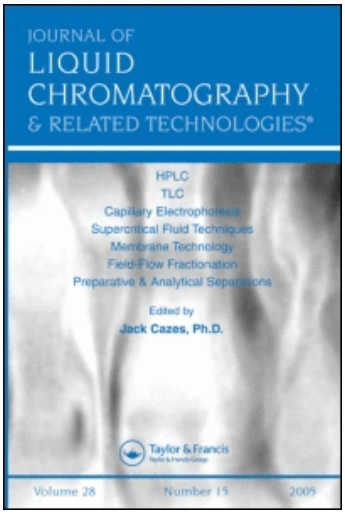

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ANALYSIS OF METRIBUZIN AND ASSOCIATED METABOLITES IN SOIL AND WATER SAMPLES BY SOLID PHASE EXTRACTION AND REVERSED PHASE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

A method to analyze metribuzin, 4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one, and its major metabolites, deaminated metribuzin, DA, 6-(1,1-dimethylethyl)-3-(methylthio) -1,2,4-triazin-5(4H)-one, diketometribuzin, DK, 4-amino-6-(1,1-dimethylethyl)-1,2,4-triazin-3,5(2H,4H) -dione and deaminated diketometribuzin, DADK, 6-(1,1-dimethylethyl)-1,2,4-triazin-3,5(2H,4H)-dione in soil and water samples by reversed-phase thin layer chromatography (RPTLC) is described. Soil samples were extracted with MeOH:0.01 *M* $CaCl_2 \cdot 2H_2O$ (4:1) and the extracts filtered to remove the sediment. The extract then was diluted with deionized water and eluted through a C18, solid phase extraction (SPE) cartridge. Water samples (150-mL) were eluted directly through C18, SPE cartridges. Both soil and water extracts were chromatographed on pre-coated RPTLC plates. The concentration of metribuzin was determined by UV densitometry at 290 nm. Recoveries of metribuzin from soil samples fortified at 2 mg kg^{-1} ranged from 73-86%. Recoveries from water samples fortified at 10 and 100 μg L^{-1} ranged from 85 to 92%. The detection limit of the method for metribuzin is 30 nanograms.

INTRODUCTION

Metribuzin is triazine herbicide that is used extensively in Louisiana and states in the mid-south for weed control in sugarcane and has been detected in groundwater (1). The triazines are the most widely applied herbicide class with an estimated market size of 1,425 million dollars (2). Although the triazines exhibit a low degree of toxicity to humans and animals (3), the carcinogenic potential of these compounds is still unclear. Metribuzin has been identified as an endocrine disrupter (4). The USEPA has evaluated the carcinogenic potential of metribuzin and has placed it in group D, indicating inadequate human and animal evidence of carcinogenicity. Recently, a comprehensive search was made of the STORET water quality data base, which is maintained by the Office of Water, U.S. Environmental Protection Agency (1). In this search, metribuzin was detected in 343 of 3,208 groundwater samples.

The concerns for water quality have led to increased pesticide monitoring programs throughout the U.S. The cost of these programs has often been prohibitive, thereby limiting the number of samples. In attempt to address these issues several analytical techniques have been investigated that retain the sensitivity of traditional methods such as gas chromatography (GC) and high pressure liquid chromatography (HPLC), while decreasing the cost and sample analysis time. One technique that has received considerable attention is enzyme immunoassay. This technique is rapid and cost effective (5). The principal disadvantage is its lack of specificity, due to cross reactions (6). The technique however, is well suited for screening large numbers of samples. Positive samples later can be confirmed by another technique, such as GC or HPLC.

In recent years, thin layer chromatography (TLC) has also received increased attention as a quantitative analysis tool (7). This emphasis has arisen partly from the significant advances that have been made in TLC stationary phases. Analytical TLC plates now are available in a wide range of normal and reversed phase sorbents, in both the standard and high performance (HPTLC) mode. Also, the availability of low cost scanning densitometers has increased the

utility of TLC as a quantitative tool. Although it is unlikely that TLC will replace GC and HPLC as the method of choice for pesticide analysis, numerous situations exist where TLC would provide a viable, rapid, low cost alternative to these methods. TLC may be particularly useful for sample screening. Potential advantages of TLC include: 1) high sensitivity, 2) minimum sample preparation time, and 3) low cost.

