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High-performance chiral separation of fourteen triazole fungicides by sulfated β-cyclodextrin-mediated capillary electrophoresis

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Abstract

In this paper, sulfated β-cyclodextrin-mediated capillary electrophoresis (CE) is evaluated as a new approach for the chiral separation of triazole-type fungicides. The 14 fungicides investigated were bitertanol, cyproconazole, difenoconazole, diniconazole, flutriafol, hexaconazole, myclobutanil, paclobutrazol, penconazole, propiconazole, tebuconazole, tetraconazole, triadimefon and triadimenol. Under the optimal conditions, excellent enantioseparation was achieved for all the 14 fungicides, including those fungicides containing two chiral centers. To our knowledge, this is the only system to date that offers outstanding enantiodiscrimination towards all triazole-type fungicides. The impact of the molecular structures of the triazole compounds on their migration behavior was studied. Similar to other chemical systems involving host–guest complexation, the interaction between sulfated β-cyclodextrin and the triazole compounds was found to be affected by a variety of factors, including electrostatic force, hydrogen bonding, steric effect and hydrophobicity. These factors, coupled with the countercurrent electroosmotic flow (EOF), were believed to be the major forces behind the exceptional chiral selectivity.

Keywords: Enantiomer separation; Pesticides; Triazoles

1. Introduction

Triazole derivatives represent the most important category of fungicides to date, thanks to their excellent protective, curative and eradicant power against a wide-spectrum of crop diseases. Triazole fungicides have a common structural moiety, the 1,2,4-triazole ring, which is connected to a hydrophobic backbone through position 1. Typically, the hydrocarbon backbone has a substituted phenyl group at one end, and an alkyl group or a different substituted phenyl group at the other end. As a consequence, asymmetrical carbons are generally present at the position(s) immediate and/or vicinal to the triazole rings. This makes chirality almost ubiquitous for triazole-type fungicides. Our statistical survey based on the Pesticide Manual shows that, of the 24 currently available triazole fungicides, 23 of them possess at least one chiral center [1]. Considering that the chiral centers of triazole fungicides are located close to the 1,2,4-triazole ring, a key template in the binding of the fungicides to their target sites, chirality is expected to play a crucial role in the bioactivities of triazole fungicides. This has actually been proven by numerous bioassay results [2].

With the exception a few cases such as diniconazole and uniconazole, most triazole-type fun-

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gicides are presently marketed in their racemic forms, making the single isomers of these fungicides practically unavailable to common researchers. Thus, chiral separation of the racemates is a fundamental aspect in the study of the different bioactivities and environmental fates of the individual enantiomers.

The vast majority of chiral separations of triazole fungicides were performed by high-performance liquid chromatography (HPLC), for which widely different chiral stationary phases (CSPs), including Pirkle-type phases [3–7], chiral cavity phases (particularly various cyclodextrin-based phases) [8] as well as helical polymeric phases (cellulose derivatives) [9] were used. Enantioseparation of triazole fungicides was also implemented with gas chromatography (GC) on a Chirasil-Val (diamide phase) capillary [10]. In addition to traditional chromatographic means, cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) has recently been employed for the same purpose [11–13].

It was noticed that, while quite a few chromatographic and electrophoretic systems had been claimed to be promising, the chiral separation of triazole-type fungicides was demonstrated only with a very limited number of compounds, namely, bitertanol, diclobutrazole, paclobutrazol and triadimenol. The chiral separation of the remaining triazole fungicides has rarely been investigated. It is likely that the chiral separation systems developed only worked with a narrow spectrum of triazole fungicides. In other words, there was a lack of a generally applicable chiral separation scheme for the whole cluster of triazole fungicides.

The omnipresence of chirality for triazole fungicides necessitates a chiral separation system that is effective for all of them. Bearing this objective in mind, the current work was initiated. Our effort was focused upon various CE techniques, because chiral separation by CE essentially involves optimization of the running electrolyte, which allows one to rapidly determine the best chiral separation medium from a large number of potential choices [14–17].

In this paper, we present results on the chiral separation of 14 triazole fungicides using a sulfated β-cyclodextrin (S-β-CD)-mediated CE system. To gain further insight into the stereospecific interactions between S-β-CD and triazole compounds, the effects of the molecular structures of triazole compounds on their migration behaviors were also investigated.

