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Evaluation of new cellulose-based chiral stationary phases Sepapak-2 and Sepapak-4 for the enantiomeric separation of pesticides by nano liquid chromatography and capillary electrochromatography

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ABSTRACT

Two novel polysaccharide-based chiral stationary phases (CSPs), known as Sepapak-2 (cellulose tris(3-chloro-4-methylphenylcarbamate)) and Sepapak-4 (cellulose tris(4-chloro-3-methylphenylcarbamate)), have been evaluated in this work for the chiral separation of a group of 16 pesticides including herbicides, insecticides and fungicides. The optimization of the mobile phase employed in nano-liquid chromatography (nano-LC) enabled the chiral separation of seven pesticides on Sepapak-2 and of nine pesticides on Sepapak-4. Due to the fact that Sepapak-4 gave better results, this column was selected to compare nano-LC and capillary electrochromatography (CEC) under the same conditions that consisted in the use of a 90/9/1 (v/v/v) ACN/H_2O/ammonium formate (pH 2.5) background electrolyte (BGE). As expected, both the efficiency and the chiral resolution obtained in CEC experiments were higher than in nano-LC for all the analyzed compounds. The analytical characteristics of the CEC developed methodology were evaluated in terms of linearity, LODs, LOQs, precision, selectivity, and accuracy allowing its application to the quantitation of metalaxyl and its enantiomeric impurity in a commercial fungicide product marketed as enantiomerically pure (metalaxyl-M) and in soil and tap water samples after solid phase extraction (SPE). The determined amount of metalaxyl-M was found to be a 26% above the labeled content and it contained an enantiomeric impurity of a 3.7% of *S*-metalaxyl was determined.

1. Introduction

Pesticides are active compounds used in agriculture to control pest being the number of commercially available pesticides very high. Many pesticides are chiral compounds (approximately 25% of them) and in most cases one of the enantiomers presents the pesticide activity while the other can present different activity toward the target organism [1–3]. In these cases, the use of enantiomerically pure pesticides would result in a major effectiveness in controlling insects or weeds in agriculture and reducing environmental risks. Other reason for using enantiomerically pure pesticides is that whereas the active enantiomer has the desired effects on target species, the other enantiomer may have adverse effects on some non-target organisms [3]. Moreover, biotic processes such as microbiological transformation are commonly enantioselective and the use of racemic pesticides can result in different environmental fates, resulting, from an environmental point of view, one enantiomer safer than the other [4,5]. Therefore, the search for new and effective methods for the separation and determination of pesticide enantiomers is necessary in order to optimize enantioselective production processes, assessing the enantiomeric purity of commercial formulations, and monitoring their presence in the environment or into different types of matrices.

An example of particular interest is the fungicide metalaxyl [(*R*/*S*)-methyl-N-(2-methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate], employed to control plant diseases caused by pathogens of the *Oomycota* division [6]. It has been demonstrated that the activity of the *R*-enantiomer (metalaxyl-M) is around 1000 times higher than that of the *S*-enantiomer [7]. In addition, the degradation of metalaxyl enantiomers in environment is also clearly enantioselective. In fact, the *S*-enantiomer has shown a faster degradation in vegetables being this enantiomer active for less time, whereas in the case of soils the first enantiomer being degraded/decomposed is the *R*-enantiomer [8].

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CEC and nano-LC with packed capillary columns have been considered very promising techniques for chiral separations because of their high efficiency, low sample requirements, low solvent and stationary phase consumption, and improved sensitivity when coupling with mass spectrometer. However, the lack of commercially available chiral columns is the main drawback of these techniques. As a consequence, the preparation of efficient stationary phases for chiral separations is required. The use of polysaccharide derivatives as CSPs offers the advantage of availability and the easy derivatization of the hydroxyl group. This leads to many different derivatives being one of the most effective the phenylcarbamate derivatives [9]. In addition, the introduction of electron-donating (methyl) and electron-withdrawing (chlorine) groups, results in an improved enantiomer recognition [10–12]. Cellulose tris(3-chloro-4-methylphenylcarbamate) (Sepapak-2 or Lux Cellulose-2) and cellulose tris(4-chloro-3-methylphenylcarbamate) (Sepapak-4 or Lux Cellulose-4) are novel CSPs which combine both electron-donating and electron-withdrawing substituents in the phenylcarbamate group. The enantioresolution ability of these novel CSPs has been tested in different works using commercially available columns. Sepapak-2 CSP has been employed for the enantioseparation of several drugs by normal phase-LC (NP-LC) [13–15], reversed phase-LC (RP-LC) [13,16] and polar organic solvent chromatography (POSC) [17,18], for amino acids using supercritical fluid chromatography (SFC) [19] and for atropisomeric biphenyls using NP-LC [20] with good results. Regarding the enantioresolution ability of Sepapak-4 CSP, it has only been demonstrated for pharmaceutical compounds by NP-LC [13–15,21,22], RP-LC [13,21] and POSC [17,22]. To the best of our knowledge, these new CSPs have never been employed for pesticide enantioseparations.

Using nano-LC and CEC with these packed capillary columns, only two works have been reported employing Sepapak-2 [23,24] and Sepapak-4 [24] for a group of pharmaceutical compounds. Recently, our research group has employed the CSP Sepapak-2 for the chiral separation of a group of FMOC-amino acids by nano-LC and CEC [25].

The aims of the present work were to study the chiral resolving power of these new polysaccharide-based CSPs, Sepapak-2 and Sepapak-4, for the enantiomeric separation of a group of 16 pesticides including herbicides, insecticides and fungicides and to show the advantages of CEC versus nano-LC in terms of efficiency and resolution. Finally, with the purpose of demonstrating the potential and reliability of this CSP, a CEC method was employed to achieve the chiral determination of the fungicide metalaxyl in a commercial formulation as well as in environmental samples (soil and tap water).

2. Experimental

2.1. Chemicals and samples

All reagents employed for the preparation of buffers, capillary columns, and samples were of analytical grade. Acetonitrile (ACN), methanol (MeOH) and hexane were from Scharlau (Barcelona, Spain), formic acid and acetic acid were from Riedel-de Haën (Seelze, Germany), boric acid was from Fluka (Buchs, Switzerland), ammonium hydroxide and sodium hydroxide were from Merck (Darmstadt, Germany) and ethyl acetate from Panreac (Barcelona, Spain). Water used to prepare all solutions was purified in a Milli-Q system from Millipore (Bedford, MA, USA).

