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~~Performance of s~~Solid-phase extraction based on cation-exchange sorbents followed by liquid chromatography-high resolution mass spectrometry ~~for the~~to determin~~eation of~~ synthetic cathinones in urine

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Abstract

Purpose ~~In recent years, the consumption of new psychoactive substances has increased considerably. Among the different substances included in this group, c~~Cathinone derivates have become very popular as legal highs~~. Moreover, these substances~~ and are easily obtained on the market as “bath salts”, getting around the legislation due to their modified structure based on banned substances. The aim of this project is to evaluate two mixed mode cation-exchange sorbents in solid-phase extraction (SPE) ~~to~~and their efficacy in pretreating urine samples for the determination of synthetic cathinones by liquid chromatography-high resolution mass spectrometry (LC-HRMS). ~~using Orbitrap as the analyser.~~

Methods A method for determining a group of eleven trending synthetic cathinones in urine using SPE followed by LC-HRMS using Orbitrap as analyser was developed and validated.

Results In the extraction step, two different cation-exchange sorbents were evaluated and compared, a weak one (Oasis WCX) and a strong one (Oasis MCX). Better results were obtained for Oasis MCX in terms of recoveries and matrix effects (lower if a clean-up step was applied). Method quantification limits were set at 0.2 ng mL^{-1} and method detection limits were between 0.04 ng mL^{-1} and 0.16 ng mL^{-1}.

Conclusions The results obtained enabled the studied compounds to be quantified at the usual levels at which they are present in urine samples. Moreover, these limits were lower than the ones found in the literature using similar extraction strategies, which shows that the reported strategy can be a useful tool in forensic and toxicological analysis.

Keywords

Synthetic cathinones

Solid-phase extraction

Cation-exchange sorbents

Liquid chromatography-high resolution mass spectrometry

Urine samples

Introduction

The number of people who take drugs is increasing every year since some of the new substances that appear on the market are not prohibited [1]. Nowadays, new psychoactive substances (NPS) are the trending drugs. They are a type of synthetic drug that includes synthetic cathinones and cannabinoids, among others. From 2009 to 2016, 106 countries and territories have reported the emergence of 739 different NPS [2]. NPS can be found under different names, for instance: “legal highs”, “bath salts” and “research chemicals”. They can be bought easily and legally because they are modified compounds which are not prohibited by law [3]. The reason they are not banned is because the process of updating the law can take more than a year due to all the requirements, and by the time one substance is banned, another one is introduced onto the market [4].

This project is focused on synthetic cathinones, which represent a significant portion of the NPS market. They are stimulants obtained from the substance cathinone and they can be found in the khat plant [5]. They are β-keto phenethylamines and structurally they are very similar to amphetamine or methamphetamine. The effects of cathinone consumption are similar to the effects produced by other drugs; for example, paranoia, hallucinations, increased friendliness, increased sex drive, panic attacks and excited delirium [5, 6]. These compounds have similar effects to other more expensive drugs and are easily obtained on the Internet.

When synthetic cathinones are taken, they are eliminated through the urine and they can reach superficial waters. Thus, the presence of cathinones in environmental and biological samples has been widely studied [7–16]. In the case of biological samples, urine is commonly used to determine the presence of drugs because it is easy to sample and also due to the mid-window of detection [17]. Cathinones are absorbed in the tissues and due to their lipidic solubility, they are present in the liver prior to their elimination through the urine, where, in some cases, they have been found at low levels of ng mL^{-1} 24 hours after consumption [18]. Apart from urine, there are other biological samples, such as saliva, breath, blood, sweat and hair, that have also been studied [16, 19–23]. In this study, we have focused on the parent compounds of the synthetic cathinones since in previous studies they were found in environmental water samples, so they probably are not or are only partially metabolised [11, 15].

Nowadays, the analysis of this type of drug is a subject of interest of several authors [8–13, 20, 21, 24, 25]. Synthetic cathinones can be determined either by liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE). GC is used in forensic and clinical analyses in some laboratories [7, 26]. However, the main drawback of this technique is that most cathinones need a derivatization prior to being analysed and they can decompose due to the high temperatures usually used [26]. The use of CE is interesting from the point of view of chiral separation, since the enantioseparation can be achieved by simply adding a chiral selector to the background electrolyte [19, 27, 28]. However, the most prevalent technique used for the separation of cathinones is LC because it ~~overtakes~~ overcomes the disadvantages of the other techniques [9–13, 20, 21, 29]. In addition, most of the studies focused on determining cathinones in urine, use a mass spectrometer (MS) detector, and more precisely, tandem MS using triple quadrupole (QqQ) [14, 30]. However, the literature in which the detection is performed with high resolution mass spectrometry (HRMS) is limited to a few articles using Q-Orbitrap [9, 10] and quadrupole time-of-flight (QToF) [12, 13]. This present research is focused on using Orbitrap to contribute to increasing the studies in this area.

