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ORIGINAL

A Simple HPLC–UV Approach for Rapid Enantioseparation of Cathinones, Pyrovalerones and Other Novel Psychoactive Substances on a 2.5-µm Cellulose Tris-(3,5-dimethylphenyl-carbamate) Column

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Abstract
The misuse of so called novel psychoactive substances is still a challenging problem worldwide. A special attribute of a lot of these compounds is a chiral centre enabling two possible enantiomers probably related to different pharmacological and toxicological properties. The goal of the present study was to present a simple and isocratic HPLC–UV method for enantioseparation of mainly cathinone and pyrovalerone derivatives as well as selected representatives of amphetamines, ketamines, benzofuries, phenidines, phenidates, morpholines and thiophenes. A Waters Acquity UPC2® Trefoil™ CEL1 2.5 µm, 3.0×150 mm column served as chiral stationary phase by means of cellulose tris-(3,5-dimethylphenylcarbamate) as chiral selector. Mobile phases consisted either of *n*-hexane/*n*-butanol/diethylamine (100:0.3:0.2) or *n*-hexane/diethylamine (100:0.2). The method was found to be applicable for rapid simultaneous chiral separations of cathinone derivatives, to determine enantiomeric elution orders, to detect positional isomers and to identify real-life samples. Also, a repeatability study was performed successfully. 78 out of 95 compounds were separated in their enantiomers successfully, 51 of them within 6 min. It was shown that all NPS bought from online vendors or seized by police were traded as racemic mixtures.

Keywords 2.5-µm CSP · HPLC–UV · Chiral separation · Tris-(3,5-dimethylphenyl-carbamate) · Cathinones · Novel psychoactive substances

Introduction

Synthetic novel psychoactive substances (NPS) are compounds, which are subject of slight alterations of well-known illicit drugs. They are mostly produced in China to help the consumers worldwide to circumvent law [1]. Since 2008, these compounds have flooded the worldwide drug market in sequential generations. The EMCDDA (European Monitoring Centre for Drugs and Drug Addiction) stated in its annual report that to date 730 different NPS have emerged [1]. Among them, the four main categories of NPS represent synthetic cannabinoids, stimulants, opioids and antidepressants. As so called "bath salts" or "plant fertilizers", synthetic cathinones represent a heterogeneous and broad subclass of amphetamine-like compounds derived from cathinone. This parent compound possesses stimulating effects and is a natural substance in the khat plant. During the past 10 years, more than 130 completely new cathinone derivatives were synthesized to circumvent law; this widespread substance class represents the second largest group of NPS after cannabinomimetics [1, 2]. Synthetic cathinones, also called beta-keto amphetamines, possess a stimulating effect on the central nervous system (CNS) generated by an increased dopamine and norepinephrine release [3].

Worldwide, the emergence of NPS has been reported meanwhile by 111 countries to the UNODC (United Nations Office on Drugs and Crime) by the end of 2017 [4]. The largest number of compounds has been reported from Asia, Europe and North America. However, there are limited valid

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data concerning NPS for other regions of the world. As the main factor for this enormous occurrence, the progress of the World Wide Web can be referred being used by both online vendors and consumers for distribution and information search. Drug fora or social networks provide information about effect or way of consumption of the drug compounds [5].

Synthesized in underground laboratories in Asia and Eastern Europe and claimed as "legal highs" or "research chemicals", NPS are intended to imitate and to replace already well-known illicit drugs such as amphetamine, *N*-methamphetamine or LSD. Circumventing law is the main intention of the manufacturer because the compounds are often not persecuted by international drug controls [6].

A huge number of NPS, particularly of the stimulants, is chiral. To date, there is much knowledge about pharmacological effects and potencies of chiral active pharmaceutical ingredients (APIs). Pharmacological and toxicological drug testings during the development process of a chiral API have to be performed for each enantiomer. However, for NPS there is limited valid data regarding the toxicology of their single enantiomers or of their degradation products. By now, referred examples of NPS showing different pharmacological effects are the substances amphetamine, methamphetamine, methcathinone and mephedrone [7–10]. NPS are sold as racemic mixtures, because stereoselective synthesis is expensive and difficult to perform. Due to these unsafe and not controlled circumstances, it is of great interest and importance to develop chiral analytical as well as chiral preparative separation methods.

