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HPLC–DAD WITH DIFFERENT TYPES OF COLUMN FOR DETERMINATION OF β-CYFLUTHRIN IN PESTICIDE FORMULATIONS

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SUMMARY

Reversed-phase (RP) and normal-phase (NP) high-performance liquid chromatography (HPLC) with diode-array detection (DAD) have been used to determine and quantify the biologically active diastereomers of the pyrethroid insecticide β-cyfluthrin in suspension concentrate (SC) and emulsifiable concentrate (EC), Responsar SC 025 and Bulldock 025 EC, respectively. Analysis was performed with three different types of analytical column – Hypersil ODS (25 cm × 0.46 cm, 5 μm), LiChrosorb CN (25 cm × 0.4 cm, 5 μm), and LiChrosorb Si 60 (25 cm × 0.4 cm, 5 μm). Use of the LiChrosorb Si 60 column resulted in a high value for the multiple correlation coefficient, satisfactory accuracy of the results obtained, and good reproducibility of retention time and peak area.

INTRODUCTION

Synthetic pyrethroids (SP) are important insecticides used in agriculture, forestry, horticulture, public health, and in houses [1]. The substances are neuropoisons which act on the axons in the peripheral and central nervous system [2]. All known SP have chiral structures and comprise four or eight enantiomers [3]. Enantiomers of the same diastereomer have the same physicochemical properties but probably different biological properties [4,5]. The biological activity of a chiral pesticide enantiomer mixture may be limited to only one enantiomer, with the activity of the other enantiomers being less effective, inactive, or different [6].

3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid cyano-(4-fluoro-3-phenoxyphenyl)-methyl ester (IUPAC), common name β-cyfluthrin (ISO), is a synthetic pyrethroid insecticide, and one of the stereo-

isomers of the parent compound cyfluthrin (Fig. 1). β-Cyfluthrin and cyfluthrin have the same toxicological profile, but the acute toxicity of β-cyfluthrin is approximately 2 to 5 times that of cyfluthrin [7]. β-Cyfluthrin is

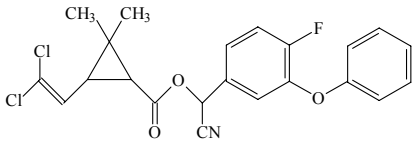

Fig. 1

The chemical structure of β-cyfluthrin

a mixture of four diastereomers, and their ratio, expressed as a percentage of the sum of the four diastereomers is 98.7% (*cis* diastereomer I, max. 2.0%; *cis* diastereomer II, 30.0-40.0%; *trans* diastereomer III, 3.0% and *trans* diastereomer IV, 57.0-67.0%) [7]. β-Cyfluthrin consists predominantly of two diastereomeric pairs (*cis* diastereomer II and *trans* diastereomer IV) of enantiomers in a 1:2 ratio [8]. It is usually assumed that only these two enantiomers have biological activity and contribute directly to biological performance against pest organisms [9].

β-Cyfluthrin acts as a contact and stomach poison. Biochemically, β-cyfluthrin has a complex mode of action and affects normal nerve function with combined rapid knock-down effects with long-lasting efficacy and long residual activity. It is not systemic in plants. Application of this pesticide is based on its wide range of activity. It is, therefore, used in agriculture, horticulture (field and protected crops), and viticulture. It is also used against migratory locusts and grasshoppers, mosquitoes, and in public health and hygiene [10,11]. It has been found that β-cyfluthrin is highly immobile and moderately persistent in alluvial soil [12]. The pyrethroids are rapidly metabolised in the human body by cleavage of the central ester link and oxidation of the resulting alcoholic component, yielding carboxylic acids. These acids are excreted, partly conjugated, in the urine [13].

Analysis for determination of β-cyfluthrin in EC and SC pesticide formulations has been performed by normal-phase HPLC with *n*-heptane–*tert*-butyl methyl ether (TBME) 95:5 (*v*/*v*) as mobile phase, UV detection at 235 nm, and external standardization, according to the CIPAC handbook method [14]. A standard reference AOAC method for determination of the total amount of cyfluthrin in liquid and solid formulations is based on iso-

