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Environmental analysis of polar herbicides in complex organic-rich matrices by high performance liquid chromatography atmospheric pressure ionization mass spectrometry (HPLC-API-MS)

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

ENVIRONMENTAL ANALYSIS OF POLAR HERBICIDES IN COMPLEX ORGANIC-RICH MATRICES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ATMOSPHERIC PRESSURE IONIZATION MASS SPECTROMETRY (HPLC-API-MS)

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in

FORENSIC SCIENCE

by

Luis Arroyo-Mora

2003

To: Dean R. Bruce Dunlap
College of Arts and Sciences

This thesis, written by Luis Arroyo-Mora, and entitled Environmental Analysis of Polar Herbicides in Complex Organic-Rich Matrices by High Performance Liquid Chromatography Atmospheric Pressure Ionization Mass Spectrometry (HPLC-API-MS), having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

Rudolf Jaffe

Jose R. Almirall

Piero R. Gardinali, Major Professor

Date of Defense: November 20, 2003

The thesis of Luis Arroyo-Mora is approved.

Dean R. Bruce Dunlap
College of Arts and Sciences

Dean Douglas Wartzok
University Graduate School

Florida International University, 2003

DEDICATION

To my wife Tatiana, who deserves all my respect and admiration

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The opportunity for professional and personal growing at FIU was one of the greatest experiences in my life as a graduate student.

First of all, I would like to thank God for his constant blessing in every aspect of my existence. Without him we are just dust in the wind.

Many people are part of this written document. The support and continuous dedication of my major professor Dr. Piero Gardinali during every step of my research definitely makes him a real "Daddy" at FIU. Thanks for believing in me and keeping the faith. I value your friendship and good will for both Tatiana and me during our time here at FIU. Thanks for also sharing all your expertise, and for teaching me not only chemistry but most importantly your own philosophy of life.

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ABSTRACT OF THE THESIS

ENVIRONMENTAL ANALYSIS OF POLAR HERBICIDES IN COMPLEX ORGANIC-RICH MATRICES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ATMOSPHERIC PRESSURE IONIZATION MASS SPECTROMETRY (HPLC-API-MS)

by

Luis Arroyo-Mora

Florida International University, 2003

Miami, Florida

Professor Piero R. Gardinali, Major Professor

A comprehensive forensic investigation of sensitive ecosystems in the Everglades Area is presented. Assessing the background levels of contamination in these ecosystems represents a vital resource to build up forensic evidence required to enforce future environmental crimes within the studied areas. This investigation presents the development and validation of a fractionation and isolation method for two families of herbicides commonly applied in the vicinity of the study area, including phenoxy acids like 2,4-D, MCPA, and silvex; as well as the most common triazine-based herbicides like atrazine, prometyne, simazine and related metabolites like DIA and DEA. Accelerated solvent extraction (ASE) and solid phase extraction (SPE) were used to isolate the analytes from abiotic matrices containing large amounts of organic material. Atmospheric-pressure ionization (API) with electrospray ionization in negative mode (ESP^-), and Chemical Ionization in the positive mode ($APCI^+$) were used to perform the characterization of the herbicides of interest.

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ENVIRONMENTAL FATE, TRANSPORT, AND EFFECTS OF PESTICIDES IN COASTAL ENVIRONMENTS

Coastal zones in the United States host a vast variety of human activity. The inevitable increase of population that occurs in these areas, has introduced a lot of contaminants main through industrial wastewater sewage and particulate transport.

One of the majors concerns in present day life linked to the population increase is the creation of efficient food production systems. The State of Florida is one the richest places for farming because of its warm climate. Approximate 3600 km^2 of land are used for agricultural purposes. In South Florida, 2000 km^2 are devoted for the production of sugar cane (80%), and the other 20 % is used mainly for vegetables, rice and sod (Miles and Pfeuffer 1997). Nursery and floriculture represents another two sources of incomes to the State of Florida. Two of the most important agricultural zones in Florida are the Homestead Agricultural Area (HAA) and the Everglades Agricultural Area (EAA). Together these areas provide a large part of the vegetables supply within US during the winter season.

Because of the economic importance, the mentioned agricultural lands are used intensively which means that they required high amounts of pesticides and herbicides for crop protection. Although agrochemical applications are being regulated is inevitable that a portion of these pesticides will impact surface waters and other important biological resources. In addition, large amounts of pesticides and herbicides are directly applied to waters systems for control of undesirable insects (specially for mosquito

control), plants and weeds. Those pesticides if persistent are carried to adjacent areas by water movement.

Several mechanisms describe the fate and transport of the pesticides in coastal environments (Albaiges 1989). Figure 1 describes the basic ways in which a toxicant could be introduced in such environment. It is important to notice that the distribution and fate of pesticides are determined by different variables that include the nature of the pesticide, its sorption, and the surrounding environmental variables such as pH, temperature, humidity, content of organic matter, presence of aquatic life, and sediments. (Grover 1988; Neilson 1994) Once a pesticide is applied, small to large portions could be carried by the wind to non-target areas. This is called the drift or volatilization of the pesticides and it represents one of the transport mechanisms normally found in pesticides spreading.

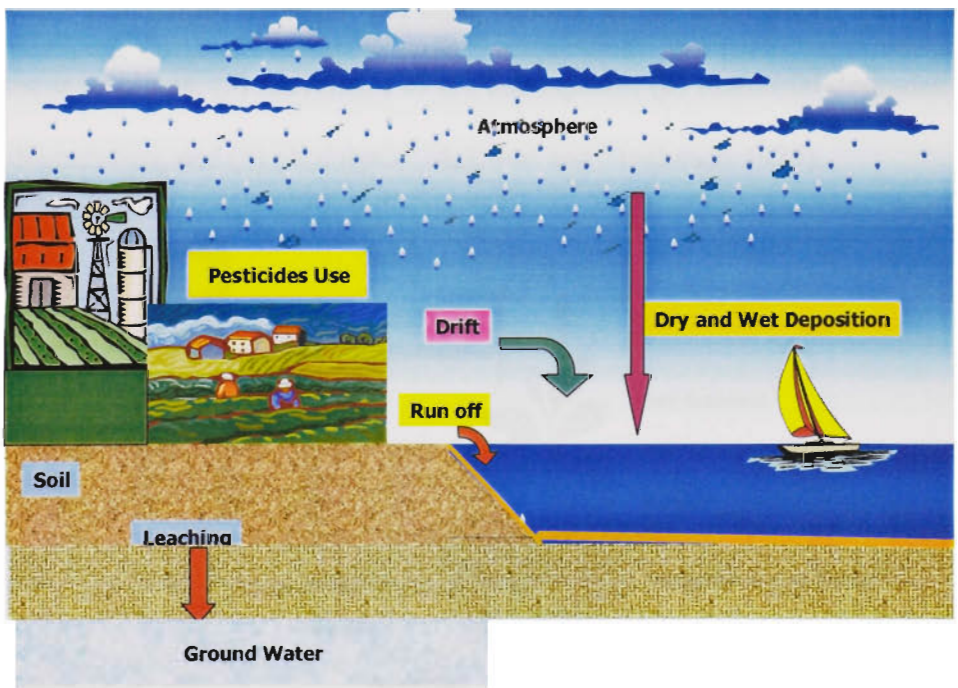


Figure 1: Fate and distribution of pesticides in coastal environments

The atmospheric fallout in which the rain is the carrier of the pesticide is another mechanism. Several manufacturing industries use pesticides in their process and their effluents may content significant residues of pesticides. Municipal sewage contains the combined discharges from industrial plants and agriculture residues from some urban runoff. All of them could contribute to the release of herbicides residues. Also because of the handling of pesticides, the storage and transportation could generate large-scale industrial spills, which can produce major impact in highly sensitive ecosystems normally present in coastal zones.

As primary agricultural resources, soils are the first point of contact of pesticides. Chemical, biological, and physical forces control the fate of pesticides in soils. The factors that influence the behavior and fate of pesticides after contact with soil are shown in figure 2. These include: (1) adsorption to clay and organic matter, (2) leaching with the downward percolation of water, (3) volatilization to the atmosphere, (4) uptake by soil organisms or plants, (5) movement to the atmosphere, (6) microbial degradation, (7) chemical degradation, and (8) photolysis (McEwen and Stepheson 1979).

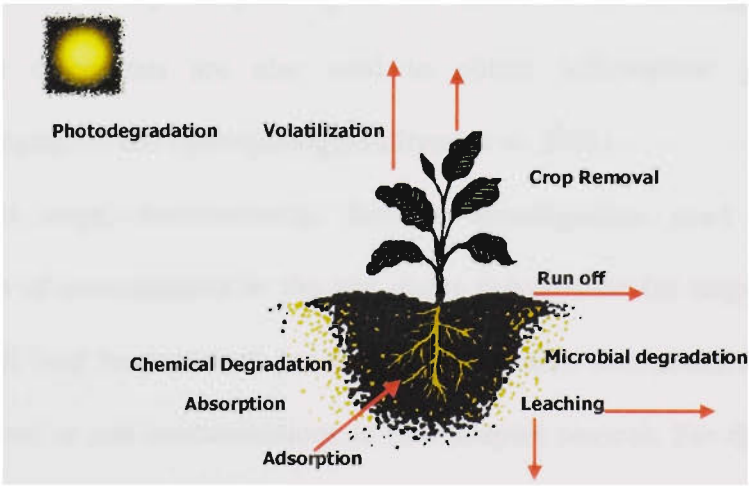


Figure 2: Mechanism that influences the behavior of pesticides in soils

The dispersion and transport of pesticides residues into the environment by surface runoff from agricultural lands or flooding has been a major concern in the past 20 years. This is probably the principal mechanism of transport of pesticides present in the agricultural areas of South Florida. With the plans for increasing the water flow in the CERP and the requirements for the creation of Storm Treatment Areas (STA's), both anthropogenic and agricultural effluents discharges will impact important areas of South Florida such as ENP or BNP. For these reasons, the study and chemical monitoring of these pesticides and herbicides is crucial and of high priority to guarantee ecosystem sustainability.

ENVIRONMENTAL FORENSICS

Environmental forensics is a relatively new branch of Forensic Sciences that involves interdisciplinary approaches and investigative techniques associated with analytical and atmospheric chemistry, environmental fate assessment, and environmental law. It also includes different techniques such as aerial photography, statigraphy, isotopic analysis, and computer modeling for determining potential origin source (Morrison 2000; Murphy and Morrison 2002). Depending on the nature of the investigation, several applied scientific disciplines are also used to obtain information: geochemistry, toxicology, oceanography, and hydrogeology(Sullivan et al. 2001).

As a first stage, environmental forensic investigations need to look for background levels of contaminants in the site under surveillance (or target site). This scientific data will help to document pre-impact information that could demonstrate a relationship between *in situ* contaminations to new suspect sources. For these reason, a comprehensive forensic investigation has been implemented, by the Southeast

Environmental Research Center (SERC), at Florida International University in order to evaluate the background levels of contaminants that could be impacting the ecosystems found in the Everglades National Park and Biscayne Bay National Park. This forensic investigation pretends to define the identity, spatial distribution, and potential exposure concentration of organic biocides within South Florida ecosystems at risk.

At present there is limited information about the concentration of both organic and inorganic contaminants in South Florida with the exception of mercury and nutrients. This situation has created increased concern over to potential hazards associated with the exposure of sensitive protected species to these contaminants. Since the Everglades restoration requires the delivery of potentially contaminated water due to efforts devoted to the Comprehensive Everglades Restoration Project (CERP) and in support of future management plans, environmental forensic data on the occurrence, source identification, and temporal variation of chemical stressors is needed for all compartments of South Florida ecosystems.

The systematic examination of background levels of contaminants is essential to determine suspect sources of chemical contamination, the timing of releases, and spatial distribution of the toxicants. This analytical background information constitutes an invaluable mean to the detection and control of possible environmental crimes such as illegal dumping and violation of restricted practices within the protected areas. The agricultural zones that surround these parks, Homestead Agricultural Area (HAA) and Everglades Agricultural Area (EAA) are considered potential contaminant sources not only because of the increasing number of poorly regulated nurseries contributing run off

of many different pollutants to protected ecosystems but also for the high usage of pesticides for crop protection.

PHENOXY ACID HERBICIDES

Phenoxy acid herbicides (shown in table 1), were introduced as selective weed killers at the end of the World War II and since then they have been extensively used to control the growth of grass, weeds and broadleaf in a wide variety of crops. The general structure of a phenoxy acid herbicide is shown below:

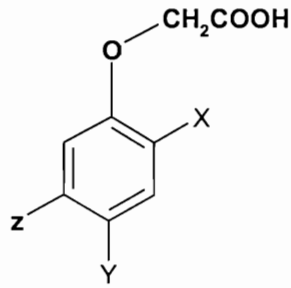
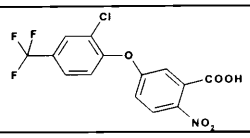
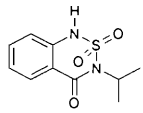
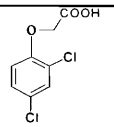
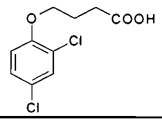
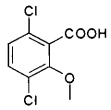
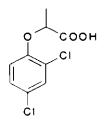
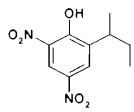
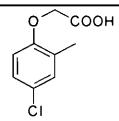
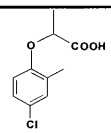
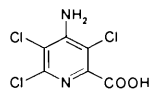
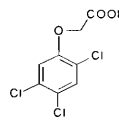
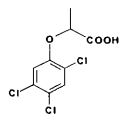


Figure 3: Basic chemical structure of phenoxy acid herbicides

The discovery of 2,4-D (X = Y = Cl) precipitated the greatest single advance in the science of weed control and one of the most significant in agriculture (Bovey and Young 1980). This herbicide was found to be very effective and selective at very low rates of applications (0.5-1.0 kg/hectare) for the control of broadleaf weeds in cereals and motivated the research for other similar chemicals that could be used as selective herbicides (McEwen and Stepheson 1979). The mode of action of phenoxy acid herbicides is based on their ability to mimic natural auxin, β-indoleacetic acid, producing an abnormal lethal growth in the affected vegetation.

Table 1: Phenoxy acid herbicides and related compounds

#	Compound	Structure	Molecular Weight
1	Acifluorfen $C_{14}H_7ClF_3NO_5$ CAS # 50594-66-6		360
2	Bentazon $C_{10}H_{12}N_2O_3S$ CAS # 25057-89-0		240
3	2,4-D $C_8H_6Cl_2O_3$ CAS # 94-75-7		220
4	2,4-DB $C_{10}H_{10}Cl_2O_3$ CAS # 94-82-6		248
5	Dicamba $C_8H_6Cl_2O_3$ CAS #1918-00-9'		220
6	Dichlorprop $C_9H_8Cl_2O_3$ CAS #120-36-5		234
7	Dinoseb $C_{10}H_{12}N_2O_5$ CAS # 88-85-7		240
8	MCPA $C_9H_9ClO_3$ CAS # 94-74-6		200
9	MCPP (Mecoprop) $C_{10}H_{11}ClO_3$ CAS # 7085-19-0		214
10	Picloram $C_6H_3Cl_3N_2O_2$ CAS # 1918-02-1'		240
11	2,4,5-T $C_8H_5Cl_3O_3$ CAS # 93-76-5		254
12	2,4,5-TP (Silvex) $C_9H_7Cl_3O_3$ CAS # 93-72-1		268

Most commercial formulations of phenoxy acid herbicides are usually in the form of esters or amine salts to improve their solubility in oil or water respectively. The amine salts are highly soluble in water, but the ester formulations are most soluble in oil. Ester formulations are more active than amines (Kearney and Kaufman 1975). 2,4-D acid and its metal or amine salts are not very volatile, but the ester formulation varies from low to high volatility.

Large scale application of phenoxy acid herbicides have great advantages for crop yield but also constitute a threat to sensitive environments in close contact with heavily used agricultural areas. The widespread use and preferential application of these herbicides over other active ingredients makes them one of the most important agricultural pesticides ever used. Leaching from fields is the main source driving polar pesticides into canals, rivers and lakes. Thus, soils and sediments are a direct link between the quality of water and ecological receptors. It has been shown that phenoxy acid herbicides are relatively less persistent in soils and water but can accumulate in bottom sediments (Cserhati and Forgacs 1998). Usually the interaction of herbicides with soils and sediment is stronger than with water and therefore the monitoring of these matrices is also very important as indicator of contamination.

The volatility of the ester formulations gives them the opportunity to move away from the target site several days after the initial application has been made. Volatilization problems have led to the complete destruction of nearby crops under proper favorable whether conditions and many states, including Florida, have totally banned the use of high-volatile ester formulations in sensitive areas. Largely due to applications of phenoxy herbicides in South Florida on sugarcane and drift or volatilization to nearby tomato

crops and the subsequent destruction of crops, the Florida Department of Agriculture and Consumer Services enacted the Organo-Auxin Herbicide Rule in 2003, banning the use of some formulations of these herbicides in the state (Ducar et al. 2003).

In spite of the enactment of this rule, the occurrence of these pesticides is still a concern because of the use of alternative formulations and their application for structural pest control, rights of way maintenance (including canals), golf courses, pastures, nurseries, and private homes.

TRIAZINE HERBICIDES

The herbicidal properties of the s-triazines were discovered in 1952 by a research group of J.R. Geigy in Switzerland. The selective action of these compounds was first reported in 1955 (Kearney and Kaufman 1975). Atrazine, simazine, prometryn and ametryn and related s-triazines are shown in table 2. These compounds gained major recognition in agriculture in the 1950's and today are still widely used. The s-triazines currently in use have a chlorine, methoxy, or methylthio group attached to R1, R2 or R3 , as is shown in the general structure below:

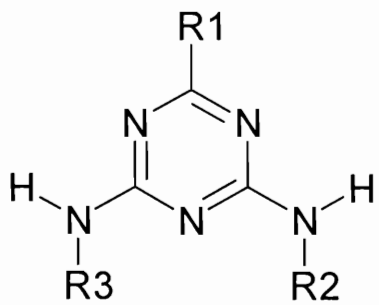
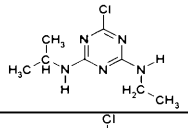
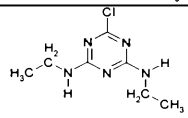
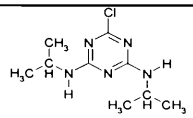
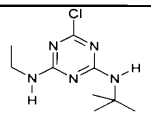
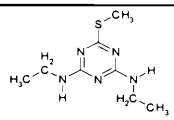
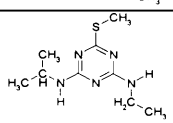
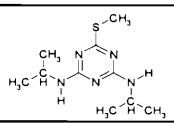
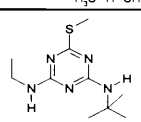
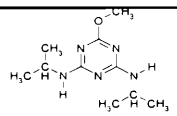
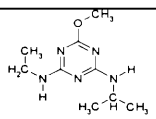
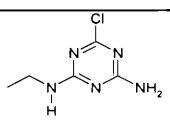
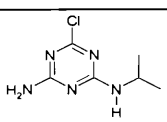


Figure 4 : Basic chemical structure of s-triazine herbicides

Table 2: Triazine-based herbicides and common metabolites.

#	Compound	Structure	Molecular Weight
1	**Atrazine** $C_8H_{14}ClN_5$ CAS # 1912-24-9		215
2	**Simazine** $C_7H_{12}ClN_5$ CAS # 122-34-9		201
3	**Propazine** $C_9H_{16}ClN_5$ CASS # 139-40-2		229
4	**Terbutylazine** $C_{10}H_{18}ClN_5$ CAS # 5915-41-3		229
5	**Simetryn** $C_8H_{15}N_5S$ CAS #1014-70-6		213
6	**Ametrin** $C_9H_{17}N_5S$ CAS # 834-12-8		227
7	**Prometryn** $C_{10}H_{19}N_5S$ CAS # 7287-19-6		241
8	**Terbutryn** $C_{10}H_{19}N_5S$ CAS # 886-50-0		241
9	**Prometon** $C_{10}H_{19}N_5O$ CAS # 1610-18-0		225
10	**Atratone** $C_9H_{17}N_5O$ CAS # 1610-17-9		211
11	**Deisopropylatrazine (DIA)** $C_5H_8ClN_5$ CAS # 1007-28-9		174
12	**Desethylatrazine (DEA)** $C_3H_4ClN_5$ CAS # 3397-62-4		187

The physico-chemical properties of the s-triazines are determined mainly by the substituent in position R1 that is most often –Cl (-azine), -SCH_3 (tryn), and OCH_3 (-ton). Their basicity increases in the order of chloro<methylthio<methoxy-s-triazines (Pacakova et al. 1996). These compounds are insoluble in water and the normal formulations are in the form of wetable powders, granules and liquids, which facilitates the transport and applications of the herbicides. Atrazine is one the most widely used herbicide in the world. It is used in the production of corn, sorghum, sugar cane, pineapples, macadamia nuts and for industrial weed control. Atrazine is applied worldwide and in 1998 it was the most widely used corn herbicide in the US.

Two of the most important degradation products of the triazine herbicides are the desisopropyl-atrazine (DIA) and the desethyl-atrazine (DEA), which are shown in table 2. DIA and DEA the dealkylated metabolites, are produced by microbiological transformation. The monitoring of these metabolites and parent compounds is important in several environment compartments such as sediments, surface water and groundwater, and the Environmental Protection Agency (EPA), then a maximum allowable limit of 3 parts per billion for triazines in drinking waters. However, no official limits have yet been set for the degradation products in the United States(Roilag et al. 1996).

Triazine herbicides have been extensively used in South Florida for sugar cane and winter vegetables. In the case of Atrazine approximately half million of kilograms of active ingredient are applied every year for these crops. (Crowford 1999). This situation requires the attention of both government and local authorities for its proper surveillance.

EXTRACTION AND CLEANUP TECHNIQUES OF PESTICIDES IN COMPLEX MATRICES

The environmental analysis of pesticides at trace levels in complicated matrices represent a major challenge for analytical chemists and forensic scientists. The different environmental compartments -water, soil, sediment and biota-, in which the pesticides could partition, will determine not only the behavior and fate of analytes in such biological compartments, but also their extraction and analysis. Traditional analytical protocols for pesticides residue analysis in complex matrices require several common steps. The compounds of interest must be extracted from the sample, isolated from the matrix, concentrated and sometimes transformed before they could be run by a suitable instrumental technique (Barcelo 2000).

The presence of organic compounds in environmental waters could be both the results of naturally occurring compounds and anthropogenic compounds. Traces amounts of pesticides are normally found in ground and surface waters at low ppb levels for most contaminants. Sediments on the other hand, accumulate natural and anthropogenic products from overlying water and integrate different inputs in time and space.

The primary objective of the sample preparation step is to provide a sample fraction enriched in all the analytes of interest, and as free as possible of other matrix components. In the case of waters several steps could be conducted: i) extracting traces of analytes from aqueous media; ii) concentrating these traces, iii) removing other compounds from the matrix which have been co-extracted and that may interfere in the further chromatographic analysis (Barcelo 2000). During the process of selecting the

best strategy for sample pre-treatment, different physicochemical properties of the analytes of interest should be considered, for example the pKa and the Kow. Also the nature of the matrix and the level of concentration required (ppm, ppb or ppt).

Water is a relatively easy matrix to handle; soils and sediments are a complex living dynamic assemblage of chemical components both organic and inorganic (Steinheimer 2000). The presence of organic matter in different percentages in the soils/sediments makes the isolation of pollutants a much more complex task. Several extraction techniques have been used for trace enrichment in both aqueous and solid matrices.

