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Enantiomeric separation of Novel Psychoactive Substances by capillary electrophoresis using (+)-18-crown-6-tetracarboxylic acid as chiral selector

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Abstract

In the recent years, hundreds of Novel Psychoactive Substances (NPS) have entered both the European and the global drug market. These drugs, which are mainly used for recreational matters, have caused serious social problems. Every year, the spectrum of these misused drugs is enlarged by new derivatives, which are produced by modifications of basic structures of already well-known substances. Additionally, a lot of them possess a stereogenic center which leads to 2 enantiomeric forms. The fact that the pharmacological effects and potencies of the enantiomers of these chiral NPS may differ can be assumed from a broad spectrum of active pharmaceutical ingredients. For this reason, analytical method development regarding enantiomeric separation for these classes of substances is of great pharmaceutical and medical interest. The aim of this work was to create an easy-to-prepare chiral capillary electrophoresis method for the enantioseparation of NPS which contains a primary amino group by means of (+)-18-crown-6-tetracarboxylic acid as chiral selector. Novel Psychoactive Substances were purchased at various Internet stores or represent samples seized by Austrian police. The effects of selector concentration, the electrolyte composition, and the addition of organic modifiers to the background electrolyte on enantioseparation were investigated. Under optimized conditions, the use of 20-mM (+)-18-crown-6-tetracarboxylic acid, 10-mM Tris, and 30-mM citric acid buffer at pH 2.10 turned out to be effective. Fifteen of 24 tested NPS were resolved in their enantiomers within 15 minutes. It was found that all NPS were traded as racemic mixtures.

KEYWORDS

(+)-18-Crown-6-tetracarboxylic acid, amphetamine, benzofuries, capillary electrophoresis, Novel Psychoactive Substances (NPS), primary amines

1 | INTRODUCTION

Since the last decade, globalization and the further technological progress of the Internet modified the drug market in a drastic way. A huge number of NPS still float the European as well as the global drug market. By the end of 2016, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) monitored more than 620 NPS that have appeared in Europe's drug market and which are often not covered by international drug

controls.[1] These compounds are mainly produced in Eastern Europe or in Asian countries and are promoted and sold via the Internet as "plant food," "research compounds," or "bath salts" worldwide. Being misused mainly for recreational matters, the spectrum of these substances is continuously enlarged by new derivatives, which are produced via molecular structure modification of already existing compounds. Novel Psychoactive Substances compete with illicit drugs such as cocaine, opium, or cannabis. In chemical context, these drug molecules belong to different compound classes like phenetylamines, arylamines, piperazines, or cathinone derivatives. In most cases, they are sold as "legal" replacements for prohibited drugs. Because of their explicit labeling with disclaimers like "not for human consumption," Internet stores sell the compounds with a very low risk of law enforcement. The simple access to NPS via the World Wide Web is an immense challenge and makes the control of the global drug situation more difficult. Also, online stores offer dubious data about identity and purity, which should be critically scrutinized about their correctness. These data collected by mass spectroscopy or nuclear magnetic resonance are displayed on vendors' websites pretending quality assurance of the provided NPS.

An interesting attribute of some of these drug molecules is the presence of an asymmetric carbon atom in their chemical structure. This plays a very important role in their pharmacological and toxicological behavior because stereogenic centers lead to 2 different enantiomeric forms with potentially different pharmacological effects and potencies. The fact that enantiomers may exhibit different effects is well known for a broad spectrum of chiral active pharmaceutical ingredients. With respect to illicit drugs, native amphetamine represents an example, where the *S*(+) enantiomer shows higher potency than its corresponding R(−) enantiomer.[2] Regarding methamphetamine, the *S*(+) enantiomer also shows higher CNS-stimulating effects, and for methcathinone, the *S*(−) enantiomer leads similarly to higher stimulation.[3,4] However, for the majority of NPS, there are few data yet available for potential differences of the effects of the enantiomers. Therefore, analytical method development for the enantiomeric separation of these substances is of great importance.

