An electrokinetic supercharging approach for the enantiodetermination of cathinones in urine samples by capillary electrophoresis

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Abbreviations: **BGE,** background electrolyte; **CD,** cyclodextrin; **EKS**, electrokinetic supercharging; **CE,** capillary electrophoresis; **FASI**, field amplified sample injection; **GC-MS,** gas chromatography-mass spectrometry; **LC-MS,** liquid chromatography-mass spectrometry; **LE,** leading electrolyte; **LLE**, liquid-liquid extraction; **LOD,** limit of detection; **LOQ,** limit of quantification; **MeOH**, methanol; **MDPV**, methylenedioxypyrovalerone; **NPS**, new psychoactive substances; **TE**, terminating electrolyte; **t-ITP**, transient isotachophoresis;

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Abstract

This paper presents a strategy based on electrokinetic supercharging (EKS) in combination with capillary electrophoresis (CE) for the enantiodetermination of a group of cathinones in urine samples after sample pretreatment with liquid-liquid extraction (LLE). The background electrolyte (BGE) consisted of an aqueous solution of 70 mM of monosodium phosphate at pH 2.5 containing 8 mM 2-hydroxypropyl β-cyclodextrin (β-CD) and 5 mM β-CD, which acted as chiral selectors. To solve capillary electrophoresis's lack of sensitivity, we used EKS as the preconcentration strategy. We also tested several parameters affecting this strategy, i.e. the choice of suitable leading and terminating electrolytes, their injected volumes, and the sample injection times and voltages. The highest response enhancement was achieved by electrokinetically injecting the sample (10 kV, 120 s) between a leading electrolyte (LE) of 25 mM HCl and a terminating electrolyte (TE) of 75 mM tetrabutylammonium bromide, both of which were hydrodynamically injected at 50 mbar for 40 s. The method was validated by analysing spiked urine samples. The linear range went from 15 to 250 ng/mL, limits of detection (LODs) were between 4 and 8 ng/mL, and RSDs were below 11 % in terms of intra-day and inter-day repeatability. This is therefore an efficient method for the enantiodetermination of cathinones in urine samples by forensic laboratories.

1. Introduction

Novel psychoactive substances (NPS) are a challenging problem worldwide. These compounds are widely available on various websites but, unlike classical synthetic drugs such as cocaine and amphetamines, knowledge of their health effects is limited. One of the most common types of NPS reported in the literature are cathinones [1]. These synthetic derivates from cathinone, a beta-ketone amphetamine naturally found in the leaves of the khat plant, currently represent a more affordable and accessible alternative to other well-known illicit drugs such as amphetamines [1,2].

Cathinones are usually ingested or nasally insufflated [3]. After their consumption, they can be found in the organism either metabolized or in their pure form at low concentrations. Highly sensitive methods generally based on liquid chromatography-mass spectrometry (LC-MS) [4–11] or gas chromatography-mass spectrometry (GC-MS) [12–15] have been developed to analyse these compounds in biological samples such as hair [8], oral fluids [9,11], blood [10,12] or, more commonly, urine [4–7,12–15], which is both easy to collect and non-invasive and has a detection window that can last weeks.

An important feature of cathinones is their chirality. The presence of an asymmetric carbon in their structure implies the presence of two enantiomers, only one of which displays biological activity in the human body while the other is inactive or may exhibit unwanted side effects [2]. In fact, the stimulant effects of cathinones are mainly attributed to the *S*(-) form [2,3,16,17]. There is therefore a need for analytical methods that allow the enantiodetermination of these compounds. In recent years, CE has been found to be an excellent technique for this purpose since enantioseparation can be achieved simply by adding a chiral selector to the background electrolyte (BGE) [18,19]. Several compounds have been successfully used as chiral selectors in CE. With cathinones, the compounds usually used for that purpose are cyclodextrins [20–23]. However, despite all the advantages of CE (mainly simplicity and the low consumption of sample and reagents), CE's lack of sensitivity, especially when coupled with UV detection, limits its application when low detection limits are needed.

To improve sensitivity, authors have proposed various preconcentration strategies based on stacking principles. With these strategies, a large volume of sample is usually injected into the capillary and the analytes are then focused into a narrow zone [24,25]. Although sensitivity is increased by these approaches, it can sometimes be limited. To increase sensitivity, numerous authors have proposed combining two or more preconcentration techniques. One example of this is EKS, which combines field-amplified sample injection (FASI) with transient isotachophoresis (t-ITP) [26–33]. EKS overcomes the main drawback of FASI – the limited amount of sample that can be injected before excessive band broadening affects the separation quality – because the extra t-ITP step involved can preconcentrate such bands. To perform EKS, the analytes are electrokinetically introduced between a LE, which contains ions with a higher mobility than the analytes, and a TE, which contains ions with a lower mobility than the analytes. Under these conditions, the analytes can migrate more quickly in the TE zone and more slowly in the LE zone, so they become focused on the LE/TE interface. This produces an important gain in sensitivity and several authors have reported high sensitive enhancement factors (SEFs) in the determination of various kinds of analytes using this preconcentration strategy in combination with CE [26–33]. Some of these applications have used EKS-CE as the preconcentration method for analysing biological samples. For example, enhancement factors between 160 and 600 were obtained in the determination of tamoxifen and its metabolites in human plasma [30] and between 2193 and 2976 in the determination of biogenic amines in mice brain [31]. Both these studies based their pretreatment on LLE.

The aim of the present study is to develop a sensitive method based on EKS-CE for the chiral determination of four cathinones in urine samples. To the best of our knowledge, no study has

yet been based on EKS for the enantiodetermination of this kind of compounds in biological samples.

2. Materials and methods

2.1. Reagents and standards

All reagents were of analytical reagent grade. Cyclohexane, methanol (MeOH), methyl tert-butyl ether and toluene were purchased from J.T. Baker (Deventer, Netherlands). Ammonium hydroxide 28%, β-CD 97%, 2-hydroxypropyl β-CD, hydrochloric acid 37 %, phosphoric acid 85 %, potassium chloride 99 %, monosodium phosphate 99 %, sodium acetate 99 %, NaOH 97 %, tetrapropylammonium bromide 98 %, tetrabutylammonium bromide 98% and tetrapentylammonium bromide 99 % were acquired from Sigma-Aldrich (Saint Louis, MO, USA). NaCl 99% was purchased from Fluka Honeywell (Morris Plains, NJ, USA). Milli-Q water was obtained with a water purification system from Veolia Water (Paris, France).

