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Hollow-fiber liquid-phase microextraction of amphetamine-type stimulants in human hair samples

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ABSTRACT

A fast method was optimized and validated in order to quantify amphetamine-type stimulants (amphetamine, AMP; methamphetamine, MAMP; fenproporex, FPX; 3,4-methylenedioxymethamphetamine, MDMA; and 3,4-methylenedioxyamphetamine, MDA) in human hair samples. The method was based in an initial procedure of decontamination of hair samples (50 mg) with dichloromethane, followed by alkaline hydrolysis and extraction of the amphetamines using hollow-fiber liquid-phase micro extraction (HF-LPME) in the three-phase mode. Gas chromatography–mass spectrometry (GC–MS) was used for identification and quantification of the analytes. The LoQs obtained for all amphetamines (around 0.05 ng/mg) were below the cut-off value (0.2 ng/mg) established by the Society of Hair Testing (SoHT). The method showed to be simple and precise. The intra-day and inter-day precisions were within 10.6% and 11.4%, respectively, with the use of only two deuterated internal standards (AMP-d5 and MDMA-d5). By using the weighted least squares linear regression ($1/x^2$), the accuracy of the method was satisfied in the lower concentration levels (accuracy values better than 87%). Hair samples collected from six volunteers who reported regular use of amphetamines were submitted to the developed method. Drug detection was observed in all samples of the volunteers.

1. Introduction

Amphetamine-type stimulants (ATS) are a group of substances comprised of synthetic stimulants from the amphetamines-group (including amphetamine and methamphetamine) and the ecstasy-group substances (3,4-methylenedioxymethamphetamine-MDMA and its analogues) [1,2]. The United Nations Office on Drugs and Crime (UNODC) estimates that the global annual prevalence for ATS substances ranged between 0.3% and 1.3% of people aged 15–64 in 2009. For the ecstasy-group, annual prevalence was estimated at between 0.2% and 0.6% of the same age-range population [1].

ATS are most commonly swallowed, injected or smoked, but can also be snorted. Amphetamine is the prototype of this class of compounds with central and peripheral stimulant activity [3]. Methamphetamine (also known in the illicit market with the names "crystal meth," "speed," "ice") is the most widely manufactured ATS, with the United States of America reporting a large number of detected illicit laboratories [1]. Fenproporex was an anorectic drug highly prescribed in Brazil until its prohibition at the end of 2011. Nevertheless, it is still used illicitly as stimulant by some Brazilian truck drivers with the aim to maintain their extensive work schedule and stay awake [4]. MDMA is the main active component found in ecstasy pills although other analogue compounds (e.g., 3,4-methylenedioxyamphetamine-MDA) as well as other stimulants can also be present in these products [5].

In the last years, hair analysis has gained a lot of attention and become an important tool to detect therapeutic and illicit drug use or chronic exposure to environmental toxicants [6,7]. Some methods have been described in the scientific literature for the determination of amphetamines alone or in combination with other drugs of abuse in hair samples. Gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) are the main techniques used in the majority of these methods. Sample preparation techniques used in these chromatographic methods include solid-phase extraction (SPE) [8–10], liquid–liquid extraction (LLE) followed by SPE [11], small-volume liquid extraction [12], and micropulverized extraction [13–15]. Some other miniaturized methods such headspace solid-phase microextraction (HS-SPME) and triple phase suspended droplet microextraction (SD-LPME) have also been used in the development of new methods [16–18].

Hollow-fiber liquid-phase microextraction (HF-LPME), a relatively new miniaturized technique, has gained considerable interest in a broad field of the analytical area. Simplicity, rapidity, less sample manipulation, and low consumption of organic toxic solvents (low microliter range) are some advantages of its use over conventional extraction techniques. Especially in the

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three-phase mode, HF-LPME can provide a great enrichment and sample clean-up, reducing or eliminating potential problems from matrix components [19,20].

