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Enzymes: The New Water/Wastewater Treatment Chemical

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Enzymes: The New Water/Wastewater Treatment Chemical

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Enzymes: The New Water/Wastewater Treatment Chemical

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Pharmaceuticals and personal care products (PPCPs) are detected routinely in raw and treated municipal wastewater. Conventional wastewater treatment processes are not effective in removing PPCP; therefore, treated wastewater discharges are one of the main entry points for PPCPs into the aquatic environment, and eventually into drinking water supplies. The use of laccase-catalyzed oxidation for removing low concentrations of PPCPs from municipal wastewater after primary treatment is investigated. Oxybenzone was selected as a representative PPCP. Like many other PPCPs, oxybenzone is not recognized directly by the laccase enzyme. Therefore, mediators were used to expand the oxidative range of laccase, and the efficacy of this laccase-mediator system in primary effluent was evaluated. Eight potential mediators were investigated. The greatest oxybenzone removal efficiencies were observed when 2,2'-azino-bis(3-ethylbenzthiazoline-6sulphonic acid) (ABTS), a synthetic mediator, and acetosyringone (ACE), a natural mediator, were present. An environmentally relevant concentration of oxybenzone (10 μg/L) in primary effluent was removed below the detection limit after two hours of treatment with ABTS, and 95% was removed after two hours of treatment with ACE. Several mediator/oxybenzone molar ratios were evaluated at two different

initial oxybenzone concentrations. Higher mediator/oxybenzone molar ratios were required at the lower (environmentally relevant) oxybenzone concentrations, and ACE required higher molar ratios than ABTS to achieve comparable oxybenzone removal. The oxidation mechanisms and kinetics of the ACE mediator was evaluated. A better understanding of the mediator oxidation process would lead to a better design of the laccase-mediator system. An alternative laccase-mediator treatment configuration, which allows the enzyme and mediator to react prior to coming in contact with the target PPCP, was investigated. This treatment configuration shows promise for further development since it might reduce laccase and mediator requirements. Oxidation byproducts generated by the laccase-mediator system were characterized and compared to those generated during ozonation. Enzymatic treatment generated byproducts with higher mass to charge (m/z) ratios, likely due to oxidative coupling reactions. The results of this study suggest that, with further development, a laccase-mediator system has the potential to extend the treatment range of laccase to PPCPs not directly recognized by the enzyme, even in a primary effluent matrix.

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Chapter 1 - Introduction

The overall goal of this research is an investigation of the use of oxidoreductase enzymes for the removal of pharmaceuticals and personal care products (PPCPs) from municipal wastewater. Specifically, this research investigated the possible application of laccase–catalyzed oxidation to the removal of PPCPs from municipal wastewater. Oxybenzone was selected as a representative PPCP. Oxybenzone, like many other PPCPs, is not oxidized directly by the laccase enzyme. Mediators were used to expand the oxidative range of laccase. The efficacy of this laccase–mediator system was evaluated. This investigation focused primarily on municipal wastewater primary effluent (effluent from the primary clarifier in a conventional municipal wastewater treatment plant) because, if eventually implemented in municipal wastewater treatment plants, placing enzymatic treatment after the primary clarifier would allow for potential removal of byproducts in subsequent treatment processes.

1.1 MOTIVATION

The scarcity of water will ultimately hinder economic development in the United States. Development of new water supplies lags population growth and current supplies are being exhausted, thereby exacerbating the water supply problem (Loraine and Pettigrove, 2006). Water reuse, the intentional beneficial use of water for a second time, is becoming essential. Water reuse demands a higher effluent quality from wastewater treatment plants, particularly with respect to contaminants of emerging concern such as PPCPs. These recalcitrant compounds are widespread in the environment. PPCPs are detected routinely in wastewater effluents, natural water systems, and drinking water supplies at levels ranging from a few ng/L to a few µg/L. The primary means by which these microconstituents enter ecosystems is municipal wastewater that has been

contaminated via excretion, flushing of unused medications, and our daily use of personal care products (Kolpin et al., 2002; Barber et al., 2006).

Conventional wastewater treatment processes are not particularly effective for PPCP removal (Auriol et al., 2006), resulting in many of these microconstituents being discharged to surface waters. Consequently, freshwater sources of drinking water now contain many of these recalcitrant compounds (Kolpin et al., 2002). PPCPs have the potential to cause adverse physiological and developmental effects at trace levels (Khetan and Collins, 2007) and have already been found to cause physiological changes in some aquatic organisms (Vajda et al., 2008). PPCPs thus pose a potential health risk and represent a significant barrier to widespread acceptance of water reuse. Since passage of the Water Pollution Control Act 35 years ago, wastewater treatment has been designed to solve an earlier set of environmental problems (oxygen depletion and eutrophication in natural waters), but not to reach the high quality effluent required by water reuse. Modern problems require new treatment solutions.

1.2 RESEARCH CHALLENGE

Partial removal of some PPCPs is often achieved in current water and wastewater treatment systems, although the majority of the compounds remain soluble in the effluent streams (Auriol et al., 2006). Several advanced physical/chemical and microbiological treatment technologies have been proposed to remove PPCPs from water.

Although certain advanced physical/chemical treatment processes (*e.g.,* ozonation, advanced oxidation processes (AOPs), reverse osmosis, and adsorption) can effectively remove PPCPs from wastewater (Snyder et al., 2007), these technologies have certain disadvantages. Ozonation, AOPs, and reverse osmosis are quite energy intensive. Reverse osmosis and adsorption concentrate pollutants and change their phase,

respectively, rather than destroying them (Auriol et al., 2006). Ozonation and AOPs can form undesirable byproducts as a result of reactions with both target PPCPs and natural organic matter (Ikehata et al., 2006). Microbiological treatment is another attractive alternative if the PPCPs can be completely mineralized to carbon dioxide and water. However, such systems have difficulty treating the low levels of PPCPs typically found in wastewater sources, and long acclimation times are common (Alcalde et al., 2006). Developing a more robust treatment process that can effectively remove a wide range of PPCPs from either drinking water or wastewater is critical.

Mineralization of some aromatic-containing compounds such as PPCPs to carbon dioxide and water has been observed to take place in nature via the action of white rot fungi (Bumpus et al., 1985). The first step in the degradation of these compounds is their oxidation by oxidoreductase enzymes secreted by the fungi. Laccase, an oxidoreductase enzyme commonly secreted by white rot fungi, catalyzes the oxidation of certain aromatic compounds, particularly phenolic compounds, using molecular oxygen as the terminal electron acceptor. Laccase exhibits substrate specificity for some PPCPs; however laccase cannot directly oxidize many others (Cañas and Camarero, 2010). However, the presence of low molecular weight mediators (usually low molecular weight phenolic compounds) has been reported to enhance/expand laccase's oxidative ability (Cañas and Camarero, 2010; Kunamneni et al., 2008a). Laccase is able to oxidize these mediators to free radicals, which react non-specifically, providing highly effective removal (transformation) of a wide spectrum of compounds. The enzymatic treatment investigated in this research utilizes a laccase-mediator system, which is expected to be capable of oxidizing a broader spectrum of PPCPs.

Laccase-catalyzed oxidation has been investigated for the removal of organic contaminants. Direct oxidation of phenols in industrial effluents from olive oil mills,

petroleum refineries, pulp and paper mills, and wine distilleries by laccase has been demonstrated (Berrio et al., 2007; Ko and Fan, 2010; Steevensz et al., 2009; Strong and Burgess, 2007). Moreover, direct oxidation of several emerging organic contaminants (including several PPCPs) by laccase in buffered ultrapure water has been successfully demonstrated (Lu et al., 2009; Lloret et al., 2010; Kim and Nicell, 2006; Cabana et al., 2007a). Most of these compounds contain phenolic moieties, which is why they were directly oxidized by laccase without the addition of mediators. Several emerging organic contaminants that are not directly oxidized by laccase have been successfully oxidized by the laccase-mediator system in buffered ultrapure water (Hata et al., 2010; Lloret et al., 2010; Sei et al., 2008; Camarero et al., 2008; Johannes and Majcherczyk, 2000b). Thus, the enzyme laccase has the potential to effectively oxidize these recalcitrant compounds, without the necessity of maintaining the viability of a particular microbial community or utilizing other expensive or energy intensive approaches. The laccase oxidation will transform the target compounds to oxidation byproducts.

Previous studies were performed in the context of PPCP removal from municipal wastewater, but the experiments were conducted in ultrapure buffered solutions, and most were at relatively high PPCP concentrations. In this research, the laccase-mediator system was evaluated for removing trace concentrations of a PPCP not directly oxidized by laccase from municipal wastewater solutions. Laccase-catalyzed oxidation (like ozonation and AOPs) transforms the target compounds, generating oxidation byproducts. As part of this study, byproduct formation also was evaluated and compared to that of ozonation. Mediators contribute significantly to the laccase-mediator oxidation process. Consequently, the mechanisms and kinetics of the laccase oxidation of mediators were evaluated. A better understanding of the mediator and laccase oxidation mechanisms and kinetics would allow a better design of the laccase-mediator treatment system. Finally, a

new treatment configuration of the laccase-mediator system was attempted to yield better performance. The new treatment configuration allows the enzyme and the mediator to interact prior to coming in contact with the target contaminant.

Both transformation of the parent compound and the removal of byproducts are desirable. The greatest advantage of laccase-catalyzed oxidation is its potential for implementation in municipal wastewater primary effluent, which might allow removal of the enzyme oxidation byproducts in subsequent conventional treatment processes, such as activated sludge followed by secondary clarification. This removal could occur by biodegradation, precipitation followed by sedimentation, or adsorption to the biomass present in secondary treatment. The biodegradation of laccase oxidation byproducts of bisphenol A was demonstrated by Nakamura and Mtui (2003). Also, several authors have reported enzyme-catalyzed oxidative coupling of organic substrates, including laccase-catalyzed oxidative coupling of acetaminophen to larger molecular weight oxidation byproducts (Huang et al., 2005; Lu et al., 2009). The products of oxidative coupling reactions are usually less hydrophilic particularly as the molecules become larger (Lu et al., 2009) and thus may be easier to remove from the aqueous phase.

Primary effluent contains relatively high concentrations of organic and inorganic constituents (in comparison to secondary effluent and buffered ultrapure water), and these constituents could affect the efficacy of the laccase-mediator system. In particular, a primary effluent matrix would be expected to demand higher mediator and/or enzyme concentrations. These issues were explored in this research.

1.3 RESEARCH APPROACH

The performance of the laccase-mediator system for removing PPCPs from primary effluent was evaluated in this study using oxybenzone (a representative PPCP

not directly oxidized by laccase) in the following stages: (i) mediator screening experiments to evaluate the need for a mediator and determine which mediators performed best; (ii) initial experiments in primary effluent to determine if the laccase-mediator system could effectively remove oxybenzone from a primary effluent matrix (despite the presence of other organic and inorganic constituents); (iii) further experiments in primary effluent investigating the effect of the mediator/oxybenzone molar ratio; (iv) evaluation of the laccase-mediator system at environmentally relevant oxybenzone concentrations in primary effluent; (v) characterization of the enzyme oxidation byproducts and comparison of these byproducts with those generated by ozonation; (vi) evaluation of the mechanisms and kinetics of laccase oxidation of mediators, and (vii) assessment of an alternative treatment configuration to optimize the laccase-mediator system, allowing the enzyme and the mediator to interact prior to coming in contact with the target contaminant.

Oxybenzone ((2-Hydroxy-4-methoxyphenyl)-phenylmethanone) absorbs and dissipates UV radiation, and is therefore commonly used in sunscreens and cosmetic products. Oxybenzone was selected as a representative PPCP for three primary reasons. First, despite being a phenolic compound, oxybenzone is not directly oxidized by laccase and so was a good candidate for study in the laccase-mediator system. Second, oxybenzone demonstrates estrogen-like activity *in vitro* and *in vivo* (Schlumpf et al., 2004; Calafat et al., 2008). Adverse health effects in humans have not been reported; however, dermal and oral administration of oxybenzone to rats and mice has resulted in alterations in liver, kidney, and reproductive organs (Calafat et al., 2008). Third, measurable oxybenzone concentrations in conventional wastewater treatment plant effluents have been reported (Kim et al., 2007; Snyder et al., 2007). Snyder et al., (2007)

reported relatively high concentrations ranging from 37 to 3,810 ng/L in untreated municipal wastewater and in primary effluent.

1.4 DISSERTATION STRUCTURE

Chapter 1 introduces the problem that is being addressed and outlines a set of objectives for this research. Chapter 2 describes some general background related to PPCPs in water systems, human health and ecological effects, enzymatic treatment, and reaction mechanisms of oxidoreductase enzymes. Chapter 3 describes the methodologies followed to design, set up, and optimize the experiments. Chapter 4 summarizes and discusses the experimental results that respond to the objectives of this work. Chapter 5 contains conclusions of this research as well as suggestions for future research.

Chapter 2 – Literature Review

Water will ultimately limit the economic growth of many regions all over the world. Intentional water reuse will be necessary to satisfy demand. The public is keenly aware that pharmaceuticals and personal care products (PPCPs) are in wastewater, river systems and even drinking water. These microconstituents enter the ecosystem primarily through wastewater. Such compounds are known to cause negative environmental consequences, and public health concerns have been suggested. They constitute a major obstacle to water reuse. Enzymes, particularly oxidoreductase enzymes can be used as an alternative treatment technology to remove PPCPs at environmentally relevant concentrations (ng/L - µg/L), even from wastewater.

2.1 PPCPs IN WATER SYSTEMS

The use of PPCPs in the United States continues to rise rapidly. The number of drugs prescribed in 2009 set a record at 3.9 billion prescriptions, and annual sales in 2009 reached $300.3 billion (Bartholow, 2010). These pharmaceuticals can enter the aquatic environment via human excretion or via flush-disposal of unused medication. In a U.S. Geological Survey study performed in 2002, pharmaceutical products were detected in 80% of the 139 rivers tested across 30 states in the United States (Kolpin et al., 2002). Effluents from wastewater treatment plants are the main point source for PPCPs to enter the environment because these compounds are not completely removed in conventional wastewater treatment plants.

Auriol et al. (2006) reported that conventional physical/chemical and biological treatment systems at water and wastewater treatment plants are not particularly effective for removing PPCPs. Coagulation processes with aluminum and ferric salts are not successful at removing PPCPs. Chlorination removes some PPCPs, but results in

formation of chlorinated byproducts that exhibit carcinogenicity and/or mutagenicity. Biological treatment processes such as activated sludge systems, trickling filters, and anaerobic treatment accomplish some PPCP removal although these compounds are often not completely degraded. The majority of the PPCPs are non-polar and hydrophobic; therefore, most of the removal observed in biological treatment systems is due to adsorption onto the sludge rather than biodegradation. Consequently, high concentrations of PPCPs are observed in water released by dewatering sewage sludge and in digested sludge. Aerobic treatment achieves higher removal efficiencies than anaerobic treatment, and high hydraulic residence times and solid retention times enhance the removal efficiency, particularly in aerobic processes. Nevertheless, most PPCPs pass through the treatment systems without being removed (Auriol et al., 2006).

Incidental and unplanned water reuse is occurring naturally and goes unrecognized by the public. Many communities currently use surface water sources that are subject to a significant number of upstream discharges of treated wastewater. The unplanned indirect reuse of wastewater in the public drinking water supply is widespread and increasing; consequently, freshwater sources of drinking water contain many of the same recalcitrant constituents of public health concern found in wastewater effluent discharges. The pathway from discharge of wastewater effluents to the intake for drinking water treatment plants is rather direct in several regions across the United States including the entire southwest, Florida, and Virginia (Sedlak et al., 2000). Twenty-three percent of regulated effluent discharges in the U.S. enter streams receiving little dilution (less than 10-fold dilution), and this percentage rises to 60% under low-flow conditions. In some cases such as the Trinity River, Texas, 90% of the flow is composed of effluent discharges under low-flow conditions (Brooks et al., 2006).

PPCPs have been detected in raw and treated drinking water supplies at levels ranging from a few ng/L to a few µg/L. The Metropolitan Water District of Southern California, which supplies water to approximately 18 million people, detected phthalate esters, sunscreens, clofibrate, clofribric acid, ibuprofen, triclosan, and N,N-Diethyl-meta-toluamide (DEET) in the raw water, and also detected many of these compounds in the finished drinking water supplies (Loraine and Pettigrove, 2006). Similarly, 16 pharmaceutical compounds (e.g., medicines for pain, infection, high cholesterol, and heart problems) were detected in Philadelphia's treated drinking water supply, and six different pharmaceuticals were detected in Washington, D.C. treated drinking water (Donn et al., 2008).

2.2 HUMAN HEALTH AND ECOLOGICAL EFFECTS

The effects of PPCPs on ecological systems and human health depend not only on the quantity of compounds that are discharged through wastewater effluents, but also on the degree of degradation of these compounds in the natural system. Biotransformation is an important removal mechanism for these compounds, but biodegradation does not take place at low contaminant concentrations (Sedlak et al., 2000). PPCPs account for a tiny fraction of the total organic matter present and their low concentrations are insufficient to allow microorganisms to successfully biodegrade these compounds. Consequently, PPCPs persist in natural systems.

Pharmaceutical compounds are designed either to be toxic to infectious organisms (bacteria, fungi, and parasites), or to be active and interact with receptors in humans and animals; therefore, it seems reasonable that these compounds will have some effect, particularly for chronic exposures when PPCPs are present in aquatic systems (Khetan and Collins, 2007). The potential adverse health and environmental effects of PPCPs

even at a few ng/L concentrations create uncertainties about water reuse. As described by Khetan and Collins (2007), the three most important toxicological effects exhibited by PPCPs are as follows: i) killing of cells, ii) mutation of DNA leading to cancer, iii) disruption of chemical signaling controlling cellular development. The latter toxicological effect, also known as endocrine disruption, behaves completely differently than the previous two. Endocrine disrupting compounds (natural or synthetic) alter the hormonal systems that enable the organisms to communicate and respond to their environment. The disruption process occurs at low (ng/L) rather than at high concentrations (Diamanti-Kandarakis et al., 2009). As reported by Diamanti-Kandarakis et al. (2009) endocrine disrupting compounds have effects on reproduction, cardiovascular diseases, metabolism and obesity, breast development and cancer, and prostate cancer.

Some of the observed toxicological effects of PPCPs in aquatic systems are described as follows. Antibiotics allow microorganisms to gene transfer into antibiotic-resistant pathogens. Bacteria in raw wastewater are more resistant to antibiotics than bacteria elsewhere (Khetan and Collins, 2007). Similarly, several pharmaceuticals have shown endocrine disrupting effects modifying reproduction and affecting cellular development (Khetan and Collins, 2007).

Estrogenic hormones such as 17 α-ethinyl estradiol (a component of birth control pills) cause endocrine disruption in fish. Feminization of male fish exposed to ng/L concentrations of these hormones has been observed (Sedlak et al., 2000). Vajda et al. (2008) reported fish feminization downstream of a wastewater treatment plant effluent discharge in Boulder City, containing a complex mixture of endocrine disrupting compounds including 17β-estradiol, 17 α-ethinyl estradiol, alkylphenols, and bisphenol A resulting in a total estrogen equivalent concentration of 31 ng/L. The percentage of

male fish downstream was 50% that of the upstream. Approximately, 20% of intersex fish (with both ovarian and testicular tissue) were reported downstream the effluent wastewater discharge while intersex fish were not found upstream the wastewater treatment plant discharge. Antiepileptic and anticonvulsant drugs such as carbamazepine are also suspected to have endocrine disrupting effects (Khetan and Collins, 2007). Triclosan, a personal care product used as an antimicrobial agent, is broadly used in deodorants, soaps, dermatological preparations, and dental products. *In vitro* studies on rat and human have shown that triclosan might affect offspring and disturb metabolic systems and hormone homeostasis (Allmyr et al., 2006). Oxybenozone, another personal care product, is commonly used as a sunscreen agent in several cosmetic products. Oxybenzone absorbs and dissipates ultraviolet radiation. Even though human exposure to oxybenzone was not associated with adverse health effects, studies using rats and mice demonstrated alterations in liver, kidney, and reproductive organs when oxybenzone was administered (Calafat et al., 2008). Oxybenzone exhibits in vivo and in vitro estrogen-like activity (Schlumpf et al., 2004).

A relationship between exposure to endocrine disrupting compounds and diseases in humans still remains unknown. The main reason is because individuals and populations are not exposed to the same types and concentrations of endocrine disrupting compounds over time (Diamanti-Kandarakis et al., 2009). Some studies aimed to link the exposure to certain endocrine disrupting compounds (by measuring their concentrations in human urine) with chronic diseases. A significant relationship was found between concentration of bisphenol A in urine and cardiovascular diseases, diabetes, and abnormal concentrations of liver enzymes (Lang et al., 2008). In addition, mixtures of many pharmaceuticals may have synergistic effects, exhibiting greater toxicity than would be predicted by the additive contribution of the single compounds. Thus, although

the impacts of human exposure to low dose mixtures of pharmaceuticals are not fully understood, what is known about these compounds gives reason for serious concern (Khetan and Collins, 2007).

2.3 PPCPs Treatment Technologies

PPCPs have negative environmental effects and a potential negative health effect in humans; therefore, wastewater effluents must be treated properly prior to discharge. PPCPs exhibit some chemical similarities in that most of them exhibit aromatic character. Aromatic compounds are extremely stable and unreactive with reagents that usually attack other unsaturated organic compounds. The great stability of aromatic containing compounds results mainly from the resonance energy of the aromatic ring due to the much lower energy of its bonding molecular orbitals compared to the uncombined atomic orbitals (W. Brown and Foote, 2002). PPCPs are designed to be persistent, so this is the main reason why they accumulate in the environment. Substances that were prohibited decades ago such as polychlorinated biphenyls (PCBs) remain at relatively high concentrations in the environment, and they can be even detected in animals or humans (Diamanti-Kandarakis et al., 2009).

Although PPCPs found in water sources contain some chemical structure similarities, these compounds exhibit a diverse range of physical/chemical properties such as acid dissociation constants (pK_a), water/octanol partitioning coefficients (log K_{ow}), and water solubility (S) (Snyder et al., 2007). Therefore, a treatment technology for successfully removing all of these compounds from water sources should consider the diversity of the physical/chemical properties of PPCPs. Conventional physical/chemical and biological treatment systems are not effective at removing PPCPs (Auriol et al., 2006). Removal of PPCPs is possible with advanced physical/chemical treatment

technologies such as chemical oxidation, adsorption on activated carbon, UV treatment, ozonation, advanced oxidation, and reverse osmosis. However each of these processes has disadvantages including high costs, byproduct formation, difficulty treating low levels of PPCPs, and in some cases, concentrating the compounds into a different phase.

Membrane filtration and adsorption onto activated carbon can effectively remove PPCPs from water and wastewater sources (Snyder et al., 2007; Khetan and Collins, 2007). Microfiltration (MF) and ultrafiltration (UF) membranes are not particularly effective in rejecting PPCPs. MF and UF are effective when used as a part of a membrane bioreactor (MBR). Reverse osmosis (RO) filtration is very effective at removing PPCPs although trace concentration of some compounds are still detected in RO effluents (Snyder et al., 2007). Snyder et al. (2007) demonstrated that both powdered activated carbon (PAC) and granular activated carbon (GAC) were very effective at removing PPCPs from water sources, with removal efficiencies greater than 90%. These technologies have certain disadvantages. The retention and adsorption capacity of membranes and activated carbon decreases with operation time. The presence of natural organic matter (NOM) and biofouling can decrease the performance of these systems (Khetan and Collins, 2007). Membrane filtration, particularly RO, remove organic and inorganic compounds into a concentrate stream (brine) that requires proper disposal. Similarly, adsorption onto activated carbon requires disposal of the spent activated carbon. Moreover, the high pressure requirements for membrane filtration and either the continuous addition of PAC or the regeneration of GAC involve a significant operational cost (Snyder et al., 2007).

Ozonation and other advanced oxidation processes (AOPs) are also effective at removing PPCPs from water and wastewater (Khetan and Collins, 2007; Snyder et al., 2006; Auriol et al., 2006). Ozone reacts with organics through a direct reaction with

molecular ozone. The addition of hydrogen peroxide (H_2O_2) involves the formation of highly reactive hydroxyl radicals (OH•), which react less selectively than molecular ozone and with faster reaction rates (Snyder et al., 2006). Full mineralization of PPCPs is not achievable at typical (economically feasible) ozone (O_3) doses used in water treatment applications, resulting in the formation of several oxidation byproducts (Khetan and Collins, 2007; Ikehata et al., 2008; Snyder et al., 2006). Other organic and inorganic constituents coexist with PPCPs and at much higher concentrations than the PPCPs in water and wastewater sources; therefore, hydroxyl radicals are consumed by the other constituents in the water matrix (Auriol et al., 2006; Snyder et al., 2006). Even though estrogenicity (measured as *in vitro* cellular bioassays) was readily removed from wastewater (Snyder et al., 2006), oxidation byproducts may exhibit an equal or even higher toxicity than parent compounds (Ikehata et al., 2008; Khetan and Collins, 2007; Snyder et al., 2006). Limited information is available regarding oxidation byproduct identities, toxicity, and biodegradability (Ikehata et al., 2008). There is no a single advanced water/wastewater treatment technology capable of removing trace concentrations of PPCPs to less that the detection limit of current analytical instruments. Toxicological studies are relevant to set up appropriate treatment goals (Snyder et al., 2006). Nevertheless, ozonation and AOPs have proven to be among the most viable methods for oxidizing PPCPs in water and wastewater.

Even the most effective treatment technology cannot completely remove PPCPs below the detection limit of modern analytical instrumentation. Consequently, the efficacy of a treatment technology (as well as the determination of treatment goals) should be established based on toxicity determinations (bioassays) rather than on final target concentrations. No single bioassay will be effective for the wide variety of PPCPs present in water and wastewater. Among PPCPs, endocrine disrupting compounds (such

as oxybenzone) might be the most problematic, since toxicological and ecological effects have been demonstrated at trace level concentrations (ng/L) of these compounds (Khetan and Collins, 2007). By assessing the reduction ofthe toxicity introduced by endocrine disrupting compounds, treatment goals and treatment performance can be more precisely defined. Cellular bioassays such as the human breast carcinoma *in vitro* assay, and the functional recombination yeast assay, are capable of determining the endocrine disrupting toxicity of a reaction mixture. These bioassays have been successfully applied to evaluate the performance of several oxidation treatment systems for PPCPs (Snyder et al., 2006; Auriol et al., 2008).

2.4 ENZYME CATALYSIS

Enzymatic treatment is an attractive alternative for removing PPCPs from wastewater effluents. Enzymes have been recently used for many beneficial uses such as a green alternative for industrial processes and products such as detergents, textiles, food and beverages, and can become so in the treatment of water. Enzymatic systems have low energy requirements, easy process control, and can operate over a wide range of pH, temperature, and ionic strength (Cabana et al., 2007b). Enzymes also are less likely to be inhibited by substances that may be toxic to living organisms (Karam and Nicell, 1997). In addition, recent biotechnological advances allow rapid and inexpensive production of appropriate enzymes (Kunamneni et al., 2008a). Novozymes Corp. supplies laccase enzymes at concentrations of approximately 1,000 U/mL at $30/Kg of enzyme; one unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of ABTS per minute at 37^{o}C.

Enzymes are biological catalysts; that is, enzymes increase reaction rates of chemical reactions without being consumed. The thermodynamic requirements of the

reaction that is catalyzed are not affected. That is, a chemical process needs to be thermodynamically favorable in order to be catalyzed by enzymes. Enzymes speed up reaction rates by factors of 10^6 to 10^{12} (Voet and Voet, 2004). An advantage of enzyme catalysis, compare to chemical catalysis, is that enzymes catalyze reactions at mild conditions such as temperatures below 100^oC, atmospheric pressure, and neutral pH (Voet and Voet, 2004).

Substrates bind to enzymes due to non-covalent interactions such as van der Waals forces, electrostatic attraction, hydrogen bonding, and hydrophobic interactions. The intermolecular interactions provide the enzyme a high degree of specificity for certain substrates (Voet and Voet, 2004). A substrate-binding site consists of a hole in the surface of an enzyme molecule that is complementary in shape to the substrate. Some amino acid residues located in the surface of the enzyme active site are capable of interacting with the substrate. Substrates that do not fit into the active site or have functional groups unable to interact with the active site cannot bind to the enzyme (Voet and Voet, 2004). Enzymes catalyze many reactions in association with small molecules (cofactors) which act as mediators between the enzyme and the substrates. Cofactors may be organic molecules known as coenzymes. Coenzymes are chemically modified by the enzymatic reactions in which they participate. To complete the catalytic cycle, the coenzyme must be returned to its original state (Voet and Voet, 2004).

Most of the processes that happen in metabolic pathways involve enzyme catalysis. Oxidation-reduction reactions in organic chemistry require breaking covalent bonds (a covalent bond means that an electron pair is shared between two atoms). In breaking a covalent bond, the electrons of the electron pair can either stay with one of the atoms (heterolytic bond cleavage) or split in a way that one electron stays with each of the two atoms (homolytic bond cleavage). Enzyme catalyzed oxidation-reduction

reactions happen mainly by homolytic bond cleavage, producing free radical compounds (Voet and Voet, 2004).

2.4.1 Kinetics of Enzyme Catalysis

The rate at which enzyme catalysis happens depends on several factors such as: i) the concentration of the substrate (the higher the substrate concentration, the higher the chances to have enzyme-substrate collisions), ii) the temperature (the higher the temperature, the higher the molecular motion, and therefore collisions between enzyme and substrate are more probable) , iii) the presence of inhibitors (preventing the enzyme from binding to the substrate or competing for the enzyme), and iv) pH (strongly affects the interaction between the substrate and the active site).

The model proposed for studying enzyme kinetics is presented as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_2} P + E$$

E = enzyme
S = substrate
ES = enzyme-substrate complex
P = product

$$K_M = \frac{k_{-1} + k_2}{k_1} \qquad \text{Eq. (2.1)}$$

K_M = Michaelis constant

When the substrate concentration is in great excess with respect to the enzyme concentration, the second step shown in the previous reaction (the enzyme-substrate complex transformation to the final product) controls the kinetics of the overall process. Equation 2.2 describes the overall reaction rate for that process. At the enzyme and

substrate relative concentrations previously described, it can be assumed that the concentration of the enzyme-substrate complex [ES] remains constant over time (equation 2.3). The overall reaction is considered to be at steady state. Johnson (2009) suggested that a substrate is in great excess with respect to the enzyme when the following expression applies: $[\mathrm{E}]_T < \frac{K_M}{5}$ where $[E]_T$ refers to the total enzyme concentration.

$$v = \frac{d[P]}{dt} = k_2[ES] \qquad \text{Eq. (2.2)}$$

v = reaction velocity

$$\frac{d[ES]}{dt} = 0 \qquad \text{Eq. (2.3)}$$

The rate of disappearance of the enzyme-substrate complex, and the mass balance for the enzyme concentration can be described by equations 2.4 and 2.5, respectively:

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] \qquad \text{Eq. (2.4)}$$

$$[E]_T = [E] + [ES] \qquad \text{Eq. (2.5)}$$

By combining equations 2.3, 2.4 and 2.5, the following expression can be derived $[ES] = \frac{[E]_T[S]}{K_M + [S]}$, and the overall reaction velocity can be obtained by substituting this term into equation 2.2, as described in equation 2.6.

$$v = \left(\frac{d[P]}{dt}\right) = k_2[ES] = k_2\frac{[E]_T[S]}{K_M + [S]} \qquad \text{Eq. (2.6)}$$

The term $k_2[E]_T$ is defined as the maximum velocity of the catalyzed reaction V_{max}. Substituting this term into equation 2.6 yields equation 2.7.

The Michaelis constant K_M can be defined as the substrate concentration at which the reaction velocity is half the V_{max}. Therefore, the smaller the K_M values of an enzyme, the better the enzyme performs at lower substrate concentration.

$$v = \frac{V_{max}[S]}{K_M + [S]} \qquad \text{Eq. (2.7)}$$

Equation 2.7 is known as the Michaelis-Menten equation and is followed to determine enzyme kinetics. The initial reaction velocity, the velocity when less than 10% of the substrate is consumed, is commonly used to determine the kinetic parameters. Factors such as byproduct enzyme inhibition, and enzyme deactivation, can be minimized by using initial velocities (Voet and Voet, 2004). Once the kinetic parameters are determined, substrate concentrations over time can be predicted.

2.5 OXIDOREDUCTASE ENZYMES

White rot fungi (WRF) produce oxidoreductase enzymes, particularly peroxidases and oxidases, which are capable of oxidizing a wide range of recalcitrant aromatic compounds such as phenols, biphenols, anilines, benzidines, and polycyclic aromatic hydrocarbons (PAHs) (Loske et al., 1990). WRF also secrete a great variety of low molecular weight compounds that act as mediators, which enhance the activity of the enzymes for degrading recalcitrant compounds. Exocellular oxidoreductase enzymes secreted by WRF include the following: (i) Lignin peroxidase (LiP); (ii) Manganese-dependent peroxidase (MnP); (iii) Versatile peroxidase (VP); and (iv) Laccase (the only oxidase). These enzymes are produced via secondary metabolism since they are not used for energy generation purposes. Secretion of the enzymes is induced by limited nutrient levels (low carbon and nitrogen concentrations). Moreover, under limited nutrient levels, peroxidases induction can be increased by oxidative stress (e.g. presence of hydrogen

peroxide) (Li et al., 1995). Oxidoreductase enzymes are relatively non-specific biocatalysts with respect to aromatic compounds (Cabana et al., 2007b).

Oxidoreductase enzymes (laccases or peroxidases) are biological catalysts that promote the oxidation of aromatic-containing compounds by either oxygen (laccases) or hydrogen peroxide (peroxidases), increasing the oxidation reaction rates. The substrates are oxidized and either oxygen (laccases) or hydrogen peroxide (peroxidases) is reduced. Laccases and peroxidases each have their own advantages. Laccases require only dissolved molecular oxygen for catalytic activity whereas peroxidases need hydrogen peroxide. For full-scale water treatment processes, oxygen is readily available, and thus is an easy co-substrate to provide (Riva, 2006). Oxidoreductase enzymes are now readily available commercially at relatively low cost, but they can also be prepared as described in Cabana et al. (2007a).

2.5.1 Oxidoreductase Enzymes - Mechanisms

Peroxidases and laccases are capable of oxidizing a wide range of aromatic compounds producing free radicals through a single electron oxidation in the presence of hydrogen peroxide or oxygen, respectively. The mechanisms presented in Figure 2.1 indicate that laccases promote the oxidation of four substrate molecules per molecule of oxygen consumed, while peroxidases promote the oxidation of two molecules of substrate per molecule of hydrogen peroxide consumed.

Laccase	**Peroxidase**
(Aromatic $\leftrightarrow$ Aromatic • + e^-)	(Aromatic $\leftrightarrow$ Aromatic • + e^-)
($4e^- + 4H^+ + O_2 \leftrightarrow 2H_2O$)	($2e^- + 2H^+ + H_2O_2 \leftrightarrow 2H_2O$)
$4Aromatic + 4H^+ + O_2 \leftrightarrow 4Aromatic\bullet + 2H_2O$	$2Aromatic + 2H^+ + H_2O_2 \leftrightarrow 2Aromatic\bullet + 2H_2O$

Figure 2.1 – Laccase and peroxidase oxidation scheme

Laccase is one of a small group of oxidases that can use the full oxidizing capacity of oxygen and reduce it to water. Laccase contains copper, which is essential for the catalytic activity of these enzymes (Lontie, 1984; Solomon et al., 1996). The redox potential of the fungal laccase (0.5 V – 0.8 V) is higher than the aquatic redox potential value of the Cu(II)/Cu(I) system (0.15V). As described by Riva (2006), the copper ion is able to interact with certain amino acids of the laccase enzyme forming a coordination complex, which forces a particular geometry that leads to a higher enzyme redox potential. The oxidation catalyzed by laccase proceeds by the monoelectronic oxidation of suitable substrate molecules such as phenols and aromatic amines to the corresponding free radicals (Riva, 2006). The substrate specificity of laccase for certain aromatic compounds can be explained by the geometry and chemical nature of the enzyme active sites (Piontek et al., 2002). The active site of the enzyme provides a suitable environment for the binding of the substrate and their emerging free radical intermediates (Piontek et al., 2002). Substrates that bear the –OH or $–NH_2$ functionality and that are not too wide for the active site are recognized and dragged inside the active site (Tadesse et al., 2008).

Oxidation rates depend on the ability of the enzyme to accept electrons and the ability of the substrate to give electrons; that is, for the same enzyme, the lower the redox potential of the substrate the higher the enzyme activity. Substrates with lower redox potential are oxidized faster by the enzyme (Piontek et al., 2002). Solomon et al. (1996) suggested that the rate-determining step for the substrate oxidation by the enzyme might be the very first interaction between the substrate and the enzyme active site in which the rate of electron transfer increases as the redox potential of the substrate decreases.

2.6 LACCASE OXIDATION AND THE LACCASE-MEDIATOR SYSTEM

Laccase catalyzes the oxidation of certain aromatic compounds, particularly phenolic compounds. The performance of laccase's direct oxidation has been evaluated for a variety of organic contaminants containing phenolic moieties. Phenols from olive oil mills, petroleum refineries, pulp and paper mills, and wine distilleries have been successfully removed (transformed) by direct laccase oxidation (Berrio et al., 2007; Ko and Fan, 2010; Steevensz et al., 2009; Strong and Burgess, 2007). Moreover, laccase's direct oxidation of several emerging organic contaminants containing phenolic moieties (including several PPCPs) has been successfully evaluated in buffered water solutions. Compounds investigated include acetaminophen (Lu et al., 2009), diclofenac, estradiol, estrone, ethynilestradiol (Lloret et al., 2010), triclosan (Kim and Nicell, 2006; Cabana et al., 2007a), bisphenol A, and nonylphenol (Cabana et al., 2007a).

Laccase is able to directly interact with some PPCPs (particularly phenolic compounds) although it cannot directly oxidize many other PPCPs (Riva, 2006; Kunamneni et al., 2008a; Cañas and Camarero, 2010). For example, the personal care product oxybenzone, despite its phenolic moiety, is not directly oxidized by laccase. Substrates that are not directly oxidized by laccase might be too large to fit into the enzyme active site, might have functional groups that do not interact appropriately with the enzyme's active site, or they might have too high a redox potential to be directly oxidized (Riva, 2006). However, low molecular weight compounds (usually phenolic compounds with low molecular weights of approximately 200 g/mol) are used by laccase to accelerate and expand laccase's oxidative ability (Riva, 2006; Kunamneni et al., 2008a; Cañas and Camarero, 2010). These compounds are known as mediators. Laccase is able to oxidize these mediators to free radicals, which react non-specifically, providing highly effective removal (transformation) of a wide spectrum of compounds including

non-typical enzyme substrates (Riva, 2006). Some emerging organic contaminants that are not directly oxidized by laccase, but can be effectively oxidized by the laccase-mediator system in buffered water solutions include carbamazepine (Hata et al., 2010), naproxen (Lloret et al., 2010), anthracene (Sei et al., 2008), benzo[a]pyrene, phenanthrene (Camarero et al., 2008), and pyrene (Johannes and Majcherczyk, 2000b).

2.6.1 Mechanisms of the Laccase-mediator System

Galli and Gentili (2004) reported that laccases are able to affect the oxidation of compounds that cannot be directly recognized by the enzyme. They reported that low molecular weight compounds with low redox potentials were used to mediate the oxidation between laccase and the target compounds. Galli and Gentili (2004) investigated the oxidation of several different mediators by laccase such as N-hydroxyphthalimide (HPI), violuric acid (VLA), and 1-hydroxybenzotriazole (HBT). The oxidized form of the mediators reacts with the target compounds, primarily by free radical oxidation routes through hydrogen-atom transfer (HAT). Mediators can thus expand the activity and range of laccase.

As shown in Figure 2.2a for a substrate that can be directly oxidized by laccase (substrate fits into the active site, does not exhibit steric effect, and has an appropriate redox potential), the oxygen activated form of the laccase enzyme can oxidize the substrate to a final stable oxidized product. Figure 2.2b shows how the mediators work. The mediator is oxidized by electron abstraction producing a free radical (Aromatic • free radical) which can pursue several different routes (Weber and Huang, 2003; Galli and Gentili, 2004): i) the mediator can go back to its reduced form oxidizing the target compound by a typical electron transfer oxidation route (similar to oxidation with chemical oxidants); ii) the mediator can go back to its reduced form by free radical

routes; that is, abstracting an hydrogen atom from the target compound (hydrogen-atom transfer, or HAT) or by nucleophilic attack of the free radical by the target compound; or iii) the mediator can couple either with each other or with the target compound (depending on the reaction conditions) to form dimers, oligomers, and eventually high molecular weight polymers (not shown in Figure 2.2b). These mechanisms are not exclusive, and a combination of the previous reaction mechanisms also can be expected. If the mediator follows the latter pathway, described in item iii), it will be consumed as a part of the oxidation reaction. Target compounds that cannot be directly oxidized by the enzyme might be transformed (oxidized) by the action of the oxidized form of the mediators.

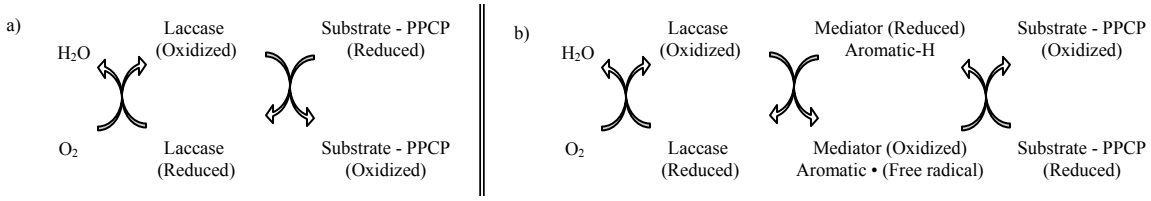


Figure 2.2 – Schematic representation of redox cycles for substrate oxidation: a) without chemical mediator; b) with the addition of a chemical mediator

Cantarella et al. (2003) also suggested that target compounds not directly recognized by the active site of laccase can still be oxidized by using mediators as shown in Figure 2.2b. The oxidation can proceed either by electron transfer routes or by free radical routes. Hydrogen-atom transfer mechanisms (HAT) by mediators is perhaps what makes the laccase oxidation process such a good treatment alternative for removing recalcitrant aromatic PPCPs from water sources. Oxidation of the carbon-hydrogen (C-H) bonds in hydrocarbons is one of the most difficult tasks in synthetic organic chemistry (Cantarella et al., 2003). The high redox potential of the hydrocarbon substrates makes

that transformation hard to perform. As suggested by Cantarella et al. (2003), laccase oxidation is able to tackle this problem by HAT. Hydrogen atoms can be abstracted from the recalcitrant compound (along with its electron) by a mediator free radical derived from a mediator laccase oxidation. The energy of the C-H bond of the resulting product (mediator-abstracted hydrogen atom), is higher than the C-H energy of the initial recalcitrant substrate, making this mechanism energetically favorable (Cantarella et al., 2003). After the hydrogen abstraction, the recalcitrant target compound is transformed to a radical that can continue reacting to form a stable final product (Cantarella et al., 2003). Free radical oxidation routes might be the true mechanisms responsible for the oxidation of recalcitrant compounds such as some PPCPs by the enzyme-mediator system.

2.6.2 Oxidative Coupling

Laccase oxidation of selected types of low molecular weight mediators at specific experimental conditions may lead to the formation of dimers, oligomers, and eventually large molecular weight polymers. As described by Huang et al. (2005), low molecular weight phenolic compounds in particular can undergo extensive oxidation coupling and polymerization via oxidation by naturally occurring extracellular oxidoreductase enzymes such as laccases. Phenoxyl radicals are generated through a single electron oxidation that can couple by ether and/or C-C bonds to form polyphenolic products (Huang et al., 2005). These products can continue to serve as substrates for further oxidative coupling, making large polymers that can eventually precipitate out of solution (particularly at high mediator concentrations). Some non-phenolic compounds (e.g., some PPCPs) can be incorporated into such polymeric precipitation products by adsorption onto the polymer and/or by covalent bonding with the polymer (Huang et al., 2005). Weber and Huang (2003) stated that the substrates converted via enzymatic mediation to radicals can then

either couple with each other (self-couple) or bind to other reactive substances present in the system (cross-couple). Cross-coupling may occur through radical binding or nucleophilic addition mechanisms between two different active substrates. It is also possible for cross-coupling reactions to occur between an active substrate and an inert chemical if the free radicals generated from the active substrates transfer to the inert chemical via hydrogen abstraction or free-radical addition (Weber and Huang, 2003). Therefore, removal of inert chemical contaminants can be achieved by one or both of two possible mechanisms: i) cross-coupling reactions with phenoxyl radicals enzymatically generated from phenolic substrates, or ii) adsorption of the inert chemical by precipitated polymeric products resulting from phenol self-coupling reactions. Precipitation of the laccase-produced polymers is observed when working at high mediator concentrations (approximately 10 mM or higher), at high salts concentrations in solution, and at lower pH, especially in the range from pH 5 to pH 3. As the solution pH decreases, acidification (protonation) of the produced polymer reduces their ionic character and increases their tendency to precipitate (Huang et al., 2005).

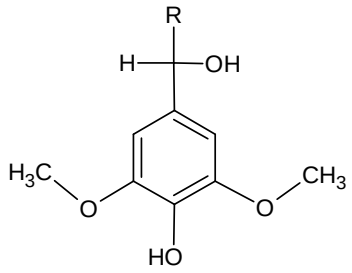
Kupriyanovich et al. (2007) demonstrated that a low molecular weight phenolic compound, ferulic acid (FA) (commonly used as a mediator), can be oxidized by the action of laccase to form phenoxyl radicals that couple together to form large polymers. The phenoxyl radical of ferulic acid exists in several resonance forms making their formation a thermodynamically favorable process. Polymerization proceeds by interaction of these free radicals. The dynamic conditions of the synthesis affect the structure of the polymer (one time introduction of the monomer (bulk polymers) versus gradual introduction of the monomer (end-wise polymers)) (Kupriyanovich et al., 2007). The isolated polymeric products can be precipitated and exhibit a solid powder-like

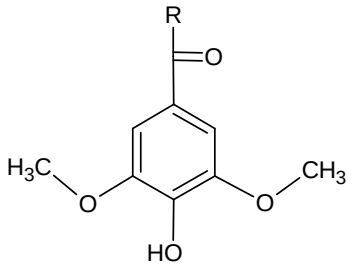
characteristic colored from cream to dark brown. Their solutions exhibit a distinguishing yellow color (Kupriyanovich et al., 2007).

2.6.3 Persistent Radical Compounds

Free radicals are compounds with unpaired electrons in the outermost molecular shell configuration. Although most free radicals are generally highly reactive and decay extremely fast, other free radicals exhibit stability and are long lived (Caldwell and Steelink, 1969; Johannes and Majcherczyk, 2000a; Griller and Ingold, 1976; Scott et al., 1993). These types of compounds are called persistent free radicals as opposed to transient radicals. Transient radicals have half lives of less than 10^{-3} seconds as opposed to persistent radicals, which may have half lives ranging from seconds to years (Griller and Ingold, 1976). The persistence of a radical compound depends not only on its chemical nature, but also on its environment. The presence of other compounds such as radical scavengers could strongly impact the half lives of the radical species. Steric factors have been shown to be the main cause of radical persistence (Griller and Ingold, 1976).

Oxidoreductase enzymes (either laccases or peroxidases) are able to oxidize certain phenolic compounds producing long lived phenoxyl radicals. Caldwell and Steelink (1969) investigated the oxidoreductase oxidation of syringyl derivatives. The following compounds were investigated with R = H, CH_3, and CH_2CH_3.





The enzyme oxidation of these compounds in aqueous solutions at a physiological pH produced persistent free radicals with long half lives of up to 30 minutes.

Caldwell and Steelink (1969) also demonstrated that the phenoxyl radicals produced in the process decay following first order kinetics to yield stable compounds with higher oxidation potentials. The final compounds did not undergo further oxidation. The maximum phenoxyl radical concentration in aqueous solution was determined to be approximately 30% of the concentration of the initial phenolic substrate. Results clearly indicated that phenoxyl radicals are important long lived intermediates in aqueous solutions.

