Stereoselective Degradation of Diclofop-Methyl During Alcohol Fermentation Process

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ABSTRACT Stereoselective degradation of Diclofop-methyl (DM) has been found in alcohol fermentation of grape must and sucrose solution with dry yeast. A method was developed for separation and determination the two enantiomers of DM during the fermentation process by high-performance liquid chromatography based on cellulose tri-(3,5-dimethylphenyl-carbamate) chiral stationary phase. The results showed that the enantiomers of DM degraded following the first-order kinetics in the sucrose solution and the degradation of DM enantiomers in grape must were biphasic (slow-fast-slow process). In the sucrose solution, half lives of (+)-(*R*)-DM and (−)-(*S*)-DM were calculated to be 8.5 h and 3.1 h, respectively. In the grape must, half life of (+)-(*R*)-DM was calculated to be 41.7 h while (−)-(*S*)-DM was 16.0 h. The result was that (−)-(*S*)-enantiomer degraded faster than the (+)-(*R*)-enantiomer in both alcohol fermentation. The results also showed that the differences of the enantioselective degradation of DM depended on the fermentation matrix. DM was configurationally stable in fermentation, showing no interconversion of (−)-(*S*)- to (+)-(*R*)- enantiomer, and vice-versa. *Chirality 23:424–428, 2011.*

KEY WORDS: chiral pesticide; enantioselective degradation; diclofop-methyl; fermentation

INTRODUCTION

Fermented food is prepared by microorganisms (or enzymes), which can alter the properties of the food (e.g., wine or bread) and it plays an important role in our daily life.[1] It is recognized as a kind of safe and nutrient foodstuff.[2] However, with wide usage of organic agrochemicals in agriculture, the material of fermented food has been contaminated with residues of pesticides.[3,4] Typically, in the vineyard, because of the occurrence of ruderal, vermin and epiphyte, pesticides are used to control the pest and reduce economic loss.[5,6] In those cases, these pesticides may be present in the grapes used for the wine-making process and even in the final wine.[7,8] In the previous studies, the presence of pesticides was associated with stuck and sluggish fermentations and also with problems in malolactic fermentation.[9–11] In this case, the persistence of pesticide residues in the wine might affect the heath of the consumers.[12] Considering this problem, more and more laws and regulations have been established to limit the residues in wines and other fermented food. Moreover, rising studies have been carried out to study the detection method for the residues and the metabolites of these pesticides.[13–17] Unfortunately, these studies ignored the chirality of some pesticides and the potential risk resulting from the chiral enantiomers.

It is well known that more than 25% of the frequently used pesticides are chiral and they consist of one or more pairs of enantiomers.[18] Usually, most of them are treated as one compound and used as racemic form or a mixture of enantiomers. The case also occurs in most analysis of pesticide residues. However, a number of works have shown that the enantiomers of these pesticides have different behaviors in bioactivity, toxicity, metabolism and degradation.[19–22] Therefore, it is necessary to take chirality into account during evaluating the risk of pesticide residues. Nevertheless, in the past few years, no work has been reported on the stereoselective degradation of the chiral pesticides during wine-making process.

Diclofop-methyl (DM), methyl-(*RS*)-2-{4-(2,4-dichlorophenoxy)phenoxy} propionate, which contains a chiral carbon in the molecule as shown in Figure 1, is a herbicide in phenoxy propionate group.[23] Its absolute configuration was confirmed with (+) rotation of the *R*-enantiomer and (−) rotation of the *S*-enantiomer by previous study.[24] It has been reported that (+)-(*R*)-DM showed significantly higher herbicidal activity by foliar application than the (−)-(*S*)-enantiomer, but less difference by soil application.[23] The two enantiomers of DM was also reported that (−)-(*S*) enantiomer of DM was similar to or higher than the (+)-(*R*) form in toxicity to algae, depending on specific species.[21]

In this study, a simple method was established to research the stereoselective kinetics of DM during the alcohol fermentation process caused by dry yeast. A developed model of degradation was applied in the fermentation and the enantiomers of DM were separated and determined by high-performance liquid chromatography-chiral stationary phase (HPLC-CSP) technology. Grape must and sucrose solution were chosen as two different kinds of matrix to investigate the enantioselective degradation of DM during the alcohol fermentation process. Chiral stability of the two enantiomers of DM was also studied in both kinds of matrix.

