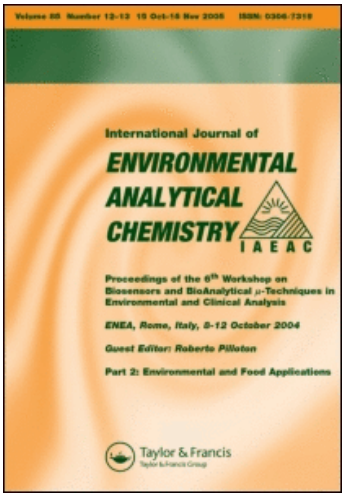

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Derivatization of Humic Compounds: An Analytical Approach for Bound Organic Residues

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DERIVATIZATION OF HUMIC COMPOUNDS: AN ANALYTICAL APPROACH FOR BOUND ORGANIC RESIDUES

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Bound residues of pesticides and their metabolites are defined as being nonextractable with organic solvents, but partly extractable together with the humic matrix by NaOH or other solvents suitable to extract humic compounds. Recently, an improvement in humus extraction from soils was achieved upon derivatization of the organic matter with silylating reagents at room temperature. By this method 70–90% of the organic carbon or nitrogen either from soil or from humin became soluble in organic solvents. The extracts were analyzed by means of ^{13}C NMR-spectroscopy. The spectra were well resolved with signal-separation of less than 1 ppm. The extracted humic compounds were of rather low molecular weight, ranging from 300 to 4000 to 6000 d or more.

^{14}C-labeled residues of pesticides or other xenobiotics found to be nonextractable after exhaustive organic solvent extraction became readily dissolved along with most of the humic matrix using this derivatization procedure. Between 60–80% of ^{14}C anilazine residues or of ^{14}C-labeled chlorinated phenols or anilines originating from both previously solvent extracted soil samples or from humin became solubilized in organic solvents.

KEY WORDS: Soil humus, derivatization with silylating reagents, ^{13}C NMR-spectroscopy, ^{14}C-labeled pesticides, bound pesticide residues, extraction upon silylation.

INTRODUCTION

Soil-bound residues of plant protection agents, their metabolites or of other xenobiotics are defined as being unextractable with organic solvents, but to be partly extractable together with the humic matrix by NaOH. Most of the studies about 'bound residues' are conducted with ^{14}C-labeled compounds, and ^{14}C-activity can be located in humic and fulvic acids or remains in the humin. Using organic solvents

this ^{14}C-activity cannot be removed, however, from those fractions as either parent compounds or their metabolites.

The ongoing discussion[1,2,3] indicates that organic xenobiotics can be sorbed or bound to humic compounds by various types of forces ranging from van der Waals forces, hydrogen bonds, charge transfer complexes and covalent binding. Hydrophobic sorption of nonpolar residues, trapping of molecules in a molecular sieve formed by humic materials, has been suggested[4,5]. Several of these mechanisms may operate with chemicals in soil leading to reversible sorption phenomena or to unextractable residues[6].

EXPERIMENTAL

Ten gram of dry soil or of humin was shaken with 100 mL 0.1 N NaOH under N_2 for 2 hours and sonicated for 3 min with a 300 W Labsonic 1510 sonifier (Fa. Braun, Melsungen, FRG). The suspension was immediately frozen and then lyophilized. Afterwards it was suspended in 40 mL dry acetone or tetrahydrofuran, and 5 mL of freshly distilled chlorotrimethylsilane was added under cooling with icewater. Then the suspension was shaken (100 rpm) under N_2 for 24 h and exclusion of moisture. The dark brown solution was immediately filtered. After several washings of the residue, the solvent, excess of the silylating reagence was evaporated i.v. at low temperature. Due to disturbance by silylated metalhydroxides and other paramagnetic compounds, the residue was dissolved in $CHCl_3$ and agitated for 10 min with icewater. The $CHCl_3$ was removed from the water-layer, dried and completely evaporated. Then the residue was dissolved in neat $CDCl_3$ and used for ^{13}C NMR measurements. The spectra were measured in 5mm tubes with a Bruker AC 200 NMR spectrometer at 50.3 MHz for ^{13}C under broad band decoupling.

