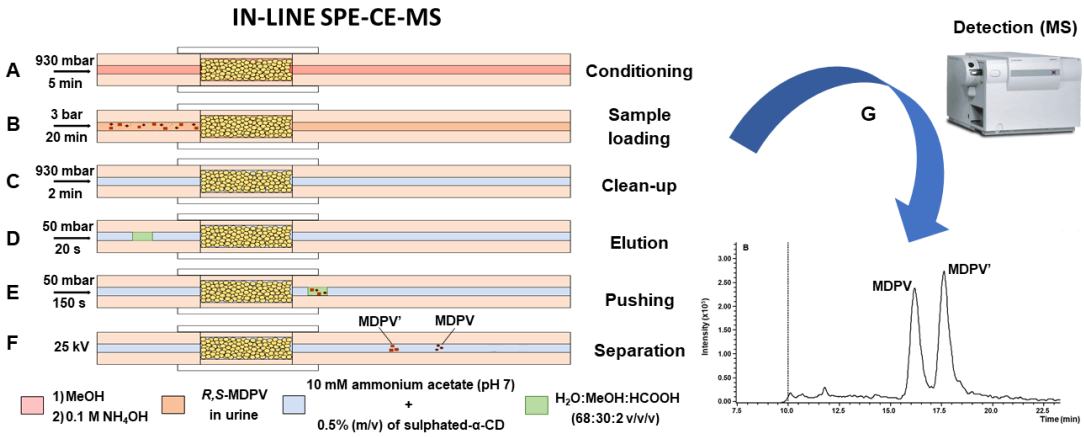
IN-LINE SPE-CE-MS
A
930 mbar
5 min
Conditioning
B
3 bar
20 min
Sample loading
C
930 mbar
2 min
Clean-up
D
50 mbar
20 s
Elution
E
50 mbar
150 s
Pushing
F
25 kV
MDPV'
MDPV
Separation
1) MeOH
2) 0.1 M NH4OH
R,S-MDPV in urine
10 mM ammonium acetate (pH 7)
+
0.5% (m/v) of sulphated-α-CD
H2O:MeOH:HCOOH (68:30:2 v/v/v)
Detection (MS)
G
MDPV
MDPV'
Intensity (x10⁵)
Time (min)

Novelty Statement

We consider this work to be novel because this is the first time that in-line solid phase extraction capillary electrophoresis has been successfully combined with chiral capillary electrophoresis-mass spectrometry.

Highlights

- In-line SPE is combined with chiral CE-MS for the sensitive analysis of drugs of abuse.
- An anionic CD is used to resolve MDPV enantiomers.
- CD counter migration and segmented acquisition ensures excellent MS performance.
- The method is validated for the analysis of MDPV in urine samples.
- Figures of merit are remarkable, including high enrichment factors.

Enantiodetermination of *R,S*-3,4-methylenedioxypyrovalerone in urine samples by high pressure in-line solid-phase extraction capillary electrophoresis-mass spectrometry

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Abstract

This study presents for the first time an in-line solid-phase extraction capillary electrophoresis-mass spectrometry (SPE-CE-MS) method for the enantiodetermination of drugs of abuse in urine samples. The enantioseparation of *R,S*-3,4-methylenedioxypyrovalerone (*R,S*-MDPV) was achieved with a 10 mM ammonium acetate BGE (pH 7) that contained 0.5% (m/v) of sulphated-α-CD as chiral selector. At these pH conditions, this CD was negatively charged, which prevented its entrance into the mass spectrometer since it migrates in the opposite direction. To improve sensitivity, an in-line SPE-CE-MS method using high pressure for sample introduction (i.e. 20 min at 3 bars) was developed. Furthermore, the conditioning procedure and the first part of the electrophoretic separation were performed by switching off the nebulizer gas and the ionization source voltage to avoid non-volatile contaminant arrival into the mass spectrometer. The developed methodology was validated by analyzing urine samples, which required a very simple liquid-liquid extraction (LLE) sample pretreatment. Linearity ranged from 30 to 250 ng mL^{-1}, limit of detection (LOD) was 10 ng mL^{-1}, relative standard deviation (RSD) values were below 10.5% in terms of intra-day and inter-day precision and the relative error values were below 9% for peak areas accuracy.

Keywords: Capillary electrophoresis mass spectrometry; Enantiodetermination; In-line preconcentration; Cathinones; Sulphated cyclodextrins; Urine analysis

Abbreviations: ACN, acetonitrile; **BGE,** background electrolyte; **CD,** cyclodextrin; **CE,** capillary electrophoresis; **EIE,** extracted ion electropherogram; **GC,** gas chromatography; **LC,** liquid chromatography; **LLE**, liquid-liquid extraction; **LOD,** limit of detection; **MeOH**, methanol; **MDPV**, 3,4-methylenedioxypyrovalerone; **MS,** mass spectrometry; **NPS**, new psychoactive substances; **THF,** tetrahydrofuran; **TIE,** total ion electropherogram;

1. Introduction

The production of alternatives to controlled drugs of abuse is a constant practice in the illicit marketplace. Among these alternatives, in recent years it has stood out the increase in consumption of synthetic derivatives of cathinone, an alkaloid naturally found in khat's leaves. This increase is mainly due to the similar euphoric effects of these cathinones, but lower prices and easier acquisition than amphetamines [1–3]. For this reason, the interest of the police and health authorities in the detection and determination of these substances has grown, as it is reflected in the emergence of extensive literature related to their analysis [4–18].

