Journal of Chromatography, 637 (1993) 167–173
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 937

Simultaneous determination of the herbicides glyphosate, glufosinate and bialaphos and their metabolites by capillary gas chromatography–ion-trap mass spectrometry

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(First received September 25th, 1992; revised manuscript received January 2nd, 1993)

ABSTRACT

A sensitive gas chromatographic (GC)–ion-trap mass spectrometric (IT-MS) method has been developed to determine simultaneously the herbicides glyphosate, glufosinate and bialaphos and their major metabolites. A single-step derivatization is achieved at 80°C for 30 min with the reagent N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide in dimethylformamide, which introduces the *tert.*-butyldimethylsilyl group at active hydrogens and gives only a single peak for each compound. The derivatives of three herbicides, their metabolites and nineteen amino acids were simultaneously chromatographed and well separated in a single run on a DB-1 fused-silica capillary column. Each *tert.*-butyldimethylsilylated derivative produces an easily interpretable mass spectrum dominated by unique M − 15, M − 57, M − 85 and M − 159 fragment ions. The limits of detection were estimated to be 10–20 ng for glyphosate and glufosinate and their metabolites, and 500 ng for bialaphos, by GC–IT-MS. On the other hand, using GC analysis with flame ionization detection, glyphosate and glufosinate and their metabolites were detectable at levels of approximately 100 ng, but bialaphos could not be detected at a level of 5000 ng.

INTRODUCTION

Glyphosate [N-(phosphonomethyl)glycine] (GLYP), glufosinate [DL-homoalanine-4-yl-(methyl) phosphinic acid] (GLUF) and bialaphos [L-2-amino-4-(hydroxy)(methyl) phosphonoyl]butyryl-L-alanine (BIAL) are broad-spectrum, non-selective herbicides with an increasing number of international users, including in Japan. And there is great interest in the need to determine these herbicides and their major metabolites, aminomethyl phosphonic acid (AMPA), 3-methylphosphinico propionic acid (MPPA) and L-2-amino-4-[(hydroxy)(methyl) phosphinoyl] butyric acid (AMPB, enantiomer of DL-GLUF), in physiological, food, plant, water and soil samples. Their chemical structures and proposed metabolic pathways are shown in Fig. 1.

Various approaches to the analyses of GLYP, GLUF and BIAL and their metabolites have been taken. These include gas chromatography (GC) after chemical derivatization [1–7], high-performance liquid chromatography (HPLC) utilizing pre- or post-column fluorogenic labelling [8–14] and thin-layer chromatography [7, 15–19]. However, these methods are not suitable for simultaneous determination. Recently, the simultaneous detection of the herbicides by paper chromatography [20] has been demonstrated.

This paper describes the simultaneous preparation and analysis of *tert.*-butyldimethylsilyl (*t*BDMS) derivatives of the herbicides and their

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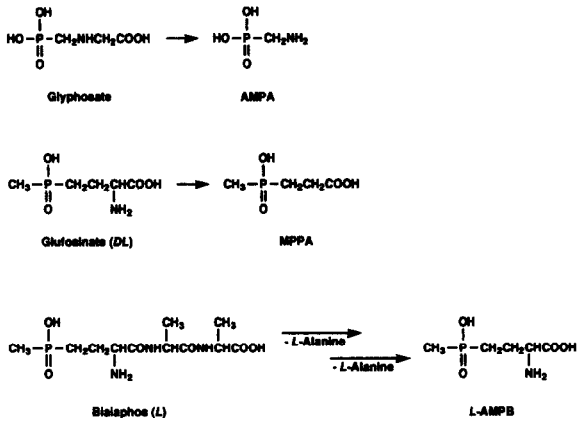

Fig. 1. Proposed metabolic pathways of glyphosate, glufosinate and bialaphos.

