Microsampling with Solid Phase Extraction Cartridge: Storage and Online Mass Spectrometry Analysis

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ABSTRACT: This study aims to introduce the concept of utilizing solid phase extraction (SPE) cartridge for remote biofluid collection followed by direct sample analysis at a later time. For this, dried matrix spot was prepared in a syringe, in the form of SPE cartridge for the first time to enable small biofluid collection (microsampling), storage, shipment, and online electrospray ionization (ESI) mass spectrometry (MS) analysis of the stored dried samples. The SPE sorbents were packed into ESI syringe and the resultant cartridge was used for sampling small volumes (<20 μL) of different complex biological fluids including blood, plasma, serum, and urine. The collected sample was stored in the dry-state within the confinement of the SPE sorbent at room temperature and analyte stability (e.g., diazepam) was maintained for more than a year. Direct coupling of the SPE cartridge to MS provide excellent accuracy, precision, and sensitivity for analyzing illicit drugs present in the biofluid. The corresponding mechanism of wrong-way positive ion generation from highly basic elution solvents was explored. Without chromatography, our direct SPE-ESI-MS analysis technique afforded detection limits as low as 26 pg/mL and 140 pg/mL for raw urine and untreated plasma, respectively. These promising results prove that the new syringe-based SPE cartridge can serve as a good alternative to conventional microsampling techniques in terms of analyte stability, ease of operation and versatility, and analytical sensitivity and speed.

INTRODUCTION

Dried matrix spots (DMS) sampling methods such as dried blood spots (DBS), dried plasma spots (DPS), dried serum spots (DSS), and dried urine spots (DUS) have recently gained increased interest in biomedical research, disease diagnosis, and pharmaceutical application.[1,2] Herein, we describe a procedure for adapting syringe for electrospray ionization (ESI) as a solid-phase extraction (SPE) cartridge for microsampling biofluids (≤50 μL), room temperature storage of collected samples, and online analysis of the dried samples using ESI mass spectrometry (MS). Blood samples collected onto filter paper as DBS have been used extensively in newborn screening (NBS) for almost 60 years.[3,4] Recently, DBS sampling has been extended to pharmacokinetic and toxicokinetic studies, therapeutic drug monitoring, and metabolite profiling.[5] This microsampling technique is a good alternative to conventional venipuncture (>0.5 mL) sampling of whole blood since it is less invasive, inexpensive, and offers simplified means of collection, transportation, storage, and processing of blood. Compared with liquid blood, DBS are relatively more stable and less reactive in the dried matrix since enzymes and other pathogens are deactivated during the drying process. For analytes that can be detected in both blood and urine, the DUS sampling method may be preferred since DBS suffers from hematocrit effects and difficulty in determining blood-to-plasma ratios. For these reasons, urine is the matrix of choice for drug screening allowing non-invasive detection of the drug and its metabolites with high sensitivity. DPS prepared in paper provides a simpler means (among other methods) to deal with hematocrit effects cause by variation of spot size in DBS due to varying red blood cell levels in different patients.

Quantitative assays for dried biofluids assume that the DMS punch provides volumetric samples that are comparable in volume to those obtained by liquid measuring tools. Unfortunately, this assumption is challenged by hematocrit and chromatographic effects, making it necessary to use stringent mathematical calculations[6,5] and radioactive tracers[7,5] to effectively estimate the volume of biofluid in the punched sample. Also, challenges in sample recovery from DMS often lead to dilution, which culminate in the use of sophisticated instrumentation that has the corresponding required sensitivity for analysis of the diluted samples. Moreover, almost all DMS samples must be dried several hours to immobilize the biofluid in the cellulose matrix; this ensures safe shipping minimizing risks of infection while also avoiding possible contamination of sample during shipment.

Aside from the traditional DMS microsampling methods, newer platforms have been developed that provide solutions to some of the challenges listed above. For example, pre-cut dried blood spot (PCDBS) can reduce hematocrit effect, improve sample utilization, and minimize carryover issues between the punches.[8] Volumetric absorptive microsampling (VAMS) overcomes hematocrit effects by collection precise volumes of biofluid into an absorbent tip with reproducible internal porous volume.[9,10] Recently, the biofluid sampler approach was introduced where cellulose fabric substrate is coated with porous sol-gel sorbents to decrease maximum sample volume, reduce hematocrit effect, and improve physical adsorption of sample.[11] Like the traditional DMS microsampling methods, these new methods also require several hour of drying in ambient air, as well as subject to dilution since several steps are required to

recover and extract analyte for offline analysis. Solid-phase extraction (SPE) methods are traditionally employed to integrate analyte extraction, enrichment, and isolation from sample matrices. Therefore, in principle, SPE should be able to reduce sample dilution when combined with microsampling. Unfortunately the use of SPE cartridge as a microsampling and storage device is rare.[12] By virtue of the mode of operation, solid-phase microextraction (SPME) fibers serve as efficient microsampling platforms (for different matrices including surface water, tissues, and biofluids)[13] and offer online analyte desorption and analysis capabilities.[14] Here too, the SPME fiber is typically not considered as a storage medium for adsorbed analytes for prolong periods.