For the preparation of 'soil bound residues' hundred gram of soil was mixed with 50 or 100 ppm of the respective ^{14}C-labeled compound and incubated at 60% WHC under aeration with moist CO_2-free air at 22°C for 8 weeks. Volatile ^{14}C from the ^{14}C-labeled compounds in the outgoing air stream was adsorbed in a cotton filter moistened with paraffin oil. Released CO_2 was trapped in dilute NaOH and analyzed for total and radioactive carbon. After incubation the soil was exhaustively extracted with organic solvents. The extracted soil was treated with chlorotrimethylsilane as described. Upon extraction of aliquots of the soil with 0.1 N NaOH and centrifugation to separate humic and fulvic acids, the residual and lyophilized humin was accordingly treated with chlorotrimethylsilane.

Distribution of molecular weights in silylated extracts from soil or humin was determined by gel permeation chromatographic separation as described by Kern[18]. The extracts were dissolved in tetrahydrofuran and analyzed by HPLC on microgel columns (Chrompak, Netherlands). THF was used as an eluent and polystyrene standards for calibration. The chromatograph was equipped with a UV-detector and with a detector for radioactivity.

RESULTS AND DISCUSSION

Soil Organic Matter Extraction Upon Silylation and Analytical Results

In recent years our understanding about the chemical nature of humic compounds has progressed considerably[7]. The limited solubility of soil organic matter in sodium hydroxide or sodium phosphate solutions, however, still hampers their analyses. Although by supercritical fluid extraction using organic solvents of different polarities larger amounts of humic compounds became dissolved, this method has the disadvantage that only specific fractions such as aliphatics, polysaccharides or more humic acid like compounds were isolated[8,9,10]. Due to the high temperatures applied for extraction, a partial destruction of humic polymers or of 'bound xenobiotics' may be possible.

Recently, an improvement for humus extraction from soil or from humin was achieved by derivatization of the organic matter with silylating reagents at room temperature[11,12]. After evaporation of the solvent and the excess of silylating reagent, the remaining reaction products probably contained most of the soil organic carbon. An exact estimation simply by carbon determination, however, was not possible, since trimethylsilylation of functional groups in organic and inorganic compounds falsified the C-balance. Since more than 70% of the nitrogen was solubilized too, a similar amount of solubilized C could also be assumed. Isolated humic or fulvic acids were completely dissolved upon derivatization. Similarly, most of the organic carbon remaining in the humin fraction of a soil after its exhaustive extraction with NaOH could be removed after treatment with the silylating reagents.

^{13}C NMR-spectroscopy turned out to be an effective tool and was central to the 'nondegradative' approach for elucidating humic structures. Usually humic compounds are measured either as solutions in deuterated NaOH or using the CP-MAS method. Although ^{13}C NMR-spectroscopy led to great progress in our understanding of the structure of humic compounds, signals in these NMR-spectra were not well resolved due to interferences by dissolved paramagnetic heavy metals or radicals. Therefore, the ^{13}C spectra generally show broad peaks which can hardly be assigned to distinct carbon species. An example of typical ^{13}C NMR spectra of humic and fulvic acids is shown in Figure 1.

The spectral range from 5–200 ppm is divided into four ranges of chemical shifts: 5–46 ppm designates the aliphatic region, 46–110 ppm the C—O/C—N region, 110–160 ppm the aromatic region and 160–200 ppm the carboxylic and C=O regions[14].

^{13}C NMR-spectra of humic compounds upon silylation show enhanced resolution when compared to those shown in Figure 1. An example for silylated humic compounds extracted from several soils is given in Figure 2.

