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Simultaneous enantioselective determination of triazole fungicides in soil and water by chiral liquid chromatography/tandem mass spectrometry

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ABSTRACT

The manuscript concerns the development and validation of a novel and sensitive multi-residue method for simultaneous enantiomeric analysis of 8 triazole fungicides (tetraconazole, fenbuconazole, epoxiconazole, diniconazole, hexaconazole, triadimefon, paclobutrazol, and myclobutanil) in soil and water using chiral liquid chromatography coupled with tandem mass spectrometry. The separation and determination were performed using reversed-phase chromatography on a cellulose chiral stationary phase, a Chiralcel OD-RH (150 mm × 4.6 mm) column, under isocratic conditions using a mixture of ACN-2 mM ammonium acetate in water (55/45, v/v) as the mobile phase at 0.45 mL/min flow rate. The effects of three cellulose-based columns and three amylose-based columns on the separation were also investigated. The QuEChERS (acronym for quick, easy, cheap, effective, rugged and safe) method and solid-phase extraction (SPE) were used for the extraction and clean-up of the soil and water samples, respectively. Parameters including the matrix effect, linearity, precision, accuracy and stability were undertaken. Under optimal conditions, the mean recoveries for all sixteen enantiomers from the soil samples were 76.4–108.1% with 2.6–12.0% intra-day relative standard deviations (RSD) and 4.2–14.1% inter-day RSD at 5, 25 and 50 μg/kg levels; the mean enantiomer recoveries from the water samples were 81.2–106.5% with 2.1–11.5% intra-day RSD and 3.4–13.6% inter-day RSD at 0.25, 0.5 and 2.5 μg/L levels. Coefficients of determination $R^2 \geq 0.9989$ were achieved for all studied analytes in the soil and water matrix calibration curves within the range of 1.0–125 μg/L. The limits of detection (LOD) (S/N = 3) for all enantiomers in the soil and water were less than 1.0 μg/kg or μg/L, whereas the limit of quantification (LOQ) (S/N = 10) did not exceed 3.0 μg/kg or μg/L. The results of the method validation confirm that this proposed method is convenient and reliable for the enantioselective determination of the enantiomers of triazole fungicides in soil and water.

1. Introduction

Triazoles are a class of systemic fungicides that contain the 1,2,4-triazole moiety and are used to control a variety of fungal diseases on fruit, vegetable, legume, and grain crops, both as pre- and postharvest applications [1]. They were introduced as pesticides in the mid-1970s, because of their excellent antifungal activity, and a relatively low resistance risk, triazoles are becoming the most important class of fungicides [2]. Their highly fungicidal effect against many different fungal diseases including powdery mildews, rusts, and many leaf-spotting fungi is a result of the inhibition of cytochrome (CYP) P-450 dependent C14 demethylation of lanosterol, an intermediate in ergosterol biosynthesis [3]. From the toxicological point of view and as a consequence of the ability to inhibit enzymes involved in the biosynthesis of steroid hormones, the triazole fungicides can potentially produce endocrine-related side effects on humans and wildlife [4]. Accordingly, approximately half of the triazole fungicides are included in the priority list of chemicals developed within the EU Strategy for Endocrine Disrupters. In addition, their characteristics, such as high chemical and photochemical stability, low biodegradability and easy transport in the environment, make them persistent in soil and water [5,6].

Triazole fungicides are typically comprised of imidazole, hydroxy (keto) group, and substituted benzyl. Most of them have stereogenic centers and they consist of one or two pairs of enantiomers (Fig. 1). However, most of the triazole fungicides are commercialized as racemate products and released into the environment as an equimolar mixture of enantiomers. Enantiomers of chiral triazoles fungicides usually differ in their bioactivities due to the fact that interactions between chiral molecular and

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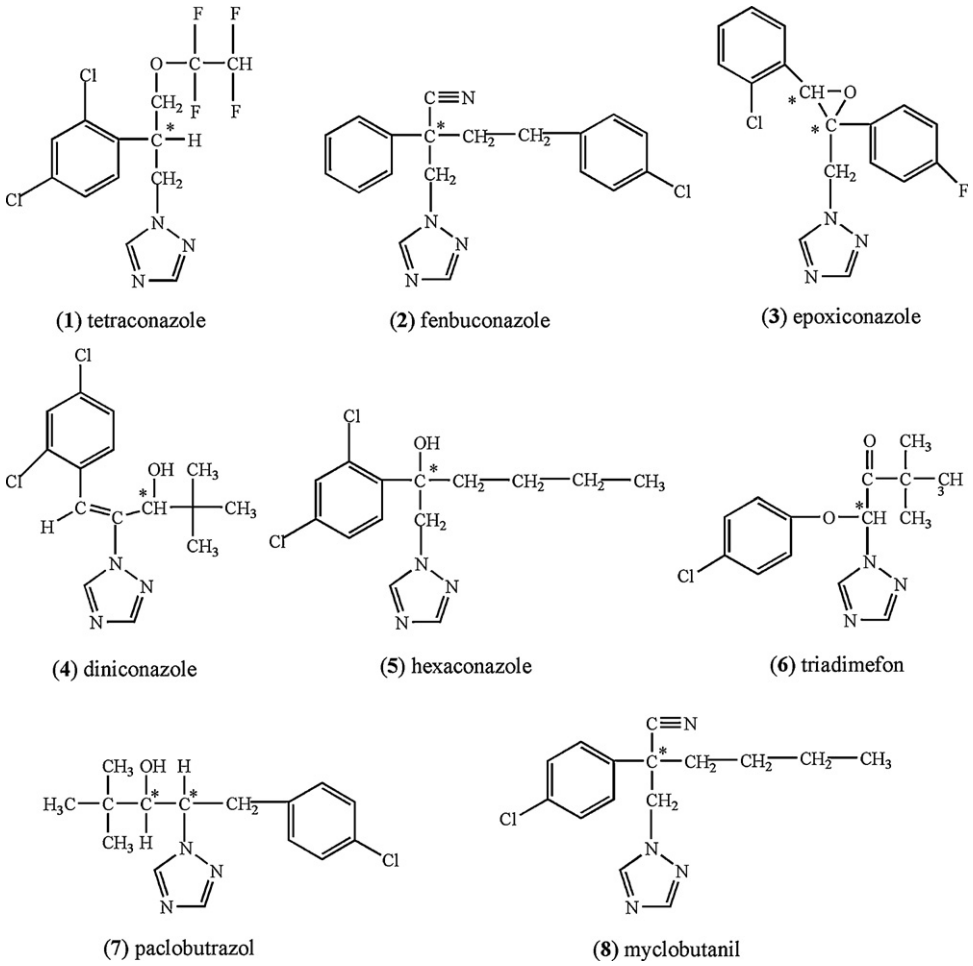

Fig. 1. The chemical structures of chiral triazole fungicides studied (*chiral center).

