Exhibit 39, #11

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Mixed-Mode Sorption of Hydroxylated Atrazine Degradation Products to Soil: A Mechanism for Bound Residue

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This study tested the hypothesis that sorption of hydroxylated atrazine degradation products (HADPs: hydroxyatrazine, HA; deethylhydroxyatrazine, DEHA; and deisopropylhydroxyatrazine, DIHA) to soils occurs by mixed-mode binding resulting from two simultaneous mechanisms: (1) cation exchange and (2) hydrophobic interaction. The objective was to use liquid chromatography and soil extraction experiments to show that mixed-mode binding is the mechanism controlling HADP sorption to soils and is also a mechanism for bound residue. Overall, HADP binding to solid-phase extraction (SPE) sorbents occurred in the order: cation exchange ≫ octadecyl (C_{18}) ≫ cyanopropyl. Binding to cation exchange SPE and to a high-performance liquid chromatography octyl (C_8) column showed evidence for mixed-mode binding. Comparison of soil extracted by 0.5 M KH_2PO_4, pH 7.5, or 25% aqueous CH_3CN showed that, for HA and DIHA, cation exchange was a more important binding mechanism to soils than hydrophobic interaction. Based on differences between several extractants, the extent of HADP mixed-mode binding to soil occurred in the following order: HA > DIHA > DEHA. Mixed-mode extraction recovered 42.8% of bound atrazine residues from aged soil, and 88% of this fraction was identified as HADPs. Thus, a significant portion of bound atrazine residues in soils is sorbed by the mixed-mode binding mechanisms.

Introduction

Hydroxylated atrazine degradation products (HADPs: hydroxyatrazine, HA; deethylhydroxyatrazine, DEHA; and deisopropylhydroxyatrazine, DIHA) are a major class of atrazine degradation products that form in the environment via chemical, biological, and photolytic mechanisms (*1*–*3*). Although formation of HADPs has been widely regarded as a major detoxification pathway for atrazine (*4*, *5*), relatively little is known about the fate of HADPs in the environment because of a lack of viable analytical methods for their isolation from soils or sediments.

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Decomposition studies of [^{14}C]triazines typically show that a large fraction of the residue is tightly bound to soil (30–70%) (*6*–*11*). These residues have been attributed to initial hydrophobic interactions with soil organic matter followed by aging of the residues to form irreversibly bound residues by covalent bonding or chemical incorporation into organic matter (*8*). HADPs have been shown to comprise a significant portion of the bound residues formed from atrazine degradation in soils. In a long-term field study of [^{14}C]atrazine fate in soil, Capriel et al. (*7*) showed that HADPs were the main products remaining after 9 years. Bound HADPs and atrazine accounted for about 3% and 1%, respectively, of the atrazine applied (*7*). Supercritical fluid extraction of [^{14}C]atrazine bound residues from incubations of 1 or 9 years contained greater amounts of HA than atrazine (*12*). Extraction of the soil incubated for 1 year showed that 31% of the bound residue was hydroxyatrazine and 17% was atrazine (*12*). Supercritical fluid extraction of samples from the study by Capriel et al. (*7*) gave a different distribution of bound residues than originally reported. Khan (*12*) reported that 0.4 ppm hydroxyatrazine and 0.3 ppm atrazine were extracted from the bound residues, representing 4% and 3%, respectively, of the atrazine applied. Thus, the specific extraction method resulted in different recoveries of bound atrazine residues.

An operational definition of bound residues also exists based on the inability of solvents or solvent mixtures to extract pesticides and their degradation products from soil (*13*–*16*). For triazine herbicides and their degradation products, aqueous methanol or acetonitrile has been most commonly used for soil extraction of residues following incubation in soil (*9*–*11*, *14*, *17*). These aqueous-organic solvent mixtures often do not quantitatively extract HADPs from soil because of their low solubility in these extractants (*18*). Incubation studies reporting bound atrazine residues based on soil extraction by aqueous methanol or acetonitrile possibly have HADPs that can be recovered by some other extracting agents. A key point of this study was to demonstrate that the extraction of bound atrazine residues from soil can be greatly enhanced if the extracting solution is tailored to an understanding of the specific binding mechanisms of HADPs to soil. In the context of this study, bound residues are defined as atrazine residues that are not extractable using aqueous–organic solvent mixtures.

Soils in which aqueous methanol or acetonitrile results in HADP recoveries of 50% or less indicate that hydrophobic interaction and hydrogen bonding sorption mechanisms do not always predominate (*12*, *13*, *19*, *20*). Extraction of HADPs from soils has also utilized acids or acidified organic solvents in an attempt to increase extraction efficiency (*18*, *21*–*23*). The use of acidity was designed to increase the solubility of the HADPs by protonating them. However, acidic solutions give varying recovery of HADPs from soils. Muir and Baker (*18*) reported HADP recoveries of 22–68% from spiked soils incubated for 5 or 21 days by sequential extraction using aqueous methanol followed by acidified methanol. Poor recovery efficiency for this method was, in part, due to the derivatization reaction used for gas chromatographic analysis of the HADPs. Weber and Best (*23*) recovered HA from two soils incubated for 5 months using 30 h Soxhlet methanol extraction followed by glacial acetic acid extraction for 30 h. Glacial acetic acid recovered 28–31% of the HA, and the total recovery for the methanol and acetic acid extractions was 85–88% of the applied HA. More recently, supercritical fluid extraction has been used for recovery of atrazine and its degradation products from soil (*12*, *24*). Despite the extensive work on the triazine herbicides, no generally accepted method for extraction of HADPs from soil has emerged because the

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mechanisms controlling their binding to soils are not clearly understood.

