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Multi-milligram resolution and determination of absolute configuration of pentedrone and methylone enantiomers

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ABSTRACT

The enantioresolution of pentedrone and methylone was carried out at a multi-milligram scale by liquid chromatography on a Chiralpak AS® stationary phase. The excellent enantioresolution using this column allowed to collect highly pure enantiomeric fractions, achieving enantiomeric ratios higher than 98%. An overall recovery of 72% was achieved for pentedrone enantiomers and 80% for methylone. Furthermore, the absolute configuration of the enantiomers of both cathinones was determined for the first time by electronic circular dichroism (ECD) spectroscopy, with the aid of theoretical calculations, as (+)-(*S*) and (−)-(*R*)-pentedrone, and (−)-(*S*) and (+)-(*R*)-methylone.

1. Introduction

At the beginning of the twenty-first century, “legal highs” arrived to the market *via* “smartshops” or Internet, with high consumer acceptance [1]. The compounds most representative in “legal highs” are synthetic analogs of cathinone, one of the major components present in the leaves of *Catha edulis* (Khat) plant [2]. Synthetic cathinones are sold as “plant food” or “bath salts” and labeled “not for human consumption”, in order to circumvent the law, since they are designed for recreational use [2–4]. They are the second largest group of new drugs monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), after synthetic canabinnoids, with 118 detected in total, including 14 reported for the first time in 2016 [5]. Nevertheless, new synthetic cathinones continue to emerge on the market [5]. Hallucinations, paranoid agitation, tachycardia, hypertension, acute liver and/or kidney failure, are some of the adverse effects resulting from chronic abuse of this group of compounds, being potentially dangerous to consumers' health [2,6].

A high number of papers describing the biological and toxicological effects of synthetic cathinones have been published in recent years [7–14]; however, the influence of the stereochemistry of these compounds in their activities was not studied. In fact, despite the growing interest regarding synthetic cathinones, there are only few examples of studies concerning the potential enantioselectivity of these compounds [15], as observed for methcathinone [16], mephedrone [17–19] and MDPV [20–23] enantiomers. It should be highlighted that enantioselectivity studies have an important role in forensic toxicology, as many illicit drugs, including cathinones, are chiral and their pharmacodynamics or pharmacokinetics biological responses could differ between enantiomers [24]. It is absolutely necessary obtain both enantiomers with very high enantiomeric purity to perform enantioselectivity studies. The single enantiomers can be obtained either by enantioselective synthesis or by a preparative method for the resolution of a racemate or mixture of enantiomers [15,25,26]. The improvement of chromatographic instrumentation and the development of efficient chiral stationary phases (CSPs) have made the enantioresolution by preparative liquid chromatography (LC) a first choice, considering its robustness, versatility, speediness, and simplicity [27]. For determination of enantiomeric purity, LC using CSPs has become the most useful and widely used method [28–30].

Nowadays, numerous CSPs are available [31–34]; however, among the enantioresolution studies of synthetic cathinones found in literature

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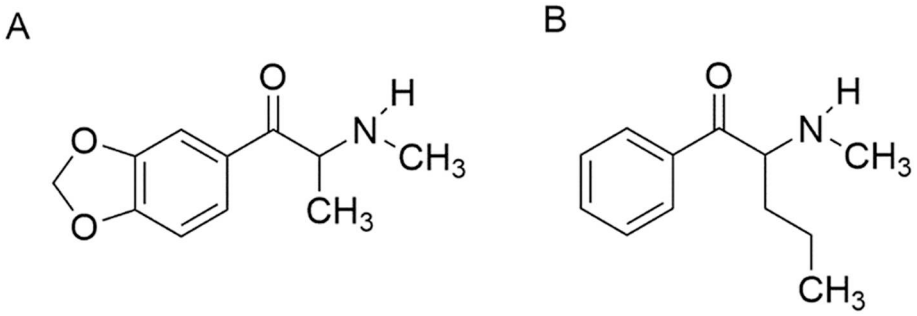

Fig. 1. Chemical structures of methylone (A) and pentedrone (B).

[15,35,36] polysaccharide-based CSPs proved to be one of the most versatile and efficient [21,37,38]. Actually, based on the referred published works and on our own experience, the polysaccharide-based CSPs are the most successfully and broadly applied, even for both analytical [21,39–41] and preparative [21,40,42,43] enantioresolutions.