In literature, successful attempts for enantioseparation of NPS were presented by different high-performance separation techniques such as high-performance liquid chromatography,[5-14] gas chromatography (GC),[9,12,15-18] and supercritical fluid chromatography.[19] Also with capillary electrophoresis as well as with capillary electrochromatography as electrophoretically driven methods, positive results were achieved.[7,17,19-31] For successful enantioseparation, different chiral selectors such as cyclodextrins, polysaccharide derivatives, and chiral ion-exchange type stationary phases were chosen. Chiral crown ethers turned out to be a suitable chiral selector class for primary amines.[32-35] Crown ethers with their macrocyclic polyether ring systems were first introduced by Pedersen in 1967.[36] For enantioseparation by CE, particularly (+)-18-crown-6-tetracarboxylic acid turned out to be an appropriate chiral selector. Being used as an additive to the BGE, successful chiral separation of 4 amino acids was first shown by Kuhn in the 1990s.[32] Chiral recognition is based on the formation of host-guest inclusion complexes between the chiral selector and the analyte containing a primary amino group. Additionally, ion-dipole interactions can be taken into account. The different complex stability constants of the mixed host-guest complexes with R-enantiomer and S-enantiomer and the different electrophoretical mobilities of the complexes in CE are responsible for the enantiomeric discrimination. A scheme of such a tripod interaction between (+)-18-crown-6-tetracarboxylic acid molecule and a primary amine is shown in Figure 1. However, (+)-18-crown-6-tetracarboxylic acid was not commercially available for a certain period of time.[34] Because the purchase of this chiral selector has been continued, the aim of this work was to investigate its chiral separation ability for NPS with primary amino structure.

Because many NPS are not yet commercially available from official distributors of chemicals, they were either purchased from Internet shops or represent samples seized by Austrian police. According to our present knowledge, apart from successful enantioseparation of amphetamine and para-chloroamphetamine by CE using (+)-18-crown-6-tetracarboxylic acid as chiral additive,[26] there is no publication about chiral separation of NPS with this chiral selector. Therefore, we tested amphetamines, benzofurans, and some other psychoactive substances being traded as NPS as primary amines for this purpose.

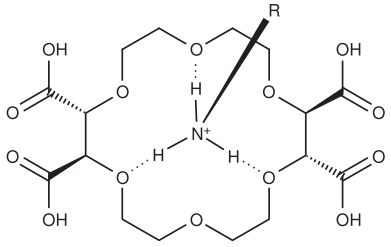


FIGURE 1 Schematical diagram showing a tripod interaction between (+)-18-crown-6-tetracarboxylic acid and a primary amine

2 | MATERIALS AND METHODS

2.1 | Chemicals and solutions

All chemicals were of analytical grade. (+)-18-Crown-6-tetracarboxylic acid was purchased in the past from Merck-Schwachhardt (Hohenbrunn, Germany). Tris [Tris(hydroxymethyl)-aminomethane] was bought from BioRad Laboratories (Hercules, California). Citric acid was purchased from Merck (Darmstadt, Germany). Methanol was from VWR Chemicals (Fontenay-sous-Bois, France). Nanopure water was prepared inhouse (Millipore, Darmstadt, Germany).