MATERIALS AND METHODS

Chemicals and Regents

Aether, sodium chloride, Anhydrous sodium sulfate and Sucrose (analytical grade) were purchased from commercial sources. n-Hexane (HPLC grade) and 2-propanol (HPLC grade) were obtained from Fisher Scientific

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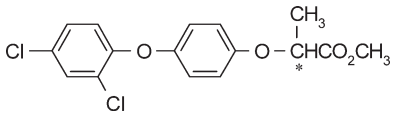

Fig. 1. The enantiomers of Diclofop-methyl (* Chiral center).

(Fair Lawn, NJ). Rac-DM (>99.0% purity) was provided by the China Ministry of Agriculture Institute for Control of Agrochemicals. The two enantiomers of DM were prepared by HPLC with a preparative column based on cellulose tri-(3,5-dimethylphenyl-carbamate) chiral stationary phase (CDMPC-CSP), and the enantiomeric purities of (+)-(*R*)-enantiomer and (−)-(*S*)-enantiomer were 98.5 and 99.0%, respectively.

Grapes and Dry Yeast

The test grapes were purchased from a vineyard in Beijing (China), and were not treated with DM in the last 3 years. The dry yeast was provided by Angel Yeast, and it is highly active devoted to wine.

Grape must Fermentation Experiment

To reach a proper activity, the dry yeast was activated in a 5% sucrose solution (m/m) at 37°C for 30 minutes. First-class grapes were selected and washed with pure water. When the surface was dry, the grapes were flayed and crushed by a juice extractor. After 24-h centrifugation with cold settlement, pellucid must was obtained and then sterilized by pasteurization. DM was added into the grape must with a concentration of 5 mg L^{-1}, according to the total volume of the must. Oscillate in order that DM was dispersed evenly throughout the grape must.

In Wang's study,[25] at different time intervals, the concentrations of the added pesticide changed irregularly because of the volatilization of the must and the produce of bubbles during the fermentation. To avoid these cases and get a true rate of degradation of the two enantiomers of DM, exact amount (15 mL) of the must spiked with DM was respectively loaded into a 50 mL hermetic fermenter (each fermenter for one sampling point). At last, the must was inoculated with activated yeasts in a ration of 0.5 g yeast per 100 g must to initiate the alcoholic fermentation. All the samples were stored at 25°C.

Single enantiomers assay was performed to study whether there were any configuration reversal of the two enantiomers during the fermentation process. Other treatments were the same as described above with the two enantiomers of DM were separately added into the grape must at a concentration of 5 mg L^{-1}.

Contrast experiments were set without addition of DM while other operations were carried out the same condition.

Sucrose Solution Fermentation Experiment

The other experiment was carried out similarly to the procedures mentioned above. The only difference is that we used a 20% sucrose solution (m/m) instead of the grape must to make a comparison of the two kinds of matrix. Meanwhile, the contrast experiments were set. Another contrast experiments were set without addition of the yeast while other operations were the same.

Sample Preparation

The analyzed samples were collected at different time intervals after the addition of the yeast and immediately stored at −20°C to interrupt the fermentation process. Each time, three fermenters were collected from each treatment as repeat. For extraction, the sample was transferred into a 50 mL polypropylene centrifuge tube. Wash the fermenter with total 20 mL aether to eliminate residues for three times. Combine the aether with the analyzed sample in the centrifuge tube. Then 2 g sodium chloride was added to promote the stratification of the must and aether. The tube was stirred for 4 minutes on a vortex mixer and centrifuged at 2425*g* for 3 minutes. The extraction was repeated three times, and the extracts of aether were passed through a funnel with about 10 g anhydrous sodium sulfate to a pear-shaped flask. At last, 10 mL aether was used to wash the anhydrous sodium sulfate. Remove most of the aether by vacuum evaporation at 45°C to about 1 mL and evaporate under a stream of nitrogen.

