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Enantioseparation of Imazalil Residue in Orange by Capillary Electrophoresis with 2-Hydroxypropyl-β-cyclodextrin as a Chiral Selector

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Chiral resolution of imazalil, a fungicide, was performed by capillary electrophoresis (CE) using 2-hydroxypropyl-β-cyclodextrin as a chiral selector. Factors affecting the chiral resolution and migration time of imazalil were studied. The optimum running conditions were found to be 5 mM ammonium dihydrogenphosphate−50 mM phosphate buffer (pH 3.0) containing 4 mM 2-hydroxypropyl-β-cyclodextrin with an effective voltage of +25 kV at 20 °C using direct detection at 200 nm. Under these conditions, the resolution (Rs) of racemic imazalil was ∼6. The extraction of imazalil from orange samples was done with acetonitrile under basic conditions. The extract was purified with a solid-phase extraction cartridge (Sep-Pak plus PS-2) and was analyzed by the above CE method. Eight orange samples were analyzed, and imazalil was detected in seven samples. In four of these seven oranges, the level of (−)-imazalil was the same as that of (+)-imazalil, but in the other three oranges, the level of (−)-imazalil was found to be lower than that of (+)-imazalil, suggesting that (−)-imazalil was degraded more quickly than (+)-imazalil in oranges.

KEYWORDS: Enantioseparation; imazalil; fungicide; capillary electrophoresis; orange

INTRODUCTION

About 25% of agrochemicals are reported to be chiral molecules, although a few of the new commercial formulations contain only the active enantiomer (*1*, *2*). The two enantiomers of a chiral molecule possess the same physicochemical properties, but the biological activity may be mainly due to only one enantiomer. Microbial degradation, metabolic pathways, biological uptake, and toxicity can exhibit stereoselectivity. There has been increasing interest in the different environmental processes and stereoselective biological behavior of the (+)- and (−)-enantiomers of agrochemicals. To monitor the stereoselective processes of the agrochemicals in organisms and in the environment, analytical methods for the separation of enantiomers of agrochemical compounds are needed.

Capillary electrophoresis (CE) is a recently developed and powerful analytical technique with a wide range of applications. The availability of many chiral selectors makes CE an important tool for chiral analysis as previously reviewed (*3*−*10*). Of these selectors, cyclodextrins (CDs) and their derivatives have been most widely applied in CE for the separation of enantiomers of many compounds. In a process called inclusion complexation, the cavity of the native or derivatized CDs hydrophobically interacts with the hydrophobic part of the compounds, such as an aromatic ring. This process plays an important role in the stereoselective interaction. The migration times of analytes in CE were well characterized by Guttman et al. (*11*). Wren and Rowe (*12*) developed a theoretical model relating the mobility to the concentration of a CD selector.

Imazalil, 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole (**Figure 1**), can control a wide range of fungal diseases on fruits, vegetables, and ornamentals by inhibiting ergosterol biosynthesis (*13*). Thus, it is widely used as a postharvest fungicide. Imazalil has been reported to have a cytotoxic effect (*14*), an ability to induce P450 isoforms (*15*), and an inhibitory activity against CYP19 aromatase (*16*−*18*), which catalyzes the conversion of androgens to estrogens. In Japan, the maximum permitted imazalil concentration is 5 ppm for citrus fruits such as oranges, lemons, and grapefruits and 2 ppm for bananas. A number of investigations on the determination of imazalil in fruits and vegetables have been conducted by gas chromatography (GC) (*19*−*26*), high-performance liquid chromatography (HPLC) (*26*−*29*), and CE (*30*). The enzyme-linked immunosorbent assay technique (*31*) has also been proposed. Imazalil has an asymmetric carbon and is normally used as a racemic mixture. In investigations of CDs, the enantiomers of agrochemicals including imazalil in standard solutions by CE using anionic CD derivatives (*32*) or by GC using CD derivative chiral stationary phases (*33*) have been

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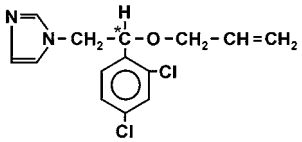

Figure 1. Structural formula of imazalil. *C represents an asymmetric carbon.

separated. To our knowledge, the enantiomeric separation of imazalil residues in real samples such as citrus fruits has not previously been studied, and it is not known whether there are enantioselectivities for toxic and antifungal activities of imazalil or not. Because oranges are eaten all over the world, it is important to determine relative concentrations of imazalil enantiomers in fruits after spraying with a racemic mixture and also the above enantioselective activities of imazalil. The aim of the present study was to develop a method for the chiral separation of imazalil using capillary electrophoresis with CD and to determine the enantioselectivity of (+)- and (−)-imazalil residues in orange.