Amphetamine, D-amphetamine, 4-methylthioamphetamine, 2,5-dimethoxyamphetamine, 4-dimethoxyamphetamine, 4-methoxyamphetamine, 4-bromo-2,5-dimethoxyamphetamine, 4-methylendioxyamphetamine, 4-(2-aminopropyl)benzofuran, 1-(2,3-dihydro-1-benzofuran-4-yl)propan-2-amine (4-APDB), and 7-(2-aminopropyl)benzofuran were purchased from LGC Standards (Wesel, Germany). Because a part of the analytes were not commercially available from official suppliers because of their novelty, they were bought from diverse shops in the Internet. 3-Fluoroamphetamine, 5-(2-aminopropyl)benzofuran, 6-(2-aminopropyl)benzofuran, and 5-(2-aminopropyl)indole were obtained from www.sensearomatic.com. 2,5-Dimethoxy-4-chloroamphetamine and 1-(2,3-dihydro-1-benzofuran-5-yl)propan-2-amine were purchased from www.deboralabs.com. 1-(2,3-Dihydro-1-benzofuran-6-yl)propan-2-amine was purchased from www.officialbenzofury.com. Thiopropamine was bought from www.rcnetchemicals.com. 6,7-Methylendioxy-2-aminotetraline and 4-fluoroamphetamine represent real-life samples seized by Austrian Police. Prior to chiral separation experiments in CE, the substances were characterized by GC-electron impact mass spectrometry and NMR if necessary. The optimized, final BGE was prepared by dissolving 20-mM (+)-18-crown-6-tetracarboxylic acid in a mixture of 10-mM Tris and 30-mM citric acid in ultrapure water at a pH of 2.10. Prior to measurements, the solution was degassed by ultrasonification and was filtered through a 0.45-μm pore size nylon filter (Carl Roth, Karlsruhe, Germany). With respect to the comparability of the separation factors, α_{app} (t_2/t_1) is shown in this work because with (+)-18-crown-6-tetracarboxylic acid the EOF was slower than the compounds, and therefore, negative α_{eff} ($(t_2\text{-}t_0)/(t_1\text{-}t_0)$) values would have been generated.

2.2 | Instrumentation

A fully automated 3^DCE system (Agilent Technologies, CA, USA) equipped with a diode array detector was used for the experiments. Measurements were performed in 50-μm ID-fused silica capillaries with a total length of 68.5 cm and an effective length of 60 cm, purchased from MicroQuartz (Munich, Germany). Detection was performed via on-column measurements of UV absorption at 208 nm. All measurements were carried out at ambient temperature. Before and after each run, the capillary was flushed with 0.2-M NaOH, water, and BGE, respectively. Samples were injected dynamically by applying a pressure of 10 mbar * 5 seconds on the inlet vial.

2.3 | Sample preparation

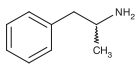
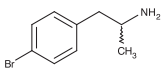
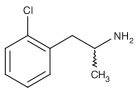
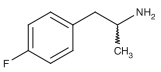
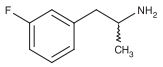
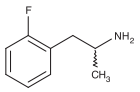
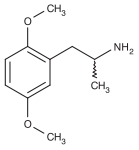
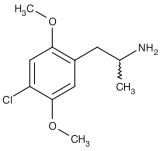
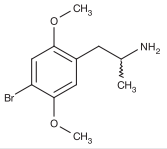
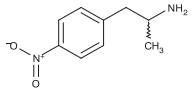
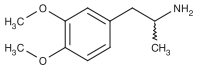
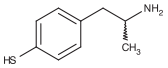
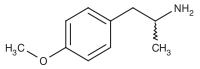
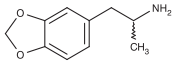
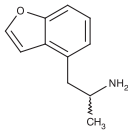
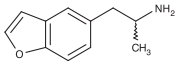
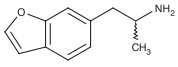
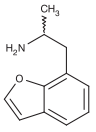
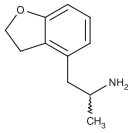
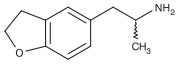
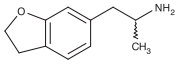
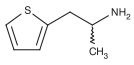
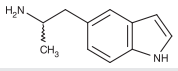
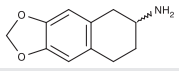
Each analyte (1.0 mg) was dissolved in 1 mL of nanopure water. To accelerate the dissolving process, the samples were put on an ultrasonic bath for 1 minute prior to filtration, analog to the procedure the BGE was prepared and filtered through a 0.45-μm pore size filter (Carl Roth, Karlsruhe, Germany).

