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Studies on Bound ^{14}C-Chlorsulfuron Residues in Soil

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The cause for phytotoxicity of bound residues of chlorsulfuron (2-chloro-*N*-[[4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino] carbonyl]benzenesulfonamide) to rotational crops is unknown. This study was conducted to determine the formation of nonextractable (bound) residues of chlorsulfuron in soil, and the distribution of bound residues in different organic matter fractions. The results showed that over 150 days, the extractable fraction of ^{14}C-residues decreased to 25.1% of applied chlorsulfuron, while bound residues concurrently increased to 47.1%. The distribution of ^{14}C-bound residues in soil organic matter fractions followed an order of humic acid (HA) < humin < fulvic acid (FA). Although the most bound residues were detected in the FA fraction, the amount associated with the humin fraction increased with time. After soil treatment by autoclaving, it was found that bound ^{14}C-chlorsulfuron residues became available again in the soil. One of the released products was 2-amino-4-hydroxyl-6-methyl-1,3,5-triazine (identified by GC−MS), which is a degradation product of chlorsulfuron.

KEYWORDS: Chlorsulfuron; bound residues; phytotoxicity; herbicides; degradation

INTRODUCTION

Chlorsulfuron, a sulfonylurea herbicide, is used for controlling broadleaf weeds in small grains (*1*, *2*). Chlorsulfuron is very effective when used at very low application rates, usually in the range of 10 to 40 g/ha for wheat, oats, and barley (*3*). The sulfonylurea family has been the focus of much research and development (*4*), and the physical and chemical properties, toxicology, mode of action, plant uptake and metabolism, and environmental fate of chlorsulfuron are well documented (*5*−8). The half-life of chlorsulfuron in soil is 1−2 months under field conditions (*9*). Although the residues of chlorsulfuron in soil are expected to be very low, damage to sugar beet crops has been reported two years after chlorsulfuron application (*6*). It is known that a large fraction of pesticides in soil can form "bound" residues that are not extractable with solvents. A recent study showed that chlorsulfuron could still cause injury to rice seedling and pea when in the bound state (*10*), which indicated that bound chlorsulfuron residues were bioavailable to these plants. However, mechanisms for the bioavailability and phytotoxicity of chlorsulfuron-derived bound residues have not been properly elucidated.

The objectives of this study were to (1) evaluate the formation of bound residues of chlorsulfuron in soil; (2) determine the distribution of chlorsulfuron bound residues in different organic matter fractions; and (3) establish the identity of bound residues that were released by autoclaving.

MATERIALS AND METHODS

Chemicals and Soil. ^{14}C-chlorsulfuron was synthesized by the Institute for Applications of Atomic Energy, Chinese Academy of Agricultural Science, Bejing, China. The specific activity of ^{14}C-chlorsulfuron was 6.07×10^{10} Bq/kg, and its radiochemical purity was >96%. The structure of chlorsulfuron is shown in **Figure 1**. Here, the fourth carbon of the triazine ring is radiolabeled.

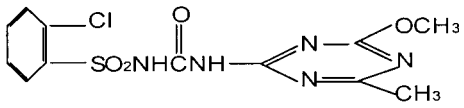

Figure 1. Chemical structure of chlorsulfuron.

The soil was collected from the experimental farm of Zhejiang University. The soil is a loamy type, with organic matter content of 2.14%, and silt, sand, and clay contents of 39, 43, and 18%, respectively. The soil pH was measured to be 7.7 (in water).

Preparation of Soil Containing ^{14}C-chlorsulfuron Bound Residues. Portions of 50 g (oven-dry weight basis) of air-dried soil were weighed into flasks to which 0.5 mL of methanol containing 1.215×10^5 Bq of ^{14}C-chlorsulfuron was added to give a concentration of 2.43×10^3 Bq/g soil. After the soil was mixed thoroughly and the solvent was evaporated under air, water was added to bring the water content to 70% of the maximum water holding capacity. The flasks were loosely stoppered with cotton wool and incubated at 26 °C in the dark. Distilled water was added every 2 days to maintain the soil water content. The samples were sacrificed in triplicates at the time intervals of 10, 30, 60, 90, 120, and 150 days after treatment.

