Table IX. Stability Test Results with 8f

(1) differential thermal analysis
endotherm peaks: 113 °C
exotherm peaks: 216 °C
(2) spark sensitivity: 50% fire point = 0.650 J (HMX standard = 0.7 J)
(3) rotary friction: negative (4-kg load at 3000 rpm)
(4) impact sensitivity: 50% fire point = 100 cm (Bureau of Mines apparatus, 2-kg weight)
(5) drop weight test: 50% fire point = 5–6 in. (Janaf standard tests, 2-kg weight); RDX = 7 in.
(6) explosive power: 0 cm^3/g (Trunzl block test, No. 8 blasting cap); RDX = 25 cm^3/g

well-defined conditions of impact, shock, vibration, friction, sparks, flame, or heat. Shock sensitivity and detonation studies, as well as thermal stability and rotary friction tests, were carried out on all intermediates and most products. The results indicate that all intermediates **1–3** and **5** are very sensitive to shock and impact [see also Inman et al. (1964)]. By use of proper precautions, compounds such as **8f** can be handled safely. Stability tests results with **8f** are included in Table IX.

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Verification of Structures of Chloroethyl *N*-Heptafluorobutyryl Derivatives of Glyphosate and (Aminomethyl)phosphonic Acid by Chemical Ionization and Electron Impact Mass Spectrometry

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Glyphosate (a highly effective herbicide with a low mammalian toxicity) is currently being studied on a wide range of agricultural commodities. The most commonly utilized residue procedure which quantitates this compound and its major metabolite, (aminomethyl)phosphonic acid, depends on the formation of the methyl *N*-trifluoroacetyl derivatives of both compounds following an extensive cleanup procedure. This study reports on the structural verification of the chloroethyl *N*-heptafluorobutyryl derivatives which were synthesized, resulting in the simplification of this residue analysis. Chemical ionization mass spectrometry with both isobutane and methane reagent gases is used to obtain reliable molecular weights for both derivatives. Electron impact mass spectrometry is then used to identify the fragmentation of the compounds.

Glyphosate [*N*-(phosphonomethyl)glycine] is the active ingredient of the herbicidal formulation ROUNDUP (a product of Monsanto Co.). This is an effective, broad-spectrum, nonselective herbicide which has a low mammalian toxicity (LD_{50} = 4320 mg/kg). These factors have led to the widespread field trial study of this compound on a variety of agricultural commodities. A sensitive, widely applicable residue analysis for glyphosate and its major metabolite, (aminomethyl)phosphonic acid, is therefore necessary for the quantitation of these two compounds in a wide range of treated crops. A procedure developed by Monsanto and suggested by the Environmental Protection Agency (U.S. EPA, 1977) has been adapted and used in this laboratory since 1975 (Thompson et al., 1980). This method involves extensive cleanup including solvent partitioning, charcoal elimination of pigments, large volume anion exchange, and separation of the pesticide and its metabolite by cation exchange. This is followed by separate acylation with trifluoroacetic anhydride, alkylation with diazomethane, and detection by gas chromatography with flame photometric detection. Reproducible results are sometimes difficult to obtain with this tedious methodology, especially on crops which present extreme cleanup problems (i.e., blueberries, strawberries, and okra).

A recent study from this laboratory (Guinivan et al., 1982) has reported the use of aqueous gel permeation chromatography and the formation of two new derivatives, presumably the chloroethyl *N*-heptafluorobutyryl esters, of glyphosate and (aminomethyl)phosphonic acid as a means for simplifying the complex existing procedures for the analysis of residues on blueberries. Smaller sample

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handling volumes and simultaneous purification, derivatization, and detection of the pesticide and its metabolite resulted in considerable savings in analysis time. The use of chloroethylation and heptafluoroacylation also aids the detection of the two compounds by electron capture gas chromatography.