3 | RESULTS AND DISCUSSION

Enantioseparation by chiral crown ethers requests the presence of a primary amine group in the chiral analyte. Because this is the case for the drug compound classes of amphetamines and benzofurans, they were subject to further investigations. The chemical structures of the analytes are pictured in Table 1.

The application of (+)-18-crown-6-tetracarboxylic acid for chiral separation of primary amines was first reported in the literature in the 1990s.[32] Based on the work of Gübitz and Schmid, who used this chiral selector for enantioseparation of racemic glycyldipeptides and diastereomeric dipeptides, separation ability was tested for the aforementioned substance classes being abused as NPS.[37] For preliminary experiments, 3,4-dimethoxyamphetamine and 4-APDB were chosen as model compounds. An aqueous solution of 20-mM (+)-18-crown-6-tetracarboxylic acid (pH 1.99) served as electrolyte. In this case, both substances were resolved in their enantiomers. After these promising results, the effect of different BGEs as well as different concentrations of the chiral selector on enantioseparation was checked. With a BGE of 10 mM of (+)-18-crown-6-tetracarboxylic acid, 10-mM Tris solution, and 10-mM citric acid (pH 2.47), baseline separations were obtained. For this reason, the above-mentioned Tris/citric acid buffer was chosen for further measurements. The use of 20-mM (+)-18-crown-6-tetra-carboxylic acid in this BGE (pH 2.22) led again to better separation results in combination with only slightly higher migration times. Furthermore, an increase of citric acid from 10 to 30 mM to acidify the BGE showed better separation results. For this reason,

TABLE 1 Chemical structures of the NPS investigated in this study

Amphetamines			
			
Amphetamine	4-Bromoamphetamine (4-BA)	2-Chloroamphetamine (2-CA)	4-Fluoroamphetamine (4-FA)
			
3-Fluoroamphetamine (3-FA)	2-Fluoroamphetamine (2-FA)	2,5-Dimethoxyamphetamine (DMA)	2,5-Dimethoxy-4-chloroamphetamine (DOC)
			
4-Bromo-2,5-dimethoxy-amphetamine (DOB)	4-Nitroamphetamine (4-NA)	3,4-Dimethoxyamphetamine	4-Methylthioamphetamine (MTA)
			
	4-Methoxyamphetamine	3,4-Methylendioxyamphetamine (MDA)	
Benzofuries			
			
4-(2-Aminopropyl)-benzofuran (4-APB)	5-(2-Aminopropyl)-benzofuran (5-APB)	6-(2-Aminopropyl)-benzofuran (6-APB)	7-(2-Aminopropyl)-benzofuran (7-APB)
			
1-(2,3-Dihydro-1-benzofuran-4-yl) propan-2-amine (4-APDB)	1-(2,3-Dihydro-1-benzofuran-5-yl) propan-2-amine (5-APDB)	1-(2,3-Dihydro-1-benzofuran-6-yl) propan-2-amine (6-APDB)	
Other psychoactive substances			
			
Thiopropamine	5-(2-Aminopropyl)-1-methyl-ethylamine (5-API)	6,7-Methylenedioxy-2-aminotetralin (MDAT)	

20-mM (+)-18-crown-6-tetracarboxylic acid, 10-mM Tris solution, and 30-mM citric acid (pH 2.10) and a voltage of 30 kV to the cathode were used as final conditions to test a set of 24 NPS. Fifteen NPS of different compound classes were partially or even baseline separated within 15 minutes. Separation data are shown in Table 2. An electropherogram of the chiral separation of 3,4-dimethoxyamphetamine is given in Figure 2.

