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Comparison of different chiral selectors for the enantiomeric determination of amphetamine-type substances in human urine by solid-phase extraction followed by capillary electrophoresis-tandem mass spectrometry

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Keywords

Amphetamine-type substances

Cation-exchange sorbents

CE-MS/MS

Chiral selectors

Human urine

Abbreviation: ACN, acetonitrile; **ADHD,** attention-deficit hyperactivity disorder; **AMP,** amphetamine; **ATS,** amphetamine-type substances; **CMT,** counter-current migration technique; **COL. E,** collision energy; **LLE,** liquid-liquid extraction; **MAMP,** methamphetamine; **MEOH,** methanol; **SFC,** supercritical fluid chromatography

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Abstract

The present study develops a method for the enantioseparation of a group of amphetamines and their metabolites in urine by capillary electrophoresis coupled to tandem mass spectrometry (CE-MS/MS). Amphetamines present a chiral centre and thus two enantiomers, which is important from a toxicological point of view because they may have different pharmacokinetic and pharmacological properties. It is therefore essential to find suitable methods to distinguish both enantiomers. Today the use of CE is becoming more important in this field since, with the simple addition of a chiral selector to the background electrolyte, the enantioseparation can easily be achieved. However, when CE is coupled to MS, the use of volatile chiral selectors and compatible background electrolytes or other strategies such as the counter-current migration approach are required to avoid contamination of the ion source from non-volatile species. In the present study we use the latter strategy to evaluate six different chiral selectors using CE-MS/MS. As a sample pre-treatment, two cationic-exchange sorbents – Oasis WCX and Oasis MCX – are compared for the urine pre-treatment. Using this method it was possible to achieve the complete chiral separation of the amphetamines under study with detection limits ranging between 0.8 and 1.5 ng mL^{-1} and method quantification limits between 2.0 and 8.0 ng mL^{-1}. Matrix-matched calibration curves up to 150 ng mL^{-1} were used to cover the usual concentration ranges at which amphetamines have generally been found in toxicological and forensic analyses.

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1. Introduction

The World Drug Report of 2020 stated that 0.5% of the adult European population had taken amphetamine-type stimulants (ATS) the previous year, this being the second most-consumed group of drugs. It also highlighted that consumption is increasing year by year [1,2]. ATS include some of the most popular drugs in the illegal market, such as amphetamine (AMP), methamphetamine (MAMP) and 3,4-methylenedioxymethamphetamine (MDMA or ecstasy) [2,3]. These drugs have a symmetric carbon in their molecule and so they may occur in the form of R and S enantiomers with different pharmacokinetic, pharmacological and toxicological properties [4]. In some cases, as both enantiomers may produce different effects, their control and legality are different. This is why their proper identification in the forensic field is so important [4]. For example, although AMP is widely known as an illegal drug, it also has a medical application. The prescribed AMP is S-AMP or its prodrug Lisdexamphetamine, which is used to treat adults with attention-deficit hyperactivity disorder (ADHD) [5,6]. In the case of MAMP, the R-enantiomer is known to be a nasal decongestant in Vicks VapoInhaler devices [7]. In the synthesis of pharmaceuticals, the aim is to obtain the single active enantiomer responsible for the therapeutic properties, but many of them are marketed as racemates, with still small percentages of the other enantiomer. In the case of illicit drugs, racemates are normally found together with other additives because clandestine laboratories do not usually isolate the enantiomer that functions as a central nervous system stimulant for economic reasons [8].

Once ATS are taken they are metabolized in the body, and consequently these compounds and their metabolites can be found in different biological matrices. Thus when biological samples are analysed, apart from MAMP, their main metabolites p-hydroxymethamphetamine (OHMA) and AMP can be found. Similarly, MDMA can also give rise to their metabolites 4-hydroxy-3-methoxymethamphetamine (HMMA) and 3,4-methylenedioxyamphetamine (MDA) [9,10]. Therefore it is important to be aware that AMP can be found due to drug intake and also due to the metabolization of MAMP. In the present study AMP, MAMP, MDMA and MDA are the compounds chosen for studying both parent compounds and some of their main metabolites. Not all the different biological samples used to detect drug consumption present the same detection times (detection window) and, depending on the aim of the intended study, it might be better to analyse one matrix rather than another. Urine is one of the most frequently used biological samples and has a wide detection window stretching from days to weeks. In this biological sample ATS can be found ranging from low levels of ng mL^{-1} to values higher than 1000 ng mL^{-1} [9,11–15]. The main advantages of urine are that huge quantities of sample are easily obtainable, no trained personnel are needed to collect it, and the parent drugs and their metabolites can be found in higher concentrations compared to other matrices [16–18].