Again the well defined signals were located within the four ranges of chemical shifts which are usually distinguished in the ^{13}C-spectra of humic compounds. Absorptions with separations of less than 1 ppm can be distinguished. A group of signals around 1 ppm originates from trimethylsilyl-groups in different chemical

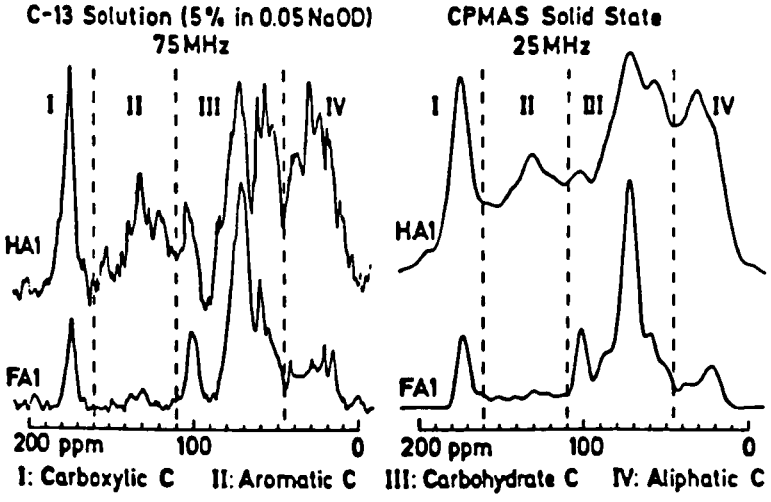


Figure 1 ^{13}C NMR-spectra of humic and fulvic acids from a Mollisol Rendoll[13].

environments. Numerous distinct signals are located in the aliphatic region from 5–46 ppm and can be assigned to CH_3—, CH_2— or CH— groups. Other signals from 46–110 ppm represent carbons in the C—O/C—N region and are assigned to those from carbohydrates, ethers or peptides. A triplet centered at 77.06 ppm arises from the $CDCl_3$ used as solvent for spectra aquisition. Other numerous resonances between 115–145 ppm belong, according to their chemical shifts, to aromatic carbons in different chemical environments, and those between 145–160 ppm to phenolic carbons. Distinct signals around 200 ppm originate from carbons in carboxyl or C=O groups.

A comparison of the spectra of the four silylated soil samples mediates the impression of a general close similarity among the extracted humic compounds. Sometimes groups of identical signals are present in each of the humic extracts from the four soils. Sometimes signals differ slightly in intensities or chemical shift values (e.g. those around 110 ppm). Spectra of lyophilized NaOH-extracts, representing the humic and fulvic acid fractions also had, upon silylation, a similar appearance to those of the spectra obtained from the whole soil. Resonances, however, are less numerous. Spectra of the humin, extracted from the silylated soil residue upon previous NaOH-extraction, also have a similar distribution in their resonance signals.

Humic compounds are mostly considered as polydisperse systems having a wide range of molecular weights, varying from a few hundred daltons in fulvic acids, to as much as several hundred thousand daltons for humic acids[15]. Methodologies for determining molecular sizes and weights of humic compounds, however, depend strongly upon experimental conditions because they undergo aggregation reactions[16]. The authors[16] as well as Wershaw[17] therefore, propose that particles of similar chemical structure are linked together by weak bindings to form mixed aggregates or micelles.

