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Direct chiral separations of the enantiomers of phenylpyrazole pesticides and the metabolites by HPLC

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Abstract

The enantiomeric separation of the enantiomers of three phenylpyrazole pesticides (fipronil, flufiprole, ethiprole) and two fipronil metabolites (amide-fipronil and acid-fipronil) were investigated by high-performance liquid chromatography (HPLC) with a CHIRALPAK® IB chiral column. The mobile phase was *n*-hexane or petroleum ether with 2-propanol or ethanol as modifier at a flow rate of 1.0 mL/min. The influences of mobile phase composition and column temperature between 15 and 35°C on the separations were studied. All the analytes except ethiprole obtained complete enantiomeric separation after chromatographic condition optimization. Fipronil, flufiprole, amide-fipronil, and acid-fipronil obtained complete separation with the best resolution factors of 2.40, 3.40, 1.67, and 16.82, respectively, but ethiprole showed no enantioselectivity under the optimized conditions. In general, *n*-hexane with 2-propanol gave better separations in most cases. The results showed decreasing temperature and content of modifier in the mobile phase resulted in better separation and longer analysis time as well. The thermodynamic parameters calculated according to linear the Van't Hoff equation indicated the chiral separations in the study were enthalpy-driven. Fipronil and its two chiral hydrolyzed metabolites obtained baseline separation simultaneously under optimized conditions.

KEYWORDS

chiral separation, enantiomers, HPLC, phenylpyrazole pesticides

1 | INTRODUCTION

Chiral separations have been one of the major issues in the area of pesticides. The enantiomers of chiral pesticides usually exhibit similar physical and chemical properties but different biological behaviors.[1] Many chiral pesticides are manufactured and applied as racemates, and therefore equal amounts of enantiomers are released to the environment.[2] In order to study the stereoselective bioactivity, toxicity, and environmental behavior of chiral pesticides and determine the chiral purity of enantiomeric enriched products, there is an urgent need for developing chiral analytical methods.[3]

The choice of chiral stationary phases (CSPs) is undoubtedly the key point contributing to successful separation. CSPs based on the phenylcarbamates or benzoates derivatives, such as cellulose-tris(3,5-dimethyl-phenyl-carbamate) and amylose-tris(3,5-dimethyl-phenyl-carbamate), are the most widely used CSPs among the polysaccharide-based CSPs due to their excellent ability towards recognizing various chiral compounds.[4,5] Both coated and immobilized polysaccharide-CSPs have been developed. Both the CSPs with polysaccharide derivatives coated and immobilized on silica have been widely used, but the chiral recognition ability of immobilized CSPs is slightly reduced compared to the corresponding coated CSPs; however, the immobilized CSPs can be used under a wider range of mobile phases.[6] Polysaccharide-based chiral columns have been frequently used for analytical and preparative-scale separations of enantiomers in liquid chromatography and several related techniques, such as capillary electrochromatography, nano-liquid

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chromatography, and super-subcritical fluid chromatography.[7] High-performance liquid chromatography (HPLC) is now an effective and the most used method for individual enantiomer determination and preparation in small amounts.

Fipronil (*R,S*)-{5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile} (Figure1) is the first phenylpyrazole insecticide introduced for pest control, which is a γ-aminobutyric acid (GABA)-disrupting insecticide with low toxicity to mammals and selective toxicity to insects.[8] It is chiral, with two individual enantiomers (*S*-(+)) and (*R*-(-)) and they usually have different bioactivity or toxicity.[9] For example, the toxicity of the *S*-(+)-fipronil enantiomer to *Ceriodaphnia dubia* was 3-fold higher than that of the *R*-(-)-enantiomer.[10] For the control of certain pests such as mites, fleas, lice, etc., *S*-(+)-fipronil is more effective.[11]

Fipronil has been widely used for the protection of crops, such as cotton and rice. However, it is highly toxic to aquatic organisms, particularly crustaceans, and many insects have developed high resistance.[12,13] To reduce the resistance risk and the side effects to nontarget aquatic organisms, many fipronil derivatives have been synthesized and commercialized, such as flufiprole{1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-5-[(2-methyl-2 -propen-1-yl)amino]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile} and ethiprole {5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(ethylsulfinyl)-1*H*-pyrazole-3-carbonitrile} (Figure 1), which are also chiral.[12] Since the introduction of the modifying group, flufiprole and ethiprole have a lower toxicity or higher activity than fipronil.

