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A CAPILLARY MICELLAR ELECTROKINETIC CHROMATOGRAPHY METHOD FOR THE STEREOSELECTIVE QUANTITATION OF BIOALLETHRIN IN BIOTIC AND ABIOTIC SAMPLES

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Highlights

- A high resolution stereoselective CE methodology is proposed for bioallethrin
- Application to the analysis of bioallethrin stereoisomers in a commercial formulation is shown
- Toxicity parameters were obtained using bioallethrin real concentrations
- Toxicity of bioallethrin was higher than that of esbiol in algae
- Total inhibition of germination was observed for bioallethrin and esbiol

ABSTRACT

A capillary micellar electrokinetic chromatography (MEKC) method was developed enabling the stereoselective separation of the insecticide bioallethrin. The use of sodium deoxycholate bile salt and acetyl-β-cyclodextrin (acetyl-β-CD) made possible the separation of bioallethrin stereoisomers with a high enantioresolution (7.4) in a short analysis time (6.5 min). The analytical characteristics of the developed method were evaluated in terms of linearity, accuracy, precision, and limits of detection (LOD) and quantitation (LOQ) showing a good performance for the quantitation of bioallethrin stereoisomers with LODs of 0.2 and 0.3 mg/L. The developed method was applied to the stereoselective analysis of a commercial bioallethrin pediculicide formulation

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and to evaluate the toxicity of bioallethrin stereoisomers on the growth of the unicellular freshwater green alga *Pseudokirchneriella subcapitata* and on the germination of the higher plant *Sorghum bicolor* (non-target organisms). The analysis of the commercial pediculicide showed a good agreement between the contents determined for the two stereoisomers and those labelled in the commercial samples. Different toxic responses and biodegradation profiles were found for each stereoisomer in ecotoxicity assays. The mixture of S/R stereoisomers of bioallethrin resulted more toxic than S-bioallethrin for green algae, with EC50 values of 1.10 ± 0.06 for the mixture and of 1.73 ± 0.05 mg/L for the pure S-biallethrin (esbiol). Germination of plants seeds was also affected.

Keywords: bioallethrin / capillary electrophoresis / micellar electrokinetic chromatography/ cyclodextrins / pyrethroid insecticide / toxicity / non-target organism

1. Introduction

Stereochemistry is an important issue in environmental analysis because the stereoisomers of agrochemicals or pollutants can have different activities, toxicities or degradation rates [1, 2]. When an agrochemical contains several chiral centers in its structure (for instance pyrethroids), the different routes followed to synthesize it may affect the ratio in which its isomers are present so that it is essential to determine the amount of each isomer before assessing its biological activity. Moreover, when a mixture of the isomers of an agrochemical is used, if only one of the stereoisomers has the desired activity, an unnecessary amount of the agrochemical is released to the environment to obtain the same result as if only the active isomer would be employed [3]. As a consequence, the use of pure agrochemical formulations demanded by regulatory agencies can avoid the undesirable effects of the use of some stereoisomers that may have toxic effects against non-target organisms [4, 5]. On the other hand, stereochemistry is not usually considered when analyzing environmental pollutants and this originates that the measurement of ecotoxicity, bioavailability and accumulation would be made improperly.

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A correct evaluation of environmental risk derived from the use of pesticides needs a revision of toxicity data reported. In fact, the toxicity parameters were usually estimated using nominal concentration of pollutant and the real concentration in the exposition assay was generally not measured. As a consequence, and as reported by Katagi for synthetic pirethroids [6], probably the EC50 values are lower than those corresponding to the real degradation in the environment, both in abiotic and biotic conditions. Maund *et al.* reviewed and compared toxicity data for synthetic pyrethroids on non-target organisms [7]. Authors found that the ecotoxicity of pyrethroids is high for aquatic and terrestrial non-target arthropods being fishes also sensitive, but significant differences in toxicity values from µg/L to mg/L were revealed and the discrepancies were attributed to dissipation and mitigation in field experiments. In any case, more attention should be paid on the non-target organisms, and considering the stereochemistry of most agrochemicals and pollutants, the impact of stereoisomers must be studied more in deep, taking into account diffuse contamination, mobility and bioaccumulation of these compounds.

Bioallethrin ((*RS*)-3-allyl-2-methyl-4-oxocyclopent-2-enyl (1*R*,3*R*)-2,2-dimethyl -3-(2-methylprop-1-enyl) cyclopropanecarboxylate) is a 1:1 mixture of [1R, trans; 1R] and [1R, trans; 1S] of the allethrin, an insecticide belonging to the pyrethroids family which is frequently used in agriculture, forestry, house-hold and public health. Bioallethrin is bioaccumulative, persistent in soil, slowly degradable in the environment and moderately toxic by skin and ingestion absorption. Exposure to large doses by either of the two absorption pathways can cause nausea, lack of coordination, tremors, convulsions, muscle paralysis, as well as, damage to the liver as it acts as an endocrine disruptor. Furthermore, bioallethrin is a central nervous system stimulant due to its interaction with the sodium channels in the membranes of neuronal cells [8, 9]. The isomer [1R, trans; 1S] of bioallethrin (esbiol) originates severe damage to blood immunocompetent cells, being this effect higher when combined with piperonyl butoxide, an insecticide often found in commercial formulations of bioallethrin [10].

Although some articles and two book chapters published in the literature provide interesting information about analysis of pyrethroids by chromatographic and electromigration methods [3, 11-17], the literature dealing with the stereoselective separation of bioallethrin is scarce. The separation of

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bioallethrin stereoisomers was carried out by HPLC using different types of chiral columns [18-21], GC [20], and capillary micellar electrokinetic chromatography (MEKC) [22]. Cayley and Simpson reported the baseline separation of bioallethrin isomers using an ionic Pirkle column and a mobile phase of 0.1% propan-2-ol in hexane in 30 min being S-bioallethin (esbiol) the first-eluting stereoisomer [18]. ÔI *et al.* separated allethrins isomers (including bioallethrin) with a good resolution using a Sumichiral OA-4600 column and hexane-dicloroethane-ethanol (500/30/0.5 v/v/v) mixture as mobile phase in 48 min [19]. Kutter and Class carried out the separation of four *trans*-allethrin isomers into three baseline separated peaks on a chiral β-CD HPLC column. In addition, eight isomers of allethrin gave rise to six partially resolved peaks by GC in 90 min when a coupled column consisting of permethylated-β-cyclodextrin and achiral DB 1701 was employed [20]. Zhou *et al.* separated bioallethrin stereoisomers by HPLC with two synthesized new cellulose chiral stationary phases and studied the influence of isopropyl alcohol concentration in a hexane mobile phase obtaining resolutions ≤ 1.6 [21]. As far as we know, only one work has been published dealing with the stereoselective separation of bioallethrin by MEKC [22]. In that work, a sodium cholate/hydroxypropyl-β-cyclodextrin mixture in phosphate buffer at pH=7.0 was employed. By using this BGE, a resolution value of 2.4 for bioalletrhin isomers was achieved in ~ 8.6 min [22]. The developed methodology was applied to the analysis of water samples fortified with different pesticides including bioallethrin. It is also interesting to point out the research work carried out by Chu *et al.* [23] which described both a MEKC and a microemulsion electrokinetic chromatography (MEEKC) methodologies to perform the stereoselective separation of esbiothrin, that is an approximately 1:3 mixture of [1R, *trans*; 1R] and [1R, *trans*; 1S] allethrin isomers [23]. The results obtained demonstrated that both methods provided similar results in terms of resolution (Rs ≈ 3), analysis time (≈ 15 min) and LODs although better separation efficiencies were reached in the presence of the microemulsion.

