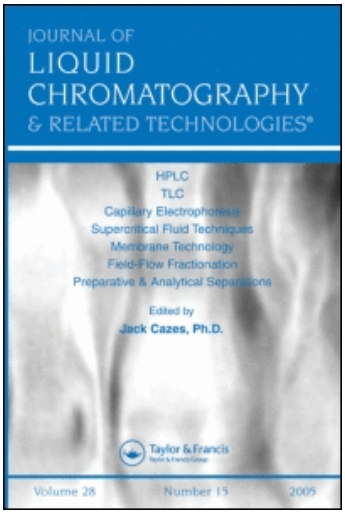

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Chiral HPLC Resolution of Neutral Pesticides

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Chiral HPLC Resolution of Neutral Pesticides

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ABSTRACT

The direct HPLC resolution of two synthetic neutral pesticides, Metolachlor and Ethofumesate, was studied on silica gel coated with cellulose tris-(3,5-dimethylphenylcarbamate) derivative (Chiracel OD-H). The specific composition of the mobile phase has allowed the full separation of the four Metolachlor stereoisomers, which had not previously been resolved on other chiral stationary phases. The isomer elution sequence of both resolved pesticides was determined by ^{1}H NMR and polarimetric analyses. The chromatographic system, coupled with

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mass-spectrometric detection, was used to determine the isomeric ratio of Metolachlor stereoisomers after incubation in soil.

Key Words: Herbicides; Metolachlor; Ethofumesate; HPLC; Chiral separation; Pesticides.

INTRODUCTION

Several chiral synthetic herbicides are marketed either as a single, biologically active isomer or as a mixture of stereoisomers. It is well known that the biological activity and toxicity of these compounds are strongly related to the absolute configuration of the molecules,[1,2] so chiral separation methods are necessary for an accurate specification of the stereoisomeric composition of the formulated products. In this respect, HPLC and, more recently, capillary zone electrophoresis (CZE) have proven to be the most efficient techniques. In fact, they provide not only fast separations, but also a large spectrum of suitable chiral selectors, and show negligible epimerisation of the sample during the analysis run.[3–6]

The present work deals with the chiral HPLC separation of stereoisomers of two neutral pesticides, Metolachlor and Ethofumesate (Fig. 1), on a chiral stationary phase (CSP) based on the tris-(3,5-dimethylphenylcarbamate) derivative of cellulose (Daicel Chiracel OD-H column).

Metolachlor [*N*-(1′-methyl-2′-methoxy)-*N*-chloroacetyl-ethyl-6-methyl-aniline] is a widely used herbicide. Its chirality is due to the presence of an asymmetric carbon in one of the alkylic substituents at the nitrogen in the imide group (C*) and to the atropisomerism generated by the hindered rotation around the aryl carbon–nitrogen bond (a). The resulting four stereoisomers exhibit a very different herbicidal activity. This activity is mainly influenced by the configuration at the chiral centre, the (*S*)-isomers being the most active, and, in particular, the isomer with configuration (C**S*, a*S*).[7] The stereoselective synthesis of the (C**S*) isomer pair has recently been obtained,[8] and mixtures of these two diastereoisomers (S-DUAL) are marketed by Syngenta. However, several products containing all the four isomers, usually commercialised as a mixture with other herbicides, are still diffused in Europe. A partial resolution of the Metolachlor four stereoisomers has been obtained by GC-CSP,[9] γ-CD-MEKC,[10] and in normal phase (NP) HPLC on phenyl-carbamate modified polysaccharides stationary phases (Chiracell OD-H and Chiralpack-AS).[3] Only the combination of reverse phase (RP) achiral and NP-chiral HPLC allowed the separation of the four stereoisomers.[11] RP-chromatography on a modified cellulose chemically bonded to silica (OD-R) did not give satisfactory results.[9]

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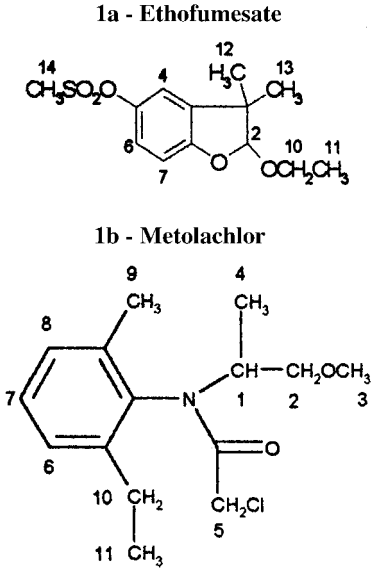

Figure 1. Chemical structure of the examined herbicides.

