APPLICATION NOTE

YMC EUROPE GMBH

LC-MS compatible Separation of the Fungicide Spiroxamine

Spiroxamine is a systemic fungicide, which was brought to the market by Bayer CropScience. The substance is a mixture of diastereomers A and B again consisting of 4 enantiomers A1, A2, B1 and B2 (fig. 1).

Chiral Method for LC-MS

Bayer CropScience was looking for a new state-of-the-art LC-based chiral separation for spiroxamine that can be coupled to LC-MS/MS in the background of analyses of residues of spiroxamine at trace concentrations. Therefore, this meant a preference for reversed phase (RP) conditions should be investigated. Further, the aim was to perform the separation of the four isomers in less than 30 minutes. It was intended to first develop a separation application using LC-UV and then to transfer the application to LC-MS/MS.

YMC accepted the challenge and developed a new chiral application on a CHIRAL ART Amylose-SA column using water, ethanol and diethylamine.

LC-MS compatible Separation of the Fungicide Spiroxamine

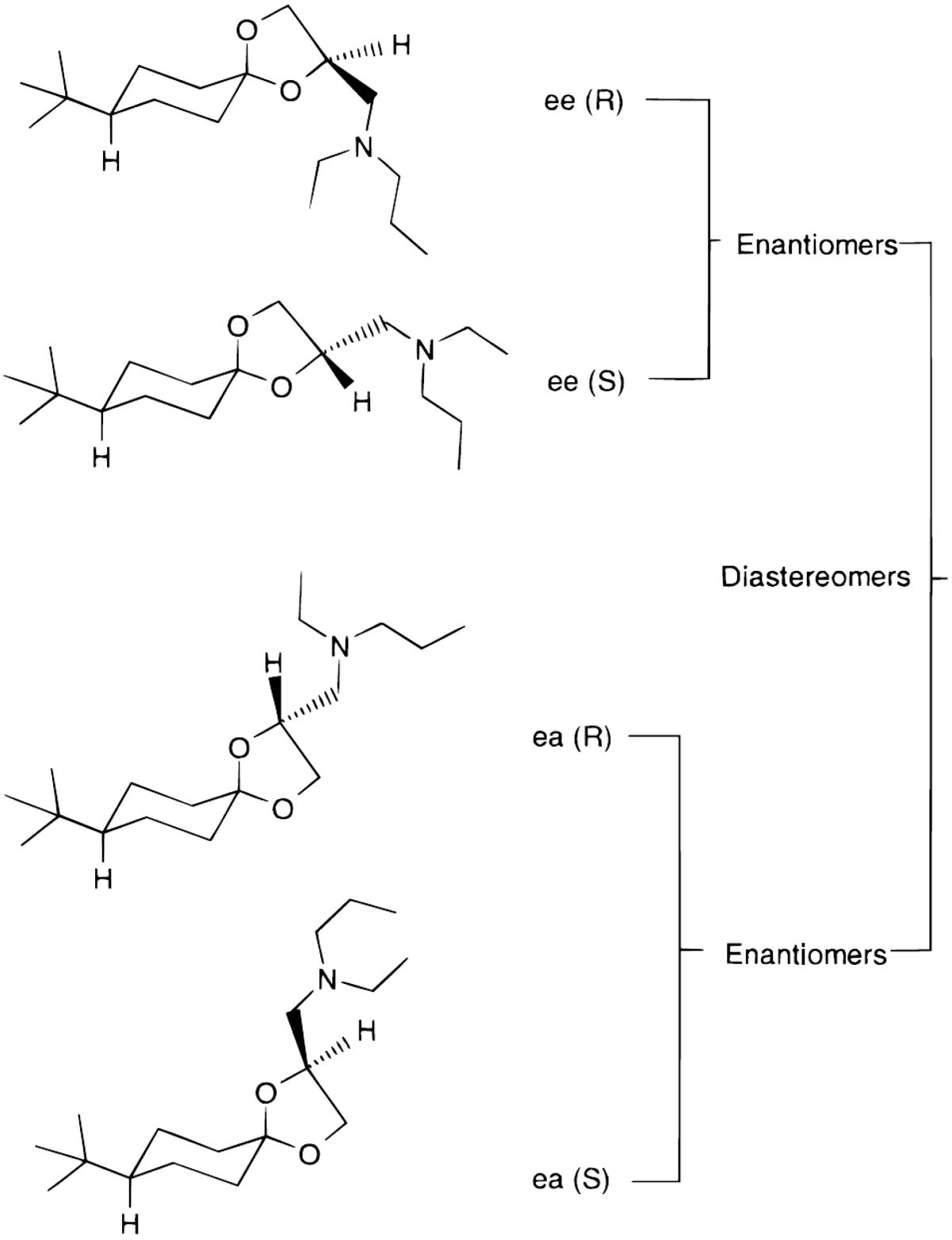

Fig. 1: Isomers of spiroxamine [1], diastereomer A: log P_{ow} = 2.79 (at 20 °C), diastereomer B: log P_{ow} = 2.92 (at 20 °C), pK_a value = 6.9.

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Existing Chiral Normal Phase Separation

Bayer CropScience had already developed a chiral method to separate these enantiomers and diasteromers respectively from each other [1]. However, at the time of the development only coated polysaccharide phases were available, which are only suitable for normal phase (NP) conditions. The resulting method was performed on a Chiralcel® OD-H column with n-heptane and isopropanol as eluents.

In a first step YMC was able to reproduce this separation on a corresponding CHIRAL ART Cellulose-C column using the same chiral selector (fig. 2).

