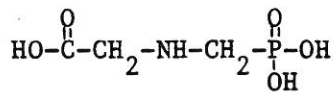

N-(phosphonomethyl)glycine

CAS 1071-83-6

I. Monsanto Co., St. Louis, MO 63166 - Method submitted with Pesticide Petition 5F1536. See Method I.

Glyphosate and its major metabolite, aminomethylphosphonic acid, can be isolated from aqueous extracts of various samples--barley, corn, cotton, oats, rice, soil, sorghum, soybeans, water, and wheat. The isolated residue is further purified by ion exchange chromatography, and the compounds are converted to the corresponding N-trifluoroacetyl methyl esters, which are determined by GLC. Two derivatization techniques are described.

In an EPA method tryout, glyphosate and its major metabolite were added to soybean samples. The recoveries of glyphosate, added at the 0.1 and 0.2 ppm levels, were 55-70%. The recoveries of the metabolite, added at the 0.1 ppm level, were 45 and 60%. Apparent residues in the crop were <0.01 ppm.

Product application: barley, corn, forage grasses, oats, rice, soil, sorghum, soybean forage and hay, water, wheat

Detection limit: 0.05 ppm (50 g sample), 0.10 ppm (25 g sample), 2.5 ppb (1 kg water sample)

SELECTED REFERENCES

1. Moye, H.A., & Boning, A.J., Jr. (1979) A Versatile Fluorogenic Labelling Reagent for Primary and Secondary Amines: 9-Fluorenylmethyl Chloroformate. Anal. Lett. 12(B1), 25-35.

Glyphosate and aminomethylphosphonic acid are derivatized with 9-fluorenylmethyl chloroformate, a reagent which possesses a fluorescent moiety and is commercially available in pure form. The reaction proceeds rapidly under alkaline aqueous conditions, and excess reagent is removed by partitioning. The derivatized compounds are separated on a μCarbohydrate (pentylamine) or μNH_2 column by anion exchange HPLC with fluorometric detection.

2. Moye, H.A., & St. John, P.A. (1980) A Critical Comparison of Pre-Column and Post-Column Fluorogenic Labeling for the HPLC Analysis of Pesticide Residues. In Pesticide Analytical Methodology, J. Harvey, Jr., and G. Zweig (Eds), ACS Symposium Series, No. 136, American Chemical Society, Washington, DC, pp. 90-102.

The 9-fluorenylmethyl chloroformate reagent (ref. 1) and the o-phthalaldehyde-mercaptoethanol system (ref. 3) were chosen to study the relative merits of precolumn and postcolumn fluorogenic labeling when used for the determination of glyphosate and its major metabolite, aminomethylphosphonic acid. The Dowex 50W-X8 cation exchange column described in ref. 3 was used in all cases for cleanup and separation of glyphosate and aminomethylphosphonic acid before derivatization. The 9-fluorenylmethyl chloroformate reagent also derivatizes alcohols under the conditions that were used and, therefore, could be expected to react with crop coextractives to produce possible interferences; this was observed for the cantaloupe aminomethylphosphonic acid fraction. Since the o-phthalaldehyde-mercaptoethanol system is a primary amine-specific reagent, fewer potential interferences ought to be expected. Amino acids, as well as several naturally occurring phosphonic and sulfonic acids, did not interfere with the postcolumn fluorogenic labeling determination of the early eluting aminomethylphosphonic acid; an unidentified component, which eluted after aminomethylphosphonic acid and did not interfere at the 0.1 ppm level, was observed for several crops.

3. Moye, H.A., Miles, C.J., & Scherer, S.J. (1983) 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. J. Agric. Food Chem. 31, 69-72.

Residues of glyphosate and its major metabolite, aminomethylphosphonic acid, are extracted from fruits and vegetables with water. A cation exchange column (Dowex 50W-X8) is used for liquid chromatographic cleanup; the glyphosate and aminomethylphosphonic acid are collected in separate fractions. Each compound is determined separately by HPLC with fluorometric detection on an anion exchange column by using postcolumn fluorogenic labeling with o-phthalaldehyde-mercaptoethanol. Two different anion exchange columns (13.5 µm Aminex A-27 and 7-10 µm HA-X10) were used; either column type is suitable for the determination of glyphosate or aminomethylphosphonic acid. Postcolumn oxidation with calcium hypochlorite is used to convert glyphosate to a primary amine (presumably ammonia and/or glycine) for the derivatization step. With this method, residues of glyphosate and aminomethylphosphonic acid can easily be measured at the 0.05 ppm level; recoveries were ≥60%.