Additionally, the enantiomeric migration order for native amphetamine was found to be R(−) before S(+)

TABLE 2 Separation results of the tested enantiomers using (18)-crown-6-tetracarboxylic acid

Compound	t_1 (min)	t_2 (min)	α_{app}	R_s
Amphetamine	10.40	10.58	1.017	1.29
4-FA	10.50	10.70	1.018	1.65
3-FA	10.11	10.22	1.011	0.86
2-FA	10.91	10.98	1.006	0.54
3-NA	13.11	13.42	1.024	2.18
4-BA	11.75	11.86	1.010	0.93
2-CA	12.16	12.69	1.044	5.07
DOC	12.79	–	–	–
MTA	12.06	–	–	–
DMA	11.46	–	–	–
3,4-Dimethoxyamphetamine	14.46	15.11	1.046	4.65
4-Methoxyamphetamine	11.33	–	–	–
DOB	13.44	–	–	–
MDA	10.48	–	–	–
4-APB	11.84	12.01	1.015	1.19
4-APDB	14.12	14.85	1.051	4.20
5-APB	10.89	–	–	–
5-APDB	13.50	13.74	1.018	2.00
6-APB	12.48	–	–	–
6-APDB	10.99	11.17	1.016	1.54
7-APB	12.73	12.97	1.019	1.66
Thiopropamine	9.70	9.87	1.018	0.94
5-API	11.09	11.30	1.018	1.69
MDAT	11.28	–	–	–

Conditions: 20-mM (+)-18-crown-6-tetracarboxylic acid, 10-mM Tris, 30-mM citric acid, pH 2.10, cassette temperature: 20°C, applied voltage: 30 kV to the cathode, injection: 10 mbar for 5 seconds, samples: 1 mg/mL in water.

as shown in Figure 3. Enantiomeric migration order was tested by spiking a sample of racemic amphetamine with S(+)-amphetamine. Because no further pure enantiomers of the other analytes were commercially available, determination of further EMOs was not possible.

Substances which showed no chiral discrimination under the optimized conditions were subject of further separation experiments: A BGE of 40-mM (+)-18-crown-6-tetracarboxylic acid, 10-mM Tris solution, and 30-mM citric acid (pH 2.00) was used. The significantly higher amount of chiral selector and the further decrease of pH did not show positive results. Similarly, addition of organic modifiers such as methanol to the BGE did not result in any positive impact on enantioseparation or analysis time.

The optimized method was also found to be suitable for simultaneous enantioseparation of different analytes. An example is given in Figure 4 showing the analytes 2-CA, 5-APDB and 4-APDB. Furthermore, this approach can not only be used to separate enantiomers but also to distinguish clearly between different positional isomers. This is shown by 3 fluorinated amphetamine derivatives in Figure 5. Obviously, the analytes only differ in the position of the fluorine atom but show different migration times. This approach is advantageous because the positional isomers can hardly be distinguished by GC-MS due to their equal molecular weights. Finally, a validation to prove the robustness of the method was performed. Results are shown in Table 3, where both intraday and interday repeatability by means of 4-APDB were tested in 5 single runs, respectively. As the result for intraday measurements, the RSD of the migration times was less than 0.65% and for the resolution factor 4.30%. The RSD of the day-to-day measurements for the migration times was below 1.41% and for the resolution factor 8.19%. In our opinion, these are acceptable values for the performed CE experiments.