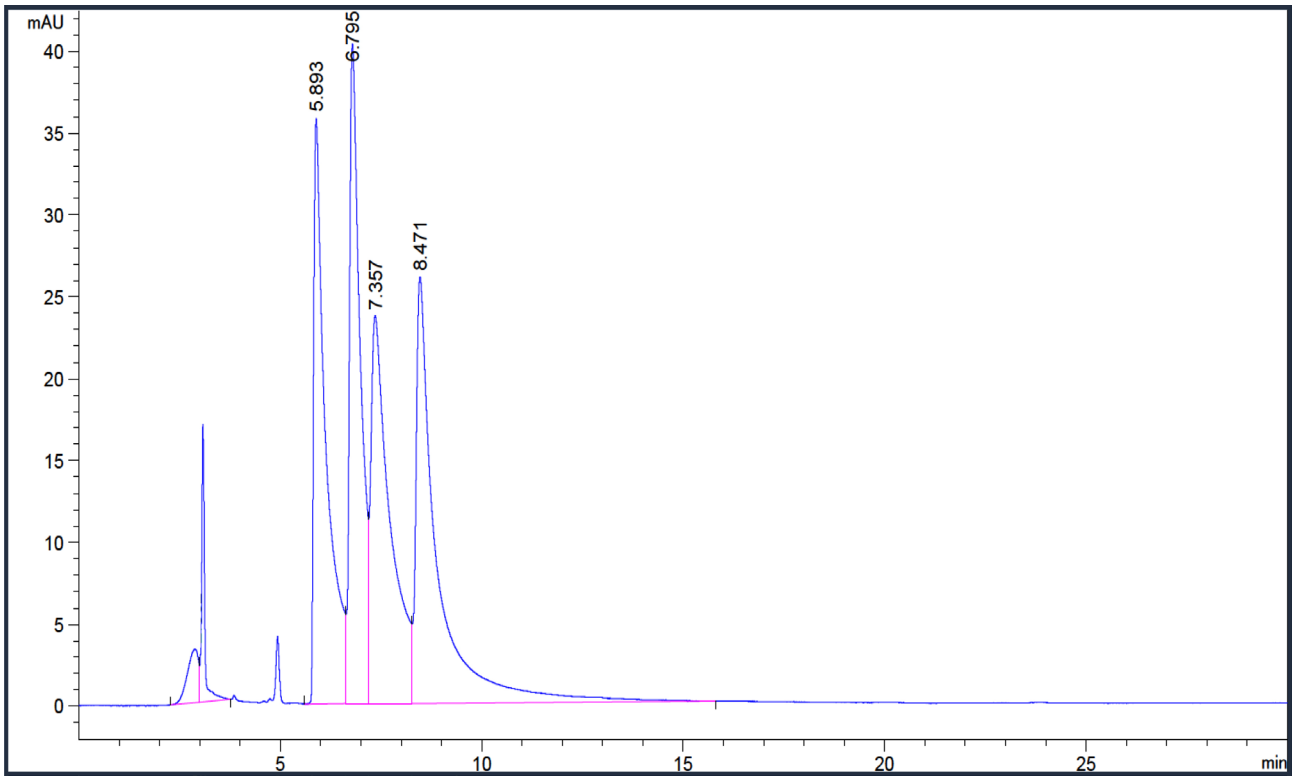

*Fig. 2: Normal phase separation on **CHIRAL ART Cellulose-C** using the existing published method.*

Column:	CHIRAL ART Cellulose-C 5 µm (250 x 4.6 mm ID)
Part No.:	KCN99S05-2546WT
Eluent:	*n*-heptane/isopropanol (99.9/0.1)
Flow rate:	1.0 ml/min
Temperature:	25 °C
Detection:	UV at 210 nm
Injection:	20 µL (0.1%)

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Chiral RP Screening

To overcome the requirement of RP conditions, YMC tested all immobilised YMC chiral phases, namely CHIRAL ART Amylose-SA, Cellulose-SB and Cellulose-SC, with RP eluents. A sufficient separation could only be obtained on a CHIRAL ART Amylose-SA column (fig. 3). A particle size of 5 µm and a dimension of 250 x 4.6 mm were used for each column.