Liquid-Liquid Extraction (LLE)

Liquid-Liquid Extraction is based in the preferential partition of organic compounds between the aqueous phase and an immiscible organic solvent. The efficiency of the extracting solvent depends on the affinity of the compound for this solvent as measured by the partition coefficient, on the ratio of the volumes of each phase, and on the number of the extraction steps. Solvent selection for the extraction of environmental samples is related to analyte nature. Non-polar or slightly polar solvents are generally used. LLE is a very simple, batch wise methodology for most non polar organics, but in the case of polar and water soluble organic compounds like triazines and phenoxy acid herbicides, the extraction is generally more difficult to accomplish (Barcelo, 2000).

Liquid-Solid Extraction (LSE)

Liquid Solid Extraction (LSE) is a technique that is used for the extraction of organic contaminants from soil, sediments or biota. Soxhlet extraction is a typical extraction system, which allows an exhaustive extraction of the analytes. In this system, the soil sample is poured in a container made of porous fiber material and positioned over a boiling solvent. Even though this technique has the advantage of being exhaustive, it has the drawback of requiring large amounts of solvent and being time consuming (6 or more hours per sample). Soxhlet also generates dirty extracts, and the sample preparation time involved in the clean up of the extracts rise the cost of analysis. In fact, sample preparation for LSE has been estimated to constitute about two-thirds of the total time of analysis (Pawliszyn 2002).

Accelerated Solvent Extraction (ASE)

The accurate chemical analysis of small amounts of organic pollutants requires the use of a powerful, till versatile, extraction technique. Solvent extraction is the main technique used for the extraction of organic compounds. However, in order to improve the efficiency of this procedure, repetitive extractions are usually required, which translates in the consumption of larger amounts of solvents such as CH_2Cl_2, hexane or methanol. The possibility of reducing the consumption of toxic solvent for pesticides analysis has become a topic of discussion in recent years (Richter et al. 1996; Wan and Wong 1996; Gan et al. 1999). As it was mentioned before, the isolation of pesticides from solid environmental samples such sediments, is carried out traditionally by exhaustive extraction of the sample using liquid-liquid extraction techniques (LLE).

However, the large volume of highly purified solvents associated with high cost of both purchase and disposal, and the long extraction times involved, have caused an increasing demand for new techniques which can minimized this problem (Bjorklund et al. 2000).

Accelerated Solvent Extraction (ASE) is a promising technique that could be used to speed the extraction process. A typical ASE system consists of a high-pressure stainless steel extraction cell, and a solvent delivery and handling system. By controlling temperature and pressure the organic solvent is kept in liquid state above its boiling point. These subcritical conditions favor the efficient extraction of the analytes. Figure 5 shows a diagram of the regular accelerated solvent extraction system.

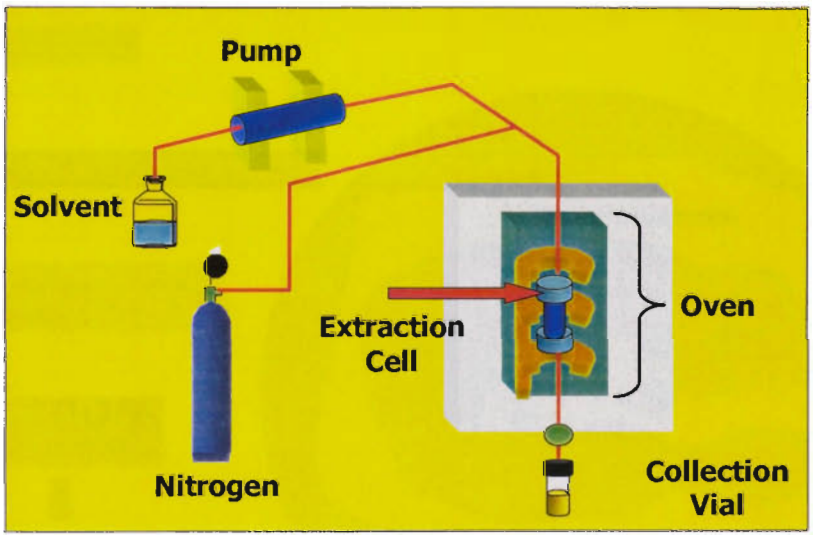


Figure 5: Diagram of an accelerated solvent extraction system (ASE)

Pre-treatment of the sample prior it's loading into the stainless steel cell is a regular step in ASE. For solid samples, like sediments or soils, a drying agent is mixed with the sample in order to remove water and help to improve the extraction efficiency. Diatomaceous earth or anhydrous sodium sulfate is usually used for this purpose. Three

main stages could be distinguished during the ASE process: the pre-extraction step which takes between 5-10 minutes (heating and filling of the cell), the static extraction period when the analytes are released from the sample, usually takes between 5-20 minutes; and the final flushing step. During the last step the analytes are removed from the extraction cell and transferred to a collection vial using an inert gas such as nitrogen, as purging gas.

In the case of sediments or soils samples, several steps are associated with the extraction of the analytes. These steps are shown in Figure 6 for a soil or sediment particle surrounded by an organic solvent (Bjorklund et al. 2000).

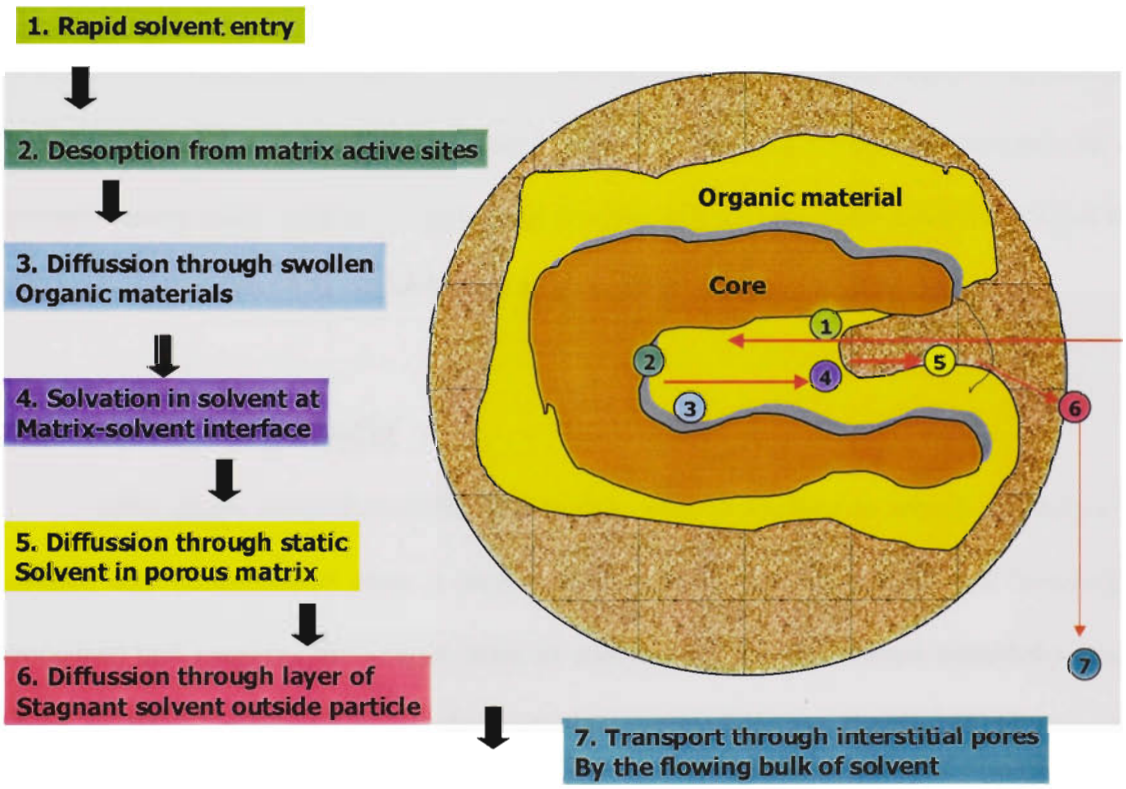


Figure 6: Extraction steps for a sediment sample using ASE (modified from Bjoklund, 2000)

Two parameters have a strong influence in this extraction process: temperature and pressure. Both variables control the transport of the analytes from the matrix to the bulk fluid. Increasing the temperature results in increase solubility, diffusion rate, and mass transfer, while viscosity and surface tension decrease. This also serves to improve the sample wetting and matrix penetration (Richter et al. 1996). The high pressure is used mainly to keep the solvent from boiling. It has been demonstrated that the pressure has a minor role for resulting recoveries. However, the use of a high pressure is justified during the filling of the cell to force its entrance through the small pores of the sample. Typical sample size for soils/sediments are in the range of 10-30 g, and the total volume of solvent required for extraction is in the 20-60 ml range. Extraction times are in the order of 20 minutes range per sample. These features provide ASE with superior advantages over conventional extraction techniques, in terms of time and amount of solvent. As an example, using ASE instead of the typical Soxhlet extraction, saves around 85 % of the solvent consumption (400 ml) and reduces up to 90 % of the time of analysis.

Solid Phase Extraction (SPE)

Solid phase extraction (SPE) is a widely used procedure in which an analyte is isolated and concentrated from a sample matrix. It is also described as a "clean-up" procedure that prepares the analyte prior to analysis so that the desired sensitivity range of an analytical method can be obtained. The compounds are isolated from complex mixtures by proper selection of a variety of sorbent chemistries (reversed phase, mixed phase, normal phase, and ion exchange) (Leon-Gonzalez and Perez-Arribas 2000).

SPE works through the interaction of three components: a) sorbent or stationary phase, b) solute and c) mobile phase. The sorbents are typically loaded into disposable syringe-shaped columns or cartridges. Depending on the analyte of interest the sorbent could be polar, moderately polar or non-polar.

A typical SPE sequence involves four steps: First, the SPE column is prepared to receive the sample (conditioning). This is usually done to activate the sorption sites before the sample is passed through the media. Once the cartridge is active, the sample is loaded using vacuum at a specific sample rate and the analytes of interest are trapped. The third step consists of the elimination of interferences with the use of proper solvents. Finally the polar solutes are removed or eluted using solvents of decreasing polarity like methanol, acetone or acetonitrile that will be compatible with the instrumentation available for determination. Upon elution from the SPE cartridge, a gentle stream of nitrogen is used to concentrate and or to dry the sample before the identification and quantification analysis.

Compared to the classic liquid-liquid extraction methods using separator funnel or preparative HPLC purification it offers several advantages like:

- Reduced lab time
- Easy manipulation
- Lesser amount of solvent required, no disposal of large quantities of organic solvents required
- Higher concentration factor
- No problem with the miscibility of solvent
- Easy adaptable for very selective extraction

- Easy to automate
- Avoids problems such as incomplete phase separations, less-than-quantitative recoveries and emulsion formation as encountered in liquid-liquid extractions

SPE has been developed as an alternative to LLE for the analysis of herbicides. Separation, purification, concentration and solvent exchange of solutes from solution is possible. The coupling of this clean up procedure to modern analytical techniques like HPLC or GC represents a viable way of matrix simplification-analysis. Polar herbicides such as phenoxy acid herbicides and triazines are common target analytes that could be determined by SPE procedures (Butz et al. 1994; Geerdink et al. 1997; Sabik and Jeannot 1998; Peruzzi et al. 2000; Shen and Lee 2003). However, most methods are developed for aqueous matrices and there is few references for sediments or soils.

TRADITIONAL INSTRUMENTAL METHODS FOR ANALYSIS OF POLAR HERBICIDES

The forensic characterization of pesticides at trace levels requires the separation and proper identification of residues in the target biotic and abiotic matrices. Therefore, High Performance Liquid Chromatography (HPLC) and Capillary Gas Chromatography (GC) are the two favorite separation techniques for the analysis of acidic herbicides in water or soil/sediment matrices.

Capillary GC is the analytical method with the greatest resolution power and is the preferred technique for pesticides analysis. The easy coupling of sensitive and selective detectors such electron capture detectors (ECD), nitrogen phosphorous detectors

(NPD), flame-photometric detectors (FPD), and mass spectrometry detectors (MSD), makes it ideal for identification and quantification of organic pollutants. Because of their high polarity and low volatility acidic herbicides are not directly amenable to GC analysis and have to be derivatized (Hodgeson 1994; Sanchez-Brunete et al. 1994). Many authors have reported the analysis of chlorophenoxy acid using different derivatization techniques (Butz and Stan 1993; Hodgeson et al. 1994; Pena and Silveira 1997; Catalina et al. 2000). However methylation by diazomethane and derivatization using pentafluorobenzyl bromide (PFBBr), are the most commonly used derivatizing agents for the determination of phenoxy acid herbicides and other pesticides in part per billion range by GC.

Triazines can also be analyzed using gas chromatography coupled to many detector systems, such as the flame ionization detector (FID) or nitrogen-selective detectors like the NPD. (Sabik and Jeannot 1998). However, triazines degradation products are polar and they are not amenable to direct analysis. Therefore derivatization is also required: eg. silylation, alkylation, acylation and methylation have been used. GC/MS systems have the advantage over regular detectors of provide structural confirmation of the triazines and their degradation products (Berg et al. 1995; Hernandez et al. 1997).

The derivatization step required in GC analysis of polar compounds has the disadvantages of being time consuming and complex. In addition, some derivatization agents are water sensitive and highly toxic. Fortunately, more flexible techniques like liquid chromatography could overcome these limitations. HPLC is well suited to the direct, and non-destructive analysis of more polar herbicides. Indeed, parent compounds

and also metabolites can often be included in the chromatographic separation. HPLC is, however, less useful for screening purposes than GC, mainly because of its relative low separation efficiency (Liska and Slobodnik 1996; Hernandez et al. 1997). Also, detection of polar and small compounds is difficult because of low sensitivity. In the case of s-triazines, the HPLC determinations are possible because of their strong absorbance in the UV-region. However, the lack of selectivity of the UV-detectors represents an analytical concern due to high incidence of matrix interferences. Reverse phase is the regular mode of separation of the triazines (Pacakova et al. 1996; Hernandez et al. 1997; Hernandez et al. 1998).

Considering the high polarity and low volatility of the phenoxy acid herbicides High Performance Liquid Chromatography (HPLC) provides a better alternative to gas chromatographic-based methodologies. HPLC coupled with UV-detectors has been reported for the analysis of these herbicides (Hogendoorn et al. 1999). This author reports limits of detection in the 1 ppb range for some herbicides like MCPA and Mecroprop.

A large amount of work has been traditionally devoted to the determination of polar phenoxy-acetic herbicides in aqueous samples using many different extraction/clean up approaches and both GC and HPLC as instrumental techniques (Kim et al. 1991; Ferrer et al. 1997; Lee et al. 1998; Li and Lee 2000). However, fewer articles are available describing their analysis in complex matrices like soil or sediments (Hunter and Carroll 1982; Hogendoorn et al. 2001; Luque-Garcia and Luque de Castro 2002).

The methodology reported by the Environmental Protection Agency (EPA) for the analysis of chlorinated herbicides (method 8151 A), has been used for many government

agencies and private laboratories as a guide to perform the analysis of these polar compounds. This method is suitable for aqueous, soil and waste matrices and describes the extraction (ultrasonic or shaker extraction), derivatization and gas chromatography conditions for the analysis of these herbicides. However, depending on the interferences that could be present in the matrix, the precision and accuracy of the results could be severely affected in particular with regard to analyte recovery. In addition, a derivatization step with diazomethane is needed, which represents a safety hazard due to the carcinogenic and explosive nature of the reagent. The extraction technique used within this method is reported as effective but prone to interferences and losses because of the use of alkaline conditions and is very common to observe large differences in the behavior of several analytes target in the method. Other disadvantages of the traditional methods of extraction are that they are time consuming and require high amounts of chlorinated solvents. To overcome some of this problems, a modernized extraction technique is also recommended by the EPA in method 3545A which uses pressurized fluid extraction (PFE), followed by clean up and derivatization before the gas chromatography determination. EPA also suggested an HPLC UV method for the analysis of phenoxy acid herbicides (EPA-555).

Although HPLC provides added advantages over GC methods in soil/sediment monitoring, the main weakness is its lack of specificity for confirming the presence of the analytes because of interferences coming from the matrix. The possibility of give a false positive is a concern with UV-based methods. This situation does not provide enough degree of confidence, which is fundamental in any forensic investigation. Therefore, the

use of analytical methodologies such as LC/MS that allows a clear and unique confirmation of the analyte is relevant and necessary.

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC/MS)

The coupling of a liquid chromatograph with a mass spectrometer (LC/MS) has been increasingly used in recent years for environmental screening of target pollutants at trace levels (Cappiello and Famiglini 1995; Chiron et al. 1995; Aguilar et al. 1999). The most important development in LC/MS instrumentation was the creation of interfaces for atmospheric-pressure ionization (API) capable of handling the high liquid flows often used in liquid chromatography (Niessen 1998; Niessen 1999).

The operational principle of an API interface and ion source for LC/MS is shown in figure 7, and comprises three basic steps: a) nebulization and charging; b) desolvation; and c) ion evaporation. Nebulization is either performed pneumatically, i.e. in heated nebulizer atmospheric pressure chemical ionization (APCI) or by means of the action of a strong electrical field, i.e. in electrospray (ESI) (Niessen 1999). Nebulization and charging occur as the HPLC effluent with analyte ions in solution emerges from the tip of the nebulizing needle, which is at ground potential, into a semi-cylindrical electrode to which high voltage is applied. The potential difference between the nebulizer and the counter-electrode produces a strong electric field that charges the surface of the emerging liquid and forms a fine spray of charged droplets.

During the desolvation step, the charged droplets are attracted toward the capillary sampling orifice through a counter flow of heated nitrogen drying gas, which shrinks the droplets and carries away uncharged material.

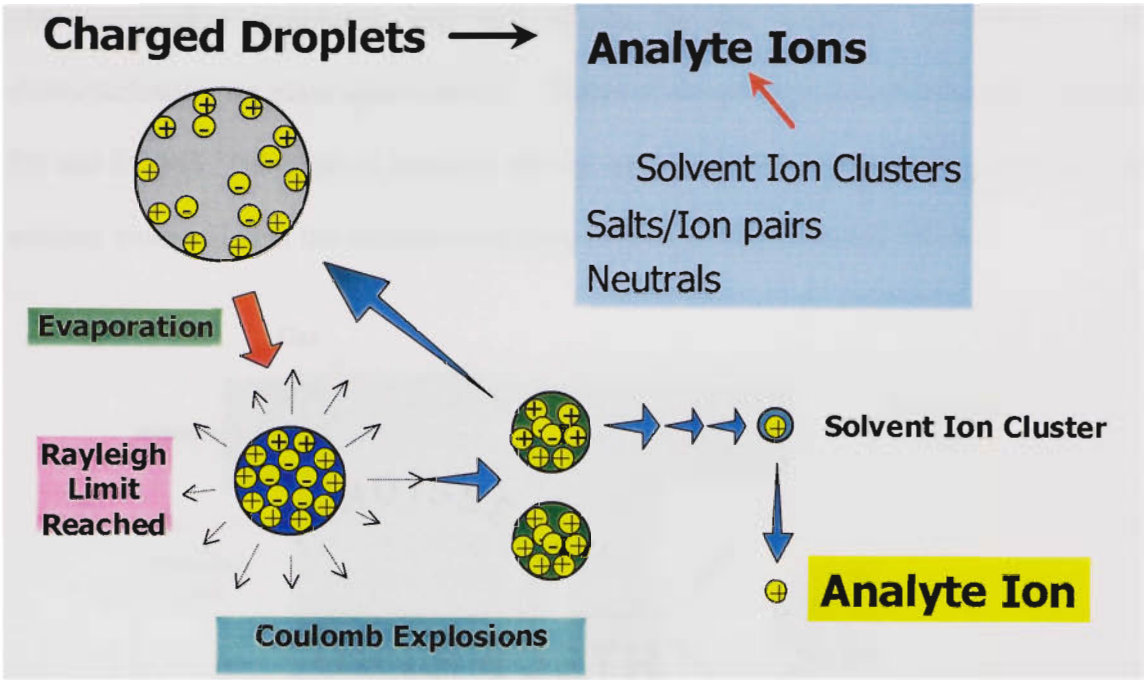


Figure 7: Operative principle of an API interface

The final step is ion evaporation. Here the droplets continue to shrink until the repulsive electrostatic (Coulombic) forces exceed the droplet cohesive forces, leading to droplet explosions. This process is repeated until analyte ions are ultimately desorbed into the gas phase, driven by strong electric fields on the surface of the microdroplets. The emerging gas phase ions are then passed through the capillary sampling orifice into the low-pressure region of the ion source, and transported to the mass analyzer (Niessen 1999).

Polar, low molecular organic molecules like the phenoxy acid herbicides are ideal for low-level detection using API interfaces. The sensitive and selective response of the MS detector at low concentration levels plus the capability of simultaneous detection of both positive and negative ions avoids the derivatization steps required for other

chromatographic techniques and still allows for the versatile quantification and characterization by mass spectrometry.. Figure 8 shows a typical "Z-shaped" interface for and LC/MS. This type of interface allows the use of mobile phases at controlled pHs, making more efficient the ionization of organic compounds.(Niessen 1999)

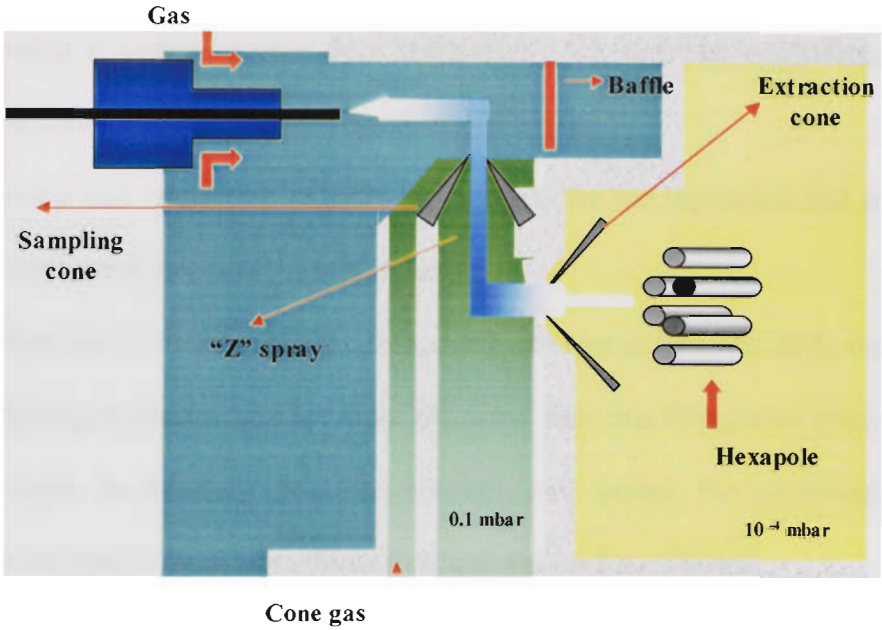


Figure 8. Shematic representation of a Z-electrospray LC-MS interface (adapted from Niessen 1999)

OBJECTIVES

Because of the limitations of the available methodology for the detection and quantification of the compounds of interest, successful completion of the proposed forensic investigation will provide an analytically robust, selective and sensitive method for the analysis of polar herbicides in complex environmental matrixes by using a combined ASE-SPE-LC/MS strategy. Application of the method to samples collected within ENP and BNP will provide environmental forensic evidence to be used for source

mitigation, regulation and future management of resources affected by CERP. In addition, the availability of "baseline" data will help in the prevention and enforcement of future environmental crimes.

