Application Note

Forensic and Toxicology - Criminalistics

Separation of Enantiomers of Amphetamine-Related Drugs and Their Structural Isomers

Using the Agilent 1260 Infinity II SFC and Detection by Coupled Mass Spectrometry

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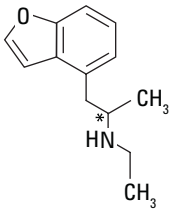
Abstract

This Application Note demonstrates the separation of enantiomers of amphetamine-related drugs and their positional isomers as well as enantiomers of the same compounds. This separation was performed using chiral phase columns with the Agilent 1260 Infinity II SFC System. The qualitative detection and quantitative determination of both the structural isomers and enantiomers was achieved using the Agilent 6495 triple quadrupole MS and the Agilent 6150 single quadrupole MSD.

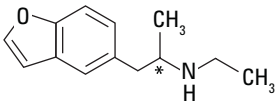
Introduction

The isomeric compounds 4-, 5-, and 6-EAPB are psychedelic drugs structurally related to amphetamine, and belong chemically to the class of benzofuran compounds (Figure 1)[1]. The three compounds are structural isomers comprising one chiral center; therefore, each exists in two enantiomeric forms.

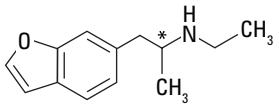
This Application Note demonstrates the separation of these structural isomers and their enantiomers by chiral chromatography. This separation was achieved using the Agilent 1260 Infinity II SFC System coupled to mass spectrometry for qualitative and quantitative determination.



1-(Benzofuran-4-yl)-N-ethylporpan-2-amine
(4-EAPB)



1-(Benzofuran-5-yl)-N-ethylporpan-2-amine
(5-EAPB)



1-(Benzofuran-6-yl)-N-ethylporpan-2-amine
(6-EAPB)

Figure 1. Formulae of 4-, 5-, and 6-EAPB. Each of the isomeric compounds also has a stereo center (asterisk), and thus exists in two enantiomeric forms.

Experimental

Instruments

Agilent 1260 Infinity II SFC/MS System comprises:

- Agilent 1260 Infinity II SFC Control Module (G4301A)
- Agilent 1260 Infinity II SFC Binary Pump (G4782A)
- Agilent 1260 Infinity II SFC Multisampler (G4767A)
- Agilent 1260 Infinity II DAD with high-pressure SFC flow cell (G7115A)
- Agilent 1260 Infinity II Multicolumn Thermostat (MCT) (G7116A) with
- Agilent InfinityLab Quick Change 4-position/10-port four-column selection valve (p/n 5067-4287)
- Agilent 1260 Infinity II Isocratic Pump (G7110B) and SFC/MS Splitter kit (G4309-68715)
- Agilent 6495 Triple Quadrupole MSD with Agilent Jet Stream and iFunnel Technology
- Agilent 6150 Single Quadrupole MSD with Agilent Jet Stream

SFC Method for the separation of all six isomers (enantiomers and structural isomers) on Column 1

Parameter	Value
SFC Flow	3 mL/min
Modifier	Methanol + 0.1 % NH_3 aq.
Isocratic	10 % modifier
Column temperature	20 °C
BPR Temperature	60 °C
BPR Pressure	200 bar
Total run time	7 minutes
Injection	5 µL
Feed speed	400 µL/min
Overfeed volume	4 µL
Needle wash	3 seconds methanol

SFC Method for the separation of the three structural isomers on Column 2

Parameter	Value
SFC Flow	2.5 mL/min
Modifier	Methanol + 0.1 % NH_3 aq.
Isocratic	11 % modifier
Column temperature	30 °C
BPR Temperature	60 °C
BPR Pressure	200 bar
Total run time	3.5 minutes
Injection	1 µL
Feed speed	400 µL/min
Overfeed volume	4 µL
Needle wash	3 seconds methanol

Instrumental setup

The recommended configuration of the Agilent 1260 Infinity II Analytical SFC System with Agilent LC/MS Systems was described previously[2].