Regarding the ecotoxicity of bioallethrin, it has scarcely been studied on non-target organisms. Toxicity data of both bioallethrin and esbiol on fishes have been reported on different database [24, 25] but the values of EC50 are very different ranging from μg/L to mg/L.

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The aim of this work was to develop a rapid analytical methodology by MEKC for the separation of bioallethrin stereoisomers, to apply this methodology to the quantitative analysis of bioallethrin in commercial insecticide formulations and to evaluate the toxicity of its stereoisomers on non-target organisms as unicellular freshwater green alga *Pseudokirchneriella subcapitata* and on the higher plant *Sorghum bicolor.*

2. Materials and methods

2.1. Reagents and samples

Boric acid, sodium hydroxide and sodium taurocholate (STC) were purchased from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) was from Merck (Damstadt, Germany). Urea and methanol were from Scharlab (Barcelona, Spain). Sodium deoxycholate (SDC), sodium cholate (SC) and bioallethrin were supplied by Fluka (Buchs, Switzerland). Esbiol was supplied from Santa Cruz Biotechnology (Heidelberg, Germany). Water used to prepare solutions was purified through a milli-Q System from Millipore (Bedford,MA, USA).

Carboxymethylated-β-cyclodextrin (CM-β-CD) (DS~3), Sulfated-β-cyclodextrin (Sulfated-β-CD) (DS~7-11), 6-monodeoxi-6-monoamine-β-cydlodextrin, β-cyclodextrin (β-CD), 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) (DS~0.6), Heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD) and γ-cyclodextrin (γ-CD) were purchased from Fluka. Methyl-β-cyclodextrin (Me-β-CD) (DS~10.-14.7), Heptakis (2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) from Sigma. Carboxymethylated-γ-cyclodextrin (CM-γ-CD) (DS~3), Acetyl-γ-cyclodextrin (Ac-γ-CD) (DS~7), 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD), Methyl-γ-cyclodextrin (Me-γ-CD) (DS~12), and Acetyl-β-cyclodextrin (Ac-β-CD) (DS~7-8) were provided by Cyclolab (Budapest, Hungary).

MicroBioTest Inc. (Mariakerke/Ghent, Belgium) provided algal beads of *Pseudokirchneriella subcapitata*, *Sorghum bicolor* seeds, dissolving matrix and growing media.

The commercial formulation analyzed (pediculicide spray) was acquired in a drug store of Alcalá de Henares (Madrid, Spain).

2.2 CE-UV conditions

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An HP^{3D}CE instrument from Agilent Technologies (Palo Alto, CA, USA) with a diode array detector (DAD) working at 220 nm with a bandwidth of 4 nm, and a response time of 1 s was employed. The HP^{3D}CE ChemStation (Agilent Technologies) was used to control the CE system. Uncoated fused-silica capillaries of 50 μm I.D. (375 μm O.D.) with a total length of 58.5 cm (50 cm effective length) were from Polymicro Technologies (Phoenix, AZ, USA).

New capillaries were rinsed (applying 1 bar) with methanol for 5 min, 1 M NaOH for 25 min followed by 5 min with Milli-Q water and finally 60 min with the separation buffer (BGE). Each capillary was conditioned each day with 0.1 M NaOH for 10 min, Milli-Q water for 5 min and BGE for 25 min, rinsed between injections with 0.1 M NaOH for 4 min, Milli-Q water for 2 min and BGE for 4 min and with Milli-Q water for 5 min, 0.1 M NaOH for 5 min and 5 min Milli-Q water at the end of the day.

2.3. Preparation of stock and sample solutions

In order to prepare buffer solutions, the appropriate amount of boric acid was dissolved in Milli-Q water and the pH adjusted at pH 8.0 with 1 M NaOH. Milli-Q water was added to complete the volume necessary to reach the desired buffer concentration (100 mM). The appropriate amounts of different CDs, surfactants and urea were dissolved in the buffer solution to obtain the BGEs.

The preparation of stock standard solutions of bioallethrin and esbiol was carried out by dissolving each standard in methanol up to a final concentration of 2000 mg/L and 1000 mg/L, respectively. Sample solution of the pediculicide spray of bioallethrin was prepared by diluting the appropriate amount in methanol to obtain a concentration of approximately 1000 mg/L of bioallethrin (taking into account the labelled amount). Finally, standards and sample solutions were diluted in methanol in order to obtain the desired concentration.

All the solutions were stored in brown glass flasks at 4°C in the dark until use. Before injection in the CE system they were degassed in an ultrasonic bath and filtered through 0.45 μm pore size nylon filters from Scharlab (Barcelona, Spain).

2.4 Toxicity study

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An Ibercex F cultivation chamber from Ibercex S.L. (Spain), which includes light and temperature controls, was employed to carry out the incubation of organisms. Culture media was sterilized using a Presoclave II from Selecta (Spain). Handling of media and organisms was done into a laminar flow cabinet Telstar AV-30/70 (Telstar S.L. (Spain)). A spectrophotometer UV-VIS 1800 from Shimadzu (Japan) was used to measure the absorbance during preparation of inoculum of algae. Natural fluorescence of chlorophyll in algal culture was measured using a Fluoroskan FL, Thermo Fisher (Finland), fixing excitation and emission wavelengths at 450 and 672 nm, respectively. Twenty upper reading per well was done at room temperature.

