Cubosomic Supramolecular Solvents: Synthesis, Characterization and Potential for High Throughput Multiclass Testing of Banned Substances in Urine.

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ABSTRACT

This paper was intended to efficiently extract multiclass prohibited substances in human sport drug testing by using supramolecular solvents (SUPRASs) made up of cubosomes. These SUPRASs, here firstly reported, were synthesized by the salt-induced coacervation of 1,2-hexanediol in urine. The formation of square and rounded cubosomes with a size range of 140-240 nm was confirmed by electron microscopy. These nanostructures consisted of 1,2-hexanediol, salt and a high water content (36-61%, w/w). Their applicability in multiclass determinations was investigated by the extraction of 92 prohibited substances (log P from - 2.4 to 9.2) belonging to ten categories of the World Anti-doping Agency (WADA) list. Variables influencing both recoveries and matrix effects were optimized. Cubosomic SUPRASs showed a high extraction efficiency and interference removal capability which was attributed to their large hydrophilicity and surface area. Both features were superior to that of other eleven SUPRAS that were based on sponge droplets and inverted hexagonal aggregates and to that of conventional organic solvents. A sport drug testing method based on cubosomic SUPRASs-LC-ESI-MS/MS was proposed and validated. Around 82-95% of drugs were efficiently extracted (recoveries 70-120%) in urine samples and 81-92% did not present matrix effects. Method detection limits (0.001-4.2 ng mL^{-1}) were all far below WADA`s limits. The proposed SUPRAS-based sample treatment is as simple as QuEChERS but the distinctive features of cubosomes confer them high capability in multiclass determinations.

INTRODUCTION

The list annually published by the World Anti-Doping Agency (WADA) on prohibited substances in sports encompasses a large number of compounds covering a wide range of polarity and physicochemical properties.[1] This list is not exhaustive and extends to their metabolites or biomarkers and any substance showing similar structure or effect. So, hundreds of substances are of potential interest in anti-doping testing programs.

According to WADA´s rules, drug testing in doping control laboratories involves the use of Initial Testing Procedures (ITPs) for the screening of all the substances covered by the Prohibited List. [2] Then, confirmatory methods are applied to the analysis of positive samples. Most of ITPs involve multiclass screening and they are mainly based on liquid chromatography coupled to both high and low resolution mass spectrometry (LC-HRMS, LC-MS/MS).[3] Increasing sample throughput and shortening turnaround and reporting times, while guaranteeing utmost specificity and sensitivity, have been long sought-after goals in drug testing laboratories.[4,5]

A major current limitation to develop multiclass ITPs is related to sample preparation. At present, three strategies are primarily followed for this purpose; solid phase extraction (SPE), liquid-liquid extraction (LLE) and matrix dilution (dilute-and-shoot, D&S, methods), each with advantages and drawbacks.[6,7]

D&S methods, based on direct injection of urine after dilution with a buffer (viz 1:1 to 1:25 v/v), allow for little to none sample workup, increased throughput and reduced cost. However, by diluting the sample, the sensitivity is only adequate for the screening of substances that are easily ionizable and for which permitted detection levels in urine are high (e.g. diuretics, stimulants and narcotics). Other drawbacks include retention time shifts, which reduce identification capability, and peak shape changes. [8] So, the use of a more extensive sample preparation based on LLE or SPE is yet the more reliable approach for the multiclass screening of sport drugs.

LLE is widely used because of its great simplicity. However, extractions are mainly based on analyte polarity and, given that water-immiscible solvents are used as extractants, LLE offers very poor recoveries for polar and very polar compounds. [6] Both, the use of acid and basic conditions and the reconstitution of the evaporated extracts with the original urine sample increase the multiclass screening

capability of LLE. However, these strategies drastically reduce sample throughput or increase matrix effects. [9]

SPE is, in general, more appropriate than conventional LLE for the development of comprehensive ITPs because of the possibility of using mixed-mode sorbents that offer different interaction mechanisms for prohibited substances. As for LLE, reconstitution of the SPE extract with the original urine sample increases its multiclass screening capability.[10-13] However, SPE involves multiple steps (conditioning, loading, washing, elution and evaporation) and costs are high compared to LLE.

Within the analytical chemistry field, the search for new solvents that replace the conventional ones in analytical extractions has been particularly active in the last years.[14] Among them, supramolecular solvents (i.e. nanostructured liquids produced in colloidal suspensions of amphiphilic compounds by sequential self-assembly and coacervation, **Figure S1** in Supporting Information), feature outstanding properties for efficient LLE of multiclass substances. [15,16] Thus, they offer: (A) *different polarity microenvironments* where drugs may solubilize through mixed-mode mechanisms; (B) *multiple binding sites* that facilitate high extraction yields at low SUPRAS/urine ratios; and (C) *large surface area* that provides fast solute mass transfer from the sample.

Despite these characteristics, application of SUPRASs to multicomponent determination has barely been exploited. Some applications involving the extraction of more than ten components include PAHs,[17] phenols,[18] surfactants,[19] bisphenols,[20] and perfluorinated compounds.[21] Among the SUPRASs investigated for this purpose, those based on alkanols[20,22] and alkanediols[21] are particularly promising given that alcohol groups do not ionize in ESI and have negligible signal in LC-ESI-MS/MS.[23] To the best of our knowledge, application of SUPRASs in multiclass determinations is testimonial (e.g. quantification of residual solvents in pharmaceutical formulations[24]).

In this paper, SUPRASs based on 1,2-hexanediol were firstly synthesized, characterized and their capability for extracting multiclass banned substances in urine was investigated. Our working hypothesis was that this amphiphile has a great potential to produce highly hydrophilic nanostructures able to solubilize efficiently very polar substances, while keeping their capability for solubilizing the nonpolar ones. This hypothesis is supported by the fact that aggregation of double-headed amphiphiles is limited by steric hindrance among them, which results in aggregates where the hydrocarbon chains remain

highly folded and wetted.[25] On the other hand, 1,2-hexanediol, unlike other longer alkyl chains alkanediols,[21] is highly soluble in water and consequently its coacervation can be performed in aqueous media, which should additionally contribute to the formation of highly hydrophilic supramolecular nanostructures. Furthermore, this SUPRAS is also advantageous from a green chemistry perspective since it does not require the use of organic co-solvents for formation.

