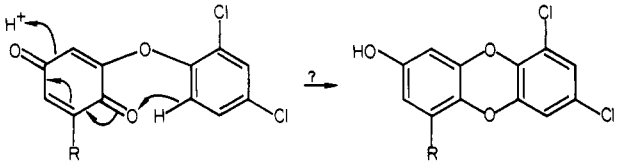
the photodecomposition of *p*-chlorophenoxyacetic acid by Crosby and Wong (1973), but the mechanism of this process for the reactions in Figure 2 is not clear.

The phenoxy quinones 3 and 4 are interesting structures because of the possibility of their conversion to dibenzodioxins 11 by an acid-catalyzed cyclization mechanism such as



3, R = H
4, R = Cl

11, R = H, Cl

Although hydroxy chlorinated dibenzodioxins have not been characterized in terms of their toxicological properties, the existence of a possible mechanism for their formation from 2,4-dichlorophenoxyacetic acid (2,4-D) in soil may be of consequence for the environmental impact of the use of 2,4-D. Conditions for this transformation are being explored in our laboratories.

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Coupling Reactions of 2,4-Dichlorophenol with Various Anilines

Shu-Yen Liu, Robert D. Minard, and Jean-Marc Bollag*

Cross-coupling between various aromatic compounds in the presence of an enzyme can lead to hybrid products. When a fungal phenol oxidase was incubated with 2,4-dichlorophenol and various halogenated anilines, the formation of hybrid oligomers was determined. Mass spectrometric analysis indicated two types of trimers which consisted of a phenoxyquinone dimer coupled with an aniline molecule or a quinone coupled with two aniline molecules. Since the incubation of anilines alone with the fungal phenol oxidase did not cause the formation of oligomeric products, it was assumed that the coupling of aniline to enzymatic products of 2,4-dichlorophenol has to be of chemical origin. Quinone products from the enzyme reaction of 2,4-dichlorophenol were found to react with the anilines in the absence of phenol oxidase. This indicated clearly that both enzymatic and nonenzymatic causes were responsible for the formation of cross-coupling products.

Oxidation and coupling of phenols is known to occur either through enzymatic or nonenzymatic systems. Enzymatic polymerization of phenolic compounds is an important reaction for the biosynthesis of a wide range of natural products (Taylor and Battersby, 1967) and is important as a key reaction in the humification process (Flaig et al., 1975).

In soil, phenolic and other aromatic degradation products of lignin, together with microbially synthesized phenolic compounds, can form complexes with amino compounds and undergo enzymatic or chemical polymerization to form humic acid type polymers (Haider et al., 1975). If humic acids are formed by the polymerization of phenols and other products, it seems reasonable to assume that opportunities will exist for the incorporation of xenobiotic substances into humus substances. The cross-coupling reactions between 2,4-dichlorophenol and phenolic humus constituents in the presence of a fungal laccase serve as a model for this hypothesis (Bollag et al., 1980).

Haider et al. (1965) studied the linkage of amino acids and peptides with phenols during oxidation with phenol oxidases. The binding to nitrogen of amino acids was found mostly in the oxidation products of dihydroxyphenols like catechol, protocatechuic acid, and hydroquinone. Ladd and Butler (1966), synthesizing model phenolic polymers from catechol and *p*-benzoquinone, also concluded that amino compounds could be incorporated into the polymers, and they compared the resulting products with humic acids.

Many of the microbially synthesized phenols can be oxidized to semiquinonoid or quinonoid intermediates which strongly react with other phenolic or nitrogenous substances. The same reaction can take place with either naturally occurring or xenobiotic compounds.