Similar derivatives of glyphosate and (aminomethyl)phosphonic acid have been identified by nuclear magnetic resonance and mass spectral studies (Rueppel et al., 1976, 1977). These *n*-butyl *N*-trifluoroacetyl esters were produced (as in the Monsanto method mentioned earlier) by first acylating the molecules and then alkylating with freshly prepared diazo reagents. The new derivatives reported from this laboratory involve alkylation first with commercially purchased boron trichloride (BCl_3)–2-chloroethanol and then acylation with heptafluorobutyric anhydride. This is the first report on the use of a Lewis acid catalyzed alkylation reagent for the derivatization of this herbicide and its metabolite. The derivatized molecules are later extracted from an aqueous environment with hexane. This extraction procedure can potentially serve as a major solvent partitioning cleanup step. Crops which do not present formidable cleanup difficulties could possibly be analyzed with little prederivatization purification. This study was conducted to verify the presence of the chloroethyl *N*-heptafluorobutyryl esters of glyphosate and (aminomethyl)phosphonic acid. Evidence from chemical ionization mass spectrometry and electron impact mass spectrometry verifies the molecular weights and proposed structures of these derivatives.

EXPERIMENTAL SECTION

Reagents. Pesticide-grade hexane (Mallinckrodt) was used. The heptafluorobutyric anhydride was obtained from the Pierce Chemical Co. (Rockford, IL) and BCl_3–2-chloroethanol (10% w/v) was supplied by Applied Science (State College, PA). Standard glyphosate was a gift from Monsanto Co. and (aminomethyl)phosphonic acid was purchased from Sigma Chemical Co. (St. Louis, MO).

Gas Chromatograph. An automatically integrating, microprocessor-controlled, Hewlett-Packard Model 5840A gas chromatograph equipped with a ^{63}Ni electron capture detector and a 327 cm × 4 mm i.d. packed glass column of 10% DC-200 on Gas-Chrom Q (Applied Science, State College, PA) was utilized. The instrument was operated under the following parameters: 95% argon–5% methane carrier gas (flow rate 60 mL/min), 1- or 5-L injection volumes, an injection port temperature of 250 °C, a column oven temperature of 220 °C, and a detector temperature of 300 °C.

Mass Spectrometer. A Finnigan 4021 quadrupole GC/MS/DS instrument in the positive ion mode was used. Samples were introduced through the gas chromatograph equipped with a 327 cm × 4 mm i.d. packed 10% DC-200 column under the following conditions: a helium carrier flow of 20 mL/min, an injection volume of 1 μL, an injection port temperature of 250 °C, and a column oven temperature of 220 °C. Electron impact mass spectra were obtained at 70 eV. Separate chemical ionization studies were conducted with isobutane and methane reagent gases.

Derivatization of Standards. A sample containing 1.0 μG/μL of both derivatives was prepared by adding 1.5 mL of BCl_3–2-chloroethanol to a tube containing 1 mG each of glyphosate and (aminomethyl)phosphonic acid. The tube was sealed with a Teflon-lined cap and heated at 110 °C for 45 min. The excess reagent was evaporated in a boiling water bath under nitrogen for 30 min. Heptafluorobutyric anhydride (200 μL) was added to the tube, and the tube was sealed and heated to 110 °C for 45 min.

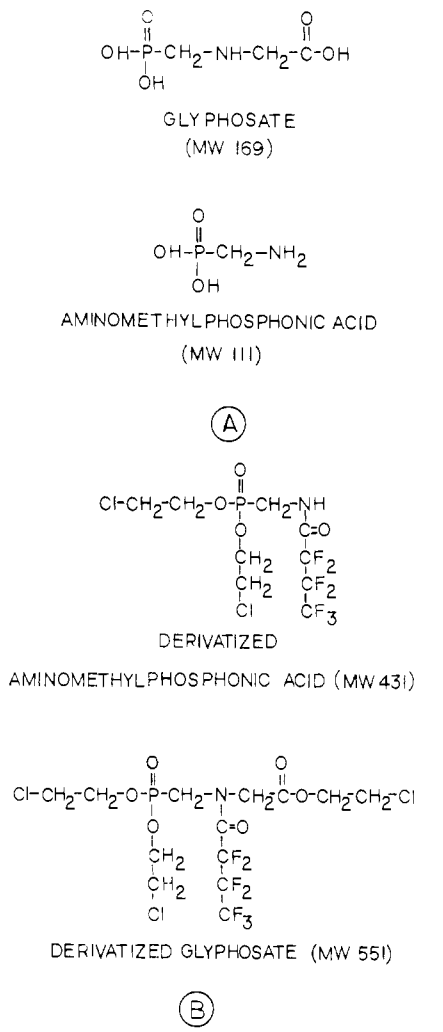

Figure 1. The structures for (A) glyphosate and (aminomethyl)phosphonic acid and (B) the proposed derivatives of these two compounds.