As part of our ongoing efforts to create contained ion sources,[15-21] we sought to pack SPE sorbents into a syringe and use this assembly as a SPE cartridge for microsampling of biofluids. We anticipated that the containment of the SPE sorbent in ESI syringe will provide three-dimensional (3D) space/encloser to effectively store the collected biofluid under ambient conditions without the need for low temperatures during sample transportation and prolong storage for biobanking purposes. Collectively, we envision microsampling with SPE cartridge to have the following *six* advantages over other microsampling techniques, namely to: ***1***) it avoids the several hours of drying time to enable samples to be shipped immediately after collection. This objective is achieved by entrapping sample in the SPE sorbent, which is in turn contained in the ESI syringe providing the encloser needed for safe delivery, as well as avoiding sample contamination; ***2)*** microsampling with SPE cartridge increases analyte stability. Previous studies from our laboratory have consistently shown that drying biofluid as 2D disk (e.g., DBS) in a porous medium predisposes the sample to oxidative stress that leads to rapid degradation of labile analytes.[22-24] As will be described later, SPE sorbent containing the biofluid is sandwiched between two frits, offering a 3D medium within which the labile analytes can be stored, thereby limiting exposure to light and air; ***3***) elimination of large sample volume requirements since the SPE sorbent can serve to concentrate analyte, reduce matrix effect, and offer improved analyte recovery, all enabling sensitive detection; ***4)*** it overcomes hematocrit and chromatographic effects since graduated SPE cartridges and pre-weighted SPE sorbents are used; ***5)*** like SPME, the proposed SPE-ESI-MS platform will combine sampling, sample preparation, and analyte pre-concentration in a single experiment. In addition, our method avoids extra waiting times typically found in PCDBS and SPME (e.g., equilibrium extraction of analyte into the SPME fiber requires minutes to hours of waiting time) making self-sampling at home possible; and ***6)*** the process simplifies MS analysis through the direct online ESI process where the SPE cartridge is coupled to MS without additional extraction and/or separation steps. This is contrary to traditional procedures in which the analyte concentrated on the SPE sorbent is typically eluted and subjected to liquid chromatography (LC) or gas chromatography (GC) before final quantification by MS.

Perhaps the RapidFire system from Agilent is the closest platform to the method described here. RapidFire provides high throughput MS analysis of SPE cartridge with high sensitivity.[25,26] However, obvious advantages such as the flexibility to customize our method for any type of analysis, including routine laboratory experiments and remote sampling followed by analysis at a later time provide some important additional benefits. In the course of our studies, particularly during the year-long stability studies, two reports[27,28] have appeared in the literature that utilized syringes packed with sorbents for extraction and MS analysis. These focused on enabling 1) effective on-line, on-site analysis of cosmetics in organic solvents[27] and 2) analysis of pesticides in vegetable oils by enabling direct injection onto high pressure liquid chromatography (HPLC) column followed by MS analysis.[28] Nevertheless, to the best our knowledge, our syringe-based SPE-ESI-MS platform represents the first attempt to adapt SPE cartridges for biofluid sampling, storage, shipment, and direct analysis. That is, unlike the syringe approaches described above, our approach shows extraordinary stability for biofluids stored at room temperature, something that is not available in any other microsampling platform. For example, diazepam and cocaine, previously determined to degrade in DMS samples, were observed to be stable for more than 50 weeks (>1 year) when whole serum sample were stored in the SPE device in the dry-state and at room temperature. In addition, prior separation step (i.e., HPLC) is not required for analysis of the collected biospecimens. The SPE microsampling platform itself can be adopted for any commercially available ESI source for direct MS analysis. We applied the platform for sampling and analysis of raw urine, plasma, and whole blood and achieved limit of detection as low as 26 pg/mL and 140 pg/mL for cocaine in untreated urine and plasma samples, respectively. Acceptable accuracy (trueness) and precision were also obtained demonstrating quantitative recovery and online ESI-MS analysis directly from the custom made SPE cartridge. Furthermore, the mechanism of ionization in positive-ion mode from a highly basic NH_4OH (pH 10) eluent without pH adjustment is discussed. The promising results presented here illustrate a new concept for applying SPE and MEPS (microextraction by packed sorbent)[29] cartridges that does not involve the typical immediate clean-and-use procedure. Rather, our results show that these cartridges can serve to allow reliable remote sample and storage option for various medical application, especially those involving resource-limited settings.

EXPERIMENTAL SECTION

Creation of customized syringe containing solid phase extraction sorbent.

A home-made SPE cartridge (i.e., customized ESI syringe containing SPE sorbent) was constructed through a dry-wet packing process in which 10 mg of SPE mixed-mode cation-exchange (MCX), polymeric cation-exchange (PCX), or hydrophilic-lipophilic balance (HLB) sorbents were separately placed into 1 mL plastic syringes (**Figure 1**). To accomplish this, a precut frit was placed at the bottom of the syringe followed by a specific SPE sorbent. Then, a second frit was placed on top of the SPE sorbent. A plunger was used to manually push (three times) the frit/SPE sorbent assembly all the way to the bottom of the syringe to ensure firm and close packing as possible. This dry packing process was proceeded by wet packing in which methanol (1 mL; three times) was flushed through the sorbent, after which the frit/SPE sorbent assembly was pressed again (each time the methanol solvent was added) with the plunger using maximum force toward the bottom of the syringe. After this dry-wet packing process was completed, the SPE cartridges were kept in a fume hood to dry for 24 h. Note: commercial SPE cartridges are designed to operate

properly under vacuum and their conical shape does not allow the use of plunger. Free moving plungers (as used in this study) are needed for connection SPE cartridge to syringe pump during ESI.

Sample collection/introduction.

Optimization of mixed-mode cation-exchange (MCX)-based SPE cartridge. Varied sample volumes (20 − 200 µL) of the biofluid were placed onto SPE cartridge, followed by 20 µL internal standard (IS) solution. Then, 200 µL of 4% phosphoric acid was added to ionize both analyte and sorbent for electrostatic retention on the MCX SPE sorbent. Any mixture of solution present on the top of the frit was mixed and pushed down by the plunger for proper absorption onto the sorbent. This method of sample introduction was utilized for process optimization where biofluids were processed and analyzed immediately without drying.

