APPLICATION NOTE

Chiral LC-MS-Analysis of the Fungicide Tebuconazole

Tebuconazole is a chiral racemic fungicide used as a pesticide as well as a wood preservative. Bayer AG (Crop Science Division), the manufacturer of this agent, was looking for an effective LC-MS compatible method for separating Tebuconazole. Therefore, a reversed phase separation mode was required and the mobile phase needed to contain acid for ionisation. Due to the expected hydrophilic matrix in plant extracts, the retention factor k´ should be no less than 3.

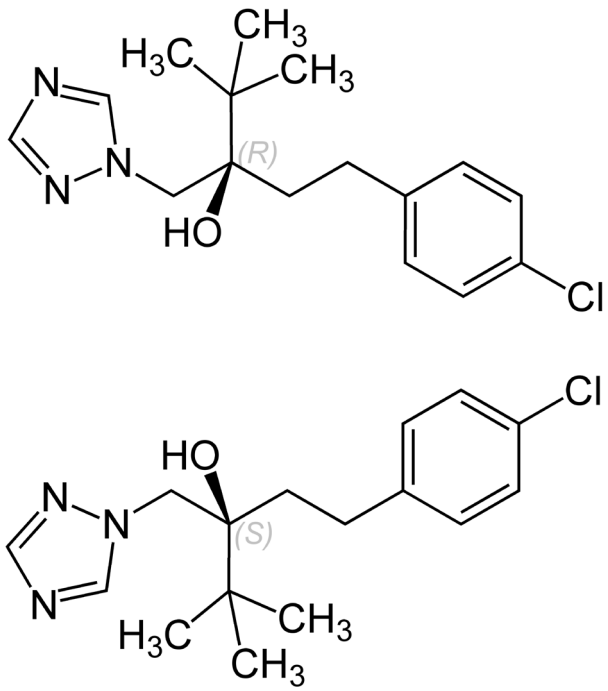

Figure 1: Tebuconazole, a triazole, is a racemic mixture, pka-values: 2.27, 13.86.

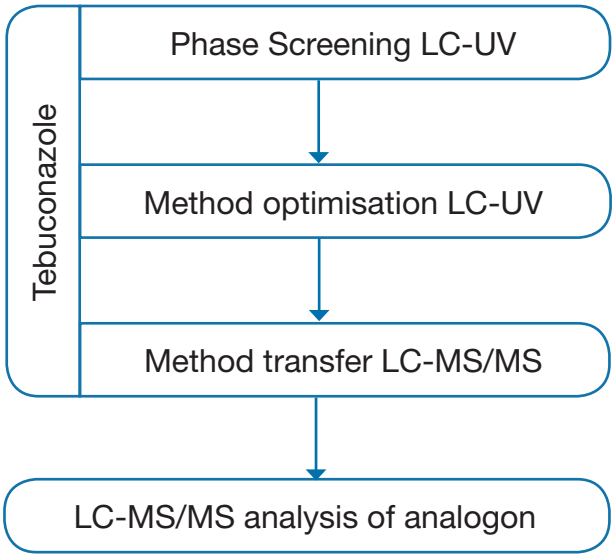

Figure 2: Method development strategy

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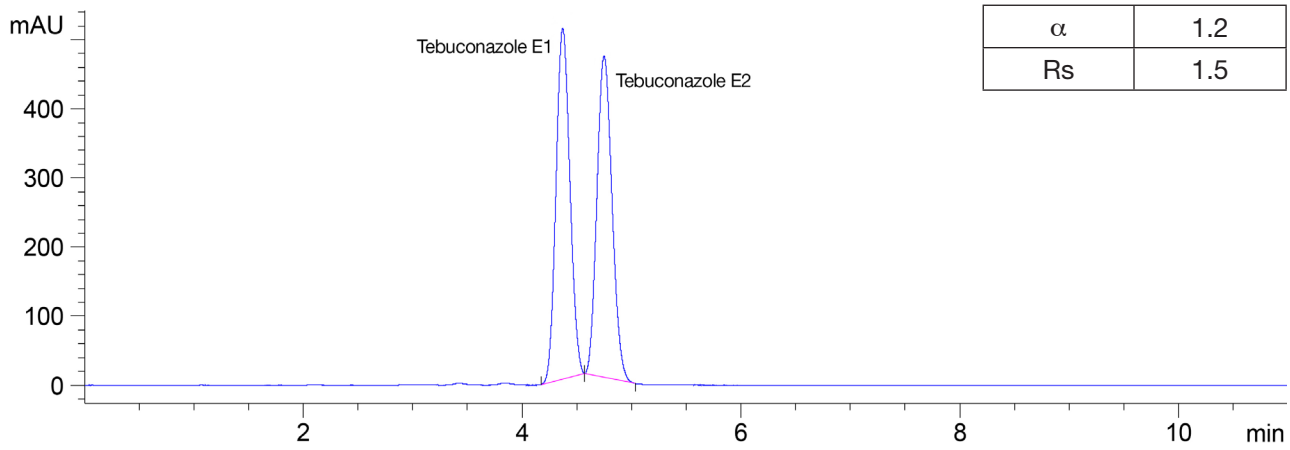
1. Phase Screening

