Determination of Total Residues of Chlorphenamidine [*N'*-(4-Chloro-*o*-tolyl)-*N*,*N*-dimethylformamine] in Plant and Soil Material by Colorimetry and Thin-Layer and Electron Capture Gas Chromatography

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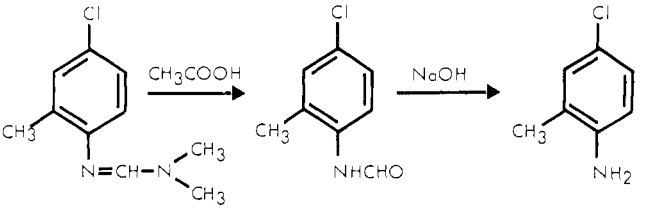
Chlorphenamidine [*N'*-(4-chloro-*o*-tolyl)-*N*,*N*-dimethylformamidine] and its potential metabolites and/or conjugates are hydrolyzed to 4-chloro-*o*-toluidine in the presence of plant and soil material by successive treatments with acetic acid and sodium hydroxide, respectively. The aromatic amine is steam distilled and extracted into isooctane. Diazotization and coupling of 4-chloro-*o*-toluidine with *N*-ethyl-1-naphthylamine yields a stable purple dye, which, after column chromatography on cellulose, is measured colorimetrically. To verify identity of chlorphenamidine, the azo-dye is separated and visualized on a cellulose thin-layer plate. Alternatively, 4-chloro-*o*-toluidine is diazotized and iodinated. The iodine derivative is determined by electron capture gas chromatography. A further potential metabolite of chlorphenamidine, 2,2'-dimethyl-4,4'-dichloroazobenzene, is reduced to 4-chloro-*o*-toluidine in the presence of zinc and acid 1,4-dioxane and likewise determined by gas chromatography. Recoveries in various plant and soil materials have been observed to vary between 75 and 100% for all three quantitative methods described.

Chlorphenamidine [*N'*-(4-chloro-*o*-tolyl)-*N*,*N*-dimethylformamidine] is a new acaricide developed by both CIBA Ltd. (Galecron) and Schering AG (Fundal). The biological effects and mode of action of this compound have been described in a series of papers by Dittrich (1966a,b, 1967a,b, 1968, 1969). Plant metabolism experiments have recently been reported by Sen Gupta and Knowles (1969). These authors observed that degradation of chlorphenamidine in and on apple seedlings proceeded at a relatively slow rate. Metabolites characterized from this plant material included *N'*-(4-chloro-*o*-tolyl)-*N*-methylformamidine, *N*-formyl-(4-chloro-*o*-toluidine), and 4-chloro-*o*-toluidine. Conjugation of the free toluidine to form *N*-(2-methyl-4-chlorophenyl)-D-glucosylamine was described as a tentative pathway.

In further experiments, Knowles *et al.* (1969) observed the formation of small amounts of the symmetrical azo derivative of 4-chloro-*o*-toluidine by *in vitro* peroxidatic catalysis. Although the authors suggested that during *in vivo* degradation of chlorphenamidine in plants and animals 4-chloro-*o*-toluidine was not present in sufficient quantities to allow the formation of 2,2'-dimethyl-4,4'-dichloroazobenzene, a sensitive residue method was required by regulatory authorities to verify the absence of the azo derivative in crop and soil material.

The chemical properties and chromatographic methods for separate determination of residues of the parent compound and of its 4-chloro-*o*-toluidine-containing metabolites are described by Kossmann *et al.* (1971). These procedures are too tedious and time-consuming for routine residue analysis of large series of plant and soil samples. Methods were therefore developed that account in a single procedure for the acaricide and for all its 4-chloro-*o*-toluidine-containing breakdown products and/or conjugates.

Chlorphenamidine (I) and its metabolites are hydrolyzed to 4-chloro-*o*-toluidine (III) by successive treatments with acetic acid and sodium hydroxide, respectively.



(I) (II) (III)

The hydrolysis product is steam distilled and extracted into isooctane by means of the distillation-extraction head originally described by Bleidner *et al.* (1954). Diazotization of 4-chloro-*o*-toluidine and coupling with *N*-ethyl-1-naphthylamine yields a stable purple dye which is determined colorimetrically. Before colorimetric measurement, interfering azo-dyes derived from aromatic plant or soil amines are removed by chromatography on a cellulose column. The colorimetric method is sensitive to 0.05 ppm as calculated for a 50-g plant or soil sample.

If required, the identity of residues of chlorphenamidine may be verified by subjecting the azo-dye of 4-chloro-*o*-toluidine to thin-layer chromatography on cellulose, instead of applying it to a column. This semiquantitative procedure is sensitive to about 0.1 ppm.

