A Simplified High-Performance Liquid Chromatographic Residue Procedure for the Determination of Glyphosate Herbicide and (Aminomethyl)phosphonic Acid in Fruits and Vegetables Employing Postcolumn Fluorogenic Labeling

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An analytical procedure for residues of the herbicide glyphosate [*N*-(phosphonomethyl)]glycine and its metabolite [(aminomethyl)phosphonic acid] in fruits and vegetables is described. After water extraction and cleanup by cation-exchange liquid chromatography, high-performance liquid chromatography is used as the determinative step, employing postcolumn fluorogenic labeling with *o*-phthalaldehyde–mercaptoethanol. Residues can easily be measured at the 0.05-ppm level with recoveries always above 60%, usually higher. The simplicity of the procedure allows for relatively high sample throughput compared to existing procedures.

Glyphosate [*N*-(phosphonomethyl)glycine] (GLYPH) is a nonselective postemergence herbicide with wide application in weed control which when used at prescibed rates does not injure crops planted immediately after treatment (Sprankle et al., 1975). Its rapid translocation from plant foliage to underground parts, high solubility, and low mammalian toxicity contribute to its growing popularity as a herbicide. (Aminomethyl)phosphonic acid (AMPA) has been shown to be the major metabolite in plants, water, and soil (Sprankle et al., 1978).

Residue procedures employing gas chromatography (GC) after chemical derivatization (Sprankle et al., 1978; "Pesticide Analytical Manual", 1980), thin-layer chromatography (TLC; Sprankle et al., 1978; Young et al., 1977), and high-performance liquid chromatography (HPLC) have been reported (Moye and St. John, 1980). All suffer from difficulties associated with the extensive and laborious sample cleanup required (GC), multiple derivatizations (GC), lack of sensitivity and/or semiquantitative nature (TLC), and crop interference with AMPA (HPLC). Possibly as a result of extensive sample manipulation, low recoveries (<60%) at fortification levels of 0.1 ppm and lower have been reported ("Pesticide Analytical Manual", 1980; Thompson et al., 1980) as well as broad and ill-defined peaks (Thompson et al., 1980). Additionally, one procedure requires a multiramp GC column program and still does not resolve the AMPA peak from interferences normally found in blueberries (Guinivan et al., 1982).

This report describes a procedure based upon the approach previously described (Moye and St. John, 1980) with improvements in amount of sample required, sample throughput, chromatographic efficiency, resolution and sensitivity, and reagent consumption. Extraction of fruits and vegetables is performed with water; cleanup is achieved by a single cation-exchange column, and detection is performed by postcolumn fluorogenic labeling of the appropriate primary amine with *o*-phthalaldehyde (OPA) and mercaptoethanol (MERC). Conversion of GLYPH to a primary amine (presumably ammonia and/or glycine) is achieved postcolumn by oxidation with calcium hypochlorite.

MATERIALS AND METHODS

Instrumentation. A schematic of the instrumental arrangement is shown in Figure 1. It was constructed from a Waters Associates Model 6000 solvent delivery system (Milford, MA), a Rheodyne Model 7010 injector with 20-μL loop (Applied Sciences, State College, PA), a 4 mm × 25 cm stainless steel column packed with 13.5-μm Aminex A-27 anion-exchange resin (Bio-Rad Laboratories, Richmond, CA) or 7–10-μm HA-X10 (Pierce Chemical Co., Rockford, IL), two Milton Roy Model 196-0066-001 reagent pumps (Riviera Beach, FL), and a Gilson Spectra/Glo fluorometer equipped with a 15-μL cell and OPA filters for excitation at 360 nm and emission at 455 nm (Middleton, WI). Isothermal column conditions (62 °C) were maintained with a small oil bath (Model PYI, Bench Scale Equipment Co., Dayton, OH); chromatograms were recorded on a Soltec No. 2615 strip chart recorder (50 mV). Oxidant (calcium hypochlorite) and fluorogenic reagent (OPA-MERC) were pumped and mixed with the HPLC eluant via Model CJ3031 Kel-F "t"s (Laboratory Data Control, Riviera Beach, FL) and 1.6 mm o.d. × 0.5 mm i.d. Teflon tubing (Dixon Medical Products, Dixon Heights, PA). A 10.6-m delay coil of this tubing (0.79-mL volume) at ambient temperature was used to provide a delay time of about 60 s before entering the Kel-F "t" into which the OPA-MERC reagent was pumped; a 1-m length of tubing carried the mixture to the fluorometer.

