# Studies on the Nature and Identity of Bound Chloroaniline Residues in Plants

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When rice plants grown hydroponically were treated with [$^{14}$C]-3-chloroaniline or [$^{14}$C]-3,4-dichloroaniline, more than 40% of the $^{14}$C was found in the roots in isolated lignin fractions. A coniferyl alcohol polymerization system was developed for the preparation of a model copolymerization to yield synthetic chloroaniline–lignin copolymers. Gel permeation chromatography, mass spectrometry, $^{13}$C NMR, and pyrolysis–gas chromatography were used to study the structure of the synthetic copolymers. Results indicated that chloroanilines may be bonded covalently to lignin via 1,6 addition to a quinone methide intermediate during the lignin synthesis. The $\alpha$-carbon in the lignin side chain (i.e., the benzylic carbon) is the most likely chloroaniline nitrogen binding site.

The conjugation of pesticide chemicals and/or their metabolites with natural macromolecules in animals, plants, and soil organic matter to form the "bound" or unextractable residues has been reviewed (Kaufman et al., 1976). Chloroaniline compounds are known degradation products of substituted phenylamides (Kearney and Kaufman, 1969). A large number of phenylamide herbicide, fungicide, and insecticide chemicals are applied annually to the agricultural environment. Several studies have been reported on the fate of chloroaniline and anilide compounds in animals, plants, and soil (Parke, 1960; Leber and Freudenthal, 1976; Still and Mansager, 1975; Frear, 1975; Still et al., 1976; Balba etal., 1977; Chin et al., 1964, 1970, 1973; Bartha, 1971; Hsu, 1975). Most investigators have encountered the problem of bound residues with chloroaniline compounds. In most cases, however, the nature and identity, biological effect, and environmental fate of the bound residue fraction remain unknown. Only a few reports have indicated that lignin is the major bound residue fraction of aniline and chloroaniline compounds in plants (Chin et al., 1964, 1970, 1973; Yih et al., 1968). The present research is an attempt to understand the molecular nature and identitiy of bound 3-chloroaniline and 3,4-dichloroaniline residues in plants.

## MATERIALS AND METHODS

**$^{14}$C-Radiolabeled Chloroaniline Compounds.** [$^{14}$C]-3-Chloroaniline was obtained by the alkaline hydrolysis of [*phenyl*-$^{14}$C(U)]-3-chloroacetanilide, purchased from Pathfinder Laboratories, Inc., St. Louis, MO (3.74 mCi/mmol). [$^{14}$C]-3,4-Dichloroaniline (1 mCi/mmol) was prepared by the alkaline hydrolysis of [*phenyl*-$^{14}$C(U)]-*N*-(3,4-dichlorophenyl)-*N'*,*N'*-dimethylurea (diuron) synthesized Tanaka (1970). [$^{14}$C]-3-Chloroaniline and [$^{14}$C]-3,4-dichloroaniline were purified by TLC. Desired specific radioactivity was obtained by the addition of either 3-chloroaniline (bp 112 °C/16 mmHg) or recrystallized 3,4-dichloroaniline (mp 71–72°C) to the corresponding [$^{14}$C]chloroaniline.

**Treatment of Plants.** Thirty-day-old rice plants (Nato) were grown hydroponically in vermiculite trays and treated with radioactive chloroaniline compounds. Treating solutions contained either $5 \times 10^{-5}$ M [$^{14}$C]-3-chloroaniline (sp act. 50.63 $\mu$Ci/mmol) or $5 \times 10^{-5}$ M [$^{14}$C]-3,4-dichloroaniline (sp act. 52.36 $\mu$Ci/mmol) in one-third strength Hoagland's solution. The plants were exposed to the treating solution for 15 days, removed from trays, and separated into roots and shoots.

**Isolation of Lignin Fractions from the Roots of Rice Plants.** Harvested roots from the [$^{14}$C]-3-chloroaniline treatment weighed 1500 g (fresh weight) and those from the [$^{14}$C]-3,4-dichloroaniline treatment weighed 1130 g (fresh weight). Root tissues were fractionated as shown in Figure 1. They were cut into small pieces (~2 cm), freeze-dried, and ground with a Wiley mill (20 mesh). Ground tissues were Soxhlet extracted with benzene–ethanol (2:1) and ethanol and washed with a stream of water at 50 °C (~3 L). Extracted tissues were then washed with ethanol, dried under vacuum at 50 °C, ground again with a Wiley mill (40 mesh), and ball milled under toluene and nitrogen for 4 days at 0 °C. The resultant slurry was centrifuged and the precipitate was filtered under nitrogen with suction to remove the toluene.

Bjorkman lignin was obtained from this material by the repetitive extraction with dioxane–water (9:1) as described by Freudenberg and Neish (1968). Each extraction was carried out under nitrogen at 0–4 °C with 250 mL of dioxane–water and a high-speed Omni-mixer. Extractions were repeated a minimum of 5 times or until the extract was essentially colorless. The combined dioxane–water extract was concentrated under vacuum at 45–50 °C until a brown viscous residue remained in the flask. Water (100 mL) was then added with stirring to precipitate Bjorkman lignin. The mixture was transferred to centrifuge tubes, purged with nitrogen, and centrifuged at 5 °C for 1 h at 10000*g*. The Bjorkman lignin was freeze-dried, weighed, analyzed for $^{14}$C, and stored under nitrogen in a desiccator at 0 °C in the dark.

Dioxane acidolysis lignin was prepared from the residue after the extraction of Bjorkman lignin, as described by Chin et al. (1964). The residue was mixed in a refluxing flask with 500 mL of dioxane–2 N HCl (9:1). The mixture was stirred with a magnetic stirrer, refluxed under nitrogen at 87 °C for 30 min, cooled to room temperature, centrifuged, and reextracted until no significant $^{14}$C was measured in the extract. Combined acidic dioxane extracts were evaporated under vacuum until a viscous brown residue remained. One liter of water was added to the residue with stirring until a suspension of fine particles was obtained. The particles were removed by centrifugation, washed with water, and recentrifuged. Washed particles were dissolved in dioxane–water (9:1), centrifuged to remove insoluble matter, and concentrated under vacuum at 45–50 °C to a viscous brown residue. The dioxane acidolysis lignin was precipitated by the addition of water

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with stirring. The precipitate was centrifuged at 5 °C for 1 h at 10000g, freeze-dried, weighed, analyzed for $^{14}C$, and stored under nitrogen in a desiccator at 0 °C in the dark.

