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Optimization of a rapid microwave-assisted extraction method for the simultaneous determination of opiates, cocaine and their metabolites in human hair

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ABSTRACT

A rapid and cleanup-free microwave-assisted extraction (MAE) method is proposed for the simultaneous extraction of six illegal drugs of abuse – cocaine, benzoylecgonine (BZE), cocaethylene (CCE), morphine, 6-monoacethylmorphine (6AM) and codeine – from human hair samples. The analytes were determined using high performance liquid chromatography (HPLC) with photodiode array UV detection. The influence of several variables on the efficiency of the MAE procedure was investigated in detail by a multi-objective optimization approach based on a hybrid experimental design (17 experiments) and desirability functions. Six drugs were successfully extracted from human hair with recoveries close to 100% and good reproducibility (<3.6% RSD) under the optimal MAE conditions: 11 mL dichloromethane (DCM) extraction solvent, 60 °C extraction temperature, 9 min extraction time and 0.5 mL of methanol (MeOH) added to 50 mg of the hair sample in the extraction vessels. Limits of quantification of 0.2 ng mg^{-1} were found for the studied compounds. A comparison of sample preparation procedures, including MAE, enzymatic digestion and digestion by aqueous acids, was also conducted. The results indicated that the global behaviour of sample procedures provided similar satisfactory recoveries ranging from 86 to 100%. Indeed, the MAE procedure resulted in a reduction of extraction time by 100-fold and the elimination of cleanup steps. Slightly higher recoveries of morphine, 6AM, BZE and CCE, at 1 ng mg^{-1} concentration level and cocaine at 40 ng mg^{-1} concentration level, were achieved using MAE. Lastly, the proposed MAE method was applied to several human hair samples from multidrug abusers.

1. Introduction

Hair analysis for drugs of abuse is routinely used in many countries in forensic and clinical fields [1]. Hair is an advantageous sample because of its substantially longer detection window (months to years) enabling retrospective investigation of chronic and past consumption. In addition, the collection, storage and transport of hair samples are easier and less invasive than the collection of blood or urine samples [2–4]. Moreover, hair samples may be useful even in post-mortem cases where other bodily fluids are unavailable. The drugs have been selected according to their high consumption, found in our country in recent years. Sample preparation for the extraction of illicit drugs from hair typically involves extraction with any amount of organic solvents such as methanol [3,5–12], acid [1,4,10,13–18] or basic [7,9,10,19] digestion, or enzymatic digestion [7,9,10,20–23]. The major drawback of these methods is the length of time needed to achieve efficient recoveries. Extended methanol extraction (3–18 h) with ultrasonic bath can provide lower drug recoveries as compared to other procedures [5–8]. Digestion with sodium hydroxide, which completely dissolves the hair matrix, allows the solubilisation of all drugs. Unfortunately, under these conditions, 6AM, a unique proof of heroin abuse, is hydrolysed to morphine. Therefore, the alkaline procedure cannot be recommended for drugs that are not stable under these conditions [7,24]. The use of enzymes for hair analysis targets the destruction of the hair structure, thus promoting the release of the incorporated drugs to the digestion buffer. The hair is dissolved by incubating the sample with an enzyme at 40–60 °C temperature for 6–24 h. For this purpose several enzymes, including proteinase K and pronase E, have been used [7,9,22]. The disadvantages of the enzymatic digestion of hair have been considered given the fact that the resulting digest could, under certain conditions, denature the antibodies used for the preliminary detection of drugs by immunoassays [10]. Overnight digestion in an aqueous solution of 0.01–0.5 M HCl at 40–60 °C temperature or phosphate buffer, followed by post-cleanup using solid phase extraction columns is usually the extraction method of choice [4,7,24–27]. Thus, according to results from the third proficiency

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test organized by the Society of Hair Testing (SoHT), acid hydrolysis can offer higher yields for cocaine, opiates, and their metabolites than those obtained using enzymatic hydrolysis or methanol extraction [9].

More efficient environmentally friendly techniques for the rapid analytical extraction from solid matrices are supercritical fluids (SFE) [2,7,25,28] and microwave-assisted extraction (MAE). Compared with other traditional extraction techniques, MAE presents advantages such as the reduction of extraction time and solvent volume employed. It enables partial or total automation of the analytical process, decreases analyte loss, and increases personal safety [29–31]. Commercial microwave equipment with security systems and closed vessels have made MAE an analytical technique of interest and potential since these instruments enable the simultaneous extraction of drugs of abuse at a high pressure and temperature, facilitating rapid and selective analyte desorption from complex matrices such as human plasma and urine. Moreover, the extraction yields of the analytes are equivalent to those obtained with conventional methods [32,33].

