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Ecotoxicity evaluation of tetramethrin and analysis in agrochemical formulations using chiral electrokinetic chromatography

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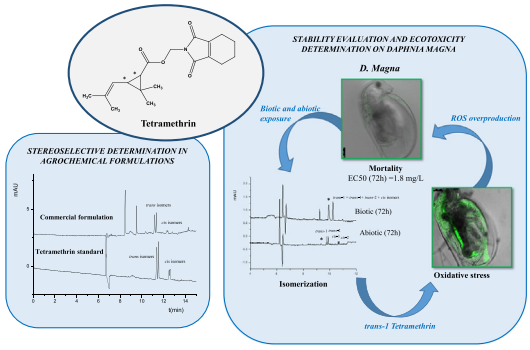
HIGHLIGHTS

- Tetramethrin isomers were separated by MEKC for the first time.
- A 95% isomerization into *trans*-1 isomer in presence of *D. magna* was observed.
- Ecotoxicity of tetramethrin on *D. magna* was attributed to the *trans*-1 isomer.
- Tetramethrin acts on aquatic microinvertebrates by oxidative stress.
- Tetramethrin enantiomeric quantitation was performed in antiparasitic formulations.

GRAPHICAL ABSTRACT



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ABSTRACT

The separation of the four isomers of tetramethrin was performed for the first time using a cyclodextrin-micellar electrokinetic chromatography methodology. Using sodium deoxycholate and 2-hydroxypropyl-β-CD as chiral selectors, tetramethrin isomers were separated with resolution values of 1.7 and 1.1 for *trans*- and *cis*-isomers, respectively, in analysis times lower than 12.5 min. Once developed and optimized, the analytical method was applied to the analysis of an antiparasitic commercial formulation and to the evaluation of the stability and ecotoxicity of tetramethrin. Using measured concentrations, the stability was assessed at enantiomeric level and the ecotoxicological parameters on *Daphnia magna* were determined. Tetramethrin presents toxicity on aquatic microinvertebrates, with EC_{50} (t = 72 h) of 1.8 mg/L. The acute toxicity of tetrametrin was attributed to the *trans-1* enantiomer. The first evidence of oxidative stress-mediated mode of action for tetramethrin on *Daphnia magna* is reported in the present work.

1. Introduction

Population growth has originated a considerable increase in the use of pesticides to reduce or control pests. Almost 30% of these compounds are chiral molecules (Carrao et al., 2020; Liu et al., 2005). Pesticide stereoisomers can present different activity, toxicity towards biological organisms or environmental persistence (Pérez-Fernández et al., 2010).

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Despite this, pesticides continue being commercialized as racemic mixtures due to difficult and high-cost stereoselective synthesis (Basheer, 2018). Pyrethroids are nowadays ones of the most used in the world and these were synthesized to enhance both the biological action and the stability of natural pyrethrins (Pérez-Fernández et al., 2010). They are persistent substances with high hydrophobicity which limits their water solubility (Feo et al., 2010) and present low toxicity to mammals but high toxicity to the aquatic organisms with lethal concentrations (LC_{50}) below 1 ppb (Hill, 1989; Mekebri et al., 2008).

Tetramethrin (1,3-dioxo-4,5,6,7-tetrahydroisoindol-2-yl)methyl-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropane-1-carboxylate) is a synthetic pyrethroid that contains two chiral centers and thus 4 stereoisomers. It is used as antiparasitic in commercial formulations for veterinary purposes. These formulations are composed of the 4 stereoisomers in a proportion 80:20 of *trans:cis* isomers, respectively. The enantiomer 1R of the *trans* isomer has more pesticide activity than the other isomers followed by the isomer 1S-*cis* (Zhe et al., 2008). Thus, the development of analytical strategies to perform tetramethrin stereoselective determination combined with toxicological studies is required in order to achieve a more realistic risk assessment of this pollutant in the environment.

The most employed analytical techniques dealing with the stereoselective separation of chiral pyrethroids are liquid chromatography (LC), gas chromatography (GC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE) (Jiménez-Jiménez et al., 2019). Despite CE is one of the most powerful separation techniques employed to carry out enantioselective separations due to its numerous advantages (low sample and reagent consumption, short analysis times, or easy to change different chiral selectors), only some works employed CE for the stereoselective separation of pyrethroids (Carrao et al., 2020; Pérez-Fernández et al., 2010; Jiménez-Jiménez et al., 2019). One of the CE modes presenting interesting characteristics for the enantiomeric separation of hydrophobic compounds such as pyrethroids, is Micellar Electrokinetic Chromatography (MEKC). A micellar phase is present in the separation medium although other selectors such as cyclodextrins, among others, can also be present (Salido-Fortuna et al., 2020). In the case of tetramethrin, the works reported in the literature concerning its chiral separation are limited. The separation of its 4 stereoisomers has only been performed using LC with UV detection (Zhe et al., 2008) and GC coupled to mass spectrometry (MS) (Corcellas et al., 2015a). In the first case, the use of a Chiralpak AD-H column enabled the baseline separation of the four isomers of tetramethrin (resolution for each of the two pairs of enantiomers was more than 2.0) within 20 min. This methodology was used for the quantitation of tetramethrin enantiomers in soil samples (Zhe et al., 2008). Regarding the use of the GC–MS approach, it enabled the simultaneous enantiomeric separation of the most common pyrethroids, including tetramethrin. The separation was completed in 74 min with resolutions higher than 0.6 in all cases (for tetramethrin enantiomers the resolution values were 1.2 and 0.9 in 32 min) (Corcellas et al., 2015a), and the methodology was useful to perform the determination of enantiomers in commercial insecticides (which contained tetramethrin along with cypermethrin and permethrin) and human breast milk samples. Just one work described the chiral separation of tetramethrin by CE, using capillary electrochromatography (CEC) (Lee et al., 2012). Under the most appropriate conditions, it was possible to obtain the enantioseparation of two of the four isomers of tetramethrin with a resolution value lower than 1.0 and an analysis time up to 18 min. Taking into account the limited resolutions or long analysis times obtained in the above-mentioned works, there is a need to develop faster stereoselective analytical methodologies enabling the separation of the four isomers of tetramethrin with adequate resolution. If these new methodologies are developed using analytical techniques respecting the principles of the green chemistry, such as CE, this could be doubly interesting since the only work reported by CEC described just the separation of two isomers of tetramethrin. Moreover, it can be highlighted that no work was reported on the use of Electrokinetic Chromatography (EKC) which is considered the most powerful separation mode in the context of CE.

