Specific Determination of Chlorphenamidine

[N'-(4-Chloro-o-tolyl)-N,N-dimethylformamidine] in Plants and

Soil Material by Colorimetry and Thin-Layer and Electron Capture

Gas Chromatography

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A specific method for the determination of residues of the acaricide chlorphenamidine [N'-(4-chloro-o-tolyl)-N,N-dimethylformamidine] is presented. The parent compound and three potential metabolites [N'-(4-chloro-o-tolyl)-N-methylformamidine, N-formyl-(4-chloro-o-toluidine), and 4-chloro-o-toluidine] are isolated and determined following thin-layer chromatography or by gas chromatography.

For quantitative determination of the compounds, a colorimetric procedure is preferred. So far the procedure has been tested in apples, pears, peaches, plums, grapes, and bean leaves. Results of analyses of field samples demonstrate that the breakdown of the acaricide takes place mainly in metabolically active tissues such as young bean leaves.

Chlorphenamidine [N'-(4-chloro-o-tolyl)-N,N-dimethylformamidine] is a new acaricide marketed under the trade names Galecron and Fundal. The compound is very effective as an ovicide as well as an adulticide. It may be used as a versatile miticide in cultivations of fruits, vegetables, and ornamentals.

The substance was introduced by Schering and CIBA information bulletins (1966), and its biological properties were discussed in several publications by Dittrich (1966a,b, 1967a,b, 1968, 1969). Chlorphenamidine belongs to a class of chemical substances not previously represented in the actual practice of plant protection. Its application as an acaricide, therefore, opens some possibilities for overcoming current problems of mite resistance against organic phosphorus compounds and other familiar miticides.

N'-(4-Chloro-o-tolyl)-N,N-dimethylformamidine (I) is a medium strength base showing a pK_B-value of 7.2 in a water/methanol 1 to 1 (v/v) mixture, and forming crystalline salts with strong acids. The hydrochloride salt, as well as the free base, is used in agricultural practice. The solubility of the base in water is 250 ppm. The hydrochloride salt is more than 50% soluble in water (Schering, 1966).

The formamidine (I) is readily hydrolyzed in weakly-acid to weakly-alkaline solutions, and is rather stable in strong acid solution. In an aqueous buffer solution of pH 7.0 containing 5% of methanol, the formamidine showed a half-life of 42 hr at 30° C. At pH 9, under otherwise equivalent conditions, a half-life of 5 hr was observed (Schering, 1964). Hydrolysis of the parent compound (I) yields N-formyl-(4-chloro-o-toluidine) (II) and ultimately 4-chloro-o-toluidine (III) (Figure 1).

The second step of hydrolysis is very slow at room temperature but is accelerated by heating with strong acids or alkali. These properties of chlorphenamidine as a base, as well as its stepwise degradation to 4-chloro-o-toluidine by hydrolysis,

are used for the quantitative determination of the substance (Geissbühler et al., 1971). Residues of chlorphenamidine in or on plant materials may be expected to be chemically hydrolyzed to some degree according to the pattern described above. In addition, degradation studies indicate that enzymatic demethylation to N'-(4-chloro-o-tolyl)-N-methylformamidine (compound IV) does occur in certain metabolically active plant tissues, such as young leaves (Sen Gupta and Knowles, 1969). Further hydrolysis of compound IV may be assumed to follow the scheme given for the parent compound.

The total residue methods published by Geissbühler et al. (1971), which are based on the colorimetric or gas chromatographic determination of 4-chloro-o-toluidine after hydrolysis, account for all the breakdown products listed in the degradation scheme, as well as for any conjugates of these substances. The method does not, however, allow separate determination of metabolites.

This report describes thin-layer and gas chromatographic procedures which permit separate determination of residue quantities of the parent compound and its potential degradation products in plant materials.

MATERIALS AND EQUIPMENT

Apparatus. The gas chromatograph used was a Model 400 F & M High Efficiency Gas Chromatograph equipped with a flame ionization detector.

Reagents. All inorganic chemicals were reagent grade, solvents were reagent grade, or redistilled. Silica gel HF 254 for thin-layer chromatography. Nopco NXZ liquid antifoam (Nopco Chemical Company, Newark, N.J.). N-Ethyl-1-naphthylamine, reagent grade (Fluka AG, Buchs, Switzerland, or Eastman Organic Chemicals, Rochester, N.Y.).