The standards of *R*,*S*-methylone, *R*,*S*-mephedrone and *R*,*S*-MDPV were purchased as hydrochloride salts with a purity of 98% from LGC Standards (Teddington, UK). *R*,*S*-4-methylephedrine was acquired as a solution of 1 mg/mL in MeOH from LGC Standards (Teddington, UK). Standard stock solutions of the cathinones (100 mg /L of *R*,*S*-4-methylephedrine, 1000 mg /L of *R*,*S*-mephedrone and 2000 mg/L of *R*,*S*-methylone and *R*,*S*-MDPV) were prepared in MeOH and stored in the freezer at -20 ºC. Working standard solutions of a mixture of all the compounds at a concentration of 20 µg/mL were prepared weekly by diluting the stock standard solutions in Milli-Q water and kept at 4 ºC. The solutions with lower concentrations were prepared daily by diluting suitable volumes of the working standard solutions in Milli-Q water.

2.2. Instrumentation

For the electrophoretic separations we used a 7100 CE System from Agilent Technologies (Waldbronn, Germany) equipped with a UV DAD. All pH measurements were carried out with a GLP 21 pH-meter from Crison (Barcelona, Spain). A Universal 32 R centrifuge from Hettich (Kirchlengern, Germany) was also used.

2.3. CE conditions

The BGE, which consisted of 70 mM of monosodium phosphate, 8 mM of 2-hydroxypropyl β-CD and 5 mM of β-CD (adjusted to pH 2.5 with concentrated phosphoric acid), was prepared by dissolving the appropriate amount of each compound in Milli-Q water.

A bared fused-silica capillary of 50 µm id and 80 cm in total length (72 cm effective length), acquired from Polymicro Technologies (Phoneix, AZ, USA), was used as separation capillary.

New capillaries were conditioned by subsequently flushing 1 M NaOH for 40 min and Milli-Q water for 10 min. At the start of each working day, the capillary was conditioned with 0.1 M NaOH for 10 min, Milli-Q water for 5 min and BGE for 5 min, all at 930 mbar. Between runs, a conditioning step was performed with 0.1 M NaOH, Milli-Q water and BGE, all at 930 mbar for 4 min. A postconditioning step was carried out at the end of each run by flushing Milli-Q water for 5 min at 930 mbar to ensure good reproducibility.

For all experiments, the capillary chamber was heated at 25 ºC and the wavelength used to detect the cathinones was 200 nm.

2.4. EKS-CE procedure

EKS-CE was performed as follows. First, a plug of LE, consisting of an aqueous solution of 25 mM of HCl, was hydrodynamically injected at 50 mbar for 40 s. The samples were then electrokinetically injected by applying a voltage of 10 kV for 120 s. The next step was to hydrodynamically inject the TE, an aqueous solution of 75 mM of tetrabutylammonium bromide, at 50 mbar for 40 s. Finally, a separation voltage of 30 kV was applied, and separation took place.

2.5. Sample preparation

Drug-free urine samples were obtained from several non-addicted volunteers. These were collected in polypropylene tubes and kept in the freezer at -20 ℃ until analysis. Pooled urine, prepared by mixing the urine samples obtained from the volunteers, was used to validate the method. Before beginning the procedure, the analytes were added to the urine to simulate a real sample.

Before electrophoretic analysis, liquid-liquid extraction (LLE) was performed as sample pretreatment for extracting target compounds from the urine samples. The procedure was as follows: 2 mL of cyclohexane were added to a 2 mL urine sample alkalized to pH 10 with 28 % ammonium hydroxide. After vortex mixing for 1 min, the samples were centrifuged for 10 min at 9000 rpm. The organic phase, containing the cathinones, was then transferred to a vial and immediately a second extraction was performed by adding another 2 mL of cyclohexane to the remaining aqueous phase and repeating the same procedure. Next, the two organic phases were combined, and the final extract was evaporated to dryness under a gentle stream of N_2. The residue was reconstituted with 2 mL of Milli-Q water (adjusted to pH 6 with concentrated HCl). Finally, a 0.45 µm PTFE syringe filter was used to filter the sample and the extract was transferred to a microvial for analysis. This pretreatment procedure took about 40 min to be completed.

3. Results and discussion

3.1. Enantioseparation by CE

The BGE consisted of an aqueous solution of 70 mM of monosodium phosphate, 8 mM of 2-hydroxypropyl β-CD and 5 mM of β-CD (adjusted to pH 2.5 with concentrated phosphoric acid). This was selected after a recent study by our research group in which the same group of cathinones were also enantioseparated [22]. For all experiments the cathinones were solved in an aqueous solution at pH 6. At this pH, the cathinones are positively charged, since they present pKa values between 8.83 and 9.13 [34], which should support their electrokinetic injection in EKS.

3.2. EKS optimization

EKS is a powerful preconcentration strategy that combines FASI with t-ITP. It involves the electrokinetic injection of a long plug of sample between a high mobility LE and a low mobility TE. Its enormous potential for preconcentrating and determining different analytes in different matrixes has been demonstrated [26–33]. However, as far as we know, EKS has not yet been tested for analysing cathinones. We therefore investigated using EKS as an on-line preconcentration strategy for improving the detection sensitivity of the target cathinones. To do so, we studied the parameters that affect the preconcentration efficiency of EKS, including the choice of suitable LE and TE, their injected volumes, and the sample injection time and voltage.

3.2.1. Optimization of the LE

An important characteristic of an effective LE is that it must have faster mobility than the target analytes [27]. The cathinones studied present electrophoretic mobilities between 22.3 x 10^{-9} and 14.1 x 10^{-9} m^2 V^{-1} s^{-1} in their cationic form. We therefore evaluated as LE different salts that contain a co-ion with high electrophoretic mobility. In particular, we evaluated the following co-ions: sodium, potassium and proton, which present the following electrophoretic mobilities 51.9 x 10^{-9}, 76.2 x 10^{-9} and 362.5 x 10^{-9} m^2 V^{-1} s^{-1}, respectively [29]. For this optimization, a standard sample containing the target compounds at a concentration of 1.25 µg/mL was electrokinetically injected by applying 10 kV for 20 s after hydrodynamic introduction of the LE (40 s, 50 mbar) at a concentration of 50 mM. The TE (40 s, 50 mbar), which consists of an aqueous solution of 50 mM of tetrabutylammonium bromide, was then added. Our results for the various LE tested are shown in Fig. 1A. For all the cathinones under study, the greater the difference between the electrophoretic mobilities of the analytes and the LE, the greater the response, being HCl which provided the best stacking efficiencies of all the LE. On the other hand, when NaCl and KCl were used as LE, the response was lower because the difference between the electrophoretic mobilities of sodium and potassium ions and the electrophoretic mobilities of the target compounds was not big enough to generate an efficient ITP state [27]. Moreover, the results were fairly similar for these two LE, probably because of the similarity between their electrophoretic mobilities. HCl was therefore selected as the most suitable LE for later experiments.