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The separation of enantiomers can be easily accomplished by capillary electrophoresis (CE) [19–24], although supercritical fluid chromatography (SFC) [11], gas chromatography (GC) [12,25–27] and liquid chromatography (LC) [13,14,28] have also been used by some authors. The main advantages of CE are its high separation efficiency and the fact that it is an environmentally-friendly technique. Its main drawback is its limited sample loading capacity, which means reduced sensitivity, especially when used in combination with ultraviolet detection [29–31]. The advantages of CE in chiral analyses are related to the rapid method development and its flexibility when it comes to choosing or changing the chiral selector and its concentration, since the enantioseparation is performed simply by adding this substance to the background electrolyte (BGE) [11,30,32–34]. However, when a chiral CE method is combined with MS it is important to take into account that, apart from volatile and low conductivity BGEs, compatible chiral selectors to prevent source dirtying should be used since this can cause problems when low sensitivity is required [29,30,34,35]. Although cyclodextrins (CD) are the most common chiral selectors used in CE, most of them are non-volatile compounds and may bring about source contamination of the MS system. This can result in the ion suppression of the MS signal and consequently the sensitivity of the MS will be lower [30,32,34,36]. Although lowering the concentration of the chiral selector can minimize these drawbacks, different strategies have been reported in the literature as regards minimizing the contamination of the ionization source [34]. One of these is the counter-current migration technique (CMT), in which the selected chiral selector migrates in the opposite direction to the electroosmotic flow (EOF) [35,36]. To achieve this, charged chiral selectors are required. Since ATS are basic compounds with pK_a values of around 10, at a lower pH than their pK_a they are positively charged. When using CMT, therefore, negatively charged chiral selectors such as sulphated-CD, phosphated-CD, sulphobutylated-CD, macrocyclic antibiotics or crown ethers are needed. This approach has been used in some examples previously reported in the literature that have focused on the chiral determination of ATS by CE-MS [21–24]. Charged CDs were used in all of them, with sulphated-CD being the most usual.

Urine is a complex sample and the matrix components may interfere in the determination of the analytes of interest. A pre-treatment should therefore be carried out when this matrix is selected for ATS determination. The most common extraction techniques used by authors for this purpose are liquid-liquid extraction (LLE) and solid-phase extraction (SPE) [11,13,14,19,20,25–27].

The main aim of this study is to develop a method for the enantiodetermination of a group of ATS and their metabolites in urine samples by SPE CE-MS/MS. For the enantioseparation of the selected ATS, six different chiral selectors are evaluated and compared using CMT in order to be able to couple the method with MS detection. As far as we know, this is the first study in which different chiral selectors are compared for the enantioseparation of ATS in urine by CE-MS/MS. Five of these chiral selectors are

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based on charged CDs, while the last is a macrocyclic antibiotic. For the urine pre-treatment, two cationic exchange sorbents – Oasis WCX (weak) and Oasis MCX (strong) – are also compared to extract the ATS from urine.

2. Materials and methods

2.1. Standards and materials

The analytical standards of AMP, R-AMP, S-AMP, MAMP, R- MAMP, S- MAMP, MDA and MDMA were purchased from Sigma Aldrich (St. Louis, MO, United States). The chiral selectors α-CD sulphated sodium salt, β-CD sulphated sodium salt, γ-CD sulphated sodium salt, γ-CD phosphate sodium salt and β-CD sulphobutylated sodium salt were obtained from Cyclolab (Budapest, Hungary). Vancomycin was obtained from LGC Standards (Teddington, UK).

Standard solutions of the ATS were prepared individually in MeOH at 100 mg L^{-1} and kept in the freezer at -20 °C. The working solutions were prepared by diluting a mixture solution containing the ATS at a concentration of 1 mg L^{-1} in MeOH with water at different concentrations. These solutions were also kept in the freezer at -20 °C.

Ultra-gradient HPLC grade methanol (MeOH) and 2-propanol (IPA) were obtained from J.T. Baker (Deventer, The Netherlands). Formic acid (HCOOH), ammonium acetate, ammonium formate and ammonium hydroxide (NH_4OH) were purchased from Sigma-Aldrich. Methanol for LC-MS was obtained from Chem-Lab (Zedelgem, Belgium). Water and acetonitrile (ACN) for LC-MS were purchased from Scharlab (Barcelona, Spain). Ultrapure water was obtained using a water purification system (Meck Millipore, Darmstadt, Germany).

The capillary used was purchased from Polymicro Technologies (Phoneix, AZ, USA). Both cation-exchange sorbents (150mg/6 mL) – the weak (Oasis WCX) and the strong (MCX) – for the SPE process were obtained from Waters Corp. (Milford, MA, United States).

2.2. Instrumentation

The instrumentation used in this study was an Agilent model 7100 series CE coupled with an Agilent 6460 series triple quadrupole mass spectrometer with an electrospray ionization (ESI) interface from Agilent Technologies (Waldbronn, Germany). For data analysis and instrumental control, Agilent MassHunter Workstation Software (version B.09.00) was used. pH measurements were performed using a GLP 21 pH-meter from Crison (Barcelona, Spain).

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2.3. CE-MS/MS conditions

The BGE consisted of 1 M HCOOH:1 M ammonium formate (5:0.1, *v/v*) with 0.2% sulphated γ-CD. The sheath liquid used in the analyses was a mixture of 10 mM ammonium acetate:MeOH (50:50). The capillary was a bared fused-silica of 50 µm with a total length of 80 cm, which was thermostatted at 25 °C. The conditioning of new capillaries (outside the MS system) consisted of flushing (930 mbar) 1 M NaOH for 40 min followed by 10 min of Milli-Q water. At the beginning of each day, the capillary was conditioned with 0.1 M NH_4OH, followed by Milli-Q water and finally BGE, each for 10 minutes. Between each analysis it was conditioned with 0.1 M NH_4OH, followed by Milli-Q water and finally BGE, each for 5 minutes. The sample was hydrodynamically injected at 50 mbar for 10 s and the separation was performed applying a voltage of 20 kV.

