# Fluorescence Detection and Determination of Glyphosate via its N-Nitroso Derivative by Thin-Layer Chromatography

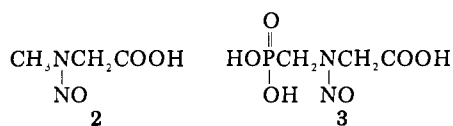J. Christopher Young,* Shahamat U. Khan, and Paul B. Marriage

A sensitive, selective, and simple procedure is described for the analysis of the herbicide glyphosate. The method involves aqueous extraction of glyphosate from the sample, in situ preparation of the N-nitroso derivative with sodium nitrite at pH 3.0, thin-layer chromatographic separation on silica gel, degradation of N-nitrosoglyphosate to aminomethylphosphonic acid with ultraviolet light, and fluorophore formation from this primary amine with fluorescamine. As little as 10 ng (45 pmol) of glyphosate could be detected, and with a minimum of sample cleanup 5 ppm was easily determined in 2-mg samples of the one plant investigated. Roots taken 1, 3, or 6 days after treatment of Canada thistle leaves with 40 mg of glyphosate per plant showed residue levels of 30–60 ppm. Recoveries in spiked samples ranged from 75% at 5 ppm to 90% at 100 ppm, and the precision of the determinations was about ±9%.

The herbicide glyphosate [N-(phosphonomethyl)glycine] (1) controls top growth and prevents shoot regrowth from

$$\underset{\underset{OH\ H}{\mid\ \ \mid}}{HO\overset{\overset{O}{\parallel}}{P}CH_2NCH_2COOH}$$

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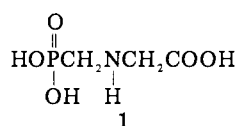
the root or rhizome system of widespread perennial weeds such as Canada thistle (*Cirsium arvense* (L.) Scop.) and quackgrass (*Agropyron repens* (L.) Beauv.). Its effectiveness is based on its ready translocation from the foliage to the underground parts (Davison, 1972; Rioux et al., 1974; Claus and Behrens, 1976; Gottrup et al., 1976; Saidak and Marriage, 1976). The amount of this translocation has been determined by radioisotope techniques employing [^{14}C]glyphosate (Sprankle et al., 1975; Claus and Behrens, 1976; Gottrup et al., 1976). Glyphosate residues in crops, soil, and water have been determined by gas chromatography involving multistep derivatization of the residues after an initial ion-exchange cleanup procedure (Comes et al., 1976). Thin-layer chromatography (TLC) has been employed with visual or autoradiographic detection for examination of glyphosate metabolism (Marvel, 1974; Gottrup et al., 1976; Putnam, 1976). Direct gas chromatographic analysis of glyphosate (Tate and Alexander, 1974) and colorimetric detection with an amino acid analyzer (Ekstrom and Johansson, 1975) have also been studied. Only in the last case has a minimum quantitation level (ca. 3.5 μg) been reported.

Since N-nitrosamino acids, such as N-nitrososarcosine (2), can be detected and determined by TLC (Young, 1977b), it was thought that glyphosate might also be analyzed via its N-nitroso derivative, 3. This paper reports

$$\underset{\underset{NO}{\mid}}{CH_3NCH_2COOH} \qquad \underset{\underset{OH\ NO}{\mid\ \ \mid}}{HO\overset{\overset{O}{\parallel}}{P}CH_2NCH_2COOH}$$

2 3

the development of such a method and its successful application to roots of glyphosate-treated Canada thistle. Bronstad and Friestad (1976) have just reported a method for glyphosate residues in natural waters based on polarography of 3.