Reference Compounds. Chlorphenamidine (I) [N'-(4-chloro-o-tolyl)-N,N-dimethylformamidine] prepared according to U.S. Patent 3,502,720. Purification by high vacuum distillation under nitrogen at 110° C and 2.5 10^{-2} Torr. Purity > 99% confirmed by tlc, m.p. 32° C, mol. weight 196.7. Compound II [N-formyl-(4-chloro-o-toluidine)] prepared according to Organic Syntheses (1955), recrystallized from methanol. Purity > 99% determined by tlc, m.p. 118–120° C, mol. weight 169.6. Compound III (4-chloro-o-toluidine) received

from Schuchardt, Munich, W. Ger. Purity 99%, m.p. 26–27° C, mol. weight 141.6. Compound IV [N-(4-chloro-o-tolyl)-N-methylformamidine] prepared according to U.S. Patent 3,502,720. Recrystallized from hexane. Purity > 97% confirmed by tlc, m.p. 91° C, mol. weight 182.7. Acetanilide (internal standard) prepared according to *Organic Syntheses* (1963), recrystallized from methanol. Gas chromatographically pure > 99%, m.p. 114° C.

PROCEDURES

Principle. Plant material is subjected to a twofold extraction. The first extraction step is carried out with methanol/hydrochloric acid, and the second one with the more lipophilic solvent mixture methanol/methylene chloride. Three partitioning steps provide sufficient cleanup of extracts. Separation of the parent compound from degradation products is accomplished by thin-layer chromatography. Following separation, the eluates are successively treated with acetic acid and sodium hydroxide, resulting finally in 4-chloro-o-toluidine. This compound is diazotized and coupled with N-ethyl-1-naphthylamine, yielding a stable azo dye for quantitation. If concentrations of metabolites greater than 0.1 ppm are present, the parent compound and compounds III and IV may alternatively be determined by gas chromatography using a flame ionization detector.

Extraction and Cleanup. Mix 100 g of chopped fresh or frozen material with 100 ml of methanol and 1 ml of concentrated hydrochloric acid (D = 1.19), and macerate in a Waring Blendor for 5 min (Figure 2). The macerate should have a pH value of 3. Filter off the first extract through Whatman No. 1 filter paper. Repeat the extraction of the residue, including the paper, with 100 ml of the solvent mixture methanol/methylene chloride (1 to 1 v/v) for another 5 min. Filter off the second extract and wash the filter residue with solvent mixture. Combine all filtered solutions. The residue consists of a colorless fibrous mass. The total extracts must be further cleaned without delay.

Evaporate the solvents by means of a rotating evaporator (30° C, 20 Torr). Mix the remaining aqueous phase with 5 g of Celite 545, and filter through a (G4)-fritted funnel. Rinse the funnel and residue three times with 10 ml of water, followed by two portions of 50 ml of methylene chloride. Saturate the mixed filtrates with sodium chloride, and cool with cubes of ice produced from demineralized water. Make the mixture alkaline with 8 ml of 5 N sodium hydroxide (pH = >14) and extract four times with 100 ml of methylene chloride in a 500-ml separatory funnel. Any emulsions which form at this stage may be broken by centrifuging at 3000 rpm for 10 min. Discard the aqueous layer.