metabolites by GC–ion-trap mass spectrometry (IT-MS). The *t*BDMS derivatives are more stable than the trimethylsilyl ones upon storage [21], easily gas chromatographed and give more informative mass spectra in both electron impact (EI) and chemical ionization (CI) mode under moderate conditions without the need for the removal or destruction of the reagent, N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) [22–26]. In earlier studies, this reagent has been shown to be a very powerful *t*BDMS donor capable of *tert.*-butyldimethylsilylating active protic functions (*i.e.*, hydroxyl, amino, carboxylic and thiol moieties) [27]. This reagent has been used for the derivatization of sulphate, phosphate and other oxyanions [27], and introduces *t*BDMS groups to inorganic acids [28], amino acids [22–25] and dipeptides [26] at sites having active hydrogens. Furthermore, this reagent allows *t*BDMS derivatization of dicarboxylic acids [29], alkylphosphonic and alkyl methylphosphonic acids [30] and organic sulphonates [31].

MTBSTFA has also been shown to be a useful reagent in the GC analysis of GLYP and its metabolite, AMPA [4], which include hydroxy, amino, carboxylic and methylphosphonic functions in their structures. Also, gas chromatographic analysis of GLUF has been reported to convert into aminoacetyl and methyl derivatives by employing acetic acid–trimethyl orthoacetate [7]. On the other hand, since it has been difficult to detect BIAL as an intact molecule by GC analysis, AMPB, which is the L-isomer of GLUF (Fig. 1), can be indirectly determined using the same GC method for GLUF as described above after acid hydrolysis of BIAL.

The present investigation reports on a simultaneous analytical method that employs the *t*BDMS derivatives of the phosphorus-containing amino acid-type herbicides, GLUF and BIAL, and their metabolites, including GLYP and AMPA, and keeps them intact.

IT-MS is capable of providing full-scan mass spectra on 1 ng of material or less. This enhanced sensitivity, coupled with GC retention indices (*I*), provides the analytical chemist with a powerful means for unambiguous identification and quantitation of trace levels of organic compounds. In this paper, the application of IT-MS to the analyses of the phosphorus-containing amino acid-type herbicides and their metabolites is described.

EXPERIMENTAL

Chemicals

GLYP was supplied by Monsanto Japan (Tokyo, Japan), and AMPA was purchased from Sigma (St. Louis, MO, USA), GLUF and MPPA were donated by Hoechst Japan (Tokyo, Japan), and BIAL and L-AMPB by Meiji Seika Kaisha (Tokyo, Japan). MTBSFTA was purchased from Pierce (Rockford, IL, USA). Nineteen amino acids were obtained from Ajinomoto (Tokyo, Japan). All other compounds were of analytical-reagent grade obtained from Wako (Osaka, Japan).

Gas chromatography–mass spectrometry

GC–MS analyses were performed on a Perkin-Elmer Model 8420 capillary gas chromatograph equipped with an ion trap detector. Chromatographic conditions for these analyses were: a DB-1 fused-silica capillary column (30 m × 0.24 mm I.D., 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA); helium carrier gas at 1 ml/min; GC oven temperature programme, 8°C/min from 100 to 300°C, held for 5 min; injector and transfer line temperature, 320°C; split/splitless injector, split mode, split ratio

1:30, and injection volume, 1 μl. The mass spectrometer was operated in the EI mode or in the CI mode using isobutane as reagent gas at a pressure that has a 3:2 ratio for *m/z* 43 to *m/z* 57. The scan range of the EI mode was from 45 to 650 u at 1 s/scan, and that of the CI mode from 70 to 650 u. Manifold temperature was 220°C. The data system was an IBM PC/AT computer with the automatic gain control software version 4.00.

Straight-chain hydrocarbons were used for the calculation of retention indices.

Gas chromatography

GC analyses were carried out with a Hewlett-Packard (Palo Alto, CA, USA) Model 5890 gas chromatograph equipped with a split/splitless capillary injector port, a flame ionization detector and an HP 3396 integrator. The capillary column used was the same as that for GC–IT-MS. The operating conditions for the chromatograph were: helium carrier gas at 1 ml/min; oven temperature, 160 to 320°C at 8°C/min; injector and detector temperature, 320°C; split ratio 1:50 and injection volume 1 μl.

