A Microanalytical Method for 4-Aminopyridine in Sunflower Seeds and Plants

by JAMES E. PETERSON
U.S. Fish and Wildlife Service
Wildlife Research Center
Denver, Colo. 80225

One of the more successful chemicals recently developed to protect crops from feeding birds is 4-aminopyridine (Avitrol 200*). Baiting fields with very small amounts of this toxicant can disperse flocks by causing the few birds that eat the baits to display vocal distress behavior that frightens away the others (GOODHUE and BAUMGARTNER 1965). 4-Aminopyridine on cracked corn baits has proved very effective for protecting ripening corn from marauding blackbirds (DE GRAZIO et al. 1971, 1972; STICKLEY et al. 1972). Preliminary tests indicate that the same baits will protect fields of ripening sunflowers, which are also subject to severe blackbird damage (PFEIFER 1968-1972). In anticipation of Federal registration of 4-aminopyridine for this use, the following method was developed to detect and estimate residues in sunflower plants and ripe seeds at levels as low as 2-3 ppb.

METHOD

Sample Preparation and Cleanup

An earlier microanalytical method for 4-aminopyridine in corn plants (PETERSON 1971) proved unsuitable for sunflower seeds, which contain 25-40% oil, plus about 3% free fatty acids and acidic amino acids. In addition, because the seed hulls are mainly cellulose, they are adsorptive when finely ground. Therefore, to extract free 4-aminopyridine from seeds, sizable amounts of water and sodium hydroxide must be included with the solvent. For simplicity, the same cleanup procedures are used for samples of sunflower plant tissues. (However, more solvent is used to extract plant tissue because of the much greater bulk of the ground, dry plant tissue.)

Grind the sample to about 30 mesh in a Wiley mill. For seeds, weigh two 25.0-g portions of the ground sample material into two 250-ml screw-cap centrifuge bottles and add 125 ml of n-butyl acetate and 20 ml of 15% (w/v) NaOH to each bottle. For plants, use four centrifuge bottles each containing 12.5 g of sample, 125 ml

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of n-butyl acetate, and 20 ml of 15% NaOH. Cap securely, shake vigorously on a wrist-action shaker for 1 hr, and centrifuge at 1,800 rpm for 10 min. This will extract about 75% of the 4-aminopyridine present, and at least part of the remainder stays in the aqueous portion of the mixture (the distribution of 4-aminopyridine between 1:1 n-butyl acetate and water is about 55:45). Although recovery could be improved somewhat by a second extraction, the solvent volumes would become unwieldy in subsequent cleanup steps.

Remove all remaining particulates in the bottles by filtering the contents through a 20- X 400-mm chromatographic tube containing a Celite 545 filter pad, about 1.5 cm thick, that has been washed with 25 ml of water and superficially dried by adding 10 ml of acetone and evaporating it with a stream of air. Air pressure at 3-5 psi will speed the filtration.

Collect the filtrate in a 250-ml graduated cylinder and note its volume. For each series of seed samples, the volume of oil extractable from 50 g of ground seeds should be determined by Soxhlet extraction. Adding this to the volume of butyl acetate used allows calculation of the sample equivalent weight recovered in the extraction step. For example, seeds containing 40% oil will contribute about 20 ml of oil to produce a total oil-solvent volume of 270 ml; thus, 230 ml of filtrate would represent 50 X 230/270, or 42.6 g. For plants, the volume of extractables is negligible and need not be considered.

Add 2-3 drops of 0.5% Bromocresol purple indicator dissolved in butyl acetate to the filtrate, transfer to a 250-ml separatory funnel (a 500-ml funnel for plant samples), and add 40 ml of distilled water. To partition the 4-aminopyridine into the aqueous phase, acidify with 4-6 drops of 25% (v/v) acetic acid added dropwise with vigorous shaking between additions. When the color is a permanent butter-yellow, the pH of the aqueous phase will be 5.2-5.5. Continue shaking for 3-4 min, then allow the phases to separate (5-10 min). To clarify the aqueous phase and break up the small amount of emulsion at the interface, withdraw all the aqueous (lower) phase and 2-3 ml of the butyl acetate phase, pass through a 20- X 400-mm chromatographic tube containing a 1.5-cm pad of Celite 545 washed as before, and collect in a 60-ml separatory funnel. Scavenge the residual aqueous phase from the butyl acetate by adding 5 ml of distilled water. Shake and again withdraw the aqueous phase and the interfacial solids and pass through the Celite pad into the 60-ml separatory funnel.

In the next step, the 4-aminopyridine is removed from aqueous solution by passage through a cation exchange resin. Since 4-aminopyridine is a relatively strong base (pK_a of the conjugate acid = 9.11), a strongly acidic resin can be used, but this also removes enough weakly basic materials to interfere with the chromatography of low-concentration samples. Therefore, the solution is passed through a small-scale column apparatus containing a weakly acidic resin.

The apparatus consists of a 50-ml culture tube (the reservoir) extended by fusing about 4 cm of 6-mm-o.d. (4-mm-i.d.) glass tubing straight down from the bottom. A second piece of 6-mm tubing about 14 cm long (the resin column) is joined to the bottom of the reservoir extension with a short rubber coupling. The lower end of the column contains a 1-cm plug of glass wool to retain the resin beads. A second rubber coupling with a screw clamp and about 4 cm of 5-mm glass tubing to serve as a delivery tube are added at the bottom. This device is clamped on a ring stand in a position that allows collecting the percolate in a 10-ml tapered centrifuge tube. To prepare the column, close the screw clamp and add about 15 ml of distilled water. When the column and reservoir are free of trapped air bubbles, add enough Amberlite IRC-50 resin beads, 20-50 mesh, to come to a depth of 11-12 cm. Open the screw clamp, allow the water to percolate through, and before air can enter the resin bed, wash with an additional 10-15 ml of distilled water. The column is now ready for use.

