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Chiral separation of metalaxyl and benalaxyl fungicides by electrokinetic chromatography and determination of enantiomeric impurities

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ABSTRACT

The enantiomers of two acylamine fungicides (metalaxyl and benalaxyl) were separated by EKC using CDs as chiral selectors. The use of 15 mM succinyl-γ-CD for metalaxyl and 5 mM succinyl-β-CD for benalaxyl dissolved in a 50 mM 2-morpholinoethanesulfonic acid buffer (pH 6.5), enabled the chiral separation of metalaxyl enantiomers in 11.5 min with a resolution of 3.1 and the enantiomeric separation of benalaxyl in 7.5 min with a resolution close to 15. Under these conditions, the two enantiomers of each of the chiral compound studied were also separated from folpet, very commonly present in fungicide formulations containing metalaxyl or benalaxyl. The analytical characteristics of the two developed methods were studied in terms of precision, linearity, selectivity, limits of detection (LODs) and limits of quantitation (LOQs) showing their suitability for the determination of these compounds in commercial agrochemical formulations. Finally, the development of an in-capillary preconcentration strategy allowed the detection of enantiomeric impurities up to 1.2% in commercial products labeled as enantiomerically pure in metalaxyl-M.

1. Introduction

The research around pesticides is a growing area in the field of analytical chemistry perhaps due to the fact that every year new pesticides are synthesized in order to achieve more effective products with a minor application rate [1]. In this sense, chirality plays an important role because it may have a great influence on the effectiveness and toxicity of the pesticide. Furthermore, when chirality is not considered and racemic compounds are employed, the use of these products may suppose the emission of a 50–75% of unnecessary product [2]. Approximately a 25% of the existing agrochemicals are chiral [3]. In some cases all the pesticide activity resides only in one of the enantiomers being the rest of them partially or totally inactive [4]. In addition, the biological response against non-target organisms may also differ a lot. Sometimes the enantiomers have similar biological activity, others one of the enantiomers produces a completely different biological response being for example much more toxic, causing malformations or having a carcinogenic effect [5,6]. This is why in order to contribute to risk reduction some of the new pesticides are formulated to contain mainly the active enantiomeric form. The use of enantiopure pesticides in place of racemic products not only supposes lower application rates but also reduces the amounts of pesticides released into the environment, prevents deployment of an inactive isomer to the biosphere, and thus reduces potential side-effects on non-target organisms [7].

From all the pesticides employed annually in Spain, approximately a 28% of them correspond to fungicide group [8]. Although the number of different fungicide types is huge, the major information about the role that chirality plays in their properties is focused in conazole and amide group.

Metalaxyl [(R,S) methyl-N-(2-methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate] (see Fig. 1a) is an acylamine fungicides (included into the amide group), being the most widely known member of this group [9]. This fungicide, synthesized in 1977, is widely used to control plant diseases caused by pathogens of the *Oomycota* division, in particular, against *Phytophthora infestans* and *Phytium ultimum* [10]. Metalaxyl possesses two enantiomers with the same mode of action, that is, both posses fungicidal activity [11]. However, it has been proved that this activity mostly originates from the R-enantiomer [12] being this one almost 1000 times more effective than the S-enantiomer [13]. Its toxicity against non-target organisms has been demonstrated to be enantioselective. Acute

Abbreviations: BGE, background electrolyte; Ac, corrected peak area; CE-β-CD, carboxyethyl-β-cyclodextrin; CE-γ-CD, carboxyethyl-γ-cyclodextrin; CM-β-CD, carboxymethyl-β-cyclodextrin; CM-γ-CD, carboxymethyl-γ-cyclodextrin; CD, cyclodextrin; DAD, diode array detector; EKC, electrokinetic chromatography; EOF, electroosmotic flow; LOD, limit of detection; LOQ, limit of quantitation; LC50, median lethal concentration; MEKC, micellar electrokinetic chromatography; MES, 2-morpholinoethanesulfonic acid; RLOD, relative limit of detection; SDS, sodium dodecyl sulphate; succ-β-CD, succinyl-β-cyclodextrin; succ-γ-CD, succinyl-γ-cyclodextrin.

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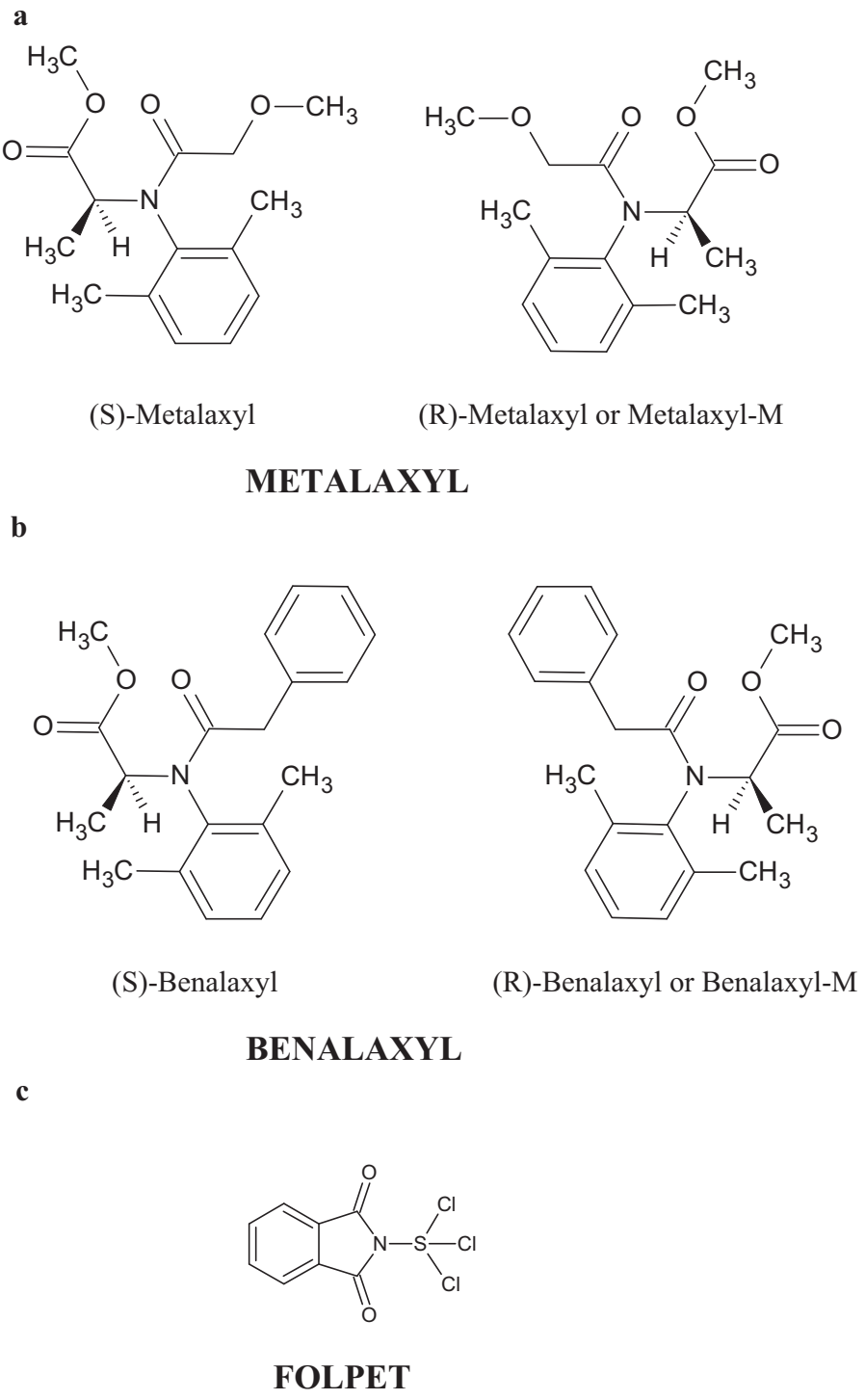