The negative impact of synthetic pyrethroids on a wide range of aquatic and benthic non-target species has been broadly reported in the literature (Zhu et al., 2020; Li et al., 2017; Lu et al., 2019; Eybe et al., 2009; Hernandez-Moreno et al., 2010; Toumi et al., 2014; Touaylia et al., 2019; Yang et al., 2020; Wang et al., 2016). The main mechanism of toxicity for this category of pollutants was related to the oxidative stress (Eybe et al., 2009; Hernandez-Moreno et al., 2010; Toumi et al., 2014; Touaylia et al., 2019; Yang et al., 2020; Wang et al., 2016). In particular, tetramethrin showed to cause acute toxic effects on fish or aquatic invertebrates at concentrations from 3.7 to 160 μg/L depending on the species, as reviewed by ECHA dossier (ECHA, 2015). For instance, several studies were carried out for *Lepomis macrochirus* ($LC_{50,\ 96h}$ 16 μg/L) (Bowman, 1990a), *Oncorhynchus mykiss* ($LC_{50,\ 96h}$ 3.7 μg/L) (Bowman, 1990b), and *Danio rerio* ($LC_{50,\ 96h}$ 33 μg/L (Seyfried, 2002a; Croce, 2006). Acute toxicity studies with *Daphnia magna* (*D. magna*) were performed under 18 h:9 h light photoperiod using a flow-through system ($EC_{50,\ 48h}$ 0.045 mg/L) (EPA Environmental Fate and Effects Division, 1992); and $EC_{50,\ 48h}$ 0.11 mg/L (ECHA, 2015; Blasberg, 1993). In another study, *D. magna* were exposed to tetramethrin under darkness in a static test system ($EC_{50,\ 48h}$ 0.16 mg/L) (ECHA, 2015; Seyfried, 2002b). All studies compiled by ECHA dossier (ECHA, 2015) were carried out with analytical monitoring of the test substance concentration at test start and end, but in terms of a single compound. At present, there is no study focused on tetamethrin stability at isomeric level. Moreover, tetramethrin was detected in biota tissues of wild fish (Corcellas et al., 2015b). However, the enantiomeric contribution on bioaccumulation was not evaluated (Corcellas et al., 2015b). It was also reported that *trans*-isomers of tetramethrin were bioacumulated on bluegill sunfish *Lepomis macrochirus* (827 L/$kg_{wet\ fish}$), but information on the *cis*-isomers is still lacking (ECHA, 2015; Saito et al., 1994; Miyamoto et al., 2015). Besides this context and the extensive literature which showed different ecotoxicological pattern depending on the specific isomer of each pyrethroid (Corcellas et al., 2015a; Corcellas et al., 2015b), no evidence exists for ecotoxicological effects on aquatic organisms with an underlying enantiomeric-selectively or the mode of action of tetramethrin, highlighting so the importance of considering the isomerism in ecological risk evaluation (Corcellas et al., 2015a).

The present work aimed to combine the development of the first methodology by MEKC to separate the four tetramethrin stereoisomers with ecotoxicity test on the microinvertebrate *Daphnia magna* to determine for the first time the real toxicological effect of tetramethrin and elucidate its mode of action from a chiral point of view. In addition, the analytical methology developed was employed to carry out the quantitative analysis of tetramethrin in a commercial formulation.

2. Materials and methods

2.1. Reagents and samples

Reagents were of analytical grade. Boric acid, sodium hydroxide, methyl-β-CD (Me-β-CD), Heptakis(2,6-di-*O*-methyl)-β-CD (DM-β-CD), tetramethrin, sodium dodecylsulfate (SDS), sodium deoxycholate (SDC), sodium taurocholate (STC) and sodium cholate (SC) were obtained from Sigma-Aldrich (Madrid, Spain). Disodium hydrogen phosphate was purchased from Panreac Química S.A. (Barcelona, Spain). Hydrochloric acid and urea were from Scharlau (Barcelona, Spain). Methanol was from Thermo Fischer Scientific (Madrid, Spain). β-CD, Heptakis(2,3,6-tri-*O*-methyl)-β-CD (TM-β-CD), 2-Hydroxypropyl-β-CD (DS ~ 3) (HP-β-CD), and γ-CD were from Fluka (Buchs, Switzerland). Acetyl-β-CD (Ac-β-CD), 2-Hydroxypropyl-γ-CD (HP-γ-CD), Methyl-γ-CD (Me-γ-CD), and Acetyl-γ-CD (Ac-γ-CD) were from Cyclolab (Budapest, Hungary). Milli-Q water (purified using a Milli-Q

system from Millipore (Bedford, MA, USA)) was used to prepare solutions.

The commercial antiparasitic formulation was acquired on a web page (https://www.mascoteros.com/) of animal care products. According to the label, it contains 2.1 g tetramethrin per liter of solution (*cis: trans*, 20:80) and excipients (1 g parahydroxymethylbenzoate per liter, 1 g parahydroxypropylbenzoate per liter).

The *Daphnia magna* specimen and the standard medium for biota culturing were supplied by the MicroBioTests (Ghent, Belgium). 2′,7′ dichlorofluorescin diacetate (H_2DCFDA) dye was from Sigma Aldrich (Spain).

2.2. CE conditions

Analyses were carried out on a 7100 CE system controlled by the ChemStation software (B. 04. 03 SP1) (Agilent Technologies, Waldbronn, Germany). Electrophoretic separations were performed in an uncoated fused-silica capillary of 50 μm ID x 58.5 cm (50 cm of effective length) purchased from Polymicro Technologies (Phoenix, AZ, USA), and employing a voltage of 20 kV and a temperature of 15 °C. Samples were injected applying a pressure of 50 mbar for 2 s. Duplicate and triplicate injections were carried out during method development and quantitative analysis, respectively. UV detection was performed at 220 ± 4 nm.

To prepare new capillaries, 1 M sodium hydroxide, Milli-Q water, and buffer solution were passed through the capillary (applying 1 bar) during 30, 10 and 60 min, respectively. Before starting to work each day, the capillary was conditioned with 0.1 M sodium hydroxide (10 min), Milli-Q water (5 min), buffer solution (15 min), and BGE (10 min). Between injections, the capillary was washed with 0.1 M sodium hydroxide, Milli-Q water, and BGE during 2, 1 and 5 min, respectively.

2.3. Solutions and samples preparation

Buffer solutions were prepared dissolving the appropriate amounts of boric acid or disodium hydrogen phosphate in water to obtain the desired concentration. Before completing the volume with water, buffer pH was adjusted with sodium hydroxide or hydrochloric acid. BGE was prepared dissolving the corresponding amount of urea, bile salt, and cyclodextrin in the buffer solution.

The proper amount of tetramethrin was dissolved in methanol to prepare the stock standard solution of this pyrethroid. The standard working solution was diluted with methanol or with 100 mM borate buffer (pH 8.0) containing 2 M urea and 100 mM SDC to achieve a concentration of 200 mg/L.

To prepare sample solutions of the commercial formulation and taking into account the amount of tetramethrin indicated in the label of the product, an appropriate volume of the solution was dissolved in buffer containing 2 M urea and 100 mM SDC to obtain a concentration of 200 mg/L.

All buffer solutions were filtered before their use through 0.45 μm pore size nylon filters from Scharlau (Barcelona, Spain).

2.4. Ecotoxicological study and stability evaluation

The acute toxicity evaluation of tetramethrin on freshwater invertebrate *D. magna* was achieved by utilizing Daphtox kit F (MicroBioTests, Gent, Belgium), following both manufacturer and Standard Guideline OCDE 202 procedures (S. G. O. 202, 2021). The base of this test is the immobilization of the organisms after 48-72 h exposure to a dilution series of the compound, with subsequent calculation of the 48-72 h ECx (effective concentration that cause x% of inhibition on biological response, where x range from 1 to 99%).

Prior to the exposure experiments, the dormant eggs were activated for 72 h, in 50 mL Standard Freshwater medium (64.75 mg $NaHCO_3$/ L, 294 mg $CaCl_2$ $2H_2O$/ L; 123.25 mg $MgSO_4$ $7H_2O$/ L; 5.75 mg KCl/L), at 20–22 °C, under light. The resulted organisms with age ≤ 24 h (neonates,) were feed with Spirulina powder 2 h before collecting then for incubation with the compound. Ten serial dilutions of tetramethrin, with final concentrations ranging from 0.39 to 200 mg/L using Standard Freshwater medium, were obtained from a concentrated solution in methanol. Methanol content in the tested solution was maintained below 0.1%. Besides the assayed concentrations are high, there are indications that concentrations of pyrethroids in suspended sediments might be larger than in the surface waters (Zhu et al., 2020; Hladik and Kuivila, 2009).