Other authors have also demonstrated the existence of persistent phenoxyl radicals produced by the oxidation of phenolic compounds by oxidoreductase enzymes. Valoti et al. (1989) demonstrated the formation of phenoxyl radicals from peroxidase oxidation of the following compounds: 2-t-butyl-4methoxyphenol (BHA), 2,6-di-t-butyl-4-methylphenol (BHT); 3,5-di-t-butyl-4-hydroxyanisole (DTBHA); and 2,4,6-tri-t-butyl-phenol (TTP). The authors reported that peroxidase oxidation of the *ortho*-di-substituted phenols (BHT, DTBHA, and TTP) produced more stable phenoxyl radicals than the mono-substituted phenols (BHA). The persistent radicals were observed for many minutes, until they decayed by dimerization routes. As also observed by Caldwell and Steelink (1969), the substitution of both *ortho* positions by the t-butyl groups prevented the destruction (decay) of the radical by *ortho* dimerization enhancing the persistency of these radical compounds. Kalyanaraman (1995) also reported the formation of persistent phenoxyl radical by the oxidoreductase oxidation of di- and tetra-substituted metoxy phenols.

The formation of persistent free radicals from the oxidation of non-phenolic compounds by oxidoreductase enzyme has also been reported by several authors (Johannes and Majcherczyk, 2000a; Scott et al., 1993; Majcherczyk et al., 1999). For example, the oxidoreductase oxidation of the non-phenolic substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6sulphonic acid) (ABTS) by abstraction of one electron producing the persistent free radical (ABTS•) has been extensively studied. This compound has a very high stability, although it can react with other compounds present in solution (Johannes and Majcherczyk, 2000a). As reported by Majcherczyk et al. (1999) the ABTS radical produced by oxidoreductase oxidation remained stable after 22 hours in a pH 3 buffered solution. At neutral or alkaline conditions, the decomposition of the ABTS radical was detected.

Although free radical compounds are generally considered to be highly reactive and to decay extremely fast, there are several free radical compounds (both phenolic and non-phenolic) that exhibit high persistence. Enzyme oxidation by oxidoreductase enzymes (either oxidases or peroxidases) will produce some of these persistent free radicals by a single electron abstraction oxidation. When designing a laccase-mediator treatment system for removing emerging contaminants through cross-coupling reactions, the generation of long-lived radicals is desirable. The higher the persistence of the mediator radicals in the reaction mixture, the higher their chances to react with the target compounds.

2.7 Removal of Enzyme Oxidation Byproducts

A significant advantage of enzymatic treatment is its potential for implementation in municipal wastewater primary effluent, which would allow the removal of enzyme oxidation byproducts in subsequent conventional treatment processes, such as activated

sludge followed by secondary clarification. This removal could occur by biodegradation, precipitation followed by sedimentation, or adsorption to the biomass present in secondary treatment.

The biodegradation of laccase oxidation byproducts from the oxidation of the endocrine disrupting phenolic compounds bisphenol A and 2,4-dichlorophenol was demonstrated by Nakamura and Mtui (2003). These compounds were oxidized by laccase, and the reaction mixture was later inoculated with activated sludge. Identical chemical oxygen demand (COD) concentrations for the mixture before and after laccase oxidation demonstrated that the enzyme oxidation transformed the parent compounds into oxidation byproducts without mineralizing them. When the reaction mixture was later inoculated with activated sludge, a decrease in the COD concentration of about 80% was observed after 3 days of treatment. That is, not only the parent compounds were oxidized by laccase, but also the oxidation byproducts were mineralized/biodegraded by a subsequent activated sludge process. Oxidation pathways have not been reported by the authors.

Several authors have reported enzyme-catalyzed oxidative coupling of organic substrates, particularly phenolic compounds (Roper et al., 1995; Huang et al., 2005; Lu et al., 2009). Huang et al. (2005) reported the effects of pH and salt concentration on the precipitation of the oligomeric and polymeric products produced by laccase oxidation of phenol. Precipitation of the coupled polymeric products was favored at higher salt concentrations and lower pH (Huang et al., 2005). Lu et al (2009) researched the laccase-catalyzed oxidative coupling of acetaminophen. Laccase oxidized the substrate acetaminophen to a free radical compound. The coupling of these radicals produced dimers, oligomers, and polymeric products. The coupling products tend to be less hydrophilic than the parent compound. Hydrophobicity tends to increase as the

molecules become larger, leading to easier removal from the aqueous phase by precipitation, coagulation and/or adsorption (Lu et al., 2009).

Placing an oxidoreductase enzymatic treatment after primary clarification and before the activated sludge process in a conventional wastewater treatment plant could potentially remove the target PPCPs and oxidation byproducts in subsequent treatment processes. Complete biodegradation of parent compounds and enzyme oxidation byproducts to CO_2 and H_2O (mineralization) is desirable, although it is expected that an important fraction of the oxidation byproducts would either precipitate as previously described or be adsorbed onto the biomass in the activated sludge process. Therefore, some of the PPCPs oxidation byproducts would end up in the biosolids. As reported by McClellan and Halden (2010), approximately 72 PPCPs were found in digested municipal sludge. Most of the biosolids produced in the United States are applied to the land as fertilizers (McClellan and Halden, 2010). Therefore, through biosolids recycling, PPCPs are released into the environment. Another concern regarding biosolids contaminated with PPCPs involves the possible uptake of contaminants into food crops growing on agricultural fields fertilized with biosolids. Even though the removal of the parent PPCPs and oxidation byproduct is highly desirable, the fate and toxicity of oxidation byproducts should be carefully analyzed.

2.8 Alternatives for Optimizing Enzyme Oxidation Treatment

Enzyme-catalyzed oxidation of PPCPs by oxidoreductase enzymes requires the supply of two main components to the reaction mixture to work effectively: oxidoreductase enzymes and mediators. The enzyme oxidation treatment system can be optimized by reducing the amount of oxidoreductase enzyme required, and by investigating the utilization of inexpensive mediator sources.

Conventional uses of enzymes in free solution can suffer from low enzyme stability and from difficulties in recovering and reusing enzymes for wastewater treatment systems of any reasonable scale. Even though the low enzyme stability does not affect the specificity or functionality of the enzyme, larger quantities of enzymes are required to maintain a relatively high activity over time. Recent biotechnological advances allow rapid and inexpensive production of enzymes appropriate for water treatment (Kunamneni et al., 2008a). Novozymes Corp. supplies laccase enzyme at a concentration of 1,000 U/mL (one unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of ABTS per minute at 37^{o}C) at $30/Kg of enzyme. While it might be possible to continuously add the enzyme in the treatment system, enzyme requirements could be reduced if enzymes could be reused; such repetitive use appears possible by enzyme immobilization. In particular, immobilizing the enzymes on epoxy-activated supports increases enzyme activity and stabilizes the three-dimensional structure of the enzymes, thereby reducing their potential for inactivation by heat, pH, organic solvents or other denaturing agents (Yinghui et al., 2002; Mateo, Abian et al., 2006; Mateo, Pessela et al., 2006). Laccase was successfully immobilized in epoxy activated supports (Sepabeads®) by Kunamneni et al. (2008b). The resulting preparation exhibited a very high activity with an excellent stability towards pH, temperature, and storage time. The final preparation retained 87% of the enzyme activity after 17 cycles of oxidation of ABTS.

Most of the mediators investigated that successfully oxidize PPCPs in the laccase-mediator system contain phenolic moieties. In research studies, mediators are generally supplied externally as a pure compound to the reactions mixtures. There is the potential for optimizing the laccase-mediator system by investigating the utilization of inexpensive mediator sources; in particular, several industries (wineries, olive oil mills, and other

food processing industries) generate waste streams that have a high content of phenolic compounds that might serve as mediators. For instance, Bustamante et al. (2005) characterized effluent discharges from several wineries. Two waste streams were analyzed: winery wastewater (comes from the water used in the different steps of wine production), and vinasse (comes from the distillation of wine, wine lees, and grape marc for the production of alcohol). The two waste streams were similar. The pH of both wastewaters was acidic, approximately 5.5 for winery effluents and 4.2 for vinasse. Soluble polyphenol concentrations ranging from 29 to 474 mg/L and 65 to 766 mg/L were observed from winery wastewater and vinasse, respectively. Beltrán et al. (1999) also reported that wine, wine lees, pressed grapes, and other agricultural wastes are usually raw material sources for distillers to produce ethanol for use as an alternative fuel. Vinasse is the main wastewater of the distillation process containing phenolic compounds at concentrations of approximately 800 mg/L.

Strong and Burgess (2008) reported that the phenolic compounds commonly found in wastewater streams from distillation processes were directly oxidized by oxidoreductase enzymes such as laccase. Jaouani et al. (2005) reported that other agricultural wastes such as the wastewater generated from the process of extracting oil from olives also contain high concentration of phenolic compounds, which were directly oxidized by the laccase enzyme. Consequently, several agricultural industries generate wastes containing high concentrations of polyphenolic compounds. These compounds are directly recognized and oxidized by oxidoreductase enzymes, so they might serve as an inexpensive source of mediators for the laccase-mediator system oxidation process.

Even though waste streams from agricultural and food-processing industries might be used as an inexpensive source of mediators, discharging an industrial wastewater into a conventional municipal wastewater treatment plant might affect the

performance of the municipal wastewater treatment plant. For instance, as described by Wang et al. (2004), wastewaters from olive oil production are characterized by an intense violet-dark brown to black color, a high degree of organic pollution (COD values up 220 g/L), an acidic pH (pH 3 to 6), a high content of polyphenols (8 g/L), and a high content of solids (up to 102.5 g/L). Some of the constituents responsible for the high COD values such as polyphenol compounds, might be inhibitory to biological oxidation processes and affect the performance of the activated sludge process in a conventional wastewater treatment plant. Moreover, the high COD values might introduce a significant concentration of readily biodegradable soluble organic compounds into the activated sludge process, increasing the biomass concentration and oxygen requirements in the aeration tank. Furthermore, the high influent solids concentration may also affect the performance of the municipal treatment system. Nevertheless, as reported in Garcia et al. (2010), the laccase-mediator system would require a phenolic mediator concentration of approximately 80 μM. Assuming a food-processing waste has a total phenolic concentration of approximately 8 mM (approximately 8 g/L), the waste stream would be diluted by a factor of approximately 1,000 to reach the required mediator concentration of 80 μM. Thus, the food-processing wastewater will be significantly diluted, minimizing its potential negative impacts on a municipal wastewater treatment plant.

2.9 SUMMARY

PPCPs are present in wastewater, river systems and even drinking water, entering the ecosystem primarily through wastewater. Such compounds are known to cause negative environmental consequences, and public health concerns have been suggested. They constitute a major obstacle to water reuse. Certain advanced treatment processes can remove PPCPs from wastewater, although they are energy intensive and generate

toxic byproducts. Enzymatic treatment using oxidoreductase enzymes such as laccase is an attractive alternative for PPCP removal. Laccase acts on a variety of substrates with chemical similarities to PPCPs, and through the use of low molecular weight mediators, the laccase's oxidation ability may be expanded. Although laccase oxidation might successfully remove the target PPCPs, byproducts also will likely be generated. Both transformation of the parent compound and the removal of byproduct are desirable. Thus, implementation of laccase oxidation in primary effluent should be investigated because the oxidation byproducts might be removed in subsequent biological treatment processes. This removal could occur by biodegradation or adsorption to the biomass present in activated sludge.

While laccase-catalyzed oxidation has been investigated for the removal of recalcitrant organic contaminants, most of the compounds evaluated were directly recognized by the laccase enzyme, and the studies were performed in ultrapure buffered solutions at a relatively high concentration of the target compounds. Laccase-catalyzed oxidation of substrates not directly recognized by laccase in more complex matrices at trace concentrations has not been proven. Hence, the evaluation of the performance of the laccase mediator system for PPCPs not directly recognized by laccase from municipal wastewater primary effluent at environmentally relevant concentrations is required.

Chapter 3 – Materials and Methods

3.1 RESEARCH DESIGN

The potential for oxidation of PPCPs by a laccase-mediator system was evaluated using oxybenzone as a representative PPCP that is not directly oxidized by laccase alone. The experiments were conducted in amber glass batch reactors at 23^{o}C. The reaction mixture for most of this research consisted of the target PPCP (oxybenzone), the enzyme laccase, and a mediator. Two initial oxybenzone concentrations of 1,000 μg/L and 10 μg/L (environmentally relevant concentrations) were evaluated. Most of the experiments were designed at an initial laccase activity of 1 U/mL (one unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of ABTS per minute at 37^{o}C). Several mediators were investigated at different concentrations. Experiments were carried out in either buffered solution or in municipal wastewater primary effluent. Laccase activity was measured using a colorimetric method, and the removal of the target compound oxybenzone was analyzed using liquid/chromatography tandem mass spectrometry (LC/MS/MS). This research was divided into the following phases:

(i) mediator screening experiments to evaluate the need for a mediator and determine which mediators performed best;

(ii) initial experiments in primary effluent to determine if the laccase-mediator system could effectively remove oxybenzone from a primary effluent matrix (despite the presence of other organic and inorganic constituents);

(iii) further experiments in primary effluent investigating the effect of the mediator/oxybenzone molar ratio;

(iv) evaluation of the laccase-mediator system at environmentally relevant oxybenzone concentrations in primary effluent;

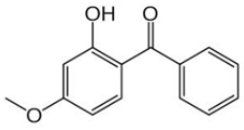
(v) characterization of the enzyme oxidation byproducts and comparison of these byproducts with those generated by ozonation;

(vi) evaluation of the mechanisms and kinetics of the laccase oxidation of mediators, and

(vii) assessment of an alternative treatment configuration to optimize the laccase-mediator system, allowing the enzyme and the mediator to interact prior to coming in contact with the target contaminant.

The personal care product oxybenzone ((2-Hydroxy-4-methoxyphenyl)-phenylmethanone) was selected as the representative PPCP. Some of the most relevant chemical properties of oxybenzone are described in Table 3.1.

Table 3.1 – Chemical characteristics of oxybenzone.

Compound	Molecular Structure	Molar Mass (g/mol)	pK_a [(a)]	Log K_{ow} [(b)]	Solubility (mg/L)
Oxybenzone		228.1	7.77[(c)]	3.79[(c)]	69[(c)]

[(a)] Acid dissociation constant. [(b)] Octanol-water partition coefficient. [(c)] (Snyder et al., 2007)

Oxybenzone absorbs and dissipates UV radiation and is therefore commonly used in sunscreens and cosmetic products as shown in Figure 3.1. Oxybenzone was selected as a representative PPCP for several reasons. First, it has potential human health and environmental effects, as it demonstrates estrogen-like activity *in vitro* and *in vivo* (Schlumpf et al., 2004; Calafat et al., 2008). Although adverse health effects in humans have not been reported, dermal and oral administration of oxybenzone to rats and mice has yielded alterations in liver, kidney, and reproductive organs (Calafat et al., 2008). Second, measurable oxybenzone concentrations in conventional wastewater treatment plant effluents have been reported (Snyder et al., 2007; Kim et al., 2007). Snyder et al.

(2007) reported relatively high concentrations ranging from 37 to 3,810 ng/L (0.161 to 16.6 nM) in raw municipal wastewater and primary effluent. A related consideration is that oxybenzone has low volatility (Henry's constant = $1.5x10^{-5}$ atm-L/mol) (NTP, 2011); compounds with reasonably high Henry's constants are volatilized in conventional secondary wastewater treatment.

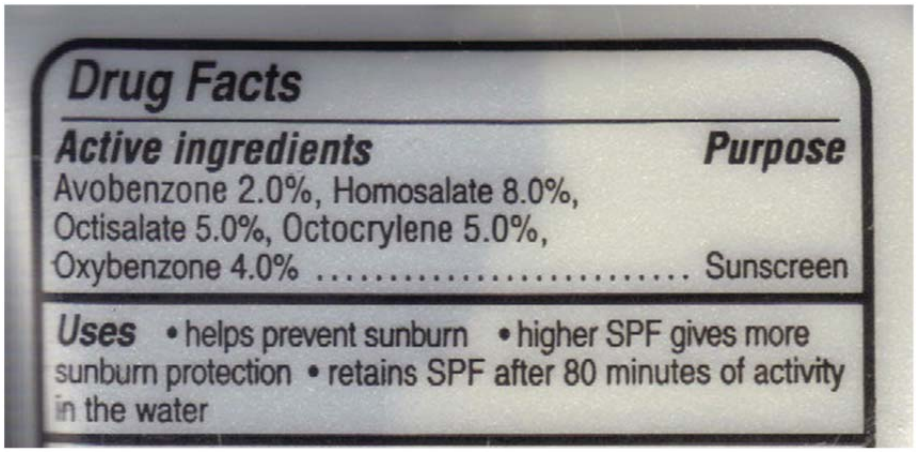


Figure 3.1 – Active ingredients of a sunscreen (CVS Pharmacy Sunscreen).

3.2 CHEMICALS

Laccase derived from *Trametes versicolor* (CAS 80498-15-3, minimum 20 units/mg), oxybenzone (CAS 131-57-7, minimum 98%), ferulic acid (FA) (CAS 1135-24-6, minimum 99%), and p-coumaric acid (PCA) (CAS 501-98-4, minimum 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2'-Azino-bis(3-ethylbenzthiazoline-6sulphonic acid) diammonium salt (ABTS) (CAS 30931-67-0, minimum 98%) was purchased from EMD Bioscience (Gibbstown, NJ, USA). Acetosyringone (ACE) (CAS 2478-38-8, minimum 99%) was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). 4-Hydroxybenzoic acid (HBA) (CAS 114-63-6) was purchased from Spectrum Chemical (Garden, CA, USA). 1-Hydroxy-

benzotirazole (HBT) (CAS 134-96-3, minimum 98%) was purchased from ScienceLab.com Inc. (Houston, TX, USA). Sinapinic acid (SIN) (CAS 530-59-6, minimum 98%) and syringaldehyde (SYR) (CAS 134-96-3, minimum 98%) were purchased from Alfa Aesar (Ward Hill, MA, USA). LC/MS grade methanol and water were purchased from JT Baker (Phillipsburg, NJ, USA). Ultrapure water was produced by filtering distilled water through a Milli-Q UV Plus water purification system (Millipore, Billerica, MA, USA).

3.3 EXPERIMENTAL PROCEDURES

3.3.1 Mediator screening experiments

Mediator screening experiments were performed to investigate the oxidative capacity of laccase acting alone on oxybenzone, as well as in the presence of several different mediators. Several experiments were performed utilizing laccase in batch reactors to analyze the oxidation of oxybenzone (disappearance of the parent compound) as a function of time. The experiments were conducted in 50-mL amber glass batch reactors containing 35 mL of reaction mixture.

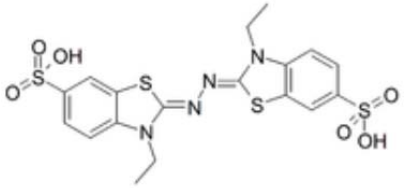
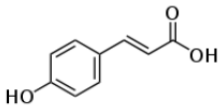
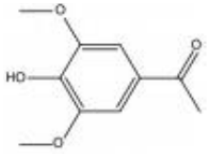
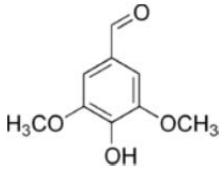
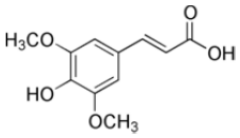
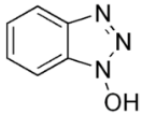
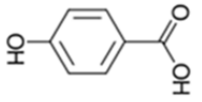
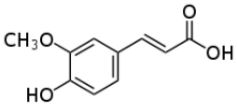
The effect of different mediators was evaluated at the same initial mediator concentration as described in Table 3.2. Molecular structures and molecular weights (MW) of the different mediators involved in this study are presented in Table 3.2. The reaction mixture had an initial oxybenzone concentration of 4.38 μM (1,000 μg/L) in 0.1 M sodium phosphate buffer at pH 7 in ultrapure water. The initial laccase activity was 1 U/mL, which is approximately equal to a concentration of 0.71 μM (assuming the molecular weight of laccase is 70 kDa) (Piontek et al., 2002). Eight mediators (ABTS, ACE, FA, HBA, HBT, SIN, SYR, and PCA) were evaluated at an initial concentration of

1 mM. The reactors were placed in a constant temperature orbital shaking water bath (Boekel Grant, Feasterville, PA, USA) at 23°C.

Table 3.2 – Experimental conditions for mediator screening experiments performed at the same initial mediator concentration.

Experimental Conditions	
Oxybenzone Concentration	4.38 µM (1000 µg/L)
Laccase Activity	1 U/mL (0.7 µM)
pH	7
Temperature	23°C
Medium	Sodium Phosphate Buffer

Mediators (1mM concentration), Molecular Weights (g/mol) are also reported (MW)

ABTS MW 514.6 (Synthetic)		**PCA** MW 164.2 (Natural)	
ACE MW 196.2 (Natural)		**SYR** MW 182.2 (Natural)	
SIN MW 224.2 (Natural)		**HBT** MW 135.1 (Synthetic)	
HBA MW 138.1 (Natural)		**FA** MW 194.2 (Natural)	

The reactors were sampled at 0 (before laccase addition), 2, 6, and 24 hours to measure the concentration of oxybenzone remaining at each time point. The samples (3 mL) were placed in 10-mL test tubes and acidified with 40 µL of 5 N hydrochloric acid to a final pH of approximately 1.5 to inactivate the enzyme as described by Auriol et al. (2007). The analytes in the acidified samples were extracted using 3 cm^3 60 mg Oasis

HLB solid phase extraction (SPE) cartridges (Waters, Milford, MA, USA). SPE was used in these experiments as a purification step, not as a concentration method. The SPE cartridges were conditioned with 3 mL of LC/MS grade methanol followed by 3 mL of LC/MS grade water. After conditioning, 2 mL of the samples were passed through the cartridges, which were subsequently rinsed with 3 mL of LC/MS grade water three times. After allowing the cartridges to dry, they were eluted with two 0.7 mL and one 0.6 mL aliquots of LC/MS grade methanol. Purified samples were analyzed using LC/MS/MS as described in Section 3.5. Samples were also collected at 0 and 48 hours to measure enzyme activity.

The effect of different mediator concentrations and different enzyme activities were also evaluated. Experiments were performed with the mediators ABTS and HBT at the experimental conditions described in Table 3.3. These experiments were almost identical to the experiments previously described, but the reactors were sampled at 0 (before laccase addition), 0.5, 2, and 4 hours to measure the concentration of oxybenzone remaining at each time point.

Table 3.3 – Experimental conditions for mediator experiments performed at different mediator concentrations.

Experimental Conditions		
Oxybenzone Concentration	4.38 μM (1000 μg/L)	
pH	7	
Temperature	23°C	
Medium	Sodium Phosphate Buffer	
Mediator	Mediator Concentration (mM)	Laccase Activity (U/mL)
ABTS	0.001 - 0.01 - 0.1 - 1	1
HBT	1 – 10	1 and 5

The laccase oxidation of the phenolic mediator FA was also evaluated. Experiments were performed in 50-mL amber glass reactors containing 35 mL of reaction mixture. The objective of these experiments was to evaluate the formation of precipitates due to polymerization reactions at high concentrations of the phenolic mediator FA. Reactions were carried out in different solutions such as distilled water, pH 7 sodium phosphate buffer solutions, and pH 4.5 ammonium acetate buffer solution, at an initial FA concentration of 10 mM and at an initial laccase activity of 5 U/mL. Blanks were performed without laccase addition and without FA mediator addition. The reactors were sampled after 48 hours of treatment. The samples (1 mL) were placed in 2-mL vials and acidified with 100 µL of 5 N hydrochloric acid. Samples were inspected to evaluate the formation of precipitates.

3.3.2 Initial experiments in primary effluent

Based on the results of the mediator screening experiments, the two mediators that yield the highest oxybenzone removal (ABTS and ACE) were selected for further experiments in primary effluent. These experiments were nearly identical to the mediator screening experiments, but they were conducted in filtered primary effluent rather than in phosphate buffer. The experimental conditions are described in Table 3.4. The primary effluent was collected weekly from the Walnut Creek wastewater treatment facility in Austin, Texas and was stored at 4°C between collection and use. It was filtered using grade 934-AH glass microfiber filters with a 1.5-µm pore size (Whatman, Piscataway, NJ, USA). The following measurements were typical of the collected primary effluent: alkalinity = 273 mg/L as $CaCO_3$, BOD = 145 mg/L, soluble COD = 112 mg/L, total COD = 194 mg/L, total NH_4^+ = 31 mg/L as N, pH = 7.4, TSS = 29 mg/L, pH after filtration = 7.67. In one set of experiments, the pH of the primary effluent was not adjusted, and in a

second set, the primary effluent was adjusted to an initial pH of 6 with 5 N hydrochloric acid. In addition to the measurements described in Section 3.3.1, pH measurements were taken at 0 and 48 hours using a pH meter (Thermo Electron Corporation, Waltham, MA, USA).

Table 3.4 – Experimental conditions for experiments performed in municipal wastewater primary effluent solutions.

Experimental Conditions	
Oxybenzone Concentration	4.38 µM (1000 µg/L)
Laccase Activity	1 U/mL
Temperature	23°C
Medium	Municipal Primary Effluent
Mediator	pH
ABTS	7.67 (unadjusted pH) 6 (initial adjusted pH)
ACE	7.67 (unadjusted pH) 6 (initial adjusted pH)

3.3.3 Effect of mediator/oxybenzone molar ratios in primary effluent

The initial experiments in primary effluent were performed at a high mediator/oxybenzone molar ratio of approximately 230. The next set of experiments focused on determining the minimum mediator/oxybenzone molar ratios in primary effluent at which the laccase-mediator system was still effective for oxidizing oxybenzone. These experiments were performed similarly to those described in Section 3.3.1, but they were conducted in filtered primary effluent adjusted to an initial pH of 6 rather than in phosphate buffer. The ABTS and ACE mediator concentrations were 87.6, 26.3, 8.76, and 2.63 µM to achieve mediator/oxybenzone molar ratios of 20, 6, 2, and 0.6, respectively. The experimental conditions are described in Table 3.5. The reactors were

sampled at 0, 0.25, 1, and 6 hours to determine the oxybenzone concentration remaining. Enzyme activity and pH measurements were taken at 0 and 6 hours.

Table 3.5 – Experimental conditions for experiments performed at different oxybenzone/mediator molar ration in primary effluent solutions.

Experimental Conditions		
Oxybenzone Concentration	4.38 μM (1000 μg/L)	
Laccase Activity	1 U/mL (0.7 μM)	
Temperature	23°C	
Medium	Municipal Primary Effluent	
pH	6 (initially adjusted)	
Mediator	Mediator Concentration (μM)	Mediator/Oxybenzone Molar Ratio
ABTS	2.63	0.6
	8.76	2
ACE	26.3	6
	87.6	20

Several control experiments were also performed by omitting one or more of the following three constituents: oxybenzone, laccase, and ACE. Two controls were performed for oxybenzone oxidation: without laccase and without both laccase and mediator. Three controls were performed for enzyme activity: without mediator, without oxybenzone, and without both mediator and oxybenzone. Samples from all of the control reactors were taken at 0 and 6 hours and measured for relevant constituents (oxybenzone and/or laccase activity).

3.3.4 Experiments at environmentally relevant concentration

Although the previous experiments at an initial oxybenzone concentration of 4.38 μM (1,000 μg/L) simplified the analytical procedures, oxybenzone and other PPCPs are typically found in municipal wastewater at much lower concentrations. Thus, a set of experiments was conducted to evaluate enzyme oxidation at a more environmentally

relevant oxybenzone concentration. Experiments were conducted in 250-mL amber glass batch reactors containing 100 mL of reaction mixture. The reaction mixture had an initial oxybenzone concentration of 43.8 nM (10 μg/L) in filtered primary effluent adjusted to an initial pH of 6. In one set of experiments, ACE mediator was added to yield mediator/oxybenzone molar ratios of 2,000, 200, and 20, corresponding to ACE concentrations of 87.6, 8.76, and 0.876 μM, respectively. In a separate experiment, ABTS mediator was added to yield a mediator/oxybenzone molar ratio of 200, corresponding to an ABTS concentration of 8.76 μM. The initial laccase activity was 1 U/mL. The experimental conditions are summarized in Table 3.6. Two controls without laccase were performed with this set of experiments, one with oxybenzone and ACE, and one with oxybenzone and ABTS. The reactors were placed in a constant temperature orbital shaking water bath at 23^{o}C. Twenty mL samples were collected at 0, 0.25, and 2 hours, placed in 50-mL amber glass vials, and immediately acidified with 300 μL of 5 N hydrochloric acid to a final pH of approximately 1.5 to inactivate the enzyme. The controls were sampled at 0 and 2 hours. The acidified samples were passed through SPE cartridges. The SPE cartridges were conditioned prior to passing samples and rinsed after passing samples, as described in Section 3.3.1. After drying, the cartridges were eluted with two 2-mL and one 1-mL aliquots of LC/MS grade methanol. The eluates were collected in 5-mL conical vials and evaporated to less than 1 mL, but not to dryness, using a Rapidvac nitrogen evaporation system (Labconco, Kansas City, MO, USA). The samples were reconstituted to 1 mL with LC/MS grade methanol and analyzed for oxybenzone concentration using LC/MS/MS as described in section 3.5. Samples from selected experiments were also analyzed for mediator concentration. Enzyme activity and pH measurements were taken at 0 and 2 hours.

Table 3.6 – Experimental conditions for experiments performed at environmentally relevant concentrations of oxybenzone.

Experimental Conditions		
Oxybenzone Concentration	43.8 nM (10 μg/L)	
Laccase Activity	1 U/mL (0.7 μM)	
Temperature	23°C	
Medium	Municipal Primary Effluent	
pH	6 (initially adjusted)	
Mediator	Mediator Concentration (μM)	Mediator/Oxybenzone Molar Ratio
ABTS	8.76	200
ACE	0.876	20
	8.76	200
	876	2000

3.3.5 Characterization of enzyme oxidation byproducts

Two experiments were performed to investigate the formation of enzyme oxidation byproducts. They were conducted in 50-mL amber glass batch reactors containing 35 mL of reaction mixture in a 0.1 M sodium phosphate buffer at pH 7 in ultrapure water. When ABTS was used as the mediator, the initial oxybenzone concentration was 43.8 μM (10 mg/L), and the ABTS concentration was 0.1 mM (ABTS/oxybenzone molar ratio of 2.3). When ACE was used as the mediator, the initial oxybenzone concentration was 218 μM (50 mg/L), and the ACE concentration was 4.38 mM (ACE/oxybenzone molar ratio of 20). The initial laccase activity was 1 U/mL. The reactors were placed in a constant temperature orbital shaking water bath at 23°C. Experimental conditions are summarized in Table 3.7. Three-mL samples collected from the ABTS and ACE reactors were acidified and purified as described in Section 3.3.1. Samples were analyzed using LC/MS in the full scan mode to detect both the

disappearance of the parent compound and the formation of byproducts as described in Section 3.5.

Table 3.7 – Experimental conditions for characterization of laccase-mediator oxidation byproducts.

Experimental Conditions		
Oxybenzone Concentration	43.6 μM (10 mg/L) - ABTS experiments 218 μM (50 mg/L) - ACE experiments	
Laccase Activity	1 U/mL (0.7 μM)	
Temperature	23°C	
Medium	Sodium phosphate buffer	
pH	7	
Mediator	Mediator Concentration (μM)	Mediator/Oxybenzone Molar Ratio
ABTS	100	2.3
ACE	4360	20

3.3.6 Characterization of ozonation byproducts

An experiment was performed to characterize the byproducts generated when oxidizing oxybenzone with dissolved ozone. An ozone generator (Yanco Industries Ltd., Burton, BC, Canada) was used to produce a 60 mg/L ozone stock solution. Ten different aliquots of the ozone stock solution were added to 50-mL vials containing 3 mL of a 100 mg/L oxybenzone solution in ultrapure water. Table 3.8 describes the ten resulting solutions. Samples were analyzed as in Section 3.5.

Table 3.8 – Initial concentrations of ozone and oxybenzone as a function of the volume of ozone stock solution added.

Ozone Stock Solution Added (mL)	Total Volume of Solution (mL)	Initial Ozone Concentration in Solution (mg/L)	Initial Oxybenzone Concentration in Solution (mg/L)	Initial Oxybenzone Concentration in Solution (mM)	Initial Ozone/Oxybenzone Molar Ratio
0	3	0	100	0.44	0
1	4	15	75	0.33	0.95
2	5	24	60	0.26	1.91
3	6	30	50	0.22	2.86
4	7	34	43	0.19	3.82
5	8	38	38	0.16	4.77
6	9	40	33	0.15	5.73
7	10	42	30	0.13	6.68
8	11	44	27	0.12	7.63
9	12	45	25	0.11	8.59
10	13	46	23	0.10	9.54

3.3.7 Laccase oxidation of ACE: Mechanism

Several experiments were performed to evaluate the mechanisms of laccase oxidation of the mediator ACE at the experimental conditions described in Table 3.9. All the experiments were conducted in 0.1 M sodium acetate buffered solution at pH 5 at 23^{o}C.

Table 3.9 – Experimental conditions for the laccase oxidation of the mediator ACE. Experiments were performed in a pH 5 sodium acetate buffered solution.

Experiment	Reaction volume (mL)	Laccase Activity (U/mL)	ACE Concentration (mM)
1	9.75	99.0	8.97
2	10.0	11.8	0.824
3	20.4	1.19	0.940
4	20.0	0.0075	1.00

The first experiment (Experiment #1) was performed to evaluate the rate of formation of the oxidation product with an absorption maximum at 520 nm from the laccase oxidation of the mediator ACE. The final goal was to relate the rate of formation of that oxidation product to the rate of disappearance of the mediator ACE. The experiment was performed at the conditions described in Table 3.9. The reaction mixture was sampled prior to the addition of laccase to the reaction mixture, after 5 and 10 minutes of treatment, and every 10 minutes of treatment after that point up to 160 minutes of treatment. The samples (300 μL) were diluted by the addition of 3 mL of distilled water. The visible spectrum of the samples (spectrum range between 450 nm and 650 nm) was measured, and the absorbance of the samples at a wavelength of 520 nm was recorded as a function of the reaction time. A UV-visible spectrophotometer (Agilent Technologies, Waldbronn, Germany) was used to obtain the UV-Visible spectrum, and to measure the absorbance at different wavelengths as described below. A 1-cm quartz cuvette was utilized to load the samples into the spectrophotometer.

Experiments #2 and #3 were performed to confirm or refute a direct relationship between the disappearance of the mediator ACE and the formation of the oxidation product monitored in Experiment #1 and to further understand the mechanisms of ACE oxidation.

Two replicates of Experiment #2 were performed. The first replicate (Experiment #2a) was used to evaluate the reaction mixture in the visible range of the spectrum, and the second replicate (Experiment #2b) was used to evaluate the UV range of the spectrum. In the first experiment (Experiment #2a), samples were taken after 30 seconds of treatment, 5 minutes of treatment, and every 5 minutes after that point up to 115 minutes of treatment. The absorbance of all the undiluted samples was recorded at a wavelength of 520 nm. The visible spectrum (spectrum range between 350 nm and 700

nm) of selected undiluted samples taken after 30 seconds, 5, 10, 20, 30, 40, 60, 80, 100, and 115 minutes of treatment was measured. In the second experiment (#2b), samples were taken after 15 seconds, 3, 5, 7.5, 10, 15, 30, 45, 60, and 100 minutes of treatment. The samples (300 μL) were diluted by the addition of 3 mL of distilled water. The UV spectrum (spectrum range between 250 nm and 340 nm) for the selected samples was measured.

Experiment #3 was conducted at a similar initial ACE as Experiment #2, but at an initial laccase activity that was one-tenth that of Experiment #2. As in the previous experiments, two replicates were performed (Experiments #3a and #3b) to evaluate different regions of the spectrum (UV and visible). In the first experiment (Experiment #3a), samples were taken prior to the onset of the reaction, and after 7, 17, 27, 37, 47, 57, 72, 87, 102, and 122 minutes of treatment. The absorbance of all the undiluted samples was recorded at 520 nm. The visible spectrum (spectrum range between 350 nm and 650 nm) was measured for all the undiluted samples. For the second replicate (Experiments #3b), samples were taken prior to the onset of the reaction, and after 5, 15, 25, 35, 45, 70, 170, and 275 minutes. The samples (300 μL) were diluted by the addition of 3 mL of distilled water. The UV spectrum (spectrum range between 250 nm and 340 nm) for the selected samples was measured.

Experiment #4 was carried out to better understand the mechanisms of the ACE oxidation by identifying the molecular weight and the absorption spectrum of the most abundant laccase oxidation products of the ACE mediator. Samples were taken before the onset of the reaction (before the addition of laccase to the reaction mixture) and after 24 hours of treatment. The samples (1 mL) were analyzed using LC/MS in the full scan mode to detect both the disappearance of the parent compound and the formation of oxidation products as described in Section 3.5.

3.3.8 Laccase oxidation of ACE: Kinetics

The kinetics of the laccase oxidation of the mediator ACE was evaluated. Experiments were performed for a range of different ACE concentrations at the same initial laccase activity in pH 5 buffered ultrapure water solutions and in pH 6 primary effluent solutions. Initial experiments were performed at pH 5 because laccase oxidation is optimal under acidic conditions (Strong and Claus, 2011). If the laccase-mediator system is intended to be directly applied to municipal wastewater primary effluent solutions, little or no acidification is desirable due to cost restrictions. Consequently, subsequent experiments in primary effluent solutions were performed at a higher pH value of 6. If the laccase-mediator system can be applied in a treatment configuration that allows the enzyme and mediator to interact in a separate reactor prior to coming in contact with the target compound in primary effluent (the "free radical generator" configuration as explained below), lower pH conditions that are closer to the optimal laccase-oxidation values can be achieved. This treatment configuration requires smaller reactors where pH control is possible and the laccase oxidation of the mediator can be conducted in buffered water solutions instead of in primary effluent solutions.

The goal of these experiments was not only to determine the oxidation kinetics of the mediator ACE, but also to evaluate the effects of a primary effluent solution on the kinetics of laccase oxidation of the mediator ACE. The initial oxidation rate of the mediator ACE at different initial mediator concentrations was evaluated, and the Michaelis-Menten equation (Section 2.4.1) was used to determine the kinetic parameters. Factors such as byproduct enzyme inhibition and enzyme deactivation can be minimized by using the initial velocity method.

The first objective of these experiments was to evaluate the kinetics of the ACE oxidation in ultrapure water buffered solutions. Several experiments were carried out at

the experimental conditions described in Table 3.10. The experiments were conducted in 50-mL batch reactors containing 20 mL of reaction mixture in a pH 5 sodium acetate buffered solution at an initial laccase activity of 0.0182 U/mL (0.019 μM) at 23^{o}C. The initial velocity of the laccase oxidation of ACE was calculated for every experiment described in Table 3.10 by measuring the disappearance of the mediator ACE as a function of time when less than 10% of the mediator was oxidized. The disappearance of ACE was evaluated by measuring the absorbance at 294 nm (absorption maximum of the mediator ACE) using the same spectrophotometer described in the previous section (Section 3.3.7). Depending on the initial ACE concentration, samples were diluted as indicated in Table 3.10, to get absorbance measurements always below 1.0 unit of absorbance in the spectrophotometer. The absorbance values were converted to concentrations, and the concentration was plotted as a function of time. The slope of the curve represents the initial velocity of the oxidation of the mediator ACE for a particular initial ACE concentration.

Table 3.10 – Experimental conditions for the Michaelis-Menten initial velocity kinetics experiments for the laccase oxidation of ACE. The experiments were performed at a laccase activity of 0.0182 U/mL in pH 5 sodium acetate buffered solutions at 23°C.

Experiment	ACE Concentrations (μM)	UV-vis Dilution factor
1	50	Undiluted
2	105	3
3	211	3
4	316	5
5	421	5
6	513	10
7	615	10
8	718	10
9	821	10
10	923	10
11	1026	15
12	1231	15

The Michaelis-Menten equation using the initial velocities method (Equation 3.1) describes the expected theoretical relationship between the initial reaction velocity and the initial substrate (ACE) concentration. If the experimental values of initial reaction velocity and initial substrate concentration fit the theoretical equation (Equation 3.1), then the kinetic parameters k_2, and K_M can be determined by non-linear regression analysis.

$$v_o = -\left(\frac{d(S)}{dt}\right)_o = \frac{k_2[E]_T[S]_o}{K_M + [S]_o} \qquad \text{Eq. (3.1)}$$

v_o = Initial reaction velocity [μM/min]

S_o = Initial substrate concentration [μM]

K_M = Michaelis-Menten constant [μM]

k_2 = Catalytic constant [μM min^{-1} $(U/mL)^{-1}$]

E_T = Total enzyme concentration [U/mL]

The second objective of the kinetics experiments was to evaluate the effects of primary effluent constituents on the rate of laccase oxidation of the mediator ACE. The experiments were performed in primary effluent solutions rather than in sodium acetate buffer. The primary effluent was collected from the Walnut Creek wastewater treatment facility in Austin, Texas and was filtered using grade 934-AH glass microfiber filters with a 1.5-μm pore size. The primary effluent was equilibrated with the atmosphere by rapid mixing of the solution for approximately 30 minutes before starting the experiments. Sodium acetate was added to the primary effluent solution up to a final concentration of 0.1 M to avoid further changes in pH during the experiments. The pH of the resulting solution was then adjusted to an initial pH of 6 with 5N hydrochloric acid. Several experiments were carried out at the experimental conditions described in Table 3.11. These experiments were nearly identical to the previous experiment performed in pH 5 sodium acetate buffered solutions. The experiments were conducted in 50-mL reactors containing 20 mL of reaction mixture in pH 6 primary effluent solutions at an initial laccase activity of 0.0169 U/mL (0.012 μM). The initial velocity of the laccase oxidation of ACE was calculated for every experiment described in Table 3.11 as previously indicated. The absorbance values were converted to concentrations, and the concentrations were plotted as a function of time. The slope of the curve represents the initial velocity of the oxidation of the mediator ACE for a particular initial ACE concentration. As in previous experiments, samples were diluted as indicated in Table 3.11 to always measure absorbance values in the spectrophotometer below 1.0 unit of

absorbance. If the experimental values of initial reaction velocity and initial substrate concentration fit the theoretical equation (Equation 3.1), then the kinetic parameters k_2, and K_M can be determined by non-linear regression analysis.

Table 3.11 – Experimental conditions for the Michaelis-Menten initial velocity kinetics experiments for the laccase oxidation of ACE. The experiments were performed at a laccase activity of 0.0169 U/mL in pH 6 primary effluent solutions at 23°C.

Experiment	ACE Concentrations (μM)	UV-vis Dilution factor
1	50	Undiluted
2	105	3
3	158	3
4	211	3
5	316	5
6	421	5
7	513	10
8	615	10
9	821	10

3.3.9 Laccase oxidation of ABTS

The laccase oxidation of the mediator ABTS (ABTS• radical formation) was evaluated for a particular set of conditions and to confirm the relatively high stability of the free radical (ABTS• radical) that is produced in the reaction.

An experiment was conducted in a 50-mL batch reactor containing 20 mL of the reaction mixture in a pH 5 sodium acetate buffered solution at an initial laccase activity of 0.08 U/mL (0.057 μM) at 23°C. Samples were taken every two minutes for the first 40 minutes of treatment, then every four minutes for the next 48 minutes of treatment, and then every eight minutes for the next 60 minutes of treatment. The disappearance of the

mediator ABTS was evaluated by measuring the formation of the oxidation product, the free radical ABTS• (Scott et al., 1993). ABTS• absorbs visible light with an extinction coefficient of $3.6x10^4$ $M^{-1}cm^{-1}$ at a wavelength of 420 nm (Scott et al., 1993). Samples were diluted to always get absorbance measurements below 1.0 absorbance units. The absorbance was measured using the same spectrophotometer described in the previous section (Section 3.3.8).

3.3.10 Free radical generator experiments

The goal of this experiment was to evaluate a different treatment configuration for the laccase-mediator system for removing the target compound oxybenzone. This new treatment configuration allows the enzyme and the mediator to react before coming in contact with the target compound, oxybenzone (Figure 3.2). The laccase enzyme and the mediator are added to a smaller batch reactor "free radical generator" reactor. The free radicals generated are then introduced to an oxidation batch reactor containing the target PPCP. Two experiments were performed to evaluate this treatment configuration using ACE and ABTS mediators at the experimental conditions described in Table 3.12.

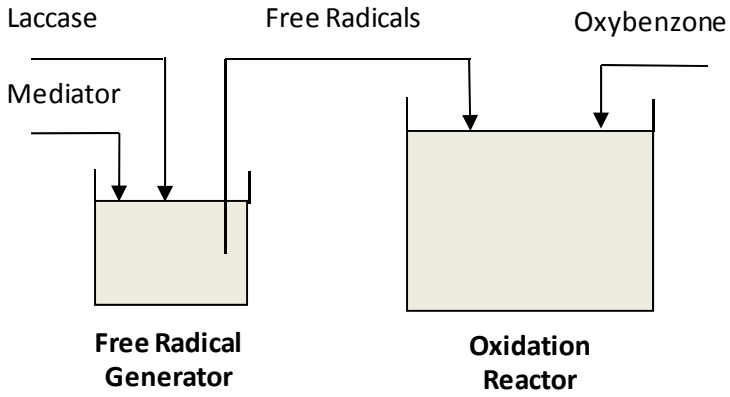


Figure 3.2 – Free radical generator treatment configuration consisting of two batch reactors: a) Free radical generator batch reactor; b) Oxidation reactor batch reactor.

Table 3.12 – Free radical generator experimental conditions.

Parameter	**Mediator**	
	ACE	**ABTS**
Free Radical Generator Reactor		
Reactor Volume	50 mL	25 mL
Mediator Initial Concentration	8.74 mM	1.00 mM
Initial Laccase Activity	6.6 U/mL	0.08 U/mL
Reaction Time	15 min	60 min
pH	3	5
Oxidative Reactor		
Reactor Volume	200 mL	250 mL
PPCP (Oxybenzone) Concentration	10 µg/L (43.8 nM)	10 µg/L (43.8 nM)
Initial Expected Oxidized Mediator (i.e., Free Radical) Concentration	210 µM	9.85 µM
Mediator/Oxybenzone Molar Ratio	4800	225
Reaction (Detention) Time	60 min	30 min
Medium	Ultrapure water	pH 5 sodium acetate buffer

The first experiment was performed using the ACE mediator. As described in Table 3.12, the free radical generator had an initial ACE mediator concentration of 8.74 mM in an ultrapure water solution adjusted to an initial pH of 3 at 23°C. The laccase enzyme was added to the free radical generator to yield an initial laccase activity of 6.6 U/mL (4.7 μM). After 15 minutes of treatment, 4 mL from the free radical generator were added into the oxidative reactor containing the target PPCP oxybenzone at an initial concentration of 10 μg/L in a ultrapure water solution with an unadjusted pH of 5.8 (Table 3.12). After the addition of the 4 mL from the free radical generator into the oxidative reactor, the oxidative reactor had an ACE free radical concentration of approximately 210 μM. The oxidative reactors were placed in the constant temperature orbital shaking water bath at 23°C. Forty-mL samples were collected at 0 and 60 minutes of treatment, placed in 50-mL beakers, and immediately acidified with 100 μL of 5N hydrochloric acid to a final pH of approximately 1.5. The acidified samples were passed through SPE cartridges. The SPE cartridges were conditioned prior to passing samples and rinsed after passing samples, as described in Section 3.3.1. After drying, the cartridges were eluted with two 2-mL and one 1-mL aliquots of LC/MS grade methanol. The eluates were collected in 5-mL conical vials and evaporated to less than 1 mL, but not to dryness. The samples were reconstituted to 1 mL with LC/MS grade methanol and analyzed for oxybenzone concentration using LC/MS/MS as described in Section 3.5.

The second experiment was conducted with ABTS mediator similar to the previous experiment. The reaction mixture in the free radical generator had the mediator ABTS at an initial concentration of 1 mM in a pH 5 sodium acetate buffered solution at an initial laccase activity of 0.08 U/mL (0.057 μM) at 23°C. After 60 minutes of treatment, 2.5 mL from the free radical generator were added to the oxidative reactor containing oxybenzone at the experimental conditions described in Table 3.12 to yield an

ABTS free radical concentration of 9.85 μM. Triplicate samples were taken at 0 and 30 minutes of treatment, and SPE was performed as described in the previous paragraph. One control without laccase was performed with this experiment.

