BRIAN A. SHIRA
Improved Monitoring of Halogenated Organic Pollutants in Natural, Iron Containing, and Bimetallic Systems
(Under the Direction of DR. JAMES L. ANDERSON)

The now notorious pesticide, 2,2-bis (4-chlorophenyl)-1,1,1-trichloroethane (DDT), was once used widely in the US. While many sites show a marked reduction in the levels of DDT, sites associated with the manufacture of the DDT still demonstrate abnormally high levels of the compound and its metabolites. Until recently, the residues DDD and DDE were thought to be the terminal residues although other metabolites have recently been identified, namely 2,2-bis (4-chlorophenyl)-1-chloroethylene (DDMU) from DDE and dichlorobenzophenone (DBP) from DDT. Little work has been done on the identification of polar degradation products of DDT.

DDA was one of the first metabolites of DDT that was isolated and it has been called "perhaps the universal DDT metabolite in microorganisms, plants, animals, and higher animals". The goals of this study were to determine if *o,p'*-DDA was present in river water samples, and to develop a method for the determination of the enantiomeric ratio of *o,p'*-DDA utilizing typical environmental laboratory equipment such as a benchtop GC/MS. The limitations of other methods as well as the success of selected ion monitoring (SIM) mode detection will be discussed.

Toxaphene is the name given to the complex mixture of polychlorinated bornanes of the average elemental composition $C_{10}H_{10}Cl_8$ that was applied as a pesticide. Toxaphene residues have not received more attention in the environmental arena due to the complexity of the mixture present in technical toxaphene, a lack of suitable standards, and the slow development of analytical methods for detection and quantitation of toxaphene.

This work reports the development of a new analytical methodology based on ESI-TOF-MS without the use of a prior chromatographic separation. Toxaphene was

incubated with zero valent iron and a bimetallic system containing magnesium and palladium to reductively dechlorinate toxaphene. Results from the ESI-TOF-MS analysis are compared to GC/ECD results for homologue distribution data and to evaluate the effectiveness of the remediative schemes.

The determination of the oxidation state of both oxidized and reduced cytochrome *c* with ESI-FTICR-MS was investigated. Through this work, it has been demonstrated that although electrochemical processes are occurring during the electrospray process, ESI-MS can preserve the oxidation state of the introduced sample.

INDEX WORDS: Zero-Valent Iron, Toxaphene, DDT, Mass Spectrometry, Electrospray Ionization, Homologue, Reductive Dehalogenation, Dechlorination, Cytochrome *c*.

IMPROVED MONITORING OF HALOGENATED ORGANIC POLLUTANTS IN NATURAL, IRON CONTAINING, AND BIMETALLIC SYSTEMS

by

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DEDICATION

To Allison, my wife, and to our son Brison.

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CHAPTER 1

INTRODUCTION

PURPOSE OF THE STUDY

This investigation originated to examine the reductive dehalogenation of pollutants to aid in the understanding of the remediation of groundwater contaminants as well as to assess the abiotic species that contribute to the natural attenuation of subsurface pollutants. It is widely known that throughout the chemical age, vast amounts of chemicals have been discharged directly into the environment, while large amounts have leaked as a result of failures of containment facilities. In an effort to protect human health as well as environmental health, remediation schemes have been developed. The effectiveness of a remediation scheme can be assessed by a multitude of parameters. Convenience generally dictates that the monitored parameter is the concentration of the pollutant. While this works quite often, there can be reactions that generate unexpected products that may or may not be of great concern. This study examines the use of iron, iron containing systems, and reactive bi-metallic systems to remediate priority pollutants. It is hoped that the outcome will be the identification of products to look for upon the cleanup of polluted sites as well as an understanding of the processes that create such products.

ORIGINALITY OF THIS STUDY

Numerous research groups have focussed on the reduction of halogenated compounds with metal system. This work focuses on the formation of products that may have been overlooked by other researchers, but are still of importance. Experiments were conducted with synthetic systems that approximated subsurface conditions, both for iron walls and natural systems. Possible remediative schemes can

vary from the complete excavation of a given site on the most aggressive extreme, to allowing natural processes to remediate the site on the most passive extreme. It is not always feasible to implement one of the above strategies. Either the remediation may be too invasive or the time required for natural attenuation to clean up the site may be too long. Due to the above limitations, remediative strategies have been developed where the contaminated groundwater plume flows through a wall of iron particles; this permeable reactive barrier serves to transform the pollutant, thus remediating the site.

SCOPE OF THE PROBLEM

Anthropogenic chemicals have become a major threat to human health as well as to ecosystems. The manufacture, distribution, disposal, and associated use and misuse of chemicals have created many noteworthy examples of acute environmental and health effects. Perhaps the most famous of these sites is the now infamous Love Canal, where an unused canal was filled with drums of toxic waste. The site was covered with earth and then housing was built on the site. Eventually families became ill from the leaking chemical drums and fumes from below their houses. Although Love Canal is an extreme example of recklessness and carelessness, it does serve as a reminder that improper disposal of chemicals not only endangers health, but also impacts business due to the expense of clean up.

For decades, large waste generators have disposed of their wastewater and waste chemicals in the least economically costly manner, regardless of the environmental impact. This was largely the case until the government began enforcement of minimum water quality standards. As the public became more aware of the environmental impact of such careless behavior, they became outraged and demanded protection. The founding of the EPA and subsequent legislation was a major step in the protection of the environment. Now some 30 years later, the "Environmental Pendulum" is beginning to

find a middle ground. The days of free dumping for businesses are over, and the punitive legislative restraints are relaxing. Under current regulatory codes companies can produce products and still make sufficient profits, while being environmentally responsible. Since more stringent environmental legislation costs businesses money, there is an inherent resistance to new legislation. Due to the huge impact of new legislation, businesses and governments alike are calling for legislation based on science. It is hoped that by basing the goals of regulation on the results of scientific studies that the outcome will be fairer to both businesses and the environment.

Groundwater is obviously susceptible to contamination as demonstrated above. In addition to groundwater, surface waters can be polluted. There are many sources for the contamination of surface water. They can be an easily identifiable source such as a wastewater discharge pipe from a factory, a point source; or they may be a less traceable source, such as runoff from agricultural land, a non-point source. Another prominent non-point source is atmospheric deposition; this has been demonstrated with acid rain as well as transport of semi-volatile pesticides. A complicating factor with atmospheric transport is the potentially vast distance between the source and the receptor. These great distances cut across international boundaries and are immune to national policies.

For a pollutant to cause an environmental or health impact, it must have both substantial toxicity at the concentration at which it is discharged and it must have some movement. The toxicity issue is quite clear; however, the flux of the pollutant is less obvious. If the pollutant is not moving then it poses little health or environmental risk as it can easily be sequestered or avoided. The transportation of pollutants in surface water and groundwater is the principal method of dispersal. It is not a pre-requisite for a chemical to be water soluble to cause serious environmental effects. In fact numerous examples exist of soil or sediment bound species accumulating in aquatic plant and animal species. A factor that can increase the impact of the pollutant is its persistence.

A compound that degrades rapidly has less time to impact the environment before its removal is complete.

A general class of chemicals responsible for a disproportionate number of pollution sites is the halogenated solvents, namely, perchloroethylene, trichloroethylene, and tetrachloroethanes. This is due in part to their widespread use and partly due to their recalcitrance. Although the use of the halogenated solvents and their subsequent disposal has been greatly reduced, there are a vast number of sites to remediate.

REMEDIATION STRATEGIES

Traditional pump and treat technology is a useful technology to pump up groundwater contaminated with halogenated solvents and strip the volatile compound out by sparging with air and trapping the vapor on a sorbent such as carbon. The cleaned ground water can then be discarded inexpensively or returned to its source. It should be noted that this technology is more useful to pump and treat the water, but less so to cleanup the site. The current feeling about pump and treat is that the technology is vital to controlling the contamination plume shape and movement, not for the removal of the pollutant. The inherent lack of water solubility of the halogenated solvents makes pump and treat inefficient for clean up. Halogenated solvents are denser than water and when they are introduced into the ground water, they form a dense non-aqueous phase lens (DNAPL) which only very slowly dissolves into the water. Thus, pump and treat does virtually nothing to remove the source contaminant. Furthermore, pump and treat technology requires constant work to maintain the pumping and treatment systems. It is not unusual for the project to run for decades, even with source removal.

Due to the cost associated with maintaining such remediative strategies, research was begun into the use of zero-valent iron walls to intercept and treat groundwater contaminated with halogenated compounds. Zero-valent iron is readily oxidized by

halogenated compounds to Fe^{2+} with the halogenated compound undergoing a reduction and subsequent elimination of a halogen atom as the corresponding anion. This reductive dehalogenation results in lower chlorinated species, which with a few notable exceptions are generally of less concern than the parent compounds.

The reaction of iron with halogenated solvents is rapid compared to the natural attenuation process. In an effort to develop better remediation strategies, further research has been in the area of bimetallic reductive/catalytic systems such as palladium/magnesium. The premise is that the magnesium acts as a reductant and the palladium salt is reduced to finely divided metallic palladium, which serves as a very efficient hydrogenation catalyst. In addition to direct electron transfer as the magnesium is oxidized, the reaction also utilizes the hydrogen gas produced to drive the hydrogenation of the pollutant.

Under natural conditions the subsurface reductants are generally Fe^{2+} sorbed onto the surface of naturally occurring iron oxides or iron oxyhydroxides. While this system has shown reductive capability, it is generally not as fast as either the iron or the Pd/Mg system. It is still quite relevant however because it most closely approximates the natural subsurface environment. Furthermore as reactive iron walls age, an oxide coating rapidly forms on the surface of the iron, generally passivating the reactive iron to some degree.

In addition to the halogenated solvents, there are other halogenated species of environmental interest. The notorious pesticide DDT; the carcinogenic PCB's; and the less well known toxaphene, chlordane, and aldrin are all polychlorinated chemicals that are recalcitrant and are being focused on for investigation as persistent organic pollutants. Preliminary work has begun on the use of iron and Pd/Mg systems for the reduction of these systems. A subset of the aforementioned chemicals also presents

some unique analytical challenges due to their complexity and subtle differences in congeners.

Evaluation of the products and pathways of these remediative strategies is the objective of this study with the intent of understanding the potential pitfalls. For the persistent polyhalogenated pesticide, toxaphene, a new analytical method based on electrospray ionization time of flight mass spectrometry has been developed to aid in monitoring of reaction progress.

CHAPTER 2

BACKGROUND

INTRODUCTION

A specific class of environmental pollutant that is responsible for a disproportionate amount of concern is the halogenated organic compounds. Halogenated aliphatic solvents have been used for decades as industrial solvents, dry cleaning fluid, and anti-knock additives in gasoline. Additionally, halogenated compounds such as DDT, chlordane, toxaphene, and mirex were found to be effective and inexpensive pesticides. The inherent persistence of these pesticides, as well as their widespread use coupled with the improper disposal of halogenated solvents, have catapulted the halogenated aliphatic compounds onto the EPA's priority pollutants list.

While several halogenated solvents are on the priority pollutant list, the halogenated aliphatic pesticides are singled out as "persistent organic pollutants", (POPs) and are the subject of current treaty talks[1]. Additionally, the U.S. EPA's Scientific Advisory Board recently suggested[2] that more information about the processes that contribute to the natural attenuation of these compounds be acquired. The physical and chemical processes that affect the fate of halogenated compounds in the environment can be grouped into two major categories, transportation or transformation.

The transportation of organic molecules in the environment is generally through phenomena such as volatilization and subsequent atmospheric transport by wind or movement in surface or ground water. While these phenomena are important to the overall impact of a pollutant, they tend to do little to mitigate the toxic effects of the pollution. As the pollution is transported, it typically is diluted and is generally

considered less toxic; however, this decrease in toxicity is coupled to a correspondingincrease in exposure. More ecosystems and humans are impacted as the pollutant is spread through the environment, albeit at a reduced concentration. The control of these macroscopic processes is generally reserved for engineers and is beyond the scope of this work.

The transformation of organic pollutants in the environment can occur through many different routes and has a tremendous effect on the impact of the organic pollutant. The transformation reactions can create compounds with different physical properties, which can affect their transport and partitioning in the environment, ultimately influencing exposure. Additionally, the compound may be advantageously transformed into a less toxic species or disadvantageously transformed into a more toxic species[3]. Common abiotic transformation processes that are observed in the environment are hydrolysis, photochemical reactions, oxidations, and reductions[4]. Chlorinated aliphatic compounds are fairly immune to hydrolysis. Although these compounds are susceptible to photochemical reactions, such reactions are of limited significance except in the upper atmosphere. These compounds are however susceptible to reduction, which generally occurs through a process termed reductive dehalogenation.

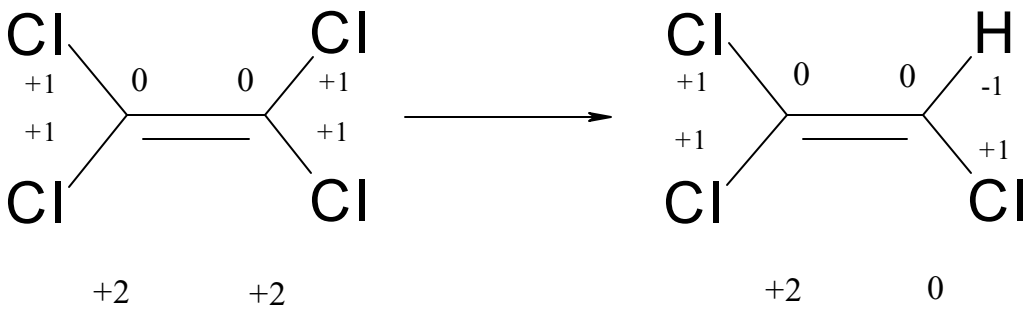
Reductive dehalogenation is the addition of electrons which results in a change of the oxidation state of the atoms involved in the process along with a release of a halogen from the molecule, generally in the form of a halide anion. Examination of the oxidation state of the carbon atoms involved in the reaction determines whether a reduction has occurred. By assigning oxidation states to the carbon atoms of an organic molecule the oxidation state of the reactants and products can be derived and compared. The general procedure for assigning the oxidation state of a carbon atom is as follows: (1) a value of +1 is assigned for each bond with a more electronegative substituent such as Cl, (2) a value of 0 is assigned for each bond to another carbon atom because their

electronegativity is identical, and (3) a value of –1 is assigned for each bond to a less electronegative substituent such as H. Once these values have been properly assigned, they are summed and yield the oxidation state of the carbon atoms. This process must be completed for both the reactant and the product so that the oxidation states may be compared. An example of the transformation of tetrachloroethylene to trichloroethylene is shown in Figure 2-1. The sum of the oxidation state of the carbons in the molecule is changed from +4 to +2 indicating that a reduction has occurred. While this example proves to be obvious, it can be difficult at first glance to differentiate between hydrolysis mechanisms and reductive mechanisms.

Dehalogenation depends on the redox potential of the molecules, which can be approximated by the strength of the carbon-halogen bond[5]. The higher the bond strength, the more reducing the system must be in order to result in transformation. The strength of the bond is dictated by the type of halogen present and influenced by other substituents and the degree of saturation of the molecule. Iodine and bromine substituents are easier to remove than chlorine. Furthermore, saturated compounds are reduced more easily than are unsaturated compounds. Because these trends are dictated by the physical and thermodynamic properties of the molecule, the same trends hold true for abiotic reductions. Due to the importance of the thermodynamics to the feasibility of reduction, numerous investigators have attempted to calculate such thermodynamic values to use as predictors[5-9].

The transformation of halogenated aliphatic compounds has been observed in a variety of systems containing anaerobic sediments[10], soils[11], sewage sludge[12], reduced transition metal cofactors[13], metal sulfides[14], and chemical reducing agents[15]. Reductive dehalogenation has been studied extensively and an excellent review has been published by Vogel et al[16]. Recent work has led Arnold and Roberts to propose[17] a

Figure 2-1 Calculation of the formal oxidation state to determine whether the transformation of tetrachloroethylene to trichloroethylene is through a reductive process.



Overall +4 +2

tetrachloroethylene trichloroethylene

more complex reaction pathway for chloroalkenes. The proposed pathway proceeds through a di-sigma-bonded surface-bound intermediate.

All of the reaction mechanisms that are proposed by Vogel initiate with electron transfer that results in a carbon centered radical.The radical intermediate can then react through at least four pathways. Hydrogen abstraction from a suitable donor atom is termed hydrogenolysis. The loss of a hydrogen atom from an adjacent carbon atom to form a carbon-carbon double bound is termed dehydrohalogenation. The addition of one radical to another is termed radical coupling; this is generally not observed in natural systems due to the low concentration of the intermediates. The last pathway is the elimination of two vicinal halides, resulting in a carbon-carbon double bond; this is termed vicinal dehalogenation. Figure 2-2 illustrates the possible outcomes following formation of the radical through the reaction mechanisms mentioned above. There are some additional reactions that the carbon-centered radical can undergo, most notably subsequent electron transfer to form a carbene. Because the product distribution observed following transformation is dictated by the mechanism, understanding the fate of the radicals is important.

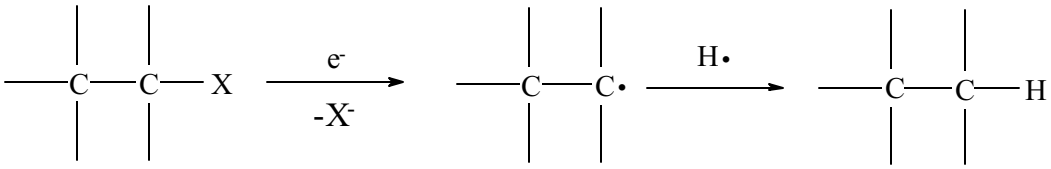
MICROBIAL PROCESSES

The impact that anaerobic biological processes have on the degradation of halogenated compounds in aquifers and sediments through reductive dehalogenation pathways is substantial. These environments are anaerobic due to oxygen depletion from the microbial reduction of organic matter. Thus, the introduction of halogenated compounds into these anaerobic zones allows biologically mediated reduction to occur.

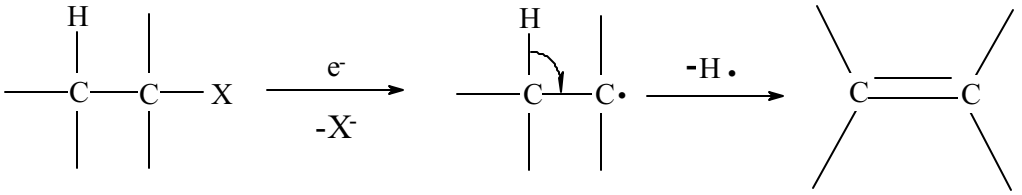
The reductive and vicinal dehalogenation reactions are dependent on the redox potential of the electron donor and the electron acceptor. To be thermodynamically

Figure 2-2 The fate of the radical formed following the initial electron transfer in reductive dehalogenation.

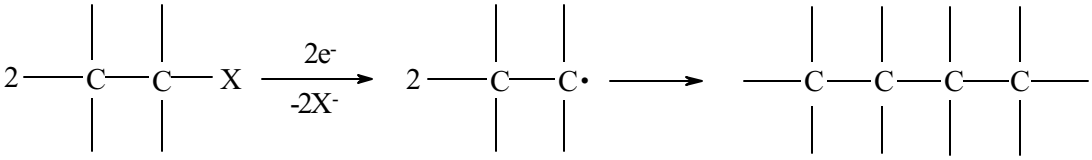
Hydrogenolysis



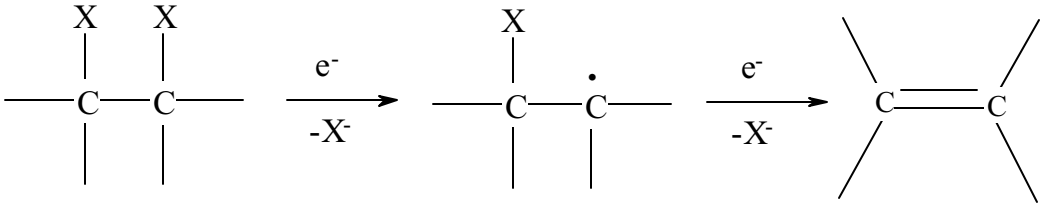
Dehydrohalogenation



Radical Coupling



Vicinal Dehalogenation

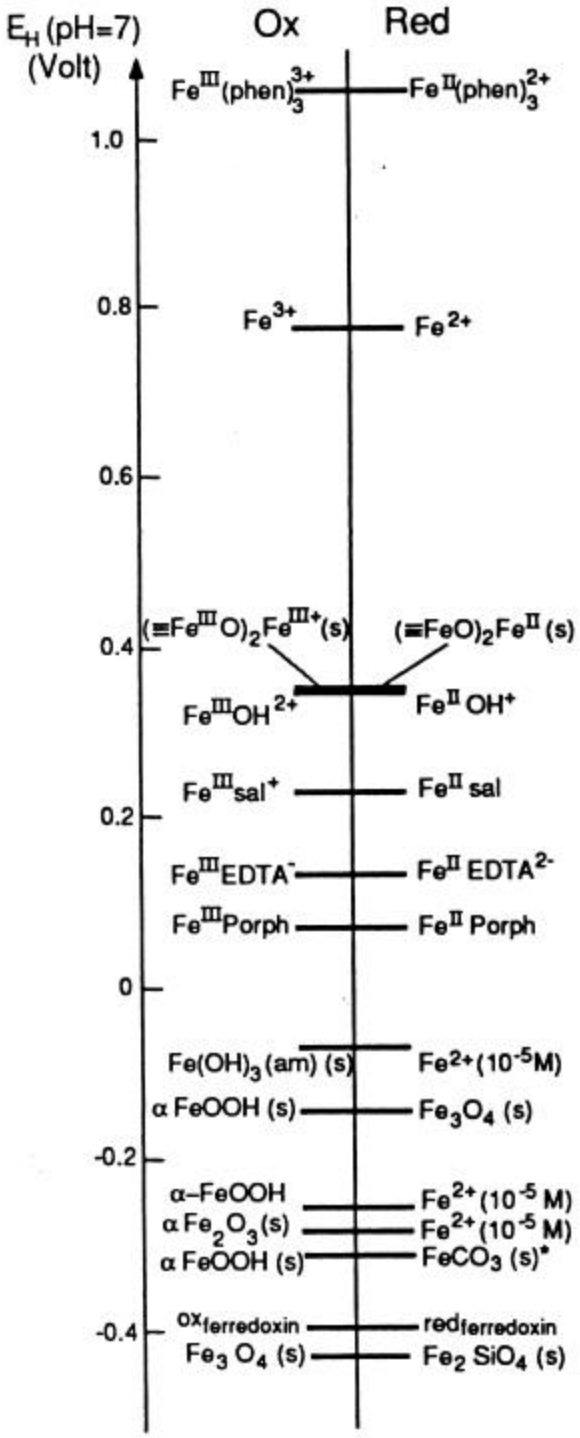


favored, the standard reduction potential (E_h, measured in volts vs. the standard hydrogen electrode) of the electron accepting reagent (halogenated compound) must be more positive than that of the electron donating reagent. This thermodynamic requirement sets some limits on the types of electron donors that can dehalogenate some compounds. Fortunately, the metalloproteins of the microbial electron transport system have sufficiently negative reduction potentials such that the dehalogenation of a wide variety of halogenated compounds becomes feasible.

Microbes have multiple pathways through which they can obtain energy from halogenated compounds[18]. Chloroaliphatics with one or two chlorine atoms per molecule may serve as a growth substrate. The microbes obtain energy by breaking down the carbon backbone and donating the resulting electrons to an electron acceptor. Conversely, compounds with three or more chlorine atoms per molecule may function as electron acceptors. The presence of the multiple chlorine atoms withdraws electrons, facilitating the accepting of electrons by the carbon skeleton. In the absence of another oxidized species, microbes utilize the highly chlorinated compounds as electron acceptors so that they may derive energy from other organic compounds such as acetate or other small organic molecules.

Figure 2-3 illustrates the electron donating ability of several common naturally occurring species. Although the energetics of the electron transfer to hexachloroethane rather than Fe (III) is more favorable, it is possible for the electron transfer to the less energetic species to occur[16]. The thermodynamics only describe one portion of the reaction. Kinetics may also control the reaction pathway. There may be a preference due to enzyme substrate specificity such that the naturally occurring species is favored. The interference of high concentrations of naturally occurring electron acceptors has been shown to inhibit the microbial reduction of these higher chlorinated species[19].

Figure 2-3 Redox potential of some environmentally important iron species, from Stumm[20].


E_H (pH=7)
(Volt)
Ox
Red
$Fe^{III}(phen)_3^{3+}$
$Fe^{II}(phen)_3^{2+}$
1.0
0.8
Fe^{3+}
Fe^{2+}
0.6
$(\equiv Fe^{III}O)_2Fe^{III+}$(s)
$(\equiv FeO)_2Fe^{II}$ (s)
0.4
$Fe^{III}OH^{2+}$
$Fe^{II}OH^{+}$
$Fe^{III}sal^{+}$
Fe^{II} sal
0.2
$Fe^{III}EDTA^{-}$
$Fe^{II}EDTA^{2-}$
Fe^{III}Porph
Fe^{II} Porph
0
$Fe(OH)_3$(am) (s)
$Fe^{2+}(10^{-5}M)$
α FeOOH (s)
Fe_3O_4 (s)
-0.2
α–FeOOH
$Fe^{2+}(10^{-5}$ M)
α Fe_2O_3(s)
$Fe^{2+}(10^{-5}$ M)
α FeOOH (s)
$FeCO_3$ (s)*
-0.4
$ox_{ferredoxin}$
$red_{ferredoxin}$
Fe_3O_4 (s)
Fe_2SiO_4 (s)

Microbes have an additional pathway through which the anaerobic degradation of chlorinated aliphatic compounds may occur. This pathway is termed oxidative and fermentative degradation. No external electron source is required because the halogenated molecule acts as its own electron donor and acceptor. The reducing equivalents are derived from degrading the carbon backbone and the presence of multiple halogen atoms allows the resulting halogenated carbon product to act as an electron acceptor. Under certain conditons the fermentative pathway may be favored over the competing reductive dehalogenation reactions. According to Dolfing[19], the availability of electron donors as well as the availability of halogenated compounds will have a significant effect on the pathways. A large excess of electron donors will result in reductive dehalogenation and conversely, the fermentative pathway will become pronounced when the availability of reducing equivalents is scarce.