Based on the types of extracting solvents used for recovery of HADPs from soil, hydrophobic interaction and, to a lesser extent, hydrogen bonding have been the presumed mechanisms by which HADPs sorb to soils. Moreover, hydrophobic interaction with soil organic matter has predominantly been considered as the primary sorption mechanism by which herbicides are retained in soils (*25, 26*). The current view of hydrophobic interaction between pesticides and soils has been described by partitioning theory that treats soil organic matter as a water-immiscible liquid phase (*25, 26*). However, soil organic matter is a solid phase with pH-dependent functional groups and a matrix of internal and external hydrophobic surfaces; therefore, in this study, the term hydrophobic interaction rather than hydrophobic partitioning is used to describe the sorption of a compound to soil organic matter via bonding mechanisms that involve interactions between the hydrophobic moieties of both the solute and the sorbent.

The predominance of partitioning theory in the herbicide sorption literature has resulted in the importance of cation exchange as a mechanism for triazine sorption to soils to be disregarded. Several studies have demonstrated the importance of cation exchange to the sorption of *s*-triazines to soil colloids (*13, 14, 19, 20, 27, 28*). Since triazines and their stable degradation products are weak bases with dissociation constants (pK_a) ranging from 1.7 to 5.3 (*27, 29, 30*), cation exchange to either clay minerals or organic matter would be expected if ambient soil–water pH is within 2 pH units of the pK_a. The solution pH near colloid surfaces has been indicated to be approximately 0.5–2 pH units lower than that of the bulk soil solution (*27*). Since HADPs have dissociation constants ranging from 4.6 to 5.2 (*28*), even at a bulk soil pH of 7 cation exchange would be a significant sorption mechanism for these compounds. Weber (*27, 29*) reported that considerable adsorption of hydroxy-, methoxy-, and methylthio-*s*-triazines to soil colloids occurred over a pH range of 2–8 with maximum adsorption at the pK_a of the compounds. In contrast, propazine adsorption to Na-montmorillonite occurred over a limited pH range of 1.5–4. The molecular structure of *s*-triazines, specifically the substituent at the 2-position (see Chemical and Standard Materials within the Experimental Section for triazine nomenclature), largely determines their basicity and sorption by cation exchange. Within a group of *s*-triazines differing only in the substituent at the 2-position, the extent of cation exchange to soils generally occurs in the following order: methylthio > methoxy > hydroxyl > chlorine (*28, 31*). Therefore, the HADPs would be expected to sorb by cation exchange to a greater degree than atrazine and its chlorinated metabolites. The importance of cation exchange to sorption and environmental fate of HADPs has been largely overlooked in the recent literature.

This study proposes the hypothesis that HADPs bind to soils by two simple sorption mechanisms occurring simultaneously: (1) cation exchange and (2) hydrophobic interaction. This is referred to as a mixed-mode model of sorption (Figure 1). The evidence consists of both chromatographic studies, using solid-phase extraction and reverse-phase liquid chromatography as well as soil extraction studies that show the release of HADPs bound by mixed-mode sorption. Finally, the mixed-mode sorption model is proposed as one of the major mechanisms for bound triazine residue in soils.

Experimental Section

Chemicals and Standard Materials. Hydroxyatrazine (HA) (2-hydroxy-4-ethylamino-6–isopropylamino-*s*-triazine), deethylhydroxyatrazine (DEHA) (2-hydroxy-4-amino-6-isopropylamino-*s*-triazine), and deisopropylhydroxyatrazine (DIHA) (2-hydroxy-4-ethylamino-6-amino-*s*-triazine) were 94–99% pure (Ciba-Geigy Corp., Greensboro, NC). Radioisotope standards were also provided by Ciba-Geigy Corp. with the following radiochemical purity and specific activity respectively: [^{14}C-U-*ring*]hydroxyatrazine, 98.6%, 1.635×10^9 Bq g^{-1}; [^{14}C-U-*ring*]deethylhydroxyatrazine, 96.1%, 7.733×10^8 Bq g^{-1}; and [^{14}C-U-*ring*]deisopropylhydroxyatrazine, 92.7%, 8.362×10^8 Bq g^{-1}. All solvents and the KH_2PO_4 used were HPLC grade. Hydrochloric acid (HCl), concentrated H_3PO_4, KCl, and NaOH were reagent grade. The KH_2PO_4 buffers used concentrated H_3PO_4 or 50–75% (w/v) NaOH to adjust pH.

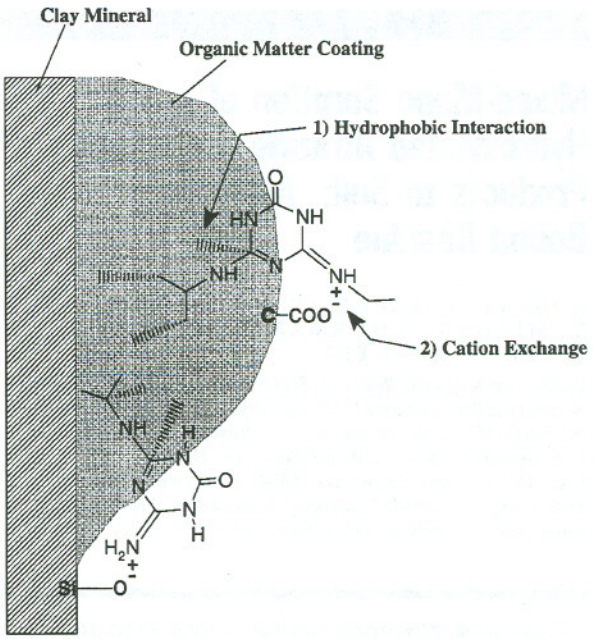

FIGURE 1. Mixed-mode model for HADP sorption to soils.

Solid-Phase Extraction. Three solid-phase extraction (SPE) bonded phases (500 mg in 2.8-mL polypropylene reservoirs; Varian, Harbor City, CA) representing different sorption mechanisms were investigated for their retention of HADPs: weak polar, cyanopropyl (CN); strong cation exchange (SCX), propylbenzenesulfonic acid (pK_a 0–1); and hydrophobic interaction, octadecyl (C_{18}). Triplicate samples containing 100 μg L^{-1} HADPs (HA, DEHA, and DIHA) in 25 mL of HPLC grade water for the CN and C_{18} bonded phases or 0.05 M KH_2PO_4, pH 2.5, for the SCX bonded phase were used to evaluate retention. Samples were passed through the cartridges at a flow rate of 3–5 mL min^{-1}, and the last 5–10 mL of the sample passing through the SPE was collected into glass test tubes. HADP concentrations were determined by HPLC following the addition of buffer (1 M KH_2PO_4, pH 7.5) to give a final concentration of 0.1 M KH_2PO_4, pH 7.5. A 30-μL portion of the buffered samples was injected for HPLC analyses. SPE cartridge conditioning was performed as describe by Lerch and Donald (*30*).