Recently, an analytical method, using a polysaccharide-based CSP for enantioresolution of several synthetic cathinones present in "legal highs", was reported by us [21]. Additionally, the semipreparative enantioresolution of the synthetic cathinone MDPV was carried out for the first time, by LC on amylose *tris*(3,5-dimethylphenylcarbamate) CSP, for further enantioselectivity studies [21]. To pursue our aim of studying the influence of stereochemistry on bioactivity/toxicity of this recent class of drugs, herein we report the development of a reliable LC method for enantioresolution at a multi-milligram scale of pentedrone and methylone (Fig. 1), two of the most commonly used synthetic cathinones worldwide. In this work, a semipreparative Chiralpak® AS column was used under normal phase elution conditions. Noteworthy, to our knowledge, no data are available in the literature for the semipreparative enantioseparation of pentedrone and methylone.

Determination of the absolute configuration of chiral molecules has been a central issue in any field where stereospecific responses are to be expected, especially in medical and forensic sciences. Electronic circular dichroism (ECD) quantum calculations have been successfully used for this purpose for a variety of organic molecules [44–47]. In this work, we identified the absolute configuration of the eluted enantiomers by comparing the experimental ECD data of LC fractions with the theoretical ECD data calculated for the cathinones' molecular models. This also helped in confirming its desired enantiomeric isolation.

2. Experimental

2.1. Materials

Twenty formulations of "legal highs" were purchased in "smartshops" during 2012 and 2013 and were fully characterized by mass spectrometry, nuclear magnetic resonance (NMR) and elemental analysis [14]. Among them, ten packages designated "Bliss" contained 100% of methylone, while ten packages of "Kick" contained 98% of pentedrone and 2% of isopentedrone. For both products, the purity was higher than 98%, and therefore they were used also as standards. All samples were purchased as powders.

n-Hexane (Hex), ethanol (EtOH), 2-propanol (2-PrOH), for LC purpose, were purchased from CARLO ERBA Reagents S.A.S (Val de Reuil, FR). Triethylamine (TEA), diethylamide (DEA), hydrogen chloride 2M in diethyl ether, and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) were of analytical grade and obtained from Sigma-Aldrich Co. (St. Louis, Missouri, USA).

2.2. Sample preparation

For the multi-milligram enantioresolution, working solutions of "legal high" products containing the respective synthetic cathinone were prepared using the mobile phase as solvent at a concentration of 1 mg/mL with further addition of 0.4% DEA. All samples were filtered through a membrane of 0.45 µm pore size before injection.

For the enantiomeric ratio (e.r.) determination, the working solutions concentration of each enantiomer of pentedrone and methylone was 0.1 mg/mL.

To evaluate the optical activity, a 2.5 mg/mL solution in EtOH of each of the enantiomers was prepared.

2.3. Instrumentation and chromatographic conditions

The LC system used was a Dionex UltiMate, equipped with a 3000 quaternary pump and a 3000 Automated Fraction Collector, a 3000 quaternary Variable Wavelength Detector with a discrete channel programmed to 254 nm. The data was analyzed with Chromeleon$^{7.0}$ software.

The chromatographic enantioseparations were carried out on an analytical Chiralpak® AS-H (150 × 4.6 mm i.d., 5 µm particles size) and semipreparative Chiralpak® AS (250 × 10 mm i.d., 5 µm particles size) columns from Chiral Technologies Europe, Daicel Chemical Industries, Ltd., Tokyo, Japan.

Optical rotation values for all enantiomers were determined on a Polartronic Universal polarimeter with a sodium lamp (SCHMIDT + HAENSCH GmBH & Co., Berlin, Germany), at 25 °C (concentrations expressed in mg/mL; solvent: EtOH). The volume of the measuring cell was 1 mL and the optical path was 10 cm.

Semipreparative chromatographic separation of the enantiomers of pentedrone and methylone was achieved through multiple injections, to obtain the desired quantities of pure enantiomers, fitted with a 500 µL loop using Hex:2-PrOH as mobile phase in different proportions according to the cathinone. Analyses were performed at 25 ± 1 °C, in isocratic mode under UV detection at a wavelength of 254 nm. The collection parameters were adjusted to collect fractions of pentedrone and methylone enantiomers with elution windows of 9 to 15 min and 16 to 31 min, respectively. The detection parameters were adjusted to a peak start threshold of 200 mAU and a peak end threshold of 200 mAU for pentedrone enantiomers. Regarding methylone enantiomers, the detection parameters were adjusted to a peak start threshold of 100 mAU and a peak end threshold of 100 mAU.