ENVIRONMENTAL REDUCTANTS

Reducing equivalents found in the environment can drive the reductive dehalogenation process even without active microbes. However, the creation of these reducing species is dependent upon microbial action[21, 22]. As the microbes utilize small organic molecules as a food source, they release the resulting electrons to oxygen, nitrate, iron, sulfate, or other oxidized species. This action forms redox zones, which are characterized by the reduced species that are present. Common reducing equivalents that are found in such anoxic environments are H_2S and Fe (II). Thus, through the natural respiration process of the microbes, the oxygen is depleted and the formation of abiotic reduced species occurs. These reduced species may react directly with the organic pollutant, or an intermediate step may be necessary. Numerous investigations into the use of electron mediators that shuttle electrons from reduced species to the oxidized species have been performed[6, 23-25]. The species that are thought to be the

primary electron mediators possess a quinone functionality and are derived from humic acids. Transition metal enzyme cofactors and functional groups are also viewed as electron transfer mediators[26-28].

Sulfide species produced through microbial processes have been shown to be active in the reduction of environmentally important species as well as halogenated compounds. Sulfide species have been shown to reduce Fe (III) minerals[29], react with natural organic matter to generate a mercaptojuglone type compound[23], reduce carbon tetrachloride[30], and form iron sulfide minerals[14, 30] and polysulfide species[31, 32] capable of direct reduction of halogenated compounds.

Solubilized Fe^{2+} does not reduce most halogenated organic pollutants directly;,if the oxidation product is an aquo iron (III) species. The reduction potential of the Fe^{2+}/ Fe^{3+} couple is much too positive to reduce many pollutants directly. However, Fe^{2+} can sorb onto mineral oxide surfaces, which greatly lowers the reduction potential of the system, primarilly by strong stabalization of the Fe(III) product species by oxygen linkages. Figure 2-3 illustrates the reduction potential of the common iron-containing redox couples. Several researchers have independently illustrated that systems with uncomplexed or free solution Fe^{2+} demonstrate very little or no reductive dehalogenation[33, 34] or even reduction of nitroaromatic compounds[35]. Systems with a mineral oxide surface such as goethite in addition to Fe^{2+} show a marked increase in pollutant transformation[33-35]. There are two accepted explanations for the increase in transformation rates with Fe^{2+}/goethite systems. The first is based on the fact that the sorption of the Fe^{2+} is by an inner sphere complex with the surface hydroxyl groups, which increases the electron density around the adsorbed Fe^{2+} [20, 33, 36]. The other explanation is that the $Fe^{3+}$is stabilized through the formation of the mineral oxide species, which reduces the redox potential. This effect can be observed in Figure 2-3.

The potential of the á-FeOOH/ Fe^{2+} couple is more negative than that of á-FeOOH/ Fe_3O_4, illustrating that the formation of a solid species of Fe^{2+} actually shifts the reduction potential towards more positive values. Stabalization of Fe(III) with oxide species serves to reduce the redox potential from 770 mV vs. SHE to -220 mV vs. SHE[36]. Clearly the thermodynamics are far more favorable if the Fe^{2+} is present with an Fe^{3+} oxide or hydroxide surface.

Even though Fe^{2+} in the presence of Fe^{3+} mineral oxides has been shown to reduce halogenated compounds, the time scale for these reactions can be lengthy, depending upon the groundwater flow, concentration and identity of the halogenated pollutant, and presence of other electron acceptors. In such cases where the naturally occurring species are not capable of pollutant transformation, active remediation methods must be employed. Due to the failure of traditional pump and treat technologies, the development of remediation methods based on reductive mechanisms has blossomed over recent years.

ZERO-VALENT IRON

Through laboratory experiments, zero-valent iron has been shown to reduce halogenated compounds[7, 37-44]. Several explanations for the mechanism of reduction have been given. Matheson and Tratnyek investigated[45] the reduction of carbon tetrachloride on iron filings. In addition to determining the effects of surface and pH, they proposed three possible reaction mechanisms. Direct electron transfer from the metal surface to the pollutant was the favored explanation, with the other hypotheses being either reduction by Fe^{2+} produced through corrosion or hydrogenolysis by H_2 that was formed from the reduction of H_2O during anaerobic corrosion. Due to the effectiveness and relatively benign nature of iron release into the environment, the use

of zero-valent iron as a reactive surface for the *in situ* reduction has been employed for the remediation of halogenated solvents and pesticides.

Engineers have developed permeable reactive barriers where contaminated ground water flows through a subsurface iron wall and is remediated. Other less elegant applications of zero-valent iron for sediment and soil contamination have also been utilized. Generally the iron is broadcast across the sediment or soil and mixed in with a rotary tiller to insure good contact with the polluted media. The area can then be periodically sampled. The benefits of zero-valent iron are the relatively low cost and toxicity of the remediation process. Unfortunately, as the iron ages it becomes passivated with iron oxides that slow the reductive process, and occasionally toxic products are created[46]. These complications limit the effectiveness of this technology.

BIMETALLIC SYSTEMS

Efforts have been made to develop faster and more effective remediative approaches based on bimetallic systems with the hope to overcome some of the limitations of the zero-valent iron approach. Bimetallic systems consist of a reactive metal to generate reducing equivalents and H_2 through the reduction of H_2O. A catalytic metal then uses the produced H_2 to promote the hydrogenation of the halogenated compound. It has been shown that these systems are capable of completely dechlorinating common solvents and pesticides to hydrocarbons[42, 46, 47]. Aluminum, zinc, iron, and magnesium have been used as the hydrogen producing metals with copper, zinc, and palladium serving as the catalyst. Fennelly and Roberts have investigated[48] several bimetallic systems with 1, 1,1-trichloroethane in an effort to determine the reaction mechanism in these systems. The use of magnesium/palladium systems has been demonstrated for the quantitative dechlorination of PCB's[49], although not in a remediative effort. The use of palladium supported on Al_2O_3 and metallic

palladium catalysts was investigated[46] and was shown to rapidly destroy several halogenated compounds, without the formation of toxic products. Current research into the long-term use of these systems along with their limitations is ongoing[46].

The identification of the successes and failures of remediation technology drives the discovery process towards better methods. This work investigates several aspects of remediation and attenuation technology. The application of these improved remediation efforts in natural systems still needs to be investigated for long-term behavior and possible complications. The use of zero-valent iron was investigated for the reductive dechlorination of toxaphene. The need for a supplemental analytical method for toxaphene determination (Chapter 4) was demonstrated. A related mass spectrometric method was also developed and applied to determine the oxidation state of the metal center of metalloproteins (Chapter 6) The use of the magnesium/palladium bimetallic system was also investigated to determine the feasibility for the reduction of toxaphene. The occurrence of the DDT metabolite [2,2-bis(4-chlorophenyl) acetic acid] (DDA) in river water is investigated in Chapter 5.

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CHAPTER 3

EXPERIMENTAL METHODS

INTRODUCTION

The development of GC/MS has provided excellent confirmatory identification for small organic molecules and has become a workhorse in the analytical laboratory. The use of the capillary GC provides excellent resolution between peaks and the mass spectrometer provides characteristic spectra, which can help to assign the identity of an analyte especially in a complex matrix[1, 2]. While these developments along with the development of benchtop instruments have provided excellent tools to the analytical chemist, occasionally other methods are needed, especially if the analyte is not amenable to GC or not suited to electron impact ionization.

Many analytes are not suitable for GC based methods. Typically limitations are encountered with polar molecules, thermally labile, or high molecular weight species. Chemical derivatization can transform some classes of molecules to more volatile species and broaden the utility of GC based methods. An alternative to GC based methods is the use of liquid chromatographic methods. Because a solvent-based mobile phase is utilized in LC, it is ideally suited to polar, thermally unstable, and high molecular weight compounds[3-5]. Unfortunately, the mass spectrometer requires gas phase ions for analysis and cannot tolerate a liquid sample. Thus, the interface between the LC and the mass spectrometer is critical.

Early interfaces included the thermospray interface, which used a heated capillary and evaporation to desolvate the analytes and generate gas phase ions, and the particle beam interface which was used to produce a monodisperse aerosol so that the

LC effluent could be introduced into a mass spectrometer[6]. The thermospray interface ionized the analyte by depositing a fraction of the solution charge onto the analyte[7]. If additives such as ammonium acetate are present, then as the solvent evaporates the analyte molecules take up charge, typical sample ions are $[M + H]^+$ and $[M + NH_4]^+$ [8]. The particle beam interface serves to desolvate the analyte, and works with a conventional ion source. As the aerosol evaporated, the lighter solvent was pumped away and the heavier analyte molecules were selected for by a momentum separator. Following the desolvation, the analyte particles struck a heated target where they disintegrated and released individual molecules that were ionized by a conventional CI or EI source. Because these interfaces overcame some of the initial problems with analyte desolvation and ionization, methods were developed for the analysis of many classes of environmentally important compounds[6, 7]. Routine methods have been developed to monitor chlorinated phenoxy acid herbicides, azo dyes, nitroso compounds, and organophosphorous pesticides. Although these methods have been successful, some limitations still remain. Thermospray lacks any fragmentation to aid in identification of the analyte and the particle beam interface cannot transport non-volatile compounds to the ion source[9]. Perhaps improvements and evolution of these interfaces and ionization methods would solve these shortcomings; however, other methods have been developed that offer better sensitivity and selectivity[9].

The development of sample ionization systems operating at atmospheric pressure greatly expanded the utility of LC/MS. In atmospheric pressure ionization (API) ions are created in a region at or near atmospheric pressure and through differential pumping and ion optics are transferred into the mass spectrometer. Two principal methods of API have come to the forefront, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).

The development of APCI methods has provided a soft ionization method for fragile molecules that fragment extensively under the far harsher electron impact ionization. Electrospray ionization allows large species with multiple ionizable sites, such as proteins, to be ionized while remaining intact. Generally the API methods have found utility for the analysis of biological molecules, particularly those of high molecular weight. The analytes that are commonly determined in environmental analysis are smaller molecules that are polar, non-volatile, and thermally unstable in the molecular weight range of 100-800. These compounds of environmental interest generally yield singly charged molecular ions under ESI or APCI[10, 11 12, 13]. Although the molecular ions are useful for the identification of some molecules, fragmentation provides identifying information about the analyte, if necessary. This can be accomplished through the use of tandem mass spectrometers, although the cost and instrument complexity are prohibitive for their routine use.

The use of collision induced decomposition (CID) in conjunction with API methods can provide structural information about the molecular ion. CID occurs in the API transport region between the capillary-skimmer or between two skimmers. The extent of the fragmentation is dependent upon the voltage difference on these two elements[14]. An increase in the voltage accelerates the ions, which causes them to undergo collisions with the bath gas, generally air or nitrogen. These collisions result in an increase in internal ion energy, which can aid in the removal of adducts and in the formation of fragments. As the voltage is increased, the internal energy exceeds that of the bond energy and decomposition of adducts or fragmentation of the molecular ion occurs. Therefore, comparable information to that of tandem mass spectrometers can be obtained without the instrument cost or complexity. The use of CID with a single mass analyzer requires high purity of the sample being introduced. Otherwise, fragment ions of multiple components can occur simultaneously, complicating analysis[15]. An

alternative is to look only at the molecular ion or ions, in the case of mixed species, and forego the structural information given by CID. Even with this limitation, the use of single mass analyzer instruments is still favored because of their simplicity, cost, ruggedness, and sensitivity.

ESI has found utility for both large molecules of biological interest and smaller species of environmental importance. Fortunately, the multiple charges that result from ESI of large biological molecules such as proteins pushes the m/z ratio of the analyte to a more workable region ca 1.5 kDA vs. ca 15 kDA. This m/z ratio, while still quite large for quadrupole instruments, is almost ideal for TOF and FTICR instruments. A discussion of the complications encountered while trying to maintain the oxidation state of metalloproteins during the ESI process is given in Chapter 6. The ESI/MS methods utilized in this research (Chapter 4) for the small molecule toxaphene are not implemented in a conventional manner and thus some extra explanation about how the electrospray process is accomplished for a small molecule without obviously ionizable sites is necessary.

ELECTROSPRAY IONIZATION

ESI is accomplished by introducing a dilute liquid sample through a small tube, capillary, or needle into a mass spectrometer[16]. Placing a potential difference of a few kilovolts between the exit of the tube and the entrance to the mass spectrometer creates an intense electrostatic field. The droplets exiting from the tube are dispersed into a fine spray of charged droplets as a result of being in this intense field. The point when the repulsive force of the like charges exceeds the surface tension of the droplet is the Rayleigh limit. At this point, the droplet undergoes a process termed “Coulombic explosion” where the surface area is increased by creating smaller droplets. These fine droplets undergo evaporation and successive “Coulombic explosions” until a gas phase

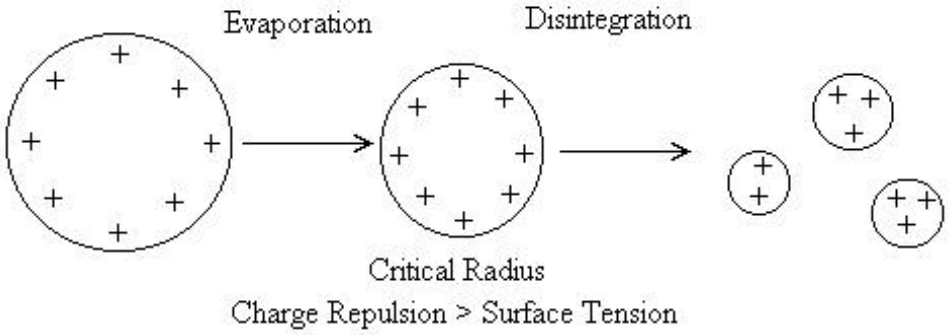
ion is created. There are two competing models that attempt to explain how the gas phase ion is created: the Charged Residue Model (CRM)[16] and the Ion Desorption Model (IDM)[11], both shown in Figure 3-1.

Both models invoke the use of Coulombic explosions to create charged droplets; the difference lies in how the ion leaves the droplet. The CRM holds that the solvent evaporates and the droplets undergo successive explosions until a single analyte is present in the droplet. The solvent then evaporates and leaves the analyte with all of the non-volatile material from the droplet. The IDM holds that the droplet ejects the analyte ion at an intermediate step prior to reaching the point of a single analyte per droplet. At some point the surface charge density is below the Rayleigh limit, so that no further Coulombic explosions will occur, but the surface field is still intense enough to overcome solvation forces and the analyte molecule is lifted from the droplet surface. It is thought that the IDM provides a better explanation of the ionization process than the CRM for molecules with molecular weight of less than 1000 kDA [17].

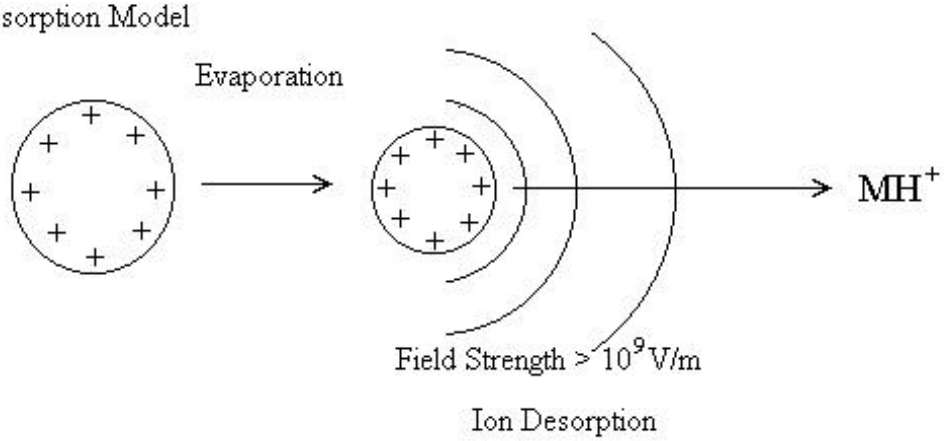
There are several properties that an analyte may posses that will enable it to more readily become a gas phase ion[18]. It may be a surface-active molecule, which based on the IDM will increase its likelihood of being ejected. It may already be ionized or be quickly ionized in solution, which facilitates its acceptance of charge, and since no barrier exists to the ionization, desolvation becomes the limiting step of the ESI process. The analyte may readily undergo adduct formation with a species in order to obtain charge. Association of Na^+ or Cl^- can assist in gas phase ion creation. Finally the molecule may undergo a redox reaction which will transfer electrons to or from the molecule. Thus the ideal analytes are ionic species or species that may be protonated or deprotonated by changing solution pH.

Figure 3-1 Cartoon depicting the Charged Residue Model (CRM) and the Ion Desorption Model (IDM) for ion formation. The CRM illustrates the fission steps leading to droplets with a single analyte molecule per droplet. The IDM shows an intermediate stage where the surface charge density is below the Rayleigh limit such that no Coulombic fission takes place but the surface field is still intense enough to desorb the analyte molecule.

Charged Residue Model



Ion Desorption Model



ELECTROSPRAY OF ENVIRONMENTALLY IMPORTANT MOLECULES

Large molecules such as proteins have numerous sites available for either protonation or deprotonation, depending upon whether positive or negative mode is utilized in the study. Many smaller molecules also have ionizable sites that allow for ionization in solution. ESI has been used in the LC/MS analysis of many compound classes including dyes, pesticides, herbicides, amine, hydroxyl, carbonyl compounds, and hydrocarbons.

The most difficult class of compounds to ionize with ESI is the hydrocarbons and polycyclic aromatic hydrocarbons (PAHs). These compounds lack sites for protonation and deprotonation; furthermore, they have low proton affinity that limits their ability to form ions with APCI[15]. The lack of functional groups also makes derivatization difficult. Fujimaki et al. have demonstrated the ionization of such non-polar species through charge exchange with solutions such as acetonitrile[19]. In their study, the ionization of fullerenes was achieved in negative mode.

The pesticide toxaphene is a mixture of polychlorinated bornanes. The molecules are not surface active and they possess no functional groups that readily undergo protonation or deprotonation. Therefore, ion formation of toxaphene in negative mode ESI is not a straightforward process. The most plausible explanations are charge transfer from the solvent, adduct formation, or dissociation of a C-H bond.

STRUCTURAL DIVERSITY OF TOXAPHENE

Toxaphene is the name given to the pesticide mixture of chlorinated bornanes. It is the most heavily used chlorinated pesticide around the world with an estimated 1.3 megatons applied to date[20]. It is produced by the exhaustive chlorination of camphene, which produces a huge number of congeners. Due to the structural diversity of technical toxaphene, numerous nomenclature systems have been developed. The IUPAC

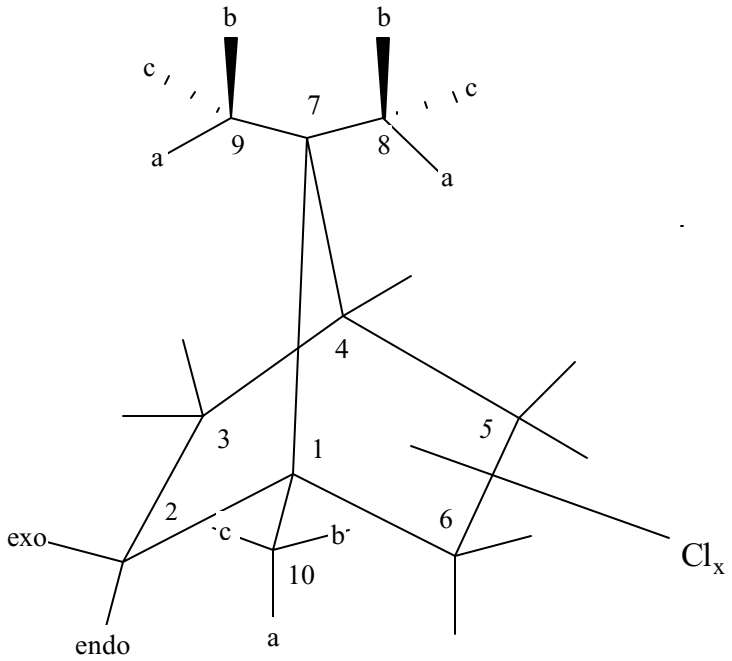
designation is based upon the numbering of the bornane skeleton with positions of chlorine atoms being denoted by letters (either a, b, or c,) for a given carbon atom. Furthermore endo and exo positions can be differentiated. Figure 3-2 shows the structure of a generic toxaphene component along with the IUPAC designations.

Hainzl et al. have done a complete treatment of the structural variety of toxaphene[21]. In contrast to purely theoretical calculations performed by Vetter[22], they were able to examine the 27 congeners that have been isolated and characterized and draw some conclusions that limit the total number of possible congeners[23]. Based on the work by Vetter[22], they concluded that there are 18 possible sites for chlorination on the bornane skeleton which would yield 32,768 possible congeners.

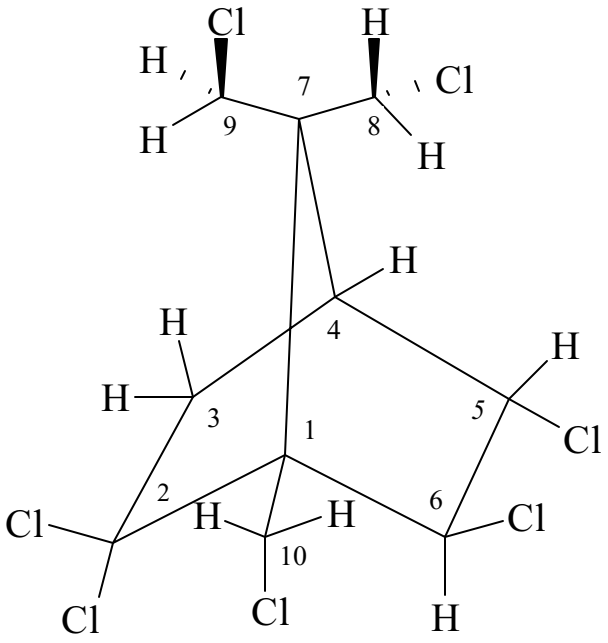
This theoretical number of congeners has some practical limits. The known intermediates 2-exo, 10-dichlorobornane and 2-exo,10,10-trichlorobornane demonstrate that all congeners start with a chlorine in the 2-exo position and at least one chlorine atom in the 10 position. Thus it is impossible for toxaphene congeners to be monochlorinated or unchlorinated[24]. As in other molecules with bridgeheads, the bridgehead at the 4 position is never substituted with chlorine. These two restrictions reduce the number of possible chlorination sites from 18 to 15. The total possible number of congeners decreases to 6144. (The calculation can be broken down to the product of the following: possible sites of chlorination on the 2,3,4,6,8,9,10 positions is 2^7, the 10 position can have 0-2 chlorine atoms for a total of 3 possible combinations, the 8 and 9 positions have the possibility of 0-3 chlorine atoms or 4 and 4) (2^7 x 3 x 4 x 4).

An additional consideration concerning positions 8, 9, and 10 is that the carbons are not free to rotate after substitution. This has been demonstrated in the NMR

Figure 3-2 The structure of the bornane skeleton with the IUPAC nomenclature system. The structure of the congener Toxicant B with its IUPAC nomenclature is shown below the chlorobornane. The unprimed numbers refer to endo substitution. The primed numbers refer to exo substitution.



Chlorobornane
$C_{10}H_{18-x}Cl_x$



Toxicant B
2,2',5,6',8c,9b,10a heptachlorobornane

spectrum of toxicant B[25]; furthermore, because position 10 is always chlorinated with at least one chlorine, its rotation is hindered. Due to the lack of free rotation, the 3 possible substitutions at the 8 and 9 positions are not considered equivalent[23]. The same argument may be made for the 10 position although it has only 2 additional substitution sites. Thus, the possible number of congeners becomes (2^7 x 2^2 x 2^3 x 2^3) = 2^{15} = 32,768.

This number may be reduced for steric reasons. Because the 8 and 9 positions are in such close proximity it is impossible for 6 chlorine atoms to occupy the 8 and 9 positions, reducing the positions available for chlorination to 14. Since the 2-exo and the 10 positions are already chlorinated when the intermediate is formed, that leaves 12 positions for chlorination and the total possible number of congeners is 2^{12} = 4096. This number includes optical isomers and can be cut in half since nonchiral analysis will not detect the presence of the optical isomers. Based on purely on this theoretical basis, there are 2050 possible congeners, however upon close examination of the 27 compounds that have been isolated, some additional real world restrictions become evident[21].

The 8 and 9 positions may hold a maximum of 3 chlorine atoms and the chlorine atoms are distributed with the following restrictions. If only one chlorine atom is present then it lies behind the 7,8,9 plane i.e. it is in the c position. If two chlorine atoms are present then one lies behind the plane and the other must be in front of the plane. If a third chlorine is present then it lies behind the plane. That makes two possible arrangements for one chlorine atom, four arrangements for two chlorine atoms, and two arrangements for 3 chlorine atoms. It has been noted that there are no congeners to date with two endo- chlorine atoms next to each other or directly opposite each other. Thus the maximum number of chlorine atoms that the ring (C_1-C_6) can hold is 6. This limits the highest order chlorobornane to undecachlorobornane. When the

aforementioned restrictions are taken into account only 1008 possible congeners exist including optical isomers.

A more realistic number can be determined if energetic reasons exclude some of the statistically possible substitutions. Hainzl ultimately concludes that 197 bornanes and camphenes with 5 or more chlorine atoms can exist in technical toxaphene in reasonable amounts[21]. A systematic application of all of the restrictions noted previously led to the total number of important chlorobornanes to be 138, comprised of 44 hexachloro-, 40 heptachloro-, 32 octachloro-, 15 nonachloro-, 8 decahloro-, and 2 undecachlorobornanes. While this does not address the relative concentrations of technical toxaphene, it does provide a reasonable estimation of the total number of congeners. These results compare well to the numbers that have been determined by other groups. Using GC/MS, Holmstead et al. identified 135 different hexachloro to decachlorobornanes, along with 42 singly unsaturated compounds[26]. Saleh detected 154 polychlorinated bornanes by GC/MS with many of those having less than six chlorines[27]. Thus, the theoretical treatment by Hainzl is in very good agreement with the best estimates from experimental data.

