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Matrix solid-phase dispersion on column clean-up/pre-concentration as a novel approach for fast isolation of abuse drugs from human hair

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ABSTRACT

A simple and fast sample pre-treatment method based on matrix solid-phase dispersion (MSPD) for isolating cocaine, benzoylecgonine (BZE), codeine, morphine and 6-monoacethylmorphine (6-MAM) from human hair has been developed. The MSPD approach consisted of using alumina (1.80 g) as a dispersing agent and 0.6 M hydrochloric acid (4 mL) as an extracting solvent. For a fixed hair sample mass of 0.050 g, the alumina mass to sample mass ratio obtained was 36. A previously conditioned Oasis HLB cartridge (2 mL methanol, plus 2 mL ultrapure water, plus 1 mL of 0.2 M/0.2 M sodium hydroxide/boric acid buffer solution at pH 9.2) was attached to the end of the MSPD syringe for on column clean-up of the hydrochloric acid extract and for transferring the target compounds to a suitable solvent for gas chromatography (GC) analysis. Therefore, the adsorbed analytes were directly eluted from the Oasis HLB cartridges with 2 mL of 2% acetic acid in methanol before concentration by N_2 stream evaporation and dry extract derivatization with N-methyl-tert-butylsilyltrifluoroacetamide (BSTFA) and chlorotrimethylsilane (TMCS). The optimization/evaluation of all the factors affecting the MSPD and on column clean-up procedures has led to a fast sample treatment, and analytes extraction and pre-concentration can be finished in approximately 30 min. The developed method has been applied to eight hair samples from poli-drug abusers and measured analyte concentrations have been found to be statistically similar (95% confidence interval) to those obtained after a conventional enzymatic hydrolysis method (Pronase E).

1. Introduction

Among different human materials used for toxicological and forensic analysis, hair offers the advantage of a substantially longer detection window (months to years) which enable retrospective investigation of chronic consumption. In addition, hair is a durable and stable matrix in which toxic substances are pre-concentrated and remain for a long time without significant alterations. Therefore, hair analysis for assessing drugs is a well established and recommended methodology in the forensic field. As reviewed by Pragst and Balikova [1], and by Hansen [2], there are numerous applications of hair analysis for assessing abuse drugs, and standardized hair testing approaches and official guidelines [3] are available for those laboratories dealing with toxicological studies.

Hair is considered a non-homogenous fiber with a complex structure [4] which determines the selective incorporation of certain compounds. In addition to the melanin content of the hair, which is one of the key factors controlling drug incorporation, the lipophilicity and the basicity of the drug also plays an important role [5]. This key factor affects the passive diffusion of drugs from blood capillaries into the growing cells, and uncharged (lipophilic) organic molecules penetration and diffusion in matrix cells is favored. However, it must be said that drug incorporation into the hair follows a multi-compartment model [5] and other important drugs incorporation ways into the hair such as absorption from sweat or sebum secretions, and also from deep skin compartments during hair shaft contribute significantly to the drugs incorporation into hair [6,7]. Other drugs, such as hydrophilic substances (molecules or ions), can reach the matrix cells after protonation (basic compounds) or deprotonation (acid compounds) [1,5]. Therefore, drug incorporation is a function of the pK_a of the compound and its melanin affinity [8,9], and it is facilitated at lower pHs of the matrix cells for basic drugs incorporation, and at higher pHs of the matrix cells for retaining acid drugs [10].

Because drug incorporation in hair is dependent on the lipophilicity and basicity of the drug, there are several extraction methods which are focused for isolating certain groups of substances from hair. Reviews on this topic show numerous extraction

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treatments mainly based on methanol, aqueous acids or buffer solutions as extracting solvents, alkaline digestions with aqueous sodium hydroxide, or enzymatic digestions (hydrolysis) [1,11,12]. Methods based on acidic hydrolysis have been reported to offer high yields for cocaine, opiates and their metabolites [13], although enzymatic hydrolysis are also recommended, mainly because the moderate pH and temperature conditions inherent to these procedures [14–19]. In addition, the possibility of speeding up the enzymatic hydrolysis procedures by using ultrasound irradiation has offered important practical advantages for shortening the whole analytical procedure [20]. Most of these sample treatment methods require a clean-up procedure for removing co-extracted substances present in the extracts. This is quite important mainly when using GC–MS as an analytical technique [21], and although several liquid–liquid and solid-phase extraction (SPE) procedures are available [1,21], the over-all procedure is long and difficult when analyzing large number of samples.

Other reported extractions for drugs are those based on supercritical fluid extraction (SFE) [22–24]. Although SFE is a quite expensive technique [1], it main advantage is the possibility of simultaneous extraction and clean-up stages [25,26]. This advantage is also offered by other modern extraction techniques such as pressurized liquid extraction (PLE) [25,26], sub-critical water extraction (SWE) or pressurized hot water extraction (PHWE) [27], and matrix solid-phase dispersion (MSPD) [28,29]. Among these extractive procedures, MSPD is simpler and cheaper because specific equipment is not required. In addition, this sample preparation procedure allows for reducing of solvent consumption, exclusion of sample component degradation, and improvement of extraction efficiency. Since the introduction of MSPD by Barker et al. [30], this technique has been used for extracting numerous organic compounds in quite different samples [28,29]. MSPD consists of sample architecture disruption by mechanical blending with a solid support bonded-phase [28,29,31], which leads after blending to a new sample matrix solid support phase in which analytes tend to be less strongly bonded. Therefore, analyte extraction can be easily performed by using less-toxic reagents/solvents (at low concentration and/or using low volumes), and under mild operating conditions (atmospheric pressure and room temperature). Therefore, integrity of target compounds is enhanced, and the procedure can be considered as an environmentally friendly method.

