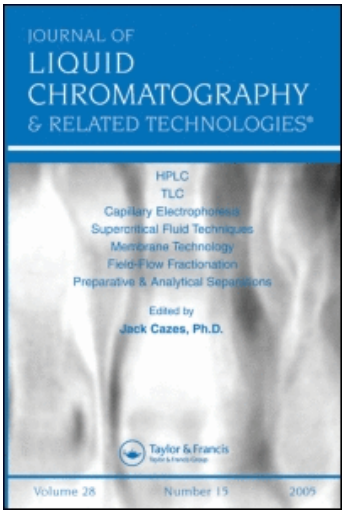

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The Determination of Metribuzin and Its Metabolites by High Pressure Liquid Chromatography

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THE DETERMINATION OF METRIBUZIN AND ITS METABOLITES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY 1,2

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ABSTRACT

A method for the determination of metribuzin and its metabolites in plant tissues has been developed using High Pressure Liquid Chromatography (HPLC). The system used involves reversed-phase chromatography on a C-18 HPLC column and a 62:38 methanol/0.05 M acetic acid mobile phase. Under these conditions, metribuzin and the three known metabolites [deaminated metribuzin (DA), deaminated diketometribuzin (DADK) and diketometribuzin (DK)] are completely resolved from each other. Detection is by UV absorbance at 254 nm.

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INTRODUCTION

Metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5 (4H)one; BAY 94337] is a systemic herbicide widely used for the control of grasses and broadleaf weeds in a variety of crops, including soybeans, potatoes, and tomatoes. The relative tolerance of plants such as soybeans to metribuzin has been attributed to their ability to convert metribuzin to less phytotoxic substances more rapidly than do more susceptible plants (1). Three metabolites of metribuzin have been identified (2-4): deaminated metribuzin (DA), deaminated diketometribuzin (DADK), and diketometribuzin (DK). The structures of these compounds are shown in Figure 1. These metabolites have been found in soybeans (1,5-7), tomatoes (8,9), potatoes (10), sugarcane (11), barnyard grass and American nightshade (12), other crops (10), and in soils (7,10,13).

Differences between cultivars in susceptibility to metribuzin injury have been observed for soybeans (5,14-25), potatoes (26,27), and tomatoes (8). These differences have been attributed to differences in metribuzin metabolism (5,8,14,25). Another area of concern involves possible phytotoxic interactions between metribuzin and other pesticides (6,9,28-32). Changes in metribuzin metabolism have been found in soybeans (6) and in tomatoes (9) which appear to have been induced by the additional pesticide.

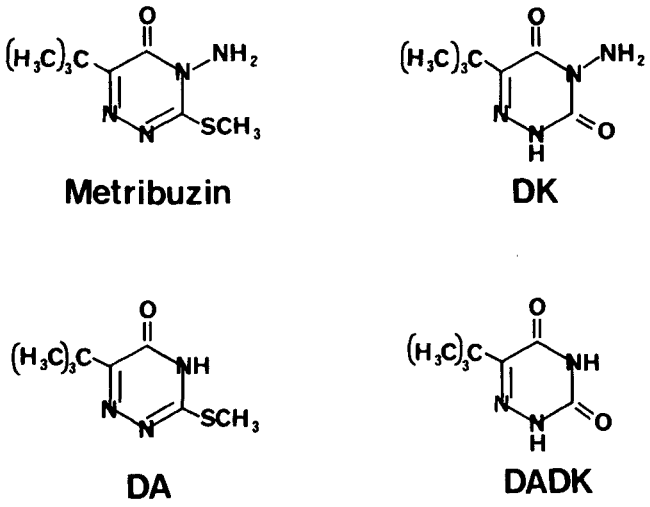

Figure 1. Structures of metribuzin and the three known metabolites

Interest in crop tolerance or susceptibility to metribuzin injury has led to the development of various research methodologies for the detection and quantitation of metribuzin and its metabolites in plant tissue and soils. Thus far, these methods include only gas chromatography (GC) and thin-layer chromatography (TLC). GC analysis of metribuzin, DA, DADK, DK may be performed with an OV-225 column using an electron-capture detector (7,10,11) or Coulson conductivity detector (10); or with a Silar-5CP column and an alkali flame ionization detector (13). Using these GC methods, extensive sample cleanup is usually required, and several

injections are sometimes required to separate all of the metabolites (10), although the use of N-specific detectors has eliminated many interferences (7).

Various thin-layer chromatographic procedures using aluminum oxide (12) or silica gel (5,6,12) are currently in use for the analysis of metribuzin and its metabolites. Many of these methods cannot resolve all of the known metabolites. Moreover, using the TLC method, quantitation must be done as a separate step, usually after the material has been removed from the plate.