For urine samples, SPE is the most ~~habitual~~ commonly used technique for extracting the analytes from the sample matrix~~,~~ as it achieves~~ing~~ high recoveries due to the different sorbents available [9, 10, 13, 14, 20, 21, 31–33]. One type of sorbent that combines capacity and selectivity ~~are~~ is mixed-mode ion-exchange sorbents. In the literature, some authors have

evaluated these types of sorbent~~s~~ ~~for~~ to extract~~ing~~ different compounds from urine samples [9, 10, 13, 32, 33]. In the determination of the opioid dermorphin by Guan *et al.* [33], they used Oasis MCX cartridge for the SPE. The authors obtained excellent extraction efficiencies in urine (between 86% and 92%) and excellent intra-day and inter-day accuracies (between 91% and 100%). ~~In the determination of~~To determine tricyclic antidepressants ~~[32]~~, ~~they~~Chambers *et al.* [32] -used an Oasis WCX as the extraction cartridge. The authors achieved recoveries from 92% to 104% and matrix effects lower than 15%. ~~Mixed-mode sorbents are widely used and it is important to compare the results obtained with them with the ones obtained with other sorbents in the determination of synthetic cathinones from urine.~~

The objective of the present study is to develop and validate a method for determining synthetic cathinones in urine by SPE followed by LC-HRMS using Orbitrap as the analyser. Based on the mentioned literature and considering the excellent recoveries reported with Oasis WCX and Oasis MCX for other drugs in urine, the present study tests and compares these two cartridges ~~are tested and compared~~ for the first time for the extraction of cathinones from urine. Therefore, it is important to evaluate these two sorbents in terms of their recoveries and matrix effects in order to obtain low detection limits. This application has not been reported before using Orbitrap, which might achieve the required concentration levels ~~found~~ of the target compounds in urine and ~~it~~ allows the retrospective analysis.~~; moreover,~~ ~~i~~It is also more affordable than other HRMS detectors.

Materials and methods

Standards and materials

The cathinone standards were purchased from LGC Standards (Luckenwalde, Germany). The cathinones chosen for this study were: 4-fluoromethcathinone (flephedrone), N-Ethylcathinone (ethcathinone), buphedrone, 2-methylmethcathinone (2-MMC), butylone, 4-methylmethcathinone (mephedrone), 4-methylethcathinone (4-MEC), beta-ethylmethcathinone (pentedrone), 3,4- dimethylmethylcathinone (3,4-DMMC), alpha-pyrrolidinovalerophenone (alpha-PVP) and methylenedioxypyrovalerone (MDPV).

Individual stock solutions of these analytes were prepared at 100 mg L^{-1}, 1000 mg L^{-1} and 2000 mg L^{-1} depending on the compound, using methanol (MeOH) as solvent, and they were kept in the freezer at -20ºC. A working mixture solution containing all the analytes at a concentration of 1 mg L^{-1} was prepared in MeOH and the diluted standard solutions were further prepared in H_2O. They were also kept in the freezer at -20ºC.

Ultra-gradient HPLC grade MeOH, acetone and water for LC-MS were purchased from J.T. Baker (Deventer, The Netherlands). Acetonitrile (ACN) for LC-MS was obtained from Chem-Lab (Zedelgem, Belgium). Formic acid (HCOOH), ammonium hydroxide (NH_4OH), hydrochloric acid (HCl), sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Ultrapure water was obtained using a water purification system (Veolia, Sant Cugat del Vallès, Spain).

Oasis WCX (weak cation-exchange) and Oasis MCX (strong cation-exchange) (150 mg/6mL~~cc~~) extraction cartridges for the SPE process were purchased from Waters Corp. (Milford, MA, United States).

Instrumentation

The method optimization was performed using a Hewlett Packard 1100 HPLC system with a diode array detect~~ion~~or (DAD) from Agilent Technologies (Waldbronn, Germany) controlled by

132 Agilent Chemstation software. The column used was a Luna Omega 5 µm Polar C_{18} (150 mm x
133 4.6 mm, 5 µm) from Phenomenex (Torrance, CA, United States) with a Security Guard from
134 Phenomenex.

135 The method validation and the sample analysis were performed using a Thermo Scientific
136 Accela 1250 UHPLC system (Bremen, Germany) equipped with an Accela Autosampler
137 automatic injector and an Accela 1250 pump coupled with a Thermo Scientific Exactive
138 Orbitrap^{TM} mass spectrometer. The HRMS instrument is also equipped with a heated
139 electrospray ionization (HESI) source and a higher-energy collisional dissociation (HCD) cell to
140 fragment the analytes.

141 **Chromatographic conditions**

142 For the LC-DAD, the temperature of the column was set at 35 ºC. The mobile phase flow rate
143 was set at 0.6 mL min^{-1} and the sample volume injection was 20 µL. The mobile phase
144 consisted of A: 0.1% HCOOH in ultrapure water and B: 0.1% HCOOH in ACN in gradient
145 mode. The gradient started at 15% B, which was held for 5 minutes and increased to 35% in 5
146 minutes, then increased to 80% in 4 minutes and to 100% in 1 minute and held for 2 minutes
147 before returning to the initial conditions in 1 minute and maintained for 4 minutes. All the
148 compounds were detected at 260±10 nm except for butylone and MDPV, which were detected
149 at 325±10 nm.

