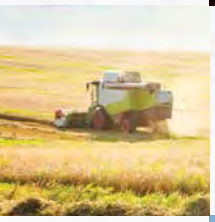
AGROCHEMICAL SOLUTIONS APPLICATION NOTEBOOK

Accelerate Synthesis, Purification, and Formulation



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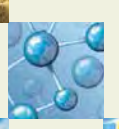
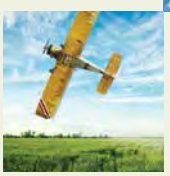
AGROCHEMICAL SOLUTIONS APPLICATION NOTEBOOK

Developing next-generation crop protection solutions

To accelerate the development of highly effective, environmentally friendly agrochemicals, laboratories must perform analyses that generate more information, are completed more rapidly and reduce overall costs. Waters advanced analytical technologies including chromatography, mass spectrometry, columns and sample preparation, and data management software, provide impactful scientific and operational benefits at key steps along the agrochemical development workflow helping manufacturers deliver next-generation crop protection solutions today.





[CHIRAL SEPARATIONS]

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Enantiomeric and Diastereomeric Separations of Pyrethroids Using UPC²

John P. McCauley, Lakshmi Subbarao, and Rui Chen
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APPLICATION BENEFITS

- Superior and faster separations of pyrethroids by UPC²™ allow for higher throughput analysis of a better quality compared to GC and LC.
- Column coupling facilitated by the low viscosity of liquid CO_2 enables high efficiency chiral separations required for resolving pyrethroid isomers. The underlying principle is easy to implement and reduces the time required for method development.
- Even with 5-µm particle packed chiral columns, the low system/extra-column volume of the ACQUITY UPC²™ System enables a more efficient diastereomeric separation of pyrethroids than LC or traditional SFC. The use of 5-µm chiral columns simplifies the method transfer from UPC² to potential downstream purification.

WATERS SOLUTIONS

ACQUITY UPC² System with a photodiode array (PDA) detector

Empower™ 3 Software

KEY WORDS

Enantiomers, diastereomers, chiral, pesticides, pyrethroid, convergence chromatography, UPC²

INTRODUCTION

Pyrethroids are synthetic compounds similar to natural pyrethrins. They are commonly used in both commercial agriculture and households as insecticides and insect repellents. Unlike other common classes of insecticides, such as organophosphates and carbamates that are acetylcholine esterase inhibitors or nicotinic acetycholine agonists, the pyrethroids act upon neuronal sodium channels for their insecticidal effect. Due to metabolic rate differences between species, many pyrethroids possess moderate to good selectivity for insect versus mammalian biology, allowing for their relatively safe application.[1]

Many pyrethroids possess multiple stereogenic centers, as shown in Figure 1. Individual stereoisomers can have vastly different biological activities. Furthermore, environmental degradation of many insecticides is enzymatic and sensitive to the stereochemical configuration. As a result, rapid, reliable, and precise determination of the isomeric ratio of these chiral pesticides is of great importance not only for product formulation, but also for the subsequent study of the pharmacokinetics, metabolism, and the environmental fates of the individual pesticide isomers.

Historically, chiral separations of pyrethroids have primarily been carried out using HPLC on chiral stationary phases (CSPs), capillary electrophoresis (CE), and gas chromatography (GC).[1] More recently, supercritical fluid chromatography (SFC) has been applied to these separations as well, often resulting in improved resolution and reduced run time. In this application note, we present the enantiomeric and diastereomeric separations of four pyrethroids with varying stereochemical complexity using a Waters® ACQUITY UPC² System.

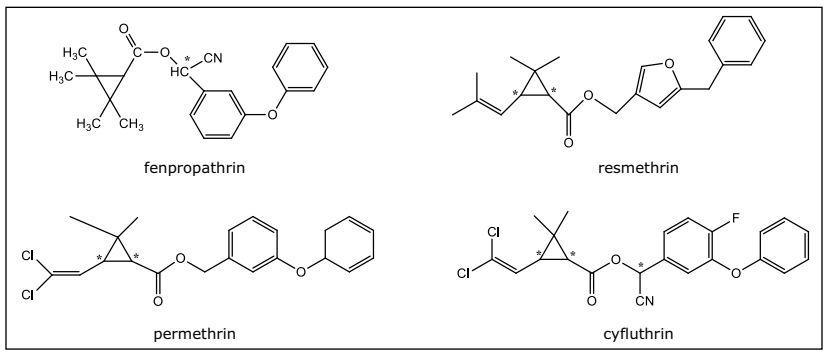

Figure 1. Structures of the pyrethroids presented in this study. The asterisks denote chiral centers.

EXPERIMENTAL

Sample description

The pesticide samples used in this study were purchased from Reidel-de Haen, Pestanal, Sigma and Fluka, with their structures shown in Figure 1. All samples were dissolved in isopropanol (IPA) for analyses.

UPC^2 conditions

System:	ACQUITY UPC^2
Detection:	PDA
Columns:	CHRALCEL OJ-H and CHIRALPAK IC (4.6 x 150 mm, 5 µm) obtained from Chiral Technologies

Data management

Empower 3 Software

Other key experimental parameters are listed in the respective figure captions.

RESULTS AND DISCUSSION

Chiral method development often starts with screening different stationary phases and co-solvent/additives. After extensive screening of fenpropathrin, an OJ-H column and methanol were selected for further optimization. Figure 2 shows the UPC^2 chromatograms of fenpropathrin at different temperatures. As the temperature increased, the solvation strength of supercritical CO_2 decreased and retention time became longer. This allowed for more interactions between analytes and the chiral selectors on stationary phases, resulting in higher resolution. While temperature is often perceived as a secondary parameter for selectivity adjustment in achiral SFC, the current example clearly demonstrates that temperature could dramatically alter the chiral resolution.

To further improve the resolution, the flow rate was lowered to 1.5 mL/min while other parameters remained the same, and the resulting chromatogram is shown in Figure 3. A baseline resolution (R_s=1.67) was achieved. Contrary to typical reverse phase or normal phase liquid chromatography for an achiral separation where a dominant interaction usually dictates retention, chiral chromatography with SFC typically results from multiple interactions between analytes and stationary phases. These interactions could have different dependence on typical experimental parameters, including flow rate, pressure, and temperature. It is, therefore, important to systematically explore all possible contributors to achieve maximum resolution.

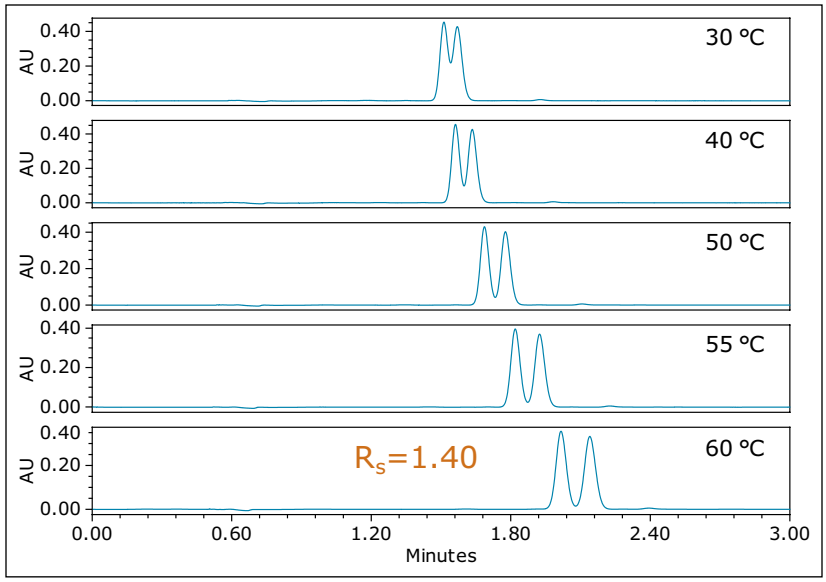

Figure 2. UPC^2 chromatograms of fenpropathrin at different temperatures on an OJ-H column (4.6 x 150 mm, 5 µm) under isocratic conditions (5% co-solvent). Flow rate was 3 mL/min. Back pressure was 120 bar. Co-solvent was methanol.

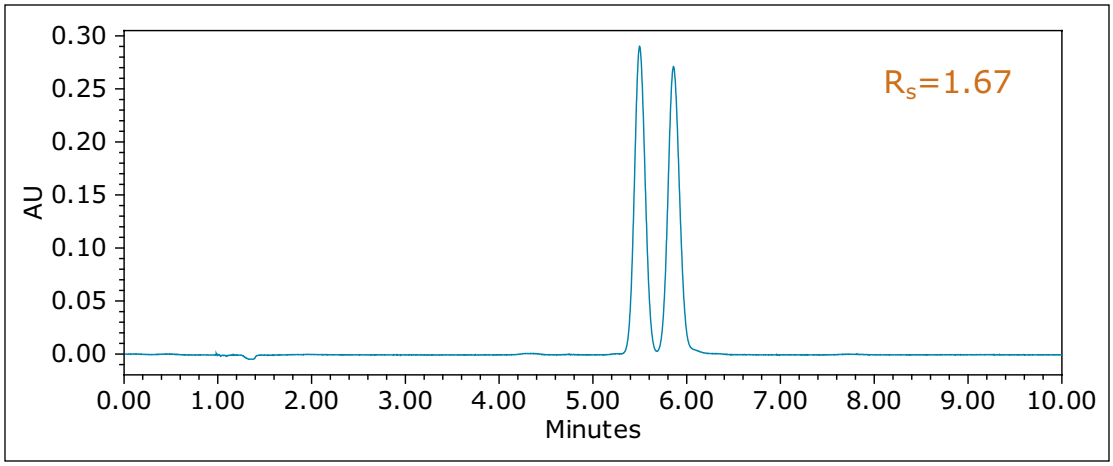

Figure 3. UPC² chromatogram of fenpropathrin on an OJ-H column (4.6 x 150 mm, 5 µm) under isocratic conditions (5% co-solvent). Flow rate was 1.5 mL/min. Back pressure was 120 bar. Co-solvent was methanol. Temperature was 60 °C.

Figure 4 shows the UPC² separation of permethrin, a pyrethroid with two chiral centers. The four isomers were baseline resolved in less than 4 min on an OJ-H column (4.6 x 150 mm, 5 µm). A 100-min GC separation resolved three out of the four isomers.[2] The four isomers couldn't be completely separated by either reverse phase or normal phase LC.[3]

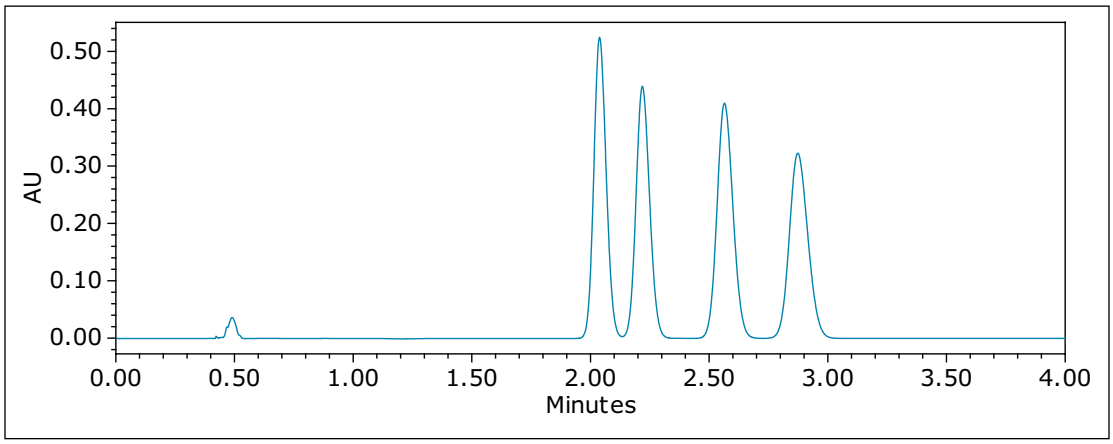

Figure 4. UPC² chromatogram of permethrin on an OJ-H column (4.6 x 150 mm, 5 µm) under isocratic conditions (7% co-solvent). Flow rate was 4 mL/min. Back pressure was 120 bar. Co-solvent was isopropanol. Temperature was 40 °C.

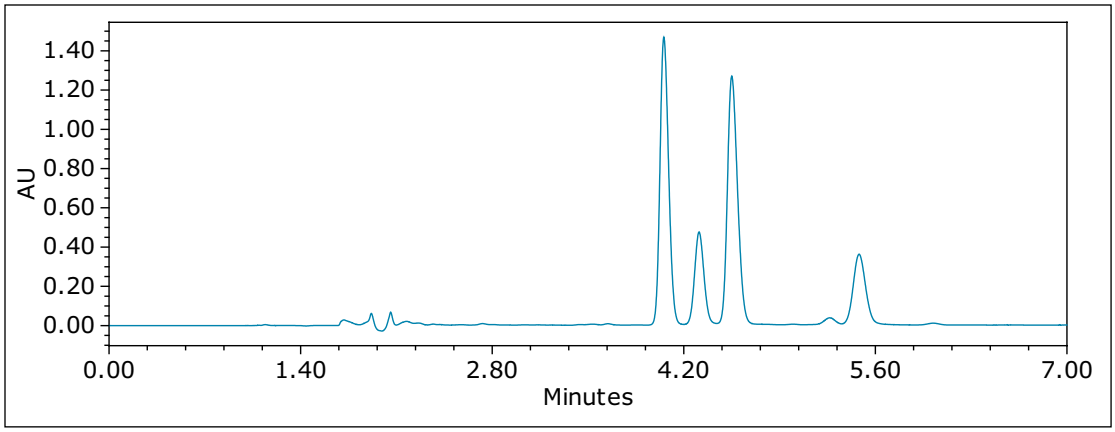

Figure 5. UPC^2 chromatogram of resmethrin on two OJ-H columns (4.6 x 150 mm, 5 µm) coupled in tandem under isocratic conditions (15% co-solvent). Flow rate was 2 mL/min. Back pressure was 120 bar. Co-solvent was isopropanol. Temperature was 30 °C.

Figure 5 shows a UPC^2 chromatogram of resmethrin, another pyrethroid with two chiral centers. Despite the structural similarity between permethrin and resmethrin, the baseline resolution of all four isomers required two OJ-H columns coupled in tandem, and a reduced flow rate. Increasing column length is one of the most fundamental and facile means to improve chromatographic resolution. Column coupling has been used for both analytical and preparative SFC,[4-5] when selectivity or retentivity was inadequate to improve resolution. Compared with HPLC, SFC holds the following unique advantage: the viscosity of liquid CO_2 under typical SFC conditions is 10- to 100-fold lower than those commonly used HPLC solvents. The low viscosity of liquid CO_2 allows for column coupling, hence, extending column length for higher chromatographic efficiency without generating a prohibitive pressure drop across columns.

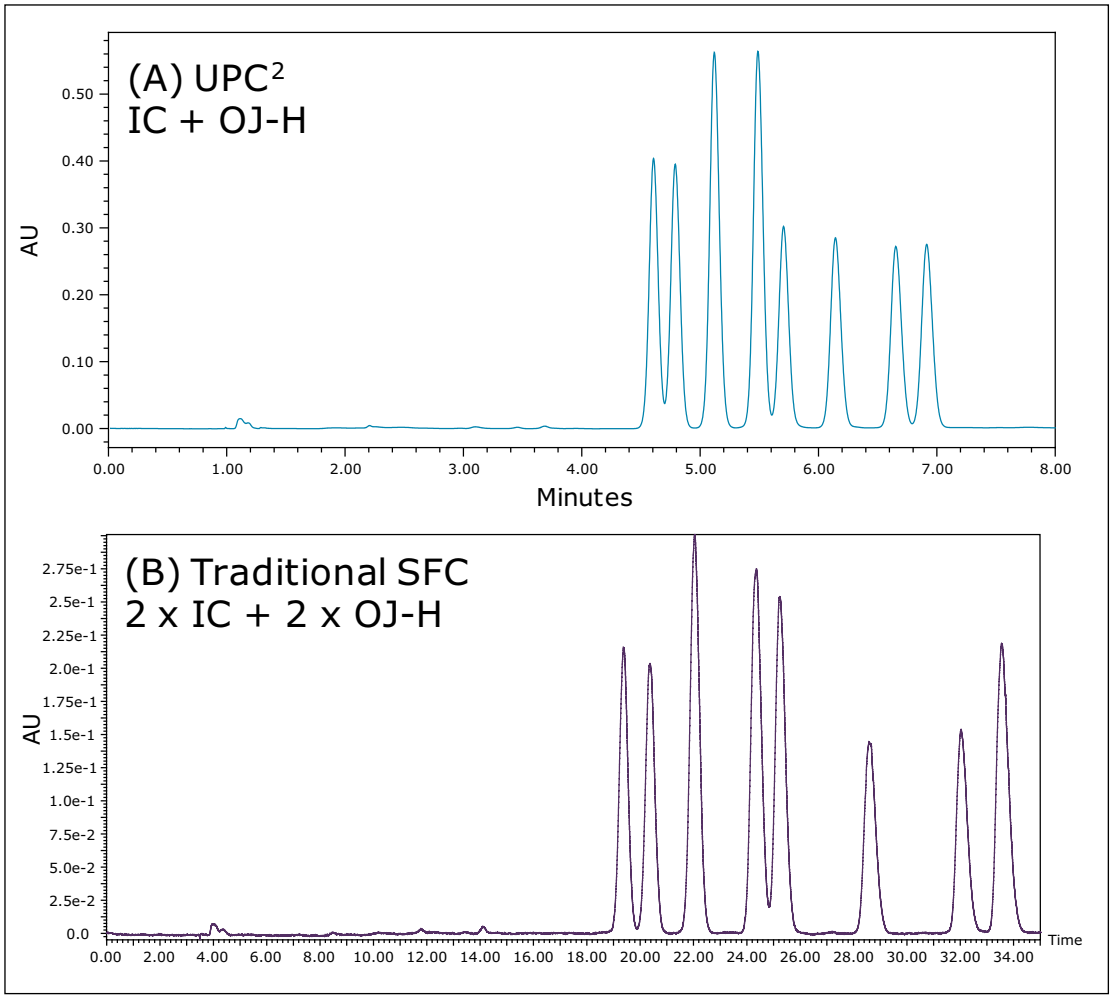

Figure 6. UPC^2 and SFC chromatograms of cyfluthrin. For the UPC^2 chromatogram, the co-solvent was isopropanol; flow rate was 3.5 mL/min; temperature was 50 °C; and back pressure was 120 bar. The gradient was 4.5% to 6% in 3 min, 6% to 10% in 4 min, and 10% for 1 min. The total analysis time was 8 min. For the SFC chromatogram, the co-solvent was isopropanol; flow rate was 3 mL/min; temperature was 45 °C; and back pressure was 120 bar. The gradient was 4% for 0.5 min, 4% to 6% in 29 min, 6% for 5 min. The total analysis time was 35 min.

Figure 6 shows the diastereomeric separation of cyfluthrin, a pyrethroid with three chiral centers, on both UPC^2 and traditional SFC systems. Similar separations were achieved on both systems. Only two coupled columns (300 mm) were used for the UPC^2 system; whereas, four coupled columns with a total length of 800 mm were required on the traditional SFC system. The low extra-column volume of the UPC^2 system greatly mitigates peak dispersion, resulting in sharper peaks, higher resolution, and significantly reduced analysis time (8 min using a UPC^2 system compared to 35 min using a traditional SFC system). The separation of cyfluthrin also exemplifies another type of column coupling. Each column offered complementary separations. Cumulatively, the coupled columns of different chemistries resulted in the simultaneous separation of all eight isomers. The coupled columns in this case essentially altered the selectivity rather than increasing column length, as illustrated in Figure 5.

CONCLUSIONS

In this application note, we demonstrated some challenging chiral separations of pyrethroids with varying stereochemical complexity using a Waters ACQUITY UPC^2 System. Due to the nature of chiral separations, it is important to systemically explore some secondary chromatographic parameters, such as temperature and flow rate, to achieve the desired resolution. The separations presented in this paper are thermally sensitive. Temperature optimization is a critical step for method development. The low viscosity of supercritical CO_2 allows for facile column coupling, between identical or different column chemistries, for highly efficient chiral separations required for resolving pyrethroid isomers. Finally, low system volume and extra-column volume of the ACQUITY UPC^2 System enables superior, faster, and more efficient diastereomeric separations of pyrethroids compared to traditional SFC.

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Diastereoselective Separation of Permethrin Using the ACQUITY UPC^2 System

GOAL

To successfully develop a diastereoselective UltraPerformance Convergence Chromatography™ (UPC^2) method for the baseline resolution of all four permethrin isomers using the Waters® ACQUITY UPC^{2}™ System.

Compared to chiral HPLC methods, UPC^{2}™ offers a complete baseline resolution of all isomers with significantly shorter run time; ideal for pesticide manufacturers who routinely perform diastereomer analyses.

BACKGROUND

Public concern about pesticide use is growing. Twenty-five percent of pesticides currently used are chiral compounds. Chirality plays an important role in the potency, toxicity, metabolic, and environmental fate of these chiral pesticides. As a result, there has been an increasing demand for stereo-selective separation techniques and analytical assays to evaluate the enantiomeric purity of pesticides.

Permethrin is a synthetic chemical widely used as an insecticide and an insect repellent. Permethrin has four stereoisomers (two enantiomeric pairs), arising from the two stereo-centers in the cyclopropane ring as shown in Figure 1. Consequently, separation and quantification of permethrin isomers can be challenging. Great effort has been devoted to developing both normal phase and reversed phase HPLC methodologies to separate permethrin, but with moderate success. As we demonstrated here, a baseline resolution of all four permethrin isomers is achieved in less than six minutes using the ACQUITY UPC^2 System.

THE SOLUTION

Various chiral stationary phases (CSPs) have been evaluated to separate permethrin using both chiral normal phase and reversed phase HPLC. Lisseter and Hambling reported the use of a Pirkle type CSP for permethrin separation under normal phase HPLC conditions. The total run time was more than 30 min and the mobile phase used was hexane with 0.05% isopropanol (*Journal of Chromatography*, 539 1991; 207-10). However, the enantiomeric *cis*- and *trans*- pairs were inadequately resolved. Shishovska and Trajkovska used a chiral β-cyclodextrin CSP for permethrin separation under reversed phase HPLC conditions with methanol and water as the mobile phase (*Chirality*, 22 2010; 527-33). The total run time was more than 50 min. The resolution of the *trans*-permethrin enantiomeric pair was <1.5. Alternatively, the CHIRALCEL OJ column under normal phase HPLC conditions was also used for permethrin separation (*Chromatographia*, 60 2004; 523-26). Our experiment, conducted under the same conditions described in Table 1, yielded three separate peaks as shown in Figure 2. This is in agreement with the reported results.

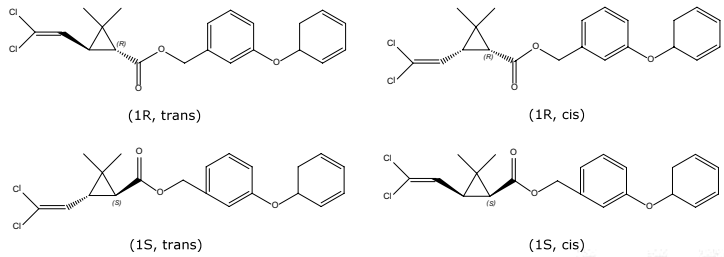

Figure 1. Chemical structures of permethrin.

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Figure 3 shows the diastereoselective separation of permethrin using the ACQUITY UPC^2 System. Baseline resolution of all four isomers was achieved in less than six minutes with a shorter OJ-H column. Results are summarized in Table 2. Overall, compared to the chiral HPLC methods, the current UPC^2 method offers a better resolution and a shorter run time.

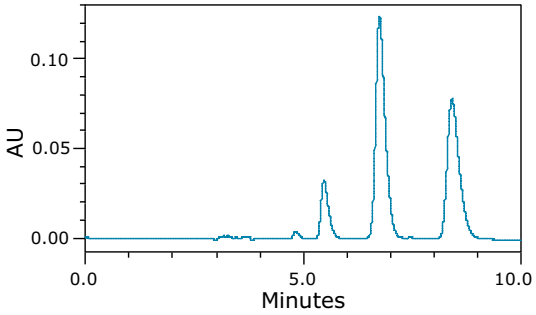

Figure 2. Chromatogram of permethrin obtained with a CHIRALCEL OJ-H column under normal phase HPLC conditions.

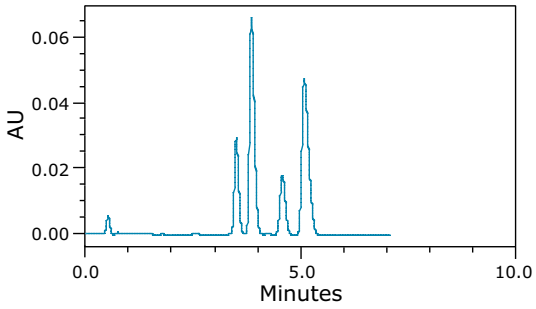

Figure 3. Chromatogram of permethrin obtained with a CHIRALCEL OJ-H column under UPC^2 conditions.

	Normal phase HPLC	UPC^2
Flow rate (mL/min)	1	4
Mobile phase	Hexane:ethanol=90:10	CO_2:methanol:DEA=95:5:0.2
Back pressure (bar)	n/a	120
Temp. (°C)	Ambient	40
Column	CHIRALCEL OJ-H (4.6 x 250 mm, 5 µm)	CHIRALCEL OJ-H (4.6 x 150 mm, 5 µm)
Sample conc.	2 mg/mL	
Injection volume (µL)	10	

Table 1. Key experimental parameters.

Peak	Retention time (min)	K'	α	Resolution	USP tailing factor
1	3.509	5.66			1.12
2	3.862	6.33	1.12	1.80	1.31
3	4.582	7.69	1.22	3.25	1.12
4	5.089	8.66	1.13	1.92	1.50

Table 2. Retention time, retention factor (K'), selectivity (α), resolution, and USP tailing factor of permethrin obtained under UPC^2 conditions with a CHIRALCEL OJ-H column.

SUMMARY

Successful diastereoselective separation of permethrin was demonstrated using the Waters ACQUITY UPC^2 System. Baseline resolution of all four isomers was achieved in less than six minutes. Compared to chiral HPLC methods, the UPC^2 method offers a better resolution and a shorter run time. The UPC^2 method also eliminated the need for toxic hexane often used in normal phase HPLC methods. The ACQUITY UPC^2 System is ideal for pesticide manufacturers who routinely perform diastereomer analyses.

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Enantiomeric and Diastereomeric Resolutions of Chiral Pesticides by ACQUITY UPC^2 with UV Detection

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APPLICATION BENEFITS

- Improved enantiomeric and diastereomeric resolution and shorter analysis times resulting in higher sample throughput and reduced solvent consumption when compared with normal phase separations.
- Reliable and reproducible measurement of the enantiomer and/or diastereomer ratios to accurately determine the correct application rates.

WATERS SOLUTIONS

ACQUITY® UltraPerformance Convergence Chromatography™ System (UPC^2®)

ACQUITY UPLC® Photodiode Array (PDA) Detector

Empower® 3 Software

KEY WORDS

Chiral pesticides, UPC^2, enantiomer, diastereomer, chiral separation, chiral resolution, metolachlor, metalaxyl, difenoconazole.

INTRODUCTION

The development of analytical methods for the separation of chiral compounds is important in many areas of research because it is well known that enantiomers can react differently in a chiral environment. Biochemical reactions can be stereo or enantioselective – and while one enantiomer may deliver the desired effect (referred to as the eutomer) to the target species, the other enantiomer may be less effective to the target or completely ineffective. It is estimated that 30% of pesticides on the market today have optical isomers and there are reports that 40% of the pesticides used in China are chiral.[1,2] The study of enantioselectivity is important to the agricultural chemicals manufacturing industry since the knowledge of the efficacy of each individual enantiomer could facilitate a significant reduction in the total amount of pesticide applied. In order to improve our knowledge of enantioselectivity, analytical methods that provide reliable and reproducible separations in a rapid time frame are needed. Supercritical fluid chromatography (SFC) has become known as an effective chiral separations technique possessing many advantages over conventional high performance liquid chromatography (HPLC).[3,4] The properties of a supercritical fluid allow high efficiency separations with shorter analysis times to be achieved. The structural complexity of new pesticides is increasing, which means that there is a greater likelihood that multiple chiral centers may be present in a molecule[5,6,7] and high efficiency techniques are needed to perform successful separations.

In this application note, we present the enantiomeric and/or diastereomeric separations of three pesticides: metalaxyl-M (a phenylamide fungicide), S-metolachlor (acetanilide class of herbicides), and difenoconazole (a triazole fungicide). Metalaxyl has one chiral center, while metolachlor and difenoconazole have two chiral centers. The structures are shown in Figures 1 to 3. Separations were performed using Waters® ACQUITY UltraPerformance Convergence Chromatography System (UPC^2). Convergence chromatography is a complimentary separation technique to liquid chromatography, providing orthogonal selectivity and using supercritical CO_2 as the primary mobile phase.

EXPERIMENTAL

Instrumentation

Separations were done using the ACQUITY UPC^2 System. Detection was by photodiode array (PDA) in combination with single wavelength detection. Empower 3 Software was used for chromatographic data processing.

Sample Preparation: The pesticide standards, supplied by Syngenta, were prepared in 2-propanol.