3.4 ENZYME ACTIVITY ASSAY

Laccase activity for all experiments was determined following the methods described by Auriol et al. (2007) with some minor modifications. Enzyme activity was measured by determining the oxidation rate of a substrate, ABTS, to its final product, the radical ABTS•. The rate of disappearance of ABTS was determined by measuring the rate of formation of the radical ABTS•. ABTS• absorbs visible light with an extinction coefficient of $3.6x10^4$ $M^{-1}cm^{-1}$ at a wavelength of 420 nm (Scott et al., 1993). Enzyme solutions were diluted to obtain enzyme activities of approximately 1.0×10^{-3} U/mL in assay mixtures; one unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of ABTS per minute at 37°C. In addition to an enzyme solution, an assay mixture contained 5.0 mM ABTS in 0.1 M sodium acetate buffer at pH 5 at 37°C. Assay mixtures were placed in a 96-well polypropylene round bottom microplate (Greiner Bio-One, Monroe, NC, USA) with a well volume of 300 μL. The microplate was incubated in an orbital shaker (New Brunswick Scientific, Edison, NJ, USA) at 37°C, and the absorbance was measured approximately every 1.5 minutes for approximately 15 minutes using a microplate reader (Biotek, Winooski, VT, USA). The radical ABTS• exhibits a characteristic green color since ABTS• also absorbs visible light at 420, 625, and 728 nm. As observed in Figure 3.3, the cells that contained the higher enzyme activity (top of the figure), exhibited a very intense green color due to the high concentration of the radical ABTS•.

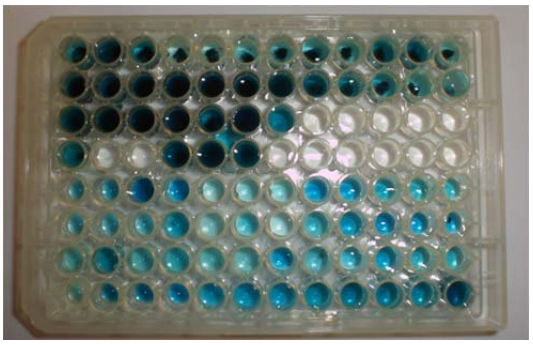


Figure 3.3 – 96-well polypropylene microplate after performing the enzyme activity test.

The absorbance can be converted to concentration by applying the Beer-Lambert law as observed in Equation 3.2 (assuming that the extinction coefficient is known). This implies that the absorbance becomes linear with the concentration. The concentration can be converted to μmoles of ABTS• by knowing the volume of the sample. Consequently, the μmoles of ABTS• can be plotted as a function of time as shown in Figure 3.4. From the slope of this graph, the amount of μmoles of ABTS• produced per minute can be determined; that is the units of enzyme activity can be determined since one unit of enzyme activity (U) is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of ABTS per minute at 37^{o}C. Triplicates were performed when analyzing the laccase activity. The average relative standard deviation for the laccase activity method was equal to 4.00%.

$$Absorbance = (extinction\ coefficient)(path\ length)(concentration) \qquad \text{Equation 3.2}$$

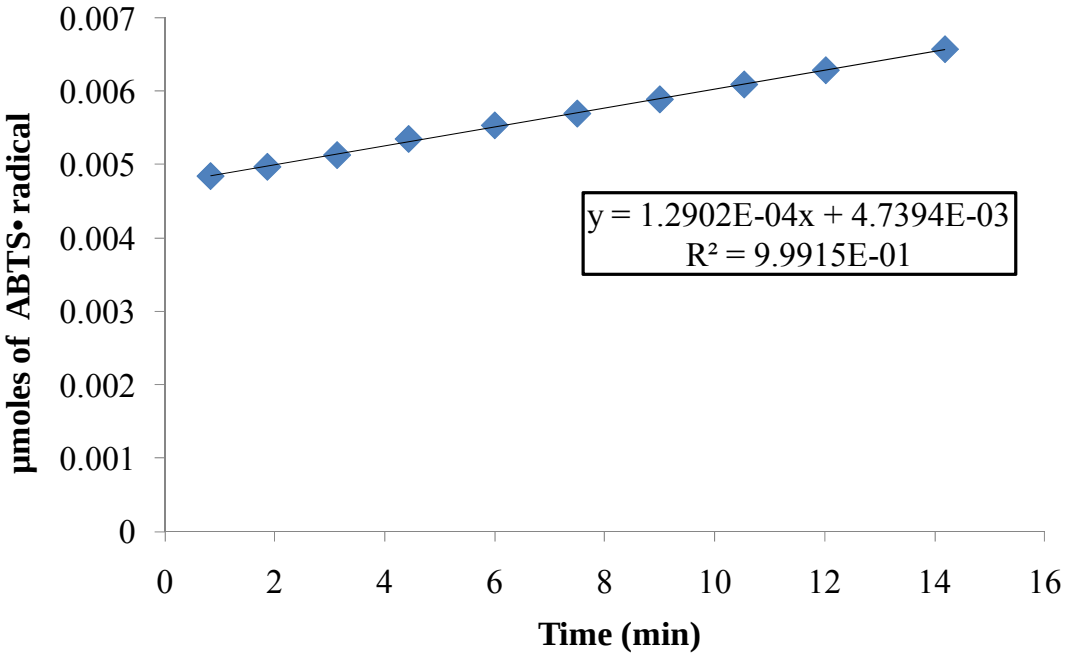


Figure 3.4 – Determination of laccase activity. The slope of the graph yield the rate at which ABTS• radicals are produced in µmoles of ABTS•/minute; that is, units (U) of enzyme activity.

3.5 LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY ANALYSIS

The quantitative analyses of oxybenzone, ABTS, and ACE for all experiments were performed using liquid chromatography/tandem mass spectrometry (LC/MS/MS), as in Vanderford et al. (2003). The analytes were isolated using a Shimadzu 150×4.6 mm C18 column with a particle size of 5 µm and a binary gradient of methanol and water. A Finnigan Surveyor autosampler, a Finnigan Surveyor mass spectrometer pump, and a TSQuantum mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) were used. Electrospray ionization in the positive mode was the ionization source. Different methods were employed to measure the analytes, as described in Table 3.14. The relative standard deviations of the methods used for determining oxybenzone, ABTS,

and ACE concentrations at the different experimental conditions are also reported in Table 3.14.

The characterization of oxybenzone oxidation byproducts and ACE mediator oxidation byproducts was performed using the same instrument setup described above, but a Finnigan Surveyor photodiode array (PDA) detector was incorporated. Also, the mass spectrometer was used in the full scan mode rather than in the tandem MS/MS mode. The scan range selected for the mass spectrometer full scan was between m/z ratios of 30 and 1,500. The PDA detector was programmed to scan in the ultraviolet-visible range between 200 and 800 nm.

Table 3.14 – LC/MS/MS analytical parameters for oxybenzone, ABTS, and ACE.

	Oxybenzone Higher Range	**Oxybenzone Lower Range**	**ABTS**	**ACE**
Liquid Chromatography				
Injection Volume (µL)	10	20	20	20
Flow Rate (µL/min)	700	350	700	700
Gradient:				
Oxybenzone Higher Range: 5% methanol held constant for 3 min, increased linearly to reach 80% at 10 min, held constant at 80% for 10 min, stepped up to 100% and held constant for 11 min. At the end of each run, the methanol was stepped down to 5% and held constant for 4 min.				
Oxybenzone Lower Range, ABTS, and ACE: 5% methanol held constant for 6 min, increased linearly to reach 80% at 18 min, held constant at 80% for 4 min, stepped up to 100% and held constant for 9 min. At the end of each run, the methanol was stepped down to 5% and held constant for 4 min.				
Mass Spectrometry				
Collision Energy (V)	20	29	40	20
Collision Gas Pressure (mTorr)	1.0	1.0	1.5	1.5
Ion Spray Voltage (V)	4,200	4,000	2,700	4,000
Ion Source Temp. (^{o}C)	400	400	400	350
Precursor-Product Ion Mass/Charge (m/z) Ratio	229.0 – 150.9	229.0 – 150.9	514.7 – 230.0	197.2 – 140.0
Method Detection Limits (MDLs), Recoveries, and Relative Standard Deviations (RSDs)				
Standard Curve Range (µg/L)	0 – 1,000	0 – 200	0 – 90,000	0 – 15,000
Standard Curve R^2 Values	0.9914 – 0.9997	0.9937 – 0.9980	0.9932 – 0.9917	0.9906 – 0.9989
MDL (µg/L)	15	0.25	132	6
Mean Recovery by SPE (%)	81 (in buffer) 79 (in MWPE)	44	53	58
RSD (%)	6	5	5	9

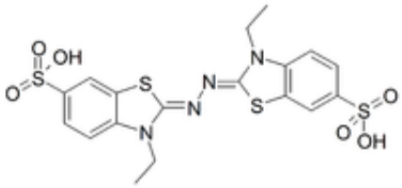
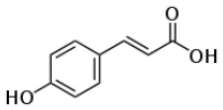
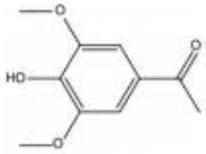
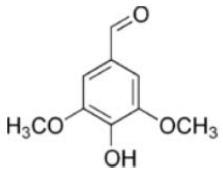
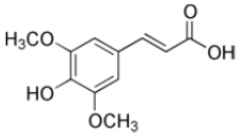
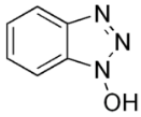
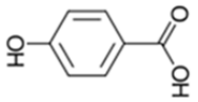
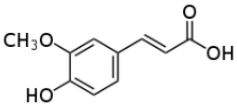
Chapter 4 – Experimental Results

4.1 MEDIATOR SCREENING EXPERIMENTS

The objective of these experiments was to evaluate the effects of several possible mediators on laccase oxidation of oxybenzone. The mediators that exhibited the best oxybenzone removal were selected for performing further experiments in the next phases of this research.

The effectiveness of several different mediators was evaluated at the same experimental conditions described in Table 4-1. As shown in Figure 4.1, experiments performed without a mediator yielded no removal of oxybenzone. In the presence of ABTS or ACE mediators at 1mM concentration, oxybenzone was completely oxidized (below the instrument detection limit) within 24 hours of treatment. With SIN mediator at the same initial mediator concentration, a small fraction of oxybenzone (approximately 6%) remained after 24 hours of treatment (Figure 4.1), and oxybenzone was completely removed after 48 hours of treatment (data not shown). The other mediators evaluated at an initial concentration of 1mM (FA, HBA, HBT, PCA, and SYR) did not perform as well. For instance, HBA and PCA mediators did not yield any removal of oxybenzone after 24 hours of treatment. In experiments with HBT, SYR, and FA mediators, 80%, 77%, and 38% of the oxybenzone remained after 24 hours of treatment, respectively. Although most of the mediators evaluated at the conditions previously described exhibited some oxidation of oxybenzone, ABTS, ACE, and SIN were the most effective.

Table 4.1 – Experimental conditions for mediator screening experiments performed at the same initial mediator concentration.

Experimental Conditions			
Oxybenzone Concentration	4.38 µM (1000 µg/L)		
Laccase Activity	1 U/mL (0.7 µM)		
pH	7		
Temperature	23°C		
Medium	Sodium Phosphate Buffer		
Mediators (1mM concentration)			
ABTS (Synthetic)		PCA (Natural)	
ACE (Natural)		SYR (Natural)	
SIN (Natural)		HBT (Synthetic)	
HBA (Natural)		FA (Natural)	

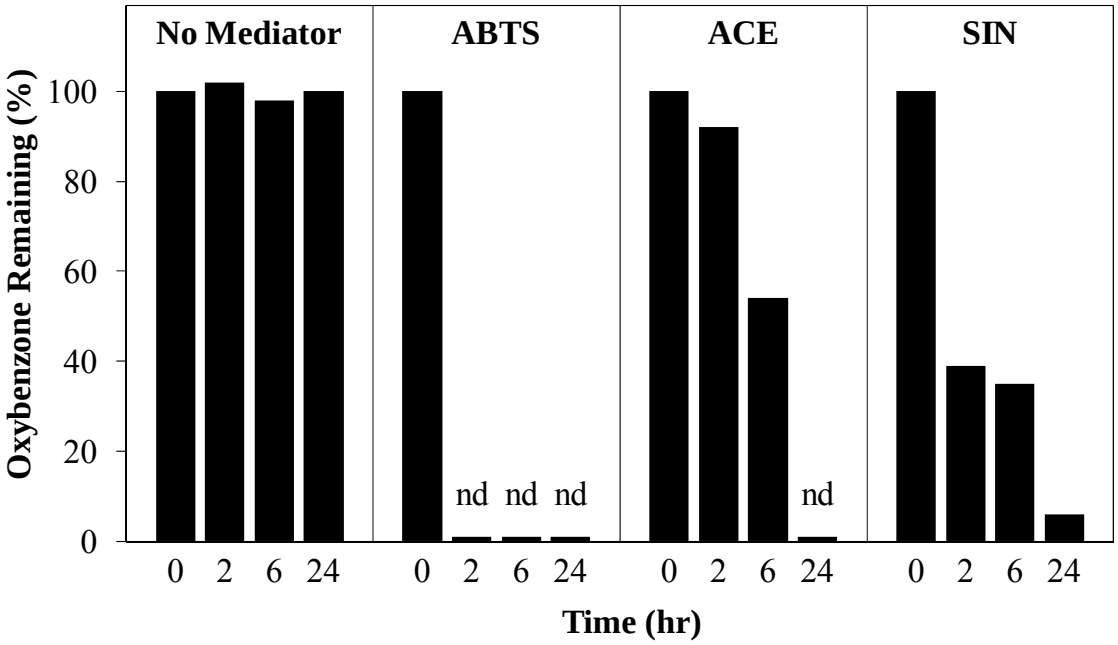


Figure 4.1 – Results from selected mediator screening experiments. (Conditions shown in Table 4.1; nd means not detected).

The main disadvantages of using a synthetic mediator, such as ABTS, are high costs and potential toxicity. On the other hand, many phenolic compounds (e.g., ACE) are naturally occurring and environmentally friendly mediators. Such phenolic mediators can be obtained at low cost due to their abundance in nature, as well as their presence in several industrial wastes (Kunamneni et al., 2008a).

The oxidation of oxybenzone generally caused some loss of enzyme activity, but the relationship was quite different for different mediators. As described in Figure 4.2, when no mediator was added, no oxidation occurred, and no enzyme activity was lost in 48 hours. When a mediator was present and no oxybenzone was removed, as was the case in the PCA and HBA experiments, the loss of enzyme activity was very small (between 5% and 15%) after 48 hours of treatment. When greater oxidation of

oxybenzone was observed (experiments with ACE, HBT, SIN, and SYR mediators), the loss of enzyme activity was more pronounced, ranging from 34% to 47%. When ABTS was used as the mediator, the loss of enzyme activity was even more significant at approximately 64%. In contrast, FA mediator achieved a relatively high removal of oxybenzone, but only a small fraction of the initial enzyme activity was lost (5%). As reported by Kunamneni et al. (2008b), the enzyme activity (when no mediator was added and no oxidation occurred) remained approximately constant after 24 hours of treatment at pH values of approximately 7 and temperatures of 30^{o}C. According to Cabana et al (2007b), higher enzyme inactivation might be caused by free radicals that are produced by the laccase oxidation of the mediator (byproduct enzyme inhibition).

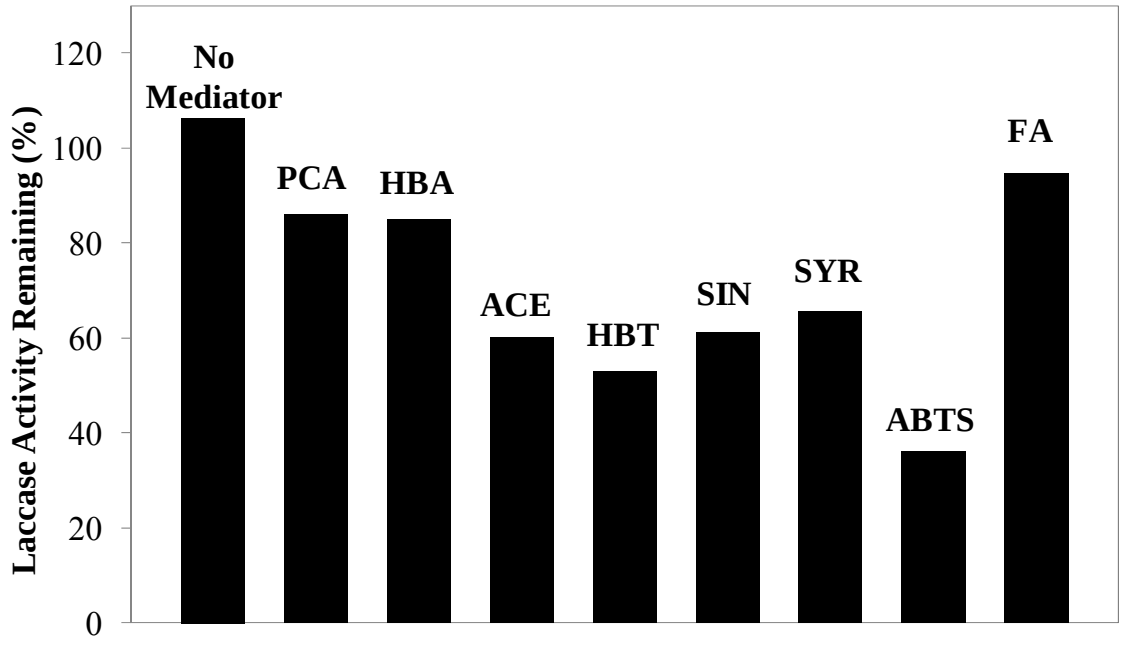


Figure 4.2 – Residual laccase activity after 48 hours of treatment for the selected mediators in the mediator screening experiments.

The effect of different mediator concentrations at different enzyme activities was also evaluated for ABTS and HBT mediators at the experimental conditions described in Table 4.2. As described in Figure 4.3, experiments carried out with the mediator ABTS yielded almost complete removal of oxybenzone after 4 hours of treatment for the optimized mediator addition at an enzyme activity of 1 U/mL. ABTS concentrations higher than 0.001 mM were required to completely oxidize oxybenzone. Lower ABTS concentrations were ineffective in removing the parent compound. The results of these experiments suggest that a minimum mediator concentration is required for the removal of the target compound, and that the removal of the target compound increases with increasing mediator concentration.

Table 4.2 – Experimental conditions for mediator experiments performed at different mediator concentrations.

Experimental Conditions		
Oxybenzone Concentration	4.38 μM (1000 μg/L)	
pH	7	
Temperature	23°C	
Medium	Sodium Phosphate Buffer	
Mediator	*Mediator Concentration (mM)*	*Laccase Activity (U/mL)*
ABTS	0.001; 0.01; 0.1; & 1	1
HBT	1 & 10	1 and 5

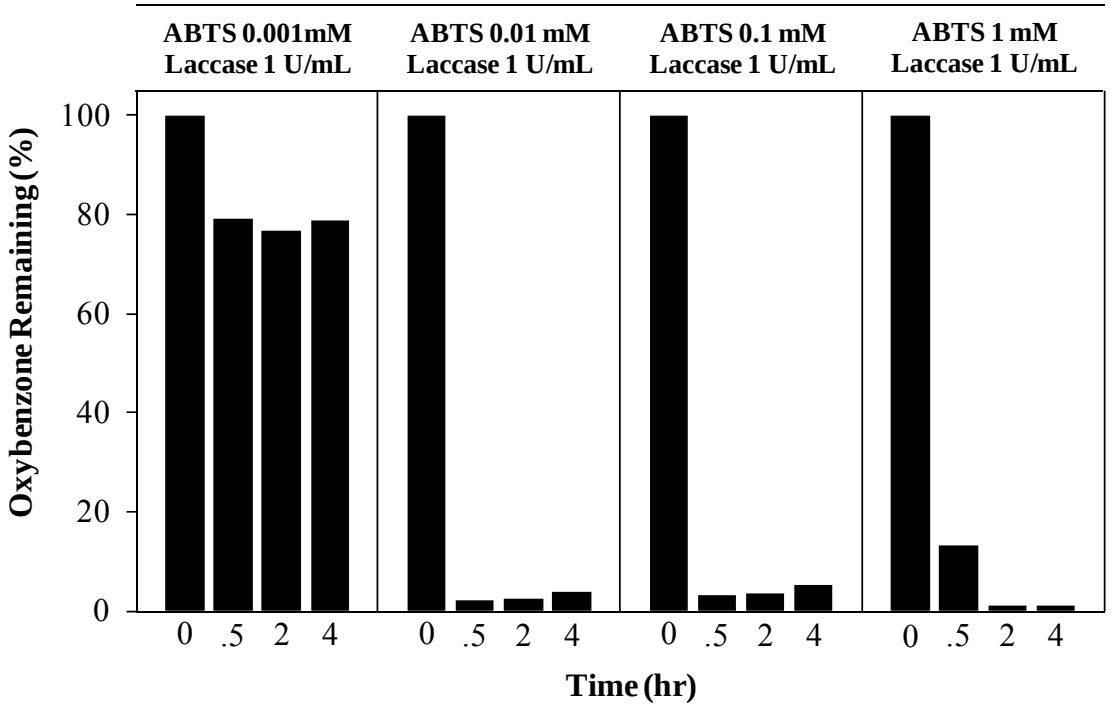


Figure 4.3- Oxybenzone removal experiments conducted with ABTS mediator. (Conditions shown in Table 4.2)

Experiments performed with the HBT mediator at different mediator concentrations and enzyme activities (Table 4.2) are described in Figure 4.4. As previously observed, experiments performed with the addition of HBT mediator at a concentration of 1 mM exhibited almost no removal of oxybenzone after four hours of treatment despite the initial enzyme activity. As the concentration of the HBT mediator increased from 1 mM to 10 mM, the removal of oxybenzone increased with increasing initial enzyme activities. HBT concentrations lower than 10 mM are ineffective for removing the target compound despite the enzyme activity.

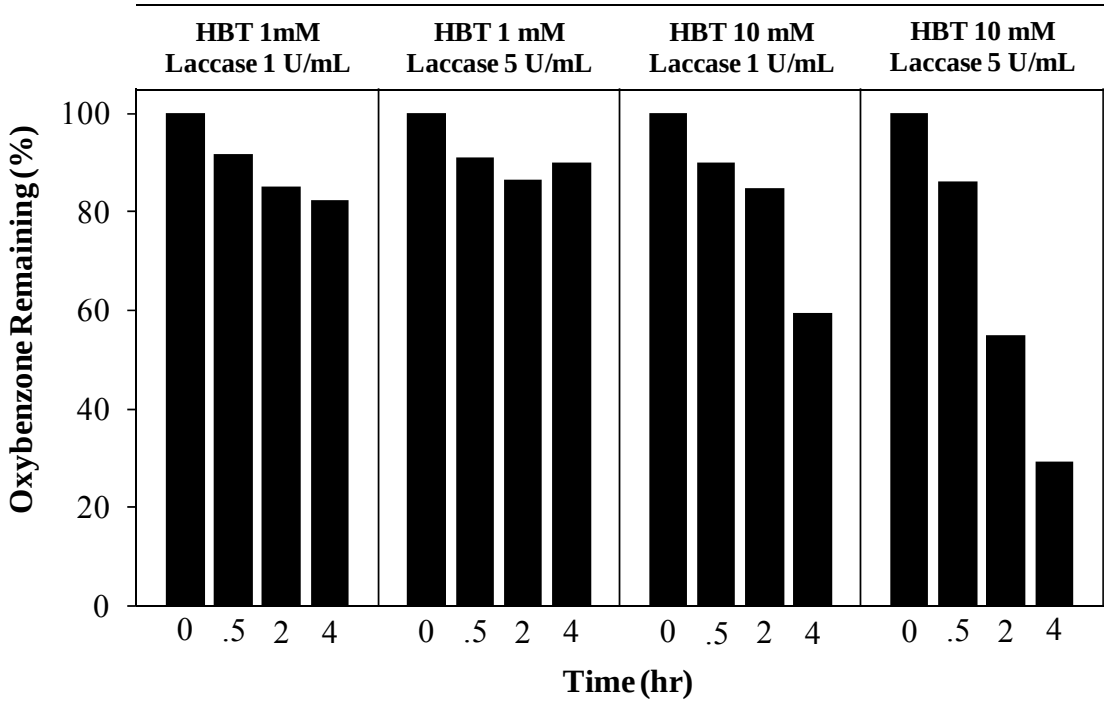


Figure 4.4- Oxybenzone removal experiments conducted with HBT mediator. (Conditions shown in Table 4.2)

As described by Huang et al. (2005), laccase oxidation of some mediators (particularly low molecular weight phenolic compounds) can lead to extensive coupling and polymerization reactions. Laccase oxidation of phenolic mediators generates phenoxyl radicals through single electron abstraction. Phenoxyl radicals can couple to each other or to other non-phenolic or inert compounds (for instance, the target contaminants to be removed) producing dimers, oligomers, and eventually large molecular weight polymers. When working at high mediator concentrations, the polymers can be precipitated out of the solution by either increasing the salt concentration or by reducing the pH (Huang et al., 2005). That is, the removal of an inert contaminant (that does not directly react with laccase) can be achieved either by adsorption onto the polymer produced by self-coupling of the phenolic mediator or by

cross-coupling reaction with the laccase-generated phenoxyl radicals (forming a covalent bonded with the phenoxyl mediator polymer).

The oxidation of the phenolic mediator FA was evaluated at a relatively high initial mediator concentration of 10 mM, and at an initial laccase activity of 5 U/mL. The reaction was performed in different solutions such as sodium phosphate buffer at pH 7, ammonium acetate buffer at pH 4.5, and distilled water. Samples taken from the reaction mixture were acidified with concentrated hydrochloric acid. Figure 4.5 shows the results obtained after 48 hours of treatment. Vials 1 to 3 and vials 7 to 9 in Figure 4.5 correspond to blanks performed where no FA mediator and no laccase was added to the reaction mixture, respectively. Vials 4, 5, and 6 correspond to the acidified reaction mixture (with laccase and FA addition) after 48 hours of treatment for the experiments performed in pH 7, pH 4.5, and non buffered distilled water solutions, respectively. The formation of a precipitate is clearly observed in these three vials. That is, laccase oxidation of the phenolic mediator FA, through the formation of phenoxyl radicals, leads to intensive coupling reactions producing large molecules that can be precipitated out of the solution by decreasing the solution pH. Similar results were published by Kupriyanovich et al. (2007). They obtained precipitated product from the laccase oxidation of FA which exhibited a solid powder-like characteristics colored from cream to dark brown. The performance in these experiments confirmed that laccase is effective in producing precipitatable polymers through phenoxyl radical reactions as suggested by other authors (Huang et al., 2005; Kupriyanovich et al., 2007). The phenoxyl radicals produced by the laccase oxidation may be used to oxidize the target contaminants.

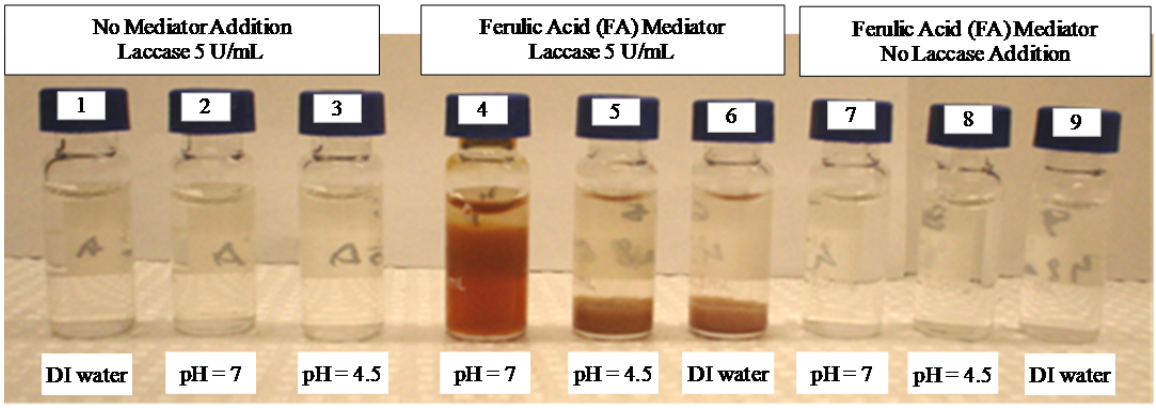


Figure 4.5 FA oxidation experiments performed at an initial FA concentration of 10 mM and at an initial enzyme activity of 5 U/mL. Experiments were performed in distilled water solutions (DI water), pH 7 sodium phosphate buffered solutions (pH = 7), and pH 4.5 ammonium acetate buffered solutions (pH = 4.5).

Most of the mediators investigated in this section were directly recognized and oxidized by the enzyme laccase at enzyme activities as low as 1 U/mL (approximately 0.7 μM). When working at the same initial mediator concentrations and same initial laccase activities the mediators ABTS and ACE exhibited the best performance for oxidizing the target compound oxybenzone. A minimum mediator concentration is required for oxidizing the target compound. The higher the mediator concentration, the higher the removal of the target compounds. Laccase oxidation of some mediators (particularly phenolic mediators such as FA) produces free radicals through single electron abstraction, and these radicals can undergo extensive coupling producing polymers. These polymers can be precipitated out of solution by decreasing the pH. The oxidation of the target compounds when using mediators (that is, by the laccase-mediator system) may occur by an interaction between the mediator free radical and the target compound.

4.2 INITIAL EXPERIMENTS IN PRIMARY EFFLUENT

The two mediators that exhibited the best performance oxidizing oxybenzone in the mediator screening experiments (ABTS and ACE) were selected for further experiments in primary effluent. The objective of these experiments was to evaluate the performance of the laccase-mediator oxidation with ABTS and ACE mediators for the removal of oxybenzone in a more complex solution such as a municipal wastewater primary effluent. Implementation of the laccase oxidation in municipal wastewater primary effluent is desirable, since the potential byproducts generated by laccase oxidation might be removed in subsequent conventional treatment processes, such as activated sludge followed by secondary clarification.

Experiments were performed at the conditions described in Table 4.3. The initial pH of the primary effluent was 7.67. When this pH was not adjusted, it took 24 hours for complete oxybenzone removal (below the instrument detection limit) in the presence of ABTS mediator (Figure 4.6). In the presence of ACE mediator, no removal of oxybenzone was observed after 24 hours of treatment (Figure 4.6). These results contrast with the high removals achieved in the mediator screening experiments (Figure 4.1). One possible reason for this difference is that the pH of the reaction mixture was buffered at 7 in the mediator screening experiments, whereas the pH increased from 7.67 to over 8.5 during the 48-hour primary effluent experiments with ABTS and ACE. Thus, the lower performance of the laccase-mediator system in these experiments might not be due to matrix effects, but rather to the higher pH of the primary effluent. Kim and Nicell (2006) found that the optimal pH range for laccase's direct oxidation of triclosan was between 4 and 6, and that above pH 7, oxidation decreased dramatically. Auriol et al. (2007) reported that the optimum pH for laccase's direct oxidation of several estrogens was approximately pH 6 in synthetic water (a buffered solution in ultrapure water), and they

also observed a significant decrease in oxidation above pH 7. In all reported cases known to us, the greatest direct oxidation by laccase occurred at pH between 3 and 6, suggesting that pH affects laccase activity (Cabana et al., 2007b). If laccase activity is reduced when using the laccase-mediator system, mediator radical generation slows down, thereby slowing down oxybenzone oxidation.

Table 4.3 – Experimental conditions for experiments performed in municipal wastewater primary effluent solutions.

Experimental Conditions	
Oxybenzone Concentration	4.38 µM (1000 µg/L)
Laccase Activity	1 U/mL
Temperature	23°C
Medium	Municipal Primary Effluent
Mediator	*pH*
ABTS	7.67 (unadjusted pH) 6 (initially adjusted)
ACE	7.67 (unadjusted pH) 6 (initially adjusted)

To investigate the pH effect further, these experiments were repeated after adjusting the initial pH of the primary effluent to 6.0. As shown in Figure 4.6, much more rapid oxybenzone removal was achieved for both ABTS and ACE mediators; no oxybenzone was detected after two hours of treatment. The pH still increased over the course of the experiments, and the pH values after 48 hours of treatment were 8.02 and 7.36 for ABTS and ACE, respectively. With ACE mediator, the oxybenzone removal observed with the initial pH of 6 in primary effluent was even better than the removal observed in the mediator screening experiment, performed in a buffered pH 7 solution. Auriol et al. (2007) reported that a municipal wastewater secondary effluent matrix did not have a significant impact on direct laccase oxidation of several estrogens, as

compared to synthetic water. Our experimental results in this laccase-mediator system confirm that, despite the higher concentration of organics in primary effluent, the matrix had little effect on oxybenzone removal.

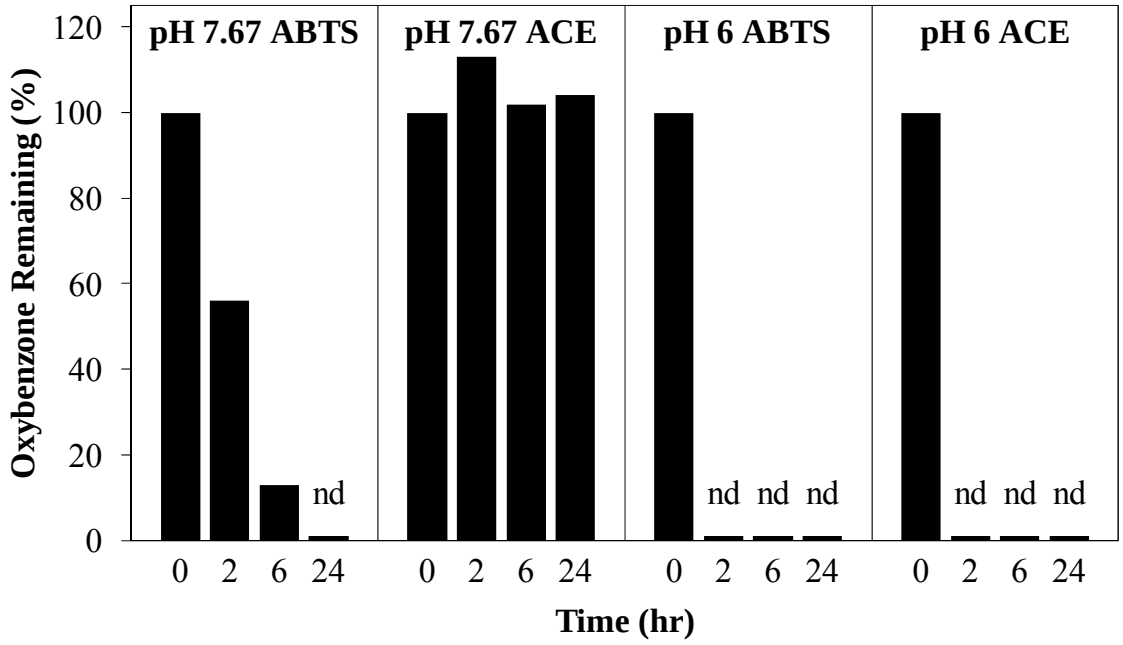


Figure 4.6 – Initial experiments in primary effluent at initial pH values of 7.67 and 6 with ABTS mediator and ACE mediator. (Conditions shown in Table 4.3)

As discussed further in following sections, it seems likely (although not proven in this research) that a wastewater matrix would demand higher mediator/oxybenzone molar ratios than would be required in ultrapure water, due to competition for oxidized radicals of the mediator by other organics. At the high mediator/oxybenzone molar ratios used in these experiments (approximately 230), more than enough mediator was present to meet the demands of oxybenzone and other components of the matrix. Thus, in this set of experiments, the effect of initial pH was much more significant than the matrix effect introduced by the primary effluent.

Austin's wastewater has relatively high alkalinity, with an average of 273 mg/L as $CaCO_3$ in the samples in our experiments. When a system is opened to the atmosphere, the alkalinity remains constant, but the carbonate speciation and pH change as the solution achieves equilibrium with the carbon dioxide in the atmosphere. For Austin's wastewater, that process led to a loss of carbonic acid (and therefore total carbonate) and a consequent rise in the pH. As shown in Figure 4.7, in a separate experiment with no enzymes but constant mixing, the pH rose from 6.0 to 7.94 in a six-hour period, proving that the rise in pH in our experiments was not due to enzymatic reactions but only to gas/liquid equilibration. Such a pH rise from gas transfer would not occur in an actual wastewater treatment plant reactor, because the surface area per unit volume of fluid is dramatically lower in full-scale reactors. In addition, this result suggests that the necessity to lower the pH would be much less in a full-scale reactor. A more detailed explanation of the equilibration of the primary effluent with the atmosphere (including pH equilibrium calculations) is in Appendix A.

Typical pH values for domestic municipal wastewater in the U.S. range from pH 6.7 to pH 7.5 with an average value of pH 7.0 (Qasim, 1999), and typical alkalinity values range from 50 to 200 mg/L as $CaCO_3$ with an average of 100 mg/L as $CaCO_3$ (Qasim, 1999). The pH and alkalinity values observed in Austin's municipal wastewater are considerably higher than the average values in the U.S. Thus, the acidification requirements for municipal wastewaters from any other city in the U.S. would be significantly less than that required for Austin's wastewater.

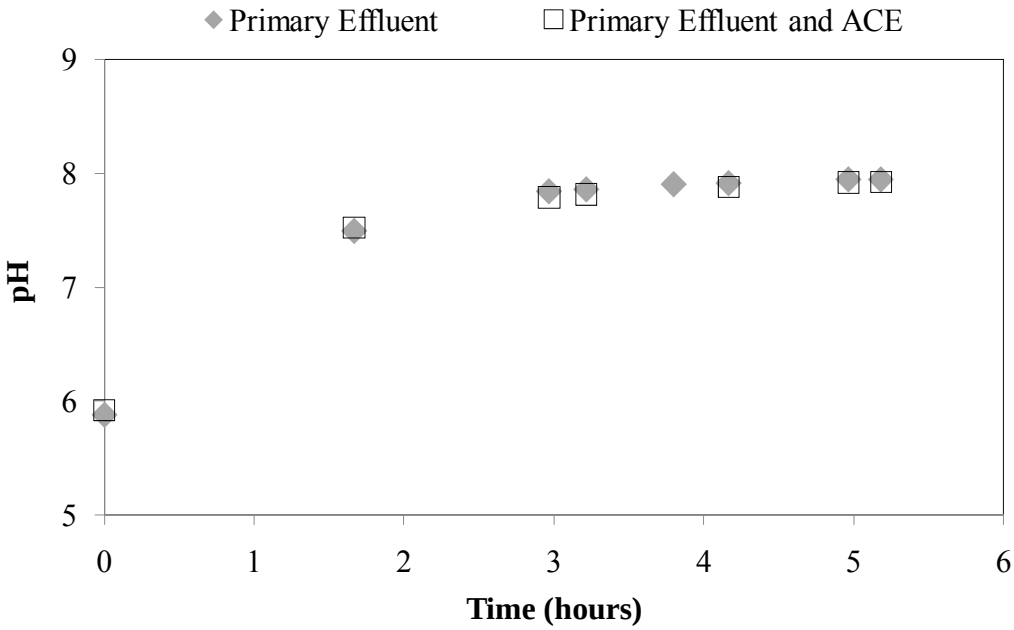


Figure 4.7 – Equilibration of primary effluent with the atmosphere. Experiments performed in primary effluent solutions with no enzyme addition. The pH of the reaction mixture was initially adjusted to 6.

As noted above, no loss of enzyme activity was observed in the mediator screening experiments when no mediator was present. As observed in Figure 4.8, in the control experiments performed in primary effluent without mediator, no oxybenzone was oxidized, but enzyme activity losses of 35% and 16% were observed at initial pH values of 7.67 and 6, respectively after 48 hours of treatment. These results indicate that interaction with other constituents of the primary effluent led to some inactivation of the enzyme. For instance, laccase might have been biodegraded, or inactivated by common organic/inorganic wastewater constituents such as sulfite, sulfide, nitrite, thiosulfate, copper, iron, cyanide, and halogen ions (Kim and Nicell, 2006b). Moreover, the different enzyme activity losses observed at the two initial primary effluent pH values suggest that higher pH conditions contribute more to enzyme destabilization. For both ABTS and

ACE mediators, the enzyme activity losses observed in experiments performed in primary effluent were similar to those observed in experiments performed in phosphate buffer at pH 7. Thus, the enzyme destabilization produced by the free radical oxidation byproducts (byproduct enzyme inhibition) when working at relatively high mediator concentrations of 1 mM is more significant than the destabilization produced by common organic wastewater constituents.

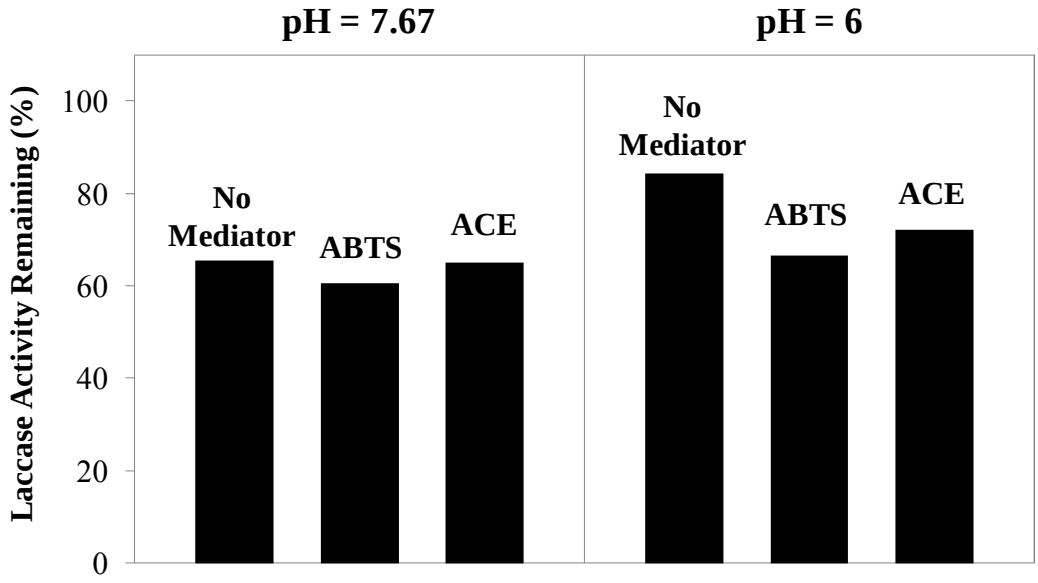


Figure 4.8 – Residual laccase activity after 48 hours of treatment for the selected mediators in the primary effluent experiments.

4.3 EFFECT OF MEDIATOR/OXYBENZONE MOLAR RATIOS IN PRIMARY EFFLUENT

After determining that the laccase-ABTS and laccase-ACE systems could oxidize oxybenzone in primary effluent at a high mediator/oxybenzone molar ratio of 230, the objective of the next set of experiments was to determine the lowest molar ratios at which the oxidation would still be effective. The experiments were performed at the experimental conditions described in Table 4.4.

Table 4.4 – Experimental conditions for experiments performed at different oxybenzone/mediator molar ration in primary effluent solutions.

Experimental Conditions		
Oxybenzone Concentration	4.38 μM (1000 μg/L)	
Laccase Activity	1 U/mL (0.7 μM)	
Temperature	23°C	
Medium	Municipal Primary Effluent	
pH	6 (initially adjusted)	
Mediator	*Mediator Concentration (μM)*	*Mediator/Oxybenzone Molar Ratio*
ABTS	2.63	0.6
	8.76	2
ACE	26.3	6
	87.6	20

As can be seen in Figure 4.9, the ABTS mediator was more effective at lower molar ratios than the ACE mediator. The laccase-ABTS system achieved significant oxidation of oxybenzone at ABTS/oxybenzone molar ratios as low as 2, at which only 6% of the oxybenzone remained after six hours of treatment; complete and very rapid oxidation occurred at higher molar ratios. Substantial oxybenzone removal (85%) was achieved at an ACE/oxybenzone molar ratio of 20 after only one hour of treatment, but no additional removal was achieved in six hours.

As can be seen in the ACE mediator results in Figure 4.9b, most of the oxybenzone removal generally occurred in the first 15 minutes of the reaction. A similar trend is evident with ABTS mediator, except that the majority of the removal occurred in the first hour. In both cases, the enzyme-mediator system apparently lost its ability to oxidize the target compound after a certain point. During this initial period, the pH of the reaction mixture was still below 7 although the final pH after six hours of treatment was approximately 7.8 (consistent with the information provided in Figure 4.7).

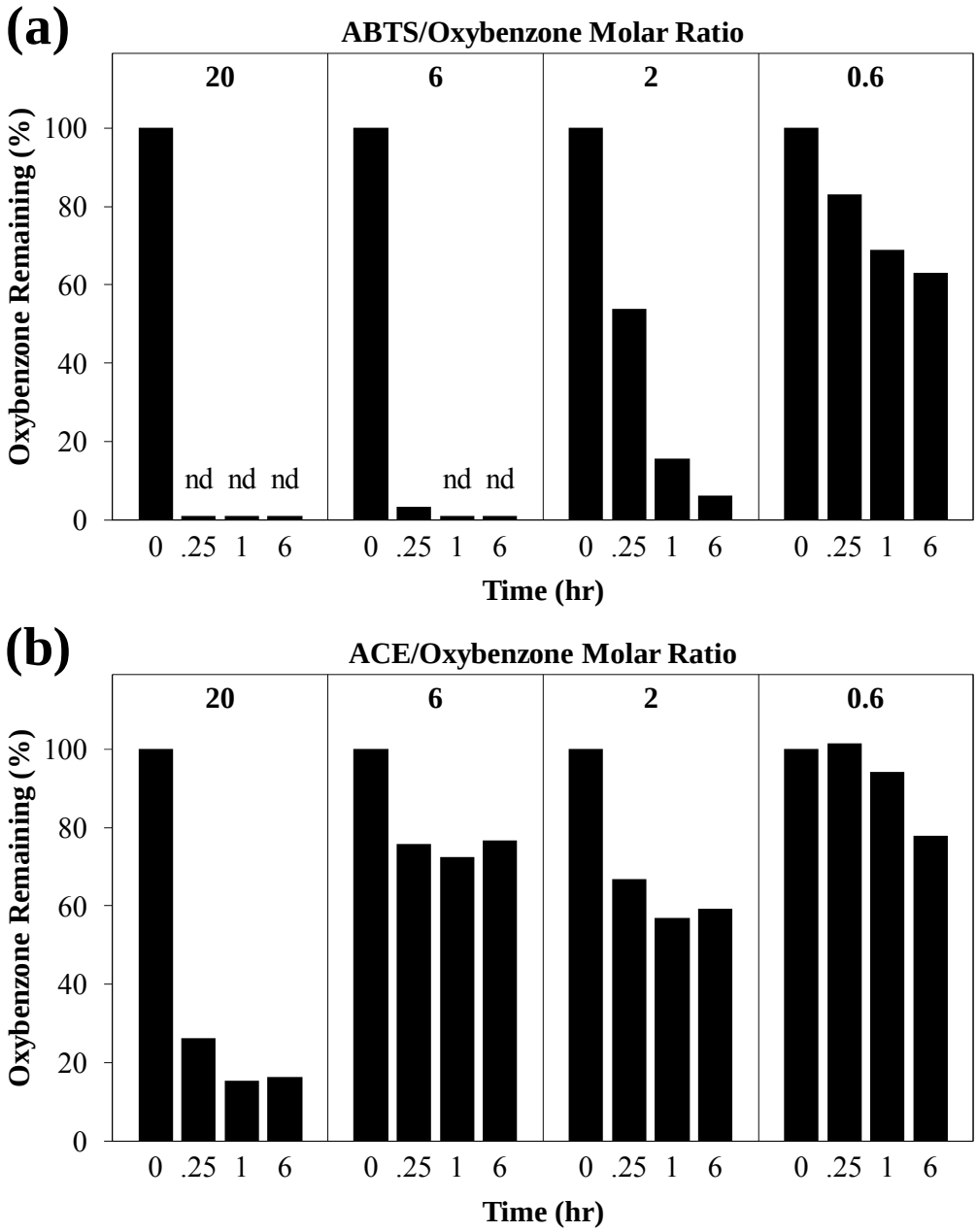


Figure 4.9 – Mediator/oxybenzone molar ratio experiments conducted with (a) ABTS mediator and (b) ACE mediator. Experiments were performed in filtered primary effluent at 23°C, with an initial oxybenzone concentration of 4.38 μM, and an initial laccase activity of 1 U/mL.

To investigate further whether the pH increases were reducing the amount of oxybenzone oxidation, the experiments with ACE/oxybenzone molar ratios of 20 and 6 were repeated with the pH held constant at 6 by adding hydrochloric acid as the reaction progressed. The results of these experiments (shown in Figure 4.10) were not totally different from those without pH control, indicating that pH rises in the range of 6.0 to nearly 7.8 did not affect oxybenzone removal under these conditions. Thus, the observed decline in oxybenzone oxidation was not likely due to high pH, but rather to consumption of the mediator, as shown below.