Clarify and dry the combined organic extracts with 30 g of anhydrous sodium sulfate (in many cases some suspended substances and parts of the aqueous phase will remain in the solvent). Reextract the organic phase four times with 50 ml of 1 N sulfuric acid. Combine the acid extracts. The remaining organic layer (solution A) is retained for the determination of compound II. Saturate the combined sulfuric acid extracts with sodium chloride and cool with ice cubes. Rinse into a 500-ml separatory funnel, add 100 ml of methylene chloride, and make alkaline with 25 ml of 10 N sodium hydroxide (pH = 13–14). Shake the mixture and separate the layers. Repeat the partitioning step three times with 50-ml portions of methylene chloride. Combine the extracts and dry with 20 g of anhydrous sodium sulfate. Solution B, thus prepared, is then used for thin-layer chromatography and subsequent colorimetric measurement, or for gas chromatographic analysis.
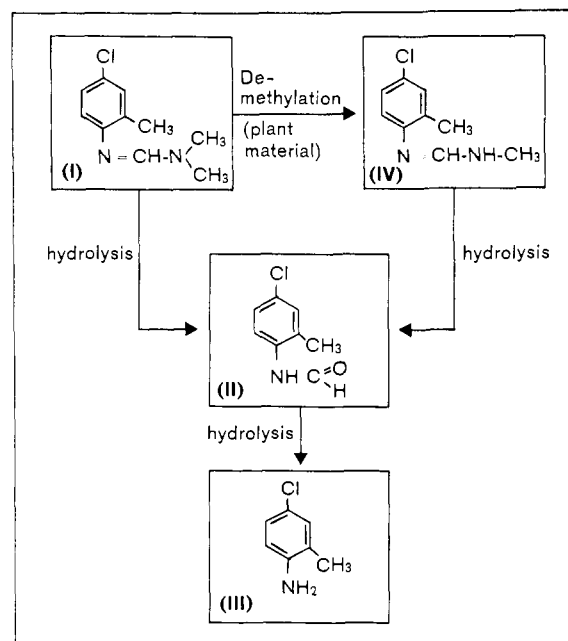

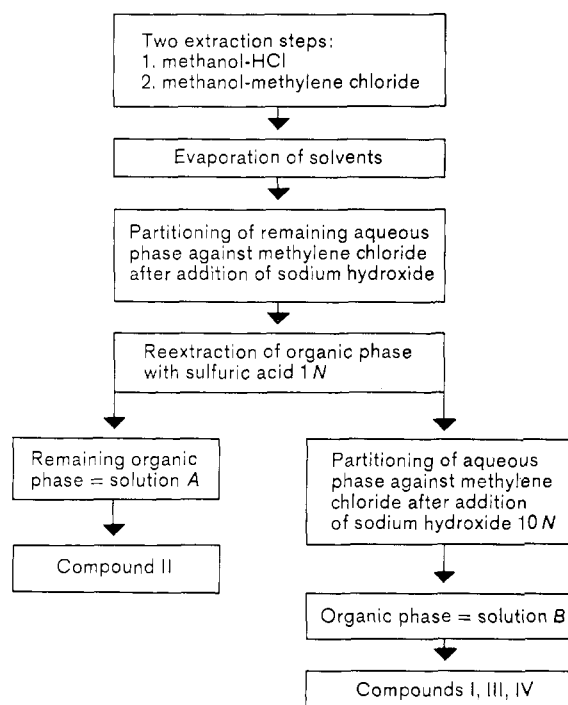Figure 1. Pathway of chemical and enzymatic degradation of chlorphenamidine

Figure 2. Scheme of extraction from plant material and purification of chlorphenamidine and its potential metabolites

Thin-Layer Chromatography (Compounds I, III, IV). Add two drops of concentrated hydrochloric acid/methanol (1 to 9 v/v) mixture to solution B and reduce the volume to about 15 ml by means of a rotating evaporator (40° C, 20 Torr). Transfer the residue into a Kuderna-Danish evaporative concentrator and reduce the solvent to 0.5 ml by distillation, without vacuum, at 60° C. Apply one half of the concentrate, corresponding to 50 g of plant material, to a thin-layer plate coated with a 250 μ layer of silica gel. For comparison, spot a concentrate of untreated plant material (control sample) and reference solutions containing 5 μg each