Although MSPD has been largely used for isolating numerous organic compounds [28,29,32], including organometallic species [33,34], the application for extracting drugs from forensic materials such as hair has not been tested yet. The objective of the current work has been the novel application of MSPD for extracting basic abuse drugs (cocaine, BZE, codeine, morphine and 6-MAM) from human hair samples. A clean-up procedure based on SPE was on column interfaced with the MSPD procedure for a fast abuse drugs isolation, clean-up and pre-concentration before GC–MS measurement. Variables affecting the MSPD process were fully studied by application of an experimental design approach.

2. Experimental

2.1. Apparatus

GC–MS analysis was performed with a Hewlett-Packard Model 6890 gas chromatograph (Hewlett-Packard, Avondale, PA), equipped with a HP-5 capillary column (30 m 0.22 mm I.D., 0.33 μm film thickness of cross-linked 5% phenyl methyl silicone) and a HP 5973 mass spectrometer as a selective detector. A Nahita glass mortar (50 mL capacity) with a glass pestle (Auxilab S.L., Beriáin, Navarra, Spain) was used for sample dispersion. Dispersed sample were packaged in 10 mL Injekt plastic syringes (Braun, Melsungen, Germany), between 10 mL polyethylene frits (Supelco, Bellefonte, PA, USA), and elution was forced by using a Visiprep TM DL vacuum manifold from Supelco (Bellefonte, USA). Other pieces of equipment were: 65 mm powder funnels from Barloworld Scientific (Stone, Staffs, UK), a Raypa UCI-150 ultrasonic cleaner bath (ultrasounds frequencies of 17 and 35 kHz and programmable for temperature and time) from R. Espinar S.L. (Barcelona, Spain), an ultracentrifuge Laborzentrifugen model 2K15 (Sigma, Osterode, Germany), an Orion 720A plus pH-meter with a glass–calomel electrode (Orion, Cambridge, UK), a Boxcult incubation camera (Selecta, Barcelona, Spain) coupled with an agitator Rotabit (Selecta), Univeba and Digiterm 3000542 thermostatic bathes (Selecta), a Reax 2000 mechanical stirrer (Heidolph, Kelheim, Germany), a VLM EC1 metal block thermostat and N_2 sample concentrator from VLM (Leopoldshöhe-Greste, Germany), and Oasis HLB syringes (3 cm^3, 60 mg) and Oasis HLB cartridges (225 mg) from Waters (Milford, MA, USA). Chemometrics package was Statgraphics Plus V 5.0 for Windows, 1994–1999 (Manugistics Inc., Rockville, MD, USA).

2.2. Reagents

Ultrapure water of resistance 18 MΩ cm^{-1} was obtained from a Milli-Q purification device (Millipore Co., Bedford, MA, USA). Pronase E, acetonitrile (gradient grade), methanol (gradient grade), 1,4-dithiothreitol (DTT), sodium hydroxide, potassium chloride, boric acid, acetic acid, chlorotrimethylsilane (TMCS) and N-methyl-tert-butylsilyltrifluoroacetamide (BSTFA) were from Merck (Poole, UK). Hydrochloric acid 37% was from Panreac (Barcelona, Spain). Ammonium hydroxide was from Scharlau (Barcelona, Spain). TRIS–hydroxymethyl-aminomethane (TRIS) was from Sigma–Aldrich (Stemheim, Switzerland). Diatomaceous earth, 95% SiO_2; C18 octadecyl-functionalized silica gel; and active magnesium silicate (Florisil), 60–100 mesh, used as dispersing agents, were from Aldrich Chemical Co. (Milwaukee, WI, USA). Alumina, aluminium oxide 90 active neutral (alumina N), 70–230 mesh (also used as a dispersing agent) was from Merck, while sea sand (washed) QP, SiO_2 was from Panreac (Barcelona, Spain). Drug stock standard solutions were prepared from cocaine, BZE, codeine, morphine and 6-MAM from Lipomed (Arlesheim, Switzerland). Deuterated drug stock standard solutions were prepared from cocaine-d_3 in acetonitrile, BZE-d_3 in methanol, codeine-d_3 in methanol, morphine-d_3 in methanol and 6-MAM-d_3 in methanol from Cerillant (Texas, USA).

2.3. Hair sample pre-treatment

Hair samples were obtained from poli-drug abusers from an addiction research centre in Santiago de Compostela. Hair about 2–3 cm long (approximately 0.5 g in weight) was cut with round-point scissors from the vertex posterior region of the scalp. To establish the limit of detection of the method drug-free scalp hair from laboratory staff volunteers was used.

All samples were decontaminated to remove residues of hair care products as well as sweat, sebum and dust typically present on hair, substances that can worsen the analytical noise/background ratio. In addition, the decontamination process also removes any drug potentially introduced through passive contamination [1]. Therefore, the decontamination procedure consisted of a mechanical stirring of hair in a diluted soap solution (physiological pH) for 30 min at room temperature, and finally, mechanical stirring with Milli-Q water several times. The successful removal of the external contamination of hair was proved through the negative result after the analysis of the last washing solution. The decontaminated hair samples were then oven dried at 40 °C for 24 h, and finally cut into small segments and pulverized in a vibrating zircon ball mill for 20 min. This last step ensures homogeneity of the sample. Pulverized hair specimens (mean particle size around 50 μm measured by

laser diffraction) were finally kept in either sealed glass or plastic bottles before analysis.