The aim of the present study was to develop a method for the determination of amphetamine-type stimulants (amphetamine, AMP; methamphetamine, MAMP; fenproporex, FPX; 3,4-methylenedioxymethamphetamine, MDMA and 3,4-methylenedioxyamphetamine, MDA) in human hair samples using three phase hollow fiber liquid phase microextraction (HF-LPME) and gas chromatography/mass spectrometry (GC–MS). Chemical structures of these amphetamine-type stimulants are shown in Fig. 1. To the best of our knowledge, this is the first study reporting the determination of amphetamines in hair samples by means of HF-LPME. The validated method was successfully applied to samples collected from six volunteers (in-patients of a rehabilitation clinic).

2. Experimental

2.1. Reagents and standards of reference

Amphetamine (1-phenyl-2-aminopropane), fenproporex ((±)-3-([α-methylphenethyl]amino)propionitrile), methamphet amine (1-phenyl-2-methylaminopropane), 3,4-methylenodioxy methamphetamine (N-α-dimethyl-1,3-benzodiozole-5-ethana mine), and 3,4-methylenedioxyamphetamine (α-methyl-1,3-benzodioxole-5-ethanamine) solutions (1 mg/mL) in methanol and the internal standards (amphetamine-d5 and 3,4-methylenodioxymethamphetamine-d5) were purchased from Cerilliant Analytical Reference Standards (Round Rock, TX, USA). Sodium hydroxide, hydrochloric acid, and sodium chloride were purchased from Merck (Darmstadt, Germany), trifluoroacetic anhydride (TFAA), and ethyl acetate was purchased from Sigma–Aldrich (MO, USA).

2.2. Preparation of standard solutions

Working solutions of AMP, MAMP, FPX, MDMA, and MDA and the internal standards (IS) AMP-d5 and MDMA-d5 at concentrations of 10 μg/mL and 1 μg/mL were prepared with methanol in volumetric glassware. Stock solutions were stored refrigerated (2–8 °C) when not in use.

2.3. Instrumentation

Hollow-fiber Q3/2 Accurel KM polypropylene (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany). GC–MS analyses for amphetamines were performed using a gas chromatograph model *Focus GC* coupled with an ion trap mass selective detector (MSD) model *Focus Polaris Q* (Fischer Scientific, Bremen, Germany). Chromatographic separation was achieved on a HP-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness) using helium as the carrier gas at 0.6 mL/min in a constant flow rate mode. Injections were made in the splitless mode. The MSD was operated by electronic impact (70 eV) in scan mode (70–300 *m*/*z*). The injector port and interface temperature was 270 °C. The oven temperature was maintained at 70 °C for 2 min, programmed at 10 °C/min with a hold at 190 °C for 1 min; programmed again at 20 °C/min with a hold at 270 °C for 1 min (run time 20 min). The acceptance criteria of qualification were: retention time within 2% compared with standards analyzed in the same batch, and the mass spectrum should have a good visual match to that of the standards. The following ions were chosen for quantification of the analytes: AMP (140); MAMP (154), FPX (193), MDMA (162), and MDA (162).

2.4. Head hair samples

Authentic head hair samples were obtained from six volunteers who reported regular use of amphetamines (more than three times a week). Hair samples were cut with scissors as near as possible to the scalp in the posterior vertex region. The subjects provided informed consent prior their participation in the study. The protocol of study was previously approved by the Faculty of Pharmaceutical Sciences Ethics Committee, University of Sao Paulo, Brazil (Ethics Protocol Approval no. CEP 550/10). Before the analyses, the specimens were stored under dry conditions at room