To date, enantioseparations of NPS have been shown via different chromatographic and electrophoretic methods. Gas chromatography (GC) [11–16], high-performance liquid chromatography (HPLC) [17–23], supercritical fluid chromatography (SFC) [24–26], and capillary electrophoresis (CE) or capillary electrochromatography (CEC) [27–35] are meanwhile common techniques to identify NPS as powder samples as well as in biological matrices.

During the past years, various successful chiral separation methods using different substituted polysaccharide-based chiral stationary phases for pharmaceutical active compounds were presented [36–39]. Additionally, successful applications applying the mentioned CSPs for enantioseparation of NPS have been reported. Aturki et al. used amylose tris(5-chloro-2-methylphenylcarbamate) in 2014 for the chiral separation of new cathinone derivatives by CEC [31]. In 2016, Silva et al. presented a semipreparative HPLC method applying amylose tris(3,5-dimethylphenylcarbamate) for the enantioseparation of 3,4-methylenedioxypyrovalerone (MDPV) [40]. Taschwer et al. employed a HPLC method for the enantioseparation of NPS in 2017 using a CSP consisting of cellulose tris(3-chloro-4-methylphenylcarbamate) [20]. Furthermore, Kadkhodaei et al. used cellulose tris(3,5-dichlorophenylcarbamate) in 2018 [19] and later amylose tris(3,5-dimethylphenylcarbamate) [41] for successful enantioseparation of various different NPS classes. However, conventional HPLC systems equipped with chiral analytical columns packed with 3 µm particles were used for this purpose. In continuation of this project, a novel HPLC column with a smaller particle size should yield in significant gain in efficiency.

For these reasons, the goal of the presented work was to develop a rapid HPLC–UV method with a Waters Acquity UPC2® Trefoil™ CEL1 2.5 µm, 3.0 × 150 mm column containing the chiral selector cellulose tris-(3,5-dimethylphenyl-carbamate) for the enantioseparation of mainly cathinone derivatives, pyrovalerone derivatives and selected further NPS. In this context, the impact of 2.5-µm CSP particle size on analysis time and separation efficiency should be studied.

Materials and Methods

Chemicals and Solutions

All chemicals were of analytical grade. *n*-Hexane was purchased from VWR International (Fontenay-sous-Bois, France), *n*-heptane was obtained from Fluka AG (Buchs, Switzerland). Isopropanol (IPA) and *n*-butanol were from Chem-Lab NV (Zedelgem, Belgium). Diethylamine (DEA), sodium hydroxide (0.1 mol L^{-1}), sodium sulfate and Milli-Q-Water (HiPerSolv CHROMANORM) were from VWR International (Vienna, Austria).

Because of their novelty, most of the analytes were commercially not available from official suppliers. As a consequence, most of them were obtained from different online drug vendors. All applied pure enantiomers were prepared by a semipreparative HPLC method (unpublished results) in a multi milligram scale. Furthermore, some racemic analytes not available from online stores were synthesized in our laboratory in small quantities for scientific purposes. Additionally, some real-life samples were seized by Austrian police. The sources of all investigated substances are listed in Table A1 (see Electronic Supplementary Material).

An identity check for all analytes was performed by gas chromatography–electron ionization mass spectrometry (GC–MS) prior to the measurements. If necessary, the substances additionally were identified via nuclear magnetic resonance (NMR).

Mobile phases were prepared by mixing *n*-hexane, *n*-butanol and DEA in a ratio of 100:0.3:0.2 or *n*-hexane and DEA in a ratio of 100:0.2. They were degassed by ultrasonification for at least 2 min.

Chromatographic Conditions

Separation experiments were performed with an Agilent 1260 Series Infinity II Liquid Chromatograph allowing a back pressure up to 600 bar. Data evaluation was carried out via a ChemStation for LC 3D Systems Rev. C. 01.07SR2[255] (Agilent Technologies, Waldbronn, Germany) software. The chromatograph was equipped with an autosampler and a diode array detector. UV absorbance was measured at a wavelength of 230 nm. Analysis was performed at ambient temperature and under isocratic conditions with flow rates ranging from 1.0 to 2.0 mL min^{-1}. Injection volume was 1 µL.

A Waters Acquity UPC2® Trefoil™ CEL1 2.5 µm, 3.0 × 150 mm column containing cellulose tris-(3,5-dimethylphenyl-carbamate) as chiral selector served as chiral stationary phase.