cratic reversed-phase HPLC with 55% (*v*/*v*) acetonitrile in water as mobile phase, UV detection at 230 nm, and decanophenone as internal standard [15]. Slahck developed an LC method with a cyano 100A (250 mm × 4.6 mm, 5-μm particle) column for determination of total cyfluthrin active ingredient content in technical and formulated products with dioctyl phthalate as internal standard [16]. Quantitative estimation of β-cyfluthrin by gas–liquid chromatography with Ni^{63} electron-capture detection (ECD) has been reported by Saikia and Gopal [17]. Leicht et al., using GC with an HP-1 capillary column and mass-selective detection (MSD), completely separated the four pairs of diastereomers of cyfluthrin [18]. Liu and Gan used GLC with an HP-5 column and both ECD and MSD for separation and analysis of the diastereomers and enantiomers of cypermethrin and cyfluthrin [19]. Four commonly used pyrethroids, including cyfluthrin, have been separated into their enantiomers by enantioselective HPLC on two connected Chirex columns with hexane–1,2-dichloromethane–ethanol as mobile phase [20]. Lisseter and Humbling used Pirkle ionic and covalent columns to separate the enantiomers of cypermethrin and cyfluthrin; under optimum conditions eight peaks were obtained, although most were not well separated [21]. Other authors using chiral-phase HPLC for separation of pyrethroid enantiomers proved that several investigated peaks were not completely separated to baseline [22].

Available HPLC and GC methods for quantification of pyrethroids require sophisticated instrumentation which is not readily available or cost-effective. Most of these methods require use of expensive speciality columns, gradient elution and relatively long retention times. Another difficulty is poor availability of enantiomer standards, which are hard to synthesize or purify [19]. The objective of the work discussed in this paper was, therefore, to investigate the possibility of developing a simple, practical and sensitive HPLC method suitable for accurate analysis of the major and biologically active diastereomers (*cis* diastereomer II and *trans* diastereomer IV) of the pyrethroid insecticide β-cyfluthrin in different pesticide formulations, for example Responsar SC 025 and Bulldock 025 EC.

EXPERIMENTAL

Reagents and Chemicals

HPLC-grade acetonitrile, methanol, *n*-hexane, and dichloromethane were supplied by Sigma–Aldrich (Deisenhofen, Germany). Water was deionized then distilled from glass apparatus. All solvents and solutions for

HPLC analysis were degassed in an ultrasonic bath before use. The pesticide formulations Responsar SC 025 and Bulldock 025 EC, and the pure analytical standard of β-cyfluthrin (98.7%) were provided free of charge by Bayer (Germany).

Preparation of Stock and Standard Solutions

β-Cyfluthrin stock solution for reversed-phase HPLC (Method I), was prepared by dissolving 0.0179 g analytical standard in methanol in a 25-mL volumetric flask. Working standard solutions were prepared fresh for each day of analysis, by diluting the stock solution with 70:30 (*v*/*v*) acetonitrile–water or 80:20 (*v*/*v*) methanol–water.

Primary stock solutions of β-cyfluthrin for normal-phase HPLC were prepared by dissolving 0.0341 g (Method II) and 0.0327 g (Method III) analytical standard in *n*-hexane in a 25-mL volumetric flask and storage under refrigeration at 4°C. Individual working solutions were prepared daily by serial dilution of 0.35, 0.50, 0.65, 0.80, 1.00, 1.50, 2.00, 2.50, 3.00, and 3.50 mL stock solution in 80/20 (*v*/*v*) *n*-hexane–dichloromethane (Method II) or 0.1, 0.2, 0.3, 0.4, 0.5, 1.00, 1.50, 2.00, 2.50, 3.00, and 3.50 mL stock solution in 40:60 (*v*/*v*) *n*-hexane–dichloromethane (Method III) in 10-mL volumetric flasks.

Calibration plots for β-cyfluthrin were obtained by triplicate analysis (20 μL) of the working solutions. The areas and heights of the peaks and the corresponding amounts of β-cyfluthrin were used to construct the plots, using the least-squares method of Omega statistics software with external standard multilevel calibration by linear fitting [23].

Preparation of Sample Solutions

For reversed-phase chromatography sample solutions of the pesticide formulations were prepared by dissolving Responsar SC 025 (0.5695 g) and Bulldock 025 EC (0.5532 g) in methanol in 25-mL volumetric flasks. For normal-phase chromatography sample solutions were prepared by dissolving 1.3079 g and 1.4469 g Responsar SC 025 and 0.908 g and 1.0122 g Bulldock 025 EC in *n*-hexane in 25 mL volumetric flasks. The solutions were degassed for 20 min in an ultrasonic bath and then 1 mL (for RP HPLC) and 3.5 mL and 2.0 mL (for NP HPLC) were transferred to 10-mL volumetric flasks and dissolved with the appropriate solvents. All the sample solutions were filtered through 0.45 μm Spartan-T syringe filters. Triplicate HPLC analysis was performed on 20 μL of each of these solutions.