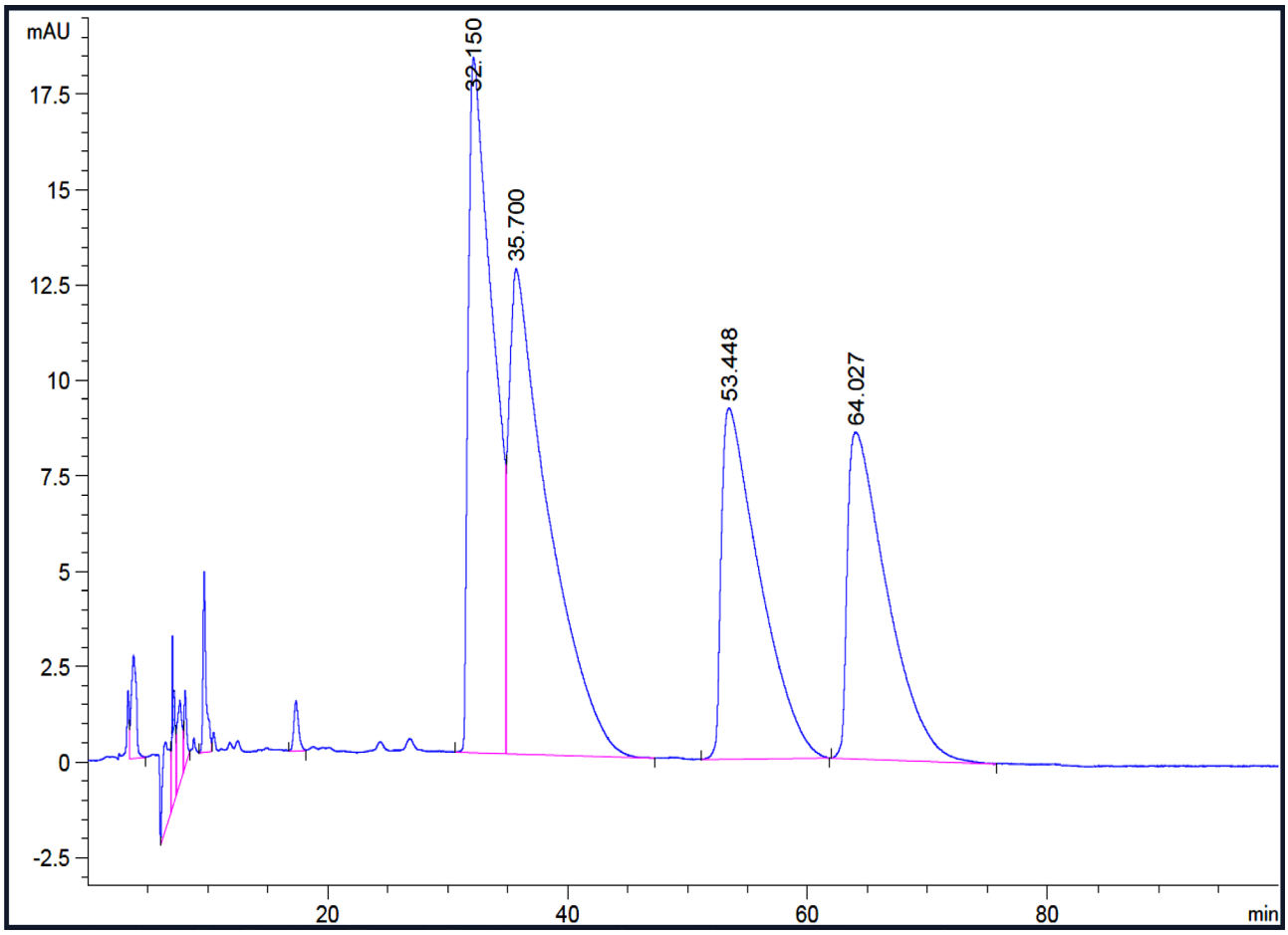

*Fig. 3: Normal phase separation on **CHIRAL ART Cellulose-C** using the existing published method.*

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Separation in less than 20 Minutes

In order to achieve the aim of a separation in less than 30 minutes, the column length and ID were reduced. In addition, 3 µm particles were used instead of 5 µm to increase the resolution.

The ionisation in MS detection can be improved by using an additive, but the addition of acetic or formic acid was found not useful as no retention could be observed. However, the addition of diethylamine resulted in an even better peak shape.

It was not only possible to separate all 4 of the isomers from each other, but also the separation time could be reduced to just 20 minutes. Therefore, all requirements could be fulfilled.