In order to identify the most appropriate selectivity for this compound, YMC used three RP-stable immobilised polysaccharide phases:

- CHIRAL ART Amylose-SA
- CHIRAL ART Cellulose-SB
- CHIRAL ART Cellulose-SC

CHIRAL ART Cellulose-SC was the most suitable column for further method development.



α	1.2
Rs	1.5

Figure 3: Separation of Tebuconazol enantiomers on **CHIRAL ART Cellulose-SC** (KSC99S05-1546WT, 5 µm particle size, 150 mm × 4.6 mm), 1 mL/min, 25°C, 5 µL (1 mg/mL) injection volume, water/acetonitrile (40/60), 220 nm.

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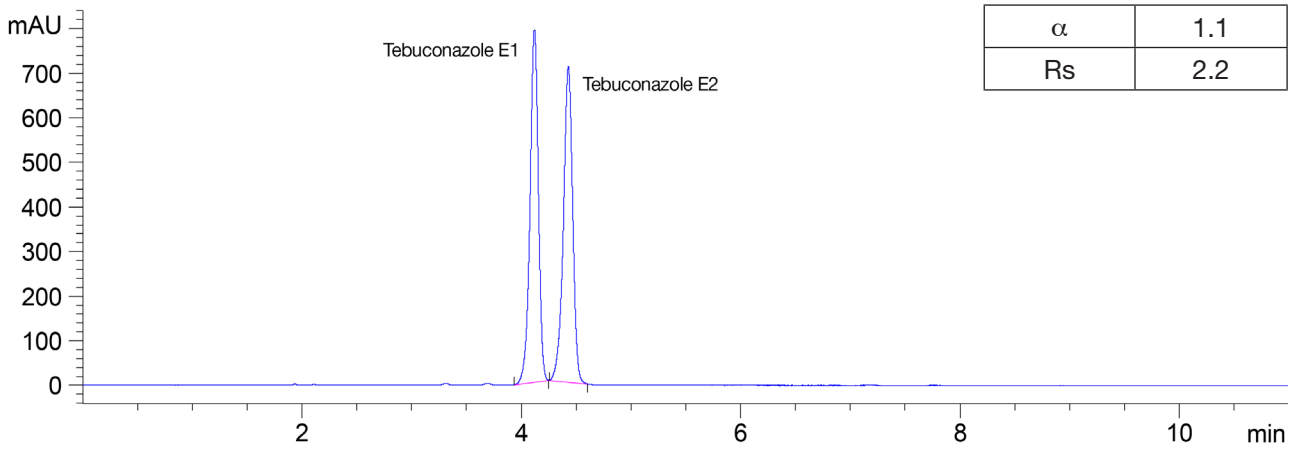
2. Method Optimisation

