THE FUNGICIDE VINCLOZOLIN: HYDROLYSIS AND CHEMICAL PERSISTENCE ON PEA LEAVES

by

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The fungicide vinclozolin: hydrolysis and chemical persistence on pea leaves

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ABSTRACT

The hydrolysis of vinclozolin, a dicarboximide fungicide, at 35 C and pH 4.5-8.3 and its persistence on pea leaves (Pisum sativum L.) were studied under laboratory conditions.

Three degradation products of hydrolysis were detected. They were identified as 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid (M1), 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2), and 3,5-dichloroaniline (M3). The identity of M3 was confirmed by gas chromatography-mass spectrometry. M1 and M2 were isolated from a 0.1 M phosphate buffer of pH 7.0 after incubation with 500 mg of vinclozolin at 35 C for 7 days. After purification their identities were confirmed by solid probe mass spectrometry, proton and ^{13}C nuclear magnetic resonance. Furthermore, the identity of M1 was confirmed by unambiguous evidence from x-ray crystallography of its ethyl ester.

The disappearance of vinclozolin on hydrolysis at 35 C followed simple pseudo-first-order kinetics from pH 4.5-8.3. The pseudo-first-order rate constants, second-order rate constants and half-lives were determined. Vinclozolin was much more susceptible to hydrolysis at basic than at acidic pH and the pseudo-first-order rate constant was directly proportional to the hydroxide ion concentration. Using the Arrhenius plot of log rate constants at 13 C, 20 C, 26 C and 35 C vs. 1/T, the energy of activation for hydrolysis of vinclozolin at pH 7.0 was calculated to be 97.2 K Joules mol^{-1}, and the frequency factor to be 3.467×10^{15} h^{-1}.

The conversion of vinclozolin to M1 on hydrolysis was reversible and the forward reaction was favored by basic pH whereas the reverse reaction was favored by acidic pH. Based on the kinetic data a degradation pathway was proposed for the hydrolysis of vinclozolin. On hydrolysis the 2,4-oxazolidinedione ring opens to yield both M1 and M2 independently. M3 was a minor degradation product and was detected in the hydrolysis mixtures after at least 21 days incubation in buffers of pH 4.5-8.3 at 35 C.

Vinclozolin persisted on pea leaves for 21 to 46 days under laboratory conditions. Its persistence was higher with Ronilan® 50 WP, a commercial formulation of vinclozolin, than with an acetone solution. However, most of the Ronilan® deposits were easily dislodged by rinsing with water, indicating that Ronilan® was susceptible to weathering. The dissipation of vinclozolin on leaves was linear and the calculated half-life was 33.1 days for Ronilan® and 13.4 days for the acetone solution. Translocation of vinclozolin was not detected in pea plants after its application to one of the leaflets and the degradation products, M1, M2, and M3, were not detected in either treated or untreated plant tissues.

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TABLE OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

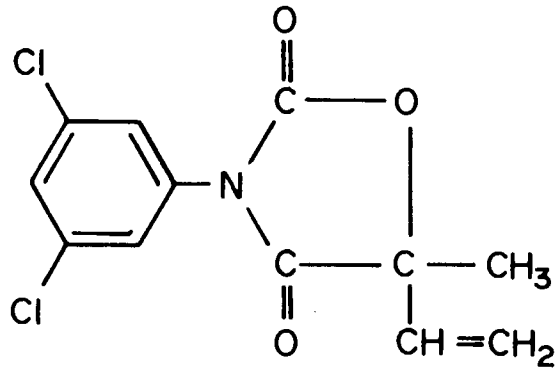
1. LITERATURE REVIEW

1.1. Physical and Chemical Properties of Vinclozolin

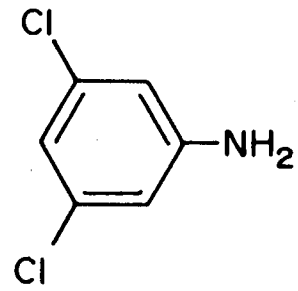
Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione] (Fig. 1) was introduced by BASF AG of Germany in 1975 under the code number BAS 35204F. It is a white crystal with a melting point of 108 C. At 20 C its vapor pressure is 1 x 10^{-7} mm Hg which is considered non-volatile as a pesticide; it is slightly soluble in water (1.0 g/L) and ethanol (14 g/L), olive oil (15 g/L) and non-polar solvents such as cyclohexane (9 g/L), but highly soluble in polar and aromatic solvents such as ether (63 g/L), ethyl acetate (253 g/L), chloroform (319 g/L), acetone (435 g/L) and benzene (146 g/L) (Spencer 1982).

The fungicidal properties of vinclozolin against Botrytis cinerea were first reported by Pommer and Mangold (1975), and by Hess and Locher (1975). Subsequent research showed that vinclozolin is effective in the control of diseases in grapes, fruits, vegetables, ornamentals, hops, rapeseed and turfgrass caused by Botrytis spp., Sclerotinia spp. and Monilinia spp. (Defloor and Scholtens 1976; Holz 1977; Moehlen and Scholtens 1979; Lebrun and Viard 1979; Vanachter et al. 1979; Van Achter et al. 1980; Entwistle and Munasinghe 1981; Kiso 1981; Kodama 1981; Pearson and Riegel 1983; Jennrich 1983; Kritzman 1983; Dueck et al. 1983; Jones 1986; Lee and Wu 1986). Since its introduction vinclozolin has been widely used in Europe for the control of fungal diseases. This fungicide is currently registered in the U.S. but not in Canada.

Figure 1. Structural formulae of vinclozolin and 3,5-dichloroaniline.



VINCLOZOLIN



3,5 – DICHLOROANILINE

1.2. Tolerance and Resistance Development

The use history of vinclozolin as a protectant fungicide in agriculture is rather short, but isolates of fungal pathogens tolerant to vinclozolin have already been developed under laboratory conditions. Leroux et al. (1977) selected strains of Botrytis cinerea Pers. tolerant to vinclozolin from spores of a benzimidazole-sensitive strain isolated at Versailles on grape. These strains were also tolerant to quintozene and dicloran but remained sensitive to benzimidazoles and other fungicides. From a benzimidazole-tolerant strain isolated at Colmar on vine shoots they selected strains resistant to vinclozolin while their tolerance to benzimidazoles always remained. The same group selected strains of B. cinerea resistant to vinclozolin by exposing spores to the fungicide at sublethal concentrations ranging from 30-100 µg/ml in the laboratory (Leroux et al. 1979). Dennis and Davis (1979) isolated some strains of B. cinerea from the fruit of vinclozolin-treated strawberry plants. These strains were tolerant to high concentrations of vinclozolin. The isolates were able to grow on potato dextrose agar containing 1000 ppm vinclozolin. Pappas et al. (1979) reported that an isolate of B. cinerea insensitive to vinclozolin was obtained from strawberries which had been inoculated with a benomyl-insensitive isolate of the pathogen and sprayed with iprodione. This isolate remained insensitive to benomyl and was as virulent as a benomyl-sensitive isolate. These findings indicate that many of the widespread benomyl-insensitive isolates may give rise to isolates also

insensitive to dicarboximides such as vinclozolin and iprodione. B. cinerea strains resistant to vinclozolin were also isolated from vineyards treated extensively with dicarboximide fungicides in the Canton of Geneva (Pezet 1982). After exposure to sublethal concentrations of vinclozolin in culture under laboratory conditions, other pathogenic fungi, namely Sclerotium bataticola (Buonaurio 1984), Monilinia fructicola (Ritchie 1982), Sclerotium cepivorum (Littley and Rahe 1984), Aspergillus nidulans, Cladosporium cucumerinum and Penicillium italicum (Fuchs et al. 1984) have been found to develop resistance.

Strains of Aspergillus nidulans, Botrytis cinerea, Penicillium expansum, and Ustilago maydis resistant to 3,5-dichlorophenyl cyclic imide fungicides (dichlorzoline, iprodione, procymidone, and vinclozolin) showed cross-resistance to other aromatic compounds such as acenaphthene, tecnazene and quintozene (Leroux et al. 1983). Littley and Rahe (1984) suggested that cross-resistance of S. cepivorum among the dicarboximides and other structurally similar fungicides such as dicloran and PCNB may have been due to the N-substituted 3,5-dichlorophenyl structural subunit they all share.

The mechanisms and genetic basis of resistance are still unclear. Beever (1983) reported that resistance to dicarboximides and aromatic hydrocarbons is often accompanied by increased sensitivity to high osmotic pressure. Grindle (1983) also observed that mutants with the greater resistance to dicarboximides display greater sensitivity to osmotic pressure. All this circumstantial evidence points to the cell wall-plasma membrane complex as the site of biochemical lesion in dicarboximide-resistant mutants.

In spite of a lack of understanding of the genetic basis which confers resistance of a fungal strain to various fungicides, many researchers use the term cross-resistance to describe simultaneous resistance to various fungicides within one fungal strain. According to Georgopoulos (1977), the term should be used only to indicate resistance to two or more fungicides mediated by a single gene. Otherwise, the term multiple resistance should be used to indicate resistance mediated by two or more genes in the same fungal strain, each of which confers resistance to one compound.

Tolerance or resistance is defined as a stable and inheritable adjustment by a cell to external or endogenous toxic or inhibitory agents (Sevag et al. 1955). Any unstable, noninheritable changes resulting from exposure to a toxicant are considered as phenotypic adaptations (Georgopoulos and Zaracovitis 1967). Resistance to vinclozolin, which showed up in fungi grown under optimal condition in the laboratory by continuous exposure, may not necessarily occur in the field. In field trials on strawberries treated 5 times with vinclozolin during 1977 to 1979, Maraite et al. (1980) detected resistance of B. cinerea in only 3 of 799 samples. However, in September 1979, a decrease of vinclozolin efficacy was observed in strawberries after 11 sprays with the fungicide. Resistance to vinclozolin was detected in up to 100% of the B. cinerea samples collected in the treated plots. The resistance spread to untreated plots or to those treated with unrelated fungicides, and resistant B. cinerea was detected in 12 of 23 samples collected in 1980.

Declining efficacy against B. cinerea of vinclozolin and other dicarboximides has also been observed in Greece (Panayotakou and Malathrakis 1983) but not in Italy (Gullino et al. 1986; Antonacci et al. 1986; Faretra et al. 1986). In surveys carried out on Crete, in 1980 and 1981, a considerable proportion of the resistant strains were found in plastic houses where tomatoes, eggplants and cucumbers were grown. From each of the plastic houses sampled, usually only resistant or only susceptible strains were isolated. There were acute disease control difficulties in three of the plastic houses with resistant strains. Monitoring in vineyards of northern Italy from 1981-1985 revealed resistance in B. cinerea in 27-50% of the vineyards surveyed each year. However, the number of bunches with resistant conidia was only 0.13-0.65% and only moderate resistance to vinclozolin was recorded indicating that vinclozolin resistance is not of major importance in this region. In Apulia, annual treatments with 3-4 sprays of vinclozolin were applied for gray mold control from 1981-1985. There was no evidence of decrease in effectiveness with prolonged use. Based on the results obtained in a 2-year investigation on distribution of benzimidazole- and dicarboximide-resistant strains, benomyl-resistant strains were found in 50% of the vineyards surveyed in 1983 and 70.4% of the vineyards surveyed in 1984. No vinclozolin-resistant strains were found.

Strains of Monilinia fructicola, the causal agent of brown rot, resistant to vinclozolin have been isolated from peach orchards in Australia (Penrose et al. 1985), New Zealand (Elmer and Gaunt 1986), and the United States (Sztejnberg and Jones 1978). The isolates

resistant to vinclozolin were resistant *in vitro* to iprodione and procymidone as well. In New Zealand, 19% of 1292 isolates surveyed were insensitive to dicarboximides. Some isolates grown in media amended with 1000 mg/L of vinclozolin showed no growth reduction. One of these isolates was pathogenic to peach flowers and nectarine fruit despite application of 500 mg/L vinclozolin before inoculation.

1.3. Mode of Action

Vinclozolin is a relatively new addition to the group of protectant fungicides, the dicarboximides. The first dicarboximide fungicide, dichlozoline [3-(3',5'-dichlorophenyl)-5,5-dimethyl-oxazolidine-2,4-dione], was developed in 1971 by the Sumitomo Chemical Company Limited of Japan but was withdrawn from the market soon after its introduction. Three other fungicides, iprodione [3-(3,5-dichlorophenyl)-*N*-(1-methylethyl)-2,4-dioxo-1-imidazoline carboximide], procymidone [*N*-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide], and myclozolin [3-(3,5-dichlorophenyl)-5-(methoxy-methyl)-5-methyl-2,4-oxazolidinedione] are also classified in this group.

The mode of action of the dicarboximides is not yet clear. Buchenauer (1976) did some preliminary studies on the mode of action of vinclozolin. He found that it was highly effective against *B. cinerea* and *Sclerotinia sclerotiorum*. It was as toxic to a benomyl-resistant strain of *B. cinerea* as to a benomyl-sensitive

strain. Vinclozolin was more inhibitory to mycelial growth than to spore germination. Free fatty acids were accumulated in vinclozolin-treated sporidia of Ustilago avenae whereas triglyceride synthesis was inhibited. Vinclozolin also strongly inhibited multiplication of U. avenae. Fritz et al. (1977) investigated the mechanism of the antifungal action of vinclozolin. They also reported that vinclozolin inhibited mycelial growth and spore germination of B. cinerea. At 1 ppm it did not inhibit spore respiration and protein synthesis but DNA and RNA syntheses were depressed, and there was accumulation of fatty acids and triglycerides which contradicted the findings of Buchenauer (1976). Albert (1978) observed a characteristic swelling of hyphal cells grown in nutrient-medium amended with about 1 ppm vinclozolin and the conidia burst after 6 h in the amended solution. The bursting of conidia was pH-dependent whereas bursting of mycelium was not pH-dependent. He hypothesized that cell wall synthesis is inhibited by vinclozolin and suggested that this hypothesis would predict the production of spheroplasts in certain osmotic solutions. The conversion of B. cinerea conidia into spheroplasts in isotonic nutrient solutions in the presence of vinclozolin was subsequently confirmed (Albert 1981).

Inhibition of mycelial growth and spore germination by vinclozolin was further demonstrated by Eichhorn and Lorenz (1978). They showed that vinclozolin acted as a contact fungicide. It was fungicidal in solid as well as liquid media against germ tubes. However, it was fungicidal against spores and mycelium in liquid media only. In solid media it was fungistatic against spores and mycelium. Pappas and

Fisher (1979) compared the mechanisms of action of vinclozolin, procymidone, iprodione and prochloraz against B. cinerea. They did not observe any significant inhibitory effects of vinclozolin on DNA and RNA synthesis as reported previously by Fritz et al. (1977). Contrary to the findings of the effects of vinclozolin on cell wall formation by Albert (1978), their results showed that vinclozolin inhibited chitin synthesis only at concentrations in excess of the ED_{50} value for mycelial growth. Incorporation of labelled chitin precursors into cell walls was barely affected, suggesting that it is unlikely that inhibition of chitin production is the primary site of action of vinclozolin. The cause of changes in fungal triglycerides and fatty acid concentrations have been attributed to dicarboximides. Buchenauer (1976) observed that vinclozolin reduced triglyceride synthesis in Ustilago avenae whilst Fritz et al. (1977) reported that vinclozolin caused accumulation of triglycerides and fatty acids in B. cinerea. Pappas and Fisher (1979) showed that vinclozolin inhibited triglyceride production whereas iprodione did not. Thus, no general mechanism for vinclozolin against various fungal pathogens or for dicarboximides as a group could be established.

The dicarboximides (vinclozolin, iprodione, procymidone and myclozolin) and the substituted aromatics (hexachlorobenzene, quintozene, chloroneb and dicloran) are remarkably similar in their action against pathogenic fungi and numerous examples of cross-resistance have been observed for these fungicides (Leroux et al. 1977; Leroux et al. 1979; Leroux et al. 1983). All these compounds interfere with mitotic division and cause mitotic

instability in Aspergillus nidulans resulting in increased frequency of mitotic recombination of diploid colonies (Georgopoulus et al. 1979). Georgopoulos et al. concluded that the effects of dicarboximides on the chromosomes, and possibly on the mitotic spindle, is a main reason for their fungitoxicity. However, this conclusion seems premature because such effects could be secondary, or reflect action at only one of a number of sites.

While this survey provides some leads on the mechanism of action of vinclozolin toxicity against pathogenic fungi, no metabolic step has yet been identified that can be unequivocally proposed as the primary target of its mode of action.

1.4. Effect on Non Target Organisms

Vinclozolin is of low mammalian toxicity. Some acute oral LD_{50}s are as follows: to rat 6400 mg/kg for the pure compound and 10000 mg/kg for technical vinclozolin; to guinea pig approximately 8000 mg/kg for technical compound; acute interperitoneal to mouse approximately 5000 mg/kg and to guinea pig 3000 mg/kg; acute dermal to rabbit 2000 mg/kg. It is slightly toxic to fish with LC_{50} (96 h) to trout 85 ppm, and to orfe 63 ppm. It is not toxic to bees or earthworms when used according to the label (Spencer 1982).

Chiesara et al. (1982) evaluated vinclozolin and its epoxide for mutagenicity. Their results showed that vinclozolin without metabolic activation was not mutagenic according to in vitro test with five strains of Salmonella typhimurium and one strain of Schizosaccharo-

myces pombe. The same test organisms showed mutagenicity with vinclozolin epoxide and with the parent compound after metabolic activation with liver microsomes prepared from rats induced with Aroclor 1254. However, when they tested urine samples collected from rats fed with 100 mg/kg of vinclozolin, no mutagenicity was observed.

Vinclozolin is generally considered to be non-phytotoxic but some adverse effects on plants have been demonstrated. Seed treatment of white Spanish and Goldberg onions with vinclozolin at 100 g/kg of seed reduced germination by at least 40% and severely stunted the growth of seedlings germinated on moistened filter paper. Similar rates of vinclozolin severely reduced field emergence and retarded growth of onions (Wicks and Philp 1985). At the cellular level, vinclozolin showed cytogenetic effects in vitro on meristematic cells of Allium cepa (Escalza et al. 1983). At 10^{-3} M, vinclozolin inhibited cell division and induced morphological alteration in the development of mitosis, but no such effects were observed at 10^{-4} M. The morphological alterations mainly affected the arrangement of chromosomes in forming the equatorial plate in the metaphase and subsequent migration to the poles. At 10^{-4} M, vinclozolin strongly induced chromosomal aberrations but only slightly increased the frequency of sister chromatid exchange. Escalza et al. hypothesized that the cytogenetic effects observed could have resulted from reactions between vinclozolin and chromosomal and spindle proteins without a damaging effect on DNA.

Since vinclozolin is extensively used in Europe for disease control in strawberries and grapes, its effects on fruit quality have

been evaluated. Davidek et al. (1981) compared strawberries with or without vinclozolin treatment. The flavor of untreated fruit was preferred to that of fruit treated with the fungicide. There were differences in the content of reducing sugars, volatile fatty acids and titratable acidity in individual samples; and gas chromatographic profiles of volatile substances isolated from treated and untreated berries also differed. In grapes, it appeared that vinclozolin did not affect the wine making process. Sapis-Domercq et al. (1977) observed that there was no difference in the yeast development during fermentation between juice from treated and untreated grapes; and the contents of alcohol, sugar and acid were similar in the resulting wines. However, some effect on flavor congeners was observed. Subsequent studies by Barbero and Gaia (1979) further confirmed that neither the fermentation of musts of grapes treated with vinclozolin nor the yeast population were affected by as many as four vinclozolin treatments.

1.5. Residues of Vinclozolin

Methodology has been developed for the determination of vinclozolin residues after its application. Crop samples such as strawberries, grapes, apples, pears, and samples of soil were extracted with organic solvent (hexane, acetone chloroform, dichloromethane, acetonitrile or benzene) (Zanini et al. 1980; Leone et al. 1981; Lemperle et al. 1982; Boccelli et al. 1982; Taccheo et al. 1984). The crude extracts were cleaned on either alumina or Florisil and vinclozolin

was determined with a gas chromatograph equipped with an electron capture detector. All these methods provided satisfactory recovery of the parent compound ranging from 71% to 116%, but none was applicable to determine the degradation products derived from vinclozolin. Loss of vinclozolin in silver ion-loaded alumina for cleanup was frequently experienced (Lokke 1979). To reduce the loss the eluting solvent must be cooled to 0 C and the rate of elution increased to 10-20 ml/min by applying light pressure with nitrogen at the top of the column. The modified procedure improved the recovery of vinclozolin to better than 90%. Pietrogrande et al. (1983) used a cleanup column comprised of a mixture of sodium sulfate (10 g), Florsil (10 g), Celite (8 g), attaclay (4 g), and activated carbon (1 g) to improve purification. Their method was successfully applied to residue determination in 78 apple samples and 38 strawberry samples. Thin layer chromatography was used in detecting vinclozolin extracted from water, soil and sunflower seed (Krasnykh et al. 1982). For confirmation, capillary GC/MS was used to identify vinclozolin and its metabolite on kiwi fruit (Holland et al. 1983).

High pressure liquid chromatography was used in the analysis of vinclozolin in must and wine. Residues were extracted from wine by liquid/liquid partition with isooctane (Allende 1981), dichloromethane (Lemperle et al. 1982), light petroleum (Cabras 1983), or by adsorption column chromatography with Amberite XAD-2 (Spitzer and Nickless 1981). The method of Cabras (1983) was capable of determining the degradation product of vinclozolin, and 3,5-dichloroaniline. However, results of analysis of wine samples were

not included in his paper and there was no mention if 3,5-dichloroaniline had been detected in wine. The sensitivity of high pressure liquid chromatographic methods was adequate for analyzing vinclozolin in wine, and residues could be detected at concentration as low as 0.01 ppm.

Vinclozolin has been used extensively in Europe to control pathogenic fungi in small fruits, vegetables and grapes since the late 1970's. Residues of vinclozolin have been detected in various commodities. When this fungicide was applied at rates from 125-750 g AI/ha to strawberry plants, residues on fruits were proportional to the application rate. The half-life varied from year to year. It was 12 to 13 days in one year and 22 days for the following year and the residues in fruits ranged from 1.5 to 5 ppm (Zenon-Roland and Gilles 1978). Van Wambeke et al. (1980) studied residues on greenhouse tomato fruits after 1 to 3 applications at 2-week intervals with vinclozolin at 500-2000 g AI/ha. Residues ranging from 0.11 ppm to 8.60 ppm were detected 13 days after the last application and the cumulative effect due to repeated application was clearly demonstrated. Since the tolerance of vinclozolin in vegetables is 2 ppm in most countries of the European Economic Community, it is imperative to revise the 14-day preharvest interval for tomatoes grown under greenhouse conditions. Similar residue problems in greenhouse tomatoes were also reported by Cabras et al. (1985). They detected 2 ppm of vinclozolin on tomato fruits 21 days after the last application at 1500 g AI/ha, which exceeded the legal tolerance of 1.5 ppm set by Italian law. They suggested that revision of the preharvest interval

of 21 days currently established in Italy may be necessary. Depending on the number of applications and application rate, residues in greenhouse lettuce may exceed the tolerance of 2 ppm. Melkebeke et al. (1980) reported that residues ranging from 0.2 ppm to 1.3 ppm were found 61 days after the last of three applications of vinclozolin at 1500 g AI/ha. Preplanting soil treatment with vinclozolin at 4000 g AI/ha resulted in low levels of residues. However, soil treatment at 4000 g AI/ha before planting followed by two foliar treatments at 3000 g AI/ha resulted in residues exceeding the legal tolerance of 2 ppm with a 4-week preharvest interval. The influence of application methods, application rates and different formulations on vinclozolin residues of lettuce was further evaluated by Van Wambeke et al. (1980) and Melkebeke et al. (1985). No residue problem was indicated when the recommended application rate and preharvest interval were followed. Indeed, only a few residue violations were detected in lettuce based on the survey conducted in Belgium from 1980 to 1981 (Galoux 1981). One hundred lettuce samples were analyzed in Dec. 1980, and January-March in 1981. Only three samples contained vinclozolin residues that exceeded the tolerance of 2 ppm.

Residue studies in rape were conducted in Germany (Rexilius 1983). Treatment with vinclozolin at 750 g/ha at the end of May resulted in 0.1 ppm residues in seeds and 0.5 ppm in straw at harvest. A 56-day preharvest interval was therefore recommended to allow the residues to fall below tolerance levels (<0.1 ppm) in the seeds.

Vinclozolin residues dissipate rapidly from grapes. Del Re et al. (1980) determined the rate of dissipation after the last of 7 treatments, each at 700 to 1400 g AI/ha and reported a half-life of 7.5 to 9 days. Shorter half-lives were reported by Gennari et al. (1985). The calculated half-life ranged from 1.2 days to 4.6 days after spraying with the recommended rate and from 2.0 days to 4.9 days with double the recommended rate. Musts and wine produced from vinclozolin-treated grapes contained residues. The concentrations varied greatly between studies. According to Allende (1981) the concentration of vinclozolin in must from treated grapes decreased during fermentation from 0.5 ppm to 0.01 ppm. Lemperle et al. (1982) surveyed the Rülander, Müller-Thurgau, and White Burgundy varieties grown in Germany during 1976, 1979 and 1980 growing seasons. At vintage, the residues ranged from 0.3 to 4.4 ppm, and in the wine from none detected to 0.8 ppm, which all fell within the tolerance. Studies conducted in Austria also showed residues in musts and wines below the legal tolerance (Fida and Womastek 1983).

Vinclozolin underwent photolysis when irradiated for 14 days with a medium pressure mercury vapor lamp (400 W) in a borosilicate glass system (Clark and Watkins 1984). Five photolytic products had been isolated from a methanolic solution and identified, namely 3,5-diclorophenylisocyanate; 3,5-dichloroaniline; methyl 3,5-dichlorophenyl carbamate; 3-(3-chlorophenyl)-5-methyl-5-vinyl-oxazolidine-2,4-dione; and methyl (3,5-dichlorophenyl) (2-hydroxy-2-methyl-1-oxo-buten-3-yl) carbamate. The major photolytic product obtained from a benzene solution was 3-(3-chlorobiphenyl)-5-methyl-5-

vinyl-oxazolidine-2,4-dione. However, it is not yet known to what extent, or if, any of these photolytic products are formed in the environment. Des-vinyl vinclozolin had been detected on kiwi fruit treated with vinclozolin (Holland et al. 1983) at a concentration (0.16 ppm) of approximately 10% of the level of the parent compound (1.6 ppm). However, this metabolite has not been observed at more than 0.01 ppm on a number of other treated fruits including blueberries and strawberries. Since there is significant difference among plant species in the formation of des-vinyl vinclozolin, it suggests that this metabolite is more likely to be produced biologically than photochemically in the plant.

The phenomenon of enhanced degradation of pesticides following repeated application was observed as early as the late 1940's for 2,4-D, MCPA and 2,4,5-T, but it is only recently that the consequences of this phenomenon to agriculture have become apparent. For example, the activity of the herbicide EPTC decreased in some soils receiving annual treatments, and more rapid degradation of this herbicide in the previously-treated than the previously-untreated soils was demonstrated (Rahman et al. 1979; Obrigawitch et al. 1982; Obrigawitch et al. 1983). Similarly reduced efficacy after repeated applications was observed with the insecticide carbofuran (Felsot et al. 1981; Felsot et al. 1982) and with the fungicide iprodione (Entwistle 1983). In order to confirm the possibility of enhanced degradation of iprodione in soil, Walker et al. (1986) studied the degradation of iprodione and vinclozolin in soil. They found that there was a progressive increase in degradation rates with successive treatments for both fungicides.

The times for 50% loss for the first and second applications were approximately 23 and 5 days, respectively. However, in a soil which had relatively low pH (5.0) degradation of both compounds occurred only slowly and the rate of degradation of a second application was the same as that of the first. This observation suggests that soil pH has a profound effect on the degradation of vinclozolin.

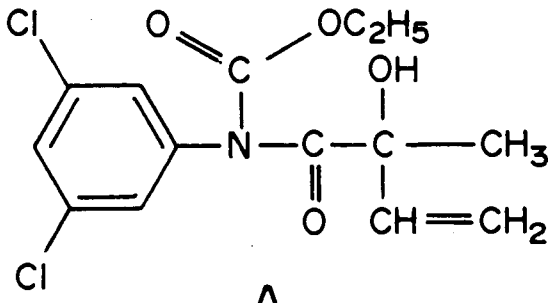
Little was known about the degradation of vinclozolin in the environment when these thesis studies on hydrolysis were undertaken in 1983. Two degradation products were detected and isolated in late 1983. At about the same time Clark (1983) reported that the oxazolidine ring of vinclozolin was opened in ethanolic and methanolic solutions and water suspension. The reaction products in ethanol and methanol were ethyl- and methyl-N-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl carbamates, respectively (Fig. 2A and Fig. 2B). Two products were identified from water suspension, N-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (Fig. 2C) and its decarboxylation product N-3,5-dichlorophenyl-2-hydroxy-2-methylbut-3-enamide (Fig. 2D). Clark synthesized these two products and tested them *in vitro* against *B. cinerea*. He found that these two compounds were non-inhibitory against mycelial growth. Cabras *et al*. (1984) studied the degradation of dicarboximide fungicides in wine and reported that the disappearance of vinclozolin showed pseudo first-order kinetics at 30 C. The rate constant was higher at pH 4.0 than pH 3.0. Two products were detected in the wine treated with vinclozolin, but 3,5-dichloroaniline was not found in any of the treated wine after incubation at 39 C for 92 days. They

hypothesized that the two products were the same ones reported previously by Clark (1983) which resulted from opening of the oxazolidine ring and decarboxylation. Their subsequent studies (Pirisi et al. 1986) succeeded in isolation and identification of one of the two products, namely N-3,5-dichlorophenyl-2-hydroxy-2-methylbut-3-enamide but failed to isolate N-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl-1-carbamic acid which they hypothesized previously as an "intermediate" for the formation of the enamide (Cabras et al. 1984). The authors did not offer any explanation as to why they were able to detect both products by reverse phase high pressure liquid chromatography in their previous studies but failed to isolate the "intermediate" in their subsequent studies.

