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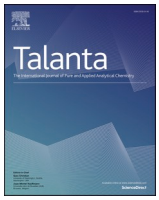

Microsampling and enantioselective liquid chromatography coupled to mass spectrometry for chiral bioanalysis of novel psychoactive substances

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ABSTRACT

In this paper, the development of efficient enantioselective HPLC methods for the analysis of five benzofuran-substituted phenethylamines, two substituted tryptamines, and three substituted cathinones is described. For the first time, reversed-phase (eluents made up with acidic water-methanol solutions) and polar-ionic (eluent made up with an acetonitrile-methanol solution incorporating both an acidic and a basic additive) conditions fully compatible with mass spectrometry (MS) detectors were applied with a chiral stationary phase (CSP) incorporating the (+)-(18-crown-6)–tetracarboxylic acid chiral selector. Enantioresolution was achieved for nine compounds with α and R_S factors up to 1.32 and 5.12, respectively.

Circular dichroism (CD) detection, CD spectroscopy in stopped-flow mode and quantum mechanical (QM) calculations were successfully employed to investigate the absolute stereochemistry of mephedrone, methylone and butylone and allowed to establish a (*R*)<(*S*) enantiomeric elution order for these compounds on the chosen CSP.

Whole blood miniaturized samples collected by means of volumetric absorptive microsampling (VAMS) technology and fortified with the target analytes were extracted following an optimized protocol and effectively analysed by means of an ultra-high performance liquid chromatography-MS system. By this way a proof-of-concept procedure was applied, demonstrating the suitability of the method for quali-quantitative enantioselective assessment of the selected psychoactive substances in advanced biological microsamples. VAMS microsamplers including a polypropylene handle topped with a small tip of a polymeric porous material were used and allowed to volumetrically collect small aliquots of whole blood (10 μL) independently from its density. Highly appreciable volumetric accuracy (bias, in the −8.7–8.1% range) and precision (% CV, in the 2.8–5.9% range) turned out.

1. Introduction

The enantioselective analysis of illicit substances in different matrices (including biological and environmental samples as well as samples from seizures) is of prior importance since it allows to obtain relevant information concerning, *inter alia*, (i) the distinction between their legal and illicit use; (ii) the distinction between their direct consumption or their presence as a result of the metabolic fate of other compounds; (iii) the identification of the synthesis protocols applied in clandestine laboratories, along with the chemical identity of the employed precursors; (iv) the monitoring of changing patterns of drugs abuse; (v) the differentiation between their direct consumption and the deliberate disposal of unused material in the sewage; and (vi) the estimation of the actual environmental risk [1–17].

More in detail, as far as the first two points in the above list are concerned [points (i) and (ii)], the case of enantiomeric composition of

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amphetamine and methamphetamine is highly explanatory [4,6–8,15]. Going forward in the list [points (iii) and (iv)], it is very well known that the methods used for the illegal synthesis of many psychostimulants may differ both in their precursors and in the enantiomeric composition of the resulting products and are subject to dynamic change due to limitations of the selected precursor(s) [7,12,13]. The last two points in the list [points (v) and (vi)] enter the frame of the so-called "sewage epidemiology" also known as "wastewater-based epidemiology" [1,2,4,5]. This approach is based on the idea that screening for drugs which are consumed by subjects, eliminated through biological fluids and reach wastewater sewage treatment facilities may be one of the fastest, most accurate ways to assess a community's drug use. According to this "sewage forensics" approach, the capacity to define the enantiomeric composition of chiral illicit drugs in the environment is of great importance as it allows to distinguish illicit drug abuse from direct disposal of unused material. Moreover, since the activity and toxicity of these compounds are often dependent on stereochemistry, it is of fundamental importance to understand the influence of wastewater treatment processes in the (stereo)selective degradation of chiral drugs to improve the performance of wastewater treatment plants (WWTPs) and to protect the receiving aquatic environment [1,2,4,5].

Many of the investigation related to the above points have been mostly addressed to the group of amphetamines, while little is still known about other classes of illicit drugs, especially as far as the so-called novel psychoactive substances (NPSs) are concerned. This justifies the necessity to develop methods enabling the easily accessible and accurate enantioselective analysis of other classes of psychoactive substances than amphetamines in different matrices [16–20].

With this aim, we have developed very efficient enantioselective liquid chromatography (LC) methods for the analysis of ten compounds (**1**–**10**, Fig. 1), five of which (compounds **1**, **2**, **5**–**7**) belong to the benzofuran-substituted phenethylamine class, two (compounds **3**, **4**) to the family of substituted tryptamines, while the remaining three (compounds **8**–**10**) are substituted cathinones. For the first time, both reversed-phase (RP) and polar-ionic (PI) conditions fully compatible with mass spectrometry (MS) detectors have been applied with a unique chiral stationary phase (CSP) incorporating the crown-ether type chiral selector shown in Fig. 2 [21–23]. To the best of our knowledge, this is the first study in which the above CSP has been used for the efficient enantioseparation of compounds **1**–**10**.