Coacervation of 1,2-hexanediol was tried in the presence of an inorganic salt. Phase diagrams, chemical composition and the resulting nanostructures were studied. The capability for multiclass determination was investigated by extracting ninety-two prohibited substances (or their metabolites) belonging to ten categories (S1-S9, P1) of the WADA´s list (**Table S1,** in Supporting Information).[1] They covered a great number of functionalities (e.g. alcohol, carboxyl, ether, ester, ketone, primary/secondary/tertiary amines, amides, sulfonyls, etc.), and a wide range of polarity (log P from - 2.4 to 9.2). A sample treatment based on 1,2-hexanediol SUPRASs was optimized and both the recoveries and matrix effects were compared with those obtained by applying 11 SUPRASs synthesized from water-insoluble alkanols (C6-C10)[22] and alkanediols (C8-C10).[21] A method for the screening of banned substances in urine based on cubosomes-based SUPRAS-LC-MS/MS was developed and validated. Below, the most representative results are presented and discussed.

EXPERIMENTAL SECTION

Reagents and Solutions

All solvents and reagents were of high purity grade and their suppliers are specified in the Experimental Section of Supporting Information (SI). The suppliers for the ninety-two sport drugs and deuterated internal standards (IS) used in this study are shown in **Table S2**. Stock solutions for the individual drugs and the IS (1, 100 or 1000 μg mL^{-1}) were prepared in methanol and stored at -20ºC. A multicomponent standard solution of the selected doping drugs at concentrations of 100-fold their respective Minimum Required Performance Level (MRPL)[26] (**Table S3**) was prepared in methanol. A IS mixture solution at 2 μg mL^{-1} each was also prepared in methanol. Intermediate and working solutions of drug mixtures

were monthly prepared by appropriate dilution of stock solutions in methanol and they were kept at -20ºC until use.

Samples

Human urine samples were collected into 100 mL-clean plastic containers (Sage Products, Crystal Lake, IL) from volunteers that were duly informed about the process, their rights and other considerations. Details on urine collection and treatment is specified in the Experimental Section of SI.

Synthesis and Characterization of Cubosomic SUPRASs from 1,2-Hexanediol.

The formation of SUPRASs from 1,2-hexanediol was tried directly in the urine by addition of Na_2SO_4. For this purpose, the salt (0.1-2 M) was dissolved in the urine into 15 mL centrifuge tubes and then the 1,2-hexanediol (1-20%) was added. The total volume for the ternary mixture was fixed at 10 mL. The mixture was vortex-shaken for 5 min to facilitate the contact between their components, and then centrifuged (3000g, 10 min) to accelerate phase separation. Experiments were performed in duplicate at 25 ºC. Region boundaries in the phase diagrams for SUPRAS formation were assigned by visual observation of two immiscible liquid phases that corresponded to the equilibrium solution (at the bottom) and the SUPRAS (at the top).

The volume of SUPRAS generated under different synthetic conditions was calculated by measuring its height in the cylindrical centrifuge tube with a digital calliper from Medid Precision, S.A. (Barcelona, Spain). Non-linear regression was used to fit a model for the prediction of the volume of SUPRAS as a function the concentration of 1,2-hexanediol and Na_2SO_4 in the bulk solution. The statistics package Statgraphics Centurion XVI.II was used to fit a model. The density of SUPRASs synthesized under different conditions was calculated by weighting a given volume of coacervate in an analytical balance. All experiments were conducted in duplicate.

The chemical composition of the SUPRASs was determined as follows. The content in water was measured using a coloumetric Karl Fischer titrator from Metrohm (Herisaus, Switzerland). The concentration of Na_2SO_4 incorporated into the SUPRAS was quantified by measuring the turbidity of barium sulfate (LP2000 Turbidity Meter from Hanna Instruments, Guipúzcoa, Spain) after dilution with water and addition of $BaCl_2$ and a stabilizing solution (method EPA 9038). Finally, once known the

concentration of water and sulfate in the SUPRAS, the concentration of 1,2-hexanediol was calculated by difference.

The presence of coacervate droplets in the SUPRASs was investigated with a light microscope (Leica model DME; Wetzlar, Germany) equipped with an automatic photocamera, using the bright field.

Investigation of the morphology of the aggregates in the SUPRAS droplets was undertaken by scanning electron microscopy (SEM). For this purpose, the SUPRAS (~10 µL) was fixed with glutaraldehyde and then it was embedded with a 6% aqueous agarose solution. After that, the sample was washed three times with sodium cacodylate, and stained with OsO_4 (1%) for contrast enhancement. The sample was subsequently dehydrated with a graded acetone series (30, 50, 70, 80, 90, 100 %) and then subjected to critical point drying. Finally, the sample was placed onto a SEM specimen stub using double-sided carbon tape and it was coated with Au/Pd. The accelerating voltage was set at 10 kV.

Optimization of the SUPRAS-based Sample Treatment in ITPs

First, the influence of the hydrophilicity of different SUPRAS nanostructures on the extraction efficiency of doping drugs and on the removal of interferences was comparatively investigated. For this purpose, different types of SUPRASs were synthesized directly in urine from alkanols (C6-C10), to give rise to inverse hexagonal aggregates, and from alkanediols (C8-C10), to form sponge-like SUPRAS. Fortified and unfortified hydrolysed urines and distilled water blanks were subjected to SUPRAS extraction, both in the presence and absence of 1M Na_2SO_4, and the results were compared to those obtained from standard solutions prepared in SUPRASs.

