Comparative Detection of Fluorinated Xenobiotics and Their Metabolites through ^{19}F NMR or ^{14}C Label in Plant Cells

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The metabolism of a new fluorinated fungicide [*N*-ethyl-*N*-methyl-4-(trifluoromethyl)-2-(3,4-dimethoxyphenyl)benzamide] by *Acer pseudoplatanus* cells was studied concurrently through the use of ^{19}F NMR and with ^{14}C-labeled compound. Approximately 10 fluorinated compounds were observed by ^{19}F NMR in the culture; 5 of them were identified. The detection limit was <1 μM. Methanol appeared to be a useful solvent as it was able to dissolve the lipophilic active ingredient as well as the more polar derivatives. However, the use of chloroform allowed a better separation of the NMR signals for the derivatives fairly soluble in this solvent. The metabolism of the studied compound mostly occurred through ring demethoxylation and N-dealkylation. The more lipophilic derivatives diffused into the culture medium; in contrast, the more hydrophilic ones remained inside the cells. This work shows that the sensitivity and high resolution power of liquid ^{19}F NMR spectroscopy confirm its potential usefulness to study the metabolism of xenobiotics.

Keywords: *Acer pseudoplatanus L.; ^{19}F NMR; fungicide; metabolization; plant cells*

INTRODUCTION

Detailed studies concerning pesticide metabolism by plants are required to ensure that the legally authorized substances cannot accumulate in crops and that formed metabolites are devoid of toxic or ecotoxic activity. These studies are generally carried out with the use of ^{14}C-labeled products, the metabolites of which need to be isolated from the plant extracts, following very complex and time-consuming procedures.

A great number of pesticides, herbicides, fungicides, and insecticides are fluorinated. In this case, metabolism studies in plants are in theory possible with the use of ^{19}F NMR. This is a relatively sensitive method with a large chemical shift range which, in principle, allows an identification of fluorinated metabolites inside highly complex mixtures without sophisticated cleanup of the extracts, since generally there is no background of natural fluorinated metabolites. ^{19}F NMR has been largely used to follow the metabolic fate of fluorinated drugs and their spatial distribution in animals and humans by *in vivo* measurements and analysis of body fluids (Malet-Martino and Martino, 1989, 1992). More recently, this method has been used in humans for the detection of flurbiprofen and its metabolites in urine (Wade et al., 1990), of antidepressors and antipsychotic agents (Karson et al., 1993; Komoroski, 1994), and also of 5-fluorouracil and its metabolites in tumors (Presant et al., 1994). It has also been used for metabolism studies of fluorinated drugs in animals (Hashimoto et al., 1991; Gilbert et al., 1992) or plants (Parisot et al., 1991).

In food and agricultural sciences, ^{19}F NMR was used for the detection of fluorinated pesticide residues in food (Mazzola et al., 1984; Mortimer and Dawson, 1991; Mortimer et al., 1994) or fluorinated pesticide photodecomposition products in water (Mabury and Crosby, 1995). However, detailed ^{19}F NMR studies of fluorinated pesticide metabolism in plant material have not been carried out to date. The purpose of the present work was to study the metabolism of a fluorinated fungicide by plant cells, with the use of either ^{14}C-labeled compound and radioactivity detection or ^{19}F NMR to establish the advantages and the limitations of the latter method.

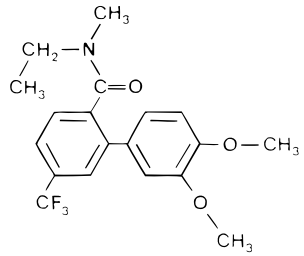

Figure 1. Structure of the *N*-ethyl-*N*-methyl-4-(trifluoromethyl)-2-(3,4-dimethoxyphenyl)benzamide.

MATERIALS AND METHODS

Chemicals. Rhône-Poulenc Agro (Lyon, France) synthesized the fungicide active ingredient (AI) *N*-ethyl-*N*-methyl-4-(trifluoromethyl)-2-(3,4-dimethoxyphenyl)benzamide (Figure 1) and several compounds of the same chemical family, the N-demethylated derivative (*N*-deCH_3), the N-deethylated one (*N*-deC_2H_5), the N-unsubstituted amide (*N*-didealkyl), the unsubstituted acid (free acid), and a monodemethoxylated derivative (3-mono-deOCH_3). The AI (100 mM) was first dissolved in dimethyl sulfoxide (DMSO), and cells were treated with AI at 50 μM, a nonphytotoxic concentration. The labeled fungicide (^{14}C label on the two phenyl rings and on CF_3; specific activity, 680.8 GBq/mol; radiochemical purity, 99.8%) was a gift from Rhône-Poulenc Agro.