receptors in biological system may be stereospecific. For example, the *R*-enantiomer of diniconazole and uniconazole shows stronger fungicidal activity than the *S*-enantiomer, whereas the latter has higher plant growth regulating activity [7,8]. The (−)-*threo-1S,2R* enantiomer of triadimenol shows the highest fungitoxicity among the four stereoisomers (up to 1000-fold more active than the other three) and the activities of four optical isomers of paclobutrazol also differ greatly [9]. Furthermore, due to different biological activity, chiral triazole fungicides can differ in toxicity. It is well-known that enantiomers from the same compound have identical physicochemical properties and abiotic degradation rates in an achiral environment [10], whereas their individual toxicity, biological activity, effects on nontarget organisms, and the environmental fate have been shown to differ [10–14]. Enantioselectivity plays an important role in the environmental fate and ecological risks of a chiral compound [15], as many environmental processes are enantioselective [14,16]. As a result, the enantiomeric composition may be changed by enantioselective degradation over time. In many cases, only one enantiomer is being decomposed, while the other enantiomer is being accumulated in the environment. However, in most cases, enantiomers are always treated just as one compound in conventional analysis [17]. Achiral analysis gives only partial information; thus, traditional risk evaluations are unreliable if enantioselective behaviors occur. As a consequence, developing enantioselective separation and enantiomeric analytical methods for chiral triazole fungicides is of great significance to facilitate the evaluation of the risks posed by these fungicides to humans, animals, and the environments.

In the past, the vast majority of enantioselective separations and analysis of triazole fungicides were performed by high-performance liquid chromatography (LC) using UV detection [18–22]. Nevertheless, the high specificity and sensitivity posed by the residue analysis at low levels of enantiomers in very complex environmental matrices is a significant analytical challenge with UV detection mainly due to problems associated with their separation from other interfering compounds. As is well known, liquid chromatography–tandem mass spectrometry (LC–MS/MS) detection is an effective alternative technique that overcomes many of the shortcomings inherent to current methods. Some of the advantages of LC–MS/MS include the combination of highly selective separation of LC with the sensitivity and specificity of tandem MS detection. In many cases, this combination generally allows for a simple sample preparation procedure, which is advantageous compared with other techniques. Currently, there is a trend toward converting the UV-based chiral LC methodologies into more sensitive mass spectrometry-based approaches without losing the enantioselectivity of the assay [23]. As long as the enantiomers of interest to an assay are chromatographically resolved, further selectivity may not be required on part of the chiral stationary phases (CSPs) due to the unique specificity of MS/MS detection, which allows for the simultaneous quantification of a series of chiral compounds enantiomers as well as their metabolites in environment matrices.

Although numerous chiral LC–MS/MS methods have been applied to the enantioselective analysis of pharmaceuticals stereoisomers in recent years [24–27], the applications of these

Table 1
Optimized MRM conditions for analysis of triazole fungicides by UPLC–MS/MS.

Analyte	CV (V)	Quantification ion transition	CE 1 (eV)	Confirmatory ion transition	CE 2 (eV)
(±)-Tetraconazole	30	372.2→159	25	372.2→70	20
(±)-Fenbuconazole	30	337→70	22	337→125	30
(±)-*cis*-Epoxiconazole	25	330.3→121.2	28	330.3→123.2	20
(±)-Diniconazole	35	326.1→70	25	326.1→159	30
(±)-Hexaconazole	32	314.4→70	24	314.4→159	30
(±)-Triadimefon	25	294.3→197	18	294.3→225	18
(±)-(2*R*, 3*R*; 2*S*, 3*S*)-Paclobutrazol	35	294→70	20	294→125	20
(±)-Myclobutanil	35	289.2→70	20	289.2→125	20

Notes: CV, cone voltage; CE, collision energy.

methods in chiral pesticides are still scarce in the literature [28,29]. For these reasons, we explored a new robust analytical technique to simultaneous determine sixteen enantiomers of triazole fungicides using a reversed-phase Chiralcel OD-RH column coupled with tandem MS in this study. To the best of our knowledge, the current report is the first to present the simultaneous enantioselective analysis of chiral pesticides as well as triazole fungicides in environment samples (soil and water) using chiral LC–MS/MS. Additionally, the establishment of multi-residue methods is crucial to obtain information regarding the cumulative presence of several groups of analytes at a particular place and time. This is of great importance as a synergistic effect of different fungicides on soil or aquatic life might occur and has to be investigated. The analytical method developed was validated by its application to the analysis of authentic samples.

2. Material and methods

2.1. Chemicals and reagents

Racemic tetraconazole (1), epoxiconazole (3), diniconazole (4), hexaconazole (5), triadimefon (6), paclobutrazol (7), myclobutanil (8) (all purity >96%) were purchased from China Standard Material Center (Beijing, China). Racemic fenbuconazole (2) (99.9% purity) were obtained from Rohm and Haas Co., Ltd. (Philadelphia, PA, USA). Note that in spite of two chiral centers (four theoretical enantiomers, two diastereoisomers), the two fungicides epoxiconazole and paclobutrazol are produced as one enantiomeric pair. To be specific, epoxiconazole (CAS Reg. No. 133855-98-8) is a mixture of 2*R*, 3*S*- and 2*S*, 3*R*-enantiomers, and paclobutrazol (CAS Reg. No. 76738-62-0) is a mixture of 2*R*, 3*R* and 2*S*, 3*S*-enantiomers. In the following, for clarity we use the terms *cis*-epoxiconazole. In addition, for paclobutrazol, the absolute configuration of dextrorotatory (+) is (2*R*, 3*R*)- whereas the levorotatory (−) is (2*S*, 3*S*)-enantiomer [30]. HPLC-grade acetonitrile (ACN) was purchased from Sigma–Aldrich (Steinheim, Germany). HPLC-grade ammonium acetate was purchased from Tedia (Fairfield, OH, USA). Analytical grade NaCl, $MgSO_4$, methanol and ACN were purchased from Beihua Fine-chemicals Co. (Beijing, PRC). Ultra-pure water was obtained from a Milli-Q system (Bedford, MA, USA). Cleanert C18 cartridges (500 mg/6 mL) were purchased from Supelco Technologies Inc. (Bellefonte, USA). octadecylsilane (C18, 40 μm) sorbents were obtained from Agela Technologies Inc. (Tianjin, PRC). The mobile phase solvents were distilled and filtered through a 0.22 μm pore size filter membrane (Tengda, Tianjin, PRC) before use.