A solid-phase-extraction method was introduced to clean up interfering substances. The column (Silica, 500 mg, 6 mL) was preconditioned by rinsing with 5 mL ethyl acetate and then 5 mL n-hexane and equilibrated with 10 mL mixture of ethyl acetate and n-hexane (1/20, v/v). The sample of dry extract was dissolved in 3 mL mixture of ethyl acetate and n-hexane (1/20, v/v), and then the solution passed through the equilibrated SPE column by gravity. The column was eluted with additional 10 mL mixture of ethyl acetate and n-hexane (1/20, v/v). All of the eluates was collected in a glass tube and evaporated to dryness under a stream of nitrogen at 40°C. In the end, the extracts were dissolved with 1.0 mL of 2-propanol for later HPLC analysis.

Apparatus and Chromatographic Conditions

The HPLC system for this study was Agilent 1200 series HPLC equipped with a G1322A degasser, G1311A pump, G1329A column compartment, UV detector and a 20 μL sample loop (Wilmington, DE). AT-930 heater and cooler column attemperator (Titanjin Automatic Science Instrument, China) was used to control the column temperature. The signal was received and processed by an Agilent Chemstation. To separate the enantiomers of DM, a chiral column based on CDMPC-CSP was used. The CDMPC-CSP was prepared according to the literature and packed into an expert column [150 mm × 4.6 mm (I.D.)]. The chromatographic separation was conducted at 20°C. The mobile phase applied was a mixture of n-hexane and 2-propanol (96:4, v/v), a flow rate at 0.5 mL min^{-1} and a detection wavelength at 230 nm. Inject volume was 20 μL. In this condition, the *rac*-DM was separated into (−)-(*S*) and (+)-(*R*)-enantiomers while the first eluted enantiomer was (−)-(*S*)-form, and the second one was (+)-(*R*)-form, according to the previous study.[26]

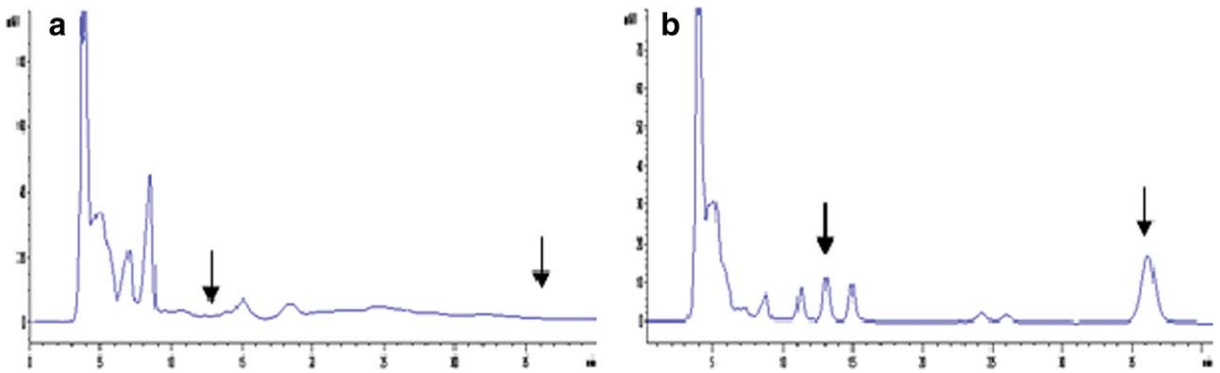

Fig. 2. Representative chromatograms of extracts from (**a**) free grape must after 48 h, (**b**) grape must fortified with *rac*-DM after 48 h treatment. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