A Polytron homogenizer, Model PCV-2 (Brinkmann, Westbury, NY), fitted with a blade-type generator was used for crop extractions. Water was removed from extracts by using a Buchi Model R at maximum aspiration (Brinkmann).

Glassware and Reagents. Cation-exchange column cleanup was performed with 30 cm × 1.9 cm i.d. columns equipped with 250-mL reservoirs and detachable Teflon stopcocks. Exactly 50 g of Dowex 50W-X8, 100–200 mesh, was packed before equilibration with 200 mL of 0.01 M HCl at 2.5 mL/min.

The mobile phase for the Aminex column was 0.09 M H_3PO_4–0.01 M H_2SO_4 delivered at 0.5 mL/min (GLYPH analysis); the HA-X10 column (AMPA analysis) was eluted with 0.02 M KH_2PO_4 buffer at pH 5.0 and 0.5 mL/min.

The cleavage reagent for GLYPH analysis was prepared by dissolving 0.5 g of HTH (Olin Co.) in 0.5 L of deionized water; 2.0 mL of this solution was diluted to 200 mL with buffer, which was prepared by dissolving 34.8 g of K_2HPO_4 and 23.2 g of NaCl in 2 L of deionized water. The OPA–MERC fluorogenic reagent was made by dissolving 100 g of H_3BO_3 (impalpable powder, Mallinckrodt) and 72 g of KOH in 1 L of deionized water. Additions of 1.0 mL of MERC and 0.8 g of *o*-phthalaldehyde (Aldrich) dissolved in 10 mL of methanol completed the reagent.

Except during the reagent optimization studies the calcium hypochlorite was pumped at 0.2 mL/min and the

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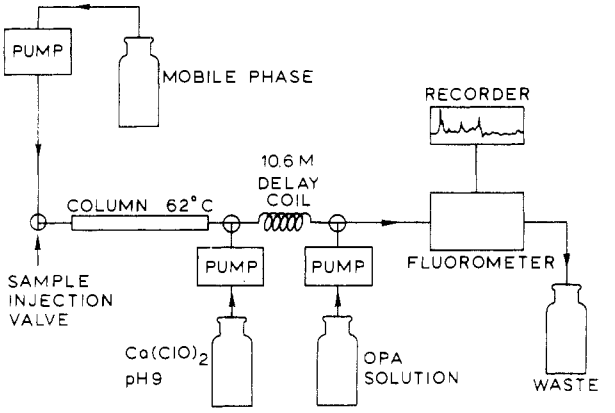

Figure 1. Schematic of postcolumn fluorogenic labeling HPLC for the analysis of GLYPH and AMPA. See the text for details.

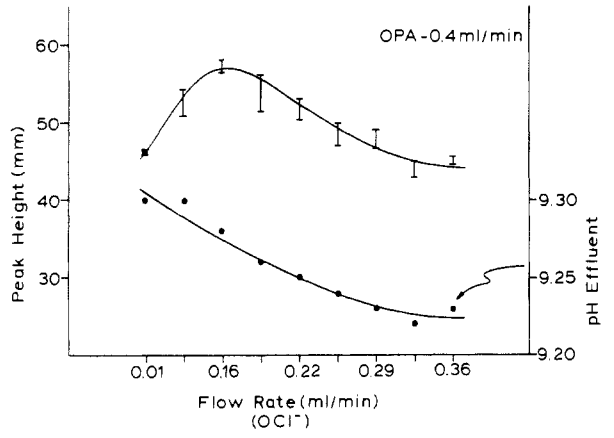

Figure 2. Effect of calcium hypochlorite flow on GLYPH (5 ng) response and effluent pH. OPA–MERC flow held at 0.4 mL/min.

