induced mass spectrum of structure IV showed that a major fragment ion appears at m/e 152 and a high-resolution mass measurement on this ion establishes its composition as $HOOC–C_6H_4–NHNH_2^+$. Further work is in progress to isolate this compound from *A. bisporus*, the cultivated commerical mushroom.

Registry No. I, 619-67-0; II, 3705-42-8; III, 71426-47-6; IV, 69644-85-5; agaritine, 2757-90-6.

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Analysis of Glyphosate Residues in Kiwi Fruit and Asparagus Using High-Performance Liquid Chromatography of Derivatized Glyphosate as a Cleanup Step

A procedure was developed for the analysis of glyphosate herbicide in kiwi fruit and asparagus. The method consisted of extraction with a water–chloroform mixture, cleanup of the water extract by anion-exchange and gel permeation chromatography, derivatization with trifluoroacetic anhydride followed by diazomethane, further cleanup of derivatized glyphosate by silica gel HPLC using a methyl *tert*-butyl ether–tetrahydrofuran solvent gradient, and determination of the appropriate HPLC fraction by GLC using a phosphorus flame photometric detector. Recoveries averaged 87% for kiwi fruit fortified at 0.10–0.25 ppm and 80% for asparagus fortified at 0.05–0.10 ppm.

Glyphosate [*N*-(phosphonomethyl)glycine] is a broad-spectrum postemergent herbicide having wide utility for weed control. To support applications for U.S. registrations on specialty food crops, the national IR-4 pesticide clearance program must obtain residue data for glyphosate on each crop for which registration is sought. In carrying out residue analysis on kiwi fruit and asparagus, interferences were encountered that precluded straightforward adaption of GLC-based residue analytical methods for glyphosate ("Pesticide Analytical Manual", 1977; Guinavan et al., 1982) at the required limits of detections. We thus made several modifications in the sample preparation steps of published procedures and added a cleanup of the *N*-trifluoroacetyl *O,O,O*-trimethyl derivative of glyphosate using silica gel adsorption HPLC (Wehner et al., 1984) in order to decrease GLC interferences. With these changes a detection limit of approximately 0.05 ppm was achieved with both kiwi fruit and asparagus.

MATERIALS AND METHODS

Glassware and Reagents. Glass distilled water and nanograde or equivalent distilled-in-glass solvents were used throughout, with the exception of methyl *tert*-butyl ether and tetrahydrofuran (THF), which were UV grade (Burdick and Jackson, Muskegon, MI). Anion-exchange chromatography used Amberlite IRA-401S C.P. resin (Mallinckrodt, St. Louis, MO) converted from the chloride form to the bicarbonate form by washing 0.75 kg of resin in a suitable column with 15 L of 1 M ammonium bicarbonate (flow rate 10 mL/min) followed by 2 L of distilled water. Prepared resin (20 g) was added to 15 mL of distilled water in a 1.8 × 30 cm glass column equipped with a stopcock and a 2.5 cm diameter flaired section at the column head to serve as a solvent reservoir. The column was prewashed with 300 mL of 1 M ammonium bicarbonate solution at 5 mL/min followed by 300 mL of distilled water at 10 mL/min. An 8-cm head of water was left on top of the resin and then a 1-L separatory funnel was attached to the column inlet with a rubber stopper. Gel permeation chromatography used Bio-Gel P-2 resin (50–100 mesh, Bio-Rad, Richmond, CA) preswelled by soaking in distilled water. A 2.6 × 40 cm SR-25 glass column equipped with two SR-25 adapters (Pharmacia, Piscataway, NJ) was packed completely with a resin–water slurry. The column was then connected via 1.14 mm (i.d.) P.T.F.E. tubing to a Milton-Roy minipump and a Pharmacia SRV-3 injection valve. The column flow rate was maintained at 3 mL/min with pH 2.1 distilled water (ca. 1.2 mL of concentrated hydrochloric acid in 2 L of water) and calibrated each week by injecting 100 000 cpm of [^{14}C]glyphosate (Monsanto Chemical Co., St. Louis, MO, who also supplied unlabeled glyphosate standard) in 5 g of kiwi fruit or asparagus extract and collecting and counting 25-mL fractions. The usual elution pattern for a 5-g extract involved discarding the first 100 mL of eluate and collecting the next 45 mL. The column was flushed with distilled water, previously adjusted to pH 2.1 with concentrated hydrochloric acid, for at least 3 h after sample collection and kept on standby with a flow of 0.25 mL/min when not in use. Diazomethane, prepared in ether solution by a standard procedure from Diazald (Aldrich, Milwaukee, WI), was kept at −10 °C prior to use.

