ENVIRONMENTAL APPLICATIONS OF CHIRAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A commercial pharmaceutical analysis chiral method development kit (Chirex Column Kit A, Phenomenex) was used to analyze six pesticide stereoisomer mixtures. The pesticides were selected from the 1994-1995 National Water Quality Assessment Program (NAWQA) covering New Mexico and Texas. Three stereoisomer mixtures were separable on three different columns. The instrumental detection limit and detection linear range of chiral high performance liquid chromatography (HPLC) of these analytes were determined. The efficient extraction (> 90%) from soil of one chiral mixture was demonstrated. The resolution of a shorter kit column versus an analytical column was also compared.

Key words: *chiral, chromatography, HPLC, pesticide, stereoisomer*

INTRODUCTION

More than 1.1 billion pounds of pesticides are used in the United States each year to control weeds, insects, and pests in agricultural and non-agricultural settings (Barbash and Resek, 1996). The increasing use of synthetic organic pesticides raises many concerns about potential adverse effects on the environment and human health, and increases the importance of analyzing pesticides in the soil, water, and air (Barceló, 1991; Müller and Buser, 1995). To assess the environmental impact of pesticides and reduce the risk of public exposure, all aspects of pesticide chemistry need to be well understood and studied.

Approximately a quarter of the hundreds of pesticides in use are asymmetric or chiral (Milne, 1995). Chiral molecules may exist as mixtures of non-superposable mirror images or enantiomers. Enantiomers have different chemical and physical properties in asymmetric environments. The desired biological activity of a chiral pesticide enantiomer mixture may be limited to only one enantiomer, with the activity of the other enantiomer being less effective, inactive, or different (Ariens et al., 1988; Buser and Müller, 1995; Venis, 1982; Faller *et al*., 1991; Renner, 1996). For example, some bacteria only degrade one enantiomer of Mecoprop (*R*) (Tedd et al., 1994; Ludwig et al., 1992; Falconer et al., 1995; Iwata *et al*., 1998). The toxicity of the (*R*)-enantiomer of Fonofos, an organophosphorus pesticide, in mice is greater than the toxicity of its mirror image, (*S*)-Fonofos (Kurihara et al., 1997). Ignoring the existence of enantiomers can lead to incorrect toxicological, distribution, and degradation data. Ongoing research is aimed at identifying chiral pesticides, determining their distribution, and measuring their degradation.

Chromatography is the most common method for enantiomeric analysis. Gas chromatography (GC) is the primary method used in pesticide analyses (Grosser et al., 1993). However, high-performance liquid chromatography (HPLC) is more suitable for larger, non-

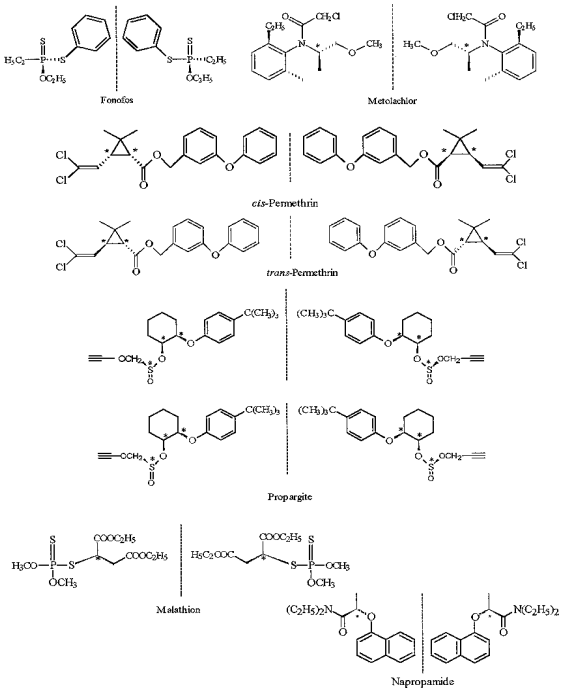

Figure 1. Structures of the stereoisomers of the six chiral pesticides from the NAWQA study of Texas and New Mexico.

volatile, polar, and thermally labile pesticides (Buser and Müller, 1995; Farran et al., 1996). Also, HPLC can tolerate large-volume injections of aqueous samples, rendering it ideal for screening water samples (Grosser et al., 1993). In fact, the number of official U.S. Environmental Protection Agency (EPA) methods using HPLC grew to more than 40 approved and draft methods by 1993 (Grosser et al., 1993).