Alternatively, the hydrolysis product 4-chloro-*o*-toluidine is analyzed by a derivative/gas chromatographic technique which is based on the Sandmeyer iodination reaction (Baunok

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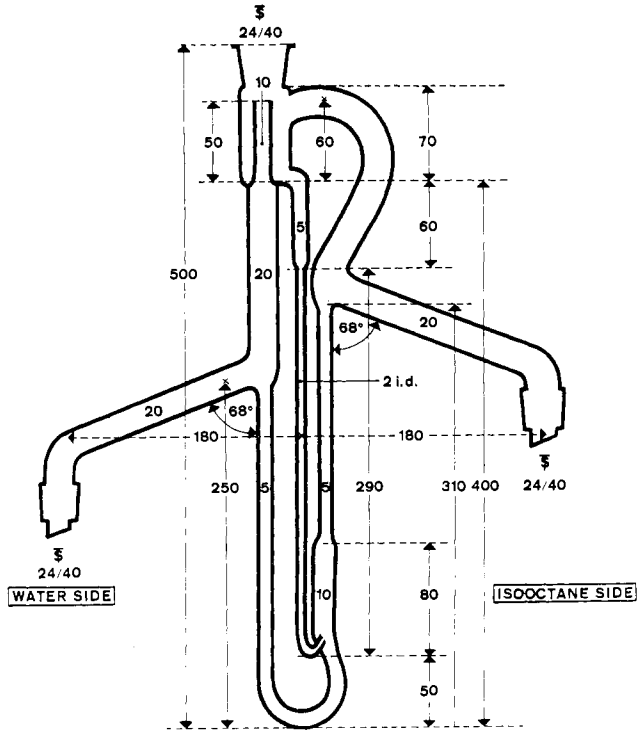
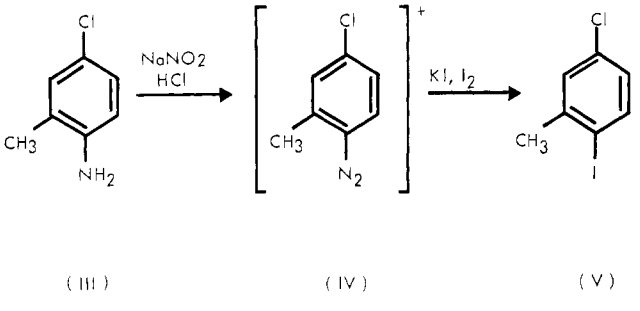


Figure 1. Dimensions (in mm) of distillation/extraction head according to Bleidner *et al.* (1954) as modified by W. Heizler of CIBA Ltd., Basle, Switzerland. Sidearm and upper vertical tube on water side were isolated with tightly wound asbestos strings to prevent cooling of vapor

and Geissbühler, 1968). In this method, the aromatic amine (III), after hydrolysis and steam distillation/extraction, is diazotized (IV) and the diazo moiety is then exchanged for iodine by potassium iodide treatment, using iodine as a catalyst.



(III) (IV) (V)

The iodinated derivative (V) is measured by electron capture gas chromatography. The method will detect 10 pg of pure standard or at least 0.05 ppm in terms of the original acaricide in soils and crops.

The principle of the methods outlined above is very similar to that of the colorimetric and gas chromatographic procedures commonly applied to urea herbicides (Baunok and Geissbühler, 1968; Bleidner *et al.*, 1954; Dalton and Pease, 1962; Geissbühler and Gross, 1967; Lowen *et al.*, 1964). However, direct alkaline hydrolysis to the aromatic amine, as described for substituted ureas, was found to be incomplete within reasonable treatment periods and was thus replaced by the two-step hydrolysis, involving the formation of *N*-formyl-(4-chloro-*o*-toluidine) (II) as an intermediate (Kossmann *et al.*, 1971). In addition, *N*-(1-naphthyl)ethylenediamine, normally used as a coupling reagent, was observed to give excessively high control (untreated sample) values with several crops, and was consequently replaced by *N*-ethyl-1-naphthylamine.

Residue analysis for 2,2′-dimethyl-4,4′-dichloroazobenzene is started by extracting the crop material with acetone and transferring the azo-derivative into *n*-hexane. After column cleanup on silica gel, 2,2′-dimethyl-4,4′-dichloroazobenzene is reduced to 4-chloro-*o*-toluidine in 1,4-dioxane by addition of zinc and hydrochloric acid. The aromatic amine is then iodinated and determined by electron capture gas chromatography, as described above. The method will detect 10 pg of 4-chloro-*o*-toluidine, or 0.01 ppm in terms of 2,2′-dimethyl-4,4′-dichloroazobenzene in crops.

EXPERIMENTAL

Apparatus. Distillation-extraction head according to Bleidner *et al.* (1954). A modified version of this apparatus, which permits more rapid operation, was designed by W. Heizler of CIBA Ltd. The dimensions of this modification are given in Figure 1.

Chromatographic columns, 40 × 2 cm i.d. with coarse porosity disk G-2, outlet vacuum adapter, and ground ⅋ 24/40 outlet joint. For preparing cellulose columns, the cellulose powder (Schleicher & Schuell No. 123) was suspended in about twice its volume of 1 *N* hydrochloric acid. The suspension was stirred or kept under vacuum to eliminate air bubbles, and was then poured into the glass column. The cellulose was allowed to settle and tightly packed to a final height of 10 cm by using vacuum and additional portions of hydrochloric acid. The column head was not allowed to run dry until the separation of azo dyes was completed.