Our interest focused on anilinic compounds which are often intermediary degradation products of various pes-

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Table I. Cross-Coupling of 2,4-Dichlorophenol and Various Halogenated Anilines in the Presence of a Fungal Laccase

aniline reacting with 2,4-dichlorophenol	color formation in enzyme assay mixture	colored product as detected by TLC,[a] R_f value	M^+ of hybrid products	probable composition[b]
2-chloroaniline	orange	0.78	ND[c]	
3-chloroaniline	orange	0.78 (XI)	392	2A + 1Q
4-chloroaniline	orange	0.78 (XII)	392	2A + 1Q
2,4-dichloroaniline	red	0.82 (VIII)	460	2A + 1Q
		(IX)	461	1A + 1Q + 1P
		(VII)	427	1A + 1Q (−Cl) + 1P
3,4-dichloroaniline	red	0.82 (XIII)	460	2A + 1Q
		(IX)	461	1A + 1Q + 1P
2,3-dichloroaniline	red	0.82	ND	
2,5-dichloroaniline	red	0.82	ND	
4-chloro-2-methylaniline	red	0.84 (XIV)	420	2A + 1Q
2-bromoaniline	orange	0.81	ND	
4-bromoaniline	orange	0.81 (XV)	480	2A + 1Q

[a] Solvent system: ether-hexane (4:1 v/v). [b] A, aniline; P, phenol; Q, quinone. [c] ND, not determined.

ticides. The fungal laccase from *Rhizoctonia praticola* did not catalyze the oxidation of anilines (Sjoblad and Bollag, 1977), but incubation of the enzyme with phenols in the presence of anilines caused the formation of oligomeric hybrid products. This report describes the probable enzymatic and chemical causes which are responsible for the formation of cross-coupling products.

MATERIALS AND METHODS

A phenol oxidase was isolated from the growth medium of the fungus *R. praticola* and purified as previously described (Bollag et al., 1979). Substrate at a concentration of 100 μg/mL in 0.1 M phosphate buffer (pH 6.9) was incubated with 0.5 unit/mL enzyme. When two substrates were incubated together, a concentration of 50 μg/mL of each was used. One unit of enzyme was defined as that amount which caused a change in optical density of 1.0 per minute at 468 nm when 2,6-dimethoxyphenol served as substrate. After 2 h of incubation at 30 °C, the reaction mixture was extracted with an equal volume of methylene chloride. The organic phase was dried over anhydrous sodium sulfate, and the solvent was evaporated in vacuo at 30 °C. The residues obtained were redissolved in a small volume of methylene chloride and examined for metabolic products by thin-layer chromatography (TLC) and high-performance liquid chromatography (LC). Residues obtained from an assay mixture using boiled enzyme were included as control.

TLC was carried out on silica gel F-254 plates with a layer thickness of 0.25 mm (Brinkman Instruments, Inc., Westbury, NY). A preparative silica gel F-254 plate with a layer thickness of 0.5 mm was used for isolation of products. A solvent system of hexane–dioxane (85:15 v/v) was used for separation of 2,4-dichlorophenol derivatives.

High-performance LC was performed with a Waters Associates (Milford, MA) Model 6000 pump equipped with a U6K injector and a 440 UV detector operating at the wavelength of 254 nm. The column used was a 30 cm × 3.9 mm (i.d.), packed with normal phase μ-Porasil column, particle size 10 μm (Waters Associates, Milford, MA). Separation of metabolic products was achieved with a solvent composition of 95% hexane and 5% dioxane at a flow rate of 1.2 mL/min. The samples were first passed through a Florisil column (Supelcosil-ATF-120, 100–200 mesh, Supelco, Inc., Bellefonte, PA) equilibrated with methylene chloride, and the column was then eluted with the same solvent unless otherwise stated. For isolation of hybrid products from 2,4-dichlorophenol and 2,4-dichloroaniline, the sample in methylene chloride was placed on a Florisil column equilibrated with hexane and the first 1.5 mL of hexane eluant was discarded. This process caused a considerable reduction of the 2,4-dichlorophenol monomer and some of its oligomeric products. Subsequently, the sample was eluted with methylene chloride and analyzed by high-performance LC.

Product identification was based on electron impact mass spectrometric analysis at 70 eV with sample introduction by direct insertion probe on an AEI MS-902 mass spectrometer. The ions referred to in the results are those containing the ^{35}Cl isotope only. The chlorine isotope patterns are compatible with the compositional formulations.