Derivatization

Solutions (1 mg/ml) of GLYP, GLUF, BIAL and their metabolites and nineteen amino acids in distilled water were prepared. Aliquots (20 μl) of these solutions were transferred into 1-ml PTFE-lined screw-capped derivatization vials. The solvent was evaporated under a gentle stream of nitrogen at 40°C. To the dry residue were added 50 μl of MTBSFTA and 50 μl of dimethylformamide. The mixture was sonicated for 2 min at room temperature, then heated at 80°C for 30 min. After cooling to room temperature, aliquots of the solution containing the derivatives were used directly for GC–IT-MS.

In some experiments the reaction time was varied from 30 to 120 min, and *n*-docosane for quantitative analysis was used as an internal standard. A Pierce Reacti-Thermo block heater was used to heat samples during derivative preparation.

RESULTS AND DISCUSSION

Derivatization reactions

As the reagent employed for *t*BDMS derivative synthesis of amino acids was a mixture of MTBSTFA and acetonitrile, dimethylformamide or tetrahydrofuran [23], a mixture of MTBSTFA and dimethylformamide (1:1) was used for *t*BDMS derivatives of those herbicides and their metabolites in this study. The time courses for the derivatization of GLYP, GLUF and BIAL and their metabolites at 80°C are shown in Fig. 2. For three herbicides and their metabolites, as would be expected, the incubation time of 30–60 min produced almost the highest yields of *t*BDMS derivatives. Sonication of the reaction mixtures for 2 min at room temperature before incubation has an enhancing effect on the responses, reaction rate and reproducibility of *t*BDMS derivatives as compared with those that were not sonicated. In some cases, no peak or fewer peaks of *t*BDMS derivatives were unexpectedly detected without sonication. The resulting derivatives were found to be stable at least for a few days in a refrigerator (data not shown).

Gas chromatographic separation

The *t*BDMS derivatives of GLYP, AMPA, GLUF, MPPA and BIAL eluted as single chro-

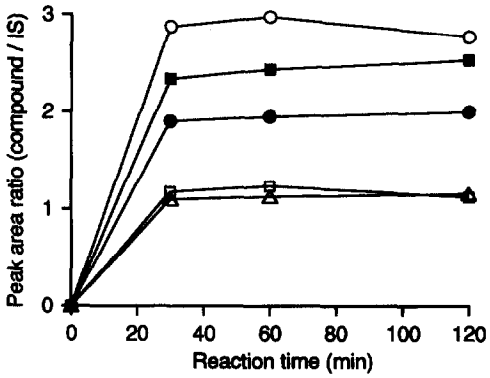

Fig. 2. Kinetics curves for glyphosate, glufosinate and bialaphos and their metabolites at 80°C. The reaction mixture consisted of glyphosate (8 μg, ○), AMPA (5 μg, ●), glufosinate (10 μg, □), MPPA (10 μg, ■), bialaphos (250 μg, △) and *n*-docosane [internal standard (IS), 10 μg] in 100 μl of MTBSTFA–dimethylformamide (1:1). A 1-μl aliquot was injected into the GC–IT-MS system. For GC–IT-MS conditions, see the Experimental section.

matographic peaks, and all peaks showed almost complete baseline separation using a DB-1 fused-silica capillary column. The GC separation of the *t*BDMS derivatives of an amino acid standard mixture presented the same good results. Fig. 3 shows the typical reconstructed total ion chromatograms of the mixture of the herbicides and their metabolites (top) and the mixture of nineteen amino acids (bottom). Of the herbicides studied, each peak showed chromatographic separation and also could be distinguished from all the amino acids studied. The retention data of the *t*BDMS derivatives of the herbicides and amino acids studied on a DB-1 fused-silica capillary column is given in Table I in ascending order of retention index.