Software

- Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, Rev. C.01.07 SR3
- MassHunter LC/TQ Acquisition Software, Version B.08.02
- MasHunter Optimizer Software, Version B.08.02
- MassHunter Source and iFunnel Optimizer Software, Version B.08.02
- MassHunter Quantitative Software, Version B.08.00
- MassHunter Qualitative Software, Version B.07.00 SP1

Columns

1. Chiral Technologies, CHIRALPAK AD-H, 250 × 4.6 mm, 5 µm
2. Chiral Technologies, CHIRALPAK AD-3, 150 × 4.6 mm, 3 µm

Chemicals

All solvents were purchased from Merck, Germany.

Samples

Separate stock solutions of 4-, 5-, and 6-EAPB (1 ppm in methanol) were used in dilution, as outlined in the text.

MS Method for triple quadrupole MS and single quadrupole MS

Parameter	Value
Make up composition	Methanol/water (95/5) + 0.2 % formic acid
Make up flow	0.4 mL/min
Electrospray Ionization with Agilent Jet Stream Ion Source	
Drying gas	150 °C, 11 L/min
Sheath gas	350 °C, 12 L/min
Nebulizer	45 psi
Capillary	2,500 V
Nozzle	0 V
iFunnel*	High-pressure RF: 90 Low-pressure RF: 70
Triple quadrupole parameters	
ESI Polarity	positive
Scan type	MRM
Transitions	2
Cycle time	502 ms
ΔEMV	+200 V
Single quadrupole parameters	
ESI Polarity	positive
Scan type	SIM (*m/z* 204.1)
Dwell time	590 ms
Fragmentor	70 V
Gain	1.0

* Only for triple quadrupole

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell (ms)	Fragmentor (V)	Collision energy (V)	Cell acc. voltage (V)
4/5/6-EAPB	204.1	159.1	250	380	12	1
4/5/6-EAPB	204.1	131.1	250	380	22	1

Results and Discussion

Separation of enantiomers, and quantitative determination by triple quadrupole MS

A method developed for the chiral separation of D- and L-amphetamine[3] was chosen as the starting point for developing a separation method for the six isomers of 4-, 5-, and 6-EAPB. This method immediately led to a promising separation of all six possible stereoisomers (Figure 2), with all six isomers being partially separated between 2.8 and 6.0 minutes. This separation was then further optimized due to the incomplete separation.

Method parameters for Figure 2

Parameter	Value
Column	Chiralpak AD-H, 4.6 × 250 mm, 5 µm
Column temperature	20 °C
Mobile phase	10 %B (EtOH + 0.1 % NH_3 aq.)
Flow rate	4 mL/min
Injection volume	5 µL
Sample	10 ppb each in MeOH

The ethanol content of modifier B was varied in the next step, which did not result in improved separation. Reducing the content of modifier B led to higher retention times with broader peaks and a lower resolution, especially of the later eluting peaks (data not shown). The effect of column temperature on the separation of the enantiomers was also examined (Figure 3).

Method parameters for Figure 3

Parameter	Value
Column	Chiralpak AD-H, 4.6 × 250 mm, 5 µm
Column temperature	20, 30, 40, and 45 °C
Mobile phase	10 %B (EtOH + 0.1 % NH_3 aq.)
Flow rate	4 mL/min
Injection volume	5 µL
Sample	10 ppb each in MeOH

