

NOVEL BLUE MUSSEL (*Mytilus edulis*) EXTRACT INHIBITS POLYPHENOL OXIDASE
IN FRUIT AND VEGETABLE TISSUE

By

ROBERT SCHWARTZ BENT

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2009

© 2009 Robert Schwartz Bent

To my family.

ACKNOWLEDGMENTS

First and foremost, I thank my family for their unconditional support in this journey through graduate school. It would have been impossible without their help. I thank Dr. Marshall and my committee for their time, effort, and personal attention over the last three years. I thank Emilia for her patience and company through many late nights in the lab. Finally, I thank Dr. Kurt Schulbach for his incredible dedication to this project and for the numerous lessons he taught me during my time working with him.

TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS	4
LIST OF TABLES	7
LIST OF FIGURES	8
ABSTRACT	11
CHAPTER	
1 INTRODUCTION	13
2 LITERATURE REVIEW	17
Problems Surrounding PPO	17
Biochemistry of PPO Reactions	18
Plant PPO	20
Properties of Apple, Banana, Potato and Avocado PPOs	24
Arthropod PPO	26
Hypotaurine	29
Blue Mussel	30
Inhibition of Enzymatic Browning	30
Objectives and Hypothesis	33
3 MATERIALS AND METHODS	38
Machine Vision Analysis of Color Development	38
Preparation of Inhibitor from Blue Mussel	38
Preparation of Puree for Testing Browning Inhibition of Blue Mussel Extract	39
Machine Vision Analysis	40
Enzyme Kinetics	40
Polyphenol Oxidase Preparation	40
Determination of IC ₅₀	42
Electrophoretic Separation of PPO Isoforms	43
4 RESULTS AND DISCUSSION	45
Machine Vision Analysis of Color Development	45
Enzyme Kinetics	48
Comparison of Novel Inhibitor to Other Browning Inhibitors	50
5 CONCLUSIONS	75
APPENDIX ADDITIONAL DATA	77

LIST OF REFERENCES90
BIOGRAPHICAL SKETCH101

LIST OF TABLES

<u>Table</u>		<u>page</u>
2-1	pH and temperature optima for apple, banana, potato, and avocado PPOs, as well as substrate specificities ordered from highest to lowest	35
3-1	Camera settings for Machine Vision experiments	44
4-1	Kinetic parameters for apple, avocado, banana, and potato	53

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
2-1 Locations within a plant cell of phenolic compounds, as well as polyphenol oxidase and peroxidase enzymes (Toivonen and Brummell 2008).	35
2-2 Mechanism of action for PPO upon its substrate compounds (Toivonen and Brummell 2008).	36
2-3 PPO-catalyzed oxidation of L-DOPA to melanin.....	37
4-1 Untreated samples in the first apple trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas	54
4-2 Untreated samples in the first apple trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas.....	55
4-3 Treated samples in the first apple trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas.....	56
4-4 Treated samples in the first apple trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas	57
4-5 Untreated samples in the second apple trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas	58
4-6 Untreated samples in the second apple trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas.....	59
4-7 Treated samples in the second apple trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas	60
4-8 Treated samples in the second apple trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas.....	61
4-10 Untreated samples in the first banana trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas.....	63
4-12 Treated samples in the first banana trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas.....	65
4-9 Untreated samples in the first banana trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas	62
4-11 Treated samples in the first banana trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas	64

4-13	Apple and avocado: comparison between control and treated L* values at each time point	66
4-14	Banana and potato: comparison between control and treated L* values at each time point	67
4-15	Apple kinetics plots.....	68
4-16	Avocado kinetics plots.....	69
4-17	Banana kinetics plots	70
4-18	Potato kinetics plots	71
4-23	Results of native gel electrophoretic separation of crude PPO isolates.....	74
4-19	Apple: plots of percent activity vs. inhibitor concentration to determine IC50.....	72
4-20	Avocado: plot of percent activity vs. inhibitor concentration to determine IC50	72
4-21	Banana: plot of percent activity vs. inhibitor concentration to determine IC50.....	73
4-22	Potato: plot of percent activity vs. inhibitor concentration to determine IC50.....	73
A-1	Untreated samples in the first avocado trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas	77
A-2	Untreated samples in the first avocado trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas.....	78
A-3	Treated samples in the first avocado trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas	79
A-4	Treated samples in the first avocado trial; 24-72 hours and one week; color blocks and percentage surface areas.....	80
A-5	Untreated samples in the first potato trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas	81
A-6	Untreated samples in the first potato trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas.....	82
A-7	Treated samples in the first potato trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas	83
A-8	Treated samples in the first potato trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas	84

A-9	Apple: browning progression in treated and untreated samples during Machine Vision experiment.....	85
A-10	Avocado: browning progression in treated and untreated samples during Machine Vision experiment.....	86
A-11	Banana: browning progression in treated and untreated samples during Machine Vision experiment.....	87
A-12	Potato: browning progression in treated and untreated samples during Machine Vision experiment.....	87
A-13	Time zero SAS output for apple machine vision L* data.....	88
A-14	Time 24 hours SAS output for apple machine vision L* data.....	88
A-15	Color block reference legend.....	89

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

NOVEL BLUE MUSSEL (*Mytilus edulis*) EXTRACT INHIBITS POLYPHENOL OXIDASE
IN FRUIT AND VEGETABLE TISSUE

By

Robert Schwartz Bent

May 2009

Chair: Maurice R. Marshall

Major: Food Science and Human Nutrition

Enzymatic browning in processed fruits and vegetables, mediated by the catalyst polyphenol oxidase (PPO; EC number 1.14.18.1), is a significant source of waste of both financial and other resources. Though there are a number of traditional browning inhibitors in commercial use today, no single inhibitor has yet been found that can be successfully used in every application. Our goal was to characterize the anti-browning and kinetic properties of a novel PPO inhibitor isolated from blue mussel (*Mytilus edulis*).

To isolate the inhibitor, frozen mussels were thawed and the drip loss extracted. The mussel meats were also squeezed to extract the aqueous contents. The liquid from the mussels was filtered through a glass filter followed by a 0.45 micron filter, both under vacuum. Following filtration, the liquid was dialyzed using 500 dalton molecular weight cutoff membrane for 24 hours against distilled water, including three water changes. After dialysis, the extract was run through a Sephadex G-25 size exclusion chromatography column, which eluted two fractions. The fraction that eluted second was freeze-dried and the resulting powder reconstituted when needed for experimentation. Apple, banana, avocado, and potato PPOs were extracted from the crops into an acetone powder and were then reconstituted for use at a ratio of

1g powder to 50 mL of 0.1 M $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.2 buffer. The mixture was stirred at 4°C for 30 minutes, centrifuged at 12000xg for 30 minutes, and the supernatant collected.

Machine Vision was used to study the inhibitory effects of the new compound on purees of the four crops mentioned earlier. 3 grams of puree was mixed with 1.5 mL of mussel extract reconstituted to a volume of 5 mL with distilled water. Pictures were taken in a lightbox to control illumination at times 0, 1, 5, 10, 30, 60, and 120 minutes, as well as 24, 48, 72 hours and 7 and 14 days. Color analysis was performed using LensEye software to determine percentage surface area covered by each of 4096 color blocks. Average L^* values also were recorded and analyzed for changes over time. Kinetic analysis was conducted using Dixon and Cornish-Bowden plots in concert, to determine the types of inhibition, as well as Prism software to perform non-linear regression in order to ascertain the kinetic parameters K_m , V_{max} , and K_i .

Analysis of the data from the Machine Vision studies showed that the inhibitor reduced browning in all four substrates, though not equally well in all. L^* values showed that browning was significantly reduced in all crops tested but avocado by one hour. Avocado showed a significant decrease in browning by 24 hours. Kinetic analysis showed that in the cases of apple and avocado PPO, the novel compound acts as a competitive inhibitor, while in avocado and potato, it is a mixed inhibitor. In addition, the K_i values calculated for the inhibitor in all crops tested compared favorably to other PPO inhibitors.

Over the last couple of decades, much research has attempted to find suitable inhibitors for PPO in processed crop products. This novel inhibitor shows a number of qualities, including strong inhibition on a number of different forms of PPO as well as long lasting inhibition, that make it a good option for many food products.

CHAPTER 1 INTRODUCTION

Polyphenol oxidase (EC number 1.14.18.1), also known as tyrosinase, plays a number of roles in various ecological systems. It has been widely studied as a browning agent in fruits and vegetables (Akisoe and others 2005, Gandía-Herrero and others 2005, Spagna and others 2005). Despite its generally negative reputation regarding browning, it also plays a positive role in the production of tea, coffee, and cocoa, all of which require polyphenol oxidase (PPO)-mediated browning. It appears as well in crustacean and fungal systems (Jaenicke and Decker 2003). In these species, as well as in some insects, PPO catalyzes reactions necessary for the proper development of the organism.