Analytical Residue Method for the Determination of Glyphosate and Aminomethylphosphonic Acid Residues in Forages, Grains, Soil, and Water

Monsanto Chemical Co., St. Louis, MO 63166
(Method C, Aug. 1, 1975)

Principle

The combined residue that may result from the use of glyphosate as a nonselective herbicide can be isolated from aqueous extracts of environmental samples by elution from appropriate ion exchange resins. Further purification of the isolated residue fraction plus final conversion of the parent molecule and its major metabolite to the corresponding N-trifluoroacetyl (TFA) methyl ester compounds results in molecules suitable for quantitative determination by GLC, using a phosphorus-specific flame photometric detector.

The derivatization steps given in this method utilize a methyl pseudourea as the methylating agent. An alternative procedure, using diazomethane, is also given for those laboratories not desiring to spend the time required to prepare and use the much less hazardous methyl pseudourea.

It is important to note that laboratories desiring to check this method by spiking distilled or deionized water must follow the procedure given for distilled water. In addition, steps in this procedure cannot be checked individually. Any step-by-step checkout must include the AG 50W-X8 step before derivatization. The sensitivity of the method is 0.05 ppm (50 g sample), 0.1 ppm (25 g sample), and 2.5 ppb (1 kg water sample).

The method described determines residues of glyphosate and aminomethylphosphonic acid, its major metabolite, in the following samples: soybean (forage, hay, grain); cotton (forage, hay, seed); wheat (forage, straw, grain); corn, field (forage, fodder, grain); corn, sweet (forage, fodder, grain - kernels plus cob with husk removed); barley (forage, straw, grain); oats (forage, straw, grain); sorghum (forage, fodder, grain); rice (forage, straw, grain); water (environmental, distilled); soil (light, medium, heavy).

Caution

Derivatization steps should be performed in a well ventilated fumehood as several reagents are highly corrosive, toxic, and explosive. Avoid reagent contact with hands by wearing laboratory or surgical-type gloves during acylation. Wear safety glasses at all times.

Apparatus

(a) Rotary evaporator. - Calab.

(b) Chromatographic tubes. - 2.2 x 30 cm, with 250 ml reservoir; 1.2 cm od (1.0 cm id) x 20 cm with 3 x 8 cm reservoir and 1.5 cm delivery tip. Sizes are given as outside dimensions.

(c) Food chopper. - Hobart.

(d) Blender. - Explosion-proof (Waring).

(e) Funnel. - 60 ml Pyrex with medium porosity fritted disk.

(f) Centrifuges. - (1) Basket type (International Equipment Co.). (2) Automatic refrigerated centrifuge, Sorvall/RC2B Superspeed (Sorvall), or equivalent.

(g) Gas chromatograph. - Tracor MT-220 or 222 with phosphorus-specific flame photometric detector.

Reagents

Use deionized water throughout.

(a) Charcoal. - Darco G-60 (Matheson, No. CX 645).

(b) Ion exchange resins. - (1) Cation exchange resin. - AG 50W-X8, analytical grade, 200-400 mesh, hydrogen form (Bio-Rad Laboratories). Divide 1 lb bottle of resin in half and wash each half 3 times with 500 ml deionized water. Store resin under deionized water until use. (Note: Ion exchange resins obtained from Bio-Rad Laboratories are renamed from the corresponding Dowex designation. Thus, Dowex 50-X8 becomes AG 50-X8. Frequently, the designations are shortened, e.g., to Dowex 50 and to D-50.) (2) Anion exchange resin. - Duolite A-101D, 20-50 mesh, chloride form (Nopco Division of Diamond Shamrock Co., Redwood City, CA). Slurry resin with deionized water and transfer to suitable size chromatographic column with medium porosity fritted support disk and Teflon stopcock at exit end. Elute resin with 1M ammonium bicarbonate until aliquot of eluate leaves no visible residue after triple evaporation with 50 ml portions of deionized water. This will require ca 13-15 L ammonium bicarbonate/lb resin. Wash resin with 4 bed volumes of deionized water. Remove excess liquid and store until ready for use.

(c) Glass fiber paper. - 3.7 cm (Reeve Angel).

(d) Ammonium bicarbonate solutions. - 0.2, 0.5, and 1.0M.

(e) Solvents. - Reagent grade n-butanol, chloroform, and tetrahydrofuran (THF); Nanograde methanol and methylene chloride; petroleum ether.

(f) Trifluoroacetic acid and trifluoroacetic anhydride. - Aldrich or Eastman.

(g) Cuprous chloride. - Fisher Scientific.

(h) Dicyclohexylcarbodiimide. - Aldrich or Eastman.

(i) Phosphoric acid. - Anhydrous (Matheson, No. PX1003).

(j) Sodium sulfate. - Anhydrous, reagent grade.

(k) Florisil. - 60-100 mesh (Floridin Co.).

(l) Alumina. - Adsorption-type neutral, Brockmann activity I, 80-200 mesh (Fisher Certified).