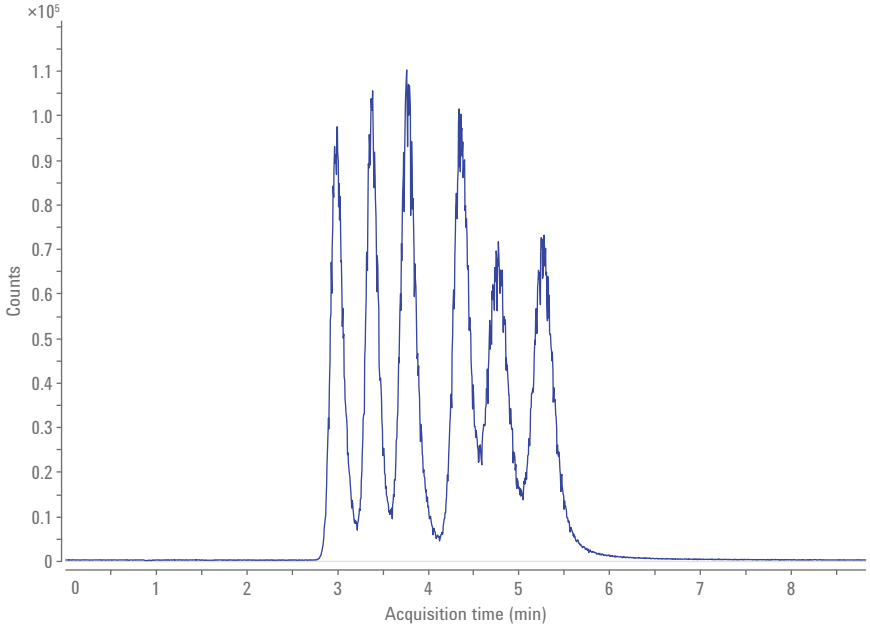


Figure 2. Initial method for the separation of all six isomers of 4-, 5- and 6-EAPB.

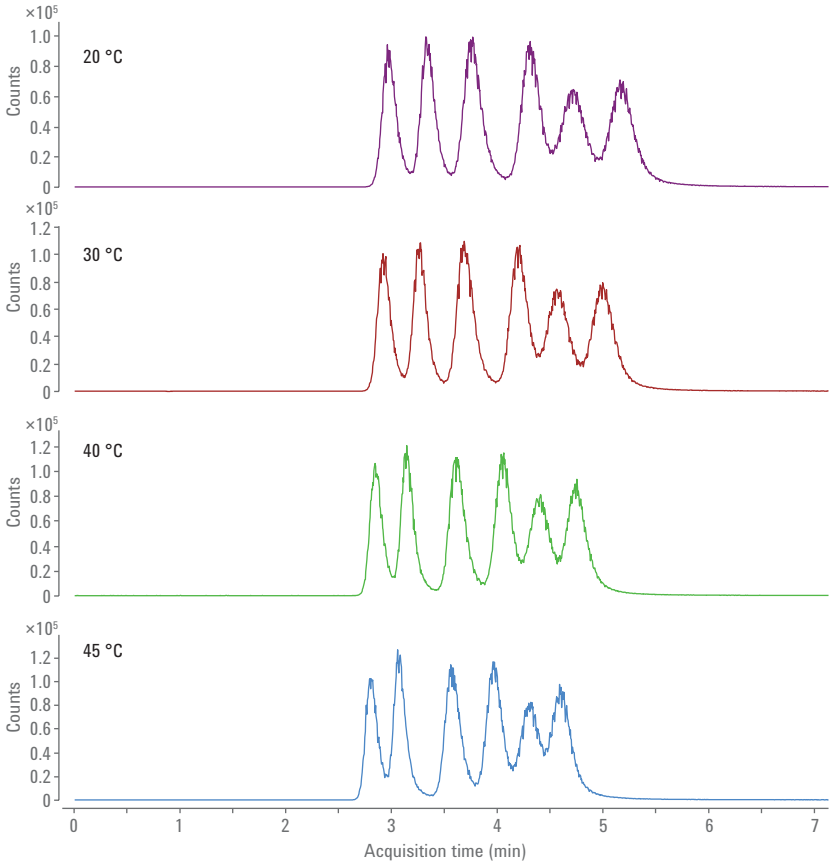


Figure 3. Separation of all six isomers of 4-, 5-, and 6-EAPB depending on the column temperature.

The highest resolutions were achieved at the lower temperatures of 20–30 °C, and the resolution decreased when using higher temperatures. Different solvents were also tested to improve the resolution between the six isomers. When methanol was used as a modifier instead of ethanol, the peaks eluted earlier, between 2.0 and 3.5 minutes, and with better peak shape. However, the peaks were still not completely resolved (Figure 4).