Specific objectives of this research are:

- Develop a comprehensive trace method for the isolation and concentration of polar herbicides in complex organic rich matrices
- Develop and optimize a LC/API-MS method for the separation and detection of the analytes at low part per billion levels.
- Perform an overall evaluation of the method as an environmental forensic tool by analyzing sediment samples from Biscayne Bay and Everglades National Parks, in order to identify potential sources and assess the prevalent transport mechanisms of these contaminants to and within the study area.

CHAPTER II. EXPERIMENTAL

CHEMICALS

Phenoxy Acid Herbicides Analysis

Methanol (CH_3OH), glacial acetic acid ($C_2H_4O_2$), water (H_2O), acetone (C_2H_6CO), and methyltertbutyl-ether (MTBE, $C_5H_{12}O$) of HPLC grade or equivalent quality were obtained from Fischer Scientific (Suwannee GA, USA). Formic acid (HCOOH) reagent grade was obtained from Fischer Scientific (Suwannee GA, USA).

The acidic herbicides 2,4-D ($C_8H_6Cl_2O_3$), MCPA ($C_9H_9ClO_3$), 2,4,5-T ($C_8H_5Cl_3O_3$), 2,4-DB ($C_{10}H_{10}Cl_2O_3$), Mecoprop ($C_{10}H_{11}ClO_3$), Silvex ($C_9H_7Cl_3O_3$), Dichlorprop ($C_9H_8Cl_2O_3$), Dicamba ($C_8H_8Cl_2O_3$), Dinoseb ($C_{10}H_{12}N_2O_5$), Bentazone ($C_{10}H_{12}N_2O_3S$), Acifluorfen ($C_{14}H_7ClF_3NO_5$) and Picloram ($C_6H_3Cl_3N_2O_2$), were obtained as solids at 99 % purity from Chem Service (West Chester PA, USA). The internal standards used were 2,4-dichlorophenyl acetic acid 99 % (DCAA, $C_8H_6Cl_2O_2$) obtained as solid from Chem Service (West Chester PA, USA) and a solution of 2,4-D $^{13}C_6$ 100µg/ml (Dr. Ehrenstorfer, GmbH, Augsburg,Germany).

Stock, working and calibration Solutions

Approximately 6 mg of each one of the solid standards were weighed individually in a Cahn-33 microbalance (Cahn Instruments CA, USA), and were added to a 10 mL amber volumetric flask and dissolved with MeOH. These c.a. 600 µg/ml stock solutions were stored in the dark at –20 °C. The working standard solutions mixtures were

prepared to a concentration c.a. 50 µg/ml by adding 790 µl of each individual stock solution into a 10 ml amber flask. These working standard solutions were used to prepare an 8 level calibration curve between 0.15 and 4 µg/m (see table 3). The internal standard solution of DCAA or 2,4-D $^{13}C_6$ was added to each calibration solution to a concentration of 2 µg/ml. Stock solutions were replaced every 3 months.

Table 3. Preparation of the phenoxyacid calibration solutions.

Solution	Concentration of working solution [a]	Volume of working solution [a]	Volume of internal standard [b]	Final volumen	Final concentration of analytes
CS0	0	0	400 µl	10 ml	0
CS1	50 ug/ml	30 ul	400 µl	10 ml	0.15 µg/ml
CS2	50 ug/ml	50 ul	400 µl	10 ml	0.25 µg/ml
CS3	50 ug/ml	60 ul	400 µl	10 ml	0.30 µg/ml
CS4	50 ug/ml	100 µl	400 µl	10 ml	0.50 µg/ml
CS5	50 ug/ml	200 µl	400 µl	10 ml	1.0 µg/ml
CS6	50 ug/ml	400 µl	400 µl	10 ml	2.0 µg/ml
CS7	50 ug/ml	600 µl	400 µl	10 ml	3.0 µg/ml
CS8	50 ug/ml	800 µl	400 µl	10 ml	4.0 µg/ml

[a]Working solution is a mix of 2,4-D; 2,4,5-T, acifluorfen, silvex, picloram, mecroprop, 2-4,D-B; bentazon, dicamba, dichlorprop, dinoseb and MCPA.
[b] Internal standard solution at 50 µg/ml

Spiking solutions

Mixtures of phenoxy acid herbicides at 20 µg/ml were prepared by adding approximately 333 µl of 600 µg/ml stock solutions to a 10 ml amber flask and diluted with methanol. The internal standard solutions of DCAA or 2,4-D $^{13}C_6$ were also prepared at 20 µg/ml and added to all samples at a constant concentration of 2 µg/ml.

Triazine-Based Herbicides Analysis

Triazine Herbicides: Ametryne ($C_9H_{17}N_5S$), Terbutryn ($C_{10}H_{19}N_5S$), Simetryn ($C_8H_{15}N_5S$), Prometryn ($C_{10}H_{19}N_5S$), Atraton ($C_9H_{17}N_5O$), Atrazine ($C_8H_{14}ClN_5$), Prometon ($C_{10}H_{19}N_5O$), Propazine ($C_9H_{16}ClN_5$), Simazine ($C_7H_{12}ClN_5$), Terbutylazine ($C_9H_{16}ClN_5$), were obtained as a neat solution TP-619 Mix of 500 µg/ml, from Chem Service (PA, USA). Solid standards of Irgarol 1051 99.0 % ($C_{11}H_{19}N_5S$), M1 99 % ($C_8H_{15}N_5S$), Desisopropyl atrazine 98 % ($C_5H_8ClN_5$), Desethyl atrazine 97.5 % ($C_6H_{10}ClN_5$) were obtained from Dr. Ehrenstorfer (GmbH, Augsburg, Germany). The internal standard Atrazine D_5 (C_8 $H_{14}N_5D_5Cl$) was obtained as a neat solution of 100 µg/ml from Dr. Ehrenstorfer (GmbH, Augsburg, Germany).

Stock and calibration solutions

Approximately 5 mg of Irgarol 1051 99%, 5 mg of M1 99%, 6 mg of Desisopropyl atrazine 98 %, and 6 mg of Desethyl atrazine 97.5 % were weighed individually in a Cahn-33 microbalance and were added to a 10 ml individualt amber volumetric flasks and dissolved with acetone in the case of Irgarol 1051 and M1, and

methanol for the atrazine metabolites to yield a concentration of c.a. 500-600 µg/ml for each standard. Five hundred micro liters of the Irgarol 1051 and M1 stock solutions were taken and added to a 25 ml independent amber volumetric flask and diluted with methanol for a final concentration of approximately 10 µg/ml for each compound (Working Solution A and B). In the case of the atrazine metabolites 390 µl of each stock solutions were added together to a 25 ml amber volumetric flask and diluted with methanol for a final concentration of 10 µg/ml of each metabolite (Working Solution C). Five hundred micro liters of the TP-619 triazine-based herbicides mixture of 500 µg/ml were taken and added to a 25 ml amber volumetric flask with methanol to obtain a final concentration of 10 µg/ml (Working solution D). Five hundred micro liters of 100 µg/ml of Atrazine D_5 neat solution were taken and diluted with methanol in a 5 ml amber volumetric flask for a final concentration of 10 µg/ml. These stock and working solutions were stored in the dark at –20 °C. The calibration standard solutions were prepared in the range of 0.05 to 2.5 µg/ml by adding increasing amounts of each one of the 10 µg/ml solutions A, B, C and D, from 50 µl up to 2500 µl (eight calibration points). The concentration of the internal standard (Atrazine D_5), was kept constant at a concentration of 0.5 µg/ml. (see table 4 for details)

Spiking solution

Mixtures of triazine-based herbicides and metabolites the in the range of 20 µg/ml were prepared from stock solutions and diluted with methanol. The internal standard solutions concentration (Atrazine D_5), was kept constant at a concentration of 0.5 µg/ml.

Table 4. Preparation of the triazines calibration solutions.

Working solutions	Calibration solutions							
	CS0	**S1**	**CS2**	**CS3**	**CS4**	**CS5**	**CS6**	**CS7**
A[a]	0 µl	50 µl	100 µl	250 µl	500 µl	1000 µl	2000 µl	2500 µl
B[b]	0 µl	50 µl	100 µl	250 µl	500 µl	1000 µl	2000 µl	2500 µl
C[c]	0 µl	50 µl	100 µl	250 µl	500 µl	1000 µl	2000 µl	2500 µl
D[d]	0 µl	50 µl	100 µl	250 µl	500 µl	1000 µl	2000 µl	2500 µl
Internal standard[e]	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl
Final concentration of internal standard	0.5 µg/ml	0.5 µg/ml	0.5 µg/ml	0.5 µg/ml	0.5 µg/ml	0.5 µg/ml	0.5 µg/ml	0.5 µg/ml
Final concentration of analytes	0 µg/ml	0.05 µg/ml	0.10 µg/ml	0.25 µg/ml	0.50 µg/ml	1.0 µg/ml	2.0 µg/ml	2.5 µg/ml

[a] solution A contains ~10000 µg/ml of Irgarol

[b] solution B contains ~10000 µg/ml of M1

[c] solution C contains ~10000 µg/ml of DIA and DEA

[d] solution D contains ~10000 µg/ml of atratone, prometrone, atrazine, simazine, symetrin, ametryn, prometryn, terbutryn, propazine and terbutilazine.

[e] solution E contains ~ 100 µg/ml of atrazine D_5

SAMPLES

Sample Collection

Samples from Everglades National Park were collected from 5 transect using a small Eckman dredge or an acrylic device (see figure 9). The first transect follows an east-west direction across the northern boundary of the park which receives water from the Water Conservation Areas to the north of the Park and lies just south of Tamiami Trail, the major highway in this area. The second transect follows a north-south direction across the eastern boundary of the park, this transect lies closest to the Homestead Agricultural Area (HAA). The third transect is located in the drainage basin of the C-111 canal which flows through most of the HAA. The last two transects follow the Shark Slough and Taylor Slough which flow in a south-west direction from the north and east boundaries, respectively (see Figure 9). At each sampling site, 2.5 x 12" cores were collected from a $100m^2$ area and consolidated as one representative sample in combusted glass jars with Teflon lined lids. All samples were collected and kept frozen from the site of collection at – 25 °C until the time of analysis.

Samples from Biscayne National Park were collected from areas near land along the channel from Black Point Marina, off the channel from Turkey Point Nuclear Power Plant, near the outflows from the main inland canal, as well as from sites near Elliot Key, a barrier island approximately 10 km offshore. At each site 3 samples were collected using the mentioned dredge system and poured directly into combusted glass jars with Teflon lids, consolidated and stored for analysis. All sediment/soil samples were collected in combusted, solvent rinsed, Teflon lined glass containers. Samples were kept refrigerated from the time of collection to the arrival to the laboratory.

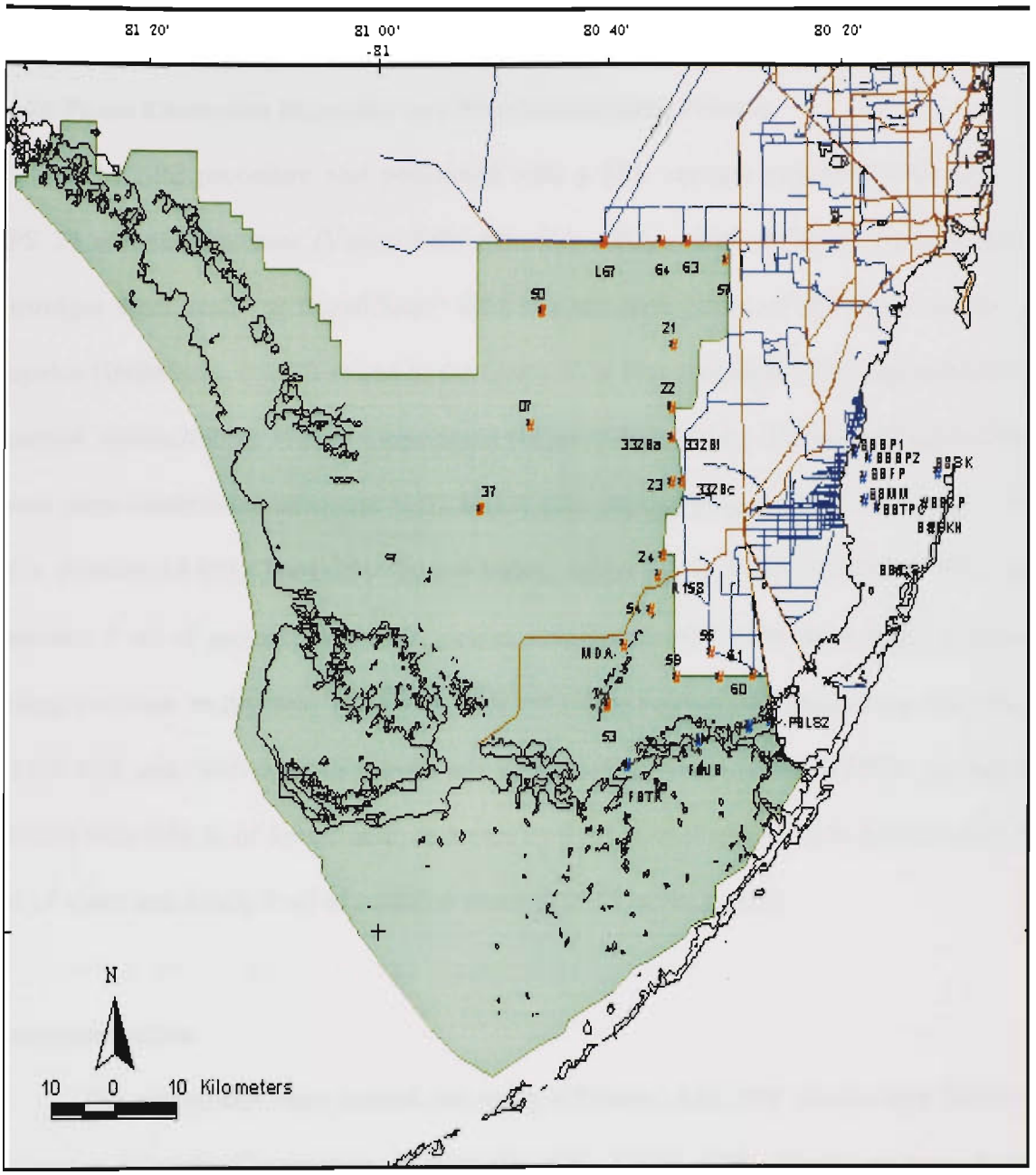


Figure 9: Sampling sites within the Everglades and Biscayne Bay National Parks.

MATERIALS

Solid Phase Extraction Materials and Pre-Conditioning Process

The SPE procedure was performed with a SPE vacuum manifold VAC ELUT SPS 24 column processor (Varian Palo Alto CA, USA). Two different types of SPE cartridges were used: a) SupelClean™ ENVI™ Carb SPE Tubes of 500 mg, 6 ml from Supelco (Bellefonte, PA USA) and b) the Oasis HLB Plus cartridges (225 mg) polymeric material obtained from Waters Corporation (Milford MA, USA). The SupelClean™ SPE tubes were conditioned using the VAC ELUT SPS 24, applying vacuum and using 5 ml of a mixture of CH_2Cl_2:MeOH (80:20) twice, followed by 5 ml of MeOH. Without vacuum, 5 ml of ascorbic acid 1 % passed through the SPE tube three times wihtout taking the tubes to dryness. The Oasis HLB cartridges were conditioned using the VAC ELUT SPS unit, with 5 ml of a fresh mix of methyl-tert-butyl-ether (MTBE): methanol (90:10) with 0.01 % of formic acid; followed by 5 ml of methanol (0.01% formic acid); 5 ml of water and finally 5 ml of acidified water (0.25 % sulfuric acid).

Instrumentation

The extractions were carried out using a Dionex ASE 200 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA, USA) with 33 ml stainless-steel extraction cells.

The liquid chromatograph-mass spectrometer system used in this investigation included a Thermo-Finnigan (Thermo-Finnigan San Jose CA, USA) P4000, quaternary pump, an AS 4000 auto-sampler and a Navigator *aQa* quadrupole mass spectrometer (50-

1500 Da). The LC/MS was run either under negative ion Electrospray Ionization (ESP-) or Positive Ion Atmospheric Pressure Chemical Ionization (APCI+), depending on the herbicide family studied.

ANALYTICAL METHODOLOGY

Mass Spectrometer Optimization for Phenoxy Acid Herbicides

Three different parameters were optimized by flow analysis of the individual herbicides at a concentration of 20 μg/ml in the mass spectrometer: cone voltage; probe temperature and pH of the mobile phase. The cone voltage was evaluated between 5 and 40 volts. The probe temperature was tested at five different conditions: 250, 280, 300, 320, 350°C. The third parameter to be considered was the effect of the mobile phase composition in terms of additive (modifier) strength. Several mobile phase modifiers were tested such as 0.1 % trifluoroacetic acid (TFA), 0.1 %ammonium acetate, and 0.1 % to 1% acetic acid.

Chromatographic Separation of Phenoxy Acid Herbicides

The HPLC separation was performed in a Zorbax XDB C_{18} Column (250 x 4.6 mm x 5 μm) (Agilent Technologies, Palo Alto CA, USA) using MeOH and acetic acid 1% as phase modifier. A gradient elution at 0.5 ml/min from 75:25 (Methanol: acetic acid 1%) to 82:18 for a total run time of 25 minutes was used. The column temperature was kept at 30 °C.

Chromatographic Separation of Triazine-Based Herbicides

The HPLC separation was performed in a Zorbax XDB C_{18} Column (250 x 4.6 mm x 5 μm) (Agilent Technologies, Palo Alto CA, USA) using MeOH and acetic acid 1% as phase modifier. A gradient elution at 0.5 ml/min from 60:40 (Methanol: acetic acid 1%) to 90:10 returning to original contidions for a total run time of 35 minutes was used. The column temperature was kept at 30 °C.

PHENOXY ACID HERBICIDES SAMPLE PREPARATION

The sample preparation used in the initial phenoxy acid study involves a total of 8 different methodologies in two different strategies in order to evaluate the extraction efficiencies. Methods A through C compare the extraction of sediments using NaOH 0.3N and sonication by LLE as well as coupled to two different SPE cleanup methodologies. In the second approach, methods D through H, an accelerated solvent extraction (ASE) procedure was evaluated using different extraction solvents and in combination with two different SPE sorbents. Details of each of those methods are presented below.

Method A: Basic Extraction – LLE with CH_2Cl_2

Twenty-five grams of sediment sample were measured and spiked with 500 μl of the phenoxy acid spiking mixture solution of 50 μg/ml. One hundred milliliters of NaOH 0.3 N was added, and the sample was sonicated for 30 minutes and filtered using combusted Whatman GF/C glass fiber filter. The extract was later acidified with H_2SO_4 to pH <2 and extracted with 3 x 50ml of CH_2Cl_2. The extracts were dried with Na_2SO_4

and concentrated using a rotary evaporator. Further concentration involved bringing the sample extract to dryness using a stream of clean dry nitrogen and reconstitution to 1 ml with MeOH before LC/MS analysis.

Method B: Basic Extraction - SPE-Carbon Cartridge Clean up Procedure

Twenty-five grams (25g) of sediment sample were measured and spiked with 500 µl of `the phenoxy acid spiking mixture solution of 50 µg/ml. One hundred mililiters of NaOH 0.3 N was added and the sample was sonicated for 30 minutes and filtered using a combusted Whatman GF/C glass fiber filter. The extract was later acidified with H_2SO_4 to pH <2 and diluted up to 1000 ml in a Erlenmeyer flask. The sample was passed through a pre-conditioned Graphitized Carbon SPE cartridge at a flow of approximately 15 mL/min under vacuum. The analytes were eluted using 1 x 1.5 mL of MeOH collected as fraction 1. The second fraction was collected after elution with 2 x 6 ml of dichloromethane, methanol and trifluoroacetic anhydride, CH_2Cl_2:MeOH:TFA (80:20:0.01). Both fractions were concentrated up to 400 µL under nitrogen flow in a block heater at 38 °C and then mixed. The final volume prior to injection was adjusted to 1 ml with methanol.

Method C: Basic Extraction - SPE-OASIS HLB Clean up Procedure

The sample was prepared and extracted using the same procedure described in method B. The extracts were then passed through a pre-conditioned Oasis HLB SPE cartridge at a flow of approximately 15 mL/min under vacuum. The analytes were recovered with 2 x 6 ml MTBE:MeOH (90:10), 0.01 % Formic Acid. The fraction was

dried under N_2 in a heated block at 45 °C and the residue was reconstituted in 1 ml of MeOH.

Method D: ASE-Water-Carbon SPE

Approximately 10 g of dry sediment (mixture of sediment and diatomaceous earth) sample were spiked with 100 µl of the phenoxy acid mix and poured in a 33 mL extraction cell of the ASE system. The extraction program in the ASE 200 was the following: oven temperature: 100 °C; pressure: 1500 psi; oven heat up time: 5 minutes; static time: 5 minutes; flush volume: 60 % of extraction volume cell; nitrogen purge: 150 psi for 60 seconds; solvent: D.I. Water. The extracted volume (approximately 45 ml) was acidified with H_2SO_4 to pH <2 and passed through a pre-conditioned Carbon SPE cartridge. The elution procedure was the same as described in method B.

Method E: ASE-Water-SPE-OASIS HLB

The sample preparation and ASE extraction method was the same used in method D. The extracted volume (approximately 45 ml) was acidified with H_2SO_4 to pH <2 and passed through a pre-conditioned OASIS HLB SPE cartridge. The elution procedure was done with 2 x 6 ml MTBE:MeOH (90:10), 0.01 % Formic Acid. The fraction was dried under N_2 in a heated block at 45 °C and the residue reconstituted in 1 ml of MeOH.

Method F: ASE-Basic-Carbon SPE

Approximately 12 g of sediment sample were mixed with diatomaceous earth (DE) and spiked with 100 µl of the phenoxy acid mix. The sample was poured in a 33 mL

extraction cell of the ASE system. The ASE extraction was performed as described in method D but using NaOH 0.3 N as the extraction solvent. The extracted volume (approximately 45 ml) was acidified with H_2SO_4 to pH <2 and passed through a pre-conditioned Carbon SPE cartridge. The elution procedure was the same as explained in method B.

Method G: ASE-Acetone-HOAc-Carbon SPE.

The same procedure followed in F with the exception of the extraction solvents. A mixture of Acetone-HOAc (80:20) was used instead of NaOH 0.3 N. The extracted volume was concentrated using rotary evaporator to approximately 20 ml. D.I. Water was added to complete a volume of 200 ml and this was passed through the pre-conditioned carbon disk SPE cartridge. The elution procedure was the same as explained in method F.

Method H: Final Conditions of Extraction and Clean up of Phenoxy acid herbicides: ASE-Acetone:Acetic Acid (80:20)-Oasis HLB SPE

Sediment samples (c.a. 15 g) were mixed with combusted diatomaceous earth. After approximately 15 minutes of mixing, the sample was transferred to a 33 ml stainless steel ASE extraction cell. The extractions were carried out using a Dionex ASE 200 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA, USA) with 33 ml stainless-steel extraction cells, using a mixture of acetone:5 % HOAc (80:20 %). A system pressure of 2000 psi and an extraction temperature of 100 °C were used. A static

time of 5 minutes, flush volume of 60 % of extraction cell volume; nitrogen purge of 150 psi for 60 s were used to complete the extractions.

