (5.6-6.5 cm) 32-36 pg/mg (6.6-7.5 cm)		[6]
Amobarbital	Female	23	Single dose	3 months	25 cm	50 mg	< LOQ (1-2cm) 0.09 ng/mg (2-3cm)		[21]
Quetiapine	Female	-	-	6 months	30 cm	10 mg	0.011 ng/mg (7-9 cm) Negative (0-7 cm)		[22]
GHB	Female	6	3 g (0.08g/kg)	-	16 cm	10 mg	40.9 ng/mg (0-2 cm)		[23]
Triazolam	Female	30	Single or few doses	34 days (hair dyed few days before sampling)	-	10 mg	1 pg/mg (0-2 cm) Negative (2-4 cm) Negative (4-6 cm)		[24]
Clonazepam	Females x2	-	-	5 weeks	-	20 mg	<u>Victim#1</u> 15.4 pg/mg (0-2 cm) 5.3 pg/mg (2-4 cm) 1.6 pg/mg (4-6 cm)	<u>Victim#2</u> 11.9 pg/mg (0-2 cm) 1.3 pg/mg (2-4 cm)	[25
GHB and morphine	Female	24	-	Several months	20 cm	20 mg	<u>GHB</u> 5 ng/mg (0-3 cm) 4 ng/mg (3-6 cm) 3 ng/mg (6-9 cm) 4 ng/mg (9-12 cm) 1 ng/mg (12-20 cm)	<u>Morphine</u> 1 ng/mg (0-12 cm)	[26]
Acepromazine	Female	29	Single dose	1.5 months	17 cm	-	31 pg/mg (0.5-2.5 cm)		[27]

Finally, solid-phase extraction (SPE) has been commonly used for sample cleanup after solvent extraction of the targeted DFSAs. As an example, a HybridSPE-phospholipid cartridge was used to extract zolpidem and its main metabolite [34] and strata X-C cartridges were used to extract amphetamine-type stimulants from hair samples [35]. On the whole, large solvent volume (mL) /hair weight (g) ratios are required (e.g. usually around 100 [32]), hard operating conditions are usual (e.g. solvent extraction at 50 °C for 1 h [34]), long extraction times are sometimes needed (e.g. 115 min, excluding residual-drying and reconstitution steps [29]), and SPE-based sample cleanup makes DFSA studies expensive [36], [37]. Furthermore, these methods focus on structurally related drugs that have similar polarity but they do not cover the extraction of different families of DFSA drugs in a single procedure. So, the development of generic and simpler hair sample treatments able to efficiently extract multiclass DFSA substances, which include different families of compounds covering a wide polarity range, is of interest for investigators and forensic toxicologists of DFSA incidents.

Supramolecular solvents (SUPRASs) have been recently proposed as generic, efficient, simple and quick procedures for the extraction of multiclass substances covering a wide polarity range [37]. SUPRASs are best defined as nanostructured liquids obtained by the self-assembly and coacervation of amphiphiles [38], [39]. They have been employed in the extraction of several drug classes from various biological samples, namely amphetamine-type stimulants from oral fluid, urine, serum, sweat, breast milk and hair with extraction recoveries between 87% and 111% [40]. Cubosomic supramolecular solvents were also efficiently applied for multicomponent extraction of 92 doping substances from urine [37]. In DFSA cases, SUPRASs have been proved to provide a green, simple and fast process to efficiently extract DFSA substances from human urine (recoveries 79%-119%) [39].

In this paper, the potential of cubosomic SUPRASs to extract multiclass substances is tested for the development of a generic sample treatment for extraction of DFSA compounds in hair. For this purpose, various DFSA families like benzodiazepines, amphetamines, cocaine, and other miscellaneous compounds, which cover a wide polarity range (log P from − 0.3 to 7.0), were selected. **Table S1** lists the chemical

structures, molecular formulas, and physico-chemical parameters for the studied DFSA compounds.

The capability of SUPRASs to overcome major limitations of current extraction methods for determination of DFSA in hair (e.g. restricted to structurally related drugs, large solvent consumption, hard operating conditions, need for SPE clean-up and time-consuming) derives for their intrinsic properties. Regarding extraction, SUPRASs offer [38]: (a) *Different polarity microenvironments* where solutes spanning wide polarity ranges can be simultaneously solubilized. Thus, contrarily to conventional solvents, SUPRASs have the ability to extract substances through mixed mechanisms (e.g. hydrogen bonding, dipole–dipole, ionic, etc. in the polar region and dispersion, π-π, etc. in the nonpolar region). (b) *Multiple binding sites* owing to the huge concentration of amphiphiles in the SUPRAS (0.1–1 mg/µL). As a result, solutes can be extracted at low SUPRAS/hair ratios, thus increasing sensitivity and, in most cases, avoiding the evaporation of extracts, which results in saving both time and costs. (c) *Large surface area*, because the coacervate droplets keep as individual entities in the SUPRAS, so fast solute mass transfer can be obtained in extraction processes. Regarding sample cleanup, SUPRASs can be tailored for exclusion of macromolecules through physical and chemical mechanisms [38], so DFSA extraction and sample cleanup can be integrated in a single step.

In conclusion, SUPRAS have the potential to offer a more selective, efficient, and environmentally friendly approach to extract illegal drugs from hair samples and to constitute a promising alternative to the use combined of liquid–liquid extraction and solid-phase extraction in drug analysis. The main results of this study are discussed below.

2. Material and methods

2.1. Chemicals and drugs

All the chemicals were of high analytical grade. Methanol (MeOH) was purchased from VWR-Prolabo Chemicals (Bois, France). Ultra-high-quality water was generated from a Milli-Q water purification system (Millipore-Sigma, Madrid, Spain). 1,2-Hexanediol was supplied by Sigma-Aldrich (St. Louis, MO, USA). The following compounds were all purchased from LGC GmbH (Luckenwalde, Germany): alprazolam,

bromazepam, flunitrazepam, diazepam, lorazepam, lormetazepam, nitrazepam, temazepam, clonazepam, zolpidem tartrate, methiopropamine hydrochloride, cocaethylene (benzoylmethylecgonine), methamphetamine, cocaine, benzoylecgonine, fentanyl, rac-MDEA (rac-3,4-Methylenedioxy-N-ethylamphetamine), and ketamine hydrochloride. Scopolamine hydrobromide trihydrate was supplied by DR.EHRENSTORFER (Augsburg, Germany). Clorazepate dipotassium and (±)-MDMA (3,4-Methylenedioxy-methamphetamine) were obtained from Supelco (USA). 3,4-Methylenedioxyamphetamine (±)-MDA was obtained from Cerilliant (Round Rock, TX, USA). 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid was obtained from Cerilliant. The deuterated internal standards diazepam-d5, nitrazepam-d5, methamphetamine-d14, benzoylecgonine-d3, and scopolamine-d3 were all supplied by Sigma Aldrich (Barcelona, Spain).

2.2. Hair samples

Hair samples from volunteers were collected according to the "Ethics Committee of Andalusian's Biomedical Research" and the Declaration of Helsinki. All volunteers were duly informed about the process and their rights. Hair samples for method optimization and validation were collected from four healthy volunteers with no history of drugs consumption. The samples were obtained from the vertex posterior region of the head and cut as close to the scalp as possible, following the recommendations of the SoHT [28]. A pooled human hair sample from the four volunteers was prepared and stored in aluminum foil at room temperature until use. Application of the method to the determination of DFSA substances in hair was proved by analyzing unfortified human hair from eight volunteers. Collection was performed following the same procedure that specified before, and they were independently analyzed.

2.3. Hair decontamination

The hair surface decontamination step started by washing the collected hair samples with ultrapure water by gently mixing for 5 min followed by another washing step using dichloromethane for 2 min. The excess of washing solvent was removed, and the hair samples were air-dried for 24 h to allow the evaporation of any residual

solvent. After the hair samples were completely dried, they underwent a pulverized process for 4 min at a vibrational frequency of 28 s−1 using a mixer mill MM-301 from Retsch (Asturias, Spain).

2.4. Extraction of DFSAs with cubosomic SUPRAS

The cubosomic SUPRAS was formed by dissolving 1,2-hexanediol (6 mL) in 30 mL of H_2O (1 M Na_2SO_4) in a 50 mL polypropylene centrifuge tube by vortex-shaking for 5 min and then centrifugation (4,000 g, 10 min). Then, two liquid phases were separated; the SUPRAS at the top was collected and stored in closed polypropylene tubes at room temperature, and the equilibrium solution was reused for a new SUPRAS synthesis. The volume of SUPRAS that was formed in these conditions (around ~8 mL) was enough to treat approximately 26 hair samples. This volume can be modified at will by increasing the volume of 1,2-hexanediol. A volume of 300 µL of SUPRAS was added to 25 mg of a decontaminated hair sample and the 192 mixture was vortex stirred for 5 min in 2-mL Eppendorf tubes and then centrifuged at 14,000 rpm for 10 min. A volume of 150 µL of SUPRAS was withdrawn using a micropipette and it was made up to 500 µL with distilled water before LC-MS/MS analysis. A schematic of the SUPRAS-based sample treatment is represented in **Figure 1**, which shows SUPRAS formation (**Fig. 1A**) and SUPRAS-based extraction of DFSA substances (**Fig. 1B**).

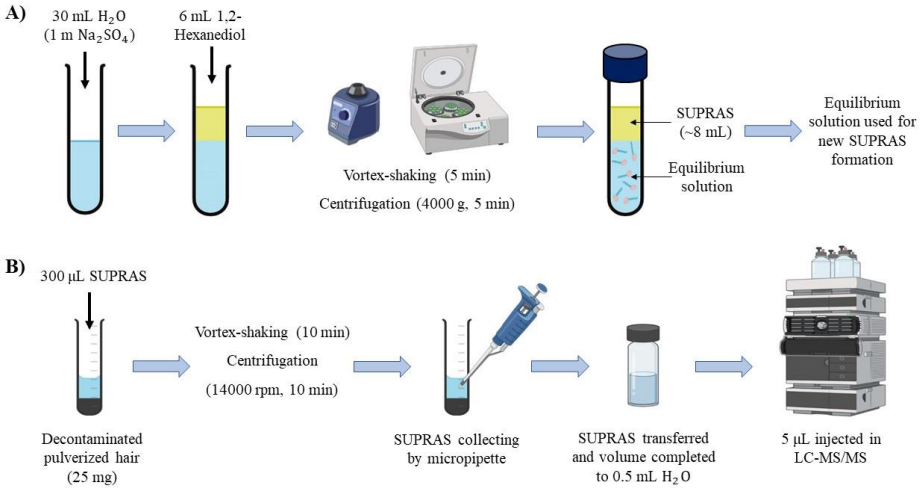


Figure 1. *Procedural steps for SUPRAS formation and extraction of DFSA substances from hair samples. Some images were created with BioRender.com.*

2.5. LC-MS/MS analysis

Measurements were carried out using an Agilent Technologies 1200 series LC (Palo Alto, CA, USA) coupled to a hybrid triple quadrupole/linear ion trap (Applied Biosystems MSD Sciex, 4000 QTRAP, Foster City, CA, USA) equipped with a Turbo V Ion Source (TIS). Data were processed using the Analyst 1.5.1 software. The stationary phase was a Raptor FluoroPhenyl 2.7 µm (100 × 3.0 mm) provided by RESTEK (Bellefonte, Pennsylvania, USA), that was operated at 35ºC. The mobile phase consisted of 0.1% formic acid in water (solvent A), and 0.1% formic acid in methanol (solvent B) and the gradient conditions are specified in **Table S2**. The flow rate was 250 µL/min, and the injection volume was 5 µL. The source was operated in positive ionization mode with the following parameters: source gas temperature 400 °C, capillary voltage: 4500 V, nebulizer gas pressure: 50 psig, drying gas pressure: 50 psig, curtain gas pressure 20 psig; declustering potential: 30 V. MRM transitions (quantifier and qualifier ions), corresponding internal standard and detection parameters for each compound are given in **Table S3**.