Standards of racemic pesticides resmethrin, diniconazole, and fenpropathrin were from Chem Service (West Chester, PA, USA) and λ-cyhalothrin, β-cyfluthrin, *cis*-bifenthrin, metalaxyl, benalaxyl, hexaconazole, myclobutanil, tebuconazole, dichlorprop, mecoprop, α-cypermethrin, and flutriafol from Fluka. Uniconazole was acquired as uniconazole-P (*R*-isomer) from Chem Service (West Chester, PA, USA). The structure of all the studied pesticides is shown in Fig. 1.

The commercial formulation analyzed, containing only metalaxyl-M was acquired in an agrochemical shop in Fuenlabrada (Madrid, Spain). According to the labeled data, this product contained 465 mg/L of metalaxyl-M.

2.2. Instrumentation

A HP^{3D}CE system from Agilent Technologies (Palo Alto, CA, USA) with a diode array detector (DAD) was employed for all the experiments. The CE system was equipped with an external pressure device able to apply up to 12 bar. Instrument control and data acquisition were performed with the HP^{3D}CE ChemStation software. Injections were made by pressure (10 bar × 0.2 min) and immediately after the sample injection a plug of the mobile phase was injected (10 bar × 0.2 min). In nano-LC experiments a pressure of 12 bar was used for the separation and for CEC experiments a voltage of −10 kV was employed in order to obtain analysis times similar to those obtained in nano-LC experiments and an external pressure of 12 bar was applied in both buffer reservoirs to avoid bubble formation. UV detection was performed at 210 ± 2 nm and all the experiments were performed at a working temperature of 25 °C.

Separations were performed in a fused-silica capillary from Polymicro Technologies (Phoenix, AZ, USA) of 100 μm inner diameter (ID) with a total length of 32.5 cm in which the length of the packed bed was 24.0 cm. For the preparation of the packed columns a LC-10AS Shimadzu HPLC pump was used (Duisburg, Germany) and an ultrasonic bath Ultrasons-H from J.P. Selecta (Barcelona, Spain) was employed for submerging the capillary during the packing procedure to assure a homogenized packing.

A pH-meter model 744 from Metrohm (Herisau, Switzerland) was used to adjust the pH of the separation buffers.

The cartridges employed for SPE were silica cartridges supplied by Varian (Middelburg, The Netherlands) for soil samples and C-18 (Discovery DSC-18, Discovery DSC-18 LT and LC-18) cartridges supplied by Supelco (Bellefonte, PA, USA) for tap water samples.

2.3. Preparation of chiral stationary phases

The chemical structures of the chiral selectors used in these experiments are shown in Fig. 2. Cellulose tris(3-chloro-4-methylphenylcarbanate) and cellulose tris(4-chloro-3-methylphenylcarbamate) were synthesized as described previously [10,11]. The polysaccharide derivatives were dissolved in tetrahydrofuran and coated in the amount of 25% (w/w) onto aminopropylsilanized spherical silica particles with 5 μm nominal particle size and 100 nm nominal pore size.

2.4. Preparation of capillary columns

The capillary columns were packed by using the slurry packing method previously described by Chankvetadze [26]. Briefly, the inlet end of a 100 μm ID capillary was connected to an HPLC column, which was used as reservoir for the slurry of the packing material. First, the capillary was flushed with MeOH during 30 min for cleaning the capillary wall. After this, the outlet end of the column was connected to a commercial HPLC mechanical frit in order to retain the stationary phase. Meanwhile the stationary phase (20 mg) was suspended in approximately 2.5 mL of 80/20 (v/v) MeOH/H_2O, sonicated during 30 min and transferred into the reservoir. The capillary was packed at 350 bar for a length of about 30 cm while the capillary is submerged in the ultrasonic bath. Following, the column was flushed with 50:50 (v/v) MeOH/H_2O for around 1 h and the frits

