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DEVELOPMENT AND OPTIMIZATION OF HPLC METHODS FOR THE CHIRAL SEPARATION OF ARYLPYRAZOLE, CHLOROACETANILIDE AND ORGANOCHLORINE PESTICIDES: CASE STUDIES OF BENZOBICYCLON, ACETOCHLOR AND CHLORDANE.

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ABSTRACT

This work is centered on the development and optimization of High-Performance Liquid Chromatographic methods for the separation of chiral pesticides, particularly benzobicyclon, chlordane and acetochlor employing Agilent Technologies Infinity II chiral HPLC system equipped with a UV – 2000 detector. The stereoisomers were successively separated using a standard analytical column of dimensions 250 mm X 4.6 mm. The Cis– stereoisomers of chlordane exhibited partial baseline separation while the Trans – enantiomers were fully separated. Benzobicyclon and acetochlor enantiomers attained full baseline separation under optimized conditions. Chlordane and benzobicyclon were analyzed using normal phase chromatography [NP]^c with a mobile phase of 90% Normal hexane and 10% Isopropanol, while acetochlor was analyzed by reverse phase chromatography [RP]^c using 70% methanol and 30% water. Benzobicyclon was later spiked into soil sample and subsequently extracted using Environmental Protection Agency (EPA) methods and the extracts were further analyzed with a shorter analytical column of dimensions 75 mm X 4.6 mm. The HPLC separation revealed that the longer column provided more efficient separation for the benzobicyclon stereoisomers than the shorter column as reflected by high separation factors (α). The high recovery rate of the stereoisomers of benzobicyclon and the internal standard from the soil extracts confirmed the effectiveness of the method of extraction and the HPLC system employed.

Key words: HPLC (High performance Liquid Chromatography), Chiral, enantiomers, benzobicyclon, chlordane, acetochlor

INTRODUCTION

High performance Liquid Chromatography (HPLC) is an important analytical technique in organic chemistry and environmental sciences, specifically for the separation of chiral molecules that are critical for analyzing complex mixtures. This study is designed to determine and optimize HPLC methods for the chiral separation of specific pesticides, with emphasis on benzobicyclon, chlordane and acetochlor. Benzobicyclon, an arylpyrazole herbicide is valued for its selective herbicide activity, thus making the chiral resolution of its stereoisomers essential for evaluating both its effectiveness and safety [1]. Chlordane, an organochlorine pesticide, is a persistent environmental contaminant with substantial health hazards, necessitating specific analytical methods to monitor its enantiomers [2]. Acetochlor, a member of the chloroacetanilide herbicides is extensively used to control weeds and precise chiral separation is essential to evaluate its herbicidal efficacy and environmental impact [3]. Furthermore, benzobicyclon was spiked into an organic- free soil sample, and the herbicide was recovered through extraction processes. The

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extracted sample was further subjected to HPLC chiral separation to assess the effectiveness of the chiral column employed in the analysis and to evaluate the recovery rate of the waste management method used [4]. This approach combined with the study's focus on chlordane, benzobicyclon and acetochlor, addresses the problems of selecting appropriate chiral stationary phases, optimizing mobile phase conditions and guaranteeing method validation for exactness and reproducibility [5]. The objective is to improve analytical methods for these pesticides, contributing to enhanced environmental safety and regulatory compliance by providing a better comprehension of their efficacy, environmental performance and persistence.

MATERIAL AND METHODS

The analysis of the pesticides were conducted using normal and reverse phase chromatography with HPLC. The UV detector was adjusted to 270 ± 5 nm for the separation of benzobicyclon and acetochlor enantiomers. While a wavelength range of 230 ± 5 nm was maintained for the separation of chlordane stereoisomers [6]. Soil recovery was assessed according to EPA solid waste recovery methods.