2.6. Method validation

Calibration curves (n = 8) were established by running a series of standard solutions in water: SUPRAS 70:30 v/v containing the analytes at concentrations in the range 0.01-200 ng/mL and the ISs (20 ng/mL). The correlation between peak areas and concentration was determined by linear regression. Estimated method detection (MLOD) and quantification (MLOQ) limits were calculated from the instrumental detection and quantification limits by taking into account the sample weight and the diluted SUPRAS extract volume used in the analytical process.

For method validation, pooled hair samples from four healthy volunteers were used after decontamination and pulverization as mentioned before. Recoveries and matrix effects were evaluated by spiking hair samples at three different concentration levels (20, 800, and 2000 pg/mg) while the internal standard concentration was 800 pg/mg in all samples. Three sets of experiments were prepared: **set A:** hair spiked with the target analytes at the three levels; ISs, added before SUPRAS extraction, **set B**: hair sample spiked with the target analytes; IS, added after SUPRAS extraction (at the final dilution step), and **set C**: no sample was added and the target analytes and ISs were

directly spiked to the SUPRAS which was also diluted before analysis. The comparison of the relative peak areas ($A_{DFSA\ substance}/A_{IS}$) × 100 for set A with respect to set B was employed for the calculation of the extraction recovery. Relative matrix effects were calculated as [($A_{DFSA\ substances}/A_{IS}$)× 100] − 100 for set B with respect to set C.

Selectivity was investigated by analyzing nine blank hair samples (25 mg). The blank hair samples were analyzed and checked for any peak interfering with the detection of the analytes or of the ISs and for verifying potential interferences or adverse matrix effects during the early validation phase.

Method precision was evaluated in terms of 240 inter-day and intra-day precision and expressed as relative standard deviation (RSD, %). Hair samples (25 mg) were spiked at three different concentration levels: high at 2000 pg/mg, medium at 800 pg/mg, and low at 100 pg/mg, all with 800 pg/mg of IS. The samples were analyzed daily (inter-day precision, *n*=6, for each concentration level) and on three different days (intra-day precision, *n*=18 for each concentration level). Precision was considered acceptable if the RSD value was equal to or below 20%.

3. Results and discussion

3.1. Cubosomic SUPRAS-based extraction of DFSA substances

The amphiphile 1,2-hexanediol self-assembles in salty water mixtures and separates as a new liquid phase (i.e., SUPRAS) by spontaneous coacervation at room temperature, which is in equilibrium with the salty water solution that contains the amphiphile at the critical aggregation concentration. Coacervation also occurs in hydro-organic media such as tetrahydrofuran-water in the presence of salt. The synthesized SUPRASs are made up of cubosomes, with a size range of 140−240 nm and a high-water content (36−61%, w/w), and they have been proved as highly efficient for the extraction of a wide polarity range of prohibited substances in doping control [38] . So, they were selected for developing a generic sample treatment for determination of DFSA substances.

Optimization of the SUPRAS-based sample treatment procedure was carried out by extracting hair samples (25 mg), previously fortified with the 23 investigated substances at a concentration of 4,000 pg/mg and the ISs at a concentration of 800 pg/mg. All the experiments were performed in triplicate.

The extraction efficiency for DFSA 264 substances of SUPRASs synthesized from 1,2-hexanediol in salty water (Method A) and salty THF (10%, v/v)-water (Method B) was firstly investigated. Both, the SUPRAS (300 µL) and the equilibrium solution (500 µL) were added to the sample; the first one for extraction of the DFSA substances and the second one for wetting the hair sample. In both cases, the SUPRAS was collected, transferred to a glass LC vial, and diluted to 500 µL of H2O for LC-MS/MS measurement. **Table 2** shows the absolute recoveries obtained for the selected DFSA substances along with their corresponding standard deviations. It was found that extraction efficiency was similar for both extraction methods (A and B) and that only four DFSA substances were efficiently extracted in the two types of SUPRASs. So, the SUPRAS obtained in salty water, which is organic solvent free, was selected for further investigations. Given that many of the DFSA substances are moderately polar, they can distribute between the SUPRAS and the equilibrium solution. Due to this, the extraction of all DFSA substances was efficient by adding only the SUPRAS phase (Method C). The results depicted in **Table 2** show that the direct extraction with SUPRAS gave good recoveries for all the 23 studied substances ranging from 86% for bromazepam up to 102% for THC-COOH.

Table 2. Recoveries and standard deviations (%, mean ± SD, *n*=3) obtained for the selected DFSA drugs in human hair extracted under different experimental conditions.

Analyte	Method A	Method B	Method C
Benzodiazepines and z-hypnotic drug			
Alprazolam	68 ± 6	64 ± 8	92 ± 6
Bromazepam	51 ± 15	70 ± 10	86 ± 5
Clonazepam	68 ± 6	64 ± 9	92 ± 2
Clorazepate	71 ± 6	57 ± 2	92 ± 7
Diazepam	83 ± 6	69 ± 5	95 ± 3
Flunitrazepam	65 ± 5	58 ± 5	98 ± 7
Lorazepam	68 ± 12	66 ± 7	99 ± 5
Lormetazepam	69 ± 7	60 ± 5	92 ± 4
Nitrazepam	70 ± 5	67 ± 10	92 ± 7
Temazepam	61 ± 6	55 ± 1	92 ± 6
Zolpidem	61 ± 4	60 ± 7	91 ± 4

Cocaine and cocaine metabolites			
Cocaine	63 ± 3	58 ± 6	91 ± 9
Benzoylecgonine	64 ± 6	70 ± 11	89 ± 3
Cocaethylene	66 ± 6	72 ± 6	98 ± 3
Amphetamine derivatives			
Methamphetamine	61 ± 2	59 ± 7	99 ± 6
Methiopropamine	43 ± 3	35 ± 11	87 ± 7
MDMA	66 ± 3	83 ± 3	92 ± 5
MDA	43 ± 4	56 ± 1	92 ± 3
MDEA	62 ± 6	55 ± 3	95 ± 6
Miscellaneous compounds			
Scopolamine	44 ± 3	52 ± 8	97 ± 9
Fentanyl	63 ± 6	56 ± 7	94 ± 5
Ketamine	63 ± 4	54 ± 7	94 ± 11
THC-COOH	77 ± 3	50 ± 7	102 ± 8
Extraction efficiency range	43%-83%	35%-83%	86%-102%
< 50%	3	1	0
50% - 69%	16	18	0
70% - 120%	4	4	23

Once method C was selected as optimal, both the hair sample size (10 and 25 mg) and the SUPRAS volume (200, 300, and 400 µL) were optimized (**Table 3**). Severe significant differences in the extraction recoveries were not observed with the two sample sizes and the three SUPRAS volumes. In order to choose the best conditions and highlight minor significant differences, Tukey tests were performed. Best conditions (highest extraction efficiency) at the lowest SUPRAS:sample ratio was obtained with 300 uL SUPRAS and 25 mg of hair (recovery range: 86%-102%).

3.2. SUPRAS method validation

Calibration curves are specified in **Table 4**, with 200 ng/mL as the maximum concentration tested for the studied drugs/metabolites. Calibration parameters (slope ± standard deviations, SD, and determination coefficient, R2), estimated method detection and quantification limits (MLOD and MLOQ), and the drug cut-off values for each DFSA compound are listed in **Table 4**. The determination coefficients of the

calibration curves were in the interval 0.990–0.999. The MLOD ranged between 0.1 and 24.2 pg/mg, while MLOQ varied in the range 0.4-80 pg/mg. All the MLOQ values were below the drug cut off concentrations for the studied drugs recommended by the Society of Hair Testing, except for THC-COOH, for which the cut-off value is 0.2 pg/mg and the reached MLOQ is 40 pg/mg in our method. Lower MLOQ for THC-COOH was reached when using GC-MS/MS (0.04 pg/mg) [41].

Table 5 shows the absolute and relative method recoveries and matrix effects of the drugs along with their SD at three concentration levels. The absolute recoveries for the three concentration levels were all within the acceptable range and varied between 68% ± 3 and 105% ± 9. The relative recoveries were between 78% ±2 and 126 ±4. Matrix effects were evaluated in terms of signal suppression and enhancement, and values were mostly within the acceptable range of −20 to +20%, except for cocaine for which signal suppression was observed at the three concentrations levels (−36.3%, −20.1%, and −27.6%). Moreover, signal suppression (−43%) was also observed for THC-COOH at the low-level concentration.

Table 3. Recoveries and standard deviations (%, mean ± SD) for the selected DFSA drugs in human hair extracted under different experimental conditions.

Analyte	Hair sample size (10 mg)			Hair sample size (25 mg)		
	Volume of SUPRAS					
	200 µL	300 µL	400 µL	200 µL	300 µL	400 µL
Benzodiazepines and z-hypnotic drug						
Alprazolam	86 ± 5	93 ± 5	88 ± 2	85 ± 6	92 ± 6	100 ± 4
Bromazepam	74 ± 11	79 ± 15	81 ± 3	72 ± 13	86 ± 5	85 ± 4
Clonazepam	88 ± 7	92 ± 12	86 ± 10	84 ± 8	92 ± 2	93 ± 6
Clorazepate	82 ± 5	90 ± 6	88 ± 7	78 ± 6	92 ± 7	96 ± 1
Diazepam	93 ± 6	88 ± 8	89 ± 6	83 ± 8	95 ± 3	97 ± 9
Flunitrazepam	99 ± 6	95 ± 10	91 ± 4	93 ± 9	98 ± 7	103 ± 3
Lorazepam	86 ± 4	80 ± 8	90 ± 4	87 ± 9	99 ± 5	96 ± 1
Lormetazepam	96 ± 8	94 ± 8	87 ± 5	87 ± 6	92 ± 4	101 ± 2
Nitrazepam	**76 ± 4 AB**	**77 ± 9 AB**	**79 ± 4AB**	**71 ± 8 B**	**92 ± 7A**	**91 ± 5A**
Temazepam	84 ± 4	93 ± 12	89 ± 4	87 ± 6	92 ± 6	97 ± 3
Zolpidem	90 ± 8	92 ± 9	89 ± 7	86 ± 8	91 ± 4	92 ± 4
Cocaine and cocaine metabolites						
Cocaine	**82 ± 6 B**	**104 ± 9 A**	**102±6 AB**	**96 ± 8 AB**	**91 ± 9 AB**	**100±11AB**
Benzoylecgonine	**73 ± 3 B**	**83±14 AB**	**87±11 AB**	**70 ± 9 B**	**89 ± 3 AB**	**97 ± 6 A**
Cocaethylene	87 ± 7	91 ± 11	92 ± 1	86 ± 11	98 ± 3	93 ± 5
Amphetamine derivatives						
Methamphetamine	84 ± 8	95 ± 8	94 ± 3	86 ± 6	99 ± 6	99 ± 5
Methiopropamine	106 ± 10	118 ± 10	98 ± 5	76 ± 10	87 ± 7	94 ± 8
MDMA	**72 ± 2 B**	**102±13 A**	**96 ± 11 A**	**72 ± 6 B**	**92 ± 5 A**	**89 ± 4 AB**
MDA	**62 ± 3 C**	**106 ± 2 A**	**113±10 A**	**80 ± 1 BC**	**92 ± 3 AB**	**109 ± 4 A**
MDEA	92 ± 7	95 ± 6	96 ± 13	84 ± 11	95 ± 6	96 ± 10
Miscellaneous compounds						
Scopolamine	107 ± 3	103 ± 8	92 ± 6	90 ± 14	97 ± 9	103 ± 12
Fentanyl	94 ± 5	103 ± 14	94 ± 5	92 ± 3	94 ± 5	98 ± 2
Ketamine	102 ± 11	112 ± 7	103 ± 3	104 ± 4	94 ± 11	112 ± 5
THC-COOH	**67 ± 12 B**	**83 ± 4 AB**	**86 ± 8 AB**	**72 ± 7 AB**	**102 ± 8 A**	**87 ±11 AB**
Recovery range	**62-107%**	**77-118%**	**79-113%**	**70-104%**	**86-102%**	**85-112%**

SD: Standard deviation (n=3). SUPRAS formation conditions: 83.3% v/v H_2O (1M Na_2SO_4) and 16.6% v/v 1,2-Hexanediol. Letters show significant differences between experimental conditions with Tukey tests. Only those compounds for which significant differences were observed are shown (conditions sharing the same letters are not significantly different).