A 1-mL volume of water was added to the tube in order to convert the anhydride to its acid. The acid is then converted to the ammonium salt with the addition of 1 mL of 5% aqueous ammonia. The derivatized glyphosate and (aminomethyl)phosphonic acid were extracted from the aqueous phase with 4 mL of hexane. This extraction was repeated twice. The hexane was passed through a small bed of anhydrous sodium sulfate and then concentrated to 1 mL before analysis by mass spectrometry. Standards ranging from 0.015 to 15 μG/mL were prepared by the same procedure.

RESULTS AND DISCUSSION

The structures for glyphosate and (aminomethyl)phosphonic acid are shown in Figure 1A, and Figure 1B shows the structures of the proposed derivatives. All hydroxyls and the amino group must be derivatized on both compounds before the molecules are sufficiently volatile to be analyzed on a nonpolar GC packing, such as 10% DC-200. Figure 2 shows the electron capture gas chromatogram of a derivatized sample containing both pesticide and metabolite (2.6 μG/mL, 1 μL injected). The peak at 2.62 min is the derivatized metabolite, and the derivatized glyphosate is retained 14.29 min. The identity of the peaks is verified by preparing separate standard curves for both compounds. A five-point standard curve (micrograms per milliliter vs. integrated area) for (aminomethyl)phosphonic acid gives a 0.9962 linear coefficient of determination, and a corresponding value of 0.9927 is found

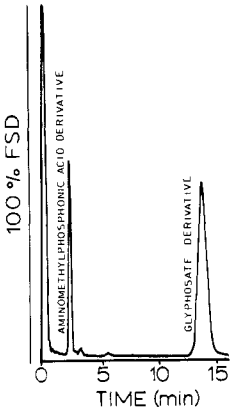

Figure 2. Chromatogram of a 2.6 μG/mL (1 μL injected) sample containing both derivatized (aminomethyl)phosphonic acid and glyphosate (attenuation 2^{12}). The retention times are 2.62 min for the metabolite and 14.29 min for the herbicide.

for the glyphosate standard curve. The minimum level of detectability is 0.015 μG/mL (5 μL injected) for the pesticide and 0.025 μG/mL (5 μL injected) for the metabolite. This evidence shows that the derivatives are formed in a manner which varies linearly with concentration over the 0–15 μG/mL concentration range.

The reconstructed ion current plot for the sample analyzed by GC–MS (1 μG/μL in both compounds, 1 μL injected) is shown in Figure 3. The reconstructed ion current is plotted against mass spectral scan numbers and retention times, and peaks are identified by scan number. As discussed below, the compound causing the peak at scan no. 986 is the acylated, triple alkylated glyphosate (M_r 551). This assumption is supported by the isobutane chemical ionization mass spectrum of this compound (Figure 4), showing the $(M + 1)^+$ ion at m/z 552, along with the m/z 554 and m/z 556 ions. The ratio of $(M + 1)^+$, $(M + 3)^+$, and $(M + 5)^+$ is 100:97.4:18.5. This pattern is consistent with what would be expected for a molecule containing three chlorine atoms. The methane chemical ionization mass spectrum of this compound gives the $(M + 1)^+$ (m/z

Table I. Probable Fragment Ions Occurring from the Electron Impact of Derivatized Glyphosate

ion fragment	m/z
$(ClC_2H_4)_2PO_3CH_2NC(O)C_3F_7CH_2CO_2C_2H_4$	516/518
$(ClC_2H_4)_2PO_3CH_2NC(O)C_3F_7CH_2C(O)^+$	472/474
$(ClC_2H_4)_2PO_3CH_2NC(O)C_3F_7CH_2^+$	444/446
$(ClC_2H_4)_2PO_3CH_2NCH_2CO_2CH_2CH_2Cl^+$	354/356/358
$PO_3CH_2NC(O)C_2F_4CH_2CO_2^+$	293/295
$ClC_2H_4PO_2CH_2NC(O)CF_2CH_2^+$	246/248
$ClC_2H_4PO_3CH_2NCH_2^+$	185/187
$CF_2CF_2CF_3^+$	169
$CF_2CF_3^+$	119
$CF_2CF_2^+$	100
$PO_3CH_2^+$	93
$ClC_2H_4O^+$	79/81
CF_3^+	69
$ClCH_2CH_2^+$	63/65
$CH_2NC(O)^+$	56
$NC(O)^+$	42
$CH_2C(O)^+$	42

