A Mircoanalytical Method for 4-Aminopyridine in Corn Plant Tissues

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INTRODUCTION

Agriculturists have long sought simple, inexpensive means of protecting low-profit crops such as corn from the severe damage often caused by blackbirds. One promising method is the use of cracked corn baits impregnated with the toxicant 4-aminopyridine (Avitrol 200*) (1). Because only a few blackbirds need to be affected by the toxicant to disperse the flock, the treated corn is thinly broadcast in cornfields at the rate of only about 15 gm per acre (2,3). A very desirable feature of this treatment is that the growing corn itself is not treated with 4-aminopyridine, which greatly minimizes the possibility of residues accumulating on or in the plants. However, the compound is freely soluble in water, and it could possibly be leached from treated cracked corn by rainfall and be absorbed by the growing plants. Because 4-aminopyridine is highly toxic to mammals as well as to birds, this possibility necessitated the development of an analytical method to determine residues in corn plant tissues at levels as low as 0.01 ppm.

EXPERIMENTAL

The developed analytical method for 4-aminopyridine was tested on about 80 pounds of nearly mature, untreated corn plants. These plants were prepared as described under "Sample Preparation," and the resulting large mass of homogeneous, dry, finely ground pseudo-sample material was used in determining the effeciency of extraction techniques by liquid scintillation counting of carbon-14 tagged 4-aminopyridine, and the reproducibility of analytical results by spiking and recovery.

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Since most of the materials extracted by acetonitrile are easily eliminated by simple liquid-liquid partitioning, this solvent was selected. Several polar solvents such as methanol, ethanol, acetone, and acetonitrile proved very efficient in Soxhlet extraction of 4-aminopyridine from spiked pseudo-samples. However, the alcohols and acetone removed so much additional material that clean-up would have been difficult or impossible.

Although Soxhlet extraction was very efficient, it had to be abandoned because of the instability of 4-aminopyridine in the presence of corn extractives. Liquid scintillation counting of carbon-14 tagged material showed that Soxhlet extraction with ammoniacal acetonitrile removed essentially 100% of the radioactive materials but destroyed 99.7% of the 4-aminopyridine, determined by recounting the sample extract following liquid-liquid partition clean-up. The extraction method adopted, a single "shake-out" extraction with acetonitrile, gives less than perfect recovery but does not require heat and prolonged extraction times. With this method, however, the distribution of 4-aminopyridine between extracting solvent and finely divided sample material is influenced to some degree by the amount of moisture present in the air-dried sample and by the average particle size of the sample material. Therefore, two samples must be run, the second one overspiked by about the amount present in the unspiked sample, so that extraction efficiency can be determined and compensated for.

Microcoulometry was found to be the most reliable means for quantitating 4-aminopyridine at low levels. With the proper choice of a gas chromatograph column, derivatization is unnecessary; and since the nitrogen content of 4-aminopyridine is nearly 30%, as little as 50 ng may be estimated reliably. The original intent was to utilize the great sensitivity of the electron capture detector to sense a chlorine-containing derivative of 4-aminopyridine (N-(4-pyridyl)-dichloroacetamide, easily produced by reacting with dichloroacetyl chloride). As little as 0.1 ng of derivatized 4-aminopyridine was readily detectable by this means, and with pure standards the derivative could be produced and chromatographed very reproducibly. Electron capture proved unsuitable for quantitation, however, because even after a final TLC clean-up, enough impurities of plant origin remained so that the degree of acetylation of 4-aminopyridine was intolerably variable. Therefore, this detection means is used only for identity confirmation (Figure 1).

Recovery achieved by this analytical method was tested by spiking aliquots of the pseudo-sample material with 0.01, 0.05, 0.10, and 0.50 ppm of 4-aminopyridine and analyzing in replicate; the results are shown in Table 1. It is obvious that the recoveries measured at the lowest spiking level (0.01 ppm) are somewhat high.