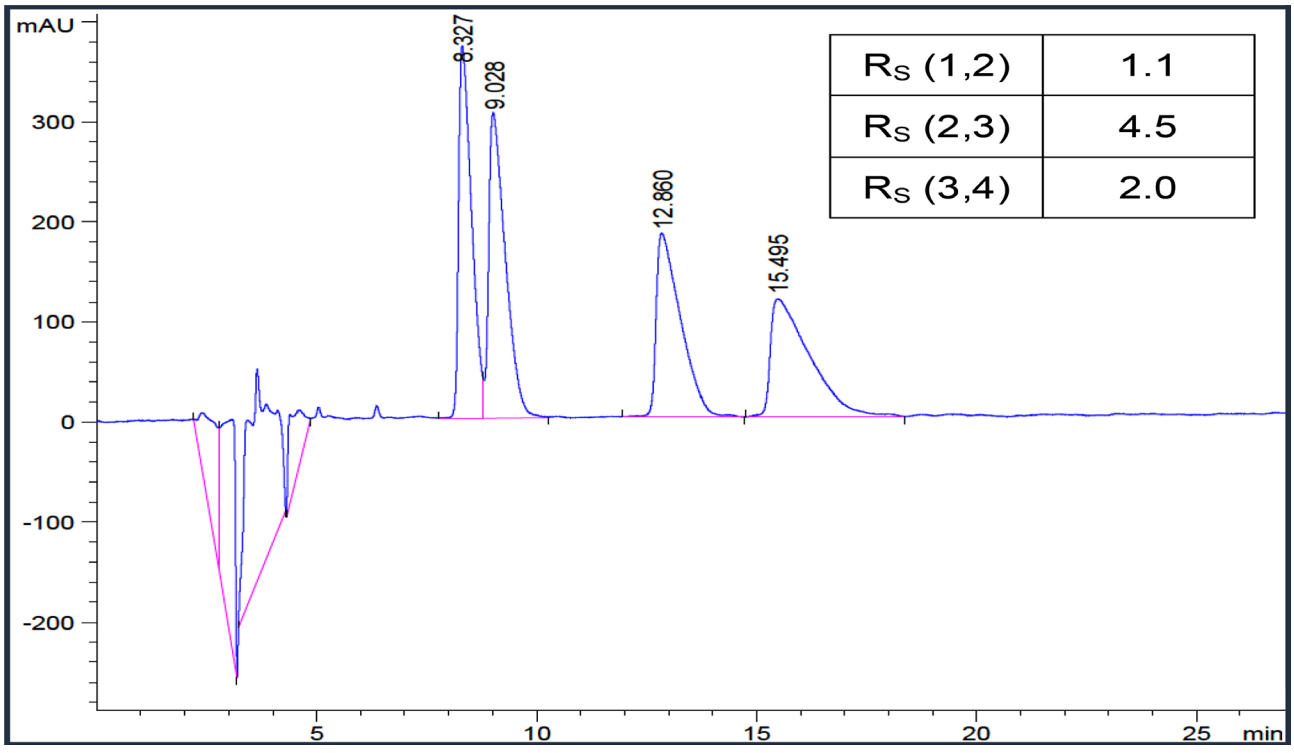

*Fig. 4: Optimised method for spiroxamine on **CHIRAL ART Amylose-SA**, 3 µm.*

Column:	CHIRAL ART Amylose-SA 3 µm (150 x 3.0 mm ID)
Part No.:	KSA99S03-1503WT
Eluent:	water/ethanol/DEA (27.5/72.5/0.1)
Flow rate:	0.25 ml/min
Temperature:	30 °C
Detection:	UV at 210 nm
Injection:	10 µL (10 mg/mL)

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Transfer from LC-UV to LC-MS/MS

At Bayer CropScience the application provided by YMC was set up on a LC-MS/MS instrument consisting of an Agilent 1290 UPLC system (binary and isocratic pump), a CTC autoinjector (Axel Semrau) and a Sciex API6500 high-end triple-quadrupole mass spectrometer.

The diethylamine used in the YMC UV-application had to be substituted by a 10 mM ammonium carbonate solution (pH 9.5). The diethylamine strongly influenced the ionisation process and “quenched” the MS-signal to about 99%.

To improve the ionisation 1% formic acid in methanol/water 50/50 was introduced post-column into the eluent flow coming from the chiral column (“change” of pH value from weak alkaline to weak acidic protonating spiroxamine; pK_a 6.9).

Final Set-up

Final conc. at LOQ:	1 µg/L spiroxamine	= 0.54 µg/L A-Isomer; 0.46 µg/L B-Isomer
		= 0.27 µg/L A1 enantiomer
		= 0.27 µg/L A2 enantiomer
		= 0.23 µg/L B1 enantiomer
		= 0.23 µg/L B2 enantiomer

HPLC Column: CHIRAL ART Amylose-SA 3 µm (150 x 3.0 mm ID)

Eluent:	A/B (25/75)
	A: Water/Ethanol 9/1 + 10 mM ammonium carbonate (pH ~9.5)
	B: Water/Ethanol 1/9 + 10 mM ammonium carbonate
Flow:	0.3 mL/min
Inj. Volume:	1 µL

Iso-pump: post-column make-up via T-peace with 0.3 mL/min 1% formic acid in water/methanol 50/50.

MS-MS conditions: Multiple-reaction-monitoring (MRM) mode in ESI positive, MRM 298-144 for quantitation and MRM 298-100 for confirmation.