Fig. 1. Structure of the two enantiomers of (a) metalaxyl, (b) benalaxyl and structure of (c) folpet.

toxicities of racemic metalaxyl and R-metalaxyl to *Daphnia magna* were determined and compared by Chen and Liu [14]. The median lethal concentration (LC50) values for racemic and R-metalaxyl to *Daphnia magna* showed the clear difference in the toxicity of the two enantiomers against this organism. The degradation of metalaxyl enantiomers in environment is also clearly enantioselective. Once in environment enantiomeric relations usually change, due to the microbial degradation observed in most samples. In this situation, for example in soils R-enantiomer of metalaxyl uses to show a faster degradation [9,15–17] but this behavior might change with the conditions of the soil analyzed (i.e. soil pH) [18]. In vegetable samples the behavior is just the opposite than in soil and the S-enantiomer showed a faster degradation [9]. The same degradation pattern has also been studied through the formation of the corresponding metabolite during the process. The acid metabolite is the main breakdown product, both for metalaxyl and metalaxyl-M [19]. In sunflower plants metalaxyl was converted into its acid metabolite with retention of configuration; in fact metalaxyl-M was converted only into an R-acid metabolite without inversion of configuration, so the formation of this metabolite can also be a

way of monitoring the enantioselective degradation of metalaxyl [13]. Finally, in rabbit tissues such as liver, plasma and kidneys a more rapid degradation of S-metalaxyl has also been observed [20].

Benalaxyl [methyl-N-phenylacetyl-N-2,6-xylyl alaninate] (see Fig. 1b) is a systemic fungicide belonging to the acylamine family, with protective, curative and eradicant action [21]. It was first time synthesized in 1981 with the aim of controlling *Oomycetes*, particularly fungi of the family *Peronosporaceae* [22]. Benalaxyl has one chiral center in the alkyl moiety and so it has two different enantiomers that have shown very different fungicidal, biological and degradation patterns. In fact, it is known that as for metalaxyl, the fungicidal activity is mainly residing in the R enantiomer, being the S one almost inactive [23]. The degradation of racemic benalaxyl in water, soils, and different plants is enantioselective. In this context, S-benalaxyl showed a faster degradation in plants such as tomato, tobacco, sugar beet and capsicum plants [24], while the R-benalaxyl showed a faster degradation in soils [24,25]. Finally, for earthworm in paper contact test, after 48 h of exposure, the calculated LC50 values of the R-form and S-form were 4.99 and 6.66 $\mu g/cm^2$ respectively, demonstrating the clear differences in the toxicity of the two enantiomers against this non-target organism [26].

Usually, these two chiral fungicides are used in combination with protectant type fungicides such as copper or folpet [27]. Folpet [N-(trichloromethylthio)phthalimide] (see Fig. 1c) is a non chiral compound relative to dicarboximide fungicide group.

The presence of these pesticides in foods is regulated. Thus, the maximum residual levels (MRLs) [28] are between 0.05 and 10 mg/kg for metalaxyl (being 0.05 mg/kg for grain cereals, asparagus, pea, potato, beet, sunflower seeds, soya and carrot and 10 mg/kg for hop and hot pepper), between 0.02 mg/kg (potatoes) and 0.5 mg/kg (hot pepper and tomato) for benalaxyl, and 0.1 mg/kg for potatoes and 50 mg/kg for lettuce for folpet.

Although different separation techniques have been employed for the chiral separation of metalaxyl and benalaxyl, only two works have been published by CE, both referred to the chiral separation of metalaxyl fungicide [16,29]. In the case of benalaxyl, to our knowledge, no chiral separation has been performed employing this separation technique. In the methods developed for metalaxyl by CE, UV detection was employed and cyclodextins (CDs) were employed as chiral selectors according to the high discrimination power of these compounds to achieve enantiomeric separations [30–34]. Thus, Jarman et al. [16] achieved the chiral separation of metalaxyl enantiomers by micellar electrokinetic chromatography (MEKC) employing sodium dodecyl sulphate (SDS) as surfactant and 40 mM γ-CD as chiral selector. However, baseline separation was not achieved for this fungicide under the selected conditions. Santilio and Dommarco [29] employed electrokinetic chromatography (EKC) for the same purpose with succinyl-β-cyclodextrin (succ-β-CD) as chiral selector allowing the enantiomeric separation of metalaxyl in approximately 19 min with a resolution of 1.68.

The purpose of this work was the development of chiral methods suitable for the determination of metalaxyl and benalaxyl enantiomers in commercial samples and enabling the determination of the selected fungicides in binary mixtures with folpet, another fungicide very common in commercial formulations containing metalaxyl or benalaxyl. Finally, an in-capillary preconcentration strategy will be developed to detect minor enantiomeric impurities in commercial agrochemical formulations labeled as enantiomerically pure in metalaxyl-M.