Method parameters for Figure 4

Parameter	Value
Column	Chiralpak AD-H, 4.6 × 250 mm, 5 µm
Column temperature	20 °C
Mobile phase	10 %B (MeOH + 0.1 % NH_3 aq.)
Flow rate	4 mL/min
Injection volume	5 µL
Sample	10 ppb each in MeOH

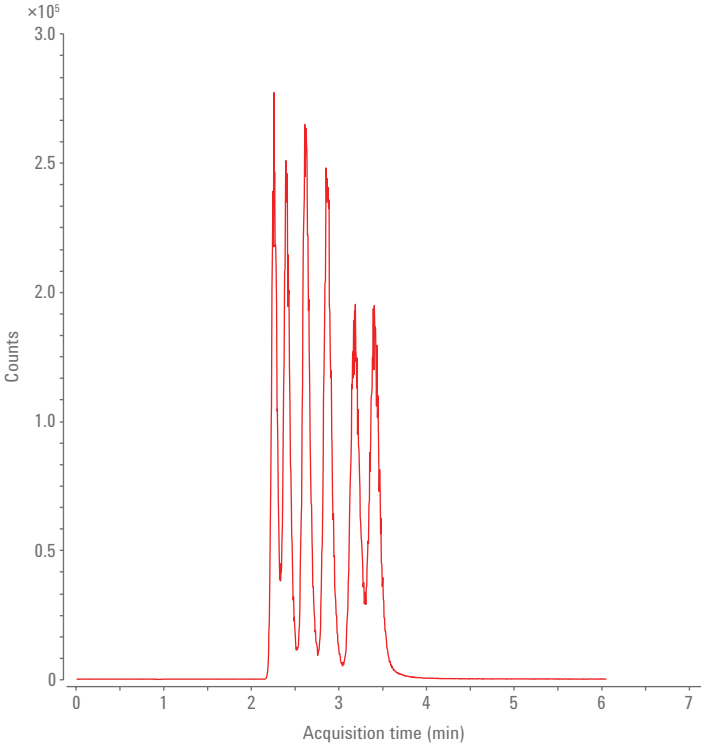


Figure 4. Method for the separation of all six isomers of 4-, 5-, and 6-EAPB with methanol as modifier.

Finally, it was found that 10 % methanol (with 0.1 % NH_3 aq.) as modifier gave the best separation at a flow rate of 3 mL/min (Figure 5).

Method parameters for Figure 5

Parameter	Value
Column	Chiralpak AD-H, 4.6 × 250 mm, 5 µm
Column temperature	20 °C
Mobile phase	10 %B (MeOH + 0.1 % NH_3 aq.)
Flow rate	3 mL/min
Injection volume	5 µL
Sample	10 ppb each in MeOH

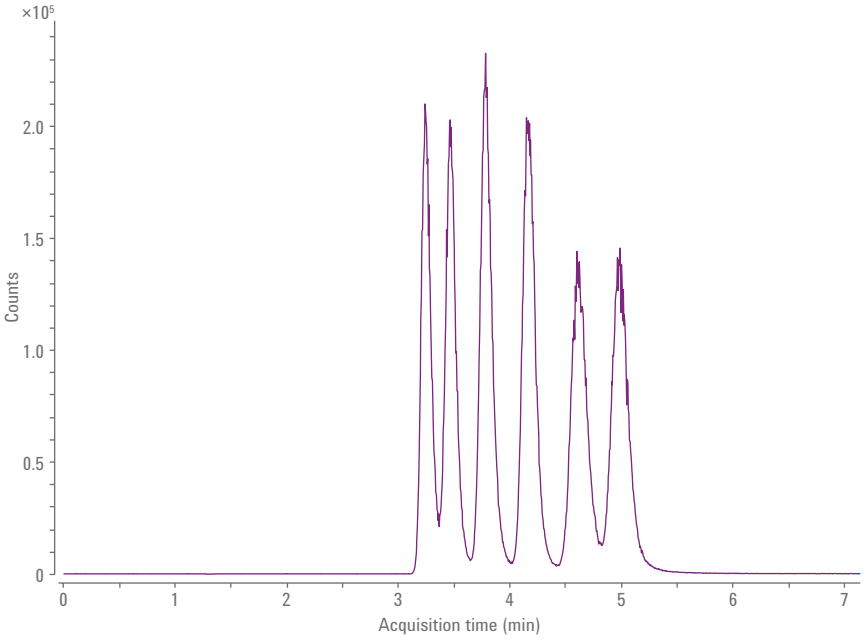


Figure 5. Optimized separation of all six isomers of 4-, 5-, and 6-EAPB.