The concentration of LE can also influence the t-ITP process. On the one hand, the concentration of LE must be high enough to provide effective sample stacking (at least 50 times higher than the concentration of the target compounds). On the other hand, a highly concentrated LE can negatively affect the current during CE separation due to an increase in the Joule effect [27]. To find a proper LE concentration, different concentrations of HCl were tested (25, 50, 75 and 100 mM) under the same conditions as those used to study the nature of the LE, except that the concentration of the analytes was lower, i.e. 400 ng/mL in this case. Fig. 1B shows that the stacking efficiency decreased for all cathinones as the LE concentration increased. Despite that trend, when lower concentrations of LE were evaluated (below 25 mM), reproducibility problems were observed. The optimum LE concentration was therefore set at 25 mM for later experiments.

Finally, we also studied the effect of the amount of LE by evaluating its hydrodynamic injection for different times (10, 40, and 70 s). All experiments were performed under the same conditions as the previous optimization but with an aqueous solution of 25 mM of HCl as the LE. Fig. 1C shows that when LE injection time increased, the peak areas for all target compounds increased. However, note that a high LE volume can reduce the resolution between cathinones. This is due to a more prolonged t-ITP zone in the capillary before the destacking step, which reduces the capillary length available for CZE separation [26,30]. In our case, although there was not so much loss in resolution at high injection times, we observed current instability problems when using 70 s. To prevent further current disturbances, we therefore selected 40 s as the optimum value.

3.2.2. Optimization of the TE

The ideal TE has a lower electrophoretic mobility than the analytes. Tetra-substituted ammonium salts could therefore become optimal TE candidates since the tetra substitution increases the size of these salts and considerably reduces their electrophoretic mobility. For example, tetrabutylammonium bromide has been used successfully as TE for analysing melamine in milk powder and liquid milk by EKS-CE [29]. We therefore tested the applicability of three tetra-substituted ammonium salts as TE, i.e. tetrapropylammonium bromide,

tetrabutylammonium bromide, and tetrapentylammonium bromide. For this optimization, a standard sample containing the analytes at a concentration of 200 ng/mL was electrokinetically injected after the hydrodynamic injection of LE under the above optimized conditions. The TE at a concentration of 50 mM was then injected at 50 mbar for 40 s. Our results are shown in Fig. 2A. With tetrapentylammonium bromide, the chiral separation of the cathinones was completely distorted, and with this TE the resolution between cathinones was negatively affected, and for some of them a peak overlap was observed, so this TE was discarded. For the other two TE, the best response for all compounds was obtained with tetrabutylammonium bromide. This can be attributed to the greater difference between the corresponding mobility of this salt and the mobilities of the analytes.

TE concentration can also affect the t-ITP process. As with LE, TE concentration must be high enough to provide effective sample stacking but low enough to avoid increasing the Joule heating effect [27]. We tested various concentrations of tetrabutylammonium bromide (25, 50, 75 and 100 mM) under the same experimental conditions as those in the previous study. As we can see in Fig. 2B, when the concentration increased from 25 to 75 mM the peak areas increased but at higher TE concentrations the response slightly decreased. We therefore selected 75 mM as the optimal TE concentration.

Finally, we also evaluated TE injection time. The TE zone must be large enough to ensure efficient sample stacking [30]. However, if this zone is too large, it reduces the capillary length available for the separation of the stacked analytes and separation efficiency may be seriously affected [26,30]. The TE injection time was optimized by testing different times (10, 40, and 70 s). For this study, we used the same conditions as for the optimization of TE concentration. As expected, our results (see Fig. 2C) show that the response was highest when 70 s was used as the TE injection time. However, under these conditions some current disturbances were detected. To prevent these problems, we selected 40 s as the TE injection time.

3.2.3. Sample injection optimization

We also evaluated the effect of the sample injection conditions (voltage and time). In theory, a longer sample injection time means that a larger sample volume can be introduced into the capillary, thus increasing detection sensitivity. However, a longer injection time can also decrease the resolution between the analytes, thus hindering their enantioseparation. It is important, therefore, to consider both the gain in sensitivity and good separation efficiency. In this study, the LE was first injected at the previous optimized conditions and a standard sample containing the analytes at a concentration of 100 ng/mL was then injected by applying 10 kV between 20 and 140 s. Finally, the TE was hydrodynamically injected under the optimum conditions. The analytes repsonses are shown in Fig. 3A. As we can see, when the sample injection time increased, the response obtained for all compounds gradually increased. However, when the injection time exceeded 120 s, current disturbances were observed. It was therefore not possible to evaluate longer injection times. Fig. 3B shows the resolutions obtained in this study. Resolution slightly decreased as the injection time increased, but the values obtained remained practically constant. We therefore concluded that we could introduce a large amount of sample without losing much resolution. The effect of sample injection voltage on peak sensitivity was also tested. A decrease in the injection voltage could induce higher current stability, which could allow for longer electrokinetic injection times and a higher sample volume [27]. The effect of using a lower injection voltage was therefore also evaluated. To do so, an injection voltage of 2 kV was tested between 300 and 500 s (since the voltage was lower, the injection time was increased to obtain similar responses to those obtained by applying 10 kV). At sample injection times above 460 s, current disturbances occurred and the response for all analytes was lower than when 10 kV was applied for 120 s.

The electrokinetic injection conditions selected to achieve efficient sample stacking and high detection sensitivity were an injection voltage of 10 kV and an injection time of 120 s.

3.3. Sensitivity enhancement factor

To determine the increase in the response of the compounds obtained with EKS-CE compared to CE, we calculated the sensitivity enhancement factors in terms of peak areas (SEF_{area}) and peak heights (SEF_{height}). To do so, we compared the peak areas and peak heights obtained under the optimized EKS conditions with those obtained by a CE procedure in which the sample was hydrodynamically injected at 50 mbar for 5 s.