Several authors have reported TLC systems for the analysis of metribuzin and other triazines. Plant extracts containing metribuzin and its metabolites, DA, DK and DADK were analyzed by TLC on silica gel plates with a mobile phase of chloroform/dioxane (95:5 v/v) (8). In a related study, metribuzin present in benzene extracts of plant tissue was analyzed by TLC on silica gel G plates using chloroform/dioxane (9:1 v/v) and on cellulose F plates using benzene as the mobile phase (9). Water extracts of plant tissue containing metribuzin were analyzed on silica gel G using *n*-butanol:ethanol:water (40:11:19 v/v/v) and chloroform/dioxane (9:1 v/v) (9).

Water samples containing atrazine and simazine were extracted with chloroform and chromatographed on silica gel G using a mobile phase of chloroform/acetone (9:1 v/v). Recoveries from samples fortified at a concentration of 10 ppb were 86% for atrazine and 83% for simazine (10). Water samples fortified with triazine and chlorophenoxy herbicides at a concentration of 10 ppb were analyzed by C18, solid phase extraction (SPE) and TLC. Atrazine and simazine were analyzed on silica gel GF plates with a mobile phase of chloroform/acetone (9:1 v/v). Silvex, 2,4-D and 2,4,5-T were determined on silica gel GF plates with a mobile phase of hexane/glacial acetic acid/diethyl ether (72:30:18 v/v/v). Recoveries ranged from 70-88% for the triazines and 93-100% for the chlorophenoxy herbicides (11). More recently, soil samples containing atrazine and its metabolites, hydroxyatrazine, deethylatrazine and depropylatrazine were extracted with MeOH and analyzed by reversed phase, C18, high performance thin layer chromatography (RP-HPTLC). Recoveries for soils fortified at a concentration of 2 ppm ranged from 87-97% (12). This paper describes

a simple and direct method for the analysis of soil and water samples containing metribuzin and its metabolites DA, DK and DADK using SPE and RPTLC.

MATERIALS

Soils

The soils investigated included surface horizons (A or Ap) from a Commerce silty clay (fine-silty, mixed, nonacid, thermic Aeric Fluvaquent), and an Ocholocknee sandy loam (coarse-loamy, siliceous, acid, thermic Typic Udifluvent) from East Baton Rouge parish, Louisiana, an Evesboro loamy sand (coarse-loamy, siliceous, mesic Aquic Hapludult) from Sussex county, Delaware and a Conover loam soil (loamy, mixed, mesic Udollic Ochraqualf) from Ingham county, Michigan. The horizon designation Ap indicates that the soil has been cultivated and the designation A indicates that the soil has not been cultivated. The moisture content at 0.33b is taken to be an estimate of the field capacity moisture content of the soil in question. Surface horizons The Ochlocknee, Evesboro, Conover and Commerce soils were used in recovery experiments and varied in clay content from 12 to 26% and in organic matter content from 0.9 to 1.8%. Selected characteristics of these soils are presented in Table 1.

Herbicides

The herbicide used was metribuzin [93-94% pure], 4-amino-6- (1,1-dimethyl ethyl)-3- (methylthio)-1,2,4-triazin-5(4H)-one. In addition, three of its major metabolites were studied, deaminated metribuzin [99.6%, DA], 6-(1,1-dimethyl ethyl) -3-(methylthio)-1,2,4-triazin-5(4H)-one, diketometribuzin [98.7%, DK], 4-amino- 6-(1,1-dimethylethyl) -1,2,4-triazin-3,5(2H,4H)-dione and deaminated diketo metribuzin, [84.8%, DADK], 6-(1,1-dimethylethyl)-1,2,4-triazin-3,5 (2H,4H)-dione (Figure 1). Parent metribuzin and metabolites were obtained from the Miles Corporation, Kansas City, MO. Standard solutions of metribuzin were prepared in HPLC grade MeOH at concentrations of 0.01, 0.1 and 1.0 $\mu g\ \mu L^{-1}$.