High-Performance Liquid Chromatography

HPLC was performed with a Perkin–Elmer system equipped with a binary LC pump (Perkin–Elmer model 250), an injection valve with 20-μL sample loop, and a UV diode-array detector (DAD) (Perkin–Elmer model 235). Data processing and integration were performed with Omega software [23]. A constant column temperature was maintained by use of a Spark Holland "Mistral" (type 880) column thermostat. Compounds were separated on a 25 cm × 0.46 cm, 5-μm particle, Hypersil ODS column (Sigma–Aldrich), on a 25 cm × 0.4 cm, 5-μm particle, LiChrosorb CN column (Merck), and on a 25 cm × 0.4 cm, 5-μm particle LiChrosorb Si 60 column (Merck).

The Hypersil ODS column with methanol–water, 80:20 (*v*/*v*), or acetonitrile–water, 70:30 (*v*/*v*), as mobile phase, at a flow rate of 1.0 mL min^{-1} and a column temperature of 40°C, was used for reversed-phase (RP) chromatography (Method I). UV detection was performed at 220 nm. Peaks were identified by comparison of retention times with that of β-cyfluthrin standard; identities were confirmed by comparison of the characteristic spectra acquired by use of the DAD.

Normal-phase (NP) chromatography was performed with the LiChrosorb CN column and *n*-hexane–dichloromethane, 80:20 (*v*/*v*), as mobile phase at a flow rate of 1.2 mL min^{-1} (Method II). The column temperature was maintained at 25°C and the DAD was operated at 265 nm.

To achieve the best separation of β-cyfluthrin on the LiChrosorb Si 60 column the mobile phase was *n*-hexane–dichloromethane 40:60 (*v*/*v*) (Method III). The mobile phase flow rate was 1.5 mL min^{-1}. Other conditions were identical with those of Method II.

RESULTS AND DISCUSSION

Separation of two major diastereomers (*cis* diastereomer II, 30.0–40.0%, and *trans* diastereomer IV, 57.0-67.0%) of β-cyfluthrin from pesticide formulations Responsar SC 025 and Bulldock 025 EC was performed on three different analytical columns – Hypersil ODS, LiChrosorb CN, and LiChrosorb Si 60 for Methods I–III, respectively, with different mixtures of acetonitrile and water, of methanol and water, and of *n*-hexane and dichloromethane as mobile phases.

Under the conditions tested on the Hypersil ODS column β-cyfluthrin furnished four peaks that were well separated to baseline. Overlay of

the spectra of the four peaks revealed the same spectral characteristics; the purity index was 1.1 (Fig. 2).

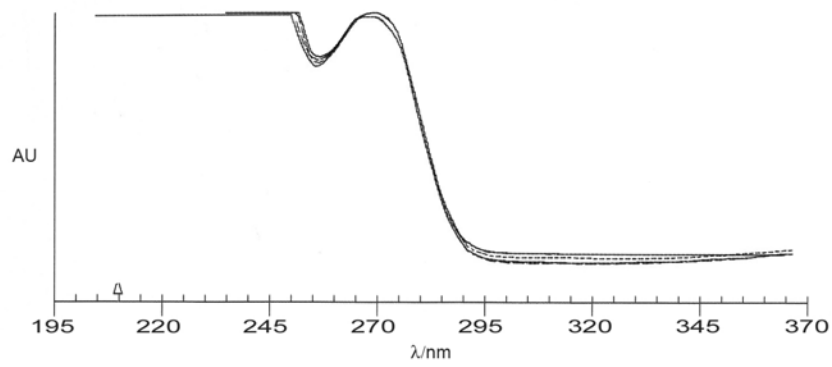

Fig. 2

Overlaid spectra of the four diastereomers of β-cyfluthrin (purity index 1.1), separated on the Hypersil ODS column