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Sample Preparation. A chopped crop sample (20 g) was blended for 1 min with water (100 mL) and chloroform (30 mL) in a 250-mL centrifuge bottle by using a Tekmar (Cincinnati, Ohio) Model SDT sonic blender. Asparagus samples required the addition of 0.25 mL of 70% acetic acid. The mixture was centrifuged at 3000 rpm for 10 min by using an International Equipment basket centrifuge and the aqueous layer decanted to a 500-mL Erlenmeyer flask. An additional portion of water (100 mL) was added to the centrifuge bottle, and the extraction, centrifugation, and decanting procedures were repeated. To the combined aqueous extract was added 200 mL of additional water along with enough 5% ammonium hydroxide solution to adjust the pH to 4.5. This solution was then added to the anion-exchange column through the 1-L separatory funnel so that the elution rate was 5 mL/min. The column was then eluted with distilled water (200 mL) at 5 mL/min followed by 150 mL of 0.4 M ammonium bicarbonate (150 mL) at 3 mL/min. The ammonium bicarbonate eluate was collected in a 1-L round-bottom flask and subsequently concentrated just to dryness by rotary evaporation at 45 °C. The sample was taken just to dryness 3 additional times with 35-mL portions of water each time to remove all traces of ammonium bicarbonate ("Pesticide Analytical Manual", 1977). The residue was quantitatively transferred to a 15-mL centrifuge tube with water and centrifuged if necessary to sediment suspended solids.

An aliquot of the supernatant representing 5.0 g of crop tissue was then added to the Bio-Gel gel permeation column and eluted at 3 mL/min with pH 2.1 distilled water previously adjusted to pH 2.1 with concentrated hydrochloric acid. The column elution pattern varied somewhat but, typically, involved discarding the first 100 mL of eluate after sample injection and collecting the next 45 mL in a 500-mL round-bottom flask. The eluate was evaporated to near dryness by rotary evaporation at a temperature of 45 °C or slightly less and then quantitatively transferred with several water rinsings to a 125-mL Erlemeyer flask equipped with a 24/40 ground glass joint. The sample was again evaporated to near dryness on a rotary evaporator at 45 °C, and then the last traces of moisture were removed under a gentle stream of N_2 until a viscous liquid persisted that solidified with time.

Derivatization. To the residue from the Bio-Gel column was added 1.5 mL of trifluoroacetic acid (99%, Aldrich). The flask was then stoppered and swirled until all the residue dissolved (ca. 20 min). Trifluoroacetic anhydride (1.5 mL, Aldrich, 99%) was then added, and the flask was stoppered, swirled, and allowed to stand at room temperature for at least 8 h. The solution was then evaporated to dryness under a gentle stream of N_2. Methanol (1.5 mL) was added with swirling to dissolve the residue and the solution was then allowed to stand 5 min. The diazomethane–ether solution was then added in ca. 2-mL portions with swirling following each addition until a yellow color persisted for 1 h. The solution was then concentrated to ca. 0.5 mL under N_2, transferred to a 15-mL glass-stoppered centrifuge tube with 5 × 0.5 mL aliquots of methanol, and again concentrated to 0.5 mL under N_2. Dichloromethane (3 mL) and 4% aqueous sodium sulfate solution (1.5 mL) were added, and the resulting mixture was shaken for 2 min. The dichloromethane layer was then transferred to a calibrated sedimentation tube with 2 × 3 mL portions of additional dichloromethane to complete the transfer. The solution was concentrated slowly to 0.2 mL with N_2.

HPLC Cleanup. The HPLC consisted of two Altex (Berkeley, CA) Model 110A pumps, Model 410 solvent programmer, LDC (Riviera Beach, FL) Spectromonitor I variable-wavelength UV detector (set at 220 nm), with range = 0.08–0.64 AUFS, Rheodyne injector (Model 7125, Cotati, CA) with a 50-μL loop, and a Linear (Model 155, Irvine, CA) recorder with the chart speed set at 20 cm/h. The column was a Partisil 10 column (Whatman, 10 μm, 25 cm × 4.6 mm i.d.) and the flow rate was 1 mL/min. The system is more fully described by Wehner et al. (1984).