The optimized ion source parameters were as follows: gas temperature, 200 ºC; gas flow rate, 2 L min^{-1}; nebuliser pressure, 7 psi; and capillary voltage, 4000 V in positive mode. Multiple reaction monitoring (MRM) was the acquisition mode chosen and the two most intensive transitions between the parent and product ions were selected (Table 1). Two different windows were set: the first without acquisition, while the second acquired the 4 ATS. The fragmentor was set at 100 V for both windows, while the collision energies (Col. E) were between 4 and 15 eV.

2.4. Urine collection and preparation

For the urine analysis, pooled mixed urine samples were obtained from non-addicted laboratory staff, both women and men of different ages. The samples were collected in polypropylene tubes and kept in the freezer at -20 °C. For the urine pre-treatment, an SPE was performed using 150 mg Oasis WCX cartridges. First they were activated with 5 mL of MeOH and conditioned with 5 mL of phosphate buffer at pH 7 (0.10 M of NaH_2PO_4 and 0.06 M of Na_2HPO_4). Then 25 mL of a mixture of urine diluted with phosphate buffer at the same pH was loaded and washed with 4 mL of MeOH. Finally, the analytes were eluted with 4 mL of 5% HCOOH in MeOH. The extracts were evaporated to dryness under a gentle stream of nitrogen and reconstituted with 0.5 mL of water. They were then filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter prior to their analysis in the CE-MS/MS system.

2.5. Validation

The method validation was performed following European guidelines for workplace drug testing in urine [37]. It was based on linearity, sensitivity, instrumental detection and quantification limits (IDLs and IQLs), method detection and quantification limits (MDLs and MQLs), apparent recoveries (R_{app}), matrix

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3 164 effect (ME), precision as repeatability (intra-day) and reproducibility (inter-day), selectivity, stability and
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5 165 accuracy. In all cases the retention time had a tolerance of ±2.5% and an ion ratio of ±20% [38].
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7 166 The instrumental linearity was investigated using different calibrator concentrations of a mixture of ATS
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9 167 neat standards of between 60 and 3500 ng mL^{-1} by evaluating the determination coefficient (r^2). IDLs
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11 168 were defined as the lowest detectable point with a signal-to-noise (S/N) ratio >3 and IQLs as the lowest
12 169 concentration in the calibration curve with S/N >10.
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14 170 The method linearity was studied using a matrix-matched calibration curve of between 2 and 150 ng mL^{-1}
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16 171 of a mixture of ATS in urine. The linearity, MDLs and MQLs criteria were the same as for the
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18 172 instrumental parameters, but in this case for extracted samples. The R_{app}, ME, repeatability and
19 173 reproducibility were studied at three different levels of concentration – 10 ng mL^{-1} (low), 50 ng mL^{-1}
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21 174 (medium) and 100 ng mL^{-1} (high) – by using five replicates (n=5). Repeatability and reproducibility were
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23 175 evaluated in terms of relative standard deviation (%RSD), the former being obtained by analysing five
24 176 replicates on the same day and the latter by analysing one replicate on five different days.
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26 177 The selectivity and specificity were studied by evaluating the possible endogenous and exogenous
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28 178 interferences that may be present in urine samples from different specimens. The stability was studied by
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30 179 reanalysing the three calibrator levels (10, 50 and 100 ng mL^{-1}) in urine every 10 hours for a total of 40
31 180 hours at room temperature. The method's accuracy was evaluated in terms of error by analysing three
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33 181 blind samples spiked at different concentrations by a member of the laboratory staff before all the
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35 182 experimental procedures. After analysing the samples, the concentration of ATS was calculated with the
36 183 matrix-matched calibration curve and compared to the spiked concentration.
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3. Results and discussion

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3.1. Separation and detection optimization

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43 186 The MS/MS parameters were individually evaluated with standard solutions of each ATS at 1 mg L^{-1}.
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45 187 Because ATS are basic compounds, the positive mode was used to choose the precursor ion of each
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47 188 analyte. The MRM mode was applied to collect the monitoring and quantitative ions. The fragmentor
48 189 voltage was investigated between 50 and 200 V and the collision energies between 0 and 30 kV. The two
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50 190 most abundant transitions of each ATS were chosen to confirm their identification. In Table 1, the
51 191 different precursor and product ions of the analytes are shown at a fragmentor voltage of 100V for all the
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53 192 compounds. The source conditions were optimized for the CE-MS/MS system using the source
54 193 optimization software, i.e. capillary voltage (4000 V), gas temperature (200 °C), gas flow rate (2 L min^{-1})
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194 and nebulizer (7 psi). Finally, two different windows of detection were created, the first without
195 acquisition and the second acquiring the four ATS under study.

