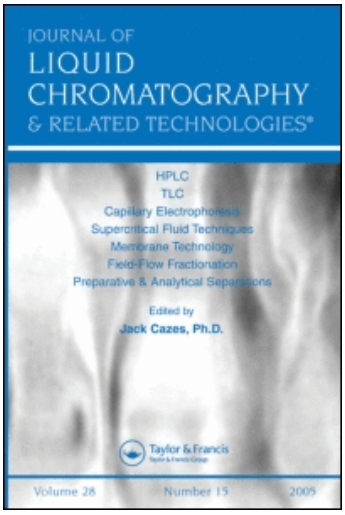


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# Analysis of Atrazine and Associated Metabolites by Reverse-Phase High Performance Thin Layer Chromatography

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# ANALYSIS OF ATRAZINE AND ASSOCIATED METABOLITES BY REVERSE-PHASE HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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**ABSTRACT**

A method to analyze parent atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) and its major metabolites in soils by reverse-phase high performance thin layer chromatography (RP-HPTLC) is described. Atrazine is extracted with methanol, filtered, concentrated by evaporation and chromatographed on pre-coated RP-HPTLC plates. The concentration of atrazine is determined via densitometry. Additional clean-up of the extract is not required. Recovery of atrazine from fortified samples ranged from 87-97%. The detection limit of the method is 20 nanograms.

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## INTRODUCTION

Groundwater pollution from agricultural sources has become a topic of great national and global concern. In the United States, 96% of the rural domestic water supply and 37% of the public water supplies are supplied from groundwater (1). In Delaware, 60% of the total population and 100% of the rural population is supplied with drinking water from groundwater sources (2). Contamination of groundwater, while indirectly affecting millions of people, is a particularly serious problem in those rural areas where agricultural activity is high. Unfortunately, these are the areas that are usually dependent on groundwater as their major drinking water source (3).



Atrazine is used extensively in Delaware and other Atlantic Coastal Plain states and has been detected in groundwater. 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 (4). In this search, atrazine was detected in 343 of 3,208 groundwater samples. This herbicide was also found in groundwater in a recent Delaware study that investigated the effect of tillage on herbicide leaching (5). In this study, atrazine was detected in a majority of monitoring wells in both conventional and no-tillage plots at concentrations up to 54 $\mu g\ L^{-1}$.

The public health implications of groundwater contamination from herbicides are unclear. The levels that have been detected in groundwater to date are generally in the $\mu g\ L^{-1}$ range and are below acute toxicity levels. However, the long term health effects of this exposure are generally unknown. A recent review of several studies demonstrated that the mortality from some types of cancer is significantly higher in rural residents of many corn belt states (3). The EPA has developed a classification scheme in an attempt to further evaluate the carcinogenic potential of herbicides (4). In this ranking atrazine is classified as a possible human carcinogen. This indicates that evidence of carcinogenicity has been suggested from animal studies, but insufficient data is available for humans. These results are not conclusive proof that a relation exists between herbicide contamination of groundwater and increased incidence of cancer, but they do indicate that further research into herbicide environmental fate and toxicology is warranted.

The most commonly utilized methods to conduct herbicide analysis of soils are gas chromatography (GC), high performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS). All of these methods have certain advantages and disadvantages. Methods that depend solely on GC analysis with

traditional detectors, such as electrical conductivity (EC) or nitrogen/phosphorus (N/P), generally require extensive sample clean-up and preparation (6). In addition, several chromatographic runs may be required because different compounds require different detectors. In addition, a separate method is generally required for confirmation. Some of these difficulties can be overcome by employing GC/MS, in which simultaneous separation and confirmation is performed on one instrument (6). Also, because the MS acts as a general detector, switching between detectors is not necessary. However, significant sample clean-up is still required. Methods that depend on HPLC analysis offer high sensitivity and resolution (7), but also require several steps of clean-up and preparation (7, 8). The principle disadvantage of both GC/MS and HPLC is their cost, although a further disadvantage in total sample analysis time may also be argued.



Several methods have recently been investigated in an attempt to develop a cost effective method that retains the accuracy and precision of GC/MS or HPLC. Enzyme immunoassay (EIA) appears to offer a promising alternative. The method is rapid and also yields results comparable to GC (9). The principle disadvantage of this technique is its lack of specificity, due to cross

reactions (10). This necessitates the use of a separate technique for confirmation, thus increasing the cost and sampling time.