MATERIALS AND METHODS

Chemicals. 2-Hydroxypropyl-β-cyclodextrin (average degree of substitution = 7), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin, and heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin were obtained from Sigma (St. Louis, MO). Hydroxypropyl-γ-cyclodextrin (average degree of substitution = 4.2) and hydroxypropyl-α-cyclodextrin (average degree of substitution = 4.2) were obtained from Aldrich (Milwaukee, WI). γ-Cyclodextrin, α-cyclodextrin, imazalil, and other chemicals (analytical grade) were obtained from Wako Pure Chemicals (Osaka, Japan).

Apparatus for CE. Electrophoretic experiments were carried out using a capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany). Samples were injected at a pressure of 50 mbar for 2 s. Separation was performed in a fused-silica bubble cell capillary of 64.5 cm (effective length 56 cm) × 75 μm ∅ (Agilent Technologies). The capillary was kept at 20 °C. The analytes were detected at 200 nm. The power supply was operated in the constant-voltage mode, at +25 kV, and the substances migrated toward the negative pole. The resulting current was ∼75 μA.

Apparatus for HPLC. The HPLC system consisted of a Hitachi (Tokyo, Japan) pump model L-6300, a Rheodyne (Cotati, CA) manual injector, a Shimadzu column oven model CTO-10AC, a Shimadzu (Kyoto, Japan) photodiode array detector model SPD-M10AV, and a Jasco (Tokyo, Japan) polarimetric detector model OR-990.

Preparation of Imazalil Enantiomers. Imazalil enantiomers were separated by HPLC using a Chiralcel OB column (4.6 mm ∅ × 250 mm, Daicel Chemical Industries, Tokyo, Japan) thermostated at 25 °C. Hexane/ethanol/triethylamine (96:3.5:0.5) was used as a mobile phase at a flow rate of 1 mL/min. Two fractions, corresponding to the (+)- and (−)-enantiomers of imazalil, were separately collected. The purities of the (+)- and (−)-imazalils collected were more than 99.8% enantiomer excess (ee) and 99.0% ee, respectively, where ee is defined as the difference between the amount of the two enantiomers in a mixture divided by their total.

Buffer and Sample Preparation for CE. The background electrolyte (BGE) in the electrophoretic experiments, unless stated otherwise, was 5 mM ammonium dihydrogenphosphate−50 mM phosphate buffer (pH 3.0) containing 4 mM 2-hydroxypropyl-β-CD (2HP-β-CD) and was filtered with a 0.22 μm filter before use. Purified water was obtained from a Toray (Mishima, Japan) ultrapure water system. Stock solutions of 1000 mg/L racemic, (+)- and (−)-imazalils were separately prepared in acetonitrile, stored at −20 °C, and diluted to 20 mg/L before use.

Valencia oranges (California) were purchased from a local market. Fifty grams of chopped orange sample containing skin and pulp was homogenized with 50 g of purified water using a Microtec (Funabashi, Japan) Physcotron homogenizer model NS 51 for 1 min. Then, a 10 g portion of the homogenate was weighed and homogenized with 30 mL of acetonitrile and 1 mL of 1 M NaOH using the homogenizer for 1 min. To determine the recovery, 50 μL of 200 mg/L imazalil was added to a 10 g portion of the homogenate. The suspension was centrifuged at ∼1200*g* for 5 min, and the supernatant was obtained. The pellet was reextracted twice with 30 mL of acetonitrile. The supernatants were combined in the 200 mL round-bottom flask and were concentrated to a volume of <10 mL with a rotary evaporator. After addition of 20% (v/v) acetonitrile (30 mL), the solution was applied to a Sep-Pak PS-2 cartridge (Nihon Waters, Tokyo, Japan). The cartridge was washed with 20 mL of 30% (v/v) acetonitrile and was dried under vacuum by aspiration. The adsorbed materials were eluted with acetonitrile and concentrated to dryness under rotary vacuum evaporation. The resulting residue was dissolved in 0.5 mL of 50% (v/v) acetonitrile and was analyzed by the above CE method.

Table 1. Resolution of Imazalil Enantiomers Using Different CDs as Chiral Selectors

cyclodextrin	resolution (Rs)	migration time (min) (−)-imazalil	(+)-imazalil
without CD	NS[a]	9.62	
α-CD	NS	16.23	
HP-α-CD	0.65	17.57	17.70
β-CD	3.91	13.93	14.59
2HP-β-CD	6.03	13.00	13.89
2,6-di-*O*-methyl-β-CD	2.23	18.74	19.22
2,3,6-tri-*O*-methyl-β-CD	2.08	10.52	10.73
γ-CD	1.38	11.81	11.96
HP-γ-CD	2.06	11.44	11.65

[a] Not separated.