2.4. Matrix solid-phase dispersion (MSPD) on column solid-phase extraction (SPE) clean-up procedure

Hair samples (approximately 0.050 g) together with 25 μL of an internal standard solution containing 10 μg mL^{-1} of each deuterated analyte (cocaine-d_3, BZE-d_3, codeine-d_3, morphine-d_3, and 6-MAM-d_3) were blended thoroughly with 1.80 g of alumina (dispersing agent) in a glass mortar for 5 min using a glass pestle to obtain a homogeneous mixture. The mixture was quantitatively transferred by using a powder funnel to a 10 mL syringe containing a polyethylene frit, and after sample-dispersing agent mixture transfer a second polyethylene frit was placed at the top of the syringe. Finally, the mixture between the frits was slightly compressed with the syringe plunger for air removal and for avoiding preferential channels. A previously conditioned Oasis HLB cartridge (2 mL methanol, plus 2 mL ultrapure water, plus 1 mL of 0.2 M/0.2 M sodium hydroxide/boric acid buffer solution at pH 9.2) was then attached to the end of the MSPD syringe for on column analytes retention. In this way, the eluted target drugs isolated from hair matrix were on column adsorbed onto the solid support of the SPE cartridge. Elution with 4.0 mL of 0.6 M hydrochloric acid was forced by using a vacuum manifold. After extraction and SPE, the SPE cartridge was separated from the MSPD syringe, and it was rinsed by passing 2 mL of 95/5 Milli-Q water/methanol and 2 mL of 78/20/2 Milli-Q water/methanol/ammonium hydroxide, and then vacuum dried for 10 min. Finally, the adsorbed analytes were eluted with 2 mL of 2% acetic acid in methanol.

2.5. Derivatization procedure

The methanol extracts were evaporated under a stream of N_2 at 40 °C to dryness. The dry extract was then derivatized with 40 μL of BSTFA/TMCS 99/1 at 100 °C for 20 min, and kept at room temperature for 20 min before analysis.

2.6. Matrix solid-phase dispersion (MSPD) on column solid-phase extraction (SPE) clean-up procedure for assessing analytical performances

The MSPD procedure used when assessing analytical performances (calibration curves, intra-day precision, inter-day precision and analytical recovery) was similar to that shown in Sections 2.4 and 2.5 but, hair samples were previously spiked with the target analytes at different concentration levels. Therefore, for calibration curves, for inter-day precision, and intra-day precision experiments, approximately 0.050 g of hair samples were mixed with 25 μL of an internal standard solution (10 μg mL^{-1} of each deuterated analyte) and with 25 μL of different standard solutions containing 0.8, 3.2 or 32 μg mL^{-1} of each target analyte, which gave after blending with 1.80 g of alumina, elution with 4.0 mL of 0.6 M hydrochloric acid, on column SPE extraction and derivatization with 40 μL of BSTFA/TMCS, analyte concentrations of 0.5, 2.0, and 20.0 ng mg^{-1}, respectively. Similarly, analytical recovery studies were performed with hair samples (0.050 g) spiked with 25 μL of an internal standard solution (10 μg mL^{-1} of each deuterated analyte) and 25 μL of standards at 3.2 and 19.2 μg mL^{-1} of each target analyte, which gave analyte concentrations in the BSTFA/TMCS extract of 2.0 and 12 ng mg^{-1}, respectively.

2.7. Pronase E enzymatic hydrolysis procedure [35]

Pulverized hair samples (approximately 0.05 g) were weighted into centrifuge tubes and 500 μL of a DTT solution (12 mg mL^{-1} in TRIS/HCl buffer solution 0.1 M/0.1 M, pH 7.2) were added, and the mixture was then incubated at 40 °C for 2 h. Afterward, 500 μL of a solution containing 2.0 mg mL^{-1} of Pronase E in TRIS/HCl buffer solution 0.1 M/0.1 M (pH 7.2) was added and the mixtures were again incubated at 40 °C for 12 h. After ultracentrifugation at 3000 rpm for 15 min, the supernatant was spiked with 20 μL of an internal standard solution containing 10 μg mL^{-1} of each deuterated analyte (cocaine-d_3, BZE-d_3, codeine-d_3, morphine-d_3, and 6-MAM-d_3) before SPE clean-up (Oasis HLB syringes), evaporation to dryness and derivatization as described above.

Table 1
Retention times and qualifier and quantifier ions *m/z* for analytes and deuterated derivates.

Compound	Retention time (min)	Ion *m/z*
Cocaine	12.5	182[a], 198, 303
Cocaine-d_3	12.5	185[a], 201, 306
BZE[b]	13.2	240[a], 82, 361
BZE-d_3[b]	13.2	243[a], 85, 364
Codeine[b]	15.1	178[a], 371, 234
Codeine-d_3[b]	15.1	181[a], 374, 237
Morphine[b]	15.7	429[a], 236, 414
Morphine-d_3[b]	15.7	432[a], 239, 417
6-MAM[b]	16.6	399[a], 287, 340
6-MAM-d_3[b]	16.6	402[a], 290, 343

[a] Quantifier ion.
[b] Analyte-TMS derivates.

2.8. Gas chromatography–mass spectrometry measurements

Cocaine, BZE, codeine, morphine and 6-MAM separation/determination was performed by GC–MS using the splitless injection mode (2 min) and an injection volume of 2 μL. The injection port was heated at 240 °C, while the temperature of the ion source was set at 300 °C. The column temperature program consisted of maintaining an initial temperature at 90 °C for 1 min, then raising it by 30 °C/min to reach 190 °C, and remaining at 190 °C for 1 min. The temperature was then increased to 260 °C at 8 °C/min and remained at this temperature during 5 min. Finally, oven temperature was increased to 290 °C and this temperature was maintained for 5 min to clean the column. The carrier gas was helium at a flow rate of 1 mL/min. The mass spectrometer uses electron impact ionization (70 eV). Compounds were identified by using the retention time and the relative abundance of three confirming ions with respect to the target. Quantitative data were obtained by selected ion monitoring (SIM) for each compound and internal standard. Retention times and ion currents at *m/z* for monitoring cocaine, BZE, codeine, morphine and 6-MAM, and the deuterated derivates are listed in Table 1. Deuterated derivates (concentration of 6.25 mg L^{-1} in the final BSTFA/TMCS extract) were used as internal standards. Calibrations have covered target compounds concentrations between 0 and 20 μg g^{-1}.

