Journal of Chromatography A 1701 (2023) 464061

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Supramolecular solvents for making comprehensive liquid-liquid microextraction in multiclass screening methods for drugs of abuse in urine based on liquid chromatography-high resolution mass spectrometry

Soledad González-Rubio[a], Noelia Caballero-Casero[a,*], Ana Ballesteros-Gómez[a], Darío Cuervo[b], Gloria Muñoz[b], Soledad Rubio[a]

[a] *Department of Analytical Chemistry, Instituto Químico para la Energía y el Medioambiente, Marie Curie Building (Annex), Campus of Rabanales, Universidad de Córdoba, Córdoba 14071, Spain*

[b] *Doping Control Laboratory. Institute of Health Carlos III, C/ Pintor el Greco S/N, Madrid 28040, Spain*

A R T I C L E I N F O

Article history:
Received 2 April 2023
Revised 5 May 2023
Accepted 8 May 2023
Available online 9 May 2023

Keywords:
Screening methods
Supramolecular solvents
LC-ESI-TOF
Doping control
Urine

A B S T R A C T

Multiclass screening methods involving hundreds of structurally unrelated compounds are becoming essential in many control labs and research areas. Accurate mass screening of a theoretically unlimited number of chemicals can be undertaken using liquid chromatography coupled to high resolution mass spectrometry (LC–HRMS), but the lack of comprehensive sample treatments hinders this unlimited potential. In this research, the capability of supramolecular solvents (SUPRAS) for making comprehensive liquid-liquid microextraction (LLME) in multiclass screening methods based on LC–HRMS was firstly explored. For this purpose, a SUPRAS made up of 1,2-hexanediol, sodium sulphate and water was synthesized directly in the urine and applied to compound extraction and interference removal in the screening of eighty prohibited substances in sports by LC-electrospray ionization-time of flight mass spectrometry. Selected substances included a wide range of polarities (log P from -2.4 to 9.2) and functionalities (e.g. alcohol, amine, amide, carboxyl, ether, ester, ketone, sulfonyl, etc.). No interfering peaks were observed for any of the 80 substances investigated. Around 84–93% of drugs were efficiently extracted (recoveries 70–120%) and 83–94% of the analytes did not show matrix effects (±20%) in the ten tested urines. Method detection limits for the drugs were in the interval 0.002–12.9 ng mL^{-1}, which are in accordance with the Minimum Required Performance Levels values established by the World Anti-Doping Agency. The applicability of the method was evaluated by the screening of thirty-six blinded and anonymized urine samples, previously analyzed by gas or liquid chromatography-triple quadrupole. Seven of the samples lead to an adverse analytical finding in line with the results obtained by the conventional methods. This research proves that LLME based on SUPRAS constitutes an efficient, economic, and simple sample treatment in multiclass screening methods, an application that is unaffordable for conventional organic solvents.

© 2023 The Authors. Published by Elsevier B.V.
This is an open access article under the CC BY-NC-ND license
(http://creativecommons.org/licenses/by-nc-nd/4.0/)

1. Introduction

Screening of multiclass substances has become relevant for control laboratories that are routinely confronted with hundreds of substances for which maximum permitted levels have been set and decisions on positive and negative samples have to be taken quickly (e.g. agrifood or anti-doping labs) [1,2]. Screening methods are also relevant for environmental monitoring, epidemiological studies, exposomics, metabolomics, etc. where the detection of as many toxics as possible in a single analysis is highly valuable [3,4].

High resolution mass spectrometry (HRMS), particularly combined with liquid chromatography, is currently the technique of choice for target, suspect and non-target screening of thousands of compounds. Instruments such as Orbitrap and time-of-flight (TOF) provide both high mass accuracy and resolution in full scan mode,

* Corresponding author.
E-mail address: a42caasn@uco.es (N. Caballero-Casero).

https://doi.org/10.1016/j.chroma.2023.464061
0021-9673/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

enabling accurate mass screening of a theoretically unlimited number of chemicals [5].

By its very nature, multiclass screening methods should be comprehensive, and given the huge number of analyses usually required, they should allow high throughput sample processing and be cost-effective and green. In this respect, one of the greatest challenges of screening methods is how to reduce matrix effects while preventing the loss of chemicals with very different physicochemical properties during sample purification, and do so with good sensitivity. Thus, although sample extraction and purification should be minimal and as non-selective as possible, matrix components can strongly influence the sensitivity of screening methods, and consequently, a compromise is required [3].

The dilute and shoot (D&S) approach is theoretically ideal for screening analysis because information on sample composition remains unchanged. However, although D&S may work for substances that present very high efficiency in the analytical instrument for which dilution does not compromise sensitivity, it suffers from significant and variable matrix effects, particularly in biological and environmental samples (e.g. urine, serum, surface water, etc.) [3]. So, this approach gives low sensitivity and repeatability for screening methods and consequently, most of them still require an effective sample treatment for both sample concentration and removal of matrix interferences.

Solid phase extraction (SPE) is currently the technique of choice for sample preparation in screening methods owing to the variety of available sorbents. However, the obtained information will be highly dependent on the type of selected sorbent, as excellently proved in a recent publication dealing with the unknown screening of urban waters [6]. Thus, if sample processing is intended to extract compounds covering a wide range of physicochemical properties, the recommended strategy is to use a multilayer cartridge combining several phases [5,7], or using different sorbents in parallel and then mixing the eluates [8]. However, SPE is far from simple, fast, cost-effective and green. Thus, all the required steps (conditioning, washing, eluting) require the consumption of organic solvents (e.g. a total volume of 20–30 mL per sample) and most of the times final eluates have to be evaporated. Unfortunately, organic solvent-based extraction, particularly liquid-liquid extraction (LLE), although faster than SPE, is not a viable solution for multiclass screening since extraction will be conditioned by the similarity of the polarity of analytes and solvents [9]. On the other hand, tailored neoteric solvents such as ionic liquids [10] or deep eutectic solvents [11] have not yet been applied in multiclass screening methods.

Because of the distinctive features of supramolecular solvents (SUPRASs), their application in the development of innovative sample treatments in analytical chemistry is deserving closer attention in the last few years [12]. Thus, the different polarity microenvironments present in the amphiphilic nanostructures of SUPRASs render them excellent candidates for the extraction of analytes in a wide polarity range [13]. By their own nature, SUPRASs are non-selective extractants, as required in screening methods, however, they can be tailored to remove major matrix macrocomponents (e.g. proteins, humic acids, carbohydrates, etc.), which reduces interferences and variability among samples [14]. On the other hand, the high number of binding sites derived from the large concentration of amphiphile in the SUPRAS, together with the mixed mechanisms offered for solute solubilisation, allow efficient extractions using low SUPRAS volume, which makes unnecessary sample extract evaporation. Moreover, SUPRAS tailoring has allowed progress in making them greener [15] and compatible with LC-ESI-MS/MS [16] and ambient mass spectrometry [17]. To the best of our knowledge, SUPRASs have not been used for screening of multiclass substances by LC–HRMS that, as mentioned above, is the current technique of choice for screening methods.

