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# Direct high-performance liquid chromatographic method for enantioselective and diastereoselective determination of selected pyrethroic acids

Wolfgang Bicker, Michael Lämmerhofer*, Wolfgang Lindner

*Christian Doppler Laboratory for Molecular Recognition Materials, Institute of Analytical Chemistry, University of Vienna, Währingerstraße 38, A-1090 Vienna, Austria*

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**Abstract**

This study reports on the direct HPLC stereoisomer separation of selected pyrethroic acids employing commercial cinchona alkaloid derived chiral stationary phases (CSPs). *cis*/*trans*-Chrysanthemic acid (*cis*/*trans*-CA), *cis*/*trans*-chrysanthemum dicarboxylic acid (*cis*/*trans*-CDCA), *cis*/*trans*-permethrinic acid (*cis*/*trans*-PA), and fenvaleric acid (FA) were resolved into the individual stereoisomers, i.e. enantiomers and diastereomers as well. To achieve satisfactory baseline separation an optimisation of the variables of the chromatographic method including chemical structure of the cinchona carbamate CSP, mobile phase composition, and flow rate was required. All four stereoisomers of PA were successfully separated in a single run ($\alpha_{cis} = 1.20$, $\alpha_{trans} = 1.26$, critical $R_s = 1.65$) with an acetonitrile (ACN)-based polar-organic eluent. The complete baseline resolution of all CA stereoisomers succeeded in polar-organic ($\alpha_{cis} = 1.20$, $\alpha_{trans} = 1.35$, critical $R_s = 3.03$) as well as in acetonitrile-based reversed-phase media ($\alpha_{cis} = 1.24$, $\alpha_{trans} = 1.22$, critical $R_s = 2.73$). The latter elution mode was also found to be suitable for the enantio- as well as diastereoselective resolution of CDCA ($\alpha_{cis} = 1.09$, $\alpha_{trans} = 1.50$, critical $R_s = 1.43$), which is to the best of our knowledge the first reported enantiomer separation of this analyte. The enantiomers of FA could be baseline separated employing also reversed-phase mode ($\alpha = 1.16$, $R_s = 2.91$). These separation methods may be applied for quality control processes in the production of stereoisomerically pure insecticides as well as stereoselective toxicokinetic studies, as CDCA, PA, and FA are suitable biomarkers for monitoring human pyrethroid burden.


*Keywords:* Enantiomer separation; Pyrethroic acids

## 1. Introduction

Naturally occurring *pyrethrins* and their synthetic analogues *pyrethroids* are powerful insecticides that in the last decades increasingly have replaced organochlorine pesticides like DDT for pest control due to their relatively low mammalian toxicity and low environmental persistence.

Pyrethrins, the biologically active components of extracts of *tanacetum cinerariaefolium*, are enantiomerically pure 4-oxo-cyclopent-2-enyl ester derivatives of either chrysanthemic acid (CA), termed pyrethrins I, or of pyrethric acid, termed pyrethrins II, and were used for insecticidal purposes for more than a century. However, as these substances have only low photostability and are thus rapidly degraded after application, so-called synthetic "pyrethroids" possessing advantageous physico-chemical properties and additionally greater insecticidal activity were developed since the 1950s, to allow a wider use in agriculture and home gardens [1–5]. Like pyrethrins, most of the established pyrethroids are esters containing various acid and alcohol entities. Chirality may reside not only in the acid component, which is most often a derivative of 2,2-dimethyl-cyclopropane-1-carboxylic acid, but also in the alcohol moiety. For that reason, the applied insecticide is very often a mixture of several stereoisomers [3].

The impact of the stereochemistry of pyrethroids on their insecticidal potency and mammalian toxicity is well-known. The configuration at the stereogenic centres of the carboxylic moiety and/or the alcoholic part of a pyrethroid

* Corresponding author. Tel.: +43-1-4277-52323; fax: +43-1-4277-9523.
*E-mail address:* michael.laemmerhofer@univie.ac.at (M. Lämmerhofer).

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can determine qualitatively and quantitatively biological activity. For instance, a main mammalian pathway of biotransformation, i.e. enzymatically controlled cleavage of the ester bond, is known to implicate stereoselectivity [3,6–9] and leads to carboxylic acid type metabolites, which are excreted renally, partly in conjugated form. These compounds can serve as suitable biomarkers for monitoring pyrethroid burden. Several diastereoselective GC assays were recently developed for this purpose [10,11]. On the other hand, only one LC–MS method for the determination of a mammalian pyrethroid metabolite (3-phenoxybenzoic acid) was reported [12].

The use of the established non-enantioselective assays for the study of the metabolism of pyrethroids and for the comprehensive assessment of potential negative health effects caused by pyrethroid exposure may be inappropriate owing to the aforementioned stereoselectivity in biological activity. The problem of non-stereoselective analysis may be ascribed to 'false positives' arising from measuring a benign isomer and reporting it as if it is harmful.

Additionally, for the production of enantiomerically enriched or pure pyrethroids, enantiomers of so-called "pyrethroic acids" are utilised as precursors, which are either synthesised by stereoselective approaches or by resolution of the respective racemates [3,13,14]. Hence, stereoselective chromatographic separation methods of pyrethroic acids are worthwhile also for industrial quality control processes, or even for preparative scale production of single enantiomers thereof.

In this study, the HPLC stereoisomer separation of four pyrethroic acids, i.e. chrysanthemic acid (CA, 2,2-dimethyl -3- (2- methylprop -1- enyl) - cyclopropanecarboxylic acid), chrysanthemum dicarboxylic acid (CDCA, 3-[(1*E*)- 2-carboxyprop-1-enyl]- 2, 2 - dimethylcyclopropanecarboxylic acid), permethrinic acid (PA, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid), and fenvaleric acid (FA, 2-(4-chlorophenyl)-3-methylbutanoic acid) is presented (Fig. 1).

Table 1 summarises a selection of important industrial insecticides of the pyrethrin and pyrethroid family and their corresponding pyrethroic acid intermediates employed for their industrial production. The overview gives also information on the chemical nature of the acidic biomarkers resulting from the mammalian metabolism of these insecticides, for which the developed stereoselective methods might be of relevance.