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Example Chromatogram with Enantiomer A1 at LOQ in Grape Matrix Extract

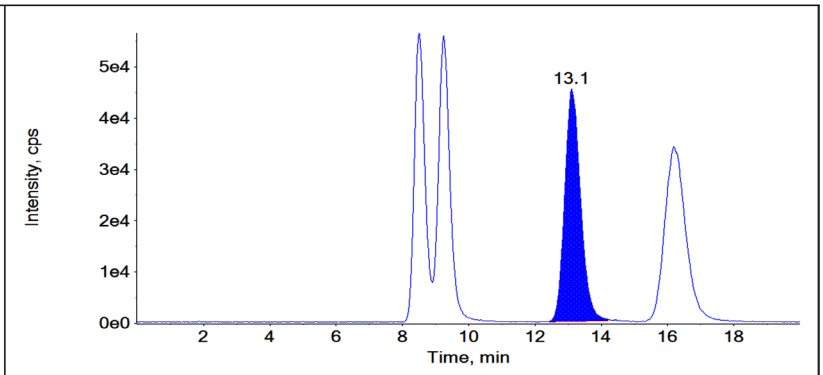
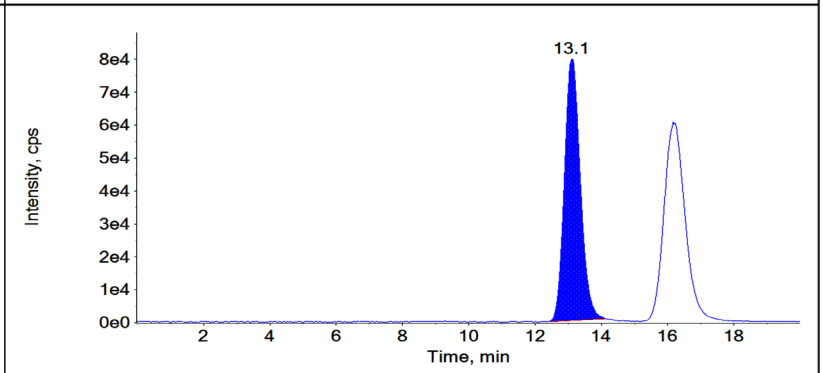
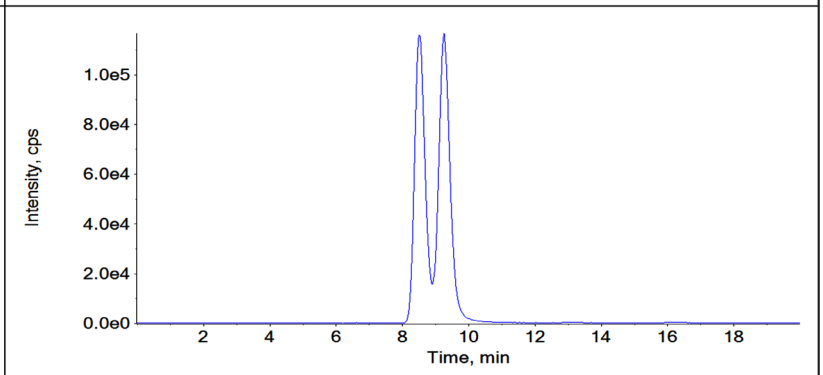
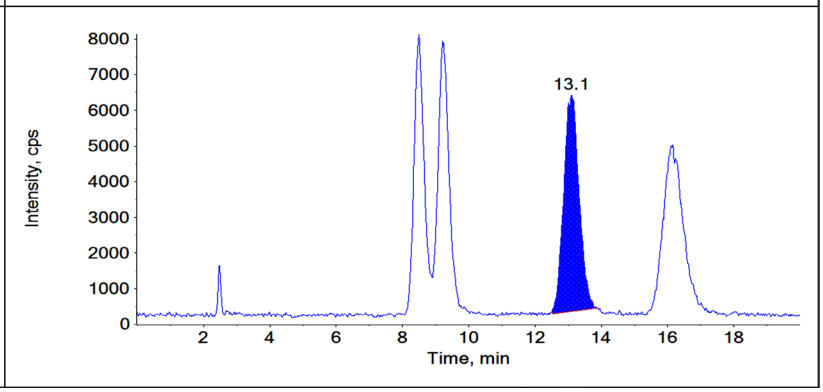
Sample Name: ***spiroxamine 10 µg/L (A1, A2, B1, B2 je 2.5 µg/L)*** *Mass(es):* *298.100/144.100 Da* *Acq. Date:* *2/18/2016 2:52:53 PM* *Retention time:* *13.1 min* *Area:* *1497813 counts* *Height:* *45347 cps*	
Sample Name: **spiroxamine-A 10 µg/L (A1, A2 je 5 µg/L)** Mass(es): 298.100/144.100 Da Acq. Date: 2/18/2016 3:13:40 PM Retention time: 13.1 min Area: 2631872 counts Height: 79489 cps	
Sample Name: **spiroxamine-B 10 µg/L (B1, B2 je 5 µg/L)** Mass(es): 298.100/144.100 Da Acq. Date: 2/18/2016 3:34:27 PM Retention time: 0.00 min Area: 0 counts Height: 0 cps	
Sample Name: **spiroxamine 1 µg/L in WTFR** Mass(es): 298.100/144.100 Da Acq. Date: 2/18/2016 3:55:14 PM Retention time: 13.1 min Area: 195256 counts Height: 6075 cps	