I—0.4 ppm chlorphenamidine (parent compound)
III—0.1 ppm 4-chloro-o-toluidine
IV—0.4 ppm N'-(4-chloro-o-tolyl)-N-methyl formamidine
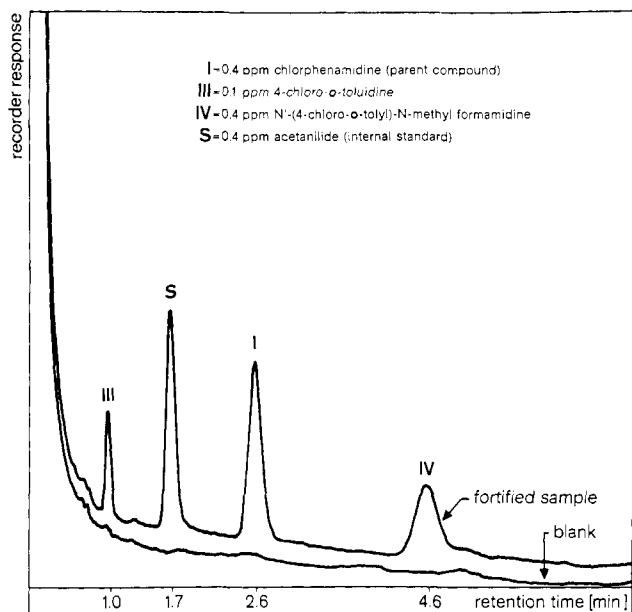S—0.4 ppm acetanilide (internal standard)

Figure 3. Gas chromatogram of a fortified grape sample in comparison to untreated control. Aliquots representing 50 mg of fortified grapes or of untreated control sample were injected. The fortified sample contained 20 ng of compounds I, IV, and 5 ng of compound III. Gas chromatographic conditions "b" (see Table II). Electrometer sensitivity 1.28×10^{-10} AFS

Table I. Gas Chromatographic Conditions for Determination of Chlorphenamidine and Some Degradation Products in Residue Samples

Column	1.1 m (3 mm i.d.) 5% Ucon LB 550 X (polypropylene glycol) and 0.5% potassium hydroxide on Chromosorb G, 60–80 mesh, silanized	
Chart speed	1.5 in./min	
	conditions a	conditions b
Column temperature	215° C	205° C
Injection port temperature	240° C	240° C
Detector temperature	260° C	255° C
Carrier gas	150 ml He/min	80 ml He/min
T_R-values		
Compound III	0.5 min	1.0 min
Acetanilide (internal standard)	0.9 min	1.7 min
Compound I	1.4 min	2.6 min
Compound IV	2.4 min	4.6 min

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of the parent compound, compound III, and compound IV. Equilibrate the prepared plate for 30 min in a solvent-saturated tank using benzene/diethylamine (95 to 5, v/v). After equilibration, develop the plate according to the ascending technique to a height of 12 cm. Remove the plate from the tank and air-dry at room temperature in a hood. Expose the plate to ultraviolet light at 254 nm. The reference compounds show a distinct fluorescence quenching.

The following R_f values may be expected: Chlorphenamidine (parent compound) 0.6; compound III, 0.4; and compound IV, 0.2 to 0.3. Locate the reference compounds and mark the corresponding zones of the plant material concentrates. Scrape off the adsorbent of each zone. The parent compound and compound IV are eluted with 10 ml of an acetone/methanol (1 to 1, v/v) mixture in small portions. Elute compound III with 20 ml of 1 N hydrochloric acid in

small portions. The solution of compound III is subjected directly to colorimetric determination. Evaporate the organic solvents from solutions of the parent compound and of compound IV, respectively, after adding one drop of concentrated hydrochloric acid to each solution. Dilute the aqueous residue with 2 ml of 1 N acetic acid and hydrolyze at 90° C for 1 hr. After cooling, add 2 ml of 5 N sodium hydroxide and heat to 90° C for another hour. Avoid any loss of aromatic amine by using a high efficiency condenser. After hydrolyzing and cooling carefully, add 5 ml of 4 N hydrochloric acid through the condenser. Transfer the hydrolyzate with an additional 11 ml of 1 N hydrochloric acid to a 50-ml volumetric flask.

Distillation/Extraction (Compound II). Reduce the volume of solution A to dryness in a rotating evaporator (30° C, 20 Torr) using a 500-ml flask. Add 200 ml of 2 N sodium hydroxide and 2 ml of antifoam solution. Subject the mixture to continuous hydrolysis and distillation/isooctane extraction for 2½ hr (Geissbühler et al., 1971). The isooctane extract thus obtained will contain the 4-chloro-o-toluidine moiety, which is successively extracted with a 10-ml and two 5-ml portions of 1 N hydrochloric acid. Combine the extracts for colorimetric determination.