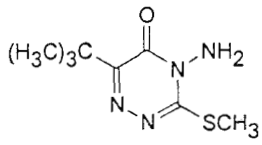
TABLE 1

Selected Soil Properties for the Evesboro, Ocholocknee, Conover and Commerce Soils.

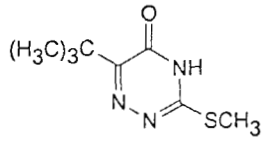
Soil Series Horizon	pH§	OM	Particle Size Sand	Silt	Clay	0.33 Bar
		----------%----------				
Evesboro						
Ap	5.3	0.94	71	16	13	9.6
Ocholocknee						
A	4.9	1.09	67	21	12	15.0
Conover						
Ap	6.6	1.79	60	20	20	20.6
Commerce						
Ap	5.2	1.52	41	33	26	29.0

§ - **pH** = pH of 1:1 soil/deionized water suspension, **OM** = soil organic matter content (13), **Particle Size** (14), **0.33 Bar** = water content (g/100 g dry soil) at 0.33 bars pressure.

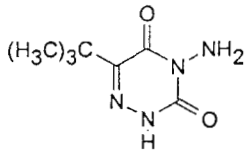
Standards for the metribuzin metabolites DA, DK and DADK were also prepared in HPLC grade MeOH at concentrations of 0.1 and 1.0 μg μL^{-1}. All solvents used for TLC were analytical grade and deionized water (≥ 18 mohm-cm) was obtained with a nanopure deionization system (Barnstead/ Thermolyne Corp., Dubuque, IA).



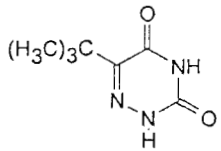
Metribuzin



DA



DK



DADK

FIGURE 1. Chemical structures for metribuzin, DA, DK and DADK.

METHODS

Water Extraction Procedures

Two techniques were investigated to extract water samples. In the first method, water samples (500 mL) were extracted in a 1-L separatory funnel with 3 x 25-mL dichloromethane. The organic fractions were combined and then dried through anhydrous sodium sulfate. The sodium sulfate was rinsed with a final 25-mL of dichloromethane and the dried organic fraction was then evaporated under a gentle air stream. The residue was reconstituted in 100 µL of HPLC grade MeOH for analysis. In the second method, water samples (150 mL) were filtered through C18, SPE cartridges (SEP-PAK®, Waters Associates, Milford. MA). Prior to filtration, the cartridges were activated with 2-mL of HPLC grade MeOH and 5-mL deionized water. After filtration, the columns were rinsed with 2-mL of HPLC grade MeOH to elute the sorbed metribuzin. The samples were dried under a gentle air stream and then re-constituted in 100-µL MeOH.

Soil Extraction Procedures

Soil (50 g) was transferred to 250-mL erlenmeyer flasks and extracted with 150-mL MeOH:0.01*M* $CaCl_2 \cdot 2H_2O$ (4:1) on a rotary shaker (200 RPM) for 18 hours. The suspension was then filtered through Whatman #4 filter paper. The soil was washed with an additional 50 mL of MeOH:0.01*M* $CaCl_2 \cdot 2H_2O$ and the filtrates were combined. At this stage two techniques were employed. In the first method the extracts were evaporated to dryness under vacuum at 60° C, redissolved in MeOH, transferred to 20-mL vials and redried under a gentle air stream. Prior to analysis, samples were reconstituted in 200 µL MeOH. In the second method 5-mL of the extract was diluted with 145-mL of deionized water and filtered through an activated C18, SPE cartridge. Metribuzin and any associated metabolites were eluted from the cartridge with 2-mL MeOH. The extract was dried under an air stream and reconstituted in 100 µL MeOH.