Table II. Probable Fragment Ions Occurring from the Electron Impact of Derivatized (Aminomethyl)phosphonic Acid

ion fragment	m/z
$Cl(CH_2CH_2O)_2P(O)CH_2NHC(O)CF_2CF_2CF_3^+$	396/398
$ClCH_2CH_2OP(O)CH_2NHC(O)CF_2CF_2CF_3^+$	352/354
$OP(O)CH_2NHC(O)CF_2CF_2CF_3^+$	289
$(ClCH_2CH_2O)_2P(O)CH_2NHC(O)^+$	262/264/266
$CH_2NHC(O)CF_2CF_2CF_3^+$	226
$CH_2OPO_2CH_2NHC(O)CF_2^+$	200
$CF_3CF_2CF_2C(O)^+$	197
$ClCH_2CH_2O_2P(O)CH_2NH^+$	171/173
$CF_3CF_2CF_2^+$	169
$NHC(O)CF_2CF_2^+$	143
$CF_2CF_2C(O)^+$	128
$CF_3CF_2^+$	119
CF_2CF_2	100
$ClCH_2CH_2O$	79
CF_3	69
$ClCH_2CH_2$	63/65
$CH_2NHC(O)$	57
$ClCH_2$	49/51
$NHC(O)$	43

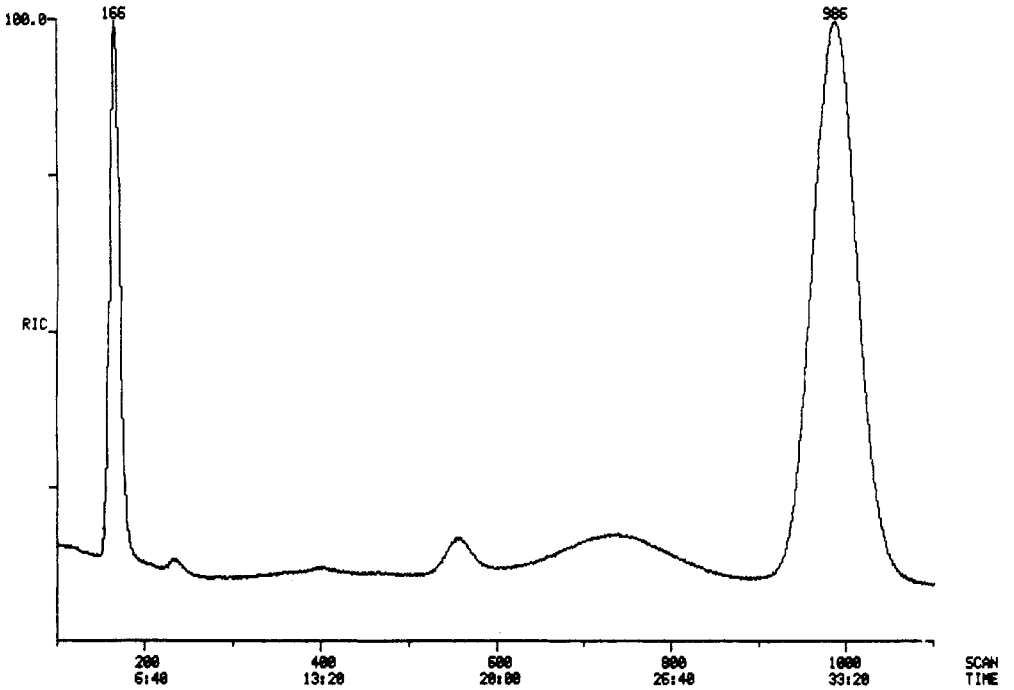

Figure 3. Reconstructed ion current plot for a derivatized sample [1.0 μG/μL in both glyphosate and (aminomethyl)phosphonic acid] introduced into the mass spectrometer through the gas chromatograph. The carrier gas is methane (20 mL/min) and the column temperature is 220 °C.

