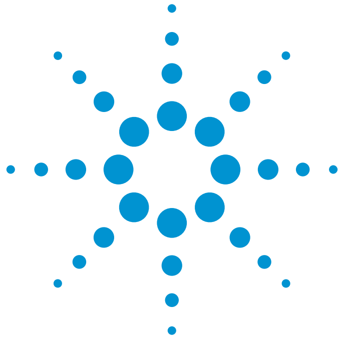

Development of a Method for the Chiral Separation of D/L-Amphetamine

A Quantitative Determination by SFC/MS in an Authentic Whole Blood Sample

Application Note

Forensic & Toxicology, Criminalistics

Authors

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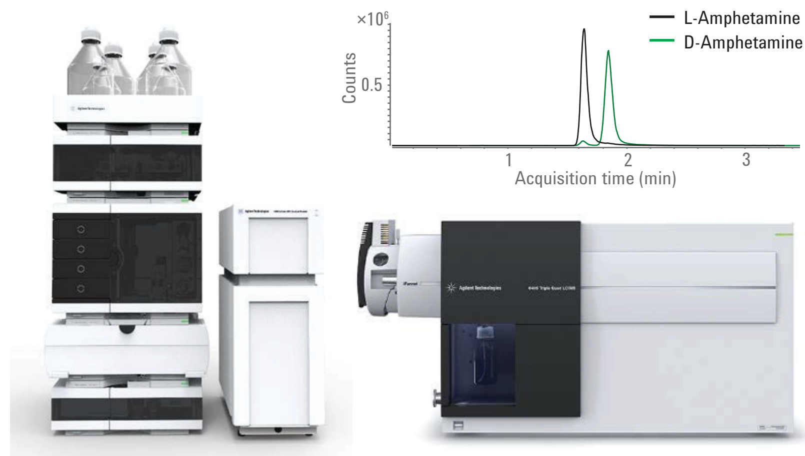
Abstract

This Application Note demonstrates method development for the chiral separation of D/L-amphetamine using an Agilent 1260 Infinity II SFC System. The Agilent 6495 triple quadrupole MS demonstrates the quantification of these two chiral enantiomeric forms. The method development process is described, and the final analytical method was used for the determination of calibration curves and the limit of quantification using a triple quadrupole MS. The analysis of a processed authentic whole blood sample is shown.



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Introduction

The compound D/L-amphetamine occurs in two chiral enantiomeric forms (Figure 1). The D-amphetamine isomer is the more active, and pharmaceutically produced in enantiomeric pure form[1].

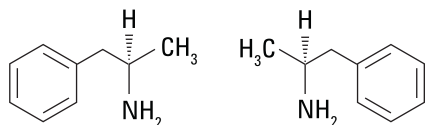

Figure 1. Formula of D-and L-amphetamine.

In forensic toxicology, amphetamine can be qualitatively and quantitatively determined in bodily fluids by chromatographic methods such as GC and HPLC coupled to mass spectrometry[2].

This Application Note demonstrates the development of a fast analytical SFC/MS method for the separation of D- and L-amphetamine and its quantitative determination using a triple quadrupole mass spectrometer. This analytical method can distinguish between the quantitative amount of D-amphetamine from medical use, and the amount of D/L-amphetamine from illegal sources. Finally, this method was verified for use in forensic toxicology by the analysis of an authentic extracted whole blood sample.

Experimental

Instruments

An Agilent 1260 Infinity II SFC/MS System comprising:

- 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) (G7116B) with four-column selection valve
- 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

Instrumental setup

The recommended configuration of the Agilent 1260 Infinity II SFC System with Agilent LC/MS Systems was described earlier[3].

Software

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

Columns

- Chiral Technologies, CHIRALPAK AD-H 150 × 4.6 mm, 5 µm
- Chiral Technologies, CHIRALPAK AD-H 250 × 4.6 mm, 5 µm
- Chiral Technologies, CHIRALPAK IA 250 × 4.6 mm, 5 µm
- Chiral Technologies, CHIRALPAK IC 250 × 4.6 mm, 5 µm
- Chiral Technologies, CHIRALPAK ID 250 × 4.6 mm, 5 µm

Chemicals

All solvents were purchased from Merck, Germany.