**Incorporation of Chloroaniline Compounds into Synthetic Lignin: A Model System.** Coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol) was prepared from 4-hydroxy-3-methoxycinnamic acid (ferulic acid) as described by Balba and Still (1978) and polymerized with or without [$^{14}C$]chloroaniline compounds. Three 2-L reservoirs (A, B, and C) were used to feed a proportioning pump. In flask A, 0.85 g of coniferyl alcohol (4.725 mmol) and 40 mg of horseradish peroxidase (EC 1.11.1.7, type II, purchased from Sigma Chemical Co.) were dissolved in phosphate buffer (pH 7.2, 0.01 M). In flask B, 0.5 mL of 29–30% hydrogen peroxide from a freshly opened bottle (~4.7 mmol) was dissolved in phosphate buffer (pH 7.2, 0.01 M). In flask C, 4.725 mmol of [$^{14}C$]chloroaniline compound was dissolved in phosphate buffer. Flask C was omitted when coniferyl lignin was the desired product. Equal volumes were used in the three reservoirs to maintain a constant molar addition ratio. The volumes depended on the solubility of chloroaniline compounds. 3-Chloroaniline was dissolved in 250 mL of phosphate buffer (pH 6.2, 0.01 M). 3-Chloroacetanilide required twice the volume of the same buffer (500 mL) for complete solution. To dissolve 3,4-dichloroaniline, it was necessary to use 500 mL of pH 3 buffer in flask C. In this case, 500 mL of phosphate buffer, pH 8, was used in flasks A and B, respectively, in order to keep the pH of the final reaction at 6–7.

The three solutions were simultaneously pumped at 0.44 mL/min into a three-necked round-bottom flask (3 L) containing 10 mg of peroxidase, and 15 mg of vanillyl alcohol dissolved in 100 mL of phosphate buffer (pH 7.2, 0.01 M) was added as an initiator. The reaction was kept under nitrogen and stirred continuously during the addition of reactants. The reaction flask was covered with a black blanket to avoid possible photochemical reactions. The reaction mixture was stirred for 10 h after the addition of reactants. The contents of the flask were transferred to centrifuge tubes, purged with nitrogen, and centrifuged for 1 h at 10000g. The supernatant was removed. The precipitate was washed with 0.01 N HCl to remove free aniline and recentrifuged. The acid wash was added to the supernatant pool. The precipitate was washed twice with distilled water, centrifuged, freeze-dried, weighed, analyzed for $^{14}C$, and stored in a desiccator under nitrogen at 0 °C in the dark. The average molecular weights and the N % and C % were determined by Schwarzkopf Microanalytical Laboratory, Woodside, NY.

**Acetylation of Lignin or Synthetic Chloroaniline–Lignin Copolymer Fractions.** Microstyragel (Waters Associates) columns used for the gel permeation chromatography of natural and synthetic lignin fractions were compatible with solvents of limited polarity. Therefore, it was necessary to acetylate lignin samples prior to chromatographic separation on these columns. Samples of isolated lignin or synthetic chloroaniline–lignin copolymer fractions (100 mg) were dissolved in 3 mL of acetic anhydride and 0.1 mL of pyridine. The reaction mixtrue was refluxed for 1 h, made alkaline by slowly adding an excess of 10% solution of sodium carbonate, and extracted several times with chloroform. The chloroform extract was filtered through anhydrous sodium sulfate and evaporated to dryness, and the residue was dissolved in tetrahydrofuran for gel permeation chromatography.

**General Techniques.** A Packard Tri-Carb Model 3375 liquid scintillation spectrometer was used for the determination of $^{14}C$. The $^{14}C$ in liquid samples was determined by counting an aliquot in 10 mL of Insta-gel (Packard Instrument Co.). In solid samples, the $^{14}C$ was determined by combustion with a Packard Tri-Carb 306 oxidizer and liquid scintillation counting of evolved $^{14}CO_2$. Quench corrections were made by the external standard method. Chromatographic spearations were obtained by gas–liquid chromatography (GC) and high-performance liquid chromatography (HPLC). A Micro-Tech-Tracor gas chromatograph with a flame ionization detector and 1:1 precalibrated splitter was used for the separation, isolation, and tentative identification of compounds. U-shaped glass columns, 122 cm long and 0.8 cm o.d., were packed with either 3% OV-17 on 60–80-mesh Gas-Chrom Q or 3% Silar-9CP on 100–200-mesh Gas-Chrom Q. Operating conditions were as follows: detector temperature, 280 °C; inlet temperature, 250 °C; either helium or nitrogen carrier gas flow rate, 50–60 mL/min. Column temperatures varied according to experimental needs.

Synthetic and natural lignin fractions were analyzed by pyrolysis–gas chromatography (Py–GC). For microsamples, a ribbon pyroprobe with a temperature control and an interface was attached to the inlet of the gas chromatograph (Chemical Data Systems, Inc., Oxford, PA). A dimethylformamide solution (10–20 μL) of lignin sample (≃100 μg) was placed on the pyroprobe ribbon with a microsyringe. The sample was heated at 100–150 °C under nitrogen to remove the solvent. The probe was then inserted into the interface, and the sample was pyrolyzed at 700 °C for 2 s. Helium was the carrier gas for all Py–GC samples. Pyrolysis products were collected from the gas chromatograph for $^{14}C$ determination and identification. A special pyrolysis system described by Balba et al. (1979) was used for the pyrolysis of macrosamples (100 mg). The sample was placed in a platinum boat and pyrolyzed under helium at 550 °C in a modified combustion tube. The pyrolysate was then collected, analyzed for $^{14}C$, and chromatographed for the identification of the pyrolysis products.