Table 4. Drug cut-off values, calibration parameters, determination coefficient and method detection and quantification limits for the investigated DFSA compounds.

Analyte	Drug cut-off* (pg/mg)	Calibration curve (ng/mL)	Linear Equation	Slope ± SD (mL/ng)	Determination coefficient (R^2)	MLOD (pg/mg)	MLOQ (pg/mg)*
Benzodiazepines and z-hypnotic drug							
Alprazolam	50	0.1-200	y = 0.0267x - 0.049	0.0266±0.0008	0.994	1.2	4
Bromazepam	50	0.4-200	y = 0.0102x - 0.0179	0.0102±0.0003	0.993	4.7	16
Clonazepam	50	0.2-200	y = 0.0236x - 0.0662	0.0236±0.0009	0.990	2.4	8
Clorazepate	50	0.2-200	y = 0.0199x - 0.0136	0.0199±0.0004	0.997	2.4	8
Diazepam	50	0.4 -200	y = 0.0161x - 0.0217	0.0161±0.0005	0.994	4.7	16
Flunitrazepam	50	0.2-200	y = 0.0419x - 0.043	0.0419±0.0010	0.997	2.4	8
Lorazepam	50	1.0-200	y = 0.0266x + 0.0421	0.0266±0.0008	0.997	12.2	40
Lormetazepam	50	0.2-200	y = 0.0491x + 0.0059	0.0491±0.0006	0.999	2.4	8
Nitrazepam	50	0.2-200	y = 0.0429x - 0.0599	0.0429±0.0017	0.990	2.4	8
Temazepam	50	1.0-200	y = 0.0555x - 0.0663	0.0555±0.0016	0.995	12.2	40
Zolpidem	50	0.01-200	y = 0.21897x - 0.4595	0.2187±0.0067	0.994	0.1	0.4
Cocaine and cocaine metabolites							
Cocaine	500	0.1-200	y = 0.0817x - 0.08	0.0817±0.0026	0.994	1.2	4
Benzoylecgonine	50	0.05-200	y = 0.0319x - 0.0281	0.0319±0.0010	0.994	0.6	2
Cocaethylene	50	0.05-200	y = 0.1433x - 0.0877	0.1433±0.0035	0.996	0.6	2
Amphetamine derivatives							
Methamphetamine	200	1.0-200	y = 0.0526x - 0.0881	0.0526±0.0017	0.995	12.2	40
Methiopropamine	n/a	0.1-200	y = 0.0817x + 0.1009	0.0817±0.0011	0.999	1.2	4
MDA	200	2.0-200	y = 0.0386x - 0.0697	0.0388±0.0015	0.996	24.2	80
MDMA	200	0.1-200	y = 0.0146x + 0.0264	0.0146±0.0006	0.991	1.2	4

MDEA	200	0.05-200	y = 0.0793x - 0.0281	0.0793±0.0014	0.998	0.6	2
Miscellaneous compounds							
Fentanyl	200	0.2-200	y = 0.0438x - 0.0465	0.0438±0.0013	0.994	2.4	8
Scopolamine	n/a	1.0-200	y = 0.0483x - 0.076	0.0483±0.0016	0.993	12.2	40
Ketamine	500	0.4-200	y = 0.008x - 0.0094	0.0080±0.0002	0.995	4.7	16
THC-COOH	0.2	1.0-200	y = 0.0097x - 0.0464	0.0097±0.0003	0.997	12.2	40

* Recommended cut-off concentrations by the European Guidelines for Workplace Drug and Alcohol Testing in Hair.

** The method limit of quantification was set as the lowest point of the calibration curve for each drug/metabolite.

As far as the selectivity of the method is concerned, peaks in chromatograms from the nine negative hair samples were compared to those obtained for the same hair spiked values. No interfering peaks were observed for any of the 23 DFSA substances investigated in the nine hair samples analyzed. **Figure S1** shows, as an example, the extracted ion chromatograms obtained for 314 some of the studied DFSA substances from a hair sample, both fortified (A) and unfortified (B) by measuring the quantifier transitions. **Figure S1** also shows the extracted ion chromatograms for the same sample but measuring the qualifier transitions.

The method precision was evaluated in terms of intra-day precision and inter-day precision at three different concentrations (**Table S4**) and expressed as relative SD (RSD, %). The repeatability and reproducibility were in the ranges of 5–13% and 5-16%, respectively.

3.3. Hair sample analysis

The proposed method was applied to hair samples of eight volunteers. Among volunteers, four of them had a history of drug consumption. The results obtained are listed in **Table 6**. As expected, the main cannabis metabolite THC-COOH was detected in three volunteers recognized as consumers of cannabis (i.e., 1, 2 and 8) being volunteer 8 who had the highest concentration (0.59±0.07 ng/mg). The concentrations found for THC-COOH are comparable to results from a recent study where 126 hair samples were analyzed and 54 samples were positive for both THC and THC-COOH with a concentration range for THC-COOH between 0.04 and 0.85 ng/mg (median: 0.31 ng/mg) [42]. In another recent study, the concentration levels of THC-COOH in hair of patients treated with medical cannabis was on average 0.32 ng/mg [43].

Cocaine was detected in three volunteers (1, 2, and 4) at concentration levels of 0.11 ± 0.01 ng/mg, 1.20 ± 0.05 ng/mg, and 5.94 ± 0.04 ng/mg, respectively. These cocaine concentrations indicated that volunteer 2 is a moderate cocaine user and that volunteer 4 is considered a heavy cocaine user according to a classification previously reported that established levels for light users (0.5-3 ng/mg), moderate users (3.1-10 ng/mg) and heavy users (10.1-40 ng/mg) [44]. The concentration of cocaine and metabolites in hair can anyway significantly vary, and it is subjected to the usage patterns and the interval since the last consumption. For example, a study on 18 hair

Table 5. Absolute and relative method recoveries and matrix effects at three different concentrations of abuse drugs.

Compound	Absolute recovery ± SD (%)			Relative recovery Mean ± SD (%)			Matrix effect		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Benzodiazepines and z-hypnotic drug									
Alprazolam	82 ± 9	92 ± 10	94 ± 1	89 ± 8	110 ± 7	103 ± 1	-16.8	-0.5	-4.9
Bromazepam	85 ± 8	80 ± 1	103 ± 5	106 ± 5	97 ± 8	116 ± 10	20.8	8.5	-9.7
Clonazepam	79 ± 4	95 ± 6	88 ± 1	104 ± 6	116 ± 1	98 ± 5	-1.3	-14.6	-23
Clorazepate	78 ± 6	95 ± 4	96 ± 3	97 ± 6	115 ± 4	107 ± 7	4.4	17.7	2.7
Diazepam	97 ± 4	99 ± 16	87 ± 6	114 ± 7	108 ± 6	96 ± 8	8.3	-14.2	-2.3
Flunitrazepam	83 ± 5	98 ± 4	87 ± 4	98 ± 12	117 ± 6	95 ± 5	7.1	-0.3	-2.4
Lorazepam	68 ± 3	102 ± 7	87 ± 7	84 ± 3	122 ± 4	97 ± 12	0.8	7.8	6.7
Lormetazepam	96 ± 7	103 ± 11	91 ± 1	113 ± 9	121 ± 1	100 ± 1	18.7	9.3	2.0
Nitrazepam	88 ± 7	102 ± 9	87 ± 3	110 ± 7	120 ± 3	96 ± 7	14	12.5	4.3
Temazepam	96 ± 5	105 ± 9	92 ± 1	114 ± 16	126 ± 4	101 ± 1	19.3	2.9	-2.3
Zolpidem	82 ± 9	100 ± 8	95 ± 1	102 ± 15	119 ± 6	106 ± 4	2.3	-21.4	-13.8
Cocaine and cocaine metabolites									
Cocaine	82 ± 9	101 ± 4	85 ± 1	110 ± 4	122 ± 4	95 ± 4	-36.3	-20.1	-27.6
Benzoylecgonine	91 ± 4	94 ± 13	85 ± 8	111 ± 4	117 ± 18	109 ± 12	10.1	3.6	15.9
Cocaethylene	75 ± 6	103 ± 9	92 ± 2	93 ± 4	124 ± 4	103 ± 6	11.3	-19.1	-5.6
Amphetamine derivatives									
Methamphetamine	89 ± 5	103 ± 8	91 ± 1	91 ± 7	115 ± 8	97 ± 1	10.4	-8.5	-4.4
Methiopropamine	100 ± 4	100 ± 10	95 ± 1	117 ± 5	117 ± 6	98 ± 3	1.6	-1.7	-0.7
MDA	89 ± 13	97 ± 11	93 ± 1	103 ± 13	93 ± 14	96 ± 3	-4.9	1.2	-8.3
MDMA	80 ± 9	97 ± 7	89 ± 12	99 ± 12	108 ± 4	94 ± 10	-7.6	-12.9	-12.5
MDEA	64 ± 5	96 ± 4	92 ± 5	78 ± 2	113 ± 4	104 ± 8	-1.2	11.2	8.0
Miscellaneous compounds									
Fentanyl	105 ± 3	98 ± 8	89 ± 2	117 ± 1	117 ± 6	98 ± 4	17.1	-7.1	-4.6
Scopolamine	75 ± 7	117 ± 12	84 ± 7	87 ± 4	109 ± 5	86 ± 9	-3.1	-2.2	-13.6
Ketamine	94 ± 12	94 ± 15	110 ± 4	114 ± 8	98 ± 7	121 ± 6	0.5	-2.2	-1.2
THC-COOH	90 ± 3	101 ± 10	81 ± 1	106 ± 10	104 ± 7	89 ± 2	-43	-9.4	-6.6

samples recorded cocaine concentrations in the range 0.05-68.67 mg/ng [45]. In another recent study in hair, cocaine levels varied from 0.20 to 9.51 ng/mg [46].

Benzoylecgonine was the major detected metabolite of cocaine in hair. The concentration for the three positive samples (1, 2, and 4) were 0.053 ± 0.004, 0.26 ± 0.01 and 2.36 ± 0.05 ng/mg, respectively. The first concentration value is lower than the drug cut-off concentration. These values were in agreement with those reported in another study from 90 hair samples in which the concentrations ranged between 0.19 and 5.77 ng/mg [44].

Cocaethylene, the other cocaine metabolite, was detected to a lesser extent and was only observed in two hair samples (2 and 4) at 0.063 ± 0.007 and 0.0183 ± 0.0003 ng/mg, respectively. The second value is lower than the drug-cut off value of cocaethylene (0.05 ng/mg). Similar concentrations of cocaethylene were recorded in previous studies (0.05-2.46 ng/mg) [46].

Two amphetamine derivatives, MDMA and MDEA were detected in two of the volunteers (i.e., 2 and 4) at a concentration of 0.7 ± 0.1 and 0.160 ± 0.002 ng/mg, respectively, with the latter, at a lower level than the drug cut-off concentration (0.2 ng/mg). It is common to detect MDMA (ecstasy) in hair samples without the detection of other amphetamine group substances, such as methamphetamine and MDA, as reported before in hair samples by Johansen et al [47], where the MDMA concentration in different hair segments was in the range of 0.2-5.9 ng/mg. The detected concentration of MDMA in our study was in agreement with the mentioned study and also with another one from hair specimens in which 8 were positive for 362 MDMA with a concentration range of 0.0743 - 3.6545 ng/mg [35].

