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Residue Analysis and Degradation Studies of Fenbuconazole and Myclobutanil in Strawberry by Chiral High-Performance Liquid Chromatography−Tandem Mass Spectrometry

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ABSTRACT: A simple and sensitive enantioselective method for the determination of fenbuconazole and myclobutanil in strawberry was developed by high-performance liquid chromatography−tandem mass spectrometry (HPLC-MS/MS). Fenbuconazole and myclobutanil residues in strawberry were extracted with acetonitrile containing 1% acetic acid, and an aliquot was cleaned up with PSA (primary and secondary amine) and C_{18} sorbent. The direct resolution of fenbuconazole and myclobutanil enantiomers was performed on a cellulose tris (3,5-dimethylphenylcarbamate) column using acetonitrile−0.1% formic acid solution (60:40, v/v) as the mobile phase. Quantification was achieved using matrix-matched standard calibration curves, and the limits of quantification for fenbuconazole and myclobutanil enantiomers in strawberry were both 2 μg/kg. The method was successfully utilized to investigate the probable enantioselective degradation of fenbuconazole and myclobutanil in strawberry. The results showed that the degradation of the fenbuconazole and myclobutanil enantiomers in strawberry followed pseudofirst-order kinetics ($R^2 > 0.97$). The results from this study revealed that the degradation of fenbuconazole in strawberry was not enantioselective, while the degradation of myclobutanil was enantioselective, and the (+)-myclobutanil showed a faster degradation than (−)-myclobutanil in strawberry, resulting in the relative enrichment of (−)-myclobutanil in residue. The results could provide a reference to fully evaluate the risks of these two fungicides.

KEYWORDS: Degradation, enantioselectivity, fenbuconazole, myclobutanil, strawberry

INTRODUCTION

The enantioselectivity behavior of chiral pesticides has received more attention at the forefront of chemistry and toxicology research.[1−5] As is well-known, enantiomers with the identical physicochemical properties may have different bioactivity and toxicity, and the biological transformation of chiral compounds is often enantioselective.[1,4] To better evaluate the environmental risk and food safety, it is useful to develop enantiomeric analysis methods of chiral pesticides and perform enantioselective biodegradation studies.

Fenbuconazole and myclobutanil are two important chiral triazole fungicides used against powdery mildew of cereal, vegetables, and fruits.[6,7] They contain the cyano-group, have an asymmetrically substituted C atom (Figure 1), and consist of a pair of enantiomers. The enantiomeric separation of myclobutanil was achieved by high-performance liquid chromatography (HPLC) with polysaccharide type chiral stationary phases (CSPs) under normal phase conditions or reversed phase conditions using ultraviolet (UV) detection.[8−11] Recently, Tian et al.[10] developed a method for the determination of myclobutanil enantiomers in water and soil by HPLC-UV. Li et al.[12] presented a method for the simultaneous enantioselective determination of fenbuconazole and its two metabolites in soil and water by chiral liquid chromatography−tandem mass spectrometry (HPLC-MS/MS). To our knowledge, no study has been reported about enantioselective determination of fenbuconazole and myclobutanil in fruits until now. Accordingly, enantioselective degradation of fenbuconazole and myclobutanil in fruits has received little attention. Strawberries are cultivated and eaten almost all over the world, and fenbuconazole and myclobutanil are widely used to control plant diseases including those of strawberries. So, it is important to determine relative concentrations of fenbuconazole and myclobutanil enantiomers in strawberries after spraying with a racemic mixture.

Reversed phase HPLC-MS/MS has been used extensively in drug metabolism and pharmacokinetic studies of enantiomers for high selectivity, high sensitivity, and simple preliminary treatment.[13−15] However, research papers concerning chiral pesticide separation by reversed phase HPLC-MS/MS are limited.[12,16] In this study, an analytical method was presented for the enantioanalysis of fenbuconazole and myclobutanil in strawberries. Moreover, this research was conducted to investigate the possible enantioselective residue behavior of fenbuconazole and myclobutanil in strawberries using reversed phase HPLC-MS/MS on a cellulose CSP.