The Rio Grande Valley study unit of the U.S. Geological Survey National Water Quality Assessment Program (NAWQA) conducted a study of the occurrence and distribution of pesticides in the surface water of our region of New Mexico and Texas (Healy, 1996). The study selected 40 pesticides and their metabolites on the basis of national usage, and the development and cost-effectiveness of analytical procedures. Seven of the 40 selected pesticides are chiral. The stereoisomer mixtures of Fonofos, Malathion, Metolachlor, Napropamide, Permethrin, and Propargite are shown in Figure 1.

Although the enantiomers of chiral pesticides may have different biological properties in the environment, the NAWQA study completely ignored stereochemistry. This may be due to either a lack of knowledge of the potential problem or a lack in methods or technology to analyze chiral pesticides. Current U.S. EPA methods (507 and 508) use GC to detect some of these pesticides in drinking water but do not separate stereoisomers of chiral pesticides. For our work we selected six chiral pesticides from the NAWQA study to demonstrate the application of chiral HPLC in pesticide analysis. Though one of the seven chiral pesticides studied in NAWQA, α-Lindane was excluded because our HPLC detector depends on UV (ultra violet-visible) absorption.

Since no single chromatography column can separate all compounds, to find suitable stationary phases and separation conditions can be expensive and time consuming. Thus we used the shorter columns of a commercial chiral HPLC method development kit to economically survey the efficiency of normal and reverse-phase systems. The survey was used to guide the purchase of more expensive and longer analytical columns.

We used Soxhlet extraction (EPA Solid Waste Test Methods, SW-846 Method 3540C (Understanding Environmental Methods, CD-ROM, 1998) to demonstrate the recovery and analysis of a chiral pesticide mixture from soil

samples. This method is applicable to the quantitative extraction of nonvolatile and semivolatile organic compounds from solids such as soil, relatively dry sludge, and solid waste in preparation for a variety of chromatographic procedures. Soxhlet extraction uses relatively inexpensive glassware, once loaded requires little hands-on manipulation, and provides efficient extraction. However, it uses fairly large volumes of solvent and is rather time consuming (16 to 24 hours). Combined with SW-846 Method 3540C, the chiral HPLC analysis methods presented are a beginning to study the different bioactivity, biodegradation, accumulation, and distribution of pesticide enantiomer pairs in soil, surface water, and groundwater.

EXPERIMENTAL

Materials

Racemates (1:1 enantiomer mixtures) of Metolachlor, Napropamide, and Permethrin were kindly provided by Norvatis (Greensboro, N.C.) and Zeneca (Richmond, Calif.). Mixtures of Fonofos, Propargite, and Malathion were purchased from Chem Service (West Chester, PA.). HPLC grade solvents (isopropanol, hexane, acetonitrile, and water) were obtained from Aldrich Chemical Co., Milwaukee, Wis. All materials were used without further purification.

Chromatography

Liquid chromatography was performed using a Spectra-Physics HPLC system consisting of a P2000 gradient pump and a UV2000 detector. Data acquisition, storage, and analyses were performed using Winner on Windows (WOW) software.

The Chirex™ Chiral Method Development Kit A (Phenomenex, Inc., Torrance, Calif.) used in this work contained five 50 x 3.2 mm normal and reverse-phase HPLC columns: Chirex™ 3001, 3005, 3010, 3014, and 3020. An additional Chirex™ 3001 analytical size (250 x 4.6 mm) column was also purchased. All HPLC injections consisted of 1µL into a 20µL loop.

Extraction and Concentration

The Soxhlet extractor (Kimax, Fisher Science) had a 40 mm ID with a 500-mL round bottom flask. Soil samples were contained in cellulose thimbles (Whatman, Fisher Science).

A Kuderna-Danish (K-D) apparatus was used to concentrate extracts. It included a 10-mL graduated concentrator tube, a 500-mL evaporation flask attached to the concentrator tube with springs, and a Snyder column. A solvent vapor recovery system (Kontes, Fisher Science) was attached to the top of the Snyder column to reduce emissions and minimize waste.

