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Journal of Chromatography A, 859 (1999) 133–142

JOURNAL OF CHROMATOGRAPHY A

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High-performance liquid chromatography study of the enantiomer separation of chrysanthemic acid and its analogous compounds on a terguride-based stationary phase

M. Dondi[a], M. Flieger[b], J. Olsovska[b], C.M. Polcaro[a], M. Sinibaldi[a,*]

[a]*Istituto di Cromatografia, CNR-Area della Ricerca di Roma, P.O. Box 10, I-00016 Monterotondo Stazione, Roma, Italy*

[b]*Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnskà 1083, C-142 20 Prague 4, Czech Republic*

Received 5 May 1999; received in revised form 6 August 1999; accepted 12 August 1999

Abstract

The direct enantioseparation of chrysanthemic acid [2,2-dimethyl-3-(2-methylpropenyl)-cyclopropanecarboxylic acid] and its halogen-substituted analogues was systematically studied by HPLC using a terguride-based chiral stationary phase in combination with a UV diode array and chiroptical detectors. Isomers with (1*R*) configuration always eluted before those with (1*S*) configuration. The elution sequence of *cis*- and *trans*-isomers was strongly affected by mobile phase pH, whereas the enantioselectivity remained the same. Conditions for the separation of all the enantiomers were also examined. This method was used for monitor the hydrolytic degradation products of Cyfluthrin (Baythroid) in soil under laboratory conditions.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Pyrethroids; Chrysanthemic acid; Terguride

1. Introduction

The new synthetic pyrethroid insecticides are of growing importance in both agricultural and domestic applications usage because of their high insecticidal activity, low toxicity to mammalian cells and adequate stability upon exposure to air and light [1]. Their chemical structures (Fig. 1) show that the asymmetric carbon atoms at positions C-1, C-3 and C-α give rise, in principle, to eight stereoisomers, two pairs of which have *cis*- and *trans*-configuration with respect to the plane of the cyclopropane ring. The individual isomers of pyrethroid esters differ widely in biological activities [2,3]. High insecticide toxicity is generally associated with the (1*R*) configuration of the chiral cyclopropane ring, adjacent to the carbonyl group [4]. Enantiomers differing in configuration at C-α also differ in toxicity [5]. Pyrethroid insecticides are synthesised and sold in the form of a single, most active isomer, or in mixtures containing two, four or eight different stereoisomers.

A considerable amount of information is available on the metabolism of synthetic pyrethroid insecticides in soil, plants and animals. Kaufman [6] studied the degradation of permethrin in soil, indicating the hydrolysis to permethrinic acid and 3-phenoxybenzyl alcohol as the principal mechanism of degradation. Similar results have been reported in studies of cypermethrin [7], fenpropathrin [8] and decamethrin [9]. Decamethrin was found to undergo several degradation reactions within 6 weeks of

*Corresponding author.