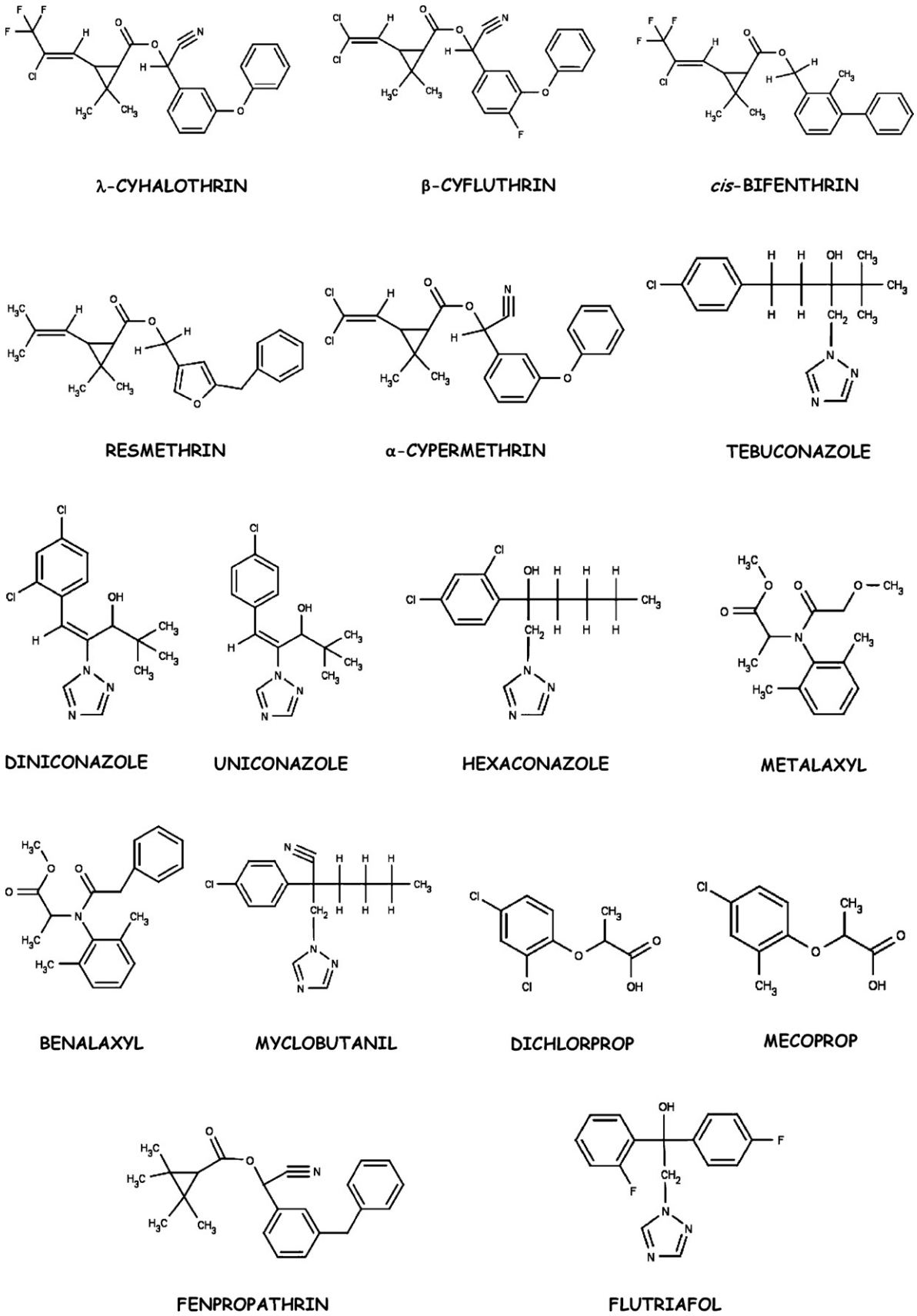
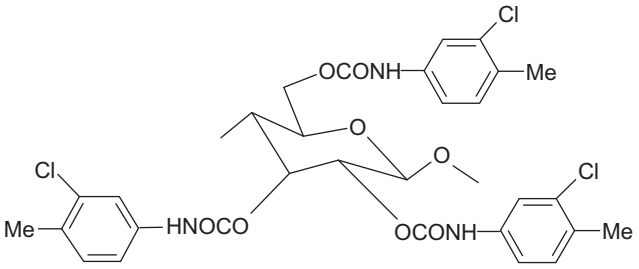
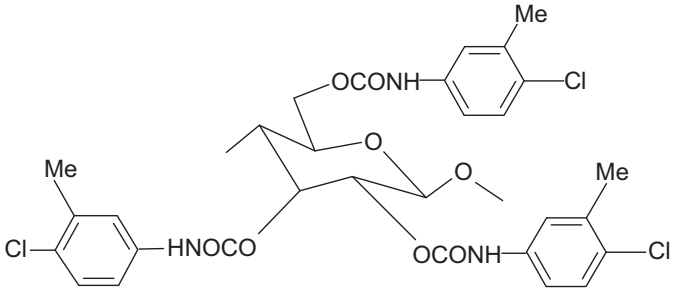


Fig. 1. Structure of the chiral pesticides selected for the evaluation of novel chiral stationary phases.



a) SEPAPAK-2: cellulose tris(3-chloro-4-methylphenylcarbamate)



b) SEPAPAK-4: cellulose tris(4-chloro-3-methylphenylcarbamate)

Fig. 2. Structure of chiral selectors (a) Sepapak-2 (cellulose tris(3-chloro-4-methylphenylcarbamate)) and (b) Sepapak-4 (cellulose tris(4-chloro-3-methylphenylcarbamate)).

were prepared by heating the two ends of the packed area with a home-made electrical wire filament heater (700 °C, 4–5 s) resulting in a packed length of 24 cm. Finally, the capillary was turned over and the excess of stationary phase was removed by flushing the capillary with the separation buffer. The same capillary was employed for all the experiments both in CEC and in nano-LC. The detection window was prepared just next to the outlet frit by removing the polyimide coating on the capillary with the same heater that was employed to make the frits.

2.5. Procedures

Buffers were prepared by dissolving the appropriate amount of formic acid, acetic acid or boric acid in Milli-Q water and adjusting the pH to the desired value with 1 M or 0.1 M NaOH. The separation media (mobile phases or BGEs) were prepared by mixing the buffers with ACN and MeOH or water in the selected proportions. Stock standard solutions of pesticides were prepared by dissolving the appropriate amount of the compound in ACN up to a final concentration of 2000 mg/L. To prepare the working solutions, different aliquots were diluted in 80/20 (v/v) ACN/H_2O to obtain concentrations of 200 mg/L of racemate (only *R*-isomer in the case of uniconazole-P) for column evaluation and between 3 mg/L and 500 mg/L for the calibration by the external standard method.

Before first use, the capillary was rinsed with the corresponding separation medium during approximately 1 h and 15 min between runs. At the end of each day the capillary was rinsed with 80/20 (v/v) ACN/H_2O for 15 min.

2.6. Sample treatment

Soil [8] and tap water samples [27] were extracted employing previously described methods with slight modifications. Briefly, 100 g of dried soil were spiked with the commercial pesticide product and mechanically shaken for 1 h. 50 g of the spiked soil was twice extracted with MeOH (2 × 50 mL) and the extract was evaporated to 5 mL. 20 mL of distilled water was added to the residue and then reextracted with ethylacetate (3 × 40 mL). The organic phases were collected and anhydrous sodium sulfate was added to remove all the remaining water. The extract was filtered and evaporated until just to dryness and it was redissolved in 2 mL hexane. It was purified by SPE with a silica column conditioned with hexane. After sample loading the column was rinsed with 5 mL of hexane, 5 mL of 5/95 (v/v) ethylacetate/hexane, 5 mL of 10/90 (v/v) ethylacetate/hexane, and 5 mL of 15/85 (v/v) ethylacetate/hexane and finally the analytes were eluted with 25 mL of 20/80 (v/v) ethylacetate/hexane. The eluate was evaporated, redissolved in 500 μL 80/20 (v/v) ACN/H_2O, and injected in the CE system.