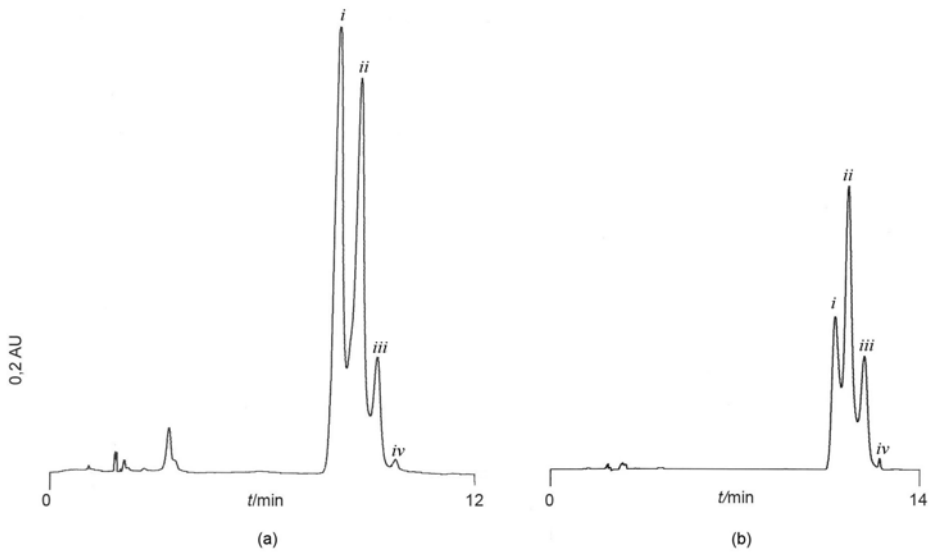

Fig. 3

Chromatograms obtained from β-cyfluthrin on the Hypersil ODS column with (a) methanol–water, 80:20 (*v*/*v*), and (b) acetonitrile–water, 70:30 (*v*/*v*), as mobile phase. The flow rate was 1.0 mL min^{-1}, the column temperature 40°C, and UV detection was performed at 220 nm

The retention times of the resolved peaks were 8.81 min (peak i), 9.23 min (peak ii), 9.65 min (peak iii), and 10.06 min (peak iv) for methanol–water as mobile phase (Fig. 3a). The relative peak areas were 38.78, 42.56, 13.59, and 2.65, respectively, for peaks i–iv. Analysis of β-cyfluthrin on the Hypersil ODS column with acetonitrile–water as mobile phase also gave four completely separated peaks with retention times of 10.87, 11.37, 12.01, and 12.46 min for peaks i–iv, respectively. The relative areas of the resolved peaks were 26.02, 49.27, 21.62, and 1.46%, respectively, for peaks i–iv (Fig. 3b).

The total peak area of the four diastereomers was essentially the same (97.5 and 98.37%), in agreement with literature data [7], but the relative peak areas did not correspond to the expected peak-area ratio for each of the diastereomers of β-cyfluthrin. It is evident that cyfluthrins in protic organic solvents, for example methanol, are susceptible to isomerization which may be fairly rapid [24]. It is therefore possible that under the chromatographic conditions described the biologically active β-cyfluthrin isomers (*cis* diastereomer II and *trans* diastereomer IV) were partly isomerised into inactive isomers (*cis* diastereomer I and *trans* diastereomer III). Similar isomerization was observed by Leicht et al. when cyfluthrin isomer I was incubated in methanol or methanol–water at room temperature in the dark [18]. Another cause of this unusual selectivity on the Hypersil ODS column might be direct interaction between β-cyfluthrin diastereomers and the phase.

The absorption spectrum of β-cyfluthrin in the *n*-hexane–dichloromethane mixture is similar to that in the acetonitrile–water and methanol–water mixtures (Fig. 2); all contain a band with maximum absorption at approximately 267 nm, but the chromatographic peak obtained at 267 nm is less intense than that obtained at 220 nm. Absorption of *n*-hexane and dichloromethane at 220 nm is significant, however. Monitoring was therefore performed at 265 nm to simplify data handling for β-cyfluthrin in *n*-hexane–dichloromethane in Methods II and III.