Standard stock solutions (100 mg/L) of racemic triazole fungicides were prepared in pure ACN. A standard mixture solution, with all 8 triazole fungicides, was prepared in ACN at 1 mg/L, of each pesticide. This solution was used as spiking solution and also to prepare the standard solutions to obtain the calibration curves, by dilution with ACN or matrix extract. Standard working solutions at 2, 10, 25, 50, 100 and 250 μg/L concentrations (1, 5, 12.5, 25, 50 and 125 μg/L for each enantiomer) were prepared from the stock solution by serial dilution. Correspondingly, matrix-matched standard solutions were obtained at 2, 10, 25, 50, 100 and 250 μg/L concentrations (1, 5, 12.5, 25, 50 and 125 μg/L for each enantiomer) by adding blank soil and water sample extracts to each serially diluted standard solution. All solutions were protected against light with aluminum foil and stored in a refrigerator in the dark at −20 °C. The working standard solutions underwent no degradation for 3 months.

2.2. Ultra performance liquid chromatography–tandem mass spectrometry

A Waters Acquity UPLC™ system (Milford, MA, USA) consisting of the Acquity UPLC™ binary solvent manager and the Acquity UPLC™ sample manager was used for the separation of analytes. A Chiralcel OD-RH (150 mm × 4.6 mm i.d., Daicel, Japan) column with 5 μm particle size was used for the separation of the enantiomers of chiral triazole fungicides.

A simultaneous enantiomeric analysis of triazole fungicides was performed using the Chiralcel OD-RH column after injection of a 10 μL volume standard working solution. The separation was carried out isocratically using solvent A (HPLC-grade ACN) and solvent B (2 mM ammonium acetate in ultrapure water) in a 55:45 (v/v) ratio and 0.45 mL/min flow rate for 25 min. The column was kept at 25 °C and the temperature in the sample manager was kept at 4 °C.

A triple quadrupole (TQD) mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) source was used to quantify triazole fungicides. The analyses were performed in the positive mode with a 3.0 kV capillary voltage, 120 °C source temperature, and a 350 °C desolvation temperature. A 50 L/h cone gas flow and 500 L/h desolvation gas flow were used. The nebulizer gas was 99.95% nitrogen, and the collision gas was 99.99% argon with a pressure of 2×10^{-3} mbar (2×10^{-5} MPa) in the T-Wave cell. The Masslynx NT v.4.1 (Waters, USA) software was used to collect and analyze the data obtained.

MS analyses were performed in the multiple reaction monitoring (MRM) mode, measuring the fragmentation of the protonated pseudo-molecular ions of triazole fungicides. MS/MS detection was performed in the positive ionization mode, and the monitoring conditions were optimized for triazole fungicides. After investigation of several dwell times in the 20–100 ms range, a dwell time of 40 ms per ion pair was used to maintain the high sensitivity of the analysis, and a number of data points across the chromatographic peak were required. The choice of fragmentation products for each substance based on the most intense signal and the optimization of cone voltages, energy collisions, and other instrument parameters was done individually in continuous-fowled mode through a direct infusion of standard solutions at concentrations of 50 μg/L into the stream of the mobile phase. All other ESI and MS parameters were optimized individually for each target compound and were listed in Table 1. Quantitation was conducted using the more abundant

ion transition, whereas the less abundant ion transition was used for identification. These settings were utilized for all subsequent studies.

2.3. Sample preparation

Water and soil (sandy loam) samples from trial plots were obtained from the Institute of Plant Protection located in the Haidian region in Beijing. These matrices did not contain the target analytes. The soil samples were placed in polyethylene bags, whereas the water samples were placed in plastic bottles. The samples were transported to the laboratory and stored in the dark at less than −20 °C until analysis. The soil samples were collected at depths of 0–30 cm at 15 randomly selected points. After collection, the soil samples were air-dried at room temperature, homogenized, and passed through a 2 mm sieve. The treated samples were kept in the dark until analysis, which was carried out within a few days.

2.3.1. Water

Water (100 mL) samples at three concentration levels of fortification were prepared by the addition of appropriate amounts of fungicides standard solutions. After standing for 2 h at room temperature to distribute the pesticide evenly and to give them time to interact with the sample matrix, 100 mL of the aliquot was slowly passed through a C18 SPE cartridge at a flow rate of about 4 mL/min. The cartridge was previously activated by flushing with 2 × 5 mL methanol, followed by 2 × 5 mL purified water. The samples were loaded into the SPE cartridge and were dried under a vacuum (0.2 MPa) for 15 min. The retained analytes were eluted with 10 mL methanol. The organic solvent was then evaporated to dry using a rotary evaporator (30 °C, 0.09 MPa). The obtained residue was redissolved in 2 mL ACN and filtered using a 0.22 μm Nylon syringe filter for chromatographic injection.

2.3.2. Soil

Dried, finely homogenized soil samples (10 ± 0.1 g) were weighed in a 50 mL Teflon centrifuge tube with screw caps. Appropriate concentrations of a mixture working standard solution with all 8 pesticides were added to the tube. The tubes containing the spiked samples were vortexed for 30 s and allowed to stand for 2 h at room temperature to allow the interaction between the compounds and the matrix to take place in order to obtain samples that would resemble natural soils as much as possible. Then, 5 mL water and 10 mL ACN were added, and the mixtures were vigorously shaken for 30 min at 25 °C in a water bath shaker (Dongming Medical Instrument, Harbin, China). Subsequently, 4 g anhydrous magnesium sulfate ($MgSO_4$) and 1 g sodium chloride (NaCl) were added. The tubes were capped and immediately vortexed vigorously for 3 min and then centrifuged for 5 min at relative centrifugal force (RCF) 2599 × *g*. Afterward, 1.5 mL of the ACN (upper) layer was transferred into a single-use 2 mL centrifuge tube containing 150 mg anhydrous $MgSO_4$ and 50 mg C18. The samples were again vortexed for 1 min and centrifuged at 2077 × *g* RCF for 5 min. The resulting supernate was then filtered using a 0.22 μm Nylon syringe filter for chromatographic injection.

2.4. Method validation

The method was validated to evaluate the performance in accordance with a conventional validation procedure that includes the following parameters: specificity, linear range, limit of detection (LOD) and limit of quantification (LOQ), matrix effect, accuracy, precision and stability.

Ten blank samples (soil and water) were analyzed to verify the absence of interfering species at about the retention time of the analytes. The linearity of the method was determined by analyzing the standard solutions and the different matrices in triplicate at six concentrations, ranging from 1.0 to 125 μg/L. A satisfactory linearity is obtained when the correlation coefficient (R^2) is higher than 0.9989 based on the measurement of the analyte peak areas. Blank analysis was performed to check interference from the matrix. The slope ratios of the linear calibration functions were calculated to differentiate between the extraction efficiency and the matrix-induced signal suppression/enhancement (SSE). The SSE caused by matrix effects was determined.

The matrix-dependent LOD and LOQ of the method were determined using the blank and calibration standards of the soil and water matrices. The LOD for the enantiomers of triazole fungicides is the concentration that produces a signal-to-noise (S/N) ratio of 3, whereas the LOQ is defined based on a S/N ratio of 10 is estimated from the chromatogram corresponding to the lowest point used in the matrix-matched calibration.