A Waters Associates HPLC system with two pumps, a solvent programmer, and a UV detector (254 nm) was used for high-performance liquid chromtography. Two Microstyragel columns (6.4 mm i.d. × 30 cm) were used for gel permeation chromatography (GPC). A 500 Å pore size column and a 1000 Å pore size column were connected in series. Tetrahydrofuran was used as the eluting solvent at a flow rate of 1 mL/min. Different molecular weight fractions of the acetylated lignin or copolymers were collected from the second cycle chromatogram. A μBondapak C-18 column (6.4 mm × 30 cm) was used for adsorption chromatography. A linear water–acetonitrile gradient from 10 to 100% acetonitrile was used. Mass spectrometry was used to verify proposed chemical structures. A Varian CH-5 mass spectrometer was used for isolated samples collected from the gas chromatograph, and a Hewlett-Packard 5992 gas chromatograph–mass spectrometer was used for direct GC–MS analysis. The $^{13}C$ NMR studies were used to elucidate the possible aniline binding site(s) in the synthetic copolymers. A 20% solution of lignin and [$^{14}C$]-3-chloroaniline lignin copolymer was prepared in dioxane-$d_8$–$D_2O$ (9:1). $^{13}C$ NMR spectra were obtained with a Bruker WH-90 NMR spectrometer operated by Fourier transform. The protons were decoupled and ~100 000 transients were accumulated in the long-term averaging mode.

## RESULTS AND DISCUSSION

**Association of the Bound Chloroaniline Residues with Lignin.** Still et al. (1976) concluded that 30% of the

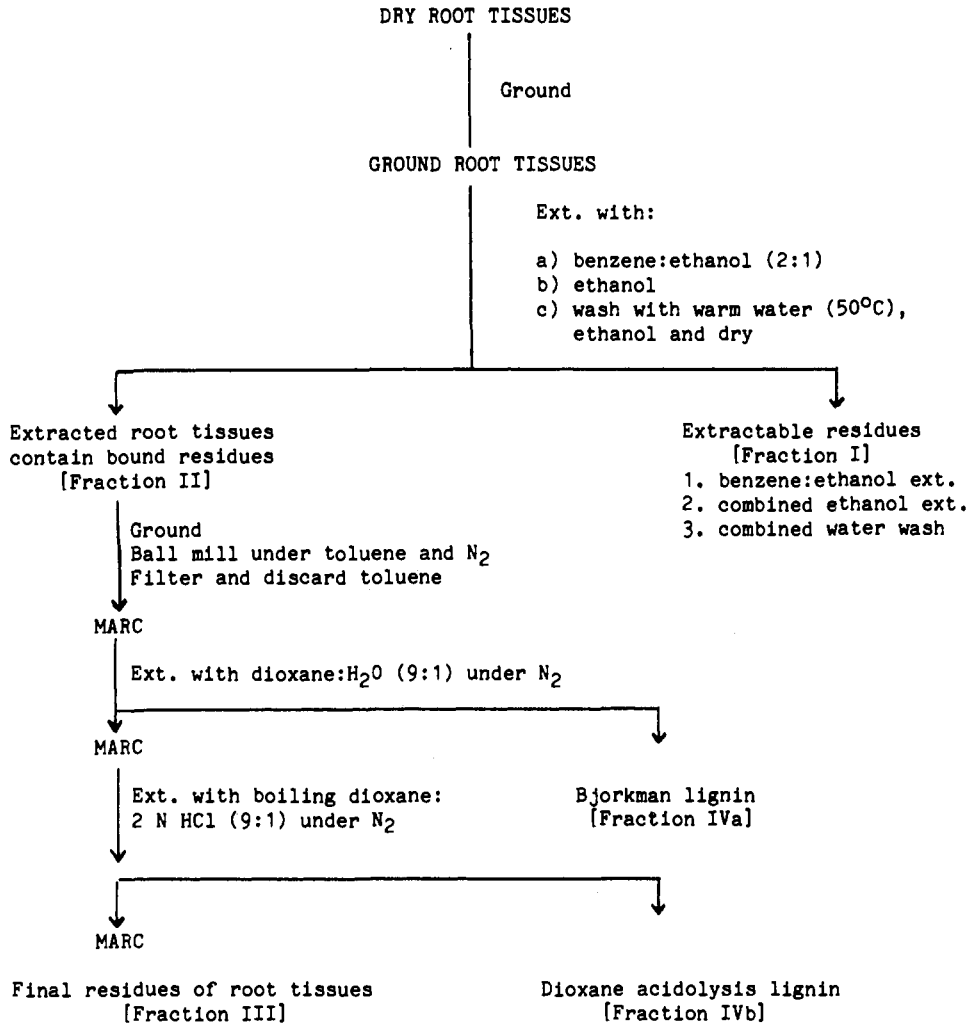



**Figure 1.** Fractionation of root tissues.

[$^{14}$C]-3,4-dichloroaniline applied in hydroponic solution to rice plants was incorporated in the root tissues as an unextractable (bound) residue. This residue remained after exhaustive sequential extraction with nonpolar and polar solvents. When the fraction was analyzed by using the Association of Official Agricultural Chemists (1965) indirect method for the determination of lignin, 65.4% of the $^{14}$C was in the plant lignin. Although the weight of isolated lignin recovered by this method is generally considered as an acceptable measurement of lignin content, the chemical structure of the residual material is completely different from that of the natural lignin due to the vigorous conditions required. Accordingly, this fraction was not used for structural studies.

Bjorkman lignin or milled wood lignin (MWL) preparations have been used as standards for structural investigations of intact lignin. However, those portions of lignin that are grafted to polysaccharides or large molecules are excluded in Bjorkman lignin extraction. Dioxane acidolysis was used to obtain excluded lignin fractions. Such preparations undergo only limited molecular alteration due to the hydrolytic conditions used (Lai and Sarkanen, 1971). Studies on the distribution of $^{14}$C in the roots of rice plants treated with [$^{14}$C]chloroaniline compounds showed that lignin fractions (Bjorkman and dioxane acidolysis lignin preparations) contained 40.4 and 56.1% of the total $^{14}$C in the plants that were treated with 3-chloroaniline and 3,4-dichloroaniline, respectively (Table I). On an equal molar basis using specific activities of [$^{14}$C]chloroaniline compounds and an arbitrary average molecular weight of

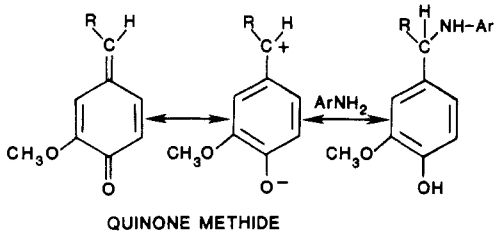
Table I. Distribution of $^{14}$C in Isolated Fractions from Roots of [$^{14}$C]Chloroaniline-Treated Rice

| treatment | extractable residues (fraction I),[a] % | combined lignin fractions (fractions IVa and IVb), % | final residues (fraction III), % |
|---|---|---|---|
| [$^{14}$C]-3-chloroaniline | 32.0[b] | 40.4 | 27.5 |
| [$^{14}$C]-3,4-dichloroaniline | 33.6 | 56.1 | 10.3 |

[a] Figure 1. [b] Percentages are relative to the recovered $^{14}$C (114.1% for 3-chloroaniline and 108.1% for 3,4-dichloroaniline).