By having a closer look at the stereochemistry of the pyrethroic acids depicted in Fig. 1 it is seen that CA, CDCA, and PA each contain two stereogenic centres in 1 and 3 position of the cyclopropane ring, thus resulting in two pairs of enantiomers in which the spatial orientation of the C-3 substituents relative to the C-1 carboxylic acid moiety at the cyclopropane ring is either in "cis" or "trans" configurational arrangement. This terminology is commonly preferred over *Z* and *E* descriptors as well as specification of both absolute configurations. For example, pyrethrins

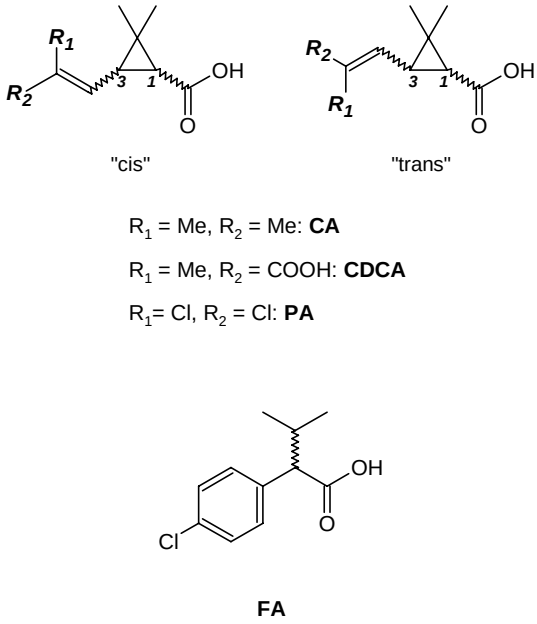


Fig. 1. Structures of the analytes investigated: chrysanthemic acid (CA), chrysanthemum dicarboxylic acid (CDCA), permethrinic acid (PA), fenvaleric acid (FA).

having natural (1*R*,3*R*)-configuration are classified as (1*R*)-*trans* isomers.

A few studies previously reported on the direct HPLC enantiomer separation of the pyrethroic acids CA, PA, and FA [15–21]. In contrast, to our knowledge no enantioselective HPLC, CE, CEC, or GC separation assay for CDCA was published yet, which is, however, an important biomarker of potential stereoselective metabolism of several pyrethroids.

For example, Chiralcel OD, Chiralcel OF, and Chiralpak AS were found to be suitable for the enantiomer resolution of *cis*/*trans*-CA and *cis*/*trans*-PA in the normal-phase mode [15,17]. Such conditions, however, might be disadvantageous for bioanalytical assays and aqueous samples.

Table 1
Pyrethrins and pyrethroids as well as their corresponding carboxylic acid building blocks (pyrethroic acids) and main acidic mammalian metabolites

| Pyrethroid (selection) | Pyrethroic acid | Biomarkers of metabolism[a] |
|---|---|---|
| Natural pyrethrum extract | CA, Pyrethric acid | CDCA |
| Allethrin<br>Phenothrin<br>Resmethrin<br>Tetramethrin | CA | CDCA |
| Cyfluthrin<br>Cypermethrin<br>Permethrin | PA | PA |
| Fenvalerate | FA | FA |

[a] Acidic metabolites resulting from oxidation of the alcoholic part of the pyrethroid are not considered here.

The same limitation holds for the enantiomer separation of *trans*-CA, *trans*-PA, and FA on brush-type chiral stationary phases (CSPs) with a chiral selector entity based on a triazine derivative of L-valyl-L-valyl-L-valine isopropyl ester in the normal-phase mode [18]. On the contrary, when such brush-type CSPs, e.g. based on amino acid derived selectors, were attempted for the enantiomeric resolution of pyrethroic acids in the reversed-phase mode, only a CSP based on L-*tert*-leucine-*N*-dinitrophenylurea (Sumichiral OA-3200) was found to be suitable to give satisfactory results for *cis*- and *trans*-CA as well as *cis*- and *trans*-PA [19]. In addition, supercritical fluid chromatography allowed separation of the enantiomers of *cis*-PA on ChirasilDex [21]. On the other hand, the (+)-terguride-based CSP previously tested for the enantioselective separation of CA and PA has its primary operational mode under reversed-phase conditions, but failed to separate *cis*-CA enantiomers, while *trans*-CA and *cis*- as well as *trans*-PA enantiomers could be resolved [16,20].

Herein, novel enantioselective anion exchangers based on carbamoylated cinchona alkaloid derivatives as molecular recognition entities (selectors) of the chiral stationary phase for the diastereoselective as well as enantioselective separation of CA, CDCA, PA, and FA are evaluated. The developed methods are intended to be applied: (i) for the semipreparative chromatographic production of single stereoisomer standards, and (ii) as stereoselective assay for metabolic and toxicological studies of carboxylic acid type pyrethroid metabolites.

## 2. Materials and methods

### 2.1. Materials

The chiral stationary phases used within this study were supplied by Bischoff Chromatography (Leonberg, Germany) and are also commercially available from Iris Technologies (Lawrence, KS, USA), or Mac-Mod Analytical (Chadds Ford, PA, USA). Fig. 2 depicts the structures of the investigated CSPs that are based on cinchona alkaloid derived chiral selectors, i.e. *tert*-butylcarbamoyl quinine (*tBu*-CQN; tradename: ProntoSIL AX QN-1), *tert*-butylcarbamoyl quinidine (*tBu*-CQD; ProntoSIL AX QD-1), 2,6-diisopropylphenylcarbamoyl quinine (DIPP-CQN; ProntoSIL AX QN-2), and 2,6-diisopropylphenylcarbamoyl quinidine (DIPP-CQD; ProntoSIL AX QD-2). All CSPs were packed into 150 mm × 4 mm i.d. stainless steel columns.

Acetonitrile (ACN) and methanol (MeOH), both of HPLC grade, were obtained from Fisher Scientific (Loughborough, UK). Analytical grade aqueous ammonia solution ($NH_3$ aq.), glacial acetic acid (HAc), and ammonium acetate ($NH_4Ac$) were purchased from Fluka (Buchs, Switzerland).

The test solutes chrysanthemic acid, permethrinic acid, and fenvaleric acid, cf. Fig. 1, were obtained by basic hydrolysis of chrysanthemic acid ethyl ester (Aldrich, Vienna, Austria), permethrin (the 3-phenoxyphenyl methyl ester of PA; Riedel de Haën, Seelze, Germany), and fenvalerate (the α-cyano-(3-phenoxyphenyl) methyl ester of FA; Riedel de Haën), respectively.

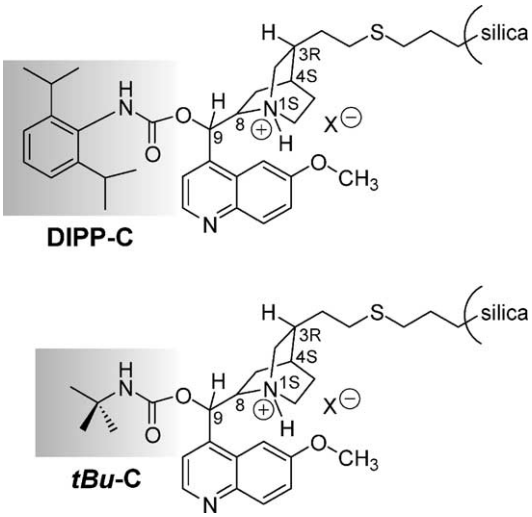


Fig. 2. Structure of the CSPs investigated: *tBu*-C: *tert*-butylcarbamoyl moiety; DIPP-C: 2,6-diisopropylphenylcarbamoyl moiety; QN: quinine (8*S*,9*R*); QD: quinidine (8*R*,9*S*).