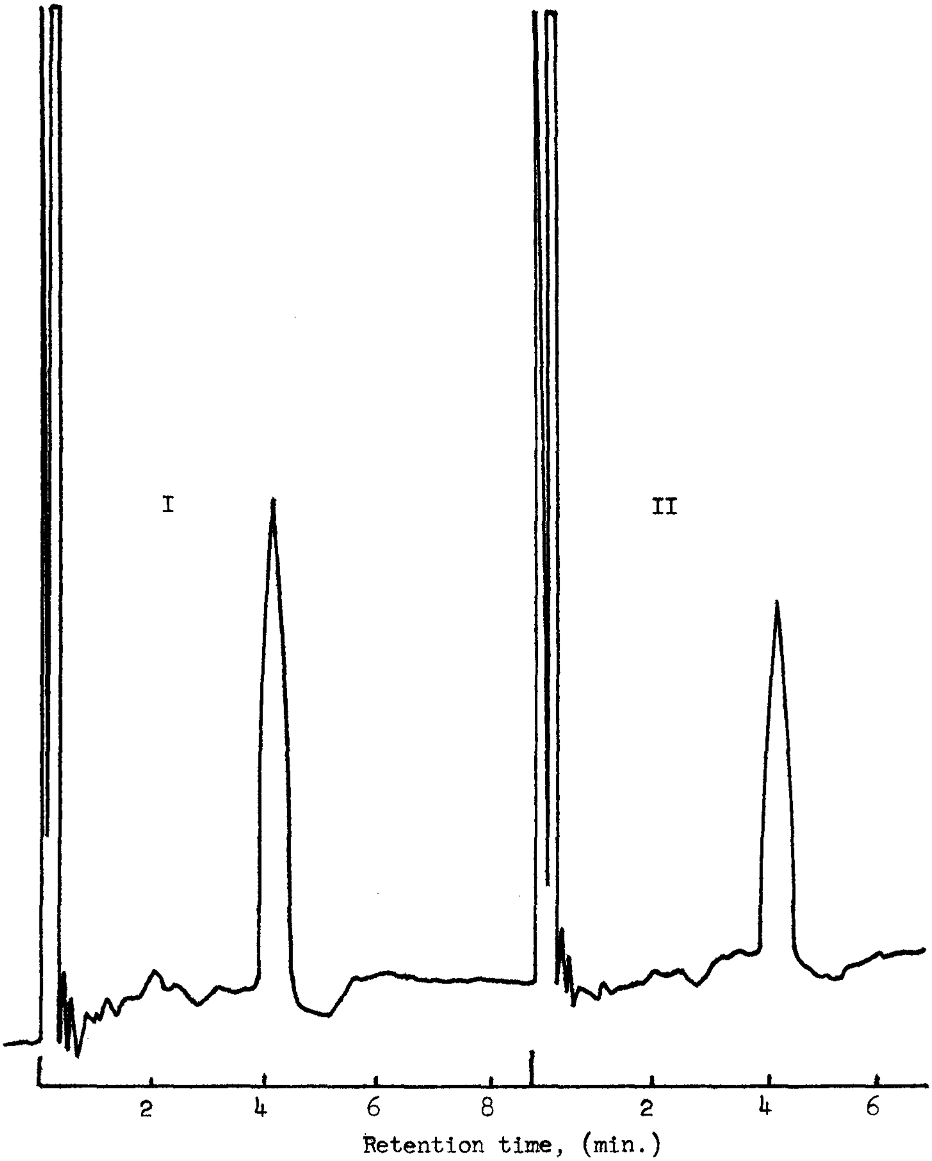

Figure 1. Gas chromatograms of derivatized 4-aminopyridine standard (I) and derivatized purified residue of actual sample-derived 4-aminopyridine (II). (The sample had contained approximately 0.05 ppm of 4-aminopyridine.)

TABLE 1

Spike Level	Overall Recovery, %*	Mean of 5 Analyses, %*
0.01 ppm	39, 53, 55, 60, 62	54
0.05 ppm	38, 39, 43, 46, 51	43
0.10 ppm	40, 47, 50, 52, 54	48
0.50 ppm	48, 49, 50, 51, 53	50

* All values rounded to nearest percent.

This is explained, as noted on the "blank" sample chromatogram (Figure 2a.), by the presence of residual interfering materials that contribute to the area response of 4-aminopyridine when large samples (10 gm equivalent) are chromatographed. At the higher spiking level of 0.05 ppm and higher, the contribution of this spurious response becomes unnoticeable, and "reference-like" chromatograms are produced (Figures 2b., 2c.).

Table 1 shows also, that regardless of the level at which the samples are spiked, the average recovery is very nearly 50%. Testing with carbon-14 tagged 4-aminopyridine showed that 58.8% of the material is removed by the shake-out extraction method. When the loss due to the liquid-liquid partition (11%) is subtracted, the maximum quantity remaining would be slightly greater than 52%. Thus there are almost no unaccountable losses in clean-up and chromatography as sample concentrations reach 0.10 ppm and higher.