Melkebeke et al. (1986) studied the chemical hydrolysis of vinclozolin between pH 3.0 and pH 11.0 and reported the half-lives and rate constants for the disappearance of the parent compound. However, they did not mention if any degradation products were detected. It is apparent that a comprehensive hydrolysis study is required to investigate not only the disappearance of vinclozolin but also the isolation and identification of any degradation products, so that the mechanism of hydrolytic degradation can be better understood.

Since the molecular structure of vinclozolin consists of a 2,4-oxazolidinedione and a 3,5-dichlorophenyl moiety it was hypothesized that on hydrolysis the oxazolidinedione ring may open and 3,5-dichloroaniline (Fig. 1) could be produced. 3,5-dichloroaniline

Figure 2. Structural formulae of ethyl-N-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl carbamate (A); methyl-N-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl-1-carbamate (B); N-((2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (C); and N-3,5-dichlorophenyl-2-hydroxy-2-methylbut-3-enamide (D).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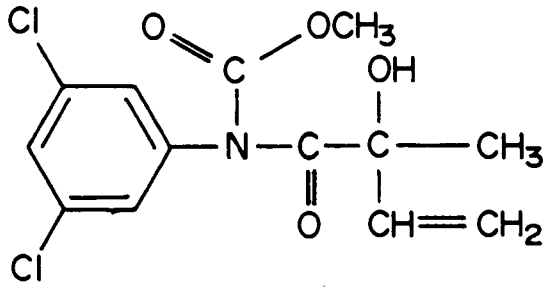
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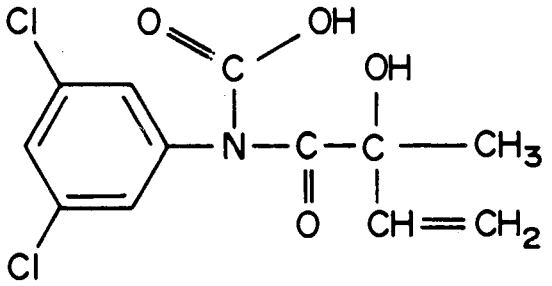
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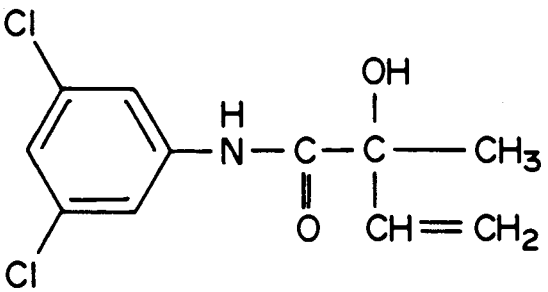
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is a chlorinated aromatic amine which may be toxic to higher animals and its possible formation on hydrolysis is important to environmental toxicologists. There is little information on the toxicity of 3,5-dichloroaniline but some of the chlorinated aromatic amines such as 4-chloro-2-methylaniline, 2,4,6-trimethylaniline and 2,4,5-trimethylaniline are known to cause tumors in experimental animals (Hill et al. 1979; Weisburger et al. 1978). Furthermore, vinclozolin, as a protectant fungicide, must be applied on the host plants before inoculation to prevent infection, or be applied periodically to reduce the rate of epidemic development. The primary goal is to reduce the number of successful infections by applying just enough fungicides so that the epidemic does not progress at a rate which will result in a yield-reducing level of disease. For vinclozolin to be an effective protectant fungicide it must be persistent enough on the host plants. Since most fungus infections occur during rainy periods, vinclozolin must be resistant to hydrolysis and to physical removal by water through erosion or solution, and stable against oxidation and photolysis. Therefore it is the objective of this research to study under laboratory conditions the hydrolysis of vinclozolin at various pHs and temperatures; to isolate and identify the degradation products; to determine if 3,5-dichloroaniline would be formed on hydrolysis; and to study the persistence of vinclozolin and Ronilan® 50 WP, a commercial formulation of vinclozolin, in garden pea, Pisum sativum L. (cv Improved Laxton's Progress).

2. MATERIALS AND METHODS

2.1. Preparation of Vinclozolin

2.1.1. Isolation

Two g of Ronilan® 50 WP were divided into four aliquots of approximately 500 mg each in 50-ml centrifuge tubes. Each aliquot was extracted with 25 ml of glass-distilled acetone for one h in an ultrasonic water bath. After extraction the acetone suspensions were centrifuged at about 2000 rpm in a bench-top centrifuge for 10 min and the supernatants were passed through glass fibre filter paper. The combined filtrates were again centrifuged and filtered until the acetone solution was clear. The acetone solution was concentrated to about 50 ml in a flash evaporator at 38 C followed by the addition of 450 ml of glass distilled water. At 4 C vinclozolin crystals were allowed to precipitate from the aqueous solution, which contained 10% of acetone. The precipitates were filtered through glass fibre filter paper and recrystalized in hexane at 4 C. Crystals of vinclozolin were harvested by filtering through glass fibre filter paper, and dried under a gentle stream of nitrogen over-night at room temperature. The purity of the crystals was determined by high pressure liquid chromatogrphy (HPLC) and gas liquid chromatography (GLC).

2.1.2. Determination of Purity

An analytical standard of vinclozolin with purity of 99.7% was obtained as a reference from the US Environmental Protection Agency at Research Triangle Park, NC 27711, U.S.A. The purity of the vinclozolin prepared from Ronilan® 50 WP was assayed by reverse phase HPLC and GLC. 3,5-dichloroaniline with purity of 98% was obtained as a reference from the Aldrich Chemical Co. at Milwaukee, WI 53201, U.S.A.

2.1.2.1. High Pressure Liquid Chromatography

A solution containing vinclozolin at 5 µg/ml and 3,5-dichloroaniline at 1.5 µg/ml in glass-distilled methanol was analyzed with a Varian model 5000 high pressure liquid chromatograph equipped with a Hewlett Packard Model 1040 A high speed spectrophotometric detector. The operating parameters were as follows:

Column	Varian Micro Pak MCH-10, 30 cm x 4 mm i.d.
mobile solvent system	72% methanol and 28% water, isocratic at 1 ml/min
UV detector wavelength	212 nm ± 2 nm

UV-visible absorption spectra were measured at up-slope, apex and down-slope of each chromatographic peak.

Under the conditions described, the absolute retention times of vinclozolin and the degradation product, 3,5-dichloroaniline, were 8.82 min and 6.32 min respectively.

2.1.2.2. Gas Liquid Chromatography

A solution of vinclozolin at 0.02 µg/ml in glass distilled hexane was analyzed with a Hewlett Packard Model 5890 gas liquid chromatograph equipped with a Ni-63 electron capture detector. The operating parameters were as follows:

Column DB-17 fused silica capillary column, 30 m x 0.253 mm i.d. from J & W Scientific, Inc.

Column head pressure 140 kPa with a total flow of 40 ml helium/min.

Column temperature programmed from 70 C to 225 C at 20 C/min with the final temperature held for 15 min.

Injector temperature 225 C; detector temperature 300 C.

Make-up gas 5% methane in argon at 20 ml/min. Injection splitless.

Under the conditions described, the absolute retention times of vinclozolin and 3,5-dichloroaniline were 10.37 min and 7.04 min respectively.

2.2. Hydrolysis of Vinclozolin

2.2.1. Incubation in Phosphate Buffers

Three phosphate buffers were prepared by adjusting the pHs of two 100-ml solutions of 0.1 M NaH_2PO_4, using 0.1 M Na_2HPO_4 to bring them to pH 5.0 and pH 7.0, and by adjusting the pH of a 100-ml solution of 0.1 M Na_2HPO_4 with 0.1 M NaOH to pH 9.0. A stock solution of vinclozolin at 1000 µg/ml was prepared in glass-distilled methanol. Aliquots of 1 ml of the stock solution were thoroughly mixed with the buffers in 100-ml volumetric flasks to give concentrations of 10 µg of vinclozolin/ml. These three solutions were incubated at 20 C in a water bath (Lab-Line Instruments, Inc., Melrose Park, Illinois, U.S.A.). The volumetric flasks were wrapped in aluminum foil to shield them from light.

2.2.2. Degradation Products

After incubation at 20 C for 7 and 30 days, an aliquot of 20 µl of each incubated solution was injected directly into a high pressure liquid chromatograph for the determination of vinclozolin and any degradation products formed in the aqueous solutions, including 3,5-dichloroaniline. The procedures were as described under 2.1.2.1.

The degradation product, 3,5-dichloroaniline was detected by GLC after incubation at 20 C for 30 days. Aliquots of 50 ml of each solution were extracted three times with 10 ml of glass-distilled

dichloromethane. Each combined extract was dried with anydrous Na_2SO_4 which had been heated at 120 C in an oven over-night prior to use. The dried extracts were concentrated to 1 ml in a flash evaporator at 38 C and then analyzed by gas chromatography-electron capture (GC-EC) as described under 2.1.2.2. and by gas chromatography-mass spectrometry (GC-MS).

The GC-MS analysis was performed with a Hewlett Packard Model 5890 gas chromatograph equipped with a Model 5970 mass selective detector. The operating parameters of the gas chromatograph were as described under 2.1.2.2. The mass selective detector temperature was 250 C and the scanning mass range was from 40 amu to 450 amu.

2.3. Isolation of Degradation Products M1 and M2

2.3.1. Incubation

A 0.1 M phosphate buffer of pH 7.0 was prepared by adding 0.1 M NaH_2PO_4 solution to 0.1 M Na_2HPO_4 solution until pH 7.0 was obtained. Five hundred mg of crystalline vinclozolin were added to one L of 0.1 M buffer of pH 7.0 and heated to about 60 C until dissolved. The buffered vinclozolin solution was incubated at 35 C in a water bath for 7 days.

At the end of the incubation, the amount of vinclozolin remaining in the buffered solution was determined by HPLC as described under 2.1.2.1. When degradation of more than 90% of the added vinclozolin

was indicated, the two degradation products designated as M1 and M2 were isolated from the buffered solution.

2.3.2. Isolation

The buffered solution was removed from the water bath, allowed to cool to room temperature and then extracted three times with 250 ml of glass-distilled dichloromethane. The combined extracts were dried with anhydrous Na_2SO_4 and the degradation product so isolated was designated as M2.

After isolation of M2, the aqueous solution was acidified to about pH 1.0 with conc. HCl. The acidified solution was extracted three times with 250 ml of glass-distilled dichloromethane. The combined extracts were dried with acidified anhydrous Na_2SO_4 and the degradation product so isolated was designated as M1. The acidified anhydrous Na_2SO_4 was prepared by washing anhydrous Na_2SO_4 with glass-distilled acetone saturated with conc. H_2SO_4 and then drying at 120 C in an oven over-night. The dichloromethane solution containing M1 was concentrated to dryness in a flash evaporator at 38 C and M1 was crystalized twice in chloroform. The crystals were harvested by filtering through glass fibre filter paper, dried under a gentle stream of nitrogen for 2 h, and then stored in a desiccator before the melting point was determined and the structure identified.

A chromatographic column (30 cm x 2.5 cm i.d.) was prepared by packing from the bottom with a glass wool plug, 2 cm of anhydrous Na_2SO_4, 8 cm of Florisil deactivated with 2% water, 2 cm of anhydrous

Na_2SO_4, and another glass wool plug. The packed column was prewashed with 25 ml of 40% dichloromethane in hexane. The dichloromethane solution containing M2 was concentrated to about 8 ml in a flash evaporator at 38 C, followed by the addition of 12 ml of glass-distilled hexane. The resultant solution was transferred quantitatively to the prewashed column and then eluted three times with 100 ml, 50 ml and 25 ml of 40% dichloromethane in hexane. The third eluate was concentrated just to dryness in a flash evaporator at 38 C; the residues were dissolved in acetone and analyzed by GC-EC. When the presence of degradation product M2 was indicated, the column was eluted with 125 ml of 10% acetone in dichloromethane. The eluate containing degradation product M2 was concentrated just to dryness in a flash evaporator and the M2 was crystallized twice in 5% benzene in hexane. The crystals were harvested by filtering through glass fibre filter paper, dried under a gentle stream of nitrogen for 2 h, and then stored in a desiccator before determination of the melting point and identification of the structure.

2.3.3. Preparation of Ethylated M1

2.3.3.1. Generation of Diazoethane

Diazoethane was prepared from its precursor, N-ethyl-N'-nitro-N-nitrosoguanidine, obtained from the Aldrich Chemical Co. at Milwaukee, WI 53201, U.S.A. Three huhndred mg of the precursor in 5 ml of glass-distilled ether reacted at room temperature with 2 ml of 5N NaOH

solution in a gas bubbler. One of the two side arms of the gas bubbler was connected to a gentle flow of nitrogen and the other arm connected with Tygon® tubing to a Pasteur pipet for dispensing diazoethane. Since diazoethane is highly toxic, mutagenic, carcinogenic, and potentially explosive, all reactions involving the preparation and use of diazoethane were carried out in an efficient fumehood and behind a safety shield.

2.3.3.2. Ethylation

One hundred mg of M1 were dissolved in 5 ml of glass-distilled dichloromethane in a graduated, glass-stoppered reaction tube and cooled to about 4 C in an ice-water bath. Freshly generated diazoethane was gently purged with nitrogen into the dichloromethane solution until a pale yellow color appeared, indicating the presence of excess diazoethane. The reaction mixture was evaporated just to dryness under a gentle stream of nitrogen in a heating block at 40 C. The ethylated product was crystallized twice in 5% benzene in hexane. The crystals were harvested by filtering through glass fibre filter paper, dried under a gentle stream of nitrogen for 2 h, and then stored in a desiccator before determination of the melting point and identification of the structure.

2.3.4. Purity Determinations

2.3.4.1. Melting Point Determination

Melting points of M1 and its ethylation product, and M2 were determined with a Dynamic Optics Model AAHT melting point apparatus.

2.3.4.2. HPLC of M1, Ethylated M1 and M2

The purity of the degradation products M1 and M2, and ethylated M1 was determined by high pressure liquid chromatography. A methanolic solution of each compound at 100 µg/ml was prepared and an aliquot of 20 µl was injected for analysis. The operating parameters were as described under 2.1.2.1. with minor modification in the mobile solvent system. The mobile phase consisted of 72% methanol and 28% 0.05 M phosphate buffer of pH 3.3 instead of 72% methanol and 28% water. UV-visible absorption spectra were measured at up-slope, apex and down-slope of each chromatographic peak.

2.3.4.3. GC-MS of M2

A solution of M2 at 10 µg/ml in acetone and an aliquot of 2 µl was analyzed by GC-MS with a Hewlett Packard Model 5890 gas chromatograph equipped with a Model 5970 mass selective detector. The detector temperature was 250 C and the scanning mass range was from 40 amu to 450 amu. The operating parameters of the gas chromatograph were as described under 2.1.2.2.

2.3.5. Identification of Degradation Products

The structures of hydrolytic degradation products M1 and M2 were elucidated by analyzing data obtained from solid probe mass spectrometry, proton and ^{13}C NMR spectrometry and X-ray crystallography of the ethylation product of M1.

2.3.5.1. Mass Spectrometry

A Kratos Model MS 50 mass spectrometer was used to obtain mass spectra for vinclozolin, M1 and its ethylation product, and M2. The temperature of the solid probe was 120 C. A Hewlett Packard Model 5890 gas chromatograph equipped with a Model 5970 mass selective detector was also used to obtain mass spectra for the same compounds. The operating parameters of the GC-MS were as described under 2.3.4.3.

2.3.5.2. Nuclear Magnetic Resonance (NMR) Spectrometry

Proton and ^{13}C NMR spectra of vinclozolin, M1 and its ethylation product, and M2 were obtained respectively on a Bruker Model WP-400 spectrometer operating at 400 MHz and a Varian Model XL-300 spectrometer operating at 75 MHz. A 5-mm broadband probe was used in both spectrometers. Solutions of each compound were prepared in d-dichloromethane or d-chloroform, to which a small amount of tetramethylsilane (TMS) was added for reference.

2.3.5.3. X-Ray Crystallography

A crystal of the ethylation product of M1 was selected for crystal structure determination at 22 C. The X-ray crystallographic analysis was done in the Chemistry Department of the University of British Columbia by Dr. J. Trotter.

2.4. Kinetic Studies on the Hydrolysis of Vinclozolin

2.4.1. Preparation of Buffered Solutions

Buffered solutions of 0.01M were prepared with sterilized deionized water as follows:

0.01 M NaH_2PO_4 adjusted to pH 4.5 with 0.01 M H_3PO_4

0.01 M NaOAc adjusted to pH 4.5 with 0.01 M HOAc

0.01 M NaH_2PO_4 adjusted to pH 5.5 with 0.01 M Na_2HPO_4

0.01 M NaOAc adjusted to pH 5.5 with 0.01 M HOAc

0.01 M NaH_2PO_4 adjusted to pH 6.5 with 0.01 M Na_2HPO_4

0.01 M NaOAc adjusted to pH 6.5 with 0.01 M HOAc

0.01 M Na_2HPO_4 adjusted to pH 7.0 with 0.01 M NaH_2PO_4

0.01 M $Na_2B_4O_7$ adjusted to pH 7.0 with 0.01 M HCl

0.01 M Na_2HPO_4 adjusted to pH 7.5 with 0.01 M NaH_2PO_4

0.01 M $Na_2B_4O_7$ adjusted to pH 7.3 with 0.01 M HCl

0.01 M Na_2HPO_4 adjusted to pH 8.0 with 0.01 M NaH_2PO

0.01 M $Na_2B_4O_7$ adjusted to pH 8.3 with 0.01 M HCl

2.4.2. Incubation of Vinclozolin in Buffered Solutions

Aliquots of 1 ml of the stock solution of vinclozolin at 1000 µg/ml in methanol were thoroughly mixed with the buffered solutions prepared as described under 2.4.1. The final concentration of vinclozolin in the buffered solution was 10 µg/ml. The controls were similarly prepared with methanol alone. Aliquots of approximately 2 ml of the buffered solutions were transferred to 2-ml brown ampules which were then sealed under nitrogen. All sealed ampules were incubated at 35 C in a water bath in darkness, except those of pH 7.0 which were incubated separately at 13 C, 20 C and 26 C in an environmental chamber and at 35 C in a water bath in darkness.

2.4.3. Incubation of M1 in Buffered Solutions

Aliquots of 1 ml of the stock solution of M1 at 1000 µg/ml in methanol were thoroughly mixed with the buffered solutions at pH 4.5 and 8.0, prepared as described under 2.4.1. The final concentration was 10 µg/ml, and the controls were similarly prepared with methanol alone. Aliquots of approximately 2 ml of the buffered solutions were transferred to 2-ml brown ampules which were then sealed under nitrogen. The sealed ampules were incubated at 35 C in a water bath in darkness.

A separate incubation experiment at pH 8.0 was similar. After incubation at 35 C for 24 h, one half of the samples were acidified with 0.01 M H_3PO_4 to pH 4.5 and then incubated at 35 C in a water bath in darkness.

2.4.4. High Pressure Liquid Chromatography

After incubation for various periods the concentrations of vinclozolin, M1, M2 and 3,5-dichloroaniline were determined by HPLC. Aliquots of the incubation mixtures were directly injected into the high pressure liquid chromatograph without extraction and cleanup. The operating parameters of the high pressure liquid chromatograph were as described under 2.3.4.2. with minor modification that 0.05 M phosphate buffer of pH 3.3. was used instead of water in the mobile solvent system.

Quantification was based on an external standard. Detector response was calibrated daily with analytical standards. Calculation was based on average peak areas of these external standards, which were injected before and after the sample.

2.5. Persistence in Pea Plants

2.5.1. Growing Plants

Garden pea, Pisum sativum L. (cv. Improved Laxton's Progress) were seeded in square pots (10 cm x 10 cm x 9 cm deep) and grown in a greenhouse. Twelve days after seeding the plants were used to study the persistence of vinclozolin and Ronilan® 50 WP.

2.5.2. Treatment of Pea Leaflets

A solution of vinclozolin at 2500 µg/ml was prepared in acetone and a similar solution of Ronilan® 50 WP containing 2500 µg/ml of vinclozolin was prepared by suspending 0.5 g of the formulation in 100 ml of water. After thorough mixing, four 5-µl aliquots were diluted individually with 2 ml methanol and the actual concentration of vinclozolin was determined by HPLC as described under 2.3.4.2. The mean concentration of vinclozolin was 2.60 µg/ml which was comparable to the theoretical concentration of 2.50 µg/ml. The standard deviation among the four aliquots was 0.21 µg/ml.

Twelve-day-old pea plants, each with only one pair of leaflets were used to study the persistence of the compounds. Fifteen 5-µl aliquots of the treatment solution were applied to one of the paired leaflets in each plant. All treated plants were maintained in an environmental chamber in which the temperature varied from 24 to 27 C and the RH from 40% to 70%. The photoperiod was 12 h each of light and darkness.

At various intervals after treatment, both the treated and untreated leaflets were removed from the plant. The surface of the treated leaflet was rinsed with 10 ml of water which was applied dropwise with a disposable Pasteur pipet. Both the treated and untreated leaflets and the rinsing were analyzed for vinclozolin and its degradation products M1, M2 and 3,5-dichloroaniline.

2.5.3. Analysis of Rinsings

Leaflet rinsings were acidified to approximately pH 1.0 with a few drops of 2.5% H_3PO_4 and then extracted three times with 10 ml glass-distilled dichloromethane in a 125-ml separatory funnel. The combined extracts were dried with acidified anhydrous Na_2SO_4, concentrated just to dryness in a flash evaporator at 38 C, and the residues were dissolved in 1 ml methanol for HPLC analysis as described under 2.4.4.

2.5.4. Analysis of Leaflets

2.5.4.1. Extraction of Leaflet

Each untreated leaflet and treated leaflet after rinsing from the vinclozolin-treated plant, and the leaflet of the untreated plant which served as the control, were extracted separately in a Polytron homogenizer with 50 ml of glass-distilled acetone for 1 min. Ten g of acidified anhydrous Na_2SO_4 were added to the mixture for extraction. The extracts were filtered through a Büchner funnel lined with Whatman glass fibre filter paper. The filter cake was extracted once more with 25 ml glass-distilled acetone and the combined extracts were evaporated just to dryness in a flash evaporator at 38 C. The residues were dissolved in 5 ml of 1:1 (V:V) mixture of dichloromethane and cyclohexane for cleanup by gel filtration column chromatography.

2.5.4.2. Gel Filtration Column Chromatography

A Pharmacia Column Model SR 25 (Pharmacia Fine Chemicals, Sweden) (45 cm x 2.5 cm i.d.) was packed with Bio-Beads S-X12 (Bio-Rad Laboratories, CA 94804, U.S.A.). The beads were swelled in a 1:1 (V:V) mixture of dichloromethane and cyclohexane over-night before packing the column. An Eldex Model B-100-S high pressure pump (Eldex Laboratories, Inc., San Carlos, CA 94070, U.S.A.) was used for solvent delivery and a Valco sample injection valve (Valco Instruments Co., Houston, TX 77024, U.S.A.) equipped with a 5-ml injection loop was used to inject samples onto the column. After introduction of a sample the column was eluted with the 1:1 (V:V) mixture of dichloromethane and cyclohexane. Fraction 1, consisting of the first 68 ml, was discarded and fraction 2, consisting of the next 150 ml, was collected. It contained the vinclozolin and its degradation products.

2.5.4.3. Isolation of M1

In order to isolate M1, fraction 2 was extracted three times with 25 ml of 0.1 M Na_2HPO_4 solution. The combined aqueous extracts containing M1 were back-extracted with 25 ml of dichloromethane. After acidification to approximately pH 1.0 with 2.5% H_3PO_4, the combined aqueous extracts were extracted three times with 25 ml of dichloromethane to isolate M1. The combined dichloromethane extracts were dried with acidified anhydrous Na_2SO_4, and then evaporated just to dryness in a flash evaporator at 38 C. The residues of M1 were

dissolved in 1 ml of methanol for HPLC analysis, which was conducted as described under 2.4.4.

The combined organic extracts which contained vinclozolin, M2 and 3,5-dichloroaniline were dried with acidified anhydrous Na_2SO_4 and then evaporated just to dryness in a flash evaporator at 38 C. The residues were dissolved in 1 ml of glass-distilled dichloromethane and analyzed by GC-MS.

2.5.4.4. Determination by GC-MS

Vinclozolin, M2 and 3,5-dichloroaniline were detected and quantified with a Hewlett Packard Model 5890 gas chromatograph equipped with a Model 5970 mass selective detector. The operating parameters for the column and the injector were as described under 2.1.2.2. Qualitative and quantitative analysis were carried out by selected ion monitoring. Four specific ions characteristic of each compound were selected for monitoring and they were as follows:

Vinclozolin 198, 212, 285 and 287 amu

M2 71, 72, 161 and 163 amu

3,5-dichloroaniline 90, 126, 161 and 163 amu

Response of the mass selective detector was calibrated daily with analytical standards. Quantification was based on the average peak areas of these external standards, which were injected before and after the sample.

2.5.5. Evaluation of Analytical Methods

Stock solutions of vinclozolin, M1, M2 and 3,5-dichloroaniline at 1000 µg/ml, 100 µg/ml and 20 µg/ml were prepared in glass-distilled acetone. Two 100-ml aliquots of water were fortified individually with one ml of the appropriate stock solution to give fortification levels of 10 ppm and 1 ppm for each compound. Four replicates of 10 ml of each fortified water were extracted and analyzed as described under 2.5.3. to determine their recoveries.

Ten g of pea leaflets were fortified with vinclozolin, M1, M2 and 3,5-dichloroaniline at concentration levels of 10 ppm, 1 ppm and 0.2 ppm by adding 0.1 ml of the appropriate solutions in acetone. One h after fortification four replicates at each fortification level were extracted, cleaned and analyzed as described under 2.5.4. to determine the recovery of each compound.

3. RESULTS AND DISCUSSION

3.1 Preparation of Vinclozolin

Approximately 835 mg of vinclozolin were isolated from 2 g of Ronilan® 50 WP indicating a yield of slightly over 80%. Two solutions of 5 µg/ml in glass-distilled methanol and 0.02 µg/ml in glass-distilled hexane were analyzed by HPLC-UV and GC-EC respectively; a single peak resulted, which was vinclozolin. All three normalized UV-visible spectra collected at up-slope, apex and down-slope of the peak were identical indicating that pure vinclozolin had been isolated from the commercial Ronilan®. Furthermore, similar area counts were obtained with the vinclozolin prepared as described and the reference obtained from the U.S. Environmental Protection Agency (EPA) when these materials were analyzed by HPLC-UV or by GC-EC. Since the purity of the reference from EPA was known to be 99.7%, the purity of the vinclozolin isolated was therefore approximately 99%. The vinclozolin isolated from Ronilan® provided all the materials needed for these studies.

3.2 Hydrolysis of Vinclozolin

When 10 µg/ml of vinclozolin in three 0.01 M phosphate buffers of pH 5.0, 7.0 and 9.0 were incubated at 20 C for various periods the concentration decreased steadily with time. The disappearance rate of vinclozolin was fastest at pH 9.0, slower at pH 7.0 and slowest at pH

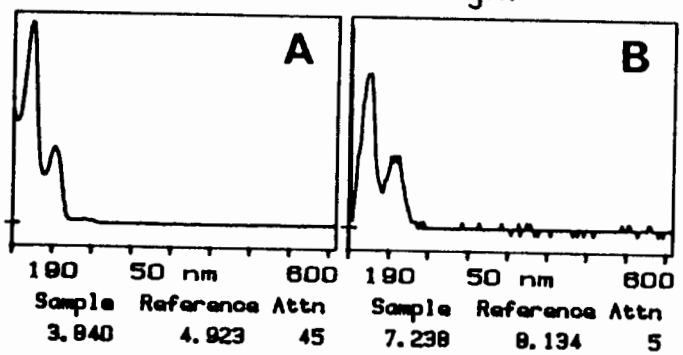
5.0. At pH 9.0 vinclozolin disappeared so fast that only about 50% remained after incubation for 1.5 h and it could not be detected at the limit of 0.1 µg/ml after incubation for 3 h.

Analysis of the incubation mixtures by HPLC showed two new peaks (Fig. 3), one with an absolute retention time of approximately 7.24 min (peak B) and the other of approximately 3.48 min (peak A) which was very close to the retention time of the injected solvent carrier for the HPLC analysis. However, the UV-visible spectrum of peak A showed an absorption maximum of 214 nm which was slightly above the UV cut-off of methanol (205 nm), indicating the presence of a degradation product from vinclozolin. Further evidence to support this hypothesis was provided by the observations that a longer absolute retention time of peak A was obtained by acidifying the water used in the mobile solvent system of the HPLC. Chromatograms of the HPLC analysis of 10 µg/ml of vinclozolin incubated in 0.01 M phosphate buffers of pH 7.0 at 20 C for 7 days are given in Figures 3 and 4. Unknown peak A had an absolute retention time of 3.84 min when the mobile solvent system comprised 72% methanol and 28% water (Figure 3). This retention time changed to 5.04 min when the mobile solvent system comprised 72% methanol and 28% 0.05 M phosphate buffer of pH 3.3 (Figure 4). The longer retention obtained for peak A with acidified water in reverse phase HPLC suggests that compound A probably has a carboxylic group which remains in unionized form at acidic pH, because in reverse phase HPLC the non-polar compounds have a much stronger interaction with the stationary phase of the column resulting in longer absolute retention times. In all subsequent HPLC analyses 0.05 M phosphate buffer of pH 3.3 was used instead of water in the mobile solvent system.

Figure 3. Chromatogram of HPLC analysis of 10 µg/ml of vinclozolin incubated at 20 C in 0.01 M phosphate buffer of pH 7.0 for 7 days (Mobile solvent syste consisted of 72% methanol and 28% water); and UV-visible absorption spectra of degradation products A and B.