2000 for the isolated lignin, 0.04 mol of 3-chloroaniline and 0.08 mol of 3,4-dichloroaniline were incorporated per mol of lignin. Incorporation of 3,4-dichloroaniline was greater than that of 3-chloroaniline in lignin fractions IVa and and IVb but less than that of 3-chloroaniline in the residual carbohydrate fraction III (Figure 1). One explanation of these data is to consider the fact that 3-chloroaniline is more prone to aryl hydroxylation than 3,4-dichloroaniline. Therefore, the resulting phenolic hydroxyl groups may conjugate preferentially with carbohydrates while 3,4-dichloroaniline may conjugate preferentially with lignin.

Know precursors of lignin are 4-coumaryl, coniferyl, and sinapyl alcohols. Under the influence of oxidative enzymes, mainly laccases and peroxidases, these alcohols undergo polymerization to form lignin. Freudenberg and his group




QUINONE METHIDE

R=-CH[$CH_2OH$][$OC_6H_3$(o-$OCH_3$)(p-CH:CH $CH_2OH$)]

**Figure 2.** Postulated mechanism of arylamine ($ArNH_2$) incorporation into lignin via addition of the quinone methide intermediate.

Table II. Incorporation of [$^{14}$C]Chloroaniline Compounds into Lignin by Copolymerization with Coniferyl Alcohol

| [$^{14}$C]chloroaniline compd | molar ratio of [$^{14}$C]-chloroaniline: coniferyl alcohol | molar ratio of chloroaniline: polymer as calculated by | | |
|---|---|---|---|---|
| | | sp act. | Cl % | N % |
| 3-chloroaniline | 1:1 | 1.19[a] | 1.17 | 1.52 |
| 3-chloroaniline | 1:10 | 0.29 | | |
| 3,4-dichloroaniline | 1:1 | 1.68[a] | 1.94 | 2.43 |
| 3-chloroacetanilide | 1:1 | 0.44 | | |

[a] Average molecular weights of copolymers were determined by osmometry: 3-chloroaniline–lignin copolymer = 1015; 3,4-dichloroaniline–lignin copolymer = 1320.

established a model system for studying coniferyl alcohol polymerization. It was proposed that the polymerization proceeds via a free radical mechanism. Quinone methide was found to be an initial intermediate in the polymerization process (Freudenberg and Neish, 1968). These same investigators found that enols add to quinone methide via a 1,6 addition. Accordingly, it is possible that chloroaniline compounds also undergo 1,6 addition to a quinone methide intermediate with the nucleophilic nitrogen bonded to the electrophilic carbon of the quinone methide and the hydrogen shifted to the oxygen (Figure 2). Results obtained from studies with model coniferyl alcohol–chloroaniline polymerization are consistent with such a hypothesis. The degree of incorporation of [$^{14}$C]chloroaniline compounds into synthetic lignin is shown in Table II.

When 3-chloroaniline was acetylated, incorporation dropped from 1.19 to 0.44 mol of chloroaniline compound/mol of polymer. These data suggested that the free amine group plays an important role in the incorporation of 3-chloroaniline into the synthetic coniferyl lignin. These data also agreed with the postulated (1,6) addition of the aromatic amine to a quinone methide intermediate. The incorporation of 3-chloroacetanilide may be due either to the formation of aryl hydroxyl groups that add to the quinone methide or to the partial hydrolysis of the acetanilide to the free amine. When the ratio of 3-chloroaniline to coniferyl alcohol was shifted from 1:1 to 1:10, there was a concomitant decrease from 1.2 to 0.3 in the molar ratio of chloroaniline incorporated into the polymer molecules. However, the decrease was not in a linear relationship with the ratio of the reactants and may indicate a saturation limit for chloroaniline incorporation.

A comparison of the incorporation of 3-chloroaniline and 3,4-dichloroaniline into synthetic lignin indicated that the degree of incorporation of 3,4-dichloroaniline (1.68) was slightly higher than that of 3-chloroaniline (1.19). Blocking the para position with chlorine appears to improve the incorporation. The higher degree of incorporation of 3,4-dichloroaniline also occurred in the natural lignin isolated from the roots of chloroaniline-treated rice (Table I). This result may be attributed to the possible para hydroxylation of 3-chloroaniline. A second reactive site in the aniline ring may permit the addition of a second quinone methide intermediate. The addition may be either an intermolecular addition producing a copolymer of higher molecular weight, as found by gel pemeation chromatography, or an intramolecular addition resulting in a lower degree of incorporation.

**Possible Binding Site(s) of Chloroaniline Compounds with Synthetic and Natural Lignin.** Lignin, natural or synthetic, does not have a defined or ordered structure like other natural polymers such as polysaccharides or proteins. Lignin structure is rather unpredictable due to the variations in the structures of the monomers, possible sites of bonding in each monomer, and the degree of polymerization. Freudenberg's schematic formula for spruce lignin is used frequently as a lignin structural model (Freudenberg and Neish, 1968). In this structure, the chloroaniline compounds may bind with a monomer side chain or aromatic ring structure, or it may be trapped inside the cage of the lignin molecule without chemical bonding, i.e., an inclusion type of association as was suggested by Chin et al. (1964).

Figures 3A and 4A show the gel permeation chromatograms of acetylated synthetic lignin–[$^{14}$C]chloroaniline copolymers. These chromatograms included a major fraction that contained 82.0 and 83.3% of the chromatographed $^{14}$C for 3-chloroaniline– and 3,4-dichloroaniline–lignin copolymers, respectively (see shadowed areas). This major fraction was followed by three relatively minor smaller molecualr weight $^{14}$C-labeled fractions including the respective chloroacetanilides. It is not known if the acetylated [$^{14}$C]chloroanilines were originally free in the synthetic copolymer or were liberated from the copolymer during the acetylation process. The elution curve was symmetrical and indicated a uniform distribution of molecular weights around the average molecular weight. Measurements of $^{14}$C along the HPLC elution curve of the synthetic copolymer suggested that 3-chloroaniline was incorporated in larger copolymer molecules than 3,4-dichloroaniline. However, this is contrary to the osmometric measurements which showed the 3,4-dichloroaniline–lignin copolymer to be slightly larger (Table II).