Samples

Solutions of D-amphetamine, L-amphetamine, and D/L-amphetamine were prepared in methanol according to the related concentrations of the described calibration curve from individual stock solutions (stock solution: 1 ppm in methanol).

A processed, authentic whole blood sample was provided (see Acknowledgments).

Sample preparation

The authentic whole blood sample was processed by protein precipitation with acetonitrile and diluted 1:1,000/1:100/1:10 with mobile phase B (ethanol + 0.1 % aq. NH_3) before analysis.

Final SFC method

Parameter	Value
SFC flow	4 mL/min
Modifier	Ethanol + 0.1 % NH_3(aq)
Isocratic	10 % modifier
Column temperature	20 °C
BPR temperature	60 °C
BPR pressure	200 bar
Total run time	3 minutes
Injection	5 µL
Feed speed	400 µL/min
Overfeed volume	4 µL
Needle wash	3 seconds methanol

MS triple quadrupole method

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	170 °C, 16 L/min
Sheath gas	300 °C, 9 L/min
Nebulizer	60 psi
Capillary	2,500 V
Nozzle	500 V
iFunnel	High-pressure RF: 80, low-pressure RF: 60
MS parameters	
ESI polarity	Positive
Scan type	MRM
Transitions	2
Cycle time	502 ms
ΔEMV	+200 V

Compound name	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell (ms)	Fragmentor (V)	Collision energy (V)	Cell accelerator voltage (V)
D/L-Amphetamine	136.1	119.1	250	380	7	1
D/L-Amphetamine	136.1	91.1	250	380	17	1

Results and Discussion

A racemic amphetamine standard solution (100 ppb) was screened against four different chiral stationary phase columns and two organic modifiers (see Experimental, columns 2 to 5). Because amphetamine is a basic compound, a basic additive, 0.1 % aqueous ammonia, was added to methanol and ethanol, which were used as the CO_2 modifier. In the initial method development steps, different isocratic separations were carried out on all the columns. The experiments resulted in an initial separation on column 2 (Figure 2). The separation of both enantiomers became, in tendency, better with decreasing amount of modifier. Baseline separation under chosen conditions was not possible with methanol.

To achieve a better separation of both amphetamines, ethanol as a solvent of weaker elution strength, was tested with column 2 (Figure 3). A clear separation of both enantiomers was obtained for modifier concentrations below 10 %B. The enantiomers eluted between 3.5 and 4.5 minutes at 10 %B, and between 7 and 9 minutes for a modifier concentration of 6 %B.

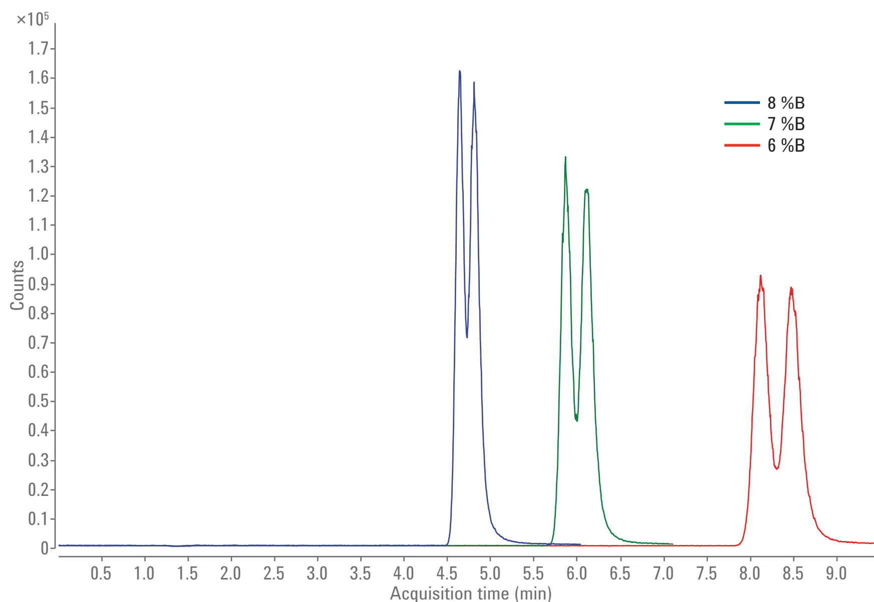

Figure 2. Chiral separation of D/L-amphetamine enantiomers (100 ppb) using different modifier content (modifier B: MeOH+ 0.1 % NH_3(aq), flow rate: 3 mL/min, column temperature: 20 °C, column: CHIRALPAK AD-H 4.6 × 250 mm; 5 µm).

