

azole-resistance in *Aspergillus fumigatus* has been found in Western European countries as well as in the Asia-Pacific due to the use of fungicides in agriculture to treat cereal crops and wheat. Thus, the risk of endocrine effects was considered in farmers and greenhouse workers from preparing azole spray mixtures.⁴

An important overlooked phenomenon characteristic of many antifungal agents is their chirality. Enantiomers of the same drug have different biological properties¹² leading to enantiomer-dependent effects on human metabolism, as well as occurrence in and biological effects on the environment.^{13–15} However, despite several papers published on the enantiomer-dependent fate and effects of several pharmaceuticals, the role of stereochemistry of most antifungal agents in the context of their fate and effect remains unknown. One of the reasons for this is the lack of available sensitive and selective analytical methods that can differentiate between enantiomers of the same pharmaceutical. Though several chiral methods have been developed to analyse chiral pharmaceuticals in the environment, high-performance liquid chromatography (HPLC) is the most commonly used technique. Chiral drugs are present in the environment at trace levels and in very complex matrices. Therefore, HPLC tandem mass spectrometry with triple quadrupole (QqQ) needs to be used for sensitive targeted identification and quantification. High resolution mass spectrometry such as QTOF can also be used for retrospective analysis and suspect screening, albeit with usually lower sensitivity. There are many factors which influence chiral recognition. These include the type of chiral selector, as well as mobile phase composition. HPLC-MS/MS has been applied in the analysis of enantiomers of antifungal agents in human serum using albumin (HSA), α 1-acid glycoprotein (AGP), cellulose, and amylose columns. The occurrence of antifungal agents and their enantiomers was reported in raw wastewater, sludge, soil, and fruit samples.^{16–24}

Although the presence of antifungal agents in the environment has become a major clinical and public health problem,¹ only a few reports have been published on the investigations of antifungal agents in China,¹⁶ Germany,²⁵ Switzerland,⁸ Ireland,²⁶ Belgium,²⁷ Spain²⁸ and UK.²⁹ Additionally, there is a lack of research in metabolism and transformation of chiral and achiral antifungal agents in the environment. Thus, this paper's objective is to introduce a new analytical method for combined separation of achiral and chiral antifungal agents and their metabolites with the utilization of chiral chromatography coupled with triple quadrupole tandem mass spectrometry to enable comprehensive profiling of wide-ranging antifungal agents and their metabolites in environmental matrices.

2. Materials and methods

2.1 Materials

HPLC-grade methanol (MeOH), acetonitrile (ACN), isopropanol (IPA), ammonium formate (NH₄HCO₂), ammonium acetate (NH₄OAc), formic acid ($\geq 96\%$) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (UK). Ultrapure water was obtained from a Milli-Q system (UK). Achiral antifungal agents

clotrimazole, fluconazole, hydroxy-tebuconazole, naftifine, prochloraz and terbinafine were purchased from Sigma Aldrich (UK). *N*-Desmethyl-carboxyterbinafine was purchased from Toronto Research Chemicals (Canada). Chiral antifungal agents used as racemates, (\pm)-econazole, (\pm)-ketoconazole, (\pm)-miconazole, (\pm)-propiconazole, (\pm)-prothioconazole-desthio, (\pm)-tebuconazole and hydroxy-tebuconazole, were purchased from Sigma Aldrich. (\pm)-Epoconazole, (\pm)-*N*-deacetyl ketoconazole and (\pm)-prothioconazole were purchased from Toronto Research Chemicals (UK). Stereoisomerically pure 2*R*,3*S*-voriconazole was purchased from Sigma Aldrich (UK). The deuterated standards terbinafine-d7, naftifine-d3, (\pm)-miconazole-d5, and (\pm)-econazole-d6 were purchased from Toronto Research Chemicals (Canada). (\pm)-Ketoconazole and (\pm)-voriconazole-d3 were purchased from Sigma Aldrich (UK). All chemicals used were of high purity ($\geq 97\%$). The target antifungal agents in this work were selected based on UK prescription data, usage of fungicides in the UK and occurrence in the environment. Table 1 shows the analytes with their CAS number, structure, molecular formula, molecular weight, p*K*_a, and log *P* along with their application.

2.2 Sample collection and preparation

River water, wastewater effluent and influent samples were collected in South West England in PTFE bottles as 24 h flow proportional composite samples (influent and effluent wastewater) or grab samples (river water) and placed in a cool box with ice during the transport from the site of sampling to the laboratory. Once in the laboratory, and after adjustment to pH 7 and addition of internal standards (to give the following concentrations: 1 ng mL⁻¹ in wastewater and 0.5 ng mL⁻¹ in river water or 100 ng mL⁻¹ in SPE extracts), samples were subject to filtration and solid-phase extraction (SPE) as described below.