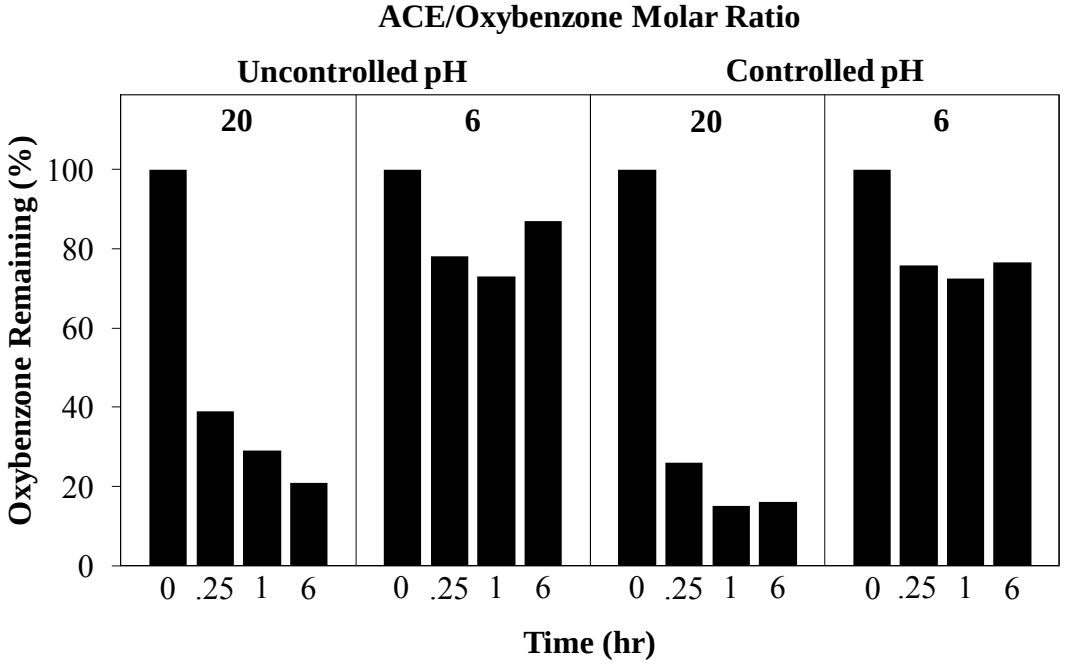


Figure 4.10 – ACE/oxybenzone molar ratio experiments conducted with and without pH control. Experiments were performed in filtered primary effluent at 23°C, with an initial oxybenzone concentration of 4.38 μM, and an initial laccase activity of 1 U/mL. In the uncontrolled pH experiments, the pH rises from 6.0 to nearly 7.8.

As shown in Figure 4.11, enzyme activity assays suggest that the enzyme was still very active after 6 hours of treatment in the presence of both mediators (e.g., 78% and 72% of the initial enzyme activity remained for ABTS and ACE, respectively, at mediator/oxybenzone molar ratios of 20). The oxidation of oxybenzone still caused some loss of enzyme activity, but not as pronounced as in the previous section. There are two possible explanations for the higher laccase activity remaining in these experiments. First, the final enzyme activity was measured after 6 hours of treatment instead of after 48 hours of treatment. Second, the mediator concentrations used in these experiments were considerably lower than in the previous section. Higher enzyme inactivation might be caused by free radicals that are produced by the laccase oxidation of the mediator (byproduct enzyme inhibition). The reaction time could have a stronger effect on enzyme destabilization than byproduct enzyme inhibition at the relatively low mediator concentrations of these experiments. In the 6-hours experiments, the enzyme was not exposed long enough to the primary effluent matrix to be destabilized.

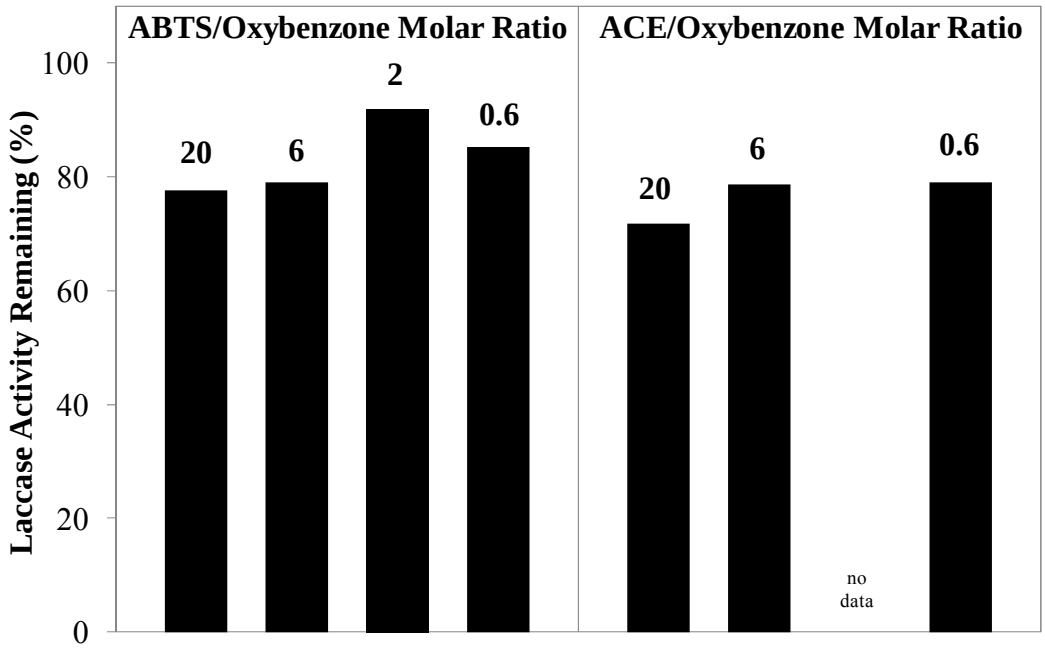


Figure 4.11 – Residual laccase activity after 6 hours of treatment for the mediators ABTS and ACE at the mediator/oxybenzone molar ratios described in the figure.

4.4 EXPERIMENTS AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS

Once it was determined that the laccase-mediator system was able to oxidize oxybenzone in primary effluent at relatively low mediator/oxybenzone molar ratios, experiments were performed at a significantly lower initial oxybenzone concentration of 43.8 nM (10 µg/L). This concentration is much closer to an oxybenzone concentration of 4 nM (900 ng/L), which would be typical in raw municipal wastewater or primary effluent (Snyder et al., 2007). The experimental conditions of these experiments are described in Table 4.5.

Table 4.5 – Experimental conditions for experiments performed at environmentally relevant concentrations of oxybenzone.

Experimental Conditions		
Oxybenzone Concentration	43.8 nM (10 µg/L)	
Laccase Activity	1 U/mL (0.7 µM)	
Temperature	23°C	
Medium	Municipal Primary Effluent	
pH	6 (initially adjusted)	
Mediator	*Mediator Concentration (µM)*	*Mediator/Oxybenzone Molar Ratio*
ABTS	8.76	200
ACE	0.876	20
	8.76	200
	876	2000

Comparing the results of experiments performed at an environmentally relevant oxybenzone concentration (Figure 4.12a) to those obtained at a high oxybenzone concentration (Figure 4.9) demonstrates that, for both ABTS and ACE, much higher mediator/oxybenzone molar ratios are required at a lower oxybenzone concentration to achieve comparable oxybenzone removal (e.g., a molar ratio of 2,000 versus 20 for ACE, and a molar ratio of 200 versus 2 for ABTS). There appears to be a minimum effective mediator concentration, as the same absolute mediator concentration yielded similar results at the two different oxybenzone concentrations. For instance, experiments performed at an ACE concentration of 87.6 µM achieved significant oxybenzone removal at both the 10 µg/L (43.8 nM) and the 1000 µg/L (4.38 µM) initial oxybenzone concentrations (corresponding to ACE/oxybenzone molar ratios of 2,000 and 20, respectively). Similarly, at an ABTS concentration of 8.76 µM, significant removal was observed at both the 10 µg/L (43.8 nM) and the 1000 µg/L (4.38 µM) initial oxybenzone

concentrations (corresponding to ABTS/oxybenzone molar ratios of 200 and 2, respectively).

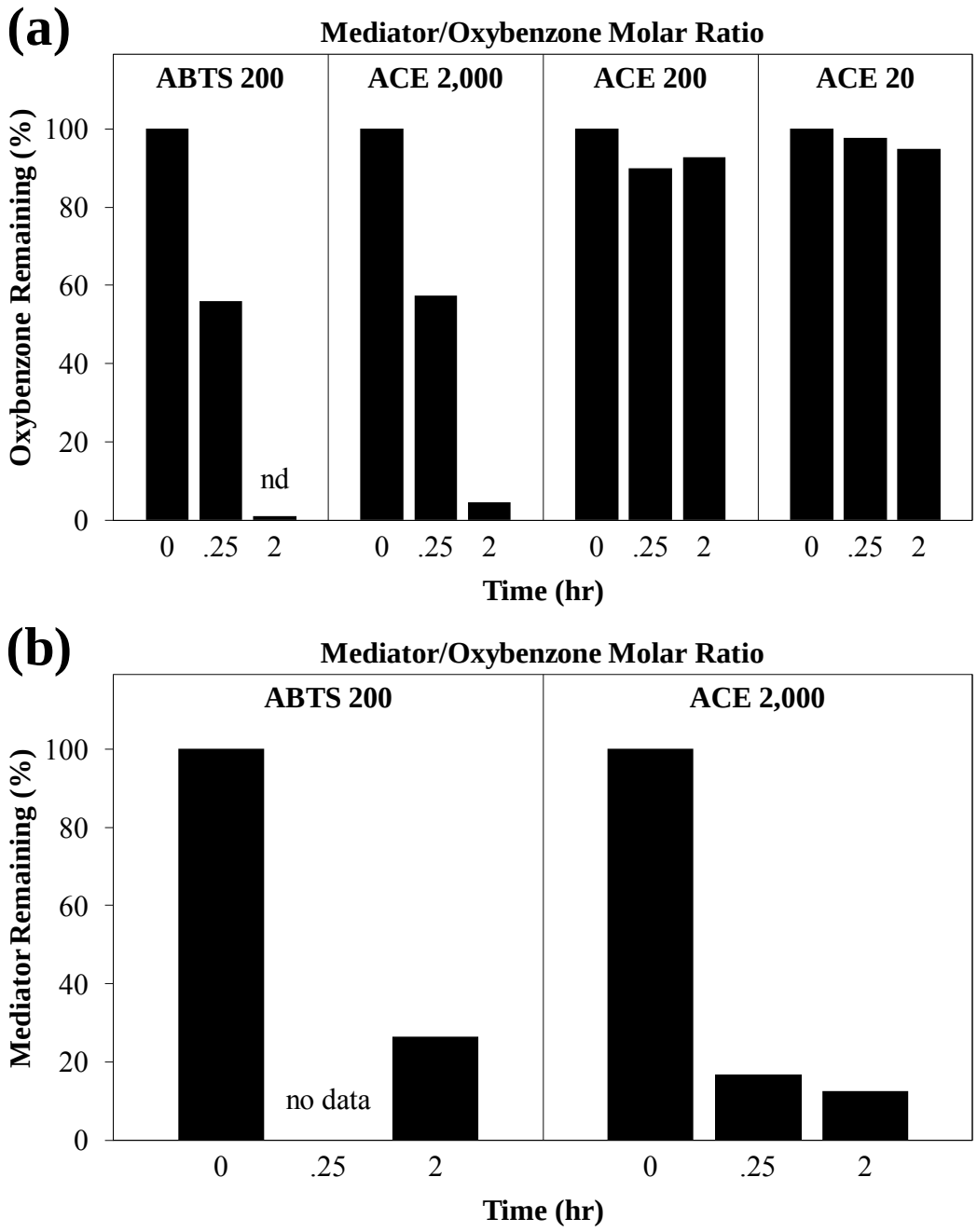


Figure 4.12 – Experiments at environmentally relevant oxybenzone concentration: (a) oxybenzone remaining and (b) mediator remaining in selected experiments. Experiments were performed in filtered primary effluent at 23°C, with an initial oxybenzone concentration of 43.8 nM and an initial laccase activity of 1 U/mL.

The requirement for higher mediator/oxybenzone molar ratios when working at low oxybenzone concentrations could be due to organic compounds in the primary effluent matrix other than oxybenzone reacting with the oxidized mediator, although it was not proven in this research. Laccase is very specific for its substrate (the mediator), but mediators might not necessarily be as specific for their target compound (oxybenzone). Free radicals produced by laccase's oxidation of mediators react non-specifically with compounds through hydrogen abstraction, radical-radical reactions, and electron transfer reactions (Kunamneni et al., 2008b). Therefore, some mediator radicals might be consumed by competing organic components, and this effect might be more pronounced when less oxybenzone is present in the solution.

Mediator concentration was also measured in two of these experiments (ACE/oxybenzone molar ratio of 2,000 and ABTS/oxybenzone molar ratio of 200). The results shown in Figure 4.12b clearly indicate that both mediators are consumed to a significant extent in the enzyme oxidation. Most of the ACE was consumed in the first 15 minutes of reaction. Likewise, 74% of the ABTS was consumed in two hours (perhaps earlier, but no data are available for the sample at 15 minutes). Although we have not yet investigated the oxidation mechanisms and pathways of ABTS and ACE mediators in primary effluent, we hypothesize that the majority of mediator consumption is due to side reactions between mediator radicals and organic constituents present in the primary effluent. The reduction in mediator concentration over time is likely the reason that oxybenzone removal slows down.

Although the laccase-mediator system was able to effectively remove oxybenzone at an environmentally relevant concentration, relatively high mediator concentrations (8.76 μM ABTS and 87.6 μM ACE) were required. The treatment implications of adding high mediator concentrations are discussed later in this chapter.

As observed in Figure 4.13, enzyme activity assays suggest that laccase activities remained approximately unaltered after 2 hours of treatment in the presence of both ABTS and ACE mediators at the different mediator/oxybenzone molar ratios evaluated. The reaction time for these experiments was just 2 hours, instead of the 6 or 48 hours of previous experiments; that is, the enzyme was not exposed long enough to the primary effluent matrix to be destabilized. Moreover, the concentration of mediator evaluated was lower than in previous experiments.

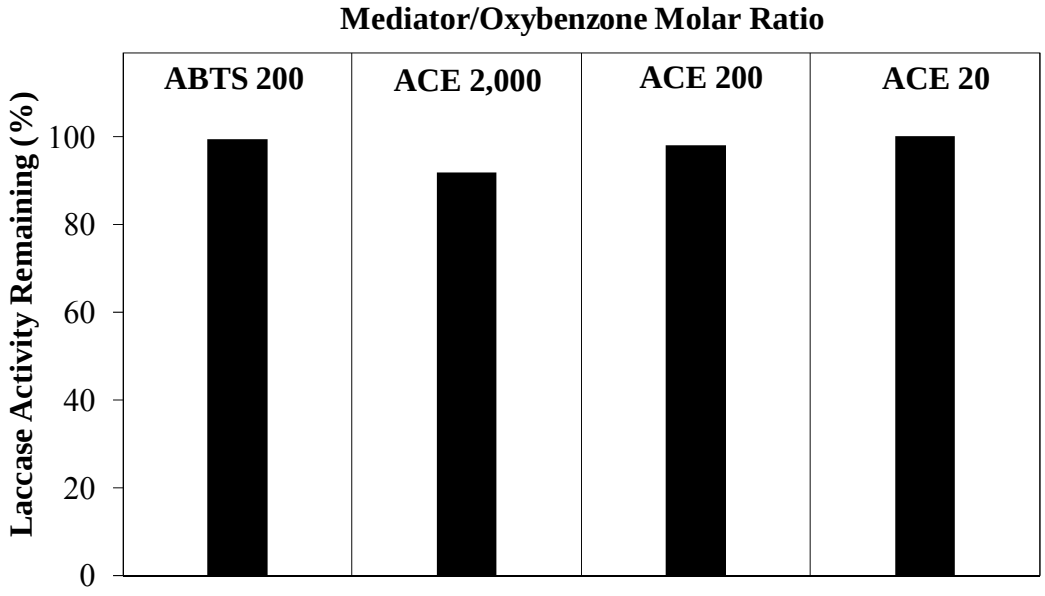


Figure 4.13 – Residual laccase activity after 2 hours of treatment for the mediators ABTS and ACE at the mediator/oxybenzone molar ratios described in the figure.

4.5 CHARACTERIZATION OF ENZYME OXIDATION BYPRODUCTS

Although the laccase mediator system was capable of completely removing oxybenzone, it is important to delineate the major products of this enzyme oxidation. To characterize the oxidation byproducts, experiments with the laccase-ABTS and laccase-ACE systems were performed in a pH 7 buffer solution at the experimental conditions

described in Table 4.6. These experiments do not necessarily reflect the full spectrum of byproducts we would see at an environmentally relevant oxybenzone concentration in a primary effluent matrix. However, qualitative conclusions can be drawn about what types of byproducts can be expected due to mediator-oxybenzone reactions.

Table 4.6 – Experimental conditions for characterization of laccase-mediator oxidation byproducts.

Experimental Conditions		
Oxybenzone Concentration	43.6 μM (10 mg/L) - ABTS experiments 218 μM (50 mg/L) - ACE experiments	
Laccase Activity	1 U/mL (0.7 μM)	
Temperature	23°C	
Medium	Sodium phosphate buffer	
pH	7	
Mediator	*Mediator Concentration (μM)*	*Mediator/Oxybenzone Molar Ratio*
ABTS	100	2.3
ACE	4360	20

The results from the laccase-ABTS experiment clearly indicate the disappearance of oxybenzone, the reduction of the ABTS concentration, and the formation of an enzyme oxidation byproduct that did not exist at time zero. Figure 4.14 shows the chromatograms of the photodiode array detector (PDA) for samples taken prior to the onset of the reaction and for samples taken after 5, 15, 60, 120, and 240 minutes of treatment. Figure 4.15 shows the chromatograms for the same samples, but the chromatograms were obtained using a mass spectrometer detector rather than a PDA detector.

After just 5 minutes of treatment, both chromatograms (Figure 4.14c and Figure 4.15c) show three main peaks: ABTS at a retention time (RT) of 8.01 min, the enzyme

oxidation byproduct at a RT of 9.73 min, and oxybenzone at a RT of 14.24 min. The enzyme oxidation byproduct did not exist at time zero, but it is clearly observed after only 5 minutes of treatment. After 30 minutes of treatment (Figure 4.14e to 4.14h and Figure 4.15e to 4.15h), both chromatograms show the complete disappearance of oxybenzone peak at a RT of 14.24 min, an increase in the response of the enzyme oxidation byproduct (peak at a RT of 9.45 min), and a decrease in the ABTS concentration (peak at a RT of 7.92 min). After 30 minutes of treatment, the oxybenzone peak was no longer present. As the reaction progressed, the response of the oxidation byproduct increased, and the response of ABTS decreased.

The mass-to-charge ratio (m/z) of the different compounds involved in a particular sample can be determined from the mass spectrometer chromatograms. Figure 4.16a shows the PDA and the mass spectrometer chromatograms for a sample taken after 5 minutes of reaction. From the mass spectrometer chromatogram (bottom of Figure 4.16a) the mass-to-charge ratio of the main constituents of the sample was determined. As observed in Figure 4.16b, it was confirmed that the mediator ABTS has a (m/z) value of 514.9, oxybenzone has a (m/z) value of 229, and the major enzyme oxidation byproduct has a (m/z) value of 740.9. Since the molar mass of oxybenzone is 228.24 g/mol, and the molar mass of ABTS is 514.62 g/mol, it is suspected (although not confirmed in our experiments) that the main oxidation byproduct consists of one oxybenzone molecule coupled to one ABTS molecule. A scheme with the proposed mechanism for the formation of the laccase-oxidation byproduct is shown in Figure 4.17. The suspected molecular structure of the laccase-oxidation byproduct is also suggested in Figure 4.17, although it was not confirmed. Nuclear magnetic resonance (NMR) spectroscopy can be performed to confirm the molecular structure of the oxidation byproduct.

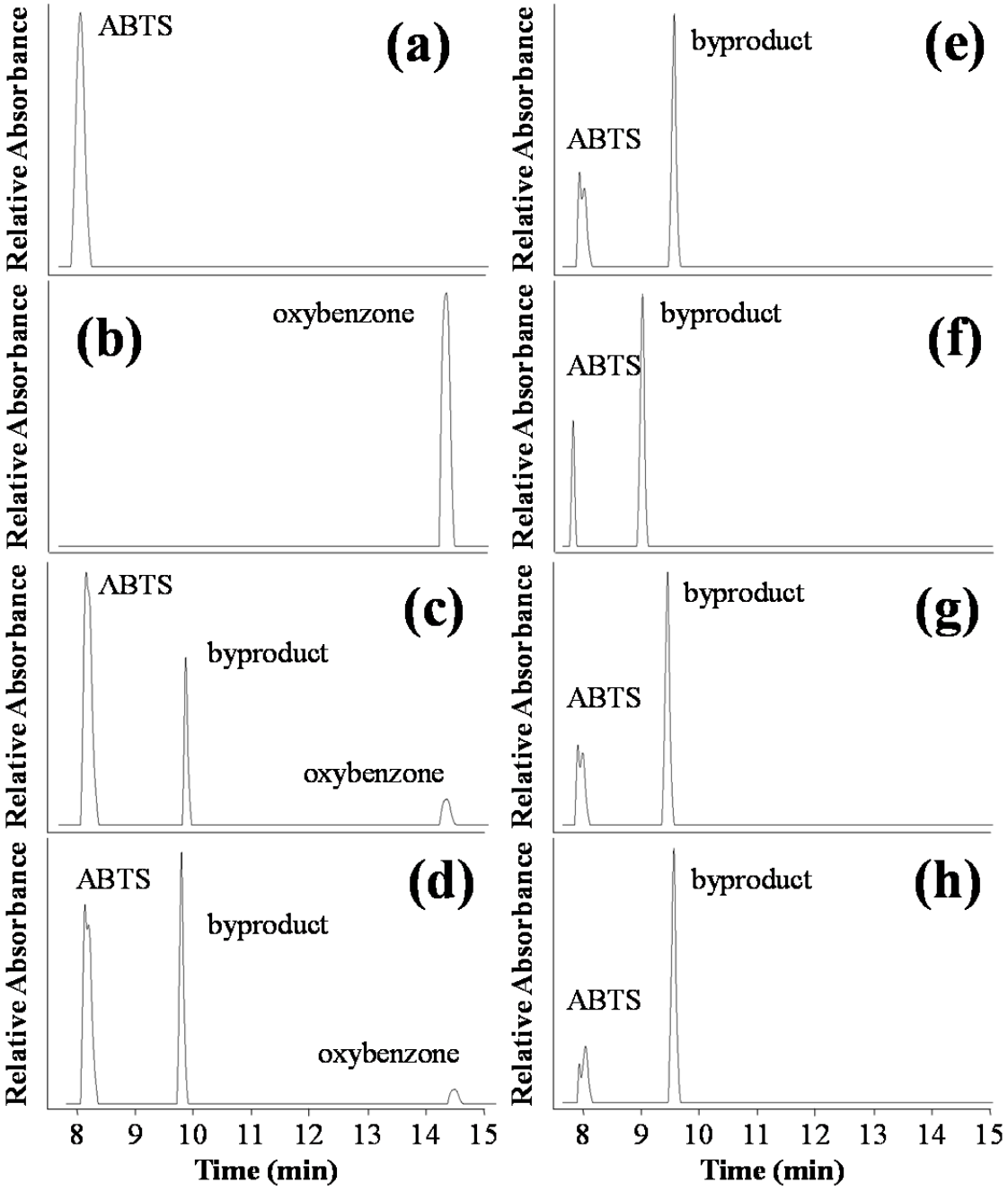


Figure 4.14 – Enzyme oxidation byproduct formation for the laccase-ABTS system: photodiode array detector (PDA) chromatograms for samples taken before starting the reaction (a) ABTS and (b) oxybenzone, and for samples taken (c) after 5 minutes of treatment, (d) 15 minutes of treatment, (e) 30 minutes of treatment, (f) 1 hour of treatment, (g) 2 hours of treatment, and (h) 4 hours of treatment.

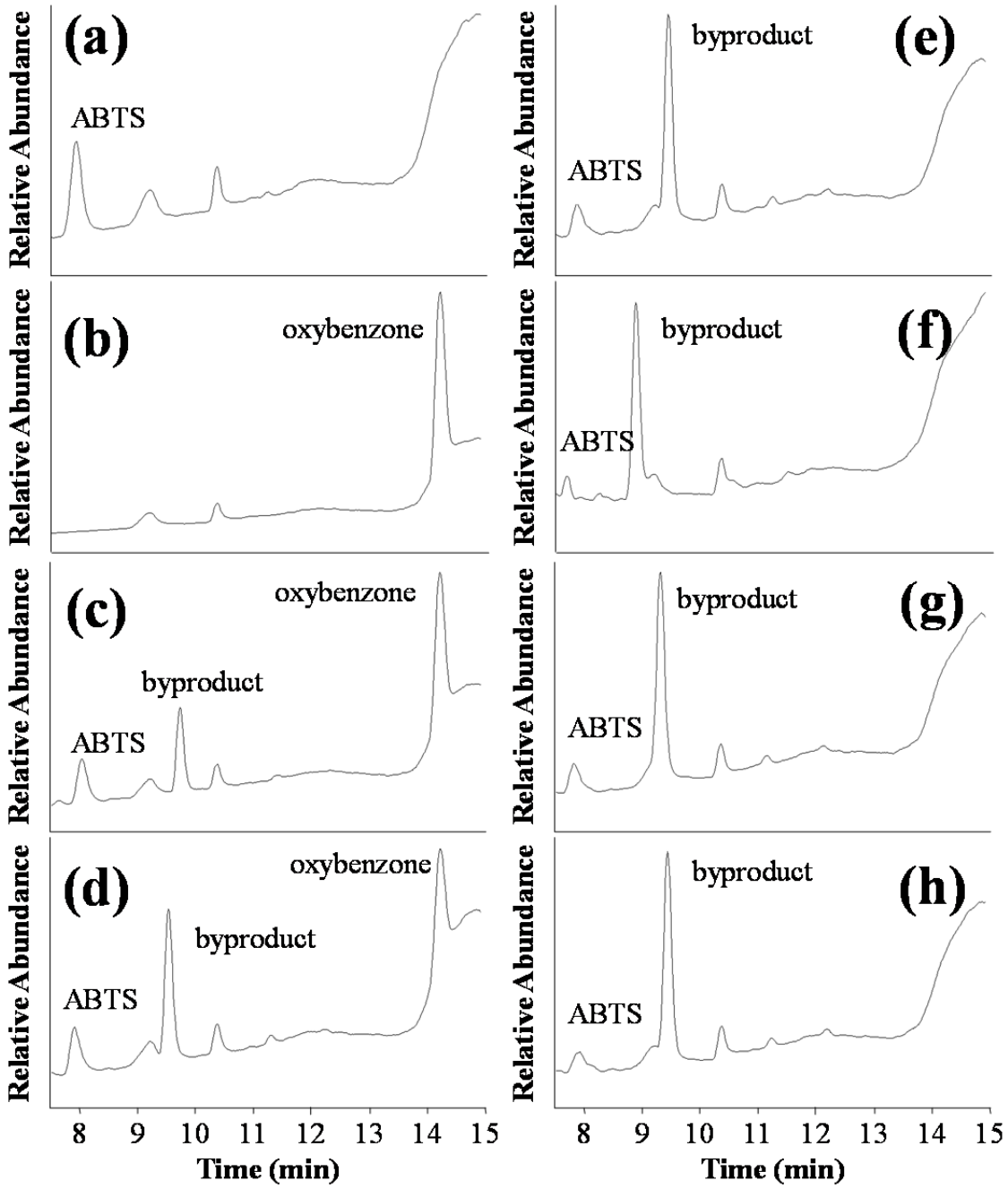


Figure 4.15 – Enzyme oxidation byproduct formation for the laccase-ABTS system: mass spectrometer chromatograms for samples taken before starting the reaction (a) ABTS and (b) oxybenzone, and for samples taken (c) after 5 minutes of treatment, (d) 15 minutes of treatment, (e) 30 minutes of treatment, (f) 1 hour of treatment, (g) 2 hours of treatment, and (h) 4 hours of treatment.

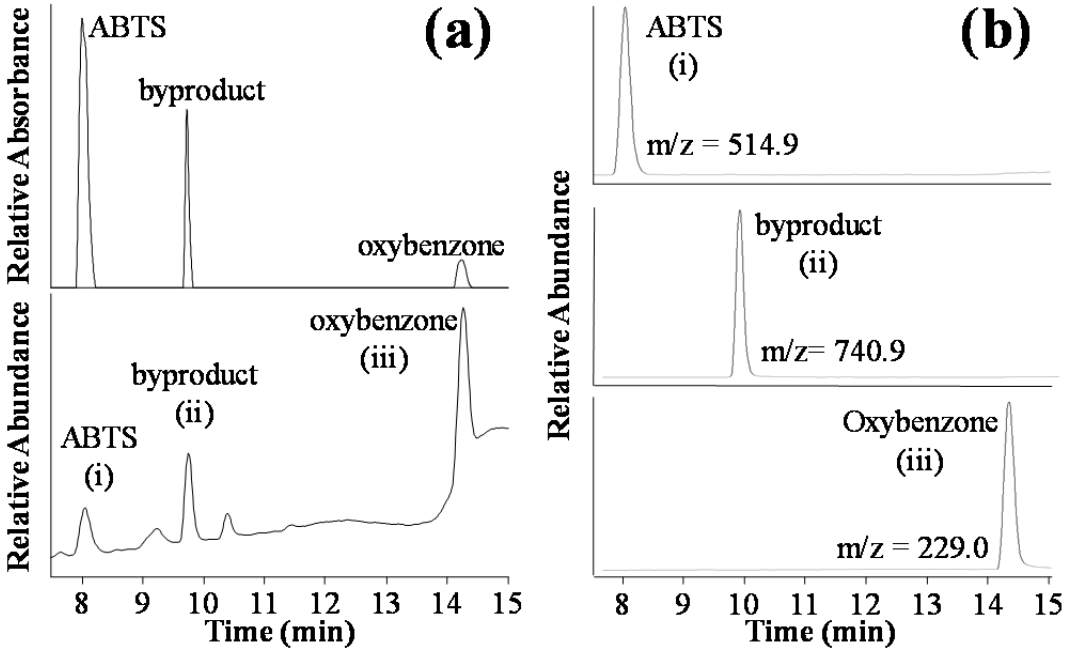


Figure 4.16 –Enzyme oxidation for the laccase-ABTS system: a) chromatograms for samples taken after 5 minutes of treatment (the top chromatogram is from the photodiode array detector, and the bottom chromatogram is from the mass spectrometer detector), and (b) mass spectrometer chromatograms identifying the m/z ratios of the most abundant constituents in the sample.

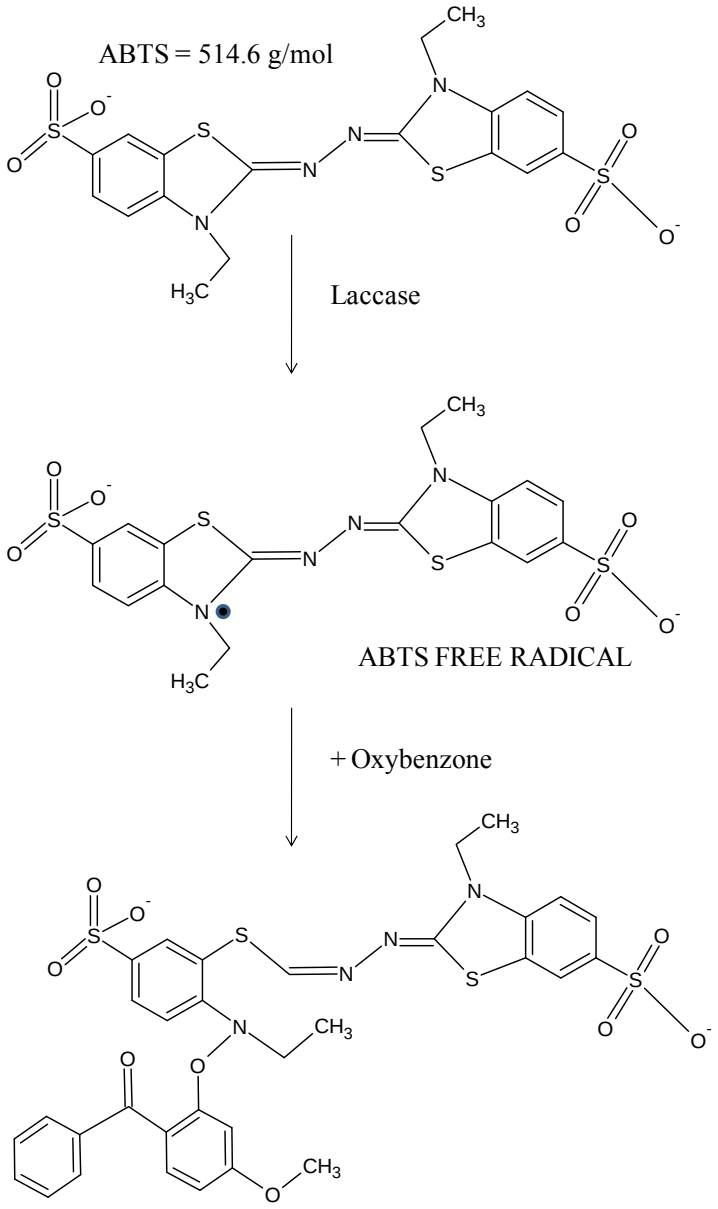


Figure 4.17 – Scheme with the proposed mechanism for the laccase-ABTS oxidation of oxybenzone. The molecular structure for the major laccase oxidation byproduct with a molar mass of 740.8 g/mol is suggested.

The response using the PDA detector for every compound (area of the peak) at the different reaction times was computed from the PDA chromatograms. As observed in

Figure 4.18, the responses of ABTS and oxybenzone decreased as a function of time while the response of the byproduct increased. The response of ABTS decreased until complete disappearance of oxybenzone was observed. After that point, the response of ABTS reached a constant value. A similar trend was observed for the oxidation byproduct. The response of the byproduct increased until oxybenzone was completely consumed. After that point, the response of the byproduct reached a constant value. The disappearance of the target compound oxybenzone is strongly related to the consumption of the mediator ABTS and to the formation of the oxidation byproduct. These results confirmed the previous suspicion that in fact the produced byproduct consists of one oxybenzone molecule coupled to one ABTS molecule. The oxidation of the target compound might happen through the formation of the mediator radical and the later coupling of the radical to the target compound to form a stable oxidation byproduct.

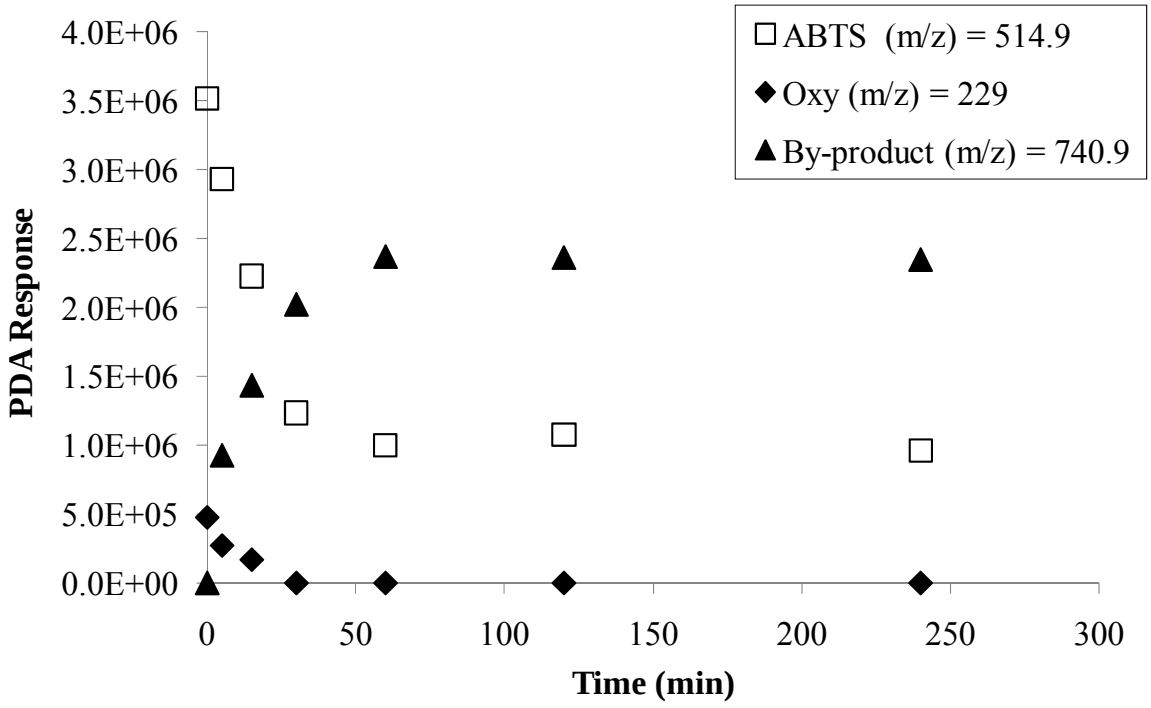


Figure 4.18 – Computed PDA responses from the PDA chromatograms for ABTS, oxybenzone and the oxidation byproduct.

The results from the laccase-ACE experiment were quite different from those with ABTS and clearly indicate the formation of several enzyme oxidation byproducts that did not exist at time zero. Figure 4.19 shows the chromatograms of the PDA detector for samples taken before the enzyme addition (Figure 4.19a) and for samples taken after 15, 30, 60, 120, and 360 minutes of treatment (Figure 4.19b to 4.19f). Figure 4.20 shows the results for the same samples, but the chromatograms were obtained using a mass spectrometer detector rather than a PDA detector.

At time zero (Figure 4.19a and 4.20a), both chromatograms show two main peaks: ACE at a RT of approximately 10.26 min and oxybenzone at a RT of approximately 14.35 min. After 15 minutes of treatment (Figure 4.19b to 4.19f and Figures 4.20b to 4.20f), both chromatograms show a decrease in the oxybenzone

concentration, a decrease in the ACE concentration, and the formation of several enzyme oxidation byproducts at retention times between 9 and 14 min. As the reaction progressed, the responses of the oxidation byproducts generally increased, and the responses of oxybenzone and ACE decreased.

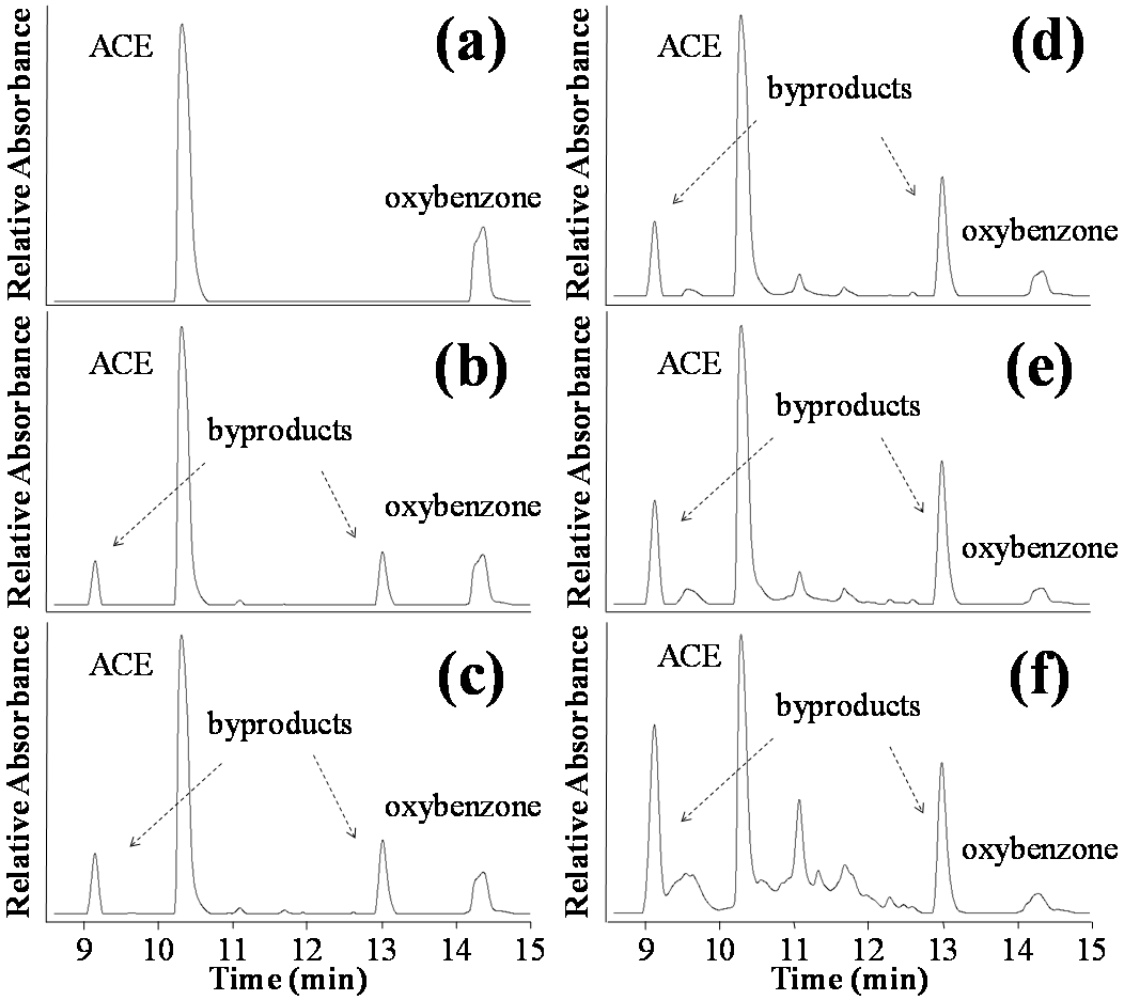


Figure 4.19 – Enzyme oxidation byproduct formation for the laccase-ACE system: photodiode array detector (PDA) chromatograms for samples taken (a) before the enzyme addition, and for samples taken (b) after 15 minutes of treatment, (c) 30 minutes of treatment, (d) 1 hour of treatment, (e) 2 hours of treatment, and (f) 6 hours of treatment.

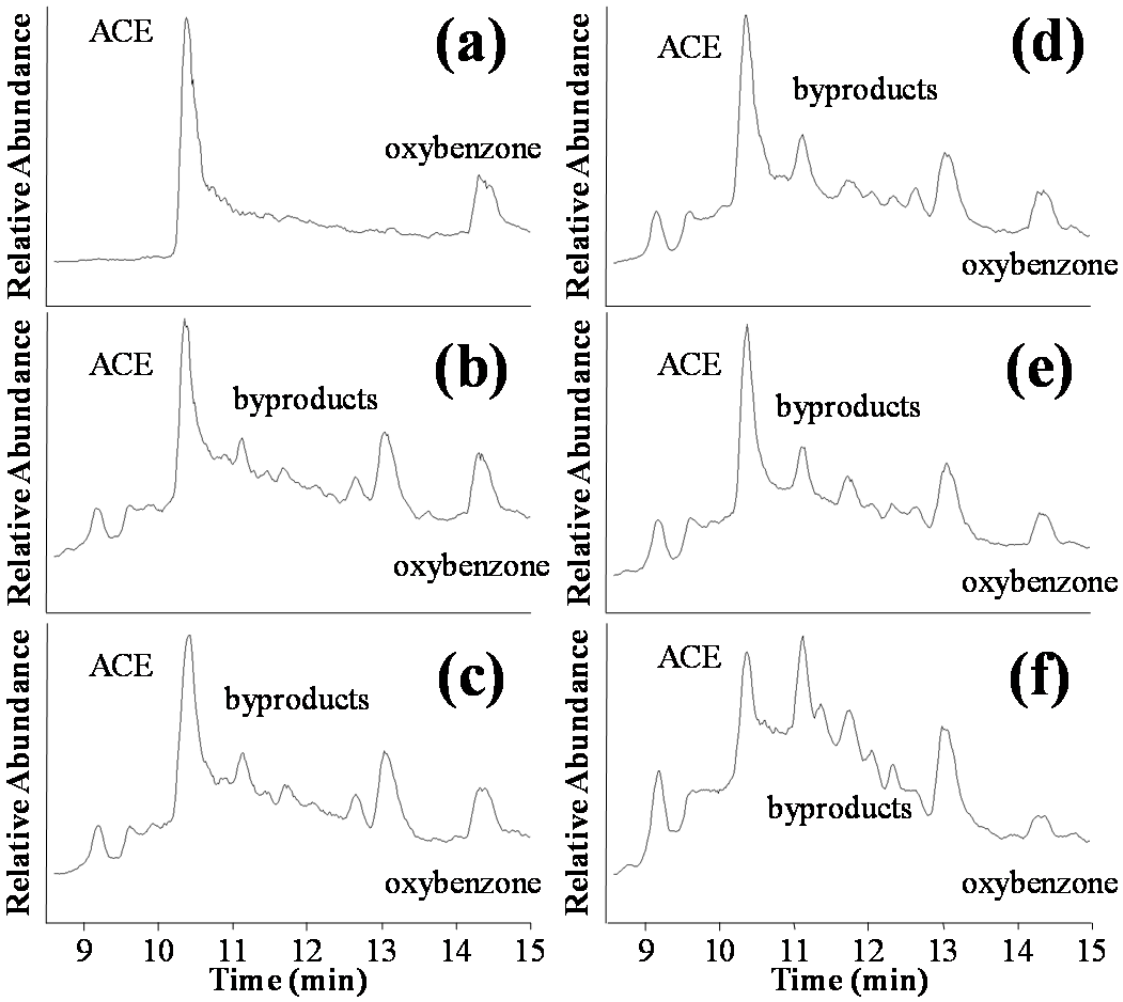


Figure 4.20 – Enzyme oxidation byproduct formation for the laccase-ACE system: mass spectrometer chromatograms for samples taken (a) before the enzyme addition, and for samples taken (b) after 15 minutes of treatment, (c) 30 minutes of treatment, (d) 1 hour of treatment, (e) 2 hours of treatment, and (f) 6 hours of treatment.

The response from the PDA detector for oxybenzone and ACE at the different reaction times was computed from the PDA chromatograms, and the results are shown in Figure 4.21. The response of the mediator ACE and of the target compound oxybenzone decreased as a function of time. As observed in the laccase-ABTS system, the disappearance of the target compound oxybenzone in the laccase-ACE system was

related to the disappearance of the mediator ACE. That is, for the laccase-ACE system, it can also be inferred that the oxidation of the target compound occurs through the previous formation of the mediator radical and the later oxidation (coupling) of the target compound by the oxidized form of the mediator.

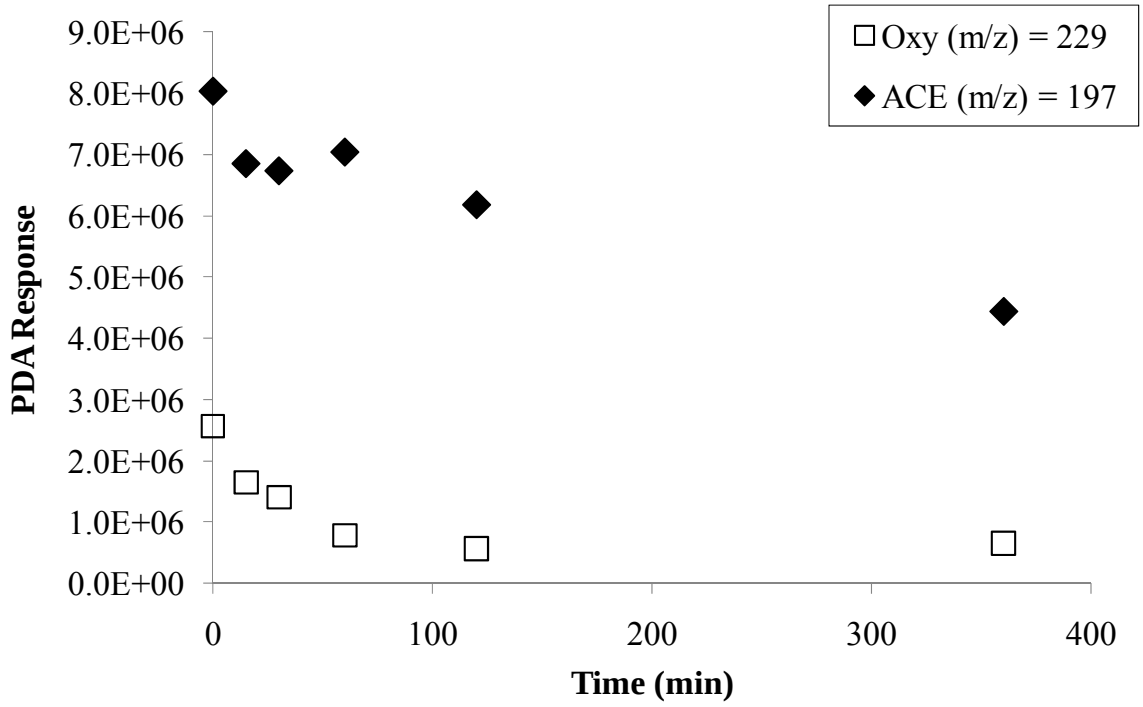


Figure 4.21 – Computed PDA responses from PDA chromatograms for ACE and oxybenzone.