For tap water samples 50 mL were spiked with the commercial product and they were passed through a C-18 SPE cartridge. The cartridge was previously conditioned with 3 mL MeOH and 3 mL water and after sample loading, the analyte was eluted with 5 mL MeOH. The extract was evaporated to dryness, redissolved in 500 μL of 80/20 (v/v) ACN/H_2O, and injected in the CE system.

To prepare the commercial formulation for its analysis, an appropriate volume of product was diluted in 80/20 (v/v) ACN/H_2O to obtain a concentration of approximately 75 mg/L of metalaxyl-M considering the labeled amount.

2.7. Data treatment

The values of areas, migration or retention times and resolution were obtained using the ChemStation software. Corrected peak areas were used for data treatment in order to obtain better precision.

The presence of matrix interferences was investigated by employing the *t*-test for comparison of two calibration lines. If the *p*-value was up to 0.05 (for a confidence level of 95%) it was considered that there were no significant differences between calibration lines.

Finally, the percentage of enantiomeric impurity in commercial formulations was determined as the corrected area of the peak of the impurity divided by the sum of the corrected areas of both enatiomers ((corrected $area_{Min}$/corrected $area_{Min}$ + corrected $area_{Maj}$) × 100).

Experimental data analysis and composition of graphs were carried out using Excel Microsoft Office 2007, Statgraphics Plus 5.1 and Origin 6.0 software.

3. Results and discussion

3.1. Evaluation of the enantioselectivity of Sepapak-2 and Sepapak-4 toward pesticides by nano-LC and optimization of the mobile phase

The evaluation of the enantioselectivity of Sepapak-2 and Sepapak-4 as chiral stationary phases in nano-LC was performed by the injection of the selected 16 pesticides. These chiral stationary phases may be used both in normal [13] and reversed phase mode [23] and there are even some works in which POSC has been used [18,22].

First, the CSP Sepapak-2 was tested in both RP-LC and NP-LC. Different mobile phases were tested in reversed phase mode, all of them containing ACN as organic modifier at a percentage of 80%. ACN was chosen as organic modifier due to its low viscosity and low UV-cut-off, although other solvents such as methanol, ethanol or 2-propanol could be added [13]. The other 20% was composed of water and a percentage of buffer at different pHs. Table 1 shows the results obtained for Sepapak-2 column using mobile phases at different pHs from 2.5 to 8.0. Due to the fact that the studied compounds are not ionizable (λ-cyhalothrin, resmethrin, *cis*-bifenthrin, β-cyfluthrin, metalaxyl, benalaxyl, α-cypermethrin, and fenpropathrin; all of them pyrethroids pesticides) or only partially ionizable, the change in the mobile phase pH did not show notable influence on the enantioselectivity and similar results were obtained at different pH values. However, a slight separation was obtained for the enantiomers of myclobutanil at higher pHs while at lower pHs no separation was observed for this compound. The best resolutions were found at the highest pH (80/19/1, v/v/v, ACN/H_2O/sodium borate; pH 8.0), which allowed the chiral separation of six of the pesticides tested. In the normal phase mode, ethanol and 2-propanol are the most suitable mobile phase modifiers due to their miscibility with hexane or heptane. However, 2-propanol usually provides better selectivities because it interacts less with the stationary phase through hydrogen bonding and thus it does not compete for the active sites with the analytes [21]. For this reason, a 75/25 (v/v) 2-propanol/hexane mobile phase was evaluated with Sepapak-2 chiral stationary phase. However, only dichlorprop (t_2 = 17.42 min and *Rs* 1.39) was enantiomerically separated under these conditions. Surprisingly this pesticide was not enantiomerically resolved in any of the tested reversed phase conditions. In conclusion, reversed phase mode was selected to perform the following experiments because apart from the negative results obtained in normal phase mode it gave poor conductivities and thus it cannot be employed in CEC.

The same experiments were performed for Sepapak-4 column in reversed phase mode and the results obtained are summarized in Table 1. In this case, as with Sepapak-2, the change in the mobile phase pH did not offer significant differences for the pesticides studied. Thus, since the objective was to conduct experiments in CEC, the lowest pH was selected because for aminopropyl silica it will result in a high EOF [23] and consequently, shorter analysis time.

It has to be remarked that despite the similarities in the structures of the two CSPs evaluated, their resolution power was quite different and Sepapak-4 enabled separating three or two more compounds (depending on the pH selected) and with higher resolutions than Sepapak-2, allowing the separation of 9 out of the 16 pesticides studied. As it can be observed, Sepapak-2 allowed to the separation of all studied compounds containing an azole group, as well as both containing a tertiary amine (metalaxyl and benalaxyl) being indicative of the potential of this CSP for the enantioseparation of this type of compounds.

Both CSPs were also evaluated using MeOH instead of water in the mobile phase. As it can be observed in Table 2, the employ of MeOH resulted in the decrease of the retention times, as well as a decrease in *Rs* on both for Sepapak-2 and Sepapak-4.

Due to the fact that Sepapak-4 provided the chiral resolution of a high number of compounds and the values of resolutions achieved were better, this column was selected for further experiments in nano-LC. The influence of ACN percentage in the mobile phase was studied. Since the compounds studied were highly hydrophobic, the decrease in ACN content may lead to their precipitation. To avoid these problems the following experiments were performed with a mobile phase containing MeOH instead of water although the presence of MeOH, as explained above, resulted in a decrease of resolution. The content of MeOH added was less than 40% in all the experiments because it has been previously reported in the literature that mobile phases containing 50–100% MeOH in mixtures with ACN were not successful due to unstable current during analysis [23]. Table 3 represents the results obtained for Sepapak-4 when employing different ACN proportions in the mobile phase. As it can be observed the resolving power of this column increased when increasing the percentage of ACN. The increase in the MeOH amount in the mobile phase negatively affects the enantioseparation because MeOH appears to be strong competitor for hydrogen bonding in the chiral sites of the stationary phase [22].