The chromatograms (Figs 4 and 5) obtained for β-cyfluthrin on the LiChrosorb CN and Si 60 columns show the two diastereomers were well resolved to baseline. The peaks of interest were sharp and symmetrical. The β-cyfluthrin standard gave two peaks with identical retention times, with iii and iv the predominant peaks. The relative peak-area ratio (35.46% with RSD = 0.85% and 61.84% with RSD = 0.76%, $n = 24$) for peaks iii and iv corresponds to the peak-area ratio expected for the two major diastereomers of β-cyfluthrin. The predominance of peaks iii and iv in the

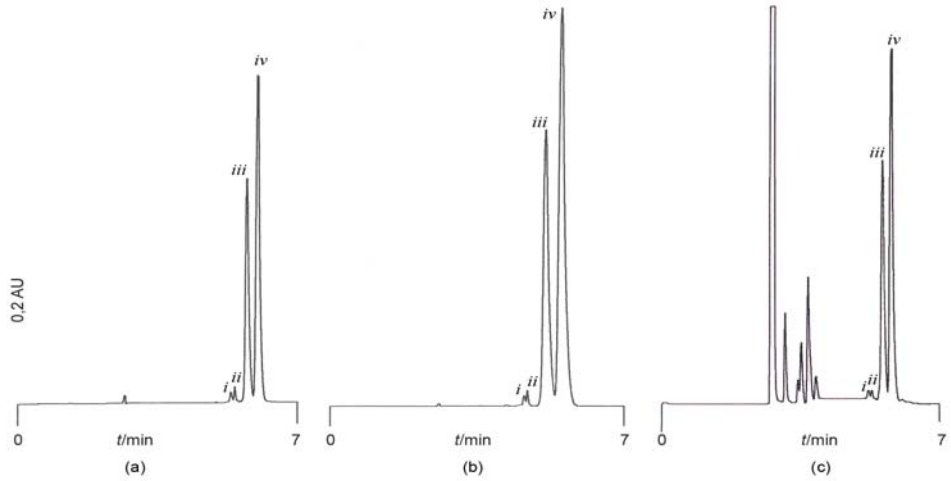

Fig. 4

Chromatograms obtained from β-cyfluthrin standard solution (a), pesticide formulation Responsar 025 SC (b), and pesticide formulation Buldock 025 EC (c) on the LiChrosorb CN column. The mobile phase was *n*-hexane–dichloromethane, 80:20 (*v*/*v*), the flow rate 1.2 mL min^{-1}, the column temperature 25°C, and UV detection was performed at 265 nm

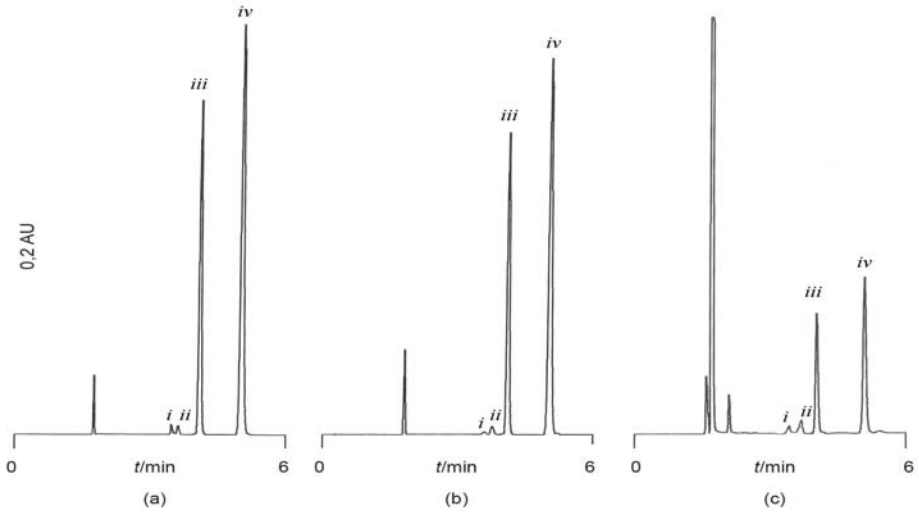

Fig. 5

Chromatograms obtained from β-cyfluthrin standard solution (a), pesticide formulation Responsar 025 SC (b) and pesticide formulation Buldock 025 EC (c) on the LiChrosorb Si 60 column. The mobile phase was *n*-hexane–dichloromethane, 40/60 (*v*/*v*), the flow rate 1.5 mL min^{-1}, the column temperature 25°C, and UV detection was performed at 265 nm

chromatograms obtained for β-cyfluthrin suggests that peak iii was the second *cis* diastereomer, leaving peak iv as the second *trans* diastereomer. Again it is known that the specific order of elution of the diastereomers of some synthetic pyrethroids, for example cypermethrin and cyfluthrin, depends on the linearity of their structure, and this may be used for tentative peak assignment in the absence of individual isomer standards [19]. Because of the structural linearity of the β-cyfluthrin diastereomers and the postulate that the *cis* diastereomer is eluted before the *trans* diastereomer [20], it can be assumed that the first peak in the chromatogram of β-cyfluthrin (Figs 3a and 3b) is that of *cis* diastereomer II (30.0–40.0%) and the second peak is that of *trans* diastereomer IV (57.0-67.0%).