Method conditions

Metalaxyl-M

Separation mode:	Gradient
Column:	ChiralpakIA-3 4.6 x 150 mm, 3-µm
Co-solvent:	2-propanol
ABPR:	2000 psi/138 bar
Flow rate:	4.0 mL/min
UV detection:	215 nm
Column temp.:	55 °C.
Injection volume:	1 µL

S-metolachlor

Separation mode:	Gradient
Column:	Chiralpak IA-3 4.6 x 150 mm, 3 µm
Co-solvent:	2-propanol
ABPR:	2000 psi/138 bar
Flow rate:	2.5 mL/min
UV detection:	220 nm
Column temp.:	35 °C
Injection volume:	2 µL

Difenoconazole

Separation mode:	Isocratic
Column:	Chiralcel OD-3 4.6 x 150 mm, 3 µm
Co-solvent:	2-propanol/Butan-1-ol (70/30)
ABPR:	2000 psi/138 bar
Flow rate:	2.0 mL/min
UV detection:	235 nm
Column temp.:	35 °C
Injection volume:	2 µL

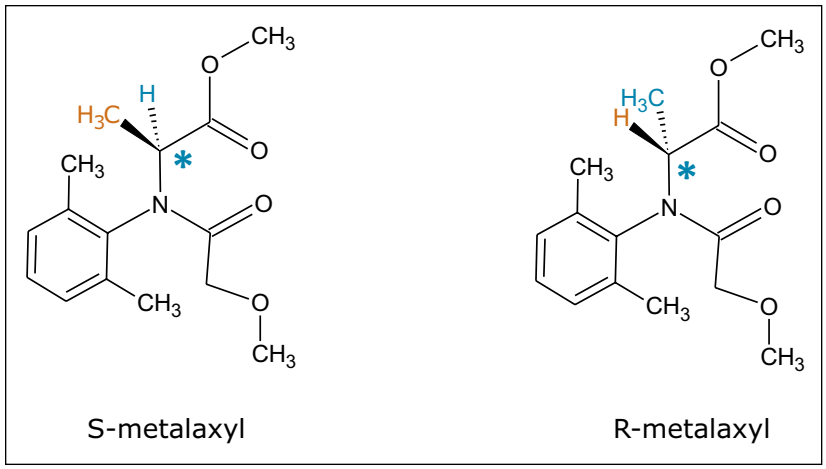

Figure 1. Structures of the two metalaxyl enantiomers. The asterisk indicates the stereogenic center.

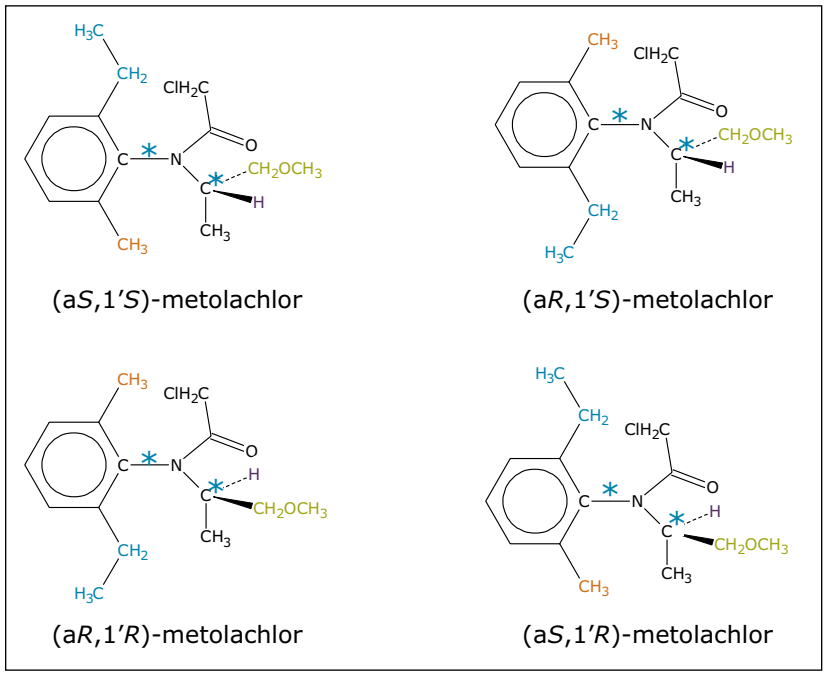

Figure 2. Structures of the four stereoisomers of metolachlor. The asterisks indicate the stereogenic centers.

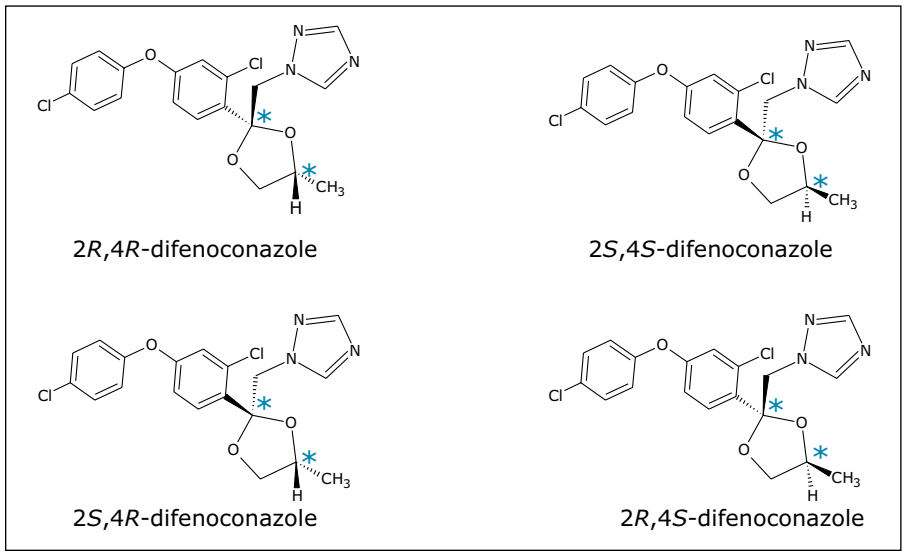

Figure 3. Structures of the four stereoisomers of difenoconazole. The asterisks indicate the stereogenic centers.

RESULTS AND DISCUSSION

The method development for the standard pesticides began by using a generic screening gradient with a number of chiral columns and co-solvents. This screening step can be completed rapidly due to the shorter analysis times that are possible with this technique. The combination of co-solvent and column that produced the most promising separation for each compound was then optimized. The selectivity in a chiral separation can change markedly by varying the temperature, pressure, and flow rate.[8] Gradient and isocratic separations were evaluated; both resulted in successful separation of the stereoisomers for each compound. The separation that showed optimum resolution (R_s) is reported here. The optimized separations for the standard racemic mixture of metalaxyl and the biologically active R-enantiomer,[9] metalaxyl-M are shown in Figure 4.

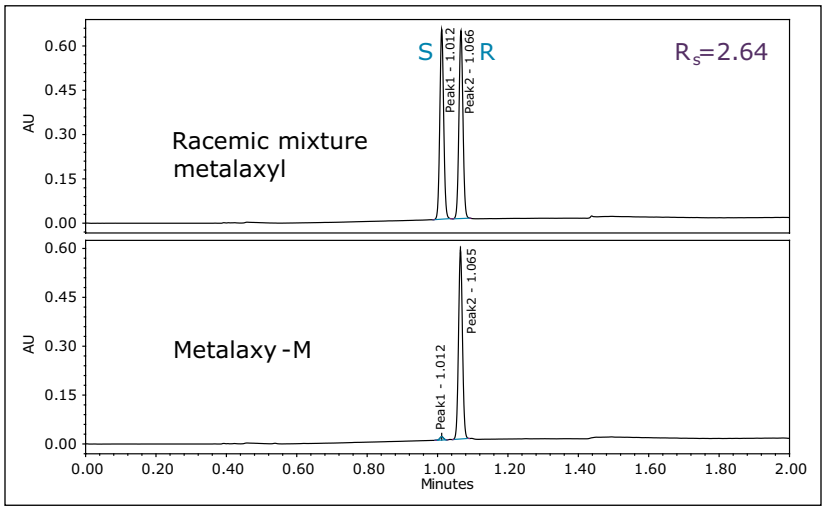

Figure 4. ACQUITY UPC² UV chromatogram at 215 nm, S, and R enantiomers of a racemic mixture of metalaxyl and metalaxyl-M standards.

The R_s between the S and R enantiomers was 2.64 (Table 1). A comprehensive survey of enantioselective separations of chiral pesticides in the literature, published in 2009, reported a normal phase isocratic method using hexane/ethanol (60/40) for the resolution of metalaxyl enantiomers. The R_s was reported to be 1.94 with both enantiomers eluting in just under 15 minutes.[10,11]

	$R_{S1,2}$	$R_{S2,3}$	$R_{S3,4}$
Metalaxyl-M	2.64	–	–
S-metolachlor	2.36	1.74	1.62
Difenoconazole	3.50	1.50	2.05

Table 1. Resolution for the stereoisomers of metalaxyl-M, S-metolachlor, and difenoconazole.

In the case of metolachlor, 95% of the herbicidal activity is reported to come from the S pair of enantiomers.[12,13] The separation of all four stereoisomers of metolachlor is shown in Figure 5 with the minimum R_s being 1.62 between peaks 3 and 4, (Table 1). The baseline resolution of the stereoisomers of metolachlor using a normal-phase separation with hexane/diethyl ether (91/9) was also reported in the 2009 article. A figure from this publication showed all four stereoisomers eluting between 20 to 30 min.[10,14] The ACQUITY UPC^2 methods reported here have significantly shorter run times for both metalaxyl and metolachlor. In addition, potentially hazardous solvents are not required.

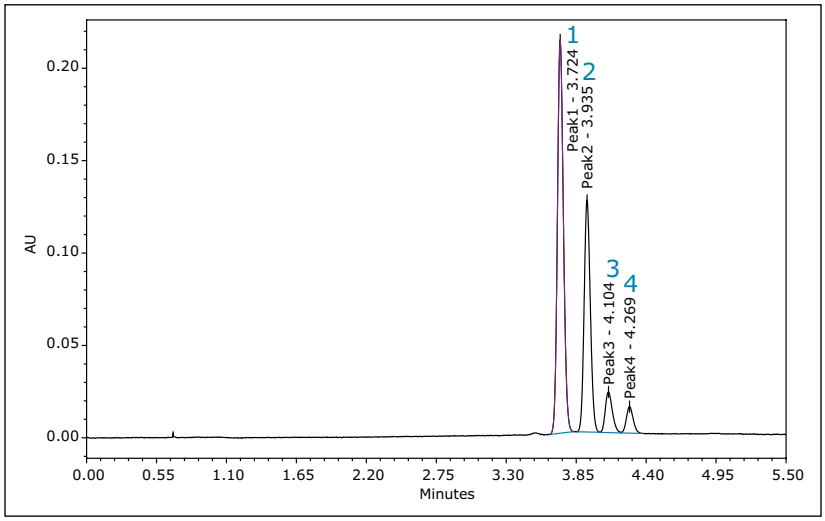

Figure 5. ACQUITY UPC^2 UV chromatogram at 220 nm using gradient elution of the S and R enantiomeric pairs of standard S-metolachlor.

The separation of the stereoisomers in a racemic mixture of difenoconazole is shown in Figure 6. The minimum R_s of 1.5 was between peaks 2 and 3. A normal-phase separation of the stereoisomers of difenoconazole using hexane/ethanol (90/10) was reported in the previously mentioned paper, resolution of the four stereoisomers took place between 30 and 55 minutes. Baseline separation (R_s>1.5) was not achieved between peaks 1,2 or 2,3 in the reported method.[10,15]

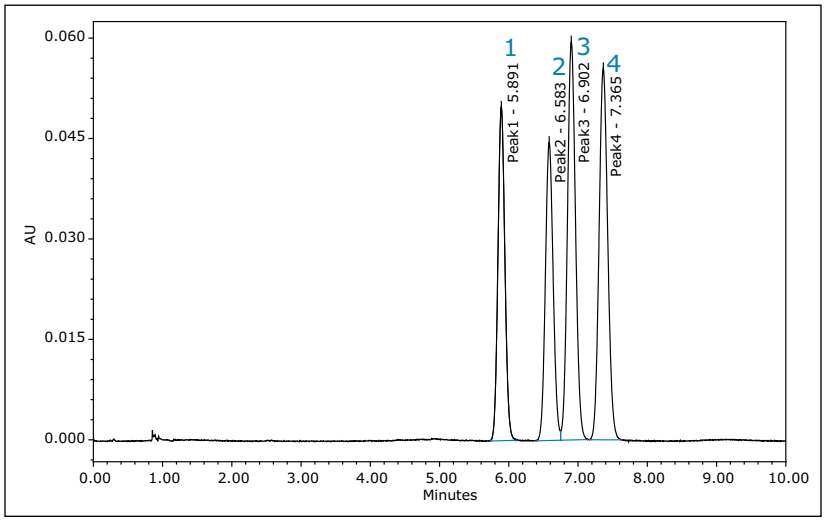

Figure 6. ACQUITY UPC^2 UV chromatogram at 235 nm using isocratic elution of the stereoisomers of difenoconazole.

The optimized ACQUITY UPC^2 methods allowed increased sample throughput and improved enantiomeric and diastereomeric resolution. In the case of metalaxyl-M (Figure 4), both enantiomers eluted in one minute; for S-metolachlor all four stereoisomers had eluted in 4.5 minutes (Figure 5). The resolution of the four difenoconazole stereoisomers used isocratic elution in under eight minutes (Figure 6), six times faster than some normal-phase methods reported in the literature. Reproducibility data (n=6) for retention time, area, area %, and height gave % RSD's less than or equal to 1.22 for all the stereoisomers of each compound, (Tables 2 to 4).

Metalaxyl-M %RSD (n=6)				
	Rt	**Area**	**%Area**	**Height**
S	0.04	1.18	1.10	0.77
R	0.03	0.61	0.03	0.66

Table 2. %RSD for six replicate injections of metalaxyl-M.

S-metolachlor %RSD (n=6)				
	Rt	**Area**	**%Area**	**Height**
Peak 1	0.11	0.22	0.09	0.77
Peak 2	0.11	0.19	0.09	0.22
Peak 3	0.07	0.33	0.27	0.29
Peak 4	0.08	1.20	1.22	0.36

Table 3. %RSD for six replicate injections of S-metolachlor.

Difenoconazole %RSD (n=6)				
	Rt	**Area**	**%Area**	**Height**
Peak 1	0.06	0.35	0.13	0.34
Peak 2	0.37	0.50	0.24	0.47
Peak 3	0.44	0.26	0.08	0.45
Peak 4	0.41	0.23	0.12	0.44

Table 4. %RSD for six replicate injections of difenoconazole.

CONCLUSIONS

In this application note we have shown the analysis of chiral pesticides using ACQUITY UPC.[2] ACQUITY UPC[2] allows high efficiency separations that can significantly increase the sample throughput when compared with traditional normal-phase separations.[10] The time taken to develop a method from the column and co-solvent screening step to the final optimized method is decreased. The methods described here use supercritical CO_2 as the primary mobile phase and predominantly 2-propanol as the organic modifier. The need to use large volumes of potentially hazardous solvents is reduced as is the cost associated with solvent waste disposal. The %RSD's obtained were comparable to those obtained by UPLC®/UV methods.

The study of enantioselective toxicity and environmental fate has previously been a challenge due to the difficulty in resolving chiral compounds. The benefit of having faster analytical methods to resolve chiral compounds means that critical information pertaining to their stereoselective behavior can be obtained more rapidly.

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Stereoselective Separation of Triazole Fungicides Using the ACQUITY UPC^2 System and ACQUITY UPC^2 Trefoil Chiral Columns

Marian Twohig and Michael O'Leary
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved enantiomeric and diastereomeric resolution.
- Shorter analysis times resulting in higher sample throughput and reduced solvent consumption compared with normal phase separations.
- Reliable and reproducible measurement of the enantiomeric and/or diastereomeric ratios.

WATERS SOLUTIONS

ACQUITY UPC2® System

ACQUITY UPC^2 Photodiode Array (PDA) Detector

Empower® 3 Software

ACQUITY UPC2 Trefoil™ AMY 1 Column

ACQUITY UPC2 Trefoil CEL1 Column

KEY WORDS

Chiral pesticides, UPC^2, enantiomer, diastereomer, chiral separation, chiral resolution, triazole fungicide, SFC, supercritical fluid chromatography, chiral columns

INTRODUCTION

The development of analytical methods for the separation of chiral compounds is important in many areas of research, as it is well known that different enantiomers are selectively biologically active.[1] Biochemical reactions can be diastereo or enantioselective. While one isomer may deliver the desired effect to the target species, the other enantiomer may be less effective to the target, completely ineffective, or cause undesirable effects. Additionally, it is known that different isomers can have very different environmental fates. It is estimated that 20 to 30% of pesticides on the market today have optical isomers, and there are reports that 40% of the pesticides used in China are chiral.[1,2] The study of enantioselectivity is important to the crop protection industry, since the knowledge of the efficacy of each individual enantiomer could facilitate a significant reduction in the total amount of pesticide applied.

In order to improve our knowledge of the stereoisomeric compositions of these substances, analytical methods that provide reliable and reproducible separations in a rapid time frame are necessary. Supercritical fluid chromatography (SFC) is known as an effective chiral separations technique that has many advantages over conventional high performance liquid chromatography (HPLC).[3,4] The properties of the supercritical fluid, such as low viscosity and high diffusivity, allow for the achievement of very high efficiency separations with shorter analysis times.[5]

In this application note we present the enantiomeric and/or diastereomeric resolutions of 12 triazole fungicides (Figure 1) using Waters® Trefoil Column Technology. Trefoil Columns use a modified polysaccharide chiral stationary phase (CSP) with a 2.5 µm particle designed for broad-spectrum chiral selectivity. Resolutions were performed using an UltraPerformance Convergence Chromatography™ (UPC^2®) System. Convergence chromatography is a complimentary separation technique to liquid chromatography, that provides orthogonal selectivity, and uses supercritical CO_2 as the primary mobile phase.

EXPERIMENTAL

Instrumentation

All separations were performed using the ACQUITY UPC^2 System. Detection was by ACQUITY UPC^2 Photodiode Array (PDA) Detector. Empower 3 Software was used for chromatographic data acquisition and processing.

Sample preparation

Racemic pesticide standards were purchased from AccuStandard (New Haven, CT) The pesticide standards were prepared in methanol at a concentration of 1 mg/mL with the exception of cyproconazole and uniconazole which were purchased as 100 µg/mL stocks in methanol and acetonitrile respectively.

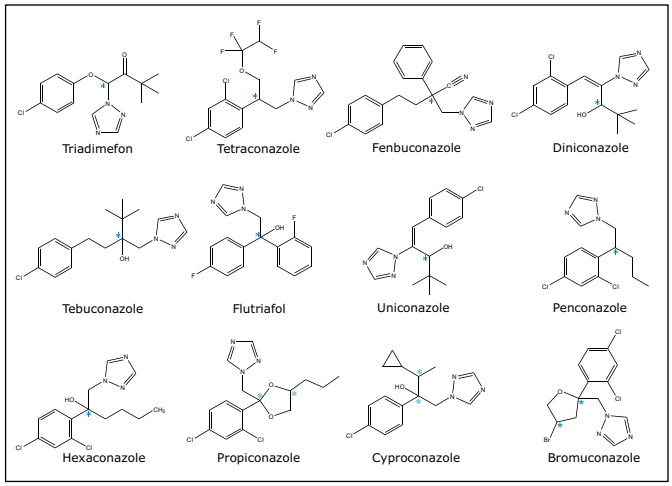

Figure 1. Structures of 12 triazole fungicides.
** denotes the stereogenic center/s.*

Summary of method conditions

	Column	Co-solvent (B)	ABPR psi/bar	Flow rate mL/min	Column temp. °C
Triadimefon	ACQUITY UPC^2 Trefoil AMY1	Methanol	1600 psi/110 bar	3.5	35
Tetraconazole	ACQUITY UPC^2 Trefoil AMY1	Methanol	1990 psi/137 bar	3.0	15
Fenbuconazole	ACQUITY UPC^2 Trefoil AMY1	Methanol	2300 psi/159 bar	3.0	35
Diniconazole	ACQUITY UPC^2 Trefoil AMY1	Methanol	1990 psi/137 bar	3.0	10
Tebuconazole	ACQUITY UPC^2 Trefoil AMY1	Methanol	2500 psi/172 bar	2.5	45
Flutriafol	ACQUITY UPC^2 Trefoil AMY1	Methanol	1990 psi/137 bar	2.5	10
Uniconazole	ACQUITY UPC^2 Trefoil AMY1	50:50 2-propanol/ethanol	2200 psi/152 bar	3.0	35
Penconazole	ACQUITY UPC^2 Trefoil AMY1	50:50 2-propanol/ethanol	1990 psi/137 bar	3.0	35
Hexaconazole	ACQUITY UPC^2 Trefoil AMY1	50:50 2-propanol/ethanol	2500 psi/172 bar	3.0	35
Propiconazole	ACQUITY UPC^2 Trefoil AMY1	50:50 2-propanol/ethanol	3000 psi/207 bar	1.5	20
Cyproconazole	ACQUITY UPC^2 Trefoil CEL1	Methanol	2200 psi/152 bar	3.0	35
Bromuconazole	ACQUITY UPC^2 Trefoil CEL1	Methanol	1500 psi/103 bar	2.0	45

Table 1. Summary of selected analysis conditions used in the study. The ACQUITY UPC^2 Trefoil AMY1 and CEL1 column dimensions were 3.0 x 150 mm, 2.5-µm.

RESULTS AND DISCUSSION

Method development for the stereoselective resolution of the technical grade fungicides began by using a generic screening gradient with a number of chiral columns and co-solvents, for example methanol, ethanol, 2-propanol, or mixtures of each. The ACQUITY UPC^2 System has multi-column switching capabilities and a choice of four co-solvents. The screening step can be completed rapidly, due to the shorter analysis times that are possible using this technique. The combination of the co-solvent and column that produced the most promising separation for each compound was then selected for further optimization. The selectivity in a chiral separation can change markedly by varying the temperature, pressure, and flow rates.[5]

Separations in chiral chromatography typically result from multiple interactions between analytes and stationary phases. These interactions can be influenced differently by changing the experimental parameters to produce desired changes in the chromatography. Consequently, each parameter including temperature, pressure, and flow rate should be systematically evaluated to investigate the individual effects each change can have on the compound resolution. A summary of the selected analysis conditions is shown in Table 1.

The chromatograms resulting from the optimized gradient separations of the racemic mixtures of triadimefon, tetraconazole, fenbuconazole, diniconazole, tebuconazole, and flutriafol are shown in Figure 2. In each case, the optimum column was a Trefoil AMY1 (3.0 x 150 mm, 2.5-µm , p/n 186007460), and the optimum co-solvent was methanol.

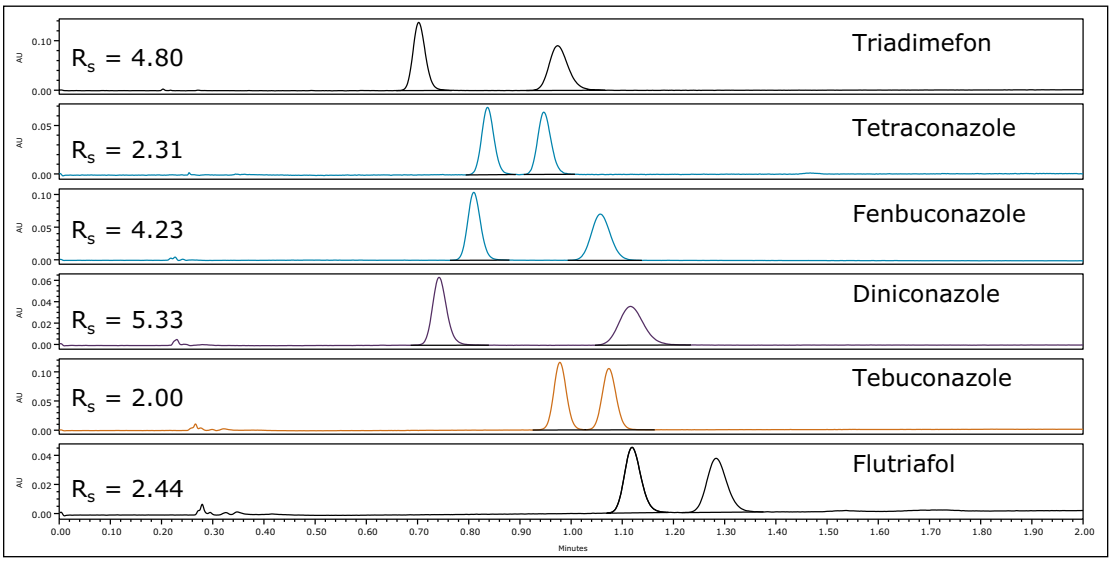

Figure 2. ACQUITY UPC^2 UV chromatograms showing the enantiomeric resolution of the triazole fungicides using an ACQUITY UPC^2 Trefoil AMY1 Column (3.0 x 150 mm, 2.5-µm), with methanol as a co-solvent. The USP resolution (R_s) values obtained are also listed (left).

Baseline R_s was achieved for all pesticides in less than 1.5 minutes. Optimized resolutions for the racemic mixtures of uniconazole, penconazole and hexaconazole are shown in Figure 3. The optimum column in these cases was also a Trefoil AMY1, 3.0 x 150 mm, 2.5-µm, and the optimum co-solvent was 50:50 2-propanol/ethanol. Baseline resolution was achieved rapidly (less than 1.2 min) for the enantiomers of each triazole fungicide.

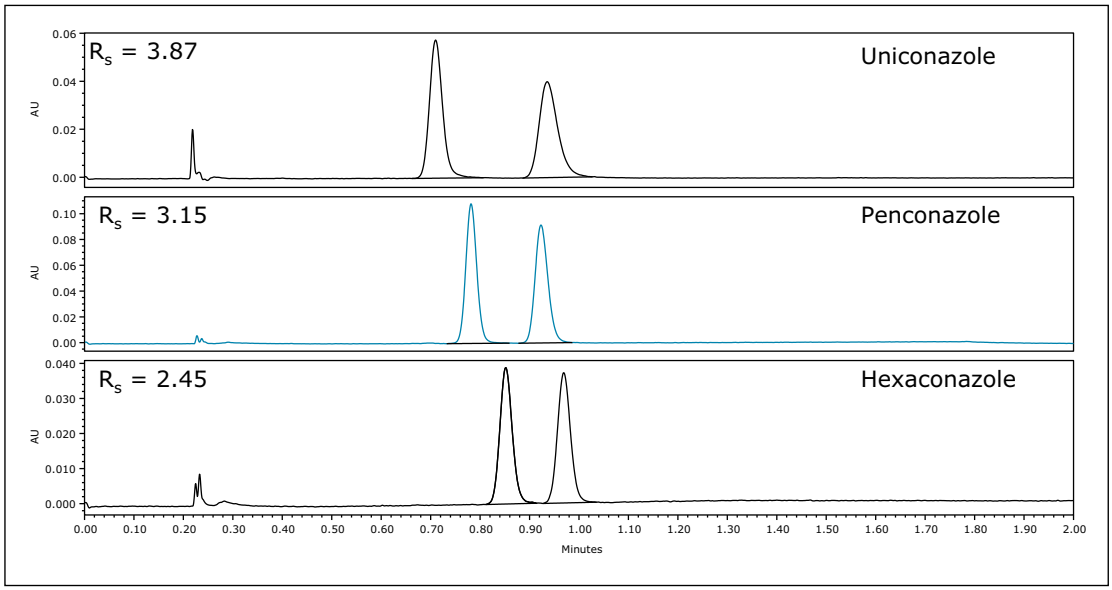

Figure 3. ACQUITY UPC² UV chromatograms showing the enantiomeric resolution of the triazole fungicide standards using an ACQUITY UPC² Trefoil AMY1 Column (3.0 x 150 mm, 2.5-µm), with 50:50 2-propanol/ethanol as a co-solvent. The R_s values achieved are also listed (left).

The chiral resolutions of propiconazole, cyproconazole, and bromuconazole, each with two chiral centers in their chemical structures, are shown in Figure 4. Despite the increase in the stereochemical complexity in the structures of these compounds, R_s values of >1.75 were achieved for all of the stereoisomers of each pesticide. The chiral separation of propiconazole is possible in less than 2.8 minutes using the ACQUITY UPC² Trefoil AMY1 CSP Column (3.0 x 150 mm, 2.5-µm, p/n 186007464).

The optimum column in the case of cyproconazole and bromuconazole was the ACQUITY UPC² Trefoil CEL1, 3.0 x 150 mm, 2.5-µm. The resolution of both triazole fungicides is possible in less than 4.5 minutes.

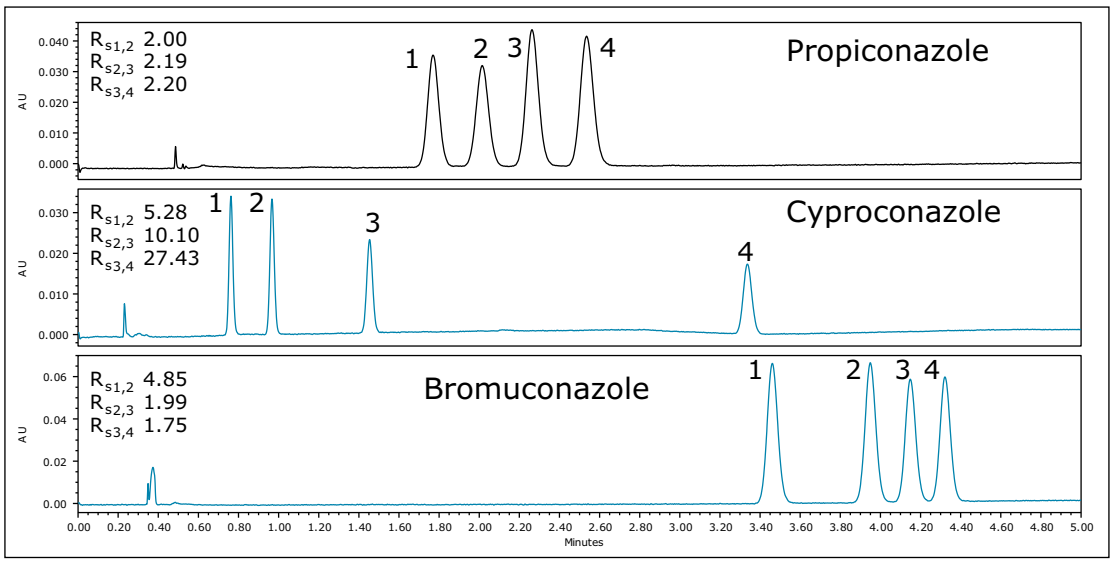

Figure 4. ACQUITY UPC² UV chromatograms showing the resolution of the enantiomers and diastereomers present in pesticide standard mixtures using an ACQUITY UPC² Trefoil AMY1 Column (3.0 x 150 mm, 2.5-µm) with 50:50 2-propanol/ethanol as a co-solvent for propiconazole, and an ACQUITY UPC² Trefoil CEL1 Column (3.0 x 150 mm, 2.5-µm) for cyproconazole and bromuconazole, with methanol as a co-solvent. The R_s obtained between the stereoisomers are also listed (left).