Synthetic cathinones are usually found in the illicit marketplace or at internet labelled as "bath salts" and 3,4-methylenedioxypyrovalerone (MDPV) is one of their major ingredients [1,2,19]. This cathinone, which is a pyrrolidine derivative of pyrovalerone, acts as dopamine and norepinephrine reuptake inhibitor [3,19,20]. After producing the stimulating effect, the remaining compound is excreted from the body and can be found at low concentration levels in biological samples, such as urine, plasma or hair [6–18]. Therefore, the determination of MDPV in urine requires the use of high sensitive methods, and there are several examples in the literature that allow reaching the necessary levels, mainly based on gas chromatography (GC) [15–18] or liquid chromatography (LC) [10–13] with mass spectrometry (MS) or tandem MS detection.

MDPV presents an asymmetric carbon in its chemical structure. Consequently, it exists as two enantiomers that can have different biological activity. Indeed, it has been demonstrated that the *S*-form is a more potent reuptake inhibitor of dopamine and norepinephrine than the *R*-form [20]. Therefore, the enantioseparation of MDPV enantiomers can be of interest for clinical, toxicological and forensic purposes. However, this chiral separation is usually difficult or expensive to achieve by LC, which typically requires a specific chiral column [14], or by GC, which needs a derivatization step that increases the analysis time [18]. In this sense, capillary electrophoresis (CE) offers an interesting alternative, as the enantioseparation can be achieved by simply adding a chiral selector in the BGE [21], being cyclodextrins (CDs) one of the preferred choices for the enantioseparation of cathinones by CE [4–9].

Despite the numerous advantages of CE, one of its major drawbacks is related with the relatively low sensitivity, as well as the limited selectivity with conventional UV detection. To solve these issues, different strategies have been proposed, including the hyphenation of CE with MS. CE-MS has been successfully used for the analysis of several kind of chiral compounds [4,5,22–27], including cathinones in standard solutions [4,5]. However, it requires the use of a volatile and low conductivity BGE with an appropriate chiral selector to obtain a good separation and stable electrospray, while preventing the mass spectrometer contamination [24]. It is well known that CDs can cause a significant signal suppression and an increase in the noise due to the contamination of the ionization source [24]. Therefore, it is important to develop strategies to prevent the entrance of incompatible chiral selectors into the mass spectrometer. Two strategies have been mainly highlighted in the literature to achieve chiral CE-MS: the counter migration technique [4,5,24–27] and the partial filling technique [4,5,22–24]. In the counter migration technique, charged chiral selectors that migrate in the opposite direction to the analytes and the mass spectrometer are employed. In the partial filling technique only a part of the capillary (e.g. 70-90%) is filled with the BGE containing the chiral selector avoiding its entrance into the mass spectrometer. Another complementary strategy is to switch off the ionization source during the conditioning step and/or part of the electrophoretic separation. Under these conditions, none of the compounds reaching the ionization source, including non-volatile chiral selectors, are ionized, hence they do not enter into the mass spectrometer [27].

The sensitivity in CE-MS may still be insufficient to reach the low levels at which MDPV is usually found in urine samples (ng mL^{-1}). Limits of detection (LOD) can be further decreased with the

application of a sample preconcentration strategy, such as the in-line coupling of solid-phase extraction to CE (in-line SPE-CE) [6,7,28–30]. In this strategy, the in-line SPE microcartridge, which contains an appropriate sorbent, is an integral part of the separation capillary, allowing the introduction of a large volume of sample to retain the target analytes. Then, after washing and filling the capillary with background electrolyte (BGE), the analytes are eluted in a small volume of an appropriate solution, resulting in sample clean-up and concentration enhancement before the electrophoretic separation and detection [6,7,28–30]. In-line SPE-CE-UV has been described for the enantiodetermination of cathinones in urine [6,7], but to the best or our knowledge in-line SPE-CE-MS has never been demonstrated for the enantiodetermination of cathinones or any other type of compounds in biological matrices.

The aim of this study was to develop an in-line SPE-CE-MS method for the sensitive enantioselective determination and unambiguous identification of chiral compounds in urine samples. The enantiodetermination of the drug of abuse *R,S*-MDPV was studied in urine samples. Different strategies were evaluated to ensure compatibility between the conditions required for an appropriate in-line preconcentration, enantioseparation and MS detection, demonstrating the feasibility of chiral in-line SPE-CE-MS for the first time.