Microsampling with MCX-based SPE cartridge. For microsampling purposes, optimized volumes of 5 µL whole blood and 20 µL plasma, serum, or urine was separately placed inside the SPE cartridge. Note: sample volumes were optimized as maximum volumes that can be trapped inside the 10 mg sorbent used in cartridge fabrication (larger amount of sorbent may be used to allow higher sample volume capacity). Then, 20 µL of 4% phosphoric acid was immediately added and mixed with biofluid. The free moving plunger was then used to push down the sample onto the sorbent. After this, the sample is ready for storage or shipment without any additional drying step. Since the sample stays in the sorbent for an extended time during shipment (or storage), efficient kinetic exchange between sample and sorbent functional groups is achieved before sample becomes completely dry. It is important to note that traditionally sample loading onto SPE cartridge is carried out rapidly (about one droplet per second) ignoring the fact that sample must interact with sorbent sufficient time. We overcome this SPE challenge by combining loading and drying into a single step. That is, sample is trapped inside of the porous sorbent and not allowed to pass through, which permit sample to dry slowly and hence obtain optimal analyte retention. When ready for analysis, the dry sample was wetted and

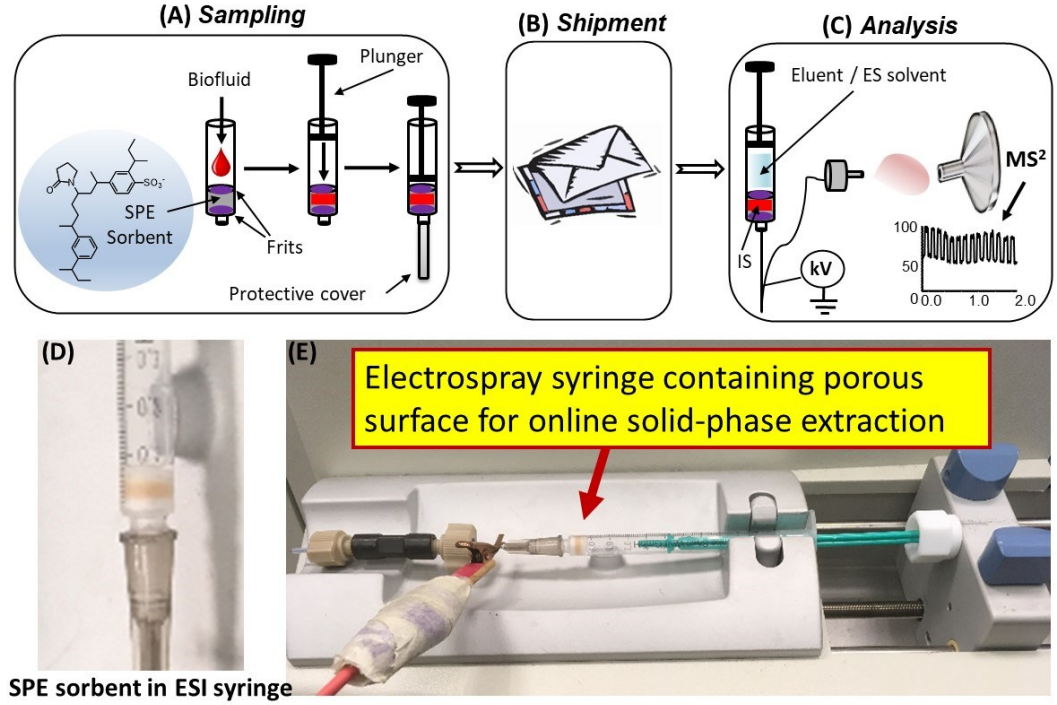

Figure 1. Schematics illustrating (**A**) a syringe packed with SPE sorbent to form a cartridge with the help of a free moving plunger; the graduated SPE cartridge is used for microsampling of biofluids, which can be (**B**) shipped by mail immediately upon sample loading without any waiting steps. This is followed by (**C**) online ESI-MS analysis of biofluid samples after washing. Sampling is achieved by introduction of a biofluid into the syringe-based SPE device and its trapping within 3D confinements of the SPE sorbent through a simple plunger push. Before shipment, protective cover should be applied as shown in (**D**). During MS analysis, internal standard (IS) is introduced into the SPE cartridge followed by the application of eluent/electrospray solvent, and then connected to electrospray ionization source through a stainless steel blunt needle that allows direct current (DC) high voltage to be applied to the spray solvent as illustrated in (**E**).

activated by 200 µL of 4% phosphoric acid. Then, a mixture of 20 µL of the internal standard solution and a second portion of 200 µL of 4% phosphoric acid were loaded onto the SPE sorbent by the help of a plunger. When available, a vacuum can be employed to help with this loading process.

Online electrospray ionization from MCX-based SPE cartridge. After activation with phosphoric acid, the sample was washed with 200 µL water containing 2% formic acid to remove matrix proteins, followed by a second-round washing process using 200 µL methanol to remove neutral and acidic matrices. The SPE cartridge was then connected to ESI source via stainless steel blunt needle. The cartridge acted as the ESI syringe where 200 µL acetonitrile/methanol (60:40, vol./vol.) containing 5% of ammonium hydroxide was used as eluent and ESI spray solvent. This solvent composition is able to neutralize functional groups thereby disrupting the electrostatic interaction to initiate analyte elution. Elution and wash volumes were optimized for sufficient residence time. Optimized conditions allowed efficient mobile phase interaction with stationary

phase. The solvent was injected into mass spectrometer with optimized 50 µL/min flowrate and assisted by 140 psi of nebulizer gas (N_2) pressure. Note: analyte elution can be achieved with the SPE cartridge in horizontal or vertical position, contrary to elution from commercial SPE cartridges in which the vertical position is typically used.

Hydrophilic-lipophilic balance (HLB)-based SPE cartridge. For sample collection using HLB-based SPE cartridge, 20 µL of biofluid was placed into the cartridge followed by 200 µL of 4% phosphoric acid, and then sample was mixed properly and loaded onto the sorbent by the plunger. For MS analysis, sample washing was performed using 200 µL of 5% MeOH in water (two times). The SPE cartridge was then connected to ESI source via stainless steel blunt needle and filled with 200 µL of MeOH. The solvent was injected into mass spectrometer at 50 µL/min flowrate and 140 psi of nebulizer gas pressure.

Polymeric cation-exchange (PCX)-based SPE cartridge. Sample loading onto PCX-based cartridges followed similar procedure used for MCX and HLB sorbents. Except that we used this cartridge to investigate the possibility of detecting analyte without washing. Acetonitrile was used as elution/spray solvent. See Supporting Information for details. Conditions for SPE process for each sorbent used in this study were adapted from manufacturer with some modifications.[30,31]

Mass spectrometry.