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PII: S0021-9673(99)00872-9

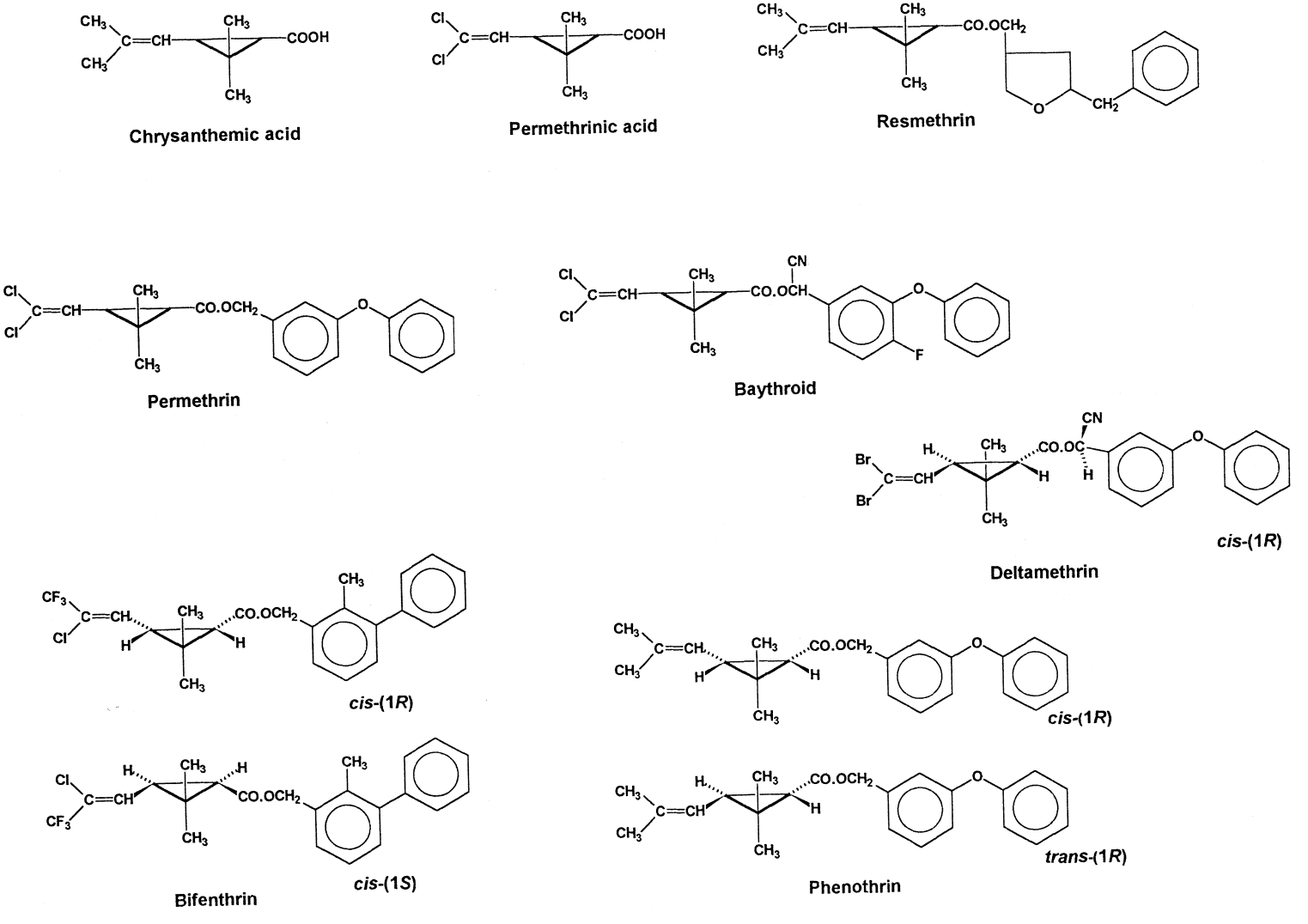

Fig. 1. Chemical structures of the examined compounds.

application to cotton leaves, including *cis-trans* interconversion and hydrolysis to the corresponding free cyclopropyl acids [10].

Environmental pyrethroid degradation is mediated by enzymes or other biologically active systems and involves reactions in chiral centres. This may lead to the stereoselective formation of metabolites [11]. Therefore, a quantitative determination of each isomer is required to describe the degradation of preparations and to assess the importance of residues. For example, interconversion reactions of phenoxyalkanoic acid herbicides in soil and water have recently been studied by Buser et al. [12,13]. These authors investigated the racemization rate of dichlorprop [2-(2,4-dichlorophenoxypropionic acid)] and mecoprop [2-(4-chloro-2-methylphenoxypropionic acid)] in soil, and found a significant enrichment of the (*S*)-isomer in the residues, irrespective of whether the racemic (*RS*) or enantiomeric pure (*R*)-form was incubated. To our knowledge, no similar study has so far been carried out on synthetic pyrethroid insecticides, despite the fact that their hydrolysis metabolites, i.e. corresponding free acids, exhibit a higher mobility in soil than the parent compounds [9]. These compounds may represent a broad class of chiral pollutants. The lack of available data may reflect the limited number of methods developed to distinguish enantiomeric metabolites of pyrethroids. In fact, pyrethroid free acid enantiomers are currently separated by either GLC of their diastereoisomers formed by reaction with a chiral agent [14], or HPLC using urea-type [15] or cellulose derivative-based CSPs [16]. This stimulated us to examine the enantioseparation of chrysanthemic acid and its halogen-substituted analogues on a terguride-based stationary phase. This CSP has recently been used successfully for the separation of 2-aryloxypropionic acids (herbicides) [17], and the recognition mechanism for 2-arylpropionic acids has been studied in detail [18]. A method for the determination of the hydrolysis degradation products of Cyfluthrin (Baythroid) in soil is also reported.

2. Materials and methods

2.1. Instrumentation

Chromatography was performed using a modular instrument consisting of a Series 400 Model (Perkin Elmer, Norwalk, CT, USA) solvent delivery pump equipped with a Rheodyne 7125 Model injection valve (10 or 50 μl injection loops). Detection was carried out with the following detectors: a 2550 Model (Varian, Walnut Creek, CA, USA) variable wavelength detector, a SPD-M6A Model (Shimadzu, Kyoto, Japan) UV–VIS photo-diode array detector, and a Polar-monitor (Büchi, Milan, Italy) detector (flow-cell volume 40 μl).