Sensitive analytical technology for the targeted assay is necessary. One of the most widely used procedures – gas chromatography–mass spectrometry – requires prior derivatization of the non-volatile analytes [1,3,9,22,27,34,35]. Liquid chromatography [6–8,12–14,20] and capillary electrophoresis [36] combined with mass spectrometry have emerged as effective and useful approaches in this context, since they allow a large number of analytes to be separated with no derivatization requirements. Despite its advantages, LC–MS/MS remains limited to a small number of forensic toxicological laboratories because of its high cost [7]. The obtained results of drugs analysis by HPLC with photodiode array UV detection [33,37] were comparable to those provided by MS-based methods [38,39].

To the best of the authors' knowledge – at the time of preparation of this paper – no previously published peer review literature detailing the application of MAE for drugs determination in hair matrices was available. In this paper, a new method for extracting cocaine, CCE, BZE, 6AM, morphine and codeine from hair using microwave energy was developed. An experimental design with variations in time, volume of dichloromethane (DCM) extraction solvent, volume of methanol (MeOH) solvent modifier and temperature was employed to optimize the MAE conditions with minimal experimental effort [40–42]. The method presented here is advantageous in terms of total extraction time and handling steps, without the need of any supplemental cleanup. The optimal MAE process was compared with other conventional procedures and then the proposed method for determination of opiates, cocaine and metabolites in hair samples spiked with drugs was used in the validation process. Moreover, the method was applied for the determination of drugs in real-life hair samples from forensic cases involving several drugs of abuse and its robustness was evaluated in terms of recovery.

2. Experimental

2.1. Reagents

Standards of morphine, 6AM, cocaine, CCE and BZE were supplied by Cerilliant (Round Rock, TX, U.S.). Pronase E, dithiotreitol (DTT), sodium hydroxide, ammonium hydroxide (25%), hydrochloric acid (37%), potassium hydroxide, sodium hydrogen phosphate, potassium dihydrogen phosphate, boric acid, acetic acid, gradient-grade acetonitrile, methanol, chloroform, isopropanol and dichloromethane were purchased from Merck (Darmstadt, Germany). Tris(hydroxymethyl) aminomethane clorhydrate (Tris–HCl buffer) was from Scharlau (Barcelona, Spain). Purified water was obtained from a Milli-Q water system from Millipore (Le Mont-sur-Lausanne, Switzerland). Oasis HLB and MCX cartridges were from Waters® (Milford, MA).

2.2. Instruments and apparatus

Microwave-assisted extraction was performed with an ETHOS PLUS MPR300/12S from Milestone® (Agrigento, Italy) equipped with a solvent detector. The pressurized microwave oven was able to extract 12 samples simultaneously in PTFE-lined closed vessels under the same conditions (temperature and pressure), with simultaneous magnetic stirring of the sample and solvent inside.

The analyses of the extracts were performed on a Model 2695 liquid chromatograph from Waters® connected to a Model 996 diode array UV–vis detector (DAD). Data were processed with Millennium software 32® v. 3.05.01 for Windows 98. Samples were injected onto an XTerra® RP8 column (250 mm × 4.6 mm i.d., 5 μm particle size) supplied by Waters®. In order to optimize peak resolution in the chromatograms and achieve efficient separation of the analytes in a reasonably short time (20 min), elution was performed in the gradient mode, using a flow rate 0.8 mL min^{-1} and a mobile phase consisting of a mixture of acetonitrile (A) and 20 mM phosphate buffer (pH 6.5) (B) set at a variable gradient program: 0–5 min, 10% A and 90% B; 5–7 min, 15% A and 85% B; 7–10 min, 20% A and 80% B; 10–15 min, 35% A and 65% B; 15–22 min, 50% A and 50% B; 22–25 min, 10% A and 90% B. Sensitivity was optimized by using the wavelengths of maximal chromatographic response for the analytes (*viz.* 233 nm for cocaine, BZE and CCE; 285 nm for morphine, codeine and 6AM) [33].

2.3. Hair sample preparation

Real hair samples were collected from 46 people ranging in age from 17 to 63 years (23 men and 23 women). All of the subjects were screened positive for drug abuse. Drug-free control hair was taken from 10 volunteers who had never used drugs and spiked with a standard solution containing all the drugs before extraction. The hairs were collected from the vertex posterior area as close as possible to the scalp and submitted to an initial procedure of decontamination by washing three times in 5 mL of a 0.1% solution of neutral soap (Tween 80), for 10 min each wash, rinsing three times with 5 mL of distilled water to eliminate any external contamination. The last wash cycle was analysed to exclude external contamination. After drying at 40 °C, the hair was cut into 1-mm segments. Then, 50 mg were weighted into a PTFE-lined extraction vessel.

2.4. Microwave extraction procedure

The nature of solvent (DCM) and the effect of the presence of a polar modifier like MeOH were fixed according to preliminary experiments and the results of a previous study on the determination of eight drugs of abuse from plasma samples [32]. The other experimental conditions were established on the basis of the results from the optimization studies. In the optimized method, 0.5 mL of MeOH was added to 50 mg of hair sample in the extraction vessels, being in equilibrium with the matrix before the addition of 11 mL of DCM. Extraction was carried out in 9 min at 60 °C. Following extraction, the vessel contents were filtered and the organic solvent was removed for evaporation to dryness under a N_2 stream at 40 °C. Finally, the dry extract was reconstituted in 100 μL of mobile phase and a 20 μL aliquot was injected into the chromatographic system for analysis.