The herbicides and their metabolites emerged from the non-polar SE-30 capillary column in order of increasing molecular mass of their derivatives, the same as those of amino acid derivatives [23]. MPPA, the metabolite of GLUF that has the smallest derivatized molecular mass ($M_r = 380$), has the shortest retention time ($I = 2031$), followed by threonine ($I = 2041$), BIAL, the largest derivative ($M_r = 665$) of the herbicides studied, was observed to have the longest retention time ($I = 2642$), followed by tyrosine ($I = 2649$). In these experimental conditions, sufficient resolution was achieved when the I differences between two adjacent peaks was 5

TABLE I

RETENTION INDICES OF GLYPHOSATE, GLUFOSINATE, BIALAPHOS AND THEIR METABOLITES AND NINETEEN AMINO ACIDS AS THEIR *tert.*-BUTYLDIMETHYLSILYL DERIVATIVES

For GC–IT-MS conditions, see the Experimental section.

Compound	Retention index
MPPA	2031
AMPA	2100
Glyphosate	2436
Glufosinate (=AMPB)	2550
Bialaphos	2642
Alanine	1537
Glycine	1553
Valine	1684
Leucine	1726
Isoleucine	1756
Proline	1783
Methionine	1960
Serine	2000
Threonine	2041
Phenylalanine	2094
Aspartic acid	2164
Hydroxyproline	2196
Glutamic acid	2282
Lysine	2389
Arginine	2498
Histidine	2593
Tyrosine	2649
Tryptophan	2938
Cystine	3208

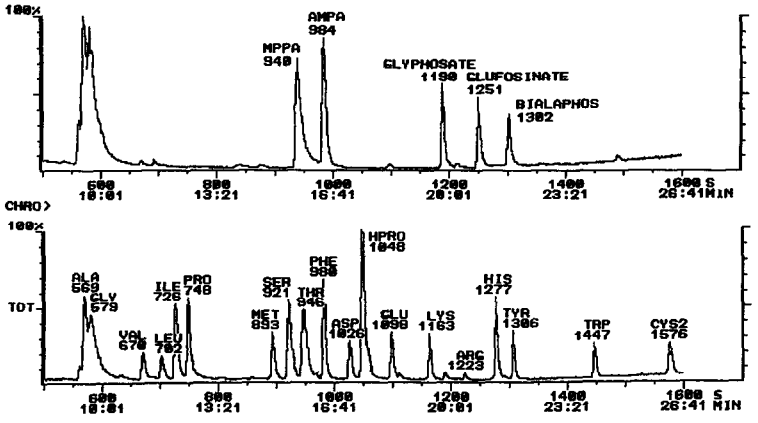

Fig. 3. Total ion chromatograms of the mixture of the herbicides (top) and nineteen amino acids (bottom) as *tert.*-butyldimethylsilyl derivatives. Amino acids: ALA = alanine; GLY = glycine; VAL = valine; LEU = leucine; ILE = isoleucine; PRO = proline; MET = methionine; SER = serine; THR = threonine; PHE = phenylalanine; ASP = aspartic acid; HPRO = hydroxyproline; GLU = glutamic acid; LYS = lysine; ARG = arginine; HIS = histidine; TYR = tyrosine; TRP = tryptophan; CYS2 = cystine. Numbers of peaks are retention times (s). For GC–IT-MS conditions, see the Experimental section.

units or more. Therefore, MPPA, AMPA and BIAL could be distinguished from threonine, phenylalanine and tyrosine by their retention times and mass spectra.

Minimum detectable amounts

The minimum detectable amounts were determined until a signal-to-noise ratio of 3 was reached by GC–MS and GC or a full mass spectrum was measured by GC–MS (Table II). In this way, the minimum detectable amounts obtained were 10–20 ng for GLYP and GLUF and their metabolites and 500 ng for BIAL using their peaks or full mass spectra on GC–IT-MS analyses. On the other hand, the detection limits of gas chromatographic analysis were 60–300 ng for GLYP, GLUF and their metabolites, but 5000 ng of bialaphos gave no peak by the GC method. The sensitivity of the GC–IT-MS method was about ten-fold greater than that of the GC method.