2. Materials and methods

2.1. Reagents and standards

The standard of *R,S*-MDPV was provided as a hydrochloride salt with a purity of 98% by LGC Standards (Teddington, UK). An individual 2000 mg L^{-1} stock solution was prepared in MeOH and was kept in the freezer at -20 °C. This stock solution was stable for 6 months. Working standard solutions were prepared weekly by diluting the stock solution in water and were stored at 4 °C. The solutions with lower concentrations (≤1 mg L^{-1}) were prepared daily by diluting the working standard solutions in water.

Acetic acid (glacial), acetone 99.8%, ammonium acetate 98%, ammonium formate 97%, ammonium hydroxide 25%, dioxane 99.8%, formic acid 99%, propan-2-ol 99.9%, sodium hydroxide 98% and tetrahydrofuran (THF) 99,9% were supplied by Sigma-Aldrich (Saint Louis, MO, USA). Acetonitrile (ACN), methanol (MeOH) and water, all of them of LC-MS grade, were provided by PanReac Applichem (Barcelona, Spain). Sulphated-α-CD 98%, sulphated-β-CD 98% and sulphated-γ-CD 98% were supplied as sodium salts by Cyclolab (Budapest, Hungary).

2.2. Instrumentation

The pH measurements were performed with a Crison 2002 potentiometer and a Crison electrode 52-03 from Crison Instruments (Barcelona, Spain). Centrifugal filtration was carried out in a 5417R centrifuge from Eppendorf Ibérica (Madrid, Spain). A Vortex Genius 3 from Ika (Staufen, Germany) was used for agitation.

2.3. BGE and sheath liquid solutions

The BGE consisted of a 10 mM ammonium acetate aqueous solution (pH 7) that contained 0.5% (m/v) of sulphated-α-CD. As sheath liquid solution for CE-MS was employed a hydroorganic mixture of 60:40 (v/v) propan-2-ol:water with 0.25% (v/v) of formic acid.

All solutions were degassed for 10 min by sonication and filtered through a 0.20 µm nylon filter from Micron Separations Inc (Westborough, MA, USA) before use.

2.4. CE-UV

CE-UV experiments were performed at 25 °C with a 7100 CE System from Agilent Technologies (Waldbronn, Germany) equipped with a spectrophotometric diode-array detector (DAD). Bare fused-silica capillary of 50 µm id and 80 cm of total length (72 cm effective length) were provided by Polymicro Technologies (Phoneix, AZ, USA).

All capillary rinses were done flushing at 930 mbar. Before the first use, the capillary was activated with NaOH 1 M (40 min) and water (10 min). At the beginning of each working day, the capillary was conditioned with NaOH 0.1 M (10 min), water (5 min) and BGE (5 min). Between each run, the capillary was rinsed with NaOH 0.1 M (5 min), water (5 min) and BGE (5 min). At the end of each run, the capillary was postcoditioned with water (5 min). The sample was injected at 50 mbar for 10 s and 25 kV (positive polarity, cathode in the outlet) were applied for the electrophoretic separation. The BGE voltage vial was refreshed after each analysis to ensure maximum repeatability. Instrument control, data acquisition and data processing were performed using ChemStation Software from Agilent Technologies.

2.5. CE-MS

CE-MS experiments were performed at 25 °C using a HP^{3D} CE system coupled with an orthogonal G1603A sheath-flow interface to a LC/MSD Ion Trap SL mass spectrometer from Agilent Technologies. The sheath liquid was delivered at a flow rate of 3.3 µL/min by a KD Scientific 100 series infusion pump from KD Scientific (Holliston, MA, USA). Full scan mass spectra were acquired from 100 to 500 m/z in positive (ESI+) mode, and MDPV was detected as a singly charged molecular ion ($[M+H]^+$ 276.2). To avoid the unnecessary entrance of interfering compounds in the mass spectrometer, MS acquisition was split in two segments, each one with a different ESI voltage and nebulizer gas (N_2) pressure for the ionization source. Segment 1 conditions (0 V and 2 psi) were applied since the beginning of the capillary conditioning until minute 10 of the CE separation, when they were automatically switched to segment 2 conditions (4000 V and 7 psi). The rest of parameters were the same in both segments. The drying gas (N_2) flow rate and temperature were 2 L/min and 300°C, and capillary exit, skimmer, octopole 1, octopole 2, octopole radiofrequency, lens 1 and lens 2 voltages were set at 115.2 V, 48.4 V, 15.9 V, 0 V, 50 V, -9.1 V and -77.9 V respectively, with the trap drive at 41.9 (arbitrary units). This last group of parameters was automatically optimized infusing at 50 mbar through the separation capillary a 100 µg/mL MDPV standard solution. Instrument control, data acquisition and data processing were performed using CE/MSD Trap Software from Agilent Technologies.