Three replicates, each one containing 5 neonates daphnids, were incubated with doses between 0.39 and 200 mg/L of tetramethrin. Exposure was done in a 24-well plate containing 10 mL/well of the toxicant dilution. Control assays, containing only test medium were also included. The test plate was incubated for 72 h, at 20–22 °C, in darkness. Each well was daily checked for immobilization during 72 h and the numbers of mobile organisms were visually determined. Individuals that were non-responsive 15 s after softly agitation and settled on the bottom of the test well were considered as immobile (S. G. O. 202, 2021).

The immobilization percentage at incubation time of 24, 48 and 72 h was calculated using Excel software. The EC_{10}, EC_{20}, EC_{50} and EC_{90} of tetramethrin resulting from the acute toxicity test were estimated with median-effect-isobologram mathematical statement, as programmed in the CompuSyn software (Amariei et al., 2017).

The overall oxidative stress produced by tetramethrin in *D. magna* was investigated as Reactive Oxygen Species (ROS), employing H_2DCFDA assay (Valimaña-Traverso et al., 2019). ROS generation was observed by fluorescence at λex/λem = 350–600 nm using confocal laser scanning microscopy, CLSM (Leica TCS SP5, Germany). The green-fluorescence intensity was quantified by imaging treatment with ImageJ software.

The stability behavior of tetramethrin was evaluated in presence of daphnids and without microinvertebrate, using an interval of concentration between 12.5 and 200 mg/L of tetramethrin in culturing media. For this, initial and final concentration at 72 h of incubation in darkness conditions was determined applying the chiral methodology developed in the present work.

2.5. Data treatment

The Chemstation software (Agilent Technologies) was employed to obtain the migration times and resolution values (Rs). Graphs containing various electropherograms were composed in OriginPro 8.0 software. Excel Microsoft and Start-graphics Centurion XVII were used to perform experimental data analysis.

3. Results and discussion

3.1. Development of a MEKC methodology for the chiral separation of tetramethrin

Due to the intrinsic characteristics of tetramethrin, it is a neutral and hydrophobic compound, one of the first choices to perform its stereoselective separation is the use of chiral anionic surfactants which enabled, for the one hand, to achieve the enantioseparation and, on the other hand, to improve the solubility of hydrophobic compounds (Poole and Poole, 1997). Thus, the potential of three different chiral anionic surfactants (SDC, SC, STC) as the sole chiral selectors to carry out the separation of the tetramethrin stereoisomers was performed. All these experiments were accomplished using borate buffer (100 mM, pH 8.0) containing 2 M urea and 50 mM of each surfactant, 15 °C, and 20 kV. However, none of the bile salts used allowed the stereoselective separation of tetramethrin.

Thus, cyclodextrin modified MEKC approaches (CD-MEKC) based on the combination of neutral CDs (β-CD, TM-β-CD, HP-β-CD, γ-CD, Me-β-

CD, DM-β-CD, Ac-β-CD, HP-γ-CD, Me-γ-CD, Ac-γ-CD) with anionic chiral (SDC, SC, STC) and non-chiral (SDS) surfactants were performed employing 15 mM of each cyclodextrin and 50 mM of each surfactant. When SDS or STC were mixed with different CDs, the enantioseparation of tetramethrin was not achieved, whereas a chiral discrimination was observed employing mixtures of SC/TM-β-CD (Rs_{trans} 1.4, Rs_{cis} 2.9; analysis time 10.4 min), SC/HP-β-CD (R*strans* 0.7, Rs_{cis} 0.6; analysis time 11.4 min), and SC/γ-CD (Rs_{cis} 1.0, Rs_{trans} 0.6; analysis time 9.7 min) (see Table 1). In the first two systems, *trans* isomers migrated before *cis* isomers whereas using SC/γ-CD a reversal migration order was observed. Even though the CD-MEKC system based on the mixture SC/TM-β-CD provided higher resolution values between the pairs of *trans* or *cis* enantiomers, a peak overlap was observed between the second-migrating *trans* enantiomer and the first-migrating *cis* enantiomer.

On the other hand, the combinations SDC/TM-β-CD (Rs_{cis} 1.7, Rs_{trans} 1.3, analysis time 11.4 min), SDC/HP-β-CD (Rs_{trans} 1.7, Rs_{cis} 1.1; analysis time 12.5 min) not only gave rise to the chiral separation of tetramethrin isomers but also slightly improved the above-mentioned results. This fact could be expected since it is known that deoxy forms of bile salts present a higher hydrophobic character and that they exhibit a better behavior towards hydrophobic compounds like pyrethroids (Crego et al., 2000). As it can be observed in Table 1, *cis* isomers migrated before *trans* isomers using the system SDC/TM-β-CD while a reversal in the migration order was obtained when SDC/HP-β-CD was employed.

Bearing in mind the results obtained, the CD-MEKC systems based on the mixtures SDC/TM-β-CD and SDC/HP-β-CD were chosen to achieve the stereoselective separation of tetramethrin. As Table 1 shows, the migration order is different in both systems since *cis*-isomer migrated first when the TM-β-CD was combined with SDC while it migrated in the second place when the CD employed is HP-β-CD.

Afterward, the influence of buffer composition, in terms of pH and concentration, on the chiral separation, was investigated keeping constant the concentrations of urea (2 M), TM-β-CD or HP-β-CD (15 mM), SDC (50 mM), and applying a voltage of 20 kV at 15 °C. Thus, 100 mM borate buffer at pH 8.0 and pH 9.0, and 100 mM phosphate buffer at pH 7.0 were evaluated. A high pH value led to a worse separation for both SDC/TM-β-CD and SDC/HP-β-CD combinations. Using SDC/TM-β-CD, broad-peaks and an overlapping between the second-migrating *trans* enantiomer and the first-migrating *cis* enantiomer were observed while employing the mixture SDC/HP-β-CD the peak shape was worse and the resolution values decreased (Rs_{cis} 1.0, Rs_{trans} 0.6). On the contrary, the use of phosphate buffer at pH 7.0 did not provide the chiral separation of tetramethrin in less than 20 min for any combination. Thus, borate buffer at pH 8.0 was maintained as separation buffer for further experiments. Then, the effect of the ionic strength of the buffer was investigated in the range from 50 to 150 mM. The use of a 50 mM borate buffer for both CD-MEKC systems originated an increase in analysis times (~2 min) while the enantioresolution values (Rs_{cis} 1.7, Rs_{trans} 1.3 for SDC/TM-β-CD; Rs_{trans} 1.7, Rs_{cis} 0.8 for SDC/HP-β-CD) were close to those obtained using a 100 mM concentration. On the other hand, 150 mM borate buffer caused a decrease in the resolution values (Rs_{cis} 1.4, Rs_{trans} 1.3 for SDC/TM-β-CD; Rs_{trans} 1.5, Rs_{cis} 0.6 for SDC/HP-β-CD). compared to a 100 mM concentration of the buffer. Hence, the best results were obtained using a 100 mM borate buffer at pH 8.0.