Canned tomato juice submitted to forensic examination was diluted with four volumes of water, and centrifuged. An aliquot of the supernatant was dried *in vacuo*, and the residue after washing with acetone was subjected to GC–MS according to this method. GLYP was identified and confirmed by GC–IT-MS as the *t*BDMS derivative. Glyphosate was adulterated in the specimen at the level of 13.7 ± 0.5 mg/ml ($n = 3$)

TABLE II

LOWER DETECTION LIMITS OF GLYPHOSATE, GLUFOSINATE, BIALAPHOS AND THEIR METABOLITES AS THEIR *tert.*-BUTYLDIMETHYLSILYL DERIVATIVES BY GC–IT-MS (ng)

GC–IT-MS and GC conditions as in the Experimental section. ++ = Detected easily; + = detected; − = not detected.

Compound	GC–IT-MS		GC
	Peak	Full mass spectrum	
Glyphosate	10 (+)	10 (−)	100 (++)
AMPA	10 (++)	10 (+)	60 (++)
Glufosinate	20 (+)	20 (+)	300 (++)
MPPA	20 (+)	20 (+)	100 (++)
Bialaphos	500 (+)	500 (+)	5000 (−)

by GC using peak area *versus* detector response. The calibration curve was plotted for concentration of 1–100 μg per vial.

Mass spectra

Although few ion-trap mass spectra of organic compounds have been published, the fragmentation patterns obtained are, in most respects, very similar to those obtained on conventional quadrupole and magnetic-sector instruments. By obtaining both EI and CI ion-trap mass spectra, the expected structures of *t*BDMS derivatives were confirmed for the herbicides and their metabolites.

In the CI mode, quasi-molecular ions (MH^+) of *t*BDMS-derivatized GLYP and GLUF and their metabolites were revealed as the base peaks. The EI mass spectra of GLYP, AMPA, GLUF, MPPA and BIAL as *t*BDMS derivatives are shown in Fig. 4 and their diagnostic ions are compiled in Table III. Ionization in the EI mode produced for these derivatives two strong [M − 57 as the base peak; loss of $C(CH_3)_3$, m/z 73; $Si(CH_3)_3$] and less intense (M − 15; loss of CH_3) ions, as was observed for the derivatives of the oxyanions and amino acids [23–25,27]. No molecular ion was observed for any derivative except GLYP. The molecular or quasi-molecular ion of *t*BDMS-derivatized BIAL (m/z 665 or 666) could not be confirmed because the scan range of the IT-MS system is from m/z 45 to 650.

M − 15 and M − 57 ions for GLYP, AMPA and GLUF indicated that three *t*BDMS groups were introduced into their molecules, and two *t*BDMS groups were introduced into the metabolite of GLUF, MPPA. An m/z 364 ion of GLUF, due to [M − 156(COO*t*BDMS)], suggests that the secondary amino group after introducing one *t*BDMS group into the primary amino moiety is not silylated in the same manner as GLYP and AMPA.

The mass spectrum of the derivatized BIAL, a kind of dipeptide, in the EI mode showed two characteristic fragment ions, m/z 364 and 324. These dominant ions in the EI spectrum of *t*BDMS-derivatized dipeptides originate from the N-terminal amino acid. The base peak ion m/z

