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The measurement of ecstasy in human hair by triple phase directly suspended droplet microextraction prior to HPLC-DAD analysis

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ABSTRACT

New pre-concentration technique, triple phase suspended droplet microextraction (SD-LPME) and liquid chromatography-photodiode array detection was applied to determine ecstasy, MDMA (3,4-methylendioxy-N-methylamphetamine) in hair samples. In this research MDMA in hair was digested and after treatment extracted. The effective parameters were investigated and method was evaluated. Under the optimal conditions, the MDMA was enriched by factor 98.11. Linearity (r = 0.9921), was obtained in the range of 10–15,000 ng mL^{-1} and detection limit was 0.1 ng mL^{-1}.

1. Introduction

MDMA (3,4-methylenedioxy-N-methylamphetamine), nowadays, most commonly known by its street name, ecstasy (often abbreviated E, X, or XTC), is a semi-synthetic member of the amphetamine class of psychoactive drugs [1]. It is structurally similar to amphetamines, but has quite different emotional effects. Ecstasy is sold as tablet, capsule or in powder from. Tablets are the most common form and tablets or pills are usually stamped with a logo or brand. Pills with the same logo can be in different sizes, colors and shapes [2]. Case reports of exaggerated responses and death associated with its use suggest that some individuals are at an increasing risk of toxicity [3,4]. So it is important to determine the concentrations of the drug in biological system in the medical and judicial case.

Over more than 20 years hair analysis for drugs has been gaining increasing attention and recognition in various toxicological fields [5] Ingested drugs circulate in a person's bloodstream and are deposited in the hair follicle and entrapped in the core of the hair shaft as it grows out from the hair follicle. Normal growth rates for human hair are approximately half an inch per month. Hair samples are less invasive and easier to collect, store and dispatch compared to blood and urine samples [6,7].

However, the biological component is complex and analytes are usually present at low concentration in body. Therefore, sample pre-concentration and cleanup must be carried out before analyte can be determined by high-performance liquid chromatography (HPLC) or gas chromatography (GC). During recent years, several different microextraction techniques have been presented almost for all small sample volumes [8–13]. Some of these techniques have been used successfully as the preparing method in the determination of drug residues by HPLC, GC and capillary electrophoresis (CE) [14–18]. Recently, LLLME was developed as a novel and disposable method for sample preparation [8,19]. Because of higher extraction efficiency and significant sample cleanup, LLLME combined with HPLC has been successfully used for pre-concentration and cleanup of complicated samples, such as abused drugs [20]. Newly in the field of liquid phase microextraction, Yangcheng et al. developed a new sampling method termed directly suspended droplet micro extraction (DSDME) [21].

Several methods have been developed for ecstasy determination after sample preparation. Analysis may be performed by applying immunoassay [22], gas chromatography [23,24], capillary electrophoresis [25,26] or liquid chromatography (LC) [27–30]. In this work, we used a novel application of DSDME method, based on a three-phase extraction system which is compatible with HPLC. In triple phase DSDME the acceptor solution is an aqueous phase providing a three-phase system, where the analyte is extracted from an aqueous sample, through the thin layer of organic solvent, and into an aqueous acceptor droplet. After the extraction, pre-concentrated analyte was directly introduced into HPLC for further analysis.