Since the concentration of the chiral selector is a crucial parameter for chiral separations (Chankvetadze, 1997), the influence of SDC and TM-β-CD or HP-β-CD concentrations was studied to select those that enabled to achieve the better separation of tetramethrin isomers. First, the effect of SDC concentration was investigated from 25 mM to 75 mM keeping constant the concentration of the CD at a value of 15 mM. As Figs. 1A and 2A show, an increase in the concentration of SDC from 50 mM to 75 mM led to higher analysis times (19 min for HP-β-CD; 16 min for TM-β-CD) but also to a worse enantioseparation since *trans*/*cis* isomers overlapped when TM-β-CD was used, and a worse separation between *cis* isomers with HP-β-CD was obtained. Employing the lower SDC concentration only two peaks corresponding to *trans* isomers were observed for TM-β-CD, while no separation was obtained for tetramethrin isomers using HP-β-CD. Therefore, 50 mM SDC was chosen. Keeping constant this value, the effect of both CDs concentrations was investigated using values of 10, 15, and 20 mM. Regarding the use of HP-β-CD in combination with SDC, the best separation was achieved employing a concentration of 15 mM since, as it can be seen in Fig. 1B, lower concentrations gave rise to a decrease in the resolution between the enantiomers of *trans* and *cis* tetramethrin, while a worse peak shape was observed at higher concentrations. Concerning the use of TM-β-CD in the CD-MEKC system (Fig. 2B), a concentration of 20 mM provided only the separation of *trans* enantiomers whereas a concentration of 10 mM led to a better separation between *cis*/*trans* isomers but a worse separation between the pair of enantiomers. Based on these results, 15 mM of both CDs was the concentration preferred.

Finally, under the best experimental conditions (borate buffer (100 mM, pH 8.0), 2 M urea, 50 mM SDC and 15 mM of CD), the influence of the temperature and the applied voltage was studied. First, the influence of temperature was evaluated between 15 °C and 25 °C keeping a voltage of 20 kV. Using the SDC/TM-β-CD system, only the use of 15 °C allowed the enantioselective separation of the four tetramethrin isomers (Rs_{cis} 1.7, Rs_{trans} 1.3) (see Fig. 2C). Regarding the use of SDC/HP-β-CD, the highest resolutions (Rs_{trans} 1.7, Rs_{cis} 1.1) were obtained at 15 °C at the expenses of longer analysis times (~12.5 min) (see Fig. 1C). Then, keeping constant the temperature (15 °C), the effect of different separation voltages was studied. While an increase in the applied voltage from 20 kV to 25 kV and 30 kV caused a decrease in analysis times in both methodologies, resolution values were also decreased in both cases (see Figs. 1D and 2D). Thus, a voltage of 20 kV was chosen to achieve the chiral separation of tetramethrin. Fig. 3 shows the electropherograms obtained using both SDC/TM-β-CD and SDC/HP-β-CD systems under the optimized conditions. As this figure shows, the use of TM-β-CD led to the separation of the four isomers of tetramethrin in less than 11.4 min with good Rs values (1.3 for *trans* isomers and 1.7 for *cis* isomers), whereas the use of HP-β-CD enabled Rs values of 1.7 and 1.1 for *trans* and *cis* isomers, respectively, and analysis times of 12.5 min. However, the separation between *cis*/*trans* isomers was not good enough when the system SDC/TM-β -CD was used since the peaks corresponding to the second-migrating *cis* enantiomer and the first-migrating *trans* isomer overlapped.

Table 1
Migration times and resolution values obtained in the separation of tetramethrin using different CD-MEKC systems.

SC			SDC	
TM-β-CD	HP-β-CD	γ-CD	TM-β-CD	HP-β-CD
$t_{trans\text{-}1}$ = 10.1 min	$t_{trans\text{-}1}$ = 8.2 min	$t_{cis\text{-}1}$ = 8.6 min	$t_{cis\text{-}1}$ = 10.9 min	$t_{trans\text{-}1}$ = 11.3 min
$t_{trans\text{-}2}$ = 10.2 min	$t_{trans\text{-}2}$ = 8.3 min	$t_{cis\text{-}2}$ = 8.7 min	$t_{cis\text{-}2}$ = 11.0 min	$t_{trans\text{-}2}$ = 11.4 min
Rs_{trans} = 1.4	Rs_{trans} = 0.7	Rs_{cis} = 1.0	Rs_{cis} = 1.7	Rs_{trans} = 1.7
$t_{cis\text{-}1}$ = 10.2 min	$t_{cis\text{-}1}$ = 11.3 min	$t_{trans\text{-}1}$ = 9.5 min	$t_{trans\text{-}1}$ = 11.2 min	$t_{cis\text{-}1}$ = 12.3 min
$t_{cis\text{-}2}$ = 10.4 min	$t_{cis\text{-}2}$ = 11.3 min	$t_{trans\text{-}2}$ = 9.6 min	$t_{trans\text{-}2}$ = 11.3 min	$t_{cis\text{-}2}$ = 12.4 min
Rs_{cis} = 2.9	Rs_{cis} = 0.6	Rs_{trans} = 0.6	Rs_{trans} = 1.3	Rs_{cis} = 1.1