150 In the case of the LC-HRMS, the mobile phase composition, flow rate, gradient elution and
151 column temperature were the same as for LC-DAD. For the HRMS, the gas flow rates and
152 temperature parameters were optimised to obtain the highest response for all the analytes with
153 positive ionisation and the following optimised parameters were used: sheath gas 60 AU;
154 auxiliary gas 5 AU; capillary voltage, 30 V; spray voltage, 2 kV; tube lens voltage, 80 V;
155 skimmer voltage, 24 V. The heater and capillary temperatures were set at 400ºC and 350ºC,
156 respectively. The probe position adjustment was side to side 0, vertical B and micrometre 1.

157 For data acquisition, two time windows were used in positive mode (0-11.75 min and 11.75-15
158 min) with two alternating scan events in each window: full scan at 50,000 FWHM with 250 ms
159 of injection time for the first one and fragmentation scan at 10,000 FWHM with 50 ms of
160 injection time for the second one. The optimum collision voltages were 15 eV for the first
161 window and 25 eV for the second one. The optimization was performed in full scan at high
162 resolution in a mass range between 60 *m/z* and 300 *m/z*.

163 **Urine collection and preparation**

164 The pooled urine samples were obtained from nonaddicted volunteers, mixing the urine of
165 different individuals, so we included urine from women and men of different ages. They were
166 collected in polypropylene tubes and kept in the freezer at -20ºC. Before their analysis, 5 mL of
167 a mixture of urine and phosphate buffer solution adjusted at pH 6 (0.15 M NaH_2PO_4 and 0.05 M
168 Na_2HPO_4) at a ratio of 50:50 (*v/v*), were loaded in a 150 mg Oasis MCX cartridge after its
169 activation with 5 mL of MeOH and its conditioning with 5 mL of phosphate buffer solution (pH
170 6). After the sample loading, the cartridge was washed with 2 mL of MeOH, and finally, the
171 analytes were eluted with 2 mL of 5% NH_4OH in MeOH. Then, 100 µL of 1% HCl in MeOH
172 were added to the methanolic solution. The extracts were finally evaporated to dryness under a
173 gentle stream of N_2, reconstituted with 1 mL of mobile phase at initial conditions, filtered
174 through a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter and then transferred to a vial
175 for their analysis.

176 **Validation**

For the instrumental validation (LC-HRMS), the linearity, which was studied at 8 different concentrations (0.1, 0.5, 1, 10, 25, 50, 100 and 250 ng mL^{-1}), detection limits and quantification limits (IDLs and IQLs) were evaluated. Due to the minimal noise when Orbitrap is used, IDLs were considered as the lowest detectable concentration with a signal intensity higher than 1 x 10^3 for the protonated molecule and for the fragments, while IQLs were defined as the lowest point of the calibration curve.

In the case of the method validation, the linearity, repeatability (intra-day), reproducibility (inter-day), method detection limits and method quantification limits (MDLs and MQLs), extraction recovery (R_{SPE}), matrix effect (ME) and apparent recovery (R_{app}) were studied [34]. ~~For the compound confirmation, t~~The retention time, the ion ratio and the error mass were considered to confirm the presence of the compound ~~taken into account~~ [35]. The retention time had to correspond ~~at~~ to a tolerance of ±2.5 %, the ion ratio ~~at~~ ±20 % and the error mass ~~at~~ to a maximum of 5 ppm.

The linearity of the method based on a matrix-matched calibration curve was studied for each compound by evaluating the coefficient of determination (r^2) of urine samples spiked prior to the SPE process with cathinones at different concentrations: 0.05, 0.1, 0.2, 0.4, 1, 4, 20, 40, 60, 100, 150 and 200 ng mL^{-1}. The repeatability and reproducibility values, in terms of relative standard deviation (% RSD), were obtained by analysing five replicates of urine samples spiked at 40 ng mL^{-1} prior to the SPE on the same day and on five different days, respectively. MDLs corresponded to a concentration of the cathinones in urine whose signal intensity was higher than 1 x 10^3 for the protonated molecule and for the fragments. MQLs were defined as the lowest point in the matrix-matched calibration curve.

The % R_{SPE} was considered to be the recoveries obtained in the SPE procedure alone and were calculated as the ratio between the concentration obtained when a urine sample was spiked before the SPE procedure and that obtained from direct injection of the standard in the LC-DAD.

The ME was calculated using the following expression:

$$\% ME=[(C_{spiked}/C_{STD})x100]-100 \quad \text{(Equation 1)}$$

Where C_{STD} is the concentration of the standard injected and C_{spiked} is the concentration obtained from the urine extract spiked at the same concentration as C_{STD} after the SPE, both injected in the LC-HRMS. A negative value of % ME means a signal suppression and a positive value is related to a signal enhancement.

The % R_{app} was determined as the recovery of the entire method and it was calculated from the ratio between the concentration obtained when a urine sample was spiked before the extraction procedure and that obtained from direct injection of the standard in the LC-HRMS.

Results and discussion

Separation and detection

The Luna Omega 5 µm Polar C_{18} column (150 mm x 4.6 mm i.d., 5 µm) from Phenomenex with a precolumn was chosen to separate the cathinones because of its selectivity and good retention for polar analytes. The composition of the mobile phase was A: 0.1% HCOOH in ultrapure water and B: 0.1% HCOOH in ACN, which was selected based on the literature [9, 11]. ACN and MeOH were studied as mobile phase B solvents, obtaining a better separation in the case of ACN.