(m) O-Methyl-N,N'-dicyclohexyl pseudourea. - Omit if diazomethane derivatization is used. Prepare as follows: Fit 250 ml round-bottom flask with reflux condenser and drying tube charged with 97 g (0.47 mol) freshly distilled dicyclohexylcarbodiimide and 16.5 g (0.52 mol) anhydrous methanol. Place solution in 30°C water bath and slowly stir with magnetic stirrer. Add 50 mg cuprous chloride (0.5 mmol); after short induction period solution warms to reflux. Let solution and water cool to room temperature and stir reaction mixture overnight. IR can be used to monitor for carbodiimide, since carbodiimides display intense absorption between 2150 and 2100 cm^{-1} for N=C=N stretching vibration. Generally it has been necessary to add 2 ml methanol and to heat reaction mixture at 50°C for several hours to consume final traces of dicyclohexylcarbodiimide. When no IR absorption in 2150-2100 cm^{-1} region is present, cool solution and add 100 ml petroleum ether. Filter solution through paper and then pass through 1.1 cm (od) x 12 cm column of alumina to help remove all cuprous chloride. Wash alumina with 50 ml petroleum ether, remove petroleum ether on rotary evaporator, and vacuum-distill (bp 90-95°C, 0.05 mm) product to give 93.5 g (85%) of desired pseudourea. This compound may be stored at room temperature for several months without noticeable decomposition. The O-methyl-N,N'-dicyclohexyl pseudourea may crystallize on standing (literature mp 32-33°C).

Preparation of Standard Solutions

(a) Fortification solutions. - Separately weigh and dissolve 0.1000 g glyphosate and metabolite in 100.0 ml 0.2M ammonium bicarbonate. Dilute as needed for sample fortification. Store at 4-6°C.

(b) GLC standard solutions. - See Diazomethane Derivatization if diazomethane is to be used. (1) Glyphosate (N-TFA trimethyl ester of glyphosate). - Assemble reflux apparatus consisting of 50 ml recovery flask with S neck for reflux condenser fitted with drying tube. Perform all of following heating and evaporation steps in well ventilated fumehood. Weigh 0.0125 g glyphosate and transfer to recovery flask. Add 12.5 ml trifluoroacetic acid. Using electric heating mantle, slowly warm mixture to dissolve glyphosate. Add 13.5 ml trifluoroacetic acid anhydride, reassemble reflux-drying assembly, and gently reflux 15 min. Remove heat source, let solution cool to room temperature, disconnect reflux-drying assembly, and slowly evaporate mixture to dryness under stream of dry nitrogen. Redissolve residue in 8 ml 4% methanol in THF containing 0.075 mg anhydrous phosphoric acid/ml. Add 100 mg O-methyl-N,N'-dicyclohexyl pseudourea in 2 ml THF and tightly stopper flask. Heat solution 16 hr in 80°C sand bath. (Formation of droplet on bottom of stopper indicates that flask is not leaking.) Cool flask to room temperature, transfer contents to 25 ml volumetric flask, and dilute to volume with portions of methanol rinses of reaction flask. The methanol dissolves any dicyclohexyl urea which may have precipitated during esterification. Concentrated stock solution contains N-TFA trimethyl ester equivalent to 500 µg glyphosate/ml. Prepare dilutions containing the equivalent of 2.0, 1.0, 0.5, and 0.25 µg by diluting 0.40, 0.20, 0.10, and 0.05 ml stock solution to 100.0 ml with THF. Store solutions at 4-6°C.

(2) Metabolite (N-TFA dimethyl ester of aminomethylphosphoric acid). - Weigh 0.0125 g aminomethylphosphonic acid and transfer to 50 ml recovery flask with reflux condenser and drying tube. Add 12.5 ml trifluoroacetic acid and gently warm solution on electric heating mantle until metabolite is dissolved. Cool to room temperature, add 13.5 ml trifluoroacetic anhydride, tightly stopper

flask, and let stand 30 min at room temperature with occasional swirling. Slowly evaporate mixture to dryness under stream of dry nitrogen. Redissolve residue in 8 ml 4% methanol in THF containing 0.09 mg anhydrous phosphoric acid/ml and continue as described above for parent compound.

Sample Preparation

Each crop sample requires slight modifications of basic analytical procedure. To simplify this method, the operating instructions for each step are described below and specific details are given in Table 1.

(a) Soil. - Weigh 25 g well mixed and screened (-8 mesh) sample whose water content was previously adjusted to or determined to be 10-20%. (Extraction efficiency is somewhat less with relatively dry samples.) Transfer sample to 250 ml polypropylene centrifuge bottle. Add 150 ml 0.5M ammonium hydroxide, cap, and place on reciprocal shaker (or equivalent agitation) for 15 min. Centrifuge 15 min at 11,000 rpm. Decant supernate into 1 L container. Repeat above steps 4 more times, combining supernates after each cycle and discarding soil cake after last cycle. Filter combined extracts by slowly pouring suspension through desk-top basket centrifuge fitted with double strip of Whatman No. 2 paper. (Resulting filtrate will be free of particulate matter but will be cloudy.) Dilute final filtrate with deionized water to 1800 ml. Sample is now ready for A-101D column chromatography.

