Fall 2007

Hyphenated HPLC-MS technique for analysis of compositional monosaccharides of transgenic corn glycoprotein and characterization of degradation products of diazinon, fonofos and aldicarb in various oxidation systems

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HYPHENATED HPLC-MS TECHNIQUE FOR ANALYSIS OF COMPOSITIONAL MONOSACCHARIDES OF TRANSGENIC CORN GLYCOPROTEIN AND CHARACTERIZATION OF DEGRADATION PRODUCTS OF DIAZINON, FONOFOS AND ALDICARB IN VARIOUS OXIDATION SYSTEMS

by

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A DISSERTATION

Presented to the Faculty of the Graduate School of the

UNIVERSITY OF MISSOURI-ROLLA

In Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

2007

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Dr. Yinfa Ma, Advisor	Dr. Craig Adams, Co-Advisor
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PUBLICATION DISSERTATION OPTION

This dissertation consists of the following five articles that have been published or submitted for publication as follows:

Pages 11-34 was published in the JOURNAL OF CHROMATOGRAPHIC SCIENCES.

Pages 35-57, 58-75, and 76-94 are intended for submission to ENVIRONMENTAL SCIENCE AND TECHNOLOGY.

Pages 95-120 are intended for submission to WATER RESEARCH.

ABSTRACT

The studies of this dissertation are composed of two sections. The first one deals with the analysis of compositional monosaccharides of transgenic corn glycoproteins. The method used in this study involves derivatization of monosaccharides with two fluorophores followed by HPLC/fluorescence detection for quantitative studies, and by HPLC/SSI/MS for identification confirmation of individual monosaccharide. The derivatization process adds a moderate polar moiety to monosaccharides, changing their structures and improving the separation of derivatized monosaccharides on C-18 column, as well as increasing the detection sensitivity dramatically. Moreover, derivatization process also adds a proton receptor to monosaccharide molecules, making them much easier to be ionized by ESI, SSI, APCI, etc. The second section investigates the degradation processes of several pesticides including diazinon, fonofos and aldicarb in various oxidation systems. This oxidation process may result in the formation of oxidation byproducts from the parent pesticides, which could be more toxic than the parent pesticides. Diazoxon and 2-Isopropyl-6-methyl-4-pyrimidinol (IPMP) were detected as the degradation products of diazinon; fonofos oxygen analog was identified to be the degradation product of fonofos, while thiophenol and phenyl disulfide were identified as the alkaline hydrolysis products of fonofos; aldicarb sulfoxide, aldicarb sulfone and N-chloro-aldicarb sulfone were detected as the degradation products of aldicarb. Quantitative studies are also carried out to provide information for kinetics of these oxidation processes.

ACKNOWLEDGMENTS

This work would not have been possible without the help and guidance from my advisor and mentor, Dr. Yinfa Ma, and my co-advisor and mentor, Dr. Craig Adams. Their incredible scientific and practical ability and vast amount of knowledge has been an inspiration throughout my pursuit of this degree.

I would also like to thank my exceptional doctoral committee members, especially Dr. Paul Nam, who provided fundamental and operational information of, as well as technical support to, the mass spectrometer. He helped me a lot in solving problems both in research and instrumentation. I would also like to thank Dr. Shubhender Kapila, Dr. Philip Whitefield, and Dr. Yue-wern Huang for their continual support and encouragement in the pursuit of my degree.

The projects during my study at UMR have been supported by Monsanto Protein Technologies and the AwwaRF Foundation. My thanks are given to them for their financial support.

I am grateful for having extraordinary colleagues/co-authors during my study and research. Honglan Shi, who is an exceptional senior chemist in Environmental Research Center at University of Missouri-Rolla, provided a wide variety of help, support and suggestions. Evelyn Chamberlain gave me many help and background knowledge and information in the area of environmental engineering. Thank you for the opportunity to publish and work together with you.

I would like to express my appreciation to Joseph Council, Mike Myers, and Dave Satterfield for their assistance in repairing and technical support to the instruments. Many thanks are also given to Donna Riggs, Carol Rodman, and Kathy Eudaly for their kind help in organization and managing of various issues of my study.

I would like to thank a very special person, Lin Yang. Without her unconditional love, understanding, and constant support every day, this dissertation would not be possible. She endured more pressure than me during my study. Finally, I would like to thank my daughter, Jessie Wang, although she is only four years old. Her existence always reminds me to take the responsibility as a father myself to support my family.

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INTRODUCTION

Glycans or oligosaccharides are found in a wide variety of proteins, including enzymes, immunoglobulins, carriers, hormones, toxins, lectins, and structural proteins. In recent years, glycoprotein research has drawn increasing interest in the fields of biotechnology, clinical chemistry, biochemistry, pharmaceutical and food sciences. Glycoprotein play important roles in biological processes including antigenicity, transport, folding, recognition, defense or decoy functions, cellular adhesion, blood clotting, immunological protection, structural support, and others. Glycosylation of proteins is one of the most common and important post-translational modifications found in secretory proteins.

Glycoproteins are a class of proteins that exhibit extraordinary complex structure due to the presence of the surface carbohydrates. The structure diversity is derived not only from different linkages between proteins and carbohydrates, but also from the composition and the structure of the carbohydrate units. Transgenic corn offers an attractive and cost effective mean for large-scale production of therapeutic glycoproteins suitable for pharmaceutical purpose. The particular glycoprotein produced by transgenic corn should not contain glycans because the glycosylation sites have been genetically altered. A sensitive and reliable analytical method is needed to determine the glycans and monosaccharides in the glycoproteins for quality control purposes.

To determine whether a protein is glycosylated, the first step is to identify and quantify the compositional monosaccharides in the glycoprotein. Several methods have been developed for analyzing monosaccharides derived from glycoproteins.

High performance liquid chromatography (HPLC) with various detectors, such as UV/vis, fluorescence, electrochemical and mass spectrometry (MS), has become a more popular method for monosaccharide analysis. Reversed phase HPLC analysis of derivatized monosaccharide with UV/vis or fluorescence detection is one category, and anion exchange HPLC with different types of detection is another. For irrefutable identification of the monosaccharides released from glycoproteins, MS has also been used in conjunction with HPLC. Thermospray and electrospray were utilized as the ionization source for liquid chromatography/mass spectrometry (LC/MS) but reduced sensitivity is often encountered for direct sugar analysis. Sonic spray ionization (SSI) can be an alternative ionization source for LC/MS analysis of glycans and monosaccharides. SSI is a softer ionization technique compared to electrospray, therefore, it is more efficient in formation of molecular ions and provides a possibility of MS characterization of the monosaccharides. Since this ionization source operates with much lower temperature and no needle voltage, it is well suited for the analysis of thermally labile and unstable compounds like carbohydrate. So far it has been successfully applied only to a limited number of compounds in the environmental and bioanalytical field, and analysis of derivatized monosaccharides by LC-SSI-MS has not been reported.

In this study, the HPLC-fluorescence and LC/SSI/MS based techniques for compositional monosaccharide analysis of glycoproteins in transgenic corn are described. The monosaccharides are released from glycoproteins by acid hydrolysis and derivatized with a fluorophore, and a reversed-phase HPLC with fluorescence detection is used to identify and quantify these monosaccharides, and each monosaccharide is further

confirmed by LC/SSI/MS. This approach showed an advantage over others in that it is possible to perform HPLC with fluorescence and MS detection simultaneously.

Many organophosphorus compounds, either as esters or thioesters of phosphoric and thiophosphoric acid, are commonly used as herbicides non-specific insecticides (i.e., insectacetyl-cholinesterases). Organophosphours pesticides can also affect the nervous systems ofhumans.

The oxidation byproducts of these organic compounds may have a greater, similar, or lesser toxicity than the parent compound, depending on the specific modifications occurring in the chemical structure. Because of the exposure of humans to these degradates in drinking water, as well as to the environment through wastewater discharges, determination of the identity and nature of these compounds under different oxidative environments is crucial. Previous studies have primarily focused on the hydrolysis of organophosphorus pesticides at different pH levels and temperatures. The catalytic or inhibitory effects of oxides surfaces, dissolved metals and metal-containing surfaces, metal oxides and natural dissolved organic matter and mixing on the hydrolysis of organophosphorus pesticides have also been investigated.

Among these organophosphorus pesticides, diazinon has been commonly used as a pesticide since 1952 due to its inhibition of the acetylcholinesterases of most kinds of insects. It has been applied in different types of cultivation such as fruit trees, rice, sugarcane, corn, tobacco, and horticultural plants. Diazinon is considered moderately toxic with a fish-based LC_{50} (lethal concentration for 50% fish kill) of 4.4 mg/L in killifish within 48 hours. However, diazinon can deteriorate to harmful substances, including monothiotepp (O, S-TEPP) and sulfotepp (S, S-TEPP), at high temperature and

under certain other conditions. These degradates are known to be highly toxic and to have a strong inhibitory effect on cholinesterase enzyme systems. Preliminary studies have found that the half-life ($t_{1/2}$) of diazinon hydrolysis was largely dependent on pH and temperature. In addition, some catalysts such as ferrihydrite, goethite, and hematite, were also found to have considerable influence on the rate of hydrolysis under different conditions. Previous studies identified 2-isopropyl-6-methyl-4-pyrimidinol (IPMP) as a major hydrolysis product under both acidic and basic conditions.

The oxidation of diazinon by free chlorine and ozone has been studied by several research groups. For example, in one study, ozonation of diazinon produced diazoxon which was further hydrolyzed to diethyl phosphate and IPMP. It was also demonstrated that ozonation was feasible for achieving nearly complete degradation of diazinon within 1 hour, and that the process was almost independent of pH, temperature, and alkalinity.

Diazoxon has a LC_{50} of 0.22 mg/L in killifish in 48 hours, showing much higher toxicity than diazinon itself. Photocatalytic degradation of diazinon was also carried out with TiO_2 as the catalyst; diazoxon and IPMP were also identified. In these studies, diazinon and its degradation products were separated and detected by a variety of analytical techniques such as gas chromatography with a nitrogen-phosphorus detector or a flame ionization detector, semi-micro liquid chromatography-mass spectrometry (LC/MS).

The use of oxidants in drinking water treatment is common for disinfection, oxidation of inorganic and organic contaminants, taste and odor control, and microflocculation. Based on a thorough literature search, there are no comprehensive and/or comparative studies that have investigated the oxidation systems most commonly

used in water treatment plants involving treatment of diazinon with free chlorine, ozone, monochloroame, and chlorine dioxide, as well as ultraviolet light (UV). Therefore, this study was conducted to investigate the oxidation products of diazinon with this wide array of oxidants and UV, including identification of byproducts. Diazinon and its oxidation products were separated with high performance liquid chromatography, and identified and quantified by using a sonic spray ionization ion trap mass spectrometer (HPLC/SSI/MS). This study provides important information regarding the formation of degradates from diazinon during disinfection and other oxidative processes. This information will be important from the perspectives of monitoring and exposure, human and environmental health, and development of treatment options.

Fonofos (fonofos, O-ethyl S-phenyl ethylphosphonodithioate; CAS 944229) is a dithiophosphonate pesticide used to control lepidopterous insects in corn, potatoes, and peanuts. This highly toxic chemical interferes with the nervous system by inhibiting an enzyme, cholinesterase. Symptoms of fonofos exposure may be delayed for a few minutes after exposure to up to twelve hours. Early symptoms include blurred vision, headache, and dizziness. Skin contact often brings about sweating and muscle twitching. Eye contact causes tearing, pain, and blurring. Ingestion may cause nausea, abdominal cramps, and diarrhea.

Determination of fonofos residue has been accomplished by various approaches in different matrices. Fonofos residue in honey and honeybees was determined by various extractions followed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LCAPCI-MS), or electrospray ionization-mass spectrometry (LC-ESI-MS). Capillary electrophoresis with cyclodextrin chiral selectors

was also used to determine the enantiomers of fonofos in aerobic soil slurries. Solid phase micro-extraction, followed by gas chromatography with mass spectrometry, was used to determine various pesticides (including fonofos) in a confined atmosphere and whole human blood.

Determinations of biologically-derived fonofos were reported by some researchers. An off-line coupling of thin-layer chromatography (TLC) with ESI-MS for routine determination of pesticides in toxicology and forensic medicine was reported. A comprehensive two-dimensional gas chromatograph with flame ionization detection was constructed and evaluated for the fast separation and analysis of fonofos extracted from human serum.

Determination of fonofos in water was also reported by using nano-HPLC coupled with direct-electron ionization mass spectrometry, and by using filtration and solid phase extraction, followed by gas chromatography/mass spectrometry (GC/MS) with large-volume injection. Fonofos, when used in agriculture, may transfer to the ground and underground water system, although a large part of it may have been absorbed and filtered by the soil or other absorbents. The fonofos residue can be degraded by free chlorine, the oxidant most frequently used by water treatment plants during the disinfection process, to produce oxidation byproducts. These byproducts may be more toxic than fonofos itself. Because of the potential human health implications, it is crucial that the oxidation byproducts of fonofos be identified and that both fonofos and its oxidation byproducts be monitored during in the water treatment process.

Studies on the metabolism of fonofos in biological systems have also been carried out. Peracid oxidation of fonofos in organic solvent systems has been studied by several

researchers. However, the free chlorine, H_2O_2 and permanganate oxidation of fonofos in water treatment plants have not yet been reported up to now. This study investigated the oxidation of fonofos by these oxidants in an aqueous buffer using HPLC coupled with quadrupole ion trap mass spectrometry, and the identified oxidation byproduct of fonofos was further confirmed using TLC and MS/MS. The results of this study can serve as a useful reference for water treatment plants.

Fonofos is fairly water insoluble but has a moderate persistence (40 day field half-life) in soil. Fonofos readily hydrolyses but transformation products were not identified. The method of spray application for fonofos has the potential for run-off contamination of surface water and the moderate persistence can cause groundwater contamination at certain sites. Fonofos has been found in groundwater at 0.01 to 0.1 µg/L, surface water at 0.01 µg/L.

A few studies have isolated transformation products of fonofos. However, both of these studies were in biological systems. The identification of transformation products of hydrolysis outside a biological tissue has not been investigated and is an important piece of information, particularly to water treatment facilities which utilize raised pH processes, where hydrolysis can become an important transformation reaction.

In this study, hydrolysis reactions were carried out at several high pH's in phosphate buffered water systems to simulate treatment processes. Fonofos and two hydrolysis products were separated, identified and quantified using two hyphenated methods. Thiophenol was separated with high performance liquid chromatography and identified and quantified by sonic spray ionization ion trap mass spectrometry (HPLC/SSI/MS). Phenyl disulfide was separated using gas chromatography and

identified and quantified using mass spectrometry (GC/MS). This study was intended to investigate the hydrolysis products of fonofos, emphasizing on identifying transformation products, mechanism and relative reaction rate.

Aldicarb [2-methyl-2(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime], an active ingredient in the pesticide TEMIK®, is a soil pesticide used in the agricultural sector worldwide for over 30 years for the control of insects, mites, and nematodes. Some of the most important uses of this product in the U.S.A. have included citrus, cotton, sugar beets, potatoes, pecans and peanuts. The commercial product is a granular formulation, which is incorporated into the soil at the time of application. After applied into the soil, it is solubilized and distributed by the groundwater, and absorbed by the roots and translocated throughout the plant and, and serves as a systemic pesticide.

The discovery of aldicarb residues in drinking water on Long Island, New York, in 1979 and later in other areas of the U.S.A. has resulted in many research and monitoring programs being conducted by university, regulatory agency and industry scientist. Such activities have included potable well monitoring studies, laboratory experiments, field research studies and computer modeling. Many of these activities have been conducted by or in cooperation with the producer and registrant of aldicarb.

It was reported that aldicarb would degrade to produce aldicarb sulfoxide and aldicarb sulfone in a variety of soil types under both field and laboratory. Thus, studies on the degradation of aldicarb under various conditions became crucial in understanding the degradation mechanisms and pathways and monitoring and removing of aldicarb and its degradates from the environment. The metabolites were determined by assay for radioactivity. It was found that aldicarb produced aldicarb sulfoxide, aldicarb sulfone,

aldicarb sulfoxide oxime, aldicarb sulfoxide nitrile, aldicarb sulfone oxime, and two unknowns. On the other hand, aldicarb sulfone nitrile and aldicarb sulfone acid were detected as the two major degradates of aldicarb sulfone under aerobic and anaerobic soils. The aerobic and anaerobic degradation rates for aldicarb were measured in soil samples collected at different depths, and the concentration changes of its two toxic oxidation products, aldicarb sulfoxide and aldicarb sulfone were determined to estimate the first-order rate constants for concurrent oxidation and hydrolysis of aldicarb, aldicarb sulfoxide and aldicarb sulfone, and for the loss of total carbamate residues. Hydrolysis of aldicarb, aldicarb sulfoxide and aldicarb sulfone in Floridan groundwater was observed with the rates decreased in the order sulfone > sulfoxide >> aldicarb. In addition, hydrolysis rates of aldicarb, aldicarb sulfoxide and aldicarb sulfone were measured at ppb levels in aqueous solution by using liquid-liquid extraction followed by gas chromatography with flame ionization detector (FID) and nitrogen-phosphorus detector (NPD). Biotransformation of is another pathway for degradation of aldicarb. Kazumi et al [10] described the studies in which the aldicarb biotransformation happening in sediment was mainly via an oxidation pathway in the presence of O_2, while in the absence of O_2, the biodegradation took place through a hydrolytic pathway. It was also reported that not only aldicarb, aldicarb sulfoxide and aldicarb sulfone at the applied dose to soils did not inhibit microbial growth, but also the microbial component in soil had a significant role in the degradation of these compounds. In fact, some researchers reported the capability of soil microorganisms to use the carbamate pesticides as a source of carbon and nitrogen for growth. Other factors affecting chemical and microbial degradation of aldicarb was

investigated, and it was shown that temperature was the most important variable affecting the degradation rate of aldicarb and its carbamate metabolites in surface soils.

Other than gas chromatography, the analysis of aldicarb and its carbamate metabolites included RP-HPLC followed by post-column derivatization and fluorescence detection, UV detection and mass spectrometry.

The use of oxidants in drinking water treatment is common for disinfection, oxidation of inorganic and organic contaminants, taste and odor control, and microflocculation. Due to the possibility of transporting aldicarb and its carbamate metabolites into drinking water via many different ways, the degradation byproducts and possible degradation mechanisms of aldicarb, aldicarb sulfoxide and aldicarb sulfone at different water treatment conditions must be systematically investigated to guide the water treatment system, to minimize environmental and human health effects.

However, no report was found through a thorough literature search to investigate the oxidation of aldicarb and its carbamate metabolites in water treatment plant involving treatment with monochloroamine, chlorine dioxide, permanganate, hydrogen peroxide, ozone and UV radiation. In this paper, a comprehensive study is conducted to analyze the oxidation byproducts of aldicarb in various oxidation systems by using HPLC/ESI/MS and to determine the removal of aldicarb by using HPLC/UV, as well as the oxidation reaction features in terms of brief mechanism and relative reaction rate. This study, along with our preliminary screening studies, provides practical information for understanding the kinetics and mechanism of the oxidation for different oxidants. Moreover, pesticides monitoring in water and control or choice of disinfection combination can be beneficial from this study.

PAPER

1. Compositional Monosaccharide Analysis of Transgenic Corn Glycoproteins by HPLC with Fluorescence Detection and LC-MS with Sonic Spray Ionization

Abstract

Transgenic corn offers an attractive cost effective mean for the large-scale production of engineered glycoproteins suitable for pharmaceutical purposes. A glycoprotein expressed in transgenic corn theoretically should not contain glycans because glycosylation sites have been genetically altered. A sensitive and reliable analytical method is developed to investigate this particular protein for the presence of glycans by monitoring the monosaccharide composition. Identification and quantitation of low-level monosaccharides in the glycoprotein hydrolyzate were accomplished by derivatization prior to high performance liquid chromatography (HPLC)-fluorescence and liquid chromatography- sonic spray ionization-mass spectrometry (LC-SSI-MS) analyses. LC-SSI-MS was used to confirm the results from HPLC-fluorescence analysis and to positively identify the compositional monosaccharides. N-Acetylglucosamine, glucose, mannose, arabinose, xylose, and sialic acid were found in the transgenic corn derived glycoprotein at less than one moiety per protein which indicated heterogeneity of the particular glycoprotein. In addition to the compositional analysis of low level monosaccharides in glycoprotein by HPLC fluorescence, the utility of SSI for the LC/MS analysis of derivatized monosaccharides was demonstrated in this paper.

Introduction

Glycans or oligosaccharides are found in a wide variety of proteins, including enzymes, immunoglobulins, carriers, hormones, toxins, lectins, and structural proteins. The most commonly occurring monosaccharides in glycans include glucose, mannose, galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine, and sialic acids [1].

In recent years, glycoprotein research has drawn increasing interest in the fields of biotechnology, clinical chemistry, biochemistry, pharmaceutical and food sciences [2-6]. Glycoprotein play important roles in biological processes including antigenicity, transport, folding, recognition, defense or decoy functions, cellular adhesion, blood clotting, immunological protection, structural support, and others. Glycosylation of proteins is one of the most common and important post-translational modifications found in secretory proteins [7-14].

Glycoproteins are a class of proteins that exhibit extraordinary complex structure due to the presence of the surface carbohydrates. The structure diversity is derived not only from different linkages between proteins and carbohydrates, but also from the composition and the structure of the carbohydrate units.

Transgenic corn offers an attractive and cost effective mean for large-scale production of therapeutic glycoproteins suitable for pharmaceutical purpose. The particular glycoprotein produced by transgenic corn should not contain glycans because the glycosylation sites have been genetically altered. A sensitive and reliable analytical method is needed to determine the glycans and monosaccharides in the glycoproteins for quality control purposes.

To determine whether a protein is glycosylated, the first step is to identify and quantify the compositional monosaccharides in the glycoprotein. Several methods have been developed for analyzing monosaccharides derived from glycoproteins. Even though direct analysis of monosaccharides by gas chromatography (GC) has been reported [15, 16], derivatization of monosaccharides is preferred to enhance the volatility of monosaccharides for GC analysis [17-21]. However, the instability of some of the derivatives renders this method less than ideal for accurate quantification of monosaccharide content.

High performance liquid chromatography (HPLC) with various detectors, such as UV/vis, fluorescence, electrochemical and mass spectrometry (MS), has become a more popular method for monosaccharide analysis. Reversed phase HPLC analysis of derivatized monosaccharide with UV/vis or fluorescence detection is one category [22-25], and anion exchange HPLC with different types of detection is another [25-28]. For irrefutable identification of the monosaccharides released from glycoproteins, MS has also been used in conjunction with HPLC [29-32]. Thermospray and electrospray were utilized as the ionization source for liquid chromatography/mass spectrometry (LC/MS) but reduced sensitivity is often encountered for direct sugar analysis [30, 33-35]. Sonic spray ionization (SSI) can be an alternative ionization source for LC/MS analysis of glycans and monosaccharides. SSI is a softer ionization technique compared to electrospray, therefore, it is more efficient in formation of molecular ions and provides a possibility of MS characterization of the monosaccharides [36, 37]. Since this ionization source operates with much lower temperature and no needle voltage, it is well suited for the analysis of thermally labile and unstable compounds like carbohydrate. So far it has

been successfully applied only to a limited number of compounds in the environmental and bioanalytical field [36, 37], and analysis of derivatized monosaccharides by LC-SSI-MS has not been reported.

In this paper, the HPLC-fluorescence and LC/SSI/MS based techniques for compositional monosaccharide analysis of glycoproteins in transgenic corn are described. The monosaccharides are released from glycoproteins by acid hydrolysis and derivatized with a fluorophore, and a reversed-phase HPLC with fluorescence detection is used to identify and quantify these monosaccharides, and each monosaccharide is further confirmed by
LC/SSI/MS. This approach showed an advantage over others in that it is possible to perform HPLC with fluorescence and MS detection simultaneously.

Experimental

Materials

The glycoprotein was monoclonal antibody (IgG) expressed in corn and provided by Monsanto Protein Technologies (St. Louis, MO, USA). This transgenic corn protein (product designation PR 390) was determined to be homogeneous by SDS-PAGE. It was engineered in such a way that asparagine, normally N-glycosylated in mammalian cells, was substituted with alanine, therefore, the protein should not be glycosylated. Nevertheless, the protein is suspected to be O-glycosylated and non-enzymatically glycosylated as well. As a glycosylated reference standard, bovine fetuin of highest purity

was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Glucose (Glc), galactose (Gal), mannose (Man), arabinose (Ara), xylose (Xyl), fucose (Fuc), glucosamine (GlcN), galactosamine (GalN), N-acetylneuraminic acid (Neu5Ac), *o*-phenylenediamine (OPD) dichloride and anthranilic acid (2-aminobenzoic acid; AA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium cyanoborohydride was from Fluka Chemie GmbH (Steinbeim, Switzerland). Ultrafree-MC centrifugal filter devices with Biomax-30 membrane was purchased from Millipore (Bedford, MA, USA). Ultra-pure water was prepared with Synergy 185 system (Millipore, Bedford, MA, USA). HPLC-grade acetonitrile and tetrahydrofuran were purchased from Fisher Scientific (Pittsburgh, PA, USA). Other reagents and solvents were of reagent grade.

Purification of glycoprotein samples

1.0 mL of transgenic corn glycoprotein (9.33mg protein/mL) or 5.1 mg of bovine fetuin (dissolved in 1.0 mL H_2O) was filtered through the Biomax-30 membrane using an Ultrafree-MC Centrifugal Filter Device to remove constituents with molecular weight less than 30,000. The protein residues collected on the filter was washed with 2.0 mL of ultra pure water. The purified glycoprotein was recovered from the filter with 1-5 mL of ultra pure water.

Acid Hydrolysis of glycoprotein for neutral and basic monosaccharides analysis

A 100 μL aliquot of purified protein sample was mixed with 500 μL of 20% trifluoroacetic acid in a 2.0 mL screw-cap glass vial. The mixture was heated at 100ºC for hours. After the completion of hydrolysis, the sample was freeze-dried.

Derivatization of neutral and basic monosaccharides with anthranilic acid

The neutral monosaccharides were derivatized by reductive amination with the anthranilic acid following a procedure described previously [23]. First, a solution of 4% sodium acetate·$3H_2O$ and 2% boric acid in methanol was prepared. Thirty mg of anthranilic acid and 20mg of sodium cyanoborohydride were then dissolved in 1.0 mL of this methanol-acetate-borate solution.

Lyophilized glycoprotein hydrolyzate or 10 μL monosaccharide standard (0.25~100 μg/mL) was dissolved in 40 μL of 1% freshly prepared sodium acetate·$3H_2O$. The solution was mixed with 50 μL of the derivatizing reagent in a screw-cap glass vial and heated at 80ºC for 1 hour. After cooling to ambient temperature, the samples were centrifuged and 10 μL of the supernatants were injected onto the HPLC column.

HPLC-fluorescence analysis of AA-monosaccharide derivatives

A Hewlett-Packard 1090 HPLC instrument (Palo Alto, CA, USA) with a Shimadzu RF-551 fluorescence detector (Columbia, MD, USA) was used for analysis of anthranilic acid derivatives of monosaccharides. The separation conditions were similar to the reported method with some modifications [23]. A Waters C18 column (300 × 3.9mm i.d., 5μm, Milford, MA, USA) was used at ambient temperature with a flow rate of 1.0 mL/min. Solvent A consisted of 0.4% n-butylamine, 0.5% phosphoric acid, and 1.0% tetrahydrofuran in water, and solvent B consisted of 50% solvent A and 50% acetonitrile. The HPLC separation was performed at 5% B for 15 min followed by a linear gradient to 15% B at 50 min. After each run, the column was washed with mobile phase B for 15 minutes, and equilibrated with the initial mobile phase for 10minutes. Fluorescence

detection was carried out at an excitation wavelength of 230 nm and an emission wavelength of 425 nm.

LC/MS analysis of AA-monosaccharide derivatives

The Hitachi M-8000 3DQ LC/MS_n system (San Jose, CA, USA) was used for the MS confirmation of monosaccharides released from hydrolysis of glycoprotein samples. The HPLC condition used for HPLC-fluorescence experiment was utilized with minor changes in mobile phase composition and gradient program. Solvent A consisted of 0.12% n-butylamine, 0.12% formic acid, and 1.0% tetrahydrofuran in water, and solvent B consisted of 50% of solvent A and 50% of acetonitrile. The HPLC separation was performed at 100% A for 30 min followed by a linear increase to 20% B at 60 min. The 1.0 mL/min effluent from the column was split through a tee connector and only 0.2 mL/min was directed to the SSI/MS. A divert valve was placed right before the ionization source to remove the HPLC fractions containing salts and excess AA derivatizing reagent and prevent the contamination of ionization source and MS. The SSI parameters were set to the following optimized values: nitrogen sheath gas at 3 kgf/cm_2, 0 kV capillary voltage, 45 V drift plate, 30 V focus plate, 200ºC cover plate temperature, 150ºC aperture 1 temperature. Ion trap MS was operated at following conditions: 500 ms accumulating time, 0.072V accumulation voltage, 48.2 amu low mass cut off, 250-350 amu scan range. The remaining (0.8 mL/min) flow of the HPLC effluent was directed to a UV detector set at 250 nm.