Ethofumesate, [2-ethoxy-2,3-dihydro-3,3′-dimethyl-benzofurane-5-yl-ethane-sulfonate], is a herbicide currently used in tobacco and sugar beet cultivation.[12] Chiral separation of Ethofumesate has been recently obtained by CZE using a charged β-cyclodextrin derivative as the selector.[13]

EXPERIMENTAL

Instrumentation

Chromatography was performed using a modular instrument consisting of a 114 Model (Beckman, USA) solvent delivery pump, equipped with a Rheodyne 7125 Model injection valve and connected either to a SPD-M6A Model (Shimadzu, Kyoto, Japan) UV-VIS photo-diode array detector or to a polarimetric HPLC detector (Büchi, Milan, Italy) with a flow cell path length of 0.25 dm and cell volume of 40 μL. Mass spectrometry analyses were carried out on a Navigator Model (Finnigan, Manchester, UK) single-quadrupole instrument, equipped with an atmospheric pressure ionization (API) source, using an ApcI inlet and coupled in series to the UV detector. NMR analysis of Metolachlor and Ethofumesate isomers was performed

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with a Bruker AMX600 spectrometer (Karlsruhe, Germany) operating at 600.13 MHz with a Bruker z-gradient probehead. Optical activity [α] measurements were carried out using a Model 241 (Perkin Elmer, Norwalk, CT, USA) spectropolarimeter at the Istituto Superiore di Sanità (Rome, Italy).

Chemicals

Standard isomers of Metolachlor and Ethofumesate (racemic mixtures) were purchased from LabService Analytica (Milan, Italy). The Metolachlor diastereoisomeric mixtures (C*R) and (C*S) were a gift from Syngenta Crop protection Agency (Basel, Switzerland). All solvents of HPLC grade, and diethyl ether (DEE) of RP grade (without stabilizing agents) were obtained from Carlo Erba (Milan, Italy). Sulphobutylated β-cyclodextrin was supplied by Fluka (Buchs, Switzerland).

Chiral Separation and Identification of Isomers by Spectropolarimetric and NMR Analysis

Separation of the stereoisomers was carried out on a CSP based on tris-(3,5-dimethylphenylcarbamate) derivative of cellulose (Chiracel OD-H 150 × 4.6 mm, 5 μ particle diameter, Daicel, Japan). A mixture of 1% isopropanol (IPA) in *n*-hexane was used as the eluent for the chiral resolution of Ethofumesate. Both UV and polarimetric detectors were used. Resolved enantiomers were collected for NMR studies.

Mixtures of either IPA or diethyl ether at varying concentrations in *n*-hexane were used for the chiral separation of Metolachlor. In order to avoid peroxides formation, recently acquired DEE was used: after opening, solvent bottles were stored in a dark and fresh place. Sample solutions were prepared by dissolving approximately 1 mg of pesticide in 1 mL *n*-heptane. UV detection was done at 230 nm. N-Heptane was used as void volume marker (V = 2.02 mL).

Semipreparative scale resolution of Metolachlor diastereoisomers (C*S) was carried out on a Chiracel OD-H column, using *n*-hexane/IPA (99/1 v/v) as the mobile phase (first eluted peak: $k = 2.48$; second peak: $k = 2.94$, at 0.5 mL/min). A few milligrams (2–4 mg) of each pure stereoisomer were then analysed by spectropolarimetric and NMR techniques.

Each (C*S) stereoisomer of Metolachlor was dissolved in methanol (1 mg/mL) and the corresponding optical rotation value was measured at 365 nm. Configuration was assigned to the separated stereoisomers on the

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basis of data reported by Moser et al.[7] The less retained compound, with a negative value of [α], was identified as (C*S, aR), whereas the more retained compound, with a positive value of [α], was identified as (C*S, aS).