Chrysanthemum dicarboxylic acid was prepared for the most part following a synthesis protocol described by Leng et al. [22]. Thus, chrysanthemic acid ethyl ester was oxidised with selenium dioxide to the corresponding aldehyde and was subsequently converted to the di-ester derivative of chrysanthemum dicarboxylic acid by reaction with sodium cyanide and manganese dioxide in methanol. After purification and diastereomer separation of the di-ester by flash chromatography basic hydrolysis yielded diastereomerically pure *cis*-CDCA (yield 28%) and *trans*-CDCA (yield 35%). $^1$H-NMR measurements of *cis*-CDCA and *trans*-CDCA were each performed with 10 mg of the respective sample dissolved in $d_6$-DMSO (Aldrich). Obtained shift values were virtually identical to those reported previously [22,23].

### 2.2. Instrumentation and HPLC conditions

HPLC experiments were carried out on an Agilent HP1090 high-performance liquid chromatograph equipped with a diode array detector (Agilent, Waldbronn, Germany). All runs were performed in isocratic mode at 25 °C with detection at 230 nm (reference wavelength 360 nm). The elution order was determined on-line by coupling of an optical rotation detector (Jasco OR-990, Biolab, Vienna, Austria). Unless otherwise stated, the flow rate was kept at 1.00 ml $min^{-1}$. The sample concentration was about 2 mg $ml^{-1}$ and the injection volume was 10 μl.

## 3. Results and discussion

### 3.1. CSP and mobile phase screening

Owing to the acidic nature of the analytes shown in Fig. 1 they should be amenable to separation on carbamoylated cinchona alkaloid based CSPs by enantioselective anion exchange. Besides the primary ionic interaction other binding increments support the selector–solute interaction and thus enantiorecognition. Amongst other effects the carbamate residue may play a key role as a steric interaction site for the successful enantiomer separation [24]. Such a steric interaction is in particular for the present solutes of utmost importance, since they lack both hydrogen bonding sites and strong π–π-interaction sites as well, which are usually favourable for the stereodiscrimination capability. Hence, the cinchona based CSPs have been made available in a variety of different carbamates, two of them commercially: *tert*-butyl carbamate (*tBu*-C) and 2,6-diisopropylphenyl carbamate (DIPP-C) of quinine (QN) and quinidine (QD) as well, cf. Fig. 2.

In general, it is difficult to foresee which of those chiral anion exchangers is most suitable in terms of enantiomer resolution capabilities for the present pyrethroic acids. All four CSPs depicted in Fig. 2 were therefore screened utilising four different eluents: methanol-based (mobile phase A) and acetonitrile-based (B) reversed-phase conditions with ammonium acetate buffer (hydroorganic mixture with 80% organic modifier adjusted to apparent pH of 6.0), as well as methanol-based (C) and acetonitrile-based (D) polar-organic phases containing 0.25% acetic acid (HAc). It is noted that the primary anion-exchange retention mechanism persists also in the polar-organic mode and acetate represents the counter-ion. In Table 2 the obtained results for the enantiomer resolution of the pyrethroic acids under investigation are summarised.

At first glance it becomes evident from the results of Table 2 that the enantiomers of *cis*/*trans*-CA, *cis*/*trans*-PA, *trans*-CDCA, and FA can be well separated both under reversed-phase as well as polar-organic eluent conditions. Even without further optimisation a baseline enantiomer separation (defined by $R_s > 1.5$) was easily achieved at least on one of the complementary CSPs for all analytes except for *cis*-CDCA. The latter analyte was only partially resolved in reversed-phase media (mobile phase B) on the DIPP-CQN CSP ($R_s = 0.82$).

The most outstanding influential parameter appeared to be the type of carbamate functionalisation of the cinchona alkaloid derived chiral stationary phase. Despite lower overall affinity and retention, in the majority of cases the aromatic CSPs DIPP-CQN and DIPP-CQD provided significantly higher values of enantioselectivity than the aliphatic *tBu*-CQN and *tBu*-CQD (Table 2).

For example, employing 0.25% HAc in acetonitrile as eluent (mobile phase D) the enantiomers of *cis*-CA were baseline separated on the *tBu*-CQN CSP within 3 min total run time ($k_1 = 0.70$, $\alpha = 1.34$, $R_s = 2.17$) and the separation was further improved on DIPP-CQN CSP ($k_1 = 0.65$, $\alpha = 1.87$, $R_s = 4.95$). Similar trends were observed for all other analytes, cf. Table 2.

Unlike other selectors derived from natural chiral pool compounds (such as cellulose, proteins, macrocyclic antibiotics, cyclodextrins) cinchona alkaloid selectors do exist in two quasi opposite configurational forms: quinine derivatives having (1*S*, 3*R*, 4*S*, 8*S*, 9*R*) configuration and quinidine analogues with opposite configuration at the stereogenic centres C-8 and C-9, cf. Fig. 2. Since the stereogenic centre at the carbon C-9 exerts stereocontrol the elution order may be reversed on the both opposite alkaloid derivatives, which are actually diastereomers but often behave like enantiomers, therefore called pseudo-enantiomers.

In fact, elution order for CA, CDCA, and PA was consistently (+) before (−) on QN-based CSPs for *cis* as well as *trans* stereoisomers and was reversed by changing to the corresponding QD-systems, regardless of type of carbamate residue. For FA the same trend was observed, but, as a single exception, on the *tBu*-CQN CSP employing mobile phase D elution order was (−) before (+) and no reversal was observed on the *tBu*-CQD CSP. This fact as well as non-equal levels of enantioseparation factors $\alpha$, especially for DIPP-CQN and DIPP-CQD CSPs, despite comparable selector loadings indicate that they are not real enantiomers. Individual stereoisomers of pyrethroic acids with known absolute configurations as reference compounds for the assignment of absolute configurations of the herein separated stereoisomers were not available. In the SciFinder Scholar Database absolute configurations are reported for various optical isomers of pyrethroic acids (Table 3). These data could be helpful to assign the absolute configurations based on the optical rotation data of the present study. Unfortunately, the solvents that have been used for the optical rotation measurements have not been specified nor any original literature. Therefore, an assignment of absolute configurations based on the tabulated SciFinder data still needs to be confirmed and validated.

In stereoselective liquid chromatography, the molecular recognition process is mediated by the mobile phase which therefore determines to a large extent the success of enantiomer separation. The four selected eluents have been supposed to be a good choice for an initial screening, as they are to some extent complementary. In particular, the different solvatochromic properties of methanol and acetonitrile containing mobile phases may lead to significant enantioselectivity differences as a result of distinct solvation of functional groups and binding sites, respectively, and/or of diastereomeric selector–solute complexes. This is indeed the case as evident from the results of the screening presented in this work.