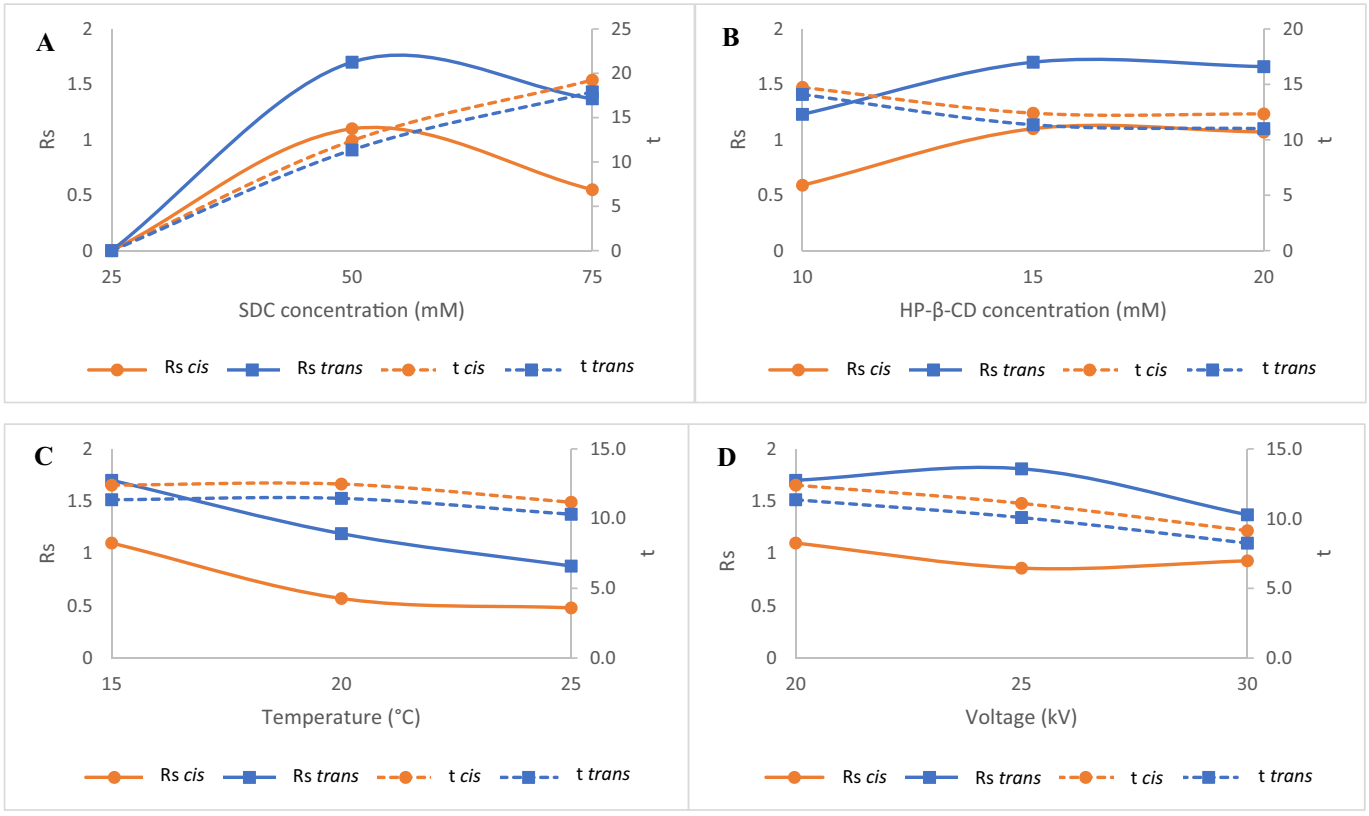

Fig. 1. Variation of the analysis time and enantiomeric resolution of tetramethrin isomers as a function of: (A) SDC concentration; (B) HP-β-CD concentration; (C) temperature; (D) voltage. Experimental conditions: uncoated fused-silica capillary, 58.5 cm (50 cm to the detector window) x 50 μm ID; BGE, the corresponding concentrations of CD and SDC in 100 mM borate buffer (pH 8.0); injection by pressure 50 mbar for 2; UV detection at 220 nm. Dotted lines represent the variation of the analysis time corresponding to the separation of the two stereoisomers of each isomer *trans* and *cis* and continuos lines to the variation of enantiomeric resolution. In addition, square and circles correspond to *trans* and *cis* isomers respectively.

It should be highlighted that the opposite enantiomeric migration order was observed when using the two CD-MEKC systems. While *cis* isomers were the first-migrating with the SDC/TM-β-CD combination, *trans* isomers were the first ones with SDC/HP-β-CD. This phenomenon may be attributed to the different interactions established in chiral selector-analyte complexes (Chankvetadze, 2018). Taking into account that the system SDC/TM-β-CD did not allow a good separation between *cis*/*trans* isomers, and that HP-β-CD was cheaper than TM-β-CD, the system SDC/HP-β-CD was chosen to perform the quantitative analysis of tetramethrin in commercial formulations.

The analysis time (12.5 min) and the stereoselective resolutions (1.7 and 1.1 for *trans* and *cis* isomers, respectively) obtained in the present work represent a reduction of the analysis time compared to the previous HPLC and GC methods (20 and 32 min, respectively) with higher resolutions with respect to the GC and the CEC methods previously reported, which only allowed in the case of CEC the separation of two stereoisomers. The present work constitutes the first tetramethrin stereoselective methodology reported by Electrokinetic Chromatography, the most powerful technique in the context of CE to achieve chiral separations under sustainable conditions (low volumes of solvents and samples required and no need of a chiral chromatographic column).

All the above-mentioned experiments were done using tetramethrin solutions at a concentration of 200 mg/L in methanol. However, reproducibility problems on the values of peak areas were observed probably due to the limited solubility of tetramethrin. Although these reproducibility problems did not affect the values of migration times nor enantiomeric resolutions (method development), they should be avoided to achieve the quantitative analysis. With this aim, standard working solutions were diluted from the tetramethrin stock solution (in methanol) using a borate buffer (100 mM) containing 2 M urea and 100 mM SDC. Under these conditions, reproducibility problems on peak area values were overcome.

3.2. Quantitative analysis of tetramethrin in an antiparasitic formulation

Before applying the CD-MEKC methodology to the tetramethrin quantitation in a commercial formulation, its analytical characteristics were assessed to demonstrate the method suitability. First, the method linearity was evaluated by the external standard calibration method. The linearity was set from 5 calibration levels from 25 to 125 mg/L of tetramethrin which corresponds to 20 to 100 mg/L for *trans* isomers and from 5 to 25 mg/L for *cis* isomers. As Table 2 shows, correlation coefficients >0.994 for both isomers were achieved. Confidence intervals for the slopes did not include the zero value and for the intercept included the zero for a 95% confidence level for both, *trans* and *cis* isomers. Moreover, an ANOVA test was performed to corroborate that experimental data fit to a linear model (*p*-values >0.05 for *trans*- and *cis*-tetramethrin).

The standard addition calibration method was employed to determine possible matrix effects by adding different amounts of tetramethrin standard (from 50 to 200 mg/L) to the commercial formulation which contained 200 mg/L tetramethrin according to the label. In this way, 5 calibration levels, from 0 to 160 mg/L for *trans* isomers, and from 0 to 40 mg/L for *cis*-tetramethrin were employed. From the comparison of the confidence intervals for the slopes achieved by both calibration methods, it was possible to determine that there were statistically significant differences between the slopes and, thus, there were matrix interferences. Therefore, to perform the quantitative analysis of tetramethrin in the commercial formulation, the standard additions calibration method was required.