196 Afterwards, the sheath liquid composition and the BGE for the ATS CE separation were evaluated. To
197 this end a mixture of AMP, MAMP and MDMA at 500 ng mL^{-1} and an 80 cm bared fused silica capillary
198 of 50 µm were used. These initial experiments were performed without the addition of the chiral selector
199 to avoid possible contamination of the MS detector.

200 The sheath liquid was studied first, since its composition and flow rate can have a strong influence on the
201 efficiency of the ionization process and detection sensitivity. It can also affect the CE separation process
202 and analyte migration times due to the possible formation of ionic boundaries [39]. Initial experiments to
203 optimize this parameter focused on evaluating its composition. For this, 10 mM ammonium acetate as
204 BGE at 20 kV of separation voltage was used. Under these conditions, two different sheath liquids were
205 evaluated: IPA:H_2O:HCOOH (60:39:1, *v/v/v*) and 10 mM ammonium acetate:MeOH (50:50), both at a
206 flow rate of 0.4 mL min^{-1}. This selection was based on the literature dealing with the determination of
207 ATS by CE-MS [21,23,40]. From the results obtained it was observed that better efficiency and higher
208 peak intensities were obtained with the mixture of 10 mM ammonium acetate:MeOH (50:50), so further
209 experiments were performed with this composition. Afterwards, the optimal flow rate was evaluated by
210 comparing 0.3, 0.4 and 0.5 mL min^{-1} and the best peak efficiency was obtained with 0.4 mL min^{-1}, so this
211 was the value chosen.

212 Two different BGEs – 10 mM ammonium acetate and 1 M HCOOH – were evaluated in terms of
213 resolution. This was performed considering their compatibility with the MS system and taking into
214 account that they are both widely employed BGEs in the determination of ATS by CE-MS [21,35,40,41].
215 Table 2 shows the resolution between the different ATS included in this study using the two
216 aforementioned BGEs. It was observed that the use of formic acid provided better peak resolution than
217 ammonium acetate. Based on these results, a combination of 1 M HCOOH:1M ammonium acetate (5:0.1,
218 *v/v*) was then tried, and with this BGE the resolution was the same as for 1 M HCOOH. Another
219 experiment involved changing ammonium acetate for ammonium formate in combination with formic
220 acid. This mixture provided lower resolution values than the previous one. Finally, a concentration of 0.5
221 M formic acid was tried in combination with ammonium acetate and ammonium formate. With these two
222 BGEs the worst resolution values were obtained and, moreover, the migration times were higher than
223 with the combination of 1 M HCOOH:1M ammonium acetate (5:0.1, *v/v*). Finally, comparing all the
224 obtained results, we were able to conclude that the best resolution values were obtained with 1M HCOOH
225 and the combination of 1 M HCOOH:1M ammonium acetate (5:0.1, *v/v*). However, when comparing the

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3 226 peak efficiency values for both, we observed that they were slightly better for the mixture. Therefore the
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5 227 combination of 1 M HCOOH:1 M ammonium formate was the BGE chosen.
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7 228 The addition of a chiral selector to the BGE was then evaluated. As mentioned earlier, when using CE-
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9 229 MS/MS it is important to take into account the chiral selector characteristics and use them at the
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11 230 minimum concentration to reduce or avoid their entering the MS system [34]. Although for CE there is a
12 231 wide variety of chiral selectors, with the most commonly used being native CDs, when the detection
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14 232 system is an MS the choice of these selectors is restricted because, depending on their characteristics, they
15 233 may cause signal suppression and contamination of the MS detector. Moreover, as mentioned in the
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17 234 Introduction, certain strategies exist to minimize these problems. In the present study CMT was chosen.
18 235 When using this strategy it is important to select chiral selectors capable of migrating in the opposite
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20 236 direction to the detector, thereby avoiding their entering the MS since this could produce loss of
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22 237 sensitivity. Moreover, as a complementary tool, during the sample injection the MS ion source can be
23 238 turned to waste mode to prevent the chiral selectors from entering the ion source [34]. Therefore, to
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25 239 comply with CMT requirements a set of chiral selectors was chosen that had previously been used in
26 240 various studies in which different ATS were enantioseparated. Specifically, the substances selected for
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28 241 investigation were α-CD sulphated sodium salt, β-CD sulphated sodium salt, γ-CD sulphated sodium salt,
29 242 γ-CD phosphate sodium salt, β-CD sulphobutylated sodium salt and vancomycin [39,42,43]. Different
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31 243 percentages of these chiral selectors ranging between 0.1 and 1% were evaluated. The concentration
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33 244 levels were low to minimize the possible suppression of the ionization of the analytes, which could lead to
34 245 a severe loss of sensitivity. When using the 0.1% level of chiral selector in the BGE, it was only possible
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36 246 to achieve the enantioseparation of the ATS under study with sulphated γ-CD. However, MAMP was not
37 247 separated at baseline with this chiral selector at this concentration level. For 0.2%, only with sulphated γ-
38
39 248 CD and sulphobutylated β-CD was it possible to achieve the enantioseparation of the analytes, although
40 249 regarding the latter, the separation between enantiomers was not at baseline. For the higher percentages of
41
42 250 all the chiral selectors tested, the peaks were not enantiomerically separated and indeed were distorted.
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44 251 Based on the obtained results, therefore, a combination of 0.1% of sulphated γ-CD with 0.1% of
45 252 sulphobutylated β-CD was also investigated, and under these conditions it was possible to achieve
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47 253 complete separation between the two enantiomers for each of the ATS under study. The resolution values
48 254 obtained for the ATS enantiomers with these chiral selectors that provided partial or total
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50 255 enantioseparation are shown in Table 3. Figure 1 shows the electropherograms of the enantioseparation of
51 256 the ATS using 0.1% sulphated γ-CD (A), 0.1% sulphated γ-CD + 0.1% sulphobutylated β-CD (B), 0.2%
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53 257 sulphated γ-CD (C) and 0.2% sulphobutylated β-CD (D).
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258 It can be seen that the best resolution results were obtained with 0.2% of sulphated γ-CD. For this CD the
259 values were the higher for AMP and MDMA, whereas for MAMP the resolution obtained was the same
260 as or similar to that obtained with the combination of 0.1% of sulphated γ-CD with 0.1% of
261 sulphobutylated β-CD or 0.2% sulphobutylated β-CD. Therefore, 0.2% of sulphated γ-CD was chosen as
262 the optimum chiral selector in the present study.