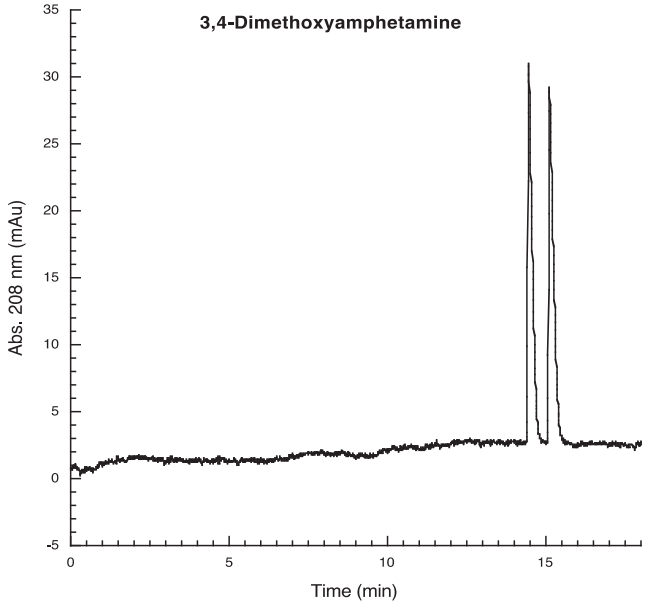


FIGURE 2 Single chiral separation of 3,4-dimethoxyamphetamine using the optimized BGE. Conditions: 20-mM (+)-18-crown-6-tetracarboxylic acid, 10-mM Tris, 30-mM citric acid, pH 2.10, cassette temperature: 20°C, applied voltage: 30 kV to the cathode, injection: 10 mbar for 5 seconds, sample: 1 mg/mL in water

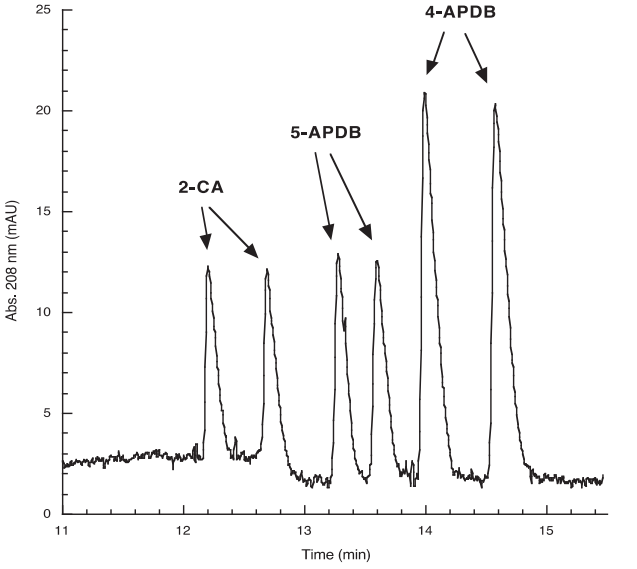


FIGURE 4 Simultaneous chiral separation of 2-chloroamphetamine, 1-(2,3-dihydro-1-benzofuran-4-yl)propan-2-amine, and 1-(2,3-dihydro-1-benzofuran-5-yl)propan-2-amine. Conditions: 20-mM (+)-18-crown-6-tetracarboxylic acid, 10-mM Tris, 30-mM citric acid, pH 2.10, cassette temperature: 20°C, applied voltage: 30 kV to the cathode, injection: 10 mbar for 5 seconds, sample: 1 mg/mL in water