MATERIALS AND METHODS

Chemicals and Materials. HPLC grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). HPLC grade water was supplied by Wahaha (Hangzhou, China). Formic acid (≥96.0% purity) was purchased from TEDIA (Fairfield, United States). Acetic acid (≥99.7% purity) was obtained from Sigma-Aldrich (St. Louis, MO). Silica-based sorbents including C_{18} (40 μm particle size) and primary secondary amine (PSA) (40 μm particle size) were obtained

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from Agilent (DE). All other chemicals were of analytical reagent grade and obtained from commercial sources.

Analytical standards of (±)-fenbuconazole (purity 99.0%) and (±)-myclobutanil (99.5%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock standards (approximately 100 mg/L) of the individual fungicides were prepared by dissolving the reference compounds in acetonitrile. Working standards at lower concentrations were prepared by serial dilution of the stock standards.

Field Experiments. The field experiments were conducted under greenhouse condition during February and March 2011. Seven plots of working areas for strawberries were prepared at the experiment field in Zhejiang Academy of Agricultural Sciences (Hangzhou, China), each with 30 (10 × 3) m^2, and the buffer zone was set up between plots. Fenbuconazole and myclobutanil were carried out in different plots. Three plots for each fungicide were applied with three replicates, and the other one was used as the control (without fungicides). These plots had never been treated with fenbuconazole and myclobutanil for more than 3 years. Average temperatures were 25.0 ± 10.0 °C at the greenhouses during application of pesticides. The application of fenbuconazole (24% emulsifiable concentrate) was in the dosage of 100 g a.i./ha (gram of active ingredient per hectare), and myclobutanil (20% microemulsion) was in the dosage of 75 g a.i./ha. Three representative samples (approximately 500 g in each sample) from each plot were collected on day 0 (2 h after application) and 1, 3, 5, 7, 9, 15, and 21 days after spraying. Strawberry samples were homogenized by a blender (Philips, China) after removal of the stem and then stored at −20 °C until they were analyzed. These samples were stored about 1 month before analysis.

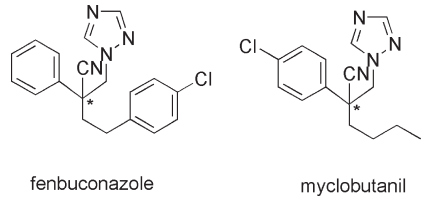

Figure 1. Chemical structures of fenbuconazole and myclobutanil. *C represents an asymmetric C.

LC-MS/MS Parameters. LC-MS/MS analyses were performed on a TSQ Discovery triple quadrupole mass spectrometer and a Surveyor liquid chromatograph (Thermo Fisher Scientific, United States). Thermo Fisher Xcalibur 2.0.7 software was used to control the instrument and collect and analyze data.

LC Profile. Separation was carried out on a chiral column Lux Cellulose-1 [cellulose tris (3,5-dimethylphenylcarbamate)] supplied by Phenomenex (Torrance, United States). The column was sized 150 mm × 2.0 mm i.d. packed with 3 μm particles. The mobile phase consisted of 60% (v/v) acetonitrile and 40% (v/v) 0.1% formic acid solution. The flow rate was set at 0.2 mL/min, the column oven temperature was set at 25 °C, and the utosampler temperature was set at 4 °C. The injection volume was 5 μL, and the total run time was 15 min.

MS/MS Conditions. The ESI-MS/MS (electrospray ionization coupled with tandem mass spectrometry) interface was operated in the positive ion mode. The ESI source conditions were as follows: ion spray voltage, 4200 V; spray needle temperature, 350 °C; sheath gas (N_2), 35 (arbitrary) units; auxiliary gas (N_2), 15 U; and collision gas (Ar), 1.5 mTorr. Multiple