CSPs based on crown ether selectors were originally designed for the

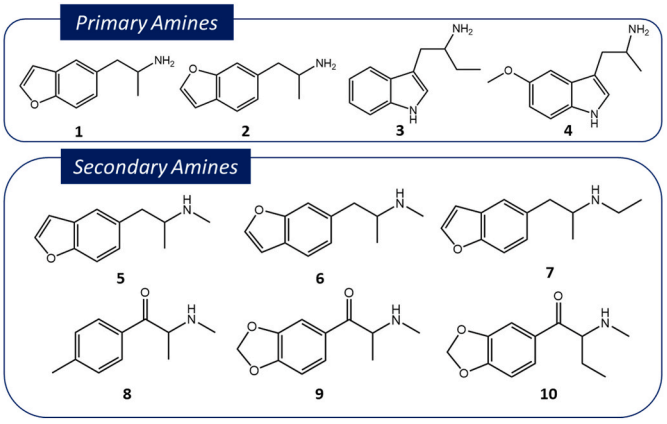

Fig. 1. Structure of the investigated analytes. Compounds **1**–**10** are respectively: 5-(2-aminopropyl)benzofuran (5-APB; **1**), 6-(2-aminopropyl)benzofuran (6-APB; **2**), α-ethyltryptamine (AET; **3**), 5-methoxy-α-methyltryptamine (5-MeO-aMT; **4**), 1-(benzofuran-5-yl)-*N*-methylpropan-2-amine (5-MAPB; **5**), 1-(benzofuran-6-yl)-*N*-methylpropan-2-amine (6-MAPB; **6**), 1-(benzofuran-5-yl)-*N*-ethylpropan-2-amine (5-EAPB; **7**), 4-methylmethcathinone (mephedrone, 4-MMC; **8**), 3,4-methylenedioxy-*N*-methylcathinone (methylone, MDMC; **9**) and β-keto-*N*-methylbenzodioxolylbutanamine (butylone; **10**).

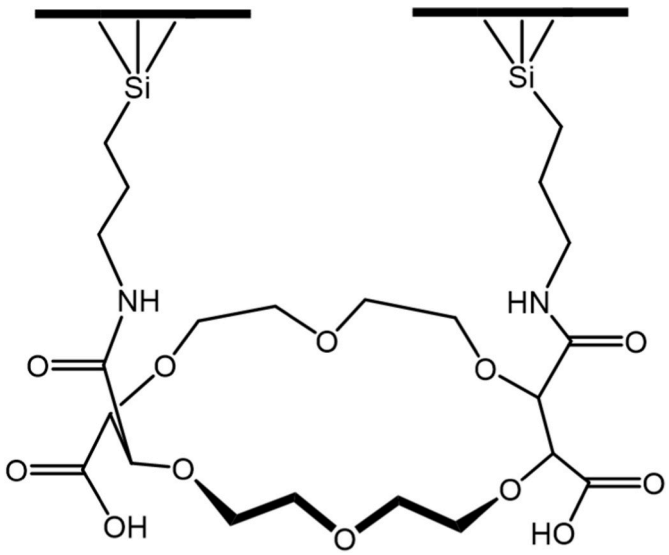

Fig. 2. Structure of the employed chiral stationary phase.

enantioselective analysis of primary amines including, *inter alia*, α-, β-, and γ-amino acids, *N*-monoalkyl amides of α-amino acids, primary amines and amino alcohols, fluoroquinolones, α-amino carbonyl compounds, aminophosphonic acids, di- and tri-peptides [21–23]. For these compounds, the complexation of the ammonium ion ($R–NH_3^+$) inside the cavity of crown ether selector *via* three $^+NH\cdots O$ hydrogen bonds is believed to be essential for the chiral recognition [22,23]. Consequently, an acidic compound added to the mobile phase is of outmost importance as it favours the protonation of the amino group and the following tripodal "host-guest" complexation of the analyte with the chiral selector. The complexation of the primary ammonium group inside the cavity of the 18-crown-6 ring allows the establishment of additional enantioselective interactions between the crown ether side groups, such as the carboxylic acid group and the alkyl spacers (the later to a lesser extent), and the side-chain residuals of the analytes. In particular, the two free carboxylic acid groups of the CSP act as "chiral barriers", participating in enantioselective hydrogen bonding or ionic interactions, as clearly evidenced by NMR studies [24] and X-ray crystallographic studies [25].

The proposed chiral recognition mechanism for the resolution of secondary amines on this CSP is not so different from that previously seen for the resolution of primary amino compounds. Indeed, for secondary amines, such the pharmaceutically relevant beta-blockers albuterol, atenolol, pindolol and propranolol, and the bronchodilator methoxyphenamine [23,26,27], just to cite some, two $^+NH\cdots O$ hydrogen bonds between the protonated secondary ammonium ions ($RR'-NH_2^+$) of analytes and the crown ether ring oxygens are believed to be at the basis of the complex formation. The host-guest complexation is accompanied by enantioselective additional hydrogen bonds or ionic interactions between the carboxylate group of the CSP and analytes [23,26,27].

According to literature, the enantioselective analysis of the benzofuran-substituted phenethylamine **1**, **2**, **5**–**7** was indeed performed by Taschwer and co-workers with a commercially available cellulose-based CSP under PI conditions and UV detection [28]. Only a partial resolution ($R_S < 1.0$) was obtained for these compounds. Commercial polysaccharide-based CSPs containing either an amylose- or a cellulose-based chiral selector were successfully employed by Albals and co-workers with normal-phase (NP) and PI eluents to obtain the complete enantioresolution of **2** [29]. UV detection was used to monitor the analysis which was performed with hardly compatible MS conditions. Conversely, no publications on the LC enantioseparation of the two substituted tryptamines **3** and **4** are so far available. A different situation

regards the three cathinone analogues **8**–**10**: both the Whelk-O1 CSP and different polysaccharide-based enantiorecognition materials (with cellulose or amylose derivatives as chiral selectors) were successfully used under NP condition by Perera and co-workers and [30], even though with hardly MS compatible mobile phases. For these compounds, UV detection was selected. A Table summarizing the chromatographic performances obtained in just mentioned studies [28–30] is reported as supplementary material (Table 1S).

All the aforementioned problems and analytical challenges justify the need for analytical methods allowing the accurate and precise determination of single enantiomers, even more so in innovative biological samples requiring superior performance in terms of sensitivity and selectivity, as well as on new methods allowing for unprecedent enantioseparations. In the present work, method optimization for the enantioseparation of compounds **1**–**10** (Fig. 1) using the crown ether-based CSP displayed in Fig. 2 is described. The application of circular dichroism (CD) spectroscopy and quantum mechanical (QM) calculations based on density functional theory (DFT) to the determination of the absolute stereochemistry of NPSs and the assessment of the enantiomeric elution order according to a well-established procedure [31–34] are discussed as well. Finally, in order to prove the applicability of the developed method and its full compatibility with MS-based bioanalysis platforms, whole blood miniaturized samples collected by means of volumetric absorptive microsampling (VAMS) technology [35, 35] and fortified with the target analytes were extracted following an optimized, original protocol and effectively analysed by means of an ultra-high performance liquid chromatography (UHPLC)-MS system in order to perform a proof-of-concept, quali-quantitative enantioselective assessment of the selected NPS in advanced biological microsamples. In fact, VAMS technology has been successfully tested for the non-chiral analysis of other NPSs [37–39] and classic drugs of abuse [36], proving to be a promising tool in the pharmaco-toxicological analysis field [40].