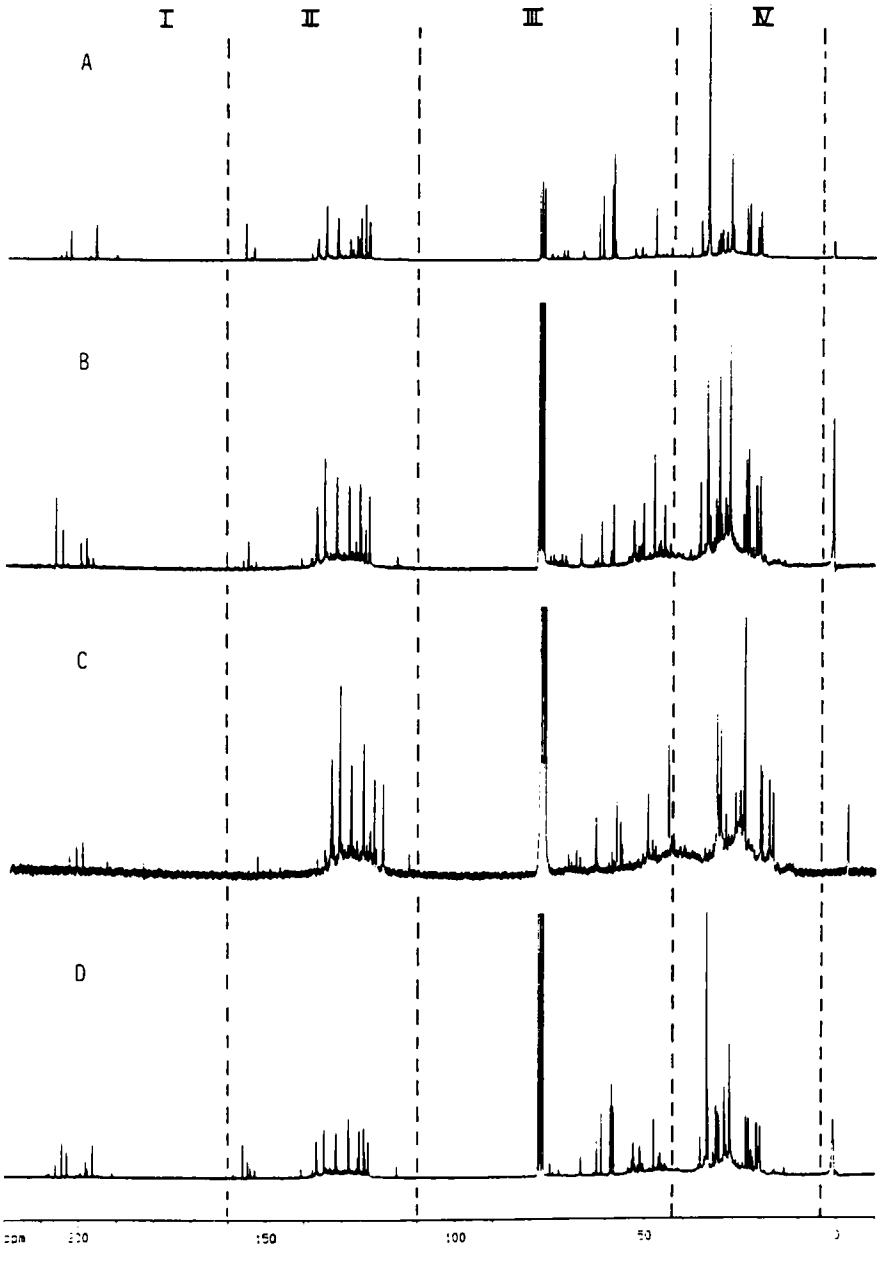


Figure 2 ^{13}C NMR-spectra of silylated soil samples; (A) Podzolic soil, (B) Chernozem, (C) Brown Earth and (D) Andosol[11,12].