Mild-acid hydrolysis of glycoprotein for sialic acid analysis

A 50 μL aliquot of purified glycoprotein sample was mixed with 50 μL 0.5 M $NaHSO_4$ in a screw-cap glass vial. The mixture was heated at 80 ºC for 20 min and allowed to cool to ambient temperature.

Derivatization of sialic acids with OPD

Sialic acids were labeled with OPD to produce fluorescent quinoxaline derivatives via an optimized procedure previously reported [23]. An aliquot (0.1 mL) of standard sialic acid solutions (0.20 - 4.0 μg/mL) or mild-acid hydrolyzates of glycoprotein samples were mixed with 0.1 mL of the 20 mg/mL OPD in 0.25 M $NaHSO_4$. The mixtures in glass screw-cap vials were heated at 80ºC for 40 min. After cooling to ambient temperature, the samples were centrifuged and 10 μL of the supernatants were injected onto the HPLC column.

HPLC-fluorescence analysis of sialic acid

A Hitachi L-7000 series HPLC system (San Jose, CA, USA) was used for the analysis of the OPD derivatives of sialic acid. The separation conditions were similar to the reported method with some modifications [23]. A Waters C18 column (300 × 3.9mm i.d., 5μm, Milford, MA, USA) was used at ambient temperature with a flow rate of 1.0 mL/min. Solvent A consisted of 0.15% n-butylamine, 0.5% phosphoric acid, and 1.0% tetrahydrofuran in water, and solvent B consisted of 50% solvent A and 50% acetonitrile. HPLC separation of OPD derivatives of sialic acids was isocratic with a solvent mixture of 89%A + 11% B. After each run, the column was washed with mobile phase B for 15

minutes, and equilibrated with the initial mobile phase for 10 minutes. The excitation and emission wavelength for the fluorescence detector was 230 nm and 425 nm, respectively.

Results and Discussion

HPLC-fluorescence analysis of neutral and basic monosaccharides

Initially a mixture of eight monosaccharides commonly-occurring in glycoproteins was used in the optimization of method [38]. HPLC separation and fluorescence detection of AA-derivatized monosaccharide standards and monosaccharides hydrolyzed from transgenic corn glycoprotein are shown in Figure 1. A number of large peaks from excess derivatizing reagent and artifacts were present but separated clearly from monosaccharide peaks and did not interfere in the quantification. Glucosamine and neutral monosaccharides including mannose, glucose, arabinose and xylose were found in the glycoprotein sample. Galactosamine and galactose levels were below the detection limits of 0.25 and 1.0 ng, respectively. All of the AA-monosaccharide peaks in the chromatogram were confirmed by standard addition method. Since the glycoprotein sample contained no galactosamine, it was employed as an internal standard for accurate quantitation of other monosaccharide constituents. Calibration curve of each AA-derivatized monosaccharide was generated using peak area ratio and the regression coefficients (r^2) of all curves ranged between 0.9983 and 0.9999. The limit of detection was calculated as the minimum amount of monosaccharide giving

a signal to noise ratio of 3. The limits of detection on column for monosaccharides were as following: 0.25 ng for glucosamine and 1.0 ng for neutral monosaccharides.

Validation of the method was conducted by monitoring recoveries of spiked monosaccharides. The results are shown in Table 1. The monosaccharide recoveries were relatively lower for the highest level in the study, most likely due to the insufficient fluorescent labeling of monosaccharides. However, the specified derivatization condition should be adequate for the transgenic corn glycoprotein whose monosaccharide concentrations are expected to be very low. The validity of the method was also evaluated with fetuin whose monosaccharide composition of glycoproteins is already well known. The fetuin monosaccharide content determined with the method is shown in Table 1 and the results are similar to the published data [39-43].

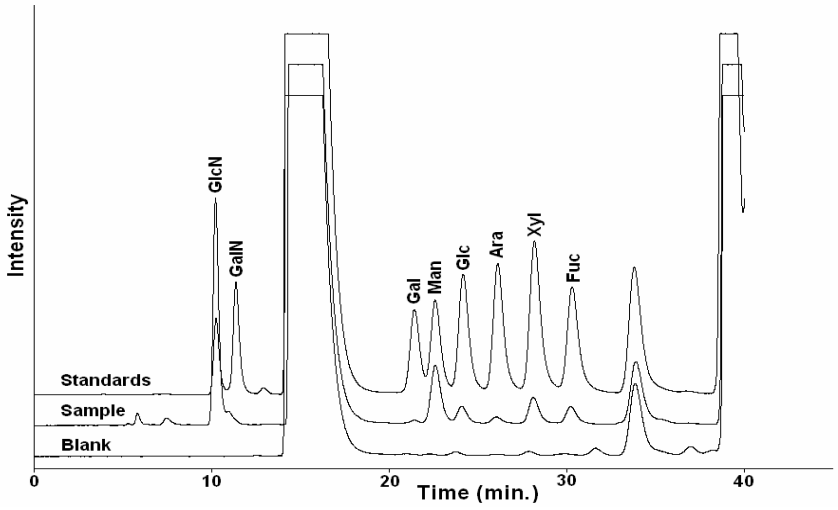

Figure 1. HPLC-fluorescence profiles of AA-derivatized neutral and basic monosaccharides from a standard mixture, a transgenic corn glycoprotein sample and a blank. Experimental conditions: C-18 column; Solvent A consisted of 0.4% n-butylamine, 0.5% phosphoric acid, and 1.0% tetrahydrofuran in water, and solvent B consisted of 50% solvent A and 50% acetonitrile. The HPLC separation was performed at 5% B for 15 min followed by a linear increase to 15% B by 50 min. Excitation wavelength: 230 nm; emission wavelength: 425 nm.

The neural and basic monosaccharide contents in glycoprotein sample from transgenic corn are shown in Table 2. The contents of individual monosaccharides were measured in μg/mg protein sample. The number of monosaccharide molecules per glycoprotein molecule was calculated based on the molecular weight (150,426 dalton) of transgenic corn glycoprotein (number of GlcN molecule per glycoprotein = content ×150,426/179; and number of neutral monosaccharide molecule = content ×150,426/180). This molecular weight was determined previously from the deconvolution of positive ion ESI-MS spectrum obtained for the same glycoprotein [44].

Table 1. Recovery of different monosaccharides.

	% Recovery (n=3)						
Spiked amount (ng) (amount injected)	GlcN	Gal	Man	Glc	Ara	Xyl	Fuc
2 – 5	83.2	89.8	87.6	105	78.5	83.9	87.0
RSD	5.4	3.5	3.7	3.3	4.3	8.6	5.1
10 – 20	81.5	79.6	80.0	96.2	76.3	72.6	79.6
RSD	2.2	4.4	4.7	9.9	1.7	8.9	4.6
50 – 100	66.4	66.5	65.9	75.8	62.0	54.6	65.3
RSD	4.8	5.0	6.6	10.0	5.1	7.9	6.6

Table 2. Neutral and basic monosaccharide composition of transgenic corn glycoprotein determined as concentrations and numbers of monosaccharide per protein (n=3).

	Glucosamine	Mannose	Glucose	Arabinose	Xylose
Monosaccharides content (μg/mg protein)	0.062 ± 0.002	0.728 ± 0.047	0.600 ± 0.158	0.350 ± 0.012	0.377 ± 0.006
# monosaccharide molecules per glycoprotein	0.052 ± 0.001	0.609 ± 0.039	0.502 ± 0.132	0.293 ± 0.009	0.316 ± 0.005

HPLC-fluorescence analysis of sialic acid

Representative HPLC-fluorescence chromatograms of sialic acid from standard and glycoprotein sample are shown in Figure 2.

The calibration curve regression coefficient (r^2) of sialic acid was 0.9998. The detection limit was 0.5 ng. The sialic acid content of the fetuin was determined to be 71.3 μg/mg protein and agreed well with the expected value (Table 3). The sialic acid content of glycoprotein sample from transgenic corn was in average 0.072 μg/mg protein. The number of sialic acid molecules per protein molecule was then calculated to be 0.035 based on the previously determined molecular weight of the particular transgenic corn glycoprotein.

Confirmation of the monosaccharides found in glycoprotein samples by LC-MS

For LC-MS analysis of AA-derivatized monosaccharides, the mobile phase solvents and gradient used for HPLC-fluorescence separation were slightly modified to accommodate SSI-MS operation. The concentrations of buffers in the mobile phase were lowered and the length of initial isocratic condition was increased. Accordingly the total time necessary for complete separation of all eight monosaccharide derivatives was greatly increased as can be seen in the UV detected chromatogram in Figure 3.

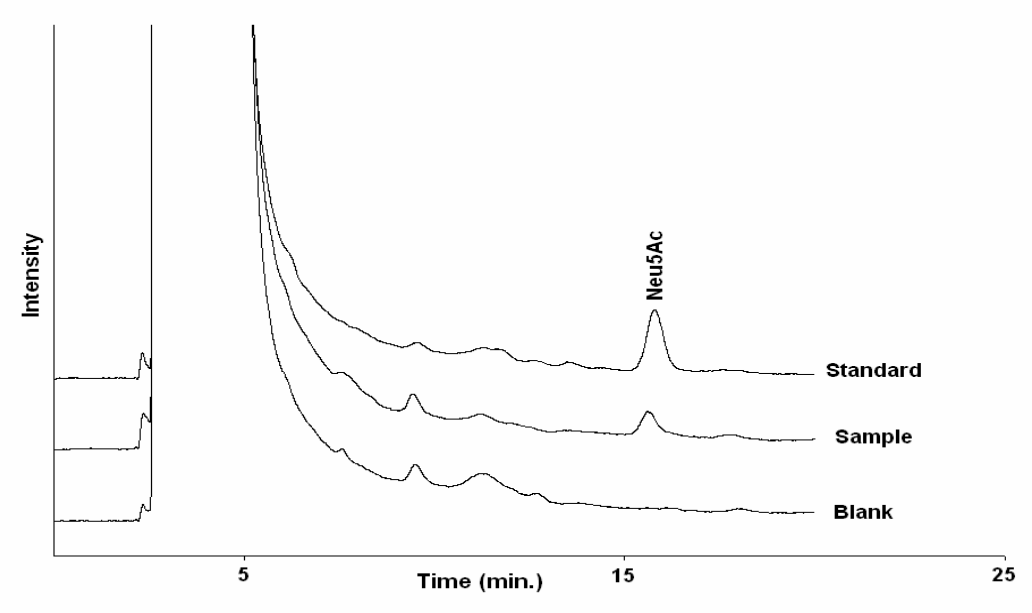

Figure 2. HPLC-fluorescence profiles of OPD-derivatized sialic acids from sialic acid (Neu5Ac) standard, a transgenic corn glycoprotein sample and a blank. Experimental conditions: C-18 column; Solvent A consisted of 0.15% n-butylamine, 0.5% phosphoric acid, and 1.0% tetrahydrofuran in water, and solvent B consisted of 50% solvent A and 50% acetonitrile. The separation was performed at an isocratic (89%A + 11% B) elution. The excitation and emission wavelength for the fluorescence detector was 230 nm and 425 nm, respectively.

Table 3. Monosaccharide composition of fetuin determined with current method and compared with published data.

	Monosaccharide Content (μg/mg protein)				
	GlcN	GalN	Gal	Man	Neu5Ac
Current Method	45.3-48.5	7.2-7.9	30.5-33.7	21.7-22.9	69.1-73.4
Reported [45, 46]	26.7-56.0	5.4-7.0	34.9-45.9	23.0-30.5	70.0-76.0

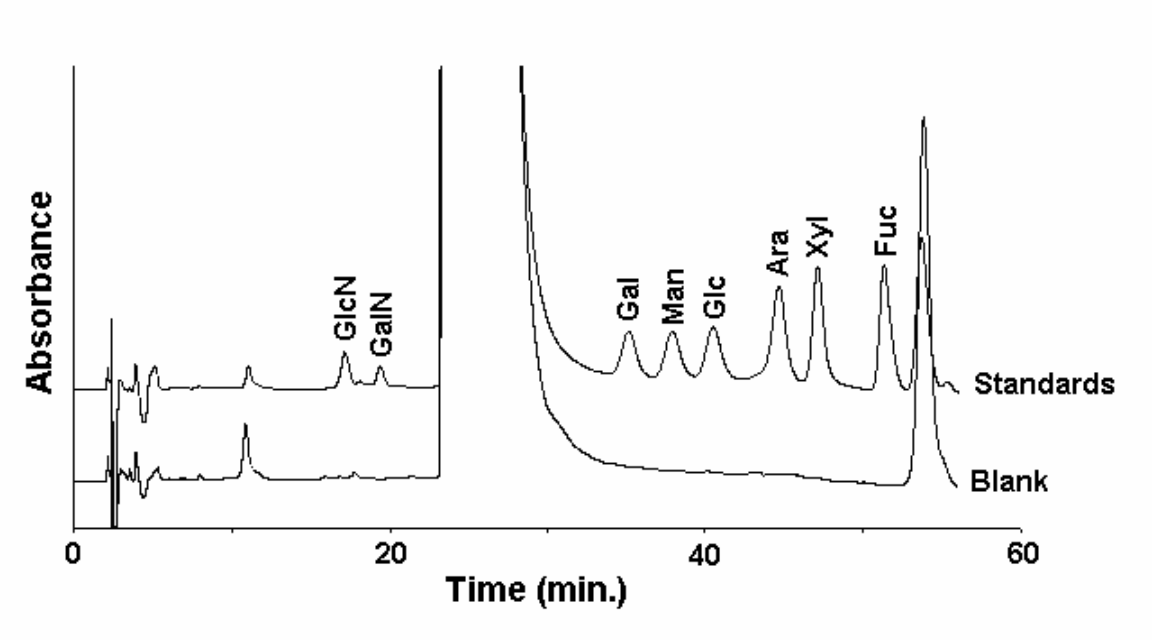

Figure 3. HPLC chromatogram of AA-monosaccharide derivatives with UV detector. Experimental conditions: The analytical column was the same as that for HPLC-fluorescence experiments. Solvent A consisted of 0.12% n-butylamine, 0.12% formic acid, and 1.0% tetrohydrofuran in water, and solvent B consisted of 50% of solvent A and 50% of acetonitrile. The HPLC separation was performed at 100% A for 30 min followed by a linear increase to 20% B by 60 min. The UV detector wavelength was set at 250 nm.

The 1/5 split of same LC effluent was directed to SSI-MS, but diverted to waste during the time segments when two large peaks corresponding to excess derivatization reagent and byproduct eluted. Non-retained solvent front segment was also diverted to waste to prevent the contamination of ionization source and MS from non-volatile buffer salt used in the derivatization of sample. Resulting total ion chromatogram of monosaccharide standards from LC-SSI-MS is shown in Figure 4. The extracted ion chromatograms corresponding to different monosaccharides are also displayed in Figure 4 (a-d). Based on the derivatization reaction process involving reductive amination of monosaccharides [45, 46], the positive ion SSI-MS should yield a protonated molecular

ion at *m/z* 301 for galactosamine and glucosamine derivatives which are optical isomers. Similarly a protonated molecular ion at *m/z* 302 is expected for AA-derivative of optical

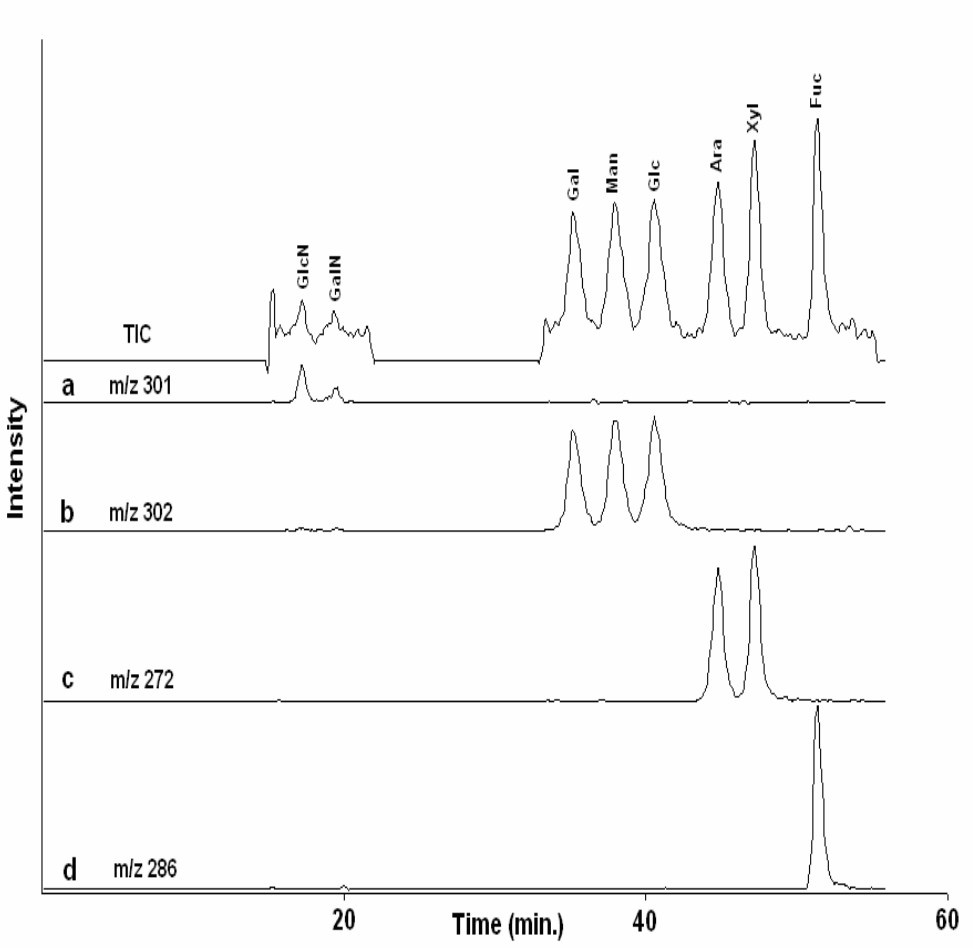

Figure 4. Reconstructed ion chromatograms of AA-derivatives of monosaccharide standards. Experimental conditions: HPLC condition was same as given in Figure 3. The SSI/MS parameters were optimized conditions stated in the Experimental section.

isomers; galactose, mannose and glucose. Another set of optical isomers, arabinose and xylose, should produce a protonated molecular ion at *m/z* 272. The protonated molecular ion of AA-derivatized fucose should have *m/z* 286.

The LC-SSI-MS analysis of the transgenic corn glycoprotein sample was performed with same conditions and the result is shown in Figure 5. Comparing the

retention time, standard addition, and mass spectra obtained for monosaccharide derivatives, glucosamine, mannose, glucose arabinose, and xylose in glycoprotein samples of transgenic corn were identified. Based on the concentrations of monosaccharides and the numbers of monosaccharide molecules determined per glycoprotein molecule, it is highly unlikely that the particular protein samples of transgenic corn contain any known N-linked or O-linked glycans. All of the N-linked oligosaccharides should have a common pentasaccharide core consisting of three mannose and two N-acetylglucosamine residues [45, 46]. Absence of any N-linked glycans in the particular transgenic corn derived glycoprotein was also confirmed by a previous hydrolysis study with an enzyme specific for N-linked glycans [44]. The protein also lacked the core structural component of O-linked glycans, N-acetylgalactosamine. Each sugar residue found in the transgenic corn glycoprotein was less than one unit per glycoprotein, which suggests the heterogeneous nature of this particular glycoprotein sample. It implies that not all of the glycosylation sites of protein molecules were genetically blocked, leading to the post glycosylation of some proteins. Another possibility could be the random linking of individual sugar to the amino acid residues by an unknown mechanism that require further investigation.

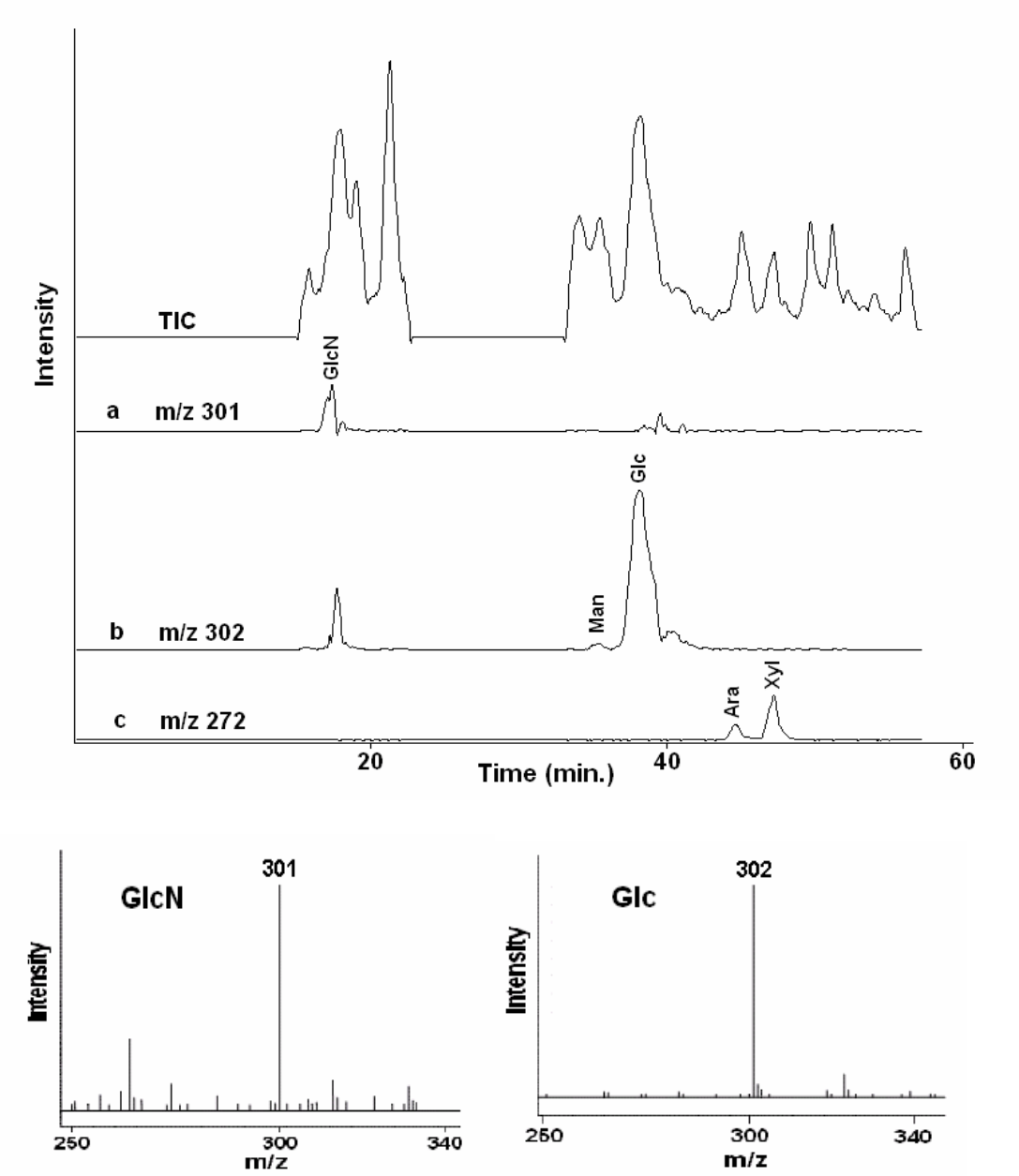

Figure 5. Reconstructed ion chromatograms and selected mass spectra corresponding to AA-derivatives of monosaccharides in transgenic corn glycoprotein sample. Experimental conditions were same as described in Figure 4.

Conclusions

The compositional monosaccharides in glycoproteins of transgenic corn were quantitatively determined and identified following derivatization of protein hydrolyzate by reversed-phase HPLC with fluorescence detection and positively confirmed by LC/MS with SSI source. The numbers of individual monosaccharide molecules bonded to the transgenic corn glycoprotein were found to be less than one for each glycoprotein, which indicated that these monosaccharides may come from the heterogeneous glycans in the glycoproteins or maybe randomly linked to some amino acid residues in the proteins. Although this phenomenon deserves further study, it implied that not all of the glycosylation sites in the protein molecules were genetically blocked.

Acknowledgment

The authors sincerely thank the financial support from Monsanto Protein Technologies to this work.

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2. Comprehensive Investigation of Degradation Products of Diazinon by Various Oxidation Systems Using High Performance Liquid Chromatography Coupled with Ion Trap Mass Spectrometer

Diazinon, an organophosphorus pesticide, is commonly used in agriculture resulting in their occurrence in drinking water supplies. The disinfection process using different oxidants for the treatment of water provides the opportunity to degrade diazinon to byproducts that may pose more or less risk than the parent. Based on the results of previous screening studies, a comprehensive study was performed involving diazinon treatment with free chlorine (FC), monochloramine (MCA), chlorine dioxide (ClO_2), hydrogen peroxide, and UV radiation to identify its degradation products. FC exhibited strong oxidation capacity than the others studied, while MCA showed the weakest oxidation ability among them. Both IPMP and diazoxon were formed as the degradation products of diazinon by oxidation with free chlorine, MCA, and chlorine dioxide. 2-Isopropyl-6-methyl-4-pyrimidinol (IPMP), but not diazoxon, was identified as an oxidative byproduct from treatment with UV light and hydrogen peroxide at very high dosages. UV light and hydrogen peroxide only act as catalysts for the hydrolysis of diazinon, while the others function as both oxidants and catalysts.

Introduction

Many organophosphorus compounds, either as esters or thioesters of phosphoric and thiophosphoric acid, are commonly used as herbicides non-specific insecticides (i.e.,

insectacetyl-cholinesterases). Organophosphours pesticides can also affect the nervous systems ofhumans.

The oxidation byproducts of these organic compounds may have a greater, similar, or lesser toxicity than the parent compound, depending on the specific modifications occurring in the chemical structure. Because of the exposure of humans to these degradates in drinking water, as well as to the environment through wastewater discharges, determination of the identity and nature of these compounds under different oxidative environments is crucial. Previous studies have primarily focused on the hydrolysis of organophosphorus pesticides at different pH levels and temperatures [1-3]. The catalytic or inhibitory effects of oxides surfaces [4, 5], dissolved metals and metal-containing surfaces [6-9], metal oxides [10, 11] and natural dissolved organic matter and mixing [12] on the hydrolysis of organophosphorus pesticides have also been investigated.

Among these organophosphorus pesticides, diazinon has been commonly used as a pesticide since 1952 due to its inhibition of the acetylcholinesterases of most kinds of insects. It has been applied in different types of cultivation such as fruit trees, rice, sugarcane, corn, tobacco, and horticultural plants. Diazinon is considered moderately toxic with a fish-based LC_{50} (lethal concentration for 50% fish kill) of 4.4 mg/L in killifish within 48 hours [13]. However, diazinon can deteriorate to harmful substances, including monothiotepp (O, S-TEPP) and sulfotepp (S, S-TEPP), at high temperature and under certain other conditions. These degradates are known to be highly toxic and to have a strong inhibitory effect on cholinesterase enzyme systems [14-16]. Preliminary studies have found that the half-life ($t_{1/2}$) of diazinon hydrolysis was largely dependent on pH

and temperature [2, 3]. In addition, some catalysts such as ferrihydrite, goethite, and hematite, were also found to have considerable influence on the rate of hydrolysis under different conditions [10]. Previous studies identified 2-isopropyl-6-methyl-4-pyrimidinol (IPMP) as a major hydrolysis product under both acidic and basic conditions [1, 2, 17].

The oxidation of diazinon by free chlorine and ozone has been studied by several research groups. For example, in one study, ozonation of diazinon produced diazoxon which was further hydrolyzed to diethyl phosphate and IPMP [18]. It was also demonstrated that ozonation was feasible for achieving nearly complete degradation of diazinon within 1 hour, and that the process was almost independent of pH, temperature, and alkalinity [19].

Diazoxon has a LC_{50} of 0.22 mg/L in killifish in 48 hours, showing much higher toxicity than diazinon itself [13]. Photocatalytic degradation of diazinon was also carried out with TiO_2 as the catalyst; diazoxon and IPMP were also identified [20]. In these studies, diazinon and its degradation products were separated and detected by a variety of analytical techniques such as gas chromatography with a nitrogen-phosphorus detector [12] or a flame ionization detector [21], semi-micro liquid chromatography-mass spectrometry (LC/MS) [22].

The use of oxidants in drinking water treatment is common for disinfection, oxidation of inorganic and organic contaminants, taste and odor control, and microflocculation. Based on a thorough literature search, there are no comprehensive and/or comparative studies that have investigated the oxidation systems most commonly used in water treatment plants involving treatment of diazinon with free chlorine, ozone, monochloroame, and chlorine dioxide, as well as ultraviolet light (UV). Therefore, this

study was conducted to investigate the oxidation products of diazinon with this wide array of oxidants and UV, including identification of byproducts. Diazinon and its oxidation products were separated with high performance liquid chromatography, and identified and quantified by using a sonic spray ionization ion trap mass spectrometer (HPLC/SSI/MS). This study provides important information regarding the formation of degradates from diazinon during disinfection and other oxidative processes. This information will be important from the perspectives of monitoring and exposure, human and environmental health, and development of treatment options.