2.2. Reference compounds

Racemic chrysanthemum monocarboxylic acid ethyl ester [2,2-dimethyl-3-(2-methylpropenyl)cyclopropanecarboxylic acid ethyl ester], and (±) *cis*- and (±) *trans*-chrysanthemum carboxylic acids were purchased from Sigma (St. Louis, MO, USA). Bifenthrin [2-methylbiphenyl-3ylmethyl-(1*R,S*)-*cis*-3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylate], permethrin [(3-phenoxybenzyl)-(1*RS*)-*cis*-*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate], resmethrin [(5-benzyl-3-furylmethyl-(1*RS*)-*cis*-*trans*-2,2-dimethyl-3-(2-methylpropenyl)cyclopropanecarboxylate], phenothrin [(3-phenoxybenzyl)-(1*R*)-*cis*-*trans*-2,2-dimethyl-3-(2-methylpropenyl)cyclopropanecarboxylate] and deltamethrin [(*S*)-α-cyano-3-phenoxybenzyl-(1*R*)-*cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate] were obtained from LabService Analytica (Milan, Italy). (±) *cis*- and (±) *trans*-Permethrinic acid [3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylic acid] and Cyfluthrin (Baythroid) [(*RS*)-α-cyano-4-fluoro-3-phenoxybenzyl-(1*RS*)-*cis*-*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclo-propanecarboxylate] were a gift from Bayer (Leverkusen, Germany). Cyfluthrin is a mixture of eight stereoisomers that contains four pairs of diastereoisomers, the composition of which is [in accordance with Certified Assays provided by Bayer Pflanzenschutz Zentrum Monhein, Leverkusen, reference substance 96020ELB02, sum of I–IV isomers: 96.2%] *cis*-isomer I: [(1*R*,3*R*-α*R*)+(1*S*,3*S*-α*S*)]=22.8% (±0.09); *cis*-isomer II: [(1*R*,3*R*-α*S*)+(1*S*,3*S*-α*R*)]=17.2% (±0.09); *trans*-isomer III: [(1*R*,3*S*-α*R*)+(1*S*,3*R*-α*S*)]=33.3% (±0.10); *trans*-isomer IV: [(1*R*,3*S*-α*S*)+(1*S*,3*R*-α*R*)]=22.9% (±0.2).

The esters were hydrolysed in a 0.1 *M* sodium hydroxide methanol solution at room temperature for 48 h. The alkaline mixture was acidified with aqueous diluted (20% v/v) sulphuric acid and the corresponding free acid was then extracted with ethyl acetate and dried under vacuum.

2.3. Chromatographic procedure

The chiral stationary phase was prepared by reaction of glycidoxypropylsilanized silica gel (particle size 5 μm, average pore diameter 100 Å, Macherey-Nagel GmbH, Düren, Germany) with a solution of (+) (5*R*,8*S*,10*R*) 1-(3-aminopropyl)-terguride (Fig. 2) in methanol, according to the procedure described by Flieger et al. [19]. The material was packed into a stainless-steel tube (150×4.6 mm. I.D.) by conventional procedures. All solvents and other reagents used were of HPLC or analytical grade, and obtained from Carlo Erba (Milan, Italy).

Acetate buffers were prepared by adding potassium hydroxide to the acetic acid solution, monitoring the pH by means of a Crison 2000 micro pH meter, then filtering the mixture through Millipore (Bedford, MA, USA) GS type (0.22 μm) filter-disks.

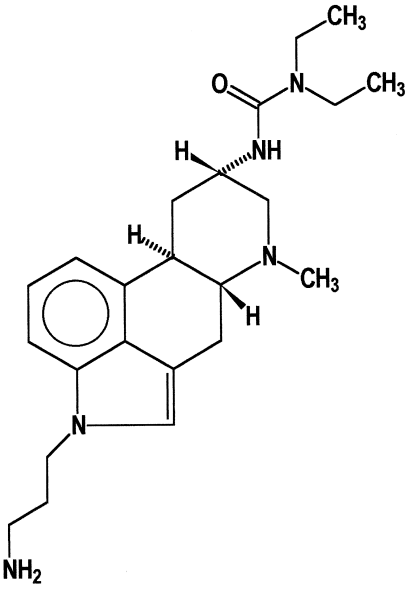

Fig. 2. Structure of (+) (5*R*,8*S*,10*R*) 1-(3-aminopropyl)-terguride.