2-Chloroaniline, 3-chloroaniline, 4-chloroaniline, 2,4-dichloroaniline, 3,4-dichloroaniline, 2,3-dichloroaniline, 2,5-dichloroaniline, 4-chloro-2-methylaniline, and chlorohydroquinone were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). 2-Bromoaniline and 4-bromoaniline were purchased from Eastman Kodak Co. (Rochester, NY). The solid anilines were recrystallized twice from petroleum ether and treated with active charcoal before use.

RESULTS

In previous experiments a great number of phenolic compounds were polymerized by an extracellular fungal enzyme which was isolated from *R. praticola* (Sjoblad et al., 1976; Bollag et al., 1977; Sjoblad and Bollag, 1977). However, with all the anilines listed in Table I, no observable reaction occurred in the presence of the enzyme (Sjoblad and Bollag, 1976). Incubation of aniline with the purified laccase did not cause any change of the substrate as determined by TLC and high-performance LC.

However, when the halogenated anilines were incubated with 2,4-dichlorophenol and the fungal enzyme, color formation was observed (Table I) which was not detected in an enzyme assay with 2,4-dichlorophenol as the only substrate. The appearance of the color served as a primary indication of a cross-coupling reaction between the various anilines and 2,4-dichlorophenol. A distinct color ranging from orange to red usually developed within 30 min of the enzyme assay, and the color intensified for all anilines during further incubation.

Figure 1 depicts a thin-layer plate on which the products from an enzyme assay with 2,4-dichlorophenol and 2,4-dichloroaniline were analyzed. It is evident that the colored spot with an R_f value of 0.40 is detectable only in the presence of both substrates and no reaction occurred during incubation with the boiled enzyme. The products from 2,4-dichlorophenol with R_f values of 0.23 and 0.48 were identified by mass spectrometry as a dimer (M^+ 322) and a phenoxyquinone (M^+ 268) [2-(2,4-dichlorophenoxy)-1,4-benzoquinone (Minard et al., 1981)], respectively, while the spot with an R_f value of 0.69 contains the original

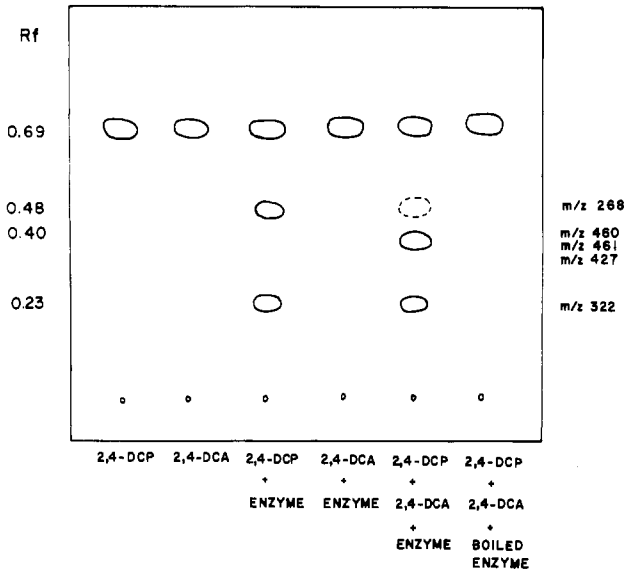

Figure 1. Thin-layer chromatographic analysis of the methylene chloride extract from enzyme assays with separate or combined incubation of 2,4-dichlorophenol and 2,4-dichloroaniline as substrates. Development solvent: hexane–dioxane (85:15 v/v).

compound, a phenoxyquinone (M^+ 302) [2-(2,4-dichlorophenoxy)-6-chloro-1,4-benzoquinone] and several other products.

The results were confirmed when the methylene chloride extract of an enzyme assay mixture with 2,4-dichlorophenol alone as the substrate or in combination with 2,4-chloroaniline was analyzed by high-performance LC. With this technique it was easier to collect each compound separately, to reinject into high-performance LC for further purification, and then to analyze by mass spectrometry.