This work was intended to explore the potential of SUPRASs for comprehensive multiclass extraction in screening methods based on LC–HRMS. For this purpose, the application of SUPRASs to the testing of banned substances in urine in doping control test in sport, was considered a proof of concept of this strategy since antidoping control is affected by the major challenges facing multiclass screening methods. Thus, substances prohibited in sports include a large number of compounds belonging to 10 categories (the World Anti-Doping Agency (WADA) Prohibited List that cover a wide range of polarity, physicochemical properties, and threshold concentrations [18–20]. This list also extends to their metabolites and any substance showing a similar structure or effect. So, hundreds of substances are of potential interest in anti-doping testing in WADA-accredited laboratories. On the other hand, urine composition shows large inter- and intra-individual variability. From an operational point of view, Initial Testing Procedure (ITP) is the first step in doping control laboratories according to WADAś guidelines [21], and only Presumptive Adverse Analytical Findings (PAAF) would be subjected to confirmatory methods.

The research here developed included the selection of eighty prohibited substances and/or their metabolites from the 10 categories of the WADA list (Table S1); the selection and optimization of the SUPRAS-based LLE on the basis of SUPRAS efficiency for multiclass extraction and removal of matrix effects; and the validation of the method according to WADA guidelines. The suitability of this method for screening analysis was explored in the analysis of thirty-six blinded and anonymized urine samples received from the anti-doping control laboratory Institute of Health Carlos III (ISCIII), located in Madrid. To the best of our knowledge, SUPRAS have not been applied so far in wide screening analysis based on LC–HRMS.

2. Experimental

2.1. Reagents and solutions

All solvents and reagents were of high purity grade. Methanol (MS grade) was supplied by Fisher Scientific (Madrid, Spain). Sodium sulphate, formic acid and 1,2-hexanediol were provided by Sigma-Aldrich (St. Louis, Missouri, USA). The enzyme β-glucuronidase from *E. coli* K12 (140 units per mL) was supplied by Roche Diagnostics GmbH (Mannheim, Germany). Ultra-high-quality water was generated from a Milli-Q water purification system (Millipore-Sigma, Madrid, Spain). A pH 7 buffer solution was prepared by mixing 10.88 g of KH_2PO_4 and 14.24 g of $Na_2HPO_4 \cdot 2H_2O$ in 200 mL of Milli-Q water. The buffer was transferred into a bottle of glass and was kept at 2–8 ºC. Table S2 lists the suppliers for the analytical standards of the eighty drugs and metabolites selected as well as the deuterated internal standards (IS). Stock solutions for the individual drugs and the IS (at concentrations of 1, 100 or 1000 μg mL^{-1}) were prepared in methanol and stored at −20ºC. A multi-component standard solution of the selected doping drugs at concentrations of 50-fold their respective Minimum Required Performance Level (MRPL) was prepared in methanol [19]. An IS mixture solution at 2 μg mL^{-1} each was prepared in methanol as well. Intermediate and working solutions of doping drug mixtures were prepared by appropriate dilution of stock solutions in methanol and they were stored at −20 ºC for at least one month.

2.2. Urine samples

Spot urine samples were collected from 20 volunteers at the Institute of Chemistry for Energy and Environment (Córdoba) and were used for both optimization of the SUPRAS-based extraction and method validation. All volunteers were informed of the purpose of the sample collection, their rights and other concerns. The

Fig. 1. Schematic of the SUPRAS-based sample treatment for screening analysis of multiclass substances in urine by LC-ESI-TOF.

data protection policy and management of biological samples were according to the Ethics Committee of Andalusianś Biomedical Research. The collection of urine samples was carried out in clean plastic containers of 100 mL (Sage Products, Crystal Lake, IL). For method optimization, a pooled urine sample was obtained by mixing ten spot urines at equal proportion. The other ten spot urine samples were used individually for method validation. The samples were centrifuged for 15 min at 1800 g and when not immediately analysed, they were stored at −20 ºC. Before analysis, samples were subjected to enzymatic hydrolysis. For this purpose, 1 mL-sub-sample aliquot was mixed with 50 µL of the KH_2PO_4/Na_2HPO_4 buffer solution and 25 µL of the β-glucuronidase enzyme solution. Then, the urine was shaken in a vortex for 5 min and it was incubated in a water bath at 55 ºC for 1 h. Finally, the urine was kept in a closed glass bottle at 4 ºC until its use. To prove the applicability of the method for the screening of the banned substances, 36 blind-urine samples were kindly provided by the WADA-accredited doping control laboratory of Madrid. The samples were labelled on the basis of their pH, sex and specific gravity. All of them were non-spiked urine. No information about the identity of the athletes and the presence/absence of sport drugs was given. Table S3 shows the physicochemical parameters for each of these urine samples. Before analysis, they were hydrolyzed following the same protocol specified above and properly stored at −20 ºC.

2.3. Sample treatment based on supras

The hydrolyzed urine samples (1 mL), containing 20 ng mL^{-1} of the IS specified in Table S2, were mixed with Na_2SO_4 (142 mg) in Eppendorf microtubes of 2 mL. Once the salt was solubilized, 1,2-hexanediol (200 µL) was added. The mixture was vortex-shaken for 5 min at 2000 rpm and then, centrifuged at 3000 g for 10 min. The SUPRAS extract (~250 μL) was withdrawn using a microsyringe, transferred to a sealed glass vial and mixed with an equal volume of Milli-Q water for subsequent LC/TOF-MS analysis. Fig. 1 shows a scheme of the SUPRAS-based sample treatment.

2.4. LC–HRMS analysis of doping drugs

Separation and quantification of the sport drugs were conducted using an Elute UHPLC coupled to a Time-of-Flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source (ESI) operating in positive and negative modes. A perfluorophenyl (PFP) column (2.7 μm, 100 mm×3.0 mm) from RESTEK (Bellefonte, Pennsylvania, USA) was used as the stationary phase. The mobile phase consisted of solvent (A) 0.05% formic acid in water and (B) 0.025% formic acid in methanol. The elution program was the following: isocratic conditions (95% A, 5% B) for 0.5 min, linear gradient from 5% to 100% of B in 27.5 min, and then isocratic conditions at 100% of B for 2 min. Finally, initial conditions were re-equilibrated for 5 min. The injection volume was 5 µL and the column temperature was kept constant at 40ºC. The flow rate was 0.25 mL min^{-1}. The acquisition method was developed in full-scan MS mode. For both positive and negative polarity modes, optimal source parameters were the following: end plate offset, 500 V; capillary voltage, ±3500 V; nebulizer gas pressure, 4.0 bar; dry gas, 10.0 L/min; dry temperature, 220 ºC; vaporizer temperature, 350 ºC. Automatic instrument recalibration was performed at the beginning of each analysis by direct infusion of the calibration solution to ensure mass accuracy. The acquisition parameters were set for *m/z* range from 100 to 1500 at a scan rate of 4 scans/s. An in-house spectral library was built by direct injection of individual analytical standards of the selected sport drug under the aforementioned chromatographic and spectral conditions. The in-house library included retention time and MS spectral information for more than 90 sport drugs and was used as a test to demonstrate the usefulness of the proposed strategy for suspect screening analyses. Detection of compounds was done by searching the acquired sample analyses against the in-

house spectral library on the basis of the following criteria: mass error below 5 ppm and retention time match (±0.1 min). Table S2 shows the theoretical and experimental masses for each compound, as well as their mass errors. Also, retention times for samples and standards in the library, and retention time errors are included. Urine samples and controls (e.g. blanks, spiked urine samples) were analysed at random in batches of 45 samples under the described conditions. All data were acquired and processed using the Compass DataAnalysis 5.3 and TASQ 2.2 software (Bruker Daltonics, Bremen, Germany).

2.5. Method validation

The method based on SUPRAS-LC-TOF-MS was validated according to the guidelines set forth by the World Anti-Doping Agency (WADA). The following parameters were evaluated using 10 spot urine samples; linearity, selectivity, extraction recoveries, matrix effects, detection and quantification limits, carry over, and SUPRAS extract stability.