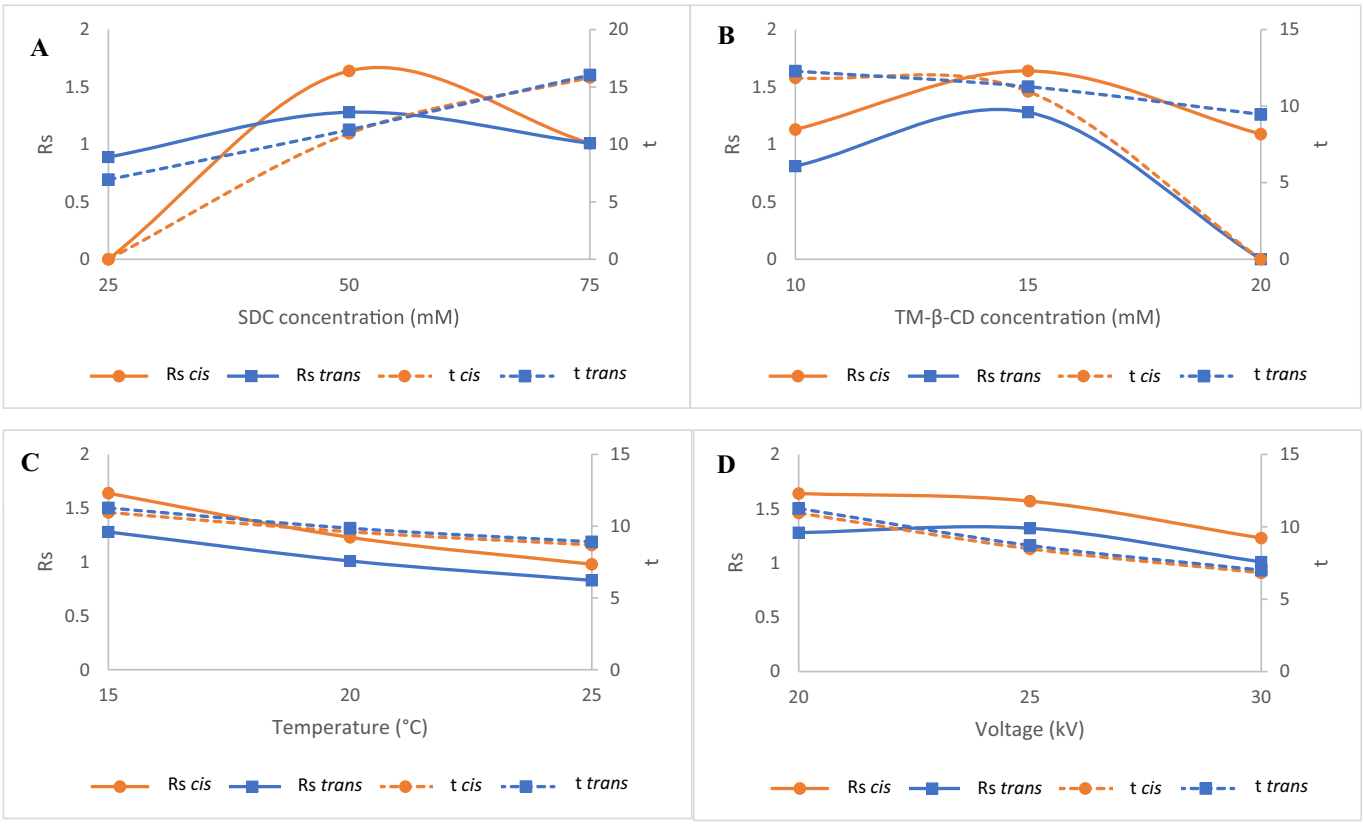

Fig. 2. Variation of the analysis time and enantiomeric resolution of tetramethrin isomers as a function of: (A) SDC concentration; (B) TM-β-CD concentration; (C) temperature; (D) voltage. Experimental conditions: uncoated fused-silica capillary, 58.5 cm (50 cm to the detector window) x 50 μm ID; BGE, the corresponding concentrations of CD and SDC in 100 mM borate buffer (pH 8.0); injection by pressure 50 mbar for 2; UV detection at 220 nm. Dotted lines correspond to the variation of analysis time and continuos lines to the variation of enantiomeric resolution. In addition, square and circles correspond to *trans* and *cis* isomers respectively.

Precision was assessed (through instrumental and methodological repeatability and intermediate precision) adding a known amount of tetramethrin standard to the commercial formulation containing a constant concentration. Regarding instrumental repeatability, RSD values for migration times were 0.1% and 0.3% for *trans* and *cis* isomers, respectively, while RSD values for peak areas were 2.2% for *trans* isomers and 3.9% for *cis* isomers. Regarding method repeatability, RSD values for peak areas of 4.7% and 6.3 for *trans* and *cis* isomers, respectively, were obtained, while RSD of 0.2% were achieved for migration times for both isomers. Finally, for intermediate precision, RSD values were below 8.2% and 2.0% for peak areas and migration times, respectively.

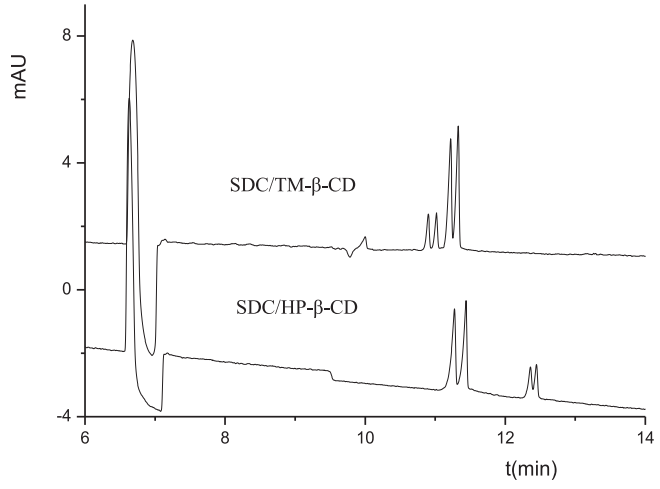

Fig. 3. Electropherograms corresponding to the chiral separation of tetramethrin under the best experimental conditions. Experimental conditions: uncoated fused-silica capillary, 58.5 cm (50 cm to the detector window) x 50 μm ID; BGE, 15 mM of the corresponding CD and 50 mM DSC in 100 mM borate buffer (pH 8.0); applied voltage, 20 kV; temperature, 15 °C; injection by pressure 50 mbar for 2; UV detection at 220 nm.

The recovery obtained for tetramethrin when known concentrations of the standard solution (25–100% of the nominal concentration of tetramethrin of 200 mg/L) were added to the commercial formulation, was employed to determine the accuracy. Recoveries of 99 ± 9 for *trans* isomers and 102 ± 8 for *cis* isomers were obtained (Table 2).

LODs were 1.30 mg/L for *trans* isomers and 0.97 mg/L for *cis* isomers, whereas LOQ values of 4.5 mg/L and 3.2 mg/L were obtained for *trans* and *cis* isomers, respectively.

The method was applied to the analysis of an antiparasitic commercial formulation (spray). According to the labeled amount, a diluted sample of the spray was injected containing a concentration of 200 mg/L (80:20 *trans*/*cis*). Fig. 4 illustrates the electropherograms obtained in the analysis of the commercial samples and a standard solution of tetramethrin. Migration times in the commercial formulation are shortened probably due to the composition of the formulation. The results obtained for the content of tetramethrin in the antiparasitic formulation were 40 mg/L for *trans* isomers and 6 mg/L for *cis* isomers, which corresponded to percentages of 25% and 16%, respectively, with respect to the labeled content.

3.3. Stability evaluation and ecotoxicity determination of tetramethrin on *Daphnia magna*

The performance of the method to carry out the analysis of the culture samples related to stability and ecotoxicity studies was assessed. A standard addition calibration was established adding 5 known amounts of tetramethrin standard (20–60 mg/L) to the medium. Taking

Table 2
Analytical characteristics of the developed methodology for the determination of tetramethrin in an antiparasitic commercial spray.

	Trans-tetramethrin	*Cis*-tetramethrin
External standard calibration method[a]		
Linear range	20–100 mg/L	5–25 mg/L
Intercept + t*Sa	0.996 ± 3.619	0.374 ± 1.237
Slope + t*Sb	0.336 ± 0.055	0.398 ± 0.075
r	0.996	0.995
P-value of ANOVA[b]	0.147	0.089
Standard addition calibration method[b]		
Linear range	0–160 mg/L	0–40 mg/L
Intercept + t*Sa	7.788 ± 1.375	1.417 ± 0.770
Slope + t*Sb	0.196 ± 0.014	0.224 ± 0.031
r	0.9992	0.9971
Precision	RSD %	
Instrumental repeatability[c] ($n = 3$)	t, 0.1 – A, 2.2	t, 0.3 – A, 3.9
Method repeatability[d] ($n = 9$)	t, 0.2 – A, 4.7	t, 0.2 – A, 6.3
Intermediate precision[e] (n = 9)	t, 1.8 – A, 7.1	t, 2.0 – A, 8.2
	% Mean recovery	
Accuracy[f]	99 ± 9	102 ± 8
LOD[g]	1.30 mg/L	0.97 mg/L
LOQ[h]	4.50 mg/L	3.23 mg/L

[a] Five standard solutions at different concentrations levels injected in triplicate.
[b] Addition of five known amounts of tetramethrin standard to an antiparasitic spray solution at a constant concentration.
[c] Instrumental repeatability was calculated from six consecutive injections of a commercial sample solution at a concentration of 200 mg/L spiked with tetramethrin standard at a concentration of 100 mg/L.
[d] Method repeatability was determined using the value obtained for three replicates of the commercial formulation solution spiked with 100 mg/L of tetramethrin standard injected in triplicate on the same day.
[e] Intermediate precision was calculated by injecting three replicates of the commercial formulation solution spiked with 100 mg/L of tetramethrin standard in triplicate during three consecutive days.
[f] Accuracy was studied as the mean recovery achieved when a commercial formulation solution was spiked with known amounts of tetramethrin standard (from 50 to 200 mg/L).
[g] LOD calculated for a S/N ratio of 3.
[h] LOQ calculated for a S/N ratio of 10.

into account the ratio 80:20 *trans-cis* tetramethrin isomers, the linear ranges employed were from 16 to 48 mg/L for *trans* isomers ($y = 0.062\ x - 0.10$; $s_b = 0.002$, $s_a = 0.06$) and from 4 to 12 mg/L for *cis*-isomers ($y = 0.080\ x + 0.07$; $s_b = 0.004$, $s_a = 0.03$). Correlation coefficients were > 0.996.