Figure 2 shows the extracted ion chromatograms obtained for cocaine in volunteer 1, cocaethylene and MDMA for volunteer 2, benzoylecgonine and MDEA for volunteer 4 and THC-COOH for volunteer 8.

Table 6. Concentrations of DFSA drugs found in eight human hair samples analyzed with the developed cubosomic SUPRAS-LC-MS/MS method.

#	Sex	Age	Hair sample length	Hair Concentration (ng/mg) (mean ± SD)					
				THC-COOH	Cocaine	Benzoylecgonine	Cocaethylene	MDMA	MDEA
1	Female	25	5 cm	0.18 ± 0.03	0.11 ± 0.01	0.053 ± 0.004*	-	-	-
2	Male	26	2-3 cm	0.51 ± 0.02	1.20 ± 0.05	0.26 ± 0.01	0.063 ± 0.007	0.7 ± 0.1	-
3	Female	39	8 cm	-	-	-	-	-	-
4	Female	56	5-6 cm	-	5.94 ± 0.04	2.35 ± 0.05	0.0183 ± 0.0003*	-	0.160 ± 0.002*
5	Male	39	4 cm	-	-	-	-	-	-
6	Female	40	6 cm	-	-	-	-	-	-
7	Male	36	4-5 cm	-	-	-	-	-	-
8	Female	60	7 cm	0.59 ± 0.07	-	-	-	-	-

*Lower than the drug cut-off concentration.

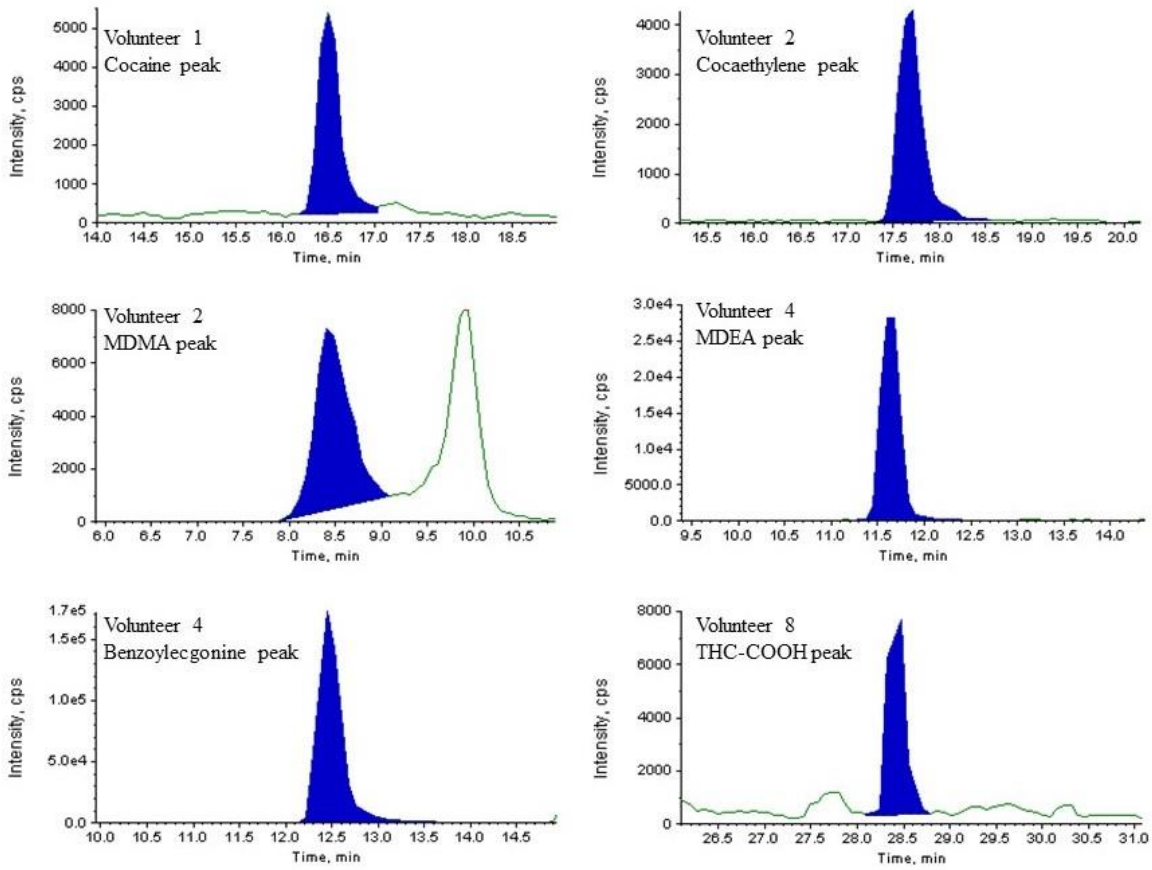


Figure 2 *Extracted ion chromatograms for selected positive hair samples.*

4. Conclusions.

A single-step extraction method based on the formation of an organic solvent-free SUPRAS of 1,2-hexanediol has been optimized and validated to extract 23 DFSA substances including benzodiazepines, z-hypnotic drugs, cocaine metabolites, amphetamine derivatives and other miscellaneous compounds from hair samples of sexual assault victims. Extracts were directly analyzed by LC-MS/MS after simple dilution with water. The developed method efficiently extracts (>86%) all the targeted substances without using any organic solvent making it an eco-friendly method. The SUPRAS synthesis was simple enough and fast as it consists of a mixture of 1M Na_2SO_4 H_2O and 1,2-hexanediol that is easily prepared with vortex shaking and centrifugation steps at room temperature. Moreover, the method was equally efficient regardless of the hair sample size used (10 or 25 mg). The SUPRAS method was considered rapid in comparison to other hair treatment processes since incubation, clean-up or evaporation/reconstitution steps were not required.

Declarations

Informed consent Volunteers' information sheet was given to all the participants, and samples were collected with their consent. Urine samples were collected according to the "Ethics Committee of Andalusian's Biomedical Research" and the Declaration of Helsinki.

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.

Acknowledgments

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Conflict of 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.

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SUPPORTING INFORMATION

Hair analysis of selected drug-facilitated sexual assault substances using green supramolecular solvent extraction and LC-MS/MS analysis

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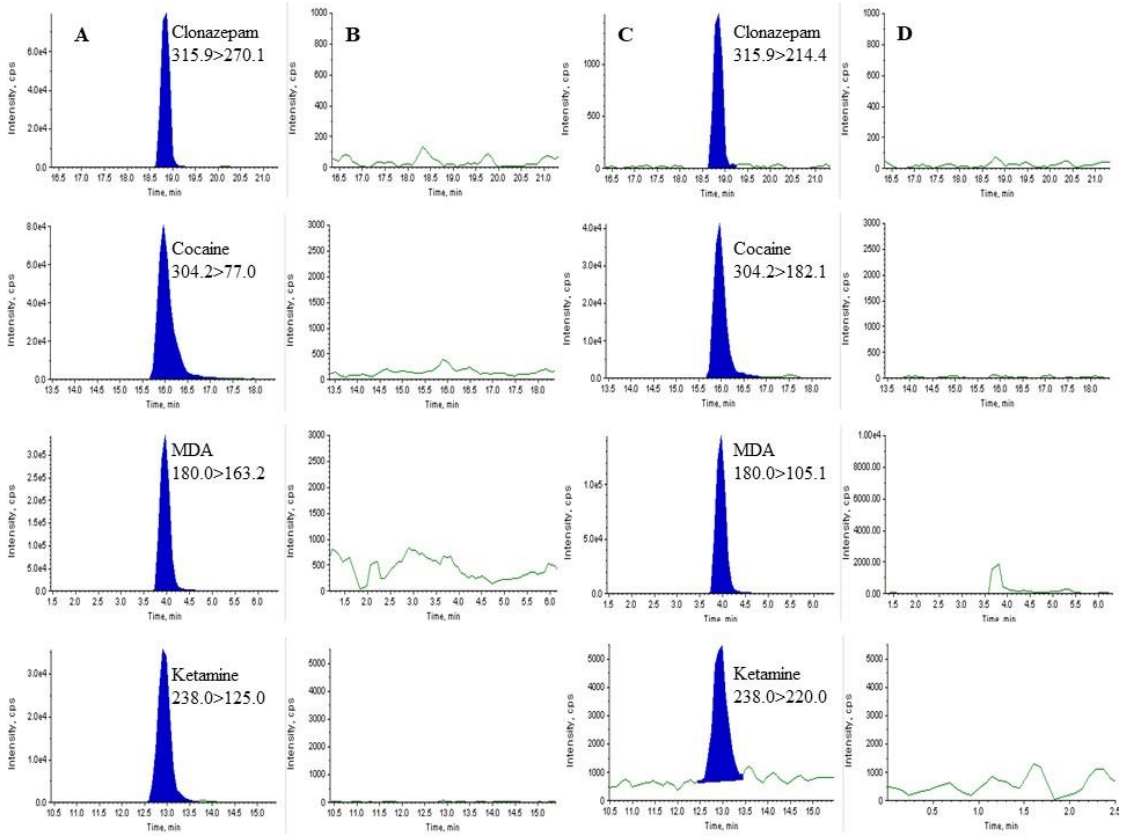
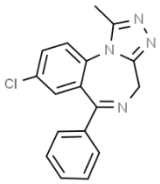
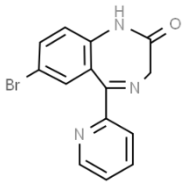
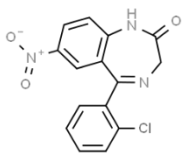
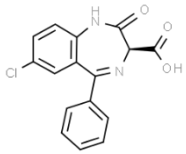
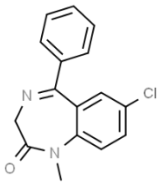
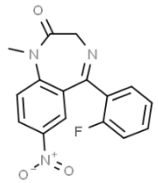
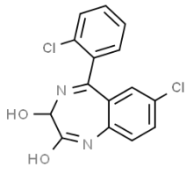
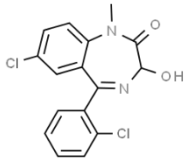
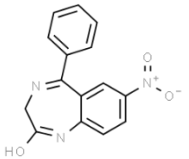
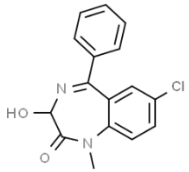
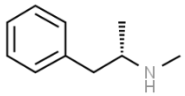
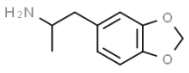
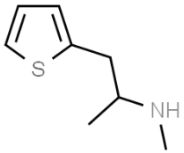
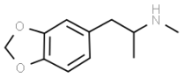
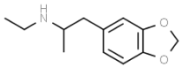
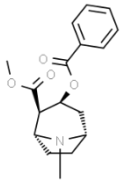
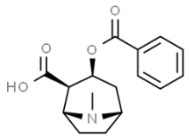


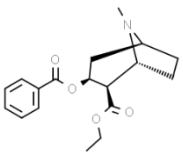
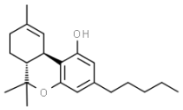
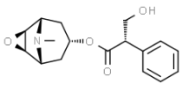
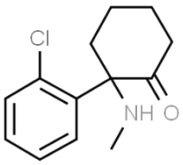
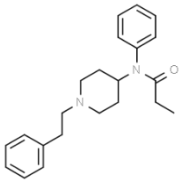
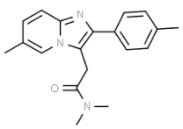
Figure S1. *Extracted ion chromatograms of the quantifier (A and B) and qualifier (C and D) transitions of four representative DFSA substances from hair (A and C) fortified at the 100 ng/mL level and (B and D) unfortified negative hair sample. Quantifier transitions were Clonazepam (315.9→270.1), Cocaine (304.2→77.0), MDA (180.0→163.2), and Ketamine (238.0→125.0). Qualifier transitions were Clonazepam (315.9→214.4), Cocaine (304.2→182.1), MDA (180.0→105.1), and Ketamine (238.0→220.0).*

Table S1. Chemical structures, molecular formulas, and different parameters of interest for the selected DFSA substances or their metabolites.