3.2. Comparison between nano-LC and CEC

The use of EOF as driving force in separation techniques supposes important advantages such as a plug-like flow profile, independence of the EOF of the particle size and geometry, etc. [29]. Furthermore, it is supposed that CEC experiments provide better efficiencies and resolution values. However, there is no reason to perform CEC experiments instead of nano-LC experiments unless this improvement is observed [30]. In addition, good reproducibility on retention times, areas and selectivities must also be obtained.

Due to the use of aminopropyl silica as support of the CSP, the anodic EOF is generated on the surface of silica particles. Thus, reversed polarity was used in order to obtain an adequate mobility of the studied compounds. With the aim of comparing the results obtained by nano-LC and CEC, a separation voltage of −10 kV was selected because with this voltage the retention times obtained by both techniques were quite similar.

Considering all the results described in the previous section, a 90/9/1 (v/v/v) ACN/water/ammonium formate pH 2.5 mobile phase was selected to carry out the comparison between nano-LC and CEC experiments. Fig. 3 shows as an example the separation obtained by nano-LC and CEC for the pesticides resolved under these conditions. With this mobile phase the enantiomers of eight compounds were resolved with resolutions above 0.6 and in less than 28 min (see Table 4).

Regarding the number of theoretical plates per meter, as it can be observed in Table 4, the values drastically increased with CEC (53,899–278,301 plates m^{-1}) compared with nano-LC (37,818–58,878 plates m^{-1}). This improved efficiency translates into an increase in the chiral resolution for most compounds showing the benefits of the use of the EOF as driving force compared with the use of pressure.

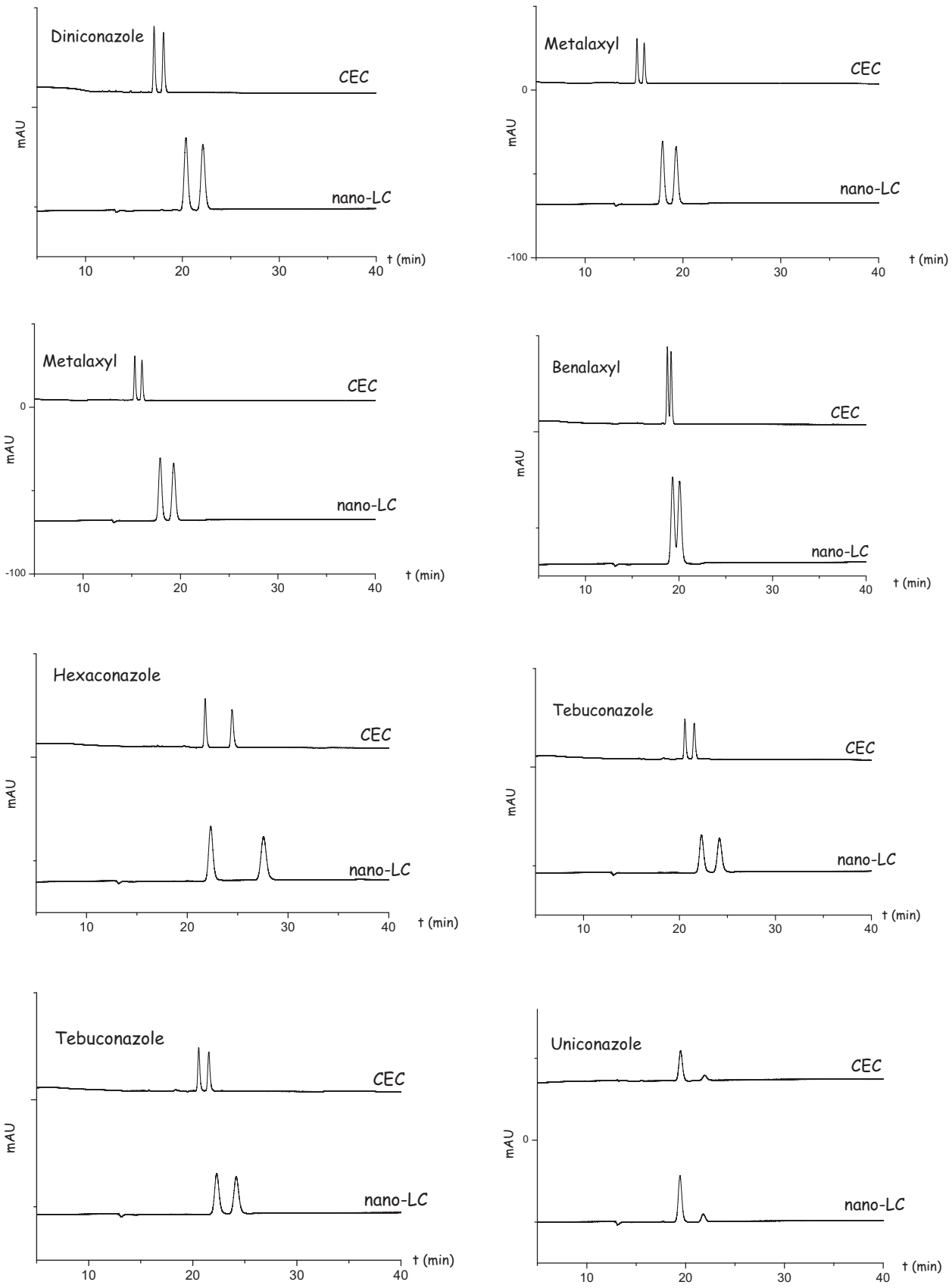


Fig. 3. Comparison of nano-LC chromatograms and CEC electrochromatograms of the enantiomerically resolved pesticides when employing Sepapak-4 chiral stationary phase. Experimental conditions: capillary, 100 μm ID with 24 cm packed and 32.5 cm total length; mobile phase or BGE, 90/9/1 (v/v/v) ACN/H_2O/500 mM Ammonium formate (pH 2.5); temperature, 25 °C; separation voltage in CEC, −10 kV with a 12 bar pressure in both inlet and outlet vials; separation pressure in nano-LC, 12 bar; injection, 10 bar × 0.2 min of sample followed by a plug of mobile phase at 10 bar × 0.2 min.

Table 1
Retention time and enantioresolution of selected pesticides by nano-LC using different mobile phases and with two different chiral stationary phases (Sepapak-2 and Sepapak-4).

