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Development of A Reversed-Phase High Performance Liquid Chromatographic Method for Efficient Diastereomeric Separation and Quantification of Cypermethrin, Resmethrin and Permethrin

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Abstract

An efficient and simple reversed phase high performance liquid chromatographic method (RP-HPLC) for diastereomeric separation and quantification of cypermethrin, resmethrin, and permethrin has been developed. Separation was performed on Phenomenex Luna C18, (4.6 x 150 mm, 5 µm; end capped column). Satisfactory separation of diastereomers was obtained for the three pyrethroids studied. Good reproducibility of retention time and peak area were achieved. Detection was performed with UV diode array detector (UV-DAD) at a wavelength of 220 nm. Most peaks were base-separated with Rs values ranged from 1.6 to 4.5 for most peaks. The optimum mobile phase was composed of a mixture of acetonitrile, methanol, and water with a mixing ratio of 1:3:1, respectively. The regression coefficients (R^2) were 0.9991, 0.9951 and 0.9964 with relative standard deviations (RSD%) of 1.95, 2.89 and 1.87, for cypermethrin (CYP), resmethrin (RES) and permethrin (PER), respectively.

Keywords: Cypermethrin, resmethrin, permethrin, RP-HPLC, pyrethroids, diastereomeric separation.

Introduction

Synthetic pyrethroids (SPs) are potent insecticides and they are of increasing importance as they have been replacing older classes of insecticides[1]. Each SP is, however, composed of a mixture of 2–4 enantiomeric pairs (diastereomers)[2,3]. Several studies have shown that SPs enantiomers differ significantly in their biological activities and toxicity[4–7]. In addition, it has experimentally been proved that SPs are enantioselectively degraded by microorganisms[8,9], which affects the distribution patterns of SPs in the SPs-treated fields[10-12]. Hence, bioaccumulation of different SPs enantiomers in a particular area will depend on its content of microorganisms and their types[13,14].

As environmental samples are highly complex, it is important that the analytical method used is capable of providing reasonable separation of peaks of analytes of interest especially when such analytes are composed of several diastereomers. Several HPLC and GC methods have been reported for quantification of pyrethroids[15,16]. However, most of these methods require use of expensive speciality columns and gradient elution with relatively long retention times. Although spectrometric methods are easy to operate[17], they are not suitable for multiresidue analysis, especially with the rapid growth in the use of hazardous chemicals[18-20].

Gas chromatography with electron capture detector and normal-phase high performance liquid chromatography (NP-HPLC) are the most used analytical techniques for SPs separation. However, reversed-phase high performance liquid chromatographic method (RP-HPLC) reported, so far, for the separation of SPs don not provide efficient diastereomeric separation. The objective of the work discussed in this paper was, therefore, to investigate the possibility of developing a simple, practical and sensitive RP-HPLC method suitable for accurate separation and quantification of diastereomers of three SPs, viz., cypermethrin (CYP), resmethrin (RES) and permethrin (PER) as model SPs. The separation was achieved on an achiral RP-HPLC column (C_{18}). The effects of factors governing chromatographic separation of SPs diastereomers were discussed. Further, the effect of the nature of injection solvent on the peak shape was evaluated by injecting the analytes in different organic solvents. In addition, the optimized chromatographic method was further evaluated by analysing SPs in real water samples.

Material and Methods

Structural Description of Three SPs: Resmethrin's IUPAC name is: (5-benzyl-3-furylmethyl (1RS,3RS;1RS,3SR)-2,2-dimethyl-3-(2-methylpropyl-1-enyl) cyclopropanecarboxylate). Resmethrin (RES) is a racemic mixture of four isomers: [1R, trans], [1R, *cis*], [1S, *trans*], [1S, *cis*]. The composition ratio of the four enantiomers in technical RES is roughly 4:1:4:1. Some RES isomers have common names, for example, the [1R, *cis*]-isomer is called cismethrin and the [1R, *trans*]- isomer is known as bioresmethrin. As for biological activities, the [1R, *trans*]-isomer possesses the highest insecticidal activity among all RES isomers followed by the [1R, *cis*]- isomer[21]. Permethrin's IUPAC name is: (3-phenoxybenzyl (1RS,3RS; 1RS,3SR)-3-(2,2 dichlorovinyl) -2, 2-dimethyl-cyclopropane carboxylate).