Experimental Section

Reagents and Chemicals. Diazinon (99.0%) was purchased from Riedel-deHaën (Germany). 2-Isopropyl-6-methyl-4-pyrimidinol (IPMP, 99.5%) and diazinon-O-analog (diazoxon, 96%) were purchased from ChemService (West Chester, PA, USA). Formic acid (96%, ACS grade), hydrogen peroxide solution (30%) and sodium hypochlorite solution (available chlorine ≥4%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC grade), acetonitrile (HPLC grade), water (HPLC grade), sodium hydroxide (98.3%), ammonium chloride (certified ACS, 99.5%), and sodium phosphate (dibasic, 99%) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Sodium phosphate (monobasic, 99%) was purchased from Aldrich (Milwaukee, WI, USA). The pH for the experiments was adjusted with either 1 N H_3PO_4 or NaOH.

Methods. All pH measurements were obtained with an Accumet XL 15 pH meter using an Accumet AccuCap combination pH electrode from Fisher Scientific (Pittsburgh, PA). All oxidation and hydrolysis experiments were conducted at a constant temperature

(23.5 ± 1 °C). Oxidation of diazinon, diazoxon, and IPMP was carried out individually in the same manner, if not specified otherwise, with an initial concentration of 0.5 mg/L (1.64 μM), 0.47 mg/L (1.63 μM), and 0.25 mg/L (1.64 μM), respectively. Terbuthylazine was chosen as an internal standard when quantitation was performed. Before each injection for HPLC/MS analysis, 0.95 mL of the reaction medium was mixed with 0.05 mL of an internal standard solution (4.0 mg/L, in pH 7.01 NaH_2PO_4 buffer).

A Hitachi M-8000 3DQ LC/MS_n system with a sonic spray ion (SSI) source (San Jose, CA, USA) was used for the HPLC/MS analysis of diazinon and its degradation products. A Supelco C18 column (150 × 2.1mm i.d., 5μm, Bellefonte, PA, USA) was used for the separation, at an ambient temperature, with a flow rate of 0.25 mL/min Solvent A consisted of 0.1% formic acid in water (pH 2.70), and Solvent B was acetonitrile. The HPLC separation was performed at 10% B for 3 min followed by an increase to 95% B at 3.1 min for 6.9 min. After elution of 95% B, a drop to 10% at 10.1 min for 14.9 min was carried out. A diversion valve was placed immediately before the ionization source to remove the HPLC fractions containing salts, and to prevent the contamination of the ionization source and MS. The SSI parameters were set to the following optimized values: nitrogen sheath gas at 3 kgf/cm_2, 0 kV capillary voltage, 45 V drift plate voltage, 30 V focus plate voltage, 150ºC cover plate temperature, and 120ºC aperture 1 temperature. Ion trap MS was operated at the following conditions: 500 ms accumulating time, 0.088V accumulation voltage, 44.53 amu low mass cut off, and 44.53-450 amu scan range.

Free Chlorine (FC) Oxidation System. The concentration of free chlorine in a sodium hypochlorite stock solution was determined with the Hach DPD Method 8221

using AccuVac ampuls obtained from the Hach Company (Loveland, CO, USA). A twenty μL diazinon stock solution (in MeOH, 0.5 mg/mL) was spiked to 20 mL NaH_2PO_4 buffer (pH 6.60) in a 100-mL bottle (reactor). The initial concentration of diazinon was 0.5 mg/L (1.64 μM). 90 μL of 0.11 mg/mL hypochlorite solution was spiked to an initial FC concentration of 0.49 mg/L initiate a reaction. The reactor was wrapped with aluminum foil to prevent exposure to light, and was mixed at 150 rpm. Samples were taken at different times (and FC exposures) for analysis of diazinon, diazoxon, and IPMP by HPLC/MS analysis. FC oxidation of diazoxon and IPMP were carried out in the same manner as that of diazinon.

Monochloroamine (MCA) Oxidation System. MCA stock solutions were prepared from ammonium chloride and sodium hypochlorite at a molar ratio of 1.05:1 at pH 11 [23]. The concentration of a MCA stock solution was determined by using the total chlorine method (via Hach DPD Method 8167; Loveland, CO, USA) and confirming that no free chlorine concentration remained. This MCA stock solution was diluted 10 times to form a working stock solution. An amble glass reactor of containing 5 mL NaH_2PO_4 buffer solution (at pH 7.01) and 0.5 mg/L diazinon (1.64 μM) was spiked with 25 μL of the MCA stock solution for an initial MCA concentration of 0.9 mg/L. Samples were taken at different times (and exposures) for HPLC/MS analysis of diazinon, diazoxon, and IPMP. MCA oxidation of diazoxon and IPMP was carried out in the same manner as for diazinon.

Chlorine Dioxide (ClO_2) Oxidation System. Gaseous chlorine dioxide was produced using a Bench-Scale ClO_2 Generator (CDG, Bethlehem, PA). The concentration of ClO_2 in the generated saturated ClO_2 solution was determined by a Cary 50 Conc UV-

Visible Spectrophotometer (Varian Australia PTY LTD, Australia) at 360 nm. This ClO_2 solution was diluted 10 times to create the working stock solution. An amble glass vial (reactor) of 5 mL NaH_2PO_4 buffer solution (pH 7.01), which contained 0.5 mg/L diazinon (1.64 μM) was spiked with 48 μL working solution for an initial ClO_2 concentration of 3.0 mg/L. Samples were taken at different times (and ClO_2 exposures), followed by HPLC/MS analysis of diazinon, diazoxon, and IPMP. ClO_2 oxidation of diazoxon and IPMP was carried out individually in the same manner as that of diazinon.

Ozone (O_3) Oxidation System. Ozone was generated using a Model GLS-1 PCI-WEDECO (Environmental Technologies, West Caldwell, NJ, USA) ozone generator from compressed oxygen. The ozone gas stream was bubbled from a stone diffuser into buffered Milli-Q water with pH adjustment to 5.41. A Cary 50 Conc UV-Visible Spectrophotometer (Varian Australia PTY LTD, Australia) at 260 nm was then used to independently monitor the decay and concentration of the aqueous ozone. Four amble glass vials of 5.0 mL NaH_2PO_4 buffer solution (pH 5.41) containing 0.5 mg/L diazinon (1.64 μM), were each spiked with 0.5, 1.0, 2.5, or 10.0 mL of saturated O_3 solution resulting in initial O_3 concentration of 2.5, 4.6, 9.3, and 18.5 mg/L. The O_3 oxidation of diazoxon was performed in the same way as that of diazinon, with an initial concentration of 0.47 mg/L (1.63 μM). The concentration of components in the reaction media was normalized in terms of the initial reaction medium volume.

Hydrogen Peroxide (H_2O_2) Oxidation System. An amble glass vial (reactor) of 5 mL NaH_2PO_4 buffer solution (pH 7.01), which contained 0.5 mg/L diazinon (1.64 μM), was spiked with 224 μL H_2O_2 solution (30%) to initiate a reaction resulting in an initial H_2O_2= concentration of 13,000 mg/L. The reaction continued for 4 hours prior to

sampling and HPLC/MS analysis of diazinon, diazoxon, and IPMP. H_2O_2 oxidations of diazoxon and IPMP were carried out in the same manner.

UV Oxidation System. A 254-nm low-pressure mercury-vapor lamp (Pen Ray Model 90-0004- 01,254 nm, 1.0 W; UVP Inc., Upland, CA) was used for the UV photo-degradation study. Three amble glass vials (reactors) of 5 mL NaH_2PO_4 buffer solution (pH 7.01), each containing 0.5 mg/L diazinon (1.64 μM), were exposed to the UV lamp for 10, 25, and 45 seconds, respectively, by placing the 0.9 cm diameter lamp down the centerline of the vial. The diameter of the reactor was 1.9 cm, and the length of the lamp in the liquid was 2.5 cm. Based on a volume weighted mean radius for the fluid, the fluence was 8.9 mW/cm_2 for the system. The reaction medium was stirred with a small stirring bar during the UV exposure. The UV oxidation of diazoxon and IPMP were performed in the same manner as that of diazinon.

Results and Discussion

HPLC/MS. In order to confirm the two products observed in our study, standard diazoxon and IPMP were also analyzed by HPLC/MS. For example, Figure 1 showed the mass spectra of diazoxon and IPMP obtained from the total ion chromatogram for the free chlorine oxidation of diazinon. The same mass spectra patterns were obtained from diazoxon and IPMP standards as shown in their extracted ion chromatograms (Figure 2).

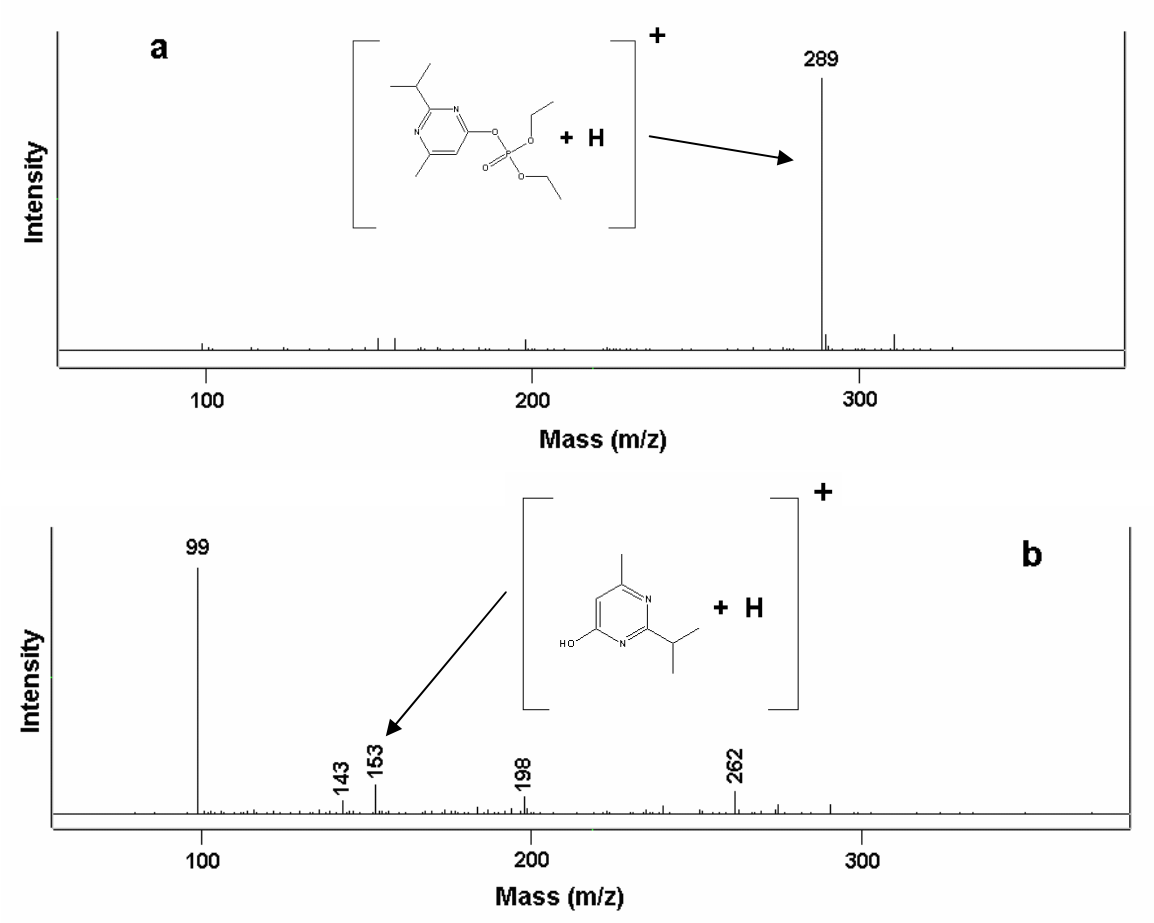

Figure 1. Mass spectrum of (a) diazoxon (MW: 288) and (b) IPMP (MW: 152) in the FC oxidation of diazinon system.

In order to assure that terbuthylazine was stable as an internal standard under the conditions in this study, terbuthylazine was treated with FC at pH 12.0 for 12 hours. The treated terbuthylazine solutions were then injected into LC/MS for analysis. The resulting relative standard deviations (RSD) of the peak areas before and after the treatment were less than 10%. Thus, terbuthylazine was determined to be a viable internal standard with the persistence at the experimental conditions, and also to have the characteristics of a similar retention time as that of the sample molecules (not shown), and an appropriate molecular weight (229 for the ^{35}Cl containing molecule).

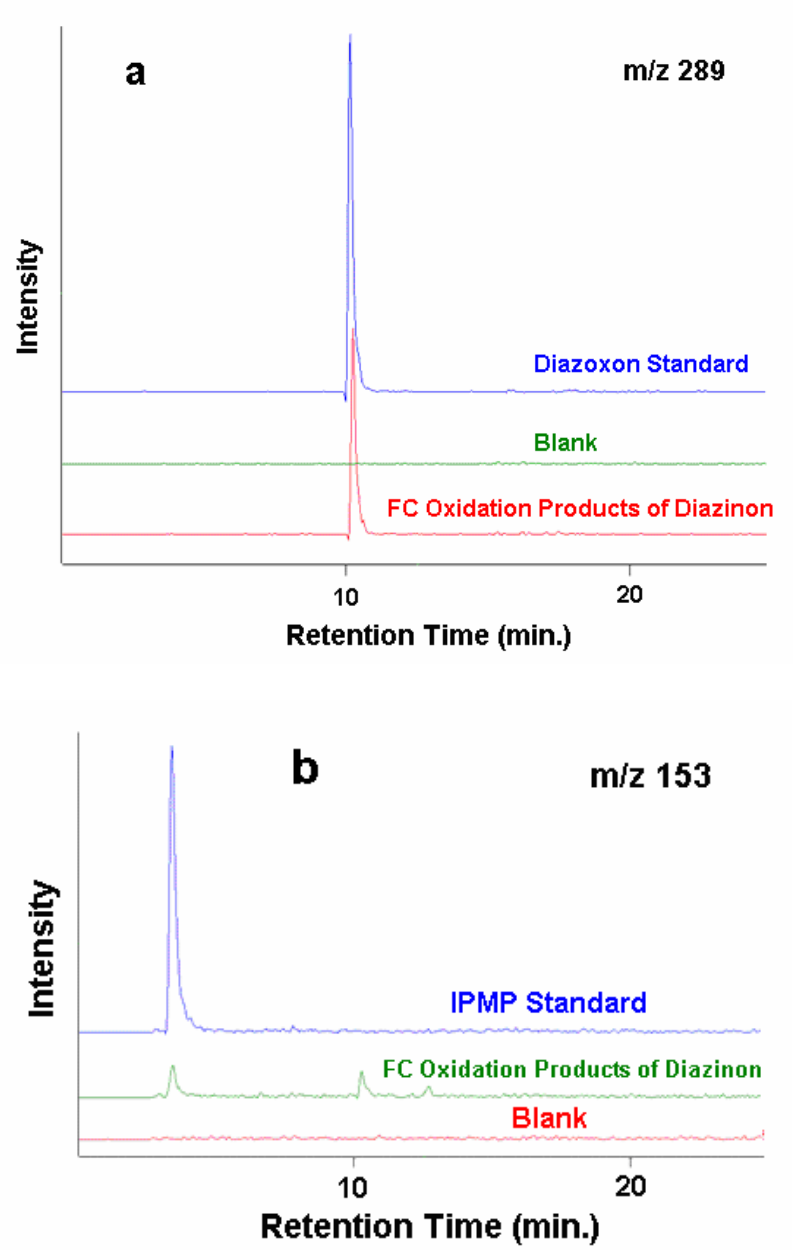

Figure 2. Extracted ion chromatograms at (a) m/z 289 and (b) m/z 153.

In order to determine and quantify diazinon, diazoxon, and IPMP in the oxidation reaction media, calibration curves were established for each of these components. Terbuthylazine was used as the internal standard with a spiked concentration of 2.0 mg/L. Terbuthylazine was also used for HPLC/MS analysis of diazinon oxidation systems. The

regression coefficient (R_2) values of calibration curves for diazinon, diazoxon, and IPMP were all greater than 0.995. The method detection limits were 0.005 mg/L for both diazinon and diazoxon, and 0.01 mg/L for IPMP.

Free Chlorine (FC). A typical CT for 3-log Giardia cyst inactivation with FC is approximately 60 mg/L·min (pH 7, 20°C) [24]. Based on the results shown in Figure 3a, it was observed that diazinon was oxidized to produce diazoxon and IPMP with a free chlorine exposure much less than these typical values. Based on a molar balance, diazoxon and IPMP were effectively the only degradates formed (to a chlorine exposure of 28 mg/L·min), and were formed in approximately a 4:1 molar ratio, respectively (Figure 3a).

The diazoxon and IPMP concentrations were both relatively constant after formation suggesting that diazoxon and IPMP were much more resistant to FC oxidation than diazinon (Figure 3a). This observation was confirmed in separate experiments under the same conditions, but with diazoxon and IPMP as the initial reactants. In both experiments, diazoxon and IPMP were observed to be relatively nonreactive with FC (Figures 3b and 3c, respectively). This suggests that diazinon is readily converted to diazoxon and IPMP during chlorination which, once formed, may be resistant to further oxidation by chlorine.

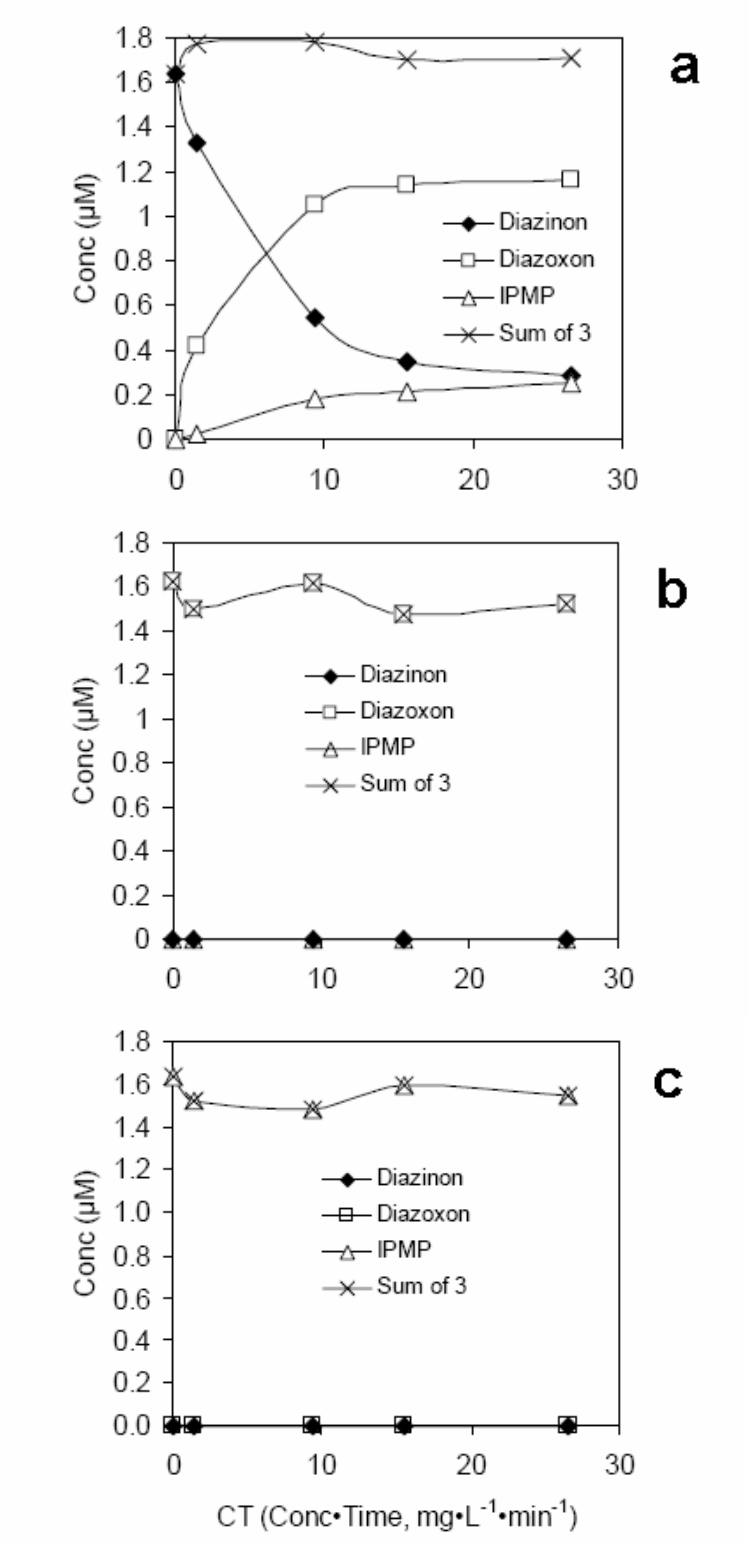

Figure 3. Free chlorine oxidation profiles of (a) diazinon system (initial concentration of diazinon: 1.64 μM), (b) diazoxon (initial concentration of diazoxon: 1.63 μM), and (c) IPMP system (initial concentration of IPMP: 1.64 μM). The experimental conditions are stated in the experimental section.

Monochloramine (MCA). A typical CT for 3-log Giardia cyst inactivation with MCA is approximately 1100 mg/L·min (pH 7, 20°C) [24]. Chloramination experiments of diazinon showed that diazinon could be oxidized to a limited degree to diazoxon and IPMP with MCA exposure several times greater than typical dosages (Figure 4a). Diazoxon and IPMP were formed in approximately a 3:1 molar ratio (Figure 4a). Both diazoxon and IPMP were relatively persistent to MCA oxidation during the experiments with their individual exposures (Figure 4b and 4c, respectively). Thus, while somewhat higher than typical MCA concentration appear to be needed to form appreciable diazoxon and IPMP, once formed they appear resistant to further oxidation by MCA.

Chlorine Dioxide (ClO_2). A typical CT for 3-log Giardia cyst inactivation with ClO_2 is approximately 15 mg/L·min (pH 7, 20°C) [24]. ClO_2 oxidation experiments of diazinon were conducted with 30 and 480 minute contact times corresponding to approximately 90 and 1440 mg/L·min CT exposures. Even with these exposures of 6 and 96 times typical disinfection exposures, only limited conversion of diazinon was observed (43 and 65%, respectively) (Table 1). Diazoxon was observed as a minor oxidation byproduct at both exposures, while a trace of IPMP was observed for the longer reaction time (Table 1).

Both diazoxon and IPMP were observed to be relatively stable and resistant to oxidation by ClO_2 in the experiments in which they were individually oxidized (Table 1). These results suggest that diazinon would not be appreciable degraded in typical ClO_2 disinfection situations. Further, if formed or present in a source water, diazoxon and IPMP would not be degraded by ClO_2.

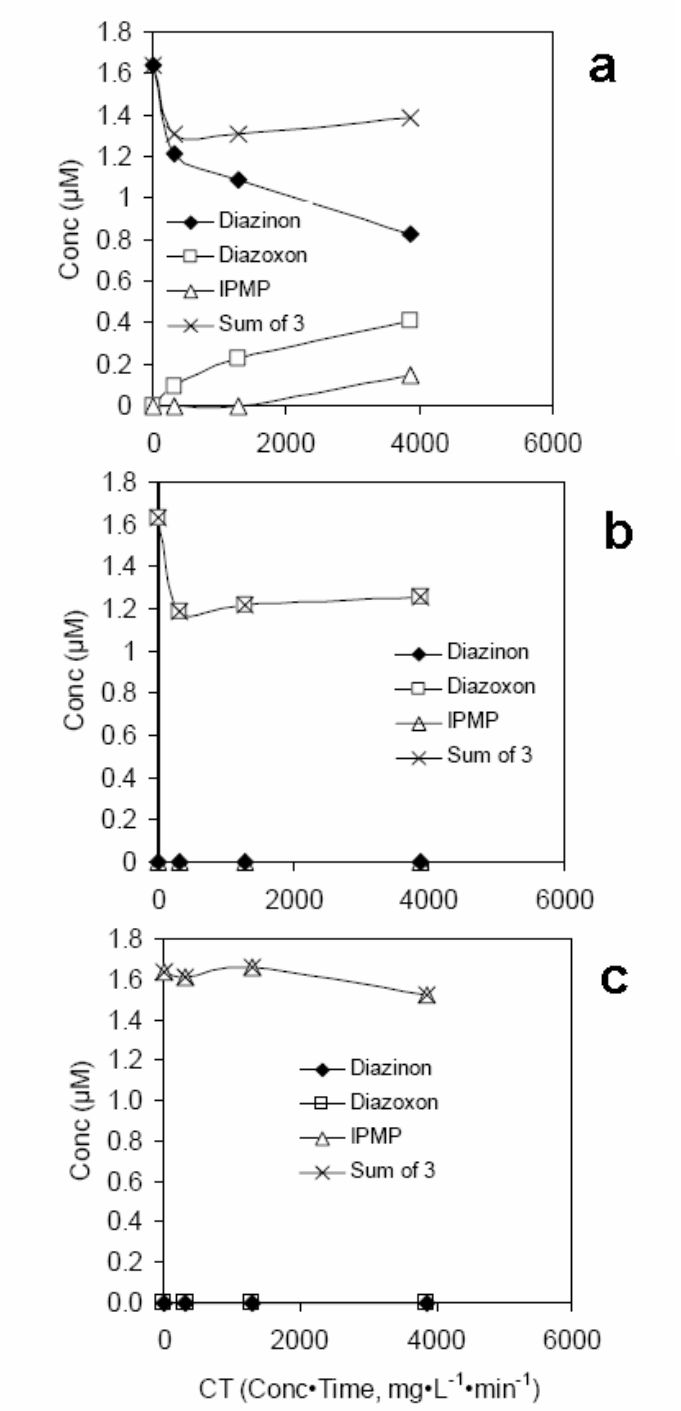

Figure 4. MCA oxidation profile of (a) diazinon (initial concentration: 1.64 µM), (b) diazoxon system (initial concentration: 1.63 µM), and (c) IPMP system (initial concentration: 1.64 µM).

Table 1. Concentration of the components in ClO_2 oxidation of diazinon system (initial concentration of diazinon: 1.64 µM).

	Reaction time (min)	Concentration (µM)		
		Diazinon	Diazoxon	IPMP
Diazinon-only spike (1.64 µM)	30	0.931	0.106	nd
	480	0.572	0.188	0.093
Diazoxon-only spike (1.63 µM)	30	nd	1.58	nd
	480	nd	1.49	nd
IPMP-only spike (1.64 µM)	30	nd	nd	1.52
	480	nd	nd	1.56

Ozone. A typical CT for 3-log Giardia cyst inactivation with O_3 is approximately 0.7 mg/L·min (pH 7, 20°C) (24). O_3 oxidation experiments of diazinon showed that diazinon was readily reactive with ozone and formed both diazinon and IPMP (Figure 5a). Additionally, oxidation of both diazinon and IPMP individually showed that both compounds were reactive towards ozone (Figure 5b and 5c). These results suggest that diazinon would be expected to be partially removed during ozonation, and that both diazinon and IPMP would likely be formed. However, with higher ozone dosages, the degradates concentrations may also be lowered through further oxidation by ozone.

