ENANTIOSELECTIVE DETERMINATION OF CATHINONES IN URINE BY HIGH PRESSURE IN-LINE SPE-CE

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ENANTIOSELECTIVE DETERMINATION OF CATHINONES IN URINE BY HIGH PRESSURE IN-LINE SPE-CE

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Abbreviations: **DCM**, dichloromethane; **MeOH**, methanol; **MDPV**, methylenedioxypyrovalerone; **MCX,** major cation exchange; **NaOH**, sodium hydroxide

Keywords: Cathinones / Chiral CE / In-line solid phase extraction / Oasis HLB / Urine analysis

Abstract

This work presents a strategy based on the in-line coupling of SPE and CE for the chiral determination of cathinones (R,S-mephedrone, R,S-4-methylephedrine and R,S-MDPV) in urine samples, using a sample pretreatment based on LLE. The chiral separation of the compounds is achieved by adding a mixture of 8 mM 2-hydroxypropil β-CD and 5 mM β-CD to the BGE, which consists of 70 mM of monosodium phosphate aqueous solution at pH 2.5. In the in-line SPE device Oasis HLB was the selected sorbent, and to reduce analysis time and LODs several parameters affecting the in-line SPE system were evaluated, such as pressure, sample injection time and dimensions of the SPE device. The highest preconcentration factors were achieved by using 3 bar of injection pressure for 20 min with an in-line SPE device of 2 mm length and 150 µm of id. The developed method was applied to determine the studied compounds in spiked urine samples. The LODs obtained were between 3 and 8 ng/mL, and these levels were below the usual concentrations at which these drugs are present in urine from cathinone abusers. Thus, the optimized method has the potential to be applied for toxicological and forensic purposes.

1. Introduction

In recent years, the consumption of synthetic derivatives of cathinone, an alkaloid naturally found in the leaves of the khat plant, has increased due to its stimulating properties and low price compared with amphetamines [1–3]. These synthetic cathinones can be found, for example, as “bath salts” on the internet with a label stating “not for human consumption”. They are currently one of the most commonly abused drugs and for this reason there has been an associated increase in interest on the part of the police and health authorities to detect and control these substances. This is reflected in the emergence of an extensive literature on the detection and determination of cathinones [4–17].

Mephedrone and methylenedioxypyrovalerone (MDPV) are major components of these bath salts and both present chiral centres, which implies the presence of two enantiomers (R and S) [11,12,16,18,19]. Each enantiomer can present different pharmacokinetic and pharmacodynamic behaviour and produce different effects on the organism [1,18,19]. Moreover, the enantiomers ratio could provide information about the drug synthetic route and help in tracking its production [7]. For these reasons is important the enantioseparation of these compounds.

CE has proven to be an appropriate technique for performing chiral separation relatively easily, since it is possible to achieve enantioseparations by simply dissolving a chiral selector in the BGE [11,12,16,20–23]. Cyclodextrins are by far the most popular chiral selectors used due, to their low toxicity, high solubility in virtually all aqueous background electrolytes, UV transparency and high commercial availability. Indeed a number of different studies in the literature have reported the use of CDs as chiral selectors to bring about the enantioseparation of cathinones [11,12,16,24,25]. To offset against the well-known advantages of CE, is the fact that one of its main limitations is its inherent poor sensitivity. To overcome this, different preconcentration strategies have been developed, and in recent years, there has been much

interest in the use of the in-line coupling of SPE with CE (in-line SPE-CE), in which the preconcentrator is an integral part of the CE system [12,26–34]. There are various designs used for the preconcentrator in in-line SPE-CE, the most popular being the fritless packed bed [12,28–34]. With this approach the preconcentrator, a small piece of capillary (2-4 mm) filled with SPE sorbent, is placed between an inlet (5-10 cm) and a separation capillary (50-100 cm) of a narrower id than the preconcentrator. The sorbent particles are retained in the packed bed because of their size, which is slightly larger than the inner diameter of the separation capillary.

The main advantages of this preconcentration strategy are that it is easy to automate and all the solvent eluted from SPE is analysed by CE, and this contributes to increasing sensitivity. However, to achieve low detection limits a large sample loading time (between 30-60 min) is generally needed at the pressure values normally used for injection (50 mbar) [34], which implies a long analysis time, and this can be a serious drawback for routine analysis. One way to reduce analysis time while maintaining sensitivity may be by increasing the injection pressure. This is because at high pressures a large volume of sample is injected and crosses the SPE device. Therefore a large amount of analytes could be retained in a shorter time than when using low pressures. In addition, the dimensions of the SPE (length and diameter) can also have an effect on the sensitivity of the in-line SPE-CE system [32]. If there is an increase in the length, diameter or both, the amount of sorbent particles inside the in-line SPE device will consequently increase, and thus more analytes could be retained in the sorbent as long as the breakthrough volume is not exceeded.