The laccase-ACE system produces several more byproducts than the laccase-ABTS system. From the mass spectrometer chromatograms, (bottom of Figure 4.22a), the m/z ratios of the most abundant oxidation byproducts were determined, and are shown in Figure 4.22b. At least twelve major byproducts were noted. The different behavior of the two mediators evaluated (ACE and ABTS) can be explained considering the chemical characteristics of the two mediators. Laccase oxidation of ABTS produces

very stable radicals (Johannes and Majcherczyk, 2000a; Majcherczyk et al., 1999) which are not able to couple with each other probably due to steric effects. On the other hand laccase oxidation of phenolic compounds such as ACE produces different resonance forms of the phenoxyl radicals (Caldwell and Steelink, 1969) which might be not as stable as the ABTS radical, and might decay by coupling to each other (Valoti et al., 1989) and to other compounds forming a variety of oxidation byproducts (as observed in Figure 4.22b).

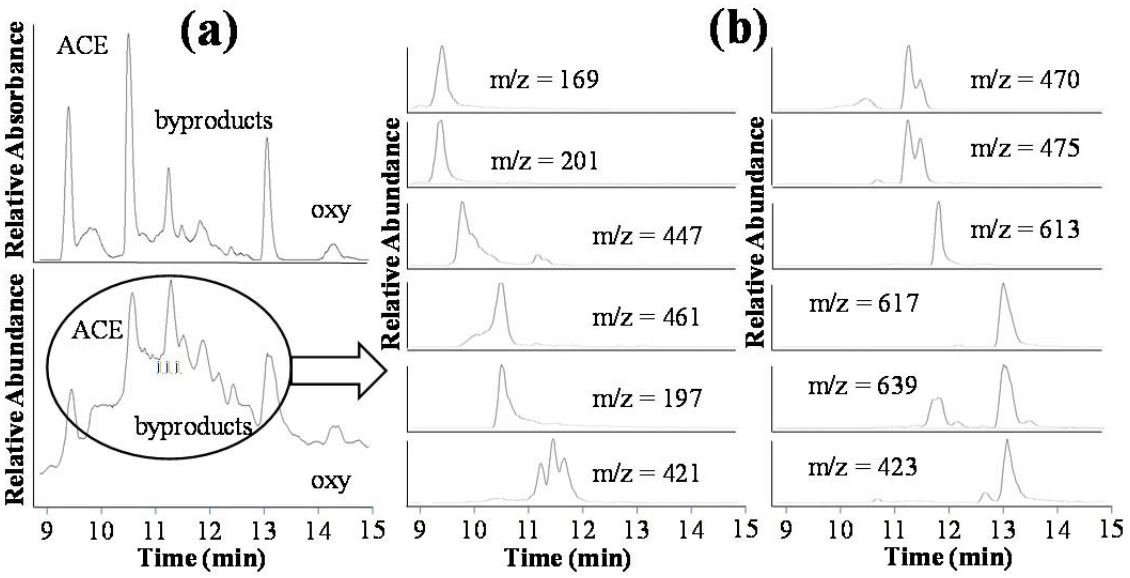


Figure 4.22 – Enzyme oxidation byproduct formation for the laccase-ACE system: a) chromatograms for samples taken after 6 hours of treatment (the top chromatogram is from the photodiode array detector, and the bottom chromatogram is from the mass spectrometer detector), and (b) mass spectrometer chromatograms identifying the m/z ratios of the most abundant constituents in the sample.

Figure 4.23 displays the responses of the major oxidation byproducts over the course of the experiment. The mass spectrometer chromatograms were used to compute the responses of the identified byproducts. The PDA chromatograms could not be used

as in the laccase-ABTS system due to the difficulties for isolating the byproducts using the PDA detector. The instrument responses of most of the byproducts increased as the reaction progressed; however, the responses of a few byproducts did eventually start to stabilize or decrease.

Far more oxidation byproducts were identified in the presence of ACE mediator than in the presence of ABTS mediator. Most of these detected byproducts have m/z ratios higher than the m/z ratio of oxybenzone (229). For a wastewater treatment application, producing high molar mass byproducts would be acceptable, since they should be more hydrophobic and more easily removed from the aqueous phase by precipitation followed by sedimentation or filtration (Huang et al., 2005), or by physical adsorption to other constituents in the wastewater, such as biomass. However, the laccase-oxidation byproducts would still be present in the waste sludge in this case. Consequently, the oxidation byproducts might be observed in the water released during sludge dewatering or remain in the digested sludge. In either case, the fate and toxicity of byproducts would need to be further examined. Mineralization of laccase oxidation byproducts to carbon dioxide and water was demonstrated by Bumpus et al. (1985) and by Nakamura and Mtui (2003). Therefore, laccase oxidation byproducts present in the waste sludge might be eventually biodegraded in the digestion process.

Since the molar mass of oxybenzone is 228.24 g/mol, and the molar mass of ACE is 196.20 g/mol, it is suspected (although not confirmed in our experiments) that most of these oxidation byproducts consist of oxybenzone molecules coupled to different oxidation forms of ACE molecules. The byproduct with a m/z ratio of 169 is an exception, as it seems to be an oxidized form of ACE. Enzyme-catalyzed oxidative coupling of phenolic compounds has been reported by several authors (Weber and Huang, 2003; Kunamneni et al., 2008a). These authors suggested that laccase oxidizes

organic substrates to free radicals, which undergo oxidative coupling reactions, producing dimers, oligomers, and polymers.

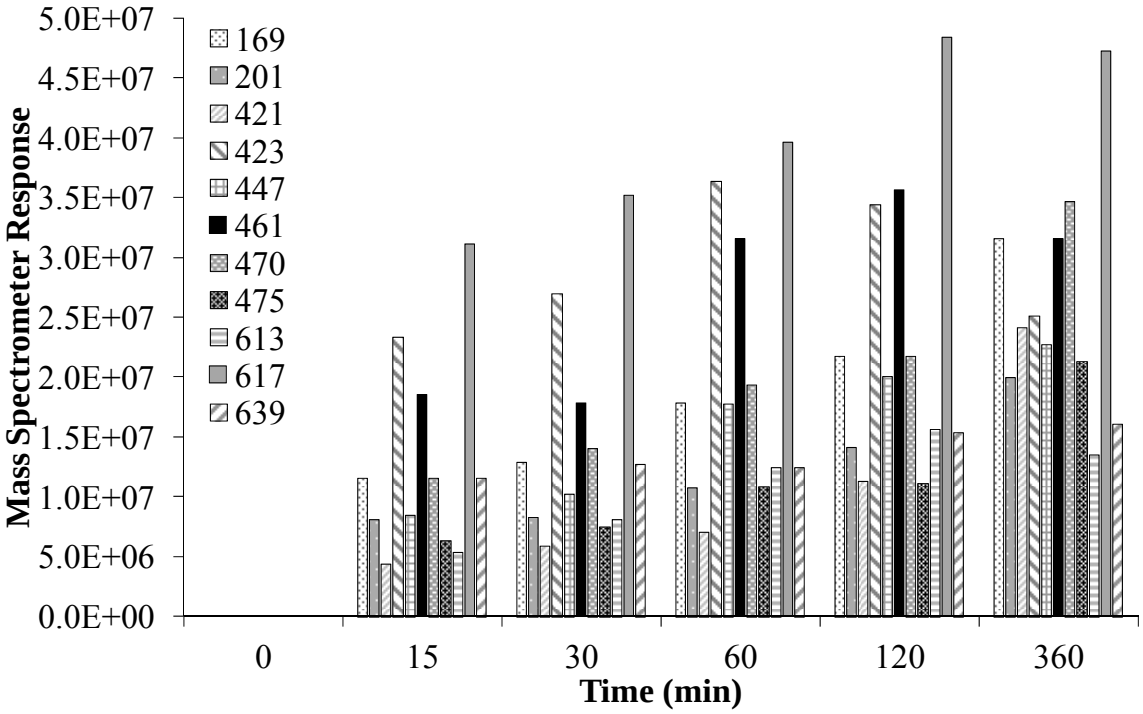


Figure 4.23 – Enzyme oxidation byproduct formation for the laccase-ACE system: mass spectrometer responses of the most abundant byproducts detected as a function of time. The m/z ratios of the byproducts represented are shown in the legend, and from top to bottom, they correspond to the bars from left to right.

In this research, formation of byproducts via laccase oxidation of just one personal care product, oxybenzone, was evaluated for two mediators - ABTS and ACE. The oxidation byproducts had higher molecular weights than that of oxybenzone, indicating oxidative coupling reactions between the mediator and the target compounds. Byproduct characterization of other PPCPs oxidized by laccase with and without the

addition of mediators has been investigated by several researchers. Hata et al. (2010) investigated byproduct formation from the laccase-oxidation of carbamazepine using the mediator HBT. At least four oxidation byproducts were observed, with two of them having larger molecular weights than the parent compounds, indicating cross-coupling reactions between HBT and carbamazepine. Lu et al. (2009) evaluated byproduct formation from the laccase-oxidation of acetaminophen without the addition of mediators. Byproducts with larger molecular weights than the parent compounds were observed, indicating oxidative radical-radical coupling reactions. Huang and Weber (2005) also observed the formation of oxidative coupling byproducts with larger molecular weights than the parent compound for the laccase oxidation of bisphenol A without the use of mediators. Weber and Huang (2003) observed the formation of cross coupling oxidative products from the peroxidase oxidation of phenanthrene with the addition of phenol as mediator. Larger molecular weight oxidation byproducts were produced.

Laccase-oxidation of target PPCPs (with and without the use of mediators) occurs through cross-coupling radical reactions producing oxidation byproducts with larger molecular weights. Bumpus et al. (1985) and Nakamura and Mtui (2003) demonstrated that laccase-oxidation byproducts can be successfully biodegraded to carbon dioxide and water. Thus, if the laccase oxidation system is applied upstream of secondary treatment in a municipal wastewater treatment plant, laccase-oxidation byproducts eventually might be biodegraded in the activated sludge process. Since most of the oxidation byproducts consist of large molecules with high molecular weights, an important fraction of these byproducts might eventually be adsorbed onto the activated sludge or precipitate out from the aqueous phase. Therefore, some oxidation byproducts might still be present in the waste sludge in which case they might eventually be released by during sludge

dewatering, sewage remain in the digested sludge, or eventually be biodegraded in aerobic or anaerobic digesters.

4.6 CHARACTERIZATION OF OZONATION BYPRODUCTS

In the previous section it was demonstrated that laccase-oxidation generates oxidation byproducts. Removal of emerging contaminants such as oxybenzone is commonly achieved by an advanced oxidation process (*i.e.,* one that involves the generation of hydroxyl radicals). Ozonation is one of the most viable treatment alternatives for emerging contaminants; however ozonation generates oxidation byproducts with unknown identities, toxicity, and biodegradability (Ikehata et al., 2008). The objective of this section is to demonstrate that ozonation, one of the most common treatment alternatives for removing PPCPs, generates several oxidation byproducts, when applied to oxybenzone. Experiments were performed at different initial ozone/oxybenzone molar ratios ranging from 0 to approximately 10 in distilled water solutions. A more detailed explanation of the experimental conditions is described in Section 3.3.6.

The byproducts of oxybenzone ozonation displayed in Figure 4.24 and Figure 4.25 provide a useful comparison with the byproducts generated from enzyme oxidation shown above. Figure 4.24 and Figure 4.25 show the chromatograms of the PDA and mass spectrometer detectors, respectively. As shown in Figures 4.24a and 4.25a, before the addition of ozone, (initial ozone/oxybenzone molar ratio of 0), only oxybenzone was present (peak at a RT of 22 min). After the addition of ozone at an initial ozone/oxybenzone molar ratio as low as 1.91 and sufficient time for all of the ozone to be dissipated, both detectors clearly indicate the formation of several byproducts (Figures 4.24b and 4.25b). As the initial ozone/oxybenzone molar ratio increased from 1.91 to

9.54 (Figures 4.24c to 4.24f and Figures 4.25c to 4.25f), the concentration of oxybenzone decreased (peak at a RT of 22 min) with the consequent formation of ozonation byproducts with retention times between 13 and 22 min. Even at the highest ozone dose applied (initial ozone/oxybenzone molar ratio of 9.54) oxybenzone was still observed in the mass spectrometer chromatogram (Figure 4.25f), and several ozonation byproducts were still observed in the PDA and mass spectrometer chromatograms (Figures 4.24f and 4.25f). That is, even working at relatively high ozone doses, full mineralization of the target compound was not achieved and oxidation byproducts persisted.

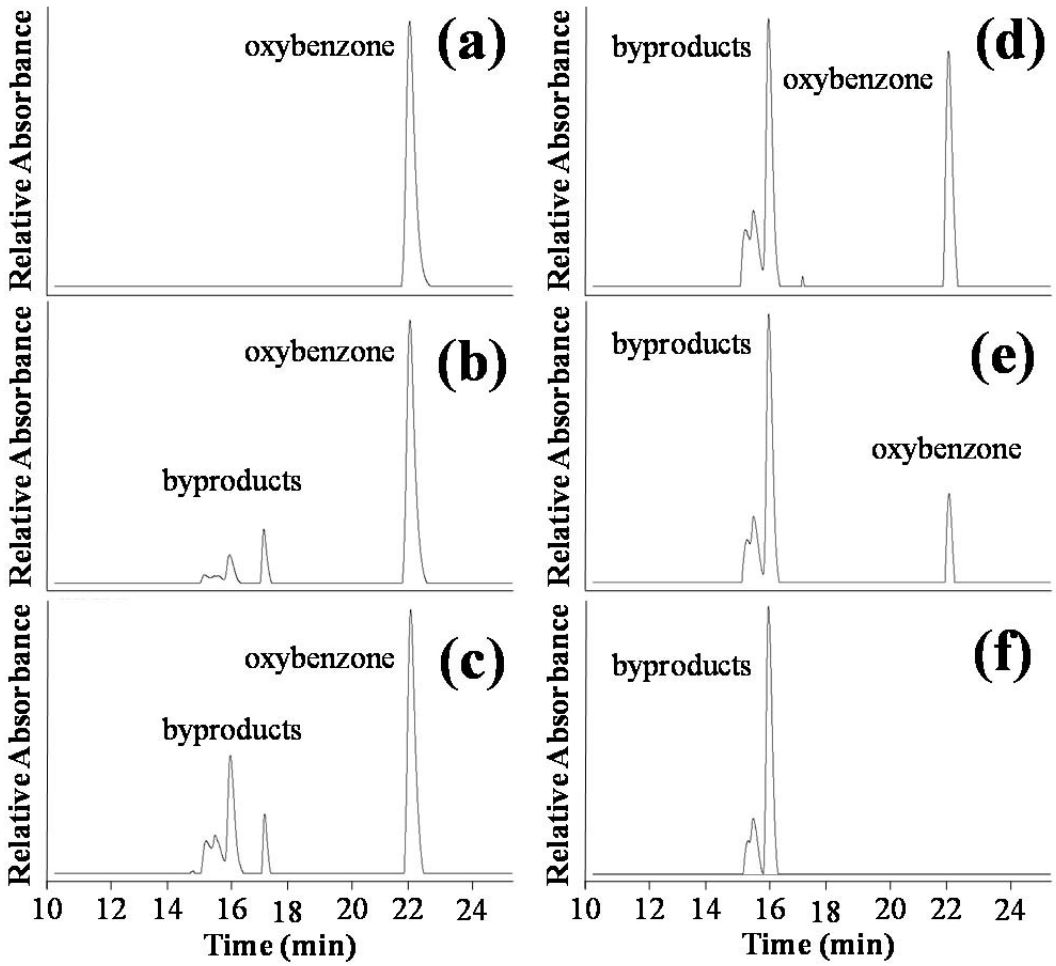


Figure 4.24 – Ozonation byproduct formation: PDA chromatograms for samples with (a) no ozone addition, (b) initial ozone/oxybenzone molar ratio of 1.91, (c) initial ozone/oxybenzone molar ratio of 3.82, (d) initial ozone/oxybenzone molar ratio of 5.73, (e) initial ozone/oxybenzone molar ratio of 7.63, (f) initial ozone/oxybenzone molar ratio of 9.54.

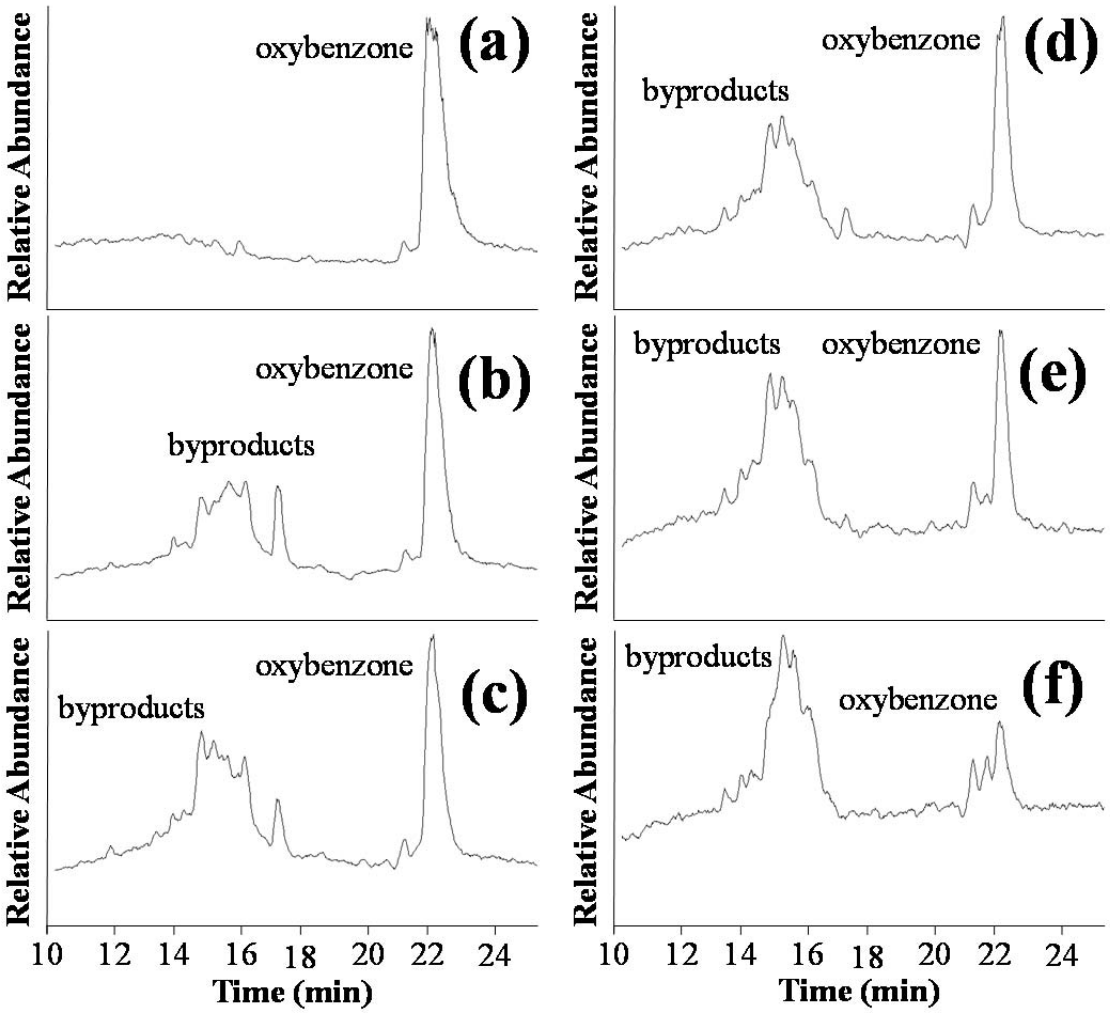


Figure 4.25 – Ozonation byproduct formation: mass spectrometer chromatograms for samples with (a) no ozone addition, (b) initial ozone/oxybenzone molar ratio of 1.91, (c) initial ozone/oxybenzone molar ratio of 3.82, (d) initial ozone/oxybenzone molar ratio of 5.73, (e) initial ozone/oxybenzone molar ratio of 7.63, (f) initial ozone/oxybenzone molar ratio of 9.54.

Based on the mass spectrometer chromatogram, (bottom of Figure 4.26a) the m/z ratios of the most abundant ozonation byproducts were determined, and the eight most common byproducts are shown in Figure 4.26b. Ozonation byproducts with the following m/z ratios were characterized: 193.1, 203, 207, 209, 223, 233, 237, 245, 249,

251, 265, 277, 291, 305, and 307. Therefore, although ozonation was able to almost completely remove oxybenzone, at least 15 oxidation byproducts were produced.

The m/z ratio distribution of the ozonation byproducts is different from that of the enzyme oxidation byproducts. Byproducts with higher m/z ratios were produced in the enzymatic treatment, likely due to oxidative coupling reactions between free radicals generated by laccase oxidizing the mediator and oxybenzone. Byproducts from ozonation had lower m/z ratios with values very similar to the molar mass of the parent compound.

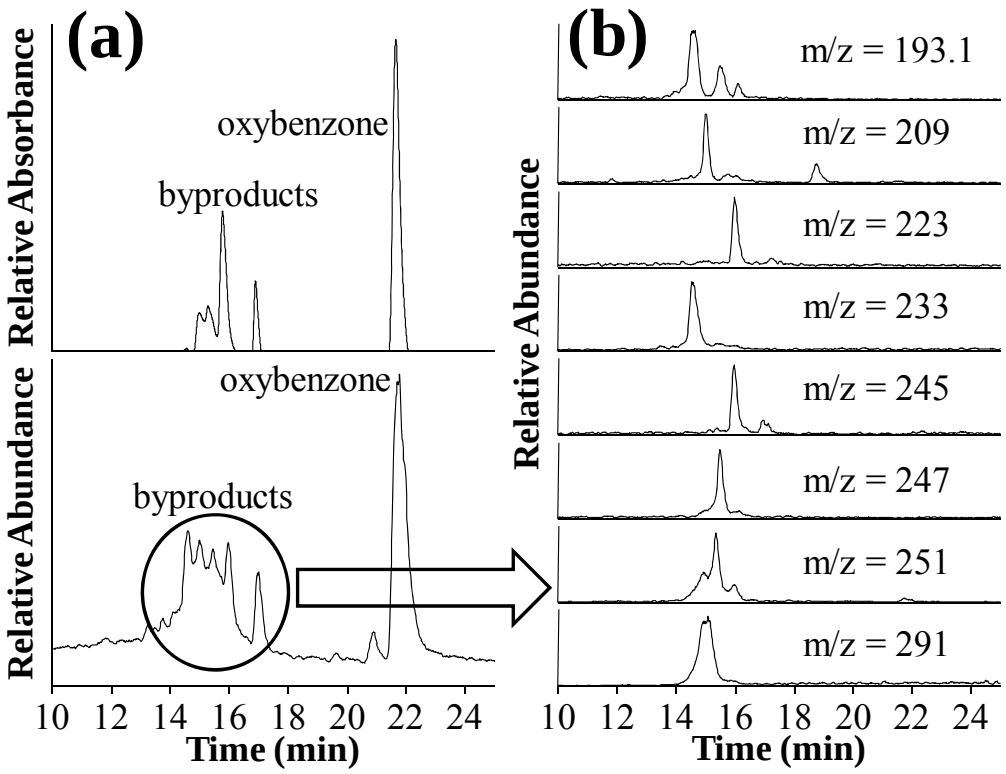


Figure 4.26 – Ozonation byproduct formation: chromatograms for samples with (a) an initial ozone/oxybenzone molar ratio of 3.82 (the top chromatogram is from the photodiode array detector, and the bottom chromatogram is from the mass spectrometer detector). (b) shows chromatograms for specific ranges of m/z ratios, capturing the eight most abundant byproducts.

4. 7 LACCASE OXIDATION OF MEDIATORS: MECHANSIMS AND KINETICS

Previous results demonstrated that laccase cannot directly oxidize a representative PPCP such as oxybenzone. The addition of low molecular weight mediators (either phenolic or non-phenolic compounds) expanded the oxidation ability of laccase through the formation of mediator free radicals. The oxidation of oxybenzone was achieved by using two different mediators: ABTS (a non-phenolic synthetic mediator), and ACE (a phenolic natural mediator). The use of a mediator was required to achieve the ultimate goal of removing the target compound. Due to the chemical diversity of PPCPs, it is thought that several PPCPs would require the addition of mediators to be oxidized successfully. That is, mediators play a fundamental role in this process. Consequently, it is essential to understand the mechanism of the laccase-oxidation of mediators. Moreover, an understanding of the mediator's oxidation kinetics is desirable to be able to predict the rate of formation of the mediator free radicals at different conditions of enzyme activity, mediator initial concentration, and solution composition. A better understanding of both the laccase-oxidation mechanism of mediators, and the mediator oxidation kinetics would allow making improvements in the design of the laccase-mediator system for the removal of emerging contaminants such as PPCPs.

4. 7.1 Laccase Oxidation of ACE: Mechansim

The goal of these experiments was to evaluate the laccase-oxidation of the mediator ACE to better understand the mechanisms of ACE oxidation. Several experiments were performed at different initial ACE concentrations and laccase activities. Changes in the reaction mixture were monitored by scanning the UV and visible spectrum using a UV-visible spectrophotometer, and by analyzing some samples using liquid chromatography/mass spectroscopy.

The first experiment (Experiment #1) was performed at a relatively high laccase activity of 99 U/mL and at a relatively high mediator concentration of 8.97 mM in a pH 5 sodium acetate buffered solution. The goal of this experiment was to relate the rate of formation of a specific oxidation product to the rate of disappearance of the mediator ACE. Based on preliminary results, it was suspected that the selected experimental conditions promote ACE laccase-oxidation reactions that can be monitored in reaction times of a few hours, at concentrations that can be simply detected by the analytical instruments involved. Prior to the onset of the reaction, the spectrum of ACE was evaluated as shown in Figure 4.27. ACE absorbs UV radiation with an absorption maximum at a wavelength of approximately 290 nm. After the enzyme addition, the formation of a red soluble product in the reaction mixture was observed. The intensity of the red color increased as the reaction progressed. Analyzing the UV-visible spectrum of the samples after the laccase addition, an absorption maximum in the visible light range at a wavelength of approximately 520 nm was observed. The complementary color to the wavelength absorbed (520 nm) is red. That is, the laccase oxidation of ACE produced a characteristic oxidation product that absorbs visible light at a wavelength of 520 nm. After the laccase addition, the reaction mixture was monitored at approximately 10-minute intervals for a total reaction time of 160 minutes. Changes in the visible spectrum are described in Figure 4.28. The absorbance of the reaction mixture at 520 nm was recorded as a function of time as described in Figure 4.29. The formation of the red oxidation product increased until reaching a constant value after approximately 150 min of treatment.

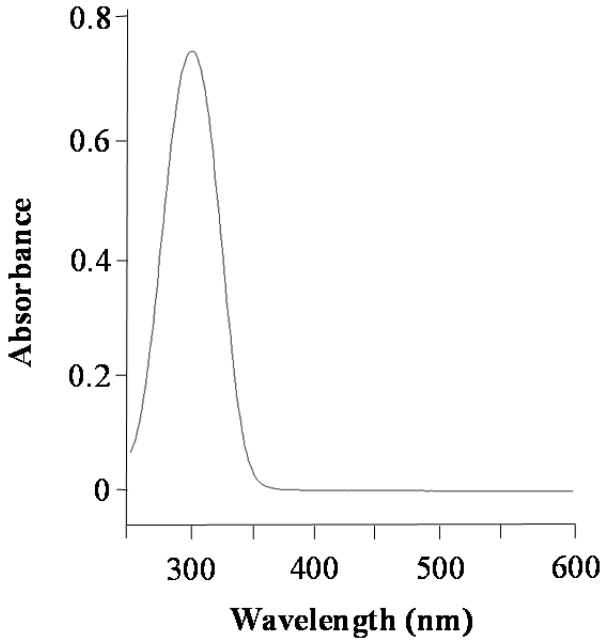


Figure 4.27 – UV-Visible absorption spectrum of the mediator ACE

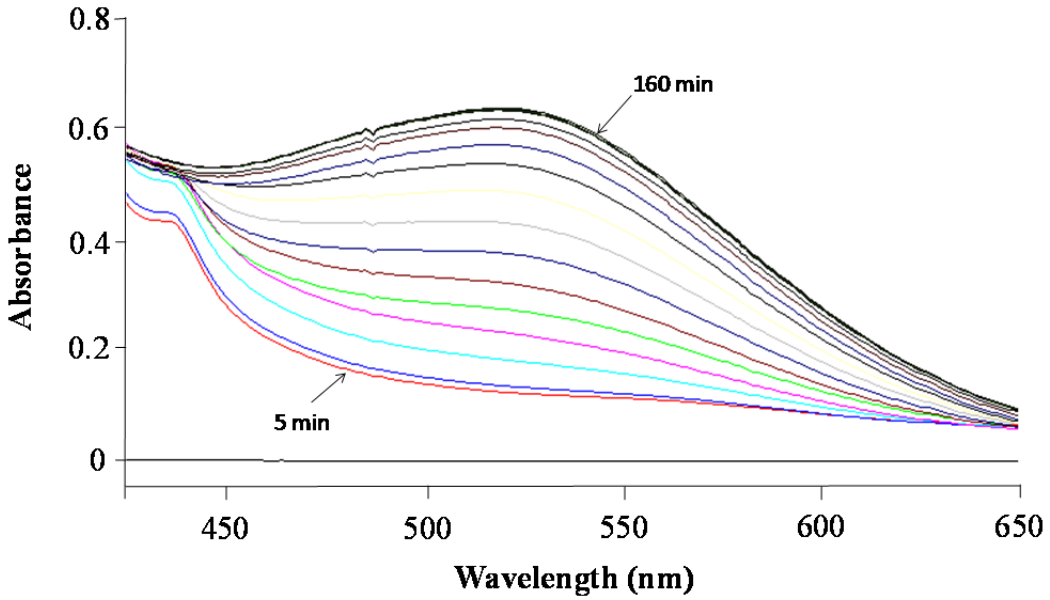


Figure 4.28 – Visible absorption spectrum for the oxidation of ACE as a function of reaction time for samples taken between 5 and 160 minutes of treatment

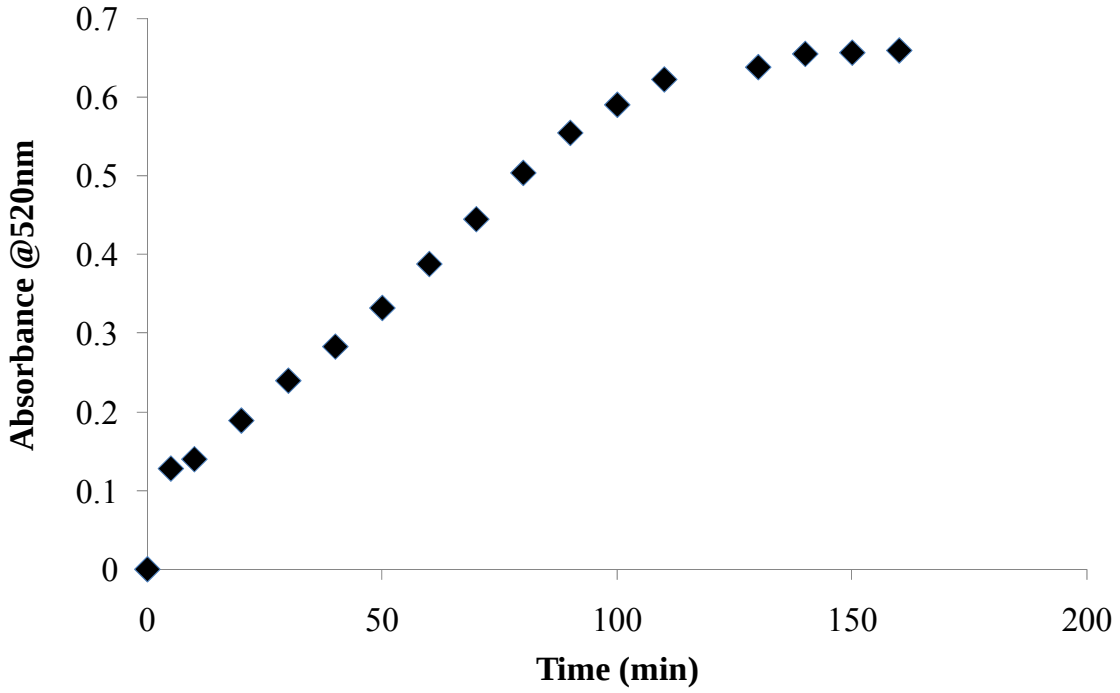


Figure 4.29 – Changes in the absorbance of ACE measured at 520 nm as a function of the reaction time for samples taken between 5 and 160 minutes.

At the experimental conditions evaluated, the complete oxidation (transformation) of ACE to the observed final product occurred in approximately 150 minutes. Based on the research performed to date, it was suspected that the rate of disappearance of the mediator ACE was the same as the rate of formation of the oxidation product. That is, the rate of ACE disappearance might be inferred from the rate of formation of this oxidation product.

Two other experiments (Experiment #2 and Experiment #3) were performed to confirm or refute the direct relation between the disappearance of the mediator ACE and the formation of the product that absorbs visible light at 520 nm. The first experiment (Experiment #2) was performed at an initial ACE concentration of 0.8243 mM and an initial laccase activity of 11.75 U/mL in a pH 5 sodium acetate buffered solution. The

second experiment (Experiment #3) was performed at an initial ACE concentration of 0.94 mM, and at an initial laccase activity of 1.188 U/mL in a pH 5 sodium acetate buffered solution. The absorbance of the reaction mixture at 520 nm was monitored for approximately 2 hours for both experiments. If the mediator ACE is directly converted to the red oxidation product that absorbs at 520 nm, the rate of formation of the oxidation product should be comparatively proportional to the laccase activity. The experimental results observed in Figure 4.30 indicated that the rates of formation of the oxidation product were very similar for the two experimental conditions investigated, despite the differences in the initial laccase activities (1 U/mL and 10 U/mL). Therefore, the rate of disappearance of the mediator ACE by laccase cannot be directly related to the rate of formation of the red oxidation product that absorbs visible light at 520 nm.

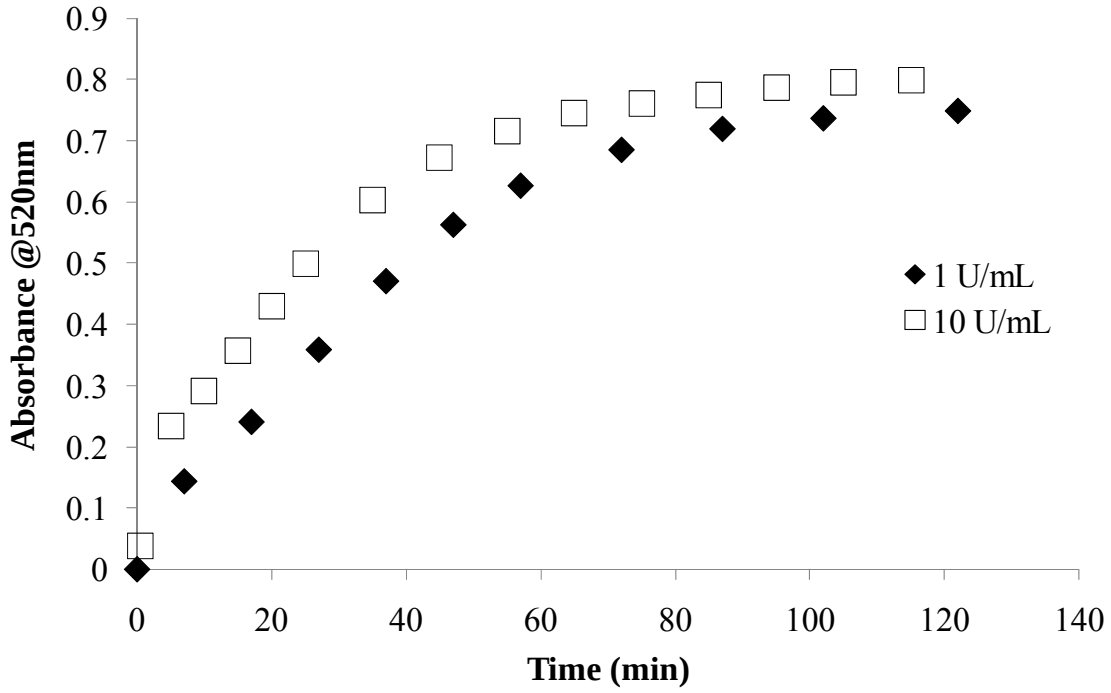


Figure 4.30 – Changes in the absorbance of the mediator ACE at 520 nm as a function of the reaction time for experiments performed at an initial laccase activity of 1 U/mL (♦) and 10 U/mL (□).

The UV and visible spectrum of the two experimental conditions previously described (Experiment #2 and Experiment #3) were analyzed to further understand the mechanism of ACE oxidation. The formation of other oxidation products (intermediates) generated by the laccase-oxidation of ACE were evaluated. Figure 4.31 and Figure 4.32 show the UV and visible spectrum for the two experiments as the reaction progress.

As observed in Figure 4.31a (UV spectrum), as the reaction progresses the intensity of the absorption maximum at 290 nm decreased until disappearance within 5 to 10 minutes of treatment. After that point, a new absorption maximum was observed at 275 nm. As observed in Figure 4.27, the absorption maximum at 290 nm corresponds to the mediator ACE. That is, from the UV absorption spectrum it can be concluded that the mediator ACE was completely consumed within 5 to 10 minutes of treatment instead of

the 100 minutes previously suggested by Figure 4.30. Moreover, after the ACE mediator was completely consumed, an oxidation product started to develop with an absorption maximum in the UV region at approximately 275 nm.

The visible spectrum for the same experimental conditions was also analyzed. As observed in Figure 4.31b (visible spectrum), a new absorption maximum was observed at a wavelength of approximately 400 nm after 5 min of treatment. The intensity of the absorption maximum at 400 nm increased in the first 5 minutes of treatment. After that point, the intensity remained constant until approximately 10 minutes of treatment; then, the intensity continuously decreased. As the intensity of the absorption maximum at 400 nm decreased, another absorption maximum was developed at 520 nm. The absorption maximum at 520 nm corresponded to the red oxidation product previously described. The absorption maximum at 520 nm was first observed after 10 minutes of treatment, when the absorption maximum at 400 nm started to decrease.

Comparing the two regions of the spectrum (UV Figures 4.31a and visible 4.31b) it can be observed that as the absorption maximum at 290nm (ACE mediator) decreases (Figure 4.31a), the absorption maximum at 400 nm increases (Figure 4.31b). The rate of disappearance of the absorption maximum at 290 nm was very similar to the rate of formation of the absorption maximum at 400 nm. Moreover, the absorption maxima at 520 nm and at 275 nm started to develop once the mediator ACE was almost completely consumed (at 290 nm) and the product with an absorption maximum at 400 nm exhibited its maximum absorption intensity. The rate of formation of the absorption maximum at 520 nm and 275 nm were very similar to the rate of disappearance of the absorption maximum at 400 nm.

Based on the previous results, the mechanism of the laccase oxidation of ACE can be proposed for the first time. The laccase-oxidation of the mediator ACE involves the

formation of at least three different species with absorption maxima at 275 nm, 400 nm, and 520 nm. The disappearance of the ACE mediator (290 nm) is directly related to the appearance of a product at 400 nm. Therefore, it may be concluded that the mediator ACE is first oxidized by laccase to an intermediate product that exhibits an absorbance maximum at 400 nm. The disappearance of that intermediate oxidation product is directly related to the formation of at least two final products with absorption maxima at 275 nm and 520 nm. Laccase oxidation occurs through a single electron removal from the substrate (Kunamneni et al., 2008a) producing free radical compounds. That is, the produced intermediate might be a mediator free radical that may be later transformed into at least two final oxidation products.

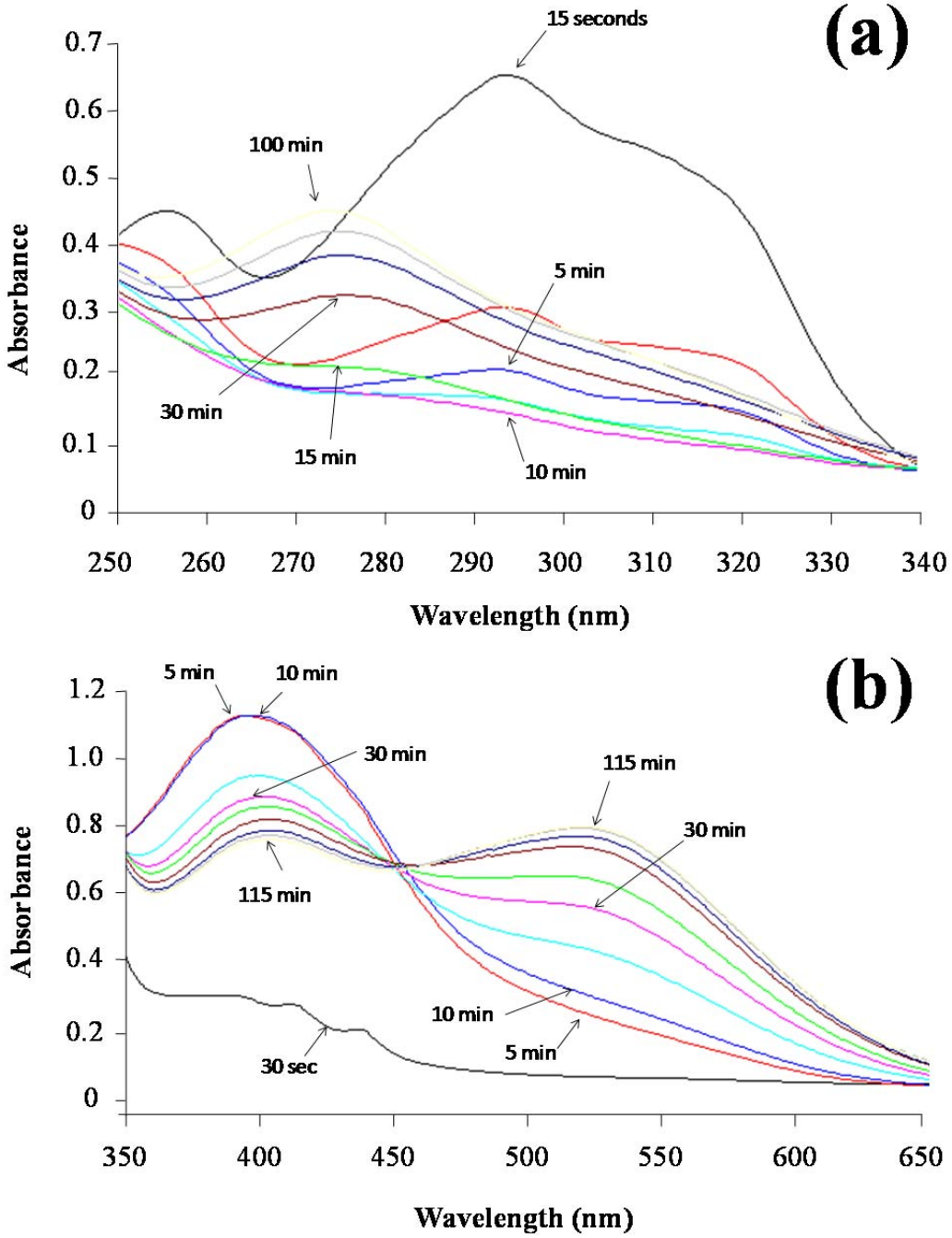


Figure 4.31 –UV-Vis spectrum of ACE oxidation: a) UV spectrum for samples taken after 15 sec, 3, 7, 7.50, 10, 15, 30, 45, 60, and 100 min; b) Visible spectrum for samples taken after 30 sec, 5, 10, 20, 30, 40, 60, 80, 100, and 115 min. Experiments were performed at an ACE concentration of 0.823 mM and at a laccase activity of 11.75 U/mL in a pH 5 sodium acetate buffer.

The disappearance of the mediator ACE occurred faster than the formation of the oxidation product at 520 nm. The rate of formation of the red final product with an absorption maximum at 520 nm cannot be directly related to the disappearance of the mediator ACE. The proposed ACE oxidation mechanism might explain the results obtained in Figure 4.30. At the same initial ACE concentration, increasing laccase activity did not increase the rate of formation of the product at 520 nm. Increasing laccase activity will increase the rate of disappearance of ACE (absorption maximum at 290 nm), and the rate of formation of the intermediate product (absorption maximum at 400 nm), but not the rate of formation of the final product at 520 nm. The rate of formation of this product might depend on the rate of disappearance of the intermediate oxidation product (absorption maximum at 400 nm).

Similar results were obtained analyzing the UV-visible spectrum of the second experiment (Experiment #3) performed at a lower initial laccase activity. As observed in Figure 4.32, the same trends as in Figure 4.31 were observed but at a slower rate. The absorption maximum at 290 nm (ACE) decreased as the intensity of the absorption maximum at 400 nm (intermediate product) increased. The rate of disappearance of the mediator ACE is very similar to the rate of formation of the intermediate product. As the intermediate product disappeared, two final oxidation products were produced at 520 nm and 275 nm. The rate of disappearance of the intermediate product seems to be related to the rate of formation of the final products.

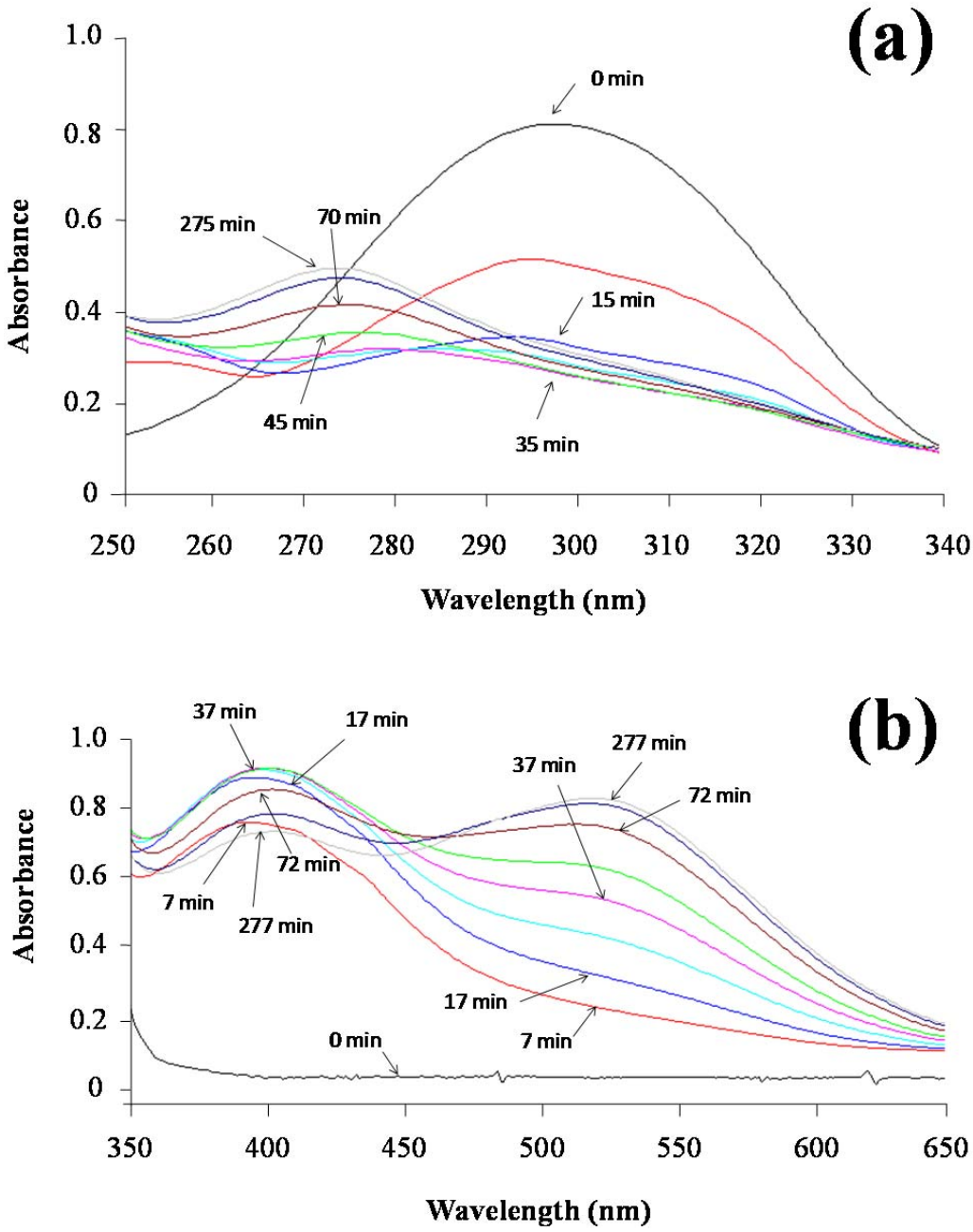


Figure 4.32 – UV-Vis spectrum of ACE oxidation: a) UV spectrum for samples taken after 0, 5, 15, 25, 35, 45, 70, 120, and 275 min; b) Visible spectrum for samples taken after 0, 7, 17, 27, 37, 47, 72, 122, and 277 min. Experiments were performed at an ACE concentration of 0.94 mM and at a laccase activity of 1.188 U/mL in a pH 5 sodium acetate buffer.