Main drugs involved in DFSA	**[a]Chemical structure**	**Molecular formula**	**[b]Molecular weight (g/mol)**	**[b]Partition coefficient, log *P***	**[b,c]p*K*a acidic**	**[b,c]p*K*a basic**	**[b]H-bond donors**	**[b]H-bond acceptors**
Alprazolam		$C_{17}H_{13}ClN_4$	308.8	2.1	18.3	5.08	0	3
Bromazepam		$C_{14}H_{10}BrN_3O$	316.15	1.7	12.24	2.68	1	3
Clonazepam		$C_{15}H_{10}ClN_3O_3$	315.17	2.4	11.89	1.86	1	4
Clorazepate		$C_{16}H_{11}ClN_2O_3$	314.72	3.3	3.32	-0.64	2	4
Diazepam		$C_{16}H_{13}ClN_2O$	284.74	3.0	n/a	2.92	0	2

Flunitrazepam		$C_{16}H_{12}FN_3O_3$	313.28	2.1	n/a	1.7	0	5
Lorazepam		$C_{15}H_{10}Cl_2N_2O_2$	321.2	2.4	10.61	-2.2	2	3
Lormetazepam		$C_{16}H_{12}Cl_2N_2O_2$	335.2	2.4	10.68	-2.2	1	3
Nitrazepam		$C_{15}H_{11}N_3O_3$	281.27	2.2	11.9	2.61	1	4
Temazepam		$C_{16}H_{13}ClN_2O_2$	300.74	2.2	10.68	-1.4	1	3
Methamphetamine		$C_{10}H_{15}N$	149.23	2.1	n/a	10.01	1	3

3,4-Methylenedioxy-amphetamine (MDA), Tenamfetamine		$C_{10}H_{13}NO_2$	179.22	1.6	n/a	10.01	1	3
Methiopropamine		$C_8H_{13}NS$	155.26	1.9	n/a	n/a	1	2
3,4-Methylenedioxy-methamphetamine (MDMA), Ecstasy		$C_{11}H_{15}NO_2$	193.24	2.2	n/a	10.14	1	3
3,4-Methylenedioxy-N-ethylamphetamine (MDEA)		$C_{12}H_{17}NO_2$	207.27	2.5	n/a	10.22	1	3
Cocaine		$C_{17}H_{21}NO_4$	303.35	2.30	n/a	8.85	0	5
Benzoylecgonine (metabolite of Cocaine)		$C_{16}H_{19}NO_4$	289.33	-0.3	3.15	9.54	1	5

Cocaethylene (metabolite of Cocaine)		$C_{18}H_{23}NO_4$	317.4	2.7	n/a	n/a	0	5
11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid		$C_{21}H_{30}O_2$	314.5	7.0	9.34	-4.9	1	2
Scopolamine		$C_{17}H_{21}NO_4$	303.35	0.9	15.15	6.95	1	5
Ketamine		$C_{13}H_{16}ClNO$	237.72	2.2	18.78	7.45	1	2
Fentanyl		$C_{22}H_{28}N_2O$	336.5	4.0	n/a	8.77	0	2
Zolpidem		$C_{19}H_{21}N_3O$	307.4	2.5	n/a	5.65	0	2

[a]Chemical structures obtained from Chemspider, [b]Values obtained from PubChem, [c]Values obtained from DrugBank, n/a: non-available value.

Table S2. Stationary phase and mobile phase parameters.

Phase	**Specification**			
Stationary phase				
Packing material	Raptor FluoroPhenyl			
Column length	100 mm			
Inside diameter	3.0 mm			
Particle size	2.7 µm			
Mobile phase				
Composition	Solvent A: 0.1% formic acid in H_2O.			
	Solvent B: 0.1% formic acid in MeOH.			
Gradient conditions	**Total time (min)**	**Flow rate (µL/min)**	**A%**	**B%**
	5.0	250	90	10
	0.5	250	90	10
	25.0	250	20	80
	26.0	250	10	90
	36.0	250	10	90

Table S3. MS parameters and internal standard used for analysis of the target drugs.

Drug	Precursor ion	Quantifier Qualifier	DP (volts)	CE (volts)	CXP (volts)	Internal standard
Alprazolam	309.1	281.2	91	39	6	Diazepam-d5
		205.3	91	37	10	
Bromazepam	318.0	182.1	51	37	16	Diazepam-d5
		209.2	51	37	16	
Clonazepam	315.9	270.1	126	35	14	Nitrazepam-d5
		214.4	126	35	14	
Clorazepate	271.0	140.1	65	50	18	Diazepam-d5
		165.0	65	50	18	
Diazepam	285.1	154.3	101	47	10	Diazepam-d5
		193.3	101	39	12	
Flunitrazepam	314.0	268.1	111	37	22	Nitrazepam-d5
		239.1	111	37	22	
Lorazepam	320.9	274.9	66	33	20	Diazepam-d5
		229.0	66	33	20	
Lormetazepam	334.9	288.9	81	33	16	Diazepam-d5
		317.0	81	33	16	
Nitrazepam	282.1	236.2	5	35	4	Nitrazepam-d5
		180.1	5	35	4	
Temazepam	301.2	255.2	91	33	16	Diazepam-d5
		177.3	91	57	8	
Zolpidem	308.1	235.2	98	47	10	Diazepam-d5
		236.1	98	47	10	
Methamphetamine	150.0	91.0	46	17	8	Methamphetamine-
		119.1	46	17	8	d14
Methiopropamine	155.9	97.0	46	23	8	Scopolamine-d3
		58.10	46	13	8	
3,4-Methylenedioxy-	180.0	163.2	42	15	9	Methamphetamine-
amphetamine (MDA)		105.1	42	32	8	d14
3,4-Methylenedioxy	194.2	163.0	62	19	10	Methamphetamine-
methamphetamine		105.2	62	37	8	d14
(MDMA)						
3,4-Methylenedioxy-	208.2	163.2	31	15	2	Methamphetamine-
N-ethylamphetamine		132.9	31	21	2	d14
(MDEA)						
Cocaine	304.2	77.0	36	89	12	Benzoylecgonine-d3
		182.1	36	14	12	
Benzoylecgonine	290.2	168.2	30	29	12	Benzoylecgonine-d3
(metabolite of		105.0	30	55	12	
Cocaine)						
Cocaethylene	318.2	196.1	10	27	20	Benzoylecgonine-d3
(metabolite of		82.1	10	43	4	
Cocaine)						
Fentanyl	337.1	105.0	30	36	9	Diazepam-d5
		188.0	30	22	9	
Scopolamine	304.1	156.0	66	31	8	Scopolamine-d3

		138.1	66	23	8	
Ketamine	238.0	125.0	60	20	4	Diazepam-d5
		220.0	60	10	4	
11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid	345.1	327.3	136	23	6	Diazepam-d5
		299.2	136	27	22	
Diazepam-d5	290.0	154.0	96	41	24	-
		198.0	111	47	14	
Nitrazepam-d5	287.1	241.1	16	37	40	-
		185.1	16	34	40	
Methamphetamine-d14	164.0	98.0	45	22	20	-
		130.0	45	10	20	
Benzoylecgonine-d3	293.0	171.0	60	30	8	-
		105.0	91	43	8	
Scopolamine-d3	307.1	141.1	30	26	20	-
		159.0	30	16	20	

Table S4. Intra- and inter-day precision for DFSA compounds in hair, at the three different concentrations low, medium, and high (100, 800, and 2,000 pg/mg) using the proposed SUPRAS-LC-MS/MS method.

Analytes	Intra-day precision (*n*=6), RSD%			Inter-day precision (*n*=18), RSD%		
	Low	Medium	High	Low	Medium	High
Benzodiazepines and z-hypnotic drug						
Alprazolam	13	9	5	12	12	13
Bromazepam	12	13	7	12	10	14
Clonazepam	7	12	7	14	8	11
Clorazepate	9	5	5	13	9	12
Diazepam	12	13	7	14	12	10
Flunitrazepam	8	7	11	8	9	11
Lorazepam	11	12	15	16	8	13
Lormetazepam	14	7	7	14	8	8
Nitrazepam	7	10	8	16	10	11
Temazepam	5	10	6	12	7	11
Zolpidem	9	7	10	10	6	11
Cocaine and cocaine metabolites						
Cocaine	9	6	10	12	9	13
Benzoylecgonine	10	9	6	12	9	13
Cocaethylene	12	13	8	9	8	15
Amphetamine derivatives						
Methamphetamine	10	5	10	12	6	13
Methiopropamine	11	12	9	10	5	10
MDA	13	11	12		15	15
MDMA	9	6	10	10	12	13
MDEA	7	9	9	12	9	9
Miscellaneous compounds						
Fentanyl	12	10	10	10	5	12
Scopolamine	11	10	9	14	12	13
Ketamine	7	10	14	14	11	15
THC-COOH	5	7	6	12	12	9

CONCLUSIONS

The main conclusions that can be drawn from the critical revision related to the *Analysis of conventional and nonconventional forensic specimens in drug facilitated sexual assault by liquid chromatography and tandem mass spectrometry,* presented in ***Chapter I*** are summarized below:

1) The selection of forensic specimens is a crucial aspect for forensic scientists in DFSA cases. Early sample collection is crucial due to the rapid metabolism and elimination rates of many DFSA substances. Blood and urine are the most collected biological specimens, and a delay in blood collection can lead to missed detection of administered substances. Factors such as the circumstances of the case, whether it is antemortem or postmortem, specimen availability, and target drugs influence the selection of biological specimens. Postmortem redistribution of drugs may impact the choice of the appropriate matrix. Early sample collection, particularly for benzodiazepines, is crucial for positive analytical findings.
2) The most used extraction strategies in DFSA analysis are liquid-liquid extraction (LLE) and solid phase extraction (SPE). LLE removes proteins and provides some clean-up but requires the use of hazardous organic solvents and labour-intensive steps. Ethyl acetate is the most employed organic solvent for LLE, and recoveries vary depending on the specific compounds. Alternative extraction methods, such as ionic liquid-based microextraction and dispersive liquid-liquid microextraction (DLLME), have been proposed for faster and more environmentally friendly analysis. Electromembrane extraction (EME) is a promising method that applies an electric field for the migration of charged substances through a liquid membrane.
3) SPE is widely used in DFSA analysis as it provides sample clean-up, preconcentration, and extracts suitable for LC-MS analysis. Mixed-mode SPE sorbents, combining reverse phase and ion exchange mechanisms, are commonly used for the extraction of a wide range of compounds. Alternative sorbents, such as porous silica coated with zirconia or highly selective molecularly imprinted polymers (MIPs), have been proposed for specific compounds.
4) LC-(ESI)MS/MS is considered the most suitable technique for the determination of illegal drugs and pharmaceutical substances in human matrices. GC-MS instruments are still widely implemented in routine laboratories due to their lower

cost and the availability of universal GC-MS libraries. LC-MS/MS offers advantages such as shorter run times, less extensive sample preparation, and the ability to correct matrix effects with deuterated internal standards. CE-MS has limited use in forensic drug analysis but shows potential for the analysis of charged drugs with low reagent and sample consumption.