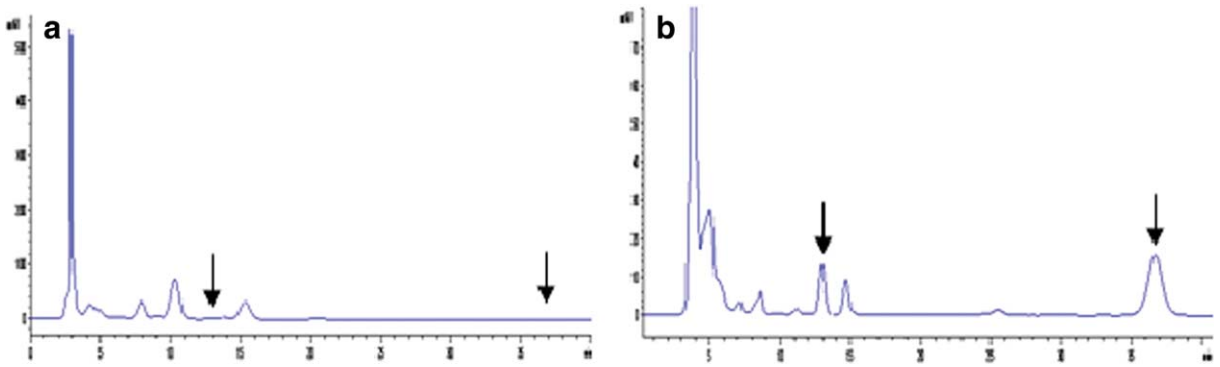

Fig. 3. Representative chromatograms of extracts from (**a**) free sucrose solution after 12 h, (**b**) sucrose solution fortified with *rac*-DM after 12 h treatment. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Calibration Curves and Assay Validation

A series of *rac*-DM standard solutions (0.2, 1, 5, 10, 50, and 100 mg L^{-1} each enantiomer) for linearity of the two enantiomers were prepared by diluting the stock standard solution with 2-propanol and inject volume was 20 μL. Calibration curves were generated by plotting the peak area of each enantiomer versus the concentration of the enantiomer. The standard deviation (S.D.) and the relative standard deviation (R.S.D.) [R.S.D. = (S.D./mean) × 100%] were calculated at the entire calibration range using Microsoft Excel.

Recovery estimate was conducted at three levels of concentration at different time intervals of the fermentation. For the recovery experiment, extra samples were incubated at the same condition as the experiment mentioned above. The standard solution were added into the contrast samples of grape must and 20% sucrose solution and gotten final concentrations equivalent to 0.2, 1, and 5 mg mL^{-1}, respectively. The recovery of the method was estimated by comparing the ratio of the peak area of each enantiomer extracting from the samples to the standard solution of an equivalent amount. The lowest possible standard on the calibration curve was accepted as the limit of quantitation (LOQ). The calibration curves and recovery validation studies were all repeated three times ($n = 3$).

The enantiomer fraction (EF) was used to measure the enantioselective degradation of DM enantiomers in the fermentation process. Using EF to represent the stereoselectivity was more meaningful and exact than using conventional enantiomeric ratio (ER). EF was defined as following equation:

$$\mathrm{EF} = \text{peak areas of the } (-)/[(-) + (+)] \qquad (1)$$

The EF value ranges from 0 to 1 and the racemate represents EF = 0.5.