2.4.1. Toxicity on green algae

The de-immobilization of *P. subcapitata* algal cells was achieved following the manufacturer's recommendations. The determination of multigenerational exposure toxicity was achieved in Erlenmeyers flask through the algal growth inhibition test described in OECD Test Guide 201 open system [26]. Algal cells were first cultivated in 25 mL shaken flasks (inoculum) in which algal biomass was determined by absorbance at 670 nm. During exposure toxicity, samples were daily taken to follow the growing of algae population and bioallethrin concentration. Algal cells were also cultivated in microplates, according to previous works [27-29]. Plates and flasks were incubated in a growing chamber at 22 ± 2 ºC under continuous light irradiation and light intensity controlled at 6000 lux. *P. subcapitata* growth was monitored during 72 h, measuring the *in vivo* fluorescence emission of chlorophyll in microplates.

Bioallethrin or esbiol were added to algal culture media and diluted to the concentration assayed. In parallel with exposition assay, a control assay without bioallethrin was incubated, and blank assays without algae to measure the background fluorescence were prepared. Each condition was replicated four times in three independent series of assays.

The biological responses obtained from algae cultivation were evaluated using the median effect/combination index (CI)-isobologram equation as reported by Chou [30]. The calculation is based on the median-effect principle that assumes that there is a univocal relationship between dose and effect regardless the number of compounds and their mechanism of action. The median effect equation is as follows:

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$$\frac{f_a}{1-f_a}=\left(\frac{D}{EC_{50}}\right)^m$$

where D is the dose affecting a fraction f_a and EC50 represents the dose at which a 50 % effect (growth inhibition) is obtained. The exponent m represents the sigmoidicity of the dose-effect curve, where m = 1 for hyperbolic, m > 1 for sigmoidal and m < 1 for negative sigmoidal dose-effect curves. The EC50 (potency) and m (shape) values for each stereoisomer were determined by computing experimental f_a and D values. We can also estimate the EC20 values. The dose D used for toxicity calculation is the real concentration (average value of the concentrations of each stereoisomer in the period of time in which these are constant) in culture media determined by the CD-MEKC methodology developed in this work.

2.4.2. Toxicity on plant

Toxicity on plants was evaluated through seeds germination of the higher plant *S. bicolor*, included in the list of the OECD guideline for testing of chemicals on terrestrial plants [31]. Assays were carried out into 24-well incubation plates, adding 1 mL of liquid and 1 seed per well. The Steinberg medium for fitotoxicity test was used (OECD Test Guide 221 and ISO 20079) [32, 33].

Similar to algae toxicity test, control assays without bioallethrin or esbiol and blank assays without seeds were incubated. The nominal concentration assayed was 10 mg/L of bioallethrin or esbiol, and solutions were prepared by dilution of the concentrated solutions in methanol. Each condition was replicated four times in three independent series of assays. Incubation was done at 22 ± 2 ºC under continuous light irradiation (6000 lux) during six days. Germination was daily observed.

2.5. Bioallethrin biodegradation

Algae cells and plants seeds were incubated using their specific media and bioallethrin or esbiol (biotic conditions) to carry out stability control. The incubation was carried out using flasks with higher working volume to facilitate

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sampling. Samples were daily taken, filtered by a membrane of 0.22 μm pore size and analyzed according to the analytical methodology developed in this work.

3. Results and discussion

3.1 Development of an analytical methodology for the stereoselective separation of bioallethrin by CE.

Taking into account that bioallethrin is a neutral compound in all the pH range with a low solubility in water (log P_0 = 4.7) [34], the easiest strategy to try its stereoselective separation was the use of a charged pseudophase. Thus, the discrimination power of three different anionic cyclodextrins (CM-β-CD, CM-γ-CD and Sulfated-β-CD) and one cationic cyclodextrin (6-monodeoxi-6-monoamine-β-CD) was investigated at a concentration of 15 mM in 100 mM borate buffer (pH 8.0). These experiments were carried out using a working temperature of 20 ºC, a separation voltage of 20 kV, and a hydrodynamic injection (50 mbar during 2s) when a solution of bioallethrin (200 mg/L) was analyzed. Under these conditions, the solubilization of bioallethrin did not take place even in presence of high concentrations of urea (2 and 5 M) or using different organic modifiers (methanol, acetonitrile, propan-2-ol or dimethylformamide) at percentages ranging from 10 to 50 %.

The next strategy investigated to achieve the separation of bioallethrin stereoisomers was the use of a CD-MEKC system. In this case, one of the most important factors is the adequate selection of the mixture of cyclodextrins and surfactants. Several anionic bile salts (SC, SDC, and STC) and SDS were tested alone and in combination with ten neutral cyclodextrins (β-CD, Me-β-CD, DM-β-CD, TM-β-CD, Ac-β-CD, HP-β–CD, γ-CD, Me-γ–CD, HP-γ-CD, Ac-γ-CD). To carry out these experiments, a 100 mM borate buffer (pH 8.0), 50 mM surfactant, 15 mM cyclodextrin and 2 M urea was used being the working temperature 15 ºC and the separation voltage 20 kV. Urea was added to the BGE in order to improve the solubilization of bioallethrin and the peak shape.

The results obtained are summarized in **Table 1** which shows the analysis time and the resolution achieved in each analysis. As it can be observed, only six of the different surfactant-cyclodextrin mixtures tested enabled to reach values of resolution higher than 1.5. In all these cases, the

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surfactant used was a bile salt so that the stereoselective discrimination observed may be justified by a synergic effect between the bile salt and the cyclodextrin. However, even though the use of the mixtures SC/Ac-β-CD or STC/β-CD provided good resolution values (2.8 and 1.9, respectively) in short times, the separation efficiency and sensitivity was poor being the combination of SDC with four different cyclodextrins (β-CD, Me-β-CD, HP-β-CD and Ac-β-CD) the conditions enabling the separation of bioallethrin stereoisomers with the highest values of resolution. Among them, the mixture SDC/Ac-β-CD gave rise to the best resolution (Rs = 9.0) in just 13 min so that this mixture was chosen for further studies. Although the acetyl derivatives of cyclodextrins are not very common selectors in CE, they have been successfully applied for the isomeric separation of different types of compounds such as pharmaceuticals [35, 36], peptides [37] or amino acids [38].

Once selected the most suitable combination bile salt/cyclodextrin, the effect of the concentration of both selectors was evaluated. First, three concentrations of Ac-β-CD (10, 15 and 20 mM) were tested. As **Figure 1 A** shows, the stereoselective discrimination increased from 10 to 15 mM, where the highest resolution was achieved (Rs = 9.0), and decreased from 15 to 20 mM. Then, 15 mM was chosen as the optimum CD concentration. Regarding SDC concentration, it was varied from 25 to 100 mM (**Figure 1 B**). At the lowest concentration, bioallethrin stereoisomers were not separated whereas using the highest value the current intensity generated into the capillary was too high. As a consequence, establishing a compromise between resolution and analysis time and taking into account that peak symmetry and separation efficiency improved using 75 mM SDC, this value was chosen as the optimum concentration.