To the best of our knowledge, this paper is the first to describe a method using high pressure liquid chromatography (HPLC), for the separation of metribuzin and its metabolites. The method allows the separation and quantitation of these compounds in a single step, without the need for extensive sample cleanup.

MATERIALS AND METHODS

Equipment

The HPLC system consisted of two Waters 6000A pumps (Waters Associates, Milford, MA), a Waters 440 UV detector set at 254 nm, and a Waters 660 Solvent Programmer. Retention times and peak areas were determined with a Waters Data Module 730. The column used was a Dupont Zorbax ODS (C-18) column, 5 micron particle size, 4.6 mm I.D. x 25 cm length (Dupont Instruments Analytical Division, Wilmington, DE).

Flow rates were 1.0 ml/min, and separations were done at room temperature unless otherwise stated. Sample cleanup was carried out on Waters C-18 Sep-Paks.

Standards and Solvents

Samples of metribuzin, DK, DADK, and DA were obtained from Mobay Chemical Corp, Ag. Chem. Div., Kansas City, MO. Stated purities for these compounds were 99.6%, 95%, 95%, and 95%, respectively. Solvents used in this study were HPLC-grade methanol (Fisher Scientific, Co., Fairlawn, NJ), HPLC grade tetrahydrofuran (THF) (Burdick and Jackson Laboratories, Inc., Muskegon, MI), HPLC-grade acetic acid (J.T. Baker Chemical Co., Phillipsburgh, NJ) and deionized water (Darco Water Systems, Inc., Durham, NC). The organic solvents were filtered through a 0.5 micron Millipore filter, type FH (Millipore Corp., Bedford, MA); the water was filtered through a 0.45 micron Millipore filter, type HA.

Plant Culture

Soybean seeds [Glycine max. (L.) Merr. "Ransom"] were germinated on paper towels. After germination, seedlings were transferred to 125-ml flasks containing 100 ml of half-strength Hoagland's solution (33). Soybean plants were cultured in a growth chamber at a light intensity of 12 klux. Day length and temperature were maintained at 16 h and 27°C, respectively.

Tissue Extracts

Four day-old plants were partitioned into leaves, stems, cotyledons, and roots. The wet weight of the combined plant parts were 0.48 g, 0.29 g, 1.19 g, and 0.47 g respectively. Each plant part was extracted with methanol in an omni-mixer. The homogenate was then filtered and the filtrate was evaporated in a flash evaporator to a final volume of 2 ml.

For control samples, 1 ml of each extract was diluted to 10 ml with methanol. For "spiked" samples, 1 ml of each extract was diluted to 10 ml with methanol to which a mixture of standards had been added. Concentrations of the standards in the spiked extracts were approximately 1.5 ng/µl DK, 1.2 ng/µl DADK, 1.9 ng/µl DA and 1.2 ng/µl metribuzin. In order to remove any strongly retained material which might be present, a 2 ml portion of each extract was filtered through a Waters C-18 Sep-Pak which had previously been rinsed with methanol. Recoveries of DK, DADK, DA, and metribuzin were greater than 99% by HPLC analysis (UV detection) before and after Sep-Pak filtration. Sep-Pak filtered control and spiked extracts were injected directly into the liquid chromatograph.

RESULTS AND DISCUSSION

Using HPLC, separation of a mixture of metribuzin, DK, DADK, and DA can be achieved in 66:34 methanol: 0.05 M

acetic acid (HOAc) and a flow rate of 1 ml/min, giving an overall analysis time of 8 minutes for the mixture (Figure 2A). The separation of the same standard mixture in 62:38 methanol/0.05 M HOAc is shown in Figure 2B. While this has the effect of increasing the analysis time to approximately 15 minutes per sample, it has been found to give better separation of the metabolite peaks from naturally occurring tissue components. The retention times of DK, DADK, metribuzin, and DA under these conditions are 5.63, 6.46, 8.56 and 9.56 min, respectively.

Chromatograms shown in Figure 3 were obtained by injecting 8 µl of the control and spiked leaf, stem, cotyledon, and root extracts. For the spiked extracts, the chromatograms shown correspond to approximately 12 ng DK, 10 ng DADK, 15 ng DA, and 10 ng metribuzin injected. As can be seen from these chromatograms, each plant organ has a different pattern of early eluting (i.e., polar) components, but these components do not seriously interfere with the analysis under these conditions. Peak areas from naturally-occurring components which coelute with metabolite peaks could be subtracted from the total peak areas if control tissue samples were run. To prevent build-up of any strongly retained materials on the HPLC column, the column was purged with tetrahydrofuran for an hour after the end of each workday, at a flow rate of 1 ml/min.