Fig. 5: Determination of enantiomer A1 in grape matrix extract, 4th repetition of each chromatogram [all chromatograms kindly provided by S. Stuke, Bayer CropScience].

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Chromatographic Performance Parameter

Study No.:
Column Type: YMC CHIRAL ART Amylose-SA 3 µm, (150 x 3.0 mm ID)
File name:
Evaluated Sample: 10 µg/L spiroxamine (mixtures of 4 enantiomers)

Col. Length [mm]	**150**
Col. ID [mm]	**3**
Pre-Col. Length [mm]	**0**
Pre-Col. ID [mm]	**2.1**
Particle Size d_p [µm]	**3**
Col. Porosity (filling with mobile phase)	**0.70**
$V_{d\ col}$ [µL]	**742.1**
$V_{d\ Pre\text{-}col}$ [µL]	**0.0**
V_{loop} [µL]	**1**
$V_{capillaries}$ [µL]	**5.7**
V_{total} [µL]	**748.7**
flow [µL/min]	**300**
$t_{d\ cal.}$ [min]	**2.496**

theor. Plates N: $$N = 5.54\left(\frac{t_R}{w_{0.5}}\right)^2$$

Peak capacity n: $$n = 1 + \frac{\sqrt{N_{max}}}{4}\ln(1 + k_{max})$$

Capacity Factor: should be: > 3 best: 4-10
Separation Factor α: k'_2/k'_1 always >1
Peak Resolution:
0.0 = co-eluting
0.6 = 12% peak overlapping
1.0 = 2% peak overlapping
1.5 = baseline separated

Analyt = Order of Elution	t_R [min]	k'-Value	W_H [min]	W_H [S]	St. Dev. σ [S]	Theor. Plates N	Plate Hight H [µm]	plates/m N/m	Separation Factor $\alpha_{n,n+1}$	Peak Resolution $RS_{n,n+1}$	k'-$_{max}$ u. N_{max}	Peak capacity n
spiroxamine enantiomere B1	8.50	2.41	0.3385	20.31	8.628	**3494**	42.9297	23294			k_{max}	
spiroxamine enantiomere B2	9.24	2.70	0.3570	21.42	9.099	**3712**	40.4083	24747	1.12	1.22	5.49	
spiroxamine enantiomere A1	13.10	4.25	0.5080	30.48	12.948	**3685**	40.7064	24566	1.57	4.47	N_{max}	**29**
spiroxamine enantiomere A2	16.19	5.49	0.6708	40.25	17.098	**3228**	46.4698	21519	1.29	2.71	3712	

Chromatographic characteristics:

Peak capacity n **29**
theor. plates/m **24747**

Literature

[1] Etzel WA, Gau W, Krämer W, Stelzer U, Weissmüller J, Assignment of the Stereochemistry of Spiroxamine by Two-Dimensional NMR Spectroscopy and Stereoselective Chemical Synthesis, Magnetic Resonance in Chemistry, 36, 64-68 (1998).