Sample Preparation

All NPS samples consisted mainly as their hydrochloric acid salts. To release the free base of the analytes, 3.0 mg substance each was dissolved in 0.5 mL Milli-Q-Water and treated with 50 µL sodium hydroxide (0.1 mol L^{-1}). After the dissolving step, the free base was extracted with 3.0 mL *n*-hexane each and dried over a small quantity of sodium sulfate. The final concentration of each sample was about 1.0 mg mL^{-1}. Additionally, all samples were degassed in an ultrasonic bath for 2 min.

Results and Discussion

Chiral stationary phases containing polysaccharide esters in combination with differently substituted phenylcarbamates are widely used for enantioseparations of pharmaceuticals, agrochemicals and divers other substances. The according chiral separation principle is based on the higher-order secondary structure of the polysaccharides. Interactions of the enantioselective cavities of the immobilized selector with the analyte enantiomers are responsible for enantiomeric discrimination. Further interaction mechanisms such as π–π-, hydrophobic- and dipol–dipol interactions, inter- and intramolecular hydrogen bondings and steric interactions of the esters and the carbamate moieties have to be taken into account for their chiral separation ability [36–38]. Because of successful application attempts with differently substituted polysaccharide columns for enantiomeric separation of NPS within our research group [5, 19, 20], a Waters Acquity UPC2® Trefoil™ CEL1 2.5 µm, 3.0 × 150 mm column containing cellulose tris-(3,5-dimethylphenyl-carbamate) as chiral selector was studied.

All investigated chemical structures of the analytes are given in Table A1 (see Electronic Supplementary Material). They represent derivatives of different parent structures such as cathinones (A-B, F), pyrovalerones (C-I), amphetamines (J), ketamines (K), phenidines and phenidates (L-O), morpholines (P), thiophenes (Q-S) and benzofuran derivatives (T-U). They are all available for public via the internet for being misused for recreational purposes.

Chiral separation experiments were initiated using the normal phase mode with a mobile phase consisting of *n*-hexane/IPA/DEA (90:10:0.1). The ratio of the origin mobile phase ingredients was chosen on basis of previous studies. As model substances, two early emerged cathinone derivatives available since 2010, namely 3,4-DMMC and 4-MMC were successfully resolved into their enantiomers. A resolution of 1.83 for 3,4-DMMC and 0.83 for 4-MMC was obtained. Additionally, both substances were eluted within 1.3 min. However, an increased interaction of the substances with the CSP connected with longer retention times demands a higher percentage of *n*-hexane. In the first step, the composition of the mobile phase was changed to *n*-hexane/IPA/DEA (95:5:0.1) and in the second step to (99:1:0.1). For both mobile phases, resolution (R_s) values as well as retention times of the model compounds increased as shown in Table 1. Furthermore, *n*-hexane was replaced by *n*-heptane to increase the lipophilic property of the mobile phase. However, with *n*-heptane, even slightly worse chromatographic resolution was obtained in combination with nearly identical retention times. For this reason, *n*-hexane was used again for further experiments. A continued increase of *n*-hexane in combination with a slight increase of the DEA amount resulted in constantly increasing chromatographic resolution in combination with longer retention times. For a mobile phase consisting of *n*-hexane/IPA/DEA (100:0.3:0.2), R_s values of 13.00 for 3,4-DMMC and 6.16 for 4-MMC were obtained. As a further optimization step, the effect of alternatives of IPA was studied. Using the equal ratio of 100:0.3:0.2, IPA was replaced by *n*-butanol. Again, both chromatographic resolution and retention times of the enantiomers increased giving R_s values of 17.90 for 3,4-DMMC and 8.05 for 4-MMC, respectively. The chromatogram of the single chiral separation of 3,4-DMMC with *n*-hexane/*n*-butanol/DEA (100:0.3:0.2) as mobile phase is given in Fig. 1 and an overview of the mobile phase optimization is presented in Table 1. Because of the high resolution in combination with acceptable retention times, this mobile phase was chosen to test a set of 49 cathinone derivatives and a set of 19 pyrovalerone derivatives. With these measurements, 33 cathinone derivatives and 11 pyrovalerone derivatives were separated in their enantiomers, successfully. Separation results are given in Tables 2 and A2 (see Electronic

Table 1 Effect of mobile phase composition on enantioseparation and elution time using the model substances 3,4-DMMC and 4-MMC