First, the DAD detector was used for the experiments to evaluate the two SPE sorbents. The cathinones were identified by individual injections and the optimal wavelength of each compound was studied to achieve the maximum response. The optimal wavelength for most compounds was 260 nm; however, for butylone and MDPV it was 325 nm, and hence, the data acquisition was performed at both wavelengths. It is important to mention that all the compounds were chromatographically separated except for flephedrone and ethcathinone, which had the same retention time and the same optimal wavelength. Although different gradients were tested, it was not possible to separate them without considerably increasing the analysis time. Therefore, with LC-DAD it was only possible to include one of them. Ethcathinone was chosen due to its prevalence in drug users in comparison with flephedrone [5]. Nevertheless, both of them were determined using LC-HRMS because of their different protonated molecules, as shown in the following section.

As already mentioned in the introduction section, the main aim of this study was to develop a method able to determine low concentration levels of synthetic cathinones in urine as these drugs are usually at low levels of ng mL^{-1} 24 hours after consumption [18]. Thus, in order to achieve this objective, a determination with LC-HRMS using Orbitrap as analyser was applied. The first step consisted in the optimization of the different parameters that can affect the ionization and the transfer to the Orbitrap analyser. The transfer parameters optimised were capillary voltage (25-60 V), tube lens (60-120 V), skimmer (15-40 V) and capillary temperature (250-350 ºC). The structure of cathinones allows them to be ionised preferably in positive mode. The optimised ionization parameters were sheath gas (10-60 AU), auxiliary gas (2-10 AU), spray voltage (2-5 kV), heater temperature (300-400 ºC) and probe position adjustment – side to side (-1 to +1), vertical (A, B, C or D) and micrometre (0-2). In addition, the HCD energy was optimised (10-60 eV) in order to observe two fragment ions for each compound, as shown in Table 1 and in agreement with other studies [9, 11, 36]. The optimum parameters are detailed in the experimental part. The fragmentation parameter was optimised by direct injection of individual standards at 1 mg L^{-1} in H_2O:MeOH (85:15, *v/v*) along with the mobile phase at 65% A and 35% B. All the cathinones had an optimal collision energy of 15 eV, except alpha-PVP and MDPV for which it was 25 eV. The difference in the collision energies can be explained because of the presence of pyrrolidinyl substituents in alpha-PVP and MDPV, which are not present in the other compounds.

The chromatographic conditions optimised for LC-DAD were the same used in LC-HRMS. Even that some compounds are not separated by their retention time, with the use of HRMS, they can be ~~separated~~ discriminated according to their protonated molecule and fragments (Table 1).

The ILDs and IQLs were evaluated under the optimal conditions found for LC-HRMS. ILDs ranged from 0.10 ng mL^{-1} to 0.40 ng mL^{-1} and the IQLs were 0.5 ng mL^{-1} for all the cathinones under study. Moreover, good linearity between the concentration points of IQL and 250 ng mL^{-1} with r^2 higher than 0.999 was obtained with standards.

Solid-phase extraction procedure optimization

In the analysis of urine, SPE is one of the techniques most frequently used to extract and preconcentrate the cathinones [20, 21]. Cathinones have an amino group so cation-exchange sorbents are suitable for this type of compounds, which have been previously demonstrated by other authors who have used this kind of sorbents to extract these compounds from environmental and biological samples [9, 11]. Their pKa values are between 7.3 and 8.2, due to the aforementioned amino group, which means that working at values of pH lower that these pKa values, cationic interactions might be present with the sorbent. In this study, two different cation-exchange sorbents were compared, a weak one (Oasis WCX) and a strong one (Oasis

MCX). These experiments were performed first with standards and then with urine by using LC-DAD. Once the optimal protocols were achieved, they were evaluated by LC-HRMS.

Oasis WCX

The initial protocol for Oasis WCX was conditioning with 5 mL of MeOH followed by 5 mL of phosphate buffer solution at pH 7 (0.10 M of NaH_2PO_4 and 0.06 M of Na_2HPO_4), loading with 5 mL of the buffer solution (pH 7) containing the cathinones at a concentration of 2 mg L^{-1}, washing with MeOH and elutinge with 5% HCOOH in MeOH. At pH 7 all the cathinones are in their cationic form and this allows the interaction with the sorbent since the carboxyl group is also deprotonated. Fixing the conditioning and the loading steps, the clean-up and the elution steps were optimised to achieve the highest recoveries with the minimal elution solvent volume. For this, different volumes of elution solvent were tested without the clean-up step. In particular, values of 2 mL, 4 mL and 5 mL of 5% HCOOH in MeOH were evaluated. It was observed that 2 mL of 5% HCOOH in MeOH were enough to ensure the complete elution of all the cathinones under study with recovery values between 90% and 104%.