RESULTS AND DISCUSSION

Calibration Curves and Assay Validation

Good linear calibration curves were obtained over the concentration range of 0.2–100 μg g^{-1} ($n = 5$) for both (+)-(*R*)-DM ($y = 38.637\times+0.177$, $R^2 = 0.9992$) and (-)-(*S*)-DM ($y = 38.653\times+0.154$, $R^2 = 0.9998$). The mean recoveries of the two enantiomers from the two kinds of matrix were determined at different time intervals at three fortification levels. At different time intervals, recoveries for grape must samples of *rac*-DM at 0.2, 1, and 5 μg g^{-1} ranged from 78.4% ± 1.3% to 86.7% ± 2.2%, and for sucrose solution ranged from 81.7% ± 2.2% to 88.3% ± 3.4%. The limit of quantification (LOQ) for both enantiomers in all samples was found to be 0.4 mg L^{-1}. The limit of detection (LOD) for both enantiomers in the two kinds of matrix was 0.1 mg L^{-1}. The two enantiomers were separated completely and there were no endogenous interference peaks eluted at the same retention times of the two enantiomers (Figs. 2 and 3). So this method applies to detect the two enantiomers of DM in the grape must and sucrose solution during the whole fermentation process.

Biodegradation of DM in the Grape must Fermentation Process

The concentration of the two enantiomers of DM decreased with the time elapsed. However, different concentrations of the two enantiomers of DM were detected in the same sample point after adding yeast, that was the concentrations of (+)-(*R*)-DM much higher than (−)-(*S*)-DM. Figure 4 showed the concentration of the two enantiomers during the process of the alcohol fermentation. The degradation of (+)-(*R*)-DM and (−)-(*S*)-DM was biphasic (slow-fast-slow process). Thus, the three-order polynomial regression analysis model quoted by Zhu was used to describe the degradation process for DM during the grape must fermentation process.[27] Curves of concentration in grape must (C(t), mg L^{-1}) versus incubation times (t, days) were regressed by eq. 2 (Excel 2003, Microsoft), A_0, A_1, A_2, and A_3 are special constants. Based on the definition of the half-life ($T_{1/2}$, hour), when the t was $T_{1/2}$, the $C(t)$ was half of A_0 ($A_0/2$). So we got eq. 3 and half-lives were calculated according to eq. 3.

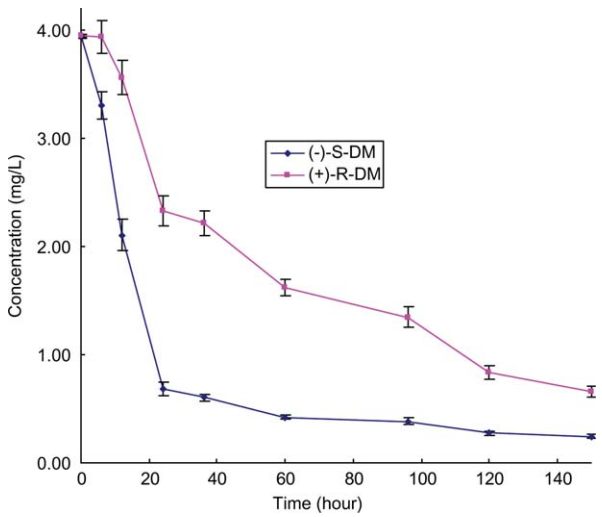

Fig. 4. Degradation linear (concentration versus time curves) of DM enantiomers in the grape must. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1. Regressive functions of two enantiomers in test matrices

Matrix	Enantiomer	Regressive functions[a]	R^2	Half-life (hours)[b]
Grape must	(+)	$C(t) = -2E\text{-}06t^3 + 0.0007t^2 - 0.0753t + 4.1359$	0.9742	41.7
	(−)	$C(t) = -6E\text{-}06t^3 + 0.0017t^2 - 0.1452t + 3.8255$	0.9517	16.0
Sucrose solution	(+)	$C(t) = 5.0368e^{-0.0817t}$	0.9404	8.5
	(−)	$C(t) = 4.3543e^{-0.2211t}$	0.9715	3.1

[a]The regressive functions were obtained based on the mean value of three replicates.
[b]Significant differences ($P < 0.05$, Student's paired *t*-test) are indicated with different alphabets in the same test material.