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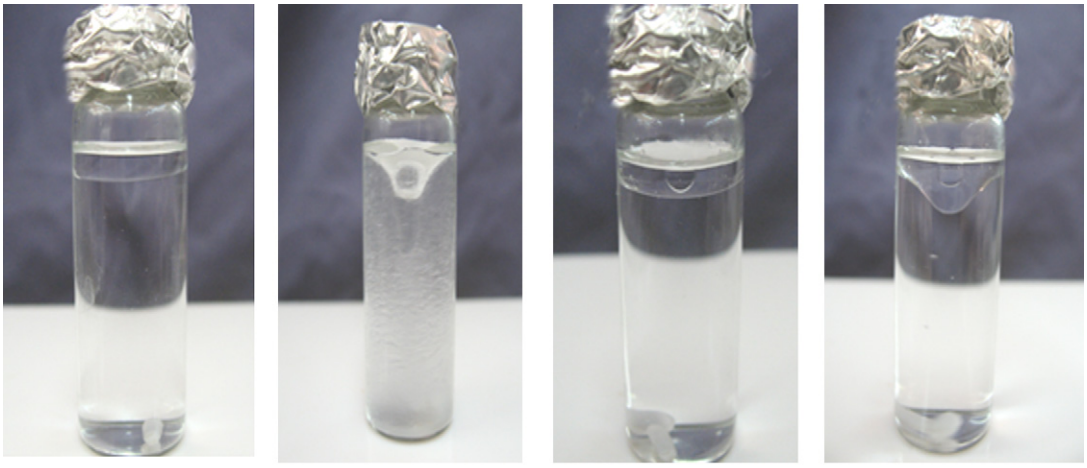

Fig. 1. Microextraction device; different steps in the developed method: (a) before turning on the magnetic stirrer, organic solvent is added to the aqueous donor phase; (b) magnetic stirrer on, extraction occurring (T_1); (c) micro-droplet addition with a microsyringe, magnetic stirrer off; (d) magnetic stirrer on, back extraction occurs (T_2) after the back extraction, droplet is withdrawn with the microsyringe.

2. Experimental

2.1. Chemicals and reagents

Mercedes type ecstasy tablets were provided from the police force (Mashhad, Iran). In order to extract 1-octanol was obtained from Applichem (Germany). HPLC-grade acetonitrile, methanol and other chemicals were purchased from Merck (Darmstadt, Germany) and used without further purification. Stock solution of MDMA was prepared by dissolving the 8.18 mg of tablet powder in 10 mL methanol. Standard sample containing MDMA at 5.0 μg mL^{-1} was provide by dilution of stock solution in de-ionized water which was from Samen Pharmacy (Mashhad, Iran) and they were stored at 4 ± 0.5 °C. Triton X-100, Brij 58 and Brij 72 were from Aldrich.

2.2. Instrumentation

2.2.1. HPLC system and conditions

The binary HPLC system used in this work was a Knauer (Germany, d-14163) containing UV-detector S2600, a port sample injection valves equipped with a 20-μL loop. Separation was accomplished using a 100/5-RP-18 column with 4.6 mm diameter, 250 mm length, from Knauer (Germany). The mobile phase, water, acetonitrile, and methanol, optimized on (80:15:5, v/v) was degassed by own system degasser and delivered two pumps S1000. The flow rate of the mobile phase was 0.8 mL min^{-1} and UV detection wavelength was set as 254 nm.

2.2.2. Directly suspend droplet LLLME procedure

For the first step, the sample solution (5 mL adjusted to pH 11 with NaOH 0.1 M) was placed within a 6 mL glass vial. 0.1 mL surfactant, Triton X-100 was added too. Then 350 μL organic solvent was added and a stirring bar was (2 mm × 7 mm) placed in the solution. An aluminum foil was used to cover the lid of the vial during extraction to prevent the evaporation of the organic phase. Then the mixture was put on a Yellow line (USA) heater and magnetic stirrer and was agitated for 3 min at 1000 rpm. In the later step a 100 μL flat-cut HPLC microsyringe (Knauer, Germany) was used to introduce the acceptor phase (10 μL droplet of de-ionized water adjusted to pH 12 with NaOH 0.1 M) to the top center position of the immiscible organic solvent. The mixture was stirred at 600 rpm for 20 min to cause back-extraction. After this period the micro-droplet was picked up by the same HPLC microsyringe and was injected into the HPLC system. The experimental microextraction setup was shown in Fig. 1.

2.3. Hair sample and analysis

A bulk of blank hair, necessary for method development and validation, was obtained from hairdresser's shop. The absence of ecstasy was verified. The hair samples containing ecstasy were collected from XTC droplet consumers (in TC center, Mashhad, Iran). The cases of said were under therapeutic treatment.

