ANALYTICAL APPLICATIONS OF IMMUNOASSAYS IN ENVIRONMENTAL AND AGRICULTURAL CHEMISTRY: STUDY OF THE FATE AND TRANSPORT OF HERBICIDES

by

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This work is dedicated to the memory of my loving parents,
Quiteria and Roberto (Sr.) Aga
who inspired me through the years
to achieve the impossible and aim for the unreachable.

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INTRODUCTION

The number of pesticides, (e.g. herbicides, insecticides, and fungicides) and their use have increased dramatically over the past three to four decades, revolutionizing agricultural practices and improving crop yields. The increased use of pesticides has been mirrored by an increased public concern about the effect of pesticides on the environment and their appearance in drinking water supplies. As public concern about the pesticide issue increases, the pressure to provide new information and guidelines on the fate of pesticides in the environment has become a monumental task for government and industry, which consequently has led to unprecedented demands on analytical laboratories.

Analysis of pesticides has been based primarily on conventional chromatographic techniques such as gas chromatography (GC) and high-performance liquid chromatography (HPLC). Although these techniques are sensitive and reproducible, they are cumbersome, time consuming, and expensive. Interest in immunoassay techniques for the detection of environmental contaminants has grown steadily over recent years because of the unique advantages they can offer. The use of immunoassay has been exploited in clinical laboratories due to its ease of use, low cost, and ability to process large numbers of samples in a short period of time. In clinical and biological chemistry, immunoassay is often used as an analytical method because of its sensitivity, specificity, ease of automation, and general applicability. Environmental laboratories are now faced with similar challenges to those of their clinical counterparts, and obviously immunoassay is an option that needs be seriously

considered. The potential of immunochemical technology for pesticide residue analysis in various matrices such as soil, water, fruits, plants, urine, and blood has been previously examined (Hammock *et al.*, 1986; Harrison *et al.*, 1988; Vanderlaan at al., 1988; Sherry, 1992; Van Emon and Lopez-Avila, 1992).

Environmental monitoring is the key to gaining knowledge on the fate and transport of pesticides and the impacts of contamination caused by modern agricultural practices. Water-quality monitoring is essential for understanding the condition of water and related resources and for providing a basis for the adoption of decisions that promote the wise use and management of water resources. Inexpensive analytical techniques, such as enzyme-linked immunosorbent assays (ELISA), would allow a more extensive sampling and analysis, which in turn will contribute to the characterization and identification of temporal and spatial changes in water-quality attributable to pesticides.

OBJECTIVES AND SIGNIFICANCE OF THE STUDY

The primary objectives of this research were to characterize, validate, and improve several immunoassays (ELISA) for herbicide analysis, and to demonstrate their applicability in the studies on the fate and transport of herbicides in the environment. There has been an increased number of commercially available ELISA kits for herbicide analysis. However, most of these kits are insufficiently characterized with respect to sample matrix effects, and potential cross-reactivity towards structurally related compounds, such as herbicide degradation products. Very few ELISA kits have been validated by conventional methods for their applicability in analyzing real environmental samples. The lack of sufficient characterization and validation may be attributed to the limited laboratory resources and access to analytical standards of herbicide metabolites and degradates. Oftentimes, the effect of different matrices are overlooked because of the limited type of environmental samples available to the manufacturer. Sometimes an ELISA for a herbicide may have an excellent performance for a particular sample matrix, but may fail to do so for another type of matrix. Therefore, in this study, interference due to the presence of other herbicides and their degradation products, and other factors that may potentially affect the performance of ELISA, such as percent dissolved organic matter and solvents used for sample extraction, were investigated. ELISA's for the most widely used herbicides, such as atrazine, alachlor, metolachlor, and 2,4-D, were used to analyze samples from various sources which are representative of the natural environment. These include samples from lakes, rivers, reservoirs, precipitation, and

ground-water wells. Selected samples were confirmed by gas chromatography/mass spectrometry (GC/MS) or by high-performance liquid chromatography (HPLC) to show the validity of the immunoassay results.

First, commercially available ELISA kits for alachlor, metolachlor, atrazine, and 2,4-D were validated by comparing results with GC/MS or HPLC, and by characterizing their cross-reactivity with structurally related herbicides and their metabolites. Solid-phase extraction (SPE) was used as a pre-concentration step for atrazine to improve the detection limit of the ELISA for the measurement of low-levels of atrazine in rain and other pristine water samples. Also, SPE was coupled with ELISA to develop a more specific analysis for alachlor and its cross-reacting metabolite, alachlor ethanesulfonic acid (ESA). An SPE method was used to effectively separate alachlor and its ESA metabolite in order to quantitatively measure the concentration of each compound using the same polyclonal antibodies which recognize both alachlor and ESA.

Since the commercially available ELISA for 2,4-D was not sensitive enough for the detection of this herbicide at concentrations commonly found in natural water samples, polyclonal antibodies were produced against 2,4-D by immunizing New Zealand White rabbits. The polyclonal antibodies generated were used for ELISA and for preparation of immunoaffinity chromatography columns. The use of SPE as a preconcentration step for low-level analysis of 2,4-D was not successful because of the simultaneous isolation of humic and fulvic acids which interfered with both ELISA and HPLC analysis. The use of immunoaffinity columns for sample preparation of water samples containing low concentrations of 2,4-D reduced the problems associated with humic and fulvic acids.

This study also demonstrated how immunoassay could be effectively used both as a screening and a quantitative tool for environmental analysis. In conjunction with this study, sensitive and selective analytical methods for herbicides and their metabolites were developed using ELISA, SPE, GC/MS, and HPLC. A combination of the above techniques were employed for the study of the occurrence and distribution of herbicides in lakes, rivers, reservoirs, and rain, and for the analysis of soil, soil water, and runoff water from field plots used in a herbicide dissipation study.

This research also identified important degradation products of the widely used chloroacetanilide herbicides, alachlor and metolachlor. Using the combination of analytical methods developed above, the relative mobility and persistence of both the parent herbicides and metabolites were cost-effectively determined in the experimental field plots at the Kansas State University Agricultural Research Station, Topeka, Kansas. In addition, the leaching potentials of the four chloroacetanilide herbicides, acetochlor, alachlor, metolachlor, and propachlor were compared. Field dissipation studies on the fate and transport of herbicides provide direct valuable data to support results from laboratory experiments which may or may not be a true representation of the actual behavior of herbicides in the environment. Results of the experiments under field conditions provide more representative data, and are used to supplement current pesticide mobility studies required by the United States Environmental Protection Agency (USEPA) to support product registration.

Because of the widespread contamination of the aquatic environments on a local, regional, and global scale, there is a need to improve the ability to measure the extent and understand the causes of these impacts in order to effectively manage and

protect natural resources. Without monitoring, it will not be possible to characterize the status of the resources or identify temporal and spatial changes in water quality caused by human and naturally occurring factors. Also, monitoring is needed to assure that the decisions made by regulatory agencies and the actions taken achieve their intended objectives effectively and economically, and as a result, provide the best management of the natural resources.

Regulatory agencies, in response to public demands, mandate extensive monitoring of environmental samples, water resources, and food. These analyses are formidable, time-consuming, and costly. In many instances immunoassays have been shown to save time, labor, and equipment costs. The timely results that immunoassay provides is of great value because oftentimes pollution impacts are detected only after they have become severe and widespread. Lack of information may result in costly delays in making or revising regulatory decisions. Demonstrating the feasibility of immunoassays both as complementary and primary analytical methods for many environmental studies will enable technical reviewers and other potential users to raise the level of confidence in accepting the immunoassay technology.

GENERAL BACKGROUND

A. Antibodies and their Production

The immune system comprises several different types of cells to protect animals from infectious organisms. One group of white blood cells, lymphocytes, secretes proteins that bind in a highly specific manner to foreign molecules. These proteins are called antibodies (also known as immunoglobulins) while the foreign molecules are called antigens. Antibodies are glycoproteins found in the globulin fractions of serum and in tissue fluids. Antibodies are produced by mammalian lymphocyte B cells, usually in conjunction with T-helper cells, as part of the immune system's response to foreign substances (Steward, 1984).

There are five classes of immunoglobulins, IgG, IgM, IgA, IgD, and IgE. The predominant immunoglobulin in serum is IgG which has an approximate molecular weight of 160,000 daltons. It has been shown that all five classes of immunoglobulins share a common basic structure composed of two light chains and two heavy chains, linked by disulfide bonds and non-covalent forces. Presently, the three-dimensional structure of an antibody molecule is well-known and is usually represented as a Y-shaped molecule. Porter (Porter, 1967) demonstrated that the two arms of the Y-shaped antibody were identical and were able to combine with the antigen, hence these were called Fab fragments (fragment antigen binding). On the other hand, the tail was found to have the ability to crystallize and was subsequently called the Fc portion (for fragment crystallizable). This fragmentation of IgG is shown in Figure I. The amino acid sequences of the N-terminal regions of both heavy

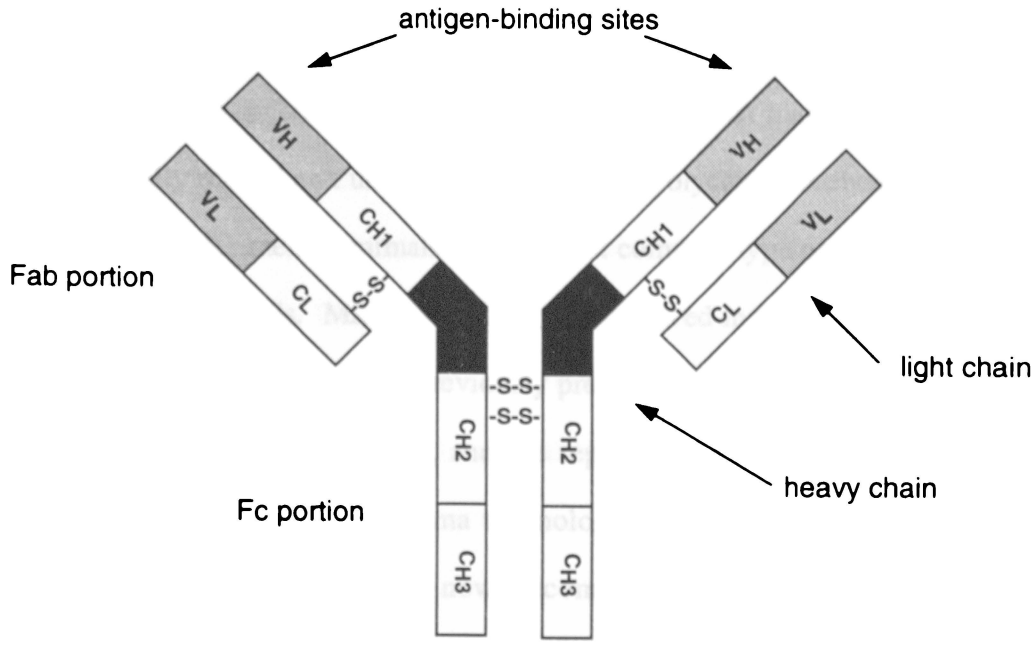


Figure I. General structure of an antibody.

(V_H) and light chains (V_L) show exceptional degrees of variability and have been termed the hypervariable regions or complementarity determining regions (CDR) and are directly involved in the formation of the antigen-binding site. The majority of the chemical structure of each immunoglobulin class is very similar and is referred to as the constant region.

Since immunoassays utilize antibodies as analytical reagents, it is vital to obtain antibodies specific for an individual compound that may be present in milieu of structurally related and unrelated compounds. Polyclonal antibodies obtained by immunizing experimental animals are the most common type used in environmental immunoassay methods. Many of the reagents involved in the production and use of polyclonal antibodies that were previously prepared by various research laboratories are now commercially available, and the repertoire of such reagents continues to increase. However, the hybridoma technology developed by Kohler and Milsten (Kohler and Milsten, 1975) is now becoming popular in the development of immunochemical methods for environmental analysis. The high initial cost of developing monoclonal antibodies, which previously hampered their use, has now become less important when compared to the expense of repeatedly validating assays that use polyclonal antibodies and the comfort of having a conceptually immortal cell line that can provide an unlimited supply of uniform antibodies.

The stimulation of the immune system of an animal can be accomplished by introducing an antigen with sufficient structural complexity, high molecular weight (usually >3,000 daltons), and one that contains a region in which the B cells will recognize as foreign. An antigen which can elicit immune response is specifically termed an immunogen. Although they may be foreign, small molecules with a

molecular weight of <1000 daltons are generally not immunogenic. Most environmental contaminants, including herbicides, fall in this range and cannot be used to directly stimulate antibody production. This limitation can be overcome by physically coupling the small molecule to a larger, more immunogenic molecule. The small molecule in this arrangement is called a hapten and the molecule to which it is coupled is referred to as a carrier. A wide range of proven methods are available for conjugation of haptens to their carriers, most of them using common commercially available reagents. These have been summarized previously (Sherry, 1992) and many examples of their use in pesticide immunoassay development exist (Fleeker, 1987; Goodrow *et al.*, 1990, 1995; Wittman and Hock, 1991; Schneider and Hammock, 1992). Because the structure of the conjugated haptens determines the assay specificity, hapten synthesis and the choice of conjugation are critical steps in assay development.

B. Enzyme-linked Immunosorbent Assay

Antibodies are the key reagent in immunoassays, and they, more than the other reagents, determine an assay's characteristics. Immunoassays are based upon the fundamental principle that antibodies prepared in animals can recognize and bind with exquisite specificity the antigen that stimulated their production. The use of antibodies as analytical reagents started back in the 1950's when Yalow and Berson successfully demonstrated the measurement of picogram levels of human insulin in samples of body fluids using immunological assays (Yalow and Berson, 1960). Since then, various immunoassays for detecting hundreds of molecules of endogenous and exogenous origin have been described.

Enzyme-linked immunosorbent assay (ELISA) is an analytical method that has recently become popular for the analysis of pesticide residues in water and soil (Van Emon and Lopez-Avila, 1992; Hammock, 1990; Vanderlaan *et al.*, 1990). Although the method has been around for many years and has been used for clinical and biomedical applications, this method is relatively new in the field of environmental and agricultural analysis.

Enzyme immunoassays can be divided into two main categories: homogeneous and heterogeneous assays. These categories are further subdivided into competitive and non-competitive assays. Competitive heterogeneous ELISA is the most popular format for pesticide residue analysis. In this format, either the antibody or the antigen is immobilized on a solid support. Typically, it is the detecting antibody that is attached to the solid support. The target analyte and an enzyme-labeled target analyte (enzyme-conjugate) compete for the binding sites of the immobilized antibody. The ratio of free analyte to enzyme conjugate determines the amount of enzyme conjugate that will bind to the antibody. After unbound materials are removed, an enzyme substrate is added, which in turn is converted by the enzyme to a colored product. The color intensity of the product is inversely proportional to the amount of analyte in the sample since the free analyte prevents the enzyme-conjugate from binding. For a more comprehensive overview of the many immunoassay formats the reader is referred to other publications (Bannister *et al.*, 1991; Porstmann and Kiessig, 1992).

C. Herbicide Use and Residue Analysis

In the United States, herbicides, which are used to control weeds and grasses, account for more than 60% of the total volume of pesticides used annually on cropland. Application of herbicides to cropland accounts for two-thirds of the total volume of herbicides used in the country (Gianessi and Puffer, 1991). The total herbicide use is approximately 460 million pounds active ingredients per year. Table I lists the eight most heavily used herbicides in U.S. crop production by weight.

It is difficult to predict the consequences of herbicide applications with respect to water contamination as many factors are involved in their dissipation from soil. These include physical processes as well as chemical, photochemical, and biological processes. Leaching of herbicides through the soil is an important consideration as it is this process which largely leads to the pollution of water supplies (Mills and Thurman, 1994). Once chemicals have reached groundwater, significant degradation is unlikely because of decreased microbial activity. Run-off from cropland has also been held responsible for the occurrence of pesticides in surface water. Previous studies by the U.S. Geological Survey (USGS) (Goolsby and Battaglin, 1993) have shown that large amounts of the herbicides applied to recently planted fields can be carried off of cropland with storm run-off. Depending on the physicochemical properties of the particular herbicide, they could persist for long periods of time in surface water, and could affect the suitability of public water supplies and pose a long-term hazard to aquatic life.

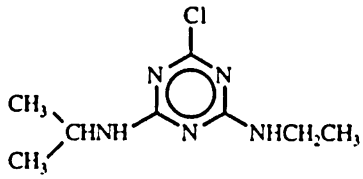
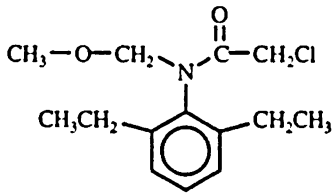
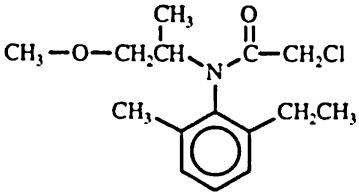
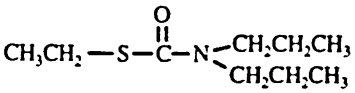
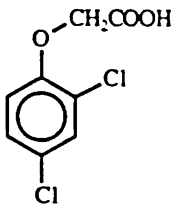
Other studies have shown that herbicides and other organic compounds can be transported into the atmosphere by various processes. In the atmosphere, these compounds can be dispersed by air currents and redeposited on the land surface,

lakes, and streams by rainfall, snow, and dry deposition, often at considerable distances from their origin. For example, the transport of chlorinated insecticides and polychlorinated biphenyls (PCB's) into the Great Lakes is believed to be largely from atmospheric sources (Eisenreich *et al.*, 1981). Herbicides have been reported in fog (Glotfelty *et al.*, 1987) and in rainfall in the midwestern U.S. (Richards *et al.*, 1987; Capel, 1991; Nations and Hallberg, 1992; Williams *et al.*, 1992), eastern U.S. (Wu, 1981; Glotfelty *et al.*, 1990), and in Europe (Buser, 1990; Trevisan *et al.*, 1993). Atmospheric transport and deposition may lead to low-level but widespread contamination. The major concern with pesticides as micropollutants of water is the hazards involved with exposure over long periods of time.

Knowledge of the occurrence, distribution, and transport of herbicides in the environment is of great value, both in the understanding of the fate and behavior of these contaminants, and in response to increased public concern. Due to the widespread contamination of rivers, streams and reservoirs (Thurman *et al.*, 1991, 1992; Leonard, 1988,; Glotfelty *et al.* 1984.; Squillace and Thurman 1992), constant monitoring of water bodies and groundwater wells is necessary to make sure that the maximum contaminant levels (MCL), or drinking-water standards, are not exceeded. MCL is the maximum permissible level of a contaminant in water delivered to any user of a public water system. The USEPA has established the MCL for herbicides based on annual average concentrations and they are legally enforceable under the Safe Drinking Water Act (USEPA, 1986). For several herbicides that do not have MCL's, Health advisory levels (HAL) have been established. MCL's and HAL's for some of the most widely used herbicides are listed in Table I. Excessive accumulation of agricultural chemicals in surface water may pose health and/or

eutrophication problems, rendering the water unfit for drinking, recreation, industry, or fishery purposes.

Table I. Herbicide active ingredients with greatest annual crop use in the U.S. (amounts in 1987/89) and their heath-based limits for drinking water.

Active Ingredient (AI)	Total Annual Use[1] (million lbs AI)	MCL[2] (µg/L)	HAL[2] (µg/L)
 **Atrazine**	64	3	3
 **Alachlor**	55	2	---
 **Metolachlor**	50	---	100
 **EPTC**	37	---	70
 **2,4-D**	33	70	70

[1] Resources for the Future (1991); [2] U.S. Environmental Protection Agency (1992)

CHAPTER I

Coupling of ELISA with Solid-Phase Extraction to Improve Detection Limits and Accuracy of Analysis

INTRODUCTION

Solid-phase extraction (SPE) and enzyme-linked immunosorbent assay (ELISA) are two analytical techniques that recently have found wide application in environmental chemistry. For example, ELISA has been applied to the analysis of herbicide residues in soil (Bushway *et al.*, 1988; Goh *et al.*, 1991; Leavitt *et al.*, 1991) and water (Thurman, et al, 1990, 1992; Aga and Thurman, 1993, 1994). It is a sensitive, fast, and cost-effective technique that can be conducted both in the laboratory and in the field. The advantages of ELISA compared to gas and liquid chromatography have been recently recognized by the USEPA, and guidelines for evaluating immunoassay kits for environmental monitoring are being developed. However, many of the applications of ELISA to complex environmental matrices are limited to screening of samples due to the potential bias of analysis toward false-positive results (Feng, *et al.*, 1990a,b; Thurman *et al.*, 1990; Baker *et al.*, 1993). A false positive result by ELISA is the detection of the analyte at concentrations above the detection limit of a reference method, such as GC/MS, when it is not actually detected by the reference method. False positive results may be caused by interference from structurally similar compounds which cross-react with the antibody. The performance of some immunoassays may be significantly affected not only by cross-reacting compounds, but also by matrix components that interfere with the assay detection system and the antibody-antigen interactions. Less commonly, false negative results (analyte not detected by ELISA but detected by GC/MS) are observed when the analyte concentration in the sample is close to the assay's detection limit. These problems may

be reduced by using a simple and rapid sample-preparation technique before ELISA, like the solid-phase extraction method.

SPE is used extensively as a clean-up procedure for clinical and environmental samples (Tippins, 1988; McDonnald, 1991). While ELISA is used to detect known substances by using the specificity of the antibodies, SPE is used to separate unknown substances by mechanisms similar to that of HPLC (Morris and Ruthann, 1988). The complimentary features of these two techniques may be combined to provide a selective and sensitive analytical method. This would be analogous to a chromatographic analysis where the SPE cartridge is the column and ELISA is the detector. With properly designed SPE procedures, closely related compounds may be separated, and proper interpretation of immunoassay results can be made. Moreover, because SPE is also a pre-concentration technique, the overall detection limit of the SPE-ELISA method may be improved by several orders of magnitude. SPE can be automated easily for water analysis (Castellanni *et al.*, 1990; Meyer *et al.*, 1993); hence the reproducibility of immunoassay will not be compromised. In addition, cross-reactivity of a structurally similar compound could become a positive aspect of ELISA because the same antibody may be used to quantify the cross-reacting compound after it has been concentrated and separated from the main analyte by SPE.

The specific analytes of interest in this study are atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) and alachlor (2-chloro-2',6'-diethyl-N-(methoxymethyl)acetanilide), two of the most extensively used herbicides in the Midwestern United States. The detection of these herbicides in rain water and other samples from pristine environments at trace levels (<1 µg/L) (Goolsby *et al.*, 1993b) demands a sensitive assay such as SPE-ELISA. In addition, the alachlor metabolite,

ESA (2-[(2,6-diethylphenyl)(methoxymethyl)amino-]-2-oxoethanesulfonic acid), is an important target compound for the development of a sensitive method that combines SPE and ELISA because of its frequent interference in the measurement of alachlor by ELISA (Baker, 1993).

Atrazine (a triazine herbicide) is the most heavily used herbicide in the United States, followed by alachlor (a chloroacetanilide herbicide) (Table I). In recent monitoring studies (Thurman *et al.*, 1991, 1992; Goolsby *et al.*, 1993a) it was found that there is widespread herbicide contamination of surface waters in the 11-state area of the United States, called the "Corn Belt". Not only did atrazine and alachlor exceed their MCL's, but they also persisted throughout the year in many of the reservoirs that had been sampled (Goolsby *et al.*, 1993a,c). In addition to the parent herbicides, the alachlor metabolite, ESA, has been the most frequently detected second to atrazine. Both of these herbicides have been classified as possible human carcinogens, and their MCL's are 3.0 µg/L for atrazine, and 2.0 µg/L for alachlor (EPA, 1992). There are no published studies on the toxicity of ESA in animals, but EPA's preliminary toxicological studies on ESA suggest that the metabolite is not mutagenic and does not bioaccumulate or undergo further metabolism at the levels commonly found in surface and ground water (Klein, 1993). However, ESA needs to be monitored constantly because it is relatively persistent and highly mobile (Goolsby *et al.*, 1993c), and thus may occur widely in ground water. Studies on the occurrence and long-term effects of polar metabolites in the environment has been limited by the difficulty of their analysis. The availability of an ELISA method for polar metabolites such as ESA would make extensive studies more feasible and less expensive.

In addition to the widespread detection of herbicides in surface waters, herbicides have also been reported in rain and fog (Glotfelty *et al.*, 1987, 1990; Nations and Hallberg, 1992; Williams *et al.*, 1992). In a recent study on the long range transport and deposition rate of herbicides, both atrazine and alachlor were detected in concentrations ranging from 1 to 3 ug/ L (Goolsby *et al.*, 1993b). In studies where the volume of sample is limited, like precipitation samples, immunoassay techniques are of great value because of the small sample requirement for the analysis. Sensitive analytical methods is also required for research involving the fate of the trace amounts of pollutants that are deposited in pristine environments through atmospheric transport.

The specific objectives of the research described herein were to: (1) evaluate the cross-reactivities of commercially available atrazine and alachlor ELISA's toward structurally-related herbicides and their commonly found metabolites, (2) examine different organic solvents for their compatibility in coupling SPE with ELISA, (3) understand the principles involved in separating parent herbicides and ionic metabolites by SPE, and couple SPE and ELISA for analysis of alachlor and ESA; and (4) apply the method to samples of surface water, ground water, and rain water, and compare the results with gas chromatography/mass spectrometry (GC/MS) and high-performance liquid chromatography with photodiode-array detection (HPLC/PDA).

EXPERIMENTAL

Isolation and Concentration of Atrazine and Alachlor by Solid-Phase Extraction for Part-per-trillion Analysis by ELISA

Analytical standards for atrazine was obtained from Supelco (Bellefonte, PA) while alachlor was from Monsanto Agricultural Co. (St. Louis, MO), both of which have 99.9% purity. Low-levels of atrazine and alachlor were isolated and concentrated from water samples using an automated solid-phase extraction procedure with a Millipore Workstation (Waters, Milford, MA) as described previously (Meyer *et al.*, 1993). In this procedure, the SPE cartridges that were used (Sep-Pak from Waters-Millipore, Milford, MA) contained 360 mg of 40-mm C_{18}-bonded silica. The C_{18} cartridges were washed sequentially with 2 mL methanol (Burdick and Jackson, Muskegon, MI), 6 mL ethyl acetate (Fisher, Springfield, NJ), 2 mL methanol and 2 mL distilled water. All solvents used were HPLC grade. One hundred milliliters (100 mL) of sample were passed through the pre-washed cartridge at a flow rate of 10 mL/min. The cartridge was then eluted with 3 mL of ethyl acetate followed by a transfer step to remove the ethyl acetate (top layer) containing the analytes from the residual water (bottom layer) in the eluate. The ethyl acetate was then evaporated to dryness under a stream of nitrogen at 45 °C using a Turbovap (Zymark, Palo Alto, CA) and then reconstituted with 1 mL of 20/80 (%v/v) methanol/water for analysis by ELISA. For analytes more volatile than atrazine, such as alachlor, evaporation must be conducted with care to prevent loss of analyte.

The preparation of samples for GC/MS analysis was performed in a similar SPE procedure, except that a 500-mL sample was used. However, each sample and standard were first spiked with 20 ng of internal standard, deuterated atrazine-D_5 (USEPA, Cincinnati, OH), prior to extraction. In addition, the ethyl acetate eluate was spiked with a second standard, Phenanthrene-D_{10} (EPA, Cincinnati, OH), prior to evaporation to 100 µL. Deuterated atrazine-D_5 was used as an internal standard for GC/MS quantitation and deuterated phenanthrene-D_{10} was used for a qualitative confirmation of the peak-retention times of the analytes for further confirmation. Standard stock solutions were prepared in methanol.

SPE Procedure for the Separation of Alachlor and ESA

The procedure for the separation of alachlor and ESA is illustrated in Figure 1.1. The SPE procedure was similar to that described above, except a 100 mL-water sample was used and after elution with ethyl acetate the cartridge was eluted with methanol to remove ESA from the cartridge. The methanol extract, which contained ESA, was collected in a separate test tube. Both ethyl acetate and methanol fractions were evaporated to dryness under nitrogen at 45 °C using a Turbovap (Zymark, Palo Alto, CA). The ethyl acetate fractions, which contained alachlor, were reconstituted with 1 mL of 20/80 (%v/v) methanol/water and the methanol extracts, with 5 mL distilled water for analysis by ELISA.

The preparation of samples for GC/MS and HPLC confirmation was performed in a similar SPE procedure. The ethyl acetate extracts were spiked with phenanthrene-D_{10}, and evaporated to about 100 µL for the analysis of alachlor by GC/MS. The

methanol extract for HPLC analysis was spiked with metribuzin, evaporated to dryness, and then redissolved in 100 µL of 10 mM phosphate buffer/methanol (20/80) mixture. Metribuzin (EPA Pesticide Chemical Repository, Research Triangle Park, NC) was used as the external standard for HPLC quantitation.

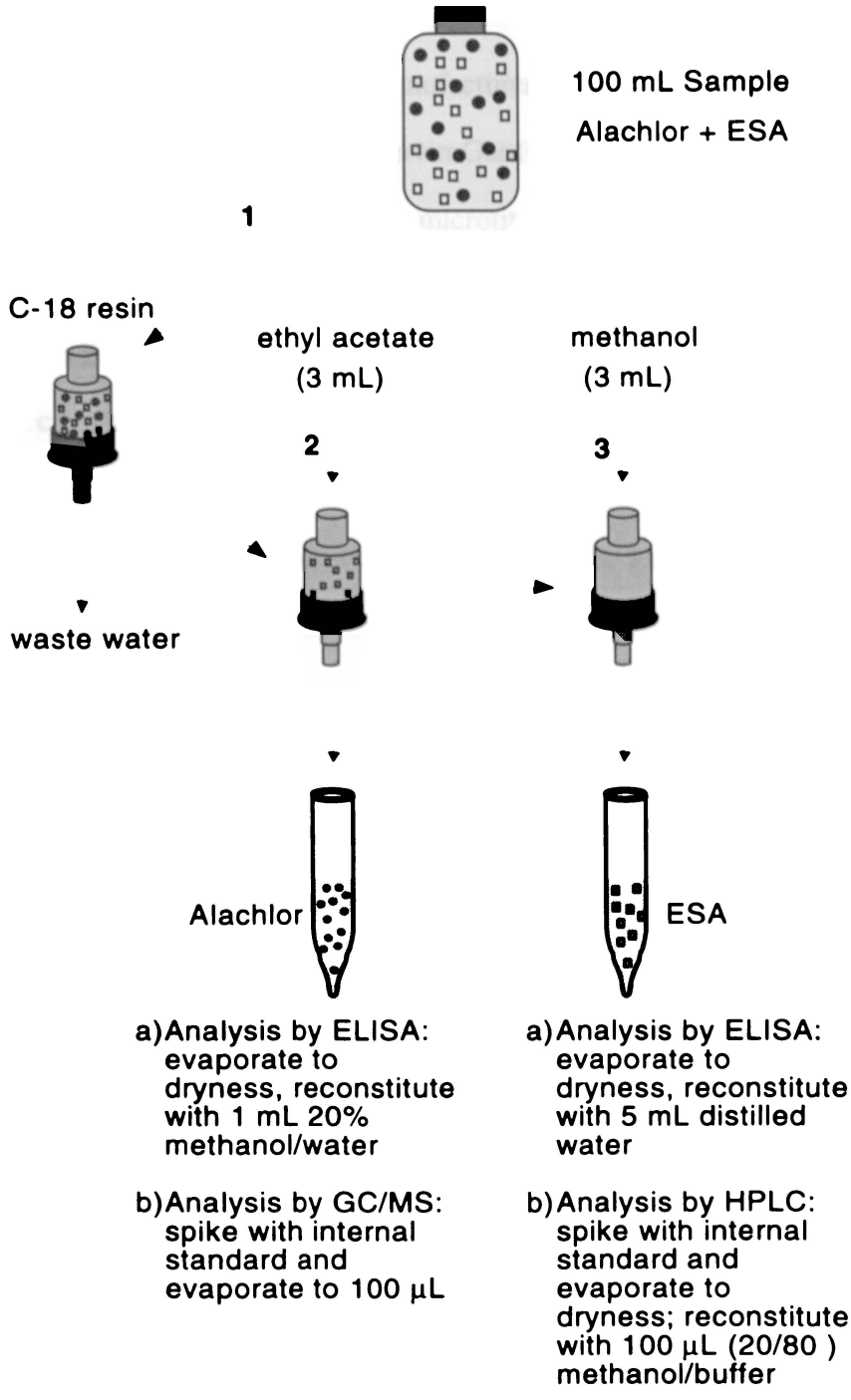


Figure 1.1. Flow-chart for the separation and analysis of alachlor and alachlor ESA by SPE-ELISA.

ELISA Procedure

ELISA kits from two different manufacturers were used to analyze atrazine and alachlor in the solid-phase eluate. The EnviroGard™ ELISA (Millipore, Bedford, MA) had antibodies adsorbed on the wells of a microtiter plate, whereas the RaPID™ assay (Ohmicron Corp., Newtown, PA) had antibodies covalently attached to magnetic particles. Both ELISA's employed polyclonal antibodies.

The cross-reactivity of the atrazine and alachlor ELISA kits towards triazine and acetanilide herbicides, and their respective metabolites, were determined. Solutions of each compound were prepared at concentrations of 0, 1.0, 5.0, 10, 100, and 1000 µg/L in water, and each solution was analyzed in duplicate using both ELISA kits.

For EnviroGard ELISA, 80 µL of sample and 80 µL of hapten-enzyme conjugate (Horseradish peroxidase-herbicide conjugate) were mixed in the well, and the mixture was incubated at 30 °C in an orbital shaker (200 rpm). After 1 h, the plate was rinsed five times with deionized water, and excess water was removed. One hundred sixty (160) µL of color reagent (1:1 mixture of 0.02% hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine) was added. The color was allowed to developed for 30 min, and then stop solution (40 µL, 2M sulfuric acid) was added. Optical densities were read at 450 nm on a Vmax microplate reader using Softmax software (Molecular Devices, Menlo Park, CA). Concentrations of the analytes were calculated using the 4-parameter-fit data reduction.

For the RaPID assay, 200 µL of sample was mixed with 250 µL of hapten-enzyme conjugate and 500 µL of antibody-coated paramagnetic particles. This mixture was incubated for 30 min at room temperature, and then a strong magnetic field was

applied. The excess reagent was removed. Five hundred (500) µL of color reagent was added next, and the mixture was incubated for 20 min at room temperature. This was followed by addition of 500 µL stop solution. The optical densities were read at 450 nm using the RPA-I RaPID Photometric Analyzer (Ohmicron Corp., Newtown, PA). The concentrations were calculated using the Ln/Logit B transformation.

Analysis of ESA in actual water samples was performed using the alachlor RaPID assay, with the same procedure described for alachlor except that the kit was calibrated with ESA standards prepared in distilled water at concentrations of 0, 1.0, 5.0, and 20 µg/L. The concentrations of the atrazine and alachlor were calculated using calibration standards of 0, 0.1, 1.0, and 5.0 µg/L.

GC/MS Analysis

GC/MS analysis was performed on a Hewlett-Packard Model 5890A GC (Palo Alto, CA) and 5970A mass selective detector (MSD). For the low-level analysis of atrazine and alachlor, standard solutions were prepared using 500 mL distilled water spiked with varying amounts of standard solutions of the analyte and a constant amount of deuterated atrazine (20 ng atrazine-D_5). The detector was operated in selected ion monitoring (SIM) mode. For atrazine, the calibration curve was prepared using the area-ratio of the 200 amu base peak of atrazine and 205 amu base peak of atrazine-D_5. Confirmation of atrazine was based on the presence of the molecular ion peak (215 amu) and another fragment ion (173). For the quantitation of alachlor, the area-ratio of the 188 amu base peak to the 205 amu atrazine-D_5 was used. Confirmation of alachlor was based on the presence of the molecular ion peak

(269) and a second fragment ion (160). A retention time match within ±0.2% relative to phenanthrene-D_{10} was also used to assure the quality of the chromatographic conditions.

A 12-m, HP-1 capillary column made of cross-linked methylsilicone with a film thickness of 0.33 mm and 0.2 mm i.d. (Hewlett-Packard, Palo Alto, CA) was used for separation. Helium was used as the carrier gas with a flow rate of 1 mL/min and a head pressure of 35 kPa. The samples were injected in the splitless mode by an auto-injector. The injection volume was 2 µL. The column temperature was held at 60 °C for 1 min, then increased at 6 °C/min to 250 °C and held at this temperature for 10 min. Injector temperature was held constant at 280 °C.

HPLC/PDA Analysis

The HPLC analysis of the methanol extracts for the confirmation of ESA was performed in a HP model 1090 series II liquid chromatograph with a photodiode-array (PDA) detector (Hewlett-Packard, Palo Alto, CA). An HPLC method was developed and optimized for the separation of alachlor ESA and another alachlor metabolite, oxoacetic acid. The HPLC was equipped with a 4.6 mm x 100.0 mm reversed-phase column packed with 3 µm particle size, 120 A pore size, C_{18} Hypersil ODS (Keystone Scientific, Bellefonte, PA). The mobile phase consisted of 34% HPLC-grade methanol and 66% 10 mM Na_2HPO_4 (pH 7.0 buffer) prepared in nanopure water. The flow rate of the mobile phase was 1.2 mL/min and the separation temperature was 60 °C. The sample injection volume was 80 µL. ESA was monitored at a wavelength of 200 nm with a 4 nm bandwidth. The reference wavelength was set at 450 nm with a 80 nm

bandwidth. To confirm the identity of the ESA, the ultraviolet (UV) spectra were scanned from 190 to 400 nm, and the spectra matched to standard spectra in a customized automated library search.

RESULTS AND DISCUSSION

Coupling SPE and ELISA

Methanol, acetonitrile, methylene chloride/isopropanol (80/20, %v/v) and ethyl acetate were investigated as elution solvents for SPE. All four solvents were effective eluents of alachlor and atrazine from the C_{18} resin, with approximately 100±5% recovery, based on GC/MS analysis at the 1.0-µg/L level (Table 1.1). However, natural organic acids (humic substances) in environmental samples, which could potentially alter the performance of ELISA, were eluted to varying degrees by each solvent and were shown as a percentage of color in the extract (Table 1.1). Methanol was the most effective eluent of the natural organic acids based on the optical density measurements of the yellow-colored extracts. Ethyl acetate gave the lowest recovery of natural organic acids in the eluate relative to methanol, acetonitrile, and methylene chloride/isopropanol. Although there is no evidence that the natural organic acids interfere with the ELISA used for atrazine and alachlor, it is desirable to have less organic acids in the eluates particularly in cases where confirmation by GC/MS or HPLC is required. The lower polarity of ethyl acetate compared to the other solvents examined is an important property of an elution solvent because it can selectively elute the target analytes from the C_{18} resin. Due to the lower polarity of ethyl acetate the more polar metabolites that may cross-react with the ELISA were retained in the column.

Since the binding of analyte to the antibody may be affected substantially by the presence of organic solvents, a quantitative solvent exchange of the eluting organic

solvent to an aqueous solvent is required. Solvent exchange was performed by complete evaporation of the eluate to dryness followed by reconstitution with the aqueous solvent. Both methylene chloride/isopropanol and ethyl acetate were good elution solvents because both solvents are immiscible with water and thus can be easily evaporated to dryness. Methanol and acetonitrile are both water-miscible, thus it was difficult to evaporate the eluate to dryness because of the co-eluted water from the cartridge. Ethyl acetate was selected as the best elution solvent because in addition to its effectiveness in eluting the analytes, it provides cleaner extracts and is easily solvent exchanged.

Table 1.1. Suitability of various eluting and coupling solvents for SPE-ELISA.

eluting solvent	% recovery[a] of atrazine and alachlor	% color[b] in extract (humic substances)	solubility in water	suitable for SPE-ELISA
methanol	100 ± 5	20	miscible	no
acetonitrile	100 ± 5	10	miscible	no
methylene chloride/ isopropanol (80/20, %v/v)	100 ± 5	8	immiscible	yes
ethyl acetate	100 ± 5	2	immiscible	yes

[a]Recovery at 1.0 µg/ L from 100-mL sample based on GC/MS analysis. [b]Color was measured as the optical density at 400 nm.

Evaporation of the eluate to dryness was performed at 45 °C under a stream of nitrogen without the loss of atrazine or alachlor. However, at temperatures greater than 50 °C, losses of both herbicides were observed. Figure 1.2 shows the recovery of atrazine and alachlor as a function of evaporation temperature. The decrease in recovery was more pronounced for alachlor, with losses of up to 50% when the temperature was raised from 50 °C to 60 °C. The vapor pressures of atrazine and alachlor are 0.04 mPa (20 °C) and 2.9 mPa (25 °C), respectively. Thus, the greater volatility of alachlor (about 70 times) results in more rapid losses of analyte. These suggest that a compound's vapor pressure and the evaporation temperature are critical in the method design.

The final step in coupling SPE and ELISA involved reconstitution of the analytes in a mostly aqueous solvent for ELISA. Table 1.2 shows the recovery of atrazine using distilled water and methanol/water as solvent. Reconstitution with distilled water alone gave low recoveries, with only 28 to 80% of the spiked atrazine recovered. Losses probably were due to incomplete dissolution of the analyte. The least amount of methanol that resulted in a nearly complete recovery was 20% (v/v) in water. In fact, even with 20% methanol, a slight decrease in recovery was observed at 50 ng/L. Fortunately, the ELISAs used in this study tolerate minor amounts of methanol (10-20%) without loss of antibody-antigen interaction and desorption of antibodies from the microtiter plate or the magnetic beads. It is important to evaluate each ELISA for its tolerance to methanol because some ELISAs are disrupted by less than 5% while others can tolerate over 50% methanol. The calibration standards should have the same solvent composition as the unknowns because the organic solvent

in the matrix shifts the dose-response curve of the immunoassay, which may lead to an overestimation or underestimation of the analyte concentration. Hence, complete evaporation of the eluate was essential to allow the solvent composition of all samples to be consistent in terms of methanol fraction before it could be analyzed by ELISA.

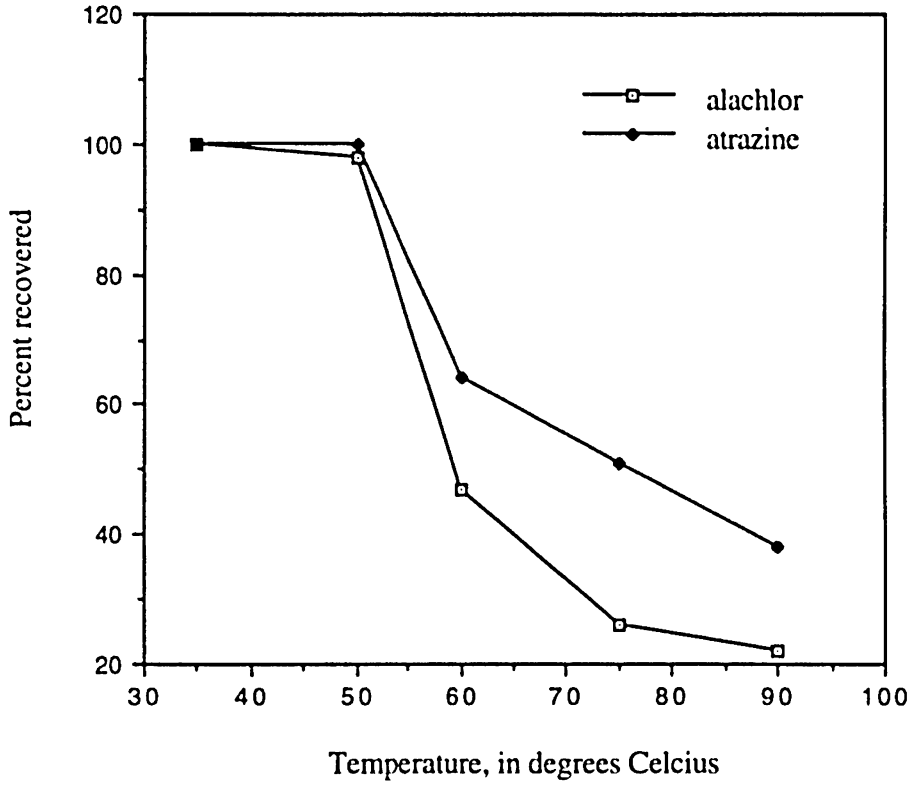


Figure 1.2. Recovery of atrazine and alachlor as a function of evaporation temperature during solvent exchange performed under a stream of nitrogen.

Table 1.2. Recovery of atrazine from dried ethyl acetate extract using distilled water and methanol/water.

concentration of spiked atrazine in 100 mL distilled water (ng/L)	% atrazine recovery using distilled water	% atrazine recovery using 20/80 (%v/v) methanol/water
1.0	80 ± 25	100 ± 6
5.0	87 ± 21	95 ± 5
10.0	77 ± 17	95 ± 5
25.0	56 ± 20	95 ± 8
50.0	28 ± 12	90 ± 7

Recovery of SPE-ELISA

Table 1.3 shows the recovery of atrazine and alachlor from spiked distilled water samples by SPE-ELISA using two kinds of ELISA kits; one is based on a microtiter plate and the other is based on magnetic particles. Analysis of the extracts from the atrazine-spiked distilled water at concentrations 1.0, 5.0, 10.0, 15.0, 20.0, 25.0, and 50 ng/L showed 79 to 120% recovery. These recoveries did not differ significantly with previous work at micrograms per liter (µg/L) concentrations (Thurman *et al.*, 1990). It is known that specific sorption sites on the silica matrix of the C_{18} resin are capable of retaining nitrogen-containing herbicides by mixed-mode interactions, where reversed-phase and ion-exchange mechanisms are combined (Mills and Thurman, 1992). Therefore, it was possible that these specific interactions may cause poor recovery of the analytes at concentrations in nanograms per liter (ng/L). However, the recovery data in Table 1.3 show that specific interactions did not cause any problem in the recovery of the analytes when ethyl acetate was used as the elution solvent.

Except for the concentration of 1.0 ng/L, good reproducibility was obtained with both ELISA forms, with relative standard deviation (RSD) ranging from 2 to 12% for the magnetic particle-based ELISA and 5 to 24% for plate-based ELISA. Because of the large RSD obtained for the 1.0-ng/L standards (>50%), the detection limit was set at 5.0 ng/L for the SPE-ELISA method using both ELISA forms based on the guidelines set by the American Chemical Society Committee on Environmental Improvement (Keith *et al.*, 1983).