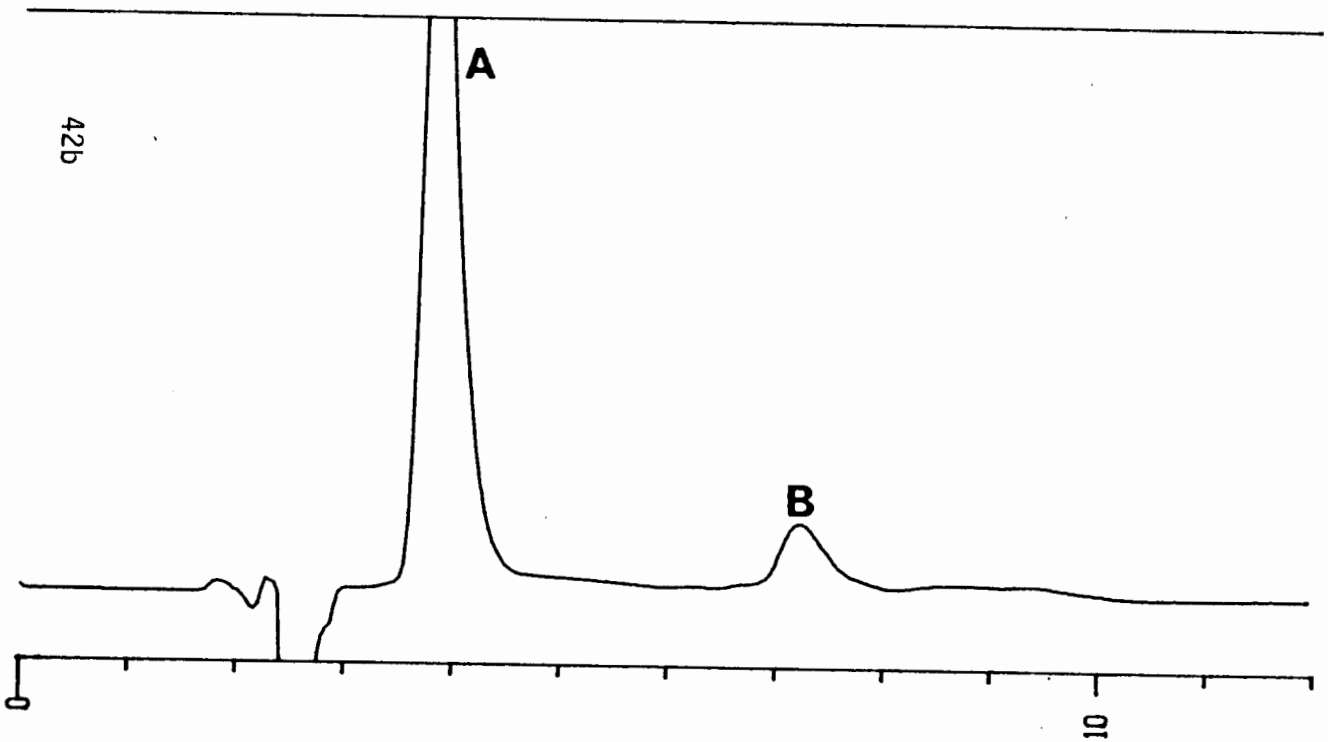
File: RAWDAT
Date: 08/08/1985

HYDROLYSIS
origin



PH 7.0 AND 20 C
sample id.

7—DAY INCUBATION



analysis S. SZETO
inj. vol. 20 MICROLITERS
" 72% METHANOL
" 28% WATER
stat. ph. C 18
column 25 CM X 5 MM I.D.
Inj. Time: 15:46
Attn [mAU]: 30.0 (39.4)
Zero%: 10%
Signal: A: 2.8 Set

Wavelength:
1. 210. 4
2. 212. 4
3. 254. 4
4. 260. 80
5. 280. 4
6. 320. 20
7. 450. 50
8. 550. 100

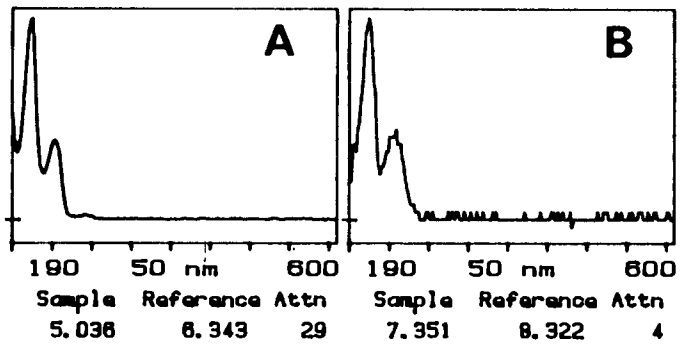
Figure 4. Chromatogram of HPLC analysis of 10 µg/ml of vinclozolin incubated at 20 C in 0.01 M phosphate buffer of pH 7.0 for 7 days (Mobile solvent system consisted of 72% methanol and 28% 0.05 M phosphate buffer of pH 3.3); and UV-visible absorption spectra of degradation products A and B.

File: RAWDAT
Date: 08/08/1985

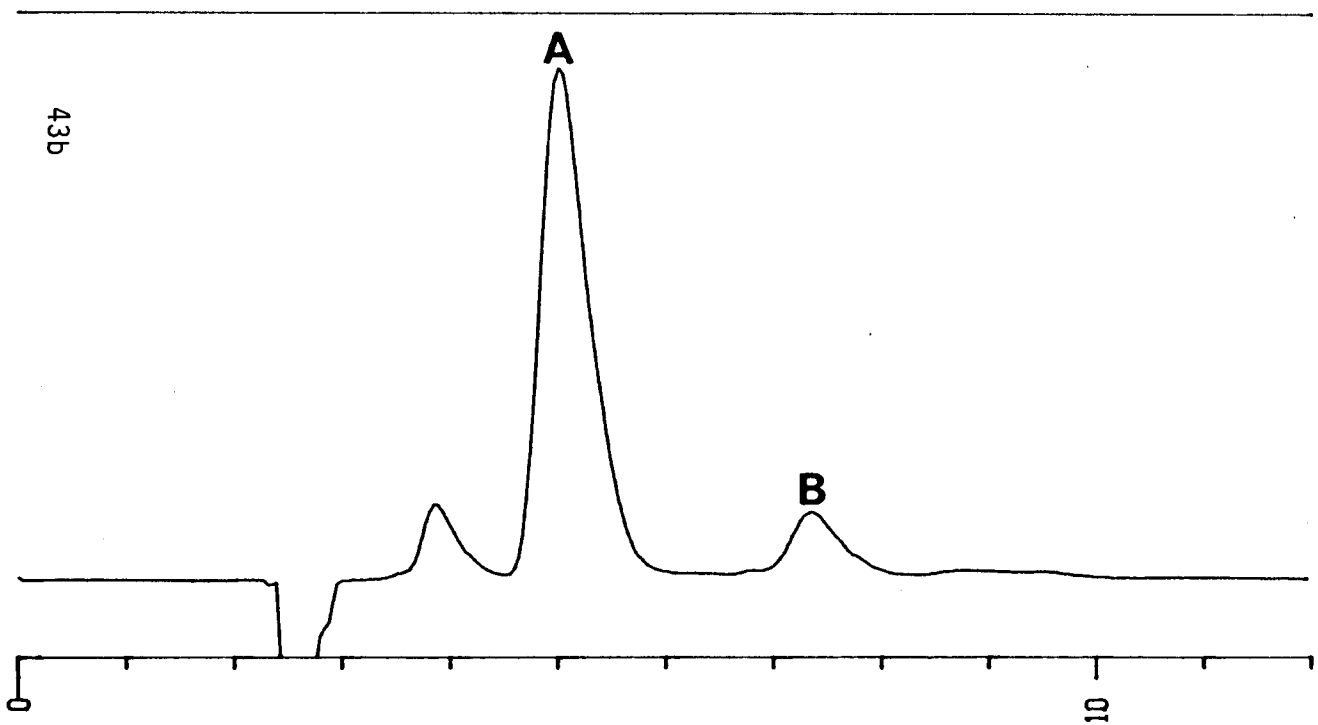
HYDROLYSIS
origin

PH 7.0 AND 20 C
sample id.

7—DAY INCUBATION



Sample	Reference	Attn	Sample	Reference	Attn
5.036	6.343	29	7.351	8.322	4



Time [min]

analysis S. SZETO
inj. vol. 20 MICROLITERS
'' 72% METHANOL
'' 28% PH 3.3, 0.05M
stat. ph. C 18
column 25 CM X 5 MM I.D.
Inj. Time: 13:55
Attn [mAU]: 30.0 (24.4)
Zero%: 10%
Signal: A: 2,8 Set

Wavelengths
1 . 210, 4
2 . 212, 4
3 . 254, 4
4 . 280, 80
5 . 280, 4
6 . 320, 20
7 . 450, 50
8 . 550, 100

At the three pHs tested, 3,5-dichloroaniline was detected as a minor product of degradation. After 30 days incubation at 20 C its concentration in the incubation mixture was less than 0.3 µg/ml. The identity of 3,5-dichloroaniline was confirmed by GC-MS. An authentic reference standard was obtained from the Laboratory Services Division of Agriculture Canada in Ottawa. Under the gas chromatographic conditions as described under 2.2.2. of the Materials and Methods Section the absolute retention time of 3,5-dichloroaniline was 7.04 min. Its detection as a degradation product was confirmed by retention time of both GC and HPLC, and by its mass spectrum where the presence of the following characteristic major ions (in amu): 62, 63, 90, 99, 125, 126, 134, 161 (molecular ion), 163 and 165, were observed.

3.3. Isolation and Identification of Degradation Products

3.3.1. Isolation and Purity Determinations

Two degradation products A and B (Figures 3 and 4), detected in the study on the hydrolysis of vinclozolin were designated as M1 and M2 respectively.

Approximately 250 mg of M1 and 150 mg of M2 were obtained by incubating 500 mg of vinclozolin in 0.1 M phosphate buffer of pH 7.0 at 35 C for 7 days. About 100 mg of M1 were used in the preparation of ethylated M1. M1, ethylated M1 and M2 are solids at room temperature. Their melting points are 131.5-132 C, 112.5-113 C and 113.5-114 C, respectively. The narrow melting point ranges of all three

compounds indicate that high purity was obtained upon recrystallization. The purities of M1 and ethylated M1 were further evaluated by HPLC analysis with UV absorption at 212 nm. The liquid chromatogram of each compound showed only one major peak, the area of which accounted for 98% to 99% of the total peak areas. Furthermore, normalized UV-visible absorption spectra taken at up-slope, apex and down-slope of the peak were identical, indicating that the peak was derived from a single compound. It was apparent that M1 and ethylated M1 were chromatographically pure and their purities were about 98%.

The purity of M2 was evaluated by GC-MS with the mass scanning range from 40 amu to 450 amu. Only one major peak was indicated, the area of which accounted for 96% to 97% of the total peak area. Mass spectra taken at up-slope, apex and down-slope of the peak were identical, indicating high peak purity. Based on the melting point determination and the results of GC-MS analysis it was evident that M2 was chromatographically pure and its purity was 96% to 97%.

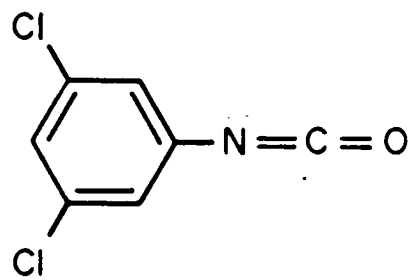
Analyses of M1 and ethylated M1 by GC-MS were unsuccessful. No peak was indicated in GC-MS runs of M1. Based on the HPLC analysis of M1 there were strong indications that M1 contained a carboxylic group which rendered direct gas chromatography unsuccessful. Analysis of ethylated M1 by GC-MS showed one major and two minor peaks, indicating its on-column thermodegradation. The major peak was identified and confirmed as vinclozolin. Confirmation was achieved by comparing the absolute retention time (10.37 min) and the presence of major ions characteristic of authentic vinclozolin obtained from the EPA (178, 187, 198, 200, 212, 213, 214, 215, 285 and 287 amu). One of the two

minor peaks was identified and confirmed as 3,5-dichloroaniline by comparing its absolute retention time (7.04 min) and characteristic ions (62, 63, 90, 99, 125, 126, 134, 161, 163, and 165 amu) with those of the authentic standard. The identity of the other minor peak (absolute retention time 5.01 min) was not confirmed with an authentic standard. However, its characteristic ions of 187, 159 and 124 amu strongly suggest its identity to be 3,5-dichloroisocyanatobenzene (Figure 5). The ratios of the isotopes indicated the presence of two chlorines. Other spectral indicators that were consistent with 3,5-dichloroisocyanatobenzene were the molecular ions at 187 amu ($C_7H_3Cl_2NO$), ions at 159 amu (loss of C = O for a total of 28 amu), and ions at 124 amu containing one chlorine (loss of C = O and one Cl for a total of 63 amu).

3.3.2. Identification of Degradation Products

Vinclozolin consists of a 3,5-dichlorophenyl and a 2,4-oxazolidinedione moiety (Fig. 1). It was hypothesized that on hydrolysis the oxazolidinedione ring may open and 3,5-dichloroaniline could be produced. In fact 3,5-dichloroaniline was identified and confirmed as a minor product; and two major products, M1 and M2 were isolated from the hydrolysis of vinclozolin which might have resulted from the cleavage of the 2,4-oxazolidinedione ring. According to the review by Clark-Lewis (1958) it is generally believed, without proof, that the hydrolysis of 3-alkyl-2,4-oxazolidinedione will lead to amides, whereas the hydrolysis of 3-phenyl-2,4-oxazolidinedione yields

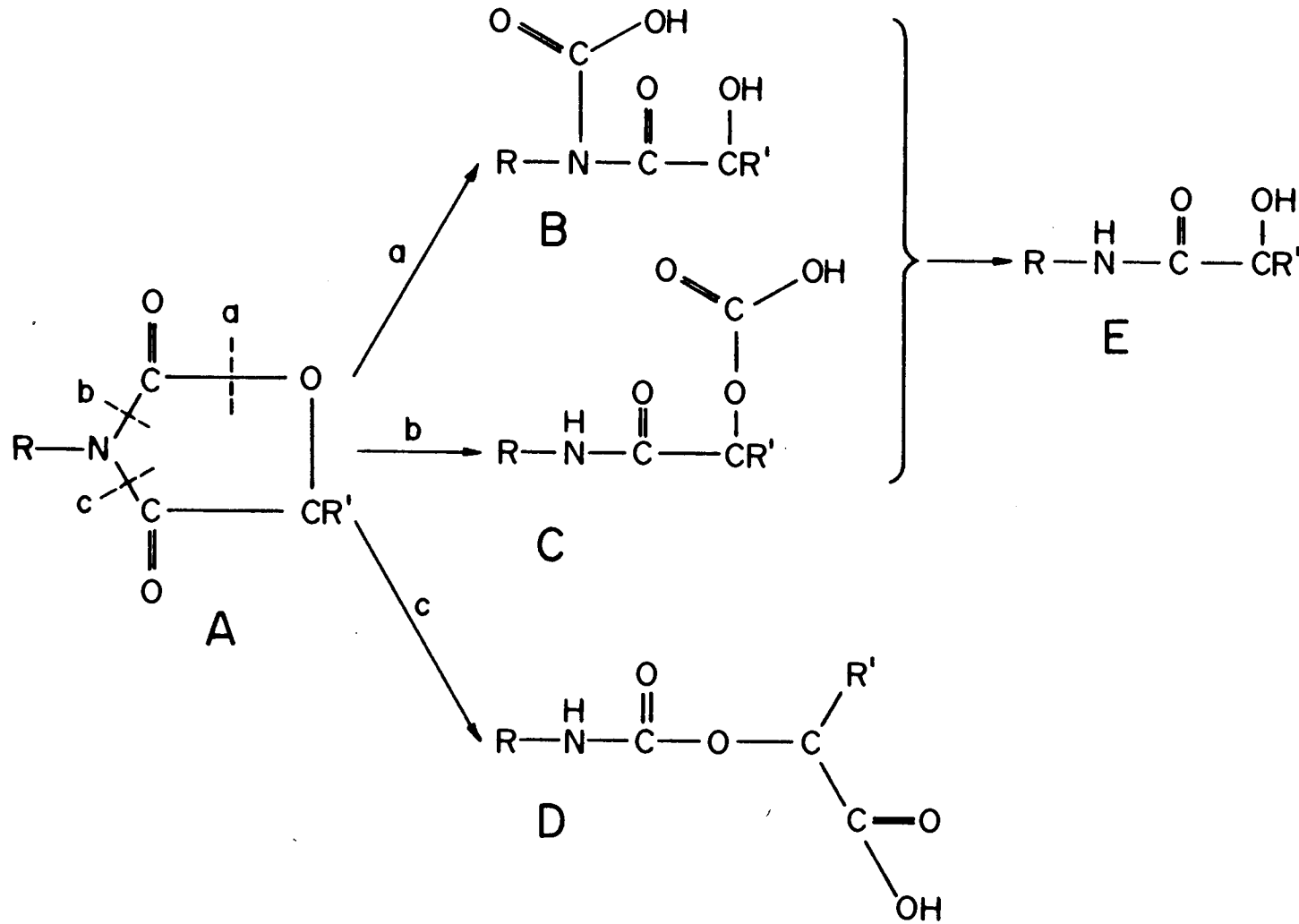
Figure 5. Structural formula of 3,5-dichloroisocyanatobenzene.



3,5 - DICHLOROISOCYANATOBENZENE

M.W. 187

Figure 6. A hypothetical pathway summarized by Clark-Lewis (1958) for the hydrolysis of 2,4-oxazolidinediones (A) leading to the formation of urethans (D) and amides (E) via the intermediates of carbamic acids (B) or alkyl hydrogen carbonates (C).

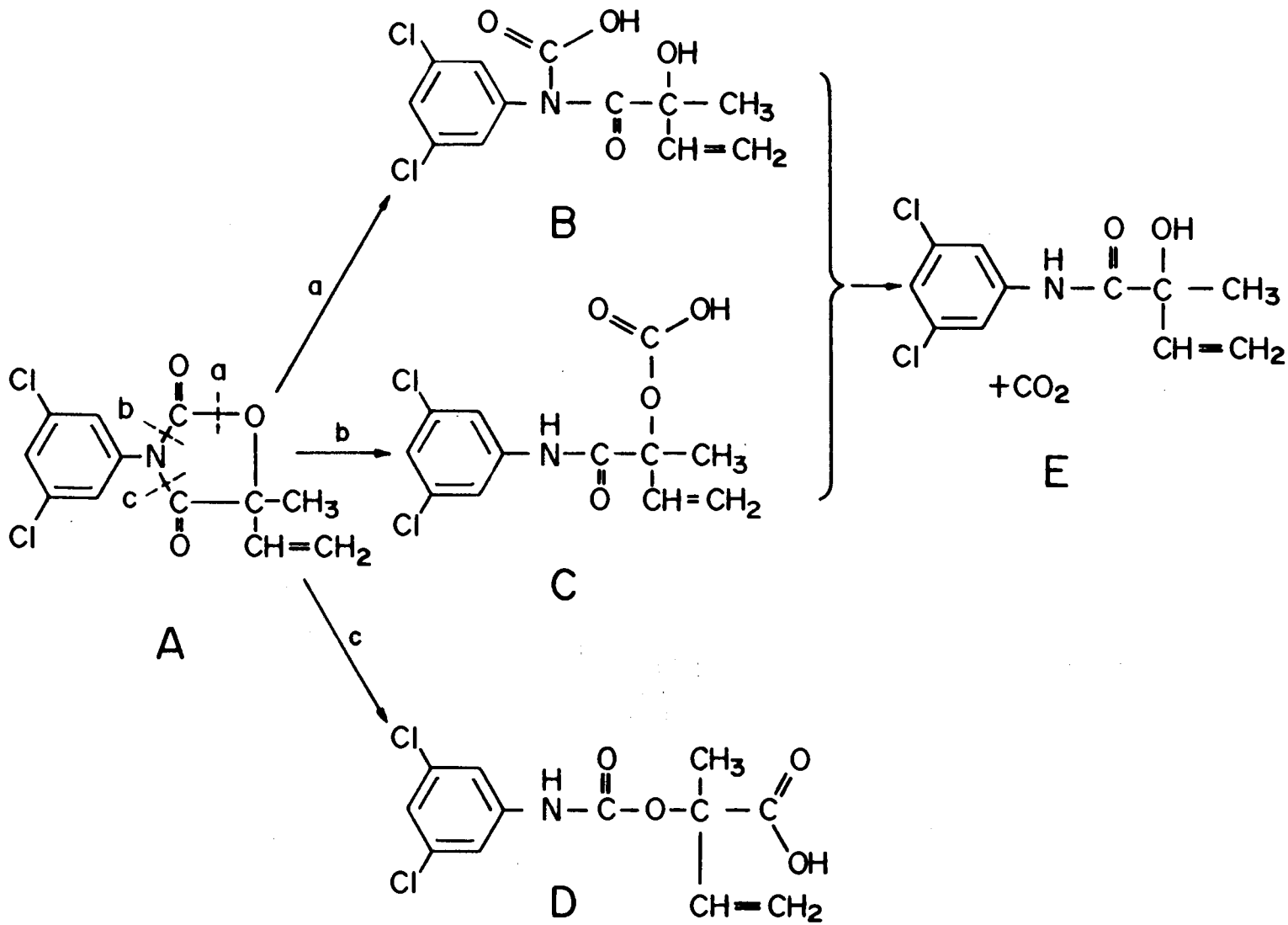

A
B
C
D
E
a
b
c

phenylurethans (Fig. 6). For vinclozolin it was hypothesized that upon hydrolysis the 2,4-oxazolidinedione ring may open to yield 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid (Fig. 7D) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (Fig. 7E) via the intermediates of N-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (Fig. 7B) or 3-[(3,5-dichlorophenyl) carbamoyl]-3-methyl-propenyl hydrogen carbonate (Fig. 7C). The behavior in chromatography of M1 indicated that M1 probably had a carboxylic group. Therefore, M1 could be the phenylurethan (Fig. 7D) or one of the intermediates, namely the carbamic acid (Fig. 7B) or the hydrogen carbonate (Fig. 7C), and M2 could be the enanilide (Fig. 7E).

3.3.2.1. Identification of M2

Solid probe mass spectral data of M2 are given in Table 1. The ratios of the isotopes indicated the presence of two chlorines and the major ions were consistent with 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (Figure 8). Ions at 259 amu represented the molecular ion. Ions at 217 amu indicated the loss of $-CH_3$ (15 amu) and ($-CH=CH_2$ 27 amu) for a total of 42 amu. Ions at 187 amu indicated the ion fragment of $C_6H_3Cl_2-N=C=O$ which was commonly found in mass spectra of dichlorphenyl urea herbicides such as linuron, diuron and neburon (Figure 9) (Hites 1985). Ions at 161 amu indicated the 3,5-dichloro-aniline ion fragment resulting from a single proton transfer coupled with elimination of a neutral particle (Figure 10) (Schlunegger 1980). The dichloroaniline ion fragment was observed in mass spectra

Figure 7. A hypothetical pathway of hydrolysis of vinclozolin (A) leading to the formation of 2-[(3,5-dichlorophenyl) carbamoyl] oxy-2-methyl-3-butenoic acid (D) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (E) via the intermediates of N-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (B) or 3-[(3,5-dichlorophenyl)carbamoyl]-3-methylpropenyl hydrogen carbonate (C).


A
B
C
D
E
a
b
c
+CO2

of many dichlorophenylanilides such as N-(3,4-dichlorophenyl)-2'-methyl-2',3'-dihydroxypropionamide; N-(3,4-dichlorophenyl)-2'-methylvaleramide; and N-(3,4-dichlorophenyl)-2'-methyl-3'-hydroxyvaleramide (Figure 11) (Safe and Hutzinger 1973). Ions at 71 amu indicated the ion fragment of $C(CH_3)(CH=CH_2)OH$.

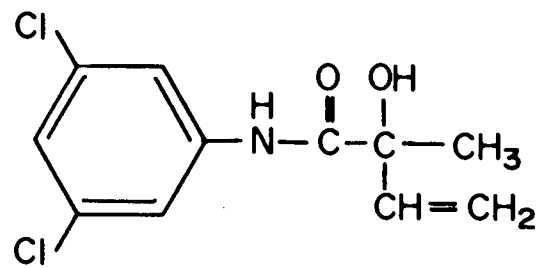
The proton NMR spectrum of M2 was consistent with 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide as indicated by the following chemical shifts (in ppm): 1.58, singlet, 3H (aliphatic CH_3); 2.48, singlet (OH); 5.25-5.48, doublet of doublets (vinylic CH_2); 6.13-6.25, doublet of doublets (vinylic CH), 7.10, triplet, 1H (aromatic CH at the para position); 7.58, doublet 2H (aromatic CH at the ortho positions); 8.60, singlet (NH) (Figure 12). The peaks of OH and NH protons were very broad and appeared at varying positions depending upon solute concentrations and solvent conditions which may be attributed to the occurrence of both intra- and inter-molecular hydrogen bonding. Similarly, the ^{13}C NMR spectrum of M2 was also consistent with 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide as indicated by the following chemical shifts (in ppm): 26.2 (CH_3); 81.1 (-OH); 118.2 (vinylic CH_2); 146.5 (vinylic CH); 139.8 (aromatic carbon attached to NH moiety); 128.2 (aromatic carbon at the ortho positions); 146.3 (aromatic carbon at the meta positions); 123.0(aromatic carbon at the para position); 178.5 (carbonyl) (Figure 13).

Based on the spectral data from MS and proton and ^{13}C NMR, the degradation product M2 isolated from hydrolysis of vinclozolin was identified as 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide.

Table 1. Solid probe mass spectral data of M1, ethylated M1 and M2.

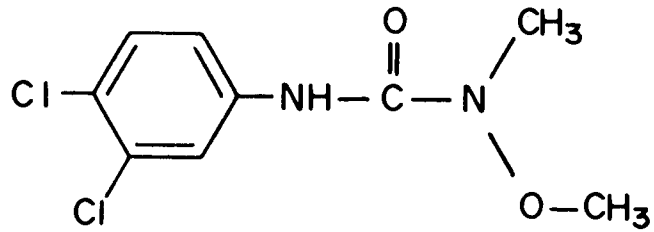
Compound	Major ions in amu (% intensity)					
M1	303(1.4)	285(3.4)	187(100)	161(50.7)	99(16.8)	71(59.6)
Ethylated M1	331(4.9)	285(4.6)	189(30.6)	187(48.3)	161 (9.3)	127(22.6)
	99(89.8)	71(100)				
M2	259(29.0)	217(17.2)	187(25.9)	161(13.8)	71(100)	

Figure 8. Structural formula of 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide.

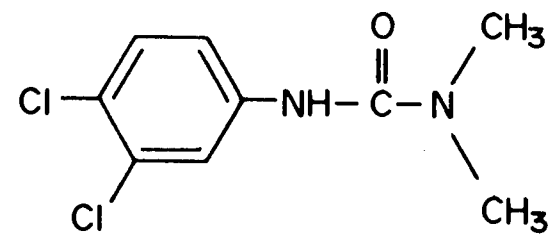


M. W. 259

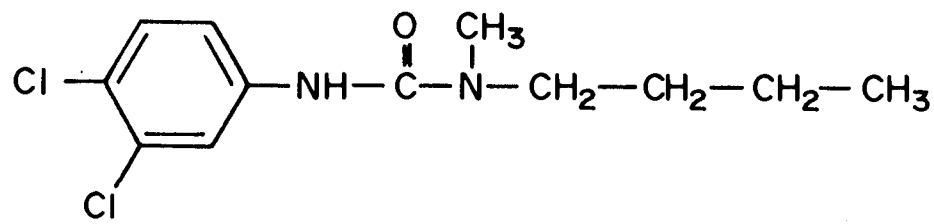
Figure 9. Structural formulae of linuron, diuron and neburon.



Linuron M. W. 248

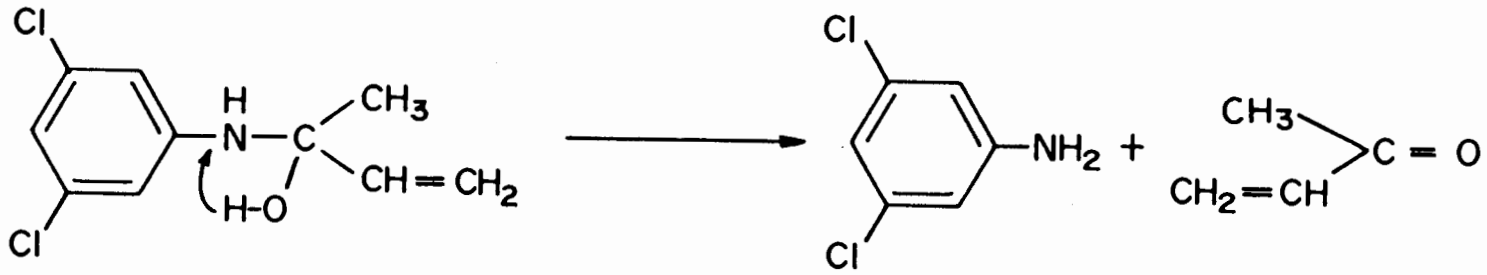


Diuron M. W. 232



Neburon M. W. 274

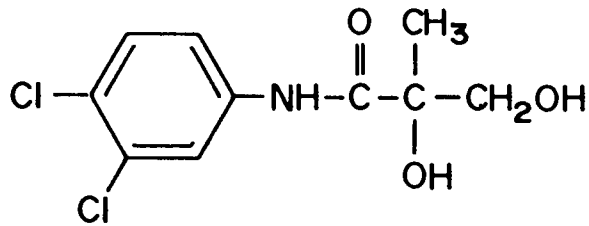
Figure 10. Rearrangement of 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanalide (A) yielding 3,5-dichloroaniline ion fragment (B).