(b) Water. - Generally, water from natural sources will be free of particulate matter; however filtration or centrifugation in desk-top centrifuge may be necessary to avoid obstructing flow through ion exchange column. Measure $\geq$250 but $\leq$1 L sample and proceed to A-101D column chromatography.

(c) Forages and grains. - (1) Grinding. - Grind frozen samples in Waring blender (grains) or Hobart chopper (green and dry forages) with Dry-Ice. Do not grind cottonseed samples. Keep ground sample overnight in ventilated cold room or refrigerate to let Dry-Ice sublime. Thoroughly mix ground sample or cottonseed and proceed to pre-extraction (grains and cottonseed) or aqueous extraction (forages) step below.

(2) Pre-extraction. - Weigh amount of sample given in Table 1 into 1 qt Waring blender. Add first portion of first solvent as listed and blend 1 min. Filter sample through Whatman No. 1 paper in Büchner funnel into 1 L flask, using vacuum. Rinse blender cup with 2-3 ml appropriate solvent and drain onto existing cake. If only 1 extraction is required, if the solvent extraction is last in a series of solvent extractions, or if a new solvent is to be used, vacuum-dry cake 15 min. For second and further solvent extractions, transfer cake back into blender cup and proceed as above. To facilitate later filtrations, add paper from first filtration with cake for second and further extractions. Vacuum-dry last filter cake 15 min. Discard all filtrates from pre-extractions.

(3) Aqueous extraction. - Transfer weighed or preextracted sample as determined from Table 1 to clean Waring blender cup, add specified amount of deionized water, and blend 1 min. Determine from Table 1 whether refrigerated or basket centrifuge is to be used. For basket centrifuge, slowly pour suspension into desk-top centrifuge fitted with strip of Whatman No. 2 paper. Collect filtrate and blender cup rinses, and refilter through same filter cake

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Table 1. Conditions for the determination of glyphosate and its major metabolite

Crop	Sample wt, g	Pre-extns[a] A	Pre-extns[a] B	Pre-extns[a] C	Centrifugation: Water xtn	Centrifugation: Type	Centrifugation: rpm x 10^3	Centrifugation: Time, min	AG 50W-X8 Column: Glyphosate ml discarded	AG 50W-X8 Column: Glyphosate ml collected	AG 50W-X8 Column: Metabolite ml discarded	AG 50W-X8 Column: Metabolite ml collected
Barley												
grain	50		1 x 300		1 x 400	refrig.	12.5	30	15	10	5	30
green forage	50				1 x 250	basket			14	10	6	30
straw	25				1 x 400	basket			14	10	6	30
Corn												
grain (field)	50	2 x 300	2 x 300		2 x 250	refrig.	11.0	30	14	10	6	30
grain (sweet)[b]	50			2 x 500	1 x 250	refrig.	11.0	30	14	10	6	30
forage	50				1 x 250	basket			13	10	7	30
fodder	25				1 x 400	basket			12	10	8	30
Cotton												
grain	25		1 x 300		1 x 400	refrig.	12.5	50	12	10	8	30
forage	50				1 x 250	basket			14	10	6	30
hay	25				1 x 400	basket			14	10	6	30
Oat												
grain	25		1 x 300		1 x 400	refrig.	12.5	30	13	10	7	30
forage	50				1 x 250	basket			15	10	5	30
straw	25				1 x 500	basket			14	10	6	30
Rice												
grain	50		1 x 300		1 x 400	refrig.	12.5	30	13	10	7	30
forage	50				1 x 250	basket			15	10	5	30
straw	25				1 x 400	basket			15	10	5	30
Soil	25		See Method						10	8	16	20
Sorghum												
grain	50		1 x 300		1 x 400	refrig.	12.5	30	13	10	7	30
forage	50				1 x 250	basket			15	10	5	30
fodder	25				1 x 400	basket			15	10	5	30
Soybean												
grain	25	2 x 300	2 x 300		2 x 250	refrig.	12.5	50	13	10	7	30
forage	50				1 x 250	basket			14	10	6	30
hay	25				1 x 400	basket			14	10	6	30
Water												
environmental	1000								9	9	17	25
distilled	1000								5	12	17	25
Wheat												
grain	50		1 x 300		1 x 400	refrig.	11.0	30	14	10	6	30
forage	50				1 x 250	basket			14	10	6	30
straw	25				1 x 400	basket			14	10	6	30

[a] Solvent A = n-butanol saturated with water; B = methanol-chloroform (2+1); and C = methanol-chloroform (1+2).