2. Experimental

2.1. Standards and chemicals

The 14 triazole fungicides, with purities higher than 98%, were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Among these referential standards, bitertanol, paclobutrazol and triadimenol were predominantly in their threo-diastereoisomeric form, whilst diniconazole was predominantly in the form of its *R*-configuration isomer (nearly 90%), with its *S*-isomer as an optical impurity. S-β-CD, with degrees of substitution ranging between seven and eleven, was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals and solvents were common brands of analytical-reagent grade or better, and were used as received. Water was collected from a Barnstead Nanopure Ultrafiltration unit (Boston, MA, USA).

Stock standards (approximately 1000 ppm) of the individual fungicides were prepared by dissolving the reference compounds in methanol. The samples to be injected were at a typical concentration of 50 ppm, and were made by diluting the individual stock solutions with the running electrolyte. The running electrolyte was prepared by dissolving exact amount(s) of S-β-CD (and urea when specified) into an appropriate volume of water, then adjusting the pH to 3.0 using 1 *M* phosphoric acid. All buffers were filtered through 0.45 μm Whatman nylon filters (Clifton, NJ, USA).

2.2. Instrumentation

All experiments were conducted on a CE-L1 capillary electrophoresis system, from CE Resources, Singapore (Singapore). CE-L1 was a modular system consisting of an autoinjector with a 50-position sample carousel, a dual polarity high-voltage power supply, and a variable-wavelength UV–Vis detector with an on-capillary detection cell. The system was computer-controlled, with an integrated software package allowing for comprehensive hardware management and data analysis. Throughout the experi-

ments, a negative high voltage of 18 kV was employed. UV detection was performed at 220 nm.

The fused-silica capillary (50 μm I.D.×360 μm O.D). was supplied by Polymicro Technologies (Phoenix, AZ, USA). A length of 60 cm was cut, and a UV detection window was created at 53 cm downstream of the capillary. Prior to actual sample separation, the capillary was equilibrated by flushing it with running buffer for about 10 min. Activation of the capillary by alkaline solution was found unnecessary, probably because the EOF was significantly suppressed by the acidic buffer, and thus it did not exert a significant impact on the separation.

3. Results and discussion

3.1. Enantioseparation of triazole fungicides with neutral CD-mediated capillary zone electrophoresis (CZE) and MEKC

The chiral separation of triazole fungicides was first explored under CZE mode. In acidic phosphate buffers and with various kinds of neutral CDs, these compounds were always detected as tailing peaks that were slightly ahead of dimethyl sulfoxide (DMSO), the EOF marker. This suggested that the basicity of triazole fungicides was too weak to be utilized for CZE separation. In a subsequent attempt using the CD-MEKC mode, with sodium dodecyl sulfate (SDS) as the micelle-forming surfactant, chiral separation was achieved with some of the triazole fungicides. These included bitertanol, cyproconazole, hexaconazole, propiconazole, tebuconazole and triadimenol (electropherograms not shown). For the other triazole fungicides, enantiomeric separation was unsuccessful in spite of numerous attempts.

The inability of the CD-MEKC mode to chirally separate all of the triazole fungicides was believed to be due to several reasons. First, it is well known that, in CD-MEKC separation mode, the migration behaviors of individual analytes are determined by their competitive distributions into the three ''phases'' (water, CD and micelles) [18]. Thus, for those hydrophobic analytes that were strongly incorporated into the SDS micelles, their chances of interacting with the chiral-discriminating CD molecules would be reduced. This eventually led to poor chiral recognition. Secondly, it has been noted previously that, in an SDS-based CD-MEKC system, the hydrophobic tails of SDS monomers might be able to penetrate into the cavities of CD [12], which could pose an adverse effect on the entrance of other solutes into the CD cavities. In both scenarios, those triazole compounds that had the weakest interactions with the CD derivative would be affected the most.

3.2. Enantioseparation of triazole fungicides using a S-β-CD-mediated CE system

The failure of CZE and CD-MEKC modes to achieve the chiral separation of all triazole fungicides prompted us to explore the feasibility of including charged chiral selectors in the running electrolytes. The utility of various charged CD derivatives in CE-type chiral separations has been reviewed previously [19]. The uniqueness of this type of separation system is that the charged CD derivatives act not only as ''pseudostationary phases'', like the micelles in MEKC separation, but also as chiral discriminating agents, as found in the various neutral CDs. Therefore, it allows the chiral separation of both charged and neutral compounds.