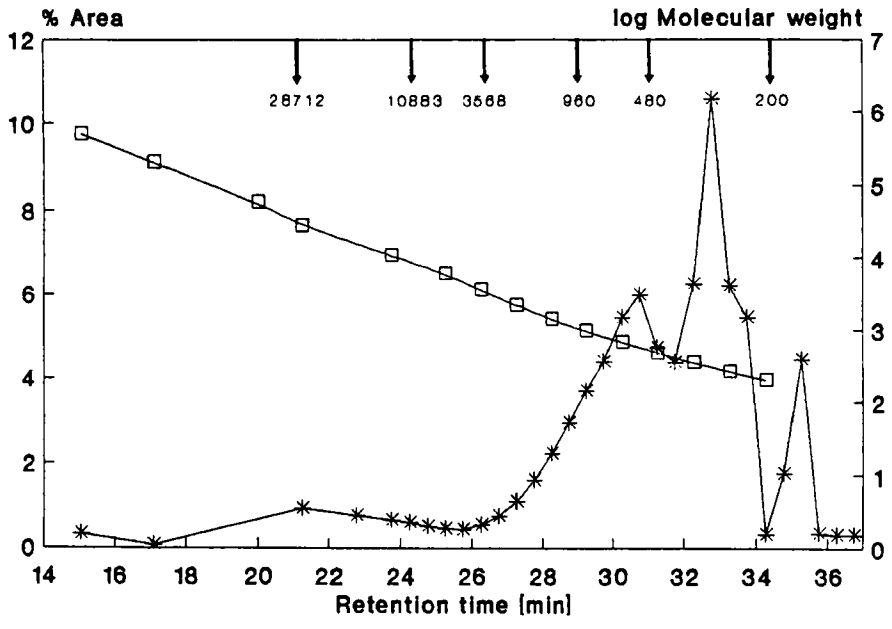


Figure 3 Molecular weight distribution of a silylated Brown Earth dissolved in tetrahydrofuran[11,12].

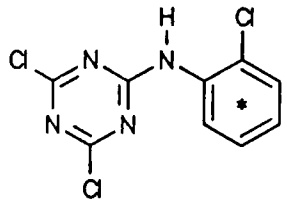
The molecular weight distributions of silylated extracts from soils or humus fractions were determined by gel-chromatographic separation[18]. It showed (Figure 3) that the majority had molecular weights between 300 to about 4000 d. Another small fraction was uniformly distributed between 6000 to 100,000 d. A rather discrete fraction at about 160 d probably belongs to hexamethyldisiloxane, a byproduct formed in the reaction of chlorotrimethylsilane with NaOH.

Our results may indicate a disaggregation by splitting of weak intermolecular bindings. Although we cannot yet exclude cleavage reactions of covalent bonds during the derivatization procedure, we propose that the trimethylsilylation of functional groups in the humic compounds protect smaller units from being aggregated into larger macromolecular structures. Wershaw[17] presented a new model for the structure of humic compounds in soils or sediments. In this model, humic materials are considered as membrane-like aggregates in which partially decomposed plant-derived compounds are held together by weak bonding mechanisms, such as π-complexing, hydrogen-bonding and hydrophobic interactions. According to our results this model seems to have good prospects.

Extractability of "Soil-Bound-Residues" Upon Silylation

The second part of this contribution deals with the improvements in extractability of residues from organic xenobiotics, which can be considered by definition as

Common Name : *Anilazine*
Trade Name : *®Dyrene (Bayer AG)*
Chemical Name : *4,6-Dichloro-N-(2-chloro-phenyl)-1,3,5-triazin-2-amine*
CAS-Reg. : *[101-05-3]*
Structural Formula



• *denotes position of the radiolabel*

Molecular Weight : *275.5*
Specific Activity [MBq/mg] : *1.97*
Hydrolysis in Buffers

pH 4	*22°C*	$t_{1/2}$	*30 d*
pH 7	*22°C*	$t_{1/2}$	*33 d*
pH 9	*22°C*	$t_{1/2}$	*22 h*

Water Solubility [g/l,20°C] : $8 \cdot 10^{-3}$
Octanol/Water
Partition Coefficient; logPow : *3.02*

Figure 4 Physical and chemical data of Anilazine.

"soil-bound-residues". Studies about these residues were generally made by application of distinct ^{14}C-labeled chemicals applied to soil.