Proton NMR spectra of the Metolachlor and Ethofumesate fractions were recorded with a soft presaturation of the HOD residual signal. Chemical shifts were reported with respect to a trace of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) used as an internal standard. In order to assign the proton spectra, COSY-gs (gradient enhanced proton correlation spectroscopy) experiments[14] were performed using the following experimental parameters: time domain = 512 words in F1 and 1024 words in F2, data matrix 512 × 512, relaxation delay = 1.2 s, number of scans = 1 and dummy scans = 4. Data were processed in the magnitude mode. NMR studies of Metolachlor isomers enantiomeric purity were performed on 1 mM solutions; in fact, at higher concentration this compound forms $\pi-\pi$ complexes in D_2O solution.[15]

Ethofumesate and Metolachlor pure isomers, and mixtures of the stereoisomers, were also analyzed by NMR using cyclodextrins as chiral selectors. In fact, the different interaction between the enantiomers and the cyclodextrin can induce a chemical shift non equivalence between the signals of the different enantiomeric forms.[16,17] In particular, the sulphobutylether derivative of β-cyclodextrin (β-CD-SBE) turned out to be a suitable chiral selector for both compounds.

Metolachlor Spiked Soil Samples Incubation, Purification, and CSP-HPLC/MS Analysis

Freshly collected soil, obtained by removing a portion of top soil in the CNR Research Area (Clay 28%, Sand 32%, and Slime 40%), was air dried and passed through a 2 mm sieve. This material was divided into six 10 g aliquots, each of which was humidified with 2 mL of distilled water. Three soil samples were spiked with a solution of *rac*-Metolachlor in methanol (mixture of four isomers) (5 ppm), whereas the other ones were spiked with a solution of Metolachlor-S (mixture of two isomers) (2.5 ppm).

Sample extractions were performed after 0, 30, and 60 days, respectively. For the whole period of incubation, soil samples were kept in the dark at room temperature and periodically humidified with small amounts of water. Samples were extracted following the procedure reported in literature,[9] which consisted of soil extraction with methanol, centrifugation of the mixture, addition of a KCl water solution to the supernatant; removal of the organic solvent by vacuum evaporation, extraction of the aqueous solution with ethyl acetate. The extract was then evaporated under a nitrogen stream. The final residue was dissolved in 200 μL of *n*-hexane-diethyl ether (1 : 1 v/v)

and injected into a nitrile HPLC column (250 × 4,6 mm I.D., 5 μ particle diameter) (Restek Corporation, Bellefonte, PA), using a mixture of *n*-hexane-DEE (97 : 3 v/v) as the eluent and a flow rate of 1 mL/min. The four isomers were eluted as a single peak ($t_r = 20$ min). The separated fraction volume was reduced to about 50 μL under a stream of nitrogen and injected into the chiral column for enantioselective analysis (*n*-hexane–DEE 9 : 1 v/v), at a flow rate of 0.8 mL/min with MS detection. The experimental conditions of the spectrometer were as follows: mass range 200–350 amu; source temperature 150°C; probe temperature 500°C; fragmentor voltage +30 V and corona discharge 1.8–2 kV. N_2 drying gas flow was 10 L/min.