In the environment, fipronil will generate several toxic metabolites, such as desulfinyl-fipronil, sulfide-fipronil, sulfone-fipronil, and amide-fipronil by reduction, oxidation, or photolysis after application (Figure 1).[14] Among them, amide-fipronil is the major one. The degradation of fipronil to amide-fipronil and acid-fipronil does not change the original chiral center in the molecules, so they are also chiral. The metabolites may prove to be of greater environmental risk than fipronil itself.

Desulfinyl-fipronil is more acutely toxic to rats than fipronil, with an LD_{50} value only 1/6 of fipronil, and the toxicity of sulfone-fipronil is about 6 times higher than fipronil to fleas.[15] The half-life of sulfone-fipronil is 25 times longer than fipronil in rabbits.[16] The LC_{50} of desulfinyl-fipronil to *C. dubia* is more than 20 times compared to fipronil.[10] HPLC is still the most effective method of chiral separation of phenylpyrazole pesticide currently. It has been reported that complete enantioseparation of flufiprole and ethiprole were performed using reversed-phase chromatography with a Lux Cellulose-2 column and Rs of flufiprole reached 7.65 under the optimized conditions.[17,18] But few literatures reported the chiral separation and the enantioselective toxicity of the chiral metabolites.

CHIRALPAK® IB is a polysaccharide-based chiral column with cellulose-tris(3,5-dimethyl-phenyl-carbamate) immobilized on the surface of silica gel and the corresponding coated column is CHIRALCEL OD. It has been reported that the chiral separations of the enantiomers of vinclozolin, betaxolol, metoprolol, bisoprolol, and bevantolol hydrochloride on CHIRALPAK® IB have been done by HPLC.[19–21]

In this work, the chiral separations of the enantiomers of three phenylpyrazole pesticides (fipronil, flufiprole, ethiprole) and two fipronil metabolites were studied by CHIRALPAK® IB chiral column on HPLC. The influencing factors such as the mobile phase composition and column temperature on the chiral separations were studied.

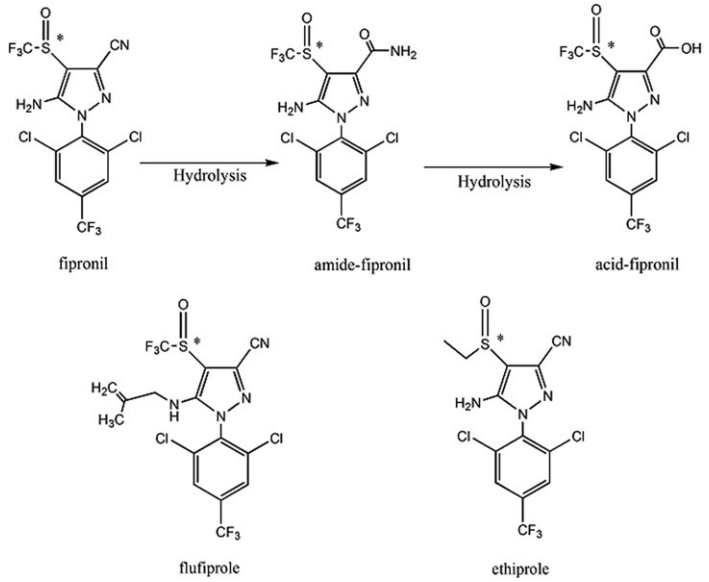

FIGURE 1 Chemical structures of the chiral compounds studied

2 | MATERIALS AND METHODS

2.1 | Materials

Separations were performed on CHIRALPAK® IB (250 mm × 4.6 mm I.D., particle size of 5 μm, Daicel Chemical Industries, Japan).