A bare fused-silica capillary of 50 µm id and 80 cm of total length (Polymicro Technologies) was used for all the CE-MS separations. All capillary rinses were performed flushing at 930 mbar. New capillaries were activated with NaOH 1 M (40 min) and water (10 min) with the capillary outside of the CE-MS interface needle. Between days, the capillary was conditioned with ammonium hydroxide 0.1 M (10 min) and water (5 min). Between each run, the capillary was rinsed with ammonium hydroxide 0.1 M (5 min), water (5 min) and BGE (5 min). At the end of each run, the capillary was postcoditioned with water (5 min). The sample was injected at 50 mbar for 10 s and 25 kV (positive polarity) were applied for the electrophoretic separation. The BGE voltage vial was refreshed after each analysis to ensure maximum repeatability.

2.6. In-line SPE procedure

The construction of the in-line SPE particle-packed fritless microcartridge was based on the procedure described in [7]. All bare fused-silica capillaries were provided by Polymicro Technologies. Briefly, a small piece of capillary (2 mm, 150 µm id) filled with 60 µm Oasis HLB sorbent particles from Waters Corp. (Milford, MA, USA) was placed between the inlet (8 cm, 50

µm id) and the separation capillary (72 cm, 50 µm id). As the id of the separation capillary was smaller than the sorbent particle size no frits were necessary to prevent sorbent bleeding. A PTFE tubing (250 µm id, Saint Gobain, Courbevoie, France) was used to connect the different capillary fragments.

The in-line SPE-CE procedure consisted of the following steps. First, the capillary was conditioned at 930 mbar with MeOH and 0.1 M ammonium hydroxide, both for 5 min. Then, the standard solutions or urine sample extracts were introduced at 3 bars for 20 min. Before the elution, the capillary was washed and filled with BGE at 930 mbar for 2 min. The water:MeOH:formic acid (68:30:2 v/v/v) eluent was injected at 50 mbar for 20 s and pushed through the capillary with BGE at 50 mbar for 150 s. Finally, 25 kV (positive polarity) were applied for the electrophoretic separation. The rest of conditions were as indicated for CE-MS.

2.7. Sample pretreatment

Urine samples were collected in polypropylene tubes from healthy volunteers, with the appropriate approval of the Ethical and Scientific Committees of the UB. A pool was prepared for method development. The pooled and the individual samples were fractionated and stored at -20 °C until analysis.

A liquid-liquid extraction (LLE) sample pretreatment, based on a LLE procedure described in [7], was applied before the in-line SPE-CE-MS analysis. First, the urine samples were alkalized to pH 10 with 25% ammonium hydroxide. Then, 0.5 mL of ethyl acetate/propan-2-ol (4:1 v/v) were added to 0.5 mL of alkalized urine sample. After vortex mixing for 1 min, samples were centrifuged for 10 min at 9000 rpm. The organic phase, containing the MPDV, was transferred to a polypropylene vial, and then a second extraction of the aqueous phase was performed by adding again 0.5 mL of ethyl acetate/propan-2-ol (4:1 v/v). Then, both organic phases were combined and dried under a gentle stream of N_2. Finally, the residue was reconstituted with 0.5 mL water (adjusted to pH 10 with 25% ammonium hydroxide) and passed through a 0.20 µm nylon syringe filter before the analysis.

3. Results and discussion

3.1. BGE selection

The selection of an appropriate BGE is especially critical in CE-MS. The BGE must present low conductivity and be volatile to prevent salt precipitation in the ionization source, poor electrospray stability, ionization suppression and contamination of the mass spectrometer [24]. Moreover, the BGE must ensure an appropriate analyte ionization in solution to guarantee a proper separation and migration towards the detector. Since MDPV is a weak base, which presents a pKa value of 9.13 [31], at acidic or neutral BGE conditions it is positively charged and migrates towards the detector in the cathodic end of a bare fused silica capillary. In our previous work, we used a BGE of 70 mM monosodium phosphate aqueous solution at pH 2.5 with a mixture of 8 mM 2-hydroxypropil β-CD and 5 mM β-CD for the enantiodetermination of a group of cathinones, including MDPV, by in-line SPE-CE-UV [7]. However, these BGE conditions were rapidly discarded due to the low compatibility of phosphate BGEs with CE-MS and the presence of neutral CDs that will continuously arrive to the mass spectrometer pushed by the electroosmotic flow. As an alternative to the acidic phosphate BGE, four different aqueous solutions were tested, in particular: 10 mM formic acid (pH 3), 10 mM acetic acid (pH 3.5), 10 mM ammonium formate (pH 6.5) and 10 mM ammonium acetate (pH 7). For all of these BGEs the electric current was below 50 µA that is mandatory to prevent electric arcing between the CE-MS interface needle and the mass spectrometer entrance [25]. When the acidic BGEs were used, the shape of the MDPV peak was distorted. This distortion was not observed with the

ammonium salt solutions, but the acetate BGE allowed to detect MDPV in the shortest migration time and was selected for further experiments.

3.2. Chiral separation

In recent years, several studies based on CE-UV have demonstrated the chiral separation of cathinones by employing several CDs as chiral selectors [4–9]. However, the selection of the chiral selector to add in the BGE can be a critical issue in CE-MS [4,5,22–27]. In this sense, it is important to find strategies to avoid the entrance of these non-volatile substances in the mass spectrometer. Among these strategies, partial filling [4,5,22–24] and counter migration techniques [4,5,24–27] are the main approaches.