Mobile phase	Compound	t_1 (min)	t_2 (min)	α	R_s
n-Hexane/IPA/DEA (90:10:0.1)	3,4-DMMC	1.14	1.23	1.08	1.83
n-Hexane/IPA/DEA (95:5:0.1)	3,4-DMMC	1.34	1.51	1.12	2.86
n-Hexane/IPA/DEA (99:1:0.1)	3,4-DMMC	2.18	2.80	1.28	7.82
n-Heptane/IPA/DEA (99:1:0.1)	3,4-DMMC	2.17	2.75	1.27	7.30
n-Hexane/IPA/DEA (99.5:0.5:0.1)	3,4-DMMC	2.49	3.29	1.32	9.41
n-Hexane/IPA/DEA (99.5:0.5:0.2)	3,4-DMMC	2.66	3.64	1.37	10.58
n-Hexane/IPA/DEA (100:0.3:0.2)	3,4-DMMC	3.23	4.83	1.50	13.00
n-Hexane/*n*-butanol/DEA (100:0.3:0.2)	3,4-DMMC	4.46	7.91	1.78	17.90
n-Hexane/IPA/DEA (90:10:0.1)	4-MMC	1.16	1.20	1.03	0.83
n-Hexane/IPA/DEA (95:5:0.1)	4-MMC	1.36	1.44	1.06	1.47
n-Hexane/IPA/DEA (99:1:0.1)	4-MMC	2.16	2.42	1.12	3.56
n-Heptane/IPA/DEA (99:1:0.1)	4-MMC	2.16	2.40	1.11	3.31
n-Hexane/IPA/DEA (99.5:0.5:0.1)	4-MMC	2.46	2.79	1.13	4.11
n-Hexane/IPA/DEA (99.5:0.5:0.2)	4-MMC	2.60	2.99	1.15	4.71
n-Hexane/IPA/DEA (100:0.3:0.2)	4-MMC	3.04	3.67	1.21	6.16
n-Hexane/*n*-butanol/DEA (100:0.3:0.2)	4-MMC	4.22	5.43	1.29	8.05

Conditions: column: Trefoil® CEL1 2.5 µm, chiral selector: cellulose tris-(3,5-dimethylphenyl-carbamate), 150×3 mm, ambient temperature, flow: 1 mL min^{-1}, UV: 230 nm, injection: 1 µL

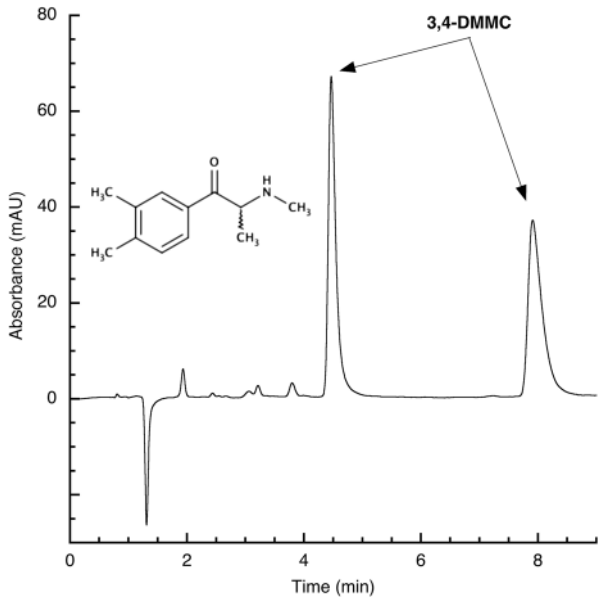

Fig. 1 Chiral separation of 3,4-DMMC phase *n*-hexane/*n*-butanol/DEA (100:0.3:0.2) as mobile phase. Conditions: column: Trefoil® CEL1 2.5 µm, 150×3 mm, chiral selector: cellulose tris-(3,5-dimethylphenyl-carbamate), mobile phase: *n*-hexane/*n*-butanol/DEA (100:0.3:0.2), ambient temperature, flow: 1.0 mL min^{-1}, UV: 230 nm, injection: 1 µL