2. Materials and methods

2.1. Reagents and samples

All reagents employed for the preparation of background electrolytes (BGEs) and samples were of analytical grade. Boric acid and urea were supplied from Fluka (Buchs, Switzerland), sodium hydroxide and 2-morpholinoethanesulfonic acid (MES) from Merck (Darmstadt, Germany) and methanol was purchased from Scharlab (Barcelona, Spain). Water used to prepare all solutions was purified in a Milli-Q system from Millipore (Bedford, MA, USA). Carboxymethyl-β-CD (CM-β-CD, degree of substitution (DS), average number of substituents on one CD ring ~ 3) and sulfated-β-CD (DS ~ 12) were supplied from Fluka. Carboxymethyl-γ-CD (CM-γ-CD), carboxyethyl-γ-CD (CE-γ-CD), carboxyethylβ-CD (CE-β-CD, DS ~ 3), succinyl-γ-CD (succ-γ-CD), succinyl-β-CD (succ-β-CD, DS ~ 3.5) and sulfated-γ-CD were from Cyclolab (Budapest, Hungary).

Standards of racemic metalaxyl, metalaxyl-M, racemic benalaxyl, benalaxyl-M and folpet were supplied from Sigma (St. Louis, MO, USA). The structure of the enantiomers of metalaxyl and benalaxyl and the structure of folpet are shown in Fig. 1. The commercial fungicide formulations were acquired in agrochemical shops in Fuenlabrada (Madrid, Spain) and Haro (La Rioja, Spain). Three commercial formulations were analyzed containing only metalaxyl-M, metalaxyl-M with folpet and racemic benalaxyl with folpet. According to the labeled data, sample 1 (liquid) contains 465 mg/L of metalaxyl-M, sample 2 (solid) contains 4.8% of metalaxyl-M and 40% of folpet and sample 3 (solid) contains 8% of racemic benalaxyl and 50% of folpet.

2.2. Apparatus

A $HP^{3D}CE$ system from Agilent Technologies (Palo Alto, CA, USA) with a diode array detector (DAD) was employed for the experiments. Instrument control and data acquisition were performed with the $HP^{3D}CE$ ChemStation software. Separations were performed in an uncoated fused-silica capillary of 50 μm i.d. (375 μm o.d.) with a total length of 58.5 cm (50 cm to the detector) purchased from Polymicro Technologies (Phoenix, AZ, USA). UV detection was performed at 210 ± 2 nm. A pH-meter model 744 from Metrohm (Herisau, Switzerland) was used to adjust the pH of the separation buffers. All the solutions were degassed in an ultrasonic bath Ultrasons-H from J.P. Selecta (Barcelona, Spain).

2.3. Procedure

Before first use, the new capillary was rinsed with methanol for 5 min, 1 M NaOH for 30 min, followed by 5 min with water and finally 60 min with the separation buffer at 15 °C. After each run the capillary was rinsed with 0.1 M NaOH for 2 min and with BGE for 5 min in order to maintain an adequate repeatability between injections.

Running buffers were prepared by dissolving the appropriate amount of boric acid or MES in Milli-Q water and adjusting the pH to the desired value with 1 M and 0.1 M NaOH. The final volume was adjusted by adding Milli-Q water to get the desired buffer concentration. BGEs were prepared by dissolving the appropriate amount of different CDs, and urea in the running buffer.

Stock standard solutions of racemic metalaxyl, metalaxyl-M, racemic benalaxyl, benalaxyl-M and folpet were prepared by dissolving the appropriate amount of the compound in methanol up to a final concentration of 2000 mg/L. To prepare the working solutions, different aliquots were diluted in methanol to obtain concentrations of metalaxyl, benalaxyl and folpet between 20 and 400 mg/L (this concentration is referred to racemic mixture in the case of metalaxyl and benalaxyl) for the calibration by the external standard method. When the standard addition calibration method was employed, different amounts of standard solutions of metalaxyl, benalaxyl and folpet were added to a commercial sample of known concentration. For the optimization of the enantiomeric

separation of metalaxyl and benalaxyl enantiomers a 200 mg/L standard solution was employed. For the determination of enantiomeric impurity of metalaxyl, working solutions were prepared by diluting the appropriate amount of stock standard solution in a mixture BGE:H_2O 50:50 (v/v).

To prepare the commercial formulations for their analysis, different procedures were followed depending on the physic state of the sample. For solid samples (samples 2 and 3) the appropriate amount of formulation was weighed (to obtain a solution of approximately 2000 mg/L of chiral amide fungicide), dissolved in methanol, and sonicated during 15 min. To prepare the liquid sample (sample 1) an appropriate volume of product was diluted in methanol to obtain a concentration of approximately 4000 mg/L of active ingredient (metalaxyl-M). Working solutions were prepared by diluting these samples in methanol or BGE:H_2O 50:50 (v/v) to obtain a concentration of approximately 200 mg/L of racemic benalaxyl or 100 mg/L of metalaxyl-M.

All the solutions were stored at 4 °C in the dark and they were filtered with a Nylon 0.45 μm pore size filter from Titan (Eatontown, NJ, USA) before their injection in the CE system.

2.4. Data treatment

The values of areas, migration times and resolution were obtained using the ChemStation software. For data treatment corrected peak areas (Ac) were used to compensate the differences in the electrophoretic conditions of each analyte and to obtain better reproducibility of data [35]. Limits of detection (LODs) ($3.3\,S_a/b$) and limits of quantitation (LOQs) ($10\,S_a/b$) for each enantiomer or compound were determined from the calibration line using the standard error of the intercept (S_a) and the slope (b) [36]. The values achieved were experimentally checked using the S/N ratio equal to 3 and 10 for LODs and LOQs respectively.

Relative limit of detection (RLOD) is defined as the minimum amount of impurity that can be detected as a function of the amount of main enantiomer and it was determined employing the following expression [37]:

$$\mathrm{RLOD} = \frac{\mathrm{LOD\ Impurity}}{C\,\mathrm{Main\ Enantiomer}} \times 100$$

where $\mathrm{LOD_{Impurity}}$ is the limit of detection for the minor enantiomer (impurity) and $C_{\mathrm{Main\ Enantiomer}}$ corresponds to the concentration of the major compound or main enantiomer.