OPA–MERC at 0.4 mL/min for GLYPH analyses. For analyses of AMPA the hypochlorite pump was turned off and the OPA–MERC pump left at 0.4 mL/min.

Reagent Optimization. The effect of hypochlorite flow on GLPH response and effluent pH is shown in Figure 2. Flow through the A-27 column was held at 0.5 mL/min along with an OPA–MERC flow of 0.4 mL/min. Optimization of the OPA–MERC flow was also conducted for GLYPH analyses (Figure 3) with hypochlorite held at 0.32 mL/min and for AMPA analyses (Figure 4) with hypochlorite turned off.

Residue Procedure. Exactly 25 g of crop, 25 mL of chloroform, and 50 mL of water were placed in a 250-mL polyethylene centrifuge bottle. Fortification was performed at this point for recoveries. After the contents were blended for 30 s at medium power with the Polytron the tubes were centrifuged at 10000*g* for 20 min. The supernatant was filtered through Whatman No. 1 into a 500-mL round-bottom flask followed by 2 × 20 mL rinses of the centrifuge bottle. Rotary evaporation at 40 °C reduced the volume to about 5 mL which was transferred to a 10-mL volumetric tube with 2 × 2 mL rinses. Adjustment to pH 2 with concentrated HCl prepared the sample for cation-exchange chromatography.

Placement of the sample at the top of the 50-g cation-exchange column was accomplished by layering, after which elution by gravity feed with 0.01 M HCl at 2.5 mL/min was conducted. Eluant was added to the reservoir during the chromatography to maintain flow. The first 60 mL was discarded; the second 60 mL contained all of the GLYPH. Exactly 60 mL more was discarded, and the last 100-mL fraction contained all of the AMPA. Each of the two collected fractions were separately rotary evaporated to dryness and transferred to volumetric tubes by 3 × 1.0 mL water rinses. Each tube was adjusted to pH 3–8 with 10 M KOH, made to 5.0-mL volume, and filtered through glass fiber filter paper (Whatman GF/F) by means of a syringe and Swinney adapter.

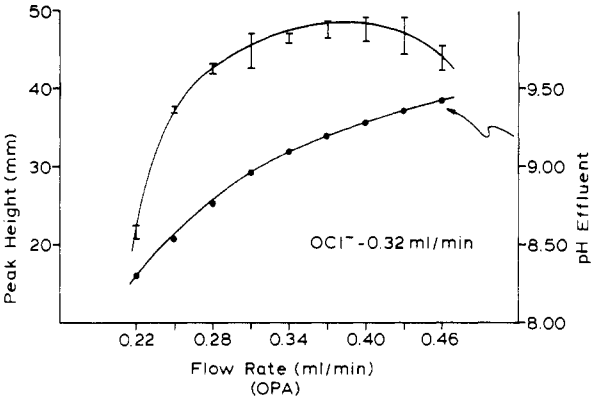

Figure 3. Effect of OPA–MERC flow on GLYPH (5 ng) response and effluent pH. Calcium hypochlorite flow held at 0.32 mL/min.

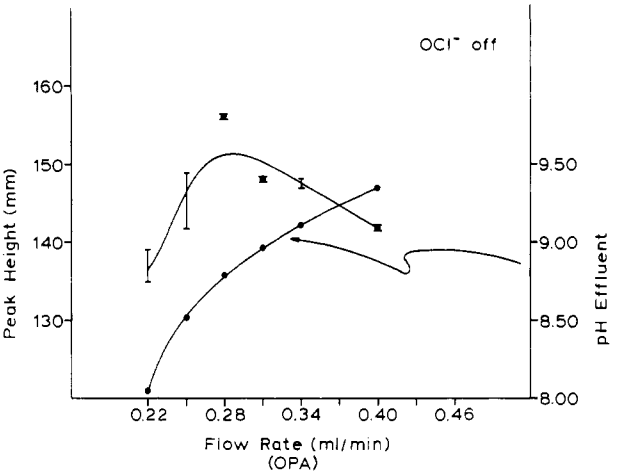

Figure 4. Effect of OPA–MERC flow on AMPA (5 ng) response and effluent pH. Calcium hypochlorite pump turned off.