Table I. Solvent Systems Used as Developers for Thin-Layer Chromatography

Designation	Solvents (ratios)
A	95% Ethanol–benzene–water (4:1:1)
B	Methanol–chloroform (4:1)
C	Acetonitrile–95% ethanol–glacial acetic acid–water (12:4:3:1)
D	1-Propanol–water (1:1)
E	95% Ethanol–water (7:3)
F	95% Ethanol–concentrated ammonium hydroxide (7:3)
G	95% Ethanol–benzene (4:1)
H	Hexane–ether–methylene chloride (5:7:10)

The basis for the method described in this paper is: (1) aqueous extraction of glyphosate from the sample, (2) in situ preparation of the corresponding N-nitroso derivative 3 with sodium nitrite at pH 3.0, (3) TLC separation, (4) photolytic cleavage of 3 with UV light to afford a primary amine, and (5) formation of a fluorophor with the primary amine specific reagent fluorescamine (Udenfriend et al., 1972).

EXPERIMENTAL SECTION

Materials. Glyphosate, as its isopropylamine salt (Roundup) was obtained from Monsanto Commercial Products Company (St. Louis, Mo.); fluorescamine, pH 9.0 buffer solution, Florisil, and Eastman Chromagram Sheets (13179) 0.1 mm silica gel without fluorescent indicator from Fisher Scientific; XAD-2 ion-exchange resin from British Drug House; and other reagents and solvents of reagent grade from commercial sources.

Preparation and Detection of N-Nitrosoglyphosate (3). To a stirred solution of 13.7 mg (0.06 mmol) of glyphosate isopropylamine salt in 2 mL of 0.12 N hydrochloric acid was added 20 mg (0.29 mmol) of sodium nitrite. After 1 h in the dark the mixture was transferred to a 100-mL volumetric flask and made up to volume with acetone. This standard solution and serial dilutions from it were kept refrigerated when not in use. An aliquot of this solution was spotted on a TLC plate, developed in 95% ethanol–benzene–water (4:1:1), irradiated with ultraviolet (UV) light, sprayed with fluorescamine reagent and viewed under UV light, or sprayed with the diphenylamine–palladium chloride reagent and irradiated for 10 min. A sample of 3 for comparison purposes was prepared by the method of Alt (1975).

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Thin-Layer Chromatography. The solvent systems used in this study are given in Table I. Plates were sprayed sequentially with fluorescamine (0.1 mg/mL in acetone) and triethanolamine (10% in methylene chloride), or with a freshly prepared 1:1 mixture of o-aminodiphenyl (OADP) (1% in 95% ethanol) and 20% aqueous sulfuric acid, or with a freshly prepared 4:1 mixture of diphenylamine (1.5% in 95% ethanol) and palladium(II) chloride (0.1% in 0.2% aqueous sodium chloride), or with phosphomolybdic acid (PMA) (10% in 95% ethanol).

Ultraviolet Irradiation. In some instances plates were irradiated for 10 min with UV light using the apparatus previously described (Young, 1976) and viewed under long-wave UV light. Fluorescence intensities of **3** developed and visualized with fluorescamine reagent were measured using a TLC fluorescence spectrophotometric scanner as reported previously (Young, 1976). Measurements were made for 20, 40, 60, and 80 ng spots and eleven 50 ng spots to obtain a calibration curve and the precision, respectively.

Identification of Products from Photolysis of *N*-Nitrosoglyphosate. Solutions containing 100 ng each of **3**, aminomethylphosphonic acid (**4**), glycine (**5**), **3 + 4**, and **3 + 5** were spotted, irradiated, developed in a variety of solvent systems, sprayed with fluorescamine reagent, and viewed under UV light. Solutions containing 100 ng each of **3**, **4**, and **3 + 4** were spotted, irradiated, overspotted with 1 μL of pH 9.0 buffer, developed in a 0.01% solution of fluorescamine in hexane–acetone (4:1), redeveloped in solvent system B, sprayed with triethanolamine solution, and viewed under UV light.