Table 1.3. Recovery of atrazine and alachlor from spiked distilled water by SPE-ELISA (n=10).

concentration of herbicide in 100 mL water (ng/l)	% recovery
atrazine (by magnetic particle)	
1.0	120 ± 55
5.0	96 ± 7
10.0	98 ± 7
15.0	94 ± 12
20.0	89 ± 8
25.0	97 ± 2
50.0	79 ± 11
atrazine (by microtiter plate)	
1.0	112 ± 54
5.0	103 ± 10
10.0	97 ± 24
15.0	86 ± 23
20.0	89 ± 11
25.0	92 ± 14
50.0	86 ± 5
alachlor (by microtiter plate)	
5.0	103 ± 24
10.0	83 ± 16
25.0	99 ± 7
50.0	91 ± 12

Figure 1.3 shows graphs of the atrazine and alachlor standard curves obtained from SPE-ELISA compared with the actual concentration of the analyte spiked into the distilled water. For the atrazine curve, the correlation coefficient was 0.98, and the regression line slope was 0.79 for the magnetic particle-based ELISA (Figure 1.3A), whereas the plate-based ELISA showed a correlation coefficient of 0.98 and a regression line slope of 0.87 (Figure 1.3B). The detection limit of the SPE-ELISA method was 5.0 ng/L, but atrazine concentrations of less than 5.0 ng/L could be determined quantitatively if the volume of water used for SPE is increased. A previous study (Thurman *et al.*, 1990) showed that only a 10% breakthrough of atrazine from the 360-mg C_{18} resin was observed after 2,000 mL of the sample was passed through the cartridge. This result indicates that it is possible to use sample volumes greater than 100 mL and still get high recoveries of atrazine, as long as the volume does not exceed the breakthrough capacity of the SPE cartridge.

The application of SPE-ELISA for low-level analysis was also demonstrated for alachlor. Recovery studies for alachlor from spiked distilled water at concentrations of 5.0, 10.0, 25.0, and 50.0 ng/L by SPE-ELISA showed that 83 to 103% of the alachlor can be recovered (Table 1.3). A plot of the concentrations of alachlor recovered by SPE-ELISA against the concentration in the spiked water (Figure 1.3C) has a correlation coefficient of 0.98 and a regression line slope of 0.92. The detection limit for alachlor using this method was established at 5.0 ng/L, but again a lower detection limit may also be achieved by using larger sample volumes for SPE. Thurman and co-workers, (1990) showed that alachlor remained adsorbed on the C_{18} resin after 7,000

mL of sample was passed through the SPE cartridge with no observed breakthrough. Thus, an even lower detection limit for alachlor by SPE-ELISA is attainable.

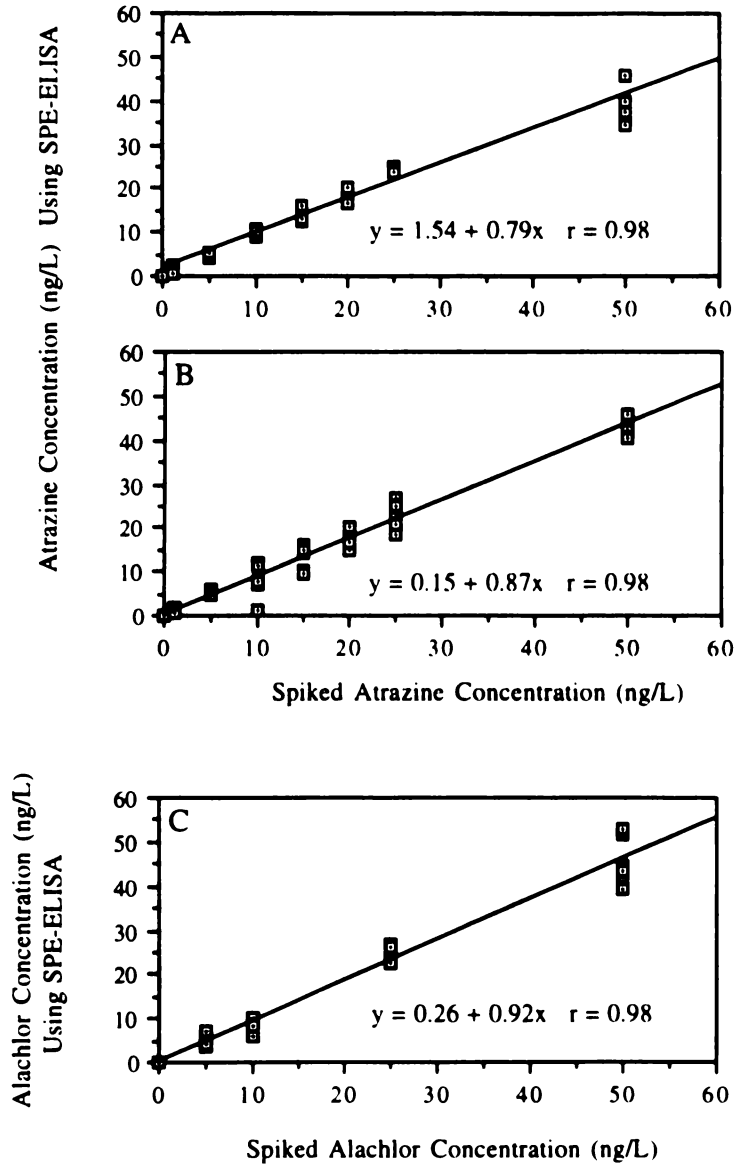


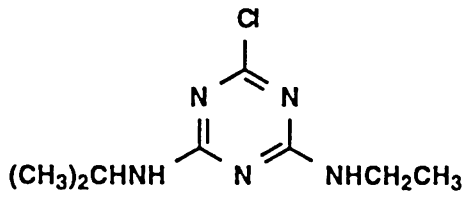
Figure 1.3. Recoveries of varying concentrations of atrazine from spiked distilled water using SPE-ELISA with (A) magnetic particle-based and (B) microtiter plate-based immunoassay and (C) recovery of varying concentrations of alachlor from spiked distilled water using SPE-ELISA with microtiter plate-based immunoassay.

Cross-reactivity of Two Atrazine ELISA's Towards Triazine Herbicides and Metabolites

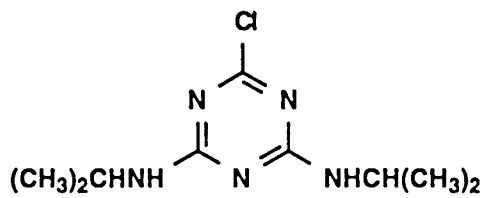
The cross-reactivities of the antibodies used in the atrazine ELISA with other structurally related compounds were examined. Cross-reactivities were expressed as the IC_{50}, which is the concentration of the compound that causes 50% inhibition of the labeled hapten, i.e. a 50% decrease in the absorbance of the negative control. The least detectable dose (LDD), which is the concentration that causes 90% decrease in absorbance was also determined. Since both ELISA forms were competitive binding assays, they were most sensitive for the compound with the lowest value for IC_{50}. The two forms of ELISA had slightly different sensitivity toward the analytes and were found to vary in cross-reactivity towards their metabolites and other structurally similar compounds.

The IC_{50} for atrazine on the RaPID ELISA was 0.93 µg/L, whereas the IC_{50} on the EnviroGard ELISA was 0.42 µg/L, indicating a slightly lower detection limit for the latter (Figure 1.4). Other triazine compounds that may give significant response are prometon and propazine, and to a lesser extent simazine and deethylatrazine. These data suggest that one must be careful in using ELISA for analysis of samples from areas of substantial triazine usage which may contain other triazine compounds. The data in Figure 1.4 also suggest that the alkyl side chains greatly affect the binding of the molecule to the antibody, as evident in the 10-fold decrease in cross-reactivity of deethylatrazine compared to atrazine. Moreover, the decrease in reactivity is even more pronounced for deisopropylatrazine. These results suggest that the alkyl group, specifically the isopropyl side-chain, is a group of key importance in the binding

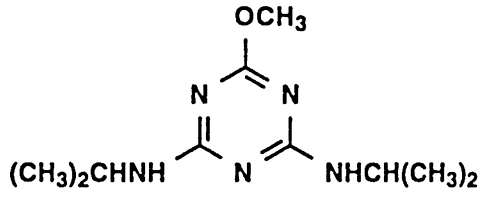
reaction. The 10-fold difference in binding between propazine (with isopropyl-amino groups) and simazine (with ethyl-amino groups) is further evidence of this hypothesis. Cyanazine has a very small cross-reactivity to both atrazine ELISA's. It is important to recognize that although the two ELISA's are most specific to atrazine, high amounts of any of these structurally similar compounds could affect the analysis.



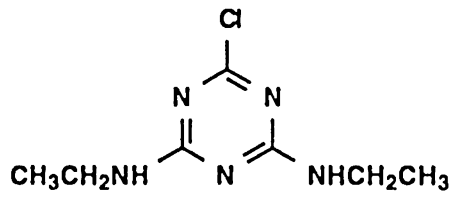
Atrazine
IC_{50}=0.93 , 0.42
LDD= 0.06 , 0.02



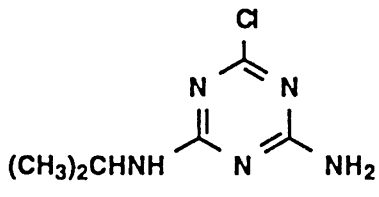
Propazine
IC_{50}= 0.83 , 0.49
LDD= 0.03 , 0.03



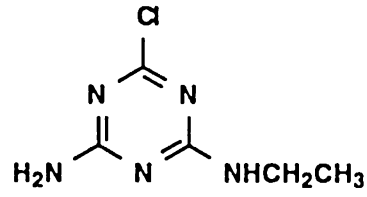
Prometon
IC_{50}= 2.4, 1.5
LDD= 0.09 , 0.06



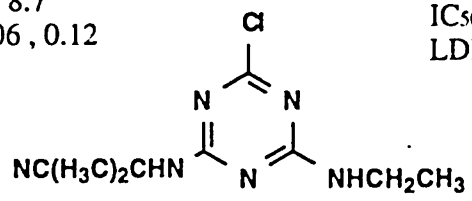
Simazine
IC_{50}= 7.5 , 3.1
LDD= 0.42 , 0.23



Deethylatrazine
IC_{50}=10.6, 8.7
LDD= 0.06 , 0.12



Deisopropylatrazine
IC_{50}= 160 , 84
LDD= 3.2 , 7.8



Cyanazine
IC_{50}= 454 , 31
LDD= 3.1 , 1.0

Figure 1.4. Cross-reactivities of antibodies with triazine herbicides and their metabolites (in µg/L). The first value is for the Atrazine RaPID assay kit (magnetic particle) and the second value is for the EnviroGard Triazine kit (microtiter plate).

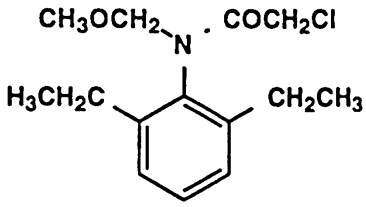
Cross-reactivity of Two Alachlor ELISA's Towards Acetanilide Herbicides and Metabolites

The cross-reactivities of the antibodies used in the RaPID and EnviroGard alachlor-ELISA's with other acetanilides and alachlor metabolites were measured (Figure 1.5). Both ELISA's were most sensitive for alachlor, with LDD of 0.07 µg/L for the RaPID assay and 0.06 µg/L for the EnviroGard assay. The RaPID assay had an IC_{50} for alachlor of 0.89 µg/L whereas the EnviroGard assay had an IC_{50} of 0.75 µg/L. Of the compounds tested for cross-reactivity with the alachlor-ELISA, ESA had the highest cross-reactivity, with an IC_{50} of 5.4 µg/L and an LDD of 0.19 µg/L for the RaPID assay and an IC_{50} of 1.7 µg/L and an LDD of 0.12 µg/L for the EnviroGard kit. The 2-[2,6-diethylphenyl)(methoxymethyl)amino-]-2-oxoacetic acid (OXA) had an IC_{50} of 335 and an LDD of 21 µg/L for theRaPID assay, and an IC_{50} of 66 µg/L and an LDD of 2.7 µg/L for the EnviroGard kit.

The cross-reactivity of the anti-alachlor antibodies with ESA has caused problems in previous water-quality surveys for alachlor by ELISA because of false-positive detections of alachlor (Baker *et al.*, 1993). Furthermore, it has been reported that the ELISA for alachlor does not correlate well with GC/MS and gives more than 10% false positives near the limit of detection, with a concentration range of 0.10 to 0.20 µg/L (Feng *et al.*, 1990a,b). This poor correlation has been attributed to the presence of high ESA levels as confirmed by liquid chromatography with tandem mass spectrometry (LC/MS/MS) (Macomber *et al.*, 1992). The ESA and OXA are two major soil metabolites of alachlor (Sharp, 1988); however, only ESA appears to have sufficient cross-reactivity to cause significant interference with ELISA.

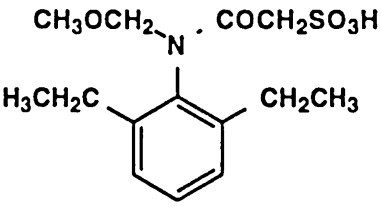
Other soil metabolites and precursors of alachlor, such as hydroxydiethylacetanilide and chlorodiethylacetanilide, respectively, did not show any cross-reactivity up to a concentration of 1000 µg/L. Metolachlor, another chloroacetanilide herbicide, showed low cross-reactivity, with an IC_{50} of 109 µg/L and 27 µg/L for the RaPID and EnviroGard assays, respectively. This cross-reactivity pattern suggests that the binding of alachlor toward the antibody is affected by the presence of the methoxymethyl side chain and the alkyl group in the ring. These results are not surprising because the antibodies against alachlor are usually generated by the use of an alachlor-protein conjugate, formed through the chlorine-bearing carbon of alachlor via a thioether bond (Feng *et al.*, 1992a). The antibodies developed for alachlor recognize ESA to a significant degree relative to the other metabolites because the sulfur atom in ESA is similar in size to the chlorine atom.

The overall performance of the two ELISA kits (magnetic particle-based and microtiter plate-based) were comparable in terms of detection limits and cost-effectiveness. The small magnetic beads have the benefit of a larger surface area to volume ratio when compared to the microtiter plate which uses a plane surface for the immunochemical reaction. Consequently, the magnetic beads have faster assay kinetics due to shorter diffusion distances. This results in a slightly better precision and shorter analysis time than the microtiter plate. However, the assay by the magnetic particle ELISA involves more steps than the microtiter plate ELISA because of repeated incubations and washing steps.



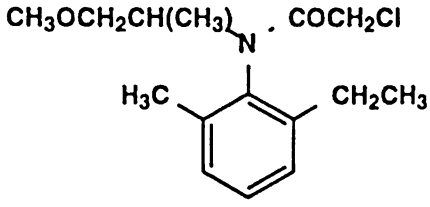
Alachlor

IC_{50}= 0.89 , 0.75
LDD= 0.07 , 0.06



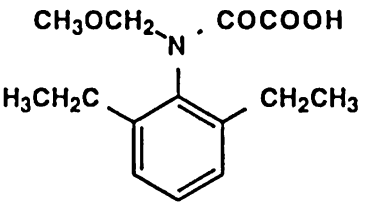
Ethanesulfonic acid (ESA) metabolite

IC_{50}= 5.4, 1.7
LDD= 0.19 , 0.12



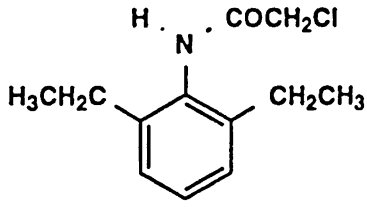
Metolachlor

IC_{50}= 109 , 27
LDD= 3.0 , 1.2



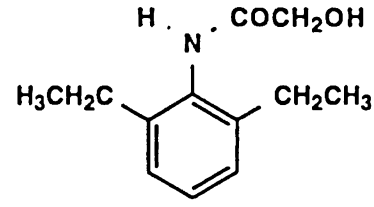
Oxoacetic acid (OXA) metabolite

IC_{50}= 335 , 66
LDD= 21 , 2.7



Chlorodiethylacetanilide

No cross-reactivity at <1,000 µg/L



Hydroxydiethylacetanilide

No cross-reactivity at <1,000 µg/L

Figure 1.5. Cross-reactivities of antibodies with chloroacetanilide herbicides and alachlor metabolites (in µg/L). The first value is for the alachlor RaPID assay kit (magnetic particle) and the second value is for the EnviroGard alachlor kit (microtiter plate).

Comparison of SPE-ELISA and GC/MS Analysis for Alachlor and Atrazine

The applicability of the SPE-ELISA method for the analysis of natural water samples was tested in a reconnaissance study on the long-range transport of herbicides. Samples were collected from the lakes of Isle Royale National Park, Michigan, located in Lake Superior near the United States-Canadian border. This island is located approximately 1,000 kilometers away from the so-called "Corn-Belt" of the United States. This park is protected from excessive human influence by its Congressional designation as a Wilderness Area and its United Nations designation as an International Biosphere Reserve. Any atrazine present in the hydrologic system of Isle Royale would be from precipitation only, hence, concentration of atrazine in the lakes, if any is present, are expected to be at trace levels.

Table 1.4 shows the concentrations of atrazine in water samples from six lakes in Isle Royale National Park as determined by SPE-ELISA and GC/MS. The correlation coefficient between magnetic particle-based ELISA and GC/MS was 0.96, with a linear regression coefficient of 0.89 (Figure 1.6A). The correlation coefficient between microtiter plate-based ELISA and GC/MS was 0.93, and the regression coefficient was 0.95 (Figure 1.6B). The non-zero y-intercept of the line may be attributed to the presence of some atrazine metabolites in the lake samples which cross-react with the atrazine-ELISA. However, the cross-reactivity of the major atrazine metabolites, deethylatrazine and deisopropylatrazine, is small relative to atrazine as shown in GC/MS analysis. In addition, these metabolites are present at concentrations significantly less than atrazine. With these considerations in mind, the SPE-ELISA method was useful for atrazine analysis in lake-water samples from Isle Royale National Park.

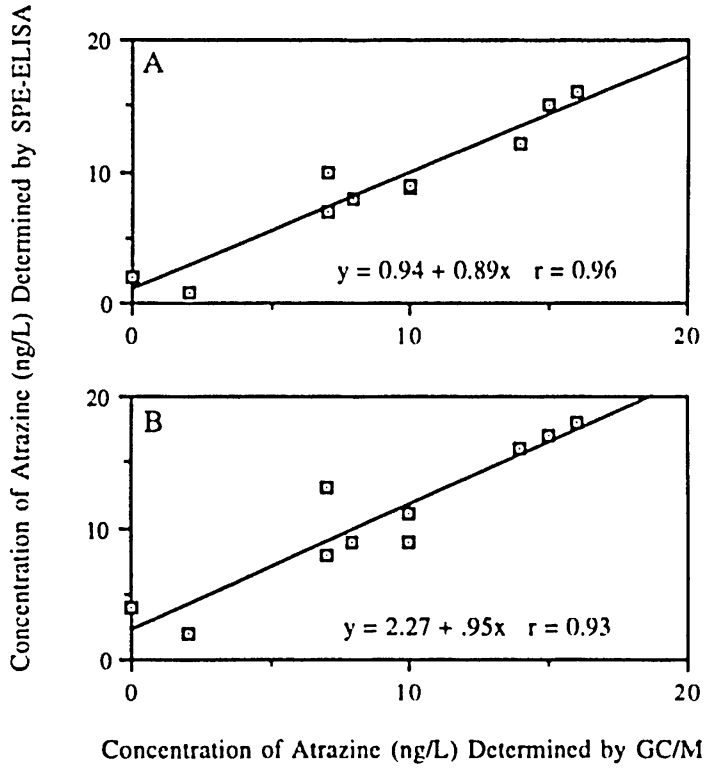


Figure 1.6. Comparison of atrazine concentrations in 10 lake-water samples from Isle Royale National Park determined by (A) magnetic particle-based and (B) microtiter plate-based SPE-ELISA with atrazine concentrations determined by GC/MS.

Table 1.4. Analysis of water samples from lakes in Isle Royale National Park: ELISA versus GC/MS for samples analyzed in duplicate by each method.

sample site	average atrazine concentration (ng/L)		
	RaPID assay	EnviroGard	GC/MS
Benson Lake (surface)	8.0	9.0	8.0
Benson Lake (outlet)	<5.0	<5.0	<5.0
Richie Lake (surface)	8.7	11.0	9.5
Siskiwit Lake (deep)	16.0	18.0	16.0
Siskiwit Lake (surface)	12.0	16.0	14.0
Wallace Lake (surface #1)	<5.0	<5.0	<5.0
Wallace Lake (surface #2)	5.6	6.8	6.2
Wallace Lake (outflow)	8.8	8.5	9.3
Whittlesey Lake (deep)	10.0	13.0	7.0
Wood Lake (deep)	15.0	17.0	15.0

To further demonstrate the applicability of the method, pristine water and snow samples from various sources outside the corn-belt were analyzed (Table 1.5). No atrazine was detected in any of the snow samples from North Fremont Glacier, WY and the waterfalls of Feldtmann Ridge, MI. This further supports the conclusion that the SPE-ELISA method is useful for both positive and negative results in surveys of triazine contamination outside the corn-belt. Moreover, the method could be substituted for the more expensive and time-consuming analysis by GC/MS, or at least reduce the sample load by screening to eliminate negative samples. The method is being used routinely to monitor herbicide contamination in rainfall at Isle Royale National Park. A field-portable SPE-ELISA set-up was designed, so that onsite analysis of atrazine in natural water can be performed.

Table 1.5. Concentration of atrazine in snow and water samples from pristine sources: Survey using RaPID atrazine ELISA.

sample site	no. of samples	average atrazine concentration (ng/L)
Sturgeon River, MN	3	16
Kawishiwi River, MN	3	13
Au Sable River, MI	3	7.5
Potomac River tributary, VA	5	<5.0
Feldtmann Ridge (waterfall), MI	5	<5.0
North Fremont Glacier, WY	7	<5.0

Fourteen ground-water samples from various sampling sites were analyzed for alachlor using the SPE-ELISA method and GC/MS method. In contrast with previous report by Baker and co-workers (1993) , a high correlation of alachlor concentrations by ELISA and GC/MS was obtained, with no occurrence of false positive nor false negative results. Figure 1.7 shows a correlation coefficient of 0.97 and a regression coefficient of 1.07 between the two methods. Furthermore, when the same ground-water samples were directly analyzed for alachlor by ELISA without prior SPE, 28% of the samples were false positives and no correlation with GC/MS results was observed. This is a direct evidence that SPE removes most of the important cross-reacting species causing the discrepancy between the results of GC/MS and ELISA for alachlor. Indeed, the SPE-ELISA method is a novel analytical tool for herbicide analysis not only because it provides lower detection limits, but also more reliable results than previously reported immunoassays.

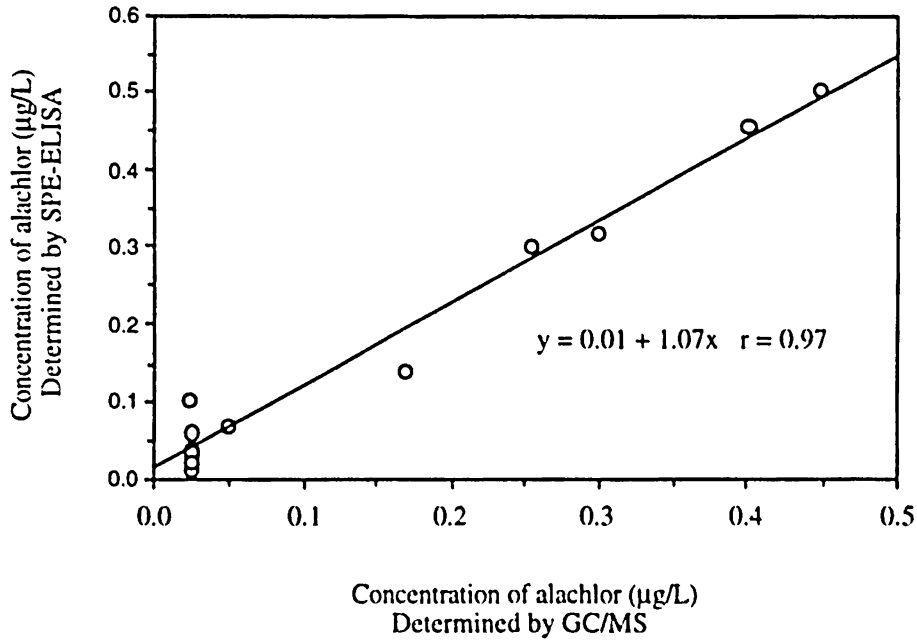


Figure 1.7. Comparison of alachlor concentrations in 14 ground-water samples determined by magnetic particle-based SPE-ELISA with alachlor concentrations determined by GC/MS.

Isolation and Separation of Alachlor and its ESA Metabolite by SPE

Both alachlor and ESA were isolated quantitatively from 100 mL water by adsorption onto a 360 mg C_{18} resin. However, in contrast to alachlor, ESA had considerably less capacity for sorption on the C_{18} resin. This was observed from a breakthrough experiment where a 1.0 µg/L aqueous solution of alachlor or ESA was passed through a C_{18} resin, and the waste from the resin was collected at a 10 mL increment and analyzed by ELISA for alachlor or ESA. Only 10% breakthrough (the waste contained 0.1 µg/L alachlor) was observed for alachlor after 7,000 mL of the solution were passed through the resin (Table 1.6). On the other hand, a 10 % breakthrough was observed for ESA after 175 mL of sample were passed through the resin, and a 100 % breakthrough (the waste contained 1.0 µg/L ESA) was observed after 750 mL of the solution was used. This difference in capacity is caused by the greater solubility of ESA in water. Likewise, OXA had a small capacity on the C_{18} resin, with a 10 % breakthrough beginning at 150 mL, and a 100 % breakthrough observed after 750 mL of water has been passed through the resin. A more soluble herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), was also examined. It was found that 2,4-D had a very small capacity for sorption, with a 10 % breakthrough observed after only 10 mL, and a 100 % breakthrough observed after 90 mL of sample was passed through the resin. These results suggest that the isolation of ionic compounds on C_{18} resin is possible, but this isolation technique is quite dependent on the aqueous solubility of the analyte. Thurman and others (1978) reported that ionic character was critical in the sorption of organic acids onto a macroporous acrylic resin and was a

Table 1.6. Breakthrough capacity (mL) of Sep-pak C_{18} cartridges for alachlor and other ionic compounds.

compound	10% breakthrough	100% breakthrough
alachlor	>7,000	----
ESA	175	750
OXA	150	750
2,4-D	10	90

function of chain length. The same concept appears to apply to the isolation of ionic metabolites on C_{18} resin because both isolations involve hydrophobic interactions.

The separation of alachlor and ESA occurs in the elution step. Alachlor is eluted first with ethyl acetate without removal of ESA from the resin, and then ESA is eluted with methanol. The recovery of spiked ESA from distilled water when analyzed by SPE-ELISA ranged from 91 to 99% in the methanol extract and none in ethyl acetate (Table 1.7). Alachlor was recovered from 97 to 105% in the ethyl acetate extract and 0% in the methanol extract. Hence, there is a complete separation of ESA and alachlor by SPE. After separation and concentration by SPE, it was possible to use the alachlor-ELISA for quantitation of ESA due to the sufficient cross-reactivity of the latter towards the anti-alachlor antibody. The OXA co-eluted with ESA in methanol as observed from HPLC data, but because OXA has almost no cross-reactivity with the alachlor-ELISA, it does not interfere with the analysis of ESA. The mechanism of differential solubility in ethyl acetate for ESA and OXA is probably a result of the fact that the ionic organic molecules require a cation to be eluted with them, either a sodium or calcium ion for most natural waters. Apparently, the solubility of ESA and OXA in ethyl acetate is low, probably because of their ionic character and because of the necessity for their counter ion and water of hydration to be present in solution. The later elution of ESA and OXA was accomplished by using a more polar solvent such as methanol. The result is a separation of parent compound and metabolites for ELISA. The importance of this finding is that many ionic compounds, particularly metabolites of herbicides, may be separated from parent compounds by this procedure.

Table 1.7. Recovery and precision of alachlor and ESA from a C_{18} resin by sequential elution with ethyl acetate and methanol by SPE using the alachlor RaPID Assay Kit (n=7).

Concentration (µg/L) of Fortified Distilled Water	% Alachlor Recovered (in ethyl acetate extract)	% ESA Recovered in methanol extract)
A. 0.20 ESA	0	92 ± 10%
B. 0.050 alachlor + 0.10 ESA	100 ± 10%	97 ± 10%
C. 0.10 alachlor + 0.050 ESA	97 ± 10%	99 ± 10%
D. 0.025 alachlor + 0.025 ESA	105 ± 10%	91 ± 10%
E. 0.20 alachlor	98 ± 10	0

Analysis of Alachlor and Alachlor ESA by SPE-ELISA Method

The detection limit of the RaPID assay for alachlor using the SPE-ELISA method described in Figure 1.1 is 0.005 µg/L. This is 20-fold lower than the detection limit of the conventional ELISA (detection limit is 0.1 µg/L). ESA was eluted with methanol and was analyzed by ELISA using the alachlor-RaPID assay but calibrated with ESA standards. The calibration curve for ESA was linear for concentrations ranging from 1.0 to 20 µg/L. As a result of the preconcentration of samples by SPE, the detection limit for ESA can be as low as 0.1 µg/L if a 100-mL sample is used and the dried extract is diluted to 5 mL water for ELISA. It should be noted that solvent exchange of the organic solvent to an aqueous medium was performed prior to ELISA because methanol may affect the antibody-antigen interaction and produce an erroneous result. The RaPID assay kit tolerates 20% methanol, but the reproducibility of the EnviroGard alachlor kit was affected, with increased %CV observed using 20% methanol in the sample matrix. The greater sensitivity of the EnviroGard ELISA to methanol may be due to the fact that the antibodies in this assay were passively adsorbed to the surface of the microwells, whereas in the RaPID assay antibodies were covalently coupled to the magnetic particles (Lawruk *et al.*, 1992).

The automated workstation for SPE acts like a liquid chromatograph with a fraction collector. The isolation and separation occurs when the sample is pumped through the cartridge, followed by sequential elution with ethyl acetate and methanol and collection of fractions for analysis by immunoassay, GC/MS, or HPLC. The automated SPE procedure is capable of reproducing the separation within ±10%.

The ability of SPE to separate alachlor and ESA was demonstrated on ground-water samples from 12 different locations. The presence of both alachlor and ESA in the ground-water samples resulted in an overestimation of alachlor when they were analyzed directly by ELISA. Four out of these 12 samples produced false-positive detection of alachlor by ELISA but negative detection by GC/MS (detection limit of 0.05 µg/L), and 8 samples had significantly higher alachlor concentrations by ELISA compared to that by GC/MS (Table 1.8). The discrepancy between the amount of alachlor measured by GC/MS and that measured by direct ELISA was proportional to the amount of ESA in the sample. However, when the water samples were passed through SPE and the ethyl acetate eluates were analyzed for alachlor by ELISA as described above, the concentration of alachlor agreed with that obtained from GC/MS. In addition, analysis of the methanol eluates by ELISA showed high levels of ESA whenever there was a discrepancy between ELISA and GC/MS for alachlor concentrations. This indicates further that ESA, which caused the false alachlor concentration in ELISA, was recovered in the methanol and not in the ethyl acetate extracts. These results suggest that the SPE-ELISA method is quite effective for the separation and analysis of alachlor and ESA.

Table 1.8. Analysis of ground-water samples for alachlor and ESA by ELISA, SPE-ELISA, and GC/MS.

sample	alachlor concentration (µg/L)			ESA Concentration (µg/L)
	direct analysis by ELISA	GC/MS	SPE-ELISA	by SPE-ELISA
1	0.6	0.45	0.50	0.06
2	0.16	<0.05	0.04	0.83
3	0.92	<0.05	0.05	2.18
4	0.33	<0.05	0.03	1.65
5	1.83	0.07	0.10	4.80
6	1.10	0.21	0.39	1.88
7	0.22	<0.05	0.04	1.10
8	8.60	1.50	1.26	3.50
9	0.22	0.06	0.09	0.91
10	2.85	0.52	0.61	9.32
11	1.79	0.36	0.33	4.29
12	4.63	2.95	2.78	5.41

Natural water samples were analyzed for ESA by SPE-ELISA and the results were confirmed by HPLC with photodiode array detection. Figure 1.8A shows the correlation of ESA analysis by HPLC and SPE-ELISA for 22 surface-water samples from reservoirs in the Midwestern United States. The correlation coefficient of 0.93 demonstrates that the two methods correlate well and the slope of 0.69 indicates that ESA concentrations by HPLC were generally lower. The smaller ESA concentrations indicated by HPLC analysis may be attributed to high levels of humic and fulvic acids that were coextracted in the methanol fraction. The high amount of humic and fulvic acids caused the increased baseline of the chromatograms which sometimes buried the smaller ESA peaks. This caused difficulty in measuring ESA at lower concentrations. The detection limit of the HPLC method was set at 0.10 µg/L. Ground-water samples from several wells in the Midwestern United States were analyzed by HPLC and results showed better correlation (r = 0.97) with ELISA (Figure 1.9B) which again may be the result of the smaller amounts of humic and fulvic acids in the samples. Also, a higher slope (1.2) compared with that observed from the surface-water samples was obtained. The samples from the various wells and reservoirs varied in ESA concentration from less than 0.1 to greater than 10 µg/L.

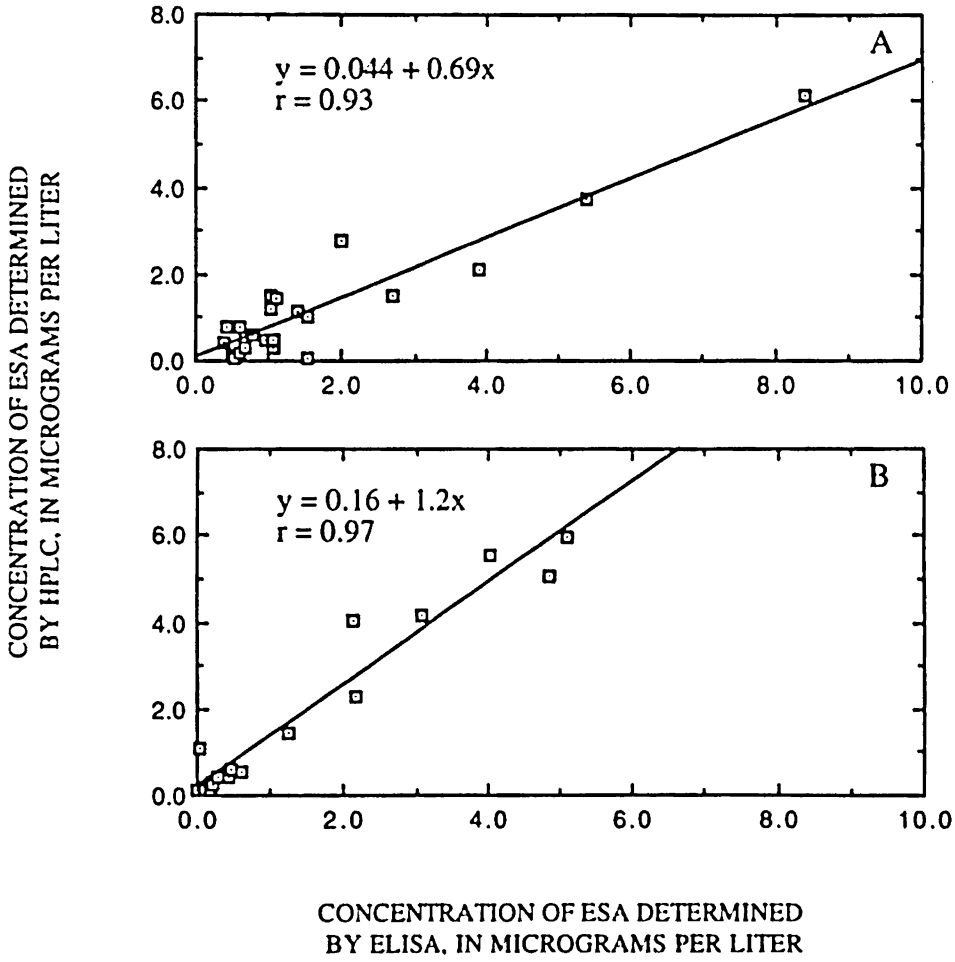


Figure 1.8. Concentrations of ESA in 22 surface-water samples (A) and 20 ground-water samples (B) determined by HPLC and SPE-ELISA using the alachlor RaPID Assay.

CONCLUSIONS

The coupling of SPE and ELISA resulted in a reproducible immunoassay method for trace analysis of two important herbicides in pristine water, atrazine and alachlor. The procedure utilizes SPE to remove some of the organic substances that could potentially interfere or cross-react with ELISA. The use of SPE to concentrate trace levels of the target analytes resulted in an assay with a detection limit of 5.0 ng/L and a relative standard deviation of ±10%. The comparison of the magnetic particle- and microtiter plate-based ELISA showed that both forms worked well, but that the precision was better using magnetic particles because of faster reaction kinetics resulting from a larger surface area to volume ratio. The SPE-ELISA method correlated well with the more expensive conventional GC/MS method for both atrazine and alachlor. This inexpensive SPE-ELISA method can measure herbicide concentrations in nanograms per liter accurately and may be used in the field to perform real-time analysis.

The cross-reactivity data that resulted from this study is important in the interpretation of results from analysis by ELISA. Most of the commercial ELISA kits for pesticide analysis have limited data on cross-reactivity with metabolites because of the unavailability of the standards or the lack of information on the occurrence of possible compounds that will cause interference.

Alachlor and ESA were effectively separated by sequential elution with ethyl acetate and methanol using SPE. The SPE method is a simple and elegant procedure because clean-up, concentration, and separation occur in a single step. Because ESA

cross-reacts strongly with the anti-alachlor antibody, it was possible to analyze ESA using the ELISA kits originally designed for alachlor analysis. The method was viable for the analysis of both surface- and ground-water samples and comparable to GC/MS and HPLC analyses, for alachlor and ESA, respectively. A detection limit of 0.005 µg/L for alachlor and 0.10 µg/L for ESA, with a precision of ±10%, was achieved by coupling SPE and ELISA.

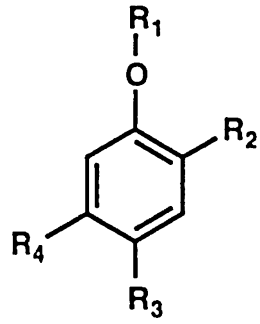
CHAPTER II

Development of ELISA and Immunoaffinity Chromatography for the Analysis of 2,4-D in Water Samples

INTRODUCTION

The phenoxyalkanoic acids are herbicides that are highly toxic to most annual and perennial dicotyledonous species and relatively nontoxic to monocotyledonous plants (Loos, 1975). Applied as foliar postemergence treatments they are readily absorbed by the plants through their foliage and roots and then translocated throughout the plant. Their systemic interference with nucleic acid metabolism causes massive disruption of the translocation system by induced cellular proliferation and results in abnormal growth or accumulation of photosynthates in the growing parts with subsequent starvation and death (Loos, 1975). For a more comprehensive discussion on the mode of action of these herbicides on plants, the reader is referred to excellent reviews on this topic (Loos, 1975; Penner and Ashton, 1966).

The molecular structure of the phenoxyalkanoic acid herbicides consists of halogen, primarily chlorine, and an alkyl substituted aromatic ring bonded to a carboxylic moiety through an ether linkage (Figure 2.1). These important herbicides are formulated as substituted amines, esters, and alkali metal salts. Currently there are eight such compounds in general use throughout the world, with 2,4-dichlorophenoxyacetic acid (2,4-D) being the most extensively used (Loos, 1975; Gianessi and Puffer, 1991). Although the major use of phenoxyalkanoic herbicides is associated with weed control in dry land vegetation, some of them are also used to kill aquatic weeds in lakes and ponds. The most extensive use of phenoxyalkanoic acid herbicides in water is in the production of rice (CAST, 1975).



Compound	R_1	R_2	R_3	R_4
2,4-D	CH_2COOH	Cl	Cl	H
2,4,5-T	CH_2COOH	Cl	Cl	Cl
MCPA	CH_2COOH	CH_3	Cl	H
2,4-DB	$CH_2CH_2CH_2COOH$	Cl	Cl	H
MCPB	$CH_2CH_2CH_2COOH$	CH_3	Cl	H
Dichloroprop	$CH(CH_3)COOH$	Cl	Cl	H
Fenoprop	$CH_2(CH_3)COOH$	Cl	Cl	Cl
Mecoprop	$CH(CH_3)COOH$	CH_3	Cl	H

Figure 2.1. General structure of phenoxyalkanoic acid herbicides.

Studies have shown that any phenoxyalkanoic herbicide, whether applied as ester or dimethylamine salt formulations, will be transformed to the respective phenoxyalkanoic anion in soil. Because the anionic form of the phenoxyalkanoic herbicide is water soluble and not considered to be highly adsorbed to soil organic matter (Kuhnt, 1993), it can readily leach to greater soil depth and can also be removed from treated areas by flood water and runoff. These processes could result in contamination of rivers and other bodies of water.

As shown in Table I, 2,4-D is the fifth most heavily used herbicide in the U.S. Approximately 33 million pounds of 2,4-D active ingredient is used annually in U.S. crop production (Gianessi and Puffer, 1991). This herbicide is a potent broad-leafed weed killer, but unfortunately also extremely toxic to grapes, cotton, tomatoes and other susceptible crops (Sherwood *et al.*, 1970). Therefore, severe injury to crops outside the treated areas can occur from spray drift, leaching and/or runoff. Hence, it has become increasingly important to analyze qualitatively and quantitatively for trace concentrations of 2,4-D in the environment.

The acute toxicity of the phenoxyalkanoic acids in mammals is low, as indicated by the LD_{50} values (dose of the toxicant that will kill 50% of the test organisms within a designated period of time) for rats or mice in Table 2.1. However, there have been studies which link 2,4-D with the development of Non-Hodgkins' Lymphoma (NHL), a cancer of the lymph system, among farmers that have been exposed to 2,4-D (Sharp and Salleh, 1993). As an important pollutant, 2,4-D is monitored regularly in municipal water and ground water. The legally established MCL for this herbicide in potable water is 70 µg/L (EPA, 1992).

Table 2.1. Physical and toxicological properties of phenoxyalkanoic acid herbicides (Chau *et al.*, 1982)

Chemical name (Common Name)	Solubility at 20-25 °C (g/100 mL) Water	Acetone	Ethanol	pK (25 °C)	Acute oral LD_{50}(mg/Kg)
2,4-Dichlorophenoxyacetic acid **(2,4-D)**	620-900	85	130	2.6-3.3	375 rat
2,4,5-trichlorophenoxyacetic acid **(2,4,5-T)**	251-280	soluble	55	2.6-3.1	500 rat
4-chloro-2-methylphenoxyacetic acid **(MCPA)**	825-1600	soluble	153	2.9-3.3	700 rat
4-(2,4-dichlorophenoxy)butyric acid **(2,4-DB)**	46	10	soluble	6.0	1960 rat
4-(2-methyl-4-chlorophenoxy) butyric acid **(MCPB)**	44-48	20	15	6.2	700 rat
2-(2,4-dichlorophenoxy)propionic acid **(dichloroprop)**	350	60	soluble	2.9-3.0	800 rat
2-(2,4,5-trichlorophenoxy)propionic acid **(2,4-5-TP; fenoprop; silvex)**	140	18	soluble	2.83	650 rat
2-(4-chloro-2-methylphenoxy) propionic acid **(mecoprop)**	895	soluble	soluble	3.1-3.2	650 mouse

Development of a procedure for the analysis of both the acid and ester forms of 2,4-D and the other phenoxyalkanoic herbicides is difficult, and the analysis is too complicated and lengthy for general purposes. Hence, many investigations focus only on the analysis of the acid form of 2,4-D, which is sufficient for monitoring because the ester forms of the phenoxyalkanoic herbicides are rapidly hydrolyzed in water (Bailey *et al.*, 1970) and in soil (Smith, 1972; 1976).

Due to the nonvolatility and polarity of 2,4-D, it is unfit to be analyzed directly by GC without derivatization. Although GC with electron-capture detection (ECD) is the conventional method for 2,4-D analysis, the use of HPLC with ultraviolet detection has been reported as well (Di Corcia *et al.*, 1989; Bogus *et al.*, 1990; Loconto, 1991). Conventional methods for quantitation of 2,4-D involve liquid-liquid extraction, acid-base partitioning, chemical derivatization, and purification. Although there have been a few examples where reversed-phase solid-phase extraction was employed to isolate phenoxyalkanoic acids (Hoke *et al.*, 1986; Wells and Michael, 1987; Bogus *et al.*, 1990; Loconto, 1991), in practice, liquid-liquid extraction is the method most frequently used for water analysis. The extraction procedure involves a tedious process and use of large volumes of organic solvents, most of which are highly toxic, such as benzene, methylene chloride, and chloroform. Adjustment of pH of the water sample to less than pH 2.0 is an important step because this allows the partitioning of the herbicidal acids into the organic solvent phase. Together with the herbicides, other acidic compounds such as other carboxylic acids and phenols are also extracted and can cause interferences. If interference from these compounds is severe, further clean-up may be required before analysis can be performed. Therefore, quick, easy, and economical methods are

needed for analysis of 2,4-D in large number of samples. Immunochemical assays represent an effective alternative to instrumental methods.

The utility of immunoassays for the analysis of 2,4-D in water and urine samples from exposed workers have been demonstrated by Hall and co-workers (Hall *et al.*, 1989). Concentrations of 2,4-D in water and urine samples were quantitated with an indirect ELISA with a working range of 100-10,000 ng/mL and with a radioimmunoassay (RIA) procedure with a working range of 50-10,000 ng/mL. Another application of a RIA for the biological monitoring of 2,4-D in exposed workers in agriculture and forestry has been reported recently (Knopp and Glass, 1991). Furthermore, a pair of enzyme immunoassays, which compliment each other in terms of specificity for the phenoxyalkanoic herbicides, has been described (Fleeker, 1987). Fleeker developed two immunoassays using two distinct immunogens and enzyme ligands. The use of two sets of antibodies with different specificities to screen for 2,4-D in ground water and municipal and river water reduced the number of false positive responses. However, because of the poor detection limits of the available immunoassay methods, a large number of false negatives often result when the immunoassay is used for screening. A solution to this problem is either to develop a more sensitive immunoassay method, or a more efficient preconcentration step which eliminates the use of large volumes of organic solvents, and reduce the interferences associated with liquid-liquid or solid-phase extraction.