Extraction and Determination. The soil samples were dried at 50 °C for 24 h; preliminary experiments showed that there were not any ^{14}C losses during the drying. The samples were then extracted with methanol using Soxhlet extractors at 65 °C for 24 h. Three cycles were performed in an hour. The extract was concentrated to 10 mL with a rotary evaporator. Then 0.1 mL of the extract was added into 10 mL of scintillation cocktail to measure the radioactivity of extractable residues on a liquid scintillation counter (LSC, Packard 1900CA, Packard, CT). The counting error was controlled at 5%.

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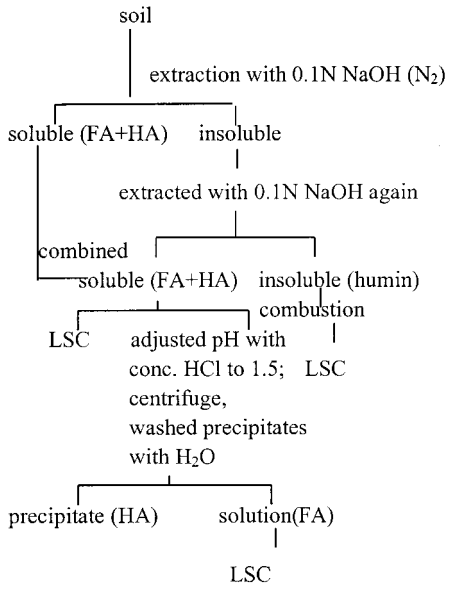

Figure 2. Flowchart of procedures used for fractionation of organic matter-associated bound ^{14}C-chlorsulfuron residues.

The extracted soil samples, containing only nonextractable ^{14}C-residues, were air-dried to remove the solvent. Aliquots (1 g) of these samples were subjected to combustion on a Biological Oxidizer OX-600 (R. J. Harvey Instruments, Hillsdale, NJ), and the evolved $^{14}CO_2$ was trapped with 10 mL of methanol/ethanolamine (875:125, v/v) solution. After mixing with 5 mL of scintillation solution (toluene/PPO/POPOP,1000 mL:5 g:50 mg), the samples were analyzed by LSC for activity associated with bound residues.

Fractionation of Soil Organic Matter. The extracted soil was fractionated into humic acid (HA), fulvic acid (FA), and humin using a conventional method (*11*). The procedures are outlined in **Figure 2**. Briefly, 10 g of soil was extracted overnight with 50 mL of 0.1 N NaOH solution using an orbital action shaker at 200 rpm. After the soil was centrifuged at 4000 rpm for 15 min, the insoluble soil residue was extracted with 25 mL of 0.1 N NaOH solution and centrifuged again. The supernatant, containing FA and HA fractions, was combined with the first extract and was made to volume. Then 0.5 mL of the supernatant was added into 10 mL of scintillation cocktail for measurement of radioactivity. The insoluble soil residue, containing humin fraction, was oven-dried at 50 °C for 24 h, then combusted as mentioned above.

The supernatant solution was adjusted to pH 1.5 with concentrated HCl to separate the HA and FA fractions. After settling overnight, the solution was centrifuged at 5000 rpm for 30 min. The supernatant, containing only the FA fraction, was made to the volume, and 0.5 mL of supernatant was transferred into 10 mL of scintillation cocktail for radioactivity measurement.

Release of Bound Residue by Autoclaving. A total of 10 g of soil was weighed into a flask and spiked with 100 μL of ^{14}C-chlorsulfuron methanol solution at a rate of 200 Bq/g soil. At 0, 20, and 120 days after incubation at 26 °C, triplicate samples were removed and extracted, using the steps given above, to produce soil samples containing only bound residues. The specific activity of these bound residue samples was determined after combustion and LSC analysis. In one set of samples, 7.5 mL of distilled water was added, but the other set was not wetted. All the soil samples were autoclaved at 121 °C for 1 h. After drying at 50 °C, the autoclaved samples were again extracted with methanol using Soxhlet extractors for 24 h as mentioned above. The extract was concentrated to 10 mL with rotary evaporation, and the radioactivity of extract was measured by LSC. To identify structures of the released residues which were previously bound, the extract was co-chromatographed with ^{14}C-chlorsulfuron in a mobile phase made of acetone and hexane (4:1, v/v, fortified with 0.1% acetic acid). A homemade TLC plate coated with silica gel GF_{254}, 20 cm in length and 5 cm in width, was used in the experiments. The developed plates were scanned for radioactive spots using a radioactive TLC scanner (FJ2109 β,γ TLC scanner, Xi'an 262 factory, Xi'an, China).