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CHAPTER 4

IMPROVED MONITORING OF TOXAPHENE DEGRADATION BY ELECTROSPRAY IONIZATION TIME OF FLIGHT MASS SPECTROMETRY

INTRODUCTION

Toxaphene is the name given to the complex mixture of polychlorinated bornanes of the average elemental composition $C_{10}H_{10}Cl_8$ that was applied as a pesticide. Toxaphene was once the most extensively used pesticide in the United States and in many other parts of the world. Prior to the ban on its use issued by the U.S. Environmental Protection Agency in 1982, approximately 0.45 megatons were produced. Estimates of global production and use to date are at 1.3 megatons[1]. Although toxaphene has not been widely used in the US since its ban in 1982, it has remained in use in regions of the former USSR, Eastern Europe, Central and South America, and Africa. Toxaphene was the subject of recent talks to ban persistent organic pollutants (POPs) throughout the world, mainly because the environmental impact of toxaphene is not limited solely to areas where it was applied. Runoff has contaminated fish and other marine life, and toxaphene has been detected in the Great Lakes, attributed to global transport[2, 3]. As the warm air of tropical and sub-tropical regions rises, it carries with it the semi-volatile toxaphene. The warm air cools as it moves toward the poles, therefore depositing the toxaphene in the cooler regions of the globe. This global transport together with the biomagnification of the congeners in fatty tissues of fish, seals, and polar bears has been the subject of numerous studies[2, 4-6].

Thus, the use of toxaphene and the cleanup of toxaphene-contaminated sediments and soils are of interest to the global population.

Toxaphene is produced by the controlled chlorination of camphene. A Wagner-Meerwein rearrangement of camphene leads to the formation of the intermediate exo-2, 10-dichlorobornane. Further chlorination leads to mainly polychlorinated bornanes[7]. According to Vetter, there are 32768 possible congeners[8], that number being based solely on the number of sites available for chlorination. Hainzl et al. have extensively treated the structural variety of toxaphene[9]. Through their detailed analysis of steric factors, they have concluded that there are 138 possible enantiomeric pairs of bornanes with five to eleven chlorine atoms. This number is more than the number that has been resolved to date by high-resolution gas chromatography. After separating the toxaphene into five fractions using HPLC on silica gel, Holmstead et al. found[10] more than 300 penta- to decachlorobornanes and bornene/camphene isomers by GC/MS. So the theoretically based number of 138 congeners suggested by Hainzl et al.[9] appears to be a reasonable approximation of the number of congeners present in technical toxaphene. Toxaphene residues have not received more attention in the environmental arena due to the complexity of the mixture present in technical toxaphene, a lack of suitable standards, and the slow development of analytical methods for detection and quantitation of toxaphene[11].

Most of the toxaphene standards that are available from standard suppliers are derived from material originally manufactured by Hercules Chemical[12]. Although this is a readily available supply of standard material, the homologue distribution in technical toxaphene does not accurately reflect the homologue distribution that is found in the environment following weathering or reductive dechlorination[13]. Additionally, because toxaphene is such a diverse mixture, the congeners that are present vary from

manufacturer to manufacturer[14]. Several attempts have been made to age and weather toxaphene standards by irradiation with UV light, to better approximate the congener distribution of environmental samples[15-17]. Parlar and coworkers have developed synthetic schemes to obtain 27 specific congeners, which have greatly increased the quantitative ability of gas chromatographic based methods[18-21].

Gas chromatographic methods are generally considered to be the best separation and analysis methods for toxaphene. Standard detection methods such as flame ionization detection (FID) are not highly sensitive to toxaphene and are not specific. Due to the high degree of chlorination, electron capture detectors (ECDs) provide excellent sensitivity for toxaphene; however, if other chlorinated pesticides are encountered, then they too will have a large response. Additionally, ECDs as well as FIDs provide little structural information except for that which can be derived from retention times. Electron impact ionization (EI) GC/MS techniques provide separation and some degree of identifying information, although the EI spectra for all of the congeners are very similar and the sensitivity is low.

Several researchers have worked on reducing the interferences that are commonly encountered during the EI GC/MS analysis of environmental samples. Hites et al. have developed[22] an automated routine to subtract out any contribution from the commonly encountered pesticide chlordane. Saleh has developed[23] a SIM method that looks for an ion at m/z 159; this ion is due to a tropylium type rearrangement of dichlorobornane and is very specific for the chlorinated bornanes. Unfortunately the use of the method of Saleh causes a loss of information about the degree of chlorination of the homologues detected, along with other structural information.

Methods based on gas chromatography with electron capture negative ion mass spectrometry (GC-ECNI-MS) provide excellent sensitivity and confirmatory spectra that

generally give the $[M - HCl]^-$ fragment, although there is some debate about the consistency of the $[M - HCl]^-$ fragment formation from congener to congener. A promising approach based on a short column GC-ECNI-MS (SC-GC-ECNI-MS) was recently developed[24] which used a 2 m GC column to desolvate the toxaphene sample and provide reproducible ionization conditions. All of these methods suffer from the fact that inlet temperatures above 150 ^{0}C can cause loss of chlorine in the inlet, prior to the chromatographic separation[25]. Despite the potential problems, currently accepted methods of analysis are based upon GC methods with ECD detection[14].

Other methods may be applicable to toxaphene analysis. Reports of electrospray ionization mass spectrometry (ESI-MS) of hydrocarbons and PAHs without ionizable functional groups do exist in the literature[26, 27]. However, most involve prior sample treatment or the addition of a charge transfer reagent to ionize the molecules. Research into the use of ESI for analysis of small environmentally relevant molecules has lagged far behind research into its use for biomolecules[28]. Furthermore, the use of negative ion mode determinations has been outpaced by positive ion mode analysis[29, 30]. To date, the use of ESI has not been applied to the polychlorinated pesticides. In the absence of fragmentation, mass spectrometric methods have a difficult time discriminating between isomers; however, this limitation does not preclude the use of mass spectrometry in the analysis of toxaphene.

Due to the toxicity and global environmental impact, numerous remediation schemes have been developed for toxaphene. Zero valent iron has been used to reductively dechlorinate the congeners, yielding less harmful congeners with lower chlorine content. Methods based on enhanced composting technology, where indigenous microflora are used to reductively dechlorinate toxaphene, have also been implemented. The use of bimetallic systems where one metal acts as a reductant and the

other metal as a catalyst has not been applied to the remediation of toxaphene at any real world sites, although its utility has been demonstrated for other uses[31].

This work reports the development of a new analytical methodology based on ESI time-of-flight mass spectrometry (ESI-TOF-MS) without the use of a prior chromatographic separation. Toxaphene was incubated with zero valent iron or a bimetallic system containing magnesium and palladium to reductively dechlorinate toxaphene. Results from the ESI TOF/MS analysis are compared to GC/ECD results for homologue distribution data and for evaluation of the effectiveness of the remediative schemes.

EXPERIMENTAL

A toxaphene standard was obtained as the neat mixture of congeners from ChemService. A working solution containing 1000 ppm toxaphene by weight was prepared in pesticide grade methanol (JT Baker). Other solvents, acetone and 2-propanol, were all HPLC grade from Fisher. Water was 18 MΩ deionized water from a Barnstead Nanopure water system. Acetic acid and hydrochloric acid were reagent grade from Fisher. Iron filings (40 mesh) and magnesium metal turnings (8-10 mesh) were obtained from Fisher. No surface pretreatment was performed on the magnesium because the magnesium had no visible surface oxide coating. Potassium palladous chloride was used as received from the vendor. The iron was washed in degassed 6 M HCl and then triply rinsed with degassed 18 MΩ water.

The reduction of toxaphene was carried out in 60 mL serum bottles with Teflon lined rubber stoppers. The reaction vessels were continuously agitated with a wrist-action shaker at an agitation rate of 150 cycles/minute to insure a constant rate of mass transport. The reactions were sampled at selected times by removing 1.5 mL of solution. The samples were sealed in bottles with Teflon lined caps and stored at 4 ^{o}C until analysis to minimize any further reaction. For the reductions with Mg/Pd, the

serum bottle was charged with 5 g of magnesium turnings, 33.6 mg potassium palladous chloride, 10 mL of a 1000 ppm toxaphene standard in methanol, 10 mL water, and 100 μL acetic acid, with a total solution volume of 50 mL The final composition of the reaction mixture was 80:20 methanol: water and 200 ppm toxaphene. The water was added last along with the 100 μL of acetic acid, to initiate the reaction. For reductions with zero-valent iron, solutions were prepared in a similar manner. The serum bottle was charged with 10 g of acid washed iron, 5 mL of a 1000 ppm toxaphene standard in methanol, 20 mL methanol, and 25 mL water, with a total solution volume of 50 mL The final composition of the reaction mixture was 50:50 methanol:water, 50 ppm toxaphene, and 10 g of acid washed iron. It was found that immediately after sampling, the withdrawn zero-valent iron aliquots air-oxidized to form an orange precipitate believed to be Fe_2O_3, and the magnesium samples showed a small amount of white precipitate believed to be MgO.

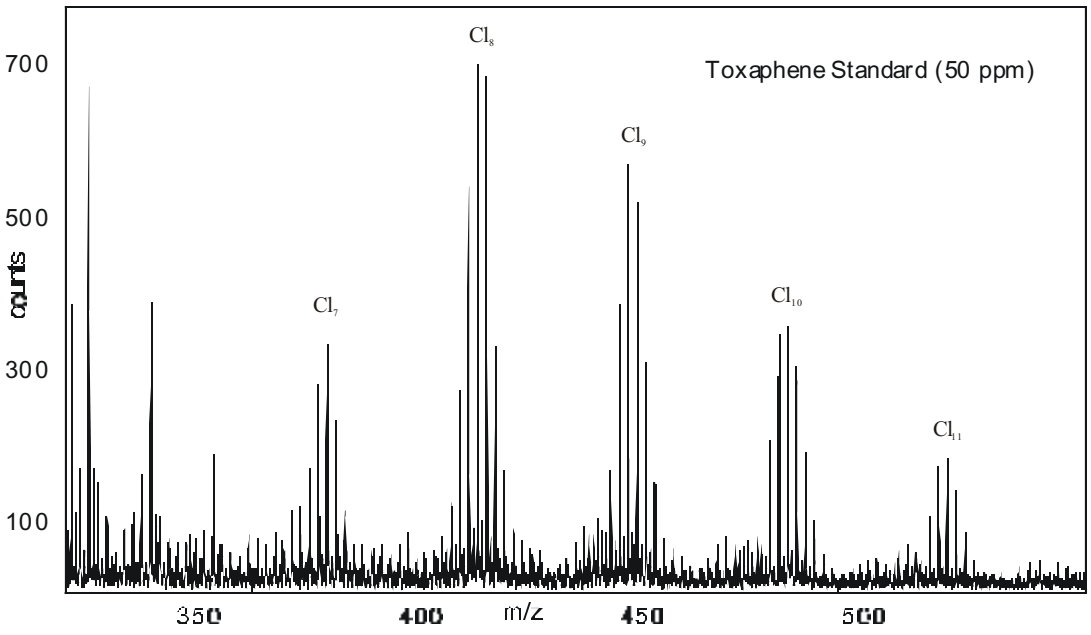
The ESI-TOF-MS analysis was conducted on a PE Biosystems Mariner Biospectrometer. The samples were ionized by nanoelectrospray ionization with a maximum flow rate of 133 nL/min. The nanoelectrospray tips were fabricated locally using 100 μm inner diameter fused silica capillary tubing (Polymicro Technologies, Phoenix, Arizona). The tips were pulled by heating with a hand held micro-torch (Microflame, Inc., Minnetonka, Minnesota) while applying a constant force of 0.112 N. Spray voltages ranged between –1500 V and –2000 V. The nozzle potential was 80 to 130 V and the skimmer was set to a potential of 14 V. The nozzle temperature was kept in the range 100 to 140 ^{o}C, to aid in the desolvation of the samples and to avoid thermal decomposition of toxaphene.

RESULTS AND DISCUSSION

Figure 4-1 shows the mass spectrum following ESI-TOF-MS analysis for a 50 ppm methanol/water toxaphene initial blank that was prepared in the same manner as the reaction vessels except that iron was omitted. The observed peaks illustrate two effects, the presence of chlorine isotopes which are separated by 2 m/z and homologues with varying numbers of chlorine atoms, which are separated by 34 m/z. The relative intensities of the observed homologue peaks, with a most abundant peak having a m/z of 413 for the $[M - H]^-$ fragment, correspond to the theoretical distribution of the toxaphene homologues. It has been widely reported that toxaphene is principally heptachloro- to nonachlorobornanes. These experimental results illustrate this fact directly without the need for reconstructing the spectrum, unlike the case for ECNI-MS analysis. ECNI-MS yields mainly the $[M - HCl]^-$ fragment[32, 33], which needs to be accounted for when determining the homologue distribution. In the study by Coelhan using SC-GC-ECNI-MS[24], toxaphene was also introduced without a prior chromatographic separation, and the m/z of the largest peak was 377. After adding the 36 amu to account for the loss of HCl, this makes octachlorobornanes the principal homologues observed in toxaphene by ECNI-MS. In addition to determining the principal components in technical toxaphene, the relative ratios of homologue abundance can be of interest, especially because the environmental reduction of toxaphene occurs through the loss of chlorine atoms, ignoring any ionization efficiencies or other factors for the moment. To a first approximation the homologue distribution can be based on the relative abundance of the most abundant peak for each set of isotope peaks (after accounting for loss of HCl in the case of ECNI-MS).

Although this analysis will lead to some bias towards the lower chlorinated species, both methods should exhibit the same bias and can still be directly compared. The bias arises because the isotope distribution patterns vary depending on the number

Figure 4-1 ESI-TOF-MS spectrum of 50 ppm toxaphene blank.


Toxaphene Standard (50 ppm)
Cl8
Cl9
Cl7
Cl10
Cl11
700
500
300
100
counts
350
400
m/z
450
500

of chlorine atoms present. For instance the most abundant peak for a compound containing seven chlorine atoms per molecule will be 31.8% of the total isotope distribution, and that of a compound containing eleven chlorine atoms per molecule will 26.3%. A more accurate approach would be to sum the isotope distributions for a given number of chlorine atoms.

Calculating relative abundance through the use of summed isotope peaks can be problematic for two reasons. First there is a contribution to the chlorinated bornanes from some unsaturated components, which will reduce the mass by 2 amu and skew the isotope distribution; this interference can be distinguished from the unsaturated chlorobornanes through the use of high-resolution mass spectrometry[34]. Secondly, for ECNI-MS, fragmentation of the higher chlorinated species occurs such that they appear as lower chlorinated species and will interfere with quantitation. This "mass leakage" has been discussed[11] in the literature previously. There is also evidence that the formation of the $[M - HCl]^-$ fragment in ECNI-MS is congener specific[33] so that the general assumption about the principal ion formed in ECNI-MS may not be valid for all of the species. The ECNI-MS approach has been noted to overestimate the higher chlorinated species, and be less sensitive to the lower chlorinated species[33, 35]. It is unknown whether or not any such inconsistencies are encountered in the ESI-TOF-MS approach.

Due to the structural similarity of the chlorobornanes, the electrospray ionization efficiency is expected to be relatively constant. Although the overall singal intensity would vary as nanospray tips and tip positions were changed, the day to day relative standard deviation of the relative abundances of the homologues in standard preparations generally agreed within 8%. Through run deviation between standards on the same day were generally under 4% It has been stated that for structurally related compounds of the same class, the abundance of the species seen in the mass spectra

following ESI should reflect the solution concentration of the various analytes[36]. Furthermore, there is little evidence of fragmentation following ESI. Fragmentation in ECNI-MS is evidenced by the isotope distribution, which does not show the spacing of 2 amu that would be dictated by the presence of numerous chlorine atoms. The presence of another isotope distribution intermingled with the expected distribution results from the loss of Cl. Thus for a given level of chlorination (Cl_8), there will be a distribution dictated by the $[C_{10}H_{10}Cl_8 - HCl]^-$ ion, and another distribution appearing 1 amu lower and occurring from "mass leakage" from higher chlorinated species corresponding to $[C_{10}H_9Cl_9 - HCl - Cl]^-$. This behavior is not observed in the ESI spectrum, which is consistent with a soft ionization method that generally yields the molecular ion and no fragmentation.

Aside from the SC-ECNI-MS method of Coelhan[24] and this ESI-TOF-MS method, all of the methods for the determination of toxaphene are based on the separation of the individual congeners and their identification. Due to the sheer number of congeners, it is difficult to resolve the peaks from one another in a typical GC chromatogram. Even if all of the congeners are resolved, the signal is spread throughout so many channels that there is a corresponding loss in sensitivity. A perfectly resolved chromatographic separation would consist of 138 peaks, whereas the mass spectrometric peaks correspond to roughly 5 sets of isotope distributions. With six important isotope peaks per molecule, that makes only 30 peaks to sum. Furthermore, there are no interferences due to coelution, because the separations are based on the mass of the ions formed, not on a partitioning phenomenon. Some investigators have used multidimensional GC methods to separate and identify congeners, although the samples under investigation in these cases generally involved a biological origin. The environmental and biological processes that lead to bioaccumulation select for specific congeners, and tend to simplify the chromatographic profile by reducing the number of

peaks that are present. Ultimately, the failure of chromatographic techniques arises from their reliance on dissimilar partitioning to effect a separation. The components of technical toxaphene are all from the same class of compounds and many are isomers, so that the partitioning behavior is likely to be nearly identical. Thus, the complete separation of all of the components is nearly impossible.

Although complete separation of the components in technical toxaphene is nearly impossible, GC based methods are still considered to be among the more reliable and robust methods[14]. The large hump or mountain that appears in the chromatogram is a characteristic of technical toxaphene and commonly makes quantitation difficult, even for experienced analysts. The elution order of the components observed on GC columns is generally as expected, with the lower chlorinated species eluting earlier and the higher order chlorinated species eluting later[13].

The variability of technical toxaphene is highly dependent upon the reaction conditions and time used to generate the mixture. It has been shown that the congener distribution can vary greatly from manufacturer to manufacturer, and unless standards are from the same sources, quantitation can be greatly skewed[14]. The degree of weathering (exposure to sunlight, heat, and moisture) can alter the homologue distribution significantly. Thus, it is difficult to generate standards that are of the same congener composition as a sample taken from the environment. A joint attempt between Hercules and the USEPA was made to improve the reproducibility of GC/ECD methods[14]. The major drawback to the method employed by Carlin[1] et al. is that specific peaks are used in the quantitation, without knowing the identity and the associated chemical properties of those components. By choosing several peaks across the hump of the toxaphene peak, an attempt is made to quantitate congeners of all degrees of chlorination accurately. The use of later eluting peaks is suggested because they are separated from other organochlorine pesticides and are more immune to matrix

interferences. Although multiple peaks are used to quantitate the toxaphene, no attempt is made to use later eluting peaks for higher chlorinated species and earlier peaks for the lower chlorinated species. Unfortunately the choice of peaks is largely arbitrary and does not account for the fact that the toxaphene congeners have been found to undergo reductive dehalogenation with reaction rates in the relative order of Cl_{11}>Cl_{10}>Cl_9, Cl_8> Cl_7, etc[37]. Thus the use of later eluting peaks tends to underestimate the overall toxaphene concentration because of the faster reaction of the higher chlorinated homologues. The use of earlier eluting peaks tends to overestimate the concentration of toxaphene due to the formation of the lower chlorinated species through reductive dechlorination, their slower reaction rate with respect to the higher chlorinated species, and interferences with other organochlorine pesticides. For these reasons a great deal of variability between contract laboratories was found upon analysis, although the average % RSD was ultimately reduced to 26 % following the introduction of appropriate standards and guidance concerning peak selection for quantitation[14].

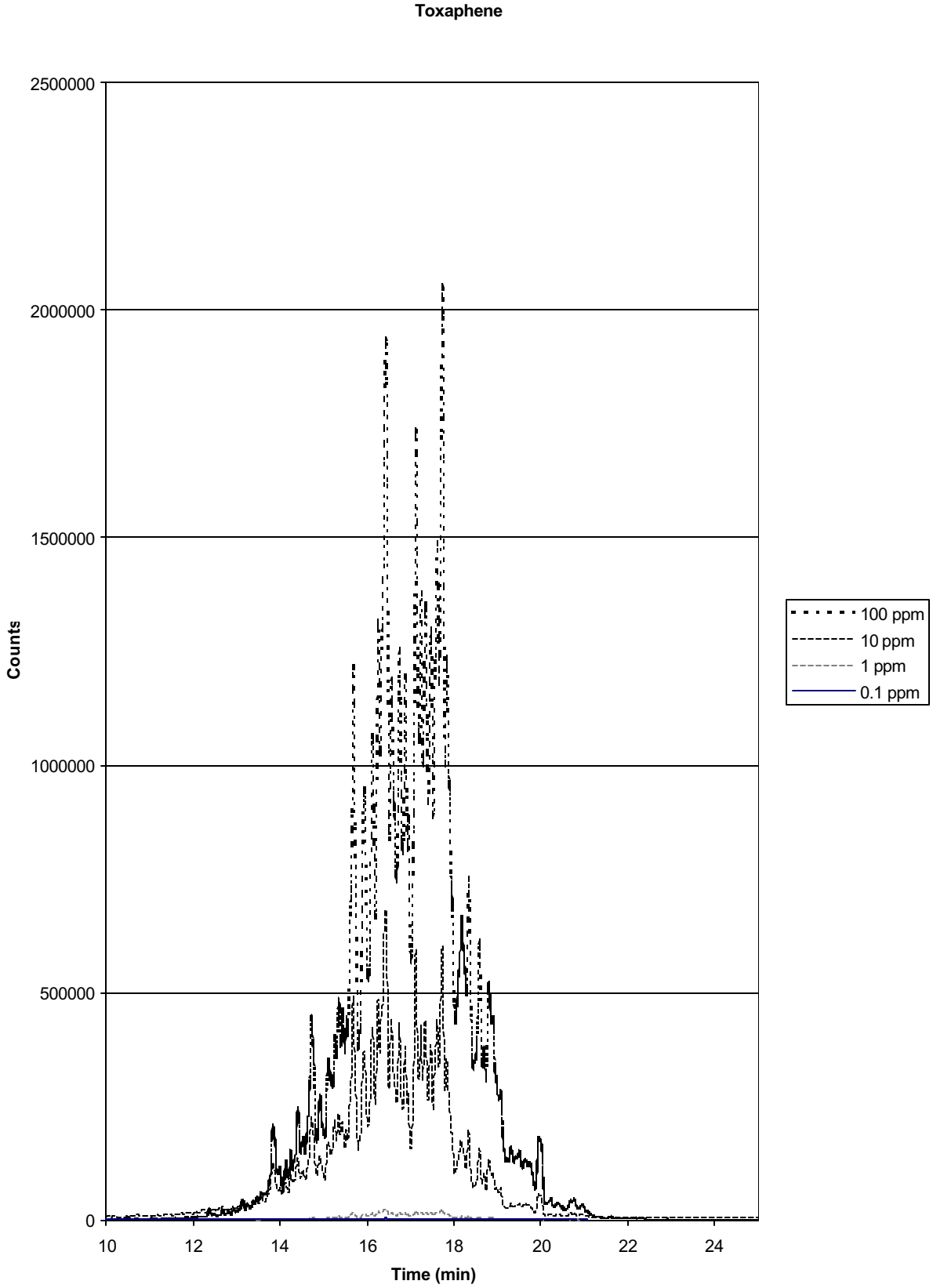
The acceptability of GC/ECD methods for quantitation is evidenced by the fact that these are the only approved methods for the analysis of toxaphene. Even the GC-ECNI-MS methods have not been validated and approved for use in the United States. Since toxaphene is regulated as a mixture, environmental determinations simply express the total concentration of residues. In addition to the determination of the concentration of the toxaphene in environmental samples, it is worthwhile to examine the homologue distribution. By knowing the homologue distribution, several inferences can be made. The degree of remediation can be assessed, the toxicity of the sediments can be determined, and the timeframe of the natural attenuation processes may be assessed. Quantification of total residues may not provide any information about these processes; however, a marked decrease in concentration along with a shift to lower chlorinated species would be excellent confirmation of remediation. Absence of homologue

distribution data prevents determinations based solely on concentration data from accurately reflecting the impact or toxicity of the mixture. Both the SC-GC-ECNI-MS and the ESI-TOF-MS techniques can give homologue distributions; however, the ESI technique does not suffer from some of the problems of the SC-GC-ECNI-MS method, particularly mass leakage.

Due to the nature of the nano-electrospray process, quantitation is a serious problem. Maintaining identical electrospray conditions is difficult and even small changes in sample composition can have tremendous effects on the suppression of one or more analyte peaks. Any perturbation to the tip, tip position, or flow rate will change the rate at which ions are created and collected. Several studies have attempted to use ESI for quantitative purposes. These methods generally involve the use of another analytical technique, such as UV-visible spectroscopy, to perform the quantitation, and the ESI data are utilized for confirmation of the analyte identity. Even the use of an internal standard to ratio the analyte against can be complex. The introduction of an additional species can cause competition for charge, which will prevent the complete ionization of one or more components. It has been stated that for analytes of similar structure, the relative intensity exhibited in the ESI mass spectrum will be reflective of the relative solution concentrations of the species[36]. So the relative intensity of the homologues observed with the ESI-MS approach should reflect the relative distribution of the homologues in solution.

Because the application of ESI to the analysis of toxaphene is a new methodology, the results were compared to the standard GC/ECD method. Standards of varying concentration were analyzed to confirm that there is no concentration dependent shift in the peak profile for toxaphene. The chromatograms from that analysis are shown in Figure 4-2. Since the profile is the same for each of the standards, any change in the peak profile is indicative of a transformation of one or more of the

Figure 4-2 GC/ECD Chromatograms of various concentrations of toxaphene standards. No discernable shift in the peak profile is observed with a change in concentration.