According to Lee and Whitaker (1995), over 50% of fruit losses occur due to enzymatic browning. The fruit and vegetable industry is a steadily growing sector that supplies a large amount of nutritious food eaten in the US. In 2002, fruit and berry production supplied \$11.2 billion in farm cash receipts to growers. Vegetable and melon sales accounted for \$12.8 billion (Kipe 2004). Supporting these large sales numbers, 76% of families in the US buy fresh-cut produce at least once a month, a commodity highly susceptible to enzymatic browning, (Gov't of Ontario 2006). This trend of increased sales and consumption is not confined to the US. In 2002, Canada produced \$517 million in fruit crops. Canadians also showed a per capita consumption of 72 kg of fruit per year. This figure is 19% higher than one decade earlier (Gov't of Saskatchewan 2005).

The largest producer of apples in the world has, for the past few years, been China. In 2004, China produced over 23 million metric tons of apples. The US came in second with almost five million metric tons of apples produced. The value of only the apples exported from the US in 2004 was over 383 million dollars (USDA Economic Research Service 2007a).

Banana production is an economically important crop to many countries, including the US. The highest production of bananas comes from India, with almost 17 million metric tons of bananas grown in 2004. One of the largest exporters of bananas in the world is Ecuador which, in 2004, exported over a billion dollars worth of bananas (USDA Economic Research Service 2007b).

Avocado production is a major economic factor for Mexico, which produced just under a million metric tons of avocados in 2004. The US produced over 162,000 metric tons during the same year. The value of avocados exported by Mexico in 2004 was over 211 million dollars (USDA Economic Research Service 2007c).

In the case of potatoes, China is again the largest producer with over 70 million metric tons in 2004. The US comes in fifth with just over 20 million metric tons. Potato exports from the US accounted for 72 million dollars, though other countries totaled far more in potato sales. The Netherlands, which produced only about 7.5 million metric tons of potatoes, sold over 497 million dollars worth of potatoes in 2004 (USDA Economic Research Service 2007d). Damage to potato crops due to impact injuries and the consequent PPO-mediated browning could cause over 20% product loss (Storey and Davies 1992).

Clearly, given the large amounts and values of these four commodities produced every year throughout the world, the potential for economic loss due to enzymatic browning is significant. The large numbers of fruits and vegetables produced, as well as their value in the marketplace, demonstrate why enzymatic browning losses every year garner great interest in finding a widely applicable, safe, and economical means of browning inhibition. While there are a number of methods to help prevent enzymatic browning, not all of these are useful in fresh-cut fruit and vegetables, either due to taste, safety, or regulatory concerns.

In addition to fruit and vegetable losses, postharvest melanosis of crustaceans, specifically lobster and shrimp, also causes severe monetary losses. Although melanosis does not actually degrade the taste or safety of the crustaceans, buyers will reject the products simply due to the brown or black discoloration (Marshall and others 2000).

The prevalence of aquaculture as a method for producing seafood is growing throughout the world, especially in China, Chile, and Thailand. In addition, the US interest in aquaculture is significant. The value of US aquaculture products in 2001 was 935 million dollars (Harvey 2004).

Prevention of post-mortem degradation of shellfish species has historically been of great interest due to the high perishability of the food products created from marine animals. Commercial, temperature-dependent methods for preservation of aquatic species include storage in flake ice, refrigerated seawater, brine solutions, and more recently, modified atmospheres (Aubourg and others 2007). As well, sulphites are a very common and powerful chemical PPO inhibitor used in seafood preservation. However, sulphites have been implicated in allergic reactions in asthmatics (López-Caballero and others 2007). A chemical PPO inhibitor that does not cause adverse reactions in consumers could be beneficial to many food industries, including seafood products. In addition, the combination of a powerful anti-browning compound in conjunction with current commercial preservation methods may yield even better results than present commercial practices.

As mentioned previously, a number of methods for inhibition of PPO-mediated browning exist. Yet none is universally applicable, and most show clear limitations in their usefulness as commercial browning inhibitors. Sulphites are a very effective browning inhibitor, but are banned in fresh fruit and vegetable products. Ascorbic acid is another very useful browning

inhibitor that is widely used commercially, especially in conjunction with citric acid. The ascorbic acid works as a pH modifier, bringing the food product out of the pH range that is optimal for the function of PPO. It also acts as a reducing agent. When PPO oxidizes its o-diphenol substrates to o-quinones, ascorbic acid reduces the latter back to the former, slowing the browning process. Unfortunately, the ascorbic acid oxidizes to dehydroascorbic acid, which has no anti-browning property. Citric acid acts as a chelator, binding the copper atoms essential to the proper function of PPO (Jiang and others 1999). The anti-browning effect of ascorbic acid is limited; however, to the time that it maintains its reducing ability. Thus, foods preserved with ascorbic acid eventually brown after all the ascorbic acid is oxidized to dehydroascorbic acid.

The search for a more effective, safe, and economical PPO inhibitor is of great interest to fruit, vegetable, and seafood industries. The potential benefit of economic savings by way of a reduction in product loss due to enzymatic browning is large. An inhibitor was found in blue mussel and is presently being identified. It shows a number of properties, which may make it useful in the prevention of enzymatic browning. Research was performed to examine the effectiveness of the blue mussel inhibitor on a number of different fruits and vegetables (apple, banana, avocado and potato) known to be prone to enzymatic browning.

Since sulphites are still used postharvest in the seafood industry to prevent melanosis in many aquatic animals, it was necessary to determine that no sulphites were applied to the frozen mussels used in this project prior to their freezing and packaging. To this end, live blue mussels were acquired from Canada and tested for inhibition of apple PPO. The inhibitory strengths of the extracts from the live and frozen mussels were the same, indicating that no sulphites were added to the frozen mussels during processing.

CHAPTER 2 LITERATURE REVIEW

Problems Surrounding PPO

Polyphenol oxidase is one name for a class of enzymes that catalyze the oxidation of o-monophenols to o-diphenols and o-diphenols to o-quinones. The oxidation reaction also uses molecular oxygen as substrate. There is large variation in PPO structure and function between different species of plants and animals. It is found in almost every class of plant and animal, as well as fungi and bacteria. PPO types are generally classified as either having mainly monophenolase (a.k.a. tyrosinase) or diphenolase (a.k.a. catechol oxidase) activity, though many PPOs exhibit both types of activity.

PPO is located in numerous organelles within the cells of plants and animals. It has been found in chloroplasts of photosynthetic plants, aerial roots of orchids, the leaves and seeds of coffee, in the mycelium and extra cellular matrix of fungi, and in the skin under the exoskeleton of crustaceans (Mayer 2006, Opoku-Gyamfua and others 1992). Figure 2-1 shows some of the locations in which phenolic substrates of PPO and the enzymes themselves have been found in plants.

Generally, PPO is separated, by way of cellular compartmentalization, from its phenolic substrates. Still, physical injury to a plant can cause the breakdown of these intracellular barriers, allowing for the contact of PPO and its substrates. Physical injury can include puncture wounds, impact injuries, abrasions, and commercial processing steps, including cutting, grinding, and pureeing.

As PPO is present in almost all crops, it is a problem for numerous food commodity industries. Some crop industries that battle losses due to enzymatic browning include apple, avocado, banana, cucumber, grape, pineapple, mango, peach, apricot, eggplant, cabbage, lettuce,

potato, water chestnut, carambola, and strawberry (Yoruk and Marshall 2003, Yueming and others 2004, Teixeira and others 2008, Chisari and others 2007). Large volumes of crop products are wasted every year due to quality loss caused by PPO-mediated browning. The search for safe, cheap, and effective inhibitors for PPO draws great interest because of the widespread economic damage done by this single class of enzymes.

In addition to plants, PPO also causes quality degradation in postharvest seafood products. Some marine species that undergo enzymatic browning after harvest include lobster, prawns, and shrimp (Slatery and others 1995, Aubourg and others 2007, Simpson and others 1997). PPO is activated and deactivated throughout the life-cycle of many marine species as a shell-hardening agent and possibly as a disease-resistance mechanism (Yoruk and Marshall 2003). However, after harvest polyphenol oxidase causes the common problem known as “black spot” in many marine species. Although not a food safety or taste concern, the visual quality of the products and therefore marketability are significantly reduced by the black spots that appear on the flesh of the animal.

Biochemistry of PPO Reactions

Polyphenol oxidase is a copper-dependant oxidase enzyme that acts upon monophenol and o-diphenol compounds, oxidizing them into o-quinones. For the reactions to take place, PPO must come into physical contact with both molecular oxygen as well as substrate molecules. These reactants are normally separated from each other by way of intracellular compartmentalization. Under conditions of injury, whether in nature or during commercial processing, the breakdown of intracellular barriers can allow the three reactants to interact (Toivonen 2004).

Depending on which substrate the enzyme is acting on, there will be either one or two main steps catalyzed by PPO to generate the final o-quinone structure. If the substrate is a

monophenolic compound, such as tyrosine, the first reaction is the oxidation of the monophenol to an o-diphenol. Once the o-diphenol has been formed, or if the substrate begins as an o-diphenol, such as catechol, the final step catalyzed by PPO is the oxidation of the o-diphenol into an o-quinone. Following the formation of the o-quinone product, numerous non-catalyzed oxidative condensation reactions take place during which the o-quinone polymerizes with amino acids, phenolic compounds, proteins, and other o-quinones. As the polymerization continues, the large, final products are the brownish melanins commonly seen on untreated, fresh-cut fruits and vegetables (Martinez and Whitaker 1995). Figure 2-2 diagrams a simplified version of the reactions catalyzed by PPO, leading from the monophenol substrate to the oxidized o-quinone product.