The values of SEF_{area} were 1314, 1313, 722 and 940 and the values of SEF_{height} were 1213, 1310, 993 and 1161 for *R*,*S*-mephedrone, *R*,*S*-methylone, *R*,*S*-4-methylephedrine and *R*,*S*-MDPV, respectively, for standard solutions. Fig S1. of the supplementary material presents the comparison of two electropherograms obtained for a standard sample containing the studied compounds at a concentration of 30 µg/mL hydrodynamically injected at 50 mbar for 5 s (A), and for a standard sample containing the studied compounds at a concentration of 30 ng/mL, obtained by the optimized EKS-CE procedure (B). As it can be seen, despite the difference between the standards concentrations for both cases, the obtained responses for each cathinone were very similar. These results demonstrate the power of preconcentration with EKS as a stacking strategy and clearly show that this dual on-line focusing strategy significantly improved sensitivity for the cathinones under study.

3.4. Urine sample pretreatment

This method was used to analyse urines samples. Before doing so, however, we needed to develop a sample pretreatment because of the co-ions that may be present in the sample matrix and that can affect the EKS procedure [35].

LLE was tested as the sample pretreatment because in previous studies it was shown to be effective in extracting cathinones from urine samples [4–6,12–15,22]. Several organic solvents were evaluated to achieve optimum extraction. These were cyclohexane, dichloromethane (DCM), ethyl acetate/isopropanol (4:1), hexane, methyl tert-butyl ether and toluene. The LLE procedure comprised the following steps. First, the urine samples were alkalized to pH 10. Then, 2 mL of organic solvent were added to 2 mL of the urine sample. Next, samples were vortex mixed for 1 min and then centrifuged for 10 min at 9000 rpm. After separating the organic phase, the extraction procedure was repeated by adding another 2 mL of organic solvent. The organic extracts were then combined and evaporated to dryness under a gentle stream of N_2. The residue was then reconstituted with 2 mL of Milli-Q water at pH 6 and filtered before analysis using EKS-CE. Our results show that the only organic solvents that successfully extracted the target compounds were toluene and cyclohexane.

Recovery values were calculated as the ratio between the response obtained for a urine sample spiked with the analytes at a concentration of 100 ng/mL after all the procedure was performed and the response obtained for a standard sample containing the cathinones at a concentration of 100 ng/mL when the LLE step was not used. The recoveries ranged from 29 to 80 % and from 50 to 98 % for toluene and cyclohexane, respectively. Cyclohexane was therefore selected as the extraction solvent.

Fig. 4 shows the electropherogram obtained under the optimum conditions: a urine sample spiked with the target compounds at a concentration of 100 ng/mL and performing EKS-CE after an LLE procedure in which cyclohexane was used as organic solvent.

3.5. Method validation

The suitability of this EKS-CE procedure was tested by validating the method in terms of linearity, selectivity, intra-day and inter-day repeatability, accuracy, limits of detection and limits of quantification (LOQs) in accordance with the *Guidance for the validation of analytical methodology and calibration of equipment used for testing of illicit drugs in seized materials and biological specimens* [36].

All validation studies were carried out by employing pooled urine samples collected from non-addicted volunteers spiked with a known amount of the target compounds. Table 1 shows the values obtained for the validation process.

Linearity was evaluated using a matrix match calibration curve in a concentration range between 15 and 250 ng/mL for mephedrone and between 20 and 250 ng/mL for the other cathinones under study. Under these conditions, correlation coefficients (r^2) were above 0.990 for all compounds.

Selectivity was tested by analysing 10 blank urine samples from different individuals. When these samples were evaluated using the method we have developed, compounds that could interfere with the target analytes were not observed, as can be seen in Fig 4A.

The precision of the whole method was tested in terms of intra-day and inter-day repeatability at three concentration levels, i.e. 20 ng/mL, 100 ng/mL and 250 ng/mL. Intra-day repeatability was evaluated on the same day by analysing five replicates of urine spiked at each concentration level. Inter-day repeatability was evaluated on five different days by analysing five replicates of urine spiked at each concentration level. The results, expressed as relative standard deviation (RSD), are shown in Table 1. Since the method presented RSD values below 10 % and 11 % for repeatability and reproducibility, respectively, the method proved to be satisfactory in terms of precision.

Because of the lack of availability of real urine samples from cathinone abusers, and to prove the accuracy of the method, the relative errors of the peak areas (% RE) were evaluated at three concentration levels, i.e. 20 ng/mL, 100 ng/mL and 250 ng/mL. The values were calculated by analysing five replicates of urine spiked at each concentration level and using the following equation:

$$\%\ RE = \frac{|\text{experimental average response} - \text{theoretical response obtained in the calibration curve}|}{\text{theoretical response obtained in the calibration curve}} \cdot 100$$

Table 1 shows the relative errors. All values were below 10 % for all three concentration levels.

LODs were calculated using a signal-to-noise criterion of 3, while the LOQs were established as the lowest point of the linear range. LODs for the target compounds were 4, 8, 7 and 6 ng/mL for mephedrone, methylone, 4-methylephedrine, and MDPV, respectively. The LODs were higher to those achieved when cathinones were determined in urine samples using methods based on LC-MS (LODs from 0.25 to 5 ng/mL [4,5,7]). In general, they were also slightly lower than those obtained with methods based on GC-MS (LODs between 1 and 50 ng/mL [12–14]). An important advantage of this strategy compared to many methods available in the literature is that the analytes could be enantioseparated. Our research group previously presented methods that were also based on CE for the enantiodetermination of cathinones using preconcentration techniques combined in-line with CE. With one of these methods we used a chromatographic preconcentration technique (in-line SPE) [22], while with the other we used an electrophoretic preconcentration technique (FASI) [37]. As we expected, the LODs obtained with the strategy developed in the present study were lower than with FASI. This is because EKS is a combination of FASI and ITP and greater sensitivity is normal with a dual preconcentration technique. On the other hand, the LODS were similar to those we obtained with in-line SPE. An important advantage of the EKS-based methodology, however, is the

shorter analysis time involved. For the in-line SPE-CE method, sample loading time was high. Also, since the in-line SPE device was homemade, we also need to consider the time taken to construct it. In general, therefore, EKS is a powerful preconcentration strategy that combines the power of preconcentration of a large electrokinetic injection with t-ITP to achieve low LODs relatively simply and easily.

4. Concluding remarks

An effective EKS-CE method for the enantiodetermination of four cathinones in urine samples was developed and validated for the first time.

Our SEF_{area} and SEF_{height} values, which ranged from 940 to 1314 and from 993 to 1310, respectively, highlight the preconcentration potential of the EKS methodology. This technique can achieve the enantiodetermination of the studied analytes with high preconcentration factors and low LODs simply by performing an in-line preconcentration procedure after LLE extraction.