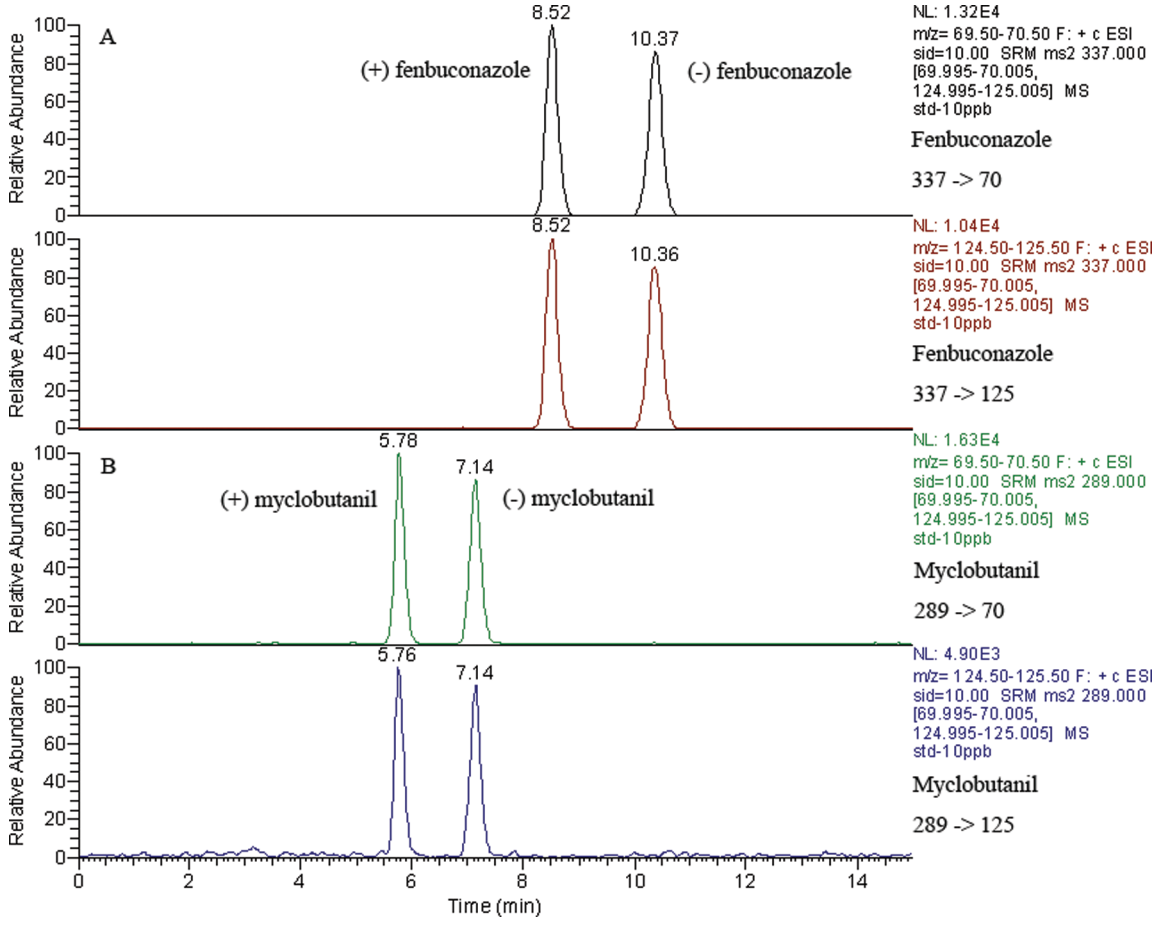

Figure 2. Typical MRM chromatograms of (A) (±)-fenbuconazole (10 μg/L) and (B) (±)-myclobutanil (10 μg/L) in standard solution.

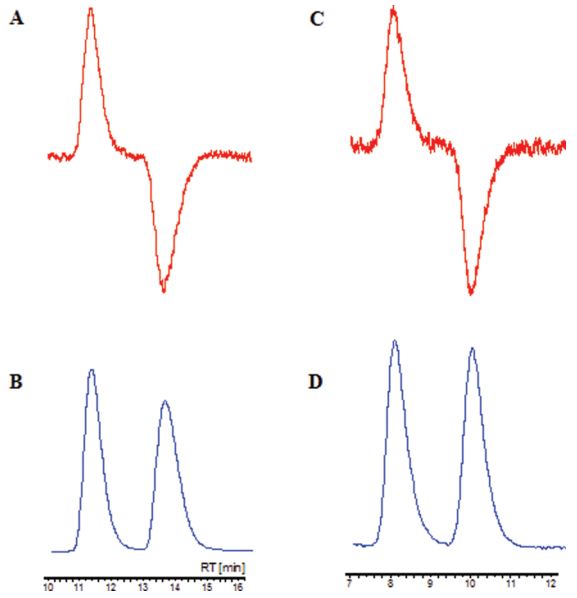

Figure 3. HPLC chromatograms for enantiomer separations, UV monitoring at 254 nm, Lux Cellulose-1 column, acetonitrile−0.1% formic acid (60:40, v/v). (A) CD chromatogram for fenbuconazole, (B) UV chromatogram for fenbuconazole, (C) CD chromatogram for myclobutanil, and (D) UV chromatogram for myclobutanil.