A standard of hair about 5 mm in diameter was cut from close to the scalp at the vertex posterior area. Samples 2–4 cm long was selected for analysis.

There is always the possibility that a drug in hair does not originate from consumption but has been incorporated from external sources. Therefore, prior to analysis of hair samples a decontamination strategy has to be performed and washing solutions.

The hair (2.0 g) was washed with different solvents as follow: 20.0 mL dichloromethane, 15.0 mL acetone, 15.0 mL methanol, 10.0 mL methanol, at room temperature for 5.0 min and then it was dried [20].

Finally, hair samples were cut into approximately 1.0 mm pieces and digested by the following steps: 2.0 mL methanol as an extracting solvent as added to 50 mg of hair, in a 10.0 mL screw-cap tube. The pH was adjusted to 7.4 by phosphate buffer solution. The samples were incubated at 50 °C for 5 h [31]. In case of a remaining solid matrix, extracts were filtered. Then the remaining solid matrix was filtered and rinsed with 0.5 mL ethanol. And it was added to the extracted solution.

3. Results and discussion

3.1. Theoretical considerations

Liquid–liquid–liquid microextraction technique involves three phases for doing two processes: in the first time MDMA from MDMA aqueous solution as the donor phase was extracted into the organic phase (secondary phase) because of its affinities to the organic solvent, and then MDMA inter rapidly into to the aqueous micro-droplet as the acceptor phase. According to basicity of ecstasy (pK_b = 3.68) pH of the donor phase was adjusted in a way the neutral molecules of analyte were formed and reduce their sol-

Table 1
Characteristics of organic solvents.

Solvent	Density, g cm^{-3}	Solubility in water, g L^{-1}	Surface tension, Dyne cm^{-1}	Viscosity C.P., 25 °C	Log $P_{o/w}$
1-octanol	0.83	0.0003	27.50	10.64	3.0
Toluene	0.87	0.53	28.5	0.59	2.69
n-hexane	0.659	0.013	18.4	0.31	3.9

ubility in the donor phase and collected in organic solvent. The neutral form of analyte was ionized again at the organic phase—the aqueous acceptor phase interface because of the acidity of acceptor phase and then ionized analyte entered into the acceptor phase. The theory of method has been well described in the many articles [10,32–34].

3.2. Enrichment factor (EF) and recovery

The enrichment factor was calculated by the following equation:

$$\mathrm{EF} = \frac{C_{\mathrm{a,final}}}{C_{\mathrm{d,initial}}} \quad (1)$$

where, $C_{\mathrm{a,final}}$ and $C_{\mathrm{d,initial}}$ respectively, the final concentration of the analyte in the acceptor phase and the initial concentration of the analyte in the donor phase. The recovery (R) was calculated according to the following formula:

$$R = \frac{C_{\mathrm{d,determine}}}{C_{\mathrm{d,initial}}} \times 100 \quad (2)$$

where, $C_{\mathrm{d,determine}}$ and $C_{\mathrm{d,initial}}$ are the concentrations of the analyte in the aqueous sample, which are determined using the LLLPME coupled with HPLC technique and the concentration of the analyte originally added in the hair sample, respectively [35].

3.3. Optimization method

3.3.1. Organic solvent

Choosing the most suitable organic solvent is very important for achieving a good selectivity and extraction efficiency. This factor is very critical for three-phase microextraction. The organic phase must therefore be immiscible with both the acceptor and donor phase. Also, the analyte in the sample solution (donor phase) should have high partition coefficient into the organic solvent. On the other hand, the solubility of the analyte should be lower in the organic solvent compared to the acceptor phase, in order to achieve a high degree of recovery of analyte in the acceptor phase. It should also have high viscosity to hold the micro-droplet, and lower density than water to lay it over the aqueous sample solution. In this study, three different organic solvents were investigated namely: toluene, n-hexane and 1-octanol (see Table 1) [36–38]. Therefore, 1-octanol with high viscosity and other suitable characterizations for this work were selected.