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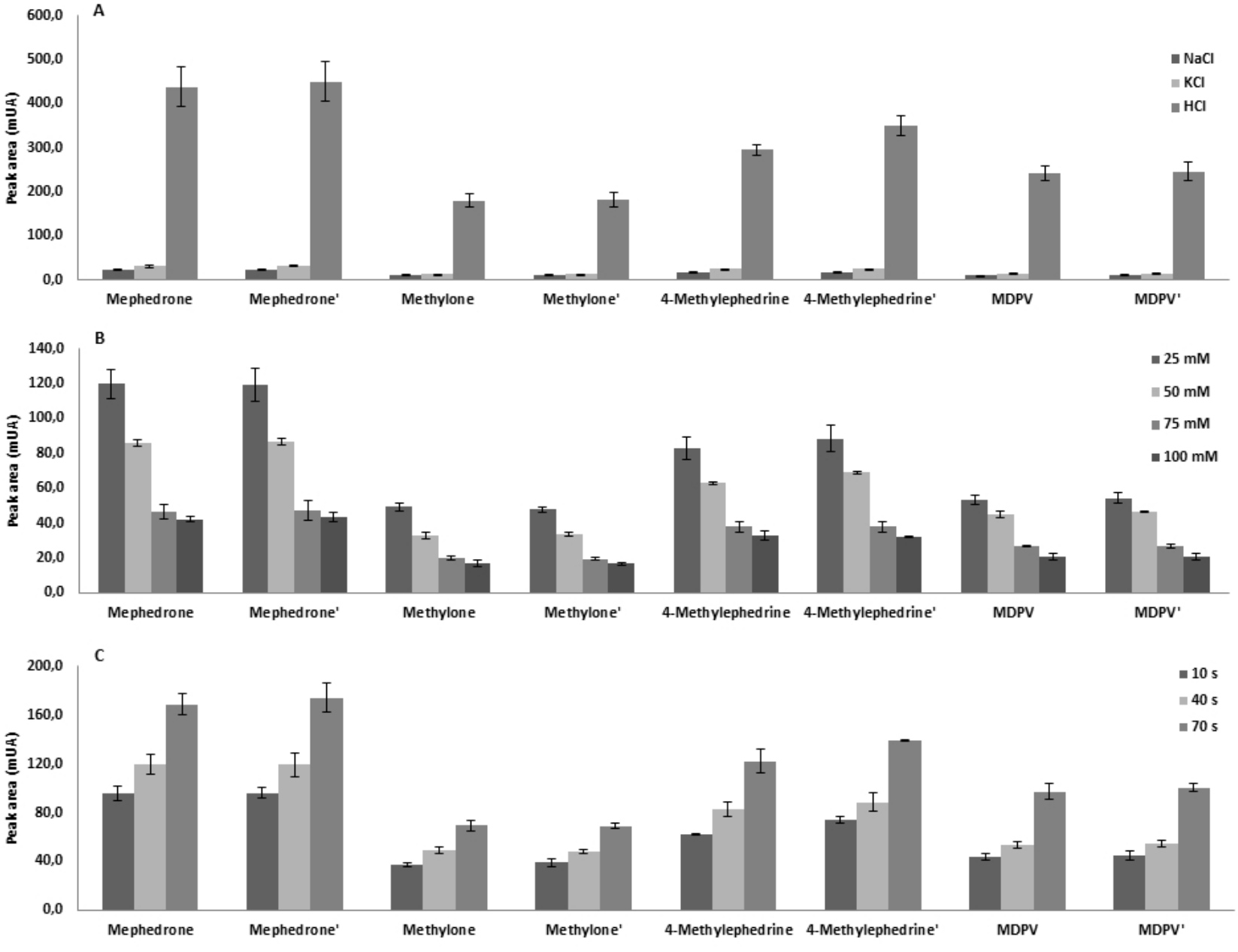
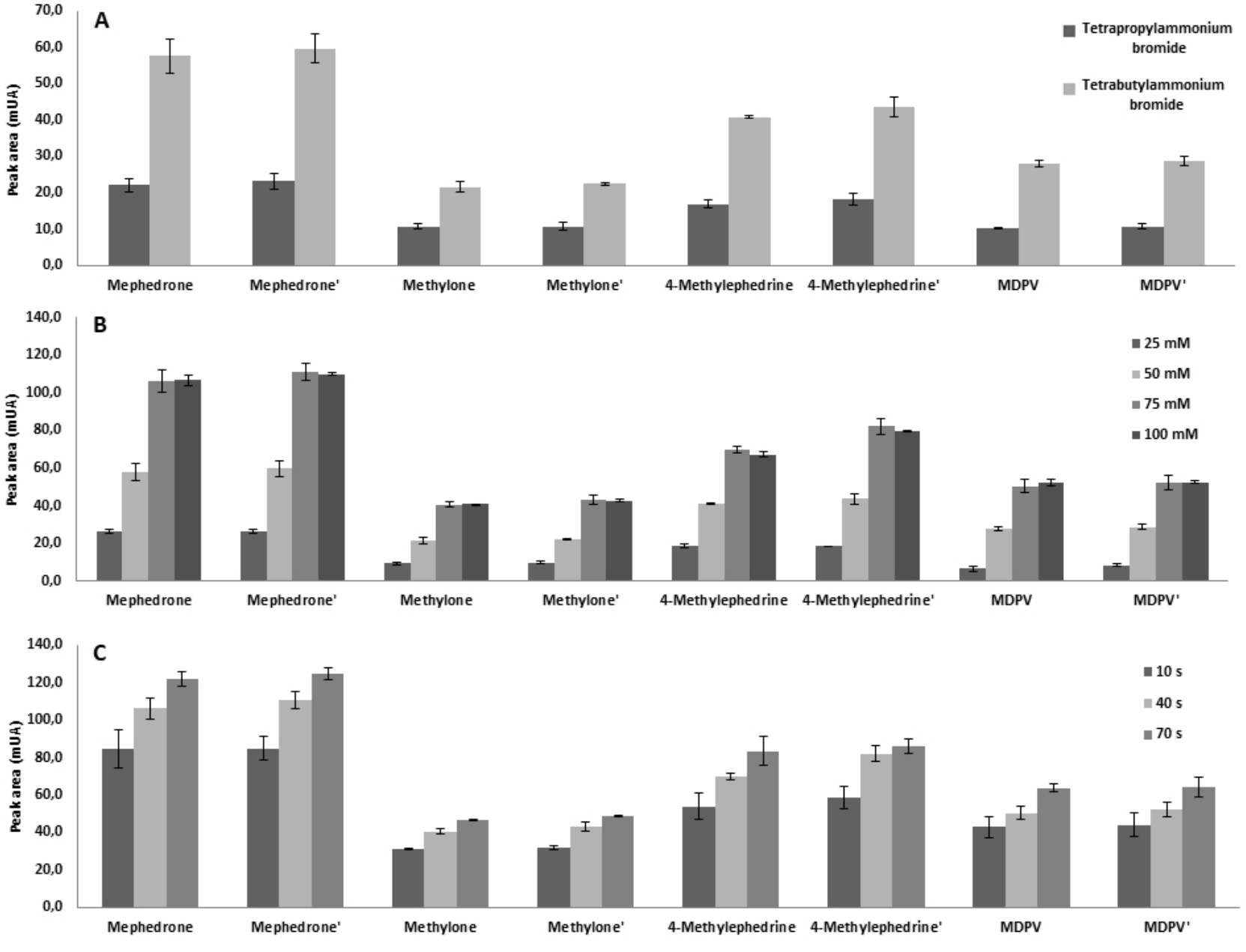
Figure 1. Optimization of the LE nature and volume. A) Optimization of LE nature. For this optimization, different LE at a concentration of 50 mM were injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 1.25 µg/mL for 20 s and applying 10 kV, 50 mM of tetrabutylammonium bromide solution was then injected at 50 mbar for 40 s. B) Optimization of LE concentration. For this optimization, a solution of HCl at different concentrations was injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 400 ng/mL for 20 s and applying 10 kV, a 50 mM of tetrabutylammonium bromide solution was then injected at 50 mbar for 40 s. C) Optimization of LE time of injection. For this optimization, a solution of 25 mM of HCl was injected at 50 mbar for different injection times. After the electrokinetic injection of a standard sample of 400 ng/mL for 20 s and applying 10 kV, a 50 mM of tetrabutylammonium bromide solution was then injected at 50 mbar for 40 s.