A review of the literature indicates that when using normal phase high performance liquid chromatography (HPLC), the chiral resolution of propiconazole is possible in 34 min, and the enantiomeric resolution of tebuconazole ranged from 17 to 45 min.[6-11] Similar resolutions were achieved for propiconazole and tebuconazole using traditional SFC, but the analysis times were reduced to 10 minutes and 10.5 minutes, respectively.[4]

The literature search also revealed two reviews[6,8] showing that the chiral resolutions of the test compounds using UPC^2 can be achieved much faster compared to reverse phase[12-19] (3 to 30X), normal phase[6-11] (8 to 40X), and conventional SFC[4,20] (3 to 10X) separations.

The optimized ACQUITY UPC^2 methods developed in this work allow increased sample throughput and improved enantiomeric resolutions, especially when compounds with multiple chiral centers are analyzed.

Reproducibility data (n=8) for retention time, area, area%, height, and USP resolution for bromuconazole are shown in Table 2. The %RSD's were less than or equal to 0.60% for all of the stereoisomers.

Bromconazole %RSD (n=8)					
	tR	Area	%Area	Height	R_s
Peak 1	0.10	0.57	0.36	0.56	
Peak 2	0.07	0.47	0.15	0.38	0.27
Peak 3	0.07	0.51	0.16	0.42	0.28
Peak 4	0.07	0.48	0.28	0.50	0.60

Table 2. %RSD for eight replicate injections of bromuconazole.

CONCLUSIONS

The study of enantioselectivity is important to the crop protection industry since the knowledge of the efficacy of a more biologically active individual enantiomer could facilitate a significant reduction in the total amount of pesticide applied and result in a more marketable product. The rapid enantioseparation of chiral pesticides has previously been challenging due to the difficulty in chromatographically resolving them in short analysis times. This application note highlights a more rapid chromatographic methodology for enantiomeric and diastereomeric separation and detection by using a combination of ACQUITY UPC^2 and Trefoil chiral columns. The result was a highly efficient stereoselective separation of 12 triazole fungicides using two CSP's. Further, the methodology shown in this work improves the sample throughput compared with LC-based chiral separations.[5-19] The %RSD's (n=8) for retention time, area, area%, height, and USP resolution for bromuconazole were less than or equal to 0.60% for all of the stereoisomers. These methods use supercritical CO_2 as the primary mobile phase and alcohol modifiers as the co-solvents. The need to use large volumes of potentially hazardous solvents that are routinely used in normal phase chiral separations is reduced, as well as the cost associated with solvent waste disposal.

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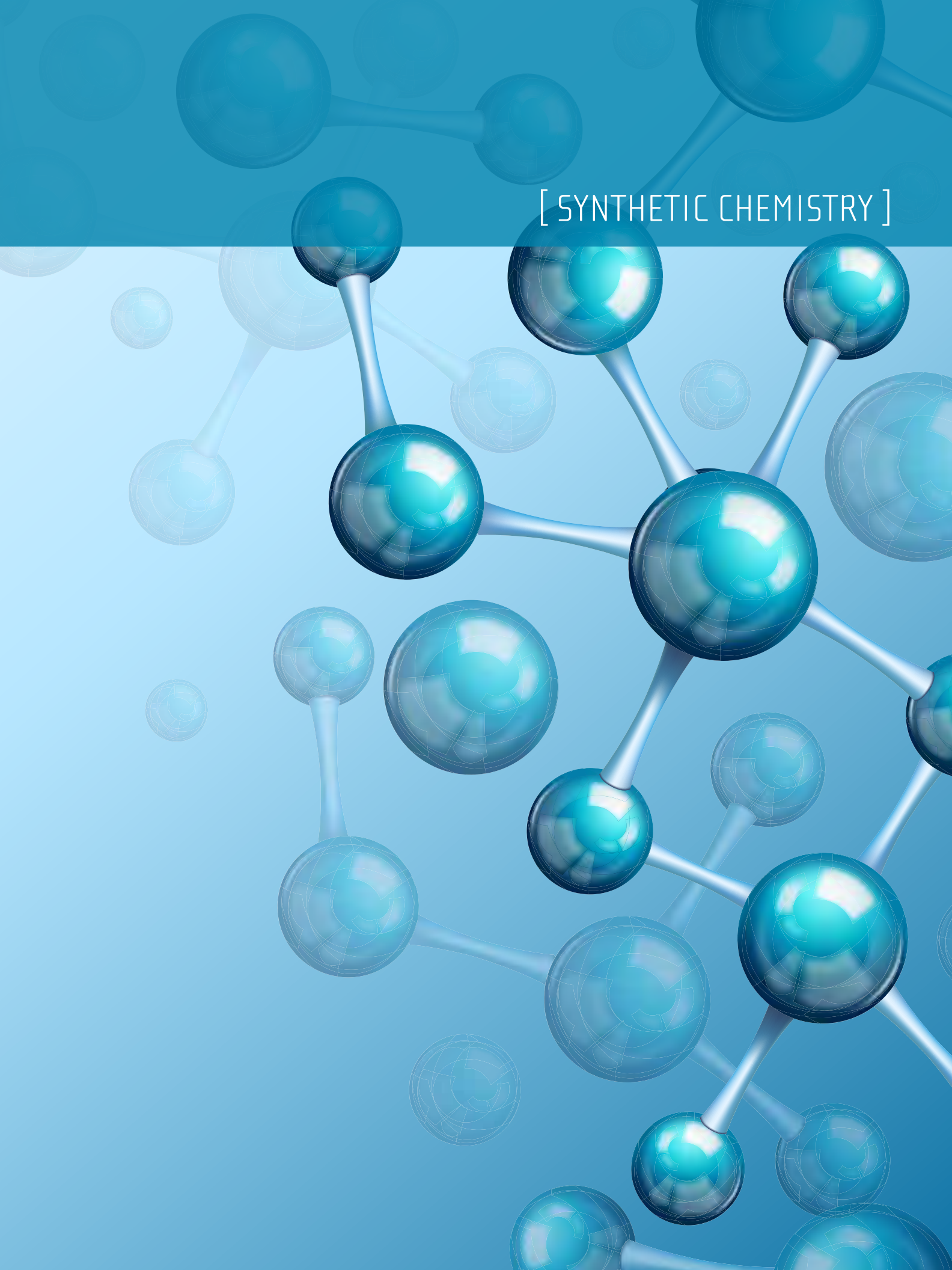

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Characterization of Impurities in the Fungicide Flutriafol Using UNIFI and UPLC-ToF-MS^E

Marian Twohig, Michael O'Leary, Peter Lee, and John McCauley
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- A comprehensive approach to the characterization of structurally related components in technical grade flutriafol.
- A rapid chromatographic separation that provides maximum component resolution.
- Accurate mass precursor and product ion information collected simultaneously increase confidence in the compound assignments.

WATERS SOLUTIONS

Screening Platform Solution with UNIFI®

ACQUITY UPLC® I-Class System

ACQUITY UPLC Photodiode Array (PDA) Detector

Xevo® G2-XS QTof Mass Spectrometer

CORTECS® Columns

KEY WORDS

Impurity identification, pesticide, impurity, agrochemical, pesticide, synthesis

INTRODUCTION

The profiling, identification, and quantification of impurities plays a critical role at all stages of both the pesticide development and manufacturing processes, and are essential requirements for the registration of crop protection products. Impurities in agrochemical products are regulated in order to ensure product safety and compliance. The structure of impurities present at or greater than 0.1% in the technical grade active substance must be identified to ensure that the overall safety of the formulated product is well understood.[1] Impurities of toxicological significance are of particular interest.

During the development process, different routes of synthesis may be evaluated, each producing its own distinct impurity profile. It is critical that these synthesis routes are thoroughly investigated with an aim to identify (or elucidate) relevant impurities alerted by the threshold criteria. Analytical investigation is often performed using a combination of LC-MS and other techniques that provide structural information, such as NMR spectroscopy. Modern, high-sensitivity exact mass instruments with time-of-flight (Tof) technology are capable of extremely fast acquisition rates and offer the opportunity to dramatically simplify the process of impurity data capture and analysis. These high acquisition rates allow mass spectrometry to be coupled together with UltraPerformance Liquid Chromatography (UPLC®), thereby greatly facilitating the analysis of complex samples. Additionally, Waters' proprietary Q-Tof™ technology enables the simultaneous collection of low and high collision energy (CE) data, otherwise referred to as "MS^E",[2] allowing the capture and interpretation of precursor and product ion data from a single analytical injection. As this data is collected with a high degree of mass accuracy, elemental compositions can be obtained for both intact molecular ions (*e.g.*, $M+H^+$) and structurally significant fragments greatly aiding structural elucidation.

EXPERIMENTAL

Instrumentation

Sample preparation

The stock solution of flutriafol (1 mg/mL) was prepared in methanol.

UPLC conditions

UPLC system:	ACQUITY UPLC I-Class
Column:	CORTECS C_{18} 2.1 x 100 mm, 1.6 µm
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid
Flow rate:	0.6 mL/min
Column temp.:	50 °C
Injection volume:	1 µL
Gradient:	0 min 10% B, 10 min 90% B return to initial conditions

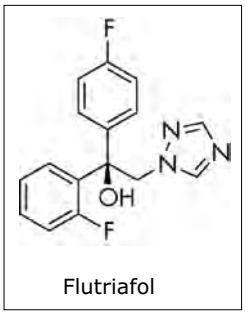
PDA conditions

Detector:	ACQUITY UPLC PDA
Wavelength:	210 to 400 nm
Sampling rate:	20 Hz

MS conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI+
Analyzer mode:	MS^E in resolution mode
Capillary voltage:	1.0 kV
Cone voltage:	25 V
Desolvation gas temp.:	450 °C
Source temp.:	150 °C
Cone gas flow:	50 L/hr
Desolvation gas:	900L/hr
MS^E low collision energy (CE):	4 eV
MS^E high collision energy ramp:	15 to 30 eV
MS scan range:	50 to 950 *m/z*
Scan time:	0.15 s

In this application note, Waters® ACQUITY UPLC I-Class System coupled to the Xevo G2-XS QTof and the ACQUITY UPLC PDA Detector were used to analyze technical-grade flutriafol material. Flutriafol, shown in Figure 1, is a triazole fungicide that is used in the treatment of a broad spectrum of diseases in cereal crops.[3] The Screening Platform Solution with UNIFI was used to screen the sample and expedite the discovery of unknown components of interest, as well as to confirm the presence of the active ingredient (AI). Using the data evaluation tools available in UNIFI, relationships between the unknown components were easily visualized and the impurity relationship to flutriafol was hypothesized. The MS data from the impurity profile can be later be used to further refine the synthetic strategy.



Flutriafol

Figure 1. Structure of flutriafol.

RESULTS AND DISCUSSION

UPLC separation using the CORTECS C_{18} Column provided maximum component resolution. Three minor chromatographic peaks (peaks 1–3) and flutriafol (peak 4) were detected in the UV at 220 nm, Figure 2. PDA detection allowed the area% contributions of peaks 1–3 to be determined relative to the AI, where they each exceeded the threshold of >0.1%. MS detection was used to perform the initial structural elucidation of the impurities.

In the component summary of the UNIFI review window, candidate masses were tabulated (Figure 3) and the identified components are listed.

The precursor and product MS^E spectral data for each candidate mass are automatically extracted and can be viewed simultaneously in the spectrum window (Figure 3). If adducts for the precursor ion are present they are automatically assigned. UNIFI also performs a *fragment match* when a structure is available, intelligently assigning likely structures to high CE fragments.

The flutriafol was identified and represented as an extracted ion chromatogram (XIC) using specific information such as compound name, retention time (t_R), mol files, and fragment ions that were generated from prior analyses of the fungicide and entered into the UNIFI Scientific Library. This approach can be used to build the analysis method prior to data acquisition (Figure 4). UNIFI's Scientific Library can store structures and begin retaining information associated with the individual library entries. Parent compounds and impurities of interest can be stored in the library for later searching and retrieval.

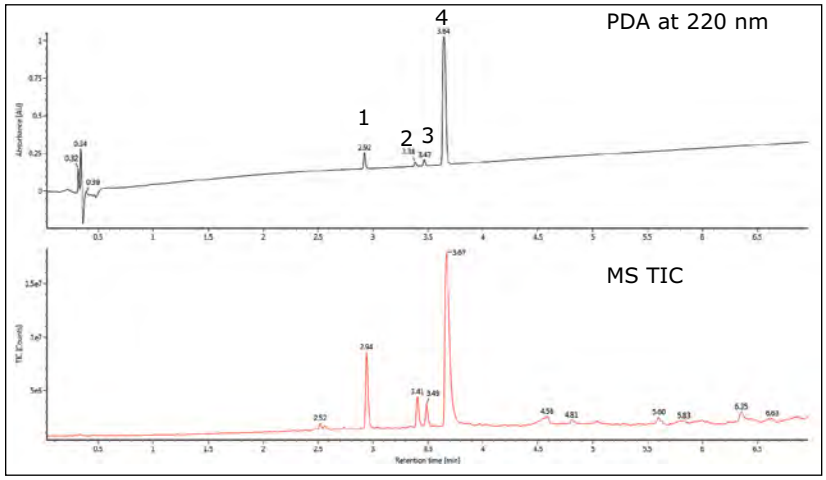


Figure 2. PDA chromatogram at 220 nm (top), showing flutriafol (peak 4) and three minor components (peaks 1–3). MS total ion chromatogram (TIC) is shown beneath.

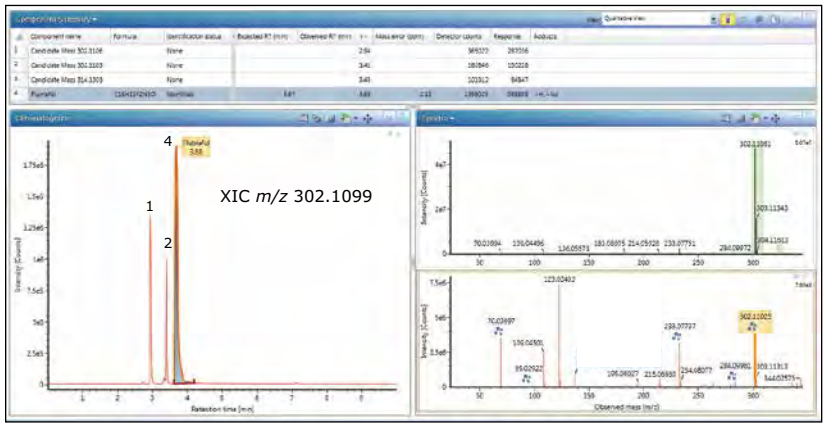


Figure 3. UNIFI review window shows the component summary, extracted ion chromatograms (XIC) for the identified flutriafol (m/z 302.1099), and the isobaric components 1 and 2. MS^E fragmentation and intact precursor spectral information for flutriafol are also shown.

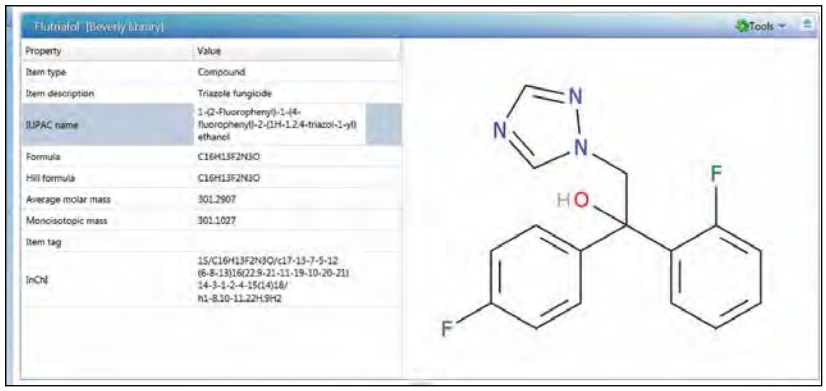


Figure 4. UNIFI Scientific Library entry for flutriafol.

The peaks labeled 1 and 2 from Figure 2 eluted at 2.94 min and 3.41 min respectively. Both components were extracted as XICs, as the two peaks have an *m/z* that is within the flutriafol target mass tolerance of 3 ppm set in the processing method (Figure 3).

Candidate masses can be investigated using the elucidation toolset in UNIFI which contains a comprehensive suite of structural elucidation tools that can be utilized by selecting any candidate mass from the component summary list. The elucidation toolset allows precursors and fragment ions to be interrogated more intensively. This includes *ChemSpider* search (*www.chemspider.com, The Royal Society of Chemistry*), isotope modeling, and elemental composition tools. Discovery tools for related ions using mass defect, common fragment, and neutral losses are also available.

The structural elucidation tools indicated that both components 1 and 2 had identical elemental composition to the flutriafol, $C_{16}H_{13}F_2N_3O$ (Figure 5). In addition the high energy MS^E data indicated that the components had common fragment ions at different ratios compared with the AI (Figure 3). The existence of the high energy product ion data along with the intact precursor increased the confidence in the identifications.

Based on these observations it is likely that components 1 and 2 are isomers of the AI.

Using UNIFI's common fragment search function, components that share common structural features can be efficiently extracted from the data. By selecting prominent fragments in the spectrum of component 1, including the signature fragment originating from the triazole ring *m/z* 70.0399 and *m/z* 109.0450, we can see an additional chromatographic peak at t_R 3.49 minutes was extracted using the same product ions, as shown in Figure 6.

This component (peak 3), has an *m/z* of 314.1303 and has been tabulated in the component summary, it was investigated in the same way as the previous example (Figure 7).

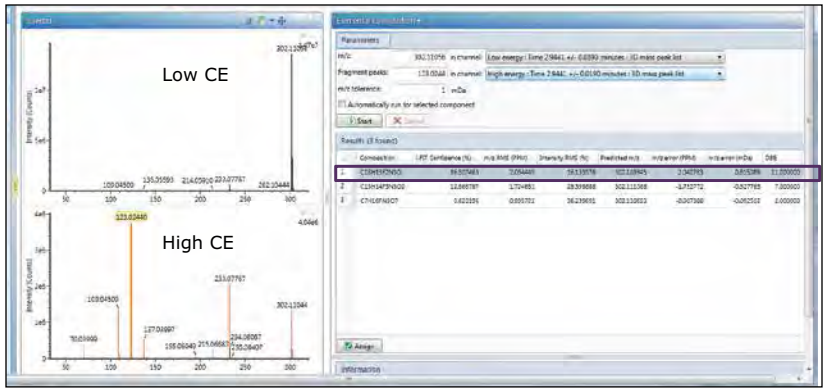


Figure 5. The elemental composition $C_{16}H_{13}F_2N_3O$ of the candidate mass m/z 302.1106 (highlighted in the purple rectangle) is determined using the elucidation toolset. The precursor (top) and product ion spectra (beneath) are shown at the left of this screen capture.

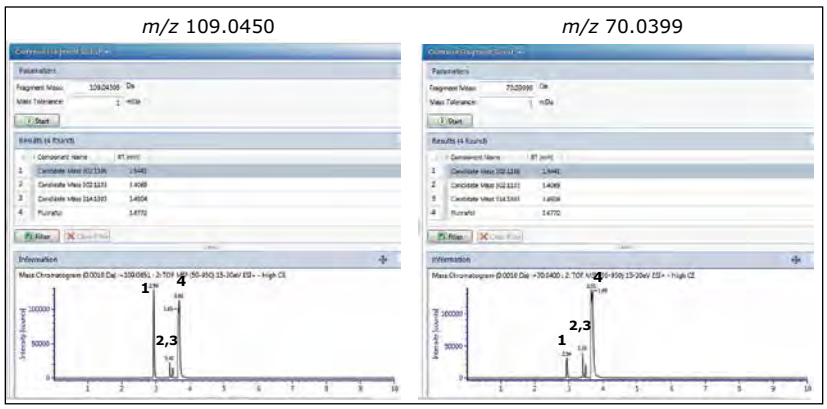


Figure 6. Common fragment search for m/z 109.0450 and 70.0399, which was used to efficiently search the technical grade flutriafol for components with common structural features.

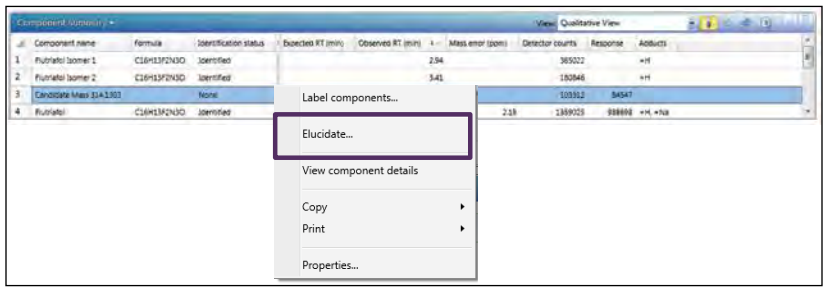


Figure 7. The unknown mass m/z 314.1303 is sent to the elucidation toolset for further investigation.

The elemental composition of the unknown mass was determined to be $C_{17}H_{16}FN_3O_2$. The common fragment search function indicated the component shared common structural characteristics with the flutriafol. The elemental composition of component 3 was searched using the *ChemSpider* Search function, which is embedded in the elucidation toolset.

A suitable match was not made based on the inability to assign all fragments, including the common fragments simultaneously within the same structure proposal. Therefore, an alternative route to assign the structure was carried out, using a combination of elemental composition for the individual fragmentation spectra of component 3, as well as confirmation of the fragments using MS/MS, knowledge of the synthetic route, and a comparison with the elemental composition of the fragmentation spectra for the flutriafol. The proposed structure was then used to assign probable structures to the product ions (Figure 8).

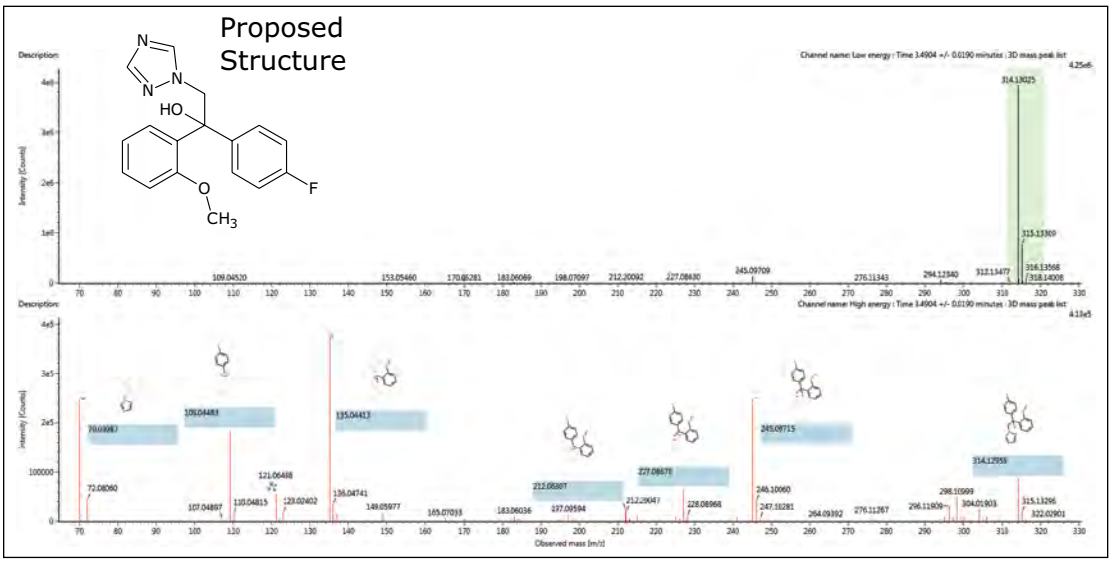


Figure 8. Structure proposal for component 3, MS^E intact precursor and fragmentation spectra with UNIFI's fragment match structural assignments.

Based on the general structural proposal (inset) the fragments were assigned using the UNIFI fragment match tool. Absolute confirmation of the structure would require scale up, trace isolation followed characterization using appropriate techniques, such as NMR spectroscopy.

The components can be added to the UNIFI Scientific Library, including any known information (retention time, known fragments, etc.) so that they can be tracked across further syntheses.

CONCLUSIONS

During chemical synthesis residual starting products, intermediates, or reaction by-products can lead to impurities in the final technical grade materials. Identification of the components can help to optimize the synthetic process. Minor components present in the technical grade flutriafol were detected in the PDA at 220 nm. The unidentified components were characterized using UPLC coupled with ToF MS^E and the UNIFI Screening Platform Solution. Two components were tentatively identified as isomers of the active ingredient, and a third unknown component was also observed. Using the elucidation toolset within UNIFI, the unknown component was found to have structural features that were common to flutriafol. Based on the elemental composition of the fragments, knowledge of the synthetic route, and the structure of the active ingredient, a structure for the unknown component having *m/z* 314.1303 was tentatively proposed. This tentative structure is in agreement with all of the data that is currently available. Future impurity isolation studies will allow for the definitive determination of the structures of these impurities.

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Enantioseparation of Metalaxyl Using ACQUITY UPC^2 and Small-Scale Purification Using the Investigator SFC System

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved enantiomeric and diasteriomeric resolution and shorter analysis times can be obtained using the ACQUITY UPC^2® System, resulting in higher sample throughput and decreased analytical method development time.
- The Investigator SFC System is a versatile and cost-effective system for laboratories routinely performing chiral analysis and small-scale purification.
- A typical workflow for small-scale chiral purification, from method development, method optimization, and scale-up, to post-purification analysis can be accomplished rapidly.
- This workflow is ideal for industries facing escalating pressure for improved productivity while adhering to strict deadlines and budget constraints.

WATERS SOLUTIONS

ACQUITY® UltraPerformance Convergence Chromatograph System (UPC2®)

ACQUITY UPLC® Photodiode Array (PDA) Detector

Investigator SFC System

Analytical-2-Prep Column Oven

2998 Photodiode Array (PDA) Detector

ChromScope™ Software

KEY WORDS

SFC, chiral pesticides, chiral purification, small scale purification, enantiomer, metalaxyl, agrochemicals

INTRODUCTION

Knowledge of the efficacy of the individual enantiomers of pesticides is of great interest to agrochemical companies. The benefits of using these products can be realized only when the optimal application rates are determined and when the appropriate metabolic and toxicological evaluations are performed. However, the ability to separate the enantiomers is required in order to study the effects of each individual isomer. Experiments concerning the steroeselective behavior of the individual pure isomers of chiral pesticides depend on the development of the necessary preparative separation technology that enables them to be readily separated and isolated as single stereoisomers.

Although traditionally dominated by liquid chromatography (LC), there is an increasing trend toward using supercritical fluid chromatography (SFC) to replace normal phase LC for chiral purifications from the semi-preparative scale up to the kilogram scale. Due to the higher diffusivity and lower viscosity of supercritical fluid, SFC often provides a three- to eight-fold faster separation than normal phase HPLC, resulting in a measurable increase in productivity. Compared to normal phase LC, SFC also offers unique selectivity, less organic solvent consumption and related waste removal, smaller collection volume, and faster post-purification dry-down time. Generally speaking, SFC is as a more cost-effective and environmentally friendly preparative chromatographic technique.

Development of SFC purification begins with analytical method development to identify optimal stationary phases and mobile phases. This step can be completed rapidly using the Waters® ACQUITY UPC^2 System, and the resulting method can then be scaled up for preparative purifications using the Investigator SFC System.

Combined with the technical advantages of SFC, this workflow is ideal for agrochemical companies facing escalating pressure to discover, develop, and release new products into the marketplace while adhering to strict deadlines and budget constraints.

Metalaxyl, a phenylamide fungicide, with one chiral center in its chemical structure (Figure 1), owes its fungicidal activity predominantly to the R enantiomer.[1] In this application note, we present the chiral resolution of metalaxyl using the ACQUITY UPC^2 System, followed by scaling to a semi-preparative purification using the Investigator SFC System.

EXPERIMENTAL

Analytical chromatography

Analytical system:	ACQUITY UPC^2
Column:	Chiralpak IA-3, 4.6 x 150 mm, 3 µm
Flow rate:	3.5 mL/min
Mobile phase composition:	4% 2-propanol in CO_2
ABPR:	2000 psi/138 bar
Column temp.:	55 °C
Detector:	ACQUITY UPLC PDA
UV detection:	215 nm

Preparative chromatography

Prep system:	Investigator SFC
Column:	Chiralpak IA, 10 x 150 mm, 5 µm
Flow rate:	12 mL/min
Mobile phase composition:	8% 2-propanol in CO_2
ABPR:	1600 psi/110 bar
Column temp.:	30 °C
Detector:	2998 PDA
UV detection:	215 nm

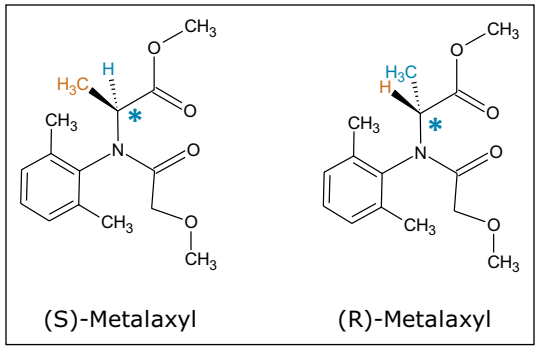

Figure 1. Structure of the metalaxyl enantiomers. The asterisk denotes the stereogenic center.

Instrumentation

Analytical experiments were performed on an ACQUITY UPC^2 System with PDA detection at 215 nm. Semi-prep experiments were performed on the Investigator SFC System. The system consists of the following: Fluid Delivery Module (FDM), Automated Back Pressure Regulator (ABPR), Alias Autosampler, 10-port Analytical-2-Prep Column Oven, 2998 PDA Detector, make-up pump, and six-position Fraction Collection Module. The system is controlled by ChromScope Software.

Sample preparation

A racemic (R, S)-metalaxyl standard was obtained from Sigma-Aldrich (St. Louis, MO). The sample was dissolved in methanol at 1 mg/mL and 20 mg/mL, for analytical and preparative injections, respectively.

Analytical chromatography

A Chiralpak IA-3 (4.6 x 150 mm, 3 µm) was used for the analytical separation.