To evaluate these strategies, we tested different conditions by CE-UV to avoid the unnecessary contamination of the mass spectrometer. For the partial filling technique different negatively charged CDs were tested as chiral sectors, namely sulphated-α-CD, sulphated-β-CD and sulphated-γ-CD. Anionic CDs are good candidates for the partial filling and the counter migration approaches because in positive polarity mode (cathode in the outlet) they would migrate in the opposite direction to the mass spectrometer. For this study, the capillary was first flushed with BGE without CD and then, prior to the sample injection (75 μg mL^{-1} MDPV, 10 s at 50 mbar) and voltage application (25 kV), 50, 60, 70, 80 or 90% of the total capillary length was filled with BGE containing 1% (m/v) of CD. However, under the studied conditions it was not possible to achieve the baseline enantioseparation of *R,S*-MDPV. Then, the partial filling approach was discarded. Alternatively, the same anionic CDs were evaluated for the counter migration approach. For this study, the conditions were the same as before, excepting for the capillary that was completely filled with BGE containing 1% of the studied sulphated-CDs before the sample injection. Sulphated-α-CD and sulphated-β-CD allowed the baseline separation of the enantiomers of *R,S*-MDPV, while sulphated-γ-CD was not able to do it. As the best resolution was achieved with sulphated-α-CD, this CD was the chosen chiral selector for further experiments with the counter migration approach.

As sulphated-CDs are ionic compounds, at high concentration they can negatively affect the CE-MS performance [25,27]. Therefore, lower concentrations of sulphated-α-CD in the BGE, namely 0.25, 0.5, 0.75 and 1% (m/v) were tested. With 0.25% (m/v) of this CD in the BGE we got only partial separation of both enantiomers, but with 0.5% (m/v) they were baseline resolved. In view of these results, 10 mM of ammonium acetate at pH 7 containing 0.5% (m/v) of sulphated-α-CD was selected as the optimized BGE. Interestingly, when 0.5% (m/v) of sulphated-α-CD was added to the acidic BGEs (i.e. 10 mM formic acid (pH 3) or 10 mM acetic acid (pH 3.5), the MDPV enantiomers were not detected, even with an analysis time of over an hour. This definitely discarded the use of acidic volatile BGEs in combination with negatively charged CDs for the enantioseparation of *R,S*-MDPV in the counter migration approach.

As it has been mentioned in the introduction section, another complementary strategy to avoid the entrance of contaminant compounds, including non-volatile chiral selectors, into the mass spectrometer is to switch off the ionization source during the conditioning step and part of the electrophoretic separation [27]. Therefore, the MS acquisition was split into two segments. First, the ionization was switched off since the beginning of the capillary conditioning until minute 10 of the CE separation, by setting the ESI voltage and the nebulizer gas pressure to the minimum possible values (i.e. 0 V and 2 psi, respectively). Then, ionization was switched on to detect the enantioseparation, by setting both parameters to the typical values in CE-MS (i.e. 4000 V and 7 psi, respectively). Under these conditions, the mass spectrometer could be operated for an extended period of time without implementing any specific maintenance procedure, apart from the typical weekly cleaning of the ionization source recommended in routine operation. Fig. 1 shows the extracted ion electropherogram (EIE) by (segmented) CE-MS for a 75 μg mL^{-1} MDPV

standard solution with the optimized BGE. As can be seen, at these conditions the baseline enantioseparation of the *R,S*-MDPV was successfully achieved (resolution was 3.8).

3.3. In-line SPE-CE-MS optimization

The in-line SPE-CE-MS procedure was developed taking into account the (segmented) CE-MS method and an in-line SPE-CE-UV method for the enantiodetermination of a group of cathinones that we described in a previous work [7]. In that research, an in-line SPE fritless microcartridge of 2 mm length and 150 µm of id packed with Oasis HLB sorbent particles was used in combination with sample introduction at high pressure (3 bars) to achieve high enrichment factors, namely between 6,000 and 8,000.

The initial conditions for in-line SPE-CE-MS preconcentration were based on the optimum conditions found in that previous research [7]. These conditions were essentially the same as described for the final in-line SPE-CE-MS optimized conditions in the experimental section, except for the use of an eluent of 2% (v/v) of formic acid in MeOH. However, using this eluent composition, the chiral separation of the MDVP enantiomers was not achieved. This can be explained due to the different BGE conditions. The enantioseparation in CE is achieved due to the differences between the stability of the complexes formed by the CD and the enantiomers. Furthermore, it has been demonstrated that the presence of an organic modifier in the BGE can decrease the cathinone/CD binding constant, resulting in a decrease of the resolution between enantiomers [5]. Therefore, elution conditions required a careful optimization to ensure the highest enrichment factors while maintaining an appropriate enantioseparation.