Cell Suspension Cultures. *Acer pseudoplatanus* L. cells were cultured under sterile conditions in Erlenmeyer flasks on a gyratory shaker (80 rpm), at 25 °C, under continuous light with a photon fluence rate of 50 μmol m^{-2} s^{-1}. The nutrient medium was prepared according to the method described by

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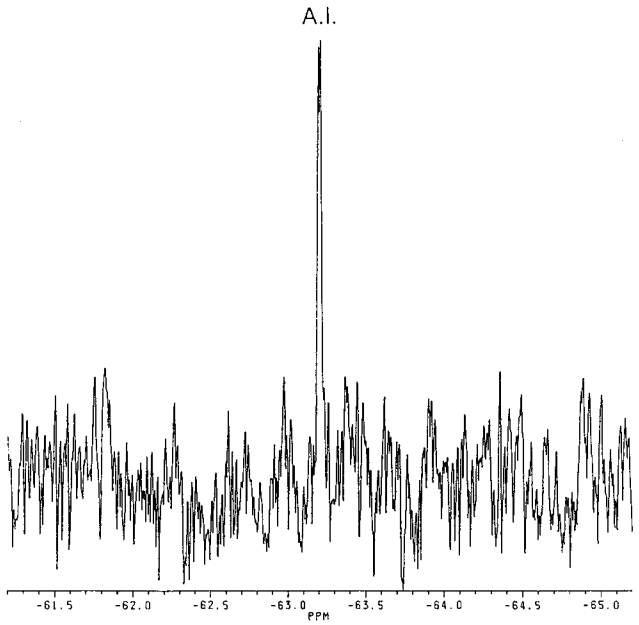

Figure 2. ^{19}F NMR spectrum of the AI at 5 μM. Solvent was $CHCl_3$.

Lescure (1966) and was a modification of Lamport's medium (Lamport, 1964). The AI was added at the start of the culture. Six days later, the cells and the medium were separately extracted by acetone.

NMR Spectroscopy. ^{19}F NMR spectra were recorded at 376.5 MHz with a Bruker AM400 spectrometer equipped with a 5 mm ^{19}F/^{1}H probehead. The sample volume was always 0.7 mL. About 30% of the solvent, chloroform or methanol, was deuteriated to provide a field lock signal. $CFCl_3$ was added as a chemical shift reference (0.00 ppm) and as a concentration reference at 10.8 (0.1% v/v) and 1 mM. The probe temperature was maintained at 25 °C. The ^{19}F 90° pulse length was 11.7 μs. Two different tube rotation frequencies (10 and 40 Hz) were used to control for spinning side-band artifacts.

RESULTS

(A) ^{19}F NMR Spectroscopy: Perfecting the Technique. *Sensitivity.* NMR is a relatively insensitive technique. So, first, the detection limits allowing a study comparable to ^{14}C radioactive labeling had to be evaluated. Among NMR sensitive nuclei, ^{19}F is well placed since it has a 100% natural abundance and a rather high gyromagnetic ratio (94% of the proton, ^{1}H). In proton NMR of a complex mixture, as a crude cell extract, the electronic noise is easily eclipsed by "chemical noise" and by dynamic range problems from the many proton-containing constituents. One has therefore to use sensitivity-losing multidimensional and/or selective excitation methods. These problems do not arise in ^{19}F NMR, since normally there are no naturally occurring fluorinated metabolites.

We tested sensitivity on our spectrometer with the AI. With a 5 μM solution of the trifluorinated substance, we obtained the spectrum shown in Figure 2 with a signal-to-noise ratio (S/N defined as signal peak amplitude divided by rms noise amplitude) of 10.5 and a line width (LW) of 3 Hz doubled by an exponential window function. A total of 3600 scans were recorded in 4.0 h. The repetition time was 4.0 s (about $3T_1$) and the pulse angle 90°. These parameters allow a reliable quantification. However, the S/N may be enhanced (here by a factor 1.26) by using a shorter repetition time (RT) of about $3/\pi$LW (here 0.3 s) and the corresponding Ernst pulse angle $\alpha = \arccos[\exp(-RT/T_1)]$ (here 36°) (Ernst and Anderson, 1966).

Table 1. ^{19}F NMR Characteristics of the Active Ingredient and of the Parent Compounds Dissolved in Chloroform or Methanol

	chloroform			methanol		
	δ^a			δ^a		
compound	alone	mixed	$T_1{}^a$ (s)	alone	mixed	$T_1{}^a$ (s)
AI (double peak)	−63.205	−63.204	1.42	−62.494	−62.482	1.23
	−63.217	−63.214	1.41	−62.503	−62.491	1.26
3-mono-deOCH$_3$	−63.247	−63.253	1.43	−62.528	−62.519	1.21
N-deCH$_3$	−63.298	−63.312	1.45	−62.521	−62.511	1.19
N-deC$_2$H$_5$	−63.311	−63.329	1.47	−62.544	−62.536	1.23
N-didealkyl	−63.370	−63.390	1.46	−62.544		
free acid	−63.449	−63.467	1.24	−62.633	−62.595	1.20

^{a} δ values are given ± 0.001 ppm; T_1 values are given ± 0.01 s.