[b] Sweet corn grain samples include the cob.

to remove additional particulate matter. Rinse filter mat and all receivers with deionized water. Dilute combined filtrate and rinses to 1800 ml. Sample is now ready for A-101D column chromatography. For refrigerated centrifuge, evenly divide extract between two or three 250 ml polypropylene centrifuge bottles. Wash blender cup with 50-75 ml deionized water, using wash to balance centrifuge bottles. Centrifuge pairs should be balanced within 3 g to avoid excessive vibration. Centrifuge extracts according to time and rpm settings shown in Table 1. Decant supernate through powder funnel plugged with glass wool. Where second extract is required (Table 1), transfer solid residue to blender cup, using two 50 ml portions of deionized water rinses of each centrifuge bottle. Blend 1 min and repeat balancing, centrifuging, and decanting steps described above. After extract has finished filtering, rinse glass wool plug and side of funnel with 2-3 ml water. After dilution to 1800 ml, extracted sample is ready for A-101D column chromatography.

Column Chromatography (A-101D Bicarbonate Form)

Using 2.2 x 30 cm column plugged with glass wool, add 7-8 cm deionized water to column. Measure and transfer 25 ml A-101D resin to column. Pre-wash with 100 ml 1M ammonium bicarbonate followed by three 100 ml rinses with deionized water.

Transfer diluted sample to ion exchange column and let sample flow through at 600-800 ml/hr. Follow sample with three 100 ml portions of deionized water. Discard all eluates. Elute parent molecule and metabolite from column with 0.5M ammonium bicarbonate. Use 6 separate 25 ml portions for forage, grain, or soil samples and 7 separate 25 ml solution portions for water samples. Combined 150 ml solution from forage, grain, or soil samples is ready for charcoal treatment. Combined 175 ml solution from water sample is ready for evaporation.

Charcoal Treatment

Do not use for water samples.

Add 2.0 g charcoal to A-101D fraction collected above and mechanically shake 15 min. Filter mixture, with suction, through medium fritted glass funnel fitted with glass fiber pad. Wash bottle and filter with two 10 ml portions of 0.5M ammonium bicarbonate. Combine filtrates and washes in 500 ml round-bottom flask.

Evaporation

Evaporate contents of 500 ml round-bottom flask to dryness on rotary evaporator and 50°C water bath. Wash sides of flask with 50 ml deionized water and evaporate again. Repeat 50 ml wash and evaporation 2 more times. Dissolve final residue in 5 ml deionized water. Sample is now ready for AG 50W-X8 column chromatography.

Column Chromatography (AG 50W-X8 Hydrogen Form)

Prepare AG 50W-X8 column by transferring ca 12.0 ml previously water-washed resin to 1.2 cm od x 20 cm column with 1.5 cm delivery tip containing 5-10 cm deionized water and glass wool plug. Let resin settle and wash column with 75 ml deionized water from siphon and reservoir. (Siphon apparatus can be beaker

placed ≥3" higher than column; 0.062" id polyethylene tubing can be used for water flow, with glass tubing inserted in rubber stopper placed on column.) Using disposable pipet, adjust resin column length to 14.5 cm.

Place dissolved residue from evaporation step (5 ml) on column with disposable pipet. Let first 5 ml sample aliquot enter column. Follow sample with two 5 ml rinses (one at a time) of flask, using same disposable pipet. Just as last of total transfer volume enters column, connnect overhead siphon and continue elution with deionized water. Using graduate, discard and collect fractions as given in Table 1. Fraction collected first contains parent molecule and fraction collected second contains metabolite.

Note: Proceed to Diazomethane Derivatization, Procedural Modifications, if diazomethane is to be used.

Transfer 2 sample volumes to separate 50 ml recovery flasks, add 0.5 ml (glyphosate) or 0.2 ml 1M ammonium bicarbonate (metabolite), and evaporate to dryness on rotary evaporator, using 25 C water bath. Slowly increase bath temperature to 40 °C. The 2 portions are now ready for derivatization. (Flasks with dry evaporated residue should show white crusty film; this is desirable.)

Derivatization With Methyl Pseudourea

See Caution.

(a) Glyphosphate. - "Dry" contents in recovery flask containing final evaporated residue by gentle stream of dry nitrogen to remove last traces of moisture. Add 1 ml trifluoroacetic acid to dry flask and let stand 5 min with occasional swirling to rinse sides of flask. Add 1.1 ml trifluoroacetic anhydride, firmly stopper flask with glass stopper, and place 10 min in 50°C water bath. Periodically swirl reaction mixture during heating period (3-4 times). Remove flask, let cool to room temperature, and remove stopper. Evaporate contents to dryness under gentle stream of dry nitrogen. Add 4 ml 4% methanol in THF containing 0.15 mg anhydrous phosphoric acid/ml. Add 80 mg O-methyl-N,N'-dicyclohexyl pseudourea in 1 ml THF, tightly stopper flask, and heat 16-18 hr in 80°C sand bath. Cool to room temperature and transfer solution to 5 ml graduated tube. If necessary, adjust volume to 5 ml, using 4% methanol in THF. Sample is ready for GLC.