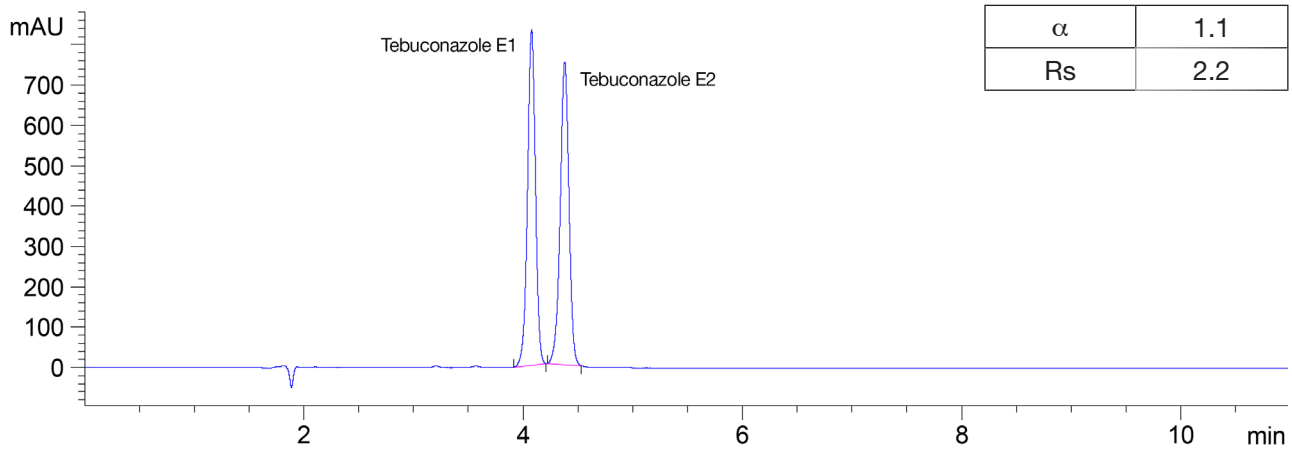
In a first step, a smaller column ID and smaller particle size (3 µm) were selected, which led to improved peak shape.



α	1.1
Rs	2.2

Figure 4: Separation of Tebuconazol enantiomers on **CHIRAL ART Cellulose-SC** (KSC99S03-1503WT, 3 µm particle size, 150 mm × 3 mm), 0.43 mL/min, 25°C, 2 µL (1 mg/mL) injection volume, water/ acetonitrile (40/60), 220 nm.

To enable ionisation for the MS-detection, formic acid was added to the mobile phase. As expected, the influence on the separation itself was marginal.



α	1.1
Rs	2.2

Figure 5: Separation of Tebuconazol enantiomers on **CHIRAL ART Cellulose-SC** (KSC99S03-1503WT, 3 µm particle size, 150 mm × 3 mm), 0.43 mL/min, 25°C, 2 µL (1 mg/mL) injection volume, water with 0.1% formic acid / acetonitrile (40/60), 220 nm.

Chiral LC-MS-Analysis of the Fungicide Tebuconazole

The Crop Science Division of Bayer AG expects hydrophilic matrix components in Tebuconazole samples. To avoid co-elution of Tebuconazole with those components, the retention factor of the enantiomers should be increased. This was achieved by reducing the acetonitrile percentage of the mobile phase from 60% to 40%. The retention factor increased to 6.3, which fulfilled the desired criteria.