Figure 2A shows the various peaks from high-performance LC separation of the products from an assay with only 2,4-dichlorophenol as the substrate. The mass spectral data indicate that peak I contains two compounds with M^+ (molecular ion) 448 (a dehalogenated trimer) and M^+ 608 (a dehalogenated tetramer); peak II also comprises two products, M^+ 482 (a trimer) and M^+ 642 (a tetramer); peak III consists of the substrate 2,4-dichlorophenol (M^+ 162) and chlorobenzoquinone (M^+ 142); peaks IV and VI are the phenoxyquinones IV (M^+ 302) and VI (M^+ 268) (see Figure 3) previously identified (Minard et al., 1981); peak IVa remains unidentified, and peak X (M^+ 322) is a carbon–carbon or carbon–oxygen coupled 2,4-dichlorophenol dimer.

The high-performance LC elution pattern of a methylene chloride extract from an enzyme assay with 2,4-dichlorophenol and 2,4-dichloroaniline is presented in Figure 2B. The peaks of Figure 2A could also be found here (although in different proportions) and they could be confirmed as identical except for peak V which has M^+ 161 and corresponds to 2,4-dichloroaniline. In addition, three new peaks, VII, VIII, and IX, were found. The molecular ions of the three compounds as determined by EI and CI revealed that peak VII is a trimer with M^+ 427 and peaks VIII and IX are trimers with M^+ 460 and 461, respectively. The mass spectra of the three products allowed the conclusion that they are hybrid trimers (see Figure 3).

The fact that 2,4-dichloroaniline is not oxidized by the fungal enzyme suggests that the formation of hybrid products between 2,4-dichlorophenol and the aniline is likely due to a chemcial reaction of 2,4-dichloroaniline with 2,4-dichlorophenol derivatives. Further evidence of a nonenzymatic reaction was obtained when the individually isolated products (peaks III, IV, and VI, Figure 2A) from an enzyme assay with 2,4-dichlorophenol were each combined with 2,4-dichloroaniline, and it could be established that the quinones III, IV, and VI (Minard et al., 1981) reacted with the aniline as indicated by the formation of a reddish compound.

When the two isolated quinones IV and VI were separately mixed with 2,4-dichloroaniline in 0.1 M phosphate buffer (pH 6.9), a reddish color developed within 10 min. The reaction mixture was extracted with methylene chloride and analyzed by high-performance LC. The products from the (dichlorophenoxy)quinone (VI) and the (trichlorophenoxy)quinone (IV) with 2,4-dichloroaniline had retention times identical with those of peaks VII and IX in Figure 2B, respectively. Mass spectral data indicated the formation of hybrid products with 2,4-dichloroaniline addition. With the quinone VI, 2,4-dichloroaniline formed a compound with M^+ 427 (VII), and with quinone IV, a