Finally, the effect of different instrumental parameters on the separation was also evaluated. First, to obtain the maximum sensitivity without loss of separation, a study on the variation of the resolution with the hydrodynamic injection was carried out. Several pressure values (25 and 50 mbar) and injection times (2, 4 and 6 s) were tested. The most symmetrical and efficient peaks were obtained using an injection of 50 mbar x 2 s. The influence of the temperature on the resolution of bioallethrin was also evaluated. To do that, the temperature was studied from 15 ºC to 25 ºC (**Figure 1 C**). The results obtained showed that the analysis time decreased when increasing the working

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temperature but the resolution was slightly lower (changed from 8.1 at 15 ºC to 7.8 at 25 ºC). Considering that the resolution obtained for bioallethrin stereoisomers was high and the migration time was lower, 25 ºC was chosen as working temperature. Finally, the influence of the applied voltage was also investigated. **Figure 1 D** show the variation of the resolution of bioallethrin stereoisomers when this parameter ranged from 20 to 30 kV. It can be observed that an increase in the separation voltage originated a decrease in the analysis time (from 12 to 6.5 min) and a slightly variation of the resolution. A separation voltage of 30 kV (current intensity of 40 μA) was chosen since it enabled to obtain a high resolution (Rs = 7.4) in a short analysis time (6.5 min).

Figure 2 shows the electropherogram obtained for the separation of a standard solution of bioallethrin (200 mg/L) spiked with S-bioallethrin (100 mg/mL) under the optimized conditions. It can be observed that the first migrating isomer was R-bioallethrin which is very interesting to control this isomer as impurity in presence of a majority of the S-enatiomer if pure commercial formulations of esbiol were prepared.

3.2 Quantitative analysis of bioallethrin in commercial pediculicide formulations.

To demonstrate the potential of the developed methodology for the quantitative analysis of bioallethrin stereoisomers in a commercial pediculicide formulation, its analytical characteristics were evaluated.

The external standard calibration method was employed to evaluate linearity by plotting corrected peak areas (Ac) versus bioallethrin concentration in mg/L. Eight standard solutions of bioallethrin from 1 to 400 mg/L were injected in triplicate and checked for linearity during three different days. The linear range was established between 0.5 and 150 mg/L for each stereoisomer (between 1 and 300 mg/L for the racemate). **Table 2** groups the linear equation, the standard error for the intercept (S_a) and the slope (S_b), and the determination coefficient (R^2) for each stereoisomer. Satisfactory results were obtained in terms of linearity with R^2 values ≥ 99 %. ANOVA confirmed through the p-values (0.0910 and 0.0851 for the first (R) and the second (S) migrating isomers, respectively, for a 95% confidence level) that the experimental data fit properly to linear models.

LODs and LOQs for bioallethrin stereoisomers were calculated as 3.29 and 10 times the standard deviations of the intercepts (S_a), respectively, divided

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by the slopes of external standard calibration method [39]. For the R-bioallethrin stereoisomer a LOD of 0.2 mg/L and a LOQ of 0.7 mg/L were obtained. LOD and LOQ values for the S-bioallethrin stereoisomer were 0.3 and 0.9 mg/L, respectively (see **Table 2**).

Selectivity was verified by the injection of a sample solution of the commercial pediculicide formulation. **Figure 3** shows the electropherograms obtained for a standard solution containing 200 mg/L of bioallethrin and for the commercial sample (diluted in methanol at approximately 200 mg/L). Although a peak corresponding to other component present in the commercial formulation (piperonyl butoxide) appeared under the experimental conditions employed, it is well-separated from the bioallethrin stereoisomers which demonstrated the selectivity of the developed methodology. The standard additions calibration curve was obtained adding six known amounts of bioallethrin standard to the commercial formulation containing a constant concentration of bioallethrin (60 mg/L). A comparison between the confidence intervals for the slopes obtained by the standard additions and the external standard calibration methods for the two bioallethrin stereoisomers showed that there were not statistically significant differences between the slopes of each calibration method for a 95 % confidence level (**Table 2**). This was also confirmed through the p-values obtained by the t-test (> 0.05). These results justified the use of the external calibration method to achieve the quantiation of bioallethrin stereoisomers in the commercial formulation (no matrix interferences was observed).

The recovery obtained from six commercial sample solutions containing 40 mg/L of bioallethrin spiked with 20 or 160 mg/L of bioallethrin standard solutions was determined in order to evaluate the method accuracy. As it can be seen in **Table 2**, the mean recoveries obtained for the samples analyzed ranged from 99 to 104 %.

Repeatability, intra-day precision and inter-day precision were evaluated in order to assess the precision of the developed method. The commercial *pediculicide* formulation containing 40 mg/L of bioallethrin was spiked with 20 or 160 mg/L of bioallethrin standard solutions. Repeatability was determined from six consecutive injections of the two samples at two concentration levels. RSD values obtained were lower than 2.4 % for corrected peak areas and lower than 0.7 % for migration times. Six replicates of the two commercial sample solutions were injected in triplicate on the same day in order to assess intra-day

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precision. In this case, RSD values were lower than 5.2 and 2.1 % for corrected peak areas and migration times, respectively. Inter-day precision was evaluated injecting in triplicate three replicates of insecticide sample solutions at two concentration levels during three consecutive days. RSD values for corrected peak areas were lower than 5.7 % whereas for migration times they were lower than 3.0 %.

Finally, the developed CD-MEKC method was applied to the determination of bioallethrin stereoisomers in a commercial pediculicide spray (labelled amount, 0.66 g of bioallethrin per 100 mL sample). The average content obtained (n = 10) was 0.32 ± 0.01 and 0.323 ± 0.006 (g ± σ) for R and S-bioallethrin stereoisomers, respectively, what corresponded to percentages of 99, and 98% with respect to the labelled content. This demonstrated a good agreement between the amount of bioallethrin determined in the commercial spray by the CD-MEKC method developed and that declared as the labelled content.

3.3 Toxicity study of bioallethrin on non-target organisms.

Prior to carry out the toxicity study, the variability of the method including the incubation process with R/S-bioallethrin and esbiol in algae and seeds was evaluated by means of precision. Thus, repeatability was determined from six consecutive injections of algae and seed samples treated with R/S-bioalletrin or esbiol. RSD values lower than 2.5 % for corrected peak areas and less than 0.6 % for migration times was obtained. Three replicates of each samples (algae and seeds) incubated with R/S-bioallethrin or esbiol were injected in triplicate on the same day in order to evaluate intra-day precision. In this case, the RSD values were less than 5.6 and 2.0 % for the corrected peak areas and the migration times, respectively. Inter-day precision was calculated from three sample replicates injected in triplicate for three consecutive days. The RSD values for the corrected peak areas were lower than 6.0%, whereas for the migration times they were lower than 3.1%.