Thin layer chromatography (TLC) may also offer an excellent alternative to the aforementioned techniques. Significant advances have been recently made in TLC stationary phases, and low cost scanning densitometers have become more readily available. These improvements have greatly increased the utility of TLC as a quantitative analysis technique (11). Potential advantages of TLC include: 1) high sensitivity, rivaling HPLC, 2) minimum sample preparation time, and 3) low cost. This paper describes a simple and direct analysis of soils for atrazine and its metabolites by using reverse-phase high performance thin layer chromatography (RP-HPTLC) technique.



## MATERIALS AND METHODS

### Soils and Herbicide

The soils used in this study were sampled from the Atlantic Coastal Plain region and included two surface soils and five subsoils. Three of the subsoils were used in recovery experiments and varied in clay content from 8 to 48%. Selected characteristics of these subsoils are presented in Table 1. The four remaining

TABLE 1

Chemical and Physical Characteristics of Three Atlantic Coastal Plain Subsoils

| Soil Property+ | Klej C3 | Runclint Btg2 | Mattawan Btg2 |
|---|---|---|---|
| Organic Matter (%) | 0.04 | 0.12 | 0.19 |
| Sand (%) | 92 | 45 | 17 |
| Silt (%) | 3 | 27 | 35 |
| Clay (%) | 5 | 28 | 48 |

+ Organic matter (12), Particle size (13).



soils were not classified but all were mapped as Evesboro loamy sands with the surface soils having an Ap horizon designation and the subsoils having a Bt designation.

The herbicide used in this study was atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine). Atrazine is a weak base and will protonate at pH values near and below its pKa value (1.68). Additionally, three metabolites of atrazine were investigated: deethylatrazine (2-chloro-4-amino-6-isopropylamino-*s*-triazine),

deisopropylatrazine (2-chloro-4-ethylamino-6-amino-*s*-triazine), and hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine). Atrazine, deethylatrazine and deisopropylatrazine were obtained from Ciba Geigy, Greensboro NC. Their chemical purities were 97, 99 and 98%, respectively. Hydroxyatrazine was prepared via acid hydrolysis of atrazine (14). All standards were prepared in analytical grade MeOH with the exception of hydroxyatrazine. Hydroxyatrazine is not readily soluble in most suitable organic solvents or in neutral water (15, 16). For this reason, hydroxyatrazine standards were prepared in 0.1 M HCl.



**Extraction Procedures**

Soil (50 g, oven dry weight basis) was transferred to 250-mL erlenmeyer flasks and extracted with 150-mL MeOH on a rotary shaker (150 RPM) for 2 hours. The suspension was then filtered through Whatman #42 filter paper. The soil was washed with an additional 50 mL of MeOH and the filtrates were combined. All extracts were evaporated to dryness under vacuum at 60 °C, redissolved in MeOH, transferred to 20-mL vials and redried under nitrogen gas. Prior to analysis, samples were redissolved in 200 μL of MeOH.

**Thin Layer Chromatography**

TLC was performed on reverse-phase, Unibond Series HPTLC plates (10 x 20 cm, 150 micron thickness), (Analtech Inc., Newark, DE). Standards and sample extracts were drawn into microcapillary pipets and applied with a Nanomat III (Camag Inc.). The mobile phase for all experiments was MeOH:$H_2O$ (70:30). All solvents were analytical grade. Spotted plates were equilibrated in a horizontal chamber containing the mobile phase for 0.5 h prior to development. Plates were developed for a distance of 10 cm, dried and scanned at 222 nm with a variable wavelength Shimadzu CS900U Dual-Wavelength Flying Spot Scanner. Standard curves were analyzed by linear and polynomial regression analysis (17).



**Herbicide Recovery**

To evaluate the extraction efficiency of the proposed method, 50 g (oven dry weight basis) of the Klej, Runclint and Mattawan soils were weighed into 250-mL erlenmyer flasks. The soils were moistened to field capacity (14, 20 and 28%, respectively) and amended with atrazine at a rate of 2 mg $kg^{-1}$ soil. Samples were aged for three days and extracted and analyzed as outlined above.

**Field Samples**

To evaluate the ability of the proposed method to detect atrazine and its metabolites in non-fortified soil samples, four soils were sampled from the University of Delaware experiment station in Georgetown, DE. One surface soil and one subsoil were sampled from a field in which atrazine had been applied annually for the past two years. Corresponding samples were also taken from a field which had not received atrazine for two years. Soils were extracted and analyzed immediately as outlined before. Putative atrazine and metabolites were confirmed on the TLC plate via co-chromatography of standards and visualization with silver nitrate, Dragendorff and iodine reagents (11).