Thin Layer Chromatography

RPTLC was performed on reversed-phase, hydrocarbon impregnated uniplates, (10 x 20 cm, 250 micron thickness, Analtech Inc., Newark, DE). Standards and sample extracts were drawn into microcapillary pipettes (1, 2 and 4 µL) and applied with a Nanomat III (Camag Inc., Greenville, NC). A standard curve, which varied in concentration depending on the samples being analyzed, was included on each plate. The optimum mobile phase was determined to be MeOH:H_2O (45:55 v/v). Spotted plates were equilibrated in a vertical chamber containing the mobile phase for 0.5-h prior to development. Plates were developed for a distance of 10 cm, dried and scanned with a variable wavelength Shimadzu CS9000U Dual Wavelength Flying Spot Scanner. Standard curves were analyzed by linear regression analysis (MSTATC, East Lansing, MI).

Herbicide Recovery

To evaluate the extraction efficiency of the proposed method, 1-L of deionized water and 1-L of tap water was spiked with metribuzin at rates of 10

and 100 μg L^{-1}. Samples were analyzed as outlined above. The extraction efficiency for soil was evaluated by adding 50-g of the Evesboro, Ochlocknee Conover and Commerce soils to 250-mL erlenmeyer flasks, moistening the soil to field capacity (10, 15, 20 and 29%, respectively) and amended them with metribuzin at a rate of 2 mg kg^{-1} soil. Samples were aged for 24-hours and extracted and analyzed as outlined above.

Response to Other Herbicides

The performance of the method with other herbicides was also evaluated. Compounds evaluated included; atrazine, (6-chloro-N-ethyl-N'- (1-methylethyl) -1,3, 5-triazine-2,4-diamine), cyanazine, 2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2-methylpropanenitrile, and alachlor, 2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide. These compounds were spotted along with the metribuzin at a concentration of 2 μg to determine if they could be resolved in the proposed system.

RESULTS AND DISCUSSION

A series of experiments was conducted to determine the optimum wavelength for analysis of metribuzin and it metabolites. The UV-absorbtion spectrum of metribuzin, DA, DK and DADK was constructed by spotting 2 μg of each compound on a RPTLC plate and scanning the spots from 200 to 370 nm (Figure 2). The maximas for metribuzin, DA, DK and DADK were found to be 290, 235, 254 and 254 nm, respectively. Further analyses of each compound was performed at their respective maxima.

Several TLC systems were investigated to perform these separations. The first stationary phase investigated was a RP-HPTLC, C18, bonded phase plate. These plates proved adequate for the parent metribuzin, but not for the separation of metribuzin, DA, DK and DADK together. Various mobile phases composi-

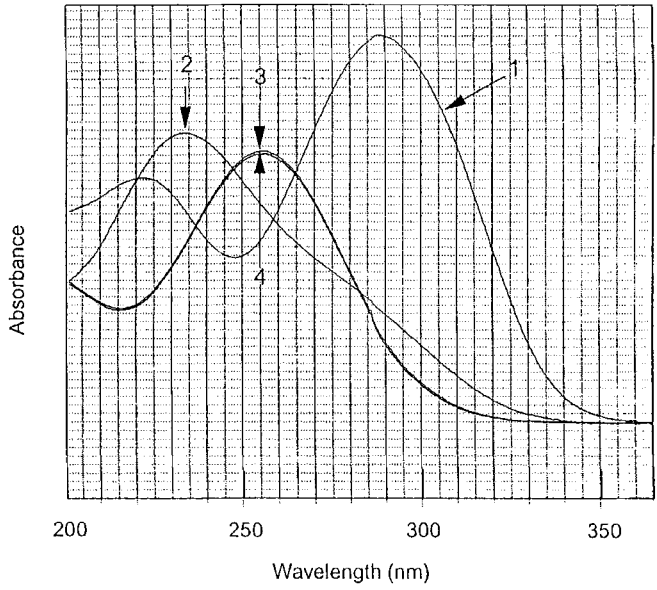


FIGURE 2. Absorbance spectrum for metribuzin (1), DA (2), DADK (3) and DK (4).