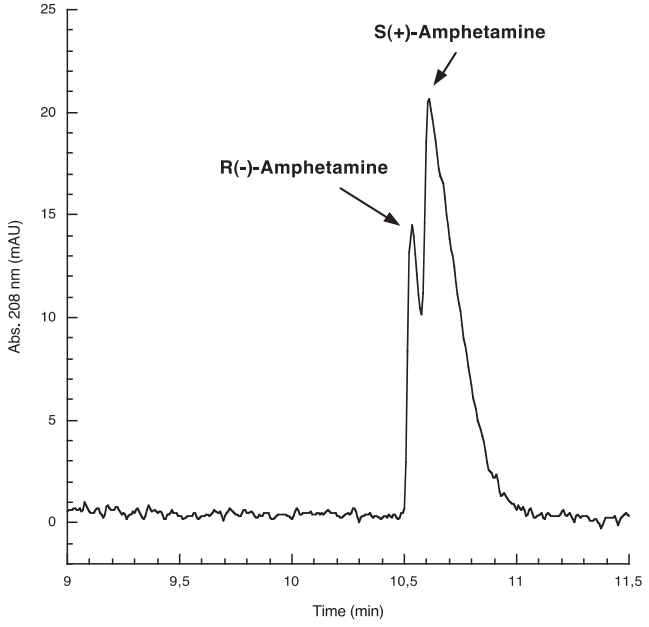


FIGURE 3 Determination of the enantiomeric migration order of racemic amphetamine via spiking a racemic amphetamine sample with S(+)-amphetamine. Conditions: 20-mM (+)-18-crown-6-tetracarboxylic acid, 10-mM Tris, 30-mM citric acid, pH 2.10, cassette temperature: 20°C, applied voltage: 30 kV to the cathode, injection: 10 mbar for 5 seconds, sample: 1 mg/mL in water

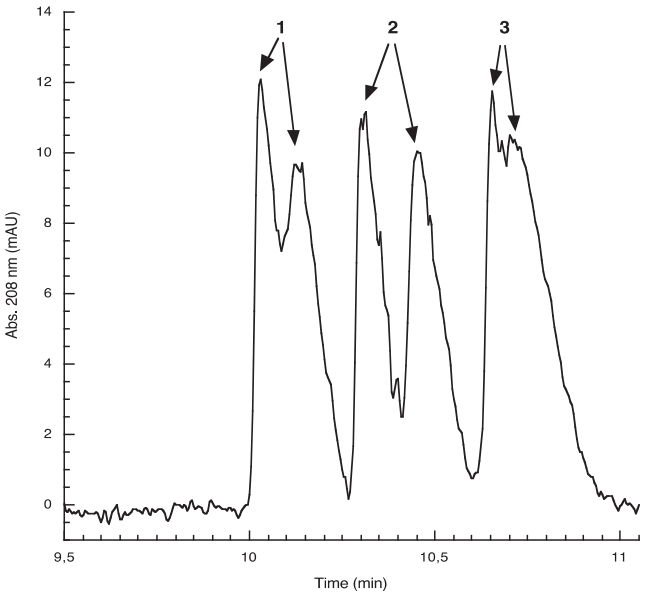


FIGURE 5 Separation of 3 positional isomers of fluorinated amphetamine (1: 3-FA; 2: 4-FA; 3: 2-FA). Conditions: 20-mM (+)-18-crown-6-tetracarboxylic acid, 10-mM Tris, 30-mM citric acid, pH 2.10, cassette temperature: 20°C, applied voltage: 30 kV to the cathode, injection: 10 mbar for 5 seconds, sample: 1 mg/mL in water

TABLE 3 Repeatability data by means of 4-APDB including retention time and resolution

Repeatability	t_1 (min)	t_2 (min)	R_s
Intraday $n = 5$	14,26 ± 0,14, RSD = 0,65%	14,96 ± 0,13, RSD = 0,61%	4,05 ± 0,23, RSD = 4,30%
Interday n = 5	14,33 ± 0,28, RSD = 1,41%	15,05 ± 0,25, RSD = 1,32%	4,03 ± 0,49, RSD = 8,19%

Conditions: 20-mM (+)-18-crown-6-tetracarboxylic acid, 10-mM Tris, 30-mM citric acid, pH 2.10, cassette temperature: 20°C, applied voltage: 30 kV to the cathode, injection: 10 mbar for 5 seconds, samples: 1 mg/mL in water.

4 | CONCLUSION

In the recent years, NPS gained an enormous popularity worldwide. Every year, the number of these mainly not scheduled substances is growing continuously. Because many of these compounds are chiral, analytical method development for enantioseparation is essential.

With the introduced approach, an easy-to-perform, fast, and reliable chiral CE method for the enantioseparation of NPS was developed; however, it is restricted to compounds with a primary amino group. Under the optimized conditions, 15 of 24 tested NPS were separated successfully within 15 minutes. Resolution factors ranged from 0.54 to 5.07.

In the future, the presented technique can be an additional useful tool for enantiomer and positional isomer separation of further emerging NPS with a primary amino group. Additionally, this approach can help to clarify the enantiomeric status of real-life samples.

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