ACN-based eluents provided significantly higher $\alpha$ values and resolution compared to their MeOH-based analogous system both in the reversed-phase as well as the polar-organic mode. This holds not only for the DIPP-CQN

Table 2
Enantiomer separation of selected pyrethroic acids on four complementary commercially available cinchona alkaloid derived CSPs employing reversed-phase and polar-organic media

| Compound | *tBu*-CQN | | | *tBu*-CQD | | | DIPP-CQN | | | DIPP-CQD | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_1$[a] | $R_s$ | $\alpha$ | $k_1$[a] | $R_s$ | $\alpha$ | $k_1$[a] | $R_s$ | $\alpha$ | $k_1$[a] | $R_s$ | $\alpha$ |
| Mobile phase A [10 mM ammonium acetate in methanol–water = 80:20 (v/v), $pH_a$ 6.0 (HAc)] | | | | | | | | | | | | |
| *cis*-CA | 1.04 | 1.70 | 1.24 | 0.74 | 1.32 | 1.24 | 0.79 | 3.06 | 1.59 | 0.80 | 2.11 | 1.40 |
| *trans*-CA | 1.76 | 1.60 | 1.13 | 1.23 | 0.99 | 1.15 | 1.27 | 2.03 | 1.27 | 1.32 | 1.16 | 1.15 |
| *cis*-CDCA | 9.95 | 0.00 | 1.00 | 6.46 | 0.00 | 1.00 | 5.08 | 0.00 | 1.00 | 4.69 | 0.00 | 1.00 |
| *trans*-CDCA | 14.24 | 1.68 | 1.13 | 9.68 | 1.40 | 1.11 | 8.35 | 2.65 | 1.26 | 7.26 | 0.81 | 1.10 |
| *cis*-PA | 4.54 | 1.71 | 1.14 | 3.71 | 1.60 | 1.14 | 3.44 | 2.26 | 1.24 | 3.44 | 1.90 | 1.22 |
| *trans*-PA | 4.79 | 1.25 | 1.10 | 3.73 | 1.18 | 1.10 | 3.47 | 2.09 | 1.23 | 3.52 | 0.78 | 1.14 |
| FA | 6.93 | 0.61 | 1.04 | 5.32 | 0.70 | 1.06 | 5.38 | 1.52 | 1.12 | 5.05 | 0.95 | 1.09 |
| Mobile phase B [10 mM ammonium acetate in acetonitrile–water = 80:20 (v/v), $pH_a$ 6.0 (HAc)] | | | | | | | | | | | | |
| *cis*-CA | 0.65 | 2.17 | 1.34 | 0.42 | 0.99 | 1.22 | 0.70 | 4.95 | 1.87 | 0.63 | 3.13 | 1.52 |
| *trans*-CA | 1.02 | 2.11 | 1.19 | 0.74 | 1.59 | 1.24 | 1.13 | 3.73 | 1.45 | 0.99 | 2.18 | 1.24 |
| *cis*-CDCA | 7.42 | 0.00 | 1.00 | 7.02 | 0.00 | 1.00 | 6.69 | 0.82 | 1.07 | 5.77 | 0.00 | 1.00 |
| *trans*-CDCA | 10.66 | 2.21 | 1.16 | 9.72 | 2.02 | 1.14 | 10.52 | 4.78 | 1.45 | 8.67 | 1.54 | 1.18 |
| *cis*-PA | 2.57 | 2.14 | 1.19 | 2.10 | 2.55 | 1.23 | 2.99 | 6.35 | 1.61 | 2.60 | 3.20 | 1.43 |
| *trans*-PA | 2.84 | 2.01 | 1.15 | 2.31 | 1.61 | 1.13 | 3.17 | 5.06 | 1.39 | 2.79 | 2.50 | 1.22 |
| FA | 3.95 | 0.00 | 1.00 | 3.02 | 1.07 | 1.08 | 4.35 | 2.32 | 1.15 | 3.70 | 1.71 | 1.14 |
| Mobile phase C [0.25% acetic acid in methanol] | | | | | | | | | | | | |
| *cis*-CA | 0.32 | 0.88 | 1.28 | 0.25 | 0.89 | 1.30 | 0.19 | 1.83 | 1.79 | 0.22 | 1.28 | 1.40 |
| *trans*-CA | 0.57 | 1.37 | 1.23 | 0.46 | 0.88 | 1.22 | 0.48 | 1.70 | 1.42 | 0.39 | 0.84 | 1.20 |
| *cis*-CDCA | 3.75 | 0.00 | 1.00 | 2.83 | 0.00 | 1.00 | 1.91 | 0.00 | 1.00 | 1.62 | 0.00 | 1.00 |
| *trans*-CDCA | 6.47 | 2.25 | 1.17 | 5.09 | 1.47 | 1.12 | 3.92 | 3.22 | 1.32 | 3.23 | 1.10 | 1.12 |
| *cis*-PA | 1.54 | 2.50 | 1.15 | 1.31 | 1.96 | 1.20 | 0.96 | 2.36 | 1.30 | 1.01 | 1.28 | 1.17 |
| *trans*-PA | 1.84 | 1.78 | 1.15 | 1.58 | 1.53 | 1.15 | 1.15 | 2.49 | 1.28 | 1.24 | 1.41 | 1.16 |
| FA | 2.72 | 0.62 | 1.04 | 2.18 | 1.06 | 1.09 | 1.69 | 1.52 | 1.13 | 1.77 | 0.83 | 1.10 |
| Mobile phase D [0.25% acetic acid in acetonitrile] | | | | | | | | | | | | |
| *cis*-CA | 1.80 | 2.40 | 1.16 | 1.74 | 2.08 | 1.18 | 1.34 | 4.80 | 1.67 | 1.41 | 3.63 | 1.41 |
| *trans*-CA | 2.60 | 3.57 | 1.22 | 2.64 | 2.74 | 1.18 | 2.23 | 4.02 | 1.38 | 2.31 | 2.57 | 1.23 |
| *cis*-CDCA | >35 | n.d. | n.d. | >35 | n.d. | n.d. | 41.00 | 0.00 | 1.00 | >35 | n.d. | n.d. |
| *trans*-CDCA | >35 | n.d. | n.d. | >35 | n.d. | n.d. | 65.04 | 4.74 | 1.52 | >35 | n.d. | n.d. |
| *cis*-PA | 6.59 | 2.63 | 1.16 | 6.61 | 3.76 | 1.26 | 5.37 | 4.97 | 1.46 | 5.62 | 3.66 | 1.39 |
| *trans*-PA | 6.88 | 3.61 | 1.22 | 7.24 | 2.68 | 1.19 | 5.84 | 4.57 | 1.41 | 6.40 | 2.55 | 1.28 |
| FA | 8.86 | 0.47 | 1.02[b] | 8.66 | 1.32 | 1.08 | 7.45 | 1.89 | 1.13 | 8.05 | 1.82 | 1.14 |

Elution order: (+) before (−) on QN-based CSPs and (−) before (+) on QD-based CSPs; *T*, 25 °C; flow rate, 1.00 ml $min^{-1}$.
[a] $k_1$: retention factor of first eluted enantiomer.
[b] Elution order: (−)-FA before (+)-FA.