The presence of matrix interferences was investigated by two different methods. The first one consisted in the comparison of the confidence interval of the slopes obtained when using the external standard calibration method and the standard additions calibration method. If the overlapping of the confidence intervals of the slopes of both calibration methods was demonstrated, no statistically significant differences between the slopes were obtained; hence the matrix did not produce systematic errors. The second method employed 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 area of the peak of the impurity Ac divided by the total area ($(\mathrm{Ac_{impurity}}/\mathrm{Ac_{impurity}} + \mathrm{Ac_{major\ peak}}) \times 100$).

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. Development of an analytical methodology for the chiral separation of metalaxyl and benalaxyl by EKC

The most critical step in the development of a chiral methodology enabling the separation of the enantiomers of a compound is the appropriate selection of the chiral selector. Due to the fact that metalaxyl and benalaxyl are neutral compounds, several charged CDs were tested at a 15 mM concentration. A screening of eight anionic CDs (CM-β-CD, CM-γ-CD, succ-β-CD, succ-γ-CD, sulfated-β-CD, sulfated-γ-CD, CE-β-CD, CE-γ-CD) was carried out to explore the chiral discrimination against the selected fungicides metalaxyl and benalaxyl. In addition, each CD was tested using two different separation buffers (50 mM borate buffer (pH 8.5) and 50 mM MES buffer (pH 6.5)) to prove the influence of buffer nature. The low solubility of pesticides in general in aqueous media and the previous experience of the research group have demonstrated the convenience of adding urea to the BGE in order to improve the shape of the peaks, the solubility on the media and the resolution achieved. For these reasons, a 2 M concentration of urea was added to the separation buffer. All experiments were performed employing a working temperature of 15 °C, with a separation voltage of 30 kV and a hydrodynamic injection (50 mbar × 4 s). The value of 15 °C was chosen because it is known that at low temperatures the interactions selector–selectand are quite more stable and better resolutions can be obtained [38]. On the other hand, a 30 kV separation voltage was applied in order to reduce the analysis times as much as possible.

Table 1 groups all the experiments performed and the chiral resolution achieved in each case. As it can be observed, among the two separation buffers tested 50 mM MES buffer (pH 6.5) produced the chiral discrimination of both compounds in most cases. No baseline separation was obtained for benalaxyl with borate buffer. This fact may occur because MES buffer allows a better solubilization of the compounds analyzed in the BGE, achieving also a better peak shape under all the experimental conditions proved. For this reason, a 50 mM MES buffer (pH 6.5) was chosen as the most adequate buffer to perform the enantiomeric separation of these two fungicides. Furthermore, in spite of the similar structures of the two compounds analyzed, metalaxyl and benalaxyl (see Fig. 1), the enantioseparation power of the CDs was very different and those CDs that enabled the chiral separation of metalaxyl did not work properly with benalaxyl and vice versa.

For metalaxyl, when using MES buffer, only two CDs offered an enantiomeric resolution higher than baseline separation,

Table 1
Enantiomeric resolution (Rs) for metalaxyl and benalaxyl enantiomers under the different experimental conditions employed.

CD	Separation buffer	Metalaxyl (Rs)	Benalaxyl (Rs)
CM-β-CD	50 mM MES (pH 6.5)	–	5.82
	50 mM borate (pH 8.5)	1.45	–
CM-γ-CD	50 mM MES (pH 6.5)	0.68	3.80
	50 mM borate (pH 8.5)	–	–
Succ-β-CD	50 mM MES (pH 6.5)	–	12.80
	50 mM borate (pH 8.5)	1.45	–
Succ-γ-CD	50 mM MES (pH 6.5)	3.10	–
	50 mM borate (pH 8.5)	–	0.55
Sulfated β-CD	50 mM MES (pH 6.5)	–	–
	50 mM borate (pH 8.5)	0.40	–
Sulfated γ-CD	50 mM MES (pH 6.5)	10.10	–
	50 mM borate (pH 8.5)	–	–
CE-β-CD	50 mM MES (pH 6.5)	0.96	–
	50 mM borate (pH 8.5)	–	–
CE-γ-CD	50 mM MES (pH 6.5)	0.58	–
	50 mM borate (pH 8.5)	–	–

succ-γ-CD and sulfated-γ-CD. However, due to the marked anionic character of sulfated-γ-CD, the mobility of this CD against the direction of the electroosmotic flow (EOF) was very high and resulted in higher analysis times than those obtained for succ-γ-CD. Benalaxyl was enantiomerically separated when employing CM-β-CD, CM-γ-CD and succ-β-CD dissolved in MES buffer. Although every CD allowed the chiral separation of benalaxyl enantiomers with resolutions higher than baseline (between 3.8 and 12.8), succ-β-CD was chosen to obtain the chiral separation of the enantiomers of benalaxyl due to the fact that the chiral resolution achieved was higher than that for the other two CDs in approximately the same analysis times. Finally, the possibility of combining the selected CDs was studied in order to obtain the simultaneous separation of metalaxyl and benalaxyl. As positive results were no obtained, it was decided to employ different experimental conditions for each fungicide taking also into account that these two compounds are not simultaneously used in any commercial formulation. In short, succ-γ-CD and succ-β-CD were selected as the most adequate CDs for the chiral separation of metalaxyl (Rs 3.1, ~11.5 min) and benalaxyl (Rs 12.8, ~18 min) respectively.

The different behavior observed for the different CDs with respect to the two compounds studied are in agreement with previous results showing that a slight change in the structure of the analyte can result in a large difference in the interaction with the CD proving the difficulty to predict and explain the success of separation from structural considerations. In fact, CDs can change their shape to interact with analytes because they posses a flexible structure [39] yielding more than one mode of interaction with the analytes.

Once chosen the most suitable CD for the enantiomeric separation of each fungicide, an optimization of the EKC methodology was carried out. First, the influence of the injection employed for the sample introduction into the capillary was studied. As the two analytes are neutral a hydrodynamic injection by pressure was employed for this purpose. Several pressure values (25 or 50 mbar) and injection times (1–4 s) were tested in order to select the injection that offered the best peak shape. Injections involving the highest sample amount (50 mbar × 4 s, 50 mbar × 3 s) gave peaks with a slight distortion. For this reason a hydrodynamic pressure injection of 25 mbar × 3 s was selected as the most appropriate.