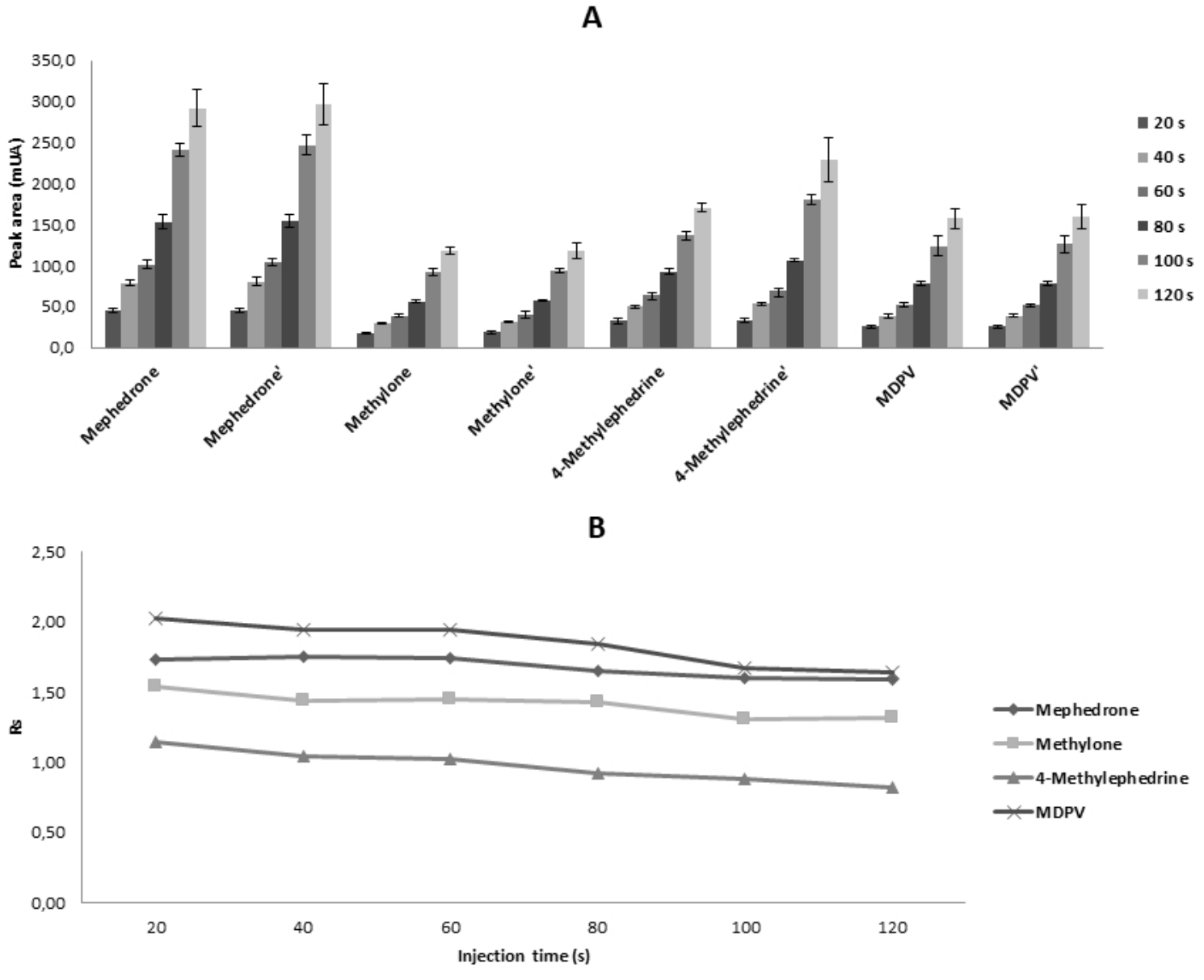
Figure 2. Optimization of the TE nature and volume. A) Optimization of TE nature. For this optimization, a solution of 25 mM of HCl was injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 200 ng/mL for 20 s and applying 10 kV, different TE at a concentration of 50 mM were then injected at 50 mbar for 40 s. B) Optimization of TE concentration. For this optimization, a solution of 25 mM of HCl was injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 200 ng/mL for 20 s and applying 10 kV, a tetrabutylammonium bromide solution at different concentrations was then injected at 50 mbar for 40 s. C) Optimization of TE time of injection. For this optimization, a solution of 25 mM of HCl was injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 200 ng/mL for 20 s and applying 10 kV, a 75 mM of tetrabutylammonium bromide solution was then injected at 50 mbar for different injection times.

Figure 3. Electrokinetic sample injection time optimization for the EKS procedure in terms of: A) peak area; and B) resolution. For this optimization, a solution of 25 mM of HCl was injected at 50 mbar for 40 s. After the electrokinetic injection of a standard sample of 100 ng/mL for different injection times and applying 10 kV, a 75 mM of tetrabutylammonium bromide solution was then injected at 50 mbar for 40 s.