3. Results and discussion

3.1. Preliminary studies. Selection of the extracting solution (eluent) and the dispersing agent (solid support)

Due to the hydrophilic nature of methanol, this organic solvent is considered an useful extractant for isolating almost all drug substances from hair [1], especially when assisting the extraction with ultrasounds [36,37]. In addition, diluted hydrochloric acid solutions are also quite popular for extracting basic drugs such as opiates, cocaine and its metabolites, as well as for amphetamines and methadone [1,38]. The first experiments were performed to know the feasibility of methanol and diluted hydrochloric acid (0.1 M) as extracting solvents for MSPD when extracting basic drugs from hair. MSPD was obtained using C18 (2.0 g) as a dispersing agent,

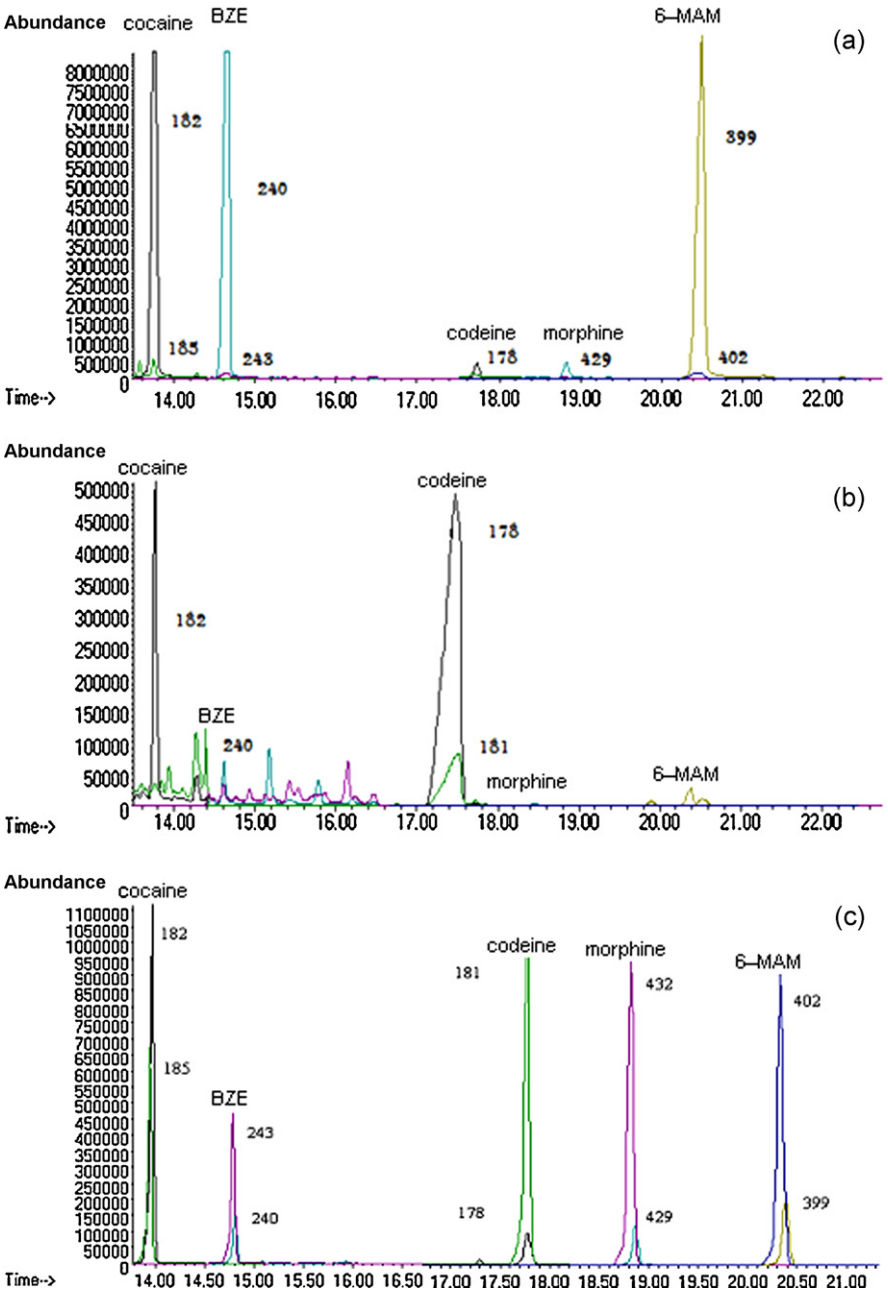

Fig. 1. Chromatograms for drugs separation after MSPD with C18 as a dispersing agent and 0.1 M hydrochloric acid (a) and methanol (b) as extracting solutions, and after conventional Pronase E hydrolysis (c): cocaine (12.5 min), BZE (13.2 min), codeine (15.1 min), morphine (15.7 min) and 6-MAM (16.6 min).