$$C(t) = A_1t^3 + A_2t^2 + A_3t + A_0 \quad (2)$$

$$A_1t^3 + A_2t^2 + A_3t + A_0' = 0 \quad (A_0' = A_0/2) \quad (3)$$

The degradation of (+)-(*R*)-DM and (−)-(*S*)-DM followed the eq. 2 with $R^2 = 0.9742$ and 0.9517, respectively (Table 1). Half life of (+)-(*R*)-DM was calculated to be 41.7 hours while (−)-(*S*)-DM was 16.0 h. Obviously, (−)-(*S*)-DM degraded faster than (+)-(*R*)-DM. A "t-test" was carried out to compare the means of the EF values in grape must with EF = 0.5. As shown in Figure 5, the EF values were observed to deviate from 0.5 ($P = 0.013$) and decreased from 0.50 (0 h) to 0.27 (150 h) which suggested there was a phenomenon of stereoselective degradation of the two enantiomers.

Biodegradation of DM in the Sucrose Solution Fermentation Process

Similar result was found in this experiment. As shown in Figure 6, the concentrations of both enantiomers were degressive and meanwhile at different time intervals, the concentrations of (+)-(*R*)-DM were also observed much higher than (−)-(*S*)-DM. First-order kinetics was assumed for the degradation of the two enantiomers in the fermentation process. Corresponding rate constants k for both enantiomers were determined by the linear range of logarithmic plots, concentration of (+)-(*R*) and (−)-(*S*) versus time t, respectively.

$$C(t) = C_0e^{-kt} \quad (4)$$

$$T_{1/2} = \ln 2/k = 0.693/k \quad (5)$$

The degradation of (+)-(*R*)-DM and (−)-(*S*)-DM followed the first-order kinetics with $R^2 = 0.9404$ and 0.9715, respectively (Table 1). Half life of (+)-(*R*)-DM was calculated to be 8.5 hours while (−)-(*S*)-DM was 3.1 h. Obviously, the degradation rate of (−)-(*S*)-DM was faster than (+)-(*R*)-DM. A t-test was carried out to compare the means of the EF values in sucrose solution with EF = 0.5. Figure 7 showed the EF values deviated from 0.05 ($P = 0.0001$). The EF value was decreased from 0.50 (0 h) to 0.04 (24 h) and this curve would be a good evidence to prove the stereoselective degradation of the two enantiomers.

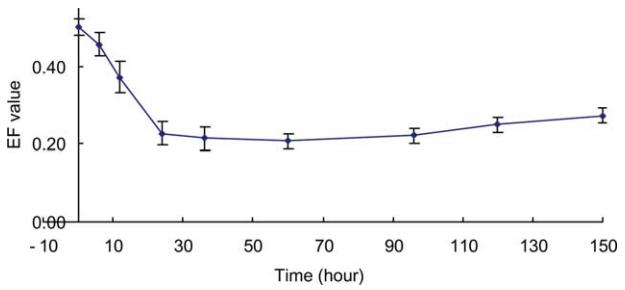

Fig. 5. EF value versus time curves of DM residues in the grape must. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Analysis of Stereoselective Degradation of DM in the Fermentation Process

DM was almost no degradation after 24 h in the sucrose solution without yeast. Compared with the case that contains yeast, it is not hard to find that the yeast plays an important role in the stereoselective degradation of DM. However, it needs further study to illustrate the primary cause of the stereoselective degradation.

Comparison of the Two Fermentation Process

The two enantiomers of DM behaved clearly stereoselective degradation during the alcohol fermentation in the two different kinds of matrix (grape must and sucrose solution). However, there are some striking dissimilarities between the two kinds of matrix due to the more complex system of the grape must than sucrose solution.