The ASE extracts (ca 45 ml) were acidified with 700 µl of concentrated sulfuric acid and centrifuged in 50 ml Teflon centrifuges tubes at 3,500 rpm for 7 minutes. The extracts were later concentrated in a Büchi rotary evaporation unit from Brinkmann (Westbury NY, USA) up to 20 ml. Fifty milliliters of 5 % solution of acetic acid was added to each concentrated extract to reconstitute de sample to 70 ml before the SPE cleaning step.

The SPE procedure was performed with a SPE vacuum manifold VAC ELUT SPS 24 column processor (Varian Palo Alto CA, USA). The Oasis HLB Plus cartridges were pre-conditioned with 5 ml of freshly mixed methyl-tert-butyl-ether (MTBE): methanol (90:10) with 0.01 % of formic acid; followed by 5 ml of methanol (0.01% formic acid); 5 ml of water and finally 5 ml of water (0.25 % sulfuric acid). An aqueous extract of 70 ml was passed through the conditioned cartridge at 1.5 ml/min. A pre-filter unit consisting of a 25 mm filter polypropylene holder with a previously combusted GF/B glass microfibre filter (Whatman Scarborough MN, USA), was used before the SPE cartridge in order to trap the fulvic/humic materials precipitated during the acidification. Analytes were recovered from the cartridge by using a mixture of MTBE:Methanol (90:10) with 0.01 % of formic acid (2 x 6 ml). The eluted fraction from the SPE was concentrated using nitrogen in a heated block (45°C) up to 500 µl and reconstituted in methanol to a final volume of 1000µl. Figure 10 summarizes the final extraction procedures selected for the analysis of phenoxyacid herbicides.

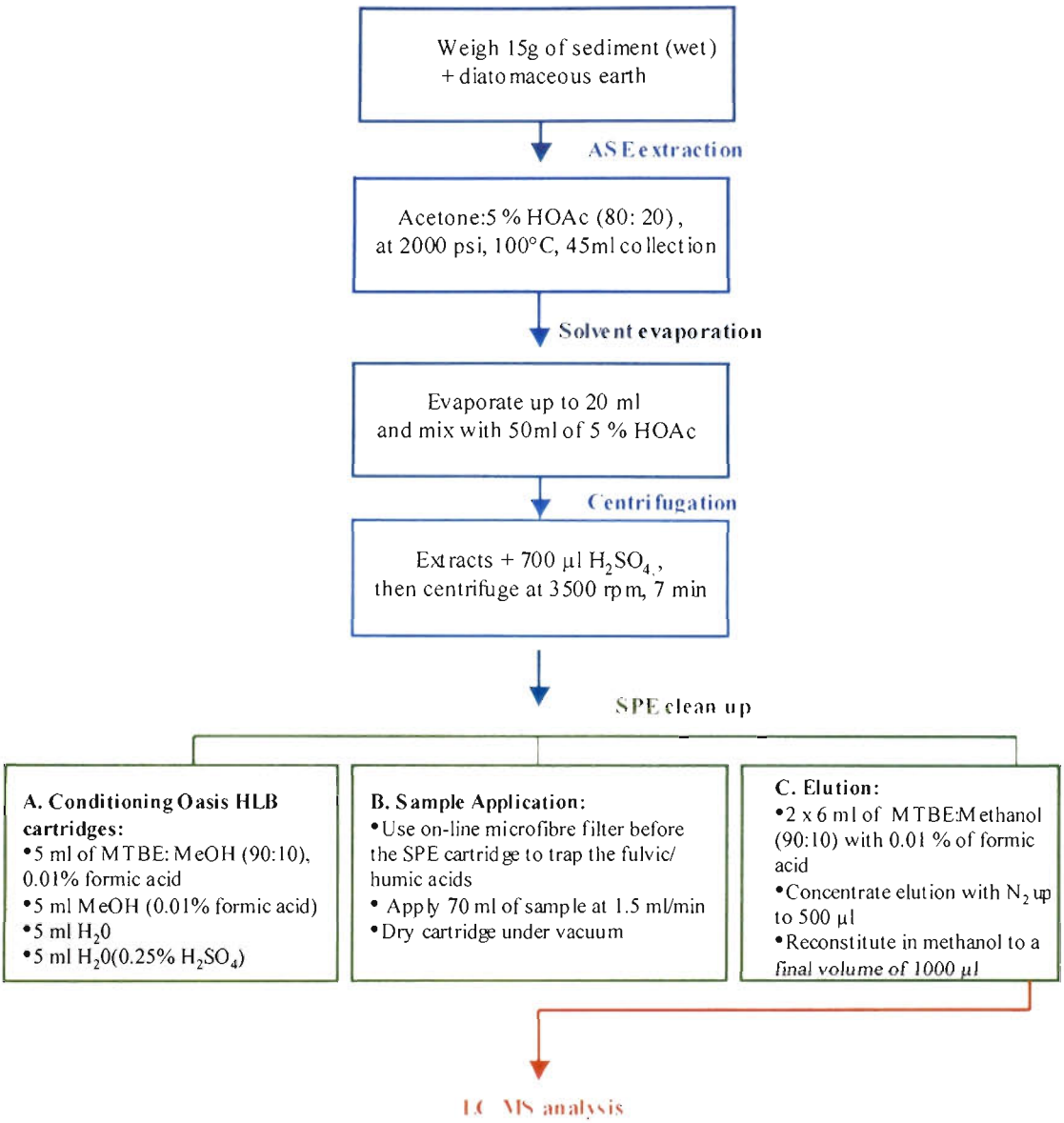


Figure 10. Scheme for the analysis of phenoxyacid herbicides in sediments.

TRIAZINE HERBICIDES SAMPLE PREPARATION

Two different methods were used in order to evaluate the extraction efficiencies for triazine herbicides.

Method A: ASE-MeOH:H2O (90:10)-Oasis SPE.

Wet sediment samples (c.a. 15 g) were mixed with combusted diatomaceous earth. After approximately 15 minutes of mixing, the sample was transferred to a 33 ml stainless steel ASE extraction cell. The solvents of choice were methanol: water (90:10). The extraction program in the ASE was the following: oven temperature: 100 °C; pressure: 1500 psi; oven heat up time: 5 minutes; static time: 10 minutes; flush volume: 60 % of extraction volume cell; nitrogen purge: 150 psi for 60 seconds. The ASE extracts (ca 45 ml) were centrifuged in 50 ml Teflon centrifuges tubes at 3,500 rpm for 7 minutes. The extracts were later concentrated in a Büchi rotary evaporation unit from Brinkmann (Westbury NY, USA) up to 10 ml. Twenty milliliters of water was added to each concentrated extract to reconstitute the sample to 30 ml before the SPE cleaning step.

The Oasis HLB Plus cartridges were pre-conditioned with 10 ml of methanol follow by 5 ml of water. An aqueous extract of 30 ml was passed through the conditioned cartridge at 1.5 ml/min using a 30 ml polypropylene syringe as support of the sample. A pre-filter unit consisting of a 25 mm filter polypropylene holder with a previously combusted GF/B glass microfibre filter (Whatman Scarborough MN, USA), was used before the SPE cartridge in order to trap the fulvic/humic materials precipitated. The elution solvent was 10 ml of CH_2Cl_2. The eluted fraction from the SPE was

concentrated using nitrogen in a heated block (45°C) up to dryness and reconstituted in methanol to a final volume of 1000µl.

Method B: ASE-Acetone:Acetic Acid (80:20)-Oasis HLB SPE

Wet sediment samples (c.a. 15 g) were mixed with combusted diatomaceous earth. After approximately 15 minutes of mixing, the sample was transferred to a 33 ml stainless steel ASE extraction cell. The extractions were carried out using a Dionex ASE 200 Accelerated Solvent Extractor with 33 ml stainless-steel extraction cells. The solvents of choice were acetone-5 % HOAc (80:20 %). A system pressure of 2000 psi and an oven heat up temperature of 100 °C were used. A static time of 5 minutes, flush volume of 60 % of extraction cell volume; nitrogen purge of 150 psi for 60 s were used to complete the conditions for extractions.

The ASE extracts (ca 45 ml) were acidified with 700 µl of concentrated sulfuric acid and centrifuged in 50 ml Teflon centrifuges tubes at 3,500 rpm for 7 minutes. The extracts were later concentrated in a Büchi rotary evaporation unit from Brinkmann (Westbury NY, USA) up to 10 ml. Twenty milliliters of 5 % solution of acetic acid was added to each concentrated extracted before the SPE cleaning step.

The SPE procedure was performed with a SPE vacuum manifold VAC ELUT SPS 24. The Oasis HLB Plus cartridges were pre-conditioned with 5 ml of a fresh mix of methyl-tert-butyl-ether (MTBE): methanol (90:10) with 0.01 % of formic acid; followed by 5 ml of methanol (0.01% formic acid); 5 ml of water and finally 5 ml of water (0.25 % sulfuric acid). The 70 ml aqueous extract of was passed through the conditioned cartridge at 1.5 ml/min using a polypropylene syringe as support of the sample. A pre-

filter unit consisting of a 25 mm filter polypropylene holder with a previously combusted GF/B glass microfibre filter (Whatman Scarborough MN, USA), was used before the SPE cartridge in order to trap the fulvic/humic materials precipitated. The elution solvent was a mixture of MTBE:Methanol (90:10) with 0.01 % of formic acid (2 x 6 ml). The eluted fraction from the SPE was concentrated using nitrogen in a heated block (45°C) up to 500 µl and reconstituted in methanol to a final volume of 1000µl.

Figure 11 shows the final methodology selected for the extraction of triazines from sediments.

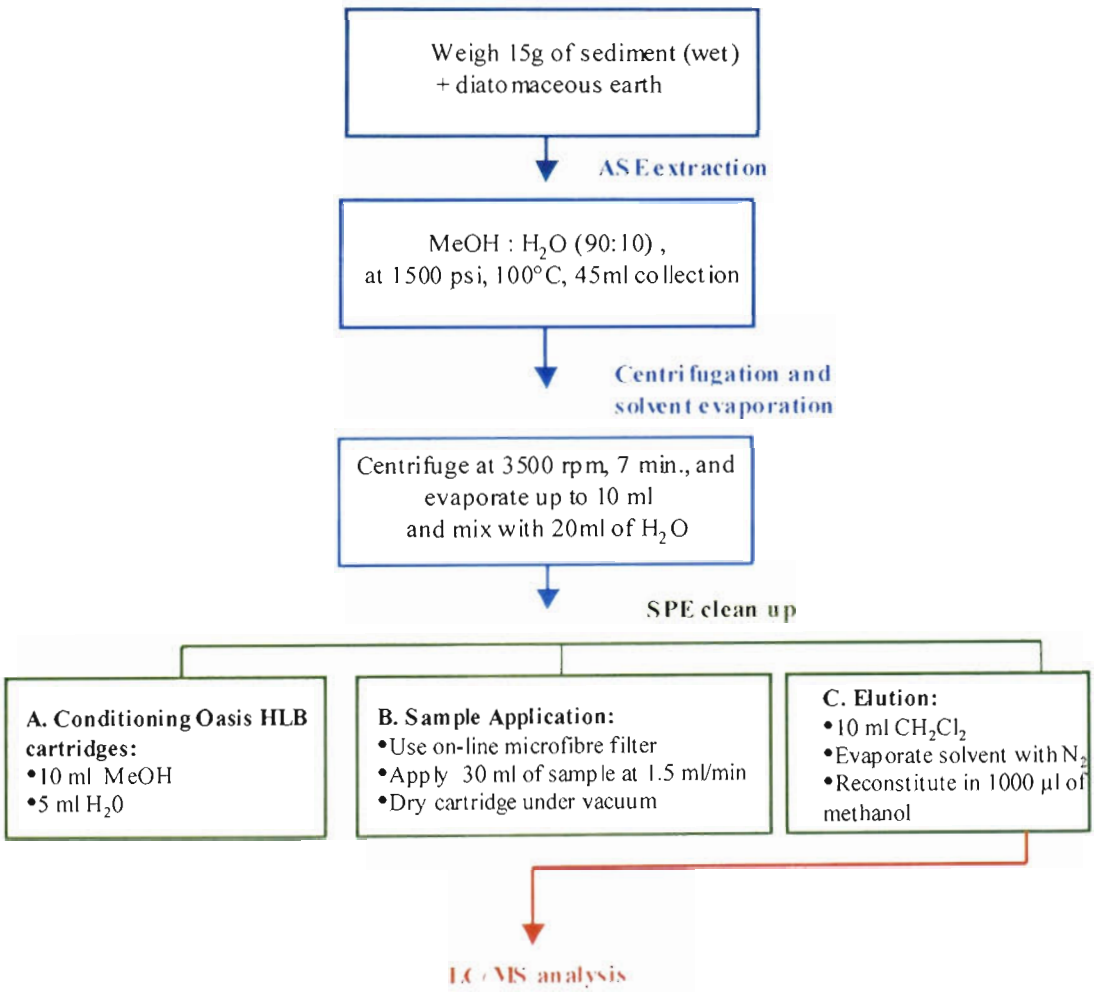


Figure 11. Scheme for the analysis of triazine-based herbicides in sediments.

CHAPTER III. RESULTS AND DISCUSSION: PHENOXYACID HERBICIDES

OPTIMIZATION AND METHOD DEVELOPMENT

LC/MS Ionization Selection and Detector Optimization

The majority of studies based on liquid chromatography mass spectrometry require the proper selection of the interface and the most favorable ionization mode for the target analytes of interest. Depending on the chemical characteristics of the compounds and their physicochemical properties, the choosing of an interface is very important for pesticides characterization.

In electrospray ionization, the eluent coming from the HPLC is pumped through a nebulizing needle that is at ground potential. Electrospray nebulization is the result of charging a liquid at a needle tip by applying a high potential between the needle and a nearby counter electrode. The formation of the aerosol depends on the competition between coulombic repulsion and surface tension (De Hoffman and Stroobant 2001). The mechanism of ionization is summarized in three main steps: a) the production of charged droplets; b) the solvent content in the droplets evaporates, which causes them to shrink to the point where the repelling coulombic forces come close to their cohesion forces, thereby causing their division; c) desorption of ions from the surface of the droplets and gas phase ion formation. The presence of a heated nitrogen gas (~300°C), helps in the process of solvent evaporation and droplet shrinkage. It is assumed that the molecule is either, positively or negatively charge before final gas-phase ion formation and that the charging of the analyte has occurred in solution (Thurman et al. 2001).

In the case of the phenoxy acid herbicides, it is expected to have good sensitivity

in the negative mode electrospray because of the presence of anionic species in solution. These pesticides are negative anions in solution and are well vaporized from the ionic state. This situation was confirmed experimentally, and concurs with the typical behavior of organic molecules as reported by Thurman in the ionization-continuum diagram, which evaluates these concepts in a practical way (Mansoori et al. 1997; Thurman et al. 2001).

In a single quadrupole mass spectrometer instrument like the one used in this research, several parameters are considered critical when an electrospray ionization interface is being used: cone voltage; probe temperature, and pH conditions. These ion source parameters should be selected in a manner that they give the best response in terms of sensitivity and selectivity.

When performing the inlet optimization process, the settings of one parameter were changed, while the other parameters were kept constant. Each one of the herbicides has different ionization behavior in the electrospray interface. For this reason, they needed to be injected separately to optimize their detection. The best approach to evaluate each one of these parameters and its influence in the nebulization and ionization process was using a flow injection technique. In flow injection, the flow from the pump passes directly to the mass spectrometer without the need to have a chromatographic column attached to the system. In this system, once the injection is done the analyte goes directly to the spectrometer without separation and a constant amount of the analyte could be delivered during the optimization. In this preliminary study the mobile phase composition was 100 % MeOH at 0.5 mL/min of flow and the standards were injected at a concentration of 20μg/ml with a volume of injection of 20 μl.

Electrospray ionization is a soft ionization technique. The possibility of losing a single proton in solution to become charged, such the case of phenoxy acid herbicides, allow the formation of a strong and abundant parent ion $[M-H]^-$, that retains molecular information.

The voltage applied to the cone is a parameter that determines not only the pattern of fractionation of the molecules but also the sensitivity of the ions of interest. The cone voltage was ramped between 5 to 40 volts. The abundances of the signals for the molecular ion $[M-H]^-$, the most intense trace, and the isotopically enriched molecular ions is shown in figure 12 for 2,4,5-T.

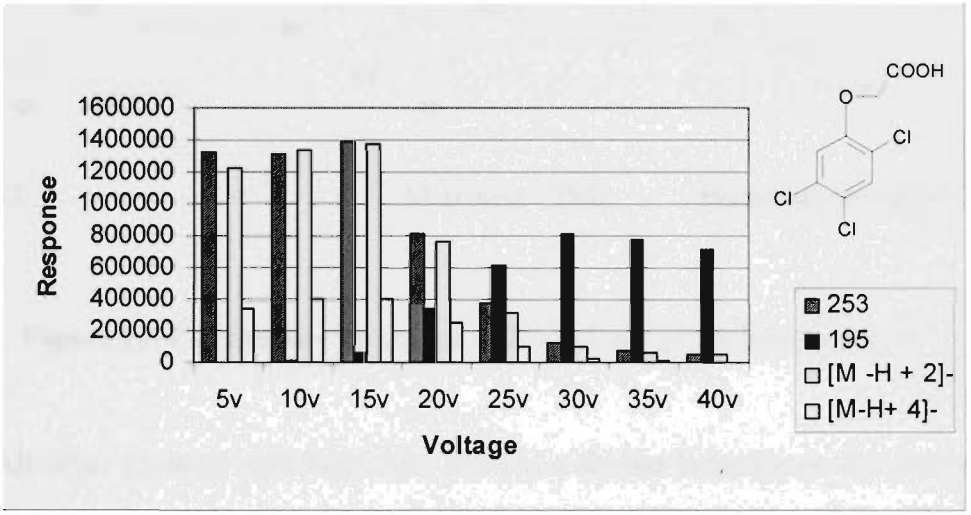
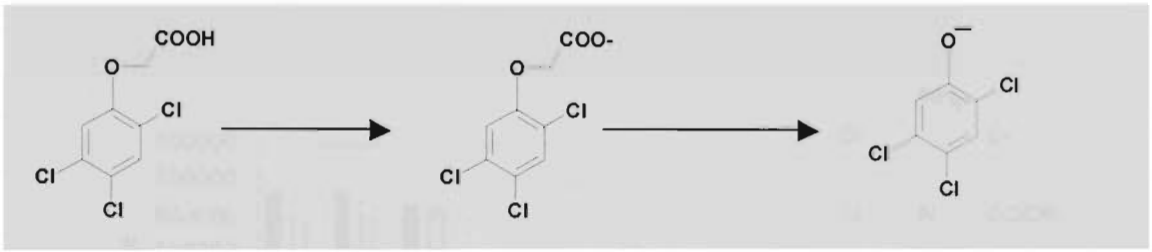


Figure 12: 2,4,5-T Cone voltage optimization

It is important to notice the effect of the cone voltage on the ionization of this herbicide. The expected $[M-H]^-$ ion corresponding to the pseudo molecular ion at m/z= 253 appears at low cone voltage values and reached a maximum in response at 15 volts. The evaluation of the presence of $[M-H+2]^-$ and $[M-H+4]^-$ in the case of 2,4,5-T (m/z of 255 and 257), is relevant because of the presence of chlorine atoms in the structure. For

these reason, the m/z ions including the isotopes are shown to describe their own behavior under the voltage conditions.

The fragment at m/z= 195 shown in figure 10 corresponds to the formation of the phenolate ion presented in figure 13. Formation of this fragment starts at 15 volts with a maximun response at 30V. However the presence of the parent m/z ion 253 and the isotopes $[M-H + 2]^-$ and $[M-H + 4]^-$, are considered analytically more important than the fragment at m/z =195 for confirmation purposes. Thus, the optimization was set to maximize their formation



2,4,5-T M-H(m/z =253) Phenolate ion (m/z=195)

Figure 13: Electrospray ionization of 2,4,5-T and phenolate formation

All other phenoxy acid herbicides showed a similar behavior on the electrospray source.

Another compound of interest in this investigation was picloram. This herbicide is not a phenoxy acid compound but it is included in the analytical evaluation as a target analyte as well as other acidic herbicides. The ion formation pattern for this molecule with the change of cone voltages is shown in Figure 14. The results demonstrate a behavior similar to 2,4,5-T. At low voltages the pseudo-molecular ion at m/z of 239 dominates the ion current but it tends to disappear quickly at relatively high cone

voltages. The isotopic information provided by the $[M-H+2]^-$ and $[M-H+4]^-$ is essential for this compound because they provided the only confirmatory evidence for this compound at the optimized cone voltage of 15 V. Also in this case, the selection of the optimum cone voltage was less critical because similar responses were observed between 10 and 15 volts. However, because most of the phenoxy acid herbicides showed a similar behavior on the electrospray source, the optimum response was achieved at 15 volts and this variation was not significant.

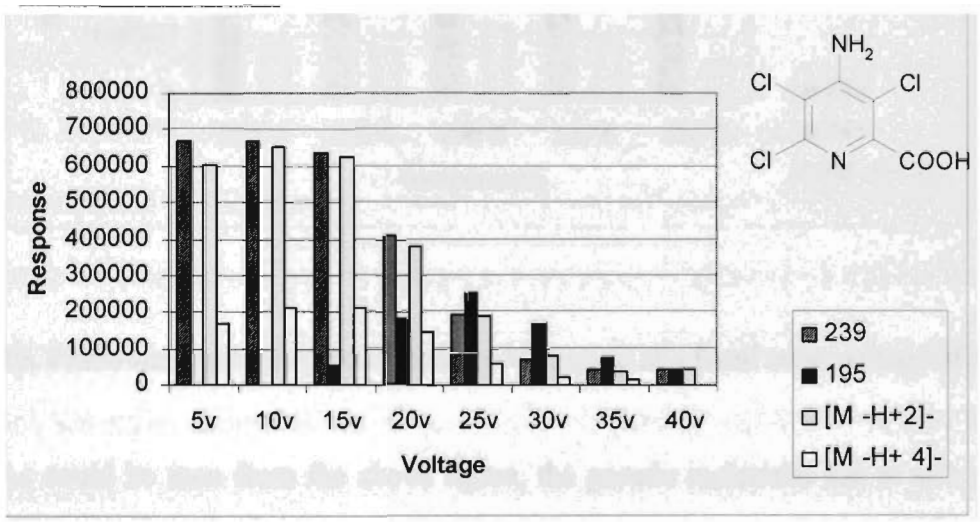


Figure 14: Picloram cone voltage optimization.

This pattern was observed in the rest of the compounds of interest both phenoxy and non phenoxy acid herbicides in the group. Ion currents were observed better at 15 V and this value was chosen as the optimum cone voltage.

The value of the cone at 15 V was then fixed in order to study the effect of the second parameter of interest: the probe temperature. Five different temperatures were tested: 250, 280, 300, 320 and 350°C. Figure 15 shows the probe temperature optimization results for mecoprop.