Acetonitrile-buffer mixture solutions were degassed under the pressure of He before use. Sample solutions were prepared by dissolving approximately 1 mg of free acid in 1 ml of methanol and were stored at 4°C until use. Volumes of 1 μl, corresponding to 0.2 μg of sample, were directly injected into the column. A detection limit <0.01 ppm was calculated by setting the UV detector at 230 nm.

2.4. Determination of the pK_a value of trans permethrinic acid

The pK_a value of permethrinic acid was calculated following the method described by Ingelse et al. [20]. The experiment was performed by means of a HP^{3D} CE system (Hewlett-Packard, Waldbronn, Germany), equipped with a diode array detector operating at 215 nm, and using a 75 μm I.D.×386 μm O.D. inner coated fused-silica capillary.

2.5. Hydrolysis of Cyfluthrin in soil

2.5.1. Incubation procedure

Table 1 shows the chemical and physical characteristics of the soil sample. The soil was air-dried (one day at 20°C), then mixed and sieved through a 4 mm screen. A portion of the soil (30 g) was placed in a jar, wetted with 9 ml distilled water and mixed, then fortified with 300 μl of a solution containing 1.08 mg of Cyfluthrin dissolved in 2 ml methanol (fortification level corresponding to 5 ppm). The soil was carefully mixed and then incubated in the dark at 20–25°C after covering the jar with aluminium foil. The jar was opened daily for a short time aeration. After 15 and 30 days, about 15 g samples were submitted for analysis. Blank examination of the soil prior to fortification showed the absence of permethrinic acids.

2.5.2. Extraction and clean up procedures

After incubation, 20 ml of methanol–1 N HCl solution (9:1, v/v) were added to the soil sample under stirring. The mixture was centrifuged at 4,000 r.p.m. for 1 h, then the clear supernatant solution was transferred to a vial to which 6 ml distilled water were added. The solution was evaporated under

Table 1
Chemical and physical characteristics of the soil

Site	pH	CEC[a] meq/100 g	% O.S.[b]	% Sand	% Lime	% Clay	Type
Roma	6.9	15.4	4.1	59.3	17.0	23.7	sandy clay loam

[a] Cation-exchange capacity.
[b] Organic substance content.

vacuum to a volume of 18 ml, and the samples were extracted with three 6 ml portions of methylene chloride. The sample solution was water-dried with anhydrous sodium sulphate, dried under stream of He and dissolved in 100 μl methanol. Ten μl aliquots were directly injected into the chiral column.

3. Results and discussion

3.1. Separation of standard enantiomers

Standards of pyrethroid free acids were obtained by hydrolysis of a series of commercially available formulas in alcoholic potassium hydroxide at room temperature (Fig. 1). Although it is known that pyrethroids do not epimerize under the alkaline conditions used here [21], we identified the resolved peaks as enantiomeric compounds by the comparison of their retention times with those of standards (as in the cases of chrysanthemic and permethrinic acids) or by UV spectra using a photo-diode array detector (phenothrin, deltamethrin).

Chromatographic data are summarised in Table 2. The enantiomer separation of three free acids derived from synthetic pyrethroid insecticides is reported in Fig. 3. The chromatograms show the ability of the CSP to resolve all types of isomers in a few minutes, whenever the group in C-1 is substituted with halogen atoms. In order to determine the enantiomer sequence in the isomeric pairs, fractions of pure enantiomers prepared by replicate injections were analysed by spectropolarimetry. These measurements allowed assigning optical rotation (+) to less retained enantiomers with (*R*)-configuration.

3.2. Effect of mobile phase composition on retention and enantioselectivity

Table 2 indicates that column selectivity strongly depends on the polarity of the radical in C-3 position to the carboxylic group. The higher the hydrophobicity of the moiety, the better the resolution of the racemate. These findings are consistent with our assumptions concerning the chiral recognition on ergot alkaloid-based CSPs [22]. In fact, the structure

Table 2
Resolution of pyrethroid hydrolysates (free acids) on terguride-based chiral stationary phase. Chromatographic conditions: column, 150×4.6 mm I.D.; eluent, 20 m*M* acetate buffer (pH 4.0)–acetonitrile (6:4, v/v); flow-rate, 0.8 ml/min. Detector UV, 230 nm. Room temperature