and 0.25 g of a pulverized hair sample that was positive for cocaine and opiates after a conventional enzymatic hydrolysis treatment [35]. The dispersing agent mass to sample mass ratio obtained was 8, and elution was performed by passing 5.0 mL of each extractant. After off-line SPE clean-up (Oasis HLB syringes, 60 mg) of the extracts, evaporation to dryness and derivatization, the chromatograms shown in Fig. 1 were obtained. Although cocaine and cocaine-d_3 should offer a similar behavior under these operating conditions, cocaine-d_3 (m/z of 185) was not totally recovered when eluting with hydrochloric acid as an extractant (Fig. 1(a)), and it was not extracted when using methanol (Fig. 1(b)). A further optimization of the experimental MSPD conditions, mainly the selection of alumina as a dispersing agent, will lead to quantitative recoveries for cocaine-d_3. In addition, BZE, morphine and 6-MAM, as well as their deuterated compounds, were only observed after hydrochloric acid elution. Codeine (m/z of 178) and codeine-d_3 (m/z of 181) were observed in both cases, but chromatographic signals after MSPD with methanol were broader (Fig. 1(b)) and quite higher than those expected for codeine concentration found in this sample after conventional Pronase E hydrolysis (Fig. 1(c)). This can be attributed to a high impurity level in the extract, even after a conventional clean-up procedure based on SPE. Therefore, the chromatographic signal at m/z of 178 can be the contribution of different compounds extracted with methanol, which offer similar m/z ratios to that given by codeine. Thus, the use of methanol was rejected, and diluted hydrochloric acid was selected for further experiments.

A screening experiment was then performed to select the proper solid support (dispersing material). Different polar materials for normal-phase MSPD were tested, including silica-based solid supports, commonly used in most of the MSPD applications [29,31]; such as bonded octadecyl-bonded silica (C18), and non-retentive supporting materials [32] (diatomaceous earth, –DE–, and sea

sand). Alumina N (aluminium oxide active neutral), used when assessing polar compounds such as sulfonamides [39], and certain pesticides [40], and Florisil (synthetic magnesia-silica gel) [33], a material that has became to be popular for normal-phase MSPD in recent years [32], were also considered. For each dispersing agent, MSPD was performed in triplicate with 0.10 g of a pulverized hair sample and 2.0 g of supporting agent (dispersing mass/sample mass ratio of 20), and eluting with 5.0 mL of hydrochloric acid 0.1 M. A blank was also prepared for each case. Fig. 2(a and b) shows that matrix dispersion with alumina gives high extracted concentrations for all target substances, concentrations that are quite similar to those obtained after an enzymatic hydrolysis treatment [35]. High extraction efficiencies, except for morphine, were also obtained when using silica-based materials (C18, sea sand and diatomaceous earth). In addition, recovered concentrations of morphine-d_3 were low when using silica based dispersing agents. Therefore, silica-based supports clearly show affinity for retaining morphine. The affinity of silica-based supports for retaining morphine (two –OH groups in its structure) can be explained by taking into account that the underivatized silanols on the surface of silica-based solid supports can interact with –OH groups by forming hydrogen bonding [41]. Regarding Florisil, low concentrations for all drugs were obtained. These low recoveries were to be expected because this material is commonly used to retain polar compounds [42]. Because alumina has offered adequate retention and subsequent elution properties with diluted hydrochloric acid for all target compounds, this material was selected as a dispersing support for further studies.

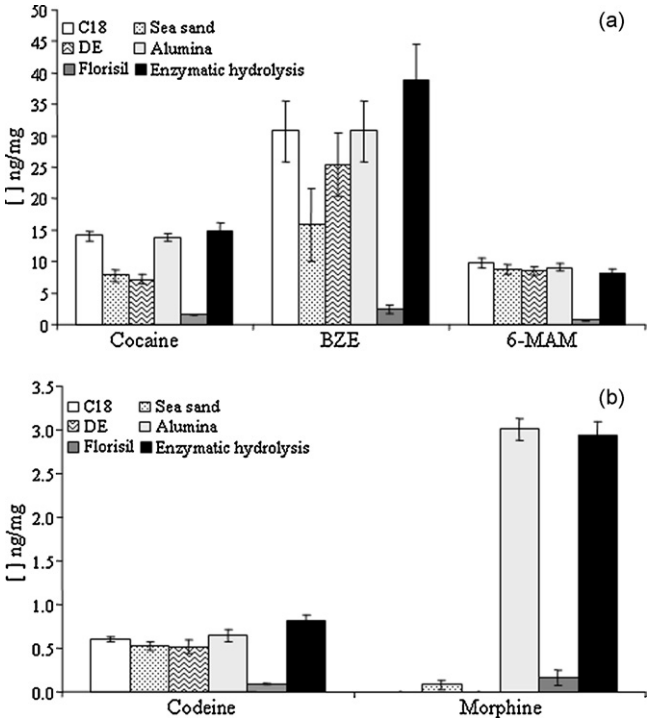

Fig. 2. Concentrations ($n = 3$) of cocaine, BZE and 6-MAM (a), and codeine and morphine (b) after MSPD with different dispersing agents: 0.1 M hydrochloric acid solution was used as an extractant, and off-line SPE clean-up/pre-concentration procedure.

3.2. Multivariate optimization of alumina based-MSPD for extracting basic drugs from hair

An orthogonal 2^3 + star central composite design (CCD) with 6 error degree of freedom, 2 centers, 2 replicates and 16 runs (Table 2)

Table 2
2^3 + star orthogonal central composite design.