Several residues of pesticides or other xenobiotics are known to have a high potential for the formation of "bound residues" within a period of weeks or months of soil incubation[19]. This tendency was shown for phenols, aromatic amines and their derivatives, carbamates and also for triazines or several organophosphates[20–23]. In some cases more than 50–80% of the compounds, applied in radioactive form, have been found to be unextractable from soil by organic solvents. The radioactivity is then mostly combined with soil organic matter fractions, such as fulvic and humic acids or remains sometimes preponderantly in the humin fraction. As well as several herbicides and insecticides, some fungicides and their metabolites also rapidly form "bound residues". An example is the fungicide Dyrene^{R}, which contains anilazine as an active ingredient (Figure 4).

If anilazine is applied in ^{14}C-labeled form within a few days after contact with the soil only 20% of the radioactivity can be extracted with organic solvents[24]. During soil incubation only very little of the radioactivity is evolved as CO_2. This was observed for both carbons of the triazine ring as well as with the aniline ring, with less than 1% CO_2 being evolved within 124 days in each cases[24,25].

According to several studies, this strong binding occurs by biotic or abiotic removal

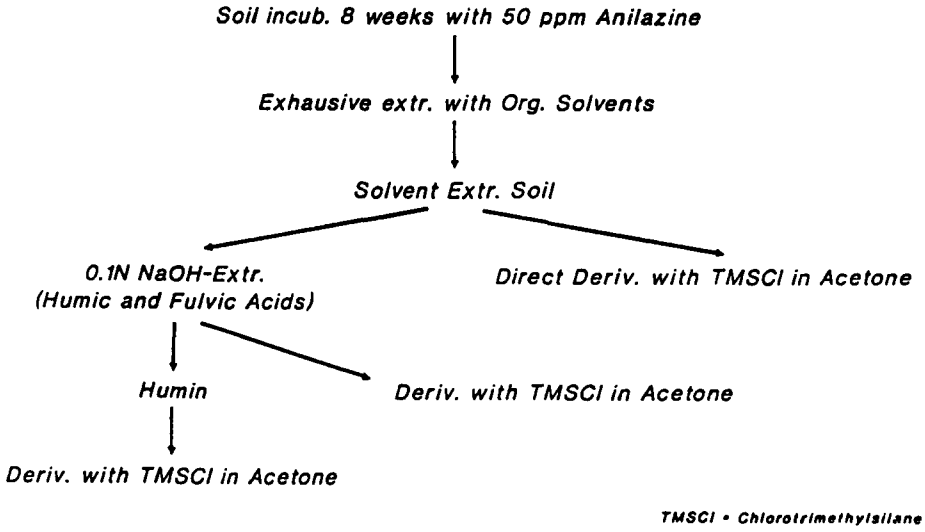


Figure 5 Extraction scheme of ^{14}C-anilazine-incubated soil.