In the chromatogram obtained from the β-cyfluthrin standard on the LiChrosorb CN and Si 60 columns it is interesting to note the appearance of two peaks (i and ii), of low intensity, eluting before the peaks (iii and iv) of the biologically active diastereoisomers. The UV spectra of the compounds generating chromatographic peaks i and ii have a band with maximum absorbance at approximately 211 nm. The overlaid spectra (Fig. 6) of peaks i and ii showed they had spectral characteristics similar to those of peaks iii and iv, the major components (purity index 1.3), suggesting it is possible to separate all four diastereomers of β-cyfluthrin on both the LiChrosorb CN and Si 60 columns under the NP conditions used.

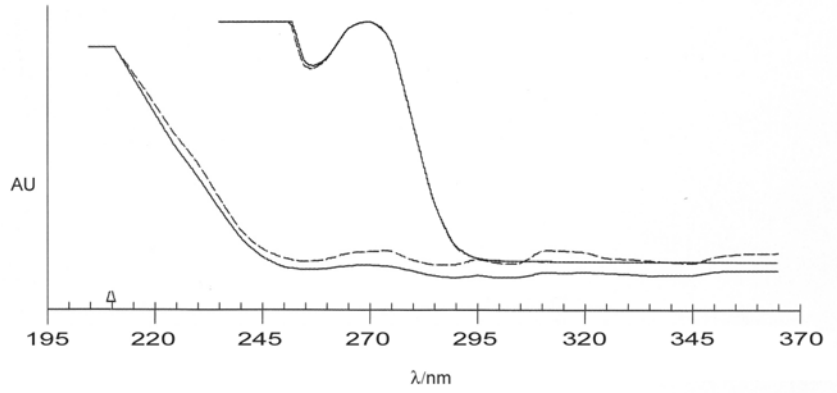

Fig. 6

Overlaid spectra of the four diastereomers of β-cyfluthrin (purity index 1.3), separated on the LiChrosorb CN and Si 60 columns

The order of elution of the diastereomers of β-cyfluthrin on the LiChrosorb CN and Si 60 columns using the chromatographic condition described should follow the order:

cis diastereomer I, *trans* diastereomer II,
cis diastereomer III, *trans* diastereomer IV

with retention time increasing from left to right.

The retention times for the two major diastereomers (peaks iii and iv) of β-cyfluthrin on LiChrosorb CN were approximately 5.61 min (RSD = 0.862%) and 6.00 min (RSD = 0.929%), respectively, and the column dead-time (t_0) was 2.73 min (RSD = 0.228%). The retention factors k_{iii} and k_{iv} were 1.05 and 1.20 and the value calculated for the separation factor (α) between adjacent peaks was 1.14.

The estimated retention times of the diastereomers of β-cyfluthrin on the LiChrosorb Si 60 column under the chromatographic condition described above were approximately 4.14 min (RSD = 0.441%) and 5.12 min (RSD = 0.301%), for peaks iii and iv, respectively. The column dead-time was approximately 1.80 min (RSD = 0.550%), and the retention factors were $k_{iii} = 1.28$ and $k_{iv} = 1.83$. For the LiChrosorb Si 60 column the separation factor (α) was 1.43.

The results from these two columns show that resolution of the diastereomers of β-cyfluthrin is better on the LiChrosorb Si 60 column than on the LiChrosorb CN column. Thus, highly efficient separation, reduced analysis time, and reduced mobile phase consumption [25] made the LiChrosorb Si 60 column more suitable for HPLC analysis of the two major and biologically active diastereomers of β-cyfluthrin. Although the literature contains descriptions of HPLC systems with similar analytical columns, they are used for separation of total cyfluthrin only [15,16].