There must be an excess of pseudourea present at all times to insure complete esterification. Presence of O-methyl-N,N'-dicyclohexyl pseudourea can easily be determined by standard flame ionization gas chromatograph and 4' column packed with 3.8% SE-30 on 80-100 mesh Chromosorb W and operated at 190°C. For derivatization of glyphosate 80 mg is enough in most cases.

Formation of large amounts of insoluble dicyclohexyl pseudourea or poor recoveries with fortified samples are signs that substantial amount of pseudourea has been consumed by other reactants and complete methylation of glyphosate has probably not taken place. If this occurs, add additional 40 mg O-methyl-N,N'-dicyclohexyl pseudourea and repeat heating step for additional 16-18 hr. To save time, check samples for presence of excess pseudourea after 1 hr heating, using flame ionization GLC. If none is present, add more pseudourea reagent and recheck. In all cases, total heating period after last addition of reagent must be 16-18 hr before GLC.

(b) Metabolite. - Add 2 ml trifluoroacetic acid to dry recovery flask and let stand 5 min with occasional swirling to rinse sides of flask. Add 2.1 ml trifluoroacetic anhydride, firmly stopper flask with glass stopper, and place in 50°C water bath for 30 min with occasional swirling. Remove flask, let cool to room temperature, remove stopper, and evaporate contents to dryness under gentle stream of dry nitrogen. Add 4 ml 4% methanol in THF containing 0.15 mg anhydrous phosphoric acid/ml followed by 40 mg O-methyl-N,N'-dicyclohexyl pseudourea in 1 ml THF, tightly stopper flask, and heat 16-18 hr in 80°C sand bath. (Flask is tightly sealed if droplet forms on bottom of stopper.) Cool flask to room temperature and transfer contents to 5 ml graduated tube. If necessary, adjust volume to 5 ml with 4% methanol in THF. Sample is ready for GLC.

Additional Cleanup

With some samples, it may be necessary to reduce background for accurate GLC quantitation. Following procedures can be used if initial GLC injections show interferences.

(a) Florisil cleanup (for glyphosate). - Concentrate 5 ml sample volume from derivatization step to 1 ml by evaporation under gentle stream of dry nitrogen. Prepare column as follows: plug restricted long stemmed (75 mm od x 150 mm) filter funnel with glass wool, add 6 cm Florisil to stem, and top with 1 cm anhydrous sodium sulfate. Pre-wash column with 5 ml THF and 5 ml methylene chloride-THF (3+1), discarding both washes. As last of second pre-wash enters column, change receiver to another 5 ml graduated centrifuge tube and add evaporated esterification solution to top of column, using disposable pipet. Rinse original centrifuge tube with 1 ml methylene chloride-THF (3+1), and add to column as last of sample enters. Repeat rinse addition second time. Continue elution with 2 additional ml methylene chloride-THF (3+1), thus bringing final volume to original 5 ml. Sample is ready for GLC.

(b) Alumina cleanup (for metabolite). - Concentrate 5 ml sample volume from derivatization step to 1 ml by evaporation under gentle stream of dry nitrogen. Prepare column as follows: Plug restricted long stemmed (75 mm od x 150 mm) filter funnel with glass wool, add 6 cm alumina to stem, and top with 1 cm anhydrous sodium sulfate. Pre-wash column with two 5 ml portions of 4% methanol-THF, discarding both washes. As last of second pre-wash enters column, change receiver to another 5 ml graduated centrifuge tube and add evaporated esterification solution to top of column, using disposable pipet. Rinse original centrifuge tube with 2 ml 4% methanol-THF and add to column as last of sample enters. Repeat rinse addition with second 2 ml 4% methanol-THF, thus bringing final volume to original 5 ml. Sample is ready for GLC.

Determination

GLC operating conditions. - (1) Glyphosate. - 6' x 1/4" od glass U-shaped column packed with 10% DC-200 on 80-100 mesh Chromosorb W (HP) or 3.8% OV-17 on 80-100 mesh Gas-Chrom Q; temperatures (°C) - column 185 (DC-200), 150 (OV-17), flash heater 230, detector 185, phosphorus filter 526 nm, gas flows (ml/min) - nitrogen 100, air 40, hydrogen 200, oxygen 20; 6 µl injection. (2) Metabolite - the GLC conditions are the same as those for glyphosate except for column temperatures: 155°C (DC-200) and 120°C (OV-17). These are approximate settings and will vary from instrument to instrument. Retention time of glyphosate or

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its metabolite is ca 2 min; 30 ng of either compound will give ca 50% full-scale deflection.