2. Materials and methods

2.1. Chemicals

All the reagents and solvents used in the study were of analytical grade. Acetonitrile (ACN), methanol (MeOH), sulfuric acid (H_2SO_4), acetic acid (CH_3COOH), triethylamine (TEA) and toluene were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany). Compounds **1**–**10** (Fig. 1) are respectively 5-(2-aminopropyl)benzofuran (5-APB; **1**), 6-(2-aminopropyl)benzofuran (6-APB; **2**), α-ethyltryptamine (AET; **3**), 5-methoxy-α-methyltryptamine (5-MeO-aMT; **4**), 1-(benzofuran-5-yl)-*N*-methylpropan-2-amine (5-MAPB; **5**), 1-(benzofuran-6-yl)-*N*-methylpropan-2-amine (6-MAPB; **6**), 1-(benzofuran-5-yl)-*N*-ethylpropan-2-amine (5-EAPB; **7**), 4-methylmethcathinone (mephedrone, 4-MMC; **8**), 3,4-methylenedioxy-*N*-methylcathinone (methylone, MDMC; **9**) and β-keto-*N*-methylbenzodioxolylbutanamine (butylone; **10**), and were obtained from Cayman Chemical (Ann Arbor, MI, USA). Water for HPLC analysis was purified with a New Human Power I Scholar water purification system (Human Corporation, Seoul, Korea). For UHPLC-MS analysis, MS-grade ACN, MeOH and CH_3COOH were employed and were purchased from Merck Life Science. Mitra® VAMS™ microsamplers (10 μL) were kindly provided by Neoteryx (Torrance, CA, USA).

2.2. Instrumentations

The HPLC-UV analyses were performed on a Shimadzu HPLC equipped with a SCL-10Avp system controller, two LC-10AD high pressure binary gradient delivery systems, a SPD-10A variable-wavelength UV–Vis detector, and a Rheodyne 7725i injector with a 20 μL stainless steel loop. The enantioselective analyses were carried out with the ChiroSil RCA(+) (Fig. 2) (250 mm × 4.6 mm I.D, 100 Å pore size, 5 μm particle diameter, incorporating the (+)–(18-Crown-6)–tetracarboxylic acid chiral selector) column from Regis® Technologies Inc. (Morton Grove, IL, USA). All the analyses were performed at a 0.5 mL min^{-1} eluent flow rate, unless otherwise stated. The column temperature was set at 25 °C with a 7956R heather/chiller thermostat (Grace, Sedriano, Italy). A 260 nm detection wavelength was selected for analyses. Toluene was selected as the unretained marker to evaluate the chromatographic parameters.

Ultra-high performance liquid chromatography-single quadrupole mass spectrometry (UHPLC-MS) analysis of whole blood VAMS microsamples was performed to prove the applicability of the optimized enantioselective chromatography conditions and their compatibility within MS-based bioanalytical platforms. The UHPLC-MS system was composed of a UHPLC Vanquish pump, Vanquish Autosampler, Vanquish column compartment, and ISQ EC single-quadrupole mass analyser (Thermo Fisher Scientific, Waltham, MA, USA). Enantioselective separations were obtained on the ChiroSil RCA(+) column, operating at 0.5 mL min^{-1} flow rate and using a 20-μL injection volume. The autosampler needle was washed with a 15/85 (v/v) MeOH/H_2O mixture for 10 s after each sample injection. Optimized MS parameters were the following: vaporizer temperature (VT), 200 °C; ion transfer tube temperature (ITT), 300 °C; sheath gas pressure (SGP), 35 psig; auxiliary gas pressure (AGP), 4.0 psig; sweep gas pressure (SWGP), 0.4 psig; source voltage, 2.0 kV. Positive ionisation mode was exploited, which resulted in extracted chromatograms for each compound from their $[M+H]^+$ ions: *m/z* 176.69 for **1**, *m/z* 176.67 for **2**, *m/z* 189.67 for **3**, *m/z* 205.62 for **4**, *m/z* 190.63 for **5**, *m/z* 190.69 for **6**, *m/z* 204.66 for **7**, *m/z* 178.72 for **8**, *m/z* 208.61 for **9**, *m/z* 222.58 for **10**. Column and autosampler temperatures were maintained respectively at 25 °C and 10 °C. Data elaboration was carried out by means of Thermo Fisher Scientific Dionex Chromeleon 7.3 Chromatography Data System software.

The enantioselective HPLC analysis with CD detection (HPLC-CD) on compounds **8**, **9** and **10** was performed on a Jasco (Tokyo, Japan) HPLC system composed of a PU-980 pump, a MD-910 photodiode array (PDA) detector and a Rheodyne (Rohnert Park, CA, USA) 7725i syringe loading injector equipped with a 50 μL sample loop, which was connected to a Jasco (Tokyo, Japan) J-810 spectropolarimeter using a HPLC flow cell (1 cm pathlength) and a Rheodyne (Rohnert Park, CA, USA) 7010 injector set up as a three-way valve for full-spectrum UV and CD measurements in stopped-flow mode. The enantioresolution of compounds **8**, **9** and **10** was performed on the ChiroSil RCA(+) column, using a MeOH/ACN (50:50, v/v) mixture containing acetic acid (10 mM) and triethylamine (10 mM) as mobile phase at a 0.8 mL min^{-1} flow rate; the elution of enantiomers was monitored using the PDA, UV and CD signals at 250 nm for compound **8** and 275 nm for compounds **9** and **10**. Stopped-flow CD and UV spectra were recorded on the most retained enantiomers in the 415-225 nm wavelength range, using a 4 nm spectral bandwidth, a 100 nm min^{-1} scanning speed, a 2 s data integration time, a 0.5 nm data interval and an accumulation cycle of 3. Spectra were blank-corrected with a stopped-flow measurement on the mobile phase and compared to the theoretical spectra of both enantiomers to determine the absolute stereochemistry of the most retained enantiomers and assess the enantiomeric elution orders.