The enantioseparation was studied using as eluent different hydroorganic mixtures compatible with MS detection. For this study, a 200 ng mL^{-1} MDPV standard solution was analyzed by in-line SPE-CE-MS with eluents containing from 10 to 40% (v/v) of acetone, ACN, dioxane, propan-2-ol, MeOH and THF, which were injected at 50 mbar for 20 s. The resolution values between both enantiomers obtained at the different tested conditions are summarized in Table 1. As can be observed, a high organic solvent content (40%) did not allow the enantioseparation and 30% (v/v) of MeOH was the highest percentage of organic solvent capable of maintaining the chiral separation. Moreover, this composition allowed the highest response for MDPV enantiomers. Taking as a reference these elution conditions (30% (v/v) MeOH at 50 mbar for 20 s), the influence of the eluent volume was also investigated, injecting the eluent at 50 mbar for 10 s or 30s. As it was expected, when the eluent volume was increased the enantioseparation was compromised, as it was also greater the amount of organic modifier in the capillary. On the other hand, when the eluent volume was reduced, the MDPV enantiomers response also decreased. Then, to evaluate if the acidification of the eluent could have a positive effect in the elution without compromising the enantioseparation, the addition of a 2% (v/v) of formic acid to the hydroorganic mixture was tested. This allowed increasing response more than 10 times without significantly compromising the chiral separation (resolution was 1.5). Therefore, the optimized eluent was selected as a mixture of water:MeOH:formic acid (68:30:2 v/v/v) injected at 50 mbar for 20 s.

To investigate the increase in the response for MDPV under the in-line SPE-CE-MS optimized conditions in comparison with CE-MS the enrichment factor was calculated. A value of 500 was obtained as the ratio between the LODs for the analysis of MDPV standards by CE-MS (4000 ng mL^{-1}) and in-line SPE-CE-MS (8 ng mL^{-1}). This confirmed the sensitivity enhancement potential of in-line SPE-CE-MS, while achieving the unambiguous identification of the separated enantiomers. However, this enrichment factor was lower than the value obtained for MDPV in our previous study by in-line SPE-CE-UV (i.e. 6,000) [7]. This can be explained due to the modifications needed in the BGE and the eluent compositions to make compatible the preconcentration and enantioseparation in in-line SPE-CE-MS, and especially due to the

differences on the eluent composition. It is well known that an eluent of 2% v/v of formic acid in MeOH [7], presents a greater elution strength from an Oasis HLB sorbent than the water:MeOH:formic acid (68:30:2 v/v/v) solution optimized in the current study.

3.4. Urine sample pretreatment

The applicability of the in-line SPE-CE-MS method was tested by analyzing urine samples. However, before this could be possible it was necessary to develop a sample pretreatment to avoid the microcartridge saturation and poor ionization efficiency due to co-extraction of urine sample matrix components [7,10–13].

A LLE procedure based on alkalizing the urine to pH 10 and extracting MDPV with an ethyl acetate/propan-2-ol mixture was applied, as in our previous study by in-line SPE-CE-UV [7]. A recovery value of 87% was calculated as the ratio between the response by in-line SPE-CE-MS for a urine sample spiked with 100 ng mL^{-1} of MDPV and a 100 ng mL^{-1} standard solution without LLE. The matrix effect after performing the developed LLE procedure was calculated using the following expression:

$$\%\ \text{Matrix effect} = \frac{C_{spiked}}{C_{standard}} \times 100 - 100$$

Where C_{spiked} is the concentration of a urine extract spiked after the LLE procedure and $C_{standard}$ is the concentration of a standard at the same concentration as C_{spiked}, both analyzed by in-line SPE-CE-MS. Applying this equation at a concentration level of 100 ng mL^{-1} of MDPV, a value of -16% was obtained, hence signal suppression was observed when urine extracts were analyzed. This matrix effect value is within the range reported in the literature for cathinone analysis in urine by LC-MS in ESI+ mode (i.e. between 3.2 and - 28%) [10–12]. From the obtained recovery and matrix effect values it was concluded that recovery of the LLE procedure was practically total, which agrees with the recovery value (i.e. 93%) obtained for MDPV when this LLE procedure was applied prior to in-line SPE-CE-UV [7].

Fig. 2 shows the total ion electropherogram (TIE) (A) and the EIE (B) by in-line SPE-CE-MS of a urine sample spiked with 100 ng mL^{-1} of MDPV and pretreated by LLE, and the EIE (C) of a 100 ng mL^{-1} MDPV standard solution. As can be seen, the TIE does not present remarkable interferences in the scanned m/z range. Furthermore, the MDPV enantiomer peaks were wider and the number of theoretical plates ($N = 16 \times (t_m/w)^2$) lower by in-line SPE-CE-MS (Figure 2C, N=14,796 and 8,119 for MDPV enantiomers) than by CE-MS (Figure 1, N=23,409 and 28,224, for MDPV enantiomers). As both the standard solution (Figure 2C) and the spiked urine sample (Figure 2B) present peak broadening by in-line SPE-CE-MS, this must be due to the in-line SPE microcartridge and not to a matrix effect. This kind of peak broadening has been described before in in-line SPE-CE [7,28–30], but good resolution between MDPV enantiomers was maintained (resolution was 1.5). It should not be either forgotten that a slight decrease in peak efficiency is always expected when moving from CE-UV to CE-MS due to the characteristics of the sheath-flow interface (N=30,625 and 40,000 by CE-UV for MDPV enantiomers).