CSP where this effect is most obvious, but with a few exceptions (*cis*-CA and FA on the *tBu*-CQN CSP) also for the other CSPs.

The use of polar-organic eluents, i.e. employing 0.25% HAc in MeOH (mobile phase C) and ACN (mobile phase D), respectively, resulted in major differences regarding retention factors compared to hydroorganic mode. Generally, elution times employing eluent C were by a factor of about five lower compared to eluent D independent of the chemical nature of CSP and analyte. This may be ascribed to the differences in the hydrogen-donor capabilities of the both organic solvents, which is clearly an important parameter in mediating the hydrogen-bond supported ionic interaction between chiral selector and analyte, thus determining chiefly the extent of retention. For the dicarboxylic acid CDCA this effect was even more pronounced, resulting in extraordinarily large run times (>120 min) on all CSPs with the ACN-based mobile phase D. All other analytes were monocarboxylic acids, and the retention factors were therefore substantially lower ($k_1 < 9$) being in an acceptable range. However, one has to keep in mind that a fast elution may be even detrimental, especially in bioanalytical assays where a problem would arise from poorly retained analytes due to interference with early eluted matrix components.

To summarise briefly, it can be stated that DIPP-functionalised CSPs (especially DIPP-CQN) are highly enantioselective towards the pyrethroic acids under investigation. In reversed-phase as well as polar-organic mode baseline separations of the enantiomers of *cis*-CA, *trans*-CA, *cis*-PA, *trans*-PA, and FA on the DIPP-CQN CSP with α values between 1.12 and 1.87 could easily be achieved with run times typically between 5 and 15 min. For CDCA retention was generally higher due to its dicarboxylic character and *cis*-CDCA enantiomers could only be resolved on the DIPP-CQN CSP in the reversed-phase mode employing the ACN-based eluent. On contrary, *trans*-CDCA is much

Table 3
Relationship between optical rotation and absolute configuration of selected pyrethroic acids (SciFinder Scholar Database, cited 19.11.2003)

| Nomenclature | CAS-number | Absolute configuration |
|---|---|---|
| (+)-*cis*-CA | 26771-11-9 | (1*R*,3*S*) |
| (−)-*cis*-CA | 26771-06-2 | (1*S*,3*R*) |
| (+)-*trans*-CA | 4638-92-0 | (1*R*,3*R*) |
| (−)-*trans*-CA | 2259-14-5 | (1*S*,3*S*) |
| (+)-*cis*-CDCA | – | – |
| (−)-*cis*-CDCA | – | – |
| (+)-*trans*-CDCA | 33383-55-0 | (1*S*,3*S*) |
| (−)-*trans*-CDCA | 72120-98-0 | (1*R*,3*R*) |
| (+)-*cis*-PA | 55667-40-8 | (1*R*,3*R*) |
| (−)-*cis*-PA | 55701-08-1 | (1*S*,3*S*) |
| (+)-*trans*-PA | 55701-03-6 | (1*R*,3*S*) |
| (−)-*trans*-PA | 55701-09-2 | (1*S*,3*R*) |
| (+)-FA | 55332-38-2 | (*S*) |
| (−)-FA | 63640-09-5 | (*R*) |

better separated into the individual enantiomers under such conditions.

The above screening provided enough information about the most promising CSP and mobile phase to be used. However, it has to be emphasised that for practical purposes some additional considerations have to be taken into account. With the exception of fenvaleric acid the other pyrethroic acids are existing as two pairs of enantiomers (*cis*/*trans*), see Fig. 1, and many pyrethroids are applied as mixtures of their diastereomers. Thus, a simultaneous separation of all four stereoisomers would be mandatory and was therefore the aim of further optimisation experiments.

### 3.2. Optimisation of the stereoisomer separation of chrysanthemic acid (CA)

Although the DIPP carbamate phases emerged from the screening as the most enantioselective ones, their diastereoselectivity was not sufficient to allow a simultaneous separation of *cis*- and *trans*-CA without overlap of bands. On contrary, *tBu*-functionalised CSPs gave more promising results in terms of diastereoselectivity and for that reason the *tBu*-CQN CSP was chosen for further optimisation using ACN-based eluents.

In the hydroorganic media, the apparent pH ($pH_a$) decisively influences the anion-exchange mechanism by controlling the degree of dissociation and protonation of analyte and chiral selector entity, respectively. A pH-study was therefore performed to gain knowledge how this parameter affects separation results (see Fig. 3).

It is seen that the eluent $pH_a$ significantly influences extent of ionic interactions between negatively charged analyte and positively charged chiral selector as evidenced by the dependencies of retention factors (Fig. 3a). Especially between $pH_a$ 6.5 and 8 retention factors of both *cis*- and *trans*-CA

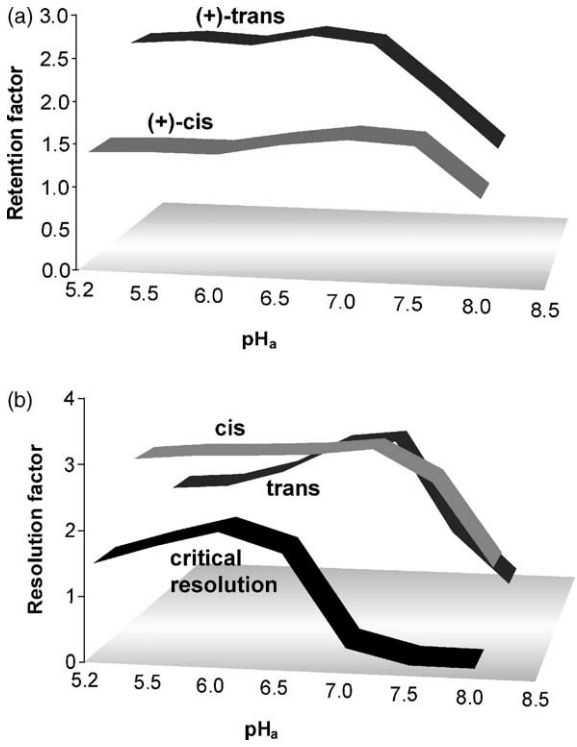


Fig. 3. $pH_a$-profile of the retention factor of the first eluted enantiomer of *cis*- and *trans*-chrysanthemic acid (CA) (a) and the respective enantioresolution and critical resolution (b) on the *tBu*-CQN CSP. Mobile phase: 10 mM HAc in ACN–water = 80:20 (v/v), $pH_a$ stepwise adjusted with $NH_3$ aq.; *T*, 25 °C; flow rate, 1.00 ml $min^{-1}$.