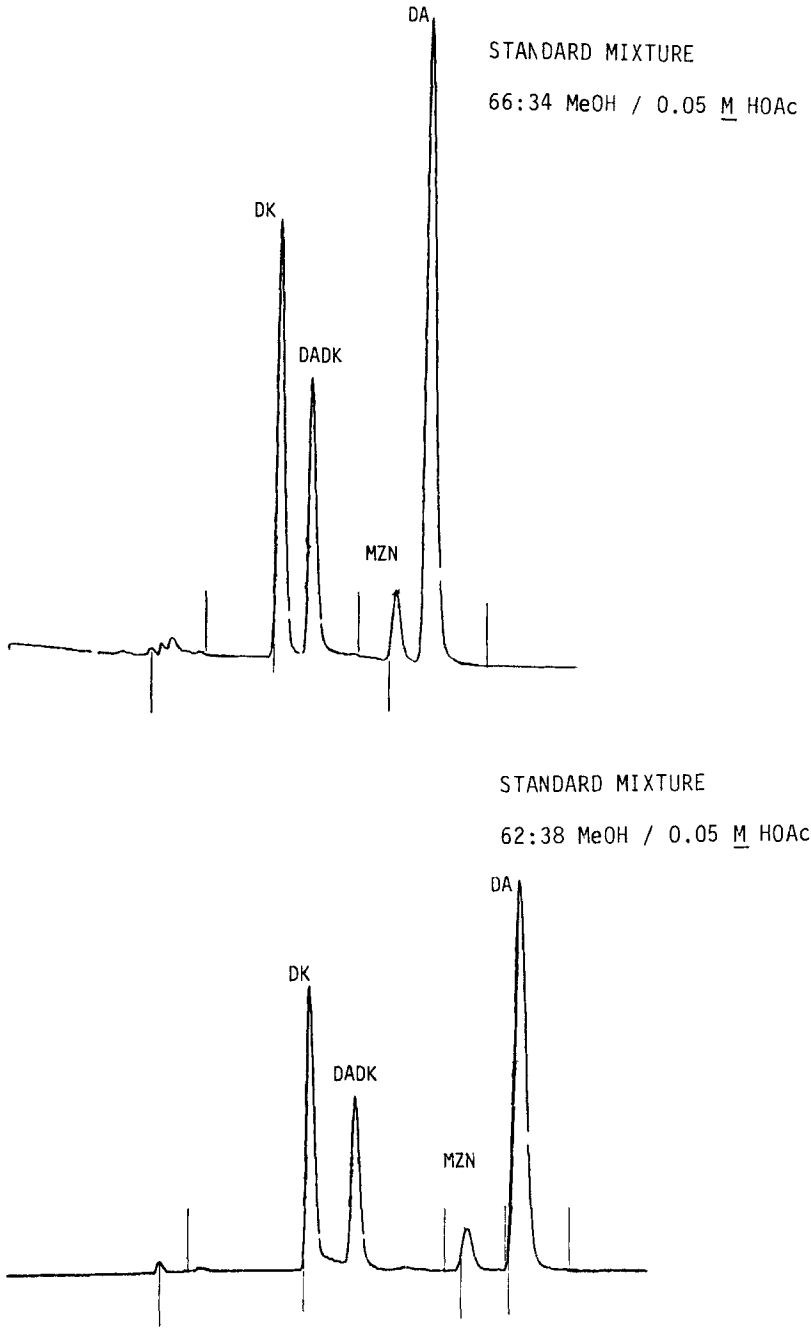

Figure 2. Chromatograms of a mixture of standards in methanol: 0.05 M acetic acid.