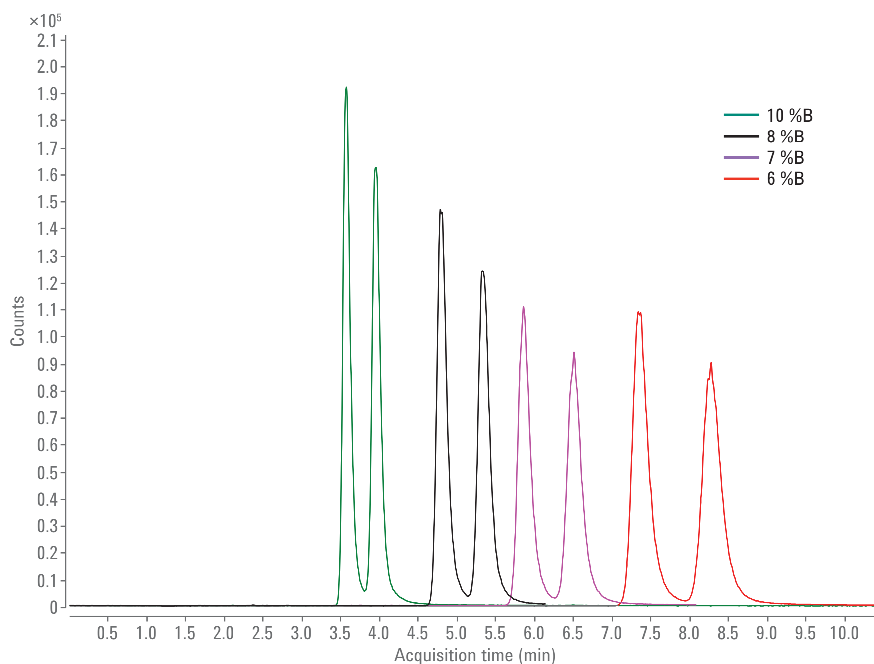

Figure 3. Chiral separation of D/L-amphetamine enantiomers (100 ppb) using different modifier content (modifier B: EtOH+ 0.1 % NH_3(aq), flow rate: 3 mL/min, column temperature: 20 °C, column: CHIRALPAK AD-H 4.6 × 250 mm; 5 µm).

In the final step, the method run time was optimized by increasing the flow rate from 3 to 4 mL/min. The increased flow rate resulted in elution of the enantiomers between 2.5 and 3.2 minutes (Figure 4). To further shorten the run time, a shorter column (column 1) consisting of the same stationary phase, inner diameter, and particle size was used (Figure 5). The change from a 250-mm column to a shorter 150-mm column led to an earlier elution of between 1.5 and 2.1 minutes for both enantiomers. Furthermore, different temperatures (40, 30, and 20 °C) were tested, and a column temperature of 20 °C achieved the highest resolution (data not shown).

