# Extractable and Nonextractable (Bound) Residues of Acifluorfen in an Organic Soil

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Acifluorfen (AC), 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid, was applied to an organic soil at 220 mg $kg^{-1}$ in a laboratory incubation study. A conventional extraction method with solvents and supercritical fluid extraction (SFE) were used to evaluate extractable and nonextractable (bound) residues of AC and its metabolites in the soil. Over an incubation period of 7 months, the soil contained 22% AC of the initially applied herbicide and the residues were almost completely extracted with aqueous methanol. Only 0.8% AC was found in the bound form. Seventy-five percent AC applied was degraded to aminoacifluorfen (AAC) (due to reduction of nitro group to amino group) of which 60% was extracted by aqueous methanol, and 15% was in the bound form, mainly associated with the humin fraction of the soil organic matter. No further transformation of AAC to other metabolites was observed.

**Keywords:** *Acifluorfen; bound residues; soil; SFE*

## INTRODUCTION

Acifluorfen (AC), 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid, is a highly effective herbicide, used in the selective control of broad-leaf weeds in soybeans, peanuts and rice (Johnson et al., 1978; Wills and McWhorter, 1981). AC is moderately soluble in water (120 mg $L^{-1}$ at 25 °C) and contains a carboxyl group with $pK_a = 3.5$ (Roy et al., 1983).

Soil adsorption of AC was investigated by Pusino et al. (1993) and Gennari et al. (1994a). In both studies, the adsorption was described by Freundlich isotherms and the adsorption capacity, expressed as Freundlich coefficient ($K_f$), was highly dependent on the soil type. $K_f$ values were positively correlated with cation-exchange capacity and decreased with increasing pH (Pusino et al., 1993). Moreover, Gennari et al. (1994a) observed that the $K_f$ values were positively correlated with the organic carbon content and the hydrogen ion concentration while no significant correlation was found between $K_f$ values and the clay content. The role of organic matter was further substantiated by the decrease of the $K_f$ value when organic matter was partly removed by $H_2O_2$ oxidation (Gennari et al., 1994a). Studies of AC interaction with humic acids (Ruggiero et al., 1992; Celi et al., 1996) showed high affinity between the humic acids and AC at pH lower than the $pK_a$ of the herbicide.

Andreoni et al. (1994) and Gennari et al. (1994b) examined the degradation of AC by different microbial cultures. They observed the transformation of AC to the amino derivative aminoacifluorfen (5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-aminobenzoic acid) (AAC). The degradation occurred in both aerobic and anaerobic conditions, but was higher in the limited-oxygen systems either in the medium with AC as sole source of carbon or through a cometabolic process. Transformation of AAC to 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-aminobenzamide and 5-[2-chloro-4-(trifluoromethyl)-phenoxy]-2-acetyl-aminobenzoic acid has been observed only in anaerobic conditions in the presence of 2-nitrobenzoate (Gennari et al., 1994b).

U.S. EPA (1989), reported the detection of denitroacifluorfen, AAC, and the acetamide of AAC in soil after 2 months of incubation under anaerobic conditions.

Gennari and Nègre (1990) examined AC degradation in soils of different physico-chemical characteristics. Conventional methods to extract AC from the soils using organic solvents were used (Gennari et al., 1990). The extraction was found to be highly dependent on soil type. Thus, 112 days after the herbicide application, the amount of AC extracted from the soils varied from 40 to 70% and was lower in the soils with high organic matter content. However, no attempt was made at that time to determine whether the AC loss was due to degradation, or due to bound residue formation which cannot be extracted with conventional solvent methods. Soil-bound residue has been defined as "that unextractable (...) pesticide residue remaining in fulvic acids, humic acids and humin fractions after exhaustive sequential extraction with non-polar organic and polar solvents" (*Fed. Regist.*, 1975). Special attention should be given to this form of residues in assessing the disappearance of pesticides in the soils. Though bound residues are not detected in routine analysis they can affect the bioavailability, persistence, and mobility of the herbicide in the soil.