5) LC separation is primarily carried out on C18 columns, but alternative phases such as biphenyl (BP) and pentafluorophenyl (PFP) columns have been proposed for improved retention of polar compounds.
6) Gradient elution with water and acetonitrile is preferred in the mobile phase, and LC-MS/MS based on triple quadrupole or QTRAP instruments is commonly used. High-resolution MS instruments, such as Orbitrap, are also proposed for DFSA analysis.

The conclusions of de investigations presented in ***Chapter II*** related to the application of *Green nanostructured liquids for the analysis of urine in drug-facilitated sexual assault cases* can be summarized in the following points:

1) Eco-friendly extraction: The use of SUPRASs, a green and eco-friendly extraction approach, reduces the reliance on organic solvents and promotes sustainable analytical practices.
2) Simplified and rapid extraction: SUPRAS extraction is a simple and rapid process, minimizing the time-consuming and costly steps associated with traditional extraction methods such as solid-phase extraction or liquid-liquid extraction.
3) Increased sensitivity: The proposed method achieves low limits of detection and quantification, enabling the detection of DFSA compounds at trace levels in urine samples.
4) Efficient solubilization of drugs in a wide polarity range: The SUPRAS-based method demonstrates the ability to extract and analyse a broad range of DFSA compounds, covering multiple drug classes.
5) High extraction efficiency: the SUPRAS made of 1,2-hexanediol exhibits high extraction efficiencies for the target compounds, ensuring accurate and reliable results.

The third section (***Chapter III***) discusses the *development and validation of a single-step extraction method for analysing drug-facilitated sexual assault (DFSA) compounds in hair samples*. The main points concluded from chapter III are as follows:

1) The study developed an eco-friendly, one-step extraction method using solvent-free SUPRAS of 1,2-hexanediol. It eliminates the need for organic solvents, it is fast and simple to perform, it does not require additional incubation or clean-up steps, and allows direct analysis by LC-MS/MS.
2) The method works equally well for 10 or 25 mg hair samples, making it versatile.
3) This method was applied efficiently to extract 23 DFSA substances from sexual assault victims' hair samples.

CONCLUSIONES

Las principales conclusiones que pueden derivarse de la revisión crítica realizada sobre *Análisis de muestras forenses convencionales y no convencionales en DFSA mediante cromatografía de líquidos y espectrometría de masas en tándem,* presentada en el ***Capítulo I***, se resumen a continuación:

1) La selección de muestras forenses es un aspecto crucial para los científicos forenses en los casos DFSA. La recolección temprana de las muestras es crucial debido al rápido metabolismo y tasas de eliminación de muchas sustancias DFSA. La sangre y la orina son las muestras biológicas que más se recolectan y un retraso en la recolección de sangre puede provocar que no se detecten las sustancias administradas. Factores como las circunstancias del caso, si es antemortem o postmortem, la disponibilidad de muestras y los fármacos diana influyen en la selección de muestras biológicas. La redistribución postmortem de fármacos puede afectar la elección de la matriz adecuada. La recolección temprana de muestras, particularmente para las benzodiazepinas, es crucial para obtener resultados analíticos positivos.
2) Las estrategias de extracción más utilizadas en el análisis DFSA son la extracción líquido-líquido (LLE) y la extracción en fase sólida (SPE). LLE elimina proteínas y proporciona cierta limpieza, pero requiere el uso de solventes orgánicos peligrosos y pasos que requieren mucha mano de obra. El acetato de etilo es el disolvente orgánico más utilizado para el LLE y las recuperaciones varían según los compuestos específicos. Se han propuesto métodos de extracción alternativos, como la microextracción basada en el uso de líquidos iónicos y la microextracción líquido-líquido dispersiva (DLLME), para un análisis más rápido y respetuoso con el medio ambiente. La extracción por electromembrana (EME) es un método prometedor que aplica un campo eléctrico para la migración de sustancias cargadas a través de una membrana líquida.
3) SPE se usa ampliamente en el análisis DFSA, ya que proporciona limpieza, preconcentración y extractos de muestras adecuados para el análisis LC-MS. Los adsorbentes SPE de modo mixto, que combinan mecanismos de intercambio iónico y de fase inversa, se utilizan comúnmente para la extracción de una amplia gama de compuestos. Para compuestos específicos se han propuesto adsorbentes

alternativos, como sílice porosa recubierta con circonio o polímeros de impresión molecular (MIP) altamente selectivos.

4) LC-(ESI)MS/MS se considera la técnica más adecuada para la determinación de drogas ilegales y sustancias farmacéuticas en matrices humanas. Los instrumentos de GC-MS todavía se implementan ampliamente en laboratorios de rutina debido a su menor costo y la disponibilidad de bibliotecas de GC-MS universales. LC-MS/MS ofrece ventajas como tiempos de ejecución más cortos, preparación de muestras menos extensa y la capacidad de corregir los efectos de la matriz con estándares internos deuterados. CE-MS tiene un uso limitado en el análisis forense de drogas, pero muestra potencial para el análisis de drogas cargadas con bajo consumo de reactivos y muestras.
5) La separación por LC se lleva a cabo principalmente en columnas C18, pero se han propuesto fases alternativas como columnas de bifenilo (BP) y pentafluorofenilo (PFP) para mejorar la retención de compuestos polares.
6) Se prefiere la elución en gradiente con agua y acetonitrilo en la fase móvil, y comúnmente se usa LC-MS/MS basada en instrumentos de triple cuadrupolo o QTRAP. También se proponen instrumentos MS de alta resolución, como Orbitrap, para el análisis DFSA.

Las conclusiones de las investigaciones presentadas en el ***Capítulo II*** con relación a la aplicación de *Líquidos ecológicos nanoestructurados para el análisis de orina en casos de DFSA* se pueden resumir en los siguientes puntos:

1) Extracción sostenible: El uso de SUPRASs permite el desarrollo de extracciones más ecológicas, reduciendo la dependencia de disolventes orgánicos y promoviendo prácticas analíticas acordes con los principios de la Química Verde.
2) Extracción simple y rápida: la extracción con SUPRASs permite el desarrollo de procesos simples y rápidos, que reducen el número de etapas requeridas en las extracciones tradicionales, reduciendo el coste y tiempo con respecto a la extracción en fase sólida o la extracción líquido-líquido.
3) Elevada sensibilidad: el método propuesto logra límites bajos de detección y cuantificación, lo que permite la detección de compuestos DFSA a niveles traza en muestras de orina.

4) Solubilización eficiente de compuestos en un amplio intervalo de polaridad: el método basado en SUPRASs tiene la capacidad de extraer una amplia gama de compuestos DFSA, que cubren múltiples clases de fármacos.
5) Alta eficiencia de extracción: los SUPRASs sintetizados a partir de 1,2-hexanodiol exhiben elevada eficiencia de extracción para los compuestos objetivo, lo que garantiza resultados precisos y fiables.

La tercera sección (***Capítulo III***) discute el *desarrollo y la validación de un método de extracción para analizar compuestos de agresión sexual facilitada por drogas (DFSA) en muestras de cabello.* Los principales puntos que se concluyen del Capítulo III son los siguientes:

1) El estudio desarrolló un método de extracción sostenible que consta de una etapa utilizando SUPRASs de 1,2-hexanediol que no requiere el uso de disolventes orgánicos. El método es rápido y sencillo de realizar, no requiere etapas de incubación ni purificación de las muestras y permite el análisis directo del extracto SUPRAS mediante LC-MS/MS.
2) El método es versátil y aplicable a muestras de diferente cantidad de cabello (10 o 25 mg).
3) Este método se aplicó de manera eficiente para extraer 23 sustancias DFSA de muestras de cabello de víctimas de agresión sexual.

Annex A

Informed consent for extraction, use and storage of biological samples.

Paciente N° _____/

CONSENTIMIENTO INFORMADO PARA EXTRACCIÓN, USO Y ALMACENAMIENTO DE MUESTRAS BIOLÓGICAS

Título del proyecto:
Nombre Investigador Responsable: Eloy Girela López / Soledad Rubio Bravo
Unidad/Departamento/Servicio: Medicina Legal y Forense / Química Analítica
Correo electrónico:

A. Hoja de Información al paciente

1. Solicitud

Le estamos solicitando que autorice la extracción y uso de muestras de sangre y/u orina y/o cabello para la realización del estudio de investigación "*Detección de sustancias por LC-MS-MS implicadas en víctimas de Sumisión Química"*.

Para que pueda tomar una decisión informada de si desea o no participar de la investigación, en este documento se describe el objetivo del estudio, sus derechos y obligaciones, los procedimientos necesarios para el estudio y los posibles beneficios y riesgos de participar en él. Tome el tiempo que necesite para leer detenidamente la información que sigue. No dude en hacer las preguntas que desee al investigador que se lo está explicando.

2. Objetivos del estudio

El propósito de esta investigación es optimizar la técnica para realizar el análisis toxicológico de sustancias implicadas en víctimas de sumisión química por LC-MS-MS mediante sistemas supramoleculares. Dicha plataforma genérica permitirá la extracción simultánea y eficiente de las sustancias implicadas habitualmente en los delitos de sumisión química, y en una fase posterior podrá aplicarse a las distintas matrices biológicas de interés (sangre, orina, pelo, uñas) y contribuir al análisis de casos reales y a la mejora y eficiencia de un protocolo de atención a las víctimas de estos delitos.

3. Procedimientos a seguir en la obtención de la muestra

Si después de hablar del estudio con su médico usted acepta participar en él, tendrá que firmar este formulario de Consentimiento Informado para someterse a la toma de muestras de **sangre y/u orina y/o cabello y/o uñas**:

- 1 tubo de sangre de 5 ml, extraída mediante una venopunción en una vena del brazo.
- 1 recipiente con orina (hasta 50ml).
- 1 mechón de cabello de la zona occipital, de aproximadamente el grosor de un lápiz (7mm de diámetro), cortado con tijera a ras del cuero cabelludo.
- Extremo distal al pulpejo de las uñas de manos.

4. Razones y uso de la recolección de muestras

Las muestras de sangre, orina, cabello y uñas que se obtengan, así como la información médica relativa a las mismas, serán utilizadas por los investigadores para optimizar la técnica analítica del estudio toxicológico, con la finalidad de identificar y cuantificar las sustancias implicadas en la comisión de un posible delito de abuso sexual por sumisión química.

No existe otro procedimiento alternativo para la determinación de sustancias mediante estudio toxicológico.

5. Riesgos

Cualquier actuación médica tiene riesgos, aunque sean mínimos. Por eso es importante que usted conozca los riesgos que pueden aparecer en este proceso o intervención.

- Sangre: Los riesgos posibles vendrían derivados de la técnica utilizada para la obtención de la muestra.
  - LOS MÁS FRECUENTES:
  - Pequeños hematomas en la zona de punción que desaparecen transcurridos unos días.
  - Dolor leve-moderado en el momento de la extracción, que suele ser de corta duración.
  - En casos excepcionales, infección en la zona de punción.
  - LOS MÁS GRAVES:

  - En algunas personas se puede producir una reacción vaso-vagal con mareo y desmayo.
- Orina: La recogida de un espécimen de orina no plantea ningún riesgo o complicación esperable.
- Cabello: La recogida de una muestra de cabello no plantea ningún riesgo o complicación esperable.
- Uñas: La recogida de una muestra de uñas no plantea ningún riesgo o complicación esperable.

6. Beneficios

Su participación en el estudio contribuirá a la caracterización y cuantificación de las sustancias empleadas en este tipo de atentados, permitiendo posteriormente el desarrollo de un protocolo de actuación clínico y médico-legal donde se favorezca una adecuada toma de muestras y detección de sustancias psicoactivas implicadas mediante estudio toxicológico.

7. Voluntariedad y revocación del consentimiento

Su participación es completamente voluntaria. Sea cual sea su decisión, no necesita dar ninguna explicación. Usted tiene derecho a retirar su consentimiento para extracción y uso de sus muestras en cualquier momento durante el estudio, en tal caso debe notificar a cualquiera de los investigadores que ya no desea que su muestra se almacene o se utilice para la investigación. No necesita dar los motivos por los que cambió de opinión. No obstante, si sus muestras ya se han analizado, los resultados seguirán formando parte de los datos globales de la investigación.