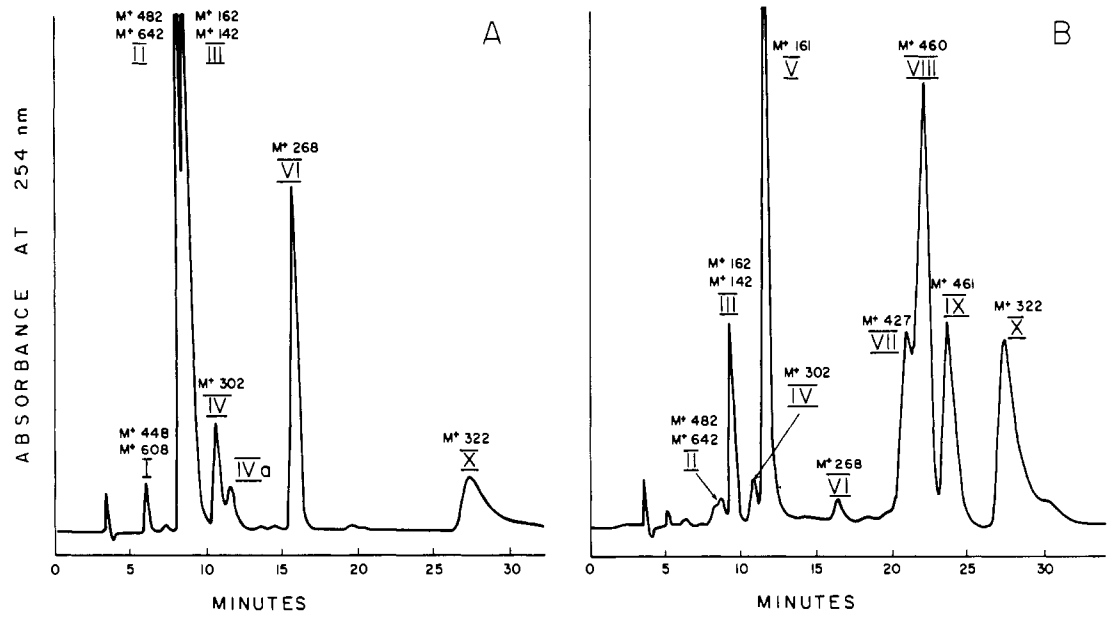

Figure 2. High-performance LC separation of the products from the methylene chloride extract of enzyme assays with 2,4-dichlorophenol (A) and 2,4-dichlorophenol combined with 2,4-dichloroaniline (B). High-performance LC conditions: solvent, 95% hexane and 5% dioxane; sample size, 10 μL in CH_2Cl_2; flow rate, 1.2 mL/min; UV detection at 254 nm; 0.5 aufs.

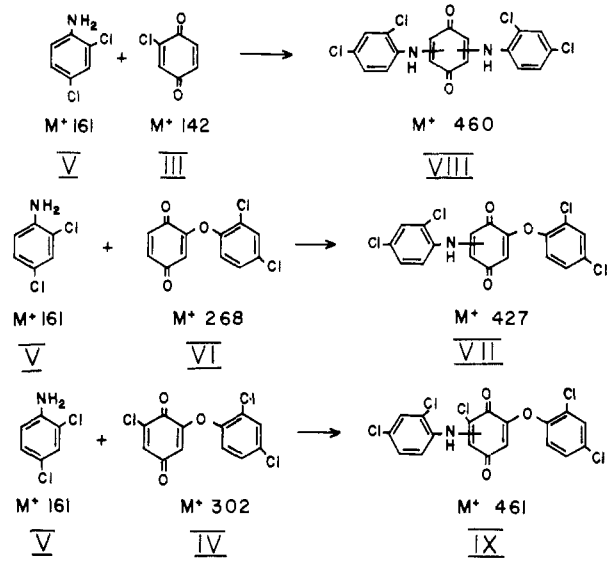

Figure 3. Reaction of 2,4-dichloroaniline with three quinones from 2,4-dichlorophenol and the probable hybrid products.

product with M^+ 461 (IX). High-resolution mass measurement yielded a composition of $C_{18}H_9NO_3Cl_4$ for VII (calculated 426.9335; found 426.9331) and $C_{18}H_8NO_3Cl_5$ for IX (calculated 460.8945; found 460.8915).

If peak III of the enzyme assay with 2,4-dichlorophenol (as shown in Figure 2A) was collected after high-performance LC separation and allowed to react with 2,4-dichloroaniline in phosphate buffer (pH 6.9), a red product was formed. When this product was extracted and analyzed on high-performance LC, a product corresponding to peak VIII (M^+ 460) of Figure 2B was obtained. It appears that this compound presents a hybrid product from chlorobenzoquinone III and two aniline molecules.

Figure 3 shows a scheme of the probable structural configurations of the three hybrid trimers (VII, VIII, and IX). The point of addition of the aniline is not shown since these hybrid products could not be isolated in sufficient quantities to allow NMR analysis which might have differentiated between the two or three positional isomers possible from the 2,4-dichloroaniline additions to the quinones.