Permethrin (PER) is a racemic mixture of two diastereomers, and both have low mammalian toxicity. The optical ratio of 1R:1S is 1:1 (racemic). Because the *trans* isomer is somewhat less toxic, the (*cis*:*trans*) isomeric ratio of 25:75 was chosen for PER products[22,23]. In addition, it has been shown that [1R, *cis*] isomer possesses the highest insecticidal activity among PER isomers followed by the [1R, *trans*] isomer[24].

Cypermethrin's IUPAC name is: ((RS)-α-cyano-3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclo propanecarboxylate). Cypermethrin (CYP) consists of eight stereoisomers[25], which form four enantiomeric pairs (diastereomers), viz., *trans* (1R-3S-αS + 1S-3R-αR), *cis* (1R-3R-αS +1S-3S-αR), *trans* (1R-3S-αR + 1S-3R-αS), and *cis* (1S-3S-αS + 1R-3R-αR). Among the eight isomers, only the 1R-3R -αS and 1R-3S -αS possess biological activity against pests and insects[26,27]. The absolute configurations of resmethrin, permethrin, and cypermethrin are shown in figure-1 with asterisks indicating asymmetric positions.

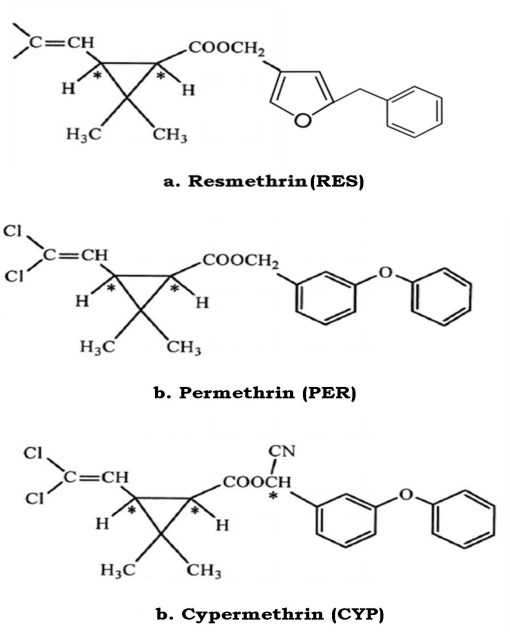

Figure-1
Chemical structures of: a) resmethrin, b) permethrin, and c) cypermethrin. Asymmetric positions are indicated with an asterisk ''*''

Materials and Reagents: Permethrin (PER) and Resmethrin (RES) (PESTANAL®, analytical reagent grade, 98.2% and 98.5% purity, respectively) were obtained from Sigma-Aldrich Co., USA. Cypermethrin (CYP) (technical grade, 95% purity) was donated by Hyderabad Chemicals Pvt. Ltd, Hyderabad, India. HPLC grade acetonitrile, acetone, methanol, and n-hexane were obtained from MERCK (Merck Specialties Pvt. Ltd, Mumbai, India). Analytical reagent grade sodium chloride (NaCl) and ethanol were obtained from MERCK (Merck Specialties Pvt. Ltd, Mumbai, India). Analytical reagent grade carbon tetrachloride, ethyl acetate, dichloromethane, n-heptane, toluene, and tetrachloroethane were obtained from RFCL Pvt. Ltd, New Delhi, India. All reagents were used without further purification.

Instrumentation: The chromatographic analysis was performed on a high performance liquid chromatograph (HPLC) (LC-20AT Prominence, Shimadzu, Japan), equipped with a binary solvent delivery system, an injection valve with a 20 µL sample loop and a UV-diode array detector model SPD-M 20A Prominence with in-line degasser. The chromatographic separation was performed on a Phenomenex Luna C_{18}, 4.6 x 150 mm, 5 µm; end capped column), purchased from Phenomenex, Inc. (411 Madrid Avenue Torrance, CA, USA). Ultrapure water, purified by a Milli-Q water purification system, Millipore (Bedford, MA, USA) was used throughout the experiments unless stated, and was collected on daily basis and degassed with a vacuum pump and further filtered through 0.45 µm membrane (Nylon, Hydrophilic, Millipore, Hyderabad, India). Samples of 20-µl volume were injected into HPLC at a mobile phase flow rate of 1.0 mL min^{-1}. A 25-µL microsyringe (Hamilton, Switzerland) was used for sample injection into the HPLC.