Chromatography

The analysis was conducted using an Agilent 1260 Infinity II Chiral HPLC system, which included a 2000 gradient pump and a UV – 2000 detector. Data acquisition, peak spectral storage and baseline modifications were performed by a software linked to the HPLC system. The analysis employed Chiralpak AD – H columns with particle sizes in the range of 3 - 5 µm and dimensions of 250 mm length by 4.6 mm internal diameter for both the normal and reverse column chromatography. A shorter analytical grade column of dimension 75 mm X 4.6 mm was employed in the separation of benzobicyclon extracts obtained from the spiked soil sample using the normal phase chromatography. A 5 µL injection was injected into a 20 µL loop for all tests [7]. Chlordane and benzobicyclon stereoisomers were separated using normal phase [NP] ^{c} chromatography and mobile phase mixture of 90% N-hexane and 10 % isopropanol in each case, while acetochlor enantiomers were separated using reverse phase chromatography [RP] ^{c} and mobile phase mixture of 70% methanol and 30% water [8]. All pesticides used in the analysis were concentrates and hence required no further purifications.

Extraction and Concentration of Soil Extract

50 g of loamy (agricultural) soil was accurately weighed by means of an analytical balance and sieved through a 100-mesh screen to remove bulky particles and clumps. The sieved soil was

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then washed with water and methanol. Thereafter, it was air dried in a hood overnight. The air dried soil was put in an oven overnight at a temperature of 105^{o}C. Benzobicyclon was chosen for the soil recovery analysis because it gave a good base-line separation in the chiral HPLC pesticide analysis. 10 mg of benzobicyclon was spiked into the soil sample and the mixture was homogenized. The spiked soil was placed in a 150 ml centrifuge tube, followed by addition of 100 mL of methanol. The sample was mixed thoroughly using a vortex mixer. Then, the mixture was centrifuged at 3000 – 5000 rpm for 15 – 20 minutes to isolate the liquid layer from the soil. The supernatant was filtered through a filtration unit furnished with 0.2 µm filter to eradicate any residual soil particles. The filtered extract was concentrated by means of a rotary evaporator. The extract was then stored in a clean, dry and chemically inert glass container, made air tight to inhibit evaporation and contamination. The stoppered glass container was preserved in a refrigerator at -20^{o}C to prevent degradation of the analyte [9].

Preparation of standard pesticide stock solution

In order to prepare standard stock solutions of the pesticides for HPLC analysis, 25 mg of each pesticide was accurately weighed using an analytical balance and transferred into a 25 mL volumetric flask. Each flask was carefully filled up to the 25 mL mark with methanol solvent, confirming complete dissolution of the compounds by graduallystirring and swirling the flasks. The solutions were then filtered using a 0.2 µm filter to remove particulates and stored in a cool, dark place to inhibit degradation [10]. This gave a standard concentration of 1 mg/mL for each pesticide. These solutions were further diluted prior to HPLC analysis [11].

Internal standard for pesticide analysis

Suitable reference compounds were chosen as internal standards for the HPLC analysis [12]. Triphenyl methane was selected as the internal standard for benzobicyclon HPLC analysis because it has comparable structure with the analyte, performs similarly in the chromatographic system and does not impede with the process [13]. 1 mg of triphenyl methane was accurately weighed and dissolved in 1 mg of methanol to produce a 1 mg/mL stock solution [14]. Hexachlorobenzene was selected as the internal standard for chlordane HPLC analysis due to its related chemical features and chromatographic performance with the analyte [15]. Both Chlordane and hexachlorobenzene are chlorinated which confirmed that they interact with the stationary phase in an analogous manner [16]. Hexachlorobenzene also gave a constant and reproducible signal which is essential for accurate quantification of chlordane. Its distinct peak which did not overlap with chlordane's peaks speeded up precise measurement [17].

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Similarly, 1 mg of hexachlorobenzene was precisely weighed and dissolved in 1 mL of propanone to prepare 1 mg/mL stock solution [18]. 1, 3 –diphenyl-2-propanol was adopted as the internal standard for the HPLC analysis of acetochlor because of its chemical properties and stability [19]. It has related retention characteristics or response factors as acetochlor under HPLC conditions which permits accurate quantification and comparison [20]. 1 mg of 1, 3 –diphenyl-2-propanol was accurately weighed and dissolved in 1 mL of methanol to prepare 1 mg/mL stock solution [21].In each case, the solutions were thoroughly homogenized and filtered to remove particulates [22]. The solutions were stored in a refrigerator at -20^{o}C to retain stability [23]. In order to prepare the calibration standards, the stock solutions were diluted to the desired concentrations and introduced into the sample matrix [24]. 1.0 mL of each internal standard solution was injected into 10 mL of each pesticide stock solution. A 5µL aliquot of each pesticide solution was then injected into a 20 µL loop for chiral HPLC analysis [25].