To document cathinone consumption it is essential to perform an analysis of these compounds in biological samples such as urine, which can be obtained non-invasively and presents a detection window that can reach weeks. In recent years urine has been successfully used as a matrix for different applications [4–10,13–15] including the identification of cathinones in

urine from victims of sexual assault [6,15] and screening studies for cathinone consumption [13,14]. These studies showed that cathinones can be found in urine at low concentration levels (ng/mL), so it is natural that very sensitive methodologies need to be developed to determine these compounds. Due to these low levels, in previous studies the determination of cathinones in urine samples was mainly achieved by the combination of a chromatographic technique with MS detection such as LC-MS [4,5,8,9,13,15] or GC-MS [6,7,10,14] after an appropriate pretreatment, generally SPE [5,7–10] or LLE [6,13,14]. However, despite the benefit of CE in enantioseparation, as far as we know there are no studies in which this technique has been used for the enantioseparation of cathinones in urine samples. This can be attributed to its lack of sensitivity. In view of this, the main aim of our study is to develop a method for the enantioselective determination of *R,S*-MDPV, *R,S*-mephedrone and *R,S*-4-methylephedrine in urine samples on the basis of in-line SPE-CE. Moreover, in an attempt to improve in-line SPE methodology, the dimensions of the SPE device concentrator and the sample loading conditions were optimized in order to achieve high preconcentration factors.

2. Materials and methods

2.1. Reagents and standards

The standards of *R,S*-mephedrone, one of its metabolites *R,S*-4-methylephedrine, and *R,S*-MDPV were acquired as hydrochloride salts with a purity of 98% from LGC Standards (Teddington, UK). Individual stock standard solutions of analytes (100 mg/L, 1000 mg/L or 2000 mg/L) were prepared by dissolving an appropriate amount of the standards in methanol (MeOH) and kept in the freezer at -20 °C. Working standard solutions of a mixture of all the compounds were prepared by diluting stock solutions in MeOH and then storing them at -20 °C. The solutions with a lower concentration were prepared daily by diluting appropriate volumes of the working standard solutions in Milli-Q water. Dichloromethane (DCM), ethyl acetate, isopropanol and MeOH, all of analytical-reagent grade, were purchased from J.T Baker (Deventer, Netherlands). Ammonium hydroxide 28%, formic acid 98%, hydrochloric acid 37%,

monosodium phosphate 99%, phosphoric acid 85%, sodium hydroxide (NaOH) 97%, β-CD 97% and 2-hydroxypropil β-CD were acquired from Sigma-Aldrich (Saint Louis, MO, USA). Milli-Q water was obtained with a water purification system from Veolia Water (Paris, France). Oasis HLB and Oasis MCX cartridges (150 mg) with an average particle size of 60 µm were obtained from Waters Corp. (Milford, MA, USA).

2.2. Instrumentation

The electrophoretic system was a 7100 CE instrument from Agilent Technologies (Waldbronn, Germany) equipped with a DAD. For all the experiments the capillary chamber was set at 25 °C and 200 nm was used for the detection of the analytes. Bared fused-silica capillaries of different id (50, 150 and 200 µm) were purchased from Polymicro Technologies (Phoenix, AZ, USA). The off-line SPE was carried out using a manifold system from Ashcroft (Stratford, CT, USA). The pH measurements were performed with a GLP 21 pH-meter from Crison (Barcelona, Spain). A Universal 32 R centrifuge came from Hettich (Kirchlengern, Germany).

2.3. Sample preparation

Urine samples were obtained from several non-addicted volunteers. They were collected in polypropylene tubes and stored at -20 °C until analysis. For method validation pooled urine was used, prepared by mixing the urine collected from the volunteers.