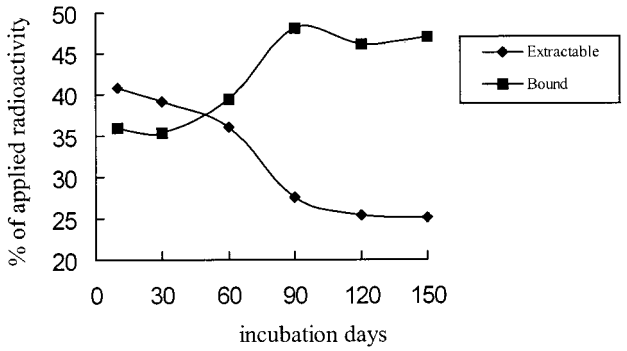

Figure 3. Dynamics of extractable and bound ^{14}C-chlorsulfuron residues in soil.

GC−MS Analysis. The extract was concentrated to about 0.1 mL with a flow of nitrogen gas at room temperature. After addition of sodium chloride (25 g in 100 mL of solution), the samples were washed twice with equal volumes of hexane by shaking vigorously for 1 min. The hexane layers were decanted, and the remaining aqueous phase was further extracted 3 times with equal volumes of chloroform by shaking vigorously for 1 min. The chloroform layers were then combined and concentrated to small volume. The final extract was transferred into 10-mL screw cap centrifuge tubes, and the solvent was removed under N_2 and reconstituted in 0.5 mL of dimethyl sulfoxide, 1.0 mL of hexane, 20 mg of NaH, and 0.15 mL of methyl iodide. The tubes were tightly closed, heated in a water bath at 35 °C for 5 min with occasional shaking, and then the excess NaH was decomposed with water. The mixture was extracted with hexane (5 mL × 3) by shaking, and the hexane layers were combined and dried under N_2 to 0.5 mL for GC−MS analysis. To preclude any artifact derived from sample handing, ^{14}C-chlorsulfuron was also added in silica, and prepared in a manner similar to that used for bound residue samples, followed by GC−MS analysis.

Analysis on GC−MS was carried out on a HP 5890A GC in tandem with a 5970B MSD. The GC column was a silica capillary column (30 m long × 0.2 mm i.d.) coated with SE-30. The injection port temperature was 240 °C. The oven temperature was initially 60 °C (3 min), and then ramped to 240 °C at a rate of 10 °C/min. The MSD had an electron energy of 70 eV.

RESULTS AND DISCUSSION

Extractable and Bound Residues of ^{14}C-Chlorsulfuron in Soil. The relative proportion of extractable and bound ^{14}C-chlorsulfuron residues in soil is shown in **Figure 3**. The results are expressed as the averaged percentage of the initially applied ^{14}C-chlorsulfuron. After 10 d of incubation, only 40.9% of the applied ^{14}C-chlorsulfuron was extractable, and 35.4% of the initial activity was detected in the bound residue form. After 150 d of incubation, the fraction of extractable residues further decreased to only 25.1%, while the fraction of bound residues increased to 47.1%. It is evident that the decreases in extractable residues were accompanied by concurrent increases in bound residues. It is also apparent that the decrease in extractable residues was relatively rapid during the initial 90 d, after which the change in relative proportion was relatively slow. Correspondingly, the fraction of bound residues increased rapidly prior to day 90, and then increased at a slow rate. The recovery of ^{14}C-residues, i.e., the total amount of extractable and bound residues, was relatively stable between 10 and 150 days after treatment, averaging 76.8 and 72.2%, respectively, which indicates that little of the ^{14}C-residues was lost to volatilization and/or mineralization from day 10 and onward.