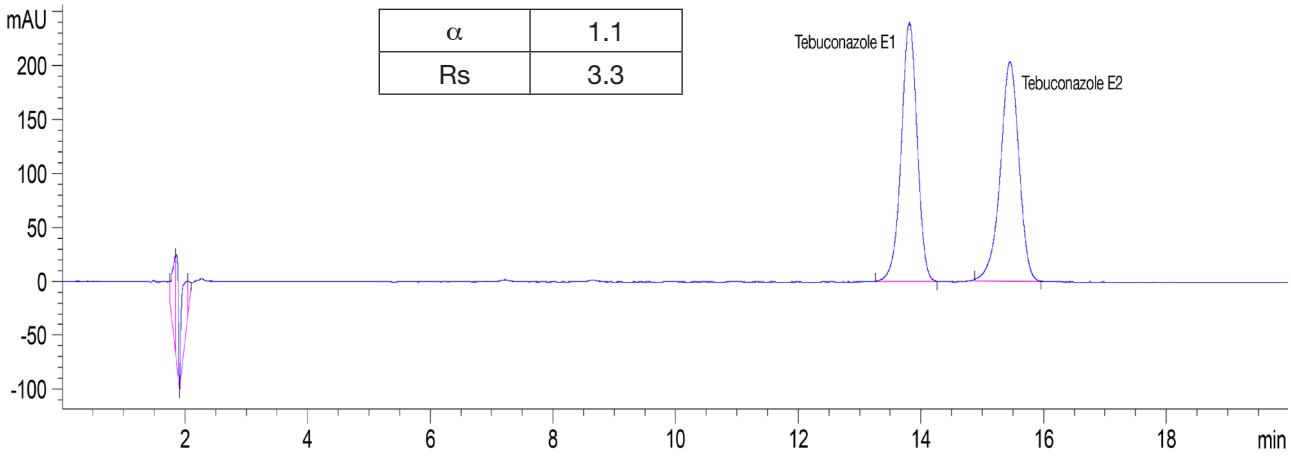


Figure 6: Separation of enantiomers Tebuconazole on **CHIRAL ART Cellulose-SC** (KSC99S03-1503WT, 3 µm particle size, 150 mm × 3 mm), 0.43 mL/min, 25°C, 2 µL (1 mg/mL) injection volume, water with 0.1% formic acid/ acetonitrile (60/40), 220 nm.

Final chromatographic conditions

Column:	CHIRAL ART Cellulose-SC 3 µm (150 x 3.0 mm ID)
Part No.:	KSC99S03-1503WT
Eluent:	water / acetonitrile / formic acid (60/40/0.1)
Flow rate:	0.43 mL/min
Temperature:	25°C
Detection:	UV at 220 nm
Injection:	2 µL (1 mg/mL)

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3. Transfer from LC-UV to LC-MS/MS and Final Set-up

At Bayer AG – Crop Science Division the application developed by YMC was transferred to a LC-MS/MS instrument consisting of a Agilent 1290 LC coupled to a Sciex API 6500 triple-quadrupole mass spectrometer.
For detection of tebuconazole the mass transitions m/z 308-70 for quantitation and m/z 308-125 for result confirmation were recorded. Tebuconazole was detected in the electrospray positive ionisation mode under optimised ion source and fragment ion transmission settings.

3.1 Results

As shown on the following tables and chromatograms superior results were obtained in regard to retention factor (5.7 and 6.5), peak resolution of 2.5 and capacity factor of more than 40.
As “prove of concept” a chiral molecule which is structure analogue to tebuconazole was chromatographed and detected with the identical set-up, only recording two more mass transitions for this additional analyte also in electrospray positive ionization mode.
For this additional chiral analyte high retention factors (4.9 and 6.2), and even better peak resolution for both enantiomers of 4.9 and again a capacity factor of more than 40 were obtained.