263 Figure 2 shows an electropherogram using the optimal conditions to determine the ATS, including in this
264 case MDA. As can be seen, the four ATS were totally enantioseparated using the CMT approach in
265 combination with sulphated γ-CD. Moreover, with the use of R and S standards for AMP and MAMP, the
266 R-enantiomer migrated earlier than the S. However, due to the lack of R/S standards for MDMA and
267 MDA, the identification of each enantiomer could not be performed for these two compounds.

268 **3.2. SPE optimization**

269 For the urine pre-treatment, SPE was the strategy using cation-exchange sorbents selected due to the
270 amino group of ATS, which are charged at a lower pH than their pKa. To this end Oasis WCX (weak) and
271 Oasis MCX (strong) sorbents were compared for the SPE process. The methodologies for both SPE
272 sorbents were adopted from a previously published paper by our group regarding the determination of
273 cathinones by LC-HRMS [44]. In the present study, the SPE procedure for both sorbents was first
274 optimized by using urine samples spiked at 200 ng mL^{-1} of each analyte to enable us to select the sorbent
275 that provided better results in terms of recovery.

276 In the case of Oasis WCX, this was first activated with 5 mL of MeOH and conditioned with 5 mL of
277 phosphate buffer at pH 7 (0.10 M of NaH_2PO_4 and 0.06 M of Na_2HPO_4). A mixture of 5 mL of
278 urine:phosphate buffer (pH 7) (50:50, *v/v*) was then loaded, washed with 2 mL of MeOH and finally
279 eluted with 2 mL of 5% HCOOH in MeOH. Following this protocol, recoveries of around 95% for all the
280 analytes were obtained. As for the Oasis MCX cartridge, the activation and conditioning consisted of 5
281 mL of MeOH followed by 5 mL of phosphate buffer at pH 6 (0.15 M NaH_2PO_4 and 0.05 M Na_2HPO_4)
282 and the loading of 5 mL of a mixture of urine:phosphate buffer (pH 6) (50:50, *v/v*). It was then washed
283 with 2 mL of MeOH and eluted with 2 mL of 5% NH_4OH in MeOH. With this protocol, recoveries
284 around 90% for all analytes were achieved.

285 Although similar recoveries were obtained with both sorbents, Oasis WCX was selected for further study
286 due to its slightly higher values (around 5% and 10% higher). As our aim was to determine low
287 concentration levels of ATS in urine samples and bearing in mind that CE is one of the most frequently
288 used techniques and has a lower sensitivity than LC, the SPE process was reoptimized to gain greater