The objective of this study was to evaluate the extractable and nonextractable (bound) residues of AC and its metabolites in an organic soil incubated for 7 months after the herbicide treatment. The distribution of bound AC and its metabolites in the organic matter fractions was also examined.

## MATERIALS AND METHODS

**Soil.** The experiment was conducted on a humic Mesisol soil obtained from the S.te Clotilde Experimental farm at the

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Agriculture Canada Research Station at St. Jean, P.Q. The soil has 45.5% organic carbon, 15.1% mineral matter (3.3% clay, 6.1% loam, 5.7% sand), a bulk density of 0.34, and a pH of 5.2.

**Chemicals.** All solvents were of pesticide grade and used as received. AC standard (97% purity) was supplied by Dr Ehrenstorfer (Augsburg, Germany). AAC was obtained by anaerobic degradation of AC by microbial cultures (Gennari et al., 1994b). The metabolite was isolated from the culture and identified by GC-MS. Stock solutions of AC (1000 mg $L^{-1}$) and AAC (10 mg $L^{-1}$) were prepared in methanol. For GC analyses, AC and AAC standards were methylated. AC and AAC solutions (1 $\mu$g $mL^{-1}$) were placed into two vials and an excess of diazomethane was added until the yellow color persisted. Diazomethane is a toxic, explosive, and unstable compound. Thus, it was prepared with special cautions each time it was needed by following the classical method described in literature (Vogel, 1988). The vials were closed and allowed to stand for 30 min. The excess of diazomethane and methanol was removed by evaporation under a gentle flow of air and the residues were dissolved in methanol, quantitatively transferred to volumetric flasks, and then analyzed by GC.

**Laboratory Experiment.** Moist soil (372 g, 200 g oven dry weight basis) was placed in one Erlenmeyer flask (2 L) to which 44 mL of methanol containing 44 mg of AC was added to give a herbicide concentration of 220 $\mu$g/g. The solvent was evaporated under air and the soil was throughly mixed. Water (238 mL) was also added to obtain a moisture concentration of 205%, corresponding to the soil field capacity. The flask was loosely stopped with cotton wool and incubated at about 23 °C in the dark. Distilled water was added as necessary to maintain the initial moisture content of the soil sample. The soil was incubated for 7 months. A control sample containing 50 g of moist soil without AC was also prepared.

**Determination of Extractable Residues.** Three portions of treated soil (25 g oven dry weight basis) were transferred from the flask to three 300 mL Erlenmeyer flasks and extracted with 100 mL of aqueous methanol (methanol:water, 80:20). The samples were shaken for 2 h and then filtered on a Whatman filter paper (No. 1) under suction. The extraction was repeated two more times and the samples filtered under suction into the same flask. The samples were then washed with aqueous methanol (3 × 75 mL) and the combined filtrate was concentrated on a rotary evaporator. Further extraction of treated soil with aqueous methanol did not remove any AC or its metabolites. Thus, the procedure employed in this study was considered to be effective in removing all the extractable residues from the soil. Extraction of AC and its metabolites from the soil with other solvent systems such as ethanol (recovery 75%), methanol-HCl 1 N (9:1) (recovery 70%), did not increase the extraction efficiency.

AC and its metabolites were then extracted from the filtrates with dichloromethane (50 mL × 3) followed by ether (50 mL × 3). The aqueous phases were successively acidified to pH 2 with 1 N HCl and extracted again with dichloromethane (50 mL × 3) followed by ether (50 mL × 3). The organic phases were collected together, evaporated to dryness, and redissolved in methanol 25 mL. The solutions were analyzed by HPLC and GC. For GC analysis, 1 mL of each solution was transferred to a vial and then methylated as described above.

The extracted soil samples were allowed to air-dry and were stored for the determination of the bound residues. An aliquot of each of these samples (1 g) was subjected to supercritical fluid extraction (SFE). The remaining soil was used for determining the distribution of nonextractable residues in the organic matter fractions.