Hydrogen Peroxide. H_2O_2 is not commonly used for drinking water disinfection. However, it is commonly used in water treatment for a wide variety of advanced oxidation and related processes. Thus, it was of interest to determine the reactivity of diazinon with H_2O_2. In there experiments, very high peroxide concentrations (e.g., 13,000 mg/L) and a long exposure (4 hours) produced only a 47% conversion of diazinon (Table 2). No diazoxon was observed, though a 14% yield of IPMP was observed. Oxidation of IPMP individually under the same conditions showed the IPMP was not reactive with

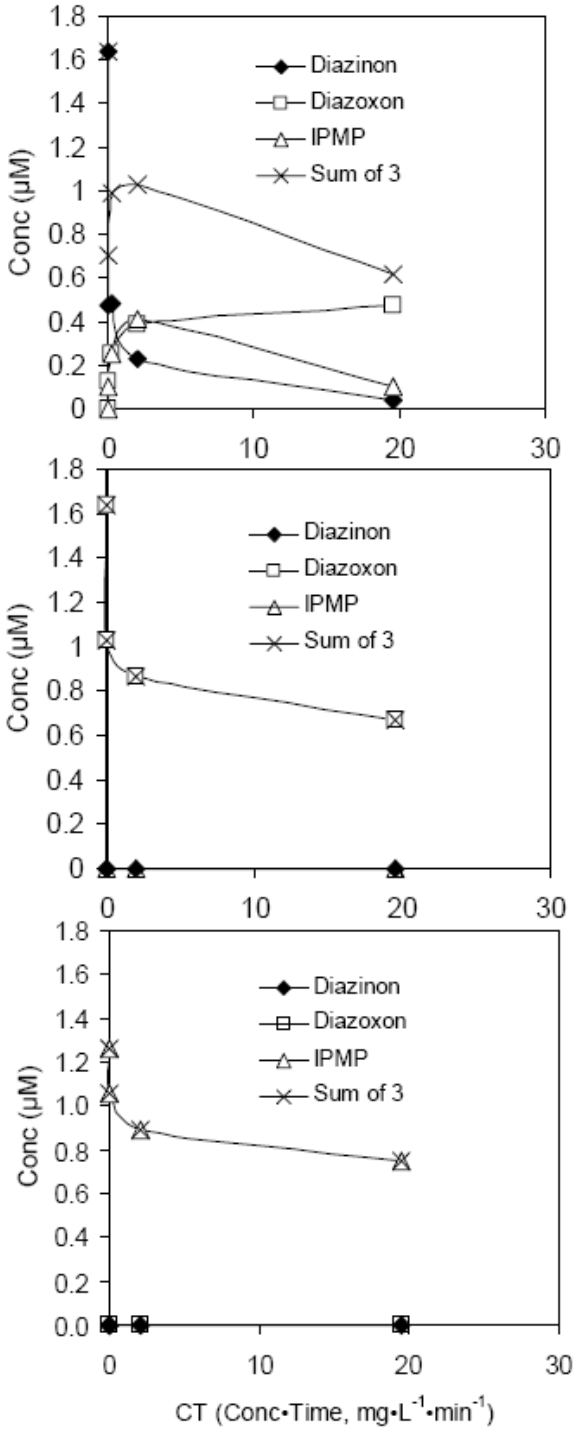

Figure 5. Ozone oxidation profiles of (a) diazinon system (initial concentration of diazinon: 1.64 μM), (b) diazoxon system (initial concentration of diazoxon: 1.63 μM), and (c) IPMP system (initial concentration: 1.64 μM).

peroxide (Table 2). Similarly, oxidation of diazoxon individually showed that it too was relatively recalcitrant to peroxide oxidation (Table 2). These results suggest that all three compounds are relative stable in the presence of H_2O_2 at disinfection dosages.

Table 2. Concentration of the components in H_2O_2 oxidation of three systems determined after 4 hours of treatment (initial concentration of diazinon: 1.64 μM; diazoxon: 1.63 μM; IPMP: 1.64 μM). Dosage: 4.25 × 105 (contact time min × concentration mg/L).

	Concentration after 4 hr ($3(10^5)$ mg/L•min)		
	Diazinon (μM)	Diazoxon (μM)	IPMP (μM)
Diazinon -nly spike (1.64 μM)	0.87	nd	0.222
Diazoxon-only spike (1.63 μM)	nd	0.93	nd
IPMP-only spike (1.64 μM)	nd	nd	1.83

UV Radiation. A typical CT for 3-log Giardia cyst inactivation with UV radiation is approximately 11 mJ/cm^2 (pH 7, 20°C) [24] though these exposures may range much higher (e.g., 40 mJ/cm^2). High UV exposures much greater than typically used for disinfection were required to achieve any significant diazinon removal (Figure 6a). Furthermore, only a very low concentration of IPMP was detected, and no diazoxon, was detected (Figure 6a). Separate experiments in which diazoxon and IPMP were exposed individually showed that these degradates were relatively stable during UV photolysis with high UV exposures (Figures 6b and 6c, respectively).

These experiments show that at very high UV dosages, diazinon may be degraded but that this is highly unlikely in a disinfection scenario. Further, if diazoxon and/or IPMP were formed by some other process (e.g., chlorination), subsequent UV disinfection would not be effective for their removal.

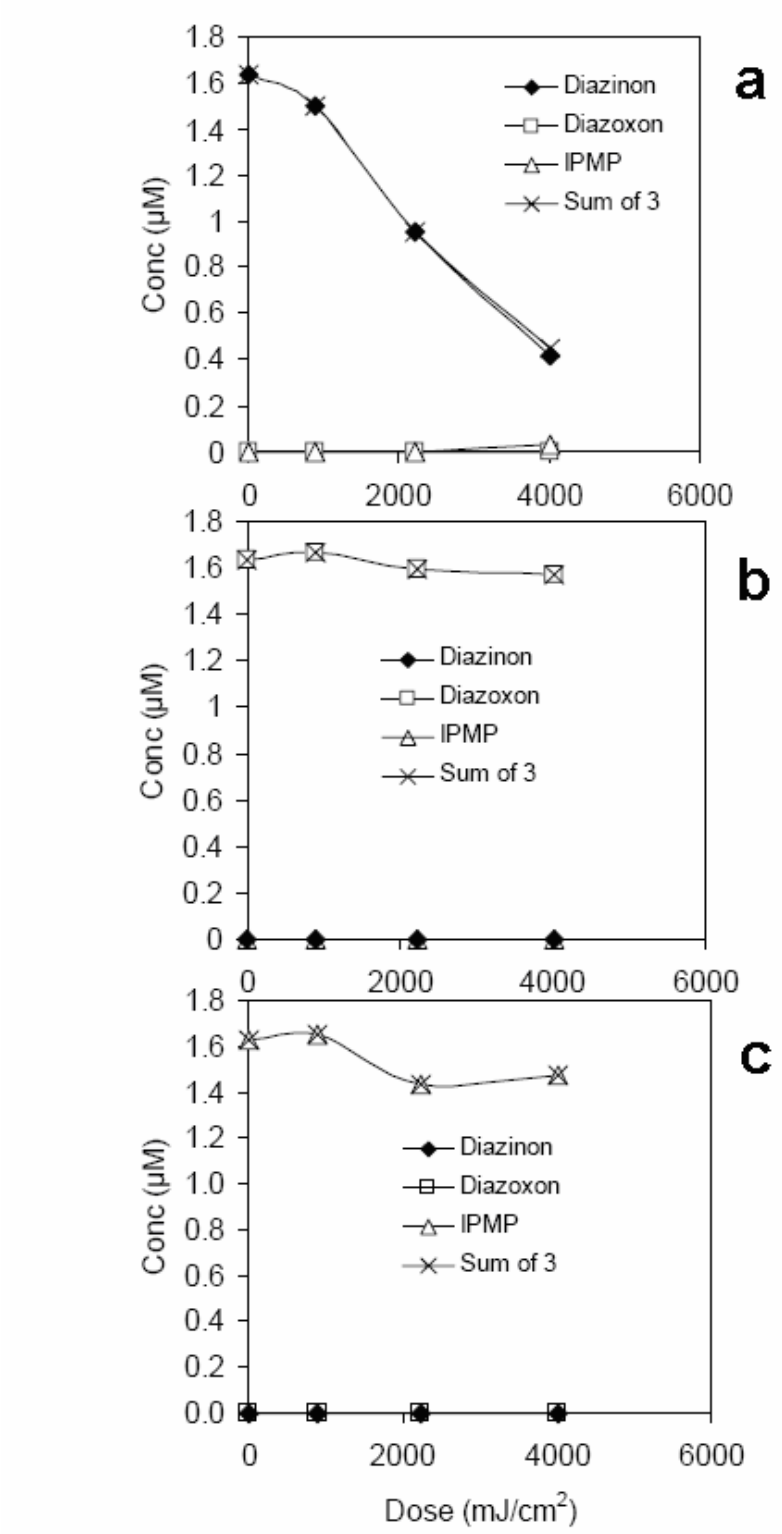

Figure 6. UV radiation profiles of (a) diazinon system (initial concentration of diazinon: 1.64 μM), (b) diazoxon system (initial concentration of diazoxon: 1.63 μM), and (c) IPMP system (initial concentration of IPMP: 1.64 μM).

Conclusions

Diazinon appears likely to be oxidized to diazoxon during chlorine and ozone disinfection, and possibly during chloramination (depending on the CT used). Little conversion of diazinon during typical ClO_2, peroxide or UV contact in a drinking water treatment plant is likely. Diazoxon is formed by an oxidative process, while it is hypothesized that IPMP is formed in an oxidative-assisted hydrolysis reaction (Figure 7).

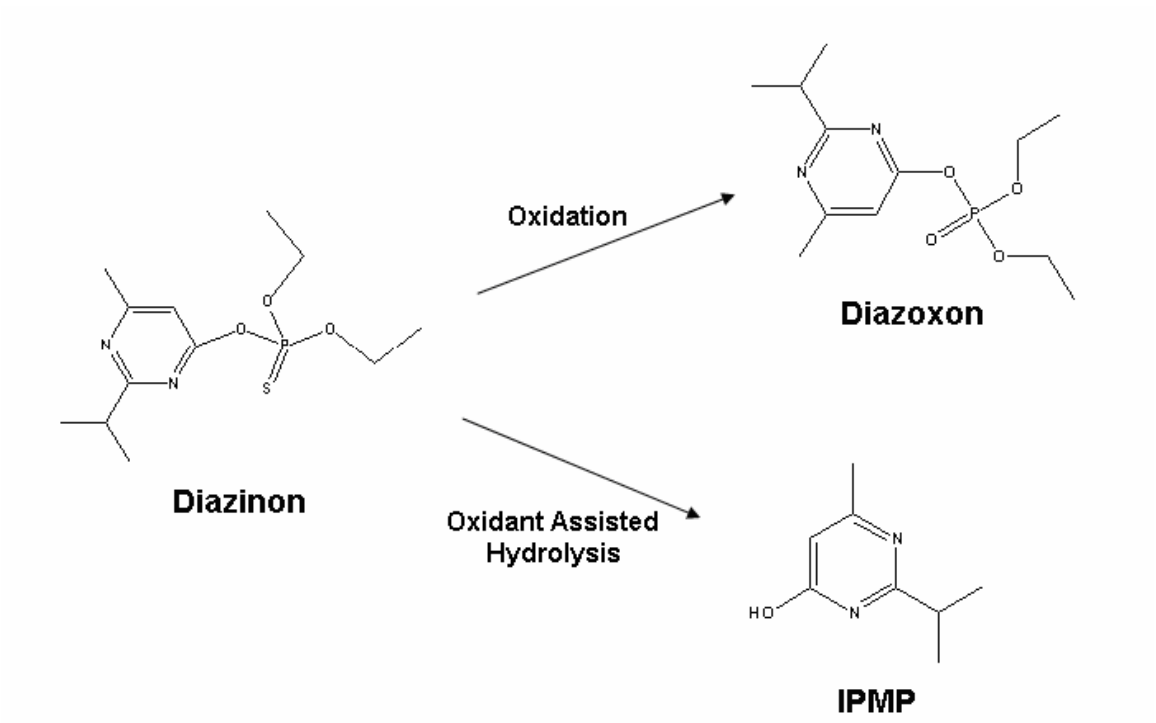

Figure 7. Proposed pathway of oxidation of diazinon.

Log K_{OW} values were estimated for diazinon, diazoxon and IPMP using KOWIN software as 3.9, 2.1, and 2.2, respectively. These estimates suggest that both diazoxon and IPMP are much more hydrophilic than diazinon and would, therefore, be expected to behave differently than diazinon. While experimental validation is required for confirmations, the significantly lower Log K_{OW} values would suggest the degradates would have a much lower propensity for adsorption to, for example, powdered or

granular activated carbon than diazinon. Baseline toxicity for fish estimates were also made using ECOWIN software which estimated LC_{50} concentrations (the concentration for 50% lethality) of 10, 317, and 134 mg/L for diazinon, diazoxon and IPMP, respectively. These estimates suggest that the degradates may be less toxic than the parent pesticide, at least to fish.

Acknowledgments

The authors thank the AwwaRF Foundation (project 3170) for financial support of this project. Additional support was provided by the Department of Chemistry; the Department of Civil, Architectural and Environmental Engineering; and the Environmental Research Center for Emerging Contaminants at the University of Missouri-Rolla. Thanks to Ms. Barbara Harris for her technical editing of this manuscript.

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3. Analysis of Oxidation Byproducts of Fonofos in Three Oxidation Systems Using High Performance Liquid Chromatography Coupled with Quadrupole Ion Trap Mass Spectrometry

In this paper, oxidation of fonofos (fonofos ; CAS 944229) by free chlorine, H_2O_2, and permanganate in an aqueous buffer (pH 7) was performed to identify the oxidation byproducts for each oxidant. High pressure liquid chromatography/mass spectrometry (HPLC/MS) was employed to separate and analyze the byproducts. Thin layer chromatography (TLC) and nuclear magnetic resonance (NMR) were also used for structure confirmation of the byproduct that identified through HPLC/MS. One byproduct, fonofos oxygen analog (phosphonothioic acid, ethyl-, o-ethyl s-phenyl ester; CAS 944218) was identified as the primary oxidation byproduct for both free chlorine and H_2O_2 systems, and no byproduct was detected for the permanganate system.

Introduction

Fonofos (fonofos, O-ethyl S-phenyl ethylphosphonodithioate; CAS 944229) is a dithiophosphonate pesticide used to control lepidopterous insects in corn, potatoes, and peanuts. This highly toxic chemical interferes with the nervous system by inhibiting an enzyme, cholinesterase. Symptoms of fonofos exposure may be delayed for a few minutes after exposure to up to twelve hours. Early symptoms include blurred vision, headache, and dizziness. Skin contact often brings about sweating and muscle twitching.

Eye contact causes tearing, pain, and blurring. Ingestion may cause nausea, abdominal cramps, and diarrhea [1].

Determination of fonofos residue has been accomplished by various approaches in different matrices. Pang *et al.* reported the determination of fonofos in grain by accelerated solvent extraction then gas chromatography-mass spectrometry or liquid chromatographytandem mass spectrometry [2]. Hirahara *et al.* used gas chromatography to establish a screening method for 186 pesticides (including fonofos residue) in 11 agricultural products [3]. Zhang *et al.* utilized a combination of gel permeation chromatography and Florisil column purification, and gas chromatography/mass spectrometry to simultaneously determine 109 pesticides (including fonofos) in unpolished rice [4].

Fonofos residue in honey and honeybees was determined by various extractions followed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LCAPCI-MS), or electrospray ionization-mass spectrometry (LC-ESI-MS) [5-7]. Wong *et al.* used solid phase extraction, followed by capillary gas chromatography with electron impact mass spectrometry in the selected ion monitoring mode [GC-MS(SIM)], to determine multiresidues of pesticides (including fonofos) in wines and malt beverages[8,9]. Capillary electrophoresis with cyclodextrin chiral selectors was also used to determine the enantiomers of fonofos in aerobic soil slurries [10]. Solid phase micro-extraction, followed by gas chromatography with mass spectrometry, was used to determine various pesticides (including fonofos) in a confined atmosphere [11] and whole human blood [12].

Determinations of biologically-derived fonofos were reported by some researchers. An off-line coupling of thin-layer chromatography (TLC) with ESI-MS for routine determination of pesticides in toxicology and forensic medicine was reported by Brzezinka *et al.* [13]. A comprehensive two-dimensional gas chromatograph with flame ionization detection was constructed and evaluated for the fast separation and analysis of fonofos extracted from human serum [14]. Russo *et al.* employed ethanol-ethyl acetate extraction followed by gel permeation chromatography clean-up step and capillary gas chromatography with negative chemical ionization mass spectrometry in the selected ion monitoring mode to analyze fonofos residue in human tissues [15].

Determination of fonofos in water was also reported by Cappiello *et al.* [16] using nano-HPLC coupled with direct-electron ionization mass spectrometry, and by Sabik *et al.* [17] using filtration and solid phase extraction, followed by gas chromatography/mass spectrometry (GC/MS) with large-volume injection. Tse *et al.* determined fonofos in water, sediment, and biota using dichloromethane or acetone/hexane extraction and micro-column silica gel chromatography cleaning-up, followed by dual capillary column gas chromatography with both nitrogen-phosphorus (NPD) and electron capture (ECD) detection [18].

Fonofos, when used in agriculture, may transfer to the ground and underground water system, although a large part of it may have been absorbed and filtered by the soil or other absorbents. The fonofos residue can be degraded by free chlorine, the oxidant most frequently used by water treatment plants during the disinfection process, to produce oxidation byproducts. These byproducts may be more toxic than fonofos itself. Because of the potential human health implications, it is crucial that the oxidation

byproducts of fonofos be identified and that both fonofos and its oxidation byproducts be monitored during in the water treatment process.

Studies on the metabolism of fonofos in biological systems have also been carried out. McBain *et al.* investigated the oxidation byproducts of fonofos in a microsomal oxidation system [19]. Onisko *et al.* identified the metabolites of fonofos in several plants by packed capillary flow fast atom bombardment tandem mass spectrometry [20]. Metabolic degradation of fonofos in potato plants was investigated by McBain *et al.* using chromatographic and autoradiographic methods [21].

Peracid oxidation of fonofos in organic solvent systems has been studied by several researchers [19, 22]. However, the free chlorine, H_2O_2 and permanganate oxidation of fonofos in water treatment plants have not yet been reported up to now. This paper investigated the oxidation of fonofos by these oxidants in an aqueous buffer using HPLC coupled with quadrupole ion trap mass spectrometry, and the identified oxidation byproduct of fonofos was further confirmed using TLC and MS/MS. The results of this study can serve as a useful reference for water treatment plants.

Experimental Section

Reagents and Chemicals. Fonofos (99.5%, CAS 944229) was purchased from ChemService (West Chester, PA, USA). Formic acid (96%, ACS grade), sodium hypochlorite solution (available chlorine ≥ 4%), and hydrogen peroxide solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC grade), acetonitrile (HPLC grade), water (HPLC grade), sodium hydroxide (98.3%), sodium phosphate (dibasic, 99%), potassium permanganate (ACS reagent), chloroform (HPLC

grade, 99.8%), 2,2,4-trimethylpentane (ACS reagent), methylcyclohexane (99.9%), isoamyl alcohol (ACS reagent), paraffin oil, and acetone (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Thin layer chromatography plates (silica gel 60 Å with fluorescent indicator) were purchased from Whatman (Florham Park, NJ, USA).

Fonofos was dissolved in methanol, with concentrations of 10 mg/mL as the stock solution, and stored in a freezer. Further dilutions of the stock solution were applied, depending on the individual experiments.

Sodium hypochlorite stock solution was prepared by diluting the sodium hypochlorite solution (available chlorine ≥ 4%) to a final concentration of 0.05% (0.5g/L). This stock solution was stored in a refrigerator.

Potassium permanganate stock solution was prepared by dissolving potassium permanganate in water with a concentration of 1.0 g/L. This stock solution was stored in a refrigerator.

Instruments. The Hitachi M-8000 3DQ LC/MS^n system with an electrospray ion source (San Jose, CA, USA) was used for the HPLC/MS analysis of fonofos and its degradation products. A Supelco C18 column (150 × 2.1mm i.d., 5μm, Bellefonte, PA, USA) was used for separation at an ambient temperature, with a flow rate of 0.25 mL/min. Solvent A consisted of 0.1% formic acid in water (pH 2.70), and solvent B was acetonitrile. The HPLC separation was performed at 10% B for 3.5 min, followed by a jump to 95% B at 3.6 min. for 9.4 min. After elution of 95% B, a drop to 10% at 13.1 min. for 14.9 min. was carried out. A divert valve was placed right before the ionization source to remove the HPLC fractions containing salts and to prevent contamination of the

ionization source and MS. The ESI parameters were set to the following optimized values: nitrogen sheath gas at 3 kgf/cm2, 4 kV for ESI probe, 30 V for drift, 25 V for focus, 450 V for detector. 200ºC for assistant gas heater, 180ºC for desolvator, 160ºC for aperture 1, 120ºC for aperture 2. Ion trap MS was operated at the following conditions: 500 ms for accumulating time, 0.069V for accumulation voltage, 41.78 amu low mass cut off, 41.78-450 amu for scan range.

Methods. All pH measurements were obtained with an Accumet XL 15 pH meter using an Accumet AccuCap combination pH electrode from Fisher Scientific (Pittsburgh, PA). All oxidation were conducted at an ambient temperature (23.5 ± 1 °C).

Free Chlorine (FC) Oxidation System. The concentration of free chlorine in sodium hypochlorite stock solution was determined with the Hach DPD Method 8221 using AccuVac ampuls obtained from the Hach Company (Loveland, CO, USA). A 50 μL aliquot of fonofos stock solution (10 mg/mL in methanol) was spiked into a 100 mL NaH_2PO_4 buffer (pH 7.01), forming an initial concentration of 5.0 mg/L (20.25 μM) for fonofos. A 1.0 mL sodium hypochlorite stock solution (free chlorine: 0.5 g/L) was spiked and the reaction was initiated. The reactor was wrapped with aluminum foil to prevent its exposure to light and was shaken at 150 rpm. Samples were taken after 2 hours, followed by HPLC/MS analysis.

Hydrogen Peroxide (H_2O_2) Oxidation System. A 50.0 μL aliquot of fonofos stock solution (10 mg/mL in methanol) was spiked into a 100 mL NaH_2PO_4 buffer (pH 7.01), forming an initial concentration of 5.0 mg/L (20.25 μM) for fonofos. A 1.0 mL hydrogen peroxide solution (30%) was spiked and the reaction was initiated. The reactor

was wrapped with aluminum foil to prevent its exposure to light and was shaken at 150 rpm. Samples were taken after 4 hours, followed by HPLC/MS analysis.

Permanganate (MnO_4^-) Oxidation System. A 50 μL aliquot of fonofos stock solution (10 mg/mL in methanol) was spiked into a 100 mL NaH_2PO_4 buffer (pH 7.01), forming an initial concentration of 5.0 mg/L (20.25 μM) for fonofos. A 1.0 mL potassium permanganate stock solution (1.0 g/L) was spiked and the reaction was initiated. The reactor was wrapped with aluminum foil to prevent its exposure to light and was shaken at 150 rpm. Samples were taken after 4 hours, followed by HPLC/MS analysis.

Thin Layer Chromatography. The reaction matrix of free chlorine oxidation of fonofos was extracted with 1.0 mL chloroform. 30 μL of the extract was spotted on a thin layer chromatography plate (with fluorescent indicator), followed by developing with a solvent consisting of 2,2,4-trimethylpentane : methylcyclohexane : isoamyl alcohol : paraffin oil : acetone (4:2:2:3:1). Visualization was performed under a UV light with a wavelength of 254 nm.

Results and Discussion

Identification of Free Chlorine Oxidation Byproducts of Fonofos: Identification by HPLC-MS. HPLC/MS was performed to search for and identify the free chlorine oxidation byproducts of fonofos. Figure 1 shows the chromatograms of blank, sample, and control. The sample experiment was carried out as described above, and the blank experiment was conducted in the same way, except the 50 μL aliquot of fonofos stock solution was replaced by 50 μL of methanol. The control experiment was also carried out

in the same manner, except the 1.0 mL sodium hypochlorite stock solution with 1.0 mL water.

By comparing the total ion chromatogram of the control with that of the blank, a fonofos peak can be identified as labeled in Figure 1. An unknown peak was found for the sample, as labeled in Figure 1. In order to confirm the fonofos peak and to identify the unknown peak, the mass spectra for both peaks are shown in Figure 2.

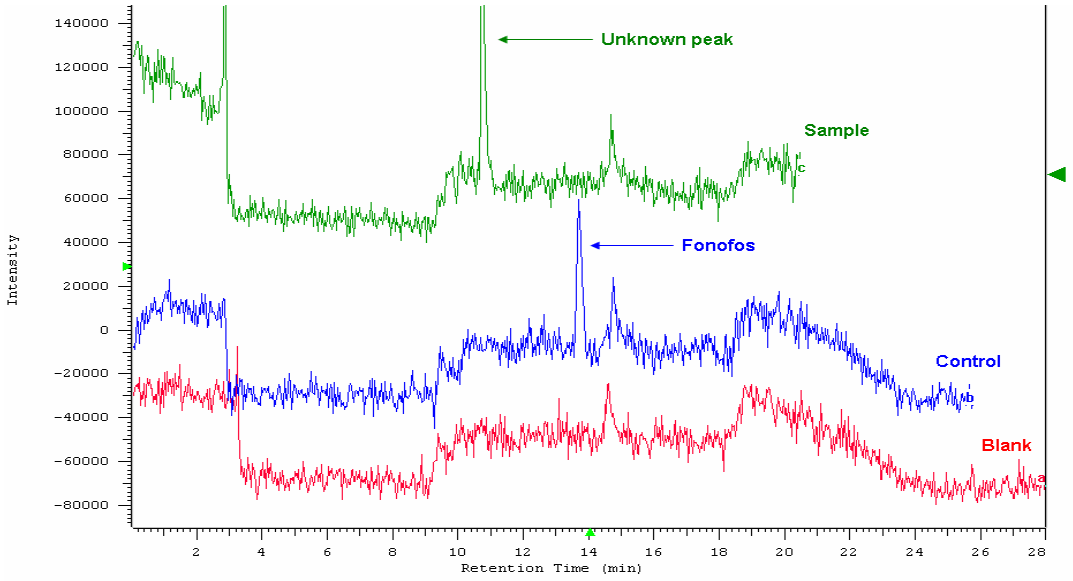

Figure 1. Total ion chromatograms of free chlorine oxidation of fonofos at pH 7.0.

Figure 2 indicates that fonofos (MW: 246) produced a protonated ion, while the unknown also produced a protonated ion with m/z 231. The extracted ion chromatograms for free chlorine oxidation of the fonofos system are presented in Figures 3 and 4, further confirming that the unknown peak readily reflected the oxidation product of fonofos.

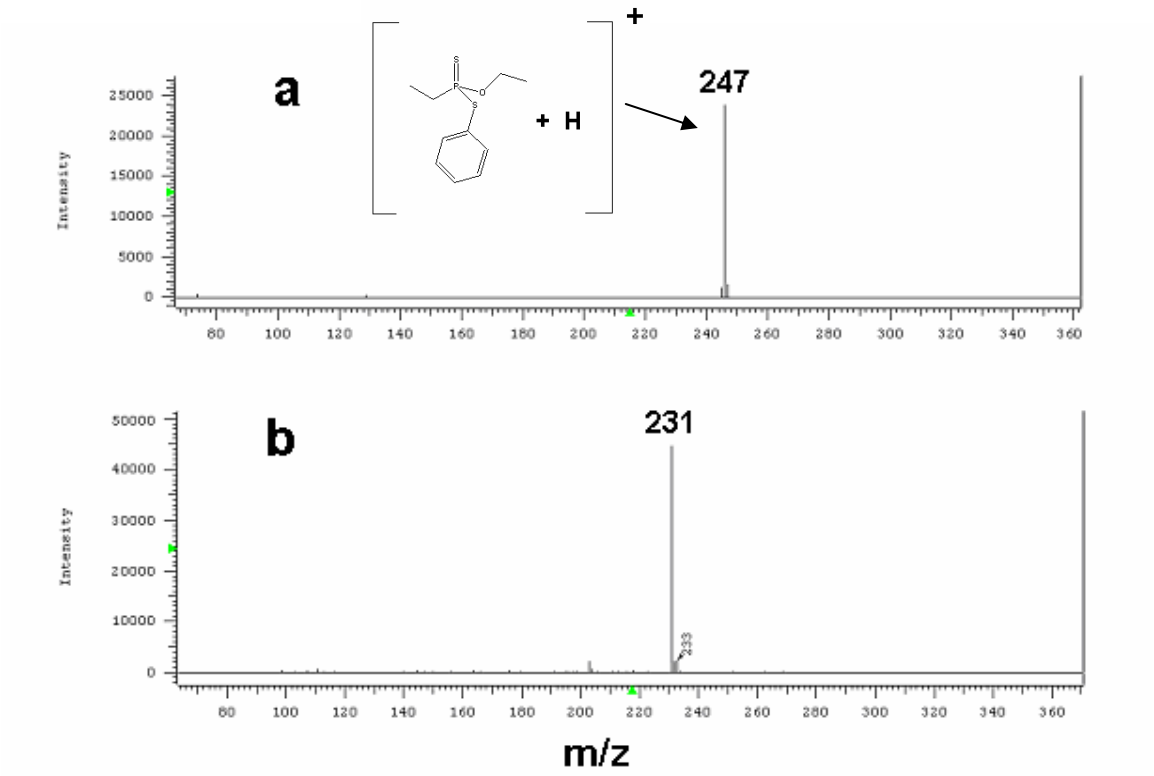

Figure 2. Mass spectra of (a) fonofos (MW: 230) and (b) unknown.