2.3. Quantum mechanical computational spectroscopy

A preliminary conformational search on compounds **8**, **9** and **10** was carried out by molecular mechanics (MM) calculations with the RDKit software (version 2020.03.1) [RDKit: Open-source cheminformatics (https://www.rdkit.org)]. The MM conformational search was performed on the cations of (*R*)-enantiomers using the ETKDG search method [41] (2000 initial conformers, 100 attempts) and the MMFF94s force field for energy minimization [42] (1000 maximum iterations, 0.05 Å RMSD clustering threshold). All conformers within a MM energy window of 10 kcal mol^{-1} from the minimum were used for geometry optimization and frequency calculations at the DFT level, using the B97D3 functional

[43], the def2-TZVP basis set [44,45], the density fitting approximation [46–48] and the IEFPCM solvation model for MeOH [49]. Conformers displaying imaginary frequencies or converging to the same geometry (identified by RMSD values for heavy atoms lower than 0.05 Å) were discarded. All optimized conformers within an electronic energy window of 2.5 kcal mol^{-1} from the minimum were further used for time-dependent DFT (TD-DFT) calculations, using the PBE0-$^1/_3$ functional [49], the def2-TZVPD [44,50,51] basis set and the IEFPCM solvation model for MeOH. Excitation energies (expressed as wavelengths, λ_j), oscillator strengths (f_j) and rotational strengths in dipole length formalism (R_j) were calculated for the first 40 electronic transitions. The theoretical UV and CD spectra of (*R*)-**8**, (*R*)-**9** and (*R*)-**10** were then determined by approximation of f_j and R_j values to Gaussian bands ($\Delta\sigma$ = 0.2 eV) [52], summation over all excited states and conformational averaging based on the Boltzmann distribution of conformers at equilibrium, as calculated from relative free energies (ΔG). The theoretical UV and CD spectra of (*S*)-**8**, (*S*)-**9** and (*S*)-**10** were simply derived by reflection across the *x*-axis. All DFT and TD-DFT calculations were performed using the Gaussian 16 software package (Rev. B.01) [53]. Detailed results are reported in Fig. S1, and Tables S2–S5 (supplementary material).

2.4. Preparation of whole blood VAMS samples

VAMS microsamplers include a polypropylene handle (about 4 cm long) topped with a small tip (about 2-mm diameter) of a proprietary polymeric porous material able to volumetrically collect 10 μL of blood independently from its density. The technology was previously tested for volumetric sampling accuracy [37]. Briefly, the average sampled volume was 10.3 μL and volumetric accuracy (bias) was in the −8.7–8.1% range, with precision (% CV) in the 2.8–5.9% range. Blank or blank fortified whole blood was obtained from healthy volunteers, and 100-μL blood aliquots were fortified with 5 μL of analyte standard solution at three different known concentrations, namely to obtain whole blood concentrations of 200 ng mL^{-1}, 500 ng mL^{-1} and 1 μg mL^{-1} of compounds **1**–**10**. The tip of a VAMS microsampler was put in contact with the surface of the fluid blood sample for 5 s, dried at room temperature (RT) for 1 h and stored at RT in a dedicated clamshell until analysis in order to avoid contact with any surface. Whole blood VAMS microsamples were thus obtained. For sample pretreatment, the VAMS tip was detached from the handle and subjected to ultrasound-assisted extraction (UAE) for 15 min in 1 mL of 0.1% CH_3COOH in MeOH. The resulting solution was quantitatively transferred into a different vial and brought to dryness exploiting a Thermo Fisher Savant SpeedVac SPD 1030 vacuum concentrator. The residue was then re-dissolved in 100 μL of mobile phase and analysed by UHPLC-MS without the need for further treatment. Resulting chromatograms were checked and compared with chromatograms from standard solutions of compounds **1**–**10**, then the overlapping of retention times and the correspondence of chromatographic parameters were verified.

3. Results and discussion

3.1. Optimization of the enantioselective LC analysis conditions

For the sake of clarity, the description of the method optimization for the analysis of primary and secondary amines is discussed in separate subsections.

3.1.1. Enantioseparation of the primary amines *1*–*4*

Being inspired by previous studies with the here employed CSP, dealing with the enantioseparation of compounds carrying primary amino groups, the analysis of the two benzofurans **1** and **2** was initially performed with a mobile phase composed of 50% (v/v) MeOH in water plus 10 mM sulfuric acid [54,55].

A resolution of 1.53 was obtained for **1**, while a worse result turned out for **2**, with R_S being 0.84 (Table 1, entries *a* and *d*). For the two isomeric analytes, the enantioselectivity was instead nearly the same under the applied eluent conditions. Data listed in Table 1 readily suggest that this result can be explained by the contribution of retention to R_S. Compound **1** is, indeed, approximately 4-fold more strongly retained than its isomer **2**, likely because the oxygen at the 5th position in the furan ring is capable to engage an additional non-enantioselective H-bond with some complementary site of the CSP, including the carboxyl groups of the chiral selector. In other words, the complexation of compound **1** with the chiral selector imposes definite conformations to compound **1** which favour additional retentive interaction with some functionalities of the stationary phase.