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3 289 preconcentration of the analytes, thereby enabling lower limits of concentration to be achieved. To this
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5 290 end the sample volume was increased and 25 mL of urine:phosphate buffer (pH 7) (50:50, *v/v*) evaluated.
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7 291 As the volume of urine was increased, the washing and elution steps were also reoptimized by increasing
8 292 their volumes. A study was made of 4 mL of MeOH and 4 mL of 5% HCOOH in MeOH respectively for
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10 293 washing and eluting. Under these conditions no losses in the washing step were observed and 4 mL of
11 294 MeOH was chosen as the optimal washing volume. Increasing the elution solvent from 2 mL to 4 mL led
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13 295 to an increase of 15% in the $\%R_{app}$, so 4 mLwas chosen as the optimal volume. The final conditions for
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15 296 Oasis WCX were therefore the activation and conditioning with 5 mL of MeOH and 5 mL of phosphate
16 297 buffer at pH 7, then loading 25 mL of a mixture of urine:phosphate buffer (pH 7), washing with 4 mL of
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18 298 MeOH, and finally eluting with 4 mL of 5% HCOOH in MeOH. Under these conditions a $\%R_{app}$ of
19 299 between 70% and 82% was achieved when a urine sample containing the ATS at a concentration of 10 ng
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21 300 mL^{-1} was analysed.
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23 301 **3.3. Method validation**
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26 302 The method using Oasis WCX was validated with urine samples by evaluating linear range, MQLs,
27 303 MDLs, repeatability (intra-day), reproducibility (inter-day), stability and accuracy.
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29
30 304 The proposed method presented IDLs from 20 to 40 ng mL^{-1} and IQLs between 60 and 125 ng mL^{-1} for
31 305 the ATS under study. The $\%R_{app}$, %ME, repeatability and reproducibility were studied at low (10 ng mL^{-1}),
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33 306 medium (50 ng mL^{-1}) and high (100 ng mL^{-1}) levels of ATS concentration in urine (Table 4). Different
34 307 blanks of urine samples from different laboratory staff members demonstrated good selectivity of the
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36 308 method because no endogenous or exogenous interferences were observed at the same migration times of
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38 309 the ATS. The exogenous interferences studied were cocaine and a group of synthetic cathinones.
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40 310 Matrix-matched calibration curves were chosen as the calibration model. The linear range was from the
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42 311 MQL to 150 ng mL^{-1} for all the compounds, with r^2 higher than 0.990 for all ATS under study. The MDLs
43 312 in urine were between 0.6 and 1.5 ng mL^{-1}, while the MQLs ranged from 2.0 to 8.0 ng mL^{-1}. It is
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45 313 important to note that these values are suitable for reaching the levels at which ATS are usually found in
46 314 urine (ng mL^{-1}). Values from 70% to 82%, from 80% to 90% and from 82% to 95% were achieved for
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48 315 $\%R_{app}$ at low (10 ng mL^{-1}), medium (50 ng mL^{-1}) and high (100 ng mL^{-1}) levels of concentration in urine
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50 316 respectively. For %ME, the values obtained at the same three concentration levels were between -26%
51 317 and -15%, -17% and -10% and -14% and -6% respectively. In addition, values below 20% of %RSD were
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53 318 obtained for repeatability (intra-day) and reproducibility (inter-day) at the levels of concentration stated
54 319 above. The analytes were shown to be stable at room temperature for 40 h with no evidence of
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320 degradation and with RSDs lower than 7% for each calibration level for the different periods of time
321 evaluated (every 10 h).

322 The method was finally tested to prove its accuracy by analysing three blind urine samples spiked by a
323 laboratory staff member with the ATS under study at 14 ng mL^{-1}, 38 ng mL^{-1} and 75 ng mL^{-1}. Accuracy
324 was calculated as the percentage of error between the concentration found when analysing these samples
325 per triplicate and the spiked concentration in the same sample. Errors were between 12% and 16% for the
326 first sample, 7% and 14% for the second and 4% and 12% for the third.

327 The developed method is comparable to other previously published studies in which different strategies
328 based on SFC, LC-MS/MS, LC-QTrap or GC-MS were used [11,13,14,27,28]. It is important to highlight
329 that in the present study, lower MDLs and MQLs are achieved compared to some of these earlier studies.
330 For example, Hegsatd *et.al.* [11] developed a method to quantify R/S-AMP in urine by SFC-MS/MS with
331 semi-automatic sample extraction. They achieved MDLs of 10 ng mL^{-1} and MQLs of 25 ng mL^{-1}. Other
332 authors who used LC-MS/MS as the analytical technique to determine ATS in urine samples also
333 obtained higher MDLs and MQLs than those obtained in the present study [13,14,28]. Although these
334 limits are higher than those obtained in the present study, they used considerably lower loading volumes,
335 less than 1 mL, even though urine is not a problematic matrix considering its availability in huge
336 quantities, so using more volume is not an issue. However, there are other examples in which the reported
337 MDLs were lower, such as the method developed by Fujii *et al.* [27], in which AMP, MAMP, MDA,
338 MDMA and other ATS were enantiomerically separated in less than 5 min. Nevertheless, one of the
339 strengths of our method is that these methodologies use more expensive equipment, whereas in the
340 present case the enantioseparation is easily achieved with the simple addition of a chiral selector to the
341 BGE. Shorter analysis times are also involved in our strategy, and lower organic solvent volumes are used
342 compared to the strategies based on SFC, GC or LC mentioned above.

343 4. Conclusions

344 A method for the enantiodetermination of a group of ATS in urine by CE-MS/MS was successfully
345 developed and validated. In the present case, using the CMT with a BGE of 1 M HCOOH:1 M
346 ammonium formate with 0.2% of sulphated γ-CD, 4 ATS including parent compounds and some of their
347 metabolites were enantioseparated. This proves the strength of the CE technique in combination with MS,
348 achieving low levels of concentrations with easy enantiomeric separation. In addition, two different
349 cationic-exchange SPE sorbents were compared in the urine pre-treatment, with Oasis WCX achieving
350 better results compared to Oasis MCX in terms of R_{app}. Finally, the method showed that it can determine
351 both enantiomers of the four compounds studied both at low levels of concentration and at the usual

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352 levels in which they can be present in urine from drug users. It is therefore demonstrated that this method
353 is a useful tool in toxicological and forensic analyses.

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358 Compliance with ethical standards

359 Conflict of interest

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

361 Ethical approval

362 All the procedures performed in studies involving human participants were in accordance with the ethical
363 standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration
364 and its later amendments or comparable ethical standards. This article does not contain any studies with
365 animals performed by any of the authors.