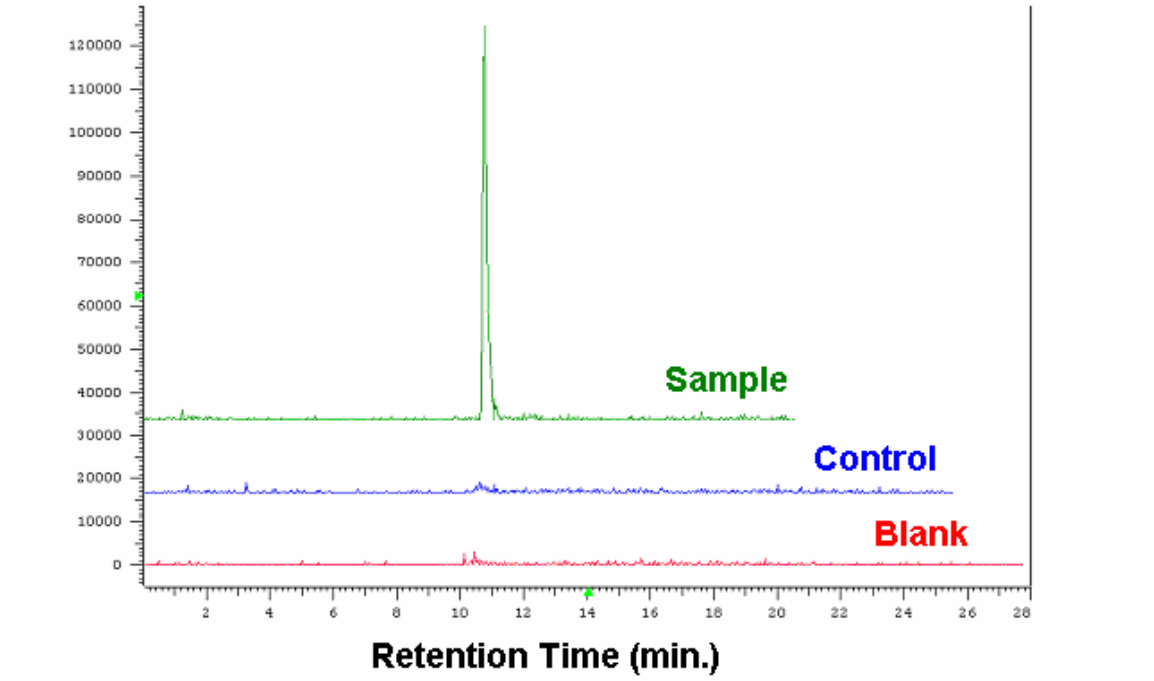

Figure 3. Extracted ion chromatograms of free chlorine oxidation of fonofos in pH 7.0 at m/z 231 (for the unknown).

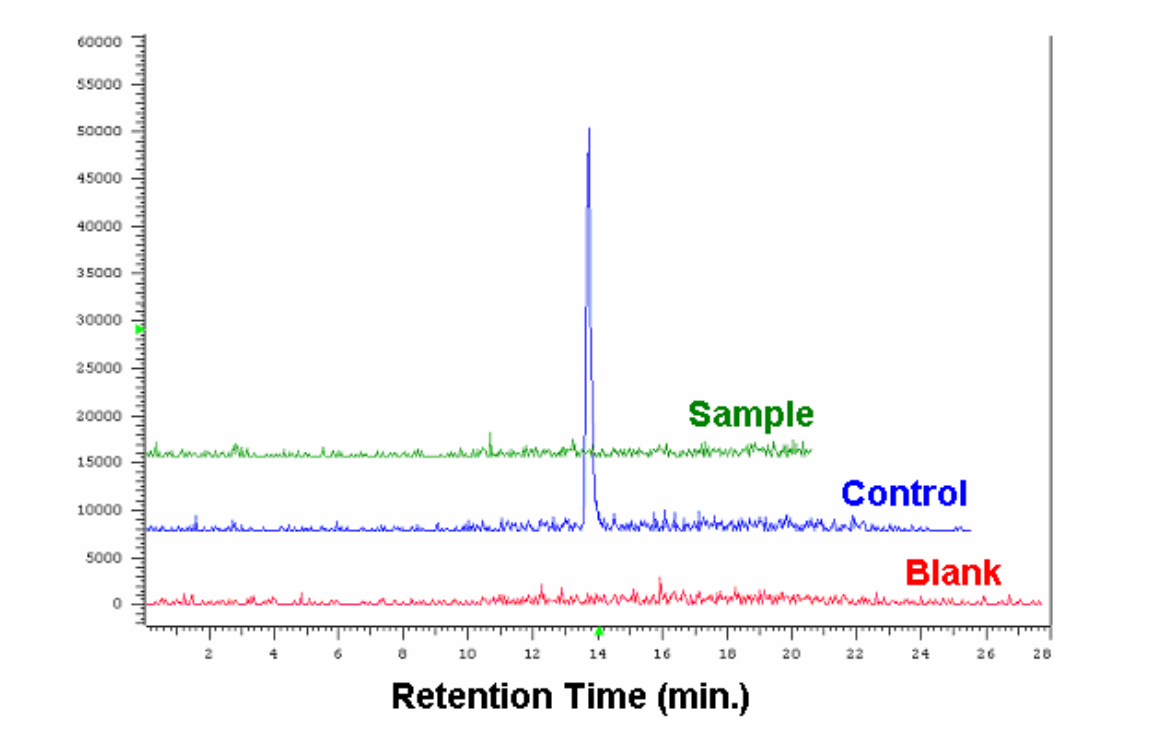

Figure 4. Extracted ion chromatograms of free chlorine oxidation of fonofos in pH 7.0 at m/z 247 (for fonofos).

Based on the m/z value and the results of other researchers [19], the unknown was proposed to be fonofos oxygen analog, as shown in Figure 5.

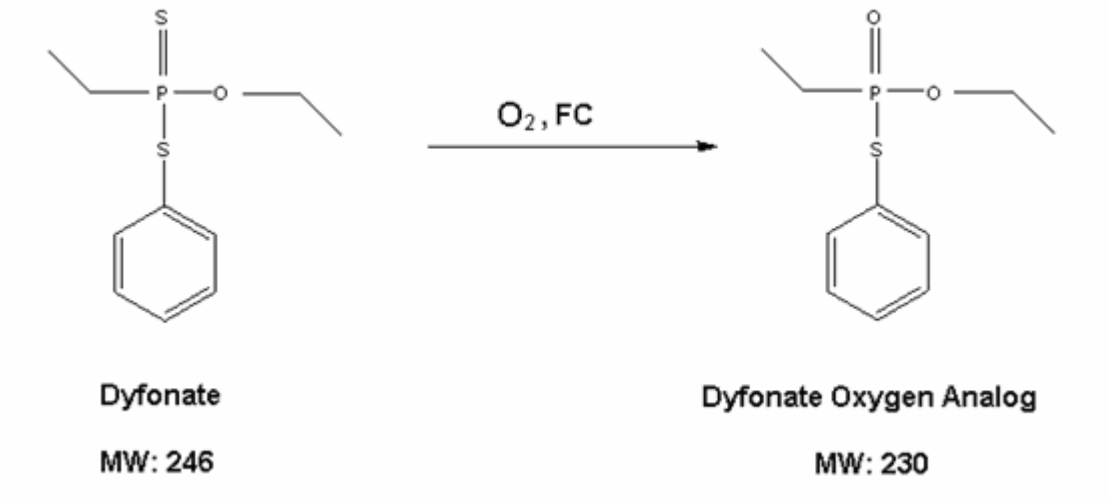

Figure 5. Proposed mechanism of free chlorine oxidation reaction of fonofos.

Identification of Free Chlorine Oxidation Byproducts of Fonofos:

Identification Confirmation by TLC-HPLC-MS. Before the TLC analysis, HPLC/MS analysis was performed to validate the efficiency of liquid-liquid extraction. A comparison of the chromatograms of free chlorine oxidation of fonofos before and after extraction showed that fonofos analog was completely extracted to chloroform (data not shown). TLC analysis of free chlorine oxidation products of fonofos showed a spot with an R_f value of 0.72, which was confirmed by scratching the spot and dissolving it in methanol, HPLC/MS analysis, indicating that the spot was really fonofos oxygen analog (data not shown). The measured R_f value was very close to the reported one [23], further confirming that the unknown in the free chlorine oxidation of fonofos is fonofos oxygen analog.

Identification of Free Chlorine Oxidation Byproducts of Fonofos:

Identification Confirmation by Tandem MS. The spot obtained by TLC was dissolved in methanol, and was injected into mass spectrometer for tandem MS analysis with the continuous flow injection mode. The mass spectrum of daughter ions obtained by CID (0.150 V for CID voltage) was shown in Figure 6. The fragmentation reaction pathway was proposed in Figure 7. Tandem MS operation provided a positive evidence for the elucidation of structure of fonofos oxygen analog.

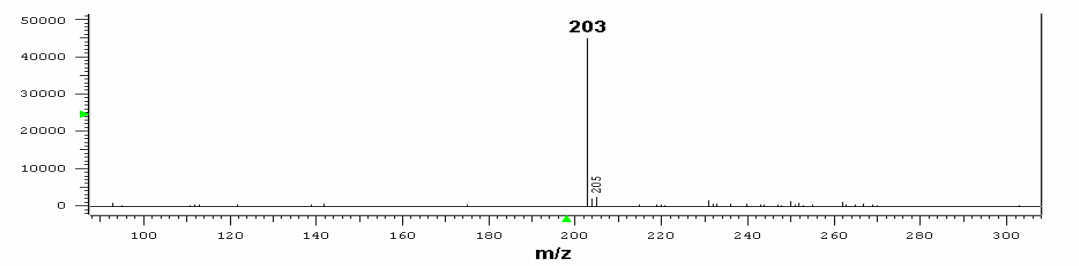

Figure 6. Mass spectrum of MS^2 of fonofos oxygen analog.

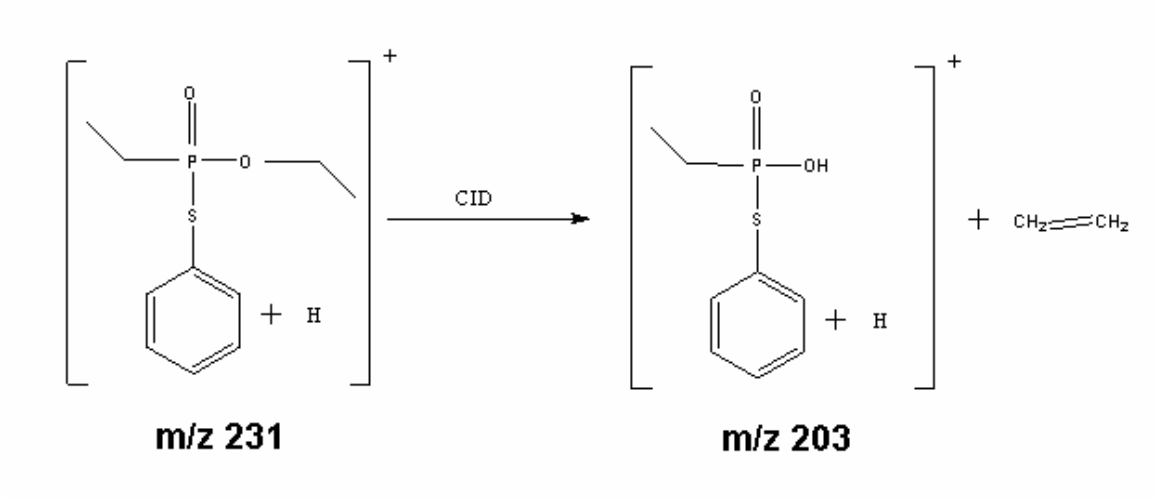

Figure 7. Proposed CID fragmentation reaction for fonofos oxygen analog.

Identification of H_2O_2 Oxidation Byproducts of Fonofos. H_2O_2 oxidation of fonofos was performed as described in the experimental section. Figure 8 shows that after 4 hours' reaction with H_2O_2, a certain amount of fonofos oxygen analog was found based on the retention time of the peak in the corresponding extracted ion chromatogram at m/z 231, while 30% of fonofos remained in the system. This indicates that H_2O_2 possesses the oxidation ability for transferring fonofos into fonofos oxygen analog.

Identification of Permanganate Oxidation Byproducts of Fonofos. The results (chromatograms not presented) showed that after 4 hours' reaction with $KMnO_4$, no fonofos oxygen analog was detected, and that fonofos remained in the same concentration in the system. This indicates that $KMnO_4$ is not able to oxidize fonofos under the experimental conditions in this study.

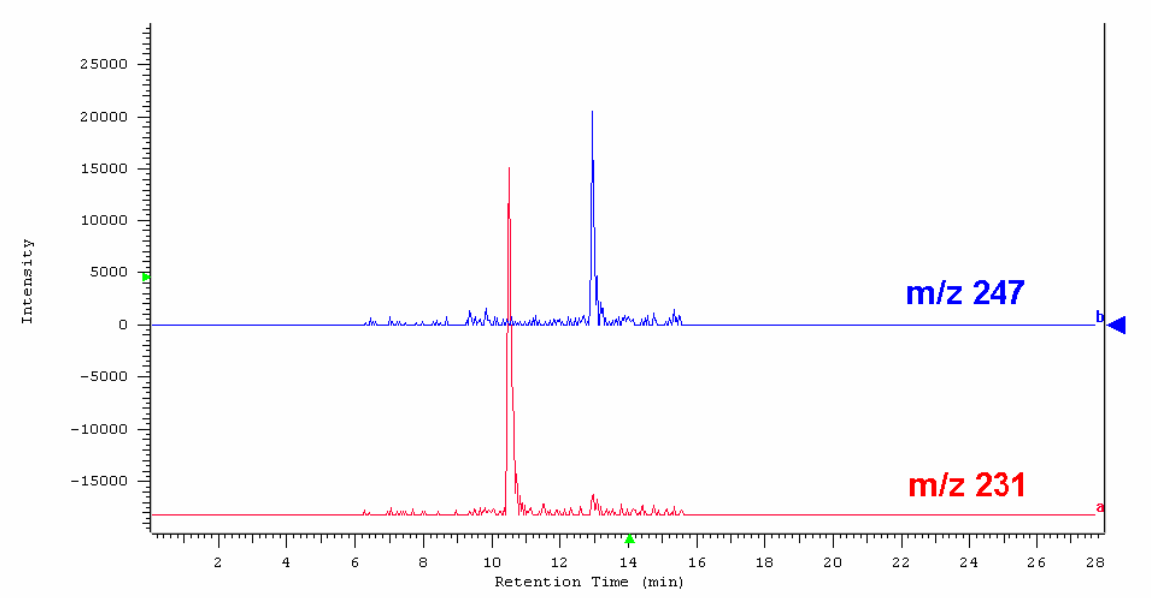

Figure 8. Extracted ion chromatograms of H_2O_2 oxidation products of fonofos at m/z 231 and 247.

Estimated Properties of Parent and Degradate. To estimate the fate and effects in the water treatment systems of the fonofos oxygen analog in comparison with the parent, fonofos, key chemical properties were estimated using chemical computational software and/or experimental literature values. First, potential dissociation constants were estimated using SPARC (SPARC Performs Automated Reasoning in Chemistry) software [24,25]. Neither fonofos nor its oxygen analog were estimated to have any ionized forms and, hence, no dissociation constants.

Log octanol-water partition coefficients (log Kow) were estimated using KOWWIN (ver. 1.65) to be 4.02 and 2.26 (both checks) for fonofos and its oxygen analog, respectively. These values compare well with literature values of 3.94 and 2.11 [26] (both checks), respectively. These values were used by WSKOW (ver. 1.36) to calculate estimated water solubilities of 10.7 and 479 (both checks) mg/L, for the parent and degradates, respectively. These data suggest that the degradates (the oxygen analog)

would most likely have a much higher affinity for the aqueous phase than the parent, fonofos. This would suggest that the degradates would have a much lower capacity to adsorb on activated carbon during water treatment or to sorb to other solids. Thus, the oxygen analog degradate would be anticipated to be significantly more difficult to treat (i.e., remove) the parent, fonofos, by either powdered activated carbon (PAC) or granular activated carbon (GAC) during water treatment.

To compare volatilities of the parent and oxygen analog degradate, HENRYWIN (ver. 3.04) was used to estimate Henry's Law constants of $1.12(10^{-4})$ and $2.41(10^{-7})$ (checks) $atm \cdot m^3 \cdot mole^{-1}$, respectively. These estimates would suggest that fonofos would be significantly more volatile than its oxygen analog. This is consistent with the estimated (and experimentally determined)

Estimates of fate in wastewater treatment plants were compared using EPIWIN (ver. 3.04). For fonofos, a total removal in a wastewater treatment plant of 27.6% was estimated based on a biodegradation removal of 0.3% and a 27.1% sorption rate to biosolids. For the oxygen analog degradates, a much lower removal of 2.4% was estimated, with biodegradation and sorption to biosolids accounting from 0.1 and 2.3%, respectively. These estimates suggest that neither the parent nor the degradate would be readily biodegradable. Furthermore, the degradate would be much less likely to be removed than fonofos during wastewater treatment, and thus, more likely to enter the environment.

Finally, toxicity of the parent and degradate were estimated using ECOSAR (ver 0.99e) which estimated a 14-day baseline toxicity LC_{50} (50-percent lethal concentration) of 5.7 and 184 mg/L, respectively. While the accuracy of computational software for

chemical properties and toxicity varies, this estimate does suggest that the oxygen analog degradate is less toxic than the parent, at least to fish. Therefore, though the oxygen analog degradate may be formed in water (and wastewater) disinfection, and may be more difficult to treat with less removed, this is partially offset from a risk perspective by the degradate's apparent lower toxicity.

Conclusions

The oxidation of fonofos in an aqueous buffer by free chlorine, H_2O_2, and permanganate was investigated. One oxidation byproduct, fonofos oxygen analog, was identified in free chlorine and the H_2O_2 system, while no oxidation byproduct was detected in the permanganate system. TLC, tandem MS and NMR experiments were performed to further confirm the identification of the free oxidation byproduct of fonofos. Due to the lower sorptive removal of degradates from water and wastewater, the exposure of humans could be greater for degradates than for the parents. However, the estimated toxicity of the degradate is lower than that of the parent. Thus, the risk associated with the degradates may be similar to that of the parents based on an exposure and toxicity risk assessment approach.

Acknowledgments

The authors are thankful for the financial support from Awwa Research Foundation (project 3170). Additional infrastructure support was provided by the Environmental Research Center, Department of Chemistry, and Department of Civil, Architectural and Environmental Engineering at University of Missouri-Rolla. Ms. Alice Fulmer serves as

the AwwaRF project officer on this project, and contributed significantly to this manuscript. Thanks to Ms. Barbara Harris for her technical editing of this manuscript.

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4. Identification of HydrolyticC Metabolites of Fonofos in Alkaline Aqueous Solutions by Using HPLC/UV and GC/MS

Organophosphorus compounds, becoming the most commonly used pesticides in agriculture, are garnering more interest toward environment and health issues associated with their usage. These compounds leach and run-off into surface and ground water supplies where they have been detected. Critical information on the transformation of these parent compounds into byproducts is lacking. In this study, fonofos hydrolysis at elevated pH's, simulating a water treatment operation or similar process, was investigated. Fonofos, an organophosphorus insecticide used to treat infestations primarily on corn, was investigated due to its greater rate of hydrolysis observed during our screening studies. The hydrolysis of fonofos was investigated at pH 10, 11, and 12 in phosphate buffered water over the course of 7 days. Two hydrolysis products, thiophenol and phenyl disulfide, were detected. Thiophenol was detected using HPLC/SSI/MS, while phenyl disulfide was detected using GC/MS. The transformation mechanism and relative reaction rates are included.

Introduction

Organophosphorus and carbamate compounds are among the most commonly used pesticides and are on the verge of replacing organochloride compounds. The increased usage of these compounds, particularly organophosphorus pesticides, raises concerns

about their environmental and human health impact which to date are poorly addressed. Even less information is known regarding the pesticide transformation products as a result of biotic and abiotic processes that occur naturally in the environment or within a water treatment facility. Some of the abiotic processes that can affect organophosphorus compounds are photolysis, hydrolysis, oxidation-reduction, and other nonselective chemical reactions [1]. The real concern on the environmental and health impacts of pesticides and their transformation products is due to the mechanism of action for organophosphorus compounds not being specific just to insect acetyl-cholinesterases but having the ability to affect the human nervous system as well. Additionally, some transformation products may have greater toxicity than the parent compounds, as shown with DDT [2]. Thus it is imperative to study the transformation of specific pesticides and identify their transformation products.

O-ethyl S-phenyl ethylphosphonodithioate, also known as fonofos or fonofos, is commonly applied to the soil as an insecticide for the control of aphids, corn borer, corn rootworm, corn wireworm, cutworms, white grubs, and some maggots on corn (95%), sugar cane, peanuts, tobacco, turf, and some vegetable crops. Fonofos is typically applied with a water carrier using ground spray equipment at 1 to 4 lb/acre. It is considered a Class I toxicity pesticide (highly toxic) based on acute oral, dermal, eye and inhalation effects [3]. Fonofos is readily absorbed through skin, gastrointestinal, and respiratory tracts [4].

Fonofos is fairly water insoluble but has a moderate persistence (40 day field half-life) in soil [5]. Fonofos readily hydrolyses but transformation products were not identified [5, 6]. The method of spray application for fonofos has the potential for run-off

contamination of surface water and the moderate persistence can cause groundwater contamination at certain sites. Fonofos has been found in groundwater at 0.01 to 0.1 μg/L [7, 8], surface water at 0.01 μg/L [9].

A few studies have isolated transformation products of fonofos [10, 11]. However, both of these studies were in biological systems. The identification of transformation products of hydrolysis outside a biological tissue has not been investigated and is an important piece of information, particularly to water treatment facilities which utilize raised pH processes, where hydrolysis can become an important transformation reaction.

In this study, hydrolysis reactions were carried out at several high pH's in phosphate buffered water systems to simulate treatment processes. Fonofos and two hydrolysis products were separated, identified and quantified using two hyphenated methods. Thiophenol was separated with high performance liquid chromatography and identified and quantified by sonic spray ionization ion trap mass spectrometry (HPLC/SSI/MS). Phenyl disulfide was separated using gas chromatography and identified and quantified using mass spectrometry (GC/MS). This study was intended to investigate the hydrolysis products of fonofos, emphasizing on identifying transformation products, mechanism and relative reaction rate.

Experimental Section

Reagents and Chemicals. Fonofos (99.5%) was purchased from ChemService (West Chester, PA, USA). 2-Nitro-*m*-xylene (99%), thiophenol (≥99%) and phenyl disulfide

(99%) were purchased from Aldrich (Milwaukee, WI, USA). Formic acid (96%, ACS grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC grade), acetonitrile (HPLC grade), water (HPLC grade), sodium hydroxide (98.3%), and sodium phosphate (dibasic, 99%) were purchased from Fisher Scientific (Pittsburgh, PA, USA). D_{10}-Phenanthrene (98 atom % D) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The pH for the Na_2PO_4 buffer was adjusted with 1 M NaOH.

Fonofos, 2-Nitro-*m*-xylene, thiophenol and phenyl disulfide were dissolved in methanol with the concentrations of 10 mg/mL respectively as stock solutions and kept at -20 ^{o}C for storage. Further dilutions of these stock solutions were applied depending on the individual experiments.

Instruments. The Hitachi M-8000 3DQ LC/MS^n system with an L-7400 UV detector (San Jose, CA, USA) and the Waters HPLC system with a PDA detector were used for the analysis of fonofos and one of its degradation products. A Supelco C18 column (150 × 2.1mm i.d., 5μm, Bellefonte, PA, USA) was used for the separation at ambient temperature with a flow rate of 0.25 mL/min. Solvent A consisted of 0.1% formic acid in water (pH 2.70), and solvent B was acetonitrile. The HPLC separation was performed at an isocratic elution with 50% A and 50% B. The UV detector was set in a programmed mode with the wavelength 236 nm from 0 to 10 minute and 240 nm from 10.1 minute to 18 minute. Injection volume was 20 μL.

An Agilent 6893 Series gas chromatograph (GC) with a 5973 Mass Selective Detector and a 7673 autosampler (Palo Alto, CA, USA) was used for this study. The carrier gas is high purity helium from Airgas (Ozark, MO) flowing at a rate of 1 mL/min. A 2 μL sample is injected in a splitless mode at an injection temperature of 280°C into a

HP-5MS capillary column from Agilent (30 m × 0.25 mm i.d., 0.25 μm film thickness). The column has an inlet pressure of 13.5 psi, total flow of 54.1 mL/min, and injector purge flow of 50.0 mL/min at 0.3 minutes. The temperature gradient is programmed as follows: starting with an initial temperature of 100°C, ramping at 40°C/min to 170°C, then 3 °C/min to 185°C, 10 °C/min to 220°C, and finally 60 °C/min to 280°C where it is held for 7 minutes. The total method time is approximately 18 minutes. The samples were scanned from 50 to 300 amu after a 3 minute solvent delay.

Methods. All pH measurements were obtained with an Accumet XL 15 pH meter using an Accumet AccuCap combination pH electrode from Fisher Scientific (Pittsburgh, PA). All experiments were carried out in duplicate.

The hydrolysis experiments were conducted at constant temperature (23.5 ± 1 °C). The hydrolysis of fonofos, thiophenol and phenyl disulfide were carried out individually under the same conditions, if not specified otherwise, with an initial concentration of 2.0 ppm (8.13 μM), 0.90 ppm (8.18 μM) and 0.45 ppm (2.06 μM), respectively. For HPLC/UV analysis, 2-Nitro-*m*-xylene was chosen as an internal standard when quantification was performed. Before each HPLC/UV injection, except for the calibration curve, 0.95 mL of the reaction medium was mixed with 0.05 mL internal standard solution (0.2 mg/mL, in methanol). This procedure will not be mentioned afterwards.

For GC/MS analysis, D_{10}-phenanthrene was chosen as an internal standard when quantitation was performed. Liquid-liquid extraction was preformed with 3.0 mL hexane added into 20 mL of reaction mixture after the hydrolysis reaction was completed. A 0.9

mL aliquot of the extract was mixed with 0.1 mL of internal standard (10 mg/L in methanol) to conduct a GC/MS analysis.

Hydrolysis of fonofos at pH 10 was performed as following: A 30 μL aliquot of fonofos stock solution (10 mg/mL in methanol) was spiked into 150 mL Na_2HPO_4 buffer (pH 10.02), forming an initial concentration of 2.0 mg/L (8.1 μM) for fonofos. An initial sample of 0.95 mL reaction media was taken and mixed with 0.05 mL internal standard solution (0.2 mg/mL, in methanol) as an initial control for HPLC/UV analysis. A 20 mL volume of reaction media was taken and mixed with 3.0 mL hexane to perform a liquid-liquid extraction as an initial control for GC/MS analysis. The remaining reaction media was distributed evenly to 5 amble vials (reactors) with 20.95 mL reaction media in each vial. Each vial was wrapped with aluminum foil to prevent light penetration and put on a shaker table at a speed of 200 rpm. At each desired time, 0.95 mL reaction media in each reactor was taken and mixed 0.05 mL internal standard solution for HPLC/UV analysis and the remaining solutions were used to conduct liquid-liquid extraction for GC/MS analysis. Hydrolysis at pH 11.00 and 12.01 was carried out using the same procedure.

The hydrolysis of thiophenol and phenyl disulfide at pH 10, 11 and 12 were carried out as the same way as that for fonofos, except that the stock solution concentrations of the thiophenol and phenyl disulfide were 4.5 mg/mL and 2.25 mg/mL, respectively. The initial hydrolysis concentrations of thiophenol and phenyl disulfide were 0.90 mg/L (8.2 μM) and 0.45 mg/L (2.1 μM), respectively.

Blank experiments were performed as the similar way as the hydrolysis of samples except that the same volume of methanol was added to replace the stock sample solutions of fonofos, thiophenol and phenyl disulfide.

Results and discussion

Identification of Hydrolysis Products of Fonofos. Hydrolysis reaction of fonofos at pH 12 was analyzed by HPLC/UV after 7 days of reaction. Figure 1 showed the chromatograms of blank, reaction mixture, standard, and internal standard (terbuthilazine). One of the hydrolysis products of fonofos had a same retention time as that of thiophenol. To confirm the identity of the product, HPLC with PDA detector was employed to provide the spectra for thiophenol peak and the product peak. Both spectra, as shown in Figure 2, are identical, implying that one of the hydrolysis products of fonofos is thiophenol. It is worth mentioning that that identification of hydrolysis products of fonofos by LC/MS was not applied because none of the hydrolysis products can be ionized with the ionization sources we have in our laboratory including electro spray (ESI), sonic spray (SSI) and atmosphere pressure (API).

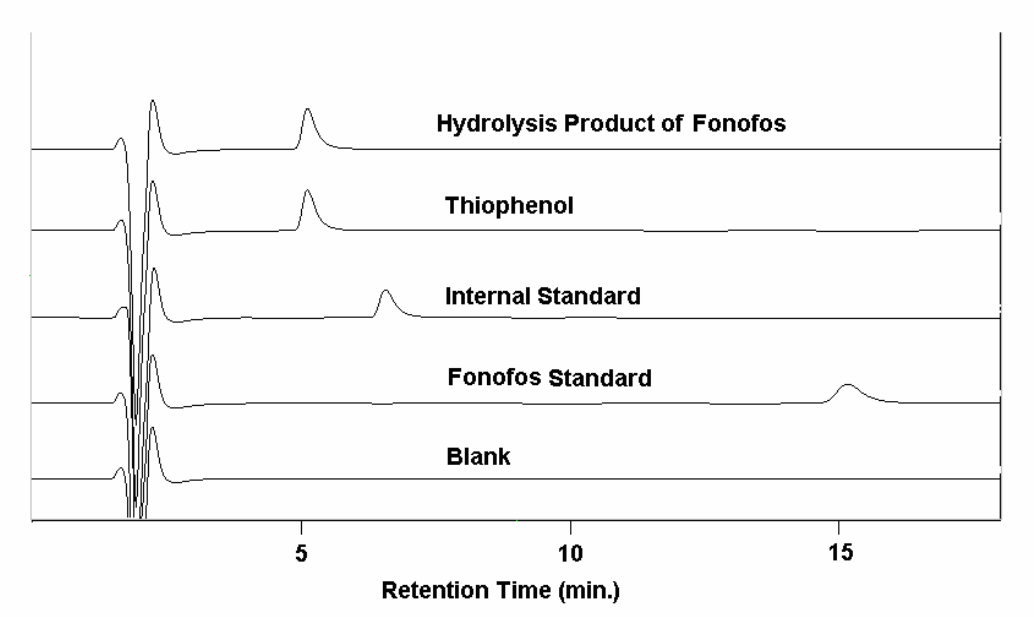


Figure 1. UV chromatograms of blank, hydrolysis product of fonofos and other standards.