After optimising the elution step, the clean-up was evaluated ~~with urine~~ with a mixture of urine:phosphate buffer solution (pH 7) (1:1, *v/v*). The aim of this step is to break the non-specific interactions between the interferences and the sorbent without breaking the ionic interactions (specific) between the analytes of interest and the sorbent. Therefore, the composition and volume of the washing solvent were optimised. MeOH was tested first due to the good results obtained in the determination of cathinones in environmental samples [11]. In particular, different volumes were evaluated: 2 mL, 4 mL and 5 mL of MeOH. Unfortunately, even with the lowest volume of MeOH used, all the analytes were eluted. One possible explanation is that urea, which is present at high concentrations in urine, might compete with the cathinones at the active ionic sites and might displace the interactions with ~~the cathinones~~them. Therefore, a change in the washing solvent was made and clean-ups with MeOH:H_2O (75:25, *v/v*) and H_2O were tested with the same volumes previously tested for MeOH. In the case of MeOH:H_2O (75:25, *v/v*), the analytes were partially lost and in the case of H_2O, there were no losses. Therefore, 2 mL of H_2O were enough to clean most of the interferences ~~in the form of~~such as salts and ionic species. From the obtained results, it can be concluded that if all the analytes are eluted with 2 mL of MeOH, then the acid (5% HCOOH) is not necessary in the elution solvent since the analytes are probably only retained by reversed-phase interactions.

After this, a mixture of urine:phosphate buffer solution (pH 7) (1:1, *v/v*) spiked with cathinones at a concentration of 2 mg L^{-1} was loaded into the cartridge, then it was washed with 2 mL of water and finally, the analytes were eluted with 2 mL of MeOH. The extraction yields using the optimised protocol for Oasis WCX were between 84% and 105%.

Oasis MCX

The initial protocol for the Oasis MCX cartridge was to use 5 mL of MeOH and 5 mL of phosphate buffer solution at pH 6 (0.15 M of NaH_2PO_4 and 0.05 M of Na_2HPO_4) for conditioning, 5 mL of the buffer solution (pH 6) containing cathinones at a concentration of 2 mg L^{-1} as loading step, MeOH for the clean-up and 5% of NH_4OH in MeOH as the elution solvent. This pH, which makes it possible for all cathinones to be in their cationic form, has been previously studied in urine and values of pH 3 and 6 have been compared. Better results were obtained at pH 6 when urine samples were analysed. As before, with the initial conditioning and loading steps fixed, the washing and elution were optimised by using LC-DAD to achieve the highest recoveries using the minimum volume of solvent.

In this case, 2 mL, 4 mL and 5 mL of 5% of NH_4OH in MeOH were ~~studied~~ examined in the elution step without the clean-up step. It was observed that 2 mL was enough to elute the analytes with recoveries between 90% and 105%. Higher elution volumes (4 mL and 5 mL) did not improve the recoveries. ~~much~~.

The washing step was evaluated with urine and volumes of 2 mL, 4 mL and 5 mL of MeOH were tested for this step. It was observed that with 2 mL of MeOH there were no losses. However, with 4 mL it could be observed that some analytes were lost and 2 mL of MeOH was chosen as the optimal washing solvent. It has been previously observed that when a 60 mg Oasis MCX cartridge in the analysis of doping compounds, 2 mL of MeOH are suitable for the washing step in urine samples [37].

Once the protocol had been optimised, a mixture of urine:phosphate buffer solution (pH 6) (1:1, *v/v*) spiked with cathinones at a concentration of 2 mg L^{-1} was loaded. The extraction recoveries using the optimised protocol for Oasis MCX were between 84% and 101%.

Comparison between Oasis WCX and Oasis MCX

As it can be seen in Fig. 1, both sorbents achieved ~~good~~ recoveries higher than 80% when they were tested by LC-DAD. Due to the ~~good~~ acceptable results obtained for the two sorbents in terms of extraction, they were compared by evaluating the clean-up step, the recoveries of the extraction procedure and the matrix effect (% ME) by using LC-HRMS. As it is well known that when an MS detector is used, the matrix effect must be considered because it can cause an enhancement or a suppression of the signal.

Firstly, ~~a study~~the performance of the two SPE sorbents was ~~performed~~ compared by loading 5 mL of the mixture urine:buffer solution (pH 6 with Oasis MCX or pH 7 with Oasis WCX) (1:1, *v/v*) containing cathinones at a concentration of 40 ng mL^{-1} without the clean-up step ~~to~~ and then comparing~~e~~ these results ~~to~~ with the ones obtained with a clean-up step. A higher % ME was obtained for Oasis WCX (between -29% and -73%), while for Oasis MCX it was between -27% and -50%. ~~It is important to mention that b~~Both sorbents had high values of % ME, which means that a clean-up in the SPE is necessary to reduce this effect. Table 2 shows the values of % ME with and without the clean-up step. A reduction of the % ME can be observed in both cases if a clean-up step is applied. Better results of % ME were obtained for Oasis MCX with the clean-up step, and therefore it was selected as the optimal sorbent for its better clean-up with MeOH.

~~It is important to mention that~~In this experiment, the SPE extracts were evaporated to dryness under a gentle stream of N_2. Cathinones are volatile in their base form and it is necessary to neutralise the basic extract solution by adding 100 µL of 1% HCl in MeOH before the evaporation [38]. Finally, after the solution had been reconstituted with 1 mL of mobile phase, it was filtered through a 0.45 µm PTFE filter and injected into the LC-HRMS.