Among the various charged chiral selectors, S-β-CD is one of the most frequently utilized. Randomly substituted S-β-CD is commercially available and is relatively inexpensive. The power of S-β-CD as a chiral selector was first recognized by Stalcup and Gahm [20], who demonstrated that, in a S-β-CD-mediated CE system, a number of pharmaceutical compounds with diverse structural and charge features could be resolved enantiomerically. The merit of S-β-CD was later demonstrated by CE-type chiral separation of a lot more compounds, including catecholamines [21], monoterpenes [22], piperoxan [23], sulonium ions [24], carbidopa [25], ormeloxifene [26], phenethylamines [27], tropa alkaloids [28] and many other basic pharmaceuticals [29].

Due to the strong negative electrophoretic mobility of S-β-CD, the chiral separation was typically performed using an acidic medium and with a negative separation voltage. The feasibility of such a system for the chiral separation of most of the triazole fungicides was revealed in the initial attempt. The most suitable running electrolyte, based on chiral

selectivity and separation time, was found to be a phosphate buffer containing 2% (w/v) S-β-CD, with the pH adjusted to 3.0. Although higher concentrations of S-β-CD favored faster separation, the concentration could not be increased unduly. This was because of the multiple-charge feature of S-β-CD, which resulted in the very high ionic strength of the electrolyte solution. Therefore, high concentrations of S-β-CD would bring about large Joule heating during the chiral separation process, which, in turn, would reduce column efficiency and the signal-to-noise ratio for UV detection.

The chiral separation of 12 of the triazole fungicides (excluding bitertanol and difenoconazole, which will be discussed in the next section) is shown in Fig. 1. To facilitate comparison of the migration times of different triazole fungicides, the chiral separation of the 12 fungicides was performed collectively in two runs. Fig. 1 shows that, besides being capable of resolving the enantiomeric isomers of the individual fungicides, the system was also quite promising in the separation of different triazole fungicides. However, the latter was not pursued further since it was not our original purpose.

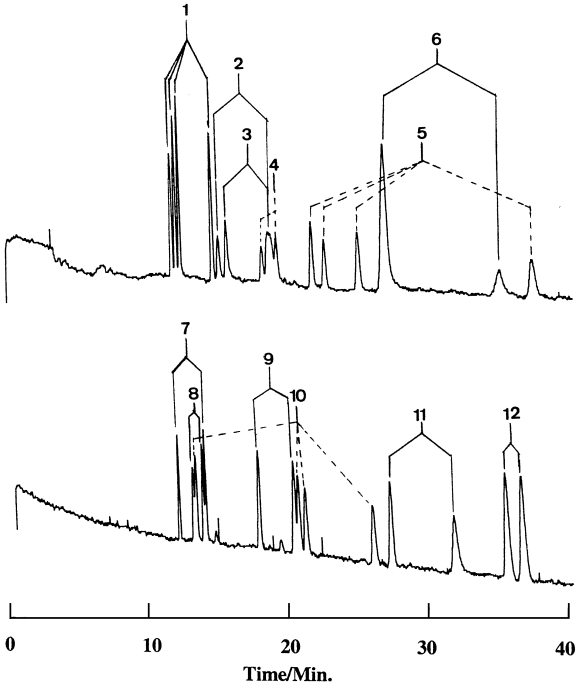


Fig. 1. Chiral separation of twelve triazole fungicides by S-β-CD-mediated CE. Running electrolyte, phosphate buffer with 2% S-β-CD, pH 3.0. High voltage, −18 kV. Capillary, 60 cm (53 cm effective length)×50 μm I.D.; UV detection, 220 nm. Peak identification: 1, cyproconazole; 2, penconazole; 3, hexaconazole; 4, tetraconazole; 5, triadimenol; 6, diniconazole (9:1 *R:S* mixture); 7, paclobutrazol (dominated by 2*RS*, 3*RS* isomers); 8, tebuconazole; 9, flutriafol; 10, propiconazole; 11, triadimefon and 12, myclobutanil.

Fig. 1 indicates that all racemic triazole fungicides were separated into their enantiomeric isomers with typical resolutions that were much greater than unity. This was true even for those fungicides with two asymmetrical centers, e.g., cyproconazole, paclobutrazol, propiconazole and triadimenol. All of these racemates were completely separated, resulting in four optical isomers. Such outstanding performance was virtually unattainable by any other existing methodology.