Interested at exploring the possibility to replace sulfuric acid with a volatile and MS compatible acidic additive, acetic acid was tested in the hydroalcoholic eluent at the same concentration (10 mM). Acetic acid has already shown the best chiral recognition results in the enantioselective analysis of β-amino acids with the same CSP [56,57]. Notably, the use of the weaker acid produced superior performance for both compounds (Fig. 3a and 3b), allowing to get the peaks of **2** fully resolved (R_S = 2.55) within a good analysis time (Table 1, entries *b* and *e*). A different effect on retention has been observed for the two compounds. It has been hypothesized [58] that the lipophilicity of the acid anion can plays a key role in the retention mechanism with this CSP. Indeed, assuming the superimposition of reversed-phase [24] and host-guest complexation mechanisms, the higher is the lipophilicity of the acid anion, the stronger should be the analyte retention because of a more lipophilic ion pair formed between the protonated form of the analyte and the acid anion. This was the case of compound **1** (Table 1, entries *a* and *b*). However, a retention behaviour coherent with the lipophilicity of the acid anion was missing for compound **2** [59] (Table 1, entries *d* and *e*). Accordingly, in this case other properties than the lipophilicity of the ion pair did contribute to the retention mechanism. Indeed, it is possible to speculate that for compound **2**, the presence of the weaker acetic acid favoured the electrostatic interaction between the positively charged nitrogen of the analyte and the negatively charged carboxylate of the chiral barrier [24]. It seems likely that even subtle differences in the analyte structure can preferentially activate one of the two mechanisms. In line with literature data, as the concentration of acetic acid was decreased, the retention factors increased, mostly for compound **2**. The separation and the resolution factor values remained constant for compound **1**, while decreased for compound **2** as the concentration of acidic modifier was decreased. The decreased ionic strength of the hydroalcoholic eluents, together with the reduced concentration of acidic additive, was already proposed to be responsible for the observed retention behaviour [56,57]. It is worth to highlight that, irrespectively to the acetic acid concentration, the applied RP method is profitably chemoselective for the enantiomers of compounds **1** and **2** (Fig. 3a and 3b), thereby allowing the simultaneous analysis of the two isomers in different matrices.

As far as compounds **3** and **4** are concerned, it is important to recall that, to the best of our knowledge, this is the first study in which the enantioseparation of these two tryptamines is described. Also in this case, owing to sample limitations, we firstly applied experimental conditions already optimized by other authors [26] for the enantioseparation of compounds sharing some structural similarities with compounds **3** and **4**. Accordingly, a mobile phase composed of 80% (v/v) methanol in water plus 10 mM sulfuric acid was firstly tested. In spite of its high retention (Table 1, entry *g*), the enantiomers of compound **3** were not resolved under these conditions. On the contrary, compound **4** experienced a good enantioseparation with a partial enantioresolution (R_S = 1.39, Table 1, entry *j*). Very importantly, replacing sulfuric acid with acetic acid (always at a 10 mM concentration) led to the enantioseparation and the partial resolution of compound **3** (R_S = 1.19; Table 1, entry *h*) in approximately 10 min of analysis. The baseline separation (R_S = 1.65; Table 1, entry *i*) of this compound was achieved lowering the flow rate to 0.2 mL min^{-1}. This lower flow rate kept almost constant the

Table 1
Chromatographic performance obtained under RP conditions for primary amines **1**–**4**, and PI conditions for secondary amines **5**–**10**. The chromatographic data are obtained from the injection of standard solutions.

Compound	Entry	Mobile Phase	Flow Rate (mL min^{-1})	k_1	k_2	N_1	N_2	α	R_S
1	*a*	A	0.5	3.93	4.72	1847	1603	1.20	1.53
	b	B		2.64	3.24	3268	2364	1.23	2.00
	c	C		2.88	3.55	2942	2224	1.23	2.01
2	*d*	A	0.5	0.39	0.47	4365	2994	1.21	0.84
	e	B		2.19	2.82	3830	2737	1.29	2.55
	f	C		3.17	3.94	2812	2138	1.24	2.08
3	*g*	D	0.5	5.73	5.73	–	–	1.00	–
	h	B	0.5	0.75	0.93	2430	2671	1.23	1.19
	i	B	0.2	1.18	1.40	4591	5047	1.19	1.65
4	*j*	D	0.5	0.68	0.84	3537	3691	1.24	1.39
	k	D	0.2	0.81	0.98	4644	4562	1.21	1.53
	l	E	0.5	2.43	2.81	1461	1217	1.16	0.96
	m	E	0.2	3.78	4.28	2505	2555	1.15	1.25
	n	E	0.1	4.28	4.81	3763	3739	1.11	1.48
5	*o*	F	0.5	9.97	10.92	9572	6328	1.09	1.77
6	*p*	F	0.5	8.36	8.99	8129	7704	1.08	1.47
7	*q*	F	0.5	11.98	11.98	–	–	1.00	–
8	*r*	F	0.5	4.80	6.22	6279	6183	1.29	4.29
9	*s*	F	0.5	5.34	7.04	7705	7371	1.32	5.12
10	*t*	F	0.5	3.76	5.49	6778	6019	1.46	6.13

A, MeOH/H_20 50:50 (v/v) + 10 mM H_2SO_4.
B, MeOH/H_20 50:50 (v/v) + 10 mM CH_3COOH.
C, MeOH/H_20 50:50 (v/v) + 5 mM CH_3COOH.
D, MeOH/H_20 80:20 (v/v) + 10 mM H_2SO_4.
E, MeOH/H_20 80:20 (v/v) + 10 mM CH_3COOH.
F, MeOH/ACN 50:50 (v/v) + 10 mM TEA + 10 mM CH_3COOH.