Crop Recoveries. Recoveries were performed by adding 0.1 mL of GLYPH and AMPA in water to the appropriate crop immediately before blending with the Polytron. Crops studied were cantaloupe, cranberry, cucumber, blueberry, Jalapeño pepper, pumpkin, and summer squash. All recoveries were performed in duplicate, except for the 0.05-ppm recoveries for cranberries which were replicated 5 times.

Cranberry Residues. Field-weathered cranberries were analyzed for GLYPH and AMPA 64 days after a 6% (wt) solution was applied by rope wicks to the mature fruit. The analyses were performed in triplicate.

RESULTS AND DISCUSSION

A prime requirement for efficient reaction to occur between the primary amine and the OPA–MERC reagent is that the solution pH be maintained between 9 and 10 (Moye et al., 1977). It can be seen from Figures 2–4 that this criteria is met for the flow rates selected, 0.4 mL/min for OPA–MERC and either 0.0 mL/min or 0.2 mL/min for hypochlorite when AMPA or GLYPH are measured, respectively. It can also be seen from Figure 2 that a hypochlorite flow of 0.2 mL/min provides near-maximum response to GLYPH while allowing for small flow fluctuations which would not affect response as greatly as if the 0.16 mL/min optimum were chosen. Similarly, the 0.4 mL/min OPA–MERC flow that was chosen provides

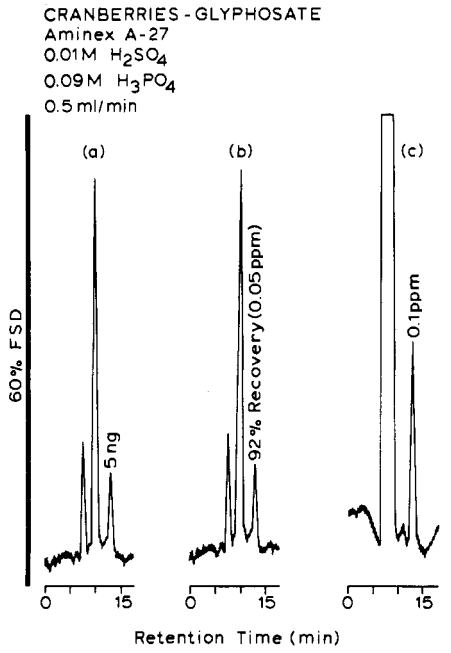

Figure 5. Glyphosate chromatograms: (a) 5 ng of standard; (b) 0.05-ppm recovery from cranberries; (c) 0.1 ppm found in field-weathered cranberries.

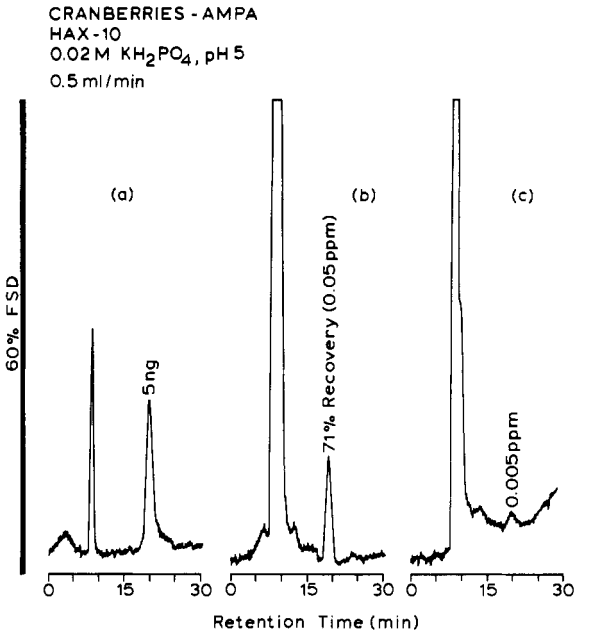

Figure 6. (Aminomethyl)phosphonic acid chromatograms: (a) 5 ng of standard; (b) 0.05-ppm recovery; (c) 0.005 ppm found in field-weathered cranberries.

near-maximum response when the hypochlorite is turned off for AMPA analyses while providing maximal response for GLYPH when the hypochlorite is turned on.