High-Performance Liquid Chromatography. A Beckman Model 338 high-performance liquid chromatography (HPLC) system (Beckman Instruments, Inc., San Ramon, CA) was used. The system consisted of two Model 110B pumps operated at a 1 mL min^{-1} flow rate, a Model 507 autosampler with a column oven at 30–35 °C and a 100-μL sample loop, and a Model 166 variable-wavelength UV detector set to 220 nm. A deactivated octyl (C_8) reverse-phase column (LC-8-DB, 5 μm, Supelco, Inc., Bellefonte, PA) with dimensions of 250 mm by 4.6 mm (i.d.) was used. Two different isocratic HPLC methods were used for HA and the *N*-dealkylated HADPs (DEHA and DIHA). For HA, the mobile phase was 2:3 CH_3OH:H_2O (v/v) (referred to as 40% aqueous CH_3OH hereafter), and the sample injection volume was 40 μL. For

DEHA and DIHA, the mobile phase was 3:17 CH_3OH:H_2O (v/v) (referred to as 15% aqueous CH_3OH hereafter), and the sample injection volume was 10 μL. HA fractions were collected from standard samples using the Beckman HPLC system and an ISCO Foxy 200 fraction collector (Lincoln, NE). Standards contained approximately 10 mg L^{-1} HA in a buffer of 3:1 0.5 M KH_2PO_4, pH 7.5:CH_3CN (v/v) (referred to as KH_2PO_4/CH_3CN hereafter).

Extraction of [^{14}C]HADPs from Freshly Spiked Soil. Surface soil (0−20 cm) of the Mexico−Putnam soil association (Mollic Albaqualf−Mollic Ochraqualf; fine, montmorrillonitic, mesic) was used for all freshly spiked soil recovery experiments. Soils were passed through a 6-mm sieve, large pieces of plant residue were removed and stored at 4 °C and 16.5% gravimetric moisture content (wet weight basis). Soil properties were as follows: 4.0% organic matter; pH (saturated extract), 5.9; sand, 18%; silt, 56%; clay, 26%; textural class, silt loam; cation exchange capacity, 12.0 cmol(+) kg^{-1}. It should be noted that extraction conditions for all freshly spiked soil experiments were not optimized for quantitation. Instead, the intent was to achieve substantial recoveries for the purpose of making relative comparisons between extractants. The initial soil experiment was a comparison of extraction recoveries of [^{14}C]HADPs by a mixed-mode extractant, KH_2PO_4/CH_3CN, and an acidified methanol extractant, 4:1 CH_3OH:0.1 M HCl (v/v). Duplicate 25-g (dry weight) soil samples for each HADP and extractant were spiked with 5000 Bq of [^{14}C]HA, [^{14}C]DEHA, or [^{14}C]DIHA. Samples were spiked with the appropriate volume (3.75−5.20 mL) of each [^{14}C]HADP in 0.002 M HCl, and the samples were thoroughly mixed with a glass stirring rod. The acidity in the spiking solution represented no more than 0.01 mequiv of H^+, which was <1% of the cation exchange capacity of the soil samples. Therefore, the soil pH was unaffected by the added acidity. Final concentrations of each HADP were 120 μg kg^{-1} [^{14}C]HA, 250 μg kg^{-1} [^{14}C]DEHA, and 220 μg kg^{-1} [^{14}C]DIHA. Spiked soils and a blank for each extractant were incubated at 22−25 °C for 72 h in 150-mL Teflon screw cap bottles. Soil moisture ranged from 26% to 29% gravimetric moisture content (wet weight basis). Alkali traps consisting of 10 mL of 2 M NaOH were placed in all samples to trap $^{14}CO_2$. A 1-mL aliquot of each alkali trap solution was mixed with 4 mL of Ultima Gold (Packard Instrument Co., Meriden, CT) scintillation cocktail for liquid scintillation counting. Recovery efficiency of each extractant was corrected for the amount of mineralization that occurred during the incubation period, but no attempt was made to correct for the formation of other degradation products during incubation. Soils were extracted twice with 50 mL of extractant for 1 h on an end-to-end shaker at about 200 oscillations min^{-1} and centrifuged for 30 min at 3500 rpm and 4 °C. The supernatant solutions were combined, filtered through a 0.2-μm Anotop 25 (Whatman International Ltd., Maidstone, England) syringe filters if necessary, and a 1-mL aliquot of extractant was mixed with 4 mL of Ultima Gold scintillation cocktail. A Packard 1900 TR (Packard Instrument Co., Meriden, CT) liquid scintillation counter was used for all ^{14}C analyses. All samples analyzed by liquid scintillation counting were corrected for quenching and background.

Additional extraction recovery experiments were designed to further determine the relative importance of hydrophobic interaction, cation exchange, and mixed-mode retention of [^{14}C]HADPs to soil. Recovery of [^{14}C]HADPs from freshly spiked soils, using a separate set of samples from the previously described experiment, were performed with the following extractants: cation exchange, 0.5 M KH_2PO_4, pH 7.5, and 0.5 M KCl; hydrophobic interaction, 1:3 CH_3CN:H_2O (v/v) (referred to as 25% CH_3CN hereafter); and mixed-mode, KH_2PO_4/CH_3CN and 3:1 0.5 M KCl:CH_3CN (v/v) (referred to as KCl/CH_3CN hereafter). The 25% CH_3CN extractant is referred to as a hydrophobic extractant for reasons of simplicity and for comparison of the contribution of 25% CH_3CN in the mixed-mode extractant. However, it is recognized by the authors that hydrogen bonding with the solute is also a potentially significant polar interaction associated with this extractant. Triplicate 25-g (dry weight) soil samples for each HADP and extractant were spiked with 5000 Bq of [^{14}C]HA, [^{14}C]DEHA, or [^{14}C]DIHA. Incubation conditions, soil extractions, and scintillation counting were performed as described above. Recovery efficiencies were also corrected for any mineralization that occurred during incubation. Mineralization rates for all freshly spiked soil experiments ranged from 3.9 to 6.6% of added [^{14}C]DIHA, from 1.3 to 3.5% of added [^{14}C]DEHA, and from 0.9 to 2.8% of added [^{14}C]HA.