2.3 Solid phase extraction

SPE was carried out using Oasis HLB cartridges (60 mg, Waters, UK). The SPE protocol is discussed in detail elsewhere.³² Briefly after filtration through a GF/F filter (0.7 μ m), 100 mL of river water or 50 mL wastewater was loaded into Oasis HLB cartridges (at 3 mL min⁻¹) and pre-conditioned with 2 mL of MeOH and 2 mL of H₂O (at 1 mL min⁻¹). The cartridges, after drying under vacuum for 30 min, were then eluted with 4 mL MeOH at 1 mL min⁻¹. The obtained eluate was subject to evaporation under nitrogen using a TurboVap evaporator (40 °C, N₂, <5 psi) and reconstituted with 500 μ L mobile phase (NH₄OAc/MeOH 1 : 99).

2.4 Chiral liquid chromatography-mass spectrometry (cLC-MS/MS)

Chromatographic separation was carried out using a chiral CHIRALCEL® OZ-RH column (5 μ m particle size, *L* × *I*. D.: 15 cm × 2.1 mm, Chiral Technologies, France) with a 2.0 mm × 2.0 mm guard filter (Chiral Technologies, France). The MS system was a triple quadrupole mass spectrometer (Xevo TQD, Waters, Manchester, UK) equipped with an electrospray



Table 1 (Contd.)

Compounds	Structure	CAS	Formula	M_w (g mol^{-1})	pK_a	Log P	Application
Prochloraz		67747-09-5	$\text{C}_{15}\text{H}_{16}\text{Cl}_3\text{N}_3\text{O}_2$	376.7	3.8	3.5	Crops
Propiconazole		60207-90-1	$\text{C}_{15}\text{H}_{17}\text{Cl}_2\text{N}_3\text{O}_2$	342.2	1.09	3.72	Crops
Prothioconazole		178928-70-6	$\text{C}_{14}\text{H}_{15}\text{Cl}_2\text{N}_3\text{OS}$	344.3	6.9	2.0	Crops
Prothioconazole-desthio		120983-64-4	$\text{C}_{14}\text{H}_{15}\text{Cl}_2\text{N}_3\text{O}$	312.19	13.25	—	Metabolite of prothioconazole
Tebuconazole		80443-41-0	$\text{C}_{16}\text{H}_{22}\text{ClN}_3\text{O}$	307.82	5.0	3.7	Crops
Hydroxy-tebuconazole		212267-64-6	$\text{C}_{16}\text{H}_{22}\text{ClN}_3\text{O}_2$	323.82	—	—	Metabolite of tebuconazole
Terbinafine		78628-80-5	$\text{C}_{21}\text{H}_{26}\text{ClN}$	327.9	8.86	5.51	Human or animal
N-Desmethylcarboxy terbinafine		99473-15-1	$\text{C}_{20}\text{H}_{21}\text{NO}_2$	307.39	—	—	Metabolite of terbinafine
Voriconazole		137234-62-9	$\text{C}_{16}\text{H}_{14}\text{F}_3\text{N}_5\text{O}$	349.31	12.71	1	Human or animal



water or wastewater (analyte conc._x) to analyte concentrations in the mobile phase (analyte conc._{QC}). The concentration of analyte in the blank river water and wastewater samples (analyte conc.₀) was subtracted from the measured concentration, to account for analyte already present in the matrix (eqn (5)). Recoveries were determined in triplicate at three different