## RESULTS AND DISCUSSION

The retention factors ($R_f$) for the various systems investigated are presented in Table 2. Although slight variation in the absolute $R_f$ was observed among experimental systems, relative trends in $R_f$ values were consistent. The observed variation is most likely related to the combined effects of intramolecular attraction and the presence of interfering compounds in the soil extracts. In each instance, however, all of the compounds were well resolved (Figure 1).

TABLE 2

Retention Factors ($R_f$) for Atrazine, Deethylatrazine, Deisopropylatrazine and Hydroxyatrazine on RP-HPTLC Plates in Various Systems.

| Compound | System+ | Rf |
|---|---|---|
| Atrazine | field | 0.40 |
| | spiked | 0.41 |
| | cochromatography | 0.43 |
| | standard curve | 0.44 |
| Deethyl atrazine | field | 0.62 |
| | spiked | 0.59 |
| | cochromatography | 0.64 |
| | standard curve | 0.62 |
| Deisopropyl atrazine | field | 0.68 |
| | spiked | 0.64 |
| | cochromatography | 0.70 |
| | standard curve | 0.68 |
| Hydroxy atrazine | field | 0.58 |
| | spiked | 0.48 |
| | cochromatography | 0.54 |
| | standard curve | 0.52 |

+ **field** = natural field samples, **spiked** = recently spiked soils, **co-chromatography** = co-chromatographed compounds, **standard curve** =compounds chromatographed alone.

Standard curves for parent atrazine and the metabolites were linear or curvilinear with the exception of that for hydroxyatrazine (Figure 1). Hydroxyatrazine samples tailed significantly on all plates, and there was not a consistent increase in area with increasing concentration. This compound has several pH-dependent

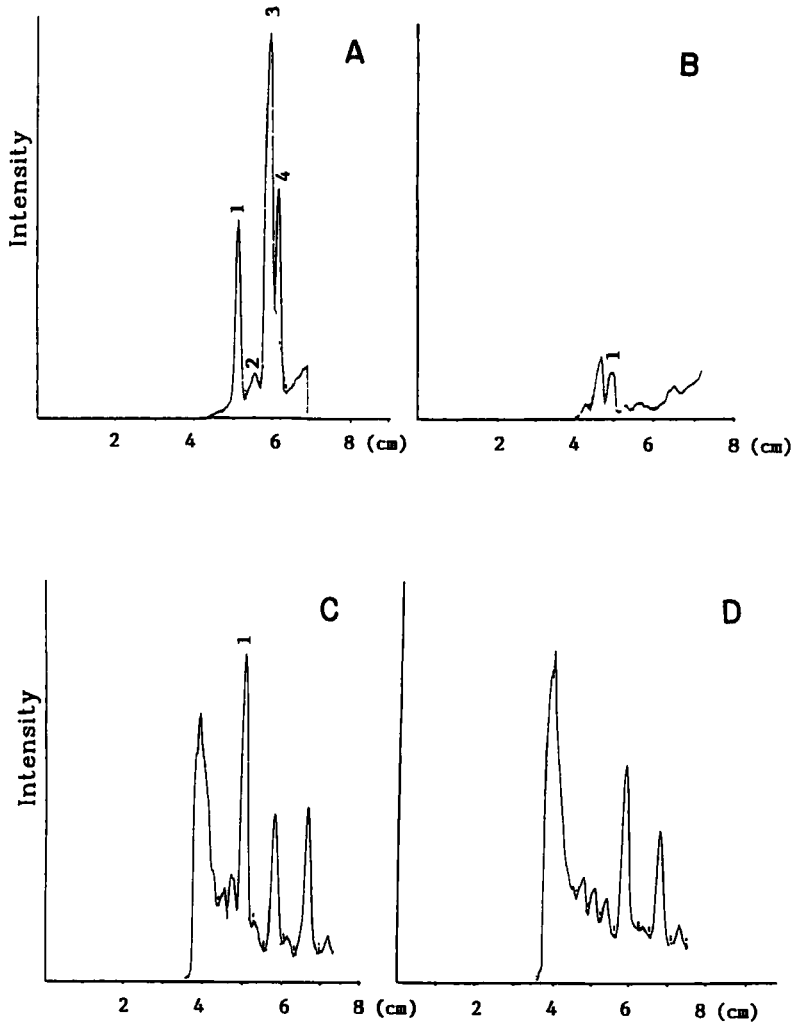


FIGURE 1. Densitometer chromatograms of A) atrazine(1), hydroxyatrazine (2), deethylatrazine (3) and deisopropylatrazine (4) , B) atrazine (1)in a non-fortified soil sample, C) atrazine (1) in a spiked soil sample, D) soil control.