The general procedures were as follows:

(A) *Fortified hydrolysed urine samples*. Aliquots of 1 mL were fortified with the doping drugs at the Minimum Required Performance Levels (MRPLs) (**Table SI3**) in two mL-microtubes Safe-Lock from Eppendorf Ibérica (Madrid, Spain). Those drugs for which Decision Limits (DLs) have been defined were tested at the MRPLs corresponding to their WADA group (i.e. salbutamol, formoterol, cathine, methylephedrine, morphine and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid were tested at 20, 20, 50, 50, 25 and 1 ng mL^{-1}, respectively). All the samples were fortified with the deuterated IS at 20 ng mL^{-1} Then, the amphiphile (1-hexanol, 1-octanol, 1,2-octanediol, 1-decanol or 1,2-decanediol) and

THF were added to the urine to give a final concentration of 20% (w/v for solid and v/v for liquid amphiphiles) and 10% THF (v/v), respectively. The water content in the urine promoted the self-assembly of the amphiphile and caused the spontaneous in situ formation of the SUPRAS. For experiments carried out in the presence of 1 M Na_2SO_4, the salt was solubilized in the urine prior to the addition of the amphiphile and THF. In the case of 1,2-hexanediol, the amphiphile was directly solubilized in the urine, given its solubility in water, and the formation of the SUPRAS was induced by Na_2SO_4. Then, the urine samples containing the respective reagents were vortex shaken (2,000 rpm, 10 min in a vortex shaker from Heathrow Scientific, Illinois, USA) and centrifuged (3,000g, 15 min in a Minicen CE 182 centrifuge from Ortoalresa, Daganzo, Madrid, Spain). All the supramolecular extracts were less dense than urine and the volume of SUPRAS obtained from the different amphiphiles was in the range 250-480 µL. The SUPRAS extracts were withdrawn with a microsyringe and an aliquot (250 µL) was diluted 1:1 with methanol. Finally, the diluted SUPRAS extracts were transferred to a sealed glass vial for subsequent LC-MS/MS analysis.

(B) *Unfortified hydrolysed urine samples*. Aliquots of 1 mL were extracted with SUPRAS as specified above. Then, the diluted SUPRAS extracts (250 µL of SUPRAS plus 250 µL of methanol) were fortified with the doping drugs at 2-fold the MRPL (Table SI3), and the deuterated IS at 40 ng mL^{-1}, and then they were analysed by LC-MS/MS.

(C) *Procedural blanks of distilled water samples*. SUPRAS were synthesized in water by following the same procedure specified in (A). SUPRAS extracts were then diluted with methanol and they were fortified with the doping drugs at 2-fold the MRPL (**Table SI3**), and the deuterated IS at 40 ng mL^{-1}. Finally, they were analysed by LC-MS/MS.

Recoveries and matrix effects for the selected doping drugs were calculated by comparison of the relative peak areas ($A_{\text{doping substance}}/A_{\text{internal standard}}$) obtained from the experiments specified in A and B, and B and C, respectively.

In addition to this comparative study, the influence of different parameters on both recoveries and matrix effects for the selected doping drugs was investigated using the cubosome based SUPRASs that were made up of 1,2-hexanediol. The variables investigated included the concentration of Na_2SO_4 (0.6-1.5

M) and 1,2-hexanediol (10-30% v/v) and the vortex-shaken extraction time (5-15 min). Experiments were conducted using fortified and unfortified urine and procedural blanks, as described above.

Recommended Procedure for Sample Treatment in ITPs Based on Cubosomic SUPRASs

Hydrolyzed urine samples (1 mL), fortified with 20 ng mL^{-1} of each of the IS specified in **Table S2**, were mixed with Na_2SO_4 (final concentration 1M) in 2-mL Eppendorf microtubes. After salt solubilization, 200 µL of 1,2-hexanediol were added. The mixture was vortex-shaken for 5 min at 2,000 rpm and then, centrifuged for 10 min at 3,000g. An aliquot of the supramolecular extract (about 250 µL) was withdrawn using a microsyringe and transferred to a sealed glass vial. Then, the aliquot was mixed with the same volume of Milli-Q water for subsequent LC-MS/MS analysis. **Figure S2** shows a schematic of the SUPRAS-based sample treatment.

LC-MS/MS Analysis of Doping Drugs

Separation and quantification of the selected doping drugs was conducted using a liquid chromatograph (Waters, Acquity H-Class, Milford, MA, USA) coupled to a hybrid triple quadrupole/linear ion trap (Applied Biosystems MSD Sciex, 5500QTRAP, Four Valley, ON, Canada) equipped with a TurboIonSpray (TIS) interface. All data were acquired and processed using the Analyst 1.6.2 Software. Chromatographic and mass spectrometric conditions are specified in the Experimental Section of SI.

Method Validation

The method based on SUPRAS-LC-MS/MS was validated in terms of selectivity, recovery, matrix effects, method detection limits, carry-over and SUPRAS extract stability by using 10 urine samples. Procedures used for method validation are specified in the Experimental Section of SI.

RESULTS AND DISCUSSION

Synthesis and Characterization of Cubosomic SUPRASs made up from 1,2-Hexanediol

SUPRAS Synthesis

Aggregation of amphiphiles to give colloidal suspensions (e.g. micelles, vesicles, etc.) is a key step for their posterior coacervation, a phenomenon through which SUPRASs are generated. The formation of micelles in aqueous solutions of 1,2-hexanediol has long been reported in the literature.[27] These micelles have a critical aggregation concentration (cac) around 0.71-0.74 M, an aggregation number of 20, a micellar mass of 2,330, and a hydrodynamic radius of 13.5 Å.[27] Their study by small-angle neutron scattering (SANS) has proved that molecules of 1,2-hexanediol exhibit a strong diol–diol interaction in solution and give spherical hydrated micelle-like aggregates with attractive interaction between them.[28] Specific ion effects on the micellization of 1,2-hexanediol has been deeply investigated.[29] Thus, its *cac* has been determined in the presence of a variety of chloride salts of monovalent and divalent cations as well as sodium salts of monoatomic and polyatomic monovalent anions. In all cases, the *cac* decreased with increased salt concentrations in the range 0-3 M. The lowest *cac* was achieved for 3 M of divalent cations (0.14-016 M) followed by 3 M of monovalent cations and anions (0.21-0.39 M).[29]

Coacervation of 1,2-hexanediol from urine was tried by destruction of the hydration layer at its head groups. There is a broad consensus that by destructing this layer, the effective area per molecule at the interface diminishes and amphiphile monomers can be packed closer together leading to aggregate growth and liquid phase separation.[30] Two salts were investigated for destruction of the hydration layer of 1,2-hexanediol, namely sodium chloride and sulfate, at concentrations between zero and their maximum aqueous solubility. These salts are nontoxic and have low cost and high stability. The study was restricted to amphiphile concentrations below 20% (v/v) since they are the concentrations typically used in analytical extraction processes.[15]

Figure 1A shows the phase diagram obtained at 25 ºC from the 1,2-hexanediol-urine-sodium sulfate mixture. The phase diagram exhibited two different regions as the concentration of salt increased up to its limit of solubility in water (~2 M); an isotropic solution and a region where two immiscible liquid phases were observed (SUPRAS formation). Coacervation did not occur under the addition of sodium chloride to the colloidal suspension of the amphiphile.