tions were investigated including; MeOH:H_2O (from 85:15 to 60:40 v/v), Acetonitrile:H_2O, MeOH:acetic acid, MeOH:tetrahydrafuran:H_2O, etc. The most promising approach appeared to be with MeOH:H_2O (60:40 v/v), however this amount of water resulted in a very slow development and still yielded an unsatisfactory separation (Figure 3a). The stationary phase was changed to a RPTLC, hydrocarbon impregnated plate. This plate allows for a greater percentage of H_2O in the mobile phase, up to 100%, without significant increases in development time. Several mobile phase combinations were evaluated starting at MeOH:H_2O (50:50 v/v) (Figure 3b). After several experiments, a mobile phase consisting of MeOH:H_2O (45:55 v/v) was found to yield the optimum separation (Figure 3c). It should be noted that water percentages greater than 55% resulted in a decreased resolution of the four compounds.

Although slight variation in the absolute retention R_f (Table 2) was observed among experimental systems, relative trends in R_f values were consistent.

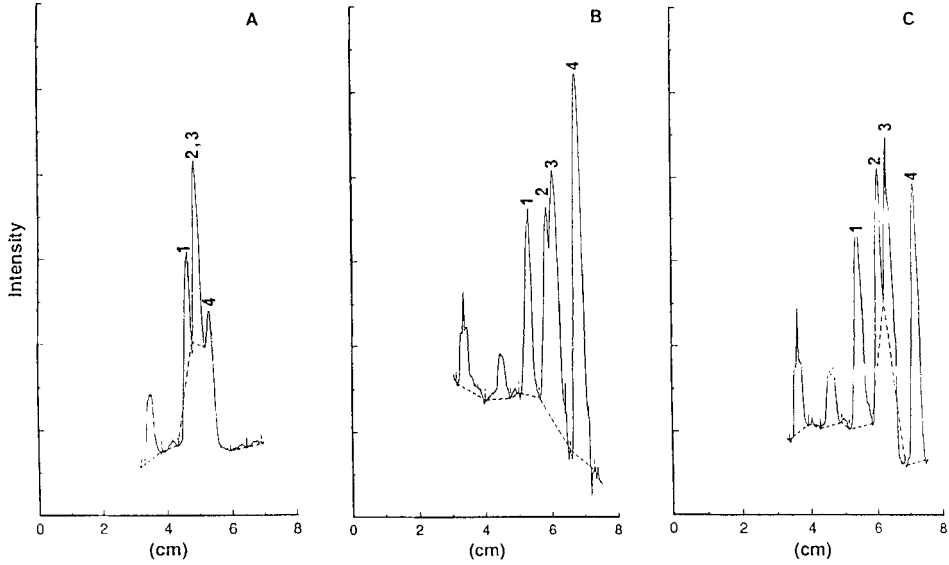


FIGURE 3. Densitometer chromatograms of A) metribuzin (1), DA (2), DADK (3) and DK (4) on a HPTLC, C18 plate, MeOH:H20 (60:40 v/v), B) metribuzin (1), DK (2), DADK (3)and DA (4) on a RPTLC plate, MeOH:H20 (50:50 v/v), C) metribuzin (1), DK (2), DADK (3)and DA (4) on a RPTLC plate, MeOH:H20 (45:55 v/v).

The observed variation is most likely related to the combined effects of intramolecular attraction between the co-chromatographed compounds and the presence of interfering compounds in the water and soil extracts. All plates were thoroughly dried before development making it unlikely that residual MeOH is causing the observed effect. It is conceivable that residual H_2O was present in the soil and water extracts, although this was not directly observed. If this was the case then the observed R_f values would tend to be lower. Despite the observed variations, all of the compounds were well resolved in all systems.