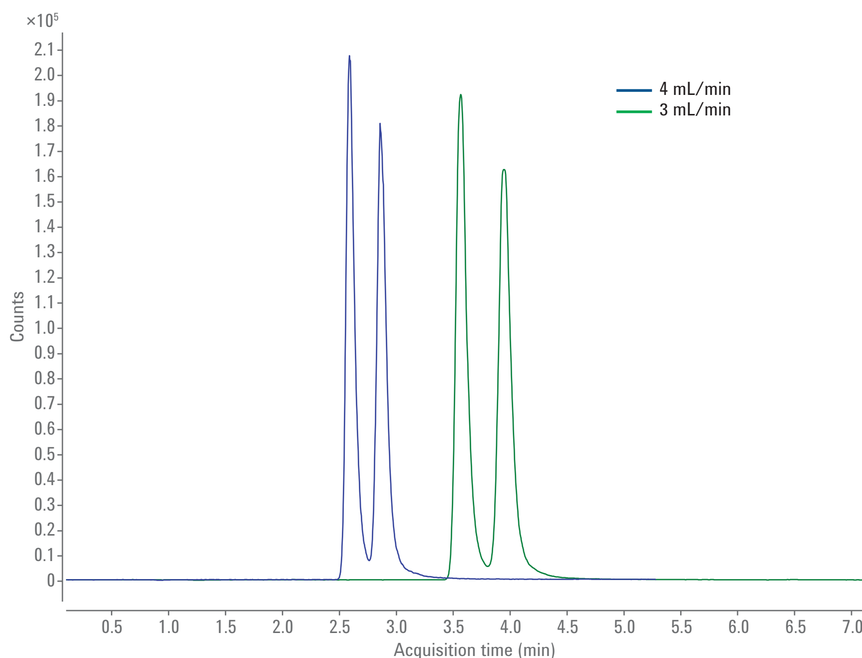

Figure 4. Chiral separation of D/L-amphetamine enantiomers (100 ppb) using different flow rates (modifier B: 10 % EtOH+ 0.1 % NH_3(aq), flow rate: 3 and 4 mL/min, column temperature: 20 °C, column: CHIRALPAK AD-H 4.6 × 250 mm; 5 µm).

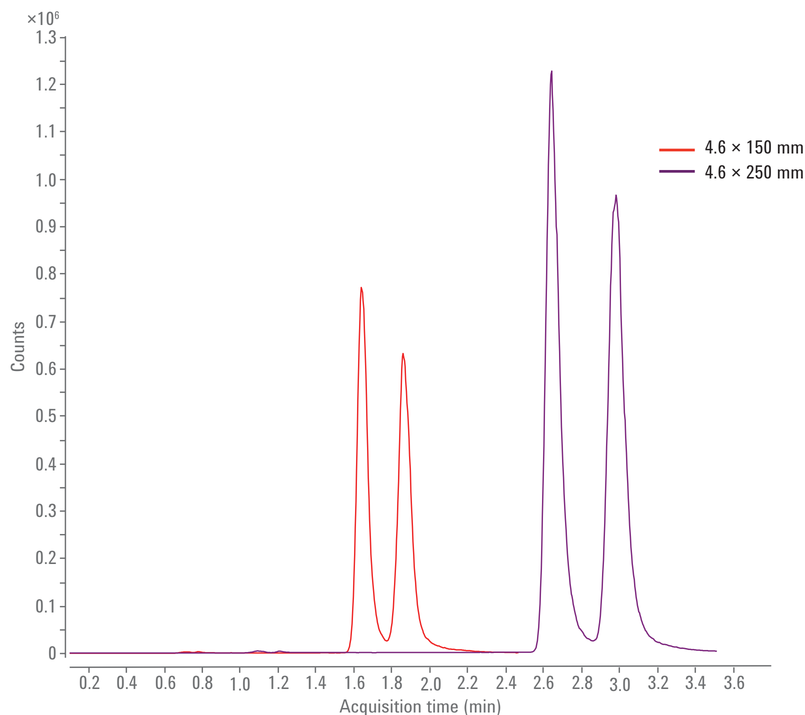

Figure 5. Chiral separation of D/L-amphetamine enantiomers (100 ppb) using different column sizes (modifier B: 10 % EtOH+ 0.1 % NH_3(aq), flow rate: 4 mL/min, column temperature: 20 °C, column: CHIRALPAK AD-H 4.6 × 250 mm and 4.6 × 150 mm, 5 µm).

Finally, the transfer to the source of the mass spectrometer includes the addition of make-up solvent for proper ionization, which was optimized to gain the maximum sensitivity. For that purpose, different backpressure settings were tested, because with the splitter setup the backpressure regulates the amount of column effluent that is transferred to the ionization source (Figure 6). For the final method, a backpressure of 200 bar was applied. The influence of the flow rate of the added make up solvent (methanol/water (95/5) + 0.2 % formic acid) was also examined, but since there was virtually no influence on sensitivity, it was kept at 0.4 mL/min. All source parameters of the MS were fully optimized to obtain the highest sensitivity (see Experimental).