Compound (as free acid)	*cis*-			*trans*-			
	$k'_{(1R)}$	$k'_{(1S)}$	α	$k'_{(1R)}$	$k'_{(1S)}$	α	R_S[a]
Chrysanthemic acid	1.92	1.92	1.0	4.14	4.45	1.08	2.1
Permethrinic acid	5.92	6.30	1.07	8.29	9.10	1.10	2.6
Resmethrin	1.92	1.92	1.0	4.14	4.45	1.08	2.1
Permethrin	5.92	6.30	1.07	8.30	9.10	1.10	2.6
Baythroid	5.92	6.30	1.07	8.29	9.10	1.10	2.6
Bifenthrin	6.71	7.64	1.14	–	–	–	–
Phenothrin	1.92	–	–	4.14	–	–	–
Deltamethrin[b]	3.45	–	–	–	–	–	–

[a] Resolution was only determined for the *trans*-isomer pairs.
[b] Mobile phase: acetate buffer–acetonitrile (4:6, v/v).

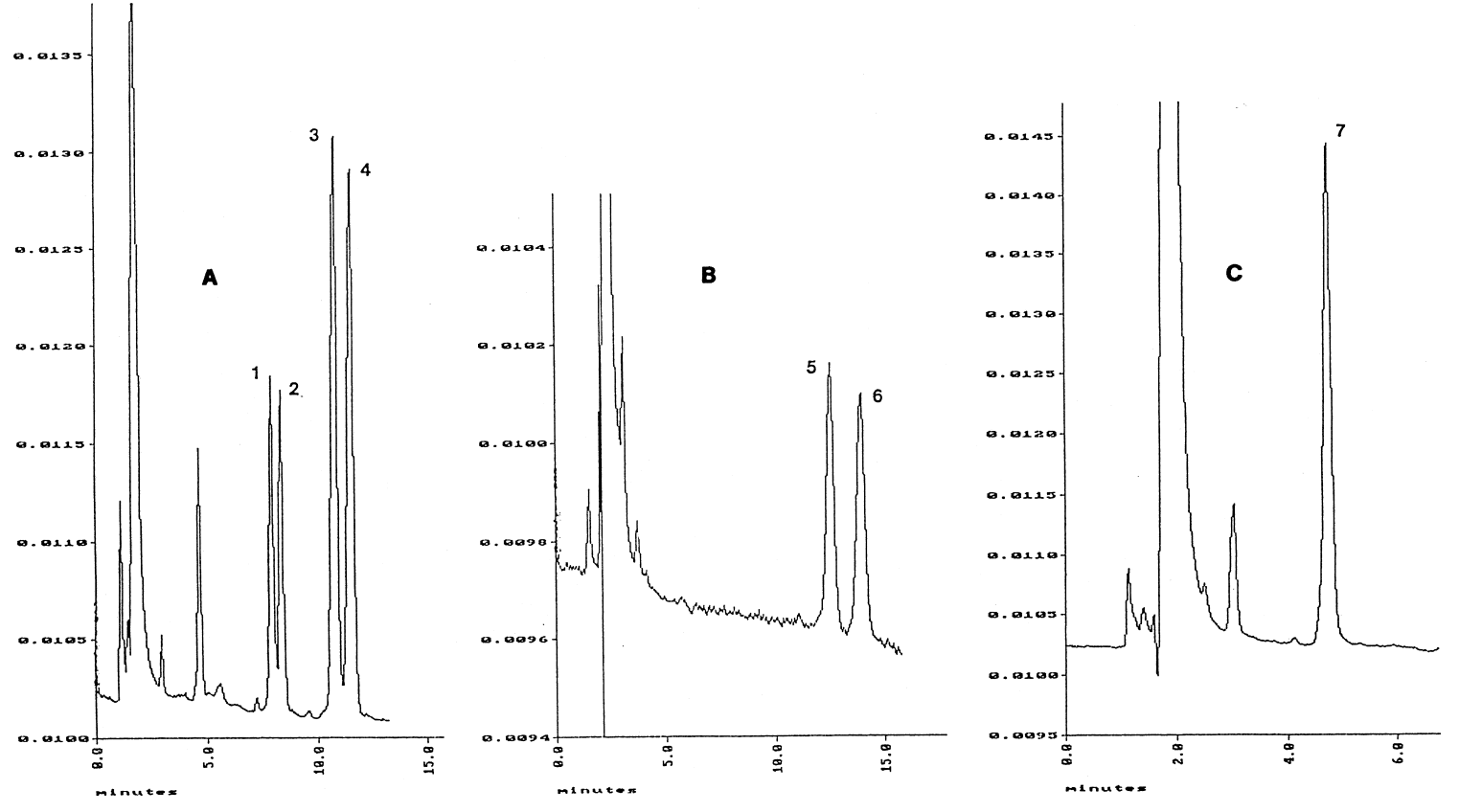

Fig. 3. Enantioseparation of pyrethroid free acids derived from hydrolysis of: (a) permethrin (1) *cis*-(1*R*), (2) *cis*-(1*S*), (3) *trans*-(1*R*), (4), *trans*-(1*S*); (b) bifenthrin (5) *cis*-(1*R*), (6) *cis*-(1*S*); (c) deltamethrin (7) *cis*-(1*R*). Chromatographic conditions, as in Table 2.