Chiral LC-MS-Analysis of the Fungicide Tebuconazole

Tebuconazole

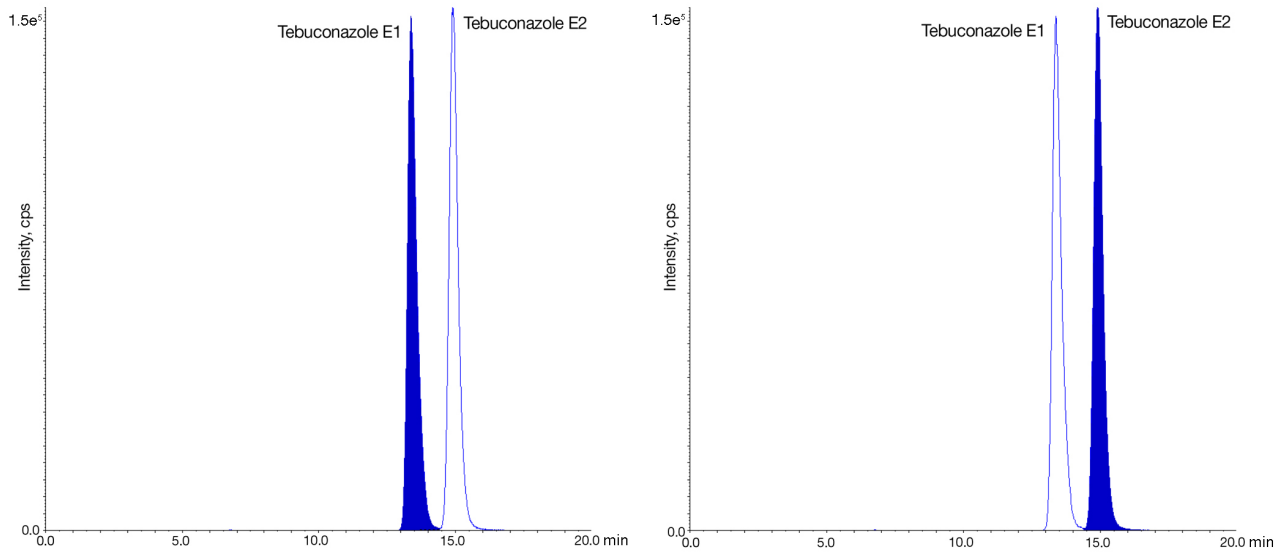

Figure 7: 250 µg/L Standard Solution; Separation of Tebuconazole enantiomers on **CHIRAL ART Cellulose-SC** (KSC99S03-1503WT, 3 µm particle size, 150 mm × 3 mm), 0.43 mL/min, 25°C, 1 µL injection volume, water with 0.1% formic acid/acetonitrile (60/40), ESI+ m/z 308-70.

Table 1: Chromatographic performance for tebuconazole enantiomer separation

Column length	150 mm
Column ID	3 mm
Particle size	3 µm
Vloop	1 µL
Vcapillaries	7.9 µL
Vtotal	857.0 µL
Flow rate	0.43 mL/min

	Enantiomer 1	**Enantiomer 2**
Retention time	13.37 min	14.89 min
Peak area	3,610,915 counts	3,629,237 counts
Capacity factor k’	5.71	6.47
Peak width at 50% height	0.351 min	0.352 min
Plate count N	8040	9916
Plate height H	18.66 µm	15.13 µm
Plates/m	53,601	66,104
Separation factor α		1.13
Peak capacity η		46

Chiral LC-MS-Analysis of the Fungicide Tebuconazole

Structure analogon (for prove of concept)

Exactly the same method on **CHIRAL ART Cellulose-SC** is suitable to investigate a structure analogon.

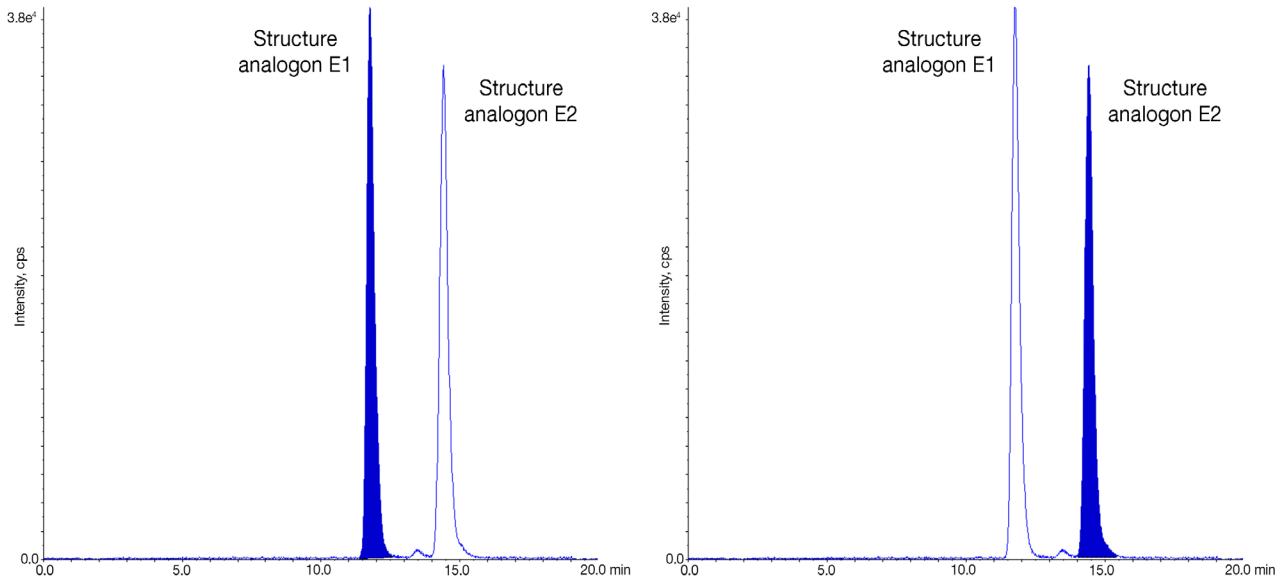

Figure 8: Separation of structure analogon enantiomers on **CHIRAL ART Cellulose-SC** (KSC99S03-1503WT, 3 µm particle size, 150 mm × 3 mm), 0.43 mL/min, 25°C, 1 µL injection volume, water with 0.1% formic acid/ acetonitrile (60/40); ESI+ m/z 312-70.