Calculation of Resolution. The resolution (Rs) of an enantiomer was calculated by using the equation

$$\mathrm{Rs} = 2(t_2 - t_1)/(w_1 + w_2)$$

where t is the migration time and w is the width of the peak at the baseline.

RESULTS AND DISCUSSION

Factors Affecting Chiral Separation. As mentioned above, CDs and their derivatives have been most widely applied in CE for the separation of enantiomers of many compounds; we selected several CDs as chiral selectors for the enantioseparation of imazalil. The effect of the type of CD on the enantioseparation of imazalil was investigated by CE using a BGE containing separately 4 mM α-CD, hydroxypropyl-α-CD (HP-α-CD), β-CD, 2HP-β-CD, 2,6-di-*O*-methyl-β-CD, 2,3,6-tri-*O*-methyl-β-CD, γ-CD, or HP-γ-CD (**Table 1**). The electroosmotic flow in an uncoated fused silica capillary at pH 3.0 was very slow. The above CDs are electrically neutral compounds, and hence they migrate very slowly at the electroosmotic velocity. The imidazole group of imazalil with a pK_b of 6.53 is protonated at pH 3.0. Thus, the analyte migrates electrophoretically to the cathode. When a charged analyte is included in the CD cavity, the inclusion complex thus formed has a charge identical with that of the free analyte but an increased molecular mass and, hence, a lower electrophoretic mobility than the free analyte. In an enantiomeric separation, free enentiomers have identical electrophoretic mobilities and the included enantiomers also probably have the same mobilities. Therefore, the separation principle of CE with CD for enantiomeric separation is the difference in inclusion complex formation constants between a pair of enantiomers and CD. The more strongly included enantiomer has a lower mobility. Imazalil was enantioseparated by the addition of each CD, except α-CD. In all instances that caused enantioseparation of imazalil, the (+)-isomer moved

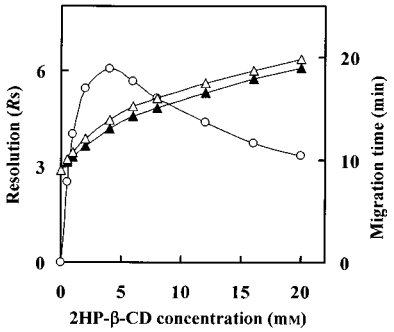

Figure 2. Effect of 2HP-β-CD concentration on the resolution and migration time of imazalil: (○) resolution (Rs) of imazalil; (△) migration time of (–)-imazalil; (▲) migration time of (+)-imazalil. The BGE was composed of various concentrations of 2HP-β-CD containing 5 mM ammonium dihydrogenphosphate–50 mM phosphate buffer (pH 3.0).

more slowly than the (−)-isomer. This might indicate that the (+)-isomer formed stronger diastereomer complexes with each CD than the (−)-isomer. Of these CDs, 2HP-β-CD was found to be most effective for the resolution of imazalil enantiomers.

The effect of the 2HP-β-CD concentration (0−20 mM) on the resolution and migration time of imazalil was studied (**Figure 2**). The migration times of imazalil enantiomers increased with increasing amounts of 2HP-β-CD, suggesting that a higher 2HP-β-CD concentration formed a more stable complex of imazalil with 2HP-β-CD. The resolution showed a maximum at 4 mM 2HP-β-CD.

The effect of the pH (2.0−5.5) of the BGE on the resolution and migration time of imazalil was studied. The migration time of imazalil remained constant with increasing pH up to 3.5 and then decreased at higher pH values. It is well-known that the velocity of electroosmotic flow is very slow in the BGE at pH values <3 and increases with increasing pH values >3. Thus, the increase in the migration time of imazalil at pH up to 3.5 can depend on the velocity of electroosmotic flow at each pH. The resolution showed a maximum at pH 3.0. It was suggested that a shorter migration time would decrease the chance of interaction between the analyte enantiomers and 2HP-β-CD as a chiral selector. The BGE contained 5 mM ammonium dihydrogenphosphate. It was found that the use of a BGE without ammonium dihydrogenphosphate caused peak tailing and that the addition of more ammonium dihydrogenphosphate caused an increase in the baseline noise in this CE system.

The effect of the capillary temperature (20−40 °C) on the resolution and migration time of imazalil was studied. A lower capillary temperature caused increases in both the resolution and migration time of imazalil. The shorter migration time at higher temperature could be attributed to a decrease of the buffer viscosity (*34*).

Therefore, the optimum BGE conditions, that is, the conditions giving both high resolution and short migration time, were found to be 4 mM 2HP-β-CD in 5 mM ammonium dihydrogenphosphate−50 mM phosphate buffer (pH 3.0) with an effective voltage of +25 kV at 20 °C.