Methods

Preparation of standards

To prepare stock standard solutions, 25 mg of each pesticide were accurately weighed and dissolved in an appropriate solvent to make a 25-mL solution. The purity of each pesticide was 96% or greater; therefore, weights were used without correction to calculate the concentration of each stock standard solution. Methylene chloride was used to dissolve Napropamide, and hexane was used to dissolve

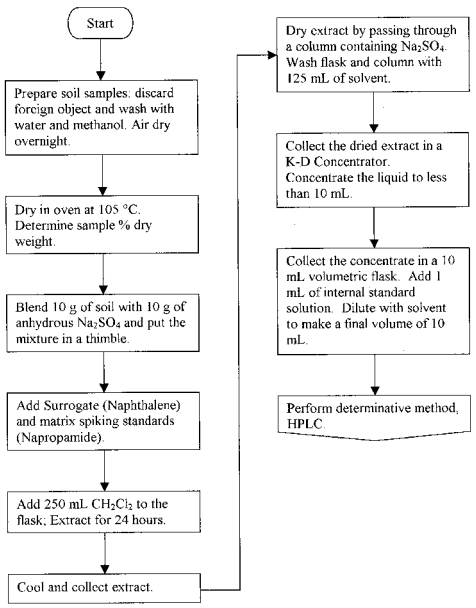

Figure 2. An outlined procedure of Soxhlet extraction of soil samples.

Fonofos, Malathion, Metolachlor, Permethrin, and Propargite. In the cases that separation of isomers was not observed by HPLC, no further standard solutions were made. Otherwise 10 mL of standard solutions of each pesticide in 0.1, 0.5, 1.0, 5, 10, 50, 100, 250, and 500 ppm concentrations were prepared from their stock solution for linear-detection range determination.

Instrumental detection limits (IDL)

The HPLC UV detector was set to a 210-nm wavelength for strong absorption by all six analyzed mixtures. For each standard solution, triplicate HPLC analyses were performed at room temperature.

Soil Recovery analysis

Local soil samples were conditioned according to EPA SW-846 Method 3500 (Understanding Environmental Methods, CD-ROM, 1998). Soil was collected, passed through a 100-mesh screen, washed with water and methanol, and air dried in a hood overnight. A 10-g soil sample was then accurately weighed and put in an oven overnight at 105 °C. The percent dry weight was determined by the following equation:

$$\%\ \text{dry weight} = \frac{\text{g of dry sample}}{\text{g of sample}} X 100\%$$

The oven-dried soil sample and 10 g of anhydrous sodium sulfate were mixed and put into a thimble. Soxhlet extraction of the soil sample was then performed as outlined in Figure 2.

For reasons to be discussed later, Napropamide was chosen for soil sample recovery analysis. A 50-mL spiking standard solution was prepared by dissolving 50 mg of pure Napropamide in methanol. The stock standard solution was stored in a Teflon-sealed container at 4 °C.

Since there is no recommendation for Napropamide analysis, naphthalene was chosen as a surrogate, a compound that is chemically similar to the analyte but is not expected to occur in an environmental sample. About 50 mg of pure naphthalene were added to a 50-mL volumetric flask and diluted with methanol to make a 50-mL surrogate standard solution. Adding 1.0 mL of the surrogate standard and 1.0 mL of spiking solution into the soil sample gave a final concentration of both surrogate and spiking standards of 100-mg/Kg soil.

This soil sample was extracted in a Soxhlet apparatus with 250 mL of methylene chloride

for 24 hours at four to six cycles/hour. After the extraction was completed, the extract was cooled and dried by passing it through a drying column containing about 10 cm of anhydrous sodium sulfate. The extractor flask and drying column were rinsed with a further 125 mL of methylene chloride. The dried extract was concentrated to less than 10 mL in a K-D apparatus in about two hours. No solvent exchange was performed. Once the K-D apparatus was cooled, the Snyder column was removed and an additional 2 mL of methylene chloride were used to rinse the flask. The concentrated extract and rinse were transferred to a 10-mL volumetric flask for HPLC analysis.