Preparation of Reference Solutions: The stock solutions of the individual standard solutions of the three SPs were prepared in HPLC-grade acetonitrile (each 10 mg L^{-1}) in amber reagent bottles and kept in the refrigerator at +4° C. Working standard solutions of concentrations ranged from 50-2000 µgL^{-1} were prepared by dilution of the above stock solutions in HPLC-grade acetonitrile and were kept in the refrigerator at +4° C.

Results and Discussion

Determination of λ_{max} of The Tested Pyrethroids: For efficient chromatographic detection of the tested pyrethroids, their chromatograms must be recorded at a wavelength at which they show maximum absorption of UV radiation. The optimum wavelength (λ_{max}) of the three pyrethroids was determined by recording their UV absorption spectrum using a spectrophotometer. The overlaid UV-spectra of CYP, RES and PER are shown in figure-2. For confirmation, the λ_{max} was also evaluated using HPLC by comparing the absorbance of the investigated pyrethroids at five different wavelengths. The advantage of recording the spectrum using HPLC is the possibility of obtaining λ_{max} of individual diastereomers of the three SPs. The results showed that *trans* forms of permethrin (PER) diastereomers and resmethrin (RES) diastereomers exhibited higher UV absorption than their counterparts *cis* forms, figure-3.

On the contrary, the *cis* forms of cypermethrin (CYP) diastereomers exhibited higher UV absorption than their counterparts *trans* forms. For the RP-HPLC analysis, the UV-detector was set at 220 nm as maximum wavelength (λ_{max}) for simultaneous determination of the three analytes as interfering peaks observed at wavelengths of 200 nm and 210 nm were eliminated at this wavelength.

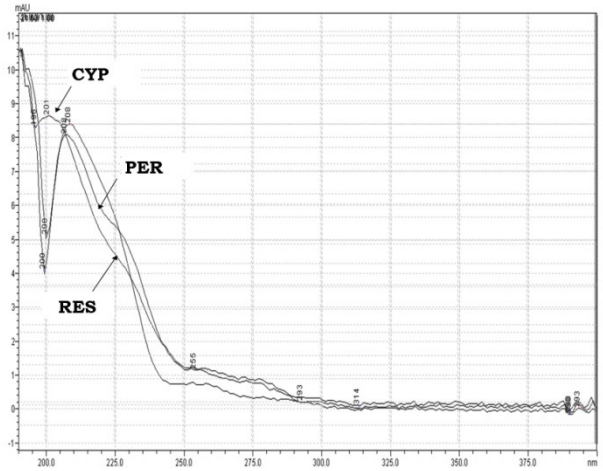

Figure-2
Overlay of UV-spectra of cypermethrin (CYP), resmethrin (RES), and permethrin (PER)

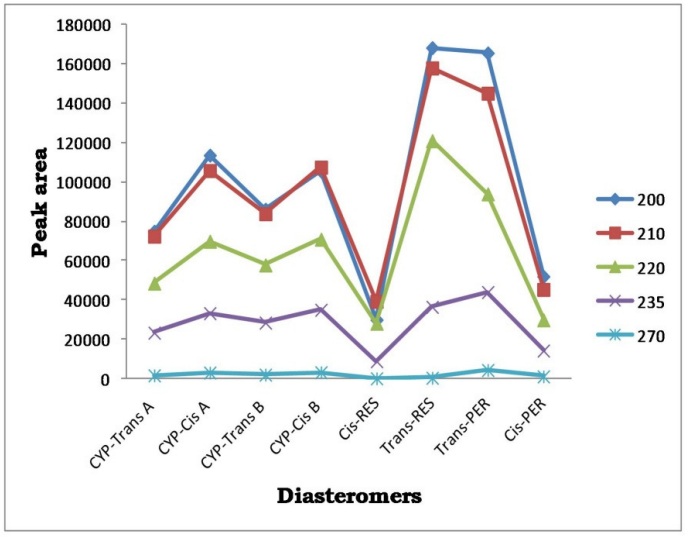

Figure-3
Optimization of detection wavelength for the investigated pyrethroids by HPLC-DAD. Note: for diastereomers' full names, please see text

Optimization of Peak Separation: Changes in the composition of the elution system (mobile phase) will generally affect both retention factor k, and selectivity factor α, but with less effect on N (number of column theoretical plates)[28]. In RP-HPLC, the elution system consists of water as one of the eluent components and an organic solvent that is usually called a "modifier".