Figure 4. Electropherograms of A) a blank of a urine sample and B) a urine sample spiked with the studied compounds at a concentration of 100 ng/mL, both obtained by EKS-CE after an LLE procedure using cyclohexane as organic solvent. The EKS procedure was performed by first injecting the LE solution (25 mM of HCl) at 50 mbar for 40 s. After, the sample was electrokinetically injected at 10 V for 120 s, and then, the TE solution (75 mM of tetrabutylammonium bromide) was injected at 50 mbar for 40 s. Peak assignments: (1, 1') *R*,*S*-mephedrone, (2, 2') *R*,*S*-methylone (3,3') *R*,*S*-4-methylephedrine and (4, 4') *R*,*S*-MDPV.


A
600,0
500,0
400,0
300,0
200,0
100,0
0,0
Peak area (mUA)
NaCl
KCl
HCl
Mephedrone
Mephedrone'
Methylone
Methylone'
4-Methylephedrine
4-Methylephedrine'
MDPV
MDPV'
B
140,0
120,0
100,0
80,0
60,0
40,0
20,0
0,0
Peak area (mUA)
25 mM
50 mM
75 mM
100 mM
Mephedrone
Mephedrone'
Methylone
Methylone'
4-Methylephedrine
4-Methylephedrine'
MDPV
MDPV'
C
200,0
160,0
120,0
80,0
40,0
0,0
Peak area (mUA)
10 s
40 s
70 s
Mephedrone
Mephedrone'
Methylone
Methylone'
4-Methylephedrine
4-Methylephedrine'
MDPV
MDPV'


A
Peak area (mUA)
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
Tetrapropylammonium bromide
Tetrabutylammonium bromide
Mephedrone
Mephedrone'
Methylone
Methylone'
4-Methylephedrine
4-Methylephedrine'
MDPV
MDPV'
B
Peak area (mUA)
140,0
120,0
100,0
80,0
60,0
40,0
20,0
0,0
25 mM
50 mM
75 mM
100 mM
Mephedrone
Mephedrone'
Methylone
Methylone'
4-Methylephedrine
4-Methylephedrine'
MDPV
MDPV'
C
Peak area (mUA)
140,0
120,0
100,0
80,0
60,0
40,0
20,0
0,0
10 s
40 s
70 s
Mephedrone
Mephedrone'
Methylone
Methylone'
4-Methylephedrine
4-Methylephedrine'
MDPV
MDPV'


A
Peak area (mUA)
350,0
300,0
250,0
200,0
150,0
100,0
50,0
0,0
20 s
40 s
60 s
80 s
100 s
120 s
Mephedrone
Mephedrone'
Methylone
Methylone'
4-Methylephedrine
4-Methylephedrine'
MDPV
MDPV'
B
Rs
2,50
2,00
1,50
1,00
0,50
0,00
20
40
60
80
100
120
Injection time (s)
Mephedrone
Methylone
4-Methylephedrine
MDPV

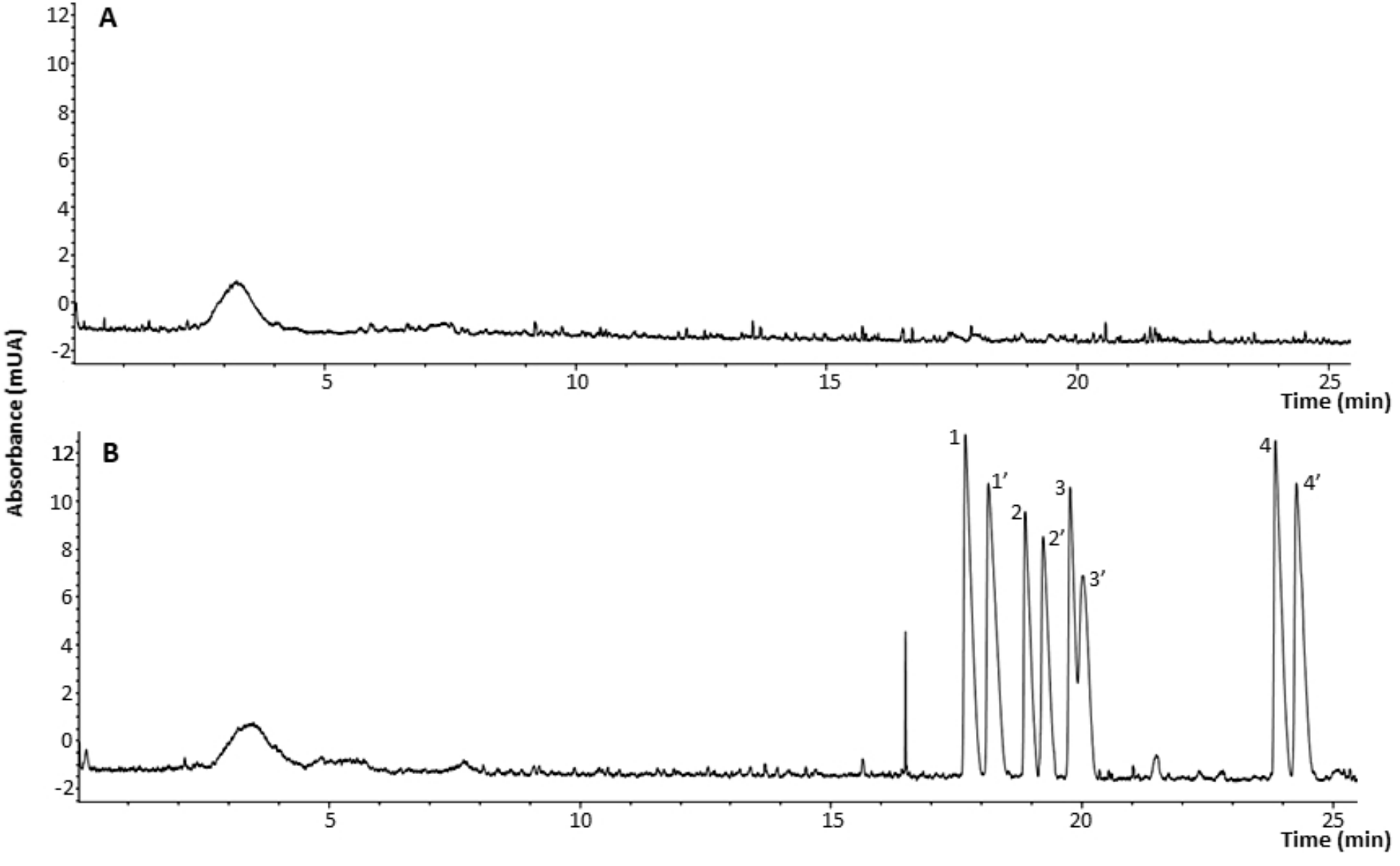

A
B
Absorbance (mUA)
Time (min)
Time (min)
1
1′
2
2′
3
3′
4
4′

Table 1. Method validation in terms of regression equations, LODs, intra-day and inter-day repeatability and relative errors obtained for urine-spiked samples from non-addicted volunteers by LLE/EKSI -CE.