Standard curves for parent metribuzin and its metabolites DA, DK and DADK were linear or curvilinear, depending on the range of concentrations investigated (Figure 4). Although the curves could be described by quadratic or cubic regression equations, these equations generally resulted in a poor fit at the

TABLE 2

Retention Factors (R_f) for Metribuzin, DA, DK and DADK on RPTLC Plates in Various Systems.

Compound	System[§]	R_f
Metribuzin	soil	0.51
	water	0.51
	cochromatography	0.52
	standard curve	0.58
DA	soil	0.79
	water	0.78
	cochromatography	0.82
	standard curve	0.83
DK	soil	0.63
	water	0.62
	cochromatography	0.63
	standard curve	0.74
DADK	soil	0.67
	water	0.67
	cochromatography	0.68
	standard curve	0.78

§ - **soil** = fortified soil samples, **water** = fortified water samples, **co-chromatography** = co-chromatographed compounds, **standard curve** =compounds chromatographed alone.

lower concentration ranges. A better fit was generally obtained if the curve was broken into two ranges, 100 to 800 ng and 1000 to 3000, 4000 or 5000 ng (Table 3). The detection limit of this method was 30 ng for spotted standards.

Recoveries of parent metribuzin from fortified water samples were highly dependent on the method employed. Water samples fortified at 10 and 100 $\mu g\ L^{-1}$ that were extracted with methylene chloride resulted in recoveries of 43 and 70%,

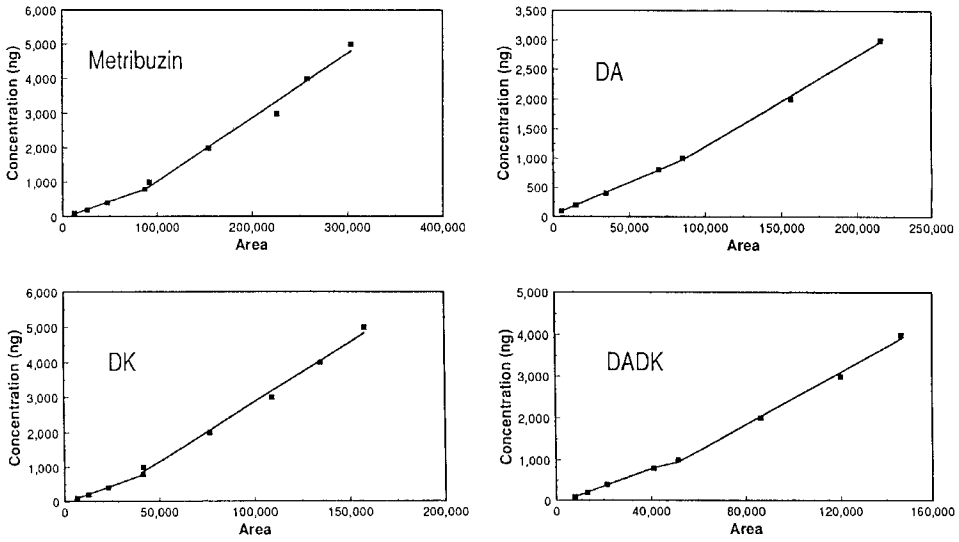


FIGURE 4. Standard curves for metribuzin, DA, DK and DADK as described by linear equations.

TABLE 3

Regression Data for Metribuzin, DA, DK and DADK.

Compound	Conc. Range	Linear r^2
	ng applied	
Metribuzin	(100-800)	0.997***
	(1000-5000	0.981***
DA	(100-800)	0.998***
	(1000-3000)	0.997***
DK	(100-800)	0.998***
	(1000-5000)	0.991***
DADK	(100-800)	0.999***
	(1000-5000)	0.996***

*** significant at P(0.001)

respectively. Recoveries of parent metribuzin from water samples analyzed by SPE techniques were significantly improved. For deionized water and tap water samples fortified at 10 $\mu g\ L^{-1}$ the recoveries were 85 and 89%. For deionized water and tap water samples fortified at 100 $ug\ L^{-1}$ the recoveries were 92 and 91%, respectively. In all cases, the RPTLC system yielded a well resolved chromatogram, free of interferences (Figure 5a). In addition to an increase in recovery, the SPE technique also resulted in significant decreases in analysis time and solvent use. The method also avoided the use of dichloromethane, a recognized carcinogen.