Day-to-day ($n = 3$) and within-day ($n = 8$) repeatability [26,27] were evaluated for retention times and peak areas of the β-cyfluthrin diastereomers from eight successive injections of analytical standards of β-cyfluthrin of concentration 1.364 mg mL^{-1} and 1.308 mg mL^{-1}. Testing of the results by ANOVA revealed there were no significant differences between the assays within and between days and indicated the very good repeatability of retention time and peak area under the conditions used for Method III, in contrast with problems with irreproducibility of retention time and peak area of peak iv observed under the conditions used in Method II (Table I).

Table I

Statistical data for intra and inter-day precision of retention times and peak areas for Methods II and III

	Peak	Within day (P = 0.05)		Between day (P = 0.05)	
		$F_{2,14}$	$F_{2,14\ (crit.)}$	$F_{7,14}$	$F_{7,14\ (crit.)}$
Method II					
Retention time	iii	2.812	3.739	1.523	2.764
	iv	0.541		5.189	
Peak area	iii	1.383	3.739	1.204	2.764
	iv	0.678		3.904	
Method III					
Retention time	iii	2.083	3.739	1.068	2.764
	iv	0.191		0.842	
Peak area	iii	0.045	3.739	0.422	2.764
	iv	2.270		0.782	

The limit of detection (LOD) for a signal-to-noise ratio of 3:1 was calculated as three times the ratio of the standard deviation to the slope (LOD = 3× SD/slope) [28]. The limit of detection in the low concentration range (0.654–65.4 μg mL^{-1}) was found to be 1.37 μg (68.5 μg mL^{-1}) for Method II and 1.11 μg (55.5 μg mL^{-1}) for Method III. The limit of quantification (LOQ = 10 × SD/slope) was found to be 4.578 μg and 3.702 μg for Methods II and III, respectively.

Calibration graphs were constructed by plotting the amount of the active ingredient (β-cyfluthrin) injected as a function of peak area and height using external standard multilevel calibration by linear fit. Statistical data for the graphs are listed in Table II. The calibration plots for each day of validation and analysis revealed linearity was good over the range 0.261 to 9.156 μg in 20 μL injected. The results obtained for the correlation coefficients (R^2) indicated that Method III should be preferred to Method II.

Accuracy was determined by the method of standard addition [29]. Sample matrix containing a known amount of the analyte was spiked at three different levels (20, 40, and 80%) and the amounts of the analyte recovered at the three concentration levels, as measured by use of the LiChrosorb CN column, were 90.4% (RSD = 4.95%, n = 3), 91.7% (RSD = 2.72%, n = 3), and 97.3% (RSD = 0.89%, n = 3), respectively. Recovery of the diastereomers of β-cyfluthrin by use of the LiChrosorb Si 60 column were

106% (RSD = 1.33%, n = 3), 93.4% (RSD = 1.10%, n = 3), and 100% (RSD = 1.06%, n = 3), respectively.

Table II

Statistical data from regression analysis for LiChrosorb CN and Si 60 columns

	Peak	Regression equation	Multiple correlation coefficient (R^2)
Method II			
Area	iii	$y = 478561x + 23824$	0.9974
	iv	$y = 775305x + 4820.8$	0.9979
Height	iii	$y = 51.164x - 12.471$	0.9977
	iv	$y = 56.058x + 1.0482$	0.9976
Method III			
Area	iii	$y = 364435x + 88747$	0.9994
	iv	$y = 596709x + 119415$	0.9986
Height	iii	$y = 68.08x + 15.361$	0.9980
	iv	$y = 84.642x + 23.938$	0.9981

The mean amounts of active substance, as the sum of both major biologically active diastereomers (*cis* diastereomer II and *trans* diastereomer IV) in the pesticide formulations Responsar 025 SC and Bulldock 025 EC were approximately 2.46% (RSD = 1.02%; n = 5), and 2.49% (RSD = 0.92%; n = 5), respectively. These amounts are in agreement with the amount declared by the manufacturer (2.5%).

CONCLUSION

The method established in this study enables simple, practical, and sensitive routine NP-HPLC analysis of the biologically active diastereomers (*cis* diastereomer II and *trans* diastereomer IV) of the pyrethroid insecticide β-cyfluthrin in the SC (suspension concentrate) and EC (emulsifiable concentrate) pesticide formulations Responsar 025 SC and Bulldock 025 EC. The method using the LiChrosorb Si 60 column had high multiple correlation coefficients and gave results of satisfactory accuracy. Reproducibility of retention times and peak areas was also good.

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