For statistical comparison of mixed-mode and acidified methanol extractants, a one-way analysis of variance (ANOVA) was conducted to determine the probability of observing a more extreme F statistic (P-value) within and between each extraction treatment. For the cation exchange, hydrophobic, and mixed-mode extractant comparisons, a two-way ANOVA using a factorial design (3 analytes by 5 extractants) was conducted to determine the P-value for extractants as a main effect and for the interaction of the main effects (analyte by extractant) averaged over all HADPs. In addition, the significance of the main effects was determined by one-way ANOVA within and between each extraction treatment. Least significant difference (LSD) values were calculated at the level of significance corresponding to the P-values from the ANOVA.

Extraction of [^{14}C]HADPs from Aged Soil. Surface soil (0−30 cm) of the Nicollet−Webster complex (Aquic Hapludoll−Typic Haplaquoll; fine-loamy, mixed, mesic) was collected from a field with no previous pesticide use history. Soils were passed through a 2.4-mm sieve and stored at 4 °C for no more than 90 days. Soil properties were as follows: 2.6% organic matter; pH (1:1 soil:water, (w/v)) 5.5; sand, 56%; silt, 24%; clay, 20%; textural class, sandy clay loam; cation exchange capacity, 14.6 cmol(+) kg^{-1}. A 150-g soil sample was spiked to a concentration of 5 μg g^{-1} of [^{14}C]atrazine containing approximately 111,000 Bq of radioactivity. Following evaporation of the methanol solution used for spiking, the sample was divided into three 50-g samples, and the moisture content was adjusted to 34.4% gravimetric moisture content (wet weight basis) to simulate saturated conditions. Samples were then incubated for 120 days at 24 °C in the dark. During the incubation period, samples were opened weekly to change the NaOH traps. Following incubation, the soils were extracted three times with 150 mL of 9:1 CH_3OH:H_2O (v/v) for determination of atrazine residues, and a portion of the samples was combusted using a Packard Model 306 sample oxidizer (Packard Instrument Co., Downers Grove, IL) for determination of bound residues. The samples were then stored frozen (−20 °C) for approximately 2 years. Frozen samples were thawed, and subsamples were combusted again for total ^{14}C determination. The remaining soil was extracted three more times with 150 mL of 9:1 CH_3OH:H_2O (v/v) to assure that only bound atrazine residues remained in the samples. The samples were then extracted two times with 100 mL of the mixed-mode extractant, KH_2PO_4/CH_3CN. The two mixed-mode supernatants were combined, the CH_3CN was removed by rotary evaporation, and the ^{14}C content was determined on a 0.5-mL aliquot by liquid scintillation counting using a RackBeta Model 1217 (Pharmacia LKB Biotechnology, Inc., Gaithersburg, MD) liquid scintillation counter. The intent of the mixed-mode extraction was not quantitative recovery but rather to demonstrate that a portion of the bound residue was sorbed by mixed-mode binding. Additional details of the aged soil samples were reported by Kruger et al. (*11*).

The mixed-mode extractable bound residue was then fractionated by HPLC to determine the amount of HADPs. The same HPLC system conditions, column, and fraction

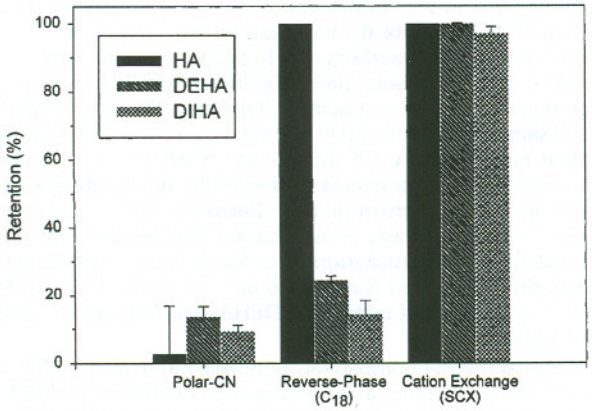

FIGURE 2. Retention of HADPs by different solid-phase extraction bonded phases. Polar-CN was cyanopropyl; reverse-phase (C_{18}) was octadecyl; and cation exchange (SCX) was propylbenzenesulfonic acid, pK_a 0−1. Sample conditions were 100 μg L^{-1} in H_2O for polar-CN and C_{18} and 100 μg L^{-1} in 0.05 M KH_2PO_4, pH 2.5 for SCX. Data from Lerch and Donald (*30*). Error bars represent the standard deviation.

collector described above were used to collect the HADP fractions. Chromatographic conditions employed a gradient mobile phase method:

mobile phase composition	time (min)
30% aqueous CH_3OH	0
30% aqueous CH_3OH	3.5
45% aqueous CH_3OH	3.6
45% aqueous CH_3OH	11.6
30% aqueous CH_3OH	11.7
30% aqueous CH_3OH	20

Sample injection volume was 50 μL, and column temperature was 35 °C. Retention times were 3.8 min for DIHA, 5.0 min for DEHA, and 14.2 min for HA. Fractions were collected from 3:00 to 4:00 min for DIHA, from 4:00 to 5:45 min for DEHA, and from 13:45 to 15:45 min for HA. Fraction time windows were verified for quantitative recovery using [^{14}C]HADPs before collection of the samples. Each sample was injected 24−28 times, and the fractions were combined, rotary evaporated to 1−2 mL, further evaporated under N_2 to dryness, reconstituted, and brought to a final volume of 2 mL with 0.01 M HCl. A 1-mL aliquot was used for ^{14}C determination by liquid scintillation counting.