3.3.2. Effect of the acceptor and donor phase pH

The pH value of both aqueous donor and acceptor phases plays an essential role in the extraction processes. They are very important and effective parameters in efficiency and enrichment factor in LLLPME. The difference in pH between donor and acceptor phase is also one of the major factors that progress the transfer of analyte from the donor to acceptor. Therefore, after survey of the pH effect in the pH range 3–12, enrichment factor increase for sample solution in pH 11 and about aqueous micro-droplet pH 5 was the best and we used.

3.3.3. Effect of the stirring rate

Stirring speed is one of the major factors that affect the extraction efficiency. Agitation of the sample is routinely applied to the mass transfer coefficient in aqueous solution and accelerates the extraction kinetics. Increasing the stirring rate can decrease the thickness of the diffusion film in the aqueous phase and improve the repeatability the extraction method [39]. Thus, the influence of the stirring rate in the range of 100–700 rpm was surveyed and in stirring speed = 600 rpm as optimized rate, enrichment factor is better. Consequently, on the basis of the stability of micro-droplet, 600 rpm was selected (see Fig. 2).

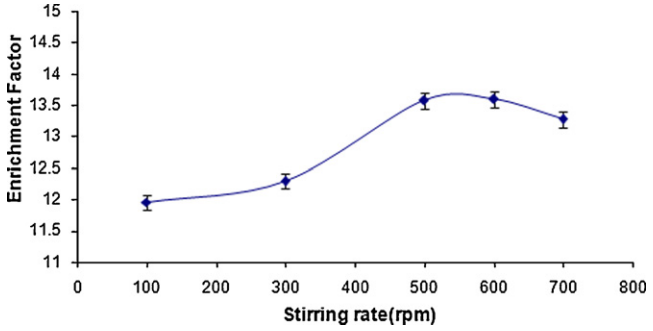

Fig. 2. Effect of stirring rate on the extraction when using DSDME technique with 1-octanol as the organic solvent; analyte concentration 5 μg mL^{-1}, sample pH 11.0, acceptor phase pH 5.0, T_1 = 30 s, T_2 = 60 s, 4.5 mL donor sample volume, micro-droplet volume 10 μL.

3.3.4. Extraction time (T_1)

In the first step, analyte extracted from the aqueous sample into the organic solvent that is a slow equilibrium process, and mass transfer is depended on time [40]. With the passage of time solute molecules have sufficient time for transfer from donor phase to interface between the donor and organic phases and collection in organic phase. Therefore, extraction time is a significant factor in the extraction efficiency. The mixture of water sample and organic solution was agitated at 600 rpm for 180 s. Due to the high degree of mixing between the donor and organic phases the mass transfer is rapid. The result was shown in Fig. 3.

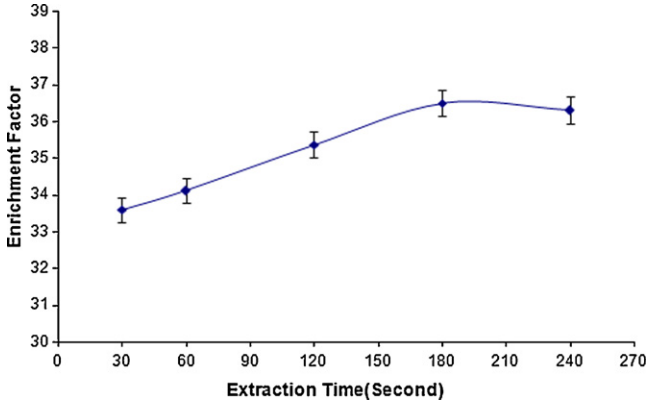

Fig. 3. The effect of extraction time (T_1) on the extraction efficiency. Other extraction conditions: analyte concentration 5 μg mL^{-1}; 1-octanol as the organic solvent; sample pH 11.0; acceptor phase pH 5.0; stirring speed 600 rpm; 4.5 mL donor sample volume; micro-droplet volume 10 μL.