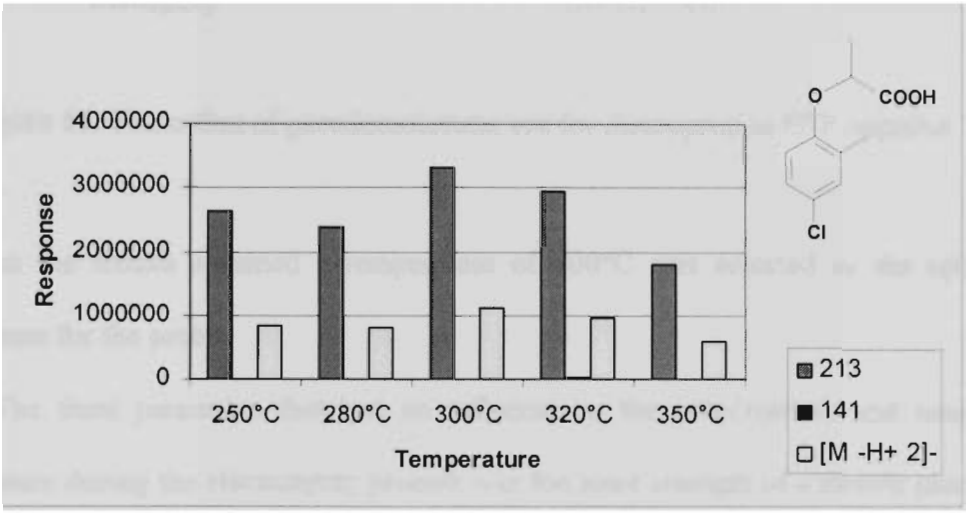
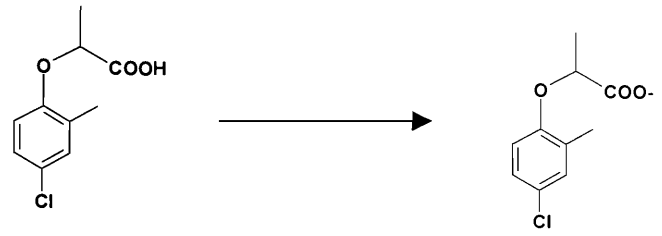


Figure 15. Probe temperature optimization for Mecoprop at a fixed cone voltage of 15 V

As could be seen from the above figure, the pseudo molecular ion at m/z = 213 and the isotope $[M-H+2]^-$ =215, show better ion production between 300 and 320 °C. It is also interesting to note that the main fragment ion m/z = 141 is not formed under these conditions of ionization.

The formation of the pseudo-molecular ion for mecroprop is show in figure 16. In general, the probe temperature at which ionization efficiencies are maximized for all the phenoxy acid herbicides was found to be 300°C.



Mecoprop $(M-H)^- = 213$

Figure 16: Formation of pseudomolecular ion for mecroprop in ESP negative

Based on the results obtained a temperature of 300°C was selected as the optimum temperature for the probe.

The third parameter that had an influence in the nebulization, and ionization performance during the electrospray process was the ionic strength of a mobile phase and the presence of phase modifiers. However, is important to notice that a delicate balance exists between chromatographic separation and ionization efficiency. In terms of separation, the acidic moiety of the phenoxy acid compounds and their low pKa values require separation using a slightly acidic mobile phase to selectively retain protonated-carboxilic acid groups. Nevertheless, the addition of a base will help in the formation of anionic species that could improve the ionization of the compound during the ESI process:

$$RCOOH + :B \longrightarrow RCOO^- + HB^+$$

In order to investigate the effect of acid-base equilibrium process on the ionization, the presence of volatiles additives such as acetic acid and ammonium hydroxide were tested, by changing the composition of a mobile phase consisting of MeOH:H_2O (50:50). Changes included the following variations: MeOH:NH_4OH 100% (50:50); and MeOH:HOAc (50:50) with the concentration of the acetic acid at 0.1% (v/v).

The change in mobile phase composition via flow injection was done measuring 3 replicates of each herbicide using the mentioned modifiers such as: HOAc 0.1 % (pH = 3.45) and NH_4OH 0.1 % (pH = 9.72). A comparison of the response to each of these modifiers under the same analysis conditions (Probe Temp = 300°C and cone voltage = 15 V) was included. An increased response with ammonium hydroxide was found in four of the twelve herbicides: 2,4,5-T; acifluorfen; dichlorprop and MCPA. However the ionization efficiency for the rest of the herbicides under basic pH was very poor. The same situation occurs when acetic acid was tested at 0.1 % concentration. Even though the acetic acid did not provide an additional enhancement of the signal at the concentration tested, it did play an important role in the chromatographic separation and therefore it was the modifier of choice. In summary, the optimization of the inlet conditions had a pronounced effect on the formation of the ionized analyte molecule in the electrospray process. On the basis of this results the optimum conditions were set up at 15V cone voltage, 300°C probe temperature and pH below 3.45 (acetic acid).

Chromatographic Separation of Phenoxy Acid Herbicides

As stated before, a number of variables control the electrospray performance: the analyte concentration, the mobile phase composition, the pH and concentration of additives. All these parameters are involved in the optimization of the chromatographic separation of the phenoxy acid herbicides.

The chromatographic separation was tested by using a reverse phase column C_{18} 250 x 4.6 mm x 5 μm, and changing the composition of the mobile phase, the flow rate and the temperature of the column in order to find the best resolution between the peaks of interest in the shortest possible time of analysis. Methanol and acetic acid 1% were chosen as the principal components of the mobile phase. The presence of an acidic modifier in the eluent, helped to avoid the ion suppression of the herbicides.

The mobile phase flow rate is another significant experimental parameter considered during the chromatographic separation. The best electrospray performance was achieved at low flow rate of 0.5 ml/min. At this flow rate, the mass flow of analyte is efficient and allows a good sensitivity of the herbicides studied. A gradient elution was optimized to allow the chromatographic separation of the twelve herbicides by LC/MS and it is presented in figure 17. The mobile phase composition consisted of a binary gradient elution of methanol and acidified water with acetic acid 1%.

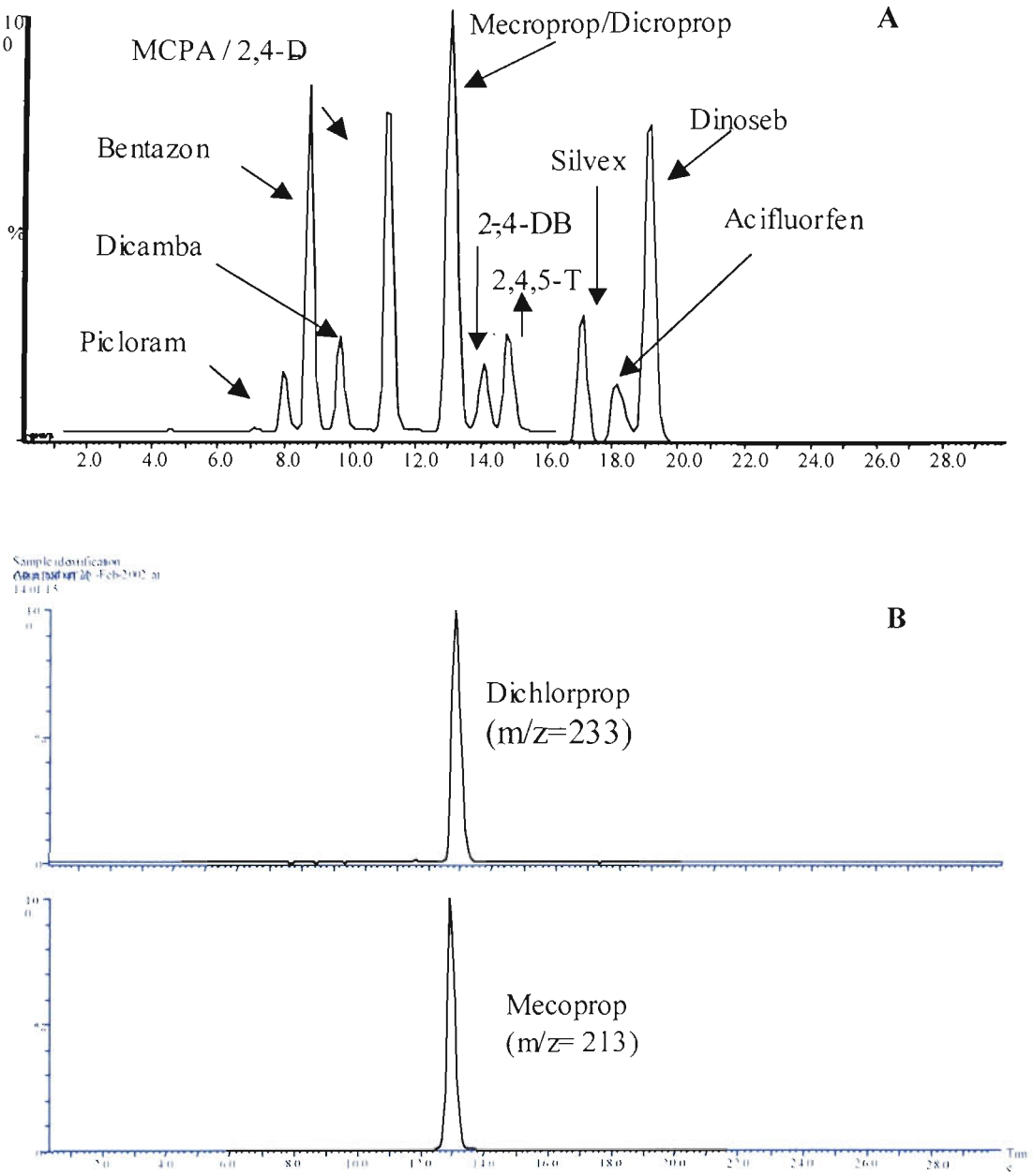


Figure 17: A. Typical chromatogram from an LC-ESP-MS analysis of phenoxy acid herbicides. B. Extracted ion for mecoprop (m/z=213) and dichlorprop (m/z =233)

The HPLC gradient program is shown in table 5. The total run time for this separation was less than 30 minutes.

Table 5: Gradient elution program for phenoxy acid separation

#	Time (minutes)	% A (Methanol)	%B (HOAC 1%) pH 2.76
1	0	75	25
2	15	82	18
3	18	75	25
4	30	75	25

Although some analytes co-elute (peaks 4,5 and 6,7), they can be easily identified using their specific molecular ion and fragments. This was the case for mecoprop and. dichlorprop (figure 14 B) where their identification is possible by means of the respective negative parent ions $[M-H]^- = 213$ and $[M-H]^- = 233$, respectively.

In order to improve the sensitivity of analysis, a single ion-monitoring program (SIM) was used. This program includes molecular and isotope ions for the phenoxyacids that are shown in table 6. Seven different SIM functions were selected with an interchannel delay of 0.02 seconds and a dwell time of 0.5 seconds per ion. The SIR monitoring mode allows the elimination of inteference background and the proper separation of the 12 peaks of interest using a gradient elution

Table 6: Single ion monitoring (SIM) program and quantifying ions for phenoxy acid

#	Compound	Formula	SIM #	Retention time (s)	Nominal Mass	Parent Ion $[M-H]^-$	$[M-H+1]^-$ (q)	$[M-H+2]^-$ (q)
1	Picloram	$C_6H_3Cl_3N_2O_2$	1	6.9	240	239	241	243
2	Bentazon	$C_{10}H_{12}N_2O_3S$	1	7.8	240	239	241	-
3	Dicamba	$C_8H_6C_{l2}O_3$	1	8.0	220	219	221	223
4	2,4-D	$C_8H_6Cl_2O_3$	3	10.3	220	219	221	223
5	MCPA	$C_9H_9ClO_3$	3	10.4	200	199	201	-
6	Dichlorprop	$C_9H_8Cl_2O_3$	4	12.6	234	233	235	-
7	Mecoprop	$C_{10}H_{11}ClO_3$	4	12.6	214	213	215	-
8	2,4,5-T	$C_8H_5Cl_3O_3$	5	13.4	254	253	255	257
9	2,4-DB	$C_{10}H_{10}Cl_2O_3$	5	13.6	248	247	249	-
10	Acifluorfen	$C_{14}H_7ClF_3NO_5$	6	15.7	361	360	362	-
11	(Silvex)	$C_9H_7Cl_3O_3$	6	16.3	268	267	269	-
12	Dinoseb	$C_{10}H_{12}N_2O_5$	7	18.9	240	239	241	-

Analytical Performance for Phenoxy Acid Herbicides

The linear dynamic range was studied under the chromatographic conditions adopted. The measurement of the response by LC-ESP-MS, was performed by injecting standards solutions of all the target analytes within the range of 0.15 to 4 µg/ml (eight calibration points), and averaging the peaks areas of the extracted ions of interest (n=3). Satisfactory linearity ($r^2 > 0.990$) was obtained for all acidic herbicides. Regression coefficients values are summarized in table 7.

Table 7: Correlation coefficients for target analytes

Analyte	r^2	Analyte	r^2
Picloram	0.996	Dichlorprop	0.994
Bentazon	0.992	2,4,-DB	0.994
2,4-D	0.999	2,4,5-T	0.998
MCPA	0.996	Silvex	0.995
Dicamba	0.993	Acifluorfen	0.995
Mecroprop	0.995	Dinoseb	0.990

Method Development for Extraction and Clean up Procedures

General Considerations

The organic content in the sediment samples varied depending on the collection site. For most of the Everglades National Park sediment samples the total organic content or TOC were high (10-85%), and therefore they are very difficult to analyze by conventional extraction methods such as liquid/liquid extraction due to the formation of complex emulsions and the interferences from co-extractants. In the case of marine sediments of Biscayne Bay, these had lower percentages of organic matter (below 8 %) but they are still a challenge for traditional extraction and clean up. Figures 18 and 19 shows the distribution of the organic matter present in the different sediments evaluated in these studies.

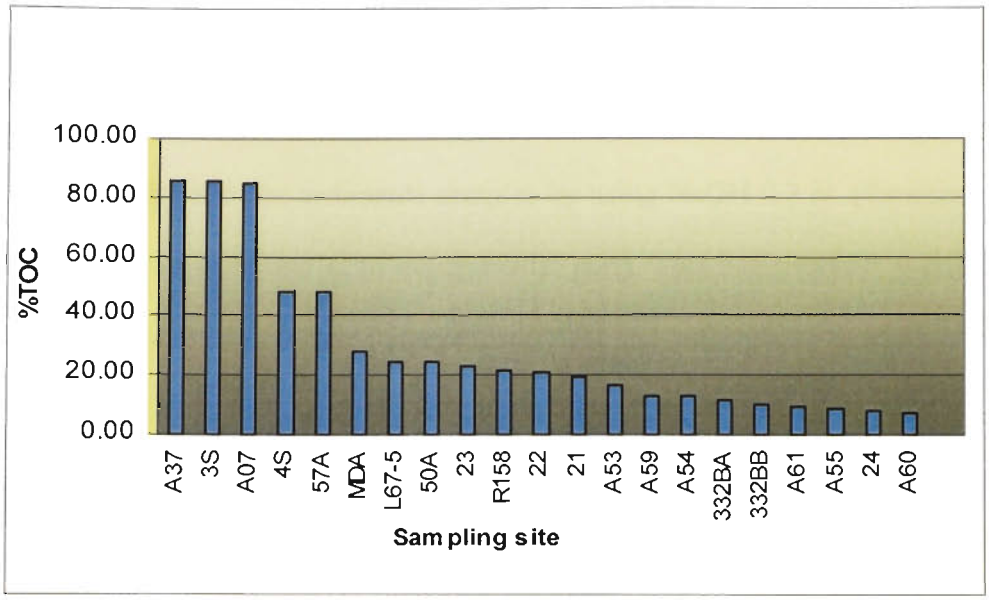


Figure 18:Total organic carbon distribution in Everglades National Park

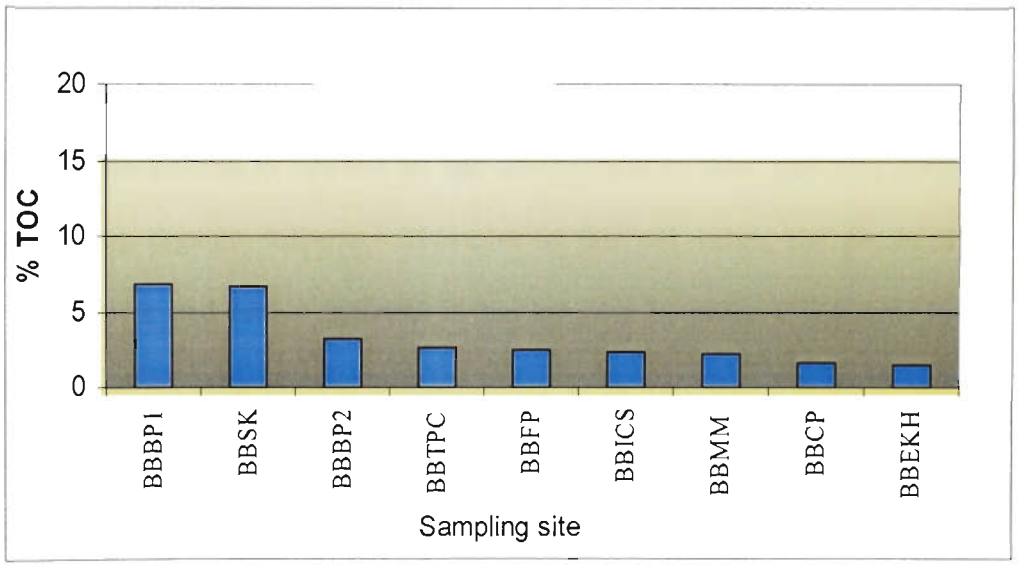


Figure 19. Total organic carbon distribution in Biscayne Bay.

Extraction and Clean Up Procedures

Different strategies and approaches were conducted in order to compare the efficiency of the extraction in sediments samples. The first one compares the hydrolysis of the different analytes in the sediments samples by using NaOH 0.3 N. (for details see description of method A, B and C in experimental part). This basic extract was further isolated and purified by liquid-liquid extraction (LLE) and solid phase extraction (SPE) with two different sorbents: graphitized carbon black and a polymeric resin. In the second approach, the use of accelerated solvent extraction (ASE) was evaluated using different extraction solvents as well as two different SPE sorbents (methods D, E, F and G). A summary of mean recoveries for the first extraction and clean up procedures is presented in table 8.

The use of NaOH as hydrolyzing agent facilitates the water-based extraction of these compounds from the sediments because these acidic herbicides were presumable present as ionizable compounds at the elevated pHs. The subsequent decreasing of the pH below 2 was needed to convert all the components to their free acid form prior to extraction using organic solvents. However at this pH value, the presence of high amounts of fulvic and humic material in the samples becomes a concern. In the first method (Method A), recoveries of approximately 40 % were found in the samples. These relatively low values reflect the effect of the matrix interferences presented in the sediment samples. The LLE technique was too laborious and time consuming to be used for routine screening purposes. As a way to reduce the complexity of extraction, the application of SPE in carbon and polymeric phases was tested.

Table 8:Comparison of percentage recoveries on sediments using LLE, and SPE with carbon or polymeric cartridges.

Compound	LLE-NaOH-CH_2Cl_2	SPE Carbon - NaOH	SPE-HLB-NaOH
Picloram	23.0	13.1	ND
Bentazon	49.8	27.2	ND
Dicamba	38.3	26.9	21.0
MCPA	34.7	40.5	24.0
2,4-D	75.1	62.8	31.0
Mecoprop	42.2	27.1	53.0
Dichlorprop	41.8	32.7	47.2
2,4-DB	44.8	6.2	55.8
2,4,5-T	41.9	19.7	53.0
Silvex	40.0	18.3	57.0
Acifluorfen	35.4	ND	10.0
Dinoseb	45.2	ND	ND
	Method A	**Method B**	**Method C**

Based on preliminary recovery information obtained in the laboratory with a graphitized carbon-based material in water samples, this sorbent material was selected and tested with the sediment matrix. Because of the inherent advantage of the simultaneous extraction of neutral, basic and acidic compound in this type of sorbent, the decision to work at neutral pH was taken. However the recoveries obtained with the basic extraction- SPE carbon (Method B), even making the adjustment of the pH, showed significant loses of 4 of the 12 analytes of interest. A possible explanation requires to consider the existence of different mechanisms of adsorption involved in the retention of the herbicides. This includes the interaction between the analyte and the sorbent itself, the presence of different groups on the benzene ring; lipophilicity of the analyte and pH.

The use of a polymeric material under the same extraction conditions instead of the carbon sorbent (Method C), showed better overall recoveries but some target analytes were still lost (see table 8). Six of the herbicides (mecroprop, diclhorprop, 2,4-DB, 2,4,5-T, sylvex and acifluorfen) shown an improvement with 1.4 to 9 times higher recoveries values but the overall recoveries are still low and highly un-reproducible. Because of these problems, the use of LLE as extraction technique was discontinued.

Coupling of Accelerated Solvent Extraction with SPE

Sediments from the Everglades are rich in humic and fulmic acids that may produce matrix interferences, complexation and co-precipitation making the extraction of polar herbicides an analytical challenge. The use of accelerated solvent extraction (ASE) was evaluated using different extraction solvents in combination with two different SPE

sorbents. A summary of the comparison of extraction recoveries obtained with the ASE-SPE methods is presented in table 9.

Table 9: Comparison of percentage recoveries on sediments using the combination of ASE and SPE under different parameters

ASE ext. solvent	**Water**	**Water**	**NaOH**	**Acetone-HOAc**	**Acetone-HOAc**
SPE cartridge	**Graph. carbon**	**HLB – Oasis**	**Graph. carbon**	**Graph. carbon**	**HLB – Oasis**
Compound					
Picloram	ND	ND	ND	30	74
Bentazon	23	ND	15	35	98
Dicamba	18	43	65	66	77
MCPA	10	28	22	56	102
2,4-D	45	45	60	54	108
Mecoprop	19	58	32	60	105
Dichlorprop	24	65	39	57	93
2,4-DB	2	23	53	35	53
2,4,5-T	21	41	39	42	76
Silvex	27	69	38	45	83
Acifluorfen	66	98	4	45	54
Dinoseb	21	ND	ND	40	71
	Method D	Method E	Method F	Method G	Method H

The recoveries of the different herbicides varied according to the polarity and pH of the extraction solvent used. The use of water had the intention of replace organic solvents as extracting agent. Its high polarity suggested that it would be the best solvent for the analysis of polar herbicides like the phenoxy acid in their ionic form. Furthermore, the use of pressure and high temperature could increase the extraction capabilities as sub-critical water, which has been shown as an effective solvent for complex matrix like sediments. Neither water at neutral pH or a 0.3 M solution of NaOH proved to be adequate to extract the acidic herbicides.

The use of an on-glass fiber filter before the SPE cartridge, proved to be necessary because of the presence of high levels of organic matter and the subsequent precipitation after the pH adjustment (see figure 20 for details). The filtration also improved the background levels in the final extraction and prevented the clogging of the cartridges, enhancing the reproducibility in recoveries.

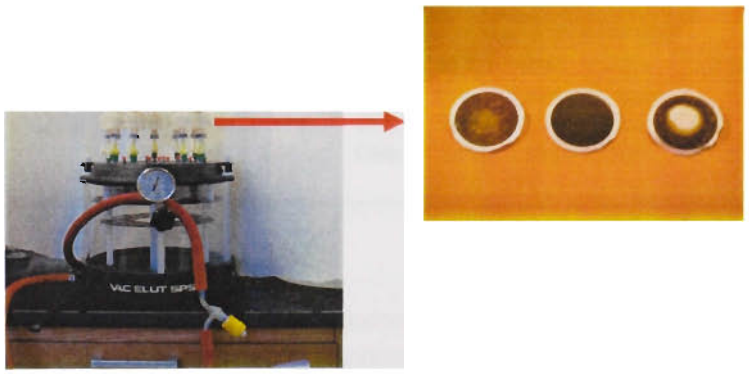


Figure 20: Solid Phase extraction system with glass fiber filter

Figure 21 shows a summarized comparison of all extraction strategies, and demonstrates the extraction capabilities of each methodology. The overall extraction of the 12 compounds is shown together in each of the columns of the figure, therefore a maximum of 1200 units in the "y" axis will represent 100% efficiency for all analytes. The combination of acetone and acetic acid for ASE extraction and polymeric cartridges for the clean up step not only improved the number of analytes recovered from the sediment but also the ease and reproducibility of the analysis of phenoxy acids as compared to other approaches.