Results and Discussion

Retention of HADPs to Liquid Chromatographic Bonded Phases. The polar CN bonded phase resulted in retention of only 2.7−13.7% of the HADPs, with HA having the lowest retention (Figure 2). Reverse-phase C_{18}-SPE resulted in 100% retention of HA while DEHA and DIHA showed very low retention. All three HADPs had a high affinity for the propylbenzenesulfonic acid bonded phase of the SCX−SPE (>97% retention). This bonded phase has also been successfully used for isolation of HADPs from water (*30, 32*). The poor retention of HADPs to the polar CN bonded phase suggested that retention by hydrogen bonding or weak hydrophobic bonding to this SPE bonded phase is insignificant. The large differences in retention of HADPs to C_{18}-SPE highlights the differences in nonpolarity exhibited by the HADPs. HA, with both alkyl side chains, possesses significant nonpolar character, resulting in an ability to hydrophobically bind to the C_{18} bonded phase. The *N*-dealkylated HADPs are more polar due to the amino group at either the 4- or 6-positions and, therefore, show considerably reduced ability to bind to a hydrophobic phase. Retention of HADPs to different SPE bonded phases showed the importance of cation exchange as a binding mechanism for all three HADPs, and

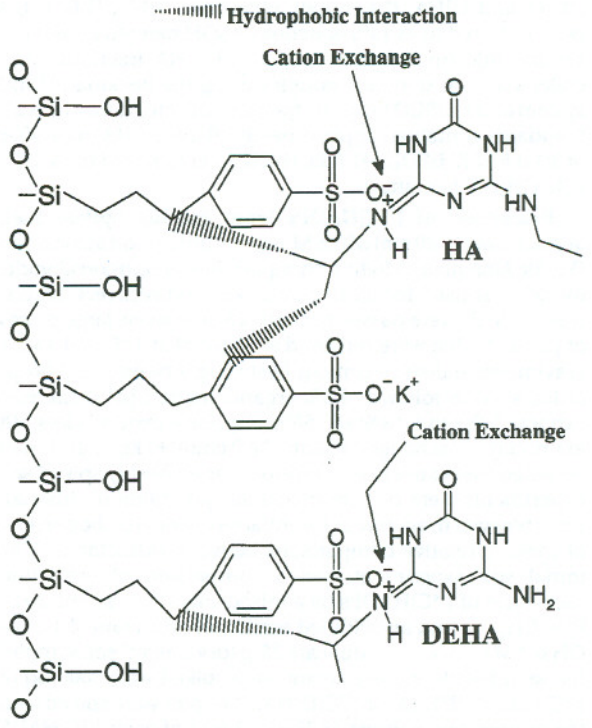

FIGURE 3. Mixed-mode retention of HADPs on propylbenzenesulfonic acid (SCX) solid-phase extraction. Sample conditions were 100 μg L^{-1} HADPs in 0.05 M KH_2PO_4, pH 2.5.

for HA, hydrophobic bonding was also an important retention mechanism. Sorption/desorption studies with triazines and H-, Na-, and Cl-exchange resins demonstrated ionic bonding to a H-cation exchange resin and physical bonding to Na-cation exchange and Cl-anion exchange resins (*12, 13, 19*).

The amphipathic nature displayed by the HADPs is manifested in their retention to the SCX−SPE as well. Lerch and Donald (*30*) reported that retention of HADPs to SCX−SPE as a function of volume varied for each HADP. As the polarity of the HADPs increases, progressively smaller volumes were required for significant breakthrough. For example, recoveries for 100- and 2000-mL treatments, respectively, were 95.3% and 95.9% for HA and 90.5% and 41.4% for DIHA. Lerch and Donald (*30*) concluded that cation exchange and secondary hydrophobic interactions with the SCX sorbent were responsible for this observation. Therefore, mixed-mode binding appears to play a role in the retention of HADPs, particularly HA, to the SCX bonded phase (Figure 3). The hydrophobic bonding depicted in Figure 3 has been restricted to the functional groups of the sorbent and the alkyl side chain of the HADPs because $\pi-\pi$ bonding between the triazine and benzene rings would not be expected to occur when HADPs are in their protonated keto form. Furthermore, there is no spectroscopic evidence that HADPs exist in a protonated enol form (*33*). Mixed-mode SPE using propylbenzenesulfonic acid alone or mixed with C_{18} as a bonded phase has been reported for isolation of other organic cations (*34, 35*).

Retention of HADPs to a reverse-phase (octyl, C_8) HPLC column showed that HA had a retention time of 13.0 min with a mobile phase of 40% aqueous CH_3OH as compared to retention times of 9.0 min for DEHA and 5.1 min for DIHA with a mobile phase of 15% aqueous CH_3OH (*30*). Retention of HADPs to C_8-HPLC follows the hydrophobicity of the HADPs, with HA most hydrophobic and DIHA least hydrophobic. This observation is also consistent with the results for HADP retention to C_{18}-SPE (Figure 2). These results further emphasize the differences in hydrophobic character exhibited by the HADPs.

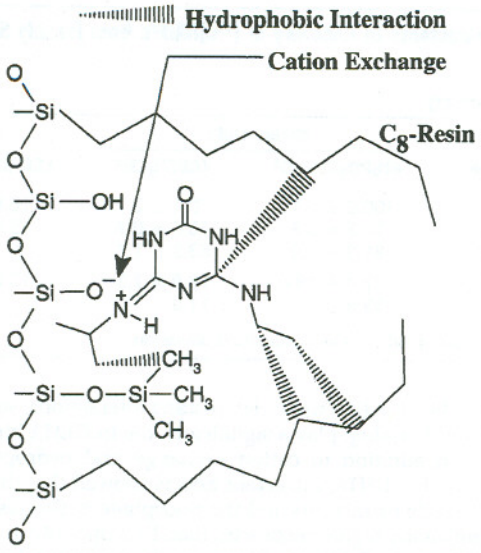

FIGURE 4. Mixed-mode retention of HA to a C_8-HPLC stationary phase occurs in the absence of sample buffer (3:1 0.5 M KH_2PO_4, pH 7.5:CH_3CN, v/v). Mobile phase was 2:3 CH_3OH:H_2O (v/v), and HA sample solution was also 2:3 CH_3OH:H_2O (v/v).