The concentration of the chiral selector is one of the factors that has more influence on the enantiomeric resolution obtained for a known compound. However, it is not possible to predict which CD concentration will provide the best enantiomeric separation for a known compound [40] and it is necessary to try experimentally several concentrations in order to find the most appropriate. In this work, the influence of the concentration of the two selected CDs was studied in the range from 1 to 25 mM. Fig. 2 shows the variation of the enantiomeric resolution and analysis times for metalaxyl (Fig. 2a) and benalaxyl (Fig. 2b) as a function of CD concentration chosen for each fungicide. For metalaxyl (Fig. 2a) the resolution achieved increases rapidly when the concentration of succ-γ-CD goes from 1 mM to 10 mM. However, higher concentrations of succ-γ-CD resulted in a chiral resolution almost constant. Nevertheless, the analysis times obtained when using 15 mM succ-γ-CD are lower than those obtained for 10 mM and the shape of the peaks was better perhaps because a major concentration of CD allowed a better solubilization of the corresponding compound. Thus, 15 mM succ-γ-CD was chosen as the most appropriate concentration for the enantiomeric separation of metalaxyl. For benalaxyl (Fig. 2b) there is a clear maximum in the enantiomeric resolution reached when changing the concentration of succ-β-CD from 1 to 25 mM. This concentration corresponds to 5 mM succ-β-CD and the analysis times at this concentration are short enough to select this value as the optimum one.

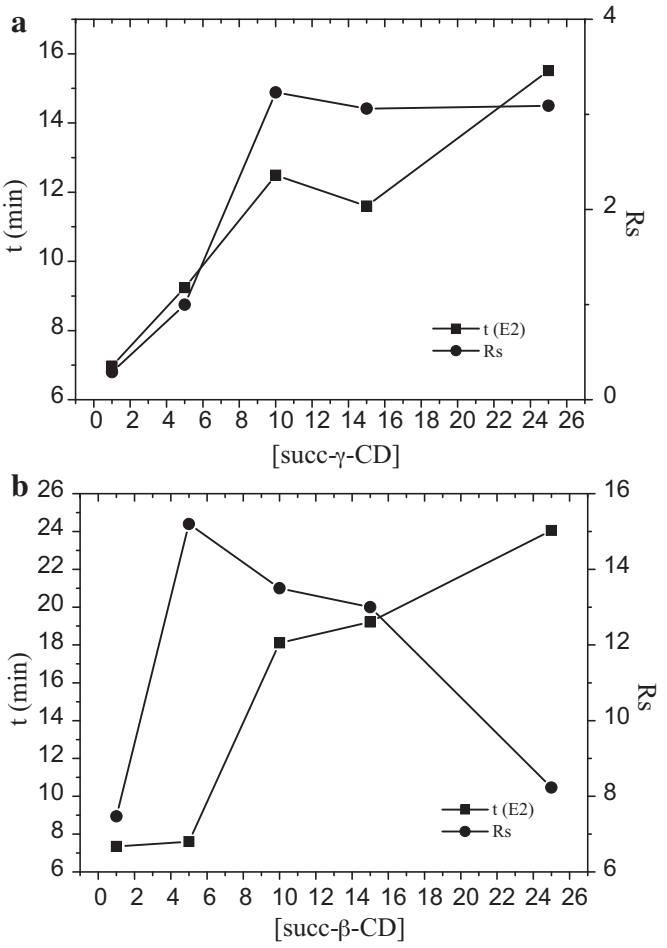

Fig. 2. Variation of the enantiomeric resolution and analysis time for (a) metalaxyl as a function of the concentration of succ-γ-CD and (b) benalaxyl as a function of the succ-β-CD concentration. Experimental conditions: BGE: CD dissolved in 50 mM MES buffer (pH 6.5) with 2 M urea; uncoated fused-silica capillary 50 μm i.d. × 50 cm (58.5 cm t.l.); injection by pressure at 25 mbar × 3 s; applied voltage: 30 kV; temperature: 15 °C; λ: 210 ± 2 nm; [metalaxyl]: 200 mg/L dissolved in methanol; [benalaxyl]: 200 mg/L dissolved in methanol.

As it has been mentioned in Section 1, it is very common to find metalaxyl or benalaxyl mixed with other fungicides able to complement their activity. Folpet is one of these fungicides so it is very important the developed chiral methods to determine not only the enantiomers of metalaxyl or benalaxyl but also folpet in the commercial products analyzed. For this reason each chiral method developed was applied to the separation of binary samples containing metalaxyl or benalaxyl and folpet. Fig. 3a shows the electropherogram obtained for a standard solution containing 200 mg/L of racemic metalaxyl and 200 mg/L of folpet dissolved in methanol under the experimental conditions chosen as optimal for the chiral separation of metalaxyl enantiomers (BGE: 15 mM succ-γ-CD dissolved in 50 mM MES buffer (pH 6.5) with 2 M urea; uncoated fused-silica capillary: 50 μm i.d. × 50 cm (58.5 cm t.l.); injection by pressure: 25 mbar × 3 s; applied voltage: 30 kV; temperature: 15 °C; λ: 210 ± 2 nm). The total separation was achieved in 12.5 min with a chiral resolution of 3.1 for the two enantiomers of metalaxyl. Fig. 4a shows the electropherogram obtained for a standard solution containing 200 mg/L of racemic benalaxyl and 200 mg/L of folpet dissolved in methanol under the experimental conditions chosen as the most appropriate for the enantiomeric separation of benalaxyl enantiomers (BGE: 5 mM succ-β-CD dissolved in 50 mM MES buffer (pH 6.5) with 2 M urea; uncoated fused-silica capillary: 50 μm i.d. × 50 cm (58.5 cm t.l.); injection

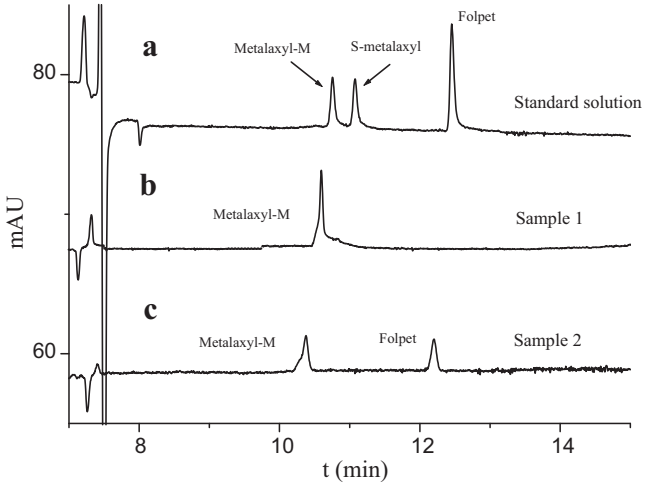

Fig. 3. Electropherograms corresponding to the separation of metalaxyl enantiomers and folpet (a) in a standard solution containing 200 mg/L of racemic metalaxyl and 200 mg/L of folpet, (b) in a commercial fungicide formulations containing metalaxyl-M (approximately 100 mg/L) alone (sample 1) and (c) in a commercial fungicide formulation containing metalaxyl-M (approximately 100 mg/L) combined with folpet (sample 2). Experimental conditions: 15 mM succ-γ-CD in 50 mM MES buffer (pH 6.5) with 2 M urea. Other experimental conditions as in Fig. 2.