8. Costos

Usted no tendrá gasto alguno relacionado con los procedimientos y materiales necesarios para la extracción y almacenamiento de sus muestras.

9. Confidencialidad y privacidad

El Investigador Responsable adoptará las medidas necesarias para garantizar la seguridad y la confidencialidad suficientes que permitan el uso correcto de las muestras biológicas almacenadas. Para garantizar la confidencialidad de su información médica, formularios, registros y muestras, se etiquetarán con su número de identificación de paciente; no se etiquetarán con su nombre, ni ninguna otra información de carácter personal. Sólo tendrán acceso a sus datos los miembros del

equipo investigador autorizados por el Comité de Ética de la Investigación de Córdoba. Se cumplirán en todo momento las recomendaciones de confidencialidad recogidas en la Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales.

10. Publicación científica y confidencialidad

Es posible que los datos y resultados derivados de este estudio puedan ser publicados en revistas y/o congresos médicos. Si esto ocurre, y en conformidad en lo establecido la Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales, en la Ley 14/1986, General de Sanidad, y en la Ley 41/2002, básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica, sus datos clínicos ya están anonimizados por lo que usted no podrá ser identificado(a).

11. Derechos del paciente y contacto

Cualquier pregunta que usted desee hacer en relación con el estudio y/o específicamente en relación con la extracción de muestras, será respondida por el Investigador Responsable cuyos datos de contacto se encuentran al inicio de este documento.

B. Consentimiento Informado. Hoja de firmas

Si alguna muestra de sangre y/o orina y/o cabello que he proporcionado para este proyecto de investigación queda sin usar o sobrante cuando se ha completado el proyecto (Marcar una opción de las siguientes):

- □ Deseo que mi muestra de sangre y/u orina y/o cabello sea destruida de inmediato.
- □ Deseo que mi muestra de sangre y/u orina y/o cabello se destruya después de ___ años.
- □ Autorizo a que mi muestra de sangre y/u orina y/o cabello sea almacenada indefinidamente.

Y si la muestra es almacenada:

- □ Autorizo a que mi muestra de sangre y/u orina y/o cabello sea almacenada y se use en investigación futura, pero sólo con el mismo objetivo del proyecto de investigación actual, relacionado con "Sumisión química".
- □ Autorizo a que mi muestra de sangre y/u orina y/o cabello sea almacenada y se use en cualquier investigación futura, de cualquier tipo que haya sido adecuadamente aprobada.

1. He recibido una explicación satisfactoria -he leído o alguien me ha leído el documento- sobre el procedimiento de extracción de mis muestras para el estudio descrito, su finalidad, riesgos y beneficios.

2. Entiendo la información recibida, mis dudas han sido respondidas y comprendo que mi participación es voluntaria.

3. Autorizo voluntariamente la recolección de muestras y su utilización para la investigación descrita anteriormente y conozco mi derecho a retirar mi consentimiento cuando lo desee, con la única obligación de informar mi decisión al Investigador Responsable del estudio.

Los espacios que siguen van escritos de puño y letra de los firmantes

Nombre y apellidos del paciente	DNI	Firma	Fecha
Nombre y apellidos de representante y relación con el paciente (si fuere pertinente)	DNI	Firma	Fecha
Nombre del investigador que explica el Consentimiento Informado	DNI	Firma	Fecha

Annex B

Communications (oral and poster contributions) presented at congresses.

1) **Poster** (Green supramolecular solvent extraction for the analysis of urine in drug-facilitated sexual assault DFSA cases).
   Authors: Nouman Almofti, Soledad González-Rubio, Ana Ballesteros, Soledad Rubio, Eloy Girela.
   Presentad in la X Reunión de la Sociedad Española de Espectrometría de Masas (X-RSEEM). Held in Córdoba on June 1st to 3rd 2022.

2) **Oral Presentation** (Analysis of hair samples using green supramolecular solvent for the extraction of selected drug-facilitated sexual assault substances by lc-ms/ms analysis).
   Presented at the XI Scientific Congress of Researchers in Training, with the title "The art of investigating", organized by the Educo and eidA3 Doctoral Schools of the University of Córdoba, held at the Faculty of Medicine and Nursing, in Córdoba on May 4th , 2023.

3) **Poster** (Development of green supramolecular solvents for the extraction of compounds for drug-facilitated sexual assault from human hair).
   Authors: Nouman Almofti, Ana Ballesteros, Soledad Rubio, Eloy Girela.
   Presentad in The I Congreso de Química Aplicada a la Energía y al Medio Ambiente (QUIEMA23) . Held in Córdoba on June 12th and 13th 2023.

1-2-3 de **junio** de **2022**

Rectorado de la Universidad de Córdoba

Curso precongreso

Espectrometría de Movilidad Iónica-Espectrometría de Masas **31 de mayo de 2022**

Sala de Grados Manuel Medina, Campus de Rabanales, Universidad de Córdoba

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Green supramolecular solvent extraction for the analysis of urine in drug-facilitated sexual assault cases

N. Almofti[1,2], S. González-Rubio[1], A. Ballesteros[1*], E. Girela[2], S. Rubio[1]

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[2] *Section of Forensic and Legal Medicine. Department of Morphological and Sociosanitary Sciences. Faculty of Medicine and Nursing. University of Córdoba, 14071 Córdoba, Spain.*

AREA: Bioanalysis

Abstract

In this work, we optimize and validate a simple, time-saving, and environmentally friendly sample preparation method based on green supramolecular solvents (SUPRAS) for the extraction of drug-facilitated sexual assault (DFSA) substances from human urine. The methodology did not involve the use of organic solvents and was fast and simple (stirring, centrifugation and dilution). Cubosomic SUPRAS were formed by the addition of 1,2-hexanediol (200 µL) to 1.0 mL of human urine containing 1 M Na_2SO_4. SUPRAS extracts were analyzed by LC-MS/MS. The method was fully validated for 25 DFSA compounds including 10 benzodiazepines, 2 z-hypnotic drugs, 6 amphetamine derivatives, 3 cocaine metabolites and 4 miscellaneous compounds. The limits of detection ranged from 0.003 to 1 ng/mL and the limit of quantification ranged from 0.01 to 2.5 ng/mL, values low enough for the established minimum required performance levels (MPRLs) of these substances. Total procedural recoveries were in the range 70-125%. This simple and green method has a great potential to be implemented for the monitorization of illegal drugs involved in DFSA cases by forensic laboratories. The applicability of the proposed method was proven by analyzing 7 human urine samples and was able to detect various targeted substances at different concentrations.

Keywords Supramolecular solvents SUPRAS, DFSA, urine, benzodiazepines, z-hypnotics, cocaine, LC-MS/MS.

Green supramolecular solvent extraction for the analysis of urine in drug-facilitated sexual assault DFSA cases

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INTRODUCTION

The quantification of substances involved in DFSA is considered a significant challenge for forensic laboratories around the world. DFSA compounds, are determined in a variety of biological matrices that includes, blood, urine and hair. In sexual assault incidences, the time-laps between the administration of the drug and the reporting of the sexual attack is usually more than 12 hours, which gives urine; as it has a longer window of detection a priority to be the sample of choice.

Sample extraction methods for the determination of DFSA substances in urine mostly include solid-phase extraction (SPE), liquid-liquid extraction (LLE) or microextraction techniques which involve multiple time-consuming and costly steps with the use of considerable volume of organic solvents.

OBJECTIVE

The main objective of this work is to develop and validate an extraction method based on supramolecular solvents (SUPRASs) to extract 23 DFSA substances that belong to different classes like benzodiazepines, z-hypnotic drugs, amphetamine derivatives, cocaine and metabolites and other miscellaneous compounds involved in DFSA cases.

In this study, we proposed and validated a simple and rapid method based on the use of SUPRAS (supramolecular solvents) for sample treatment. SUPRAS are nanostructured liquids made up by self-assembly with mixed mechanisms for multiple binding. This simple and green method has a great potential to be implemented for the monitoring of illegal drugs involved in DFSA cases by forensic laboratories.

EXPERIMENTAL

1) Optimization of SUPRAS synthesis

Three different amphiphiles based on alkanediols (C6,C8 and C10), namely 1,2-hexanediol, 1,2-otanediol and 1,2-decanediol in salty water (1 M Na_2SO_4) and the addition of a co-solvent (THF) were investigated to form different SUPRAS and evaluate their extraction capabilities. Results are shown in the table below.

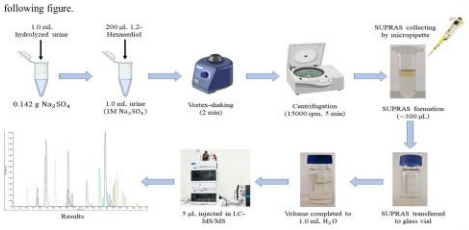
Parameter	Studied values	Optimum value
Amphiphile based on	1,2-hexanediol, 1,2-otanediol and 1,2-decanediol	1,2-hexanediol
Organic solvent volume	0, 60, 100, 140, and 200 µL.	0 µL
Amphiphile volume	100, 150, 200, 250, and 300 µL.	200 µL

2) Validation of the method

The optimized parameters were then applied for the validation method. The method was validated in terms of linearity, the limit of detection, the limit of quantification, selectivity, recovery, and matrix effect. The calibration curve was constructed in the range of 0.1xMRPL to 5xMRPLs.

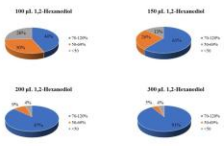
3) Application on real samples

The method was applied to 10 different authentic urine samples, as expected 8 samples were found positive for one DFSA substances and 2 were found negative. The urine samples were prepared as seen in the following figure.

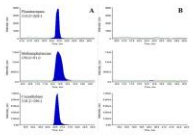


RESULTS

Recoveries for DFSA substances with SUPRAS synthesized from: (A) 1,2-hexanediol, (B) 1,2-octanediol, and (C) 1,2-decanediol.



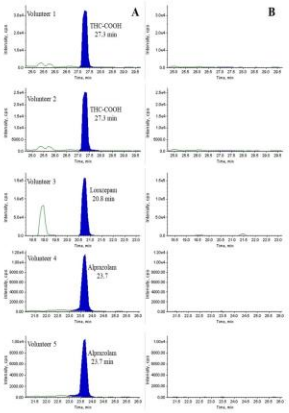
Recoveries for DFSA substances of SUPRAS synthesized from (100, 150, 200, 300 µL) of 1,2-hexanediol.



Extracted ion chromatograms for selected DFSA substances from a urine sample (A) fortified at the 1×MRPL level and (B) unfortified blank urine.

