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Stereoselective separation of sulfoxaflor by electrokinetic chromatography and applications to stability and ecotoxicological studies

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ABSTRACT

An Electrokinetic Chromatography method was developed for the stereoselective analysis of sulfoxaflor, a novel sulfoximine agrochemical with two chiral centers. A screening with fourteen negatively charged CDs was performed and Succinyl-β-CD (Succ-β-CD) was selected. A 15 mM concentration of this CD in a 100 mM borate buffer (pH 9.0), using an applied voltage of 20 kV and a temperature of 15 °C made possible the baseline separation of the four stereoisomers of sulfoxaflor in 13.8 min. The evaluation of the linearity, accuracy, precision, LODs and LOQs of the method developed showed its performance to be applied to the analysis of commercial agrochemical formulations, the evaluation of the stability of sulfoxaflor stereoisomers under biotic and abiotic conditions, and to predict, for the first time, sulfoxaflor toxicity (using real concentrations instead of nominal concentrations), on two non-target aquatic organisms, the freshwater plant, *Spirodela polyrhiza,* and the marine bacterium, *Vibrio fischeri*.

1. Introduction

The world population growth and the increased demand for food productivity have led to an increased use of pesticides, which have become an essential part of agriculture [1,2]. Specifically, since 1950 their use has increased 50-fold, which has resulted in the registration of more complex structures, followed by a higher proportion of chiral pesticides [3], whose stereoisomers can present different toxicity and persistence. In addition, one of the stereoisomers can be active while the others may be less active or present toxic effects to non-target organisms [4,5]. In these cases, the use of the pure stereoisomer or an enriched mixture of the active stereoisomer is recommended in order to minimize the negative effects of the pesticide on the environment and non-target organisms [6]. The quality control of commercial agrochemical formulations as well as the investigation of the stability and toxicity of chiral pesticides require the development of adequate analytical methodologies capable of individually analyse their stereoisomers.

Sulfoxaflor, [methyl(oxo){1-[6-(*trifluoromethyl*)−3-*pyridyl*]ethyl}-λ^6-sulfanylidene]cyanamide [1], a systemic fourth generation neonicotinoid [7] belonging to the novel insecticide class of the sulfoximines [8,9], has two tetrahedral stereogenic atoms, one carbon atom bound to the third position of the pyridine ring, and the sulfur atom. Thus, it presents two pairs of enantiomers: (R,S)-sulfoxaflor/(S,R)-sulfoxaflor and (R,R)-sulfoxaflor/(S,S)-sulfoxaflor (Fig. 1) [8].

Government protection agencies in Europe and Canada alerted on the unintended environmental consequences associated to the use of neonicotinoids insecticides pertaining to the first generations. Regulatory authorities banned these neonicotinoids insecticides and recommended the use of alternative systemic insecticides to substitute them [10-16]. Sulfoxaflor emerged as an alternative insecticide (fourth generation neonicotinoid), which is widely used in agriculture around the world [17].

Sulfoxaflor has a potent insecticidal activity across sap-sustaining insects [18,19]. It is a potent neurotoxin, affecting the nicotinic acetylcholine receptors (nAChRs) [20]. The mechanism

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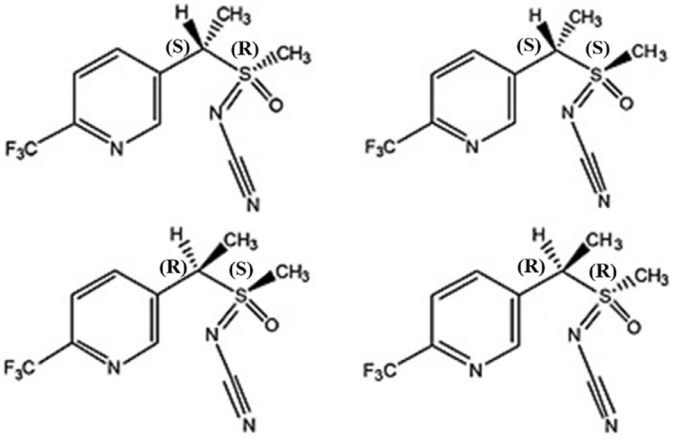

Fig. 1. Chemical structure of sulfoxaflor stereoisomers.

of toxicity eventually displays as cell collapse in exposed insects [21,22]. Due to its low cross-resistance with neonicotinoids like imidacloprid, sulfoxaflor has proven to be a potential alternative over the current neonicotinoids [23]. Nevertheless, there is an ecotoxicological risk to the environment, especially for the aquatic ecosystems to which this pollutant can easily reach by spray drift or by run-off [17]. Data on the environmental fate of sulfoxaflor are scarce. The European Chemical Agency (ECHA) reported that sulfoxaflor is stable to hydrolysis in aqueous environments, it does not undergo photolytic degradation, and is not readily biodegradable. So, this insecticide displays the potential to persist in aquatic environments [24]. A recent study indicates that sulfoxaflor presents an ecotoxicological risk to aquatic insects *Chironomus dilutes* [17].

Despite the potential of sulfoxaflor to adversely affect organisms inhabiting contaminated aquatic environments, there is no data available on the toxicities of sulfoxaflor to environmentally representative aquatic bacteria and primary producer species.

Today, sulfoxaflor is still employed and marketed all around the world as a mixture of the four stereoisomers. Only three articles conducted by Chen and co-workers reported the stereoselective analysis of this insecticide in different matrices such as soils and vegetables [8,25,26]. Using HPLC, the separation of the four stereoisomers of sulfoxaflor was performed in around 28 min with resolution values between consecutive peaks of 1.85, 1.54 and 3.08 [8]. Both ultra-performance convergence chromatography and ultrahigh-performance supercritical fluid chromatography coupled with a triple quadrupole mass spectrometer originated a considerable reduction in the analysis time to around 6 min with a minimum resolution between peaks of 1.5 [25,26].

Electrokinetic chromatography (EKC) is a Capillary Electrophoresis (CE) mode in which a chiral selector is added to the separation medium. It is a powerful tool to carry out stereoselective separations due to its numerous advantages including the easy change of the chiral selector and the variation of its concentration, the low consumption of reagents, solvents and samples, which reduces the environmental impact of the methods, and the short analysis times [27-31]. However, the separation of the four stereoisomers of sulfoxaflor has never been carried out by CE.