To further explain the mechanism of the laccase oxidation of the mediator ACE, another experiment (Experiment #4) was performed at an initial ACE concentration of 1 mM at a laccase activity of 0.0075 U/mL in a pH 5 sodium acetate buffered solution. Samples were taken before laccase addition and after 24 hours of treatment. Samples were analyzed by liquid chromatography/mass spectroscopy to characterize the ACE final oxidation products. The main goal of this experiment was to clarify the previously proposed mechanism for the laccase oxidation of ACE.

The data in Figure 4.33 clearly indicate the formation of several ACE laccase oxidation products that did not exist at time zero. Figure 4.33 shows the chromatograms of both the PDA (top) and the mass spectrometer detector (bottom) for a sample taken before laccase addition (Figure 4.33a) and after 24 hours of treatment (Figure 4.33b). At time zero (Figure 4.33a), both chromatograms show one main ACE peak. After 24 hours of treatment (Figure 4.33b), both chromatograms show a decrease in the ACE response, and the formation of several oxidation products. From the mass spectrometer chromatogram (bottom of Figure 4.33b), the m/z ratios of the most abundant oxidation products were determined and the eight most representative products are shown in Figure 4.33c.

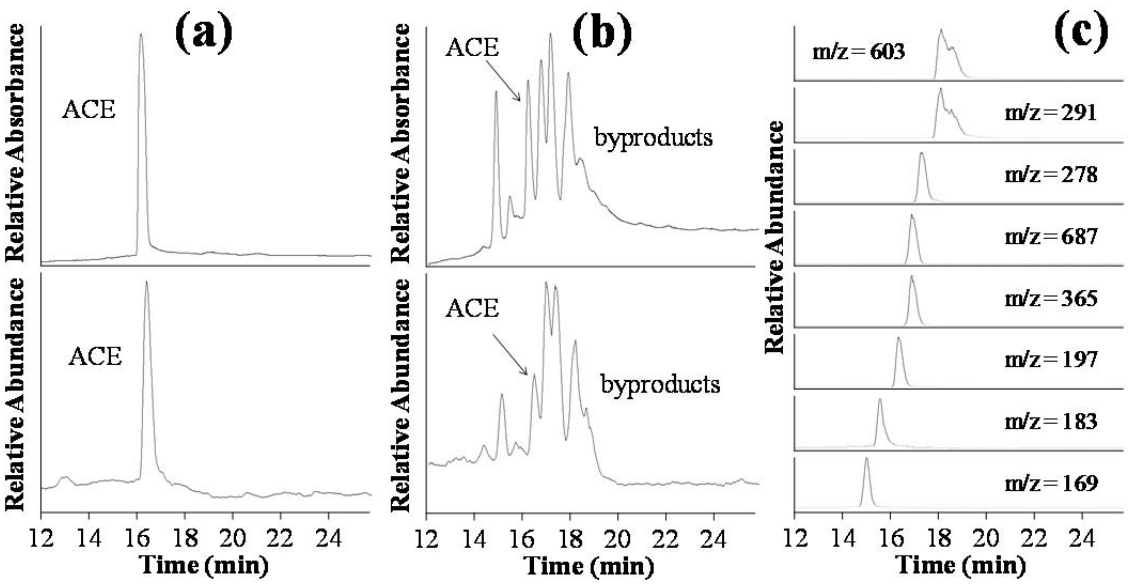


Figure 4.33 – ACE laccase oxidation products: chromatograms for samples taken (a) at time zero and (b) after 24 hours of treatment. For each sample (a & b), the top chromatogram is from the photodiode array detector, and the bottom chromatogram is from the mass spectrometer detector. Part (c) shows chromatograms for specific ranges of m/z ratios, capturing the eight most abundant products.

Figure 4.34a shows the PDA (top) and the mass spectrometer (bottom) chromatograms for a sample taken after 24 hours of treatment. The scanning wavelength range of the PDA detector was adjusted to just show the chromatograms of compounds that absorb visible light at a wavelength of 520 nm, and the results are shown at the top of Figure 4.34b. Only one peak at a retention time of approximately 16.8 minutes was observed. Similarly, the scanning m/z ratio of the mass spectrometer detector was adjusted to just show the chromatograms of compounds that contain m/z ratios from 277.5 to 278.5. The resulting chromatogram is observed at the bottom of Figure 4.34b at the same retention time. As observed from Figure 4.34b it can be concluded that the ACE oxidation product with an absorption maximum at 520 nm has an m/z ratio of

approximately 278. That is, the red final oxidation product previously described has a molar mass of 278 g/mol.

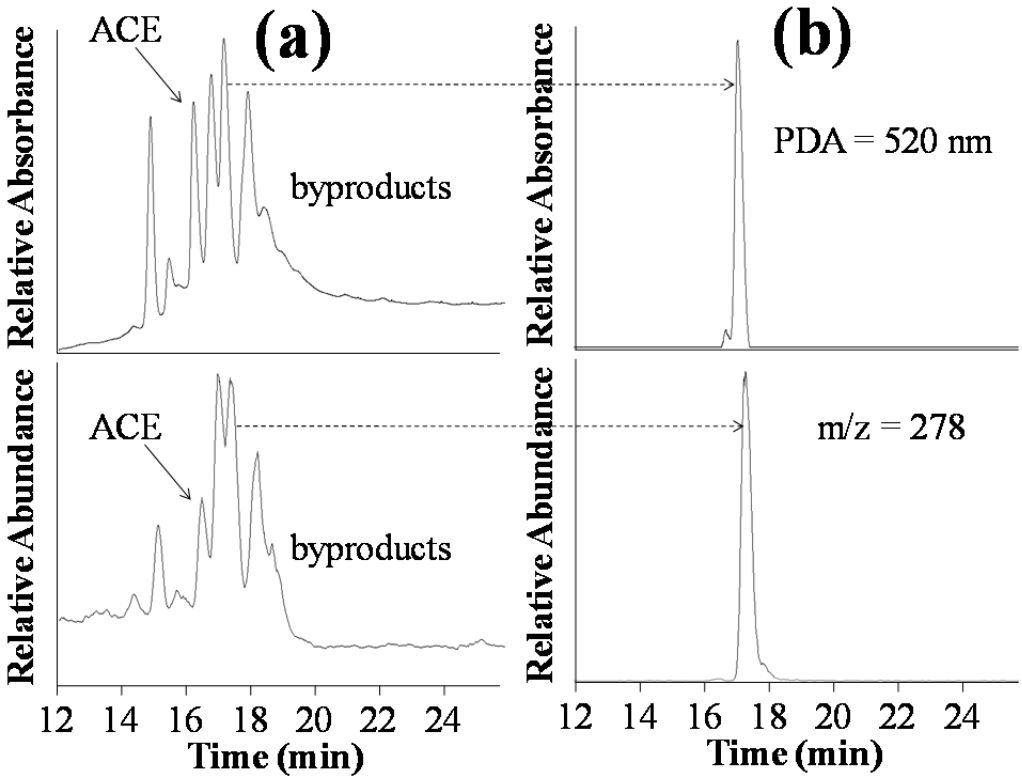


Figure 4.34 – ACE laccase oxidation products: chromatograms for a sample taken (a) after 24 hours of treatment. The top chromatogram is from the photodiode array detector, and the bottom chromatogram is from the mass spectrometer detector. (b) shows chromatograms for a specific wavelength range of 515-525 nm (top) and specific m/z ratios of 277.5-278.5 (bottom).

More information can be obtained by evaluating the PDA chromatograms for the sample taken after 24 hours of treatment. As observed in Figure 4.35, the absorption maximum of the oxidation products can be inferred from analyzing the scanning wavelength range of every peak in the PDA chromatogram. Figure 4.35a shows the overall PDA chromatogram. Figure 4.35b shows the scanning wavelength range (in the

UV range from 250 to 310 nm) for selected peaks of the PDA chromatograms. As observed from Figure 4.35b (from top to bottom) the peaks at a retention times of 16.46 min, 16.82 min, and 17.53 min, exhibited an absorption maximum at approximately 275 nm. From Figure 4.33c, the m/z ratios for these peaks are known and are also described in Figure 4.35b. That is, combining the results from Figures 4.33 and 4.35, we can conclude that the oxidation products with molar masses of 278, 291, 365, 603, and 687 g/mol exhibited an absorption maximum in the UV spectrum at 275 nm.

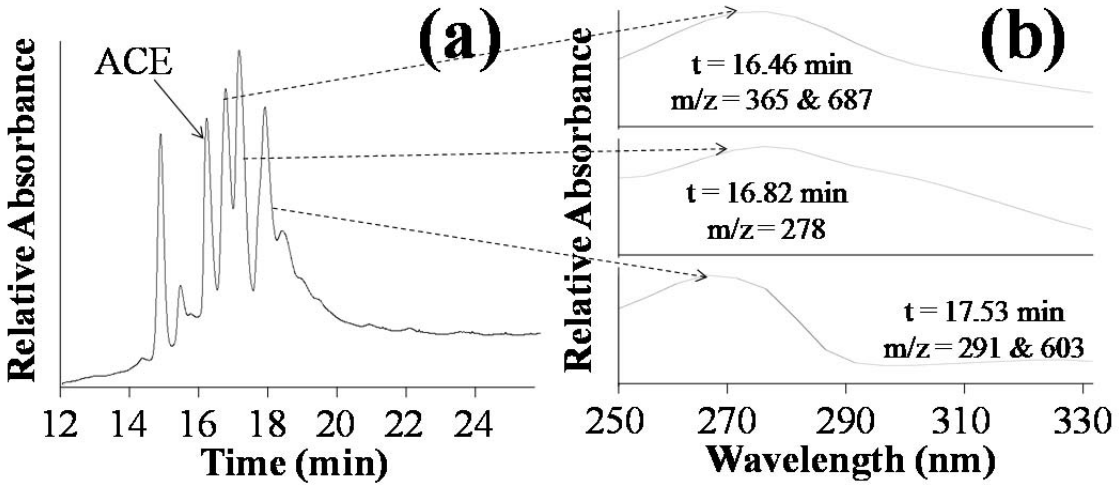


Figure 4.35 – ACE laccase oxidation products: (a) photodiode array detector chromatogram for a sample taken after 24 hours of treatment. (b) shows the absorption UV spectrum for selected peaks. From top to bottom the peaks correspond to retention times of 16.46 min, 16.82 min, and 17.53 min. The m/z ratios for the selected peaks are also shown.

In a similar way, Figure 4.36 shows the PDA chromatogram and the scanning wavelength range for the visible region of the spectrum. As observed from Figure 4.36b, the peak at retention times of 14.70 min showed an absorption maximum at approximately 400 nm, and the peak at a retention time of 16.82 min exhibited absorption

maxima at 400 nm and 520 nm. From comparing with Figure 4.33c, the m/z ratios of these peaks can be determined (and are also shown in Figure 4.36b). That is, we can conclude that the oxidation product with a molar mass of 169 g/mol exhibited an absorption maximum in the visible spectrum at approximately 400 nm, and the oxidation product with a molar mass of 278 g/mol exhibited absorption maxima in the visible spectrum both at 400 nm and 520 nm, respectively.

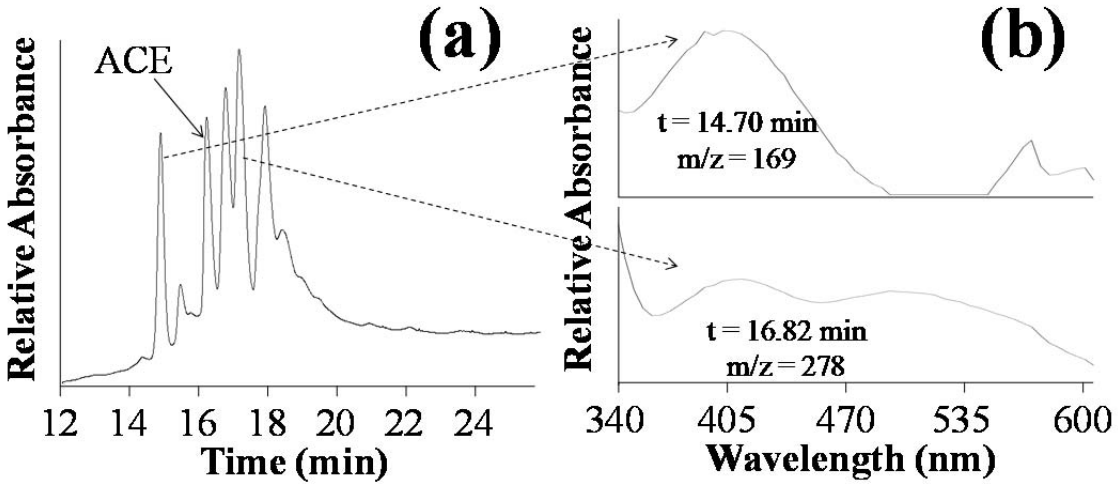


Figure 4.36 – ACE laccase oxidation products: (a) photodiode array detector chromatogram for a sample taken after 24 hours of treatment. (b) shows the absorption visible spectrum for selected peaks. From top to bottom the peaks correspond to retention times of 14.70 min and 16.82 min. The m/z ratios for the selected peaks are also shown.

Combining the experimental results from the absorption spectrum in the UV-visible region described in Figures 4.31 and 4.32 with the liquid chromatography/mass spectroscopy results described in Figures 4.33 to 4.36, the mechanisms of the oxidation of the mediator ACE by laccase can be better explained. Laccase enzymes oxidize the mediator ACE to a radical compound with an absorption maximum at 400 nm (Figure

4.31). Then, the free radicals generated by laccase oxidation react, producing oxidation products with absorption maxima of 275 nm, 400 nm, and 520 nm (Figure 4.31). The oxidation products that exhibited an absorption maximum at 275 nm have molar masses of 278, 291, 365, 603, and 687 g/mol (Figure 4.36). The final oxidation products that absorb at 400 nm have molar masses of 169, and 278 g/mol (Figure 4.36), and the final oxidation product that absorbs at 520 nm has a molar mass of 278 g/mol (Figures 4.34 and Figure 4.36).

A scheme with the proposed mechanisms is shown below. ACE is oxidized by laccase through one electron abstraction, producing a free radical compound. There are three possible resonance forms for the ACE radical as shown below. As the ACE is oxidized (decrease in absorption maximum at 290 nm), the ACE radicals are produced (increased in absorption maximum at 400 nm). After that point, the ACE radicals are transformed into diverse products (decreased in absorption maximum at 400 nm). Two different routes might be followed by the ACE radicals, as shown schematically in Figure 4.37. First, the ACE radicals might decay to form oxidation products with molar masses of 169 g/mol and 182 g/mol, as shown in the scheme below and also reported by Caldwell and Steelink (1969). The compound with a molar mass of 169 g/mol exhibits an absorption maximum at 400 nm (Figure 4.31), and that is the main reason of the residual absorption maximum at 400 nm in Figure 4.31. Second, the ACE radicals react due to oxidative coupling reactions generating oxidation products with higher molar masses than the parent ACE mediator. Some of the potential oxidation products generated by ACE radical coupling reactions are shown in the scheme below[1]. Products with molar masses of 278, 291, 365, 603 (not shown), and 687 (not shown) g/mol

[1]The molecular structures of the oxidation products are proposed based on the molecular weight information. The structures were not confirmed.

exhibited an absorption maximum at 275 nm (Figure 4.31), and the product with a molar mass of 278 g/mol also exhibited an absorption maximum at 520 nm (Figure 4.31).

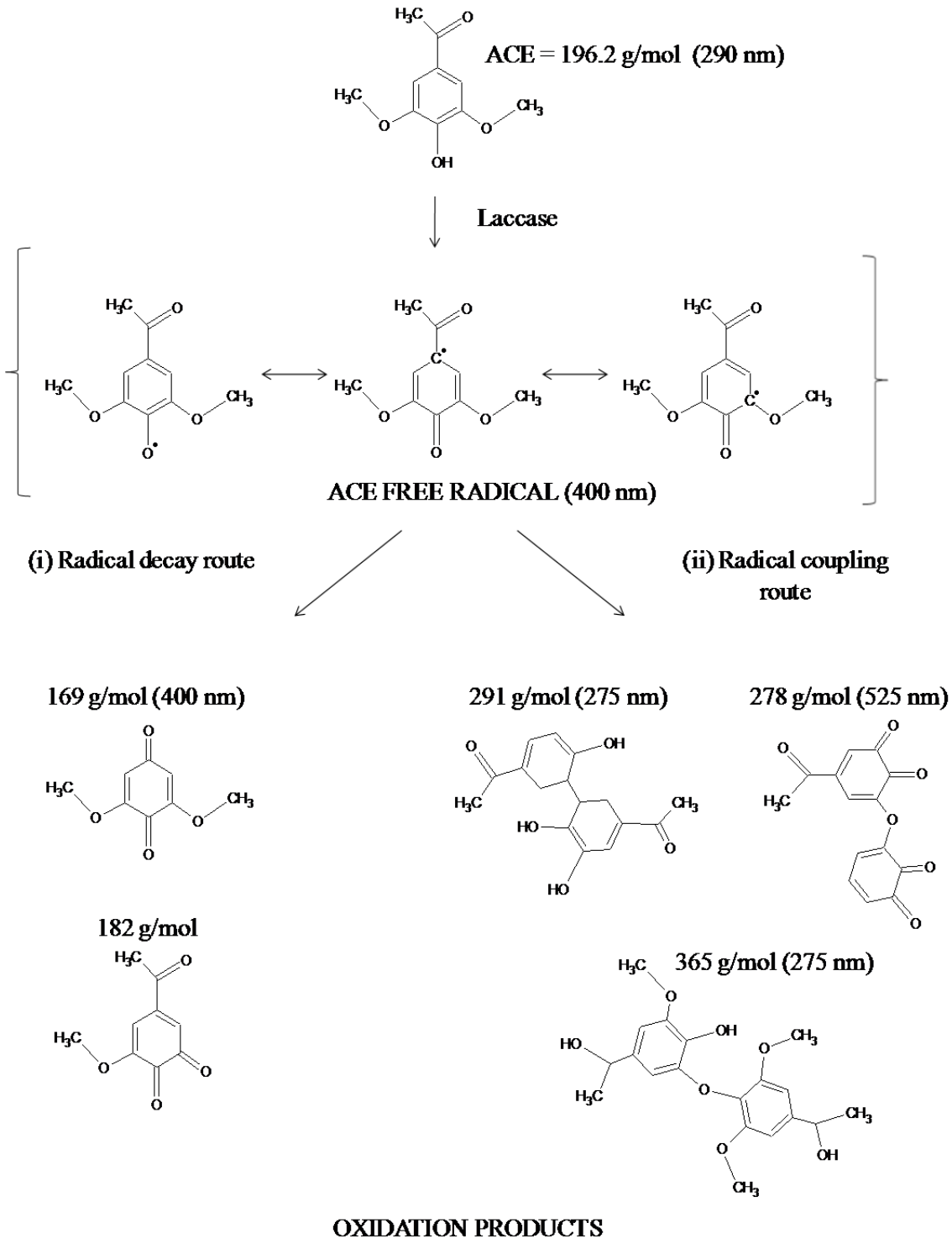


Figure 4.37 – Scheme with the proposed mechanism for the laccase-oxidation of the mediator ACE.

The evaluation of the laccase oxidation mechanism of the ACE mediator may lead to an important confirmation of this research. Laccase oxidation of suitable substrates produces free radicals that can undergo extensive coupling either with each other, or with other compounds. From previous results, it was observed that, after the ACE free radicals were produced, they reacted through coupling reactions producing compounds with larger molecular weights. Therefore, the removal of target emerging contaminants such as PPCPs by the laccase-mediator system might occur through the formation of mediator free radicals that undergo coupling reactions, transforming (oxidizing) the target contaminants into oxidation byproducts.

When using the laccase-mediator system for removing target emerging contaminants, it would be desirable to promote radical coupling reactions between the mediator free radicals and the target compounds. That is, the goal would be to design a laccase-mediator system that produces a high concentration of mediator radicals, avoiding mediator radical coupling with each other, so most of the oxidative power of the mediator radicals can be used to oxidize the target compounds. The extent of mediator self-coupling reactions depends primarily on the chemical structure of the mediator. Steric factors have been demonstrated to be the main cause of radical persistence (Griller and Ingold, 1976). That is, a mediator that exhibits steric factors will be less likely to produce free radicals that decay through self-coupling reactions. Moreover, ortho-di-substituted phenols that do not have any resonance structure such as butylated hydroxytoluene (BHT) produce more stable phenoxyl radicals which are less likely to decay through self-coupling reactions (Valoti et al., 1989). For most of the phenolic mediators, self-coupling reactions cannot be completely avoided, although changes in the laccase-mediator treatment configurations might reduce the amount of mediator radicals that decay by self-coupling. For instance, gradual introduction of the mediator into the

reaction mixture might reduce the interaction between phenolic radicals, thereby avoiding mediator losses by self-coupling reactions.

4.7.2 Laccase Oxidation of ACE: Kinetics Experiments

The removal of the target compounds would depend at some extent on the rate at which the mediator radicals can be generated by the laccase-mediator system. Therefore, an understanding of the mediator oxidation kinetics is desirable to make improvements in the design of laccase-mediator system for removing emerging contaminants such as PPCPs.

The Michaelis-Menten model (Equation 4.1) was followed to investigate the mediator ACE oxidation kinetics. The development of the Michaelis-Menten equation is described in Section 2.4.1. The initial reaction velocity (when less than approximately 10% of the substrate was consumed) was used to determine the kinetic parameters (Equation 4.3). Factors such as byproduct enzyme inhibition and enzyme deactivation can be avoided by using the initial velocities method.

$$v = \frac{V_{\max}[S]}{K_M + [S]} \qquad \text{Eq. (4.1)}$$

$$V_{\max} = k_2[E]_T \qquad \text{Eq. (4.2)}$$

$$v_o = -\left(\frac{d(S)}{dt}\right)_o = \frac{k_2[E]_T[S]_o}{K_M + [S]_o} \qquad \text{Eq. (4.3)}$$

v = Reaction velocity [μM/min]

v_o = Initial reaction velocity [μM/min]

V_{max} = Maximal velocity of the catalyzed reaction [μM /min]

S = Substrate concentration [μM]

S_o = Initial substrate concentration [μM]

K_M = Michaelis-Menten constant [μM]

k_2 = Catalytic constant [μM min^{-1} $(U/mL)^{-1}$]

E_T = Total enzyme concentration [U/mL]

Several experiments were performed at the experimental conditions described in Table 4.7 to evaluate the initial reaction velocity of the ACE disappearance at different ACE concentrations in pH 5 sodium acetate buffered solutions. Experiments in this section were designed at an initial substrate (ACE mediator) concentration in great excess with respect to the enzyme concentration. The disappearance of the substrate ACE was measured as a function of time for every experimental condition, and the initial reaction velocities were calculated. The disappearance of the ACE mediator was followed by measuring the absorbance at 294 nm (ACE exhibits an absorption maximum at approximately 290 nm). If the experimental calculated values for v_o and S_o, at a constant E_T, fit Equation 4.3, the kinetic parameters k_2 and K_M can be determined.

Table 4.7 – Experimental conditions for the initial velocity kinetic experiments performed in pH 5 sodium acetate buffered solutions.

Experimental Conditions		
Laccase activity	0.0182 U/mL (0.013 μM)	
Medium	Sodium Acetate Buffer	
pH	5	
Temperature	23^{o} C	
Experiment	*Initial ACE Concentration (μM)*	*Initial Reaction Velocity (μM/min)*
1	50	0.619
2	105	0.980
3	211	1.41
4	316	1.72
5	421	2.11
6	513	2.31
7	615	2.40
8	718	2.34
9	821	2.79
10	923	2.23
11	1026	2.55
12	1231	2.59

The Michaelis-Menten kinetic parameters k_2 and K_M were calculated by adjusting their values to minimize the sum of the squared differences between the experimental initial reaction velocity and the initial reaction velocity calculated from the Michaelis-Menten model. Minimizing the sum of the squared difference yields the values of k_2 and K_M that best fit the experimental data to the proposed model. *Microsoft Excel Solver* was used to minimize the sum of the squared difference. The experimental and calculated initial reaction velocities as well as the square difference between these two values are

presented in Table 4.8. A more detailed description of the calculations performed can be found in Appendix C.

Table 4.8 – Measured and calculated initial reaction velocities for the kinetic experiments performed in pH 5 sodium acetate buffered solutions

Experiment	Experimental initial reaction velocity	Initial reaction velocity according to the model fit	Error (Experimental initial velocity – Predicted initial velocity)^2
	(μM/min)	(μM/min)	(μM/min)^2
1	0.619	0.568	0.00260
2	0.980	0.997	0.00029
3	1.41	1.51	0.01034
4	1.72	1.83	0.01285
5	2.11	2.04	0.00401
6	2.31	2.18	0.01602
7	2.40	2.30	0.01112
8	2.34	2.39	0.00304
9	2.79	2.46	0.10568
10	2.23	2.53	0.08503
11	2.55	2.58	0.00048
12	2.59	2.66	0.00435
		TOTAL:	0.256

The values of k_2 and K_M that best fit the experimental data to the proposed equation are:

$k_2 = 173$ [μM min^{-1} (U/mL)$^{-1}$] or $k_2 = 4.04$ s^{-1}

$K_M = 227$ μM

Specificity constant (k_2/K_M) = 0.0178 μM^{-1}s^{-1}

$R^2 = 0.95$

The values from the Michaelis-Menten model are plotted together with the experimental data in Figure 4.38. The good agreement of the model and the experimental results suggest that the Michaelis-Menten model described by Equation 4.3 is a reasonable description of the laccase oxidation of the ACE mediator at the experimental conditions evaluated.

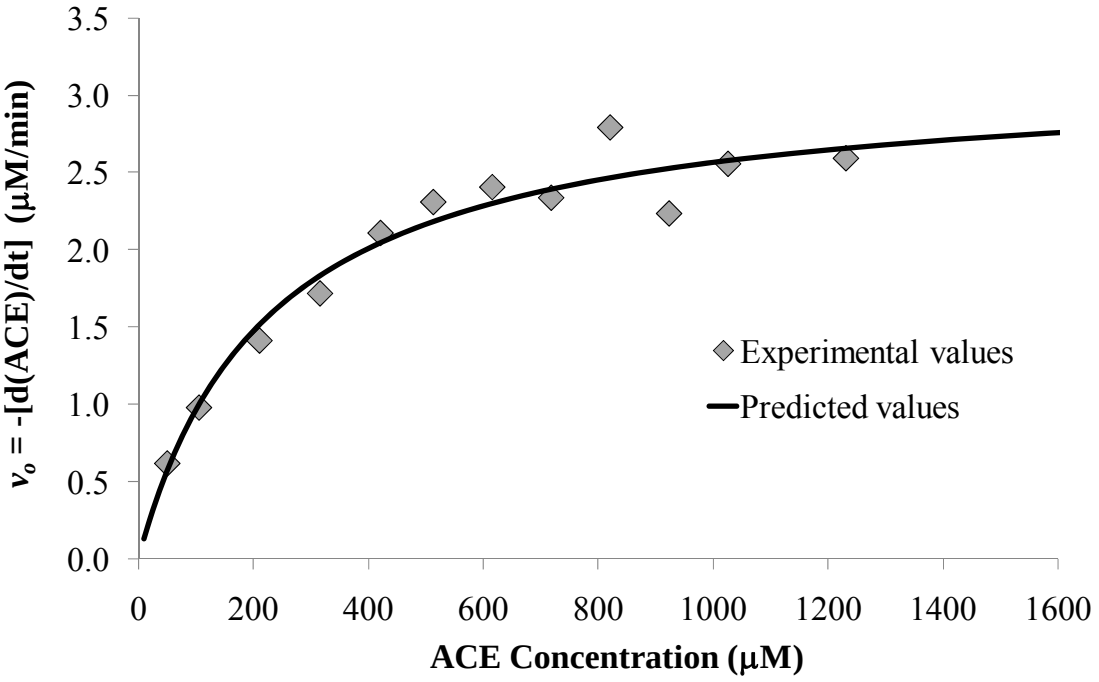


Figure 4.38 – Experimental and calculated values of the initial reaction velocities as a function of the substrate (ACE) concentrations. Experiments were performed at an initial laccase activity of 0.0182 U/mL in pH 5 sodium acetate buffered solutions at 23°C.

In this research, the laccase-mediator system was evaluated for removing PPCPs from municipal wastewater primary effluent solutions. Therefore, it would be advantageous to evaluate the effect of a primary effluent matrix on the oxidation kinetics of mediators. The laccase activity might be affected by the presence of some constituents

in the primary effluent solutions, which might decrease the oxidation rate of mediators. Similar experiments were performed as previously described, but in primary effluent solutions rather than in sodium acetate buffered solutions. The experimental conditions of these experiments are summarized in Table 4.9. The disappearance of the substrate ACE was measured as a function of time, and the initial reaction velocities were calculated. The pH of the primary effluent solutions was adjusted to 6 to emulate the conditions of the oxybenzone removal experiments performed in primary effluent solutions (Sections 4.2, 4.3 and 4.4). The Michaelis-Menten model (Equation 4.3) was followed to investigate the mediator ACE oxidation kinetics in primary effluent solutions. If the experimental values for v_o and S_o, at a constant E_T fit the Equation 4.3, the kinetic parameters k_2 and K_M can be determined.

Table 4.9 – Experimental conditions for the initial velocity kinetic experiments performed in pH 6 primary effluent solutions.

Experimental Conditions		
Laccase activity	0.0169 U/mL (0.012 μM)	
Medium	Primary Effluent Solution	
pH	6	
Temperature	23^{o} C	
Experiment	*Initial ACE Concentration (μM)*	*Initial Reaction Velocity (μM/min)*
1	50	0.297
2	105	0.416
3	158	0.540
4	211	0.771
5	316	0.876
6	421	0.875
7	513	1.019
8	615	0.932
9	821	0.992

The Michaelis-Menten kinetic parameters k_2 and K_M were calculated by adjusting their values to minimize the sum of the squared differences between the experimental initial reaction velocities and the calculated initial reaction velocities from the Michaelis-Menten model. *Microsoft Excel Solver* was used to minimize the sum of the squared difference. The experimental and calculated initial reaction velocities as well as the square difference between these two values are presented in Table 4.10. A more detailed description of the calculations performed can be found in the Appendix D.

Table 4.10 – Measured and calculated initial reaction velocities for the kinetic experiments performed in pH 6 primary effluent solutions

Experiment	Experimental initial reaction velocity	Initial reaction velocity according to the model fit	Error (Experimental initial velocity – Predicted initial velocity)^2
	(μM/min)	(μM/min)	(μM/min)^2
1	0.297	0.286	0.00013
2	0.416	0.480	0.00414
3	0.540	0.604	0.00411
4	0.771	0.694	0.00592
5	0.876	0.815	0.00373
6	0.875	0.893	0.00031
7	1.019	0.941	0.00607
8	0.932	0.981	0.00239
9	0.992	1.04	0.00201
		TOTAL:	0.0288

The values of k_2 and K_M that best fit the experimental data to the proposed equation are:

$k_2 = 74.0$ [μM min^{-1} (U/mL)$^{-1}$] or $k_2 = 1.74$ s^{-1}

$K_M = 169$ μM

Specificity constant (k_2/K_M) = 0.0103 μM^{-1}s^{-1}

$R^2 = 0.95$

As shown in Figure 4.39, a good agreement between the model and the experimental results suggest that the proposed model is a reasonable description of the laccase oxidation of the ACE mediator in primary effluent solutions.

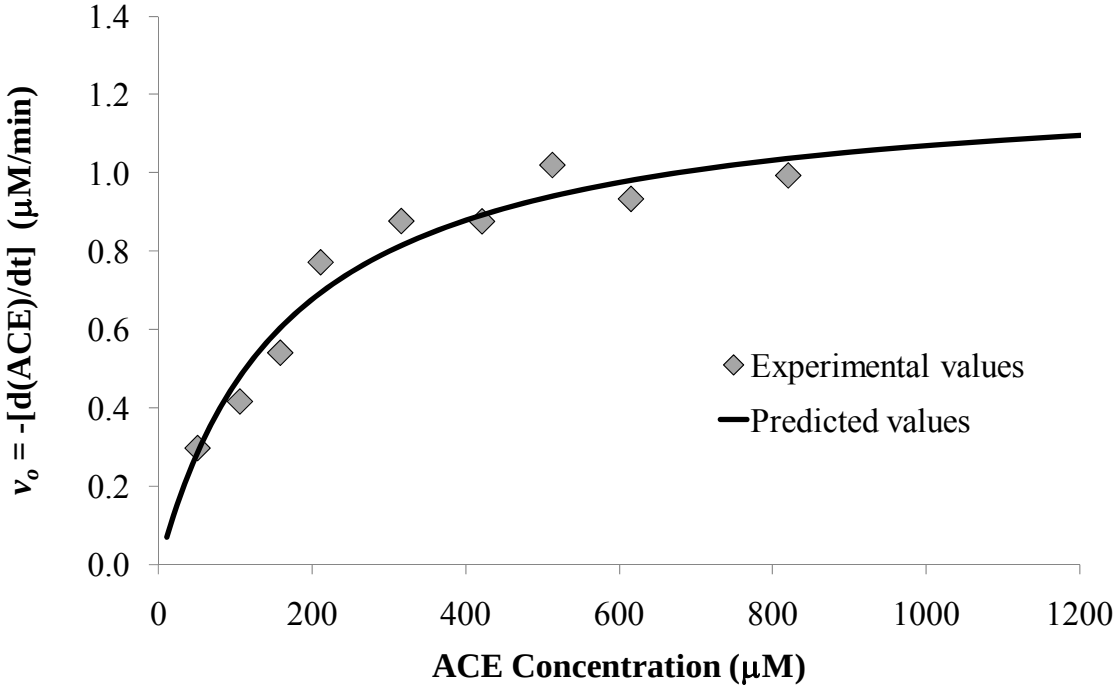


Figure 4.39 – Experimental and calculated values of the initial reaction velocities as a function of the substrate (ACE) concentrations. Experiments were performed at an initial laccase activity of 0.0169 U/mL in pH 6 primary effluent solutions at 23°C.

Once the kinetic parameters k_2 and K_M were determined for buffered and primary effluent solutions, the rate of disappearance of the mediator ACE predicted by the Michaelis-Menten model (Equation 4.3) can be calculated as a function of the mediator concentration. As shown in Figure 4.40, the rate of disappearance of the mediator ACE in buffered solutions is higher than the rate of disappearance in primary effluent solutions at the same initial laccase activity. The results observed in Figure 4.40 were expected since a primary effluent matrix contains several organic and inorganic constituents that may inhibit the activity of the laccase enzyme. The experiments performed in buffered solutions were conducted at one unit of pH lower (pH 5) than the experiments performed in primary effluent solutions (pH 6). Therefore, even though it is expected that primary

effluent solutions exhibit a stronger effect on the oxidation rate, the differences in pHs needs to be addressed in this discussion. It was reported by several authors (Strong and Claus, 2011; Kunamneni et al., 2008b) that laccase activity is optimal at acidic pH values, and the optimum pH may range from 3.0 to 4.5 (Strong and Claus, 2011). Laccase would exhibit a higher activity at lower pHs; consequently, a small fraction of the observed reduction in the rate disappearance of ACE in the primary effluent experiments could be assigned to the pH differences between experiments. For instance, Kim and Nicell (2006) reported losses in laccase activity of approximately 20% when the solution pH was increased from 5 to 6 in the same buffered solution. Thus, a decrease in the oxidation rate of approximately 20% can be expected when experiments are conducted at pH 6 as compared to experiments conducted at pH 5.

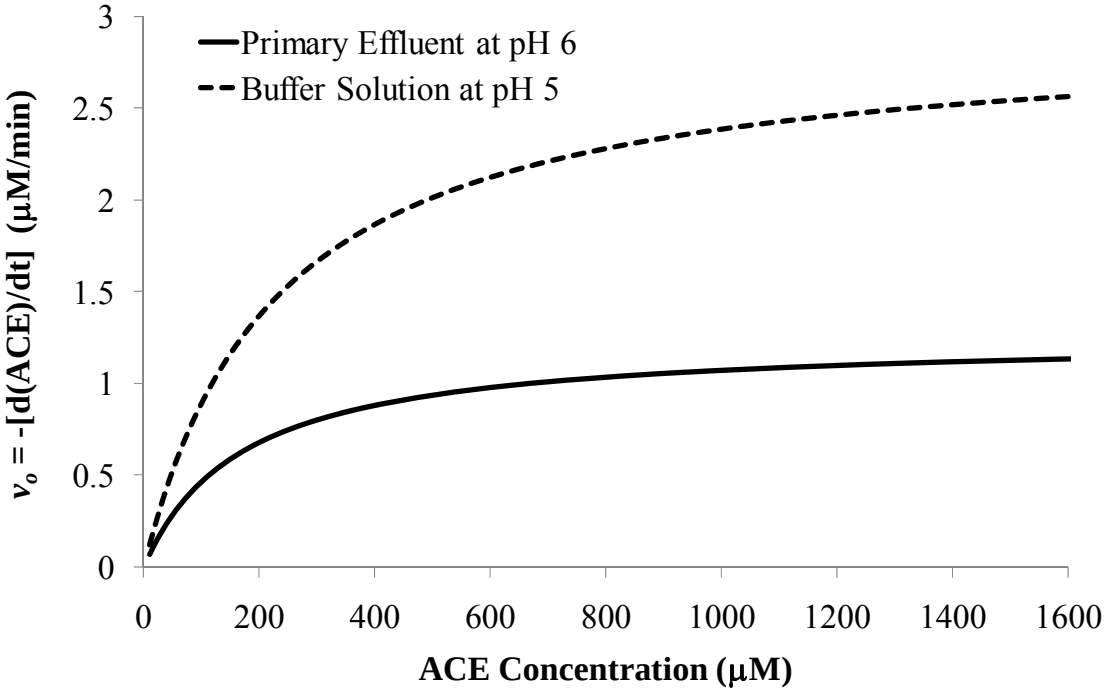


Figure 4.40 – Rate of disappearance of the mediator ACE predicted by the Michaelis-Menten model as a function of the mediator ACE concentration. A laccase activity of 0.0169 U/mL was used in these models.

One of the advantages of determining the oxidation kinetics of the mediators (kinetic parameters k_2 and K_M) is that the extent of the mediator oxidation as a function of time can be predicted for a given set of conditions such as ACE initial concentration, laccase activity, temperature, and solution composition. The Michaelis-Menten equation (Equation 4.3) can be integrated, and the substrate (mediator) concentration can be predicted as a function of time.

The extent of the ACE oxidation for the experiments performed for oxybenzone removal in primary effluent solutions (Sections 4.2 and 4.3) can be evaluated. Depending on how fast the ACE mediator is oxidized, the removal of the target PPCP might be affected.

Figure 4.41 exhibits the predicted extent of the ACE oxidation in a primary effluent solution at the same experimental conditions as the experiments performed to evaluate the removal of high oxybenzone concentrations (1,000 μg/L) in a primary effluent solution at an initial pH of 6 (Section 4.2 - Figure 4.6). As observed in Figure 4.6, oxybenzone was completely removed after 2 hours of treatment. Figure 4.41 shows that after approximately 20 minutes of treatment the entire mediator ACE would be oxidized. Therefore, two hours of treatment was enough detention time for achieving the complete oxidation of the mediator. The mediator ACE in the pH 6 primary effluent solution was completely oxidized before the first sample was taken after two hours of treatment. At that point, the entire ACE was converted to the free radical form and could react with the target compound oxybenzone.

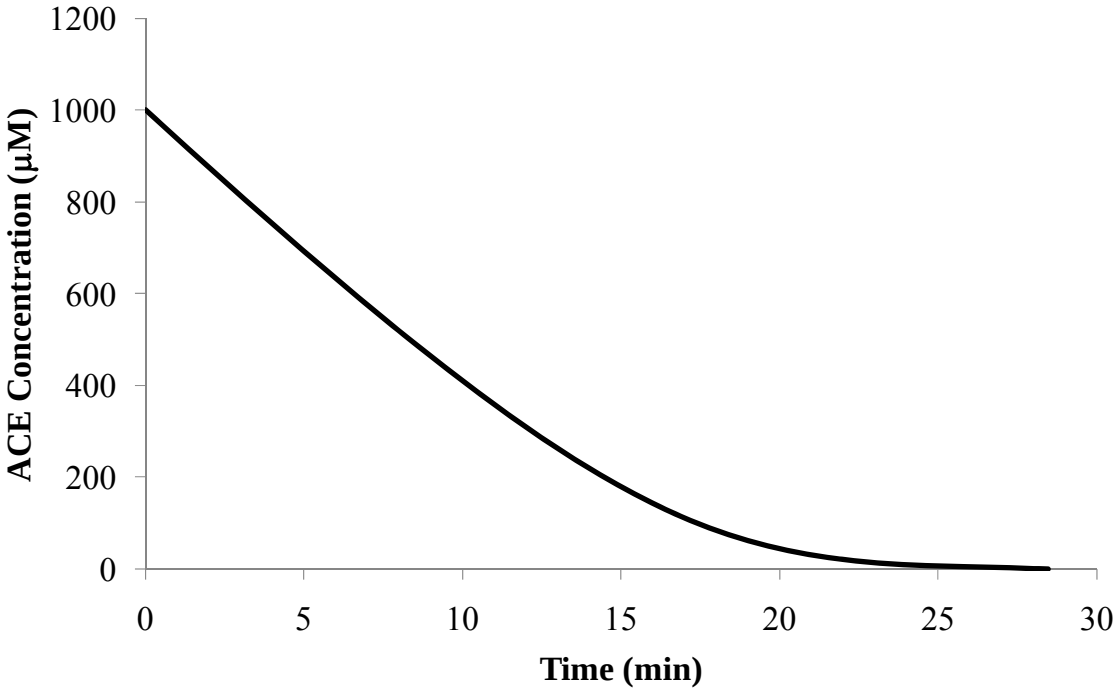


Figure 4.41 – Extent of the predicted mediator ACE oxidation as a function of time from the integrated form of the Michaelis-Menten equation. The plot was obtained for an initial ACE concentration of 1000 μM, a laccase activity of 1 U/mL in a pH 6 primary effluent solution.

Similarly, the extent of the predicted ACE oxidation was evaluated at the same experimental conditions as the experiments performed to evaluate the effect of ACE/Oxybenzone molar ratios in primary effluent solutions (Section 4.3 - Figure 4.9b). Figure 4.42 shows the extent of the predicted ACE oxidation as a function of time for ACE/Oxybenzone molar ratios of 20, 6, 1, and 0.6. The molar ratios previously described correspond to ACE concentrations of 87.6, 26.3, 8.76, and 2.63 μM, respectively. As observed in Figure 4.9b most of the oxybenzone removal generally occurred in the first 15 minutes of reaction. Figure 4.42 shows that, after approximately 10 minutes of treatment, the entire ACE mediator would be oxidized. That is, the mediator ACE in the pH 6 primary effluent solution was completely oxidized before the

first sample was taken for all the ACE/Oxybenzone molar ratios evaluated. At that point, the entire ACE was converted to the free radical form and could react with the target compound oxybenzone.

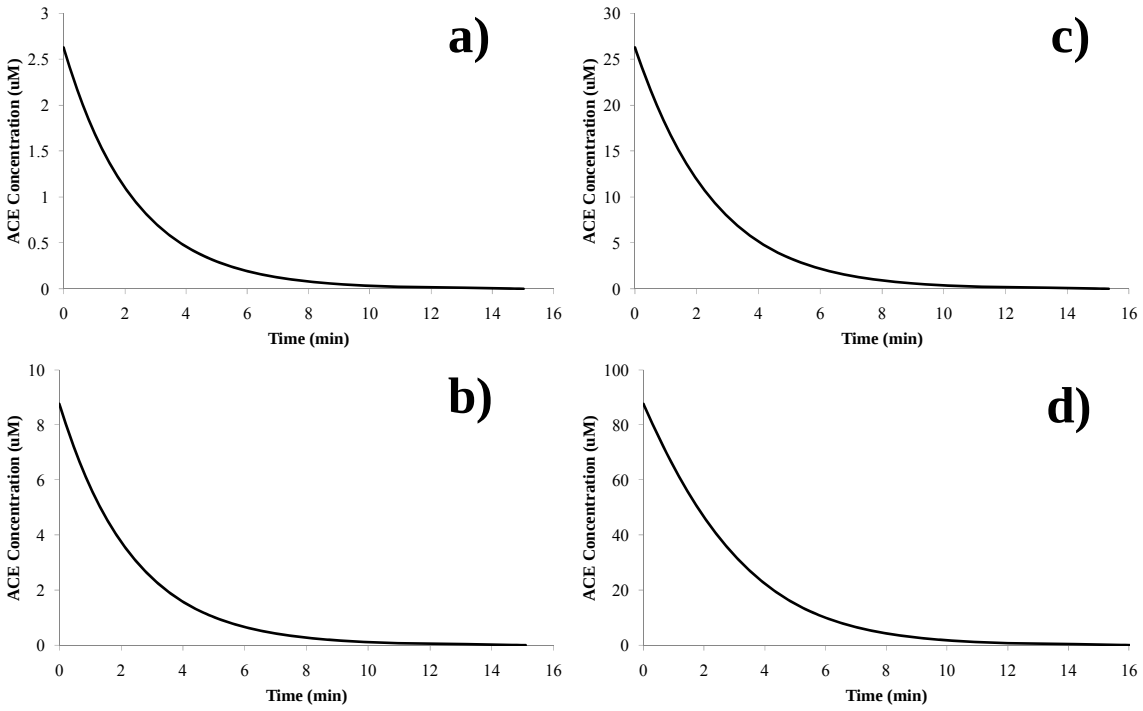


Figure 4.42 – Extent of the predicted mediator ACE oxidation as a function of time from the integrated form of the Michaelis-Menten equation. The plots were obtained for initial ACE concentrations of (a) 2.63 µM, (b) 8.76 µM, (c) 26.3 µM, and (d) 87.6 µM, a laccase activity of 1 U/mL in pH 6 primary effluent solutions.

If the kinetic parameters for a particular set of conditions are known, the Michaelis-Menten equation can be integrated, and the extent of the mediator oxidation (or free radical production) can be predicted as a function of time. Experiments that involved the laccase-mediator systems can be better designed by knowing how fast the mediators are oxidized (or the free radicals are produced). Limitations of the Michaelis-

Menten model include the following: (i) The Michaelis-Menten model can be applied when the substrate (mediator) concentration is in great excess with respect to the enzyme concentration. Johnson (2009) suggested that a substrate is in great excess with respect to the enzyme when the following expression applies: $[E]_T < \frac{K_M}{5}$ where $[E]_T$ refers to the total enzyme concentration, and K_M to the Michaelis-Menten constant; and (ii) laccase inhibition by oxidation byproducts and/or enzyme deactivation may affect the predicted extent of the mediator oxidation as the reaction progresses.

The kinetic experiments previously described were conducted at a total enzyme concentration of approximately 0.012 μM. The total enzyme concentration $[E]_T$ was significantly lower than the $K_M/5$ ratio for the two experimental conditions evaluated ($K_M/5$ ratios of 45 μM and 38 μM were obtained for the experiments performed in pH 5 buffered solutions and in pH 6 primary effluent solutions, respectively.) That is, it can be concluded that the substrate was in great excess with respect to the enzyme, and the Michaelis-Menten model was correctly proposed.