Fipronil, flufiprole, ethiprole (>95%) were provided by Institute for Control of Agrichemicals Ministry of Agriculture (Beijing, China) and Dalian Raiser Pesticides (Dalian, China). Amide-fipronil and acid-fipronil (>95%) were synthesized by the Lab of Pesticide Residual Analysis and Environmental Toxicology of China Agricultural University (Beijing, China). All reagents were of analytical grade (Beijing Chemical Reagents, Beijing, China). The eluents were distilled or filtered by 0.45-μm film before use.

2.2 | Instrumentation

Separations were performed with an Agilent 1200 Series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a G1322A degasser, a G1311A pump, a 20-μL sample loop, a G1378A injector, a G1316A column compartment, and a G1315B diode array detector. The signal received was processed by an HP1100 workstation.

2.3 | Chromatographic conditions

The mobile phase was *n*-hexane or petroleum ether, and 2-propanol or ethanol was added as modifier. The flow rate was 1.0 mL/min and injection volume was 20 μL. Chiral separations were performed at a temperature of 15–35°C with detection wavelength of 230 nm. All the sample solutions were separately prepared in 2-propanol at a concentration of 100 mg/L. An optical rotation detector was used to determine the elution orders.

The parameters of retention factor *k'*, separation factor α, and resolution factor Rs were calculated to evaluate the resolution.

3 | RESULTS AND DISCUSSION

3.1 | Chiral resolutions using *n*-hexane/2-propanol or ethanol as mobile phase

The enantiomeric separations of the enantiomers of fipronil, flufiprole, ethiprole, amide-fipronil, and acid-fipronil were performed using *n*-hexane as mobile phase with 2-propanol or ethanol as modifier at a flow rate of 1.0 mL/min at 30°C. Table 1 shows the resolution results, the effects of the percentage of 2-propanol and ethanol in the mobile phase on the resolutions, and the elution orders. Fipronil, flufiprole, amide-fipronil, and acid-fipronil reached complete resolutions with Rs >1.5, while the two enantiomers of ethiprole could not be separated using *n*-hexane as mobile phase with 2-propanol or ethanol as modifier.

The retention factors (*k'*) and resolution factor (Rs) increased with decreasing content of 2-propanol or ethanol, indicating that a low percentage of modifier resulted in better resolutions and longer retention time as well. Fipronil, amide-fipronil, and acid-fipronil got better chiral separation using *n*-hexane/2-propanol, while the enantiomers of flufiprole got better resolution using *n*-hexane/ethanol. Compared to 2-propanol, ethanol decreased the retention of the enantiomers, resulting in narrow peaks.

For the chiral separation of fipronil, the best resolution was obtained with Rs of 2.3 using 5% 2-propanol and complete resolution was also obtained using 5% of ethanol. The two enantiomers of flufiprole could be separated completely using 2-propanol or ethanol less than 15%, and the best Rs was 3.4 using 5% of ethanol. Among these samples, acid-fipronil exhibited the best resolution on the column, with an Rs value of 11.7 using 10% 2-propanol in *n*-hexane and the two enantiomers could be easily separated. Amide-fipronil enantiomers showed long retention on the column, and the peaks were relatively wide. The two enantiomers obtained complete resolution using 5% 2-propanol or ethanol. The typical chromatograms for the chiral separations are shown in Figure 2.