All samples were analyzed by a Thermo Fisher Scientific Velos Pro LTQ mass spectrometer (San Jose, CA, USA). MS parameters applied were as follows: 4 kV spray voltage, 200 °C capillary temperature, 5 mm distance from an ion source to MS inlet, 3 microscans, 100 ms ion injection time, 60% S-lens voltage. Spectra were recorded for at least 60 s, yielding an average of 600 individual scans. MS data collecting and processing were performed by Thermo Fisher Scientific Xcalibur 2.2 SP1 software. Tandem MS with collision induced dissociation (CID) was utilized for analyte identification. For the CID experiments, 30% (manufacturer's unit) and 1.5 Th (mass/ charge units) for isolation window of normalized collision energy were selected.

Materials and Reagents.

Acetonitrile (99.9%), ammonium bicarbonate (99%), *n*-butylamine (99.5%), cortisone (98%), and methanol (99.9%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Ammonium hydroxide (28-30%), hydrochloric acid (37.5%), potassium hydroxide (85%), and formic acid (88%) were supplied by Fisher Scientific (Pittsburgh, PA, USA). Ammonium chloride (99.5%) was obtained from Acros Organics (Geel, Belgium). 100 µg/mL solution of cocaine-D3 and 1.0 mg/mL standard solutions of benzoylecgonine, cocaine, diazepam, diazepam-D5, (±)-methamphetamine, and (−)-Δ^9-tetrahydrocannabinol were provided by Cerilliant (Round Rock, TX, USA). Single-donor human blood, single-donor human plasma, single-donor human serum, and single-donor human urine were all purchased from Innovative Research (Novi, MI, USA). MCX SPE bulk sorbent (30 µm particle size) and HLB bulk sorbent (30 µm particle size) were supplied by Waters (Milford, MA, USA). PCX SPE bulk sorbent (45 µm particle size) and frits (6.4 mm diameter) for SPE cartridges were acquired from Agilent Technologies (Santa Clara, CA, USA). 1 mL plastic syringes were provided by Ace Glass (Vineland, NJ, USA). Micro Essential Laboratory ultrasensitive pH paper (Hydrion UltraFine; Brooklyn, NY, USA) was used for pH measurements. 18.2 MΩ water was employed for all water-based solutions (Milli-Q water purification system, Millipore, Billerica, MA, USA).

RESULTS AND DISCUSSION

Optimization

Figure 2 displays a typical workflow and timeline for sample loading, elution, and MS analysis by conventional procedure for offline SPE-LC-ESI-MS analysis. This process is contrasted with a summary of the workflow involved in our proposed online SPE-ESI-MS approach. The traditional offline SPE-MS protocol requires significant amount of time (>40 min) even when utilized without conditioning and equilibration steps. As can be observed, most of the time is spent on separation (LC) and SPE sorbent conditioning and equilibration. Since we skipped conditioning and equilibration prior to analyte elution and avoided LC completely, our method yields results in <5 min. **Figure 3** shows multiple reaction monitoring (MRM) elution profiles for cocaine (MW 303 Da) and its metabolite, benzoylecgonine (MW 289 Da) spiked in serum and loaded onto MCX-based SPE cartridge. **Figure 3A** compares the profile of cocaine (*m/z* 304) to that of its internal standard (IS) cocaine-D3 (*m/z* 307) spiked at 300 and 500 ng/mL concentration levels, respectively. As shown, the elution/ionization process is stable and quantitative, with approximately 3X more signal from cocaine-D3 than cocaine. Assuming comparable ionization efficiencies, the data indicate that cocaine is quantitatively eluted from the MCX sorbent using 200 µL