Linearity was determined using the IS method within the interval 0.1xMRPL to 2xMRPL (see MRPL values in Table S4) by fortifying SUPRAS prepared in distilled water. *Selectivity* was evaluated by analysing 10 negative urine samples and checking for interfering peaks in the chromatograms. *Extraction recoveries* for each urine were investigated by comparing relative peak areas ($A_{analyte}/A_{IS}$) obtained for analytes in samples spiked before extraction (at the MRPL) with those obtained in SUPRAS spiked after extraction (at 2xMRPL to consider sample concentration). In the case of endogenous substances (e.g. testosterone), the obtained relative peak area ($A_{analyte}/A_{IS}$) for a non-spiked sample was subtracted from the obtained relative peak area ($A_{analyte}/A_{IS}$) in spiked samples. *Matrix effects* were determined in terms of signal suppression and enhancement. For this purpose, the relative peak areas ($A_{analyte}/A_{IS}$) of urine samples spiked after SUPRAS extraction (2xMRPL) were compared to those obtained in SUPRAS blanks prepared in distilled water spiked at 2xMRPL. *Precision* was evaluated in terms of repeatability and within-laboratory reproducibility. For this purpose, the analysis of a spiked urine sample (at the MRPL) was repeated six times for three consecutive days ($n = 18$), and the relative peak areas ($A_{analyte}/A_{IS}$) were compared. Repeatability, expressed as relative standard deviation (RSD), was calculated as the square root of the average value of the intra-day variances, and within-laboratory reproducibility as the square root of the inter-day variance. To calculate the *method detection* (MDLs) *and quantification* (MQLs) *limits* urine samples were spiked at 0.5xMRPL, 0.25xMRPL, and 0.1xMRPL. MDLs and MQLs were defined as the lowest level at which a compound could be detected with a signal-to-noise ratio (S/N) greater than 3 and 10, respectively. *Carry over* was evaluated by analysing a blank sample after analysis of a sample fortified at 10xMRPL. *SUPRAS extracts stability* was evaluated for three consecutive days for a urine sample fortified at the MRPLs, by storing the sample at 4 ºC after analysis.

2.6. Screening analysis methods used for comparison in the analysis of urine samples

The thirty-six urine samples specified in Table S3 were subjected to the screening procedure based on SUPRAS-LC-TOF-MS (Sections 2.3 and 2.4). Results were compared to those obtained by the ISCIII lab, which uses three routinely protocols for the screening of the substances included in this study. The first protocol aims to determine anabolic agents (S1), some antiestrogenic substances (S4), some conjugated stimulants (S6), narcotics (S7) and cannabinoids (S8) and consists of the extraction of the hydrolysed urine with tert–butyl methyl ether, evaporation of the extract to dryness, and derivatization with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), followed by analysis with GC-QQQ-MS in MRM mode. The second protocol aims to determine unconjugated stimulants and consists of the extraction of the urine with ethyl acetate, evaporation of the extract and analysis by GC–MS in the SIM mode. The third protocol is intended to detect some anabolic agents (S1), beta-2 agonists (S3), antiestrogens (S4), diuretics (S5), some conjugated stimulants (S6), cannabinoids (S8), glucocorticoids (S9) and beta-blockers (P1) and consists in the 5-fold dilution of the hydrolysed sample and analysis by LC-QQQ in the MRM acquisition mode.

3. Results and discussion

3.1. Selection of multiclass substances for evaluation of supras in screening methods based on lc-hrms

As commented in Introduction, doping control has ideal characteristics to test the potential of SUPRAS in screening methods using LC–HRMS. Thus, screening is always the first step in WADA-accredited labs and only those urine samples with PAAF are subjected to confirmatory analysis. This screening, which involves hundreds of substances covering a wide range of physico-chemical properties and concentrations, should be reliable and avoid false negative and positive results while being simple, fast, cost-effective and green. The combination of different methods involving low resolution mass spectrometry coupled to LC and GC have been traditionally used for this purpose, and although the use of LC–HRMS is progressively having more prominence in this field, its potential is not yet fully exploited, partly because of the lack of comprehensive sample treatments.

Table 1 shows representative methods reported in the last decade for the testing of banned substances in urine by LC–HRMS [9,22–35]. None of these methods was applied to the 10 drug categories (S1-S9, P1) of the WADA list, and many of them did not report studies about matrix effects. The typical three strategies used in general screening methods for sample treatment (e.g. SPE, LLE and dilute and shoot) have been also used in doping control. Although no information is generally reported on the interval of the polarity of the substances selected for screening, LLE methods using diethyl ether, methyl tert–butyl ether or ethyl acetate are not effective for extraction of polar substances [26,32,34], and addition of original urine to the solvent extract is sometimes used for extending the range of polarity of the detected analytes [34]. As expected, direct injection of urine [31] or dilute and shoot methods [27–29] are affected by significant interferences, unless high dilution factors are applied. SPE, commonly using mixed sorbents [9,23–25], has been the most straightforward approach for sample treatment in doping screening analysis, although, as above mentioned, the SPE procedure is far from being simple, fast and green.

In this research, we selected eighty substances or their metabolites belonging to the 10 drug categories of the WADA list (Table S1). The target compounds were selected with the aim of covering a wide range of polarities (log P from −2.4 to 9.2), functionalities (e.g. alcohol, amine, amide, carboxyl, ether, ester, ketone, sulfonyl, etc.) and acid/base characteristics (pK_a acid: 2.8 to 18.6; pK_a basic: −6.9 to 12.5). So, this selection was considered representative for investigating the potential of SUPRAS for LLE extraction in screening methods based on LC–HRMS.

3.2. SUPRAS selection and optimization

Among SUPRAS properties, the following three characteristics were highlighted for SUPRAS application in screening analysis. (1) *High hydrophilicity*, which should favour the extraction of very polar substances while keeping the ability to solubilize nonpolar

Table 1
Sample preparation strategies and analytical parameters obtained for representative methods reported in the last decade for screening of banned substances in urine using LC-HRMS.