Solutions containing 10 μg of **3**, 5 μg of glyoxylic acid (**6**), and **3 + 6** were spotted, irradiated, developed in solvent system B, sprayed with OADP, heated at 105 °C for 10 min, and viewed under UV light. These solutions were also spotted, irradiated, overspotted with 5 μg aniline, after 10 min developed in solvent system B, irradiated, sprayed with fluorescamine reagent, and viewed under UV light.

Aqueous solutions containing 1 mg of **3** or 25 μg of formaldehyde were uniformly spotted over the entire surface of a silica gel plate (1 × 20 cm) and dried at room temperature for 45 min or at 105 °C for 15 min. The plates were placed inside a quartz tube (1.5 × 35 cm), continually flushed with a slow stream of dry nitrogen, and irradiated 10 min with UV light. The effluent from the tube was bubbled into 10 drops of solutions of 2,4-dinitrophenylhydrazine (DNPH) (0.5% in 2 N hydrochloric acid) or 5,5-dimethyl-1,3-cyclohexadione and piperidine (10% and 1% respectively in 50% aqueous ethanol). Precipitates, if any, from DNPH solution were separated, dissolved in ethyl acetate, spotted alongside authentic formaldehyde 2,4-dinitrophenylhydrazone, developed in solvent system H, sprayed with PMA, and heated for several minutes at 105 °C. Plates spotted with **3** or acidic aqueous sodium nitrite not treated with glyphosate were treated in a similar manner and the effluents bubbled into a solution of saturated aqueous barium hydroxide.

Glyphosate Treatment of Canada Thistle. Canada thistle plants of the variety *arvense* (*mite*) were started from 7.5 cm root sections (Moore and Frankton, 1974). Roots were cut from plants that had been vegetatively propagated from a single original specimen. After 1 month in the greenhouse, thistle seedlings were transplanted into root boxes 5.1 cm × 21 cm and 76 cm deep similar to those designed by Muzik and Whitworth (1962), containing sterilized potting soil. Complete soluble fertilizer was applied to the soil in the root boxes 1 month after transplanting. When these plants had reached the bud

stage (10 weeks old), they were treated early in the day with commercial glyphosate at 40 mg of active ingredient (approximating a field rate of 4 kg/ha) per plant in 5 mL of water applied to the upper surfaces of leaves and to stems with an artist's brush. Supplementary fluorescent and incandescent light was provided for 6 h in the evening to give a day length of approximately 15 h.

Extraction and Analysis. At 1, 3, and 6 days after treatment, shoots were removed and the roots excavated from the top, middle, and bottom 23-cm sections of soil in the boxes. These roots were washed, surface dried, and frozen until examined further. Prior to extraction, the thawed roots were patted dry, weighed, cut into short segments, and ground in a mortar. The ground roots were macerated in a blender (Sorvall Omni-Mixer) with sufficient water to provide maximum disruption of the roots. The blended material was extracted with 200 to 300 mL of water, depending on the weight of roots, for 30 min in a boiling water bath, filtered by centrifugal filtration (Filtermatic, Chemical Rubber Company) through 3.2 μm pore filter paper, and the residue on the filter rinsed with warm water. After the final extract was brought to 450 mL, aliquots (4 mL) were removed and evaporated to dryness at 60 °C under reduced pressure. Some untreated samples were spiked during blending or after final extraction.

The dried root extracts were dissolved in 2 mL of 0.12 N hydrochloric acid, centrifuged, the supernatant removed, and washed with methylene chloride (6× 3 mL). The aqueous phase was then treated with 20 mg of sodium nitrite, stirred in the dark for 1 h, and washed again with methylene chloride (6× 3 mL). The latter methylene chloride extracts were stored in a hood in a well-stoppered bottle for proper disposal with other chemical carcinogens. Aliquots containing 20, 40, and 60 ng of **3** and an aliquot of the sample aqueous layer were spotted, developed in solvent system A, dried, irradiated, sprayed with fluorescamine reagent, and viewed under UV light. Then an aliquot of the sample, estimated to contain ca. 40 ng of **3** was spotted alongside 30, 35, 40, 45, and 50 ng of standard and analyzed as above.