Internal standard for the soil extract

50 mg of 1, 2-diphenylethane was dissolved in 50 mL of methanol to prepare a 1, 2-diphenylethane stock solution with a concentration of 1 mg/mL [26]. From this stock solution, a working internal standard solution was developed by diluting it to 1 µg/mL using methanol [27]. 10 µL of the 1 µg/mL 1, 2-diphenylethane solution was then added to 1 mL of the concentrated soil extract, introducing 10 ng (Nano-gram) of the 1, 2-diphenylethane into the extract [28]. The mixture was homogenized carefully to ensure uniform distribution. 5 µL of a mixture of this extract and the internal standard was then injected into a 20 µL loop for chiral HPLC analysis [29]. The HPLC UV-detector was set at 270 ± 5 nm [30].

RESULTS AND DISCUSSION

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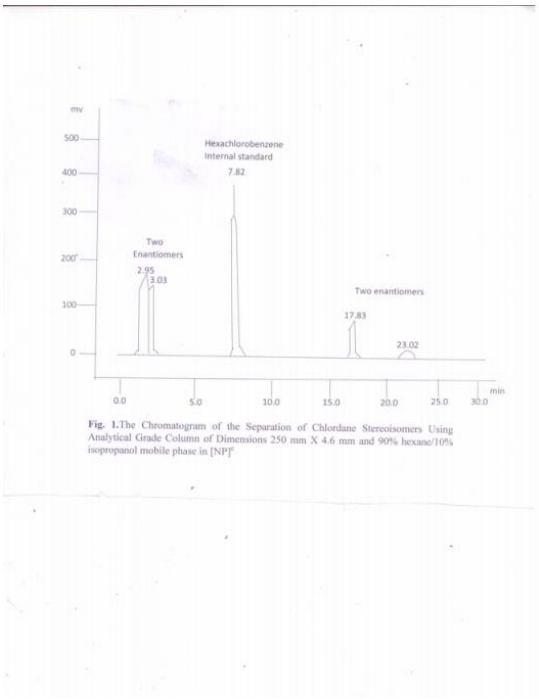

Fig. 1. The Chromatogram of the Separation of Chlordane Stereoisomers Using Analytical Grade Column of Dimensions 250 mm X 4.6 mm and 90% hexane/10% isopropanol mobile phase in [NP]

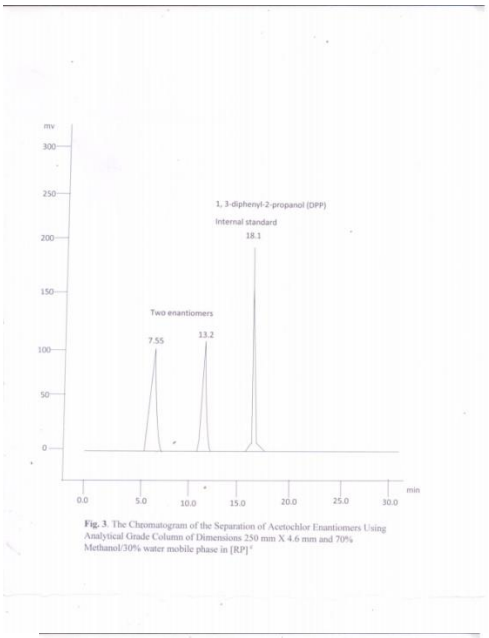

Fig. 3. The Chromatogram of the Separation of Acetochlor Enantiomers Using Analytical Grade Column of Dimensions 250 mm X 4.6 mm and 70% Methanol/30% water mobile phase in [RP]