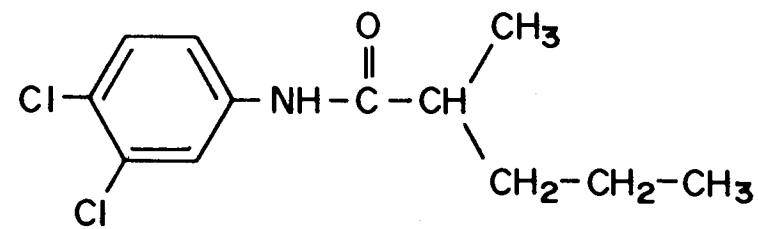
A

B + C = O

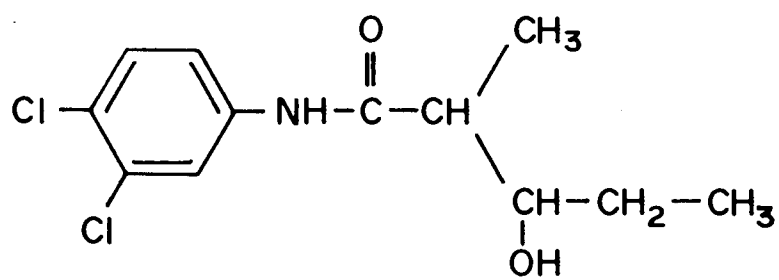
Figure 11. Structural formulae of N-(3,4-dichlorophenyl)-2'-methyl-2',3'-dihydroxypropionamide (A); N-(3,4-dichlorophenyl)-2'-methylvaleramide (B); and N-(3,4-dichlorophenyl)-2'-methyl-3'-hydroxyvaleramide (C).



A



B



C

Figure 12. Assignments of proton NMR chemical shifts (in ppm) to 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide.

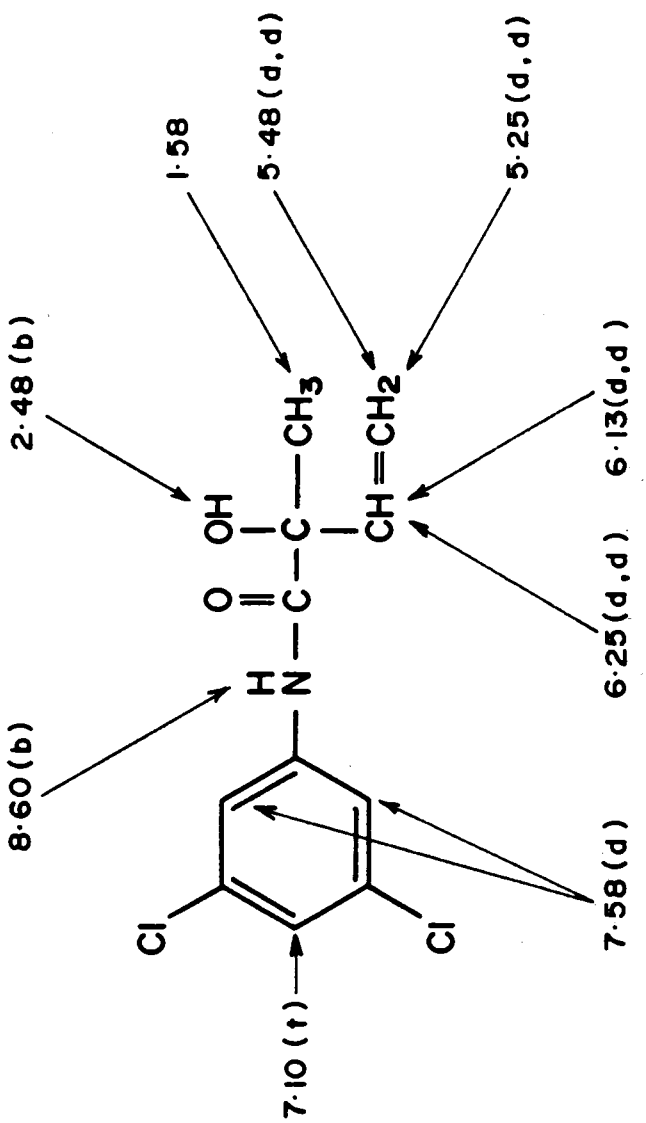
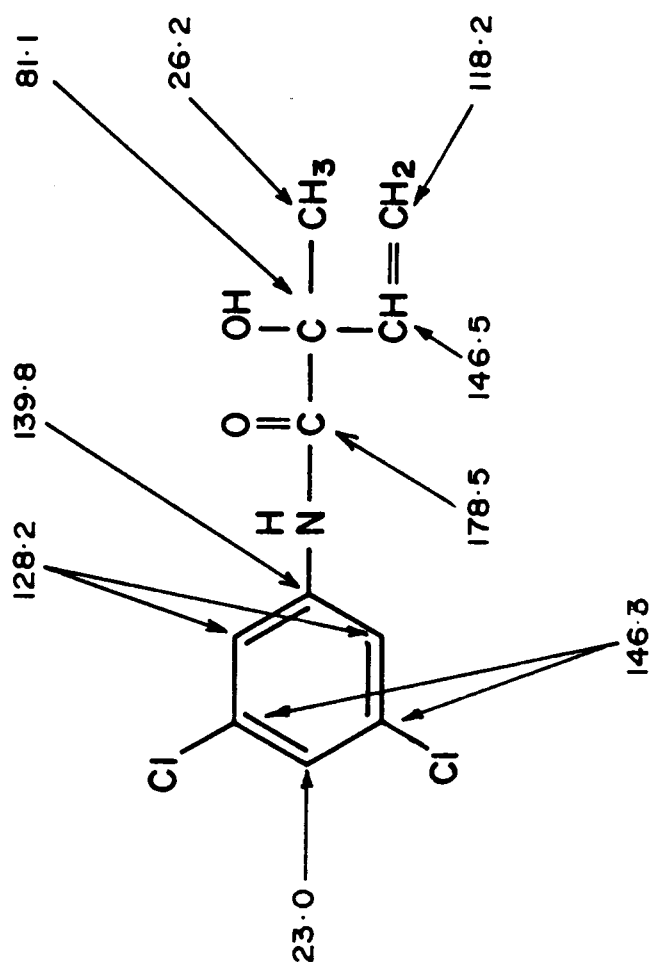

1·58
5·48 (d, d)
5·25 (d, d)
2·48 (b)
6·13 (d, d)
6·25 (d, d)
8·60 (b)
7·58 (d)
7·10 (t)
Cl
Cl
OH
O
CH3
CH=CH2
H
N

Figure 13. Assignments of ^{13}C NMR chemical shifts (in ppm) to 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide.


81·1
26·2
118·2
OH
CH3
CH2
C
CH
146·5
O
C
139·8
178·5
H
N
128·2
146·3
Cl
Cl
123·0

3.3.2.2. Identification of M1

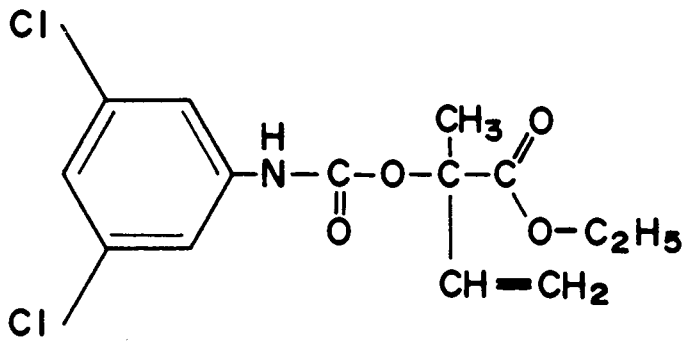
Solid probe mass spectral data of M1 are given in Table 1. The ratios of the isotopes indicated the presence of two chlorines and the major ions were consistent with 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid (Fig. 7D). Ions at 303 amu represented the molecular ion. Ions at 285 amu indicated recyclization to yield vinclozolin by eliminating water (18 amu). Recyclization of M1 to yield vinclozolin was also demonstrated simply by heating M1 at 120 C for 5 min. Ions at 187 amu indicated the ion fragment of $C_6H_3Cl_2$-N=C=O which was commonly found in mass spectra of dichlorophenyl urea herbicides. The mass spectrum of M2 also showed this ion fragment of 187 amu. Ions at 161 amu indicated the 3,5-dichloroaniline ion fragment resulting from a single proton transfer coupled with elimination of a neutral particle as discussed previously in the identification of M2. Ions at 99 amu indicated the ion fragment of $C(CH_3)(CH=CH_2)COOH$ and ions at 71 amu indicated the ion fragment of $O\text{-}CH(CH_3)(CH=CH_2)$ resulting from loss of CO_2 from ion fragment $-O\text{-}C(CH_3)(CH=CH_2)COOH$, a product of α cleavage along the carbonyl carbon (Creswell et al. 1972).

The solid probe mass spectral data of M1 were inconsistent with N-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (Fig. 7B) and 3-[(3,5-dichlorophenyl)carbamoyl]-3-methyl-propenyl hydrogen carbonate (Fig. 7C). The carbamic acid can not undergo re-arrangement to yield the 3,5-dichloroaniline ion fragment of 161 amu because there is no -NH moiety in its molecular structure (Schlunegger

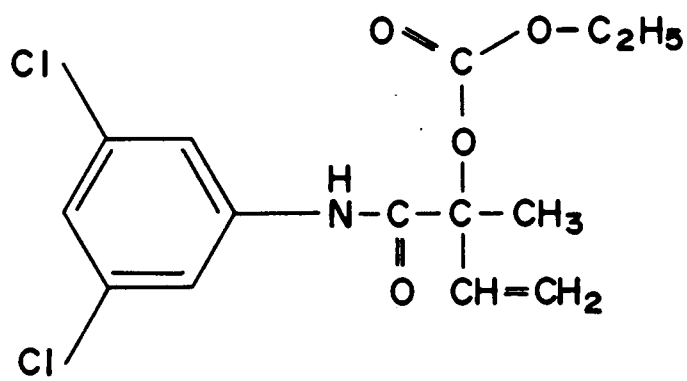
1980). Fragmentation of the hydrogen carbonate can not be expected to yield major ions at 99 amu which were present in the mass spectra of M1.

Solid probe mass spectral data of ethylated M1 (Table 1) were consistent with the ethyl ester of the butenoic acid (Fig. 14A). The ratios of the isotopes indicated the presence of two chlorines. Ions at 331 amu represented the molecular ion. Ions at 285 amu indicated recyclization to yield vinclozolin by eliminating ethanol (C_2H_5OH, 46 amu). Recyclization of ethylated M1 to yield vinclozolin in GC-MS analyses has been discussed previously under 3.3.1. Ions at 189 amu were attributed to fragmentation of vinclozolin. Ions at 187 amu indicated the ion fragment of $C_6H_3Cl_2$-N=C=O. Ions at 161 amu indicated the 3,5-dichloroaniline ion fragment resulting from a single proton transfer coupled with elimination of a neutral particle. The intensity of the ion at 161 amu (9.3%) was much lower than that observed in solid probe mass spectrum of M1 (Table 1). Ions at 127 amu indicated the ion fragment of $C(CH_3)(CH=CH_2)COOC_2H_5$ resulting from α cleavage along the carbonyl carbon. Ions at 99 amu indicated further elimination of ethylene ($CH_2=CH_2$,28 amu) from ions at 127 amu. Ions at 71 amu indicated the ion fragment of $OCH(CH_3)(CH=CH_2)$ which was also observed in M1. Certain aspects of the solid probe mass spectral data of ethylated M1 were inconsistent with the ethyl esters of hydrogen carbonate (Fig. 14B) and carbamic acid (Fig. 14C). Fragmentation of the ethylester of hydrogen carbonate cannot be expected to yield major ions at 99 amu which were present in the mass spectra of ethylated M1. Ethyl ester of the carbamic acid cannot

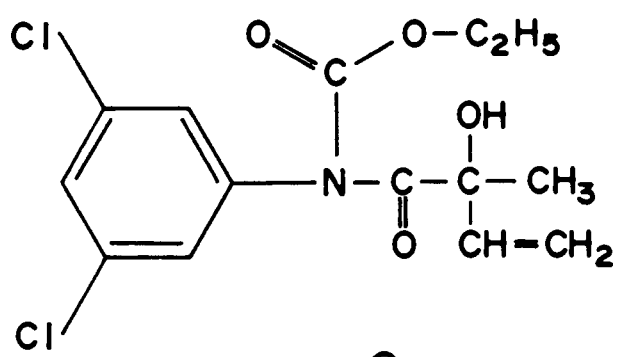
Figure 14. Possible structures of ethylated M1: (A) 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid; (B) 3-[(3,5-dichlorophenyl)carbamoyl]-3-methyl-propenyl hydrogen carbonate; and (C) N-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl-1-carbamic acid.



A



B



C

undergo rearrangement to yield the 3,5-dichloroaniline ion fragment at 161 amu because there is no -NH moiety in its molecular structure.

The proton NMR spectrum of M1 was consistent with the butenoic acid (Fig. 7D) as indicated by the following chemical shifts (in ppm): 1.72, singlet, 3H (aliphatic CH_3); 5.26-5.48, doublet of doublets (vinylic CH_2); 6.24-6.30, doublet of doublets (vinylic CH); 7.10, triplet, 1H (aromatic CH at the para position); 7.60, doublet, 2H (aromatic CH at the ortho positions); 9.22, singlet (NH); 2.80 and 11.4, singlet (carboxylic proton) (Figure 15). The peaks of NH and carboxylic protons were very broad. The fact that the carboxylic proton peaks appeared at varying positions, namely 2.80 and 11.4 ppm, may be attributed to the occurrence of both intra- and inter-molecular hydrogen bonding. Similarly the ^{13}C NMR spectrum of M1 was also consistent with the butenoic acid as indicated by the following chemical shifts (in ppm): 22.7 (CH_3); 115.9 (vinylic CH_2); 142.5 (vinylic CH); 80.6 [OC*(CH_3)(CH=CH_2)COOH]; 152.9 (carbamate carbonyl); 172.7 (carboxylic carbon); 138.5 (aromatic carbon attached to NH moiety); 117.4 (aromatic carbon at the ortho positions); 123.0 (aromatic carbon at the para position); 135.8 (aromatic carbon at the meta positions) (Figure 16). The ^{13}C NMR spectrum of M1 was inconsistent with the carbamic acid (Fig. 7B). Chemical shift of the ortho carbons of carbamic acid would be in the range of 125-128 ppm. Many N-(3,5-dichlorophenyl)-dicarboximides showed chemical shifts at about 125 to 128 ppm for carbons ortho to the nitrogen (Bremser 1985; Bremser and Fachinger 1985). For example, the chemical shift for the two ortho-carbons was 125.6 ppm for vinclozolin (Fig. 17). The actual measure-

ment of the chemical shift of the two ortho carbons of M1 was 117.4 ppm which did not agree with the predicted chemical shift for the carbamic acid. The ^{13}C NMR spectra of M1 were inconsistent with the hydrogen carbonate (Fig. 7C) in that the chemical shift of 172.7 ppm (carboxylic carbonyl) was inconsistent with the carbonate carbonyl. Examples of some carbonates, such as dimethyl carbonate, diethyl carbonate, diphenyl carbonate and dibutyl carbonate showed chemical shifts from 152.0 ppm to 156.5 ppm for the carbonyl carbon (Levy and Nelson 1972). Furthermore, it is well established that both the carbamates and the hydrogen carbonates are unstable and decarboxylation occurs easily (Morrison and Boyd 1959); and decarboxylation of both the carbamic acid and the hydrogen carbonate lead to the formation of M2 (Fig. 7E).

^{13}C NMR spectrum of ethylated M1 was consistent with the ethyl ester of 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid as indicated by the following chemical shifts (in ppm): 14.0 (CH_3 of the ethyl group); 62.2 (CH_2 of the ethyl group); 23.1 (CH_3); 80.6 [$OC^*(CH_3)(CH=CH_2)COOC_2H_5$]; 151.4 (carbamate carbonyl); 171.4 (carboxylic carbon); 116.2 (vinylic CH_2); 136.6 (vinylic CH); 139.6 (aromatic carbon attached to NH moiety); 116.8 (aromatic carbon at the ortho positions); 123.4, (aromatic carbon at the para position); 135.1 (aromatic carbon at the meta positions) (Fig. 18).

Based on the spectral data of MS, proton and ^{13}C NMR, the degradation product M1 isolated from hydrolysis of vinclozolin was identified as 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid.

Figure 15. Assignments of proton NMR chemical shifts (in ppm) to 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid.

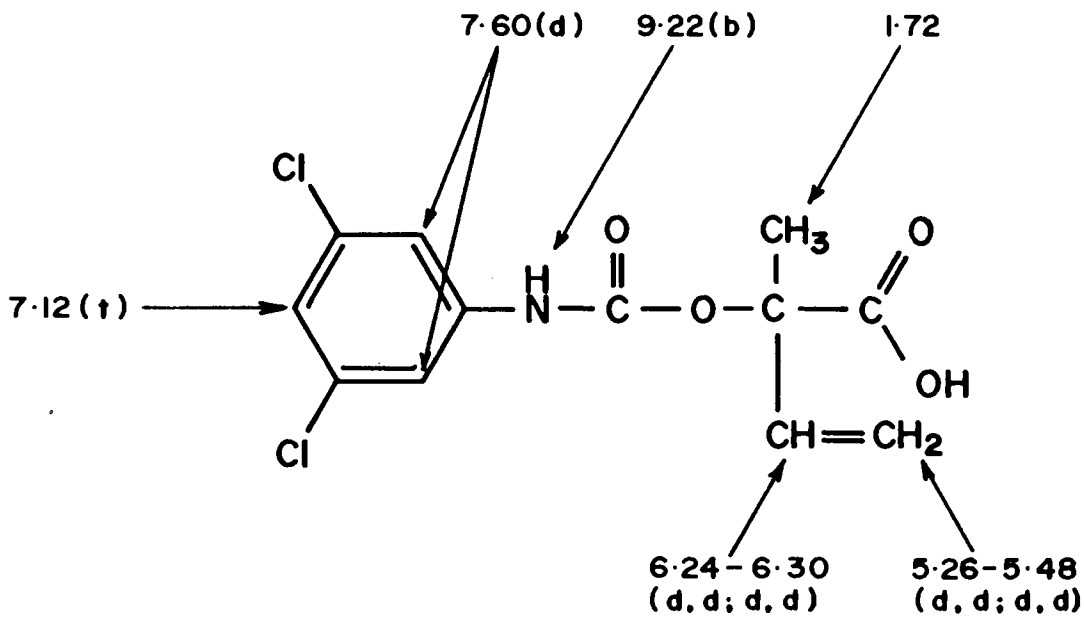

7·60(d)
9·22(b)
1·72
7·12(t)
Cl
Cl
H
N
O
C
O
CH3
C
O
C
OH
CH=CH2
6·24-6·30
(d, d; d, d)
5·26-5·48
(d, d; d, d)

Figure 16. Assignments of ^{13}C NMR chemical shifts (in ppm) to 2-[(3,5-dichlorophenyl) carbamoyl] oxy-2-methyl-3-butenoic acid.

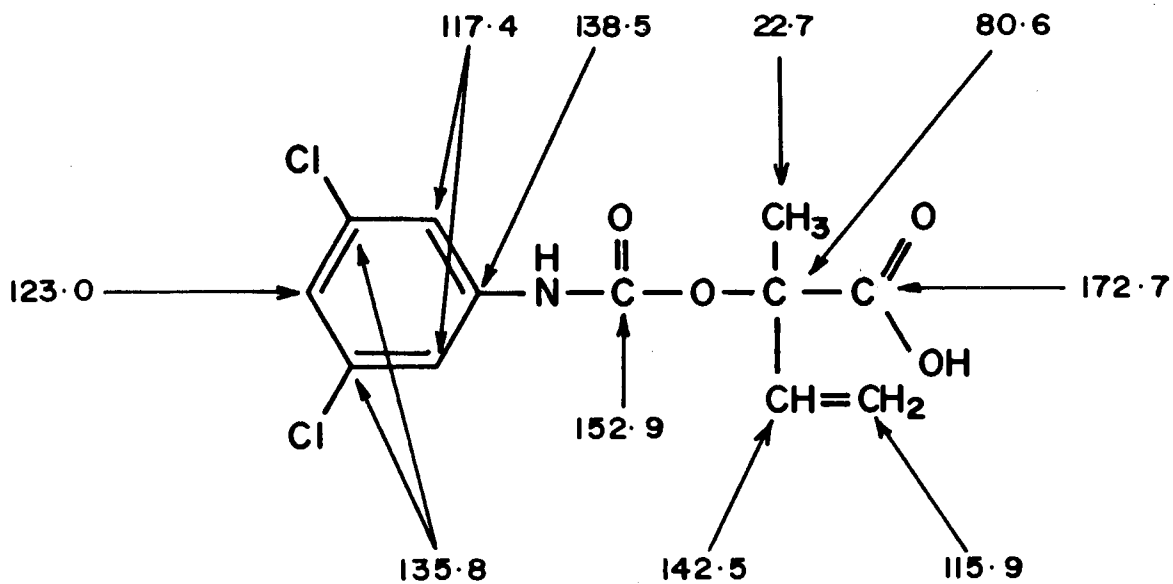

117·4
138·5
22·7
80·6
Cl
O
CH3
O
123·0
H
N
C
O
C
C
172·7
OH
Cl
152·9
CH=CH2
135·8
142·5
115·9

Figure 17. Assignments of ^{13}C NMR chemical shifts (in ppm) to vinclozolin.

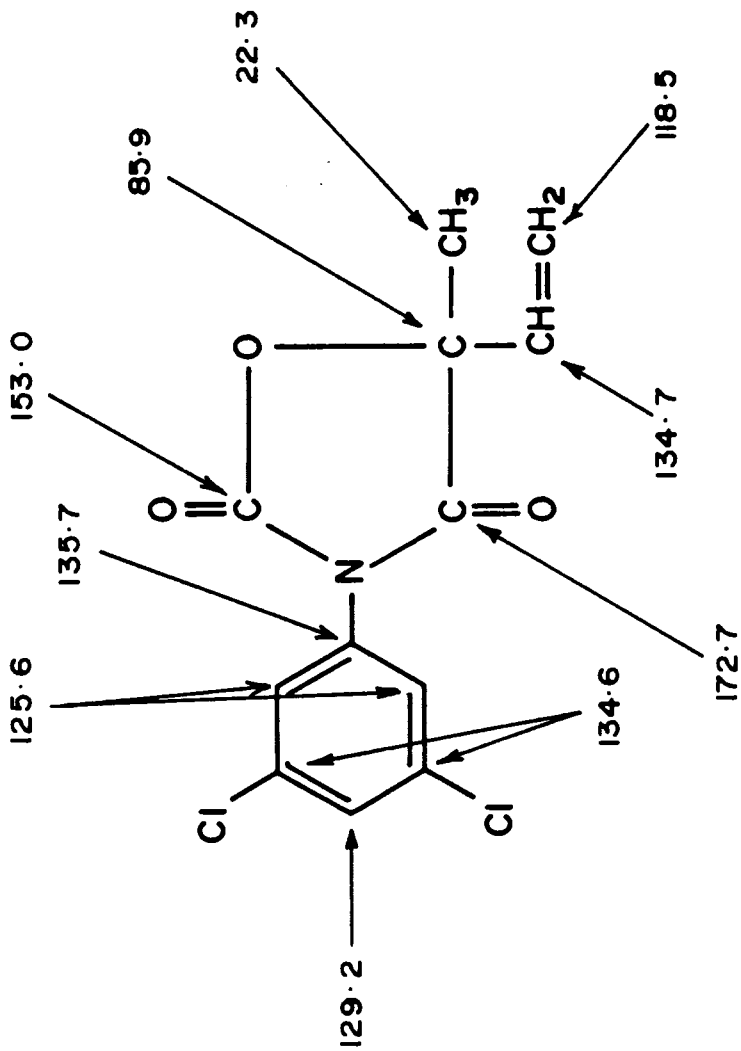
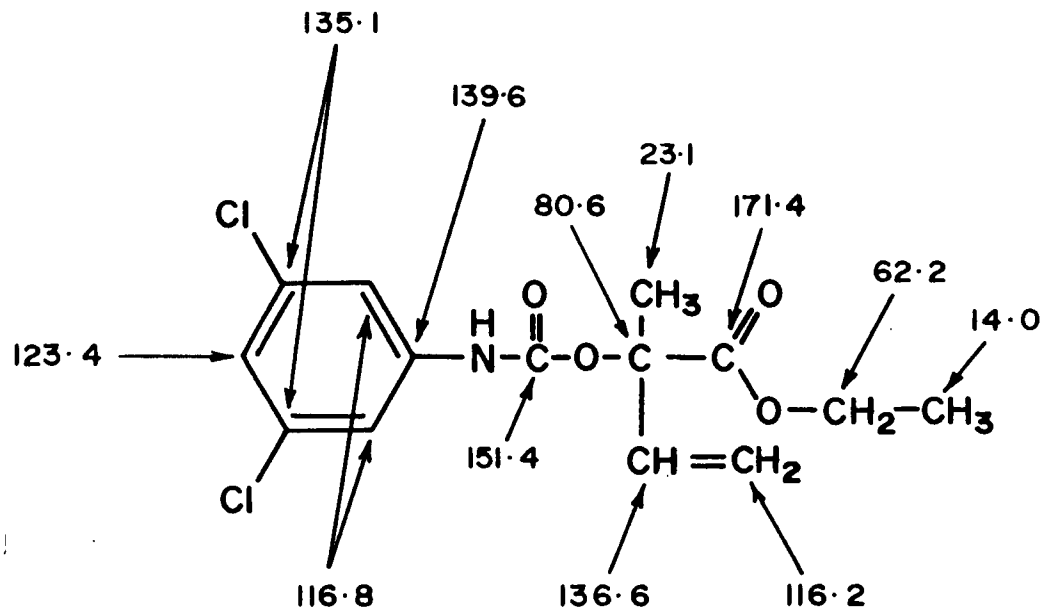

22·3
85·9
118·5
153·0
134·7
135·7
172·7
125·6
134·6
129·2
CH3
CH=CH2
O
C
N
Cl
Cl

Figure 18. Assignments of ^{13}C NMR chemical shifts (in ppm) to the ethyl ester of 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid.


135·1
139·6
23·1
80·6
171·4
62·2
14·0
123·4
Cl
Cl
H
N
O
C
O
C
CH3
C
O
O
CH2
CH3
151·4
CH
CH2
116·8
136·6
116·2

Clark (1983) studied the stability of vinclozolin in the presence of ethanol, methanol and water. He synthesized what he believed to be N-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (Fig. 7B) by modifying the method of Sumida et al. (1973a) for its 1-oxopropan-2-yl analogue. Vinclozolin (0.5 g) in a mixture of 12.5 ml of acetone: water (4:1) was reacted with NaOH (1 g) in an ice bath for 1.5 h. The solution was then acidified to pH 1 with conc. HCl and the precipitate was removed and recrystallized twice from chloroform. Based on the method of synthesis by Clark it is highly likely that the compound he synthesized is the same compound as M1 isolated from the hydrolysis of vinclozolin. Indeed, the mass spectrum and the proton NMR spectrum he presented are similar to those of M1. It is apparent that there are disagreements in structure elucidation. The proton NMR data are consistent with either the carbamic acid or the butenoic acid. The chemical shifts of the OH and NH proton may appear at varying positions due to intra- and intermolecular hydrogen bonding. However, the ^{13}C NMR and mass spectrometry data are consistent with the butenoic acid but inconsistent with the carbamic acid as discussed previously. For example, mass ion at 161 amu with 63% relative intensity was reported by Clark. This major ion is inconsistent with the carbamic acid structure he proposed because it cannot undergo rearrangement to yield the amine ion as previously discussed. Finally, X-ray crystallography data of ethylated M1 gave unambiguous evidence of the identity of M1 as 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid.

Bond lengths and bond angles with estimated standard deviations are given in Tables 2 and 3 respectively. The stereo view of the ethyl ester of 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid is given in Figure 19.

3.4. Kinetics of Hydrolysis of Vinclozolin

3.4.1. Disappearance of Vinclozolin

Kinetic studies on the hydrolysis of vinclozolin were conducted in aqueous buffered solutions from pH 4.5 to pH 8.3. Its disappearance at 35 C followed simple pseudo-first-order kinetics. The pseudo-first-order plots of ln Co/C vs. incubation time for the hydrolysis of vinclozolin are given in Figures 20-24, where Co = initial concentration and C = concentration at time t. Table 4 summarizes these data.

Buffer catalysis was not observed. At pH 4.5, 5.5, 6.5 and 7.0 the observed rates at each pH were similar between the two buffers (Figs. 20-23). The disappearance of vinclozolin at 35 C was very fast at basic pH but much slower at acidic pH. At pH 8.3 the half-life was 0.62 h whereas at pH 4.5 it was about 530 h. The log observed rate vs. pH for the hydrolysis of vinclozolin is plotted in Figure 25. A linear relationship was indicated between the logarithm of the observed rate and the pH from 4.5 to 8.3. Using a least-squares method a linear regression was calculated as follows:

log observed rate = 0.7450 pH - 3.1562 ($r = 0.9967^*$, $n = 12$)

It was evident that the rate of disappearance of vinclozolin was

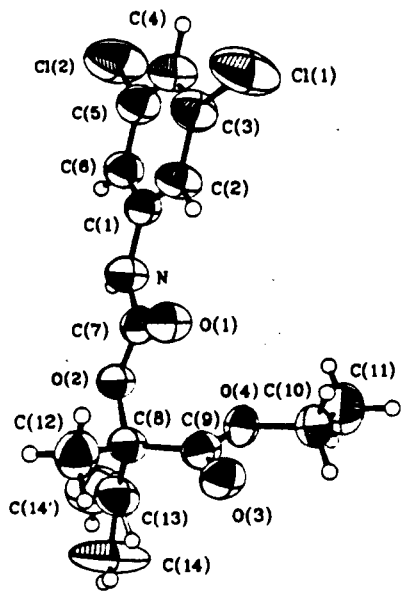
Table 2. Bond lengths (Å) of ethylated M1 with estimated standard deviations in parentheses.