The fractions of each enantiomer, obtained after three days of multiple collection, were combined followed by evaporation of the mobile phase solvents, under reduced pressure. Then, a small amount of HCl on diethyl ether (2M) was added dropwise to each enantiomer to precipitate. Working solutions for each enantiomer of pentedrone and methylone were prepared at a concentration of 0.1 mg/mL. Twenty microliters of each solution were injected, in triplicate, on the analytical column under the optimized chromatographic conditions, to

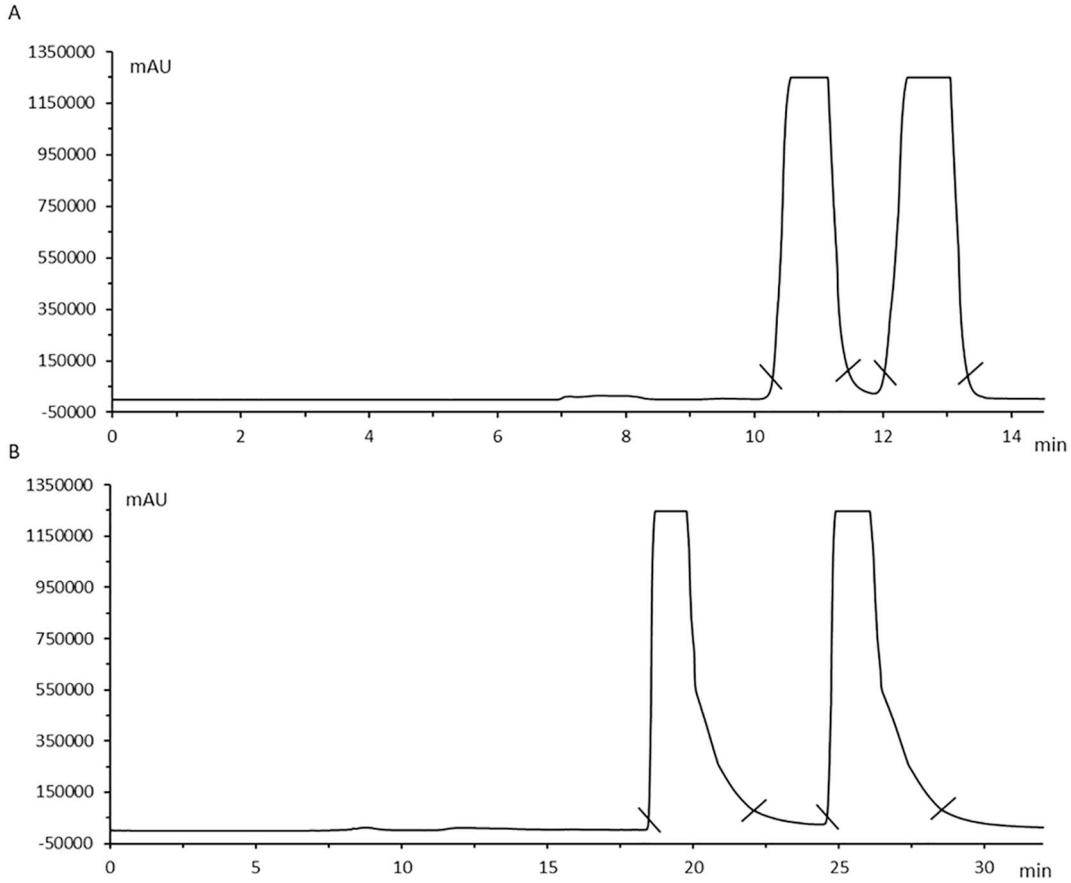

Fig. 2. LC enantioseparation chromatograms of pentedrone (A) and methylone (B). Chromatographic conditions: Chiralpak® AS-H column, mobile phase Hex/2-PrOH (97:3, v/v) for A and Hex/2-PrOH (85:15, v/v) for B, flow rate 2 mL/min, UV detection 254 nm.

measure their enantiomeric purity (e.r.). The e.r. was determined by the relative percentages of the peak areas [48] and expressed as percentage.

2.4. GC–MS analysis

GC–MS analysis was performed according to Araújo et al. [14] with some adaptations. Briefly, methanolic extracts of collected fractions of single enantiomers and synthetic cathinone standards were prepared at 100 µg/mL and were evaporated to dryness under nitrogen flow. Fifty microliters of MSTFA were added to the dried residue and were incubated at 40 °C for 20 min. After cooling to room temperature, 1 µL was injected into the GC–MS apparatus.