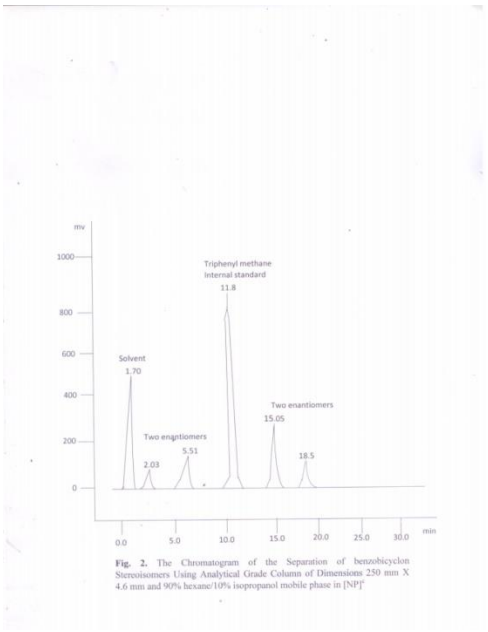

Fig. 2. The Chromatogram of the Separation of benzobicyclon Stereoisomers Using Analytical Grade Column of Dimensions 250 mm X 4.6 mm and 90% hexane/10% isopropanol mobile phase in [NP]

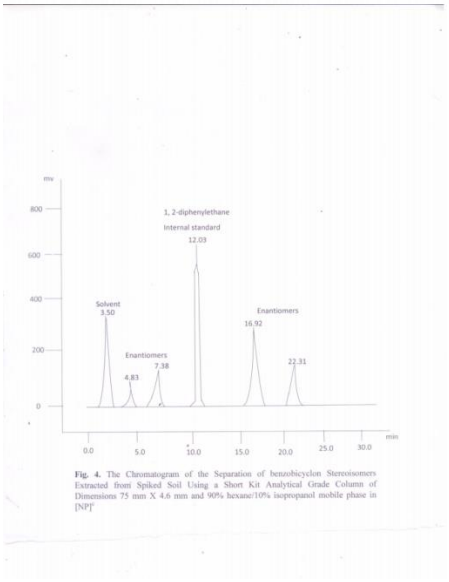

Fig. 4. The Chromatogram of the Separation of benzobicyclon Stereoisomers Extracted from Spiked Soil Using a Short Kit Analytical Grade Column of Dimensions 75 mm X 4.6 mm and 90% hexane/10% isopropanol mobile phase in [NP]

Table 1: Separation of the Stereoisomers of Chlordane Using Column (250 mm X 4.6 mm)

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			Retention time in minutes			
Separation column	%Mobile phase	Flow rate	Cis 1	Cis 2	Trans 1	Trans 2
250 mm X 4.6 mm [NP]c	90% hexane and 10% isopropanol	1.0 mL/min.	2.95	3.03	17.83	23.02

Table 1 above shows the separation of the stereoisomers of chlordane in normal phase chromatography. The Cis-enantiomers were partially separated with retention time of 2.95 and 3.03 minutes respectively, while the Trans-enantiomers were completely separated with retention time of 17.83 and 23.02 minutes respectively. The Chromatogram of the separation of chlordane stereoisomers is shown in Fig. 1.

Table 2: Separation of the Stereoisomers of Benzobicyclon Using Column (250 mm X 4.6 mm)

			Retention time in minutes			
Separation column	% Mobile phase	Flow rate	Cis 1	Cis 2	Trans 1	Trans 2
250 mm X 4.6 mm [NP]c	90% hexane and 10% isopropanol	1.0 mL/min.	2.03	5.51	15.05	18.5

Table 2 above shows the separation of the stereoisomers of benzobicyclon in normal phase chromatography. The Cis-enantiomers were separated with retention time of 2.03 and 5.51 minutes respectively, while the Trans-enantiomers were separated with retention time of 15.05 and 18.5 minutes respectively. Complete base –line separation was obtained for the four stereoisomers. The Chromatogram of the separation of benzobicyclon stereoisomers is shown in Fig. 2.

Table 3: Separation of the Enantiomers of Acetochlor Using Column (250 mm X 4.6 mm)

			Retention time in minutes	
Separation column	% Mobile phase	Flow rate	Cis	Trans
250 mm X 4.6 mm [RP]c	70% methanol and 30% water	1.0 mL/min.	7.55	13.2

Table 3 above shows the separation of the enantiomers of acetochlor in reverse phase chromatography. The Cis-enantiomer was separated with retention time of 7.55 minutes, while the Trans-enantiomer was separated with retention time of 13.2 minutes. Complete base –

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line separation was obtained for the two enantiomers. The Chromatogram of the separation of acetochlor enantiomers is shown in Fig.3.