Fractions that contained the highest concentration of $^{14}$C were rechromatographed on μBondapak C-18 to isolate selected molecular species (Figures 3B and 4B). Peaks that contained the highest concentrations of $^{14}$C were collected and mass spectra were obtained (Figures 3C and 4C). Mass spectra of isolated molecular species of acetylated 3-chloroaniline–lignin or 3,4-dichloroaniline–lignin copolymers (Figures 3C and 4C) showed chlorinated ion fragments at $m/e$ 304 or 338, respectively. Further fragmentation of these ion fragments suggested that peaks at $m/e$ 304 and 338 corresponded to structures I and II, respectively. the relative abundance of the molecular ions and ion fragments in the mass spectra of *N*-alkylbenzylanilines (Stenhagen et al., 1974) were compared to support this contention. It was noticed that, in many cases, as the size of the alkyl group increased, the relative abundance of the molecular ion decreased and was associated with a noticeable increase in the $(M - R)^+$ fragment. In the proposed structures I and II, a large alkyl group associated with the rest of the lignin molecule was present and an expected fragment was obtained. As a general rule, cleavage of *N*-alkylarylamines occurs at the C–C bond next to the nitrogen atom (Silverstein and Bassler, 1967), and

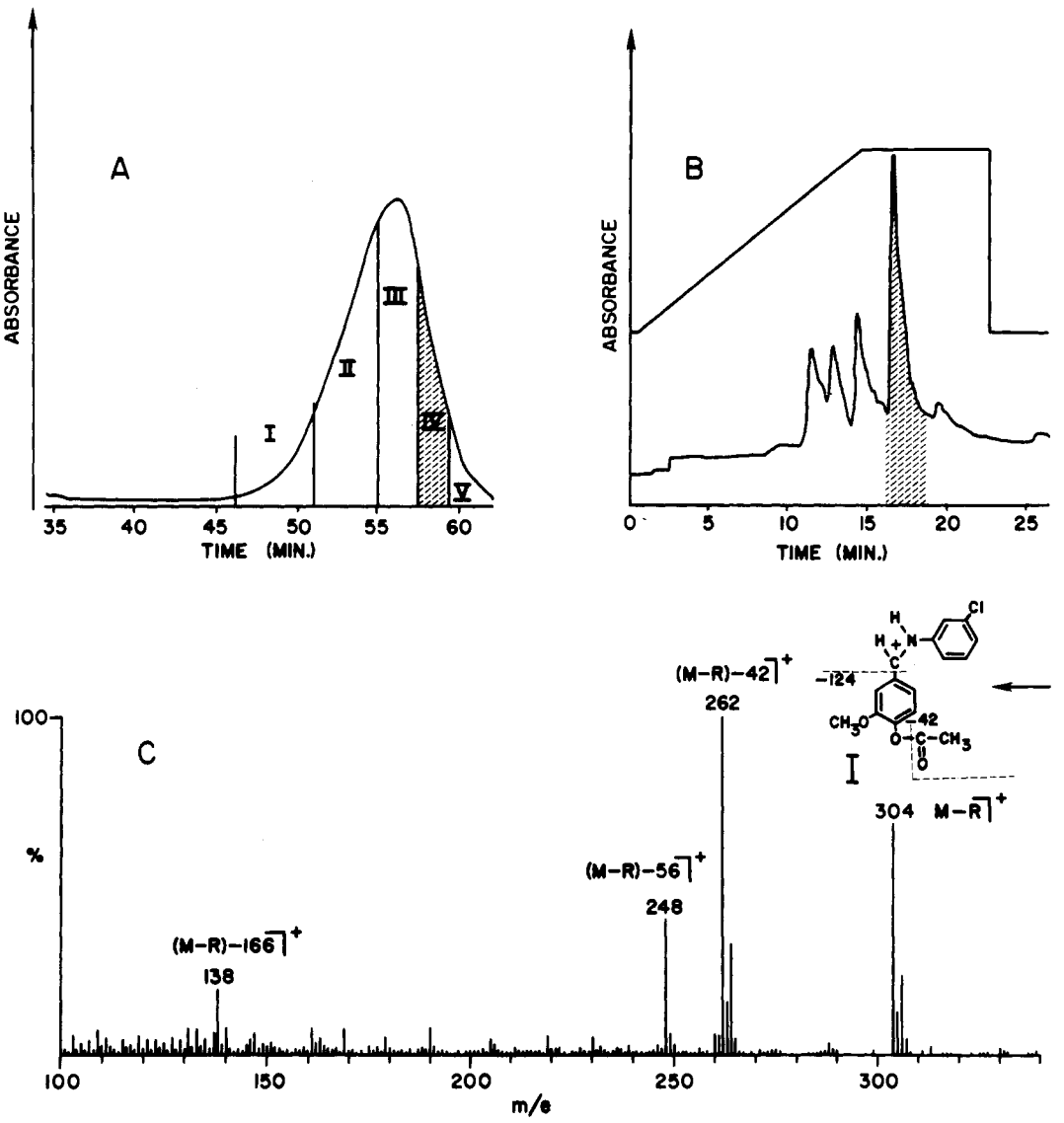



**Figure 3.** (A) Gel permeation chromatogram (GPC) of the synthetic acetylated 3-chloroaniline–lignin copolymer. (B) Reverse-phase adsorption chromatogram of fraction IV collected from the GPC. (C) Mass spectrum of the isolated shadowed peak in (B).

the alkyl group usually leaves as an undetected radical. These results agree with the hypothesis of a 1,6 addition to the quinone methide intermediate as a possible mechanism of chloroaniline incorporation.