The compounds eluted between 3.2 and 5.5 minutes. The compounds were identified by comparison to racemic single standards of 4-, 5-, and 6-EAPB (Figure 6). Under the optimized conditions:

- 4-EAPB elutes between 3.1 and 3.7 minutes
- 5-EAPB elutes between 3.6 and 4.6 minutes
- 6-EAPB elutes between 4.4 and 5.4 minutes

In particular, 5- and 6-EAPB were successfully separated by the developed method, which was the aim for the development of this method.

After optimization of the MS source and all related MS parameters, calibration curves were created for 4-, 5-, and 6-EAPB between 100 ppt and 100 ppb (Figure 7), with three replicates for each calibration level. The linearity, R^2, showed values of 0.9998, 0.9993, and 0.9990 for 4-, 5-, and 6-EAPB, respectively. The limit of quantification (LOQ) was determined at 100 ppt for a signal-to-noise ratio (S/N) of 10. The limit of detection (LOD) was determined at 30 ppt for an S/N of 3.

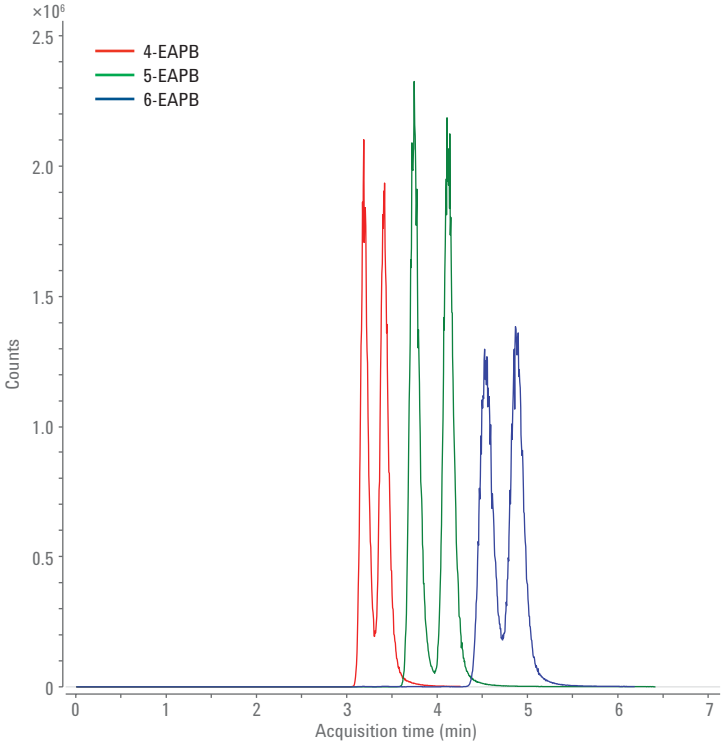


Figure 6. Identification of the enantiomeric compounds 4-, 5-, and 6-EAPB by injection of their racemic single standards.