A sample of root extract spiked with 250 ppm of glyphosate was dissolved in 10 mL of dilute hydrochloric acid and split into five equal portions. Each portion was treated with 20 mg of sodium nitrite and sufficient hydrochloric acid to give final pHs of 2.6, 2.8, 3.0, 3.2, and 3.4, respectively. After 1 h, equivalent aliquots were taken and examined by TLC as above.

Column Chromatography. A sample of root extract spiked with 25 ppm of glyphosate was nitrosated as above. After 1 h the sample was taken to dryness under reduced pressure and the residue dissolved in 10% water in acetonitrile (1 mL). This solution was transferred to a microcolumn prepared by placing 0.8 g of Florisil in a disposable Pasteur pipet and prewashing with 10% water in acetonitrile. The column was eluted successively with 90, 80, 50, and 0% acetonitrile in water (10 mL each), the eluates concentrated to 1 mL, and aliquots examined by TLC.

The above procedure was repeated except a micro XAD-2 ion-exchange column was used. Another sample was dissolved in water (1 mL), placed on a micro XAD-2 ion-exchange column, and eluted with water (10 mL). The eluate was taken to dryness under reduced pressure, nitrosated as above and an aliquot examined by TLC.

Safety Precautions. Many *N*-nitrosamines are potent carcinogens (Druckrey et al., 1967). The *N*-nitrosamines derived from amino acids such as sarcosine and proline are
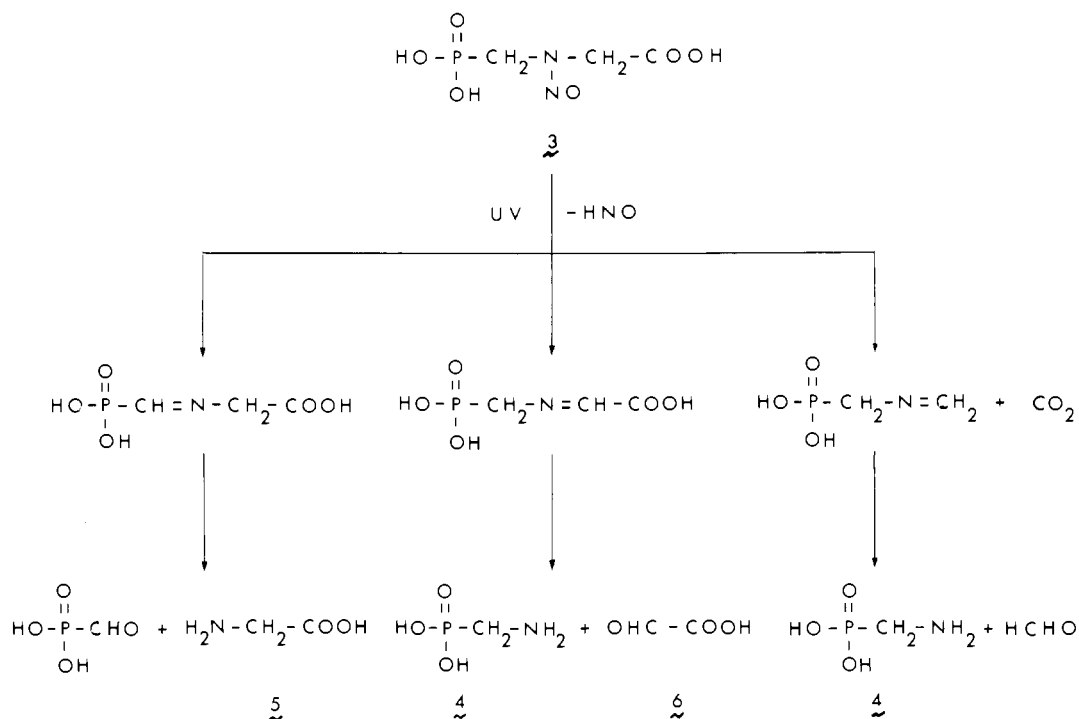
Figure 1. Possible photolytic degradation pathways for N-nitrosoglyphosate.

weakly and not carcinogenic, respectively (Druckrey et al., 1967). Thus, although the carcinogenic properties of N-nitrosoglyphosate are unknown at this time, safety precautions such as those outlined in the National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens (DHEW, 1976) to prevent skin contact and inhalation must be exercised at all times.