WADA class (number of drugs)	Sample treatment	Chemicals involved in sample treatment	Limits of detection (ng mL^{-1})	LC-HRMS technique	Recoveries (%)	Matrix effects (%)	Refs.
S1, S5, S6, S7, S8, P1 (124)	· Hydrolysis · SPE (Cation exchange/C8) · Solvent evaporation	MeOH: 3 mL	27 out of 124 drugs no detected at their MRPL value.	LC-TOF	33–98 (median 58%). Five compounds 0–9%	Ion suppression of 50% for compounds at retention times below 2 min. An unspecified decrease in intensity for the rest of the compounds.	[23]
S1, S3, S4 (29)	· Hydrolysis LLE · Solvent evaporation	Diethyl ether: 5 mL	>0.1	LC–Orbitrap	23–97 (mean: 80)	No reported	[26]
S4, S5, S6, S7, P1 (103)	· Dilute and shoot (D&L)	Dilution factors from 2 to 10	1–500	LC-QTOF		73% of compounds presented ion suppression	[27]
S1, S3, S4, S5, S6, S7, S8, S9, P1 (197)	· Hydrolysis · Mixed-mode SPE (HCX and HCA cartridges) · Solvent evaporation	MeOH: 4 mL MeOH/water 50/50: 1mL	6 out of 20 drugs deeper studied for validation could not be detected at their MRPL values.	LC-TOF	83–115%	No reported	[24]
S1, S3, S4, S5, S6, S7, S9, P1 (241)	· Hydrolysis · LLE · Solvent evaporation	Diethyl ether: 5 mL	<1–100	LC-TOF	1–103%	Ion suppression depended on the specific urine matrix that was analyzed, resulting in great variability between the six different matrices tested.	[33]
S1, S3, S5, S6, S7 (56)	· Hydrolysis · Solvent extraction · Clean up based on SPE (Oasis MCX cartridge)	Diethyl ether; MeOH: 3 mL Acetone/water 1/1: 3 mL Ammonia-ethyl acetate: 3 mL	$CC_{\alpha}{}^{b}$=2.5–192	LC-TOF	68–143	No reported	[32]
S5, S6 (122)	· D&S	Dilution factors of 2,4, 10, 100	For diuretics: 25–250. For stimulants: 5–500	LC–Orbitrap		From −56 to +68.4	[28]
S1, S2, S3, S5, S6, S7, S9 (182)	· Hydrolysis (1 h, 50ºC) · Solvent extraction · SPE (mixed-mode cation exchange) · Solvent evaporation	MeOH (0.5 mL) 3% ammonium hydroxide in MeOH:acetonitrile (50:50, v/v, 3 mL) MeOH in water (20%, v/v, 1 mL)	0.5–200	LC-Q/Orbitrap	No reported	No reported	[25]
S1, S3, S4, S5, S6, S7, S9 (189)	7 different sample procedures: · SPE (PLEXA polymeric cartridge) · SPE (Oasis HLB cartridge) · SPE (Discovery DSC-18 cartridge) · SPE (Oasis MCX mixed mode cartridge) · SPE (Oasis MAX mixed mode cartridge) · LLE · Sorbent-supported liquid extraction (SLE) (Chem Elut cartridges)	SPE PLEXA MeOH:MeCN (50 :50, 8 mL) ; 5% MeOH in ultra pure water (4 mL) ; MeOH/water (10:90(v/v), 0.5 mL) SPE Oasis HLB and SPE C18 MeOH (8 mL); 5% MeOH in MilliQ water (4 mL); MeOH/water (10:90(v/v), 0.5 mL) SPE MCX MeOH (8 mL); 5% NH4OH in MeOH (4 mL); MeOH/water (10:90 (v/v), 0.5 mL) SPE MAX MeOH (8 mL); 2% formic acid in MeOH (2 mL); MeOH/water (10:90(v/v), 0.5 mL) LLE Methyl tert–butyl ether (5 mL); MeOH/water (10:90 (v/v), 0.5 mL) SLE Methyl tert–butyl ether (6 mL); MeOH/water (10:90 (v/v), 0.5 mL)	No reported	LC-TOF	Compounds with recoveries >50% by using: SPE PLEXA : 185 SPE Oasis HLB:178 SPE C18: 174 SPE MCX:146 SPE MAX : 157 LLE : 83 SLE : 99	Moderate signal suppression between 10% and 30%, for 85% of the compounds tested.	[9]
S4, S5, S6, (27)	· D&S	Dilution factor of 4	<5–100	LC-Q/Orbitrap		ion suppression ≥50% for 75% of the compounds	[29]

(*continued on next page*)

Table 1 (*continued*)

WADA class (number of drugs)	Sample treatment	Chemicals involved in sample treatment	Limits of detection (ng mL^{-1})	LC-HRMS technique	Recoveries (%)	Matrix effects (%)	Refs.
S1, S3, S4, S5, S6, S7, S8, S9, P1 (200)	· SPE (Bond Elut PLEXA cartridge) · Solvent evaporation	MeOH:MeCN (1:1.8 mL); Ultra pure water (4 mL); 5% MeOH in water (4 mL); MeOH:water (1:90, v/v, 0.5 mL)	0.005–7.6	LC-TOF	70–120	20% of the compounds presented matrix effects.	[22]
S1, S2, S3, S4, S5, S6, S7 (200)	· Urine direct injection		0.1–25	LC-Q/Orbitrap		150 out of 200 compounds presented matrix effects (75%).	[31]
S1, S3, S4, S5, S6, S7, S8, S9, P1 (81)	· D&S	Water/MeCN (95/5) at two levels (1:20 and 1:50)	LOQ: 5	NanoLC-Q-Orbitrap		Negligible matrix effects (0–10%) at a factor dilution of 1:50.	[30]
S1, S2, S3, S4, S6, S7, S8, S9, P1 (304)	· Hydrolysis · LLE · Sample freezing (−80ºC) · Mixture of reconstituted extract and original urine	Ethyl acetate: 5 mL	0.025–12.5	LC-Q/Orbitrap/MS	2.1–101	Only 62 substances were measured for matrix effects and all of them presented significant effects.	[34]
S1, S6, S7, S8, S9, P1 (300)	· Hydrolysis · SPE · Extract evaporation · Reconstitution	Methanol: 5.5 mL	0.5–100	LC-Q/Orbitrap/MS	0.6–185.7	0.04–5.28 (RSD,%)	[35]

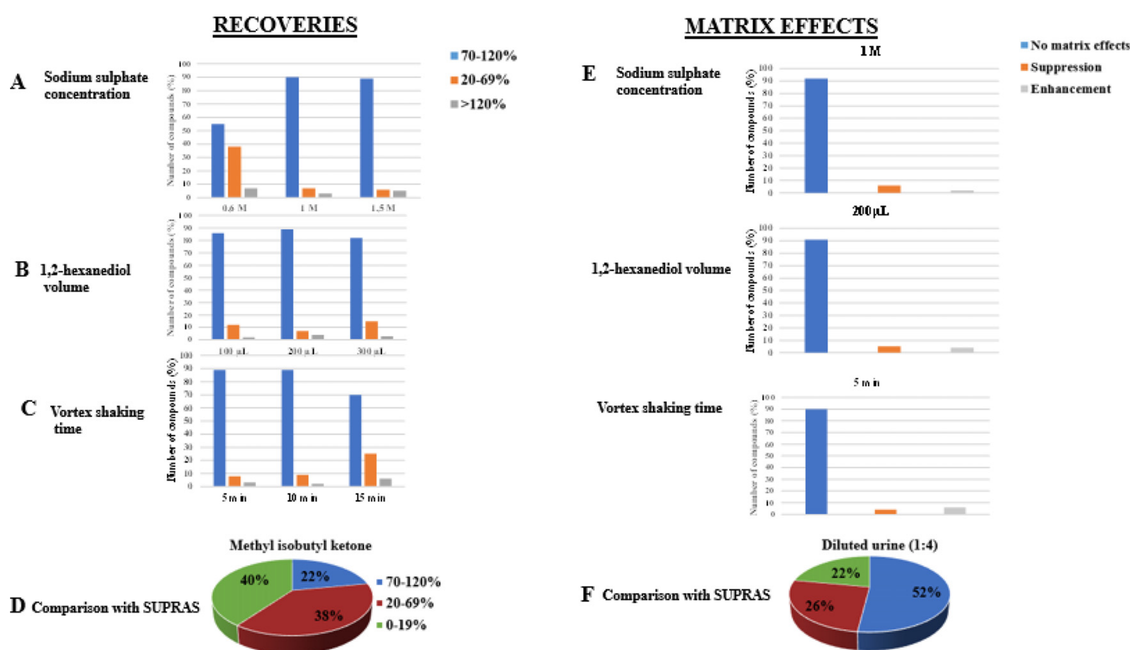

Fig. 2. (A–D) Percentage of banned substances with recoveries in the ranges of 70–120% (blue), 20–69% (red) and >120% (green) as a function of the experimental conditions used for SUPRAS formation: (A) sodium sulphate concentration, (B) volume of 1,2-hexanediol, and (C) vortex-shaking time, and (D) for methyl isobutyl ketone extraction. (E-F) Percentage of banned substances showing no matrix effects (±20%, in blue), signal suppression (red) and enhancement (green) for (E) SUPRAS formed in different experimental conditions and (F) analysing diluted urine (1:4).

compounds in the hydrophobic chains of the amphiphilic nanostructures. (2) *Negligible signal in LC-ESI-TOF(MS),* which encourages the use of amphiphiles containing polar groups that do not ionize in ESI. (3) *No need for organic cosolvents in sample treatment,* which would make the process advantageous from a green chemistry perspective.