Supplementary Material). Resolution for the separated substances ranged from 0.93 for dimethylone to 17.90 for 3,4-DMMC as cathinone derivatives. For pyrovalerone derivatives, resolutions were obtained from 1.52 for 4-M-PrC to 8.92 for α-PPP. Due to the fact that most of the substances were still resolved within short retention times, a further mobile phase improvement step was investigated. To create a maximum of interaction time of the analytes with the CSP under the application of *n*-hexane, a mobile phase without any addition of a hydrophilic component was investigated by means of *n*-hexane/DEA (100:0.2) as mobile phase. As a consequence, further nine cathinone derivatives and one additional pyrovalerone derivative were resolved. Resolutions for the separated cathinone derivatives using a mobile phase of *n*-hexane/DEA (100:0.2) ranged from 1.13 for pentylone to 4.04 for 4F-NPP. Regarding the pyrovalerone derivative 4-Cl-PVP, a chromatographic resolution of 1.25 was feasible. Chiral separation results with *n*-hexane/DEA (100:0.2) as mobile phase are presented in Table A3 (see Electronic Supplementary Material). A comparison regarding chiral separation ability and prolonged retention times between the finally used mobile phases is given in Fig. 2 by means of 4F-NPP.

Additionally, further substance classes of NPS comprising selected representatives of amphetamines, ketamines, phenidines, phenidates, morpholines, thiophenes and benzofuranes were tested without any further method optimization. As mobile phases both, *n*-hexane/*n*-butanol/DEA (100:0.3:0.2) and *n*-hexane/DEA (100:0.2), were used. Best results obtained are shown in Table A4 (see Electronic Supplementary Material). Resolutions for the chirally separated substances ranged from 0.47 for the amphetamine derivative 4-FMA up to 9.27 for the thiophene derivative thiothinone. As an example, a chromatogram of the chiral separation of thiothinone is given in Fig. 3.

Table 2 Enantioseparation results of a set of 49 cathinone derivatives

Compound	t_1 (min)	t_2 (min)	α	R_s
4-MC	14.67	24.67	1.68	13.88
4-MMC	4.22	5.43	1.29	8.05
3-MMC	4.96	5.42	1.09	3.15
3,4-DMMC	4.46	7.91	1.78	17.90
Methedrone	11.98	n.d	–	–
2-CMC	5.03	n.d	–	–
3-CMC	3.50	3.85	1.10	3.10
4-CMC	3.96	4.43	1.12	3.60
4-EMC	3.85	5.34	1.39	10.21
4-FMC	3.95	4.34	1.10	3.19
3-FMC	3.56	n.d	–	–
2-FMC	2.95	n.d	–	–
4-BMC	4.36	4.90	1.12	3.66
Buphedrone	3.21	3.37	1.05	1.63
4-Methylbuphedrone	2.66	3.22	1.21	6.36
Pentedrone	2.71	n.d	–	–
Ethcathinone	2.97	3.15	1.06	1.68
4-EEC	2.47	2.72	1.10	3.28
3-CEC	2.30	2.45	1.06	2.06
4-CEC	2.46	2.75	1.12	3.55
N-Ethylbuphedrone	2.02	2.08	1.03	0.97
N-Ethylpentedrone	6.45	9.18	1.42	6.68
N-Ethylhexedrone	1.78	n.d	–	–
4-MPD	2.18	n.d	–	–
Amfepramone	1.82	n.d	–	–
DL-4662	6.41	9.13	1.42	7.21
3-MEC	2.82	3.07	1.09	1.98
4-MEC	2.62	3.15	1.20	5.64
N-Propcathinone	1.20	1.34	1.12	3.03
4-MPC	2.84	3.16	1.11	3.19
4-CPRC	2.47	2.72	1.10	3.23
4F-NPP	1.16	n.d	–	–
4-CIC	2.73	n.d	–	–
4-CBC	2.17	n.d	–	–
Methylone	11.58	n.d	–	–
Dimethylone	3.54	3.62	1.02	0.93
2-AIMP	22.08	25.24	1.14	3.79
Butylone	6.12	6.72	1.10	3.04
N,*N*-Dimethylbutylone	2.71	n.d	–	–
Pentylone	5.12	n.d	–	–
Ethylone	6.02	6.36	1.06	1.76
5-ME	6.03	6.38	1.06	1.75
N-Ethylpentylone	2.84	3.16	1.11	3.20
4-MBC	2.62	3.13	1.19	5.37
5-PPDi	1.74	n.d	–	–
bk-IVP	1.56	1.90	1.22	5.53
DOMC	22.05	25.14	1.14	3.74
5-BPDi	1.74	n.d	–	–
4-CDC	1.50	1.59	1.06	1.71