3.3.1 Toxicity on green algae Pseudokirchneriella subcapitata

Following the procedures described in the experimental section, the dose-effect relationship parameters for the growth of *P. subcapitata* in presence of R/S-bioallethrin and esbiol were calculated. Values of EC50 of 1.73 ± 0.05

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and 1.10 ± 0.06 (value ± confidence interval at 95 %) for esbiol and R/S-bioallethrin, respectively, were determined using the median effect/combination index (CI)-isobologram equation (see *section 2.4.1*). The m and R^2 values were 1.982 and 0.979, respectively, for esbiol and 1.152 and 0.978 for R/S-bioallethrin. In addition, the EC20 values (which represent the Lower Observed Effect Concentration (LOEC)) were also calculated achieving values of 0.85 ± 0.03 and 0.33 ± 0.01 for esbiol and R/S-bioallethrin, respectively.

According to the EC50 and EC20 values calculated, the toxicity was 1.6-fold higher or 2.6-fold higher for R/S-bioallethrin than for esbiol which showed a higher potential damage at low concentration for R/S-bioallethrin compared to the pure S-isomer. Taking into account the EC50 values obtained and according to the EU criteria [40], both substances could be classified as toxic to algae, although the toxicity of R/S-bioallethrin is higher than esbiol.

The results obtained were similar to the values reported for other synthetic pyrethroids on *P. subcapitata*; for instance, Burkiewicz *et al.* obtained an EC50 value of 2.56 mg/L for deltamethrin [41], and Miyamoto *et al*. obtained EC50 values from 2.6 to 110 mg/L for methofluthrin and its eight major metabolites [42]. Then, R/S-bioallethrin and esbiol can cause similar damage on algae population than other pyrethroids considered most toxic and persistent.

The significant differences found in the ecotoxicity data reported for bioallethrin on aquatic non-target organisms [24, 25] which ranged from µg/L to mg/L could be due not only to the use of different conditions in the assays but also to the use of a nominal concentration to obtain the toxicity values instead of the real concentration.

3.4.2. Toxicity on plant Sorghum bicolor

The results obtained showed a significant reduction in seeds germination in presence of R/S-bioallethrin and esbiol. In fact, *S. bicolor* showed 100 % inhibition of seeds germination in presence of 10 mg/L of both the R/S-bioallethrin and esbiol in the culture media. In both cases, it was observed the germination of 4 or 5 seeds of 5 replicates in control assay (without toxicant) whereas a complete inhibition of germination was observed in 6 days of exposition assays.

Unfortunately, no toxicity data for bioallethrin have been found in bibliography. Maund *et al.* indicated that considering the relevance mode of

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action of synthetic pyrethroids on insects, they have not adverse effects on plants [7]. The only data found on different plants is for the pyrethroid d-phenothrin [24]. No toxicity effect was observed on terrestrial plants (wheat, sugarbeet, soybean or tomato) but it is very toxic to the aquatic plant *Lemma gibba* (EC50 185 µg/L). The difference in toxicity levels found between terrestrial and aquatic plants may be due to different test conditions. According to the protocols for terrestrial plants [31], they should be germinated using soil where the negative effects of toxic substance may be attenuated similarly to the natural process (adsorption in soil matrix and microbial biodegradation). On the contrary, in the toxicity assays for aquatic plants, the toxic substances must be dissolved into the liquid media [32].

3.4.3. Bioallethrin biodegradation

The CD-MEKC methodology developed was also useful to evaluate changes in the concentration of bioallethrin stereoisomers under biotic conditions. Evaluation of stability of R/S-bioallethin and esbiol, was carried out in parallel with the biodegradation study. No significant transformations of isomers in solution were observed in both cultivation media used during the exposition period. Stability data were in accordance to the values reported by World Health Organization in 2005 [43].

Figure 4 shows the concentration profile of R and S stereoisomers of bioallethrin in the two cultivation media. During the contact with algae cells (**Figure 4a**), R-bioallethrin stereoisomer was almost completely depleted at 1 day of incubation, while S-bioallethrin stereoisomer persisted in the solution even at the end of assays. In contrast, concentration profiles for incubation with plants seeds (**Figure 4b**) are different, because both stereoisomers were depleted in a similar way and they were not completely removed from aqueous media at the end of exposition period. This fact can also be observed in **Table 3** which shows the fractional conversion (percentage of initial concentration removed from the media at final time) under biotic conditions both for the R and S-stereoisomers from an R/S mixture of bioallethrin and esbiol. From these data it is clear that esbiol (pure S-bioallethrin isomer) in contact with algae or seeds showed higher depletion rates compared to that obtained in biollethrin.

Furthermore, the presence of algae increased the disappearance of stereosiomers, especially R-bioallethrin which could be bioaccumulated in algal

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cells. The S-isomer in bioallethrin persists in solution at the end of exposition time and probably the bioaccumulation in algae biomass is lower. This effect was pointed out by Katagi *et al.* who showed that algae can absorb and metabolize different pesticides [44]. Another probable effect in bioallethrin is the competition between both stereoisomers, but this phenomenon must be investigated more in deep.

4. Concluding remarks

The use of SDC and acetyl-β-CD as pseudostationary phases in CD-MEKC enabled the separation of the bioallethrin stereoisomers with a resolution of 7.4 in 6.5 min. The analytical characteristics of the developed methodology were evaluated and showed a good performance for the quantitation of bioallethrin in a commercial pediculicidal formulation for which the concentrations determined were in good agreement with the labelled amounts. In addition, the analytical procedure was useful to obtain toxicity parameters for bioallethrin on non-target organisms and also to evaluate changes in concentration of toxic compounds during exposition assays. The toxicity parameters obtained in this work were calculated using real concentrations of bioallethrin in aqueous solution. Although bioallethrin and esbiol could be classified as toxic to algae, the toxicity of racemate is higher. 100 % inhibition of seeds germination was obtained on plant for both racemate and pure S-bioallethrin. Finally, esbiol showed higher depletion rates than S-isomer in bioallethrin in contact both with algae and plants. The results of this work also show the interest that the preparation of pure isomeric formulations presents to limit their environmental impact.

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Figure captions

Figure 1. Variation of the resolution values and analysis time for bioallethrin stereoisomers as a function of: (A) concentration of Acetyl-β-CD, (B) concentration of SDC, (C) temperature and (D) voltage. Experimental conditions: BGE: Acetyl-β-CD, SDC, 2 M Urea, 100 mM borate buffer (pH 8.0), λ: 220 nm ± 4 nm, uncoated fused-silica capillary 50 μm x 50 cm effective length, hydrodynamic injection: 50 mbar x 2 s. Bioallethrin: 200 mg/L. □ time and ● resolution.