RESULTS AND DISCUSSION

SFC purification often starts with gradient screening of multiple chiral stationary phases and mobile phases. After the column and solvent conditions that provide the highest enantioselectivity for the analyte are determined, the gradient method is converted to an isocratic separation. The ACQUITY UPC^2 System has multi-column switching capabilities and a choice of four co-solvents. The method development process can be completed rapidly due to the shorter analysis times that are possible using this technique. The IA-3 column yielded the highest resolution between the enantiomeric pair (Figure 2). It was, therefore, chosen for the remaining experiments.

Isocratic conditions are necessary for employing stacked injections in preparative SFC for high productivity. Resolution and run time, the two competing factors for overall productivity, should be carefully considered when developing an appropriate isocratic method. Typically, a higher percentage of co-solvent leads to a shorter run time but also to reduced resolution. Resolution is also affected in preparative chromatography by larger mass/volume injections. A successful isocratic method often involves a compromise between run time and resolution which is determined experimentally. In this case, an isocratic eluent containing 4% co-solvent appeared to be a suitable choice on the analytical scale.

Scale up

The major differences between the analytical and semi-prep run parameters are due to the different particle sizes used in the columns (3 µm versus 5 µm), and to the differences in system volumes between the UPC^2 and the Investigator systems. The run parameters were adjusted to account for these differences to give favorable resolution, loading and run times.

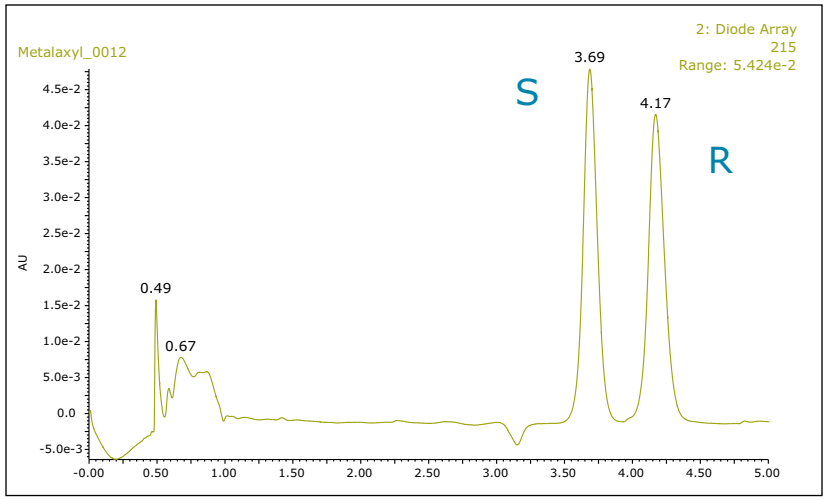

Figure 2. ACQUITY UPC^2 PDA extracted wavelength chromatogram (215 nm) using an analytical IA-3 column.

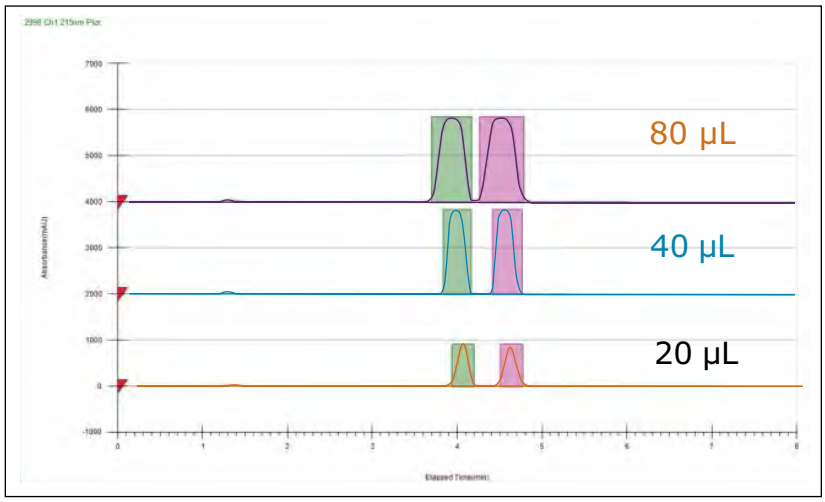

Figure 3. SFC chromatograms of the (R, S)-metalaxyl obtained using a 10-mm I.D. IA column with injection volumes of 20 µL, 40 µL, and 80 µL. The sample concentration was 2 mg/mL.

A loading study was then conducted on a 10-mm I.D. x 150 mm IA column to determine the maximum loading for preparative runs. The results are shown in Figure 3. The injection volumes used were 20 µL, 40 µL, and 80 µL. At 80 µL, the metalaxyl enantiomers were still separated. The maximum effective loading was 80 µL or 1.60 mg in mass per injection.

Stacked injection is an effective means to improve productivity without compromising chromatographic efficiency. Figure 4 shows the SFC chromatograms of (R, S)-metalaxyl (20 mg/mL) from a sequence of five stacked injections. Each injection was 80 µL of a 20 mg/mL racemic solution. A total of 8 mg (4 mg for each enantiomer) was purified in the five stacked injections over 20 minutes. The total solvent use was 20 min x 8% x 12 mL/min = ~19 mL. Performed under isocratic conditions, injections are made during the course of chromatography so that the first peak from a subsequent injection elutes off the column adjacent to the last peak of the preceding injection. Additionally, resolution is affected in preparative chromatography by larger mass/volume injections. The chromatographic space is efficiently populated by peaks from consecutive injections, resulting in noticeable savings of both time and solvents. ChromScope Software manages the injection and collection intervals such that fractions are predictably collected efficiently.

Sample purification was confirmed by analyzing the two collected fractions using the UPC^2 isocratic analytical method and the resulting chromatograms are shown in Figure 5. Both fractions showed >99% purity. Absolute configurations were assigned using a commercially available standard of metalaxyl-M (the (R) isomer).

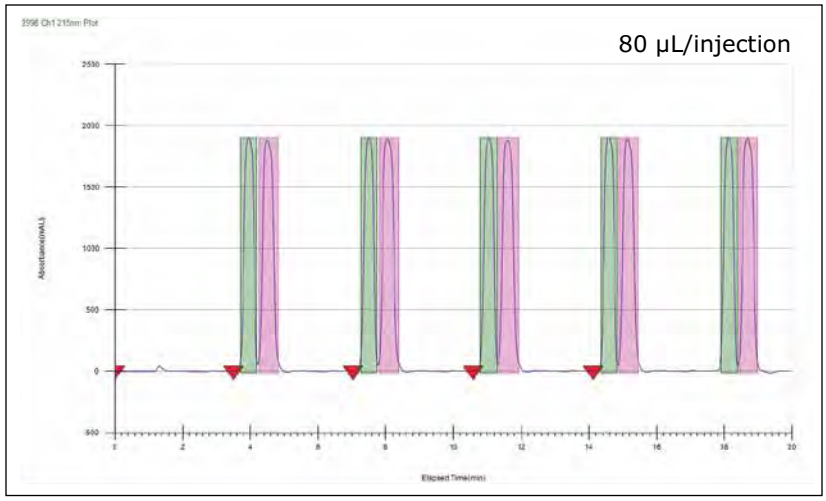

Figure 4. SFC chromatogram of the (R, S)-metalaxyl sample from a sequence of five stacked injections. The shaded areas represent collected fractions. The red triangle represents the injection mark.

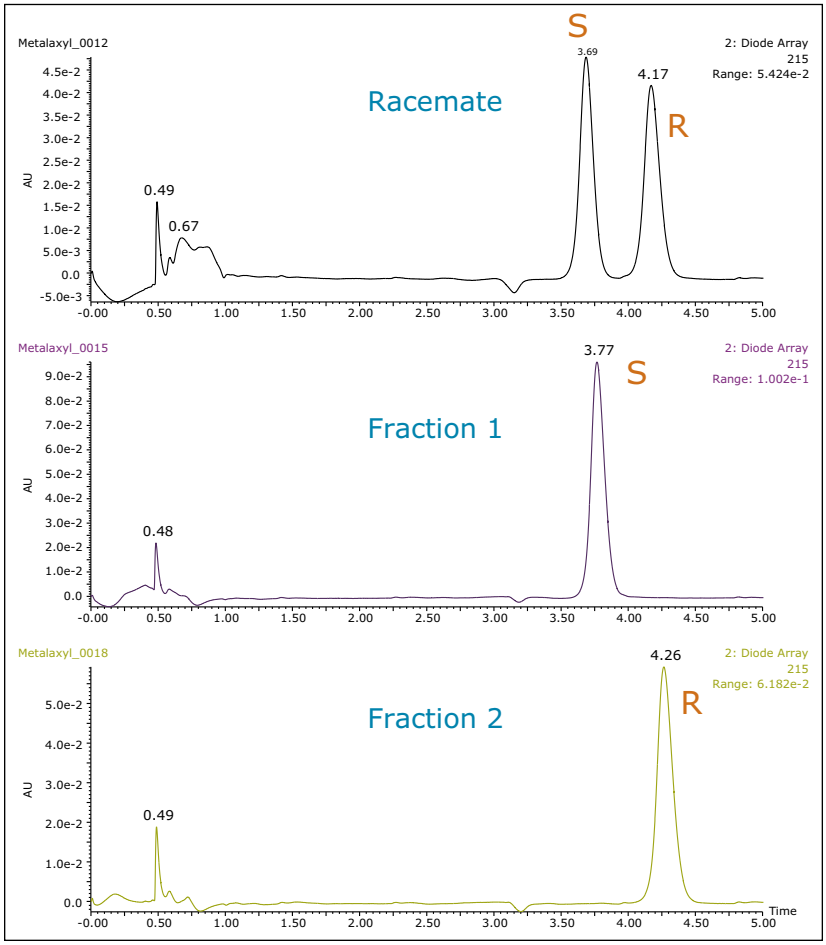

Figure 5. Analytical SFC chromatograms of the collected fractions showing >99% enantiomeric purity.

CONCLUSIONS

Using racemic metalaxyl as a model compound, the Investigator SFC System was shown to be a powerful tool in meeting the challenges of chiral separation and purification on a single platform. Chiral screening using the ACQUITY UPC^2 System quickly determined the optimal column for the separation of R- and S-metalaxyl. An isocratic method was developed to take advantage of stacked injection purification and the method was easily scaled up following logical principles concerning the preservation of resolution, maintaining reasonable run times and the maximization of loading. Stacked injections using the SFC technique allowed for the purification of 24 mg/hour, yielding high purity fractions of the metalxyl enantiomers in a smaller volume of organic solvent than would be traditionally used in LC purification. Longer sequences of a larger number of stacked injections could also be employed to give more purified product. The complete workflow of SFC in chiral screening, method development, scale-up, and efficient small scale prep using stacked injections has been successfully demonstrated using UPC^2 in conjunction with the use of the Investigator SFC System, meeting the increasing demand for fast chiral resolutions. Reduced solvent consumption coupled with reduced dry-down time has a positive impact on the laboratory by increasing the throughput while reducing the cost of processing each sample.[2]

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Isolating Trace Impurities for Structural Elucidation in a Commercial Fungicide Formulation Using Preparative SFC

John McCauley and Marian Twohig
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Supercritical fluid chromatography (SFC) offers high speed and efficiency, as well as the environmentally sustainable benefit of lower solvent consumption.
- This workflow is ideal for industries facing escalating pressure for improved productivity while adhering to strict deadlines and budget constraints.
- Improved enantiomeric and diasteriomeric resolution and shorter analysis times can be obtained using the ACQUITY UPC^{2}® System.

WATERS SOLUTIONS

ACQUITY® UltraPerformance Convergence Chromatography System™(UPC2®)

SFC 80q Preparative System

Prep 100q SFC System

2489 UV/Visible (UV/Vis) Detector

2998 PDA Detector

ACQUITY UPC2 BEH Column

ACQUITY UPC2 Trefoil™ AMY1 Column

Viridis® SFC Column

MassLynx® Software

SQ Detector 2

KEY WORDS

Agrochemicals, SFC, pesticides, chiral purification, enantiomer, propiconazole, triazole fungicide, impurity identification, trace impurity, NMR, structural characterization

INTRODUCTION

Trace impurities in synthetic products that interact with human end users or may have an undesirable environmental fate are regulated by various government agencies such as FDA and EPA. As a result, impurity separation and their structural identification are important research functions for many industries, including pharmaceutical, agrochemical, food, and consumer products. Full chemical identification requires structural elucidation of the separated compound using high resolution mass spectrometry (HRMS). However, MS alone is often insufficient to unambiguously identify a compound, especially in the case of isomers. This often necessitates obtaining the isolated pure compounds of interest using purification procedures for study using NMR spectroscopy.

In this application note, we describe how a workflow to achieve the full structural elucidation of trace impurities can be implemented using preparative supercritical fluid chromatography to isolate trace impurities. A commercial formulation of the fungicide propiconazole was used as an example to demonstrate this workflow.[1] Propiconazole has the structural potential for the existence of several stereoisomers. The propiconazole product used contains isomers (Figure 1) and also related trace impurities at approximately the 1% level (Figure 2). Some propiconazole impurities have previously been structurally identified.[2] The SFC based workflow described here is generally applicable for impurity isolation and offers many advantages, including high speed and efficiency, fast dry-down, quick turnaround time, as well as the environmentally sustainable benefit of lower solvent consumption.[3]

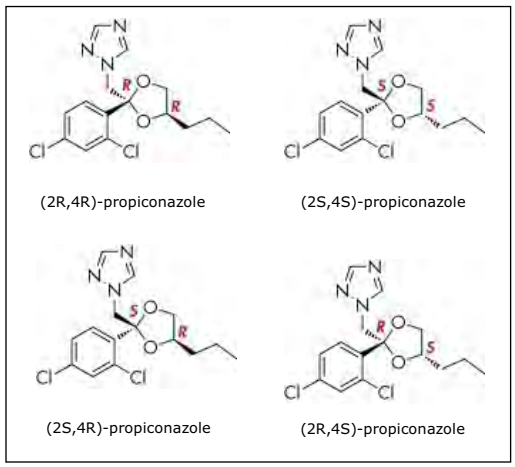

Figure 1. Structures of the propiconazole stereoisomers.

EXPERIMENTAL

Sample preparation

A representative sample was obtained from a commercial formulation containing 1.55% of propiconazole and its trace impurities, using liquid-liquid extraction with dichloromethane (DCM) and a 5% sodium bicarbonate solution ($NaHCO_3$).

Analytical and preparative chromatography conditions

(See Table 1)

Mass spectral detection was performed on a Waters SQ Detector 2 in ESI mode scanning from 200 to 700 Da with a capillary voltage of 3 kV, cone voltage of 30 V, source temp. of 150 °C, and a desolvation temp. of 450 °C using an extracted ion at 342 Da, $(M+H)^+$.

NMR methods

^1H ^{13}C, and 2D NMR spectra were acquired at 400 MHz using a Bruker Avance III spectrometer at the COSMIC Laboratory at Old Dominion University in Norfolk, VA. Assignments were based on chemical shifts, splitting patterns, integration of the peaks, coupling constants, coupling patterns in a 2D COSY spectrum (Correlated Spectroscopy) and one-bond carbon-proton coupling correlations observed in an HSQC spectrum (Heteronuclear Single Quantum Coherence) edited for carbon multiplicities. In a 2D NOESY spectrum (Nuclear Overhauser Effect Spectroscopy), NOE (Nuclear Overhauser Effect) was observed between the protons at carbons 11 and 14 of the triazole and the protons on carbon 6. The same NOE signal was observed in a 1D NOE spectrum with selective irradiation of the triazole protons in impurity peak 1 from the ACQUITY UPC^2 BEH Column. There were no equivalent NOE signals observed in impurity peak 2.

	Achiral UPC^2 Analysis	Chiral UPC^2 Analysis	Achiral SFC 80 Prep	Chiral SFC 100 Prep
Separation mode	Isocratic	Isocratic	Isocratic	Isocratic
Column	ACQUITY UPC^2 BEH 3.0 x 150 mm, 1.7-µm	ACQUITY UPC^2 Trefoil AMY1 3.0 x 150 mm, 2.5-µm	Waters Viridis BEH 19 x 150 mm, 5-µm	Chiralpak IC or AD-H 30 x 150 mm, 5-µm
Co-solvent/ Method conditions	Methanol 6% Methanol in CO_2	2-Propanol/Ethanol 10% iPrOH/EtOH in CO_2	Methanol 5% Methanol in CO_2	Methanol IC: 18% Methanol in CO_2 AD-H: 6% Methanol in CO_2
ABPR	120 bar	137 bar	120 bar	120 bar
Flow rate	1.5 mL/min	2.0 mL/min	70 mL/min	100 mL/min
UV detection	222 nm	222 nm	222 nm	222 nm
Column temp.	40 °C	45 °C	40 °C	40 °C

Table 1. A summary of analytical UPC^2 and preparative chromatographic conditions.The use of both the SFC 80q Preparative System and the Prep 100q SFC System was based on the dimensions of the columns used in this study.

RESULTS AND DISCUSSION

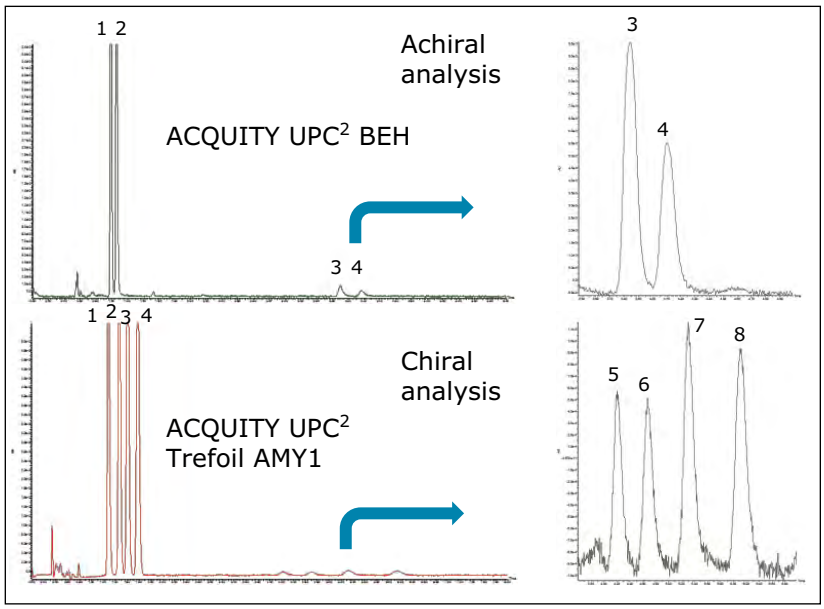

Figure 2. Achiral UPC^2 analysis of the propiconazole in the pesticide formulation sample using an ACQUITY UPC^2 BEH Column showing two peaks for the propiconazole and two impurity peaks (top trace). Lower trace: chiral analysis using an ACQUITY UPC^2 Trefoil AMY1 Column showing four peaks for propiconazole and four peaks for the trace impurities.

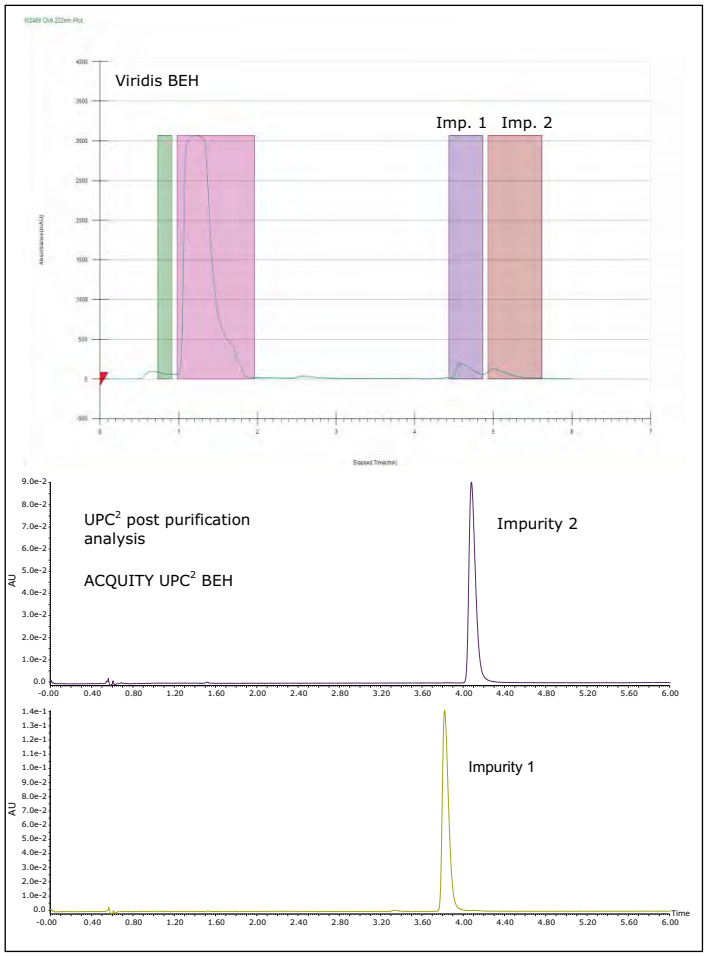

Figure 3. Preparative collections of trace impurity peaks using a 19 mm I.D. Viridis BEH Column (top trace). UPC^2 analysis of the two impurity peak batches resulting from the SFC 80q preparative analysis demonstrate that these collections resulted in >98% purity for each impurity fraction (lower trace).

A commercial fungicide formulation (150 mL) containing propiconazole as the active ingredient at a concentration of 1.55% was suspended in 5% $NaHCO_3$ solution then extracted with DCM three times. The combined extracts were dried over sodium sulphate (Na_2SO_4), filtered, then concentrated. The expected amount (2.3 g) of active ingredient (AI) was contained in 4.5 g of crude extract that contained other inactive ingredients including various surfactants. These crude extracts were then analyzed using UPC^2. In both achiral and chiral modes (Figure 2) trace impurities at <1% of the amount of the original 1.55% claim were observed.

Methods were then developed to scale separations to preparative chromatography. The achiral prep chromatography was carried out using an SFC 80q Preparative System with a 19 mm I.D. Viridis BEH Column. The analysis showed that the two impurity peaks were obtained in 98% purity (Figure 3).

Sufficient pure material was collected both to enable full structural assignments of the trace impurities as well as to carry out further chiral separations of each peak into their individual enantiomers. MS analysis confirmed that the two isolated impurity peaks were isobaric with each other as well as with the main ingredient suggesting that they were indeed structural isomers.

^{1}H, ^{13}C, 2D, and NOE NMR experiments revealed that these two impurities differed from propiconazole itself by the nitrogen attachment point of the triazole moiety to the methylene group on the dioxolane ring (Figure 4). This result is clearly evident due to symmetry in the NMR. The propiconazole, being attached at the N adjacent to the other N is not symmetric, while in the impurity the N has a C atom adjacent on both sides of the nitrogen, giving symmetry to the triazole moiety and a simpler NMR. The assignment of the cis and trans isomers results from the fact that a strong NOE is seen in both a 2D NOESY NMR experiment as well as in a 1D NOE spectrum between the protons attached to carbons 11 and 14 and those on carbon 6 in the isomer assigned as cis, while this same NOE was not observed in the NMR spectrum of the isomer assigned as trans.

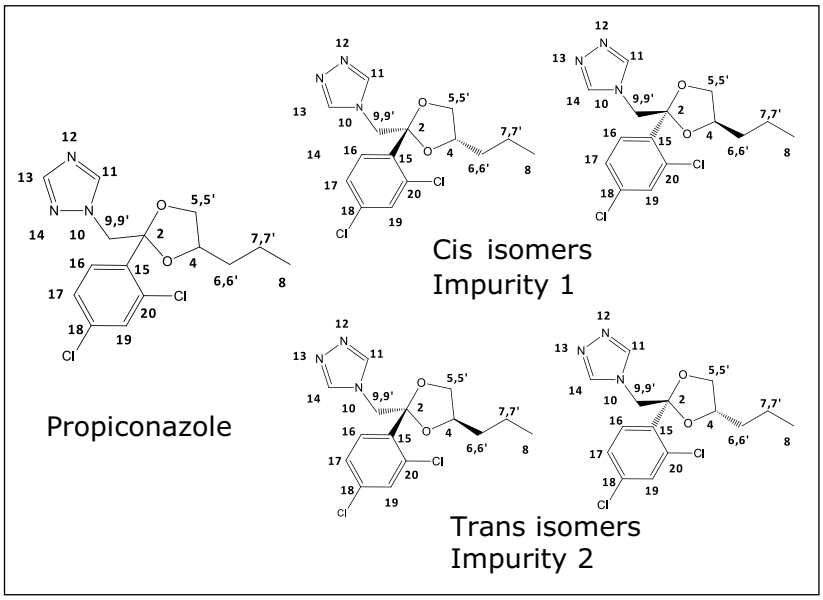

Figure 4. Structures shown for propiconazole (left) and for its impurities (top right, cis isomers, Impurity 1 from Figure 3), and (bottom right, trans isomers, Impurity 2 from Figure 3). Note that the parent propiconazole triazole is connected at a different nitrogen atom.

Having completed the full structural assignments for the two impurity peaks that could be resolved with achiral chromatography, attention was next turned to preparatively separating the enantiomers for both the cis and trans isomers (Figure 5). While all four isomers can be resolved in a single run analytically on a small particle size chiral column, in this case two different column chemistries were required for the separation of each enantiomeric pair upon scale up. The cis isomer separated on the IC column chemistry while the trans isomer required an AD-H column. All four of the possible impurity stereoisomers were then obtained in their pure form.

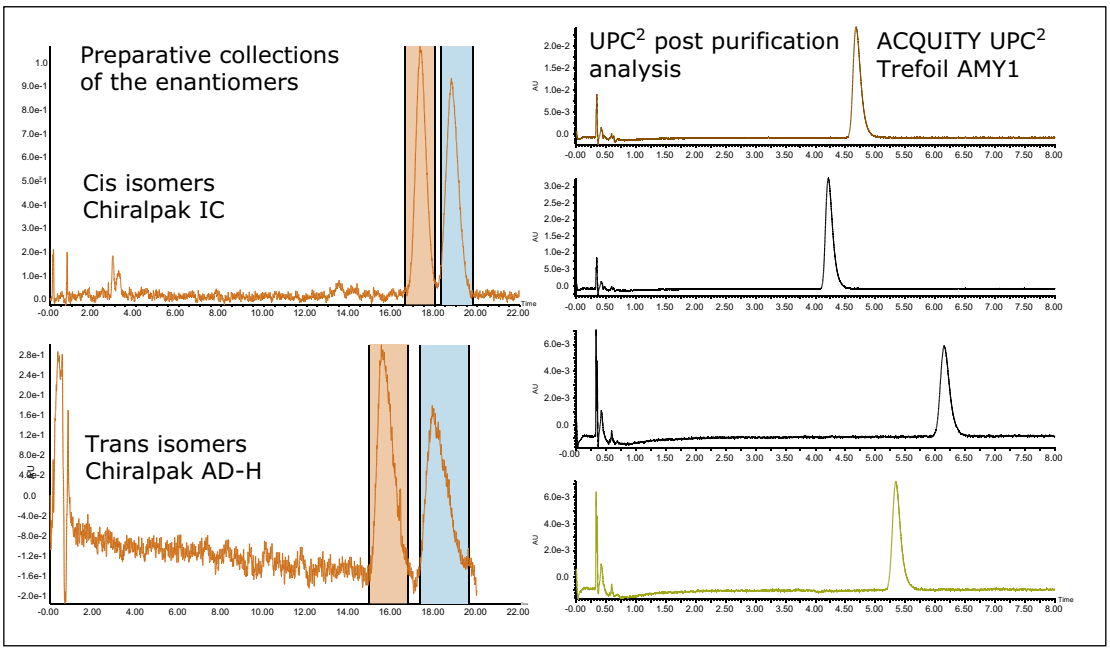

Figure 5. Preparative collections of the enantiomers of the cis isomer on the Chiralpak IC Column and of the trans isomer on the Chiralpak AD-H Column (left) using the Prep 100q SFC System. UPC^2 chiral analysis demonstrates that all four of the possible impurity stereoisomers were each preparatively isolated in the enantiopure form with >98% enantiomeric excess (right).

Chiral analysis using UPC^2 demonstrated that each isomer had an enantiomeric excess >98%. The cis isomer was comprised of the 2R, 4S and the 2S, 4R isomers, while the trans isomers contained the 2R, 4R and the 2S, 4S isomers. Although the assignment of the absolute configurations for each of the stereoisomers was beyond the scope of this study, these isolated impurities do in fact possess the same structures as those found in a previous study.[2] The capability to have all four of the impurity stereoisomers in hand in their pure form, in sufficient supply, then allows for the unambiguous assignment of the mechanistic potencies and toxicities of each individual impurity species. This capability then enables improved knowledge of the efficacy and safety of the product mixture ingredients.

CONCLUSIONS

ACQUITY UPC^2 analyses of actual product mixtures, using both chiral and achiral column chemistries are useful for both impurity profiling and also for developing separation methods suitable for eventual scale up to preparative separations.

Trace impurities, as in this case <1% of a 1.55% formulation of the active ingredient, can be efficiently isolated in the amounts needed for structural elucidation and other needed studies using preparative SFC.

The isolated impurities can be obtained in their pristine forms, thus enabling the simple application of HRMS, 1D, and 2D NMR spectroscopic studies, allowing for the full structural elucidations of these trace impurities.

Initially isolated impurities can be further separated into their enantiomeric pairs through the implementation of chiral stationary phase preparative SFC with high efficiency.

The assigned structures for all of the isolated impurities were in agreement with information in the prior literature.[2]

Acknowledgement

Special thanks for the intellectual contributions of James E. Hall, NMR Spectroscopist, Old Dominion University, Norfolk, VA.

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[FORMULATION]

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Chiral and Achiral Profiling of a Pesticide Formulation Using the ACQUITY UPC² System and the ACQUITY QDa Detector

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APPLICATION BENEFITS

- Chiral and achiral method development and sample analysis can be performed on the same system.
- Improved enantiomeric and diasteriomeric resolution and shorter analysis time compared with normal phase separations resulting in higher sample throughput.
- Reliable and reproducible measurement of the enantiomer and/or diasteriomer ratios.
- Detection and chiral resolution of structurally related formulation components.