reaction monitoring (MRM) was applied to determine fenbuconazole and myclobutanil. A syringe pump was used to provide a constant analyte infusion (5 mg/L, 5 μL/min) into the LC eluent via a T-connection. The full-scan mass spectra and the MS/MS spectra were acquired to obtain two transitions (product ions) for each fungicide. Fenbuconazole and myclobutanil show the base peak at $[M + H]^+$ in Q1 scan mode. The collision energy (CE) was optimized to produce the most sensitive and stable product ion. For fenbuconazole, transition m/z 337 > 70 and m/z 337 > 125 were used for quantification and confirmation, respectively, and collision energies were 20 and 20 eV, respectively. For myclobutanil, transition m/z 289 > 70 and m/z 289 > 125 were used for quantification and confirmation, respectively, and collision energies were 15 and 20 eV, respectively.

Determination of the Elution Order. At a given wavelength, the individual enantiomer of a chiral compound corresponded to a specific circular dichroism (CD) signal, and CD was frequently used to distinguish a pair of enantiomers. In this work, a Jasco LC-2000 series HPLC system (Tokyo, Japan) with a variable wavelength CD-2095 CD was used to distinguish the elution order of enantiomers by determining the optical rotation of each enantiomer. The chiral separation was performed under the same LC conditions as HPLC-MS/MS. The elutions were monitored at 254 nm for fenbuconazole and myclobutanil.

Samples Extraction and Purification. Sample extraction and cleanup procedures followed the buffered QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method[17−19] or AOAC Official Method 2007.01.[20] The homogenized strawberry samples (15.0 g/sample) were weighed into individual 50 mL polypropylene centrifuge tubes, followed by the addition of 15 mL of acetonitrile containing 1% acetic acid. After they were mixed by vortexing for 1 min, 6 g of $MgSO_4$ and 1.5 g of CH_3COONa were added. Then, the tube was shaken vigorously for 5 min using a vortex mixer and centrifuged at 6000 rpm for 5 min. One milliliter aliquot of the acetonitrile extracts (upper layer) was transferred into a 2 mL centrifuge tube containing 50 mg of PSA, 50 mg of C_{18}, and 150 mg of anhydrous $MgSO_4$ for cleanup. After it was shaken and centrifuged, a 0.5 mL aliquot of the upper layer was transferred into a 2 mL centrifuge tube containing 0.5 mL of water. The resulting solution was filtered through 0.22 μm filter for HPLC-MS/MS analysis.

Table 1. Linearity for Fenbuconazole and Myclobutanil Enantiomers

compd	concn range (μg/L)	standard solution linear equation, R^2	matrix-matched linear equation, R^2
(+)-fenbuconazole	1−500	$Y = 38156X +$ 33467, 0.9995	$Y = 34012X +$ 76441, 0.9973
(−)-fenbuconazole	1−500	$Y = 39540X -$ 12425, 0.9994	$Y = 35204X +$ 93428, 0.9982
(+)-myclobutanil	1−500	$Y = 39267X -$ 9276.3, 0.9998	$Y = 35365X +$ 50266, 0.9986
(−)-myclobutanil	1−500	$Y = 38354X -$ 16499, 0.9997	$Y = 34698X +$ 63975, 0.9991

Method Validation. A series of standard solutions of fenbuconazole and myclobutanil for linearities were constructed using mixed working standard solutions at concentrations of 2, 5, 10, 50, 100, 200, 500, and 1000 μg/L. According to a procedure described in Samples Extraction and Purification, a blank matrix was prepared, and a series of matrix-matched calibration standards with the same concentrations were also prepared.

Fenbuconazole and myclobutanil were added to untreated control samples at four concentration levels (2, 5, 50, and 500 μg/kg for each enantiomer based on five replicates). The samples were left for 1 h to equilibrate the analytes in the sample. The fortified samples were analyzed based on the described procedure, and the recoveries were calculated. The precision of the method was determined by the repeatability and expressed by the relative standard deviation (RSD).