Sensitivity is proportional to the concentration, the number of equivalent nuclei, the square root of the number of scans (or experimental time), the square of the magnetic field, and the inverse line width. S/N = 4 gives 99.99% probability of the presence of a real signal. It is obtained in 22 min with the 5 μM solution. Each 2-fold dilution necessitates a quadrupling of the experimental time. This means that a concentration of 0.2 μM of the trifluorinated compound (0.14 nmol) is still detectable in 24 h. This corresponds to 0.6 μM of a monofluorinated molecule. This result is comparable to recently published data (Mortimer et al., 1994).

Peak Separation. The second criterion on the utility of ^{19}F NMR as an analytical method is its ability to separate the peaks stemming from the different metabolites. In principle, ^{19}F chemical shifts are very sensitive to the fluorine's chemical environment. This results in a chemical shift range of approximately 500 ppm. However, the CF_3's used here are an exception since they resonate in a narrow 10 ppm range. Even this was experimentally sufficient; the ^{19}F chemical shifts were able to monitor chemical changes taking place far away from the CF_3 site. The exact peak positions and their separation depend on the solvent (Table I).

For a mixture of the AI and five of its metabolites we found a separation of at least 0.013 ppm (=4.9 Hz) in $CHCl_3$ (Figure 3). This is greatly superior to the obtainable spectral resolution, which is better than 1 Hz.

For the same mixture in CH_3OH, the chemical shift range was notably reduced. Therefore, two peaks (*N*-deC$_2$H$_5$ and *N*-didealkyl) had the same frequency and two other ones (*N*-deCH$_3$ and mono-deOCH$_3$) were only separated by 0.007 ppm (=2.7 Hz). Overlapping in a mixed solvent chloroform/methanol (1:1 v/v) was even stronger. So, it might be necessary to experiment with different solvents to find an optimal peak separation inside the solubility limits.

Signal Identification. The simplest way of attributing the signal peaks to different molecules is based on the chemical shifts. Since the exploited differences are small, one needs a sure internal reference. We used $CFCl_3$, which is well soluble in the solvents used and which is the standard zero for the chemical shift scale, but another molecule with a known stable chemical shift would do as well. The chemical shifts obtained are compared to the ones coming from pure solutions of the synthetic metabolites. These standards have to be established separately for each solvent since the chemical shift changes due to interactions with the solvent. Here we have a global 0.7 ppm shift between $CHCl_3$ and CH_3OH as well as specific effects depending on the molecular polarity. The latter one changes even the

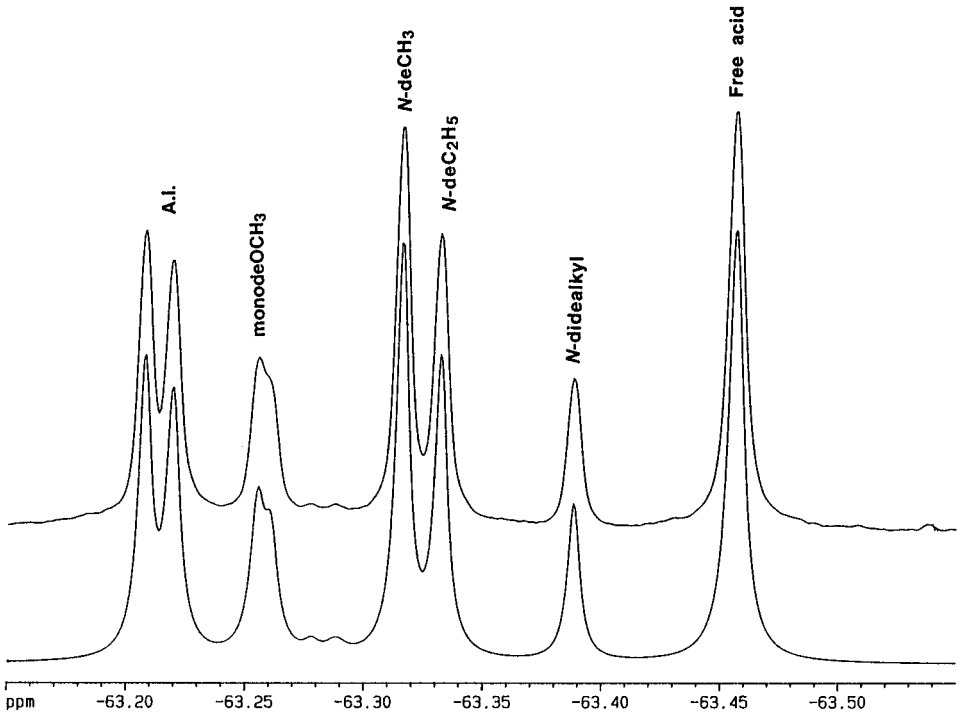

Figure 3. ^{19}F NMR spectrum of the AI and related compounds, mixed at different concentrations in $CHCl_3$. The upper trace shows the ^{19}F NMR spectrum of a mixture of the AI and five of its metabolites at different concentrations. The lower trace shows the calculated deconvolution of the spectrum. The concentrations of the different compounds were calculated from the deconvolution data with $CFCl_3$ (1 mM) as a reference. From left to right, the results are as follows: AI, 2.57 mM calculated/2.50 mM actual concentration; 3-mono-deOCH$_3$, 1.25/1.25 mM; *N*-deCH$_3$, 1.84/1.88 mM; *N*-deC$_2$H$_5$, 1.31/1.25 mM; *N*-didealkyl, 0.68/0.62 mM; free acid, 2.33/2.50 mM.