For a final confirmation of the SFC/MS method, commercially available separate D- and L-amphetamine standards in enantiomeric pure form were analyzed. Single peaks resulted for the respective enantiomers: the L-amphetamine eluted at 1.632 minutes and the D-amphetamine at 1.860 minutes (Figure 7).

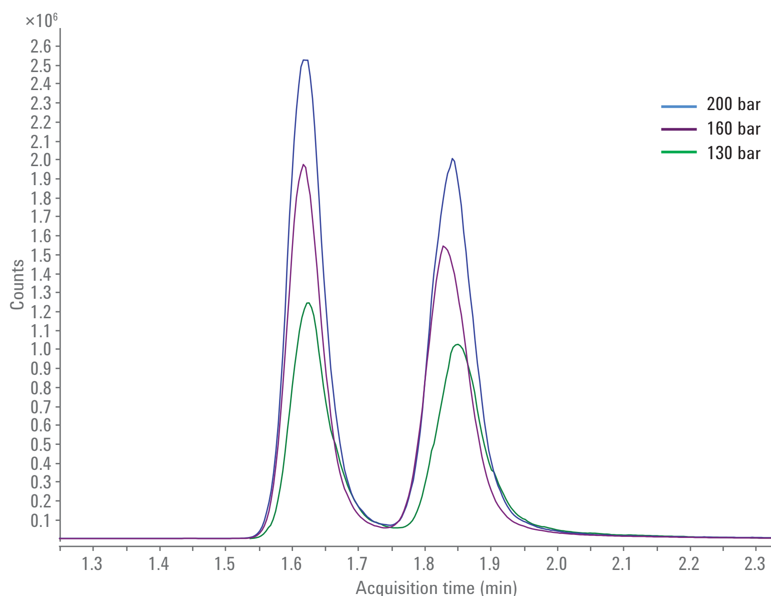

Figure 6. Optimization of SFC backpressure settings to maximize MS sensitivity.

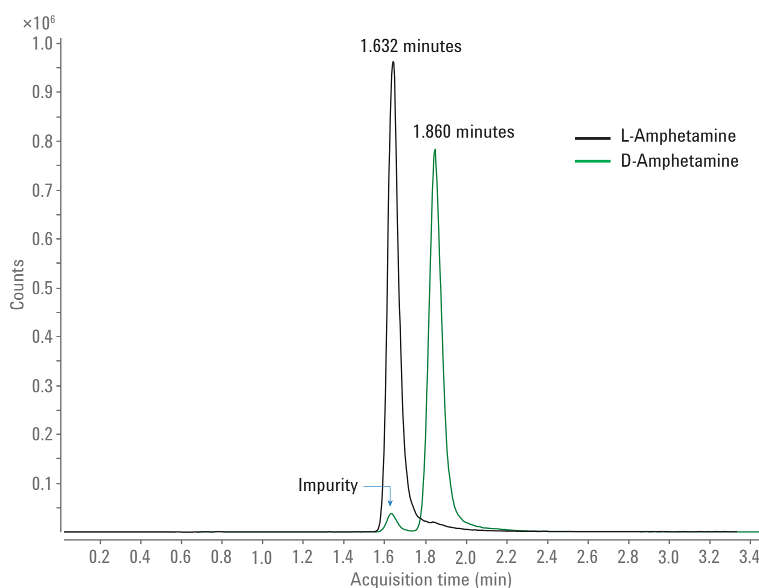

Figure 7. Chiral separation of commercially available enantiomerically pure standards of D-amphetamine and L-amphetamine (100 ppb) with the developed chiral method. The arrow indicates an impurity of the L-enantiomer in the standard of the D-amphetamine.