Conditions: column: Trefoil® CEL1 2.5 µm, 150×3 mm, chiral selector: cellulose tris-(3,5-dimethylphenyl-carbamate), mobile phase: *n*-hexane/*n*-butanol/DEA (100:0.3:0.2), ambient temperature, flow: 1 mL min^{-1}, UV: 230 nm, injection: 1 µL

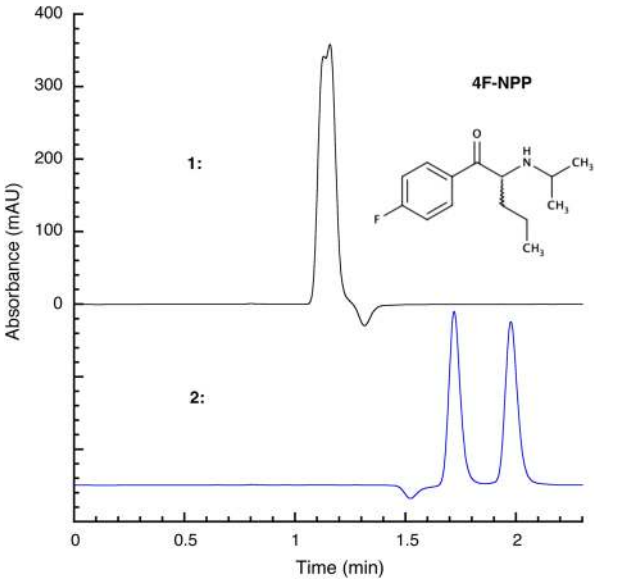

Fig. 2 Chiral separation of 4-F-NPP. Conditions: column: Trefoil® CEL1 2.5 µm, 150×3 mm, chiral selector: cellulose tris-(3,5-dimethylphenyl-carbamate), mobile phase 1: *n*-hexane/*n*-butanol/DEA (100:0.3:0.2), mobile phase 2: *n*-hexane/DEA (100:0.2), ambient temperature, flow: 1.0 mL min^{-1}, UV: 230 nm, injection: 1 µL

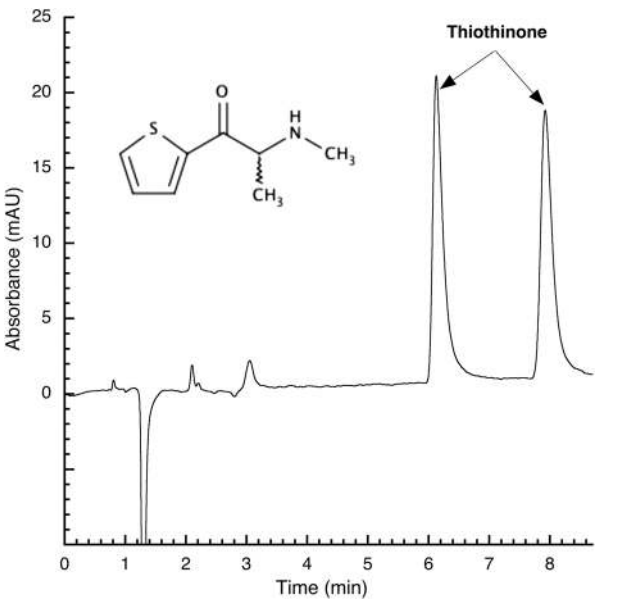

Fig. 3 Chiral separation of thiothinone. Conditions: column: Trefoil® CEL1 2.5 µm, 150×3 mm, chiral selector: cellulose tris-(3,5-dimethylphenyl-carbamate), mobile phase: *n*-hexane/*n*-butanol/DEA (100:0.3:0.2), ambient temperature, flow: 1.0 mL min^{-1}, UV: 230 nm, injection: 1 µL

In addition to the single analyte measurements, the method was checked for simultaneous chiral separations. In Fig. 4 an example of a rapid simultaneous chiral separation of six different cathinone derivatives, namely