Chromatography columns, 40 × 1.5 cm i.d. with coarse sintered disk G-2 (for cleanup of symmetrical azo-derivative of chlorphenamidine). After half-filling the tube with *n*-hexane, 10 g of silica gel (Woelm, activity grade 1) were added, and the column head was layered with 1 g of anhydrous sodium sulfate. The column was rinsed with 20 ml *n*-hexane before applying the plant extract.

GAS CHROMATOGRAPHY. The apparatus used was a Model 402 F&M High Efficiency Gas Chromatograph equipped with a pulsed-type tritium electron capture detector. The 1200 × 3 mm i.d. borosilicate glass column was packed with 10% QF-1 on 80–100 mesh Gas Chrom Q, and was maintained as follows: oven 120° C, isothermal; inlet 125° C; detector 200° C; pulse interval 150 μsec.; range × attenuation 10 × 64 (128); purge gas 5% methane in argon at 37 ml/min. The carrier gas was 5% methane in argon at 23 ml/min.

Chemicals and Reagents. Organic solvents were reagent (nano-) grade or redistilled in an all glass apparatus.

N-Ethyl-1-naphthylamine, reagent grade (Fluka AG, Buchs, Switzerland, or Eastman Organic Chemicals, Rochester, N.Y.). The 2% ethanolic solution prepared was kept for no more than 3 days.

Potassium iodide/iodine solution: 2.5 g iodine were dissolved in 50 ml of a 10% aqueous potassium iodide solution. The solution was replaced every second day.

STANDARDS. Chlorphenamidine was dissolved in ethanol (100 μg/ml). For recovery experiments, the solution was further diluted with the same solvent. The stock solution of 4-chloro-*o*-toluidine was prepared by dissolving 10 mg of the aromatic amine in 5 ml ethanol, and diluting with 1 *N* hydrochloric acid to 100 ml (100 μg/ml). This stock solution was further diluted with 1 *N* hydrochloric acid for establishing colorimetric and gas chromatographic calibration curves.

The symmetrical azo derivative, 2,2′-dimethyl-4,4′-dichloroazobenzene, was dissolved in benzene (100 μg/ml) and further diluted with the same solvent for recovery experiments and establishing calibration curves.

Cellulose thin-layer plates, 20 × 20 cm, thickness of layer 250 μ, cellulose powder for tlc MN 300 without binder (Macherey, Nagel & Co., 516 Düren, Germany).

Nopco NXZ liquid antifoam (Nopco Chemical Company, Newark, N.J.).

Hydrolysis, Steam Distillation/Extraction. The entire fresh or frozen laboratory crop sample (1 to 2 kg) was finely chopped with a Hobart- or Bauknecht-type food chopper and mixed thoroughly. A 50-g aliquot was placed into a 1 l. heavy duty Pyrex round-bottomed flask, together with several boiling chips, 150 ml distilled water, 50 ml 2 *N* acetic acid, 6 g sodium acetate, and 3 ml of Nopco antifoam solution (pH of the mixture = 5.0 ± 0.2). The flask was attached to a water-cooled reflux condenser, and the mixture was heated for 90 min with an oil bath (170–180° C) or an electrical heating device of similar heating capacity. With the condenser still in place, the hydrolyzate was allowed to cool at room temperature for 15 min, then the flask was immersed into an ice bath for an additional 10 min.

Meanwhile the distillation-extraction head was prepared as follows. 100 ml of isooctane were placed into a 250-ml round-bottomed flask and attached to the upper arm (Fig. 1). The U-tube was first half-filled with distilled water and then with isooctane (this procedure prevented organic solvent from flowing into the digestion flask). The condenser was moved from the digestion flask to the distillation-extraction head. After adding 80 ml of 10 *N* sodium hydroxide to the acid hydrolyzate, the digestion flask was immediately connected to the lower arm. Operation was started by heating both flasks at such a rate that equal amounts of water and isooctane were condensed (this can be judged by the size of the solvent globules which are constantly formed in the feeding capillary). Alkaline hydrolysis and steam distillation/extraction were continued for 150 min.

Colorimetric Method. After completion of the distillation/extraction process, the isooctane solution was allowed to cool to room temperature and then transferred to a 250-ml separatory funnel, followed by rinsing the round-bottomed flask with several small volumes of organic solvent. The isooctane was extracted with three 10-ml portions of 1 *N* hydrochloric acid by vigorously shaking the separatory funnel for at least 2 min during each extraction. The aqueous extracts were collected in a 50-ml glass- or Teflon-stoppered volumetric flask.

For diazotization, 2 ml of a 1% sodium nitrite solution were added to the acid-extract. The latter was swirled and allowed to stand for 10 min. Excess nitrite was then destroyed by adding, under vigorous shaking, 2 ml of a 10% sulfamic acid solution. The flask was allowed to stand for a further 5 min and occasionally shaken to insure complete destruction of nitrite.