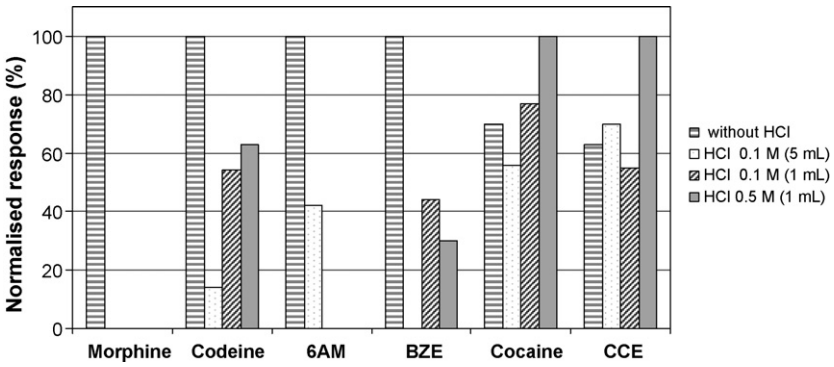

Fig. 1. Influence of hydrochloric acid presence on responses obtained for each drug by MAE.

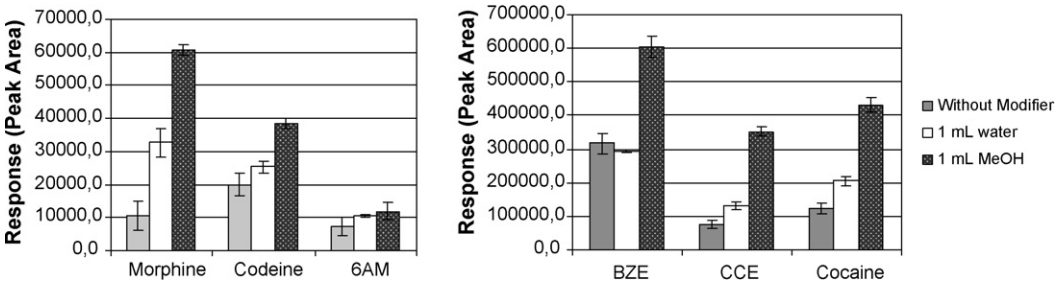

Fig. 2. Effect of polar modifier utilization on the extraction efficiency of MAE.

3. Results and discussion

3.1. Preliminary experiments

Preliminary studies were carried out in order to decide on the extraction solvent to be used, the type of modifier to be added to the sample before extraction, as well as to examine the effect of HCl on the MAE yields.

Three organic solvents were studied to select the ones that would be most suitable in this case. Chloroform, MeOH and DCM were tested, using MAE general conditions of 80 °C and 6 min. Owing to their electric characteristics, it was expected that the larger the dielectric constant and the dipole moment of the solvent, the more optimal the thermal energy would be [29]. However, the extracting selectivity and the ability of the medium to interact with microwaves can be modulated by the interactions of the matrix itself with microwaves. In this case, maximum efficiency was observed when DCM (dielectric constant, 8.9, and dipole moment, 1.16 Debye) was used as a solvent since all the drugs were quantitatively extracted. In contrast, they could not be extracted using MeOH (dielectric constant, 32.6, and dipole moment, 2.87 Debye) or chloroform (electric constant, 5.5, and dipole moment, 1.1 Debye).

The use of microwave energy for accelerating the acid hydrolysis of human hair for extracting illicit drugs was tested, using MAE general conditions (80 °C, 6 min and 10 mL DCM) by adding different volumes and concentrations of HCl in extraction vessels and evaluating the effect on MAE yields. The results ($n = 2$) were compared with those obtained by MAE without HCl (Fig. 1) and it was observed that several drugs were not extracted when acid hydrolysis was used simultaneously with MAE. In the case of MAE without HCl, microwave heating was sufficient for the disruption of weak hydrogen bounds promoted by the dipole rotation of the molecules [29], providing the best extraction responses.

Table 1
Experimental plan for hybrid design and responses obtained (areas) in each experiment for drugs.