RESULTS AND DISCUSSION

Chiral recognition on cellulose derivatives based CSPs has to be ascribed to the processes that involve stereoselective formation of hydrogen bonds between the analyte and the urea groups of the polysaccharidic chains outside and inside the chiral cavities. Therefore, eluents with high aprotic properties are, in general, used with these CSPs. The mobile phase constituted of mixtures of *n*-hexane and IPA, allowed the baseline separation of the two Ethofumesate enantiomers with a high selectivity factor ($\alpha = 2.2$; (+) enantiomer: $k = 4.5$; (−) enantiomer: $k = 10.1$ at 1 mL/min). Figure 2 shows the chiral separation of Ethofumesate on Chiracel OD-H with (A) UV (LOD of the racemic mixture = 12 ng) and (B) polarimetric (LOD of the racemic mixture = 20 μg) detection. The ^{1}H NMR spectra of the (+)- and (−)-enantiomers, as well as, the ^{1}H spectrum of rac-Ethofumesate in the presence of β-CD-SBE in D_2O are reported in Fig. 3. The addition of β-CD-SBE induces chemical shift non equivalence between the signals of the two enantiomeric forms and allows achieving a good separation of the two forms in the rac-Ethofumesate. Moreover, the comparison between the ^{1}H spectrum of (−) and (+) enantiomers (Fig. 3a, b) and that of the racemic mixture (Fig. 3c) in the presence of β-CD-SBE, allows the ^{1}H assignment of each enantiomeric form and confirms the enantiomeric purity of the eluted fractions; in fact, the ^{1}H spectrum of the separated fractions shows the presence of only one enantiomer.

According to the literature,[3] Metolachlor is resolved in only three peaks when mixtures of *n*-hexane and IPA are used as the eluent. Thus, due to its high ability to form hydrogen bonds with the selector,[18] IPA does not appear to be a suitable mobile phase component to achieve the full resolution of Metolachlor. However, if another hydrophobic solvent is used, the herbicide can be resolved in four peaks. Figure 4 shows the resolution of Metolachlor stereoisomers using eluents with different concentration of DEE in *n*-hexane.

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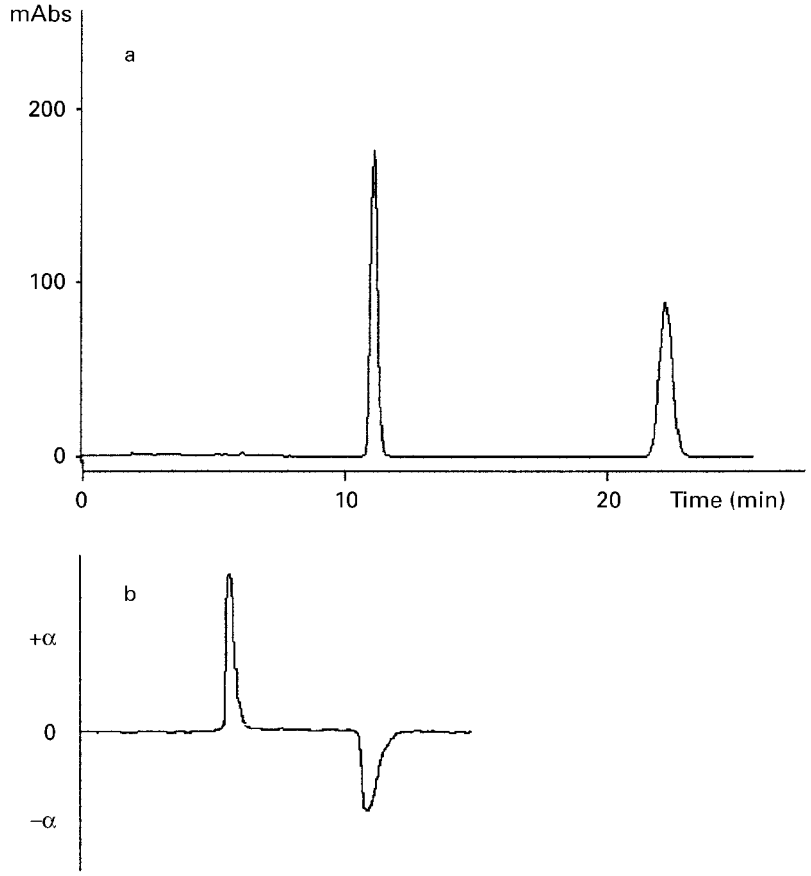

Figure 2. Resolution of Ethofumesate on Chiracel OD-H using 1% 2-propanol in *n*-hexane as the mobile phase with (a) UV, 230 nm; and (b) polarimetric detection. Flow-rate, 0.6 mL/min. Room temperature.