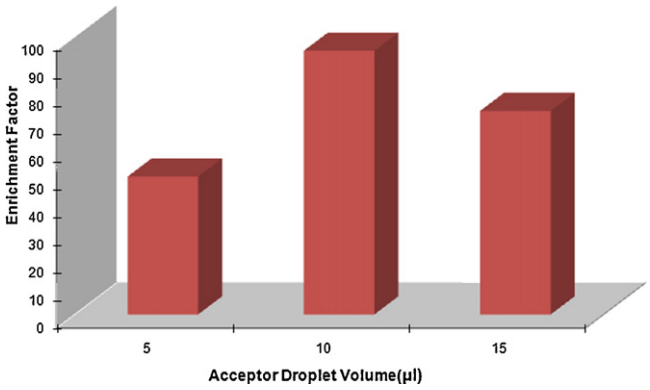

Fig. 4. The effect of micro-droplet volume on the extraction efficiency. Extraction conditions: analyte concentration 5 μg mL^{-1}; 350 μL 1-octanol as organic solvent; T_1 = 180 s, back-extraction time (T_2) = 20 min; stirring speed 600 rpm; 5 mL donor sample volume.

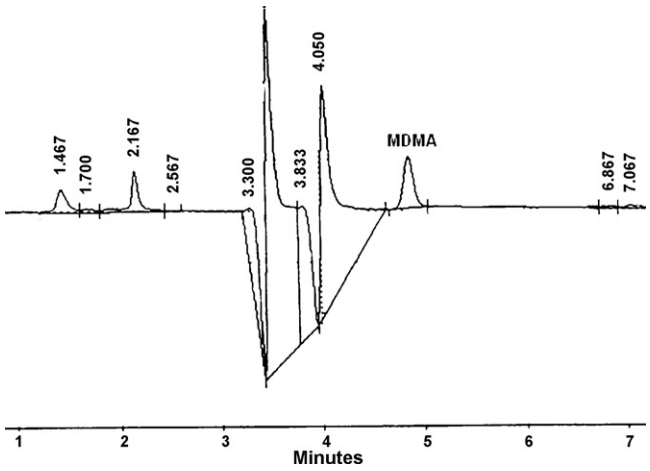

Fig. 5. The chromatogram of real sample, hair of drug consumers after extraction under optimal conditions.

3.3.5. Back-extraction time (T_2)

Suspended droplet LLLME is not an exhaustive extraction technique. Although maximum sensitivity is attained at the equilibrium, complete equilibrium needs not to be attained for accurate and precise analysis [40,41]. Increasing this time causes increased extraction and leads to progressed enrichment factor. However, longer extraction time will result in the dissolution of extracted analyte in the organic phase and instability of the droplet especially under stirring. We have tested different back-extraction times from 60.0 s to 20.0 min. On this basis, 20.0 min was selected as optimal back-extraction time for the experiment and after 20.0 min micro-droplet is dissolved.

3.3.6. Phases volume

In the present work, the phase volume of donor and acceptor solution and organic solvent was optimized. The enrichment factor can be improved by increasing the volume ratio of donor and acceptor phases [16,41–43]. The results however indicated that the best extraction efficiency was obtained when the donor acceptor ratio phase more than 100-fold. With attention to selected glass vial shape and volume, 5 mL volume for aqueous sample solution was better than any other (between 4, 4.5, and 5 mL donor phase volumes). For studying droplet volume, de-ionized water samples of 5, 10, 15 μL were exposed separately for 20 min to the aqueous solution (see Fig. 4). Recovery decreased with the relative size of the micro droplet. With smaller droplets the area to volume ratio was greater than for the larger droplets and mass transfer can take place more easily with a smaller droplet. Also the greater droplet due to stirred solution was wasted. Thus, a 10.0 μL drop volume was chosen for further work. Meanwhile the organic phase volume was kept on 350 μL.