The specific objectives of this study were to: (1) produce polyclonal antibodies against the herbicide 2,4-D using rabbits, (2) to characterize the specificity of the polyclonal antibodies generated, (3) to optimize an ELISA method for 2,4-D using the antibodies produced from the rabbits, and (4) to examine the potential of using the antibodies for immunoaffinity chromatography to isolate and concentrate 2,4-D from water samples.

EXPERIMENTAL

Preparation of 2,4-D-Protein Conjugates

To prepare the immunogen for antibody production, it was necessary to conjugate 2,4-D (Ultra Scientific, North Kingstown, RI) to a carrier protein such as Purified Protein Derivative (PPD) (Lederle Laboratories, Pearl River, NY). The conjugation of 2,4-D to PPD (2,4-D-PPD) was achieved through the -COOH group of 2,4-D according to the enhanced carbodiimide coupling method described by Staros and co-workers (Staros *et al.*, 1986). The reaction was performed by first activating 2,4-D with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Pierce, Rockford, IL) using sulfo N-hydroxysuccinimide (sulfo-NHS) (Pierce, Rockford, IL) as enhancer. The molar ratio of PPD:2,4-D was 1:20. First 4 mg of 2,4-D was dissolved in 10 mL of 0.1M phosphate buffer (PB), pH 7.4, containing no azide. Then, sulfo-NHS was added stepwise to a final concentration of 5.8 mM (12.6 mg). After obtaining a homogeneous mixture, EDC was added to a final concentration of 0.1M (195 mg) with stirring. Finally, 1 mg of PPD was added, and the reaction was allowed to proceed for 24 hrs at room temperature with gentle and constant stirring. The reaction mixture was then extensively dialyzed against 0.01 M phosphate-buffered saline (PBS, pH 7.4), to remove excess EDC, sulfo-NHS, and 2,4-D.

To prepare the coating antigen for the screening of antisera, 2,4-D was conjugated to bovine serum albumin (BSA) (Pierce, Rockford, IL) using the same carbodiimide method described above. The final concentrations of the reagents were

the same as above, except for the molar ratio of 1:35 for BSA (70 mg):2,4-D (9 mg) in the reaction mixture. The 2,4-D-BSA conjugate was purified by dialysis.

To prepare the labeled hapten for competitive ELISA, horseradish peroxidase (HRP) (Biozyme Laboratories Int., San Diego, CA) was conjugated to the haptens following the same EDC coupling procedure above, except that the reaction was allowed to proceed at room temperature for 4 hrs, and then at 4 °C for an additional 24 hrs. Two labeled haptens were synthesized, one was prepared by conjugating 2,4-dichlorobutyric acid (8 mg) with HRP (4 mg) (2,4-DB-HRP), and the other was prepared by reacting 2,4-D (4 mg) with HRP (4 mg) (2,4-D-HRP). The total volume of each reaction mixture was 3 mL and the final concentrations of EDC and sulfo-NHS were the same as described above. The general reaction for the above protein conjugations may be represented in Figure 2.2.

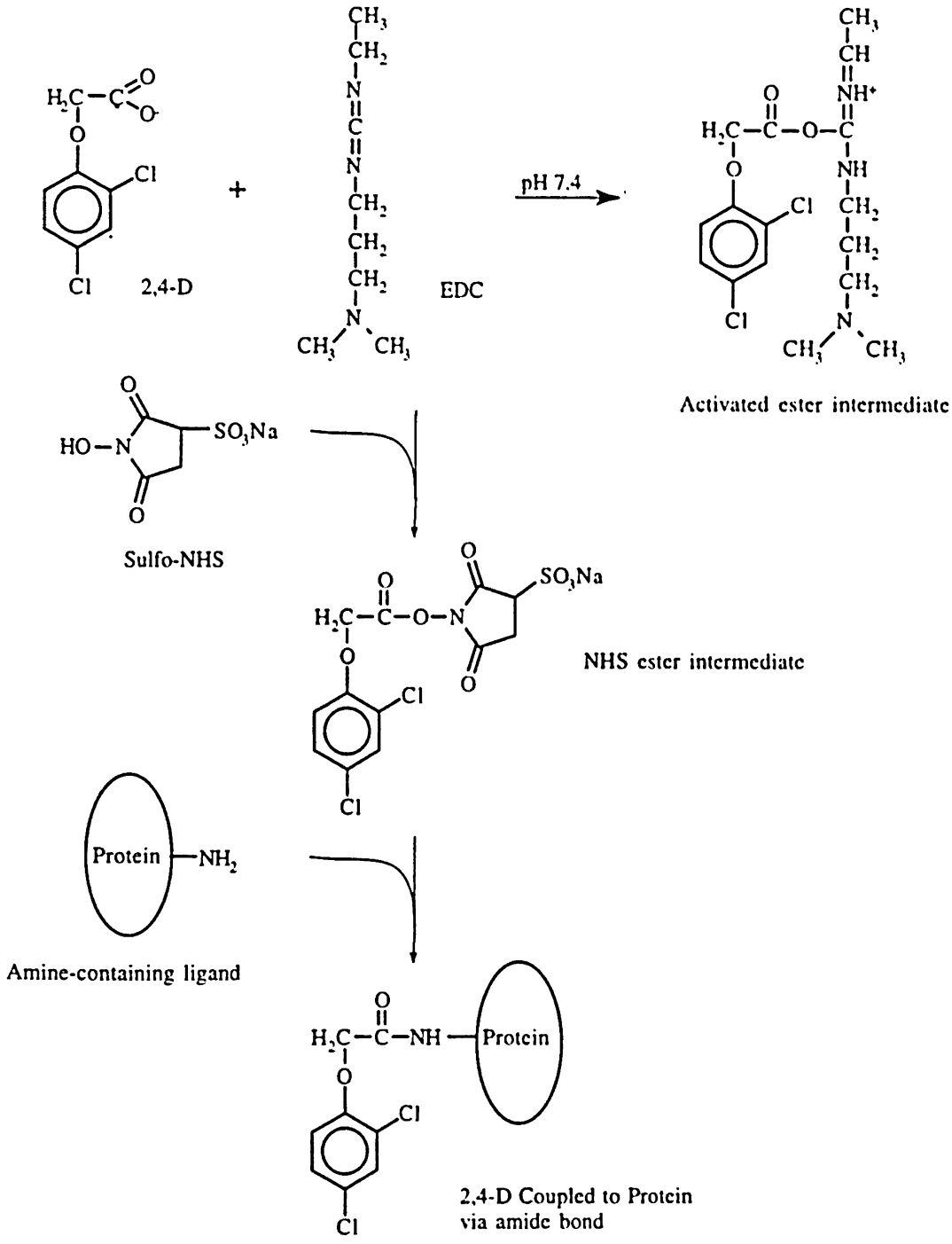


Figure 2.2. Coupling of 2,4-D with proteins via enhanced carbodiimide reaction.

Immunization

Polyclonal antibodies against 2,4-D were generated by immunizing two female New Zealand white rabbits with the prepared 2,4-D-PPD immunogen according to a standard protocol (Ausubel *et al.*, 1992). One week prior to the first immunization, each rabbit was presensitized with 200 μg of Bacillus Calmette-Guerin (RIBI Immunochem Research Inc., MT) via the intramuscular (200 μL) route. After seven days, the rabbits were immunized intradermally at five sites, each site with 100 μL of a 1:1 suspension of 2,4-D-PPD in Freund's complete adjuvant (Gibco Laboratories, Grand Island, NY). At two-week intervals, two booster shots with 500 μL of 2,4-D-PPD in Freund's incomplete adjuvant (3:1) at each site were given to each rabbit intramuscularly. Nine weeks after the first immunization, the rabbits were bled from their ear arteries. Immunization was continued every two weeks, and bleeding of the rabbits was done 7-9 days after each immunization. After 16 months, serum was collected from a terminal bleed of both rabbits. The titer of each pool of antiserum was determined by an ELISA method described below.

Isolation of Serum and Purification of Antibodies

Immediately after each bleeding procedure, the blood was allowed to clot at room temperature for about 1 hr, then stored at 4 °C overnight to allow the clot to contract. Then, the clot was detached from the walls of the container, and centrifuged for 30 min at 2500 g. The supernatant (antiserum) was carefully transferred to a plastic container for storage at -20 °C.

The immunoglobulin fraction from the antisera was separated by saturated ammonium sulfate (SAS) precipitation following the procedure described by Goers

(Goers, 1993). The precipitation of the γ globulin fraction from the serum (about 25-30 mL per blood collection) was achieved by slowly adding finely crushed powder of ammonium sulfate (Fisher Scientific, Pittsburgh, PA) up to a 40-45% saturation (24-27 g $(NH_4)_2SO_3$/100 mL serum at room temperature). Constant stirring and slow addition of $(NH_4)_2SO_3$ were necessary to avoid localized high concentrations of the salt that may cause precipitation of nonimmunoglobulins. After precipitation was complete the mixture was centrifuged and the supernatant was discarded. The precipitate was washed 3 times with cold 50% SAS using a volume that is at least 10 times the volume of the precipitate.

The IgG fraction was obtained by anion-exchange chromatography using a diethylaminoethyl column, DEAE-Sephacel (Pharmacia Biotech Inc., Piscataway, NJ). First, the precipitate obtained from SAS precipitation was redissolved in 10 mL 0.05 M phosphate buffer (PB), pH 8.0. This was dialyzed against 2 L PB at 4 °C for three days, changing the buffer every 6-7 hrs. After dialysis, the absorbance of a diluted aliquot of the sample was measured at 280 nm and the protein concentration was calculated using an extinction coefficient of 1.35 L/g.cm (Goers, 1993). The rest of the sample was then applied to a previously prepared DEAE column (35 mL bed volume) and eluted with 0.05 M PB, pH 8.0. The eluate was collected at 5-mL fractions. The absorbance of each fraction was monitored at 280 nm, and the first eluting fractions with absorbance greater than 0.3 were pooled together and stored for future use.

ELISA Method 1 : Determination of Titer and Apparent Affinity Constant

A modified ELISA procedure (Beatty *et al.*, 1987) was used to determine the titer of the antiserum and the apparent affinity constant of the DEAE-purified antibodies. A 96-well microtiter plate was coated overnight at 4 °C with either 100 µL of 2,4-D-BSA conjugate or BSA (10 µg/mL) dissolved in coating buffer (0.1 M carbonate buffer, pH 9.35). The plate was washed four times with 0.01 M PBS containing 0.05% Tween 20 (Sigma, St. Louis, MO) (wash buffer) and patted dry on a stack of paper towels. Then 150 µL/well of wash buffer containing 0.2% BSA (ELISA diluent buffer) was added and incubated at 37 °C to block the remaining active sites on the plastic surface. After 1 hr of incubation, the plates were washed four times with wash buffer and patted dry. To all wells, 100 µL of ELISA diluent buffer was added. Then, to the first column, 100 µL of diluted serum or DEAE-purified antibody solution was added and mixed thoroughly by pipetting and dispensing the liquid 7x in the same well using an 8-channel pipette. Without changing the tips, 100 µL of the liquid was withdrawn from the first column and transferred to the second. These transferring and mixing steps were repeated up to the 11th column, after which 100 µL of sample from the wells in the 11th column were discarded. This procedure resulted in a serial dilution of the antiserum or the antibody solution in all 11 columns, but left the 12th column free of antibody (blank or negative control). The plate was covered with a plastic lid and incubated for 1 hr at 37 °C.

After 1 hr of incubation, the plate was washed with wash buffer and patted dry. Then, 100 µL of horseradish peroxidase (HRP)-labeled goat-anti-rabbit IgG (Gt x Rbt-HRP, Organon Teknika Corp., Durham, NC) (1:10,000 dilution) was added and incubated for 1 hr at 37 °C. The plate was washed four times with nanopure water

(Barnestead, Nanopure II Sybron, Barnestead, MA) and patted dry. A color reagent consisting of a 1:1 mixture of tetramethyl benzidine and hydrogen peroxide (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was prepared, and 100 µL of this mixture was added to each of the 96 wells. After 20 min of color development, the enzyme reaction was quenched with 50 µL of 1 M HCl. The optical density was read at 450 nm using a 96-well plate reader (Vmax, Molecular Devices, Menlo Park, CA).

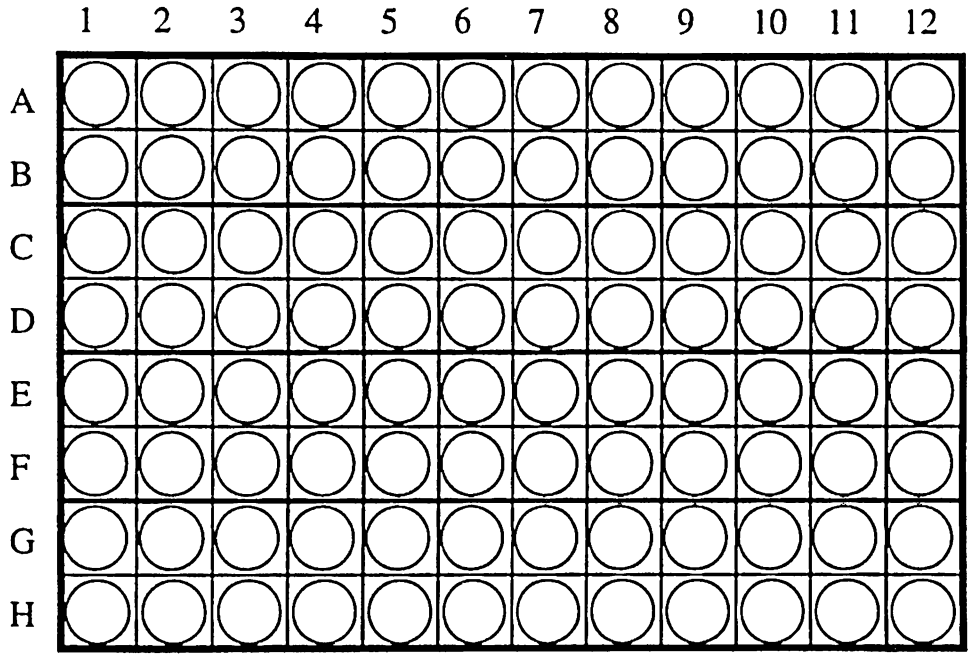
ELISA Method 2 : Optimization of Coating IgG and Enzyme-Conjugate Dilution

Format 2.1 (Direct Coating)

A two-dimensional ELISA was performed to determine the optimum concentration of coating antibody and labeled hapten for the competitive ELISA. First, a microtiter plate was coated directly with 150 µL of the anti-2,4-D antibodies at increasing dilutions from column 1 to 11 using the coating buffer (Figure 2.3). The plate was incubated overnight, washed, and blocked with BSA as described before. To perform the assay, 75 µL of distilled water were placed in each well, and mixed with 75 µL of various dilutions of freshly prepared enzyme-conjugates (labeled haptens:2,4-D-HRP or 2,4-DB-HRP diluted with ELISA diluent). The dilution of the labeled hapten was increased (in duplicate wells) from row A to row H of the microtiter plate. The plate was incubated for 1 hr at 37 °C, then washed and patted dry. Then, 150 µL of color reagent was added to each well and color was allowed to develop for 20 min. The enzyme reaction was stopped by adding 1 M HCl, and the absorbances were read at 280 nm as described above. Each combination of antibody and labeled hapten that resulted in an absorbance of ~1.0 was further examined to obtain the optimum condition for ELISA.

Format 2.2. (Pre-coating with Protein A)

A microtiter plate was coated overnight at 4 °C with 150 µL of 10 µg/mL solution (in coating buffer) of Fc-binding grade protein A (ICN Biomedicals Inc., Irvine, CA). The plate was washed four times with the wash buffer and patted dry. Then, 150 µL of various dilutions of anti-2,4-D antibodies (in ELISA diluent buffer) were placed in each well as shown in Figure 2.3 and incubated for 1 hr at 37 °C. The plate was washed four times with wash buffer and patted dry. Following the procedure above, the optimum concentration of the coating antibody and labeled hapten were determined.



Column	IgG Dilution	Row	HRP-Conjugate Dilution
1	1:200	A	1:5,000
2	1:400	B	1:5,000
3	1:800	C	1:10,000
4	1:1,600	D	1:10,000
5	1:3,200	E	1:15,000
6	1:6,400	F	1:15,000
8	1:12,800	G	1:20,000
9	1:25,600	H	1:20,000
10	1:51,200		
11	1:102,400		
12	BLANK		

Figure 2.3. Two dimensional ELISA.

Preparation of Immunoaffinity Chromatography Columns

To prepare immunoaffinity columns for the purification of anti-2,4-D antibodies, 2,4-D molecules were coupled with adipic acid hydrazide-activated agarose support (Pharmacia Biotech Inc., Piscataway, NJ) via the enhanced EDC method. The gel (20 mL) was reacted with 25 mg sulfo-NHS and 1 g EDC, followed by the addition of 25 mg 2,4-D. The coupling reaction was allowed to proceed overnight at room temperature with constant and gentle shaking. This procedure linked 2,4-D with the hydrazide group through the formation of an amidine linkage (Figure 2.4). After the reaction, the gel was washed thoroughly with water and PB pH 7.4, and was packed in 5 mL bed volume disposable plastic columns (Pierce, Rockford, IL).

To prepare immunoaffinity columns for the isolation of 2,4-D from water samples, a site-directed immobilization procedure (Hermanson, 1992) was used to attach the anti-2,4-D antibodies to the solid support. This method coupled antibodies through the carbohydrate chains coming off the heavy chains to an adipic acid hydrazide-activated agarose support (Figure 2.5). First, 150 mg antibody (IgG solution in 0.1 M PB buffer, pH 7.4) was oxidized by adding 150 mg of sodium *meta*-periodate (Sigma, St. Louis, MO) and gently mixing it in a test tube wrapped in aluminum foil (reaction is light-sensitive), for 30 min at room temperature. The oxidized IgG was then purified by desalting the mixture on a 50-mL Sephadex G-25 (Pharmacia Biotech Inc., Piscataway, NJ) column equilibrated with 0.1 M PB, pH 7.4. The fractions were collected in 5-mL increments and the absorbances were monitored at 280 nm. The first fractions contained the oxidized IgG. The gel was then washed with PBS and packed in 5 mL plastic columns.

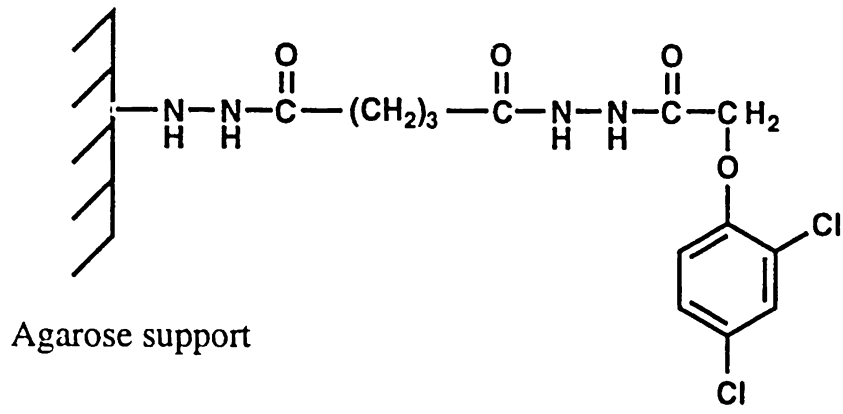


Figure 2.4. 2,4-D Immunoaffinity Column Support for Antibody Purification.

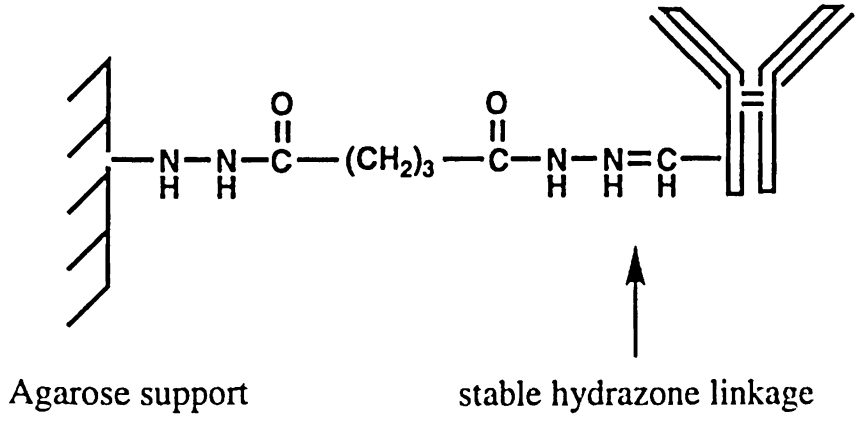


Figure 2.5. Immunoaffinity column for the isolation of 2,4-D from water samples using polyclonal antibodies.

RESULTS AND DISCUSSION

Optimization of the ELISA Conditions

The antisera obtained by immunizing the rabbits with 2,4-D-PPD conjugate were screened against 2,4-D-BSA coated on microtiter plates. Using ELISA method 1, the titers of antisera and the apparent affinity constant of purified IgG were determined. The antisera from Rabbit 1 had titers ranging from 2×10^4 to 9×10^4, while rabbit 2 had slightly higher titers ranging from 1×10^5 to 3×10^5. Because the titers did not vary significantly over the period of 11 months of blood collection, all antisera from each rabbit were pooled together and purified by SAS precipitation, followed by DEAE ion exchange chromatography.

The antibodies purified by DEAE ion exchange chromatography did not show any cross-reactivity toward BSA. The average apparent affinity constant of the antibodies for 2,4-D was found to be 2×10^9 for Rabbit 1 and 4×10^9 for Rabbit 2. Further purification of the antibodies by immunoaffinity chromatography using the 2,4-D column described above did not improve the binding constant of the antibodies towards 2,4-D. Instead, the apparent affinity constant dropped to 3×10^7 and 1×10^7, for Rabbit 1 and 2, respectively. The cause of the decrease in affinity constant is not known. No further attempt to repeat the experiment was done. Therefore, all the antibodies used in this study were purified only by ion exchange chromatography.

Using the antisera from Rabbit 1, the ELISA format 2.1 (direct coating with antibodies) and format 2.2 (precoating with protein A) were compared. This study showed that precoating with Fc-binding protein A results in enhanced binding of 2,4-D

to the antibodies. Pre-coating the plates with protein A reduced the amount of coating IgG and HRP-conjugate necessary for the negative control (blank) to produce an absorbance of ~1.0. This absorbance was achieved using a 1:1,000 dilution of the IgG stock solution (concentration of 1.7×10^{-5}), and 1:15,000 dilution of the labeled hapten (2,4-D-HRP or 2,4-DB-HRP). On the other hand, directly coating the plates with the antibodies required a dilution of 1:200 of the same IgG stock solution and 1:2,000 of the labeled hapten to produce the same absorbance (~1.0) for the negative control. Results from ELISA format 2.1 and 2.2 showed that antibodies obtained from Rabbit 2 did not bind with 2,4-D in solution. Apparently, the high titers of the antisera from Rabbit 2 was a result of very strong affinity of the antibody to the linker between the hapten and the protein (2,4-D-BSA) used for coating the plates. Similarly, in the competive ELISA, the free 2,4-D was not capable of competing with the 2,4-D-HRP or 2,4-DB-HRP conjugate, hence no decrease in absorbance was observed with increasing 2,4-D concentration in solution. Therefore, only the antibodies from Rabbit 1 were used for the succeeding studies.

Figure 2.6 shows the difference between a competitive ELISA for 2,4-D using a protein A-precoated plate (Fig. 2.6A) and an IgG-directly coated plate (Fig. 2.6B). Protein A specifically binds to the Fc region of the IgG molecule, and orients the Fab region (binding sites) towards the solution. This IgG orientation facilitates efficient binding between the target analyte in solution and the IgG coated on the plate. Consequently, the amounts of coating IgG and labeled hapten necessary for an effective ELISA were significantly reduced, which in turn lead to a lower IC_{50} for 2,4-D. The IC_{50} is the concentration of the antigen required to inhibit the binding of the labeled hapten to the antibodies by 50%. A lower IC_{50} value means a more effective

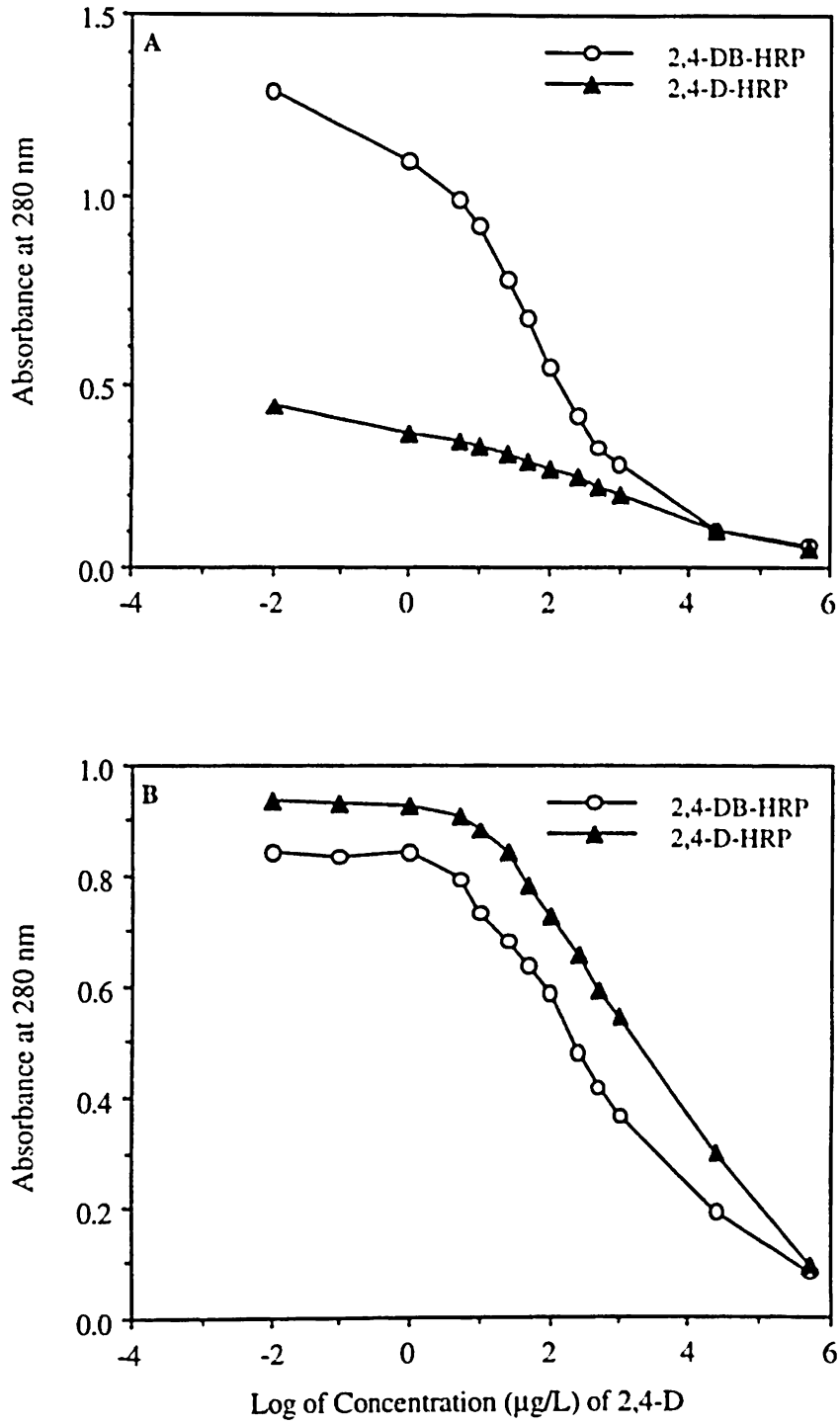


Figure 2.6. Comparison of ELISA with Protein A (A) and Direct Coating (B), and the Effect of the Structure of the Hapten-HRP Tracer on the Sensitivity of ELISA.

binding of the antigen to the IgG molecules. Using the protein A-precoated plate, the IC_{50} for 2,4-D was 45 µg/L with 2,4-DB-HRP conjugate, and 200 µg/L with 2,4-D-HRP conjugate as labeled hapten. On the other hand, for the plates directly coated with IgG, the IC_{50} for 2,4-D was 178 µg/L with 2,4-DB-HRP, and 355 µg/L with 2,4-D-HRP conjugate as labeled hapten.

The improved sensitivity of the assay using 2,4-DB-HRP compared to 2,4-D-HRP as labeled hapten indicates that the alkyl chain of the phenoxyalkanoic acid molecule, bonded to the aromatic ring via an ether linkage, plays an important role in the binding of the antigen to the antibody. The longer alkyl chain length of the 2,4-DB molecule results in an increased binding of the labeled hapten. This may be due to a decreased steric hindrance between the 2,4-D molecule and the antibody binding site. This increased binding also suggests that the antibodies have affinity toward the spacer arm that linked the hapten (2,4-D) to the foreign protein (PPD) used for immunization. A similar pattern has been observed (Eremin *et al.*, 1993) when varying spacer arm lengths were used to prepare fluorescent-labeled haptens to study the influence of the structure of the labeled haptens on the sensitivity and specificity of a polarization immunofluoroassay for 2,4-D. Eremin and co-workers reported that increasing the spacer arm length increased the binding of the labeled hapten to the antibody, and decreased the specificity of the assay for 2,4-D. However, in a competitive binding assay, care must be observed so that the labeled hapten does not bind too strongly to the antibody such that displacement by the analyte becomes highly unfavorable. Therefore, it is important to examine a variety of different haptens for conjugation to an enzyme to optimize the sensitivity of the assay.

Cross-reactivity Studies

The cross-reactivity of the anion exchange purified antibodies against 2,4-D and related phenoxyalkanoic acid herbicides was determined by ELISA method 2.2 (precoating with protein A) using the optimum dilution for IgG and 2,4-DB-HRP conjugate. To calculate for the % cross-reactivity, the absorbances (A) were first converted to %B/Bo values for normalization according to the following formula:

$$\%B/Bo = \frac{[(A - A_{excess})]}{[(A_{blank} - A_{excess})]} \times 100\%$$

where A_{excess} is the absorbance of the 500,000 µg/L solution of the target compound and A_{blank} is the absorbance of the labeled hapten in distilled water. The IC_{50} for each compound was determined graphically as the concentration that causes 50% reduction in the absorbance of the negative control (%B/Bo = 50%). Then the % cross-reactivity of each compound was calculated based on a 100% cross-reactivity of 2,4-D as shown below:

$$\%\text{Cross - reactivity} = \frac{IC_{50} \text{ of } 2,4\text{ - D}}{IC_{50} \text{ of target compound}} \times 100\%$$

Table 2.2 shows the LDD, IC_{50}, and % cross-reactivity of 2,4-D and other structurally related herbicides towards the polyclonal antibodies generated from Rabbit 1. The IC_{50} for 2,4-D was 45 µg/L with an LDD of 0.5 µg/L. The antibodies exhibited strong cross-reactivity towards phenoxyalkanoic acids with longer alkyl chains, such as 2,4-DB (250%) and MCPB (112%), and also with the isopropyl (150%) and methyl (150%) ester forms of 2,4-D. On the other hand, replacement of chlorine with methyl

Table 2.2. Binding characteristics of the antibodies from Rabbit 1 towards phenoxyalkanoic acid herbicides.

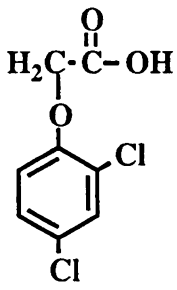
	Compound tested	LDD_{90} (µg/L)	IC_{50} (µg/L)	% Cross-reactivity
1.	2,4-D	0.5	45	100
2.	MCPA	5.0	105	43
3.	2,4-DB	1.0	18	250
4.	MCPB	1.3	40	112
5.	2,4-D isopropyl ester	1.3	30	150
6.	2,4-D methyl ester	1.9	30	150
7.	2,4,5-T	40	355	8.0
8.	Dicamba	NR	NR	NR
9.	2,4,5-T anisole	NR	NR	NR
10.	2,4-D anisole	NR	NR	NR
11.	2,4-D phenol	NR	NR	NR
12.	2,4,5-T phenol	NR	NR	NR

NR= No reactivity at concentrations $\leq$ 1,000 µg/L

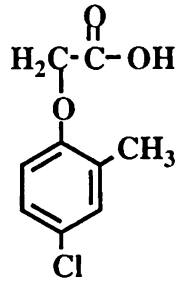
group in the aromatic ring, such as in MCPA, reduced the % cross-reactivity to less than 50%. Furthermore, addition of a third chlorine to the ring, such as in 2,4,5-T, resulted in an almost complete loss of cross-reactivity. The cross-reactivity pattern shown in Figure 2.7 suggests that although the binding of the antibodies is greatly influenced by the substitution in the aromatic ring, the length of the alkyl moiety makes a more important contribution. This can be further supported by the fact that no cross-reactivity was observed from 2,4-D anisole, 2,4-D-phenol, 2,4,5-T phenol, 2,4,5-T anisole, and dicamba, up to the highest concentration tested (10,000 μg/L).

According to a previous study (Lachman *et al.*, 1986), the use of PPD as a carrier for antibody production provides very powerful T-cell help, and yet gives rise to virtually no antibody response against itself. PPD is a protein component of tuberculin prepared from the culture supernatants of *Mycobacterium tuberculosis*. It is not a pure protein, but the molecular mass of its main component is 10,000. It has been reported that coupling small peptides to PPD gives rise to good titers of anti-peptide antibody. For these reasons, 2,4-D was coupled to PPD and used for immunization. In general, antibodies produced in response to an immunogen may recognize either the carrier protein, the hapten, or a site combining parts of both. The use of PPD conceivably reduces, if not eliminates, the unnecessary response to the carrier molecule. The results of this study show that the antibodies produced in response to 2,4-D-PPD recognize the linker and the chlorinated aromatic ring, resulting in an assay which is not highly specific for 2,4-D, but recognizes other members of the phenoxyalkanoic acid herbicides. However, this is not necessarily a bad characteristic of the antibodies especially if they are used in a class-specific screening assay. Because the herbicidal usage of 2,4-DB or MCPB is generally lower than that of 2,4-D and MCPA, the

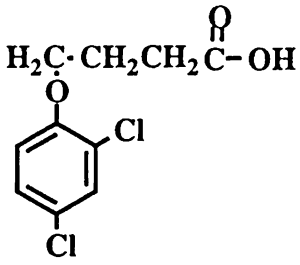
favored cross-reactivities of the antibodies towards the former compounds may even be an asset rather than a liability. The ability of the antibodies to recognize the substitution in the aromatic ring, in combination to the phenoxyalkyl group is important because many environmental pollutants have chlorinated aromatic rings, such as polychlorinated biphenyls (PCB's). The fact that the antibodies were not able to detect dicamba and the dichloro- and trichloro- phenols and anisole (Table 2.2), suggest that the di-chlorinated ring and the phenoxyalkanoic group must be both present in a molecule in order for it to bind with the anti-2,4-D antibodies. Therefore, chlorinated aromatic rings alone, such as PCB's, will not be detected by these antibodies. The antibodies described here are thus suitable for screening water samples for the most commonly used phenoxyalkanoic acid herbicides at levels above the LDD listed in Table 2.2. The LDD is the lowest detectable dose of a particular compound which result in a 90% reduction in the absorbance of the negative control. The data in Table 2.2 show that 2,4-D has the smallest LDD value (0.5 μg/L), although 2,4-DB, MCPB, and the esters of 2,4-D have comparable LDD's ranging from 1.0 to 5.0 μg/L.



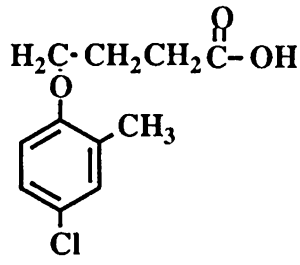
2,4-D
Cross-reactivity = 100%



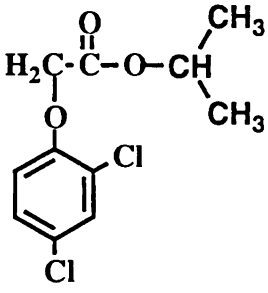
MCPA
Cross-reactivity = 43%



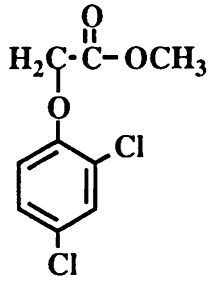
2,4-DB
Cross-reactivity = 250%



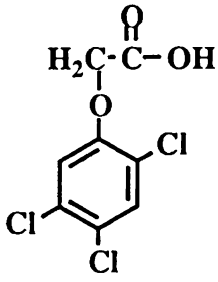
MCPB
Cross-reactivity = 112%



2,4-D isopropyl ester
Cross-reactivity = 150%



2,4-D methyl ester
Cross-reactivity = 150%



2,4,5- T
Cross-reactivity = 8%

Figure 2.7. Cross-reactivity of the antibodies toward phenoxyalkanoic acid herbicides.

Analysis of Water Samples by Competitive ELISA

To asses the potential of the ELISA method for the analysis of field samples, the optimum dilutions for coating IgG (1:1,000, with protein A pre-coating) and 2,4-DB-HRP (1:15,000) were used. First the linear working range of the assay was determined by preparing a calibration curve using 2,4-D concentrations ranging from 0.01 to 500,000 µg/L in distilled water. Analysis of these calibrator samples resulted in a sigmoidal curve (Figure 2.8A) typical for ELISA, with a linear range at concentrations ranging from 1.0 to 50 µg/L (Figure 2.8B).

Distilled water and samples taken from river, field runoff, and ground-water, were spiked with 2,4-D at concentrations of 1.0, 5.0, and 10.0 µg/L. The spiked and unspiked samples were analyzed directly by ELISA, without any sample preparation, in quadruplicates in the same microtiter plate. The concentrations of the samples were calculated based on a calibration curve prepared from concentrations of 0, 1.0, 2.0, 5.0, 10, 25, and 50 µg/L. The average coefficient of variation obtained from quadruplicate analysis was 5.86% which indicate good reproducibility within a plate.

Table 2.3 shows the recovery of 2,4-D from the spiked field samples. All the unspiked samples have no detectable 2,4-D concentration by ELISA. The % recovey of 2,4-D from the fortified samples using the ELISA method ranged from 95 to 150%, with the highest recovery observed on the runoff samples at 1.0 µg/L level. These unusually high recoveries may be due to the possible interference from high concentrations of dissolved organic carbon (DOC) present in the samples, as indicated by the yellow coloration of the water samples. The effect of matrix interference was more pronounced for the surface water than ground-water samples at lower 2,4-D concentrations.

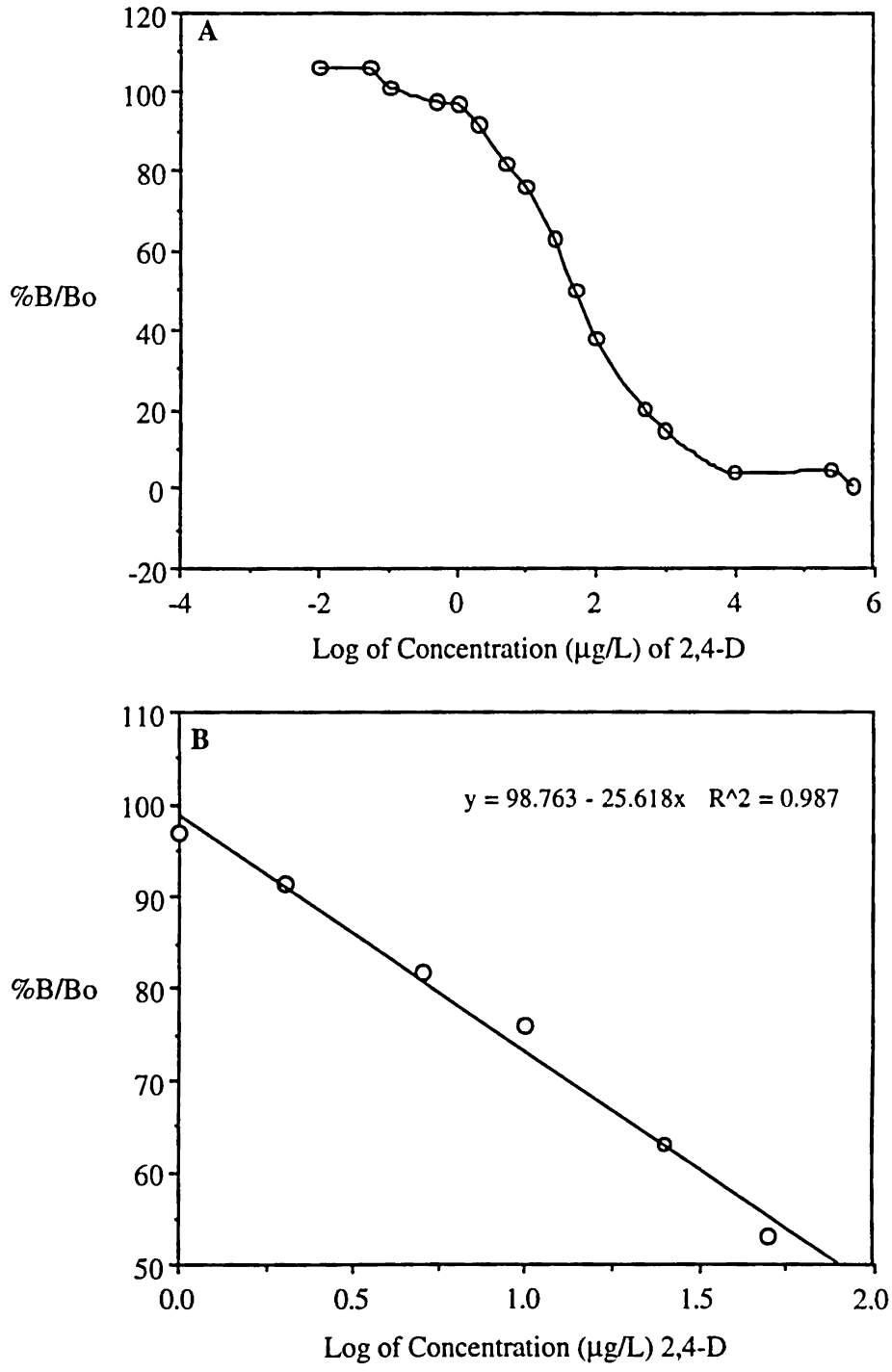


Figure 2.8. Standard curve for competitive ELISA (A), and linear range for 2,4-D ELISA (B). Data points represent the means of quadruplicates.

Table 2.3. Analysis of 2,4-D in natural water samples by ELISA.

Sample	Amount of 2,4-D added, (µg/L)	[a] Amount of 2,4-D measured by ELISA	% Recovery
Lake water	1.0	1.2 ± 0.5	120
	5.0	5.6 ± 0.8	112
	10	11 ± 0.9	110
Field runoff	1.0	1.5 ± 0.6	150
	5.0	6.1 ± 0.7	122
	10	12 ± 0.8	120
ground water	1.0	1.1 ± 0.3	110
	5.0	4.9 ± 0.6	98
	10	10 ± 1.0	100
Distilled water	1.0	0.95 ± 0.16	95
	5.0	5.3 ± 0.5	106
	10	10 ± 0.9	100

[a] average of 4 replicates

The limit of detection (LOD) of the ELISA for 2,4-D was calculated as three times the standard deviation about the mean measurement of the field blank dose signal. The result from 14 determinations of the field blanks from various sources (lake, runoff, ground-water) gave an LOD of 2.5 μg/L. It is important to recognize that this LOD, calculated based on the guidelines set for data evaluation by the ACS Committee on Environmental Improvement (Keith *et al.*, 1983), is five times higher than the previously determined LDD of the ELISA method (0.5 μg/L) listed in Table 2.2.

It is interesting to note that the LDD of an ELISA, which is determined from the 90% reduction in the absorbance of the enzyme-labeled hapten in distilled water, is typically what manufacturers of pesticide ELISA kits report as the LOD of the assay. One commercial 2,4-D ELISA kit (Ohmicron, Newtown, PA), for example, is claimed to have an LOD of 0.7 μg/L. However, actual analysis of natural water samples conducted in our laboratory using the ELISA kit resulted in more than 50% false negatives (compared to HPLC analysis) at concentrations below 1.0 μg/L. Moreover, most of the product inserts provide fortification data at concentrations that are much higher than the reported LOD. The 2,4-D ELISA kit from Ohmicron (Newtown, PA), for example, provides data on the % recovery at 5.0 μg/L as the lowest level of fortification, although the kit's reported LOD is 0.7 μg/L. The true detection limit of this commercial assay, based on the said guidelines, was determined in our laboratory to be 2.0 μg/L, which is comparable to that of the ELISA developed in this investigation.

With the objective of improving the detection limit of the 2,4-D ELISA, the water samples were concentrated by SPE, as described in Chapter I. Since 2,4-D has a

pKa of 2.6-3.3 at 25 °C (Sirons *et al.*, 1982), it is anionic at the pH levels typical for surface and ground water samples (pH 6-9). Therefore, it was necessary to adjust the samples to pH 2.2 to protonate 2,4-D and effect its adsorption onto the C_{18} resin used for SPE. A consequence of lowering the pH is enhanced co-extraction of other dissolved organic acids, such as humic substances, that are present in natural waters. Humic substances are polymeric organic acids of molecular weight ranging from 500 to 5000 and are polyelectrolytes of carboxylic, hydroxyl, carbonyl, and phenolic functional groups (Thurman, 1985). They comprise 50-75% of the DOC and are the major class of organic compounds in natural waters. The average pKa of dissolved humic substances is 4.2, therefore, at pH 2.2 the majority of the humic fractions are unionized and will adsorb effectively on the C_{18} resin. During elution with ethyl acetate, most of the adsorbed humic substances were eluted with 2,4-D as was evident by the yellow coloration of the eluate.