Similar results were also obtained from the halogenated anilines which are listed in Table I. The colored products were extracted with methylene chloride from the enzyme assay mixture and isolated by TLC. For further purification the reddish compounds were extracted with methylene chloride from the thin-layer plates and rechromatographed before they were analyzed by mass spectrometry. The molecular ions of the compounds analyzed indicated the formation of trimeric hybrid compounds. Their probable structures are presented in Figure 4. The reaction of 3-chloro- and 4-chloroaniline with 2,4-dichlorophenol in the presence of the fungal enzyme formed compounds with M^+ 392 (XI and XII) which indicated the addition of two aniline molecules to chlorobenzoquinone (III). Similarly, the products from 4-bromoaniline and 4-chloro-2-methylaniline reacting with 2,4-dichlorophenol had molecular ions of m/z 480 (XV) and m/z 420 (XIV), respectively. In a manner analogous to that of 2,4-dichloroaniline, incubation of 2,3-dichloroaniline with 2,4-dichlorophenol in the presence of the fungal enzyme yielded two hybrid trimers with molecular ions of m/z 460 and m/z 461, whose structures must be analogous to compounds VIII and IX, respectively. In all cases, the halogen isotope patterns were consistent with the assigned partial structures.

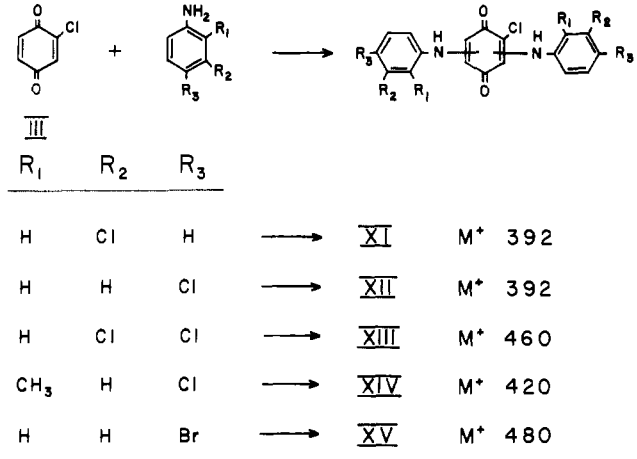

Figure 4. Scheme of hybrid products resulting from the combined incubation of 2,4-dichlorophenol and halogenated anilines in the presence of a fungal phenol oxidase.

DISCUSSION

The reported studies were performed with 2,4-dichlorophenol, but numerous other phenols are also oxidized by the fungal phenol oxidase and consequently form radicals, quinones, and polymers which can react with substituted anilines. Therefore, the described reaction could serve as a model for explaining the binding or complexing of anilines to soil organic matter.

Various investigations are known which describe the chemical reaction between quinones and anilines. Willstätter and Majima (1910) described the condensation of aniline with quinone in lightly acid solution to dianilinoquinone which could be reduced to anilinoquinone with the simultaneous release of an aniline molecule. Suida and Suida (1918) studied the reaction of *p*-benzoquinone with different anilines under various conditions. They found that *p*-benzoquinone with anilines in a slightly acid solution formed only "monoanilidochinones" while an ethanolic solution caused the formation of "dianilidochinones". Numerous "anilidochinones" were characterized in their composition and coloration.

Although similar products were obtained and reported in more recent work, the binding and incorporation of xenobiotic anilines into soil constituents was not considered. Several researchers (Haider et al., 1965; Ladd and Butler, 1966) described the formation of humic-like substances by condensation of aromatic polyphenols with amino acids and peptides by way of quinone oxidation. Similar reactions may occur with aniline- or phenol-based pesticides or their degradation intermediates.

The formation of oxidative coupling products with an extracellular fungal enzyme has been previously demonstrated (Sjoblad and Bollag, 1977; Bollag et al., 1980; Minard et al., 1981), and now we show that enzymatic products react chemically with substances which are not attacked by the enzyme. This phenomenon indicates further the large array of hybrid or cross-coupling products which can be produced in nature. Recently, we extracted enzyme-like catalysts from soil capable of causing oxidative coupling (Suflita and Bollag, 1980), providing additional support that this process appears to be of major importance in the soil environment.