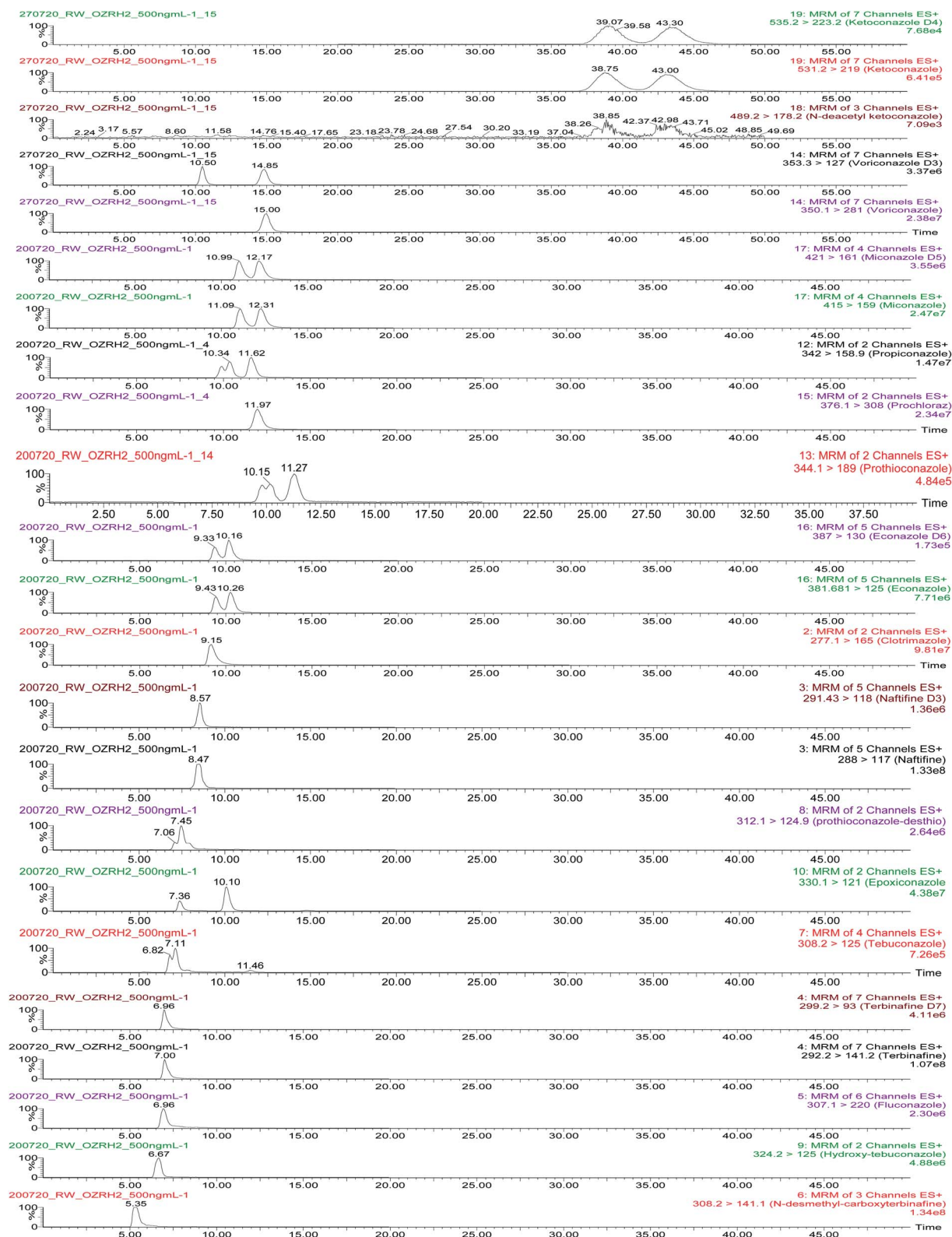


Fig. 1 Separation of antifungal agents by cLC-MS/MS.





Table 3 Instrumental performance data for selected antifungals and metabolites in the mobile phase

Compounds	Retention time	Linear range (ng mL ⁻¹)	R ²	EF	R _s	IDL (ng mL ⁻¹)	IQL (ng mL ⁻¹)	Intra-day		Inter-day	
								Accuracy [%]	Precision [%]	Accuracy [%]	Precision [%]
Clotrimazole	9.3	0.02–500	0.997		0.007	0.02	107	7	111	11	
Econazole E ₁	9.52	600–1000	0.997	0.52	0.02	0.08	103	2	104	5	
Econazole E ₂	10.4	600–1000	0.997		0.002	0.04	95	1	96	5	
Epoxiconazole E ₁	7.41	0.04–500	0.997	0.55	1.87	0.03	98	11	92	9	
Epoxiconazole E ₂	10.24	600–1000	0.998		0.006	0.02	97	9	98	9	
Fluconazole	6.96	0.02–500	0.995		0.04	0.1	95	2	105	2	
Ketoconazole E ₁	33.69	600–1000	0.997	0.52	0.4	1.2	96	2	96	5	
Ketoconazole E ₂	37.46	1.2–400	0.998		0.2	0.9	115	1	111	7	
N-Deacetyl ketoconazole (DAK) E ₁	33.54	500–1000	0.991								
N-Deacetyl ketoconazole (DAK) E ₂	39.22	500–1000	0.997	0.49	6.4	21.3	101	6	102	5	
Miconazole E ₁	11.24	500–100	0.998	0.52	11.6	38.6	90	2	94	8	
Miconazole E ₂	12.46	0.03–500	0.997		0.01	0.03	111	4	101	9	
Naftifine	8.17	0.03–500	0.993		0.01	0.03	108	1	108	2	
Prochloraz	10.89	0.004–1000	0.997		0.001	0.004	93	2	88	4	
Propiconazole E ₁	10.54	0.02–900	0.997	0.51	0.005	0.02	105	1	105	6	
Propiconazole E ₂	11.47	0.06–1000	0.998		0.02	0.06	94	4	98	12	
Prothioconazole E ₁	10.3	0.04–1000	0.998	0.52	0.01	0.04	97	1	91	11	
Prothioconazole E ₂	11.67	2.9–1000	0.997		0.9	2.9	90	4	99	14	
Prothioconazole-desthio	7.45	2.8–1000	0.999		0.8	2.8	96	2	99	6	
Tebuconazole	7.11	0.4–700	0.98		0.1	0.4	98	3	96	5	
Hydroxy-tebuconazole	6.67	0.1–1000	0.993		0.04	0.1	93	1	96	6	
Terbinafine	7.05	0.07–1000	0.997		0.02	0.07	108	4	99	10	
N-Desmethyl-carboxyterbinafine	5.84	0.01–1000	0.998		0.002	0.01	98	3	95	6	
Voriconazole	16.17	0.07–300	0.996		0.01	0.07	109	5	106	7	
		500–1000	0.992				100	2	98	3	
		0.02–1000	0.998		0.006	0.02					

concentrations, and then averaged. The analyte was spiked into the sample matrix with the internal standard, before filtration and SPE as described above.³³

$$\text{Overall relative recovery (\%)} = \frac{\text{Analyte conc.}_x - \text{analyte conc.}_0}{\text{Analyte conc.}_{\text{QC}_x}} \times 100 \quad (5)$$