The stability evaluation of tetramethrin under abiotic conditions was performed from 0 to 72 h. In this range, no changes in the tetramethrin isomers were observed so that it was stable. However, in presence of daphnids (biotic conditions), tetramethrin suffered over 95% isomerization of *trans*-2 isomer and *cis*-isomers into *trans*-1 isomer (see Fig. 5A). The remaining/missing 5% could be bioaccumulated into the microinvertebrate's bodies. This result shows that toxicological effects of tetramethrin on aquatic organisms can be attributed to its *trans*-1 isomer.

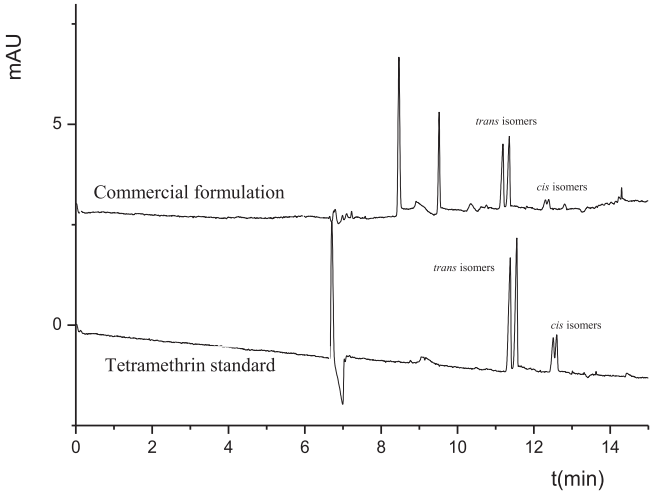

Fig. 4. Electropherograms obtained for tetramethrin standard and commercial formulation under the best experimental conditions using HP-β-CD. Other experimental conditions as in Fig. 3.

The toxic effects of tetramethrin on freshwater crustacean *D. magna* were defined in terms of immobilization and were expressed as EC_{10}, EC_{20}, EC_{50} and EC_{90} at different exposure times (24, 48 and 72 h) (Table 3) under darkness and static conditions, being our work the first evidence of those ecotoxicological parameters. In addition, the possible mode of action for tetramethrin in aquatic microinvertebrates was suggested for the first time in this work. Measured (instead of nominal) concentrations were used for the ecotoxicity parameters estimation.

Regarding the EC_{50} obtained in our work, tetramethrin is identified as toxic compound for aquatic ecosystems. The values obtained in the present article can be compared with those reported in the literature only for EC_{50} and an exposure time of 48 h (a value of EC_{50} 0.16 mg/L was reported in a static system and darkness conditions (ECHA, 2015; Seyfried, 2002b)). Moreover, under light photoperiod (16 h:8 h), EC_{50} values were 0.045 mg/L (EPA Environmental Fate and Effects Division, 1992) and 0.11 mg/L in flow-through system (ECHA, 2015; Blasberg, 1993) but these conditions are not comparable with the results obtained in this work. The EC_{50} ($t = 48$ h) value obtained in our work (1.52 ± 0.01 mg/L) was higher than that previously reported under similar conditions (0.16 mg/L). Unfortunately, no more information was available in the articles reported in the literature on the methodology employed for the analysis of tetramethrin in solution, or the composition of the culture medium used in exposure test that could help us to justify the differences found between both results. Results obtained in the present work are in agreement with the fact that pyrethroids and tetramethrin in particular were recognized to pose negative effects on fish and non-target invertebrates (Li et al., 2017; Lu et al., 2019; ECHA, 2015).

As can be observed in Table 3, tetramethrin toxicity presented time depending tendency. The values estimated at all effects levels (EC_{10}, EC_{20} and EC_{90}) decreased until the 72 h of incubation. This indicated that tetramethrin presents a high impact for aquatic microinvertebrates upon long-term exposure values for immobilization.

Moreover, based on the previously described stability data, our work demonstrated for the first time that the toxicity of tethrametrin in freshwater crustacean *D. magna* is attributed to the *trans*-1 isomer (see Fig. 5A). The different spatial configurations of pyrethroids induced different toxicity to aquatic organisms. For example, *trans*-permethrin presented more toxicity than *cis*-permethrin to water fleas such as *D. magna* or *Ceriodaphnia dubia* (Zhu et al., *2020*).

The possible mechanism of toxicity of tetramethrin in *D. magna* was investigated in terms of general oxidative stress produced. The ROS generation on neonates of microinvertebrates in contact with tetramethrin was recorded with CSLM and is shown in Fig. 5B. The concentrations used were the determined toxicity parameters at 24 h of exposure (EC_{10} - EC_{90} levels).

As can be seen, the specimens displayed generalized ROS formation during 24 h of exposure and were proportional with pesticide doses up to EC_{90} effect level (in mg/L). In fact, fluorescence quantified by image analysis increased 3.6-fold respect to the control assays (without pollutant) at EC_{10} effect level (in mg/L) of tetramethrin. In the same way, exposure at EC_{20} effect level of contaminant increased fluorescence 18.7-fold; while a 64.8-fold higher emission was measured at EC_{50} effect level of the pyrethroid.

At higher concentration of tetramethrin (EC_{90} effect level), ROS generation was observed mostly in the gut of neonates as indicated with arrows in Fig. 5B. Probably during exposure to high concentrations, tetramethrin entered the body of *D. magna* following two pathways. By directly absorption through their external carapace into the targeted