**Fractionation of Soil.** The remaining soil previously extracted with aqueous methanol was then treated with a conventional procedure of separation of humic acids, fulvic acids, and humin. The method, outlined in Figure 1, is analogous to that described by Schnitzer (1982). Briefly, the humic acid (HA) and fulvic acid (FA) were extracted from the previously extracted soil with 0.1 N NaOH under $N_2$. The solution was separated from the residue by centrifugation. The HA was precipitated with 6 N HCl, purified by dialyzing against distilled water for 3 days, then freeze-dried. The FA solution was kept as such for the herbicide residue extraction. The residue, containing humin (base insoluble) and the mineral fraction, was washed with distilled water, purified following the procedure for the HA, then freeze-dried. The residue was not washed with HCl and HF to remove the mineral fraction because the content of this fraction was low (15.1%) and it was constituted mainly by sand (5.7%). Alkaline extraction of humic materials from the soil with 0.1 N NaOH under nitrogen and HA precipitation with HCl did not chemically degrade the pesticide residues in the soil. Preliminary experiments revealed quantitative recoveries of AC from aqueous solutions of the herbicide in the presence of NaOH (pH 12) or HCl (pH 1).

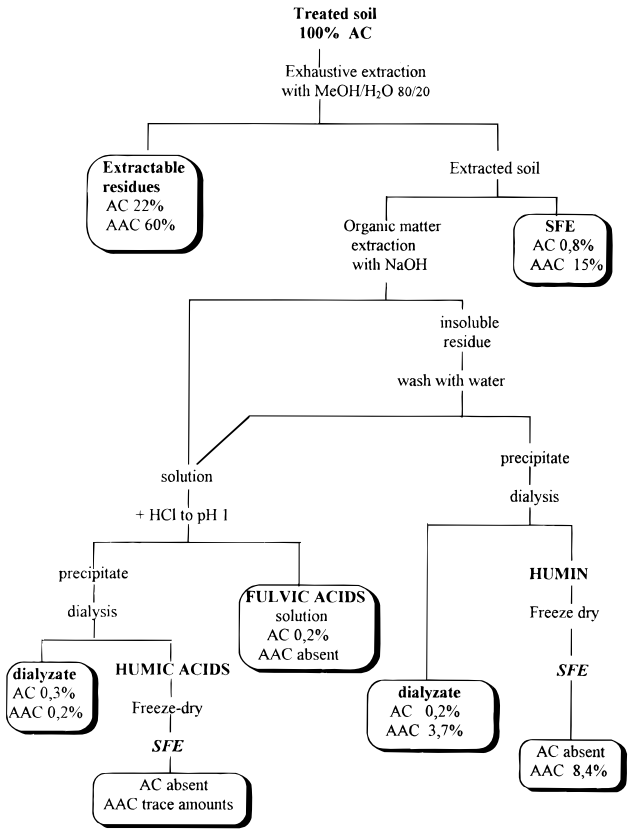


**Figure 1.** Extractable and nonextractable AC and AAC residues in the organic soil. All values were expressed as AC percentage, with average deviation <10%.

The yield of humin, HA, and FA amounted to 0.73, 0.086, and 0.009 g/g of soil, respectively. HA (0.5 g) and humin (0.5 g) were subjected to SFE as described below.

**Analysis of FA Solution and Dialysates.** AC and its metabolites were extracted from the FA solution, HA, and humin dialysates (water) by liquid/liquid separation as described for the extractable residues. The residue was dissolved in 5 mL methanol and then analyzed by HPLC. A portion of the sample was also methylated for GC analysis, by following the same method described for the standards.

**Supercritical Fluid Extraction.** The SFE system (Suprex Model SFE-50, Suprex Corp., Pittsburgh, PA) consisted of a 250 mL syringe pump, a control module for the SFE system, an extraction oven, a 5 mL extraction vessel containing the sample, and a four-part valve connected with outlet restriction (fused silica tubing, 50 $\mu$m i.d.) that was vented into the first of two glass tubes containing 30 mL of methanol. The two glass tubes containing methanol were connected in series for collection of the released material. Extraction was carried out with $CO_2$ using methanol (30% methanol in $CO_2$) which was delivered by a HPLC pump (Varian 2510). The flow rates for $CO_2$ and methanol were maintained at 1 mL/min and the extraction was carried out at 160 °C and 400 atm for 2 h after initial equilibration of the SFE system for 3 min at 160 °C and 150 atm.