In this work, the first method allowing the stereoselective separation of sulfoxaflor by EKC was developed and applied to the analysis of sulfoxaflor-based agrochemical formulations and to evaluate stereoisomers stability under abiotic and biotic conditions. Moreover, for the first time, the acute ecotoxicological effect of sulfoxaflor on representative marine and freshwater sensitive aquatic species, specifically, the bacterium *Vibrio fischeri* (*V. fischeri*) and the plant *Spirodela polyrhiza* (*S. polyrhiza*), was characterized using real (not nominal) concentrations.

2. Materials and methods

2.1. Analytical method

2.1.1. Reagents and samples

All chemicals and reagents used were of analytical grade. Sodium hydroxide and boric acid were acquired in Sigma-Aldrich (St. Louis, MO, USA). Methanol was obtained from Scharlau (Barcelona, Spain). Carboxymethyl-γ-CD (CM-γ-CD, DS $\sim$ 3.5), carboxymethyl-α-CD (CM-α-CD, DS $\sim$ 3.5), (2-carboxyethyl)-β-CD (CE-β-CD, DS $\sim$ 3.5), (2-carboxyethyl)-γ-CD (CE-γ-CD, DS $\sim$ 3.5), succinyl-β-CD (Succ-β-CD, DS $\sim$ 3.4), succinyl-γ-CD (Succ-γ-CD, DS $\sim$ 3.5), sulfated α-CD (S-α-CD, DS $\sim$ 12), sulfated γ-CD (S-γ-CD, DS $\sim$ 10), phosphated β-CD (pH-β-CD, DS $\sim$ 4) and sulfobutylated β-CD (SB-β-CD, DS $\sim$ 6.3) were purchased from Cyclolab (Budapest, Hungary). Sulfated β-CD (S-β-CD, DS $\sim$ 18) and carboxymethyl-β-CD (CM-β-CD, DS $\sim$ 3) were from Sigma-Aldrich (St. Louis, MO, USA). Heptakis-(2,3-di-O-acetyl-6-O-sulfo)-β-CD (DA-β-CD) was supplied by AnaChem (Budel, The Netherlands). Sulfobutileter-β-CD (Captisol) was from Cydex Pharmaceuticals (Lawrence, Kansas). Water used was purified through a Milli-Q system from Millipore (Bedford, MA, USA).

Racemic sulfoxaflor was obtained from Greyhound Chromatography & Allied Chemicals Birkenhead, United Kingdom). The agrochemical formulation analysed (Closer®, Dow Agrosciences S.A., Madrid, Spain) contained an 11.43% of racemic sulfoxaflor according to the label.

2.1.2. Analytical procedure

Buffer solutions (100 mM, pH 9.0) were prepared by dissolving the appropriate amount of boric acid in Milli-Q water to obtain the desired concentration. Then, the pH was adjusted with 1 M sodium hydroxide to the desired value before completing the volume with water. Background electrolytes (BGEs) containing a CD were prepared dissolving the adequate quantity of each CD in the buffer solution.

Stock standard solutions of racemic sulfoxaflor were obtained by dissolving the adequate amount in methanol to have a final concentration of 1000 mg L^{-1}. All standard solutions were kept at -20 °C. Standard working solutions were obtained from the racemic stock standard solution of sulfoxaflor by dilution in water. The preparation of commercial formulation solutions consisted of weighing the appropriate amount of sample and extracting it with water using a high intensity focused ultrasounds (HIFU) probe (model VCX130, Sonics Vibre-Cell, Hartford, CT, USA) for 5 min at 50% amplitude. The sample was centrifuged for 10 min at 4000 rpm and 25 °C and supernatants were collected. All solutions were filtered through 0.45 μm Nylon syringe filters purchased from Scharlau (Barcelona, Spain) and sonicated before analysis using an ultrasonic bath B200 from Branson Ultrasonic Corporation (Danbury, USA).

Reagents, standards and samples were weighed in an OHAUS Adventurer Analytical Balance (Nänikon, Switzerland) and the pH of the separation buffer was adjusted with a pH-meter model 744 from Metrohm (Herisau, Switzerland).

EKC experiments were achieved in an Agilent 7100 CE system from Agilent Technologies (Waldbronn, Germany) with a diode array detector (DAD) and controlled by HP ^{3D}CE ChemStation software. 50 µm I.D. uncoated fused-silica capillaries with a total length of 58.5 cm (50 cm effective length) were employed (Polymicro Technologies (Phoenix, AZ, USA)).

New capillaries were rinsed (at a pressure of 1 bar) for 30 min with 1 M sodium hydroxide, followed by 15 min with Milli-Q water and finally for 60 min with buffer solution. Every working day, the capillary was flushed at the beginning (at a pressure of 1 bar) with 0.1 M sodium hydroxide, Milli-Q water, buffer solution and

BGE during 10, 5, 20 and 10 min, respectively. With the aim of ensuring the repeatability between injections, the capillary was conditioned with 0.1 M sodium hydroxide for 4 min, with Milli-Q water for 2 min, with buffer solution for 4 min and with BGE for 3 min.

2.1.3. Analytical data treatment

The Agilent Technologies Chemstation software was employed to acquire the values of migration times, peak areas and resolution values (Rs). With the aim of having good data reproducibility, corrected peak areas (Ac), calculated as the quotient between peak area and migration time, were considered. Composition of graphs with different electropherograms, experimental data analysis and calculation of the studied parameters were performed using Origin Pro 8, Excel Microsoft and Statgraphics Centurion XVII software.

2.2. Eco-toxicological study

In order to investigate the potential toxic effects of sulfoxaflor, two acute toxicity tests using *V. fischeri* (a sensitive bacterium model for marine ecosystems [32]) and *S. polyrhiza* an important aquatic specimen in the assessment of ecotoxicity on freshwater compartments [33]) were carried out.

2.2.1. Eco-toxicological assays with V. fischeri

The acute toxicity test for the bacterium *V. fischeri* was performed using a BioTox^{TM}1243–1000 WaterTox^{TM} Standard kit (MicroBioTests, Ghent, Belgium) following the fabricant guidelines and the UNE EN ISO 11,348–3: 2007 standard method. This test established the reduction of the bio-luminescence naturally emitted by the bacterium *V. fischeri* after 15 min of contact with a dilution series of the targeted compound, with subsequent calculation of the 15-min median effective concentration, EC_{50} (concentration of the evaluated samples that, in 15 min, inhibited 50% of the bioluminescence).