The SUPRAS was less dense than the equilibrium phase under all the experimental conditions. The minimum percentage of 1,2-hexanediol required to get coacervation was 3% (v/v) that corresponds to a

concentration of 0.25 M of amphiphile. As previously reported, aqueous micelles are formed at these concentrations of 1,2-hexanediol in the presence of salts.[29]

Although the microscopic origins of coacervation still remain elusive,[30] the results for the coacervation of 1,2-hexanediol in the presence of the investigated salts (sodium sulfate and sodium chloride) are strongly related to the dehydration power of their anions. Thus, sulfate is a highly hydrated anion whereas chloride is a poorly hydrated anion, as inferred from their Jones-Dole viscosity coefficients (B); 0.208 for sulfate and -0.007 for chloride.[31] So, the water withdrawing power of sulfate is much higher than that of chloride, making it able to effectively destruct the hydration layer of the amphiphile. This basis strongly suggests that this mechanism is the dominant for 1,2-hexanediol coacervation.

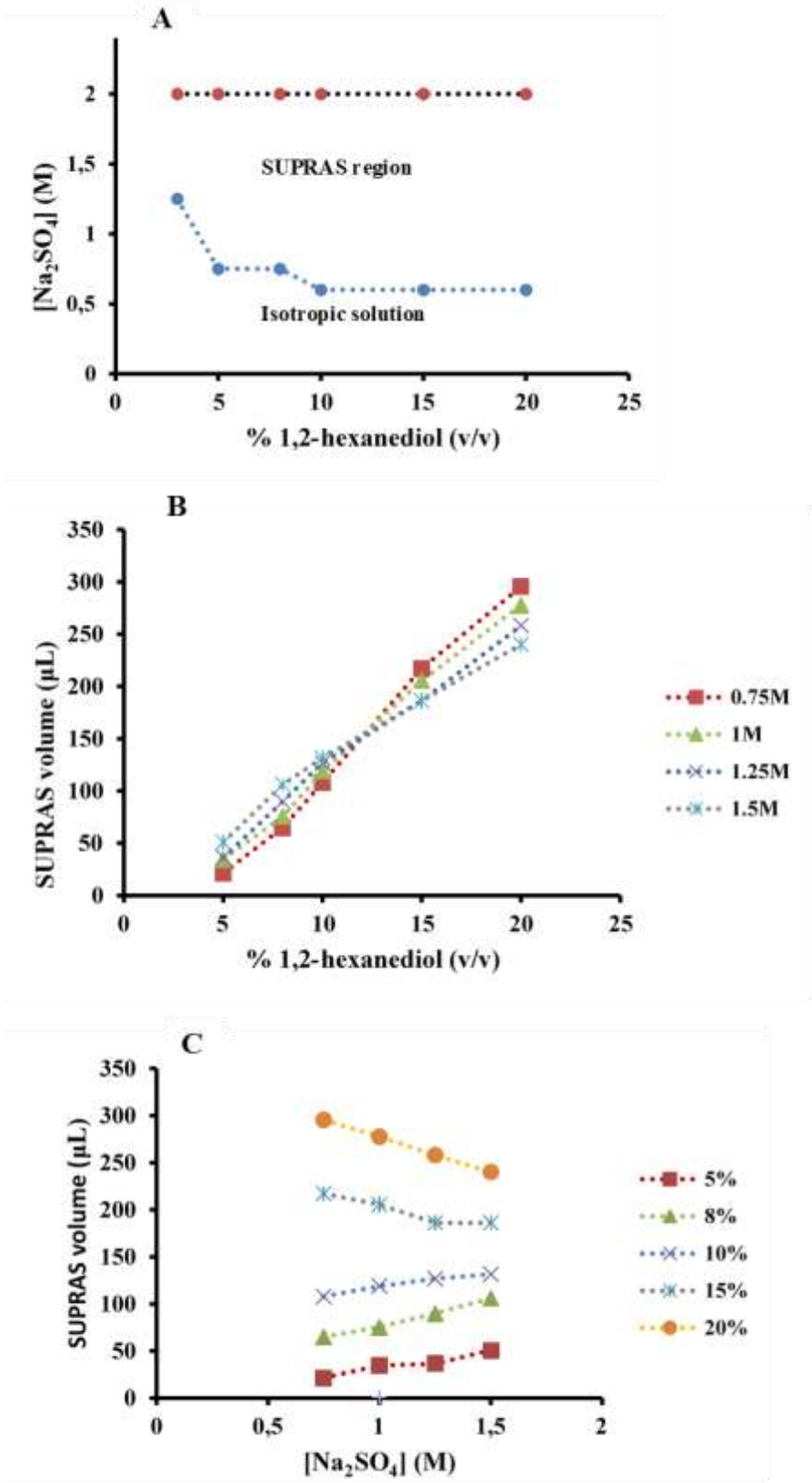

Figure 1. (A) Phase diagram for the ternary mixture 1,2-hexanediol-urine-sodium sulfate. (B) Volume of SUPRAS as a function of the initial concentration of 1,2-hexanediol (%, v/v) at different concentrations of sodium sulfate (0.75-1.5 M). (C) Volume of SUPRAS as a function of the initial concentration of sodium sulfate at different concentrations of 1,2-hexanediol (5-20%, v/v).