Before in-line SPE-CE analysis, the urine samples were alkalized to pH 10 with 28% ammonium hydroxide and then extracted in accordance with a LLE procedure. This LLE was as follows: 2 mL of a mixture of ethyl acetate/isopropanol (4:1, v/v) were added to a 2 mL alkalized urine sample. After vortex mixing for 1 min, samples were centrifuged for 10 min at 9000 rpm. The organic phase containing the analytes was transferred to a vial and a second extraction of the aqueous phase was performed by adding another 2 mL of the organic solvent mixture and repeating the same procedure. The two organic phases were then combined and 200 µL of isopropanol added. The obtained extract was dried under a gentle stream of N_2 until approximately 200 µL remained. The residue was then reconstituted to 2 mL with Milli-Q

water (adjusted to pH 10 with 28% ammonium hydroxide). The sample was filtered through a 0.45 µm PTFE syringe filter, then transferred to a microvial for analysis and finally injected into the CE instrument.

2.4. CE separation

The CE separation was performed on a fused-silica capillary with a total length of 80 cm (72 cm effective length), with an id of 50 µm and an od of 360 µm. The separation voltage was 30 kV (positive polarity). The BGE solution consisted of an aqueous solution of 70 mM of monosodium phosphate, adjusted to pH 2.5 (with concentrated phosphoric acid), containing 8 mM 2-hydroxypropil β-CD and 5 mM β-CD. Prior to its first use, the capillary was conditioned with NaOH 1 M for 40 min and for 10 min with Milli-Q water at 930 mbar. At the beginning of each working day, a conditioning step was performed with NaOH 0.1 M for 10 min, Milli-Q water for 5 min and BGE for 5 min, all of them at 930 mbar. Between runs, the conditioning step was performed with NaOH 0.1 M, Milli-Q water and BGE, all of them at 930 mbar for 4 min. Standard samples were hydrodynamically injected at 50 mbar for 5 s.

2.5. In-line SPE procedure

The construction of the in-line SPE-CE device was based on the procedure described in [12]. Briefly, between the inlet (9 cm, 50 µm id) and separation capillary (71 cm, 50 µm id) a small piece of capillary (2 mm, 150 µm id) filled with 60 µm Oasis HLB particles was placed. A PTFE tubing (250 µm id), obtained from Saint Gobain (Courbevoie, France), was used for the connection between the capillaries and the SPE device.

The in-line SPE-CE procedure consisted of various steps. First, before injection the capillary with the preconcentrator was conditioned at 930 mbar with MeOH and Milli-Q water (adjusted to pH 10 with 28% ammonium hydroxide), both for 5 min. Then the urine sample extracts were injected at 3 bars for 20 min. After the injection, a clean-up step with BGE solution at 930 mbar for 2 min was performed. The elution stage was carried out by injecting 2% (v/v) of formic acid in MeOH at 50 mbar for 20 s. Then a pushing step with BGE at 50 mbar for 200 s was

performed to displace the elution plug through the in-line SPE. Finally, 30 kV was applied for the CE separation of the analytes.

3. Results and discussion

3.1. Optimization of the chiral separation by CE

The studied cathinones present an asymmetric carbon atom in their chemical structure. As mentioned in the introduction, this plays an important role in the pharmacological and toxicological behaviour of these compounds, since the two enantiomers can have different biological effects [1,18,19]. However, and despite the fact that it would be extremely useful to have information about these differences relating to the effects of the enantiomers in the body, to date there are few data available. Therefore it is of great importance to develop analytical methods to enable the enantioseparation of cathinones. In the literature there are several successful strategies based on CE, in which the enantioseparation is achieved by simply adding a chiral selector. Of the various chiral selectors, CDs have been extensively used for a wide range of analytes [11,12,16,20–22,24,25] and, in particular, β-CD has been one of the preferred choices used in the separation of cathinones [11,12,24]. For example, Merola *et al.* [11] successfully achieved the chiral separation and determination in seized drugs of 12 cathinones by CE using a phosphate buffer containing 10 mM of β-CD. Baciu *et al.* [10] also achieved the enantioseparation of three cathinones in hair samples using the same CD at a concentration of 12 mg/mL.

Based on the results reported in these previous studies, β-CD was selected as the chiral selector for the present study. As the drugs studied are weak bases with pKa values between 8.83 - 9.13 [35], the selected BGE was a solution of 70 mM monosodium phosphate aqueous solution containing β-CD, at a concentration of 10.6 mM adjusted to pH 2.5. The studied compounds were positively charged so should migrate towards the cathode. Standard solutions containing the analytes at a concentration of 25 µg/mL were injected into a capillary without the in-line SPE device at 50 mbar for 5 s and 30 kV was applied as separation voltage.