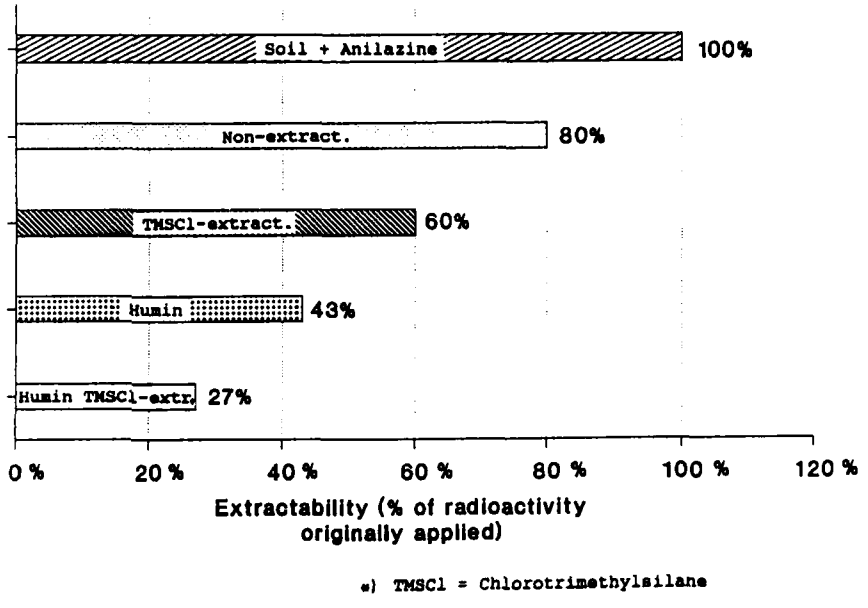


Figure 6 Extractability of "soil-bound" [phenyl-u-^{14}C] anilazine residues following derivatization with chlorotrimethylsilane.

of one of the two chlorine atoms from the triazine-ring to form a highly reactive intermediate which reacts with the soil organic matter.

We have applied anilazine, uniformly labeled by ^{14}C in the six carbons of the phenyl moiety, to different soils at a concentration of 50 $\mu g\, g^{-1}$ followed by incubation for 8 weeks. Only 20% or less of the total radioactive residue could be removed by exhaustive extraction with aqueous isopropanol, followed by several further extractions with isopropanol and CH_2Cl_2. Afterwards the solvent extracted dry soil was treated with dilute NaOH and after lyophilization it was derivatized with chlorotrimethylsilane in acetone or tetrahydrofuran for 24 hours at room temperature. The extractable radioactivity was determined either directly by liquid scintillation counting or after combustion.

Aliquots of the soil sample were first extracted with dilute NaOH to remove humic and fulvic acids and the radioactivity in the residual humin was determined. The organic matter contained in the humin fraction was also derivatized with the silylating reagent, extracted with organic solvents and measured for radioactivity. The results of these experiments are shown in Figure 6.

Eighty percent of the applied ^{14}C-activity remained in the soil after solvent extraction. Upon direct derivatization with chlorotrimethylsilane, 60% of the total applied radioactivity became solubilized, corresponding to 75% of the radioactivity remaining in the organic solvent extracted soil. Dilute NaOH extracted about 37% in the form of humic and fulvic acids, whereas 43% remained in the humin. Twenty-seven percent of the radioactivity in the humin could be removed upon derivatization which corresponded to more than 60% of its total ^{14}C-content.

A selection of ^{14}C-labeled chlorinated phenols and anilines which are known to form "bound residues", were incubated in Brown Earth for 8 weeks (Table 1). Between 20–60% of the applied ^{14}C was mineralized to CO_2 and another 8–60% could be removed from the incubated soil by organic solvent extraction. The unextractable activity remaining in soil after an 8 week incubation period ranged between 20–60%, and was highest for 2-chlorophenol and 4-chlorocatechol.

Table 1 Extractability of several 'soil bound' ^{14}C-residues following derivatization with chlorotrimethylsilane/acetone

^{14}C-Compounds 100 ppm	*% of $^{14}C_2$ Appl.*			*% of ^{14}C in Solvent-Extr. Soil*		
	% $^{14}CO_2\uparrow$ 8 Weeks	*Solv. Extract.*	*Unextr.*	*TMS/Acet. Extractab.*	*Humin*	*TMS/Acet. from Humin*
2-Cl-Phenol	22	20	58	65	52	30 (58)[1]
4-Cl-Phenol	44	10	46	53	54	32 (59)
2,4-di-Cl-Phenol	56	13	31	83	55	37 (67)
4-Cl-Catechol	31	8	61	72	46	25 (54)
2-Cl-Aniline	46	13	41	62	60	29 (48)
4-Cl-Aniline	13	63	24	82	65	42 (65)

Aerobic incubation of 100 g Brown Earth with 100 ppm of each ^{14}C-compound in 2 parallels for 8 weeks; means of 2 parallels.

[1] Values in parenthesis related to 100% ^{14}C-activity in humin.