To the diazonium salt solution were added 2 ml of the 2% *N*-ethyl-1-naphthylamine reagent. For subsequent maximum color development, 4 *M* aqueous sodium acetate was added until the solution turned orange and milky (from 4 to 8 ml of the acetate were required and the pH of the reaction mixture was about 3.5). The suspension was allowed to stand for 5 min. The azo-dye solution was then clarified and changed to a red-purple color by acidification with 2 ml of 5 *N* hydrochloric acid.

For chromatographic separation the dye solution was brought onto the cellulose column by carefully delivering it along the glass wall from a pipette. The solution was drained into the column by applying vacuum. The volumetric flask and the wall of the glass column were rinsed with small portions of a 1 *N* hydrochloric acid + glacial acetic acid (85 + 15 v/v) mixture. The dyes were now located in the top third of the cellulose column. Interferences were removed by passing through the column 100 ml of the 1 *N* hydrochloric acid + glacial acetic acid (85 + 15) mixture at a rate of about 10 ml per min (additional volumes of acid mixture ranging from 20 to 100 ml were sometimes required to remove all interfering azo-dyes derived from certain crop materials). Elution of the azo-dye of 4-chloro-*o*-toluidine, by now located approximately half-way on the column, was started by passing a 1 *N* hydrochloric acid + glacial acetic acid (1 + 2 v/v) mixture. When the front of the dye-band just reached the bottom of the column, the receiver attached to the outlet joint was changed. The toluidine dye was completely eluted with no more than 30 ml of the second acid mixture and collected in the same 50-ml volumetric flask which had been used for diazotization and coupling. The flask was filled up to the mark with glacial acetic acid. Any turbidity observed after column elution disappeared upon addition of this acid. The dye solution was observed to be stable for at least 2 hr.

Absorbance of the dye solutions was measured in a photoelectric colorimeter or spectrophotometer at 535 nm, using distilled water as a reference. The length of the light path of the measuring cells was 40 mm. The absorbance values were compared with a calibration curve established as described below. For calculating residues in terms of unchanged chlorphenamidine, the determined quantities of 4-chloro-*o*-toluidine were multiplied by a factor of 1.39.

The calibration curve was established and frequently verified by subjecting graded amounts of 4-chloro-*o*-toluidine (2 to 40 μg) to the diazotization, coupling, and chromatographic procedures described above. The desired quantities of the aromatic amine were prepared in no more than 30 ml of 1 *N* hydrochloric acid by appropriate dilution of the standard solution. Absorbance values were plotted *vs.* μg 4-chloro-*o*-toluidine on a linear scale. The curve followed Lambert-Beer's law within the range indicated, and corresponded to an absorption coefficient ϵ of 3.96×10^4 l. × mol^{-1} × cm^{-1}. Residues higher than 1 ppm chlorphenamidine were measured in a 1-cm cell. In those isolated analyses where the dye precipitated, the entire procedure was repeated with a smaller aliquot (5 to 10 g) of crop material.

Confirmatory Thin-Layer Chromatography. Diazotization and coupling of 4-chloro-*o*-toluidine were carried out as described in detail above. Instead of applying the acidified dye solution to a cellulose column, it was transferred to a 50-ml separatory funnel and extracted with 10 ml of diethyl ether. The extraction was repeated once with 5 ml of the same solvent. The ether phases were collected in a 25-ml Erlenmeyer flask and dried with 3–4 g of anhydrous sodium sulfate. The dried solution was decanted into a 15-ml tapered test tube, and the sodium sulfate rinsed with small portions of ether. The solvent was completely evaporated in a gentle stream of air, and the dye residue was taken up in 0.5 ml of acetone.

An aliquot of 5 μl of the acetone concentrate (representing 0.5 g of crop or soil material) was spotted on the starting line of a cellulose thin-layer plate. Acetone concentrates derived from control (untreated) material and appropriate standards of the 4-chloro-*o*-toluidine dye were also applied to the plate

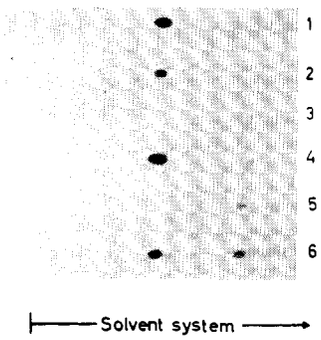


Figure 2. Thin-layer separation on cellulose of azo-dyes of 4-chloro-*o*-toluidine and of distillates derived from untreated and chlorphenamidine containing apples and orange pulp. Solvent system: dimethylformamide-1 *N* hydrochloric acid-ethanol (40:50:10). 1 = 0.5 μg 4-chloro-*o*-toluidine; 2 = 0.1 μg 4-chloro-*o*-toluidine; 3 = untreated orange pulp; 4 = orange pulp containing 1 ppm of chlorphenamidine; 5 = untreated apples; 6 = apples containing 0.1 ppm of chlorphenamidine