SUPRAS Volume

The volume of SUPRAS in the colloidal system (expressed as µL of SUPRAS per mL of urine) was a function of the concentration of both the amphiphile and the salt. As it can be observed in **Figure 1B**, this volume increased linearly with the concentration of 1,2-hexanediol, independently of the concentration of sodium sulfate investigated. The equations and coefficients of determination for these linear dependences are shown in **Table S4**. The value of the slopes of these linear graphs decreased as the concentration of salt increased (Table S4). This decrease, along with intersection point of these plots at around 12% of amphiphile (Fig. 1B), suggested that the volume of SUPRAS produced followed different dependences at high and low concentration of amphiphile. This effect was clearly illustrated by plotting the volume of SUPRAS produced as a function of salt concentration **(Figure 1C).** Thus, the volume of SUPRAS decreased and increased at 1,2-hexanediol concentrations above and below 10%, respectively, as the concentration of sodium sulfate raised. Given that low sample volumes are commonly used in doping control (typically 1 mL urine) and that a minimum of around 100 µL of SUPRAS are required in chromatographic analysis using autosamplers, we decided to work at amphiphile concentrations equal or above 10% (Figure 1C). An equation was derived to calculate the volume of SUPRAS as a function of the concentrations of 1,2-hexanediol and sodium sulfate (see the section SUPRAS volume in SI) that had a good capability of prediction (**Figure S3).**

SUPRAS Chemical Composition

All the SUPRASs produced in the region of analytical interest ([1,2-hexanediol] from 10% to 20%) were made of the three ingredients present in the colloidal suspension, namely water, 1,2-hexanediol and sodium sulfate. However, the ratios for these ingredients in the SUPRAS were dependent on the concentration of the coacervation-inducing agent (sodium sulfate), so these SUPRASs were environment-responsive and their composition can be tailored according to the salt concentration in the synthetic solution.

Table S5 shows representative results. The water and amphiphile contents in the SUPRAS respectively decreased and increased when the concentration of salt in the synthesis mixture raised. This behavior

suggests that, in addition to the destruction of the hydration layer caused by the salt, salting-out effects in the bulk solution can help to coacervation.[31] The amount of sodium sulfate in the SUPRAS progressively decreased with the increase of salt in the synthesis solution (Table S5). This behavior is in agreement with the decrease of the solubility of this salt in water-alcohol mixtures as the proportion of alcohol (i.e. 1,2-hexanediol in this case) increases.[32]

Water content in 1,2-hexanediol-based SUPRASs were above 36% (w/w) and reached concentrations as high as 61% (w/w). So, these SUPRAS have extensive hydrophilic regions in its nanostructures, which are expected to confer it a great capability for extraction of polar compounds, On the other hand, the high concentration of 1,2-hexanediol in the SUPRAS provided extensive hydrophobic environments, suitable for solubilisation of nonpolar compounds.

The amphiphile was almost fully incorporated into the SUPRAS ($\geq$ 90%) after coacervation, except for the lowest concentration of sodium sulfate (around 40% of amphiphile incorporation for 0.6 M Na_2SO_4). This means that at the boundaries between the isotropic region and SUPRAS formation (Figure 1A), coacervation only occurred partially. All SUPRASs were less dense than water, with density values in the interval 0.90-0.98 g mL^{-1} (Table S5).

SUPRAS Structure

A representative optical micrograph obtained for the SUPRAS is shown in **Figure S4**. This micrograph illustrates that the liquid phases were produced through self-assembly and coacervation. Thus, they were not continuous phases but they were made of coacervate droplets within the interval 6-7 μm (mean value 6.5 μm).

Micrographs obtained by SEM clearly showed (**Figure 2A**) that the nanostructures in the SUPRAS were cubosomes with a size in the range 140-240 nm. These square and rounded discrete nanostructures (see magnification in **Figure 2B**) consist of bicontinuous domains of water and amphiphile bilayers twisted into a three dimensional morphology (see schematic in **Figure 2C**). Cubosomes are usually obtained by top-down and bottom-up approaches from the cubic phases of some amphiphilic lipids (e.g. glyceryl monooleate and phytantriol) by addition of a suitable stabilizer to prevent their aggregation.[33] Thus, they possess the same microstructure as the parent cubic phase but have larger specific surface area. Their

high water content along with their large surface area make cubosomes highly suitable for extracting structurally unrelated compounds covering a wide polarity range. To the best of our knowledge this is the first time that SUPRAS based on cubosomes are reported. Likewise, this is the first time that cubosomes are produced by coacervation from monomeric amphiphiles, which opens the door to their production through predictable and low-energy processes.

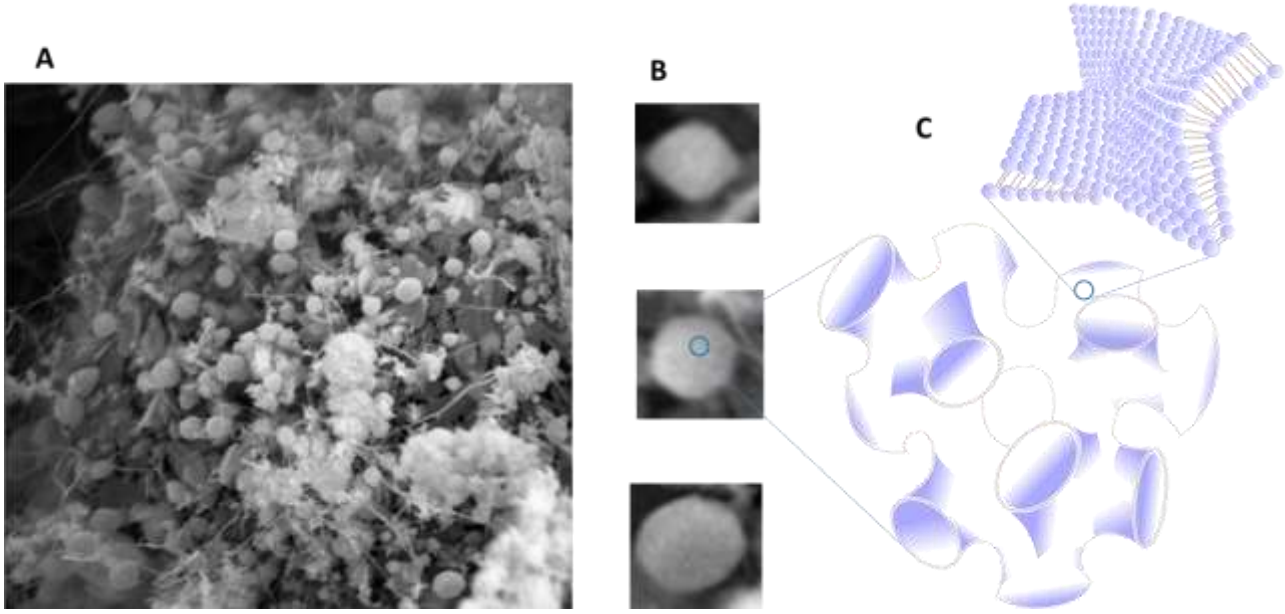

Figure 2. (A) Micrographs obtained by scanning electron microscopy for a SUPRAS produced from a mixture containing 20% (v/v) of 1,2-hexanediol and 1M Na_2SO_4. Magnification: 2 KX. (B) Amplification of some cubosomes present in (A). (C) Schematic of the bicontinuous cubic phase and the amphiphilic bilayer making up cubosomes