For optimizing the mobile phase composition, several binary and ternary mixtures of water, acetonitrile (ACN) and methanol (MeOH) were examined at 220nm as maximum wavelength (λ_{max}) and 1.0 mL/min as a mobile phase flow rate. The corresponding chromatograms obtained under various mobile phase compositions are illustrated in figure-4. Starting with acetonitrile (ACN) as the organic modifier, results showed that the mobile phase mixture of ACN:H_2O with a mixing ratio of 4:1, respectively, provides acceptable resolution for both RES and PER diastereomers, but with very poor diasteriometric separation of CYP, especially the peaks 1 and 2. So, the peak pair (peaks 1 and 2) of cypermethrin's diasteroimers was considered as the critical band pair. Therefore, for obtaining acceptable resolution of all diastereomers, acetonitrile was replaced by methanol. Mixtures of methanol and water with mixing ratios of 80:20 and 85:15 of methanol and water, respectively, were able to provide better resolution of all peaks.

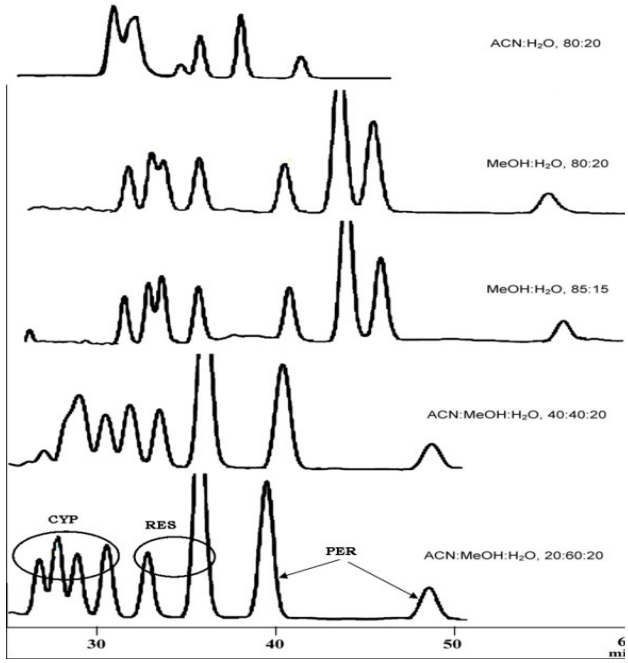

Figure-4
Optimization of mobile phase composition for optimum separation of diastereomers of cypermethrin (CYP), resmethrin (RES) and permethrin (PER), on an achiral HPLC column

However, peak resolution of the critical band of CYP diastereomers was not complete as the second and third peaks were still, slightly, overlapped. Seeking for optimum peak resolution, ternary mixtures of acetonitrile (ACN), methanol, and water were also tested. The results showed that the ACN content in the ternary mixture of the mobile phase was very critical. As mobile phase content of ACN increases, there is a reduction in runtime but, unfortunately, with simultaneous degradation in separation quality. Acceptable peak separation of all diasteriomers of the three SPs with reasonable run time was achieved at a mixture ratio of 20:60:20 of acetonitrile, methanol, and water, respectively. Most peaks were base-separated with Rs values ranged from 1.6 to 4.5, except for two band pairs (band pear of peaks 1&2 and 2&3 of CYP peaks), whose Rs values were1.0 and 1.1, respectively.

Method Validation: Working standard solutions of concentrations ranged from 50-2000 μgL^{-1} were prepared by

dilution of the stock solutions in pure HPLC grade acetonitrile. Triplicate injections of 20 µL injection volume were made for mixtures of the three compounds at each concentration under the optimized chromatographic conditions. Peak areas calculated from the respective chromatograms were plotted against sample concentration to build calibration curves.

The results showed that the method exhibited linearity in the tested range of concentrations. The regression coefficients (r^2) were 0.9991, 0.9951, and 0.9964 with relative standard deviations (RSD %) of 1.95, 2.89, and 1.87, for CYP, RES and PER, respectively. The lowest instrumental detection limit (LOD) and limit of quantification (LOQ) were determined at a signal to noise ratio (S/N) of 3 and 10, respectively, and the results showed that CYP, RES and PER have LOD and LOQ values ranging from 17–23.4 and 56–78 µg/L, respectively. The LOD and LOQ values were calculated based on injection of standard solutions without prior preconcentration. However, as determination of environmental samples involves a preconcentration step, which results in obtaining very high enrichment factors, we can then realize that the LOD and LOQ values reported here are low enough to allow detecting of these compounds at trace levels, table-1.