Analysis of lake, river, and ground-water samples fortified with low concentrations of 2,4-D (0.1, 0.2, 0.4, and 0.5 µg/L) showed recoveries ranging from 130 to greater than 200% by SPE-ELISA. Figures 2.9A-C show that the slopes of the lines of 2,4-D concentration measured by SPE-ELISA versus the spiked concentration are all greater than 1.0. The highest slope of 2.44 was observed in river samples (Figure 2.9B), followed by lake samples with a slope of 1.82 (Figure 2.9A), and ground-water samples with a slope of 1.32 (Figure 2.9C). The differences in ELISA response corresponds to the humic substances content in the samples. The concentration of humic substances varies for different natural waters. The lowest concentrations of humic substances are in ground-water and seawater, where concentrations vary from 0.05 to 0.6 mgC/L. Streams, rivers, and lakes contain from

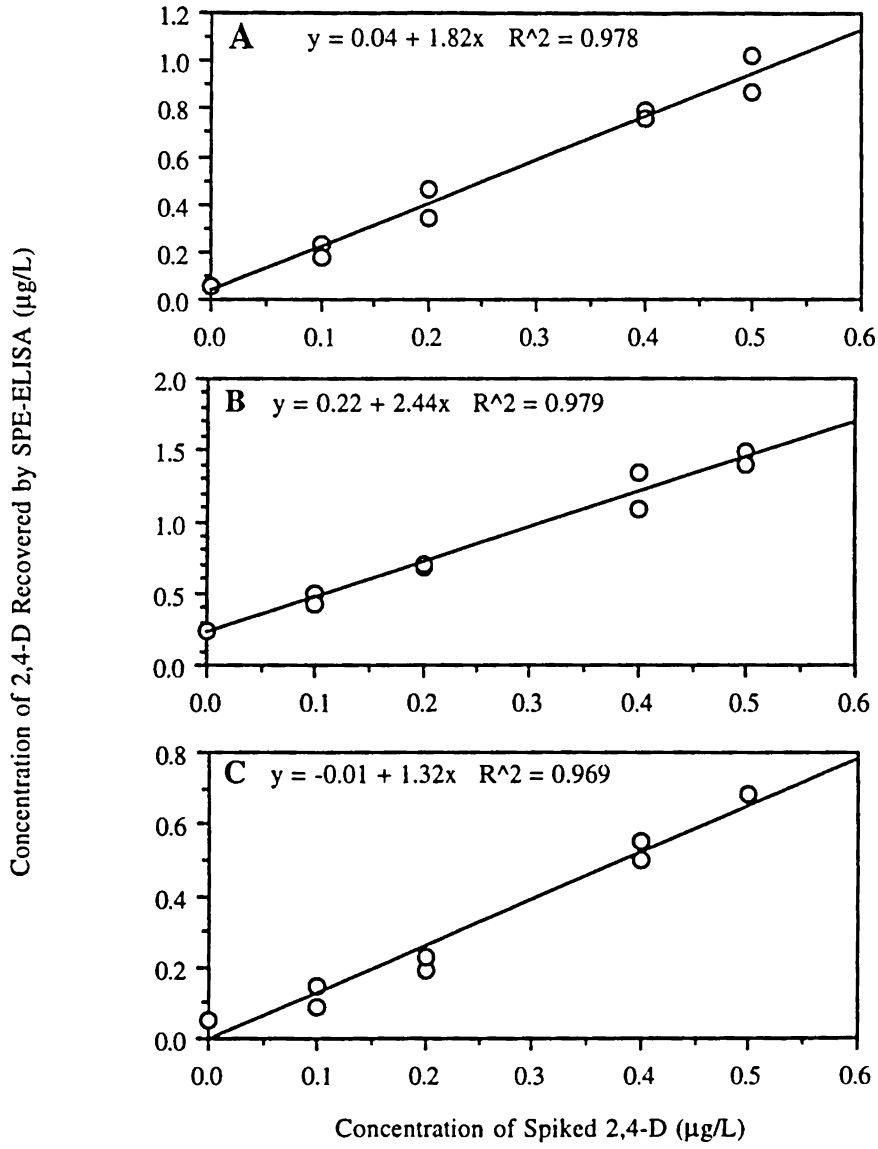


Figure 2.9. Recovery of 2,4-D from spiked lake (A), river (B), and ground-water (C) samples by SPE-ELISA.

0.50 to 4.0 mgC/L, and colored rivers and lakes have much larger concentrations from 10 to 39 mgC/L (Thurman, 1985).

The effect of DOC levels on the response of ELISA was indirectly determined by correlating the volume of the sample used for SPE on the %recovery of 2,4-D. Increasing the volume of sample subsequently increases the amount of humic substances co-extracted on the C_{18} resin. Figure 2.10 clearly indicates that the %recovery of SPE-ELISA increased logarithmically with the volume of sample used for extraction. The interference caused by the humic substances on the ELISA method for 2,4-D is apparently magnified by SPE. Unlike the SPE-ELISA method for atrazine and alachlor described in Chapter I, the method is not suitable for 2,4-D because of the necessity for prior pH adjustment which ultimately resulted in an augmented matrix effect.

Although interference of DOC on immunoassays has not been well characterized, it is speculated that overestimation of analyte concentrations is due to the interaction of the humic substances with the antibody binding site (Goh *et al.*, 1990), preventing the enzyme-conjugate from binding. For the anti-2,4-D antibodies, this postulate is supported by the fact that humic acids have chemical moieties that are very similar to the phenoxyalkanoic group of 2,4-D. Although the antibody may have a very weak affinity to the humic material, the binding can be enhanced by its relatively high concentration in the matrix compared to 2,4-D. However, the presence of high amounts of humic substances in the sample matrix does not always result in overestimation of analyte concentration by immunoassay. The effect of humic substances on the assay is governed by the characteristics of the antibody employed. Stearman and Adams (1992) for example, did not observe overestimation of atrazine

concentrations by ELISA from various soils containing 4.7% organic matter, while Stearman and Wells (1993) observed interference of up to 40 ppm of humic acid on atrazine and metolachlor assays only at low concentrations (0.1 and 0.6 μg/L, respectively) of the analyte.

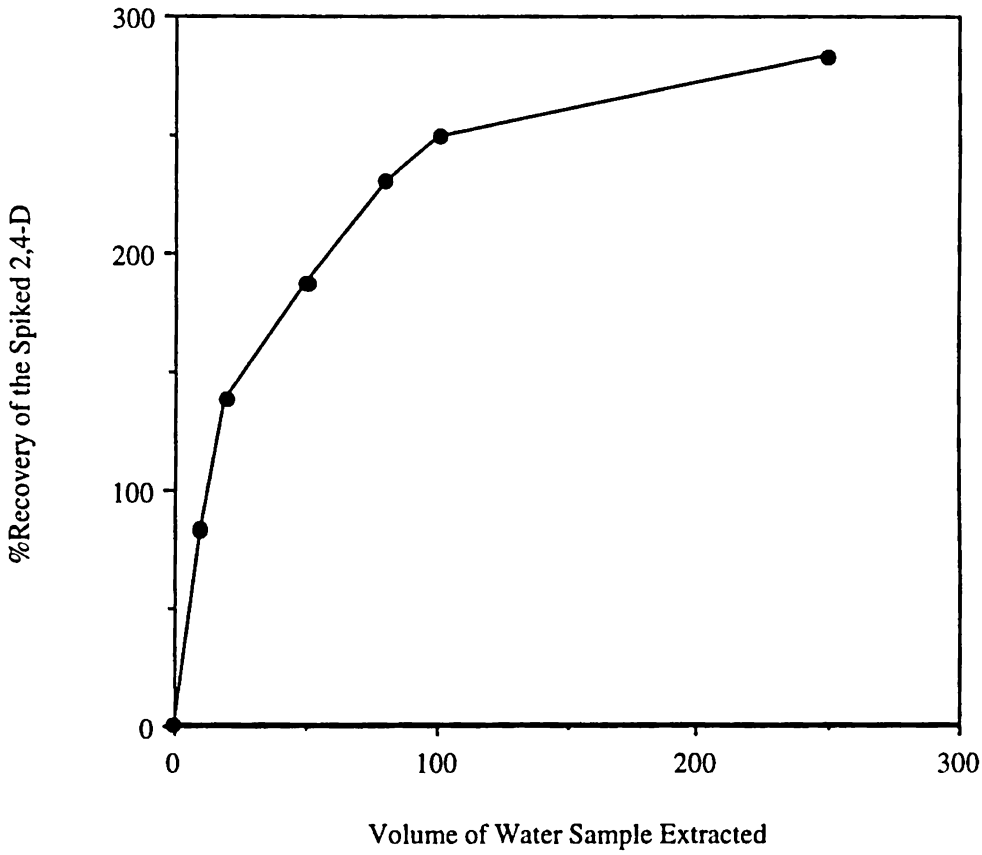


Figure 2.10. Effect of increasing volume of sample for SPE on the concentration of 2,4-D recovered by ELISA.

Immunoaffinity Chromatography For Sample Preparation

Because of the problems associated with the SPE-ELISA method, the potential application of the polyclonal antibodies against 2,4-D for immunoaffinity chromatography (IAC) was examined. The use of antibodies in IAC for sample preparation has been demonstrated successfully in many clinical and biomedical applications to simplify sample clean-up of complex biological matrices (Hendrickson and Wilson, 1994; Katz and Siewierski, 1992; Van Ginkel, 1991). Application of IAC in pesticide residue analysis, however, has not been as widespread. The first application of IAC in the isolation of a pesticide and its metabolites from various plant and animal extracts has been described only recently for imidazolinone herbicides (Wong et al., 1995).

In the present study, the use of IAC to isolate and concentrate the 2,4-D herbicide from water samples for HPLC analysis was examined. The hydrazide coupling method was used to link the IgG molecules onto the agarose gel. This procedure links the IgG through its Fc region, leaving the antigen binding sites free to interact with the antigen in the mobile phase (Figure 2.5). The coupling efficiency of this method, calculated from the absorption of the protein solution at 280 nm before and after coupling, was 67%. Each of the packed IAC columns (5 mL bed volume) contained approximately 10^{-7} moles of immobilized IgG.

After washing the packed IAC columns with PBS, 100 mL of distilled water and river water samples, fortified with 0.1µg/L ($4.5x10^{-11}$ mol) and 1.0 µg/L ($4.5x10^{-10}$ mol) 2,4-D, were passed through the columns at 0.2 mL/min. The columns were then washed with 10 mL distilled water to remove unbound materials, and eluted with 50% methanol/50% water (2 mL). The use of a lower percentage of methanol (30%) for

elution resulted in incomplete removal of the bound 2,4-D. Therefore, the amount of methanol was increased for effective elution of the bound analyte. With 50% methanol in the eluting solvent the recoveries were increased to an average of 92% (Table 2.4). These recovery data were determined by comparing the concentration obtained by IAC-HPLC with that obtained by SPE-HPLC of the fortified samples.

During the application of the water samples to the IAC columns, the waste water coming out of the column was collected to determine if breakthrough could occur using a 100 mL water sample. The waste water was adjusted to pH 2.2, and extracted by SPE for HPLC analysis. Evidently, at the levels of 2,4-D present in the samples nothing was detected in the waste water, suggesting no breakthrough. Theoretically, to obtain reproducible high recoveries, the amount of the sample loaded should be 100-fold lower than the capacity of the column (Hendrickson and Wilson, 1994). Apparently, the amount of 2,4-D loaded ($\leq 10^{-10}$ moles) is far from the capacity of the IAC columns prepared (10^{-7} moles IgG / 5 mL column).

One advantage of using the IAC over SPE for sample preparation was the substantial decrease in the co-extracted humic substances observed in the HPLC chromatograms. Figure 2.11 shows a comparison of the chromatograms of a spiked river water sample prepared by SPE (Fig. 2.11A) and IAC (Fig. 2.11B). Although the IAC did not completely eliminate the humic substances from the extract, the absorbances from the early eluting peaks were reduced several orders of magnitude in samples prepared by IAC (~250 mAU) compared to that by SPE (~2200 mAU). Consequently, the 2,4-D peak was more visible in samples prepared by IAC, whereas in samples prepared by SPE the peak was obscured by the high background absorbances resulting from the co-extracted materials. The results obtained may be

explained by the fact that in SPE, isolation is based on hydrophobic interactions, which are not selective. On the other hand, IAC takes advantage of the specificity and affinity of the antibodies toward the antigen, resulting in cleaner extracts.

Table 2.4. Recovery of 2,4-D from fortified water samples by IAC-HPLC.

Sample	Spiked concentration (μg/L)	%Recovered [a]
Distilled water	0.1	91±10
	1.0	92±8
Kansas river	0.1	88±14
	1.0	96±6

[a] Average of 2 samples

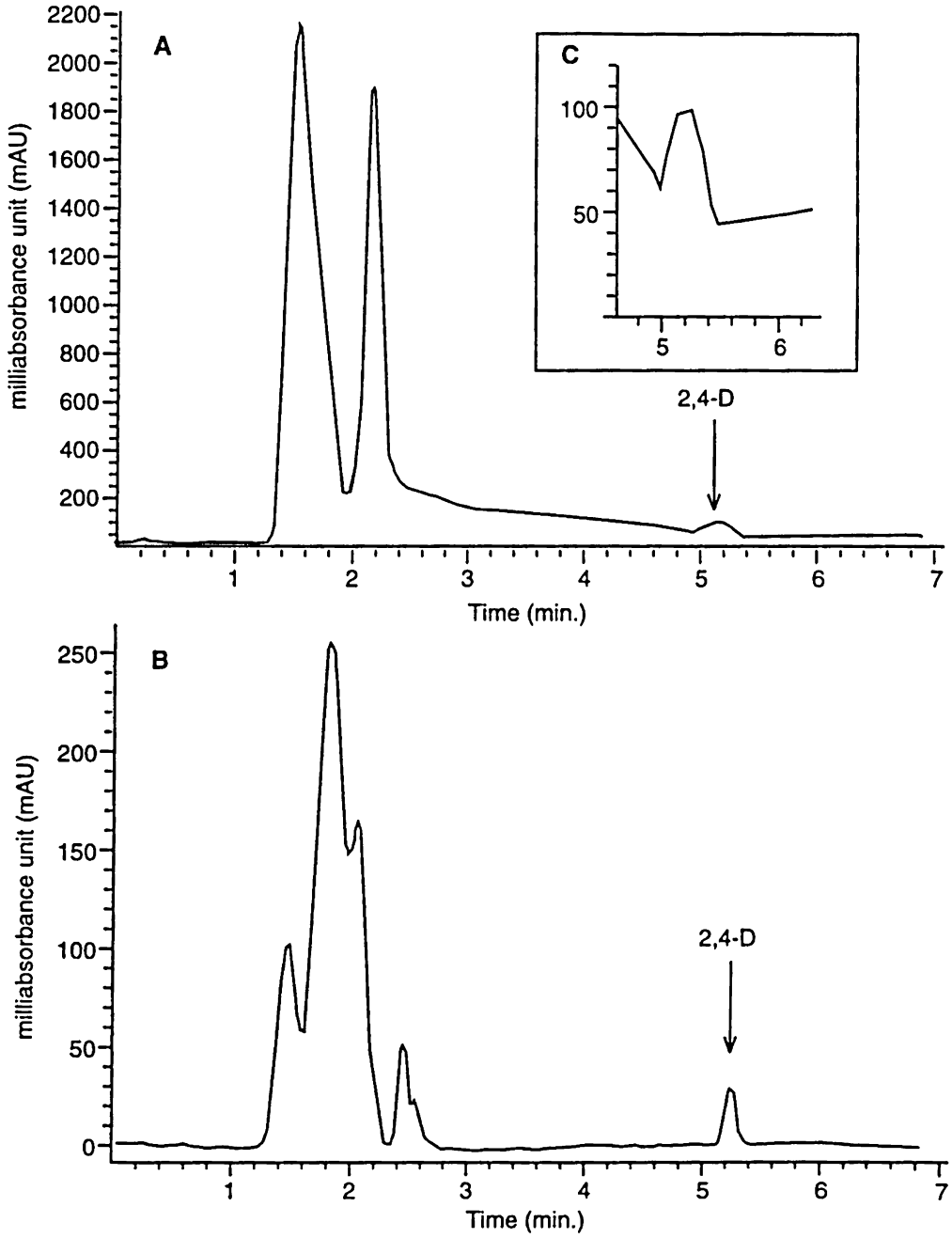


Figure 2.11. HPLC Chromatograms, with Photodiode-Array Detection (λ=202 nm), of river water sample (100 mL). The sample clean-up was (A) SPE and (B) IAC. Stationary phase: C_{18} ODS-Hypersil (100x4.6 mm, particle size 3µm, pore size 120 A); mobile-phase: 40/60 (v/v) methanol/10 mM phosphate buffer (pH 7.0); flow-rate 1.2 mL/min; temperature 40°C. Inset (C) is the 2,4-D peak of chromatogram (A) drawn with the same scale of chromatogram (B).

CONCLUSIONS

Polyclonal antibodies against 2,4-D were produced in rabbits using 2,4-D-PPD conjugates for immunization. The antiserum from Rabbit 1 has a moderately high affinity constant (2×10^{-9}) for 2,4-D and was useful for the development of a competitive ELISA suitable for screening dichloro-substituted phenoxyalkanoic acids. Although the antiserum from Rabbit 2 has a comparable affinity constant (4×10^{-9}), it was not useful for competitive ELISA. This is due to its high affinity to the linkage between the hapten and the enzyme label which prevents the binding of the free 2,4-D in solution to the immobilized antibody on the plate.

The use of the 2,4-DB-HRP conjugate resulted in a competitive ELISA with a higher sensitivity compared to the one which uses 2,4-D-HRP as labeled hapten. This may be attributed to the longer linker between the phenoxyaromatic ring and the enzyme label in 2,4-DB-HRP. The longer linker length reduces steric hindrance between the antibody and the analyte in solution. Since a portion of the polyclonal antibody population recognizes the linker, as evident in the cross-reactivity pattern, it is important not to use a spacer arm that is too long for the preparation of the labeled enzyme because this may eventually prevent binding of the free analyte. Conversely, steric hindrance must be minimized to improve the accessibility of the Fab fragment of the antibody for binding. The importance of making the binding site accessible for reaction was illustrated by using protein A to orient the antibodies coated on the microtiter plate which resulted in an increased assay sensitivity. Therefore, care must

be observed in choosing the structure of the labeled hapten to optimize the sensitivity of the assay.

Preliminary results demonstrated the potential of using IAC for preparing water samples for HPLC analysis. The use of antibodies for the isolation of 2,4-D from water resulted in a reduction of the co-extracted humic substances associated with SPE. Consequently, the HPLC chromatograms of river water samples prepared by IAC had lower baselines than those prepared by SPE. However, because IAC requires milligram amounts of antibodies, the long term supply of polyclonal antibodies is a restriction in the applicability of IAC for environmental analysis. This limitation may be circumvented if monoclonal antibodies are used for the development and optimizaion of IAC for sample preparation.

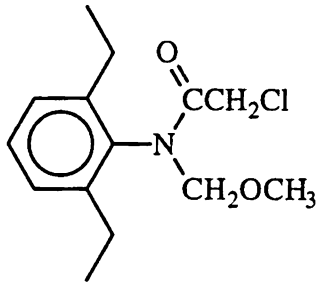
CHAPTER III

Integrating ELISA with Conventional Methods in the Study of the Fate and Transport of Chloroacetanilide Herbicides in the Field

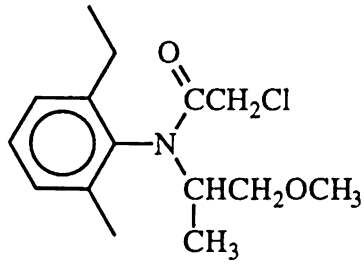
INTRODUCTION

The chloroacetanilide herbicides are among the most popular herbicides in the U.S. both for crop and noncrop use. Included in this class of herbicides are alachlor, metolachlor, propachlor, and acetochlor; all are selective herbicides used to control specific annual grasses and broadleaf weeds. Their chemical structures are shown in Figure 3.1. The basis for selective phytotoxicity of the chloroacetanilides is lack of metabolic deactivation in susceptible plants. Biochemical/physiological processes affected by the chloroacetanilides include protein and lipid synthesis, membrane phenomena, gibberellic acid-induced reactions, respiration, and cell elongation (Bucholtz and Lavy, 1978; Deal *et al.*, 1980; Marsh *et al.*, 1979; Narsaiah and Harvey 1977; Phillai *et al.*, 1979; Sloan and Camper, 1986; Warmund *et al.*, 1985; Wilkerson, 1982). These herbicides, which are not photosynthetic inhibitors, normally kill or affect susceptible weeds before emergence, without inhibiting seed germination. Table 3.1 lists some important physical properties of the four chloroacetanilides, together with their EPA classification and general use.

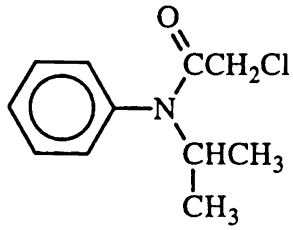
Propachlor has been registered for use in the U.S. since 1965, alachlor since 1969, and metolachlor since 1976. On the other hand, acetochlor has only been registered since 1994. The registration of acetochlor is dependent on several restrictions and conditions imposed by the USEPA in order to limit potential risks to humans and the environment. The conditional registration of acetochlor was granted by the USEPA with the objective of significantly reducing the total use of corn herbicides in the U.S. Acetochlor has a broader spectrum of activity and is applied at



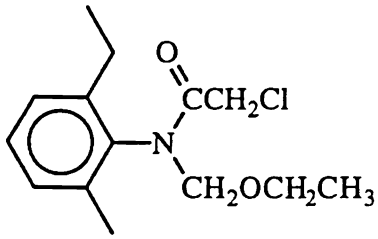
Alachlor
2-chloro-N-(2,6-diethylphenyl)-N-
(methoxymethyl)acetamide



Metolachlor
2-chloro-N-(2-ethyl-6-methylphenyl)-N-
(2-methoxy-1-methylethyl)acetamide



Propachlor
2-chloro-N-(1-methylethyl)-N-phenylacetamide



Acetochlor
2-chloro-N-(ethoxymethyl)-N-
(2-ethyl-6-methylphenyl)acetamide

Figure 3.1. Chemical structures of chloroacetanilides.

Table 3.1. Physical Properties and Use of Chloroacetanilide Herbicides ([a] Humburg *et al.*, 1989; [b] Nowell and Resek, 1994) .

Common Name of Herbicide	Solubility in Water [a]	Vapor Pressure [a]	EPA Carcinogenicity Classification [b]	General Use [a]
Alachlor	242 ppm at 25 °C	2.2×10^{-5} mm Hg at 25 °C	Group B2: Probable Human Carcinogen. (Sufficient evidence from animal studies to support causal association between exposure and cancer)	Control of annual grasses and certain broadleaf weeds and yellow nutsedge. Tolerant crops are corn, soybeans, grain sorghum, dry beans, peanuts, cotton, sunflowers, and certain ornamental and turf species.
Metolachlor	530 ppm at 20 °C	1.3×10^{-5} mm Hg at 20 °C	Group C: Possible Human Carcinogen. (Limited or equivocal evidence from animal studies and inadequate or no data in humans to support causal association between exposure and cancer)	Control of annual grasses and weeds, yellow nutsedge and certain broadleaf species in corn, cotton, nonbearing grapes, peanuts, pod crops, potatoes, safflowers, soybeans, tree nuts, and field and liner-grown wood ornamentals.
Propachlor	613 ppm at 25 °C	2.3×10^{-4} mm Hg at 25 °C	Group D: Not Classified. (There is inadequate or no human or animal evidence of carcinogenicity)	Control of most annual grasses and certain broadleaf weeds. Tolerant crops are corn, sorghum, soybeans, other legume crops, and onions.
Acetochlor	223 ppm at 25 °C	4.4×10^{-5} mm Hg at 25 °C	Group B2: Probable Human Carcinogen. (Sufficient evidence from animal studies to support causal association between exposure and cancer)	Control of annual grasses, broadleaf weeds in cabbage, citrus, coffee, green peas, maize, onion, orchards, soybeans, sugarbeet, sunflower, vineyards.

lower rates of application than the available alternatives; therefore, it is expected to substitute for many herbicides of concern, including alachlor, metolachlor, atrazine, cyanazine, and 2,4-D. An important condition for the registration of acetochlor is the completion of several prospective studies to show that acetochlor and its degradation products do not occur in surface- and ground-water.

Although there is extensive literature on field studies concerning the fate, degradation, and transport of the chloroacetanilides in various types of soil (Buhler *et al.*, 1993; Buttle, 1989; Nègre *et al.*, 1992; Patterson and Schnoor, 1992; Ritter, *et al.*, 1974; Smith and Parrish, 1993; Walker *et al.*, 1992; Wietersen *et al.*, 1993; Yen *et al.*, 1994; Zimdahl and Clark, 1982), a comparative study between alachlor, metolachlor, propachlor, and acetochlor has not yet been reported. The persistence and mobility of herbicides depend greatly on soil type, moisture, temperature, and soil microbial community. Therefore, a study on the relative rates of dissipation of chloroacetanilides under one field condition is timely and necessary.

A recent national well-water survey showed that alachlor does not leach readily to ground-water (Holden *et al.*, 1992). However, alachlor degrades to a more polar compound, alachlor ESA, which was found in ground-water in the Midwest where alachlor was applied. Alachlor ESA was detected in samples collected from rural private wells in the Midwest at concentrations ranging from 1.2 to 74 µg/L (Baker *et al.*, 1993; Kolpin *et al.*, 1993). This finding indicates that alachlor ESA is highly leachable to the ground-water. Although the USEPA's recent toxicological studies on alachlor ESA indicate that this metabolite is not mutagenic and does not bioaccumulate at the levels commonly found in surface and ground-water (USEPA, 1992), it is

important to investigate the fate of alachlor ESA in the environment because it is relatively persistent and mobile (Goolsby *et al.*, 1993c).

Metolachlor may behave in a similar fashion to alachlor because of its similarity in chemical structures. Both alachlor and metolachlor are detoxified rapidly by nonsensitive plants via conjugation with glutathione and/or homoglutathione (Breaux *et al.*, 1987; Lamoureux *et al.*, 1971). The glutathione conjugate of alachlor further degrades to the sulfonic-acid derivative as a major metabolite (Sharp, 1988). The sulfonic-acid derivative has also been reported as a major soil metabolite of acetochlor (Feng, 1991) and propachlor (Lamoureux and Rusness, 1989). However, the presence of a metolachlor sulfonic acid (metolachlor ESA) metabolite has not yet been observed either in soil, plants, nor in animals. Metolachlor ESA, if formed in the environment, may have a high potential for leaching to ground-water, as has been observed for alachlor ESA.

Due to public concern about pesticide contamination of ground-water, pesticide levels are monitored to satisfy drinking-water regulations. Nonetheless, metabolites of pesticides are not included in these monitoring programs and the importance of the fate and transport of metabolites has not been thoroughly investigated.

Herbicides dissipate from soil, but dissipation rates vary tremendously among compounds, soils, and climate. Dissipation studies under field conditions are important because the field is the real world of concern. Laboratory data help understand processes of herbicide dissipation in the field, but direct extrapolation to the field is not usually warranted. The concept of herbicide dissipation processes in soil is presented pictorially in Figure 3.2. Initially, the highest loss rates occur during

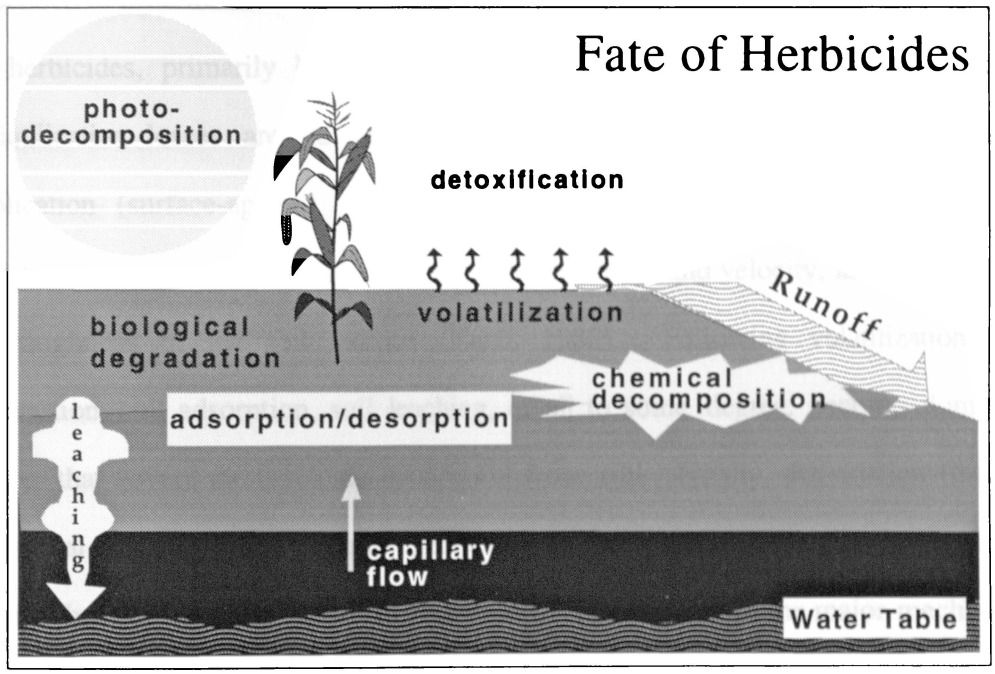


Figure 3.2. Processes influencing the fate of herbicides in the environment.

application (Seiber and Woodrow, 1983). Considerable engineering efforts have been expended to limit these losses. Second, volatilization loss apparently occurs for almost all herbicides, primarily because of their distribution over a large surface area. Volatilization losses are governed by the herbicide vapor pressure, method of application (surface-applied vs. soil-incorporated), formulation, temperature, soil moisture, soil organic matter content, relative humidity, wind velocity, and smoothness or roughness of the soil surface (Nash, 1988). Following volatilization, soil penetration, soil adsorption, soil leaching, or all to some degree, become dominant factors that govern the herbicide dissipation from soil. Finally, degradation (usually biological, but sometimes partially chemical) becomes paramount in the disappearance of most herbicides. Data supporting microbial decomposition as the major mechanism of alachlor and metolachlor disappearance have been presented (Chesters *et al.*, 1989).

Laboratory studies provide much information regarding degradation products and are the only convenient method for trapping and identifying volatile degradation compounds. Nevertheless, it is important to know the identity and concentration of degradation products actually formed in field soils following normal agricultural practices. Likewise, the mobility of herbicides can be measured in leaching experiments in the lab using soil columns. But studies of the movement of herbicides and their metabolites in soil under field conditions give useful direct information. In field dissipation studies, the soil must be sampled in layers and the concentration patterns must be completely measured in a sufficient number of replicates. Therefore, in this type of study many samples need to be analyzed. This is a perfect example where ELISA would be a valuable analytical tool because it could cost-effectively provide herbicide concentrations in a relatively large number of samples. In addition,

because of the short analysis time of ELISA, unwanted further degradation of the herbicides in soil samples during storage may be avoided. Availability of timely information of the herbicide concentrations may also prevent unnecessary extra sampling events.

The specific objectives of this study were (1) to determine the relative rates of dissipation of alachlor, metolachlor, and acetochlor, (2) to identify and confirm the formation of metolachlor ESA in soil under field conditions, (3) to determine the persistence and mobility of alachlor, metolachlor, and their sulfonic acid metabolites in soil using ELISA, GC/MS, and HPLC as complementary methods and (4) to demonstrate and evaluate the feasibility of using immunoassays in field dissipation study of herbicides.

EXPERIMENTAL METHODS

Field Dissipation Study

The field dissipation study was conducted in 1993 and 1994 at the Kansas State University, Kansas River Valley Experimental Field, located on the Kansas River alluvial flood plain, near Topeka, Kansas. In 1993, two adjacent plots were used; plot 1 was 4.6 x 30.5 m (0.0344 acre), while plot 2 was 4.6 x 18.3 m (0.0207 acre). In 1994, three new plots (east of the 1993 field plots) were used; all plots were 3.1 x 30.5 m (0.023 acre). The field plots used in this study have less than 1% slope and the water table was at a depth of approximately 5.5 m. The soil consisted of a Eudora silt-loam with a particle-size distribution of 44-62% silt, 26-50% sand, and 5-21% clay (Meyer, 1994). The pH of the soil was in the range of 6.8 to 7.8. Soil samples were submitted to Huffman Laboratories (Golden, CO) for analysis of the organic carbon content. The organic carbon contents of the soil were 0.45%, 0.22%, and 0.19%, at 15 cm, 45 cm, and 76 cm respectively.

A series of 10 suction lysimeters (Soil-Moisture Equipment Corp., Santa Barbara, CA) of 5 cm diameter were installed in the center of each plot at duplicate depths of 0.3, 0.6, 0.9, 1.2, and 1.8 m. Figure 3.3 illustrates installation and sampling of the lysimeters. To install the lysimeters, holes were cored to the desired depths. A small quantity of wet bentonite clay was poured in the holes to isolate the sampler from the soil below. Then, a small quantity of 200 mesh pure silica-sand was poured over the bentonite. The lysimeter was then inserted in the hole, followed by at least six inches of additional silica-sand around the porous ceramic cup of the soil water

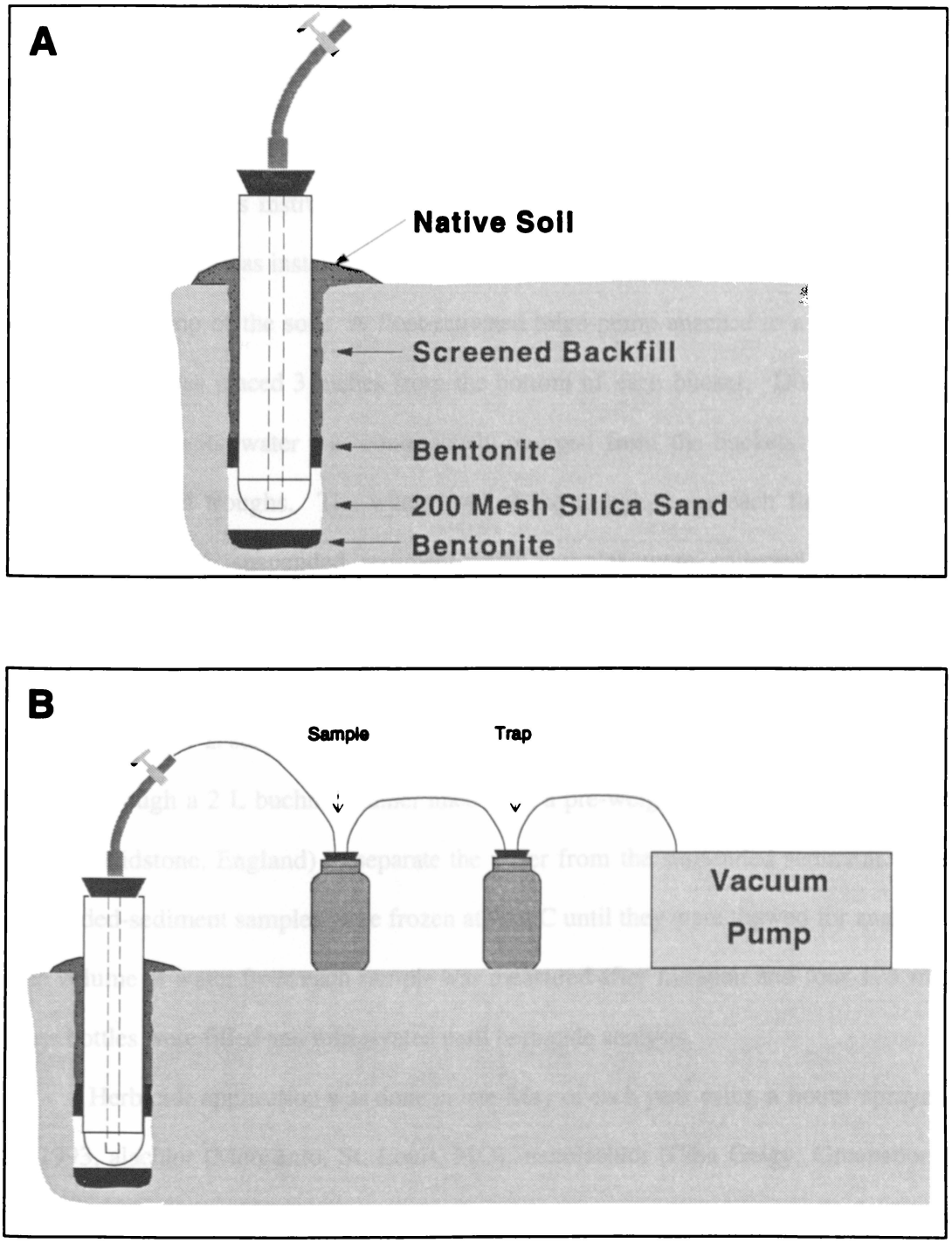


Figure 3.3. Installation of a suction lysimeter (A), and sampling of soil-pore water from the lysimeter (B).

sampler. Finally, a small quantity of bentonite was added as a plug to further isolate the ceramic cup and guard it against possible channeling of water down the outsides of the lysimeter. The remainder of the hole was backfilled with the native soil.

Each plot was instrumented with a surface-water runoff-collection system. A 19 L plastic bucket was installed at the downslope end of each plot so that the rim was even with the top of the soil. A float-activated bilge-pump attached to a battery-solar panel system was placed 3 inches from the bottom of each bucket. During surface-water runoff events, water was continuously pumped from the buckets into 1,875 L galvanized metal troughs. The water level of the trough from each field plot was recorded, and 4 L suspended sediment-water samples were collected to measure surface-water runoff losses from each plot. After each runoff event the buckets and troughs were drained and rinsed. Runoff was initiated by either rainfall or sprinkler irrigation. The 4 L suspended sediment-water samples collected from each plot were filtered through a 2 L buchner funnel lined with a pre-weighed, no. 2 Whatman filter paper (Maidstone, England) to separate the water from the suspended sediment. The suspended-sediment samples were frozen at -10 °C until they were thawed for analysis. The volume of water from each sample was measured after filtration and four 125 mL glass bottles were filled and refrigerated until herbicide analysis.

Herbicide application was done in late-May of each year using a boom sprayer. In 1993, alachlor (Monsanto, St. Louis, MO), metolachlor (Ciba Geigy, Greensboro, NC), and propachlor (Monsanto, St. Louis, MO), were applied together in duplicate plots (plots 1 and 2). In 1994, plot 1 was treated with alachlor and metolachlor, plot 2 was treated with metolachlor alone, and plot 3 was treated with acetochlor (Monsanto, St. Louis, MO). The herbicides were incorporated to a depth of 10 cm. After

incorporation, corn (*Zea mays* L.) was planted on each plot. The field lay-out is illustrated in Figure 3.4. A summary of the herbicide application rates is listed in Table 3.2, following the recommended rates of use for the particular purpose and soil type.

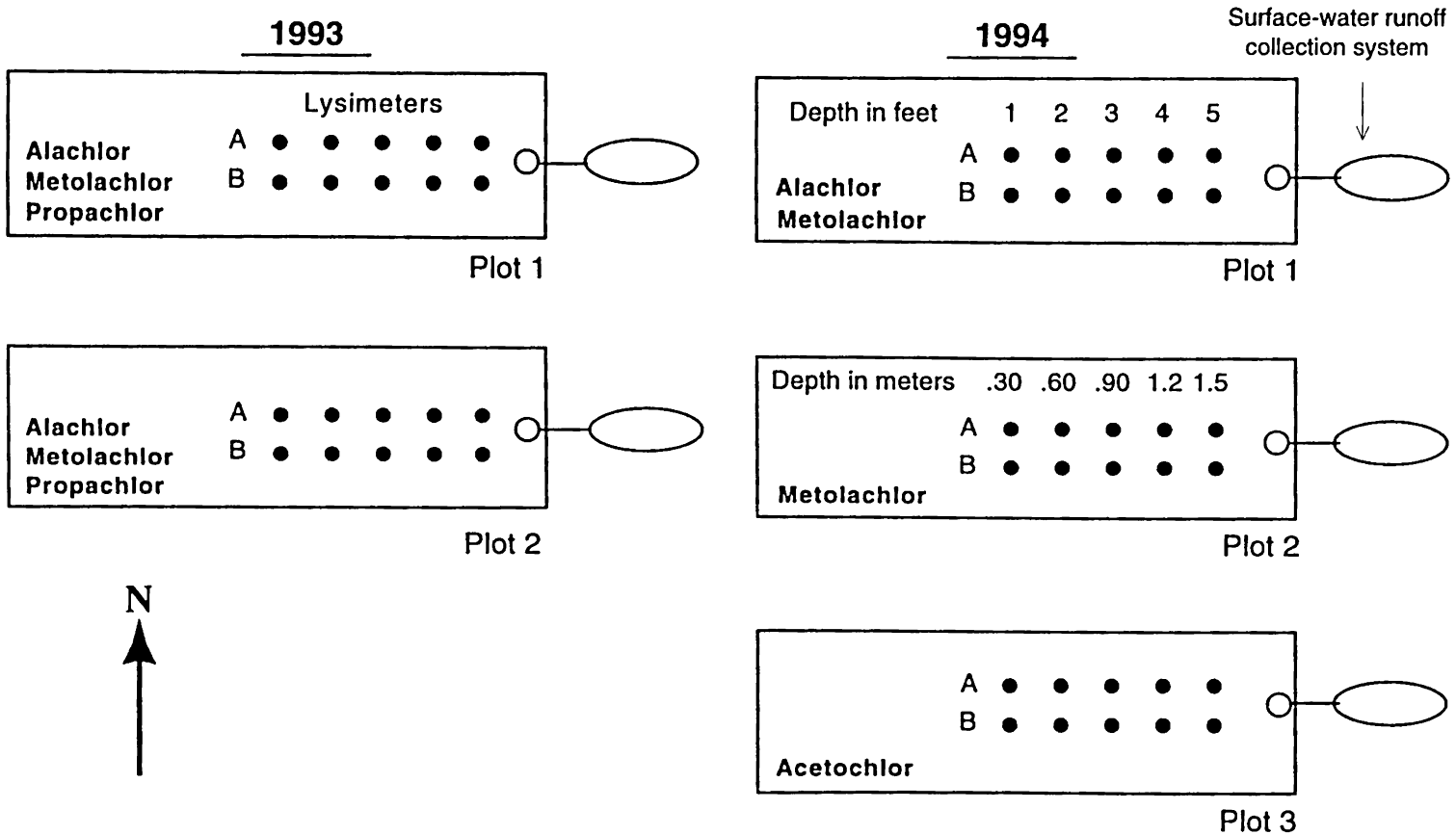


Figure 3.4. Design layout of the field plots for the chloroacetanilide dissipation study at the Topeka Experimental Farm.

Table 3.2. Formulation and Application Rates of the Chloroacetanilide Herbicides used for the Field Dissipation Study.

Trade Name	Active Ingredient	Formulation Description	Total Grams Applied	Total Pounds Applied	Plot Size (square meter)	Plot Size (acres)	Application Rate (lbs ai/acre)
Plot 1, 1993					139.4	0.0344	
Lasso MT	Alachlor	liquid	23.96 g	0.0528 lb			1.53
Dual	Metolachlor	liquid emulsifiable	23.96 g	0.0528 lb			1.53
Ramrod FL	Propachlor	flowable	35.95 g	0.0793 lb			2.30
Plot 2, 1993					83.6	0.0207	
Lasso MT	Alachlor	liquid	17.01 g	0.0375 lb			1.82
Dual	Metolachlor	liquid emulsifiable	16.97 g	0.0374 lb			1.81
Ramrod FL	Propachlor	flowable	25.50 g	0.0562 lb			2.72
Plot 1, 1994							
Lasso MT	Alachlor	liquid	23.96 g	0.0528 lb	92.9	0.0230	2.30
Dual II	Metolachlor	liquid emulsifiable	23.96 g	0.0528 lb			2.30
Plot 2, 1994							
Dual II	Metolachlor	liquid emulsifiable	23.96 g	0.0528 lb	92.9	0.0230	2.30
Plot 3, 1994							
Harness +	Acetochlor	liquid emulsifiable	17.97 g	0.0396 lb	92.9	0.0230	1.73

Soil cores of 30.5 to 152.4 cm depth from the surface were obtained using a 5 cm diameter, split-tube sampler (CME Co., St. Louis, MO), before and after herbicide application. Sampling of soil cores was continued at approximately two-week intervals. Each plot was sampled in both the east and west side. The soil cores were divided into 15 cm intervals, placed in polypropylene bags, and frozen at -10 °C until they were thawed for herbicide analysis.

Extraction Procedure for Soil

Approximately 15-20 g of soil was extracted in duplicate with 20 mL of a 75/25 (v/v) methanol/water mixture in a Teflon-lined, screw-capped test tube. This mixture was shaken to a slurry using a Vortex mixer (Daigger and Co. Inc., Wheeling, IL) and heated at 75 °C for 30 min. Then, the soil mixture was allowed to equilibrate and cool down to room temperature in a mechanical shaker for at least 1 hr. Each sample was then centrifuged and the clear supernatant was poured directly into a 40 mL vial. The extraction procedure was repeated on the same soil sample and the second supernatant was combined with the first. The combined extracts were evaporated at 50 °C using a Turbovap (Zymark, Palo Alto, CA) until only 10 mL of water remained. The concentrate was transferred to a test tube for automated solid-phase extraction using a C_{18} Sep-Pak cartridge (Waters, Milford, MA). The C_{18} cartridges were preconditioned sequentially with methanol (1 mL), ethyl acetate (1 mL), methanol (1 mL), and water (3 mL). Then the soil extracts were passed through the cartridge and eluted first with ethyl acetate (2.5 mL), followed by methanol (2.5 mL). This sequential elution separated the parent herbicides (eluted in ethyl acetate) from their

more polar ESA metabolites (eluted in methanol) as described in Chapter I. An aliquot (ca. 5 g) of each soil sample was weighed and dried to correct for the % moisture content of the soil.

ELISA Procedure for Alachlor and Metolachlor

The ethyl acetate fractions from the SPE of the soil extracts were analyzed for alachlor and metolachlor using ELISA obtained from Idetek/Quantix Systems (Sunnyvale, CA). First, the ethyl acetate was evaporated to dryness, and then reconstituted with 5 mL water. The manufacturer's recommended procedure was followed with some slight modification. Briefly, 200 µL of samples or standards and 50 µL of hapten-enzyme conjugate (Horseradish peroxidase-herbicide conjugate) were mixed in each well of the antibody-coated microtiter plate. The mixture was incubated at 30 °C in an orbital shaker (200 rpm) for 10 min. Then, the plate was rinsed five times with the wash solution (0.1 M PBS, pH 7.4) and excess water was removed. Two hundred (200) µL of color reagent (a mixture of hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine) was added and allowed to react for 10 min at room temperature. Then stop solution (50 µL, 1.5% sodium fluoride) was added. Optical densities were read at 650 nm on a Vmax microplate reader with Softmax software (Molecular Devices, Menlo Park, CA). Concentrations of the analytes were calculated using the 4-parameter-fit data reduction. Samples with herbicide concentrations exceeding the linear working range were diluted and reanalyzed.