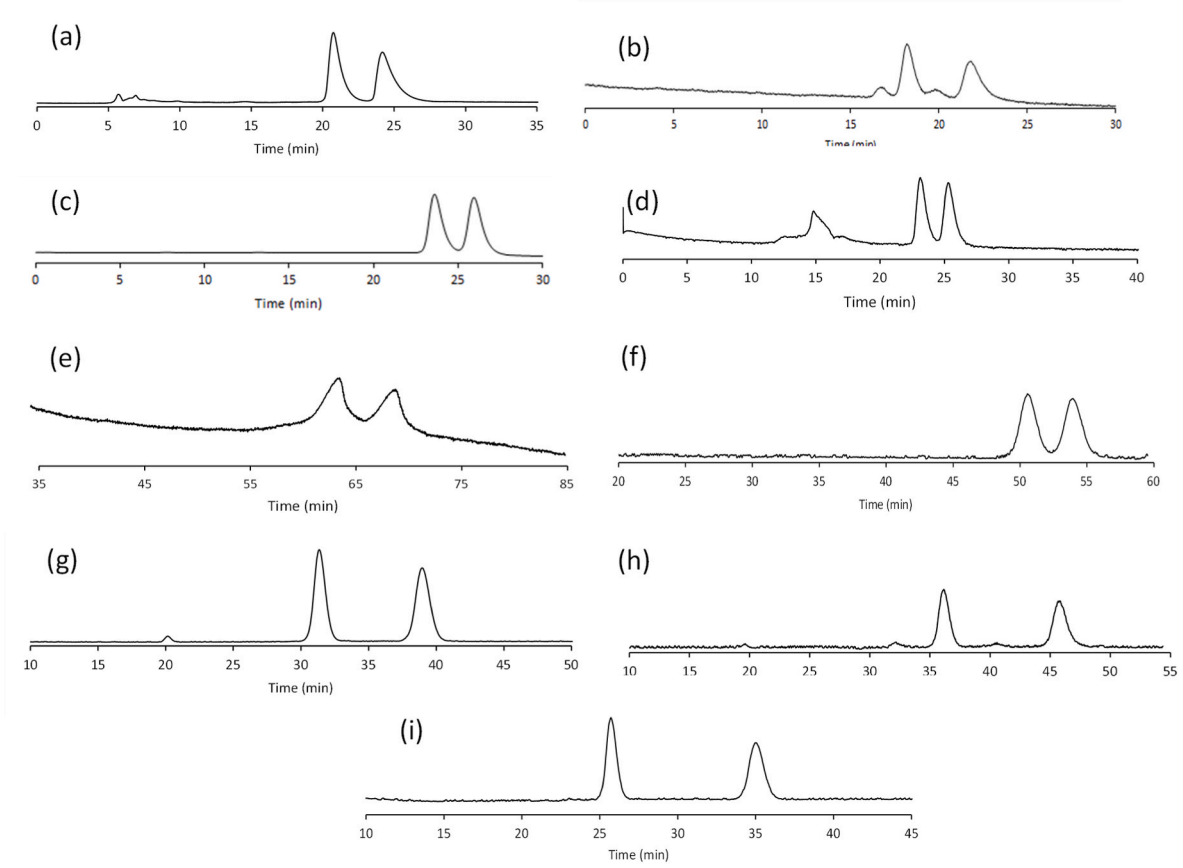

Fig. 3. Chromatograms of the investigated compounds. The analysis conditions can be derived from Table 1: (a) cmpd 1, entry *b*; (b) cmpd 2, entry *e*; (c) cmpd 3, entry *i*; (d) cmpd 4, entry *k*; (e) cmpd 5, entry *o*; (f) cmpd 6, entry *p*; (g) cmpd 8, entry *r*; (h) cmpd 9, entry *s*; (i) cmpd 10, entry *t*.

α value, while produced an analysis time suitable for practical applications (Table 1, entry *l*, Fig. 3c). This positive trend for R_S is often evident in enantioselective LC systems based on processes with slow kinetics [59,60]. In such settings, low flow rate enables the complexation processes to be completed, in turn leading to better R_S and N values. For compound **4**, the best performance was obtained with sulfuric acid and a flow rate of 0.2 mL min^{-1} (Table 1, entry *k*; Fig. 3d). Using the MS compatible acetic acid, the enantioresolution of compound **4** was gained ($R_S = 1.48$; Table 1, entry *n*) only with a 0.1 mL min^{-1} flow rate with unpractical analysis times (~120 min), thus confirming a large slope of the C-term in the Van Deemter curve and, in turn, slow adsorption/desorption kinetics. Also, for this compound the α values did not change remarkably upon a modification of the eluent flow rate. For this compound, reduced retentions with improved column efficiencies could be plausibly obtained even with acetic acid running the analysis with a column of smaller dimensions.

Data reported in Table 1 show that for the enantiomers of compound **4** retention factors increases as the eluent flow rate was reduced from 0.5 to 0.1 mL min^{-1}. This impact on the retention factors was already noted by several authors [61] and tentatively ascribed to pressure effects caused by the different flow rates, which affect the enantiomer retention mechanism. This effect is generally more evident when the mobile phase pH is close to the pK_a value of the analyte, which was however not the case in this study [62].

3.1.2. Enantioseparation of the secondary amines 5–10

Based on the works by Steffeck and co-workers [63] and Jeon & Hyun [26], for the present study we used a mobile phase containing both a protic and an aprotic organic component. Accordingly, we mixed MeOH with ACN in combination with an acidic (acetic acid, 10 mM) and a basic (triethylamine, 10 mM) additive. According to literature [63], the contemporary presence of these two additives in the PI eluent seems to be necessary since the acidic additive alone should lead to little or no retention and enantioseparation, while the amine alone should hamper analyte elution. However, due to sample limitations we did not verify the above assumptions.

With a MeOH/ACN (50:50, v/v) mobile phase also containing acetic acid (10 mM) and triethylamine (10 mM), the two benzofurans **5** ($R_S = 1.77$; Figs. 3e) and **6** ($R_S = 1.46$; Fig. 3f) were enantioresolved (Table 1, entries *o* and *p*). No separation was instead obtained for compound **7** neither with this mobile phase nor with the other few ones scrutinized during the study (data not shown). The lack of enantioseparation for **7** can be ascribed to the sterically bulkier N-ethyl group (with respect to methyl in compound **5**), which negatively alters the analyte fitting with the CSP [64].

The same PI mobile phase succeeded extremely well in the enantioresolution of the three cathinones **8**–**10** (Table 1, entries *r*–*t*; Fig. 3g-i), with rather elevated α and R_S values (up to 1.46 and 6.13, respectively, for compound **8**). The quite long retention times obtained for these three compounds is only an apparent drawback as the residence times of analyte in the column can be in principle shortened with a simple tuning of the experimental conditions or, alternatively, using the same eluent system in combination with analogous columns of smaller dimensions. However, due to limited availability of these three samples, further analyses with other experimental settings were not possible.