Distribution of ^{14}C-chlorsulfuron Bound Residues in Soil. The soil containing bound residues was fractionated according

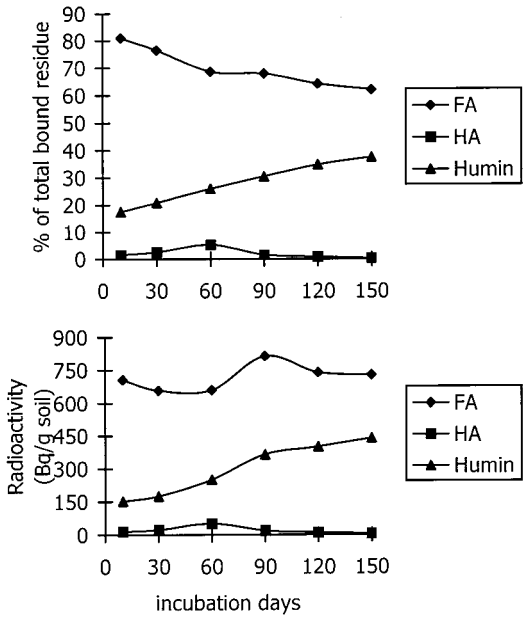

Figure 4. Distribution of bound ^{14}C-chlorsulfuron residues in soil organic matter fractions. Top, relative fraction; and bottom, absolute activity.

to the procedures outlined in **Figure 2**. The distribution of ^{14}C-bound residues in FA, HA, and humin fractions is shown in **Figure 4**. All values are expressed as the averaged percentage of total bound residues. Most of the ^{14}C-bound residues existed in the FA fraction; however, the total bound residues in the FA fraction decreased from 81.0 to 62.2% over time. Conversely, the relative fraction associated with humin increased from 17.5 to 37.5% over time. Compared to FA and humin associated radioactivity, only a small amount of bound residues was found in the HA fraction. Thus, the distribution of ^{14}C-bound residues in soil organic matter fractions follows the order FA > humin > HA. It is known that FA is made of naturally occurring water soluble and low molecular weight polyelectrolytes and is considered to be the dominant soluble organic fraction present in the soil solution under field conditions. It is also well-known that FA is present in many surface waters. Because of its low molecular weight, FA is more soluble than HA in water, which makes it a better carrier for bound residues in the soil−water−crops system (*12*). So ^{14}C-residues which are bound to FA can move and migrate easily or can be degraded by microorganisms in soil solution. Once taken up by plants, ^{14}C-residues may come loose from FA under the metabolic reaction of plants. And also, it is revealed that 34.4−40.6% of total bound ^{14}C-chlorsulfuron residues become extractable after incubation with microorganisms for 5 days, and the amount of these residues changed slightly thereafter. It is also shown that the residues existing in the FA fraction of soil organic matter would be preferentially released by microorganisms; about 82.2% of released residues is originated from the FA bound complex (*13*). These may have determined that bound residues of chlorsulfuron are still biologically available. Therefore, the association of a significant amount of bound residues with soil fulvic acids may contribute directly or indirectly to the observed injuries to certain crops from bound and aged chlorsulfuron residues.

The distribution of ^{14}C-bound residues in FA, HA, and humin as measured by specific activity (Bq/g of soil) is given in **Figure 4**. The specific activity associated with the FA and HA fractions changed slightly over time. However, the specific activity in the humin fraction increased continuously with time of incubation. Soil organic matter usually has a high content of oxygen-containing functional groups, such as −COOH, C═O, and phenolic- and enolic-OH. It is known that the content of these oxygen-containing functional groups is higher in FA than in HA (*14*). It is likely that the residues of chlorsulfuron preferentially reacted with the oxygen-rich FA fraction, and as time increased, the FA-bound ^{14}C-residues were further incorporated into humin and clay mineral fractions, resulting in gradual increases in humin. Calderbank (*15*) suggests that aging of bound residues is most likely a result of covalent bond formation, i.e., by chemical incorporation of pesticide residues into the humin fraction of soil.