The recovery assays were carried out to investigate the accuracy and precision of the method. Five replicates of the spiked samples at different levels—0.25, 0.5 and 2.5 μg/L for water and 5, 25 and 50 μg/kg for soil—were prepared on three different days. The enantiomers of 8 triazole fungicides were extracted and purified according to the above-mentioned procedure. The precision in these conditions for repeatability, expressed as the relative standard deviation (RSD), was determined by the intra- and inter-day assays.

The stability was determined in the solvent and in the matrix. The stability of the stock solutions was tested monthly by injection of a newly prepared working solution. The stability of the spiked soil and water samples (10 and 50 μg/L) for triazole fungicides and their enantiomers were evaluated monthly, and all the samples used in the stability test were stored at −20 °C. The results of the stability tests were compared with those obtained from the freshly prepared samples using the Student's *t*-test ($P < 0.05$).

Enantiomer fractions (EFs) were calculated for each set of enantiomers, and EF is defined by Eq. (1):

$$EF = (+)/(+) + (-) \quad (1)$$

where (+) and (−) are peak areas of the (+) and (−) enantiomers of analytes eluting from the Chiralcel OD-RH column (see Fig. 2). The EF values can range from 0 to 1, with EF = 0.5 representing the racemic mixture. The measured EFs ± σ for racemic standards were 0.497 ± 0.005 for tetraconazole ($n = 6$), 0.494 ± 0.007 for fenbuconazole ($n = 6$), 0.502 ± 0.004 for *cis*-epoxiconazole ($n = 6$), 0.505 ± 0.009 for diniconazole ($n = 6$), 0.498 ± 0.007 for hexaconazole ($n = 6$), 0.504 ± 0.006 for triadimefon ($n = 6$), 0.493 ± 0.008 for (2*R*, 3*R*; 2*S*, 3*S*)-paclobutrazol ($n = 6$), 0.508 ± 0.007 for myclobutanil ($n = 6$). These were not significantly different from the expected value for racemates of 0.500 (*t*-test, $P < 0.05$ for all statistical tests in this study unless otherwise indicated). These observations indicate that the resolution of the triazole fungicides was sufficient for quantification of enantiomer composition.

3. Results and discussion

3.1. Chromatographic condition optimization

LC analysis of stereoisomeric pesticides with normal or reversed phase has been extensively performed, which have been reviewed by Ye et al. [21]. Reversed-phase LC was more compatible to ESI or APCI MS than normal phase. Of the many types of the commercial CSPs used for chiral separations in LC, the polysaccharide-based CSPs are currently the most popular due to their versatility, durability and loading capacity [26,31]. They are effective under not only normal-phase conditions, but also reversed-phase conditions using the appropriate mobile phases [26]. The majority of

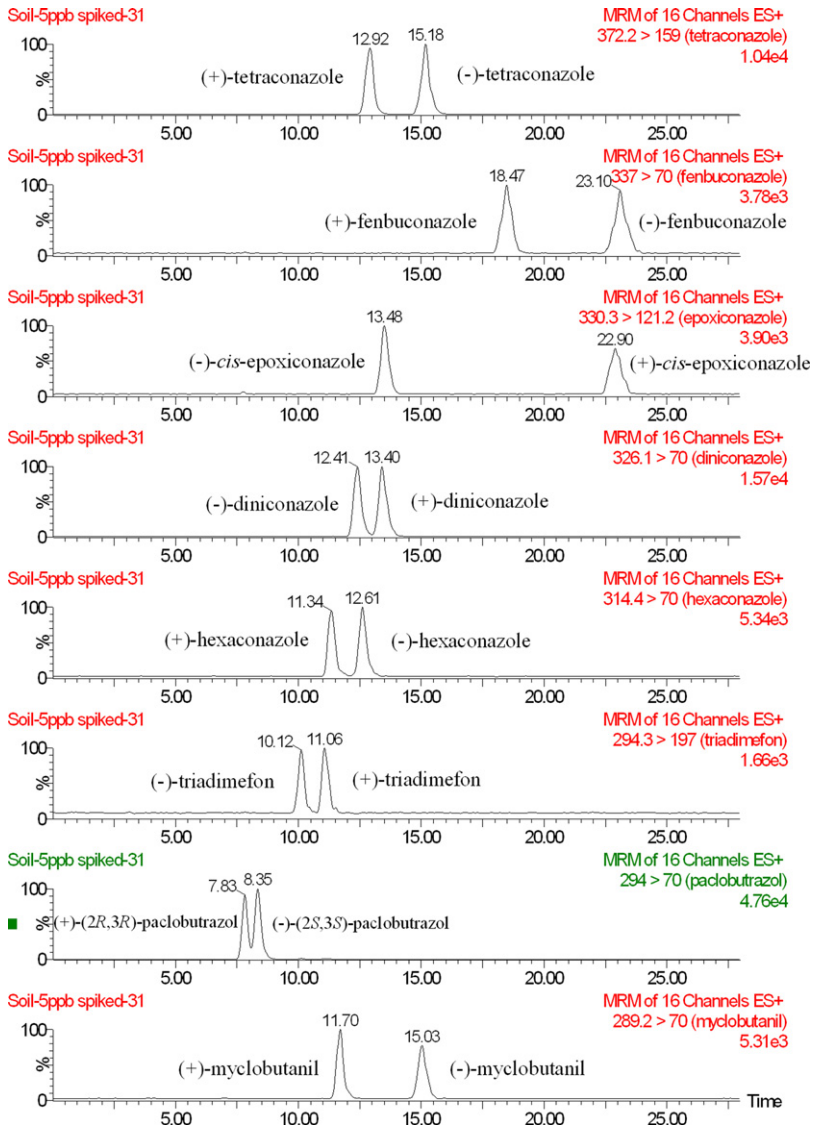

Fig. 2. Typical enantioselective LC–MS/MS MRM chromatograms of chiral triazole fungicides spiked into soil (concentration, 5 μg/kg).

polysaccharide-based CSPs employed were cellulose- and amylose-based polysaccharide columns [32].

A series of CSPs was evaluated to discriminate the enantiomers of triazole fungicides. In the preliminary experiments, the separation of triazole fungicides on three cellulose-based columns (Lux 3u Cellulose-1 and Cellulose-2, 150 mm × 2.0 mm i.d., 3 μm particle size; Chiralcel OD-RH, 150 mm × 4.6 mm i.d., 5 μm particle size) and three amylose-based columns (Chiralpak AD-RH and AS-RH, 150 mm × 4.6 mm i.d., 5 μm particle size; Lux 3u Amylose-2, 150 mm × 2.0 mm i.d., 3 μm particle size) was tested using a variety of reversed-phase mobile phase combinations. Of the six tested columns, the best chromatographic separation of all the sixteen enantiomers of 8 triazole fungicides was achieved with the Chiralcel OD-RH column (Fig. 2), which was operated under reversed-phase conditions using a mixture of ACN-2 mM ammonium acetate in water (55/45, v/v) as the mobile phase at a flow rate of 0.45 mL/min with all the resolution factor (R_S) values were above 1.46. Methanol, as one of the most frequently used modifiers for reversed-phase LC, was also investigated. However, a satisfactory enantioselective separation of the sixteen enantiomers was not achieved, and the column pressure greatly increased when methanol was used. Thus, ACN was chosen as the organic phase in the current study.