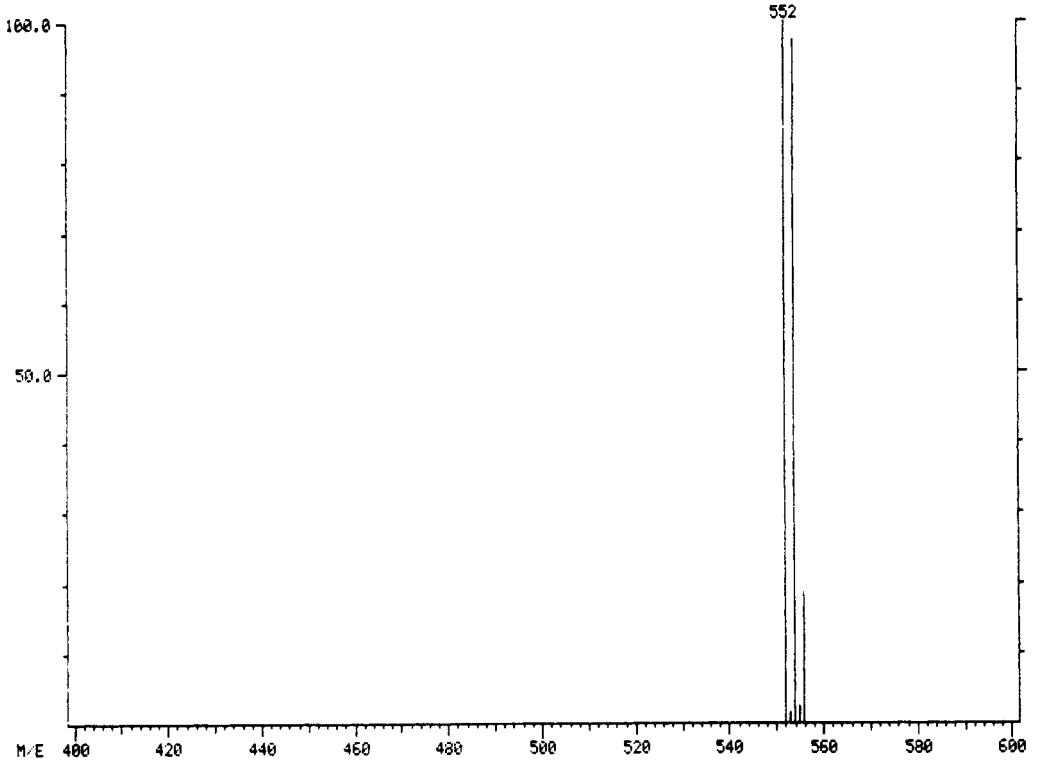

Figure 4. Chemical ionization mass spectra of derivatized glyphosate with isobutane reagent gas. The mass spectra were collected to 700 mass units, and no ions above m/z 556 were observed.