start to decrease, which may be mainly ascribed to a depletion of actual ion-exchange capacity. This, in turn, affects negatively the enantio- as well as diastereorecognition capabilities of the CSP. A constant resolution of *cis*-CA enantiomers is afforded between $pH_a$ 5 and 7, while the *trans*-CA enantiomers are even slightly better resolved between $pH_a$ 7 and 7.5. The more relevant figure for a simultaneous separation of all four stereoisomers, on contrary, is the critical resolution ($R_s^{crit}$), i.e. the resolution between neighbouring peak pairs that shows the lowest value. It is obvious from Fig. 3b that this value is highest between $pH_a$ 5.5 and 6.5, which represents also the pH range of superior robustness. Thus, it can be concluded that for CA and the *tBu*-CQN CSP a $pH_a$ between 5.5 and 6.5 is optimal in terms of separation parameters and in particular critical resolution.

Although the latter parameter turned out to be sufficient for a simultaneous separation of all four stereoisomers (cf. Fig. 3), the effect of organic modifier content (ACN) was further investigated. Since there is a reversed-phase type retention and separation mechanism superimposed upon the primary anion-exchange retention principle, the amount of organic modifier can play a predominant role for enantiomer separation and in particular for diastereoselectivity. For example, when the ACN content in the hydroorganic eluent was reduced from 90 to 70% (10 mM HAc in ACN–water, $pH_a$ 6.0) enantioselectivity of *cis*-CA was slightly increased

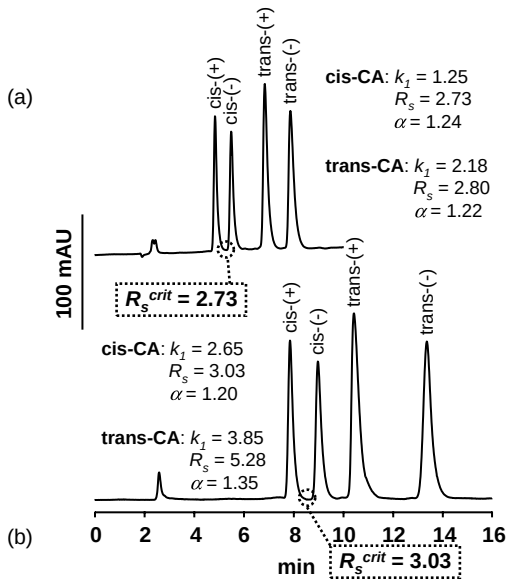


Fig. 4. Separation of CA on the *tBu*-CQN CSP under optimised reversed-phase (a) and polar-organic (b) conditions. Reversed-phase eluent: 10 mM HAc in ACN–water = 90:10 (v/v), $pH_a$ 6.0 ($NH_3$ aq.); polar-organic eluent: 0.06% HAc in ACN–MeOH = 95:5 (v/v); *T*, 25 °C; flow rate, 0.65 ml $min^{-1}$.

while the effect was negligible for *trans*-CA. Since peak efficiencies and thus resolution values, in particular $R_s^{crit}$, were worse, the mobile phase containing 90% ACN was found to be preferable, also with regard to run times, which were shorter by a factor of ∼1.5.

In recent studies, a slow linear flow velocity turned out to be favourable in terms of peak efficiencies owing to slow mass transfer kinetics. The van Deemter plots confirmed the validity of this dependency for the present separation system and analytes, and the optimal flow rates were found in the range of 0.5–0.75 ml $min^{-1}$. This resulted in a highly efficient and fast baseline separation of all four isomers of CA (run time <10 min) which is depicted in Fig. 4a.

The polar-organic mode employing ACN-based mobile phase D promised, also usefulness for CA stereoisomer separation on the *tBu*-CQN CSP. Addition of a small amount of MeOH (5%), reduction of HAc to 0.06%, and lowering the flow rate to 0.65 ml $min^{-1}$ improved the separation so that a baseline separation of all isomers of CA could be achieved within 15 min, cf. Fig. 4b.

It may be concluded that both separation modes, reversed-phase and polar-organic as well, are suitable, but polar-organic conditions seem to be a better choice due to the higher critical resolution ($R_s^{crit}$ = 3.03) compared to reversed-phase mode ($R_s^{crit}$ = 2.73).

### 3.3. Optimisation of the stereoisomer separation of permethrinic acid (PA)

Permethrinic acid has the two methyl groups of the alkenyl substituent of CA substituted by two chlorine atoms (see Fig. 1). This has a marked impact on the chromatographic behaviour: While the enantiomers of *cis*-CA had always a lower affinity towards the chiral selector than the both *trans*-CA enantiomers, PA showed in general a more complex elution pattern, with reversals occurring upon changes of experimental conditions. This behaviour unfortunately is prone to partial peak overlap affecting the diastereoselectivity while the elution order of (+)-enantiomers before (−)-enantiomers (on QN based CSPs) did not change. Hence, the optimisation of the simultaneous separation of all four PA isomers appeared to be more challenging compared to chrysanthemic acid. Similar to CA, for the separation of PA isomers optimisation attempts were performed on the *tBu*-CQN CSP with reversed-phase as well as polar-organic conditions due to the higher diastereorecognition capabilities of this CSP compared to the DIPP-CQN CSP.

Using the methanol-based reversed-phase mode the $pH_a$-value of the hydroorganic mobile phase turned out to be the major influential variable not only for enantioselectivity, but in particular for diastereoselectivity as is illustrated in Fig. 5.

Upon change of the $pH_a$ from 7.0 to 5.1, the elution order of *cis*- and *trans*-isomer pairs was inverted due to a stronger increase of retention factors for the *trans*-isomers compared to *cis*-isomers. This dependency caused a peak overlap of diastereomeric components in the intermediate pH range, e.g. $pH_a$ 6.0. Interestingly, essentially the same behaviour was observed by performing an analogous $pH_a$-study with an ACN-based eluent.

Since none of the separation conditions presented in Fig. 5 was sufficient for full resolution of all four isomers, the polar-organic mode was also tested owing to promising results of the preliminary screening study. Like for CA the addition of certain amounts of MeOH to the polar-organic eluent had a major influence on separation results (Fig. 6).