3.5. Method validation

The proposed LLE/in-line SPE-CE-MS methodology for the analysis of urine samples was validated, with spiked urine samples, in terms of selectivity, linearity, intra-day and inter-day

precision, accuracy, LODs and LOQs following the guide published by the United Nations Office on Drugs and Crime (UNODC) [32].

To evaluate the selectivity 10 blank urine samples from different individuals were analyzed after the LLE pretreatment. At these conditions, no endogenous peaks were observed at the expected migration time for the MDPV enantiomers.

A matrix-matched calibration curve in the range between 30 and 250 ng mL^{-1} was used to evaluate the linearity. As it is shown in Table 2, good results were obtained in terms of linearity as the regression coefficients (R^2) were greater than 0.99.

The intra-day and inter-day precision were evaluated at 30 ng mL^{-1}, 100 ng mL^{-1} and 250 ng mL^{-1} (n = 5 at each concentration level on the same day or on five different days, respectively). As can be seen in Table 2 the method provided good results in both cases as the obtained values, expressed as relative standard deviation (RSD), were below 9% and 10.5%, respectively. The accuracy was also investigated at the same concentration levels. For this purpose, the relative errors of peak areas (%RE) were calculated using the following expression:

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

As can be seen in Table 2 %RE values were good (below 9% for all concentration levels).

The LODs for the MDPV enantiomers were calculated by applying the signal-to-noise ratio (S/N) criterion of three, whereas the LOQ was set as the lowest concentration value of the linear range. The LOD and LOQ values were 10 ng mL^{-1} and 30 ng mL^{-1}, respectively. These LODs were suitable to reach the levels at which this cathinone is usually found in urine (ng mL^{-1}) and were similar or lower than those obtained using GC-MS for the analysis of MDPV in urine (i.e. between 5 and 30 ng mL^{-1}) [15–17]. Additionally, the obtained LODs were higher than those reported using LC-MS for the analysis of MDPV in urine (i.e. between 0.06 and 2 ng mL^{-1}) [10–13]. However, it is important to highlight that none of these alternative methods allowed the separation of MDPV enantiomers. Despite MS detection usually presents a higher sensitivity than UV detection, the LODs by in-line SPE-CE-MS were slightly higher than those obtained for the MDPV enantiomers in urine by in-line SPE-CE-UV in our recent work (i.e. 3 ng mL^{-1}) [7]. As indicated before, this can be explained due to the modifications needed to set an appropriate in-line SPE-CE-MS method for enantiomer analysis.

4. Concluding remarks

In this study we reported a successful methodology for the enantiodetermination of MDPV in urine samples by in-line SPE-CE-MS at the typical ng mL^{-1} levels at which this compound is present in this biological fluid. As far as we know, this is the first time that in-line SPE-CE has been demonstrated in combination with chiral CE-MS.

The enantioseparation of MDPV was achieved by adding 0.5% (m/v) of sulphated-α-CD to the BGE (10 mM ammonium acetate BGE (pH 7)). Due to the anionic character of this CD the chiral separation was conducted in the counter migration approach without negatively affecting the mass spectrometer performance. The MS acquisition was also segmented to appropriately switch the ionization source and prevent the entrance of non-volatile contaminants into de mass spectrometer. For the elution a water:MeOH:formic acid (68:30:2 v/v/v) solution injected at 50 mbar for 20 s was providing enrichment factors of 500 times while maintaining an appropriate enantioseparation. The potential of the method for forensic, toxicological or clinical applications

was demonstrated by validating the method for the analysis of urine samples. Remarkable figures of merit were obtained, including LODs of 10 ng mL^{-1} for both enantiomers.

In the future, novel combinations of sorbents, extraction conditions, separation capillaries, BGE compositions, separation approaches and detection conditions should be explored to further enhance sensitivity and expand the applicability of chiral in-line SPE-CE-MS that is able to provide a novel insight into enantiomer analysis at the low concentration level.

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

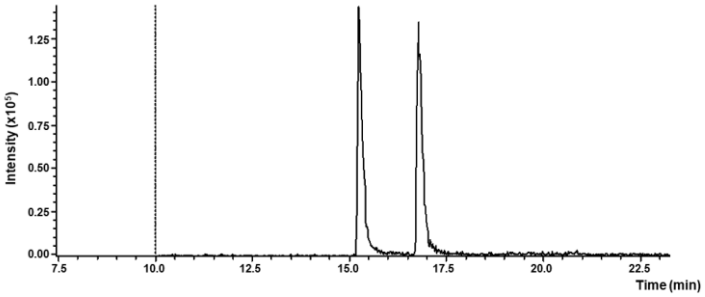

Figure 1. EIE ($[M+H]^+$ 276.2) by (segmented) CE-MS of a 75 µg/mL MDPV standard solution with the optimized BGE (10 mM ammonium acetate (pH 7) with 0.5% (m/v) of sulphated-α-CD). The ionization was switched off until minute 10.