of diastereoisomeric complexes formed between terguride and 2-aryloxypropionic acid enantiomers showed that the enantiodiscrimination is partially governed by π–π interactions, implying high selectivity toward strong π electron donators [23].

Fig. 4 shows the influence of the mobile phase parameters on the retention and enantioseparation of permethrinic acid. Fig. 4a shows the effect of the buffer pH on the retention and selectivity of the *cis*- and *trans*-enantiomers of permethrinic acid and of *cis*-(1*RS*)-chrysanthemic acid. In general, as the pH value of the buffer solution increases, the capacity and selectivity factors of all four isomers decrease. When comparing the retention plots of *cis*- and *trans*-permethrinic acids with that of *cis*-(*RS*)-chrysanthemic acid, we can observe that all permethrinic acid isomers exhibit an apparently anomalous retention behaviour in the narrow range of pH values between 4.0 and 5.0. Here, the *cis*-permethrinic acid shows an increase in retention exceeding that of the *trans*-isomer. When pH is above 5.0 the retention and selectivity again start to decrease until pH 6.0. The enantiomers thus appear to be incompletely separated. The effect observed around pH 4.5 may be explained in terms of a secondary equilibrium in correspondence with the dissociation of the acid ($pK_a = 5.02$). Presumably, this secondary equilibrium determines the variation of the polarity of the compounds and, consequently, their different adsorption on the stationary phase. The retention of solutes on ergot alkaloid-based chiral stationary phases depends on the affinity of both carbonyl and carboxylic groups toward the nitrogen atoms present in the ergoline skeleton and in the ureic side chain [18]. The extent of such affinity is a direct function of the protonation degree of the carboxylic group. The increased retention of permethrinic acid is presumably due to a higher disposability of such functions for these interactions, which are in some way masked when pH is below 4. In comparison with the *trans*-isomer, the positional *cis*-isomer has a structure more suitable to undergo this effect. A detailed study of these equilibria and related structures in solution will be reported elsewhere. However, as a consequence of such equilibria, the *cis*-isomer appears to be retained more than the *trans*-isomer when completely dissociated. On the other hand, the presumed variation of polarity of the