With these characteristics in mind, we selected SUPRASs produced directly in the urine samples by the addition of 1,2-hexanediol as the amphiphile and sodium sulphate as the coacervation-inducing agent. It has been previously reported that these SUPRASs consist of cubosomic nanostructures made up of 1,2-hexanediol, sodium sulphate and water, whose relative concentrations depend on the concentration of the coacervation-inducing agent added to the urine [16]. Thus, SUPRASs containing variable and high content of water (36–61%, w/v) can be easily synthesised at urinary concentrations of sodium sulphate in the interval 0.6–1.5 M. On the other hand, it is known that long chain alcohols do not significantly ionize in ESI [36], so it is expected they give a negligible signal in the analysis of SUPRAS extracts by LC-ESI-TOF(MS). Finally, conventional organic solvents are not necessary for SUPRAS formation or sample treatment.

The influence of the *chemical composition of the SUPRAS* on its capability for extracting the eighty drug/metabolites selected (Table S1) was investigated by adding to the pooled fortified urine sample (Section 2.2) a fixed volume of 1,2-hexanediol (200 µL), and variable amounts of sodium sulphate (final concentration 0.6–1.5 M). Fig. 2A shows the recoveries obtained for SUPRAS synthesised at three sulphate concentrations, which gave SUPRASs with

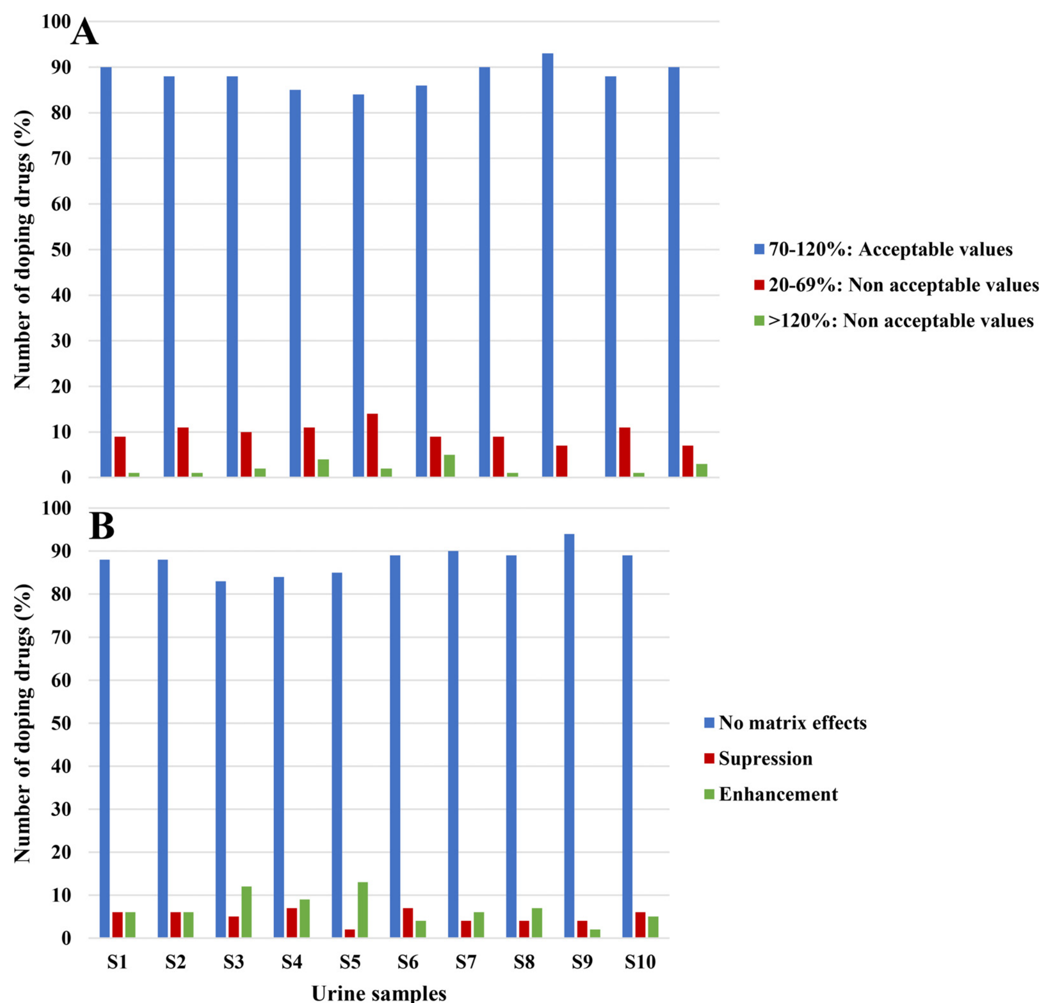

Fig. 3. Percentage of prohibited substances with (A) recoveries in the ranges of 70–120% (blue. Acceptable values), 20–69% (red. Non-acceptable values), and >120% (green. Non-acceptable values), and (B) showing no matrix effects (± 20%, in blue), signal suppression (red), and enhancement (green) for the extraction of 10 human urine samples (S1-S10) with SUPRAS.

the following composition in 1,2-hexanediol, water and sodium sulphate (%, w/v): 35.8 ± 0.7, 61±4, 5.3 ± 0.1 for 0.6 M, 58.9 ± 0.2, 39±1, 3.0 ± 0.2 for 1 M, and 62.6 ± 0.9, 36±1, 1.49±0.05 for 1.5 M. The results show that SUPRASs containing a high concentration of amphiphile (~60%, w/v) were able to efficiently extract 90% of the selected drugs (recoveries in the range 70–120%). The high extent of the hydrophilic area provided by the double-headed amphiphile and the surrounding water molecules (near 40%, w/v) were considered responsible for these results, from which it is worth noting that any of the selected drugs were extracted with recoveries below 30%.

The influence of the *volume of SUPRAS* that is produced in the urine on the extraction efficiency of the drugs was investigated by adding different volumes of the amphiphile to the pooled fortified urine sample (Section 2.2) while keeping constant the concentration of sodium sulphate (1 M). Under these conditions, the chemical composition of the SUPRAS remained unchanged but its volume varied linearly according to the following equation [16]:

$V_{SUPRAS} = (-15.26[Na_2SO_4] + 32.52)[1,2 - hexanediol] - -(-210.84[Na_2SO_4] + 273.12)$where V_{SUPRAS} is the volume of SUPRAS (µL per mL of sample), and $[Na_2SO_4]$ and [1,2-hexanediol] represent their concentration in the urine, expressed as M and%,v/v, respectively. Fig. 2B shows representative results for SUPRAS prepared from different volumes of 1,2-hexanediol. Although no great differences in recoveries were obtained in the tested interval, a higher percentage of drugs were extracted with recoveries within the optimal interval (70–120%) using 200 µL of 1,2-hexanediol and this volume was selected for further experiments.