The chromatographic analysis was performed with an EVOQ 436 GC system (Bruker Daltonics, Fremont, CA) coupled to a SCION TQ mass detector, using a capillary column Rxi-5Sil MS (30 m × 0.25 mm × 0.25 µm) from RESTEK. Helium C-60 (Gasin, Portugal) was used as the carrier gas at a constant flow rate of 1.0 mL/min. Samples were injected in split mode (ratio 1:60), the injector temperature was 250 °C (held for 20 min) and the manifold temperature was 40 °C. The oven temperature was fixed at 100 °C for 1 min, then increasing to 300 °C (rate 15 °C/min) and held for 10 min. Total separation run time was 24 min.

Mass spectra were acquired in electron impact (EI) mode and the mass ranged from 40 to 600 *m/z*.

2.5. ECD spectroscopy

Experimental ECD spectra were obtained with a Jasco J-815 CD spectropolarimeter in a 0.1 mm cuvette. Model construction, dihedral driver search and preliminary MM2 minimizations were done with Chem3D Ultra (Perkin-Elmer Inc.). All other conformational energy minimizations and spectral calculations (TDDFT method) were performed with Gaussian 16W (Gaussian Inc.) at the B3LYP/6-31G theory level for conformer ranking and at the B3LYP/6-311 + G(2d,p) theory level for final geometry optimization and Gibbs energy calculation. An IEFPCM EtOH solvation model was used in all simulations. Simulated spectral lines for each relevant conformer were obtained by Gaussian broadening (0.2 eV) of calculated ECD rotational strengths followed by summation of the resulting curves, as recommended in [49]. The selected single-conformer ECD spectral lines were then Boltzmann-averaged using weights derived from its minimal B3LYP Gibbs energies [50].

3. Results and discussion

3.1. Pentedrone and methylone multi-milligram enantioresolution

The analytical method was developed using the polysaccharide-based CSP Chiralpak® AS-H (15 cm, 4.6 mm ID, 5 µm particle size) for resolution of pentedrone and methylone enantiomers as described in a previous work [21]. Due to the excellent enantioselectivity and resolution achieved for both synthetic cathinones ($\alpha > 1.7$ and $Rs > 7.0$) [21] and considering that these cathinone derivatives are the most consumed worldwide with proven hepatotoxicity and neurotoxicity [8,10,14,51], this method was scaled-up to a semipreparative mode.

Regarding the multi-milligram enantioseparation of pentedrone and methylone a semipreparative version of Chiralpak® AS-H column (250 × 10 mm ID, 5 µm particle size) was used. The column diameter was enlarged from 4.6 to 10 mm and the separation was optimized by adjusting the sample amount from a scaling up of the analytical method [21].

The optimized mobile phase of the analytical system was transferred; however, the ionic suppressor present in the mobile phase was withdrawn and added only to the sample, allowing to easily remove the mobile phase after collection of the enantiomer fractions and to renounce the extraction procedure. A solution of 1 mg/mL was prepared in the mobile phase and 0.4% of DEA was added to the sample. The solubility thus obtained was regarded as the maximum solubility. The loading effect in semipreparative mode was examined by keeping the concentration and by varying the volume. The maximum injection volume achieved was 200 µL for pentedrone and 400 µL for methylone.

In a first step, several injections at different flow rates were performed, using Hex/2-PrOH (97:3, v/v) as mobile phase, to attempt the separation of pentedrone and methylone enantiomers in the same run. However, very high retention factors were observed for methylone enantiomers, being the chromatographic run up to 45 min. In order to overcome this situation, the polarity of the mobile phase was augmented by increasing the percentage of organic modifier (2-PrOH). Lower retention factors were observed; however, for pentedrone, the other chromatographic parameters were not satisfactory since the enantioselectivity and resolution decreased. Consequently, the enantioresolution of the two synthetic cathinones was separately performed, and a mixture of Hex/2-PrOH (85:15, v/v) was used as mobile phase for enantioseparation of methylone to shorten the purification cycle. For both cathinones the flow rate was increased from 0.5 mL/min to 2 mL/min corresponding to a choice of a higher scale-up factor to reproduce retention times. Fig. 2 shows the chromatograms obtained by semipreparative resolution of pentedrone and methylone enantiomers.