After much experimentation with isocratic mobile phases of varying pH, ionic strength, and buffer type it became obvious that the single-column anion-exchange separation of GLYPH and AMPA which has already been reported (Moye and St. John, 1980) could not be improved. As well, mobile phase gradients of decreasing pH and increasing ionic strength in order to provide adequate retention for AMPA, thereby allowing it to be fully resolved from crop interferences, while at the same time eluting GLYPH in less than 30 min could not be satisfactorily employed owing to the long equilibration times required of the microporous plastic bead resins. Consequently, two columns were employed, each kept at 62 °C and equilibrated with the appropriate mobile phase. Column switching was accomplished by simply manually disconnecting and connecting the stainless steel high-pressure fittings. While this approach requiring separate injections for the analysis of AMPA and GLYPH may appear to be unattractive it allowed for sample throughput nearly equal to that already reported for the single-column procedure. Anion-exchange columns of different particle size and manufacturer were employed simply because they were available; either column type could just as well be employed for either AMPA or GLYPH analysis. Marked reduction in GLYPH retention over that previously reported (Moye and St. John, 1980) was accomplished by making the eluant 0.01 M in H_2SO_4. This did not significantly change the effluent pH as measured at the fluorometer, allowing for full fluorescence sensitivity. Additionally, as can be seen from the chromatograms (Figures 5 and 6), a slight improvement in chromatographic efficiency was realized. A limit of detection of approximately 0.5 ng (S/N = 3) could be realized for both GLYPH and AMPA. Analytical curves were linear from 5 to 100 ng for both GLYPH and AMPA.

By employment of fluorometric detection not only are there both a theoretical advantage and real advantage in terms of sensitivity but also it is possible to meter the needed postcolumn reagents with relatively inexpensive positive displacement pulsatile pumps. Pulseless pumps or pressurized vessels fed through restrictors could be used just as well but no advantage is realized. The only requirement for eliminating the noise which may be produced from pulsatile pumps is that of ensuring homogeneity of the solution reaching the fluorometer cell; this is easily accomplished in the mixing which occurs in the 1 m × 0.5 mm Teflon tubing leading from the last "t" to the fluorometer.

If refrigerated at 4 °C both the $Ca(ClO)_2$ and OPA–MERC reagents were stable up to 1 week. After that a loss in sensitivity was observed. Since the reagent reacts with primary amines as well as ammonia all reagent reservoirs should be covered as tightly as possible and efforts made to prevent vapors of these compounds from entering the laboratory atmosphere. Impure reagents from the suppliers listed have not been a problem; however, boric acid from another supplier created high fluorescent background.

Recoveries at the 0.1-ppm level were consistently above 70% for all crops studied and for both GLYPH and AMPA, except for the 61% AMPA recovery observed on cranberries. At this level GLYPH recoveries ranged from 86 to 100% while AMPA recoveries were somewhat lower, ranging from 61 to 85%; recoveries from cranberries (mean of five replicates) were 87 and 76% for GLYPH and AMPA, respectively. Recoveries for three crops (cantaloupe, cucumber, and pumpkin) at the 1.0-ppm level ranged from 88 to 107% for GLYPH and from 88 to 89% for AMPA. Both AMPA (0.005 ppm) and GLYPH (0.1 ppm) were observed in the field-treated cranberries (Figures 5c and 6c). Although the single cation-exchange column provided good resolution of AMPA and GLYPH from sugars, pigments, and other crop interferences, occasionally samples with high sugar content, particularly blueberries, would slow the column flow. In such an instance a slight stirring of the top of the resin bed would restore flow; however, infrequently an interference would appear on the chromatogram at approximately the GLYPH retention and could be observed as a shoulder on the GLYPH peak. This problem never occurred with the vegetables studied.

Unlimited column life for the plastic bead quaternary ammonium HPLC columns was observed. After over a

year and a half of use they showed no loss in efficiency or sensitivity to AMPA or GLYPH. The extremely acidic mobile phase required for elution would not be compatible with the chemically bonded silica column packings which are prone to dissolution at such pH conditions. Reagent and HPLC pump maintenance over this period was limited to periodic flushings with deionized water to dissolve small amounts of crystalline deposits. Columns and pumps were stored for short periods (1 week) containing the mobile phase. Water was used for storage at longer periods.