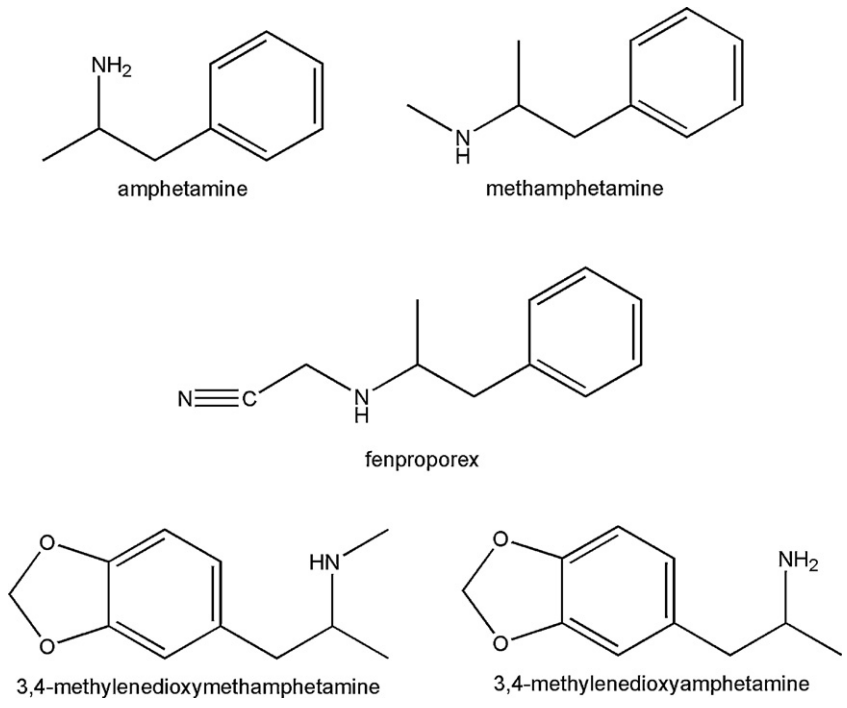

Fig. 1. Chemical structures of the amphetamine-type drugs in study.

temperature in paper envelopes. A negative control sample was obtained from a member of the laboratory staff.

2.5. Sample preparation

Hair samples were submitted to an initial procedure of decontamination by washing them twice with 2.0 mL of dichloromethane. The solvent was removed and the samples were dried and cut into small pieces of about 1 mm. An aliquot of 50 mg was submitted to alkaline hydrolysis (digestion) in a glass tube in the presence of 1.0 mL of NaOH 1.0 mol/L and 10 ng of each internal standard (AMP-d5 and MDMA-d5). The glass tube was tapped and maintained in the water bath at 70 °C for 15 min. After cooling down to room temperature, the content was transferred to an eppendorf tube (2 mL of capacity) contained 10 mg of NaCl. A 9-cm hollow fiber, filled-up with dihexyl ether in its pores, was used for extraction. The fiber lumen was filled with acceptor phase (15 μL of HCl 0.1 mol/L), and introduced into the sample solution in u-configuration. During extraction, the system was submitted to shaking of 1000 rpm for 45 min in an eppendorf mixer. After extraction, the acceptor phase was withdrawn from the fiber and dried under nitrogen stream. The residue was derivatized with 100 μL of TFAA: ethyl acetate (50:50) at 70 °C for 30 min. After cooling, the samples was dried once more (40 °C under N_2 stream) and resuspended with 50 μL of ethyl acetate. An aliquot of 1.0 μL of this solution was injected into the GC–MS system.

2.6. Optimization of the method

The study of optimization of the method was performed taking into consideration choice of organic phase, influence of acceptor phase, time for extraction, intensity of stirring/sonication, and addition of salt on the extraction yield. Fortified hair samples at a concentration of 2.0 ng/mg of each analyte were submitted to the method previously described. The efficiency of extraction was evaluated by the recovery values produced by each condition. The following parameters were studied: organic phase (dihexyl ether, xylol, and n-octanol); acceptor phase (phosphate buffer 0.1 mol/L pH 4.7; acetate buffer 0.1 mol/L pH 4.0; HCl 2 mol/L; HCl 1 mol/L; HCl 0.1 mol/L; and HCl 0.01 mol/L); time for extraction (15, 30, and 45 min); lateral shaking (1000 and 1400 rpm laterally), magnetic stirring (1000 rpm using a stirring bar), and ultrasonication for 5 and 10 min. The salting out effect was also tested by adding 10 mg of NaCl in the sample before extraction.