The electropherogram obtained under these conditions is shown in Fig. 1A. The enantiomers of *R,S*-MDPV are baseline separated, but it was not possible to achieve the enantioseparation of the other cathinones. The use of 2-hydroxypropil β-CD (Fig. 1B) instead of native β-CD improves the enantioseparation of *R,S*-mephedrone, but the other two pairs of enantiomers were not as well separated as when using β-CD. Different combinations were studied of both CDs, β-CD and 2-hydroxypropil β-CD, specifically 5 mM of both, 5 mM of β-CD and 8 mM of 2-hydroxypropil β-CD and *vice versa*, and 5 mM of β-CD and 10 mM of 2-hydroxypropil β-CD and *vice versa*. The best separation was obtained with a BGE containing a mixture of two CD, 2-hydroxypropil β-CD at a concentration of 8 mM and β-CD at a concentration of 5 mM (Fig. 1C). In view of these results, 70 mM of monosodium phosphate aqueous solution at pH 2.5 containing the dual system of CD previously mentioned was selected as BGE.

3.2. In-line SPE optimization

As explained in the introduction, cathinones can be present in urine at low concentration levels. Therefore, when using CE a preconcentration step such as in-line SPE is required prior to their determination in order to reach these low levels. Oasis HLB, a polymeric sorbent with a polar group with lipophilic and hydrophilic retention characteristics, was selected because in previous studies high preconcentration factors were obtained for cathinones [12,17]. For example, LODs between 0.2-0.10 ng/mg were achieved for the determination of cathinones in hair by in-line SPE-CE, when 40 min of injection time at 930 bar were used [12]. Despite the high sensitivity achieved with this approach, the long injection time involved can be a big drawback in routine analysis. In an attempt to reduce analysis time while retaining or even increasing sensitivity, we evaluated the pressure at which the sample is loaded into the system to reduce the injection time and the analysis time. An increase in the injection pressure means that a larger volume of sample can be introduced through the SPE device and, as long as the breakthrough volume is not exceeded, more analytes could be retained. Hence working at high pressures allows a reduction in injection time and consequently in the analysis time.

To increase the sensitivity of the method, one issue that could have an important influence is related to the dimensions of the SPE device, in particular the id of the preconcentrator and its length. An increase in these parameters may mean that the capillary containing the SPE sorbent could be filled with a higher amount of sorbent particles, and this could have a big influence on analyte retention.

3.2.1. Pressure and sample loading time

In the literature the usual sample injection pressure applied for loading the sample into the in-line SPE-CE system is 930 mbar for 30-60 min. Under these conditions, low LODs have usually been reported. For example, LODs between 20-50 ng/mL for drugs of abuse in urine applying 30 min of loading time [33] and 5-60 ng/mL for barbiturates in urine with an injection time of 60 min [31] have been obtained. However, one drawback of these strategies despite the good sensitivity achieved is the long analysis time. For this reason the effect of increasing injection pressure on the response while simultaneously reducing the sample loading time was evaluated. An important issue to consider when a high pressure value is used to load the sample is to ensure the integrity of the preconcentrator, because at high pressures it was observed that the mechanical friction could separate the PTFE tubing connection used between the separation capillary and the SPE device, and this could bring about drops in current.

For this study, a standard sample containing the analytes at a concentration of 20 ng/mL was injected at different pressures: 930 mbar, 2000 mbar, 3000 mbar and 4000 mbar for 10 min. Higher pressures were not evaluated, since the PTFE tubing connection was unstable. Fig. 2A shows the results obtained in terms of peak area values. These were as expected because as the pressure increases, more sample volume is introduced into the SPE device and thus more analytes can be retained. It can be seen that an improvement in the signal was obtained for each compound when the injection pressure was increased, with 4 bar being the pressure providing the highest peak area. The standard deviations in terms of % RSD (n = 3) were also

evaluated and we observed a slight increase in their values when the pressure increased. However, the values obtained were below 8 % even at the highest pressures, so no significant differences between results were observed.

As regards the sample loading time, different values were also tested. Specifically, we evaluated 5, 10, 20 and 30 min. Standard samples containing the analytes at a concentration of 10 ng/mL were injected at 3 bar, because at 4 bar we observed instability problems with the PTFE tubing connection at injection times higher than 10 min. As expected, an increase in the sample injection time means a signal improvement for each compound (Fig. 2B) because more sample volume is introduced. However, when the injection time was 30 min, after several (more or less 10) consecutive analyses instability problems related to the PTFE tubing connection were observed. 20 min was selected as the optimum injection time in order to avoid these problems and lengthen the life of the preconcentrator.