METHOD

Sample Preparation

Chop the plant or plants selected as a representative sample with a food chopper such as Hobart Model 8181D. Weigh approximately 1 kg of the resultant mixture, spread in a thin layer on wax paper, and air-dry to constant weight in a hood at room temperature. Calculate the water content so that "fresh weight" sample equivalent aliquots can be weighed. When the sample is dry, rough grind in a Waring-type blender, then finish with a Wiley-type mill fitted with a 40-mesh screen. (Large mature plants that contain much tough fibrous material can first be milled to smaller particles with a 20-mesh screen.) Commonly, a 100 gm aliquot (fresh weight equivalent) of the resulting finely ground sample will weigh 10-16 gm, depending on the maturity of the plants.

Extraction

Place sample aliquot (100 gm fresh weight equivalent) in a glass-stoppered 250 ml Erlenmeyer flask. Add 150 ml of extraction solvent consisting of 144 ml of acetonitrile, 3 ml of water, and 3 ml of concentrated NH_4OH (29%). Stopper the flask and

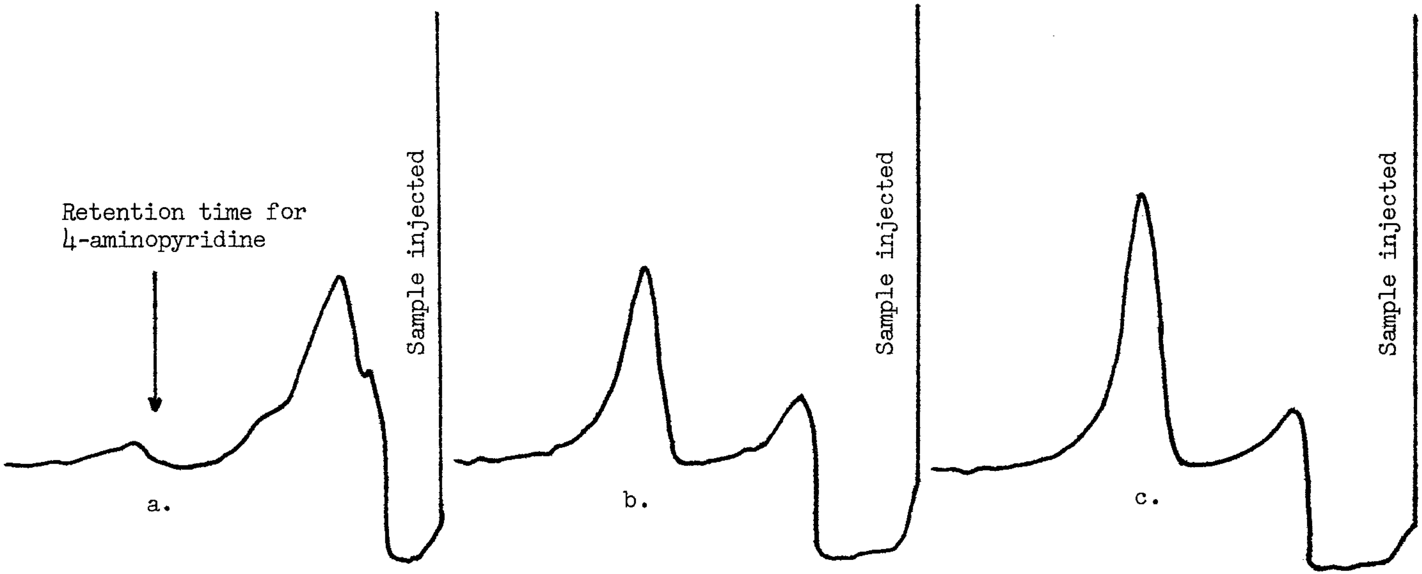

Figure 2a., 2b., 2c.
a. Gas chromatogram of sample "blank" (10 gm sample equivalent injected).
b. Gas chromatogram of sample spiked with 4-aminopyridine at 0.10 ppm (2.5 gm sample equivalent).
c. Gas chromatogram of 4-aminopyridine reference standard, 225 ng.

shake vigorously for 20 minutes. Decant and filter 75.0 ml of the supernatant, collecting the filtrate in a 50 X 90 mm evaporating dish. This volume now represents 50.0 gm of starting sample. Reduce the sample almost to dryness on a warm hot plate (ca. 60° C) in a hood in a strong current of air. As the sample reaches near-dryness, reduce the hot plate temperature to about 40° C. Continue drying until all water and acetonitrile have evaporated.