The matrix effect (ME) was calculated by comparing the concentrations of the post-spiked sample (analyte conc._{ME,x}) minus analyte concentrations in the blank (analyte conc.₀) to analyte concentrations in the mobile phase (analyte conc._{QC,x}) at the following concentration levels (eqn (6)).³³

$$\text{Matrix effect (\%)} = \left(\frac{\text{Analyte conc.}_{\text{ME},x} - \text{analyte conc.}_{\text{ME},0}}{\text{Analyte conc.}_{\text{QC}_x}} \right) - 1 \times 100 \quad (6)$$

In environmental samples, the method detection limit (MDL) was calculated using the following equation:³⁴

$$\text{MDL} = \frac{(\text{IDL}_{\text{ng/L}} \times 100)}{\text{Rec} \times \text{CF}} \quad (7)$$

In the same way, the method quantification limit (MQL) in the environmental samples was calculated as follows:³⁴

$$\text{MQL} = \frac{(\text{IQL}_{\text{ng/L}} \times 100)}{\text{Rec} \times \text{CF}} \quad (8)$$

Rec is the relative recovery of the analyte in the matrix, that is the average of the recoveries obtained at three different concentrations considering the internal standard, and CF is the concentration factor.

Method accuracy (MD) was calculated (eqn (9)) to determine how close the measured concentration (analyte conc._{x₁-x₃}) was to spiked concentrations (x) and method precision (MP) was used to measure how similar the measured concentration values were to each other (eqn (10)). The concentration of the analyte in the blank river water and wastewater samples (analyte conc.₀)_{x₁-x₃} was subtracted from the measured concentration. The standard deviation of analyte concentration is denoted by σ .

$$\text{Method accuracy (\%)} = \frac{x}{(\text{average analyte conc.}_x - \text{analyte conc.}_0)_{x_1-x_3}} \quad (9)$$

$$\text{Method precision (\% RSD)} = \frac{\sigma(\text{average analyte conc.}_x - \text{analyte conc.}_0)_{x_1-x_3}}{(\text{average analyte conc.}_x - \text{analyte conc.}_0)_{x_1-x_3}} \quad (10)$$

3. Results and discussion

3.1 Liquid chromatography-tandem mass spectrometry

Antifungal compounds and their metabolites were analysed using cLC-MS/MS in ESI+ mode. Optimised multiple reaction monitoring (MRM) transitions are presented in Table 2. Seventeen compounds were separated using a chiral CHIRALCEL® OZ-RH column (5 μm particle size, $L \times I. D.$: 15 cm \times 2.1 mm, Chiral Technologies, France) with a 2.0 mm \times 2.0 mm guard filter (Chiral Technologies, France). The following parameters were considered when selecting method conditions: analytical characteristics such as peak area, enantiomeric resolution and signal-to-noise ratio. The selected mobile phase was 10 mM $\text{NH}_4\text{OAc}/\text{MeOH}$ 1 : 99 at a flow rate of 0.075 mL min^{-1} and temperature of 25 $^\circ\text{C}$.

Mass chromatograms showing analyte and enantiomeric separations are presented in Fig. 1. Prothioconazole, econazole, miconazole, ketoconazole and ketoconazole metabolite, epoxiconazole and propiconazole were separated with R_s denoting 0.80, 0.56, 0.54, 0.65, 0.61, 1.87 and 0.82, respectively. The

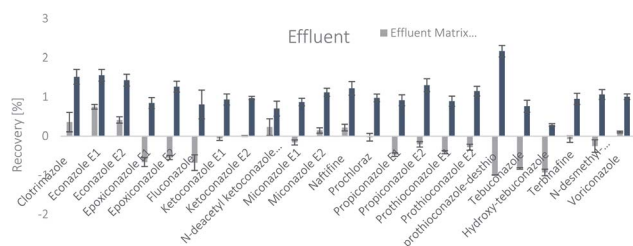


Fig. 3 SPE recovery and matrix effect of antifungal agents in effluent samples (a negative value indicates ionization suppression and a positive value indicates ionization enhancement).

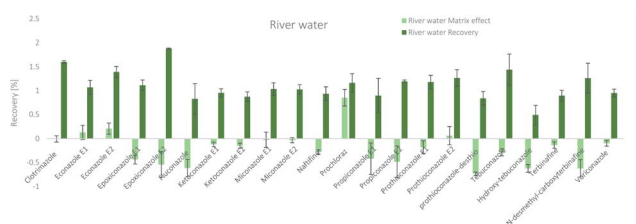


Fig. 2 SPE recovery and matrix effect of antifungal agents in river water samples (a negative value indicates ionization suppression and a positive value indicates ionization enhancement).