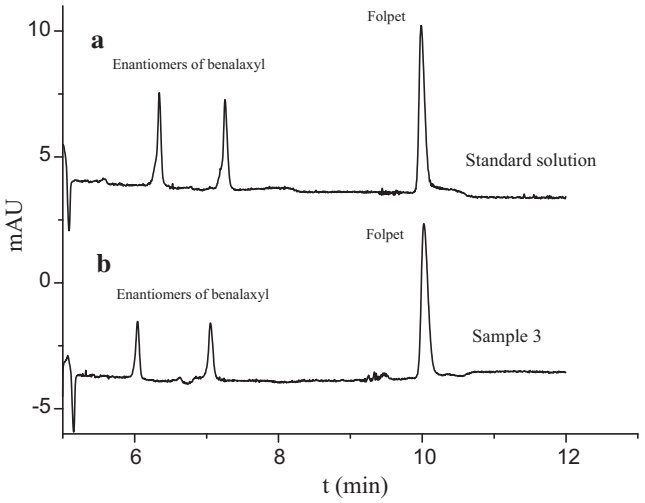

Fig. 4. Electropherograms corresponding to the separation of benalaxyl enantiomers and folpet (a) in a standard solution containing 200 mg/L of racemic benalaxyl and 200 mg/L of folpet and (b) in commercial fungicide formulation containing racemic benalaxyl (approximately 200 mg/L) and folpet (sample 3). Experimental conditions: 5 mM succ-β-CD in 50 mM MES buffer (pH 6.5) with 2 M urea. Other experimental conditions as in Fig. 2.

by pressure: 25 mbar × 3 s; applied voltage: 30 kV; temperature: 15 °C; λ: 210 ± 2 nm). The separation of the two enantiomers of benalaxyl and folpet was achieved in 10.5 min and with a chiral resolution of 14.6 for the enantiomers of benalaxyl.

3.2. Quantitative analysis of metalaxyl enantiomers, benalaxyl enantiomers and folpet in commercial agrochemical formulations

Before carrying out the quantitative determination of metalaxyl, benalaxyl and folpet in commercial fungicide products, the analytical characteristics of the corresponding methods were evaluated in terms of linearity, LODs, LOQs, precision, selectivity and accuracy. The results obtained are grouped in Table 2 for the method developed for metalaxyl–folpet mixtures and in Table 3 for the method developed for benalaxyl–folpet mixtures.

Linearity was determined by plotting Ac as a function of the concentration of each compound in the range 20–400 mg/L referred to the racemic mixture in the case of metalaxyl and benalaxyl and for the total amount in the case of folpet. A total number of seven standard solutions were prepared individually during three different days and injected by triplicate each day. These experiments allowed fixing the linear range for each compound and each method. Tables 2 and 3 present this interval, the linear equation obtained in the selected range as well as the standard errors for the intercept (S_a), the slope (S_b) and the determination coefficient (R^2). Satisfactory results were obtained in terms of linearity with $R^2 > 0.99$, moreover, ANOVA confirmed that data fit to a linear model, by the p-values (>0.05 for every linear model).

Table 2
Analytical characteristics of the developed method for the simultaneous separation of the enantiomers of metalaxyl and folpet.

Analytical characteristics	Metalaxyl				Folpet	
	First enantiomer		Second enantiomer			
Precision (RSD)						
Concentration (mg/L)	20	50	20	50	100	200
Instrumental repeatability ($n = 6$)						
Ac, RSD (%)	4.4	5.0	4.8	5.4	5.5	3.1
t, RSD (%)	0.7	0.5	0.7	0.5	0.5	0.7
Intermediate precision ($n = 9$)						
Ac, RSD (%)	5.2	4.0	6.2	5.1	5.7	2.6
t, RSD (%)	1.3	2.2	1.3	2.3	2.1	1.6
Linearity						
Linear range (mg/L)	10–100		10–100		40–300	
Linear equation	$-0.0464 + 0.0178x$		$-0.0335 + 0.0171x$		$-0.3382 + 0.0206x$	
Standard errors	$S_a = 0.0204$ $S_b = 0.0003$		$S_a = 0.0240$ $S_b = 0.0004$		$S_a = 0.0795$ $S_b = 0.0005$	
Determination coefficient (R^2)	0.998		0.997		0.997	
$b \pm t \cdot S_b$	0.0178 ± 0.0010					
p-value of ANOVA	0.6610		0.4150		0.1448	
LOD (mg/L)	3.4		4.2		12.8	
LOQ (mg/L)	11.4		14.1		38.7	
Study of matrix interferences						
p-value of t-tests						
Sample 1	0.0601		–		–	
Sample 2	0.0730		–		–	
Recovery						
Sample 1	108 ± 8					
Sample 2	104 ± 8					

Table 3
Analytical characteristics of the developed method for the simultaneous separation of the enantiomers of benalaxyl and folpet.