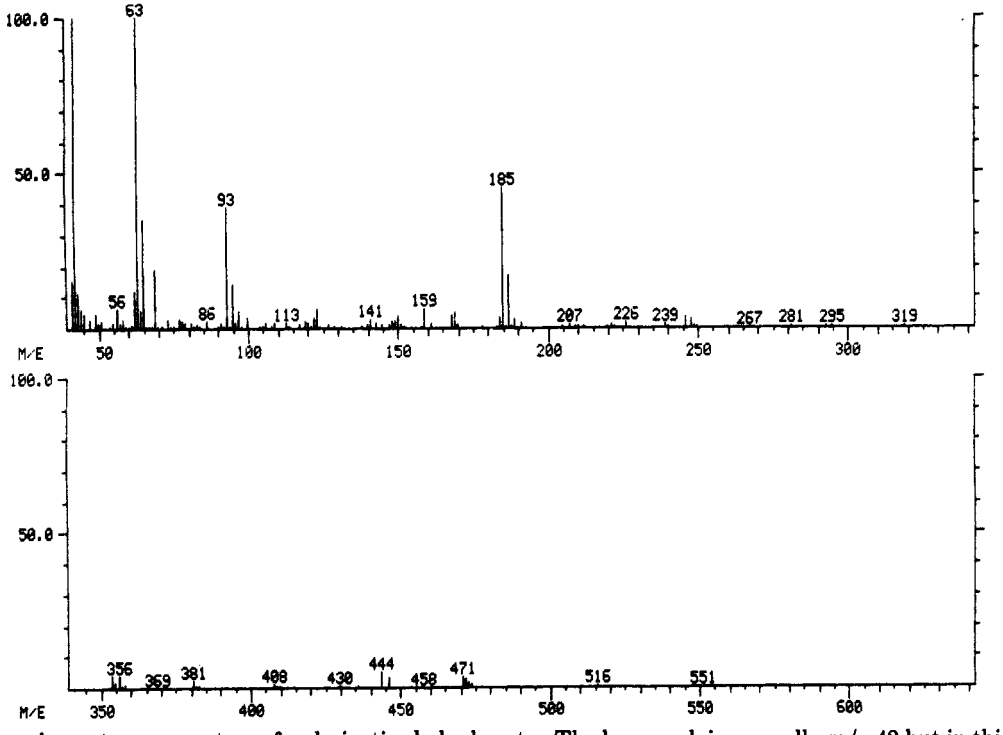

Figure 5. Electron impact mass spectrum for derivatized glyphosate. The base peak is normally m/z 42 but in this figure the scale is expanded to set the base peak at m/z 63.

552), $(M + 29)^+$ (m/z 580), and $(M + 41)^+$ (m/z 592) ions along with appropriate chlorine isotope ions. The molecular ion (m/z 551) is also present as a minor ion in the electron impact spectrum of this molecule (Figure 5). Even though the intensity of the m/z 551 ion is only 0.03% relative abundance, the M + 2 chlorine isotope ion at m/z 553 is present at 81%, the relative abundance of the molecular ion. Table I lists ions which are consistent with a reasonable fragmentation of this derivative. The ion m/z 63 and the chlorine isotope peak at m/z 65 correspond to the $ClCH_2CH_2^+$ fragment. The base peak at m/z 42 could result from the $NC(O)^+$ and $CH_2C(O)^+$ fragments. The compound causing the peak at scan no. 166 on the reconstructed ion current is probably the acylated, dual alkylated metabolite. Figure 6 shows the isobutane chemical ionization spectra of this compound. The $(M + 1)^+$ (m/z 432), $(M + 2)^+$ (m/z 434), and $(M + 4)^+$ (m/z 436) ions are present in a 100:70:5 ratio of relative abundance. This is consistent with the presence of two chlorine on the molecule. The appropriate M + 1 (m/z 432), M + 29 (m/z