It turned out that a content of 5% MeOH in ACN is optimal and allows an elution of the four stereoisomers with nearly equal band spacing ($R_s^{crit}$ = 1.42 between (+)-*trans* and (−)-*cis*). Again a reduction of the concentration of acetic acid (counter-ion) from 0.25 to 0.10% and a decrease of the flow rate to 0.65 ml $min^{-1}$ resulted in a significant improvement of the critical resolution ($R_s^{crit}$ = 1.65 between (+)-*cis* and (+)-*trans*) and a successful separation of all isomers of PA in a single run. Replacing methanol by ethanol or 2-propanol did not positively influence separation results of PA. The optimised chromatogram together with the enantiomer separation data is given in Fig. 7.

The critical resolution of the separation shown in Fig. 7a appears, although for many applications sufficient, still somewhat low. As result from the screening study it was found that the DIPP-CQN CSP provided higher enantioselectivity, while the *tBu*-CQN CSP afforded significantly better diastereoselectivity. The combination of the high enantioselectivity of the DIPP-CQN CSP with the diastereoselectivity of the *tBu*-CQN CSP by simple serial column coupling may afford an improved overall selectivity.

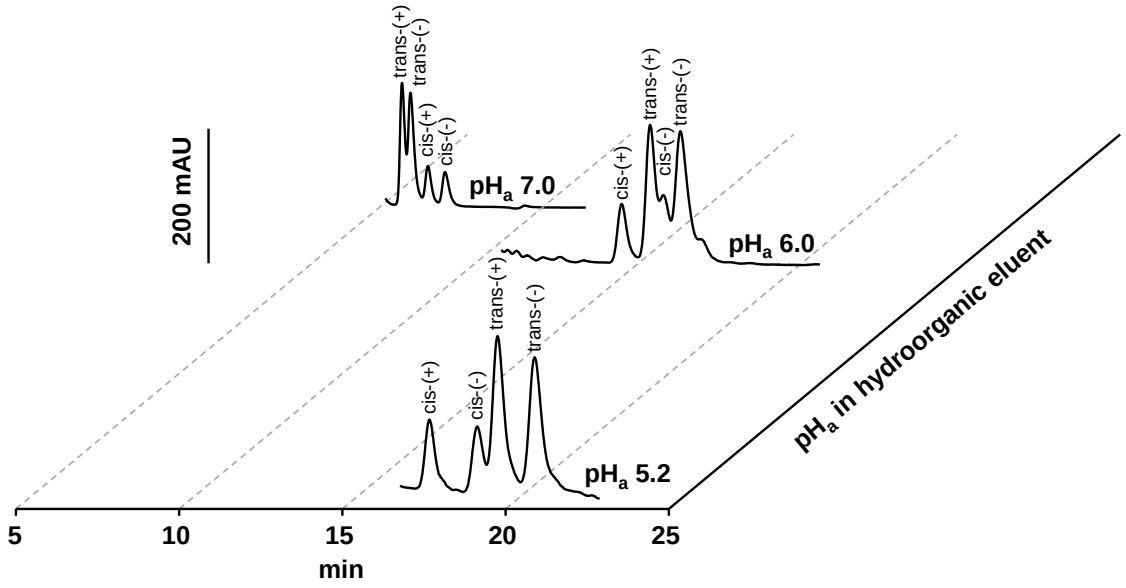


Fig. 5. pH$_a$-profile of the enantiomer separation of permethrinic acid (PA) on the *tBu*-CQN CSP. Mobile phase: 10 mM HAc in MeOH–water = 80:20 (v/v), pH$_a$ stepwise adjusted with $NH_3$ aq.; *T*, 25 °C; flow rate, 1.00 ml min$^{-1}$.

As the total length of the separation bed was now 30 cm, the back-pressure of the system increased, but due to the low viscosity of the polar-organic mode was still quite tolerable. To compensate for the longer run times that are a result of the longer separation bed the amount of HAc in the polar-organic eluent was increased to 0.25% and flow rate was set to 1.00 ml min$^{-1}$. This in-line column coupling revealed a critical resolution of 2.02 between (+)-*cis* and (+)-*trans* without further optimisation experiments (cf. Fig. 7b). Such a resolution was assessed to be satisfactory for the intended purpose.

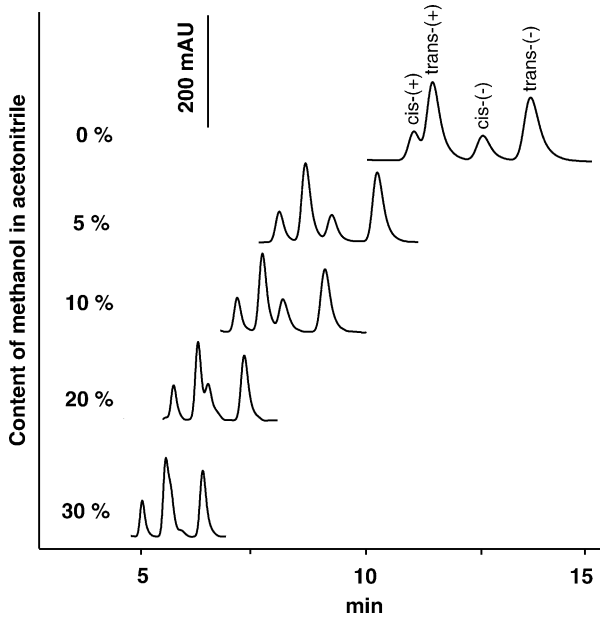


Fig. 6. Separation of PA on the *tBu*-CQN CSP in polar-organic mode. Mobile phase: 0.25% HAc in ACN containing 0–30% MeOH; *T*, 25 °C; flow rate, 1.00 ml min$^{-1}$.

### 3.4. Optimisation of the stereoisomer separation of chrysanthemum dicarboxylic acid (CDCA)

The enantiomer separations of *cis*- and *trans*-CDCA, which are important metabolites most likely formed stereoselectively, are of special interest because enantioselective separation systems for these enantiomer pairs have not yet been reported. Compared to CA and PA, CDCA experiences due to its dicarboxylic character and bivalency much stronger ionic forces and in turn a substantially higher retention in the anion-exchange process for given conditions. Moreover, the enantiomer separation of *cis*-CDCA caused considerable difficulties and was only possible on the DIPP-CQN CSP with mobile phase B ($R_s = 0.82$) as was discussed previously, while diastereoselectivity was no problem at all. On contrary, *trans*-CDCA enantiomers can easily be baseline separated on all four CSPs investigated ($R_s^{max} = 4.78$; Table 2). Hence, the optimisation studies for CDCA were carried out with focus on finding a compromise between sufficient resolution of *cis*-CDCA enantiomers and an acceptable run time. The same optimised reversed-phase type eluent that was found to be suitable for the enantiomer separation of chrysanthemic acid (cf. caption to Fig. 4) provided satisfactory separation of *cis*-CDCA enantiomers on the *tBu*-CQN CSP, but required long analysis time (110 min). An increase of the ionic strength (from 10 to 30 mM HAc) in order to accelerate the separation had as consequence a slight reduction in resolution. Although still sub-optimal with regards to run time (∼85 min), an overall quite useful separation system with reversed-phase