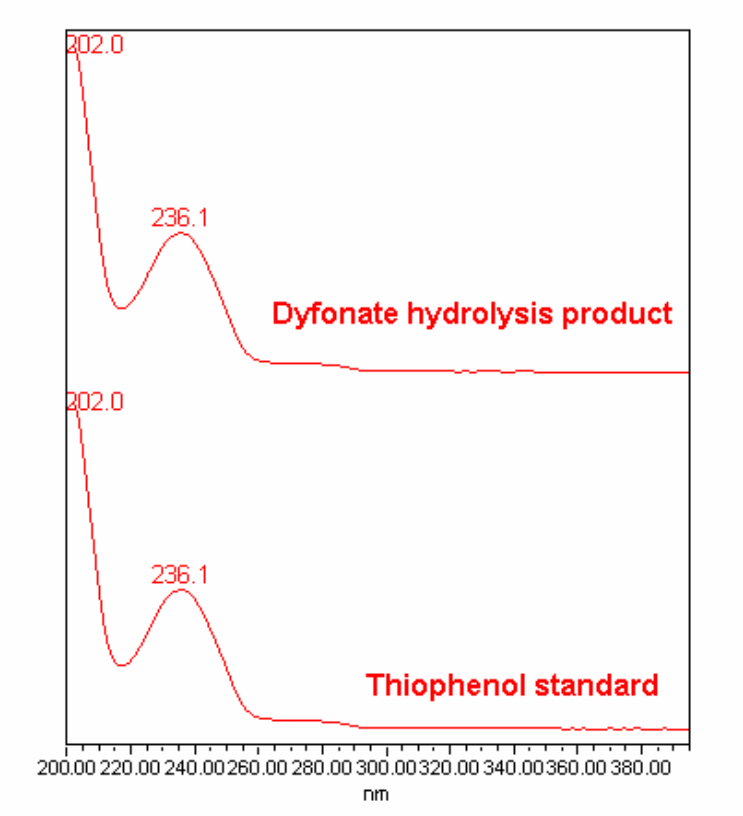


Figure 2. UV spectra of fonofos hydrolysis product and thiophenol standard extracted at the retention time of the corresponding peak.

To identify and quantify other hydrolysis products of fonofos at different pHs, hexane liquid-liquid-extraction followed by GC/MS analysis was applied. The aqueous layer was analyzed by HPLC/UV after liquid-liquid extraction of the hydrolysis of fonofos with hexane to determine the extraction efficiency. Both the fonofos and the thiophenol were no longer detectable after hexane extraction (chromatograms were not shown). This indicates that the efficiency of the liquid-liquid extraction system for both compounds was high enough for further identification. It was found that thiophenol generated at pH=12 was not ionizable either by using GC/MS. It was interesting that the hydrolysis of fonofos at lower pH such as ≤ 11, a different unknown compound was

detected. Therefore, the hydrolysis of fonofos from pH's 10-12 was systematically studied.

The hexane extract of the hydrolysis of fonofos was analyzed by GC/MS, and the total ion chromatogram (TIC) of a fonofos hydrolysis sample at pH 11.00 was shown in Figure 3a. There were two main peaks in the TIC of fonofos hydrolysis sample with the retention times labeled in the figure. The Mass spectrum of each peak was obtained by scanning each of them, and the corresponding identity of them was carried out by matching the mass spectrum with that in the database, giving the chemical name of each peak, as shown in Figure 3b and 3c.

Based on the results from Figure 3b and 3c, the first peak is fonofos peak and the second peak corresponds to the phenyl disulfide (retention time: 7.168 min). To further confirm the identity of the peak with retention time of 7.168 minute, standard phenyl disulfide was spiked to pH 7 NaH_2PO_4 buffer and extracted with hexane, followed by GC/MS analysis. The chromatogram and mass spectra were shown in Figure 4a and 4b, indicating that phenyl disulfide was the hydrolysis product of fonofos at pH 11. Combining the results in Figures 1-4, it is clear that the hydrolysis products of fonofos changes greatly with pHs. The hydrolysis of fonofos as well as its hydrolytic products at different pHs, therefore, were studied to investigate the transformation mechanism.

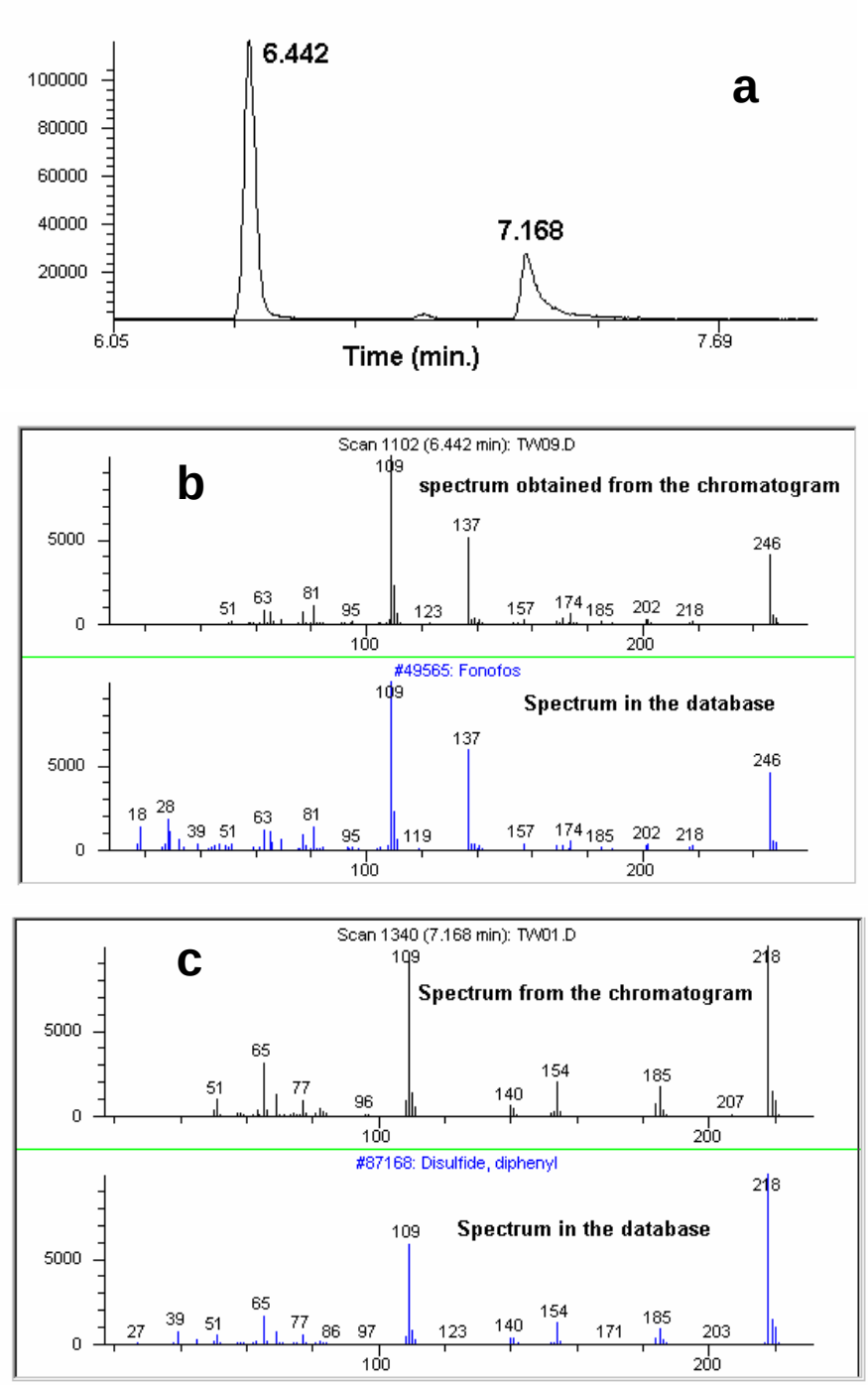


Figure 3. (a) GC/MS TIC of fonofos hydrolysis sample at pH 11.00; (b) Mass spectrum of the peak with retention time 6.442 minute and mass spectrum of fonofos in the database; (c) Mass spectrum of the peak with retention time 7.168 minute and mass spectrum of phenyl disulfide in the database. (Disulfide, diphenyl = phenyl disulfide).

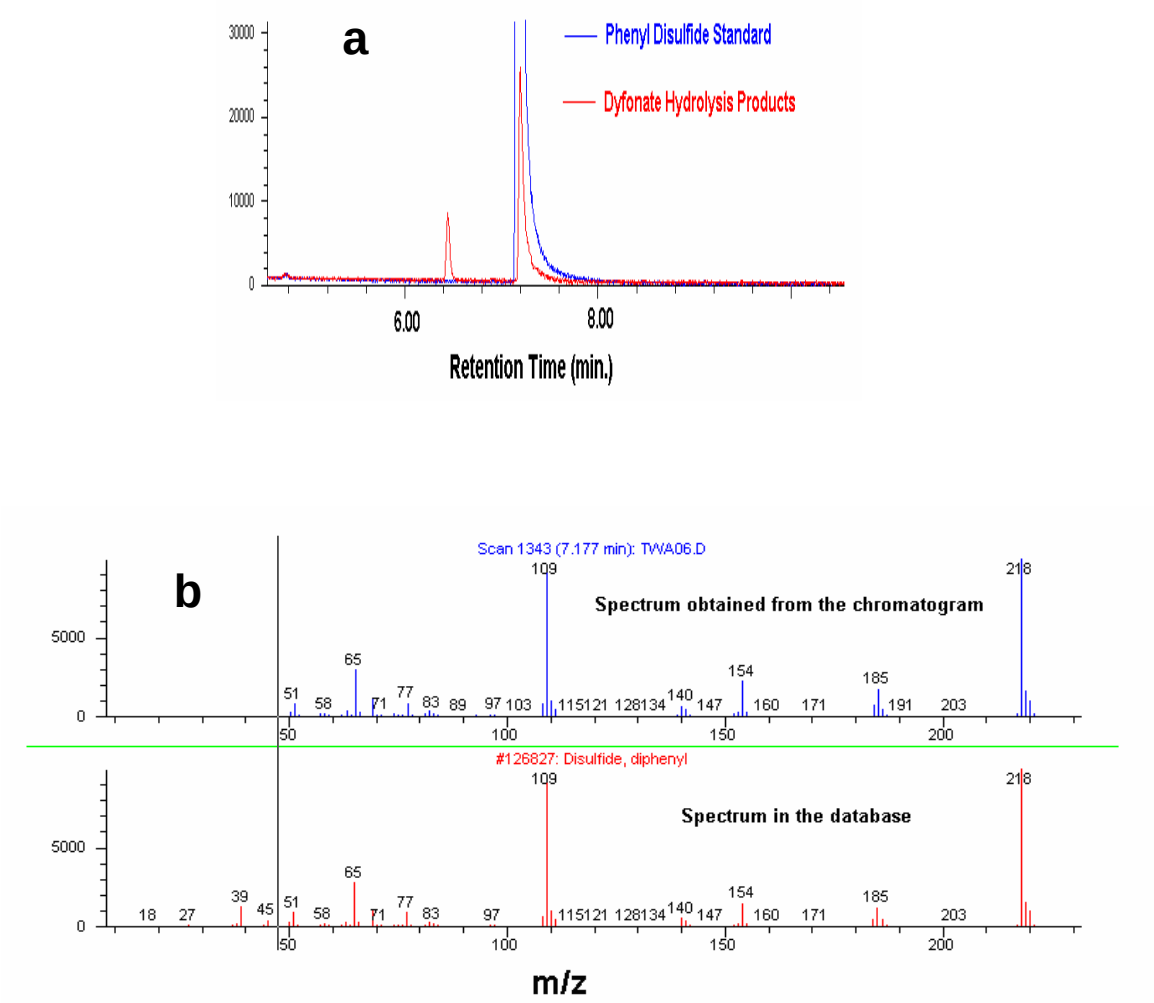
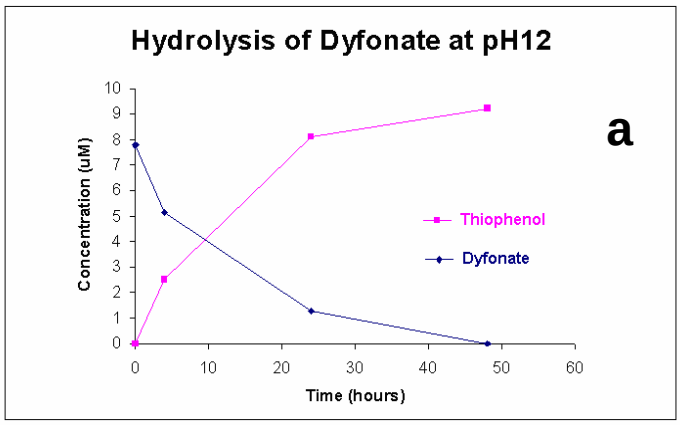
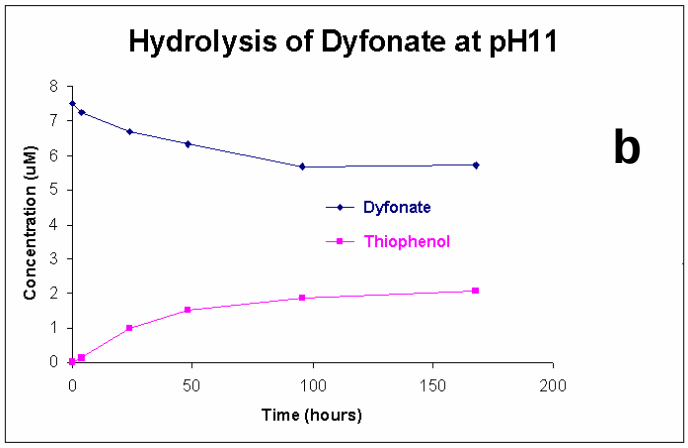


Figure 4. (a) GC/MS TIC of phenyl disulfide standard and hydrolysis product of fonofos at pH 11; (b) Mass spectrum of phenyl disulfide standard and mass spectrum of phenyl disulfide in the database.

Time Response of the Hydrolysis of Fonofos, Thiophenol and Pheny

Disulfide. A 200-hour time response study was performed. Based on the results of quantitative determinations, the concentration profiles of the components in fonofos hydrolysis reaction system were shown in Figure 5a, 5b, and 5c. We can conclude





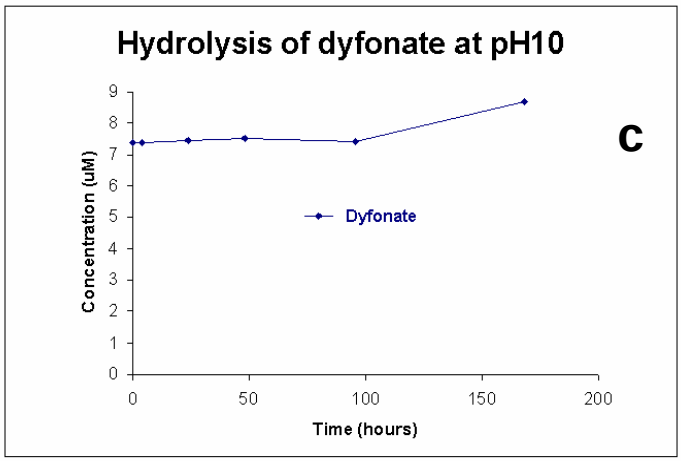


Figure 5. Concentration profile of the components in hydrolysis of fonofos system at (a) pH12, (b) pH11, and (c) pH10.

from the concentration profile in Figure 5a that fonofos is completely degraded at pH 12 to form thiophenol after 48 hours. No other degradates were detected.

Figure 5b demonstrated that the levels of both fonofos and thiophenol were maintained constant after 96 hours, and no other degradates were detected. The results demonstrate that these two components reach to an equilibrium state after a certain period of time and the hydrolysis rate is much slower compared with that at pH 12. Figure 5c showed that the concentration of fonofos was kept in a same level throughout the whole experiment at pH 10, and no degradates were detected. It indicates that fonofos is stable at pH 10.

The concentration profile of thiophenol hydrolysis at pH's from 10-12 was shown in Figure 6. Figure 6a showed that thiophenol was very stable at pH 12. Neither fonofos nor phenyl disulfide were detected, which is opposite to the situation of fonofos. When solution pH was decreased to 11, as shown in Figure 6b, it was found that the level of thiophenol gradually decreased as time, while the concentration of phenyl disulfide, which was the degradate of thiophenol, increased slowly and then kept almost constant throughout the whole experiment. No fonofos was detected. It was found interesting that the concentration of thiophenol decreased even faster at pH 10 than that of pH 11, which was shown in Figure 6c, while the concentration of phenyl disulfide, the degradate of thiophenol, increased faster accordingly than that at pH 11, then decreased slowly throughout the whole experiment. These results indicate that thiophenol is less stable at pH 10 that that at pH 11, but quite stable at pH 12. No fonofos was detected in this experiment, which means that the degradation of fonofos to thiophenol was an irreversible reaction.

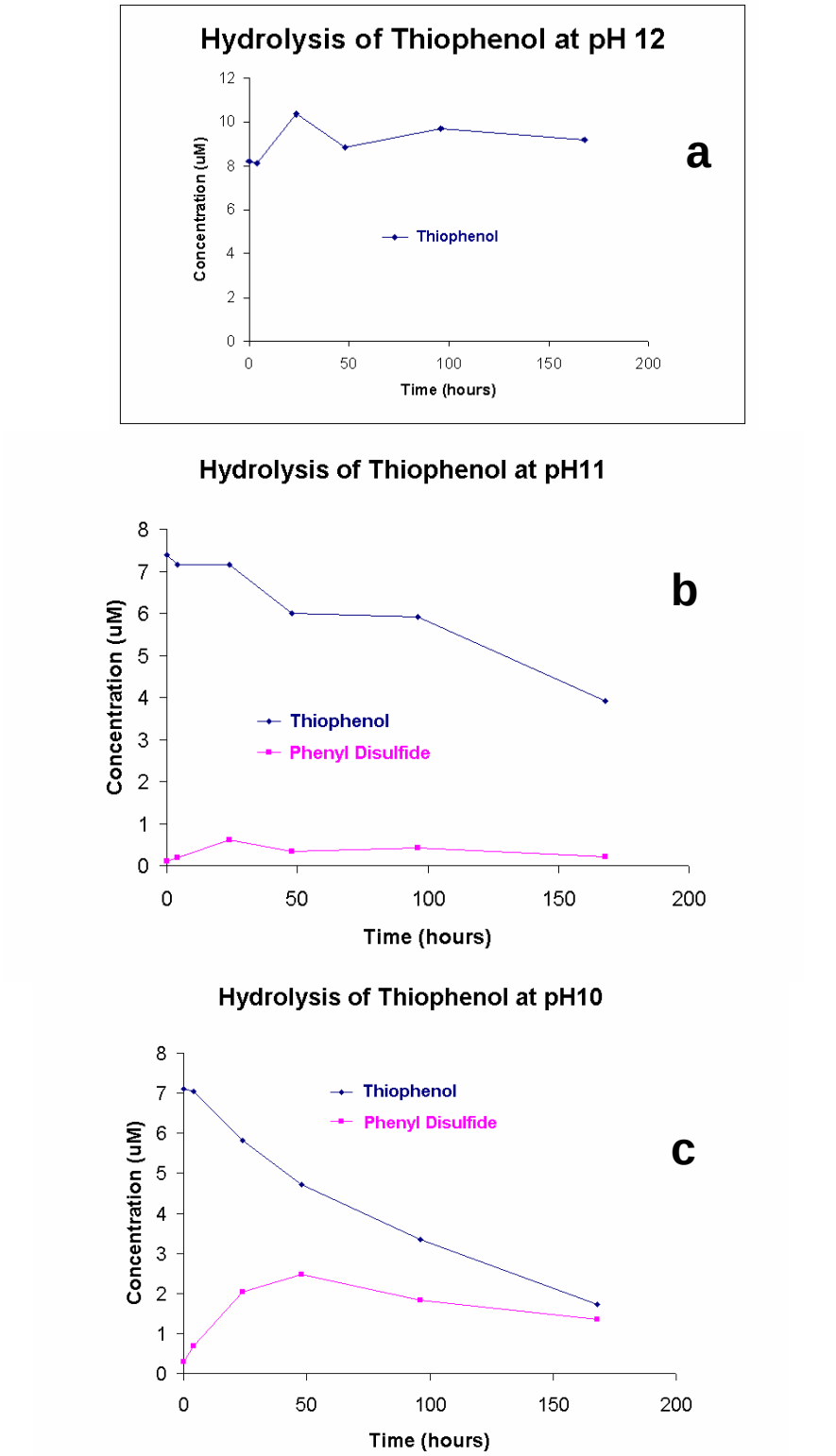


Figure 6. Concentration profile of the components in hydrolysis of thiophenol system at (a) pH12, (b) pH11, and (c) pH10.

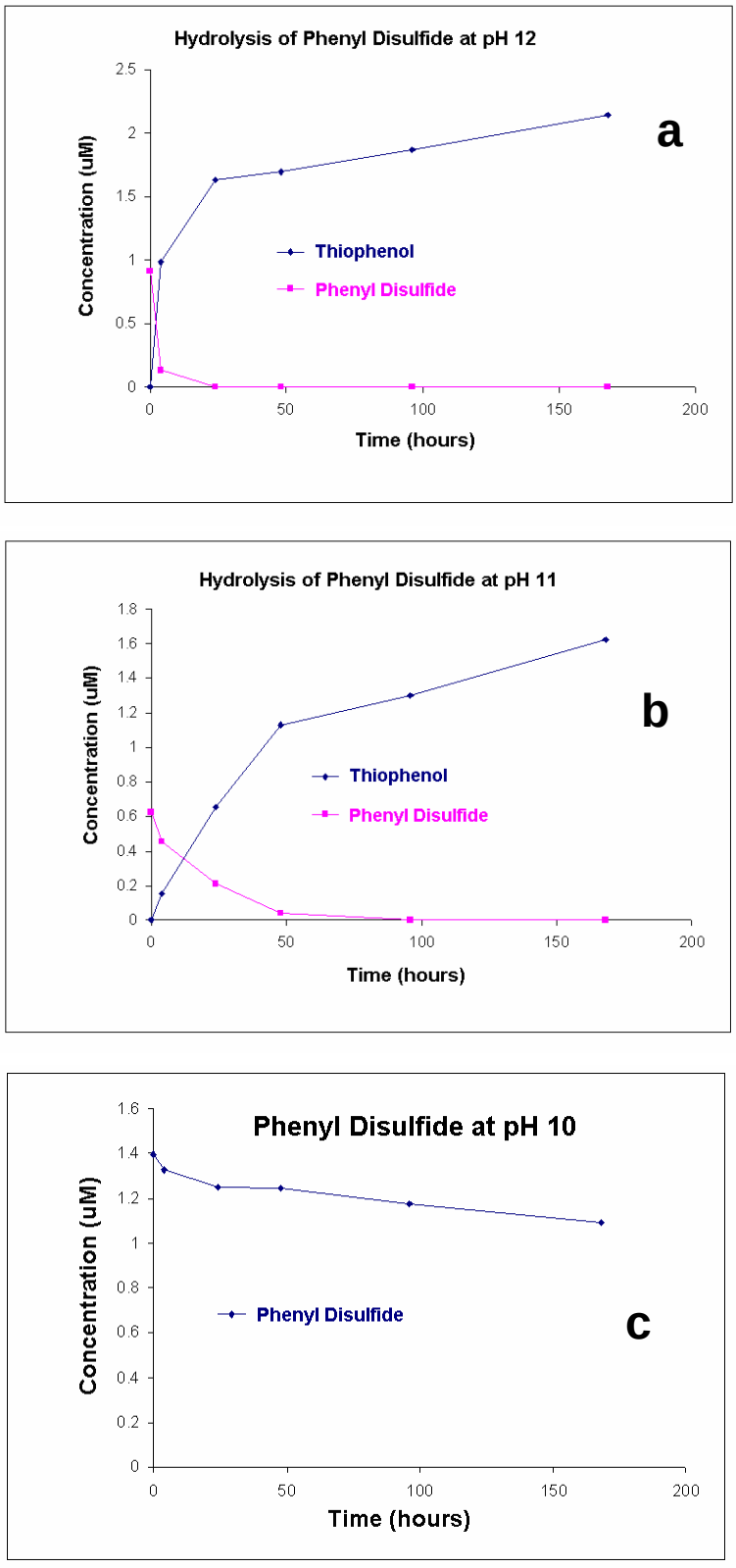


Figure 7. Concentration profile of the components in hydrolysis of phenyl disulfide system at (a) pH12, (b) pH11, and (c) pH10.

When phenyl disulfide was hydrolyzed at pH 11 and 12, it was found that phenyl disulfide degraded quickly and thiophenol was produced, as shown in figure 7a and 7b. No fonofos was detected. However, when phenyl disulfide was hydrolyzed at pH 10, no thiophenol and fonofos were detected, and the concentration of phenyl disulfide was maintained almost at the same level, as shown in figure 7, indicating that phenyl disulfide is rather stable at pH 10.

Based on the results above, we can see clearly that phenyl disulfide was not detected in the hydrolysis of fonofos at pH 11 (Figure 5b) but a significant amount of phenyl disulfide was detected in the hydrolysis of thiophenol at pH 11 and 10 (Figure 6b and 6c, respectively). The reason was that the thiophenol concentration generated through the fonofos hydrolysis was much lower than the initial thiophenol concentration in the thiophenol hydrolysis system, hence produced even lower level of phenyl disulfide, which was below the detection limit of this MS instrument for phenyl disulfide.

Pathway of the Degradation of Fonofos in Alkaline Aqueous Solution. Based on the time response studies and mass balances of fonofos hydrolysis, a pathway of the degradation of fonofos in alkaline aqueous solution is proposed, which is shown in Figure 8. In an alkaline aqueous solution, fonofos will be hydrolyzed to produce thiophenol, as shown in our study. When the pH drops below 11, thiophenol will degradate and produce phenyl disulfide. Based on our best knowledge, the formation of phenyl disulfide during the hydrolysis of fonofos has not been reported. A report indicated that phenyl disulfide can be reduced back to thiophenol using sodium borohydride followed by acidification [12]. However, formation of thiophenol from phenyl disulfide at a high pH aqueous solution (pH $\geq$ 11) was observed in our studies without any reducing agent in

the solution. According to this reaction type, it should be categorized as an oxidation-reduction reaction. However there is no reducing agent being identified. The mechanism of this reaction deserves further investigation.

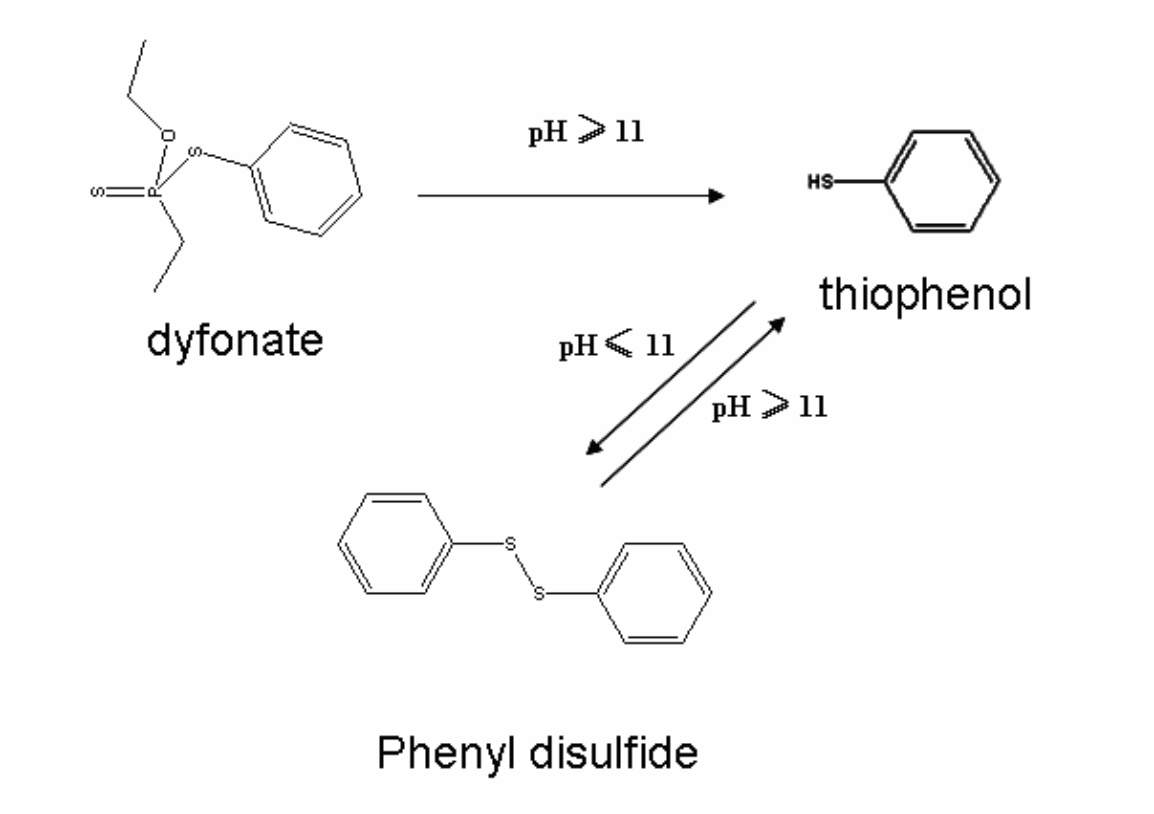


Figure 8. Proposed pathways of the degradation of fonofos in alkaline aqueous solution.