Copper plays an instrumental role in the function of PPO. The active site of all PPO compounds contains two copper ions, which in their normal states are in the form of met-PPO (Lerch 1983). Each of the two copper atoms is bound by three conserved histidine residues. Both molecular oxygen and phenolic substrates bind to the copper-containing active site (Van Gelder and others 1997). When acting as a monophenol oxidase, PPO is reduced to deoxy-PPO by oxidizing one molecule of diphenol substrate to the o-quinone structure. After this reduction, the active site binds with a molecule of oxygen, giving the third form of PPO, oxy-PPO. Monophenolic compounds can then bind to the oxy-PPO active site, forming a PPO-monophenol-oxygen complex. After the monophenol is converted to an o-quinone, it is released from the active site and the cycle begins again.

When acting as a diphenol oxidase, the first step is again the reduction of met-PPO to deoxy-PPO by the oxidation of one molecule of o-diphenol substrate. Following this reduction,

the PPO is then available to bind to a molecule of oxygen, forming oxy-PPO. Oxy-PPO then binds to a second molecule of o-diphenol, which is oxidized to the final o-quinone product.

The products and intermediate compounds of PPO-mediated oxidation of phenolic substrates can result in nutritive degradation of food products. The oxidized products of the PPO reactions can covalently bind to food proteins and amino acids, possibly changing their tertiary structure. This change in three dimensional conformation may degrade the ability of enzymes and other bioactive food proteins to perform their expected functions in the human body, since form and function are integrally tied in the case of proteins. In addition, tyrosine free radicals produced during the PPO-catalyzed browning processes can also interact with food proteins, negatively affecting their functional and nutritive characteristics (Matheis and Whitaker 1984, Prigent and others 2007).

Plant PPO

Polyphenol oxidase is essentially ubiquitous throughout the plant kingdom (Yoruk and Marshall 2003). It is located in a number of different locales within the cell, and is generally separated from its phenolic substrates. However, when the crop sustains injury, there is commonly a breakdown of intracellular compartmentalization, leading to enzyme-substrate contact and the browning reactions normally seen in fresh-cut fruits and vegetables (Toivonen and Brummell 2008).

PPO catalyzes the first two major reactions in the transformation of mono and diphenols to o-quinones, which go on to form brown melanin pigments by non-enzymatic condensation. The reactions that occur following the two PPO-catalyzed steps have historically been hard to document because they are generally insoluble (Lee and Whitaker 1995). Figure 2-4 shows the steps leading from the diphenolic PPO substrate L-DOPA to its final form in a melanin-protein complex.

Describing the possible functions of PPO in a living plant is problematic. The enzyme has been implicated in defense against pathogens and insect infestation, as well as wound healing. The intermediate hydroxyphenolic compounds and final o-quinone products of PPO-mediated oxidation reactions exhibit virucidal and bacteriocidal properties. In addition, the large polymerized phenolic complexes resulting from the non-catalyzed condensation of o-quinones and other compounds show fungicidal action (Mayer and Harel 1979, Vaughn and others 1988, Macheix and others 1990, Scalbert 1991, Shaw and others 1991, Zawistowski and others 1991).

The insoluble products of o-quinone condensation following enzymatic oxidation also may serve as wound caps, much like scars in humans (Vamos-Vigyazo 1981, Vaughn and others 1988, Zawistowski and others 1991). An insoluble blocking agent within an injury could also potentially decrease water loss following injury. Water loss is a major influence on the post-harvest quality of fruits and vegetable products. Dehydration significantly decreases the quality and salability of many commodities (Smith and others 2006, Thomas and others 2006, Porata and others 2005).

Finally, o-quinone products of enzymatic browning reactions, as well as intermediate phenolic radical compounds may modify the structure of amino acids and proteins. This scheme of modification is generally termed an “antinutritive defense” mechanism that is thought to deter insects from attacking a plant (Duffey and Stout 1996). Quinone products from PPO-catalyzed oxidation of phenolic compounds are highly reactive compounds. They are able to alkylate the amino groups of lysine and tryptophan residues, as well as other thiol groups within a protein’s structure, crosslink proteins into large, insoluble complexes, and destroy many amino acids outright (Duffey and Felton 1991, Felton and others 1989, Felton and others 1992, Pierpoint 1969, Rawel and others 2001, Rawel and others 2002). Alkylation of lysine residues within

plant proteins decreases the number of sites at which an insect's protease trypsin can attack the protein. Trypsin specifically cleaves peptide chains at the carboxyl end of lysine and arginine residues. The crosslinking of protein molecules occurs either directly across an o-quinone, or by way of a phenolic or protein free radical. The large size of the resulting protein complex causes its precipitation (Duffey and Stout 1996).

PPO has been shown to be encoded by nuclear genes, synthesized on cytoplasmic ribosomes, and remains membrane-bound and in a latent state until activated (Yoruk and Marshall 2003, Tolbert 1973, Lax and others 1984). Activation mechanisms shown to be effective *in vitro* include exposure to white and red light, treatment with trypsin or acids, aging, and the addition of the anionic detergent SDS (Tolbert 1973, Sanchez-Ferrer and others 1993, Lerner and others 1972). *In vivo* activity of PPO may be influenced by a number of different sources, including developmental age, transcription-level upregulation of PPO expression due to injury, or exposure to certain fatty acids or endogenous proteases (Van Gelder and others 1997). The size of activated and latent PPOs varies widely between species. PPOs have been isolated weighing between 32 and 200 kDa, though most exist between 35 and 70 kDa (Flurkey 1986, Fraignier and others 1995, Sherman and others 1991, Steffens and others 1994, Van Gelder and others 1997, Yang and others 2000). Some *in vitro* studies have attributed the activation of PPO to cleavage of a peptide cap covering the active site of the enzyme. In one study using broad bean, latent PPO weighing 60 kDa was observed using protease inhibitors. PPO from this crop is generally considered to be a 45 kDa protein. Following *in vitro* proteolytic cleavage of the latent enzyme using both trypsin and thermolysin, active proteins weighing 42 kDa, as well as smaller inactive fractions were observed (Robinson and Dry 1992). These data suggest that exogenous,

as well as some possibly endogenous, proteases may cleave the larger, latent form of PPO to form the smaller, active form.

In many organisms, PPO activity changes throughout the lifecycle. Generally, PPO activity has been shown to be higher in younger plants and decreases as ripening and senescence takes place (Cipollini and Redman 1999, Murata and others 1995, Serradell and others 2000, Shahar and others 1992). There are a number of factors that may contribute to this change in activity, including changes in PPO concentration, changes in concentrations of phenolic substrates for PPO, conformational changes and/or denaturation of PPO, or a decrease in concentration of PPO activators (Murata and others 1995, Laveda and others 2000). It is worth noting that the changes in PPO concentrations at different points during development may occur due to transcription-level decreases in expression of PPO genes and not simply because of denaturation or other means of protein degradation (Chevalier and others 1999, Gooding and others 2001).

Another means of increasing PPO activity in crops is through mechanical injury or infection. This increase in activity is believed to contribute not only to wound healing, but also to defense against insect and microbiological infection (Duffey and Stout 1996, Mayer and Harel 1979, Vamos-Vigyazo 1981). Commercial harvesting and processing methods pose serious threats to food crops. There is often physical damage, by way of cutting, grinding, peeling, and transport, incurred by a fruit or vegetable during these processes that eventually leads to a decline in quality such that it is no longer salable. Susceptibility to mechanical damage is influenced by a number of physical properties, including cultivar, dry matter content, mineral content, turgidity, temperature, shape, and size of the crop. In addition, vulnerability to bruising due to mechanical injury also depends on chemical factors, including PPO content and

distribution, as well as concentration and location of phenolic substrates for PPO (Partington and others 1999).

It is not clear whether this increase in PPO activity is a localized response to the wound or infection, or whether it is a systemic action undertaken throughout the plant. There is evidence that, in a number of crops, injury induces an increase in PPO activity systemically by way of increased levels of available PPO mRNA. Whether this change is due to increased transcription of PPO mRNA or a physical modification of the mRNA is unknown (Thipyapong and others 1995). On the other hand, there is evidence that the increase in PPO activity may be localized. Both PPO activity and the concentration of phenolic substances increased around the wounded tissue of potato following injury. In this study, PPO was considered to be one of a number of factors highly associated with resistance to infection (Ray and Hammerschmidt 1998).

Properties of Apple, Banana, Potato and Avocado PPOs

The enzymes that comprise the class commonly referred to as PPOs are varied by many physicochemical properties. As with any enzyme, the structure of the protein, including its tertiary characteristics, influences the ability of the PPO to interact with a specific substrate. Hence, each PPO has a different range and order of phenolic substrate specificities with which it best reacts. In addition, the family of PPOs is also widely varied in other properties, including the temperature and pH at which each specific PPO best operates.

Temperature and pH optima in enzymatic systems are determined by a number of different factors. In the case of both extreme temperature and pH, protein quaternary and tertiary structures are changed. Quaternary structure is affected by a disassociation of protein subunits from each other, or a change in the spatial arrangement of subunits to each other. Both of these modifications may change the biochemical functionality of the protein.