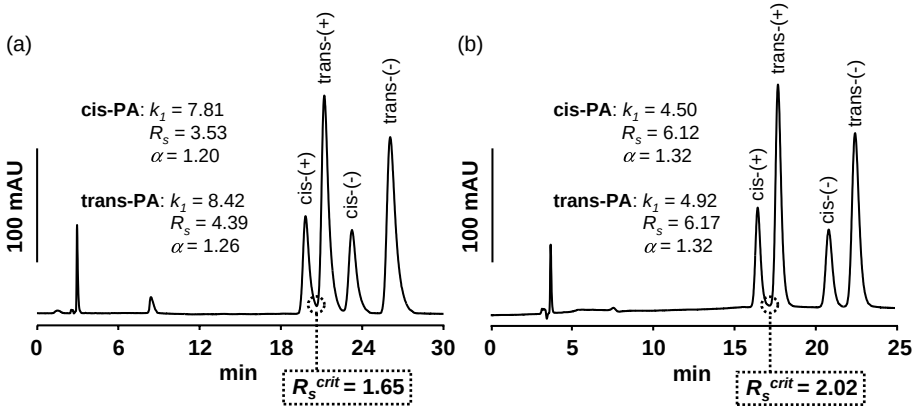


Fig. 7. Separation of PA on the *tBu*-CQN CSP under optimised polar-organic conditions. Mobile phase: 0.10% HAc in ACN–MeOH = 95:5 (v/v); *T*, 25 °C; flow rate, 0.65 ml min$^{-1}$ (a) and employing a dual column coupling separation system with a *tBu*-CQN CSP and a DIPP-CQN CSP in series. Mobile phase: 0.25% HAc in ACN–MeOH = 95:5 (v/v); *T*, 25 °C; flow rate, 1.00 ml min$^{-1}$ (b).

type eluent was the result with the critical resolution between *cis*-CDCA enantiomers ($R_S^{crit}$ = 1.43; Fig. 8). The polar-organic mode did not provide any better results.

### 3.5. Optimisation of the stereoisomer separation of fenvaleric acid (FA)

Fenvaleric acid differs considerably in its chemical structure from the other pyrethroic acids (cf. Fig. 1); inter alia, it contains only one stereogenic centre. The optimisation was therefore straightforward and the more enantioselective DIPP-functionalised CSPs, i.e. DIPP-CQN and DIPP-CQD, afforded baseline separation of FA enantiomers even with "standard" conditions employing eluent B and D (see Table 2). Although sufficient for analytical purposes, a further improvement of FA enantiomer separations in reversed-phase mode on the DIPP-CQN CSP could be obtained by reducing the ionic strength and flow rate, and increasing the content of ACN in the hydroorganic mixture (Fig. 9).

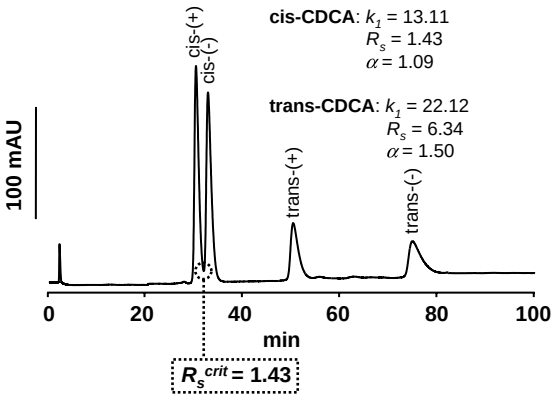


Fig. 8. Separation of chrysanthemum dicarboxylic acid (CDCA) on the DIPP-CQN CSP under optimised reversed-phase conditions. Mobile phase: 30 mM HAc in ACN–water = 90:10 (v/v), $pH_a$ 6.0 ($NH_3$ aq.); *T*, 25 °C; flow rate, 0.65 ml min$^{-1}$.

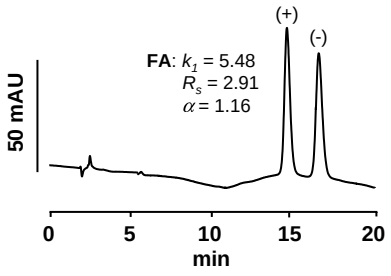


Fig. 9. Separation of fenvaleric acid (FA) on the DIPP-CQN CSP under optimised reversed-phase conditions. Mobile phase: 30 mM HAc in ACN–water = 90:10 (v/v), $pH_a$ 6.0 ($NH_3$ aq.); *T*, 25 °C; flow rate, 0.65 ml min$^{-1}$.

## 4. Conclusions

HPLC methods employing anion-exchange type chiral stationary phases based on functionalised (carbamoylated) cinchona alkaloid moieties as chiral selectors were developed for the enantiomer separation of four pyrethroic acids, i.e. chrysanthemic acid (CA), chrysanthemum dicarboxylic acid (CDCA), permethrinic acid (PA), and fenvaleric acid (FA). Based on results from a CSP and mobile phase screening (four complementary cinchona alkaloid derived CSPs and four different mobile phases) optimisation steps including type of chiral stationary phase, eluent mode (hydroorganic/polar-organic) and composition, as well as flow rate were performed. With exception of FA (only one stereogenic centre) and CDCA, the critical factor was the limited diastereoselectivity between neighbouring *cis*- and *trans*-stereoisomers of the pyrethroic acids having two stereogenic centres. Sufficient diastereo- as well as enantioselectivity was found for a *tert*-butylcarbamoyl quinine (*tBu*-CQN) based CSP that allowed the baseline separation of all four isomers of CA with reversed-phase and polar-organic media. *cis* and *trans* stereoisomers of PA were successfully resolved on *tBu*-CQN with a polar-organic mobile phase, whereas for the enantiomer separation of CDCA

and FA, respectively, 2,6-diisopropylphenylcarbamoyl quinine (DIPP-CQN) as chiral selector of the CSP employing a reversed-phase eluent was most suitable for enantiomer resolution.

As all mobile phases investigated herein are miscible with water, the presented separation conditions are well compatible with samples of bioanalytical studies. CDCA, PA, and FA are all main mammalian metabolites of pyrethroid degradation and are for that reason suitable biomarkers of pyrethroid burden. The chromatographic methods presented herein might be the basis to allow the elucidation of stereoselectivity in metabolic pathways of these xenobiotics. Hence, the present study is a preliminary work for a LC–MS assay that will be developed for toxicokinetic studies of selected pyrethroids. This topic is currently under investigation in our laboratory. Apart from this, for quality control processes the determination of the stereoisomer ratio of CA, PA, and FA might be of interest for companies that market pyrethroid containing insecticides with enriched stereoisomers.

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