Analysis of alachlor ESA from the methanol fractions was performed using ELISA and/or the HPLC method described in Chapter I. Metolachlor ESA was analyzed only by HPLC.

Analysis of Chloroacetanilide Herbicides by GC/MS

For the determination of the herbicide concentration in the water samples by GC/MS, 100 mL was used for SPE. For soil analysis by GC/MS, the soil extraction procedure described above was followed except that 250 ng of a surrogate compound (deuterated atrazine-D_5) was added to the soil prior to extraction. In addition, the ethyl acetate fraction of the soil extract was further passed through an ion-exchange SPE resin to reduce the amount of natural organic acids in the extract. This is an important step in the soil sample preparation for GC/MS analysis because these substances cause rapid degradation of the GC/MS column. The ethyl acetate fractions from the SPE elution, which contained the parent herbicides, were evaporated to about 50-75 μL for analysis by GC/MS in selected ion monitoring (SIM) mode.

A standard solution in ethyl acetate containing alachlor, metolachlor, propachlor, and acetochlor was analyzed by GC/MS under scan mode. A 12-m HP 1 (Hewlett-Packard, San Fernando, CA) cross-linked methyl siloxane column with 0.33 mm film thickness and 0.2 mm id was used. The chromatographic conditions were the same as that described in Chapter I. From the mass spectra of each compound, prominent ions that are characteristic of the particular herbicide were selected for the identification and quantitation of the herbicide under SIM mode. The ions selected for propachlor (Figure 3.5A) were the base peak, 120 amu for quantification, and 176 and 169 amu for identity confirmation. For acetochlor (Figure 3.5B), 223 amu (base peak) was chosen for

quantification, while 269 (molecular ion) was used for identity confirmation. For alachlor (figure 3.5C), 188 amu (base peak) was used for quantification, while 160 was used for confirmation. Lastly, the base peak of 162 amu was used for quantification of metolachlor (Figure 3.5D), while 238 and 240 amu were used for identity confirmation. In addition, confirmation was also based on a retention time match of ±.2% relative to the internal standard (deuterated phenanthrene-D_{10}) and on the correct ratios of the selected ions. Figure 3.6 is an example of the total ion chromatogram of the four chloroacetanilide herbicides.

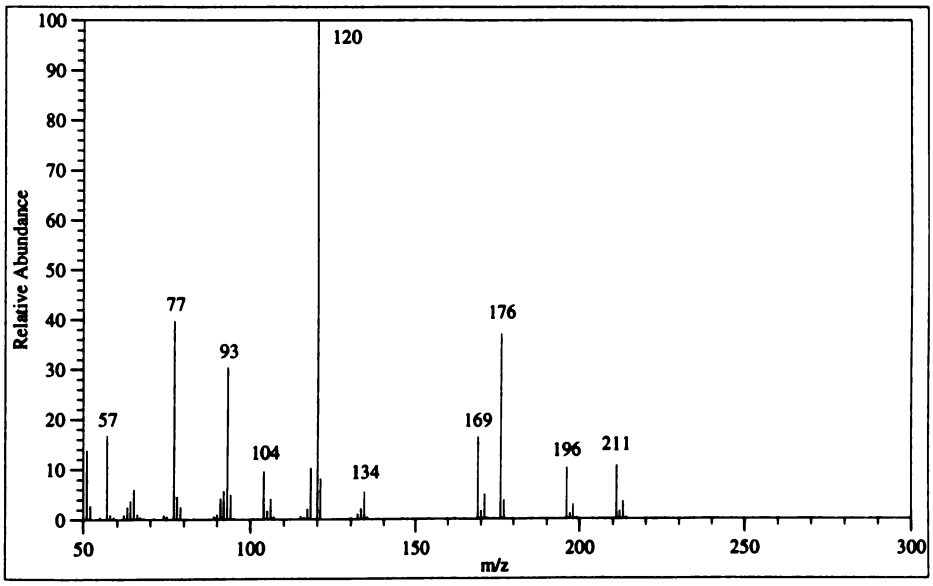


Figure 3.5A. Mass spectrum of propachlor.

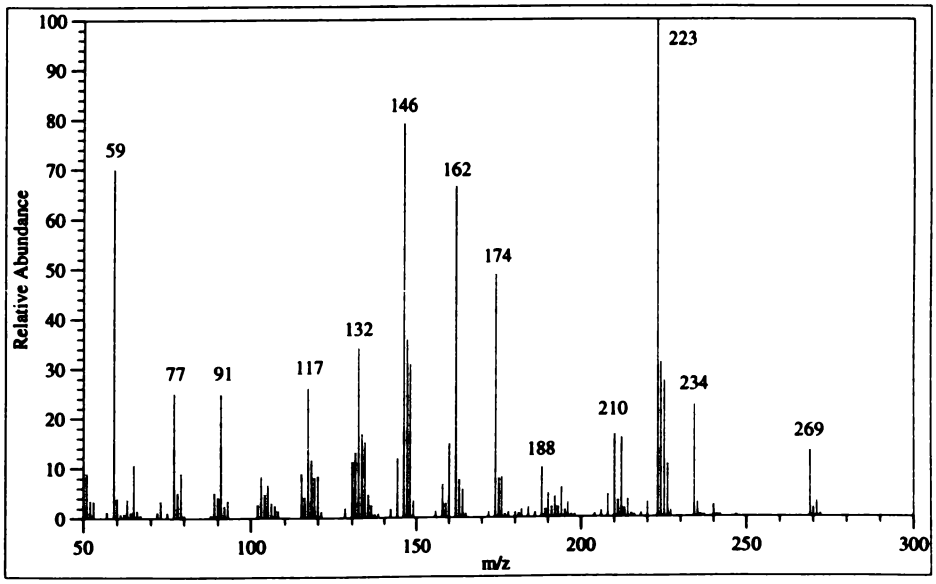


Figure 3.5B. Mass spectrum of acetochlor.

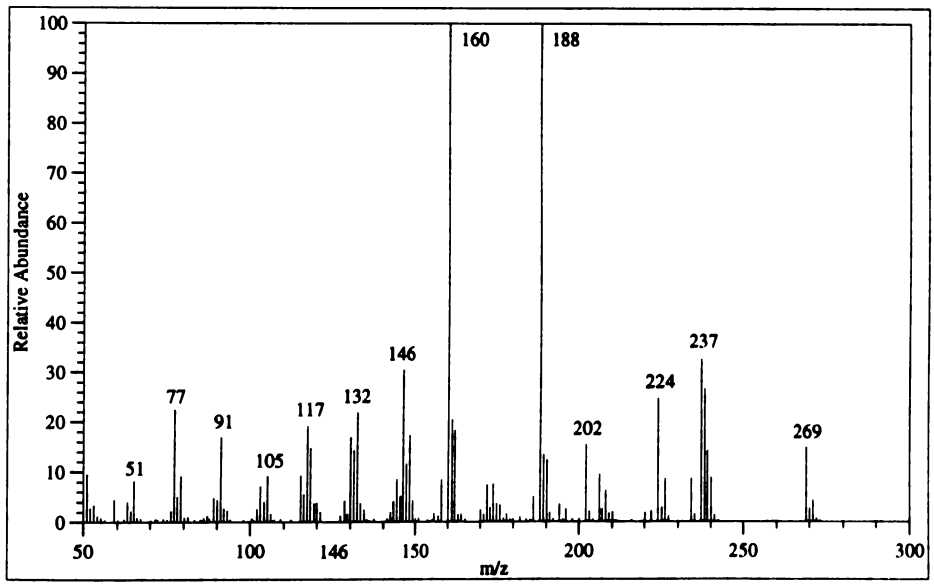


Figure 3.5C. Mass spectrum of alachlor.

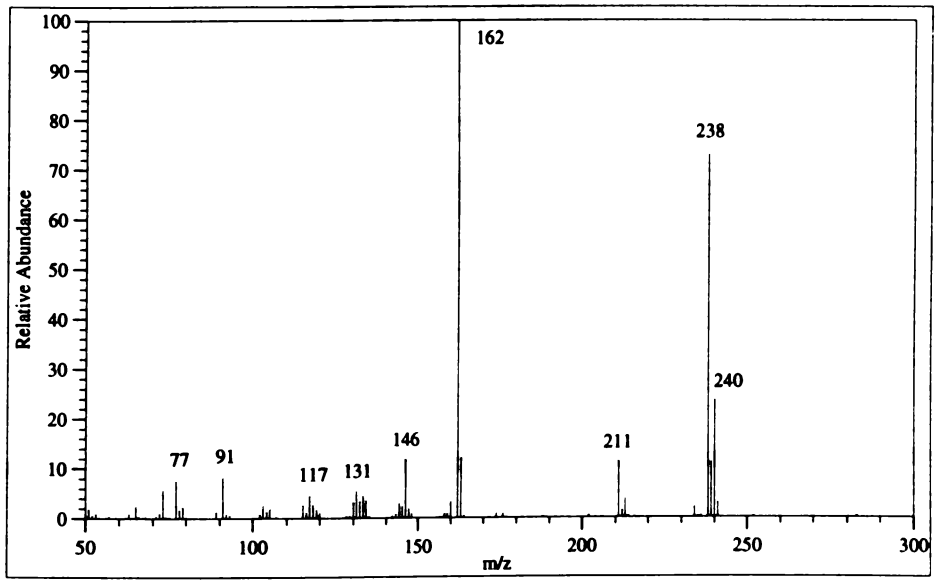
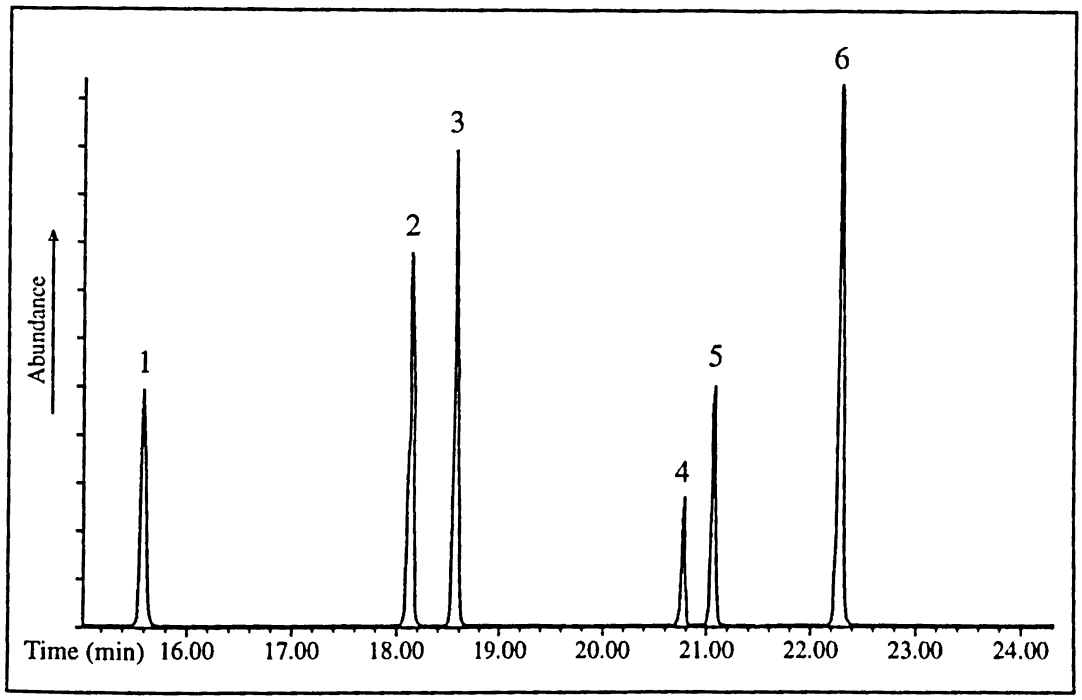


Figure 3.5D. Mass spectrum of metolachlor.



EXPLANATION

1. Propachlor
2. d5-Atrazine (surrogate standard)
3. d10-phenanthrene (internal standard)
4. Acetochlor
5. Alachlor
6. Metolachlor

Figure 3.6. GC/MS total ion chromatogram of the chloroacetanilide herbicides.

Synthesis of Alachlor and Metolachlor ESA

Alachlor ESA and metolachlor ESA were synthesized based on the procedure described by Feng (Feng, 1991). Either alachlor or metolachlor was refluxed with excess (10x more than the starting moles of the herbicide) sodium sulfite in 10% ethanol in water (100 mL) for 3 to 6 hrs or until the mixture became homogeneous. Following acidification with sulfuric acid, the product was extracted into methylene chloride. The methylene chloride was evaporated, and the reaction products were dissolved in hot ethanol. The hot ethanol mixture was filtered and allowed to stand undisturbed for recrystallization of the ESA. The white crystals that formed were collected and washed several times with cold ethanol. The chemical structures of the white crystals were confirmed as alachlor or metolachlor ESA by mass spectrometry.

Identification of Metolachlor ESA by FAB MS and FAB MS/MS

The soil extracts that showed positive for alachlor ESA by ELISA were combined and concentrated by SPE for mass spectral analysis. These samples, together with the synthesized alachlor ESA and metolachlor ESA standards, were submitted to the Mass Spectrometry Lab, Chemistry Department, University of Kansas. Analysis by negative-ion fast atom bombardment (FAB) was conducted on a Fisons/VG AUTOSPEC-Q tandem mass spectrometer (Fisons/VG Analytical Ltd, Manchester, UK). FAB experiments were performed using a cesium gun operated at 20 keV energy and 1 μA emission. Samples as methanol solutions were added to a glycerol matrix on the FAB probe. Exact mass FAB determinations were conducted at a 1:10,000 resolution using linear voltage scans. Homologous fatty acid standards served as bracketing calibration ions. FAB MS/MS was conducted using the linked

scan at a constant B/E technique. The collision gas was argon adjusted to a pressure that attenuated the precursor ion by 80%.

RESULTS AND DISCUSSION

Identification and Confirmation of Metolachlor ESA in Soil

Analysis of the combined soil extracts by negative-ion FAB MS showed the presence of the $(M-H)^-$ ions of alachlor ESA (m/z 314) and metolachlor ESA (m/z 328) (Figure 3.7). The mass spectra of the soil extracts were compared with the spectra of an authentic standard of alachlor ESA obtained from Monsanto Agricultural Co. and the metolachlor ESA synthesized in the laboratory. FAB MS/MS spectra of precursor ions (m/z 314 and m/z 328) derived from standard samples (Figures 3.8A and 3.9A) and soil extracts (Figures 3.8B and 3.8B) revealed characteristic fragment ions consistent with the structure of the alachlor and metolachlor sulfonic acids. Figure 3.10 is a structural interpretation of the fragmentation observed for the two sulfonic-acid metabolites. The product ions that are common to both metabolites are m/z 80, 94, and 121. Their source is indicated in figures 3.10A and 3.10C. A distinguishing ion unique for alachlor ESA is m/z 270, which represents the removal of the methoxymethyl group (Fig. 3.10B). On the other hand, m/z 256, which results from the analogous removal of the 2-methoxy-1-methylethyl group, is unique for metolachlor ESA (Fig. 3.10D). Soil extracts and standard-derived anions were subjected to exact mass determinations. The $(M-H)^-$ ions derived from both soil extracts and standard samples have the same mass units within experimental error and match the calculated formula mass of the sulfonic-acid metabolites (Table 3.3). The exact mass and MS/MS results indicate that both alachlor ESA and metolachlor ESA were present in the soil samples.

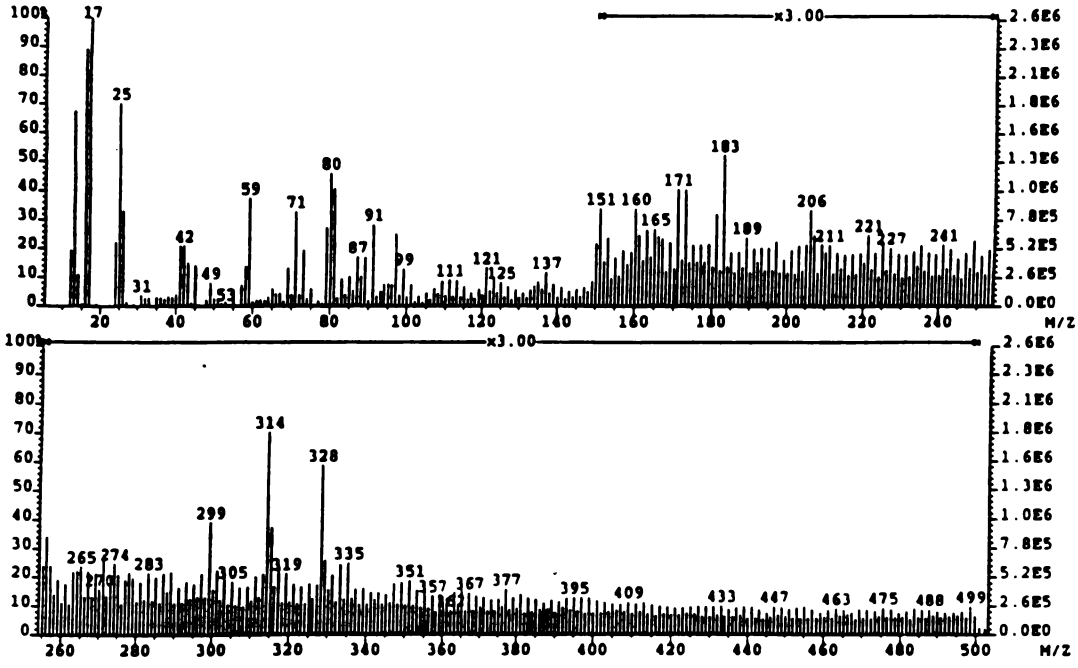


Figure 3.7. FAB MS fragmentation spectrum from soil sample extract.

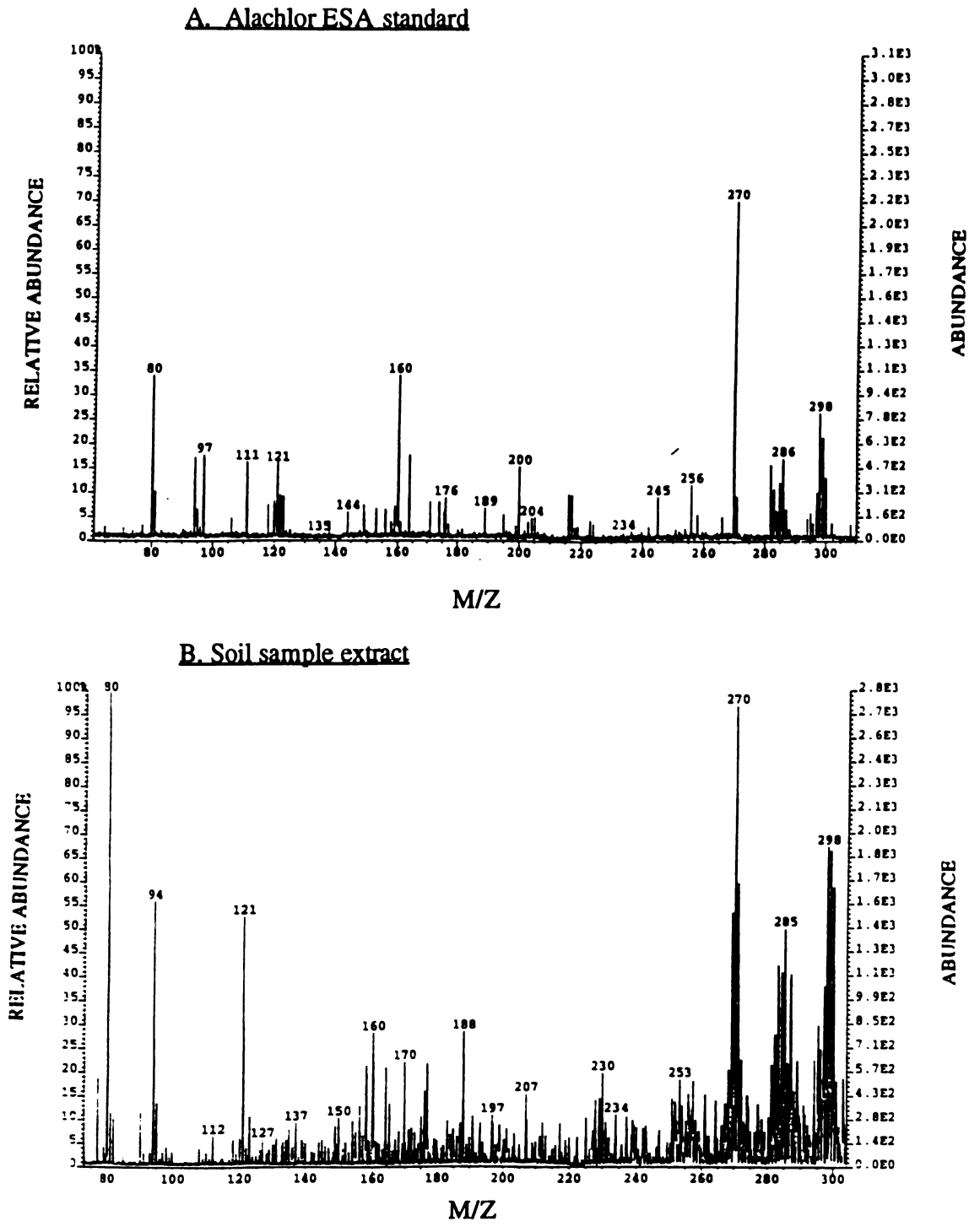


Figure 3.8. FAB MS/MS fragmentation spectrum of the negatively charged molecular ion 314 amu, from alachlor ESA standard (A), and soil-sample extract (B).

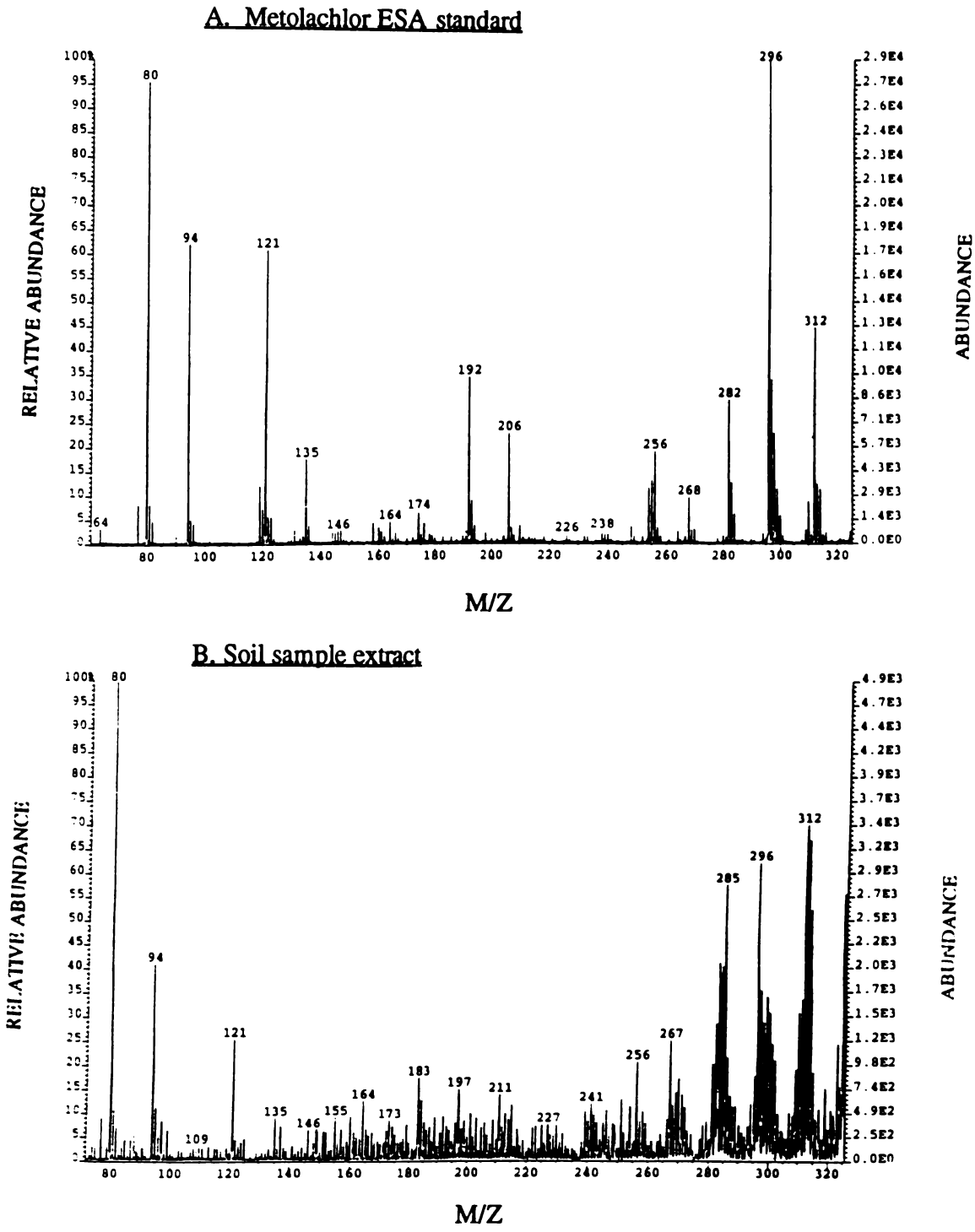
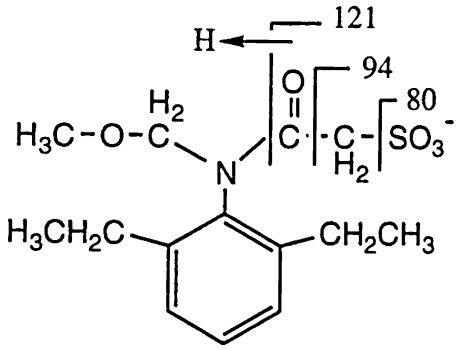
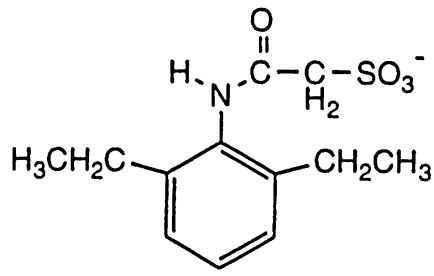


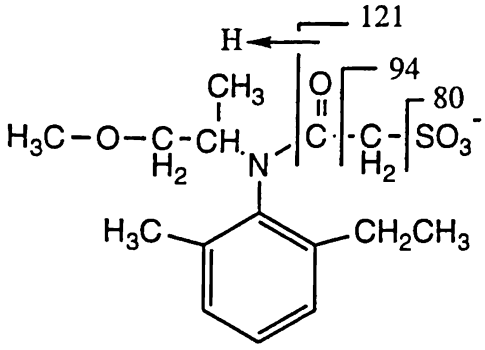
Figure 3.9. FAB MS/MS fragmentation spectrum of the negatively charged molecular ion 328 amu, from metolachlor ESA standard (A), and soil-sample extract (B).



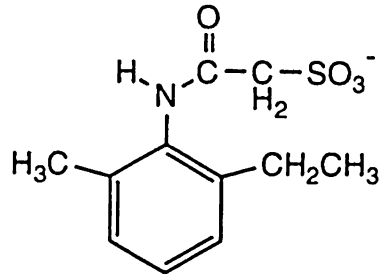
A. Alachlor sulfonic acid, molecular weight= 314



B. Negatively charged product ion = 270



C. Metolachlor sulfonic acid, molecular weight=328



D. Negatively charged product ion = 256

Figure 3.10. Structural interpretation of the fragmentation of alachlor and metolachlor sulfonic acids by FAB MS/MS.

Table 3.3. Negative-ion FAB exact mass determinations.

ion	314 amu	328 amu
formula	$C_{14}H_{20}NO_5S$	$C_{15}H_{22}NO_5S$
calculated mass	314.1062	328.1219
standard mass (*)	314.1060 +/-0.6	328.1222 +/-0.9
soil-sample mass (*)	314.1057+/-1.6	328.1234+/-4.6

(*) Mass measurement error with respect to the indicated formula, expressed as parts per million.

The evidence that metolachlor ESA is formed in soil is very important in several aspects. First, the formation of metolachlor ESA indicates that all chloroacetanilide herbicides follow a common metabolic pathway in soil. It is thought that conjugation with glutathione is a common mechanism utilized by living organisms to detoxify electrophilic xenobiotics such as pesticides, drugs, and other chemical pollutants (Lamoureuax and Bakke, 1984). It has been proposed (Feng, 1991; Lamoureux and Rusness, 1989), that glutathione conjugation is an initial pathway of acetochlor and propachlor metabolism in soil. The results of this research suggest that alachlor and metolachlor undergo the same glutathione conjugation as an initial metabolic pathway in soil. Also, the observed sulfonation of chloroacetanilides, including that of metolachlor, substantiates a previous hypothesis by Feng (1991) that conjugation of chloroacetanilide herbicides with glutathione is a common metabolic pathway shared by animals, plants, and soil microorganisms. An understanding of the formation and final metabolism of the sulfonic-acid metabolites of chloroacetanilide herbicides is necessary if glutathione conjugation is to be evaluated as a detoxification process or if the fate of pesticides is to be considered. Secondly, because the sulfonic-acid metabolites have high water solubility, they are expected to leach through the soil.

The cross-reactivity of the synthesized metolachlor ESA towards two commercially available ELISA tests for metolachlor produced no response up to the concentration tested (1000 µg/L). The design of the haptens and the method of conjugation to the carrier protein for antibody production are not available to the public. Therefore, no explanation could be made as to why metolachlor ESA is not detected by the metolachlor ELISA test, unlike alachlor ESA which has about 5-10% cross-reactivity towards the available alachlor ELISA tests. The result of the cross-reactivity

study conducted for metolachlor ESA explains why the metolachlor ELISA kits produced data which are highly correlated with GC/MS results (unpublished data).

Dissipation of Herbicides and their Metabolites in Surface Runoff

Dispersion of pesticide residues into the environment by surface runoff from agricultural lands has been a major concern for about the last 20 years. Runoff may be defined as water and any dissolved or suspended matter it contains that leaves a plot, field, or small single-cover watershed in surface drainage (Leonard, 1988). Specifically, "herbicide runoff" includes dissolved, suspended particulate, and sediment-adsorbed herbicide that is transported by water from a treated land surface.

Dissipation half-lives of herbicides may be estimated from their concentrations in the runoff water coming off the fields. Figures in 3.11 and 3.12 for example, depict the disappearance of the chloroacetanilide herbicides in the field plots during 1993 and 1994, respectively. In general, these herbicides decay exponentially and the disappearance may be interpreted using first-order kinetics. The equation for a first-order reaction is:

$$C = C_o e^{-kt} \qquad (1)$$

where C is the concentration after time t, C_o is the initial concentration, and k is the rate constant (Hurle and Walker, 1980). Thus a plot of the logarithm of concentration against time gives a straight line with a slope proportional to the rate constant. The value of the slope is taken as k and is used to calculate the half-life, $t_{1/2}$, which is the time taken for 50% dissipation. Therefore, equation (1) above becomes:

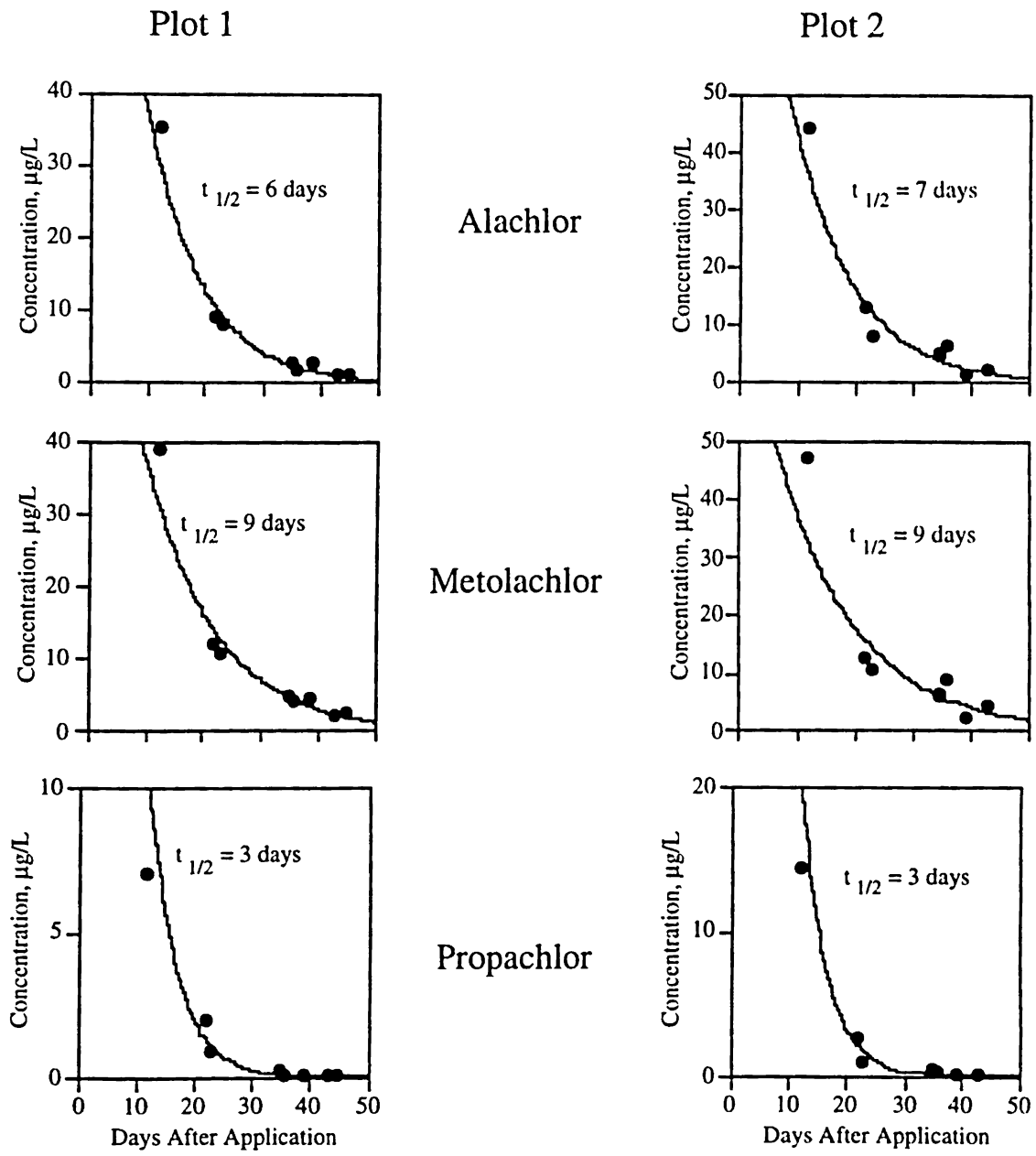


Figure 3.11. Dissipation half-lives ($t_{1/2}$) of chloroacetanilide herbicides in 1993 field plots.

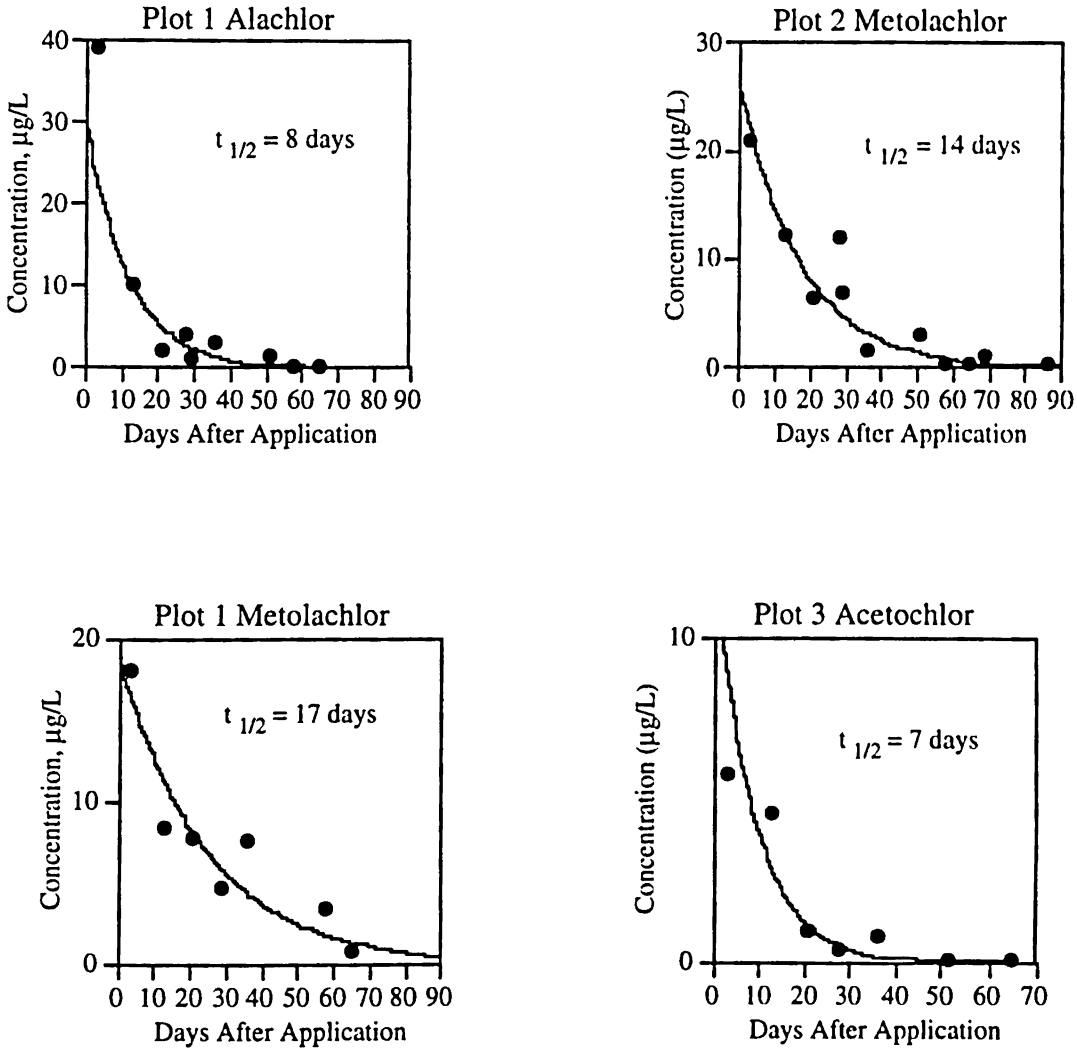


Figure 3.12. Dissipation half-lives ($t_{1/2}$) of chloroacetanilide herbicides in 1994 field plots.

$$t_{1/2} = \frac{0.6932}{k} \qquad (2)$$

It is obvious from equation (2) that the half-life in a first-order reaction is independent of the initial concentration. The half-life concept is a valuable tool in comparing rates of herbicide degradation, and because of the simplicity of first-order reaction kinetics, this rate law is widely used.

In the field dissipation study conducted in 1993, the half-lives of the herbicides applied were determined to be 6.5 days for alachlor, 9 days for metolachlor, and 3 days for propachlor (Figure 3.11). Results of the 1994 study showed slightly longer half-lives for alachlor (8 days) and particularly for metolachlor which increased to an average of 15.5 days (Figure 3.12). The newly registered herbicide, acetochlor, was evaluated in the 1994 study and resulted in a half-life of 7 days. The differences in the half-lives of alachlor and metolachlor between 1993 and 1994 may be attributed to the difference in the moisture conditions in the field during the two periods of study. The year 1993 was an extremely wet season with an average total monthly precipitation of 6.86 cm, compared to 4.22 cm in 1994, between the months of June and September. The frequency of rain events and the cumulative rain during the period of study are shown in Figure 3.13 for both years. The difference in the volume of precipitation is discernible. For example, on the 120th day after application, the cumulative rain was 71.37 cm and 41.58 cm, for 1993 and 1994, respectively.

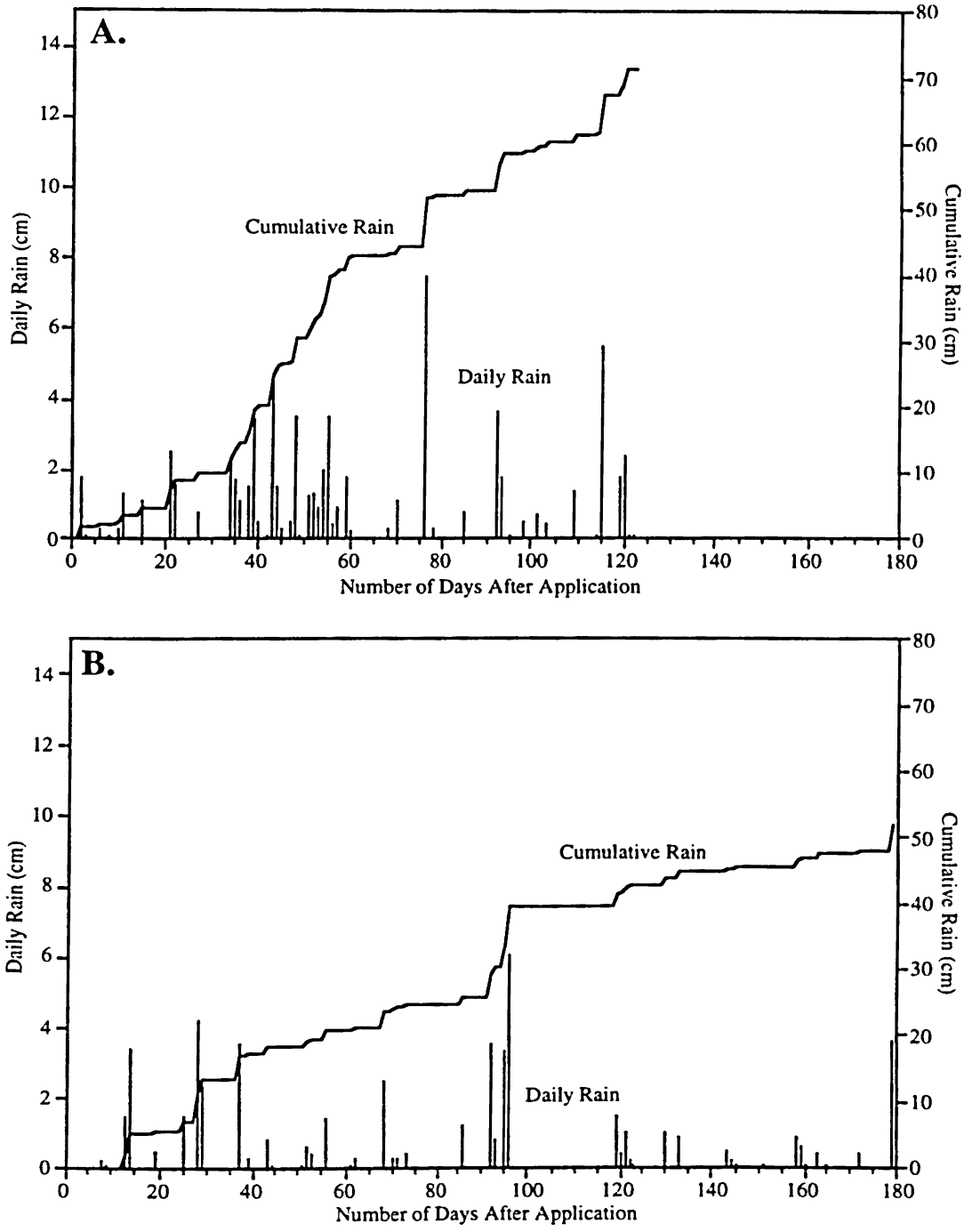


Figure 3.13. Cumulative and daily rainfall in 1993 (application date, May 27) (A) and 1994 (application date, May 25) (B) at the agricultural research station during the field dissipation study season.

The two environmental variables which have the greatest effect on biodegradation by controlling microbial activity in general are temperature and moisture content (Gerstl, 1991). It has been shown that in general the rate of herbicide degradation increased with increasing temperature within the range of mesophilic microbial activity (Hamaker, 1972). Water is required for microbial activity and controls the oxygen level in soil. Because the average monthly temperature was comparable during 1993 and 1994, ranging from 22 to 26 °C, the moisture content of the soil played a more important role in causing the difference in half-lives of alachlor and metolachlor. The result of this study agrees with previous reports showing a more favorable degradation of alachlor in soil with higher moisture content (Nègre *et al.*, 1992; Walker *et al.*, 1992).

The relative persistence of the chloroacetanilides in surface water runoff can be arranged in an increasing order: propachlor << alachlor, acetochlor < metolachlor. It is not surprising that acetochlor and alachlor have comparable half-lives because of the similarity in their chemical structure and physical properties. The very short half-life of propachlor may be attributed to the simplicity of its chemical structure (no alkyl substituents in the aromatic ring), hence, making it more easily degraded. Metolachlor on the other hand, has more carbon in the structure and has a higher solubility than either alachlor or acetochlor which may influence its adsorption and behavior in soil.

A number of pesticides are preferentially transported adsorbed to entrained sediments (Leonard, 1988). Sediment pesticide transport may be described using the adsorption partition coefficient (K_d), which is the ratio of the concentration of the pesticide in soil to that in water. Since soil organic matter is the primary soil

constituent responsible for adsorption of nonionic organic compounds, the sorption constant (K_{oc}) may be based on only the organic matter present such that :

$$K_{oc} = \frac{K_d}{f_{oc}} \quad (3)$$

where f_{oc} is the fraction of organic carbon present in the soil or sediment. A sorption constant expressed in this manner is dependent only on the pesticide and is independent of soil type (Leonard, 1988).

The K_{oc} for chloroacetanilides and the two ESA metabolites were determined by batch equilibrium experiments. In this procedure, soil samples that do not contain the herbicide in question were fortified with the specific compound at different concentrations. The samples were equilibrated with water (1:1 ratio between soil and water, v:v) for 5 days with constant shaking. After equilibration the concentration of the herbicide in the water layer was determined. The concentration of the herbicide adsorbed in the soil can then be calculated from the amount of the herbicide used for fortification and the amount dissolved in the water layer. From this experiment and the results of the organic carbon analysis, the K_{oc} of the herbicides and the two ESA metabolites were obtained and are listed in Table 3.4. The two ESA metabolites have comparable K_{oc}, 184 and 194 for alachlor ESA and metolachlor ESA, respectively. Alachlor (K_{oc} = 2154), propachlor (K_{oc}= 2218), and acetochlor (K_{oc} = 2031), have very similar K_{oc} , whereas the value for metolachlor (K_{oc} = 1018) was half of those for the other chloroacetanilide herbicides.