3.2.2. In-line SPE dimensions optimization

The dimensions of the preconcentrator, in particular its length and id, have a considerable influence on the amount of sorbent present in the SPE device, and this could therefore affect the amount of analytes retained. Hence both parameters were evaluated. For the optimization of the in-line preconcentrator dimensions, standard samples containing the analytes at a concentration of 5 ng/mL were injected for 20 min at 3 bar. In the study of the preconcentrator id, capillaries of different id – 150 µm, 200 µm and 250 µm – were tested. From the results obtained (Fig. 2C) we could see that the preconcentrator id has a great influence on the separation of the analytes. In particular, for capillaries with a higher id than 150 µm the resolution was worse. This was already noted by Jooß *et al.* [32], who also reported this behaviour when the difference between particle size and the SPE id was too high. They attributed this to problems with the absorption of analytes and their subsequent elution due to a greater dead volume. Because of this, 150 µm was the id selected.

The influence of the preconcentrator length on the response was also considered. For this purpose, different capillary lengths (2 mm, 3 mm and 4 mm) were evaluated. As can be seen in the electropherograms obtained (Fig. 2 D), the change in preconcentrator length also has a negative effect on the separation of the cathinones under study, since at greater length the resolution was worse. Therefore 2 mm was selected as the in-line SPE length.

3.2.3. Enrichment factor

To evaluate the improvement in CE sensitivity for the studied compounds under the optimized conditions using the in-line SPE preconcentration, we calculated the corresponding enrichment factors as the ratio of the LODs without using in-line SPE and the LODs using the in-line SPE device. The results are shown in Table 1, these being 8000, 7000 and 6000 for mephedrone, 4-methylephedrine and MDPV respectively. In the literature, the injection pressures used for loading the sample into the in-line SPE preconcentrator are generally lower than 1 bar, with enrichment factors of between 450 and 1000 for organic sulfonates [32] and between 125 and 700 [33] or 2610 and 2930 [29] for drugs of abuse. In the present study, the application of 3 bar as the sample injection pressure led to higher enrichment factors, thus demonstrating the potential of the reported strategy for the sensitive analysis of cathinones.

3.3. Urine extraction

The applicability of the method was demonstrated through the analysis of urine samples. To avoid interferences when urine was directly injected into the system, it was necessary to include a sample pretreatment step prior to loading the sample into the in-line SPE-CE. Two specific procedures were evaluated: off-line SPE and LLE. We based our selection on various studies that have reported a sample pretreatment prior to the analysis of cathinones in urine [5–10,13,14].

Different strategies based on the off-line SPE using strong cation exchange sorbents have been employed for the clean-up and extraction of cathinones from urine samples [5,7–10]. Glicksberg *et al.* [8], for example, obtained LODs of 1 ng/mL and 2 ng/mL for MDPV and

mephedrone respectively in urine by LC-MS after pretreatment with a strong cation exchange sorbent. LLE has also previously been used by other authors for the extraction of cathinones in urine [6,13,14]. For example, LODs between 10 and 50 ng/mL were obtained by GC-MS on urine samples after a sample pretreatment based on LLE using *N*-butyl chloride as the extractant [4].

In this work an Oasis MCX sorbent (150 mg cartridge, with a particle size of 60 µm) was selected for the off-line SPE. The procedure consisted of different steps: a conditioning step with 2 mL of MeOH, 2 mL of Milli-Q water and 2 mL of Milli-Q water adjusted at pH 5, followed by a loading step of 5 mL of sample at pH 5, then a washing step with 2 mL of 2% v/v of formic acid in MeOH and Milli-Q water, and finally the compounds were eluted with 4 mL of MeOH/NH_4OH (95:5 v/v) and the extracts were dried under a N_2 current and injected into the CE system. Under these conditions the obtained recoveries were between 65-93% for standard samples containing the analytes at a concentration of 20 ng/mL. However, when this procedure was applied to spiked urine samples, the interferences overlapped the cathinone peaks even when the sample was diluted 1:10 (urine:water). We therefore focused on optimizing a procedure based on LLE. For that purpose, the LLE strategy used consisted of the following steps. The sample (2 mL) was alkalized to pH 10 to ensure that all the compounds were in their neutral form. Then 2 mL of an organic solvent were added and the mixture was vortex-mixed for 1 min then centrifuged at 9000 rpm for 10 min, and 200 µL of isopropanol were added. Finally, the organic extract was dried under a N_2 current to a volume of approximately 200 µL. This was injected into the CE system. Different organic phases were tested for the LLE procedure: dichloromethane (DCM), hexane, ethyl acetate and isopropanol. The best recoveries were obtained with a mixture of ethyl acetate/isopropanol (4:1), with values from 71 to 108 % for standard samples containing the analytes at a concentration of 20 ng/mL. These recoveries were calculated as the ratio between the response obtained for the