3.3.7. Effect of nature and concentration of surfactant

Commonly, correct selection of surfactant is fundamental for obtaining a satisfactory pre-concentration and extraction process.

When choosing the surfactant, consideration should be given to its interaction with analytes and matrix, as well as the solubility of the analytes. So, non-ionic and zwitterionic surfactants have been applied for liquid phase separation while the use of charged surfactants species is still in question [44]. In addition, the surfactant phase is compatible with the water-organic mobile phase usually employed in HPLC and in most cases, is UV transparent too.

Surfactant concentration is an important parameter for effective extraction. The extraction efficiency of relative non-polar organic compounds can reach to about 100% even when very low surfactant concentration is used [44]. In this we have carried out a comparative study of three different non-ionic surfactants: Triton X-100, Brij 58 and Brij 72 (see Table 2). Triton X-100 (MW = 631 g mol^{-1} and C.M.C. = 0.240 mM or equal 157 mg L^{-1}) has shown better results than other surfactants. When the surfactant concentration, in the donor solution, was reached over its CMC, the extraction efficiency decreased because sample molecule interacted with micelles.

We worked on three concentration of Triton X-100: 20, 50 and 100 ppm. The results obtained indicated that Triton X-100 with 20 ppm concentration have been shown better result than the others and the enrichment factors can be increased as a function of the surfactant concentration.

4. Method validation

4.1. Analytical performance

Stock solution containing 0.818 mg mL^{-1} of MDMA was prepared, in methanol and stored at 4 °C. Standard solutions were obtained by adding calculated amounts of the stock solution into the blank hair solutions which were prepared and described previously. These working samples were used for optimization of experimental and calibration curves. In concentration range between 1.0 and 15,000 ng mL^{-1} calibration curve is drowned. The obtained calibration equation was $y = 0.0098x + 23.431$. Linearity was observed with $r = 0.9921$ for analyte. Limit of detection (LOD) were calculated as the minimum concentration providing chromatographic signals three times higher than background noise.

Limit of quantification (LOQ) was estimated as the minimum concentration preparing chromatographic signals ten times higher

Table 2
Characteristics of non-ionic surfactant.

Surfactant	Commercial name	Formular	M.W.	B.P. (°C)	Density (g cm^{-3}) at 25 °C	CMC (mM)
Polyoxy ethylene(20) cetyl ether	Brij 58	$C_{56}H_{116}O_{21}$	1123.50	35.4–39.6°	1.70	0.007
Polyoxy ethylene(2)stearyl ether	Brij 72	$C_{18}H_{37}(OCH_2CH_2)_2OH$	358.60	44–49°	0.893	0.00025
Polyoxyethylene octyl phenyl ether	Triton X-100	$C_{14}H_{22}O(C_2H_4O)_n$ n = (9–10)	625	6°	1.07	0.240

Table 3
A comparison between methods for determination of ecstasy in different samples.