TABLE 1 Chiral resolutions using *n*-hexane with isopropanol (IPA) or ethanol

Sample	IPA (%)	k_1	k_2	α	Rs	Ethanol (%)	k_1	k_2	α	Rs	Pk1/Pk2
Fipronil	20	0.61	0.75	1.24	1.03	20	—	—	—	—	–/+
	15	0.97	1.20	1.24	1.41	15	0.60	0.68	1.13	0.65	
	10	1.80	2.25	1.25	1.94	10	1.09	1.24	1.14	1.18	
	5	4.55	5.74	1.26	2.30	5	2.67	3.14	1.18	1.84	
Flufiprole	20	0.44	0.56	1.28	1.01	20	0.70	0.92	1.33	1.35	+/–
	15	0.63	0.83	1.32	1.71	15	0.65	0.86	1.32	1.72	
	10	0.99	1.38	1.40	2.64	10	1.06	1.44	1.36	2.68	
	5	3.12	3.90	1.25	1.91	5	2.22	3.07	1.38	3.40	
Acid-fipronil	20	0.52	1.95	3.73	7.09	20	0.35	0.63	1.83	2.02	–/+
	15	0.77	3.30	4.29	8.87	15	0.57	1.06	1.87	3.21	
	10	1.57	7.83	4.99	11.73	10	0.97	2.02	2.07	5.44	
Amide-fipronil	20	0.63	0.80	1.27	1.04	20	0.52	0.60	1.16	0.58	–/+
	15	1.06	1.34	1.26	1.31	15	0.62	0.76	1.22	0.81	
	10	2.12	2.67	1.26	1.49	10	1.23	1.49	1.21	1.18	
	5	6.54	8.18	1.25	1.62	5	3.56	4.34	1.22	1.67	

Chromatographic conditions: *n*-hexane/isopropanol or ethanol, 1 mL/min, 30°C.

Pk1 and Pk2 mean the first and second eluted enantiomer.

As shown in Table 1, the elution orders of fipronil, amide-fipronil, and acid-fipronil were -/+, and that of flufiprole was +/-. The elution orders did not change with the modifiers and their percentage.

3.2 | Chiral resolutions using petroleum ether/2-propanol or ethanol as mobile phase

As the tendency of the effects of the volume fraction of 2-propanol or ethanol on the separations using petroleum ether as mobile phase at 30°C was the same as in Table 1, therefore we only kept the data relating to the best separation (with 5% modifier) in Table 2. Fipronil, flufiprole, amide-fipronil, and acid-fipronil could also reach complete resolutions, and the two enantiomers of ethiprole were still inseparable. As in the *n*-hexane mobile phase, the retention factors (*k'*) and resolution factor (Rs) increased with decreasing content of 2-propanol or ethanol. 2-Propanol gave a better chiral separation of acid-fipronil than ethanol, while no significant difference was found between 2-propanol and ethanol for the chiral resolution of fipronil, flufiprole, and amide-fipronil. For fipronil, petroleum ether demonstrated a slightly stronger separation capability than *n*-hexane and the enantiomers could be completely separated using 2-propanol or ethanol less than 10%. Very similar enantioselective ability and retentions were observed using the two mobile phases for acid-fipronil and amide-fipronil.

Many factors controlled the complex process of chiral recognition. Cellulose-tris(3,5-dimethyl-phenyl-carbamate) is a well-known polysaccharide-based CSP with carbamate derivatives. The binding of the two enantiomers and the carbamate groups commonly includes hydrogen bonding, π–π and dipole–dipole interactions. Furthermore, the process that each enantiomer enters the chiral cavity of the stationary phase in different ways is also a key factor. In this study, fipronil obtained a good separation, while ethiprole (a trifluoromethyl connected with the sulfinyl group was substituted by ethyl, shown in Figure 1) exhibited no enantioselectivity. The interaction between the trifluoromethyl group of fipronil and the chiral center of the CSP was mainly the H-F hydrogen bonding. The chiral resolution of the separated compounds may also be attributed to the different magnitude of hydrogen bonding between the two enantiomers and the CSP. In addition, the structures of all analytes are similar, but only acid-fipronil owns a hydroxyl, which will generate a strong hydrogen bonding with the carbonyl of CSP.