Run	Temperature (°C)	Time (min)	DCM volume (mL)	MeOH volume (mL)	Responses (areas)					
					Morphine	Codeine	6-MAM	BEG	Cocaine	Cocaethylene
1	80	7	8	1.0	31,025	18,398	20,417	431,505	119,838	212,019
2	53	5	6	0.7	36,736	23,171	23,794	513,110	150,488	287,900
3	53	5	10	0.7	42,214	25,839	27,849	541,685	161,006	349,425
4	107	5	6	0.7	45,650	25,126	29,076	546,336	175,910	350,473
5	107	5	10	0.7	46,098	24,775	29,273	593,236	188,204	322,328
6	53	9	6	0.7	37,869	22,119	24,898	512,465	143,715	311,048
7	53	9	10	0.7	51,732	34,266	39,387	614,132	250,627	504,727
8	107	9	6	0.7	42,061	23,174	28,089	557,541	171,569	360,001
9	107	9	10	0.7	43,693	25,075	27,184	590,478	177,153	293,746
10	80	7	5	0.2	46,201	27,820	29,595	577,865	186,336	350,563
11	80	7	11	0.2	46,057	29,517	35,399	573,307	223,788	431,355
12	40	7	8	0.2	48,928	28,581	32,968	622,096	190,822	368,085
13	120	7	8	0.2	43,123	27,534	29,748	573,436	193,059	386,466
14	80	4	8	0.2	46,262	27,996	30,958	586,399	192,853	361,745
15	80	10	8	0.2	46,104	27,971	28,970	563,587	198,055	394,929
16	80	7	8	0.5	45,137	26,374	28,416	557,318	173,603	365,812
17	80	7	8	0.5	46,231	25,643	27,745	602,048	153,735	294,046

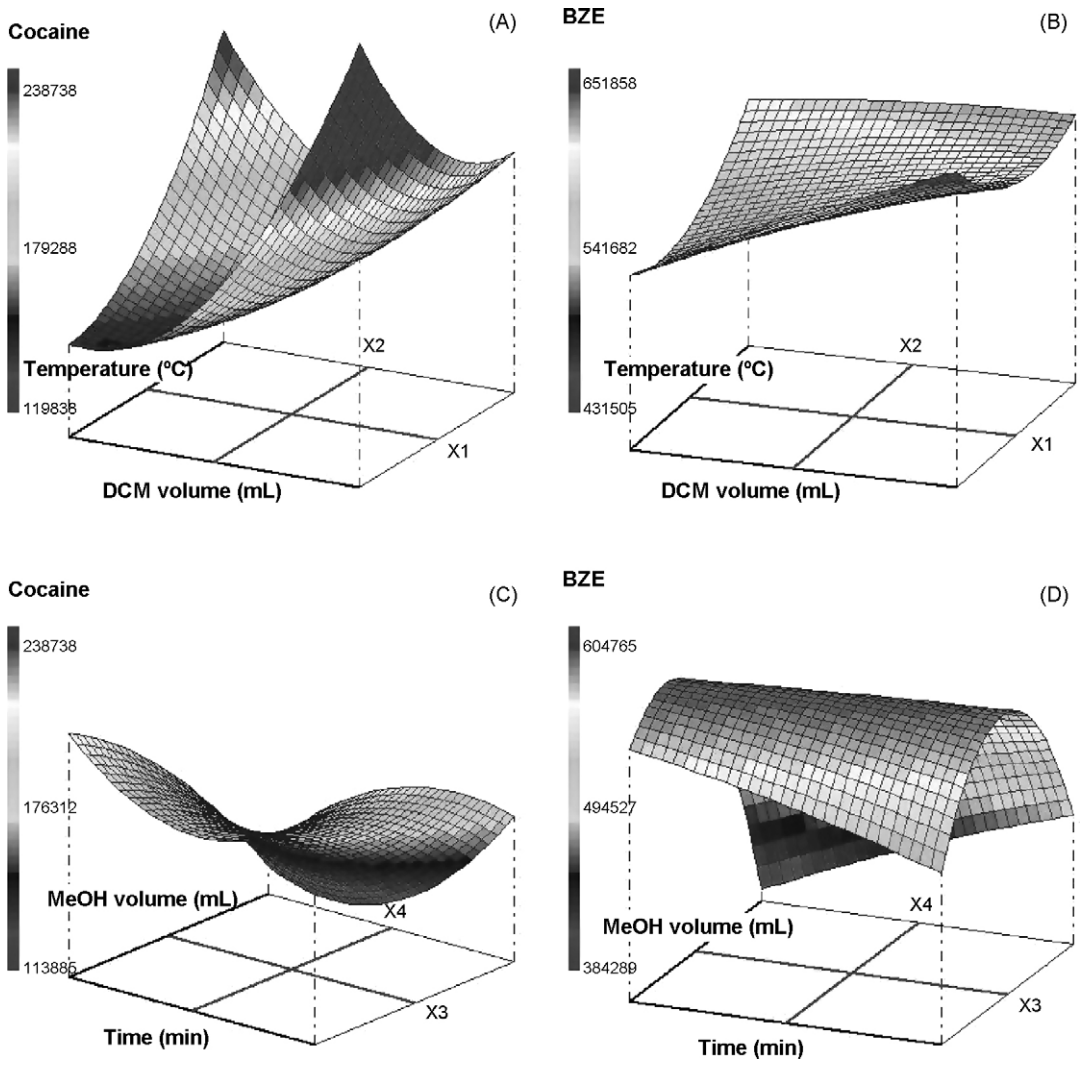

Fig. 3. Response surfaces of extraction efficiency (peak area) as function of temperature and DCM solvent volume for cocaine (A) and BZE (B) and as function of MeOH modifier volume and time for cocaine (C) and BZE (D).