Method validation

The analytical performance of the ~~developed~~ method developed was investigated under optimal conditions by evaluating the % R_{app}, % ME, linear range, the MDLs, MQLs, repeatability (intra-day) and reproducibility (inter-day). The precision parameters were studied at two concentration levels, 1 ng mL^{-1} (low level) and 40 ng mL^{-1} (high level). Table 3shows the values at the two levels of concentration. Moreover, a non-spiked urine sample was included in the analysis to substract any analyte signal present in the samples.

Values of % R_{app} at high concentration were between 69% and 82%, and these values were mainly affected by the ME. Values at low concentration were between 75% and 125% and as

stated before, they were mainly affected by the ME. In general, for both concentration levels a suppression of the signal was observed (between -8% and -28%). However, 4-MEC and alpha-PVP showed enhancement at the low concentration (12% and 21% respectively). These values of % ME are considered acceptable. Although these ME values are similar to the ones previously obtained by Concheiro *et al.*[9], who used the SOLA SCX sorbent, better recoveries of the extraction procedure can be observed in our case.

As the ME using Oasis MCX was higher than -20% in some cases at both concentration levels (1 ng mL^{-1} and 40 ng mL^{-1}), it was decided to use a matrix-matched calibration curve to deal with the ME. The linear range was from MQL to 100 ng mL^{-1} for most compounds or to 150 ng mL^{-1} for 4-MEC, pentedrone, 3,4-DMMC, alpha-PVP and MDPV, ~~obtaining~~ in all cases obtaining r^2 values higher than 0.999.

The MDLs were between 0.04 ng mL^{-1} and 0.16 ng mL^{-1} while the MQLs were 0.2 ng mL^{-1} for all the compounds. Thus, the developed method achieved lower MDL and MQL values than a previously published ~~work~~ study in which a similar strategy was reported; however, in that case, the main difference was the use of another kind of cation-exchange sorbent (Sola SCX) [9]. They achieved recoveries between 79% and 117% and detection and quantification limits of 0.25 ng mL^{-1} and 0.5 ng mL^{-1}, respectively when a Q-Orbitrap instrument was used. If the method is also compared with other HRMS methods, which used salting-out liquid-liquid extraction (SALLE) [12] or liquid-liquid extraction (LLE) [30], it can be observed that lower MDL and MQL values are obtained with the present method.

Repeatability and reproducibility were studied at both levels of concentration by analysing five spiked samples on the same day and on five different days. Values at 1 ng mL^{-1} in terms of relative standard deviation (% RSD) were between 1% and 13% for repeatability and 2% and 10% for reproducibility.

To prove the accuracy of the method, as we could not apply the method to urine samples from drug ~~ab~~users, it was used ~~applied~~ to analyse different blind samples and the concentration of the cathinones were obtained by the matrix-matched calibration curve. All the blind samples showed similar results in terms of bias, which were lower than 10% in all cases. ~~As f~~For example, one sample was spiked by a colleague at 46 ng mL^{-1} and after ~~all~~ the procedure was completed, the concentrations obtained were between 45 and 53 ng mL^{-1} for the different cathinones, ~~obtaining an~~and the average accuracy in terms of bias ~~of~~ was 7%, which ~~is considered a very good result~~shows acceptable results and ~~shows~~ proves the reliability of this method. An example of a chromatogram of a blind sample can be observed in Fig. 2.

Conclusions

A method based on SPE using a strong cation-exchange sorbent (Oasis MCX) followed by LC-HRMS using Orbitrap as analyser was successfully developed and validated for determining synthetic cathinones in urine.

Two different cation-exchange sorbents were studied to extract the cathinones from urine, a weak one (Oasis WCX) and a strong one (Oasis MCX). Oasis MCX achieved excellent recoveries (around 100%) and acceptable levels of matrix effects (reduced if a clean-up step was applied). The present method ~~accomplished~~ achieved very low MDLs and MQLs~~,~~ which are suitable for detecting these kind of compounds in urine at the usual concentration levels found. Thus, the ~~developed~~ method developed is a promising tool for forensic and toxicological analysis.

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Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

~~**Human and animal rights**~~**Ethical approval** All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

~~**Informed consent**~~ Informed consent was obtained from all individual participants included in the study.

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542

543 **Figure captions**

544 Figure 1. Comparison of the recoveries obtained by SPE when 5 mL of a standard sample
545 spiked at 40 ng mL^{-1} of each analyte were percolated through Oasis WCX and Oasis MCX
546 sorbents and analysed by LC-DAD.

547 Figure 2. Chromatogram of a urine blind sample analysed by SPE/LC-MS/MS under the
548 optimal conditions. 1: flephedrone, 2: ethcathinone, 3: buphedrone, 4: 2-MMC, 5: butylone, 6:
549 mephedrone, 7: 4-MEC, 8: pentedrone, 9: 3,4-DMMC, 10: alpha-PVP and 11: MDPV.

550

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Table 1. Retention time, protonated molecule, the two fragment ions and the ion ratios of the studied cathinones.