Pesticide	AcN/H_2O/500 mM Ammonium formate pH 2.5 80/19/1 (v/v/v)			AcN/H_2O/500 mM Ammonium acetate pH 4.5 80/19/1 (v/v/v)			AcN/H_2O/500 mM Ammonium acetate pH 6.5 80/19/1 (v/v/v)			AcN/H_2O/500 mM Sodium borate pH 8.0 80/19/1 (v/v/v)		
	t_1 (min)	t_2 (min)	*Rs*	t_1 (min)	t_2 (min)	*Rs*	t_1 (min)	t_2 (min)	*Rs*	t_1 (min)	t_2 (min)	*Rs*
Sepapak-2												
λ-Cyhalothrin	19.17	–	–	17.24	–	–	17.24	–	–	17.48	–	–
β-Cyfluthrin	21.59	–	–	19.18	–	–	19.11	–	–	19.45	–	–
Cis-bifenthrin	25.62	–	–	22.51	–	–	22.44	–	–	22.95	–	–
Resmethrin	25.04	–	–	22.47	–	–	22.39	–	–	22.85	–	–
Diniconazole	21.14	22.72	1.57	20.40	22.10	1.73	20.38	22.11	1.80	20.69	22.48	1.80
Metalaxyl	16.32	17.20	1.23	16.20	17.16	1.35	16.20	17.17	1.36	16.45	47.49	1.42
Benalaxyl	19.22	–	–	19.66	–	–	19.72	–	–	20.08	–	–
Hexaconazole	21.53	25.10	3.35	23.06	27.07	3.47	23.95	28.22	3.53	24.56	29.04	3.62
Myclobutanil	21.64	–	–	23.33	–	–	24.17	24.36	0.19	25.00	25.37	0.35
Tebuconazole	21.77	22.53	0.66	23.26	24.08	0.64	24.34	25.25	0.67	25.31	26.27	0.68
Dichlorprop	41.27	–	–	21.43	–	–	12.75	–	–	11.29	–	–
Mecoprop	31.01	–	–	21.59	–	–	12.65	–	–	11.24	–	–
α-Cypermethrin	20.82	–	–	22.90	–	–	24.68	–	–	26.26	–	–
Uniconazole	18.28	20.68	2.82	19.36	22.11	2.98	20.15	23.13	3.07	20.84	24.00	3.14
Flutriafol	15.99	–	–	16.70	–	–	17.27	–	–	17.71	–	–
Fenpropathrin	19.30	–	–	21.12	–	–	22.55	–	–	23.88	–	–
Sepapak-4												
λ-Cyhalothrin	25.51	–	–	27.91	–	–	31.48	–	–	26.14	–	–
β-Cyfluthrin	28.10	–	–	30.79	–	–	35.05	–	–	28.84	–	–
Cis-bifenthrin	32.80	–	–	36.50	–	–	42.51	–	–	33.87	–	–
Resmethrin	32.94	–	–	36.51	–	–	42.24	–	–	35.00	–	–
Diniconazole	28.02	30.44	2.30	29.60	32.38	2.50	32.42	35.70	2.67	28.48	31.21	2.52
Metalaxyl	23.55	25.17	1.86	24.38	26.15	2.00	26.20	28.26	2.19	23.77	25.52	2.03
Benalaxyl	29.16	30.11	0.82	30.85	32.01	1.00	33.92	35.33	1.11	29.55	30.73	1.06
Hexaconazole	29.85	35.65	4.84	31.33	37.81	5.14	33.88	41.29	5.38	30.35	36.79	5.27
Myclobutanil	29.32	30.26	0.82	31.63	32.62	0.90	34.26	35.52	1.00	30.23	31.27	0.93
Tebuconazole	30.38	32.54	1.91	32.84	35.36	2.04	35.26	38.05	2.11	31.20	33.64	2.08
Dichlorprop	n.d	n.d	n.d	60.75	61.90	0.53	24.60	25.02	0.50	15.101	–	–
Mecoprop	n.d	n.d	n.d	n.d	n.d	n.d	24.42	–	–	15.127	–	–
α-Cypermethrin	31.13	–	–	36.08	–	–	40.28	–	–	32.50	–	–
Uniconazole	26.04	29.27	3.19	27.96	31.69	3.42	29.39	33.44	3.56	24.47	29.94	3.57
Flutriafol	22.62	23.24	0.67	24.03	24.75	0.69	25.00	25.77	0.75	22.96	23.65	0.69
Fenpropathrin	29.48	–	–	33.28	–	–	36.45	–	–	30.125	–	–

n.d., not detected in 60 min; $Rs = 1.18\,(t_2 - t_1)/(w_{1/2,1} + w_{1/2,2})$.

Table 2
Retention times and enantioresolutions of selected pesticides by nano-LC using AcN/MeOH/ammonium formate pH 2.5 mobile phase with Sepapak-2 and Sepapak-4 chiral stationary phases.

Pesticide	Sepapak-2			Sepapak-4		
	AcN/MeOH/ammonium formate pH 2.5 80/19/1 (v/v/v)			AcN/MeOH/ammonium formate pH 2.5 80/19/1 (v/v/v)		
	t_1 (min)	t_2 (min)	*Rs*	t_1 (min)	t_2 (min)	*Rs*
λ-Cyhalothrin	8.63	–	–	12.51	–	–
β-Cyfluthrin	8.74	–	–	12.71	–	–
Cis-bifenthrin	8.92	–	–	13.10	–	–
Resmethrin	9.31	–	–	13.67	–	–
Diniconazole	11.24	11.75	1.00	15.93	16.67	1.31
Metalaxyl	10.00	10.21	0.50	14.34	14.93	1.19
Benalaxyl	10.00	–	–	14.70	–	–
Hexaconazole	12.37	14.74	3.79	17.26	20.76	5.24
Myclobutanil	11.90	12.10	0.37	16.39	16.79	0.64
Tebuconazole	12.38	13.03	1.10	17.13	18.56	2.31
Dichlorprop	28.83	–	–	n.d	n.d	n.d
Mecoprop	21.95	–	–	n.d	n.d	n.d
α-Cypermethrin	8.95	–	–	13.10	–	–
Uniconazole	10.81	11.62	1.59	15.42	16.40	1.81
Flutriafol	10.28	10.36	0.19	14.64	15.04	0.67
Fenpropathrin	8.89	–	–	13.01	–	–