Analytes	MRPLs (ng/mL)	Range (ng/mL)	Regression coefficient R^2	LOD (ng/mL)	LOQ (ng/mL)
Benzodiazepines and z-hypnotic drug					
Alprazolam	10	1-50	$R^2 = 0.999$	0.10	0.20
Bromazepam	10	1-50	$R^2 = 0.999$	0.50	1.00
Clorazepate	10*	1-50	$R^2 = 0.997$	0.10	1.00
Diazepam	10	1-50	$R^2 = 0.961$	0.05	0.20
Lorazepam	10	1-50	$R^2 = 0.995$	0.10	0.40
Lormetazepam	10	1-50	$R^2 = 0.998$	0.10	0.30
Temazepam	10	1-50	$R^2 = 0.998$	0.05	0.30
Clonazepam	5	0.5-25	$R^2 = 0.997$	0.10	0.25
Flunitrazepam	5	0.5-25	$R^2 = 0.999$	0.10	0.25
Nitrazepam	5	0.5-25	$R^2 = 0.997$	0.20	0.50
Zolpidem	10	1-50	$R^2 = 0.998$	0.003	0.01
Cocaine and cocaine metabolites					
Cocaine	50	5-250	$R^2 = 0.999$	0.05	0.15
Benzoylecgonine	50	5-250	$R^2 = 0.996$	0.05	0.15
Cocaethylene	50	5-250	$R^2 = 0.994$	0.009	0.03
Amphetamine derivatives					
Methamphetamine	10	1-50	$R^2 = 0.999$	0.50	1.00
Methiopropamine	n/a	1-50	$R^2 = 0.958$	0.30	1.00
MDA	10	1-50	$R^2 = 0.998$	0.05	0.20
MDMA	10	1-50	$R^2 = 0.995$	0.008	0.02
MDEA	10	1-50	$R^2 = 0.998$	0.01	0.04
Miscellaneous compounds					
Scopolamine	10	1-50	$R^2 = 0.999$	0.05	0.25
Fentanyl	10	1-50	$R^2 = 0.999$	0.05	0.20
THC	10	2.5-50	$R^2 = 0.998$	1.00	2.50
Ketamine	1	0.1-5	$R^2 = 0.998$	0.05	0.20

MRPLs values, calibration parameters, limits of detection, limits of quantification, and coefficients of determination (R^2).



Examples of extracted ion chromatograms (A) volunteers 1-5 urine samples containing DFSA and (B) blank human urine.

CONCLUSION

An extraction method based on SUPRAS has been developed and proved to be a suitable and efficient procedure to extract 23 compounds involved in drug-facilitated sexual assault cases from urine samples by using LC-MS/MS. The use of the proposed method provides a precise, fast, and cheap method for the monitoring and observation of DFSA cases. The proposed method was applied to 10 real samples. Alprazolam, lorazepam, and THC-COOH were detected in the urine samples of volunteers.

El **Comité Científico** de la **X Reunión de la Sociedad Española de Espectrometría de Masas (XRSEEM)**

CERTIFICA que la comunicación en formato póster

"Green supramolecular solvent extraction for the analysis of urine in drug-facilitated sexual assault cases."

Nouman Almofti, Soledad González-Rubio, Ana Ballesteros-Gómez, Eloy Girela-López, Soledad Rubio

ha sido presentado en la **X Reunión de la Sociedad Española de Espectrometría de Masas (XRSEEM),**

celebrada en el Rectorado de la Universidad de Córdoba – Córdoba, los días 31 de mayo y 1 y 2 de junio de 2022

Córdoba a 3 de junio del 2022

Dra. Encarnación Moyano
Presidenta de la SEEM y de la XRSEEM

Dra. Lourdes Arce
Presidenta de la XRSEEM

Analysis of hair samples using green supramolecular solvent for the extraction of selected drug-facilitated sexual assault substances by LC-MS/MS analysis

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2Universidad de Córdoba, Departamento de Química Analítica, Instituto de Química Fina y Nanoquímica.

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Summary

Investigations of drug-facilitated sexual assaults need to understand the victim's drug usage history. In contrast to other biological matrices, hair, is a keratinized biological matrix, providing a broad window of detection for retrospective quantitative analysis. Pharmaceutical and other illicit drugs may be extracted from hair using a variety of time-consuming and solvent-intensive extraction techniques.

In this study, we developed an effective extraction technique based on the use of a supramolecular solvent (SUPRAS) based on 1,2-hexanediol to extract benzodiazepines, zolpidem (z-hypnotic agent), cocaine and its metabolites, amphetamine derivatives, and other miscellaneous substances involved in drug-facilitated sexual assault cases.

The effectiveness of several SUPRAS extraction methods that use water or an equilibrium solution as a wetting agent has been investigated. For 91% of the tested substances, the suggested approach delivers a high extraction recovery (>86%) and acceptable matrix effects. The technique quantification limits varied from 2 to 80 pg/mg and were lower than the suggested drug cut-off levels for all the compounds under study. The method detection limits were in the range of 0.09-26.1 pg/mg.

Key words: Hair, drug-facilitated sexual assault, supramolecular solvent, LC-MS/MS.

UNIVERSIDAD D CÓRDOBA

La Vicerrectora de estudios de posgrado de la Universidad de Córdoba

Acredita que

Nouman Almofti, **ha asistido** al **XI Congreso Científico de Investigadores en Formación,** con el título *"El arte de investigar"*, organizado por las Escuelas de Doctorado Educo y eidA3 (sede Córdoba) de la Universidad de Córdoba, celebrado en la Facultad de Medicina y Enfermería, en Córdoba el día 4 de mayo de 2023 y **ha presentado la comunicación oral** titulada "Analysis of hair samples using green supramolecular solvent for the extraction of selected drug-facilitated sexual assault substances by lc-ms/ms analysis".

Fdo: Cristina Aguilar Porro

FED Formación de las Escuelas Doctorales · idep Instituto de Estudios de Posgrado · educo Escuela de Doctorado de la Universidad de Córdoba · eidA3 Escuela Internacional de Doctorado en Agroalimentación

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DEVELOPMENT OF GREEN SUPRAMOLECULAR SOLVENTS FOR THE EXTRACTION OF COMPOUNDS FOR DRUG-FACILITATED SEXUAL ASSAULT FROM HUMAN HAIR

Nouman Almofti[a,b], Ana Ballesteros[a], Eloy Girela[b], Soledad Rubio[a]

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Drug-facilitated sexual assaults (DFSAs) are violent crimes perpetrated by altering the victim's state of awareness and degree of consciousness while under the influence of a drug and have grown drastically globally in recent years. Pharmaceutical compounds (such as benzodiazepines and opioid analgesics) and other illicit drugs (such as cocaine, cannabis, and ecstasy) are commonly used in drug-rape cases. These chemicals are appealing because they have sedative and hypnotic effects on the victim's central nervous system.

In this work, we developed an effective extraction method based on the use of a supramolecular solvent (SUPRAS) based on 1,2-hexanediol to extract benzodiazepines, zolpidem (z-hypnotic agent), cocaine and its metabolites, amphetamine derivatives, and other miscellaneous substances involved in drug-facilitated sexual assault cases from human hair samples. SUPRASs produced from 1,2-hexanediol in 1M Na_2SO_4 water (Method A), 1M Na_2SO_4-THF (10%, v/v)-water (Method B), and only SUPRAS (Method C) were tested for the extraction of the the targeted DFSA substances. Moreover, different hair samples volume (10 and 25 mg) and various SUPRAS volumes (200, 300, and 400 µL) were studied and optimized.

In conclusion, we developed an extraction method based on a green 1,2-hexanediol supramolecular solvent that can efficiently extract the selected drugs from human hair samples with extraction recoveries above 86%. Moreover, the method quantification limits achieved are much lower than the drug-cutoff concentrations recommended by the Society of Hair Testing.

Hair analysis of selected drug-facilitated sexual assault substances using green supramolecular solvent extraction and LC-MS/MS

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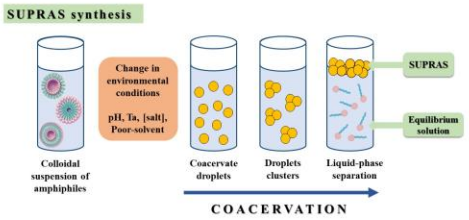
INTRODUCTION

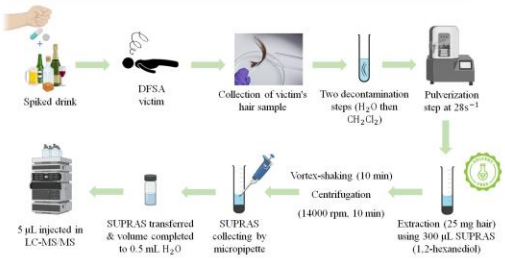
Investigations of Drug-facilitated sexual assaults DFSA incidents requested to elucidate the substances involved in the sexual crime, their concentration, and the approximate time of administration. An important aspect in most DFSA cases is the delay in reporting the incident due to various factors. For this reason, hair can incorporate and accumulate ingested drugs for a long time allowing the performance of a retrospective evaluation of the drug consumption history for up to 12 months or more.

Hair analysis has many advantages over other types of biological specimens. For instance, the collection process is painless, hair samples can be easily stored and transported, and drugs and their metabolites are considered stable in hair for a long time period.



OBJECTIVE

In this research, the potential of cubosomic SUPRASs (based on 1,2-hexanediol) to extract multiclass DFSA substances which cover a wide polarity range was selected. For this purpose, various SUPRAS extraction approaches using water and/or equilibrium solution as a wetting agent for hair samples have been studied and optimized.



MATERIAL & METHODS

The cubosomic SUPRAS was formed by dissolving 1,2-hexanediol (6 mL) in 30 mL of H_2O (1M Na_2SO_4) in a 50 mL polypropylene centrifuge tube by vortex-shaking for 5 min and then centrifugation (4000 g, 10 min). Then, two liquid phases were separated; the SUPRAS at the top was collected and stored in closed polypropylene tubes at room temperature, and the equilibrium solution was reused for a new SUPRAS synthesis.

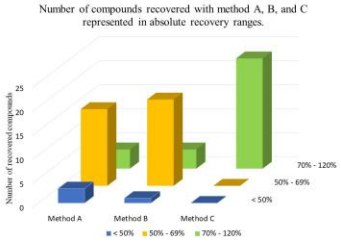
The extraction efficiency was investigated for DFSA substances from hair samples using SUPRASs synthesized from:

1) 1,2-hexanediol in salty water (Method A).
2) 1,2-hexanediol in salty THF (10%, v/v)-water (Method B).
3) 300 µL SUPRAS direct extraction (Method C).

Both, the SUPRAS (300 µL) and the equilibrium solution (500 µL) were added to the sample; the first one for extraction of the DFSA substances and the second one for wetting the hair sample.

Moreover, several SUPRAS volumes (200, 300, and 400 µL) and hair sample size (10 and 25 mg) were studied and the optimum value in terms of recovery was selected.

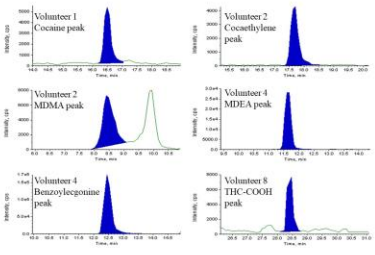
RESULTS & DISCUSSION



Method C shows a good recoveries for all the 23 studied substances (above 70%) ranging from 86% for bromazepam up to 102% for THC-COOH.

The method.

The proposed method was applied to hair samples of eight volunteers, and detected substances include; benzoylecgonine, cocaine, cocaethylene, MDEA, MDMA and THC-COOH .



CONCLUSION

- A single-step extraction method based on the formation of an organic solvent-free SUPRAS of 1,2-hexanediol has been optimized and validated to extract 23 DFSA substances including benzodiazepines, z-hypnotic drugs, cocaine metabolites, amphetamine derivatives and other miscellaneous compounds from hair samples of sexual assault victims.
- After simple and fast extraction and centrifugation steps, extracts were directly analyzed by LC-MS/MS.
- The developed method efficiently extracts (>86%) all the targeted substances without using any organic solvent making it an eco-friendly method following the green analysis criteria.
- The SUPRAS synthesis was simple enough and fast as it consists of a mixture of 1M Na_2SO_4 H_2O and 1,2-hexanediol easily prepared with vortex shaking and centrifugation steps.
- Moreover, the method was equally efficient regardless of the hair sample size used (10 or 25 mg).
- The SUPRAS method considered rapid in comparison to other hair treatment processes since incubation, clean-up or evaporation/reconstitution steps were not required.

ACKNOWLEDGEMENTS

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CERTIFICADO

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Development of green supramolecular solvents for the extraction of compounds for drug-facilitated sexual assault from human hair

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