Briefly, freeze-dried *V. fischeri* were rehydrated with the reconstitution solution in order to prepare the bacterial inoculum. Before starting the test, the optimal salinity (2%) of the bacteria suspension was osmotically adjusted using a NaCl solution (20% w/v in deionized water). The acute toxicity was determined with working concentrations varying from 0.78 to 200 mg/L obtained by diluting with 2% NaCl water solution from a stock solution of racemic sulfoxaflor (2000 mg L^{-1}) in methanol, keeping the salinity of the samples at 2% content with respect to NaCl. The pH value of the samples was recorded and adjusted to 7.0 ± 0.2, as required by the standard. The bacterial inoculum was subsequently added to each pollutant solution. Nine final concentrations of racemic sulfoxaflor were obtained and tested: 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 mg L^{-1}. The saline solution (20 g L^{-1} NaCl) was used as control. All samples were tested by triplicate.

The exposure test was achieved in white sterile 96-well microplate, at 15 °C by using Fluoroskan Ascent FL Luminometer (Thermo Fisher Scientific, Waldham, MA, USA). The light output was measured during 60 min, at intervals of 1 min. The bioluminescence inhibition percentage was calculated from the integration of the light emission curve using Origin Pro 8 software for further EC_{50} calculation.

2.2.2. Eco-toxicological assays with S. polyrhiza

The freshwater plant *S. polyrhiza* acute test was carried out using Duckweed Toxkit F^{TM} kit (MicroBioTests, Gent, Belgium) according to both the manufacturer's instructions and the International Standard ISO 20,227: 2017, with some modifications. This test established the growth reduction of the "first frond" of the plant after 96 h exposure to a dilution series of the targeted compound, with subsequent calculation of the 96 h EC_{50}.

In order to supply the biological culture for the duckweed toxicity test, the dormant vegetative buds (turions) were germinated for 72 h, in standardised Steinberg medium, under controlled conditions (25 °C, 6000 lux light) on a growth chamber (IBERCEX, Madrid, Spain). Nine working (tested) concentrations of racemic sulfoxaflor, ranging from 0.78 to 200 mg L^{-1}, were obtained, from an initial stock solution (2000 mg L^{-1} in methanol) by diluting with the Steinberg medium. For the exposure experiment, a transparent 24-well plate was filled with 2 mL per well of each tested sample, including a control (0 mg L^{-1} racemic sulfoxaflor), and subsequently inoculated with 1 freshly, heathy, and uniform frond sized plant. Each sample was tested by duplicate. The contact was performed during 96 h (25 °C, 6000 lux light, IBERCEX, Madrid, Spain). The plants were digitally photographed at 0, 24, 48, 72, and 96 h of exposition.

The growth inhibition of the duckweed was determined by area measurement of the first frond using digital image treatment (Image J software, National Institute of Health, Rasband, WS, USA). In addition, the photosynthesis efficiency, in terms of chlorophyll fluorescence (CF), was analysed via confocal recording (Leica TCS SP5 system, Germany, $\lambda_{exc}/\lambda_{em}$ = 488/595–700 nm) of its components (bud, leave, root). The intensity was estimated by processing confocal images with Image J software. The growth and CF inhibition percentages were assessed using Excel Microsoft software for further EC_{50} calculation.

2.2.3. Estimation of toxicity parameters

Acute toxicity parameters (EC_{50} and EC_{20}) of sulfoxaflor were estimated by fitting inhibition data to concentration-response curve in CompuSyn [34] using the median-effect- isobologram equation [35-37]:

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

D corresponds to a sample concentration which induces a fractional negative effect fa; Dm represents the median effective concentration (EC_{50}), and m describes the sigmoidicity to the concentration-effect curve.

2.3. Stability assessment

The stability of each stereoisomer was assessed in abiotic and biotic runs using racemic mixtures of the four isomers in each experiment. Concentrations of racemic sulfoxaflor (ranging from 0.39 to 100 and from 0.78 to 200 mg L^{-1} for marine and freshwater media, respectively) were systematically incubated in abiotic assays, in absence of light and under controlled irradiation. In parallel, same concentrations of racemic sulfoxaflor were tested in presence of each biological specimen (biotic assays).

Enantiomers concentration were evaluated at initial time and at the end of each assay (1 h for *V. fischeri*, 96 h for *S. polyrhiza*). All analyses were performed by duplicate.

3. Results and discussion

3.1. Development of an EKC method for the stereoselective analysis of sulfoxaflor

Since CDs are potent chiral selectors, fourteen CDs negatively charged at the working pH (CM-α-CD, CM-β-CD, CM-γ-CD, CE-β-CD, CE-γ-CD, Succ-β-CD, Succ-γ-CD, S-α-CD, S-β-CD, S-γ-CD, pH-β-CD, SB-β-CD, DA-β-CD and Captisol) were tested with the aim of achieving the separation of the four enantiomers of sulfoxaflor, which, in all the pH range, is neutral. In all cases, CDs were at a 10 mM concentration (except Succ-γ-CD, Captisol, CM-β-CD, and S-β-CD which were added at a concentration of 2% w/v) in

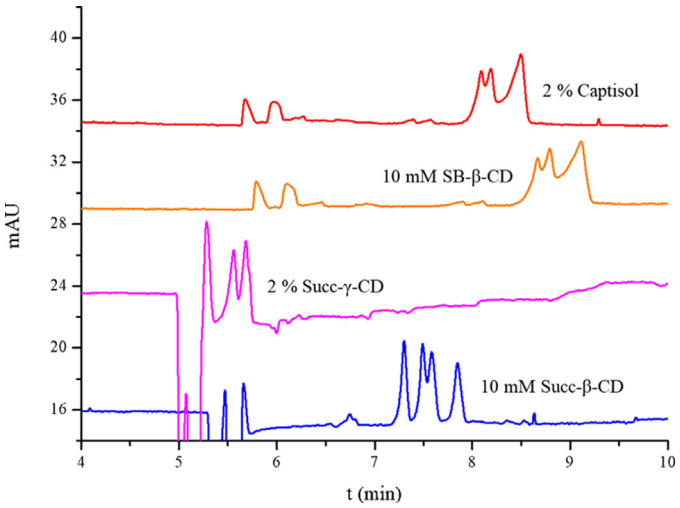

Fig. 2. Electropherograms illustrating the separation of the four stereoisomers of sulfoxaflor employing Succ-β-CD, Captisol, SB-β-CD and Succ-γ-CD as chiral selectors. Experimental conditions: 10 mM CD (Succ-β-CD and SB-β-CD) or 2% w/v CD (Captisol and Succ-γ-CD) in 100 mM borate buffer (pH 9.0); uncoated fused-silica capillary 50 µm id × 50 cm (58.5 cm to the detector); injection by pressure 50 mbar × 10 s; applied voltage 20 kV; temperature 20 °C; λ 205 ± 4 nm and [Racemic sulfoxaflor]: 200 mg L^{-1}.