Table 2: Chromatographic performance for structure analogon enantiomer separation

	Enantiomer 1	**Enantiomer 2**
Retention time	11.76 min	14.41 min
Peak area	759,339 counts	745,010 counts
Capacity factor k`	54.9	6.23
Peak width at 50% height	0.283 min	0.316 min
Plate count N	9569	11523
Plate height H	15.68 µm	13.02 µm
Plates/m	63,792	76,820
Separation factor α		1.27
Peak capacity η		49

Chiral LC-MS-Analysis of the Fungicide Tebuconazole

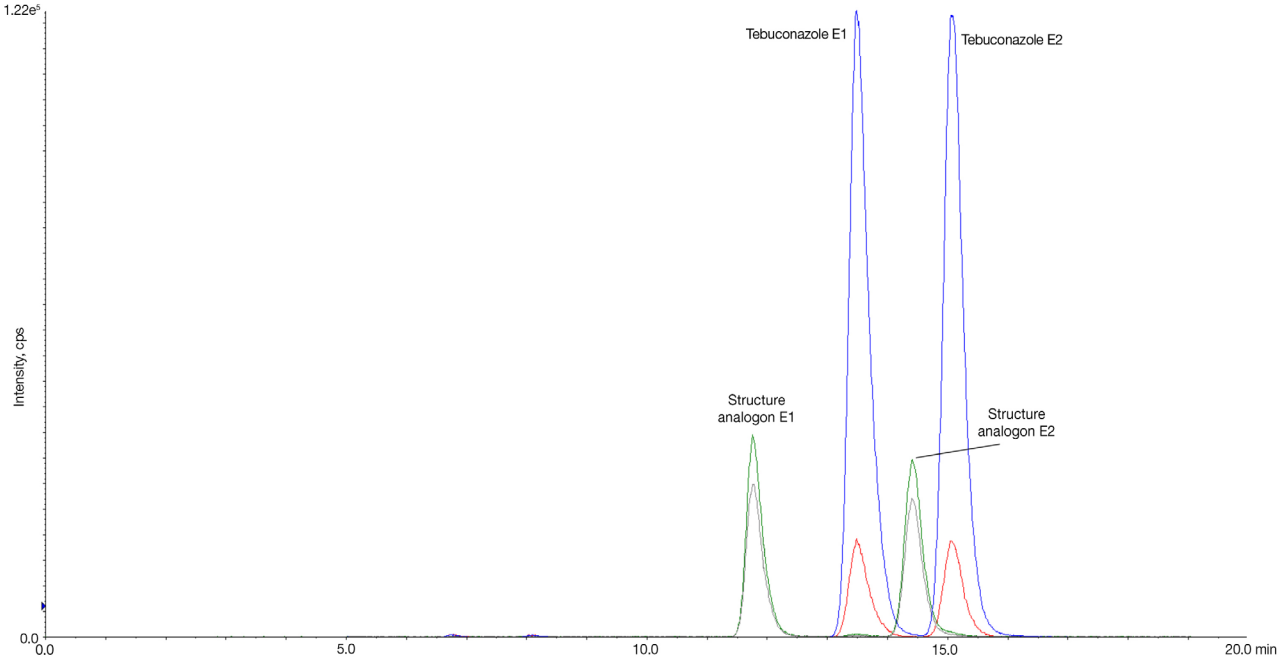

Figure 9: Combined LC-MS raw data chromatogram of tebuconazole and structure analogon enantiomers (2 MRMs each analyte).

4. Acknowledgements

YMC Europe GmbH thanks Sven Stuke (Bayer AG – Crop Science Division), who introduced the separation task, for the great teamwork and providing the valuable LC-MS data.

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