Precondition GLC column of choice each time with ≥3 prior injections of solutions representative of those to be analyzed. Use standard-sample-standard injection pattern. OV-17 or DC-200 column offers adequate capability for quantitative isolation of derivatized molecules. Final sample quantitation is based on peak height in crop or fortified sample relative to standard peak height calibration across range of expected residue and/or recovery from known additions to control samples.

Diazomethane Derivatization

Additional Reagents

(a) N-Nitrosomethylurea. - K&K.

(b) Diazomethane. - Redistilled in ether (prepared from N-nitrosomethylurea).

(c) Benzene. - Nanograde.

Preparation of GLC Standard Solutions

For diazomethane derivatization only.

(1) Glyphosate (N-TFA trimethyl ester of glyphosate). - Assemble semimicro reflux assembly consisting of 25 ml round-bottom flask with S neck for semimicro reflux condenser fitted with semimicro drying tube. Perform following heating and evaporation steps in well ventilated fumehood. Weigh 0.0050 g glyphosate and transfer to semimicro round-bottom flask. Add 5 ml trifluoroacetic acid. Using semimicro electric heating mantle, slowly warm mixture to dissolve glyphosate. Add 5 ml trifluoroacetic anhydride, re-assemble reflux-drying assembly, and gently reflux mixture 15 min. Remove heat source, disconnect reflux-drying assembly, and slowly evaporate mixture to dryness under stream of dry nitrogen. Redissolve residue in 5 ml methanol. Add 5 ml diazomethane-ether solution and let mixture stand 30 min at room temperature. Remove excess diazomethane-ether by gently warming on steam bath to residual volume of 2-3 ml. Transfer remaining solution to 5 ml volumetric flask and dilute to volume with portions of methanol rinses of reaction flask. Concentrated solution contains N-TFA trimethyl ester equivalent to 1000 µg glyphosate/ml. Prepare dilutions containing 10, 25, and 50 µg/ml by diluting 0.10, 0.25, and 0.50 ml stock solution to 10.0 ml with methylene chloride. Store solutions at 4-6°C.

(2) Metabolite (N-TFA dimethyl ester of aminomethylphosphonic acid). - Weigh 0.0100 g aminomethylphosphoric acid and transfer to 100 ml round-bottom flask. Add 10 ml trifluoroacetic acid and 10 ml trifluoroacetic anhydride. Using electric heating mantle, gently warm mixture until metabolite is dissolved. Tightly stopper flask and let stand 30 min at room temperature. Slowly evaporate mixture to dryness under stream of dry nitrogen. Redissolve residue in 7 ml benzene. Add 4 ml diazomethane-ether solution and let stand 30 min at room temperature. Remove excess diazomethane-ether by gently warming on steam bath to residual volume of 5-7 ml. Transfer remaining solution to 10 ml volumetric flask and dilute to volume with benzene rinses of reaction flask.

Concentrated solution contains N-TFA dimethyl ester equivalent to 1000 µg metabolite/ml. Make subsequent dilutions with benzene. Store solutions at 4-6 °C.

Procedural Modifications

Continue from point noted in Column Chromatography (AG 50W-X8 Hydrogen Form)

Transfer glyphosate sample volume to 50 ml recovery flask; transfer metabolite sample volume to 100 ml round-bottom flask. Add 0.5 ml 1M ammonium bicarbonate to each and evaporate to dryness on rotary evaporator, using 25°C water bath. Slowly increase bath temperature to 40°C. The 2 portions are now ready for derivatization.

Derivatization (With Diazomethane)

See Caution.

(a) Glyphosate. - "Dry" contents in recovery flask containing final evaporated residue by gentle stream of dry nitrogen to remove last traces of moisture. Add 1 ml trifluoroacetic acid to dry flask and let stand 5 min, with occasional swirling to rinse sides of flask. Add 1 ml trifluoroacetic anhydride, firmly stopper flask with glass stopper, and place 10 min in 50°C water bath. Periodically swirl reaction mixture during heating period (3-4 times). Remove flask, immediately loosen stopper, and let flask cool to room temperature. Evaporate flask contents to dryness under gentle stream of dry nitrogen. Add 1 ml methanol, swirl to rinse sides of flask, and let stand 5 min. Add enough diazomethane-ether solution to maintain distinct and permanent yellow color (3-5 ml are normally adequate). Let mixture stand 30 min at room temperature, swirling intermittently.

Remove excess diazomethane-ether by gently warming on steam bath in fumehood. Evaporate to ca 0.5 ml. Using disposable pipet, transfer remaining volume to 5 ml graduated glass-stoppered centrifuge tube. Rinse flask with small portions of methanol and transfer these to graduated tube. Adjust final volume to 1 ml. Withdraw 0.5 ml methanol solution and transfer to second graduated tube. Hold remaining 0.5 ml portion as reserve sample. Dilute 0.5 ml methanol sample aliquot to 3 ml with 5% sodium sulfate. Add 2 ml methylene chloride, stopper, and shake 2 min. Withdraw lower (methylene chloride) layer by disposable pipet. Repeat step and combine methylene chloride extracts in third graduated tube. Evaporate methylene chloride extract to 0.5 ml, using gentle stream of dry nitrogen gas. Sample is ready for GLC.