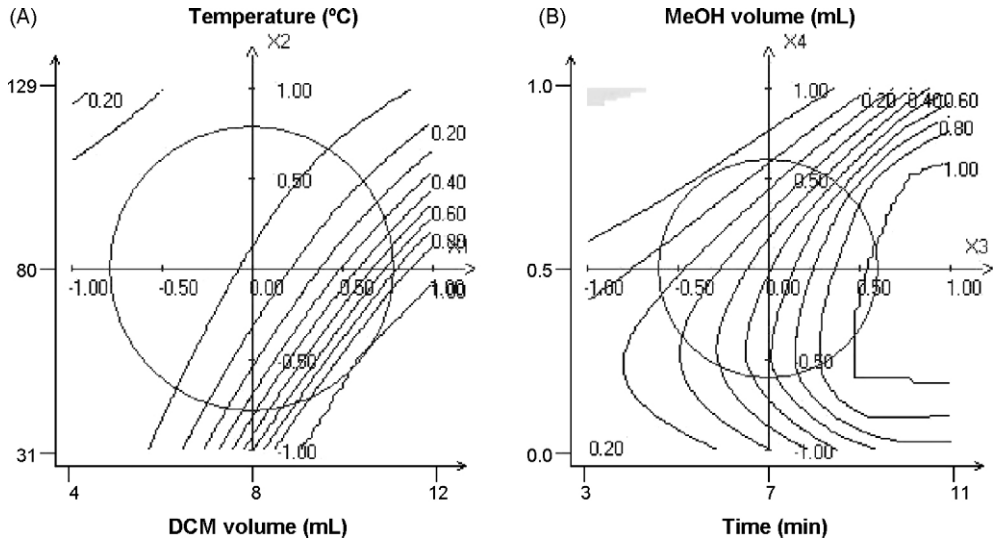

Fig. 4. Global desirability contour plots obtained in the optimization of MAE using a hybrid design.

Table 2
Comparison of the proposed method with conventional procedures: working conditions.

Enzymatic digestion (Ref. [18])	Acid digestion (Ref. [23])	Microwave-assisted extraction (this paper)
Temperature: 37 °C	*Temperature*: 60 °C	*Temperature*: 60 °C
Time: 2 h + overnight	*Time*: 12 h	*Time*: 9 min
Reagents: 500 μL of 12 mg mL^{-1} DTT and 500 μL of 2.0 mg mL^{-1} pronase E in Tris–HCl buffer 0.1 M, pH: 7.2	*Reagents*: 1 mL HCl 0.01 M, 1 mL NaOH 0.01 M, 1 mL phosphate buffer, pH: 7.0	*Reagents*: 0.5 mL MeOH as modifier and 11 mL DCM as extraction solvent
Ultracentrifugation: 5 min	*Ultracentrifugation*: 5 min	*Ultracentrifugation*: No
Cleanup: SPE (Oasis HLB) with 1 mL borax buffer, pH: 9.2	*Cleanup*: SPE (Oasis MCX)	*Cleanup*: No
Rinsed with 2 mL MeOH:water (5:95) and 2 mL NH_4OH:MeOH:water (20:2:78)	Rinsed with 2 mL water, 1 mL acetate buffer, pH: 4 and 2 mL MeOH	
Vacuum-dried for 20 min	Vacuum-dried for 20 min	
Elution: 2 mL 2% acetic acid in MeOH	Elution: 2 mL methylene chloride:isopropanol:ammonium hydroxide (80:18:2)	
Extract: Evaporated under a stream of N_2 at 40 °C to dryness	*Extract*: Evaporated under a stream of N_2 at 40 °C to dryness	*Extract*: Evaporated under a stream of N_2 at 40 °C to dryness

The suitability of MAE (at general conditions of 80 °C, 6 min and 10 mL DCM) with and without an added polar modifier prior to extraction of the studied drugs from human hair was assessed. We tested both water and MeOH as modifiers for MAE, and the results were compared to those reported for the extraction of hair with MAE in the absence of a polar modifier (Fig. 2). When MeOH was added to hair samples in the vessel before extraction, the presence of the modifier helped remove drugs from hair more easily than with unmodified MAE. Due to the responses obtained, further research work has focused on the use of MeOH.