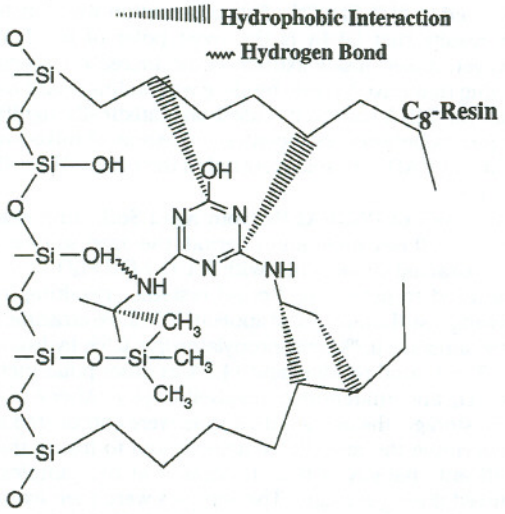

FIGURE 5. Retention of HA to a C_8-HPLC stationary phase occurs by hydrophobic interaction and hydrogen bonding when sample is buffered. Mobile phase was 2:3 CH_3OH:H_2O (v/v). HA sample solution was 3:1 0.5 M KH_2PO_4, pH 7.5:CH_3CN (v/v).

There is also the possibility of cation exchange to the silica surface of the C_8-HPLC column. For example, it was observed that injection of HA into the HPLC without sample buffer present resulted in irreversible binding (i.e., no elution after 35 min) to the silica-bonded C_8 sorbent in spite of the 40% aqueous CH_3OH mobile phase. Injection of sample buffer only (KH_2PO_4/CH_3CN) resulted in elution of HA from the column within 1 min of the measured retention time. This observation is consistent with mixed-mode binding of HA to the C_8-HPLC column (Figure 4). In the absence of sample buffer, silanol groups donate a proton to HA, creating a cation exchange site. Simultaneously, HA hydrophobically bonds with the C_8-resin to result in mixed-mode binding. In the presence of sample buffer at pH 7.5, the silanol groups do not dissociate (pK_a 9.5) (*36*), resulting in HA retention predominantly by hydrophobic interaction with some hydrogen bonding possible between a side chain amino- or ring-N and the silanol-H (Figure 5). Thus, mixed-mode binding is a primary reason for difficulties associated with reverse-phase HPLC of the HADPs using silica-based columns (*30*). Successful reverse-phase HPLC of these compounds, particularly HA, requires a neutral or acidic buffer in either the sample or the mobile phase to suppress silanol dissociation and to prevent mixed-mode binding of HADPs (*29*, *30*, *32*, *37*, *38*).

TABLE 1. Comparison of Mixed-Mode and Acidified Methanol Extractants to Recovery of [^{14}C]HADPs from Freshly Spiked Soil

	% recovery		
compd	mixed-mode[a]	acidified methanol[b]	*P*-value[c]
HA	98.9 ± 3.5[d]	45.7 ± 0.8	0.002
DEHA	82.2 ± 0.7	59.6 ± 1.1	0.002
DIHA	74.5 ± 2.5	5.28 ± 0.3	0.001
mean	85.2 ± 11.3	36.9 ± 25.2	0.002
LSD (0.005)	19.0	6.0	

[a] Mixed-mode extractant was 3:1 0.5 M KH_2PO_4, pH 7.5:CH_3CN (v/v). [b] Acidified methanol extractant was 4:1 CH_3OH:0.1 M HCl (v/v). [c] Probability of observing a more extreme *F* statistic from the analysis of variance. [d] Mean ± standard deviation.

Recovery of [^{14}C]HADPs from Freshly Spiked Soil. The results of the liquid chromatography experiments provided the impetus to test the concept of mixed-mode binding of HADPs in soils. The mixed-mode soil extractant, KH_2PO_4/CH_3CN, was originally used as the elution and sample buffer for the SCX–SPE cleanup and HPLC analysis of HADPs in water (*30*). Previous studies have used acidified solutions to recover HADPs from soils (*18*, *22*, *23*). Thus, the initial soil extraction experiment was designed to compare the mixed-mode extractant with acidified methanol. Comparison of acidified methanol to the mixed-mode extractant showed that mixed-mode extraction resulted in significantly higher recoveries for all three HADPs ($P = 0.001-0.002$) (Table 1). For mixed-mode extraction, HA recovery (98.9%) was significantly greater than DIHA (74.5%), indicating greater importance of mixed-mode binding for HA. For acidified methanol extractions, DEHA had higher recoveries than either HA or DIHA suggesting that cation exchange is less important to DEHA sorption in soil. Conversely, cation exchange appears to be critical to DIHA binding to soil as it had the lowest recovery by acidified methanol extraction (5.3%) and much higher recoveries by mixed-mode extraction. These results clearly showed the importance of mixed-mode binding of HADPs to soils, particularly for HA and DIHA. In addition, it showed that acidification of methanol can accentuate cation exchange binding of HADPs to soil and lower recovery.

In order to better distinguish the relative importance of cation exchange and hydrophobic interaction to HADP sorption, additional extraction studies were conducted using cation exchange, hydrophobic, and mixed-mode extractants. The significance of the analyte by extractant interaction ($P < 0.001$) showed that the effectiveness of the extractants varied for each HADP (Table 2). This interaction further illustrated differences between the HADPs in the degree to which hydrophobic interaction and cation exchange contribute to their retention in soils. The effect of extractant averaged over HADPs showed that the two mixed-mode and phosphate buffer (0.5 M KH_2PO_4, pH 7.5) extractants resulted in significantly greater recoveries ($P = 0.001$) than 25% CH_3CN or 0.5 M KCl (Table 2). There was no significant difference in extraction recovery averaged over HADPs between the mixed-mode extractants and phosphate buffer. Cation exchange extraction by phosphate buffer accounted for much higher recovery than hydrophobic extraction alone (72.3% versus 50.8%). This is consistent with our results using acidified methanol and previous studies showing the importance of cation exchange as a retention mechanism for hydroxy-*s*-triazines (*12*, *13*, *19*, *20*, *27*, *28*). However, the relative importance of cation exchange as compared to hydrophobic interaction had not previously been shown for the HADPs.