The best resolution of the four isomers is obtained at 9% DEE (Fig. 5). The relative peak areas in the chromatogram showed that peaks 1,4 and 2,3 are the resolved enantiomers. The addition of (C*S*, a*R*/C*S*, a*S*) standard to the racemic mixture (data not reported) demonstrates that peaks 2 and 4 correspond to the (C*S*) diastereoisomers. Moreover, spectropolarimetric and ^{1}H NMR analyses of the resolved (C*S*) isomers (see Experiment Section) allowed the identification of peak 4 (which in the studied mixture belongs to the most abundant enantiomeric pair) as the stereoisomer with absolute configuration (C*S*, a*S*) and peak 2 as (C*S*, a*R*). The elution order was

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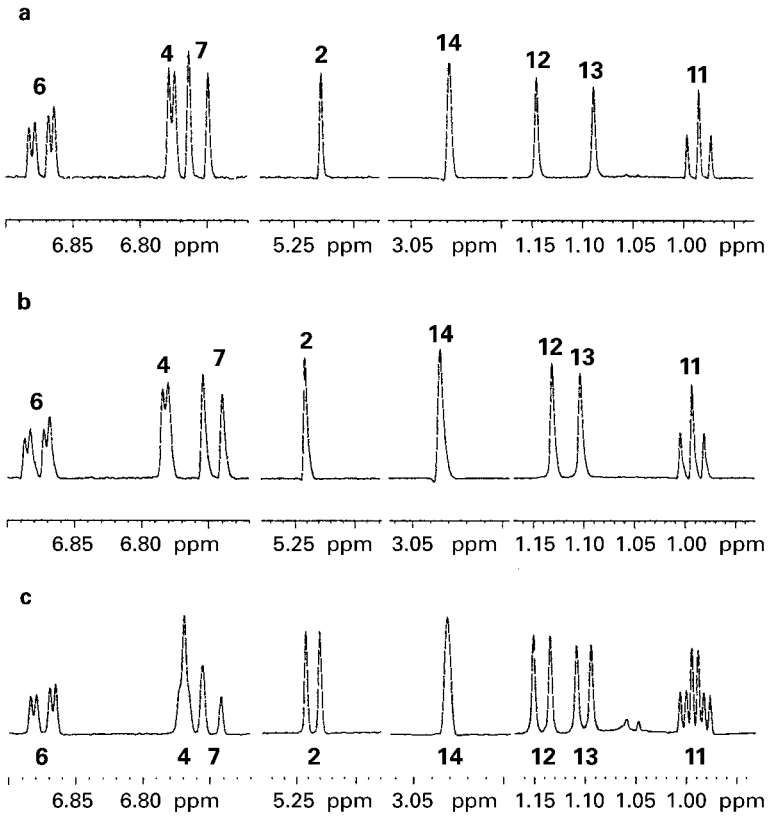

Figure 3. Expansions of the ^{1}H spectrum of (a) = (+) Ethofumesate enantiomer + β-CD-SBE, (b) = (−) Ethofumesate enantiomer + β-CD-SBE; (c) = racemic mixture + β-CD-SBE. The assignment is relative to the sketch reported in Fig. 1. Experimental conditions are reported in Experimental.

assigned as reported in Fig. 5: (1) C*R, aR ($k = 6.70$); (2) C*S, aR ($k = 7.70$); (3) C*R, aS ($k = 8.39$); (4) C*S, aS ($k = 9.03$). The LOD determined for the stereoisomers mixture was 65 ng, which corresponds approximatively to 12 ng for a single isomer.

The above mentioned separation method was extended to the analysis of spiked soil extracts, using a mass spectrometry (ESMS) detection system (LOD of the four isomers mixture = 10 ng) (see Experimental). Figure 6 shows the MS spectrum of Metolachlor. The results are summarized in Table 1. After an incubation period of $t = 30$ days, *rac*-Metolachlor showed an isomer composition similar to that corresponding at $t = 0$ day.