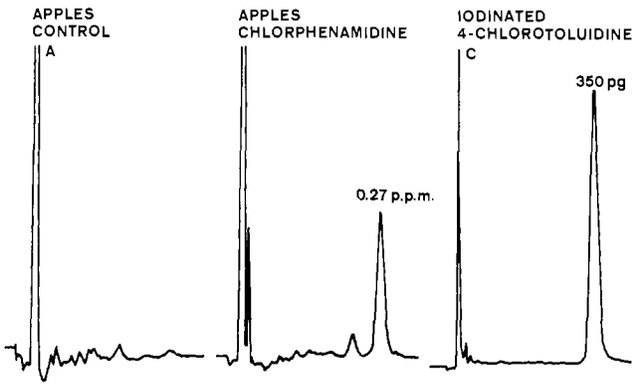


Figure 3. Electron capture gas chromatographic analysis of total chlorphenamidine residues by iodine derivatization of 4-chloro-*o*-toluidine. A = untreated apples; B = apples containing 0.27 ppm chlorphenamidine; C = standard of iodinated 4-chloro-*o*-toluidine

(Figure 2). The plate was developed for about 15 cm (3 hr) in a saturated tank with the following solvent system: dimethylformamide—1 *N* hydrochloric acid-ethanol (40:50:10). After development, the plate was removed from the tank and dried at room temperature. The R_f value of the purple 4-chloro-*o*-toluidine dye was 0.43–0.46.

Semiquantitative estimation of the amount of 4-chloro-*o*-toluidine residue was made by comparing spot size and intensity with appropriate standards. These standards, which ranged from 0.04 to 1 μg 4-chloro-*o*-toluidine, were prepared as follows: 100 μg of the aromatic amine in 30 ml 1 *N* hydrochloric acid were subjected to diazotization, coupling, and extraction as described above. From the final 0.5 ml acetone concentrate, graded volumes (0.2–5 μl) were applied to the thin-layer plate.

Gas Chromatographic Method. A 20-ml aliquot of the isooctane extract, normally representing 10 g of crop or soil material, was placed into a 50-ml separatory funnel. The solvent was extracted with two 5-ml portions of 1 *N* hydrochloric acid by vigorous shaking. The acid extracts were collected in a glass- or Teflon-stoppered 50-ml Erlenmeyer flask and cooled to 0° C in an ice bath. After adding 2 ml of 1% aqueous sodium nitrite, the mixture was allowed to stand at 0° C for 25 min. Excess nitrite was then destroyed by adding 2 ml of 10% aqueous sulfamic acid. The flask was vigorously shaken until nitrogen generation stopped.

To the ice-cold diazonium salt solution, 0.5 ml of potassium iodide/iodine solution was added, and the mixture was allowed to warm up to room temperature and to stand for 25 min under these conditions. It was then transferred to a room-temperature water bath which was heated to boiling within 15 min. The stoppers were periodically loosened to prevent excess pressure. After 5 min in boiling water, the flask was removed from the bath and allowed to cool to room temperature. Excess iodine was reduced by adding about 200 mg of sodium sulfite powder, whereupon the solution turned colorless. The reaction mixture was made alkaline with 1.1 ml of 10 *N* sodium hydroxide solution, transferred to a 50-ml separatory funnel, and extracted with 20 ml of *n*-hexane. After vigorous shaking, the aqueous phase was removed, and a few milliliters of the upper organic phase were transferred to a 10-ml screw-capped vial with a pipette.

Sporadically, interferences showed up during subsequent gas chromatography when injecting the hexane solutions as prepared above. These hexane extracts were further cleaned by shaking them with about the same volume of 1% potassium permanganate in 1 *N* sodium hydroxide solution. The mixture was allowed to stand for 1 to 2 hr before agitating it again and transferring the hexane to a clean 10-ml vial.

From each hexane solution a 1–5 μl portion was injected into the gas chromatograph. The peak height was measured and compared with the calibration curve prepared as described below. For accurate determinations, a number of standard solutions of approximately the same concentration were injected with each series of residue samples. By this procedure possible slight variations in the sensitivity of the method were eliminated. Hexane solutions derived from samples containing more than about 1.3 ppm in terms of the original acaricide were diluted with appropriate volumes of the same solvent.

Under the gas chromatographic conditions described above, the iodine derivative of 4-chloro-*o*-toluidine had a retention time of 4 min, whereby a 200 pg equivalent was found to give a response of 50% full scale deflection on the recorder (Figure 3).

For establishing the calibration curve, a standard solution of 4-chloro-*o*-toluidine in 1 *N* hydrochloric acid was prepared (1 μg/ml). A 10-ml aliquot of this solution was subjected to diazotization and iodination as described above. The final hexane solution was diluted to permit injection of 4-chloro-*o*-toluidine equivalents ranging from 20 to 500 pg. The peak heights were plotted *vs.* ng 4-chloro-*o*-toluidine on a log-log scale. The curve was found to be linear within the range indicated and followed the equation $\log h = 1.05 \log m + 1.778$ (h = peak height in cm, m = quantity of 4-chloro-*o*-toluidine in ng).