3.3 | Influence of temperature and thermodynamic parameters

Temperature is certainly an important factor both for optimization of enantioseparation and for reading the mechanisms

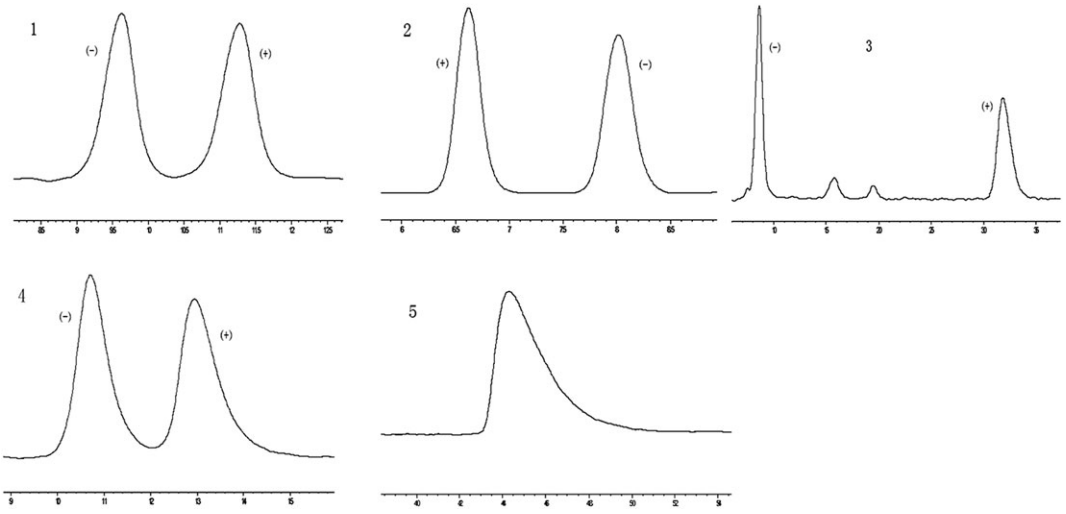

FIGURE 2 The typical chromatograms of the chiral separations of the enantiomers using *n*-hexane–2-propanol mobile phase at a flow rate of 1.0 mL/min. A, fipronil,10% 2-propanol, 25°C; B, flufiprole, 10% 2-propanol, 25°C; C, acid- fipronil, 10% 2-propanol, 25°C; D, amide-fipronil, 10% 2-propanol, 25°C; E, ethiprole, 5% 2-propanol,15°C

TABLE 2 Chiral resolutions using petroleum ether/isopropanol or ethanol (5%) as mobile phase

	IPA (5%)				Ethanol (5%)			
Sample	**k_1**	**k_2**	**α**	**Rs**	**k_1**	**k_2**	**α**	**Rs**
Fipronil	3.61	4.66	1.29	2.40	3.55	4.58	1.29	2.34
Flufiprole	3.01	3.67	1.22	1.61	3.02	3.68	1.22	1.74
Acid-fipronil	4.09	27.44	6.71	16.82	2.86	7.44	2.61	9.40
Amide-fipronil	5.78	7.34	1.27	1.56	3.32	4.07	1.23	1.67

Flow rate 1.0 mL/min; wavelength 230 nm; 30°C.

of enantiomeric recognition. The enantioselective separation was investigated with stepwise raising of the CHIRALPAK IB column temperature from 15 to 35°C in 5°C increments.

Table 3 lists the results and the chromatographic conditions. In general, low temperature resulted in better resolutions; however, longer retention times and wide peaks as well. The retention factor (*k'*), separation factor (α) and resolution factor (Rs) decreased with increasing temperature. Take amide-fipronil, for example: the separation factor (α) decreased from 1.37 to 1.24, and resolution (Rs) decreased from 1.70 to 1.16 when the temperature increased from 15°C to 35°C in the mobile phase of *n*-hexane/2-propanol (85/15). It should be noted that a lower temperature was not always conducive to separation. The best separation of acid-fipronil was obtained at 20°C with the highest Rs value of 10.3, instead of 15°C.