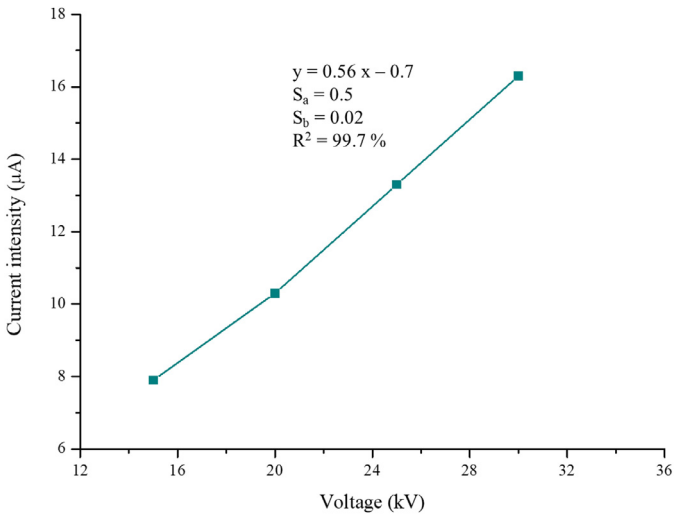

Fig. 3. Oms's plot obtained under the following experimental conditions: 15 mM Succ-β-CD, 100 mM borate buffer (pH 9.0), 15 °C, λ 205 ± 30 nm without reference. Other conditions as in Fig. 2.

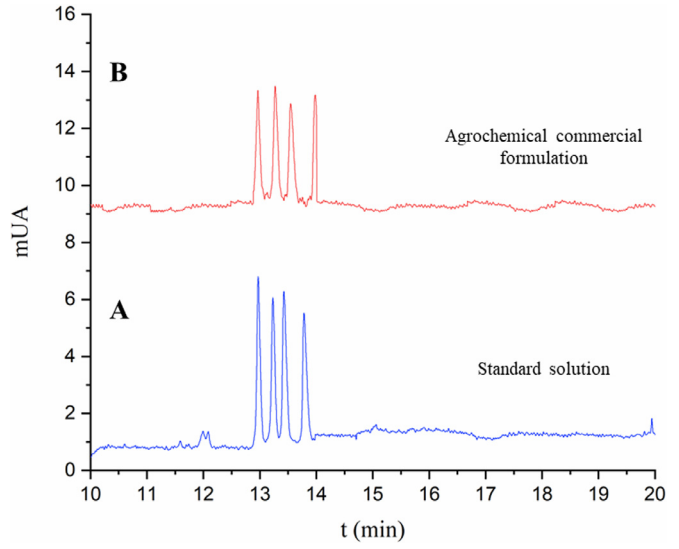

Fig. 4. Electropherograms obtained for (A) a sulfoxaflor standard solution and (B) a sulfoxaflor-based agrochemical commercial formulation solution, under the optimized conditions. Experimental conditions: 15 mM Succ-β-CD; injection by pressure 50 mbar × 8 s; temperature 15 °C; λ 205 ± 30 nm (reference off) and [Racemic sulfoxaflor]: 100 mg L^{-1}. Other conditions as in Fig. 2.

100 mM borate buffer (pH 9.0). A temperature of 20 °C and a voltage of 20 kV were employed. As can be observed in Fig. 2, only with four of the fourteen CDs tested, some chiral discrimination was observed; Succ-γ-CD lead to two peaks, SB-β-CD and Captisol to three peaks and Succ-β-CD to four peaks (although not baseline separated), corresponding to the four enantiomers of the analyte. Taking this into account and knowing that the analysis time when using Succ-β-CD was less than 8 min, this CD was chosen. With the aim of improving the resolution and the shape of the peaks, other experimental variables were optimized.

The effect of the Succ-β-CD concentration was investigated in the 5 to 20 mM range (5, 10, 15 and 20 mM). It was noted that as the CD concentration increased, the analysis time and the resolution increased too. An improvement in the separation of the 4 enantiomers was obtained for a concentration of CD of 15 mM (analysis time of 11.5 min; resolution values between consecutive peaks of 2.3, 1.2 and 2.6). Although the resolutions obtained when a concentration of Succ-β-CD of 20 mM were better, the analysis time was much higher (20.6 min). As a commitment between analysis time and resolution, 15 mM Succ-β-CD was selected.

Afterwards, some detection parameters such as the bandwidth (4, 15 and 30 nm) and the possibility of using reference wavelength (300 nm; bandwidth of the reference when selected: 100 nm) were optimized. Wavelength was set at 205 nm (bandwidth 30 nm, reference off) as the highest peak heights were acquired with these values since sensitivity increased.

Subsequently, the influence of the temperature (15, 20 and 25 °C) was studied. While an increase in temperature from 20 °C to 25 °C reduced the resolution between consecutive peaks (1.9, 0.7 and 2.1) in an analysis time of 10 min, a temperature of 15 °C gave rise to the baseline separation of the 4 stereoisomers of sulfoxaflor (resolution values between consecutive peaks of 2.1, 1.5 and 2.6) in 13.8 min. Thus, a temperature of 15 °C was selected as optimum.

With respect to the effect of the applied voltage, an increase in this parameter originated shorter analysis times (10.2 min for 25 kV and 8.0 min for 30 kV) but worse resolution values between consecutive peaks (2.0, 1.4 and 2.6 for 25 kV and 1.9, 1.3 and 2.4 for 30 kV) while a voltage of 15 kV led to better resolution values (3.3, 2.4 and 3.8) but in a much higher analysis time (23.7 min) so a value of 20 kV was chosen (current intensity 10.3 μA). Fig. 3 shows the Oms' plot which demonstrates that current intensity values were adequate. Fig. 4A shows the enantioseparation of sulfoxaflor under the optimized conditions.