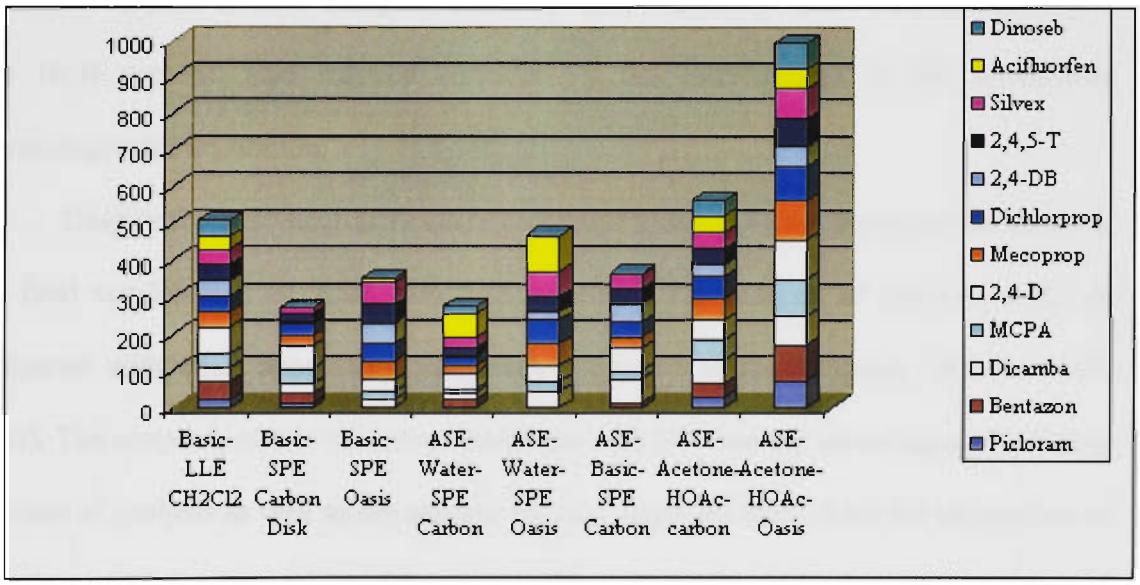


Figure 21: Comparison of the overall recovery efficiencies of the different extraction approaches studied.

Table 10 shows that relative standard deviations better than 15% were achieved for all analytes with the optimized protocol. The higher recoveries obtained using the polymeric cartridges can be explained due to the more selective interactions of the functional groups of the solid phase and the larger surface area (~800 m^2/g) increasing contact between the analyte and the polymeric sorbent (Leon-Gonzalez and Perez-Arribas 2000; Wells and Yu 2000). The vinilpyrrolidone co-polymer has also the key advantage of being highly water wettable and therefore differences in recoveries due to temporal dryness of the cartridge are no longer a problem. Also the use of MTBE as elution solvent during the final step in SPE, allowed a fast evaporation of the enriched sample under the N_2 flow or even using air at the vacuum chamber of the VAC ELUT system. The HLB sorbent, also reduced considerably the interferences in the subsequent chromatographic separation.

Despite the analytical difficulties presented in some of the experimental designs, the final combination of ASE with SPE improved the analysis of phenoxy acids as compared with other approaches previously presented in the literature. (Patsias et al. 2002). The coupling of this extraction technique with SPE has the advantages of reducing the time of analysis as well as the amount of toxic waste solvents. Also the automation of sample preparation was achieved by means of the use of the ASE which is a system that can hold up to 24 samples without any attendance of the operator.

Table 10: Recovery study for phenoxy acid herbicides in sediments samples (n=7) using the optimized ASE and SPE parameters

Herbicide	Mean Recovery %	% RSD
Picloram	74	15
Bentazon	98	12
Dicamba	77	12
MCPA	102	5
2,4-D	108	4
Mecoprop	105	11
Dichlorprop	93	7
2,4-DB	53	9
2,4,5-T	76	8
Silvex	83	9
Acifluorfen	54	11
Dinoseb	71	15

Chemical properties of acidic herbicides and effect on the overall recoveries

The selection of the extraction technique, the sorbents for SPE clean-up and the elution solvents depend not only in the analytical technique used for the detection but also on the chemical properties of the analytes.

Even though the acidic herbicides under study hold similarities in their chemical structures, the physical and chemical properties vary according to the main functional groups. Table 1 (see page 6) shows the variety of acid functional groups of the suite of acidic herbicides that include: phenol, benzoic acid, acetic acid, pyridinecarboxylic acid and diferent phenoxyacetic acids. During the SPE analysis the surface chemistry, and the sorption properties together with the chemical properties of the acidic herbicides, influence directly the way they compounds are extracted and the recovery efficiencies. Table 11 summarizes some of the properties of the target compounds. As mentioned before, SPE methods have numerous advantages over the conventional LLE. Nevertheless, the technique also has some inherent weaknesses that should be taken into account during the method development. One of the major limitations of SPE is the restricted sorption capacity of sorbents. Sample overload is one of the main causes of low recoveries and poor reproducibility for SPE. An estimation of the load capacity of the SPE sorbents is given by the calculation of the breakthrough volume (V_B). The breakthrough occurs when a solute is no longer retained by the sorbent, because the capacity of the sorbent has been reached. This is especially important in solid phase extraction because the sample is being continuously applied to the sorbent and this material must retain all the solute (Pawliszyn 2002).

Table 11: Chemical properties of the phenoxyacid herbicides

Compound	Formula	Log Kow	Koc	log Koc	pka	Aqueous solubility (mg/l)
Acifluorfen	$C_{14}H_7ClF_3NO_5$	3.70	3125	3.49	1.93	120
Bentazon	$C_{10}H_{12}N_2O_3S$	2.81	37.5	1.57	3.30	500
2,4-D	$C_8H_6Cl_2O_3$	2.83	29.4	1.47	2.60	620
2,4-DB	$C_{10}H_{10}Cl_2O_3$	3.53	100	2.00	4.80	46
Dicamba	$C_8H_6Cl_2O_3$	0.54	28.8	1.46	1.87	4500
Dichlorprop	$C_9H_8Cl_2O_3$	3.00	48.6	1.69	2.86	350
Dinoseb	$C_{10}H_{12}N_2O_5$	2.29	3544	3.55	4.50	52
MCPA	$C_9H_9ClO_3$	3.25	29.4	1.47	3.12	825
(Mecoprop)	$C_{10}H_{11}ClO_3$	3.13	48.6	1.69	3.11	620
Picloram	$C_6H_3Cl_3N_2O_2$	1.92	18.1	1.26	1.97	430
2,4,5-T	$C_8H_5Cl_3O_3$	3.31	48.6	1.69	2.20	238
2,4,5-TP (Silvex)	$C_9H_7Cl_3O_3$	3.80	80.4	1.91	3.00	140

There are several methods to estimate the breakthrough volume. The most straightforward method is using on-line ultraviolet (UV) detection of a water sample spiked with traces of a solute, which has an initial absorbance A_0. The spiked sample is passed through the SPE column. If the compound is retained by the sorbent, the effluent will have an absorbance of zero. A frontal or breakthrough curve is recorded (figure 19), beginning at a volume V_b, usually defined as 1 % A_0 up to a volume V_m ,defined as 99 % of A_0, where the effluent has the same composition as the spiked water sample (Poole

2002). Under normal conditions, the shape of the curve is logarithmic, where the inflection point is the retention volume, V_r, of the analyte (chromatographic elution volume). The parameter V_b (1% breakthrough) in very important for the pre-concentration of the analyte and could be determined by several methodologies in which the V_r is the target measurement.

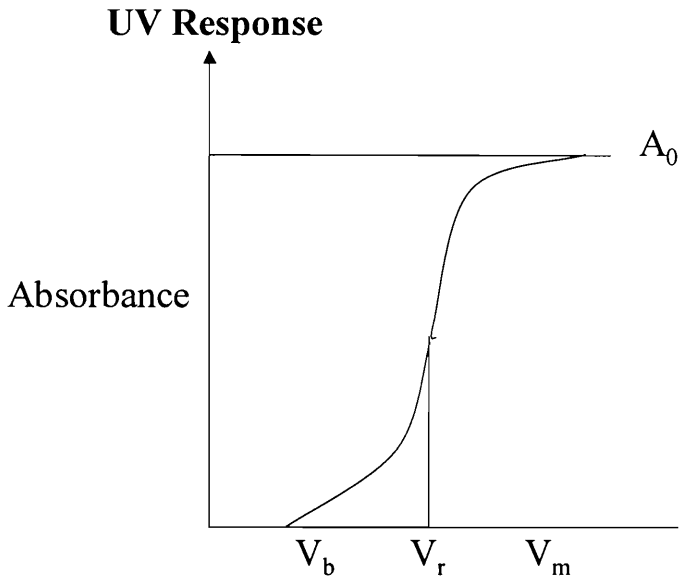


Figure 22: Typical representation of the breakthrough curve (Poole 2002)

The problem with the recording of the breakthrough curves is that it is time consuming and the reading of 1% V_r is difficult and not always accurate. As a consequence, different models have been developed to estimate the V_B based on solute and analyte properties. In this study the model proposed by Thurman, was used to estimate this volume(Thurman et al. 2001).

The model uses the octanol –water partition coefficients (log Kow) to estimate the sorbent-water retention factor (log Kw) using the following equations:

(1) $\log k_w = 0.988 * \log K_{ow} + 0.02$

(2) $Vr = Vo\ (1 + Kw)$

(3) $V_B = Vr - 2.3\ \delta$

(4) $\delta = Vo\ (1 + kw) / \sqrt{N}$

where:

$\log K_{ow}$ is the water-octanol distribution constant for each analyte

Vo is the void volume estimated from the total pore volume

δ is the standard deviation depending on the axial dispersion of the analyte and

N is the plate number for the sorbent

According with the certificate of analysis from the HLB extraction cartridges, the reported total pore volume was 1.28 cm^3/g, the cartridges employed for this study have 250 mg of sorbent weight and therefore the void volume can be estimated as follows:

$$Vo = (1.28\ mL / g) \times 0.250\ g$$

$$Vo = 0.32\ mL \text{ per cartridge}$$

The plate number used for the calculations has been estimated to be ~20 regular C_{18} or polymeric SPE cartridges (Poole 2002)

Using the reported water-octanol constants the calculated breakthrough volumes are summarized on table 12. From this table, dicamba, picloram and dinoseb presented a very low breakthrough volume, which is below the sample volume added into cartridges after ASE extraction, which is approximately to 70 ml. This low V_B could contribute to

the mean recoveries values obtained for those 3 compounds (~70 %) because that could limit the retention of analytes into the cartridge and produce some losses during the loading step. The other herbicides did not present any problem with the recoveries because all of them are above the loading volume used in the method and therefore the extraction efficiency was not affected.

Table 12:Estimated breakthrough volumes for the acidic herbicides using HLB cartridges

Herbicide	**log Kow**	**VB / (mL)**
Dicamba	0.54	0.70
Picloram	1.92	13
Dinoseb	2.29	29
Bentazon	2.81	95
2,4-D	2.83	100
Dichlorprop	3.00	145
Mecroprop	3.13	195
MCPA	3.25	257
2,4,5-T	3.31	296
2,4-DB	3.53	488
Acifluorfen	3.80	718
Silvex	3.80	893

It is important to mention that the application of the previous formulas does not adjust the model for the use of graphitized carbon bases SPE cartridges, because of the presence of other type of interaction like ion exchange that could affect the retention of the analytes (Poole 2002). The informative value of the breakthrough volume is a powerful tool when considering use SPE as a clean up process of a matrix.

Principal Component Analysis (PCA)

PCA is a method of identifying patterns in data, and expressing the data in such a way that could serve to highlight their similarities and differences. The primary objective of a principal components analysis is to reduce the amount of data when there is correlation between variables. It does not assume that the data have any particular distribution (Miller and Miller 2000).

The environmental fate of organic compounds and their interaction with biological systems are determined in part for hydrophobicity. Octanol-water and soil-water partition coefficients, K_{ow} and K_{oc}, respectively, have been the most widely used parameters in evaluating the movement and persistence of pesticides in the environment and structure-activity relationship studies (Liu and Quian 1995). In this particular study, a relationship between the pKa of each compound, the K_{ow} and the K_{oc}, were use to explain their influence in the recoveries of the herbicides under study. The PCA analysis was conducted using the properties reported on table 11. Figure 23 shows the PCA graph obtained using the Minitab software for multivariate statistics.

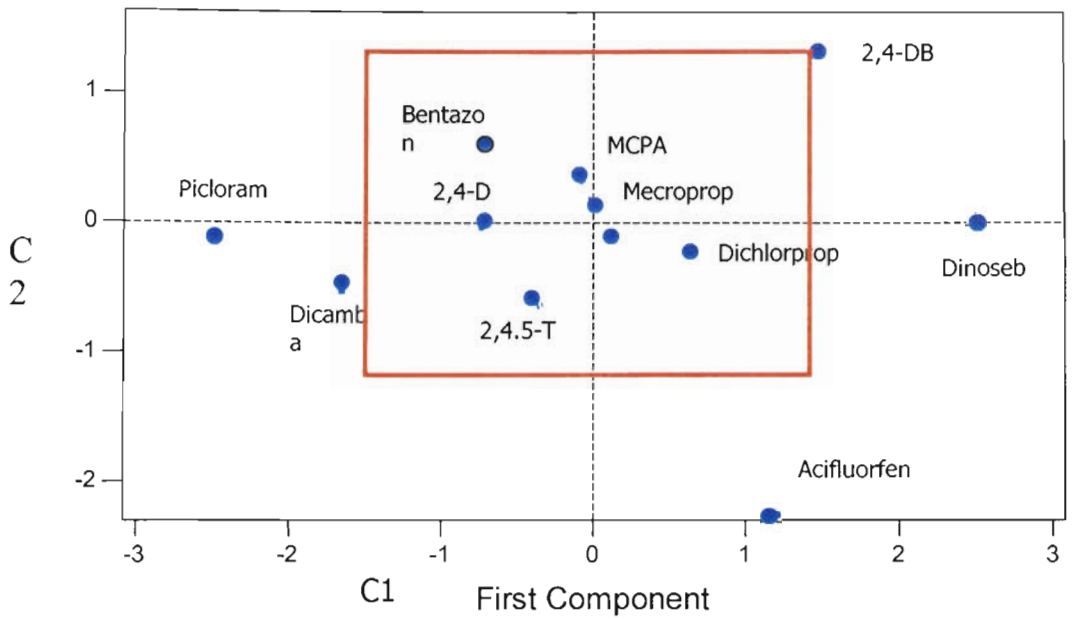


Figure 23:PCA analysis of acidic herbicides

This analysis showed a good correlation between the chemical properties of the herbicides and their recoveries. The four herbicides that have data points outside the inner square were more difficult to extract and are the ones that resulted in lower to medium recoveries: 53% (2,4-DB), 54% (acifluorfen), 71% (dinoseb) and 74% (picloram.) In addition, dicamba is near the boundaries of the square and presented mean recovery of 77%. All compounds that fit inside the square presented excellent recoveries. The chemical partitioning in the sediments is reflected in the recoveries obtained.

This correlation support the important role that values such as pKa and water-octanol distribution constants could play in the prediction of efficiencies in SPE extractions.

Those compounds that present log K_{ow} values below 1 such as picloram, are very hydrophilic while compounds with values close or above 4 are very hydrophobic. The pKa will be also influent in the extraction capabilities because the relative concentration of dissociated and non-disociated forms of ionizable analytes in aqueous solutions will be determined by the pH of the solution. The working pH of the SPE method is ~2.5 and therefore compound such as dicamba, picloram and acifluorfen, which have pKa values lower than 2, are not completely in their ionic form.

Instrument detection limits and method detection limit

In analytical mass spectrometry there are a few basic parameters that need to be assessed to determine the robustness of the analytical procedure. Two related concepts that are critical are the instrument detection limit (IDL), which evaluates the capability of detection and identification of an analyte by the mass spectrometer, and the method detection limit (MDL), which is a more valuable analytical criteria and includes the steps involved in the method. In this investigation, the instrument detection limit (IDL) was calculated by measuring the signal to noise ratio (S/N = 3) of the extracted ion chromatograms relative to a low concentration mixture of the individual herbicides. The estimation was done for each analyte by injecting seven independent mixed standards, each one by triplicate. The IDL, based on 15g of sediment sample, was set at a ranged in the low ng/g levels, depending on the analyte. The method limit of detection (MDL) is a much broader concept than the IDL, and it is defined as the minimun concentration of a substance that can be identified and measured in a real matrix with the complete

analytical method, including in this case, all the steps involved in the ASE extraction, SPE clean up and LC/MS determination (Budde 2001).

The method limit of detection was calculated at a spiked level of a sediment sample close to the IDL and 7 replicates were measured in order to account for variability of the method. The MDL results are presented in Table 13 and they were as low as 0.5 ng/g for dichlorprop to 23 ng/g for picloram. These MDLs give a sensitive screening level for the identification these herbicides in areas of low potential for contamination such the Florida Everglades and other protected environments.

There are few papers that report the detection and identification of these polar compounds in sediments or soils (Hunter and Carroll 1982; Hogendoorn et al. 2001; Luque-Garcia and Luque de Castro 2002). The proposed method, which take advantage of the combined power of ASE, SPE and LC/MS to provide an important improvement in the limits of detection given by the EPA 8151 A method (GC-ECD derivatization with Diazomethane) and the ones reported by Patsias et al (LC/UV) (Patsias et al. 2002) for sediment samples (Table 14). Elimination of derivatization reaction as part of the analytical protocol represents a great enhancement for the determination of these herbicides in particular for very complex matrix like sediments.

Table 13: Instrument detection limits (IDL) and method detection limits (MDL) for phenoxy acid herbicides

Herbicide	IDL (ng/g)	MDL (ng/g)
Bentazon	2.00	8.5
Dicamba	5.00	9.1
MCPA	0.34	1.0
2,4D	1.10	3.3
Mecroprop	0.54	1.6
Dichlorprop	0.16	0.5
2,4 DB	5.00	13.8
2,4,5-T	0.55	1.7
Silvex	0.65	1.9
Acifluorfen	2.00	3.2
Dinoseb	0.67	1.6
Picloram	10.00	23
Bentazon	2.00	8.5

Due to the constantly low MDLs and the ability to eliminate co-extractants and interferences, this method presents an excellent alternative for forensic investigation of relatively non-impacted sites like the ENP.

Table 14: Comparison of Method Detection Limits

Herbicide	Proposed method	Patsias et al	EPA 8551
Picloram	23	nr	nr
Bentazon	9	nr	nr
Dicamba	9	nr	nr
MCPA	1	50	43
2,4-D	3	40	0.11
Mecoprop	2	nr	66
Dichlorprop	0.5	40	nr
2,4-DB	14	50	nr
2,4,5-T	2	40	nr
Silvex	2	nr	0.28
Acifluorfen	3	nr	nr
Dinoseb	2	nr	nr

Analytical Quality Control

As part of the analytical protocol for the analysis of phenoxy acid herbicides, several quality control criteria were established to ensure the analytical data and the levels of concentration in the areas of interest. This is of especial importance, because of the nature and possible impact of the results in further forensic investigations.

Trace analysis requires special attention and should be conducted in a rigorous way considering the different steps in the analytical procedure. These steps include sampling, sample preparation, separation, quantification and evaluation of results.

Sampling sediments requires greater care than sampling other type of matrices. The sample must resemble the original population and also being representative. For these reason a simple sampling apparatus was used to collect the sediment samples. The sampling system consisted of a plexiglass tube that assisted a vertical penetration into the mud, and by means of vacuum the sample was collected and poured in a glass jar. This procedure helped to undisturbed the surface layer. The sampling unit was rinse before any collection of the samples. Also, the jars were previously combusted at 400°C for 6 hours, and solvent rinse to keep them free of organic contaminants.

All the sediment samples within the Everglades and Biscayne Bay National Parks were collected as a core composite sample from different stations within the same area. These core samples consisted of the top 3 inches of five 2.5 ' in a 100 m^2 area. In this way, both superficial as well as sediment column were collected. Glass jars with PTFE lined lids were used to preserve the samples and stored at 4°C before being transferred to the laboratory where they were stored in a freeze at –25°C.

Laboratory Blank Spikes (LBS)

In order to determine if the analytical method is under control, fortified laboratory blanks were run with every set of samples analyzed. The LBS are made using diatomaceous earth as sediment matrix replacement, and they are spiked with a mixture of the phenoxy acid herbicides in the ASE cell. The LBS are then processed through the

whole analytical procedure. As mention before, each batch of samples was run with an LBS. Figure 24, shows the variation of the recoveries within three different extraction batches. Good precision (less than 15 % RSD) was obtained for the inter-batch quantification of the different herbicides.

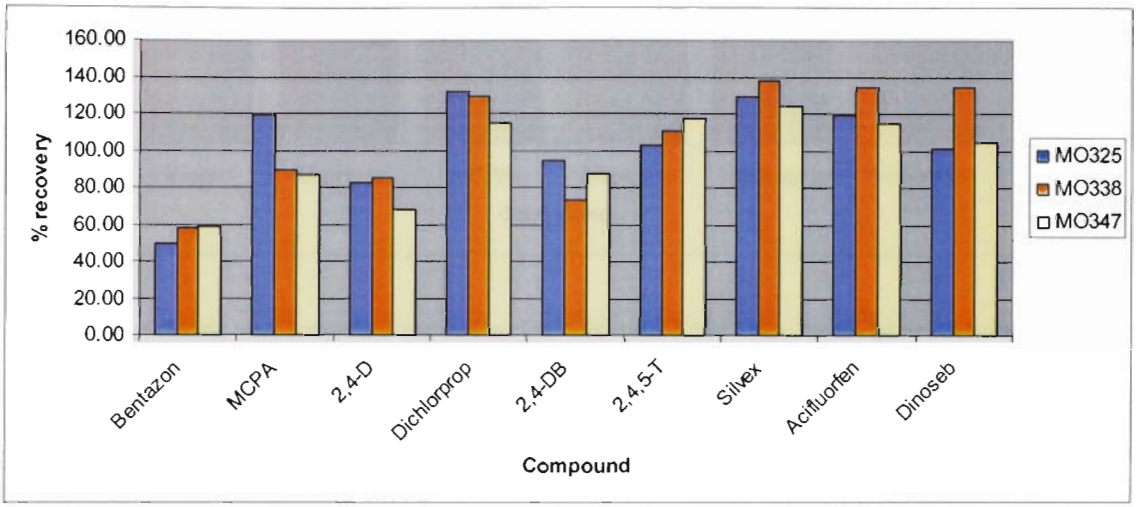


Figure 24:Interday variation of the LBS

Matrix Spike and Matrix Spike Duplicates

To assess both precision and accuracy, matrix spikes (MS) and duplicates (MSD) were analyzed. Both consisted of the use of real fortified sediment sample, spiked with a mixture of the herbicides of interest. Once again the whole analytical procedure was followed and the behavior of both control samples is shown in figure 25 for three extraction batches.

In this figure it is showed the variation of the MS and MSD within the same day (intraday) and during different days for three batches of sediments samples analyzed (interday).