		Protonated molecule $[M+H]^+$		Fragment 1			Fragment 2		
Analyte	**Rt (min)**	**Formula**	**Accurate mass *m/z***	**Formula**	**Accurate mass m/z**	**Ratio $[M + H]^+$ / F 1**	**Formula**	**Accurate mass *m/z***	**Ratio $[M + H]^+$ / F 2**
Flephedrone	6.01	$C_{10}H_{13}FNO$	182.09757	$C_{10}H_{11}FN$	164.08755	38	C_9H_8FN	149.06408	10
Ethcathinone	6.12	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	23	$C_9H_{10}N$	132.08132	12
Buphedrone	7.46	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	19	C_9H_9N	131.07350	6
2-MMC	8.72	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	30	$C_{10}H_{11}N$	145.08915	16
Butylone	877	$C_{12}H_{16}NO_3$	222.11247	$C_{11}H_{12}NO$	174.09189	14	$C_{12}H_{14}NO_2$	204.10245	17
Mephedrone	9.20	$C_{11}H_{16}NO$	178.12264	$C_{11}H_{14}N$	160.11262	34	$C_{10}H_{11}N$	145.08915	14
4-MEC	10.47	$C_{12}H_{18}NO$	192.13829	$C_{12}H_{16}N$	174.12827	30	$C_{10}H_{11}N$	145.08915	9
Pentedrone	10.90	$C_{12}H_{18}NO$	192.13829	$C_{12}H_{16}N$	174.12827	16	$C_9H_{10}N$	132.08132	10
3,4-DMMC	11.60	$C_{12}H_{18}NO$	192.13829	$C_{12}H_{16}N$	174.12827	37	$C_{11}H_{13}N$	159.10480	12
Alpha-PVP	12.11	$C_{15}H_{22}NO$	232.16959	C_7H_7	91.05478	15	C_7H_5O	105.03404	5
MDPV	12.48	$C_{16}H_{22}NO_3$	276.15942	$C_8H_{16}N$	126.12827	8	$C_8H_7O_2$	135.04461	9

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Table 2. % ~~ME~~ Matrix effect of Oasis WCX and Oasis MCX with and without clean-up step in the SPE from a mixture of cathinones at 40 ng mL^{-1} using LC-HRMS and the % RSD values in brackets.

	% ME Oasis WCX		**% ME Oasis MCX**	
n=3 40 ng mL^{-1}	**Without clean-up**	**With clean-up**	**Without clean-up**	**With clean-up**
Flephedrone	-73 (6)	-50 (5)	-50 (4)	-21 (2)
Ethcathinone	-46 (8)	-34 (4)	-48 (3)	-20 (1)
Buphedrone	-35 (8)	-28 (7)	-39 (5)	-12 (2)
2-MMC	-44 (10)	-36 (7)	-27 (5)	-9 (3)
Butylone	-29 (11)	-24 (8)	1 (10)	-12 (2)
Mephedrone	-36 (4)	-28 (9)	-37 (7)	-22 (1)
4-MEC	-50 (6)	-36 (8)	-49 (9)	-10 (12)
Pentedrone	-41 (9)	-34 (7)	-40 (6)	-20 (2)
3,4-DMMC	-42 (7)	-31 (5)	-40 (4)	-15 (2)
Alpha-PVP	-49 (7)	-34 (7)	-49 (8)	-10 (1)
MDPV	-49 (8)	-34 (5)	-45 (5)	-22 (4)

Table 3. Validation parameters of the method based on SPE with Oasis MCX followed by LC-HRMS for the determination of synthetic cathinones in urine.

Analyte	% ME[a] (n=3)	% R_{app}[a] (n=5)	% ME[b] (n=3)	% R_{app}[b] (n=5)	MDLs[c]	Linear range[c]	Repeatability[a] % RSD (n=5)	Reproducibility[a] % RSD (n=5)
Flephedrone	-24	73	-21	73	0.08	0.2-100	2	3
Ethcathinone	-21	84	-20	76	0.10	0.2-100	5	4
Buphedrone	-14	90	-12	85	0.10	0.2-100	7	6
2-MMC	-10	93	-9	88	0.10	0.2-100	7	6
Butylone	-8	96	-12	84	0.12	0.2-100	9	7
Mephedrone	-26	81	-22	76	0.08	0.2-100	13	10
4-MEC	12	115	-10	87	0.04	0.2-150	4	4
Pentedrone	-22	76	-20	74	0.04	0.2-150	6	5
3,4-DMMC	-18	76	-15	79	0.04	0.2-150	11	9
Alpha-PVP	21	125	-10	87	0.16	0.2-150	1	2
MDPV	-28	82	-22	69	0.06	0.2-150	7	6

[a] spiked at 1 ng mL^{-1} ; [b] spiked at 40 ng mL^{-1} ; [c] concentration in ng mL^{-1}