order of the peaks of *N*-deCH$_3$ and mono-deOCH$_3$. To ascertain an identification, it might be advisable to add the authentic product to the extract. The AI appears as a doublet, since it exists in two conformations due to a hindered rotation around the C−N bond. *N*-Desubstitution lowers the rotation barrier and therefore gives singlet peaks. The two conformations of 3-mono-deOCH$_3$ give a chemical shift difference about half that of the AI.

If there are $^{19}F-^{1}H$ scalar couplings of at least some hertz, a direct structure determination by multidimensional heteronuclear correlation NMR is conceivable for concentrations of at least 10 μM. Alternatively, $^{19}F-^{13}C$ correlation at natural abundance is feasable upward from a concentration of 1 mM.

Quantification. Quantification by NMR uses the fact that the signal integral is proportional to the molecular concentration. This was verified with the AI at concentrations between 0.01 and 10 mM in $CDCl_3$ with $CFCl_3$ at 1 mM as internal standard. It was found that this quantification gave routinely a precision of ±10%. If the peaks are well separated, their surface may be determined directly. If not, one has to pass by a deconvolution procedure which iteratively models the spectrum. To compare between the signal surfaces, the intensities have to be corrected for T_1 effects if RT < $5T_1$. T_1 has to be measured and the intensities have to be multiplied by $[1 - \cos(\alpha)E]/(1 - E)$ with $E = \exp(-RT/T_1)$. To obtain absolute concentrations, the integrals have to be calibrated by an internal reference signal. Herefore, it is convenient to use the chemical shift reference.

T_1 constants were measured with a fast inversion−recovery sequence (Canet et al., 1975) using a composite 180° pulse $[(90°)_x(240°)_y(90°)_x]$ (Levitt, 1984). The results are in Table 1. The relaxation times change with the solvent, but within a solvent they are remarkably stable. Thus, relative peak intensities are directly comparable without T_1 correction. The shorter T_1 values in CH_3OH seem to indicate a slightly slower overall tumbling of the molecule, which might be due to polar interactions. The only notable exception to this uniform relaxation behavior is the free acid's resonance in $CHCl_3$, which relaxes 15% more quickly than the other ones.

As a test for quantification, the spectrum shown in Figure 3 has been deconvoluted using XWIN NMR1-1 software. Since the fluorine in the different derivatives has the same T_1 (Table 1), the areas of the separated peaks are directly proportional to the number of spins. The proportionality between these integrals and the relative concentrations has been verified with a maximum divergence of 10%.

(B) The Extracts: Analytical Results. *Metabolism of ^{14}C-Labeled AI by an Acer Cell Suspension Culture.* The cells were cultivated for 6 days in the presence of 50 μM AI (this concentration being not phytotoxic). At that time, the acetonic extracts contained 96% of the radioactivity. Metabolites and the AI were shown to be present as well in the medium as in the cells. The hydroacetonic solutions were then fractionated with hexane, and the residual hydroacetonic solutions were partially evaporated, resulting in a water solution that was successively fractionated with diethyl ether and 1-butanol as shown in Figure 4. The radioactive content of the different fractions is shown in Table 2.

By thin layer chromatographic (TLC) analysis, it was shown that the AI was mostly present in the hexane fraction (Figure 5). Some metabolites were shown to be present in each fraction. The AI remained at a high level in the medium but was mixed with the more lipophilic derivatives which, surprisingly, were in far greater amounts in the medium than in cells.

Three lipophilic derivatives were present in high concentrations in the diethyl ether fraction coming from the medium (Figure 5) and were studied further.