All signals in $^{13}C$ NMR spectra of synthetic lignin corresponded closely to the published spectra of natural and synthetic lignin (Ludemann and Nimz, 1973: Nimz and Ludemann, 1974; Nimz et al., 1974) and permitted peak assignments (Figure 5A). In the aliphatic region (lower than 100 ppm) of the synthetic lignin and the 3-chloroaniline–lignin copolymer spectra (parts A and B of Figure 5), the intensity of peaks with chemical shifts at 87.3, 73.1, and 62.1 ppm increased in the copolymer spectrum relative to that of the syntheitc lignin. An additional signal at 59.5 ppm was present in the copolymer spectrum. The increase in the intensity of these peaks was proportional to the degree of 3-chloroaniline incorporation in the copolymer, as was established by comparison of the 1:10 and 1:1 copolymers. It should be noted that all the chemical shifts of the chloroaniline carbon atoms were in the aromatic region (greater than 100 ppm). Thus, observed differences in $^{13}C$ NMR spectra suggested that changes in the chemical environment of the carbon atoms in the aliphatic side chain of the synthetic lignin are due to the incorporation of 3-chloroaniline and indicated that there is a covalent bond between the chloroaniline and the side chain of the lignin molecule.

The benzylic carbon (the $\alpha$-carbon) bonded to the nitrogen of 3-chloroaniline or to the oxygen of 4-hydroxy-3-chloroaniline may have a chemical shift at 59.5 ppm. Several attempts to prepare model compounds to determine the chemical shift of the benzylic carbon when bonded to an aniline nitrogen were unsuccessful. Reductive alkylation of 3-chloroaniline or aniline with *p*-hydroxyacetophenone, vanillin, or 4-acetylvanillin were not successful. Therefore, conclusive evidence that these changes in the spectrum of the copolymer are due to a change in the chemical shift of the benzylic carbon was not obtained.

When synthetic chloroaniline copolymers were pyrolyzed in a pyrolysis tube, more than 85% of the $^{14}C$ was recovered in the pyrolysate. Analysis of the collected pyrolysates showed that 3-chloroaniline or 3,4-dichloroaniline accounted for more than 65% of the $^{14}C$ present in the pyrolysates. Pyrolysates of the isolated dioxane acidolysis lignin contained only 50% of the $^{14}C$ present in this fraction, and intact chloroaniline compounds accounted for only ~20% of the $^{14}C$ in the pyrolysates. The reason for the relatively low $^{14}C$ recoveries from the dioxane acidolysis pyrolysates is uncertain. Nearly quantitative

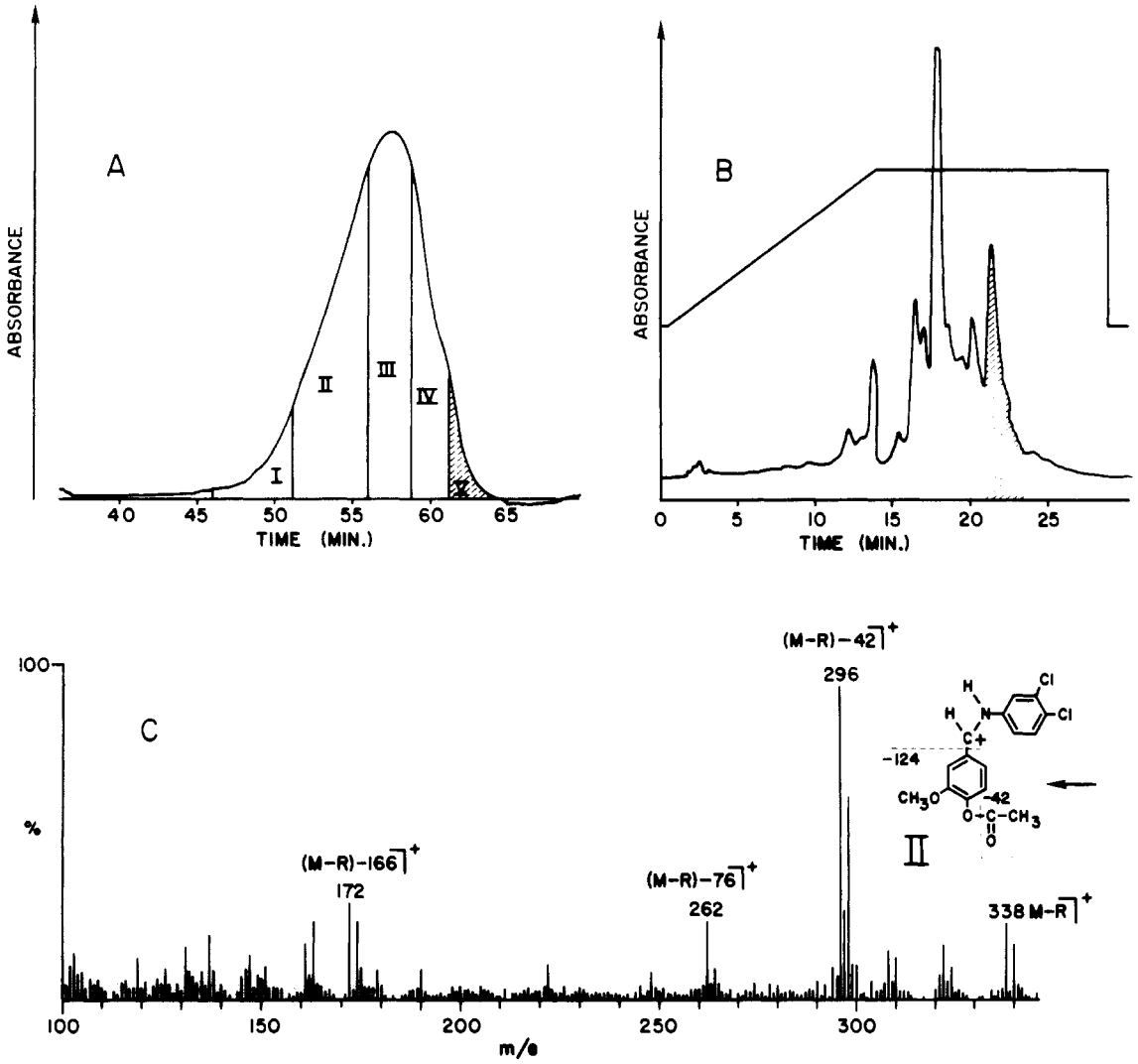



**Figure 4.** (A) Gel permeation chromatogram (GPC) of the synthetic acetylated 3,4-dichloroaniline–lignin copolymer. (B) Reverse-phase adsorption chromatogram of fraction V collected from GPC. (C) Mass spectrum of the isolated shadowed peak in (B).