Liquid-Liquid Partition

Transfer to a 250 ml separatory funnel all of the residue that will dissolve in 10 ml of n-butyl acetate (redistilled). Allow dish to dry and transfer additional residue to the funnel with 10 ml of N/10 HCl. Again allow dish to dry and transfer remaining residue with an additional 10 ml of n-butyl acetate followed by a final rinse with 8 ml of N/10 HCl. Stopper the funnel and shake vigorously for 5 minutes. Allow phases to separate and transfer aqueous phase to a second 250 ml funnel. Scavenge adhering and suspended water from the first funnel with 1.0 ml N/10 HCl, adding this to the separated aqueous phase. Add 20 ml of n-butyl acetate to the aqueous phase, shake as before, and allow phases to separate. Again scavenge residual water with 1.0 ml of N/10 HCl and add to the separated aqueous phase. Discard both ester phases. Adjust the aqueous phase to a pH of at least 11 with a few drops of aqueous 20% NaOH (w/v). Add 40 ml of n-butyl acetate and shake as before. Separate the phases and extract the aqueous phase with a second 40 ml portion of the ester. (Make certain that the aqueous phase remains at a pH of at least 11.) Discard the aqueous phase and combine the ester phases by passing them through a short column (ca. 3 inches) of powdered anhydrous Na_2SO_4 to remove suspended and dissolved water. Collect in a crystallization dish. Wash the Na_2SO_4 column with 10-15 ml of n-butyl acetate, collecting the wash in the same dish. Acidify the n-butyl acetate with 0.2 ml of glacial acetic acid. Again reduce just to dryness on a warm hot plate, taking care that the contents do not exceed 50° C. Transfer residue with acetone to a small vessel (19 X 125 mm screw-cap culture tube). Rinse dish and combine rinsings in the same vessel. Reduce just to dryness and make up to 0.4 ml with acetone. Withdraw 0.2 ml and place in a small test tube (10 X 75 mm) to use for TLC clean-up and subsequent GLC. Reserve the other portion for identity confirmation.

The liquid-liquid partitioning step removes nearly all of the plant pigments and neutral oils. When a 2:1 ratio of ester to alkaline water is maintained in the back-partition step, the overall recovery of 4-aminopyridine is 89%. However, the small amount of residual plant extractives is basic and contains nitrogen compounds that interfere with the microcoulometric detection of 4-aminopyridine. Consequently, thin-layer chromatography is used as an additional clean-up step.

TLC Clean-up

The most suitable thin-layer adsorbent is activated alumina (Eastman Chromagram type 6063 with fluorescent indicator). Use a piece of the Chromagram stock 7.5 X 20 cm and draw the origin line 3/4" from one end. Allow a span of 5 cm for the sample. Impregnate the strip with NaOH by dipping in a 0.2% solution (w/v) of NaOH in absolute methanol. When the alcohol has evaporated, apply 0.2 ml of the sample (representing 25 gm of starting sample) to the strip, with a 10 µl syringe, keeping the spots small (ca. 0.2 µl) and closely spaced in a reasonably uniform streak 3-4 mm wide and 5 cm long. Application is less tedious if the 0.2 ml of sample is reduced to ca. 50 µl before spotting. Wash down the sides of the sample tube with 50-60 µl of acetone and apply this to the sample streak also. On the same chromatogram place 0.2-1.0 µg of reference standard 4-aminopyridine. Develop the chromatogram with a mobile solvent consisting of water, methanol, and acetone (1 + 5 + 45). Good resolution is achieved when the mobile solvent migrates 13 cm (Rf of 4-aminopyridine is 0.47-0.50). Dry the chromatogram and view under filtered short-wave UV (254 mµ). (UV may cause some destruction of 4-aminopyridine, so it is desirable to mask all of the chromatogram but the strip containing the reference standard.) The 4-aminopyridine spots appear dark blue against a bright orange fluorescent background. With a soft pencil, mark the chromatogram where the standard is seen, then outline the area that will contain the sample-derived 4-aminopyridine (this will be a rectangle about 1.5 X 5.5 cm). With a chromatogram sprayer, lightly spray the developed chromatogram with a 0.5% NaOH (w/v) in water to deactivate the alumina and to allow the desired area to be scraped off without particle scattering due to static electricity. Do <u>not</u> spray so heavily that the water runs. While the chromatogram is still damp, scrape the adsorbent off the outlined area with a small chisel-shaped hardwood stick, transfer to a small (10 X 75 mm) test tube, and add 1.0 ml of methanol. Stopper the tube and break up the alumina flakes with an ultrasonic cleaner. Allow to stand for 10 minutes in a beaker of warm (40° C) water to complete the deactivation of the alumina and desorption of the 4-aminopyridine. Again agitate thoroughly and centrifuge at about 2000 rpm for 3-4 minutes. Withdraw 0.8 ml from the clear supernatant, place in a 10 X 75 mm test tube containing 2 µl of ethylene glycol to serve as a "keeper", and reduce just to dryness. Make up the dry residue to 80 µl with 1 + 1 methanol-water. Agitate to dissolve all residue.