Toxaphene
2500000
2000000
1500000
1000000
500000
0
Counts
10
12
14
16
18
20
22
24
Time (min)
100 ppm
10 ppm
1 ppm
0.1 ppm

toxaphene components. A multi-peak calibration following the method of Carlin et al.[14] can be employed. In that method, several peaks across the toxaphene peak are selected and following calibration are used to determine the concentration of toxaphene in a sample. Carlin et al. selected peaks from the middle to the latter half of the chromatogram to avoid matrix effects and interfering organochlorine residues. Because matrix effects and organochlorine contaminants are not a problem in our samples, the peaks selected for quantitation were picked across the toxaphene profile. It is known that the higher order homologues elute at later times, so that judicious choice of peaks may reflect different homologue groups. The peaks selected were at retention times of 14.7, 16.7, and 17.7 minutes. The calibration curves for each of the peaks are shown in Figure 4-3. The non-linear response of the 17.7-minute peak is expected due to its high concentration in the toxaphene standard. The observation that the response increases for peaks of longer retention times has two explanations. The higher order homologues have a greater response on the ECD than that of the lower chlorinated species; furthermore, the distribution of toxaphene is such that the maximum occurs later in the chromatogram.

Toxaphene was incubated with acid washed zero-valent iron in glass serum bottles to evaluate the effectiveness of the iron for the reduction of toxaphene. Figure 4-4 shows the mass spectrum for the sample taken after a 0.25-hour incubation of a 50 ppm toxaphene sample. There is a significant decrease in signal as compared to the 50 ppm blank (Figure 4-1). There is a change in the relative abundance of the Cl_7 and Cl_9 homologues, indicating that either the Cl_9 homologues are reacting faster or the Cl_7 homologues are being formed during the reaction, or both. An examination of the next time point, 22 hours, is shown in Figure 4-5. Examination of Figure 4-5 reveals that in addition to an overall reduction in signal, the Cl_{11} and Cl_{10} homologues are greatly reduced in intensity. Figure 4-6 corresponds to the 48 hour time point and the Cl_{11}, Cl_{10},

Figure 4-3 Calibration curves for the three peaks selected across the toxaphene chromatogram.

GC Calibration Curve for Toxaphene
3 Different Quantitation Peaks

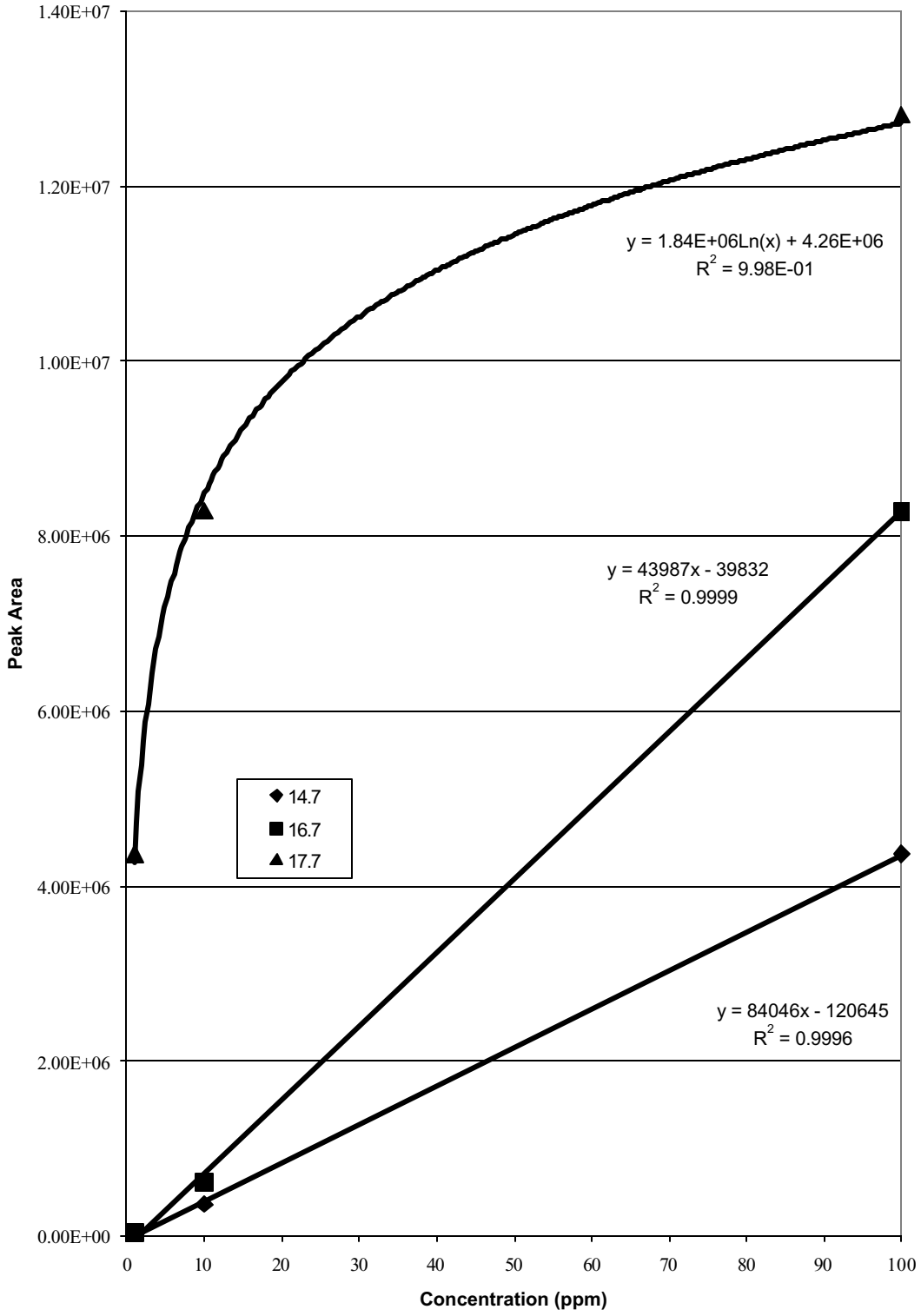


Figure 4-4 ESI-TOF-MS spectrum of toxaphene reacted with acid washed iron. Time =0.25 hours

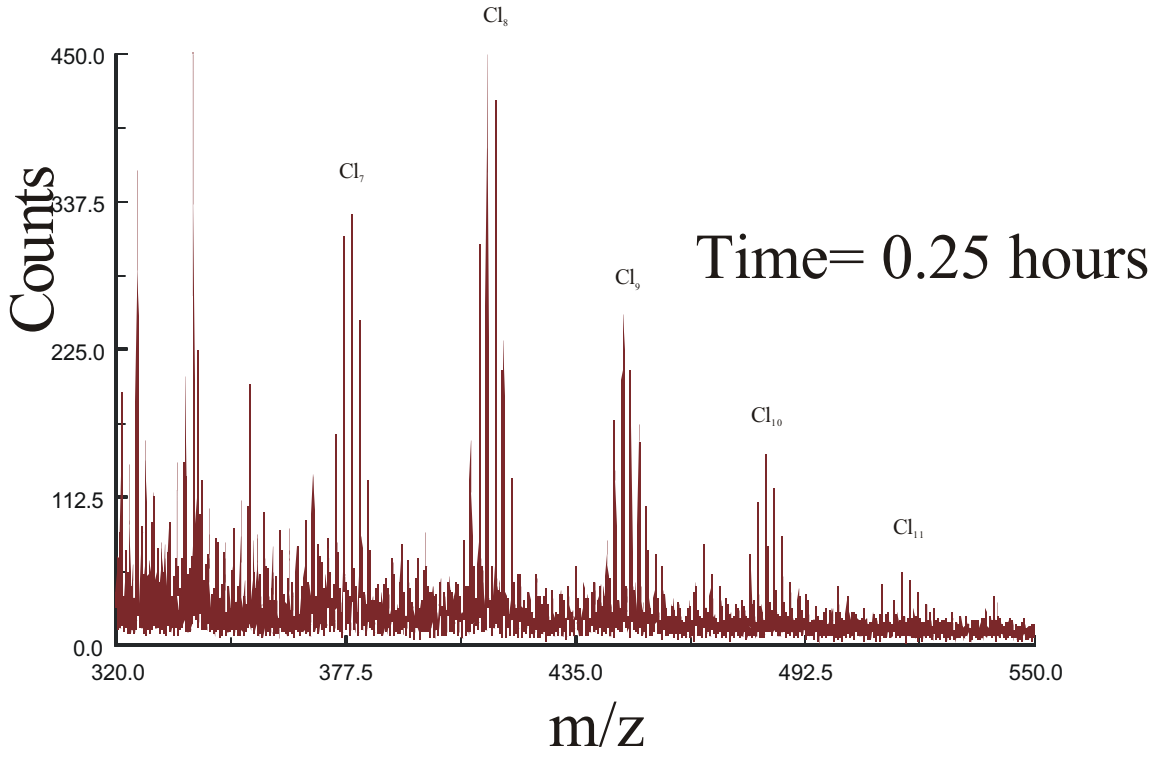

Cl8
Cl7
Cl9
Cl10
Cl11
Time= 0.25 hours
Counts
450.0
337.5
225.0
112.5
0.0
320.0
377.5
435.0
492.5
550.0
m/z

Figure 4-5 ESI-TOF-MS spectrum of toxaphene reacted with acid washed iron. Time = 22 hours

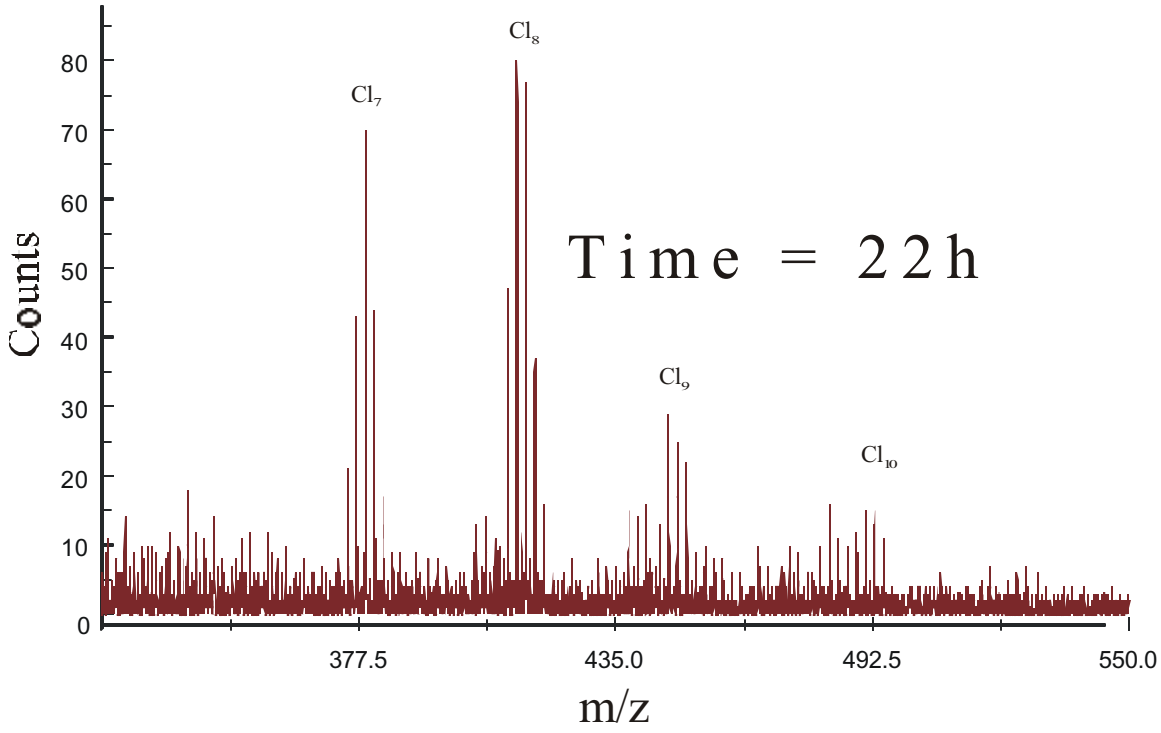
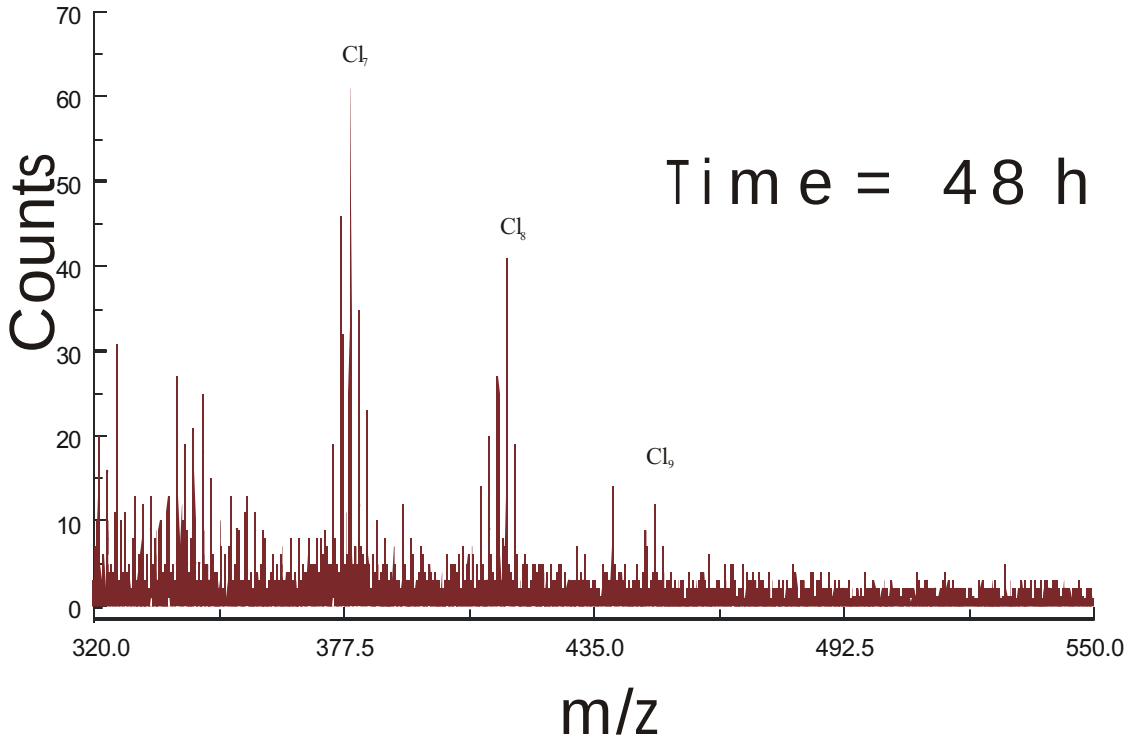

Cl8
Cl7
Time = 22h
Cl9
Cl10
Counts
80
70
60
50
40
30
20
10
0
377.5
435.0
492.5
550.0
m/z

Figure 4-6 ESI-TOF-MS spectrum of toxaphene reacted with acid washed iron. Time =48 hours


Time = 48 h
Counts
m/z
Cl7
Cl8
Cl9
70
60
50
40
30
20
10
0
320.0
377.5
435.0
492.5
550.0

and Cl_9 homologues are completely gone; furthermore, Cl_7 is the dominant homologue. Figure 4-7, 120 hours, shows an almost complete loss of signal.

Because the quantitative reproducibility of ESI is limited, it is important to correlate the findings of this study with the findings of other methods. The GC/ECD traces are an excellent indicator that the ESI methodology is valid. Unfortunately the GC methods also have limitations. Peak identity is difficult to obtain and the reported standard deviation is high; however, GC is the only accepted quantitation method for toxaphene. Figure 4-8 illustrates the decline in the abundance of the homologues with increasing incubation time with zero-valent iron. This can be directly compared to Figure 4-9, where the determination of the remaining homologues is made by the GC/ECD method. Even though the exact homologues that are chosen for the GC determination are not known, the data agree in a qualitative sense. As the reaction time progresses, the higher order homologues are reduced prior to the disappearance of the lower Cl homologues; however, all of the homologues are undergoing reduction simultaneously. The reduction in total counts, coupled with GC data, demonstrates that the concentration of all of the homologues is decreasing with time, with the Cl_7 homologue undergoing the slowest reaction. The GC chromatograms shown in Figure 4-10 correspond to the 50 ppm standard and the 0.25-hour sample. It is clear that the peaks at shorter retention time are attenuated less than the peaks at longer retention times. This seems to correlate well with the ESI data and illustrates the importance of determining homologue distribution.

Representing the change in concentration of all of the species is difficult but can be estimated by use of an approximate mass balance based on the intensities of reliably measured homologues of toxaphene as an internal standard. In this approach the signals of all of the homologues of a given mass are normalized to the sum of the signals of all

Figure 4-7 ESI-TOF-MS spectrum of toxaphene reacted with acid washed iron. Time = 120 hours

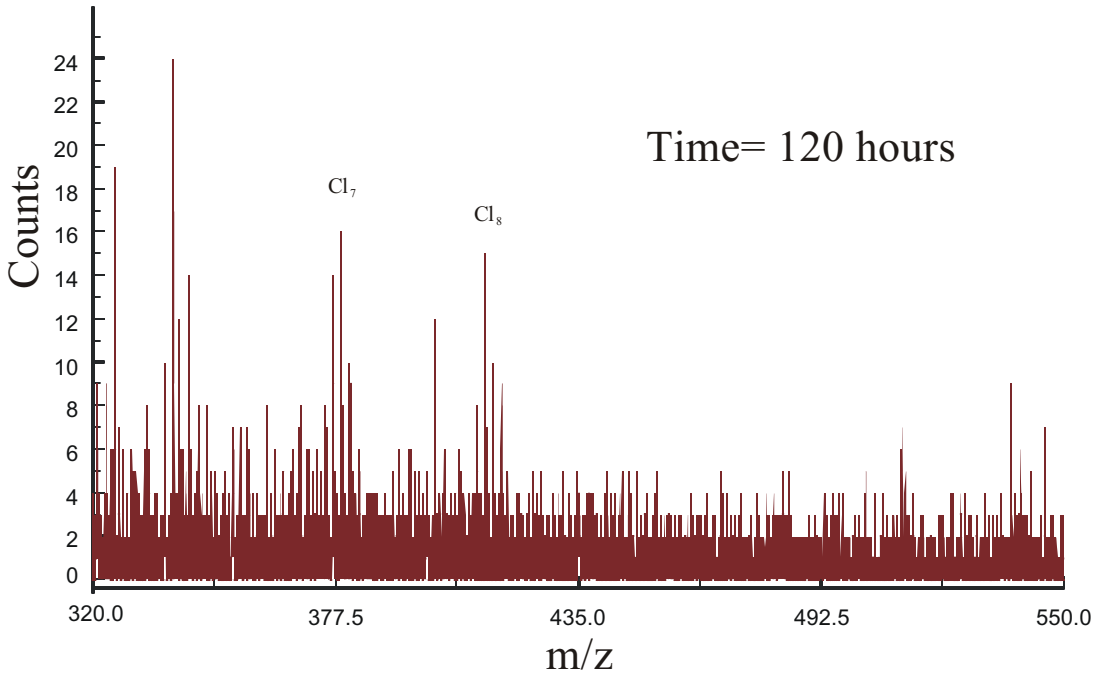

Time= 120 hours
Cl7
Cl8
Counts
24
22
20
18
16
14
12
10
8
6
4
2
0
320.0
377.5
435.0
492.5
550.0
m/z

Figure 4-8 Toxaphene ion counts for each homologue as a function of reaction time with zero-valent iron.

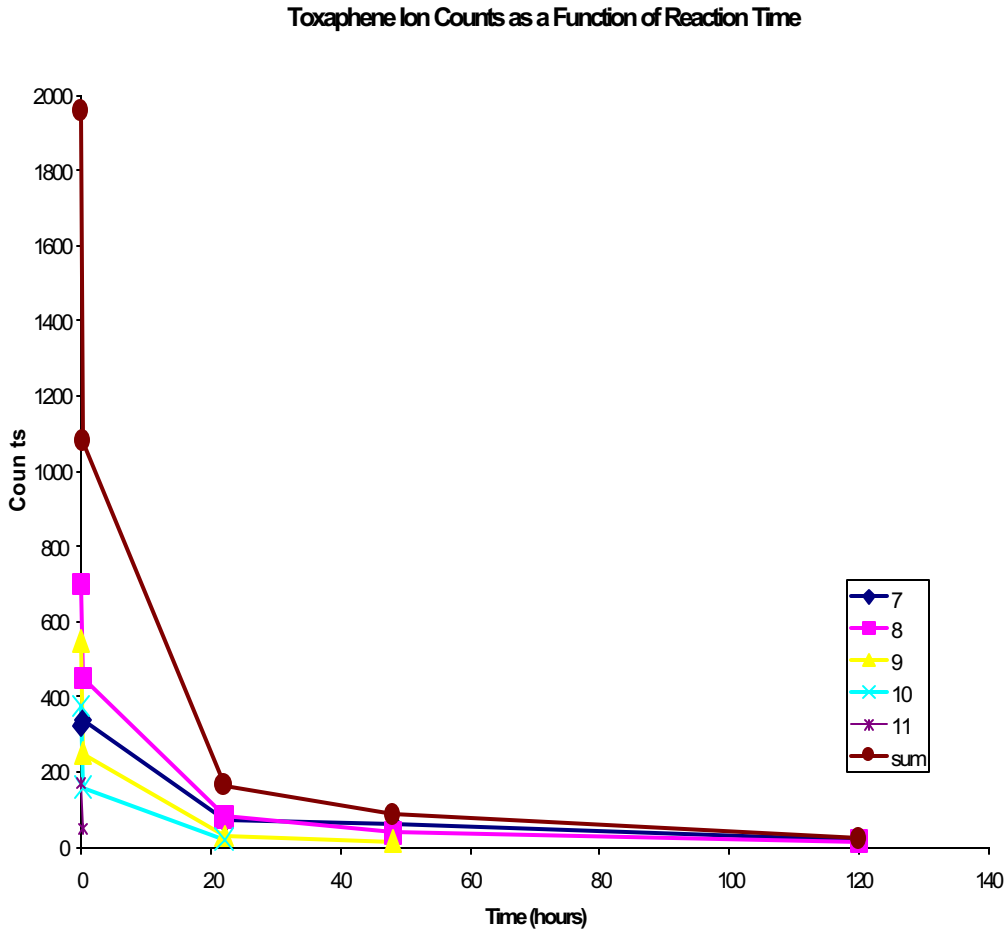

Toxaphene Ion Counts as a Function of Reaction Time
Coun ts
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
Time (hours)
7
8
9
10
11
sum

Figure 4-9 Concentration of toxaphene as determined by multi-point GC calibration.

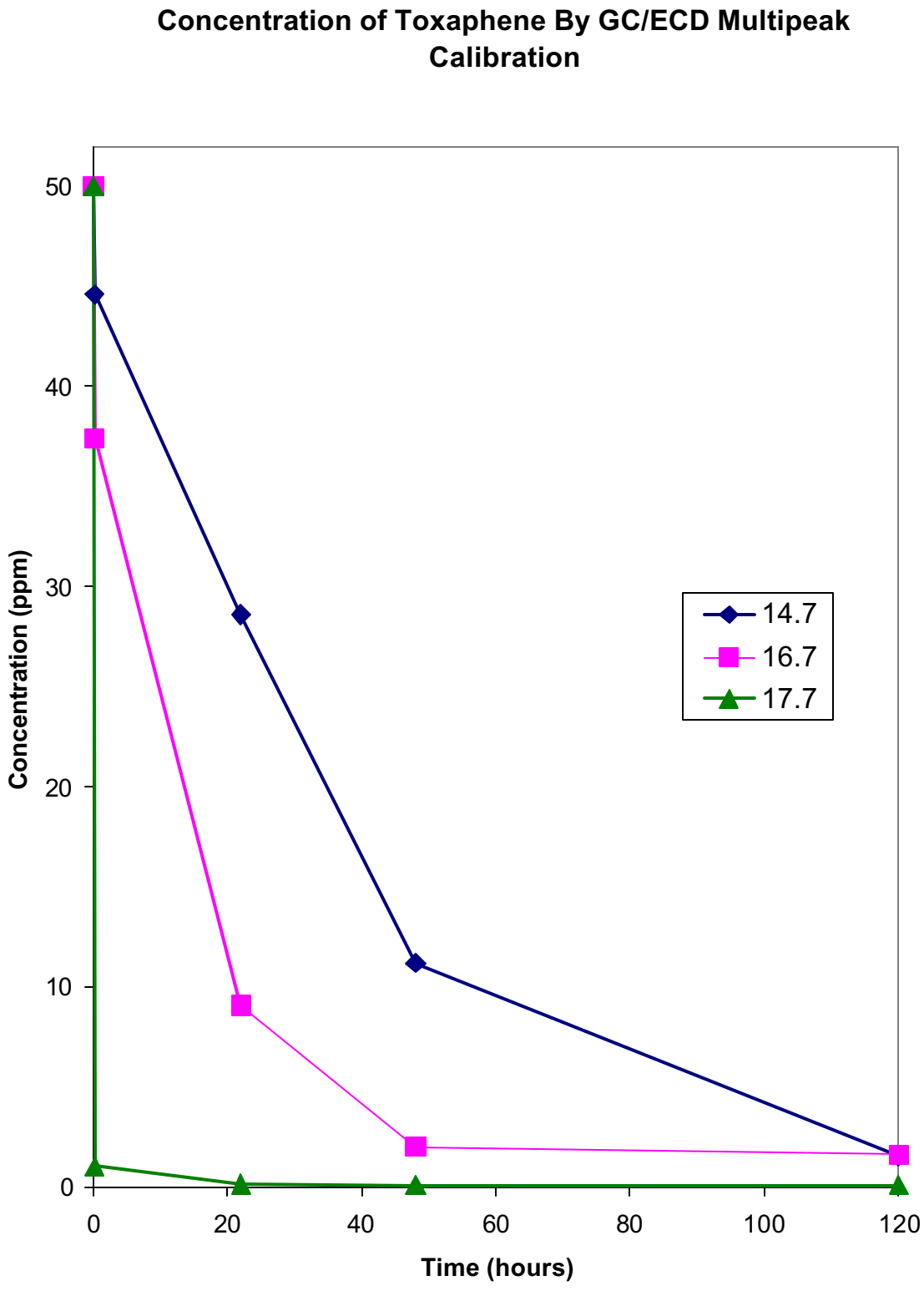
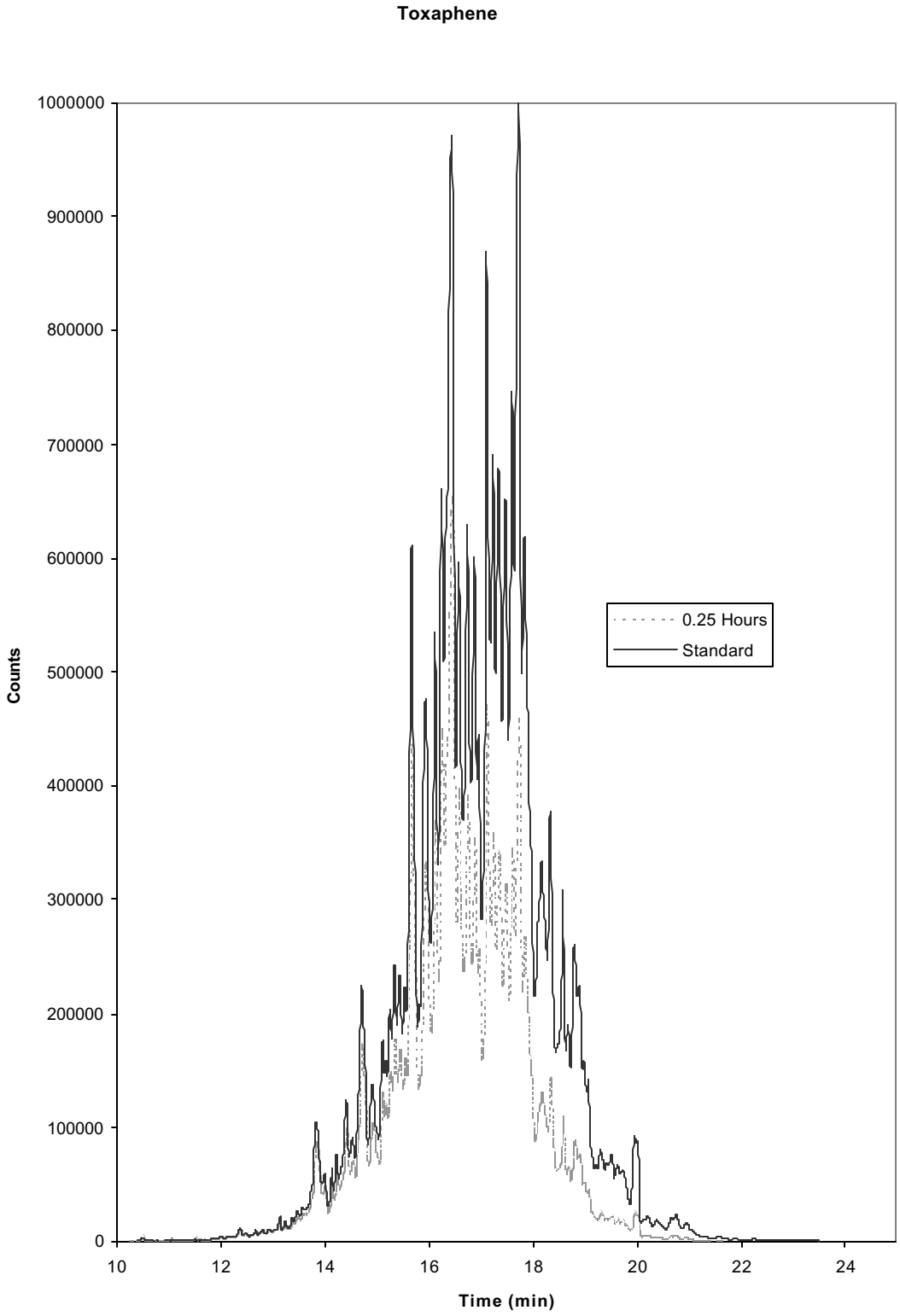

Concentration of Toxaphene By GC/ECD Multipeak Calibration
Concentration (ppm)
50
40
30
20
10
0
0
20
40
60
80
100
120
Time (hours)
14.7
16.7
17.7

Figure 4-10 GC/ECD chromatogram for 0.25 hour sample. The loss of the later eluting peaks is more pronounced, which is consistent with the ESI data.