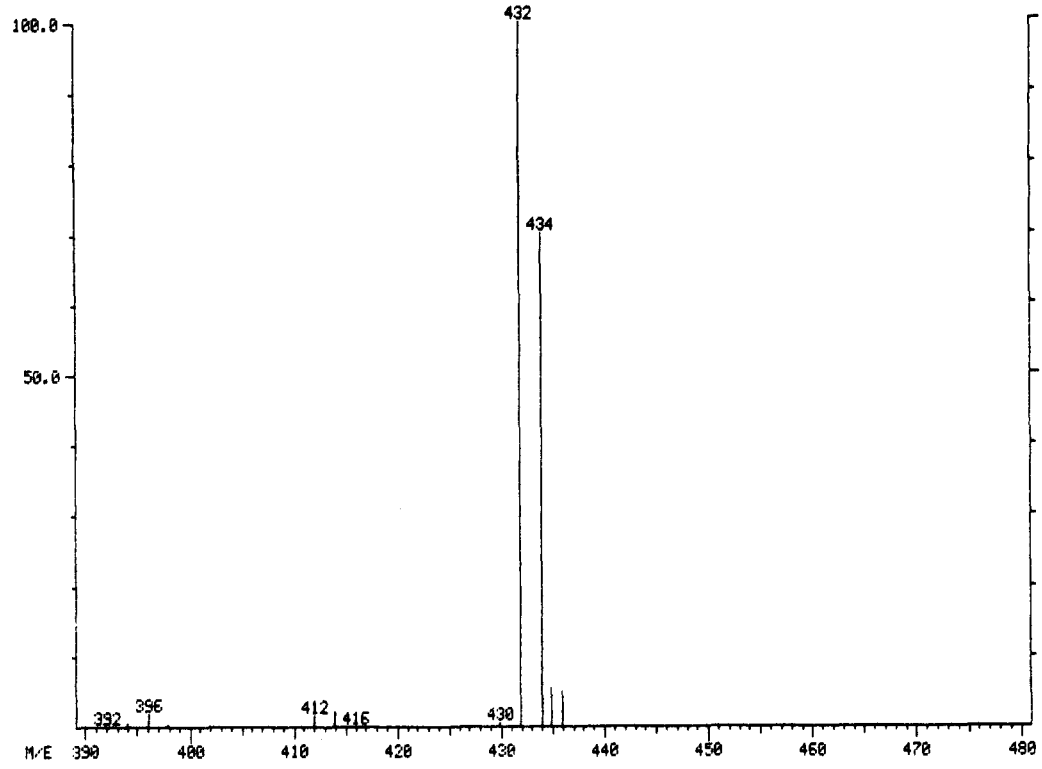

Figure 6. Chemical ionization mass spectra of derivatized (aminomethyl)phosphonic acid with isobutane reagent gas. The mass spectra were collected to 700 mass units, and no ions above m/z 436 were detected.

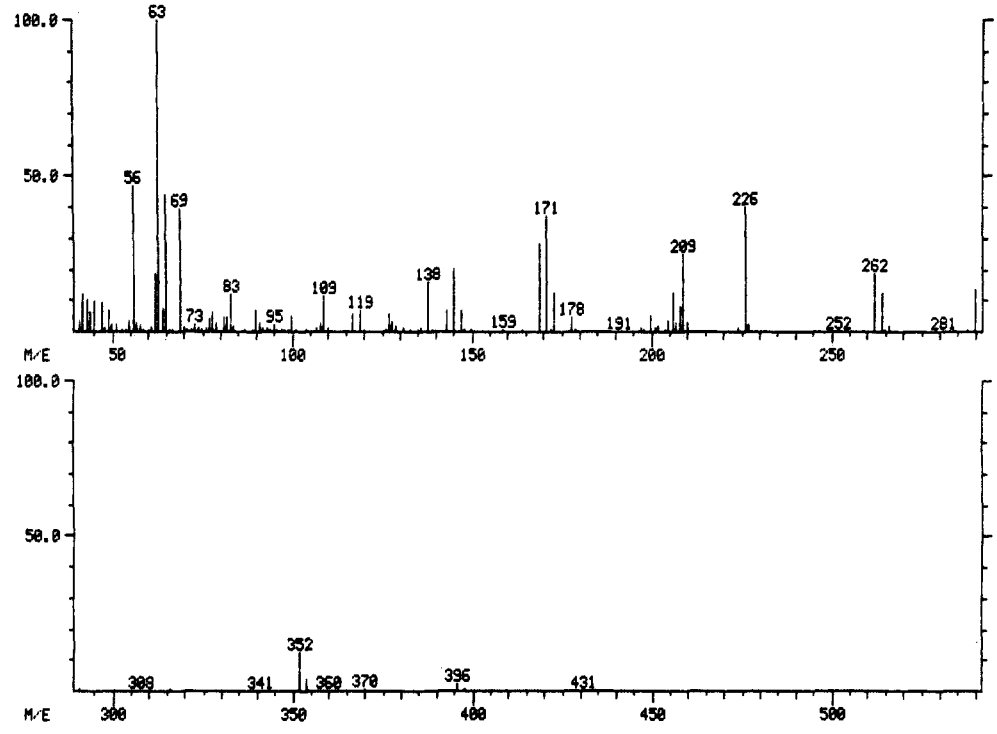

Figure 7. Electron impact mass spectrum for derivatized glyphosate. The base peak is m/z 63.

460), M + 41 (m/z 472), and chlorine isotope peaks are present in the methane chemical ionization spectrum of this compound. The electron impact mass spectrum (Figure 7) also shows the molecular ion (m/z 431) in low relative abundance. Reasonable fragment ions from the derivatized metabolite are shown in Table II. The combination of molecular weight confirmation and reasonable electron impact fragmentation patterns strongly suggests that the proposed structures for the chloroethyl *N*-heptafluorobutyryl derivatives of glyphosate and (aminomethyl)phosphonic acid are correct.

CONCLUSIONS

Published reports would suggest that alkylation followed by acylation of glyphosate and (aminomethyl)phosphonic acid would produce the structures proposed in this study. Confirmation of the structures was pursued because the derivatization steps were reversed and modified from the existing procedures. These derivatives can potentially play an important role in the purification and quantitation of

residues of this pesticide and its metabolite. Confirmation of the molecular weights of these derivatives by chemical ionization mass spectrometry and supporting evidence from reasonable electron impact mass spectrometry fragmentation patterns gives assurances that the proposed structures are correct.