standard sample at 20 ng/mL after performing the overall methodology including LLE, and the response obtained for the same standard sample without LLE.

When this strategy was applied to the analysis of urine samples, the electropherogram corresponding to a blank was free from interferences, although the recoveries obtained for this real sample were slightly lower than for the standards. Based on the results obtained in the pretreatment step, LLE was chosen for further experiments. Fig. 3 shows the electropherogram obtained under the optimum conditions for LLE/in-line SPE-CE for a urine sample spiked with the analytes at a concentration of 40 ng/mL.

3.4. Method validation

The proposed LLE/in-line SPE-CE method was validated with pooled urine from non-addicted volunteers, which was spiked with the analytes in terms of linearity, repeatability, reproducibility, LODs and LOQs. The values obtained are shown in Table 2.

The linearity was evaluated using a matrix-matched calibration curve and pooled blank urine spiked with known amounts of each compound in a range between 5 and 1000 ng/mL. As can be seen in Table 2, good linearity was obtained with regression coefficients (r^2) greater than 0.988.

The intra-day and inter-day precision (in terms of repeatability and reproducibility) was evaluated by analysing five replicates of urine spiked at two concentration levels (20 ng/mL and 100 ng/mL) on the same day and at five different days. The results, expressed as % RSD, under both intra and day-to-day conditions were mostly below 10 %. The LODs were calculated as the concentration that gave a signal-to-noise ratio (S/N) of approximately 3, while the LOQs were set at the lowest point of the linear range. The LODs obtained for the cathinones studied were between 3 and 8 ng/mL. These were comparable to those already published in previous studies. For example, various authors using a strategy based on LC-MS after a pretreatment with SPE using a strong cation exchange sorbent have reported LODs of between 1-2 ng/mL for MDPV and 1-2 ng/mL for mephedrone in urine [5,8]. LC-MS was also the strategy chosen by

Paul *et al.* [4], although in this case a salting-out liquid-liquid extraction was used as the sample pretreatment and the LODs reported were of 2 ng/mL for MDPV and 2 ng/mL for mephedrone. GC-MS has also been used to determine cathinones in urine with a strong cation exchange sorbent as a sample pretreatment, and the reported LODs were of 5 ng/mL and 20 ng/mL for mephedrone and MDPV respectively [10].

To our knowledge this is the first strategy for the chiral determination of cathinones in urine samples by capillary electrophoresis.

4. Concluding remarks

We have reported a method for the analysis of cathinones in urine which allows the determination of these compounds at the usual levels at which they are present in this kind of biological sample from drug abusers.

Under the optimized conditions, the enantioseparation of synthetic cathinones in urine by adding CDs to the background BGE was successfully achieved. The results demonstrate that using a binary CD system consisting of β-CD and 2-hydroxypropil β-CD is an effective strategy for the chiral separation of cathinones. High enrichment factors of between 6000 and 8000 were obtained for the three cathinones under study by using in-line SPE-CE. The study of the sample injection conditions and the dimensions of the preconcentrator in the in-line SPE process allows a reduction in analysis time while retaining sensitivity. Urine samples were able to be analysed by the strategy developed simply by adding a LLE procedure prior to their introduction into the in-line SPE-CE system. The LODs obtained were between 3 and 8 ng/mL, which are similar to those reported by other authors using LC or GC.

5. References

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Figure 1. Electropherograms corresponding to the chiral separation optimization of the cathinones under study in a standard sample containing the analytes at a concentration of 25 µg/mL. The BGE consisted of a solution containing 70 mM of monosodium phosphate aqueous solution at pH 2.5 and (A) β-CD 10.6 mM, (B) 2-hydroxypropil β-CD 10.6 mM, and (C) 2-hydroxypropil β-CD 8 mM and β-CD 5 mM. Peak assignments: (1, 1') *R,S*-mephedrone, (2, 2') *R,S*-4-methylephedrine and (3, 3') *R,S*-MDPV.