On the other hand, it was found (Fig. 2C) that, as usual, extraction equilibrium conditions were quickly reached (e.g. 5 min of vortex-shaking) since SUPRAS-based extraction is intrinsically a dispersive liquid extraction because of the coacervate droplets making up these solvents.

The superiority of SUPRASs compared to conventional organic solvents for extraction of multiclass substances in doping control can be inferred from the results previously reported in the literature [26,32,34], and those obtained from the extraction of the drugs selected in this study with methyl isobutyl ketone (MIBK) (Fig. 2D). Only 22% of the drugs had good recoveries, while the 40% of them had recoveries below 20% (many of them approaching zero).

Matrix effects were investigated under the same experimental conditions as those used for determining the extraction efficiency of SUPRASs. For simplicity, Fig. 2E shows only the results at the value of the variable selected as optimal. No matrix effects were detected for around 90–92% of the drugs, which is highly valuable if these results are compared with the dilution and shoot approach for the same drugs (Fig. 2F). No great differences were obtained in matrix effects as varying the chemical composi-

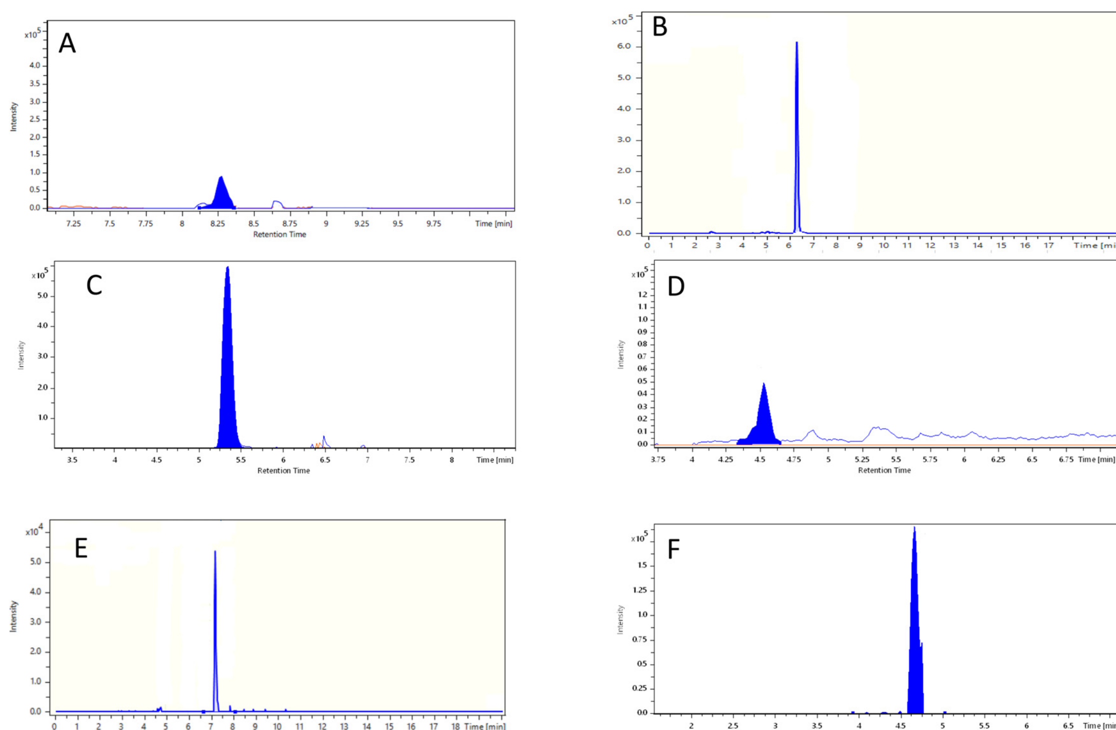

Fig. 4. Extracted ion chromatograms obtained for (A) 3-OH stanozolol, (B) furosemide, (C) ritalinic acid, (D) terbutaline, (E) bis-(4-cyanophenyl) methanol (letrazol metabolite) and (F) atenolol in the screening of the urine samples numbered as 7, 10, 15, 18, 22 and 25, respectively, using the SUPRAS-LC-ESI-TOF method.

tion or volume of the SUPRAS or the vortex-shaking time. Thus, the percentage of substances not showing matrix effects varied in the intervals 89–92%, 77–91% and 83–90% as the concentration of sodium sulphate, volume of 1,2-hexanediol and vortex-shaking time varied in the ranges 0.6–1.5 M, 100–300 µL and 5–15 min, respectively.

3.3. Method validation

Method validation was carried out according to the procedure specified in Section 2.5. Table S4 gives the values of the slopes and intercepts, along with their respective standard deviations, for the calibration curves obtained from each of the doping substances. Calibrations were run in SUPRAS prepared from distilled water. Correlation coefficients for these calibrations were calculated by linear regression and gave values in the interval 0.982–0.999. Method detection and quantification limits (MDLs and MQLs) for the drug selected were in the intervals 0.002–12.9 ng mL^{-1} and 0.007–43.2 ng mL^{-1}, respectively. Since these values were all below the respective MRPLs (Table S4), the SUPRAS-LC-QTOF method meets the requirements to be used for the screening of banned substances in doping control. The intra-day and inter-day precision were expressed as relative standard deviation and they varied in the range 0.5–16.4% and 0.9–19.7%, respectively.

The selectivity of the SUPRAS-LC-QTOF method was checked for each of the banned substances by analysing ten fortified (at the respective MRPL value) and unfortified urine samples. Figure S1 shows, as an example, the extracted ion chromatograms for urine samples fortified with banned substances belonging to different groups (S1-S9, P1) of the WADA list (A) and those from their respective blanks (B). The ordinate axes in blank chromatograms are 1 or 2 orders of magnitude below the respective chromatograms corresponding to fortified samples. No interfering peaks were observed for any of the 80 investigated doping substances in the urine samples.

With regard to recovery studies, Fig. 3A shows the whole picture of the obtained results and Table S5 depicts the specific recoveries for the tested substances in ten urine samples fortified at their respective MRPLs. Around 84–93% of drugs were efficiently extracted (recoveries 70–120%) in the urine samples. The only substance with recoveries below 30% for some urine samples was ecgonine methyl ester. It is worth noting that ritalinic acid (log P −2.4) was extracted with recoveries within the range 72–93% in the ten urines.

The study of matrix effects (Fig. 3B and Table S6) proved the high ability of the selected SUPRAS for eliminating matrix components that potentially can provoke undesired effects on the detection of the analytes. Thus, as depicted in Fig. 2B, around 83–94% of the analytes did not show matrix effects (±20%) in the ten urines. Only three compounds, acetazolamide, chlorothiazole and aminoglutethimide underwent ion suppression/enhancement effects out of this range for most of the urine samples. When we focused on the individual sample results per analyte, the matrix effect affected 35 analytes out of 80 (43.8%) in at least one sample. However, this percentage decreased down to 17.5% when only compounds with matrix effect for more than two samples were taken into account, revealing the high potential of the SUPRAS as a general sample treatment prior to screening analysis.

The selected drugs were stable in the SUPRAS extracts, kept at 4º C, for at least three days. On the other hand, these drugs were undetected in blank urine samples that were analysed following the screening of urines fortified with the 80 doping substances, at a concentration of 10-fold the respective MRPLs. Therefore, there were not carry-over effects.