Ammonium acetate was used as the buffer to obtain a better peak shape and a higher signal response in the MS/MS detector because of its volatility and compatibility. Different buffer concentrations (0.5, 1, 2, 5 and 10 mM) were investigated. The different buffer concentrations exhibited no significant effect on the enantioselective separation, whereas the MS signal response was the highest when the buffer concentration was 2 mM. Therefore, 2 mM concentration of ammonium acetate was finally chosen for the optimal condition. In addition, retention and resolution of analytes were also maintained by the flow rate of mobile phase and the temperature of separation. Out of several flow rates studied 0.45 mL/min was chosen as it provided satisfactory separation and good MS performance. An evaluation of the effect of the column temperature on chiral separation showed a slightly longer retention, better separation, and better peak shape at 25 °C compared with other temperatures (20–40 °C).

3.2. MS detection

Multi-residue analysis methods are possible today because of the availability of affordable an MS system, the power of which is the provision of structural information for recognizing non-target contaminants, increases the specificity of target pesticide

identification and achieves high sensitivity trace-level determination [33,34]. LC in combination with tandem MS has been proven to be an excellent analytical tool for multi-residual determinations in different matrices of pesticides [35–38]. In the current experiment, the enantiomers of most of triazole fungicides shared similar retention times on the same chiral column, thereby making their chromatographic separation impossible in general only under LC condition. Nevertheless, the triazole fungicides have different molecular masses and can be selected in different mass channels. Thus, the triazole fungicides enantiomers were clearly distinguished from each other under mass spectrometric conditions. No endogenous interferences were observed in the eight separated channels (Fig. 2); Thanks to the technique of LC combined with tandem MS (MRM), making the simultaneous enantioselective quantification of all the sixteen enantiomers of 8 triazole fungicides was not impaired in a single run. Table 1 lists the precursor ions and the product ions of each compound with their optimum selected collision energy.

3.3. Elution order determination of triazole fungicides enantiomers

Chiroptical properties-based detector including circular dichroism (CD) and optical rotation (OR) detectors were often used to identifying the elution orders of the enantiomers. However, CD absorption signals of enantiomers may be reversed with change of CD wavelength in two different absorption ranges because it is based on difference of absorption between left and right circularly polarized light. OR can specifically give the left (−) or the right (+) rotation information of an enantiomer because it is based on the difference in the refractive index between the left and the right linearly polarized lights [29]. In this work, the elution order of triazole fungicides, as well as their enantiomers, was determined by measuring the optical rotation of each enantiomer using reversed-phase LC coupled with an on-line OR-2090 detector (Jasco, Japan), which was performed on the same chiral column (Chiralcel OD-RH) using the ACN/water as mobile phase with the UV detection at 225 nm. The elution order of triazole fungicides was then determined as (+)-tetraconazole (12.92 min), (−)-tetraconazole (15.18 min), (+)-fenbuconazole (18.47 min), (−)-fenbuconazole (23.10 min), (−)-*cis*-epoxiconazole (13.48 min), (+)-*cis*-epoxiconazole (22.90 min), (−)-diniconazole (12.41 min), (+)-diniconazole (13.40 min), (+)-hexaconazole (11.34 min), (−)-hexaconazole (12.61 min), (−)-triadimefon (10.12 min), (+)-triadimefon (11.06 min), (+)-(2*R*, 3*R*)-paclobutrazol (7.83 min), (−)-(2*S*, 3*S*)-paclobutrazol (8.35 min), (+)-myclobutanil (11.70 min), and (−)-myclobutanil (15.03 min).

3.4. Sample preparation

A simplified QuEChERS method, which is a major development in sample preparation that involves a streamlined approach to pesticide residue analysis, was employed for soil sample extraction [39]. This method has been recently introduced as an attractive alternative method for sample preparation because of a number of advantages over traditional techniques [40]. C18 was used as the absorbent for soil samples clean-up because of cheapness and the results were satisfactory.

C18 SPE was utilized for the determination of the sixteen enantiomers of triazole fungicides from water samples. A C18 cartridge was chosen for the water sample because of its good cleanup and enrichment efficiency, good recovery, and good processing speed. Methanol was selected as the eluting solvent because of its effectiveness for the analytes. The SPE procedure was used for the pre-concentration to be able to determine the low levels of analytes normally present in water samples.

3.5. Method validation

3.5.1. Linearity, LOD, and LOQ

The linearity, analytical LOD, and LOQ were obtained using the peak areas of the product ions obtained from the MS/MS mode. As shown in Table 2, the linearity was evaluated by preparing three different calibration curves (solvent, soil and water) within the concentration range of 1–125 μg/L for each of the enantiomers of triazole fungicides. Table 2 indicates the slopes, intercepts, and coefficients of determination (R^2) of both the soil and water matrix-matched curves and the standard solution curves. Satisfactory linearities were observed for all enantiomers ($R^2 \geq 0.9989$ in all cases). The RSDs of 10 replicate determinations of the same standard solution ranged from 2% to 6%, indicating a good repeatability.

The LODs and LOQs were based on the minimum amount of target analyte that produced a chromatogram peak with a sign-to-noise ratio of 3 and a peak 10 times the background chromatographic noise, respectively. Table 2 summarizes the LODs and LOQs for triazole fungicides, as well as their enantiomers in the original samples. The LODs for the sixteen enantiomers were estimated at 0.04–1.0 μg/kg or μg/L based on five replicate extractions and analyses of spiked soil and water samples at low concentration levels, and the corresponding LOQs were 0.12–3.0 μg/kg or μg/L.

3.5.2. Matrix effect

It is well known that matrix effects (ion suppression and ion enhancement) are ubiquitous during LC–MS with ESI analysis owing to the ionization competition between co-eluting compounds in a chromatographic system [41]. Therefore, in the current study, the matrix effect on the MS/MS (MRM mode) detector using the proposed method was investigated in soil and water by comparing the standards in the solvent with the matrix-matched standards. The slopes obtained in the calibration using the matrix matched-standards were compared with those obtained using the solvent standards. Table 2 shows the slope ratio of the matrix to the solvent for each enantiomer of triazole fungicides. Matrix effect was evaluated for all the enantiomers in the two matrices. In general, no significant suppression or enhancement differences were observed for the sixteen enantiomers in water, as evidenced by the slope ratios, which were within 10% of the slope ratio of 1.0 (0.904–0.978). Therefore, the matrix effect of the sixteen enantiomers in water was negligible. However, the signal enhancements for the sixteen target compounds were typically observed in the soil matrix extracts as the slope ratios were in the range of 1.125–1.820. The best way to compensate for matrix effects is the use of isotopically labeled internal standards. However, for most pesticides these compounds are not available, and due to the high costs, the use of these standards is not always applicable to multi-residue methods. In this study, for more accurate results, calibration of enantiomers in soil samples was performed by external matrix-matched standards to eliminate the matrix effect and to obtain a more realistic determination. External pure solvent standard calibration curves were utilized for the quantification of the sixteen enantiomers in water.