Throughout a period of 14 h, multiple injections of working solutions totaling 9.80 mg of pentedrone resulted in 3.53 mg of the first eluted enantiomer of pentedrone (collected fractions P1), with a recovery of 72%, and 3.48 mg of the second eluted enantiomer of pentedrone (collected fractions P2), with a recovery of 71%. Regarding methylone, 12 mg were injected in the LC system, resulting in 4.80 mg of the first eluted enantiomer of methylone (collected fractions M1), with a recovery of 80%, and 4.74 mg of the second eluted enantiomer of methylone (collected fractions M2), with a recovery of 79% (Table 1). After this time (14 h), the enantioresolution performance of the CSP decreased, and a wash process with EtOH was needed to regenerate the column before starting a new fraction collection cycle.

The recovery rate was lower than it could have been due to the preparation of the hydrochloride of the collected fractions every three days, necessary to increase the compounds' stability.

3.2. Stability of pentedrone and methylone fractions in solution

Every day, an analysis of collected fractions (each enantiomer) was performed in the LC system to study the stability of pentedrone and methylone enantiomers in solution. It was possible to observe that they start to degrade four days after collection or after three thawing processes (Fig. 3). Thus, every three days, the hydrochloride of the collected fractions of each enantiomer were prepared and stored at −80 °C in independent vials.

Table 1
Elution order, specific rotation and enantiomeric ratios of pentedrone and methylone enantiomers at 25 °C.

Enantiomer	Elution order	e.r. (%)	$[\alpha]_D$ (c)[a]	Recovery (%)
S-(+)-pentedrone	First	98.4	+16 (2.5)	72
R-(−)-pentedrone	Second	97.8	−12 (2.5)	71
S-(−)-methylone	First	98.3	−20 (2.5)	80
R-(+)-methylone	Second	97.1	+24 (2.5)	79

[a] Specific rotation in EtOH (degrees mL/mg/dm) with c = concentration in mg/mL.

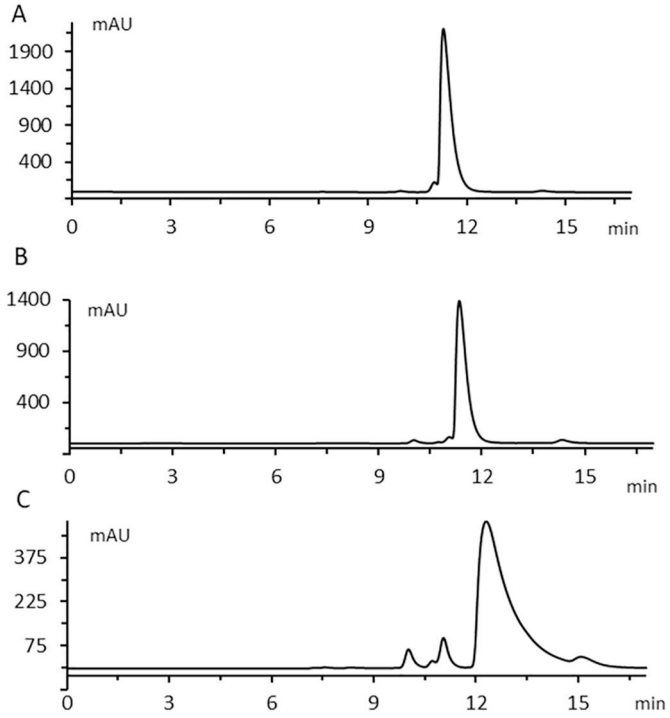

Fig. 3. Chromatograms of fraction P1 after one day (A), two days (B) and three days (C) of collection.

3.3. Enantiomeric purity and absolute configuration of pentedrone and methylone enantiomers

The determination of the enantiomeric purity for each enantiomer was performed using the optimized chromatographic conditions associated to the best enantioselectivity, according to previous work [21]. Fig. 4 shows the chromatograms obtained to measure the e.r. values. The optimized chiral LC conditions developed allowed the accurate determination of the e.r. of each enantiomer, being higher than 97% (Table 1).

The specific rotation of each single enantiomer was measured at 25 °C and the values obtained are shown in Table 1. It was possible to verify that for pentedrone the dextrorotatory enantiomer was the first eluted, while for methylone the first eluted enantiomer was the levorotatory. The elution order and specific rotation of each resolved enantiomers are presented in Table 1.