HPLC analysis

Phenanthrene (100 mg) was accurately weighed and dissolved in methylene chloride to make a 100-mL internal standard solution of 1000 mg/L concentration. To the concentrated soil extract was added 1.0 mL of internal standard solution. The final analysis solution was made up by adding methylene chloride to complete a 10-mL volume. A 1 µL aliquot of this solution was analyzed by chiral HPLC using

Table 1. Summarized results of the separation of pesticide stereoisomer mixtures using the chiral method development kit.

CHIREX COLUMN	FONOFOS	MALA-THION	METOL-ACHLOR	NAPRO-PAMIDE	PERMETHRIN	PROPARGITE
3001	No separation	No separation	No separation	α^a = 1.432 (30% ISP/H[b]; 0.5 mL/min)	2 peaks (α = 1.28) (0.25% ISP/H, 0.3 mL/min)	2 overlapped peaks (α=1.1) and 2 separated peaks (α=1.15) (0.25% ISP/H, 0.3 mL/min)
3005 (NP)[c]	No separation	No separation	No separation	No separation	2 overlapped peaks (α = 1.03) and 1 separated peak (0.25% ISP/H, 0.5 mL/min)	2 overlapped peaks (α=1.14) and 2 separated peaks (α=1.13) (0.5% ISP/H, 0.4 mL/min)
300[5] (RP)[d]	No separation	No separation	No separation	No separation	No separation	No separation
3010 (NP)[c]	No separation	No separation	No separation	α=1.25 (15% ISP/H, 0.5 mL/min)	*cis /trans* (α=1.11) (5% ISP/H, 0.5 mL/min)	2 overlapped peaks (α=1.34) (1% ISP/H, 0.5 mL/min)
3010 (RP)[d]	No separation	No separation	No separation	α=1.09 (95% acetonitrile/water, 0.4 mL.min)	No separation	No separation
3014	No separation	No separation	No separation	No separation	No separation	No separation
3020	No separation	No separation	No separation	No separation	*cis /trans* (α=1.19) (0.5% ISP/H, 0.4mL/min)	2 sets of overlapped peaks (α=1.16, 1.30) (0.5% ISP/H, 0.4 mL/min)

[a.] α: separation factor

[b.] Solvent used in normal-phase separation is isopropanol (ISP) in hexane (H). For example, 1% ISP/H represents 1% v/v of isopropanol in 99% of hexane.

[c.] NP: Normal-Phase Mode

[d.] RP: Reverse-Phase Mode

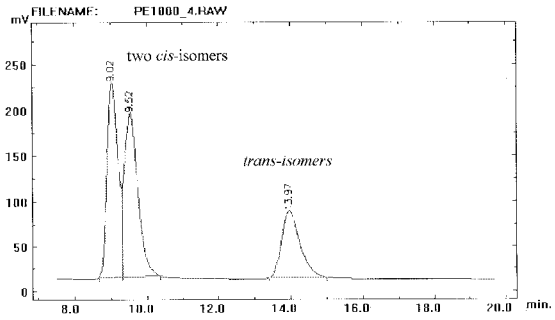

Figure 3. The chromatogram of the separation of Permethrin enantiomers on a Chirex™ 3005 column using 0.25 % isopropanol in hexane.

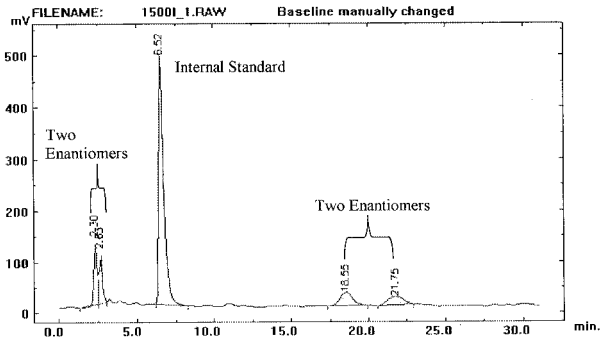

Figure 4. The chromatogram of the separation of Propargite enantiomers on a Chirex™ 3001 column using 0.3% isopropanol in hexane.

a Chirex™ 3001 analytical column with 30% isopropanol in hexane as the mobile phase.