As mentioned earlier, due to the weak protonation of their triazole rings in the present running solution, free forms of triazole fungicides tended to migrate towards the cathode (inlet) site. Therefore, any apparent mobilities directed towards the detection side should be attributed solely to the interactions between the triazole fungicides and S-β-CD. Therefore, the migration times of the fungicides were good indicators of the strength of the interactions concerned. From Fig. 1, it can be seen that, overall, greater chiral separation was associated with those molecules that exhibited weak interactions with S-β-CD. According to Wren and Rowe's model [30], there exists an optimum concentration for a chiral selector, under which, an enantiomeric pair can best be resolved. This optimum concentration is dependent on the binding strength between the chiral selector and the solute, with a weakly interacting solute normally requiring a higher concentration of chiral selector. In this context, the chiral separation of those early migrating triazole fungicides in Fig. 1 could be improved by simply reducing the concentration of S-β-CD, however, this would be at the cost of a longer separation time.

3.3. Improvement in the enantioseparation of highly hydrophobic triazole fungicides by the addition of urea

Notwithstanding its success in the chiral separation of most triazole fungicides, the background

electrolyte formed merely with phosphate buffer and S-β-CD was found to be unsuitable for the chiral separation of bitertanol and difenoconazole, the two highly hydrophobic fungicides. As shown in Fig. 2A, using the normal running electrolyte, the enantiomers of the above two fungicides were detected as distorted peaks. Such a difficulty was caused by the extreme hydrophobicity of these two triazole fungicides. Note that the water solubilities of bitertanol and difenoconazole were 2 and 10 ppm, respectively, in contrast to values of 20–200 ppm for the twelve triazole fungicides listed in Fig. 1. This made bitertanol and difenoconazole easily saturated in the running electrolyte. Therefore, to ensure a sufficiently high signal response for these two fungicides, a way of enhancing their solubilities in the running medium was needed.

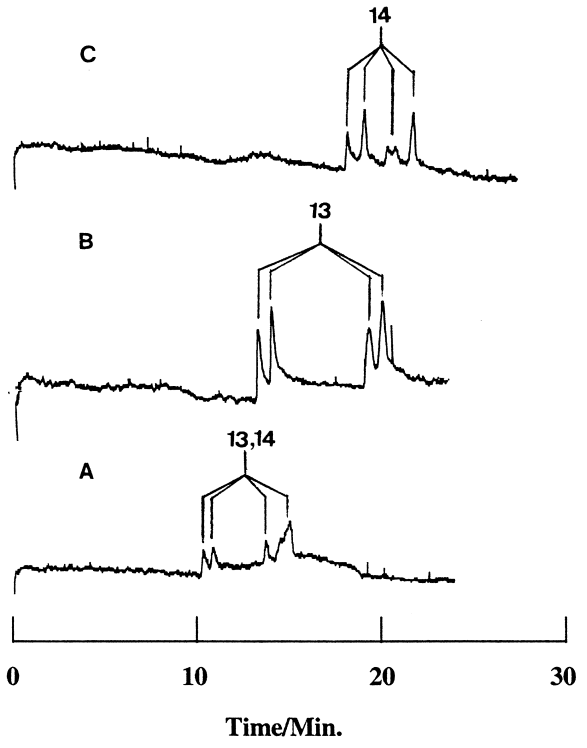


Fig. 2. Comparison of the chiral separation of the two highly hydrophobic triazole fungicides, bitertanol and difenoconazole, in the presence and absence of urea. Running electrolyte, phosphate buffer with 2% S-β-CD, pH 3.0 (A); or phosphate buffer with 2% S-β-CD and 2 *M* urea, pH 3.0 (B and C). Other conditions were the same as in Fig. 1. Peak identification: 13, difenoconazole and 14, bitertanol.

A common practice in CE studies for increasing the solubility of hydrophobic samples is to add organic solvents and/or surfactants to the running medium. Unfortunately, in the present separation system, adopting these two methods had negative implications, i.e., resulting in either prohibitively long migration times or loss of chiral resolution. For instance, if a surfactant like SDS was included in the running electrolyte, highly hydrophobic analytes, like bitertanol and difenoconazole, would partition predominantly into the SDS micelles, instead of into the S-β-CD cavity. Thus, the hydrophobic analytes would be carried rapidly to the detection site by the negatively charged SDS micelles. Without sufficient interaction with the chiral selector, loss of chiral resolution was inevitable. On the other hand, addition of an organic solvent such as methanol or acetonitrile was found to prolong the migration times of bitertanol and difenoconazole. This phenomenon might have stemmed from the competitive binding of the sample and solvent molecules to S-β-CD cavities, which acted to weaken the effective interaction between the analytes and S-β-CD.