TABLE 2. Comparison of Cation Exchange, Hydrophobic, and Mixed-Mode Extractants to Recovery of [^{14}C]HADPs from Freshly Spiked Soil

	% recovery					
	cation exchange		hydrophobic	mixed-mode		
compd	0.5 M KCl	0.5 M KH_2PO_4, pH 7.5	25% CH_3CN[a]	KH_2PO_4/CH_3CN[b]	KCl/CH_3CN[c]	LSD (.001)
HA	26.5 ± 1.2[d]	79.2 ± 6.4	55.3 ± 4.2	100.6 ± 3.1	78.4 ± 3.7	16.8
DEHA	54.0 ± 2.0	79.8 ± 2.6	72.2 ± 7.9	70.2 ± 3.3	76.6 ± 0.7	15.4
DIHA	10.8 ± 0.9	57.8 ± 1.7	24.8 ± 1.2	61.3 ± 1.9	63.5 ± 1.4	5.5
mean	30.9 ± 20.2	72.3 ± 11.5	50.8 ± 21.3	77.4 ± 18.0	72.8 ± 7.3	13.0
LSD (0.001)	8.3	20.0	25.4	13.8	11.3	

[a] 1:3 $CH_3CN:H_2O$ (v/v). [b] 3:1 0.5 M KH_2PO_4, pH 7.5:CH_3CN (v/v). [c] 3:1 0.5 M KCl:CH_3CN (v/v). [d] Mean ± standard deviation.

Differences in HADP recovery between the two cation exchange extractants, phosphate buffer and 0.5 M KCl, were attributed to the effect of pH on the exchange reaction and to the amount of organic matter dissolved. The phosphate buffer, at pH 7.5, drives the exchange reaction to completion by the deprotonation of the exchanged HADPs upon entering the solution phase. The 0.5 M KCl will not necessarily deprotonate the exchanged HADPs entering the solution phase because the extracting solution will have equilibrated with the soil pH (5.9). In addition, the phosphate buffer dissolved considerably greater amounts of soil organic matter as compared to 0.5 M KCl based on observation of the solution color. One possible explanation for why the phosphate buffer dissolved greater amounts of soil organic matter was its ability to disrupt cation bridges formed between polyvalent cations and soil organic matter. Polyvalent metals are known to precipitate soil organic matter by forming intra- and intermolecular cation bridges (*39*). Since Ca^{2+} and Mg^{2+} are the dominant exchangeable ions in these soils, an extraction system that displaces them from the humic exchange sites and removes them from solution will increase the solubility of soil organic matter by disruption of the cation bridges.

The phosphate buffer displaces Ca^{2+} and Mg^{2+} by exchange with K^+ and then drives the exchange reaction to completion by precipitation of calcium and magnesium phosphates. The replacement of these polyvalent cations with K^+ disrupts cation bridging because K^+ does not completely satisfy the negative charge of the organic colloids resulting in expansion and increased solvation (*39*). Although the 0.5 M KCl can displace some of the Ca^{2+} and Mg^{2+} by exchange, Cl^- does not precipitate them nor does it form ion pairs to any significant degree. The greater recovery of [^{14}C]HADPs with the phosphate buffer as compared to 0.5 M KCl occurred because a significant portion of the HADPs were exchanged to soil organic matter (*20*, *40*).

Within analyte comparisons between extractants showed that HA recovery was by far greatest with the mixed-mode extractant, KH_2PO_4/CH_3CN (P = 0.001) followed by the phosphate buffer and the KCl/CH_3CN mixed-mode extractant (Table 2). All three of these extractants recovered significantly more HA than hydrophobic extraction alone. Although cation exchange was a key mechanism for HA binding to soil, mixed-mode binding was more critical for HA than for the N-dealkylated HADPs. The greater extent of mixed-mode binding exhibited by HA was consistent with previous observations that HA has the highest degree of amphipathic behavior among the three HADPs, such as high retention to both C_{18}- and SCX−SPE. Differences in recovery between extractants for DEHA showed that 0.5 M KCl was significantly lower than the other four extractants (Table 2). DEHA recovery was not enhanced by mixed-mode extraction. Based on the extraction recoveries, the contribution of either hydrophobic or cation exchange bonding to soils was similar for DEHA. In addition, DEHA binding to soil appears to be the weakest of the three HADPs since all extractants used resulted in recoveries of >50% (Tables 1 and 2). This may be an indication that weak electrostatic interactions, such as hydrogen bonding, play a significant role in DEHA binding to soil in addition to cation exchange and hydrophobic bonding. For DIHA, extractant effects showed that the two mixed-mode extractants and the phosphate buffer resulted in significantly higher recoveries than hydrophobic or 0.5 M KCl extraction (Table 2). Mixed-mode extraction by KCl/CH_3CN was also significantly higher than phosphate buffer alone. DIHA showed the greatest extent of cation exchange bonding relative to hydrophobic bonding of any of the HADPs with 2.3 times more DIHA extracted by phosphate buffer as compared to 25% CH_3CN. This observation was consistent with results that DIHA is the most polar of the HADPs. However, mixed-mode extraction did increase its recovery showing that mixed-mode binding was an important mechanism for DIHA sorption. Based on statistically significant differences between extractants, the extent of mixed-mode binding of HADPs to soil occurred in the order: HA > DIHA > DEHA.