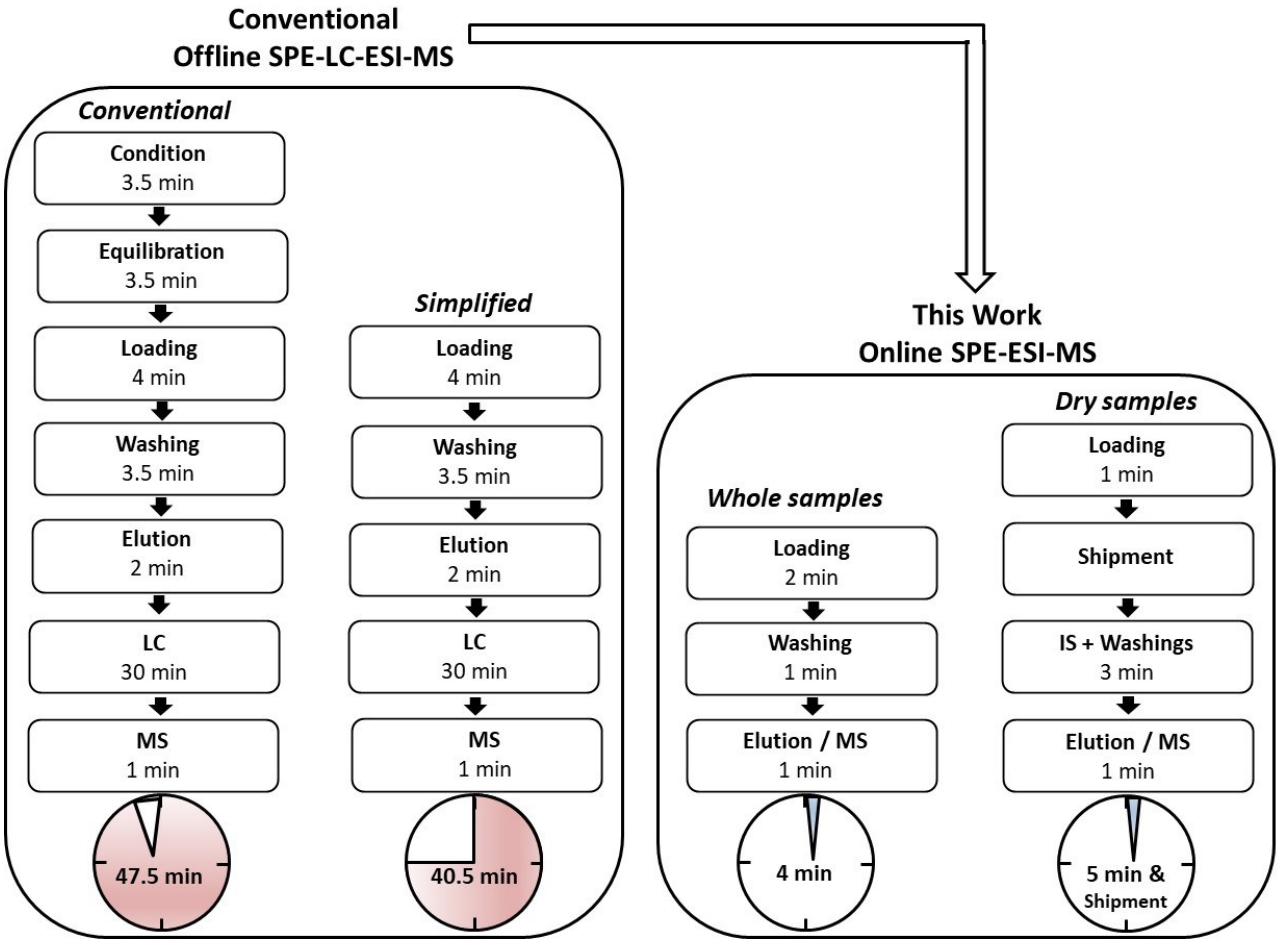

Figure 2. Comparison of workflow and timeline for offline SPE-LC-ESI-MS procedure (conventional) and to the online SPE-ESI-MS process proposed in this work. This general procedure applies to both fresh and dry biofluid sample analysis.

acetonitrile/methanol solvent (60:40, vol/vol) containing 5% of ammonium hydroxide. We observed similar elutionefficiency for cocaine in other biofluids such as whole blood, serum (**Figure S1**) and plasma (**Figure S2**), as well as fordifferent analyte, diazepam in serum (**Figure S3**). Interestingly, whole blood was analyzed without any traditional lysis or protein precipitation steps. The consistent results indicate the absence of sample coagulation that can be explained by efficient drying process attainable through the use of the porous materials of SPE sorbents.

Figure 3B compares the elution profile of hydrophobic cocaine (Log *P* 2.3) analyte to its hydrophilic benzoylecgonine (Log *P* -0.3) metabolite. Although both compounds are present in the serum matrix at equal concentrations (300 ng/mL), we observed higher ion signal for cocaine than benzoylecgonine. This result may be ascribed to the combined effects of the difference in ionization and elution efficiencies for the two analytes. Hydrophobic compound like cocaine ionizes very well in ESI and as a result is detected often at higher sensitivity than benzoylecgonine.[32-34] As stated above, acetonitrile/methanol spray (60:40, vol/vol) solvent containing 5% of ammonium hydroxide was used to neutralize the analyte and so release it from mixed-mode cation-exchange SPE sorbent. However, this basic solution (measured pH ≈ 10) can ionize benzoylecgonine due to the presence of the carboxylic functional groups, thus impeding its elution from the MCX cartridge. For this reason, we created other SPE cartridges packed with hydrophilic-lipophilic balance (HLB) sorbent to evaluate its effect on relative ion signal from hydrophobic versus hydrophilic analytes. Here too, we observed high signal for cocaine (**Figure S4A**) indicating elution efficiency played minimal role in both SPE sorbents. This leaves us to conclude that the relatively lower signal observed for benzoylecgonine, when compared with the hydrophobic cocaine analyte, due to the ionization step. Aside from analyte properties (i.e., hydrophobicity) the different spray solvents used for a different SPE sorbent can also play important role in overall ionization efficiency of different analytes. For example, we observed the acetonitrile/methanol (60:40, vol/vol) solvent mixture containing 5% of ammonium hydroxide utilized for elution from HLB cartridge to yield slightly (~2X) better ion signal (absolute) than methanol alone, which is required for MCX cartridges (**Figures S4**). Thus, unless otherwise stated, the rest of the experiments were performed using MCX cartridges. An optimized flow rate of 50 µL/min was for used for all experiments (**Figure S5**). At this flowrate, MS signal persisted for at least 7 min (**Figure S6**), which is sufficient for MS detection and quantification. Moreover, re-analysis

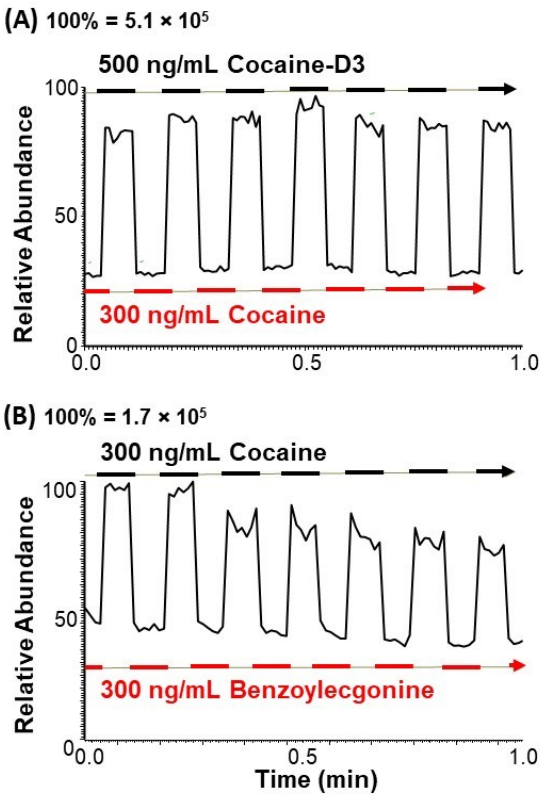

Figure 3. (**A**) MRM profile for cocaine (300 ng/mL) relatively cocaine-D3 (500 ng/mL) analyzed in 20 µL serum and (**B**) MRM profile comparing 300 ng/mL each of cocaine and benzoylecgonine in 200 µL serum. The MCX-based SPE cartridge was used for both experiments.

of the same SPE cartridge is possible for at least 3 times, and it still produces good ion signal (**Figure S7 and S8**). Such capabilities can ensure validation of results at a later time without the need to dilute sample before the initial analysis.