Figure 2. Optimization of the in-line SPE-CE, applying 30 kV as separation voltage and using a 70 mM phosphate aqueous solution, adjusted to pH 2.5, with a mixture of 2-hydroxypropil β-CD 8 mM and β-CD 5 mM as BGE: (A) pressure of injection (20 ng/mL, 10 min, in-line SPE: 2 mm length and 150 µm id), (B) time of injection (10 ng/mL, 3 bar, in-line SPE: 2 mm length and 150 µm id), (C) SPE id (5 ng/mL, 3 bar, 20 min, in-line SPE: 2 mm length), and (D) SPE total length (5 ng/mL, 3 bar, 20 min, in-line SPE: 150 µm id).

Figure 3. Electropherogram obtained under optimum conditions for LLE/in-line SPE-CE from pooled blank urine spiked with the studied compounds at a concentration of 40 ng/mL. Peak assignments: as in Figure 1.

Table 1. LODs of standard solutions, with and without in-line SPE, and their corresponding enrichment factors.

Analyte	LOD without in-line SPE (ng/mL) (A)	LOD with in-line SPE (ng/mL) (B)	Enrichment factor (A/B)
Mephedrone	4000	0.5	8000
Mephedrone'	4000	0.5	8000
4-Methylephedrine	3500	0.5	7000
4-Methylephedrine'	3500	0.5	7000
MDPV	4500	0.75	6000
MDPV’	4500	0.75	6000

Table 2. Regression equations, repeatability and reproducibility values and LODs obtained for urine samples by LLE/in-line SPE-CE.

	Mephedrone	Mephedrone'	4-Methylephedrine	4-Methylephedrine'	MDPV	MDPV'
Linearity (ng/mL)	10-1000	10-1000	10-1000	10-1000	5-1000	5-1000
Calibration curve	y = 0.1008x + 28.1572	y = 0.0985x + 24.642	y = 0.2632x + 34.5367	y = 0.3358x + 22.7856	y = 1.8842x + 66.4233	y = 4.1442x - 63.6997
r^2	0.9930	0.9954	0.9981	0.9986	0.9878	0.9975
LOD (ng/mL)	8	8	7	7	3	3
Intraday RSD of peak area (%, n=5)						
20 ng/mL	5.0	6.3	8.5	8.9	4.6	5.2
100 ng/mL	6.2	6.5	6.1	5.8	4.7	4.2
Interday RSD of peak area (%, n=5)						
20 ng/mL	5.8	9.5	11.2	12.4	7.3	7.6
100 ng/mL	6.8	7.1	7.1	8.0	6.0	5.9