3.2. Stereochemical characterization

The stereochemical characterization of the NPSs mephedrone (**8**), methylone (**9**) and butylone (**10**) was carried out by means of QM calculations based on DFT [65], enantioselective HPLC-CD analysis and stopped-flow CD measurements [33,34]. This procedure currently represents the state-of-the-art for the correct and easy-to-achieve assignment of the EEO and stereochemical characterization of new molecular entities. As a general trend, QM calculations on the (*R*)-enantiomers of the NPSs under consideration predicted similar CD profiles, with positive bands at longer wavelengths followed by stronger negative bands at shorter wavelengths. In this framework, the detection wavelengths for HPLC-CD analysis were set to match the maximum intensity of the most intense CD bands, which are predicted to be negative for (*R*)-enantiomers (250 nm for **8**, 275 nm for **9** and **10**), and stopped-flow CD spectra were recorded on the most retained enantiomers on the ChiroSil RCA(+) column to enable the evaluation of single-wavelength CD signals for both enantiomers, thus increasing the information content of the analysis. For all compounds, stopped-flow CD spectra had a positive correlation with the theoretical CD spectra of (*S*)-enantiomers, while CD chromatograms showed negative CD signals for the least retained enantiomers (Fig. 4, Figs. S2–S3, supplementary material). Consequently, the enantiomeric elution orders for compounds **8**, **9** and **10** under the experimental conditions of the HPLC-CD analysis could all be assessed as [(*R*)<(*S*)], allowing a full characterization of their absolute stereochemistry.

3.3. Whole blood VAMS analysis

In order to assess the applicability of the optimized enantioselective chromatography conditions on MS-based bioanalytical platforms, a proof-of-concept application was carried out by analysing whole blood VAMS miniaturized samples fortified with compounds **1**–**10** by means of an UHPLC-MS system. This procedure was carried out to qualify the proposed methodology based on international bioanalytical method validation guidelines [66] for the enantiomer pairs of all the considered analytes, in terms of method sensitivity by evaluating the lower limit of quantitation (LLOQ), intra-day and inter-day precision, extraction yield and matrix effect. LLOQ was evaluated as the lowest analyte concentration in VAMS samples which can be quantified reliably, with an acceptable accuracy and precision, and corresponding to 5 times the signal of a blank sample. The LLOQ value estimated for the methodology proposed herein applied to whole blood VAMS ranged from 10 to 50 ng mL^{-1}. Details are reported in Table S6 (supplementary material). Extraction yield was evaluated on blood VAMS samples fortified with analyte standard solutions at three different concentration levels (namely, 200 ng mL^{-1}, 500 ng mL^{-1} and 1 μg mL^{-1}) and subjected to the previously described VAMS extraction procedure. The obtained analyte peak areas were compared with those obtained by analysing extracts from VAMS samples fortified post-extraction with the same nominal concentrations, and absolute recoveries were expressed as percentage. Precision was determined on the same fortified VAMS samples by analysing six replicates on the same day to evaluate intra-day precision and over six different days to evaluate inter-day precision, expressed as percent relative standard deviation (RSD%). Matrix effect was evaluated by analysing three VAMS replicates, fortified post-extraction by adding known analyte concentrations at the same levels as precision assays. The mean analyte peak area for each added concentration was compared with that obtained from standard solutions at the same theoretical concentration and the resulting percentage was calculated. Satisfactory absolute recoveries (>87%) and matrix effect results (86–95%) were obtained considering all enantiomer pairs in combination with good reproducibility considering three concentrations, always obtaining RSD values lower than 10.8%. Complete results for compounds **1**–**10** are reported in Table S6 (supplementary material).

As for the information from the scientific literature regarding expected blood concentrations following the intake of compounds **1**–**10**, these are predictably sparse and mainly concerning cases of acute and fatal intoxication. Nevertheless, these have been evaluated to understand the actual usefulness of the original methodology proposed here in forensic and chemical-toxicological bioanalytical contexts. For compounds **1** (5-APB) and **2** (6-APB), a report of a death case attributed solely to **1** showed post-mortem concentration in peripheral blood of 2.5 μg mL^{-1} and in central blood of 2.9 μg mL^{-1} [67], while a case study describes a fatal intoxication following the consumption of one and a half tablets of combined **1** and **2**, whose concentrations in post-mortem