Colorimetric Determination. The hydrochloric acid solutions obtained by extraction of the isooctane phase as well as by acidification of hydrolyzates of thin-layer eluates (see above) are subjected to colorimetric analysis, as described by Geissbühler et al. (1971). To calculate the concentrations of the parent compound and the separated degradation products, the following conversion factors are used. One part of 4-chloro-o-toluidine is equivalent to 1.39 parts of chlorphenamidine, to 1.20 parts of compound II, and to 1.29 parts of compound IV. If the control samples produce blanks which significantly reduce the lower limit of detection, cleaning the azo dye solution by column chromatography on cellulose is recommended (Geissbühler et al., 1971).

Gas Chromatography. Solution B obtained by cleanup steps described above will contain the compounds I, III, IV. If amounts greater than 0.1 ppm are present in plant materials, detection by gas chromatography in a single gas chromatogram is possible. Reduce solution B by means of a rotating evaporator (30° C, 20 Torr) to 50 ml.

The remaining solvent is concentrated to 1 ml in a Kuderna-Danish evaporative concentrator (bath temperature 60° C). Rinse the apparatus with 0.5 ml methylene chloride. To 1.5 ml of concentrate add 20 μg of acetanilide, in 0.5 ml methylene chloride, as an internal standard (avoid chloroform as a solvent). Inject 1 to 3 μl of the solution thus prepared into the gas chromatograph. The gas chromatographic conditions are described in Table I. Figure 3 gives an example of a typical gas chromatogram. The selection of conditions "a" or "b" depends on the composition of the sample. Conditions "a" are preferred for determining the parent compound and compound IV; compound III is determined according to conditions "b."

RESULTS AND DISCUSSION

Formation of Artifacts. To avoid any error, it is necessary to protect the parent compound, chlorphenamidine, against hydrolyzing agents during the cleanup steps. Formation of artifacts cannot be totally excluded, however. While analyzing the sample, formation of compounds II and III must be avoided by strictly observing the analytical procedures and checking the method by complete analysis of an untreated control sample fortified with known amounts of authentic

chlorphenamidine. The order of magnitude of the formation of artifacts is shown in Table II. The results indicate a high rate of hydrolysis of compound II during analysis. By comparison, the parent compound is much more stable under the reported conditions.

A further source of error may be the storage of extracts over a prolonged period of time (Table III). If storage of extracts prior to analysis cannot be avoided, care must be taken to keep storage temperatures as low as possible (<3° C). The results listed in Table III demonstrate that compound II is slowly hydrolyzed in acid solution to compound III, independent of any enzymatic degradation in active plant tissues.

Recoveries. Reproducibility and acceptable recoveries of the thin-layer/colorimetric method depend on careful execution of the procedures described above. Insufficient control of the hydrolyzable and volatile parent compound and of its metabolites affects all steps of procedure, and may result in substantial losses. Due to the volatility of compounds I, III, and IV, evaporation of all solvents has to be carried out with extreme caution.

Some typical results of recovery experiments are tabulated in Table IV. The samples or extracts were fortified with known amounts of chlorphenamidine and of its potential metabolites. The erratic recovery values for compound III are ascribed to its volatility, its partitioning behavior, and to the reactivity of the free amino group. Recoveries for gas chromatographic detection correspond to those reported for the colorimetric method.

Limits of Detection. The thin-layer/colorimetric procedure involves two main factors, both of which limit detection and determination of chlorphenamidine and the above mentioned degradation products. The first of these factors is the capacity of the thin-layer plate and the detectability of the acaricide and its metabolites by fluorescence quenching. Samples corresponding to 50 g of plant material may be spotted on the plate, and about 1 μg of compounds I, III, or IV results in distinguishable fluorescence quenching spots after developing the plate. Another factor to be taken into account is the lower limit of absorbance to be measured upon colorimetric analysis. A 1 μg quantity of 4-chloro-o-toluidine can be determined after diazotizing and coupling with N-ethyl-1-naphthylamine using a 50-mm cell. Thus, the limits of detection for the separated compounds I, III, IV are 0.02 to 0.03 ppm.