$^{14}C$ recoveries were obtained in pyrolysates of the plant bound residue fraction (fraction II, Figure 1). These data suggested that considerable alteration in lignin structure occurred during the isolation of the acidolysis lignin fraction. These results have been reported by Balba et al. (1979). Structures of the liberated chloroanilines were verified by GC–MS of the pyrolysates. A considerable portion of the incorporated chloroanilines appeared to be bound to lignin without modification of the chloroaniline ring. The only modification of the ring structure was observed in pyrolysates of the 3,4-dichloroaniline–lignin copolymer. In addition to 3,4-dichloroaniline, there was a limited amount of 3-chloroaniline. This may indicate that 3,4-dichloroaniline undergoes dechlorination during the peroxidase copolymerization (Holland and Saunders, 1968).

Pyroprobe–gas chromatography (Py–GC) was used in initial studies on the pyrolysis of synthetic copolymers. Results of these studies were, for unknown reasons, different from those obtained with the pyrolysis tube. The major $^{14}C$ component ($\simeq$50%) in pyrolysates of the 3-chloroaniline copolymer was identified as 1,2-bis(3-chloroanilino)ethylene by mass spectral analysis (Figure 6).

A possible explanation of these results is suggested in the following proposed mechanism. During the thermal degradation, either an isocyanide or an oxime intermediate is formed by the bound aniline moiety and the benzylic carbon of the lignin side chain. These intermediates may then dimerize to produce the identified ethylene product. In order to pinpoint the carbon atom in the side chain that was binding with the aniline nitrogen, we undertook specific $^{14}C$ labeling of each carbon in the chain. [$\beta$-$^{14}C$]- and [$\gamma$-$^{14}C$]coniferyl alcohols were prepared (Balba and Still, 1978) and copolymerized with nonradioactive 3-chloroaniline. The copolymer was analyzed by Py–GC. The same pyrogram was obtained; however, the isolated bis(chloroanilino)ethylene was not radioactive. Over 90% of the $^{14}C$ in the pyrolized copolymer failed to chromatograph. Recovered $^{14}C$ fractions were either very volatile and appeared in the early parts of the pyrogram or very polar and appeared at the end of the pyrogram. Results would have been more informative and conclusive if [$^{14}C$]bis(chloroanilino)ethylene had been obtained from the pyrolysis of a [$\alpha$-$^{14}C$]lignin–3–chloroaniline copolymer. Unfortunately, circumstances did not permit the syntheis of the required [$\alpha$-$^{14}C$]coniferyl alcohol. Nevertheless, the results showed that chloroaniline binding is not to the $\beta$- and $\gamma$-carbon of the side chain which supported the 1,6-addition hypothesis.

It was the intent of this investigation to determine the molecular nature by which 3-chloroaniline and 3,4-dichloroaniline are bound in plant residues. These materials that heretofore have been known as "bound" or "nonextractable" residues have been investigated by using both intact tissue and in vitro experiments. It is under-

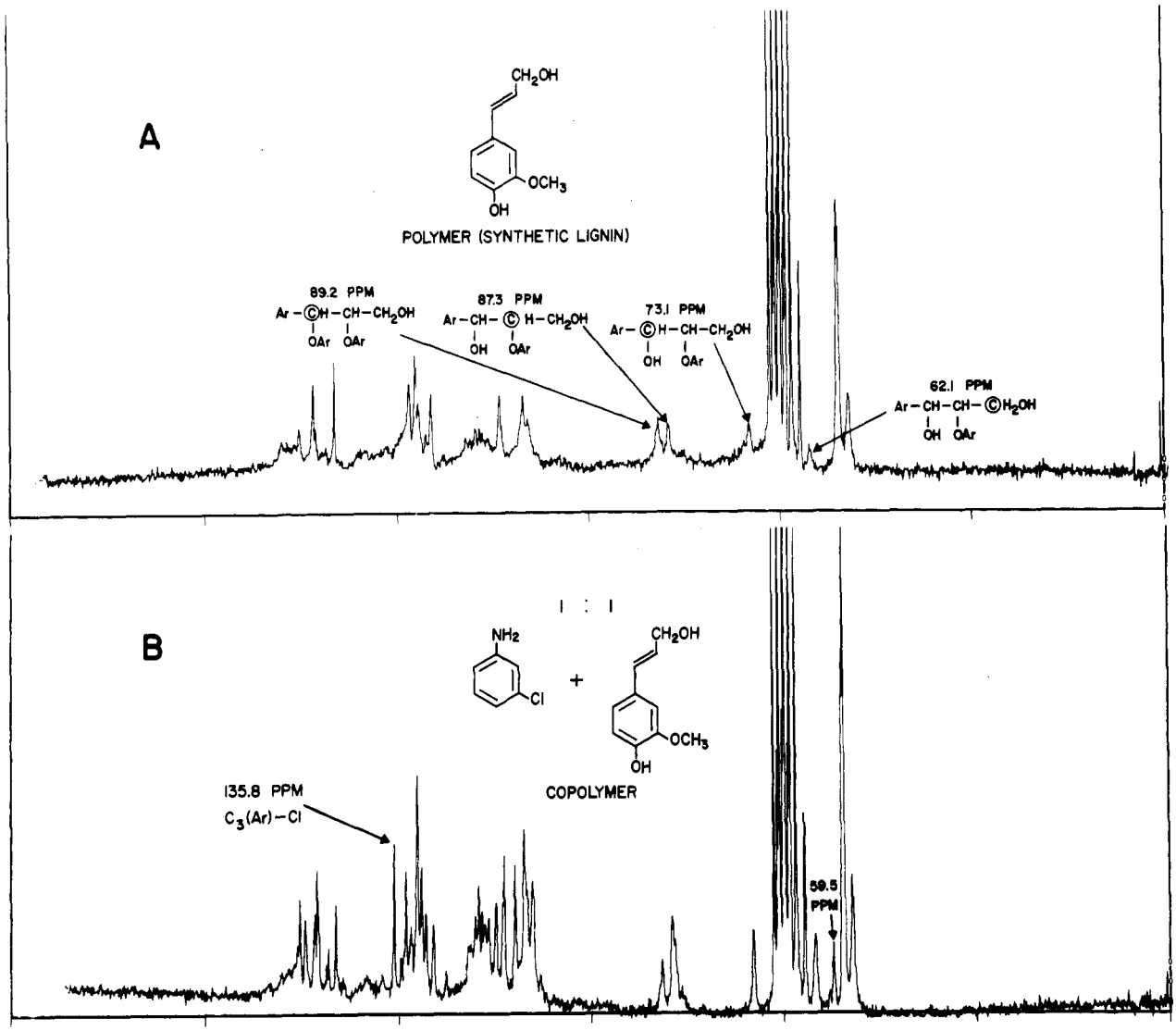



**Figure 5.** (A) Carbon-13 NMR of synthetic coniferyl lignin. (B) Carbon-13 NMR of the synthetic 3-chloroaniline–lignin copolymer.