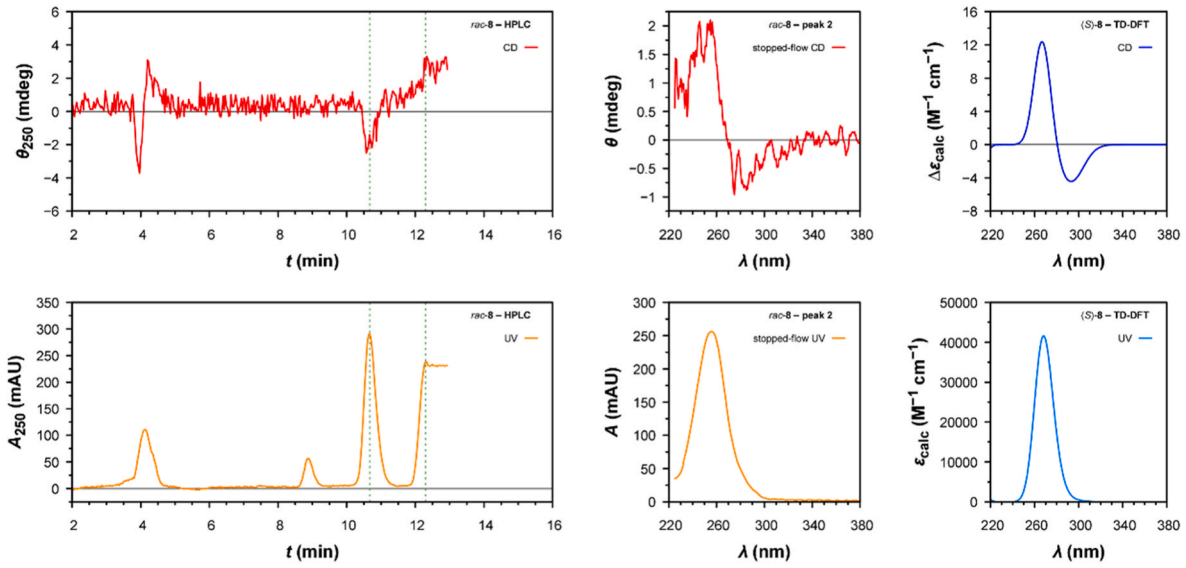

Fig. 4. Stereochemical characterization of mephedrone (8). Left: CD and UV chromatograms of rac-8 on the ChiroSil RCA(+) column (mobile phase: methanol/acetonitrile 50:50 v/v + acetic acid 10 mM + triethylamine 10 mM, 0.8 mL min^{-1}). Center: stopped-flow CD and UV spectra of the most retained enantiomer of 8. Right: theoretical CD and UV spectra of (S)-8, as calculated by TD-DFT calculations at the PBE0-$^1/_3$/def2-TZVPD//B97D3/def2-TZVP/fit level (IEFPCM solvation model for methanol).

peripheral blood were 850 ng mL^{-1} and 300 ng mL^{-1}, respectively [68]. As regards compound **3** (AET), analytical case reports in the scientific literature are very scarce, but a fatal intoxication following its intake described a blood concentration of 5.6 μg mL^{-1}, while in another fatal case involving a psychotic subject presenting agitation and hyperpyrexia, blood toxicological analysis identified **3** at 1.1 μg mL^{-1}, with the cause of death listed as malignant hyperthermia with **3** contribution [69,70]. As for compound **4** (5-MeO-aMT) no comprehensive bioanalytical investigations are reported [71]. For compounds **5** and **6** (5-MAPB and 6-MAPB, respectively), a case of acute toxicity after ingestion of **5** is reported showing serum concentrations ranging from 274 to 502 ng mL^{-1} [72]. Similar information can be retrieved for compound **7** (5-EAPB), for which a death case is reported after its consumption with other substances and whose analytical report shows blood concentration of **7** of 6.45 μg mL^{-1} [73]. Compounds **8** (mephedrone), **9** (methylone) and **10** (butylone) are among the main representatives of the cathinone derivative category [74], and considering case report examples, **8** was found at the concentration of 1.0 μg mL^{-1} in peripheral blood within a confirmed chemsex-related acute toxicity [75]; for **9**, a paper describing four post-mortem cases reported detectable levels of the compound, with heart blood concentrations of 740, 118, 60 and 1120 ng mL^{-1} [76]; compound **10** was identified and quantified at concentration of 20 μg mL^{-1} in blood [77].

From the literature data, it is evident how the original method developed and qualified herein for the enantioselective determination of NPSs in miniaturized whole blood samples obtained by VAMS technology offers a high potential for applicability in bioanalytical contexts of pharmaco-toxicological and forensic analysis and even more so it would be applicable to bulk samples originating from seizures in the context of investigations.

4. Conclusion

In this manuscript, the development of efficient enantioselective HPLC methods for the analysis of five benzofuran-substituted phenethylamines, two substituted tryptamines, and three substituted cathinones is described. For the first time, RP eluents composed of either water-MeOH solutions (with low concentrations of either acetic of sulfuric acid) or polar-ionic solutions (made up with an ACN-MeOH solution incorporating low concentrations of both triethylamine and acetic acid) were employed in combination with a CSP incorporating the (+)-(18-crown-6)–tetracarboxylic acid chiral selector. Enantioresolution was achieved for nine compounds with α and R_S factors up to 1.32 and 5.12, respectively. On this CSP, a (*R*)<(*S*) enantiomeric elution order was found for mephedrone, methylone and butylone after a full stereochemical characterization of their enantiomers by means of CD detection, stopped-flow CD spectroscopy and TD-DFT calculations.

The applicability of the optimized enantioselective chromatography methods to the study of real biological samples was appraised on a MS-based platform, through the analysis of whole blood VAMS miniaturized samples separately fortified with the compounds object of the study. In this context an UHPLC-MS system was used. Following international bioanalytical method validation guidelines for the enantiomer pairs of all the considered analytes, the enantioselective LC methods were validated in terms of sensitivity by evaluating the LLOQ, intra-day and inter-day precision, extraction yield and matrix effect. As a result, satisfactory absolute recoveries (>87%) were obtained considering all enantiomer pairs, along with good reproducibility, being RSD values always lower than 10.8%. Very appreciable LLOQ values down to 10 ng mL^{-1} were established as well.

From a comparison with literature data, clearly emerges that the unprecedented enantioselective LC methods developed and qualified herein for the enantioselective determination of NPSs in miniaturized whole blood samples obtained by VAMS technology offers a high potential for applicability in bioanalytical contexts of pharmaco-toxicological and forensic analysis and even more so it would be applicable to bulk samples originating from seizures in the context of investigations.

Credit author statement

Michele Protti: Methodology; Investigation; Validation; Writing – original draft preparation. **Ina Varfaj**: Methodology; Investigation; Validation; Writing – original draft preparation. **Daniele Tedesco**: Investigation; Software. **Andrea Carotti**: Investigation; Software; Formal analysis. **Manuela Bartolini:** Data Curation; Funding acquisition. **Alessandro Favilli**: Resources; Writing – review and editing. **Sandro Gerli:** Resources; Writing – review and editing. **Laura Mercolini**: Conceptualization; Methodology; Validation; Writing – original draft preparation; Supervision; Writing – review and editing; Project

Administration; Funding acquisition. **Roccaldo Sardella**: Conceptualization; Methodology; Validation; Writing – original draft preparation; Supervision; Writing – review and editing; Project Administration; Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2023.124332.

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