3.5.3. Precision and accuracy

The recovery and RSDs of the sixteen enantiomers were measured to validate the chiral LC–MS/MS method by spiking the blank samples with three different concentrations (0.25, 0.5 and 2.5 μg/L for water; 5, 25 and 50 μg/kg for soil) and then analyzing them in quintuplicate (Table 3). The precision of the method was determined by the repeatability and reproducibility studies, and expressed as the RSD. The intra-day precision was measured

Table 2
Comparison of matrix-matched calibration and solvent calibration (1–125 μg/L).

Compound	Calibration (matrix)	Regression equation	r^2	Slope of matrix/slope of solvent	LOD (μg/kg or μg/L)	LOQ (μg/kg or μg/L)
(+)-Tetraconazole	Solvent	$y = 40.6x - 78.236$	0.9995	–	0.6	2.0
	Soil	$y = 46.597x - 55.661$	0.9992	1.148	0.5	1.8
	Water	$y = 38.734x - 46.272$	0.9997	0.954	0.04	0.12
(−)-Tetraconazole	Solvent	$y = 41.312x - 24.306$	0.9998	–	0.6	2.0
	Soil	$y = 46.463x + 48.485$	1.0000	1.125	0.5	1.8
	Water	$y = 40.298x + 328.13$	0.9989	0.975	0.04	0.12
(+)-Fenbuconazole	Solvent	$y = 15.33x - 20.291$	0.9994	–	0.8	2.5
	Soil	$y = 21.399x - 44.473$	0.9998	1.396	0.6	2.0
	Water	$y = 14.558x - 1.195$	0.9994	0.950	0.05	0.15
(−)-Fenbuconazole	Solvent	$y = 15.698x + 3.999$	0.9992	–	0.8	2.5
	Soil	$y = 23.077x - 41.079$	0.9991	1.470	0.6	2.0
	Water	$y = 14.354x + 7.665$	0.9993	0.914	0.05	0.15
(−)-*cis*-Epoxiconazole	Solvent	$y = 17.391x - 36.891$	0.9998	–	0.8	2.5
	Soil	$y = 20.989x - 52.466$	0.9993	1.207	0.6	2.0
	Water	$y = 17.011x + 20.22$	0.9990	0.978	0.05	0.15
(+)-*cis*-Epoxiconazole	Solvent	$y = 17.003x - 38.753$	0.9996	–	0.8	2.5
	Soil	$y = 19.999x - 24.165$	0.9999	1.176	0.6	2.0
	Water	$y = 15.949x + 34.468$	0.9991	0.938	0.04	0.12
(−)-Diniconazole	Solvent	$y = 58.307x - 50.24$	0.9998	–	0.6	2.0
	Soil	$y = 79.473x - 324.28$	0.9996	1.363	0.5	1.8
	Water	$y = 54.733x - 178.51$	0.9994	0.939	0.04	0.12
(+)-Diniconazole	Solvent	$y = 57.297x - 12.098$	0.9996	–	0.6	2.0
	Soil	$y = 72.661x - 108.05$	0.9990	1.268	0.5	1.8
	Water	$y = 52.557x - 45.823$	0.9996	0.917	0.04	0.12
(+)-Hexaconazole	Solvent	$y = 21.07x - 44.423$	0.9997	–	0.8	2.5
	Soil	$y = 29.788x + 57.851$	0.9995	1.414	0.6	2.0
	Water	$y = 19.164x + 31.939$	0.9992	0.910	0.05	0.15
(−)-Hexaconazole	Solvent	$y = 20.577x - 30.99$	0.9998	–	0.8	2.5
	Soil	$y = 31.067x + 12.677$	0.9992	1.510	0.6	2.0
	Water	$y = 19.592x + 17.153$	0.9990	0.952	0.05	0.15
(−)-Triadimefon	Solvent	$y = 11.016x - 22.949$	0.9995	–	1	3
	Soil	$y = 13.444x + 33.076$	0.9993	1.220	0.8	2.5
	Water	$y = 10.584x - 46.619$	0.9994	0.961	0.06	0.18
(+)-Triadimefon	Solvent	$y = 10.447x + 4.281$	0.9997	–	1	3
	Soil	$y = 12.361x + 63.159$	0.9996	1.183	0.8	2.5
	Water	$y = 9.685x - 23.399$	0.9989	0.927	0.06	0.18
(+)-(2*R*, 3*R*)-Paclobutrazol	Solvent	$y = 82.095x - 55.648$	0.9998	–	0.6	2.0
	Soil	$y = 139.32x - 457.82$	0.9996	1.697	0.5	1.8
	Water	$y = 74.555x - 73.764$	0.9992	0.908	0.04	0.12
(−)-(2*S*, 3*S*)-Paclobutrazol	Solvent	$y = 80.39x + 212.04$	0.9997	–	0.6	2.0
	Soil	$y = 130.65x - 508.36$	0.9997	1.625	0.5	1.8
	Water	$y = 75.452x - 112.74$	0.9995	0.939	0.04	0.12
(+)-Myclobutanil	Solvent	$y = 20.023x - 16.849$	0.9999	–	0.8	2.5
	Soil	$y = 36.446x + 117.49$	0.9994	1.820	0.6	2.0
	Water	$y = 18.099x - 52.928$	0.9992	0.904	0.05	0.15
(−)-Myclobutanil	Solvent	$y = 19.676x - 14.49$	0.9996	–	0.8	2.5
	Soil	$y = 34.938x - 53.492$	0.9993	1.776	0.6	2.0
	Water	$y = 18.209x - 42.496$	0.9998	0.925	0.05	0.15