Figure 2. Electropherogram corresponding to the separation of bioallethrin stereoisomers under the optimized experimental conditions: BGE: 75 mM SDC, 15 mM Acetyl-β-CD, 2 M Urea in 100 mM borate buffer (pH 8.0); Temperature, 25 ºC; voltage, 30 kV; current intensity: 40 μA; λ: 220 nm ± 4 nm. [(S)-bioallethrin] 200 mg/L, [(R)-bioallethrin]: 100 mg/L. Other conditions as in Figure 1. (Chiral center is indicated by an asterisk).

Figure 3. Electropherograms corresponding to the separation of bioallethrin stereoisomers in a standard bioallethrin solution, and a commercial pediculicidal spray whose label indicated a centesimal composition of 0.6 g of bioallethrin. [Sample]: 200 mg/L bioallethrin; [Standard]: 200 mg/L bioallethrin. Experimental conditions as in Figure 2.

Figure 4. Concentration profile of R- and S-stereoisomers of bioallethrin in toxicity assay. A) algae. B) plant seeds. □ R-bioallethrin and ● S-bioallethrin.

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Figure 1

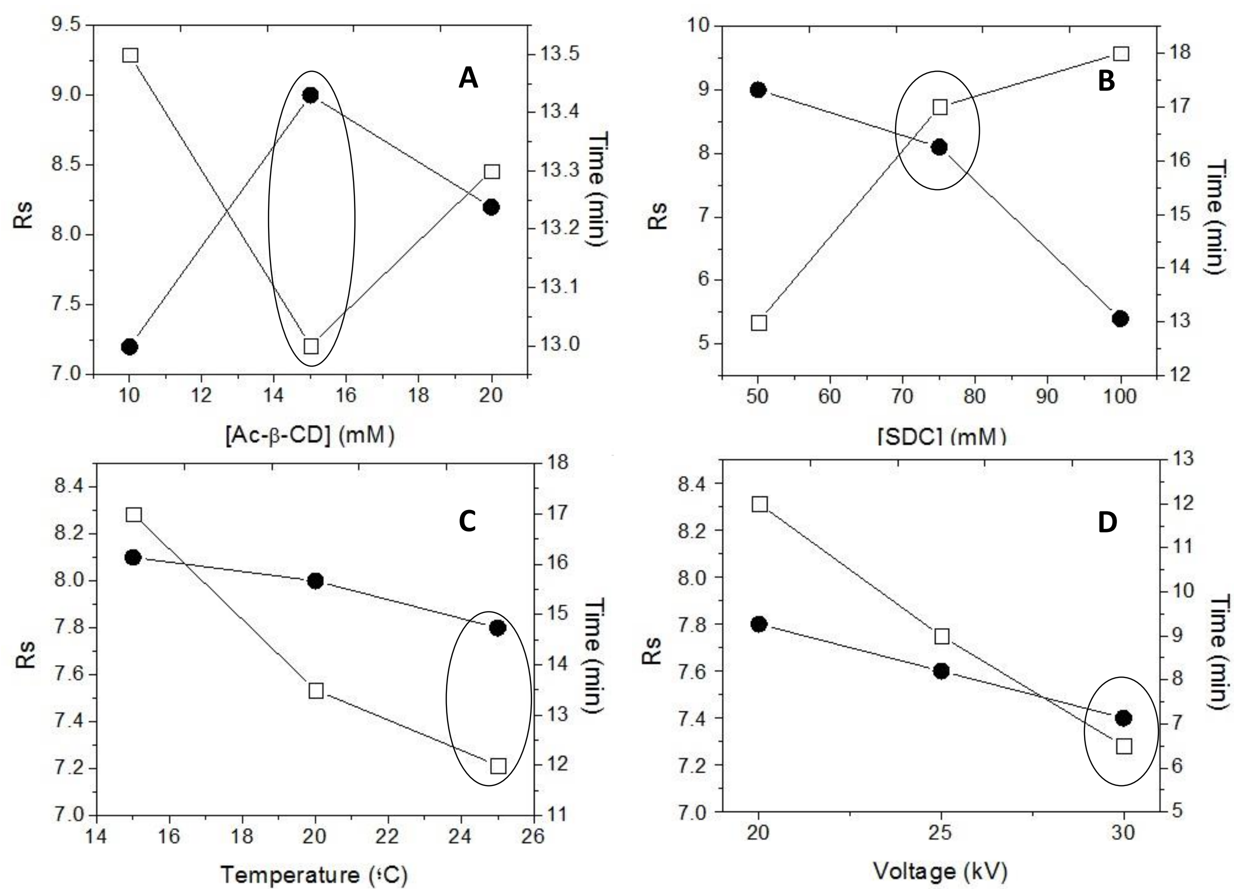

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Figure 2

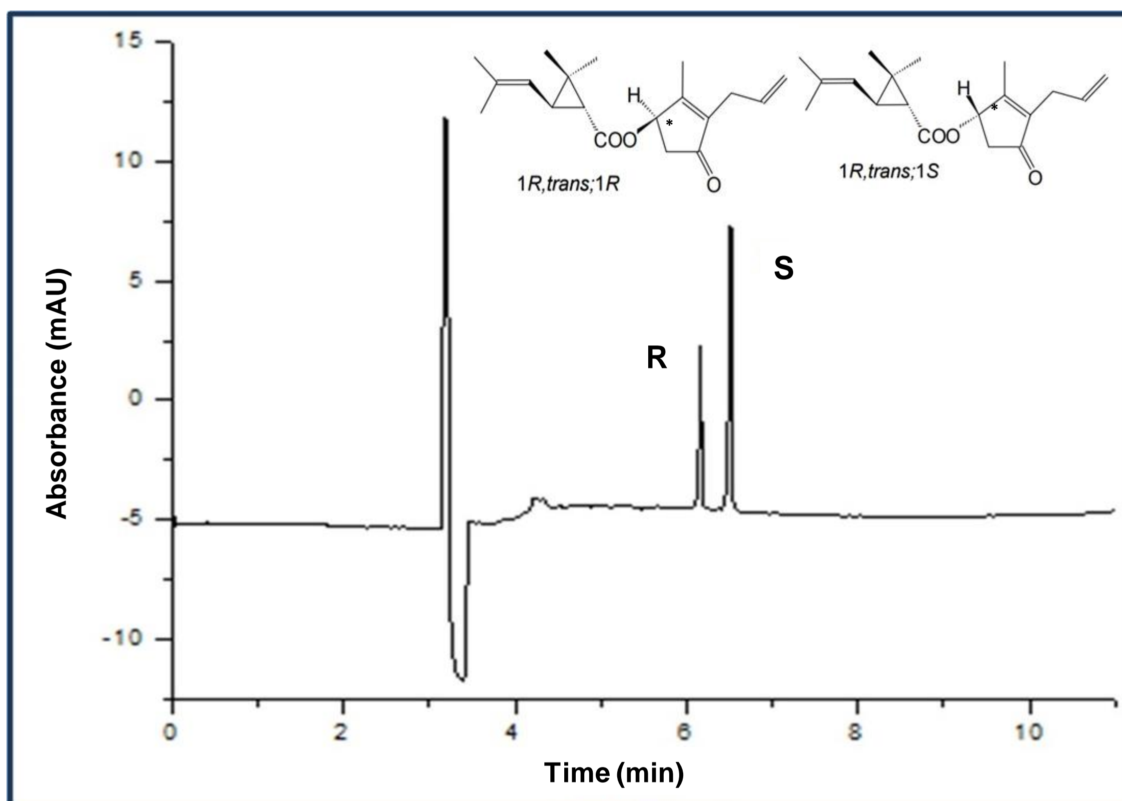

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Figure 3

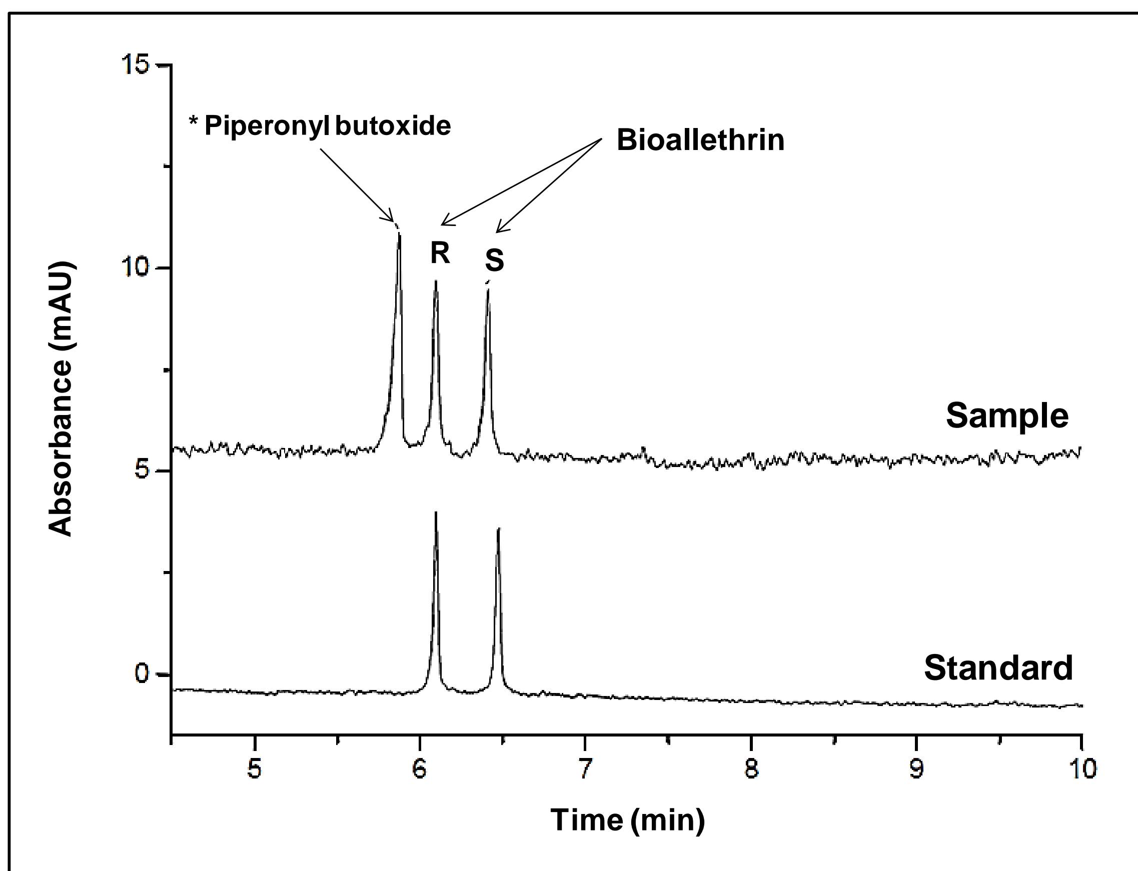

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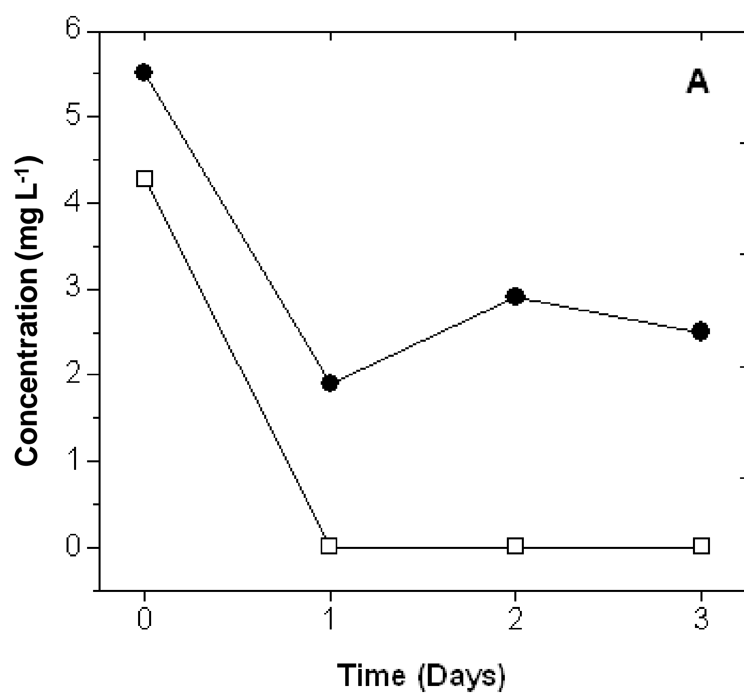
Figure 4