2.7. Validation of the method

After optimizing the method, validation took place by establishing limits of detection and quantification (LoD and LoQ), linearity, intra and inter-day precision, accuracy and recovery values as follows.

2.7.1. Limit of detection (LoD) and limit of quantification (LoQ)

The LoQ can be defined as the lowest concentration of a sample that can still be quantified with acceptable precision and accuracy. The acceptance criteria for these two parameters at LoQ were 20% (RSD) for precision and 20% for accuracy. The LoB (limit of blank) is an apparent concentration or a signal produced from blank samples. The LoD can be defined as the lowest concentration of analyte in a sample which can be reliably distinguished from the LoB and at which detectable is feasible [21,22].

2.7.2. Linearity

The study of linearity was estimated by the analyses of extracts obtained from aliquots of fortified hair, in six replicates, at the following concentrations for each analyte: 0.2; 3.0; 5.0; 7.0; and 10.0 ng/mg.

2.7.3. Precision and accuracy study

The precision and accuracy study was performed by analyzing human hair samples containing known concentrations of 1.0, 4.0, and 6.0 ng/mg of all analytes at three different and consecutive days. The analyses were performed in three replicates for each day. Precision, defined as the relative standard deviation (RSD), was determined by intra- and inter-day repetitions. Experimental concentrations were obtained using the standard calibration curves. Accuracy was expressed as a percentage of the known concentration, i.e., mean measured concentration/nominal concentration × 100.

2.7.4. Recovery

Recovery studies for AMP, MAMP, FPX, MDMA, and MDA were performed taking into consideration the possible loss of analytes during the entire procedure (degradation during hair digestion or incomplete extraction). Samples fortified with three different concentrations of amphetamines (1.0, 4.0, and 6.0 ng/mg) were analyzed according to the method previously described in five replicates (set A). The results were compared with another set of samples analyzed in five replicates for each concentration. However, for this set, the analytes were fortified to the sample immediately after the LPME procedure (set B). Absolute recovery was evaluated by comparison of the mean response obtained for the set A (processed) and the response of set B (unprocessed). The unprocessed response represented 100% recovery.

3. Results and discussion

3.1. Sample preparation

The alkaline hydrolysis of hair samples used in the present method is similar to other previously published methods [9,12]. This procedure showed to be appropriate since amphetamines are stable to heat and alkaline conditions (70 °C in NaOH 1.0 mol/L) and allowed the complete digestion of sample matrix, releasing the analytes to an aqueous solution. Afterwards, this solution could be immediately submitted to LPME for extraction of analytes because the polypropylene hollow fibers support several types of solvents and pH variations without loss of efficiency [23]. Because of the semi volatile characteristics of amphetamines, they have also been extracted from processed hair samples by using HS-SPME [17,18]. Although SPME is also a miniaturized technique that is simple and uses little or no organic solvent, it suffers from the comparatively expensive and fragile fiber with limited lifetime and the sample carry-over effect [24]. LPME, instead, eliminated the carry-over effect since the hollow fibers can be discarded after each extraction due to their low cost [25,26]. Despite the fact that LPME has a great potential for the analyses of complex biological matrices, it has been scarcely employed in forensic toxicology. Due to the simplicity of the extraction units, many samples can be processed at the same time providing a high sample throughput [20]. After the washing and digestion procedures, extractions of at least 30 samples could be finished in less than 1 h by only one analyst. The major disadvantage is the lack of automation of the process because it is a relatively new technique.