Tertiary structure is also affected by extreme temperature and pH. Low pH will tend to cause H⁺ ions to protonate carboxyl groups on some amino acids as well as bind to the unoccupied pair of electrons on the nitrogen atoms of amino groups within amino acids. These changes will disrupt the normal electrostatic interactions between amino acids that help form the tertiary structure of the protein, causing the protein to unfold. In addition, as heat is added to a molecule, the strength of hydrogen bonds decrease. As the bonds become weaker, the stabilizing force usually given to the protein's structure by these already weak bonds is lost. Acids and bases can also disrupt salt bridges usually held together by ionic charges. Finally, reducing agents have the ability to destroy the disulfide bonds formed by the oxidation of sulfhydryl groups on cysteine molecules (Ophardt 2003).

The substrate specificity of different forms of PPO is not surprising. Different crops contain various possible phenolic substrates for PPO and these different substrates contain a wide variety of structures. It is logical to assume that, over time, plants evolved such that their specific type of PPO best fit the polyphenols that exist in the highest quantities within the plant. This tight fit between enzyme and substrate would assure the highest level of enzyme activity and the greatest affinity for substrate.

It has been shown that even within a single plant; a number of different PPOs may exist within different tissues. The properties of these PPOs may vary widely. In red clover, three different PPOs exist. They differ in a number of qualities. In their latent states, the pH optima of the three PPOs range from 5.1 to 6.9. After activation by room temperature incubation for six to eight days, the activities of the three PPOs increased by 10-40 fold. Finally, the three PPOs showed different orders of substrate specificity when assayed with caffeic and chlorogenic acids,

catechol, dopamine, and other common substrates, though all showed the highest reactivity with caffeic acid (Schmitz and others 2008).

It has been shown that, within one plant, the activity of PPO can be widely variable. One study looked at the distribution of PPO activity throughout the fruits of six different varieties of Japanese apples. PPO activity was determined to be 32-78 units (U)/mL preparation in the peel, 126-430 U/mL in the apple flesh, and 383-800 U/mL in the core (Wakayama 1995). Whether these differences were due to variations in PPO structures or distribution is unclear, but both are possibilities.

These studies used four commodities grown throughout the world. Table 2-1 shows the differences in pH and temperature optima for PPO activity between the four crops. In addition, it also shows the preferred substrates for each specific PPO ordered from highest affinity to lowest.

Arthropod PPO

PPO is found throughout nature, including within the phylum arthropoda (Opoku-Gyamfua and others 1992, Chen and others 1991a). The PPOs found in arthropods, like those in plants, oxidize phenolic substrates to form o-quinone products that then polymerize into melanin compounds. However, arthropod PPOs are generally assayed for activity using L- or DL-DOPA, a substrate not usually used in plant PPO studies due to its low specificity to many plant PPOs (Opoku-Gyamfua and others 1992, Chen and others 1991b, Yoruk and Marshall 2003). Though the mechanism of action and even the structure of arthropod PPO are rather similar to those found in plants, the ways insects use the pro-oxidative properties of PPO is grossly different from plants (Opoku-Gyamfua and others 1992).

As an arthropod grows, its hard exoskeleton becomes too small for its body, and it must molt its shell. This effect is commonly seen in softshell crabs, a culinary delicacy. After molting the hard shell, PPO begins to solidify the soft outside of the animal and form the new

exoskeleton. As o-quinones are produced by the reactions catalyzed by PPO, they form cross-links between adjacent shell proteins, forming a stiff matrix of hardened proteins (Stevenson 1985). This hardening process is called sclerotization. Not surprisingly, PPO in arthropods has been shown to lie in high concentrations within the cuticles of the animals (Bartolo and Brik 1998).

PPO has been shown to exist in arthropods in both latent and activated forms. The proenzyme of PPO found in Norway lobster can be activated exogenously using trypsin (Yan and Taylor 1991). Endogenous activation is thought to be the result of a number of natural proteases, as well as one or more unknown factors that have yet to be identified (Wang and others 1994, Zotos and Taylor 1996).

PPO activity in lobsters and shrimp has been shown to be sex-dependent (Ogawa and others 1984). In addition, PPO activity also changes throughout the lifecycle of an arthropod. One study found a relationship between PPO activity and molting stage. Higher PPO activity was observed in spiny lobsters that were close to molting (Ferrer and others 1989). In addition, there are yearly peak periods for molting in the Norway lobster, though molting occurs at some level throughout the year (Farmer 1975). Danish lobster processors have also noted an increase in blackspot-related problems around September of each year (Bartolo and Brik 1998). Blackspot is a quality defect caused by PPO activity in arthropods, including shrimp, lobster and crabs. It can be brought on by injury to the animal while it is alive or by degradation or storage processes after death (Lopez-Caballero and others 2007, Ogawa 1987).

House fly (*Musca domestica*), another arthropod, has been investigated in the past as a source of new inhibitors for PPO due to the variable effects of PPO over the course of the fly's lifetime. (Yoruk and others 2003). In that study, extracts from house flies at different stages of

their lifecycle were tested for inhibitory properties against apple PPO. The most potent PPO inhibitor was isolated from third-instar larvae and 3-5 day old pupae. This compound inhibited apple PPO up to 90%.

At the outset of this investigation, a crude, aqueous extract of blue mussel was simply added to a cuvette containing 0.1M pH 5.5 phosphate buffer, apple PPO, and 0.5M catechol. Though a reduction in browning was seen immediately, the identity of the compound within the blue mussel extract that effected the inhibition of PPO was unknown for over a year (Schulbach 2008). The elucidation of the inhibitor's identity began following the successful purification of the compound through the use of high performance liquid chromatography (HPLC). With the inhibitor dissolved in methanol, HPLC-mass spectroscopy (HPLC-MS) analysis was attempted at the University of Florida's chemistry department. The methanol/inhibitor solution was assayed before HPLC-MS analysis and exhibited clear inhibition of apple PPO. However, when examined at the chemistry department, the results indicated that the solution was comprised of only pure methanol. It was hypothesized that the mass spectroscopy (MS) analysis of the inhibitor had failed due to its very low molecular weight, which was known at that time from preliminary ultrafiltration studies to be below 1000 daltons.

In a search to remedy the problem of low molecular weight, another characteristic of the inhibitor was exploited. In addition to its ability to inhibit the catalytic action of PPO, the compound also exhibited a bleaching effect on the o-quinone products of PPO and its polyphenolic substrates. The mechanism of action of the bleaching effect was hypothesized to be through the binding of the inhibitor to the colored enzyme-substrate products, creating a larger, colorless complex. Through the use of HPLC, this final colorless product was purified and sent to the chemistry department for MS analysis.

The components of the known PPO-catechol product, an o-quinone to which the inhibitor was bound, were removed from the results obtained through MS analysis of the entire colorless complex, leaving only the components of the inhibitor. The inhibitor was then identified as hypotaurine.

Hypotaurine

Hypotaurine is a sulfinic acid that, in mammals, is a metabolic precursor to taurine formed from L-cysteine (Fontana and others 2004). The metabolic pathway from cysteine to hypotaurine consists of two steps. Cysteine is first oxidized to cysteinesulfinic acid by cysteine dioxygenase. Then, cysteinesulfinic acid is likely decarboxylated to hypotaurine by cysteinesulfinic acid decarboxylase. Finally, hypotaurine is oxidized to taurine; the mechanism of that oxidation is unknown (Font and others 2001).

Hypotaurine is a small (109.15 daltons), water-soluble compound known to act as an antioxidant, binding hydroxyl radicals (Green and others 1991). It functions as an inhibitor for the sodium-dependant transport of gamma-aminobutyric acid (GABA) and β -alanine in the cerebellar granule cells of rats (Saransaari and Oja 1993). Hypotaurine also acts as a GABA neuromodulator and inhibits N-methyl-D-aspartic acid (NMDA), kainate, and quisqualate receptors (Quinn and Harris 1995, Dahchour and De Witte 2000, Kurachi and others 1983).

In preliminary experiments using fresh, unprocessed apple juice, no taste or odor changes could be detected due to the addition of hypotaurine as a browning inhibitor. Concentrations of hypotaurine in these trials were not measured, but enough hypotaurine was added to maintain the original juice color, according to the naked eye, for at least four weeks. In another set of preliminary experiments using fresh, unprocessed Thompson seedless grape (*Vitis vinifera*) juice, no odor or taste changes could be detected when hypotaurine was added to the juice at concentrations up to 200ppm (Sims 2008).

Blue Mussel

In this study, blue mussel was investigated as another potential source of PPO inhibitors. Blue mussel is a bivalve mollusk that is harvested from waters all over the world for food, and has been used for food by humans since at least 6000 BC. They grow in both marine and brackish waters with salinities as low as 4%, though their growth rate begins to drop at 18% salinity (FAO 2008). They also are the most efficient feeders of all shellfish, filtering 10-15 gallons of water per day. They consume almost all the nutritious material from this filtered water and satisfy all of their energy and nutrition needs (Batten 2008).