Gas Chromatography

The sample is now ready for GLC with microcoulometric (Dohrmann nitrogen detection. This sample volume contains the equivalent of 250 mg of sample per microliter. At a concentration of 0.1 ppm, 10 µl will provide 250 ng of 4-aminopyridine, which is sufficient to produce a response of ca. 40% F.S. on a 1 mv recorder with instrument operating parameters as follows: Temperatures- GC column, 195-210° C - GC inlet, 220° C - Inlet combustion transfer, 225° C - Center combustion, 900° C - Outlet combustion, 450° C. Range - Ohms setting, 100-150 ohms. Operate in high-gain mode. Combustion tube - Quartz (Cassill type). Catalyst - Nickel wire. Scrubber - BaO on Perlite (10-16 mesh). GC column - Chromasorb 103, 100-120 mesh in 1/4" X 3' aluminum. (Before use, condition column for 16 hours at ca. 20° C above anticipated operating temperature.) Gas flows (hydrogen) - Column, ca. 100 ml/min., Transfer, ca. 20 ml/min., Reactant, ca. 40 ml/min., Auxilliary, ca. 50 ml/min.

Identity Confirmation by Electron Capture Detection

While the microcoulometric estimation of 4-aminopyridine provides the better means of quantitation, use of another column coupled with electron capture detection provides a very sensitive means of identity confirmation and semi-quantitation. This detection means requires the formation of a chlorine-containing derivative, N-4(-pyridyl) dichloroacetamide, as follows:

Note the volume of the unused sample concentrate (see "TLC Clean-up"). Reduce this concentrate to dryness, remake to 10 times the original volume with iso-octane (dried with silica gel), and add 1.0 µl of 1% dichloroacetyl chloride in iso-octane. Wait 3-5 minutes, then make an initial chromatogram by introducing 1-2 µl into the GC to show whether the sample will require dilution or concentration. One microliter of the solution is sufficient to produce an easily identifiable response if the concentration of 4-aminopyridine in the original sample was as high as 0.01 ppm. Semi-quantitation is done in the usual fashion by comparing peak area of sample with peak area of derivatized standards (usually 1 ppm).

With the following instrument parameters, 1 ng equivalent of 4-aminopyridine will produce a peak of ca. 40% F.S. on a 1 mv recorder: Column - 1/8" X 2-1/2' pyrex packed with High Performance Chromasorb W, AW, DMCS treated, 100-120 mesh, coated with 2% Carbowax 20M. (Before use, bake column at 220° C for 16 hours with a carrier flow of ca. 10 ml/min. Condition column daily by injecting four or five 1-2 ug quantities of derivatized 4-aminopyridine standard over a span of 15-30 minutes. Temperatures - Oven, 175-180° C - Injection port, 200° C. Carrier flow (nitrogen), 25-30 ml/min. - Electrometer sensitivity, ca. 2 X 10^{-11}amps.

The derivative, N-(4-pyridyl) dichloroacetamide, is easily hydrolyzed and relatively unstable. Care must be taken to dry as thoroughly as possible the solvents used for holding the sample, the standards, and the derivatizing reagent. Derivatized standards more than 30 minutes old should not be used.

CONCLUSIONS

The analytical method described here will allow the preparation of corn plant tissue, the extraction of 4-aminopyridine from it, purification of the residues, and identification and estimation of the purified residue by thin-layer and gas-liquid chromatography using two dissimilar columns and two detection means - electron capture and microcoulometric nitrogen detectors. The method is sensitive to levels of 4-aminopyridine as low as 0.01 ppm.

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