Analytical characteristics	Benalaxyl				Folpet	
	First enantiomer		Second enantiomer			
Precision (RSD)						
Concentration (mg/L)	50	100	50	100	100	200
Instrumental repeatability ($n = 6$)						
Ac, RSD (%)	1.5	3.3	1.8	4.4	2.8	1.2
t, RSD (%)	0.2	0.2	0.2	1.9	0.3	0.3
Intermediate precision ($n = 9$)						
Ac, RSD (%)	1.8	3.3	3.2	3.6	2.8	2.8
t, RSD (%)	0.3	2.2	0.3	3.6	2.6	2.6
Linearity						
Linear range (mg/L)	10–150		10–150		40–300	
Linear equation	$-0.0514 + 0.0279x$		$-0.0489 + 0.0257x$		$0.0516 + 0.0202x$	
Standard errors	$S_a = 0.0475$ $S_b = 0.0006$		$S_a = 0.0479$ $S_b = 0.0006$		$S_a = 0.0704$ $S_b = 0.0004$	
Determination coefficient (R^2)	0.998		0.998		0.998	
$b \pm t \cdot S_b$	0.0280 ± 0.0016		0.0253 ± 0.0016			
p-value of ANOVA	0.4506		0.4932		0.1400	
LOD (mg/L)	5.1		5.6		11.5	
LOQ (mg/L)	17.0		18.6		34.8	
Study of matrix interferences						
p-value of t-test						
Sample 3	0.0508		0.0696		–	
Recovery (%)						
Sample 3S	98 ± 11		94 ± 10			

LODs and LOQs for the four enantiomers (those for metalaxyl and those for benalaxyl) and folpet were determined from the calibration line using the equations described in Section 2. For metalaxyl (Table 2) LOD values of 3.4 and 4.2 mg/L and LOQ values of 11.4 and 14.1 mg/L were obtained for the first and the second migrating enantiomer, respectively. For benalaxyl (Table 3) the LOD values were of 5.1 and 5.6 mg/L and the LOQs of 17.0 and 18.6 mg/L for the first and the second enantiomer, respectively. Finally, for folpet (Tables 2 and 3) these values were lower than 12.8 mg/L for LODs and lower than 38.7 mg/L for LOQs regardless of the method employed for its determination.

Precision of the methods was evaluated as *instrumental repeatability* and *intermediate precision*. Instrumental repeatability was determined from six repeated injections of a standard solution at two different concentration values of each compound (20 and 50 mg/L for metalaxyl enantiomers, 50 and 100 mg/L for benalaxyl enantiomers and 100 and 200 mg/L for folpet). The RSD values (%) obtained for metalaxyl enantiomers (Table 2) were lower than 0.7% for migration times and lower than 5.4% for Ac. For folpet the RSD values for migration times were lower than 0.7% and lower than 5.5% for Ac (Table 2). For the method enabling the simultaneous separation of the enantiomers of benalaxyl and folpet (Table 3) the RSD values obtained for the instrumental repeatability were lower than 1.9% for the migration times and lower than 4.4% for Ac. For folpet (Table 3) the RSD values for migration times were 0.3% for migration times and lower than 2.8% for Ac. Intermediate precision was assessed at the same concentration values for three consecutive days injecting each sample by triplicate each day. As it can be observed in Table 2 the RSD values achieved for the enantiomers of metalaxyl were under 2.3% and 6.2% for analysis times and Ac respectively. For folpet the RSD values were lower than 2.1% for migration times and than 5.7% for Ac. In the case of enantiomers of benalaxyl (Table 3) and also for the two concentration values studied the RSD values were lower than 3.3% both for migration times and Ac. With this method folpet presents RSD values lower than 2.6% and 2.8% for migration times and Ac, respectively.

The selectivity of the methods was demonstrated due to the absence of matrix interferences. For this purpose there were compared the slopes of the calibration lines obtained by the external calibration method and the standard additions method for each sample. The standard additions calibration line was obtained by spiking the commercial sample (40 mg/L of metalaxyl-M (samples 1 and 2) or 60 mg/L of racemic benalaxyl (sample 3)) with known concentrations of metalaxyl-M and racemic benalaxyl in the linear interval for each fungicide. The results were confirmed by p-value of t-test and as it can be observed in Tables 2 and 3, 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 and asses the suitability of the external calibration method for the quantitation of the three analytes in the selected commercial formulations.

Accuracy of the developed methods was determined as the recoveries achieved for the two chiral fungicides. For this purpose the commercial formulations were spiked with different known concentrations of R-metalaxyl standard solution for samples 1 and 2 and with known concentrations of racemic benalaxyl standard solution for sample 3 (six concentration levels and three determinations for each level). The mean recoveries obtained were $108 \pm 8\%$ (sample 1) and $104 \pm 8\%$ (sample 2) for R-metalaxyl, $98 \pm 11\%$ for R-benalaxyl and $94 \pm 10\%$ for S-benalaxyl.

The developed chiral methods were applied for the determination of metalaxyl-M, racemic benalaxyl and folpet in three commercial fungicide products. Fig. 3 shows the electropherograms obtained for a standard solution containing 200 mg/L of racemic metalaxyl and 200 mg/L of folpet (Fig. 3a) and for two commercial samples containing approximately 100 mg/L of metalaxyl-M (sample 1) (Fig. 3b) and approximately 100 mg/L of metalaxyl-M and 832 mg/L of folpet (sample 2) (Fig. 3c). On the other hand, Fig. 4 shows the electropherograms obtained for a standard solution containing 200 mg/L of racemic benalaxyl and 200 mg/L of folpet (Fig. 4a) and for a commercial sample (sample 3) containing racemic benalaxyl at approximately 200 mg/L and approximately 625 mg/L of folpet (Fig. 4b). The total amounts determined for each fungicide in the three commercial formulations and the labeled amounts are specified in Table 4. Only those contents referred to benalaxyl correspond to the labeled amount. In the two samples containing metalaxyl-M the determined amount is between 35 and 45% above the theoretical value and for folpet the determined amounts are much lower than those specified in the label. The differences in the determined and labeled amounts may be due to the fact that the control in agrochem-

Table 4
Determined contents of metalaxyl-M, benalaxyl and folpet in the commercial fungicide formulations analyzed (average value ± SD) ($n = 3$).

Sample	Active ingredients	Metalaxyl/benalaxyl First enantiomer (% p/p or g/L)	Second enantiomer (% p/p)	Labeled amount (% p/p or g/L)	Folpet (% p/p)	Labeled amount (% p/p)
1	Metalaxyl-M	627 ± 13 g/L	-	465 g/L	-	-
2	Metalaxyl-M + folpet	6.96 ± 0.07 (%)	-	4.8 (%)	3.1 ± 0.2 (%)	40 (%)
3	Benalaxyl + folpet	4.16 ± 0.06 (%)	3.83 ± 0.08 (%)	8 (%)	15.6 ± 0.4 (%)	50 (%)

ical formulations is mostly referred to the maximum residual concentrations in foods but not to the contents in commercial formulations.