Table 3.4. Determination of K_{oc} for chloroacetanilide herbicides and ESA metabolites.

	[a] K_{oc}		Average K_{oc}
	Depth of the soil sample used		
	0.0-0.15 cm (% OC= 0.45)	1.0-1.5 cm (% OC = 0.22)	
alachlor	2222	2085	2154
metolachlor	945	1091	1018
propachlor	2327	2110	2218
acetochlor	2295	1769	2031
alachlor ESA	178	191	184
metolachlor ESA	160	227	194

[a] average of 3 determinations; % OC = % organic carbon

The values of the K_{oc} for the herbicides under investigation suggest that alachlor, propachlor, and acetochlor are preferentially transported adsorbed to entrained sediments relative to metolachlor. Adsorbed pesticide contributes to runoff as sediment-transported pesticide and by pesticide desorption into the flowing water from either moving or stationary soil particles. It is not surprising that the values of the K_{oc} for the ESA metabolites are much lower than that of the parent herbicides because of the ionic nature of the former. The ESA metabolites may therefore be expected to have higher mobility and leaching potential in soil.

During the 1993 study, an exceptionally wet spring was experienced, with above-average rainfalls in June through August. These resulted in a large number of early season runoff events, many of which exceeded the capacity of the runoff collecting barrels used on the runoff plots. Thus, it was not possible to determine sediment loads for many of the individual storms. Therefore, only the sediments from the 1994 study were analyzed for alachlor and metolachlor.

Tables 3.5 to 3.13 show that herbicides leaving a treated area by runoff constitute only a small percentage of that applied (less than 0.5%), since most of the applied herbicide is dissipated by other processes. Generally, the herbicide concentrations were higher in the sediment than in the runoff water. However, greater total losses were associated with the greater volume of water. It must be noted though, that runoff samples were separated into water and sediment fractions several days after sampling. If any movement from the adsorbed to the dissolved phase did occur following sampling, this would mean that the concentrations of the herbicide on the

sediment are underestimates, and sediment transport is of even greater importance in herbicide loss than has been suggested here.

It can be seen in Tables 3.9 and 3.11 that the ratios of the herbicide concentration in the sediment to that in runoff water are much lower for metolachlor than for alachlor, indicating that soil sorption is more important for alachlor than for metolachlor. This is consistent with the results of the K_{oc} determination in the laboratory. The difference can be explained by the higher water solubility of metolachlor (530 ppm) compared to alachlor (242 ppm), resulting in a lower K_{oc} for the former. The longer half-life of metolachlor combined with its lower K_{oc} could explain why metolachlor is more frequently detected in surface water than alachlor (Goolsby and Battaglin, 1993a) despite the more extensive use of the latter.

The runoff data for the ESA metabolites indicate that alachlor ESA is present in the runoff water at a relatively higher concentration than metolachlor ESA even though the application rates for the parent herbicides were the same. In the 1993 study, only alachlor ESA was determined because the metolachlor ESA standard was not yet available. Figure 3.14 shows that the concentration of alachlor ESA in the runoff water reached a maximum of 48 µg/L at approximately four weeks after application. During this time the concentration of the parent herbicide was less than 5 µg/L in the runoff water.

In the 1994 study, the concentrations of alachlor ESA in the runoff were significantly lower than in 1993. Again, this can be attributed to the more frequent rainfall during the former year. Runoff begins when rainfall rates exceed infiltration rates. During the period of 1994 study, the rain was less frequent and lower in volume.

Therefore, the soluble metabolites were more likely to have been moved into the subsurface soil by infiltrating water before runoff began. Hence, there was little amount available for runoff. A comparison of the relative amounts of ESA metabolites in the runoff showed lower concentrations of metolachlor ESA than alachlor ESA (Figure 3.15). This is consistent with the longer half-life of metolachlor compared to alachlor. Moreover, leaching may be a more important dissipation process for metolachlor than microbial degradation because of its high water solubility. This hypothesis was investigated in this study and the results will be discussed below.

Table 3.5. Concentration and mass of alachlor in the runoff from each field plot during the 1993 field dissipation study.

Event Date	Days After Application	Concentration in Water (µg/L)	Total Runoff in Water (L)	Mass in Water (µg)	Percent of Alachlor in Runoff (%)
Plot 1 1993					
6/8/93	11	35.14	120	4209	0.018
6/18/93	21	8.88	143	1272	0.005
6/19/93	22	7.90	378	2984	0.012
7/1/93	34	2.37	495	1174	0.005
7/2/95	35	1.74	NA	NA	NA
7/5/93	39	2.58	>1875	>4821	>0.020
7/9/93	43	0.73	NA	NA	NA
7/11/93	45	0.94	26	25	0.000
					Total: >0.222
Plot 2 1993					
6/8/93	11	43.63	183	7952	0.047
6/18/93	21	12.71	143	1820	0.011
6/19/93	22	7.90	456	3602	0.021
7/1/93	34	4.54	469	2128	0.013
7/2/93	35	6.15	NA	NA	NA
7/5/93	39	0.97	>1875	>163	>0.001
7/9/93	43	2.05	NA	NA	NA
					Total: >0.105

Table 3.6. Concentration and mass of alachlor ESA in the runoff from each field plot during the 1993 field study.

Event Date	Days After Application	Concentration in Water (µg/L)	Total Runoff in Water (L)	Mass in Water (µg)	Percent of Alachlor ESA in Runoff (%)
Plot 1 1993					
6/8/93	11	24.20	120	2899	0.012
6/18/93	21	6.30	143	902	0.004
6/19/93	22	7.15	378	2699	0.011
7/1/93	34	48.84	495	24162	0.101
7/2/95	35	5.95	NA	NA	NA
7/5/93	39	2.43	>1875	>4556	>0.019
7/9/93	43	4.30	NA	NA	NA
7/11/93	45	3.73	26	97	0.000
					Total: >0.147
Plot 2 1993					
6/8/93	11	18.10	183	3299	0.019
6/18/93	21	6.30	143	902	0.005
6/19/93	22	5.65	456	2575	0.015
7/1/93	34	48.25	469	22612	0.133
7/2/93	35	59.66	NA	NA	NA
7/5/93	39	2.28	>1875	>4274	>0.025
7/9/93	43	4.25	NA	NA	NA
					Total: >0.330

Table 3.7. Concentration and mass of metolachlor in the runoff from each field plot during the 1993 field dissipation study.

Event Date	Days After Application	Concentration in Water (µg/L)	Total Runoff in Water (L)	Mass in Water (µg)	Percent of Metolachlor in Runoff (%)
Plot 1 1993					
6/8/93	11	38.74	120	4640	0.019
6/18/93	21	11.95	143	1711	0.007
6/19/93	22	10.62	378	4011	0.017
7/1/93	34	4.68	495	2315	0.010
7/2/95	35	3.93	NA	NA	NA
7/5/93	39	4.31	>1875	>8080	>0.034
7/9/93	43	2.17	NA	NA	NA
7/11/93	45	2.23	26	58	0.000
					Total: >0.087
Plot 2 1993					
6/8/93	11	46.93	183	8554	0.050
6/18/93	21	12.43	143	1780	0.010
6/19/93	22	10.62	456	4841	0.028
7/1/93	34	5.83	469	2731	0.016
7/2/93	35	8.69	NA	NA	NA
7/5/93	39	2.29	>1875	>4299	>0.025
7/9/93	43	4.22	NA	NA	NA
					Total: >0.145

Table 3.8. Concentration and mass of propachlor in the runoff from each field plot during the 1993 field dissipation study.

Event Date	Days After Application	Concentration in Water (µg/L)	Total Runoff in Water (L)	Mass in Water (µg)	Percent of Propachlor in Runoff (%)
Plot 1 1993					
6/8/93	11	6.99	120	837	0.002
6/18/93	21	1.93	143	277	0.001
6/19/93	22	0.90	378	339	0.001
7/1/93	34	0.15	495	75	0.000
7/2/95	35	0.06	NA	NA	NA
7/5/93	39	0.05	>1875	>94	>0.000
7/9/93	43	nd	NA	nd	NA
7/11/93	45	nd	26	nd	0.000
					Total: >0.004
Plot 2 1993					
6/8/93	11	14.30	183	2607	0.010
6/18/93	21	2.49	143	357	0.001
6/19/93	22	0.90	456	409	0.002
7/1/93	34	0.32	469	148	0.001
7/2/93	35	0.28	NA	NA	NA
7/5/93	39	nd	>1875	nd	0.000
7/9/93	43	nd	NA	nd	0.000
					Total: >0.145

Table 3.9. Concentration and mass of alachlor in the runoff from plot 1 during the 1994 field dissipation study.

Event Date	Days After Application	Concentration of Alachlor Water (µg/L)	Concentration of Alachlor Sediment (µg/kg)	Total Runoff (L)	Total Sediment (g)	Mass of Alachlor (µg) Water	Mass of Alachlor (µg) Sediment	Mass of Alachlor (µg) Total	Percent of Alachlor in Runoff (%)
Plot 1 1994									
5/28/94	3	39.11	4718.11	143	855	5601	4033	9634	0.040
6/8/94	14	9.94	453.30	65	278	647	126	773	0.003
6/15/94	21	2.07	80.59	260	NA	540	NA	>540	>0.002
6/22/94	28	3.89	NA	260	NA	1013	NA	>1013	>0.004
6/23/94	29	0.88	255.98	495	2479	435	634	1069	0.004
6/30/94	36	2.70	39.39	182	450	491	18	509	0.002
7/16/94	52	1.24	52.70	< 20	190	25	10	35	0.000
7/22/94	58	0.09	365.80	495	131	45	48	93	0.000
7/29/94	65	0.12	86.98	< 20	35	2	3	5	0.000
									Total: >0.055

Table 3.10. Concentration and mass of alachlor ESA in the runoff from plot 1 during the 1994 field dissipation study.

Event Date	Days After Application	Concentration in Water (µg/L)	Total Runoff in Water (L)	Mass in Water (µg)	Percent of Alachlor ESA in Runoff Water (%)
Plot 1 1994					
6/8/94	14	0.42	65	27	0.000
6/22/94	28	2.51	260	653	0.003
6/23/94	29	0.71	495	351	0.001
6/30/94	36	2.47	182	40	0.002
7/16/94	52	2.32	<20	<46	0.000
7/22/94	58	1.44	495	713	0.003
8/1/94	68	2.70	<20	<54	0.000
8/19/94	86	1.59	<20	<32	0.000

Table 3.11. Concentration and mass of metolachlor in the runoff from each field plot during the 1994 field dissipation study.

Event Date	Days After Application	Concentration of Metolachlor Water (µg/L)	Concentration of Metolachlor Sediment (µg/kg)	Total Runoff (L)	Total Sediment (g)	Mass of Metolachlor (µg) Water	Mass of Metolachlor (µg) Sediment	Mass of Metolachlor (µg) Total	Percent of Metolachlor in Runoff (%)
Plot 1 1994									
5/28/94	3	18.04	127.17	143	855	2584	109	2692	0.011
6/8/94	14	8.33	54.18	65	278	542	15	557	0.002
6/15/94	21	7.69	NA	260	NA	2003	NA	>2003	>0.008
6/23/94	29	4.50	56.17	495	2479	2228	139	2367	0.010
6/30/94	36	7.45	11.01	182	450	1358	5	1363	0.006
7/22/94	58	3.24	68.48	495	131	1604	9	1613	0.007
7/29/94	65	0.69	5.69	<20	35	14	<0	14	>0.000
									Total: >0.055
Plot 2 1994									
5/28/94	3	20.89	89.88	149	486	3133	44	2692	0.011
6/8/94	14	12.08	202.37	26	264	315	53	557	0.002
6/15/94	21	6.34	158.83	365	NA	2311	32	>2003	>0.008
6/22/94	28	11.95	623.20	52	204	622	85	2367	0.010
6/23/94	29	6.63	43.95	482	136	133	73	2367	0.010
6/30/94	36	1.58	74.17	130	1672	206	53	1363	0.006
7/22/94	58	0.26	15.18	339	719	54	5	1613	0.007
8/1//94	68	1.00	16.77	<20	356	20	<3	1613	0.007
8/19/94	86	0.29	8.55	<20	<177	6	<2	14	>0.000
									Total: >0.055

Table 3.12. Concentration and mass of metolachlor ESA in the runoff from each field plot in the 1994 field dissipation study.

Event Date	Days After Application	Concentration in Water (µg/L)	Total Runoff in Water (L)	Mass in Water (µg)	Percent of Metolachlor ESA in Runoff Water (%)
Plot 1 1994					
6/8/94	14	0.05	65	3	<0.001
6/22/94	28	0.56	260	146	0.001
6/23/94	29	nd	495	nd	<0.001
6/30/94	36	0.22	182	40	<0.001
7/16/94	52	0.50	<20	<10	<0.001
7/22/94	58	0.05	495	25	<0.001
8/1/94	68	0.38	<20	<8	<0.001
8/19/94	86	0.34	<20	<7	<0.001
Plot 2 1994					
5/28/94	3	0.26	149	39	<0.001
6/8/94	14	0.52	26	14	<0.001
6/16/94	21	0.26	365	95	<0.001
6/22/94	28	0.94	52	49	<0.001
6/23/94	29	0.60	482	289	0.001
6/30/94	36	0.52	130	68	<0.001
7/16/94	52	0.38	<20	<8	<0.001
7/22/94	58	0.38	338	128	0.001
7/29/94	65	0.36	312	112	<0.001
8/1/94	68	1.26	<20	<25	<0.001
8/19/94	86	0.22	<20	<4	<0.001

Table 3.13. Concentration and mass of acetochlor in the runoff from plot 3 during the 1994 field dissipation study.

Event Date	Days After Application	Concentration in Water (µg/L)	Total Runoff in Water (L)	Mass in Water (µg)	Percent of Acetochlor in Runoff (%)
Plot 3 1994					
5/28/94	3	5.78	247	1430	0.008
6/8/94	14	4.55	299	1362	0.008
6/15/94	21	0.92	130	120	0.001
6/22/94	28	0.33	182	60	0.017
6/23/94	29	nd	469	nd	0.000
6/30/94	36	0.71	885	626	0.000
7/16/94	52	nd	<20	nd	0.000
7/22/94	58	nd	338	nd	0.000
7/29/94	65	nd	312	nd	0.000
					Total: 0.020

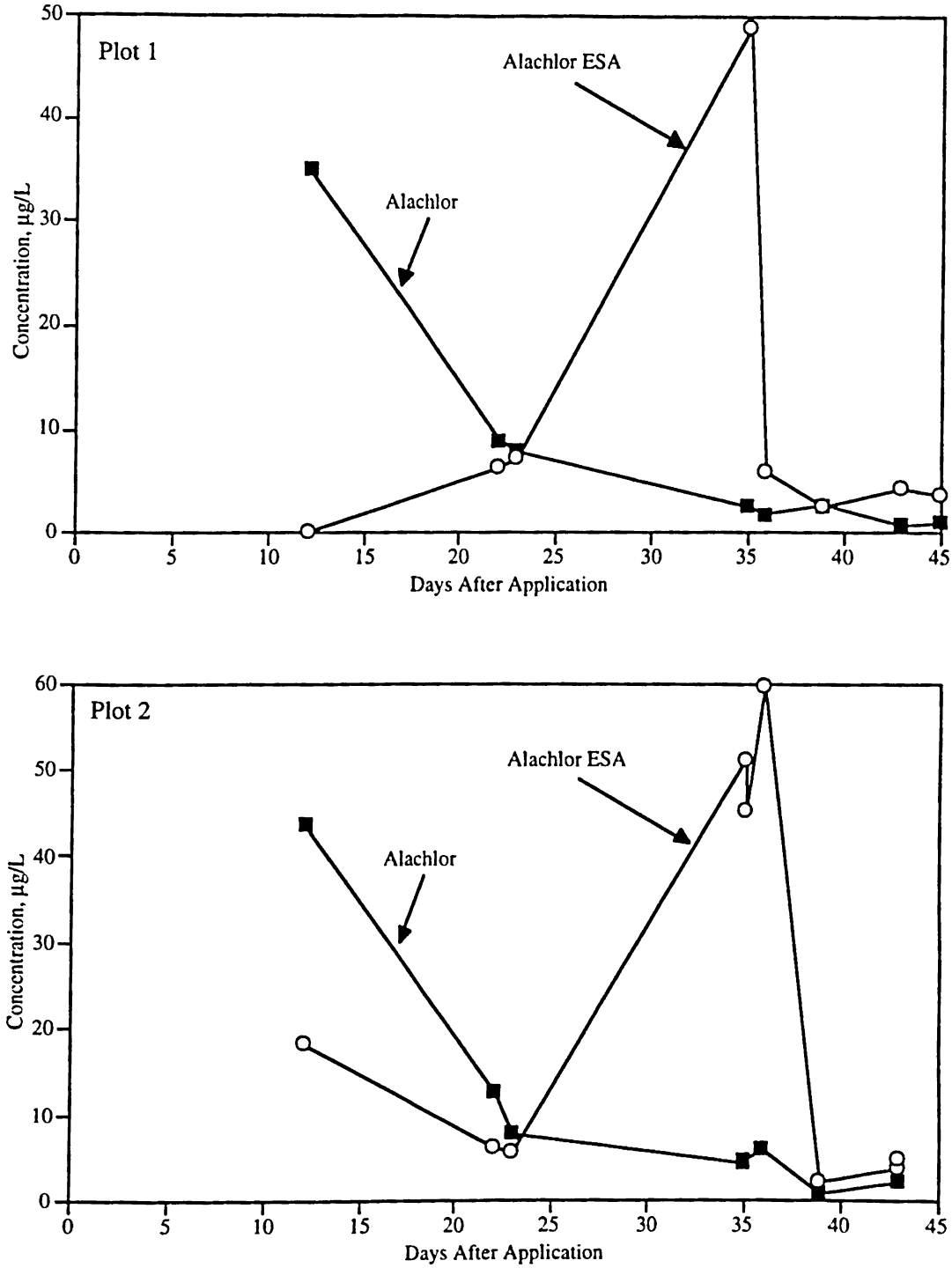


Figure 3.14. Formation of alachlor ESA metabolite with respect to the disappearance of the parent herbicide, alachlor, in plot 1 (A) and plot 2 (B) surface runoff, during the 1993 field dissipation study.

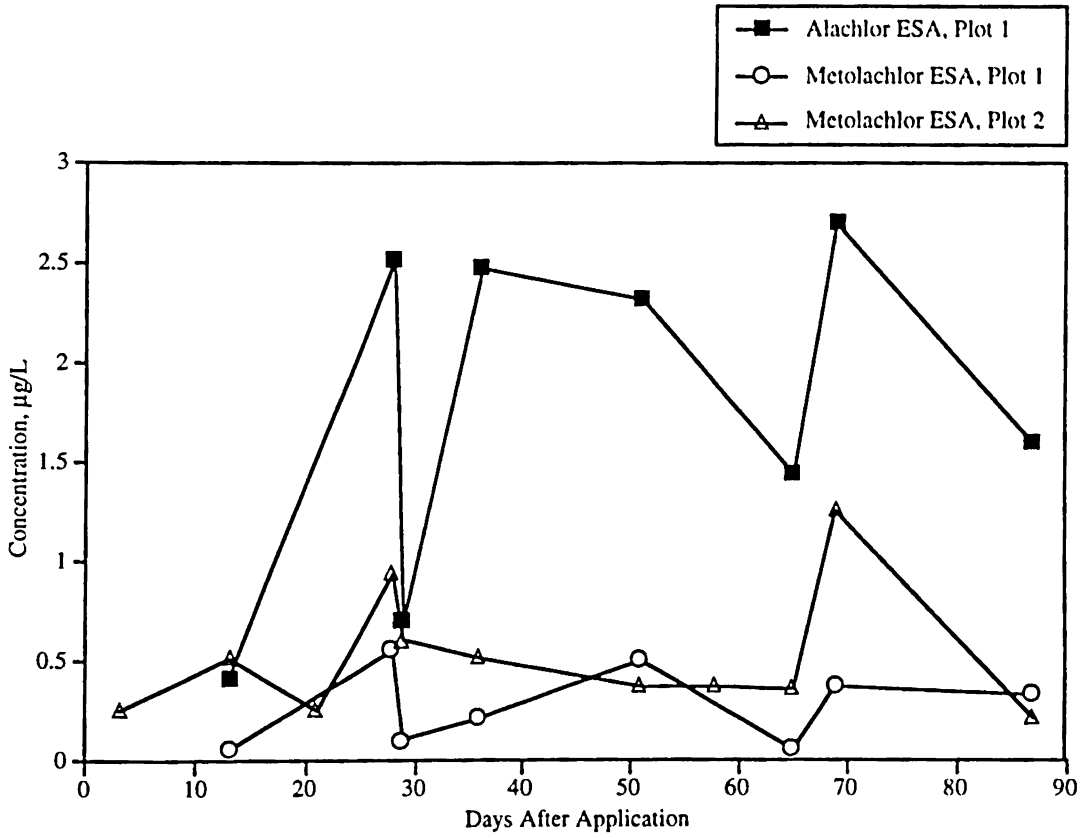


Figure 3.15. Relative formation of metolachlor ESA and alachlor ESA in soil during the 1994 field dissipation study.

Transport and Transformation of Chloroacetanilides in Soil

A. Lysimeter Samples

In the 1993 lysimeter study, a peak in concentration of alachlor, metolachlor, and propachlor in the 0.3, 0.6, and 0.9 m deep lysimeter was observed on the 14th day after herbicide treatment (Figure 3.16). The observed peaks in concentration were a consequence of a 1.25 cm rain that occurred 2 days before samples were collected, thus facilitating downward movement of the herbicides. Alachlor was detected in the 0.3 m lysimeter from day 1 at 8.23 µg/L, till day 30 at 0.07 µg/L. However, no alachlor was observed in the deeper lysimeters after day 19. On the other hand, metolachlor was detected in the 0.3, 0.6, and 0.9 m lysimeters up to day 30 at concentrations ranging from the detection limit of 0.03 µg/L to 4.84 µg/L, suggesting greater leaching of metolachlor compared to alachlor. Propachlor did not leach appreciably relative to alachlor and metolachlor. The highest concentration of propachlor (1.94 µg/L) was observed in the 0.3 m lysimeter one day after application. The concentration of propachlor in the 0.6 m lysimeter never exceeded 0.1 µg/L, while in the 0.9 m lysimeter, propachlor was only detected on the 2nd day at 0.5 µg/L, and on the 14th day at 0.12 µg/L. Even though propachlor has a higher solubility than metolachlor, no significant downward movement of propachlor was observed. This suggests that degradation is of greater importance than leaching in the dissipation process of propachlor. This is supported by the short half-life found in the surface-water runoff for propachlor. None of the herbicides were detected in the 1.2 and 1.5 m lysimeters, except for a few cases where alachlor and/or metolachlor were detected at concentrations less than 0.1 µg/L.

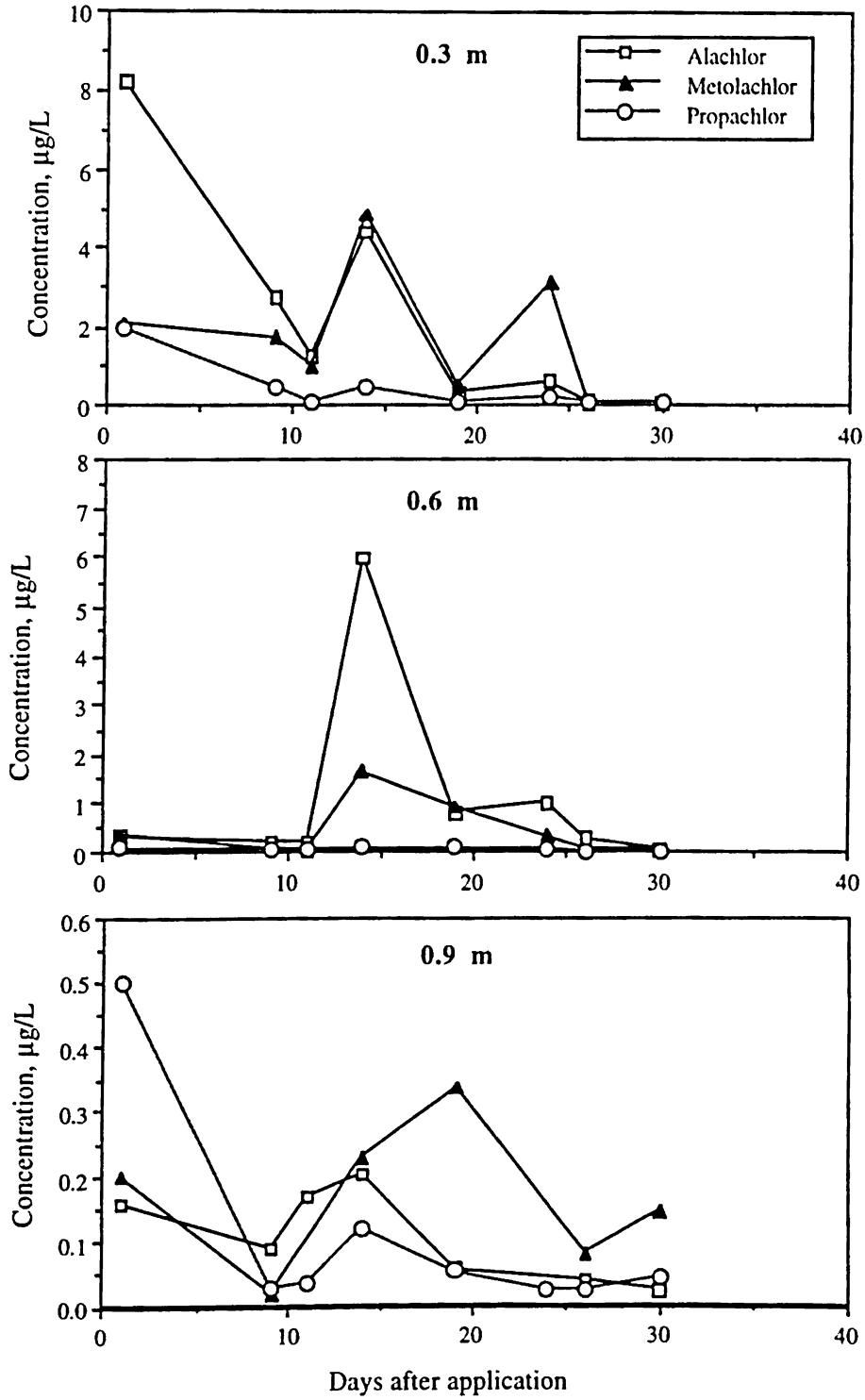


Figure 3.16. Relative transport of alachlor, metolachlor and propachlor in the unsaturated zone; collected with lysimeters at depths of 0.3, 0.6, and 0.9 m, in 1993.

In the 1994 study, the highest concentrations of alachlor (3.23 µg/L) and metolachlor (5.06 µg/L) were observed on day 3, in the 0.3 and 0.6 m lysimeters (Figure 3.17). This was a consequence of the irrigation of the corn field a day before sampling which may have caused rapid infiltration of the dissolved herbicides through the unsaturated zone. On day 23, a peak concentration of metolachlor was observed in the 0.9 m lysimeter. In fact, the metolachlor concentration (0.94 µg/L) was 3 times greater than the alachlor (0.29 µg/L) concentration. Again, this is an indication of greater downward transport of metolachlor relative to alachlor. It can also be seen that while alachlor was no longer detected in the lysimeters 30 days after application, detectable concentrations of metolachlor, ranging from 0.03 to 0.4 µg/L, were observed in some of the lysimeters up to day 100. It can be inferred from Figure 3.17 that the downward movement of alachlor and metolachlor was restricted during the 1994 study period compared to that in 1993. This is due to the lack of significant downward soil-water flux during the 1994 growing season, when evapotranspiration was greatest.

Acetochlor was hardly detected in any of the lysimeters. Except on day 23, where 0.13 µg/L and 0.05 µg/L acetochlor were observed in the 0.3 and 0.6 m lysimeter, respectively, there were no other detections of acetochlor in the lysimeters.

Alachlor ESA was observed at considerably higher concentrations than alachlor in the soil-pore water collected from the lysimeters at various depths. This indicates higher mobility and leachability of the metabolite relative to the parent in soil. This is substantiated by the results of the K_{oc} values obtained from laboratory experiments.

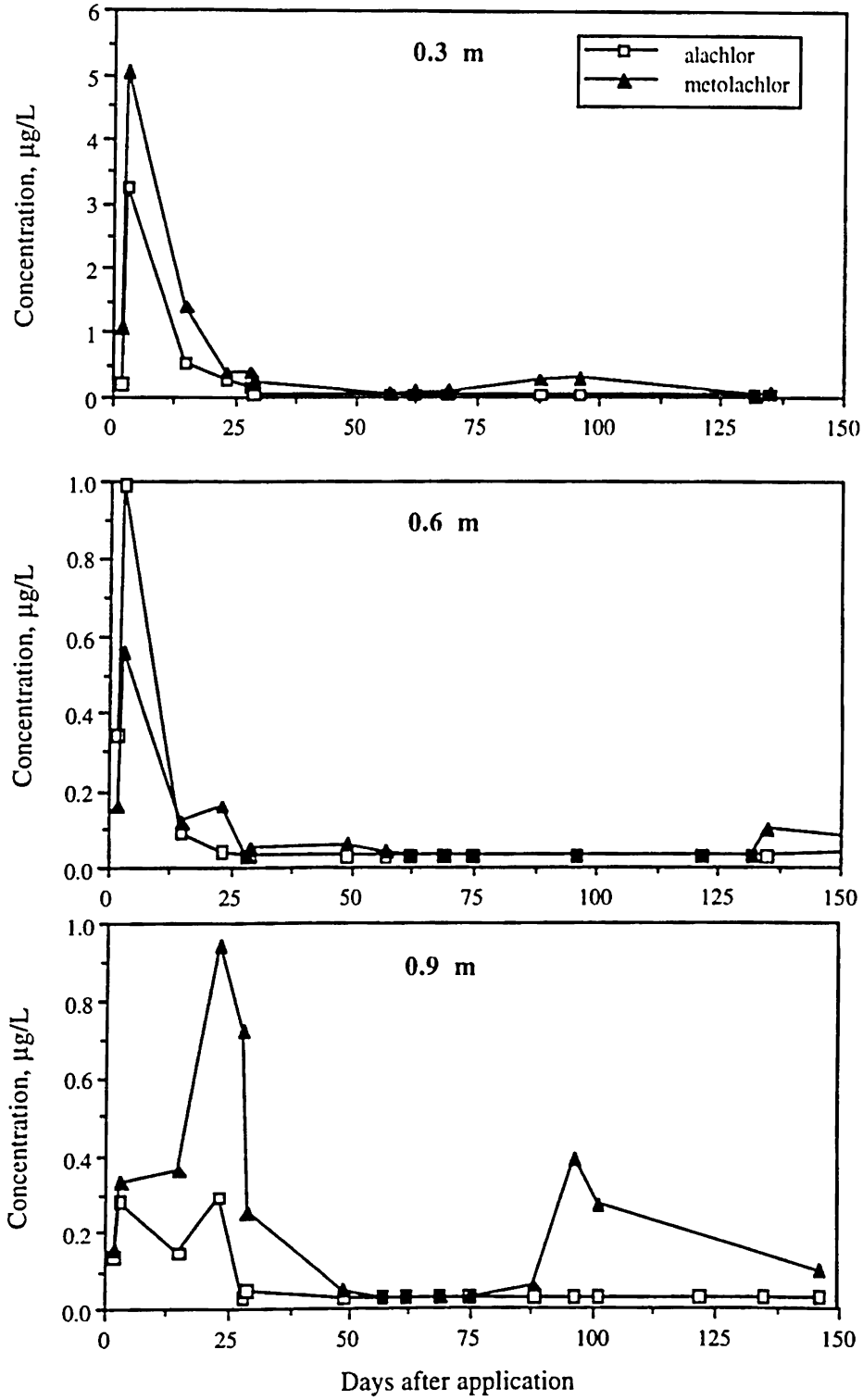


Figure 3.17. Relative transport of alachlor and metolachlor in the unsaturated zone; collected with lysimeters at depths of 0.3, 0.6, and 0.9 m, in 1994.

The concentration of alachlor ESA from the 1993 study continuously increased even after harvest (100 days after application) as can be seen in Figure 3.18. Unlike the parent herbicide, alachlor ESA leached down as far as 1.5 m, where the concentration ranged from 3 to 73 μg/L. A peak in the concentration of alachlor ESA was observed between 14-20 days after application in the 0.6, 0.9, 1.2 and 1.5 m lysimeters. This period coincides with several rain events of at least 1.25 cm experienced only a few days prior to sampling. It is interesting to note that the concentration of alachlor ESA increased dramatically in the shallow lysimeters between the samples collected on day 82 and day 136. Between these two sampling dates the corn was harvested. It is possible that corn plants took up the ESA metabolite and then released it back to the soil after harvest, resulting in a considerable increase in ESA concentration in soil. A similar observation has been reported (Lamoureux and Rusness, 1989) for propachlor ESA, which was taken up by soybean plants grown in soil treated with propachlor.

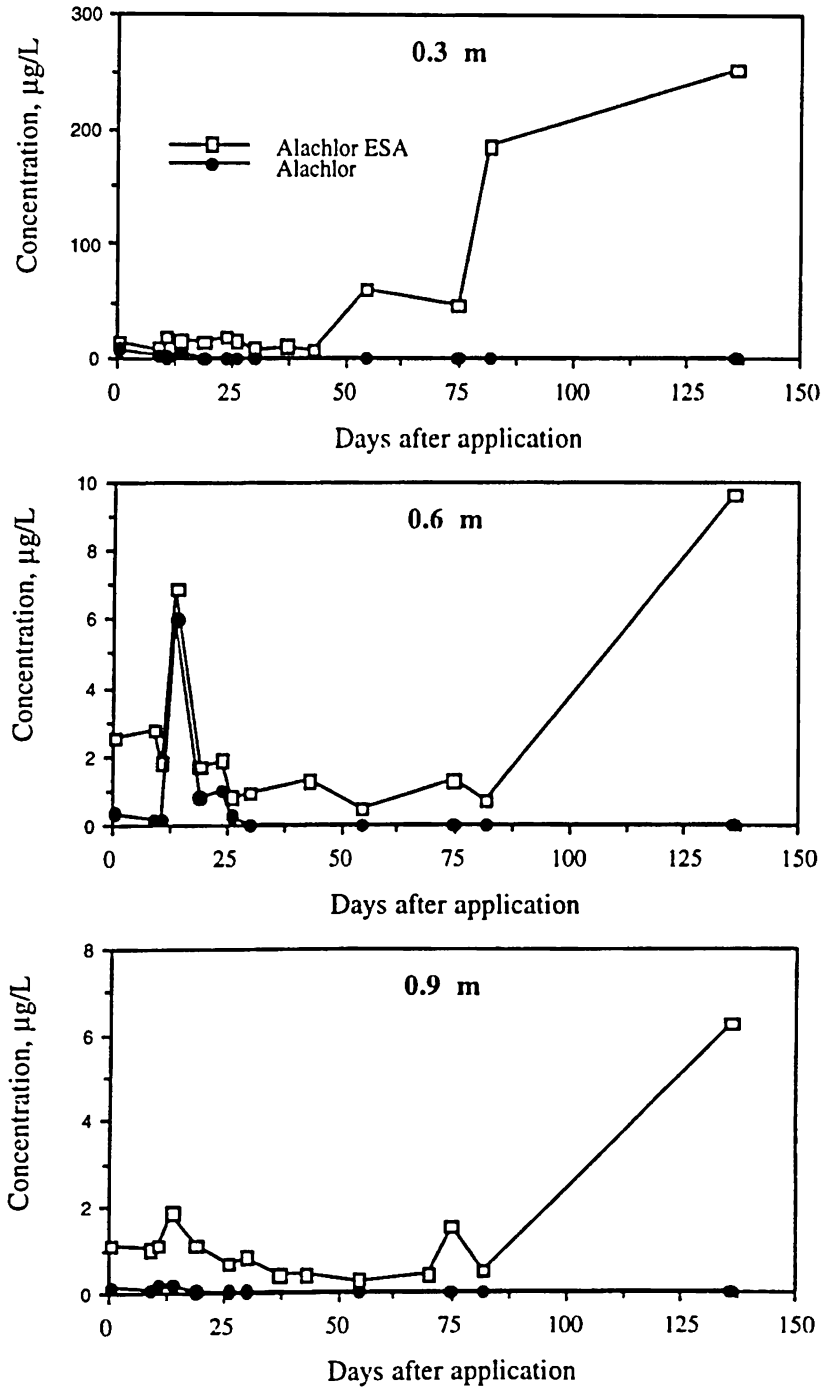


Figure 3.18. Relative leaching of alachlor and its metabolite, alachlor ESA, in the unsaturated zone; collected with lysimeters at 0.3, 0.6, 0.9, 1.2, and 1.5-m depth, in 1993.

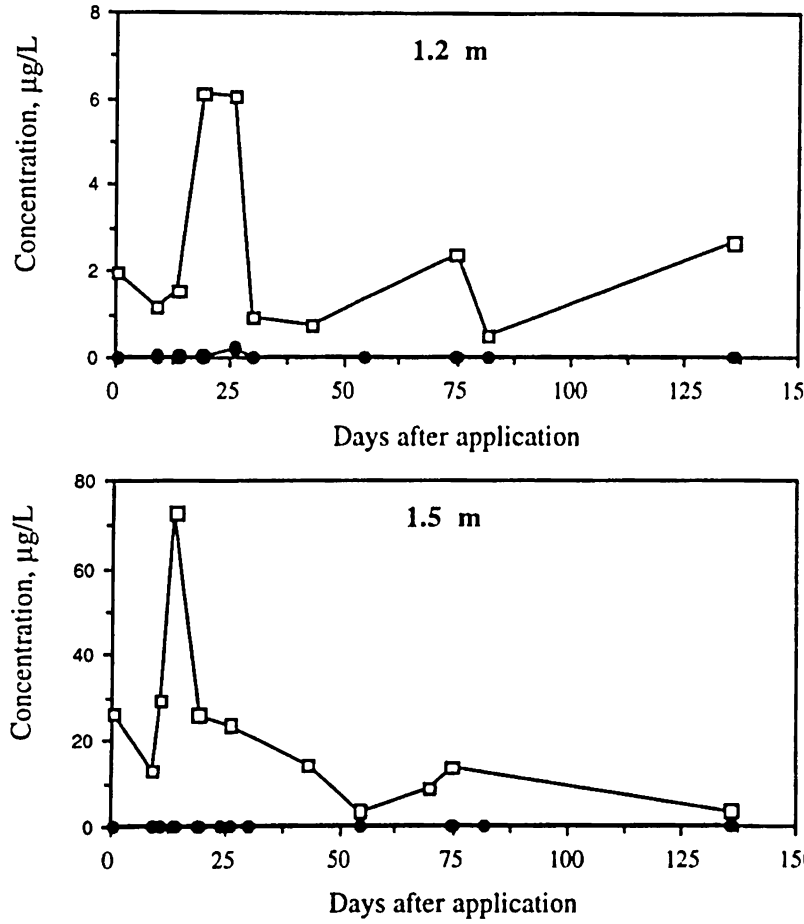


Figure 3.18 cont. Relative leaching of alachlor and its metabolite, alachlor ESA, in the unsaturated zone; collected with lysimeters at 0.3, 0.6, 0.9, 1.2, and 1.5-m deep, in 1993.

B. Soil Samples

Analysis of herbicides in soil cores complements the information provided by lysimeter samples. Whereas lysimeter samples represent the rate of transport of compounds in solution, soil cores can account for the extractable herbicides adsorbed in soil particles and those dissolved in the soil-pore water. Therefore, for the study of alachlor and metolachlor transport, soil cores from several depths of the experimental plots were also analyzed.

The results of the 1994 field study revealed that alachlor ESA is formed at a faster rate in surface soil than metolachlor ESA. For example, on day 2, the concentration of alachlor ESA in the top 15 cm of soil was 43.5 µg/Kg (Figure 3.19A), while metolachlor ESA was only 11.91 µg/Kg (Figure 3.19B). The alachlor ESA concentration represents about 3%, and metolachlor ESA about 0.4%, of the parent herbicide present in the same soil sample. Alachlor ESA and metolachlor ESA both increased progressively until they reached a peak concentration 9 to 10 weeks after application (Figures 3.20-3.22). The highest concentration of alachlor ESA detected in surface soil (0-15 cm) was 210 µg/Kg, or about 60% of the concentration of alachlor present in the same soil sample. At this period, the concentration of alachlor was less than 25% of the initial concentration applied. On the other hand, the highest concentration observed for metolachlor ESA in surface soil was 93 µg/Kg and 128 µg/Kg in plot 1 and 2, respectively. This corresponds to about 34% and 26% of the metolachlor present in the same soil sample. Like alachlor, the concentration of metolachlor during this period was less than 25% of the initial concentration applied.

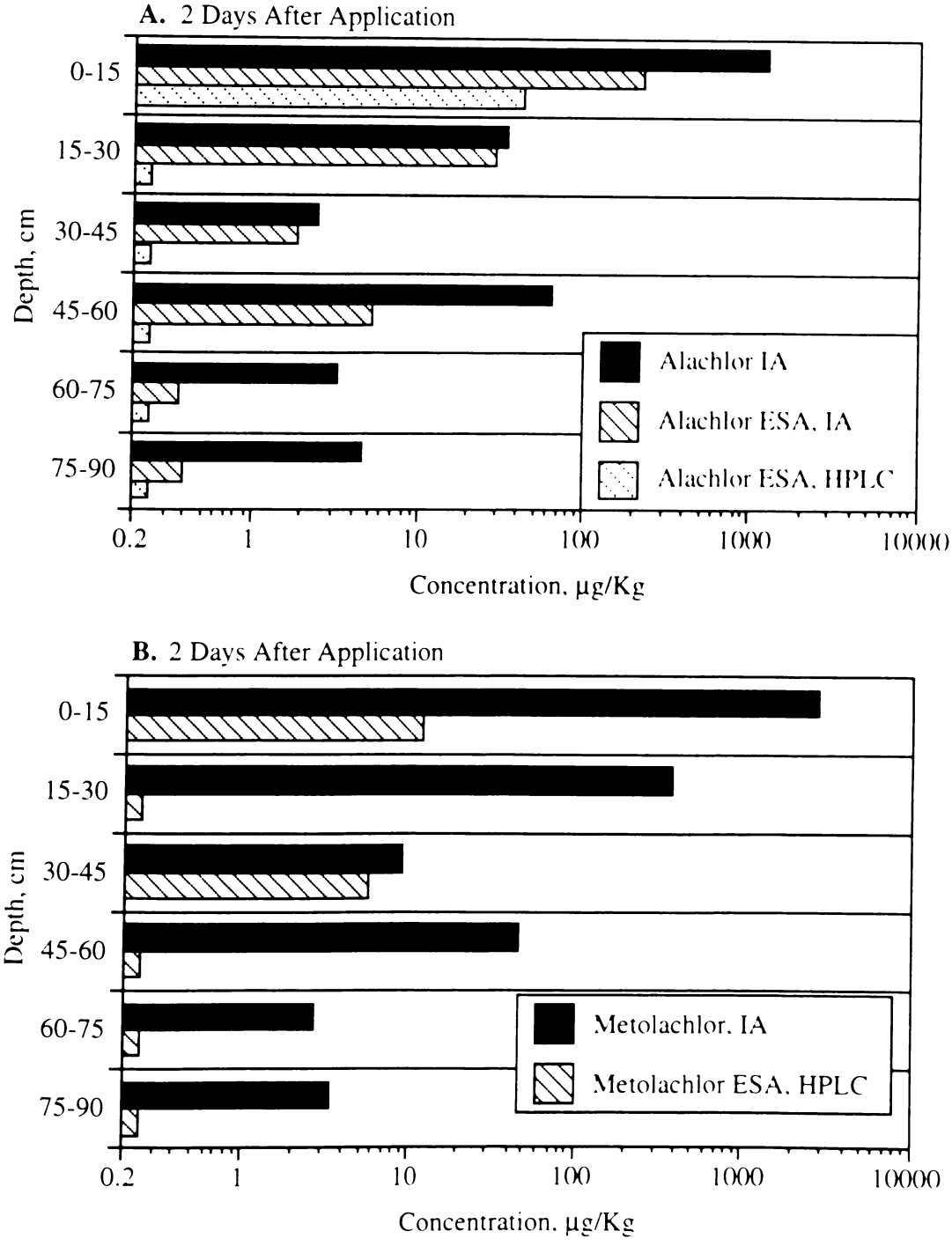


Figure 3.19. Relative concentrations of alachlor and alachlor ESA (A), and metolachlor and metolachlor ESA (B) on day 2, at different depths from the 1994 field study. Alachlor and metolachlor were determined by immunoassay (IA).

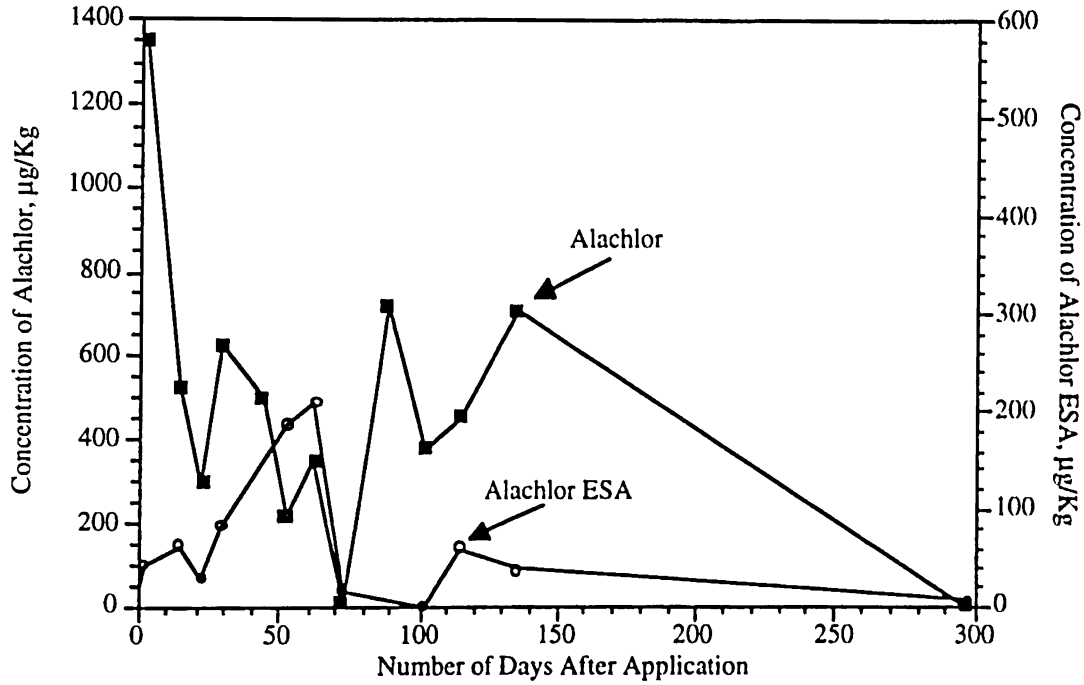


Figure 3.20. Alachlor disappearance and alachlor ESA formation in the upper 15 cm of soil (Plot 1, 1994).