Toxaphene
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
Counts
0.25 Hours
Standard
10
12
14
16
18
20
22
24
Time (min)

reliably measured homologue masses. This approach can compensate to some extent for the variability of ESI sensitivity from run to run, by comparing individual homologue signals to the initial signals for all measured homologues in the same sample at the same time. The relative concentrations can be estimated directly from the relative abundance observed in the mass spectrum, assuming similar sensitivity for all peaks. The most abundant isotope peak was chosen as a marker for each homologue. The peak abundances were tabulated and the percentage of each homologue calculated. Figure 4-11 estimates the percentage that each homologue comprises in the samples as a function of time. The drastic increase in the percentage of the Cl_7 homologue at times greater than 22 hours is due to reduction of the higher order homologues. The relative rate of disappearance is Cl_{11}>Cl_{10}~Cl_9>Cl_8>Cl_7. This relative reaction order has been observed previously in anaerobic sewage sludge[37]. Because no methods exist to accurately quantitate all degrees of chlorination present in technical toxaphene and its reaction products, relying solely on relative homologue abundance is problematic for kinetic analysis. The percentage of the homologues in each sample as shown in Figure 4-12 varies depending on which homologues are observed. As a means of illustrating the decrease in signal intensity with reaction time, the percentage of total counts relative to the initial spectra is shown. The percentage of the initial counts is divided by two to keep it on the same scale with the other data. The ESI-TOF-MS method is valid for Cl_7 and higher species but signals for lower chlorinated species are much smaller in intensity and subject to interference from other species. However, the lower chlorinated species are surely formed from the dechlorination of toxaphene, which limits the accuracy of the attempted mass balance. It is clear that the fractions appearing in homologues with more than eight chlorine atoms remains approximately constant for 48 hours, and then declines, while the fraction in the Cl_7 homologues increases. The signals after 48 hours are necessarily quite uncertain due to the low signal/noise ratio. Because

Figure 4-11 Homologue intensity at selected sample times relative to the intensity of that homologue observed in the standard. This illustrates the differing loss of intensity of the homologues due to transformation, which leads to a change in homologue distribution.

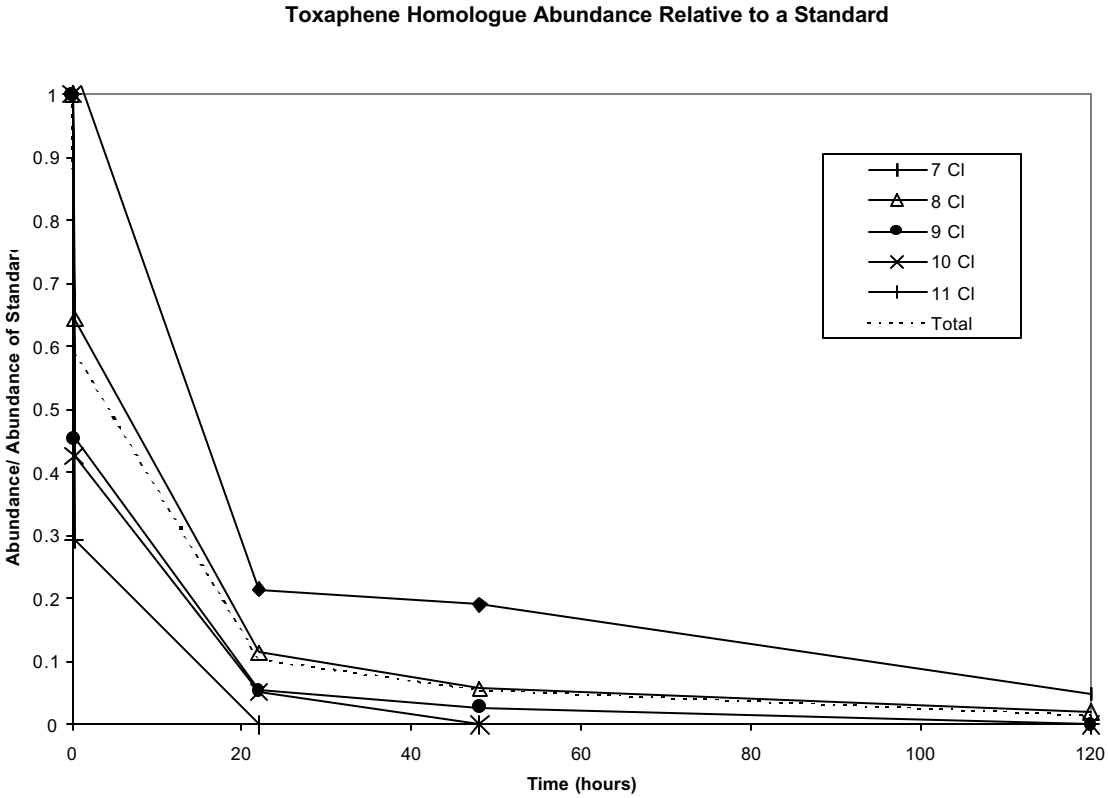
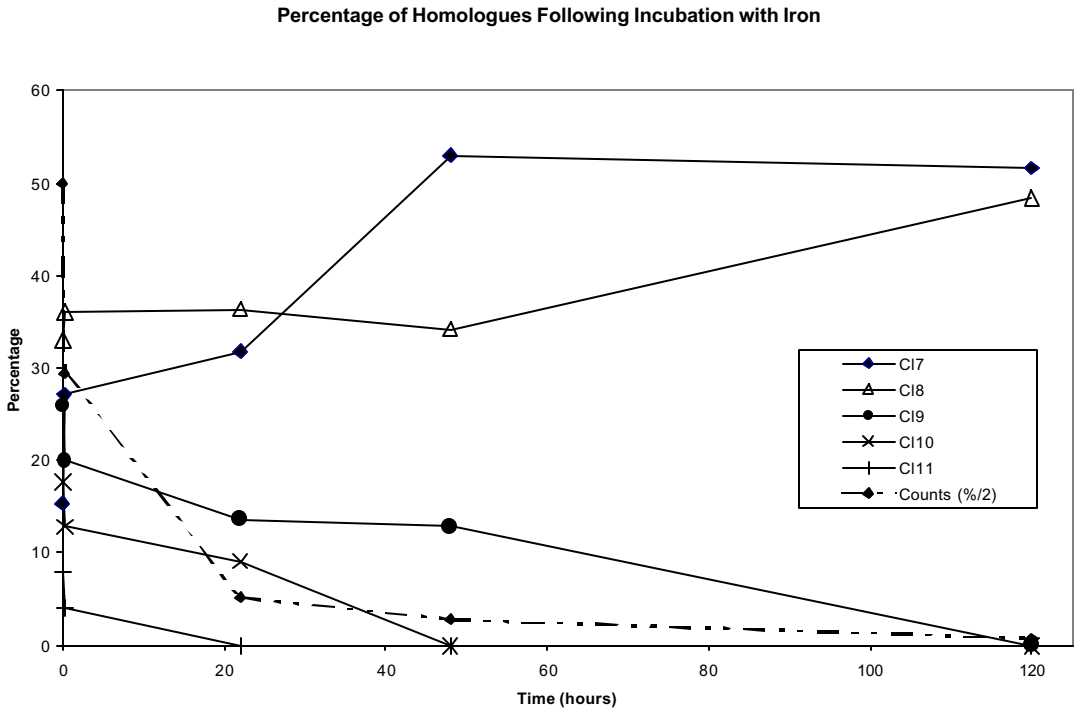

Toxaphene Homologue Abundance Relative to a Standard
Abundance/ Abundance of Standar
Time (hours)
7 Cl
8 Cl
9 Cl
10 Cl
11 Cl
Total
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
20
40
60
80
100
120

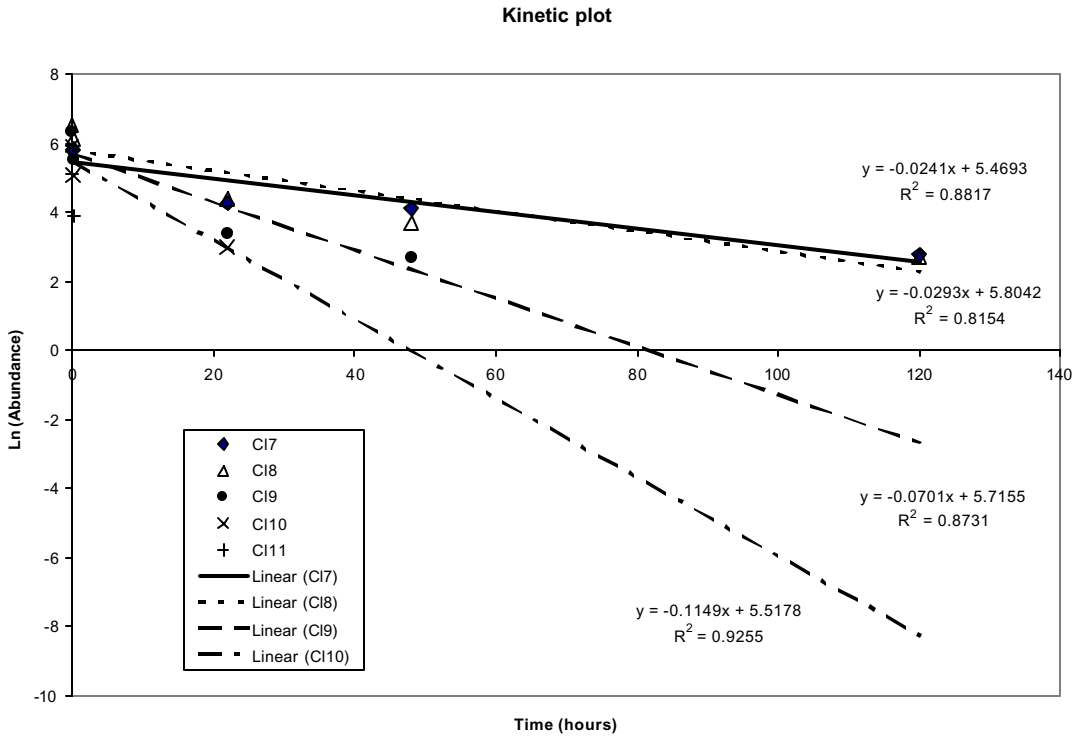
Figure 4-12 Degradation of hepta- through decachlorobornanes with zero-valent iron. The percentage that each homologue comprises in the sample is shown as a function of time.


Percentage of Homologues Following Incubation with Iron
Percentage
Time (hours)
0
10
20
30
40
50
60
0
20
40
60
80
100
120
Cl7
Cl8
Cl9
Cl10
Cl11
Counts (%/2)

the homologues with fewer than seven chlorine atoms cannot be reliably measured, the treatment becomes increasingly uncertain as dechlorination continues. The rates of disappearance of the homologues with higher chlorine number are clearly faster and fall off with decreasing chlorine number. Although homologue distribution is important, little kinetic data can be determined from relative intensities.

Kinetic plots were performed in order to estimate the rate constants for the different homologue groups. In addition to confirming that the rate of dechlorination of the homologues increases with the number of chlorine atoms on toxaphene, the analysis of the rates serves to validate the ESI data. The kinetic plots of ln (abundance) vs. time are shown in Figure 4-13. The slopes of the linear fits give the rate constants. The relative reaction rate constants are demonstrated to be $Cl_{11}>Cl_{10}>Cl_9>Cl_8>Cl_7$. With the actual values estimated to be -4.90, -0.12±0.03, -0.0701±0.019, -0.029±0.008, -0.024±0.005, respectively. The value for the Cl_{11} species is determined from the first two data points and is not considered to be entirely valid. Buser et. al.[37], determined that the relative rate consants follow the same order as observed here. They did not, however, report any actual rate constants. Except under special reaction conditions, these rates can only be used as a relative measure of homologue reactivity. The formation of lower order homologues from the reduction of higher order homologues is difficult to account for unless an excess of the highest order homologue is present or only one homologue is present initially. Deriving kinetic data for the dechlorination of toxaphene from ESI-TOF-MS is difficult. Assuming that there is no change in the ionization efficiency from sample to sample or drastic differences in homologue ionization efficiencies, a pseudo- first order model should fit the data well. Although the correlation coefficients reflect that the linear fits are not ideal, the fits are sufficient to describe the data, especially in lieu of the questionable validity of the previous assumptions. The somewhat less than ideal correlation coefficients can be attributed to

Figure 4-13 Kinetic plots of the ln(abundance) vs time. The rate constants are given by the slopes of the linear fits.


Kinetic plot
Ln (Abundance)
Time (hours)
8
6
4
2
0
-2
-4
-6
-8
-10
0
20
40
60
80
100
120
140
y = -0.0241x + 5.4693
R2 = 0.8817
y = -0.0293x + 5.8042
R2 = 0.8154
y = -0.0701x + 5.7155
R2 = 0.8731
y = -0.1149x + 5.5178
R2 = 0.9255
Cl7
Cl8
Cl9
Cl10
Cl11
Linear (Cl7)
Linear (Cl8)
Linear (Cl9)
Linear (Cl10)

appears to be very rapid at early times and passivation of the iron surface is suspected as the reaction proceeds, complicating the analysis. An additional complication arises from the formation of the lower chlorinated species from the dechlorination reaction of higher chlorinated species.

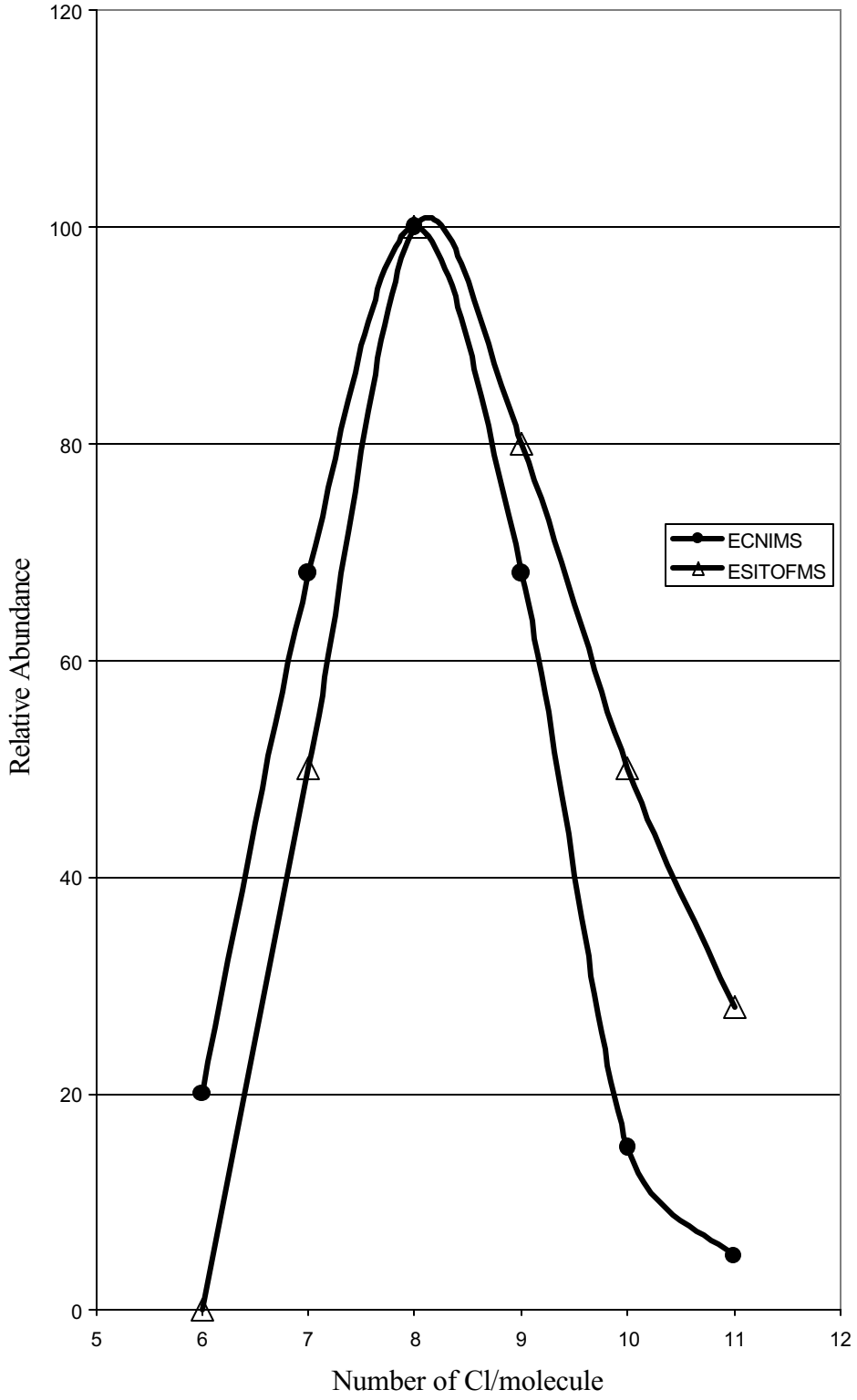
The GC data indicated that only a small amount of toxaphene remained. To verify that the reduction had occurred, a Cl^- selective electrode was used to monitor the initial and the final Cl^- concentration. There was a substantial Cl^- background in the iron system due to the acid washing with HCl. By examining the difference between the two readings, the concentration of the toxaphene added, and assuming an average of eight chlorine atoms per molecule; it was determined that 110 percent of the toxaphene had been converted to Cl^-. This additional chloride was most likely due to the release of Cl^- from the iron. The Cl^- analysis is further evidence suggesting complete dechlorination of toxaphene by zero valent iron.

The SC-ECNI-MS method of Coelhan is a drastic improvement upon chromatographic based methods and can give homologue distribution data. The comparison to the homologue distribution determined by SC-ECNI-MS and ESI-TOF-MS are shown in Figure 4-14. Both methods determine an average homologue distribution centered around $C_{10}H_{10}Cl_8$; however the SC-ECNI-MS overestimates the lower order homologues, relative to the ESI-TOF determination. It is clear, however, that there is general agreement between methods, supporting the utility of the ESI approach as a tool for monitoring homologue distribution during transformation of toxaphene, and in general both methods serve to confirm each other.

Another method of reduction was investigated to determine its effect on the homologue distribution of toxaphene. A bi-metallic system that uses magnesium metal to reduce the toxaphene together with finely divided palladium metal catalyst to catalyze the hydrogenation of toxaphene was investigated. The system served to completely

Figure 4-14 Comparison of SC-ECNI-MS results to ESI-TOF-MS results.

Comparison of Congener Distribution



reduce the toxaphene in less than 20 minutes. At the conclusion of the reduction, the ESI-TOF spectra showed little more than noise, and very little change in the homologue distributions was noted during the reduction reaction. The GC data indicated that only a small amount of toxaphene remained. To verify that the reduction had occurred, a Cl^- selective electrode was used to monitor the initial and the final Cl^- concentration. From the difference between the two readings and the concentration of the toxaphene added, the average number of chlorine atoms per molecule can be determined. It was determined that there were 8 chlorine atoms per molecule. Thus, it was concluded for the case of the Pd/Mg reduction, that the reaction was very rapid and that there is little selectivity difference for reduction between homologues.To investigate further the application of ESI to organochlorine compounds, a standard of chlordane and a PCB mixture were analyzed. No signal was observed for either analyte. It should be cautioned that this negative result does not mean that PCB and chlordane interferences can be overcome through the use of ESI-TOF-MS. It is entirely possible that the presence of other organochlorine species will compete for charge and suppress the toxaphene signal. Further investigations into the feasibility of ESI-MS of other organochlorine pesticides is needed before definitive conclusions can be made.

The ESI-TOF-MS method described here can be used to screen toxaphene samples rapidly for both the presence of toxaphene residues as well as for homologue distribution data. The ESI method confirms that unreacted toxaphene mixtures are generally of the average formula $C_{10}H_{10}Cl_8$. The congener specific fragmentation that is evidenced in the ECNI-MS based methods does not appear to have a significant impact on the ESI method. The utility of homologue distribution to evaluate the effectiveness of natural attenuation or remediative processes has also been illustrated. The application of ESI-MS methods to analytes that are not normally considered to be ideal ESI candidates due to a lack of an ionizable functional group is demonstrated. The utility of

ESI for these small molecules has not been fully explored and merits more investigation. Improvements in the quantitative aspects of ESI are also desirable. These improvements may be accomplished through the introduction of a standard concomitant with the sample in a parallel channel or the use of a flow injection analysis technique so that the sample introduction and matrix are as uniform and reproducible as possible.

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CHAPTER 5

INVESTIGATION OF THE OCCURRENCE OF DDA IN RIVER WATER

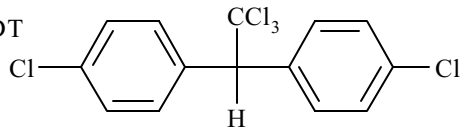
INTRODUCTION

The discovery of the insecticidal properties of 2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT)(Figure5-1) in the late 1930's and its subsequent application in the decades that followed were paramount to the prevention of insect borne disease and to the increased success of agriculture worldwide. After decades of use, evidence began to surface that DDT and its metabolites 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (DDE) and 2,2-bis(4-chlorophenyl)-1,1-dichloroethane (DDD) accumulated in higher animals and were highly persistent in the environment[1]. Ironically, it is because of the impact of DDT that Rachael Carson wrote the book Silent Spring, which is credited with beginning the environmental awakening in the 1960's. Even with public pressure mounting, DDT was still used in the United States until its ban in 1972.

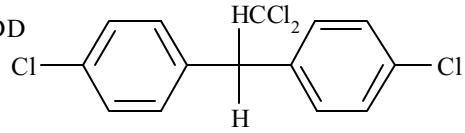
Most industrialized countries banned the use of DDT in the early 1970's except in emergency cases such as malaria and typhus outbreaks. This notable exception illustrates how successful DDT is at controlling mosquito populations and preventing disease outbreaks. Even today, however, developing countries still utilize DDT as the principal control of vector-borne diseases. Furthermore it was used in the former communist bloc countries until the early 1990's[2-5]. The cumulative global usage has been estimated at 2.6 million tons with just under half of that amount applied from 1970 to 1993[6]. As far back as 1965, DDT and its derivatives had been shown to thin the eggshells of birds, while more recent research has demonstrated endocrine disrupting effects[7-9]. The results of DDT exposure are varied. Abnormal sexual development and

Figure 5-1 Structures of the main analogues and metabolites of DDT found in the environment.