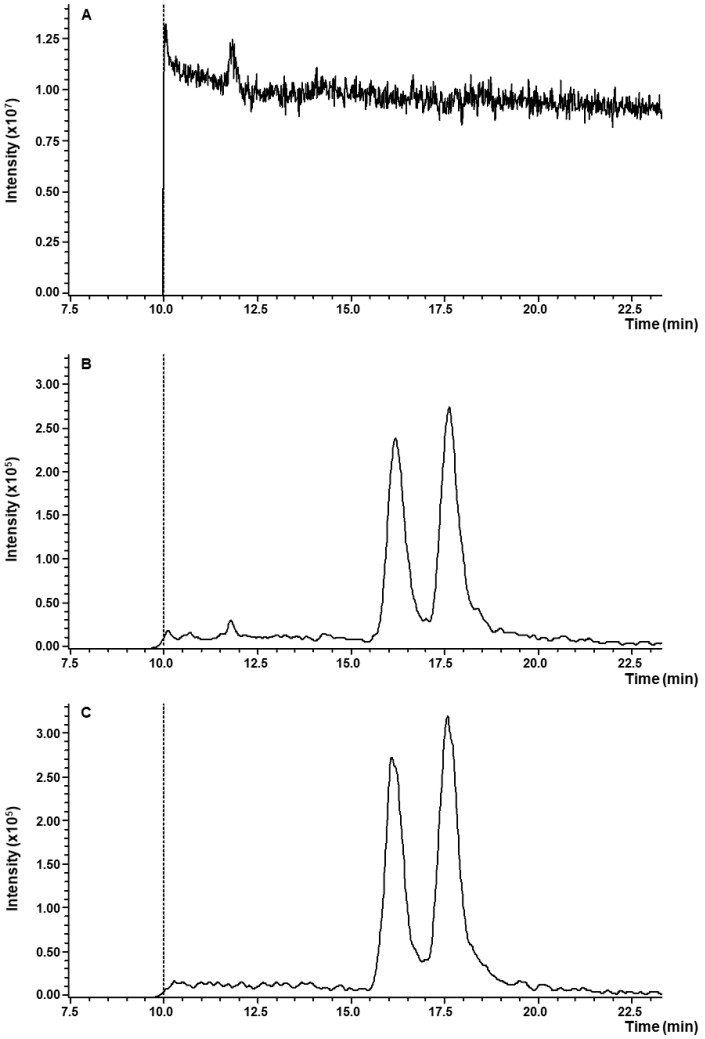

Figure 2. TIE (A) and EIE ($[M+H]^+$ 276.2) (B) of a urine sample spiked with 100 ng mL^{-1} of MDPV and pretreated by LLE, and EIE ($[M+H]^+$ 276.2) (C) of a 100 ng mL^{-1} MDPV standard solution. The ionization was switched off until minute 10. The rest of in-line SPE-CE-MS optimized conditions are indicated in the experimental section.

Table 1. Resolution of the MDPV enantiomers by in-line SPE-CE-MS with different hydroorganic eluents. A 200 ng mL^{-1} MDPV standard solution was analyzed in all cases. Resolution was calculated from the EIE as: $Rs = 2 \times \frac{t_{m2} - t_{m1}}{W_1 + W_2}$

	Percentage of the organic solvent in the hydroorganic solution (%, v/v)			
	10	**20**	**30**	**40**
Organic solvent	**Resolution between MDPV enantiomers**			
Acetone	1.1	n. e.	n. e.	n. e.
ACN	1.1	n. e.	n. e.	n. e.
Dioxane	0.6	n. e.	n. e.	n. e.
Propan-2-ol	0.6	n. e.	n. e.	n. e.
MeOH	2.0	1.6	1.5	n. e.
THF	n. e.	n. e.	n. e.	n. e.

n.e = not enantioseparated

Table 2. Method validation in terms of linearity, intra-day and inter-day precision, accuracy of peak areas and LODs obtained for spiked urine samples by in-line SPE CE-MS.

	MDPV	**MDPV'**
Linearity (ng mL^{-1})	30-250	30-250
Calibration curve	y = 84071x + 218912	y = 104393x + 4988
R^2	0.991	0.995
LODs (ng mL^{-1})	10	10
Intra-day RSD of peak area (%, n = 5)		
30 ng mL^{-1}	8.7	8.7
100 ng mL^{-1}	7.5	7.7
250 ng mL^{-1}	7.7	7.9
Inter-day RSD of peak area (%, n = 5)		
30 ng mL^{-1}	10.3	9.2
100 ng mL^{-1}	8.7	8.4
250 ng mL^{-1}	9.2	8.8
Relative error of peak area (%, n = 5)		
30 ng mL^{-1}	8.7	8.4
100 ng mL^{-1}	7.9	8.0
250 ng mL^{-1}	8.1	8.3