Mussels grown in aquaculture usually reach market size by two years. A number of aquaculture methods have been developed over time including long line or rope culture, raft culture, and on-bottom culture. However, the original form, known as intertidal wooden pole culture or “bouchots”, has been in use since at least the 13th century, when it was first used in France. Bouchots are still used for mussel aquaculture today (FAO 2008).

Blue mussels begin their development as spherical eggs measuring less than a millimeter in diameter. After 15-35 days in this larval stage, they turn into a juvenile stage called a plantigrade. After reaching a size of 1 to 1.5mm in length, the young mussels use water currents to transport themselves to established blue mussel beds, to which they affix themselves and reach sexual maturity within one to two years (Newell 1989).

Inhibition of Enzymatic Browning

Enzymatic browning has long been a problem for the fruit and vegetable industry (Lee and Whitaker 1995, Brandelli and Lopes 2005). Browning in fruits and vegetables causes losses in post-harvest product year after year, putting a heavy economic burden on the industry. Methods for browning inhibition have been of high interest because the problem stems from one class of enzymes, known collectively as polyphenol oxidases. However, despite the fact that a

single type of protein is responsible for enzymatic browning in countless varieties of fruits and vegetables, there is not a universally useful inhibition method. Some inhibitors do not work well against specific PPOs, as all PPOs are slightly different in structure. In addition, some powerful PPO inhibitors are not allowed in food products.

There are currently six popular types of browning inhibitors or inhibition methods, including reducing agents, chelators, complexing agents, acidulants, enzyme inhibitors, and enzyme treatments (McEvily and others 1992). All six of these inhibitors reduce a critical element in the browning process, which include the PPO enzyme, its phenolic substrates, the essential copper bound to the enzyme active site, and intermediate compounds during the course of the reactions.

Some of the most popular PPO inhibitors used in the food industry today are reducing agents. Reducing agents prevent the formation of polymerized melanin compounds by slowing down the buildup of o-quinone products of PPO-catalyzed oxidation reactions by reducing the oxidized o-quinones back to colorless o-diphenols. Sulfiting agents, such as sulfur dioxide, sodium sulfite, and sodium bisulfite, are very powerful reducing agents (Sapers 1993). It is also possible that they directly inhibit PPO (Lee and Whitaker 1995). However, they are banned from use in fresh fruit and vegetable products due to concerns over adverse, breathing-related problems seen in some consumers (Gomez-Lopez 2002b). Sulfites are still in use in seafood products, including shrimp, lobster, and crab, to prevent the formation of blackspot (Kim and others 2000).

A popular alternative to sulfites is ascorbic acid. Also known as vitamin C, ascorbic acid works by reducing o-quinones back to o-diphenols, preventing the polymerization of the o-quinones with other compounds to form brown melanins. Unfortunately for the fruit and

vegetable industry, while cheap and safe, ascorbic acid is generally a short-lived inhibitor (Sapers 1993). The oxidized product of ascorbic acid, dehydroascorbic acid, does not inhibit browning and is not reduced back to ascorbic acid to act again upon the o-quinones (Eskin and Robinson 2000). Ascorbic acid is commonly paired with citric acid in order to boost its anti-browning efficiency. Ascorbic acid is more stable in an acidic environment, and the low pH can bring the enzyme out of its pH optimum. In addition, citric acid may act as a chelator (Eskin and others 1971).

Another method to stop PPO-mediated browning in fruits and vegetables is the use of chelating agents. Chelators bind metal ions, removing them from the PPO molecule and preventing the enzyme from binding its substrates. PPO is a copper metalloprotein and the two copper ions associated with the active site of the enzyme are imperative to its function (Shahar and others 1992). A common commercial chelator is ethylenediaminetetraacetic acid (EDTA). It has a very strong affinity for a number of metals, including copper. One study found that EDTA prevented browning in avocado puree for up to three months (Soliva-Fortuny and others 2002). Other chelators used in the food industry include citric acid, diethyldithiocarbamic acid (DIECA), oxalic acid, and phosphates (McEvily and others 1992).

Acidification also offers a method by which enzymatic browning can be slowed. Acidifiers in foods include citric, phosphoric, lactic, malic, and ascorbic acid, among others. A low pH environment exerts a number of effects upon PPO. First, as the pH is lowered below about 4.5, PPO is generally no longer within its range of pH optima, reducing enzyme activity (Eidhin and others 2006, Unal 2007, Duangmal and Apenten 1999). This pH-mediated change in activity may be due to protonation of groups essential to catalytic action, denaturation or conformational changes in the tertiary structure of the protein, lessening its ability to bind

substrates, or a reduction in the stability of the phenolic substrates themselves (Tipton and Dixon 1983, Whitaker 1994).

Besides chemical treatments, physical methods, including freezing, heating, dehydration, modified atmosphere packaging, irradiation, pulsed electric fields, and high pressure can be used to help prevent enzymatic browning (Castro and others 2008, Kim and others 2000, Noci and others 2008, Teixeira and others 2007, Vijayanand and others 1995). These methods are not perfect since they may cause problems in product quality following treatment, such as a loss of firm texture and subcellular decompartmentalization (Diaz-Tenorio and others 2007, Macheix and others 1990).

Because no PPO inhibitor yet discovered is useful in all circumstances, interest in finding effective, safe, and economical inhibitors continues. One new facet in this vein of research is the use of natural PPO inhibitors, such as amino acids, Maillard reaction products, and cyclodextrins (Cheriot and others 2006, Kahn 1985, Sojo and others 1999). In addition, a new and extremely innovative idea in PPO inhibition research is the use of inhibitors extracted from animals in which PPO plays a role in their life cycle. On the forefront of this area of investigation was a successful study into the use of proteinous extracts from the common housefly (Yoruk and others 2003).

Objectives and Hypothesis

Another PPO inhibitor, extracted from houseflies, showed significant inhibition of apple PPO (Yoruk and others 2003). The biological functions of PPO within blue mussel and house fly have similarities, as they are both members of the phylum arthropoda (Chase and others 2000). Due to the relationship between the two organisms, the hypothesis for this project was that the inhibitor would decrease browning when used with all of the types of PPO tested.

However, it was predicted that the inhibitor would not work equally well against all PPOs, due to differences in the three dimensional structures of the PPOs.

From a review of the literature, it appeared that a large number of PPO inhibitors showed competitive-type inhibition (Richard-Forget and others 1992, Oktay and Dogan 2005, Dogan and others 2007). Therefore, it was expected that the inhibitor investigated in this study would also show competitive inhibition.

This project has a number of objectives, all of which relate to elucidating the biochemical and physical properties of a novel inhibitor of polyphenol oxidase, isolated from blue mussel. 1) To examine how effective the inhibitor is against browning in samples designed to mimic commercial processing on fresh fruits and vegetables. 2) To determine the type of inhibition taking place using enzyme kinetics plots, including Dixon and Cornish-Bowden linear plots, as well as plots fit to the Michaelis-Menten equation using non-linear regression. 3) To further elucidate the biochemical properties of the PPO inhibitor, the IC₅₀ and K_i of the inhibitor were determined when used with each of the four different types of PPO used in the project.

Table 2-1. pH and temperature optima for apple, banana, potato, and avocado PPOs, as well as substrate specificities ordered from highest to lowest.

Commodity	pH Optimum	Temp Optimum (°C)	Substrate Specificity
Apple	5.0-7.5 (1)	18-30 (1)	4-methylcatechol > catechol > pyrogallol > (-)-epicatechin > caffeic acid > DL-dopa (1)
Banana	6.5-7.0 (2,5)	30 (2,5)	Dopamine > gallic acid (7); D-dopa > L-dopa (8)
Potato	6.8 (4)	22,25 (4,6)	4-methylcatechol > caffeic acid > pyrogallol > catechol > chlorogenic acid > DL-dopa > dopamine (4)
Avocado	4.5,7.5 (3)	?	4-methyl catechol > chlorogenic acid > pyrogallol > catechol > caffeic acid > DL-DOPA (3)

Note: 1. (Eidhin and others 2006); 2. (Unal 2007); 3. (Gomez-Lopez 2002); 4. (Duangmal 1999); 5. (Yang and others 2000); 6. (Vamos-Vigyazo 1981); 7. (Yang and others 2004); 8. (Galeazzi and Sgarbieri 1981).

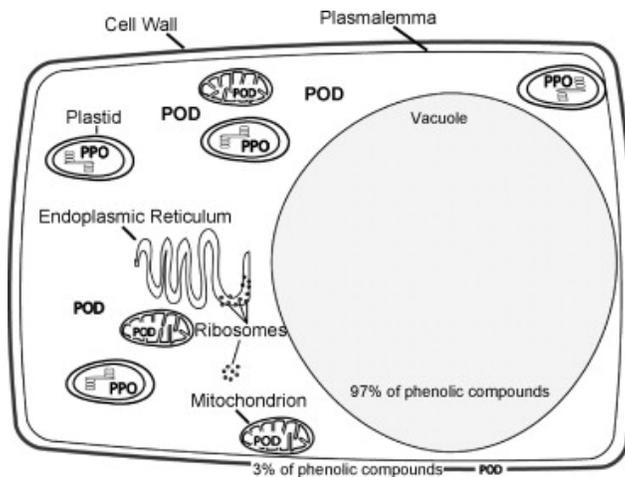


Figure 2-1. Locations within a plant cell of phenolic compounds, as well as polyphenol oxidase and peroxidase enzymes [Reprinted from Postharvest Biology and Technology, 48/1, Toivonen PMA and Brummell DA, Biochemical bases of appearance and texture changes in fresh-cut fruit and vegetables/Appearance, page 2, 2008, with permission from Elsevier].