by comparing the standard deviation of the recovery percentages of the spiked samples ran during the same day. The inter-day precision was determined by analyzing the spiked samples for three distinct days. As Table 3 shows, the method presented satisfactory mean recovery values (76.4–108.1%) and precision, with all RSD values below 14.1% at the three fortified concentration levels. For both enantiomers of tetraconazole, the mean recoveries ranged from 79.8% to 102.3% with 2.5–11.2% intra-day RSD, whereas they were from 76.4% to 101.4% with 3.1–11.5% intra-day RSD for two fenbuconazole enantiomers and from 79.4% to 103.9% with 2.3–10.7% intra-day RSD for *cis*-epoxiconazole enantiomers. The mean recoveries were 83.2–99.3% with 2.1–10.6% intra-day RSD for both of diniconazole enantiomers, 83.6–106.5% with 2.9–11.1% intra-day RSD for two hexaconazole enantiomers, and 76.5–102.9% with 3.4–9.8% intra-day RSD for triadimefon enantiomers. Finally, the mean recoveries for two (2*R*, 3*R*; 2*S*, 3*S*)-paclobutrazol enantiomers were 85.1–104.0% with 2.6–12.0% intra-day RSD, and 82.4–108.1% with 3.7–11.3% intra-day RSD for myclobutanil enantiomers. In general, the intra-day ($n = 5$) and inter-day RSDs ($n = 10$) for the proposed method ranged from 2.1% to 12.0% and 3.4% to 14.1%, respectively (Table 3). Fig. 2 presents the LC–MS/MS MRM chromatograms of the sixteen enantiomers spiked into soil. The results of the recovery studies demonstrated that this method and the chiral LC–MS/MS enantioselective analysis can achieve a satisfactory precision and accuracy for the enantiomeric analysis in soil and water. In addition, an evaluation of the stability of the sixteen

Table 3
Accuracy and precision of the proposed method in the two studied matrices.

Compound	Matrix	Spiked level (μg/kg)	Intra-day (n = 5)						Inter-day (n = 15) RSD (%)
			Day 1		Day 2		Day 3		
			Average recoveries (%)	RSD (%)	Average recoveries (%)	RSD (%)	Average recoveries (%)	RSD (%)	
(+)-Tetraconazole	Soil	5	85.4	6.5	79.8	11.2	91.2	8.8	10.9
		25	80.3	8.2	83.9	3.9	86.2	7.1	6.3
		50	90.1	3.4	89.9	6.8	92.3	5.4	5.2
	Water	0.25	88.2	9.3	94.5	8.6	91.2	6.8	8.4
		0.5	83.6	6.4	91.7	5.1	86.0	6.4	5.6
		2.5	102.3	6.1	87.1	4.3	94.5	2.5	4.4
(−)-Tetraconazole	Soil	5	81.6	9.3	82.6	6.8	90.1	10.8	9.5
		25	87.8	6.5	86.1	8.3	85.4	6.3	7.1
		50	84.0	5.5	92.3	4.3	94.8	3.1	4.6
	Water	0.25	90.5	10.1	84.3	5.1	86.2	7.2	7.8
		0.5	82.8	6.3	89.6	7.1	93.4	8.0	8.6
		2.5	98.1	8.5	95.2	4.8	91.7	4.5	6.7
(+)-Fenbuconazole	Soil	5	87.4	7.6	85.7	8.6	78.6	9.5	10.3
		25	80.8	5.8	91.5	6.9	84.3	5.6	6.6
		50	85.4	4.3	92.7	7.1	90.7	6.0	8.1
	Water	0.25	89.2	11.5	83.2	8.2	92.1	6.7	9.4
		0.5	83.6	5.8	101.4	10.7	81.9	3.1	12.3
		2.5	93.3	8.3	95.1	7.2	88.6	4.5	7.2
(−)-Fenbuconazole	Soil	5	76.4	9.3	84.6	6.7	83.3	5.9	8.2
		25	82.8	6.5	91.6	8.1	93.4	7.8	13.1
		50	90.6	4.6	92.8	7.5	85.9	6.4	6.2
	Water	0.25	91.2	6.9	83.4	9.4	87.4	4.9	9.4
		0.5	84.3	6.3	86.5	6.1	89.0	3.2	4.6
		2.5	93.5	6.0	97.6	4.8	95.2	3.6	3.8
(−)-*cis*-Epoxiconazole	Soil	5	80.8	3.8	82.5	5.6	79.5	4.3	4.3
		25	81.9	5.2	79.4	4.7	84.1	6.8	7.1
		50	86.3	7.8	91.3	6.4	92.8	5.9	7.6
	Water	0.25	86.9	8.0	83.9	6.8	91.3	9.8	8.4
		0.5	88.3	3.5	94.0	4.1	82.5	10.7	12.2
		2.5	93.2	2.3	86.1	8.2	89.6	5.1	7.3
(+)-*cis*-Epoxiconazole	Soil	5	80.1	9.1	83.1	7.9	90.5	7.6	9.5
		25	92.8	7.3	84.4	3.9	81.2	8.6	10.4
		50	85.1	3.4	92.1	6.0	87.4	4.4	5.7
	Water	0.25	83.5	6.2	91.4	5.6	88.2	7.9	8.8
		0.5	92.6	10.6	86.3	4.1	100.4	8.5	11.1
		2.5	103.9	4.4	92.6	5.9	85.8	3.4	9.7
(−)-Diniconazole	Soil	5	93.2	7.9	84.6	8.1	85.4	10.2	7.9
		25	86.5	6.8	83.5	8.6	95.6	5.8	9.8
		50	89.7	4.5	90.5	5.3	92.4	3.0	4.2
	Water	0.25	87.2	7.6	90.3	6.5	92.1	5.9	3.5
		0.5	91.8	8.3	88.6	4.2	93.5	7.1	6.3
		2.5	95.4	5.4	89.1	2.1	96.8	3.8	4.7
(+)-Diniconazole	Soil	5	86.1	6.4	84.9	7.9	90.3	8.5	5.9
		25	83.2	5.8	89.2	4.4	93.4	7.8	8.1
		50	91.5	5.6	87.7	2.8	85.8	4.1	6.2
	Water	0.25	92.5	7.5	99.3	9.6	89.4	10.6	12.4
		0.5	88.8	8.4	91.0	6.1	85.3	5.4	6.0
		2.5	93.4	3.2	92.5	5.7	94.5	4.6	3.4
(+)-Hexaconazole	Soil	5	88.7	3.8	84.5	5.6	89.5	4.3	5.2
		25	91.9	5.2	94.4	4.4	103.6	6.6	10.1
		50	96.3	2.9	105.3	6.7	92.8	5.9	9.8
	Water	0.25	87.6	11.1	90.9	7.8	106.5	9.8	13.6
		0.5	85.3	3.5	84.0	4.1	91.6	6.1	7.2
		2.5	91.2	4.6	95.1	3.0	89.1	5.2	6.1
(−)-Hexaconazole	Soil	5	94.0	9.1	83.6	8.2	90.9	7.6	9.5
		25	85.8	7.5	93.4	3.8	101.2	8.5	10.4
		50	90.1	3.3	92.6	6.1	96.4	4.5	5.7
	Water	0.25	86.5	10.1	93.4	5.5	94.3	7.9	7.8
		0.5	92.6	10.8	85.6	4.1	100.8	8.6	10.1
		2.5	94.9	4.3	104.5	5.9	89.8	3.4	9.7
(−)-Triadimefon	Soil	5	83.2	6.8	80.3	7.5	91.2	8.5	8.4
		25	85.3	8.8	76.5	3.9	82.2	7.7	6.0
		50	81.6	3.7	89.9	6.9	78.3	5.1	7.9