RESULTS AND DISCUSSIONS

Six chiral pesticides were screened with the chiral method development kit to find the optimal separation conditions for each. The results are summarized in Table 1. Isopropanol and hexane were the only two solvents tried in normal-phase chromatography, and methanol and acetonitrile were used in reverse-phase systems. In all cases normal-phase chromatographic separation was more effective than reverse-phase. Different proportions of isopropanol to hexane played an important role in the separations, while flow rate had little effect.

From the reported diastereoisomer (non-mirror image stereoisomers) ratio (3:1) of Permethrin, the *cis*-enantiomers (Figure 1) were partially separated on the Chirex™ 3005 column using 0.25% isopropanol in hexanes. The *trans*-enantiomers were not separated, as shown in Figure 3.

Though Propargite has three chiral centers, only four stereoisomers were observed because of the fixed *trans* stereochemistry of its cyclohexane ring (Welch and Szczerba, 1998). The four *trans*-isomers (Figure 1) were separated on Chirex™ 3001 using 0.3% isopropanol in hexane as shown in Figure 4. Only one pair of Propargite enantiomers was baseline separated.

Napropamide enantiomers were baseline separated on both Chirex™ 3001 and 3010 columns, using isopropanol and hexane as elutes. Figure 5 shows the chromatogram of the resolution of Napropamide on a Chirex™ 3001 column using 30% isopropanol in hexane. Because this was the best separation observed in any system, Napropamide was chosen for our soil recovery studies.

As shown in Table 1, in our hands, Fonofos, Metalochlor, and Malathion could not be effectively separated with any of the columns of our commercial chiral method development kit. The observed degradation of Malathion further complicated the analysis of this sample as shown in Figure 6.

For the pesticides that could be partially or baseline resolved, the instrumental detection limit (IDL) was determined for the best-resolved peak as shown in Table 2. All calibra-

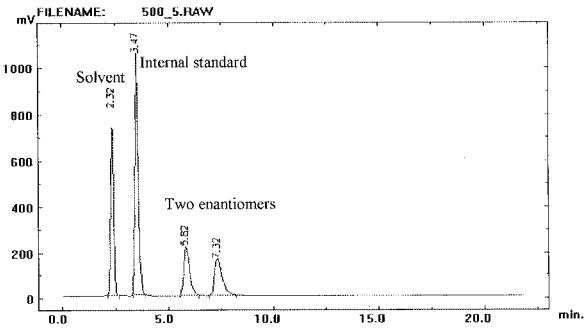

Figure 5. The chromatogram of the separation of Napropamide enantiomers on a Chirex™ 3001 (column kit) column using 30% isopropanol in hexane.

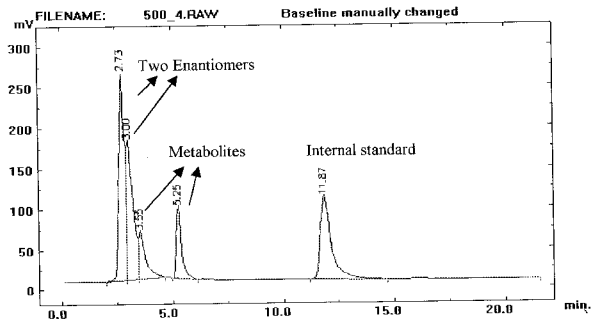

Figure 6. The chromatogram of the separation of Malathion enantiomers on a Chirex 3010™ column using 2% isopropanol in hexane.

tion curves of the pesticides had R^2 (R: correlation coefficient) values greater than 0.98.

To evaluate the performance of the total analytical process, a clean matrix, e.g. organic-free reagent soil, was spiked. Local soil was collected, washed with water and methanol, and air dried. Further oven drying only reduced the weight of the soil samples by 0.5%.

Because there was no suggested concentration or surrogate standard for Napropamide HPLC analysis by Method 3500, a 1-mL sample of 1000 mg/L (ppm) Napropamide stock solution and 1 mL of 1000 mg/L naphthalene surrogate standard solution were added to this soil sample. Naphthalene was selected as the surrogate because of its structural similarity to the analyte. The recovery of a surrogate can also be used to monitor unusual matrix effects and sample processing problems.