3.2. Analytical parameters of the EKC method

The analytical characteristics of the EKC method developed were evaluated with the purpose of applying it to the quantitative analysis of sulfoxaflor in agrochemical formulations, to study its stability in presence (biotic) and absence (abiotic) of organisms, and to predict its ecotoxicity on two non-target aquatic organisms, the duckweed, *S. polyrhiza*, and the marine bacterium, *V. fischeri*. With this aim, the linearity, precision, accuracy, limits of detection (LODs) and limits of quantification (LOQs) were evaluated. Results are grouped in Table 1.

Table 1
Analytical characteristics of the EKC method.

	First-migrating stereoisomer	Second-migrating stereoisomer	Third-migrating stereoisomer	Fourth-migrating stereoisomer
External standard calibration ($n = 9$) [a]				
Linear interval (mg L^{-1})	4–50	4–50	4–50	4–50
Slope $\pm$ $t \bullet S_{slope}$	0.087 $\pm$ 0.002	0.074 $\pm$ 0.002	0.083 $\pm$ 0.002	0.073 $\pm$ 0.002
Intercept $\pm$ $t \bullet S_{intercept}$	0.03 $\pm$ 0.07	0.04 $\pm$ 0.06	0.04 $\pm$ 0.06	0.05 $\pm$ 0.06
R^2	99.8%	99.7%	99.8%	99.7%
Standard additions calibration for commercial formulation [b]				
Linear interval (mg L^{-1})	0–35	0–35	0–35	0–35
Slope $\pm$ $t \bullet S_{slope}$	0.085 $\pm$ 0.006	0.077 $\pm$ 0.006	0.083 $\pm$ 0.007	0.077 $\pm$ 0.006
R^2	99.5%	99.5%	99.3%	99.3%
Accuracy				
p-value of ANOVA	0.3195	0.1140	0.7618	0.0844
Recovery (%) ($n = 6$) [c]	98 $\pm$ 3	96 $\pm$ 4	98 $\pm$ 5	96 $\pm$ 6
Standard additions calibration for plant culture samples [b]				
Linear interval (mg L^{-1})	0–50	0–50	0–50	0–50
Slope $\pm$ $t \bullet S_{slope}$	0.084 $\pm$ 0.003	0.073 $\pm$ 0.002	0.084 $\pm$ 0.003	0.073 $\pm$ 0.001
R^2	99.7%	99.9%	99.7%	99.9%
Accuracy				
p-value of ANOVA	0.0641	0.1105	0.5034	0.3663
Recovery (%) ($n = 3$) [c]	101 $\pm$ 2	97 $\pm$ 3	102 $\pm$ 2	96 $\pm$ 5
Standard additions calibration for vibrio culture samples [b]				
Linear interval (mg L^{-1})	0–50	0–50	0–50	0–50
Slope $\pm$ $t \bullet S_{slope}$	0.086 $\pm$ 0.005	0.072 $\pm$ 0.005	0.083 $\pm$ 0.004	0.070 $\pm$ 0.005
R^2	99.7%	99.4%	99.7%	99.4%
Accuracy				
p-value of ANOVA	0.5293	0.1966	0.6446	0.0562
Recovery (%) ($n = 3$) [c]	95 $\pm$ 5	99 $\pm$ 2	97 $\pm$ 5	97 $\pm$ 5
Precision				
Instrumental repeatability [d]				
Enantiomer concentration (mg L^{-1})	**10** **25**	**10** **25**	**10** **25**	**10** **25**
t, RSD (%)	1.6 0.4	1.7 0.4	1.6 0.4	1.7 0.4
A_c, RSD (%)	2.6 1.5	2.6 1.6	2.4 1.1	2.5 1.6
Method repeatability [e]				
t, RSD (%)	1.2 1.2	1.4 1.4	1.4 1.2	1.4 1.2
A_c, RSD (%)	2.3 1.8	2.2 2.3	2.3 2.6	2.3 2.6
Intermediate precision [f]				
t, RSD (%)	1.7 0.6	1.7 0.6	1.7 0.6	1.8 0.6
A_c, RSD (%)	1.5 4.2	1.6 3.8	1.7 5.3	1.7 4.2
LOD [g]	0.9	1.0	0.9	0.9
LOQ [h]	4.0	4.0	4.0	4.0

A_c: corrected area.
[a] Linearity was determined from nine standard solutions of racemic sulfoxaflor from 16 to 200 mg L^{-1} (from 4 to 50 mg L^{-1} for each isomer) by representing corrected peak areas (Ac) as a function of sulfoxaflor concentration in mg L^{-1}. Racemic sulfoxaflor standard solution injected by triplicate.
[b] Addition of known amounts of racemic sulfoxaflor standard solution to commercial formulation sample containing 60 mg L^{-1} of sulfoxaflor, to the culture medium of freshwater plants or to the culture medium of the marine bacterium. p value of ANOVA corresponds to the comparison of the slope obtained by the external calibration method and each of the slopes obtained for the standard additions calibration method at a 95% confidence level.
[c] Accuracy was assessed as the mean recovery obtained from a commercial formulation containing 60 mg L^{-1} of sulfoxaflor (according to the label) spiked with 70 mg L^{-1} of racemic sulfoxaflor standard solution, and from culture medium of freshwater plant and culture medium of marine bacterium solutions spiked, each, with 80 mg L^{-1} of racemic sulfoxaflor standard solution.
[d] Calculated from racemic sulfoxaflor standard solutions injected six-fold in a row at two concentration levels, 40 and 100 mg L^{-1}.
[e] Value obtained from three racemic sulfoxaflor standard solutions injected consecutively in triplicate in the same day at two concentration levels, 40 and 100 mg L^{-1}.
[f] Calculated from three racemic sulfoxaflor standard solutions injected in triplicate in three days in a row at two concentration levels, 40 and 100 mg L^{-1}.
[g] Experimentally obtained LOD ($S/N = 3$).
[h] Value corresponding to the first point of the calibration curve.