No.	Date	Matrix	Method	Detection	LOD	LOQ	Linear Range	r^2	RSD%	Recovery%	Ref.
1	2008	Human serum	HS-SPME	IMS[1]	5 ng mL^{-1}	–	20–4000 ng mL^{-1}	0.99	7.8	–	[45]
2	2008	Mouse plasma and brain	SPE	GC–EI[2]-MS	–	–	20–2000 ng mL^{-1}	–	5.3	91	[46]
					–	–	0.2–200 ng/mg	–			
3	2008	Urine	SPE	HPLC-DAD	0.1 μg mL^{-1}	–	0.2–20 μg mL^{-1}	0.99	–	–	[47]
4	2008	Plasma	–	GC–MS	2.5 μg/L	–	–	0.997	–	85.6–107.2	[48]
5	2007	Sweat	SPE	GC/MS-EI	–	2.5 ng/patch	–	–	–	–	[49]
6	2006	Oral fluid	SPE	GC/MS-EI	–	–	5–250 ng mL^{-1}	–	8.3	85	[50]
7	2006	Blood	LLE	HPLC-FLD	0.36–0.83 ng mL^{-1}	–	–	–	–	–	[51]
8	2005	Ecstasy tablet	–	HPLC	–	–	1.4–111 μg mL^{-1}	–	9	–	[52]
9	2005	Oral fluid	LLE	HPLC-FLD	2 ng mL^{-1}	10 ng mL^{-1}	–	–	–	–	[53]
10	2004	Urine	LE[3]	HPLC-FLD	15 ng mL^{-1}	–	–	–	–	85–102	[54]
11	2004	Hair	HS-SPME	GC–MS	0.7 ng/mg	–	0.1–20 ng/mg	–	7–20	–	[55]
12	2003	Urine	SPE	HPLC-UV	5.3–84 ng mL^{-1}	–	–	–	2.9–5.3	84	[56]
13	2003	Hair	LLE	GC–MS	0.03–0.08 ng/mg	–	–	–	1.5–15.7	–	[57]
14	2002	Plasma and urine	SCX[4]	HPLC-ECD	9.2 μg/L	28.2 μg/L	50–1000 μg/L	–	10.8–13.4	–	[58]
					10 μg/L	31.8 μg/L			6.5–15.3		
15	1995	Hair	LLE	GC–MS	0.1 ng/mg	–	–	–	–	–	[59]
16	1993	Urine	–	HPLC-UV	40–60 ng mL^{-1}	–	–	–	–	80–85	[60]
17	1989	Blood	LLE	GC–MS	0.12–48 ng	–	–	–	–	–	[61]
18	2009	Hair	DSDME	HPLC-DAD	0.1 ng mL^{-1}	1.0 ng mL^{-1}	1.0–15000 ng mL^{-1}	0.9921	5.395	–	Current Research

1—Ion Mobility Spectrometry.
2—Electron Impact ionization.
3—Liquid Extraction.
4—Solid Phase Strong Cation Exchange.

than background noise. Thus, LOD obtained was 0.1 and LOQ was 1.0 ng mL^{-1} too.

Enrichment factor was calculated as the proportion peak area before extraction to after that. EF was 98.11. Repeatability (RSD%) evaluated with three replicated experiments is 5.395.

As mentioned before, SD-LPME is not an exhaustive extraction method, so relative recovery was determined as the ratio of the concentration found in hair samples and distilled water samples with both samples spiked at the same concentration level, under the optimized conditions. Relative recovery for standard hair samples was 65.4.

4.2. Real sample analysis

To investigate matrix effects and applicability of the technique to biological sample, final experiments were carried out on hair sample containing ecstasy. Concentration of MDMA in the hair of drug consumers was 2.0 (ng mg^{-1} hair) with RSD% 3.19 ($n = 3$) (see Fig. 5).

5. Conclusion

The aim of the present study was to develop and validate a rapid, sensitive and reliable method for the quantitative determination of the drug abuse in human hair by HPLC. The results obtained with the method described above indicate that this methodology is a good alternative extraction technique for hydrophilic drugs in hair and offers highly interesting advantages from an analytical point of view, such as possibility of extracting and pre-concentrating the analytes of different polarities. Surfactants are less toxic and cheaper than the extractants used in LPME. The most commonly used surfactants are commercially available and, no analyte is lost in the process. The experimental operations involved in SE-LPME are very simple and the final surfactant-rich phase, if it could be diffused into the acceptor phase, is compatible with the mobile phase used in HPLC analysis. Moreover, this procedure offers several advantages over traditional extraction techniques such as; a reduction in extraction time (typically 20–45 min), also this method is economical and easy to use.

In our method, we introduced a reliable qualitative and quantitative technique for abused drugs at low level of concentration in hair. In the mean time hair sample has some advantages over the other biological samples like urine and blood, such as long time of drug residence in the sample and low risk of side effect in transferring to examiner. The review of some methods which were used for determination of MDMA in the environmental and biological samples is demonstrated in Table 3.

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