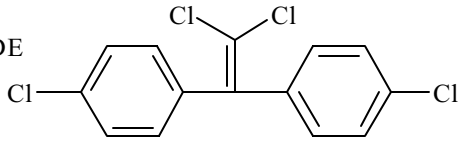
DDT



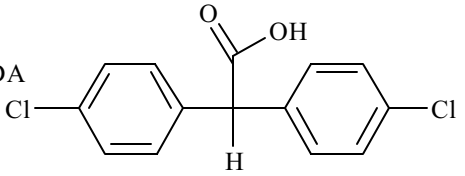
DDD



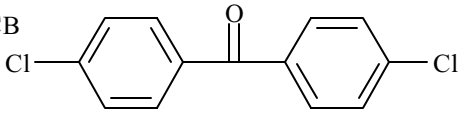
DDE



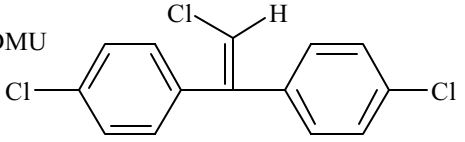
DDA



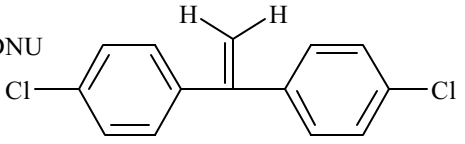
DCB



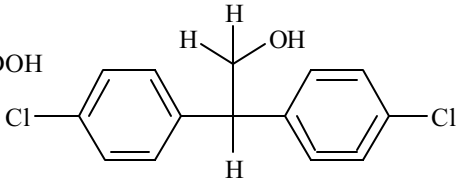
DDMU



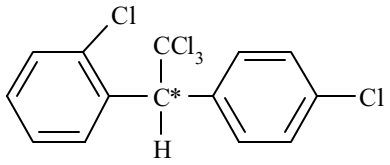
DDNU



DDOH



o,p'-DDT



* Denotes the chiral center.

impaired reproduction are among the more common effects observed[10-13]. DDT has clearly demonstrated its environmental impact. It is worthwhile to note that DDD and DDE are potent endocrine disrupters in their own right and they are also found to be bioaccumulated[1]. Thus, environmental degradation of DDT to form the metabolites DDD and DDE does little to lessen the overall impact on the ecosystem.

DDT undergoes environmental degradation by many routes, which strongly depend on the environmental conditions that are present. Under anaerobic conditions DDT is found to undergo reductive dechlorination to yield DDD[14]. Under aerobic conditions, it is dehydrochlorinated to yield DDE. At high concentrations this pathway is inhibited, presumably due to the toxicity of the DDT and DDE towards the microbial populations responsible for the degradation. Until recently, the residues DDD and DDE were thought to be the terminal residues although other metabolites have recently been identified, namely 2,2-bis(4-chlorophenyl)-1-chloroethylene (DDMU) from DDE and dichlorobenzophenone (DBP) from DDT[15]. With the notable exception of the work by Heberer[16], little has been done on the identification of polar degradation products of DDT. Recent studies have identified DDT and the nonpolar metabolites in seawater, surface water, and groundwater, but none include the polar metabolite 2,2-bis(4-chlorophenyl) acetic acid (DDA) as part of the analysis. DDA was one of the first metabolites of DDT that was isolated[17], and it has been called “perhaps the universal DDT metabolite in microorganisms, plants, animals, and higher animals”[18]. In addition to the enzyme-mediated reactions that convert DDT to DDA in plants, animals, and microbes, DDT can be converted to DDA by photo-oxidation and by chemical action[18].

CHIRALITY

Technical grade DDT was applied as a pesticide. It contained approximately 75 percent *p,p'*-DDT with the remaining 25 percent consisting of *o,p'*-DDT and small amounts of *p,p'*-DDD[19]. The *o,p'*- isomer contains an asymmetrical carbon and thus is chiral. Furthermore it is an endocrine disrupter, with demonstrated activity in avian and mammalian systems[7], and based upon the activity in rats is suspected to possess human estrogenic effects[8, 9]. Due to the increased estrogenic effect of the (-) enantiomer relative to the (+) enantiomer[8], the ratio of the two enantiomers is important to assess the overall effect of *o,p'*-DDT and its metabolites. A handful of investigations into the occurrence of the chiral compound *o,p'*-DDT and its metabolite *o,p'*-DDD have been performed. While it is generally found that as DDT degrades, the relative ratios of *o,p'* metabolites to *p,p'*- metabolites remain approximately the same as observed for technical DDT, with one study noting an overall average of 17% *o,p'* DDT in residues analyzed from fish. Heberer studied the isomeric ratios of *o,p'*-DDA to *p,p'*-DDA[16,20], but to date no studies have investigated the enantioselective occurrence of the metabolite *o,p'*-DDA.

Several plausible mechanisms exist for the preferential formation and/or removal of one enantiomer of *o,p'*-DDA relative to another. Enantiomerically preferential formation is quite possible as the formation of DDA is generally attributed to enzyme mediated reactions which degrade DDT, and this is a known pathway of DDT detoxification in mammalian systems[17,21]. Preferential transport in biological systems is another potential route of differing reaction rates for the enantiomers[22].

The now notorious pesticide DDT was once used widely in the US. While many sites show a marked reduction in the levels of DDT, sites associated with the manufacture of the DDT still demonstrate abnormally high levels of the compound and

its metabolites. Although DDT is not terribly water soluble, one metabolite, DDA, is highly water-soluble. Additionally the algicidal[23], enzyme inhibiting[24], and endocrine disrupting effects of DDA have been proven[25], although little work has been done on the relative health effects of the two enantiomers. The goals of this study were to determine whether *o,p'*-DDA was present in river water samples, and to develop a method for the determination of the enantiomeric ratio of *o,p'*-DDA utilizing typical environmental laboratory equipment such as a benchtop GC/MS.

A superfund site associated with the historical manufacturing of DDT and a nearby affected stream in Huntsville, Alabama were investigated for the occurrence of DDA, and more specifically the isomerically enriched occurrence of *o,p'*-DDA if present, as well as the enantiomeric ratio of the individual *o,p'*-DDA enantiomers.

EXPERIMENTAL

DDA was analyzed based on an analytical methodology originally developed for the determination of haloacetic acids in water. This method uses C18 solid-phase extraction (SPE) cartridges, derivatization with methanol/sulfuric acid to generate the methyl ester, and analysis with capillary gas chromatography-mass spectrometry (GC-MS) in selected ion monitoring (SIM) mode.

Methanol and methyl-tert butyl ether were HPLC grade products and used as received from Fisher (Pittsburgh, PA). Sulfuric acid was reagent grade also received from Fisher. Water was 18 M deionized water prepared on a MilliQ system. Standards of DDT, DDD, DDE, and DDA were obtained from ChemService (West Chester, PA). Solid-phase extraction was carried out on a vacuum manifold model number SPE 12G by J. T. Baker. The SPE cartridges were Supelclean LC-18 octadecyl end capped 6 mL, 500 mg cartridges from Supelco (Bellefonte, PA).

Samples were obtained from the USEPA Region 4 laboratory as part of ongoing monitoring of the Alabama site. The samples were collected in 1 L glass bottles. The samples were maintained at 4 ^{o}C until they were extracted and analyzed.

A water sample of 1.0 L was acidified with sulfuric acid to obtain a pH < 2 prior to solid-phase extraction. The SPE cartridges were conditioned by rinsing with 12 mL pH-adjusted, deionized water followed by 12 mL of methanol and a final rinse of 12 mL of pH-adjusted, deionized water. The solvents were pulled through the SPE cartridges by means of a gentle vacuum controlled by the manifold. The cartridges were not permitted to run dry throughout the conditioning and sample application procedure. To decrease sample work-up time, four cartridges were utilized in parallel for each liter of water analyzed. The water sample was flowed through the cartridge at a flow rate of approximately 6 mL/min. After the water sample was extracted, the cartridge was allowed to dry for 2 hours by pulling air through the cartridges. All of the sorbed compounds were eluted with 2 mL MTBE followed by 4 mL methanol. The cartridges at this point were devoid of the yellow color that had been obtained from the water sample and the MTBE/methanol extract was moderately yellow. The extracts were then combined and the sample volume was reduced to 10 mL by evaporating the solvent under a stream of dry nitrogen.

The derivatization was performed on these preconcentrated samples at 50 °C for 50 minutes. Approximately 1 mL of reagent grade concentrated sulfuric acid was added to produce a 10% by volume sulfuric acid/methanol solution, and the solution was thoroughly mixed with the use of a vortex mixer. This solution was then placed in a temperature-equilibrated constant temperature bath thermostated at 50 °C. Subsequent to the derivatization, 10 mL of 10% sodium sulfate in water was added to the sample to quench the reaction, and the sample was again mixed with the use of a vortex mixer. The sample was then extracted with 2 mL MTBE, mixed, and the MTBE layer removed.

The water layer was then extracted three additional times with 2 mL MTBE and the removed MTBE layers were combined. The 8 mL of MTBE extract was then concentrated under a stream of dry nitrogen to obtain a sample volume of 1 mL. Thus, the concentration factor due to the SPE and derivatization was 1000X.

An additional clean up step was employed in an attempt to remove some of the more polar river water constituents from the analytes. The derivatized sample was evaporated to dryness under a stream of dry nitrogen. The sample was then reconstituted in hexane and passed through a Waters silica gel column, and then eluted with hexane. It was found that the yellow (presumably polar) compounds were deposited on the silica column and did not elute as the column was flushed with hexane. The collected hexane was then concentrated to 300 µL and analyzed on the GC-MS in SIM mode as above. Some loss of the methyl esters was noted following this supplemental clean-up step, and so it was not utilized in the study.

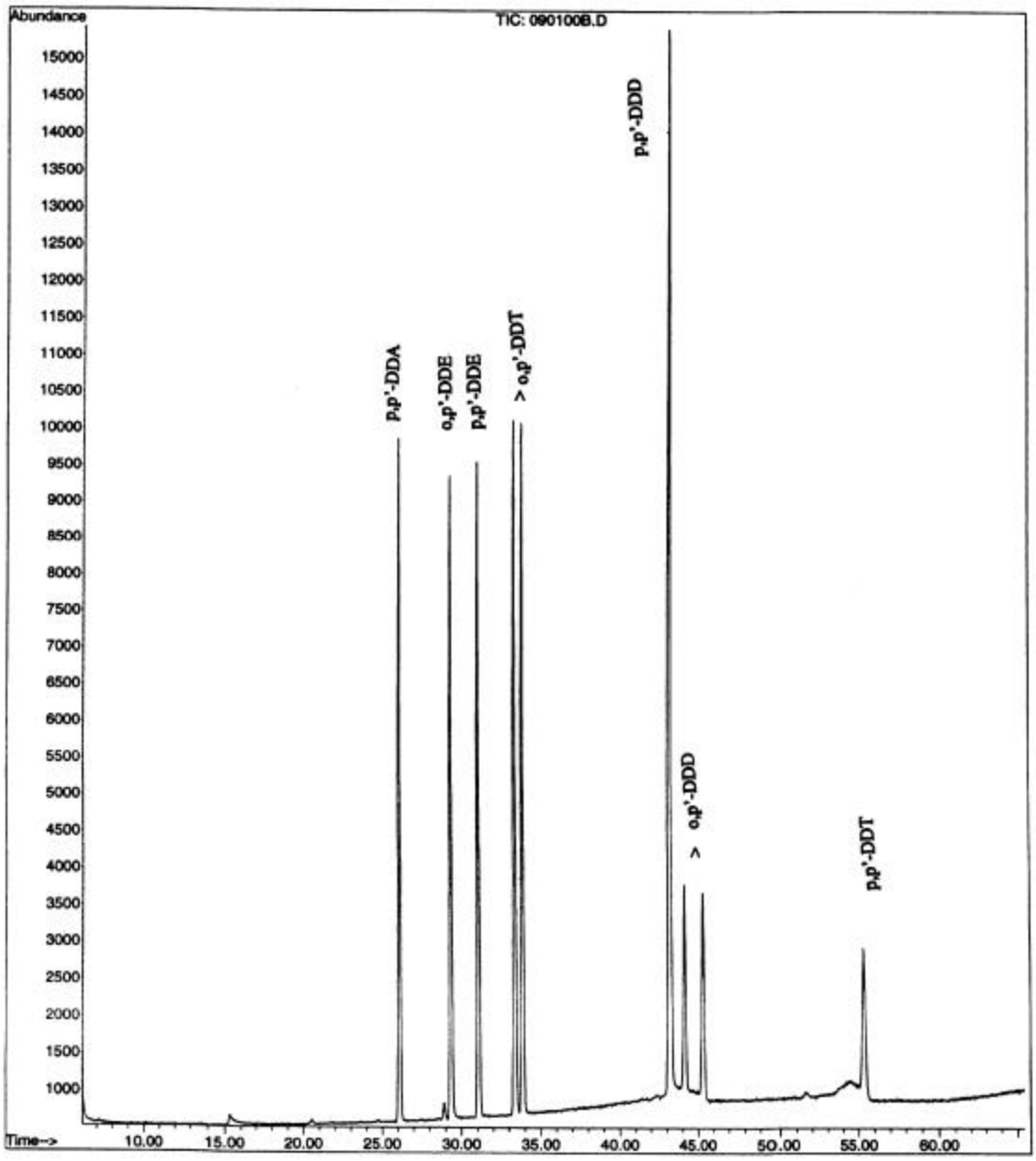
Mass spectrometric measurements were performed using a Hewlett-Packard HP5973 MSD with a HP 6890 gas chromatograph, equipped with a 30 m x 0.25 mm I.D. x 25 µm film thickness BGB-172 column (20% tert-butyldimethyl-silylated-â-cyclodextrin in 15% phenyl/ 85% dimethyl polysiloxane) (BGB Analytik AG, Anwil, Switzerland) and a 5 m x 0.25 mm I.D. bare silica retention gap pre-column (Supelco). The column temperature was initially at 180 °C with a ramp rate of 0.75 °C/min to a final temperature of 220 °C. The column was held at 220 °C for 20 minutes and then ramped at 1°C/min to a final temperature of 230 °C, which was held for 30 minutes. The inlet was set at 250 °C and 1 µL of sample was injected in splitless mode. The MSD transfer line was set to 230°C. High purity helium 99.999% was the carrier gas at a flow rate of 1.0 mL/min, corresponding to a linear velocity of 35 cm/s at 180 °C. By analyzing standards, the ions for the analytes of interest were selected to be monitored in SIM mode. Following dynamic mass calibration, the ions selected were m/z 165, 199,

235, 237, 294.1 , and 296.1 with dwell times of 75 ms/ion. It was found that this gave a detection limit of 5 µg/L in the injected sample, or 5 ng/L in the original water sample for *p,p'*-DDA. No detection limit for *o,p'*-DDA was determined as the standard was not available, although it is not expected to vary greatly from *p,p'*-DDA due to their structural similarity. An enantiomerically resolved chromatogram of the standard mix of DDT, DDD, DDE, and *p,p'*-DDA is shown in Figure 5-2.

The identification of DDA in environmental samples by gas chromatography is a difficult endeavor. The compound must first be derivatized to cause sufficient volatility for analysis. Furthermore, standard detection methods such as flame ionization detection or electron capture detection, are not highly sensitive to DDA, especially when the compound is found in a complex matrix such as river water. GC-MS holds promise for the analysis of DDA but full scan mode is also not sufficiently sensitive to DDA. By utilizing selected ion monitoring (SIM), the fragment ions for the compound of interest are selected for with many of the interferences going unnoticed as the m/z ratio of their characteristic ions are not monitored. An additional benefit of SIM mode determination is that the sensitivity is greatly enhanced, for two reasons. During a typical "full scan" mode experiment the mass filter is swept over the entire range in an effort to gain a snapshot of the entire mass spectrum for that compound as it elutes. Thus, the time spent on any individual ion is negligible. By utilizing SIM mode, the ions can be monitored for a longer duration, allowing collection of more signal for the ion(s) of interest. The second reason is mainly due to the current technological limits of chiral GC columns. Chiral GC columns exhibit more column bleed than non-chiral columns especially at elevated temperatures, and thus the SIM mode allows the majority of the column bleed to go undetected.

While SIM mode provides a marked increase in sensitivity, it suffers from a significant loss of information when compared to full scan methods. In order to gain the

Figure 5-2 A GC/MS total ion chromatogram collected in SIM mode for the DDT analogues monitored in this study. All standards were prepared in methanol. The concentrations of *p,p'*-DDA, *o,p'*-DDE, and *p,p'*-DDE are 0.9 µg/L. The concentration of *o,p'*-DDD and *p,p'*-DDD are 2.0 µg/L. The concentration of *o,p'*-DDT is 0.4 µg/L and *p,p'*-DDT is 0.2 µg/L. GC/MS conditions and program as specified in the text.


Abundance
TIC: 0901008.D
15000
14500
14000
13500
13000
12500
12000
11500
11000
10500
10000
9500
9000
8500
8000
7500
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
p,p'-DDA
o,p'-DDE
p,p'-DDE
> o,p'-DDT
p,p'-DDD
o,p'-DDD
>
p,p'-DDT
Time-->
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00

benefits of SIM mode with the spectral information and identifying capability of full scan mode, several ions must be monitored. If the monitored ions are chosen properly then this approach gives sufficient sensitivity and a reasonable amount of confirmatory information.

Judicious choice of ions also provides for detection of potential interferences. By running a sample in SIM mode and examining the limited mass spectrum produced at the retention time for the analyte (determined by standards), the identity of the compound can be determined. Interfering compounds will typically have different ratios of the characteristic ions. This approach is utilized here. It is noteworthy that the potential interferences in the river water are almost completely disregarded in SIM mode. The only interferences expected that should have similar mass spectra to DDA are the parent compounds DDT, DDD, and their respective isomers. Fortunately the chromatographic conditions can be selected such that the interfering residues of the DDX family are adequately separated from the DDA analyte peak.

SITE BACKGROUND

The Triana/Tennessee River site is approximately 5 miles southwest of Huntsville, Alabama. The site contains an eleven mile stretch of two tributaries to the Tennessee River, the Huntsville Spring Branch and Indian Creek. From 1947 to 1970 Olin Corporation operated a DDT manufacturing plant and discharged wastewater into Huntsville Spring Branch[26]. Fish became heavily contaminated from the estimated 408.8 tons of contaminated sediment.

The remedial actions consisted of diverting the stream flow around the contaminated sediments, removal of contaminated sediments, burying contaminated sediments in place, and forming a new channel for water flow. Region 4 of the

U.S.EPA is involved as part of ongoing monitoring of DDT levels in several species of fish and in the surface waters.

RESULTS AND DISCUSSION

River water samples showed differing degrees of degradation for DDT. Samples ranged from still showing DDT to showing mainly DDA residues. It should be noted that none of these samples was above current regulatory levels for DDT residues. The principal observed residue was *p,p'*-DDA. A chromatogram for a Huntsville Spring Branch water sample is shown in Figure 5-3. Note the slight resolution of *o,p'*-DDA at 19.3 and 19.5 minutes and the peak at 27 minutes corresponding to *p,p'*-DDA. The limited mass spectra for both *o,p'*- and *p,p'*-DDA are shown in Figure 5-4. The large peak at 30 minutes is a river water constituent and does not have the characteristic spectrum of the DDX family. The enantiomers exhibit an enantiomeric ratio of 1.03, indicating no extensive enantioselective differentiation of DDA. The *o,p'*-DDA residues in the river water were determined to exist at a concentration of 41 ng/L and the *p,p'*-DDA residue was determined to be 135 ng/L. The concentrations for both isomers were determined by comparisons to known standards of *p,p'*-DDA. The calibration curve for *p,p'*-DDA is shown in Figure 5-5. Since no standard for *o,p'*-DDA was available and the structures of the analogues are so similar, the *p,p'*-DDA calibration was utilized for that component. The isomeric ratio of *o,p'*-DDA to total DDA residues was found to be 0.23, which is in excellent agreement with the original ratio of the isomers of DDT in technical DDT[19]. Furthermore, this confirms Heberer's conclusion that there is little or no stereoselectivity in the formation of DDA from DDT[16]. The lack of enantiomeric preference argues for one of two possibilities. Either *o,p'*-DDA is formed from an achiral precursor such as *o,p'*-DDE, or it is formed through a chiral precursor that exists as a racemate such as *o,p'*-DDD. If the precursor is

Figure 5-3 GC/MS total ion chromatogram for a water sample from the Triana/Tennessee River site. The large peak at 30 minutes is a river water constituent, not of the DDX family. The small peaks at 40- 50 minutes are DDD and DDT peaks.

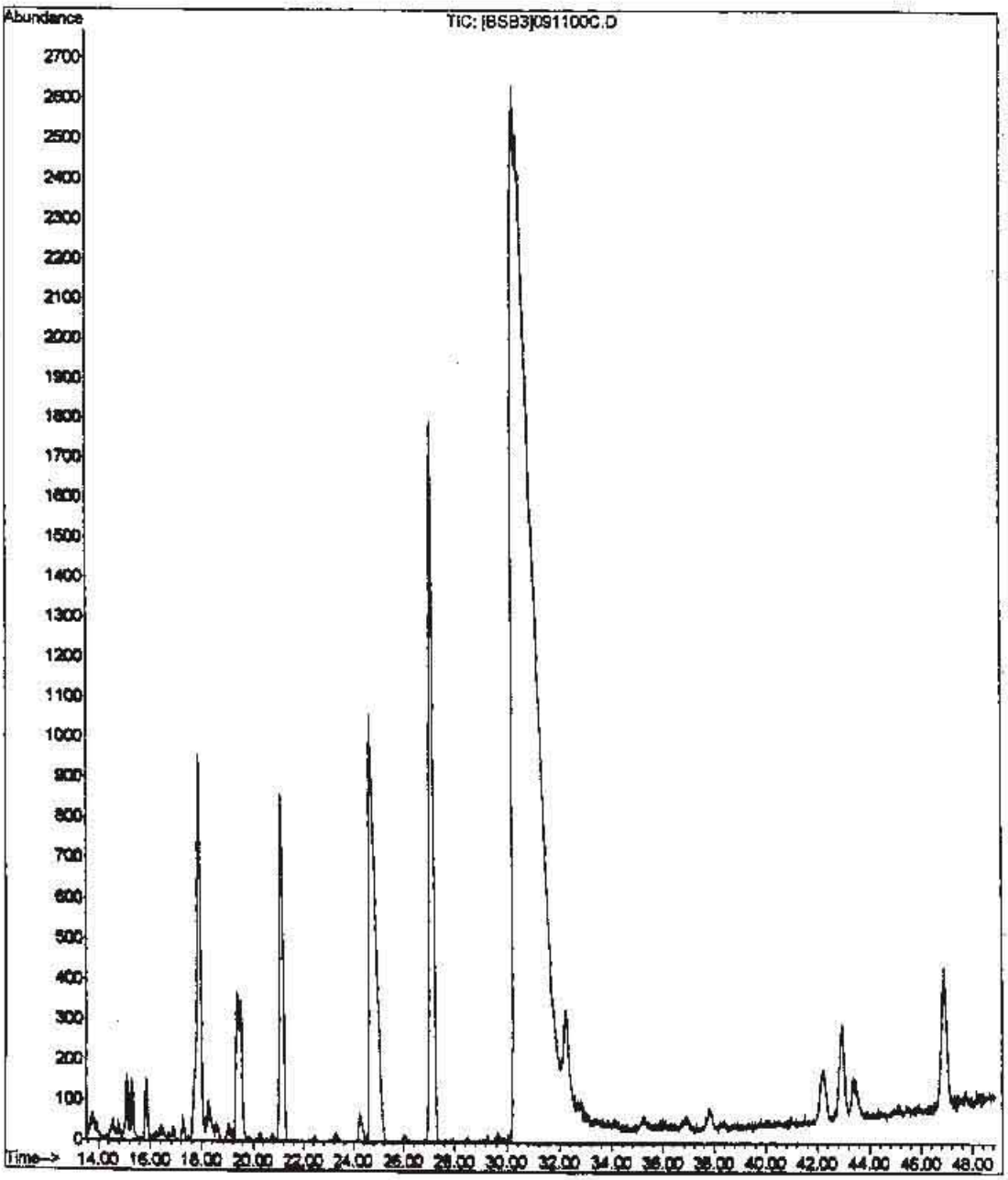
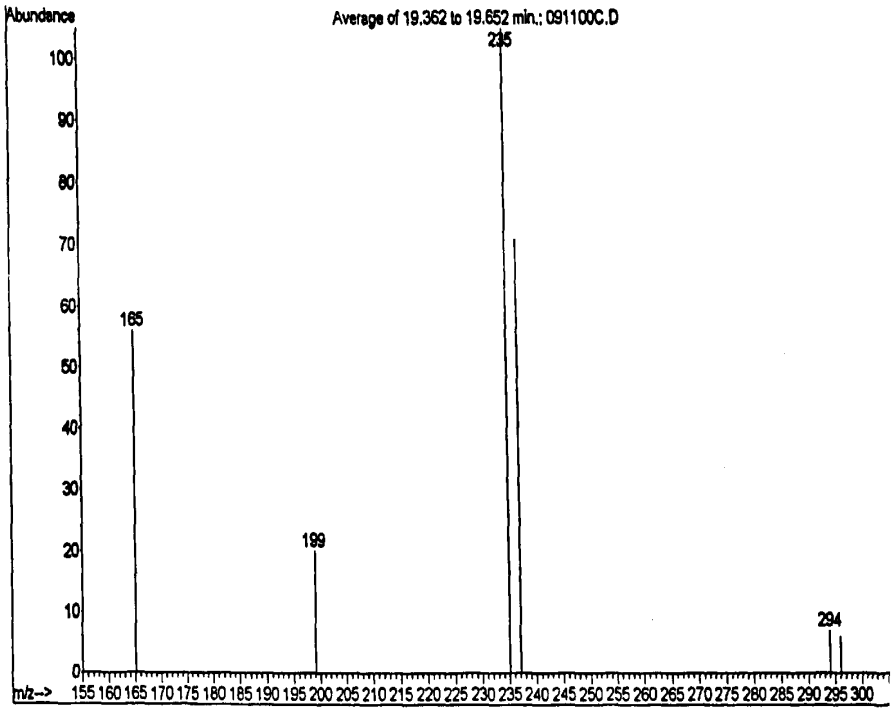

Abundance
TIC: [BSB3]091100C.D
2700
2600
2500
2400
2300
2200
2100
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Time-->
14.00 16.00 18.00 20.00 22.00 24.00 26.00 28.00 30.00 32.00 34.00 36.00 38.00 40.00 42.00 44.00 46.00 48.00

Figure 5-4 The limited mass spectra for the peaks at 19.3 to 19.5, and 27 min. From the spectra and retention times the peaks were identified as the methyl esters of *o,p'*- DDA and *p,p'*-DDA.