Optimization of the SUPRAS-Based Sample Treatment in ITPs

According to the results obtained in the previous section, the cubosomes making up the 1,2-hexanediol-based SUPRASs are highly hydrophilic (Table S5) and this should positively impact their capability for developing straightforward sample treatments in multiclass compound determinations. To check this hypothesis, the influence of the hydrophilicity of SUPRAS nanostructures on the extractability of multiclass doping drugs was initially investigated. Two types of SUPRASs having different nanostructures and hydrophilicity to that of 1,2-hexanediol-based SUPRASs were selected for this purpose. The fist type was synthesized from C6-C10 alkanols in THF-urine media, in the presence and absence of salt. It has been previously reported that these SUPRASs arrange as inverted hexagonal

aggregates where the alcohol groups surround water cavities and the THF are dispersed in the hydrocarbon chains (**Figure S5**, top).[22] The second type of SUPRAS was synthesized from C8-C10 1,2-alkanediols in THF-urine media, in the presence and absence of salt. These SUPRASs have been known to have sponge nanostructures consisting in a highly convoluted and interconnected three-dimensional network of amphiphile bilayers intersected by similarly interconnected nanometer-sized aqueous channels (**Figure S5**, bottom).[21] Under the synthesis conditions used, water content in the three types of SUPRASs were around 5%, 30% and 40% (w/w) for the SUPRAS made up of hexagonal, sponge and cubosome aggregates, respectively.

A total of eleven SUPRAS made up of cubosomes (1), sponges (4) and inverted hexagonal aggregates (6) were tested for the extraction of doping drugs. Table S1 shows the name, chemical structure and molecular formula of the ninety-two prohibited substances selected for this study, ranked by WADA category (S1-S9, P1). This table also includes different parameters of interest for their extraction behavior. The selected compounds covered a wide range of polarity (log P from - 2.4 to 9.2) and included acids, bases and neutrals.

Figure 3 shows the recoveries obtained from urine with the eleven investigated SUPRAS. Results for each SUPRAS were classified into four groups, depending on the recovery values obtained (i.e. 70-120%, 20-69%, <20% and >120%). For comparison, the results obtained from the extraction with a conventional solvent (methyl isobutyl ketone, MIBK[6]) are also included.

The worse recoveries were obtained for SUPRAS made up of alkanols in the absence of salts, for which only around 45-55% of the doping drugs were within the optimal recovery interval (70-120%). Recoveries for 1-octanol and 1-decanol considerably improved under the addition of Na_2SO_4 to the urine (around 60-70% of the drugs had good recoveries), probably because of the salting-out effect. This effect was negligible for 1-hexanol, as inferred from the similar good recoveries obtained in the absence and the presence of the salt (~50%). When considering the percentage of drugs with very low recoveries (<20 %), the best results among alkanol-based SUPRAS were obtained for 1-octanol in the presence and absence of salt, for which only around 10-15% of the drugs showed recoveries below 20%.

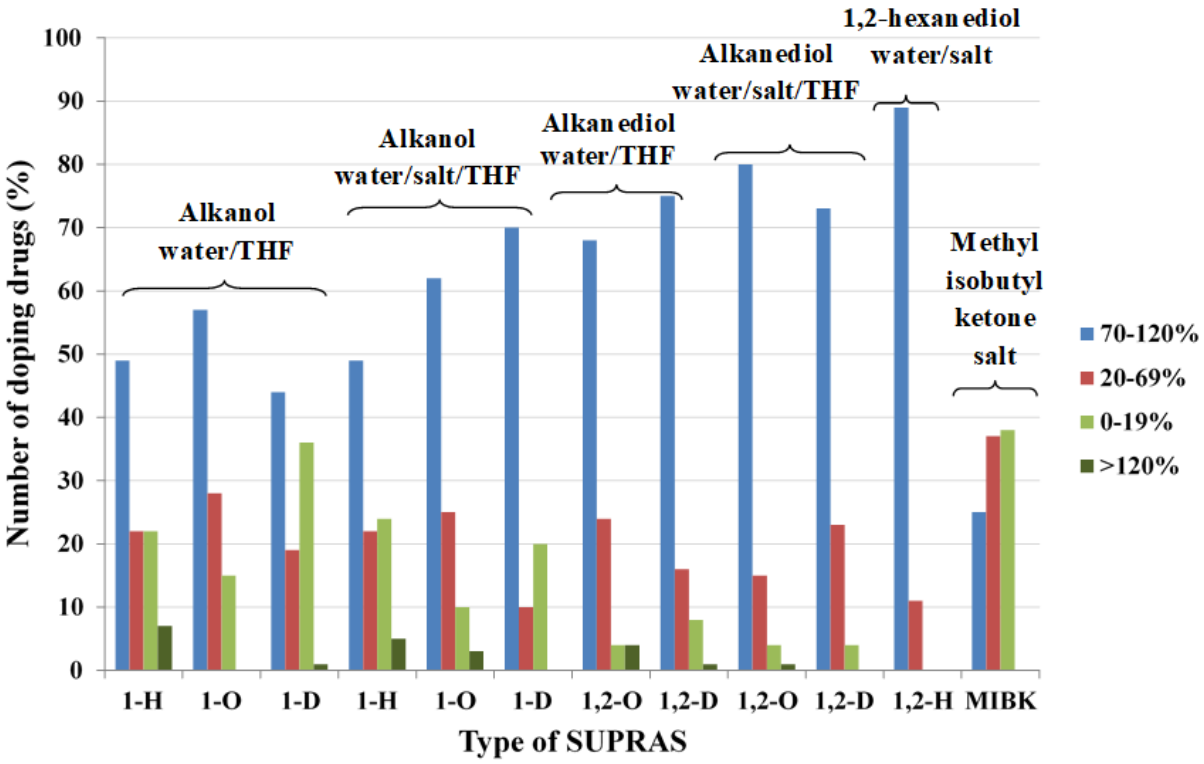

Figure 3. Percentage of prohibited substances with recoveries in the ranges 70-120% (blue), 20-69% (red), 0-19% (light green) and greater than 120% (dark green) for SUPRAS synthesized from: 1-hexanol (1-H), 1-octanol (1-O), 1-decanol (1-D), 1,2-octanediol (1,2-O), 1,2-decanediol (1,2-D), both in the absence and presence of salt, and 1-2-hexanediol (1,2-H) in the presence of salt. Results for methyl isobutyl ketone are also included.