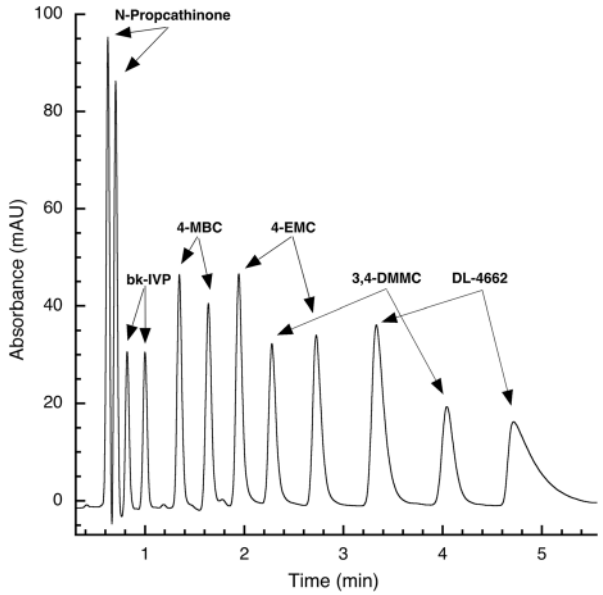

Fig. 4 Rapid simultaneous chiral separation of six different cathinone derivatives. Conditions: column: Trefoil® CEL1 2.5 µm, 150×3 mm, mobile phase: *n*-hexane/*n*-butanol/DEA (100:0.3:0.2), ambient temperature, flow: 2.0 mL min^{-1}, UV: 230 nm, injection: 1 µL

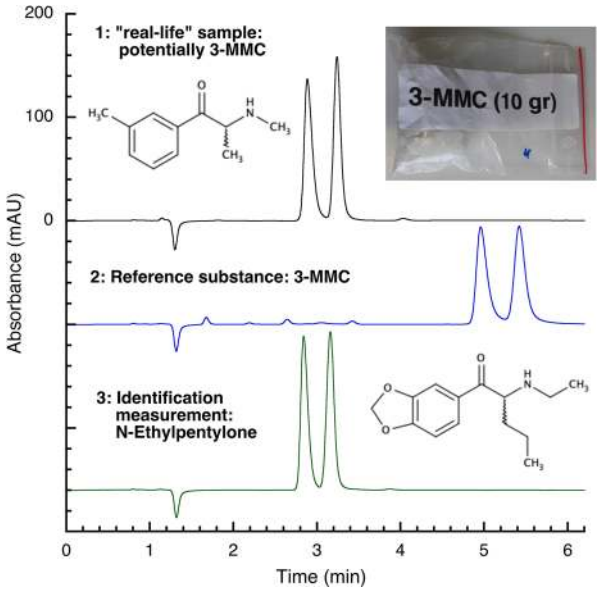

Fig. 5 Identity check of a potential 3-MMC real-life sample. Conditions: column: Trefoil® CEL1 2.5 µm, 150×3 mm, chiral selector: cellulose tris-(3,5-dimethylphenyl-carbamate), mobile phase: *n*-hexane/*n*-butanol/DEA (100:0.3:0.2), ambient temperature, flow: 1.0 mL min^{-1}, UV: 230 nm, injection: 1 µL

N-propcathinone, bk-IVP, 4-MBC, 4-EMC, 3,4-DMMC and DL-4662, is presented. All 12 enantiomers of the 6 racemic analytes were baseline separated and identified. In this case, a flowrate of 2.0 mL min^{-1} was used showing a back pressure of 310 bar. The measurement was done within less than 5.5 min. This simultaneous chiral separation example illustrates the advantages of a 2.5-µm HPLC setup with respect to short analysis time in combination with higher back pressure due to smaller particle sizes. In particular, the 2.5-µm column has the ability to give much faster enantioseparation results of chiral NPS as previously used phenylcarbamate-substituted polysaccharide columns.

Furthermore, the method was found to be applicable for positional isomer separation and to determine enantiomeric elution orders of different cathinone derivatives. An example for a successful positional isomer separation was the distinct separation of 4-methylethcathinone (4-MEC) and 3-methylethcathinone (3-MEC). A GC–MS differentiation is difficult because of their equal molecular weight. Regarding the EEO determination for cathinone derivatives, the substances 4-MEC and ethylone served as model substances. The pure enantiomers were produced by a semipreparative HPLC method (unpublished results). Sample preparation was performed by spiking the racemic analytes with their pure enantiomers. In both cases, the (−)-enantiomer was eluted prior to its corresponding (+)-enantiomer.