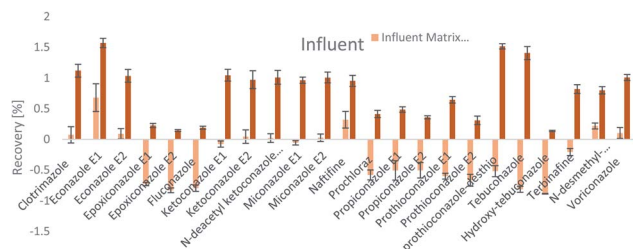


Fig. 4 SPE recovery and matrix effect of antifungal agents in influent samples (a negative value indicates ionization suppression and a positive value indicates ionization enhancement).



Table 4 Method performance data for antifungal agents and metabolites

Compounds	River water						Effluent						Influent					
	EF	R _s	MDL (ng L ⁻¹)	MQL (ng L ⁻¹)	MA (%)	MP (%)	EF	R _s	MDL (ng L ⁻¹)	MQL (ng L ⁻¹)	MA (%)	MP (%)	EF	R _s	MDL (ng L ⁻¹)	MQL (ng L ⁻¹)	MA (%)	MP (%)
Clotrimazole	—	—	2.4	7.1	85	23	—	—	4.5	15.1	69	15	—	—	6.1	20.4	86	18
Econazole E ₁	0.46	0.59	10.9	36.2	105	13	0.50	0.51	14.9	49.8	96	10	0.49	0.73	14.8	49.3	104	15
Econazole E ₂	—	—	0.6	14.4	93	8	—	—	1.3	27.9	98	12	—	—	1.7	38.6	113	7
Epoxiconazole E ₁	0.49	2.04	3.7	12.2	100	13	0.52	1.81	9.7	32.3	81	19	0.49	1.93	36.0	120.01	112	7
Epoxiconazole E ₂	—	—	1.5	5.04	94	15	—	—	4.5	15.0	72	11	—	—	39.8	132.5	109	7
Fluconazole	—	—	26.06	86.9	88	17	—	—	53.4	177.9	87	18	—	—	225.6	751.9	89	16
Ketoconazole E ₁	0.50	0.82	192.6	641.9	96	9	0.61	0.84	393.4	1311.3	61	10	0.64	0.89	351.2	1170.7	94	6
Ketoconazole E ₂	—	—	98.3	536.5	101	9	—	—	176.8	964.8	97	3	—	—	176.5	963.3	125	5
N-Deacetyl ketoconazole (DAK)	—	—	—	—	—	—	—	—	1045.1	30362.5	135	18	—	—	6315.3	21051.1	98	12
N-Deacetyl ketoconazole (DAK) E ₂	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Miconazole E ₁	0.58	0.68	4.5	14.9	86	19	0.55	0.69	10.6	35.4	95	23	0.59	1.26	9.5	31.8	95	3
Miconazole E ₂	—	—	4.9	16.4	123	24	—	—	9.05	30.2	104	22	—	—	10.0	33.4	143	31
Naftifine	—	—	0.6	1.9	87	11	—	—	0.9	2.8	89	16	—	—	1.1	3.6	97	27
Prochloraz	—	—	2.2	7.5	78	16	—	—	5.3	17.9	84	15	—	—	12.6	42.0	94	13
Propiconazole E ₁	0.48	0.81	9.4	31.5	92	17	0.50	1.01	18.5	61.9	94	9	0.52	1.09	34.8	116.1	95	9
Propiconazole E ₂	—	—	4.9	16.4	102	22	—	—	9.03	30.1	89	19	—	—	32.6	108.7	104	18
Prothioconazole E ₁	0.50	0.94	373.4	1244.6	88	5	0.48	1.21	989.9	3299.8	96	14	0.52	1.02	1363.5	4545.0	78	8
Prothioconazole E ₂	—	—	334.2	1114.2	99	19	—	—	735.3	2450.9	89	8	—	—	2720.8	9069.5	92	17
Prothioconazole-desthio	—	—	62.7	208.9	74	23	—	—	48.4	161.4	75	13	—	—	69.3	231.1	63	3
Tebuconazole	—	—	14.9	49.8	90	16	—	—	56.5	188.5	96	6	—	—	30.6	102.05	61	25
Hydroxy-tebuconazole	—	—	22.3	74.2	126	10	—	—	77.7	259.0	87	9	—	—	159.6	532.05	105	19
Terbinafine	—	—	1.02	3.4	106	17	—	—	1.9	6.4	96	6	—	—	2.2	7.4	107	9
N-Desmethyl-carboxyterbinafine	—	—	3.8	12.8	82	19	—	—	9.1	30.5	78	23	—	—	12.1	40.4	127	7
Voriconazole	—	—	3.4	11.2	103	9	—	—	6.4	21.3	100	7	—	—	6.3	21.1	107	8



results of 2 chiral center compounds (ketoconazole, ketoconazole metabolite, epoxiconazole and propiconazole) provided 2 peaks because chemical compounds in this research study are a racemic mixture of 2 enantiomers. Other racemic compounds (tebuconazole, hydroxy-tebuconazole and prothioconazole-desthio) could not be separated and are reported as the sum of two enantiomers. The method provided very good separation and peak shapes for achiral compounds.

3.2 cLC-MS/MS performance

The following parameters were measured to test instrument performance: linearity, the instrumental limit of detection (IDL), the instrumental limit of quantification (IQL), the enantiomeric fraction (EF) of chiral compounds and instrument accuracy and precision.