A considerable improvement in the recovery of doping drugs was obtained with SUPRASs made up of alkanediols compared to those based on alkanols (Fig. 3). The best results were found for 1,2-octanediol in the presence of salt, for which the 80% of the selected drugs showed recoveries in the range 70-120%. Additionally, a valuable aspect of these SUPRAS is that they increased notably the recoveries of very polar substances. Thus, only the 4% of the selected doping drugs had recoveries below the 20% as 1,2-octanediol was used as amphiphile, both in the absence and presence of salt. The best results were obtained for 1,2-hexanediol-based SUPRAS; substances within the recommended recovery range (70-120%) approached 90% and no substances were within the very low recovery range (0-19%). As expected, recoveries with methyl isobutyl ketone were low. Only around 25% of the doping drugs were within the optimal recovery interval. It is worth noting that absolute recoveries for many of the drugs

within the range 0-19% was nearly zero, despite the extraction was carried out in the presence of salt to account for the enhancement of recoveries by salting-out effects.

These results clearly show that increasing the hydrophilicity of the supramolecular nanostructures greatly increases the extractability of very polar compounds, as it can be inferred from the number of compounds falling within the interval 0-19% for each of the extractant investigated (Fig. 3). Thus, the most hydrophilic nanostructures (i.e. sponge and cubosomes) achieved the highest global recoveries for the selected compounds. On the other hand, these results also corroborate that conventional organic solvents are unable to develop efficient sample treatments for multiclass drug screening.

Given the greater extraction capability of SUPRAS based on alkanediols compared to those based on alkanols, the suitability of the formers for the removal of matrix effects was investigated. Results were compared with those obtained by a D&S approach (a urine sample was diluted 1:4 v/v with distilled water and readily analyzed). Matrix effects (**Figure 4**) were greatly reduced after SUPRAS extraction in comparison with the D&S approach. Thus, up to 89% of the drugs could be determined in absence of matrix effects under extraction with 1,2-hexanediol. So, in addition to excellent recoveries, the cubosome-based SUPRASs efficiently removed matrix effects from urine. Therefore, further optimization of the extraction process was performed for 1,2-hexanediol-based SUPRASs. The studied variables included the concentration of sodium sulfate used for inducing the formation of the SUPRAS (0.6-1M), the percentage of amphiphile added to the urine (10-30%) and the time for extraction (5-15 min). Selection of the optimal conditions were guided by both the recoveries and matrix effects obtained for doping drugs in urine. Representative results are shown in **Figure S6**. Final optimal values were 5 min extraction, 1 M Na_2SO_4 and 200 µL of amphiphile. Under these conditions, around 90 % of the drugs showed recoveries in the 70-120% range and were not affected by matrix effects.

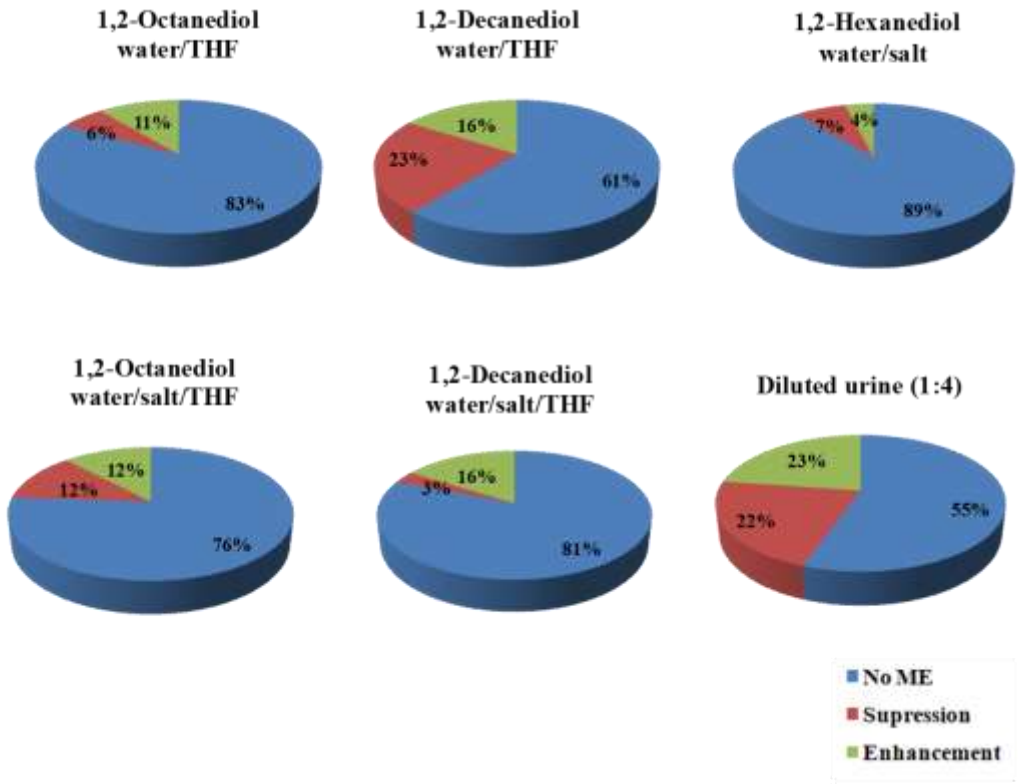

Figure 4. Percentage of prohibited substances showing no matrix effects (±20% in blue), suppression (red) and enhancement (green) for SUPRAS produced from 1,2-octanediol and 1,2-decanediol, both in the absence and presence of salt, and 1,2-hexanediol . Likewise, results obtained for diluted urine (1:4) are also shown.

Method Validation

The method developed was validated according to the procedures specified in the Experimental Section of SI. Results for this validation are discussed in SI (section Method Validation). Data obtained are shown in Table S3 and **Figure S7** (analytical figures of the method), **Figures S8-S9** (selectivity), **Figure 5A** and **Table S6** (recoveries) and **Figure 5B** and **Table S7** (matrix effects). In brief, MDLs for the banned drugs were all far below the respective MRPL[26] and decision limit[34] values, the method was selective and precise, around 82-95% of drugs were efficiently extracted (recoveries 70-120%) in urine samples, and 81-92% did not present matrix effects.