Table 3 (*Continued*)

Compound	Matrix	Spiked level (μg/kg)	Intra-day (n = 5)						Inter-day (n = 15) RSD (%)
			Day 1		Day 2		Day 3		
			Average recoveries (%)	RSD (%)	Average recoveries (%)	RSD (%)	Average recoveries (%)	RSD (%)	
	Water	0.25	91.2	7.2	85.4	8.0	87.4	6.3	8.2
		0.5	83.6	9.4	92.6	4.9	87.0	4.5	5.6
		2.5	101.3	5.3	95.1	4.0	93.7	3.4	6.3
(+)-Triadimefon	Soil	5	77.3	8.7	79.4	5.8	92.1	9.3	13.9
		25	90.8	6.8	82.7	9.3	84.9	4.7	7.6
		50	86.1	5.6	80.3	3.9	85.8	4.1	4.7
	Water	0.25	89.1	7.8	93.7	4.6	95.2	7.6	6.0
		0.5	81.2	8.5	86.4	5.2	84.3	7.5	5.2
		2.5	97.0	9.8	90.1	4.2	102.9	4.9	7.8
(+)-(2*R*, 3*R*)-Paclobutrazol	Soil	5	85.4	8.7	107.2	12.0	92.3	5.8	14.1
		25	94.6	4.8	99.3	7.9	88.0	6.0	10.5
		50	95.2	2.6	90.4	6.4	98.5	3.8	6.3
	Water	0.25	92.6	5.9	86.1	8.9	90.9	7.4	6.9
		0.5	85.1	9.9	95.2	3.1	97.7	5.2	11.3
		2.5	88.8	3.8	90.8	6.5	98.1	4.8	7.7
(−)-(2*S*, 3*S*)-Paclobutrazol	Soil	5	92.5	6.7	98.4	9.1	85.3	8.0	10.2
		25	86.4	7.2	101.6	8.5	95.1	5.4	11.8
		50	104.0	8.3	92.9	3.0	97.6	5.8	8.9
	Water	0.25	89.1	9.0	92.3	6.8	86.4	6.1	4.8
		0.5	100.4	10.5	91.6	7.0	96.0	2.9	8.2
		2.5	95.5	5.6	98.4	6.1	93.3	4.0	5.1
(+)-Myclobutanil	Soil	5	92.3	7.1	95.7	3.7	88.4	5.9	6.8
		25	108.1	5.7	101.7	4.5	96.1	5.4	11.6
		50	102.5	3.8	90.5	6.4	91.3	4.2	9.4
	Water	0.25	85.6	9.4	83.1	5.8	90.0	7.7	6.5
		0.5	93.8	5.5	82.4	6.1	88.3	4.3	10.0
		2.5	91.5	5.9	87.9	4.3	95.4	5.1	7.1
(−)-Myclobutanil	Soil	5	92.0	4.6	100.6	10.9	95.1	8.2	8.2
		25	95.3	6.2	93.1	5.3	86.7	11.3	9.5
		50	103.3	7.3	94.7	4.8	90.8	5.8	11.2
	Water	0.25	89.9	8.0	84.4	9.7	93.1	7.0	8.9
		0.5	95.8	4.8	85.5	8.5	83.9	3.9	10.7
		2.5	86.3	7.6	90.0	4.4	92.6	4.8	5.3

enantiomers of triazole fungicides was conducted, and no significant difference (P > 0.05) was observed under the solvent and matrix storage treatment as described in Section 2.

3.6. Application to real samples

A newly method for the enantioselective determination of 8 triazole fungicides was described in the currently study. The effectiveness and applicability of this method in measuring trace levels of the *rac*-fenbuconazole, myclobutanil and triadimefon were evaluated by analyzing soil samples collected from our residual study trial field (Langfang, China). Water samples were collected from the Jingmi Irrigation Canal in Beijing. A total of 30 samples were analyzed, results showed that 3, 2, and 3 positive soil samples containing enantiomers of fenbuconazole, myclobutanil and triadimefon in the range 10.49–23.54, 12.53–18.56, and 8.79–17.16 μg/kg were detected, respectively (Table 4). The enantiomers were not detected in real water samples using the proposed method. In addition, it was observed that the EF of fenbuconazole, myclobutanil and triadimefon were ranged from 0.517 to 0.531, 0.501 to 0.511, and 0.445 to 0.480 (Table 4), respectively, indicating the dissipation and of fenbuconazole, myclobutanil and triadimefon may be enantioselective in the soil, and need to be verified in further studies. It should be kept in mind that the stereoisomers of chiral triazole fungicides are independent entities with respect to many of their biological properties. Each isomer may differ in toxicity to a variety of species and may be transformed by microbes at different rates. Thus, the development of enantiomeric analysis method to measuring the enantiomers of chiral triazole fungicides, we can better characterize the mechanisms affecting their fate and better understand their risk to ecological health.

Table 4
Concentration levels and EF values of fenbuconazole, myclobutanil, and triadimefon in real soil samples.

Real Sample	Concentration (μg/kg)		EF
Fenbuconazole	(+)-enantiomer	(−)-enantiomer	–
Sample 1	16.82	15.33	0.517
Sample 2	11.97	10.49	0.531
Sample 3	23.54	21.07	0.526
Myclobutanil	(+)-enantiomer	(−)-enantiomer	–
Sample 1	18.56	17.74	0.511
Sample 2	12.60	12.53	0.501
Triadimefon	(−)-enantiomer	(+)-enantiomer	–
Sample 1	9.63	8.79	0.467
Sample 2	17.16	15.83	0.480
Sample 3	13.55	10.88	0.445

4. Conclusions

Mass spectrometry offers a significant advantage in both analyte detection sensitivity and specificity over other detector systems. It is increasingly being used in drug discovery to monitor stereospecific drug kinetics. In the present study, a very simple and reliable enantioselective method using chiral LC–MS/MS for the simultaneous quantitative determination of 8 triazole fungicides, as well as enantiomers in soil and water has been successfully

developed and validated. A series of polysaccharide-based chiral stationary phases was evaluated, and chromatographic conditions were also optimized. Extracts containing the target compounds were analyzed and validated using chiral LC–MS/MS in the ESI positive mode. The specificity, calibration curves, precision, and reproducibility were successfully determined, demonstrating the suitability of this enantioselective method for the sixteen enantiomers. However, the purpose of the current work was not only to set up a novel valid chiral LC–MS/MS method to separate the enantiomers of the triazole fungicides, but also to develop a useful way of simultaneously determining quantitatively of the sixteen enantiomers in soil and water. This novel method was developed to facilitate further studies in tracing the different bioactivities, toxicities, metabolism, and environmental behavior of each enantiomer, and finally to help minimize the risks posed by the fungicide to human health, animals, and the environment.

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