3.3. Quantitative analysis of metalaxyl enantiomers in commercial agrochemical formulations by CEC

Aimed to show the potential of Sepapak-4 in CEC, metalaxyl was selected as model compound to carry out its quantification and the determination of its enantiomeric purity in a commercial pesticide product. In order to choose the separation voltage providing the best efficiency for metalaxyl chiral separation, the Van Deemter curve for this compound was constructed in the range from −5 kV to −30 kV, employing a 90/9/1 (v/v/v) ACN/H_2O/ammonium

Table 3
Retention times and enantioresolutions of selected pesticides by nano-LC using AcN/MeOH/Ammonium formate pH 2.5 mobile phase with different proportions of AcN/MeOH in Sepapak-4 chiral stationary phase.

Pesticide	AcN/MeOH/Ammonium formate pH 2.5 60/39/1 (v/v/v)			AcN/MeOH/Ammonium formate pH 2.5 80/19/1 (v/v/v)			AcN/MeOH/Ammonium formate pH 2.5 90/9/1 (v/v/v)		
	t_1 (min)	t_2 (min)	*Rs*	t_1 (min)	t_2 (min)	*Rs*	t_1 (min)	t_2 (min)	*Rs*
λ-Cyhalothrin	13.08	–	–	12.51	–	–	12.39	–	–
β-Cyfluthrin	13.56	–	–	12.71	–	–	12.65	–	–
Cis-bifenthrin	14.00	–	–	13.10	–	–	12.91	–	–
Resmethrin	15.52	–	–	13.67	–	–	13.53	–	–
Diniconazole	15.34	15.75	0.67	15.93	16.67	1.31	16.91	17.95	1.71
Metalaxyl	14.75	15.03	0.56	14.34	14.93	1.19	14.65	15.59	1.83
Benalaxyl	15.24	–	–	14.70	–	–	14.70	14.90	0.44
Hexaconazole	15.87	17.58	3.03	17.26	20.76	5.24	19.02	24.39	6.77
Myclobutanil	15.94	16.31	0.62	16.39	16.79	0.64	17.37	17.83	0.66
Tebuconazole	16.01	16.83	1.49	17.13	18.56	2.31	18.83	21.03	3.03
Dichlorprop	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Mecoprop	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
α-Cypermethrin	14.12	–	–	13.10	–	–	12.94	–	–
Uniconazole	14.90	15.44	0.99	15.42	16.40	1.81	16.61	18.00	2.34
Flutriafol	14.36	14.48	0.30	14.64	15.04	0.67	15.83	16.44	1.02
Fenpropathrin	14.10	–	–	13.01	–	–	12.89	–	–

n.d., not detected in 60 min.

Table 4
Comparison of chiral separation of selected pesticides and efficiency by nano-LC and CEC in Sepapak-4 chiral stationary phase. Mobile phase: 90/9/1 (v/v/v) AcN/H_2O/ammonium formate pH 2.5.

	Nano-LC (12 bar)					CEC (−10 kV)				
	t_1	t_2	N_1/m	N_2/m	*Rs*	t_1	t_2	N_1/m	N_2/m	*Rs*
Diniconazole	20.37	22.12	53858	54562	2.38	17.08	18.05	171247	155400	2.72
Metalaxyl	17.92	19.30	57969	56278	2.17	13.78	14.67	278301	260804	2.48
Benalaxyl	19.32	20.07	58878	54420	1.07	18.75	19.15	270169	249601	1.31
Hexaconazole	22.33	27.56	53428	50800	5.81	21.78	24.45	236849	183583	6.42
Myclobutanil	21.25	21.78	37818	50454	0.63	19.97	20.27	202275	176680	0.79
Tebuconazole	22.30	24.17	53441	53029	2.27	20.57	21.54	223214	199426	2.57
Uniconazole	19.43	21.79	56336	56299	3.39	19.50	21.90	59502	58236	3.31
Flutriafol	17.86	18.46	52129	49575	0.92	17.83	18.45	56116	53899	0.94

$N/m = 5.54\,(t/w_{1/2})^2 \times 100/L_C$.

formate BGE at pH 2.5, at a temperature of 25 °C, and an injection of 10 bar × 0.2 min of sample with a plug of BGE at 10 bar × 0.2 min. From these experiments (see supplementary material)), it could be concluded that the optimum voltage, in which the number of theoretical plates was the maximum one and thus the efficiency of the separation was the best, was −10 kV so this value was chosen for the analysis.

Before carrying out the quantitative determination of metalaxyl in commercial fungicide products, the analytical characteristics of the developed method were evaluated The results obtained are shown in Table 5.

Linearity was determined by plotting the corrected peak area as a function of the concentration of each compound in the range 5–500 mg/L referred to each enantiomer during three different days and injected by triplicate each day. Satisfactory results were obtained in terms of linearity with $R^2 > 0.99$, the intercept not different from zero, and ANOVA confirming that data fit to a linear model (p-values > 0.05).

LODs and LOQs for the two enantiomers of metalaxyl were experimentally determined using a S/N ratio equal to 3 and 10, respectively. LODs values were of 1.4 and 1.6 mg/L and LOQs were 4.6 and 5.3 mg/L for the first and the second enantiomer, respectively. RLOD calculated considering the maximum concentration of metalaxyl tested without loss on resolution (250 mg/L) and the LOD for *S*-metalaxyl (1.4 mg/L) was 0.56%. Therefore, ∼0.56% *S*-metalaxyl could be detected.

Precision of the method was evaluated as instrumental repeatability and intermediate precision. Instrumental repeatability was determined from six repeated injections of two standard solutions (5 and 100 mg/L of each enantiomer). RSD values (%) obtained for metalaxyl enantiomers were lower than 0.2% for migration times and lower than 2.8% for corrected peak areas. Intermediate precision was assessed at the same concentration levels for three consecutive days injecting each sample by triplicate. As it can be observed in Table 5 the RSD values achieved for the enantiomers of metalaxyl were from 1.8 to 2.2% and from 4.7 to 7.0% for analysis times and corrected peak areas, respectively.