3.2. Optimization

For the optimization of the method, different organic solvents (dihexyl ether, xylol, and n-octanol) were tested for the LPME procedure. Only dihexyl ether provided measurable signals of the analytes and was chosen to be the supported liquid membrane

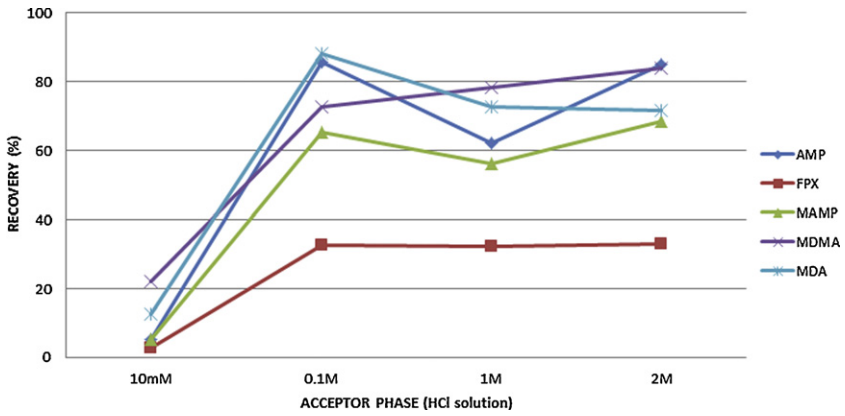

Fig. 2. Influence of the acceptor phase (HCl concentration) in the extraction yield. Other conditions of the experiment were fixed: time of extraction (45 min) and shaking (1000 rpm).

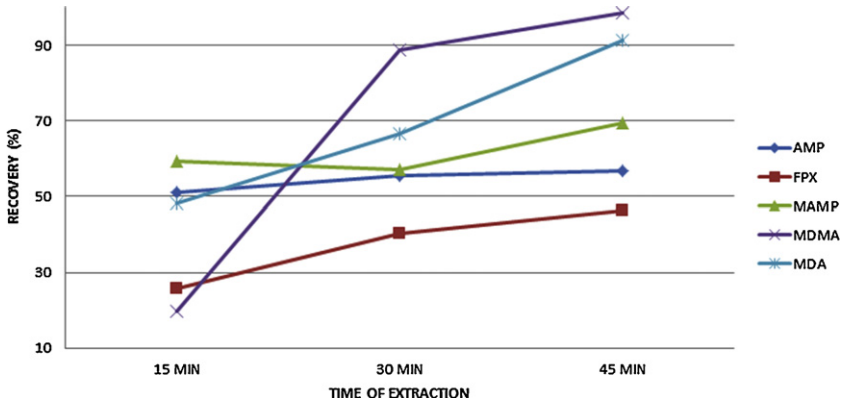

Fig. 3. Influence of the time of extraction in the response of the analytes. Other conditions of the experiment were fixed: acceptor phase (HCl 0.1 M) and shaking (1000 rpm).

in the pores of hollow fibers. In LPME, high partition coefficients are beneficial, and this can be achieved by proper selection of the organic solvent and the pH conditions of the aqueous solutions. For basic analytes as the ATS, pH in the sample should be high (preferably 3 units higher than the pK_a value of the analytes), whereas the acceptor phase should be acidic (preferably 3 units below the pK_a value of the analytes) [20]. The amphetamines analyzed in this study have pK_a values ranging from 9.41 to 9.90. Therefore, the conditions used to digest hair samples (NaOH 1 mol/L) did not demand any pH adjustments for further extraction by LPME.

As we can see in Fig. 2, there were no significant improvements in the recovery values when HCl solutions with higher concentrations than 0.1 mol/L were used as acceptor phase. With the use of HCL solution 0.01 mol/L the efficiency of extraction decreased abruptly. The response when phosphate or acetate buffer was used as acceptor phase showed to be poor and irregular and, because of this, it was not reported in the graph.

Extraction of samples at increased times led to an improvement of sensitivity. The highest time tested (45 min) provided an optimum condition to extract the analytes by HF-LPME (Fig. 3). For the different intensities and kinds of agitation (1000 and 1400 rpm lateral shaking, 1000 rpm magnetic stirring, and ultrasonication for 5 and 10 min), the most efficient was 1400 rpm lateral shaking for almost analytes in the study, with the exception of