3.3. Purity testing of metalaxyl-M in commercial fungicide formulations

The determination of enantiomeric impurities requires analytical methods sensitive enough to be able to detect minor enantiomeric impurities. In order to obtain the maximum injection volume and so the highest peak areas possible without any lost of resolution, an in-capillary preconcentration strategy (stacking) was tried. To achieve this preconcentration, the standard of racemic metalaxyl was dissolved in the same BGE employed for its chiral separation but diluted with water to 50% instead of methanol, as it was done till this moment. When a voltage is applied between the ends of the capillary, the electric field will be higher in the sample region, due to its low conductivity and consequently the electrophoretic velocity of the analytes in this region is also higher compared to the separation region. So when the analytes move to the BGE they slow down and focus in the boundary that separates the two phases [41]. By this strategy it is possible to inject a higher amount of sample without lost of resolution. In order to choose the injection time most adequate for this purpose, a study of the variation of the enantiomeric resolution with the injection time (from 3 to 20 s) at a pressure of 25 mbar was carried out. From 3 to 10 s the chiral resolution achieved is almost constant, however, from 10 s a clear lost of resolution occurs (from approximately 4 to 2.8), so a hydrodynamic injection by pressure 25 mbar × 10 s with the standard dissolved in the BGE diluted to 50% with water was selected for the determination of the enantiomeric impurity of commercial fungicide formulations.

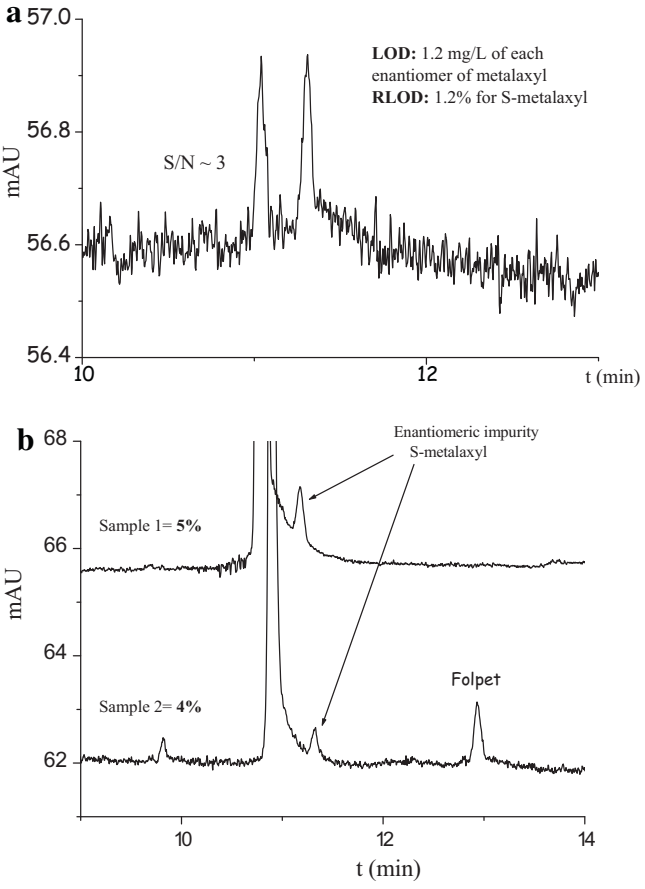

Fig. 5. Electropherograms corresponding to (a) the LOD obtained when employing normal stacking mode with the standard (1.2 mg/L) dissolved in BGE:H_2O 50:50 (v/v) for the enantiomeric separation of metalaxyl and (b) to enantiomeric impurity of S-metalaxyl in commercial fungicide formulations labeled as enantiomerically pure in metalaxyl-M. Experimental conditions: Injection by pressure: 25 mbar × 10 s; [metalaxyl-M] ~ 100 mg/L. Other experimental conditions as in Fig. 2.

The analytical characteristics of the new method were evaluated in the same terms as before achieving appropriate values for every parameter. It has to be highlighted that with this in-capillary preconcentration strategy the sensitivity of the method was improved and the LODs obtained were approximately three times lower than before (from around 4 mg/L for each enantiomer of metalaxyl to 1.2 mg/L) (see Fig. 5a). This approach enabled the detection of impurities of S-metalaxyl up to 1.2% referred to the main enantiomer.

Finally, the quantitation of the enantiomeric impurity of metalaxyl-M in two commercial fungicide formulations was performed. Fig. 5b shows the electropherograms obtained for the two commercial formulations containing only metalaxyl-M according to their label (samples 1 and 2), under the experimental conditions selected (BGE: 15 mM succ-γ-CD dissolved in 50 mM MES buffer (pH 6.5) with 2 M urea; uncoated fused-silica capillary: 50 μm i.d. × 50 cm (58.5 cm t.l.); injection by pressure: 25 mbar × 10 s; applied voltage: 30 kV; temperature: 15 °C; λ: 210 ± 2 nm, [metalaxyl-M]: 100 mg/L approximately dissolved in BGE:H_2O 50:50 (v/v)). The percentages obtained for each sample have been calculated according to the equation shown in Section 2. Impurities of 5% and 4% of S-metalaxyl were determined for samples 1 and 2, respectively.

4. Concluding remarks

A method enabling the chiral separation of benalaxyl enantiomers by CE has been proposed for first time achieving the chiral separation of the compound in 7.5 min and with a resolution near 15. These results were achieved employing a 5 mM succ-β-CD dissolved in 50 mM MES buffer (pH 6.5). On the other hand, the method proposed for the chiral separation of metalaxyl by this technique involves a clear improvement in both the analysis times and the resolutions achieved compared with the two works appeared in the literature. In this case, a 15 mM succ-γ-CD in 50 mM MES buffer (pH 6.5) was employed for the chiral separation that was achieved in 11.5 min and with a chiral resolution of 3.1. Due to the fact that these two amide fungicides are employed in commercial products mixed with other fungicides, the two chiral methods were applied to the simultaneous separation of metalaxyl or benalaxyl in binary combinations with folpet. Furthermore, the performance of the method was established in terms of precision, linearity, selectivity,

LODs and LOQs showing their suitability for their application to the determination of these three compounds in commercial fungicide formulations. Finally, an in-capillary preconcentration strategy was developed in order to improve the detection limits of the method developed for metalaxyl and applying it to the determination of enantiomeric impurity (S-metalaxyl) of metalaxyl-M in commercial products. This new method was able to detect enantiomeric impurities up to 1.2% of S-metalaxyl.

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