3.2. Optimization of the MAE procedure

We used a hybrid design of a spherical domain to optimize the responses (analyte peak area) directly related to MAE. The design consisted of three factors with five levels and one factor with four levels: extraction temperature (40, 53, 80, 107 and 120 °C), extraction time (4, 5, 7, 9 and 10 min), DCM solvent volume (5, 6, 8, 10, and 11 mL) and MeOH volume added to the sample as a modifier (0.2, 0.5, 0.7 and 1 mL). The proposed experimental design (15 experiments + 2 central points) is shown in Table 1. NEMROD©W software was used for the generation and evaluation of the experimental design [43]. The three-dimensional response surface shows the effect of two independent variables on a given response, at a constant value (central value) of the other two independent variables. Thus, for cocaine (Fig. 3A), morphine and CCE, higher responses were obtained when temperature and DCM volume were at opposite levels, whereas for codeine, 6AM and BZE (Fig. 3B) extraction efficiency was favoured by high DCM volume and low temperature values. Similar opposing behaviours were observed when temperature and time factors were considered for morphine, BZE, cocaine and CCE. Also, better responses were obtained when time and MeOH volume were at their lowest levels for 6AM and cocaine (Fig. 3C). However, middle values of MeOH volume without a significant time effect, for morphine, codeine, BZE (Fig. 3D) and CCE produced higher results. The response contour plots serve as a preliminary graphical approach to optimization, but the use of multicriteria optimization based on the construction of a desirability function for each individual response provided the identification of the best-compromise conditions for the simultaneous extraction of drugs using MAE [44]. Each individual desirability function was chosen from a family of linear or exponential continuous functions, and ranged from zero (undesirable response) to one (optimal response). The overall desirability function (D) was estimated as the geometric average of the individual desirability functions (d_i) using NEMROD©W without additional experiments. In this case, MAE efficiency was maximised ($d_i = 1$ for the highest areas) and the D function acquires its maximum value of 1 (Fig. 4) under the following optimal conditions: 60 °C, 9 min, 11 mL of DCM and 0.5 mL of MeOH.

Table 3
Precision and accuracy for analysis of drugs in fortified hair. Comparison of sample preparation procedures.

Drug	Target concentration (ng mg^{-1})	Enzymatic digestion ($n = 5$)		Acid digestion ($n = 5$)		MAE ($n = 5$)	
		1.0	40.0	1.0	40.0	1.0	40.0
Morphine	Mean concentration (ng mg^{-1})	0.86	37.9	0.88	38.3	1.00	40.00
	% Recovery	86	95	88	96	100	100
	% RSD	6.6	4.3	1.8	5.2	1.8	0.8
Codeine	Mean concentration (ng mg^{-1})	0.89	39.0	0.91	36.1	1.01	39.98
	% Recovery	89	98	91	90	101	100
	% RSD	8.1	5.7	5.1	3.0	3.6	0.4
6AM	Mean concentration (ng mg^{-1})	0.87	40.3	0.92	37.6	0.99	40.00
	% Recovery	87	101	92	94	99	100
	% RSD	6.6	6.9	6.0	7.0	2.1	0.7
BZE	Mean concentration (ng mg^{-1})	0.89	36.3	0.97	39.6	0.99	40.00
	% Recovery	89	91	97	99	99	100
	% RSD	3.4	3.4	9.0	1.1	3.1	0.6
Cocaine	Mean concentration (ng mg^{-1})	0.96	36.0	1.02	35.3	1.00	40.00
	% Recovery	96	90	102	88	100	100
	% RSD	6.5	4.0	6.7	4.2	2.7	0.6
Cocaethylene	Mean concentration (ng mg^{-1})	0.87	35.8	0.86	35.2	1.00	40.00
	% Recovery	87	89	86	88	100	100
	% RSD	6.8	6.0	1.1	2.8	2.2	0.8

3.3. MAE-HPLC/DAD method validation

The analytes were identified from their retention times (*viz.* 6.35 min for morphine, 9.22 min for codeine, 10.53 min for BZE, 11.89 min for 6AM, 14.78 min for cocaine, 15.83 min for CCE) and absorption spectra. This gives the method a high specificity as it provides information about the purity of the corresponding peaks.

Linearity was assessed by injecting each sample five times over a concentration range of 0.5–40 ng mg^{-1}. Results obtained for the correlation coefficients (R^2) varied between 0.9992 and 0.9999. Quantification was based on the standard addition method using hair samples spiked with a multistandard mixture of a known concentration, in order to avoid matrix effects. Precision was investigated studying intra-day repeatability and inter-day reproducibility. Relative standard deviations (RSD), and accuracy (relative error), were evaluated at three concentration levels (1, 10 and 40 ng mg^{-1}) to the MAE procedure described above. The results showed low RSD, from 0.98 to 6.3% ($n = 5$) for intra-day precision and from 0.43 to 3.6% ($n = 5$) for inter-day precision. Intra-day relative errors were less than 7% in all cases while inter-day relative errors were less than 4%. Quantification limits (LOQs) were calculated for a S/N ratio of 10 [45]. The LOQs achieved were 0.2 ng mg^{-1} for all the drugs. These limits are in the range of ng mg^{-1}, suggested for these drugs in the recommendations for hair testing in forensic cases by the Society of Hair Testing [46].