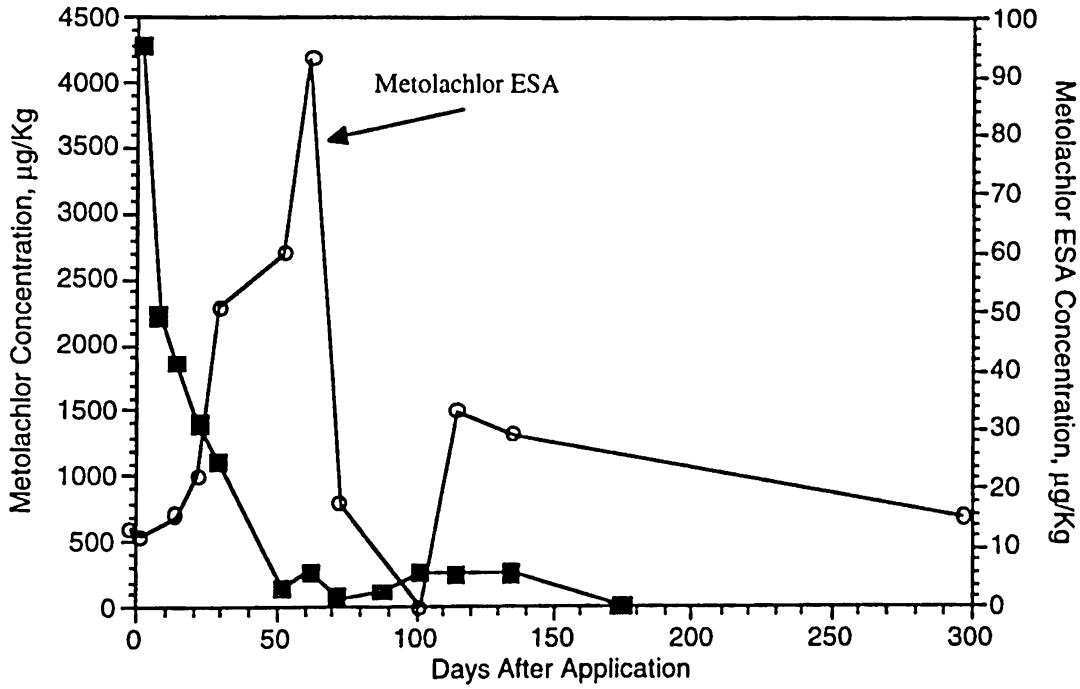


Figure 3.21. Metolachlor disappearance and metolachlor ESA formation in the upper 15 cm of soil (Plot 1, 1994).

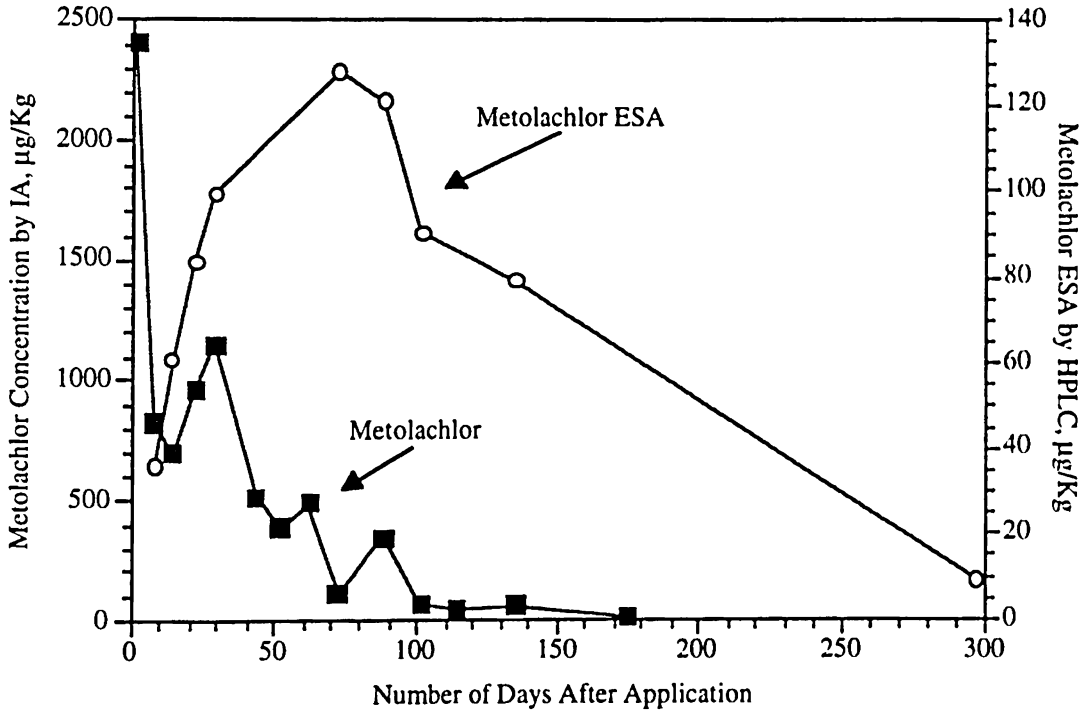


Figure 3.22. Metolachlor disappearance and metolachlor ESA formation in the upper 15 cm of soil (Plot 2, 1994).

It is important to note that except on the last day of sampling (297 days after application), the concentration of alachlor ESA was higher than metolachlor ESA by about 2 to 3 times. However, it must be pointed out that on the last day of sampling, metolachlor ESA was higher in concentration than alachlor ESA at all depths (Figure 3.23). During this sampling period, no detectable amount of the parent herbicides was present in any of the soil samples. This observed reversal of relative concentration between the two ESA metabolites may be due to the difference in the dissipation rates between alachlor and metolachlor. The disappearance of alachlor in soil was faster than metolachlor, therefore at the later period of the study there was only metolachlor remaining in soil available for microbial degradation. Consequently, on the last samples collected, metolachlor ESA was higher than alachlor ESA.

Figures 3.24-3.28 show the downward transport of the ESA metabolites relative to the parent herbicides. The relative leaching of alachlor and metolachlor is also depicted in these graphs. It is obvious from the graphs that, in general, the leaching of metolachlor was greater than alachlor. For example, metolachlor leached to 30-45 cm deep at a concentration of 80 µg/Kg on day 8, and increased to 142 µg/Kg on day 14. On the other hand, the concentration of alachlor was only 3.6 µg/Kg on day 8, and 13.3 µg/Kg on day 14 at the same depth. Also, although both compounds were detected as far down as 75 cm below the surface, the concentrations of alachlor and metolachlor found below 45 cm only exceed 3 µg/Kg once.

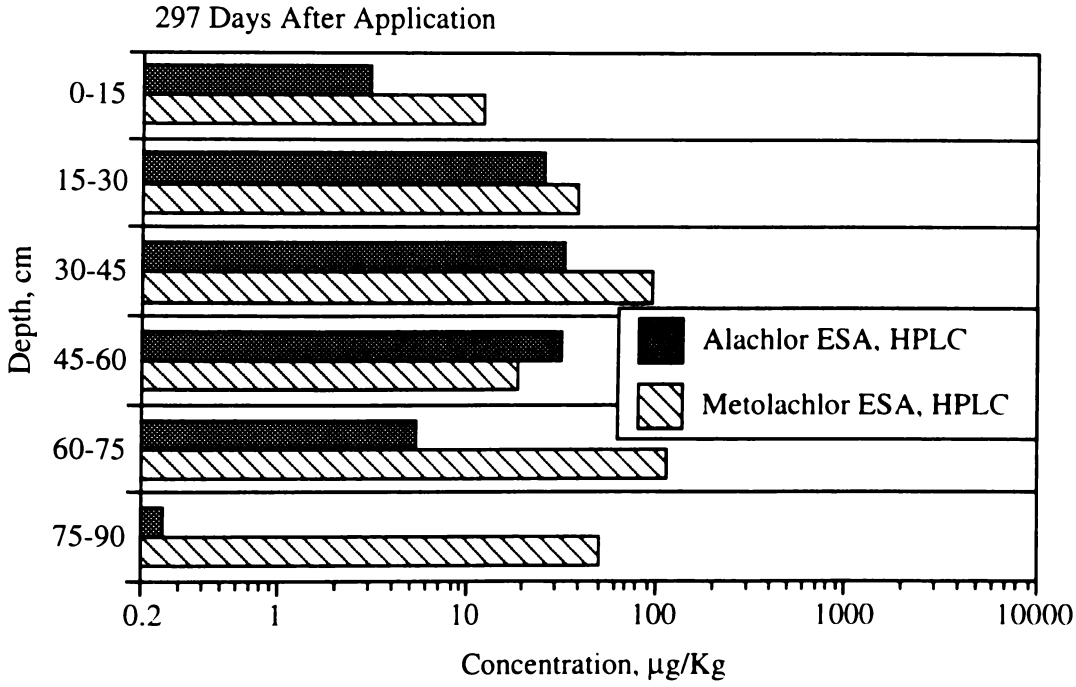


Figure 3.23. Relative concentrations of alachlor ESA and metolachlor ESA on the 297th day after application in the 1994 field study.

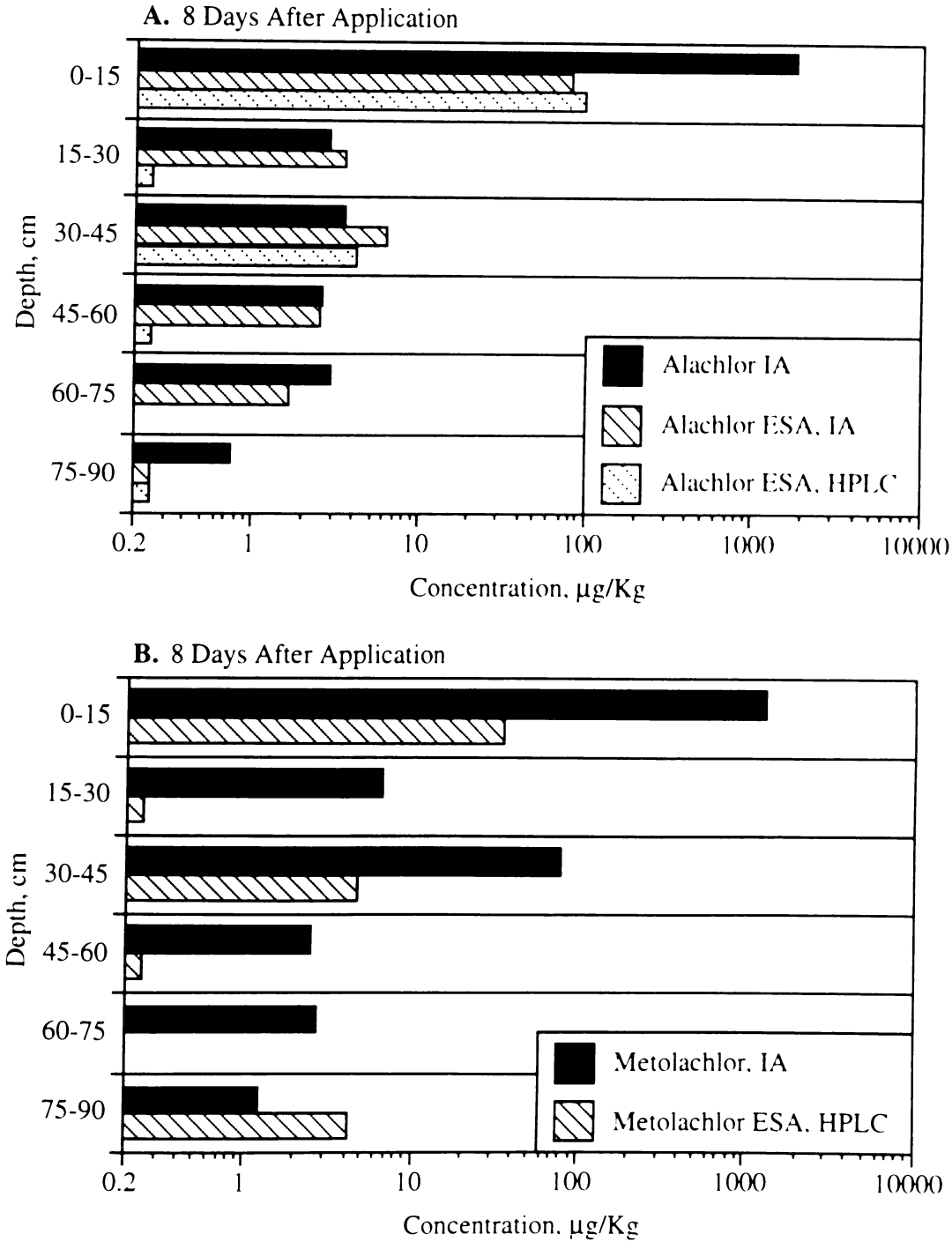


Figure 3.24. Relative concentrations of alachlor and alachlor ESA (A), and metolachlor and metolachlor ESA (B) on day 8, at different depths from the 1994 field study. Alachlor and metolachlor were determined by immunoassay (IA).

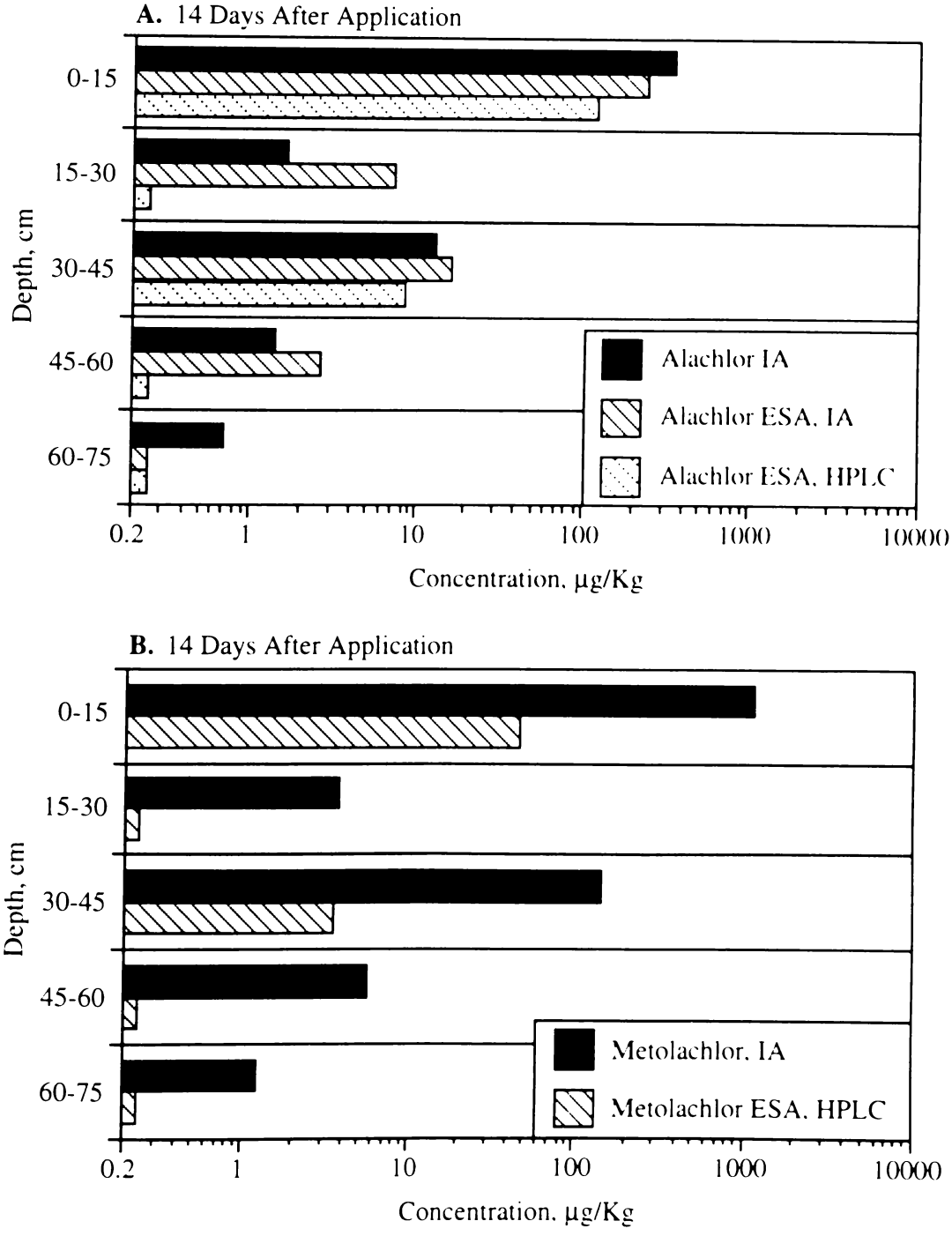


Figure 3.25. Relative concentrations of alachlor and alachlor ESA (A), and metolachlor and metolachlor ESA (B) on day 14, at different depths from the 1994 field study. Alachlor and metolachlor were determined by immunoassay (IA).

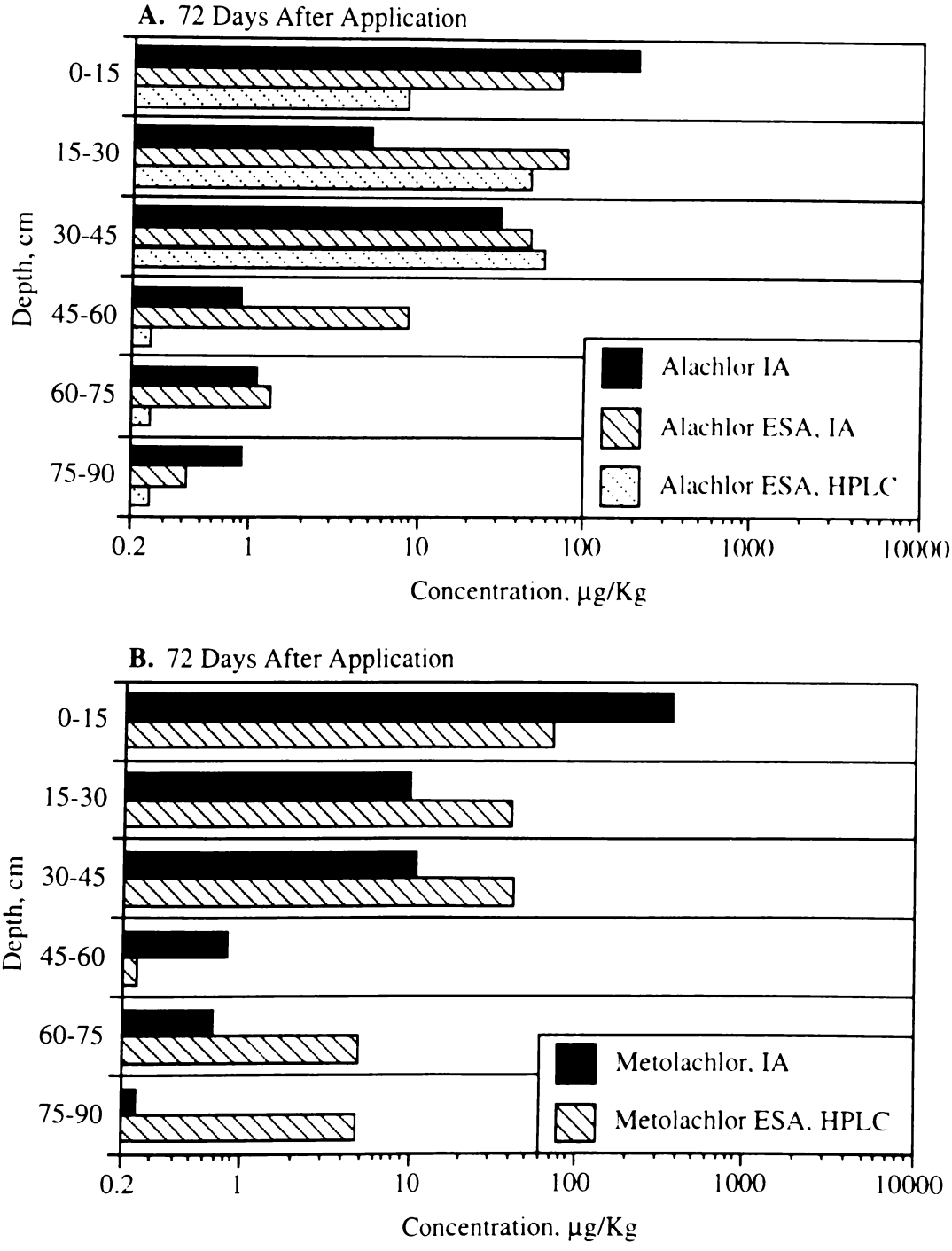


Figure 3.26. Relative concentrations of alachlor and alachlor ESA (A), and metolachlor and metolachlor ESA (B) on day 72, at different depths from the 1994 field study. Alachlor and metolachlor were determined by immunoassay (IA).

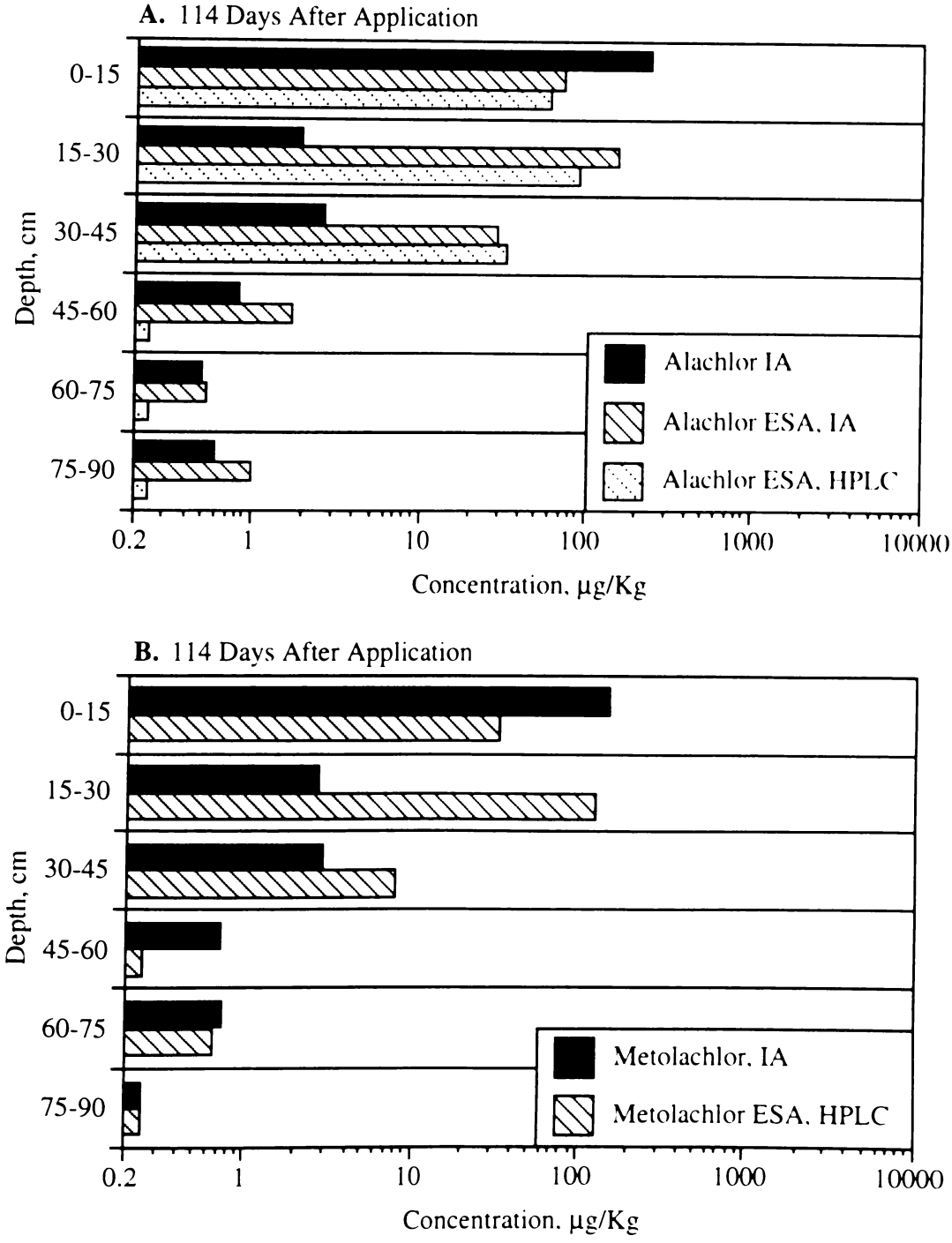


Figure 3.27. Relative concentrations of alachlor and alachlor ESA (A), and metolachlor and metolachlor ESA (B) on day 114, at different depths from the 1994 field study. Alachlor and metolachlor were determined by immunoassay (IA).

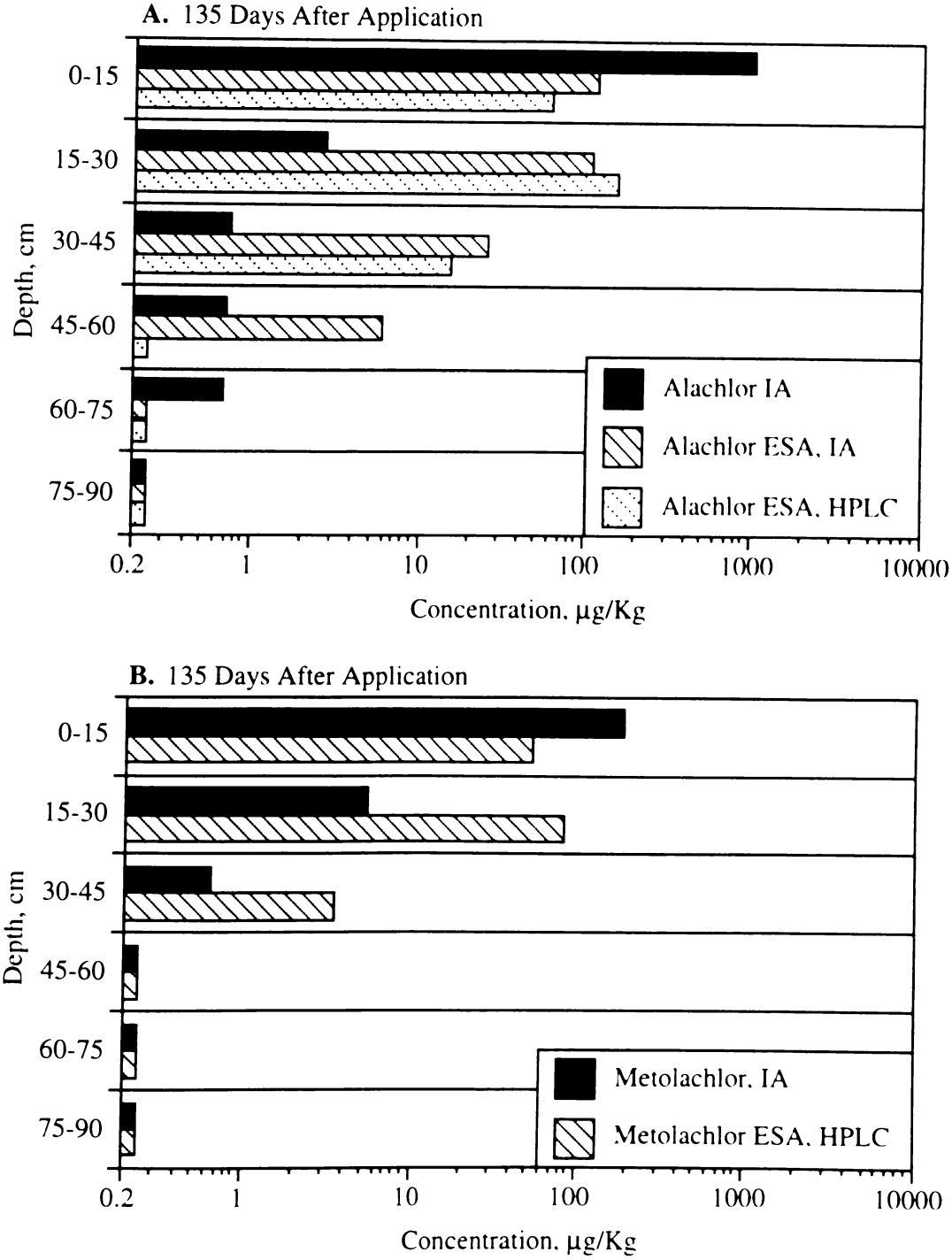


Figure 3.28. Relative concentrations of alachlor and alachlor ESA (A), and metolachlor and metolachlor ESA (B) on day 135, at different depths from the 1994 field study. Alachlor and metolachlor were determined by immunoassay (IA).

It is also discernible from the figures above that on day 2 alachlor ESA was 3% of the parent and then it increased to 10% and 20% on day 8 and 9, respectively. Metolachlor ESA started at 0.4% of the parent on day 2 and increased to 3% and 5% of the parent on day 8 and 14 respectively. Apparently, formation of metolachlor ESA is slower than alachlor ESA.

From day 72, it can be seen that the ESA metabolites started to exceed the parent herbicide concentration at all depths except at the soil surface (0-15 cm). The difference was most dramatic for days 114 and 135, in which both ESA metabolites reached approximately 100 µg/Kg in the 15-30 cm depth, while the parents were below 5 µg/Kg (Figures 3.27 and 3.28). It may also be concluded from the graphs that unlike the parent herbicides, the two ESA metabolites leached at higher concentrations and to greater depths. For example, as high as 140 µg/Kg alachlor ESA and 122 µg/Kg metolachlor ESA were observed at 60-75 cm deep on day 43. Moreover, concentrations of 13.3 µg/Kg and 18.7 µg/Kg of alachlor ESA and metolachlor ESA, respectively, have been observed at a depth of 75-90 cm on day 297. As mentioned earlier, neither alachlor nor metolachlor exceeded 3.0 µg/Kg at depths lower than 45 cm. Again, the increase in the concentration of both ESA metabolites below the soil surface observed on day 297 (Figure 3.23) may be attributed to the release of the ESA metabolites from the corn after harvest.

Evaluation of the Analytical Method and Utility of ELISA in a Field Dissipation Study.

The results presented above for soil analysis were from duplicate samples collected from each plots. Thus, for a herbicide applied in two plots, the final concentration reported was an average of 4 analyses. The concentrations of alachlor and metolachlor varied greatly at each sample location with time, which can be attributed to non-uniform application of the herbicides. The difficulty in applying herbicides uniformly was due to the variations in wind velocity, oscillation of the sprayer booms, and variation in tractor operation. Increased confidence in the average pesticide concentration could be obtained if more samples were taken. Similar problems have been encountered by other researchers when measuring pesticide contents under field conditions. For example, Taylor et al. (1971) predicted that, to reduce the coefficient of variation in half in sampling dieldrin, the number of soil cores taken would have to be increased from 8 to 30 samples per 100 m^2. However, the accuracy gained by taking more samples must be balanced against the cost of analyzing the extra samples.

The variability contributed by the overall analytical method was far less significant than the inherent variability in the soil samples. For example, in the soil analysis using ELISA, 2 aliquots from each soil sample were extracted and analyzed as A and B samples. The range of the percent coefficient of variation (%CV) between A and B samples are presented in the boxplot in Figure 3.29. It can be seen that the mean and the median of the %CV of the ELISA method for alachlor, metolachlor, and alachlor ESA are all less than 15%. The boxplot also indicates that the 75th percentile

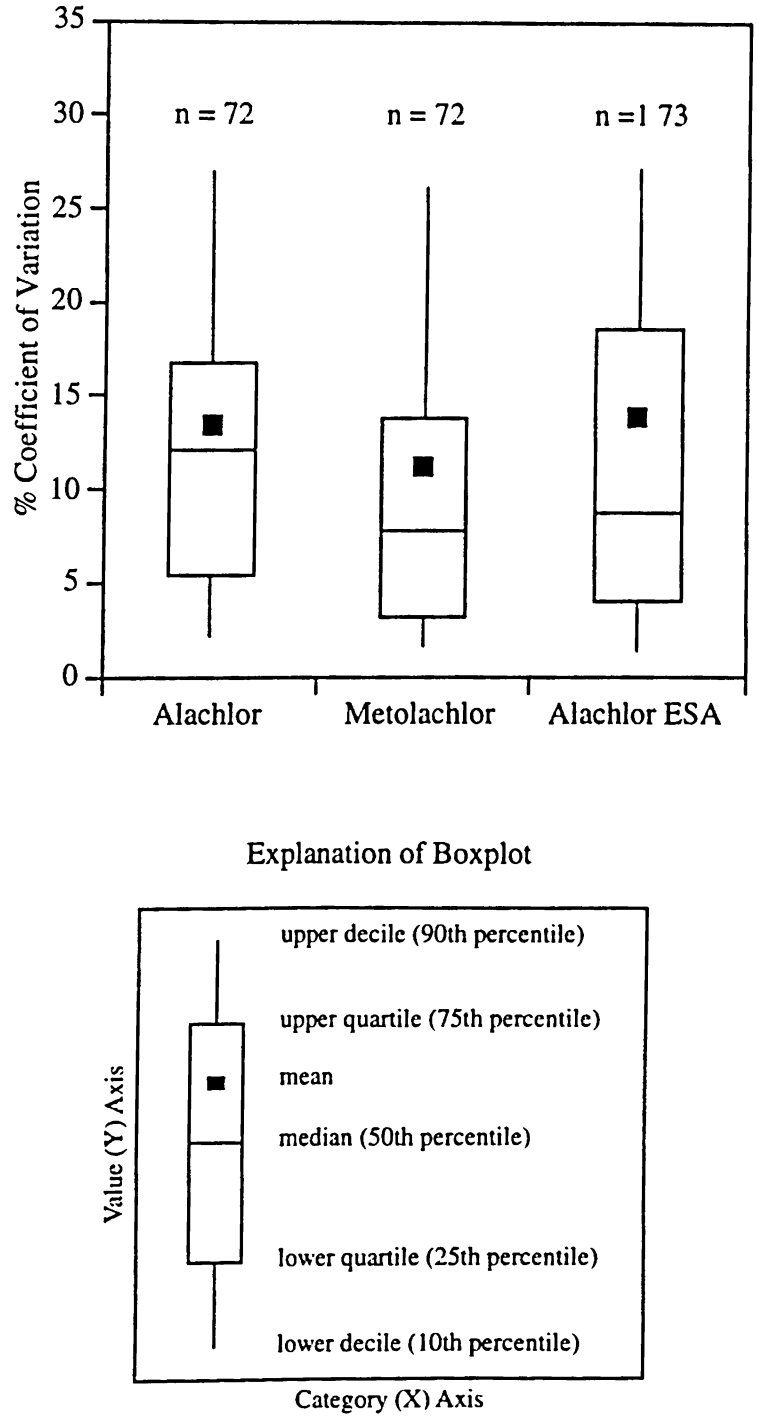


Figure 3.29. Precision of the overall SPE-ELISA method of analysis.

of the samples have CV's lower than 20%. The agreement between A and B samples were impressive considering that there were several steps in the extraction process which could contribute to the possible sources of error. It must be pointed out that for the analysis of alachlor and metolachlor, each soil extract was analyzed using two wells in a microtiter plate, and the average was used to report the concentration of the soil extract. Therefore, the well-to-well variations in ELISA has already been incorporated in the %CV shown here. Thus, it can be concluded that the SPE-ELISA method for the analysis of alachlor, metolachlor, and alachlor ESA has good precision.

The accuracy of ELISA results was determined by a comparison with GC/MS analysis of a selected set of soil samples. The values of the ELISA results were plotted against GC/MS results. Concentrations below 10 μg/L were graphed separately to clearly show the correlation between the two analytical methods at high and low concentrations. The linear correlation between ELISA and GC/MS results were better at higher concentrations (above 10 μg/Kg). For alachlor, the correlation coefficient between ELISA and GC/MS was 0.72 and 0.92, for low and high concentrations (Figure 3.30), respectively. Likewise, the correlation coefficient between metolachlor ELISA and GC/MS was 0.74 for low, and 0.88 for high concentrations (Figure 3.31). The closer agreement between ELISA and GC/MS at higher concentrations may be explained by the reduction of the matrix effects at higher sample dilutions. The complexity of the soil matrix affects the performance of both GC/MS and ELISA, although the effect is more pronounced in the latter method.

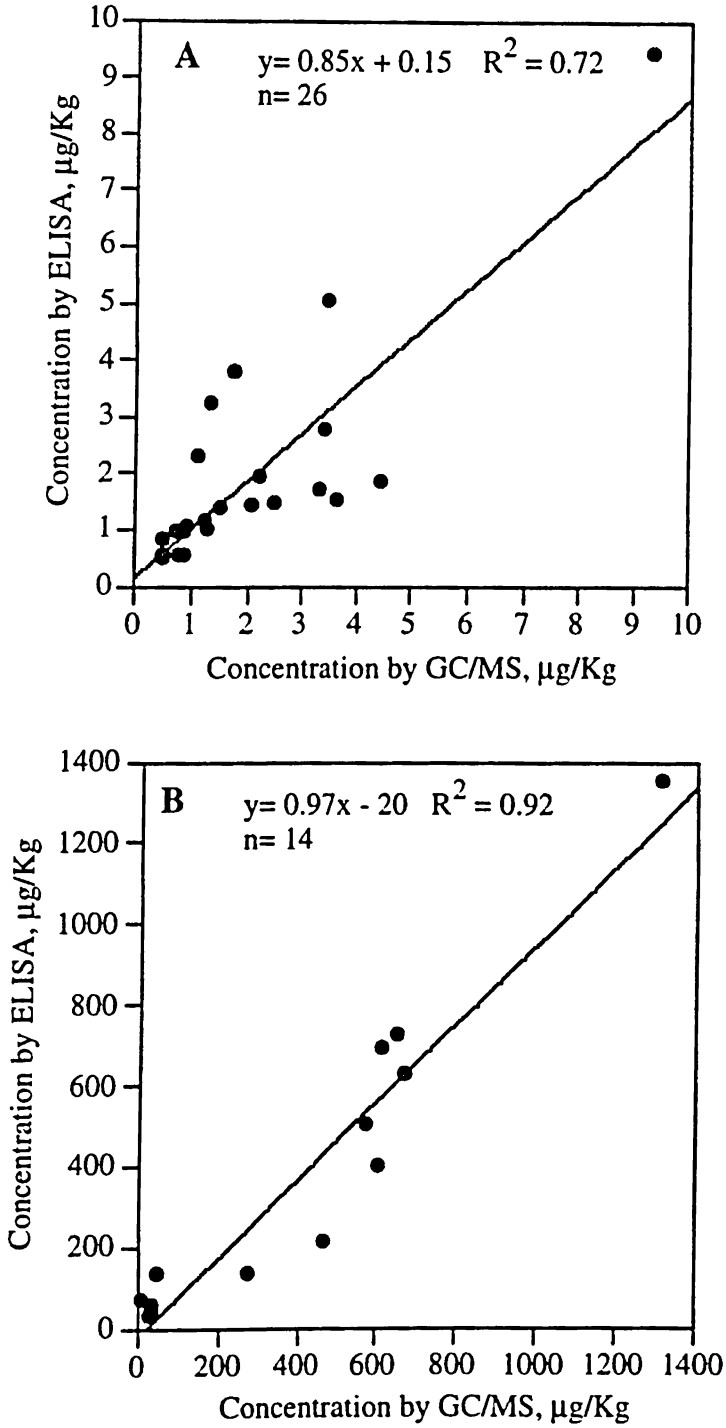


Figure 3.30. Correlation between ELISA and GC/MS results for alachlor in soil at concentrations <10 µg/Kg (A) and >10 µg/Kg (B).

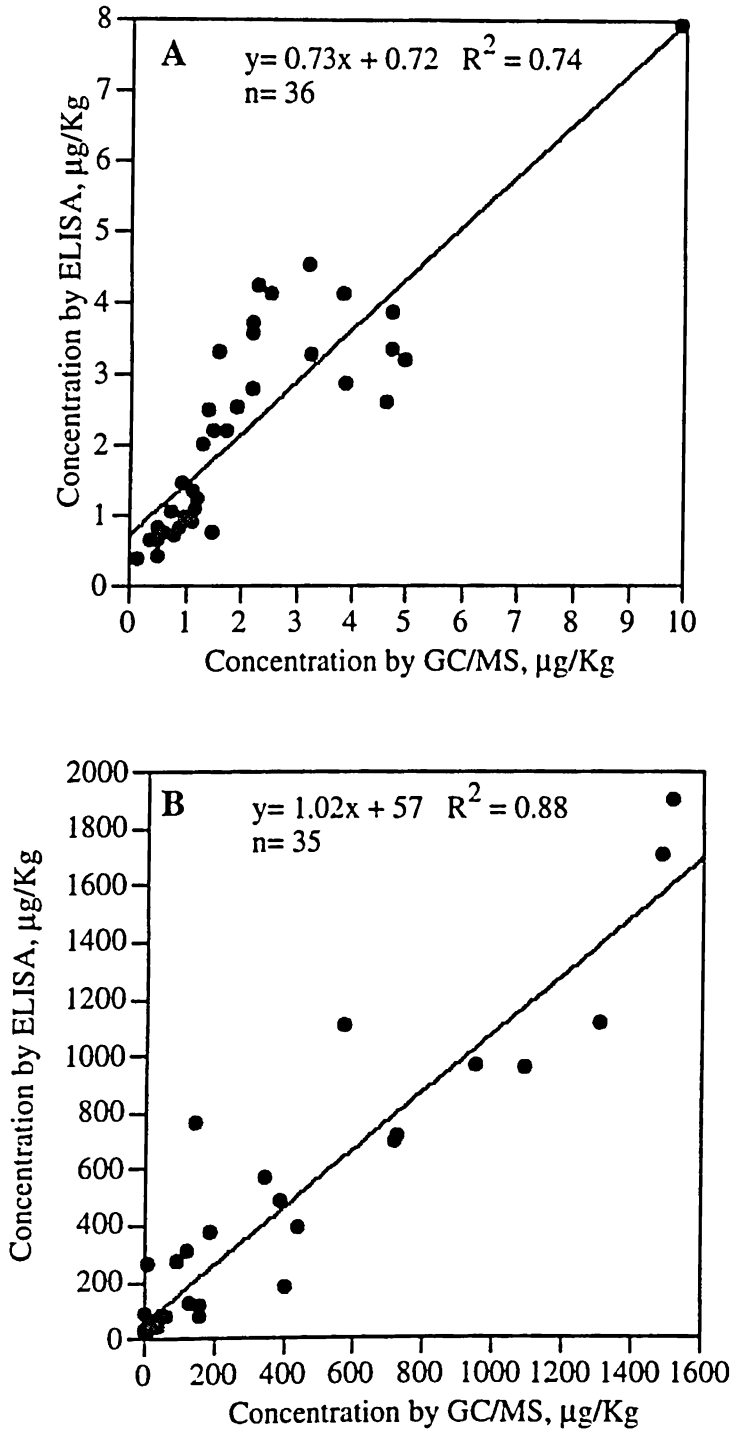


Figure 3.31. Correlation between ELISA and GC/MS results for metolachlor in soil at concentrations <10 µg/Kg (A) and >10 µg/Kg (B).

The applicability of the SPE-ELISA method for the analysis of alachlor ESA in the runoff and lysimeter samples was successfully demonstrated. High correlation between HPLC and ELISA results was obtained for water samples (discussed in Chapter I). Likewise, the ELISA method for alachlor ESA in soil was comparable with HPLC results as can be seen in Figures 3.24 to 3.28, where ELISA values were plotted side by side with HPLC values for comparison. In general, the ELISA values of alachlor ESA for the top soil were higher than the HPLC values. Two reasons could explain these discrepancies. First is the interference from humic substances present in soil. The top soil has the greatest amount of humic substances, as evident from the high yellow coloration of the methanol extract. As discussed in Chapter I, methanol effectively elutes humic acids which are adsorbed on the C_{18} resin used for SPE. Secondly, the top soil contained the highest amounts of alachlor. At very high concentrations of alachlor (> 1000 µg/Kg), breakthrough may occur when the capacity of the resin is exceeded. Since the ELISA used for alachlor ESA was more sensitive to alachlor, a small amount of alachlor in the extract could significantly magnify the values of alachlor ESA. It is very important that a complete separation of alachlor and its ESA metabolite is achieved if the SPE-ELISA method is to be used for the analysis of the latter. Nonetheless, the result of the SPE-ELISA for the analysis of alachlor ESA in soil was comparable to the HPLC values, particularly for the subsurface soil samples.

The SPE-ELISA for alachlor ESA was a useful method to complement the HPLC results, especially because the ESA metabolites do not have individual characteristic UV spectra. For instance, metolachlor ESA, alachlor ESA, and alachlor

oxanilic acid (another soil metabolite of alachlor), and other unknown transformation products, have the same UV spectra. Therefore, when chromatographic retention time is affected significantly by the complexity of the soil matrix, it becomes difficult to identify the analyte peaks. Oftentimes, the analyte peaks are also obscured by broad interfering peaks present in the soil sample. The result provided by the SPE-ELISA method therefore becomes of paramount importance in the identification and quantification of alachlor ESA. Therefore, the two methods may be used to complement each other to produce more reliable results. Furthermore, HPLC analysis of soil samples could be a tedious process. Individually filtering soil extracts and frequent troubleshooting of the instrument because of clogging becomes cumbersome in a study that requires more than just a few samples. Hence, the ELISA method becomes favorable and practical.