The sample was injected with 100% methyl *tert*-butyl ether as the eluting solvent, and immediately a gradient to 50% methyl *tert*-butyl ether–50% tetrahydrofuran was begun extending over 10 min. The 50:50 mixture was then held for 15 min. A fraction (1 mL) containing derivatized glyphosate was collected, based upon the predetermined elution time of a standard of derivatized glyphosate. The usual elution time was ca. 21 min after injection. After the above-described solvent program was completed, solvent was recycled to 100% methyl *tert*-butyl ether and held there for 30 min to allow for reequilibration.

Gas Chromatography. A Tracor (Austin, TX) Microtech MT 220 gas chromatograph equipped with a 1 m × 3 mm glass column containing 1.5% SP-2250 and 1.95% SP-2401 on 100–120-mesh Supelcoport and a Tracor flame photometric detector with a 526-nm (P-mode) filter was used. Temperatures were as follows: injector, 150 °C; detector, 170 °C; column, 110 °C (initial) and 175 °C (final) at a program rate of 10 °C/min. Gas flows were as follows: carrier gas (N_2), 70 mL/min; hydrogen, 60 mL/min; air, 80 mL/min; oxygen, 5 mL/min. Derivatized glyphosate eluted at ca. 7.5 min under these conditions. Peaks were quantitated by comparison of peak heights obtained with a Hewlett-Packard (Avondale, PA) 3390A recording integrator with those of external standards.

For confirmation, a Hewlett-Packard Model 5710A gas chromatograph with an NP-TSD detector modified for capillary work with a split injector and detector makeup gas was used. The column was a bonded-phase FSOT DB-1 column (J&W Scientific, Rancho Cordova, CA; 30 m × 0.31 mm i.d., 0.25-μm thickness). The carrier gas, helium, had a linear velocity of 29 cm/s, with a split ratio of 111 to 1. Flows to the detector were hydrogen 3, air 40, and makeup helium 35 mL/min. The bead voltage was 19 V. The injector and detector temperatures were both 200 °C. The column temperature was 150 °C.

RESULTS AND DISCUSSION

Our procedure was adopted and/or modified from several sources. Extraction was with a water–chloroform mixture (Moye et al., 1983) modified by using centrifugation to separate phases rather than filtration. Sample cleanup prior to derivatization involved use of, first, an Amberlite anion-exchange column eluted with 0.4 N ammonium bicarbonate ["Pesticide Analytical Manual" (1977) as modified] and, second, a gel permeation column eluted with pH 2.1 water [Guinavan et al. (1982) as modified]. Derivatization was done essentially as described in the "Pesticide Analytical Manual" (1977). Secondary cleanup of the *N*-trifluoroacetyl *O,O,O*-trimethyl derivative of glyphosate was adopted from the multiresidue method of Wehner et al. (1984) substituting a methyl *tert*-butyl ether–THF gradient for hexane–methyl *tert*-butyl ether. Determination of derivatized glyphosate was accomplished by packed column GLC, using a flame photometric detector in the phosphorus mode as reported in the "Pesticide Analytical Manual" (1977), with confirmation provided by capillary GLC using an FSOT DB-1 column and NP-TSD detector.

We attempted unsuccessfully to use two literature methods and several modifications of them, using kiwi fruit

Table I. Recovery of Glyphosate from Kiwi Fruit and Asparagus

sample	concentration, ppm: fortified	found	% recovery	SD (σ_n)
kiwi fruit[a]	0.10	0.092	92	
	0.10	0.074	74	
	0.13	0.12	92	
	0.25	0.23	92	
	0.25	0.21	84	
			$\bar{x}$ = 87	7.1
kiwi fruit—freezer[b]	0.20	0.19	95	
	0.25	0.14	56	
	0.50	0.44	88	
			$\bar{x}$ = 80	17.0
asparagus[a]	0.05	0.030	60	
	0.05	0.040	80	
	0.10	0.14	140	
	0.10	0.11	110	
	0.10	0.054	54	
			$\bar{x}$ = 89	36.0
asparagus—freezer[b]	0.20	0.19	94	

[a] Fortified just prior to extraction. [b] Fortified prior to storage in the freezer (−10 °C). The freezer storage period was 14 months (kiwi fruit) and 19 months (asparagus).