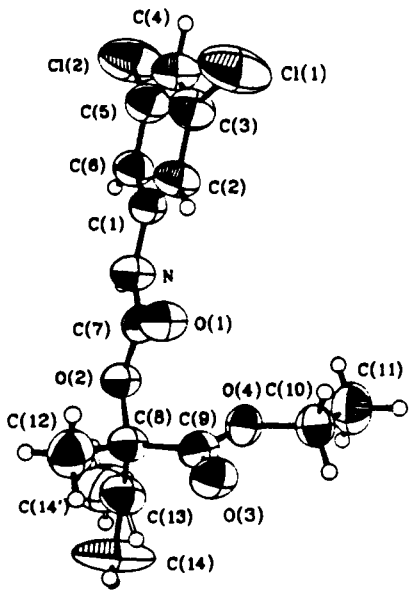
Bond	Length (Å)	Bond	Length (Å)
Cl(1)-C(3)	1.730(6)	C(1)-C(6)	1.375(7)
Cl(2)-C(5)	1.739(6)	C(2)-C(3)	1.403(7)
O(1)-C(7)	1.205(6)	C(3)-C(4)	1.360(8)
O(2)-C(7)	1.357(6)	C(4)-C(5)	1.367(8)
O(2)-C(8)	1.462(5)	C(5)-C(6)	1.387(7)
O(3)-C(9)	1.210(6)	C(8)-C(9)	1.505(7)
O(4)-C(9)	1.328(7)	C(8)-C(12)	1.509(8)
O(4)-C(10)	1.453(6)	C(8)-C(13)	1.509(8)
N -C(1)	1.407(6)	C(10)-C11)	1.452(8)
N -C(7)	1.352(6)	C(13)-C(14)	1.244(14)
C(1)-C(2)	1.374(6)	C(13)-C(14')	1.13(2)
N -H(N)	0.78(4)		

Table 3. Bond angles (deg) of ethylated M1 with estimated standard deviations in parentheses.

Bonds	Angle in deg	Bonds	Angle in deg
C(7)-O(2)-C(8)	115.5(4)	O(1)-C(7)-N	126.6(5)
C(9)-O(4)-C10)	117.6(5)	O(2)-C(7)-N	108.9(5)
C(1)-N -C(7)	127.4(5)	O(2)-C(8)-C(9)	110.1(4)
N -C(1)-C(2)	123.1(5)	O(2)-C(8)-C(12)	110.6(5)
N -C(1)-C(6)	115.4(5)	O(2)-C(8)-C(13)	104.6(4)
C(2)-C(1)-C(6)	121.5(5)	C(9)-C(8)-C(12)	112.9(5)
C(1)-C(2)-C(3)	117.3(5)	C(9)-C(8)-C(13)	107.0(5)
C(1)-C(3)-C(2)	117.8(5)	C(12)-C(8)-C(13)	111.3(5)
C(1)-C(3)-C(4)	119.2(5)	O(3)-C(9)-O(4)	123.7(6)
C(2)-C(3)-C(4)	123.0(5)	O(3)-C(9)-C(8)	123.5(6)
C(3)-C(4)-C(5)	117.4(5)	O(4)-C(9)-C(8)	112.6(5)
C1(2)-C(5)-C(4)	119.0(5)	O(4)-C(10)-C(11)	108.6(5)
C1(2)-C(5)-C(6)	118.5(5)	C(8)-C(13)-C(14)	127.5(10)
C(4)-C(5)-C(6)	122.5(6)	C(8)-C(13)-C(14')	129.0(12)
C(1)-C(6)-C(5)	118.3(5)	C(14)-C(13)-C(14')	99.5(14)
O(1)-C(7)-O(2)	124.5(5)		
C(1)-N -H(N)	118(3)	C(7)-N -H(N)	114(3)

Figure 19. Stereo view of the ethyl ester of 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid (ethylated M1).


C(4)
Cl(2)
Cl(1)
C(5)
C(3)
C(6)
C(2)
C(1)
N
C(7)
O(1)
O(2)
C(11)
O(4)
C(10)
C(12)
C(8)
C(9)
C(14')
C(13)
O(3)
C(14)


C(4)
Cl(2)
Cl(1)
C(5)
C(3)
C(6)
C(2)
C(1)
N
C(7)
O(1)
O(2)
C(11)
O(4)
C(10)
C(12)
C(8)
C(9)
C(14')
C(13)
O(3)
C(14)

dependent on hydroxide ion concentration. Melkebeke et al. (1986) studied the kinetics of the chemical hydrolysis of iprodione, vinclozolin and metalaxyl. They reported that the disappearance of vinclozolin at 60 C from pH 3.0 to pH 11.0 was pseudo-first-order and that vinclozolin was much more persistent in acidic than in alkaline pH, results which were in general agreement with those reported herein. However, the pseudo-first-order rate constants and half-lives reported by them were not in agreement. For example, they reported a pseudo-first-order rate of 1.22×10^{-4} S^{-1}, i.e., 51.1×10^{-3} h^{-1}, for the hydrolysis of vinclozolin at 25 C and pH 7.0, and the calculated half-life of 13.4 h, which differed from the rate of 35.1×10^{-3} h^{-1} and the half-life of 19.7 h for 26 C and pH 7.0 as determined in this study (Table 5). The discrepancy may be attributed to the fact that Melkebeke et al. actually determined the pseudo-first-order rates at 60 C for pH 3.0, 5.0, 7.0, 9.0 and 11.0 but their results are given at standard conditions of 25 C and 1 atm. They stated that their data had been converted to standard conditions but their methods were not described.

In order to study the influence of temperature on the rate constant of hydrolysis of vinclozolin and to establish the Arrhenius plot, pH 7.0 was selected for determination of rate constants at 13, 20, 26 and 35 C. The kinetic data including observed rate constants and calculated half-lives are summarized and given in Table 5 and Figure 26. The linear regression of the Arrhenius plot of log k vs. 1/T (Fig. 27) was as follows:

log rate = -5074 [1/T] + 15.54 ($p = 0.05$, $n = 4$, $r = -0.9698$*)

Based on the Arrhenius equation: $k = A \times e^{-E/RT}$ where E = energy

of activation, T = temperature in K, R = gas constant (8.3143 Joules mol^{-1} K^{-1}) and A = frequency factor (a constant); or ln k = ln A - E/RT, i.e., log k = log A - E/2.303R x 1/T. Therefore the slope of the Arrhenius plot = -E/2.303R. According to the linear regression of the Arrhenius plot, the slope of the plot was -5074 and the energy of activation E for the hydrolysis of vinclozolin at pH 7.0 is therefore 97.2 K Joules mol^{-1}. Since log A is equal to the intercept of the linear regression of the Arrhenius plot (15.54) the frequency factor A is therefore calculated to be 3.467×10^{15} h^{-1}. Using the Arrhenius equation and the data established for the energy of activation and the frequency factor of hydrolysis of vinclozolin at pH 7.0, the rate can be calculated for any temperature. For example, the rate of hydrolysis of vinclozolin at pH 7.0 at 28 C is calculated to be 4.82×10^{-2} h^{-1}.

By comparison with other protectant fungicides which have been used extensively for crop protection, vinclozolin appears to be more resistant to hydrolysis. According to the study of Wolfe et al. (1976), the pseudo-first-order rate constants of hydrolysis at 28 C and about pH 7.0 are: 0.234 h^{-1} for captan (pH 7.07); 0.277 h^{-1} for captafol (pH 7.17); and 0.504 h^{-1} for folpet (pH 7.14). By comparison, the value for vinclozolin at 28 C and pH 7.0 is 0.048 h^{-1} which is almost one order of magnitude lower than those of captan, captafol and folpet. The effects of pH on the hydrolysis of vinclozolin are different from those on the hydrolysis of captan. The reaction rate constant was pH dependent from pH 4.5 to pH 8.3 for vinclozolin and a linear relationship existed between log rate constant and pH (Figure 25). By comparison the reaction rate constant for the hydrolysis of captan is independent of pH

over the range of 2-6 and the average pseudo-first-order rate constant at 28 C is 6.48×10^{-2} h^{-1}. Above pH 7 the reaction rate constant is pH dependent and the mean and standard deviation of second-order rate constants for alkaline hydrolysis of captan at 28 C in the pH range 7.07-8.25 is 2.05×10^{6} M^{-1} h^{-1} ± 0.14×10^{6} M^{-1} h^{-1} (Wolfe et al. 1976). By comparison the reaction rate constant for the hydrolysis of vinclozolin was pH dependent over the pH range 4.5-8.3 (Figure 25). Using the equation "second-order rate constant (KOH^-) = pseudo-first-order rate constant/$[OH^-]$" the second-order rate constants were calculated and they are given in Table 4. The means and standard deviations of second-order rate constants for the hydrolysis of vinclozolin at 35 C are 8.12×10^{5} M^{-1} h^{-1} ± 2.42×10^{5} M^{-1} h^{-1} in the pH range 7.0-8.3 and 1.68×10^{6} M^{-1} h^{-1} ± 1.45×10^{6} M^{-1} h^{-1} over the pH range 4.5-8.3.

In the pH range of 5.5-8.0 which is typical of natural water, vinclozolin underwent hydrolysis readily. At 35 C the half-lives were about 56 h at pH 5.5 and 1.16 h at pH 8.0 (Table 4). At room temperature (20 C) and neutral pH the half-life was about 26 h (Table 5). These findings suggest that Ronilan® 50 WP, a formulation of vinclozolin, may be unstable in water especially in slightly basic water. Indeed, Ronilan® 50 WP had been shown to be unstable in water (Clark 1983). When 150 mg of Ronilan® 50 WP were stirred in 100 ml of tap water (pH 8) at room temperature 98% of vinclozolin was recovered from the suspension after 1 h but only 7% after 23 days. To ensure the efficacy of Ronilan® 50 WP it is important to prepare the spray mix freshly using water which is neutral or slightly acidic. Using water that is slightly basic to prepare the spray mix and aging of the spray mix would result in loss of efficacy from hydrolysis of the vinclozolin.

Figure 20. Pseudo-first-order plots for the hydrolysis of vinclozolin at 35 C in pH 4.5 phosphate and acetate buffers.

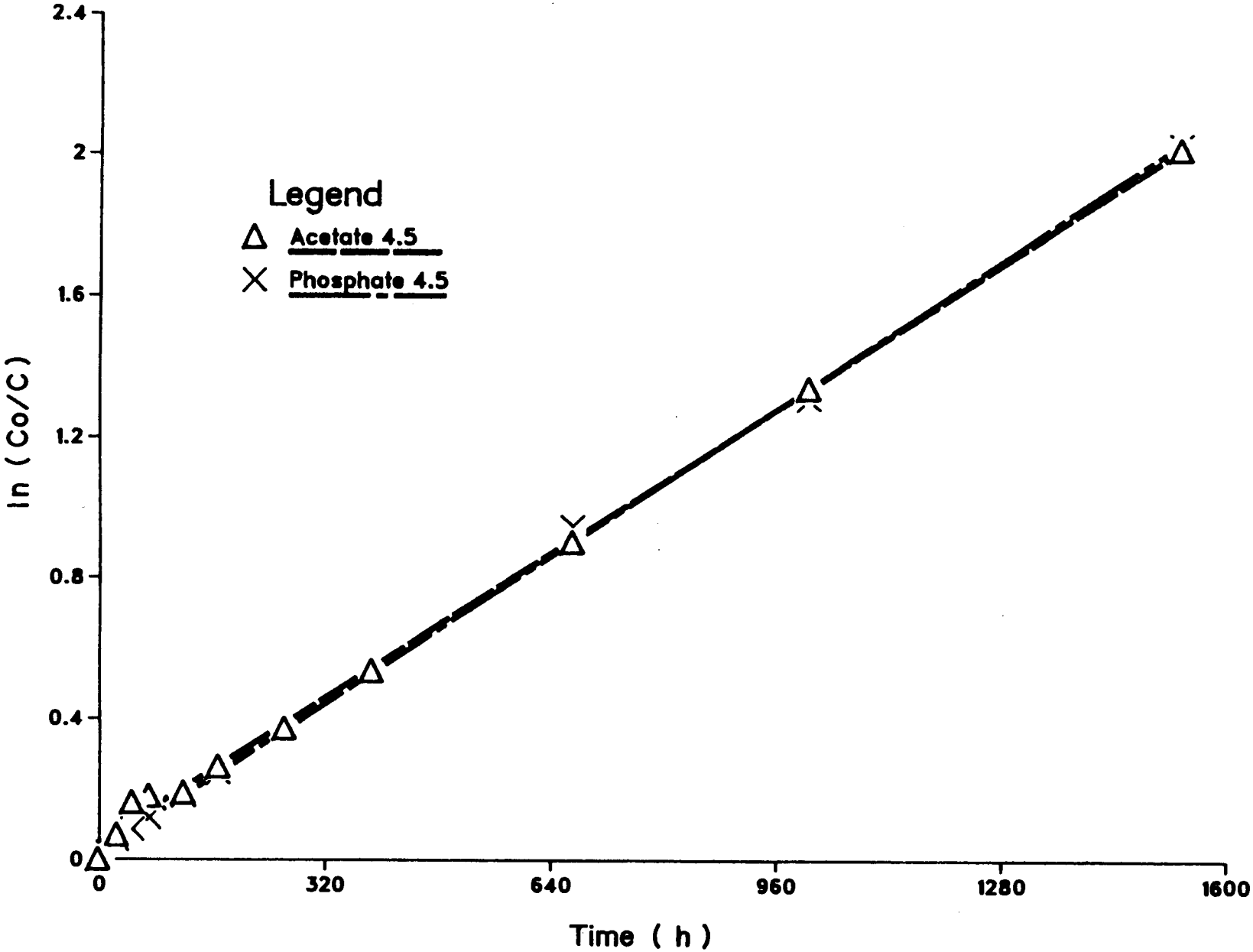

Legend
Acetate 4.5
Phosphate 4.5
ln (Co/C)
Time (h)
2.4
2
1.6
1.2
0.8
0.4
0
0
320
640
960
1280
1600

Figure 21. Pseudo-first-order plots for the hydrolysis of vinclozolin at 35 C in pH 5.5 phosphate and acetate buffers.

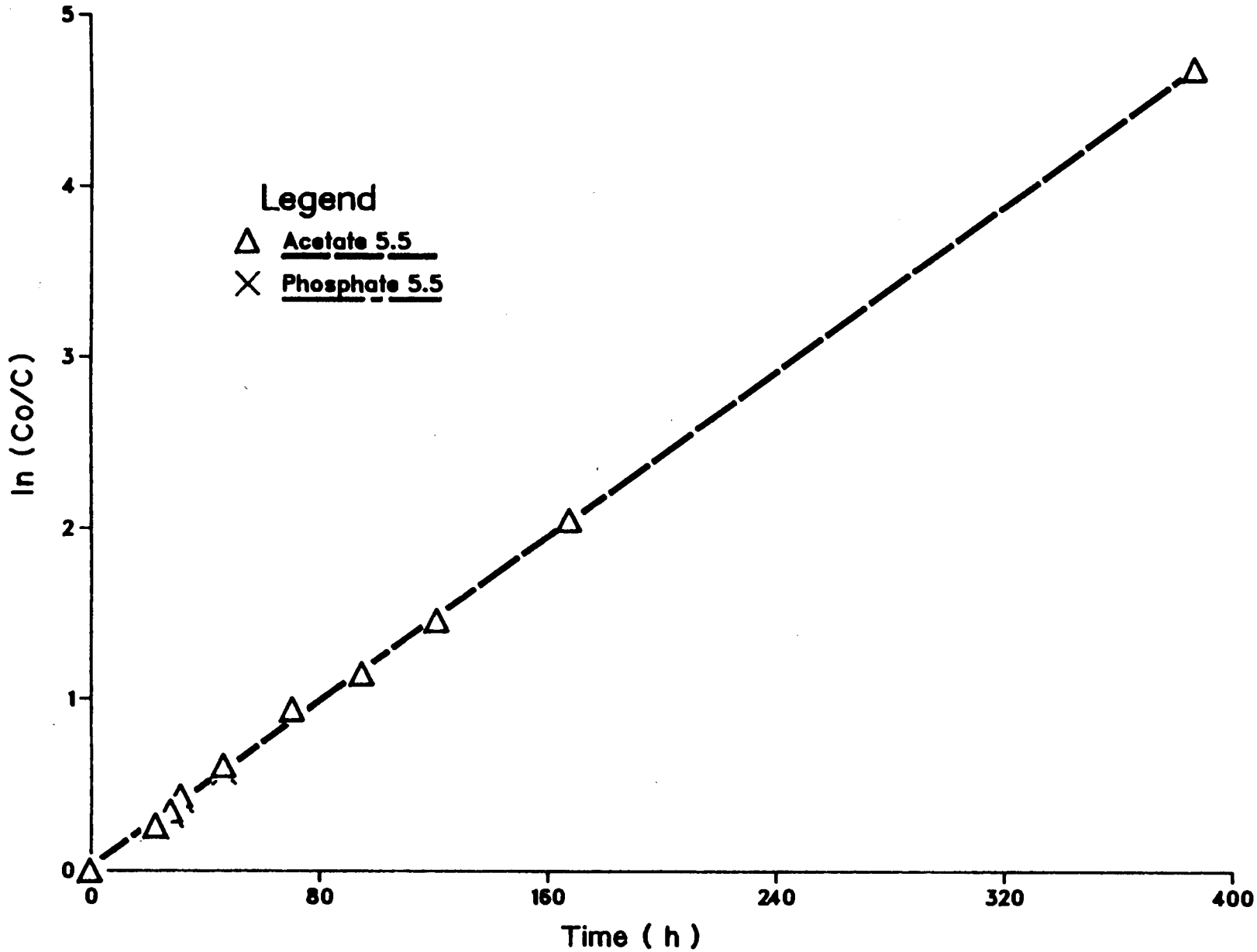

Legend
Acetate 5.5
Phosphate 5.5
ln (Co/C)
0
1
2
3
4
5
0
80
160
240
320
400
Time (h)

Figure 22. Pseudo-first-order plots for the hydrolysis of vinclozolin at 35 C in phosphate buffers of pH 6.5, 7.0 and 7.5.

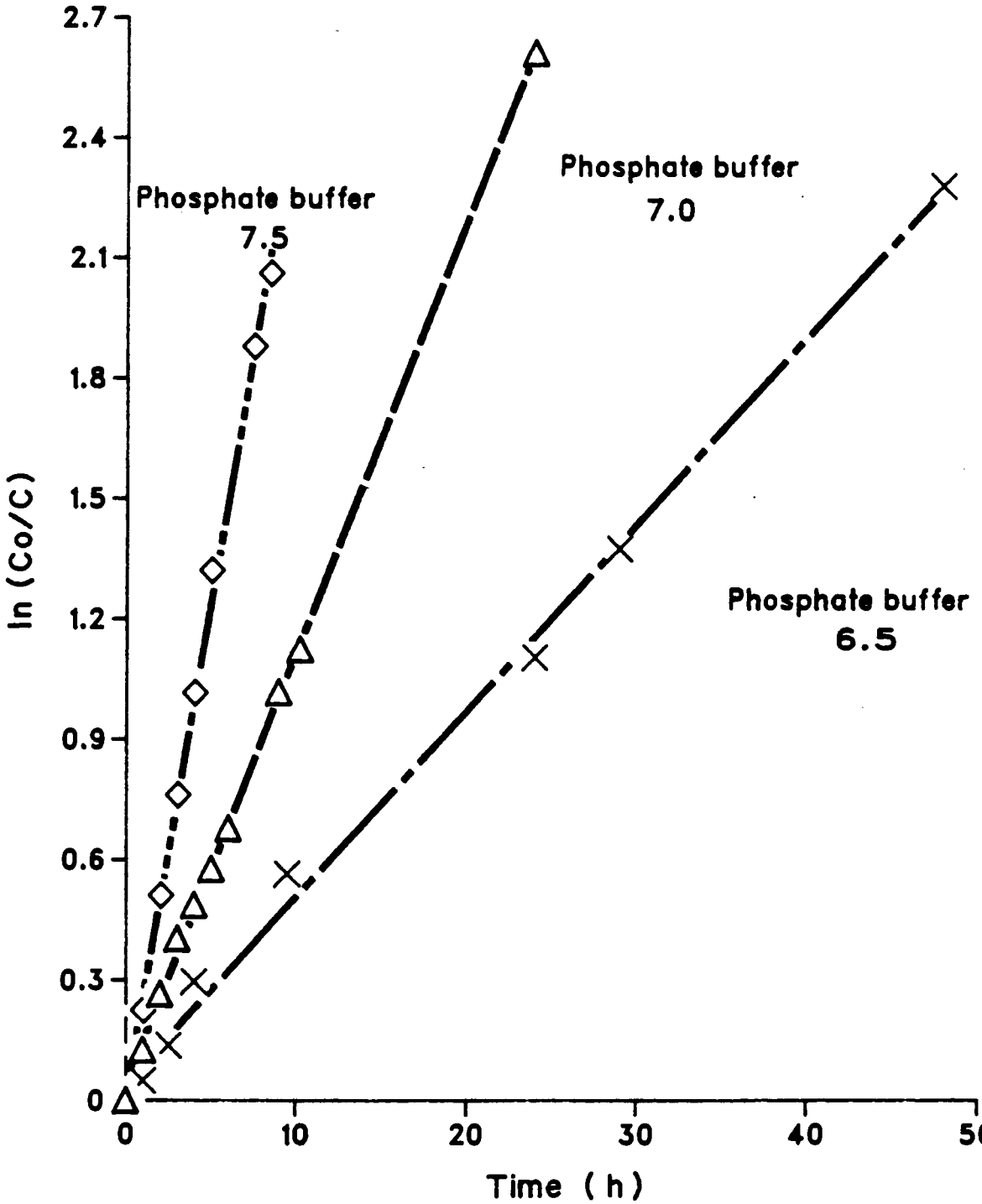

2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
ln (Co/C)
Phosphate buffer
7.5
Phosphate buffer
7.0
Phosphate buffer
6.5
0
10
20
30
40
50
Time (h)

Figure 23. Pseudo-first-order plots for the hydrolysis of vinclozolin at 35 C in pH 6.5 acetate buffer, pH 7.0 and pH 7.3 borate buffers.

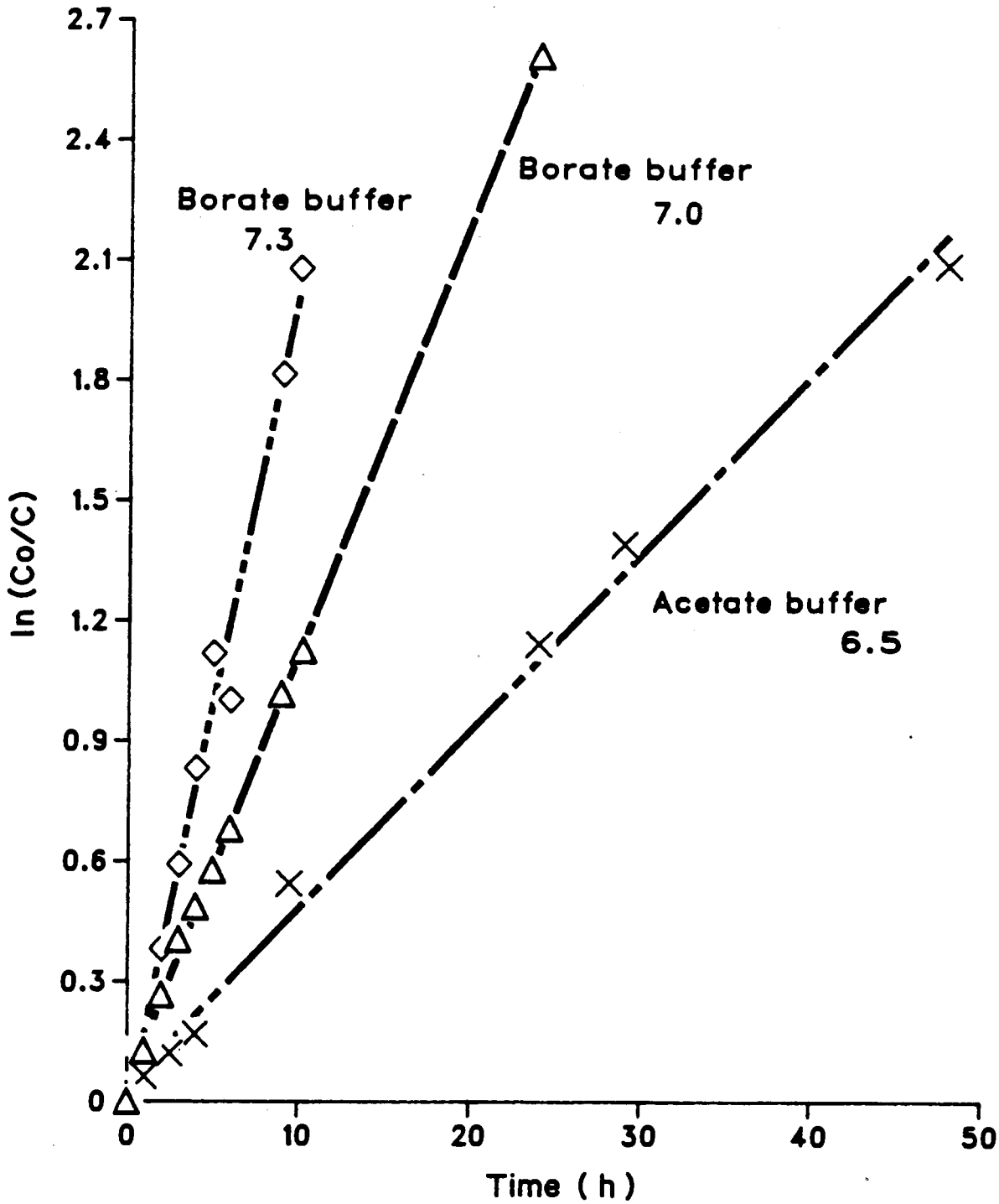

2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
ln (Co/C)
Borate buffer
7.3
Borate buffer
7.0
Acetate buffer
6.5
0
10
20
30
40
50
Time (h)

Figure 24. Pseudo-first-order plots for the hydrolysis of vinclozolin at 35 C in pH 8.0 phosphate buffer and pH 8.3 borate buffer.

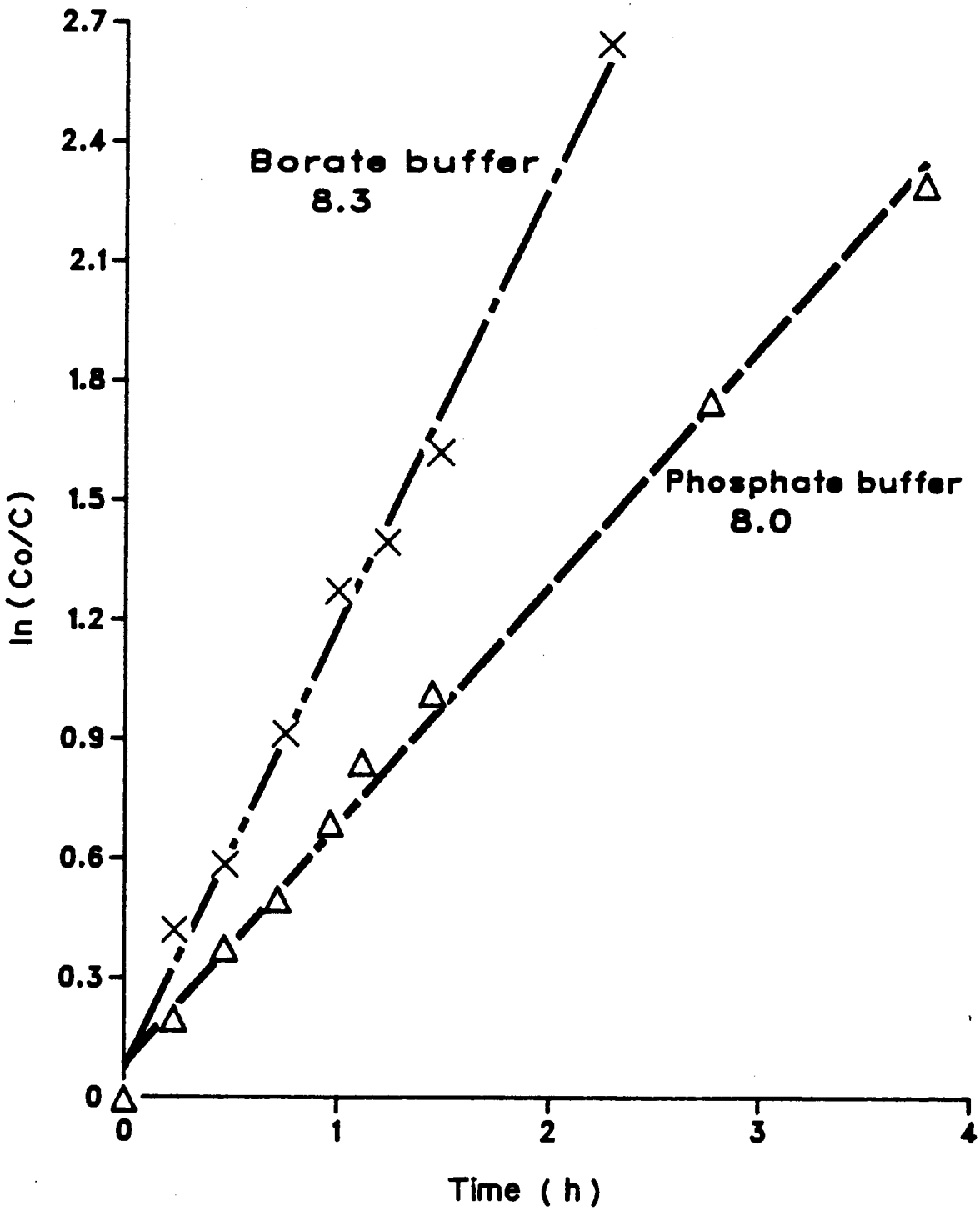

2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
ln (Co/C)
Borate buffer
8.3
Phosphate buffer
8.0
0
1
2
3
4
Time (h)

Figure 25. Profile of log rate constant vs. pH for the hydrolysis of vinclozolin at 35 C.