In general, Method 3540C, Soxhlet extraction, is considered the standard procedure for analyzing a broad range of solid samples and provides acceptable extraction efficiency for most analytes. Soil samples in this study were extracted for 24 hours. After concentrating the extracts, HPLC analysis was used to determine the amount of Napropamide recovered. The recovery rates of surrogate and Napropamide enantiomers were greater than 90%. The results are summarized in Table 3. Since the

Table 2. Instrumental detection limit (IDL) and linear range for three separable pesticide stereoisomer mixtures using a chiral method development kit.

Pesticides	IDL (ppm)	Linear Range (ppm)
Napropamide[a]	0.1	0.1-500
Permethrin[b]	50	50-500
Propargite[c]	1.0	1.0-500

a. 1st isomer peak Figure 5
b. *trans* isomer peak Figure 3
c. 3rd isomer peak Figure 4

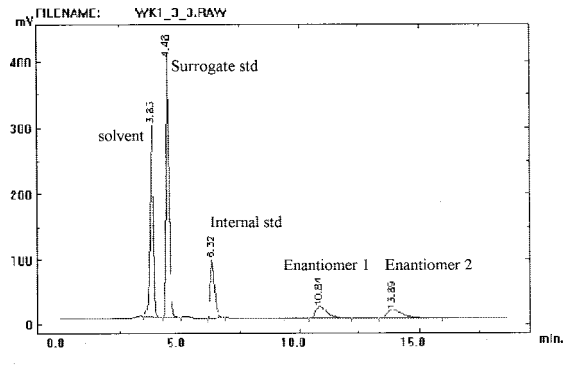

Figure 7. The chromatogram of Napropamide on an analytical Chirex™ 3001 using 30% isopropanol in hexane

recovery rate of the surrogate was greater than 94%, no unusual matrix effects were observed.

Two different sizes of Chirex™ 3001 columns were used to separate Napropamide enantiomers: a 50 x 3.2 mm (chiral method development kit, Figure 5) and a 250 x 4.6 mm (analytical column, Figure 7). The performance of both columns was compared based on their separation (α) and resolution (*Rs*) factors. These results are reported in Table 4.

CONCLUSIONS

A chiral method development kit was able to separate three of the six selected chiral pesticides. The enantiomers of Napropamide were baseline separated. Three peaks of the four Permethrin stereoisomers were observed. All four Propargite stereoisomers were separated. Along with finding the optimal separation conditions for three chiral pesticide stereosiomer mixtures, the instrumental detection limit and linear detection range were measured. The calibration curve within the linear detection range had an R^2 greater than 0.98.

As expected, the longer Chirex™ 3001 analytical column was more efficient than the shorter kit column in separating Napropamide but not to the extent of four times the cost. Nevertheless the chiral method development kit was effective in guiding the purchase of the analytical column for optimum performance.

EPA SW-846 Method 3540C was shown to be effective in recovering greater than 90% of Napropamide from spiked soil samples. Determination of the method detection limit (MDL) is still needed.

The methods developed in our work may be used to study the difference of enantiomeric bioactivities, biodegradation, accumulation, and distribution in soil, surface water, and groundwater, and to educate others on the importance of this problem.

Table 3. Recovery rates of Soxhlet extraction of surrogate and Napropamide enantiomers in soil samples.

	% Recovery[a]
Surrogate Standard	94.69 ± 4.41
Napropamide Enantiomer 1	92.46 ± 6.39
Napropamide Enantiomer 2	90.91 ± 5.61

a. 95% Confidence Level

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Table 4. Conditions, separation, and resolution factors for Napropamide resolution by short kit and analytical columns.

	Short Column	**Analytical Column**
Dimension	50 mm x 3.2 mm	250 mm x 4.6 mm
Chromatography Settings		
Mobile Phase	30% isopropanol in hexanes	30% isopropanol in hexanes
Flow Rate	0.5 mL/min	1.0 mL/min
Separation Factor (α)[a]	1.429	1.435
Resolution Factor (*Rs*)[b]	2.00	2.44

a. $$\alpha = \frac{T_{rB} - T_M}{T_{rA} - T_M}$$

T_{rA} and T_{rB} are the retention time of the two enantiomers, T_M is the retention time of the solvent front.

b. $$Rs = \frac{2(T_{rB} - T_{rA})}{(W_A + W_B)}$$

W_A and W_B are the width of the peaks of the two enantiomers.

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