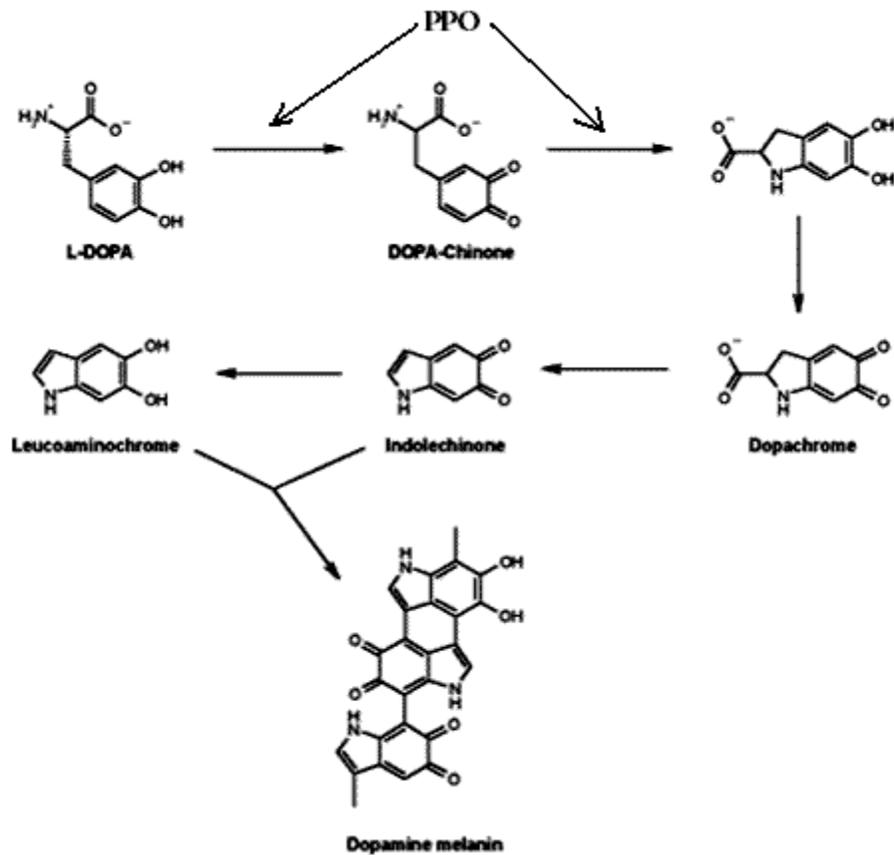


Figure 2-3. PPO-catalyzed oxidation of L-DOPA to melanin [Adapted from Double KL, Riederer P, and Gerlach M. 1999. Significance of Neuromelanin for Neurodegeneration in Parkinson's Disease. *Drug News and Perspectives* 12(6):333].

CHAPTER 3 MATERIALS AND METHODS

Machine Vision Analysis of Color Development

Preparation of Inhibitor from Blue Mussel

Crude inhibitor used in the color development studies was isolated from blue mussels (*Mytilus edulis*). Two pound bags of frozen mussels imported from China were bought from a local seafood company and thawed in warm water. The drip loss from the mussels was collected. In addition, the mussels were opened by hand and the mussel meats were wrapped in cheesecloth and squeezed to extract the liquid from the mussels. This liquid was added to the drip loss.

The liquid mussel extract was then filtered through a Whatman (Whatman Plc, Maidstone, Kent, UK) glass filter under vacuum, using celite if necessary to aid in the filtering process. It was again filtered through a 0.45um Whatman filter under vacuum.

Following filtration, the extract was poured into approximately six inch sections of 500-Da molecular weight cut off (MWCO) dialysis membrane (Spectrum Laboratory Products, Inc., New Brunswick, NJ) and dialyzed against distilled water for 24 hours with three water changes. The dialysis was performed at 4°C and the water was stirred throughout the process.

After dialysis, the mussel extract was run through a Sephadex G-25 size exclusion chromatography column (Sigma-Aldrich, Co., St. Louis, MO). Two peaks were observed, and the second peak was collected and divided into smaller containers for freeze drying. The containers were put into the freezer overnight until frozen solid and then freeze dried using a Labconco freeze dryer (Labconco Co., Kansas City, MO). Following freeze drying, each vial of extract, which contained approximately 35 mL of liquid extract, was reconstituted using 5 mL distilled water.

Preparation of Puree for Testing Browning Inhibition of Blue Mussel Extract

The fruits and vegetables used in this experiment, Hass avocado (*Persea Americana*), banana (*Musa acuminata*), red delicious apple (*Malus domestica*), and russet potato (*Solanum tuberosum*) were bought from a local supermarket. All crops were purchased at a ripeness level at which they would be eaten or prepared immediately without need for storage or further ripening. At the time of each experiment, the crop was diced into small pieces and three grams of material was transferred into a mortar already containing 1.5 mL of either pH 5.5 phosphate buffer (control samples) or reconstituted mussel extract (test samples). The mixture was ground into a homogenous puree using a pestle and then transferred into a small, round, white bottle cap. The same type of cap was used for storage and imaging throughout the Machine Vision experiments. The cap was chosen because it allowed a relatively flat, uniform surface for photographs taken from above. For each of two experiments per fruit or vegetable, three samples each of control and test samples were prepared and photographed.

Pictures of the puree were taken using a Machine Vision system comprised of two main components. The first is a light box designed to help ensure greater uniformity between pictures taken throughout the experiments. Its construction and design are described by Luzuriaga (1997). A Nikon D200 digital camera with a VR 18-200mm F/3.5-5.6 G lens at 50mm was used to photograph the samples within the sealed light box. The settings for the camera are shown in table 3-1.

Pictures were taken immediately after grinding (0 minutes), at one, five, 10, 60, and 120 minutes, as well as at one, two, and three days, and finally at one and two weeks. Samples were stored in sealed plastic containers at 4°C with a water bath inside the container to decrease drying of samples during storage. During picture taking, samples were removed from the container and the container was resealed. The ambient temperature in the room during picture

taking was 20-25°C. Following each picture, the samples were returned immediately to their storage container.

Machine Vision Analysis

Two methods of analysis were performed on the data from the photographs. The first was performed using LensEye software (Engineering and CyberSolutions, Gainesville, FL). The photographs were analyzed per pixel to determine both average L*, a*, and b* values as well as its “color block” identity. Within the software, the visible spectrum was divided into 4096 parts. Each pixel’s R,G,B value was used to determine into which color block the pixel best fit. The percentage of sample surface area taken up by each color block was then calculated. Through the specific definition and tabulation of the colors within each picture, the color development was compared over time. As various color blocks emerge and others are replaced, the shift in color and in the general intensity of the sample over time can be observed.

The second method of analysis used the average L* value from the samples at each time point to determine the general darkening of the crops over time. L* was used because it gives the most direct indication of browning. For each time point, the average L* value for the three control samples was compared to the average L* value of the three test samples at the same time point. One-way analysis of variance was calculated for each time point using SAS. Means were separated using the least significant difference test at a probability level of 0.05, also calculated using SAS.

Enzyme Kinetics

Polyphenol oxidase preparation

Enzyme kinetic experiments were performed on PPO extracted from the four crops in order to determine kinetic parameters, as well as the type of inhibition in each case. Unlike the Machine Vision experiments, the kinetics trials used purified hypotaurine (Sogo Pharmaceutical

Co., Tokyo, Japan) as the inhibitor instead of the crude mussel extract. During the course of this study, a compound, hypotaurine, was purified and identified as the browning inhibitor from blue mussels. Also note that the kinetic experiments using the hypotaurine were further complicated by the presence of a number of isoforms of PPO in each crop PPO extract because the preparation was only partially purified; however, the data could be analyzed successfully.

PPO extraction began with the whole, raw fruit or vegetable. The crop was washed, cut into large chunks and placed into a pre-chilled blender containing 400 mL of acetone at -20°C. The mixture was blended for one minute and then filtered through a Whatman #1 filter under vacuum until all of the visible wetness had disappeared from the top of the solids. The solids were then placed back into the blender with 200 mL of chilled acetone and blended again for one minute. The mixture was filtered again, and this re-extraction process was repeated another two times for a total of one primary extraction and three secondary extractions. At the end of the third re-extraction, the solids were left under vacuum overnight to dry. The resulting powder was packed under vacuum into storage bags and placed at -20°C until needed.

To extract the PPO powder for use in the experiments, one gram of powder was mixed into 50 mL of 0.1 M $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.2 buffer and stirred in 4°C for at least 30 minutes. The slurry was centrifuged at 12000xg for 30 minutes, and the supernatant was removed and filtered through glass wool. The filtrate was crude PPO extract for the enzyme kinetic experiments. The PPO extract was frozen until needed.