The cross-coupling reaction which we have described can serve as a model for demonstrating the binding of various xenobiotics to soil humus. In soil, hybridization between xenobiotics and naturally occurring compounds may easily take place, resulting in the formation of new types of pesticide residues, and this fact has to be seriously considered in explaining the fate of environmental pollutants.

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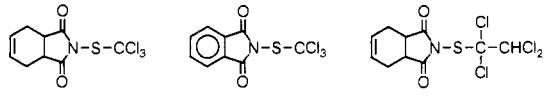
High-Pressure Liquid Chromatographic Determination of Captan, Captafol, and Folpet Residues in Plant Material

Bruno Büttler* and Wolf D. Hörmann

A specific method is described for quantitative determination of captan, captafol, and folpet residues in deciduous fruits, grapes, and cereals. The procedure involves the extraction of the samples with acetone and cleanup by partition between acetone–water and *n*-hexane. Individual cleanup procedures by column chromatography on silica are described for the separate determination of each of these fungicides. The simultaneous determination of captan, captafol, and folpet involves cleanup by gel chromatography. The fungicides are separated by high-pressure liquid chromatography on a cyano bonded phase and detected by conductivity measurement with a photoconductivity detector operated at 254 nm. Recoveries in the range of 75–120% indicate that these procedures are suitable for the residue analysis of these fungicides with detection limits of 0.02 mg/kg in fruit and grain and of 0.05 mg/kg in other plant materials.

Captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide, I], folpet [N-(trichloromethylthio)phthal-



I II III

imide, II], and captafol [*N*-(1,1,2,2-tetrachloroethylthio)-4-cyclohexene-1,2-dicarboximide, III] are three fungitoxic chemicals which have in common the phthalimide-type skeleton.

Numerous gas and thin-layer chromatographic (GC; TLC) residue methods exist. Only a few newer methods are referred to here; the publications contain further references. Pomerantz et al. (1970) described the simultaneous determination of captan, folpet, and captafol in various crops using electron capture GLC detection. Either QF-1 or XE-60 columns were used after acetonitrile extraction from fortified crops, partitioning into dichloromethane–petroleum ether, and cleanup on Florisil. With this procedure, the limit of detection for captan and folpet was 0.1 mg/kg and for captafol was 0.8 mg/kg. Barker and Flaherty (1972) improved the analytical method of Pomerantz. It was claimed that residues of folpet and captan at the 0.05 mg/kg level and of captafol at the 0.1 mg/kg level could be determined. Carlstrom (1971) reported the degradation of captan under certain gas chromatographic conditions. The possibility of decomposition of folpet under the conditions of gas chromatography made Carlstrom (1977) describe a high-pressure liquid chromatography method, using UV detection for the determination of this fungicide in formulations. Lemperle and Strecker (1971) reported the determination of folpet and captafol in grapes. They recommended that the chromatograms should not be evaluated via a standard curve because of the changing sensitivity of the electron capture detector. We too noted that the changing sensitivity of detection caused problems when determining captan, captafol, and folpet by gas chromatography, using electron capture or microcoulometric detection.

Various conditioning procedures were reported in the literature. No one gave a significant and lasting improvement in routine determination of these fungicides at the residue level in different crops. As a result of the changing sensitivity, the evaluation of the chromatograms by standard curves was not possible. The required limits of determination were often not reached. The reliable and rapid determination of residues of captafol, captan, and folpet to be an unresolved problem was also mentioned by Greve (1979).

Changing sensitivity of the electron capture detector, probably due to absorption and/or decomposition during the gas chromatographic process, was the major problem we encountered when we decided to develop a residue method based on high-pressure LC. We first developed a residue method for the determination of folpet by high-pressure LC using UV detection. Poor extinction coefficients of captan and captafol did not allow the analogous determination of captafol and captan.

At that time Tracor introduced a new selective detector for liquid chromatography on the market. This photo-

Ciba-Geigy Ltd., Agricultural Division, CH-4002 Basel, Switzerland.

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