WATERS SOLUTIONS

ACQUITY® UltraPerformance Convergence Chromatography™ System (UPC²®)

ACQUITY UPLC®
Photodiode Array (PDA) Detector

ACQUITY QDa™ Detector

ACQUITY UPC²™ BEH Column

Empower® 3 Chromatography Data Software

KEY WORDS

Chiral pesticides, UPC², enantiomer, diastereomer, chiral separation, chiral resolution, propiconazole, triazole fungicide, SFC, supercritical fluid chromatography, QDa

INTRODUCTION

Research associated with the development of new agricultural pesticide formulations centers around the design of products that provide highly effective and specific action towards the target organism with reduced application rates.[1] It is estimated that 30% of the pesticides on the market today have optical isomers.[2,3] However, the desired activities often result from one single enantiomer in the optical isomer mixtures.[4] It is therefore important to assess the enantiomeric purity of the chiral active ingredients in the formulation.[1,5] In addition, the detection, characterization, and quantitation of the other components in the formulation are necessary to support product registration.

Liquid chromatography (LC) on chiral stationary phases (CSPs), such as polysaccharide stationary phases including amylose and cellulose, has been the most commonly used chiral separation technique.[6-9] More recently, there has been an increasing adoption of supercritical fluid chromatography (SFC) on CSPs for chiral separation.[10,11] The properties of a supercritical fluid, its high diffusivity and low viscosity in particular, enable high efficiency chiral separations with shorter run times. For example, triazole fungicides, such as propiconazole (structure shown in Figure 1), are a commonly used group of pesticides because of their potent activity against a broad spectrum of crop diseases. Using HPLC, the analysis times for the diastereomeric resolution of propiconazole range from 34 to 50 min.[1,6-8] Similar resolutions were achieved for propiconazole using SFC, but the analysis times were reduced to 10 min.[11]

UltraPerformance Convergence Chromatography (UPC²) applies the performance advantages of UPLC® to SFC, combining the use of supercritical CO_2 with sub-2-µm particle columns.[12,13] UPC² is an orthogonal analytical technique to reversed-phase LC and can be used to solve complex separations challenges.

In this application note, we describe the analytical profiling of a triazole-containing pesticide formulation using UPC² and a combination of UV and mass detection. Waters® ACQUITY QDa Detector is a novel mass detector that can be integrated into existing liquid chromatography configurations in order to increase sensitivity and complement the results obtained when using only UV detectors.

EXPERIMENTAL

Instrumentation

All separations were performed on a Waters ACQUITY UPC^2 System equipped an ACQUITY UPC^2 Photodiode Array (PDA) and positive ion electrospray mass spectrometry (MS) using an ACQUITY QDa Detector. Empower 3 FR2 Software was used for data acquisition and processing.

Sample preparation

The authentic pesticide standards were made up in 50:50 acetonitrile/water. 2 grams (g) of the commercially available pesticide formulation was weighed, and 8 mL of 50:50 (v/v) acetonitrile/water was added. The resulting mixtures were sonicated for 10 minutes and the samples were syringe filtered into an autosampler vial using a 0.2-µm PVDF filter in preparation for sample analysis.

UPC^2 conditions

Achiral separation

Separation mode:	Gradient
Column:	ACQUITY UPC^2 BEH, 3.0 x 100 mm, 1.7 µm
Co-solvent (B):	Methanol
ABPR:	1990 psi/137 bar
Flow rate:	1.5 mL/min
UV detection:	220 nm
Column temp.:	35 °C
Injection volume:	0.5 µL

Formulation A achiral gradient conditions:

0 min 3% B, 4 min 30% B, 6 min 30% B, return to initial conditions.

Chiral separation

Separation mode:	Gradient
Column:	Amylose Chiral, 3.0 x 150 mm, 2.5 µm
Co-solvent (B):	50:50 2-propanol/ethanol
ABPR:	1990 psi/137 bar
Flow rate:	2.0 mL/min
UV detection:	220 nm
Injection volume:	1 µL

MS conditions

MS system:	ACQUITY QDa Detector
Ionization mode:	ESI +
Capillary voltage:	0.8 kV
Cone voltage:	10 V
Desolvation temp.:	600 °C
Source temp.:	150 °C
MS scan range:	100 to 600 *m/z*
Sampling rate:	5 Hz
Make up solvent:	98:2 MeOH/water with 0.1% ammonium hydroxide at 0.3 mL/min

PDA conditions

Detector:	ACQUITY UPC^2 PDA
Wavelength range:	210 to 400 nm
Sampling rate:	20 Hz

When MS data is combined with the UV response, it allows the analyst to determine a wider range of analytes in one analytical run with an increased level of confidence.

The formulation sample first underwent achiral separations on a sub-2-µm stationary phase, followed by chiral separations on a 2.5-µm chiral CSP. A minor isomer of the active ingredient (AI) was identified. Further chiral analyses revealed similar chirality between the minor isomer and the AI.

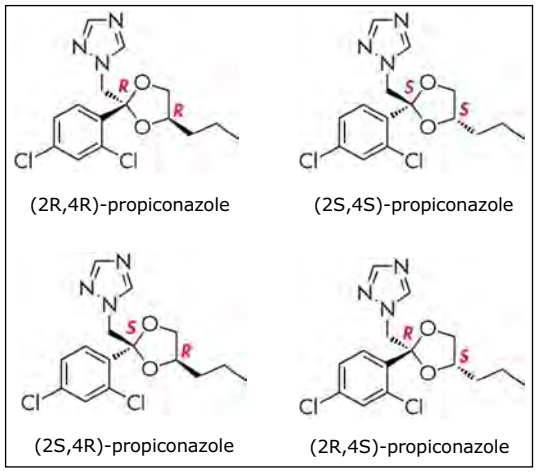

Figure 1. Structures of the propiconazole stereoisomers.

RESULTS AND DISCUSSION

Figure 2 shows the ACQUITY UPC^2 chromatograms of the propiconazole standard (lower trace) and the formulation sample (top trace) obtained using an ACQUITY UPC^2 BEH Column. The retention times of peaks 1 and 2 in the formulation sample matched those of the propiconazole standard. These two peaks correspond to the propiconazole diastereomers. It is noted, however, that there were two minor peaks (peaks 3 and 4) observed in the formulation with retention times (t_R) of 2.22 min and t_R 2.26 min, respectively.

The UV spectra of peaks 3 and 4 resemble those for peaks 1 and 2 (Figure 2), indicating their structural similarity. In addition, the four peaks resulted in identical mass spectra with base peaks at *m/z* 342 and an isotopic pattern characteristic of dichlorinated compounds. The *m/z* matched the protonated propiconazole.

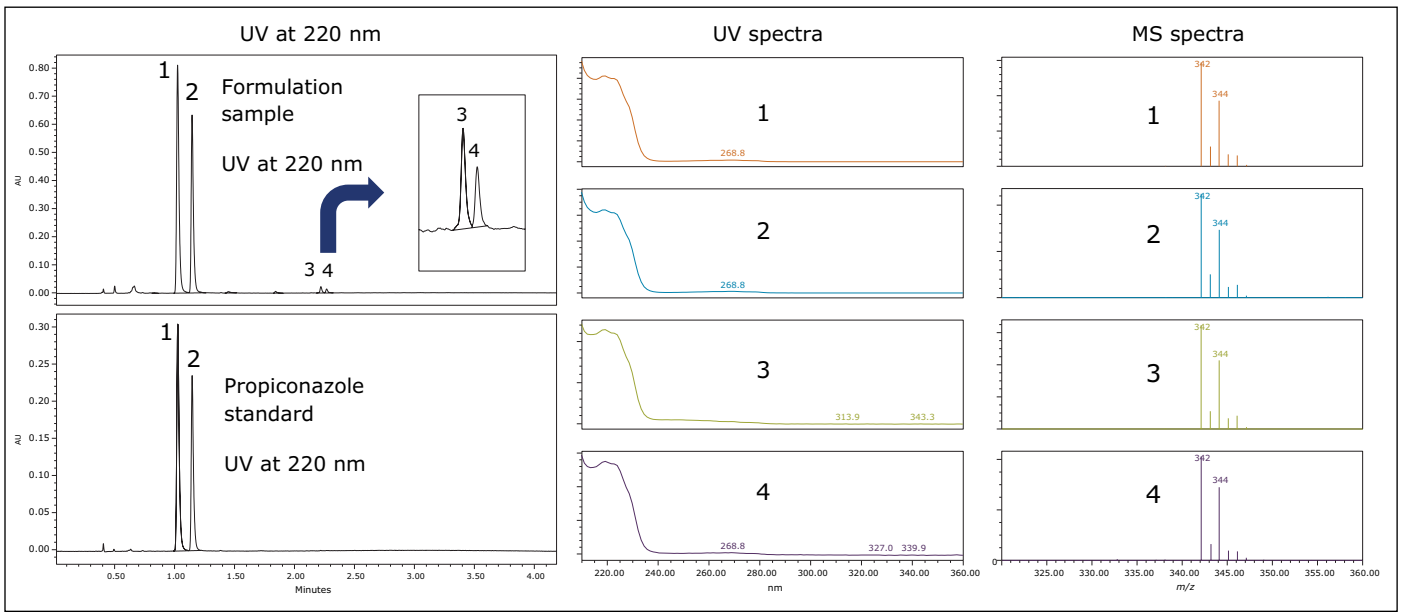

Figure 2. ACQUITY UPC² UV achiral separation of the propiconazole present in the formulation sample and propiconazole standard at 220 nm using the ACQUITY UPC² BEH Column. The UV and MS spectra for peaks 1-4 are also shown.

The ACQUITY UPC^2 System has multi-column switching capabilities and a choice of up to four co-solvents which conveniently allows both achiral and chiral method development and sample analysis to be performed on the same system. The method development process can be completed rapidly due to the shorter analysis times that are possible using this technique.

Figure 3 shows the ACQUITY UPC^2 chromatograms of propiconazole standard and the formulation sample using gradient separation on an Amylose Chiral Column. The two diastereomer peaks of propiconazole observed in Figure 2 were resolved into four individual peaks (1-4). Interestingly, the two minor isomer peaks in Figure 2 were also resolved into four peaks (5-8) in a comparable manner, indicating a similar chirality for propiconazole and the minor components.

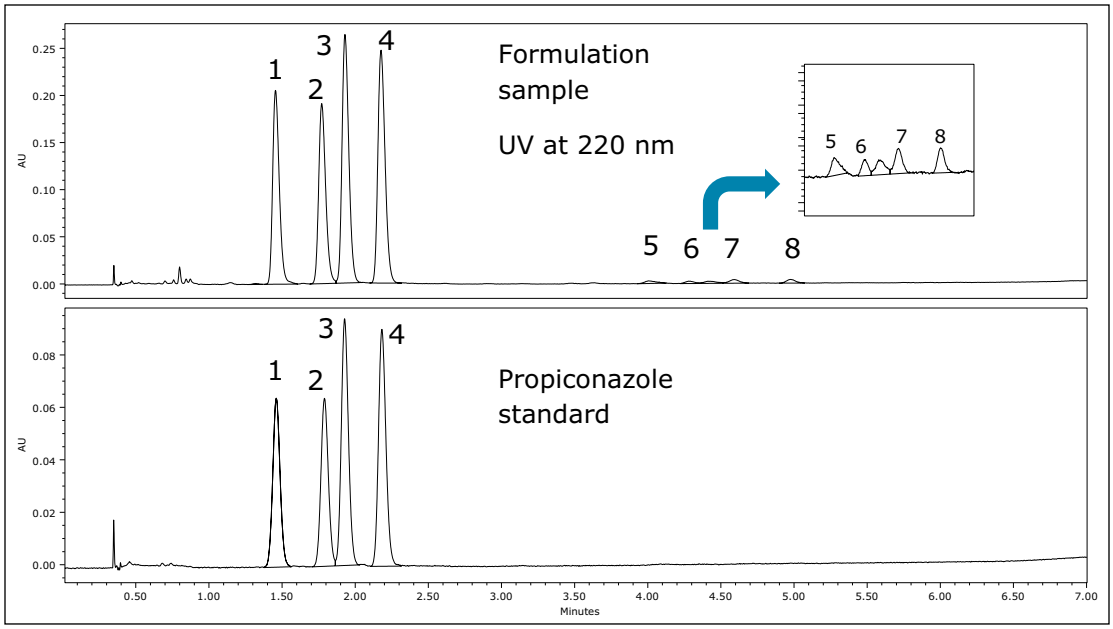

Figure 3. ACQUITY UPC^2 UV chromatogram at 220 nm showing the chiral resolution of the propiconazole stereoisomers and unknown chiral components in the formulation sample using an Amylose Chiral Column. The propiconazole standard is also shown.

Interrogation of the formulation sample data in Empower Software's mass analysis window showed similar UV and identical MS spectra for all eight peaks (Figures 4 and 5). Empower 3 Software's Mass Analysis window provides a single location to associate chromatographic peaks from all detectors used in the analysis with their corresponding spectra. The spectra from the detected peaks are time-aligned and displayed in a window above the chromatograms.

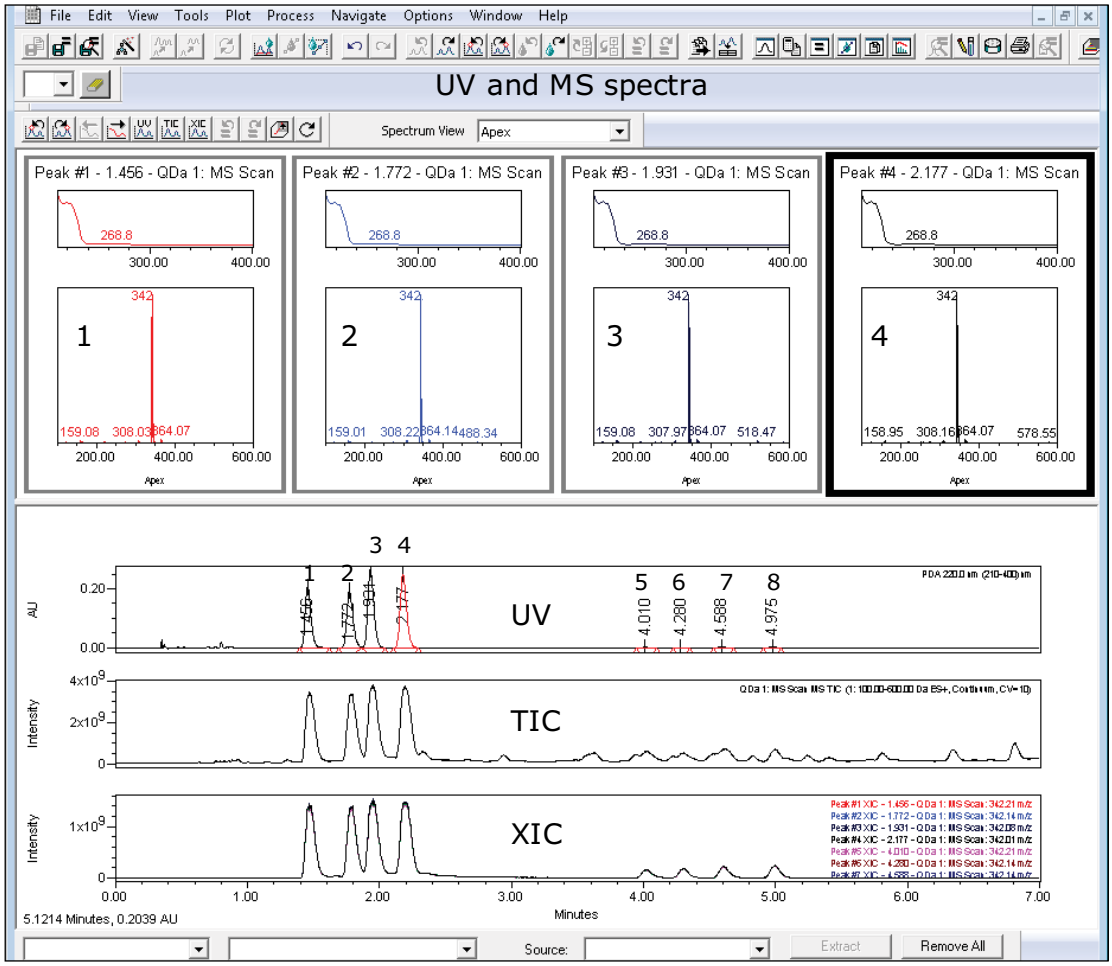

Figure 4. With Empower Software's Mass Analysis window, UV and MS spectra, along with UV and mass chromatograms and extracted ion chromatograms (XIC), can be viewed in a single window.

Matrix components visible in the UV chromatogram and the MS total ion chromatogram (TIC) of the formulation sample are clearly differentiated from the isomeric peaks of interest using an extracted ion chromatogram (Figure 5). The detection sensitivity and selectivity of the method are improved when using mass detection in combination with UV detection which is used in order to measure the enantiomeric purity of the chiral pesticide.

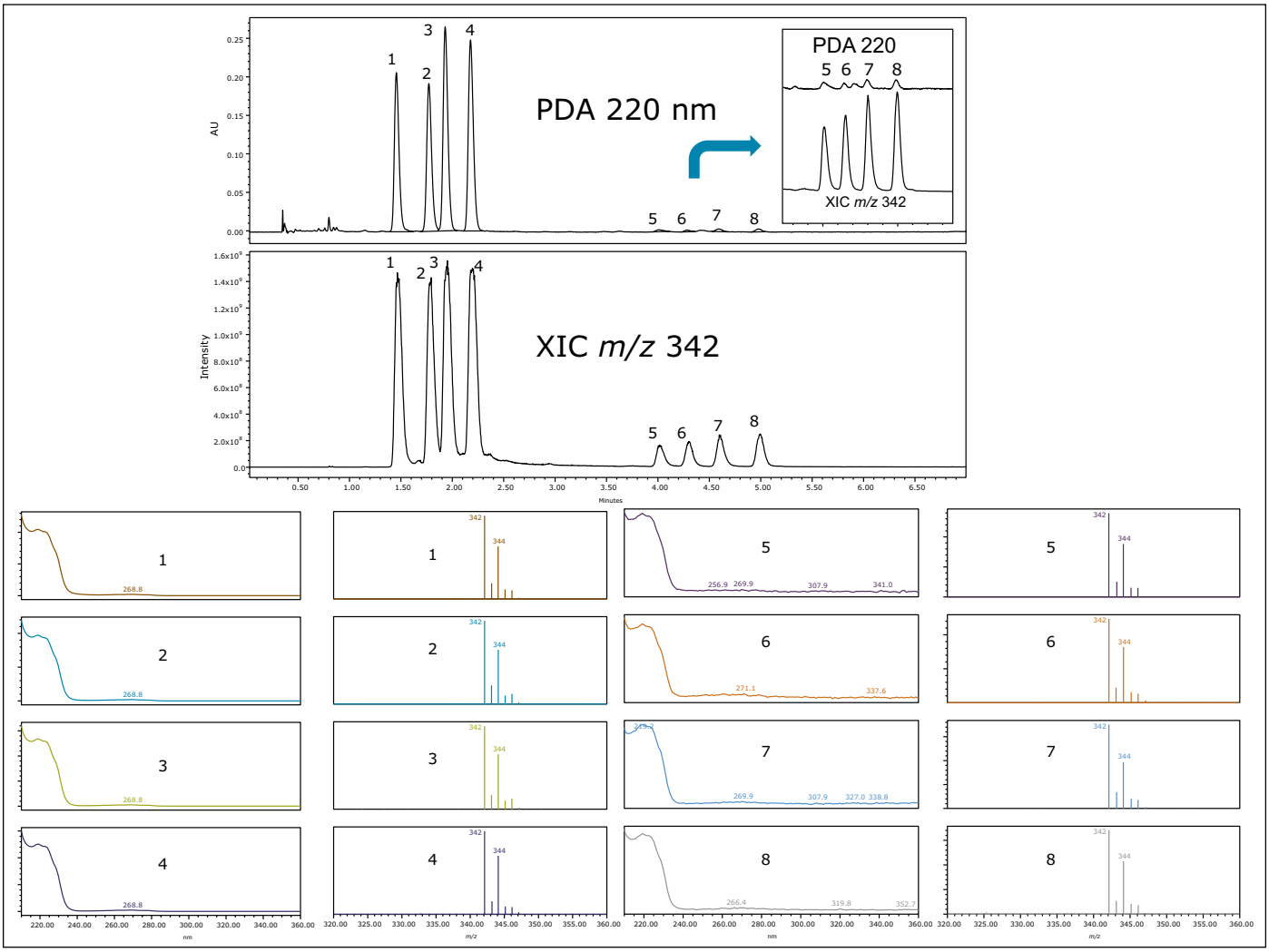

Figure 5. ACQUITY UPC² UV chromatogram at 220 nm showing the chiral resolution of the propiconazole stereoisomers and unknown chiral components in the formulation sample using an Amylose Chiral Column. The XIC of m/z 342 and the UV and MS spectra for each peak are also shown.

Based on the observations, it is postulated that the minor component is a regioisomer of propiconazole. A regioisomer of propiconazole originating from one of the nitrogens on the triazole ring was characterized by Glaser *et al.*[14] Further experiments to isolate this compound for positive identification are currently underway.

CONCLUSIONS

In this study the achiral screen of the formulated pesticide products showed that the minor components detected using UV and mass detection had similar structural characteristics to the AI, propiconazole. The minor components had the same *m/z* and shared the same isotopic pattern as the triazole fungicide, AI. Subsequent chiral resolution of the propiconazole in the formulation in combination with simultaneous mass and UV detection provided valuable spectral information which allowed the minor components to be characterized as probable stereoisomers.

The addition of mass detection as a complementary analytical detection technique enhances confidence in compound detection and identification. The ACQUITY QDa Detector provides a cost-effective means to make mass detection part of the routine analysis in laboratories that have previously relied on less selective detectors.

The ACQUITY UPC^2 System has column switching capabilities so that both chiral and achiral columns can be used with a choice of four co-solvents that are compatible with MS analysis. The chiral and achiral method development and analysis can be performed on the same system. These methods use supercritical CO_2 as the primary mobile phase. The need to use large volumes of potentially hazardous solvents is reduced compared to normal phase separations. Consequently the cost associated with solvent waste disposal can also be reduced.

The ACQUITY UPC^2 System allows high efficiency separations that can increase sample throughput compared to traditional normal-phase separations.[1,6-8] The diasteromeric resolution of propiconazole using UPC^2 took place in less than 3 minutes, which is at least 10 times faster than normal phase methods reviewed in the literature.

The study of enantioselective properties has previously been a challenge due to the difficulty in resolving chiral compounds. The benefit of having faster analytical methods to resolve chiral compounds is that critical information pertaining to their stereoselective behavior can be obtained more rapidly leading to increased laboratory productivity.

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Enantioselective Resolution and Analysis of Chiral Pesticides in Formulations by UltraPerformance Convergence Chromatography (UPC²) with UV Detection

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APPLICATION BENEFITS

- Improved enantiomeric and diasteriomeric resolution and shorter analysis times resulting in higher sample throughput and reduced solvent consumption compared to normal phase separations
- Reliable and reproducible measurement of the enantiomer and/or diasteriomer ratios in a pesticide formulation to accurately determine the correct application rates

WATERS SOLUTIONS

ACQUITY® UltraPerformance Convergence Chromatography™ (UPC²®) System

ACQUITY Photodiode Array (PDA) Detector

Empower® 3 Software

KEY WORDS

Chiral pesticides, UPC², enantiomer, diastereomer, pesticide formulations, chiral resolution, metolachlor, metalaxyl, convergence chromatography

INTRODUCTION

Agricultural chemicals ensure that we have decreased crop damage resulting in a food supply that is plentiful and of high quality.[1] In the agricultural chemicals manufacturing industry, the aim is to apply the least amount of pesticide that will give the most effective result. It is estimated that 30% of pesticides on the market today have optical isomers with reports that greater than 40% of the pesticides used in China are chiral.[2,3] Biochemical reactions are usually stereo or enantioselective and, while one enantiomer may deliver the desired effect to the target species, the other enantiomer may be less effective to the target or completely ineffective. The presence of the less active enantiomer may not contribute to the efficacy of the formulation; however, it may have an enantioselective toxic effect on a non-target species. Chiral pesticides are often formulated as either racemic mixtures or a single biologically active isomer. Chiral separation is necessary for accurate measurement of the stereoisomers in the formulated product to ensure the correct active pesticide application rates.[4,5] The study of chiral compounds has been a challenge due to the difficulty in resolving the stereoisomers. The most widely used analytical technique is separation using high-performance liquid chromatography (HPLC) with chiral stationary phases.[3,6] Supercritical fluid chromatography (SFC) is known as an effective chiral separations technique, possessing many advantages over conventional HPLC.[7,8] The properties of a supercritical fluid allow high efficiency separations with shorter analysis times. Convergence chromatography is a complimentary separation technique to liquid chromatography, providing orthogonal selectivity using supercritical CO_2 as the primary mobile phase.

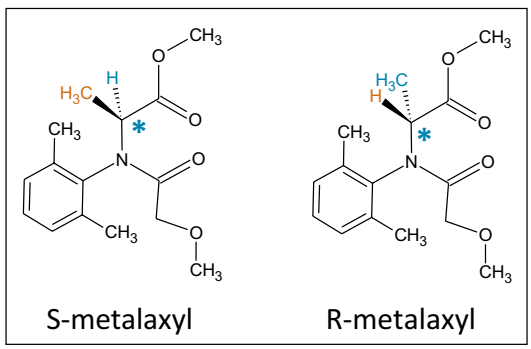

Figure 1. Structures of the two metalaxyl enantiomers. The asterisk denotes the stereogenic center.

EXPERIMENTAL

Sample preparation

The pesticide formulations (Formulation 1 and Formulation 2) were weighed, and a volume of hexane, dichloromethane, and ethyl acetate were each tested. The resulting mixtures were sonicated, and the samples were syringe-filtered into an autosampler vial using a 0.2-µm PVDF filter, ready for sample analysis. Standard compounds were made up in solvents to match the sample diluents.

UPC^2 conditions

System:	ACQUITY UPC$^{2™}$ with PDA detection
Data management:	Empower 3 Software

Metalaxyl-M

Separation mode:	Gradient
Column:	CHIRALPAK IA-3 4.6 x 150 mm, 3 µm
Co-solvent:	2-propanol
ABPR:	2000 psi/138 bar
Flow rate:	4.0 mL/min
UV detection wavelength:	215 nm
Column temp.:	55 °C
Injection volume:	2 µL

S-metolachlor

Separation mode:	Gradient
Column:	CHIRALPAK IA-3 4.6 x 150 mm, 3 µm
Co-solvent:	2-propanol
ABPR:	2000 psi/138 bar
Flow rate:	2.5 mL/min
UV detection wavelength:	220 nm
Column temp.:	35 °C
Injection volume:	2 µL

In this application note, the enantiomeric and/or diastereomeric resolutions of two pesticides, metalaxyl-M (a phenylamide fungicide) and S-metolachlor (acetanilide class of herbicides), and their analyses in aqueous-based pesticide formulations are presented. Metalaxyl (Figure 1) has one chiral center, and its biologically active form is the R enantiomer.[9] Metolachlor (Figure 2) has two chiral centers and 95% of the herbicidal activity comes from the S enantiomers.[10,11] Resolutions were performed using the Waters® ACQUITY UPC^2 System.

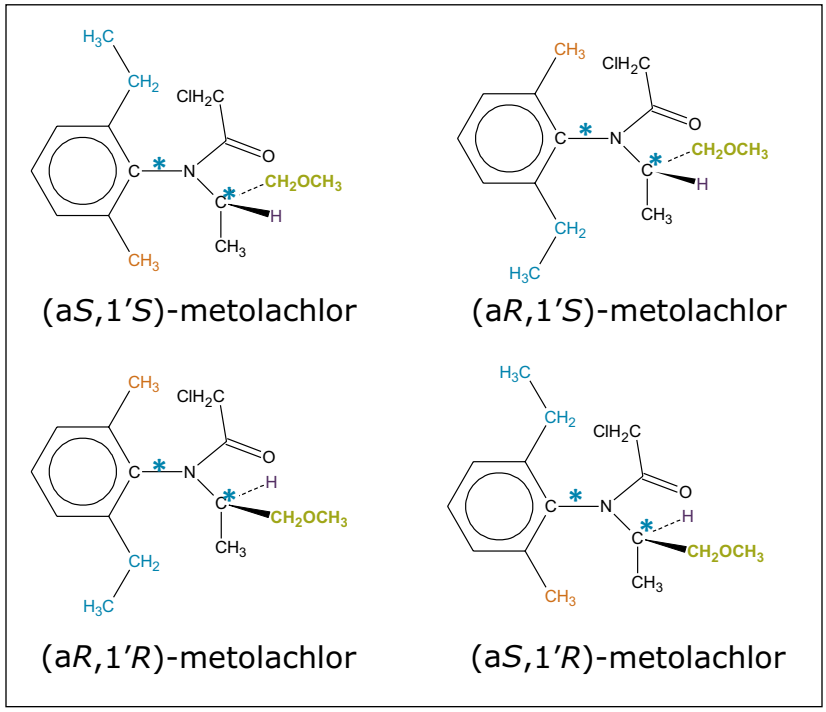

Figure 2. Structures of the four stereoisomers of metolachlor. The asterisks denote the stereogenic centers.

RESULTS AND DISCUSSION

The method development for the standard pesticides began with a generic screening gradient using a number of columns and co-solvents. The screening step can be completed rapidly due to the shorter analysis times that are possible using this technique. The combination of co-solvent and column that produced the most promising separation for each compound was then optimized. The selectivity in a chiral separation can change markedly by varying the temperature, pressure, and flow rate.[12] The optimized resolutions for the standard materials of metalaxyl-M and S-metolachlor are shown in Figures 3 and 4.