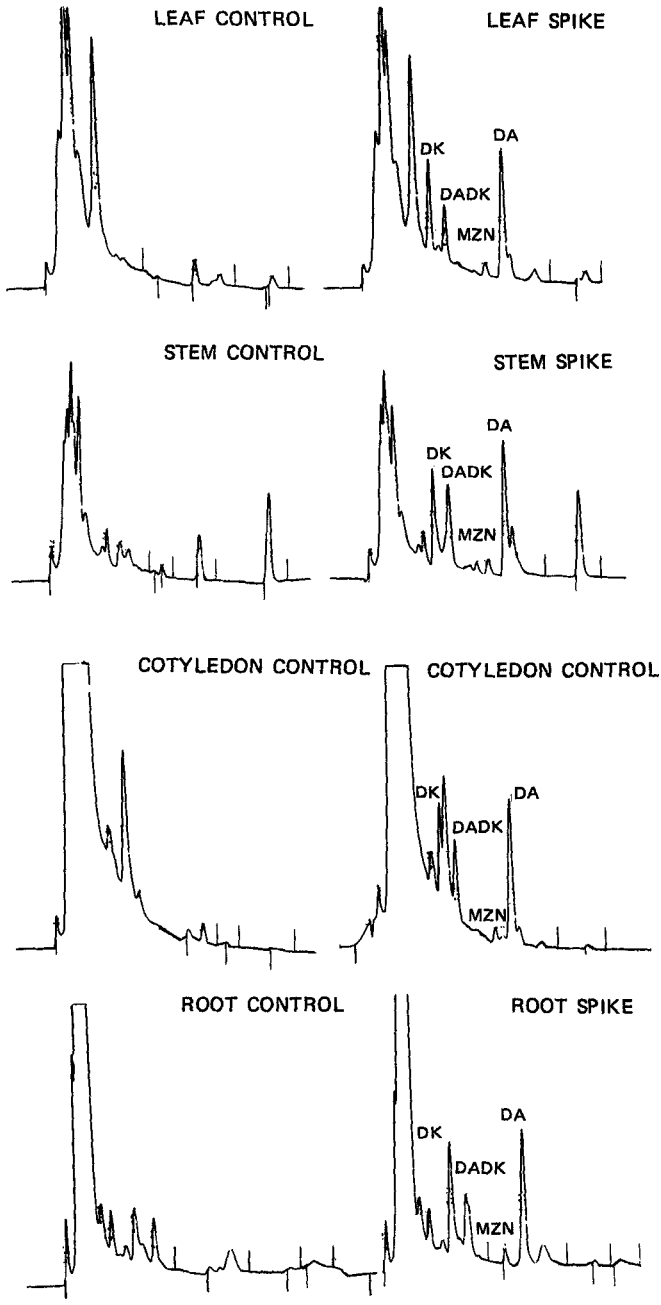

Figure 3. Chromatograms of control and spiked plant extracts in methanol:0.05 M acetic acid.

Table 1. Retention Times (min.) of the Four Standards with Different HPLC Operating Conditions.[a]

Solvent	(1:1 v/v) MeOH/H_2O			(1:1 v/v) MeOH/H_2O			(1:1 v/v) MeOH/0.05 M HOAc			(1:1 v/v) MeOH/0.05 M HOAc		
Temperature	25°C			42°C			25°C			42°C		
Flow Rate(ml/min)	1.0			1.0			1.0			1.0		
Back Pressure(psi)	3000			2000			3000			2000		
	Inj. 1	Inj. 2	Inj. 3	Inj. 1	Inj. 2	Inj. 3	Inj. 1	Inj. 2	Inj. 3	Inj. 1	Inj. 2	Inj. 3
DK	9.32	9.35	9.32	7.07	7.07	7.07	8.82	8.75	8.80	6.80	6.80	6.80
DADK	11.45	11.50	11.57	8.27	8.27	8.27	11.12	11.02	11.10	8.15	8.12	8.12
DA	12.15	17.57	18.82	11.52	11.57	11.62	21.82	21.52	21.70	23.92	23.77	23.72
MZN	21.07	21.10	21.02	14.45	14.45	14.45	19.82	19.60	19.70	13.95	13.87	13.85

[a] Void Volume in all cases is 1.90 ml (r.t.=1.90 min)

Initial work on the separation of metribuzin and its metabolites was done with a methanol:water mobile phase. Although separation could be achieved, selectivity differences were observed at room temperature between columns from the same manufacturer and instability of retention time was observed for DA (Table 1).

Two strategies were pursued in order to overcome this problem: one was to increase the temperature of the separation and the other was to lower the pH of the solvent. Table 1 shows the retention times of the compounds from repeated injections under different conditions. This data shows that an increase in column temperature stabilizes the retention time for DA and shortens overall analysis time. A similar temperature effect on selectivity was reported for the separation of trans-diethylstilbestrol from dienestrol (34). Furthermore a temperature increase from 25° to 42°C lowers the viscosity of the solvent and thereby lowers the back pressure of the system. A chromatogram obtained at 42°C of a mixture of DA, DADK, DK and metribuzin with a methanol H_2O (1:1 v/v) mobile phase is shown in Figure 4.