Table 4: Separation of the Stereoisomers of Benzobicyclon Recovered from Spiked Soil Samples Using a Shorter Analytical Column (75 mm X 4.6 mm)

			Retention time in minutes			
Separation column	% mobile phase	Flow rate	Cis 1	Cis 2	Trans 1	Trans 2
75 mm X 4.6 mm [NP]^c	90% hexane and 10% isopropanol	0.5 mL/min.	4.83	7.83	16.92	22.31

Table 4 above shows the separation of the stereoisomers of benzobicyclon in normal phase chromatography using the shorter column of dimensions 75 mm X 4.6 mm. The Cis-enantiomers were separated with retention time of 4.83 and 7.83 minutes respectively, while the Trans-enantiomers were separated with retention time of 16.92 and 22.31 minutes respectively. Complete base –line separation was obtained for the four stereoisomers. . The Chromatogram of the separation of benzobicyclon stereoisomers recovered from spiked soil samples is shown in Fig. 4.

Table 5: Comparing the Separation Efficiency of Short Column (75 mm X 4.6 mm) and Longer Analytical Column (250 mm X 4.6 mm) in Benzobicyclon Analysis.

Column Properties	**Shorter analytical column**	**Longer analytical column**
Dimension	75 mm X 4.6 mm	250 mm X 4.6 mm
Mobile Phase	90% hexane and 10% isopropanol	90% hexane and 10% isopropanol
Flow rate	0.5 mL/min	1.0 mL/min
Separation factor (α)	α cis = 3.26, α trans = 1.40, α cis – trans = 3.10	α cis = 11.55, α trans = 1.22, α cis – trans = 3.50

α cis= Separation factor of the cis isomers.

α trans = Separation factor of the trans isomers.

α cis – trans = Separation factor of the cis and trans isomers.

$$\alpha = \frac{Tr2 - Tm}{Tr1 - Tm}$$

Tr2 and Tr1 are the retention time of the pair of Cis or Trans enantiomers. Tm is the retention time of the solvent fronts. Tr2, Tr1and Tm are obtained from Table 2 and 4 respectively.

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For the longer analytical column (250 mm X 4.6mm)

$$\alpha \text{ cis} = \frac{(5.51 - 1.70)}{(2.03 - 1.70)} = 11.55$$

$$\alpha \text{ trans} = \frac{(18.5 - 1.70)}{(15.51 - 1.70)} = 1.22$$

$$\alpha \text{ cis – trans} = \frac{(15.05 - 1.70)}{(5.51 - 1.70)} = 3.50$$

For the shorter column (75 mm X 4.6 mm)

$$\alpha \text{ cis} = \frac{(7.83 - 3.5)}{(4.83 - 3.5)} = 3.26$$

$$\alpha \text{ trans} = \frac{(22.31 - 3.5)}{(16.92 - 3.5)} = 1.40$$

$$\alpha \text{ cis – trans} = \frac{(16.92 - 3.5)}{(7.83 - 3.5)} = 3.10$$

In table 5, the separation efficiency of the two analytical columns was compared using a thermodynamic term (separation factor). The data obtained showed that the longer analytical column (250 mm X 4.6 mm) proved to be a more efficient separator than the shorter column (75 mm X 4.6 mm). However the separation efficiency of the shorter column is relatively comparable to that of the longer column.

Table 6: Recovery Rates of Benzobicyclon Stereoisomers from the Spiked Soil Samples.

SAMPLES	% RECOVERY [a]
1, 2-diphenylethane internal standard	94.2 ±5.50
Cis isomer 1	92.5 ± 6.40
Trans isomer 1	90.9 ± 5.60
Cis isomer 2	93.6 ± 4.50
Trans isomer 2	91.5 ± 5.20

a = 95% confidence level (n = 3)

CONCLUSION

The HPLC methods developed for the chiral separation of benzobicyclon, chlordane and acetochlor were operational in achieving high resolution separations of the stereoisomers. The use of exact mobile phases designed for each pesticide guaranteed best separation, with the longer column offering better resolution. The method validated robustness through successful application to soil samples, accomplishing high recovery rates for benzobicyclon. These outcomes accentuate the efficacy of the chiral HPLC techniques used and their appropriateness for both laboratory and environmental sample analysis.

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