Using a Beckman Model DU 640 Ultraviolet-Visible spectrophotometer, a plot of substrate concentration vs. reaction velocity was determined, in order to find the correct substrate concentrations to use in the kinetics experiment for that crop. After the substrate concentrations were chosen, the lowest concentration was used to determine the amount of inhibitor needed to

provide a wide range of activity. A range of approximately 20% to 80% inhibition was satisfactory, though in a number of cases a wider range was used.

To determine the activity at each point, a 3 mL assay was used, containing 0.05 or 0.1 mL of PPO extract, depending on how active the particular extract was, inhibitor at the specified amount, catechol (Sigma Chemical Co., St. Louis, MO) as substrate at the specified amount for that series, and the balance in buffer at either pH 5.5 or 6.0, depending upon the pH optimum for the crop PPO being examined. In the case of avocado, 100 uL of 1% trypsin solution was also used in each cuvette in order to activate the PPO. The enzyme was added last, and the contents were covered in parafilm and shaken three times to mix the reagents. The spectrophotometer measured absorbance at 420nm for 90 seconds and the greatest initial rate was determined using the spectrophotometer's kinetic software. Each point was assayed in duplicate and each experiment was replicated.

To determine the type of inhibition taking place in the case of each crop, a Dixon plot was constructed for each trial. In addition, a Cornish-Bowden plot of substrate concentration over reaction velocity vs. inhibitor concentration was constructed in order to specifically ascertain the inhibition type (Cornish-Bowden 1974).

Prism software (Graphpad Software, Inc., La Jolla, CA) was used to determine K_m and V_{max} by nonlinear regression. K_i was determined from the results of the IC_{50} test along with the K_m using the Cheng-Prusoff equation, which reads, $K_i = IC_{50} / (1 + [S]/K_m)$ (Cheng 1973).

Determination of IC_{50}

To find the level of inhibitor that would give a 50% reduction in reaction rate, it was necessary to determine the reaction rates of assays containing one substrate concentration and one enzyme concentration while varying the amount of inhibitor. In these experiments, the substrate level used for each crop's trial was the same as the highest substrate concentration used

in the enzyme kinetics studies for that crop. All of the crops used a range of 0.038174mM to 0.61078mM of inhibitor to ensure a wide range of inhibitory strengths. Each point was assayed in duplicate and each experiment was replicated. The curve was fit using a second order polynomial equation and the IC50 was interpolated.

Electrophoretic Separation of PPO Isoforms

To determine if the PPO extract contained one or more PPO isoforms, native gel electrophoresis was conducted. Native gel electrophoresis was used to allow the staining of only the PPO proteins within the mixture by way of their natural enzymatic action. Instead of staining the gel using a protein stain, as in the case of denatured protein samples, the intact enzymes were allowed to react with their natural substrates to elucidate their locations within the gel as they formed melanin. The experiment was performed using a Bio-Rad Mini-Protean III system (Bio-Rad Laboratories, Inc., Hercules, CA).

7.5% acrylamide gels were purchased from Bio-Rad, as was the running buffer and sample buffer. The PPO extracts were diluted in a 2:1 ratio of sample buffer to sample. Eight wells were filled, two for each crop. One well for each crop was loaded with 25uL of sample and the other was loaded with 45uL. The gels were run using a Bio-Rad Powerpac 3000 voltage supply unit (Bio-Rad Laboratories, Inc., Hercules, CA) at 200 volts until the blue dye was about 2-3mm from the end of the gel. After washing the gels, they were placed in a bath of 10mM L-DOPA (Sigma-Aldrich, Co., St. Louis, MO) on a shaker plate for 10 minutes. After 10 minutes, solid catechol was added to the bath in excess to complete the staining process. The gels remained in the substrate bath until no further staining could be observed.

Table 3-1. Camera settings for Machine Vision experiments

Parameter	Value
ISO	100
Exposure	Manual
Shutter speed	1/3s F/11
Exposure compensation	-1.0 EV
Focus	Manual
Sharpening	Normal
Tone compensation	Normal
Color mode	Mode 1
Saturation	Normal
Hue adjustment	0°
White balance	Direct Sunlight
Zoom	Manual

CHAPTER 4 RESULTS AND DISCUSSION

Machine Vision Analysis of Color Development

Figures 4-1 through 4-4 show the graphical representations of color blocks emerging over time during the first apple trial for the untreated (control) and treated (exposed to hypotaurine) samples. Figures 4-5 through 4-8 display the same data from the replication of the first experiment. As there was little variation between replications for all crops, only representative data from the first experiment for each crop is shown for banana, avocado, and potato.

Two types of analysis were used to examine the data provided by the Machine Vision studies. The first was to graph the color blocks at each time point versus the percentage surface area of the sample that they covered. By overlaying these graphs by time, it is possible to observe a shift in color blocks towards the lower numbers, which indicate darker colors. A smaller shift in color blocks indicates a lower rate of browning during that time period. In the case of the first apple trial, figures 4-1 and 4-3 show a particularly large decrease in browning was seen in the samples treated with hypotaurine versus the browning seen in the untreated control samples between time points 10 minutes and 120 minutes. This inhibition of browning is indicated by the smaller range of color block values in the treated samples. Figure 4-1B shows a range of over 1400 color blocks between 10 and 120 minutes in the untreated samples. On the other hand, figure 4-3B shows that the treated samples experienced a range of only just over 300 color blocks over the same time period. Avocado and potato show the same sort of browning inhibition during the same time period. In the case of banana, the large differences in browning show up in the time period from 24 hours to two weeks. Over that period, though the range of blocks was larger for the untreated sample, the values of the blocks show the browning

inhibition. The untreated samples from 24 hours to two weeks showed values from about 2700 to 1600, as seen in figures 4-10A and 4-10B.

Over the same time period, the treated samples showed values from about 4070 to 2150, as seen in figures 4-12A and 4-12B. Hence, the treated samples were darker, though the amount of browning displayed by the treated samples from 24 hours to two weeks was greater. The difference in browning is probably due to the fact that the untreated samples had already browned significantly by 24 hours, while the browning in the treated samples had been relatively inhibited until that point.

Machine Vision data for untreated and treated banana samples during the time points 0-120 minutes are shown in figures 4-9 and 4-11. In contrast to Machine Vision data obtained using apple, as shown in figures 4-1 and 4-3, the shift in color primitives is similar between treated and untreated banana samples. The similarity between the ranges of color primitives observed during the time period 0-120 minutes indicates that there was not an effective reduction in browning taking place over those observation times.

The second analysis method used on the Machine Vision data was to compare the L* values for the treated and control samples at each time point. L* was analyzed because it is a measure of the lightness or darkness of a sample, which is the aspect of the samples' color most directly changed by PPO-mediated browning. L* values were averaged using both trials of each crop, providing six L* values per point for both treated and control samples. A one-way ANOVA was performed at each time point and the means were compared by way of the LSD test. Thus, it was determined whether or not the control and treated samples' L* values were significantly different at each time point.

As shown in figures 4-13A and 4-14B, respectively, in the case of apple and potato, the control samples were statistically different from each other at time zero, though the difference was very small. For all four crops, however, the difference between control and treated samples grew with increased storage length. Figures 4-13 and 4-14 show that the treated samples for all of the crops except avocado exhibited a significant decrease in browning beginning after one hour. The treated samples of avocado showed a significant difference starting at 24 hours. These results indicate that in all of the trials, the inhibitor was successful in slowing the browning process.

The differences in inhibition between the crops are likely due to varying tertiary structures among the various PPOs. In the same way that a particular PPO has varying affinities to specific substrates, the affinity of the inhibitor to the binding site on the surface of the PPO molecule is likely determined by its unique three-dimensional shape. Both the size and shape of a binding site can affect the affinity of one molecule to another (Bahadur and Zacharias 2007). In the case of PPO, various sizes of the enzyme have been observed, indicating possible variations in the size and/or shape of the inhibitor binding site (Flurkey 1986, Fraignier and others 1995, Sherman and others 1991, Steffens and others 1994, Van Gelder and others 1997, Yang and others 2000).

In other Machine Vision studies of PPO-mediated browning, similar results have been shown. In a study investigating the inhibitory effects of oxalic acid on browning in banana and apple slices, Machine Vision was also used to measure changes in L* values. In that study, decreases in L* value were observed over time as the fruits browned following slicing (Yoruk and others 2003). The same decrease in L* value was seen in this study following pureeing of the fruit. In the case of banana, oxalic acid treatment up to 20 mM led to approximately the

same change in L* value over the first two hours following slicing as the banana puree did in this study following grinding and treatment with hypotaurine. However, in the case of apple, even treatment with just 3 mM of oxalic acid inhibited browning slightly better during the first two hours than the hypotaurine treatment did over the same time period. In another study evaluating melanosis in shrimp, Machine Vision readings were compared with grades of melanosis given by a trained shrimp evaluator. In that study, 64 color blocks were used and 12 were designated as colors associated with melanosis. Over the first 15 days, melanosis increased linearly in the shrimp, and there was an accompanying increase in the levels of melanotic colors over the same time period (Luzuriaga and others 1997). The parallel increases in both the prominence of melanotic colors and scores of melanotic grade indicate that Machine Vision analysis is an accurate tool that can be used to quantify changes in browning-related color over time.