Conclusions

A comprehensive study on the hydrolysis of fonofos and its degradation products in alkaline aqueous solution was conducted, and fonofos can be hydrolyzed to produce thiophenol at pH 11 or higher. The formed thiophenol will react with each other to produce phenyl disulfide at pH 11 or lower. The formed phenyl disulfide will degrade back to produce thiophenol at pH 11 or higher, and this reaction was discovered at the

first time based on our knowledge. Therefore, both thiophenol and phenyl disulfide could be detected simultaneously only at pH 11 with a more sensitive MS instrument or at higher fonofos concentrations.

Acknowledgments

The authors are thankful for the financial support from Awwa Research Foundation (project 3170). Additional infrastructure support was provided by the Environmental Research Center, Department of Chemistry, and Department of Civil, Architectural and Environmental Engineering at University of Missouri-Rolla. Ms. Alice Fulmer serves as the AwwaRF project officer on this project, and contributed significantly to this manuscript.

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5. Comprehensive Studies on Aldicarb Degradation in Various Oxidation Systems Using High Performance Liquid Chromatography Coupled with Ion Trap Mass Spectrometry and UV Detection

ABSTRACT

Aldicarb, a carbamate pesticide, is commonly used in agriculture and can be naturally degraded to its carbamate metabolites, resulting in their occurrence in drinking water supplies. The disinfection process using different oxidants for the treatment of drinking water provides the opportunity to degrade aldicarb and its metabolites to byproducts that may pose more or less risk than the parents. Based on the results of previous screening studies, a comprehensive study was performed involving aldicarb and its carbamate metabolites treatment with free chlorine (FC), monochloramine (MCA), chlorine dioxide (ClO_2), hydrogen peroxide, permanganate (MnO_4^-) and UV radiation to identify their degradation products. Free chlorine, high dosage of UV radiation and permanganate exhibited stronger oxidation capacity than the others studied, while chlorine dioxide showed the weakest oxidation ability among them. Aldicarb sulfoxide was formed as the degradation products of aldicarb by oxidation with free chlorine, MCA, ozone and hydrogen peroxide. Aldicarb sulfone was identified as an oxidation byproduct of both aldicarb and aldicarb sulfoxide by permanganate. N-chloro-aldicarb sulfone was formed as an oxidation byproduct of aldicarb sulfone by free chlorine.

1. Introduction

Aldicarb [2-methyl-2(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime], an active ingredient in the pesticide TEMIK®, is a soil pesticide used in the agricultural sector worldwide for over 30 years for the control of insects, mites, and nematodes [1]. Some of the most important uses of this product in the U.S.A. have included citrus, cotton, sugar beets, potatoes, pecans and peanuts. The commercial product is a granular formulation, which is incorporated into the soil at the time of application. After applied into the soil, it is solubilized and distributed by the groundwater, and absorbed by the roots and translocated throughout the plant and, and serves as a systemic pesticide.

The discovery of aldicarb residues in drinking water on Long Island, New York, in 1979 [2] and later in other areas of the U.S.A. has resulted in many research and monitoring programs being conducted by university, regulatory agency and industry scientist. Such activities have included potable well monitoring studies, laboratory experiments, field research studies and computer modeling. Many of these activities have been conducted by or in cooperation with the producer and registrant of aldicarb.

It was reported that aldicarb would degrade to produce aldicarb sulfoxide and aldicarb sulfone in a variety of soil types under both field and laboratory [3]. Thus, studies on the degradation of aldicarb under various conditions became crucial in understanding the degradation mechanisms and pathways and monitoring and removing of aldicarb and its degradates from the environment. Richey et al [4] carried out the laboratory studies on the degradation of aldicarb in soil by separately ^{14}C labeling aldicarb at three positions (*S*-methyl, *N*-methyl, and tertiary carbon) in Norfolk sandy

loam, Lufkin fine sandy loam, and Lakeland fine sandy loam, followed by metabolizing under laboratory conditions in a metabolism chamber. The metabolites were determined by assay for radioactivity. Ou et al [5, 6] studied the aerobic and anaerobic degradation of aldicarb and aldicarb sulfone in soils. It was found that aldicarb produced aldicarb sulfoxide, aldicarb sulfone, aldicarb sulfoxide oxime, aldicarb sulfoxide nitrile, aldicarb sulfone oxime, and two unknowns. On the other hand, aldicarb sulfone nitrile and aldicarb sulfone acid were detected as the two major degradates of aldicarb sulfone under aerobic and anaerobic soils. The aerobic and anaerobic degradation rates for aldicarb were measured in soil samples collected at different depths, and the concentration changes of its two toxic oxidation products, aldicarb sulfoxide and aldicarb sulfone were determined to estimate the first-order rate constants for concurrent oxidation and hydrolysis of aldicarb, aldicarb sulfoxide and aldicarb sulfone, and for the loss of total carbamate residues [7]. Hydrolysis of aldicarb, aldicarb sulfoxide and aldicarb sulfone in Floridan groundwater was observed with the rates decreased in the order sulfone > sulfoxide >> aldicarb [8]. In addition, hydrolysis rates of aldicarb, aldicarb sulfoxide and aldicarb sulfone were measured at ppb levels in aqueous solution by using liquid-liquid extraction followed by gas chromatography with flame ionization detector (FID) and nitrogen-phosphorus detector (NPD) [9]. Biotransformation of is another pathway for degradation of aldicarb. Kazumi et al [10] described the studies in which the aldicarb biotransformation happening in sediment was mainly via an oxidation pathway in the presence of O_2, while in the absence of O_2, the biodegradation took place through a hydrolytic pathway. It was also reported [11] that not only aldicarb, aldicarb sulfoxide and aldicarb sulfone at the applied dose to soils did not inhibit microbial growth, but also

the microbial component in soil had a significant role in the degradation of these compounds. In fact, some researchers reported the capability of soil microorganisms to use the carbamate pesticides as a source of carbon and nitrogen for growth [7, 12, 13]. Kök et al [14] reported the complete removal of aldicarb by using immobilized bacteria as a degradation site/source to decrease the environmental contamination caused by pesticides. Liu et al. [15] studied the effect of anion surfactant on degradation rate of aldicarb in soil, and found that sodium dodecylbenzenesulfonate (SDBS) could accelerate the degradation of aldicarb and there was a good linear relationship between degradation rate constant and the logarithm of SDBS concentration. Other factors affecting chemical and microbial degradation of aldicarb was investigated, and it was shown that temperature was the most important variable affecting the degradation rate of aldicarb and its carbamate metabolites in surface soils [16].

Other than gas chromatography [9, 17], the analysis of aldicarb and its carbamate metabolites included RP-HPLC followed by post-column derivatization and fluorescence detection [18, 19], UV detection [10, 11, 14, 20] and mass spectrometry [21].

The use of oxidants in drinking water treatment is common for disinfection, oxidation of inorganic and organic contaminants, taste and odor control, and microflocculation. Due to the possibility of transporting aldicarb and its carbamate metabolites into drinking water via many different ways, the degradation byproducts and possible degradation mechanisms of aldicarb, aldicarb sulfoxide and aldicarb sulfone at different water treatment conditions must be systematically investigated to guide the water treatment system, to minimize environmental and human health effects.

However, no report was found through a thorough literature search to investigate the oxidation of aldicarb and its carbamate metabolites in water treatment plant involving treatment with monochloroamine, chlorine dioxide, permanganate, hydrogen peroxide, ozone and UV radiation. In this paper, a comprehensive study is conducted to analyze the oxidation byproducts of aldicarb in various oxidation systems by using HPLC/ESI/MS and to determine the removal of aldicarb by using HPLC/UV, as well as the oxidation reaction features in terms of brief mechanism and relative reaction rate. This study, along with our preliminary screening studies, provides practical information for understanding the kinetics and mechanism of the oxidation for different oxidants. Moreover, pesticides monitoring in water and control or choice of disinfection combination can be beneficial from this study.

2. Experimental

2.1. Reagents and Chemicals

Aldicarb (99.0%), aldicarb sulfoxide (98%), aldicarb sulfone (98%) were purchased from ChemService (West Chester, PA, USA). Formic acid (96%, ACS grade), hydrogen peroxide solution (30%) and sodium hypochlorite solution (available chlorine ≥4%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC grade), acetonitrile (HPLC grade), water (HPLC grade), sodium hydroxide (98.3%), potassium permanganate (certified ACS, 99.5%), and sodium phosphate (dibasic, 99%) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Sodium phosphate (monobasic,

99%) was purchased from Aldrich (Milwaukee, WI, USA). The pH for the experiments was adjusted with either 1 N H_3PO_4 or NaOH.

2.2. HPLC/ESI/MS and HPLC/UV Analysis

The Hitachi M-8000 3DQ LC/MS^n system with an electrospray ion source (San Jose, CA, USA) was used for the HPLC/MS analysis of aldicarb and its degradation products. A Supelco C18 column (150 × 2.1mm i.d., 5μm, Bellefonte, PA, USA) was used for the separation at ambient temperature with a flow rate of 0.25 mL/min. Solvent A consisted of 0.1% formic acid in water (pH 2.70), and solvent B was acetonitrile. The HPLC separation was performed at an initial 10% B followed by a gradient elution to 40% B at 15 min. then with a drop to 10% B at 15.1 min. A divert valve was placed right before the ionization source to remove the HPLC fractions containing salts and prevent the contamination of ionization source and MS. The ESI parameters were set to the following optimized values: nitrogen sheath gas at 3 kgf/cm^2, 4 kV for ESI probe, 30 V for drift plate, 25 V for focus plate, 200ºC for desolvator temperature, 180ºC for assistant gas heater temperature, 160ºC for aperture 1 temperature. Ion trap MS was operated at following conditions: 500 ms for accumulating time, 0.069V for accumulation voltage, 41.78 amu for low mass cut off, 41.78-400 amu for mass scan range. The UV wavelength for quantification was 200 nm.

2.3. Methods

All pH measurements were obtained with an Accumet XL 15 pH meter using an Accumet AccuCap combination pH electrode from Fisher Scientific (Pittsburgh, PA). All oxidation experiments were conducted at constant temperature (23.5 ± 1 °C). Oxidation of aldicarb, aldicarb sulfoxide and aldicarb sulfone was carried out individually in a same way if not specified otherwise, with an initial concentration of 5 ppm for each. Quantification was accomplished by HPLC/UV with calibration curve and external standard operation.

2.4. Free Chlorine (FC) Oxidation System

The concentration of free chlorine in sodium hypochlorite stock solution was determined with the Hach DPD Method 8221 using AccuVac ampuls obtained from the Hach Company (Loveland, CO, USA). 50 μL aldicarb stock solution (in MeOH, 1.0 mg/mL) was spiked to 10.0 mL Milli-Q water in a 20 mL amber bottle (reactor). 25 μL sodium hypochlorite stock solution (free chlorine: 4.0 mg/mL) was spiked and the reaction was initiated. The initial concentration of aldicarb was 5.0 ppm (26.3 μM). The reactor was put on a shaker for shaking at 150 rpm. Samples were taken after 2 hours, followed by HPLC/MS and HPLC/UV analysis. FC oxidation of aldicarb sulfoxide and aldicarb sulfone were carried out in a same way as that of aldicarb.

2.5. Ozone (O_3) Oxidation System

Ozone was produced using a Model GLS-1 PCI-WEDECO (Environmental Technologies, West Caldwell, NJ, USA) ozone generator and compressed oxygen. The ozone gas stream was bubbled from a stone diffuser into Milli-Q water. A Cary 50 Conc UV-Visible Spectrophotometer (Varian Australia PTY LTD, Australia) at 260 nm was then used independently to monitor the decay and concentration of aqueous ozone (initial 29.7 ppm was used in our experiments). The amber glass vial containing 5.0 mL 15 ppm aldicarb (78.8 μM) was spiked with 10.0 mL saturated O_3 solution (29.7 ppm). The concentration of aldicarb and ozone when reaction was initiated was 5.0 (26.3 μM) and 9.9 ppm, respectively. The reaction continued for 4 hours before HPLC/MS and HPLC/UV analysis. The O_3 oxidation of aldicarb sulfoxide and aldicarb sulfone were performed in a same way as that of aldicarb, with an initial concentrations of 5.0 ppm (24.2 μM) and 5.0 ppm (22.5 μM), respectively.

2.6. UV Oxidation System

A 254-nm low-pressure mercury-vapor lamp (Pen Ray Model 90-0004- 01,254 nm, 1.0 W; UVP Inc., Upland, CA) was used for the UV photo-degradation study. Three amble glass vials (reactors) of 5 mL Milli-Q water, each containing 5.0 mg/L aldicarb (26.3 μM), were exposed to the UV lamp for 2 seconds (low dosage), 10 seconds (medium dosage), and 60 seconds (high dosage), respectively, by placing the 0.9 cm diameter lamp down the centerline of the vial. The diameter of the reactor was 1.9 cm, and the length of

the lamp in the liquid was 2.5 cm. Based on a volume weighted mean radius for the fluid, the fluence was 8.9 mW/cm^2 for the system. The reaction medium was stirred with a small stirring bar during the UV exposure. The UV oxidation of aldicarb sulfoxide and aldicarb sulfone were performed in the same manner as that of aldicarb, with the concentration of 5.0 ppm (24.2 μM) and 5.0 ppm (22.5 μM), respectively.

2.7. Chlorine Dioxide (ClO_2) Oxidation System

Gaseous chlorine dioxide was produced using a Bench-Scale ClO_2 Generator (CDG, Bethlehem, PA). The concentration of ClO_2 in the generated saturated ClO_2 solution was determined by a Cary 50 Conc UV-Visible Spectrophotometer (Varian Australia PTY LTD, Australia) at 360 nm (initial 3.987 g/L was used in our experiments). An amber glass vial (reactor) of 10.0 mL Milli-Q water, which contained 5.0 mg/L aldicarb (26.3 μM), was spiked with 25 μL saturated ClO_2 solution. The initial concentration of ClO_2 was 9.97 mg/L. Samples were taken at after 4 hours of reaction, followed by HPLC/MS and HPLC/UV analysis. ClO_2 oxidation of aldicarb sulfoxide and aldicarb sulfone was carried out individually in the same manner as that of aldicarb with the concentration of 5.0 ppm (24.2 μM) and 5.0 ppm (22.5 μM), respectively.

2.8. Hydrogen Peroxide (H_2O_2) Oxidation System

An amber glass vial (reactor) of10.0 mL Milli-Q water, which contained 5.0 mg/L aldicarb (26.3 μM), was spiked with 1.0 mL H_2O_2 solution (30%) to initiate a reaction

resulting in an initial H_2O_2 concentration of 27,273 mg/L. The reaction continued for 4 hours prior to sampling and HPLC/MS and HPLC/UVanalysis. H_2O_2 oxidations of aldicarb sulfoxide and aldicarb sulfone were carried out in the same manner with the concentration of 5.0 ppm (24.2 μM) and 5.0 ppm (22.5 μM), respectively.

2.9. Monochloroamine (MCA) Oxidation System

MCA stock solutions were prepared from ammonium chloride and sodium hypochlorite at a molar ratio of 1.05:1 at pH 11 [22]. The concentration of a MCA stock solution was determined by using the total chlorine method (via Hach DPD Method 8167; Loveland, CO, USA) and confirming that no free chlorine concentration remained. An amber glass reactor of 10.0 mL Milli-Q water containing 5.0 mg/L aldicarb (26.3 μM) was spiked with 50 μL of the MCA stock solution for an initial MCA concentration of 2000 mg/L. Samples were taken after 4 hours of initiation of reaction for HPLC/MS and HPLC/UV analysis. MCA oxidation of aldicarb sulfoxide and aldicarb sulfone was carried out in the same manner with the concentration of 5.0 ppm (24.2 μM) and 5.0 ppm (22.5 μM), respectively.

2.10. Permanganate (MnO_4^-) Oxidation System

A 50 μL aliquot of fonofos stock solution (1.0 mg/mL in methanol) was spiked into 10.0 mL Milli-Q water, forming an initial concentration of 5.0 mg/L (26.3 μM) for aldicarb. A 32 μL potassium permanganate stock solution (3158 mg/L) was spiked and the reaction

was initiated. Samples were taken after 4 hours of initiation of reaction for HPLC/MS and HPLC/UV analysis. Permanganate oxidation of aldicarb sulfoxide and aldicarb sulfone was carried out in the same manner with the concentration of 5.0 ppm (24.2 μM) and 5.0 ppm (22.5 μM), respectively.

3. Results and Discussion

3.1. Identification of Oxidation Byproducts of Aldicarb in Different Oxidation Systems

HPLC/MS was the major tool for identification of oxidation byproduct of aldicarb in different oxidation systems. Under the chromatographic and mass spectrometric conditions described in the Experimental Section, total ion chromatograms of aldicarb standard and oxidation byproducts of aldicarb in free chlorine, monochloroamine, ozone, permanganate and hydrogen peroxide systems were shown in Fig. 1. From the chromatograms, it can be observed that aldicarb peak was totally gone in these five oxidation systems, among them the peak for permanganate oxidation byproduct of aldicarb showed a retention time different from that for other oxidants. Other four oxidation systems produced the byproduct with same retention times. 70% of aldicarb was removed after high dosage of UV radiation, but no degradates were detected by current method (chromatogram not shown). No significant removal (< 15%) of aldicarb was observed and no degradates were detected by current method for chlorine dioxide as well as medium and low dosage of UV radiation (chromatogram not shown).

The mass spectrum of standard aldicarb in Fig. 1 was obtained to confirm the identification of aldicarb peak. The m/z 213 ion is the sodiated molecular ion, while the m/z 116 ion is one of the fragment ions, as shown in Fig. 2. The m/z 157 and 175 ions have not been interpreted yet. This kind of fragmentation is proposed to take place with an in-source ionization mechanism.

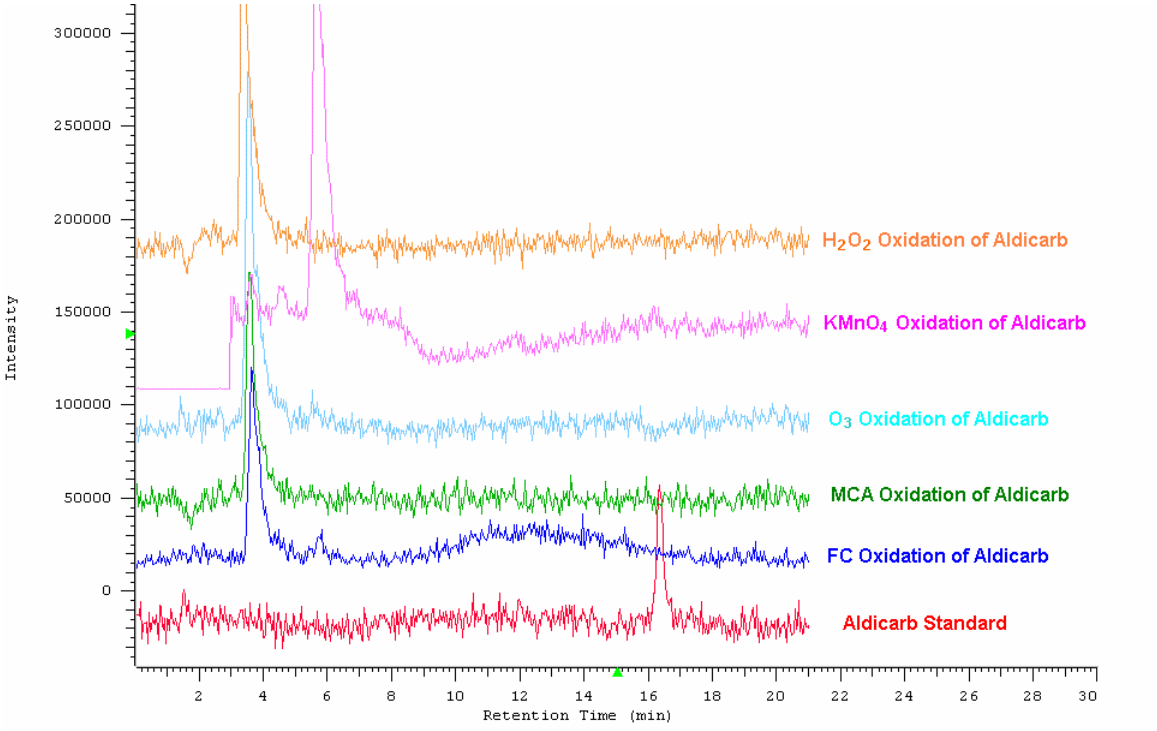

Fig. 1 - TICs of Aldicarb standard and byproducts under various oxidation systems.

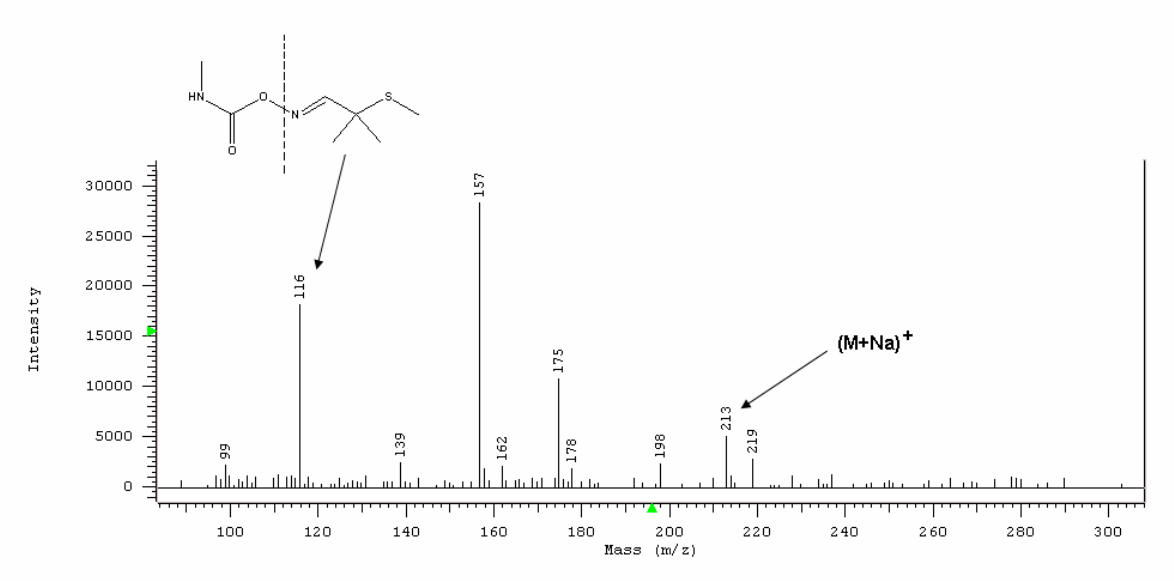

Fig. 2 - Mass spectrum of Aldicarb (MW: 190) standard

In order to identified the degradation products of aldicarb, standard aldicarb sulfoxide and aldicarb sulfone, the two carbamate metabolites of aldicarb, were analyzed by HPLC/MS, and the chromatograms of these two standards and representatives of aldicarb oxidation byproducts were shown in Fig. 3. The retention times for the standard aldicarb sulfoxide and the oxidation byproduct of aldicarb in free chlorine, monochloroamine, ozone, hydrogen peroxide systems are the same, while the retention times for the standard aldicarb sulfone and the permanganate oxidation byproduct of aldicarb are the same.

In order to confirm the identification of the oxidation byproducts of aldicarb, the mass spectrum of aldicarb sulfoxide and free chlorine oxidation byproduct of aldicarb was obtained, while the mass spectrum of aldicarb sulfone and permanganate oxidation byproduct of aldicarb was obtained, as shown in Fig. 4 and 5, respectively.

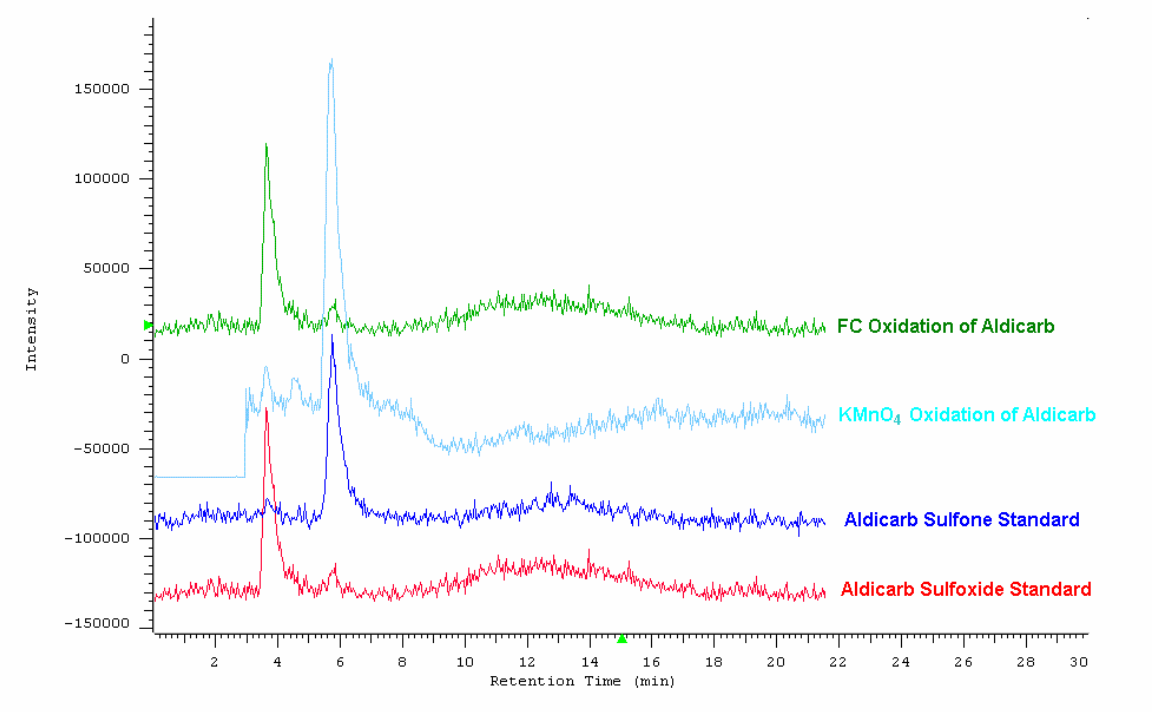

Fig. 3 - TICs of aldicarb sulfoxide and aldicarb sulfone standard, and representative byproducts of aldicarb under FC and $KMnO_4$ oxidation systems.

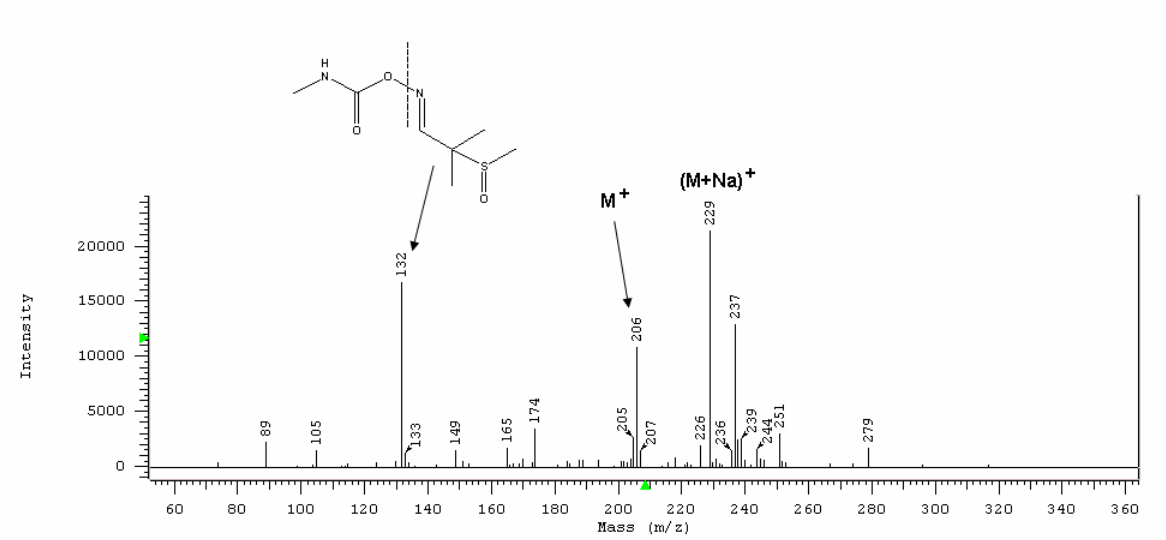

Fig. 4 - Mass spectrum of aldicarb sulfoxide (MW: 206) standard and the oxidation byproducts of aldicarb under FC, MCA, O_3 and H_2O_2 oxidation systems.