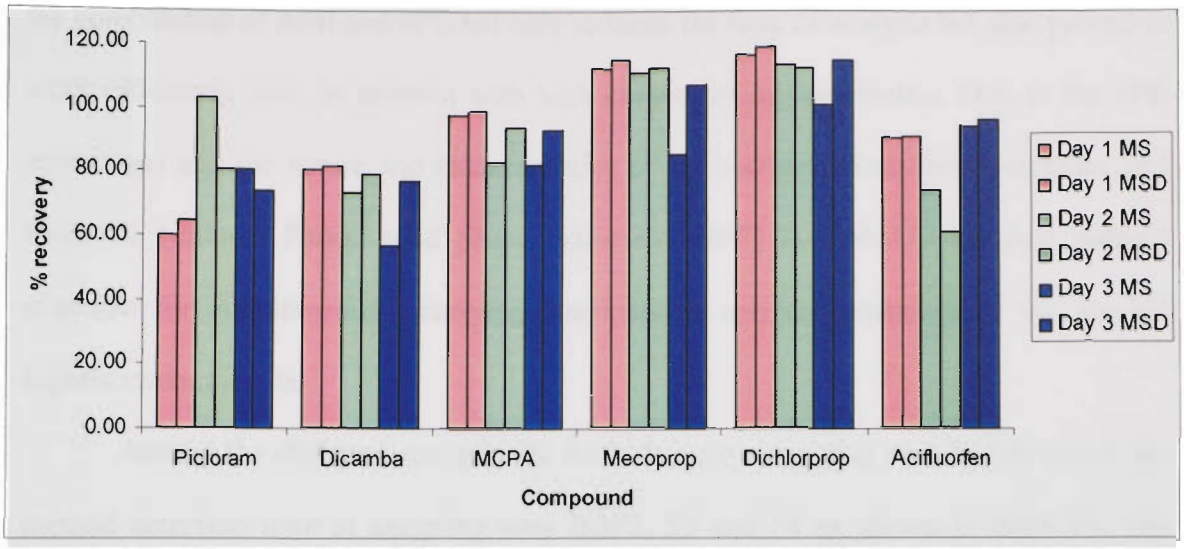


Figure 25: Intra and interday variation for MS and MSD for some phenoxy herbicides

The latter parameter was used as an indicator of the effect of the sample matrix on the recovery of the target analytes. Based on the results obtained, the precision of the method was evaluated showing values of below 15 % of RSD with the exception of picloram, which presented a higher value (19%). However the relative percent difference (RPD%) among duplicates is below 30 %. The interday variation for both MS and MSD showed also good precision and demonstrated the effectiveness of the method for the analysis of these polar compounds.

Application to real samples

Sediments from Everglades and Biscayne Bay National Parks in Florida were analyzed to assess the presence of these polar herbicides (see Figure 1 for details). The proposed method was applied to 30 different sampling sites. As was stated previously,

the combination of ASE and SPE not only reduced the time of analysis but also proved to work efficiently even in samples with high content of organic matter. Due to the SPE advantages and the nature and characteristics of the sediment from the Everglades and Biscayne National Parks, solid phase extraction (SPE) is probably the best method available for simultaneously carrying fractionation and concentration of the studied organic contaminants.

Among the different compounds studied, only mecoprop was found above the method detection limit at sampling sites BBP2, 53 and 58 as shown in table 15. The ranges of concentration for this compound were from 12 ng/g (BBP2) to 89 ng/g (ENP-53). Figure 26 shows one of the chromatogram for a positive identification of mecroprop in sample ENP-53. This chromatogram shows the effectiveness of the proposed methodology for clean up and detection capabilities of the mass spectrometer even for complex matrices.

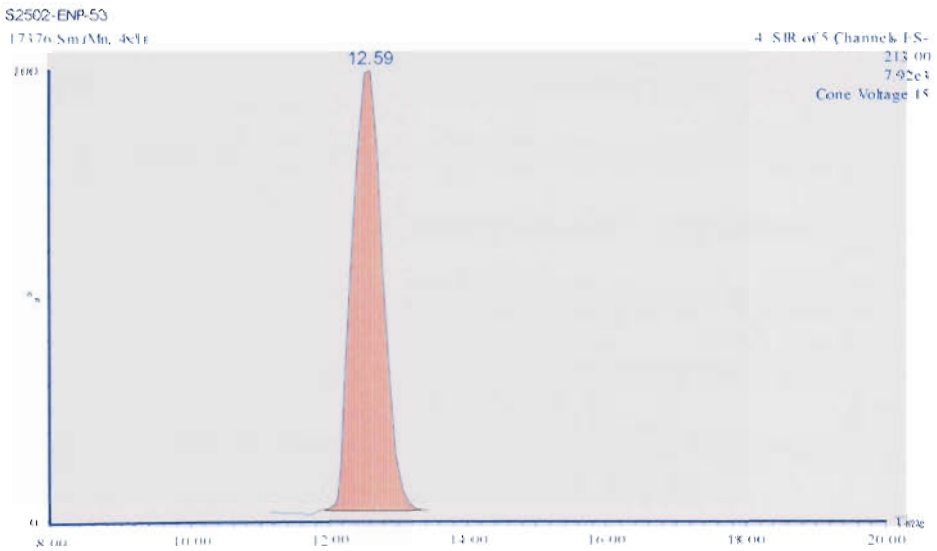


Figure 26:Extracted chromatogram for Mecoprop in sample ENP-53

Table 15 : Summary of samples analyzed for phenoxy acid herbicides within ENP and BBNP

ID	Picloram	Bentazon	Dicamba	MCPA	2,4-D	Mecoprop	Dichlorprop	2,4-DB	2,4,5-T	Silvex	Acifluorfen	Dinoseb
BBICS	*	*	*	*	*	*	*	*	*	*	*	*
BBMM	*	*	*	*	*	*	*	*	*	*	*	*
BBSK	*	*	*	*	*	*	*	*	*	*	*	*
BBP2	*	*	*	*	*	12.3	*	*	*	*	*	*
BBP1	*	*	*	*	*	*	*	*	*	*	*	*
BBCP	*	*	*	*	*	*	*	*	*	*	*	*
BBFP	*	*	*	*	*	*	*	*	*	*	*	*
BBEKH	*	*	*	*	*	*	*	*	*	*	*	*
BBTPC	*	*	*	*	*	*	*	*	*	*	*	*
332-BB	*	*	*	*	*	*	*	*	*	*	*	*
332-BAC	*	*	*	*	*	*	*	*	*	*	*	*
A-22	*	*	*	*	*	*	*	*	*	*	*	*
A-23	*	*	*	*	*	*	*	*	*	*	*	*
A-24	*	*	*	*	*	*	*	*	*	*	*	*
A-37	*	*	*	*	*	*	*	*	*	*	*	*
MDA	*	*	*	*	*	*	*	*	*	*	*	*
L-67-S	*	*	*	*	*	*	*	*	*	*	*	*
A-07	*	*	*	*	*	*	*	*	*	*	*	*
R-158	*	*	*	*	*	*	*	*	*	*	*	*
50-A	*	*	*	*	*	*	*	*	*	*	*	*
A-53	*	*	*	*	*	89.2	*	*	*	*	*	*
A-54	*	*	*	*	*	*	*	*	*	*	*	*
A-55	*	*	*	*	*	*	*	*	*	*	*	*
A-58	*	*	*	*	*	20.0	*	*	*	*	*	*
A-59	*	*	*	*	*	*	*	*	*	*	*	*
A-60	*	*	*	*	*	*	*	*	*	*	*	*
A-61	*	*	*	*	*	*	*	*	*	*	*	*
A-63	*	*	*	*	*	*	*	*	*	*	*	*
64-SD	*	*	*	*	*	*	*	*	*	*	*	*

* below MDL

CHAPTER IV. RESULTS AND DISCUSSION: TRIAZINE-BASED HERBICIDES

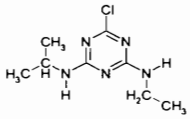
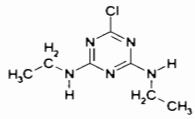
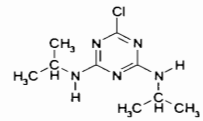
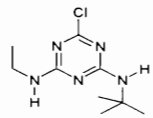
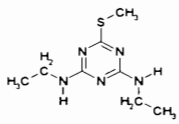
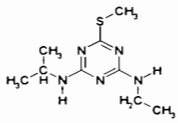
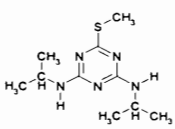
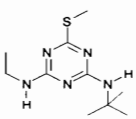
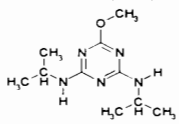
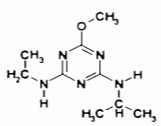
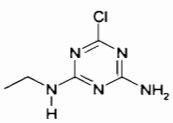
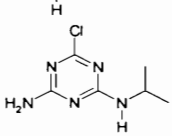
LC/MS Optimization and Chromatographic Separation

Triazine herbicides have relatively high proton affinities (750-950 kJ/mol) (Niessen 1999), and are readily amenable to solvent meditated chemical ionization. The volatility and ease of protonation of the triazine herbicides, makes them suitable for the use of atmospheric pressure chemical ionization (APCI) in the positive mode. The formation of pseudo-molecular ions like $[M+H]^+$ is possible under this conditions. The corona discharge needle present in the APCI interface, creates a stream of electrons that serve to ionize the solvent. In this study methanol was used and species like $CH_3OH_2^+$ and H_3O^+ likely to be responsible for the transfer protons to the weakly basic pesticides.

Initially a mixture of 10 triazine-based herbicides was injected directly on a C_{18} column (the same column used for the phenoxy acid herbicides), in order to investigate the ionization and fragmentation characteristics in the HPLC-APCI-MS system

The use of the APCI interface enabled direct coupling of the aQa mass spectrometer with the chromatographic column at a flow rate of 0.5 ml/min. The initial cone voltage was set at 15 volts, the corona pin at 3.5 kV, and the probe temperature at 350°C. The positive ions obtained are summarized in table 16. It is important to point out that one of the main characteristics of the APCI interface is the production of ions, where the most predominant specie is the $[M+H]^+$. Little fragmentation is normally observed under the conditions of ionization established.

Table 16: Primary ions for triazined herbicides in APCI positve

#	Compound	Structure	Exact Mass	Ion [M+H]
1	**Atrazine** $C_8H_{14}ClN_5$ CAS # 1912-24-9		215	216
2	**Simazine** $C_7H_{12}ClN_5$ CAS # 122-34-9		201	202
3	**Propazine** $C_9H_{16}ClN_5$ CASS # 139-40-2		229	230
4	**Terbutylazine** $C_{10}H_{18}ClN_5$ CAS # 5915-41-3		229	230
5	**Simetryn** $C_8H_{15}N_5S$ CAS #1014-70-6		213	214
6	**Ametrin** $C_9H_{17}N_5S$ CAS # 834-12-8		227	228
7	**Prometryn** $C_{10}H_{19}N_5S$ CAS # 7287-19-6		241	242
8	**Terbutryn** $C_{10}H_{19}N_5S$ CAS # 886-50-0		241	242
9	**Prometon** $C_{10}H_{19}N_5O$ CAS # 1610-18-0		225	226
10	**Atratone** $C_9H_{17}N_5O$ CAS # 1610-17-9		211	212
11	**Deisopropylatrazine (DIA)** $C_5H_8ClN_5$ CAS # 1007-28-9		174	175
12	**Desethylatrazine (DEA)** $C_3H_4ClN_5$ CAS # 3397-62-4		187	188

The total ion chromatogram (TIC) of the mixture is presented in figure 27. At this concentration level (150 µg/ml) all pesticides were easily detected. The initial elution program tested for this set of pesticides involved the use of methanol and acetic acid 1 % with the following linear programming: t=0, 60:40 up to 90:10 in 40 minutes and returning to original conditions at 45 minutes (see table 17).

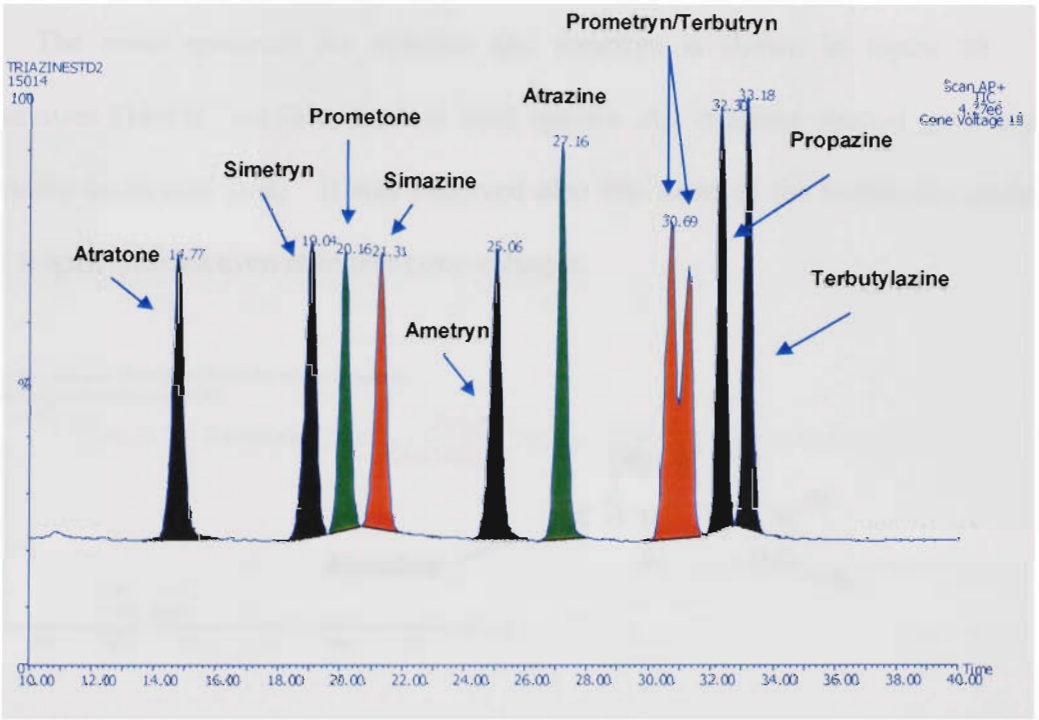


Figure 27: Full scan chromatogram for triazine herbicides mixture

Table 17: Initial gradient elution program for triazine herbicides separation

#	Time (minutes)	% A (Methanol)	%B (HOAC 1%) pH 2.76
1	0	60	40
2	40	90	10
3	45	60	40

The mass spectrum for atrazine and simetryn is shown in figure 28. The predominant $[M+H]^+$ ion is noticed in both spectra. All triazines studied gave mainly protonated molecular ions. It was observed also that none of the herbicides produced major fragmentations even at higher cone voltages.

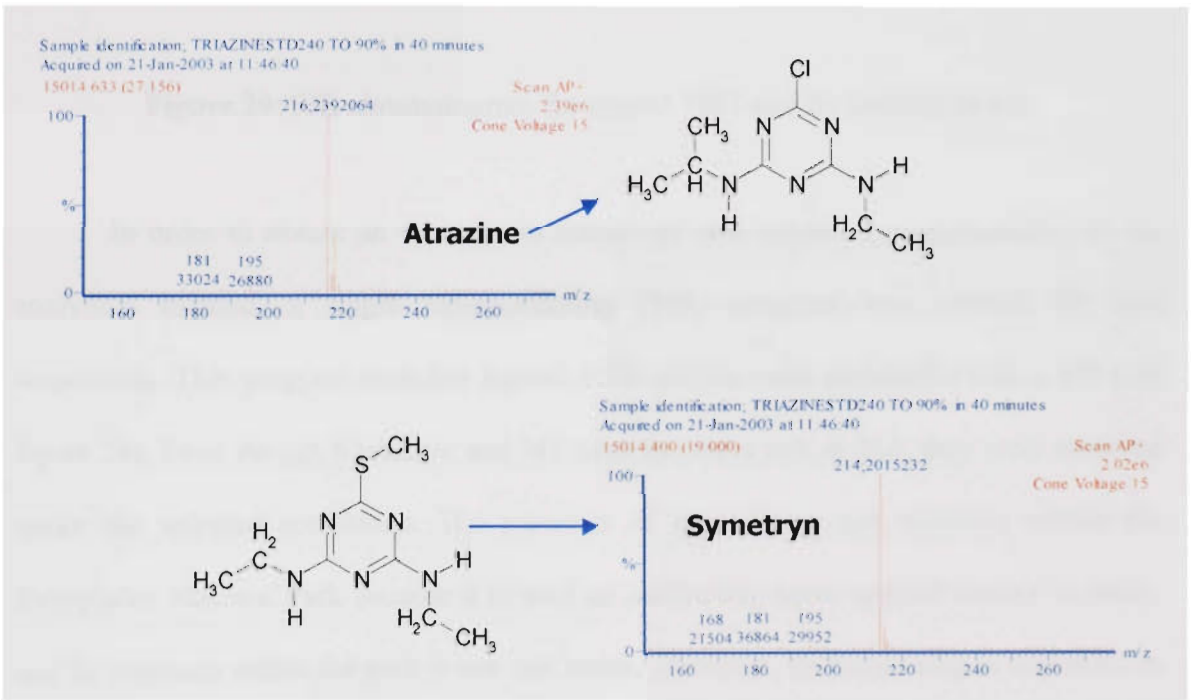


Figure 28: Mass spectrum of symetryn and atrazine herbicides

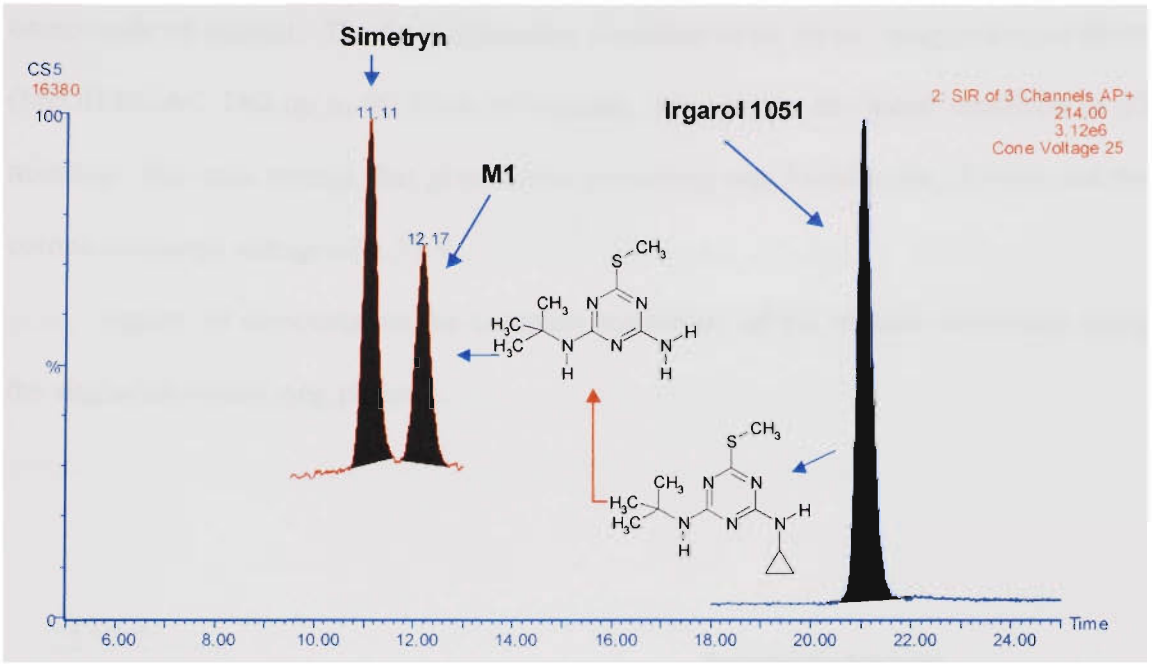


Figure 29: SIR chromatogram for irgarol 1051 and its metabolite M1

In order to obtain an increase in sensitivity and expand the applicability of the analytical method, a single ion-monitoring (SIR) program was created for data acquisition. This program included Irgarol 1051 and its main metabolite called M1 (see figure 29). Even though Symetryn and M1 have the same m/z at 214, they were resolved under the selected conditions. The presence of irgarol was not expected within the Everglades National Park because it is used an antifouling agent applied mainly in boats, and its presence within the park is not that usual. However, its monitoring is important in the Biscayne Bay National Park, because of the existence of Marinas and the frequent recreational boat activity within this area. At this point is important to mention that the chromatographic separation was optimized in terms of time of analysis. A more rapid

elution of the herbicides was achieved without altering the resolution of most of the compounds of interest. The final separation consisted of an initial composition of 60:40 (MeOH:HOAC 1%) up to 90:10 in 30 minutes, returning to the initial condition in 35 minutes. The cone voltage that gives better sensitivity was found to be 25 volts and the corona discharge voltage of 3.5 kV.

Figure 30 demonstrates the complete separation of the triazine herbicides using the single-ion monitoring program.

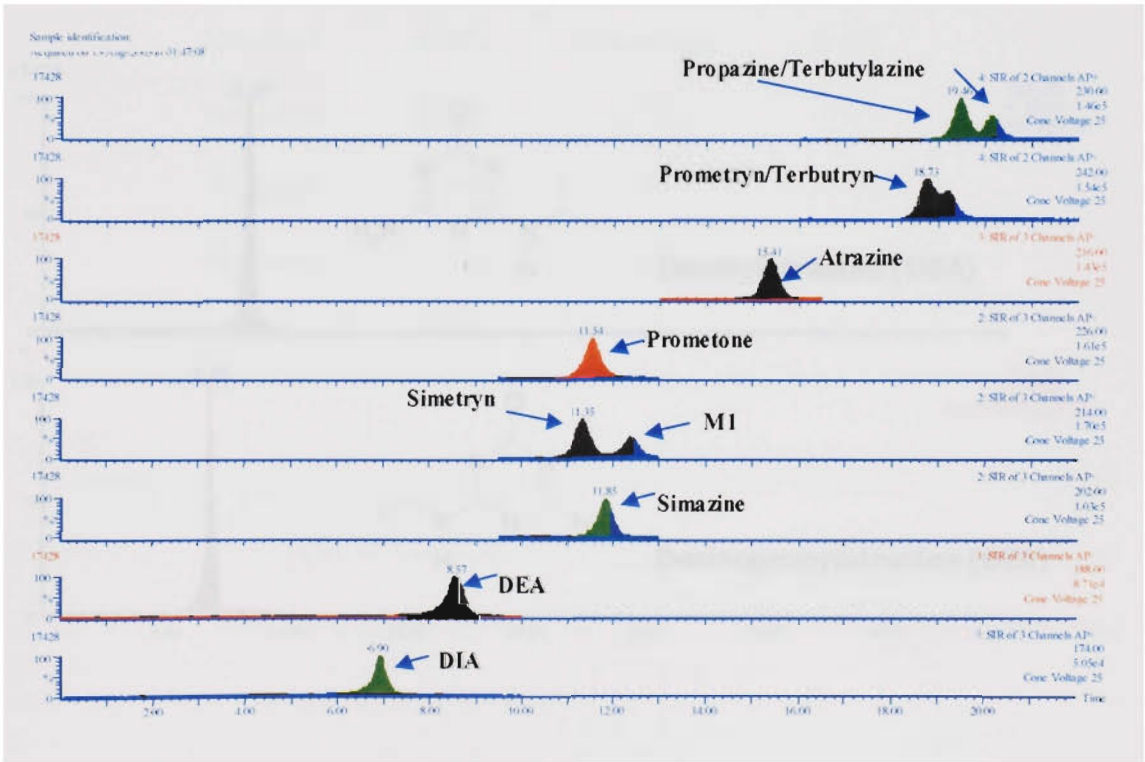


Figure 30:SIR chromatogram for most triazine and metabolites

The extracted ions shown in this figure demonstrates the capabilities of the mass spectrometer to resolve all the analytes of interest. Included in the SIR program, there are the two of the main metabolites of the atriazine: desethylatrazine (DEA) and desisopropylatrazine (DIA). A close view of these compounds is shown in figure 31. It is worth noting that there are significant differences in polarity of the metabolites compared to the rest of the triazines. This characteristic permitted the resolution of all the triazines compounds and a good chromatographic behavior during the HPLC separation since more polar compounds will have smaller retention times.