3.4. Comparison between MAE and two digestion procedures

Acid and enzymatic preparations always show higher drug recoveries compared with methanol and ultrasonic extraction. Moreover, it is not possible to differentiate between the medical intake of codeine or morphine from heroin abuse by means of alkaline digestion. Therefore, the analytical precision and recovery of the MAE technique were studied in quintuplicate and compared with enzymatic digestion and acid digestion. The results were analysed by HPLC/DAD after subjecting 50 mg of drug-free hair samples spiked at two concentration levels for each drug to the three procedures described in Table 2. Data in Table 3 shows the relative effectiveness of the three extraction procedures applied. As shown, all the tested extraction procedures gave good analytical results, with recoveries ranging from 86 to 102%. The statistical comparison between the different extraction procedures exhibited a significant effect (95% confidence level) between-methods variance (ANOVA test, using Statgraphics Plus version 5.1 for windows (Manugistics, Inc., Rockville, MD, USA)). Enzymatic and acid digestion presented similar recoveries (within 86–102% range) and RSD (within 1.1–9% range) values, while MAE recoveries were more efficient (ranging between 99 and 101%) and precision values (ranging between 0.4 and 3.6%) were lower than conventional methods. According to the ANOVA, no significant effects were found in between-drug variance ($p = 0.45$) or between-concentration level ($p = 0.22$).

Also, a multifactor analysis of variance (ANOVA) was performed to compare the results obtained with enzymatic digestion, acid digestion and MAE from real hair samples of six forensic cases. Positive results were obtained only for the two most commonly found drugs or metabolites (BZE and cocaine) (Table 4). The absence of statistical significance (95% confidence level) for the factor extraction method was demonstrated. However, the results obtained for the analysed samples were statistically different ($p \leq 0.05$) depending on the origin of the forensic case. Thus, there were two homogeneous sample groups formed for the following samples: 1, 2, 3, 4 and 6; and sample 5 constituted a separate single group. On the other hand, the results obtained for the analysed drugs were also statistically different ($p \leq 0.05$) because the concentrations range (1.67–34.63 ng mg^{-1}) for cocaine, as expected, was higher than the range obtained for BZE (0.40–2.21 ng mg^{-1}).

Table 4
Concentration range of drugs in human hair samples ($n = 6$) from forensic cases obtained by different extraction methods. ANOVA analysis demonstrating the absence of statistical significance (95% confidence level) for the factor extraction method.

Procedure	Concentration range (ng mg^{-1})	
	BZE	Cocaine
Acid digestion	0.40–1.92	1.67–28.29
Enzymatic digestion	0.57–2.21	2.12–33.86
Microwave-assisted extraction	0.42–2.03	1.76–34.63
Factor	**ANOVA**	
	F-Ratio	*p*-Value
Extraction procedure	0.11	0.90
Drug	11.74	0.00
Sample	5.72	0.00

3.5. Application of the method to hair samples from multidrug abusers

The applicability of the MAE-HPLC/DAD proposed method was checked by analysing in duplicate $N = 46$ real hair samples from drug abusers who consumed different types of drugs under study (Table 5). The only drug detected in 35 samples was cocaine; heroin was found in only 2 cases and the 2 drugs combined in 9 cases. In most of the hair samples, positive for opiates the concentration of 6AM was higher (8 cases) than that of its metabolite, morphine (6 cases). Cocaine also tended to be found in higher concentrations than its two metabolites, BZE and CCE. The overwhelming presence of BZE, in 44 cases, may be attributed to the rapid biotransformation of cocaine, while the presence of CCE in 5 cases would suggest the

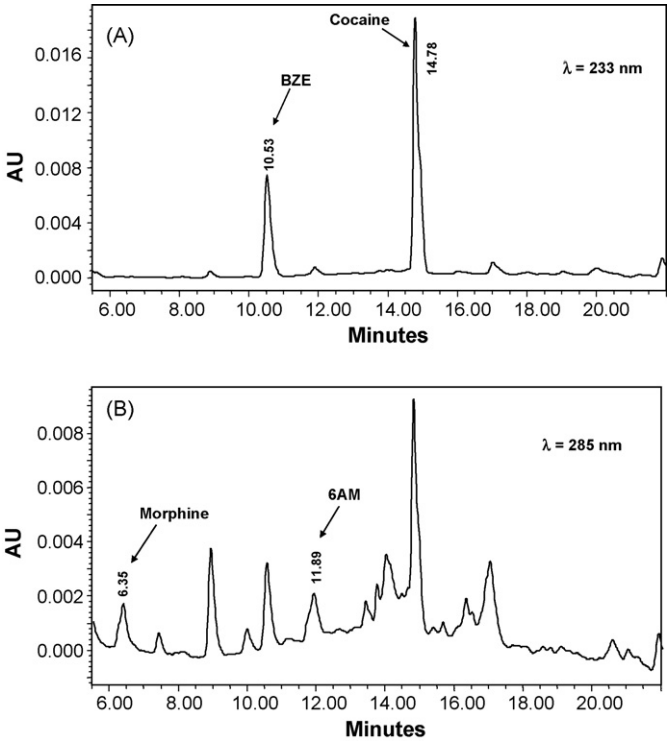

Fig. 5. Chromatograms for drug separation after MAE-HPLC/DAD method corresponding to a hair sample from consumer, monitorizing at λ = 233 nm (A) and at λ = 285 nm (B). MAE optimal conditions: 60 °C, 9 min, 11 mL DCM, 0.5 mL methanol.