Table 1. Released Activity from Bound Residues after Autoclaving (% of total bound residues)

	day 20	day 120
without water	33.6 ± 2.7	29.1 ± 2.0
with water	53.0 ± 3.9	39.0 ± 3.0

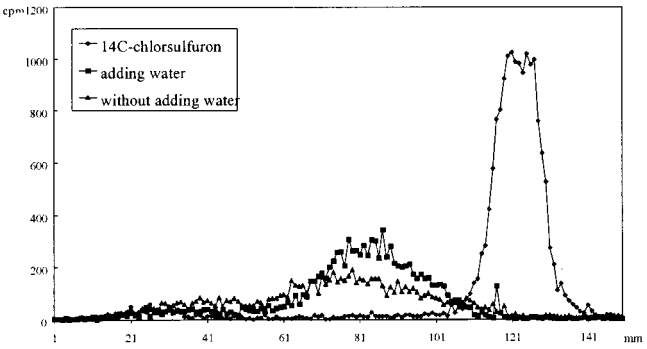

Figure 5. TLC chromatograms of bound residue samples after autoclave treatment.

Release of Bound Residue. The release of ^{14}C-activity from the bound residues following autoclave treatment is shown in **Table 1**. It is clear that the bound residues became extractable again after autoclaving. The release was also greatly enhanced by addition of water prior to the autoclave treatment. Furthermore, it appears that the shorter the incubation time, the more readily the ^{14}C-residue could be released in the treatment with water. Similar methods, e.g., high-temperature distillation (HTD) and supercritical methanol extraction, were used by other researchers for liberating pesticide residues from their bound state (*16, 17*). The working temperature and pressure in our autoclave treatment were 121 °C and 0.1 Mpa. These conditions are relatively mild compared to the conditions previously used in HTD and supercritical methanol extraction of bound residues. In previous studies where HTD was used for releasing bound pesticide residues from soil, the release rate was 50−60% (*16*). The maximum recovery from the autoclave treatment was 53.0% in this study. The autoclaving treatment does not rely on sophisticated instruments as does HTD or supercritical fluid extraction. The equipment is relatively easy to operate and provides large sample throughput. Because of the strong extraction ability of SFE, extracts contain impurities co-extracted from the sample matrix, whereas extracts from autoclaved samples are relatively clean and require little cleanup.

The extract of autoclaved bound residue samples was developed along with ^{14}C-chlorsulfuron standard on TLC plates. The TLC chromatograms for the samples incubated for different times are similar to one another, so only the chromatograms for the 20-d samples are shown in **Figure 5**. The R_f value of ^{14}C-chlorsulfuron was 0.68. Extracts from autoclaved samples invariably displayed a single broad peak with a R_f value of

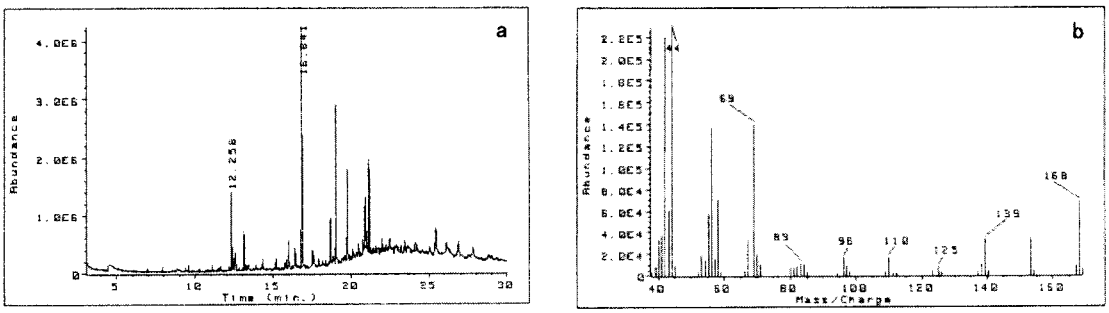

Figure 6. GC–MS profiles of bound residue products of ^{14}C-chlorsulfuron released after autoclave treatment: (a) total ion chromatogram; (b) mass spectrometer spectra.