Linearity was ensured to be adequate for all isomers since R^2 values were higher than 99% and the zero value was contained in the confidence intervals for the intercepts and not contained in the confidence intervals for the slopes (for a 95% confidence level) (Table 1). The presence of matrix interferences was studied by comparing the confidence intervals for the slopes of the external standard and the standard additions calibration methods for the commercial formulation, for the freshwater plant culture medium and for the marine bacteria culture medium using the *t*-test and comparing the slopes values using p-values. There were no matrix interferences as can be seen in Table 1 so the external calibration method was employed to the quantitation of each stereoisomer in the samples.

Precision was evaluated at two concentration levels for migration times and corrected peak areas in terms of instrumental repeatability, method repeatability and intermediate precision. RSD values obtained were between 0.4 and 1.8% for migration times and between 1.1 and 5.3% for corrected peak areas.

The accuracy of the method was studied as the mean recovery obtained for the four stereoisomers of sulfoxaflor under the conditions detailed in Table 1 showing that the 100% value was included in all cases.

3.3. Analysis of sulfoxaflor agrochemical formulations

The analysis of an agrochemical commercial formulation was carried out and the content of sulfoxaflor in this sample was determined. Fig. 4B shows the electropherograms obtained for the sample solution. Little differences in migration times were observed between standard (Fig. 4A) and sample electropherograms that could be caused by minor changes in the electroosmotic flow or the matrix sample. A content of 11.7 $\pm$ 0.3 mg per 100 mg of sample was determined, which corresponded to a percentage

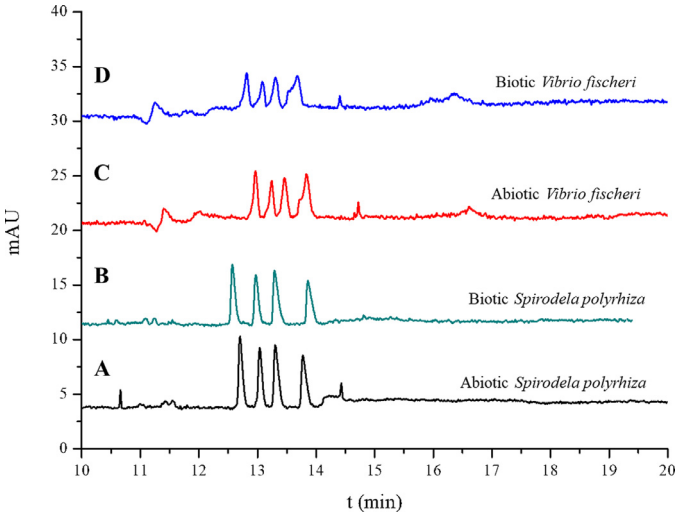

Fig. 5. Electropherograms corresponding to sulfoxaflor analysis in *S. polyrhiza* medium under abiotic (A) and biotic conditions (B); and *V. fischeri* medium under abiotic (C) and biotic (D) conditions. Initial concentration of racemic sulfoxaflor: 100 mg L^{-1}. Other experimental conditions as in Fig. 3.

of 103 ± 3 of the labelled amount. Although sulfoxaflor is nowadays commercialized as racemic mixture, these formulations need further eco-toxicological evaluation at the light of more extensive data on its environmental risk that are required, so the method developed in this work has a big potential to the control of those formulations that could be commercialized in the future based on one or various isomers.

3.4. Stability evaluation of sulfoxaflor stereoisomers

Stability of sulfoxaflor was investigated in the range from 0.39 to 100 and 0.78 to 200 mg L^{-1} using marine bacteria and freshwater plant culture media, respectively, under abiotic and biotic conditions. Initial and final real concentrations (1 h of contact in case of *V. fischeri* and 96 h of contact for *S. polyrhiza*) were determined for each stereoisomer and racemic sulfoxaflor. Fig. 5 presents the electropherograms for sulfoxaflor in *S. polyrhiza* and *V. fischeri* media under abiotic (Fig. 5A and 5C, respectively) and biotic conditions (Fig. 5B and 5D, respectively). It can be observed that the last peak in electropherograms 5C and 5D is asymmetrical but this asymmetry was not related to the presence of an organism since the same asymmetry was observed under abiotic conditions. Co-migrating of other compounds was discarded to justify this asymmetry since culture medium samples were injected without sulfoxaflor and no peaks were observed. Moreover, peak purity was 95.9% and 99.8% for electropherograms C and D, respectively. Finally, stability of sulfoxaflor [24] with the fact that the culture medium for the bacterium does not allow growing nor degradation, enable to discard a degradation of this compound originating degradation products. Fig. 6 shows that no significant differences were observed for all the stereoisomers neither for racemic sulfoxaflor since the percentage of variation for all of them decreased in the same proportion under the same specific assay conditions.

In freshwater medium used for plant growth, a minimum decay of the percentage variation of the concentration (approximately of a 3%) was obtained after 96 h of abiotic incubation (under both dark and light), indicating that neither racemic sulfoxaflor nor the stereoisomers undergo physicochemical degradation. In contrast, under biotic conditions a decrease of around a 15% of the initial concentration of racemic sulfoxaflor and all stereoisomers was found.

In the marine bacteria medium, the percentage decay of the concentrations was of about 11% in all cases after 1 h of abiotic incubation in the saline environment under dark conditions. Under biotic conditions, the percentage decay of the concentration increased to approximately a 31% for both, racemic sulfoxaflor and the four stereoisomers, twice the value obtained in presence of freshwater plant. These results suggest that despite the shorter test time, in a marine environment the concentration of sulfoxaflor in solution would be much lower than in a continental aqueous environment. In fact, the real concentrations of sulfoxaflor for *V. fischeri*