To assign collected fractions M1 and M2 to the methylone enantiomers and collected fractions P1 and P2 to the pentedrone enantiomers we employed a method that combines computational molecular modelling, ECD spectroscopy and ECD spectral simulation. Models of methylone and pentedrone were constructed based only on its protonated form since they were obtained as a solid from a hydrochloric acid solution. Furthermore, deprotonation upon dissolution of the solid in EtOH is expected to be negligible since the typically large pKa values (≈10) associated with secondary amines in water is maintained in EtOH [52]. To produce an almost exhaustive population of model conformers, all staggered orientations around single bonds were combined, with the exception of the carbonyl-phenyl bond because those groups always converged to co-planarity in preliminary MM2 minimizations. This resulted in an initial population of 18 conformers for methylone and 81 conformers for pentedrone. These conformers were then ranked by its optimized B3LYP/6-31G Gibbs energy, resulting in two relevant (lowest energy) conformations for methylone (Fig. 5-A) and four relevant conformations for pentedrone (Fig. 5-B). These models were further optimized at the B3LYP/6-311 + G(2d,p) theory level and used to simulate ECD spectra (Fig. 6). The

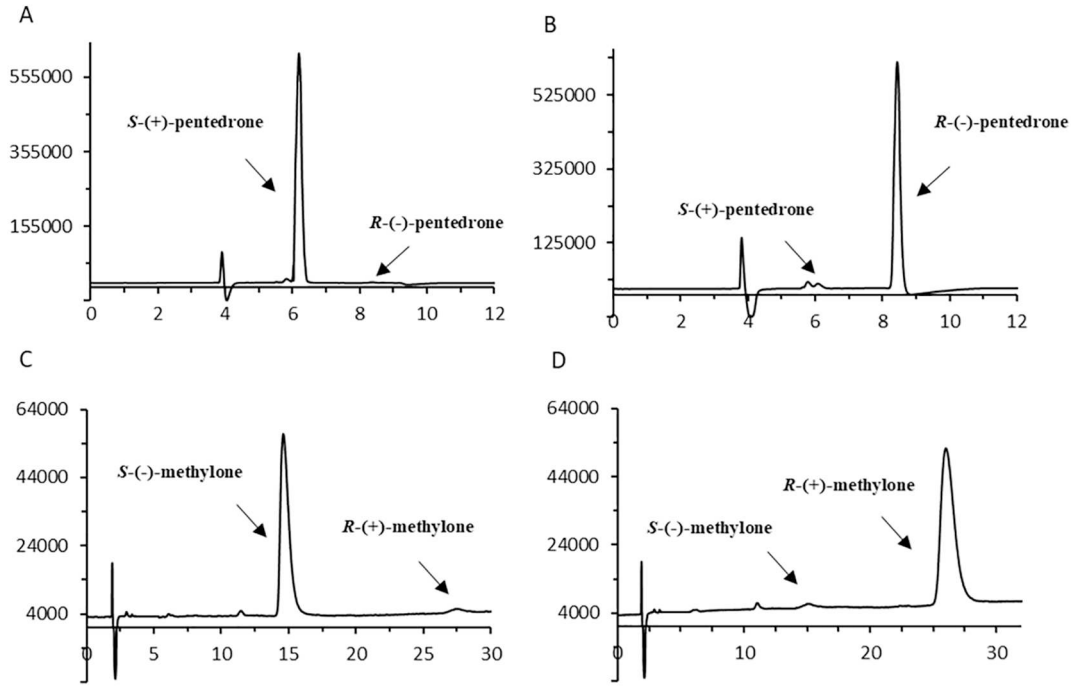

Fig. 4. Analytical chromatograms for enantiomeric ratio (e.r.) determination of collected fractions recovered from semipreparative resolution: (A) fraction P1, (B) fraction P2, (C) fraction M1 and (D) fraction M2. Chromatographic conditions used: Chiralpak® AS-H (15 cm, 4.6 mm ID, 5 µm particle size); mobile phase Hex/2-PrOH/TEA (97:3:0.1), v/v/v); flow rate 0.5 mL/min for A and B and 0.8 mL/min for C and D; detection wavelength 254 nm.