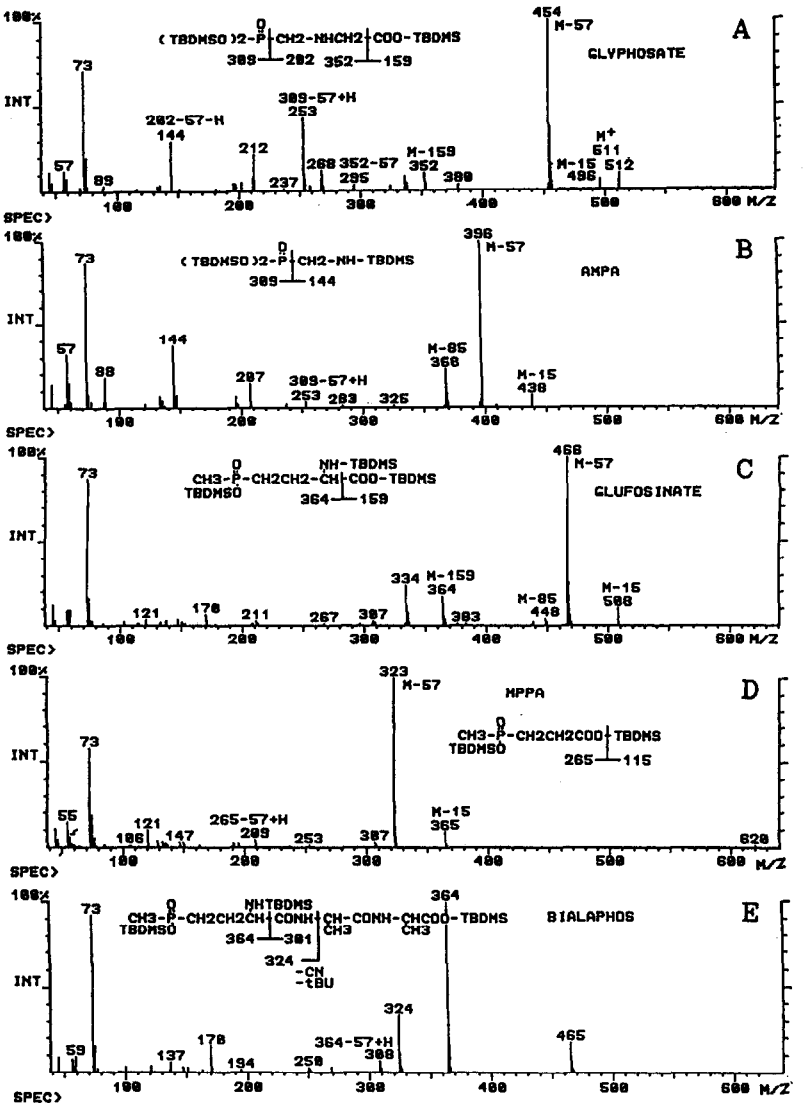

Fig. 4. EI mass spectra of *tert.*-butyldimethylsilyl derivatives of (A) glyphosate, (B) AMPA, (C) glufosinate, (D) MPPA and (E) bialaphos.

364 results from cleavage of the CH–CO bond to the N-terminal acid. This fragment ion is also found in the *t*BDMS derivatives of the corresponding amino acid, but at a lower intensity. A further peak, m/z 324, is observed at an m/z 40 less than this fragment. This fragment may arise from cleavage of the central NH–CH bond followed by loss of *tert.*-butyl and a cyanide radical. The distinctive pair of ions separated by m/z 40 clearly distinguishes the spectra of amino acid and dipeptide *t*BDMS derivatives [26].

CONCLUSIONS

A selective and sensitive GC–MS method using IT-MS as a mass detection system has been developed for the determination of GLYP, GLUF and BIAL and their metabolites. The sample preparation method is simple by derivatization with MTBSTFA and dimethylformamide. This procedure allows the determination of these herbicides and their metabolites simultaneously.

The GC–IT-MS method offers more sensitivi-

TABLE III

DIAGNOSTIC IONS IN THE EI MASS SPECTRA OF THE *tert.*-BUTYLDIMETHYLSILYL DERIVATIVES OF GLYPHOSATE, GLUFOSINATE, BIALAPHOS AND THEIR METABOLITES OBTAINED IN GC–IT-MS

BP, base peak; –, not detected.

Compound (M_r)	M^+	M – 15	M – 57	M – 85	M – 159	Others
Glyphosate (511)	511	496	454(BP)	–	352	73, 144, 212, 253
AMPA (453)	–	438	396(BP)	368	–	73, 144, 207, 253
Glufosinate (523)	–	508	466(BP)	438	364	73, 334
MPPA (380)	–	365	323(BP)	–	–	73, 121, 209
Bialaphos (665)	–	–	–	–	–	73, 170, 308, 324, 364(BP), 465

ty than the GC method. Monitoring several ions at m/z M – 15, M – 57 and M – 159 for *t*BDMS derivatives affords additional structural confirmation beyond retention time matching with a reference standard. This was found to be useful in the determination of the herbicides and discriminating them from amino acids typically found in physiological and food samples.

ACKNOWLEDGEMENTS

The author thanks Monsanto Japan, Hoechst Japan and Meiji Seika Kaisha Ltd. for the donation of the herbicides and their metabolites.

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