Direct Quantification

We applied the optimized MCX-based SPE-ESI-MS detection platform for microsampling, storage, and quantification of cocaine in raw urine and untreated plasma. Here, 200 µL of biofluid spiked with cocaine (concentration range 1 to 1000 ng/mL), 20 µL of IS (5000 ng/mL), and 200 µL of 4% phosphoric acid were loaded onto the SPE cartridge. Washing proceeded as described and elution was performed by using 200 µL acetonitrile/methanol (60:40, vol./vol.) containing 5% of ammonium hydroxide. MS analysis was achieved in MS/MS mode for both cocaine (*m/z* 304 → 182) and cocaine-d3 internal standard (IS, *m/z* 307 → 185). Calibration curves were constructed by plotting the ratio of product ion intensities for cocaine (m/z 182) and IS (*m/z* 185). Cocaine showed linear response over the entire calibration range with R^2 values of >0.99 (with $1/x^2$ weighting) for both urine and plasma matrices (**Figure 4**). Limit of detection (LOD) and limit of quantification attained were 26 pg/mL and 85 pg/mL for urine, and 140 pg/mL and 470 pg/mL for plasma, correspondingly. Sensitivity was almost twice better for urine compare to plasma (see

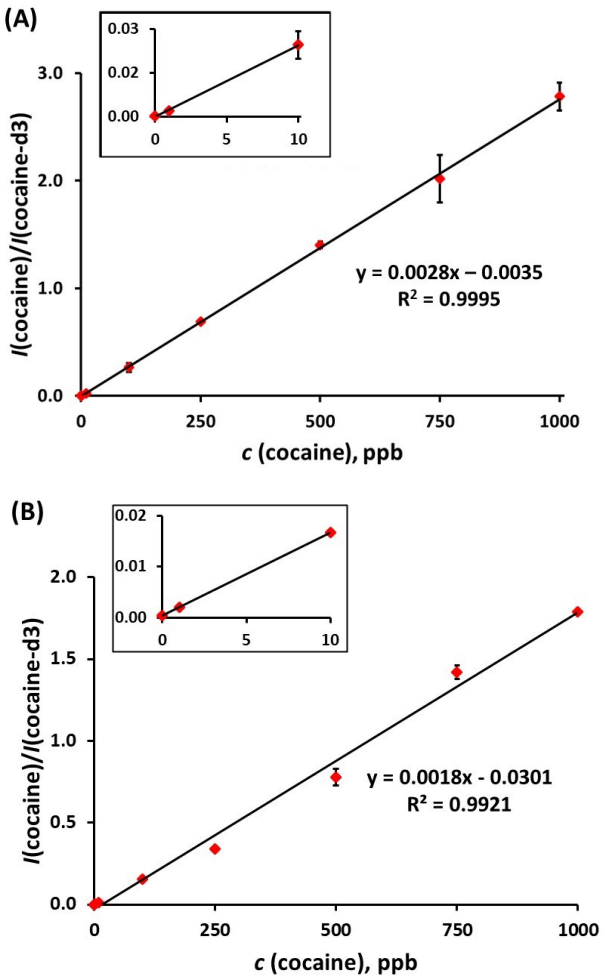

Figure 4. Quantification of cocaine in (**A**) urine and (**B**) plasma samples. 500 ng/mL of cocaine-D3 as internal standard (IS) was used for both biofluids. Data was acquired with the SPE-ESI-MS method in MRM mode (transitions at *m/z* 304 → 182 and *m/z* 307 → 185 for the analyte and IS, respectively). Error bars displays one standard deviation. Figures (**A**) and (**B**) were used to evaluate the quality control samples at 3, 30, and 300 ng/mL (N = 3) and results for % accuracy and RSD are summarized in Table 1.

calibration slope: 0.0025 versus 0.0013, respectively), which can be explained by much more complex matrices found in plasma. Excellent accuracy and precision (**Table 1**) were also achieved for quality control samples (prepared at low, medium, and high concentrations) with all results within 3% and 4% for urine and plasma, respectively. Relative standard deviation (RSD) no greater than 8% was observed for all concentrations. Note that these results were obtained without using any additional separation technique. A previous study utilized MCX sorbent in the conventional offline elution followed by ultra-performance liquid chromatography (UPLC) MS/MS to quantify cocaine in raw urine.[35] Interestingly, the analytical performance obtained from this sophisticated platform was comparable to our approach performed without the UPLC step. An accuracy of 7% was determined for the offline SPE-UPLC-MS/MS method compared with 3% for our SPE-ESI-MS/MS

Table 1. Quality control (QC) data for extracted whole urine and whole plasma samples.

Sample	QC level (ppb)	Mean (ppb)	% Accuracy	% RSD
Whole urine	3	2.95	98.2	1.4
	30	30.7	102.5	7.0
	300	304.3	101.4	4.3
			Mean 100.7	
Whole plasma	3	3.1	103.7	3.6
	30	31.0	103.3	1.8
	300	292.5	97.5	7.7
			Mean 101.5	

approach. This excellent result was achieved mainly because of the properly optimized mixed-mode polymeric sorbent MCX, which is well known for high sensitivity, selectivity, reproducibility, and robustness for extracting basic compounds with high surface area and pH stability from pH 0 to 14. Because the MCX sorbent is water-wettable, it can maintain excellent retention and recoveries even when the sorbent runs dry.

Mechanistic Consideration: The Wrong-Way Ionization

These excellent quantification results for cocaine were achieved using positive-ion mode analysis. Yet, ACN/MeOH (60/40, v/v) spray solvent containing 5% of NH_4OH (pH 10) was used. This is surprising because basic spray solutions are expected to support deprotonation and subsequent ionization in the negative-ion mode.[36] A wrong-way ionization mechanism is in operation where this basic spray solution effectively produces protonated $(M+H)^+$ ions.