All analytes showed average linearities of $r^2 > 0.997$ within the tested linearity range. Table 3 shows the r^2 and range of all selected analytes including 7 enantiomeric pairs (econazole, epoxiconazole, ketoconazole, miconazole, *N*-deacetyl ketoconazole (DAK), propiconazole and prothioconazole). However, some compounds (clotrimazole, econazole, epoxiconazole, fluconazole, ketoconazole, *n*-deacetyl ketoconazole (DAK), prochloraz, propiconazole, terbinafine and voriconazole) required two calibration curves to maintain $r^2 \geq 0.99$.

Inter-day and intra-day instrument precision were studied at three different concentrations, 10, 100 and 1000 ng mL⁻¹. As can be seen in Table 3, intra-day and inter-day instrumental precision was <15% for all compounds. Moreover, the method is characterized by high accuracy between 88 and 115% for most compounds.

The EF provided the relative ratio of enantiomers of chiral compounds. As can be seen from Table 3, EFs of econazole, epoxiconazole, miconazole, ketoconazole and its metabolite, propiconazole and prothioconazole are within 0.49–0.55 at low, medium and high concentration levels. The resolutions of enantiomers are between 0.54 and 1.87. Very good method sensitivity was achieved with IDLs ranging from 0.001 to 11.6 ng mL⁻¹ and IQLs ranging from 0.004 to 38.6 ng mL⁻¹.

3.3 SPE-cLC-MS/MS performance

The SPE methodology utilized a hydrophilic lipophilic balanced (HLB) copolymer as the extraction phase. SPE recoveries and matrix effects were calculated using eqn (3) and (4), respectively. As can be seen from Fig. 2–4, the SPE recoveries and matrix effects of antifungal agents are on average 98%. The recoveries of ketoconazole, miconazole, terbinafine, *N*-desmethyl-carboxyterbinafine and voriconazole of river water, influent and effluent samples were between 80 and 119% with deviation from 100% linked with matrix effects. Lower apparent recoveries of epoxiconazole, fluconazole, hydroxytebuconazole, propiconazole, prothioconazole, and prothioconazole-desthio in the influent are due to ion suppression as shown by the high negative percentage of matrix effects in Fig. 2–4.

Table 4 shows method performance parameters. MDLs and MQLs were calculated from eqn (5) and (6), respectively. MQLs for liquid matrices ranged from 1.9 ng L⁻¹ for naftifine in surface water, to 30362.5 ng L⁻¹ for the metabolite of ketoconazole in the effluent. The MDLs and MQLs of most analytes are low enough to measure in the environment.^{8,16,26,27,29,35–41} EFs are within 0.46–0.64. The resolutions of enantiomeric pairs are between 0.51 and 2.04 in river water, effluent and influent. Most

Table 5 Average concentrations of antifungal agents and metabolites found in several matrices

Compounds	River water (ng L ⁻¹)	Effluent (ng L ⁻¹)	Influent (ng L ⁻¹)
Clotrimazole	ND	ND	ND
Econazole <i>E</i> ₁	ND	ND	ND
Econazole <i>E</i> ₂	ND	ND	ND
Epoxiconazole <i>E</i> ₁	67.3 ± 26.5	ND	ND
Epoxiconazole <i>E</i> ₂	13.2 ± 4.4	ND	ND
Fluconazole	<MQL	101.0 ± 35.6	ND
Ketoconazole <i>E</i> ₁	ND	ND	ND
Ketoconazole <i>E</i> ₂	ND	ND	ND
<i>N</i> -Deacetyl ketoconazole (DAK) <i>E</i> ₁	ND	218.2 ± 38.6	ND
<i>N</i> -Deacetyl ketoconazole (DAK) <i>E</i> ₂	ND	ND	ND
Miconazole <i>E</i> ₁	ND	ND	ND
Miconazole <i>E</i> ₂	ND	ND	ND
Naftifine	ND	ND	ND
Prochloraz	ND	ND	ND
Propiconazole <i>E</i> ₁	32.2 ± 2.0	ND	ND
Propiconazole <i>E</i> ₂	41.3 ± 0.9	ND	ND
Prothioconazole <i>E</i> ₁	ND	ND	ND
Prothioconazole <i>E</i> ₂	ND	ND	ND
Prothioconazole-desthio	ND	ND	ND
Tebuconazole	252.4 ± 70.2	927.5 ± 2.4	115.1 ± 37.6
Hydroxy-tebuconazole	228.9 ± 54.8	ND	ND
Terbinafine	50.2 ± 6.5	ND	30.5 ± 2.4
<i>N</i> -Desmethyl-carboxyterbinafine	<MDL	ND	ND
Voriconazole	ND	ND	ND



of the compounds provided good method accuracy (61–143%) and precision (3–31%).