Analysis of 2,2′-Dimethyl-4,4′-Dichloroazobenzene. The entire fresh or frozen laboratory crop sample (1 to 2 kg) was chopped with a food chopper and mixed thoroughly. A 50-g aliquot of the mashed material was placed into the jar of the Omni-Mixer. After adding 100 ml of acetone, the mixture was macerated at high speed for 3 to 5 min. The resulting suspension was filtered through a 6-cm Buchner funnel (layered with Schleicher & Schuell "sharkskin" filter paper) by using slight vacuum. The filtrate was collected in a 250-ml pear shaped flask. The Omni-Mixer jar and the filter cake were rinsed with two 25-ml portions of acetone. The organic solvent was completely removed from the filtrate on a rotating-type evaporator (45° C). The aqueous residue was transferred to a 100-ml separatory funnel

and extracted with three 50-ml portions of *n*-hexane (the first two portions were used for rinsing the evaporation flask before extraction). The organic extracts were drained into a 250-ml Erlenmeyer flask and dried with 2 to 3 g of anhydrous sodium sulfate. The hexane solution was decanted into a 250-ml pear shaped flask. After rinsing the sodium sulfate with small portions of the same solvent, the volume of the organic solution was reduced to about 5 ml on the rotating-type evaporator (50° C).

The hexane concentrate was carefully applied to the silica gel column with a 10-ml pipette. After draining the solution into the column, the latter was rinsed by slowly passing 20 ml of *n*-hexane. The symmetrical azo-derivative was then eluted from the column with 100 ml of benzene which was percolated at the rate of 3 ml per min.

The benzene eluate was collected in a 150-ml pear shaped flask and concentrated to about 10 ml on the rotating-type evaporator (50° C). After diluting the benzene concentrate with 20 ml of 1,4-dioxane, the volume of the solvent mixture was reduced to approximately 20 ml.

For reduction of the symmetrical azo-derivative, 2 to 3 g of granular zinc and 2 ml of concentrated hydrochloric acid were added to the solvent solution. The flask was attached to a reflux condenser and heated for 30 min on a water bath at 90–95° C. The flask was removed from the water bath and, after addition of 10 ml of distilled water, the volume of the solution was reduced to about 10 ml on the rotating-type evaporator (70° C). To completely remove traces of 1,4-dioxane, a second 10-ml portion of water was added and the concentration process was repeated. The 10-ml aqueous acid concentrate, which now contained 4-chloro-*o*-toluidine, was transferred to a glass- or Teflon-stoppered 50-ml volumetric flask by using several small rinsings of 1 *N* hydrochloric acid.

Diazotization, iodination, and gas chromatography of the iodinated derivative of 4-chloro-*o*-toluidine were carried out according to the procedure described in detail above. To secure a sufficient supply of iodine, the volume of the potassium iodide/iodine solution was increased from 0.5 to 1 ml.

To prepare the standard curve, 10 ml of benzene containing 10 μg of 2,2′-dimethyl-4,4′-dichloroazobenzene were diluted with 20 ml of 1,4-dioxane and the symmetrical azo-derivative was subjected to the reduction procedure described above. The final hexane solution (20 ml) obtained after diazotization/iodination was diluted to permit injection into the gas chromatograph of 2,2′-dimethyl-4,4′-dichloroazobenzene equivalents ranging from 20 to 500 pg in no more than 5 μl of solvent. The peak heights were plotted *vs.* pg of the symmetrical azo-derivative on a log-log scale. With each series of test samples a number of standards of approximately the same concentration were injected to eliminate errors due to possible slight variations in the sensitivity of the method.

RESULTS

Colorimetric Method. The colorimetric method for determining total residues of chlorophenamidine has been used rountinely for the past 4 yr in several industrial and university laboratories. Its application covered a variety of fruit and vegetable crops, including apples, pears, plums, peaches, cherries, grapes, oranges, strawberries, tomatoes, cabbage, cauliflower, and broccoli. In addition, the method was used for determining dissipation of total chlorphenamidine residues in soil under field conditions. Typical control (untreated sample) values are summarized in Table I. These control values were observed to be below 0.1 ppm for most of the fruits and vegetables analyzed, but were higher than 0.1 ppm for a number of the soils investigated. The standard deviation of the control values of a particular crop variety or type of soil was no higher and usually less than 0.05 ppm. Consequently, when calculating the limit of determination according to Frehse and Niessen (1963), by multiplying the standard deviation with a factor of 1.5, the sensitivity of the method was found to vary between 0.05 and 0.07 ppm. In those series of analyses in which the standard deviation of the controls was 0.02 ppm or less, the sensitivity of the method was determined by the smallest amount of 4-chloro-*o*-toluidine that was to be measured with reasonable accuracy. This amount was about 2 μg, which corresponded to 0.05 ppm in terms of unchanged chlorphenamidine in a 50-g crop sample.

Recovery values observed after addition of known quantities of chlorphenamidine or 4-chloro-*o*-toluidine to the initial hydrolysis mixture are summarized in Table II. This table demonstrates that, within the range of residue concentrations examined (0.1 to 2 ppm), the average recoveries were 85% or more for all types of crops and soils investigated. During the course of many hundreds of analyses, recovery values were regularly above 75%.