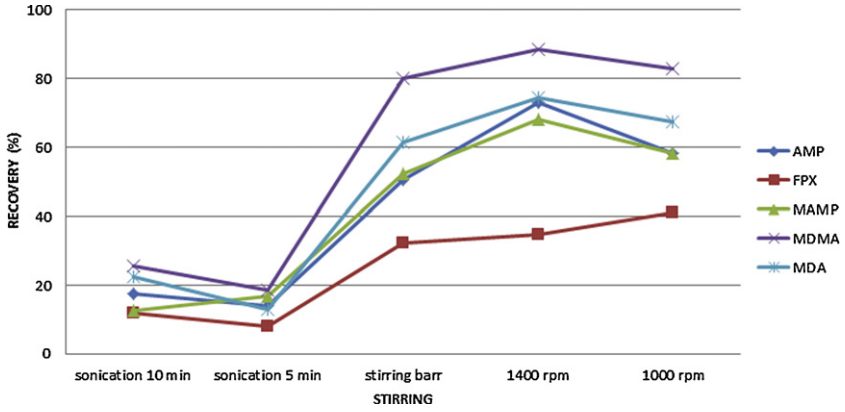

Fig. 4. Influence of stirring in the extraction yield. Other conditions of the experiment were fixed: time of extraction (45 min) and acceptor phase (HCl 0.1 M).

Table 1
Confidence parameters of the validated method for the determination of the amphetamine, methamphetamine, fenproporex, 3,4-methylenedioxymethamphetamine, and 3,4 methylenedioxyamphetamine in hair samples.

	AMP	MAMP	FPX	MDMA	MDA
Recovery (%)					
C1	56.2	51.4	38.3	77.0	61.8
C2	59.7	56.1	36.0	89.6	58.2
C3	64.2	51.7	40.5	78.8	77.6
LoD	0.02 ng/mg	0.01 ng/mg	0.04 ng/mg	0.01 ng/mg	0.02 ng/mg
LoQ	0.05 ng/mg	0.05 ng/mg	0.05 ng/mg	0.05 ng/mg	0.05 ng/mg
Intra-day precision (RSD%)					
C1	4.2	5.0	6.4	5.2	6.1
C2	2.9	5.3	8.6	10.6	7.7
C3	9.5	6.6	6.9	9.7	9.9
Inter-day precision (RSD%)					
C1	8.8	5.6	7.1	11.4	5.9
C2	6.1	9.2	6.1	8.5	6.6
C3	8.9	7.7	9.0	8.4	8.1
Accuracy (%)					
C1	91.1	87.0	88.5	91.6	88.1
C2	95.1	89.4	91.8	89.2	89.9
C3	96.6	90.1	97.9	94.9	96.0

AMP, amphetamine; MAMP, methamphetamine; FPX, fenproporex; MDMA, 3,4-methylenedioxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine; C1 = 1.0 ng/mg, C2 = 4.0 ng/mg; C3 = 6.0 ng/mg; LoD, limit of detection; LoQ, limit of quantification.

fenproporex (Fig. 4). However, 1000 rpm lateral shaking was chosen to compensate the relative low recovery obtained for this substance. These results can be explained by the mobilization of organic phase into aqueous solution (sample or acceptor phase) caused by ultrasonication. The use of magnetic bars could cause bubbles in the solution or irregular stirring and was abandoned in this study [26]. The addition of salt (10 mg of NaCl) to the sample solution did not cause a remarkable improvement in the efficiency of extraction. However, the precision improved with the use of salt and this condition was selected to the standardized method. The method provided in average an enrichment factor of 40.

3.3. Validation of the method

The confidence parameters of the validated method (LoD, LoQ, intra and interassay precision, accuracy, and recovery) for the determination of the analytes are shown in Table 1. After optimization of the extraction, the recovery values for the studied ATS ranged from 40.5 to 89.6%. These results were considered good since recoveries exceeding 80–90% are rare due to analyte trapping within the organic phase in the three-phase mode of LPME [20]. The LoD and LoQ obtained for all amphetamines were below the cut-off value (0.2 ng/mg) established by the Society of Hair Testing [27]. For quantification by HF-LPME and GC–MS, the use of deuterated labeled internal standard for each drug could be recommended to improve the precision of the method. In this work we used AMP-d5 as internal standard for the amphetamines group (amphetamine, fenproporex, and methamphetamine) and MDMA-d5 for the ecstasy-type group (MDMA and MDA). In spite of using only two deuterated analogues as internal standards, the proposed HF-LPME method for the determination of amphetamine-type drugs was shown to be precise (RSD < 15% for intra and inter-day precisions). This represented saving of deuterated standards and at same time made the procedure simpler. The use of only one deuterated standard (AMP-d5 or MDMA-d5) failed to fulfill the precision requirements for all analytes.