(b) Metabolite. - "Dry" contents of round-bottom flask containing final evaporated residue by gentle stream of dry nitrogen to remove last traces of moisture. Add 1 ml trifluoroacetic acid to dry flask, and let stand 5 min, with occasional swirling to rinse sides of flask. Add 1 ml trifluoroacetic anhydride, firmly stopper flask with glass stopper, and let stand 30 min at room temperature. Remove stopper and evaporate contents to dryness under gentle stream of dry nitrogen. Immediately add 0.7 ml benzene and swirl to rinse sides of flask. Add enough diazomethane-ether to maintain distinct and permanent yellow color (3-5 ml are normally adequate). Let mixture stand 30 min at room temperature, swirling intermittently.

Remove excess diazomethane-ether by gently warming on steam bath in fumehood. Evaporate to ca 0.5 ml. Using disposable pipet, transfer remaining volume to 5 ml graduated glass-stoppered centrifuge tube. Rinse flask with small portions of benzene and add these to tube. Adjust final sample volume to 1.0 ml. Solution is now ready for GLC.

Additional Cleanup

With some samples it may be necessary to reduce background for accurate GLC quantitation of the parent compound. The following procedure can be used to correct this situation if initial GLC injections show interferences. An additional cleanup is not needed for the metabolite.

Florisil cleanup. - Prepare column as follows: plug restricted long stemmed (75 mm od x 150 mm) filter funnel with glass wool, add 6 cm Florisil, and top with 1 cm anhydrous sodium sulfate. Pre-wash column with 5 ml methanol, followed by 5 ml methylene chloride-acetone (3+1). As last of second pre-wash enters column, change receiver to another 5 ml graduated centrifuge tube and add 0.5 ml partitioned esterification solution to top of column, using disposable pipet. Rinse original centrifuge tube with 1 ml methylene chloride-acetone (3+1) and add to column as last of sample enters. Repeat rinse addition second time. Continue elution with additional 2 ml methylene chloride-acetone (3+1). Adjust sample to original 0.5 ml volume by evaporation under gentle stream of dry nitrogen. Sample is ready for GLC.

Determination

Proceed as described for methyl pseudourea derivatization procedure.

REFERENCES

(1) Vowinkel, E. (1966) "Preparation of O-methyl-N,N'-dicyclohexyl pseudourea." Chem. Ber. 99, 1479.

(2) Vogel, A.I. (1962) A Textbook of Practical Organic Chemistry, 3rd Ed., John Wiley, New York, NY, "preparation of diazomethane".

(3) Dow Chemical Co., Midland, MI (1964) "Preparation of ion exchange resins".

EPA COMMENTS

Modifications

A Packard Model 873 gas chromatograph with a phosphorus-specific flame photometric detector was used in the method tryout. The GLC operating conditions were the same as those described in Method I. Under the stated conditions, 1 ng glyphosate and its metabolite produced 16 and 50% full-scale deflection, respectively. Thirty mg samples were injected and the results were calculated on the basis of peak heights.

Recovery Studies (EPA)

EPA conducted a trial of Method I, using soybean samples fortified at levels of 0.1 and 0.2 ppm glyphosate and 0.1 ppm metabolite. The recoveries of glyphosate were 55-70% and those of the metabolite were 45 and 60%, as shown in Table 1.

Conclusions

Both the parent and the metabolite are stable at room temperature. It is not necessary to store solutions under refrigeration.

Monsanto Co. supplied 10 g O-methyl-N,N'-dicyclohexyl pseudourea, which was the methylation reagent used in the trial. This reagent is stable at room temperature and is ready for use whenever the sample is ready for derivatization.

Each step of the method is time consuming and 4-5 days are needed for the analysis. Preliminary checks of the major steps of the method must include the AG 50W-X8 column prior to derivatization. The ammonium bicarbonate eluant for the charcoal column must be completely removed from the sample solution before chromatography on the AG 50W-X8 column; EPA used an extra distilled water and evaporation step. Much preparation and time must be invested before the analyst is ready for enforcement samples.

Table 1. Recovery of glyphosate and its metabolite from soybean samples (EPA)

Added, ppm		Rec., %	
Glyphosate	Metabolite	Glyphosate	Metabolite
0	0	< 0.01	< 0.01
0	0	< 0.01	< 0.01
0.1	0.1	55	45
0.1	0.1	62	60
0.2	0	60	0
0.2	0	70	0

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