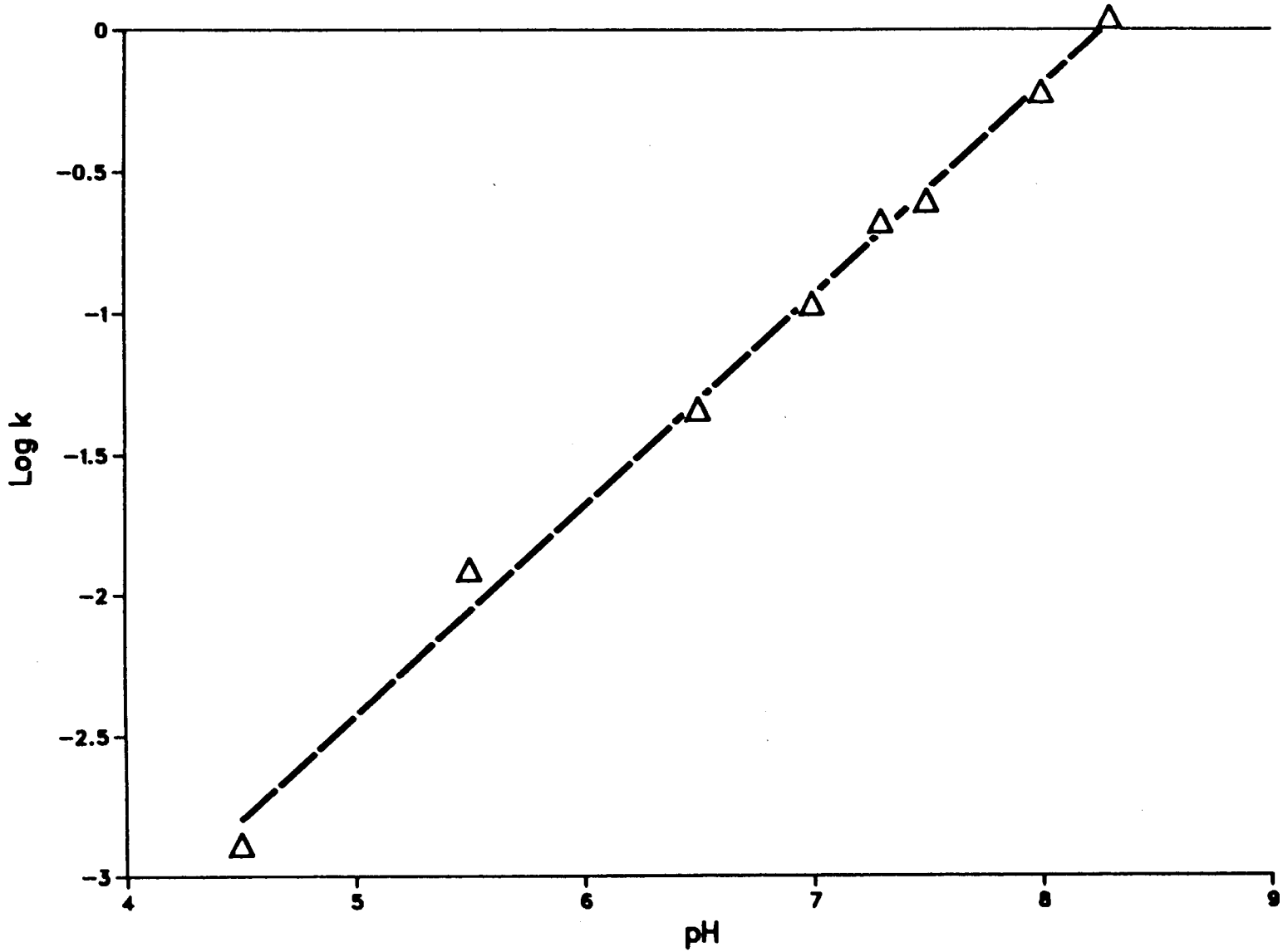

0
-0.5
-1
-1.5
-2
-2.5
-3
Log k
4
5
6
7
8
9
pH

Figure 26. Pseudo-first-order plots for the hydrolysis of vinclozolin in pH 7.0 phosphate buffer at 13, 20, 26 and 35 C.

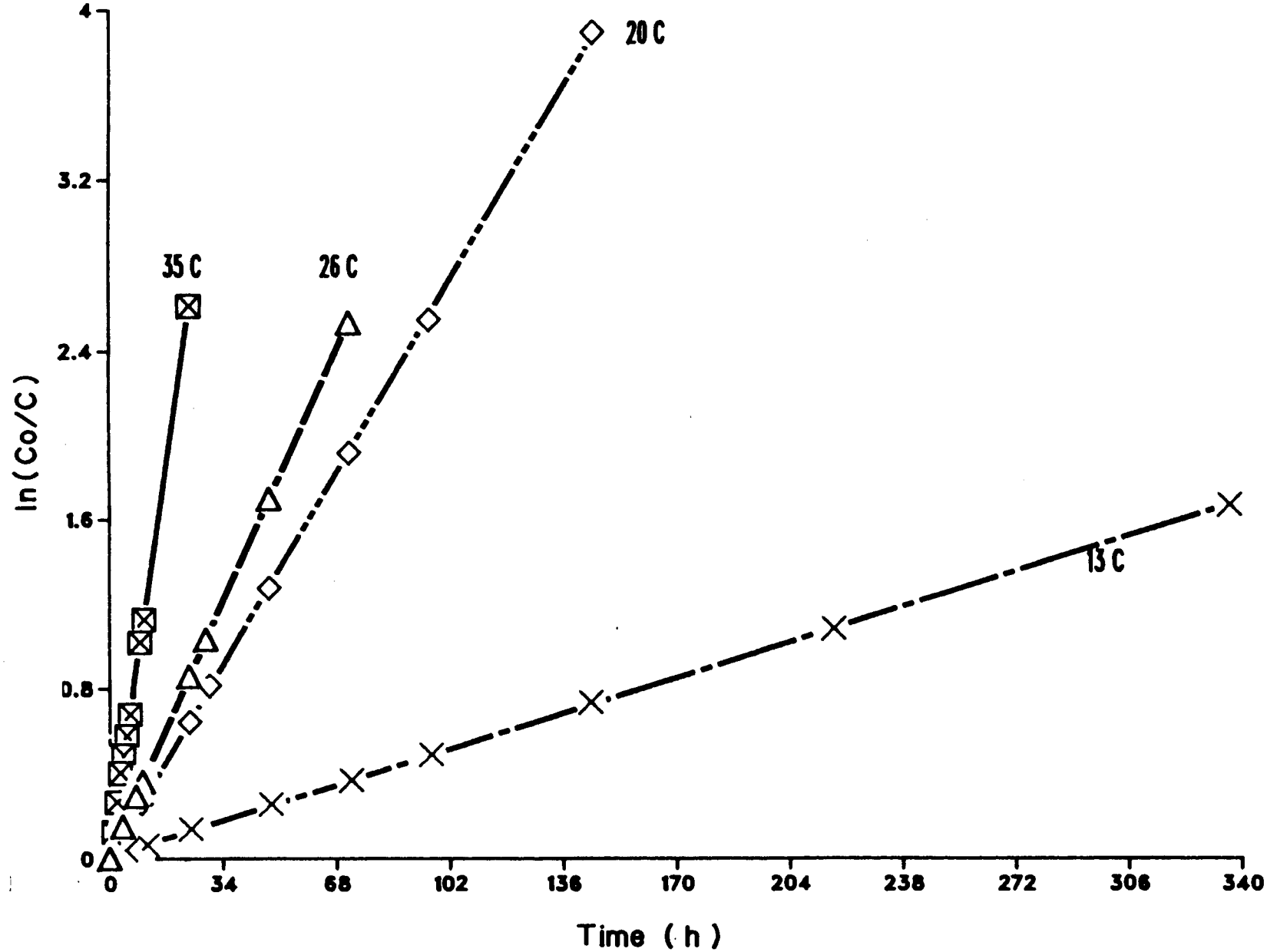

4
3.2
2.4
1.6
0.8
0
ln(Co/C)
35 C
26 C
20 C
13 C
0
34
68
102
136
170
204
238
272
306
340
Time (h)

Figure 27. Arrhenius plot of log k vs. 1/T x 1000 for the hydrolysis of vinclozolin at pH 7.0.

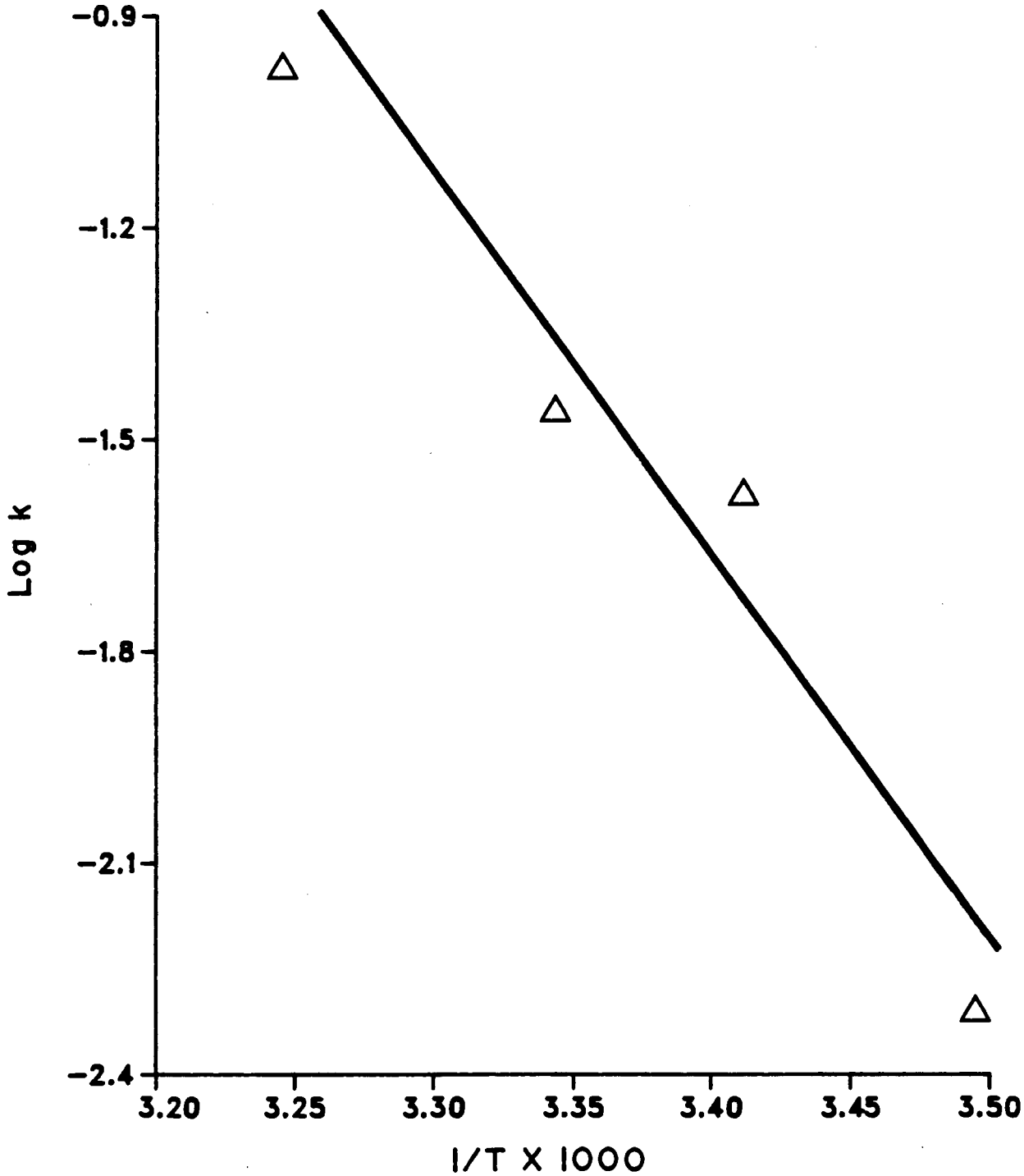

-0.9
-1.2
-1.5
-1.8
-2.1
-2.4
Log k
3.20
3.25
3.30
3.35
3.40
3.45
3.50
1/T X 1000

Table 4. Kinetic data of hydrolysis of vinclozolin at 35 C in buffered solutions.

pH	Buffer	No. of readings	Corr. coeff.	k observed[a] x $10^{-3}h^{-1}$	k_{OH^-}[b] x $10^5 M^{-1}h^{-1}$	Half-life in h
4.5	phosphate	11	0.9996	1.31	41.4	529
4.5	acetate	11	0.9991	1.28	40.5	541
5.5	phosphate	10	0.9999	12.4	39.2	56.0
5.5	acetate	10	0.9993	12.1	38.3	57.5
6.5	phospate	8	0.9980	46.4	14.6	15.0
6.5	acetate	8	0.9971	44.3	14.0	15.6
7.0	phosphate	10	0.9995	107	10.7	6.45
7.0	borate	10	0.9995	107	10.7	6.45
7.3	borate	9	0.9981	210	10.5	3.31
7.5	phosphate	8	0.9987	248	7.84	2.80
8.0	phosphate	9	0.9973	599	5.99	1.16
8.3	borate	8	0.9963	1109	5.56	0.62

[a] k observed = pseudo-first-order rate constant.

[b] k_{OH^-} = second-order rate constant.

Table 5. Kinetic data of hydrolysis of vinclozolin in 0.01 M phosphate buffer of pH 7.0 at 13, 20, 26 and 35 C.

Temperature in C	k observed $x10^{-3}h^{-1}$	Half-life in h	No. of readings	Corr. coeff.
13	4.94	140	10	0.9999
20	26.8	25.9	9	0.9999
26	35.1	19.7	8	0.9999
35	107	6.45	10	0.9995

3.4.2. Conversion Products of Vinclozolin

When vinclozolin was incubated in 0.01 M buffers of pH 4.5, 5.5, 6.5, 7.0, 7.3, 7.5, 8.0 and 8.3 at 35 C, the parent compound was gradually converted to the butenoic acid (M1), the enanilide (M2) and 3,5-dichloroaniline (M3). The concentrations of each compound at various intervals after incubation are given in Tables 6-17. Similar conversions were observed at pH 4.5, 5.5, 6.5 and 7.0 regardless of the type of buffer used for incubation, indicating that buffer catalysis did not occur in the hydrolysis of vinclozolin.

At all pHs tested a major conversion product was M1, of which the concentrations increased steadily with time. The highest concentrations of M1 in percent of the total (vinclozolin + M1 + M2 + M3) reached 70%-85% with the exception of pH 4.5 (Tables 8-17). At pH 4.5 the highest concentration of M1 reached approximately 18% of the total (Tables 6 and 7). After reaching the highest levels the concentrations of M1 decreased gradually with time. At pH 8.0 and 8.3 the incubation studies were terminated after about 4 h when the concentrations of M1 were at their highest. It was therefore not possible to conclude if the concentration of M1 would decrease gradually after reaching its highest level as observed in other pHs tested. Another major conversion product was M2. The concentrations of M2 increased steadily with time and were at their highest for all the pHs tested when the incubation studies were ended. Conversion product M3 was detected in the incubation mixtures after at least 21 days (504 h). M3 was not detected in the mixtures incubated at pH 8.0 and 8.3 because these two studies were terminated

after 3 to 4 hours. However, M3 was detected in subsequent incubation studies at pH 8.0 and 35 C with M1 as the starting material (Tables 22 and 23).

At pH 7.0 the effect of temperature on the conversion of vinclozolin was investigated (Tables 12 and 13, and 18-20). Conversion to M2 was favored by higher incubation temperature. At 13 C M2 was first detected when the concentration of vinclozolin decreased from the initial of 35.8 μM to 17.2 μM, i.e., by about 52% (Table 18). By comparison, M2 was first detected at 35 C when the concentration of vinclozolin decreased from the initial of 38.2 μM to 33.7 μM, i.e., by about 12% (Table 12). At 20 C and 26 C M2 was first detected when the concentration of vinclozolin decreased by about 47% (36.5 μM to 19.2 μM) and 26% (37.2 μM to 27.7 μM) respectively (Tables 19 and 20). The fact that conversion of vinclozolin to M2 was favored by higher temperature supports the hypothesis that M2 (Fig. 7E) was formed via the intermediate of the carbamic acid (Fig. 7B) and the hydrogen carbonate (Fig. 7C) by decarboxylation, which was promoted by higher temperature.

Vinclozolin was converted to M1, M2 and M3 on hydrolysis. The concentrations of the parent compound and the conversion products varied depending on pH, temperature and incubation time. However, the total concentrations (vinclozolin + M1 + M2 + M3) remained relatively unchanged for the duration of the incubation studies (Tables 6-17). The incubation studies at pH 4.5, 5.5, 6.5, 7.0, 7.3 and 7.5 showed that the total concentrations remained relatively unchanged after incubation for 50 days (1203 h) at pH 6.5, and for 113 days (2717 h) at pH 7.5. At pH 8.0 and 8.3 the incubation studies were terminated after about 4 h. It

was uncertain if the total concentrations remained relatively unchanged at these pHs. However, subsequent incubation studies at pH 8.0 and 35 C with M1 showed that the total concentration remained relatively unchanged after 70 days (1680 h) (Table 22). At pH 7.0 and 20 C the total concentration was approximately 94% of the initial after incubation for 494 days (Table 19); and the incubation mixture consisted of approximately 62% M1, 19% M2 and 19% M3.

The fact that the conversion products derived from the hydrolysis of vinclozolin were relatively stable in aqueous media suggests that residues may be present in wine produced from vinclozolin-treated grapes. The possibility of 3,5-dichloroaniline being produced has importance for public health workers because 3,5-dichloroaniline is a chlorinated aromatic amine which could be a potential carcinogen. Cabras et al. (1984) studied the degradation of vinclozolin in wine at pH 3.0 and 4.0. They reported that the disappearance of vinclozolin was pseudo-first-order; the rate constants at 30 C were 0.17×10^{6} S^{-1} at pH 3.0 and 0.22×10^{6} S^{-1} at pH 4.0; the half-lives were 48.5 days at pH 3.0 and 36.7 days at pH 4.0. Comparing their reported rate constants and half-lives it is evident that the reported rate constants are several orders of magnititude too high for the reported half-lives. It appears highly likely that there was a typographical error in the reported rate constants. According to their reported half-lives the rate constants should be 0.17×10^{-6} S^{-1} at pH 3.0 and 0.22×10^{-6} S^{-1} at pH 4.0. These findings were in general agreement with the kinetic data of hydrolysis of vinclozolin in buffered solutions (Table 4), indicating that the degradation of vinclozolin in wine was mainly due to

hydrolysis. At 35 C the rate constant for the hydrolysis of vinclozolin was 1.30×10^{-3} h^{-1}, i.e., 3.61×10^{-7} S^{-1}, at pH 4.5; and the half-life was about 22 days (535 h). Comparing with the corresponding data obtained from the degradation of vinclozolin in wine at 30 C and pH 4.0, the rate constant was faster and the half-life was shorter, which would be expected at the higher pH and incubation temperature.

In the study of Cabras et al. (1984), M3 was not detected as a degradation product of vinclozolin in wine. According to the results of hydrolysis of vinclozolin at pH 4.5 and 35 C, M3 was detected after incubation for 28 days. At the end of incubation, i.e., after 64 days, the concentration of M3 increased to about 2.4 μM (0.38 μg/ml) which accounted for approximately 6% of the total concentration (Tables 6 and 7). In their study the starting concentration of vinclozolin was about 10 μM which was approximately 30% of the concentration (32.4-33.8 μM) used in the hydrolysis studies (Tables 6 and 7). Taking into consideration that M3 accounted for only about 6% of the total concentration and the pH and incubation temperature were lower than those used in the hydrolysis studies, the concentration of M3 that may be present in the wine would probably be below the limit of detection of the analytical method.

M2 was the only degradation product of vinclozolin detected by Cabras et al. (1984) in their study of wine. The identification of M2 by them was based on retention time of HPLC analysis only. They speculated that M2 was derived from vinclozolin by hydrolytic opening of the ring. In a subsequent study by the same group (Pirisi et al. 1986), the identity of M2 was further confirmed by elemental analysis and proton

NMR. M2 was stable for 150 days in a medium containing by volume, distilled water:ethanol, 9:1 at pH 4.0 and 30 C. Pirisi *et al.* (1986) hypothesized that M2 was derived from the intermediate, *N*-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (Fig. 7B). Using the reference standard of the carbamic acid donated by Professor A. Del Re, Pirisi *et al.* did not detect any carbamic acid in the reaction mixture during the course of their study. They suggested that the carbamic acid was not detected probably because of the interference encountered in the HPLC analysis. A careful analysis of their HPLC method showed that the mobile solvent system consisted of 55% of acetonitrile and 45% of water with a flow rate of 1 ml/min. Using their mobile solvent system for reversed-phase HPLC the carbamic acid would be co-eluted with the solvent front as discussed previously in 3.2. It was highly likely that the carbamic acid would be isolated if acidic buffer was used in the mobile solvent system instead of water.

The rapid conversion of vinclozolin in aqueous media to M1 and M2 and the stability of these intermediate products have significant influence on the efficacy of vinclozolin against pathogenic fungi. According to Clark (1983) both M1 which he identified as the carbamic acid (see 3.3.2.2.), and M2 gave no inhibition zone in the *in vitro* test against *B. cinerea* indicating that in practice the efficacy of vinclozolin may be reduced significantly because of hydrolytic degradation in wet deposits on plants or in aged spray suspensions. Therefore it is important to emphasize again that in order to ensure efficacy, spray mixtures of vinclozolin should be prepared freshly with either neutral or slightly acidic water.

Table 6. Hydrolysis of vinclozolin in 0.01 M phosphate buffer of pH 4.5 at 35 C.

Time in hours	Concentration[a] in µM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	32.4(100)	N.D.	N.D.	N.D.	32.4(100)
26.8	30.6(85.2)	4.79(13.3)	0.46(1.5)	N.D.	35.9(110)
48.5	29.8(82.3)	5.18(14.3)	1.20(3.4)	N.D.	36.2(112)
72.6	28.8(79.8)	5.41(15.0)	1.85(5.2)	N.D.	36.1(112)
121.5	27.0(72.4)	7.06(18.9)	3.32(8.7)	N.D.	37.3(115)
170.3	25.3(68.8)	7.43(20.2)	4.02(11.0)	N.D.	36.8(114)
264.0	22.4(60.7)	6.73(18.3)	7.76(21.0)	N.D.	36.9(114)
387.3	19.0(51.8)	5.15(14.1)	12.5(34.1)	N.D.	36.7(113)
672.3	12.6(34.4)	4.88(13.3)	17.6(48.1)	1.49(4.2)	36.6(113)
1008	8.73(23.3)	3.04(8.1)	24.2(64.7)	1.49(3.9)	37.4(115)
1536	4.25(11.2)	1.25(3.3)	30.3(79.5)	2.36(6.0)	38.1(117)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 7. Hydrolysis of vinclozolin in 0.01 M acetate buffer of pH 4.5 at 35 C.

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	33.8(100)	N.D.	N.D.	N.D.	38.8(100)
26.5	31.5(87.0)	4.22(11.7)	0.52(1.3)	N.D.	36.2(107)
48.3	28.7(82.2)	5.02(14.4)	1.16(3.4)	N.D.	34.9(103)
72.3	28.2(80.5)	5.31(15.0)	1.85(4.5)	N.D.	35.4(105)
121.0	28.0(76.9)	5.68(15.6)	2.70(7.5)	N.D.	36.4(108)
170.0	26.0(71.8)	6.67(18.4)	3.55(9.8)	N.D.	36.2(107)
263.8	23.3(65.1)	5.02(14.0)	7.49(20.9)	N.D.	35.8(106)
387.0	19.8(53.5)	4.82(13.0)	12.4(33.5)	N.D.	37.0(109)
672.0	13.7(37.0)	4.62(12.5)	18.1(48.9)	0.6(1.6)	37.0(109)
1008	8.84(23.1)	3.17(8.3)	24.4(63.9)	1.86(4.7)	38.2(113)
1537	4.49(11.5)	1.06(2.7)	30.9(79.4)	2.48(6.4)	38.9(115)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 8. Hydrolysis of vinclozolin in 0.01 M phosphate buffer of pH 5.5 at 35 C.

Time in hours	Concentration[a] in µM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	33.4(100)	N.D.	N.D.	N.D.	33.4(100)
23.3	25.9(71.3)	9.11(25.1)	1.31(3.6)	N.D.	36.3(109)
28.4	24.4(67.6)	10.4(28.8)	1.31(3.6)	N.D.	36.1(108)
32.0	22.3(63.5)	11.4(32.5)	1.39(4.0)	N.D.	35.1(105)
47.0	18.9(52.8)	15.0(41.9)	1.93(5.3)	N.D.	35.8(107)
71.1	14.3(41.4)	17.6(51.0)	2.55(7.6)	N.D.	34.5(103)
96.3	10.2(29.2)	21.2(60.7)	3.47(10.1)	N.D.	34.9(104)
119.7	7.65(21.2)	24.2(67.0)	4.29(11.8)	N.D.	36.1(108)
167.3	4.28(11.8)	24.9(68.6)	7.10(19.6)	N.D.	36.3(109)
387.3	0.28(0.8)	24.8(70.0)	10.3(29.2)	N.D.	35.4(106)
1537	N.D.	13.6(36.4)	21.7(58.0)	2.11(5.6)	37.4(112)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 9. Hydrolysis of vinclozolin in 0.01 M acetate buffer of pH 5.5 at 35 C.

Time in hours	Concentration[a] in µM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	34.1(100)	N.D.	N.D.	N.D.	34.1(100)
23.0	26.3(70.9)	9.70(26.1)	1.12(3.0)	N.D.	37.1(109)
28.5	24.2(66.7)	10.8(29.8)	1.31(3.5)	N.D.	36.3(106)
31.8	22.1(63.0)	11.6(33.0)	1.39(4.0)	N.D.	35.1(103)
46.8	18.5(53.3)	14.3(41.2)	1.93(5.5)	N.D.	34.7(102)
70.8	13.3(38.3)	18.8(54.2)	2.55(7.5)	N.D.	34.7(102)
95.1	10.8(31.7)	20.2(59.2)	3.09(9.1)	N.D.	34.1(100)
121.4	7.93(22.0)	24.5(67.9)	3.71(10.1)	N.D.	36.1(106)
167.8	4.39(12.8)	24.7(71.8)	5.29(15.4)	N.D.	34.4(101)
387.0	0.32(9.2)	25.0(72.0)	9.34(18.8)	N.D.	34.7(102)
1537	N.D.	13.7(36.7)	21.5(57.6)	2.11(5.7)	37.3(109)

a M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

b % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 10. Hydrolysis of vinclozolin in 0.01 M phosphate buffer of pH 6.5 at 35 C.

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	39.6(100)	N.D.	N.D.	N.D.	39.6(100)
1.0	37.9(96.2)	1.45(3.8)	N.D.	N.D.	39.4(99.5)
2.5	34.7(91.1)	3.40(8.9)	N.D.	N.D.	38.1(96.2)
4.0	29.6(80.2)	7.29(18.8)	N.D.	N.D.	36.9(93.2)
9.5	22.6(61.1)	11.8(31.9)	2.63(7.0)	N.D.	37.0(93.4)
24.0	13.2(33.7)	22.8(58.2)	3.24(8.1)	N.D.	39.2(99.0)
29.0	10.1(25.8)	24.4(62.4)	4.59(11.8)	N.D.	39.1(98.7)
48.0	4.07(10.9)	28.1(75.3)	5.10(13.8)	N.D.	37.3(94.2)
192.7	N.D.	29.7(79.6)	7.64(20.4)	N.D.	37.3(94.2)
338.7	N.D.	27.2(76.0)	8.61(24.0)	N.D.	35.8(90.4)
505.8	N.D.	25.9(68.9)	10.3(27.4)	1.43(3.7)	37.6(94.9)
1203	N.D.	21.2(55.2)	14.3(37.2)	2.86(7.6)	38.4(97.0)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 11. Hydrolysis of vinclozolin in 0.01 M acetate buffer of pH 6.5 at 35 C.

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	37.2(100)	N.D.	N.D.	N.D.	37.2(100)
1.0	35.1(94.9)	1.85(5.1)	N.D.	N.D.	37.0(99.5)
2.5	33.1(91.4)	3.14(8.6)	N.D.	N.D.	36.2(97.3)
4.0	31.6(86.6)	4.85(13.4)	N.D.	N.D.	36.5(98.1)
9.5	21.7(58.6)	12.8(34.6)	2.47(6.8)	N.D.	37.0(99.5)
24.0	11.9(32.2)	21.5(58.3)	3.47(9.5)	N.D.	36.9(99.2)
29.0	9.30(25.1)	23.6(63.6)	4.21(11.3)	N.D.	37.1(99.7)
48.0	4.63(12.6)	27.0(73.4)	5.17(14.0)	N.D.	36.8(98.9)
193.5	N.D.	29.2(79.3)	7.64(20.7)	N.D.	36.8(98.9)
338.7	N.D.	27.4(76.3)	8.49(23.7)	N.D.	35.9(96.5)
505.8	N.D.	25.9(69.3)	10.2(27.3)	1.30(3.4)	37.4(101)
1203	N.D.	19.7(53.8)	14.4(39.3)	2.48(6.9)	36.6(98.4)

a M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

b % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 12. Hydrolysis of vinclozolin in 0.01 M phosphate buffer of pH 7.0 at 35 C.