In the calibration curve range (from 0.2 to 10 ng/mg) the phenomenon of heteroscedasticity was presented (evaluated through the F distribution), probably due to the large range considered in the study of linearity. Therefore, ordinary least square linear regression methods could result in large errors in the calculation of the drugs concentrations especially in smallest values. By using weighted least squares linear regression the sum of percentage of relative error (%RE) over the whole range indicated "goodness of fit" in the evaluation of the effectiveness of the weighting factor used ($1/x^2$) [28]. Other empirical weights such as $1/x$; $1/x^{1/2}$; $1/y^{1/2}$; $1/y$; and $1/y^2$ were also evaluated. The weighted least squares linear regression equations and coefficients of correlation were: AMP: $y = 0.71116x + 0.09679$; $r^2 = 0.9979$; MAMP: $y = 0.75407x + 0.09579$; $r^2 = 0.9982$; FPX: $y = 0.54485x + 0.01420$; $r^2 = 0.9975$; MDMA: $y = 1.8266x + 0.09625$; $r^2 = 0.9977$; MDA:

Table 2
Results of the analysis of hair samples collected from six volunteers.

Patient	Sex	Hair color	Cosmetic treatment	Results			
01	M	Dark	N		AMP: 0.12 ng/mg FPX: <LoQ		
02	F	Brown	N		AMP: 0.78 ng/mg FPX: 0.21 ng/mg		
03	F	Yellow	Dyed	S1 AMP: 0.33 ng/mg FPX: 0.24 ng/mg	S2 AMP: 0.17 ng/mg FPX: <LoQ	S3 neg	S4 neg
04	F	Brown	N		AMP: 0.18 ng/mg		
05	M	Dark	N		AMP: 6.79 ng/mg		
06	M	Dark	N		AMP: 0.41 ng/mg		

AMP, amphetamine; FPX, fenproporex; S1, S2, S3, and S4, hair segments of the same volunteer equivalent to 0–3 cm, 3–6 cm, 6–9, and 9–12 cm, respectively. Hair segments were cut from hair root.