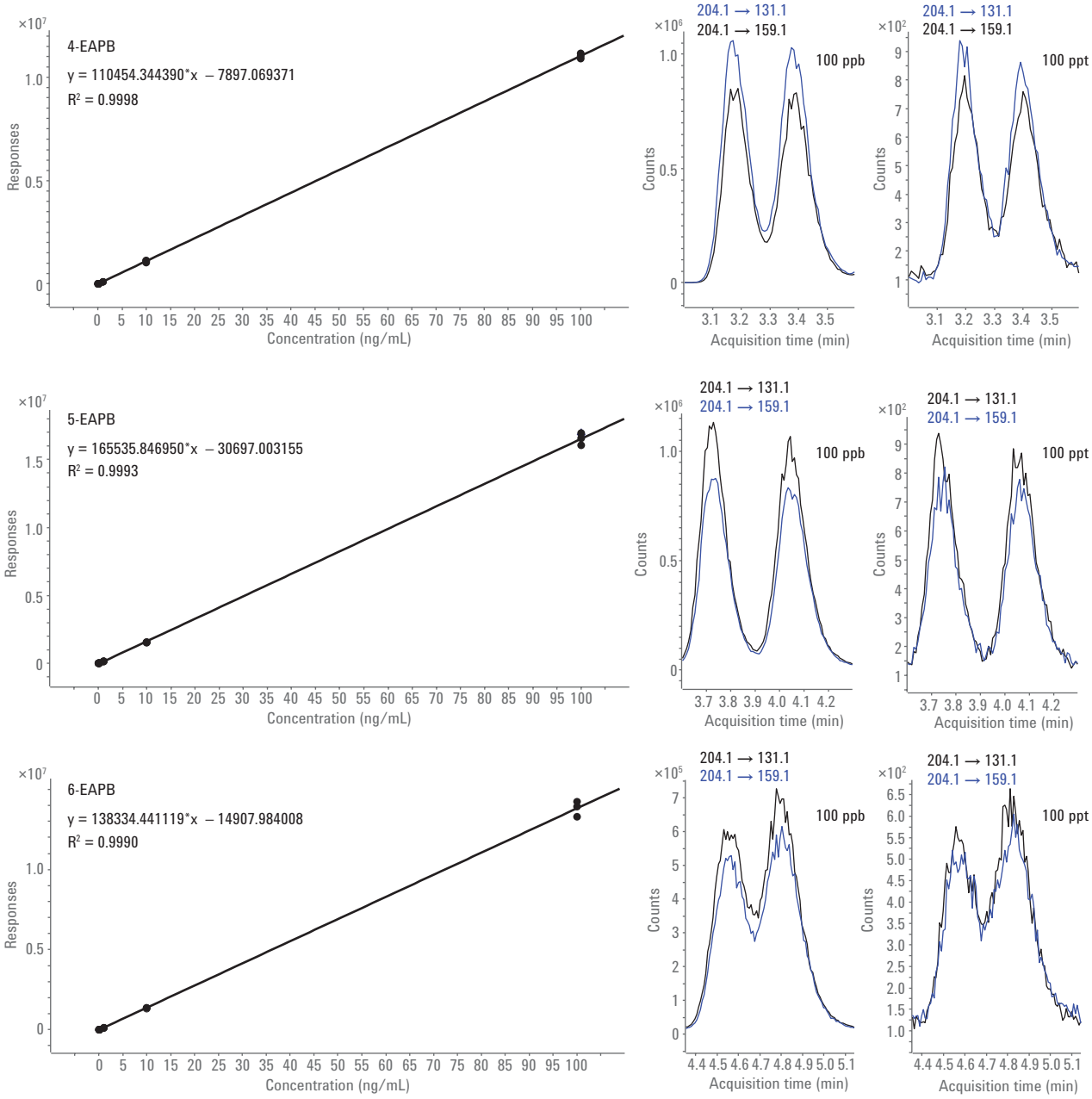


Figure 7. Calibration curves for 4-, 5-, and 6-EAPB between 100 ppt and 100 ppb. The quantifier and qualifier transition at 100 ppb and at 100 ppt (LOQ) are shown next to the individual calibration curves.

Separation of position isomers of 4-, 5-, and 6-EAPB and qualitative determination by single quadrupole MS

Another possibility for the determination of 4-, 5-, and 6-EAPB is the nonchiral separation of the structural isomers. Unfortunately, there was no existing separation method found that worked on an achiral stationary phase and separates all three structural isomers. Typically, the 4-EPAB is separated, but the other two isomers, 5- and 6-EPAB, coelute completely or elute with insufficient separation. To solve this problem, it has been attempted to separate only the isomers without separation of the enantiomers, but on a chiral stationary phase.