as the test substrate, before adopting the method described herein. The method described by Guinavan et al. (1982) for glyphosate and its major metabolite, (aminomethyl)-phosphonic acid (AMPA), on blueberries, which includes a cleanup sequence of gel permeation and cation-exchange chromatography followed by derivatization with 2-chloroethanol and heptafluorobutyric anhydride, gave an unacceptable level of GLC interference and very low recoveries when applied to kiwi fruit. It appeared that the cleanup did not remove matrix-derived materials which prevented efficient derivatization and which themselves interferred in GLC using either electron-capture, flame photometric, or NP-TSD detectors. The "Pesticide Analytical Manual" (1977) method, which also uses cation-exchange column chromatography in the cleanup, likewise gave far too high a level of GLC interference with kiwi fruit even when secondary cleanup of derivatized glyphosate was carried out by Florisil column chromatography as described with that method. Eliminating either the anion-exchange or gel permeation chromatographic cleanup steps in our method or their order of application also gave unacceptable levels of GLC interference with kiwi fluit with all GLC detectors tested (flame photometric, electron capture, and NP-TSD).

The gradient adsorption HPLC secondary cleanup was essential to successful determination of glyphosate in our kiwi fruit and asparagus samples. When this secondary cleanup step was omitted, kiwi fruit and asparagus samples produced too high a level of GLC interference to permit analysis at subppm levels (Figure 1b,c). These interferences were reduced somewhat when secondary cleanup was carried out by Florisil column chromatography as described in the "Pesticide Analytical Manual" (1977) (Figure 1d,e) but again not sufficiently for subppm determination. When HPLC cleanup was used, the level of GLC interference was negligible at the retention time of derivatized glyphosate (Figure 1f,g), although an elevated GLC background was often encountered, necessitating base-line adjustment prior to elution of the peak of interest.

Recoveries of glyphosate fortified to kiwi fruit and asparagus are listed in Table I. Mean recoveries and recovery precision in the fortification range of 0.10–0.25 just prior to extraction of kiwi fruit were quite good. Mean

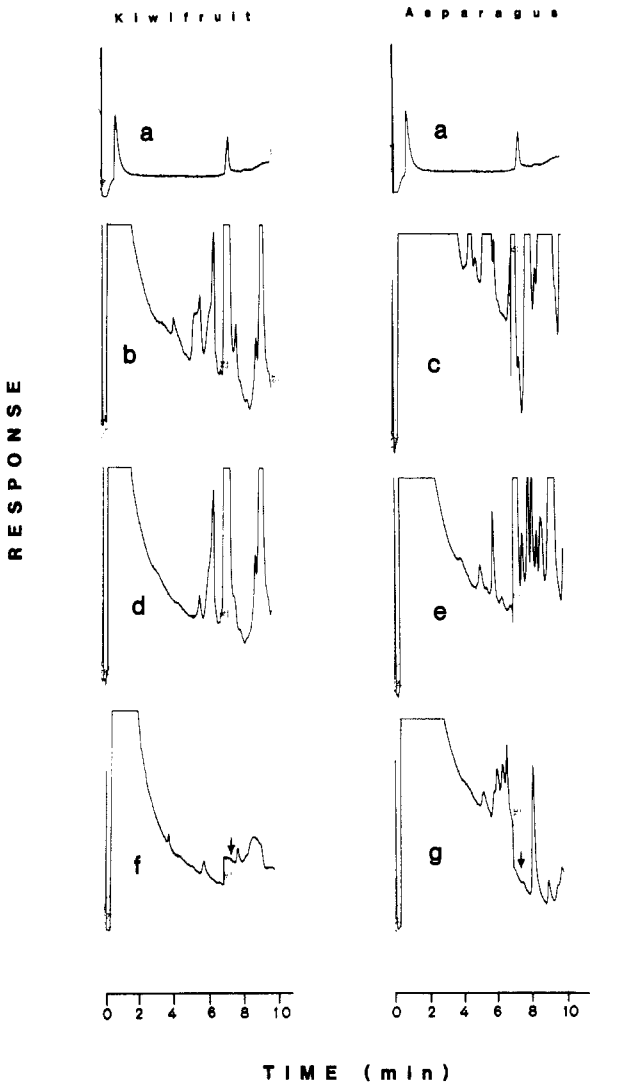

Figure 1. Gas chromatograms of (a) derivatized glyphosate standard (1.2 ng), (b) untreated kiwi fruit extract without HPLC secondary cleanup, (c) untreated asparagus extract without HPLC secondary cleanup, (d) untreated kiwi fruit extract with Florisil column chromatography secondary cleanup, (e) untreated asparagus extract with Florisil column chromatography secondary cleanup, (f) untreated kiwi fruit extract with HPLC secondary cleanup, and (g) untreated asparagus extract with HPLC secondary cleanup.

recoveries for asparagus in the fortification range 0.05–0.10 just prior to extraction were also quite good, but the precision was less than that for kiwi fruit owing apparently to the lower fortification levels used for asparagus. Fortification at 0.10 ppm for both crops produced a GLC recorder deflection of ca. 30% full scale and a signal to noise ratio of ca. 10:1. The detection limit was approximately 0.05 ppm and was verified by fortification at that level to asparagus. Although not verified by fortification, we estimated that glyphosate at 0.01 ppm would be detectable by this method in most samples of kiwi fruit and asparagus.