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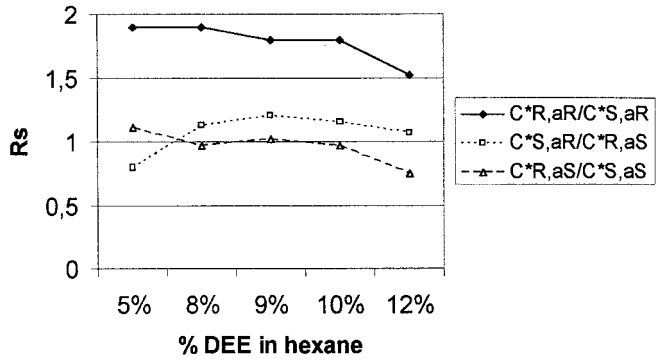

Figure 4. Effect of the DEE content in the mobile phase (DEE/*n*-hexane mixtures) on the resolution (Rs) for Metolachlor stereoisomers (Chiracel OD-H); flow rate 0.8 mL/min room temperature (20°C).

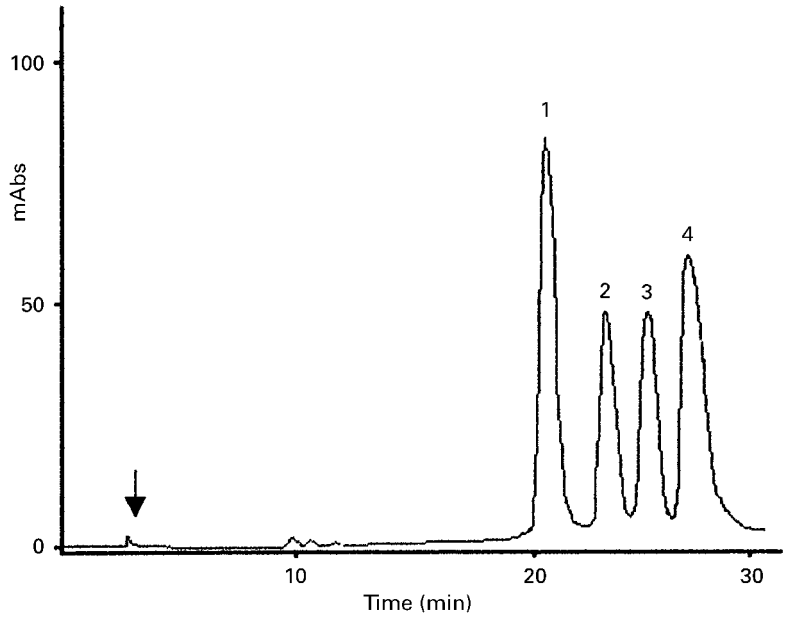

Figure 5. Separation of the four Metolachlor stereoisomers on Chiracel OD-H: (1) C**R*, a*R*; (2) C**S*, a*R*; (3) C**R*, a*S*; and (4) C**S*, a*S*. Mobile phase, 9% DEE in *n*-hexane; flow-rate, 0.8 mL/min. Detector UV, 230 nm. Room temperature (20°C). Other chromatographic conditions, as in Fig. 4.