The initial experiment, which showed some separation of the three structural isomers without showing a separation of the enantiomers was performed on a CHIRALPAK AD-3 column with methanol as mobile phase (15 % with 0.1 % NH_3 aq.) (Figure 8). To improve the partial separation of the three isomers, the methanol content was decreased in steps of 1 %. A sufficient separation with acceptable peak width and run time for the three compounds was found for a modifier content of 11 % methanol (with 0.1 % NH_3 aq.) (Figure 9).

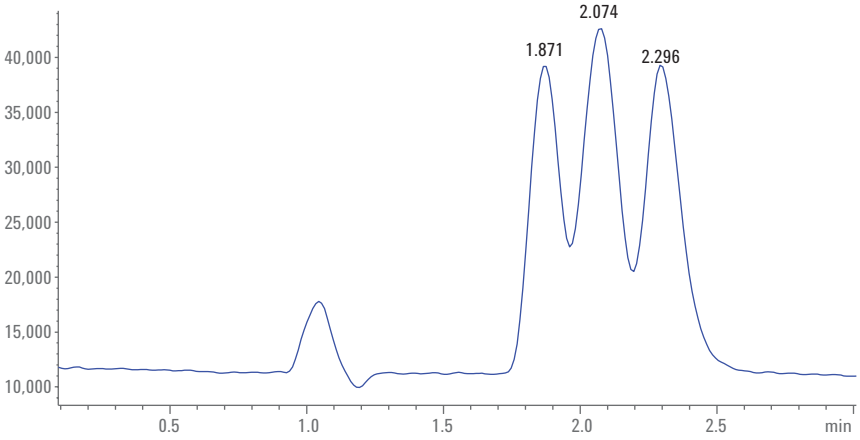


Figure 8. Separation of 4-, 5-, and 6-EAPB structural isomers with 15 % methanol as modifier.

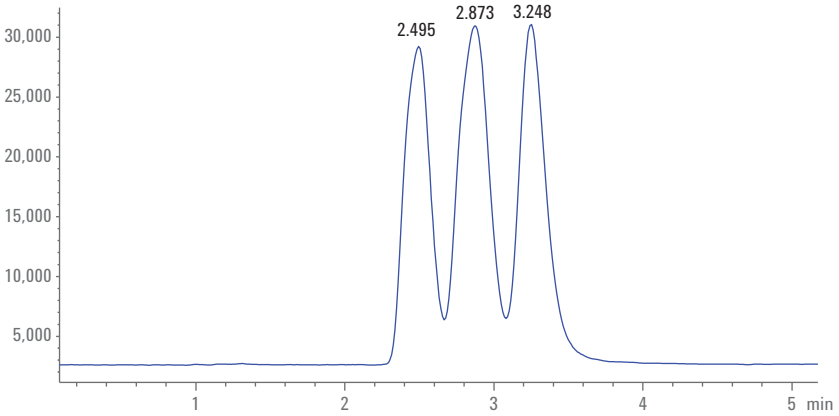


Figure 9. Separation of 4-, 5-, and 6-EAPB structural isomers with 11 % methanol as modifier.

Method parameters for Figure 8

Parameter	Value
Column	Chiralpak AD-3, 4.6 × 150 mm, 3 µm
Column temperature	30 °C
Mobile phase	15 %B (MeOH + 0.1 % NH_3 aq.)
Flow rate	2 mL/min
Injection volume	1 µL
Sample	100 ppb each in MeOH

Method parameters for Figure 9

Parameter	Value
Column	Chiralpak AD-3, 4.6 × 150 mm, 3 µm
Column temperature	30 °C
Mobile phase	11 %B (MeOH + 0.1 % NH_3 aq.)
Flow rate	2 mL/min
Injection volume	1 µL
Sample	100 ppb each in MeOH

The final method was achieved by increasing the flow rate to shorten the run time to 3.5 minutes (Figure 10). The identity of the compounds was confirmed by injection of the single standards of the three achiral isomers (Figure 11). The 4-EAPB elutes at 2.15 minutes, 5-EAPB at 2.50 minutes, and 6-EAPB at 2.83 minutes. This method could be used as quick qualitative detection of the EAPBs by a combination of SFC and a single quadrupole MS in SIM mode. For a proper quantification, the method shown for the combination of the SFC and a triple quadrupole with its additional selectivity can be used.