RESULTS AND DISCUSSIONS

Method. A concentration of 0.12 N hydrochloric acid was chosen for convenience. Addition of 10 mg of sodium nitrite per milliliter of acid gave pH 3.0, considered to be optimum for nitrosation of secondary amines (Mirvish, 1970, 1972). Nitrosation of glyphosate over the range pH 2.6-3.4 showed a maximum yield of **3** at pH 2.8-3.0. Since the rate of nitrosation is proportional to the square of the nitrous acid concentration (Kalatzis and Ridd, 1966; Mirvish, 1970), the large excess of sodium nitrite employed helped to ensure a high degree of conversion of glyphosate to **3**.

When treated with diphenylamine–palladium chloride (a reagent for detection of nitrosyl radical arising from photolysis of N-nitrosamines (Preussmann et al., 1964a,b)) and then irradiated, **3** gave pale blue-gray spots. The TLC properties of N-nitrosoglyphosate prepared by the method in this paper and by that of Alt (1975) were identical.

A linear calibration curve was obtained from N-nitrosoglyphosate for 0 to 80 ng. For quantitation, the more time consuming spectrophotometric determination of the fluorescence intensity was not necessary. One can easily distinguish between spots differing by ±5 ng at the 50 ng level, and this is at about the observed precision (±9%) for these determinations made spectrophotometrically.

Identity of Photolytic Cleavage Products. Possible products and their formation pathways from photolytic cleavage of **3** are shown in Figure 1. Photoelimination of N-nitrosamines in solution is known to proceed via imine intermediates (Chow, 1973), which in some instances can be isolated (Burgess and Lavanish, 1964). Furey and Kan (1968) and Young (1977a) have reported photochemical hydrolysis of imines to primary amines and aldehydes.

Table II. Thin-Layer Chromatographic R_f Values on Silica Gel

Compd	Solvent system					
	A	B	C	D	E	F
3	0.30	0.28	0.55	0.50		
UV irradiated 3	0.05	0.00	0.18	0.38	0.28	0.41
4	0.05	0.00	0.18	0.38		
5	0.15			0.38	0.38	0.73

To distinguish between these three possible pathways, the primary amino product was first identified by comparison of TLC R_f values of the unknown with those of aminomethylphosphonic acid (**4**) and glycine (**5**). The values given in Table II clearly indicate that **4** but not **5** is a photolysis product. Furthermore, the fluorescamine derivatives of the amino product and **4**, prepared by the method of Nakamura and Pisano (1976) had identical R_f values (0.56). It is of interest to note that **4** is a metabolite of glyphosate (Marvel, 1974).

Glyoxylic acid (**6**) standards could be detected at the 50 ng level using the OADP spray technique (Nakai et al., 1974) and at about 0.5 μg using the aniline–imine technique (Young, 1977a). However **6** was not detected as a photolysis product of **3** by either of these two methods.

The identity of volatile photolysis products was sought by spreading a relatively large quantity of **3** over the entire surface of a 1 × 20 cm plate, irradiating the plate, and trapping the volatiles with compound-specific reagents. Formaldehyde could be detected at the 25 μg level as its 2,4-dinitrophenylhydrazone (e.g., Walsh and Merritt, 1960) or as its dimedone derivative with 5,5-dimethyl-1,3-cyclohexanedione (Horning and Horning, 1946). However, it too was not detected as a photolysis product. On the other hand, carbon dioxide was detected as barium carbonate. Drying the plates at room temperature or at 105 °C had no effect on the outcome.