**Analysis of Distillates.** The distillates were evaporated to dryness and the residues dissolved in acetonitrile (5 mL). The samples were subjected to cleanup (Gennari et al., 1990) before methylation and GC analysis in order to eliminate coextracts. HPLC analysis was performed without sample cleanup.

**HPLC Conditions.** The HPLC (Varian VISTA 5500) was equipped with a variable UV detector set at 296 nm and a 25 cm stainless steel tube column packed with octadecylsilane silicagel (Beckman C18 reverse phase). The column temperature was maintained at 25 °C. The mobile phase was an isocratic solution consisting of 65% $CH_3CN$ (v/v) and 35% $H_2O$ (v/v) acidified to pH 3 with $H_3PO_4$. The flow rate was maintained at 2 mL/min. The calibration curve was obtained by injecting AC standard solutions in the range 5−50 $\mu$g/mL. The detection limits for AC were found to be 1 $\mu$g/mL.

**Gas Chromatography Conditions.** The gas chromatograph was a Varian Model STAR 3600 CX fitted with an ECD detector. The column (15 m × 0.53 mm i.d.) was coated with DB1 (1.5 $\mu$m) as stationary phase. The oven temperature was programmed at 5 °C/min from 150 to 200 °C. The detector and injector temperatures were 300 and 150 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 30 mL/min. Aliquots of methylated reference standards (2 $\mu$L) were injected for the calibration in the range 0.1−10 $\mu$g/mL and 0.1−5 $\mu$g/mL for AC and AAC, respectively. The detection limits were found to be 0.01 and 0.1 $\mu$g/mL for AC and AAC, respectively.

**Performance of the Methods.** The recoveries of the herbicide residues by solvent extraction were determined by spiking the control soil with different concentrations of AC and AAC in the range 1−200 $\mu$g/g soil. SFE of control soil and humin (both spiked with AC and AAC 1−20 $\mu$g/g) and SFE of control HA (spiked with AC and AAC 1−5 $\mu$g/g) were also carried out.

The samples were processed 10 min after the spiking as described before. The analyses were carried out in duplicate and the average values were calculated.

## RESULTS AND DISCUSSION

**Performance of the Methods.** Residue levels in soils and humic materials are reported on an oven dry basis. The recoveries of extractable AC and AAC from the control soil ranged from 96 to 98%. The residue recoveries from SFE of control soil, humin, and HA resulted in 90−92% of AC and 88−90% of AAC.

**Identification of the Compounds.** The identities of the compounds in the extracts were confirmed by comparing the HPLC and GC retention times with those of reference standards and by cochromatography. Thus, under the HPLC conditions used in this study, the compound represented by the peak at the retention time of 3.5 min was identified as AC. No other peaks were detected by HPLC. Under the GC conditions used, the peaks with retention times at 8.6 and 9.0 min were identified as AAC and AC, respectively.

**Extractable and Nonextractable Residues.** Figure 1 shows the distribution of extractable and nonextractable residues of AC and its metabolites in the soil and in its organic matter fractions. All values were expressed as AC percentage, calculated by the mean of three replications, and showed an average deviation <10%. Over an incubation period of 7 months the percent recovery of extractable AC was 22%. The presence of extractable metabolites in the treated soil was checked by HPLC and GC. It was observed that the amino derivative (AAC) was the only detectable degradation product formed under the experimental conditions used in this study and constituted 60% of the initially applied herbicide.