Urea was known for its ability to increase the water solubilities of hydrophobic compounds [31], and was also frequently employed in CE studies [32]. In view of its extremely hydrophilic nature, urea may be used to avoid the sort of problems encountered using surfactants and organic solvents. This was verified in the experiments. It was discovered that when 2 *M* urea was added to the running electrolyte, the chiral separation of bitertanol and difenoconazole was greatly enhanced (Fig. 2B and Fig. 2C). Further improvement in the chiral separation was possible by the addition of more urea, but again at a cost of longer migration times.

3.4. Study of the structural impact of triazole fungicides on their interactions with S-β-CD

For the current chiral separation system, a diversity of factors, such as electrostatic force, hydrogen bonding, steric effects and hydrophobicity, may dictate the strength of the interaction between S-β-CD and the triazole fungicides. In light of their common 1,2,4-triazole ring, which gives similar pK_a values, triazole fungicides are unlikely to show much difference in terms of their electrostatic interactions

with S-β-CD. Nevertheless, significant differences could exist in other aspects, as evidenced by their very different migration behaviors. By studying the changes in migration times related to the fungicides' structures, the influences of the different factors on the interactions between S-β-CD and triazole fungicides were determined.

Table 1 lists the structures of the 14 triazole fungicides according to the migration order of their first enantiomers. Not surprisingly, the fastest migrating triazole compounds were those fungicides that contain a 4-chlorophenyl group, a hydroxyl group and an alkyl group near the chiral center, e.g., cyproconazole, paclobutrazole and tebuconazole. It is probable that the 4-chlorophenyl group was the structural moiety that participated in the hydrophobic interaction with the cavity of the S-β-CD, while the hydroxyl group enabled the hydrogen bonding of these compounds with the rim of the S-β-CD cavity.

On replacement of the 4-chlorophenyl group with a 2,4-dichlorophenyl group (e.g., hexaconazole) or a biphenyl group (e.g., bitertanol), or removal of the hydroxyl group (e.g., penconazole and tetraconazole), or substitution of the alkyl group with a bulkier phenyl group (e.g., flutriafol), the interactions between the triazole compounds and the S-β-CD were weakened, as indicated by the extended migration times. This further indicated that the strong interactions were the result of relatively less ''crowded'' structures in combination with hydrogen-bonding functionality.

Propiconazole and difenoconazole exhibit a quite different structural pattern, in which the two asymmetrical carbons form parts of the five-membered ring. Despite their seemingly ''crowded'' structures, their interactions with the S-β-CD were rather strong, as shown by their relatively short migration times. Hydrogen bonding between S-β-CD and the two oxygen atoms in the five-membered ring might be the reason for the enhanced interaction.

Triadimenol bears much structural resemblance to paclobutrazole, except that an oxygen atom replaces a methylene group. Interestingly, such a minor difference gave rise to a significant weakening of the interaction, as suggested by the much longer migration time for triadimenol. The reason for this phenomenon was unclear. Perhaps the existence of oxygen in the vicinity of the phenyl group alters the charge density of the benzene ring, hence weakening the inclusion of the 4-chlorophenyl group into the cavity of S-β-CD. Triadimefon is chemically an ''oxidation product'' of triadimenol. The replacement of the hydroxyl group by a keto group apparently weakened the interaction with S-β-CD. This again confirmed that the hydroxyl group had a critical role in strengthening the inclusion effect.

The presence of an unsaturated hydrophobic backbone makes diniconazole unique among the triazole fungicides investigated. Its relatively long migration time was probably due to its double bond, which prevented the free rotation of the neighboring groups. As a result, the 2,4-dichlorophenyl group and the hydroxyl group might have been unable to reach a favorable configuration when interacting with S-β-CD, thus hindering the interaction.

Myclobutanil represents a scenario in which no hydroxyl group is present in the molecule, while a cyano group is attached to the chiral carbon immediately next to the 4-chlorophenyl group. This peculiar structure proved to greatly hinder the inclusion of myclobutanil into S-β-CD, as evidenced by its having the longest migration time of those compounds investigated.