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Figure legends

Figure 1. Comparison of different chiral selectors: 0.1% of sulphated γ-CD (A), a combination of 0.1% of sulphated γ-CD with 0.1% of sulphobutylated β-CD (B); 0.2% of sulphated γ-CD (C) and 0.2% of sulphobutylated β-CD (D) with a mixture of AMP, MAMP and MDMA at 500 ng mL^{-1} with a BGE of 1 M HCOOH:1 M ammonium formate and with a sheath liquid of 10 mM ammonium acetate:MeOH (50:50) at 0.4 mL min^{-1}.

Figure 2. Electropherogram of a mixture of AMP, MAMP, MDMA and MDA at 1000 ng mL^{-1} analysed by CE-MS/MS with a BGE of 1 M HCOOH:1 M ammonium formate with 0.2% of sulphated γ-CD and a sheath liquid of 10 mM ammonium acetate:MeOH (50:50) at 0.4 mL min^{-1}.

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Table 1. Migration times, precursor and product ions, collision energies and fragmentor voltages of the ATS under study.

Compound	m_t (min)	Precursor ion	Product ion 1	Col. E 1 (eV)	Product ion 2	Col. E 2 (eV)	Product ion 3	Col. E 3 (eV)
R-AMP	15.9	136.1	119	4	91	10	-	-
S-AMP	17.2							
R-MAMP	16.3	150.1	119	8	91	10	-	-
S-MAMP	16.8							
R/S-MDMA	19.1	194.1	163	10	133	15	105	15
R/S-MDMA	20.2							
R/S-MDA	18.7	180.1	163	6	135	10	133	10
R/S-MDA	20.6							

Table 2. Resolution of ATS in the BGE optimisation: 10 mM ammonium acetate; 1 M HCOOH; 1 M HCOOH:1 M ammonium acetate (5:0.1, v/v); 1 M HCOOH:1 M ammonium formate (5:0.1, v/v); 0.5 M HCOOH:1 M ammonium acetate (5:0.1, v/v); 0.5 M HCOOH:1 M ammonium formate (5:0.1, v/v).

	10 mM ammonium acetate	1 M HCOOH	1 M HCOOH:1 M ammonium acetate (5:0.1, v/v)	1 M HCOOH:1 M ammonium formate (5:0.1, v/v)	0.5 M HCOOH:1 M ammonium acetate (5:0.1, v/v)	0.5 M HCOOH:1 M ammonium formate (5:0.1, v/v)
AMP - MAMP	0.5	1.7	1.7	1.0	1.4	1.3
MAMP - MDMA	1.0	4.0	4.0	2.3	3.1	3.7
AMP - MDMA	1.5	5.7	5.7	3.3	4.6	4.6

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3 462 Table 3. Resolution between enantiomers of AMP, MAMP and MDMA with different chiral selectors at 500 ng mL^{-1}.

	AMP	MAMP	MDMA
0.1% sulphated γ-CD	2.7	0.7	1.7
0.1% sulphated-γ-CD + 0.1% sulphobutylated-β-CD	2.7	1.3	1.8
0.2% sulphated γ-CD	3.3	1.3	2.4
0.2% sulphobutylated β-CD	1.0	1.2	1.5

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3 464 Table 4. Validation parameters for the determination of ATS in urine by CE-MS/MS

Analyte	MDLs[a]	MQLs[a]	$\%R_{app}$ (n=5)			%ME (n=5)			Repeatability %RSD (n=5)			Reproducibility %RSD (n=5)		
			Low[b]	Medium[c]	High[d]	Low[b]	Medium[c]	High[d]	Low[b]	Medium[c]	High[d]	Low[b]	Medium[c]	High[d]
R-AMP	1.0	5.0	78	85	88	-18	-12	-11	8	1	10	8	3	2
S-AMP	1.0	5.0	80	86	93	-17	-12	-8	10	2	11	2	3	4
R-MAMP	1.0	8.0	82	90	95	-15	-10	-6	2	2	6	5	5	3
S-MAMP	1.0	8.0	80	84	94	-17	-13	-7	6	5	8	9	4	3
R/S-MDMA	0.6	2.0	76	86	94	-21	-10	-8	9	5	7	13	8	8
R/S-MDMA	0.6	2.0	79	85	95	-18	-13	-7	5	6	12	10	8	8
R/S-MDA	1.5	8.0	74	83	86	-22	-14	-11	18	4	11	11	2	4
R/S-MDA	1.5	8.0	70	80	82	-26	-17	-14	15	6	16	14	10	6