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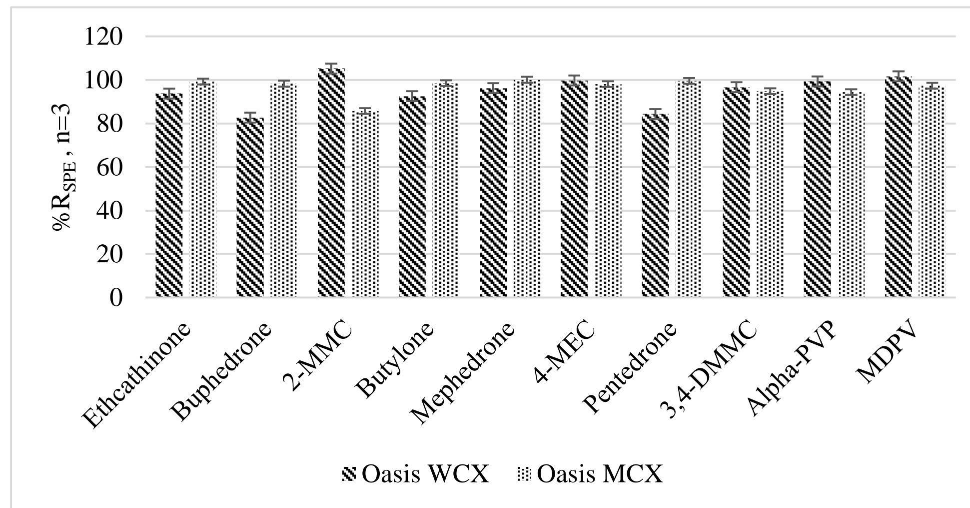

Figure2

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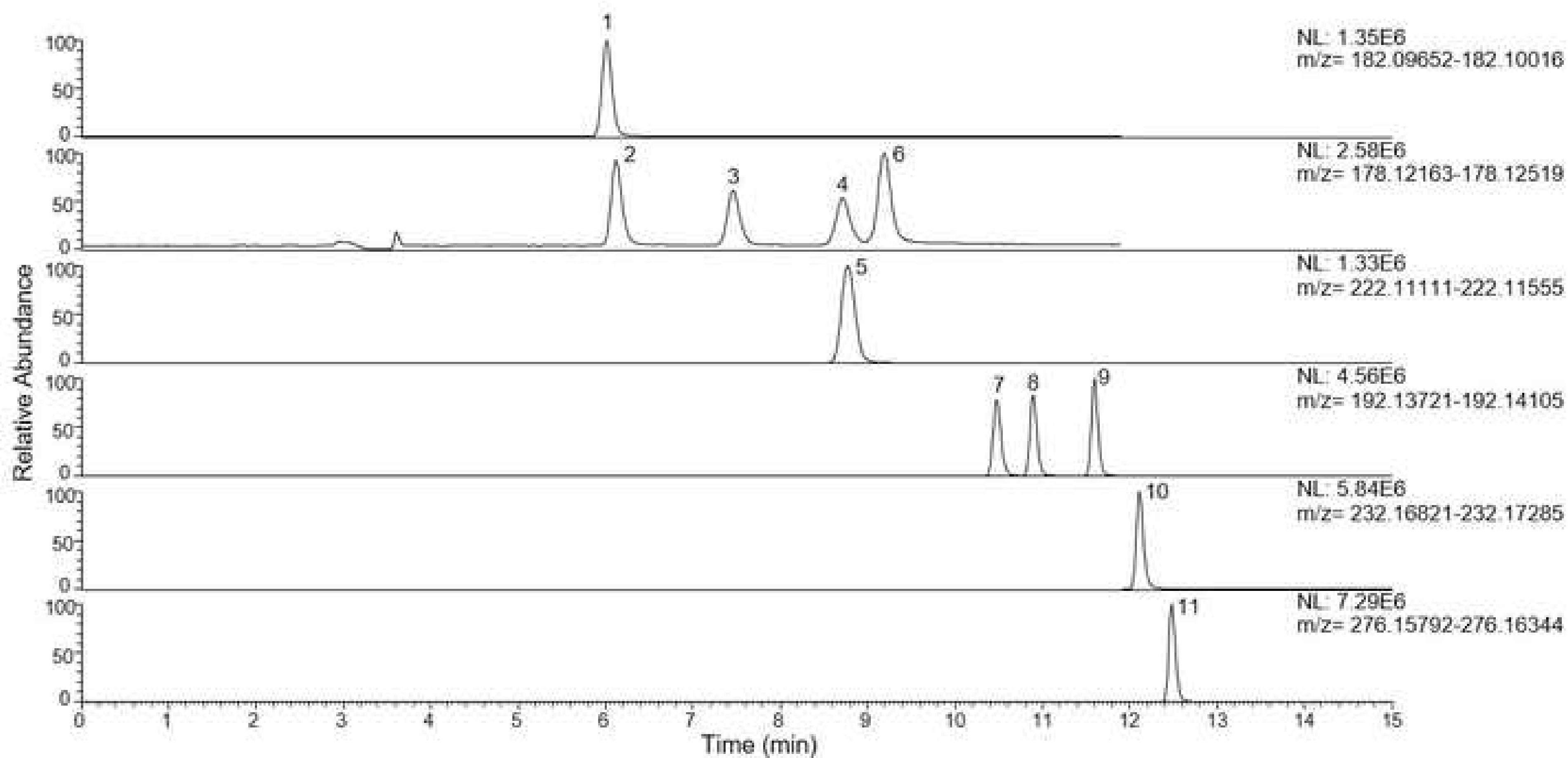

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