Time in hours	Concentration[a] in µM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	38.2(100)	N.D.	N.D.	N.D.	38.2(100)
1.0	33.7(89.6)	3.30(8.8)	0.62(1.6)	N.D.	37.6(98.4)
2.0	29.3(79.6)	6.01(16.3)	1.47(4.1)	N.D.	36.8(96.3)
3.0	25.5(69.1)	9.31(25.2)	2.05(5.7)	N.D.	36.9(96.6)
4.0	23.5(62.3)	11.5(30.5)	2.66(7.2)	N.D.	37.7(98.7)
5.0	21.5(57.6)	12.8(34.3)	2.97(8.1)	N.D.	37.3(97.6)
6.0	19.4(51.2)	14.9(39.3)	3.55(9.5)	N.D.	37.9(99.2)
9.0	13.8(36.3)	19.8(52.1)	4.40(11.6)	N.D.	38.0(99.5)
10.3	12.4(32.6)	20.7(54.5)	4.94(12.9)	N.D.	38.0(99.5)
24.0	2.81(7.4)	28.5(75.0)	6.68(17.6)	N.D.	38.0(99.5)
48.0	N.D.	29.8(79.0)	7.92(21.0)	N.D.	37.7(98.7)
72.0	N.D.	30.0(78.5)	8.19(21.5)	N.D.	38.2(100)
96.0	N.D.	30.0(78.5)	8.19(21.5)	N.D.	38.2(100)
168.0	N.D.	29.5(77.4)	8.57(22.6)	N.D.	38.1(99.7)
240.0	N.D.	29.2(75.5)	9.46(24.5)	N.D.	38.7(101)
360.0	N.D.	28.1(73.9)	9.88(26.1)	N.D.	38.0(99.5)

Table 12. (Continued)

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
504.0	N.D.	26.6(71.9)	10.4(28.1)	N.D.	37.0(96.9)
696.0	N.D.	25.2(67.4)	12.2(32.6)	N.D.	37.4(97.9)
1108	N.D.	23.8(62.3)	14.4(37.7)	N.D.	38.2(100)
1370	N.D.	20.4(51.4)	16.6(41.8)	2.67(6.8)	39.7(104)
1683	N.D.	18.6(47.4)	17.6(44.9)	2.98(7.7)	39.2(103)
2381	N.D.	15.1(38.0)	21.0(52.9)	3.60(9.1)	39.7(104)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 13. Hydrolysis of vinclozolin in 0.01 M borate buffer of pH 7.0 at 35 C.

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	35.8(100)	N.D.	N.D.	N.D.	35.8(100)
1.0	31.5(89.7)	3.07(8.7)	0.54(1.6)	N.D.	35.1(98.1)
2.0	27.4(78.7)	5.97(17.2)	1.39(4.1)	N.D.	34.8(97.1)
3.0	23.9(68.5)	9.04(25.9)	1.93(5.6)	N.D.	34.9(97.4)
4.0	22.0(62.5)	10.7(30.4)	2.47(7.1)	N.D.	35.2(98.2)
5.0	20.1(57.6)	12.0(34.4)	2.78(8.0)	N.D.	34.9(97.4)
6.0	18.1(51.0)	14.0(39.4)	3.36(9.6)	N.D.	35.5(99.1)
9.0	12.9(36.6)	18.2(51.7)	4.13(11.7)	N.D.	35.2(98.4)
10.3	11.6(32.1)	19.9(55.1)	4.59(12.8)	N.D.	36.1(101)
24.0	2.63(7.3)	27.0(75.4)	6.21(17.3)	N.D.	35.8(100)
48.0	N.D.	28.8(80.4)	7.03(19.6)	N.D.	35.8(100)
72.0	N.D.	29.1(79.9)	7.34(20.1)	N.D.	36.4(102)
96.0	N.D.	29.0(79.0)	7.72(21.0)	N.D.	36.7(103)
168.0	N.D.	28.4(77.6)	8.18(22.4)	N.D.	36.6(103)
240.0	N.D.	27.6(75.6)	8.88(24.4)	N.D.	36.5(102)

Table 13. (Continued)

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
360.0	N.D.	26.9(74.3)	9.27(25.7)	N.D.	36.2(101)
504.0	N.D.	26.2(73.0)	9.73(27.0)	N.D.	35.9(100)
696.0	N.D.	24.6(68.3)	11.4(31.7)	N.D.	36.0(101)
1107	N.D.	22.9(61.7)	14.2(38.3)	N.D.	37.1(104)
1370	N.D.	20.3(52.9)	15.6(40.6)	2.48(6.5)	38.4(107)
1683	N.D.	18.2(48.0)	16.9(44.6)	2.80(7.4)	37.9(106)
2381	N.D.	15.1(39.1)	20.3(52.6)	3.22(8.3)	38.6(108)

a M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

b % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 14. Hydrolysis of vinclozolin in 0.01 M borate buffer of pH 7.3 at 35 C.

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	36.1(100)	N.D.	N.D.	N.D.	36.1(100)
1.0	31.2(82.8)	5.12(13.6)	1.39(3.6)	N.D.	37.7(104)
2.0	24.7(66.6)	10.0(27.0)	2.43(6.4)	N.D.	37.1(103)
3.0	20.0(55.1)	13.8(38.0)	3.47(6.9)	N.D.	36.3(100)
4.0	15.8(42.9)	17.0(46.2)	4.02(10.9)	N.D.	36.8(102)
5.0	12.5(34.7)	18.9(52.5)	4.56(12.7)	N.D.	36.0(100)
6.0	10.0(27.6)	21.1(58.3)	5.10(14.1)	N.D.	36.2(100)
9.0	5.89(16.2)	23.7(65.3)	6.72(18.5)	N.D.	36.3(101)
10.0	4.53(12.3)	25.1(68.4)	7.07(19.3)	N.D.	36.7(102)
24.0	N.D.	28.0(78.0)	7.92(22.0)	N.D.	35.9(99.4)
51.8	N.D.	28.2(77.0)	8.42(23.0)	N.D.	36.6(101)
72.3	N.D.	28.4(77.2)	8.42(22.8)	N.D.	36.8(102)
96.0	N.D.	28.0(76.1)	8.84(23.9)	N.D.	36.8(102)
168.5	N.D.	27.3(75.2)	9.03(24.8)	N.D.	36.3(101)
192.5	N.D.	27.0(74.4)	9.31(25.6)	N.D.	36.3(101)

Table 14. (Continued)

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
264.5	N.D.	26.3(73.9)	9.34(26.1)	N.D.	35.6(98.7)
336.5	N.D.	26.1(72.1)	10.1(27.9)	N.D.	36.2(100)
408.5	N.D.	25.4(69.4)	11.2(30.6)	N.D.	36.6(101)
528.5	N.D.	24.8(68.3)	11.5(31.7)	N.D.	36.3(100)
672.5	N.D.	24.3(64.8)	13.2(35.2)	N.D.	37.5(104)
864.5	N.D.	24.0(63.8)	13.6(36.2)	N.D.	37.6(104)
1036	N.D.	22.8(58.6)	14.2(36.5)	1.93(4.9)	38.9(108)
1537	N.D.	20.0(50.3)	16.8(42.2)	2.98(7.5)	39.8(110)
2017	N.D.	17.6(44.9)	18.4(46.9)	3.17(8.2)	39.2(109)
2549	N.D.	16.5(40.7)	20.3(50.1)	3.73(9.2)	40.5(112)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 15. Hydrolysis of vinclozolin in 0.01 M phosphate buffer of pH 7.5 at 35 C.

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	37.2(100)	N.D.	N.D.	N.D.	37.2(100)
1.0	29.8(78.8)	6.83(18.1)	1.16(3.1)	N.D.	37.8(102)
2.0	22.3(61.1)	11.5(31.5)	2.74(7.4)	N.D.	36.5(98.2)
3.0	17.4(47.4)	15.3(41.7)	4.02(10.9)	N.D.	36.7(98.7)
4.0	13.5(36.8)	18.9(51.5)	4.32(11.7)	N.D.	36.7(98.7)
5.0	9.93(26.9)	21.4(58.0)	5.56(15.1)	N.D.	36.9(99.2)
7.5	5.68(15.2)	25.2(67.6)	6.45(17.2)	N.D.	37.3(100)
8.5	4.74(12.7)	25.6(68.6)	6.95(18.7)	N.D.	37.3(100)
24.0	N.D.	29.3(79.8)	7.37(20.2)	N.D.	36.7(98.6)
72.0	N.D.	29.3(78.1)	8.19(21.9)	N.D.	37.5(101)
96.0	N.D.	28.1(76.2)	8.84(23.8)	N.D.	36.9(99.3)
120.0	N.D.	27.9(74.6)	9.46(25.4)	N.D.	37.4(100)
168.0	N.D.	27.7(73.5)	10.0(26.5)	N.D.	37.7(101)
242.8	N.D.	27.1(73.0)	10.0(27.0)	N.D.	37.1(100)
336.3	N.D.	27.2(72.0)	10.6(28.0)	N.D.	37.8(102)

Table 15. (Continued)

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
432.8	N.D.	26.9(71.5)	10.7(28.5)	N.D.	37.6(101)
504.8	N.D.	27.0(71.2)	10.9(28.8)	N.D.	37.9(102)
578.5	N.D.	26.7(69.9)	11.5(30.1)	N.D.	38.2(103)
696.8	N.D.	25.6(68.1)	12.0(31.9)	N.D.	37.6(101)
840.8	N.D.	24.8(66.7)	12.4(33.3)	N.D.	37.2(100)
1035	N.D.	23.5(62.8)	13.9(37.2)	N.D.	37.4(101)
1563	N.D.	20.7(53.0)	15.6(40.0)	2.67(7.0)	39.0(105)
2019	N.D.	17.8(45.6)	17.8(45.6)	3.42(8.8)	39.0(105)
2717	N.D.	16.2(41.2)	19.2(48.9)	3.85(9.9)	39.3(106)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 16. Hydrolysis of vinclozolin in 0.01 M phosphate buffer of pH 8.0 at 35 C.

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	41.1(100)	N.D.	N.D.	N.D.	41.1(100)
0.233	33.6(87.5)	4.75(12.5)	N.D.	N.D.	38.4(93.3)
0.467	28.1(74.1)	9.80(25.9)	N.D.	N.D.	37.9(92.2)
0.717	24.9(63.5)	11.6(29.6)	2.66(6.9)	N.D.	39.2(95.3)
0.967	20.6(53.4)	14.9(38.6)	3.05(8.0)	N.D.	38.6(93.8)
1.117	17.6(45.4)	17.7(45.6)	3.51(9.0)	N.D.	38.8(94.4)
1.450	14.8(37.8)	20.0(51.0)	4.44(11.2)	N.D.	39.2(95.5)
2.767	7.12(18.1)	27.0(68.5)	5.25(13.4)	N.D.	39.4(95.9)
3.783	4.14(10.3)	29.5(73.6)	6.45(16.1)	N.D.	40.1(97.5)
4.300	N.D.	31.5 (80.8)	7.53(19.2)	N.D.	39.0(95.0)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 17. Hydrolysis of vinclozolin in 0.01 M borate buffer of pH 8.3 at 35 C.

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	47.4(100)	N.D.	N.D.	N.D.	47.4(100)
0.233	31.2(71.2)	12.6(28.8)	N.D.	N.D.	43.8(92.4)
0.467	26.4(57.1)	16.2(35.1)	3.59(7.8)	N.D.	46.2(97.4)
0.750	19.0(42.3)	21.9(48.8)	4.02(8.9)	N.D.	44.9(94.8)
1.000	13.3(30.8)	25.5(59.0)	4.36(10.2)	N.D.	43.2(90.5)
1.233	11.8(26.0)	28.3(62.3)	5.25(11.7)	N.D.	45.4(95.1)
1.483	9.40(21.3)	29.3(66.4)	5.44(12.3)	N.D.	44.1(93.1)
2.283	3.37(7.6)	35.0(79.0)	5.91(13.4)	N.D.	44.3(93.4)
3.650	N.D.	37.0(85.1)	6.49(14.9)	N.D.	43.5(91.8)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 18. Hydrolysis of vinclozolin in 0.01 M phosphate buffer of pH 7.0 at 13 C.

Time in hours	Concentration[a] in µM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	35.8(100)	N.D.	N.D.	N.D.	35.8(100)
4.5	34.4(96.1)	1.35(3.9)	N.D.	N.D.	35.8(100)
10.8	33.5(93.8)	2.24(6.2)	N.D.	N.D.	35.7(99.8)
24.0	31.1(87.6)	4.42(12.4)	N.D.	N.D.	35.5(99.2)
48.0	27.6(78.2)	7.66(21.8)	N.D.	N.D.	35.3(98.5)
72.0	24.7(69.8)	10.7(30.2)	N.D.	N.D.	35.4(98.9)
96.0	21.9(62.4)	13.2(37.6)	N.D.	N.D.	35.1(98.0)
144.0	17.2(49.7)	15.8(45.7)	1.62(4.6)	N.D.	34.6(96.7)
217.0	12.1(34.6)	20.5(58.6)	2.39(6.8)	N.D.	35.0(97.7)
336.0	6.74(19.5)	24.5(70.8)	3.32(9.7)	N.D.	34.6(96.7)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 19. Hydrolysis of vinclozolin in 0.01 M phosphate buffer of pH 7.0 at 20 C.

Time	Concentration[a] in µM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	36.5(100)	N.D.	N.D.	N.D.	36.5(100)
4.0 h	32.5(89.0)	4.00(11.0)	N.D.	N.D.	36.5(100)
9.5 h	28.1(77.8)	7.99(22.2)	N.D.	N.D.	36.1(98.9)
24.0 h	19.2(53.5)	14.2(39.6)	2.51(6.9)	N.D.	35.9(98.4)
30.0 h	16.1(45.2)	16.9(47.5)	2.63(7.3)	N.D.	35.6(97.6)
48.0 h	10.2(28.6)	21.7(60.8)	3.78(10.6)	N.D.	35.7(97.7)
72.0 h	5.33(15.1)	25.2(71.6)	4.63(13.3)	N.D.	35.2(96.3)
96.0 h	2.84(8.0)	27.2(77.1)	5.21(14.9)	N.D.	35.3(96.6)
145.0 h	0.74(2.1)	29.3(82.5)	5.48(15.4)	N.D.	35.5(97.3)
494 d	N.D.	21.3(62.3)	6.53(19.1)	6.34(18.6)	34.2(93.7)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 20. Hydrolysis of vinclozolin in 0.01 M phosphate buffer of pH 7.0 at 26 C.

Time in hours	Concentration[a] in μM (%)[b]				
	Vinclozolin	M1	M2	M3	Total
0	37.2(100)	N.D.	N.D.	N.D.	37.2(100)
4	32.0(86.5)	5.02(13.5)	N.D.	N.D.	37.0(99.5)
8	27.7(73.1)	8.98(23.7)	1.24(3.2)	N.D.	37.9(102)
10	25.9(67.4)	11.0(28.6)	1.48(4.0)	N.D.	38.4(103)
24	15.8(43.9)	18.2(50.6)	2.01(5.5)	N.D.	36.0(96.8)
29	13.3(36.5)	20.8(57.1)	2.32(6.4)	N.D.	36.4(97.9)
48	6.81(18.8)	24.8(68.5)	4.63(12.7)	N.D.	36.2(97.4)
72	2.95(8.1)	27.2(74.9)	6.18(17.0)	N.D.	36.3(97.7)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

3.4.3. Reaction of M1 in Buffered Solutions

Conversion of vinclozolin to the butenoic acid (M1) and the enanilide (M2) appeared to occur simultaneously at 35 C in most pHs tested (Tables 6-17). Except at pH 6.5, 8.0 and 8.3 there was a time lapse between the occurrence of M1 and M2. M1 appeared sooner than M2. During the incubation of vinclozolin in buffered solutions at pH 4.5-7.3 the concentrations of M1 increased steadily to a maximum and decreased steadily thereafter whereas the concentrations of M2 continued to increase steadily. At pH 8.0 and 8.3 both the concentrations of M1 and M2 increased steadily during the incubation study (Tables 16 and 17). These results suggest that on hydrolysis vinclozolin was first converted to M1 by opening the 2,4-oxazolidinedione ring, followed by the formation of M2. Clark (1983) identified the two degradation products of vinclozolin from water suspension to be N-(2-hydroxy-2-methyl-1-oxo-buten-3-yl)-3,5-dichlorophenyl-1-carbamic acid (Fig. 7B) and its decarboxylation product, the enanilide (M2) (Fig. 7E). However, the correct structure of the acid was 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid (Fig. 7D) as shown under 3.3.2.2. by MS, Proton and ^{13}C NMR and X-ray crystallography structural evidence. Considering the structures of the butenoic acid and the enanilide it is highly unlikely that the formation of the enanilide was via the butenoic acid as the intermediate because conversion from the butenoic acid to the enanilide would require complicated rearrangement of the molecule. Therefore it was hypothesized that vinclozolin was converted independently to both the butenoic acid and the enanilide by opening the

2,4-oxazolidinedione ring at different positions (Fig. 7). The formation of the enanilide was probably via the transient intermediates of either the carbamic acid or the hydrogen carbonate by decarboxylation. These transient intermediates were highly unstable and could not be isolated from the aqueous medium. It was hypothesized also that the position at which the ring opened would be pH dependent and the conversion from vinclozolin to the butenoic acid was reversible. There was some evidence to support this hypothesis. When vinclozolin was incubated at 35 C and pH 4.5 (Tables 6 and 7) both the butenoic acid (M1) and the enanilide (M2) were formed in about 26 h. The concentrations of M1 increased steadily to a peak after 170 h and decreased steadily thereafter whereas the concentrations of the enanilide increased steadily throughout the incubation study. Meanwhile, the concentrations of vinclozolin decreased steadily. At the end of the incubation study, i.e., after 1536 h (64 days), the concentration of vinclozolin was higher than that of the butenoic acid. However, in other pHs tested, vinclozolin was no longer detected in the buffered solution while the concentrations of the butenoic acid were at or near the peak (Tables 8-17).

To prove the above hypothesis the butenoic acid (M1) was incubated at 35 C in phosphate buffers of pH 4.5 and 8.0, and in phosphate buffer of pH 8.0 which was adjusted to 4.5 after 24 h pre-incubation. The results are given in Tables 21-23. At pH 4.5 M1 was rapidly converted to vinclozolin and the concentration of vinclozolin accounted for approximately 10% of the total (vinclozolin + M1 + M2 + M3) after 2 h. The concentrations of vinclozolin increased steadily to a maximum after

71 h and decreased gradually thereafter. The enanilide (M2) was not detected until almost 2 days after the appearance of vinclozolin and its concentration increased steadily. At the termination of the incubation study, i.e., after 1680 h (70 days), the concentration of M2 accounted for approximately 80% of the total. M3 was detected after 840 h (40 days) and its concentration increased gradually. At the end of the incubation study the concentration of M3 accounted for less than 10% of the total. During the course of the incubation study the total concentration remained unchanged. The conversion of M1 at 35 C and pH 4.5 appeared to be the reversal of the hydrolysis of vinclozolin under identical conditions (Tables 6, 7 and 21).

The disappearance of M1 in 0.01 M phosphate buffer of pH 4.5 at 35 C was pseudo-first-order (Fig. 28). The linear regression between ln Co/C and incubation time for the incubation period of 46 h showed a correlation coefficient of 0.9906 (n=5) which was significant at 95% probability. The calculated pseudo-first-order rate constant was 2.69×10^{-2} h^{-1} and the second-order rate constant was 8.51×10^{7} M^{-1} h^{-1}. During this incubation period M1 was mainly converted to vinclozolin and the rate constant was about 20 x the rate constant for the hydrolysis of vinclozolin under identical temperature and pH (1.30×10^{-3} h^{-1}). The conversion of M1 to vinclozolin reached a peak in 71 h and declined thereafter while the conversion to the enanilide (M2) increased steadily throughout the incubation study. During the incubation period from 46 h to 1680 h the disappearance of M1 appeared to deviate from pseudo-first-order as indicated by the correlation coefficient which became insignificant at 95% probability. As more than 60% of the M1 was converted to

vinclozolin after 46 h incubation the conversion from vinclozolin to M1 would then be accelerated. The time lapse of more than 22 h between the appearance of vinclozolin and M2 clearly demonstrated that M2 was not converted from M1 but from vinclozolin.

At pH 8.0, M1 was very stable in the buffered solution and the conversion product, M2 was first detected after incubation for 480 h (Table 22). The other conversion product, M3 (3,5-dichloroaniline), first appeared in the buffered solution after incubation for 840 h (40 days). The concentrations of both M2 and M3 increased steadily throughout the incubation study. Vinclozolin was never detected in the buffered solution. At the end of the 70 days (1680 h) incubation the concentrations of M1, M2 and M3 accounted for approximately 68%, 24% and 8% of the total respectively. These results clearly showed that M1 was stablized by the basic medium and that what little vinclozolin may have formed would be converted back to M1 because of the high rate constant for the hydrolysis of vinclozolin at 35 C and pH 8.0 (1.109 h^{-1}) (Table 4). Although vinclozolin was not detected it was conceivable that very minute amounts were produced and then partly converted to M2. The concentration of M2 increased slowly to the limit of detection by the HPLC analysis, so that it was first detected in the buffered solution after incubation for 480 h (20 days).

The effect of pH on the conversion of M1 to vinclozolin by recyclization was further demonstrated in a separate incubation experiment at 35 C and pH 8.0 (Table 23). After incubation for 24 h, one half of the samples were acidified to pH 4.5. Conversion of M1 to vinclozolin occurred immediately after the acidification and the

concentrations of M1, vinclozolin, M2 and M3 (Table 23) became similar to those detected in the earlier incubation studies at the pHs 4.5 and 8.0 (Tables 21 and 22).

Based on the results of the incubation studies with both vinclozolin and M1, a degradation pathway was proposed for the hydrolysis of vinclozolin leading to the formation of M1 and M2 (Fig. 29). On hydrolysis the 2,4-oxazolidinedione ring opens to yield both the butenoic acid and the enanilide. The conversion to the butenoic acid is reversible which leads to the formation of vinclozolin by recyclization. At basic pH (8.0) the forward reaction leading to the formation of the butenoic acid is favored. At acidic pH (4.5) the reverse reaction, i.e., recyclization to form vinclozolin, is favored. Formation of the enanilide occurs at pH 4.5-8.3, but is favored at acidic pH (4.5). It is conceivable that vinclozolin is converted to the enanilide by decarboxylation of the transient intermediates, the carbamic acid (Fig. 7B) and the hydrogen carbonate (Fig. 7C), but none is detected in the aqueous buffers.

3.5. Persistence of Vinclozolin on Pea Leaflets

3.5.1. Efficiency of Analytical Methods

The recoveries of vinclozolin, the butenoic acid (M1), the enanilide (M2) and 3,5-dichloroaniline (M3) from water are given in Table 24. The mean recoveries at 10 ppm and 1 ppm ranged from 86.9% to 97.5% and the method for fortified water had a high degree of precision as indicated by the small standard deviation among the four replicates at each fortification level.

Figure 28. Pseudo-first-order plot for the chemical conversion of M1, 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, to vinclozolin at 35 C in pH 4.5 phosphate buffer.

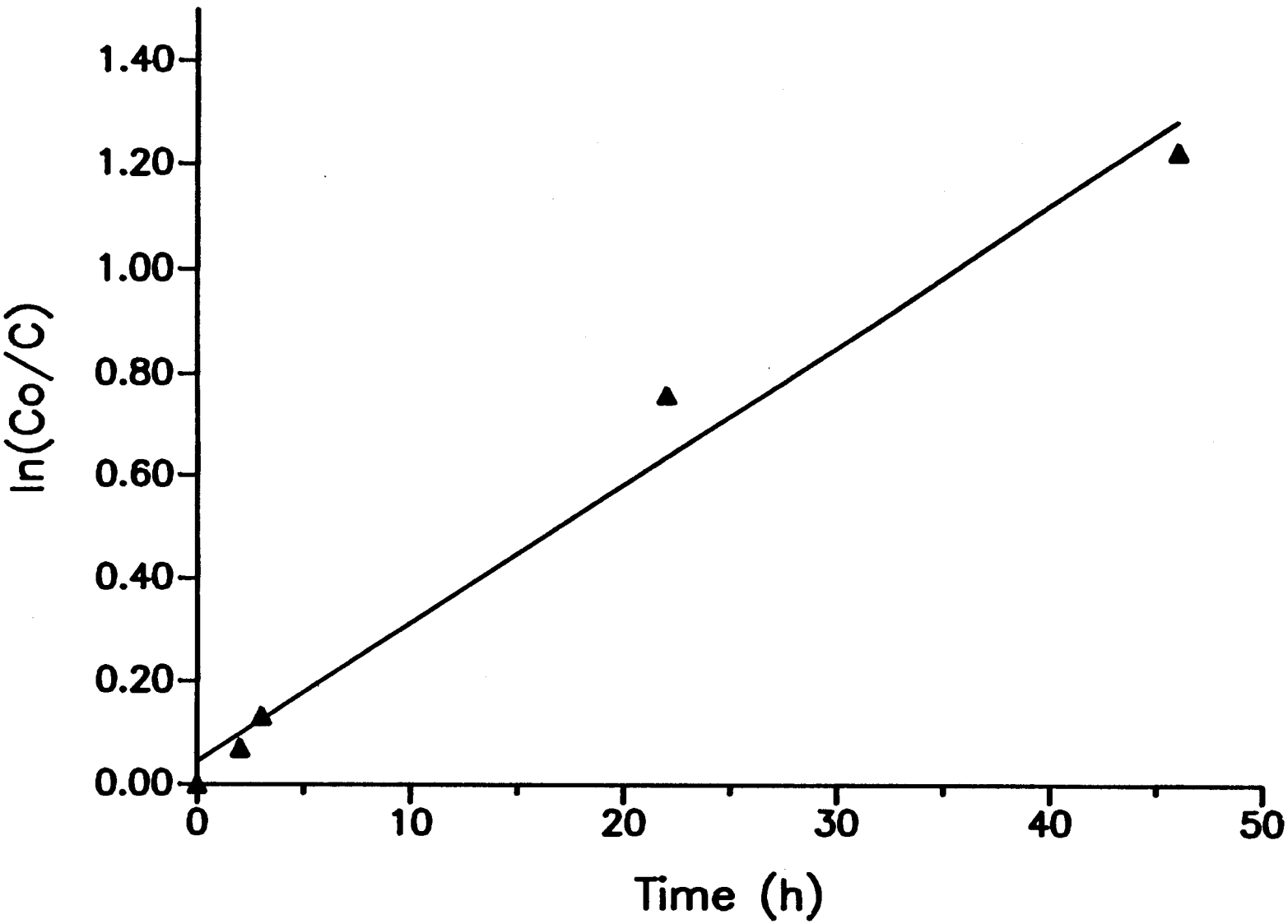

1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
ln(Co/C)
0
10
20
30
40
50
Time (h)

Figure 29. A proposed degradation pathway for the hydrolysis of vinclozolin leading reversibly to the formation of M1, 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, and to M2, 3',5'-dichloro-2-hydroxy-2-methyl-but-3-enanilide.

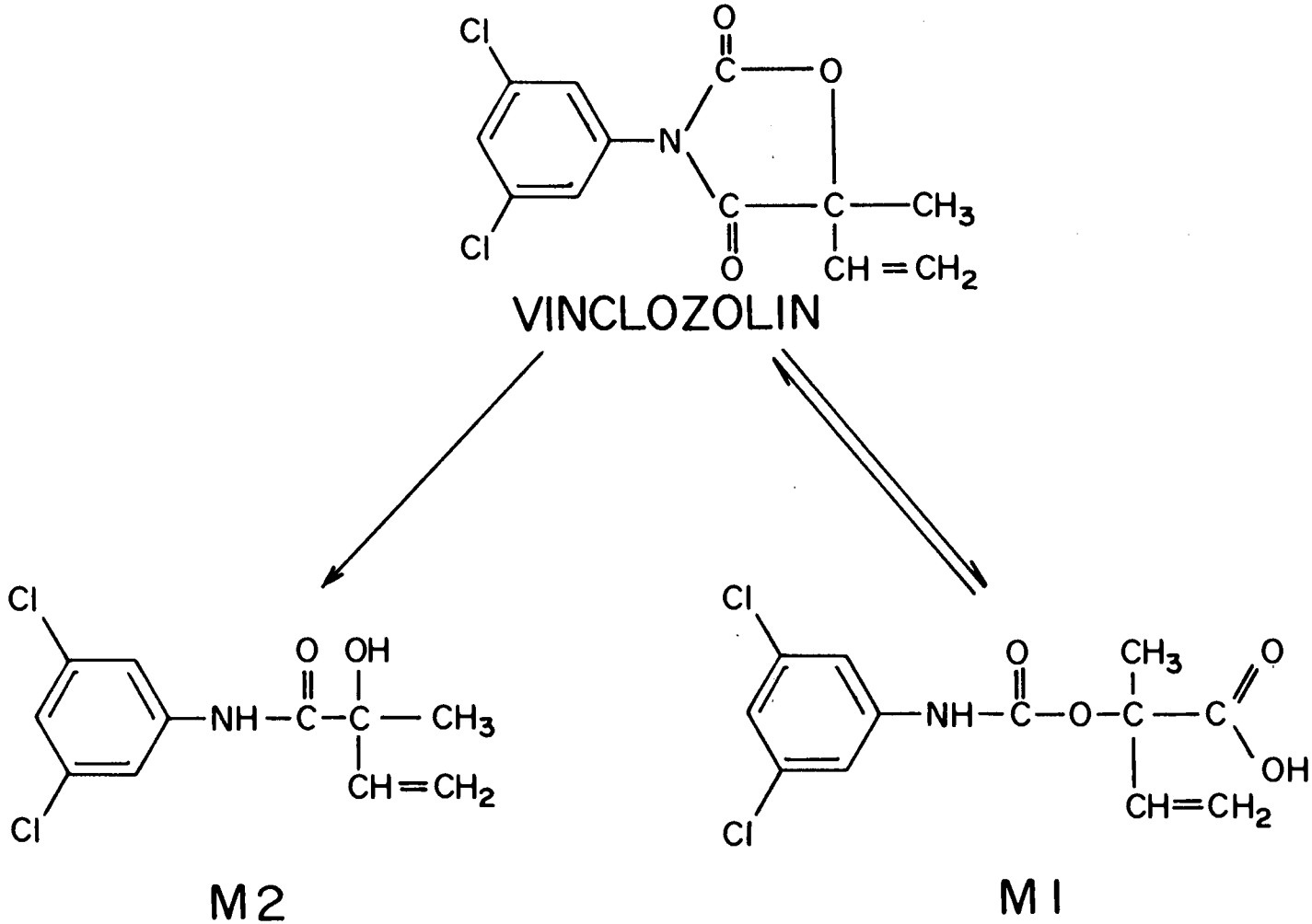

VINCLOZOLIN
M2
M1

Table 21. Chemical conversion of M1 in 0.01 M phosphate buffer of pH 4.5 at 35 C.