Run	A/H ratio	V(HCl) mL	Drugs concentrations (ng mg^{-1})					
			[HCl] M	Cocaine	BZE	Codeine	Morphine	6-MAM
1	30	8.6	0.30	4.23	9.84	0.0474	0.0626	3.58
2	30	6.0	0.30	18.9	58.5	0.593	1.57	15.7
3	40	8.0	0.10	10.9	44.6	0.432	4.97	13.6
4	40	8.0	0.50	11.5	47.1	0.702	3.70	14.0
5	40	4.0	0.10	29.0	91.6	0.804	4.83	22.9
6	17.1	6.0	0.30	6.96	15.1	0.193	0.101	5.32
7	40	4.0	0.50	31.2	89.6	0.948	5.48	25.0
8	30	6.0	0.30	17.0	51.4	0.527	2.39	14.6
9	20	8.0	0.50	7.75	12.8	0.133	0.0950	3.28
10	20	8.0	0.10	5.44	12.5	0.131	0.0287	5.84
11	30	6.0	0.56	7.20	17.1	0.210	0.143	4.23
12	30	6.0	0.043	5.22	15.2	0.136	0.196	3.73
13	42.9	6.0	0.30	5.53	13.0	0.182	0.204	5.05
14	30	3.4	0.30	10.4	25.8	0.406	0.0716	9.01
15	20	4.0	0.50	10.1	24.9	0.241	0.276	6.38
16	20	4.0	0.10	9.45	24.4	0.262	0.594	12.2
17	30	8.6	0.30	4.57	10.4	0.0368	0.0430	3.54
18	30	6.0	0.30	17.8	50.4	0.496	1.54	13.2
19	40	8.0	0.10	12.1	45.8	0.401	6.09	12.5
20	40	8.0	0.50	10.2	49.4	0.630	3.32	14.6
21	40	4.0	0.10	30.8	93.2	0.867	4.12	24.1
22	17.1	6.0	0.30	6.97	14.5	0.192	0.0930	5.29
23	40	4.0	0.50	34.5	93.0	1.16	6.16	25.6
24	30	6.0	0.30	17.5	51.6	0.494	1.04	13.0
25	20	8.0	0.50	7.49	13.4	0.136	0.0788	3.31
26	20	8.0	0.10	5.84	13.1	0.124	0.0421	6.22
27	30	6.0	0.56	8.58	15.6	0.240	0.115	4.84
28	30	6.0	0.043	5.48	15.6	0.138	0.199	3.84
29	42.9	6.0	0.30	6.34	14.5	0.201	0.172	5.71
30	30	3.4	0.30	11.0	27.6	0.414	0.0722	9.06
31	20	4.0	0.50	11.2	26.8	0.278	0.170	6.98
32	20	4.0	0.10	9.39	24.8	0.278	0.744	12.1

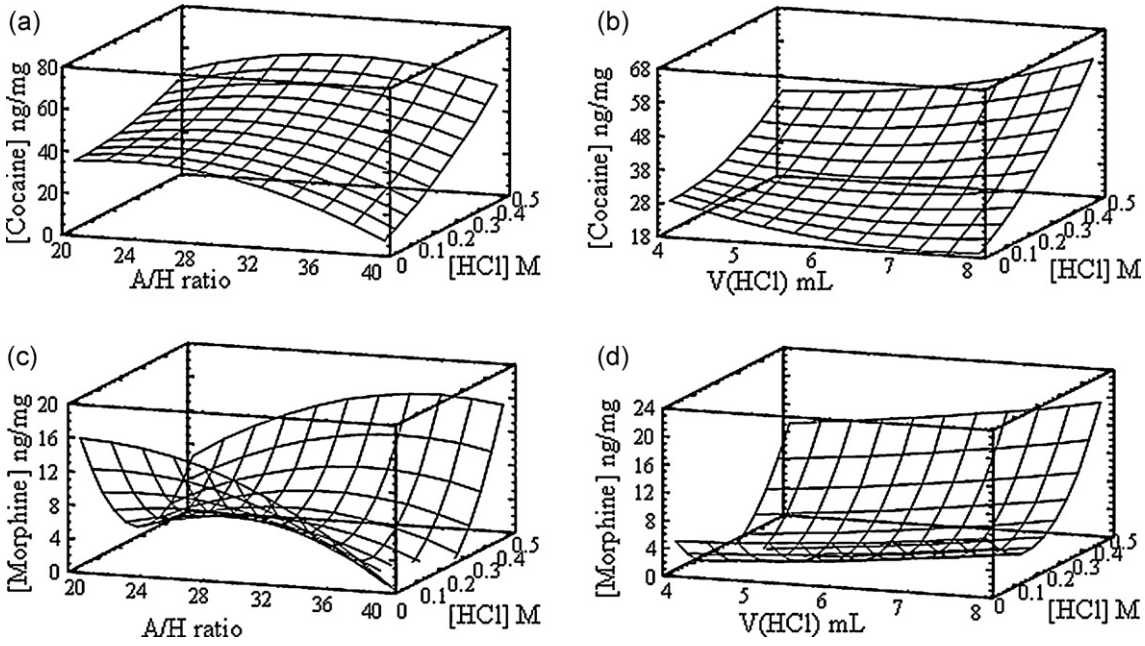

Fig. 3. Estimated response surfaces from the central composite design: cocaine (a and b) and morphine (c and d).