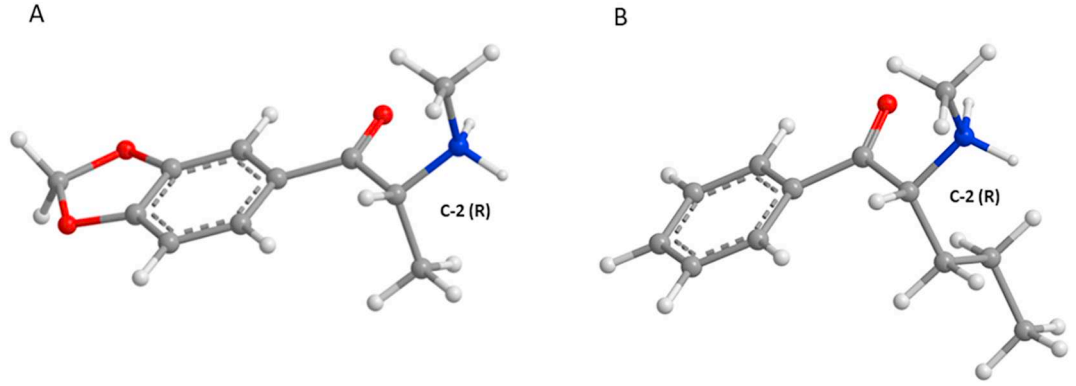

Fig. 5. A The minimal B3LYP/6-311 + G(2d,p)/EtOH Gibbs energy molecular model of amino-protonated methylone in its C-2(*R*) configuration. The presented conformation accounts for 50% of the predicted conformer population while the one obtained by 180° rotation of the carbonyl-phenyl bond accounts for 45% of conformer population. Therefore, only these two conformations were used to simulate the ECD spectrum of methylone. **B** The minimal B3LYP/6-311 + G(2d,p) Gibbs energy molecular model of amino-protonated pentedrone in its C-2(*R*) configuration. The conformation presented accounts for 54% of the predicted conformer population. The other relevant conformers contributing to the final pentedrone ECD spectrum accounted for 20%, 12% and 12% of the predicted population.

experimental ECD spectra of M1 and M2, as well as those of P1 and P2, are the almost precisely symmetric of one another, confirming very good separation between enantiomers. Comparing simulated and experimental spectra (Fig. 6) enabled the clear assignments M1/(*S*)-methylone, M2/(*R*)-methylone, P1/(*S*)-pentedrone and P2/(*R*)-pentedrone.

3.4. Analysis of pentedrone and methylone enantiomers by GC–MS

The methanolic extracts of each enantiomer of pentedrone and methylone were analyzed by GC–MS following a derivatization procedure with MSTFA. The identification of all cathinones was unequivocally confirmed since the retention times and mass spectra of each chromatographic peak coincides with the respective chromatographic peak of the derivatized standards (Fig. 7).

4. Conclusions

The enantiomers of pentedrone and methylone were successfully isolated and their absolute configuration unambiguously established, for the first time. The enantioseparation of both synthetic cathinones was performed by a LC method in a multi-milligram scale, using a polysaccharide-based chiral stationary phase, and achieving enantiomeric ratio values higher than 98%. An overall total of 3.5 mg and 4.8 mg of pentedrone and methylone enantiomers, respectively, were collected after a cycle of 14 h. The absolute configuration was established by a combined ECD spectroscopy and ECD spectral simulation, as (+)-(*S*) and (−)-(*R*)-pentedrone, and (−)-(*S*) and (+)-(*R*)-methylone. This work represents a novelty that is extremely interesting not only in LC and chirality fields but also as supply of compounds for further enantioselectivity studies on pharmacokinetic and pharmacodynamic processes.