Recovery of [^{14}C]HADPs from Aged Soil. Immediately after the 120-day incubation period, the aged soil samples were extracted three times with 9:1 $CH_3OH:H_2O$ (v/v) and combusted to determine bound residues, resulting in the following distribution: (% of applied ^{14}C): 5.8% atrazine; 0.3% deethylatrazine; 0.2% deisopropylatrazine; 0.5% hydroxyatrazine; 73% bound residue; and 14.2% as other polar metabolites, CO_2, and unidentified metabolites (*11*). After 2 years of frozen storage, the extracted samples were combusted again to determine the total ^{14}C remaining and to insure that no significant changes in the ^{14}C content of the samples had occurred during storage. The samples were then extracted three additional times with 9:1 $CH_3OH:H_2O$ (v/v), which recovered 6.0% of the ^{14}C. The reason for this second set of extractions was to ensure removal of any atrazine residues that may have become susceptible to aqueous methanol extraction in the process of freezing and thawing the samples. Following the second set of aqueous methanol extractions, extraction by KH_2PO_4/CH_3CN recovered 42.8% of the [^{14}C]-bound residue (Figure 6). HPLC fractionation of the mixed-mode extractable residues identified 88% of the ^{14}C in this fraction as HADPs with the majority identified as HA (Figure 6). Thus, these extractable bound residues were primarily HADPs formed from atrazine degradation in this soil and were bound by both cation exchange and hydrophobic interaction (mixed-mode). It should be noted that the amount of DIHA accounted for by HPLC fractionation may have been overestimated. Since DIHA elutes only 1 min after the void volume of the HPLC system, other early eluting compounds likely were included within this fraction. However, since this soil had been exhaustively extracted by aqueous methanol, the other compounds collected in the DIHA fraction would likely represent more polar HADPs, such as ammeline (2-hydroxy-4,6-diamino-*s*-triazine), ammelide (2,4-dihydroxy-6-amino-*s*-triazine), and cyanuric acid (2,4,6-trihydroxy-*s*-triazine). Ammeline and ammelide have been shown to sorb to Na-montmorillonite by cation exchange and weak elec-

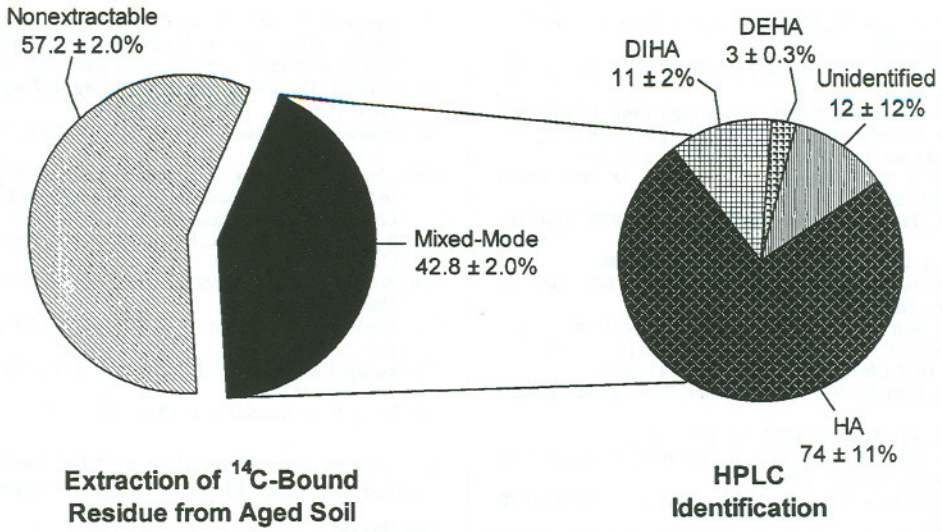

FIGURE 6. Mixed-mode extraction of bound residue from aged soil and HPLC identification of ^{14}C from the mixed-mode extractable fraction. The mixed-mode extractant was 3:1 0.5 M KH_2PO_4, pH 7.5:CH_3CN (v/v). Reported percentages represent the mean ($n = 3$) ± standard deviation.

trostatic interactions (*28*).

These results clearly showed that a significant proportion of the bound residue was simply sorbed by mixed-mode binding rather than irreversibly bound by various chemical and physical means as suggested by numerous studies (*8, 41–43*). These findings also confirm previous studies that established that ionic and physical bonding of the *s*-triazines to soil colloids depends upon the basicity and water solubility of the compounds, the pH of the system, and the type and amount of colloids present (*19, 20, 27, 28*). Furthermore, the mixed-mode extraction data were obtained by a method that had not been optimized for quantitation of HADPs in soils. Additional work with these bound residue samples showed that a third extraction by the KH_2PO_4/CH_3CN, following the two reported for Figure 6, extracted an additional 16.5%, bringing the total [^{14}C]bound residue extracted up to 59.3%. This suggested that additional extractions would likely have recovered even more bound residue. This third extraction was not used for HADP identification because of concerns associated with dilution of the ^{14}C in such a large volume of extractant, approximately 225 mL after CH_3CN evaporation. Nonetheless, the usefulness of this method for extracting HADPs and other polar triazine residues is evident, and further work to optimize this method for quantitation of HADPs in soils is currently underway.

These results refute the current view of covalent bonding as the primary mechanism for bound triazine residues in soil. The significance of these findings is that the majority of bound atrazine residues are sorbed by the mixed-mode mechanisms, and the bulk of these residues are HADPs. Therefore, past decomposition studies of triazine herbicides have likely overestimated the proportion of covalently bound residues. Future decomposition studies of triazines would benefit from the use of extraction procedures that target the appropriate soil binding mechanisms for the chlorinated and the hydroxylated residues.

The mixed-mode model of HADP binding to soils has implications to the fate of atrazine in the environment and to the geochemical mechanisms responsible for the presence of HADPs in surface water. These data suggest that the amount of HADPs formed by atrazine decomposition in soils has been underestimated by 60% or more, and it is therefore conceivable that soil-bound HADPs are the primary sink for atrazine in the environment prior to complete mineralization. With respect to the geochemistry of HADPs, sediment transport and subsequent desorption of HADPs from sediments have been implicated as a source of HADPs in streams (*32*). Further studies are underway to test the hypothesis that a significant proportion of HADPs are transported to streams sorbed to sediment.

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