The mass balance based on extractable AC and AAC did not reach 100% of applied herbicide. Since a portion of the herbicide could remain in the soil after extraction with aqueous methanol, SFE was employed in order to release the possible bound residues from the incubated soil. SFE has been used by a number of workers to extract bound residues of pesticides and/or metabolites from soils and other environmental matrices (Lopez-Avila et al., 1990; Snyder et al., 1992; Khan, 1995; Paquet et al., 1995).

In our investigation, analysis of soil extracted by SFE (Figure 1) showed that only 0.8% AC remained bound in the soil after extraction with aqueous methanol. Thus, the herbicide was almost completely extracted by polar solvents, indicating that the soil retained AC by physical forces or weak bonding, which could be cleaved easily by extraction with aqueous methanol. Similar conclusions were drawn in a previous study about the mechanisms involved in the AC interaction with HA (Celi, 1996). The herbicide appeared to be adsorbed on the HA by weak chemical bonding, probably of hydrophobic nature.

The soil contained 15% bound AAC of the total applied amount of AC as determined by SFE, indicating that the metabolite was retained from the soil by stronger forces than AC, probably because the amino group is more reactive than the nitro group.

The total recovery (extractable and bound AC + extractable and bound AAC) was 97.8%, confirming that AAC was the only degradation compound derived from AC and the amino derivative accumulated in the soil in our experimental conditions.

**Distribution of the Bound Residues in the Humic Constituents.** The distribution of AC was checked in the soil organic fractions and was as follows: 0.2% in the fulvic acid solution, 0.3% adsorbed on the humic acids, and 0.2% adsorbed on the humin. However AC was easily removed from the last two fractions by dialysis against water. This behavior may be explained with the opening of some coiled structures of the humic substances in which the herbicide could have been trapped, caused by the treatment with 0.1 N NaOH. The presence of AC in the dialysate may be due to the high desorption efficiency of this technique involving a low solid/water ratio, the daily water replacement, and the long contact time (3 days) (Jamet and Roche, 1990). Moreover, a progressive increase of the pH of the system during dialysis occurred with a promotion of the formation of the dissociated form of AC which has been seen to have more affinity to the water than to the humic surfaces (Celi et al., 1996). From these data it could be supposed that AC detected in the fulvic acid solution was not adsorbed on fulvic acids, but was in solution.

No residue of AC was extracted by SFE from the solid phases after dialysis. The total recovery of AC from the dialysates and the fulvic acid solution was 0.7% (88% of the bound residue).

AAC was not found in the fulvic acid solution; a small part was in the HA dialysate (0.2%), but the largest amount was in the humin distributed between the solid phase (8.4%) and the dialysate (3.7%). The total recovery of AAC was 8.6% (57% of the bound residue). The higher incorporation of bound residues in the humin is consistent with those reported for other xenobiotic compounds (Khan, 1982a; Khan and Hamilton, 1980; Capriel et al., 1985). Humin represents the largest humic fraction in the soil and can thus adsorb major amounts of herbicide.

Since a large amount of AAC remained in the humin fraction also after dialysis it could be supposed that the

compound could have been bound to the humin through two bond types: the first, similar to that of the adsorption of AC, easily broken by the treatment of the soil with NaOH; and another much stronger, which was broken only by SFE extraction.

**Conclusions.** Over an incubation period of 7 months in an organic soil treated with AC, the herbicide degradation was about 80% of the initially applied herbicide. AC dissipation in the organic soil was due to the reduction of nitro group to amino group with no further transformation of AAC to other metabolites. The extraction of AC by aqueous methanol recovered almost quantitatively the herbicide from the soil, indicating that the herbicide was retained by weak bonding and could be considered potentially bioavailable to both plants and fauna. Seventy-five percent AC applied was degraded to AAC, of which 60% was extracted with aqueous methanol, whereas 15% was present in the bound form mainly associated to humin.

These results are of environmental concern, as the transformation of AC to AAC was not a major detoxification step. Moreover, the observations that soil bound residues are adsorbed by plants (Khan, 1982b) and the data presented in this paper, should prompt further research to determine whether the accumulation of bound residue could affect sensitive crops grown or contribute to the contamination of the environment.

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