RESULTS AND DISCUSSION

Separation and Chiroptical Detection of Enantiomers. Under the HPLC conditions, the enantiomers of fenbuconazole and myclobutanil were separated completely (shown in Figure 2). CD spectra were recorded to compare the elution order of enantiomers, and the wavelength of 254 nm was selected in the HPLC-CD method. The chromatograms obtained (Figure 3) show the separation and optical rotation of enantiomers. So, the first eluted enantiomer was confirmed as (+)-enantiomer, while the second one was (−)-enantiomer in our study.

In addition, an optical rotatory (OR) detector was also an important chiroptical technique used to identify the elution orders of the enantiomers. Recently, Qiu et al.[11] reported the elution order of the enantiomers of fenbuconazole and myclobutanil by using an OR detector. Comparing elution orders of enantiomers on Lux Cellulose-1 chiral column under the same mobile phase, fenbuconazole and myclobutanil showed consistent CD signals with OR. CD positive (+) and CD negative (−) signals correspondingly showed OR right (+) and OR left (−).

Method Development and Validation. A QuEChERS analytical methodology has been gaining significant popularity for the determination of multiple pesticides residues in many kinds of vegetables and fruits. Validation of the method for determination of fenbuconazole and myclobutanil enantiomers in strawberry was comprised of linearity, precision, recovery, and limit of quantification (LOQ).

Good linear calibration curves for each enantiomer were obtained over the range of 1−500 μg/L. The peak areas were plotted against standard concentrations, and standard curves were in the form of $Y = AX + B$, and linear equations in standard solution and in matrix-matched solution are summarized in Table 1. The matrix effect was calculated by comparing the slope

Table 2. Recovery and RSD Values Obtained for Fenbuconazole and Myclobutanil Enantiomers in Strawberry at Four Spiked Levels

		mean recoveries (%, $n = 5$)		RSD (%, $n = 5$)	
compd	spiked (μg/kg)	(+)-enantiomer	(−)-enantiomer	(+)-enantiomer	(−)-enantiomer
fenbuconazole	2	83.8	82.6	5.42	5.20
	5	88.2	85.9	3.15	2.71
	50	85.0	83.7	4.91	4.23
	500	90.4	91.5	3.67	3.86
myclobutanil	2	82.1	80.9	4.38	4.70
	5	89.3	88.6	2.87	2.32
	50	86.5	87.7	3.24	4.05
	500	91.2	92.9	2.68	3.01

Table 3. Pesticides Residues (μg/kg) and Enantiomer Fraction (EF) in Strawberry Harvested at Different Times[a]

	fenbuconazole			myclobutanil		
days	(+)-residues	(−)-residues	EF	(+)-residues	(−)-residues	EF
0	195.2 ± 13.9	198.2 ± 14.8	0.496 ± 0.001	332.2 ± 21.1	329.6 ± 18.8	0.502 ± 0.004
1	284.3 ± 19.9	279.9 ± 21.9	0.504 ± 0.002	446.3 ± 61.4	450.9 ± 57.7	0.497 ± 0.002
3	210.2 ± 12.6	203.4 ± 8.7	0.508 ± 0.004	280.1 ± 10.7	294.4 ± 18.5	0.488 ± 0.006
5	161.0 ± 20.2	163.3 ± 22.1	0.497 ± 0.002	181.5 ± 29.0	201.6 ± 30.2	0.473 ± 0.003
7	112.5 ± 6.9	111.6 ± 5.6	0.502 ± 0.003	119.9 ± 29.8	149.2 ± 39.0	0.446 ± 0.006
9	81.5 ± 11.5	81.9 ± 10.8	0.498 ± 0.003	68.0 ± 14.1	88.0 ± 17.5	0.436 ± 0.003
15	44.4 ± 7.8	45.2 ± 6.5	0.495 ± 0.009	25.7 ± 2.6	40.6 ± 2.8	0.387 ± 0.008
21	25.0 ± 3.8	24.7 ± 3.0	0.502 ± 0.008	9.8 ± 1.8	16.6 ± 2.9	0.371 ± 0.003

[a] Values represent the means ± SDs ($n = 3$).

of matrix-matched standard curve with the slope of the standard calibration curve.[21] The slope ratios of matrix-matched to solvent-based were 0.891, 0.890, 0.901, and 0.905 for (+)-fenbuconazole, (−)-fenbuconazole, (+)-myclobutanil, and (−)-myclobutanil, respectively, so there was a little matrix effect for fenbuconazole and myclobutanil determination with HPLC-MS/MS. To remove the matrix effect error, matrix-matched standards were used as calibration standards.