4. Conclusion

Chiral separation of triazole fungicides was explored in a variety of CE running systems. The S-β-CD-mediated system turned out to be the only system that enantioseparated all of the compounds. The optimal chiral separation was obtained using a phosphate buffer containing 2% S-β-CD (pH 3.0), together with a reversed high voltage. For the most hydrophobic triazole fungicides, chiral separation was enhanced by the addition of urea.

The outstanding chiral separation ability of the above CE system was accredited to several factors. The main reason was the inherent enantiorecognition power of S-β-CD. Secondly, with S-β-CD acting as a sample carrier in the enantioseparation system, the need for other means of sample transportation, which may have had adverse effects on the chiral recognition, was eliminated. The counter-EOF operating system was believed to be a further boost to the chiral separation process.

Table 1
Structures versus migration times (min) of twelve triazole fungicides in a S-β-CD-mediated CE system[a]

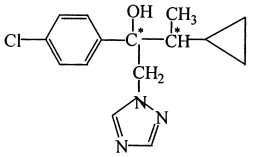
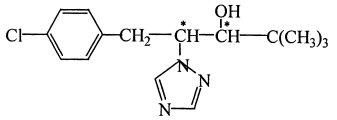
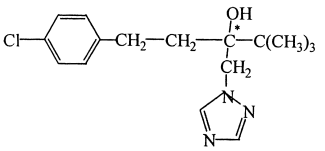
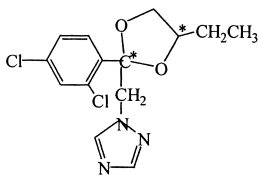
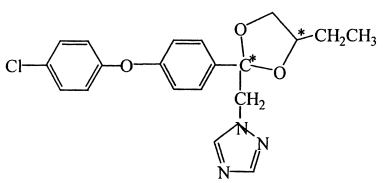
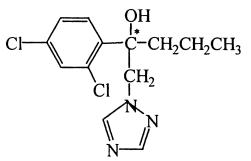
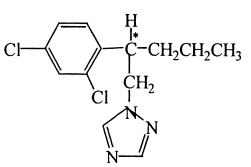
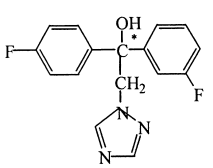
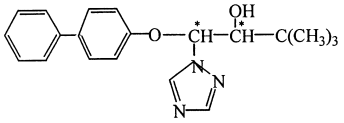
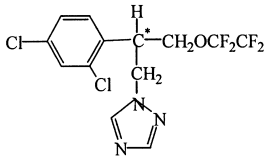
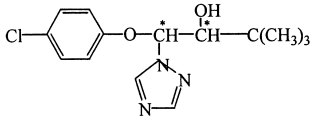
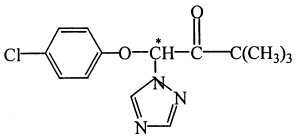
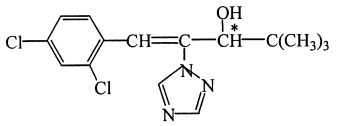
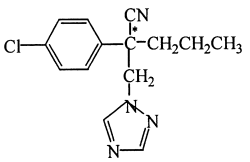
Cyproconazole		11.40/11.87/12.16/14.48
Paclobutrazole		11.57/13.29/14.21[b]/18.84[b]
Tebuconazole		12.50.13.16
Popiconazole		12.73/20.07/20.60.25.42
Difenoconazole		13.26/14.09/19.22/20.09[c]
Hexaconazole		15.41/18.68
Penconazole		15.60/18.70
Flutriafol		17.21/19.67

Table 1. Continued

Bitertanol		18.00/18.96/20.39/21.61[c]
Tetraconazole		18.19/18.84
Triadimenol		21.68/22.64/25.06/37.62
Triadimefon		26.61/31.17
Diniconazole		26.94/35.30[d]
Myclobutanil		34.78/36.03

[a] For experimental conditions, see Fig. 1.
[b] The (2*RS*, 3*SR*) isomers were detected as minor components.
[c] For experimental conditions, see Fig. 2.
[d] The *S*-isomer was detected as an optical impurity.

Success in the CE-type chiral separation of triazole fungicides may revitalize interest in the development of a chromatography-style chiral separation system. The corresponding chiral stationary phase may be created by immobilizing S-β-CD onto a supporting material (e.g. silica gel) via appropriate bonding chemistries. With the creation of such an HPLC column, constraints with respect to sample capacity can easily be overcome. This will enable the chiral separation of all of the triazole fungicides, on both analytical and preparative scales, using a single column.

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