For the quantitative determination of D- and L-amphetamine, individual calibration curves were created between 100 ppt and 100 ppb, which showed excellent linearity (Figure 8). The limits of quantitation (LOQs) were determined to be at 100 ppt at a signal-to-noise ratio (S/N) of 10 and the limits of detection (LODs) were at 40 ppt (S/N = 3). The linearity coefficients were 0.9998 and 0.9996 for L- and D-amphetamine, respectively.

To demonstrate the capability of the developed SFC/MS method, a real whole blood sample, which was prepared as described in the Experimental section, was measured using the developed method. The blood sample was diluted 1/10, 1/100, and 1/1,000 with modifier B (Figure 9 and Table 1). Table 1 gives a detailed view of the measured areas and peak heights of D- and L-amphetamine in the sample, and demonstrates that it is present *in vivo* in its racemic form, which came originally from a nonpharmaceutical drug. Figure 9 shows the corresponding chromatograms of the 1:1,000 and the 1:10 dilutions.

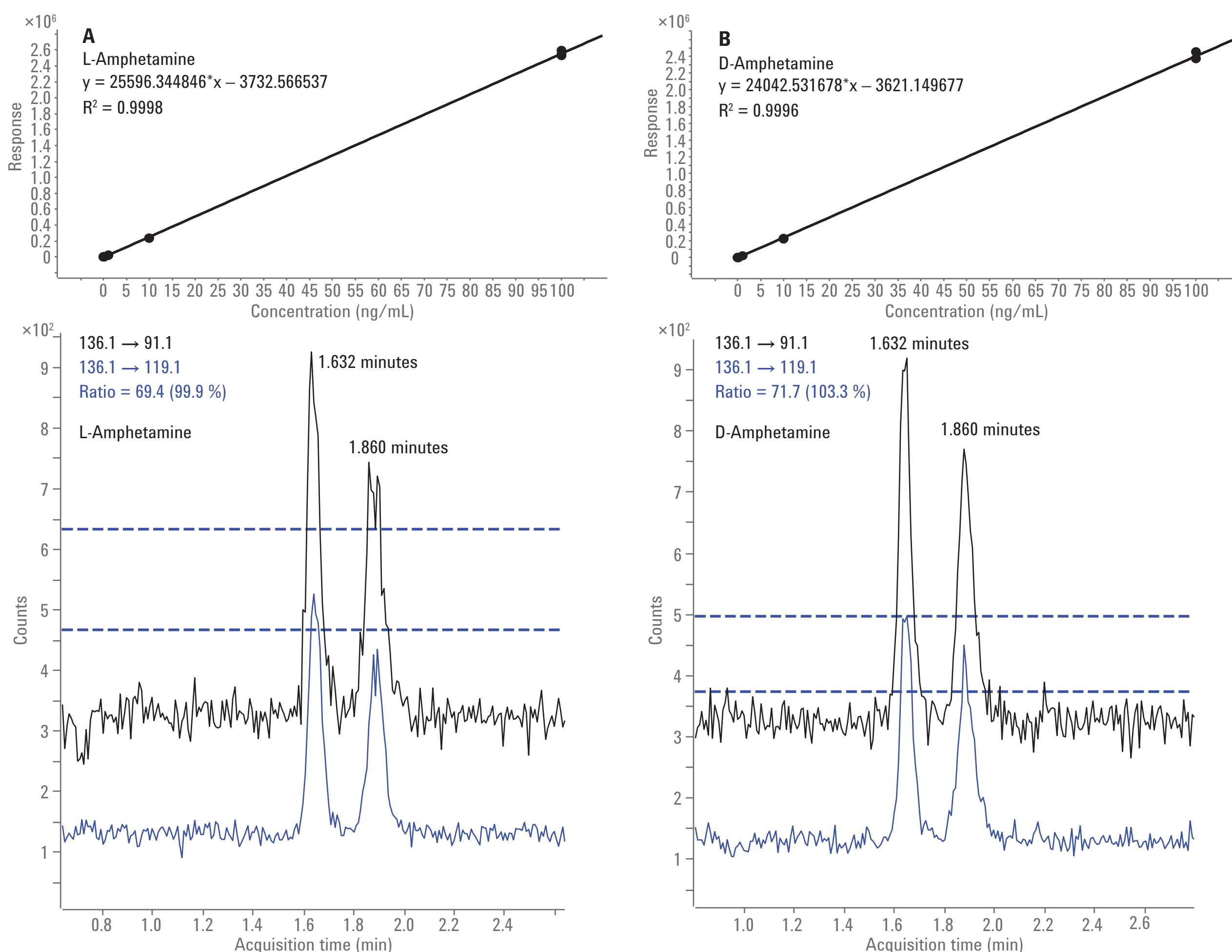

Figure 8. Calibration curves and qualifier/quantifier signal at 100 ppt for L-amphetamine, retention time 1.632 minutes (A) and D-amphetamine, retention time 1.860 minutes (B). The achieved resolution between D-and L-amphetamine was >1, and the total run time was 3 minutes.

Conclusion