The recoveries and RSD values obtained in the validation portion of the study are shown in Table 2, and the results illustrate that the method was efficient and reliable to determine the enantiomers of two fungicides in strawberries with LC-MS/MS. The limit of detection (LOD) and the LOQ were defined as the concentration giving a signal-to-noise ratio of 3 and 10. In this article, the LOD and LOQ were estimated to be 0.6 and 2 μg/kg, respectively, for each enantiomer by the analyses of spiked sample containing fenbuconazole and myclobutanil at low concentration levels with five replicate extractions.

Application To Assess Dissipation Process in Strawberries. Table 3 showed the results of degradation and the enantiomeric fraction (EF) values. After treatment, the concentration of fenbuconazole and myclobutanil increased at the first day due to the absorption process and then declined. It was assumed that the degradation of the enantiomers in plant accorded with pseudofirst-order kinetics.[22] The half-life of pesticides in plant was an important indicator of pesticide efficacy and pollution.[23] The datum (from the first day to the 21st day) showed that degradation of two enantiomers of fenbuconazole and myclobutanil in strawberry followed pseudofirst-order kinetics, and the degradation rate constants k were

Table 4. Regression Functions for the Dissipation of Fenbuconazole and Myclobutanil Enantiomers in Strawberry[a]

compd	regression functions	R^2	half-life ($t_{1/2}$, day)[b]
(+)-fenbuconazole	$C_t = 288.2\ e^{-0.1218t}$	0.9749	5.69 ± 0.16
(−)-fenbuconazole	$C_t = 284.9\ e^{-0.1210t}$	0.9769	5.73 ± 0.14
(+)-myclobutanil	$C_t = 472.9\ e^{-0.1908t}$	0.9758	3.63 ± 0.20
(−)-myclobutanil	$C_t = 468.6\ e^{-0.1631t}$	0.9770	4.25 ± 0.25

[a] The regressive functions were obtained based on the mean value of three replicates. [b] Values represent the means ± SDs ($n = 3$).

calculated from eq 1 by regression analysis (Excel 2003, Microsoft), and the half-life ($t_{1/2}$, day) was estimated from eq 2.

$$C = C_0 e^{-kt} \quad (1)$$

$$t_{1/2} = \ln 2/k = 0.693/k \quad (2)$$

EF was used as a measure of the enantioselectivity of the degradation of enantiomers in strawberry plants. Using EF to represent the enantioselectivity was more meaningful than using conventional enantiomeric ratio (ER).[24] EF is defined by eq 3. The EF values ranged from 0 to 1, with EF = 0.5 representing the racemic mixture.

$$\text{EF} = \text{peak areas of the (+)-enantiomer}/[(+)\text{-enantiomer} + (-)\text{-enantiomer}] \quad (3)$$