4. 7.3 Laccase Oxidation of ABTS

The goal of the next set of experiments was to evaluate the oxidation of the mediator ABTS by laccase. Oxidation of ABTS by oxidoreductase enzymes such as laccases and peroxidases was previously evaluated by several authors (Johannes and Majcherczyk, 2000a; Scott et al., 1993; Majcherczyk et al., 1999), so more information related to the ABTS oxidation mechanism and oxidation products identities is available.

Experiments in this part of the research were performed to confirm the relatively high stability of the ABTS radical produced by the laccase oxidation of ABTS and to evaluate the rate at which the ABTS radicals are produced for a specific set of experimental conditions. Experiments were performed at an initial ABTS concentration of 1 mM and an initial laccase activity of approximately 0.08 U/mL in pH 5 sodium acetate buffered solutions. As reported by Johannes and Majcherczyk (2000a), the colorless ABTS is oxidized by laccase by one electron abstraction to the green free radical ABTS•. The ABTS• radicals exhibit three absorption maxima in the visible spectrum at 420, 625, and 728 nm (Scott et al., 1993). The absorption of visible light in the high range of the spectrum (625 and 728 nm) confers a characteristic green color to the reaction mixture (Scott et al., 1993). Unlike in the previous ACE oxidation experiments (the disappearance of ACE was directly measured at 290 nm), the disappearance of ABTS was investigated by evaluating the formation of the ABTS laccase oxidation product, the radical ABTS•. The formation of the ABTS• radical was evaluated by measuring the absorbance at 420 nm. The rate of formation of the ABTS• radical is the same as the rate of disappearance of ABTS.

As observed in Figure 4.43, ABTS was completely oxidized to the free radical ABTS• in approximately 60 minutes of treatment at the experimental conditions previously described. The final ABTS• radical concentration was the same as the initial

ABTS concentration of 1000 μM. The ABTS• radicals remained stable in solution even after 160 minutes of treatment. The ACE radicals were not as stable as the ABTS• radicals. ACE radicals were transformed to oxidation products (through radical decay or radical coupling reactions). By comparing the molecular structure of both ABTS and ACE mediators (Table 3.2), ABTS• radicals might not couple to each other due to steric effects. Steric factors have been shown to be the main cause of radical persistence (Griller and Ingold, 1976). That is, all the oxidative power of the ABTS• radicals can be use to oxidize the target compounds.

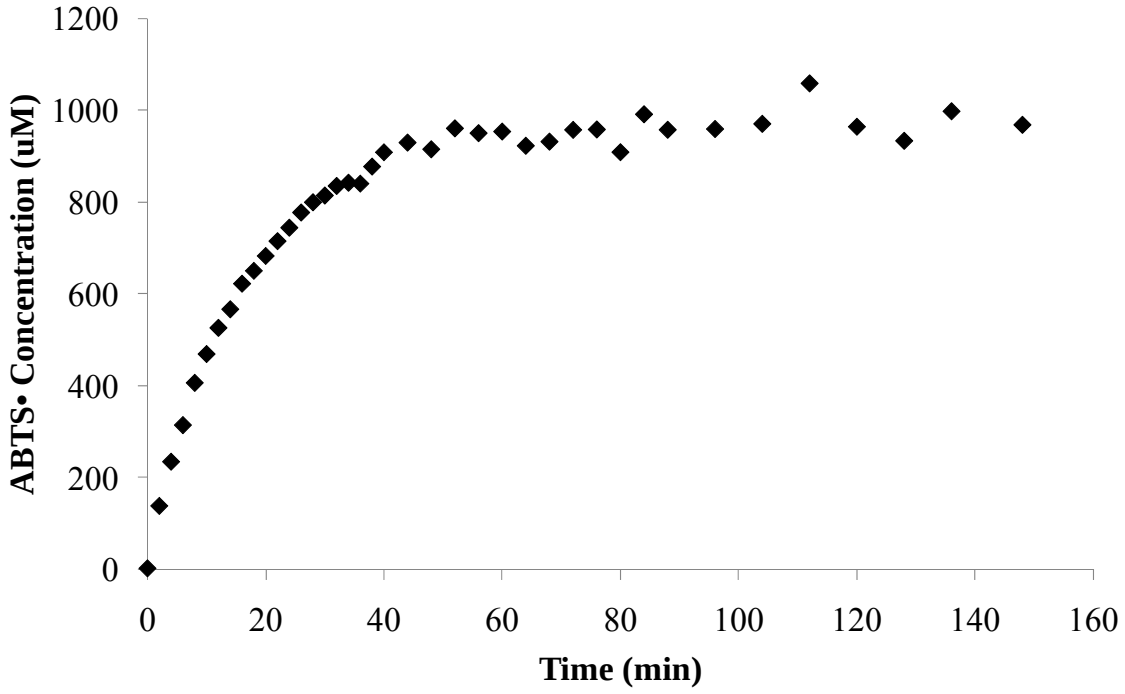


Figure 4.43 – Extent of the ABTS mediator oxidation as a function of time. The experiment was performed at an initial ABTS concentration of 1mM, and at initial laccase activity of 0.08 U/mL in a pH 5 sodium acetate buffered solution at 23°C.

The relatively high stability of the ABTS• radicals might explain the better performance of the laccase ABTS mediator system compared to the laccase ACE mediator system for removing oxybenzone in the previous sections of this research (Section 4.1, 4.2, 4.3, and 4.4). The relatively high stability (persistence) of the ABTS• radical was also reported by Scott et al. (1993), and Majcherczyk et al. (1999). Majcherczyk et al. (1999) reported that ABTS• radicals remained stable in a pH 3 buffered solution for approximately 22 hours.

The stability of the radicals will depend not only on their chemical nature, but also on the presence of other compounds such as radical scavengers that can strongly impact the half life of the radical species. As previously shown in this research, detention times of approximately 2 hours in batch reactors were required by the laccase-mediator system to completely remove environmentally relevant concentrations of a representative PPCP from primary effluent solutions. Even though ABTS radicals exhibited relatively high stability compared to other mediators such as phenolic mediators, in a two hours reaction time most of the long-lived ABTS radicals will react either with the target PPCPs or with other organic or inorganic constituents (radical scavengers) in the primary effluent municipal wastewater. The laccase-mediator system is intended to be applied after primary treatment and upstream of secondary treatment processes in a municipal wastewater treatment plant. Thus, even if some long-lived radicals persist after the laccase-mediator oxidation treatment, they will go through secondary treatment with typical hydraulic retention times of approximately 8 hours. Therefore, it is strongly suspected that the free radicals will be completely consumed in a conventional wastewater treatment system, and they will not be discharged into the receiving water thus mitigating any potential negative environmental effects of the mediator radicals.

4. 8 AN ALTERNATIVE LACCASE-MEDIATOR SYSTEM CONFIGURATION: THE FREE RADICAL GENERATOR

Laccase cannot directly react with a representative PPCP such as oxybenzone, and mediators are required to promote its removal by the laccase-mediator system. The removal of contaminants that are not directly oxidized by laccase occurs primarily due to the interaction between the mediator free radicals (generated by the laccase oxidation of the mediators) and the target contaminants. That is, an effective laccase-mediator treatment system for the removal of contaminants that are not directly recognized by laccase should be designed to deliver a high concentration of mediator radicals to interact with the target contaminants. In this research thus far, batch reactors contained a well-mixed solution consisting of the PPCP to be oxidized (oxybenzone), the laccase enzyme, and a specific mediator in primary effluent solutions. Another treatment configuration is proposed to yield better performance of the laccase-mediator system. The new treatment configuration allows the enzyme and mediator to interact prior to coming in contact with the target compound. Laccase and the mediator are added to a smaller "free radical generator" reactor (as described in Section 3.3.10) where conditions can be more easily optimized for this reaction. The free radicals generated are then introduced to an oxidation reactor containing the target PPCP. Results to date indicate that the primary effluent matrix can cause some laccase activity loss. This loss is likely due to the fact that some wastewater constituents might inactivate the enzyme. The proposed treatment configuration will allow the enzyme and mediators to react in an ultrapure water system before being exposed to wastewater constituents, likely decreasing required enzyme and mediator doses, and thereby decreasing treatment costs. In addition, the free radical generator reactor will be very small compared to the oxidation reactor. Therefore,

optimizing conditions (*e.g.*, pH) for the laccase oxidation of mediators will be easier and less expensive.

Two experiments were conducted to evaluate the performance of the free radical generator system for the removal of oxybenzone, under the experimental conditions described in Table 4.11. Laccase and mediators were added to the free radical generator. After a certain reaction time, an aliquot from this reactor was introduced to the oxidation reactor containing the target PPCP oxybenzone.

Table 4.11 – Free radical generator experimental conditions.

Parameter	**Mediator**	
	ACE	**ABTS**
Free Radical Generator Reactor		
Reactor Volume	50 mL	25 mL
Mediator Initial Concentration	8.74 mM	1.00 mM
Initial Laccase Activity	6.6 U/mL	0.08 U/mL
Reaction Time	15 min	60 min
pH	3	5
Oxidative Reactor		
Reactor Volume	200 mL	250 mL
PPCP (Oxybenzone) Concentration	10 µg/L (43.8 nM)	10 µg/L (43.8 nM)
Initial Expected Oxidized Mediator (i.e., Free Radical) Concentration	210 µM	9.85 µM
Mediator/Oxybenzone Molar Ratio	4800	225
Reaction (Detention) Time	60 min	30 min
Medium	Ultrapure water	pH 5 sodium acetate buffer

The extent of the ACE mediator oxidation can be predicted from the experiments reported in the previous section (Section 4.6.2). Figure 4.44 exhibits the predicted extent

of the ACE oxidation in the free radical generator reactor at the same experimental conditions as described in Table 4.11 (except for the pH). In the free radical generator reactor, experiments were performed at pH 3, while the predicted extent of the mediator ACE oxidation was calculated assuming a pH 5 buffered solution. As reported by Kunamneni et al. (2008) and Strong and Claus (2011), laccase is more active at pH 3 than at pH 5. That is, the ACE will be oxidized faster in pH 3 buffered solutions than in pH 5 buffered solutions. As observed in Figure 4.44, after approximately 10 minutes of treatment the mediator ACE would be completely oxidized to its radical form in pH 5 buffered solutions. The extent of the ACE oxidation should be at least the same or even higher when the reaction occurs in pH 3 buffered solutions. As observed in Table 4.11, enough detention time was provided (15 minutes) in the free radical generator experiments when using ACE mediator to completely convert the mediator to its radical form.

The experimental conditions described in Table 4.11 for the free radical generator experiment using ABTS mediator, were very similar to the experimental conditions for the experiments performed to evaluate the extent of the ABTS oxidation reported in the previous section (Section 4.6.3). The extent of the ABTS oxidation (i.e., the extent of the radical ABTS• production) can be observed in Figure 4.43. After 60 minutes of treatment the entire ABTS is converted to the radical ABTS•. That is, in the free radical generator experiments using ABTS mediator, enough detention time was provided in the free radical generator reactor (60 minutes) to completely converted the mediator ABTS to its radical form.

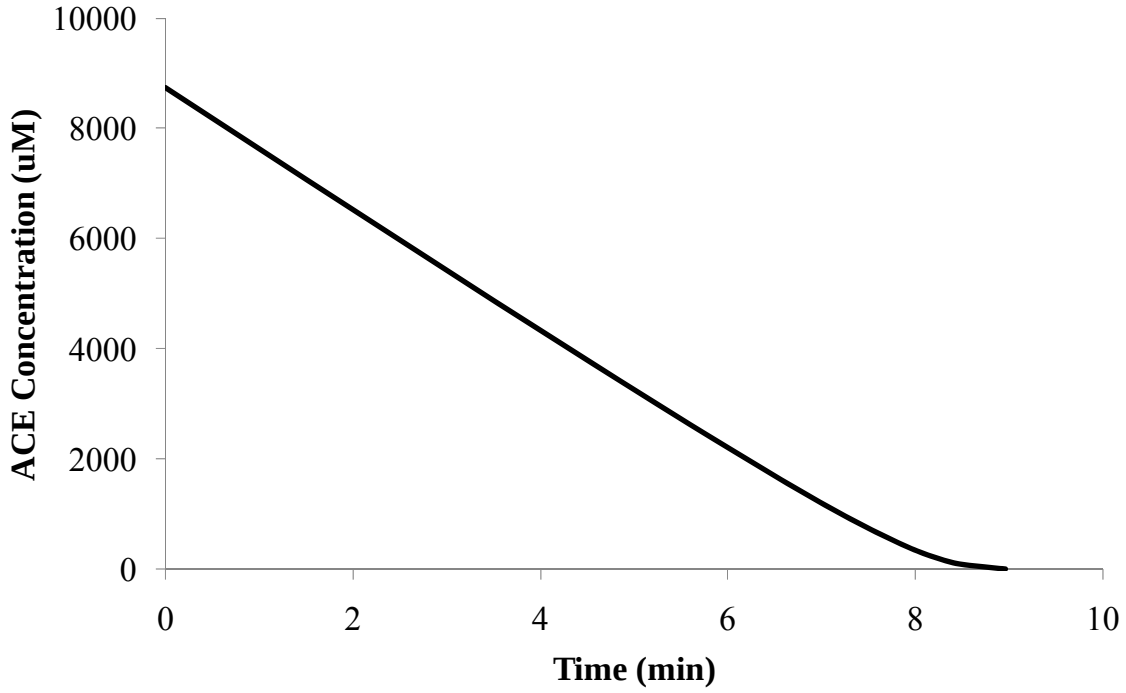


Figure 4.44 – Extent of the mediator ACE oxidation as a function of time from the integrated form of the Michaelis-Menten equation. The plot was obtained for an initial ACE concentration of 8740 µM, a laccase activity of 6.6 U/mL in a pH 5 sodium acetate buffered solution.

The experimental results for the free radical generator systems using ACE and ABTS mediators are observed in Figure 4.45. Significant removal of approximately 50% was obtained for both mediators (Figure 4.45). The free radicals generated in the free radical generator reactor were introduced to the oxidation reactor containing the target PPCP. Therefore, the removal of oxybenzone clearly indicated that the free radicals were able to react with oxybenzone. The oxidation of the target PPCP occurred due to reactions between the free radicals and the target compound.

At the experimental conditions evaluated, the free radical generator system was not effective for completely removing the target compound. The ABTS free radical performed better than the ACE free radical. The same oxybenzone removal was obtained

for ABTS and ACE mediators at mediator/oxybenzone molar ratios of 225 and 4800, respectively. Moreover, the retention time in the oxidative reactor was 30 minutes for the ABTS mediator compared to 1 hour for the ACE mediator. The better performance of the ABTS mediator compared to the ACE mediator was already observed and discussed in previous sections of this research (Sections 4.1, 4.2, 4.3 and 4.4). The lower stability of the ACE radicals compared to the ABTS radicals promote self-coupling reactions between ACE radicals, decreasing the ACE radical concentration (oxidative power) available for reacting with the target compounds.

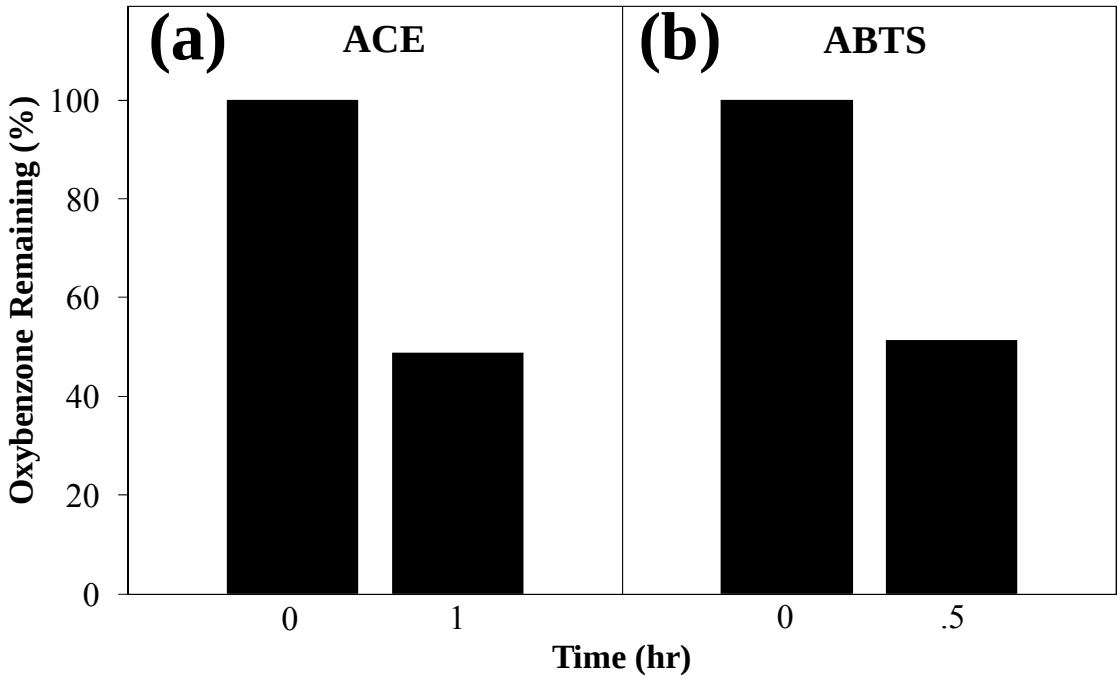


Figure 4.45 – Oxybenzone removal experiments conducted using the free radical generator system with (a) ACE mediator and (b) ABTS mediator.

Although significant removal of the target PPCP was observed, the experiments were performed in ultrapure water solutions. The performance of the free radical

generator configuration can be affected when working in more complex matrices such as primary effluents. Other organic constituents in the primary effluent wastewater might compete for the mediator free radicals.

4.9 TREATMENT IMPLICATIONS

It would be desirable to install an oxidative treatment for PPCPs before biological treatment so that biodegradable byproducts could be removed in the aeration basin. However, it is not feasible to install ozonation or AOPs before biological treatment due to the concentrations of organic matter and dissolved solids present in primary effluent. These constituents compete with target pollutants for free radicals, lowering treatment efficiency and requiring cost prohibitive oxidant doses (Ikehata et al., 2006). In contrast, enzymatic treatment, as demonstrated in this research, shows potential for eventual implementation prior to biological treatment in a conventional wastewater treatment plant. Although some wastewater constituents, such as heavy metals, organic compounds, and proteolytic enzymes, have the potential to inactivate laccase, additives and enzyme immobilization procedures have been applied with success to overcome losses in enzyme activity (Cabana et al., 2007b). In addition, recent biotechnological advances allow rapid and inexpensive production of appropriate enzymes, although further studies would be required to assess the feasibility of integrating this production with the enzyme oxidation treatment process (Cabana et al., 2007b; Rodriguez and Toca-Herrera, 2007; Kunamneni et al., 2008a).

One potential obstacle to implementation of this treatment process is the need for neutral or slightly acidic pH. However, it is not expected that acidification of primary effluent to pH 6, as was performed in this research, would be required in an actual wastewater treatment plant. As discussed in Section 4.2, acidification was needed due to the small volumes of primary effluent utilized in these laboratory experiments, with a consequent high surface area to volume ratio, which promoted rapid gas transfer. A wastewater treatment plant with neutral or slightly acidic wastewater is likely to be able to use enzymatic treatment with little to no acidification. Moreover, typical pH values for

domestic municipal wastewater in the U.S. range from pH 6.7 to pH 7.5 with an average value of pH 7.0 (Qasim, 1999), and typical alkalinity values range from 50 to 200 mg/L as $CaCO_3$ with an average of 100 mg/L as $CaCO_3$ (Qasim, 1999). The pH and alkalinity values observed in Austin's municipal wastewater are considerably higher than the average values in the U.S.

Most of the experiments in this research were performed in batch reactors with a well-mixed solution consisting of the PPCP to be oxidized, the laccase enzyme, and a specific mediator in primary effluent solutions. Alternative treatment configurations can make the laccase-mediator oxidation process more advantageous. Allowing the enzyme and mediator to interact prior to coming in contact with the target compound, would exhibit some advantages. This treatment configuration may decrease the required enzyme and mediator doses, and thereby decrease treatment costs. Moreover, the enzyme can be immobilized which would allow reuse of the oxidative activity of laccase for many cycles of oxidation, reducing the treatment cost even further. In addition, optimizing conditions such as pH for the laccase oxidation of the mediator will be easier and less expensive. A significant removal of the target PPCP (oxybenzone) was achieved using this treatment configuration, indicating that this treatment procedure shows promise for further development.

The requirement for relatively high mediator concentrations introduces an extra cost to a potential enzymatic treatment system. Although some food processing wastes could potentially be used as mediators, more research is required to determine if this would be feasible in a wastewater treatment application. Some mediators might be toxic at high concentrations. Many authors, such as Camarero et al. (2008) and Kunamneni et al. (2008), have stated that the main drawback of using synthetic mediators such as ABTS and HBT is their potential toxicity, although neither negative environmental

effects nor toxic effects for these compounds have been reported. Moreover, mediators are consumed to a great extent by the enzyme oxidation, and placing this enzymatic treatment before biological treatment in a conventional wastewater treatment plant might allow for mediator oxidation byproducts, like PPCP oxidation byproducts, to be removed in subsequent treatment processes.

Byproducts of laccase-catalyzed oxidation might be removed in subsequent biological treatment processes, but this result has not yet been confirmed in this research. This removal could occur by biodegradation or adsorption to the biomass present in activated sludge (Nakamura and Mtui, 2003). It would be desirable for byproducts to be biodegraded. If they are adsorbed to biomass or not removed at all, they will still be present in the waste activated sludge or treated wastewater, respectively. In these cases, the fate and toxicity of byproducts would need to be examined (Kinney et al., 2006; McClellan and Halden, 2010).

A potential application of this treatment technology will take advantage of the existing municipal wastewater treatment plant infrastructure with the incorporation of a laccase-oxidation reactor between the primary and secondary treatment processes. The main operational costs of the laccase-mediator treatment system are determined by the cost of the mediators and the cost of the laccase enzyme. As previously shown in this research, a phenolic mediator (ACE) concentration of 80 μM was required to remove environmentally relevant concentrations of a target representative PPCP in batch reactors from primary effluent solutions in approximately 2 hours of treatment. For a municipal wastewater treatment plant with a treatment capacity of one million gallons per day (1 MGD), and assuming plug flow reactor conditions (similar to the hydraulic behavior observed in batch reactors), 296 mol/day of a mediator will be required to reach a mediator concentration of of 80 μM in the reaction mixture. If phenol is used as a

representative phenolic mediator, 28 kg per day of phenol will be required. Phenol can be purchased at $1.5/Kg (ICIS, 2011). Therefore, the cost of the phenolic mediator would be approximately $15,000 per year for a 1 MGD plant treatment capacity. Similar calculations can be carried out to estimate the needs for the laccase enzyme. Assuming a required laccase activity of 0.01 U/mL in the reaction mixture, $37x10^6$ U/day of laccase will be required for a 1 MGD treatment plant . Novozymes Corp. provides laccase with an activity of 1,000 U/mL at approximately $30/Kg. At a laccase activity of 1,000 U/mL, approximately 30 Kg per day of laccase will be required to maintain a laccase activity in the reaction mixture of 0.01 U/mL. Thus, the cost of the laccase enzyme would be approximately $328,000 per year for a 1 MGD treatment plant .

The total operational cost of the laccase/mediator system for a 1 MGD municipal wastewater plant treatment capacity will be approximately $343,000 per year. Even though the cost of supplying a phenolic mediator is not excessively high, the use of inexpensive mediator sources such as waste streams generated by several agricultural and food-processing industries can reduce the costs related to the mediator supply. The major contribution to the laccase-mediator treatment costs, is the need for a continuous supply of the laccase enzyme. Although it was not investigated in this research, laccase requirements might be significantly reduced by reusing the enzyme. Kunamneni et al. (2008b) has successfully immobilized laccase and demonstrated that the immobilized laccase could be reused up to 17 times, retaining 87% of its oxidative capacity. Therefore, by using an inexpensive source of mediators and by immobilizing the enzyme, the treatment cost might be significantly reduced.

For comparison purposes, the operational cost of other treatment alternatives for PPCP removal was estimated as follows. The cost estimation for ozone disinfection as a tertiary wastewater treatment process for a 1 MGD plant is expected to be approximately

$74,000 per year (EPA, 1999). The ozone required to oxidize PPCPs would be expected to greatly exceed the requirements for disinfection, so the cost shown here should be considered a minimum. As for reverse osmosis (RO), a water treatment plant in Froid, Montana reported operation and maintenance costs of approximately $1,200 per million gallons of finished water (USDA, 1995). Assuming a treatment capacity of 1 MGD, the cost would be approximately $438,000 per year. The costs for a RO plant after typical wastewater treatment would be more expensive than this reported value, because greater pretreatment would be required. These cost estimates indicate that the operational cost of the laccase-mediator treatment systems is not significantly different than the operational cost of other treatment technologies. With further development, the laccase-mediator system might provide a cost-effective alternative for removing PPCPs from primary effluent sources.

Chapter 5 – Conclusions

5.1 Conclusions

- In the presence of ABTS or ACE mediators, laccase can effectively remove oxybenzone from municipal wastewater primary effluent. The mediator is oxidized by laccase to a free radical, which in turn oxidizes oxybenzone; oxybenzone is not oxidized by laccase directly. In general, ABTS, a synthetic mediator, achieves more efficient removal of oxybenzone than ACE, a natural mediator. When using the ABTS mediator lower ABTS concentration are required to achieve the same extent of oxybenzone oxidation than when using the ACE mediator.
- The laccase-mediator system can achieve complete removal of oxybenzone from primary effluent at a high initial oxybenzone concentration (4.38 μM, 1000 μg/L) and at an environmentally relevant concentration (43.8 nM, 10 μg/L).
- The extent of oxybenzone oxidation in primary effluent is dependent on the mediator/oxybenzone molar ratio. The minimum required molar ratio is dependent on the initial oxybenzone concentration; higher molar ratios are required at lower oxybenzone concentrations. Our results suggest that, in primary effluent, a specific concentration of the mediator is required, independent of the oxybenzone concentration.
- Enzyme activity was affected by three factors: (i) pH; (ii) the wastewater matrix; and (iii) reaction byproducts generated by the oxidation of the mediator by laccase. The importance of these factors varied under different experimental conditions. Greater enzyme activity was retained when experiments were initiated at pH 6 (as opposed to pH 7.67), when experiments were performed in

ultrapure water (as opposed to primary effluent), and at lower mediator concentrations.

- The laccase-mediator system does not completely mineralize oxybenzone but instead generates oxidation byproducts. In buffered ultrapure water, different mediators yielded different byproducts for the same target compound. In the presence of the ABTS mediator, one oxidation byproduct was detected. In the presence of the ACE mediator, several byproducts were detected. The m/z ratios of most of the byproducts detected were higher than that of oxybenzone, indicating that mediator free radicals and oxybenzone undergo oxidative coupling reactions.
- Ozonation of oxybenzone also produces many byproducts, but with lower m/z ratios that are similar to the molar mass of the parent compound.
- The mechanism of laccase oxidation of the mediator ACE was determined. ACE is oxidized by laccase to a free radical compound. The mediator free radicals are transformed to more stable products following two main routes: i) free radical decay (producing compounds with molecular weights similar to the parent mediator compound); and ii) oxidative coupling reactions (producing compounds with larger molecular weights than the parent mediator compound). When the reaction mixture contains just the ACE mediator and laccase (that is, no target PPCPs are present in solution), the ACE free radicals can react by self-coupling oxidation reactions producing several oxidation byproducts with larger molecular weights than the parent mediator ACE. When the reaction mixture contains a target PPCP in addition to the ACE mediator and laccase, the ACE free radicals can react by cross-coupling oxidation reactions between the ACE free radical and

the target PPCP to produce compounds with larger molecular weight than both the mediator and target PPCP.

- Laccase oxidation of the mediator ACE follows Michaelis-Menten kinetics. The extent of ACE mediator oxidation can be predicted as a function of time for a given set of experimental conditions such as ACE initial concentration, laccase initial activity, temperature, pH, and solution composition.
- The rate of laccase oxidation of the mediator ACE is decreased in a municipal wastewater primary effluent matrix. Primary effluent solutions contain several organic and inorganic constituents that inhibit laccase activity towards the substrate (ACE mediator). Higher retention times are required in primary effluent solutions to achieve the same degree of laccase oxidation of the ACE mediator than in ultrapure water buffered solutions.
- Laccase oxidation of the ABTS mediator produces very stable ABTS free radicals. ABTS free radicals do not couple with each other or decay, so the ABTS free radicals generated are available to react with the target contaminants. The high stability of the ABTS radicals as compared to the ACE radicals may explain the superior PPCP removal observed when ABTS was used as the mediator.
- A better understanding of laccase oxidation mechanisms and kinetics of mediators should enable the design of more effective and efficient treatment configurations for the removal of target emerging contaminants such as PPCPs by the laccase-mediator system. Ideally, sufficient retention time should be provided to the reaction mixture to oxidize all of the mediator to its free radical form. The oxidation of the mediator to free radicals should occur in the shortest possible

reaction time, so a high concentration of mediator free radicals are available to react with the target compounds through cross-coupling oxidative reactions.

- A laccase-mediator treatment configuration where the laccase and the mediator are added to a separate reactor, and then the free radicals generated are introduced into an oxidation reactor containing the PPCP (free radical generator configuration) yields significant removal of the target PPCP. The removal of the target PPCP occurs through cross-coupling reactions between the mediator free radical and the target PPCP. In this system, the ABTS mediator performs better than the ACE mediator. A drawback of this treatment configuration is that the enzyme cannot directly oxidize PPCPs.

Since the discharge of treated wastewater is the main entry point for PPCPs into the environment, and eventually into our drinking water, removing PPCPs from municipal wastewater would reduce the threats they pose to ecosystems, as well as reduce human exposure. Results from this study indicate that the laccase-mediator system can remove a PPCP that is not directly oxidized by the enzyme. With further development, laccase-catalyzed oxidation might eventually be implemented in municipal wastewater primary effluent for the removal of a wider variety of PPCPs. In contrast, alternative treatment methods, such as ozonation and advanced oxidation processes would be cost prohibitive in primary effluent. Although laccase-catalyzed oxidation of PPCPs shows promise, additional research will be required to design a more efficient laccase-mediator treatment configuration to remove a wider selection of PPCPs, and to determine if placing enzymatic treatment before biological treatment would yield complete removal of both the target PPCPs and the oxidation byproducts.

5.2 FUTURE WORK

Additional experiments are needed to confirm or refute the hypothesis that better performance of the enzyme-mediator system could be achieved with a treatment configuration where the laccase and the mediator are added to a separate reactor, and then the free radicals generated are introduced into an oxidation reactor containing the PPCP (free radical generator configuration). The experimental results obtained to date indicate a significant removal of oxybenzone for a specific set of experimental conditions. Other experimental conditions in the free radical generator reactor such as different initial enzyme activities, reaction detention times, mediator concentrations and pHs should be evaluated. Moreover, increasing the reaction (detention) time in the oxidation reactor might lead to higher removal of the target compounds.

The requirement for relatively high mediator concentrations introduces an extra cost to a potential laccase-mediator treatment system. It would be desirable to reduce the costs of the laccase-mediator system by investigating the use of inexpensive mediator sources. Several industries (wineries, olive oil mills, and other food processing industries) generate waste streams with a high content of phenolic compounds (concentrations up to 500 mg/L) that could serve as mediators. Several wineries in the proximity of Austin, Texas generate this kind of waste. Bustamante et al. (2005) reported that polyphenol concentrations ranged from 29 to 474 mg/L, with a mean of 140 mg/L, in 21 winery effluents. A mediator concentration in this range should be sufficient for the laccase-mediator treatment. Using a food-processing waste as a mediator source would yield an inexpensive supply of mediators. Strong and Burgess (2008) have reported success with using laccase to oxidize phenolic compounds in wine-related wastewater, indicating that laccase is highly specific for these phenolic compounds, and that winery wastewater could be used as an inexpensive source of mediators. Thus, this approach

seems promising but additional research is required to confirm if it is feasible for PPCP removal from primary effluent.

In this research, the removal of just one representative PPCP, oxybenzone, was evaluated in the laccase-mediator system. A wider range of PPCPs with different chemical structures and properties should be evaluated. It would be desirable to also evaluate the laccase-mediator system for the simultaneous removal of several PPCPs.

Finally, the fate of the laccase-oxidation byproducts should be investigated. Laccase-oxidation will transform the target PPCPs into oxidation byproducts. The performance of an activated sludge process for the removal of the laccase-oxidation byproducts should be evaluated by measuring the fate of laccase-oxidation byproducts. The success of these experiments will indicate the viability of laccase-mediator treatment for the removal of not only the parent compounds but also the oxidation byproducts.

Appendix A – Equilibration of the Primary Effluent with the Atmosphere

The observed pH increase was mainly due to the high alkalinity of Austin's wastewater and the large difference, in terms of surface area exposed to the atmosphere per unit volume of liquid, between small laboratory scale reactors and full scale reactors at wastewater treatment systems. Austin's wastewater contains a rather high alkalinity because of the source water and the disposal (in the sewer) of some softening solids from the drinking water treatment plants. As noted in the text, when we completed a separate experiment "…..with no enzymes but constant mixing, the pH rose from 6.0 to 7.94 in a six-hour period, proving that the rise in pH in our experiments was not due to enzymatic reactions but only to gas/liquid equilibration."

Many municipal wastewaters, when exposed to the atmosphere, would dissolve CO_2 into the wastewater with a consequent drop in pH. The Austin wastewater, however, is the opposite; equilibration with the atmosphere releases CO_2, causing a rise in pH. As noted in the text, the alkalinity is 273 mg/L as $CaCO_3$ and the initial pH is 7.4. Assuming that the carbonate system is the only significant weak acid/base system in the water, such a water is supersaturated with respect to atmospheric CO_2. Calculations of the expected pH of the wastewater after acidified to pH 6 and equilibration with the atmosphere ($p_{CO2} = 10^{-3.5}$) suggest that the pH could rise to nearly 8.6 which is even greater than that observed in our experiments. Calculations are shown as follows:

Initial alkalinity = 273 mg/L as $CaCO_3 = 10^{-2.26}$ N

Initial pH = 7.4

k_1 = First acidity constant for the carbonate system = $10^{-6.35}$

k_2 = Second acidity constant for the carbonate system = $10^{-10.328}$

C_T = Total carbonate concentration.

The alkalinity of the system can be calculated using Equation 1. Substituting Equation 4 and Equation 5 into Equation 1, yields Equation 6. That is, if the alkalinity and pH values are known, the total carbonate concentration C_T can be calculated by applying Equation 6.

$$Alk = -[H^+] + [OH^-] + [HCO_3^-] + 2[CO_3^{-2}] \quad \text{Eq. 1}$$

$$C_T = [H_2CO_3] + [HCO_3^-] + [CO_3^{-2}] \quad \text{Eq. 2}$$

$$\alpha_o = \frac{[H_2CO_3]}{C_T} = \frac{[H^+]^2}{[H^+]^2 + [H^+]k_1 + k_1k_2} \quad \text{Eq. 3}$$

$$\alpha_1 = \frac{[HCO_3^-]}{C_T} = \frac{[H^+]k_1}{[H^+]^2 + [H^+]k_1 + k_1k_2} \quad \text{Eq. 4}$$

$$\alpha_2 = \frac{[CO_3^{-2}]}{C_T} = \frac{k_1k_2}{[H^+]^2 + [H^+]k_1 + k_1k_2} \quad \text{Eq. 5}$$

$$Alk = -[H^+] + [OH^-] + C_T\alpha_1 + 2C_T\alpha_2 \quad \text{Eq. 6}$$

Adjusting the initial pH of the primary effluent from pH 7.4 to 6

The primary effluent solution had an initial pH of 7.4, and an initial alkalinity of $10^{-2.26}$ N. When adjusting the initial pH from 7.4 to 6, the system remained closed; therefore, C_T remained constant, but the alkalinity value changed. At the initial

conditions (*i.e.,* alkalinity = $10^{-2.26}$ N, and pH = 7.4), a C_T value of $10^{-2.22}$ N was calculated by using Equation 6.

After adjusting the pH from 7.4 to a final pH of 6 the new alkalinity value was calculated (assuming C_T remained constant) by applying Equation 6. An alkalinity value of $10^{-2.73}$ N was obtained at a C_T of $10^{-2.22}$ N and at pH of 6.

Equilibration with the atmosphere

When the system was opened to the atmosphere, the alkalinity remained constant, but the C_T value changed. That is, after the system reached equilibrium, the new pH values can be calculated by applying the Equation 1, assuming that the alkalinity remained constant. By substituting Equation 8 and Equation 9 into Equation 1, the alkalinity can be expressed as a function of just $[H_2CO_3]$ and $[H^+]$ concentrations as observed in Equation 10. Since k_H is known and the value of P_{CO2} can be assumed, the $[H_2CO_3]$ concentration is known and can be calculated as shown in Equation 7. That is, if the alkalinity of the system is known, the $[H^+]$ concentration (i.e., pH) can be calculated by applying Equation 10.

$$k_H = \frac{[H_2CO_3]}{P_{CO_2}} \qquad \text{Eq. 7}$$

$P_{CO2} = 10^{-3.5}$ atm

$k_H = 10^{-1.5}$

$$[H_2CO_3] = P_{CO_2} k_H = 10^{-5} M$$

$$k_1 = \frac{[HCO_3^-][H^+]}{[H_2CO_3]} \qquad \text{Eq. 8}$$

$$k_2 = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]} \qquad \text{Eq. 9}$$

$$Alk = -[H^+] + [OH^-] + \frac{k_1[H_2CO_3]}{[H^+]} + 2\frac{k_1 k_2[H_2CO_3]}{[H^+]^2} \qquad \text{Eq. 10}$$

Using the same alkalinity value previously calculated after adjusting the pH to 6 and before opening the system to the atmosphere (alkalinity = $10^{-2.73}$ N), the final pH of the solution (after reaching equilibrium with the atmosphere) can be calculated by using Equation 10. A final pH value of 8.6 was calculated. That is, calculations show that the pH can rise from 7.4 to nearly 8.6 which is even greater than the pH rose observed in our experiments.

Appendix B – Analysis Procedure for the Use of the LC/M

1. Record all of the following in the LC/MS notebook:
    - your name,
    - your research group,
    - the date,
    - the total number of hours of usage,
    - target analyte, and
    - concentration range.

    Please do not forget to record any of this information!
2. Make sure that the correct switch is plugged in the MS. (If you are using the IC, this should be the small one. If you are using the LC, the big one should be plugged in.)
3. Make sure that the devices that you need to use are selected.
    - Go to Instrument Configuration on the Desktop
        i. LC/MS users, select the following devices:
            a. Autosampler (Surveyor AS)
            b. MS/Pump (Surveyor MS Pump)
            c. MS (TSQ Quantum)
            d. PDA (Surveyor PDA)
        ii. IC/MS users, select the following devices:
            a. MS (TSQ Quantum)
            b. DIONEX Chromatography MS Link
4. On the MS, make sure that the vacuum switch and electronics switch are both in the on position.
5. Open the N_2 gas valve and the recirculation valve on the N_2 tank, and make sure that the N_2 pressure in the tank valve is at least 100 psi.
6. (ONLY FOR TANDEM MS/MS USERS) If you are going to use the MS in the tandem MS/MS mode, make sure that the He gas valve in the tank is open, and that the pressure is at least 20 psi.

LC/MS users

7. Make sure that you are using the appropriate column. (We have one for students from chemical engineering to use and another for EWRE students to use.)
8. Turn on the UV/VIS lamps of the PDA detector.
    - Go to Xcalibur/Instument Setup/Surveyor PDA/SurveyorPDA/Direct Control/Configuration/Turn on (both lamps)

9. Re-fill the solvent bottles with the solvents to be used. **(Never let the bottles run out of solvent!!)**
    - Bottle A: LC/MS Grade Methanol
    - Bottle B: LC/MS Grade Water
    - Bottle C: Optional
    - Bottle D: Optional
10. Prime the pump using the syringe **(making sure that you completely remove all the air from the lines from all the bottles to be used)**
    - Go to Xcalibur/Instument Setup/Surveyor MS Pump/Surveyor MS Pump/Direct Control
    - Run the pump at 1000 uL/min for approximately 10 minutes. Split the flow evenly among all of the solvents that you will use in your analysis.

<u>Now you are ready to start using the LC/MS</u>

11. On the Xcalibur software go to Sequence Setup
12. Create a new folder for storing your data on Xcalibur\DATA\Your Name
13. Fill the sequence set up table with the respective sample name, path, inst method, position, and inj vol.
14. Load your samples into the autosampler tray
15. Hit Run sequence (Yes/Yes)

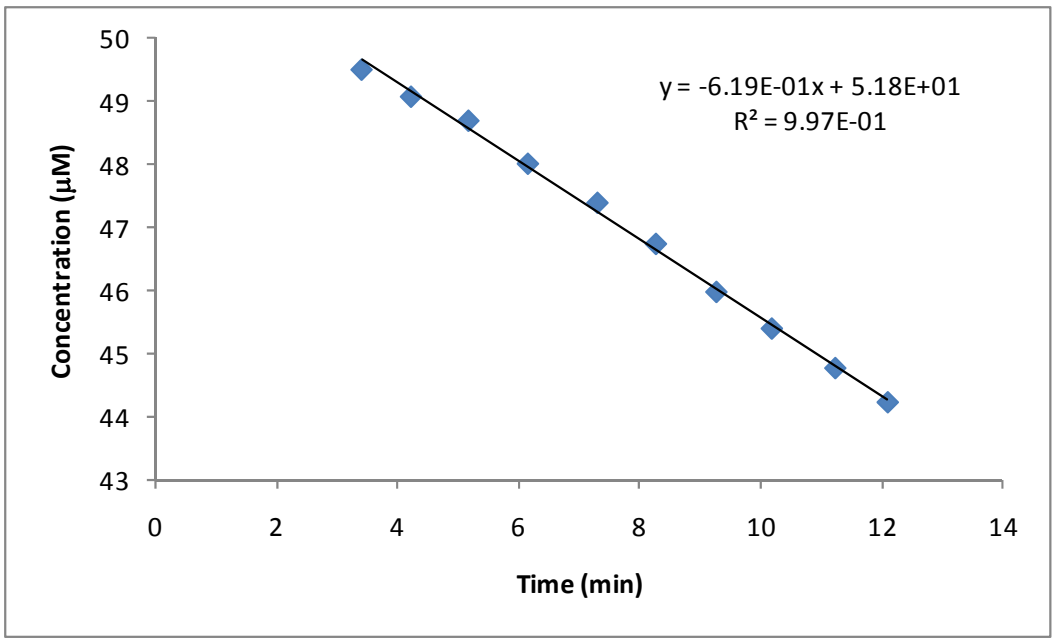
When the analysis has finished, remember to:…

1. Make sure that the pump has stopped running.
2. Turn off the UV/Vis Lamps.
3. Close the N_2 valve.

Appendix C – Experiments Performed in pH 5 Buffered Solutions

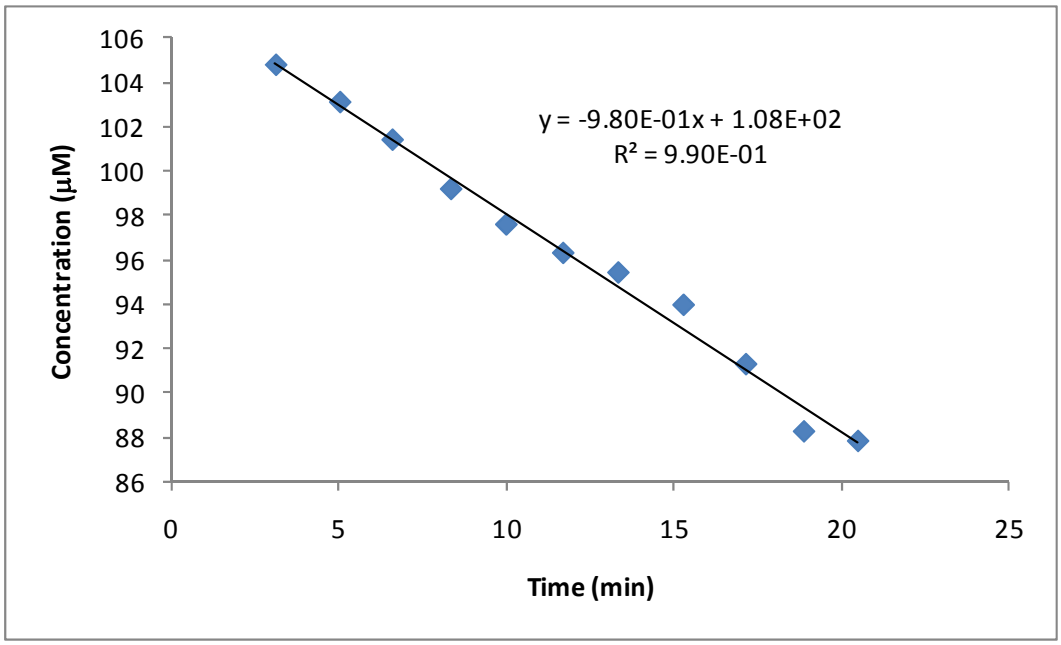
Initial ACE concentration ≈ 50 μM

Time (min)	Absorbance	Concentration (μM)
3.4	0.5235	49.5
4.2	0.5189	49.1
5.2	0.5149	48.7
6.2	0.5077	48.0
7.3	0.5011	47.4
8.3	0.4943	46.7
9.3	0.4862	46.0
10.2	0.4801	45.4
11.2	0.4734	44.8
12.1	0.4677	44.2



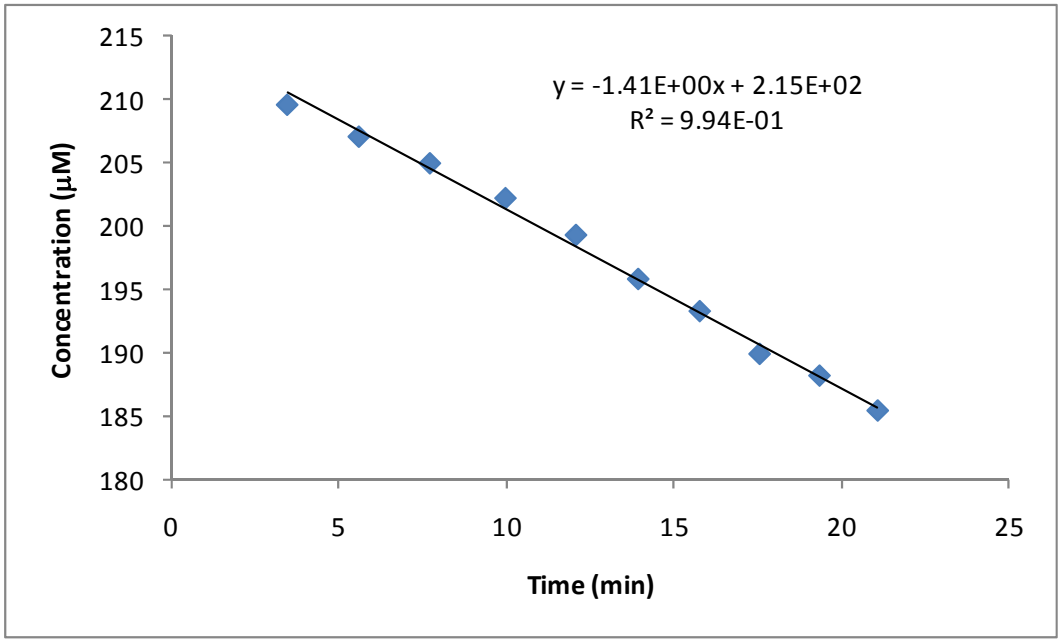
Initial ACE concentration ≈ 100 μM

Time (min)	Absorbance	Concentration (μM)
3.1	0.3675	104.8
5.0	0.3615	103.1
6.6	0.3556	101.4
8.3	0.3478	99.2
10.0	0.3422	97.6
11.7	0.3377	96.3
13.3	0.3346	95.4
15.3	0.3294	94.0
17.2	0.3200	91.3
18.9	0.3094	88.2
20.5	0.3079	87.8