In the determination of compound II, a standard deviation(s) of the control has been calculated. In accordance with the definition of Frehse and Niessen (1963), the limit of detection is set at 0.5 s, which usually corresponds to concen-

Table II. Effect of Analytical Procedure on Formation of Artifacts from Chlorphenamidine and from the Potential Metabolite Compound II

Added	Concentration ppm	Recovered as	
		Compound II, %	Compound III, %
Chlorphenamidine	10	0.4–0.5	0.1
Compound II	10	84–97	3–16

Samples were analyzed while careful attention was given to all factors necessary for preventing additional hydrolysis. Chlorphenamidine and compound II were determined (five analyses each) from an aqueous solution containing hydrochloric acid and methanol, corresponding to plant extracts usually received. Determination was made by full cleanup, thin-layer chromatography, and colorimetric measurement.

Table III. Stability of Chlorphenamidine and Compound II in Aqueous Solution (pH 3)

Added, ppm	Storage (Days)	Temperature, °C	Recovered as	
			Compound II, %	Compound III, %
Chlorphenamidine				
10	0	22 ± 2	0.5	0.1
10	30	22 ± 2	2	0.7
10	60	22 ± 2	3	3
10	30	6 ± 1	0.5	0.1
10	60	6 ± 1	0.8	1
Compound II				
10	0	22 ± 2	88	12
10	30	22 ± 2	66	34
10	60	22 ± 2	28	72
10	30	6 ± 1	97	3
10	60	6 ± 1	86	14

Hydrolysis of chlorphenamidine and/or compound II in aqueous solution of pH 3, expressed as amounts of N-formyl-(4-chloro-o-toluidine) and of 4-chloro-o-toluidine recovered after storage at varying temperatures. Analyses made by colorimetry following thin-layer chromatography separation.

trations of 0.03 to 0.1 ppm of compound II. The limits of detection by gas chromatography correspond to the values obtained by colorimetric determinations. Sufficient detector responses for quantitative evaluation are obtained with concentrations of 0.1 ppm of chlorphenamidine or compound IV, and 0.05 ppm of compound III.

Analyses of Field Samples. The methods described for determination of chlorphenamidine and its potential metabolites have been applied in a number of preliminary experiments on behavior of the active ingredient in and on different parts of plants, such as leaves, stems, or fruits. Loss and

Table IV. Typical Recoveries of Chlorphenamidine and Its Degradation Products (Compounds II, III, and IV) from Fortified Samples of Whole Fruits

Crop	Chlorphenamidine			Compound II			Compound III			Compound IV		
	Added ppm	Recovered ppm	%	Added ppm	Recovered ppm	%	Added ppm	Recovered ppm	%	Added ppm	Recovered ppm	%
Apples	0.5	0.48	96	0.1	0.04	40	0.1	0.05	50	...	...	...
	1.0	0.90	90	0.5	0.39	78	0.5	0.35	70	...	...	...
Pears	0.5	0.53	106	0.3	0.32	107	0.3	0.20	67	0.3	0.24	80
	1.0	0.86	86	0.5	0.60	120	1.0	0.56	56	0.5	0.35	70
Prunes	0.4	0.45	112	0.4	0.40	100	0.4	0.35	88	0.4	0.35	88
	0.8	0.95	119	0.8	0.85	106	0.8	0.63	79	0.8	0.73	91
Grapes	0.5	0.43	86	...	...	...	0.2	0.03	15	0.1	0.08	80
	1.0	0.72	72	...	...	...	0.2	0.13	65	0.2	0.17	85

Samples of chopped fruits (100 g) were spiked with known amounts of authentic materials. Recoveries were determined by colorimetric measurement following thin-layer chromatography separation.

breakdown of chlorphenamidine has been examined in bean leaves, grapes (stems and berries), apples, pears, peaches, and prunes. The results indicate (Table V) that a breakdown of the acaricide takes place particularly in tissues which are metabolically active, such as young bean leaves. Green stems of grapes show lower dealkylation activity, and in fruit like apples and prunes there is no significant demethylation activity detectable. A hydrolytic pathway of degradation leading to small amounts of compound II apparently occurs independent of plant tissue activities due to chemical instability of the parent compound in the presence of hydrolyzing agents.

ACKNOWLEDGMENT

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