465 [a] ng mL^{-1}
[b] 10 ng mL^{-1}
466 [c] 50 ng mL^{-1}
[d] 100 ng mL^{-1}
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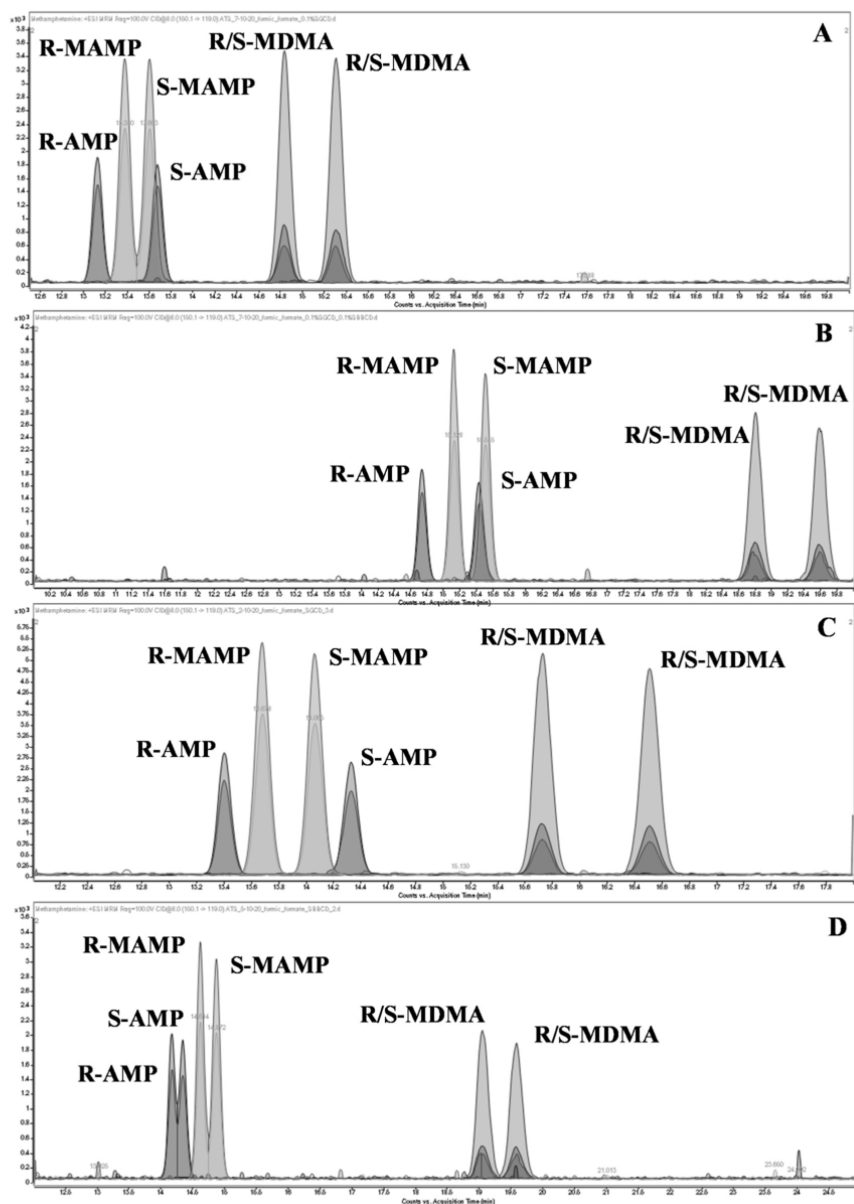

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Figure 1. Comparison of different chiral selectors: 0.1% of sulphated γ-CD (A), a combination of 0.1% of sulphated γ-CD with 0.1% of sulphobutylated β-CD (B); 0.2% of sulphated γ-CD (C) and 0.2% of sulphobutylated β-CD (D) with a mixture of AMP, MAMP and MDMA at 500 ng mL-1 with a BGE of 1 M HCOOH:1 M ammonium formate and with a sheath liquid of 10 mM ammonium acetate:MeOH (50:50) at 0.4 mL min-1.

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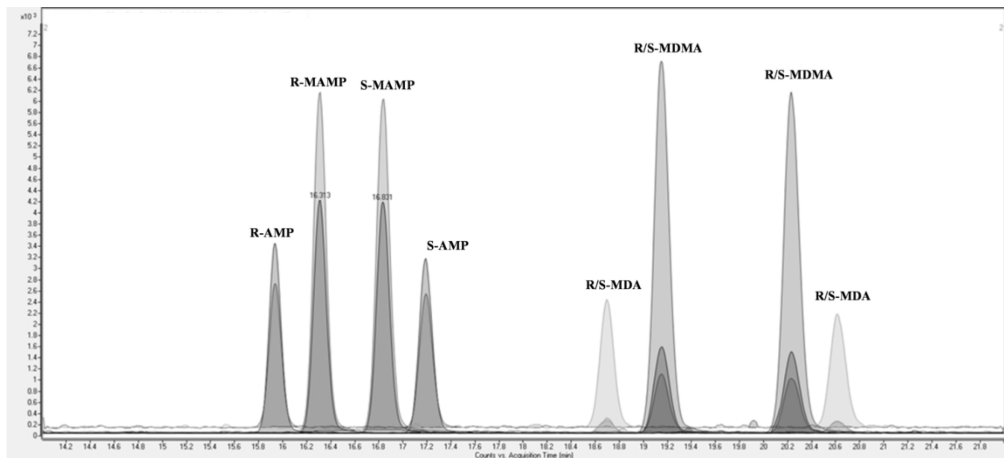

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Figure 2. Electropherogram of a mixture of AMP, MAMP, MDMA and MDA at 1000 ng mL-1 analysed by CE-MS/MS with a BGE of 1 M HCOOH:1 M ammonium formate with 0.2% of sulphated γ-CD and a sheath liquid of 10 mM ammonium acetate:MeOH (50:50) at 0.4 mL min-1.

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