Abundance
Average of 19.362 to 19.652 min.: 091100C.D
235
165
199
294
m/z-->
155 160 165 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300

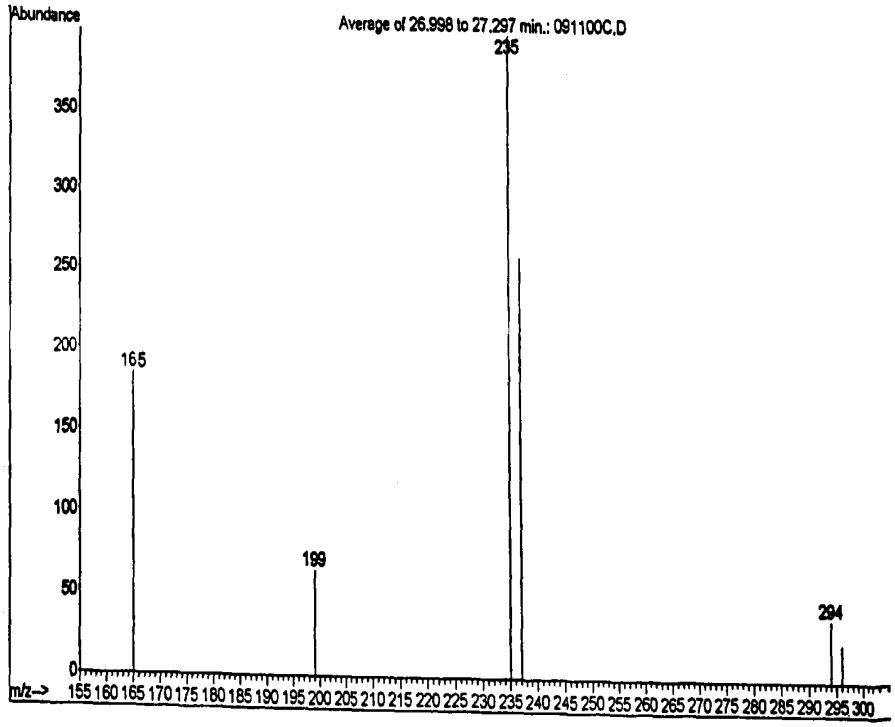
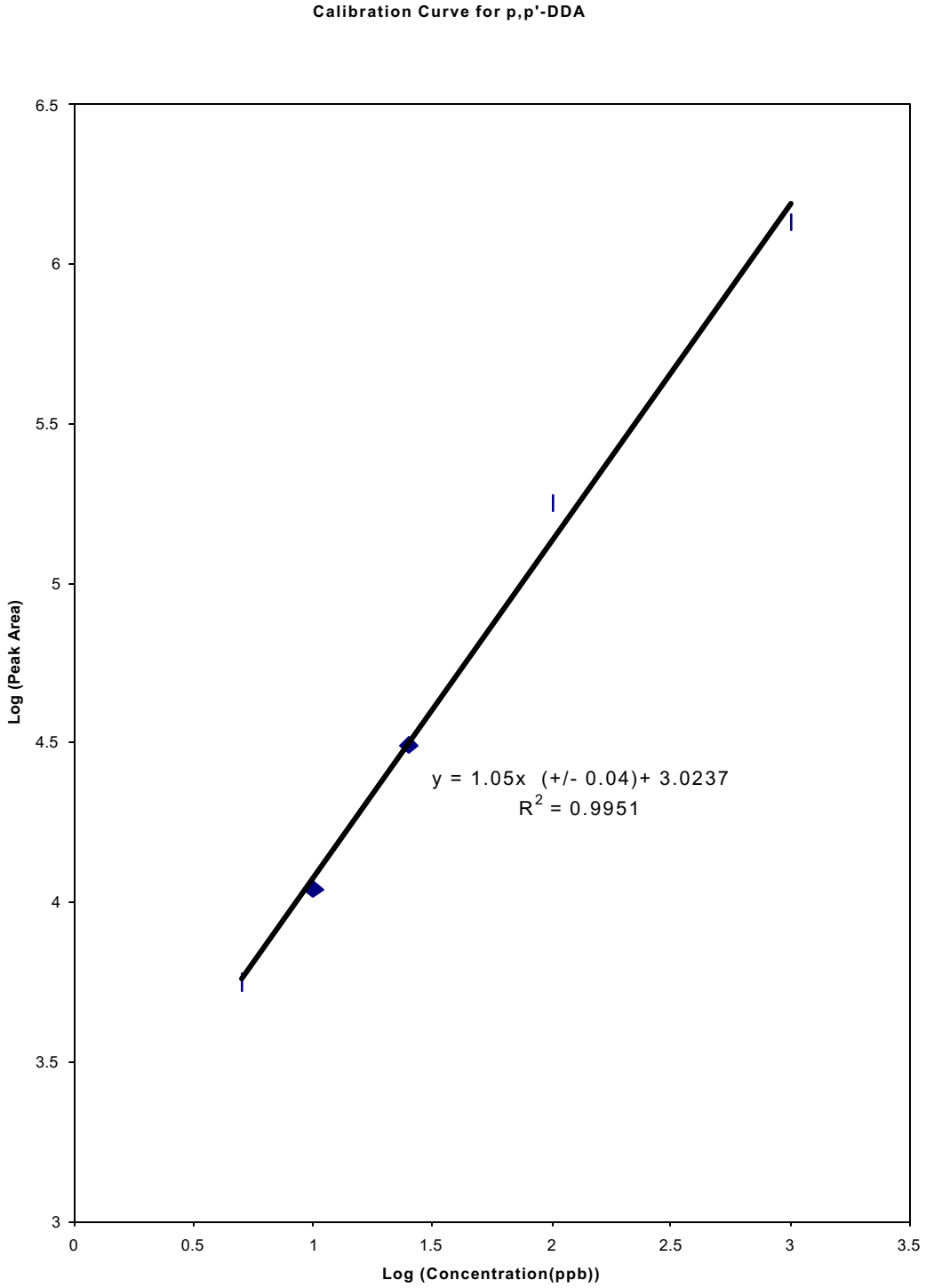

Abundance
Average of 26.998 to 27.297 min.: 091100C.D
235
165
199
294
m/z-->
155 160 165 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300

Figure 5-5 The calibration curve for *p,p'*-DDA from the GC/MS TIC. This calibration curve was also used to estimate the concentration of *o,p'*-DDA.

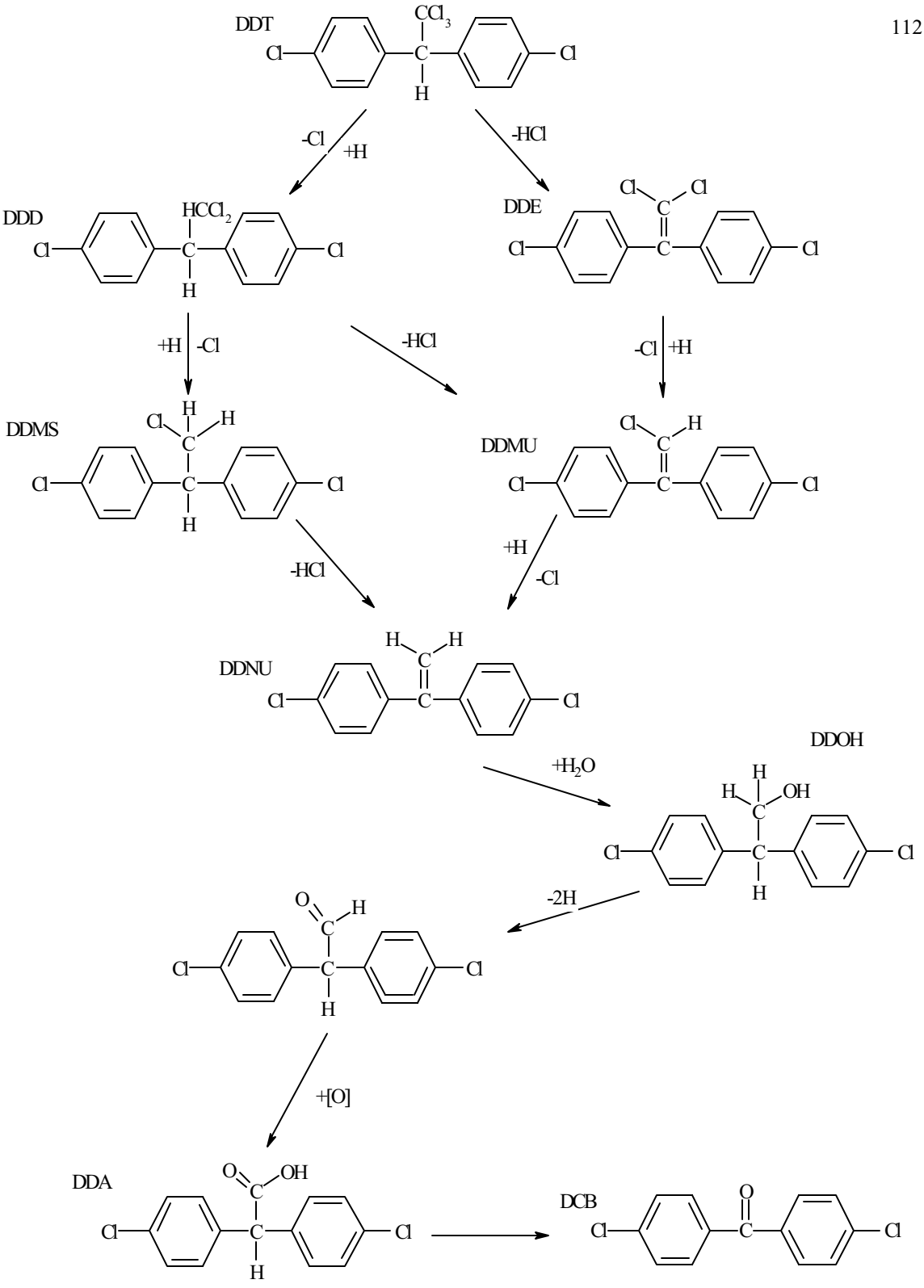

Calibration Curve for p,p'-DDA
6.5
6
5.5
5
4.5
4
3.5
3
Log (Peak Area)
0
0.5
1
1.5
2
2.5
3
3.5
Log (Concentration(ppb))
y = 1.05x (+/- 0.04)+ 3.0237
R2 = 0.9951

o,p'- DDD then the enantiomeric ratio of *o,p'*-DDA should follow the enantiomeric ratio of the precursor, which is not the case in this study as *o,p'*-DDD has an enantiomeric ratio of 2.2.

This approach enlistlisting enantiomeric ratios to assist in the determination of the origin of environmental contaminants has been used previously and may help to elucidate the origins of DDA residues[27-30]. It has also been suggested that the DDA residues are derived from DDD, especially because DDD is found to be the second most prevalent analogue in the water and the most prevalent in the sediment[16]. Figure 5-6 illustrates a pathway to DDA proposed by Heberer[16], based upon the known anaerobic metabolism of DDT[31]. Although this may be the case, some caution should be exercised because the water concentrations of the pollutants are greatly affected by the solubility and hydrophobicity of the analogues.

If DDE is the precursor of DDA, then it will tend to remain sediment bound until it is transformed into DDA, at which point it will be quickly released from the sediment. This may explain the lower concentration of DDE in the sediment, and the lack of any significant chiral selectivity for *o,p'*-DDA. The formation of *o,p'*-DDA from *o,p'*-DDE can also be mediated by abiotic reductants, in which case no enantioselective formation of *o,p'*-DDA is expected. If the reduction is mediated by enzymes, then the some enantioselective formation of o,p'-DDA is expected, regardless of whether it originates from *o,p'*-DDD or *o,p'*-DDE. The formation of DDMU from DDE was reportedly three orders of magnitude faster than the formation of DDMU from DDD[15]. Furthermore, Wedemeyer reported[32] the formation of DBP from DDMU, and DDA is the oxidation product of DBP. So the pathway appears to go from DDE to DDMU to DBP. In this case, oxidation of *o,p'*-DBP to *o,p'*-DDA is expected to produce the racemate.

Figure 5-6 Proposed pathway for the formation of DDA[16].


DDT
-Cl
+H
-HCl
DDD
DDE
+H
-Cl
-HCl
-Cl
+H
DDMS
DDMU
-HCl
+H
-Cl
DDNU
+H2O
DDOH
-2H
+[O]
DDA
DCB

Technical DDT is greater than 75% *p,p'*-DDT, and little deviation in the isomeric ratio is expected when the *o,p'*- and *p,p'*- DDT present in technical DDT are degraded to their respective products, unless the microbial populations responsible for the transformation greatly favor one isomer over the other. Since the site has not been in use for decades, it should be expected that the microbial population is well adapted to the transformation of all of the isomers and even enantiomers. Thus, any chiral selectivity of the degradation processes may have already run their course, although this is a less attractive interpretation. This long delay between pollution and analysis is also the case at Teltowkanal where Heberer noted that the ratio of *o,p'*-DDA to *p,p'*-DDA metabolites reflected that of the technical product. Furthermore it was noted that DDA was roughly five times more concentrated than DDD, the second most concentrated metabolite detected at Teltowkanal[16].

Heberer has shown that the persistence of DDT manifests itself as a buildup of DDA. The hydrophobic DDT is sediment bound and slowly reduced under anaerobic conditions. Ultimately as the DDT is transformed into more polar products, it is released from the sediment and begins to leach through the subsurface soil barrier that separates the surface waters from groundwater[16]. This study concurs that DDA was the most prevalent metabolite in the water, and a significant change in the isomeric ratio was not noted. Thus from this study and Heberer's studies, the anaerobic degradation of DDT to DDD or DDE is paramount to the formation of DDA.

This study carries the investigation a step further and examines the enantiomeric occurrence of the *o,p'*-DDA residues. By examining the enantiomeric ratio of o,p-DDA, some insight into the precursor may be gained. Furthermore this is but one example where selective environmental degradation both of enantiomers and of isomers may occur. Clearly, further research needs to be done in the area of chiral

environmental degradation as it relates to pesticides that are applied as mixtures of enantiomers or isomers.

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CHAPTER 6

DETERMINATION OF THE OXIDATION STATE OF CYTOCHROME C BY ELECTROSPRAY IONIZATION FOURIER TRANSFORM ION CYCLOTRON RESONANCE MASS SPECTROMETRY WITH CHEMICAL AND ELECTROCHEMICAL SAMPLE PREPARATION

INTRODUCTION

The electrochemistry of proteins and more specifically metalloproteins has been studied extensively. Although a great deal of work has been completed in the area of indirect metalloprotein electrochemistry with small molecules as mediators, there are a number of complicating factors that make redox proteins less than ideal candidates for direct electrochemical studies. Heterogeneous electron transfer rates to proteins are generally much slower than in small inorganic complexes, because the protein active center is generally imbedded in the interior of the protein and thus the distance from the electrode surface to the active site is larger in the case of proteins. Additionally, proteins are quite large and are slow to diffuse to the electrode surface. Furthermore, once at the electrode surface, proteins tend to adsorb strongly, blocking the surface and thus electron transfer to protein molecules in solution. Perhaps the greatest challenge of direct electrochemistry of proteins at bare electrode surfaces is the nature of the protein-electrode interaction. By examining the electron transfer *between* protein molecules, some insight into the protein-electrode interaction may be gained[1]. For such protein-protein interactions there is a commonly accepted requirement that fast and reversible formation of a donor-precursor complex is necessary. Due to the lack of functionality, the bare electrode surface possesses few of the molecular features of this precursor complex; thus the protein is not in a preferred state for redox reactions.

The use of promoters, small molecules on the electrode surface to prevent sorption of protein, has proven effective in producing electrode surfaces that are more amenable to electron transfer to the active site of the protein[2, 3]. Even without surface modification, it is possible to perform some electrochemical processes at bare metal electrodes. Irreversible reduction of cytochrome *c* has been demonstrated on platinum[4], although the mechanism is not well known. It has been suggested that this reduction is happening *via* electrogenerated hydrogen[5]. Complementary work has been done in the area of cytochrome *c* reacting directly on indium doped tin oxide electrodes. Yeh and Kuwana were the first to report success with these electrodes[6]. Their findings of direct, reversible electron transfer of cytochrome *c* by cyclic voltammetry and differential pulse voltammetry were quite surprising. Further research into the nature of these electrode surfaces has demonstrated that the amphoteric nature of surface hydroxyl groups prevents protein adsorption under carefully controlled experimental conditions such as ionic strength and pH[7-10] for suitably purified protein samples. This is in direct contrast to the strong sorption of cytochrome *c* on platinum electrodes, which leads to slow, irreversible electrochemistry in those systems. Although the electrochemical behavior of cytochrome *c* is less than ideal at bare metal electrodes, it does not preclude the use of bare metal electrodes for electrochemical reduction of cytochrome *c*. The limitations of the bare metal electrodes, namely analyte sorption and poor electron transfer, can be tolerated in some applications, especially in cases where rapid electron transfer is not required.

The first investigation of the oxidation states of myoglobin and cytochrome *c* was conducted using ESI with collision induced dissociation (CID)[11]. The entire protein was ionized by ESI and the heme group subsequently ejected from the protein backbone. The mass of the heme was determined and the oxidation state was derived. With advances in high resolution mass spectrometry, it is no longer necessary to

dissociate the active site from the protein backbone to determine the mass accurately[12-14]. Isotopic resolution of 13 kDa proteins is routinely obtained with high field electrospray ICR mass spectrometers. In fact, much work has been completed examining the oxidation state of metalloproteins containing heme and iron-sulfur clusters[15]. He et al. have reported[16] unequivocal assignment of the oxidation state of metalloproteins in the oxidized form. This work reports the determination of the oxidation state of the iron in cytochrome *c*. Cytochrome *c* was reduced chemically and the reducing agent was subsequently removed to make the sample suitable for mass spectral analysis. Additionally, the sample was reduced electrochemically and the laborious protein cleanup procedure was circumvented.

During the electrospray process many processes occur simultaneously within the Taylor cone, and sample transformation or other complications may arise. The Taylor cone is the region formed by the sample spraying from the inlet. Due to electrostatic repulsion, it is roughly in the shape of a cone. Van Berkel and co-workers have determined[17,18] that a myriad of electrochemical processes occurs during the electrospray process. Whether or not the oxidation state and conformation that is determined following the electrospray process and subsequent mass spectral analysis is indicative of the analyte in bulk solution prior to analysis is a matter open for debate. McLafferty et al. addressed[14] conformational changes following the creation of gas-phase ions. Dharmasiri and Smith have studied the conformational changes associated with oxidation state changes of the prosthetic group[19]. Van Berkel and coworkers have extensively studied[17, 18, 20, 21] the electrochemical processes that occur during electrospray ionization and they have shown that the splitting of water is the dominant electrochemical reaction in the electrospray process. It has also been demonstrated that ferrocene and polyaniline are readily oxidized during the electrospray process[17]. Furthermore, Van Berkel et al. have concluded that for a proper analyte and under

suitable electrospray conditions, complete electrochemical conversion can occur[22]. Marshall and He concluded[16] that oxygen was present during the electrospray process because metalloprotein samples were introduced as reduced species and the subsequent mass spectra determined the samples to be oxidized. Although these conclusions are almost certainly true, they do not preclude the determination of reduced metalloprotein samples by electrospray mass spectrometry.

Should a sample that has been reduced subsequently yield a signal that corresponds to oxidized metalloprotein, then the oxidant is most likely atmospheric oxygen. A reduced sample from which oxygen has been rigorously excluded should yield a signal as a reduced metalloprotein, unless electrochemical or other redox processes in the electrospray cone are responsible for the oxidation. This electrochemical conversion is unlikely for several reasons, namely the lack of an ideal electrode for metalloprotein transformation, the lack of a mediator for homogeneous electron transfer, and the protected nature of the metalloprotein prosthetic group[23]. Extensive work has been done on the electrochemical behavior of metalloproteins such as cytochrome *c*, and facile electrochemical transformation is generally not noted without the addition of a promoter molecule on the surface of the electrode or a mediator in solution to shuttle electrons between the analyte and the electrode[7, 9, 24, 25]. This work reports the preservation of the oxidation state of several metalloproteins throughout the electrospray process demonstrating that electrospray ionization can successfully preserve the solution oxidation state of metalloproteins.

EXPERIMENTAL

The mass spectra were obtained using a Bruker (Billerica, Massachusetts) BioApex FTICR mass spectrometer with a 7 Tesla magnet. The Analytica (Branford, Connecticut) ESI source used with this instrument was modified by replacing the glass capillary interface with a heated metal capillary, which was maintained at 150 °C for all

of the experiments. The samples were ionized by nanoelectrospray ionization with a maximum flow rate of 100 nL per minute. The nanoelectrospray tips were fabricated in house using 100 μm inner diameter fused silica capillary tubing (Polymicro Technologies, Phoenix, Arizona). The tips were pulled by heating with a hand held micro-torch (Microflame, Inc., Minnetonka, Minnesota) while applying a constant force of 0.112 N. Spray voltages ranged between 600 V and 1200 V (-600 V and -1200 V for negative ion mode) for the experiments. In this range, the spray voltage does not affect the observed oxidation state of the metalloproteins. The voltage was applied to the syringe needle that delivered the sample to the spray emitter, forming a liquid electrical junction[26].

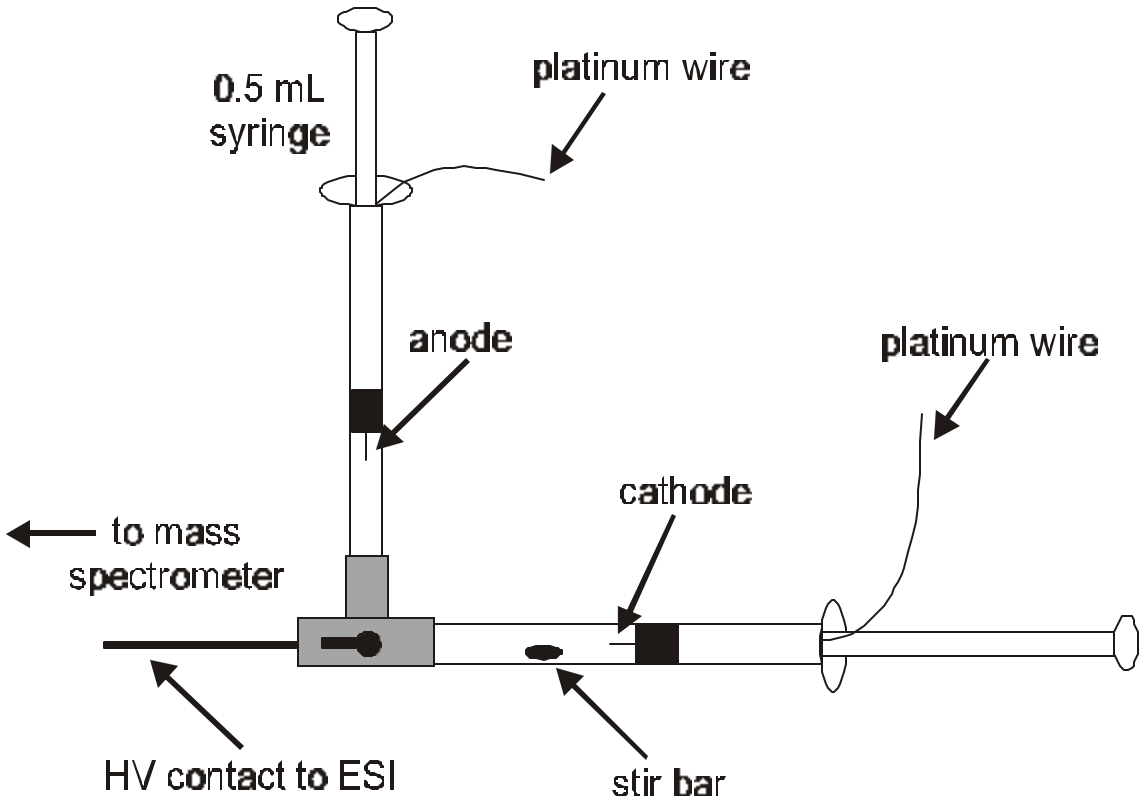
Proteins were produced by overexpression. Their production and purification have been described previously[12, 27]. Cytochrome *c* was purchased from Sigma. Protein samples were introduced at a concentration of 20 μM in water after removal of the reducing agent (see below) for chemical reduction. For electrochemical reduction (see below), cytochrome *c* was reduced (20 μM) in 5 mM ammonium acetate. Storage and trapping potentials as well as a description of the data analysis using the chi-squared test have also been described previously[12]. Exclusion of oxygen during sample handling after reducing the protein samples was imperative in obtaining mass spectra for the reduced samples. Samples that were not isolated from ambient air, and those that were not electrosprayed immediately after sample preparation were detected as oxidized protein. Only those samples which were secluded from oxygen and electrosprayed promptly after reduction were detected in the reduced form.

Chemical Reduction: Metalloprotein solutions (20 μM) were prepared for ESI by dissolving the proteins in water prior to reduction. Sodium dithionite was used to reduce all of the samples chemically. The samples were placed in a glove bag with a

microcentrifuge and degassed under a nitrogen atmosphere before reducing the proteins. The reducing agent was added to the metalloproteins in a ten fold excess and the color change was monitored for all of the samples. After chemical reduction, the reducing agent was removed prior to analysis by ultrafiltration using a microcentrifuge tube with a 5 kDa cutoff integral membrane filter (Millipore Corporation, Bedford, Massachusetts), using approximately 500 µL of degassed, 20 µM ammonium acetate solution. The protein was then resuspended in water resulting in a final concentration of 20 µM. Typically, the chemical reduction step including resuspension of the protein in water lasted 2.5 to 3 hours. The reduced protein (monitored visually) was transferred to a gas tight syringe and introduced to the mass spectrometer via nanoelectrospray ionization.