Application. The method was applied to treated Canada thistle samples, and the residue levels observed in roots are shown in Table III. The percentage of total glyphosate translocated to the root system was very similar

Table III. Glyphosate Residues in Roots of Treated Canada Thistle

Treatment, mg/plant	Days after treatment	Residues, ppm[a]				% translocation
		Top	Middle	Bottom	Total[b]	
0	1	ND[c]	ND	ND	ND	
40	1	45	15	20	30	1.8
40	3	35	50	40	40	2.5
40	6	70	35	75	60	4.3

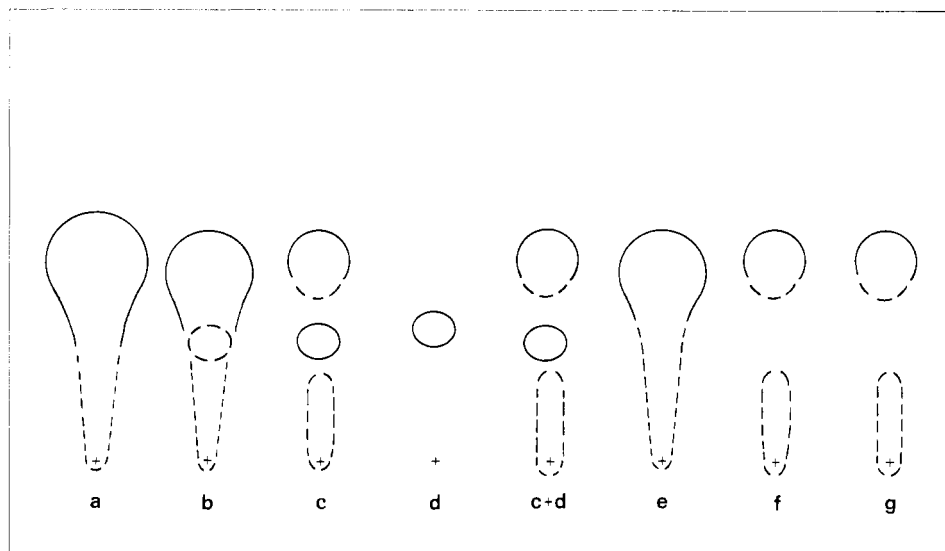[a] Values not corrected for recovery. [b] Weighted average. [c] ND, not detected.

Figure 2. Thin-layer chromatograph of glyphosate-treated and control root extracts from Canada thistle. After nitrosation of extracts, aliquots equivalent to 2 mg of root tissue containing 20 ppm of glyphosate were spotted on silica gel, developed in 95% ethanol–benzene–water (4:1:1), irradiated under UV light, sprayed with fluorescamine reagent, and viewed under long-wave UV light: (a) nitrosated extract, (b) extract washed with methylene chloride before nitrosation, (c) extract washed with methylene chloride before and after nitrosation, (d) N-nitrosoglyphosate standard, (e) nitrosated control extract, (f) control extract washed with methylene chloride before and after nitrosation, (g) same as c except developed plate not irradiated with UV.

to that observed by Sprankle et al. (1975) and Gottrup et al. (1976). Injury to shoots was apparent several days after glyphosate treatment and consisted primarily of necrotic areas on leaves and stems. These progressively became more extensive and severe following the known activity of this herbicide (Spurrier, 1973).