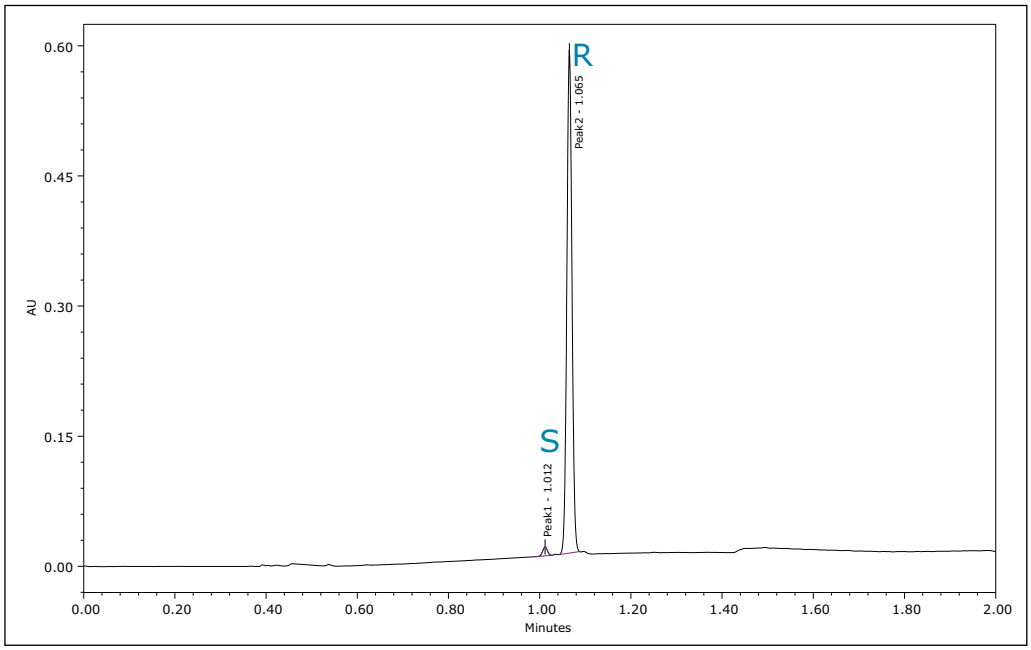

Figure 3. ACQUITY UPC²/UV chromatogram of metalaxyl-M standard at 215 nm using gradient elution of S and R enantiomers.

The relative peak areas in the chromatogram shown in Figure 4 indicate that the enantiomeric pairs for metolachlor are peaks 1,2 (S) and 3,4 (R).

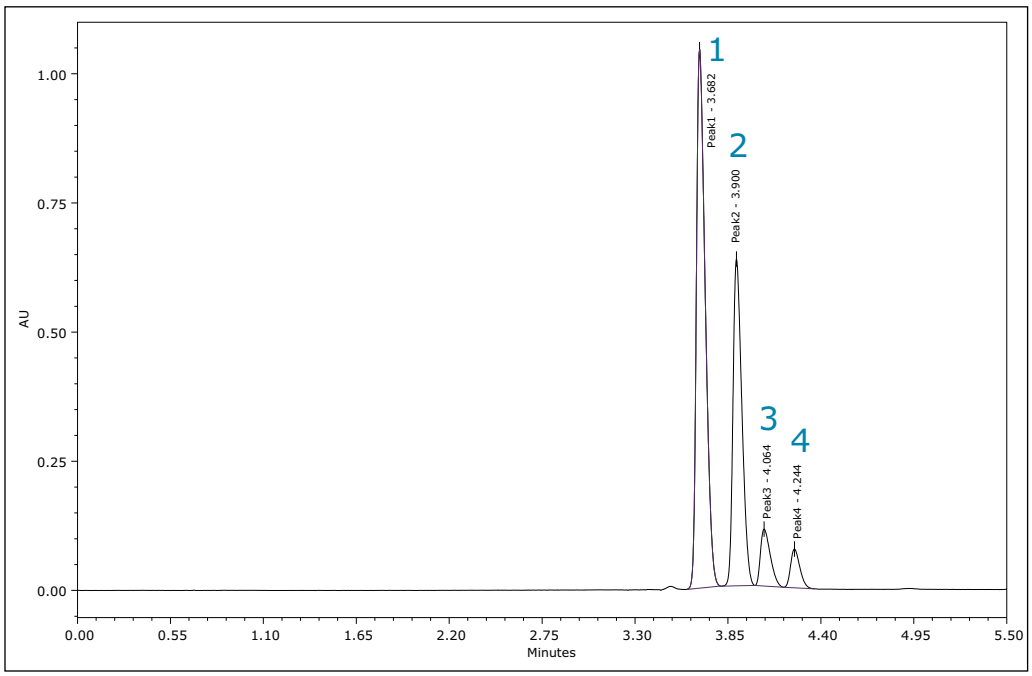

Figure 4. ACQUITY UPC²/UV chromatogram of S-metolachlor standard at 220 nm using gradient elution of the stereoisomers.

The pesticide formulations were aqueous-based and a solvent exchange was performed. Hexane, dichloromethane, and ethyl acetate were evaluated separately as extraction solvents. Hexane was more selective for less polar components in both formulations (Figures 5 and 6) and was used for further extractions. In this analysis, the goal was to resolve the stereoisomers and to reliably and reproducibly measure the ratios of the enantiomers/diastereomers.

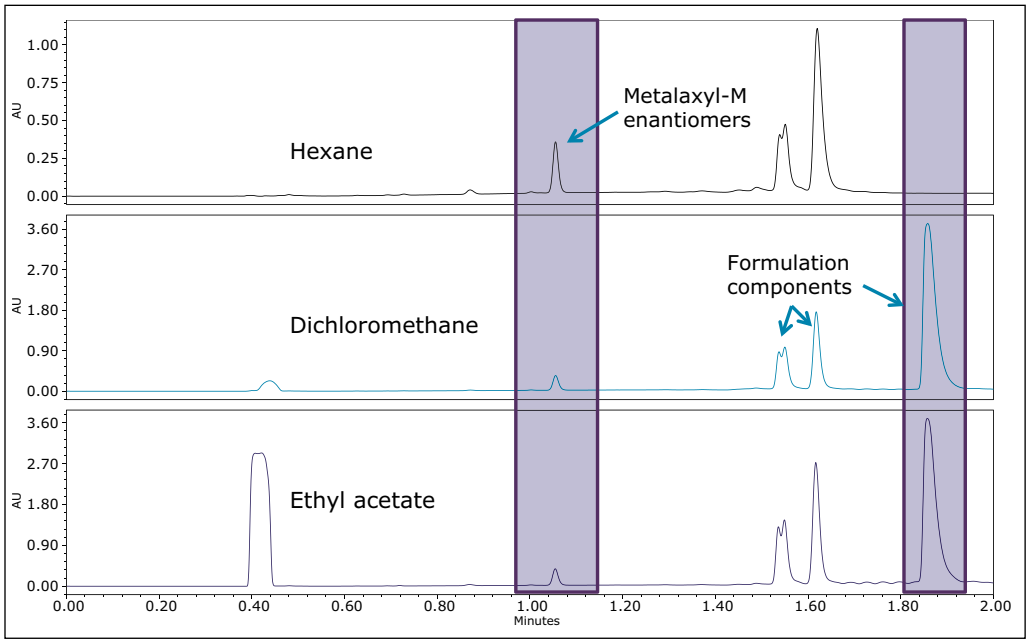

Figure 5. ACQUITY UPC²/UV chromatograms of formulation 1 at 215 nm. Hexane, dichloromethane, and ethyl acetate were each evaluated as extraction solvents for the metalaxyl-M in the formulation.

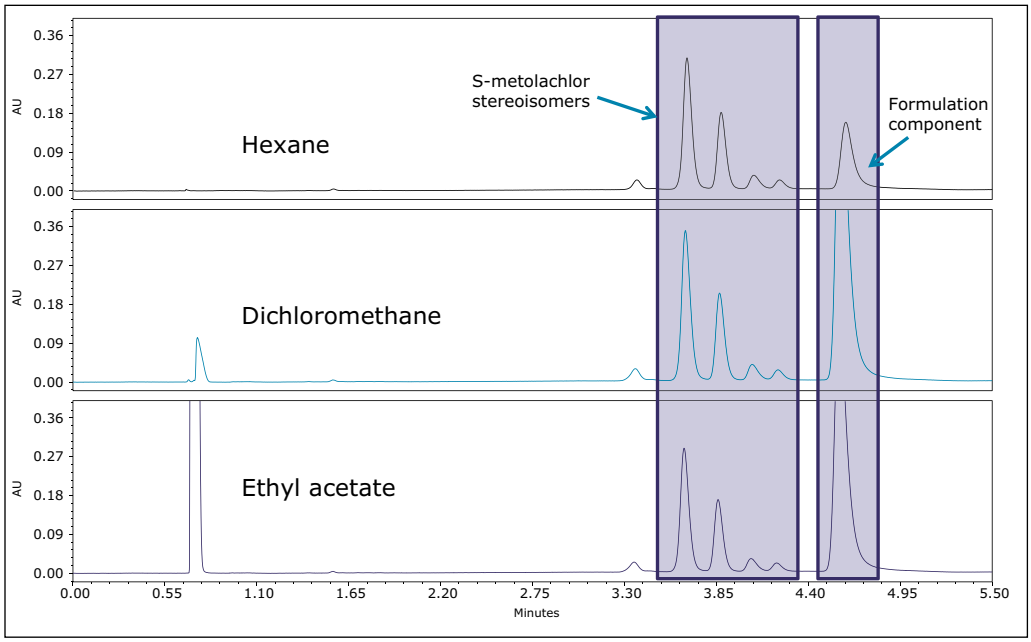

Figure 6. ACQUITY UPC²/UV chromatograms of formulation 2 at 220 nm. Hexane, dichloromethane, and ethyl acetate were each evaluated as extraction solvents for the S-metolachlor in the formulation.

The optimized UPC^2 methods allowed increased sample throughput and improved enantiomeric and diasteriomeric resolution. In the case of metalaxyl-M, (Figure 7) both enantiomers eluted in one minute with a resolution (R_s) of 2.47. A comprehensive survey of enantioselective separations of chiral pesticides in the literature, published in 2009, reported a normal phase isocratic method using 60:40 hexane/ethanol for the resolution of metalaxyl enantiomers.[6,13] The R_s was reported to be 1.94 with both enantiomers eluting in just under 15 minutes.

The four stereoisomers of metolachlor eluted in 4.5 minutes (Figure 8) with an R_s of 2.31 between peaks 1,2; R_s of 1.85 between peaks 2,3, and R_s of 1.48 between peaks 3,4. The baseline resolution of the stereoisomers of metolachlor standard using a normal phase separation with 91:9 hexane/diethyl ether was also reported in the 2009 article. A figure from this publication showed all four stereoisomers eluting between 20 and 30 minutes.[5,6]

In both of the UPC^2 methods, the stereoisomers of metalaxyl-M and S-metolachlor were sufficiently resolved from the other formulation components so that accurate and reproducible enantiomeric ratios could be obtained. Measurement of the area % for the two metalaxyl-M enantiomers over 100 injections gave %RSDs of 0.02 for R-metalaxyl and 0.85 for the lower level S-metalaxyl (data not shown).

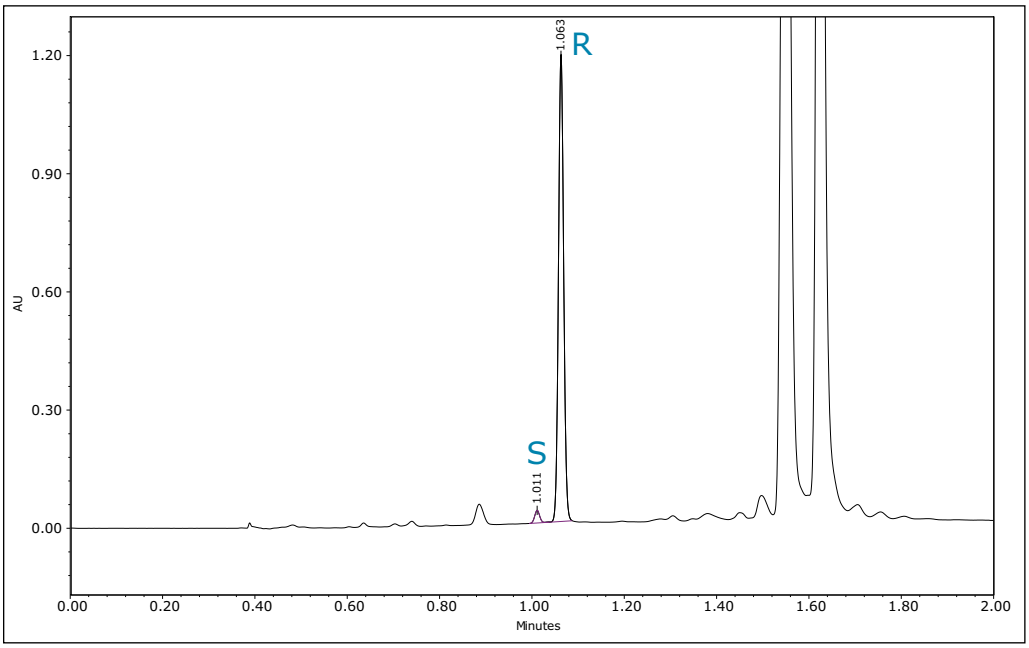

Figure 7. ACQUITY UPC^2/UV chromatogram of formulation 1 at 215 nm using gradient elution of S and R metalaxyl.

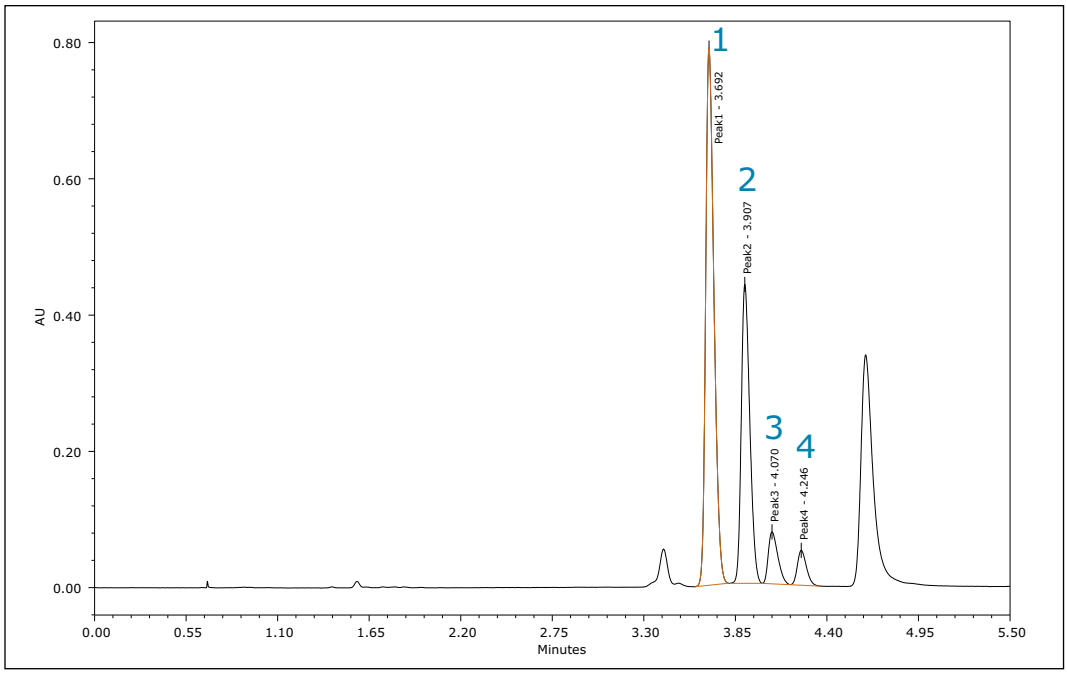

Figure 8. ACQUITY UPC²/UV chromatogram of formulation 2 at 220 nm using gradient elution of the metolachlor stereoisomers.

Reproducibility data (n=6) for retention time, area, area %, and height resulted in %RSDs less than or equal to 1.34 for all the stereoisomers of each compound. The bar chart (Figure 9) shows the mean peak areas for the S and R-metalaxyl enantiomers and the %RSDs obtained from six injections. Despite the relative difference between the peak areas for the lower level S-metalaxyl and the active R-metalaxyl, the %RSDs are comparable.

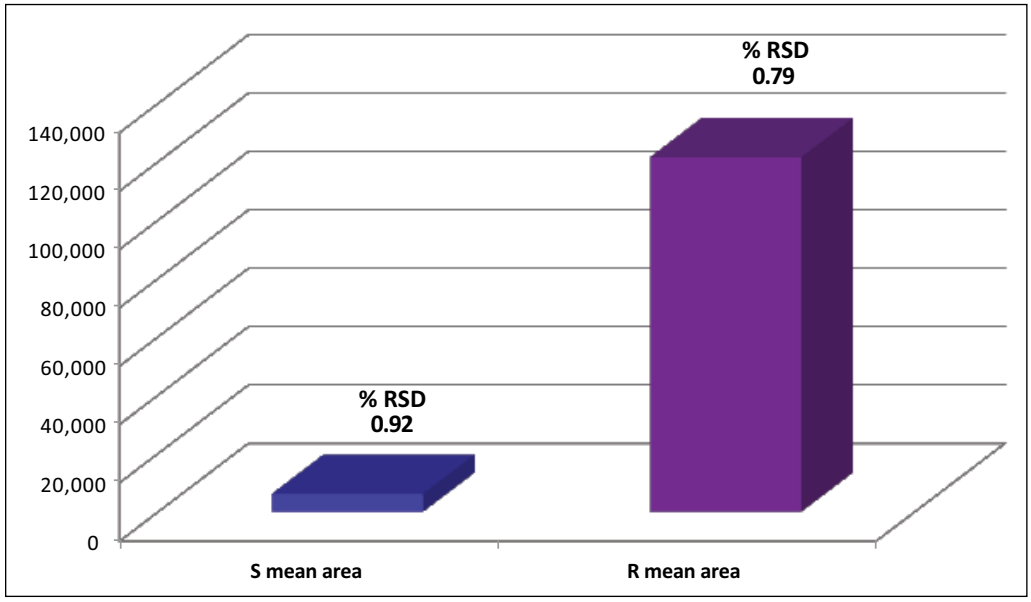

Figure 9. Comparison of the mean peak areas (n=6) and %RSDs for S and R-metalaxyl.

The mean peak areas (n=6) for the four stereoisomers of metolachlor and the %RSDs ranging from 0.55 to 1.34 are shown in Figure 10.

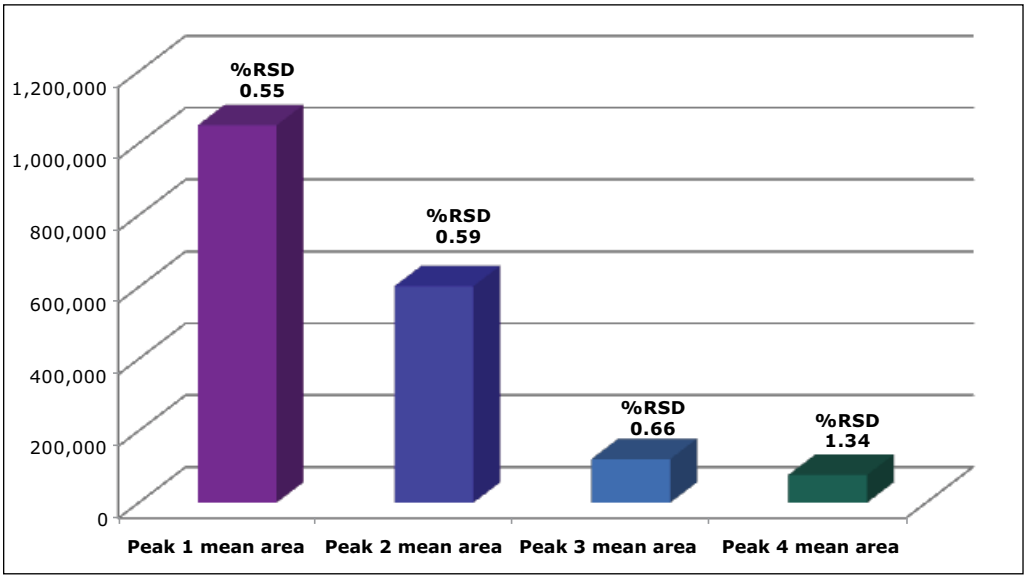

Figure 10. Comparison of the mean peak areas and %RSDs for the stereoisomers of S-metolachlor.

CONCLUSIONS

UPC^2 allows high efficiency separations that can significantly increase sample throughput. The methods use supercritical CO_2 as the primary mobile phase and 2-propanol as the co-solvent.

This application note has demonstrated the chiral analysis of two pesticides in aqueous-based formulations using UPC^2. The enantiomer and/or diasteriomer composition in the formulation was measured precisely and accurately using the UPC^2 methods that were faster than traditional normal phase methods reported in the literature allowing higher sample throughput.[6] The time to develop a chiral separation with acceptable resolution was also decreased as a result of the faster analysis times that are possible using this technique. The %RSDs for peak area, peak height, area %, and Rt were equal to those typically obtained by analyses using UltraPerformance Liquid Chromatography (UPLC®)/UV. In addition, the need to use large volumes of potentially hazardous solvents like those used in traditional normal phase chiral separations is reduced as is the cost associated with solvent waste disposal.

The study of chiral pesticides has previously been challenging due to the difficulty in resolving them in short time frames. The ACQUITY UPC^2 System overcomes this challenge by providing fast analytical methods to resolve these challenging chiral compound separations. Critical information pertaining to their stereoselective behavior can be obtained more rapidly allowing increased laboratory productivity.

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Sample Profiling of Pesticide Formulations Using UV and MS Detection for Component Identification

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved sensitivity over UV for low-level components present in the pesticide formulation
- Increased confidence in peak identification when mass detection is used with PDA detection
- Structural similarities between the active ingredient and the low-level components identified in a single analysis

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY® QDa Detector

ACQUITY UPLC Photodiode Array (PDA) Detector

Empower® 3 CDS Software

KEY WORDS

Chiral pesticides, diastereomer, pesticide formulation, impurity identification, sample profiling, propiconazole, triazole fungicides

INTRODUCTION

Agricultural chemicals decrease crop damage, resulting in a food supply that is both plentiful and of high quality.[1] For the agricultural chemicals industry, the analytical quality control of pesticide products ensures that a consistent and effective product reaches the farm.[2] More specifically, the detection, characterization, and quantitation of the active ingredient(s) as well as all other components in the formulation, including impurities and degradation products, is necessary to support product development, quality control, and product registration. The addition of a mass detector in conjunction with UV detection can increase the specificity and selectivity of methods used during analytical testing to provide additional information about a sample during a single analysis.

Waters® ACQUITY QDa Detector is a novel mass detector that can be integrated into existing liquid chromatography configurations in order to complement the results obtained by UV detectors and increase the detection selectivity. The enhanced selectivity is ideal for the detection and initial identification of low-concentration components present in samples. When MS data is combined with the UV response, it allows the analyst to determine a wider range of analytes in one analytical run with an increased level of confidence.

In this application note, optical and mass detection were combined to provide a thorough profile of a commercially available pesticide formulation concentrate. The formulated product contained the triazole fungicide propiconazole, which contains two chiral centers in its chemical structure, as shown in Figure 1. The triazole fungicides are a commonly used group of pesticides due to their potent activity against a broad spectrum of crop diseases.[3]

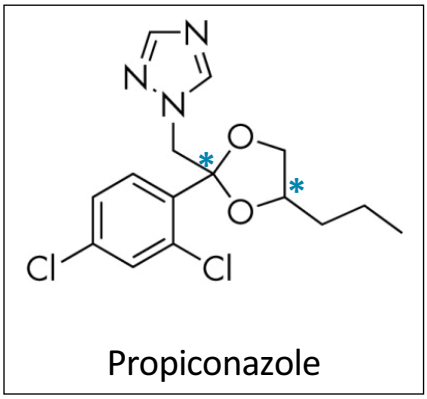

Figure 1. Structure of propiconazole. The asterisks denote the stereogenic centers.

EXPERIMENTAL

UPLC conditions

LC system:	ACQUITY UPLC H-Class
Column:	ACQUITY UPLC BEH C_{18} 2.1 x 150 mm, 1.7 µm
Column temp.:	50 °C
Injection volume:	3 µL
Flow rate:	0.60 mL/min
Mobile phase A:	10 mM ammonium formate in water
Mobile phase B:	Acetonitrile

Gradient table:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.60	70	30	6
10.0	0.60	30	70	6
11.0	0.60	10	90	6
12.0	0.60	10	90	6
12.1	0.60	70	30	1

Table 1. UPLC gradient method for analysis of the formulation.

MS conditions

MS system:	ACQUITY QDa Detector
Ionization mode:	ESI+
Capillary voltage:	0.8 kV
Desolvation temp.:	500 °C
Source temp.:	150 °C
Cone voltage:	7 V
Sampling rate:	5 Hz
MS scan range:	100 to 1000 *m/z*

PDA conditions

Detector:	ACQUITY UPLC PDA
Wavelength range:	210 to 400 nm
Sampling rate:	20 Hz

Empower 3 Software Feature Release 2 was used for chromatographic data processing.

Sample preparation

1 gram (g) of the commercially available pesticide formulation was weighed, 9 mL of 50:50 (v/v) acetonitrile/water was added. The resulting mixture was sonicated for 10 minutes, and the sample was syringe filtered into an autosampler vial using a 0.2-µm PVDF filter in preparation for sample analysis. Authentic propiconazole standard was made up in 50:50 (v/v) acetonitrile/water.

RESULTS AND DISCUSSION

A UPLC® UV chromatogram at 220 nm comparing the standard propiconazole and the propiconazole present in the formulation is shown in Figure 2. The ACQUITY UPLC H-Class System's separation of propiconazole resulted in two peaks at retention times (t_R) of 7.45 min (peak 3) and 7.54 min (peak 4). The observed peaks likely originated from the propiconazole diastereomers. The t_R's of the propiconazole standard match those in the formulation. Two minor components (peak 1 and peak 2) at t_R 6.23 min and t_R 6.43 min, respectively, were noted in the UV chromatogram of the formulation. When measured in the UV, the area% contributions from peak 1 and peak 2 were 1.20% and 0.80%, respectively.

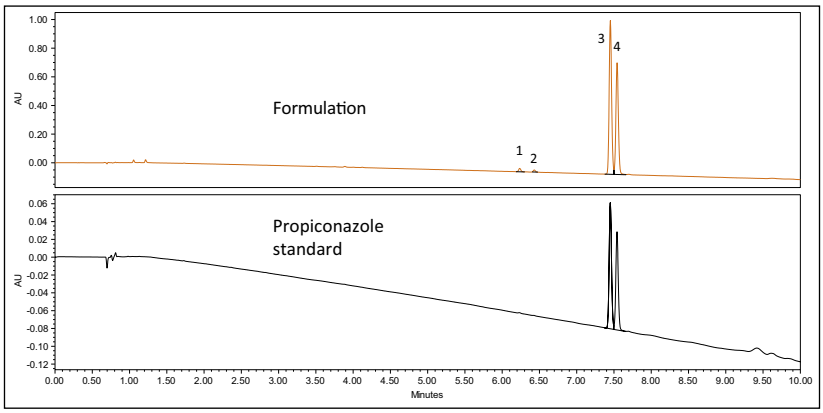

Figure 2. The ACQUITY UPLC PDA Detector's UV chromatogram of the propiconazole present in the formulation and propiconazole standard at 220 nm.

The total ion chromatogram (TIC) from the ACQUITY QDa Detector that was acquired simultaneously with the UV detector is shown in Figure 3. A protonated molecular ion, $[M+H]^+$ that corresponds to a mass-to-charge (*m/z*) ratio of 342 was observed for the propiconazole diastereomers.

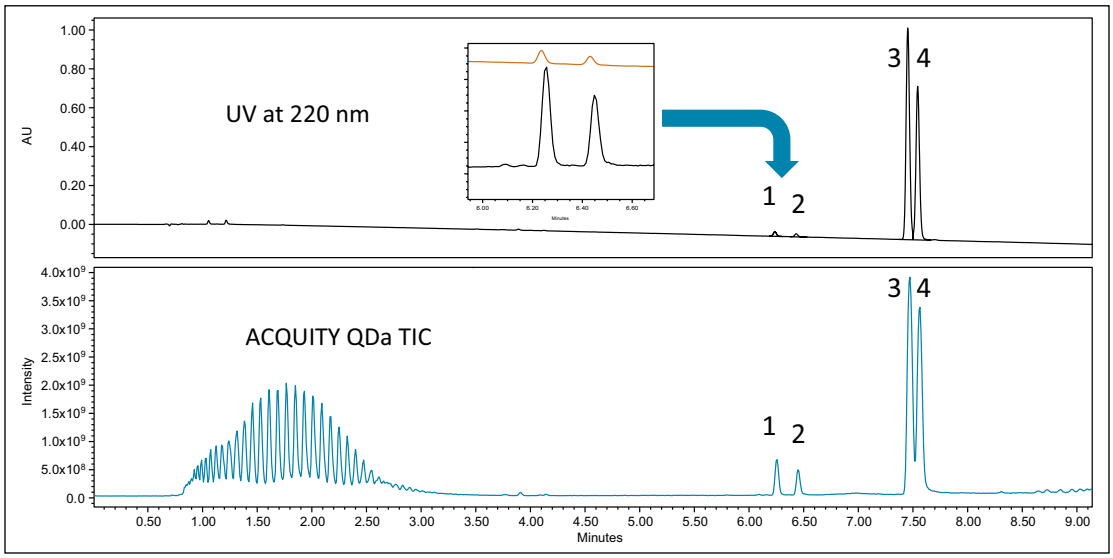

Figure 3. The ACQUITY UPLC PDA Detector's UV chromatogram of the formulation at 220 nm with the ACQUITY QDa Detector's mass chromatogram.

The signal response of all components improved in the mass chromatogram when compared with the UV chromatogram, illustrating the improved likelihood of detecting low-level components using the mass detector (inset Figure 3). The detection limits can be further improved by extracting ions of interest from the TIC to give an extracted ion chromatogram (XIC), shown in Figure 4. This enhances the confidence in compound identification. Other formulation components that could not be seen in the UV were clearly observed in the mass chromatogram, demonstrating that mass spectrometry combined with UV detection can give a more comprehensive sample profile. A series of peaks eluting between 0.80 and 3.0 min, with the *m/z* increasing by 44 amu with respect to the elution order, was observed in the mass chromatogram. The masses are consistent with a polymeric component, which is likely a surfactant present in the formulation that is used to aid in the application of the active ingredient.

Empower 3 Software's mass analysis window provides a single location to associate chromatographic peaks from all detectors used in the analysis with their corresponding spectra. The spectra from the detected peaks are time-aligned and displayed in a window above the chromatograms. The mass analysis window is shown in Figure 4.