As can be seen from Table 1 and by comparing Figures 2 and 4, acetic acid (0.05 M) in the solvent mixture gave a different elution sequence from that obtained without acetic acid. Also, in the presence of acetic acid, the relative retention time for the DA peak increased and

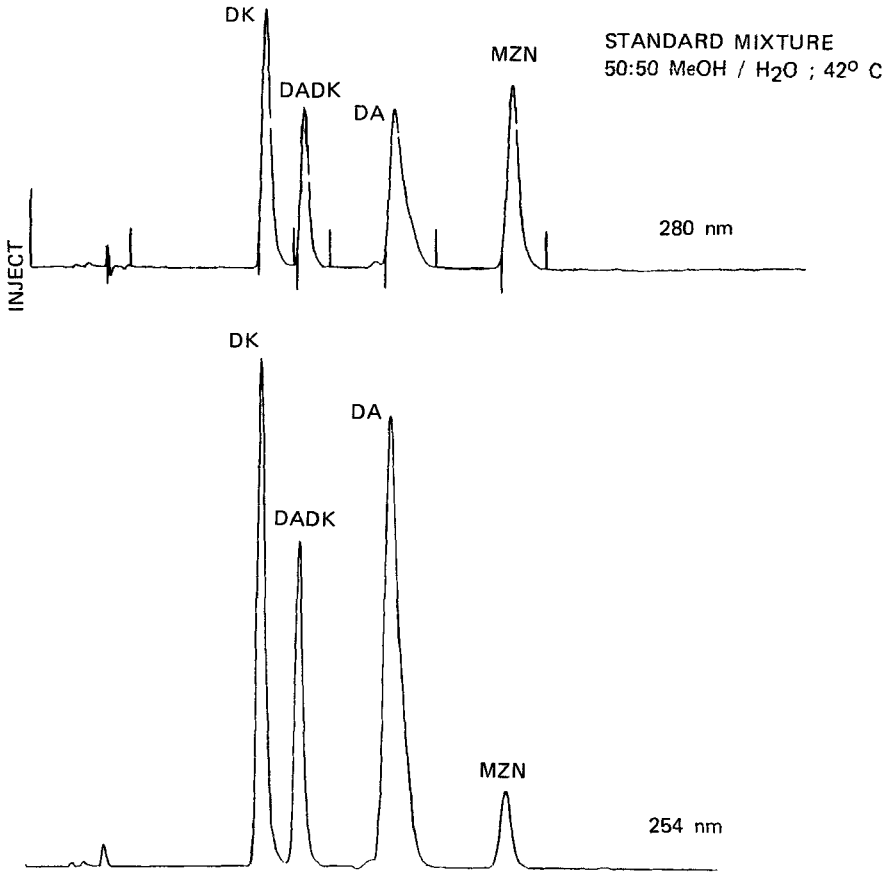

Figure 4. Chromatogram of a mixture of standards in methanol: water at 42°C with simultaneous detection at 280 and 254 nm.

temperature effects were reduced. Either modification (temperature or pH) allowed reproducible separation, so separation conditions can be chosen depending on availability and applicability to a particular sample matrix. For the separations involving plant tissue extracts described

earlier in this paper, the method involving pH adjustment was selected since it does not require a column heater and was thus considered to be more generally applicable to other laboratories.

The application of dual wavelength detection to these compounds is shown in Figure 4. While this technique is not mandatory, it can be used to increase the sensitivity of the analysis for metribuzin since the parent compound absorbs more strongly at 280 nm. In the absence of interfering compounds, detection limits at 254 nm were approximately 1 ng for DA, DK, and DADK, and 5 ng for metribuzin. At 280 nm, the detection limits were approximately 2 ng for DA, DK, DADK, and metribuzin.

CONCLUSIONS

Due to the combination of resolution, sensitivity, and ease of sample preparation, the HPLC technique described here should prove to be a convenient and rapid method for the study of metribuzin and its metabolites in plant tissue. Because isocratic conditions are used, it should be possible to automate the analysis with an automatic sampling system. The resolution obtainable with the HPLC method should also facilitate the detection and quantitation of other possible metribuzin metabolites.

With UV detection, the use of radiolabeled standards is not necessary, providing that appropriate control tissue

samples are used, and that a system can be devised to separate metabolites from normally occurring compounds. The ideal system for metabolite analysis, however, would be the use of radiolabeled parent compounds, and HPLC analysis with both a UV detector and an in-line radioactivity monitor. Radioactivity detection would be specific and selective for the radiolabeled metabolites, and thereby shorten the overall analysis time since only separation of the metabolites from each other, but not from naturally-occurring plant materials, would be required. Details of the separation were reported here in order to emphasize that this HPLC method is a relatively flexible system which should be easily adaptable for the analysis of metribuzin and its metabolites in other biological matrices.

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