Some interfering compounds were removed by washing the aqueous extract with methylene chloride both before and after nitrosation. Chloroform and ethyl acetate were also used but with less effect. The remaining compounds interfered only when an aliquot equivalent to 2.5 mg (or greater) of root tissue was spotted. In general, aliquots equivalent to ca. 2 mg of root tissue were taken for analysis. Since 10 ng of glyphosate can be detected by this method, the detection limit in Canada thistle roots is about 5 ppm. Recoveries from spiked samples ranged from 75% at 5 ppm to 90% at 100 ppm. Figures 2a–c show the cleanup effect of no prenitrosation and both pre- and post-nitrosation washings with methylene chloride, respectively. The R_f of 3 in the sample matrix is slightly lower than that of pure standard (Figure 2c + d vs. Figure 2d). Figures 2e and 2f show the interferences in control samples that have been washed with methylene chloride before and both before and after nitrosation, respectively. Figure 2g shows that when the sample in Figure 2c is examined by TLC, but without UV irradiation, 3 is not detected and no interferences appear at the same R_f.

It should be noted that other N-nitrosamines in addition to 3 may be formed during the nitrosation step. Lipid-soluble N-nitrosamines, if formed, will be extracted with methylene chloride and this solution should be carefully stored for proper disposal. A comparison of Figures 2c and
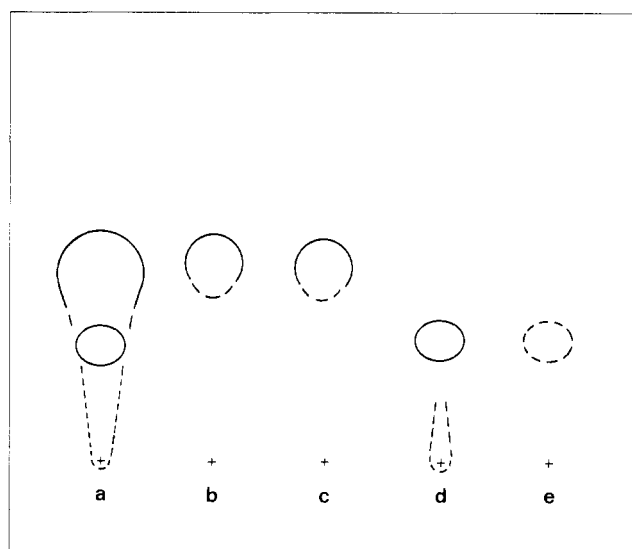

Figure 3. Thin-layer chromatograph of glyphosate-treated root extract from Canada thistle. Nitrosated extract chromatographed through column of Florisil and eluates examined by TLC: (a) extract before Florisil chromatography, (b–e) column eluted with 90, 80, 50, and 0% acetonitrile in water, respectively.

2g shows that only one spot appears after UV irradiation and thus there is only one N-nitrosamine (3) present in the aqueous extract.

In Figure 3, it is seen that interferences can be removed further, thus reducing the detection limit, by chromatography of the nitrosated extract through a short column

of Florisil. Recoveries of **3** standards after chromatography were about 90%. This extra step was not necessary in the analysis of Canada thistle roots.

Separation of interferences and other fluorescent material from **3** was not effected by chromatography through XAD-2 ion-exchange resin. Recovery of **3** was about 90% when glyphosate was nitrosated before chromatography but only 30% if chromatography preceded nitrosation.

SUMMARY

The advantages of the method reported in this paper for the analysis of glyphosate residues are: (a) sensitivity, as little as 10 ng (45 pmol) can be detected; (b) selectivity, interferences are only from surviving primary amines or those compounds affording primary amines after nitrosation and UV irradiation; (c) simplicity, extensive cleanups are not required and isolation of **3** is not necessary since an aliquot of the reaction mixture is analyzed directly; and (d) speed, once the initial extraction is completed, the remaining steps can be accomplished in several hours and many samples can be analyzed simultaneously. The other reported methods are more complex and time consuming. A disadvantage of this method is the formation of N-nitrosamines. However, the risk to chemists can be minimized if safety procedures for handling chemical carcinogens are strictly adhered to.

Since many N-nitrosamines are carcinogens, there is increasing concern over the possible formation of N-nitrosated pesticides in nature (Eisenbrand et al., 1975). This method could be applied to the direct determination of N-nitrosoglyphosate in environmental samples.

ACKNOWLEDGMENT

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