In fact, in traditional SPE experiments, eluents derived from 5% of NH_4OH (or other strong basic solutions) are not injected directly onto HPLC column. Such task is avoided for two reasons: 1) to minimize sample/mobile phase incompatibilities as basic/alkaline mobile phases are rarely used in chromatography due to dissolution of many silica-based sorbents at pH > 7 [37,38] and/or 2) to prevent ionization suppression in the positive-ion mode, especially for basic analytes. Thus, the typical concentrations of ammonium hydroxide and ammonium salts for LC-MS do not exceed 0.2% and 50 mM respectively.[38] This is often achieved by neutralizing the 5% NH_4OH eluent from the SPE cartridge with formic acid before injection onto the LC column.Since we skipped the HPLC step in our online ESI-SPE-MS, most of the negative side effects are also eliminated. The 5% NH_4OH solvent is introduced only during the online elution and ionization step, thus minimizing residence time in sorbent and subsequently limiting possible analyte instability concerns. Most importantly, we believe that the 5% of NH_4OH solution actually facilitates analyte protonation via the wrong-way ionization phenomenon.[36,39,40] Though not fully understood, the following mechanisms have been proposed to account for positive ion production in basic solution: 1) electrolytic production of protons in positive ESI,[41] 2) local acidity of the droplet surface in positive ESI,[42] 3) two-step protonation of $[M-H]^-$ ions, initially located in the droplet interior, by $[M+NH_4]^+$ species; the process is driven by evaporation of NH_3 from the droplet surface leaving behind protons,[43] 4) gas-phase proton transfer from protonated amines to neutral analyte molecules,[44] and 5) collision-induced dissociation (CID) of $[M+NH_4]^+$ ions in a de-clustering interface.[45]

Although it is now the default point to start the LC-MS method development with acidic mobile phases, we have found the 5% NH_4OH-based solution to be MS friendly. Therefore, we sought to investigate the fundamental mechanism operating in this wrong-way ionization. To avoid complicated three-component solvent system and to focus on pH effects, we used 5% of NH_4OH in MeOH solution for this investigation. First, we recorded MS spectra for cocaine in many different solvent compositions (**Figure S9**). Our own observations exclude electrolytic protonation mechanisms. This is because the use of 10% formic acid to neutralize and acidify cocaine solutions containing 5% of NH_4OH did not improve cocaine $(M+H)^+$ ion intensity (a decrease in ion yield was observed instead; **Figure S9B − C**). The same observations we recorded for cortisone in basic solutions where no improvements in $(M+H)^+$ ion intensity were achieved by acidification of ESI droplets through online exposure to HCl headspace vapor (data not shown). These results agree well with reports that suggested that solvent oxidation cannot significantly alter the pH of strongly basic solutions.[36,39] Cocaine ions ($[M+H]^+$ or $[M+K]^+$) were also not detected for basic solutions containing KOH, which also excludes possible influence of corona discharge mechanism (**Figure S9D**).[39] After addition of ammonium ions to cocaine/MeOH/KOH system, either to bulk solution (5% of NH_4OH or 0.74 M NH_4HCO_3) or droplets (headspace vapor of NH_3), cocaine ions were still not detected. This is surprising since NH_4^+ ions should partially displace some K^+ ions at droplet surface and promote ionization of cocaine. This suggested the ionization process does not occur in the droplet media but in the gas phase.

Indeed, in the absence of high abundance of protons at the surface of droplets (given high initial solution pH), the main source of protons might be due to evaporation of NH_3 from NH_4^+ according to the following reaction:

$$\mathbf{[M + NH_4]^+_{(droplet)} \rightarrow [NH_3{-}H^+{-}{-}M]_{(gas)} \rightarrow NH_{3(g)} + [M + H]^+_{(g)}}$$

The transfer of proton from ammonium ion (proton affinity, PA (NH_3) = 859 kJ/mol) to cocaine (PA = 930 kJ/mol) is thermodynamically feasible. We observed this process to be pH independent since comparable ion intensity was obtained in the presence of neutral ammonium salts (NH_4Cl and NH_4HCO_3; data not shown). Aside from favorable thermochemistry, collisions occurring during droplet transfer in the atmospheric pressure interface can provide the energy needed to evaporate NH_3 (as suggested by other researchers). For example, proton affinity of cortisone (<788 kJ/mol) is much lower than ammonia and yet protonated cortisone was observed from 5% NH4OH solution. At the same time, we did not detect any ammonium adducts even when using zero capillary and tube lens voltages (at ambient / elevated temperatures and with / without nebulizing gas). This suggests that CID in a de-clustering interface is not only the mechanism for NH_3 evaporation. Instead, we believe ammonia evaporates spontaneously. The evaporation process is facilitated by reduced solubility of NH_3 in organic solvents compare to water and by rapid evaporation of these solvents during charged droplet formation.

Stability Studies

We also investigated whether the MCX-base cartridge can serve as an effective storage medium for stabilizing labile analytes under ambient conditions without low-temperature storage. Such capability will ease shipping requirements and allow access to remote locations where resources may be limited. For this, we tested stability of cocaine and diazepam in dry serum for a period of >50 weeks (>1 year) at room temperature. Cocaine and diazepam are well known hydrolytically labile compounds that degrade quickly (within days)[23,46] when stored in the dry form at room temperature. As shown in **Figure 5A**, stable signal was recorded from cocaine stored in dry serum for first 16 weeks of storage. More than 57% of cocaine was still detected intact in the MCX-based SPE cartridge after more than year (i.e., 53 weeks) of storage in ambient air. Stability results obtained from diazepam is as illustrated in **Figure 5B**, which shows diazepam to be stable for the entire 55 weeks of storage in the dry state. This excellent stability exhibited by these hydrolytically labile analytes can be explained by the significant reduction of oxidative stress due to immediate trapping of a biofluid in SPE sorbent. This minimizes access of oxygen, moisture, and sunlight. Although, microsampling with MCX-based SPE cartridge may be more expensive compared with filter paper,[24] the significant stabilization effect achieved for such small sample volumes (<20 µL), combined with the high speed, sensitivity, accuracy, and precision with which the stored samples can be analyzed directly from the SPE cartridge make the proposed approach useful and competitive for various biomedical and pharmacological research applications. Note: this excellent stability was achieved for complex biofluid matrixes in SPE sorbents without any washing steps; if washing procedures will be applied, we are expecting even better stability for tested compounds.