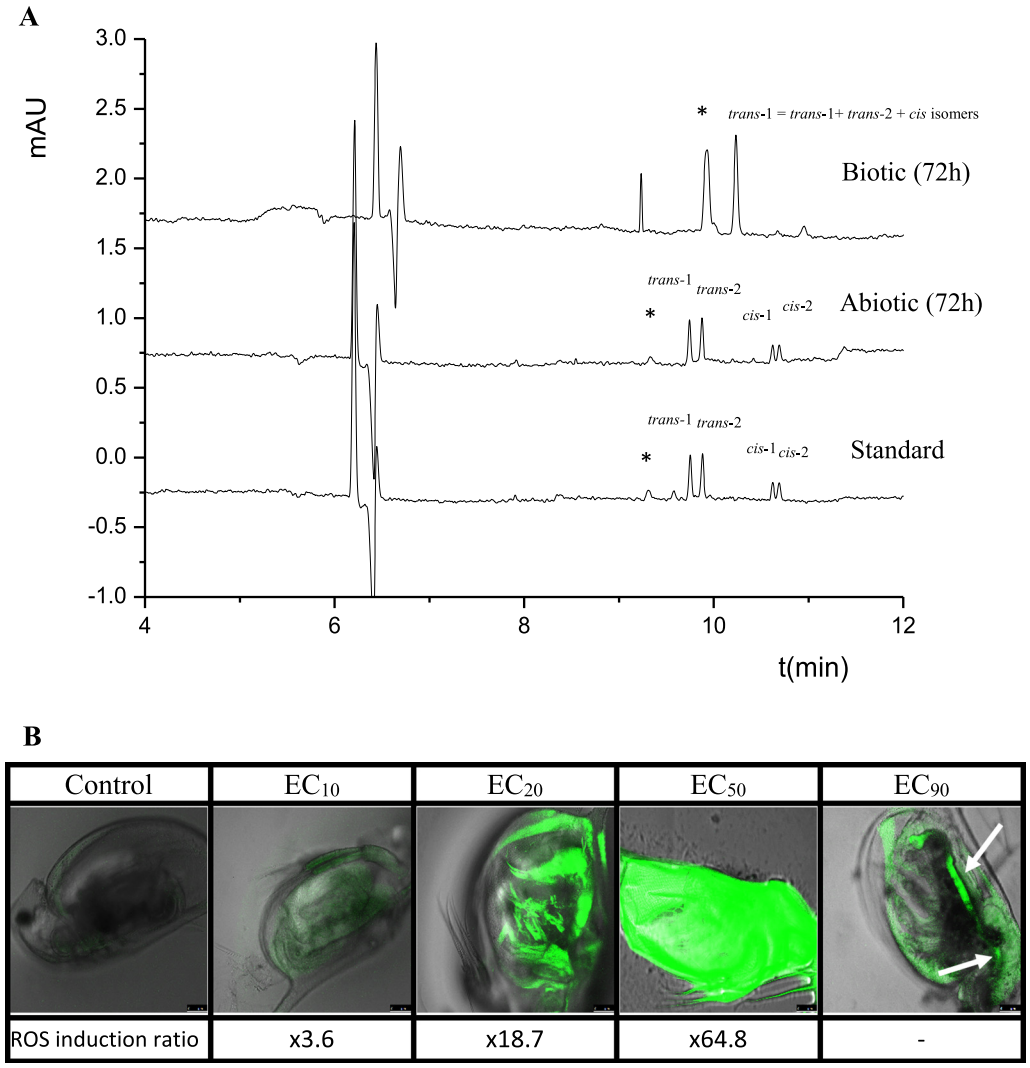

Fig. 5. (A) Analysis of tetramethrin under abiotic and abiotic conditions at 72 h. Experimental conditions as Fig. 3. * = *unknown peak corresponding to the medium.* (B) Confocal images of ROS generation in *D. magna* at 24 h of incubation at different effect levels of tetramethrin (EC_{10}, EC_{20}, EC_{50}, EC_{90}) and control.

tissue by diffusion, and by ingestion via food particles, pesticide micells, and also directly from the water phase. Both phenomena were associated with significant observed ROS generation. It can be assumed that tetramethrin can also be fixed/ embedded from the gut onto around tissue through diffusion (Eybe et al., 2009) and subsequently leading to higher toxic effects (high number of immobilized individuals), which probably explains the obtained EC_{90} determined for the immobilized number of organisms.

Both pathways seem plausible according to the relatively high lipophility of tetramethrin (Eybe et al., 2009). It has been widely reported that both types of pyrethroids (type I and type II) can induce ROS on aquatic organisms, such as fish and microinvertebrates (Zhu et al., 2020; Hernandez-Moreno et al., 2010; Toumi et al., 2014; Touaylia et al., 2019; Yang et al., 2020; Wang et al., 2016). However, this is the first experimental evidence of ROS generation on aquatic crustacean *D. magna* in contact with tetramethrin.

Table 3
Toxicological parameters (±SD) for tetramethrin on *D. magna* at different exposure times.

Exposure time (h)	EC_{10} (mg/L)	EC_{20} (mg/L)	EC_{50} (mg/L)	EC_{90} (mg/L)	r
24	5.29 ± 0.02	6.83 ± 0.02	10.57 ± 0.02	21.11 ± 0.02	0.994
48	0.49 ± 0.01	0.74 ± 0.01	1.52 ± 0.01	4.70 ± 0.01	0.947
72	0.51 ± 0.02	0.81 ± 0.02	1.80 ± 0.02	6.36 ± 0.01	0.917

4. Conclusions

A MEKC method based on the use of a chiral surfactant and a cyclodextrin was developed for the first time for the stereoselective separation of tetramethrin isomers. The separation of the four diasteroisomers was achieved using borate buffer (100 mM, pH 8.0) containing 2 M urea, 50 mM SDC and 15 mM HP-β-CD and applying a voltage of 20 kV and a temperature of 15 °C. Analysis times lower than 12.5 min with Rs values of 1.7 for *trans* isomers and 1.1 for *cis* isomers, were achieved. These results suppose a reduction of the analysis time in comparison with the previous LC and GC methods (20 and 32 min, respectively) with higher resolutions with respect to the GC and the CEC methods previously reported, which only allowed in the case of CEC the separation of two stereoisomers. The method developed in the present work constitutes the first tetramethrin stereoselective methodology reported by Electrokinetic Chromatography, which is one of the most powerful techniques to achieve chiral separations under sustainable conditions. The analytical characteristics of this methodology were assessed demonstrating its suitability for the quantitative analysis of tetramethrin to carry out for the first time stability and ecotoxicity studies at a chiral level and to achieve the quality control

of an antiparasitic commercial formulation. In this last case, the quality control was performed in 12.5 min analysis time which supposes an interesting reduction of the time needed to analyse these samples by GC (32 min) which is the only previously reported method describing the stereoselective analysis of insecticide formulations.

Stability evaluation of tetramethrin clearly demonstrated that this compound suffered over 95% isomerization of *trans*-2 isomer and *cis*-isomers into *trans*-1 isomer in presence of freshwater crustacean *D. magna*. This fact makes possible to attribute the ecotoxicity of tetramethrin on daphnids to the isomer *trans*-1. Ecotoxicity parameters EC_{10}, EC_{20}, EC_{50} and EC_{90} at different exposure times (24, 48 and 72 h) were calculated for the first time in the present work. Based on the EC_{50} at 48 and 72 h (1.52 ± 0.01 and 1.80 ± 0.02 mg/L, respectively), tetramethrin represents a toxic pollutant for aquatic environment. Moreover, this study provides the first evidence of oxidative stress-mediated mode of action for tetramethrin on aquatic microinvertebrates. This oxidative stress was generalized in the macroinvertebrate organism up to EC_{50} with further focalization on the digestive track at high effect levels (EC_{90}).

CRediT authorship contribution statement

M.Greño: investigation, methodology, formal analysis, validation, data curation, visualization, writing-original draft.

G.Amariei: conceptualization, investigation, methodology, data curation, formal analysis, visualization, writing-original draft-for ecotoxicological assessment.

K.Boltes: conceptualization, formal analysis, supervision, resources, writing-original draft, writing-review & editing, project administration, funding acquisition.

M.Castro-Puyana: conceptualization, methodology, formal analysis, resources, supervision, writing-original draft, writing-review & editing, project administration, funding acquisition.

M.A.García: conceptualization, methodology, formal analysis, resources, supervision, writing-original draft, writing-review & editing.

M.L.Marina: conceptualization, methodology, resources, supervision, writing-original draft, writing-review & editing, project administration, funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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