The solute retention and enantioselectivity obtained at different column temperatures have often been used to evaluate the contributions of enthalpy and entropy. The Van't Hoff equation can be used to calculate the thermodynamic data of enantioseparation, such as the standard enthalpy (ΔH) and entropy (ΔS) of transfer of the solute from the mobile phase to the chiral stationary phase:

$$\ln k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \Phi \quad (1)$$

$$\ln \alpha = \frac{-\Delta\Delta H^\circ}{RT} + \frac{\Delta\Delta S^\circ}{R} \quad (2)$$

where *k* represents the retention factor, *R* is the universal gas constant (8.3144 J/(mol·K)), *T* is the absolute temperature; ΔH and ΔS are the molar enthalpy and molar entropy of the adsorption; ΔΔH and ΔΔS are the differences ΔH_2–ΔH_1 and ΔS_2–S_1, and Φ is the column phase ratio. The slope and intercept are -ΔH/R and ΔS/R + lnΦ (ΔS*). For the linear plot of lnα versus 1/T, the slope and intercept are –ΔΔH/R and ΔΔS/R, respectively.

In this work, linear plots of ln*k* versus 1/T were obtained (linear correlation coefficient $R^2 > 0.97$) for all the target compounds, and the plots of lnα versus 1/T were also linear ($R^2 > 0.97$).

Van't Hoff plots were generated and the values of ΔΔH and ΔΔS were thus calculated. Table 4 shows the Van't Hoff plots and thermodynamic parameters for the chiral separations using *n*-hexane-IPA (85/15) as mobile phase. The linear plots indicate that these thermodynamic parameters are constant within the experimental temperature. No significant changes in the composition of the stationary phase, that is, the enantioselective mechanism remained unchanged.[22]

The values of ΔΔH and ΔΔS indicated that the adsorption process between solute and stationary phase was controlled by enthalpy. It should be noted that among all the chiral compounds studied, acid-fipronil exhibited the biggest absolute values of ΔΔH and ΔΔS, indicating the great difference between the two enantiomers for the interaction with the CSP, which was consistent with the fact that acid-fipronil had the best chiral separation on the column.

The force between the solute and stationary phase could be determined by the absolute value of ΔΔH. Chiral recognition is only related to the steric hindrance when the value is less than 0.1 kcal/mol. And as ΔΔH between 0.5 to

TABLE 3 Influence of temperature on the chiral separations with *n*-hexane/isopropanol 85/15

Sample	T (°C)	t_1	t_2	k_1	k_2	α	Rs
Fipronil	15	7.29	8.28	2.09	2.51	1.20	1.71
	20	7.01	7.93	2.12	2.52	1.19	1.64
	25	6.71	7.54	2.14	2.52	1.18	1.53
	30	6.50	7.27	2.30	2.69	1.17	1.46
	35	6.17	6.84	2.44	2.82	1.15	1.31
Flufiprole	15	8.02	9.71	3.09	3.95	1.28	2.30
	20	7.55	8.98	2.67	3.36	1.26	2.07
	25	7.22	8.46	2.70	3.34	1.24	1.91
	30	6.89	7.96	3.15	3.80	1.20	1.73
	35	6.52	7.40	3.37	3.97	1.18	1.44
Acid-fipronil	15	6.11	18.47	0.97	4.96	5.11	9.93
	20	5.99	16.64	0.91	4.31	4.73	10.32
	25	5.79	14.98	0.83	3.73	4.50	9.68
	30	5.61	13.64	0.77	3.30	4.29	8.87
	35	5.43	12.45	0.71	2.93	4.10	7.99
Amide-fipronil	15	7.52	9.07	1.31	1.79	1.37	1.70
	20	7.20	8.50	1.20	1.60	1.33	1.58
	25	6.95	8.07	1.12	1.47	1.30	1.50
	30	6.56	7.44	1.06	1.34	1.26	1.31
	35	6.24	6.70	0.99	1.23	1.24	1.16

Flow rate 1.0 mL/min; wavelength 230 nm.