Registry No. GLYPH, 1071-83-6; AMPA, 1066-51-9.

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Distribution of Lacinilene C and Lacinilene C 7-Methyl Ether in Cotton Plant Parts

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Lacinilene C (LC) and lacinilene C 7-methyl ether (LCME) were removed from nine cotton plant parts by exhaustive ether Soxhlet extraction and quantitated by high-performance liquid chromatography (HPLC). LCME was observed in extracts of all plant parts examined in quantities ranging from 92 $\mu g/g$ in the seed extract to 1159 $\mu g/g$ in the leaf extract. Values for LC contents varied from 23 $\mu g/g$ in the pedicel extract to 736 $\mu g/g$ in the leaf extract. LC, however, was not detected in the ether extracts of root or seed.

Stipanovic et al. (1975c) isolated lacinilene C (LC) and its 7-methyl ether (LCME) from *Gossypium hirsutum* L. bracts and revised the structure (Figure 1). Interest in LCME stems from its implication as a possible etiological agent of byssinosis, a clinical syndrome associated with inhalation of respirable dust generated during cotton processing in textile mills. Bioassays commonly used in studies of other lung diseases have been performed to ascertain the biological activity of LC and LCME. The naphthalenone, LCME, isolated from aqueous extracts of cotton dust, bracts, and stems, was identified by Stipanovic and Wakelyn (1975) as the yellow-fluorescing chemotaxin of the slow type (Lynn et al., 1974; Jeffs and Lynn, 1975; Kilburn et al., 1977; Ainsworth and Neuman, 1977; Northup et al., 1976; Ziprin and Greenblatt, 1979; Greenblatt and Ziprin, 1979a) which produced a proliferation of polymorphonuclear (PMN) leukocyte migration into airway lumen in vivo and in vitro (Kilburn et al., 1973; Lynn et al., 1974), inhibited luminol-dependent chemiluminescence of phagocytosing alveolar macrophages (Greenblatt and Ziprin, 1979b), and caused histamine release in sensitized rat mast cells (Ainsworth and Neuman, 1977; Northup et al., 1976). Lacinilene C has also been identified as an inhibitor of chemiluminescence of alveolar macrophages (Ziprin and Greenblatt, 1979; Greenblatt and Ziprin, 1979a). It has been postulated that the chemical activity of both synthetic and natural LCME is cytokinetic rather than chemotactic toward leukocytes (Jeffs and Lynn, 1978). Other investigators found that synthetic LCME was both chemotactic and cytotoxic, but the cytotoxic effect predominates (Kilburn et al., 1979, 1981).

The functions of LC and LCME in the biosystem of the cotton plant is unknown. Kilburn et al. (1977, 1981) have speculated that LCME is a glycoside naturally occurring in cotton dust and bracts. Kilburn et al. (1979) also postulated that LCME appears to be an oxidation product and therefore related to senescence. He based this on reports of LCME in cotton dust and dried bracts but observed its absence in fresh green bract. However, LCME has been shown recently to be a natural product in fresh and dried green bracts (Stipanovic et al., 1981; Beier and Greenblatt, 1981). Other researchers (Essenberg et al., 1980) have indicated that the biosynthesis of LC and its recently identified precursor, 2,7-dihydroxycadalene (Stipanovic et al., 1981; Beier and Greenblatt, 1981), was induced in the cotton plant by infection and that the latter compound is a potent bacteriocide. Jeffs and Lynn (1975) speculated that the increased incidence of LCME in aged bracts and its absence from fresh plant material may also be linked to presence of contaminating microorganisms. It is well documented that other sesquiterpenoids such as hemigossypolone (Bell and Stipanovic, 1976), hemigossypol and 6-methoxyhemigossypol (Mace, 1976; Stipanovic et al., 1975a,b; Mace et al., 1974), hemigossypolone 7-methyl ether and the heliocides H_1, H_2, H_3, H_4, B_1, B_2, B_3, and B_4 (Stipanovic et al., 1977a,b, 1978; Bell et al., 1978), and the

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