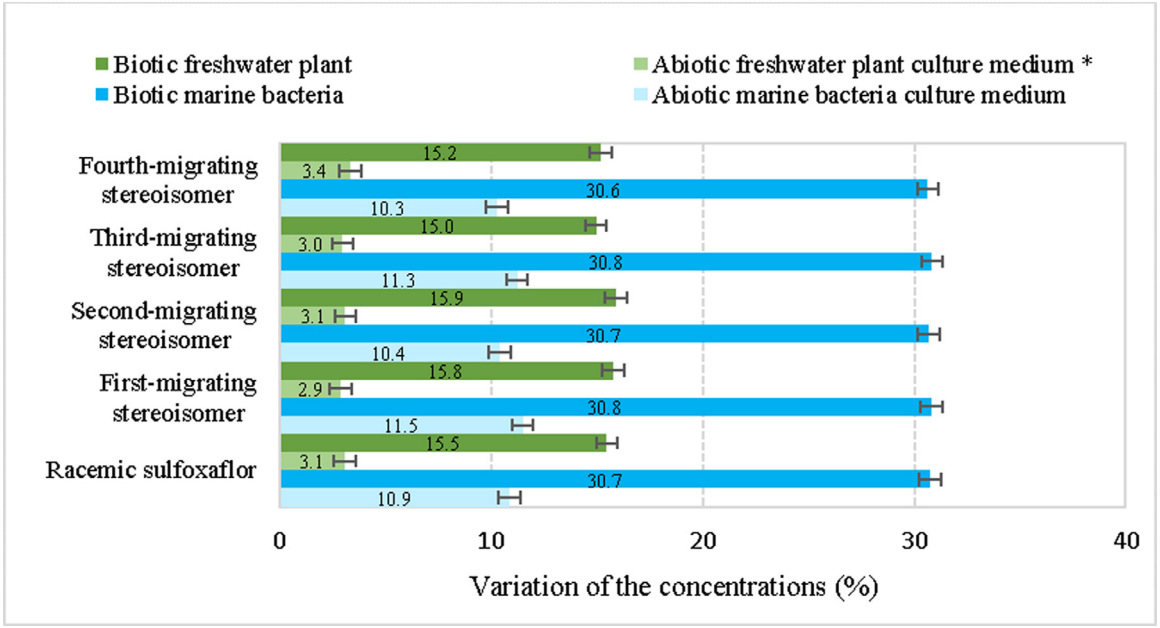

Fig. 6. Percentage decay of the real concentrations of sulfoxaflor stereoisomers and racemic sulfoxaflor with respect to nominal concentrations, evaluated under *V. fischeri* test conditions (values obtained at 1 h of contact, in presence and absence of bacteria) and *S. polyrhiza* test conditions (values obtained at 96 h of contact in presence and absence of plant with light). Error bars represent standard deviation. *Results obtained for plant without light are not shown in the Figure although they were similar to those under light.

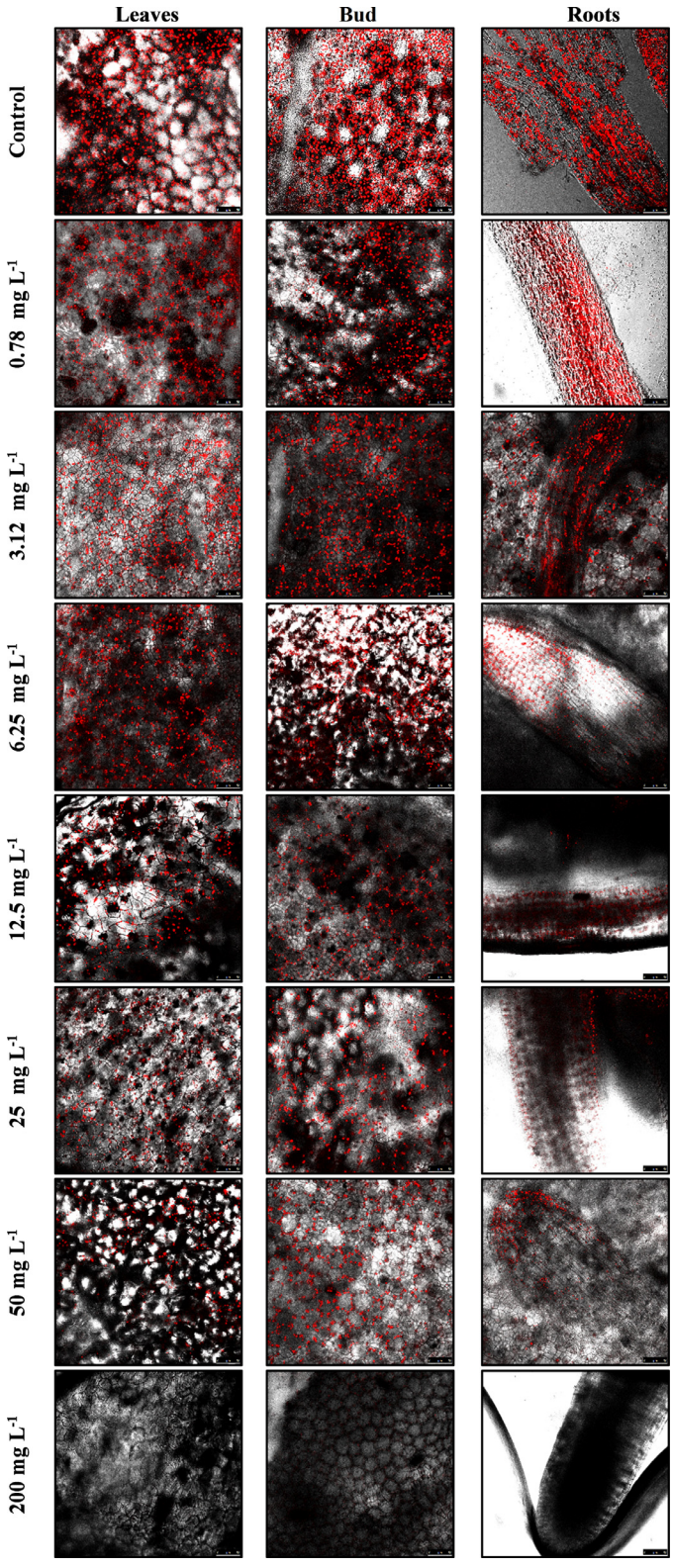

Fig. 7. Representative Confocal micrographs corresponding to chlorophyll fluorescence of *S. polyrhiza* duckweed on leaves, bud, and roots, respectively, after exposure for 96 h with racemic sulfoxaflor at concentrations between 0.78 and 200 mg L^{-1} (Scale bar represents 50 μm).

and *S. polyrhiza* exposure correspond to 69% and 85% of the nominal ones, respectively.