Additionally, the method was found to be a useful tool for fast real-life sample identity checks comparing retention times of the enantiomers. Figure 5 shows the measurement of a potential 3-MMC sample which was provided by Austrian police for an identity confirmation check. After comparison of retention times and resolution of the 3-MMC reference substance, the identity of the 3-MMC sample was not confirmed because of different retention times. The sample was rather identified as *N*-ethylpentylone via the presented 2.5-micron HPLC–UV method using a *N*-ethylpentylone reference substance. Additionally, the identity was confirmed via a GC–MS measurement.

Finally, the presented method underwent a repeatability study. Therefore, five intra- and interday measurements each using the identical HPLC–UV system were performed. *n*-hexane:/*n*-butanol/DEA (100:0.3:0.2) served as mobile phase. Three cathinone model compounds, namely 4-MMC, 3,4-DMMC and DL-4662, were chosen as analytes. In Table 3, the obtained results are given. Relative standard deviations (RSDs) for retention times were less than 0.1% and for the chromatographic resolution less than 1.5% during the intraday measurements. Regarding the interday measurements, the RSDs for retention times were less than 1.4% and for the chromatographic resolution less than 3.6%.

Conclusion

The presented study introduces a simple chiral HPLC–UV method for enantiomeric separation of a broad spectrum of cathinones, pyrovalerones and other representatives of

Table 3 Repeatability data including retention times and resolution for the model substances 4-MMC, 3,4-DMMC and DL-4662

Modelsubstance	Repeatability	t_1 (min)	t_2 (min)	R_s
4-MMC	Intraday $n=5$	4.21 ± 0.00 RSD = 0.0%	5.41 ± 0.00 RSD = 0.0%	7.8 ± 0.1 RSD = 1.5%
	Interday $n=5$	4.21 ± 0.02 RSD = 0.3%	5.46 ± 0.06 RSD = 1.1%	8.1 ± 0.4 RSD = 3.6%
3,4-DMMC	Intraday $n=5$	4.47 ± 0.00 RSD = 0.0%	7.78 ± 0.02 RSD = 0.0%	17.0 ± 0.2 RSD = 1.0%
	Interday $n=5$	4.47 ± 0.03 RSD = 0.4%	7.89 ± 0.12 RSD = 1.4%	17.2 ± 0.2 RSD = 0.7%
DL-4662	Intraday $n=5$	6.55 ± 0.01 RSD = 0.1%	9.25 ± 0.02 RSD = 0.1%	7.0 ± 0.0 RSD = 0.2%
	Interday $n=5$	6.55 ± 0.04 RSD = 0.3%	9.31 ± 0.11 RSD = 1.0%	6.9 ± 0.1 RSD = 1.5%

Conditions: column: Trefoil® CEL1 2.5 µm, 150×3 mm, chiral selector: cellulose tris-(3,5-dimethylphenyl-carbamate), mobile phase: *n*-hexane/*n*-butanol/DEA (100:0.3:0.2), ambient temperature, flow: 1 mL min^{-1}, UV: 230 nm, injection: 1 µL

NPS by means of a 2.5-micron column containing the chiral selector cellulose tris-(3,5-dimethylphenyl-carbamate).

With a chromatographic resolution ranging from 0.83 to 17.9, 78 of 95 NPS were resolved in their enantiomers, in most of the cases within short analysis time. Regarding good separation in combination with short separation times, the mobile phase containing *n*-hexane/*n*-butanol/DEA (100:0.3:0.2) was found to be optimal. The mobile phase *n*-hexane/DEA (100:0.2) served for compounds, which were not separated by the aforementioned mobile phase; however, connected with longer retention times. Furthermore, the method was found to be appropriate for rapid simultaneous chiral separations, to separate positional isomers, to determine enantiomeric elution orders and can be an additional useful technique to clarify the identity of real-life samples. The robustness of the method was demonstrated and also validated successfully via intra- and an interday repetition measurements.

With this approach, both identity and enantiomeric ratio of real-life samples can be proven. Novel derivatives of the already measured compound classes as they come up every month can also be checked via the presented method concerning their enantiomeric composition. Furthermore, the presented method could be upscaled for semipreparative scale to isolate pure NPS enantiomers. They can be subject to further studies concerning their pharmacological or toxicological behaviour. All investigated compounds were shown to be traded as racemic mixtures.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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