Recoveries of glyphosate from both crops after prolonged storage in a freezer were acceptable (Table I). This indicates no breakdown of glyphosate and no irreversible sorption to the sample matrix during freezer storage.

While our objective was to find a workable analytical method for the parent glyphosate, we briefly investigated the method's potential for determination of the AMPA metabolite. AMPA was recoverable by the same extraction and cleanup procedures used for glyphosate. Derivatized

AMPA [*O,O*-dimethyl [(trifluoroacetyl)amino]-phosphonate] had an HPLC retention time of 25 min, eluting just after derivatized glyphosate. The AMPA HPLC fraction from control asparagus samples gave only very small GLC peaks at the GLC retention time of derivatized AMPA. From the interfering peak size in comparison with that of a standard of derivatized AMPA, it appeared that AMPA analysis could proceed successfully by this method at levels of ca. 0.10 ppm and above in asparagus. However, this potential was not verified by fortification experiments.

We found that the HPLC cleanup step was a relatively simple adjunct to the analytical procedure for the two crops examined, adding approximately 2 h to the analysis time for each sample once the HPLC system was set up. It is also quite reproducible; we analyzed approximately 40 field-treated kiwi fruit and asparagus samples, including numerous repeats, at the University of California laboratory using a single HPLC column with no noteworthy change in column retention or efficiency characteristics. The selectivity afforded by HPLC cleanup suggests potential utility for glyphosate analysis on other "problem" substrates. The HPLC cleanup is not, however, essential for all crops. Experience at the Michigan State University laboratory showed that some crops (strawberries, cantaloupe, cucumber, and acorn squash) could be analyzed successfully for parent glyphosate by the procedure described herein, or minor modifications of it (a column at 5% Thermon 3000 on AW-DMCS Chromosorb W, 80–100 mesh, supplied by Shimadzu was used for GLC), without the secondary HPLC cleanup. The need for HPLC cleanup of derivatized glyphosate should thus be evaluated on an individual crop basis.

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Registry No. Glyphosate, 1071-83-6.

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A Gas Chromatographic Method for Rapid Determination of Food Additives in Vegetable Oils

A rapid and simple method for the simultaneous determination of five kinds food additives [dehydroxyacetic acid (DHA), 3(2)-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), and 4-(hydroxymethyl)-2,6-di-*tert*-butylphenol (Ionox-100)] in commercial vegetable oils was studied by means of high-resolution gas chromatogrphy with a fused silica capillary column. By use of this method, the separation between food additives and fatty components, the reproducibility of food additives' retention time, and peak area were greatly improved. Some vegetable oils contained about 4–5 ppm of DHA and less than 0.5 ppm of BHA but did not contain BHT, TBHQ, and Ionox-100 in the range of our experiments.

The addition of food additives such as 3(2)-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), dehydroxyacetic acid (DHA), *tert*-butylhydroquinone (TBHQ), 4-(hydroxymethyl)-2,6-di-*tert*-butylphenol (Ionox-100), and so on is effective for the purpose of conservation of food quality. For the determination of food additives, gas chromatographic (GC) techniques have been used since the 1960s.

Vacuum sublimation as a pretreatment for GC reported by MaCaulley et al. (1967) is convenient for decreasing the interference peaks on the chromatogram, but the analysis takes a long time. Kline et al. (1978) developed the use of a precolumn in order to prevent the appearance of oil component peaks when the diluted sample with solvents was injected directly into GC, and they measured a few ppm level of antioxidants contained in oils. But these methods have the disadvantage of not being able to determine DHA, which has been widely used.

If a column having a high power for separation of the oil components such as a capillary column and splitless mode was adopted, we thought that the DHA could be measured and the lower detection limit would be improved. Thus, studies on a rapid method for the simultaneous determination of five kinds of food additives including DHA in vegetable oils by means of a high-resolution GC with a capillary column were carried out.

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