With the screw clamp closed, transfer all the aqueous sample from the 60-ml separatory funnel to the culture tube reservoir, taking care to exclude any butyl acetate that may be present. Open the screw clamp and adjust it so that the sample percolates through the resin at a rate no faster than 1 ml/min. (The resin will swell somewhat during percolation, so the clamp will require readjustment at intervals.) Just as the last liquid enters the resin bed, close the clamp momentarily and add about 5 ml of distilled water. Open the clamp and allow this wash water to pass through the column. Discard the percolate.

The absorbed 4-aminopyridine is displaced from the resin column by regeneration with hydrochloric acid; this forms 4-aminopyridine hydrochloride, which does not volatilize when the sample is subsequently dried. Add 6 ml of 2 M HCl to the reservoir (be certain no air bubbles are trapped in the column). Open and adjust the screw clamp so that the HCl percolates through the column no faster than 0.2 ml/min. Collect the percolate in a 10-ml tapered, screw-cap centrifuge tube. Place the centrifuge tube in a water bath at 70° C, and with a stream of air delivered by a piece of 6-mm glass tubing drawn to a fine tip, reduce the percolate to dryness.

To convert the 4-aminopyridine hydrochloride back to the free base, add 0.4 ml of 10% (w/v) NaOH to the centrifuge tube. Add 8.0 ml of redistilled n-butyl acetate, cap, shake vigorously for 3-4 min, and centrifuge at 1,800 rpm for 3-4 min. Withdraw 7.8 ml of the butyl acetate phase and transfer to a second centrifuge tube. (The sample equivalent weight is now 42.6 X 7.8/8.0, or 41.5 g.) To this second tube add 1.5 ml of 1 M HCl. Again cap, shake vigorously for 3-4 min, and centrifuge at 1,800 rpm for 3-4 min. Remove and discard most of the butyl acetate phase. Withdraw 1.45 ml of the aqueous phase and transfer to a 10- X 75-mm test tube. (The sample is now equivalent to 41.5 X 1.45/1.5, or

40.1 g.) In the 70° C water bath, again reduce the sample to dryness with a stream of air. To the dry residue, add 80.2 µl of 2% (w/v) NaOH (practically, 80 µl is satisfactory). Cap the tube and roll it to completely wet all surfaces touched by the sample. For GLC analysis, inject 2.0 µl of this solution (equivalent to 1.0 g of starting sample) into the gas chromatograph.

Chromatography

Detection is by flame ionization, although an electrolytic conductivity detector could be used as well; both offer approximately the same usable sensitivity and will readily detect as little as 5 ng of 4-aminopyridine.

A GLC column that works well and will last almost indefinitely is one of 3% Carbowax 20 M and 0.1% potassium hydroxide on Chromosorb 750 support. To prepare this column packing, weigh 5.0 g of 100-200 mesh Chromosorb 750, noting its volume. To a 150-ml beaker add about twice this volume of chloroform, dissolve in it 150 mg of Carbowax 20 M, and add 0.5 ml of 1% (w/v) KOH in absolute methanol. Swirling the beaker, slowly add the support material. Under a hood, evaporate the solvent on a 40° C hot plate with a gentle current of air, swirling frequently (do not stir). When all the solvent has evaporated, place the packing in a 4-ft, 1/8-inch-o.d. stainless steel column. Before use, condition the column for about 16 hr at 185°-190° C with 30 ml/min of nitrogen flowing.

With this column, optimum chromatograph operating conditions are: oven temperature, 170° C; injector temperature, 190° C; detector temperature, 190° C; carrier (nitrogen) flow, 30 ml/min; hydrogen flow, 25 ml/min; air flow, 250 ml/min; and electrometer sensitivity (for samples containing 10-50 ng), 4 X 10^{-12} A/mV. A 2-µl sample containing 10 ng of 4-aminopyridine (equivalent to 0.01 ppm in the starting sample) will produce a recorder (1 mV) response of about 12% of full scale. Retention time is about 8 min.

With a carefully prepared, well-conditioned Carbowax 20 M/Chromosorb 750 column, detection is very linear down to about 10 ng, so results in this range may be quantitated by simply comparing sample peaks with those of reference standards. At 10 ng and below, it is best to inject varying amounts of reference standard (usually 2 ng/µl) until peak heights can be matched.

Recovery for the method usually ranges between 50% and 70%. For example, when samples of sunflower seeds (confectioner's variety) fortified with 4-aminopyridine were analyzed in triplicate, recoveries averaged 61.2% (range 58.0-63.0%) for 0.01-ppm samples, 53.7% (range 52.5-54.5%) for 0.05-ppm samples, and 58.1% (range 56.4-60.9%) for 0.10-ppm samples. However, recovery may vary somewhat with the plant variety, moisture content, average particle size, etc. Therefore, for any positive analysis, a correction factor should be derived by analyzing a second sample

overspiked with an amount of 4-aminopyridine approximately equal to that first recovered.

A simple identity confirmation may be made by thin-layer chromatography. As little as 100 ng of 4-aminopyridine can be detected by spotting the sample on a precoated, activated alumina plate with fluorescent indicator, such as Eastman Chromagram type 6063, and developing with water-saturated methyl isobutyl ketone (R_f = 0.14). Viewed under filtered UV light (254 nm) in a darkened room, 4-aminopyridine appears as a deep bluish spot on a bright orange background.

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