The greatest advantage offered by ELISA in this field dissipation study was its cost-effectiveness and short analysis time. The ELISA method was particularly useful for the soil analysis. The amount of work-time and analytical cost was cut by greater than 50% of what it would have cost if all soil samples were analyzed by GC/MS. First, the sample preparation for GC/MS requires an additional step (passing the sample through an anion exchanger) to reduce the amount of humic materials in the sample. The humic materials cause rapid degradation of the expensive GC/MS column. This additional step doubles the sample preparation time. Secondly, GC/MS analysis becomes noticeably expensive when a study involves analysis of more than 1000 samples of soil. Thus, replicate analysis is usually not feasible. ELISA, on the other hand, only costs $5 per sample. Therefore, more replicate samples may be collected and analyzed without major repercussions. Consequently, the accuracy of the

results is increased, and the variability of the soil concentrations in the field becomes less important when more measurements are made.

The ELISAs used for the analysis of alachlor and metolachlor were tested for cross-reactivity against related herbicides and known metabolites. The cross-reactivity patterns of alachlor and metolachlor ELISA are listed in Table 3.14 and 3.15, respectively. For the purpose of this study, the slight cross-reactivity of the individual ELISA against some of the related herbicides is tolerable. For instance, even if acetochlor has 2% cross-reactivity with the alachlor ELISA, it is not of concern because they were not applied on the same plot. Metolachlor has small cross-reactivity (0.7%) towards the alachlor ELISA, and vice versa. Propachlor is hardly recognized by either ELISA. In addition, the ionic metabolites of alachlor which cross-react with the assay were separated using SPE. The known metabolites of metolachlor, hydroxy-metolachlor and metolachlor morpholinone, did not cross-react with the ELISA kits. Therefore, the use of ELISA for the analysis of alachlor and metolachlor in this field dissipation study was highly reliable and plausible.

Table 3.14. Cross-reactivity of Alachlor ELISA from Quantix Systems.

Compound	IC50 (µg/Kg)	Cross-reactivity
Alachlor	0.6	100%
Acetochlor	23	2%
Butachlor	55	1%
Metolachlor	80	0.7%
Propachlor	5000	<0.1%
Alachlor oxanilic acid	5000	<0.1%
Alachlor sulfonic acid	10	6%
Metolachlor sulfonic acid	>10,000	<0.1%
Metolachlor morpholinone	>10,000	<0.1%
Hydroxy-metolachlor	>10,000	<0.1%

Table 3.15. Cross-reactivity of Metolachlor ELISA from Quantix Systems.

Compound	IC50 (µg/Kg)	Cross-reactivity
Metolachlor	1.3	100%
Alachlor	200	0.7%
Acetochlor	130	1%
Butachlor	480	0.3%
Propachlor	10,000	<0.1%
Metolachlor sulfonic acid	5,000	<0.1%
Metolachlor morpholinone	1,000	0.1%
Hydroxy-metolachlor	>10,000	<0.1%
Alachlor oxanilic acid	>10,000	<0.1%
Alachlor sulfonic acid	>10,000	<0.1%

CONCLUSIONS

In this field dissipation study, a new metabolite of metolachlor was identified in soil. This study revealed that metolachlor is transformed to metolachlor ESA, a major soil metabolite that has not been previously identified. The mobility and persistence of metolachlor ESA and alachlor ESA relative to their parent compounds was investigated. Due to the changes of molecular substituents in the structure of the herbicide, these compounds differ significantly from the parent compounds in physicochemical properties and also in their mobility and distribution in the field. Evidently, the ESA metabolites have higher leaching potentials and longer persistence in soil than the parent herbicides. The environmental significance of herbicide metabolites has hardly been explored because of the difficulty in the analysis of polar ionic compounds, such as the sulfonic acids. In fact, the presence of alachlor ESA in surface and ground water has only been recently recognized. Also, in many pesticide metabolic studies there has always been some fraction of water-soluble metabolic products that are unaccounted for due to the difficulty of their extraction and analysis.

Since upon biotic or abiotic transformation the alteration in substituents implies an alteration in the pesticide's physical properties and thus in their mobility in soil, enhanced attention must be paid to potential transformation products transported from soil to water. Methods for identifying parent compounds and metabolites, and cost-effectively analyzing them at low concentrations in complex media are desperately needed. Although immunoassay methods have their own limitations, their potential use in environmental studies can not be underestimated. The utility of ELISA in a field

dissipation study has been successfully demonstrated in this investigation. The results obtained from this study agreed with related works on the fate and transport of chloroacetanilide herbicides. For instance, in this investigation, the order of persistence of the chloroacetanilides in surface runoff was found to be : propachlor<<alachlor, acetochlor<metolachlor. The same order was reported for propachlor, alachlor, and metolachlor in a laboratory experiment using bioassay (Zimdahl and Clark, 1982). In addition, a comparative study between alachlor and acetochlor showed similar half-lives in most soil conditions (EPA, 1994), supporting the results obtained here.

To further substantiate the results of the field study on the mobility and leaching of the chloroacetanilides, and the ESA metabolites of alachlor and metolachlor, laboratory experiments were conducted to accurately determine the K_{oc} of these compounds. The analysis was performed using GC/MS and HPLC methods. The results of the K_{oc} values supported the results obtained in leaching experiments in the field. It was found that leaching of metolachlor was greater than alachlor. However, leaching of the ESA metabolites was far greater than the parent herbicides.

In this study, the use of ELISA, in conjunction with other analytical methods such as HPLC, GC/MS, and FAB MS/MS, provided important and new information on the fate and transport of the chloroacetanilide herbicides in soil. Without one or the other, this extensive study would not have been feasible. HPLC allowed the analysis and confirmation of alachlor ESA and metolachlor ESA in soil samples. GC/MS facilitated the simultaneous analysis of alachlor, metolachlor, propachlor and acetochlor in the water samples, and validated dubious ELISA results of soil analysis. FAB MS/MS confirmed the identity of the metolachlor ESA and its presence in soil,

together with alachlor ESA. Lastly, the low cost of immunoassay has made the analysis of replicate soil samples feasible, which allowed the fate and transport of the herbicides and the metabolites in soil to be followed intensively. In addition, because of the cost-effectiveness of ELISA, repetition of the field study for two years became possible. Duplication of the study provided additional information on the effect of field conditions on the persistence and behavior of the herbicides and their metabolites in the field.

So far, information on the presence of pesticide conversion products in leacheate is limited. It may be concluded that despite a great number of data available, the scientific community today is far from a quantitative understanding of all processes affecting pesticide persistence in soil. Much more information still has to be obtained both for individual processes and for their interactions and interdependencies. However, the information today in some cases gives the possibility of predicting some of the factors affecting the fate of pesticides in soil.

SUMMARY AND CONCLUSIONS

The results of this study and other investigator's reports indicate that in the development of an immunoassay for pesticides, the specificity of the assay is dictated by the structure of the hapten used for immunization and hapten conjugation to the carrier protein. However, the sensitivity of an immunoassay depends on its design and the reagents used in the assay. Sensitivity and detection limits can therefore be manipulated and optimized by varying assay parameters. For example, the sensitivity of the competitive ELISA for 2,4-D was improved by using an enzyme labeled 2,4-DB, which has a longer alkyl group for linking to the enzyme, instead of using labeled 2,4-D. In addition, precoating the plates with protein A, which orients the antibodies on the solid surface so that it can more effectively interact with the analyte in solution, results in improved assay sensitivity.

The use of simple sample preparation procedures, such as SPE, also increased the reliability as well as the detection limits of ELISA. For instance, employing SPE prior to ELISA eliminated the cross-reactivity problem in the alachlor ELISA by separating cross-reacting metabolite from alachlor. However, in coupling SPE and ELISA, important factors must be considered. First, the mechanism of adsorption/desorption of the analytes and the interfering substances on the solid phase must be understood in order to be able to predict the suitable resin and eluting solvents for the procedure. Secondly, the physicochemical properties of the analytes must be known so that the capacity of the solid phase resin is not exceeded and the analytes are not lost during solvent exchange. Thirdly, the tolerance of the ELISA for organic

solvents must be evaluated to avoid losses from incomplete dissolution of the analytes without adversely affecting the performance of the ELISA.

Selection of the immunizing hapten is the single most important factor in eliciting antibodies of desired specificity and sensitivity. Today, the design of the hapten is usually based on reasonable intuition, although generalizations have been made on how the hapten structure and conjugation affect the specificity of the antibodies. However, these generalizations have a lot of exceptions and oftentimes the production of the best antibodies is still a matter of trial and error. The use of computer modeling will become progressively important in the rational design of haptens, interpretation of the interaction of antibodies and analytes, and ultimately in the molecular engineering of antibody molecules. It is expected that molecular modeling will provide great insight into explaining assay specificity and in designing optimal haptens. Systems for the visualization, energy minimization, and comparison of molecules will be increasingly important in sophisticated hapten design to aid in planning the synthesis of the haptens.

Sample preparation is as important as the main analytical procedure. Many laboratories are trying to minimize the use of organic solvents, not only for cost-savings but for safety and environmental reasons. Sample preparation techniques which use lower volumes of solvents are beginning to attract attention. Solid-phase extraction is one of the most popular approaches because it allows trace enrichment and clean-up in one step. However, as illustrated in Chapter II of this investigation, low detection limits cannot always be obtained in contaminated surface waters owing to the many interfering compounds co-extracted and co-eluted with reversed-phase sorbents. Immunoaffinity sorbents could circumvent this limitation. Immunoaffinity

chromatography (IAC) is based on analyte-antibody interactions. These phases are much more selective than phases relying on hydrophobic interactions, adsorption, ion-exchange, etc. They do, however, rely on the availability of specific antisera to the analytes of interest and on the successful immobilization of the antisera onto a suitable solid phase. In this study, it was shown that IAC reduces matrix interferences, thus producing extracts that are cleaner and easier to quantify. The immunoaffinity approach followed by chromatography combines the specificity obtainable from immunoassays with the confirmation that can be assured from chromatographic methods. IAC is particularly valuable for the isolation of polar ionic compounds, such as metabolites of herbicides. Despite the many attractive features of IAC, it has not found popularity in the environmental field probably because of the large antibody requirement to prepare immunoaffinity columns.

Neither polyclonal nor monoclonal technologies are effective enough to produce commercial quantities of antibodies for low cost affinity chromatography and concentration systems. Recombinant antibody technology could be the solution to this problem. However, the complexity of cloning, assembling and expressing antibody molecules has hampered the advancement of this field. Considering the difficulty of the task, exceptional progress is being made. As antibodies to a wider range of pesticides become available inexpensively the IAC technique will become more popular.

For the last 20 years the acceptance of immunoassay technology in environmental chemistry has been very slow despite many clear demonstrations that it provides high quality data at a low price. Yet, in the last few years the rate of acceptance of the technology has increased dramatically. The supportive action taken

by regulatory agencies and some private sectors has helped in the promotion of immunoassay technology in the field of environmental chemistry. The USEPA, for example, sponsors an annual meeting to facilitate a dialog between immunoassay developers and users regarding the capabilities of the analytical methods and the criteria for their acceptance by regulatory agencies. Several ELISA methods are already considered "EPA approved methods" for use in analyzing samples for the Resource Conservation and Recovery Act (RCRA).

An industry association called the Analytical Environmental Immunochemical Consortium (AEIC) was formed in 1994 to establish performance standards for the use of immunochemical methods for environmental analysis. Initial efforts of this group are being directed towards establishing consistent definitions for commonly used terms, developing standardized package insert information, establishing sources of kit calibrators and quality control samples, and providing guidelines for user quality control. Furthermore, the development of a process through the Association of Official Analytical Chemists Research Institute to obtain independent evaluation of the performance of a kit will have a positive impact. As practicing analytical chemists begin to employ these assays, feedback should illustrate new applications of the technology as well as weak points in the technology that may be corrected when properly approached.

With the acceptance of immunochemical technology, more innovative concepts are now being applied for application to the environmental field. More than simply screening of environmental samples, it is likely that immunochemistry will be among the many hyphenated technologies in the analytical field. For instance, it was illustrated in this investigation that SPE-ELISA provides a more quantitative technique for the

analysis of alachlor than straight ELISA of which use is limited for screening due to its cross-reactivity towards herbicide metabolites. Other innovations have been reported such as the use of supercritical fluid extraction (SFE-ELISA) for soil sample preparation prior to the assay, high performance immunoaffinity chromatography (HPIAC), and flow-injection immunoanalysis (FIIA), each of which have been developed in response to a particular limitation encountered in the existing method. Attempts have been made in the development of biosensors and multi-analyte immunoassays for environmental analysis. Although these are ideal systems for environmental testing, these technologies are still far from routine practical use.

New techniques, applications, and alternative approaches regularly enter the field of residue analysis and frequently claim to be superior to techniques which have been developed in the past. However, only a limited few find applications outside the laboratory which developed them. The suitability of a technique is not solely determined by its analytical parameters such as specificity and reproducibility but also by non-analytical parameters such as costs and the availability of critical reagents and equipment.

Commercial ELISA kits are now available for a wide range of pesticides, but traditional analytical chemists are skeptical about their reliability for environmental analysis. Skepticism to adopt new methods is good because it is important to be able to compare analytical data among laboratories throughout the world and to compare data generated through time. The use of monoclonal antibodies, which in theory should not change characteristics through time, may reduce skepticism among environmental analytical chemists. Furthermore, availability of useful information such as the structure of the hapten used for immunization will allow the end-users to

possibly predict the cross-reactivity of the assay kits. Although most ELISA kits have product inserts that contain a list of compounds tested for cross-reactivity, they do not always contain pesticide metabolites because of the unavailability of the standards. Understanding the principles of the method and recognizing its limitations is critical in the success of this technology.

It is anticipated that successful demonstrations of the applications of immunoassays in the field of environmental chemistry will increase the confidence of analysts to employ the technology in studies were it can be a suitable analytical method. For example, it was demonstrated in this research that using ELISA as a major analytical tool in a field dissipation study allowed the investigators to be able to collect and analyze more samples, thereby increasing the accuracy of the conclusions made.

The use of immunoassays in monitoring the level of pesticide contamination in the environment could make frequent monitoring more feasible. It has been reported that the EPA-required quarterly sampling to comply with the water quality standards generally underestimates annual mean herbicide concentrations due to the seasonality of herbicide occurrence (Battaglin, in press). For example, the seasonal nature of herbicide occurrence and transport in midwestern rivers makes it difficult to calculate accurate estimates of annual mean concentrations using calendar based sampling strategies with a limited number of samples. A more accurate representation of annual mean concentrations could be obtained by sampling more frequently especially during spring and early summer runoff. For this purpose, immunoassay would be the most practical analytical tool that would give reliable results.

New pesticide registration requirements now include the submission of a low-cost immunoassay for monitoring residues of the new product in the environment.

Immunoassays could be particularly useful for newly registered pesticides which do not have designated MCLs yet. In addition, Federal water quality standards for existing pesticides may also have to be reconsidered if new knowledge on their occurrence and fate in the environment is revealed as a result of more frequent surveys.

This investigation represents an intensive study and evaluation of the immunoassay applications in environmental analysis. Factors that influence the accuracy and sensitivity of ELISAs were examined. A succesful demonstration of the analytical applications of ELISAs in field dissipation studies and water quality surveys was made. The results of this study will increase the confidence of immunoassay users on this technology and help end-users of commercial ELISA kits to understand the advantages and limitations of the immunoassay technology. It is important to recognize that immunoassay is not meant to replace conventional analytical techniques, but rather to complement them. Just like any analytical method, proper validation of results is critical in making correct interpretations.

REFERENCES

Aga, D.S., Thurman, E.M., Pomes, M.L. Determination of alachlor and its sulfonic acid metabolite in water by solid-phase extraction and enzyme-linked immunosorbent assay. *Analytical Chemistry* (1994) **66**: 1495-1499.

Aga, D.S., Thurman, E.M. Coupling solid-phase extraction and enzyme-linked immunosorbent assay for ultratrace determination of herbicides in pristine water. *Analytical Chemistry* (1993) **65**: 2894-2898.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Eds., Short Protocols in Molecular Biology (1992) John Wiley & Sons, NY, Chapter 11, 1-54.

Bailey, G.W., Thurston, A.D., Pope, J.D., Cochrane, D.R. The degradation kinetics of an ester of silvex and the persistence of silvex in water and sediment. *Weed Science* (1970) **18**: 413-419

Baker, D.B., Bushway, R.J., Adams, S.A., Macomber, C.S. Immunoassay screens for alachlor in rural wells: false positives and an alachlor soil metabolite. *Environmental Science and Technology* (1993) **27**: 562-564.

Battaglin, W.A., Hay, L.E. Effects of sampling strategies on estimates of annual mean herbicide concentrations in midwest rivers. *Environmental Science and Technology* (*in press*) .

Beatty, J.D., Beatty, B.G., Vlahos, W.G. Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. *Journal of Immunological Methods* (1987) **100**: 173-179.

Bogus, E.R., Watschke, T.L., Mumma, R.O. Utilization of Solid-Phase Extraction and Reversed-Phase and Ion-Pair Chromatography in the Analysis of Seven Agrochemicals in Water. *Journal of Agricultural and Food Chemistry* (1990) **38**: 142-144.

Brady, J.F., LeMasters, G.S., Williams, R.K., Pittman, J.H., Daubert, J.P., Cheung, M.W., Skinner, D.H., Turner, J., Rowland, M.A., Lange, J., Sobek, S.M. Immunoassay analysis and gas chromatography confirmation of atrazine residues in water samples from a field study conducted in the state of Wisconsin. *Journal of Agricultural and Food Chemistry* (1995) **43**: 268-274.

Breaux, E.J., Patanella, J.E., Sanders E.F. Chloroacetanilide herbicide selectivity: analysis of glutathione and homoglutathione in tolerant, susceptible and safened seedlings. *Journal of Agricultural and Food Chemistry* (1987) **35**:474-478.

Bucholtz, D.L., Lavy, T.L. Pesticide interactions in oats (*Avena sativa* L 'Neal'). *Journal of Agricultural and Food Chemistry* (1978) **26**:520-524.

Buhler, D.D., Randall, G.W., Koskinen, W.C., and Wyse, D.L. Water quality: atrazine and alachlor losses from subsurface tile drainage of a clay loam soil. *Journal of Environmental Quality* (1993) **22**: 583-588.

Buser, H.R. Atrazine and other s-triazine herbicides in lakes and in rain in Switzerland. *Environmental Science & Technology* (1990) **24**: 1049-1058.

Bushway, R.J., Perkins, B., Savage, S.A., Lekousi, S.J, Ferguson, B.S. Determination of atrazine residue in water and soil by enzyme-immunoassay. *Bulletin of Environmental Contamination Toxicology* (1988) **40**: 647-654.

Buttle, J.M. The role of sediment in metolachlor transport from agricultural fields, In *Sediment and the Environment*, Hadley, R.F. and Ongley, E.D., Eds., IAHS publication no. 184, IAHS: USA (1989) 11-18.

Capel, P.D. Wet deposition of herbicides in Minnesota, In *Water Resources Investigations Report 91-4034*, Mallard, G.E., and Aronson, D.A., Eds., Proceedings of U.S. Geological Survey Toxics Substances Hydrology Program Technical Meeting (1991) 334-337.

Castellani, W.J., Lente, F.V., Chou, D. Applications of robotics in the clinical laboratory. *Journal of Automatic Chemistry* (1990) **12**: 141-144.

Chester, G., Simsiman, G.V., Levy, J., Alhajjar, B.J., Fathulla, R.N., and Harkin, J.M. Environmental Fate of Alachlor and Metolachlor, In *Reviews of Environmental Contamination and Toxicology*, Ware, G.W., Ed., Springer-Verlag, New York, NY (1989) 1-65.

Council for Agricultural Science and Technology (CAST), The Phenoxy Herbicides. *Weed Science* (1975) **23**: 253p.

Deal, L.M., Reeves, J.T., Larkins, B.A., Hess, F.D. Use of an *in vitro* protein synthesizing system to test the mode of action of chloroacetamides. *Weed Science* (1980) **28**:334-340.

Di Corcia, A., Marchetti, M., and Samperi, R. Extraction and isolation of phenoxy acid herbicides in environmental waters using two adsorbents in one minicartridge. *Analytical Chemistry* (1989) **61**: 1363-1367.

Eisenreich, S.J., Looney, B.B., Thornton, J.D. Airborne organic contaminants in the great lakes ecosystem. *Environmental Science and Technology* (1981) **15**: 30-38.

Environmental Protection Agency, March 11, 1994. Questions & answers: conditional registration of acetochlor. Prevention, Pesticides and Toxic Substances (7506C).

Eremin, S.A., Lunskaya, I.M., Egorov, A.M. Influence of the structure of the tracer on the sensitivity and specificity of the polarization immunofluoroassay of 2,4-dichlorophenoxyacetic acid. *Vioorganicheskaya Khimiya* (1993) **19**: 836-843.

Feng, P.C.C. Soil transformation of acetochlor via glutathione conjugation. *Pesticide Biochemistry and Physiology* (1991) **40**:136-142.

Feng, P.C.C., Horton, S.R., Sharp, C.R. A general method for developing immunoassays to chloroacetanilide herbicides. *Journal of Agricultural and Food Chemistry* (1992) **40**: 211-214.

Feng, P.C.C., Wratten, S.J., Logusch, E.W., Horton, S.R., Sharp, C.R. Immunoassay detection methods for alachlor, In *Immunochemical Methods for Environmental Analysis*. Van Emon, J.M., Mumma, R.O., Eds., American Chemical Society Symposium Series 442 (1990a) 180-192.

Feng, P.C.C., Wratten, S.J., Horton, S.R., Sharp, C.R., Logusch, E.W. Development of an enzyme-linked immunosorbent assay for alachlor and its application to the analysis of environmental water samples. *Journal of Agricultural and Food Chemistry* (1990b) **38**: 159-163

Fleeker, J. Two enzyme immunoassays to screen for 2,4-dichlorophenoxyacetic acid in water. *Journal of the Association of Official Analytical Chemists* (1987) **70**: 874-878.

Gerstl, Z. Behaviour of organic agrochemicals in irrigated soils, In *Chemistry, Agriculture and the Environment*, Richardson, M.L., Ed. (1991) 332-369.

Gianessi, L.P., Puffer, C. Herbicide Use in the United States. Quality of the Environment Division, Resources for the Future, Washington, D.C. (1991).

Glotfelty, D.E., Williams, G.H., Freeman, H.P., Leech, M.M. Regional atmospheric transport and deposition of pesticides in Maryland, In *Long Range Transport of Pesticides,* Kurtz, D.A., Ed., Lewis Publishers, Inc., Chelsea, MI (1990) 199-221.

Glotfelty, D.E., Seiber, J.N., Lilijahl, L.A. Pesticides in Fog. *Nature* (1987) **325**: 602-605.

Glotfelty, D.E., Taylor, A. W., Inensee, A.R.; Jersey, J.; and Gle, S. Atrazine and Simazine Movement to Wye River Estuary: *Journal of Environmental Quality* (1984) **13**: 115-121.

Goding, J.W. Monoclonal Antibodies: Principles and Practice (1983) Academic Press, NY, 100-115.

Goers, J. Immunochemical Techniques Laboratory Manual, (1993) Academic Press, Inc., London, 188p.

Goh, K.S., Hernandez, J., Powell, S.J., Garretson, C., Toriano, J., Ray, M., Greene, C.D. Enzyme-immunoassay for the determination of atrazine residues in soil. *Bulletin of Environmental Contaminant Toxicology* (1991) **46**: 30-36.

Goodrow, M.H., Sanborn, J.R., Stoutamire, D.W., Gee, S.J., Hammock, B.D. Strategies for immunoassay hapten design, In *Immunoanalysis of Agrochemicals*, Nelson, J.O., Karu, A.E., Wong, R.B., Eds., American Chemical Society Symposium Series 586 (1995) 119-139.

Goodrow, M.H., Harrison, R.O., Hammock, B.D. Hapten synthesis, antibody development, and competitive inhibition enzyme immunoassay for *s*-triazine herbicides. *Journal of Agricultural and Food Chemistry* (1990) **38**: 990-996.

Goolsby, D.A., Battaglin, W.A. Occurrence, distribution and transport of agricultural chemicals in surface waters of the Midwestern United States, In *U.S. Geological Survey Open-File Report 93-418*, Compiled by Goolsby, D.A., Boyer, L.L, and Mallard, G.E. (1993a) 1-25.

Goolsby, D.A., Thurman, E.M., Pomes, M.L, Meyer, M.T., Battaglin, W.A. Occurrence, deposition, and long range transport of herbicides in precipitation in the Midwestern and Northeastern United States, In *U.S. Geological Survey Open-File Report 93-418,* Compiled by Goolsby, D.A., Boyer, L.L., and Mallard, G.E. (1993b) 75-89.

Goolsby, D.A., Battaglin, W.A., Fallon, J.D., Aga, D.S., Kolpin, D.W., Thurman, E.M. *Abstracts of the Technical Meeting,* U.S. Geological Survey Toxic Substances Hydrology Program, September (1993c) 83p.

Hall, J.C., Deschamps, R.J.A., Krieg, K.K. Immunoassays for the detection of 2,4-D and picloram in river water and urine. *Journal of Agricultural and Food Chemistry* (1989) **37**: 981-984.

Hamaker, J.W. *Organic Chemicals in the Soil Environment* (1972), C.A.I. Goring and J.W. Hamaker, Eds., New York: Marcel Dekker,. vol. 2.

Hammock, B.D., Gee, S.J., Harrison, R.O., Jung, F., Goodrow, M.H., Li, Q.X., Lucas, A.D., Székács, A., Sundaram, K.M.S. Immunochemical technology in environmental analysis, In *Immunochemical Methods for Environmental Analysis*, Van Emon, J.M., Mumma, R.O., Eds., American Chemical Society Symposium Series 442 (1990) 112-139.

Hammock, B.D., Gee, S.J., Cheung, P.Y.K., Miyamoto, T., Goodrow, M.H., Van Emon, J., Seiber, J.N. Utility of immunoassay in pesticide trace analysis, In *Pesticide Science and Biotechnology*, Greenhalgh, R. and Roberts, T.R., Eds., Proceedings of the Sixth Annual Congress of Pesticide Chemistry (1986) 309-316.

Harrison, R.O., Gee, S.J., Hammock, B.D. Immunochemical methods of pesticide residue analysis, In *Biotechnology in Crop Protection*, Hedin, P.A., Menn, J.J., Hollingworth, R.M., Eds., American Chemical Society Symposium Series 379, Washington, D.C. (1988) 316-330.

Hendrickson, T.L., Wilson, G.S. Improved clean-up method for the enkephalins in plasma using immunoaffinity chromatography. *Journal of Chromatography B: Biomedical Applications* (1994) **653**: 147-154.

Hermanson, G.T., Mallia, A.K., Smith, P.K. Immobilized Affinity Ligand Techniques (1992) Academic Press, Inc., London, 454p.

Hoke, S., Brueggeman, E., Baxter, L., Trybus, T. Determination of phenoxy herbicides using solid-phase extraction and HPLC. *Journal of Chromatography* (1986) **357**: 429.

Holden, L.R., Graham, J.A., Whitmore, R.W., Alexander, W.J., Pratt, R.W., Liddle, S.K., Piper, L.L. Results of the national alachlor well water survey. *Environmental Science and Technology* (1992) **26**:935-943.

Humburg, N.E., Colby, S.R., Hill, E.R., Kitchen, L.M., Lym, R.G., McAvoy, W.J., Prasad, R. Eds., Herbicide Handbook of the Weed Science Society of America, Sixth Edition (1989) 1-257.

Hurle, K., Walker, A. Persistence and its Prediction, In *Interactions between Herbicides and the Soil*, Hance, R.J., Ed. (1980) 83-122.

Junk, G.A., Avery, M.J., Richard, J.J. Interferences in Solid-Phase Extraction Using C-18 Bonded Porous Silica Cartridges. *Analytical Chemistry* (1988) **60**: 1347-1350.

Jung, F., Gee, S.J., Harrison, R.O., Goodrow, M.H., Karu, A.E., Braun, A.L., Li, Q.X., Hammock, B.D. Use of immunochemical techniques for the analysis of pesticides. *Pesticide Science* (1989) **26**: 303-317.

Katz, S.E., Siewierski, M. Drug residue analyis using immunoaffinity chromatography. *Journal of Chromatography* (1992) **624**: 403-409

Kaufman, D.D., Kearney, P.C. Microbial transformations in the soil, In *Herbicides: Physiology, Biochemistry, Ecology*, Audus, L.J., Ed. (1976) Academic Press, London, **2**: 29-64.

Keith, L.H., Libby, R.A., Crummett, W., Taylor, J.K., Deegan, J., Wentler, G. Principles of Environmental Analysis. *Analytical Chemistry* (1983) **55**: 2210-2218.

Klein, A.J. Monsanto Company, personal written communication, August 18 (1993).

Knopp, D., Schiller, F. Oral and dermal application of 2,4-dichlorophenoxyacetic acid sodium and dimethylamine salts to male rats: Investigations on absorption and excretions as well as induction of heptic mixed-function oxidase activities. *Archives of Toxicology* (1992) **66**: 170-174.

Knopp, D., Glass, S. Biological monitoring of 2,4-dichlorophenoxyacetic acid-exposed workers in agriculture and forestry. *International Archives of Occupational Environmental Health* (1991) **63**: 329-333.

Knopp, D., Nuhn, P., Dobberkau, H. Radioimmunoassay for 2,4-Dichlorophenoxyacetic Acid. *Archives of Toxicology* (1985) **58**: 27-32.

Köhler, G., Milsten, C. Continuous culture of fused cells secreting antibody of predefined specificity. *Nature* (1975) **256**: 495-497.

Kolpin, D.W., Goolsby, D.A., Aga, D.S., Iverson, J.L., and Thurman, E.M. Pesticides in near-surface aquifers: results of the midcontinental United States ground-water reconnaissance, 1991-92, In *U.S. Geological Survey Open-File Report 93-418,* Complied by Goolsby, D.A., Boyer, L.L., and Mallard, G.E. (1993) 64-74.

Kuhnt, G. The Euro-soil concept as a basis for chemicals testing and pesticide research, In *Fate and Prediction of Environmental Chemicals in Soils, Plants, and Aquatic Systems,* Mansour, M., Ed. (1993) Lewis Publishers, Boca Raton, FL, Chapter 8, 83-93.

Lachmann, P.J., Strangeways, L., Vykarnam, A., Evan, G. Raising antibodies by coupling peptides to PPD and immunizing BCG-sensitized animals. *Ciba Foundation Symposium* (1986) **119**: 25-40.

Lamoureux, G.L., Bakke, J.E. Formation and metabolism of xenobiotic glutathione conjugates in various life forms. In *Foreign Compound Metabolism*, Caldwell, J., Paulson, G.D., Eds., Taylor and Francis, London, UK (1984), 185-199.

Lamoureux, G.L., Rusness, D.G. Glutathione in the metabolism and detoxification of xenobiotics in plants. In *Sulfur Nutrition and Assimilation in Higher Plants*, Dekok, L.J. et al., Eds. SPB Academic Publishing, Netherlands (1993), 221-237.

Lamoureux, G.L., Rusness, D.G. Propachlor metabolism in soybean plants, excised soybean tissues, and soil. *Pesticide Biochemistry and Physiology* (1989) **34**:187-204.

Lamoureux, G.L., Stafford, L.E., Tanaka, F.S. Metabolism of 2-chloro-N-isopropyl-acetanilide (propachlor) in the leaves of corn, sorghum, sugar cane, and barley. *Journal of Agricultural and Food Chemistry* (1971) **19**:346-350.

Lawruk, T.S., Hottenstein, C.S., Herzog, D.P., Rubio, F.M. Quantitation of alachlor in water by a novel magentic particle-based ELISA. *Bulletin of Environmental Contaminant Toxicology* (1992) **48**: 643-650.

Leavitt, R.A., Kells, J.J., Bunkellmann, J.R., Hollingworth, R.M. Accessing atrazine persistance in soil following severe drought. *Bulletin of Environmental Contaminant Toxicology* (1991) **41**: 22-29.

Leonard, R.A., Herbicide in surface waters, In *Environmental Chemistry of Herbicides*, Grover, R, Ed., CRC Press, Boca Raton, FL, (1988) **I**: 45-87.

Loconto, P.R., Isolation and recovery from water of selected chlorophenoxy acid herbicides by solid-phase extraction HPLC and photo diode array detection. *Journal of Liquid Chromatography* (1991) **14**: 1297-1314.

Loos, M.A. Phenoxyalkanoic acids, In *Herbicides: Chemistry, Degradation, and Mode of Action*, Kearney, P.C. & Kaufman, D.D. Eds. (1975) Mercel Dekker, NY, **1**: 1-128.

Macomber, C., Bushway, R.J., Perkins, L.B., Baker, D., Fan, T.S., Ferguson, B.S. Determination of the ethanesulfonate metabolite of alachlor in water by high performance liquid chromatography. *Journal of Agricultural and Food Chemistry* (1992) **40**: 1450-1452.

Marsh, H.V., Bates, J., Downs, S. Inhibition of metabolite uptake and transport by alachlor. *Plant Physiol. Suppl.* (1979) 319.

McDonald, P.D. Waters Sep-Pak cartridge Applications Bibliography, Millipore Corp., Milford, Massachusetts (1991).

Meyer, M.T., Geochemistry of cyanazine and its metabolites: Indicators of contaminant transport in surface water of the midwestern United States. *Ph.D. Dissertation* (1994) University of Kansas.

Meyer, M.T., Mills, M.S., Thurman, E.M. Automated solid-phase extraction for herbicides from water for gas chromatographic-mass spectrometric analysis. *Journal of Chromatography* (1993) **629**: 55-59.

Narsaiah, D.B. Harvey, R.G. Alachlor and giberellic acid interaction on corn tissues. *Weed Science* (1977) **25**:197-199.

Mills, M.S., Thurman, E.M. Reduction of nonpoint source contamination of surface water and groundwater by starch encapsulation of herbicides. *Environmental Science and Technology* (1994) **28**: 73-79.

Mills, M.S., Thurman, E.M. Mixed mode isolation of triazine metabolites from soil and aquifer sediments using automated solid-phase extraction. *Analytical Chemistry* (1992) **64**: 1985-1990.

Morris, Z., Ruthann, K. Solid Phase Extraction for Sample Preparation, J.T. Baker Inc., (1988).

Nash, R.G. Dissipation from Soil, In *Environmental Chemistry of Herbicides*, Grover, R. Ed., (1988) CRC Press Inc., Boca Raton, Florida, I: 131-169.

National Research Council Canada, Ottawa, Phenoxy Herbicides - Their Effects on Environmental Quality, NRCC No. 16075 (1978).

Nations, B.K., Hallberg, G.R. Pesticides in Iowa precipitation. *Journal of Environmental Quality* (1992) **21**: 486-492.

Nègre, M., Gennari, M., Raimondo, E., Celi, L., Trevisan, M., Capril, E. Alachlor dissipation in soil as influenced by formulation and soil moisture. *Journal of Agricultural Food Chemistry* (1992) **40**: 1071-1075.

Nowell, L.H., Resek E.A. National standards and guidelines for pesticides in water, sediment, and aquatic organisms: Application to water-quality assessments, In *Reviews of Environmental Contamination and Toxicology*, Ware, G.W., Ed. (1994) **140**: 1-164.

Paterson, K.G., Schnoor, J.L. Fate of alachlor and atrazine in a riparian zone field site. *Water Environment Research* (1992) **64**: 274-283.

Penner, D., Ashton, F.M. Biochemical and metabolic changes in plants induced by chlorophenoxy herbicides. *Residue Reviews* (1966) **14**: 39-114.

Phillai, C.G.P., Davis. D.E., Mode of action of CGA-18762, CGA-17020, and CGA-24705. *Proc South Weed Sci Soc Am* (1975) **28**:308-314.

Porstmann, T., Kiessig, S.T. Enzyme-immunoassay techniques - an overview. *Journal of Immunological Methods* (1992) **150**: 5-21.

Porter, R.R. The Structure of Antibodies. *Scientific American* (1967) 81p.

Richards, P.R., Kramer, J.W., Baker, D.B., Kreiger, K.A. Pesticides in rainwater in the northwestern United States. *Nature* (1987) **327**: 129-131.

Rinder, D.F., Fleeker, J.R. A radioimmunoassay to screen for 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid in surface water. *Bulletin of Environmental Contaminant Toxicology* (1981) **26**: 375-380.

Ritter, W.F., Johnson, H.P., Lovely, W.G., Molnau, M. Atrazine, propachlor, and diazinon residues on small agricultural watersheds: Runoff losses, persistence, and movement. *Environmental Science and Technology* (1974) **8**:38-42.

Rubio, F.M., Itak, J.A., Scutellaro, A.M., Selisker, M.Y., Herzog, D.P. *Food and Agricultural Immunology* (1991) **3**: 113-125.

Sandmann, E.R.I.C., Loos, M.A., Van Dyk, L.P. The microbial degradation of 2,4-dichlorophenoxyacetic acid in soil. In *Reviews of Environmental Contamination and Toxicology*, Ware, G.W., Ed. (1988) Springer-Verlag, NY, **101**: 3-53.

Schneider, P., Hammock, B.D. Influence of the ELISA format and hapten-enzyme conjugate on the sensitivity of an immunoassay for the *s*- triazine herbicides using monoclonal antibodies. *Journal of Agricultural and Food Chemistry* (1992) **40**: 525-530.

Seiber, J.N., Woodrow, J.E., Methods for studying pesticide atmospheric dispersal and fate at treated areas, *Residue Reviews* (1983) **85:** 217.

Sharp, S., Salleh, A., To 2,4 D or Not to 2,4 D? *Search* (1993) **24**: 60-62

Sharp, D.B. Alachlor, In *Herbicides: Chemistry, Degradation and Mode of Action*; Kearney, P.C., Kaufman, D.D., Eds. (1988) Dekker, NY 301-333.

Sherry, J.P. Environmental chemistry: The immunoassay option. *Critical Reviews in Analytical Chemistry* (1992) **23**: 217-300.

Sherwood, C.H., Weigle, J.L., Denisen, E.L. *HortScience* (1970) B: 211.

Sirons, G.J., Chau, A.S.Y., Smith A.E. The Chlorophenoxy acid herbicides (CPHs), In *Analysis of Pesticides in Water Volume II Chlorine- and Phosphorus-Containing Pesticides*, Robinson, J.W., Chau, A.S.Y., Afghan, B.K., Eds. (1982) CRC Press, Inc., Boca Raton, FL, Chapter 3: 155-227.

Smith, A.E. Identification of 2,4-dichloroanisole and 2,4-dichlorophenol as soil degradation products of ring-labeled (14C) 2,4-D. *Bulletin of Environmental Contaminant Toxicology* (1985a) **34**: 150-157.

Smith, A.E. Identification of 4-chloro-2-methylphenol as a soil degradation product of ring-labeled (14C) mecoprop. *Bulletin of Environmental Contaminant Toxicology* (1985b) **34**: 656-660.

Smith, A.E. The hydrolysis of herbicidal phenoxyalkanoic esters to phenoxyalkanoic acid in Saskatchewan soils. *Weed Research* (1976) **16**: 19-22.

Smith, A.E. The hydrolysis of 2,4-D esters to 2,4-dichlorophenoxyacetic acids in Saskatchewan soils. *Weed Research* (1972) **12**: 364-372.

Smith, C.N., and Parrish, R.S. A field study to evaluate leaching of aldicarb, metolachlor, and bromide in a sandy loam soil. *Journal of Environmental Quality* (1993) **22**: 562-577.

Squillace, P.J., Thurman, E.M. Herbicide transport in rivers: Importance of hydrology and geochemistry in nonpoint-source contamination. *Environmental Science and Technology* (1992) **26**: 538-545.

Staros, J.V., Wright, R.W., Swingle, D.M. Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. *Analytical Biochemistry* (1986) **156**: 220-222.

Stearman, G.K., Wells, M.J. Enzyme Immunoassay Microtiter Plate Response to Atrazine and Metolachlor in Potentially Interfering Matrices. *Bulletin of Environmental Contamination and Toxicology* (1993) **51**:588-595.

Stearman, G.K., Adams, V.D. Atrazine soil extraction techniques for enzyme immunoassay microtiter plate analysis. *Bulletin of Environmental Contamination and Toxicology* (1992) **48**: 144-151.

Stevens, T.S., Skelly, N.E., Growd, R.B. Report of the Associated Referee for the Phenoxy Acid Herbicide Formulations, 91st Annual Meeting of the Association of Official Analytical Chemists, Washington, DC (1977).

Steward, M.W. Antibodies: Their structure and function. Brammer, W.J., Edidin, M. Eds., Chapman and Hall, London (1984), 96p.

Taylor, A.W., Freeman, H.P., Edwards, W.M., *Journal of Agricultural and Food Chemistry* (1971) **19**:832-836.

Thurman, E.M., Goolsby, D.A., Meyer, M.T., Mills, M.S., Pomes, M.L, Kolpin, D.W. A reconnaissance study of herbicides and their metabolites in surface water of the midwestern UnitedStates using immunoassay and gas chromatography/mass spectrometry. *Environmental Science and Technology* (1992) **26**: 2440-2447.

Thurman, E.M., Goolsby, D.A., Meyer, M.T., Kolpin, D.W. Herbicides in surface waters of the midwestern United States: The effect of spring flush. *Environmental Science and Technology* (1991) **25**: 1794-1796.

Thurman, E.M., Meyer, M.T., Pomes, M.L., Perry, C.A., Schwab, A.P. Enzyme-linked immunosorbent assay compared with gas chromatography/mass spectrometry for the determination of triazine herbicides in water. *Analytical Chemistry* (1990) **62**: 2043-2048.

Thurman, E.M. Organic Geochemistry of Natural Waters, (1985) Martinus Nijhoff/Dr W. Junk Publishers. 273-361.

Thurman, E.M., Malcolm, R.L., Aiken, G.R. Prediction of capacity factors for aqueous organic solutes adsorbed on a porous acrylic resin. *Analytical Chemistry* (1978) **50**: 775-779.

Tippins, B. Solid-Phase Extraction Fundamentals. *Nature* (1988) **334**: 273-274.

U.S. Environmental Protection Agency, (1992) Drinking Water Regulations and Health Advisories: Washington, DC, Office of Water, 13p.

Trevisan, M., Montepiani, C., Ragozza, L., Bartoletti, C., Ioannilli, E., Del Re, A.A.M. Pesticides in rainfall and air in Italy. *Environmental Pollution* (1993) **80**: 64-39.

Vanderlaan, M., Stanker, L., Watkins, B. In *Immunoassay for Trace Chemical Analysis;* Vanderlaan, M., Stanker, L.H., Watkins, B.E., Roberts, D.W., Eds., American Chemical Society Symposium Series 451 (1990) 2-13.

Vanderlaan, M.; Watkins, B.E.; Stanker, L. Environmental monitoring by immunoassay. *Environmental Science and Technology* (1988) **22**: 247-254.

Van Emon, J.M. Lopez-Avila, V. Immunochemical methods for environmental analysis. *Analytical Chemistry* (1992) **62**: 79A-99A.

Van Emon, J.M., Hammock, B.D., Seiber, J.N. Enzyme-linked immunosorbent assay for paraquat and its application to exposure analysis. *Analytical Chemistry* (1986) **58**: 1866-1873.

Van Ginkel, L.A. Immunoaffinity Chromatography, its applicability and limitations in multi-residue analysis of anabolizing and doping agents. *Journal of Chromatography: Biomedical Applications* (1991) **564**:363-384.

Varian. *Applications Bibliography Sample Preparation Products (*1992).
Walker, A., Moon, Y., and Welch, S.J. Influence of temperature, soil moisture and soil characterisitics on the persistence of alachlor. *Pesticide Science* (1992) **35**: 109-116.

Warmund, M.R., Kerr, H.D., Peters, E.J. Lipid metabolism in grain sorghum (*Sorghum bicolor*) treated with alachlor plus flurazole. *Weed Science* (1985) **33**:25-28.

Wells, M. and Michael, J. Recovery of picloram and 2,4-D from aqueous samples by reversed-phase solid-phase extraction. *Analytical Chemistry* (1987) **59**: 1739.

Weston, P.D., Avrameas, S. Proteins coupled to polyacrylamide beads using glutaraldehyde. *Biochemical and Biophysical Research Communications* (1971) **45**: 1574-1580.

Wie, S.I., Hammock, B.D. Comparison of coating and immunizing antigen structure on the sensitivity and specificity of immunoassays for benzoylphenlurea insecticides. *Journal of Agricultural and Food Chemistry* (1984) **32**: 1294-1301.

Wie, S.I., Hammock, B.D. Development of enzyme-linked immunosorbent assays for residue analysis of diflubenzuron and bay SR-8514. *Journal of Agricultural and Food Chemistry* (1982) **30**: 949-957.

Wieterson, R.S., Daniel, T.C., Fermanich, K.J., Girard, B.D., McSweeney, K., and Lowery, B. Organic chemicals in the environment: atrazine, alachlor, and metolachlor mobility through two sandy Wisconsin soils. *Journal of Environmental Quality* (1993) **22**: 811-818.

Wilkerson, R.E. Influence of sorghum (*Sorghum bicolor* cultivar G522DR) growth and gibberellin precursor biosynthesis. *Pesticide Biochemistry and Physiology* (1982) **17**:177-184.

Williams, A.L., Sweet, C.W., Peters, C. Herbicide concentrations in air and rain, In *Proceedings of the 85th Annual Meeting and Exhibition of the Air and Waste Management Association* (1992) 2-12.

Wing, K.D., Hammock, B.D., Wustner, D.A. Development of an S-bioallethrin specific antibody. *Journal of Agricultural and Food Chemistry* (1978) **26**: 1328-1333.

Wittmann, C., Hock, B. Development of an ELISA for the analysis of atrazine metabolites deethylatrazine and deisopropylatrazine. *Journal of Agricultural and Food Chemistry* (1991) **39**: 1194-1200.

Wong, R.B., Pont, J.L., Johnson,D.H., Zulalian, J., Chin, T., Karu, A.E., Immunoaffinity chromatography applications in pesticide metabolism and residue analysis. *Immunoanalysis of Agrochemicals* (1995) 235-247.

Wu, T.L. Atrazine residues in estuarine water and the areal deposition of atrazine into Rhode River, Maryland. *Water, Air, and Soil Pollution* (1981) **15**: 173-184.

Yalow, R.S., Berson, S.A. Immunoassay of endogenous plasma insulin in man. *Journal of Clinical Investigation* (1960) **39**: 1157-1175.

Yen, P.Y., Koskinen C., and Schweizer, E.E. Dissipation of alachlor in four soils as influenced by degradation and sorption processes. *Weed Science* (1994) **42**: 233-240.

Yip, G. Analysis for herbicides and metabolites. *Journal of Chromatography* (1975) **9**: 1144.

Zimdahl, R.L., Clark, S.K. Degradation of three acetanilide herbicides in soil. *Weed Science* (1982) **30**:545-548.