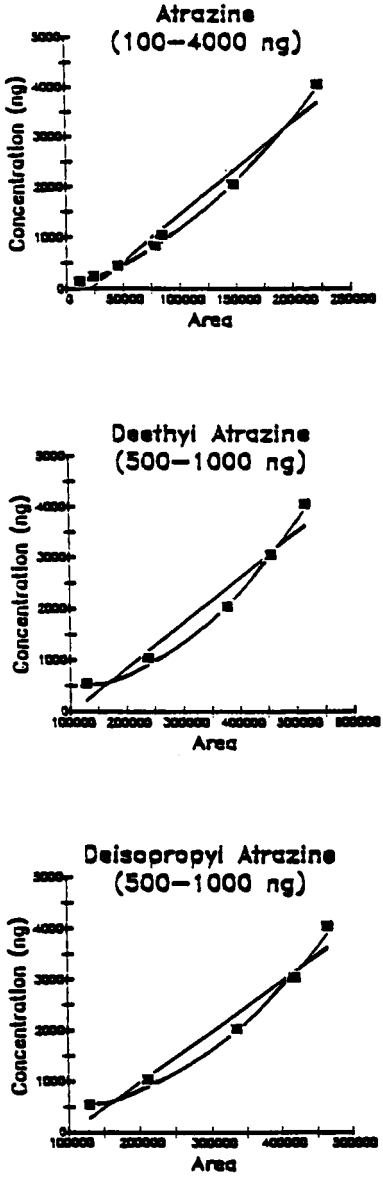


FIGURE 2. Standard curves for atrazine, deethylatrazine and deisopropylatrazine as described by linear and quadratic equations.

tautomers, including a keto and anionic form as well as the enol analog (18). Although only the enol tautomer should have been present at the experimental pH, it is possible that a small amount of the keto tautomer was also present. This possibility was supported by preliminary FTIR data (results not shown). It has been suggested that the enol and keto tautomers form a dimer in the solid phase (16). The presence of a dimer in the liquid phase would explain both the observed tailing of hydroxyatrazine and its unique solubility behavior. This possibility is currently being investigated. It was not possible to resolve the tautomers with our experimental system.



Regression analysis of the standard curves indicated that quadratic equations resulted in a more accurate representation of the results than did linear equations, especially at lower concentrations (Table 3). The detection limit of this method was 20 ng for spotted standards.

Recoveries of parent atrazine in spiked soil samples were 97, 93 and 87% for the Klej, Runclint and Mattawan soils, respectively, indicating that atrazine could be successfully extracted with MeOH alone. An effect was apparent, however, between clay content and recovery of

TABLE 3

Regression Data for Atrazine, Deethylatrazine and Deisopropyl Atrazine.

| Compound | Conc. Range | Linear $r^2$ | Quadratic $r^2$ |
|---|---|---|---|
| | ng applied | | |
| Atrazine | (100-4000) | 0.97** | 0.99** |
| Deethylatrazine | (500-4000) | 0.94** | 0.99** |
| Deisopropylatrazine | (500-4000) | 0.96** | 0.99** |
| Hydroxyatrazine | (500-4000) | NA+ | NA+ |

*** significant at P(0.001), + NA = not applicable



the parent atrazine, with recovery decreasing with increasing clay content.

Field samples having known histories of atrazine application were analyzed to determine whether the RP-HPTLC procedure could measure atrazine and its metabolites in natural soils at residue concentration levels. In particular, we wished to determine whether the extraction techniques would be effective on aged residues present in surface soils having relatively high levels of organic matter.

In the surface soil where atrazine had not been applied in two years, neither parent atrazine or metabolites of atrazine were detected (results not shown). In the subsoil at the same location atrazine was detected at 2.7 μg/kg, possibly the result of earlier application of the herbicide. Hydroxyatrazine was also present, but as previously mentioned it was not possible to confidently determine its concentration. At the location where atrazine had been applied for two years, atrazine was detected at a level of 2.4 μg in the surface soil and 4.0 μg in the in subsoil. Hydroxyatrazine was also detected in both the surface and subsoil at this location.



The described RP-HPTLC method for atrazine analysis of soils is a simple, rapid and cost effective alternative to other available procedures. The extraction procedure yielded recoveries ranging from 87-97%, and the limit of detection of the method was 20 ng. The procedure may be particularly valuable for rapid screening of soils prior to confirmatory analysis.

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