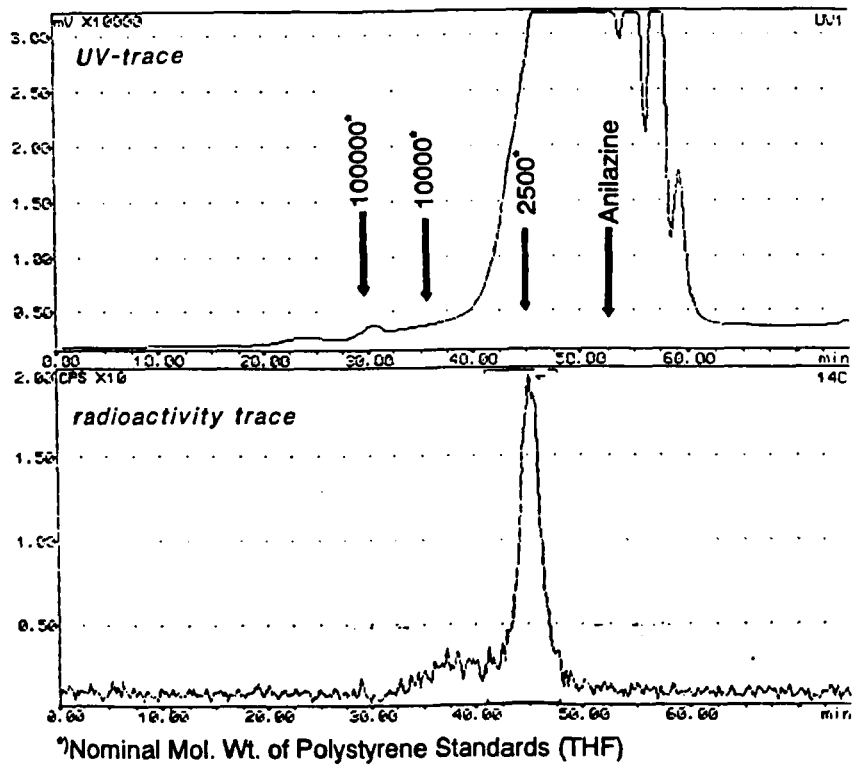


Figure 7 Gel filtration chromatography on ultrastyragel columns of the silylated extracts of aerobically incubated soil containing ^{14}C-anilazine-residues.

Generally, between 50–80% of the ^{14}C remaining as "bound compounds" in soil could be extracted upon derivatization with chlorotrimethylsilane. Furthermore, similar amounts of the ^{14}C-activities remaining in the humin could also be extracted by the same procedure.

All of the ^{14}C-compounds tested so far, including the residues from anilazine, could not be separated from the derivatized humic fractions from whole soil or humin. Several methods including thin-layer or column chromatography, HPLC or gas-chromatography failed to detect either the applied compounds or metabolites. Scanning of the column effluxes from a HPLC-gel column combined with an UV- and a radioactivity detector showed a similar molecular weight distribution as in Figure 3. The ^{14}C-activity of the unextractable anilazine residues appeared as a discrete radioactive fraction with a molecular weight of about 2.500 d (Figure 7).

CONCLUSIONS

The derivatization of humic compounds by silylating reagents looks promising as a way to a better understanding of the properties of this important group of natural

products. It furthermore, has potential to improve the analyses of the binding processes of xenobiotics or their metabolites to the humus matrix. Important in this respect seems to be the fact that the derivatization procedure results in relatively low molecular weight fractions of humic compounds containing the applied radioactivity. The monomeric radioactive compounds could not be separated from the humus matrix upon supercritical fluid extraction as formerly described for atrazine by Spiteller[26] as well as by Capriel and Haisch[27] or from prometryn upon pyrolysis as described by Khan[4]. It should be interesting to analyse the humus fractions containing ^{14}C-activity from "bound residues" by available methods for the mode of binding of the xenobiotics or their metabolites.

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