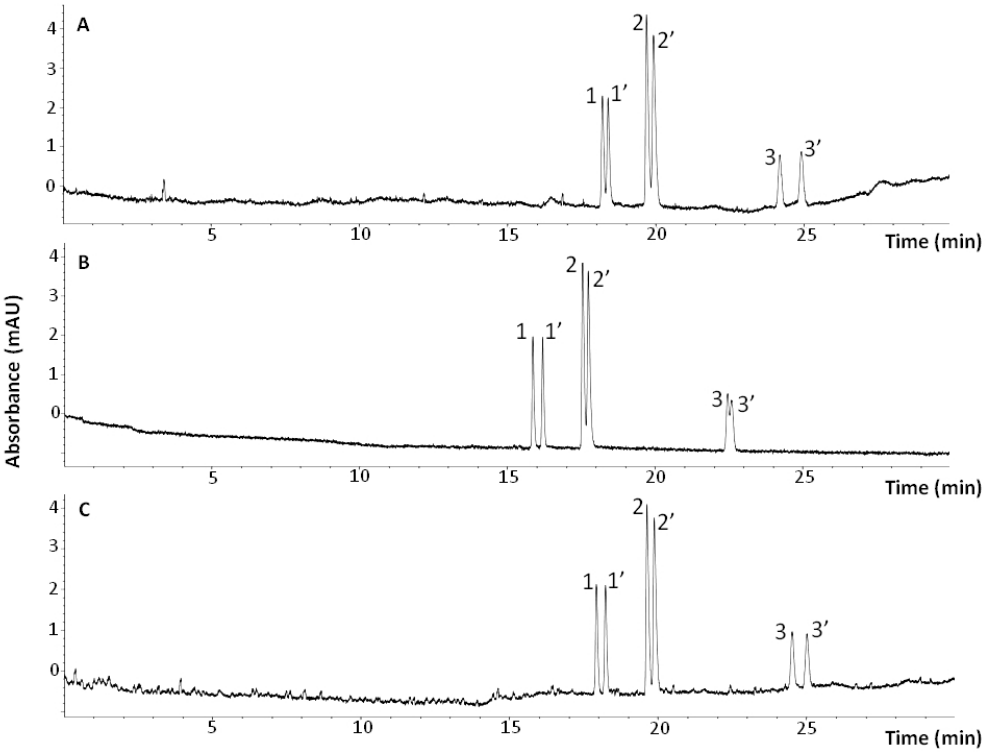

Figure 1

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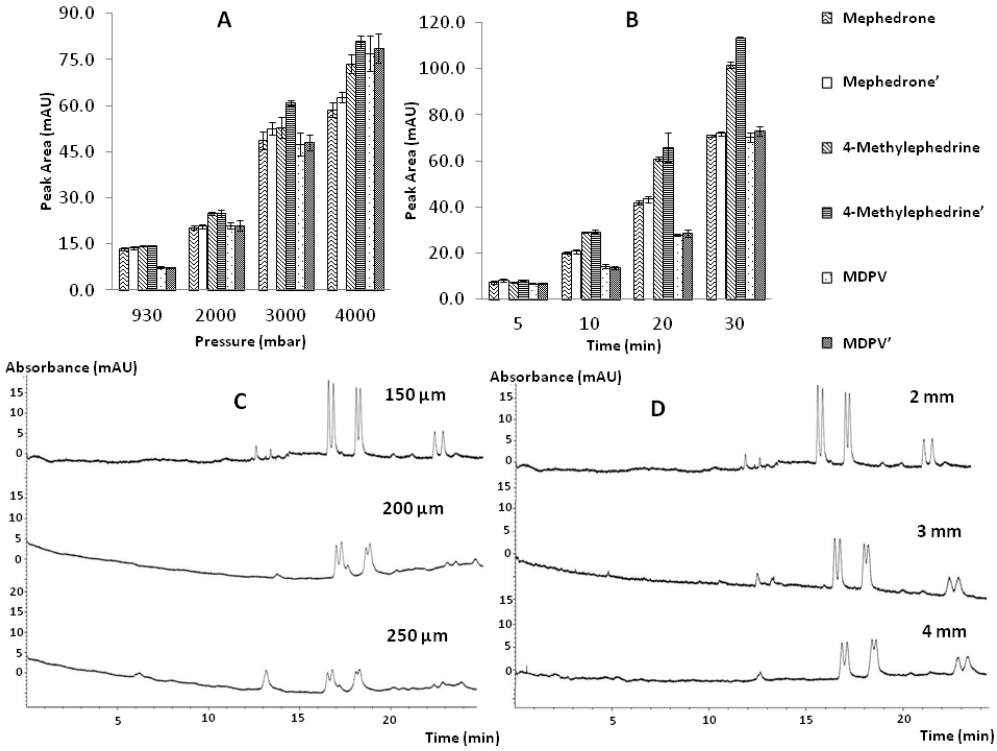

Figure 2

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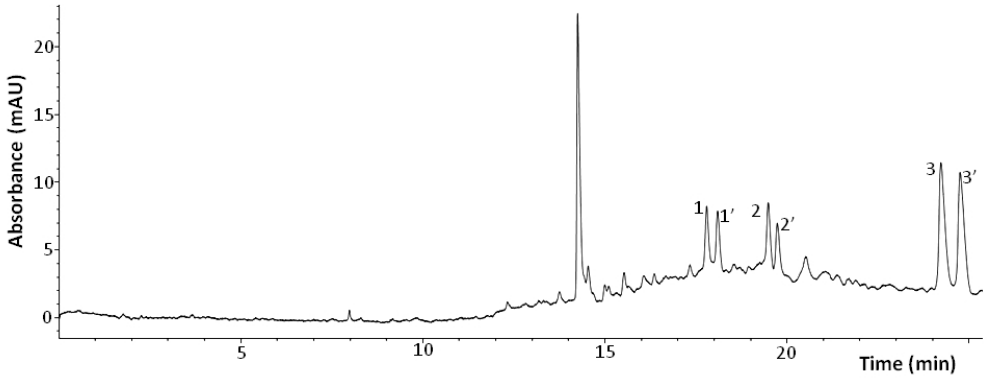

Figure 3

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