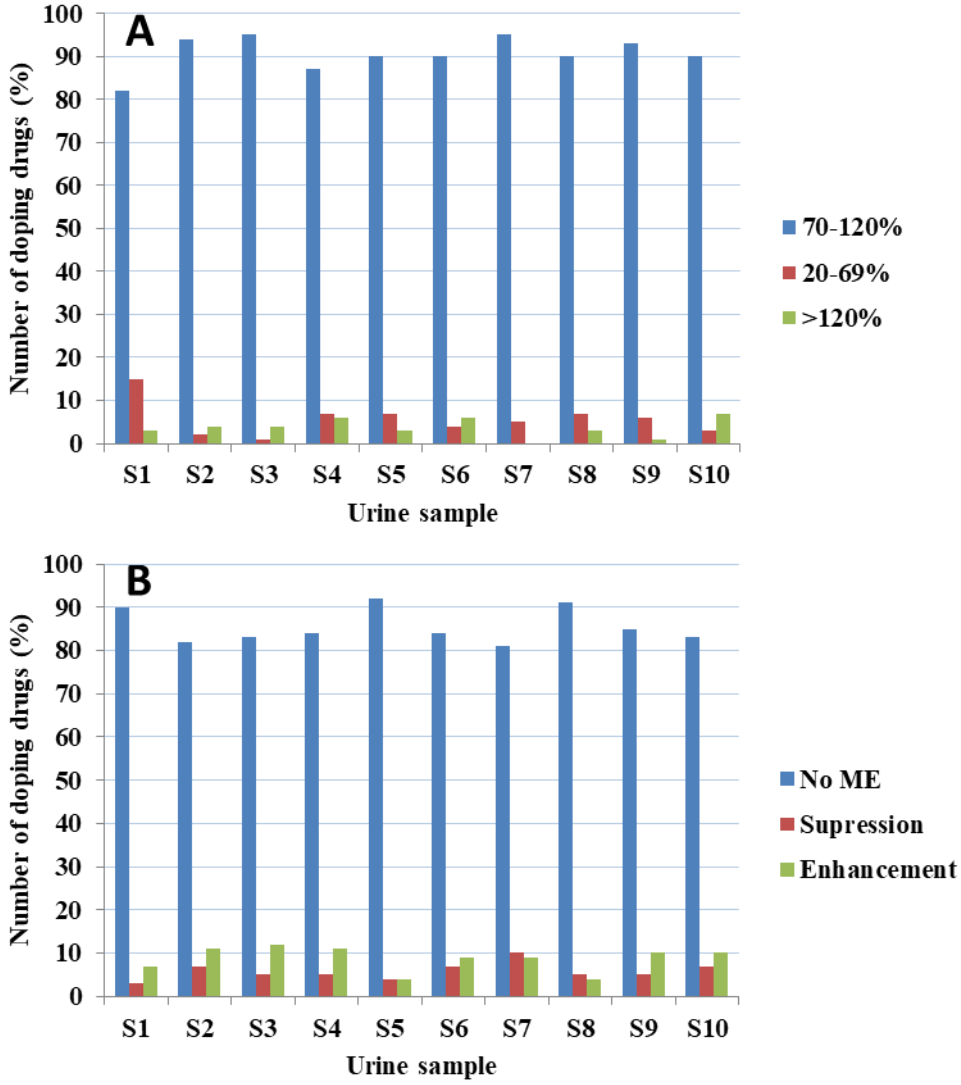

Figure 5. Percentage of prohibited substances with (A) recoveries in the ranges 70-120% (blue), 20-69% (red), and greater than 120% (green, and (B) showing no matrix effects (± 20%, in blue), signal suppression (in red) and enhancement (in green) for the extraction of ten human urines (S1-S10) with 1,2-hexanediol-based SUPRAS.

CONCLUSIONS

The use of SUPRASs tailored with the proper nanostructures provides effective multicomponent extraction of substances covering a wide polarity range. Taking into account that SUPRASs always possess hydrophobic regions, where nonpolar and medium polar substances can be solubilized, the tailoring of their hydrophilic regions seems essential to extend their application to the extraction of highly polar substances. In this respect, the cubosomic SUPRASs here reported have proved high

efficiency for extraction of polar substances with log P values up to - 2.4 and a wide range of functionalities (e.g. alcohol, carboxyl, ether, ester, ketone, primary/secondary/tertiary amines, amides, sulfonyls, etc.). These nanostructures are characterized by a high hydrophilicity, which derives from the short hydrocarbon chain and double head of the amphiphile. In our opinion, the production of highly hydrophilic SUPRASs can open the door to their effective application in multitarget liquid-liquid extractions, a field not properly covered by conventional organic solvents. Valuable practical considerations for application of SUPRASs in multiclass extractions are simplicity and high sample throughput. Regarding application of cubosomic SUPRAS to ITPs in human sport drug testing, the method here reported is simpler, faster and cheaper than SPE.

Additionally, it is worth noting that, for the first time, cubosomes have been produced from the coacervation of monomeric amphiphiles, which opens the door to their production from simple, predictable and energy-saving processes. This synthetic procedure can be of great interest in their application for drug delivery. Furthermore, the low toxicity ingredients (1,2-hexanediol, water and sodium sulfate) and simple synthetic route makes cubosomic SUPRASs comply with green chemistry principles.

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Supporting Information

Experimental methods; Equation for prediction of SUPRAS volume; Fig. S1-S2, schematics of SUPRAS synthesis and drug extraction; Fig. S3, correlation between SUPRAS volume prediction vs experimental data; Fig. S4-S5, Optical and SEM SUPRAS micrographs; Fig. S6, results for extraction optimization; Fig. S7-S9, Total and extracted ion chromatograms for drugs; Table S1-S2,

physicochemical characteristics, suppliers and MS/MS parameters for the selected banned drugs; Table S3, analytical figures of merit; Tables S4-S5, dependences of SUPRAS volume and chemical composition on synthesis ingredient ratios; Tables S6-S7, single and average recoveries and matrix effects for banned substances in 10 urine samples.

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