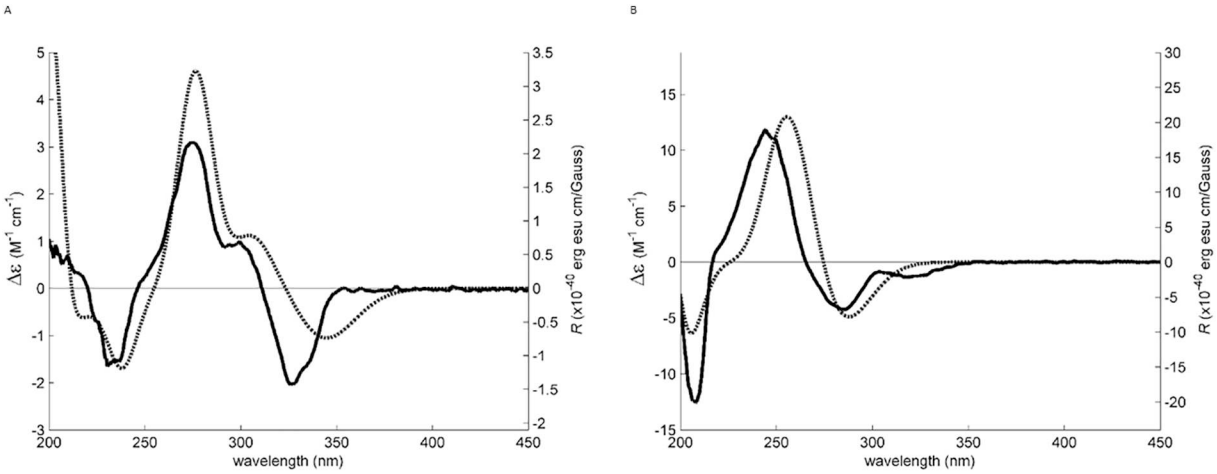

Fig. 6. Experimental ECD spectra (solid lines) of **A** methylone's M1 fraction and **B** pentedrone's P1 fraction, and simulated ECD spectra (dotted lines) of **A** methylone's C-2(*S*) and **B** pentedrone's C-2(*S*) model configurations, both in EtOH.

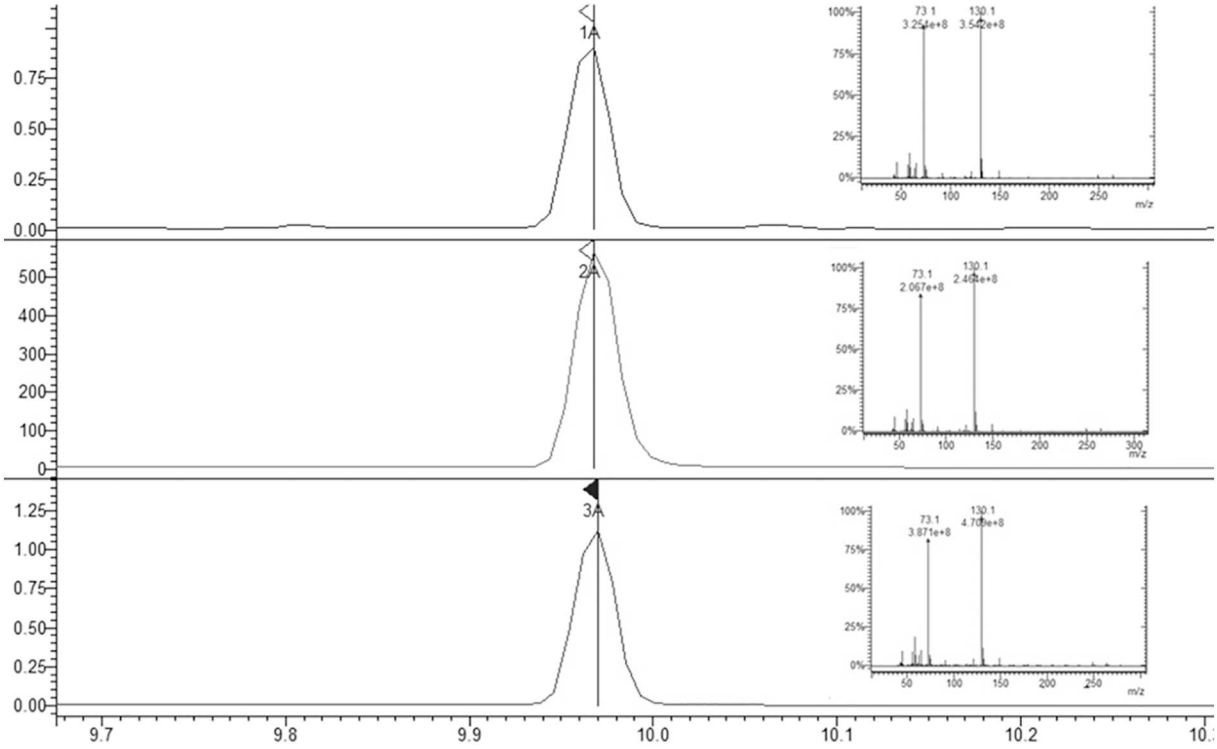

Fig. 7. Full scan chromatographic profile of methanolic extracts injected after MSTFA derivatization, indicating the potential identification of compounds based on the analysis of mass spectra of a standard of methylone. 1A: *S*-(−)-methylone, 2A: *R*-(+)-methylone, and 3A: methylone standard.

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