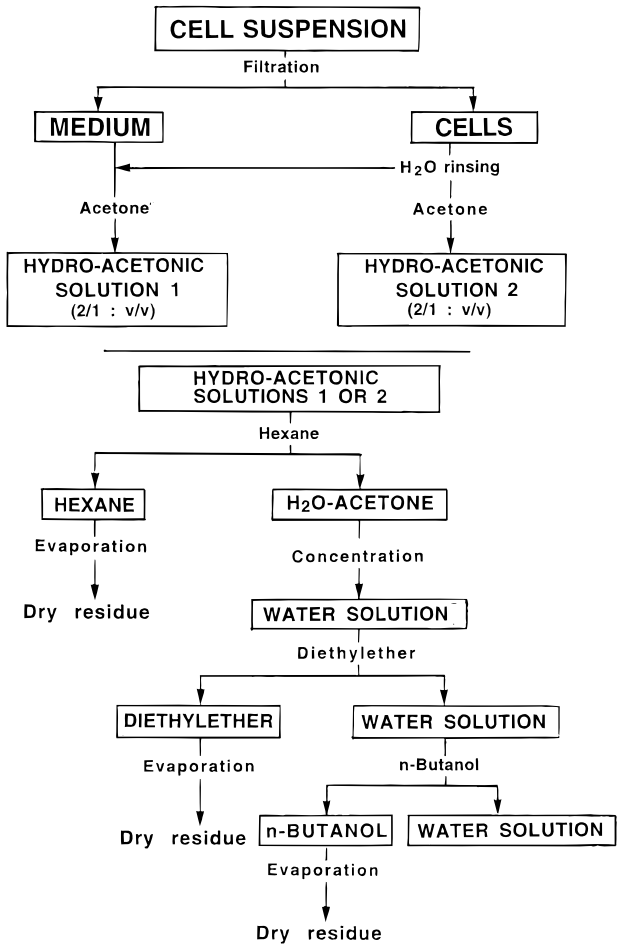

Figure 4. Extraction and separation of labeled compounds present either in the *Acer* cells or in the nutrient medium.

Table 2. ^{14}C Active Ingredient or Metabolites Contents (Percent) of the Different Fractions Obtained from an *Acer* Cell Suspension Culture Treated for 6 Days

	hexane	diethyl ether	1-butanol	water
medium, 94%	64.1	23.0	5.5	1.4
cells, 6%	0.5	0.9	4.0	0.6

The analysis by autoradiography of the diethyl ether and butanol fractions shows that, as a whole, more than 10 radioactive derivatives could be detected (Figure 5B). The derivatives contained in the residual water fraction were not further studied. The UV profile shows that AI and its metabolites represented an important part of the organic UV-absorbing compounds present in the medium in contrast with the composition of cell extracts, in which a number of natural molecules absorbing the UV light were present (Figure 5).

A comparison between the compounds of the diethyl ether fraction of the medium and the test substances was carried out in a two-dimensional system using toluene/methanol (4:1 v/v) and 100% ethyl acetate as solvents. There is a high probability that the three metabolites separated from the diethyl ether fraction of the medium were, respectively, the monodemethoxylated, N-deethylated, and N-demethylated derivatives (Figure 6). The structure of the compounds supposed to be the N-demethylated and N-deethylated derivatives isolated from the medium and purified by TLC was confirmed by mass spectrometry as shown by Figure 7. With the AI (mass = 367) and with the two studied compounds (mass = 353 and 339, respectively), fragmentation led to derivatives without $-NHC_2H_5$ or $-NHCH_3$ (mass = 309), which afterward were demethoxylated (mass = 278).

TLC and autoradiography spotted several ^{14}C-labeled compounds in the butanol fractions. However, as shown by Table 2, the amount of each of these derivatives was low. Therefore, they were only submitted to a partial analysis through R_f comparison with test substances on bidimensional TLC using toluene/methanol (4:1 v/v) and then ethyl acetate as solvents. In this system two of these derivatives had the same R_f as the N-unsubstituted derivative and the free acid, respectively.

^{19}F NMR Study of the Fractions Obtained from Acer Cells and from the Medium. The extracts and fractions analyzed by TLC and autoradiography were submitted to ^{19}F NMR analysis after evaporation to dryness and dissolution in $CHCl_3$ for the more lipophilic components and in CH_3OH for the remaining ones.

Figure 8 shows the ^{19}F NMR spectra of the hexane and diethyl ether fractions originating from the medium. In the first fraction, the AI showing its typical doublet was clearly identified. A small amount of another fluorinated compound was also present, with a chemical shift similar to that of the N-deethylated derivative. These results agree with the identifications made by two-dimensional TLC. The diethyl ether fraction gave a ^{19}F NMR spectrum containing the typical peaks of the AI, of the monodemethoxylated derivatives (two peaks might represent the 3- and 4-demethoxylated compounds), of the N-demethylated, and of the N-deethylated ones. The identification of the N-demethylated derivative was verified by the addition of the pure compound to the studied extract (Figure 9). The same verification was done for the N-deethylated derivative (result not shown). These results also agree with the composition of the diethylated fraction shown by autoradiography of the bidimensional TLC in the presence of the reference compounds (Figure 6).

The fluorinated compounds present in the butanol extracts were not soluble enough in pure $CHCl_3$. They were therefore dissolved in CH_3OH. In this solvent, the chemical shifts of the reference substances were different (Table 1). The autoradiograms of several fractions separated from the TLC of the butanol extracts show the presence of relatively high amounts of five new metabolites. In these fractions, the NMR study also shows unambiguously the presence of several new compounds. Further studies using higher amounts of these compounds would be necessary to establish their structures.