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Simple Dye Release Assay for Determining Endopectinase Activity

David R. Friend and George W. Chang*

Covalently dyed pectin is used as the basis of a simple spectrophotometric assay for the determination of endopectinase activity. The water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride is used to covalently attach the azo dye *N*-[1-[4-[(3,6-disulfo-1-naphthyl)azo]-naphthyl]]ethylenediamine (DISANED) to pectin under mild conditions (pH 4.75) to form DISANED–pectin. The dyed pectin is highly soluble in the aqueous buffer solutions used. Conditions for the use of DISANED–pectin to measure endopectinase activities in a variety of crude fungal and bacterial preparations are described. The rate of release of reducing sugars from DISANED–pectin is slower than that from unmodified pectin. Pectin methylesterase (PME) does not release measurable amounts of DISANED from DISANED–pectin. Also, addition of PME to a pectinase enzyme preparation does not alter the rate of release of dyed fragments from DISANED–pectin.

Enzymes that depolymerize pectin (polygalacturonic acid methyl ester) are known collectively as pectinases. They can be classified broadly as endodepolymerases, which act within the polysaccharide chains, and exodepolymerases, which act at the ends. Measurement of the endodepolymerizing enzymes of these two groups has usually involved comparison of the rate of decrease in viscosity with the rate of release of reducing sugars (Lim et al., 1980; Itoh et al., 1980). Although viscometric determination of endopectinase activity using capillary flow methods is quite sensitive, it is also slow and cannot be used with enzyme preparations containing particulate matter. Release of reducing sugars has been widely used to determine pectinase activity, but it cannot distinguish endo- from exodepolymerase activites and it is subject to interference by reducing substances present in crude enzyme preparations. Furthermore, reducing sugar assays are of limited use in the presence of viable organisms that can quickly metabolize the oligo- and monosaccharide products of depolymerization.

Because of these limitations in assay procedures for polysaccharide depolymerizing enzymes, alternative techniques that measure enzyme activity by the release of colored degradation products from covalently dyed substrates have been developed. Such assays are simple, rapid, and free from a great variety of chemical interferences. They have been used successfully for the measurement of α-amylase (Dougherty, 1975; Hejgaard and Gibbons, 1979), cellulase (Poincelot and Day, 1972; Ng and Zeikus, 1980), endo-1,3-β-glucanase (Philpott and Chapman, 1977), dextranase (Huang and Tang, 1976), and β-mannanase (McCleary, 1978). However, the development of a similar dye release assay for pectin and other acidic polysaccharides has been hampered by the inability to covalently attach dyes under conditions mild enough to preclude degradation. Another problem of the application of this technique to acidic polysaccharides, such as pectin, is the solubility of the dyed polymer. In preliminary experiments a two-step procedure similar to that of Huang and Tang (1976) was used. First an ethylenediamine moiety was attached to the carboxylic acid groups of pectin via a water-soluble carbodiimide. Then the free amino groups of the ethylenediamine moiety were coupled in a nucleophilic reaction with the vinyl sulfone groups of Remazol Brilliant Blue R, yielding a highly modified polymer. However, this material was nearly insoluble in the buffers used in the enzyme assays. Futhermore, the yields were low due to the rather harsh conditions of the second step (pH 9–10 for 30 min), which resulted in considerable depolymerization of the pectin.

This paper describes the development of a dyed substrate assay procedure utilizing the synthetic water-soluble azo dye DISANED. The dye is coupled to pectin in a reaction with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride to form the water-soluble pectin derivative DISANED–pectin (Figure 1). Endopectinase activity is determined by measuring the rate of release of dyed fragments soluble in 63% (v/v) ethanol.

MATERIALS AND METHODS

Preparation of *N*-[1-[4-[(3,6-Disulfo-1-naphthyl)-azo]naphthyl]]ethylenediamine (DISANED). 1-Aminonaphthalene-3,6-disulfonic acid from Fluka AG, Switzerland, and *N*-1-naphthylethylenediamine dihydrochloride (NED) from Sigma Chemical Co., St. Louis, MO, were coupled by diazotizing 1-aminonaphthalene-3,6-disulfonic acid, followed by coupling to NED. During the coupling step, 2 N NaOH was added as necessary to keep the pH between 6 and 7. After coupling was complete (30 min), the pH was adjusted to neutrality, NaCl was added

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