Direct Biofluid Analysis

Recent developments in ambient mass spectrometry put emphasis on direct analysis of complex mixtures.[47-50] As a collection medium, we investigated the possibility of analyzing the collected biofluid without prior cleaning/washing steps. Such capability will further reduce analysis time for our SPE-ESI-MS workflow down to 3 min. This will be useful for high throughput qualitative screening of samples collected from a large population. We chose to use polymeric cation-exchange (PCX), which has affinity for a wider range of chemical species (basic, acidic, and neutral analytes). To test this possibility, analytes of different chemical structures [cocaine, benzoylecgonine (MW 289 Da), methamphetamine (MW 149 Da), and

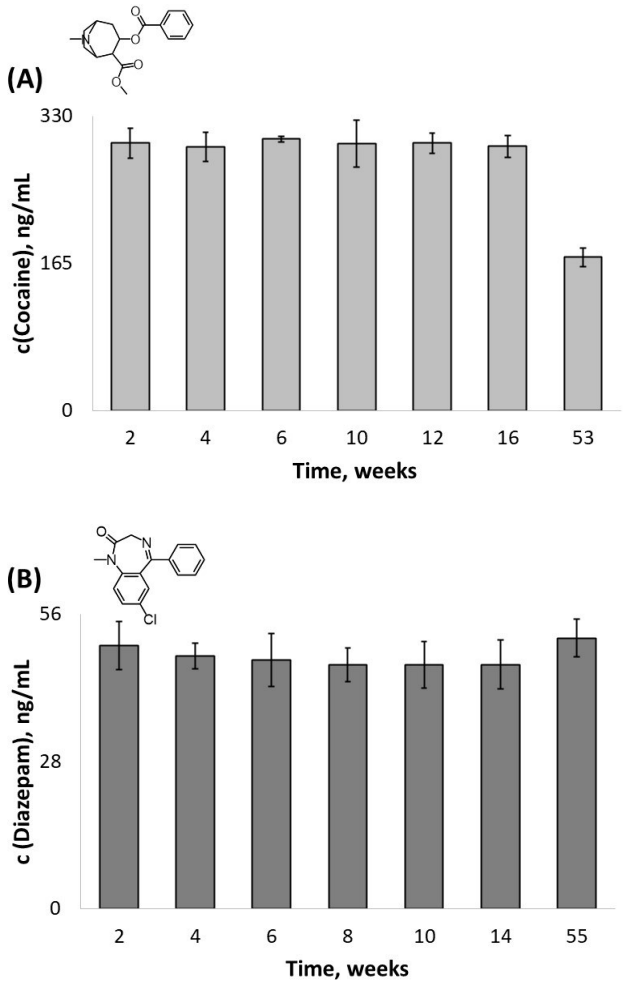

Figure 5. Stability study for (**A**) 300 ng/mL cocaine and (**B**) 50 ng/mL diazepam stored in MCX SPE cartridge for a period >50 weeks. 20 µL serum was used for each analyte and errors bars represent standard deviation of 3 replicates analysis for each analyte.

delta-9-tetrahydrocannabinol (Δ^9-THC; MW 314 Da)] were spiked into a serum sample, which was subsequently placed on the PCX-based SPE sorbent that has been packed into ESI syringe. **Figure S10** demonstrates the possibility of direct analysis of these structurally different compounds when the untreated microsampling SPE-syringe device (no condition, no equilibration, no acid addition, no washing) was subjected to ESI-MS analysis using 200 µL of acetonitrile as elution/spray solvent at 50 µL/min flowrate. As a result, all four drugs were successfully detected in full MS mode. Note: different elution solvents can be applied sequentially in the SPE cartridge to elute different analyte types for improved sensitivity.

CONCLUSIONS

In conclusion, a new syringe-based solid-phase extraction device is described that was created and optimized for effective sampling, storage, shipment, and direct MS analysis of different biofluids. Solid-phase extraction approach was applied for *in-situ* reduction of sample complexity and matrix effect and the stabilization of labile analytes (e.g., cocaine and diazepam) collected and stored on the SPE cartridge for more than a year.

Different types of SPE sorbents were successfully packed and applied for microsampling. The specific type SPE sorbent is expected to offer different mechanisms for retaining analytes of different physico-chemical properties (polarity, acidity/basicity, size, etc.) and thus affording more efficient enrichment effects. This SPE-ESI-MS(/MS) technique is highly sensitive, robust, and fast. It also enables long term stability (at least several months) for small volumes of biofluids. The results obtained from this proof-of-concept study provides confidence to apply this concept in clinical trials for samples generated across the globe to facilitate sample sharing between different analytical laboratories and for disease diagnosis in asymptomatic people. The reliability, simplicity, and ease of operation make this method very attractive, competitive, and promising for different other areas of microsampling and complex mixture analysis.

ASSOCIATED CONTENT

The Supporting Information is available free of charge at http://pubs.acs.org.

Sample collection and MS analysis by microsampling PCX cartridge; determination of cocaine in blood and serum; cocaine detection in plasma; diazepam detection in serum; analysis of cocaine / benzoylcgonine mixture in biofluids; flowrate optimization; determination of maximum spray time; reanalysis by microsampling SPE cartridge; mechanistic studies for "wrong-way-round" phenomena; biofluid analysis by PCX sorbent.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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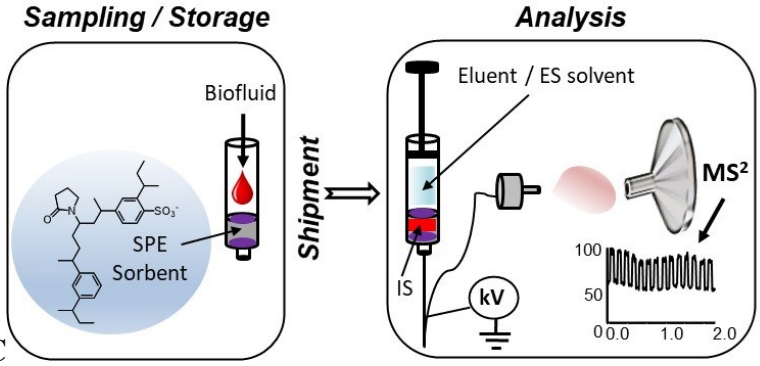
TOC


Sampling / Storage
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