Method parameters for Figure 10

Parameter	Value
Column	Chiralpak AD-3, 4.6 × 150 mm, 3 µm
Column temperature	30 °C
Mobile Phase	11 %B (MeOH + 0.1 % NH_3 aq.)
Flow rate	2.5 mL/min
Injection volume	1 µL
Sample	100 ppb each in MeOH

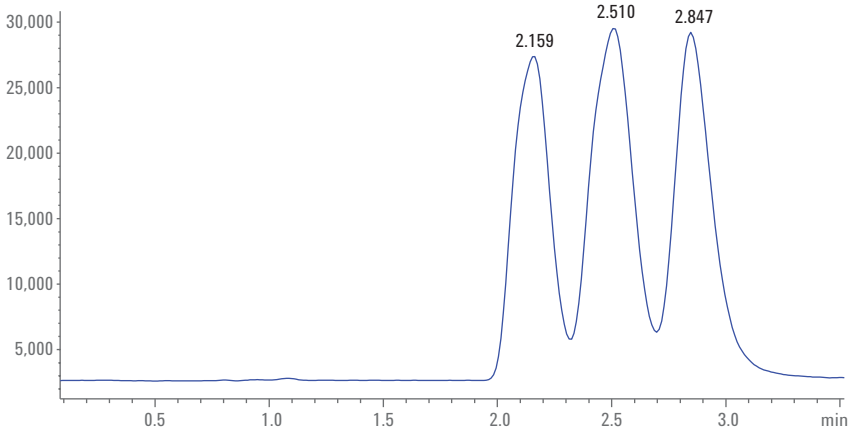


Figure 10. Final method for the separation of 4-, 5-, and 6-EAPB structural isomers.

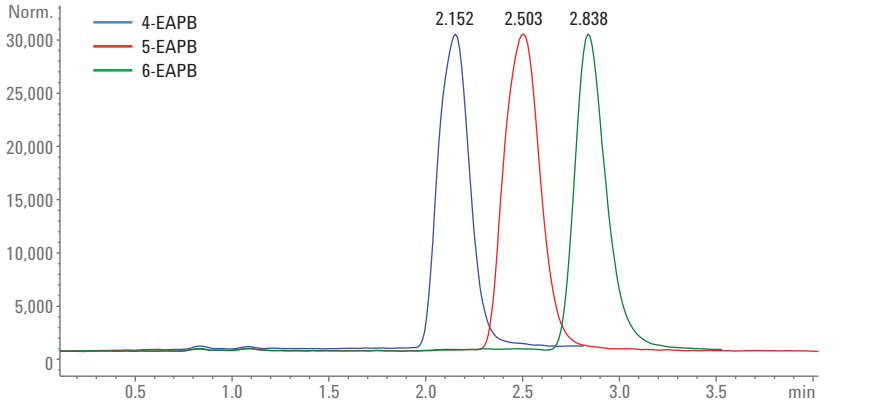


Figure 11. Overlay of the separation of individual samples of 4-, 5,- and 6-EAPB structural isomers with the final method.

Conclusion

This Application Note demonstrates the use of the Agilent 1260 Infinity II SFC for the separation of either all six possible isomers of 4-, 5-, and 6-EAPB, or the separation of only the three respective structural isomers. The detection and quantitative determination have been done by coupling the SFC either to a single quadrupole or a triple quadrupole MS. The calibration curves for quantitative analysis were performed on the triple quadruple MS and showed excellent linearity (R^2 >0.9990) and sensitivity. The LOQs were found at 100 ppt and the LODs at 30 ppt.

References

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3. M. Muelek, H. Godel, E. Naegele. Development of a Method for the Chiral Separation of D/L-Amphetamine. *Agilent Technologies Application Note*, publication number 5991-8262EN, **2017**.

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