According to the stability studies under abiotic conditions registered for racemic sulfoxaflor by the ECHA, this compound is hydrolytically and photolytically stable in aqueous conditions, at a wide range of environmentally relevant pH (5–9) [24]. These data are in agreement with the results obtained under abiotic conditions in the present study, and sulfoxaflor can be considered stable in mostly continental aquatic environments. ECHA also reported that sulfoxaflor suffered less than approximately a 3% biodegradation after 28-days study period considering this compound as not readily/rapidly degradable by freshwater aerobic bacteria [24]. No stability and biodegradability data were previously reported for racemic sulfoxaflor in marine environments, but our results show that its stability could be lower in these environments than in freshwater. No studies related with sulfoxaflor stereoisomers stability were previously reported, being this study the first one carried out with this aim.

The biotic experiments with marine specie *V. fischeri* were carried out under not growing conditions of the bacteria, so biodegradation of sulfoxaflor is very difficult to take place, but sorption of pollutant into bacterial cell could be possible and probably explain the lower concentration of pollutant found in solution under these test conditions.

3.5. Eco-toxicological profiles of sulfoxaflor in the freshwater plant S. polyrhiza and the bacterium V. fischeri

The eco-toxicological profiles of sulfoxaflor on the two considered organisms were studied for the first time. Real concentrations of sulfoxaflor were used for the determination of its toxicity. The toxicological parameters (EC_{20} and EC_{50}) for aquatic plant were estimated employing the frond growth and CF (buds, leaves and roots) end-points. The toxicity profile for marine bacterium was established using natural bioluminescence as end-point. Table 2 shows that the EC_{50} values estimated using the size of the first frond of the aquatic plant between 24 h and 96 h of exposure presented a continuous decrease trend and the same happens for EC_{20} values. The individual toxicity of sulfoxaflor stereoisomers could not be assessed due to the lack of commercially available stereoisomer standards. These results agree with the European Regulation (EC1272/2008), which states that sulfoxaflor can be classified as toxic and very toxic compound to continental aquatic environment, depending on exposure time. The high stability of sulfoxaflor in the aqueous medium and under light irradiation, benefits its continuous exposure to the duckweed leading to increased toxicity with time. Fig. 7 shows a clear change in the natural chlorophyll fluorescence emission as a function of the concentration of sulfoxaflor. The CF for buds and roots measured at 96 h incubation were affected at similar EC_{50} values obtained for plant growth (Table 2). Leaves showed the highest reduction in this biological response compared with buds and roots being EC_{50} similar to that for the first frond. EC_{20} variation profile was similar to that of EC_{50} for both endpoints.

The EC_{50} value for marine bacteria at 5 min of incubation increased at 15 min of exposure time. Similar variation pattern was observed for EC_{20} (see Table 2). The lower incidence of sulfoxaflor on the bacteria can be attributed to the reduced stability in marine environment, as described in Section 3.4 and to the low toxic sensitivity of bacteria to the pollutant. Probably the bioluminescence emission, used as endpoint for this biosensor is less affected by sulfoxaflor than in the case of the duckweed.

The results obtained in this study are the first eco-toxicological data reported for sulfoxaflor towards both, marine *V. fischeri* bacterium and freshwater *S. polyrhiza* plant.

Table 2
Toxicological parameters of sulfoxaflor on *V. fischeri* and *S. polyrhiza*.

Spirodela polyrhiza							
Evaluation of first frond					**Evaluation of chlorophyll fluorescence 96h**		
Exposure time	**24 h**	**48 h**	**72 h**	**96 h**	**Bud**	**Leaves**	**Roots**
EC20 (mg L^{-1})	0.72 ± 0.05	0.40 ± 0.10	0.33 ± 0.02	0.28 ± 0.01	0.35 ± 0.03	0.06 ± 0.01	0.99 ± 0.01
EC50 (mg L^{-1})	2.41 ± 0.02	1.30 ± 0.10	1.23 ± 0.05	0.93 ± 0.02	3.01 ± 0.02	0.95 ± 0.02	2.71 ± 0.01
Vibrio fischeri							
Exposure time	**5 min**		**10 min**	**15 min**			
EC20 (mg L^{-1})	14.27 ± 0.02		13.20 ± 0.10	44.60 ± 0.20			
EC50 (mg L^{-1})	60.10 ± 0.10		473.60 ± 0.10	507.90 ± 0.20			

EC20 and EC50 correspond to the concentration of sulfoxaflor that reduced the targeted biological endpoint with 20% and 50%, respectively. All data are expressed in base of 95% confidence interval.

Since no previous studies have been reported for comparison, the results obtained for the ecotoxicity of sulfoxaflor have been compared with the data reported for its neonicotinoid predecessor, imidacloprid. The toxicity data available for imidacloprid on primary producers such macrophytes, indicate EC_{50} values higher than 0.93 ± 0.02 mg L^{-1} (10 mg L^{-1} for *Desmodesmus subspicatus* and 740 mg L^{-1} for *Lemna minor* [38-40]), while on bacteria EC_{50} values were like the results achieved in this work [41,42], showing that the toxicity of sulfoxaflor is similar or higher than that of its predecessor imidacloprid for aquatic organisms.

4. Conclusions

A novel EKC method has been developed for the first time for the separation of the four stereoisomers of the sulfoximine insecticide sulfoxaflor. Different negatively charged CDs were tested, being Succ-β-CD the most suitable. The stereoisomers of sulfoxaflor were separated in 13.8 min with resolution values between consecutive peaks of 2.1, 1.5 and 2.6. The chiral developed methodology demonstrated its suitability for the analysis of sulfoxaflor-based commercial agrochemical formulations and to carry out the stability studies of sulfoxaflor and to predict its toxicity. The stability studies for both, biotic and abiotic conditions, revealed that sulfoxaflor is less stable in marine than in freshwater environments.

Considering the probable environmental occurrence, our investigation determined that the alternative systemic sulfoxaflor insecticide has potential to cause even higher risk to ecologically important/sensitive freshwater and marine aquatic species like *V. fischeri* and *S. polyrhiza*. Therefore, the commercially available products containing this active compound need further eco-toxicological investigation.

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.

CRediT authorship contribution statement

Sara Jiménez-Jiménez: Investigation, Methodology, Formal analysis, Validation, Data curation, Visualization, Writing – original draft. **Georgiana Amariei:** Investigation, Data curation, Visualization. **Karina Boltes:** Methodology, Formal analysis, Resources, Supervision, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **María Ángeles García:** Conceptualization, Methodology, Formal analysis, Resources, Supervision, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **María Luisa Marina:** Conceptualization, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

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