0.36−0.38, while no chlorsulfuron peak was present. It is highly likely that, because of the thermal sensitivity of chlorsulfuron (*18*), chlorsulfuron was destroyed during autoclave treatment. The relative peak size was consistently greater for the water-treatment samples than for the no-water-treatment samples.

The chemical structure of the released ^{14}C-residues was tentatively identified through GC−MS analysis after sample methylation. The freshly spiked ^{14}C-chlorsulfuron (in silica sand) showed no chlorsulfuron peak and two predominant product peaks, suggesting that chlorsulfuron broke down into products after autoclaving treatment. One of the two peaks had a molecular ion *m*/*e* of 139, which was subsequently identified as 2-methylamino-4-hydroxyl-6-methyl-1,3,5-triazine. This compound corresponded to the first eluted peak with a retention time of 12.3 min under the conditions used for analysis. Because the methyl group was added via methylation, the original compound before methylation would most likely be 2-amino-4-hydroxyl-6-methyl-1,3,5-triazine.

Extracts from autoclaved bound residue samples were analyzed by GC−MS under the same conditions. Samples from both the 20-d and 120-d periods showed peaks with the same retention times and mass spectra as those observed for the decomposed products of ^{14}C-chlorsulfuron freshly spiked in sand (**Figure 6**). This suggests that a significant component of the bound residues could be the same compound as in the freshly spiked sand, that is, the intact chlorsulfuron itself. However, this does not exclude the presence of other products, especially polar intermediates, that were not detectable by the extraction and analytical methods used in this experiment.

It has been reported that less basic compounds, such as *s*-triazines, may become cationic through protonation and can be attracted to the −COOH group of soil organic matter (*14*, *19*). When chlorsulfuron undergoes degradation in soil by both chemical hydrolytic and microbial processes (*18*), cleavage of sulfonylurea bridge occurs, resulting in the formation of a sulfonamide and an aminotriazine. The methoxy moiety on the triazine ring can also be converted to a hydroxyl group through microbial action (*6*). These triazine products could become bound to organic matter through hydrogen bonding and interactions with the −COOH group of organic matter.

A number of studies have demonstrated the potential availability of the bound residues to organisms (*20*−*22*). However, no further evidence is given to show that bound pesticide residues have direct adverse effects to organisms except for bound chlorsulfuron residues (*10*), which indicates that the formation mechanism of bound chlorsulfuron residues is different from that of other pesticides. In general there are two broad mechanisms by which organic chemicals interact with the soil colloids and become bound, viz by adsorption or by chemical reaction. Bound residues tend to lose all biological activity and become even more resistant to degradation and extraction with longer residence time in soil. This phenomenon has been referred to as "aging" of residues. It seems likely that two main mechanisms are involved in the aging process, viz redistribution of chemical from weaker to stronger adsorption sites and/or slow chemical incorporation into the humin fraction (*15*). As can be seen from **Table 1**, the shorter the incubation time, the more readily the ^{14}C-residues could be released in the treatment with water. And also, the specific activity in the humin fraction increased continuously with time of incubation. Therefore, it looks likely that ^{14}C-chlorsulfuron became bound via weaker adsorption process during the initial stage. With extension of residence time in soil, the two mechanisms of aging process mentioned above will be followed.

CONCLUSIONS

This study showed that a considerable portion of radioactivity applied as ^{14}C-chlorsulfuron became bound to soil organic matter after application. These residues are distributed predominantly in the fulvic acid and humin fractions, and less predominantly in the humic acid fraction. After partial liberation through autoclaving treatment, a significant component of the released residues was tentatively identified as the intact chlorsulfuron molecule. The fact that chlorsulfuron was associated with the water-soluble fulvic acid fraction implies that this portion of bound residues is easier to move in the soil−water−plant system and can become available to plants long after herbicide treatment. This may help to explain the observed phytotoxicity caused by aged chlorsulfuron residues to subsequently planted crops.

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