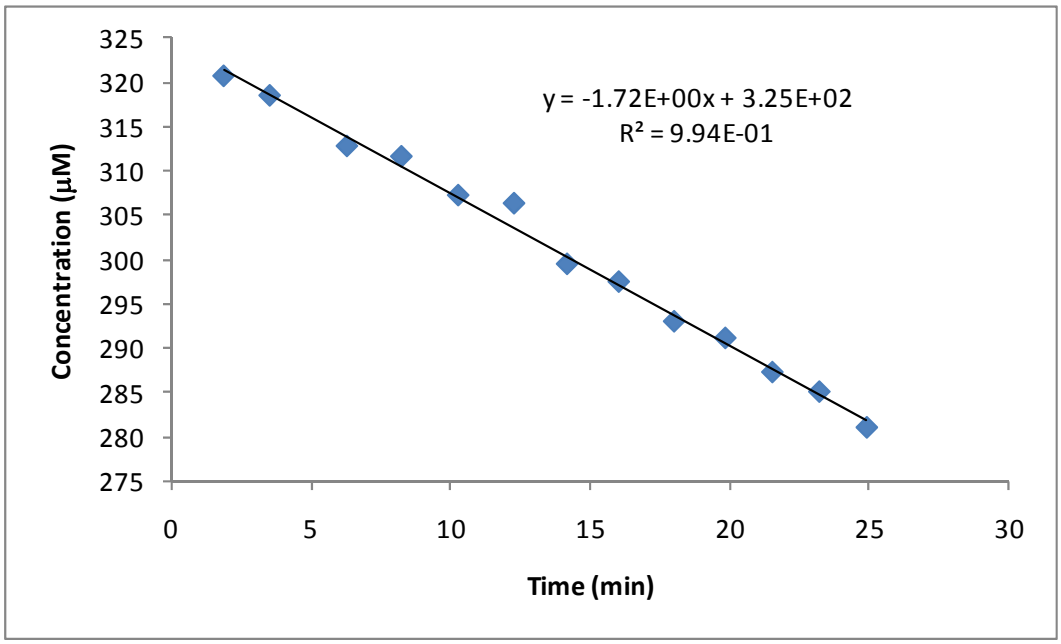
Initial ACE concentration ≈ 200 μM

Time (min)	Absorbance	Concentration (μM)
3.5	0.7196	209.6
5.6	0.7109	207.1
7.7	0.7037	205.0
10.0	0.6942	202.2
12.1	0.6842	199.3
13.9	0.6722	195.8
15.8	0.6634	193.3
17.6	0.6518	189.9
19.3	0.6459	188.2
21.1	0.6364	185.4



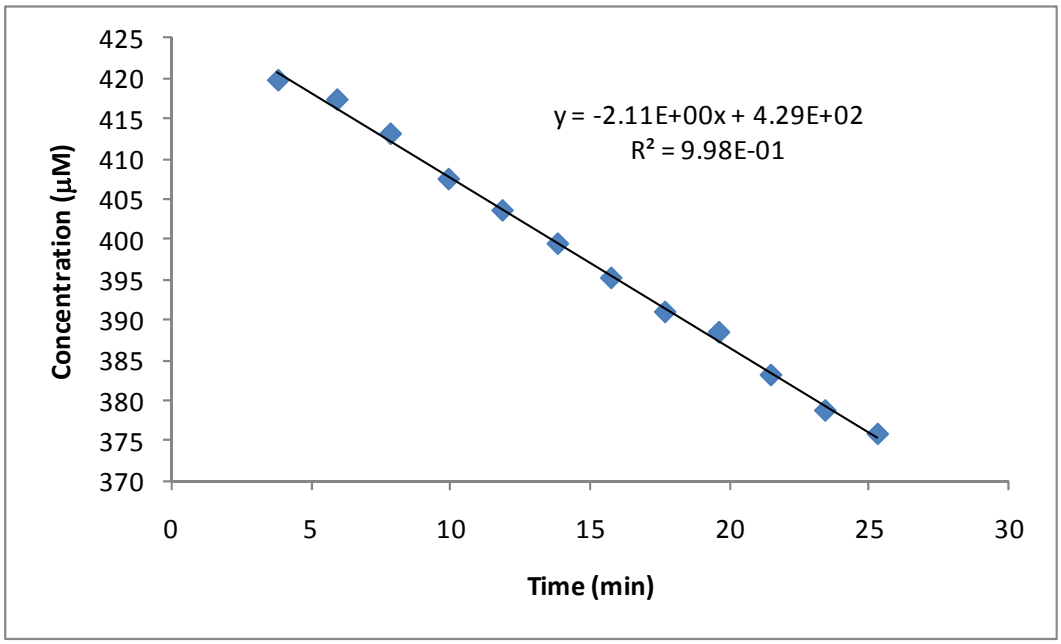
Initial ACE concentration ≈ 300 μM

Time (min)	Absorbance	Concentration (μM)
1.9	0.6667	320.7
3.5	0.6621	318.5
6.3	0.6502	312.8
8.3	0.6478	311.6
10.3	0.6387	307.2
12.3	0.6369	306.3
14.2	0.6226	299.5
16.0	0.6185	297.5
18.0	0.6092	293.0
19.9	0.6053	291.2
21.5	0.5973	287.3
23.2	0.5927	285.1
24.9	0.5844	281.1



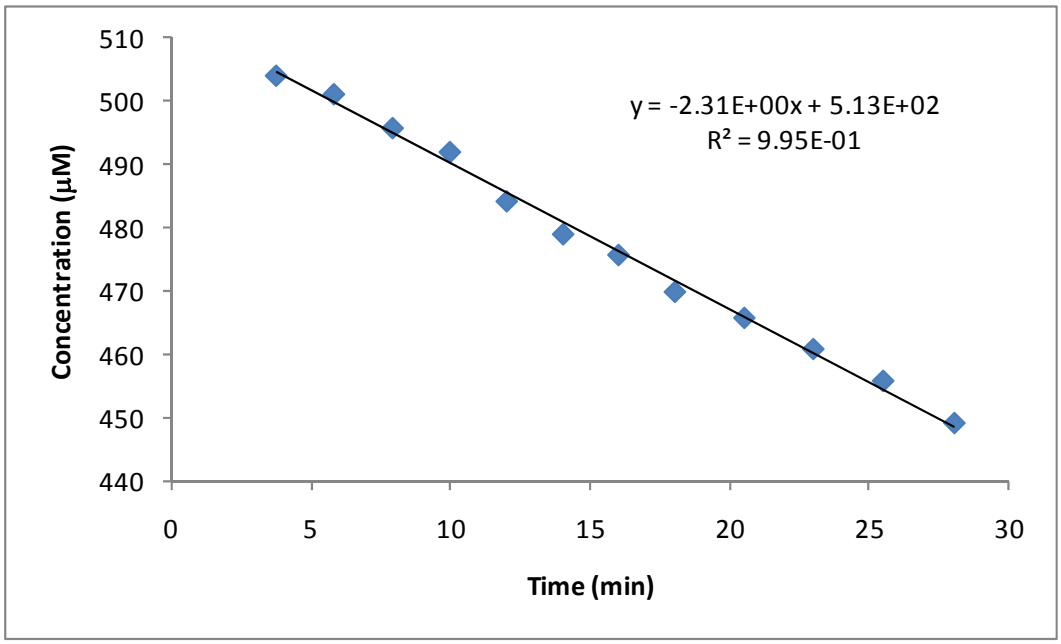
Initial ACE concentration ≈ 400 μM

Time (min)	Absorbance	Concentration (μM)
3.8	0.8786	419.8
5.9	0.8736	417.4
7.8	0.8647	413.1
9.9	0.8529	407.5
11.9	0.8448	403.6
13.8	0.8362	399.5
15.8	0.8273	395.3
17.7	0.8185	391.0
19.6	0.8132	388.5
21.5	0.8021	383.2
23.4	0.7929	378.8
25.3	0.7868	375.9



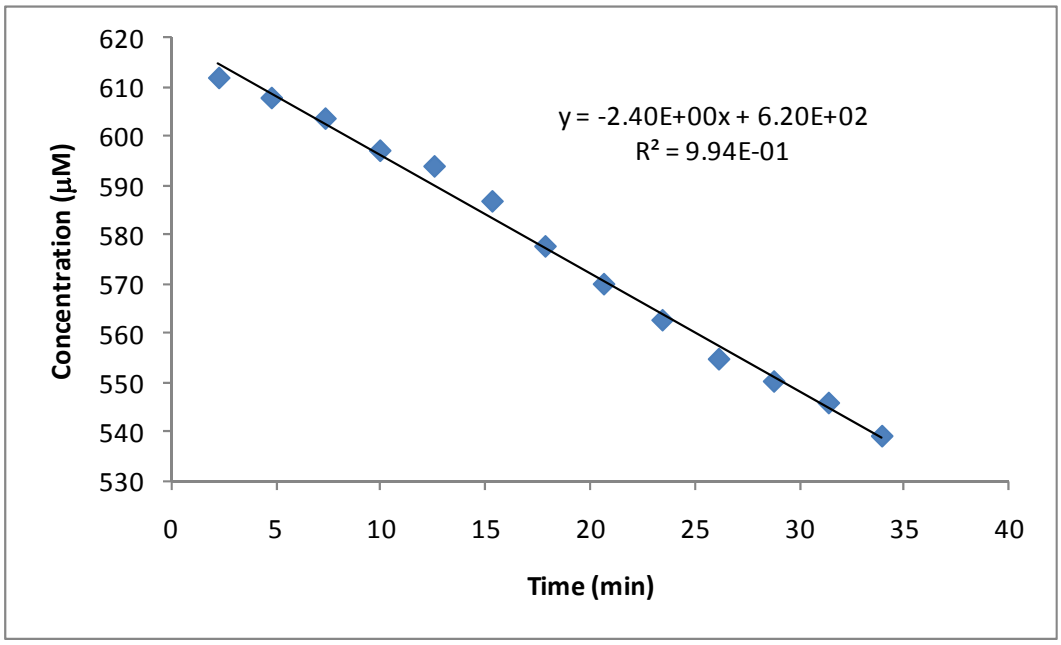
Initial ACE concentration ≈ 500 μM

Time (min)	Absorbance	Concentration (μM)
3.8	0.5275	504.2
5.9	0.5244	501.2
8.0	0.5188	495.8
10.0	0.5148	492.0
12.0	0.5067	484.2
14.1	0.5012	479.0
16.0	0.4978	475.7
18.1	0.4916	469.9
20.5	0.4873	465.8
23.0	0.4821	460.8
25.5	0.4769	455.8
28.1	0.4699	449.1



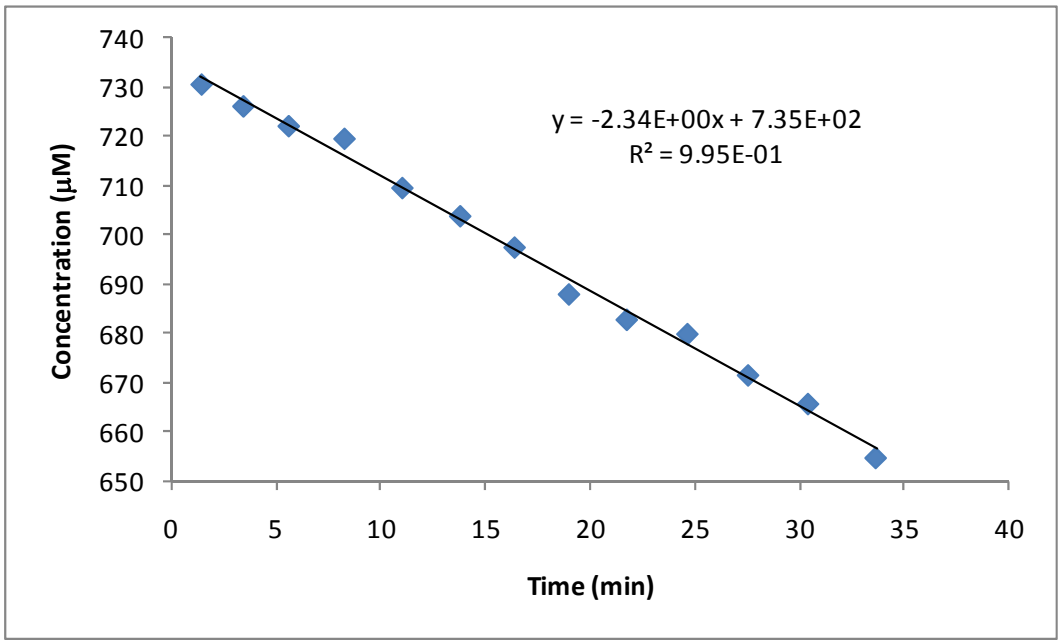
Initial ACE concentration ≈ 600 μM

Time (min)	Absorbance	Concentration (μM)
2.3	0.6382	611.8
4.8	0.6340	607.7
7.4	0.6296	603.6
10.0	0.6228	597.0
12.6	0.6195	593.9
15.3	0.6121	586.8
17.9	0.6026	577.6
20.7	0.5946	570.0
23.5	0.5869	562.6
26.2	0.5787	554.7
28.8	0.5740	550.2
31.4	0.5694	545.8
34.0	0.5624	539.1



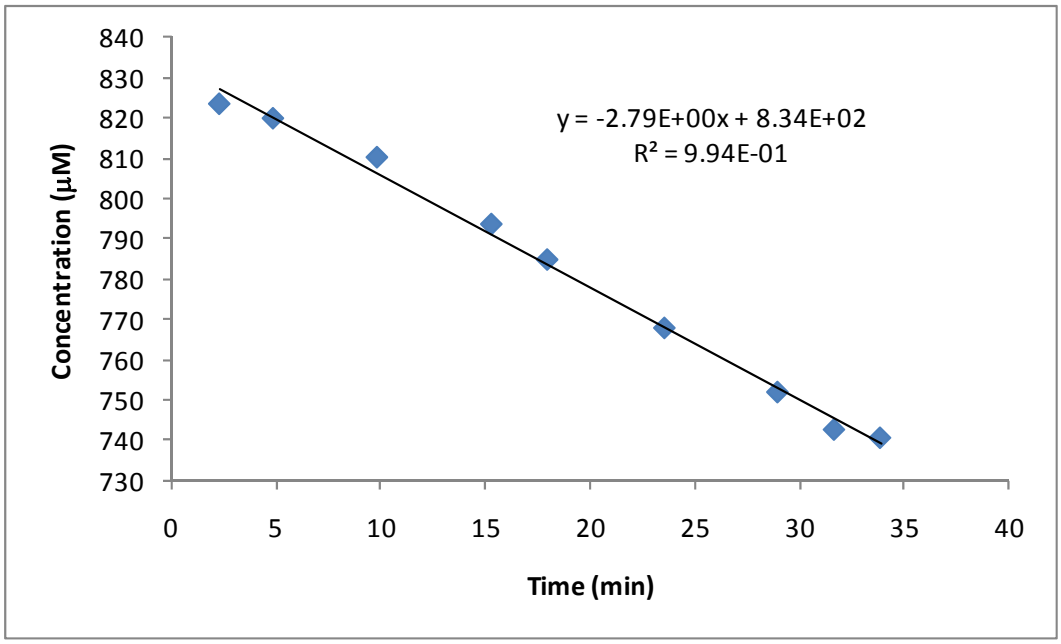
Initial ACE concentration ≈ 700 μM

Time (min)	Absorbance	Concentration (μM)
1.4	0.7478	730.7
3.4	0.7433	726.3
5.6	0.7392	722.2
8.3	0.7365	719.7
11.0	0.7263	709.7
13.8	0.7204	703.9
16.4	0.7139	697.6
19.0	0.7042	688.0
21.8	0.6989	682.9
24.7	0.6959	680.0
27.6	0.6874	671.6
30.4	0.6814	665.8
33.7	0.6702	654.8



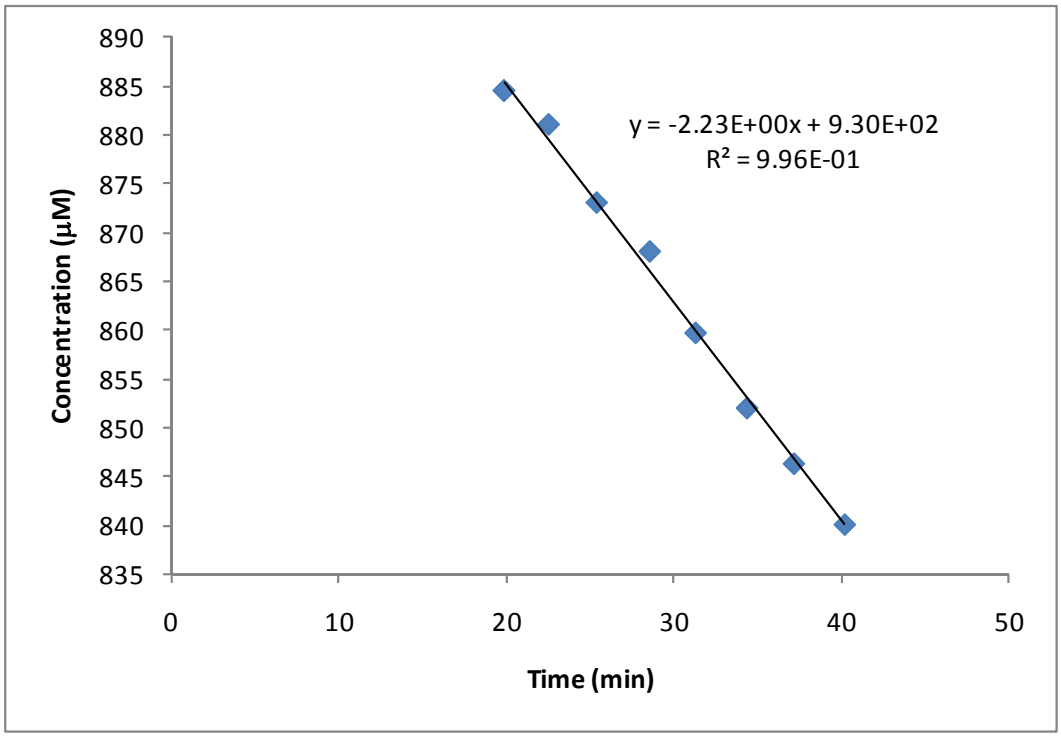
Initial ACE concentration ≈ 800 μM

Time (min)	Absorbance	Concentration (μM)
2.3	0.8438	823.3
4.9	0.8401	819.7
9.9	0.8302	810.0
15.3	0.8132	793.5
18.0	0.8042	784.7
23.6	0.7868	767.7
29.0	0.7706	751.8
31.7	0.7610	742.5
33.9	0.7589	740.5



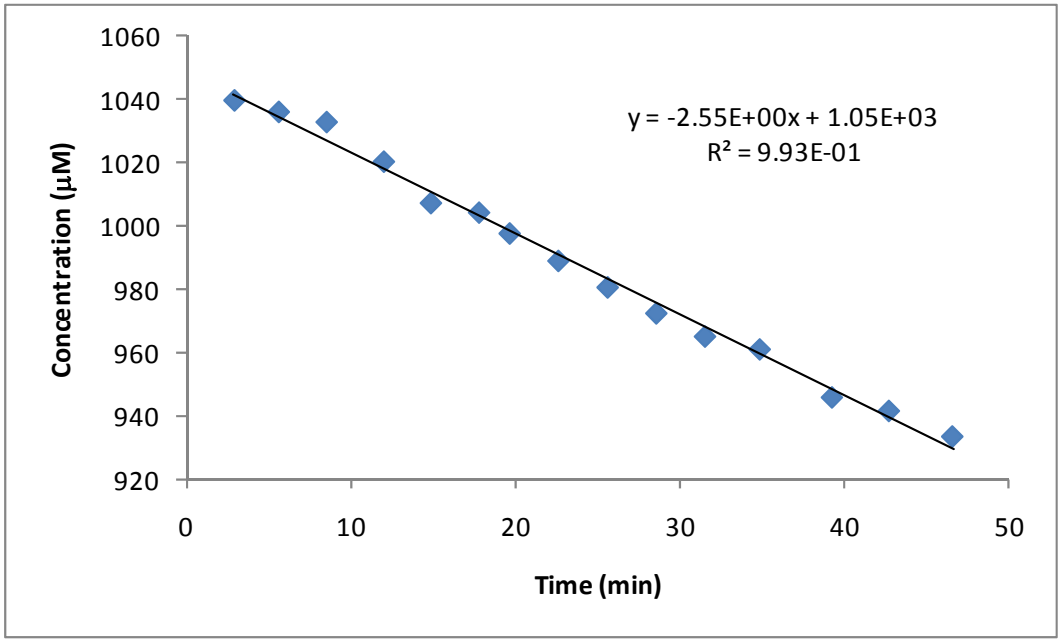
Initial ACE concentration ≈ 900 μM

Time (min)	Absorbance	Concentration (μM)
19.9	0.9293	884.5
19.9	0.9293	884.5
22.6	0.9256	881.0
25.4	0.9173	873.0
28.6	0.9120	868.0
31.3	0.9033	859.7
34.4	0.8952	852.1
37.2	0.8893	846.4
40.2	0.8827	840.2



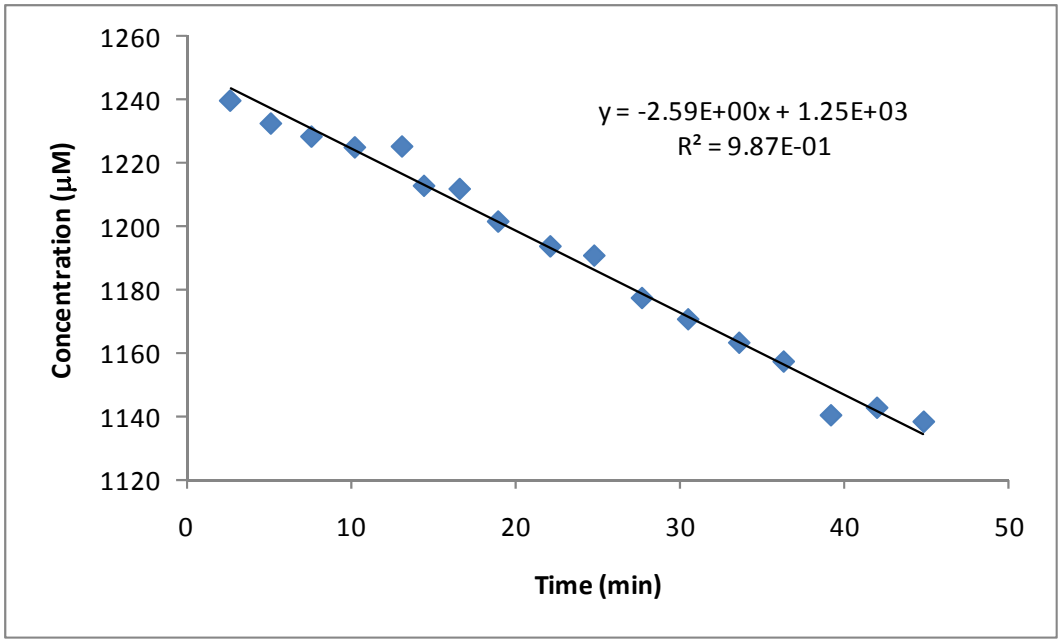
Initial ACE concentration ≈ 1000 μM

Time (min)	Absorbance	Concentration (μM)
2.9	0.7038	1039.6
5.6	0.7014	1035.9
8.5	0.6992	1032.7
12.0	0.6908	1020.3
14.9	0.6819	1007.2
17.8	0.6799	1004.2
19.7	0.6754	997.6
22.6	0.6696	989.0
25.6	0.6639	980.6
28.6	0.6584	972.5
31.6	0.6535	965.1
34.9	0.6507	961.1
39.3	0.6405	946.0
42.8	0.6376	941.7
46.6	0.6321	933.7



Initial ACE concentration ≈ 1200 µM

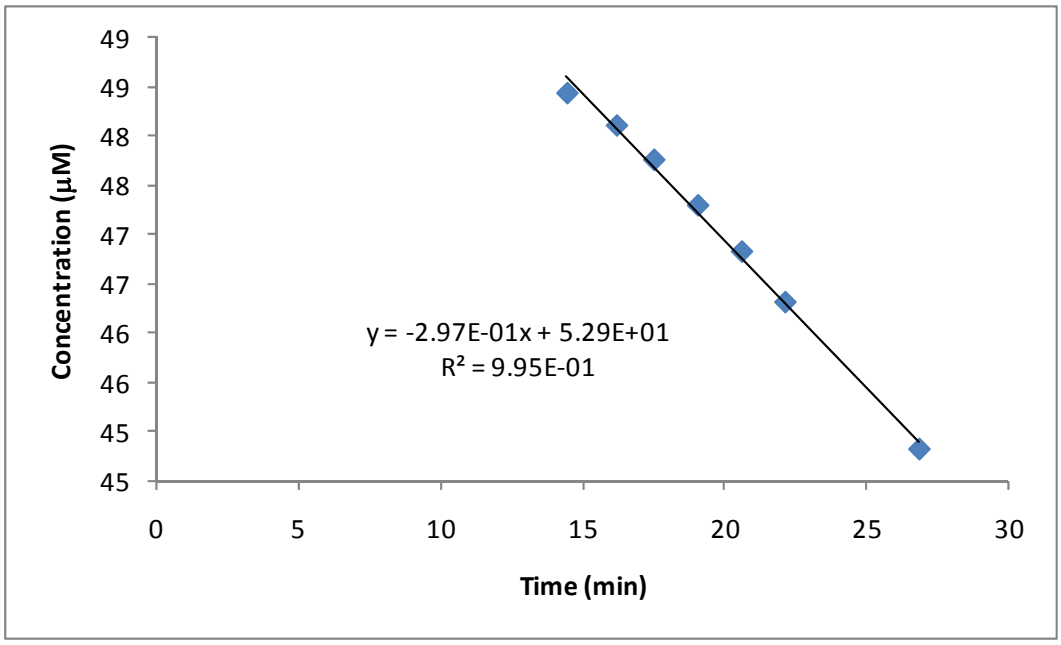
Time (min)	Absorbance	Concentration (µM)
2.7	0.8461	1240.0
5.2	0.8412	1232.7
7.7	0.8383	1228.6
10.3	0.8360	1225.2
13.2	0.8362	1225.4
14.5	0.8277	1213.0
16.7	0.8270	1212.0
19.0	0.8199	1201.6
22.2	0.8146	1193.7
24.8	0.8126	1190.8
27.7	0.8034	1177.4
30.5	0.7988	1170.6
33.6	0.7937	1163.2
36.3	0.7896	1157.2
39.2	0.7780	1140.1
42.0	0.7796	1142.5
44.8	0.7766	1138.1



Appendix D – Experiments Performed in Primary Effluent Solutions

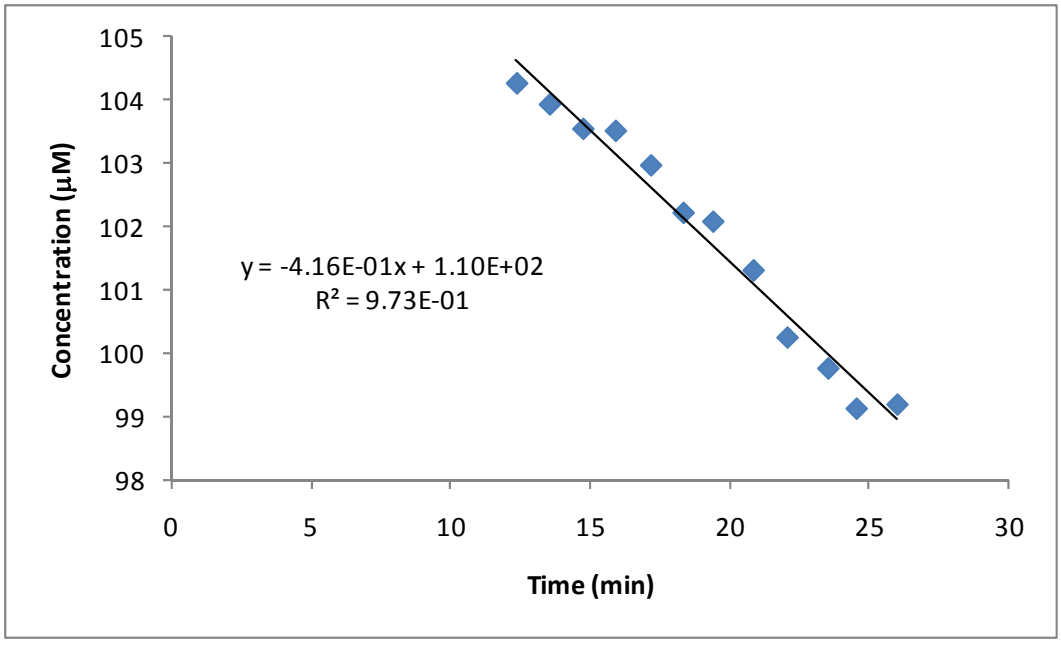
Initial ACE concentration ≈ 50 μM

Time (min)	Absorbance	Concentration (μM)
14.4	0.5208	48.4
16.2	0.5172	48.1
17.5	0.5135	47.8
19.1	0.5085	47.3
20.6	0.5035	46.8
22.1	0.4979	46.3
26.9	0.4819	44.8



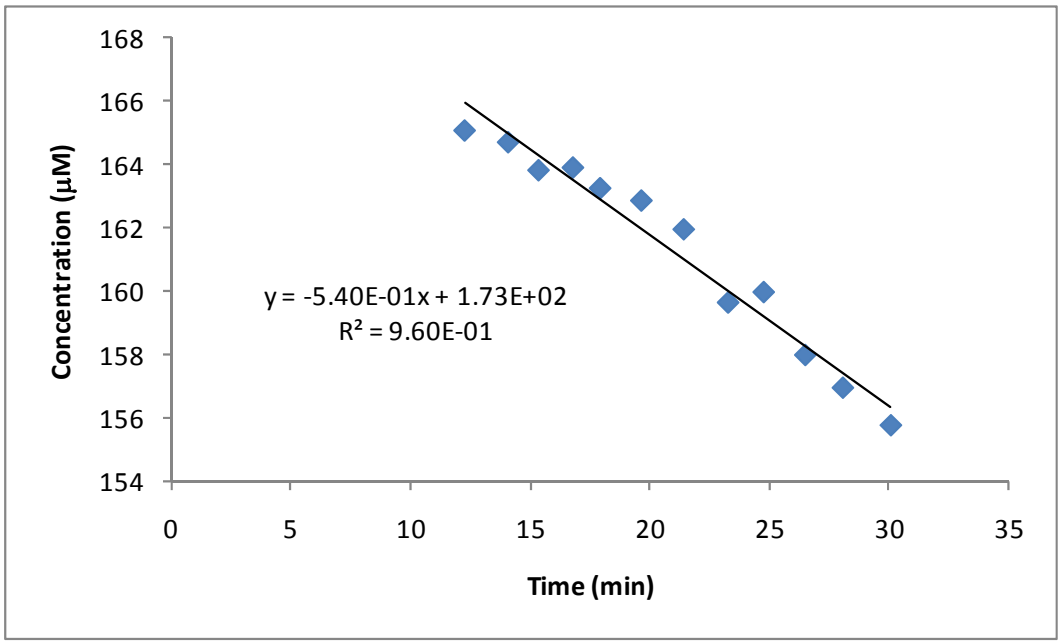
Initial ACE concentration ≈ 100 μM

Time (min)	Absorbance	Concentration (μM)
12.4	0.3693	104.3
13.5	0.3682	103.9
14.7	0.3668	103.6
15.9	0.3667	103.5
17.2	0.3647	103.0
18.3	0.3621	102.2
19.4	0.3616	102.1
20.9	0.3589	101.3
22.1	0.3551	100.3
23.5	0.3534	99.8
24.6	0.3511	99.1
26.0	0.3514	99.2



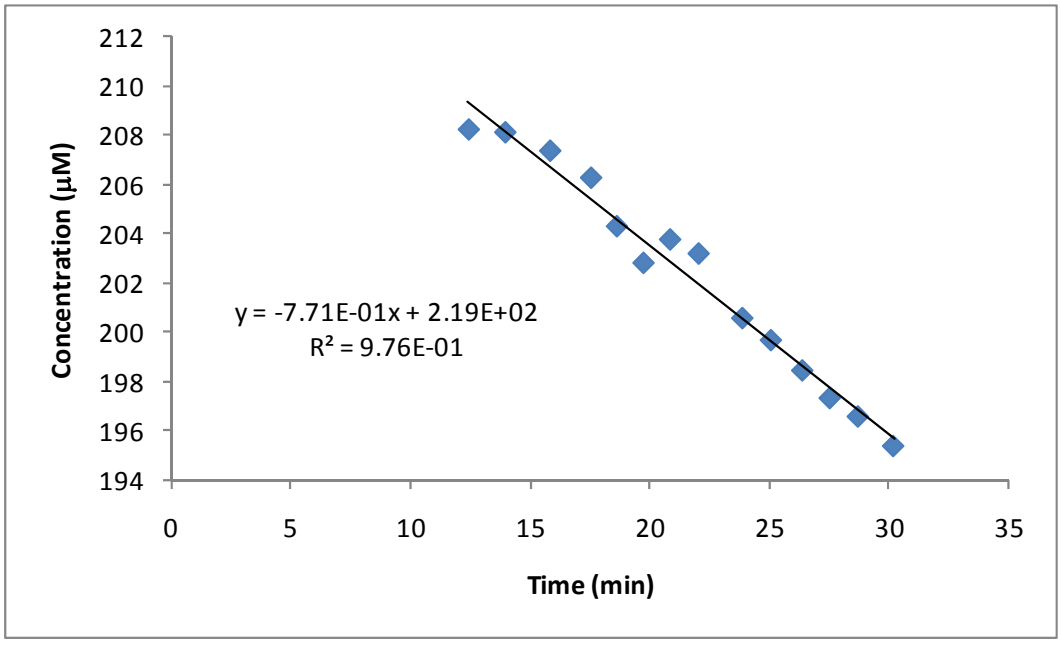
Initial ACE concentration ≈ 150 μM

Time (min)	Absorbance	Concentration (μM)
12.3	0.6057	165.1
14.1	0.6044	164.7
15.4	0.6011	163.8
16.8	0.6014	163.9
18.0	0.5990	163.2
19.7	0.5976	162.8
21.4	0.5942	161.9
23.3	0.5858	159.6
24.8	0.5870	160.0
26.5	0.5797	158.0
28.1	0.5759	156.9
30.1	0.5715	155.7



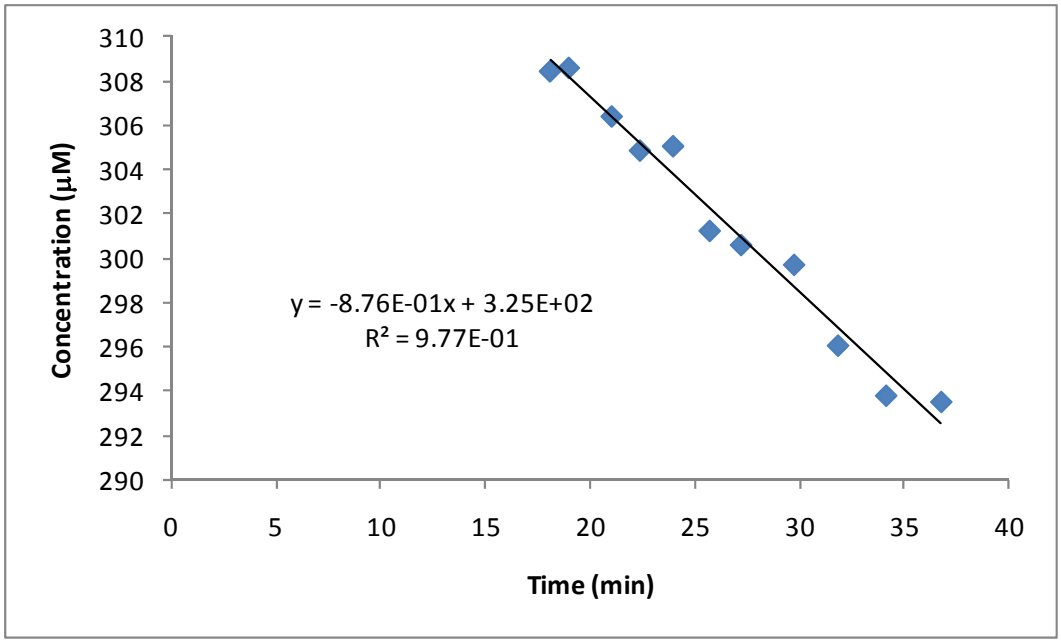
Initial ACE concentration ≈ 200 μM

Time (min)	Absorbance	Concentration (μM)
12.4	0.7396	208.2
13.9	0.7392	208.1
15.8	0.7366	207.3
17.5	0.7327	206.3
18.6	0.7257	204.3
19.7	0.7204	202.8
20.9	0.7238	203.7
22.1	0.7217	203.2
23.9	0.7124	200.5
25.1	0.7092	199.7
26.4	0.7049	198.4
27.6	0.7009	197.3
28.7	0.6982	196.6
30.2	0.6939	195.4



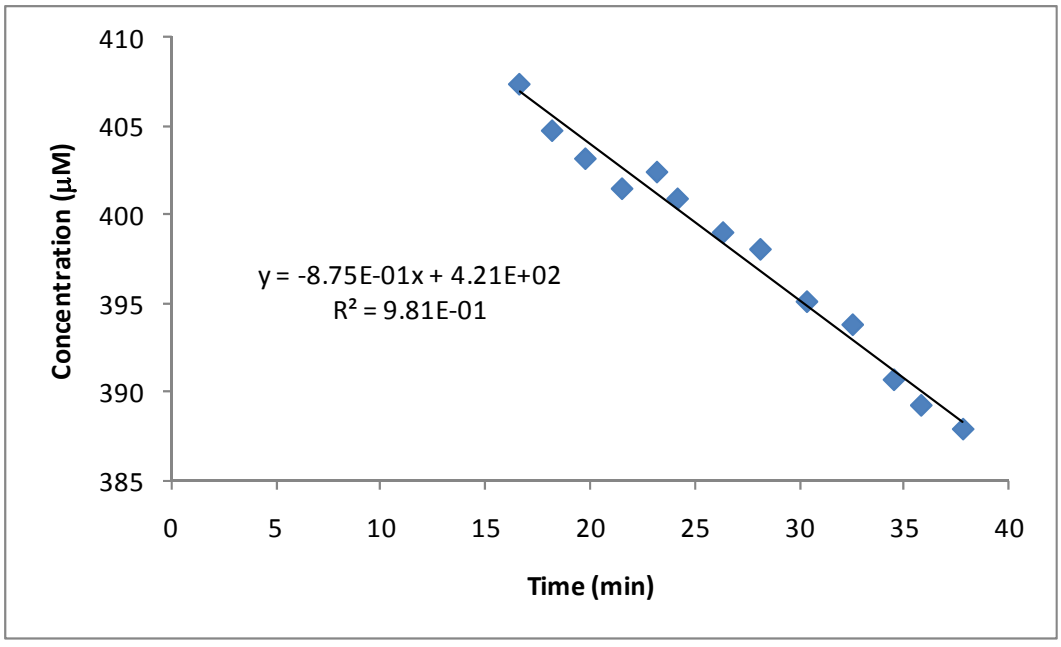
Initial ACE concentration ≈ 300 μM

Time (min)	Absorbance	Concentration (μM)
18.1	0.6715	308.5
19.0	0.6719	308.6
21.1	0.6671	306.4
22.4	0.6638	304.9
24.0	0.6642	305.1
25.7	0.6559	301.3
27.2	0.6545	300.6
29.8	0.6525	299.7
31.9	0.6446	296.1
34.2	0.6397	293.8
36.8	0.6391	293.5



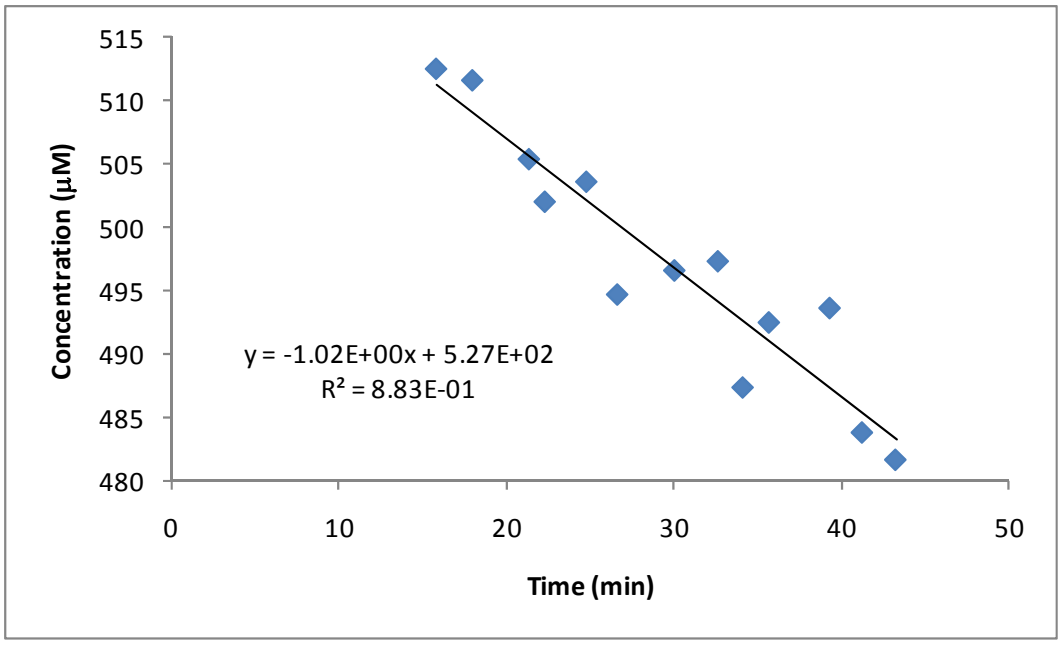
Initial ACE concentration ≈ 400 μM

Time (min)	Absorbance	Concentration (μM)
16.7	0.8975	407.3
18.2	0.8917	404.7
19.8	0.8883	403.1
21.6	0.8846	401.4
23.2	0.8866	402.4
24.2	0.8833	400.9
26.4	0.8791	399.0
28.2	0.8770	398.0
30.4	0.8706	395.1
32.6	0.8677	393.8
34.5	0.8609	390.7
35.8	0.8577	389.3
37.8	0.8548	387.9



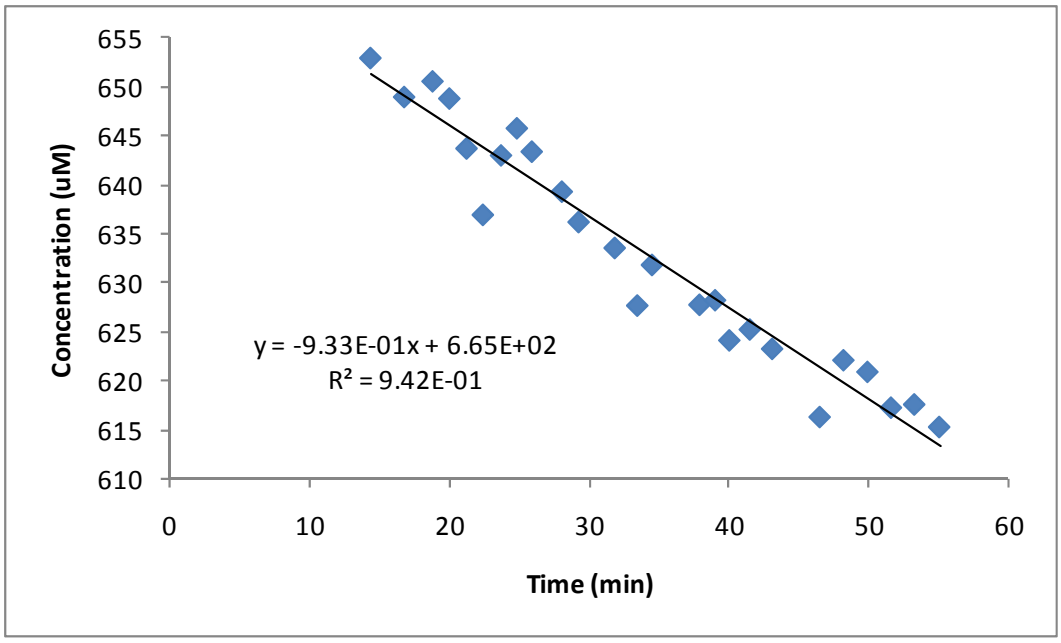
Initial ACE concentration ≈ 500 μM

Time (min)	Absorbance	Concentration (μM)
15.8	0.5591	512.6
18.0	0.5581	511.6
21.4	0.5513	505.4
22.3	0.5476	502.1
24.8	0.5493	503.6
26.7	0.5396	494.7
30.1	0.5417	496.6
32.7	0.5425	497.4
34.2	0.5316	487.4
35.7	0.5372	492.5
39.4	0.5385	493.7
41.3	0.5277	483.8
43.3	0.5254	481.7



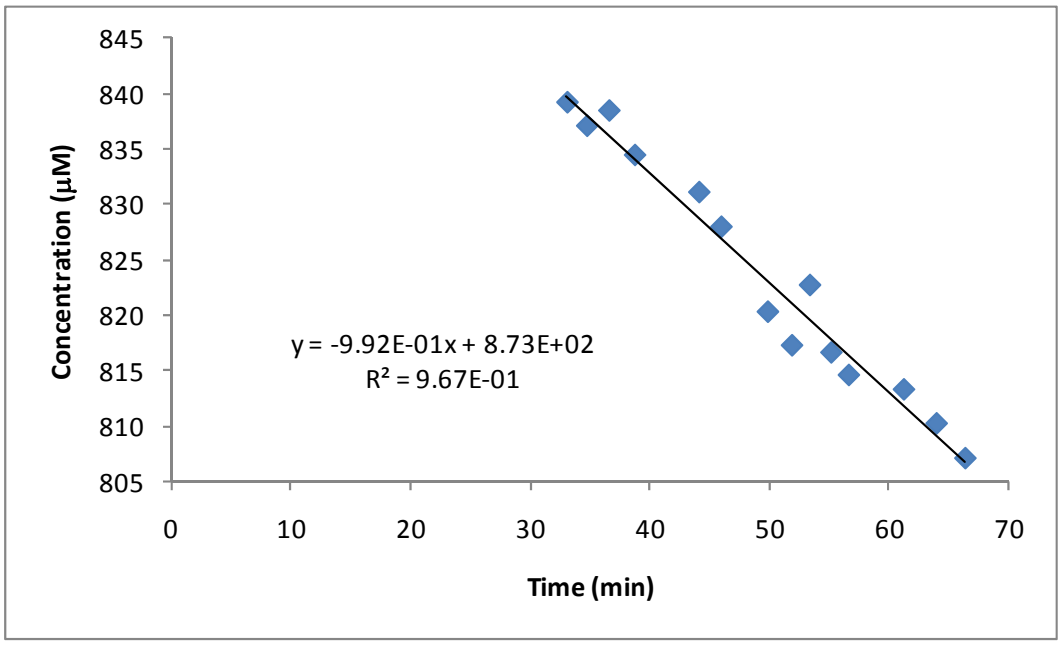
Initial ACE concentration ≈ 600 μM

Time (min)	Absorbance	Concentration (μM)
14.4	0.6949	652.9
16.8	0.6906	648.9
18.9	0.6923	650.5
20.1	0.6905	648.8
21.3	0.6851	643.7
22.5	0.6779	636.9
23.8	0.6843	643.0
24.9	0.6872	645.7
26.0	0.6847	643.3
28.1	0.6804	639.3
29.3	0.6771	636.2
31.9	0.6743	633.5
33.5	0.6681	627.7
34.6	0.6725	631.8
38.0	0.6681	627.8
39.1	0.6686	628.2
40.1	0.6643	624.1
41.6	0.6655	625.3
43.2	0.6634	623.3
46.6	0.6560	616.3
48.3	0.6621	622.1
50.0	0.6609	620.9
51.7	0.6570	617.3
53.3	0.6573	617.6
55.1	0.6549	615.3


655
650
645
640
635
630
625
620
615
610
Concentration (uM)
y = -9.33E-01x + 6.65E+02
R² = 9.42E-01
0
10
20
30
40
50
60
Time (min)

Initial ACE concentration ≈ 800 μM

Time (min)	Absorbance	Concentration (μM)
33.1	0.9464	839.1
34.7	0.9440	837.0
36.6	0.9456	838.4
38.7	0.9411	834.4
44.1	0.9373	831.0
45.9	0.9338	827.9
49.8	0.9252	820.3
51.8	0.9217	817.2
53.3	0.9279	822.7
55.1	0.9210	816.6
56.6	0.9187	814.6
61.2	0.9173	813.3
63.9	0.9138	810.2
66.3	0.9103	807.1



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Vita

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