was built for optimizing variables inherent to alumina based-MSPD: alumina mass to hair mass ratio (A/H ratio), hydrochloric acid volume (V), and hydrochloric acid concentration ([HCl]). For a fixed hair mass of 0.050 g, A/H ratio was studied using 20 and 40 as low and high cube values, respectively, which implies dispersion with alumina masses of 1.0 and 2.0 g, respectively. According to the results shown above, an A/H ratio of 20 offers adequate dispersion as well as high recovered concentrations for almost all tested drugs. The definition field for the variable hydrochloric acid volume was 4.0 mL as low value and 8.0 mL as high value. Therefore, the selected range includes the value of 5.0 mL, which offered good results in the preliminary studies. Finally, as most of the acid extraction for basic drugs involving a hydrochloric acid solution as an extractant used acid concentrations up to 0.5 M [1], hydrochloric acid concentration was studied within the 0.1–0.5 M range. The experiments from the CCD together with the drugs concentration found after each set of conditions are shown in Table 2. Quadratic terms after statistical evaluation were not statistically significant for extracting any drug, which implies that the three variables under study are independent factors. Pareto charts (not given) have shown that hydrochloric acid concentration was the most statistically significant variable (95% confidence level), offering higher recoveries when using higher hydrochloric acid concentrations. The careful study of response surfaces (some of them for cocaine and morphine given in Fig. 3) has led to compromise conditions of 0.577 M for hydrochloric acid concentration (the star value from CCD), 4.0 mL for hydrochloric acid volume (the lowest volume), and 36 for A/H ratio (1.8 g of alumina and 0.050 g of hair). As the highest concentrations were found when extracting with 0.577 M (approximately 0.6 M) hydrochloric acid, an univariate optimization within the 0.6–1.0 M range was then performed. Similar cocaine, BZE, codeine, morphine and 6-MAM concentrations were obtained for all hydrochloric acid concentrations tested, and a value of 0.6 M was finally assigned to this variable. This value is similar to those reported for hydrochloric acid concentrations when extracting basic drugs from hair, ultrasounds-assisted methods included [1]. Although partial acid hydrolysis of some basic drugs (especially cocaine and 6-MAM) can occur when extracting with solutions at high hydrochloric acid concentrations [1], experiments performed with hair samples spiked with these target compounds have not shown hydrolysis (degradation) of cocaine to BZE or 6-MAM to morphine.

3.3. On column clean-up procedure

MSPD offers as an advantage the possibility of performing extraction and clean-up operations in a single step [26]. Our objective has been to attach sequentially the MSPD extraction and the clean-up—analytes pre-concentration based on an SPE procedure. Thus, previously conditioned SPE cartridges were attached to the MSPD syringes, and results from the MSPD extraction on column SPE clean-up/pre-concentration (conditioned Oasis HBL cartridges containing 225 mg of SPE support) were compared to those obtained after MSPD or ultrasounds-assisted Pronase E hydrolysis off-line SPE (conditioned Oasis HBL syringes containing 60 mg of SPE support). After performing experiments in triplicate (Table 3), similar concentrations were found for cocaine, BZE, and codeine, although morphine and 6-MAM offered slightly higher values. This may be attributed to the support mass in SPE cartridges (225 mg), which is higher than the support mass in SPE syringes (60 mg). Therefore, conditioned SPE cartridges were attached to MSPD syringes, and analytes retention onto the SPE cartridges was on column performed to the MSPD procedure.

Table 3
Concentrations ($n = 3$) of the target compounds ($\mu g\,g^{-1}$) in a hair sample from a poli-drug abuser after ultrasounds-assisted Pronase E enzymatic hydrolysis off-line SPE, MSPD off-line SPE and MSPD on column SPE.

	Ultrasound-assisted Pronase E hydrolysis and off-line SPE (syringes, 60 mg) [18]	MSPD off-line SPE (syringes, 60 mg)	MSPD on column SPE (cartridges, 225 mg)
Cocaine	8.92 ± 0.382	9.36 ± 0.424	9.40 ± 0.406
BZE	21.7 ± 0.725	22.0 ± 1.11	22.3 ± 0.647
Codeine	0.229 ± 0.00544	0.245 ± 0.00495	0.257 ± 0.00419
Morphine	1.04 ± 0.0499	1.28 ± 0.0557	1.43 ± 0.0606
6-MAM	9.63 ± 0.168	10.2 ± 0.144	10.5 ± 0.260

Table 4
Intra-day precision, inter-day precision and analytical recovery of the method.

Added concentration ($ng\,mg^{-1}$)	RSD (%)[a]	RSD (%)[b]	Added concentration ($ng\,mg^{-1}$)	Analytical recovery (%)[c]
Cocaine				
0.50	9	9		
2.00	7	6	2.00	94 ± 3
20.0	5	5	12.0	98 ± 3
BZE				
0.50	5	5		
2.00	3	2	2.00	92 ± 3
20.0	2	1	12.0	98 ± 3
Codeine				
0.50	12	10		
2.00	5	8	2.00	87 ± 6
20.0	2	7	12.0	93 ± 4
Morphine				
0.50	13	9		
2.00	6	5	2.00	95 ± 4
20.0	2	3	12.0	97 ± 3
6-MAM				
0.50	7	6		
2.00	3	4	2.00	93 ± 8
20.0	1	2	12.0	99 ± 6

[a] Intra-day precision ($n = 5$).
[b] Inter-day precision ($n = 6$); (c) $n = 3$.

3.4. Analytical performances

Six different calibration curves performed in 6 different days were obtained by fortifying aliquots of 0.050 g of drug-free hair with standard mixtures and deuterated derivates (internal standards) at increasing concentration (covered drugs concentrations of 0, 0.5, 1.0, 2.0, 4.0, 12.0 and 20.0 $\mu g\,g^{-1}$). The mixtures were then subjected to the optimized MSPD on column clean-up and derivatization procedure described above. Results for the slopes of calibration graphs, expressed as mean ± standard deviation, were 0.274 ± 0.032, 0.184 ± 0.005, 0.273 ± 0.028, 0.250 ± 0.002, and 0.166 ± 0.029 for cocaine, BZE, codeine, morphine and 6-MAM, respectively. These results show good repeatability of the calibration curves.