	Mephedrone	Mephedrone'	Methylone	Methylone'	4-Methylephedrine	4-Methylephedrine'	MDPV	MDPV'
Linearity (ng/mL)	15-250	15-250	20-250	20-250	20-250	20-250	20-250	20-250
Calibration curve	y = 1.6034x - 10.063	y = 1.6438x - 10.793	y = 0.912x - 5.5065	y = 0.9396x - 7.7627	y = 1.1102x - 9.3257	y = 1.1751x - 10.158	y = 1.3935x - 6.4722	y = 1.389x - 7.3039
r^2	0.9903	0.9930	0.9938	0.996	0.9925	0.9922	0.9955	0.9973
LODs	4	4	8	8	7	7	6	6
Intra-day repeatability RSD in terms of peak area (%, n = 5)								
20 ng/mL	6.1	6.1	9.3	8.9	7.0	6.9	6.8	6.6
100 ng/mL	5.2	5.2	8.0	7.5	6.0	5.8	5.9	5.7
250 ng/mL	5.7	5.4	8.4	8.2	6.4	6.4	6.3	6.0
Inter-day repeatability RSD in terms of peak area (%, n = 5)								
20 ng/mL	7.4	8.0	10.3	9.7	8.1	8.5	8.7	9.3
100 ng/mL	6.7	6.5	9.2	8.9	7.8	7.4	7.6	6.7
250 ng/mL	7.3	7.4	9.9	9.9	7.8	7.7	8.1	8.5
Relative error in terms of peak area (%, n = 5)								
20 ng/mL	7.7	7.9	9.3	9.5	8.4	8.6	8.5	8.8
100 ng/mL	6.9	7.0	8.4	8.5	7.5	7.6	7.7	7.6
250 ng/mL	7.5	7.7	8.7	9.1	8.0	8.3	8.0	8.1

Declaration of interests

 The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Credit Author Statement

Albert Pérez-Alcaraz: Conceptualization, Methodology, Validation, Formal analysis, Investigation Writing - Original Draft, Visualization. **Carme Aguilar:** Conceptualization, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Marta Calull:** Conceptualization, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Francesc Borrull:** Resources, Project administration, Funding acquisition.

Supplementary material

An electrokinetic supercharging approach for the enantiodetermination of cathinones in urine samples by capillary electrophoresis

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Supplementary figures

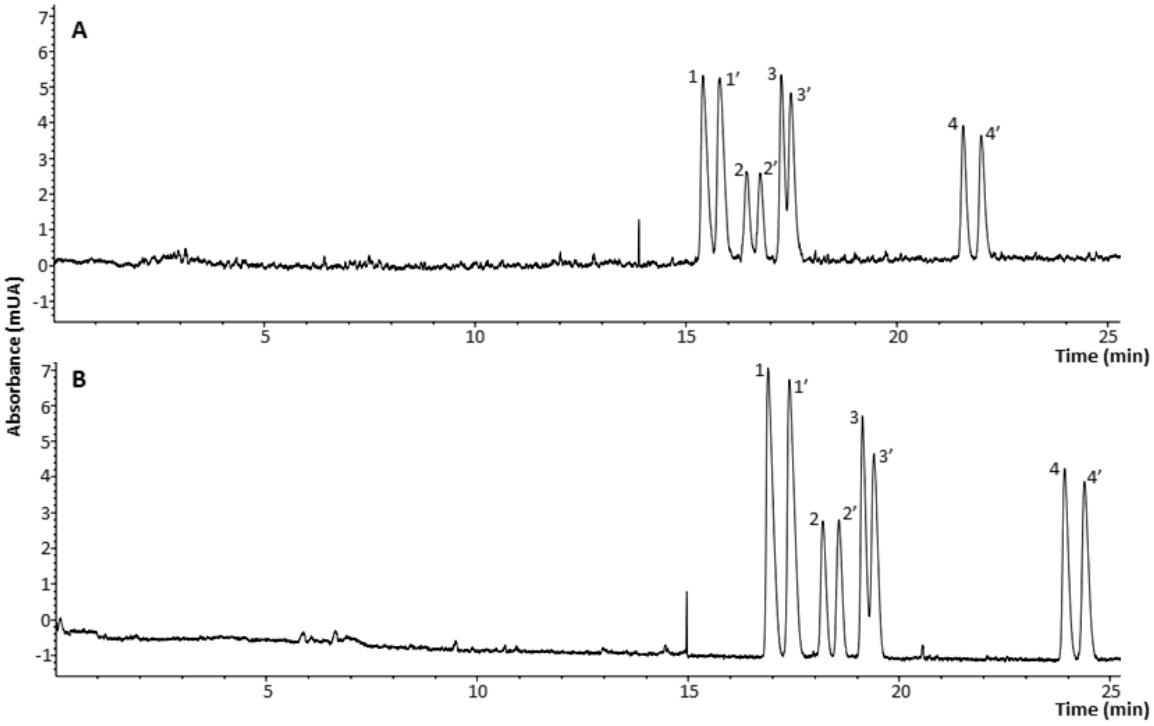

Figure S1. Comparative of the electropherograms obtained for A) a standard sample containing the studied compounds at a concentration of 30 µg/mL hydrodynamically injected at 50 mbar for 5 s and B) a standard sample containing the studied compounds at a concentration of 30 ng/mL, obtained by the optimized EKS-CE procedure. The EKS procedure was performed by first injecting the LE solution (25 mM of HCl) at 50 mbar for 40 s. After, the sample was electrokinetically injected for 120 s and applying 10 kV, and then, the TE solution (75 mM of tetrabutylammonium bromide) was injected at 50 mbar for 40 s. Peak assignments: (1, 1') *R,S*-mephedrone, (2, 2') *R,S*-methylone (3,3') *R,S*-4-methylephedrine and (4, 4') *R,S*-MDPV.