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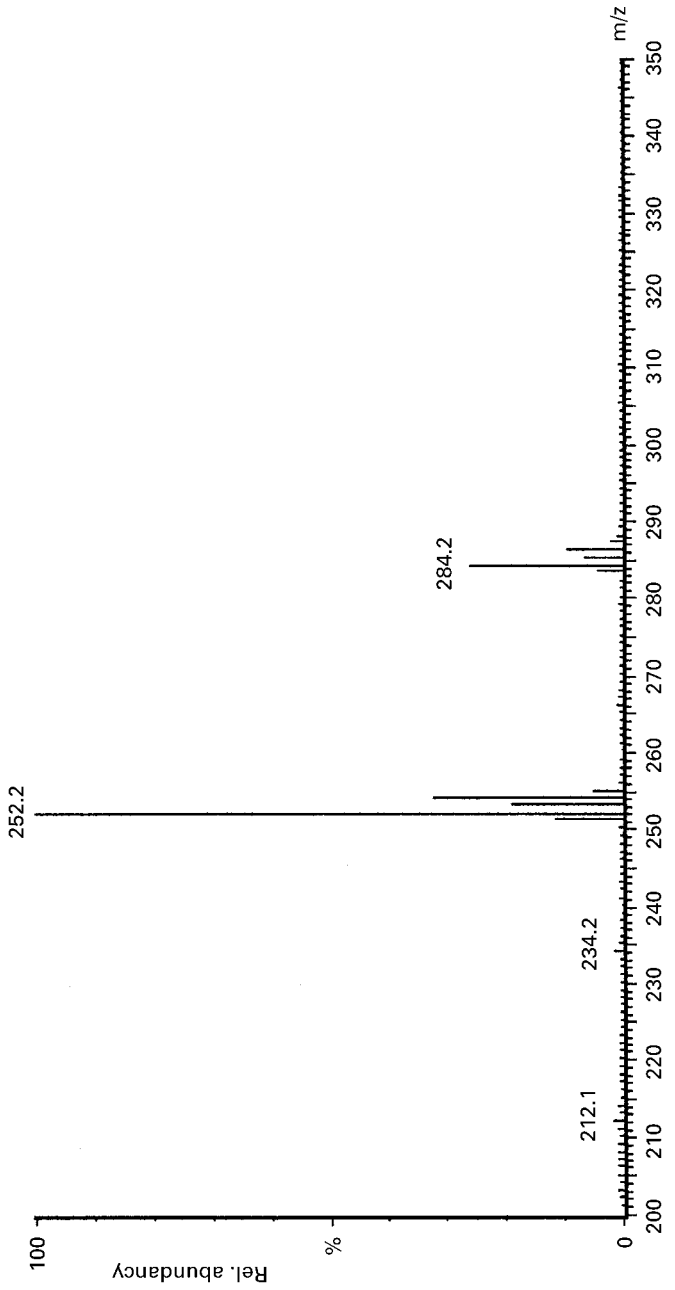

Figure 6. Mass spectra of Metolachlor obtained using a fragmentor voltage of 30 V.

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Table 1. Stereoisomeric composition of *rac*-Metolachlor and Metolaclor-*S* found in soil extracts in function of incubation time under laboratory conditions. See text for chromatographic and EMMS detection conditions.

Incubation time (days)	Stereoisomers (%)			
	(C*R, aR)	(C*S, aS)	(C*R, aS)	(C*S, aR)
rac-Metolachlor				
0	29.0 ($\pm$0.5)	29.0 ($\pm$0.4)	21.0 ($\pm$0.4)	21.0 ($\pm$0.3)
30	29.2 ($\pm$0.7)	28.8 ($\pm$0.6)	21.1 ($\pm$0.3)	20.9 ($\pm$0.3)
60	30.6 ($\pm$0.6)	34.5 ($\pm$0.5)	22.3 ($\pm$0.5)	12.6 ($\pm$0.7)
Metolaclor-*S*				
0	–	61.3 ($\pm$0.7)		38.7 ($\pm$0.8)
60	5.7 ($\pm$0.6)	63.2 ($\pm$0.5)		31.1 ($\pm$0.5)

Stereoselective degradation of the racemic herbicide was evident after $t = 60$ days, with a notable decrease in (C*R, aS) and a negligible increase in (C*S, aS) with respect to the other stereoisomers. Surprisingly, Metolachlor-*S* exhibited a different degradation trend. After 60 days of incubation, the amount of (C*S, aS) in the mixture remained almost constant, whereas the amount of the isomer (C*S, aR) decreased by about 20%. The formation of the stereoisomer (C*R, aR), not detectable in sample extracts at $t = 0$ day but present after a long incubation time, is of particular interest. Whether a change of the isomer composition of Metolachlor during incubation depends on the specific metabolism of each stereoisomer or on interconversion reactions between the four isomers, has not been investigated in the present work. However, the presence of (C*R, aR) in Metolachlor-*S* after a long period of incubation seems to demonstrate that interconversion reactions may play a part.

In conclusion, baseline separation of Metolachlor and Ethofumesate stereoisomers was achieved on Chiracel OD-H, using an appropriate modifier in *n*-hexane as the mobile phase. This newly developed method could be profitably used in the stereoselective synthesis of these herbicides and in the study of their metabolic degradation.

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