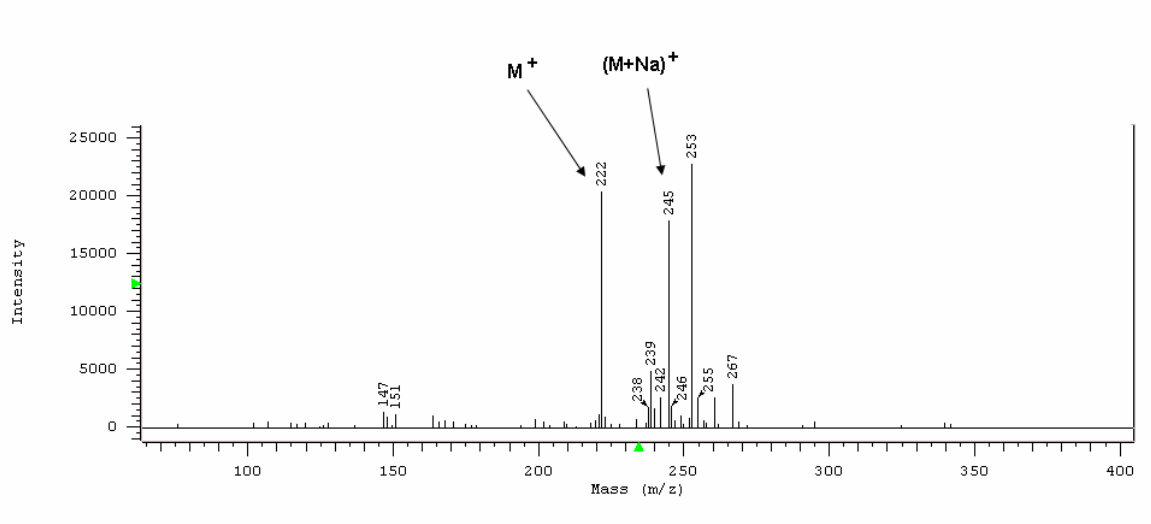

Fig. 5 - Mass spectrum of aldicarb sulfone (MW: 222) standard and the oxidation byproducts of aldicarb under $KMnO_4$ oxidation systems.

The mass spectra of aldicarb sulfoxide standard and the oxidation byproduct of aldicarb in free chlorine, monochloroamine, ozone, hydrogen peroxide systems are identical, so only one mass spectrum is presented, as shown in Fig. 4. The ion with m/z 206 is molecular ion, which is rarely observed in ESI mass spectrum. The ion with m/z 229 is the sodiated molecular ion, while the m/z 132 ion is the in-source fragment ion. Similarly, the mass spectra of aldicarb sulfone standard and the permanganate oxidation byproduct of aldicarb are identical, so only one mass spectrum is presented, as shown in Fig. 5. The ion with m/z 245 is molecular ion, while the ion with m/z 245 is the sodiated molecular ion. From Fig. 4 and 5, it can be concluded that aldicarb will be oxidized to produce aldicarb sulfoxide in free chlorine, monochloroamine, ozone, hydrogen peroxide systems, while aldicarb sulfone will be produced as an oxidation byproduct of aldicarb in permanganate system.

In order to fully understand the property of aldicarb, a comprehensive investigation of oxidation of aldicarb sulfoxide, one of the metabolites of aldicarb, was conducted, and the corresponding total ion chromatograms are shown in Fig. 6.

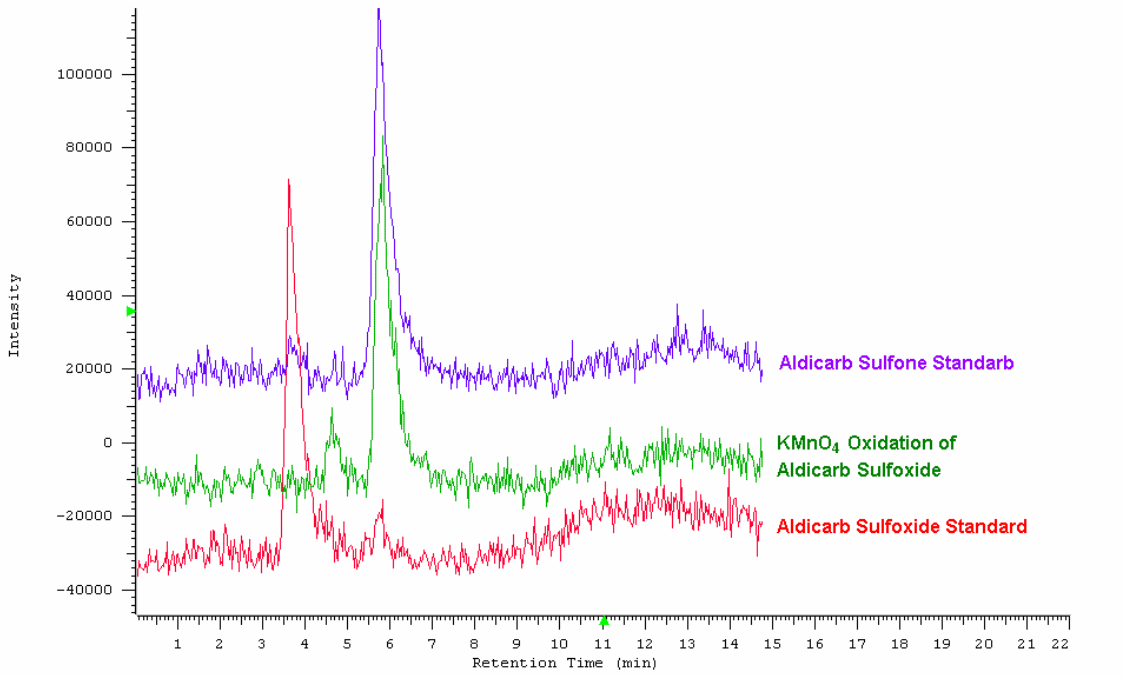

Fig. 6 - TICs of aldicarb sulfone, aldicarb sulfoxide standard and its oxidation byproduct under $KMnO_4$ oxidation system.

In Fig. 6, it can been observed that aldicarb sulfoxide was all gone after oxidation in permanganate system, and a new byproduct peak, which showed the same retention time as aldicarb sulfone peak, was presented. No degradation byproducts were detected in all other oxidation systems, despite hydrogen peroxide and high dosage of UV radiation resulted in the aldicarb sulfoxide removal with 40% and 50%, respectively. This observation shows that hydrogen peroxide oxidation and UV photodegradation of aldicarb sulfoxide are different from permanganate oxidation regarding the reaction

mechanism. The mass spectrum of the permanganate oxidation byproduct of aldicarb sulfoxide was observed to be identical to the one shown in Fig. 5, indicating that aldicarb sulfone is the permanganate oxidation byproduct of aldicarb sulfoxide.

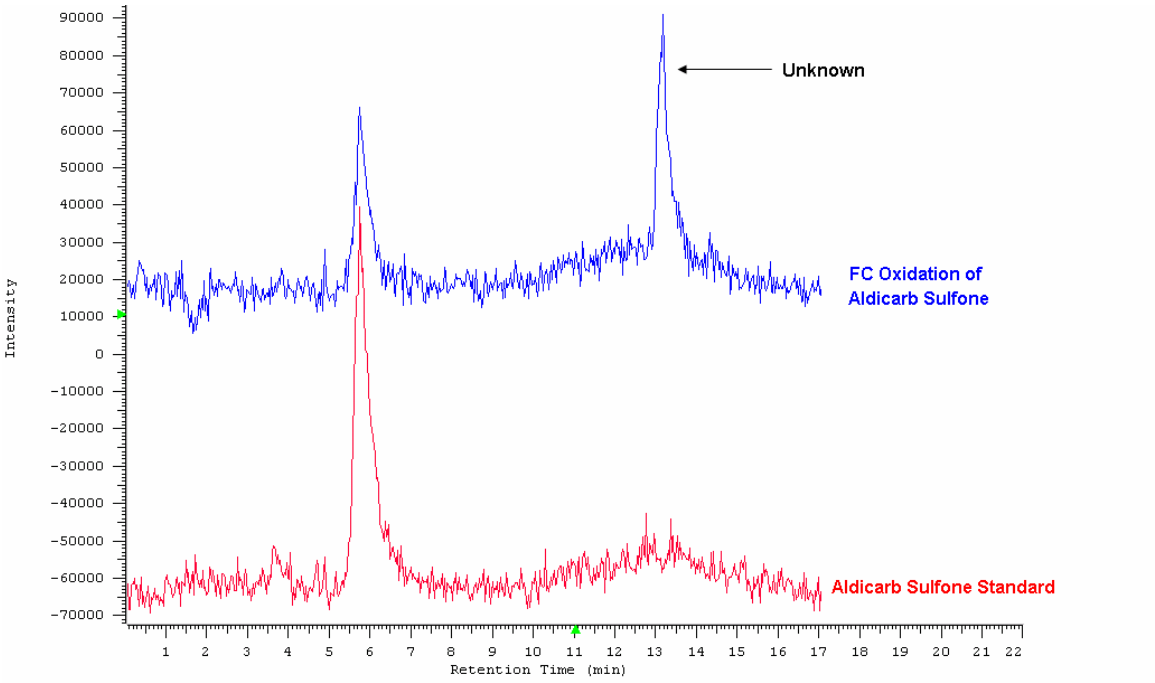

Fig. 7 - TICs of aldicarb sulfone standard and its oxidation byproduct under FC oxidation system.

Additionally, the comprehensive investigation of oxidation of aldicarb sulfone, another metabolite of aldicarb, was also carried out in various oxidation systems, and the corresponding total ion chromatograms are shown in Fig. 7. Aldicarb sulfone was partially oxidized to produce an unknown peak by free chlorine, while other oxidants except for free chlorine and high dosage of UV radiation, which removed 60% and 30% of aldicarb sulfone, respectively, did not show significant removal (<20%) of aldicarb sulfone, and no oxidation byproducts were detected.

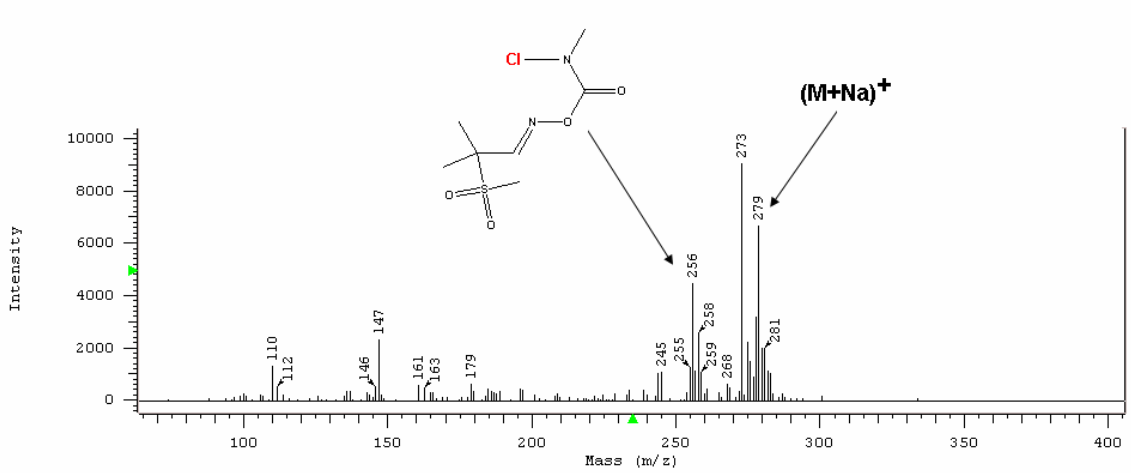

Fig. 8 - Mass spectrum of the unknown in the oxidation of aldicarb sulfone under FC oxidation system.

As usual, the mass spectrum of the unknown is shown in Fig. 8. Based on the literature [21] and the observation in this study, it is proposed that the unknown byproduct of aldicarb sulfone under free chlorine oxidation system is N-chloro-aldicarb sulfone, with the structure shown in the figure. The m/z 246 ion is interpreted to be the molecular ion, and the m/z 279 to be the sodiated molecular ion. The m/z 273 ion has not been interpreted yet.

Based on the comprehensive investigation of oxidation for aldicarb and its two carbamate metabolites, the degradation pathways are proposed, as shown in Fig. 9.

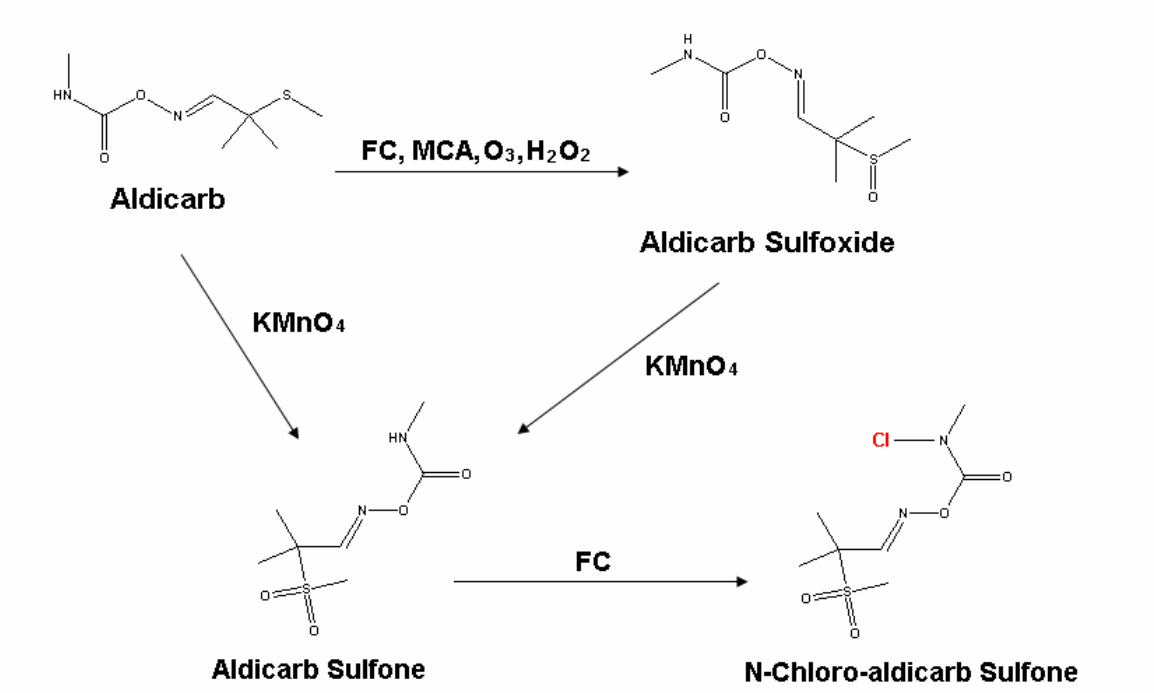

Fig. 9 - Proposed degradation pathways of aldicarb.

3.2. Quantification of Oxidation of Aldicarb and Its Carbamate Metabolites in Different Oxidation Systems

On the basis of qualitative analysis of aldicarb and its carbamate metabolites in various oxidation systems, quantification studies of aldicarb and its carbamate metabolites were performed by using HPLC/UV, as shown in Fig. 10, 11 and 12. In Fig. 10, it can be seen that all aldicarb was removed by free chlorine, monochloroamine, ozone, permanganate and hydrogen peroxide, and 70% removed by high dosage UV radiation. In Fig. 11, free chlorine, permanganate, hydrogen peroxide and high dosage of UV radiation demonstrate significant removal of aldicarb sulfoxide, but only aldicarb sulfone

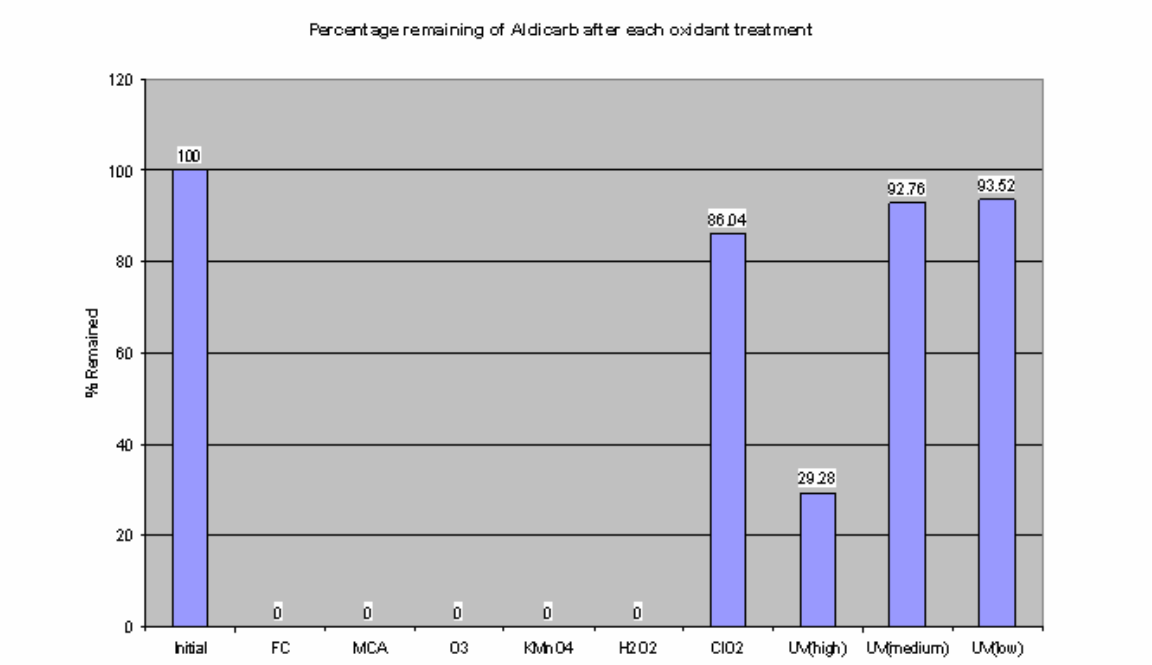

Fig. 10 - Quantification of aldicarb in terms of percentage removal in different oxidation systems.

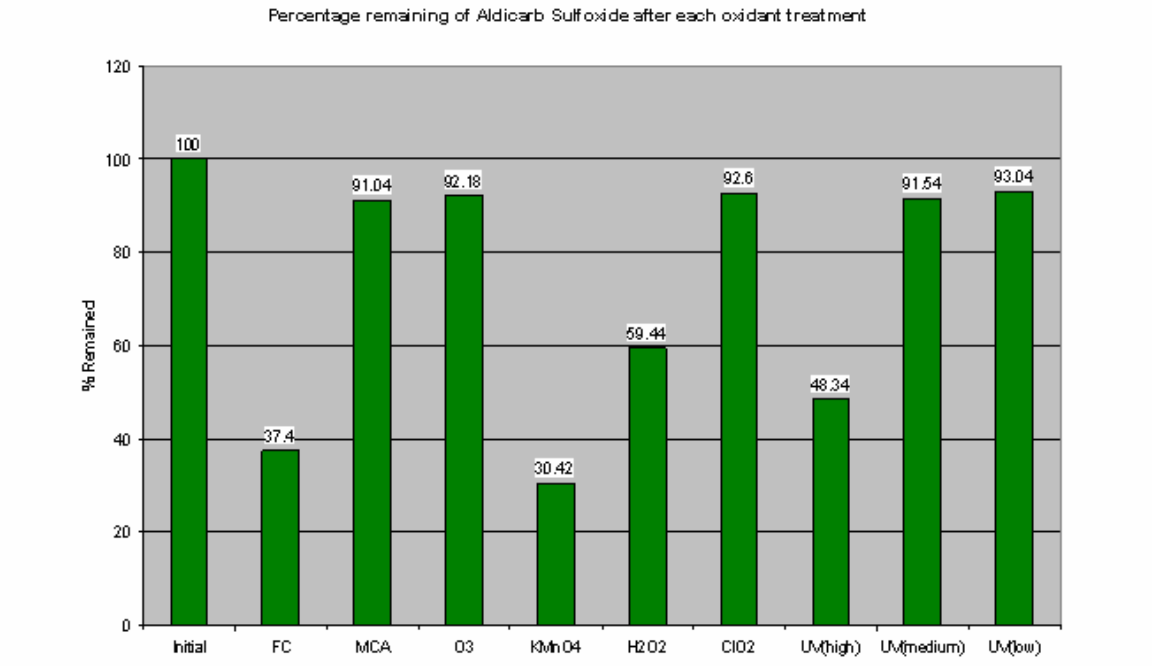

Fig. 11 - Quantification of aldicarb sulfoxide in terms of percentage removal in different oxidation systems.

was detected as the permanganate oxidation byproduct of aldicarb sulfone, no other oxidation byproducts were detected.

In Fig. 12, only free chlorine and high dosage of UV radiation demonstrate significant removal (>80% for free chlorine and >30% for high dosage of UV radiation) of aldicarb sulfone, while other oxidants do not show significant removal (<20%). Moreover, no oxidation byproducts were detected for high dosage UV radiation of aldicarb sulfone.

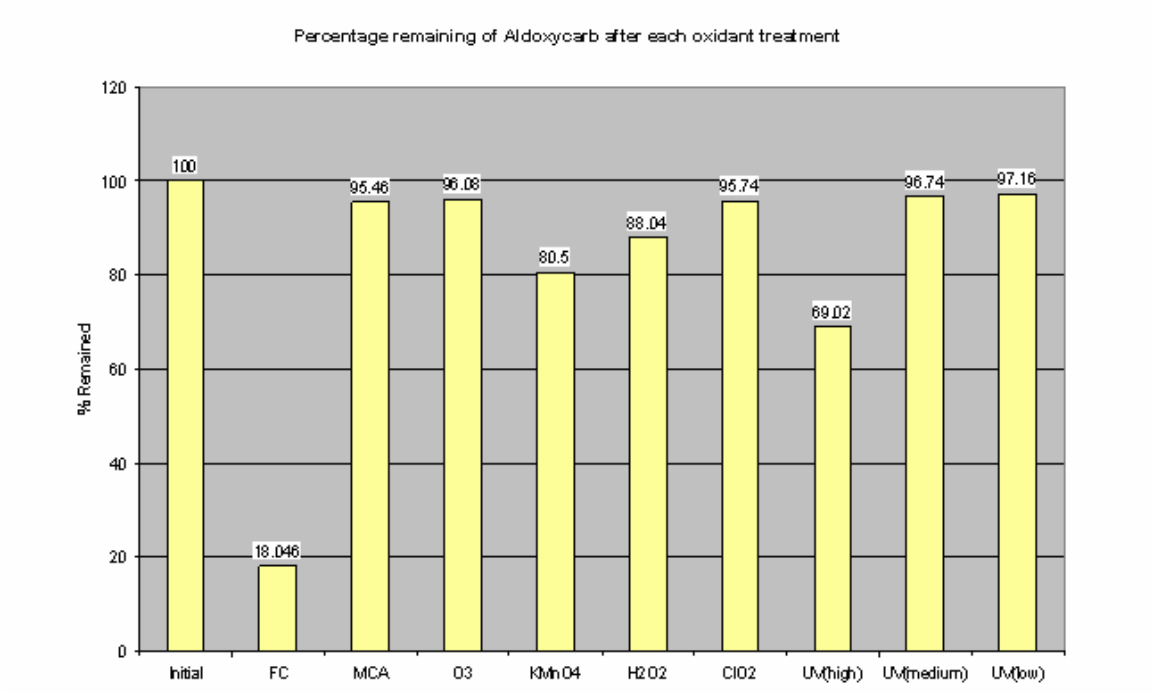

Fig. 12 - Quantification of aldicarb sulfone in terms of percentage removal in different oxidation systems.

Not only the removal of aldicarb, but also the concentrations of components in the aldicarb oxidation reaction matrices in various oxidants were determined, as shown in Fig. 13. It can been seen that only free chlorine and high dosage of UV radiation showed

an unbalanced mass, indicating that there could be other degradates existed in these two oxidation systems. Other systems showed that the detected degradates were the major degradates without other significant degradates.

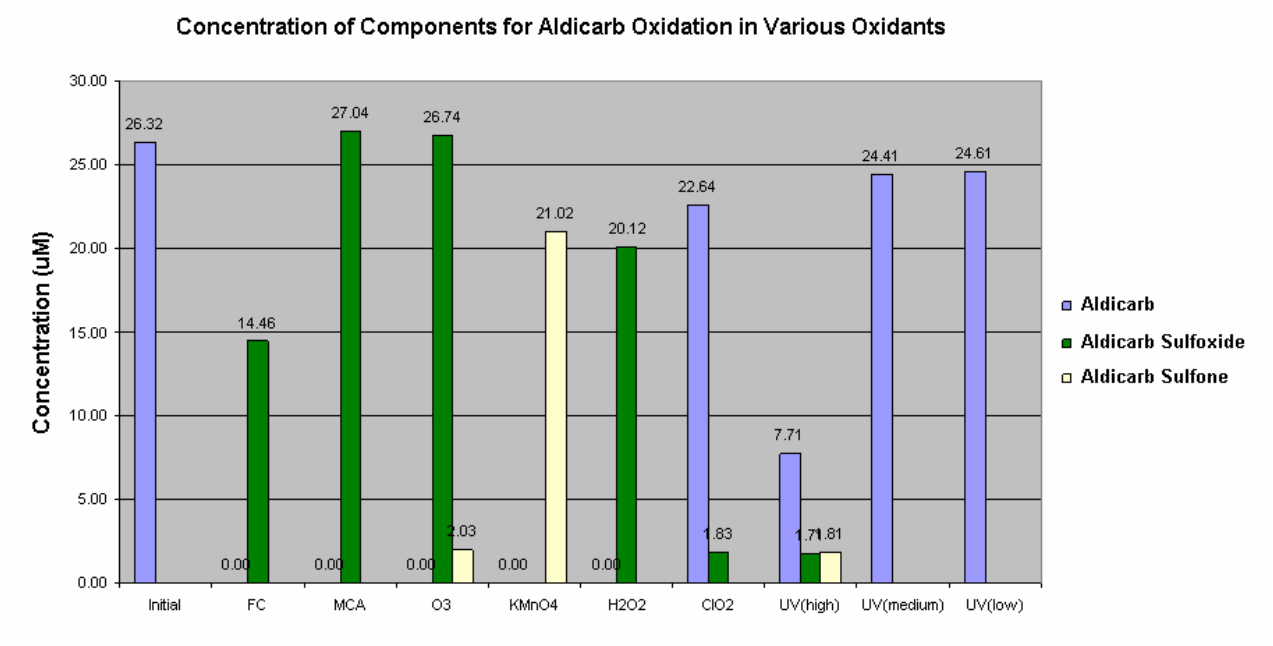

Fig. 13 - Concentration of components for aldicarb oxidation in various oxidants.

4. Conclusions

Based on the results of qualitative and quantitative analysis of oxidation byproducts of aldicarb and its carbamate metabolites in various oxidation systems, it can be concluded that aldicarb is prone to be oxidized to produce aldicarb sulfoxide by free chlorine, monochloroamine, ozone and hydrogen peroxide, and to produce aldicarb sulfone by permanganate, indicating that aldicarb is not stable in these oxidation systems. Aldicarb sulfoxide will be oxidized to produce aldicarb sulfone by permanganate, and to produce an unknown byproduct by free chlorine, meaning that aldicarb sulfoxide is more stable than aldicarb. Aldicarb sulfone is prone to be oxidized to produce N-chloro-aldicarb

sulfone by free chlorine, showing that aldicarb sulfone is more resistant to oxidants than aldicarb sulfoxide. High dosage of UV radiation displays a significant removal for aldicarb and its two carbamate metabolites, providing an ideal way of water treatment strategy.

Acknowledgments

The authors are thankful for the financial support from Awwa Research Foundation (project 3170). Additional infrastructure support was provided by the Environmental Research Center, Department of Chemistry, and Department of Civil, Architectural and Environmental Engineering at University of Missouri-Rolla. Ms. Alice Fulmer serves as the AwwaRF project officer on this project, and contributed significantly to this research work.

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