Table-1
Statistical data of calibration curves of investigating pesticides

Compound	Regression equation*	R^2	RSD (%)	LOD (µg/L)	LOQ (µg/L)
Cypermethrin	y = 121.01x + 4066.2	0.9991	1.95	22.6	75.2
Resmethrin	y = 70.951x + 4040.6	0.9951	2.89	17.0	56.7
Permethrin	y = 53.664x + 4442.1	0.9964	1.87	23.4	78.1
* y = peak area x = mass of pesticide (µg)					

For the sake of simplifying quantification and peak identification based on retention time, it is essential that the variations in values of separation parameters, i.e., k and Rs, for different analytes being analyzed for, fall within the acceptable range. The reproducibility of k, N, α, t_R and Rs through several runs is given in table-2. The results indicated clearly that the method provides high degree of precision and reliability. The retention factor k values ranging from 6.76 to 13.89 fulfil the condition of acceptable peak separation and analysis time of $0.5 < k > 20$.

The method intra-day precision (repeatability) of peak areas is very important for recovery studies based on calculation of peak areas. For this purpose, six replicates of a concentration within the linear range were analyzed. The results of repeatability of peak areas (expressed as relative standard deviation, RSD%) through several runs for the determination of SPs using the optimized RP-HPLC showed that the method precision was within the acceptable range with RSD% <10, except for the second peak of PER where RSD% was 10.2.

Effect of injection solvent: One of the most problems encountered during HPLC method development is that the solvent of standard samples is usually different from the actual injection sample, which is dictated by the extraction and preconcentration protocol applied. The injection of sample in a solvent whose viscosity is different from that of the mobile phase may cause serious distortions in early eluting bands[29]. Thus, it was worthy to check the applicability of the developed chromatographic conditions for different injection solvents. For this purpose, the three SPs were spiked into aqueous samples, which were then extracted using different solvents. The extracting solvents were n-hexane, tetrachloroethane, dichloromethane, acetonitrile, methanol, and chlorobenzene. Our results showed that, except some changes in the peaks retention times, no significant peaks distortion was observed. These results indicated that the chromatographic conditions developed are robust and can be used for several injection solvents.

Table-2
Retention times (t_R), retention factors (k), separation factors (α) and resolution (Rs) for investigated pesticides under optimized chromatographic conditions

Compound	Peak elution order	t_R (min)		Retention factor (*k*)		Selectivity factor (α)		Resolution factor (Rs)		Column Plate number (N)	
		$\bar{x}$*	RSD%	$\bar{x}$	RSD%	$\bar{x}$	RSD%	$\bar{x}$	RSD%	$\bar{x}$	RSD%
CYP	1	18.40	0.88	6.76	0.99	1.05	0.99	1.6	2.66	6691	1.77
	2	19.08	0.84	7.31	0.94	1.04	0.17	1.0	3.96	8059	1.69
	3	19.80	0.88	7.89	0.98	1.04	0.07	1.1	2.39	7742	1.77
	4	20.90	0.87	8.59	0.96	1.06	0.08	1.6	1.49	7742	1.74
RES	5	22.70	0.83	9.51	0.91	1.10	0.09	2.2	0.93	10181	1.67
	6	24.60	0.81	10.47	0.88	1.09	0.23	2.1	2.69	9679	1.62
PER	7	26.96	0.83	11.60	0.90	1.10	0.06	2.1	1.23	9614	1.67
	8	32.71	0.97	13.89	1.04	1.23	1.04	4.5	5.22	14148	1.94
* mean value (5 replicates)											

Conclusion

While normal-phase HPLC is usually most suitable for separating synthetic pyrethroids, the reversed phase-HPLC method reported by this study has proved its robustness and efficiency for diastereomeric separation of three SPs. The sensitivity and accuracy of the method were also assessed. Mobile phase consisting of ACN and water provides shorter run time but with poor diastereomeric separation. On the other hand, mobile phase consisting of MeOH and water provides longer run time but with good diastereomeric separation of all analytes except the critical diastereomeric band. A ternary mixture of ACN, MeOH and water with a mixing ratio of 1:3:1, respectively, provides the optimum diastereomeric separation of all analytes. The method exhibited very good robustness and compatibility with various injection organic solvents, and the chromatographic separation was achieved at ambient room temperature.

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