Table 5
Analytical results from $N = 46$ real hair samples from multidrug abusers using the proposed MAE-HPLC-DAD method.

Case sample number	Concentration values (ng mg^{-1})					
	Morphine	Codeine	6AM	BZE	Cocaine	Cocaethylene
1	–	2.57	9.85	–	–	–
2	–	–	–	1.85	11.52	–
3	–	–	–	0.45	3.23	–
4	–	–	–	0.42[a]	1.76	–
5	–	–	–	7.95	34.63	–
6	–	–	–	0.57	2.97	–
7	–	–	–	2.03	6.43	–
8	–	–	–	6.44	29.32	–
9	–	–	–	17.29	56.25[b]	–
10	–	–	–	3.65	23.90	–
11	–	–	–	0.76	4.54	–
12	7.42	–	4.80	36.11	171.09[b]	–
13	–	–	–	1.51	7.41	–
14	–	–	–	13.18	157.94[b]	–
15	–	–	–	3.67	25.60	–
16	–	–	–	4.29	52.99[b]	–
17	–	–	–	4.03	48.71[b]	–
18	–	–	–	31.38	141.33[b]	–
19	–	–	–	1.93	4.79	–
20	–	–	–	16.07	64.49b	–
21	3.61	1.34	–	–	–	–
22	–	–	–	7.32	29.97	–
23	–	–	–	3.63	27.30	–
24	–	–	–	0.78	–	–
25	–	–	–	2.80	5.23	–
26	–	–	–	2.93	–	–
27	–	0.31	0.94	5.22	3.16	–
28	0.62	–	–	16.35	10.68	–
29	0.71	–	–	29.11	17.43	–
30	0.85	–	2.74	4.99	3.39	–
31	–	–	3.97	0.69	2.32	0.66
32	–	–	–	0.39[a]	–	0.41
33	–	–	–	0.36[a]	–	4.32
34	3.41	8.91	12.93	4.14	5.90	2.87
35	–	–	–	15.20	7.96	–
36	–	–	–	0.34[a]	–	–
37	–	–	–	0.57	–	–
38	–	–	–	0.51	–	–
39	–	–	1.58	7.10	8.89	–
40	–	–	–	4.59	5.04	–
41	–	–	–	17.48	8.30	–
42	–	–	–	0.77	2.92	<LOQ
43	–	–	–	1.15	–	<LOQ
44	–	–	–	0.39[a]	–	1.14
45	–	–	–	0.39[a]	1.27	–
46	–	–	7.50	45.83[b]	25.05	–

–: Not detected.
[a] Results obtained by MAE using 100 mg of hair sample.
[b] Results obtained by MAE using a dilution step.

combined use of cocaine and ethyl alcohol. These findings demonstrate that 6AM and BZE are good markers of the consumption of heroine and cocaine, respectively. Fig. 5 shows the two chromatograms obtained after the optimization of the MAE process for a hair sample from a drug user, determining cocaine (27.3 ng mg^{-1}), BZE (3.6 ng mg^{-1}) at $\lambda = 233$ nm, 6AM (7.4 ng mg^{-1}) and morphine (4.8 ng mg^{-1}) at $\lambda = 285$ nm.

4. Conclusions

This paper describes a simple and fast sample preparation method based on a microwave-assisted extraction with HPLC/DAD determination and has proved that it is an efficient and quantitative procedure for the simultaneous detection and analysis of six drugs of abuse in the hair of heroin and/or cocaine users.

Application of a hybrid design and desirability functions allowed the optimization of MAE parameters. The variables investigated were extraction temperature, extraction time, solvent volume and MeOH, as modifier, volume. The total time of analysis, which is now becoming one of the most important factors, was significantly decreased when microwave energy was applied. MAE is faster (9 min) than the conventional digestion procedures and besides, simultaneous extractions can be performed in a microwave oven while only sequential extractions can be conducted with conventional hydrolysis. With regard to the straightforward methodology used, the MAE technique is clearly better than traditional procedures since MAE does not require additional cleanup steps.

The use of DAD does not require previous derivatization and improves the method's selectivity by facilitating the selection of the optimal wavelength for the maximal chromatographic response of the analytes.

Although methods such LC–MS/MS do provide high selectivity and sensitivity, not all laboratories have access to these instruments. For this reason and on the basis of the levels usually found in real samples, the method proposed here is a good analytical tool that will be useful in determining the previous history of drug abuse

and it meets all the necessary requisites to be implemented in a clinical and/or forensic Toxicology Laboratory.

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