3.4 Application to environmental matrices

The new multi-residue analytical method was applied to determine the concentration of antifungal drugs and plant fungicides in river water, influent and effluent samples collected in South West England (Table 5). The fungicide tebuconazole was found at the following concentrations: 252.4 ± 70.2 , 927.5 ± 2.4 and 115.1 ± 37.6 ng L⁻¹ in river water, effluent and influent, respectively. It is worth noting that its concentrations were higher in river water than wastewater influent indicating other than communal sources of this fungicide in the aqueous environment. Interestingly, effluent concentrations are the highest, which warrants further study regarding transformation of tebuconazole during wastewater treatment. Indeed, tebuconazole is primarily used on crops. Its metabolite, hydroxy-tebuconazole, was quantified only in the river water at 228.9 ± 54.8 ng L⁻¹ confirming its usage and environmental transformation. Terbinafine (used in both human and animal treatment) was also determined in river water (50.2 ± 6.5 ng L⁻¹) at higher concentrations than in wastewater influent (30.5 ± 2.4 ng L⁻¹). Its metabolite, *N*-desmethyl-carboxyterbinafine, was identified only in river water at <MDL indicating other than communal sources of this contaminant. Fluconazole was present at <MQL and 101.0 ± 35.6 ng L⁻¹ in river water and effluent, respectively. Epoxiconazole enantiomers (with primary usage on crops) were quantified only in river water with significant predominance of the *E*₁ enantiomer: 67.3 ± 26.5 ng L⁻¹ and 13.2 ± 4.4 ng L⁻¹ for *E*₁ and *E*₂, respectively. Propiconazole enantiomers (with primary usage on crops) were also quantified only in river water at concentrations of 32.2 ± 2.0 ng L⁻¹ and 41.3 ± 0.9 ng L⁻¹ for *E*₁ and *E*₂ enantiomers, respectively. However, only one enantiomer of deacetyl-ketoconazole was determined in effluent wastewater at a concentration of 218.21 ± 38.62 ng L⁻¹. In summary, the results of this study indicate predominance of antifungal agents in the aqueous environment with sources linked with animal and plant protection rather than usage in humans. Interestingly, chiral fungicides quantified in the river water were enriched with one enantiomer. This might have consequences in terms of their ecological effects which warrants further study.

4. Conclusions

A new multiresidue method utilizing chiral chromatography (with a chiral CHIRALCEL® OZ-RH column) and triple quadrupole tandem mass spectrometry was developed for sensitive and selective enantiomer-dependent analysis of fungicides and their metabolites in aqueous matrices such as river water and wastewater. The method showed very good linearity and range ($r^2 > 0.997$), method accuracy (61–143%) and precision (3–31%) as well as low MQLs (1.9–30362.5 ng L⁻¹). The method was applied in selected environmental samples. The following analytes were quantified: fluconazole, terbinafine, *N*-desmethyl-carboxyterbinafine, tebuconazole, hydroxy-tebuconazole,

epoxiconazole, propiconazole and *N*-deacetyl ketoconazole. They were predominantly present in the aqueous environment (as opposed to wastewater) with sources linked with animal and plant protection rather than usage in humans. Interestingly, chiral fungicides quantified in the river water were enriched with one enantiomer. This might have consequences in terms of their ecological effects which warrants further study, also focussed on identification of individual enantiomers.

Conflicts of interest

The authors declare no conflicts of interest.

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References

- 1 M. C. Fisher, N. J. Hawkins, D. Sanglard and S. J. Gurr, *Science*, 2018, **360**, 739–742.
- 2 Chapter 48. Antifungal Agents, Katzung & Trevor's Pharmacology: Examination & Board Review, 10e, AccessPharmacy, McGraw-Hill Medical, accessed 17 July 2018, <https://accesspharmacy.mhmedical.com/content.aspx?bookid=514§ionid=41817567>.
- 3 R. M. dos Santos and M. V. Dias-Souza, *Saudi J. Biol. Sci.*, 2017, **24**, 331–337.
- 4 C. R. Stensvold, L. N. Jørgensen and M. C. Arendrup, *Curr. Fungal Infect. Rep.*, 2012, **6**, 178–191.
- 5 P. H. Howard and D. C. G. Muir, *Environ. Sci. Technol.*, 2011, **45**, 6938–6946.
- 6 K. Bester, L. Scholes, C. Wahlberg and C. S. McArdell, *Water Air Soil Pollut. Focus*, 2008, **8**, 407–423.
- 7 J. P. Zubrod, M. Bundschuh, G. Arts, C. A. Brühl, G. Imfeld, A. Knäbel, S. Payraudeau, J. J. Rasmussen, J. Rohr, A. Scharmüller, K. Smalling, S. Stehle, R. Schulz and R. B. Schäfer, *Environ. Sci. Technol.*, 2019, **53**, 3347–3365.
- 8 Z. F. Chen and G. G. Ying, *Environ. Int.*, 2015, **84**, 142–153.
- 9 M. H. Haeba, K. Hilscherová, E. Mazurová and L. Bláha, *Environ. Sci. Pollut. Res. Int.*, 2008, **15**, 222–227.
- 10 M. B. Kjærstad, C. Taxvig, C. Nellemann, A. M. Vinggaard and H. R. Andersen, *Reprod. Toxicol.*, 2010, **30**, 573–582.
- 11 J. E. Woolery, E. Wombwell and M. R. Green, *Clin. Med. Insights Ther.*, 2012, **4**, CMT S5434.
- 12 L. A. Nguyen, H. He and C. Pham-Huy, *Int. J. Biomed. Sci.*, 2006, **2**, 85–100.
- 13 B. Kasprzyk-Hordern, *Chem. Soc. Rev.*, 2010, **39**, 4466.
- 14 D. Camacho-Muñoz and B. Kasprzyk-Hordern, *J. Mass Spectrom.*, 2017, **52**, 94–108.
- 15 S. Evans, J. Bagnall and B. Kasprzyk-Hordern, *Environ. Pollut.*, 2017, **230**, 368–377.
- 16 Q. Huang, Z. Wang, C. Wang and X. Peng, *Environ. Sci. Pollut. Res.*, 2013, **20**, 8890–8899.