Time in hours	Concentration[a] in μM (%)[b]				
	M1	vinclozolin	M2	M3	Total
0	33.0(100)	N.D.	N.D.	N.D.	33.0(100)
2	30.8(90.6)	3.19(9.4)	N.D.	N.D.	34.0(103)
3	28.9(87.3)	4.18(12.7)	N.D.	N.D.	33.1(100)
22	15.4(47.4)	17.1(52.6)	N.D.	N.D.	32.5(98.5)
46	9.70(29.9)	21.6(66.7)	1.12(3.4)	N.D.	32.4(98.2)
71	7.66(23.7)	22.7(70.3)	1.89(6.0)	N.D.	32.3(97.7)
144	7.36(22.2)	20.8(62.7)	5.02(15.1)	N.D.	33.2(101)
240	6.60(19.5)	19.0(56.2)	8.19(24.3)	N.D.	33.8(102)
313	5.84(17.4)	17.3(52.0)	10.2(30.6)	N.D.	33.3(101)
480	5.54(16.6)	15.9(47.7)	11.9(35.7)	N.D.	33.3(101)
672	4.95(14.6)	14.1(41.7)	14.7(43.7)	N.D.	33.8(102)
840	4.29(12.3)	12.5(35.9)	17.4(50.0)	0.62(1.8)	34.8(105)

Table 21. (Continued)

Time in hours	Concentration[a] in µM (%)[b]				
	M1	vinclozolin	M2	M3	Total
1008	2.84(8.0)	7.72(21.8)	23.3(65.8)	1.49(4.4)	35.4(107)
1176	1.91(5.3)	7.19(20.1)	25.0(69.8)	1.74(4.8)	35.8(109)
1488	1.68(4.6)	4.84(13.3)	27.5(75.5)	2.36(6.6)	36.4(110)
1680	1.45(4.0)	2.98(8.2)	29.6(81.1)	2.48(6.7)	36.5(110)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 22. Chemical conversion of M1 in 0.01 M phosphate buffer of pH 8.0 at 35 C.

Time in hours	Concentration[a] in μM (%)[b]				
	M1	vinclozolin	M2	M3	Total
0	33.0(100)	N.D.	N.D.	N.D.	33.0(100)
2	32.0(100)	N.D.	N.D.	N.D.	32.0(97.0)
3	32.4(100)	N.D.	N.D.	N.D.	32.4(98.3)
22	32.3(100)	N.D.	N.D.	N.D.	32.3(97.9)
46	32.5(100)	N.D.	N.D.	N.D.	32.5(98.6)
71	32.0(100)	N.D.	N.D.	N.D.	32.0(96.9)
144	32.3(100)	N.D.	N.D.	N.D.	32.3(98.0)
240	32.2(100)	N.D.	N.D.	N.D.	32.2(97.5)
313	31.5(100)	N.D.	N.D.	N.D.	31.5(95.3)
480	30.1(93.5)	N.D.	2.08(6.5)	N.D.	32.2(97.5)
672	29.2(91.0)	N.D.	2.90(9.0)	N.D.	32.1(97.3)
840	27.4(83.3)	N.D.	4.90(14.9)	0.62(1,8)	32.9(99.8)
1008	25.7(75.8)	N.D.	6.72(19.8)	1.49(4.4)	33.9(103)

Table 22. (Continued)

Time in hours	Concentration[a] in µM (%)[b]				
	M1	vinclozolin	M2	M3	Total
1176	25.2(72.4)	N.D.	7.64(22.0)	1.99(5.6)	34.8(106)
1488	24.2(70.8)	N.D.	7.80(22.8)	2.24(6.4)	34.2(104)
1680	23.5(67.7)	N.D.	8.42(24.3)	2.80(8.0)	34.7(105)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

Table 23. Chemical conversion of M1 in 0.01 M pH 8.0 phosphate buffer at 35 C after adjustment of pH to 4.5.

Time in hours	pH	Concentration[a] in μM (%)[b]				
		M1	Vinclozolin	M2	M3	Total
0	8.0	33.0(100)	N.D.	N.D.	N.D.	33.0(100)
24	8.0	32.1(100)	N.D.	N.D.	N.D.	32.1(97.4)
	4.5	14.5(43.9)	18.5(56.1)	N.D.	N.D.	33.0(100)
72	8.0	31.9(100)	N.D.	N.D.	N.D.	31.9(96.7)
	4.5	9.31(28.6)	21.1(64.9)	2.12(6.5)	N.D.	32.5(98.6)
192	8.0	32.0(100)	N.D.	N.D.	N.D.	32.0(97.0)
	4.5	7.43(22.4)	20.1(60.5)	5.71(17.1)	N.D.	33.2(101)
336	8.0	32.3(100)	N.D.	N.D.	N.D.	32.3(97.9)
	4.5	6.20(18.6)	16.5(49.5)	10.6(31.9)	N.D.	33.3(101)
504	8.0	29.8(92.5)	N.D.	2.39(7.5)	N.D.	32.2(97.5)
	4.5	5.35(16.1)	14.4(42.7)	13.9(41.2)	N.D.	33.7(102)

Table 23. (Continued)

Time in hours	pH	Concentration[a] in µM (%)[b] M1	Vinclozolin	M2	M3	Total
696	8.0	29.2(89.0)	N.D.	3.59(11.0)	N.D.	32.8(99.4)
	4.5	4.49(13.4)	13.2(39.4)	15.8(47.2)	N.D.	33.5(102)
1008	8.0	26.1(77.0)	N.D.	6.29(18.6)	1.49(4.4)	33.9(103)
	4.5	3.03(8.5)	7.86(22.1)	23.2(65.2)	1.49(4.2)	35.6(108)

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, M3 = 3,5-dichloroaniline, and total = vinclozolin + M1 + M2 + M3.

[b] % = vinclozolin, M1, M2 or M3 in percent of total, and total at time t in percent of initial total.

The gel filtration column of Bio-Bead S-X12 was very effective in separating plant pigments from vinclozolin and its degradation products. Using the 1:1 (V:V) mixture of dichloromethane and cyclohexane as the eluting solvent the first fraction of 68 ml which was discarded contained all the green pigments and most of the yellow pigments. Vinclozolin and its degradation products were recovered in fraction 2 of the next 150 ml which contained very little yellow pigment. The mean recoveries at 0.2 ppm, 1.0 ppm and 10.0 ppm ranged from 73.8% to 96.3% (Table 25). The recovery of M3 was only 73.8% which was relatively low but the precision was good as indicated by the small standard deviation of 3.6%.

3.5.2. Persistence on Pea Leaflets

Pea leaflets treated with Ronilan® 50 WP at 187.5 µg of vinclozolin per leaflet, equivalent to the application rate of 1.0 kg AI/ha in 400 liters of water, were analyzed for vinclozolin and its degradation products at various intervals after treatment. The results are given in Figure 30. There were 169 µg of vinclozolin present in the treated leaflet 2 h after treatment which was approximately 90% of the theoretical amount of 187.5 µg. Four days after treatment the washings of the treated leaflets were also analyzed. Vinclozolin was the only compound detected in the washings of treated leaflets and in the treated leaflets themselves. The amount recovered from the washings of treated leaflets accounted for more than 70% of the total amounts (washing + leaflet) and the amounts in the washings in percent of the total increased with time.

After 46 days the amounts of vinclozolin recovered from the washings accounted for approximately 94% of the total, and vinclozolin which remained adsorbed on the treated leaflet accounted for approximately 6% of the total. Linearity of the relationship between the total amounts of vinclozolin recovered from the treated leaflets and time was observed ($n = 12$, $r = -0.994^*$), and there was no statistically significant deviation from linearity. Based on the linear regression equation:

$$\text{Amount} = -2.575t + 169.7$$

the calculated half-life of vinclozolin on the Ronilan®-treated leaflets was 33.1 days.

The amounts of vinclozolin on pea leaflets treated with an acetone solution at the same rate at various, shorter intervals after treatment are shown in Figure 31. There were 167 μg of vinclozolin present in the treated-leaflet two h after treatment which was comparable with the initial deposits on the leaflet treated with Ronilan® (Fig. 30). There was significant difference in the persistence of vinclozolin between the two treatments. Little vinclozolin was removed by washing with water from the acetone solution-treated leaflets. The amounts of vinclozolin present in the washings accounted for only 5% to 10% of the total (washing + leaflet). Linearity of the relationship between the total amounts of vinclozolin recovered from the treated leaflets and time was again observed ($n = 8$, $r = -0.993^*$) as in the Ronilan®-treated leaflets, and there was no statistically significant deviation from linearity. Based on the linear regression equation:

$$\text{Amount} = -5.750t + 160.8$$

the calculated half-life of vinclozolin on the treated leaflets was 13.4 days.

The persistence of vinclozolin on treated leaflets was clearly influenced by the formulations used for application. The commercial formulation, Ronilan® 50 WP, was much more persistent than the acetone solution. The rate of disappearance of Ronilan® was about 45% of that of the acetone solution, so that its half-life was about 2.5 times longer. In Ronilan® the active ingredients were impregnated in the inert solid support thus reducing dissipation by volatilization. In comparison, the acetone solution of vinclozolin spread with the solvent over the surface of the leaflets. Therefore, loss of the active ingredient by volatilization and co-distillation during transpiration was significant. Biological degradation may be another important factor which contributed to the lower persistence of the acetone solution. Using acetone as the carrier promoted the penetration of the active ingredient through the waxy surfaces of the pea leaflets. Penetration of vinclozolin was evidenced by the facts that less than 10% of the chemicals were removed by water and 90% or more were extracted by organic solvent (Fig. 31). In contrast 75% to 95% of the active ingredient was removed by water from the Ronilan®-treated leaflets, indicating that only 5% to 25% of the chemicals penetrated the waxy surfaces of the treated leaflets (Fig. 30). Vinclozolin which penetrated through the waxy surfaces was subjected to biological degradation by the cells and consequently lower persistance was demonstrated.

The fact that most the active ingredient of Ronilan® 50 WP was easily removed by rinsing with water has significant influence on its efficacy. In order to prevent infection, vinclozolin, a protectant fungicide, is applied to the surface of foliage or fruit to form a

barrier between the host and the inoculum. Best protection is achieved by covering the surface with the protectant fungicide during the entire infection season. However, weathering gradually reduces the amount of residue on the surface and eventually it drops below the threshold of effectiveness. In addition plant growth further dilutes the protectant. Therefore, protective treatments must be repeated at certain time intervals. Rain as one of the important factors of weathering removes deposits from treated plants by mechanical erosion. Since the deposits of Ronilan® were easily removed by rinsing with water it was evident that this protectant fungicide would be susceptible to weathering factors such as rain and dew. To increase the effectiveness of Ronilan® spreaders and stickers may be added to improve its resistance to weathering, or the frequency and timing of treatments may be synchronized in such a way as to compensate for loss of protectants due to weathering and growth.

The persistence of vinclozolin under field conditions was lower than that under laboratory conditions as reported here because of weathering. Zenon-Roland and Gilles (1978) reported that when vinclozolin was applied at rates from 125 - 175 g AI/ha to strawberry plants, its residues on fruits were proportional to the application rate. The half-life varied from year to year. It was 12-13 days in one year and 22 days in the following year; both values were shorter than the half-life of 33 days reported here with Ronilan®. Much shorter half-lives were reported on grapes. Del Re et al. (1980) determined the rate of dissipation after the last of seven treatments with vinclozolin at 700-1400 g AI/ha and reported a half-life of 7.5-9 days. A still

shorter half-life was reported by Gennari et al. (1985), also on grapes; depending on the application rate the calculated half-life ranged from 1.2-4.9 days.

The persistence of vinclozolin was compared with that of captan, a protectant dicarboximide fungicide extensively used in agriculture. According to nine studies on apples, grapes and pears conducted in Ontario from 1981 to 1983, residues decreased significantly over a 14-day period following the last application of Captan® 50 WP, or Captan® 80 WP at 1.7, 2.8 or 3.4 kg/ha (Frank et al. 1985). Correlations between rainfall and residues were observed in five of the studies; but no correlations were observed in four studies in which little or no rain fell in the first 7 days after application. The effect of rainfall on the dissipation of captan on apples, grapes and pears outdoors was comparable with the effect of rinsing with water on removing vinclozolin residues on pea leaves reported here. These observations indicate that the wettable powder formulations of both vinclozolin and captan are somewhat susceptible to weathering.

In pea leaflets treated with Ronilan® or the acetone solution vinclozolin was the only compound detected in the tissues. The degradation products, M1, M2, and M3 were not detected with the limit of detection of 0.1 ppm (fresh wt). It was apparent that M1, M2 and M3 were not formed in pea leaves when vinclozolin was topically applied to the leaf surface. Similar observations were reported in the degradation by plants of 3-(3',5'-dichlorophenyl)-5,5-dimethyloxazolidine-2,4-dione (DDOD) (Fig. 32A), a methyl analogue of vinclozolin (Sumida et al. 1973b). Bean plants (Phaseolus vulgaris) were injected with ^{14}C-DDOD at

the stem. After 14 days, the degradation products, N-3,5-dichlorophenyl-N-∝-hydroxyisobutyryl carbamic acid (DHCA, Fig. 32B) and N-∝-hydroxyisobutyryl-3-5-dichloroanilide (HDA, Fig. 32C) were not detected in the tissues; but when DDOD was applied in nutrient solutions, both degradation products DHCA and HDA were detected in tissues of the root-treated bean plants. However, Sumida et al. (1973b) also observed that DDOD degraded rapidly to DHCA and HDA in the nutrient solution. In view of this fact they concluded that probably substantial portions of DHCA and HDA detected in the root-treated plant tissues were derived directly from DHCA and HDA in the nutrient solution rather than being products of plant metabolism. As a note of interest, it seems highly likely that the correct identity of the degradation product identified as DHCA by Sumida et al. could be 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl propionic acid (Fig. 32D) for the same reasons as described in the identification of M1 under 3.3.2.2.

Vinclozolin was not detected in any of the untreated pea leaflets in the persistence studies with Ronilan® or the acetone solution, indicating that it was non-systemic and would not be translocated from the treated leaflets to the untreated parts of the plants. In order to confirm this non-systemic property, its movement was monitored 38 days after the Ronilan® treatment and 21 days after the acetone solution treatment. All untreated parts including stems and roots were extracted and analyzed. Vinclozolin and its degradation products M1, M2 and M3 were not detected at the limit of 0.1 ppm (fresh wt). I concluded that vinclozolin is non-systemic when applied to leaves.

Table 24. Recoveries of vinclozolin and its degradation products[a] from water.

Compound	Percent recovery ($\bar{X}$ ± S.D., n = 4)	
	10.0 ppm	1.0 ppm
vinclozolin	97.5 ± 1.6	96.6 ± 2.3
M1	92.5 ± 2.2	86.9 ± 2.0
M2	95.1 ± 2.2	93.0 ± 2.4
M3	90.2 ± 3.2	90.8 ± 3.0

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide and M3 = 3,5-dichloroaniline.

Table 25. Recoveries of vinclozolin and its degradation products[a] from pea leaflets.

Compound	Percent recovery ($\bar{X}$ ± S.D., n = 4)		
	10.0 ppm	1.0 ppm	0.2 ppm
vinclozolin	96.3 ± 2.0	95.2 ± 2.8	92.9 ± 2.6
M1	85.0 ± 3.5	81.4 ± 2.7	73.8 ± 3.6
M2	95.2 ± 2.9	94.9 ± 3.4	93.5 ± 2.8
M3	86.9 ± 3.7	81.0 ± 6.4	80.5 ± 5.2

[a] M1 = 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid, M2 = 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide and M3 = 3,5-dichloroaniline.

Figure 30. Persistence of vinclozolin on pea leaves under laboratory conditions after application of Ronilan® 50 WP at 187.5 µg AI/leaflet.

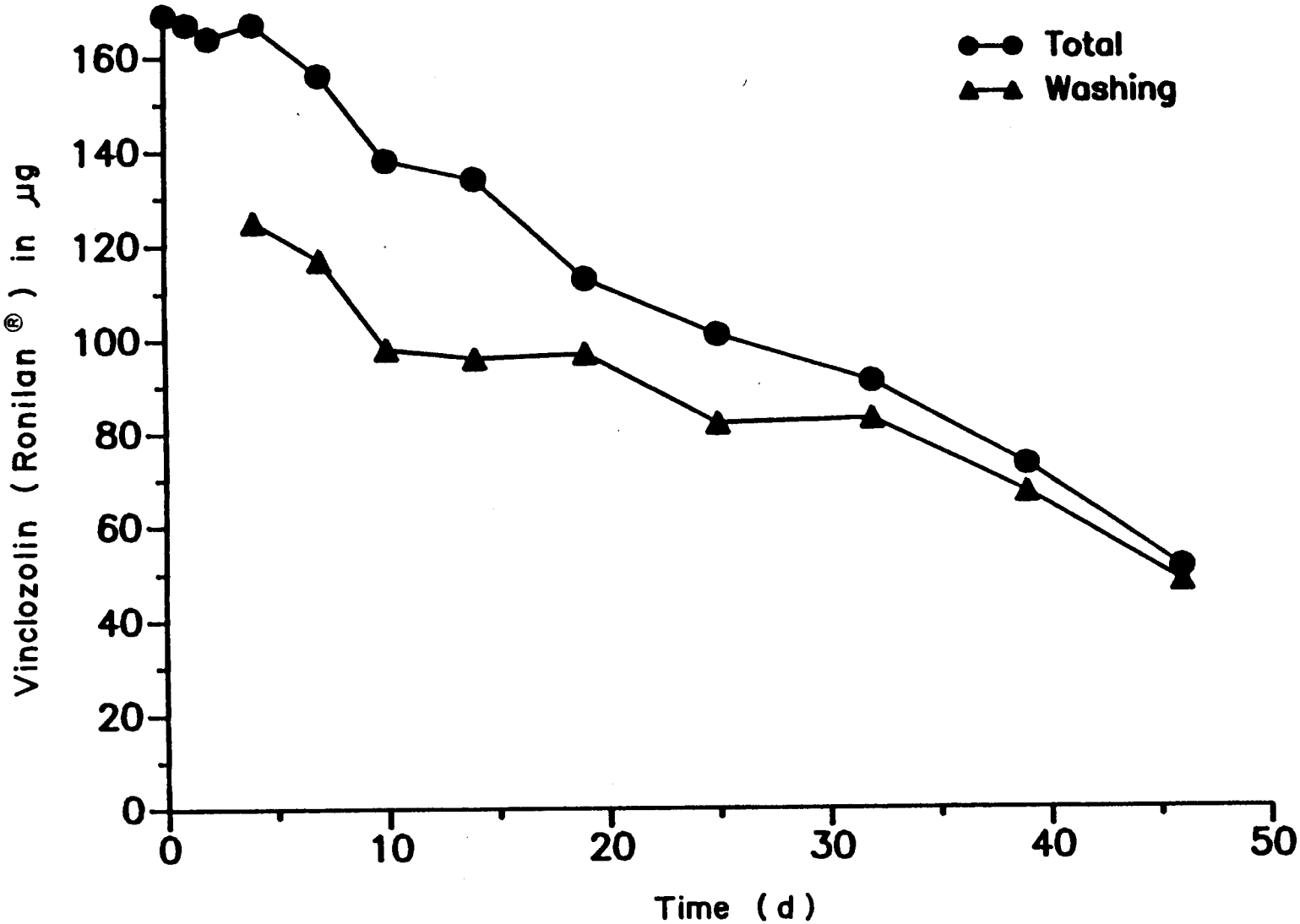

Total
Washing
Vinclozolin (Ronilan ®) in µg
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
Time (d)

Figure 31. Persistence of vinclozolin on pea leaves under laboratory conditions after application of an acetone solution at 187.5 µg AI/leaflet.

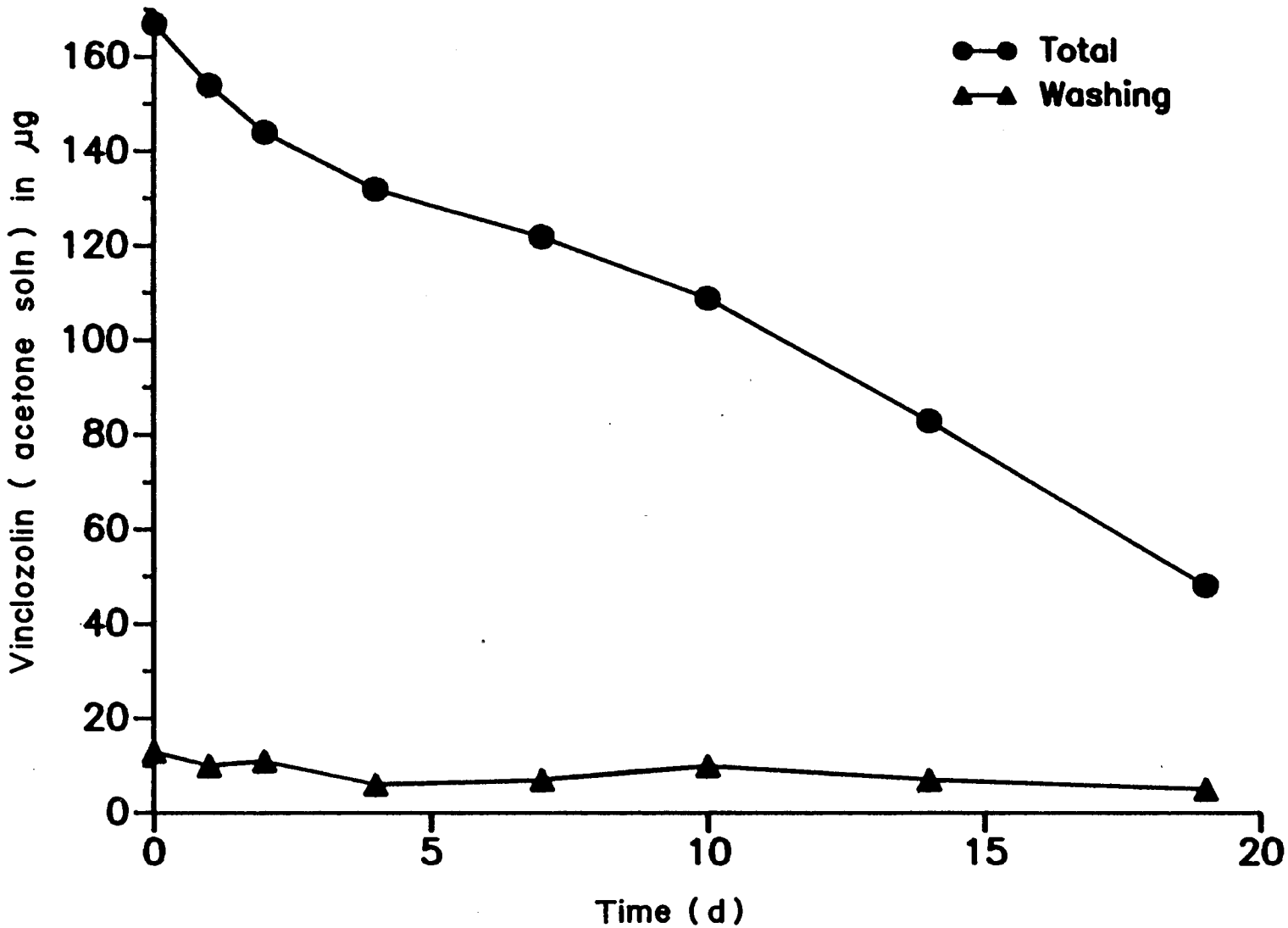
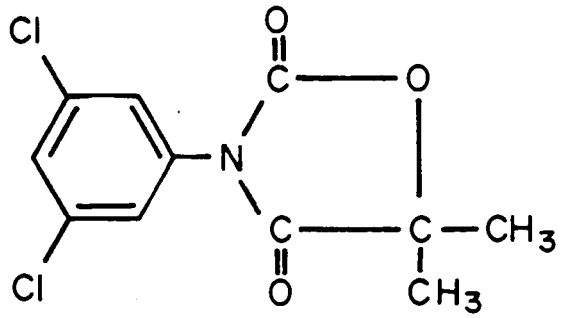
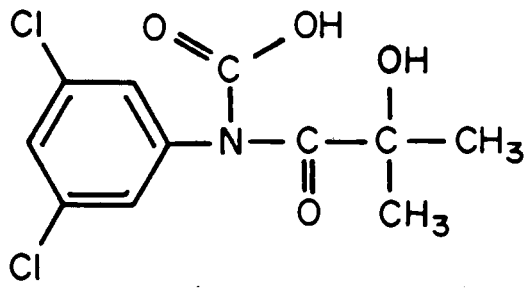

Total
Washing
Vinclozolin (acetone soln) in µg
160
140
120
100
80
60
40
20
0
0
5
10
15
20
Time (d)

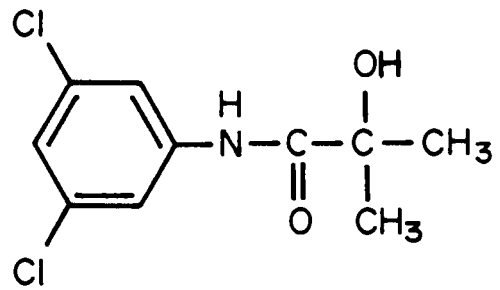
Figure 32. Structural formulae of DDOD, 3-(3',5'-dichlorophenyl)-5,5-dimethyl-oxazolidine-2,4-dione (A); DHCA, 3,5-dichlorophenyl-N-α-hydroxyisobutyryl carbamic acid (B); HDA, N-α-hydoxy-isobutyryl-3,5-dichloroanilide (C); and 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl propionic acid (D).



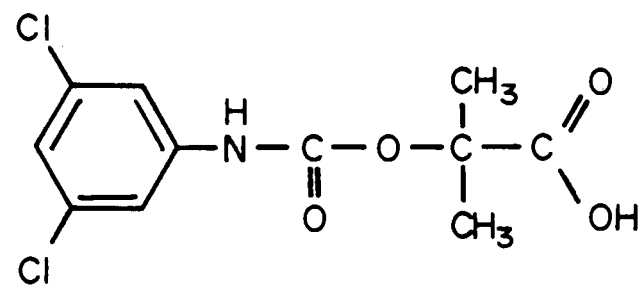
A (DDOD)



B (DHCA)



C (HDA)



D

4. CONCLUSIONS

This study on the hydrolysis of vinclozolin, a protectant fungicide, at 35 C and pH 4.5-8.3, and its persistence on pea leaves (Pisum sativum L.) under laboratory conditions, leads to the following conclusions:

1. On hydrolysis three degradation products were isolated, purified and identified. They were 2-[(3,5-dichlorophenyl)carbamoyl] oxy-2-methyl-3-butenoic acid (M1), 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2), and 3,5-dichloroaniline (M3). The identity of M3 was confirmed by gas chromatography-mass spectrometry. The structures of M1 and M2 were elucidated and confirmed by solid probe mass spectrometry, proton and ^{13}C nuclear magnetic resonance. Furthermore, the identity of M1 was confirmed by unambiguous evidence from x-ray crystallography of its ethyl ester.
2. The disappearance of vinclozolin on hydrolysis at 35 C followed simple pseudo-first-order kinetics from pH 4.5-8.3. Vinclozolin was much more susceptible to hydrolysis at basic than at acidic pH and the pseudo-first-order rate constant was directly proportional to the hydroxide ion concentration.
3. At pH 7.0 the pseudo-first-order rate constant was directly proportional to the reaction temperature. Using the Arrhenius plot of log rates at 13 C, 20 C, 26 C and 35 C vs. 1/T, the energy of activation for the hydrolysis of vinclozolin at pH 7.0 was calculated to be 97.2 K Joules mol^{-1}, and the frequency factor to be 3.467×10^{15} h^{-1}.

4. M1 and M2 were the major degradation products but M3 was a minor one which was detected in the hydrolysis mixtures after at least 21 days incubation in pH 4.5-8.3 buffers at 35 C. The finding that M3 was formed on hydrolysis of vinclozolin is important to environmental toxicologists because M3 is a chlorinated aromatic amine which may be toxic to higher animals.
5. Using M1 as the starting material for incubation studies in phosphate buffers of pH 4.5 and 8.0 at 35 C, it was demonstrated that recyclization of M1 occurred to yield vinclozolin, indicating that conversion from vinclozolin to M1 on hydrolysis was reversible. Recyclization of M1 was favored in acidic rather than in basic pH.
6. M1, M2 and M3 were stable in aqueous buffers of pH 4.5-8.0. On a molar basis the total amounts of vinclozolin plus M1, M2 and M3 remained somewhat constant throughout the incubation study for 50-113 days.
7. Based on the kinetic data a degradation pathway was proposed for the hydrolysis of vinclozolin, in which the 2,4-oxazolidinedione ring opens to yield both M1 and M2 independently. The conversion from vinclozolin to M1 was reversible and the forward reaction was favored by basic pH whereas the reverse reaction was favored by acidic pH.
8. Vinclozolin persisted on pea leaves for 21-46 days under laboratory conditions. Its persistence was higher with Ronilan® 50 WP, a commercial formulation of vinclozolin, than with an acetone solution. The dissipation of vinclozolin on leaves was linear and

the calculated half-life was 33.1 days from Ronilan® and 13.4 days from the acetone solution.

9. Ronilan® deposits were easily dislodged by rinsing with water and less than 25% were strongly adsorbed on the leaves, indicating that Ronilan® was susceptible to weathering. In comparison, deposits of the acetone solution were much more tenacious, since less than 10% were dislodged by rinsing with water.
10. Translocation of vinclozolin was not detected in pea plants after its application to one of the leaflets indicating that it is non-systemic. The degradation products, M1, M2 and M3 were not detected in either treated or untreated tissues of the same plant.

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