Enantioseparation of Imazalil in Orange. Racemic imazalil was subjected to the CE method using the above optimum conditions. Linearity ($r^2 > 0.999$) was demonstrated in the range of 0.5−25 mg/L by standard curves for (+)- and (−)-imazalils. The detection limit (S/N = 5) of both (+)- and (−)-imazalils was 0.1 mg/L. The reproducibility of five consecutive determinations was evaluated at 10 mg/L for the (+)- and (−)-imazalils. Good reproducibilities of peak areas (RSD < 2.1%) and migration times (RSD < 0.4%) were obtained. Recoveries of (+)- and (−)-imazalils using three oranges were 96.5 ± 2.53 and 96.7 ± 2.81%, respectively.

Table 2. Levels of (–)- and (+)-Imazalils in Oranges

orange	(–)-imazalil (mg/kg)	(+)-imazalil (mg/kg)
A	ND[a]	ND
B	0.49	0.50
C	0.53	0.65
D	0.57	0.59
E	0.58	0.60
F	0.82	0.83
G	0.94	1.01
H	0.27	0.37

[a] Not detected (<0.01 mg/kg).

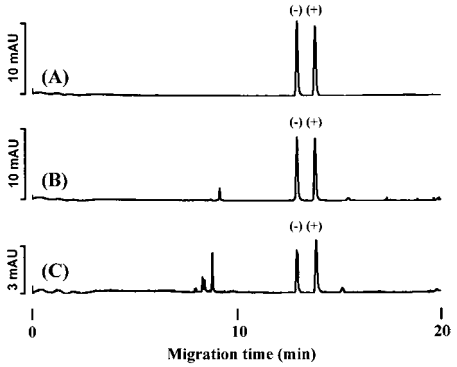

Figure 3. Electropherograms of oranges: (A) standard solution (20 mg/L racemic imazalil); (B) orange sample B in **Table 2**; (C) orange sample H in **Table 2**; (–) and (+) represent (–)- and (+)-imazalil, respectively. The BGE was composed of 4 mM 2HP-β-CD containing 5 mM ammonium dihydrogenphosphate–50 mM phosphate buffer (pH 3.0).

Using the proposed CE method, (+)- and (−)-imazalils in eight oranges were analyzed (**Table 2**). **Figure 3** shows representative electropherograms. For seven oranges (A−G), imazalil was extracted immediately after purchasing. To study the effect of mold on the enantioselectivity of imazalil residues, one orange (H) that was left to stand for 2 weeks at room temperature in the dark developed some blue mold on the outside of the orange. The total levels of (−)- and (+)-imazalils in seven oranges, except for orange A, ranged from 0.64 to 1.95 mg/kg. In four samples (oranges B, D, E, and F), each level of (−)-imazalil was the same as that of (+)-imazalil; that is, the imazalil residues in the orange samples were racemic. However, the ratios of the (−)- and (+)-isomers in three samples (oranges C, G, and H) were found to be 45:55, 48:52, and 42:58, respectively; that is, they were not racemic. The effect of pH during the extraction of imazalil on the ratios of (−)- and (+)-isomers was studied by varying the amount of 1 M NaOH added to the homogenate (100 g) of orange H with water. When 1 mL of NaOH was added, the pH was 10.5, and the levels of (−)- and (+)-imazalils were 0.27 and 0.37 mg/kg, respectively (**Table 2**). When 0.5 mL of NaOH was added, the pH was 7.5, and the levels of (−)- and (+)-imazalils were 0.25 and 0.33 mg/kg, respectively. When no NaOH was added, the pH was 4.3, and the levels of (−)- and (+)-imazalils were 0.050 and 0.067 mg/kg, respectively. These results show that lowering the pH from 10.5 to 4.3 during the extraction decreased the recovery of imazalil from orange but did not affect the ratio of (−)- and (+)-imazalils. The same results were obtained by using orange G. Although oranges F, G, and H were from the same bin, the total level of imazalil in orange H was found to be significantly

lower than the levels in oranges F and G. Moreover, the (−)/(+) ratio of imazalil in orange H was lower than the ratios in the other orange samples. Although it is unclear whether the above results are attributable to ripening or the actions of molds, the present CE study suggests that (−)-imazalil is degraded more quickly than (+)-imazalil in oranges.

In conclusion, a capillary electrophoretic method for the enantioseparation of imazalil was developed by using 2HP-β-CD as a chiral selector. Imazalil residue in orange samples was enantioseparated by the proposed CE method. Our results suggest that (−)-imazalil was degraded more quickly than (+)-imazalil in oranges.

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