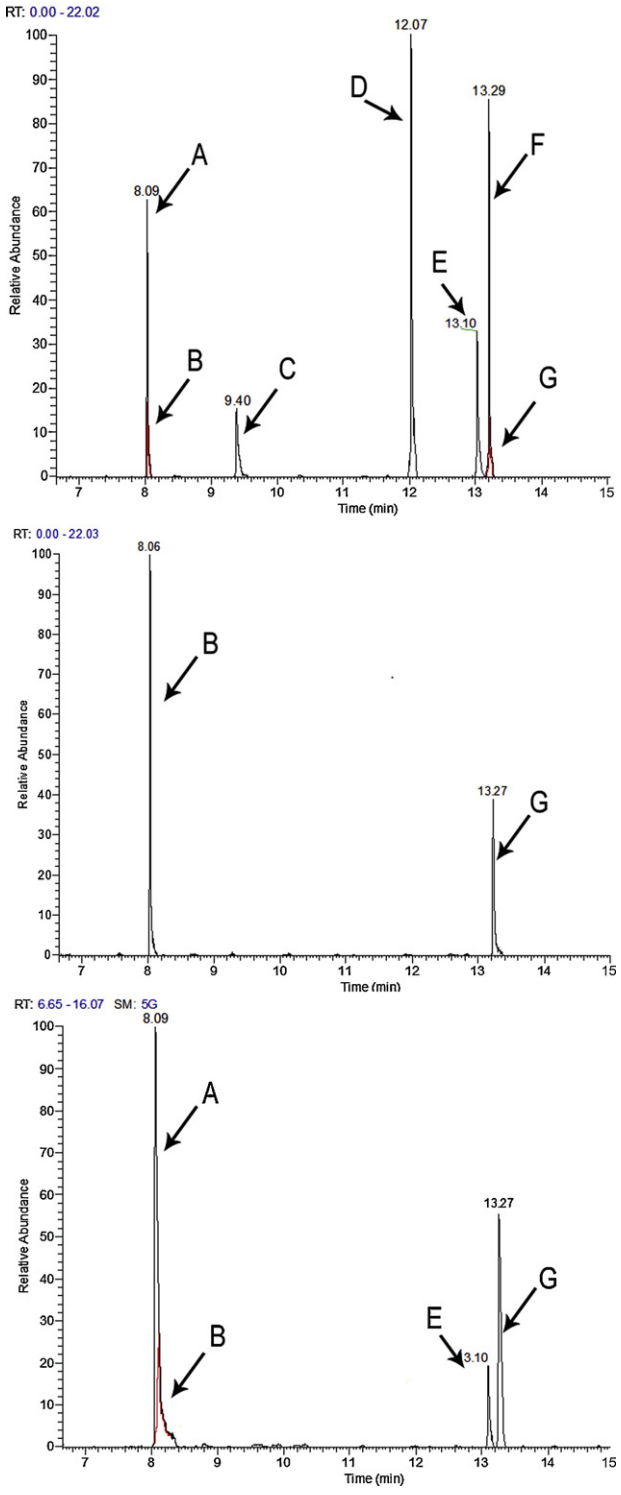

Fig. 5. Chromatogram obtained with the analysis of amphetamines in hair using HF-LPME. (1) Hair spiked at 2.0 ng/mg of (A) amphetamine, (B) amphetamine-d5, (C) methamphetamine, (D) MDA, (E) fenproporex, (F) MDMA, (G) MDMA-d5; (2) blank sample; (3) real hair sample containing 0.78 ng/mg of amphetamine, and 0.21 ng/mg of fenproporex.

$y = 2.63327x + 0.18615$; $r^2 = 0.9970$; where y and x represent the relationship between the peak area ratio (compound/IS) and the corresponding calibration concentrations, respectively. Accuracy data were determined and lay all within the acceptance interval of 15% (20% at the LoQ) of the nominal values for all analytes and concentrations.

3.4. Proof of applicability

The present developed HF-LPME and GC–MS method was applied to hair samples collected from six volunteers who reported regular use of fenproporex (at least 3 times a week). Amphetamine was detected in all samples of the volunteers since this substance is a fenproporex metabolite [29] (Table 2). Fig. 5 shows chromatograms obtained with the practical use of this method to the analyses of head hair samples (a sample spiked with 5.0 ng/mg of analytes, a blank sample, and a positive sample from the volunteer 2).

Only the volunteer 3 allowed the evaluation of the historical consumption due to her hair length. Four consecutive segments of approximately 3 cm each were cut from the hair root. The analyses suggest a recent use since detectable concentrations of both amphetamine and fenproporex were found in the proximal segments close to scalp. In spite of all the volunteers admitted fenproporex use, this substance was not detected in all samples even when amphetamine was detected. These results suggest that fenproporex seems to be incorporated less efficiently than amphetamine in this matrix. In fact, similar results were also described in rat experiments [30,31]. Also, as far as we know, this is the first report that provided data for the analysis of fenproporex in real human hair samples.

4. Conclusion

Our results demonstrated that the HF-LPME procedure is well suited to the determination of some amphetamine-type stimulants (amphetamine, fenproporex, methamphetamine, MDMA, and MDA) in human hair samples. The developed method proved to be simple and practical with little organic solvent required for analysis. This method can be promptly utilized for different purposes whenever monitoring of regular amphetamines consumption is required.

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