- 17 Q. Huang, K. Zhang, Z. Wang, C. Wang and X. Peng, *Anal. Bioanal. Chem.*, 2012, **403**, 1751–1760.
- 18 C. Knebel, T. Heise, U. M. Zanger, A. Lampen, P. Marx-Stoelting and A. Braeuning, *Food Chem. Toxicol.*, 2019, **123**, 481–491.
- 19 Z. Zhang, Q. Zhang, B. Gao, G. Gou, L. Li, H. Shi and M. Wang, *J. Agric. Food Chem.*, 2017, **65**, 8241–8247.
- 20 M. Youness, M. Sancelme, B. Combourieu and P. Besse-Hoggan, *J. Hazard. Mater.*, 2018, **351**, 160–168.
- 21 Y. Li, F. Dong, X. Liu, J. Xu, J. Li, Z. Kong, X. Chen and Y. Zheng, *J. Sep. Sci.*, 2012, **35**, 206–215.
- 22 N. Liu, F. Dong, J. Xu, X. Liu, Z. Chen, Y. Tao, X. Pan, X. Chen and Y. Zheng, *J. Agric. Food Chem.*, 2015, **63**, 6297–6303.
- 23 H. Zhang, M. Qian, X. Wang, X. Wang, H. Xu, Q. Wang and M. Wang, *J. Sep. Sci.*, 2012, **35**, 773–777.
- 24 Z. Wang, P. Zhao, J. Yu, Z. Jiang and X. Guo, *Microchem. J.*, 2018, **140**, 222–231.
- 25 A. Wick, G. Fink and T. A. Ternes, *J. Chromatogr. A*, 2010, **1217**, 2088–2103.
- 26 C. Lacey, S. Basha, A. Morrissey and J. M. Tobin, *Environ. Monit. Assess.*, 2012, **184**, 1049–1062.
- 27 J. C. Van De Steene, C. P. Stove and W. E. Lambert, *Sci. Total Environ.*, 2010, **408**, 3448–3453.
- 28 J. Casado, I. Rodríguez, M. Ramil and R. Cela, *J. Chromatogr. A*, 2014, **1339**, 42–49.
- 29 P. H. Roberts and K. V. Thomas, *Sci. Total Environ.*, 2006, **356**, 143–153.
- 30 DrugBank Online, Detailed Drug and Drug Target Information, accessed 25 January 2021, <https://go.drugbank.com/>.
- 31 PubChem, accessed 25 January 2021, <https://pubchem.ncbi.nlm.nih.gov/>.
- 32 D. Camacho-Muñoz and B. Kasprzyk-Hordern, *Anal. Bioanal. Chem.*, 2015, **407**, 9085–9104.
- 33 J. Rice, A. Lubben and B. Kasprzyk-Hordern, *Anal. Bioanal. Chem.*, 2020, **412**, 5563–5581.
- 34 L. Tonidandel and R. Seraglia, *J. Chromatogr. Libr.*, 2007, **72**, 193–210.
- 35 X. Peng, Q. Huang, K. Zhang, Y. Yu, Z. Wang and C. Wang, *Sci. Total Environ.*, 2012, **426**, 311–317.
- 36 Q. Huang, Y. Yu, C. Tang and X. Peng, *J. Chromatogr. A*, 2010, **1217**, 3481–3488.
- 37 Z. F. Chen, G. G. Ying, Y. S. Liu, Q. Q. Zhang, J. L. Zhao, S. S. Liu, J. Chen, F. J. Peng, H. J. Lai and C. G. Pan, *Water Res.*, 2014, **58**, 269–279.
- 38 A. Zgoła-Grzeškowiak and T. Grzeškowiak, *J. Sep. Sci.*, 2013, **36**, 2514–2521.
- 39 R. H. Lindberg, J. Fick and M. Tysklind, *Water Res.*, 2010, **44**, 649–657.
- 40 K. V. Thomas and M. J. Hilton, *Mar. Pollut. Bull.*, 2004, **49**, 436–444.
- 41 J. Zhang, E. Snelders, B. J. Zwaan, S. E. Schoustra, J. F. Meis, K. Van Dijk, F. Hagen, M. T. Van Der Beek, G. A. Kampinga, J. Zoll, W. J. G. Melchers, P. E. Verweij and A. J. M. Debets, *mBio*, 2017, **8**, e00791-17.