3.4. Analysis of anonymized urine samples

The SUPRAS-LC-TOF(MS) screening method was applied to the analysis of 36 urine samples taken from athletes during antidoping controls and donated by the ISCIII laboratory (Table S3). As

Table 2
Prohibited substances detected by the SUPRAS-LC-ESI-TOF method in the screening analysis of 36 blinded urine samples.

Urine internal sample number	Prohibited substances detected by SUPRAS-LC-ESI-TOF	Group of WADA list	MDL ng mL^{-1}	MRPL ng mL^{-1}	Drug concentration in the blinded urine	[a]Prohibited substances detected and confirmed by GC-MS or LC-QQQ
7	3-OH-stanozolol	S1	0.2	1	> MRPL	3-OH-stanozolol
10	Furosemide	S5	0.3	20	> MRPL	Furosemide
15	Ritalinic acid	S6	0.7	50	> MRPL	Ritalinic acid
18	Terbutaline	S3	0.1	20	> MRPL	Terbutaline
22	Bis (4-cyanophenyl) methanol[b]	S4	1.4	20	> MRPL	Bis (4-cyanophenyl) methanol
25	Atenolol	P1	0.5	50	< MRPL	Atenolol
28	Prednisolone	S9	1.5	100	< MRPL	Prednisolone

[a] Methods specified in Section 2.6.
[b] Metabolite of letrozole.

specified in Section 2.4, the identification of compounds was done by searching the acquired sample analyses against the in-house spectral library taking into account the following criteria: mass error below 5 ppm and retention time match (±0.1 min). Table 2 shows the prohibited substances detected in the different urine samples by both the SUPRAS-LC-TOF-MS protocol and the different protocols used by the ISCIII lab (Section 2.6). Besides the detection, the ISCIII lab had also confirmed the presence of these substances in the urine samples. Thus, the SUPRAS-LC-TOF-MS method was able to detect all the PAAF using a single screening analysis per sample, which proves its capability for setting reliable doping ITPs. All substances identified were non-threshold, for which minimum required performance levels (MRPLs) have been set by WADA. Table 2 includes the MDLs obtained for the detected substances using the SUPRAS-LC-TOF-MS screening method as well as their respective MRPL values and the level of concentrations found (below or above the MRPL value).

Seven out of thirty-six urine samples gave PAAF (Table 2). The SUPRAS-LC-TOF-MS method was able to detect doping drugs belonging to seven different groups of the WADA prohibited list. Figs. 4 and 2S show the extracted ion chromatograms obtained by TOF full scan for the urines with positive results.

4. Conclusions

The availability of multiclass screening methods, which are fast, simple and reliable, is highly valuable for many control labs and research areas. One of the greatest challenges to be faced in multiclass screening methods is how to be comprehensive without compromising sensitivity, selectivity and simplicity. LLME is a very simple technique, with capacity for analyte concentration and removal of interferences but conventional organic solvents can only extract substances in a limited range of polarity and they fail in extracting polar and highly polar substances, as it has been here proved for methyl isobutyl ketone. This research shows that SUPRAS-based sample treatments are a valuable strategy for screening methods involving multiclass substances covering a wide range of polarities and physicochemical properties. The SUPRAS screening method here developed features high recoveries (around 90% of the studied drugs showed recoveries in the range 70–120%) and low matrix effects (around 90% of the analytes did not show matrix effects), proving it works for an area (i.e. doping control) where multiclass analysis is mandatory. An important feature of SUPRAS-based sample treatment is the immediate applicability of this procedure in routine laboratories without significant effort, since the analytical approach does not involve any important change compared to the current practices, skills and equipment commonly used in doping control.

Declaration of Competing Interest

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

CRediT authorship contribution statement

Soledad González-Rubio: Conceptualization, Data curation, Formal analysis, Methodology, Validation. **Noelia Caballero-Casero:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Ana Ballesteros-Gómez:** Conceptualization, Writing – review & editing, Supervision. **Darío Cuervo:** Writing – review & editing. **Gloria Muñoz:** Conceptualization, Writing – review & editing, Validation. **Soledad Rubio:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

Funding credit acknowledgment

This research was supported in whole by funding from the Partnership for Clean Competition Research Collaborative (2019R1000388G). The content of this publication does not necessarily reflect the views or policies of the Research Collaborative. N. Caballero-Casero acknowledges the grant IJC2020-044941-I funded by MCIN/AEI/ 10.13039/501100011033 and by European Union NextGenerationEU/PRTR.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.464061.

References

[1] P. Pérez-Ortega, F.J. Lara-Ortega, J.F. García-Reyes, B. Gilbert-López, M. Trojanowicz, A. Molina-Díaz, A feasibility study of UHPLC-HRMS accurate-mass screening methods for multiclass testing of organic contaminants in food, Talanta 160 (2016) 704–712.
[2] M. Thevis, T. Kuuranne, H. Geyer, Annual banned-substance review: analytical approaches in human sports drug testing 2020/2021, Drug Test. Anal. 14 (2022) 7–30.
[3] K.Godlewska M.Paszkiewicz, H.L.M. Caban, A. Białk-Bielinska, P. Stepnowski, Advances in suspect screening and non-target analysis of polar emerging contaminants in the environmental monitoring, Trends Anal. Chem. 154 (2022) 116671.
[4] B. Keen, A. Cawley, B. Reedy, S. Fu, Metabolomics in clinical and forensic toxicology, sports anti-doping and veterinary residues, Drug Test. Anal. 14 (2022) 794–807.