Table I. Apparent Total Chlorphenamidine Residues Determined by Colorimetric Method in Control (Untreated) Samples of Fruits, Vegetables, and Soils

Type of Crop[a]/Soil	Number of Analyses	Average Apparent Residue, ppm	Standard Deviation, ppm
Apples	15	0.07	0.03
Pears	6	0.03	<0.01
Peaches	8	0.07	0.02
Plums	8	0.05	0.01
Strawberries	5	0.03	<0.01
Oranges, peel	15	0.08	0.05
Oranges, pulp	15	0.05	0.02
Tomatoes	4	0.04	0.02
Cabbage	10	0.05	0.04
Broccoli	10	0.05	0.03
Sandy soil	6	0.30	0.02
Clay loam	6	0.21	0.02

[a] Values obtained with a single crop variety.

Table II. Results of Recovery Experiments Conducted with Colorimetric Method for Determining Total Chlorphenamidine Residues

Type of Crop/Soil	Range of Chlorphenamidine Concentrations Added, ppm	Number of Analyses	Chlorphenamidine Recovered,[a] %
Apples	0.1–2.0	15	95 ± 8
Pears	0.2–1.0	8	94 ± 5
Peaches	0.1, 1.0	4	97 ± 9
Plums	0.2, 2.0	6	92 ± 5
Strawberries	0.4–2.0	6	93 ± 5
Oranges, peel	0.1–2.0	12	92 ± 8
Oranges, pulp	0.1–2.0	12	94 ± 7
Tomatoes	0.2, 0.4	4	87 ± 8
Cabbage	0.2, 2.0	8	97 ± 7
Broccoli	0.2, 2.0	4	101 ± 5
Sandy soil	0.2, 1.0	4	98 ± 6
Clay loam	0.2, 1.0	4	89 ± 3

[a] After deduction of mean control values.

Table III. Recoveries of Chlorphenamidine from Fortified Fruit Samples After Application of Electron Capture Gas Chromatographic Residue Method

Commodity	Chlorphenamidine Concentrations Added, ppm	Number of Analyses	Chlorphenamidine Recovered, %
Apples	0.2, 0.5, 1.0	6	105 ± 10
Pears	0.1, 1.0	4	97 ± 4
Peaches	0.2, 1.0	4	99 ± 5
Plums	0.2, 1.0, 2.0	6	87 ± 6

Table IV. Comparison of Total Chlorphenamidine Residues in Weathered Field Samples of Plums Determined by Gas Chromatographic and Colorimetric Analysis

Plum samples collected from a Swiss orchard at different time intervals after application of Galecron. Spraying concentration = 0.05% a.i. Samples analyzed twice by each method

Time Interval Days	Total Residue, ppm gc Method	Total Residue, ppm Colorimetric Method
	2.2	2.1
20	2.1	2.4
	0.9	0.8
40	0.8	0.8
	0.6	0.5
75	0.5	0.4

Table V. Recoveries of 2,2′-Dimethyl-4,4′-dichloroazobenzene from Fortified Leaf and Fruit Samples After Application of Electron Capture Gas Chromatographic Residue Method

Commodity	2,2′-Dimethyl-4,4′-dichloroazobenzene Added, ppm	Number of Analyses	Symmetrical Azo-Derivative Recovered %
Apple Fruits	0.1, 1.0	6	86 ± 6
Leaves	0.1, 1.0	4	78 ± 4
Pears	0.1, 1.0	4	82 ± 7
Oranges	0.1, 1.0	4	89 ± 6

The following two operations were found to be the main sources of error or loss during residue analyses and recovery experiments if not carried out properly according to the described procedure: too high or too low acidity of the original hydrolysis mixture by insufficient buffering with sodium acetate; incomplete extraction of 4-chloro-*o*-toluidine from isooctane by not vigorously shaking for at least 2 min the organic solvent with hydrochloric acid. In addition, it was found advantageous to run standards of the azo-dye of 4-chloro-*o*-toluidine side by side with unknowns during column chromatography on cellulose until sufficient experience was gained in the control of the various dyes to be separated and eluted from the column.

Confirmatory Thin-Layer Chromatography. A typical cellulose thin-layer plate obtained upon separation of the azo-dye of 4-chloro-*o*-toluidine from crop interferences is shown in Figure 2. The intense purple dye was easily located on the plate not only by its R_f value but also by its color which differed from that of the carmine or blue interferences. The minimum quantity of 4-chloro-*o*-toluidine that was visualized without difficulty on the plate was determined to be 0.03 to 0.04 μ. Thus, when concentrating the samples to 0.5 ml of acetone and spotting 5 μl of this concentrate (corresponding to 0.5 g of plant or soil material), the sensitivity of the thin-layer method was about 0.1 ppm in terms of the original acaricide. Depending on the quantity of coupled 4-chloro-*o*-toluidine present, the spots exhibited more or less pronounced tailing (see Figure 2). Therefore, the smallest volume possible which still produced visible spots was applied.