This Application Note demonstrates the development of a method for the fast separation of the enantiomers of D/L-amphetamine using the Agilent 1260 Infinity II SFC and a highly sensitive quantitative determination using an Agilent 6495 triple quadrupole mass spectrometer. The SFC separation was possible within a total run time of 3 minutes, showing fast analysis time. The quantitative determination was performed with LOQs below 100 ppt. Finally, a reliable determination of amphetamine in a prepared whole blood sample was shown successfully.

Acknowledgements

Thanks to Martin Josefsson and Markus Roman from the National Board of Forensic Medicine, Linkoping, Sweden for providing the processed authentic whole blood sample.

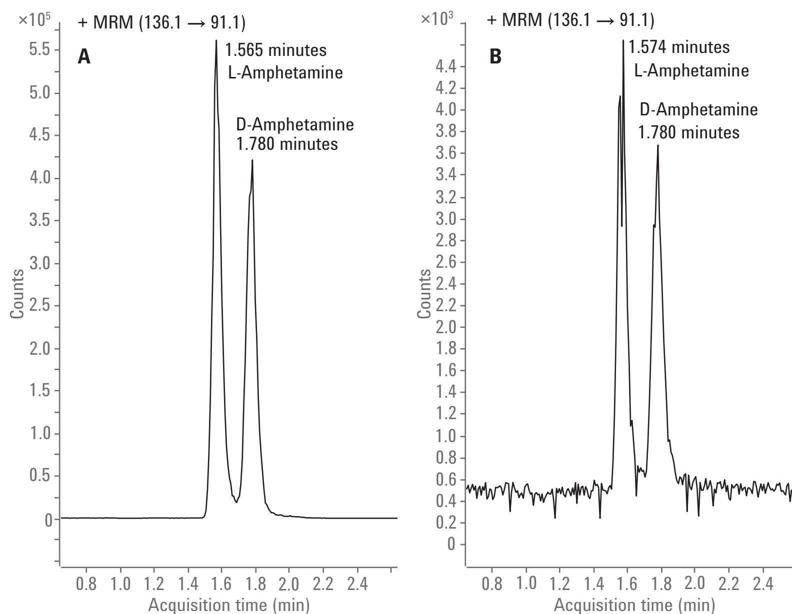

Figure 9. D/L-Amphetamine in a processed real whole blood sample in different dilutions: 1:10 (A) and 1:1,000 (B) with modifier.

Table 1. Detailed results of the measurement of the prepared real whole blood sample for D-and L-amphetamine diluted 1:10, 1:100, and 1:1,000 with the modifier.

	L-Amphetamine results			D-Amphetamine results		
Name	RT	Area	Height	RT	Area	Height
Case 2 Dil 1_1000	1.574	14,605.41	4,129.08	1.780	14,342.73	3,159.12
Case 2 Dil 1_100	1.574	178,409.64	48,475.84	1.780	165,744.26	34,952.28
Case 2 Dil 1_100	1.574	165,569.54	45,037.95	1.780	154,606.59	35,124.32
Case 2 Dil 1_100	1.565	156,671.29	44,736.99	1.780	139,086.04	32,365.24
Case 2 Dil 1_10	1.565	2,073,946.07	561,571.92	1.780	1,780,961.78	417,402.00

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Published in the USA, October 15, 2017
5991-8262EN

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