[5] V.G. Beretsou, M.-C.a Nika, K. Manoli, C. Michael, Q. Sui, L. Lundy, D.M. Revitt, N.S. Thomaidis, D. Fatta-Kassinos, Multiclass target analysis of contaminants of emerging concern including transformation products, soil bioavailability assessment and retrospective screening as tools to evaluate risks associated with reclaimed water reuse, Sci. Total Environ. 852 (2022) 158391.
[6] N. Huynh, C.Soares Peirera E.Caupos, A.Bressy J.Roux, R. Moilleron, Evaluation of sample preparation methods for non-target screening of organic micropollutants in urban waters using high-resolution mass spectrometry, Molecules 26 (2021) 7064.
[7] S. Kern, K. Fenner, H.P. Singer, R.P. Schwarzenbach, J. Hollender, Identification of transformation products of organic contaminants in natural waters by computer-aided prediction and high-resolution mass spectrometry, Environ. Sci. Technol. 43 (2009) 7039–7046.
[8] S. Moretti, F. Lega, L. Rigoni, G. Saluti, D. Giusepponi, A. Gioiello, E. Manuali, R. Rossi, R. Galarini, Multiclass screening method to detect more than fifty banned substances in bovine bile and urine, Anal. Chim. Acta 1032 (2018) 56–67.
[9] J.C. Domínguez-Romero, J.F. García-Reyes, A. Molina-Díaz, Comparative evaluation of seven different sample treatment approaches for large-scale multiclass sport drug testing in urine by liquid chromatography–mass spectrometry, J Chromatogr. A 1361 (2014) 34–42.
[10] M.J. Trujillo-Rodríguez, H. Nan, M. Varona, M.N. Emaus, I.D. Souza, J.L. Anderson, Advances of ionic liquids in analytical chemistry, Anal. Chem. 91 (2019) 505–531.
[11] M. Mushtaq, F.W. Butt, S. Akram, R. Ashraf, D. Ahmed, Deep eutectic liquids as tailorable extraction solvents: a review of opportunities and challenges, Critical Rev Anal Chem (2022), doi:10.1080/10408347.2022.2125284.
[12] S. Rubio, Twenty years of supramolecular solvents in sample preparation for chromatography: achievements and challenges ahead, Anal. Bio. Chem. 412 (2020) 6037–6058.
[13] A. Ballesteros-Gómez, M.D. Sicilia, S. Rubio, Supramolecular solvents in the extraction of organic compounds. a review, Anal. Chim. Acta 677 (2010) 108–130.
[14] A. Ballesteros-Gómez, S. Rubio, Environment-responsive alkanol-based supramolecular solvents: characterization and potential as restricted access property and mixed-mode extractants, Anal. Chem. 84 (1) (2012) 342–349.
[15] E. Romera-García, A. Ballesteros-Gómez, S. Rubio, Supramolecular biosolvents made up of self-assembled rhamnolipids: synthesis and characterization, Green Chem. 22 (2020) 6115–6126.
[16] S. González-Rubio, A. Ballesteros-Gómez, G. Muñoz, S. Rubio, Cubosomic supramolecular solvents: synthesis, characterization, and potential for high--throughput multiclass testing of banned substances in urine, Anal. Chem. 94 (2022) 4103–4111.
[17] M.J. Dueñas-Mas, A. Ballesteros-Gómez, S. Rubio, Supramolecular solvent-based microextraction probe for fast detection of bisphenols by ambient mass spectrometry, Chemosphere 294 (2021) 133719.
[18] The 2023 Prohibited ListWorld Anti-Doping Agency (WADA), The 2023 Prohibited List, 2023 https://www.wada-ama.org/sites/default/files/2022-09/2023list_en_final_9_september_2022.pdf Accessed February.
[19] Technical Document TD2022MRPL. Minimum Required Performance Levels and Applicable Minimum Reporting Levels For Non-Threshold Substances Analyzed By Chromatographic-Mass Spectrometric Analytical Methods. World Anti-Doping Agency. (WADA) https://www.wada-ama.org/sites/default/files/resources/files/td2022mrpl_v1.0_final_eng.pdf (accessed February 2023).
[20] Technical Document TD2022DLDecision Limits For the Confirmatory Quantification of Exogenous Threshold Substances By Chromatography-Based Analytical Methods, World Anti-Doping Agency (WADA), 2023 https://www.wada-ama.org/sites/default/files/2022-01/td2022dl_v1.0_final_eng_0.pdf accessed February.
[21] The International Standard for LaboratoriesWorld Anti-Doping Agency (WADA), The International Standard for Laboratories, 2021 https://www.wada-ama.org/sites/default/files/resources/files/isl_2021.pdf accessed February 2023.
[22] J.C. Domínguez-Romero, J.F. García-Reyes, F.J. Lara-Ortega, A. Molina-Díaz, Screening and confirmation capabilities of liquid-chromatography–time-of-flight mass spectrometry for the determination of 200 multiclass sport drugs in urine, Talanta 134 (2015) 74–88.
[23] M. Kolmonen, A. Leinonen, A. Pelander, I. Ojanperä, A general screening method for doping agents in human urine by solid phase extraction and liquid chromatography/time-of-flight mass spectrometry, Anal. Chem. 585 (1) (2007) 94–102.
[24] M. Kolmonen, A. Leinonen, T. Kuuranne, A. Pelander, I. Ojanperä, Generic sample preparation and dual polarity liquid chromatography-time-of-flight mass spectrometry for high-troughput screening in doping analysis Drug, Test. Anal. 1 (6) (2009) 250–266.
[25] A. Musenga, D.A. Cowan, Use of ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry for fast screening in high troughput doping control, J. Chromatogr. A 1288 (2013) 82–95.
[26] E.D. Virus, T.G. Sobolevsky, G.M. Rodchenkov, Introduction of HPLC/orbitrap mass spectrometry as screening method for doping control, J. Mass Spectrom. 43 (2008) 949–957.
[27] F. Badoud, E. Grata, L. Perrenoud, L. Avois, M. Saugy, S. Rudaz, J.L. Veuthey, Fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography–quadrupole time-of-flight mass spectrometry I. Screening analysis, J. Chromatogr. A 1216 (2009) 4423–4433.
[28] A.J. Girón, K. Deventer, K. Roels, P.V. Eenoo, Development and validation of an open screening method for diuretics, stimulants and selected compounds in human urine by UHPLC–HRMS for doping control Anal, Chim. Acta 721 (2012) 137–146.
[29] C. Görgens, S. Guddat, A.K. Orlovius, G. Sigmund, A. Thomas, M. Thevis, W. Schänzer, "Dilute-and-inject" multi-target screening assay for highly polar doping agents using hydrophilic interaction liquid chromatography high resolution/high accuracy mass spectrometry for sports drug testing, Anal. Bioanal. Chem. 407 (2015) 5365–5379.
[30] J. Alcántara-Durán, D. Moreno-González, M. Beneito-Cambra, J.F. García-Reyes, Dilute-and-shoot coupled to nanoflow liquid chromatography high resolution mass spectrometry for the determination of drugs of abuse and sport drugs in human urine, Talanta 182 (2008) 218–224.
[31] C. Görgens, S. Guddat, A. Thomas, P. Wachsmuth, A.K. Orlovius, G. Sigmund, M. Thevis, W. Schänzer, Simplifying and expanding analytical capabilities for various classes of doping agents by means of direct urine injection high performance liquid chromatography high resolution/high accuracy mass spectrometry, J. .Pharm. Biomed. Anal. 131 (2016) 482–496.
[32] R.J.B. Peters, J.E. Oosterink, A.A.M. Stolker, C. Georgakopoulos, M.W.F. Nielen, Generic sample preparation combined with high-resolution liquid chromatography–time-of-flight mass spectrometry for unification of urine screening in doping-control laboratories anal, Bioanal. Chem. 396 (2010) 2583–2598.
[33] A. Vonaparti, E. Lyris, Y.S. Angelis, I. Panderi, M. Koupparis, A. Tsantili-Kakoulidou, R.J.B. Peters, M.W.F. Nielen, C. Georgakopoulos, Preventive doping control screening analysis of prohibited substances in human urine using rapid-resolution liquid chromatography/high-resolution time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 24 (2010) 1595–1609.
[34] W. Abushareeda, A. Vonaparti, K.A. Saad, M. Almansoori, M. Meloug, A. Saleh, R. Aguilera, Y. Angelis, P.L. Horvatovich, A. Lommen, M. Alsayrafi, C. Georgakopoulos, High resolution full scan liquid chromatography mass spectrometry comprehensive screening in sports antidoping urine analysis, J. Pharm. Biomed. Anal. 151 (2018) 10–24.
[35] D. Oliveira, A. Araújo, W. Ribeiro, D. Silva, A.C. Duarte, V. Sousa, H.G. Pereira, Screening method of mildronate and over 300 doping agents by reversed-phase liquid chromatography-high resolution mass spectrometry, J. Pharm. Biomed. Anal. 195 (2021) 113870.
[36] W. Qi, Y. Wang, Y. Cao, Y. Cao, Q. Guan, T. Sun, L. Zhang, Y. Guo, Simultaneous analysis of fatty alcohols, fatty aldehydes, and sterols in thyroid tissues by electrospray ionization-ion mobility-mass spectrometry based on charge derivatization, Anal. Chem. 92 (2020) 8644–8648.