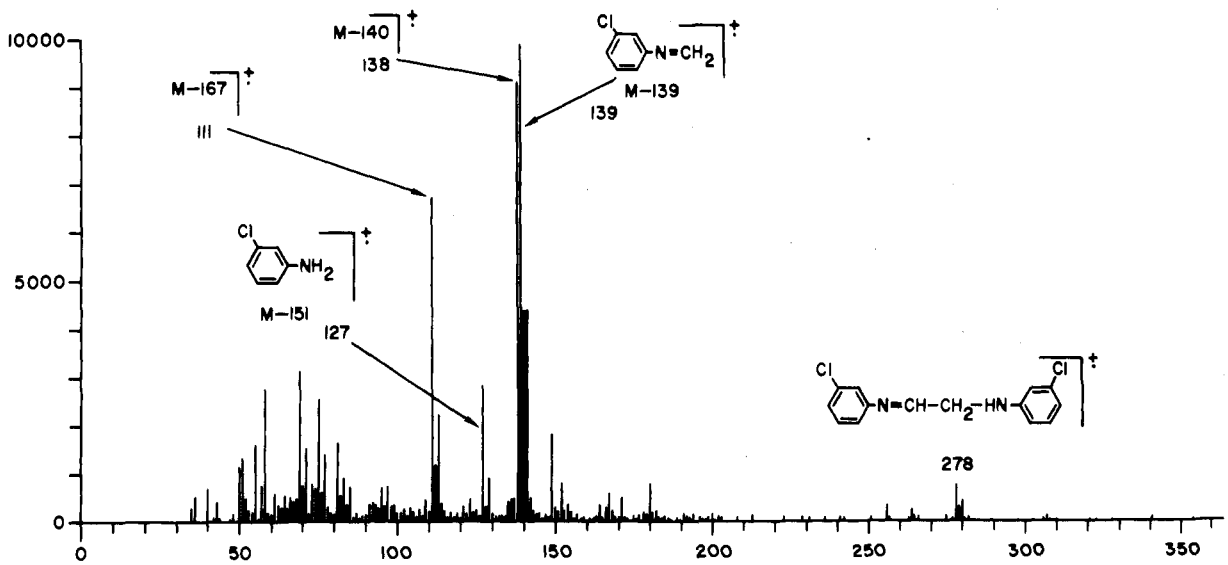



**Figure 6.** Pyrogram of the synthetic 3-chloroaniline–lignin copolymer.

stood that several chloroaniline-containing agricultural chemicals are degraded by biological systems (soil, etc.) to yield what is believed to be free chloroaniline moieties. These materials in the appropriate system possibly are available for polymerization into natural biopolymers such as lignin or soil organic matter.

To test this hypothesis, we have described an in vitro copolymerization system. This system has shown that bonding may occur via 1,6 addition to a quinone methide intermediate during synthetic lignin synthesis and that the $\alpha$-carbon of the side chain of the phenylpropenol of lignin is the most likely site of binding with the aniline nitrogen.

Similarities in the pyrolysis of natural lignin from rice roots and the synthetic lignin copolymers also suggest that the bonding of the chloroanilines may have been similar.

Mechanisms for the generation of soil organic matter have been described (Flaig, 1963). These mechanisms suggest that similar reactions take place between decomposed lignin and the amine groups of amino acids with the formation of humic substances in soil. As with lignin, investigations in soil organic matter present difficult experimental problems which have blocked rapid progress in this field. However, there is precedent for free radical and ionic intermediates which combine lignin and nitrogen components to form soil organic matter. These findings also support the conclusion that lignin, in addition to being an important structural material, also may serve as an effective reservoir for the storage of many aromatic materials in plants and soil biopolymers (Freudenberg and Neish, 1968; Flaig, 1963). Although our data do not exclude the possibility that the test chloroanilines are incorporated into lignin as inclusion products, the data suggest that the chloroaniline residues were associated with the lignin by a chemical bond.

It is the intent of the authors to share these results, pointing out the marginal successes, in an effort to stimulate research that may result in new tools and approaches so that more definitive data will be available in the future and that those social questions that arise due to the environmental involvement of these materials may be more intelligently assessed.

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# Residue Analysis of Isopropyl *N*-(3-Chlorophenyl)carbamate in Fruits and Vegetables Using High-Performance Liquid Chromatography

Ann M. Wilson, Alfred A. Bushway, and Rodney J. Bushway*

A high-performance liquid chromatographic method was developed for the determination of residues of isopropyl *N*-(3-chlorophenyl)carbamate (CIPC) in Monona potatoes at levels of 0.25–81 ppm and in beans, peas, and blueberries fortified at 0.25 ppm. CIPC was extracted with methanol, and the extract was cleaned up by being chromatographed through an acid alumina column. The average recoveries from all four commodities ranged from 64 to 102%. A study conducted to test 16 pesticides for possible interferences with CIPC demonstrated that none of the 16 cochromatographed. The lower limit of detection by using this method for beans, peas, potatoes, and blueberries is 0.12 ppm.

Isopropyl *N*-(3-chlorophenyl)carbamate (CIPC) is used primarily as a pre- and postemergence herbicide on a variety of fruit and vegetable crops and as a sprout inhibitor for potato tubers. Its varied use can be attributed to its several modes of action in plants such as the inhibition of shoot and root growth, particularly of the primary roots (Scott and Struckmeyer, 1955; Roberts, 1965; Eshel and Warren, 1967), inhibition of the photolytic activity of the chloroplasts (Moreland and Hill, 1959), inhibition of protein synthesis (Mann et al., 1965), and inhibition of mitosis

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