Recoveries of parent metribuzin from fortified soil samples were dependent on the method employed. Only the Evesboro and Conover soils were evaluated by the evaporation method. Recoveries of metribuzin by this method were 48 and 50% for the Evesboro and Conover soils, respectively. It is possible that evaporating the sample to dryness was causing the observed low recoveries. A similar result was reported for the analysis of metribuzin in soils (15). In this method, soil samples were extracted by Soxhlet in 80% MeOH and the solvent reduced by evaporation, but not taken to dryness. The remaining solvent was then extracted with benzene in a separatory funnel. Recoveries were reduced by 20% if the samples were taken to dryness. An alternate method employing SPE was investigated. This method does not include an evaporation step, but rather relies on SPE for concentration and cleanup of the sample. The recoveries by this method were 76, 73, 76 and 87% for the Evesboro, Ochlocknee, Conover and Commerce soils, respectively. For all soils, the RPTLC system resulted in well resolved chromatograms that were free of interferences (Figure 5b). Solid phase extraction not only significantly increased the recoveries, but also decreased the analysis time.

The performance of the RPTLC system was also evaluated for atrazine, alachlor and cyanazine, in combination with metribuzin. All four compounds were well resolved when co-chromatographed (Figure 5c). The R_f values for alachlor, atrazine and cyanazine were 0.26, 0.39 and 0.53, respectively.

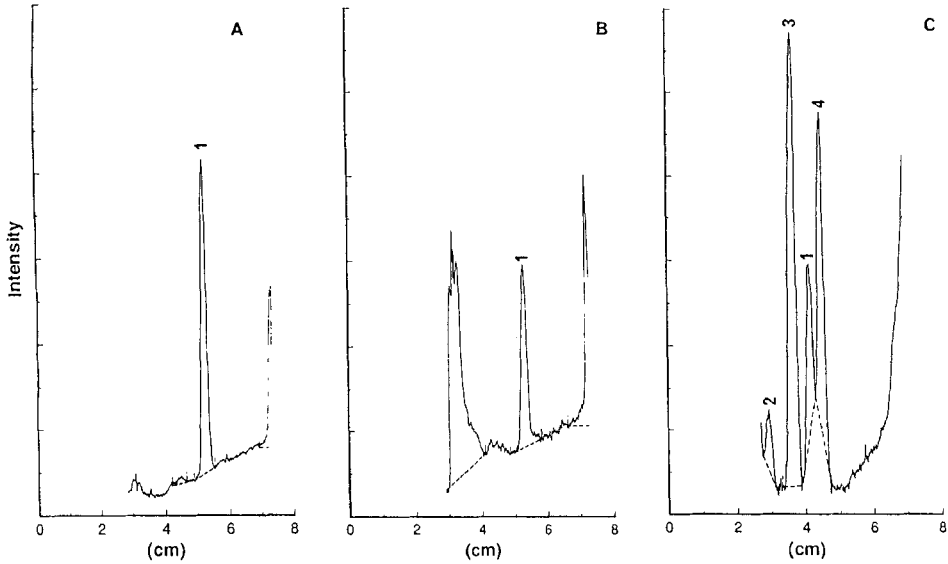


FIGURE 5. Densitometer chromatograms of A) metribuzin (1), in a tap water sample, B) metribuzin (1), in a fortified soil sample, and C) metribuzin (1), alachlor (2), atrazine (3) and cyanazine (4).

The described RPTLC method for metribuzin analysis of soil and water samples is a simple, rapid and cost effective alternative to other available procedures. The extraction procedures yielded recoveries for soils ranging from 73 to 87%, and for water samples ranging from 85 to 92%. The limit of detection of the method was 30 ng. The procedure may be particularly valuable for rapid screening of soil and water samples prior to confirmatory analysis.

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