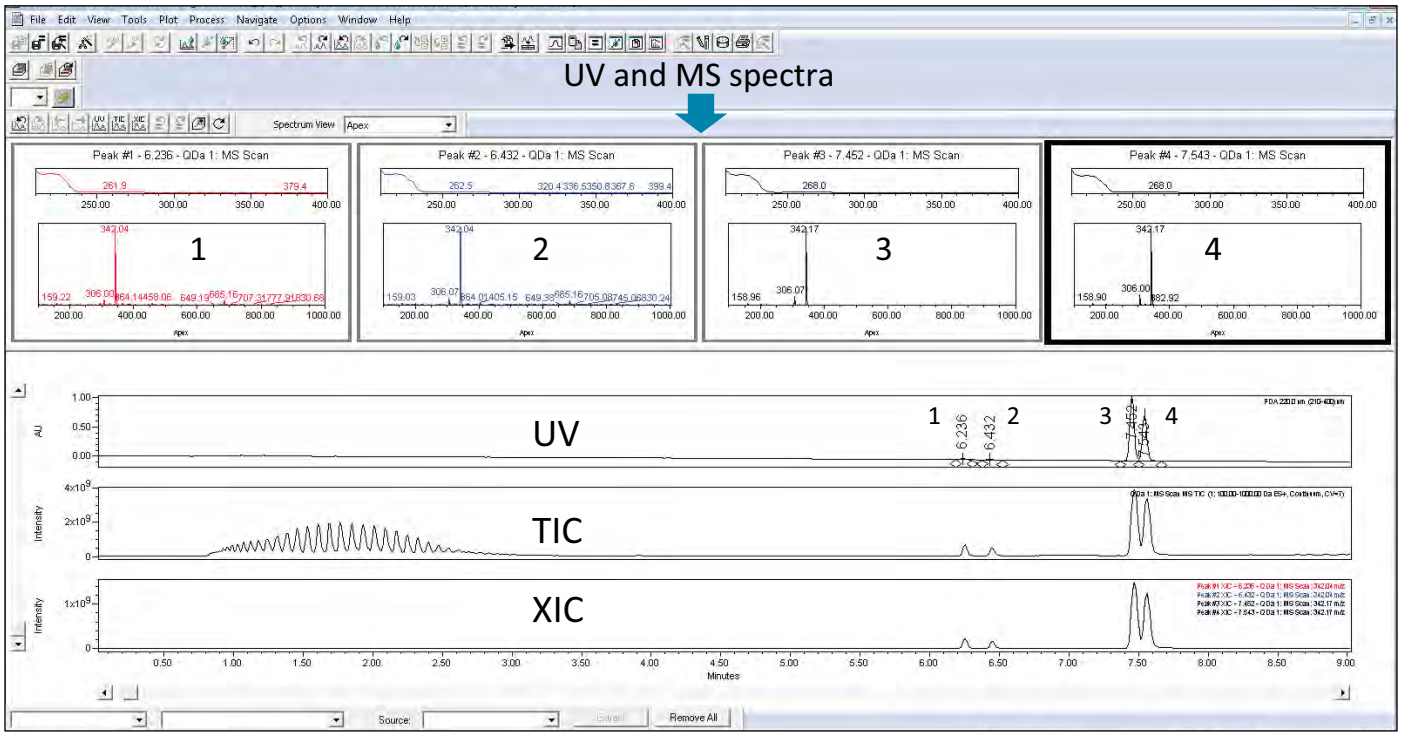

Figure 4. Empower Software's mass analysis window. UV and MS spectra, along with UV and mass chromatograms and extracted ion chromatograms (XIC), can be viewed in a single window.

Interrogation of the data in the mass analysis window indicated relationships between peak 1 and peak 2 with the active ingredient, propiconazole. The UV spectra showed the same maxima at 220 nm with an apparent shift noted in the second absorbance maxima of both peak 1 and peak 2, when compared to the spectra of the propiconazole. The mass spectra show that peak 1 and peak 2 have an *m/z* of 342 which is the same as the active ingredient. In addition, the isotopic pattern is typical of a dichlorinated compound and is identical to that of propiconazole. In a single analytical run, the unknown components were identified as having the same mass and isotopic pattern as the active ingredient. The presence of the mass detector provided additional structural information and increased the confidence in the detection and identification of the compound. The UV and mass spectra for peak 1 and peak 2 and the propiconazole diastereomers are shown in Figure 5.

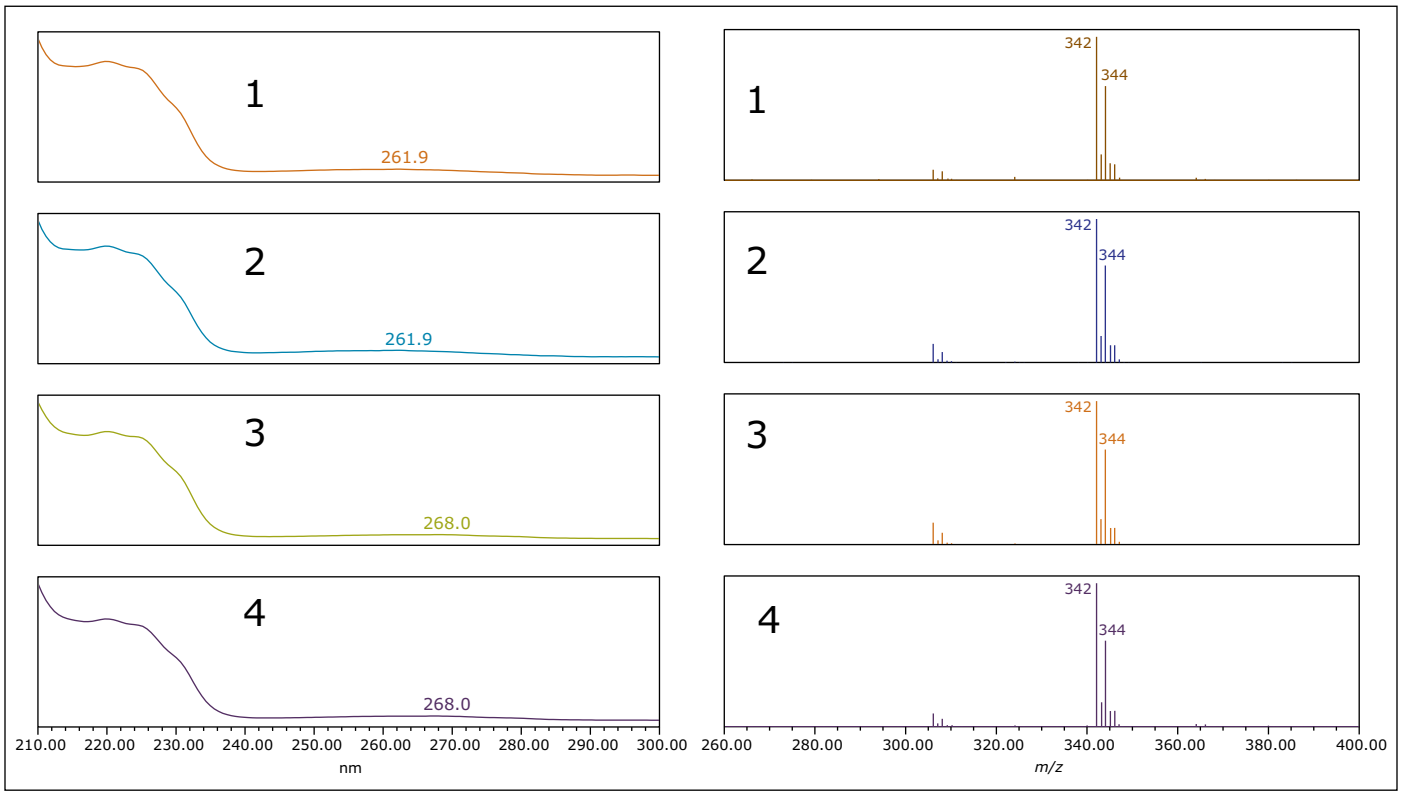

Figure 5. UV and mass spectra for peaks 1 and 2, and the propiconazole diastereomers, peaks 3 and 4, in the formulation.

CONCLUSIONS

The ACQUITY QDa Detector, in combination with PDA detection, allows for low-level components to be detected with increased confidence in pesticide formulations. The components were identified as having similar optical and structural properties to propiconazole, the active ingredient present in the formulation. Inert formulation components not seen in the UV were readily detected by the ACQUITY QDa Detector.

The Empower mass analysis window provides a single place to associate the chromatograms and spectra from all detectors used in the analysis. The consolidation of this information in one place makes data review and interpretation easy.

The addition of mass detection as a complementary analytical detection technique enhances confidence in compound detection and identification. Using the familiarity of a PDA detector, the ACQUITY QDa Detector provides a cost-effective means to make mass detection part of the routine analysis in laboratories that have previously relied on less selective detectors.

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Enantioseparation and Detection of Triazole Fungicides in Wheat Grain and Wheat Straw using the ACQUITY UPC^2 System and Xevo TQ-S

Marian Twohig, Michael O'Leary, and Claude Mallet
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Enantiomeric detection and quantitation of triazole fungicides at parts per trillion levels (ppt).
- Improved enantiomeric resolution and shorter analysis times using SFC compared with normal-phase HPLC chiral separations resulting in higher sample throughput.
- Reliable and reproducible measurement of the enantiomer ratios for use in enantioselective degradation studies.

WATERS SOLUTIONS

ACQUITY® UPC2® System

Xevo® TQ-S

Oasis® Sample Extraction Products

DisQuE™ Dispersive Sample Preparation

Sep-Pak SPE

MassLynx® Software

KEY WORDS

Chiral quantitation of pesticides, enantiomer, chiral separation, uniconazole, tebuconazole, diniconazole, flutriafol, fenbuconazole, triazole fungicide, SFC, supercritical fluid chromatography, UPC^2, enantioselective degradation studies

INTRODUCTION

The safe use of crop protection products is of paramount importance to the agricultural chemicals manufacturing industry. Extensive studies and trials are carried out in support of product registration. These studies ensure that any risks associated with using the product are characterized and properly understood so that it can be safely applied to the field. When a crop protection active ingredient (AI) contains one or more stereogenic centers in its structure the enantioselective behavior must be studied, since it is known that enantiomers can exhibit different bioactivities.[1,2] Analytical methods used to evaluate the influence of stereochemistry on the degradation dynamics, environmental fate, and final residue levels help to establish a more accurate risk assessment of crop protection products.

Liquid chromatography (LC) on chiral stationary phases (CSPs), such as polysaccharide stationary phases including amylose and cellulose, has been the most commonly used chiral separation technique.[3-6] More recently, there has been an increasing adoption of using supercritical fluid chromatography (SFC) on CSPs for chiral separation.[7,8] The properties of a supercritical fluid, its high diffusivity and low viscosity in particular, enable high efficiency chiral separations with shorter run times. For example, triazole fungicides, such as tebuconazole, structure shown in Figure 1, are a commonly used group of pesticides due to their potent activity against a broad spectrum of crop diseases. Using HPLC, the analysis times for the enantiomeric resolution of tebuconazole ranged from 13 to 45 min.[3-6] Similar resolutions were achieved for tebuconazole using SFC, but the analysis times were reduced to 10 min.[8]

UltraPerformance Convergence Chromatography™ (UPC^2) applies the performance advantages of UPLC® to SFC, combining the use of supercritical CO_2 with sub-2-µm particle columns.[9,10] UPC^2 is an orthogonal analytical technique to reversed-phase LC and can be used to solve complex separations challenges. The detection sensitivity and specificity offered by tandem MS/MS is advantageous for determining trace levels of pesticides in complex matrices like field crops or soil.[11-14]

EXPERIMENTAL

SFC conditions

SFC system:	ACQUITY UPC^2
Chiral separation:	Diniconazole, fenbuconazole, flutriafol, tebuconazole
Column:	Chiralpak IA-3, 4.6 x 150 mm, 3.0 µm
Co-solvent (B):	Methanol with 2% water and 0.1% formic acid
ABPR:	1990 psi/137 bar
Flow rate:	2.5 mL/min
Column temp.:	40 °C
Injection volume:	4 µL
UPC^2 conditions:	0 min 20% B, 2.5 min 20% B, 2.6 min 30% B, 5 min 30% B, return to initial conditions
Chiral separation:	Uniconazole
Column:	Chiralpak IA-3 4.6 x 150 mm, 3.0 µm
Co-solvent (B):	50:50 2-propanol/ethanol with 2% water and 0.1% formic acid
ABPR:	1990 psi/137 bar
Flow rate:	2.5 mL/min
Column temp.:	15 °C
Injection volume:	4 µL
UPC^2 conditions:	0 min 15% B, 4 min 15% B, 4.1 min 35% B, 5 min 35% B, return to initial conditions

MS conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI+
Capillary voltage:	2.8 kV
Cone voltage:	See Table 1
Desolvation temp.:	600 °C
Source temp.:	150 °C
Collision energy (eV):	See Table 1
MS scan range:	100 to 800 *m/z*

An AgileSLEEVE™ 30 cm x 1/16" I.D. tubing heater (Analytical Sales and Services Inc. Pompton Plains, NJ) set to 65 °C was used to heat the transfer line to the MS system. All compounds were automatically tuned by direct infusion using IntelliStart™ prior to the analysis. A summary of the optimized MRM transitions and voltages is shown in Table 1.

In this application note, ACQUITY UPC^2 and tandem quadrupole mass spectrometry were used for the trace level enantioanalysis of five triazole fungicides (Figure 1) in wheat grain and/or wheat straw. A QuEChERS (quick easy cheap effective rugged and safe) extraction modified for dry commodities was performed followed by solid phase extraction using Oasis MCX. Chiral separations using a 3.0 µm chiral CSP followed by multiple reaction monitoring (MRM) detection allowed concentrations of part per trillion (ppt) levels to be reproducibly detected and quantified.

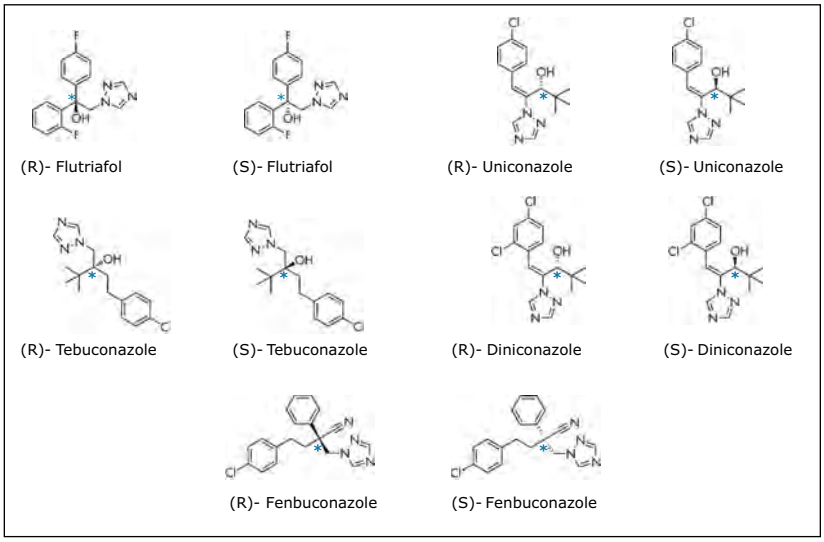

Figure 1. Structures of the target triazole fungicide enantiomers. The asterisks denote the stereogenic centers.

Instrumentation

All separations were performed on a Waters® ACQUITY UPC^2 System. Detection was by positive ion electrospray mass spectrometry (MS) using a Xevo TQ-S tandem quadrupole mass spectrometer. MassLynx Software was used for data acquisition, and TargetLynx Application Manager was used for data processing.

Sample preparation

Initial extraction (QuEChERS):

Triturated wheat straw (1 g) or wheat grain (5 g) were placed in a 50-mL polypropylene centrifuge tube. The volume of water added to the wheat straw was 9 mL with 5 mL of water added to the wheat grain, followed by phosphoric acid (100 µL) and acetonitrile (10 mL). The mixture was shaken for 20 minutes. A DisQuE Pouch for the European Committee for Standardization (CEN) QuEChERS method (Part No. 186006813) was added to the tube and shaken vigorously for 1 minute. Centrifugation at 4000 rpm followed to produce a liquid partition with a clear acetonitrile top layer. The top acetonitrile layer (5 mL) was transferred to a clean 50-mL centrifuge tube and diluted with water (45 mL) for cleanup using an Oasis MCX 3 cc, 60 mg Cartridge (Part No. 186000254).

Oasis MCX sample cleanup

Oasis MCX 3 cc, 60 mg Cartridges were conditioned with 3 mL of methanol and equilibrated with 3 mL of water. The samples were loaded in reverse phase mode into Sep-Pak 60 cc Reservoirs (Part No. 186005587) at a flow rate of 1 to 3 mL/min. After sample loading was completed the cartridge was washed with 2% formic acid in water (3 mL) followed by 100% methanol (3 mL). A collection vessel was installed and elution was achieved using 2 mL 2% ammonium hydroxide in methanol. The base containing eluent from the elution step was blown down to dryness and reconstituted in neat methanol (5 mL).

Standard and sample preparation

Working standard solutions were prepared by sequential dilution of the stock solution using acetonitrile. The working standards were spiked (in triplicate) on to the dry wheat straw/wheat grain at levels of 1, 5, and 10 ng/g. The samples were allowed to equilibrate for 30 min prior to extraction. Matrix-matched standard curves were prepared with blank matrix extracted using the same protocol.

Wheat straw and wheat grain samples were obtained from online vendors.

Table 1. MRM transitions and instrument settings for the analysis of the triazole fungicides. The primary quantitation transition is listed (top) with the confirmatory transition (bottom).

Analyte	MRM transitions	Cone voltage (V)	Collision energy (eV)
Diniconazole	326>70	16	20
	326>159	16	30
Fenbuconazole	337>70	14	18
	337>125	14	26
Flutriafol	302>70	16	16
	302>123	16	26
Tebuconazole	308>70	40	18
	308>125	40	36
Uniconazole	292>70	20	22
	292>125	20	24

RESULTS AND DISCUSSION

Enantioseparation of the five triazole fungicides

A Chiralpak IA-3, 4.6 x 150 mm, 3.0 µm was used to perform enantioseparation of the five triazole fungicides. Resolution was achieved for diniconazole, fenbuconazole, flutriafol, and tebuconazole using methanol as the co-solvent; while the chiral resolution of uniconazole was improved using a mixture of ethanol and 2-propanol (50:50 v/v). Water (2%) and formic acid (0.1%) were added directly to the co-solvents to promote ionization. A chromatogram of wheat grain directly spiked at a level of 1 ng/g and extracted using QuEChERS followed by sample cleanup using Oasis MCX is shown in Figure 2. All triazole AI's were enantiomerically resolved in less than 3.5 minutes. The United States Pharmacopeia (USP) resolution (Rs) ranged from 1.73 to 6.83.

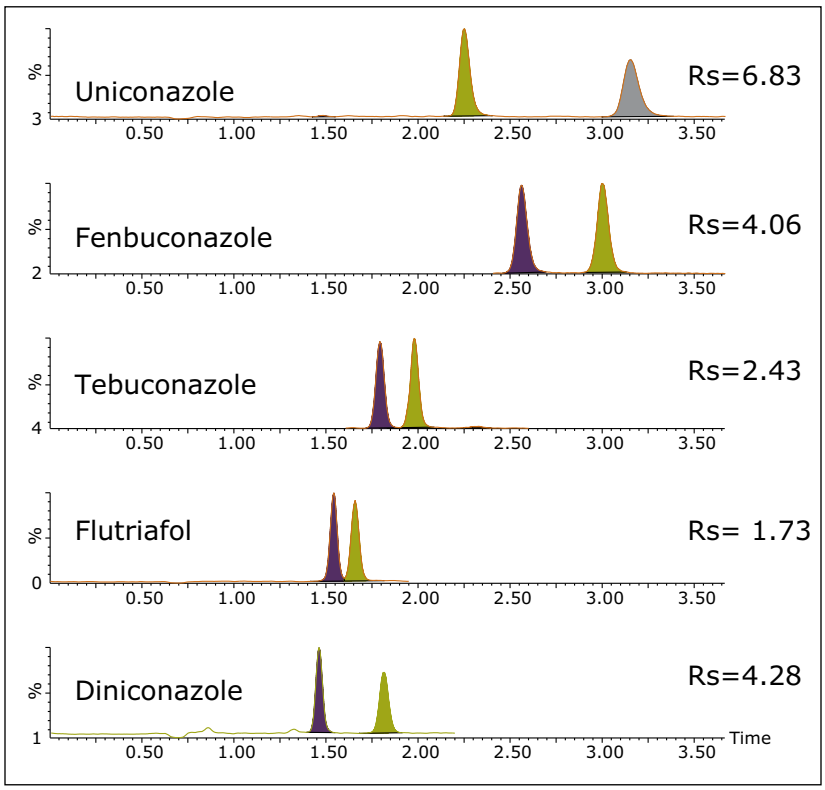

Figure 2. ACQUITY UPC²-MRM chromatograms showing the enantioseparation of five triazole fungicides pre-spiked onto wheat grain at a level of 1 ng/g and extracted using QuEChERS with Oasis MCX sample cleanup.

Linearity, accuracy, and sensitivity

The five triazole fungicides were post spiked into the wheat grain and/or wheat straw extracts. The spiked extracts were sequentially diluted with blank matrix extract to produce a series of matrix-matched curves and QC samples ranging in concentration from 0.005 to 50 ng/mL. Examples of the quantitation curves for each flutriafol enantiomer spiked into blank wheat grain extract, and for the fenbuconazole enantiomers spiked into blank wheat straw extract are shown in Figures 3 and 4 respectively. Linear calibration curves (R^2 >0.998) for each enantiomer of the target fungicides were obtained.

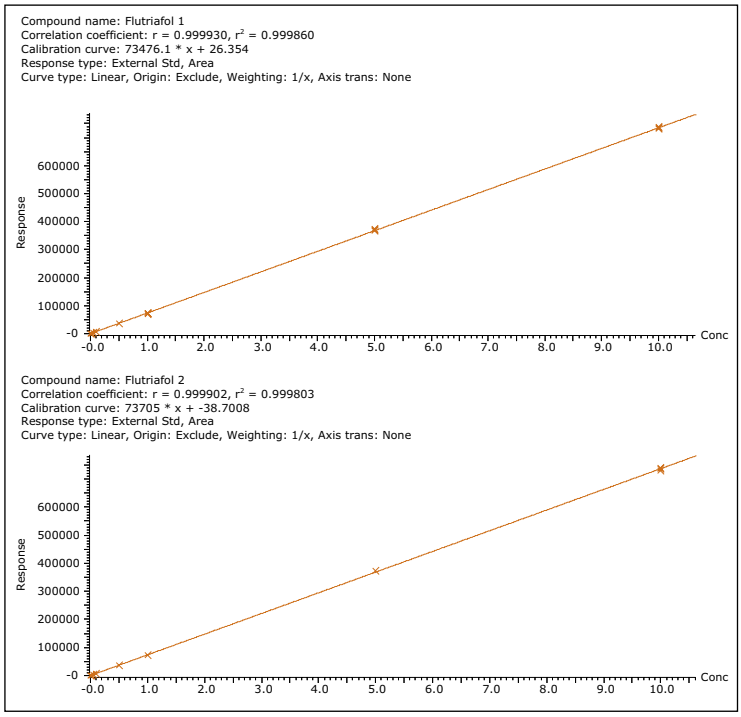

Figure 3. Wheat grain matrix-matched quantitation curves for each flutriafol enantiomer analyzed in triplicate 0.005 to 10 ng/mL.

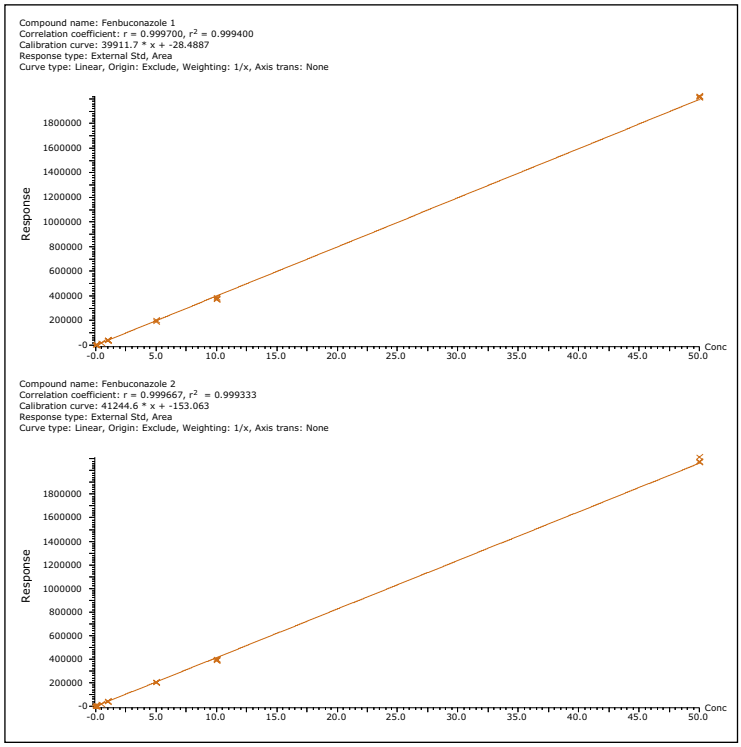

Figure 4. Wheat straw matrix-matched quantitation curves for each fenbuconazole enantiomer analyzed in triplicate 0.01 to 50 ng/mL.

To assess the accuracy of the method, quality control (QC) samples were made up in the blank extracted matrices at four concentration levels: 0.016, 0.16, 1.66, and 16.66 ng/mL. Three concentration levels were analyzed against the curves. The calculated concentrations for the QC samples were within +/- 15% of the known concentration for each enantiomer in both the wheat and straw matrices.

Examples of the blank, 0.01 ng/mL and 0.05 ng/mL level for all compounds spiked into extracted wheat grain matrix are shown in Figure 5.

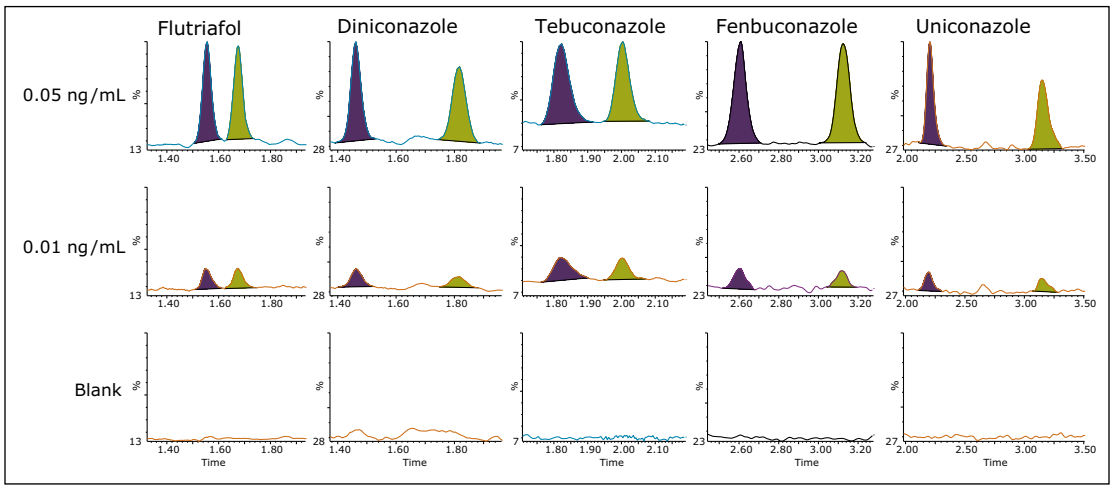

Figure 5. ACQUITY UPC²-MRM chromatograms showing the enantioseparation of five triazole fungicides spiked into blank wheat grain matrix extracted using QuEChERS, followed by cleanup using Oasis MCX. The blank, 0.01 ng/mL, and 0.05 ng/mL levels (equivalent to 0.02 ng/g and 0.1 ng/g if spiked directly on to the wheat grain pre-extraction) are shown; 4 µL injection.

Reproducibility

The precision of the technique was determined by a repeatability study (n=4) using four concentration levels 0.05 ng/mL, 1 ng/mL, 5 ng/mL, and 10 ng/mL of matrix-matched wheat grain standards, as shown in Table 2.

Table 2. Table shows the %RSD (n=4) for each enantiomer of the triazole fungicides at four concentration levels 0.05, 1, 5, and 10 ng/mL (4 µL injection).

		%RSD (n=4)			
	Enantiomer	0.05 ng/mL	1 ng/mL	5 ng/mL	10 ng/mL
Flutriafol	Peak 1	1.2	1.5	1.0	1.2
	Peak 2	2.9	1.9	0.6	2.5
Tebuconazole	Peak 1	1.3	1.3	2.8	2.0
	Peak 2	1.8	2.2	1.7	2.0
Fenbuconazole	Peak 1	1.9	0.8	2.5	1.3
	Peak 2	2.5	1.3	1.7	1.0
Diniconazole	Peak 1	2.4	2.9	2.1	1.1
	Peak 2	2.6	3.9	2.9	2.8
Uniconazole	Peak 1	1.5	1.3	1.9	1.7
	Peak 2	2.6	1.1	1.5	1.5

Internal standards were not available for the study; however the RSD's ranged from 0.6% to 3.9%. These results illustrate the reliability of the method reproducibility over a range of concentration levels.

Matrix effects and recovery

A series of standard solutions was prepared in methanol at the same concentration levels as the matrix-matched curves. The analyte response and slopes from both curves were compared. The matrix effects were calculated to within +/- 10% for each enantiomer of the target fungicides.

The average extraction recoveries from three samples fortified at 1 ng/g, 5 ng/ g, and 10 ng/g (n=3) in wheat were calculated. Recoveries in excess of 75% were obtained for each enantiomer of the pesticides analyzed in the study.

Simultaneous qualitative tools: RADAR™ and PICs

Depending on the chromatographic conditions target analytes can co-elute with endogenous matrix components which can lead to matrix effects and decreased method robustness. The Xevo TQ-S employs a proprietary scanning technology known as RADAR from which full scan (MS) and MRM (MS/MS) data can be acquired simultaneously. RADAR provides a convenient way to monitor the background matrix using its full-scan MS function. Co-eluting components can be identified at an earlier stage of the method development process.