DISCUSSION

The structural modifications due to metabolization of the AI take place far away from the observed nucleus within the molecule. Nevertheless, the fluorine chemical shift is sufficiently sensitive to detect these changes. This proves that the ^{19}F NMR chemical shift is a valuable parameter for *in vitro* identification of molecules produced by the metabolization of fluorinated xenobiotics in plant cells.

^{19}F NMR may therefore become a useful tool for detecting fluorinated compounds in complex plant extracts without difficult and time-consuming purifications. In the extracted plant material, as expected, naturally elaborated fluorinated compounds are absent. This facilitates the detection of exogenously applied fluorinated compounds, which might be either pollutants or pesticides. The method is thus especially interesting when labeled compounds cannot be used.

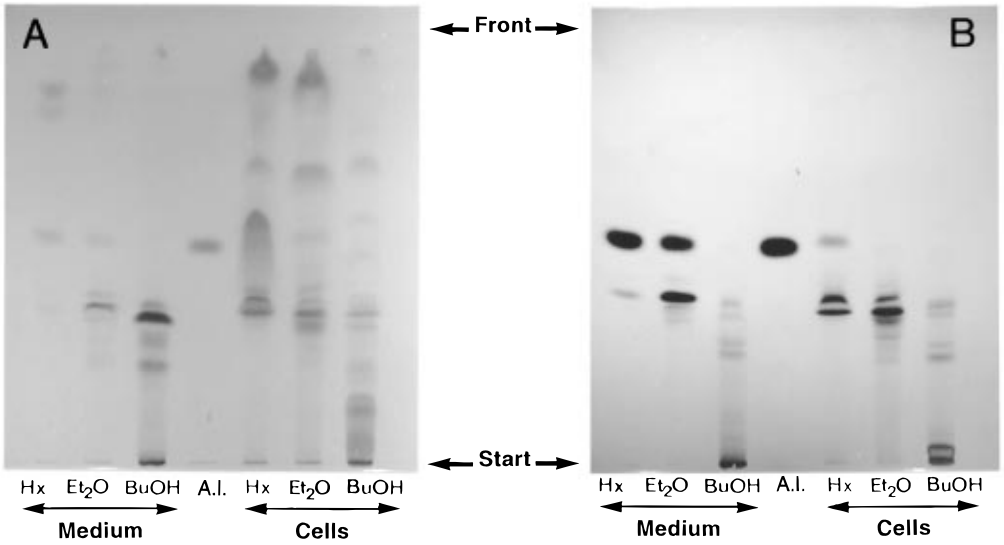

Figure 5. TLC and autoradiogram of the fractions obtained from the cells and from the medium 6 days after treatment with the AI at 50 μM: (A) thin layer chromatography (λ = 254 nm; silica Merck 60F254; solvent, toluene/methanol (4:1 v/v); (B) autoradiogram. AI, pure active ingredient; Hx, extracts from hexane; Et_2O, extracts from diethyl ether; BuOH, extracts from 1-butanol.

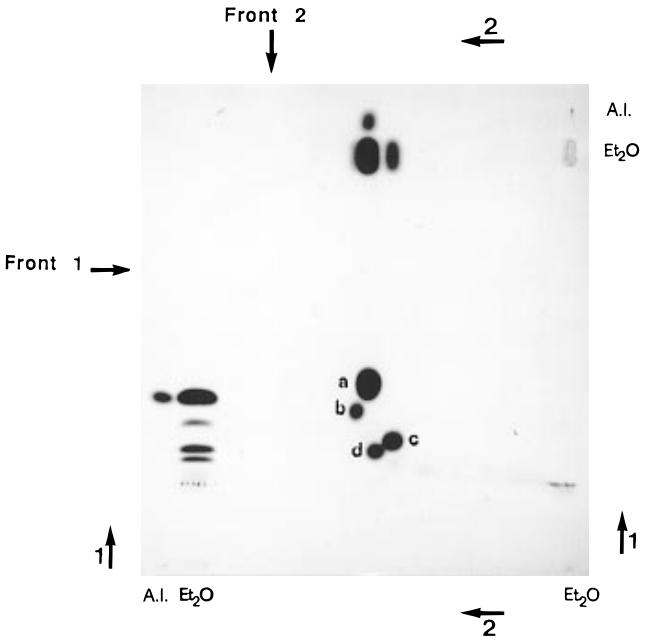

Figure 6. Two-dimensional TLC autoradiogram of the diethyl ether fraction originating from the medium: (a) pure active ingredient; (b) *N*-deCH_3; (c) 3-mono-deOCH_3; (d) *N*-deC_2H_5. Solvent 1, toluene/methanol (4:1 v/v); solvent 2, ethyl acetate.

This is the case in experiments carried out in the field and in nature, where the use of radioactive products is strictly regulated.