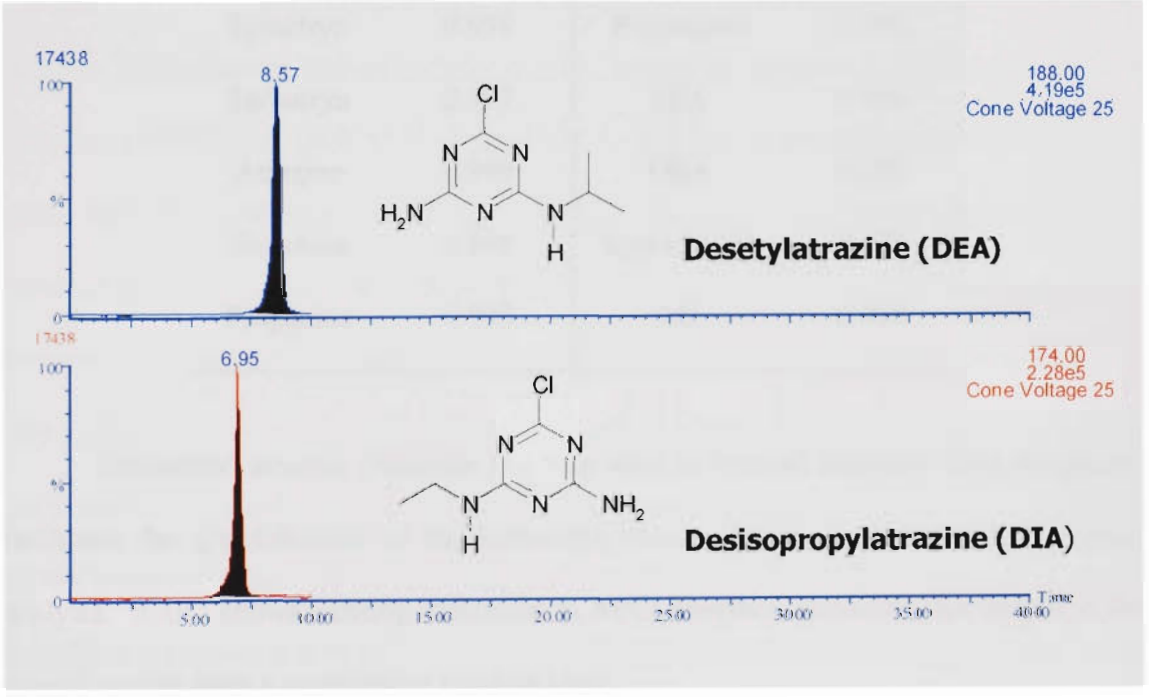


Figure 31:Extracted ion chromatogram for principal metabolites of atrazine

Analytical Performance for Triazine Herbicides

Calibration graphs were constructed with standard solutions with concentrations between 0.05-2.5 μg/ml. A linear response was observed in the range studied. The correlation coefficients are shown in table 18 and most of them are greater than 0.999 for the triazines including their metabolites

Table 18: Correlation coefficients for triazine herbicides

Analyte	r^2	Analyte	r^2
Ametryn	0.999	Terbutylazine	0.999
Prometryn	0.997	Atraton	0.999
Symetryn	0.998	Prometone	0.998
Terbutryn	0.997	DIA	0.999
Atrazine	0.999	DEA	0.999
Simazine	0.999	Irgarol 1051	0.999
Propazine	0.999	M1	0.998

Deuterated atrazine (Atrazine D_5) was used as internal standard. This compound facilitates the quantification of the herbicides because of its similarity with the target analytes. It also shows a strong ionization in APCI interface, condition that helped in the overall results from a quantitative point of view.

Method Development for Extraction and Clean up

As was previously done for the phenoxy acids herbicides, the analysis of triazines in sediment samples needs an optimized pre-concentration, clean up and separation

techniques. The successful results obtained with the ASE-SPE combination for the analysis of phenoxy acid herbicides, motivated the testing of a similar arrangement for the triazines. Because triazines are less polar in nature (except the metabolites), their extraction in the ASE system using organic solvents is more favorable than the phenoxy acid herbicides.

Because of the water solubilities of triazine herbicides and trying to avoid the use of toxic organic solvents, the ASE extraction was based on a combination of methanol:water (90:10) as extracting solvents. This mixture of solvents not only helped to increase the solubility of the herbicides but also reduced the viscosity of the solvent, situation that improved the diffusion and penetration of the solvents into the complex matrix. The exposure time of sediments with the solvent, at the selected temperature and pressure (100°C and 1500 psi), affected the wettability in the sediment. An optimized static time of 10 minutes was found to be adequate for the extraction. Since water was used as a solvent there was not need for sample drying eliminating one step in the process. The use of cellulose filters and diatomaceous earth during the packing of the ASE cells, helped to reduce the void volume and also the accessibility of the solvent into the matrix. The residual water presented at the end of the extraction was also diminished by the presence of the diatomaceous earth. The flushing of the extract by nitrogen purge, generated a volume approximate of 45 ml, and the subsequent elimination of most of the methanol from the collection vial via rotary evaporation, yielded an aqueous sample compatible with SPE. This represents a major development in the traditional analysis of triazines.

Pre-concentration of triazines

The polymeric sorbent Oasis HLB was selected as a candidate to perform the pre-concentration of the triazines because of its properties and capabilities shown in the phenoxy acid method. The easy conditioning of these cartridges with methanol and water, and its proven versatility with complex matrices, were determining factors that helped in the development of a procedure that generates an enrichment of the analytes ,as intermediate step for later analysis via mass spectrometry.

The possibility to obtain a multi-residue methodology that involves both families of compounds, phenoxy acid and triazines in a single extraction with ASE, and clean up using the SPE cartridges from Oasis, was explored in this study. Twelve independent samples from the same source (ENP-A59) were spiked and extracted using two different procedures, called method A and Method B. In method A combination of methanol and water was used as extraction solvents. while in Method B was identical to the one used for the phenoxy acids. These two methods are described in table 19.

Table 19: Triazine experimental design for method validation

	Method A	**Method B**
ASE	MeOH:H_2O (90:10)	Acetone: 5% HOAc (80:20)
SPE	Oasis HLB Elution: 100 % CH_2Cl_2	Oasis HLB Elution: MTBE:MeOH (90:10) with 0.01% Formic Acid

Both methods were compared in terms of sensitivity, precision and recovery. Sensitivity was evaluated by comparing the method detection limits (MDL). A F-test was conducted to evaluate the precision of the methodologies based on the standard deviation of the recovered analytes. In order to determine if there is a significant difference between percentages of recovery a t-test was performed (for n=6).

In order to test whether the differences between two sample variances are significant the statistical F was calculated using the following equation:

$$F = s^2_1 / s^2_2$$

The number of degrees of freedom in the numerator and denominator are n_1-1 and n_2-1, respectively. This test assumes that the population from which the samples are taken is normal (Miller, 2000). For this experiment 6 replicates were measured for each method and therefore the degrees of freedom for s_1 and s_2 was 5.

The F test allowed the comparison of the precision of both methodologies. If the null hypothesis is true, then the F value is close to 1. If the calculated value of F exceeds a certain critical value then a rejection of null hypothesis is performed. The purpose of the comparison of both standard deviations was to determine if Method A is more precise than Method B or viceversa. Thus a "one-tail" F-test was applied.

The recovery values of the spiked sediment samples (33 ng/g), as well as the precision and figures of merit are presented in table 20.

Table 20: Recoveries values for Method A and Method B for statistical evaluation

	Method A			Method B		
name	**% recovery**	**SD**	**%RSD**	**% Recovery**	**SD**	**%RSD**
Ametryn	94	2	2	153	10	6
Prometryn	96	2	2	132	8	6
Symetryn	95	3	3	131	7	6
Terbutryn	86	4	5	120	11	10
Irgarol	86	4	5	153	8	5
M1	62	12	19	75	5	6
Atrazine	88	2	2	87	2	3
Simazine	89	3	3	80	3	4
Propazine	88	1	1	90	3	3
Terbutylazine	91	2	2	100	20	20
Atratone	95	3	3	98	9	9
Prometone	95	2	2	172	14	8

The output obtained for the F-test evaluation of atraton, is presented in Table 21. Variable 1 represents the Method B and Variable 2 Method A. The value of the variance in both methods gave a calculated F value of 7.28, which is higher than the critical F value of 5.05 considering one tail. Therefore, Method A is more precise than Method B at a 95 % confidence limit for atraton. The same table reports a lower calculated F value for Irgarol 1051 of 3.56 compared with the critical value. Therefore, for this compound the precision of both methods does not differ significantly. In general terms, Method A provided best precision except for Irgarol, simazine and atrazine, where the precision did not differ significantly between both methods.

Table 21: Atratone and Irgarol 1051 F test output from Microsoft Excel

Atratone			Irgarol 1051		
	Variable 1	*Variable 2*		*Variable 1*	*Variable 2*
Mean	98.182648	94.649725	Mean	153.26266	85.993897
Variance	77.22051	10.595194	Variance	60.248634	16.909112
Observations	6	6	Observations	6	6
df	5	5	df	5	5
F	7.2882583		F	3.5630868	
P(F<=f) one-tail	0.0240027		P(F<=f) one-tail	0.0947212	
F Critical one-tail	5.0503388		F Critical one-tail	5.0503388	

A t-test is a statistical tool that is used to compare two experimental means in order to determine if there is a significant different between them. A t-test can be calculated assuming different variances, and is calculated from the means (x_1 and x_2), standard deviations (s_1 and s_2), and sample sizes (n_1 and n_2) of the two set of samples, with the formula (Miller 2000):

$$t = \frac{(x_1 - x_2)}{\sqrt{\left[\left(\frac{s_1^{\,2}}{n_1}\right) + \left(\frac{s_2^{\,2}}{n_2}\right)\right]}}$$

The t statistic is then compared to a t critical value obtained from a table, with the corresponding number of degrees of freedom (Miller 2000):

$$\text{degrees of freedom} = \frac{\left(\frac{s_1^{\,2}}{n_1} + \frac{s_2^{\,2}}{n_2}\right)^2}{\left[\frac{s_1^{\,4}}{n_1^{\,2}(n_1 - 1)} + \frac{s_2^{\,4}}{n_2(n_2 - 1)}\right]}$$

On the other hand, if the variances did not differ significantly (which can be determined by a two-tail t-test), the formula use is:

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where the variance s^2 is calculated using the formula:

$$s^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 + n_2 - 2)}$$

In both cases, if the t statistic is less than the critical value, then the sample means are not significantly different. If the t statistic is greater than the critical value then there is a significant difference between the means. For the purpose of this study, the t statistic and t critical were calculated using Microsoft Excel 2000.

A graphical comparison of the two means in the case of atriazine is observed in figure 32. The graph was obtained running a statistical program SYSTAT.

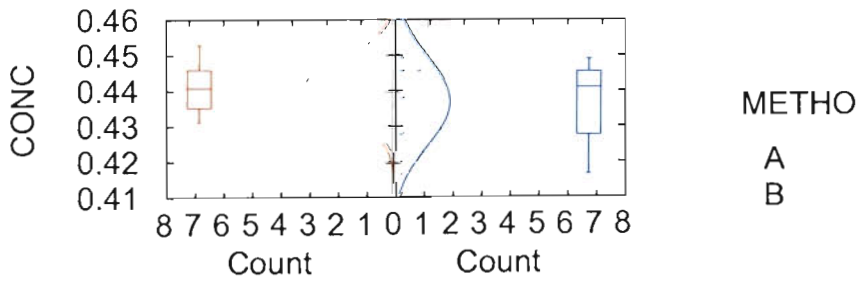


Figure 32: Distribution of concentration values for atriazine using Method A and Method B of analysis.

This figure includes the standard deviation of each set of samples, and it could be observed that the distribution of values overlap indicating that there is no significant difference between the two means for atrazine.

In the case of simazine, the situation was different. Figure 33 shows the t-test analysis graph and it shows a significant difference between the mean values obtained in the two methods A and B. Therefore there is a significant difference between the two methods of analysis.

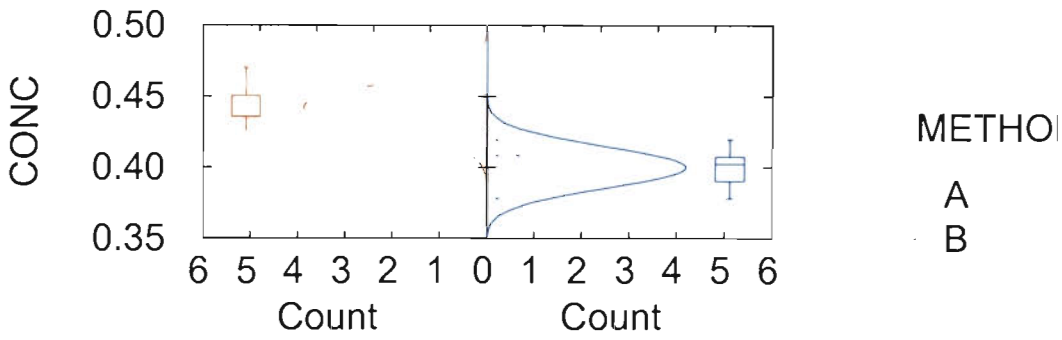


Figure 33: Distribution of concentration values for simazine using Method A and Method B of analysis

Another way to interpret the output information from Microsoft Excel is based in the p-value. At 95% confidence limit, a p-value less than 0.05 indicates that the means are significantly different, and a value greater than 0.05 specifies that the means are not significantly different (Miller 2000).

Table 22: Microsoft Excel output for t-test analysis of terbutryn

	Variable 1	*Variable 2*
Mean	0.430105	0.601065
Variance	0.000402	0.003264
Observations	6.000000	6.000000
Hypothesized Mean Difference	0.000000	
df	6.000000	
t Stat	-6.916999	
P(T<=t) one-tail	0.000226	
t Critical one-tail	1.943181	
P(T<=t) two-tail	0.000452	
t Critical two-tail	2.446914	

This table reports the value of p below 0.05, which means that there is a significant difference between the mean values and therefore the methods differs significantly.

The complete evaluation of both statistical tests is summarized in table 23. From this table could be concluded that the triazines are more efficiently extracted using Method A than Method B. In addition, Method A also gave the best precision and for this reason, it was selected as the method for the fractionation and clean up for triazine herbicides.

The method limit of detection values are shown in table 24. As could be seen the values are around 4 ng/g overall, demonstrating the high sensitivity of the proposed method for the analysis of triazine herbicides in sediment samples.

Table 23: Summary of statistical analysis for comparison of extraction and clean-up methods for triazines (n=6)

Compound name	F test Results comparison of precision (p =0.05) [2]	T-test comparison of Methods p = 0.05 [3]
A tratone	method A more precise than B	Methods A and B does not differ significantly
Prometone	method A more precise than B	Methods A and B differ significantly
Simetryn	method A more precise than B	Methods A and B differ significantly
Simazyne	does not differ significantly	Methods A and B differ significantly
M1	method A more precise than B	Methods A and B differ significantly
Atrazine	does not differ significantly	Methods A and B does not differ significantly
Ametryn	method A more precise than B	Methods A and B differ significantly
Prometryn	method A more precise than B	Methods A and B differ significantly
Tertbutryn	method A more precise than B	Methods A and B differ significantly
Propazine	method A more precise than B	Methods A and B does not differ significantly
Terbutilazine	method A more precise than B	Methods A and B does not differ significantly
Irgarol 1051	does not differ significantly	Methods A and B differ significantly

Table 24: Method Detection Limit for triazine herbicides

Herbicide	MDL (ng/g)
Ametryn	4
Prometryn	4
Simetryn	4
Terbutryn	4
Atrazine	4
Simazine	4
Propszine	4
Terbutilazine	4
Atraton	4
Prometone	4
DIA	5
DEA	5

Application to real samples

The possibility to analyze both, triazine parent compounds and metabolites in a single run at very low concentration was explored by analyzing the same sampling sites surveyed for the occurrence of phenoxy acid herbicides. Nevertheless, none of the analytes of interest or the metabolites were found at concentrations above the method detection limit for some sampling sites as shown in table 25. These results were corroborated quantifying the same samples using a GC/MS instrument. The comparison was performed using a previously optimized with GC/MS method, which detection limits were similar to the obtained by LC/MS.

Both chromatographic methods, GC/MS and LC/MS, offered good analytical performance for the analysis of complex matrices, providing good precision, good accuracy and good detection limits. However, APCI LC-MS provides the added advantage of detecting also important metabolites like DIA and DEA, offering a good alternative for the screening and quantification of these analytes at very low

concentrations in sediments and can also be applied to other environmental matrices such as water.

Table 25: Summary of results for triazines some sampling sites at ENP

ID	DIA	DEA	Atratone	Prometon	Simetryn	Simazine	Atrazine	Ametryn	Prometryn	Terbutryn	Propazine	Terbutylazine
A-21	*	*	*	*	*	*	*	*	*	*	*	*
A-22	*	*	*	*	*	*	*	*	*	*	*	*
A-23	*	*	*	*	*	*	*	*	*	*	*	*
A-24	*	*	*	*	*	*	*	*	*	*	*	*
L-67-S	*	*	*	*	*	*	*	*	*	*	*	*
A-07	*	*	*	*	*	*	*	*	*	*	*	*
A-53	*	*	*	*	*	*	*	*	*	*	*	*
A-54	*	*	*	*	*	*	*	*	*	*	*	*
A-57	*	*	*	*	*	*	*	*	*	*	*	*
A-58	*	*	*	*	*	*	*	*	*	*	*	*
A-59	*	*	*	*	*	*	*	*	*	*	*	*
A-63	*	*	*	*	*	*	*	*	*	*	*	*

* below MDL

CHAPTER V. CONCLUSIONS

PHENOXY ACID HERBICIDES

The complex nature and diverse physical-chemical characteristics, together with the high content of organic matter of the sediments studied, made the isolation, concentration and fractionation of pollutants by traditional methods a difficult task and required the development of specific analytical protocols.

Accelerated solvent extraction (ASE) proved to be a powerful and versatile extraction technique, which allowed the removal of the analytes from the sediment matrix, producing an extract that was compatible with rugged SPE clean up procedures for the final purification of the compounds of interest before measurement.

The polymeric cartridges Oasis HLB exhibited great performance for elimination of the background interferences presented in the sediments. The easy conditioning steps and the rapid setup, permitted a straightforward pre-treatment of the sediment samples once they were extracted by the ASE system. Filtering the fulvic materials did not affect the recoveries.

The breakthrough volumes estimated for the phenoxy acid herbicides were very useful to determine the limitation of the sorbent capacity of the polymeric SPE cartridges and explain the differences in recoveries of these polar herbicides.

A principal component analysis (PCA) of fundamental physical-chemical properties of the herbicides like pKa, Koc and Kow, revealed a strong influence of these properties in the partition tendency in the sediment samples and therefore in the extraction recovery.

The chromatographic separation of the phenoxy acid herbicides was successfully accomplished with gradient elution program using methanol and acetic acid 1% in a C_{18} column and by electrospray ionization in the negative mode (ESI^-). This ionization technique with selected ion monitoring, proved to be effective for the detection and quantification of phenoxy herbicides by LC/MS.

Different parameters showed a great influence in the ionization process of the mass spectrometer. As a result, the optimization of cone voltage, probe temperature and pH, as variables that control the detection, sensitivity and selectivity, were of primary importance during the method development process.

The analytical performance of the phenoxy acid quantification was evaluated through the use of internal calibration curves. The accuracy of the method was measured by running spiked samples and also the interday and intraday variation was tested for precision showing values below 15 % RSD. The MDL were as low as 0.5 ng/g for this family of compounds.

The novelty of the proposed method for the analysis of phenoxy acid herbicides was to combine ASE-SPE with LC/MS for the simultaneous analysis of 12 acidic herbicides providing several advantages over conventional extraction and detection methodologies, such as compatibility with aqueous matrixes, minimization of solvents and simplification of the complexity of the analysis in sediments samples with a high content of organic matter

Hyphenated chromatographic techniques, such as HPLC/ESI/MS offer a good alternative for the screening and quantification of the analytes at very low concentrations in sediments, and can also be applied to other environmental matrices such as water. This

method required only 15 g of wet sediment to achieve limits of detection as low as 0.5 – 23 ng/g for the suite of herbicides studied.

TRIAZINES HERBICIDES

The chromatographic separation of triazines was done using the same reverse phase column used for the phenoxy acid herbicides and practically the same solvent composition (MeOH and HOAc1 %). This set up, permitted a smooth transition to characterize the triazines in the sediment samples by LC/MS, even tough the use of a different ionization technique.

Atmospheric pressure chemical ionization (APCI) in the positive selected ion-monitoring mode, demonstrates the viability of use of a sensitive and selective ionization technique that accounts for the adequate recognition of target analytes in complex matrices.

The possibility of analyzing metabolites within the same group of compounds, and in a relative short analysis time, represent a great advantage over conventional GC/MS methodologies, which requires a previous derivatization of the polar compounds.

The combination of three analytical techniques (ASE-SPE-LC/MS) generated a rugged and adaptable strategy for the analysis of a selected suite of herbicides. The application of this approach allowed the assessment of triazines in sediment samples at concentration in the ppb ranges, and none of these herbicides were found in the agricultural areas studied.

ENVIRONMENTAL FORENSICS

The application of the analytical protocol to the samples collected within ENP and BNP provided environmental forensic evidence to be used for source mitigation, regulation, and future management of resources. Based on the chemical analysis of the sediment samples monitored, it was shown that at the moment both families of compounds do not represent an ecological risk to these highly protected ecosystems. Also the availability of these "baseline" profiles will help in the prevention and enforcement of environmental crimes.

From an environmental forensic point of view, the applications of these two analytical methodologies for the chemical characterization of herbicides in sediment samples show great advantages. Once additional biological and geochemical studies are completed, forensic scientists will be capable of perform and integral assessment of the sites at risk, and use the acquired knowledge for use in future real casework.

FUTURE WORK

During the fractionation of the phenoxy acid herbicides, the possibility of performing a two-step elution in SPE by using an extra non-polar solvent should be considered in order to improve the recoveries of the analytes.

The presence of phenoxy acid metabolites like 2,4-dichlorophenol and 2,4,5-trichlorophenol, are considered a priority for the further evaluation of the sediment samples within the parks. Their recovery study in the sediment samples, its enrichment, and clean up should be validated.

An overall assessment that comprises information from other scientific disciplines should be integrated in order to obtain a global perspective that gives information of the occurrence, spatial distribution and fate of pesticides affecting the different biological compartments in the ecosystems at the Everglades area. The data presented in this investigation represents an effort to pursuit this objective but it just embody a small portion of all the information required to achieve it. A greatest effort is needed to acquire more scientific records that strength this gap of information. Once this is accomplished, the forensic community will benefit with this portfolio but local authorities are responsible to address and support all gathered data and used it to confront different litigation issues and future management plans.

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