The limits of detection (LODs) and the limits of quantification (LOQs), based on the $3\sigma/10\sigma$ criterion [43], were calculated by analyzing eleven replicates of a blank (MSPD hydrochloric extract from a drug-free hair). LODs obtained, expressed as $\mu g\,g^{-1}$, were 0.04, 0.04, 0.02, 0.04 and 0.05 for cocaine, BZE, codeine, morphine and 6-MAM, respectively; while, LOQs were 0.13, 0.13, 0.07, 0.013, and 0.17 $\mu g\,g^{-1}$ for cocaine, BZE, codeine, morphine and 6-MAM, respectively. It can be seen that these values are low enough to apply the method to human hair from poli-drugs abusers.

Within-run precision (intra-day precision) was assessed in quintuplicate for MSPD hydrochloric extracts spiked with 0.5, 2.0 and 20 $\mu g\,L^{-1}$ of each drug. Table 4 shows the relative standard deviation (RSD); good intra-day precision has been obtained for all cases. In addition, the relative error was calculated taking into account the spiked concentration and the found concentration after analysis. Results expressed as percentages, also given in Table 4, show low intra-day relative error.

The inter-day precision and the inter-day relative error, performed in 6 different days, were also assessed for MSPD hydrochloric acid extracts spiked with 0.5, 1.0, 2.0, 4.0, and 20 $\mu g\,L^{-1}$ of each drug. Results (Table 4), expressed as RSD (%) and relative error percentage, show good values for both inter-day precision and inter-day relative error. Finally, the repeatability of the over-all procedure was assessed by subjecting eleven times a hair sample from a poli-drug abuser to the optimized MSPD on column clean-up, derivatization and GC–MS procedure. RSD values of 7, 8, 7, 7, and 9% for cocaine, BZE, codeine, morphine and 6-MAM, respectively, were obtained (drug concentrations of 19.8, 48.7, 0.534, 2.86, and 22.5 $\mu g\,g^{-1}$ for cocaine, BZE, codeine, morphine and 6-MAM, respectively).

Finally, the analytical recovery of the over-all procedure was also studied after subjecting 0.050 g of drug-free hair sub-samples spiked with low (2.0 $\mu g\,g^{-1}$) and high level (12.0 $\mu g\,g^{-1}$) of each drug following the procedure described in Section 2.6. Table 4 lists the calculated analytical recoveries for each drug at the two fortification levels and after three independent MSPD, clean-up and GC–MS determinations, and after applying the following equation:

$$\text{Analytical recovery} = \frac{[\text{drug}]_{\text{found}}}{[\text{drug}]_{\text{added}}} \times 100$$

where $[\text{drug}]_{\text{found}}$ is the found analyte (drug) concentration in the spiked hair sample, and $[\text{drug}]_{\text{added}}$ is the spiked analyte (drug) concentration. As it can be seen, good analytical recovery (values within 87–99%) was obtained for all target compounds.

3.5. Applications

The developed MSPD on column SPE for clean-up and pre-concentration procedure was applied to eight hair samples from poli-drug abusers in triplicate. In addition, each hair sample was also subjected in triplicate to a conventional enzymatic hydrolysis process [33] (reference method). Table 5 lists the minimum and maximum concentrations for each drug after GC–MS determination. All cases were positive for cocaine, BZE, codeine, morphine and 6-MAM. A *t*-paired test (95% confidence level) was applied according to the equation

$$t_{\text{calculated}} = \frac{\bar{X}_d}{SD_d} \times \sqrt{N}$$

where $\bar{X}_d$ and SD_d are the mean and the standard deviation, respectively, of the differences of drug concentrations after MSPD procedure and after the reference method. As it can be seen in Table 5, calculated *t*-values for each drug are lower than the tabulated *t*-value of 2.365 (95% confidence level, seven degrees of freedom), which confirms that results after the proposed method are statistically similar to those obtained after conventional procedures.

Table 5
Minimum and maximum concentrations of cocaine, BZE, codeine, morphine and 6-MAM in eight human hair samples from poli-drug abusers after MSPD on column SPE clean-up/pre-concentration and after conventional Pronase E enzymatic hydrolysis; and $t_{\text{calculated}}$ after *t*-paired test (95% confidence level, $n - 1 = 8 - 1 = 7$ degrees of freedom).

	Concentrations of drug ($ng\,g^{-1}$)				
	Cocaine	BZE	Codeine	Morphine	6-MAM
MSPD	1.55–27.3	0.250–74.1	0.334–2.08	0.527–4.85	1.49–32.7
Enzymatic hydrolysis	1.71–27.5	0.303–73.0	0.290–1.95	0.571–4.60	1.36–33.4
$t_{\text{calculated}}$[a]	2.025	−0.828	0.0822	−0.881	1.637

[a] $t_{\text{calculated}}$ values after *t*-paired test (95% confidence level).

4. Conclusions

The feasibility of MSPD combined with an on column SPE for clean-up and pre-concentration stages has been demonstrated to be a successful methodology for extracting basic drugs (cocaine, BZE, codeine, morphine and 6-MAM) from human hair samples. Drugs isolation from hair as well as clean-up/analytes pre-concentration can be achieved in 30 min, which is a shorter analytical time than that needed for completing conventional ultrasounds assisted hydrochloric acid extractions or conventional enzymatic hydrolysis. Alumina has been found to be a useful dispersing agent, which offers adequate retention and elution capacities for the target drugs when using diluted hydrochloric acid as an extractant. The novel application of combined alumina based – MSPD and clean-up/analytes pre-concentration SPE for isolating basic drugs from human hair has offered good repeatability and high extraction yields (extracted target concentrations statistically similar to those obtained after applying conventional enzymatic hydrolysis methods). Therefore, this methodology is a promising trend that must be fully exploited in the forensic field to assess other recreational/abuse drugs.

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