Electrochemical Reduction: The electrochemical cell was fabricated in our laboratory using two 1 mL plastic syringes connected via a T-valve, shown in Figure 6-1. The third leg of the T-valve was connected to the electrospray emitter tip so that the contents of either syringe could be selected for analysis. When the valve was set in the "off" position, the solution in the two syringes made electrical contact. The electrodes for the cathode and the anode were platinum wires that were forced through the plunger end of each syringe. This maintained a separation of the "reduction" end of the cell and the "oxidation" end of the cell and prevented mixing of oxidized and reduced protein. A micro stir bar was placed in the reduction end of the cell to aid in mass transfer to the electrode in that region. Cytochrome *c* was prepared in a solution of 2 mM ammonium acetate at a concentration of 20 µM. About 0.5 mL of the brown (oxidized) solution was transferred to the electrochemical cell. The resistance of the cell was measured at approximately 0.5 MΩ between the reducing and oxidizing electrodes. Current was maintained at 30 µA for the reduction of cytochrome *c*. This corresponded to a voltage

Figure 6-1 The electrochemical cell is illustrated and shows the arrangement of the oxidation and reduction ends of the cell connected by a T-valve. The platinum electrodes are labeled in the figure.


0.5 mL
syringe
platinum wire
anode
platinum wire
cathode
to mass
spectrometer
HV contact to ESI
stir bar

of 13.3 V across the cell, although most of this was dissipated as iR drop between the electrodes. After approximately 30 minutes, the solution in the reduction end of the capillary changed from brown to pink, indicating that the protein was reduced. The T-valve was then switched so that the oxidation end of the cell was closed. The reducing cell was placed on a syringe pump and its solution was delivered to the detectors. The voltage for electrospray was applied to the syringe needle, which forms a liquid electrical junction with the nanoelectrospray tip.

RESULTS AND DISCUSSION

ESI-FTICR mass spectrometry is sensitive to the changes in mass associated with the metalloprotein oxidation state or the disulfide bonds present within a protein. The key to the ability of ESI-FTICR to determine such small mass changes lies in the ability of the instrument to produce isotopically-resolved mass spectra, from which a monoisotopic molecular weight can be assigned. The monoisotopic molecular weight is the sum of the lowest molecular weight isotopes in the molecular formula, typically ^{12}C, ^{1}H, ^{14}N, ^{16}O, and ^{32}S. For metalloproteins an adjustment must be made to account for the most abundant isotope of the metal or metals present in the metalloprotein. Because the natural abundances of ^{56}Fe and ^{54}Fe are 91.8 and 5.82% respectively, ^{56}Fe is used in the calculation instead of ^{54}Fe. Additionally, the isotope peak for ^{54}Fe is not usually seen in the mass spectrum.

If the traditional method of molecular weight calculation of a protein is applied to a metalloprotein, then the calculation will result in molecular weights that vary depending upon the oxidation state of the metal center. This is because the calculation assumes that all of the ionizable sites in the amino acid sequence are neutral, but takes no account of the fact that metals exist in non-zero oxidation states, and differing

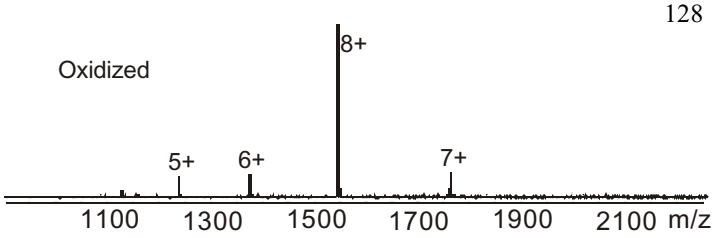
numbers of protons are required to achieve charge neutrality for the reduced and oxidized form of the metal center.

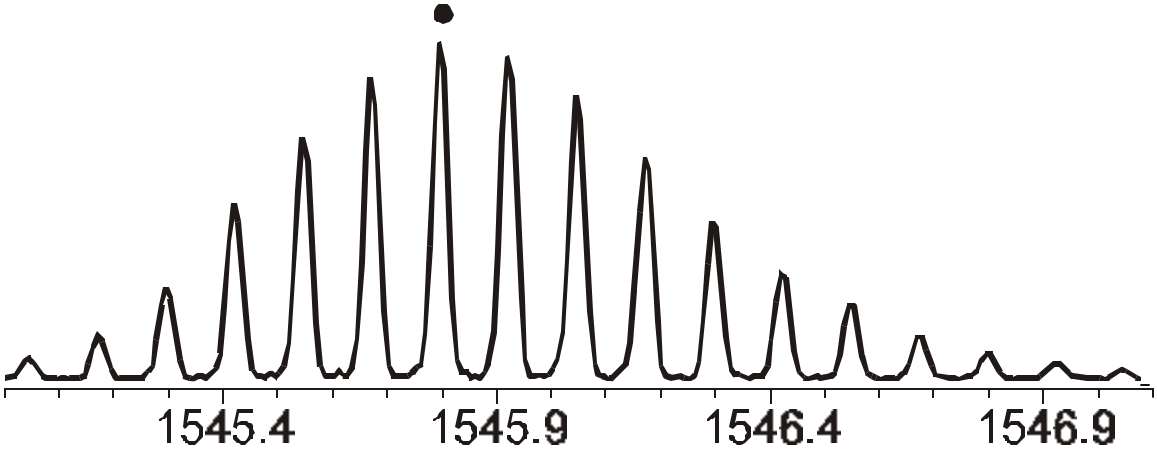
The "apparent mass" can be defined as the mass that is derived from the data with the assumption that all of the charge of an ion is due to excess of protons in positive mode or a deficit of protons in negative mode. This apparent mass differs from the actual mass by an amount corresponding to the charge present on the metal center. A comparison of the calculated and apparent mass allows the oxidation state to be determined from the difference between these two masses. This procedure is more completely explained elsewhere[12]. A brief illustration of the calculation is shown at the end of this chapter.

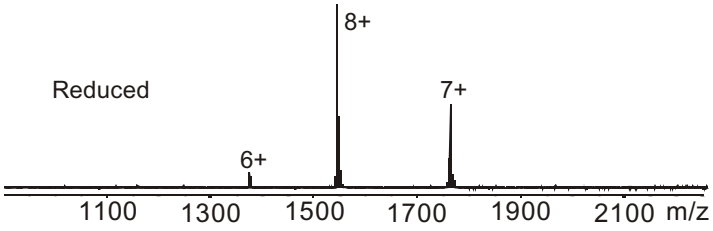
The chemical reduction of cytochrome *c* was monitored by observing the color change following the addition of sodium dithionite. The mass spectra for oxidized and reduced cytochrome *c* with a charge of +8 in positive ion mode are shown in Figure 6-2. There is clearly a shift of 1 mass unit corresponding to the gain of one H^+ for the reduced cytochrome *c*. To determine whether or not the electrospray process was affecting the oxidation state of the metalloprotein, both oxidized and reduced cytochrome *c* were run again in negative ion mode. Expansions of the -6 charge state are shown in Figure 6-3. Again the gain of one proton is noted for the reduced sample. Furthermore the oxidation state was not perturbed with positive or negative mode ionization. After determining that the data obtained from ESI-FTICR accurately reflected the oxidation state of chemically reduced cytochrome *c* in both positive and negative mode, an effort was made to circumvent the chemical reduction step with an electrochemical sample preparation method.

The marriage of bulk electrolysis and mass spectrometry is a difficult endeavor. In general terms, electrochemical sample preparation of this type requires low impedance solutions, which is generally accomplished by the addition of a conductive

Figure 6-2 The positive ion mode ESI-FTICR mass spectra of the +8 charge state for both oxidized (top) and chemically reduced (bottom) cytochrome *c* are shown. The centroid of each isotope distribution is marked with "●" and illustrates the shift in the distribution that corresponds to the change in the oxidation state of the metalloprotein.


Oxidized
8+
5+
6+
7+
1100
1300
1500
1700
1900
2100
m/z


1545.4
1545.9
1546.4
1546.9


Reduced
8+
7+
6+
1100
1300
1500
1700
1900
2100
m/z

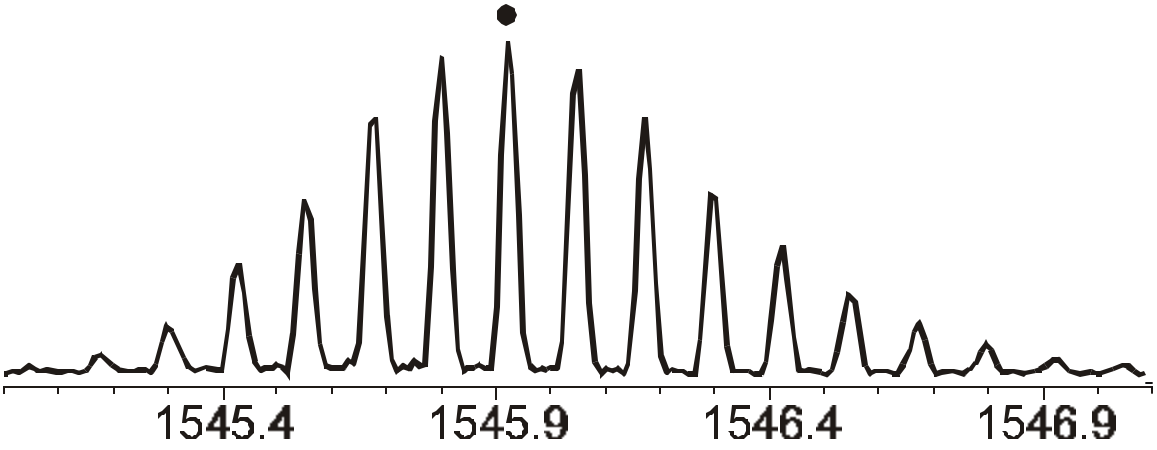
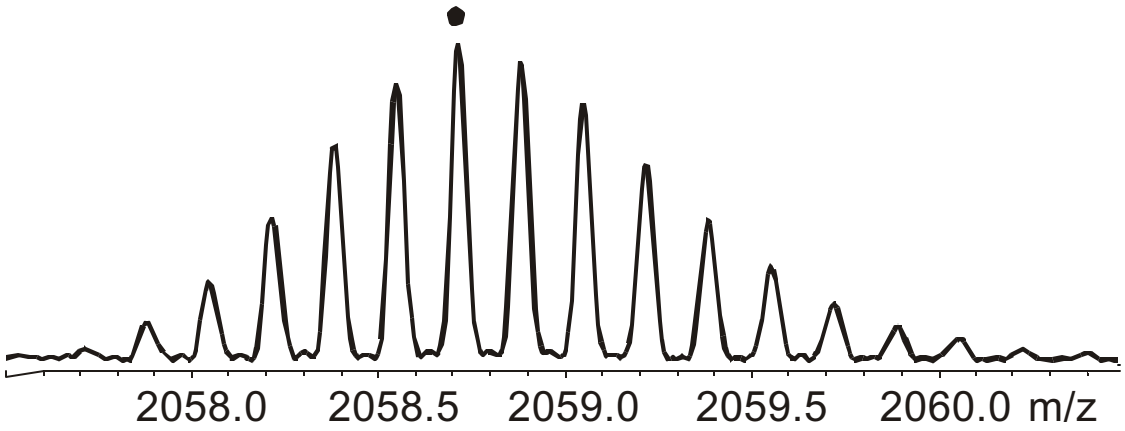
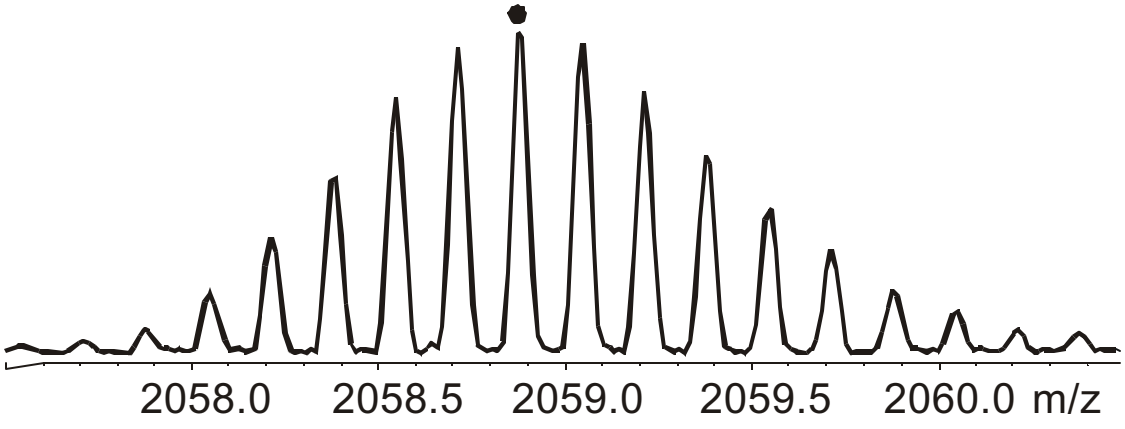

1545.4
1545.9
1546.4
1546.9

Figure 6-3 The negative ion mode ESI-FTICR mass spectra for the -6 charge state for both oxidized (top) and reduced (bottom) cytochrome *c* are shown. The centroid of each isotope distribution is marked with "●". The shift in the distribution corresponds to a change in oxidation state from Fe^{3+} to Fe^{2+} going from oxidized to chemically reduced cytochrome *c*.


2058.0
2058.5
2059.0
2059.5
2060.0 m/z


2058.0
2058.5
2059.0
2059.5
2060.0 m/z

salt; whereas electrospray ionization mass spectrometry generally cannot tolerate high salt concentrations. A suitable compromise is a system that has enough conductivity to supply the desired potential and a low enough salt concentration to avoid the suppression of analyte peaks and the formation of adduct peaks. For high impedance solutions the product of the resistance and the current flowing in the cell generates a voltage drop across the cell such that the potential realized at the electrode surface is only a portion of the applied potential. This voltage loss is commonly denoted as "iR drop". In this experimental configuration the resistance of the cell with cytochrome *c* solution was found to be ca. 0.5 MΩ with a 30 μA current. Thus most of the applied potential was dissipated as iR drop across the cell. The potential was set at a value such that a constant current flowed, in this instance 13.3 V. At this applied potential, even with significant iR drop, reduction was found to occur at the cathode with minimal hydrogen evolution. Increasing the potential even moderately caused significant hydrogen evolution and decreasing the potential slightly resulted in a drastic decrease in current.

The use of a standard three-electrode cell with a working, counter, and reference electrode was precluded due to salt leakage from the reference electrode. Even minimal sodium or potassium contamination will manifest itself as adduct peaks in the ESI mass spectrum, complicating analysis and possibly compromising mass accuracy. The above considerations led to the use of a controlled potential power supply to set the potential difference at the electrodes of a two-electrode system. In this manner, no additional sodium or potassium can enter the system from the electrochemical cell. Although a great deal of control of the electrochemical reaction is given up with this experimental configuration, it was successful for the reduction of cytochrome *c* without the need for an additional cleanup step to remove sodium and potassium.

The electrochemical reduction of cytochrome *c* was monitored visually. Cytochrome *c* is a light brown color in the oxidized state and pink in the reduced state. The full absorbance spectrum between 375 nm and 625 nm is shown in Figure 6-4. The transition at 550 nm was monitored with an UV-visible detector online prior to ionization, with the outlet of the detector connected directly to the nanoelectrospray source. An increase from 0.1410 AU to 0.4113 AU at 550 nm (pathlength = 1cm) was observed following the transformation from the oxidized to the reduced form of the protein. The resulting positive mode mass spectrum is shown in Figure 6-5. The top spectrum shows an expansion of the 7+ charge state for the oxidized form of cytochrome *c*. The same charge state for the reduced form is shown in the bottom spectrum. The shift in the isotope distribution between the two forms is due to the change in the number of protons that are observed following the electrospray process to create the 7+ charge state. The oxidized protein is determined to have 4 excess protons, from which the Fe^{3+} oxidation state is derived. The reduced form shows the addition of 5 excess protons, indicating Fe^{2+}.

The electrochemical reduction was found to have several advantages over the chemical reduction. The time needed for the electrochemical reductions was 5 times shorter than that required for the chemical reduction. This time saving is due to the avoidance of the lengthy process of removing the reducing agent by ultrafiltration. An additional benefit results from the protection from ambient oxygen. With the chemical reduction, the reductant must ultimately be removed, allowing atmospheric oxygen access to the reduced protein. With the electrochemical reduction, as long as the potential is maintained, the reducing electrode will actively scavenge oxygen and convert any oxidized cytochrome *c* to the reduced form. Although this electrochemical cell was large for protein work (0.5 mL), the volume could be reduced to a few

Figure 6-4 The UV-visible absorbance spectrum is shown for oxidized (lightweight line) and reduced (bold line) cytochrome *c* between 375 nm and 625 nm. The transition at 550 nm (pathlength = 1 cm) was monitored during on-line electrospray ionization for the electrochemically reduced sample. The concentration of cytochrome *c* calculated from these data is approximately 13 μM.

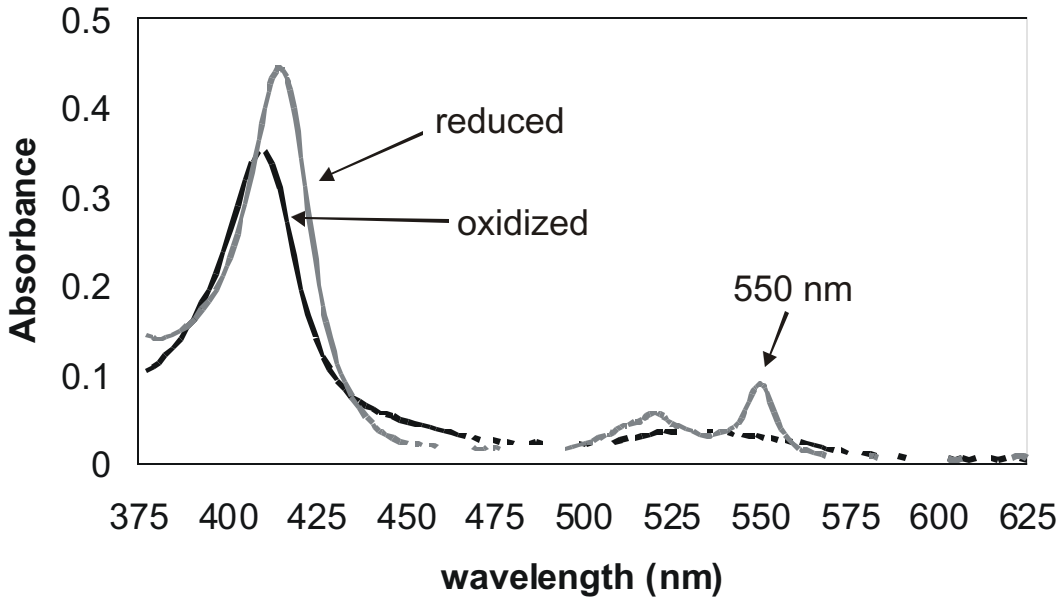
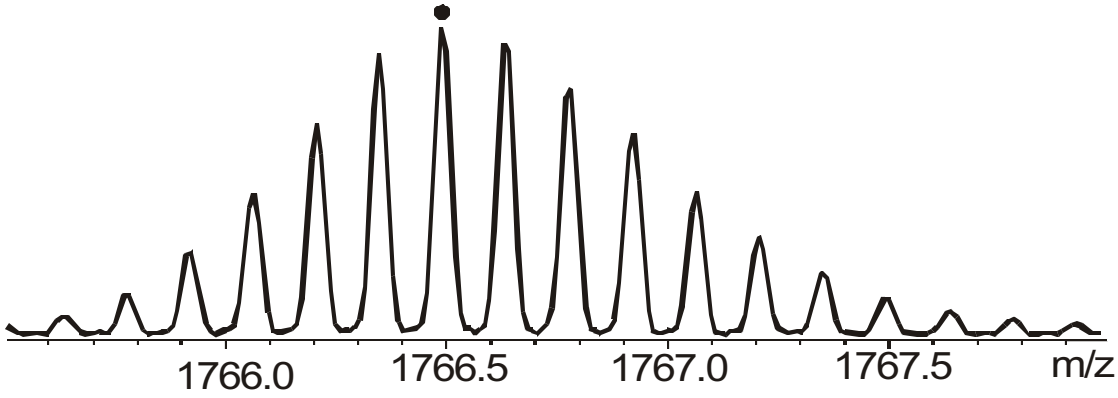
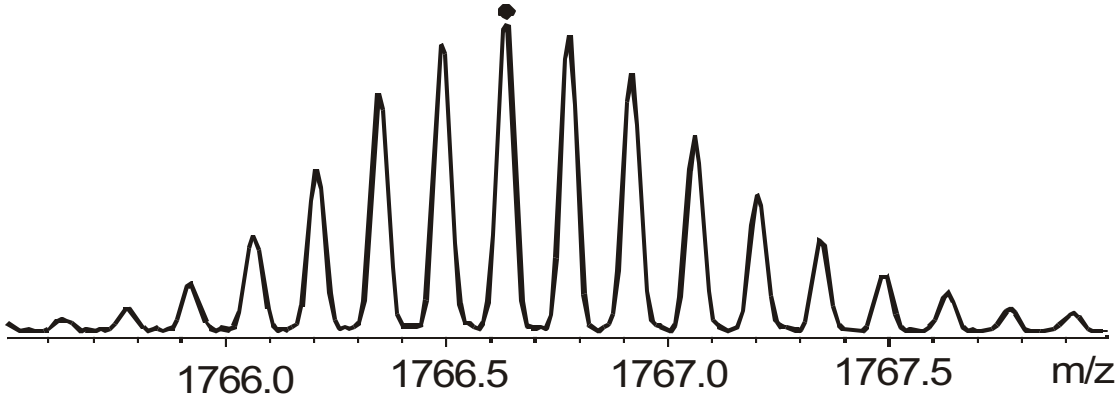

0.5
0.4
0.3
0.2
0.1
0
Absorbance
reduced
oxidized
550 nm
375 400 425 450 475 500 525 550 575 600 625
wavelength (nm)

Figure 6-5 The ESI-FTICR mass spectra for oxidized (top) and on-line, electrochemically reduced (bottom) cytochrome *c* are shown. The shift in the isotope distribution for the 7+ charge state between oxidized and reduced metalloprotein is marked by "●" at the centroid of each distribution.


1766.0
1766.5
1767.0
1767.5
m/z


1766.0
1766.5
1767.0
1767.5
m/z

microliters to provide a method for the analysis of metalloproteins that are available only in limited quantities.

Both the oxidized and reduced forms of several metalloproteins have been observed using ESI-FTICR mass spectrometry. Reduction of the metalloproteins with sodium dithionite and bulk reduction in an electrochemical cell are both feasible methods. However the electrochemical method is faster and less prone to reoxidation of the reduced protein. The mass spectra obtained for these metalloproteins accurately reflect the solution oxidation state. Neither oxidation nor reduction of metalloproteins is indicated in either positive or negative mode ESI. The electrochemical method can be conducted on-line with ESI, since no additional sample cleanup is necessary. Further design improvements incorporating a thin-layer, low-volume electrochemical cell coupled with ESI-FTICR-MS will be useful for characterizing the active oxidation states of a wide variety of metalloproteins.

CALCULATION OF PROTEIN CHARGE STATE AND MOLECULAR WEIGHT

The calculation of the molecular weight of a protein derived from the mass spectrum may not be straightforward. The mass spectrum for cytochrome *c* shown in Figure 6-2 illustrates the need to determine the charge state of the peaks in the mass spectrum. Any calculation derived from an ESI spectrum should be started with the charge state. By examining the distance between isotope peaks for a given charge state, the charge state can be determined and the molecular weight calculated. Figure 6-2 shows that the spacing between isotope peaks corresponds to a m/z difference of approximately 0.12. It is known that the isotope distribution for cytochrome *c* is due principally to ^{13}C atoms and thus the separation between isotope peaks is 1 mass unit. The known separation of isotope peaks divided by the observed separation yields the charge state of +8, (1/0.12=8).

Once the charge state is known, the apparent mass of the most abundant isotope peak is calculated. Due to the statistical probability and natural occurrence of the isotopes, the most abundant isotope peak for proteins contains several ^{13}C atoms. This is in direct contrast to small molecules where the most abundant isotope peak is generally comprised solely of ^{12}C atoms. In fact the monoisotopic peak for proteins is often not observed due to the very low abundance. The mass derived following this treatment is the "apparent mass" for the metalloprotein. It incorporates the protons that are added to obtain the charge state. For oxidized cytochrome *c*, the +8 charge state corresponds to an Fe^{3+} and 5 H^+. Thus the apparent mass should be 5 mass units higher than that obtained from computer software used to calculate the most abundant isotope peak. These calculations are based upon the known molecular formula and the natural abundance of the atoms comprising the molecule. In the same fashion the "apparent mass" for reduced cytochrome *c* should be exactly 6 mass units higher than the calculation method. This corresponds to an Fe^{2+} and 6 H^+ to arrive at the +8 charge state. It is noteworthy that this results in a difference of only 1 amu between the oxidized and reduced species, illustrating the importance of accurate high-resolution mass spectrometry for sample analysis.

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CHAPTER 7

FUTURE RESEARCH

The future of the use of ESI for non-traditional analytes lies in the ability to increase the sensitivity and reproducibility of this method. The next phase of the ESI-TOF-MS is to obtain known standards of toxaphene congeners and determine if the response is congener or homologue specific. Further research into the formation of the $[M- H]^-$ ions will help to improve the sensitivity of the method. Small percentages of chlorinated solvents may assist in this endeavor.

Due in part to the method reported here for the determination of DDA, the U.S.EPA has expressed an interest in monitoring the DDA residues at sites such as the Triana/Tennessee River site. The next phase of the study is to evaluate more water and sediment samples and attempt to correlate the formation of DDA with the disappearance of one of the other metabolites. Microcosm studies have also been discussed, although the recalcitrance of DDT and its metabolites makes these studies arduous.

The ESI-FTICR-MS determination of the oxidation state of cytochrome *c* was significant because it clearly demonstrated the use of ESI for both reduced and oxidized samples. The investigation of the redox potential range that is suitable for the ESI-FTICR-MS determination of oxidation states can be undertaken. Miniaturization of the electrochemical cell is an additional direction to be explored. The role of atmospheric oxygen may also be investigated by examining the results of oxygen saturated solutions of stable reduced proteins.