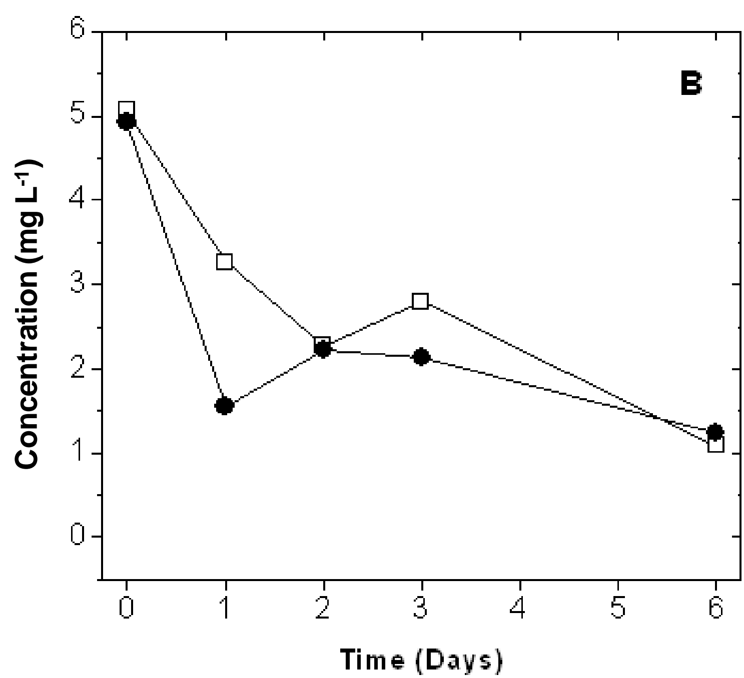


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Table 1. Resolution values (Rs) and analysis time for bioallethrin stereoisomers under the different experimental conditions employed.

	SDS		**SC**		**SDC**		**STC**	
	Rs	**Time* (min)**	**Rs**	**Time* (min)**	**Rs**	**Time* (min)**	**Rs**	**Time* (min)**
Single pseudophase	-	-	-	-	0.5	17.4	-	-
β-CD	0.6	22.6			5.6	14.7	1.9	9.8
Me-β-CD	0.8	15.7	1.0	9.5	2.3	14.7	1.1	9.7
DM-β-CD	0.5	20.2	0.9	9.7	0.6	10.0	0.8	9.5
TM-β-CD	-	-	0.7	12.2	-	-	0.7	12.0
HP-β-CD	0.6	19.1	1.3	10.1	4.5	13.3	-	-
Ac-β-CD	-	-	2.8	10.2	**9.0**	**13.0**	-	-
γ-CD	-	-	-	-	0.6	15.8	-	-
Me-γ-CD	-	-	-	-	-	-	-	-
HP-γ-CD	-	-	-	-	0.5	16.1	-	-
Ac-γ-CD	0.5	18.7	0.5	13.2	-	-	-	-

* Analysis time.
Experimental conditions: BGE, 50 mM surfactant, 15 mM cyclodextrin in 100 mM borate buffer (pH 8.0) containing 2 M urea; uncoated fused-silica capillary, 58.5 cm (50 cm to the detector window) × 50 µm ID; UV detection at 220 ± 4 nm; applied voltage, 20 kV; temperature, 15 ºC; injection by pressure, 50 mbar for 2 s.

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Table 2. Analytical characteristics of the MEKC method developed for the determination of bioallethrin stereoisomers.

	(R)-Bioallethrin First stereoisomer		(S)-Bioallethrin Second stereoisomer	
External standard calibration method[a]				
Range	**0.5 - 150 mg/L**		**0.5 - 150 mg/L**	
Linear equation	0.0125 + 0.0172 X		0.0197 + 0.0171 X	
Standard errors	S_a=0.0012 S_b=0.00007		S_a=0.0016 S_b=0.00008	
Intercept ± t x S_a	0.0125 ± 0.0029		0.0197 ± 0.0039	
Slope ± t x S_b	0.0172 ± 0.0003		0.0171 ± 0.0002	
R^2	99.9 %		99.9%	
Standard additions calibration method[b]				
Range	**0 - 110 mg/L**		**0 - 110 mg/L**	
Linear equation	0.6332 + 0.0171 X		0.5890 + 0.0175 X	
Slope ± t x S_b	0.0171 ± 0.0006		0.0175 ± 0.0006	
R^2	99.3 %		99.4 %	
Accuracy	**30 mglL**	**100 mg/L**	**30 mglL**	**100 mg/L**
Recovery[c]	101 ± 4 %	99 ± 3 %	104 ± 5 %	99 ± 3 %
Study of matrix interferences				
P-value of t test	0.7849		0.2903	
Precision	**30 mglL**	**100 mg/L**	**30 mglL**	**100 mg/L**
Repeatability[d]				
t, RSD (%)	0.6	0.4	0.7	0.3
A_c, RSD (%)	2.1	2.0	2.4	2.3
Intra-day precision[e]				
t, RSD (%)	1.5	0.9	1.9	2.1
A_c, RSD (%)	4.9	5.0	5.2	5.1
Inter-day precision[f]				
t, RSD (%)	2.0	1.1	2.8	3.0
A_c, RSD (%)	5.3	5.6	5.7	5.3
LOD	0.2 mg/L		0.3 mg/L	
LOQ	0.7 mg/L		0.9 mg/L	

Ac corrected area.

[a] Eight standard solutions at different concentration levels injected in triplicate.

[b] Adding of six known amounts of bioallethrin standard to a commercial formulation sample containing a constant concentration of 60 mg/L (as labelled amount) of bioallethrin.

[c] Accuracy was evaluated as the recovery obtained from six commercial samples solutions (n=6) containing 40 mg/L of bioallethrin (as labelled amount in commercial formulation solution) spiked with 20 or 160 mg/L of bioallethrin standard solutions.

[d] Six consecutive injections (n=6) of a commercial sample solution containing 40 mg/L of bioallethrin (as labelled amount in commercial formulation solution) spiked with 20 or 160 mg/L of bioallethrin standard solutions respectively.

[e] Six commercial sample solutions containing 40 mg/L of bioallethrin (as labelled amound in commercial formulation solution) spiked with 20 or 160 mg/L of bioallethrin standard solutions injected in triplicate respectively (n=6).

[f] Three commercial sample solutions containing 40 mg/L of bioallethrin (as labelled amound in commercial formulation solution) spiked with 20 or 160 mg/L of bioallethrin standard solutions respectively injected in triplicate in three different days (n=9).

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Table 3. Fractional conversions calculated for stereoisomers of bioallethrin and pure S-isomer (esbiol) under biotic conditions at the end of the exposition period. Data are in percentage (%). 95% confidence intervals are indicated.

Organism	Bioallethrin S-isomer (from R/S mixture)	Bioallethrin R-isomer (from R/S mixture)	Esbiol
P. subcapitata	54.61 ± 0.12	98.01 ± 0.10	82.20 ± 0.12
S. bicolor	75.52 ± 0.11	77.80 ± 0.13	96.82 ± 0.11