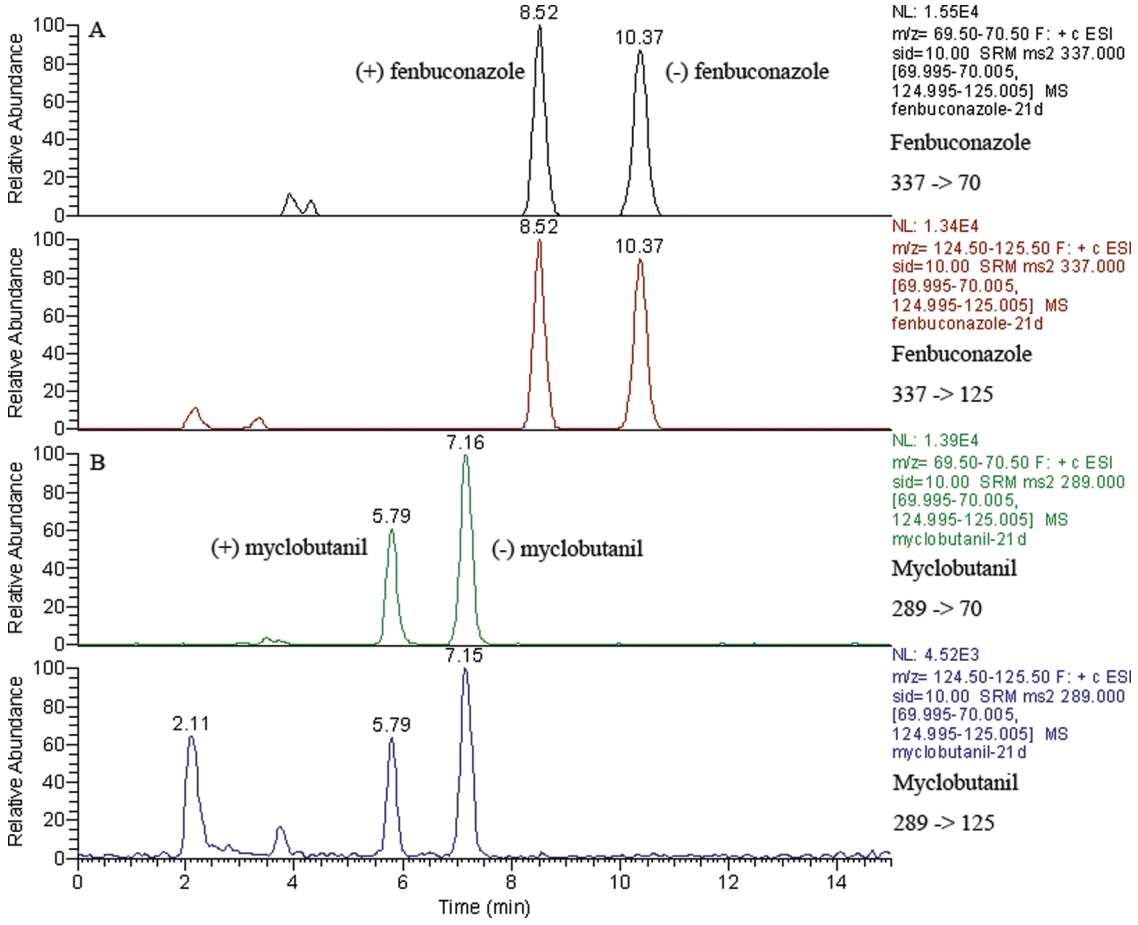

Figure 4. Representative chromatograms of extracts from strawberry harvested (A) fenbuconazole at the 21st day and (B) myclobutanil at the 21st day.

The degradation kinetics and half-lives of enantiomers are shown in Table 4.

The EF value of myclobutanil (Table 3) in test strawberry was nearly 0.5 at the first day treatment and decreased to 0.371 at the 21st day after treatment as time elapsed. The data of half-life (Table 4) showed that (+)-myclobutanil degraded faster than (−)-myclobutanil in strawberry, which resulted in strawberry enriched with (−)-myclobutanil. However, the differences of the enantioselective degradation behavior of fenbuconazole among the treated strawberry were indistinctively. The EF value of fenbuconazole (Table 3) in all of the treated strawberry was approximate 0.5. The data of half-life (Table 4) showed that two enantiomers of fenbuconazole disappeared at similar rates in strawberry. Figure 4 showed the chromatograms of the fenbuconazole and myclobutanil enantiomers in real strawberry sample harvested at the 21st day.

After the *t* test (Excel 2003, Microsoft), there was a difference for myclobutanil enantiomers in the degradation half-lives on strawberry ($p < 0.05$, Student"s paired *t* test), so enantioselectivity happened, while no obvious enantioselectivity existed in fenbuconazole enantiomers degradation behavior in strawberry.

Many chiral pesticides degrade enantioselectively in the plants,[22,23,25−30] and different fipronil enantioselectivity between Chinese cabbage[28] and water hyacinth[29] was reported. The *R*-enantiomer degraded faster than the *S*-enantiomer in Chinese cabbage. On the contrary, *S*-enantiomer degraded faster in water hyacinth. Enzyme systems in plant may play an important role in the enantioselective metabolism of many chiral compounds. Whether there is enantioselective behavior during the process of pesticide degradation in plants may be related to metabolic enzymes. Because of the current lack of knowledge concerning the enzyme systems in strawberry for myclobutanil biotransformation, the rational explanation for enantioselectivity of myclobutanil required the further investigation. This result could provide a reference to enantioselectivity study of these two fungicides on plants and further food safety evaluation.

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