The effect of matrix interferences was investigated by comparing the calibration slopes obtained by the external standard and the standard addition calibration methods. The standard additions calibration curve was obtained by spiking the commercial sample with known concentrations of racemic metalaxyl (40, 70, 100 and 130 mg/L). p-Value of t-test for comparison of two calibration lines was determined and as it can be observed in Table 5, the p-values obtained for every commercial sample were >0.05 at a confidence level of 95%. These results demonstrated the absence of matrix interferences Accuracy was determined as the recoveries achieved for metalaxyl. For this purpose the commercial formulation was spiked with different known concentrations of racemic metalaxyl standard solution (40, 70, 100 and 130 mg/L). The mean recoveries obtained were between 85.8 and 101.7% with average values of 94.0% for the first migrating enantiomer and 96.1% for the second migrating enantiomer with RSDs of 3.2 and 5.4%, respectively.

Finally, the method was applied to the determination of metalaxyl-M (*R*-metalaxyl) and its enantiomeric impurity in a commercial fungicide product. The determined amount of metalaxyl-M was 585 ± 4 mg/L that supposes an amount of 26% above the labeled content, which can be consequence of a non controlled

Table 5
Analytical characteristics of the CEC developed method.

Analytical characteristics	Metalaxyl							
	First enantiomer (impurity) (*S*-metalaxyl)				Second enantiomer (*R*-metalaxyl)			
Precision (RSD)								
Concentration (mg/L)	5		100		5		100	
Instrumental repeatability ($n = 6$)								
Ac, RSD (%)	1.6		1.6		2.8		1.9	
t, RSD (%)	0.2		0.1		0.1		0.1	
Intermediate precision ($n = 9$)								
Ac, RSD (%)	7.0		4.7		5.9		5.8	
t, RSD (%)	1.8		2.2		1.8		2.2	
Linearity								
Linear range (mg/L)	5–250				5–250			
Linear equation	$-0.488 + 0.488x$				$-0.717 + 0.455x$			
Standard errors	Sa = 0.929, Sb = 0.007				Sa = 0.887, Sb = 0.007			
Determination coefficient (R^2)	0.998				0.998			
p-Value of ANOVA	0.127				0.132			
Accuracy								
Concentration (mg/L)	+40	+70	+100	+130	+40	+70	+100	+130
Recovery (%)	93.4	86.5	97.8	100.2	94.0	93.3	97.3	101.5
Median Recovery (%)	94.0 ± 5.4				96.1 ± 3.2			
LOD (mg/L)	1.4				1.6			
LOQ (mg/L)	4.6				5.3			
RLOD (%)	1.4				1.6			
Study of matrix interferences								
p-Value of *t*-tests								
Sample 1	0.055				0.119			

RLOD = $100 \times LOD_{min}/C_{maj}$ [28].

manufacture of the commercial product. In fact, our research group found the same results in the quantification of metalaxyl in the same samples by MEKC [31]. With respect to the enantiomeric impurity, a percentage of 3.7% of *S*-metalaxyl was found.

3.4. Analysis of tap water and soil samples

The proposed CEC method was also applied to the analysis of metalaxyl in tap water and soil samples. The samples were spiked with the commercial formulation containing metalaxyl-M at concentration of approximately 150 mg/L (in the final extract) and submitted to their corresponding sample treatment described in the experimental section. For water and soil samples three different C-18 based SPE cartridges were tested but no difference was observed among them. Fig. 4 shows the electrochromatograms corresponding to soil and tap water samples spiked with the commercial product quantified above. As it can be observed, for the soil sample the main enantiomer (*R*-metalaxyl) is clearly identified but an interfering peak appeared close to the enantiomeric impurity (*S*-metalaxyl). On the other hand, for the tap water sample the extraction method employed was quite selective and the peaks appearing in the electrochromatogram corresponded only to the enantiomers of metalaxyl enabling to determine a concentration of 161 ± 2 mg/L for metalaxyl-M (in the final extract) and a percentage of 3.6% for *S*-metalaxyl.

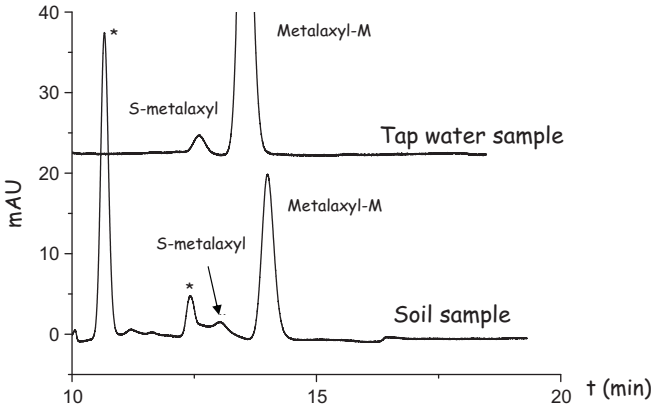


Fig. 4. Electrochromatograms obtained for tap water and soil samples spiked with the commercial product containing only metalaxyl-M at a concentration of approximately 75 mg/L (according to its label). Experimental conditions as in Fig. 3 for CEC experiments. *Unknown peaks.

4. Concluding remarks

Two novel polysaccharide-based chiral stationary phases, known as Sepapak-2 and Sepapak-4, have been evaluated for the separation of enantiomers of a group of 16 pesticides including herbicides, insecticides and fungicides. Each chiral stationary phase gave optimal results under different separation conditions reaching the chiral separation of seven pesticides on Sepapak-2 and nine pesticides on Sepapak-4. The comparison between the results obtained by CEC and nano-LC showed a clear advantage of CEC in terms of efficiency and enantioresolution power. The CEC method was applied to the determination of metalaxyl enantiomers in a commercial pesticide formulation allowing the detection of impurities up to 0.56% of *S*-metalaxyl in less than 15 min. Finally, soil and tap water samples were also analyzed previous sample treatment by SPE. Enantiomeric impurities may be easily detected in tap water samples spiked with the commercial product but an interfering peak appeared in the case of soil samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.01.035.

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