Gas Chromatographic Method. Application of the electron capture gas chromatographic method has so far been limited to fruit crops, including apples, pears, peaches, and plums. Gas chromatograms obtained from numerous control samples of these fruits did not show any significant false peaks (peak height <0.5 cm) interfering with that of the iodinated derivative of 4-chloro-*o*-toluidine. Typical chromatograms of untreated and chlorphenamidine containing apple samples are shown in Figure 3. The sensitivity of the method was found to be limited by the smallest quantity of 4-chloro-*o*-toluidine that could be diazotized and iodinated with reasonable reproducibility. This amount was determined to be 0.3 to 0.5 μg of the aromatic amine. Therefore, when subjecting an aliquot of the distillate representing 10 g of plant material to diazotization/iodination, the limit of detection of the method was 0.05 ppm, expressed as chlorphenamidine. If required, the detectability may easily be increased by extracting a larger aliquot of the original isooctane solution obtained after distillation.

The data presented in Table III demonstrate that recoveries determined by the gas chromatographic method were as satisfactory as those observed with the colorimetric procedure. Results of the two methods agreed well as shown by Table IV, which lists residue values obtained by both colorimetry and gas chromatography when analyzing weathered, chlorphenamidine-treated plum samples collected during a field experiment.

Analysis of 2,2′-Dimethyl-4,4′-dichloroazobenzene. Residue analysis of the symmetrical azo-derivative of chlorphenamidine was first attempted by direct gas chromatography of the compound without prior reduction to 4-chloro-*o*-toluidine. Unfortunately, response of the electron capture detector to the unchanged azo-compound was observed to be too weak to reach a sensitivity of 0.01 to 0.02 ppm without exhaustive and cumbersome cleanup of plant extracts. With the reduction procedure finally adopted, free 4-chloro-*o*-toluidine or other metabolites reduced to this compound by treatment with zinc in acid 1,4-dioxane might potentially interfere with the determination of the symmetrical azo-derivative. However, when chlorphenamidine and its potential metabolites, including 4-chloro-*o*-toluidine, were added to the original plant (acetone) extract, they were found not to be eluted from silica gel, together with 2,2′-dimethyl-4,4′-dichloroazobenzene under the column elution conditions chosen. Furthermore, a large number of apple leaf and fruit samples which had been treated in the field with overdoses of chlorphenamidine and thus contained high total residues of the acaricide (leaves >200 ppm; fruits 5–10 ppm) were submitted to the procedure described for the symmetrical azo derivative. None of these samples showed any significant residues of the latter compound.

As with the gas chromatographic procedure described for total residues, the sensitivity of the method used for measuring 2,2′-dimethyl-4,4′-dichloroazobenzene was limited by the smallest quantity of 4-chloro-*o*-toluidine that was to be diazotized and iodinated with reasonable reproducibility. When this quantity is taken to be 0.5 μg, the limit of detection for the symmetrical azo-derivative is 0.01 ppm in a 50-g plant sample.

Application of the residue method for 2,2′-dimethyl-4,4′-dichloroazobenzene has so far been limited to leaves of apple trees and to apple, pear, and orange fruits. Table V shows

that recoveries were higher than 75% within the range of concentrations examined.

DISCUSSION

The present residue methods for chlorphenamidine rely on hydrolysis of the acaricide to 4-chloro-*o*-toluidine without prior extraction of the crop or soil material. They therefore account for all metabolites and the only conjugate which so far has been indentified or tentatively characterized in plants (Sen Gupta and Knowles, 1969; Kossmann *et al.*, 1971). For regulatory purposes, the procedures thus measure the total residue of chlorphenamidine to be expected in edible crops, forage, and soil.

In actual spraying practice, commercial preparations contain either the free base or the hydrochloride of chlorphenamidine. Systematic recovery experiments have demonstrated that the described methods are applicable to residues derived from both types of active material.

For routine residue analysis of samples of known spraying history, the colorimetric method has been found to be entirely adequate. The procedure even has a certain specificity, since azo dyes of aromatic amines derived from a number of structurally similar pesticides (diuron, monuron, fluometuron, metobromuron, propanil, CIPC, dichloran) have been demonstrated not to interfere with colorimetric determinations owing to differing adsorptive behavior of these dyes on the cellulose column. However, if specific detection of chlorphenamidine residues in samples of ill-defined or undetermined origin and spraying history is required, the gas chromatographic procedure is preferred. This procedure will distinctly separate 4-chloro-*o*-toluidine from a large number of similar or related aromatic amines which are potential hydrolysis products of pesticides. For verifying the identity of residues determined by either the colorimetric or gas chromatographic procedure, thin-layer chromatography of the azo-dye of 4-chloro-*o*-toluidine has been observed to be a satisfactory alternative. To speed up analyses, it is recommended to divide the isooctane extract obtained after hydrolysis and steam distillation/extraction of important or critical samples into suitable aliquots. These aliquots are then subjected separately to any two of the three procedures described.

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