TABLE 4 Van't Hoff equations and thermodynamic parameters for the chiral separations

Compound	lnk = −ΔH/RT + ΔS*	ΔH (kJ/mol)	ΔS*	lnα = ΔΔH/RT+ ΔΔS/R	ΔΔH (kJ/mol)	ΔΔS (J/(mol·K))
Fipronil	$\ln k_1$ = 1153.0/T-3.8461 R^2 = 0.9862	-9.6	-3.8	lnα = 78.299/T-0.0443 R^2 = 0.9797	-0.7	-0.4
	$\ln k_2$ = 1231.3/T-3.8904 R^2 = 0.9868	-10.2	-3.9			
Flufiprole	$\ln k_1$ = 1186.4/T-3.7884 R^2 = 0.9676	-9.9	-3.8	lnα = 319.14/T-0.7992 R^2 = 0.9823	-2.7	-6.6
	$\ln k_2$ = 1505.6/T-4.5876 R^2 = 0.9869	-12.5	-4.6			
Amide- Fipronil	$\ln k_1$ = 1227.7/T-3.9960 R^2 = 0.9973	-10.2	-4.0	lnα = 432.58/T-1.1886 R^2 = 0.9905	-3.6	-9.9
	$\ln k_2$ = 1649.8/T-5.1503 R^2 = 0.9990	-13.7	-5.2			
Acid-fipronil	$\ln k_1$ = 1387.3/T-4.8375 R^2 = 0.9965	-11.5	-4.8	lnα = 957.50/T-1.7020 R^2 = 0.9897	-8.0	-14.2
	$\ln k_2$ = 2344.9/T-6.5401 R^2 = 0.9993	-19.5	-6.5			

1.0 kcal/mol, the contribution of steric hindrance would be enlarged by weak interactions such as π–π interactions and hydrogen bonding. And when the value is greater than 1.0 kcal/mol, the more retained enantiomers would suffer π–π interactions or hydrogen bonding which lead to a strong chiral separation.[23] As Table 4 shows, the chiral recognition of all compounds in this experiment except ethiprole were affected by π–π interactions or hydrogen bonding, especially acid-fipronil.

4 | CONCLUSION

The chiral separations of the enantiomers of the chiral insecticides fipronil, flufiprole, ethiprole, and the two metabolites amide-fipronil and acid-fipronil were conducted on CHIRALPAK® IB using HPLC under normal phase conditions. Complete resolutions of the enantiomers of fipronil, flufiprole, amide-fipronil, and acid-fipronil were obtained. The eluted orders were determined by a CD detector. The chiral stationary phase showed a remarkable effect on the enantiomeric separation of acid-fipronil. The resolution of the enantiomers could be improved by optimization of mobile phase composition and column temperature. There was no great difference between *n*-hexane and petroleum ether for the chiral separations. A low concentration of polar modifier in mobile phase and low temperature would result in better separations but long retention and wide peaks as well. Similar separation results were obtained when using 2-propanol or ethanol as a modifier, but 2-propanol was significantly better than ethanol for the separation of acid-fipronil enantiomers. The effect of temperature indicated that the chiral separations of these compounds were controlled by enthalpy. Ethiprole could not be separated under the present conditions, but we found ethiprole obtained complete separation with (*R,R*)Whelk-O 1 and OD column under normal conditions based on our previous work, and the chromatographic conditions need to be further studied. This study realized the simultaneous baseline separation of fipronil and its two chiral hydrolyzed metabolites under optimized chromatographic conditions. In addition, it also provided basic data of chiral separation on several phenylpyrazole pesticide enantiomers, which was conducive to evaluate the environmental behavior of phenylpyrazole pesticides on an enantiomeric level.

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