For an effective ^{19}F NMR detection, the practical sensitivity limit is between 1 and 5 μM in $CHCl_3$. Such a low concentration suggests that direct NMR analysis of the treated material could be obtained for agrochemicals containing fluorine, as the known theoretical concentrations giving the desired biological effect with several fluorinated pesticides are higher than this value. This is the case of the fungicide studied here but would also be true for herbicides such as trifluralin (Worthing, 1987). Thus, in this study, the presence of the AI and of the first metabolites was rather easily demonstrated by ^{19}F NMR. For further transformed derivatives, the amount of which decreased in the plant material, the analysis became more difficult. Such was the case of the butanolic fractions. However, their ^{19}F NMR spectra showed separated peaks with good signal to noise ratios.

The specificity of the chemical shifts obtained here for several derivatives in $CHCl_3$ or CH_3OH suggests that ^{19}F NMR may frequently give a direct identification of them in the plant material. For studying polar derivatives, the use of $CHCl_3$ is impossible and an appropriate solvent might be CH_3OH, although it was shown with some of the metabolites studied here that overlapping peaks were present.

All things considered, ^{19}F NMR seems to be able to play the role of a sensitive multiresidue analysis method, for fluorinated compounds, which could efficiently complete the GC/HPLC−mass spectrometry analysis. It might therefore rapidly become a tool for legal controls in the case of agrochemicals, in addition to its role in the field of biochemical analysis.

The study realized here brings to light several interesting physiological points. The metabolism rate of the studied fungicide by *Acer* cell suspension cultures is high: it reaches 60 nmol per day and gram of fresh weight. The first steps of metabolism concern N-desubstitution and ring demethoxylation, as shown by two facts: (a) The corresponding derivatives appeared first in the medium and in the cells (result not shown). (b) These derivatives accumulated in especially high amounts in the medium.

The combination of these two transformation processes (i.e. ring demethoxylation and N-dealkylation) leads to the possible formation of 15 derivatives. The further transformation of the lateral chain giving the free acid corresponds theoretically to 4 supplementary possible compounds. The existence of this free acid is likely since a compound having the same R_f in TLC and the same chemical shift in ^{19}F NMR as the reference acid was detected in the butanol extract.

We verified that the metabolites shown here resulted from an intracellular enzymatic process, since dead cells, or a medium from which the cells were discarded after 4 days of culture, were unable to degrade the AI. In these experimental conditions, the AI remained intact for several days and was fully extracted by hexane from the hydroacetonic solution. The fact that the metabolite composition of the medium and that of the cells presented substantial differences demonstrates