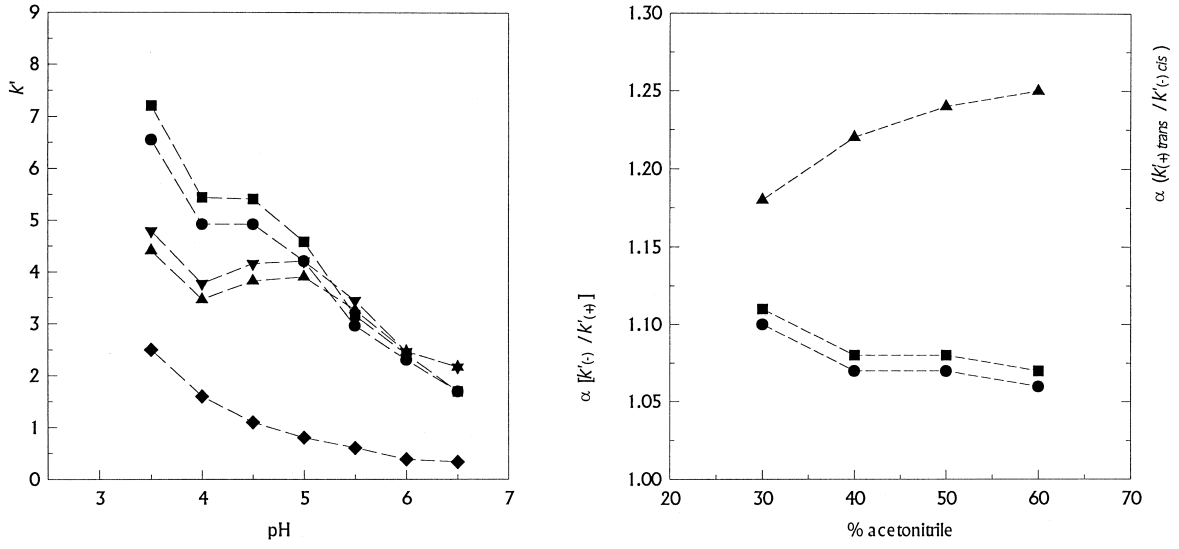

Fig. 4. Influence of the mobile phase parameters on the retention and enantioseparation of *cis*- and *trans*-permethrinic acid. (a) The effect of buffer pH for permethrinic acid (upper) and (♦) *cis*-chrysanthemic acid (lower). (b) The effect of acetonitrile on the enantioselectivity factor α [$k'_{(1S)}/k'_{(1R)}$] (lower, on the left) for *cis*- and *trans*-enantiomeric pairs of permethrinic acid, and the separation factor α [$k'_{trans\text{-}(1R)}/k'_{cis\text{-}(1S)}$] of the same acid (upper, on the right). Chromatographic conditions: column, 150×4.6 mm I.D.; eluent, (a) 20 m*M* acetate buffer–acetonitrile (1:1, v/v) [for *cis*-chrysanthemic acid a buffer–acetonitrile (6:4, v/v) mixture was used] and (b) 20 m*M* acetate buffer at pH 4.0; flow-rate, 0.8 ml/min. Detector UV, 230 nm.