In addition Product Ion Confirmation Scan (PICs) can be activated, which facilitate the collection high quality full-scan spectra during MRM acquisition, and provide an additional means of chromatographic peak identification based on MS or MS/MS spectra. Activated by a single check box in the method editor, PICS automatically triggers a product ion scan when a peak is detected by MRM.

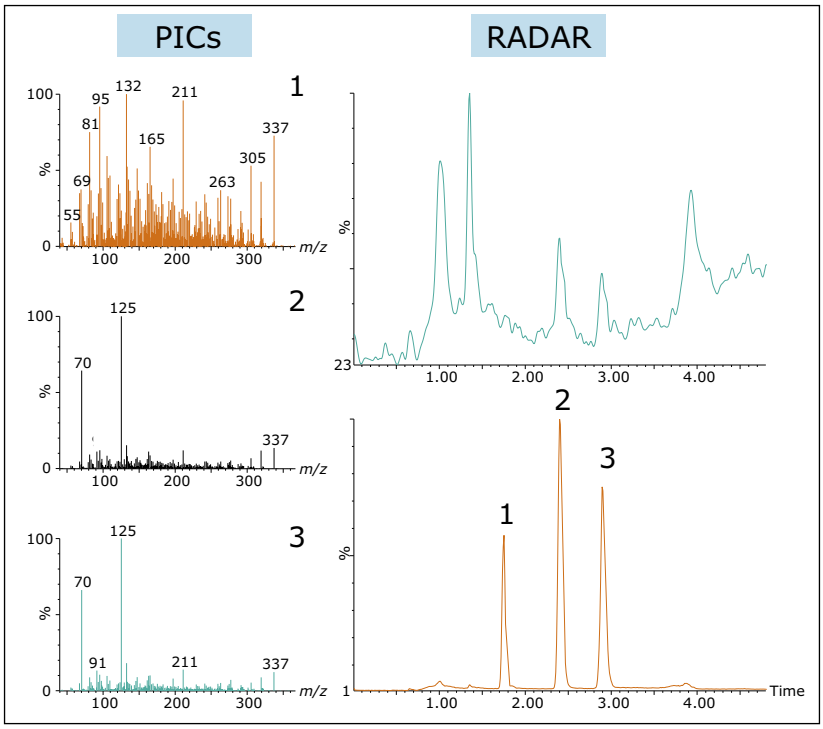

Figure 6. ACQUITY UPC²-MRM (lower trace) and RADAR chromatograms using gradient elution showing the resolution of fenbuconazole enantiomers pre-spiked onto wheat straw.

During method development for the analysis of fenbuconazole enantiomers in wheat straw the RADAR and PICs chromatograms shown in Figure 6 were acquired simultaneously with the MRM function. Peak 1 in the MRM chromatogram was isobaric (*m/z* 337) with fenbuconazole and shared a common fragment. Enantiomers were differentiated using the MS/MS PIC spectrum. The RADAR data acquired simultaneously identified components eluting closely to the analytes (Peaks 2 and 3). Changes were made to the SPE cleanup and isocratic elution was employed, which resulted in a lower spectral background and the removal of closely eluting matrix components.

CONCLUSIONS

The study of pesticide enantiomers is important as they can exhibit different bioactivities. Analytical methods that can rapidly provide information about each enantiomer at trace concentration levels can lead to a more accurate assessment of the influence of stereochemistry on the degradation dynamics, environmental fate, and final residue levels of crop protection chemicals.

In this study, the enantioseparation of five triazole fungicides was performed in less than 3.5 minutes. The Xevo TQ-S was used for detection of the rac-triazole fungicides in wheat grain and wheat straw. The results from the chiral UPC^2-MRM analysis show that trace level detection (ppt) can be achieved with good precision and accuracy over at least 3.5 orders of magnitude using this technique.

The use of RADAR, where full-scan data can be acquired simultaneously with MRM data can help identify co-eluting components that could potentially decrease the assay's robustness.

When complex matrices are analyzed, despite the specificity of MRM, matrix components give rise to signals that can be misidentified as an analyte peaks. PICs data provides an added qualitative element to the acquisition, which is useful for achieving higher selectivity, and greater confidence for peak assignment and confirmation.

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Methodology for the Identification of Pesticide Metabolites in Complex Matrices Using UPLC-ToF/MS^E and the UNIFI Scientific Information System

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Provides a comprehensive approach to metabolite identification in complex matrices using UltraPerformance Liquid Chromatography (UPLC®) and high resolution mass spectrometry in conjunction with the UNIFI® Scientific Information System
- Increased sample throughput resulting from UPLC chromatographic separations provide improved component resolution compared with high performance liquid chromatography (HPLC) separations.
- Enhanced confidence in component structural assignments via simultaneous collection of accurate mass precursor and product ion data without the need for pre-selection of precursors.
- Streamlines registration of new pesticide products by facilitating metabolite identification in complex matrices.

WATERS SOLUTIONS

Metabolite Identification Application Solution with UNIFI

ACQUITY UPLC® I-Class System

Xevo® G2- XS QTof

ACQUITY UPLC HSS T3 Column

KEY WORDS

Metabolite identification, pesticide metabolite, agrochemicals, environmental fate studies, pesticide registration

INTRODUCTION

Regulatory agencies such as the U.S. EPA require extensive documented and audited studies concerning the benefits and possible risks of using crop protection products before registration can occur.[1] For example, manufacturers must provide evidence of the potential human health and environmental effects. One such example includes the metabolic fate of the active ingredient(s) (AI) of a new product and the effects that potential metabolites can have on the overall environmental or human toxicity. Therefore, the accurate measurement of metabolites is important to assess the risk of exposure and to provide thorough product risk assessment.

Metabolite identification studies are commonly conducted by high resolution mass spectrometry using precursor and product ion data because the results improve confidence in regulatory submissions.[2] High resolution mass spectrometry techniques offer comprehensive information and intelligent software tools that can be extremely beneficial to the scientist by providing data interrogation. In some mass spectrometry technologies, a comprehensive precursor and product ion dataset is created by the use of "MS^E", which provides simultaneous acquisition of full scan precursor and product ion spectra from a single analytical injection without having to preselect precursors. This simplifies data acquisition because it does not require advanced knowledge of the analytes.[3] Data is collected in an untargeted manner with a high degree of mass accuracy. Therefore, elemental compositions can be obtained for both intact molecular ions and all fragment ions.

In this application note, atrazine (a commercially available herbicide that is well documented in the scientific literature) and its metabolites were spiked into soil extracts and used as a model to illustrate the workflow of Waters® Metabolite Identification Application Solution with UNIFI. The ACQUITY UPLC I-Class System coupled to the Xevo G2-XS QTof Mass Spectrometer were used to analyze atrazine and its metabolites in spiked soil extracts. Atrazine (Figure 1) is a triazine herbicide that is widely used to control weeds in a variety of crops including corn, sugar cane, and sorghum.[4-8] Using the data evaluation tools available with UNIFI, structural relationships between atrazine and the metabolites were easily visualized.

EXPERIMENTAL

Sample preparation

10 g of soil was weighed into a 50 mL centrifuge tube. 5 mL of water and 10 mL of acetonitrile were added to the soil sample and the mixture was shaken for 20 min. After a salting out step and cleanup of the resulting acetonitrile layer, atrazine and its metabolites were spiked directly into the soil extracts in preparation for the analysis.

UPLC conditions

UPLC system:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC HSS T3 2.1 x 100 mm, 1.8 µm [p/n: 186003539]
Mobile phase (A):	Water with 10 mM ammonium formate
Mobile phase (B):	Acetonitrile
Flow rate:	0.6 mL/min
Column temp.:	45 °C
Injection volume:	1 µL
UPLC gradient:	0 min 1% B, 1 min 1% B, 5 min 80% B, 6 min 90% B, return to initial conditions

MS conditions

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI + MS^E in resolution mode
Capillary voltage:	1.0 kV
Cone voltage:	20 V
Desolvation temp.:	400 °C
Source temp.:	120 °C
Cone gas:	50 L/hr
Desolvation gas:	900 L/hr
MS^E low collision energy (CE):	2 eV
MS^E high collision energy ramp:	17 to 45 eV
MS scan range:	50 to 950 *m/z*
Scan time:	0.15 s

Data processing

UNIFI Software was used for data acquisition and data processing.

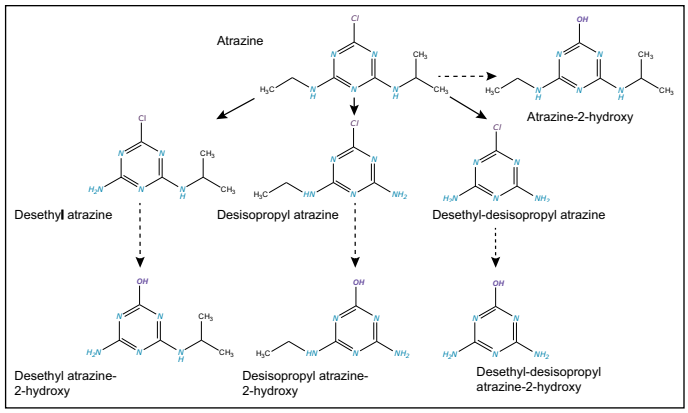


Figure 1. Examples of N-dealkylation and 2-hydroxylation metabolism of atrazine.[7]

RESULTS AND DISCUSSION

In this study atrazine was used as a model compound to demonstrate efficient metabolite identification using UPLC-MS^E and the UNIFI Scientific Information System. From the literature, metabolic biotransformation of atrazine can occur via 2-hydroxylation and N-dealkylation pathways (Figure 1).[7,9]

Conjugation with a glutathione can also occur and has been reported in the literature.[9,10] Hence, we investigated the known metabolic products as a starting point.

UNIFI workflows and data review

The UNIFI Scientific Information System is comprised of a series of workflow steps that are designed to enable thorough visualization of the entire dataset so that the information required to make a decision can easily be accessed with minimal user intervention. Users can define the process of how their data will be visualized with a single mouse click. This approach facilitates consistent, concise, and rapid review of an entire sample injection within an analysis. These steps are completely customizable and created to suit a particular analysis.

An example of a workflow designed for metabolite identification data review is shown in Figure 2.

Metabolite identification within UNIFI requires a structure of the target molecule (mol file) that can be stored in the Scientific Library. A selection of possible transformations is also required (Figure 3).

Within the UNIFI Scientific Library there are over 100 transformations. The storage and editing of custom biotransformations is also supported. UNIFI's Scientific Library is an integral part of the software package, which has is capable of not only storing structures, but also of retaining information associated with individual library entries. Parent compounds and metabolites of interest can also be stored in the library for later searching and retrieval.

In the following sections a number of workflow steps from Figure 2 will be discussed using the atrazine metabolism data set.

1. Metabolite review

Using a single left-click, the information required to view the identified metabolites in an injection is invoked within the predefined display setup in the workflow step. Metabolite Identification Review (Figure 4) shows the Component Summary, Chromatogram, and Spectral windows. The Component Summary shows a list of the metabolites that have been identified by the software. The chromatogram at the top shows XIC's of all of the identified metabolites, and the lower trace shows an XIC of the selected component; in this case, the parent pesticide atrazine. The spectra on the right side show both low and high collision energy (CE) MS^E data, as well as the likely product ion structural assignments. Two metabolites are listed below atrazine. Upon selection of the metabolites, the chromatographic, spectral, and product ion structural assignments can be viewed.

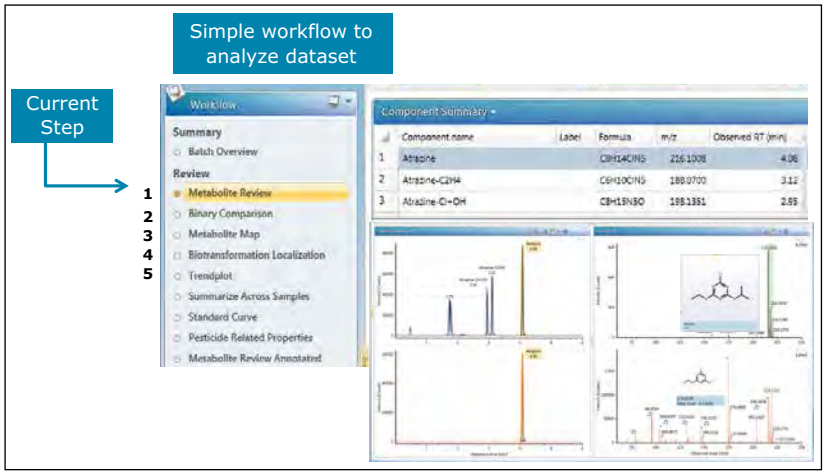


Figure 2. An example workflow to support a metabolite identification experiment within UNIFI is shown. The selected Metabolite Review workflow step presents an overview of the identified metabolites in the Component Summary. The extracted ion chromatograms (XIC) of the identified metabolites and spectra are also shown. Selecting each step in the workflow will allow complete analysis of the dataset.

Selected transformations

	Name	Delta Mass (Da):	Formula	Classifier
1	Deethylation	-28.0313	-C2H4	Phase I
2	Dechlorination	-34.9689	-Cl	Phase I
3	Oxidation	15.9949	+O	Phase I
4	Reductive dechlorination	-33.961	-Cl+H	Phase I
5	Oxidative dechlorination	-17.9661	-Cl+OH	Phase I
6	Reduction	2.0157	+H2	Phase I
7	S-Glutathione conjugation...	305.0682	+C10H15N3O6S	Phase II

Figure 3. Transformations used to identify potential metabolites.

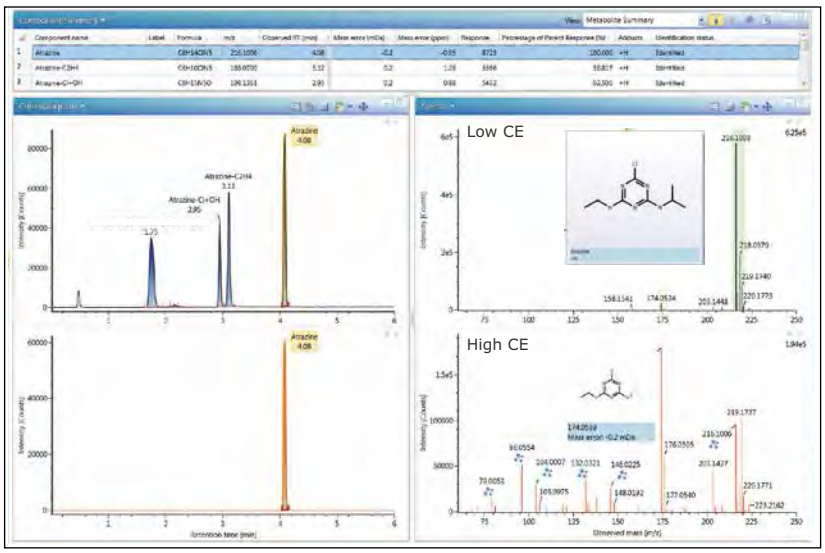


Figure 4. The Metabolite Identification Review window shows the Component Summary table, chromatograms, MS^E mass spectral data, as well as likely product ion structural assignments.

2. Binary Compare/control sample comparison

In this workflow step, a control/reference sample and an unknown sample can be automatically compared using the Binary Compare function. A metabolite with *m/z* 198.1351 at retention time (t_R) 2.95 min was identified in the Component Summary as having undergone the structural modification -Cl+OH (Figure 5). This transformation indicates that the metabolite is likely to be atrazine-2-hydroxy. The metabolite is unique to the unknown test sample as can be seen by the absence of chromatographic peaks or spectra in the top traces. The spectra can be viewed as high or low CE data. In this case, the low CE spectrum is displayed.

3. Metabolite Hierarchy Map

The Metabolite Hierarchy Map provides an overall snapshot of the metabolism based on the identified metabolites. At the center of the map resides the parent pesticide, while the identified metabolites circle the parent. The entire metabolism can be seen and useful data can be accessed from a single location including MS^E spectral data, mass fragment assignments for both the parent and all of the metabolites, and the Transformation Localization function (Figure 7).

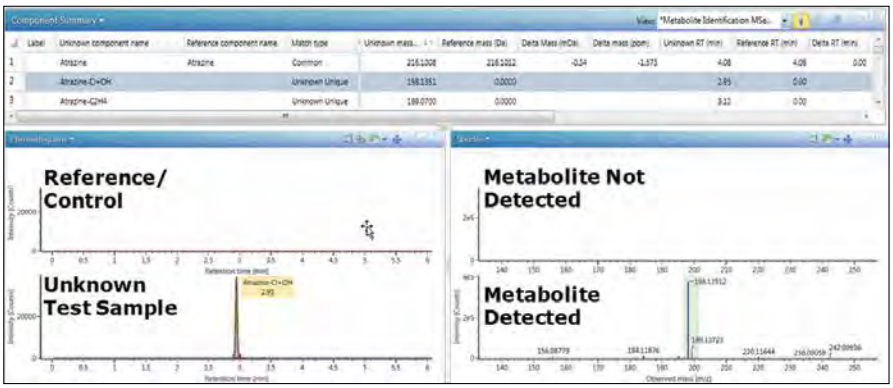


Figure 5. UNIFI's Binary Compare function shows the Component Summary, which lists the metabolites that are common or unique to the reference and unknown samples. An XIC of m/z 198.1351 produced a signal in the test sample but not in the reference. In this case, Atrazine-2-hydroxy is unique to the test sample.

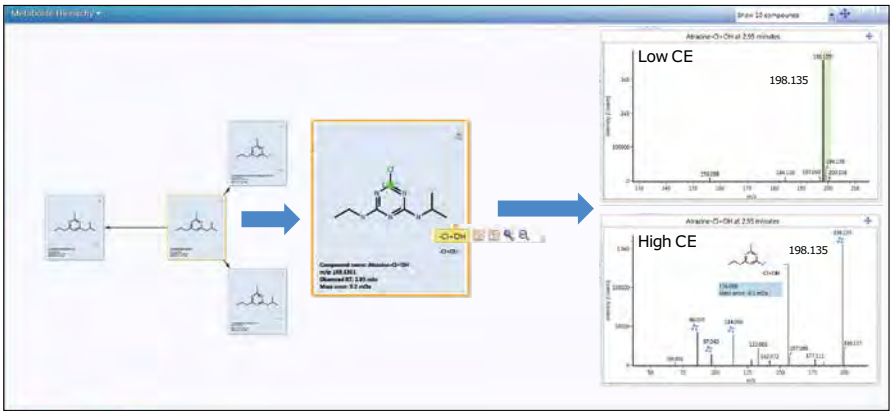


Figure 6. The Metabolite Hierarchy view allows a comprehensive overview of the entire dataset; spectral and structural data can be readily compared in a single place.

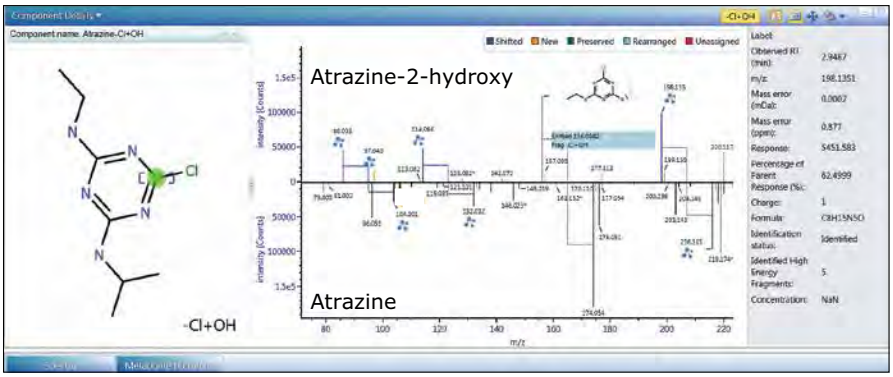


Figure 7. Integrated transformation localization performed automatically. Atrazine-2-hydroxy fragment ion spectrum (top) shown with the inverted spectrum of the parent pesticide atrazine (bottom).

4. Transformation Localization

Transformation localisation is automatically performed and gives an idea of where the position of metabolic transformation has likely occurred. An interactive interface allows for easy data review (Figure 7). The most likely location of the biotransformation within the structure is highlighted in green which indicates that there is a high probability of the transformation occurring at this site. The high energy MS^E spectrum of the metabolite on the top is compared with that of the parent pesticide in the lower spectral trace. The differences between them are highlighted and there are color-coded assignments made to the spectral peaks where UNIFI has identified relationships. In this example several mass shifts can be seen, indicated by the dark blue color that links the spectral peaks in the metabolite spectrum with those of the parent pesticide. In this case the mass shifts are due to the structural change –Cl+OH.

5. TrendPlot

With the UNIFI metabolite identification workflow each identified component in the sample can be confidently interrogated. The behavior of the metabolites relative to the parent pesticide can be observed using the TrendPlot function within UNIFI (Figure 8). To illustrate the utility of TrendPlot, a biotransformation kinetics study of atrazine was designed using samples spiked at different concentrations and at various time points. The plots depict how the fate of the primary metabolites, which appear as the parent is metabolized over time, can be monitored in the samples using the software capabilities within UNIFI.

For every point in the trend plots there is chromatographic, spectral, and structural information recorded within UNIFI. This data can be displayed in a table or as a plot view. The data shown in Figure 8 is represented as bar charts and superimposed line graphs.

The Elucidation toolset

Once the identified metabolites have been reviewed using the workflow steps, we can then investigate the data using the Elucidation toolset. Metabolites can be interrogated more intensively using the Elucidation toolset in UNIFI, which is comprised of a comprehensive suite of structural elucidation tools including ChemSpider Search from the Royal Society of Chemistry (www.chemspider.com), isotope modeling, and elemental composition tools. Discovery tools are also available for searching data for related ions using mass defect, common fragment, and neutral losses.

The MS^E data is a collected in a non-targeted way requiring no prior knowledge of the analytes. The fragment ion data can be used to search for structurally related components present in the sample.

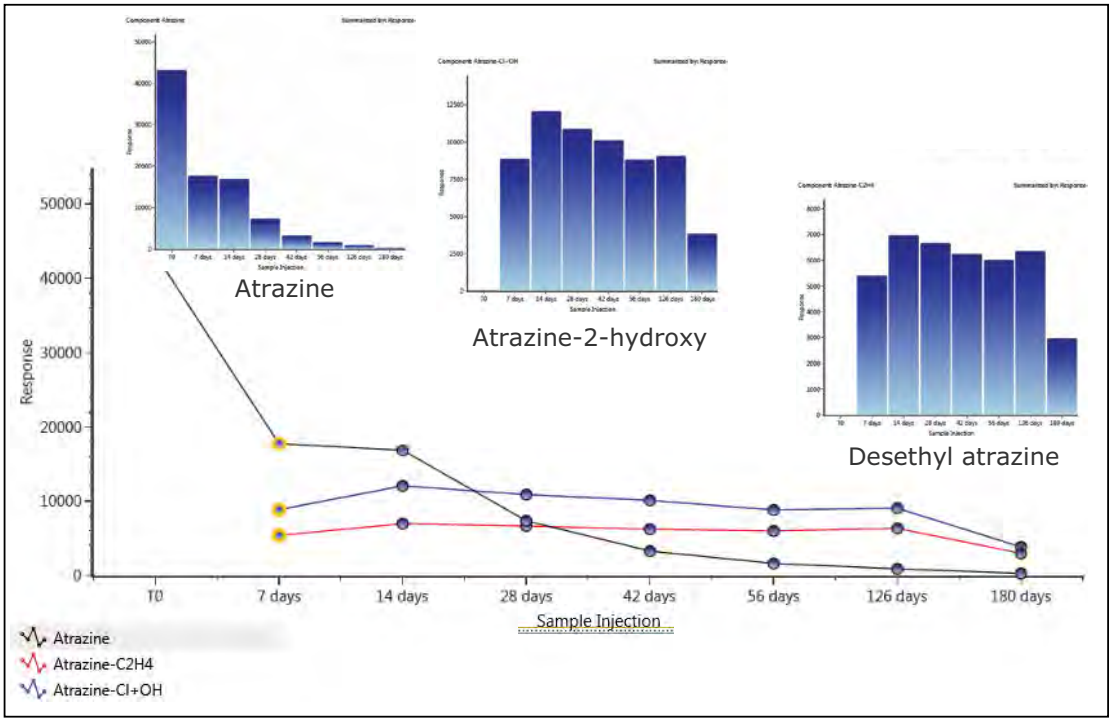


Figure 8. The TrendPlot function within UNIFI shows the graphical representation of a kinetics study made from spiked samples using atrazine and two of its metabolites: atrazine-2-hydroxy and desethyl atrazine. The graphical results shown are intended to demonstrate the results of a kinetics study.

Investigation of the metabolite identified as atrazine-C_2H_4 or desethyl atrazine, using transformation localization indicated that some product ions were preserved in the metabolite (data not shown). A fragment ion with *m/z* 79.005 was common to both the parent pesticide and desethyl atrazine.

Using UNIFI's Common Fragment Search function, components that share common structural features can be efficiently extracted from the data (Figure 9). The fragment *m/z* 79.005 was present in the product ion spectra of atrazine (Peak 3), desethyl atrazine (Peak 2), and a third component at retention time (t_R) 1.74 min (Peak 1). This component has been identified as having undergone two dealkylations, C_3H_6 plus C_2H_4 (desethyl-desisopropyl atrazine). The existence of the high energy fragment ion data along with the intact precursor provides information that allows structural relationships between the metabolites to be established in a single analytical injection.

Once the metabolites have been identified, they can be labeled and sent to the UNIFI Scientific Library where information about the t_R, accurate mass fragment ions, spectra, *etc.* can be stored for future analyses.

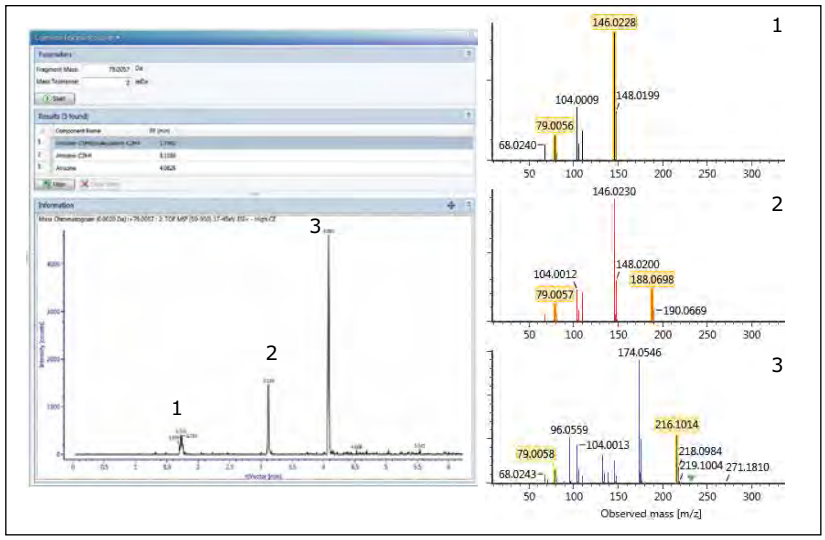
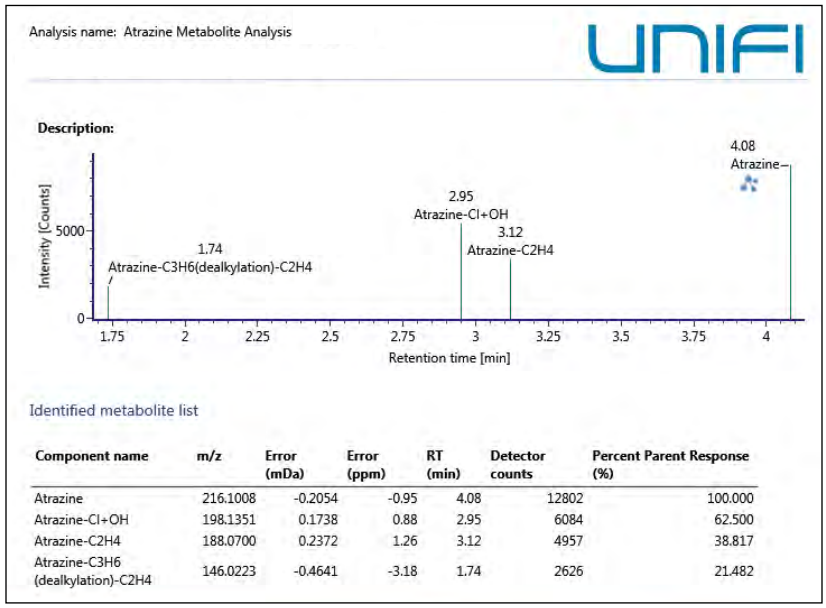


Figure 9. The UNIFI Common Fragment search is used to efficiently search the soil sample for components that have a fragment ion with m/z 79.0057 in the high CE data. Fragmentation spectra for each component (1–3) are also shown.



Component name	m/z	Error (mDa)	Error (ppm)	RT (min)	Detector counts	Percent Parent Response (%)
Atrazine	216.1008	-0.2054	-0.95	4.08	12802	100.000
Atrazine-Cl+OH	198.1351	0.1738	0.88	2.95	6084	62.500
Atrazine-C2H4	188.0700	0.2372	1.26	3.12	4957	38.817
Atrazine-C3H6 (dealkylation)-C2H4	146.0223	-0.4641	-3.18	1.74	2626	21.482

Figure 10. A summary of the atrazine metabolites found in the sample is presented in a fully customizable UNIFI report.

UNIFI reporting

Following data analysis, the organization of the data into a clear and fully customizable report is possible within UNIFI. All of the information accessed in the data review can be exported into the report including figures, graphs, tables, plots, and structural information, as shown in Figure 10. UNIFI can generate multiple reports per analysis that can each be customized to incorporate as little or as much detail as required for internal or external reports.

CONCLUSIONS

Identification of atrazine and its metabolites in spiked soil samples was successfully completed using the Metabolite Identification Application Solution with UNIFI. The use of customizable workflow steps allows data analysis to be performed more easily and efficiently with minimum user intervention. The relationships between atrazine and the metabolites were easily visualized using the data evaluation tools present within UNIFI. The workflow presented here for atrazine and its metabolites can also be applied to similar pesticide metabolite identification studies to greatly facilitate the new product registration process. The samples used in this study were formulated to test the overall analytical approach, software, and hardware, in a proof of principle analysis.

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