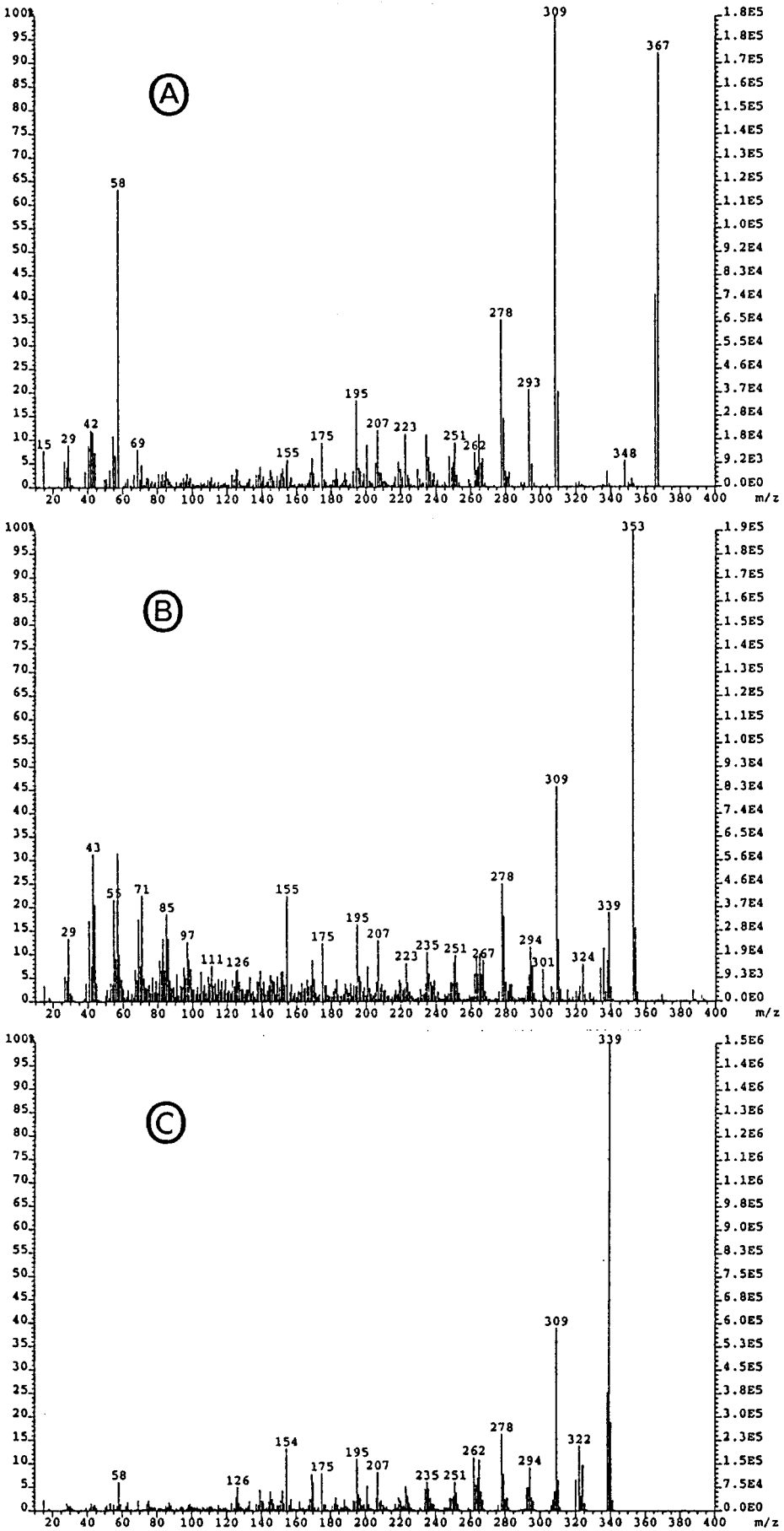

Figure 7. Mass spectra of the AI (A) and of the N-demethylated (B) and N-deethylated (C) derivatives extracted from the medium and purified by TLC.

that a selective excretion process occurred from the cells to the medium. The more lipophilic compounds were obtained in the greater amount from the medium, not from the cells. This result might be explained by a

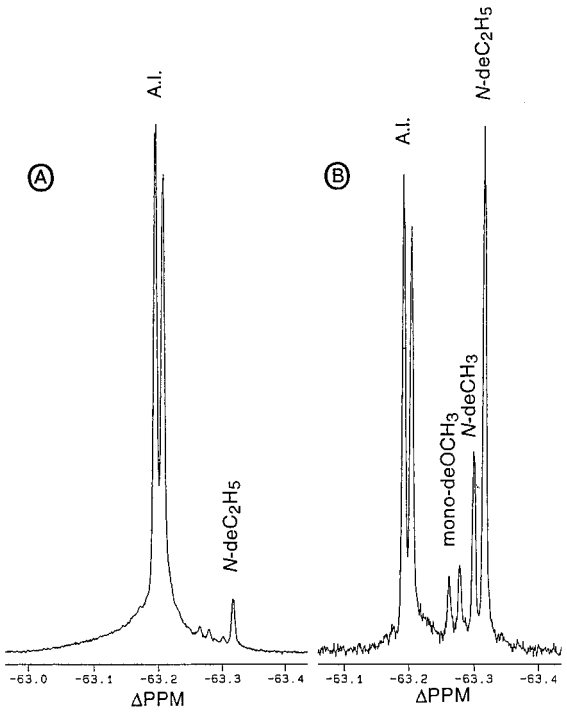

Figure 8. ^{19}F NMR spectra of the hexane (A) and Et_2O (B) fractions originating from the medium.

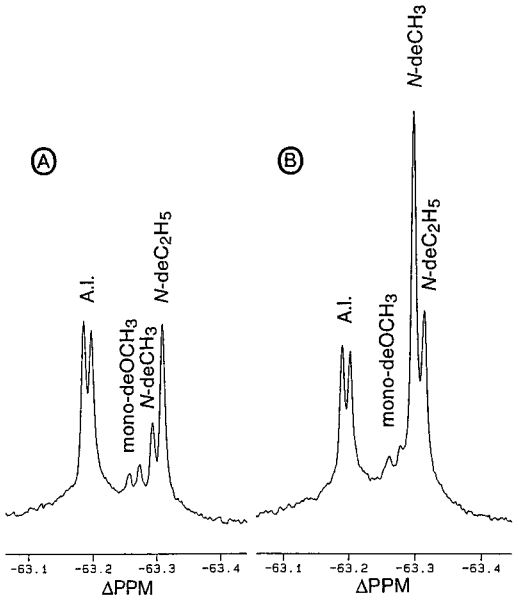

Figure 9. ^{19}F NMR spectra of the Et_2O fraction originating from the medium: (A) studied extract; (B) studied extract with addition of pure *N*-deCH$_3$.

simple diffusion/partition phenomenon under conditions in which the lipophilic phase represented by biological membranes is very small as compared to the hydrophilic phase (medium plus cell water). In contrast, the high content of hydrophilic derivatives inside the cell cannot be understood without supposing a biological segregation of these compounds preventing them from diffusing passively into the medium.

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