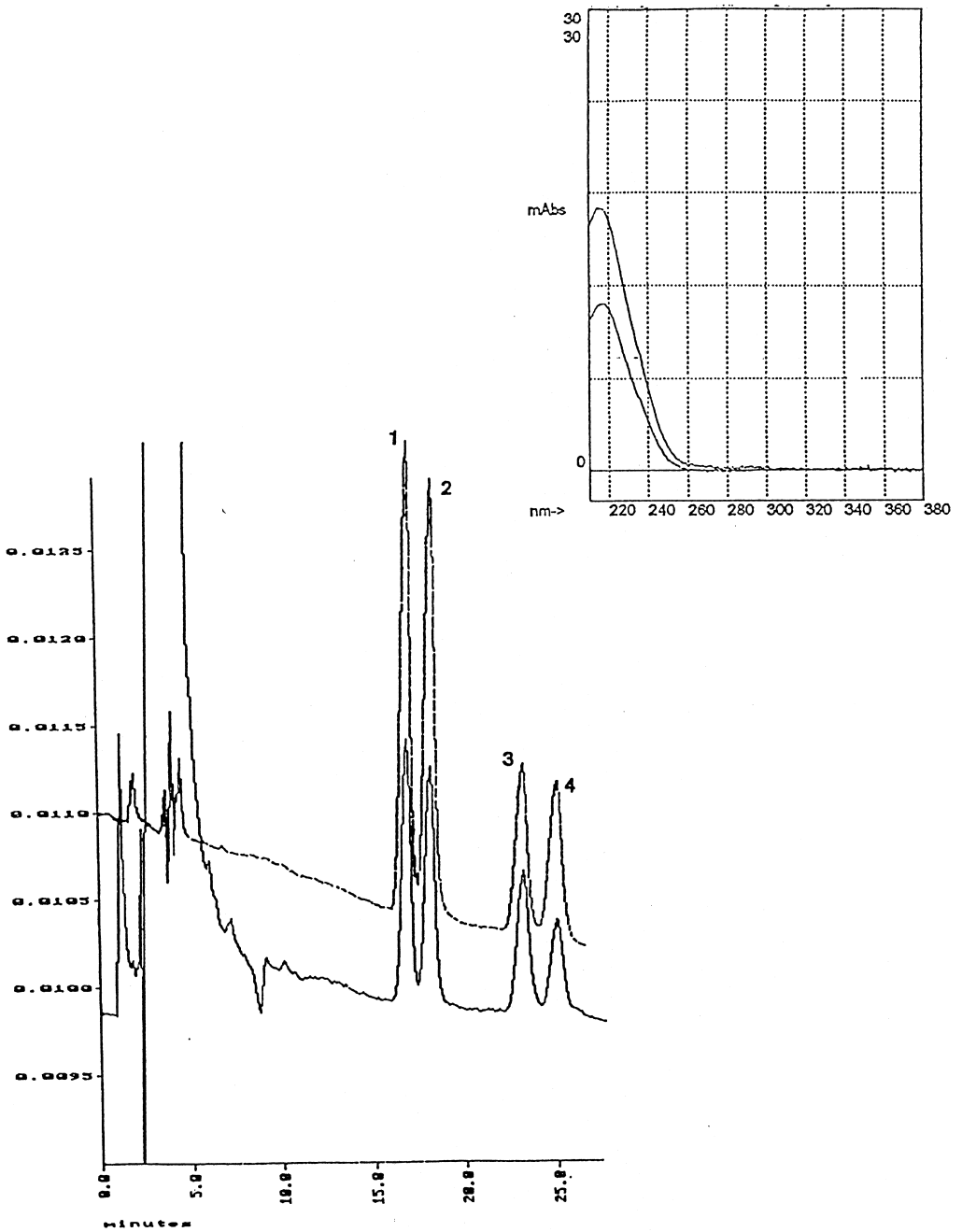

Fig. 5. Enantioseparation of: (solid line) metabolites of Cyfluthrin after 30 day incubation in soil and, (dotted line) permethrinic acid standards, (1) *cis*-(1*R*); (2) *cis*-(1*S*); (3) *trans*-(1*R*); (4) *trans*-(1*S*). Chromatographic conditions: eluent, 20 m*M* acetate buffer (pH 3.7)–acetonitrile (7:3, v/v). Detector UV, 230 nm; other conditions, as in Table 2. Upper, on the right: UV spectra of the two more retained metabolites.

Table 3
Isomeric (i.r.) and enantiomeric (e.r.) ratios of permethrinic acids found in extracts in function of incubation time of Cyfluthrin in soil under laboratory conditions. Chromatographic conditions: eluent, 20 m*M* acetate buffer (pH 4.0)–acetonitrile (6:4, v/v); detector UV, 230 nm. Other conditions, as in Table 2

Incubation time (day)	(i.r.)[a] *trans*/*cis*	(e.r.)[a]	
		trans-[(1*S*)/(1*R*)]	*cis*-[(1*S*)/(1*R*)]
0	–	–	–
15	1.02 (±0.02)	0.78 (±0.02)	0.98 (±0.02)
30	0.62 (±0.03)	0.65 (±0.03)	1.01 (±0.02)

[a] Standard deviations were calculated on the basis of triplicated injections.

molecule as a function of the buffer pH does not affect the resolution at all.

Optimal separation conditions for *cis-trans* enantiomer pairs were also studied. Fig. 4b shows that the selectivity of *cis*- and *trans*-pairs of enantiomers in the 30-60% acetonitrile (right, lower) is negligibly influenced by the organic modifier content (loss of about 2%). An improvement of the separation (10%) between the (−) *cis*- and (+) *trans*-isomers is observed with a high content of the organic solvent (left, upper). As a consequence, fast and baseline separation of all four isomers is achieved at low pH values (pH 3.5–4.0) and a high percentage of acetonitrile.

3.3. Hydrolysis of Cyfluthrin in soil

The separation of four species (solid line), arising by the hydrolysis of Cyfluthrin in soil, is shown in Fig. 5. These compounds were identified as permethrinic acid enantiomers by comparison with standards (Fig. 5, dotted line) and by UV diode array spectra. The effect of several degradation reactions within the incubation time of 30 days is shown in Table 3. General observations included: (a) interconversion from *trans*- to *cis*-isomer, which leads to a time-dependent variation of the isomeric ratio of permethrinic acids in the soil extract (0.62% of *trans*-free acids after 30 days incubation, as compared with the 1.4% total *trans*-isomers in Cyfluthrin prior to incubation). Leicht et al. [3] observed that, in protic solvents (mixtures of water and methanol), Cyfluthrin undergoes isomerization of its two active diastereoisomeric pairs *cis*-[(1*R*-α*S*)+(1*S*-α*R*)] and *trans*-[(1*R*-α*S*)+(1*S*-α*R*)] to the inactive ones; (b) a higher content of the *trans*-permethrinic acid (1*R*) enantiomer. These findings seem to be the result of the stereoselective degradation of Cyfluthrin probably due to enzymatically mediated metabolism, which leads either to a faster hydrolysis of *trans*-(1*R*) insecticide ester or a faster degradation of *trans*-(1*S*) permethrinic acid. Naturally, other reactions may also be involved. No change in the enantiomeric ratio was observed for the *cis*-isomer pair. Owing to the complexity of several interrelated reactions, a deeper insight into pyrethroid metabolism can only be obtained by studying the fate of individual enantiomers. An investigation in this direction is currently in progress.

Acknowledgements

The present study has been carried out within the framework of the Agreement on Scientific Co-operation between the National Research Council (Italy) and the Academy of Sciences of the Czech Republic. The authors are greatly indebted to Prof. A. Marino, Department of Chemistry, ''La Sapienza'' University of Rome (Italy), for providing the classified soil sample. Thanks are also due to Maura Pellegrini for technical assistance.

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