The degradation rate of DM had a remarkable distinction between grape must fermentation and sucrose solution fermentation. In the sucrose solution fermentation, the two enantiomers of DM degraded much faster than in the grape must fermentation. The half life of (+)-(*R*) in the grape must was 41.7 h while only 8.5 h in the sucrose solution. The half life of (−)-(*S*) in the grape must was 16.0 h while only 3.1 h in the sucrose solution. The reason would be that besides DM there was only sucrose could be utilized by the yeast in the sucrose solution while the grape must contain more carbon source. In this condition, DM would be used as an important carbon source to support the normal physiological activities in the sucrose solution while the grape must could provide varieties of carbon source. Much higher energy containing in the sucrose solution might be another reason for this phenomenon. The yeast might provide more energy for

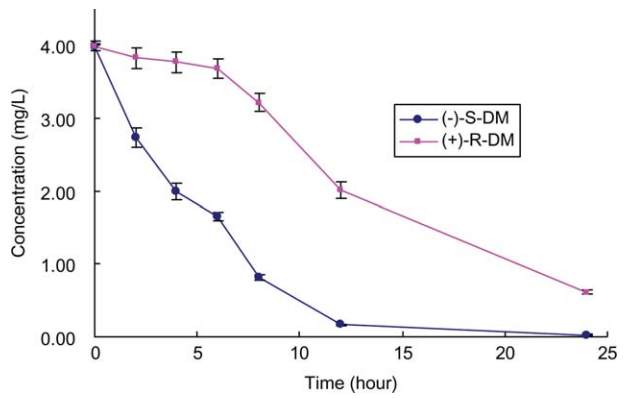

Fig. 6. Degradation linear (concentration versus time curves) of DM enantiomers in the sucrose solution. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

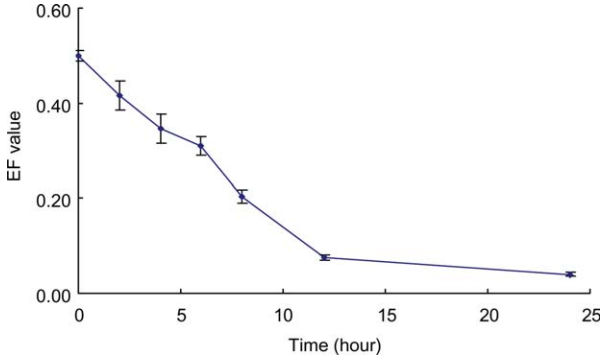

Fig. 7. EF value versus time curves of DM residues in sucrose solution. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

enzyme and get a more active state. In this state, DM would degrade faster than in grape must which contained less energy matter.

Diversities were observed from the curves of EF values in the two processes, too. In the sucrose solution, the EF values decreased from 0.50 (0 h) to 0.04 (24 h) continuously. However, in the grape must, the EF values reduced from 0.50 (0 h) to 0.21 (60 h); after this, the EF values presented a slight rise and got 0.27 at 150 h. This phenomenon might result in that the material system of the grape must was more complex than the sucrose solution. The material system did not only affect the physiological activity of the yeast but also had some actions on the pesticides.

Chiral Stability of DM in the Fermentation

Chiral stability suggests that there are no enantiomerizations of the two enantiomers during the degradation process. Simply, there is no transformation of (*S*)-enantiomer to (*R*)-enantiomer or vice versa. The two enantiomers of DM were separately studied in the two kinds of matrix in the experiment mentioned above. The trials detected the enantiomers until they degraded completely, but still no enantiomerizations were found in all trials. Based on these results, a conclusion could be drawn that enantiomerization did not occur in the enantioselective degradation of DM during the fermentation in both kinds of matrix. Further studies are required to illustrate the enantioselective mechanism of DM. The results may help to understand the detailed biological behavior of chiral pesticide better.

CONCLUSIONS

This is the first report on enantioselective degradation of chiral pesticides during the fermentation process. It indicates that enantiomers of chiral pesticides have different degraded behavior during fermentation. The result also calls for additional attention to pesticide residues on fermented food and new laws or rules that take diversities on enantiomers of chiral pesticides into consideration. Still, more research and detailed study are required in this field.

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