Enzyme Kinetics

To determine the type of inhibition in each crop, Dixon plots were constructed along with Cornish-Bowden plots. Both types of plots alone are limited. The Dixon plot cannot establish the difference between mixed and competitive inhibition, while the Cornish-Bowden plot cannot differentiate between uncompetitive and mixed inhibition. Therefore, when used in concert the two plots can unambiguously determine the type of inhibition (Cornish-Bowden 1974).

Figure 4-15 shows both the Dixon and Cornish-Bowden plots generated from the apple PPO experiments. The pattern of intersecting lines in the first quadrant in the Dixon plot and the parallel lines shown in the Cornish-Bowden plot indicate competitive inhibition. The same pattern is seen in Figure 4-16, which shows the same type of plot generated using the avocado data.

On the other hand, the results are not as clear when examining the plots of the banana and potato data. Figures 4-17 and 4-18 show the plots for banana and potato, respectively. In each

case, the Dixon plot indicates either mixed or competitive inhibition. However, in both cases, the Cornish-Bowden plot shows a pattern not expected with this analysis. These unexpected results may be due to the presence of more than one PPO isozyme within each of the PPO extracts.

Figure 4-23 shows the results of native gel electrophoresis on the four PPO isolates. Though not a high resolution separation, in all four cases it appears that there is more than one PPO isozyme in the extract. It is possible that, in the cases of potato and banana, these multiple PPO isozymes exhibit different kinetic inhibition, leading to the unexpected results seen in the Cornish-Bowden plots.

A number of sulfur-containing compounds have been evaluated as PPO inhibitors and it has been shown that they vary in their respective types of inhibition. In a study investigating the use of L-cysteine as an inhibitor for apple PPO, L-cysteine was found to act as a non-competitive inhibitor (Gacche and others 2006). Glutathione, another sulfur-containing compound that is known to inhibit PPO, was evaluated in another study using PPO isolated from artichoke. When glutathione was used with 4-methylcatechol acting as substrate for the PPO, glutathione showed mixed-type inhibition. Yet, when pyrogallol was used as the substrate instead, glutathione showed noncompetitive inhibition (Dogan and others 2005).

Finally, a third study found that p-aminobenzenesulfonamide behaved as a competitive inhibitor of wild pear PPO (Yerliturk 2008). These results are not surprising, given the fact that sulfur-containing PPO inhibitors vary in the types of inhibition effected, with differences observed even as a function of the PPO substrate used in the system. When used with apple and avocado PPOs, for example, hypotaurine exhibits competitive inhibition, like p-

aminobenzenesulfonamide. When used with banana and potato PPOs, however, hypotaurine acts as a mixed-type inhibitor.

To establish an IC₅₀ for each crop, the maximum substrate concentration used in the kinetics experiments for each fruit or vegetable was assayed in the spectrophotometer using the standard 3 mL mixture in the presence of a wide range of inhibitor concentrations. A quadratic curve was fit to the resulting data, allowing the interpolation of the IC₅₀ value, which is the concentration of inhibitor required to effect a 50% reduction in reaction velocity. Figures 4-19 through 4-22 show the data from each crop's trial. Figure 4-19 shows both the initial apple trial as well as the replication.

7 To establish K_m and V_{max} parameters for each crop, non-linear regression was performed on the untransformed data. Prism software from GraphPad Software was used to fit the data to the Michaelis-Menten model. Non-linear regression was chosen in favor of more traditional methods of K_m and V_{max} determination, such as using a Lineweaver-Burk (double reciprocal) plot because non-linear regression fits the data without modification, while the double reciprocal plot requires editing of both substrate concentration and reaction velocity data in order to construct the plot (Lineweaver and Burk 1934). The indirect analysis of the data results in a distorted distribution of error. Therefore, estimates of kinetic parameters given by fitting the data using non-linear regression are more statistically valid (Leatherbarrow 1990). Table 4-1 shows K_m and V_{max}, as well as IC₅₀ and K_i values, for each crop. K_i was calculated using the Cheng-Prusoff equation, which takes into account K_m, substrate concentration, and the IC₅₀ value (Cheng and Prusoff 1973).

Comparison of Novel Inhibitor to Other Browning Inhibitors

Compared to popular browning inhibitors that are used commercially now, hypotaurine has a number of distinct advantages. First, it is able to decrease the maximal amount of

browning in a product. In the case of ascorbic acid, another common browning inhibitor used in fresh cut fruits, only the lag phase of the browning process is affected. Because ascorbic acid slows browning, at least when inhibiting some type of PPO, solely by acting upon the oxidized reaction products. Ascorbic acid is able to reduce the products back to their colorless forms. The resulting oxidized form of ascorbic acid, dehydroascorbate, does not regenerate back to its reduced form, however, and it is no longer able to prevent browning. In time, therefore, the same level of browning will be achieved as a sample without ascorbic acid (Arias and others 2007). By contrast, the new inhibitor not only increases the period before browning is observed but also decreases the maximum browning observed, due to the fact that it inhibits the PPO molecule directly.

Another advantage of the new inhibitor over some older types of browning inhibitors is that it requires very low concentrations to achieve a high level of inhibition. In one study, a crude extract of burdock root PPO was tested against bisulfite, ascorbic acid, 4-hexylresorcinol and citric acid as browning inhibitors. At a concentration of 0.5% w/v, citric and ascorbic acids provided approximately 10% inhibition of browning activity, 4-hexylresorcinol showed approximately 85% inhibition, and bisulfite provided 100% inhibition (Lee-Kim and others 1997). It was also noted that citric acid is generally applied to foods commercially at a concentration of 0.5% to 2% w/v, which supports the argument that citric acid requires high concentrations in order to affect significant browning inhibition (Marshall and others 2000). In comparison, the inhibitor examined in this study provides over 80% inhibition to most of the PPOs tested at a concentration of less than 1 mM (0.1095% w/v).

Finally, unlike other browning inhibitors, such as ascorbic acid and EDTA, the novel browning inhibitor examined in this study can be used alone in food products instead of in

concert with other browning inhibitors. A typical commercial anti-browning cocktail for fruits and vegetables could consist of three or more acidifiers, chelators, and chemical reducing agents (Marshall and others 2000). A comparable and successful reduction in browning can be achieved by using relatively low concentrations of the novel inhibitor alone.

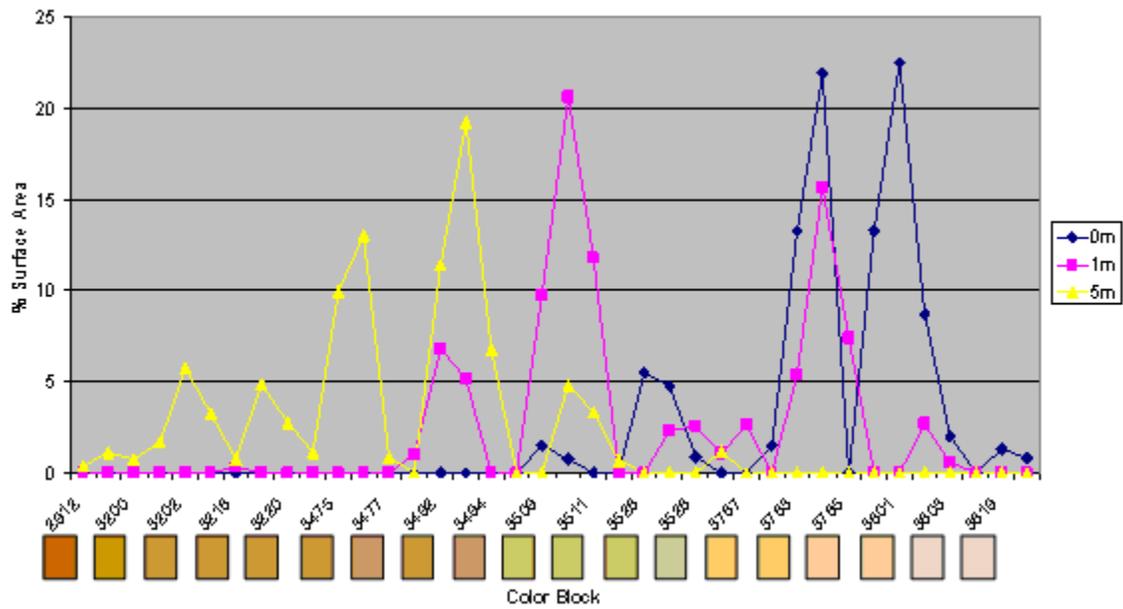
The novel compound also compares favorably to other PPO inhibitors in regards to kinetic parameters. K_i is the dissociation constant of the enzyme-inhibitor complex. A lower K_i value equates to a lower IC_{50} value, and the lower the IC_{50} value, the more strongly a compound inhibits the PPO being tested. In one study, novel PPO inhibitors were synthesized using raw materials that did not show a high level of PPO inhibition. The best PPO inhibitor synthesized during this study showed a K_i of 40 μ M (Tricand de la Goutte 2001). In comparison, the K_i values found for the novel inhibitor in this study ranged from approximately 3-14 μ M, depending on the crop from which the PPO was isolated. In another study, diethyldithiocarbamic acid was investigated as an inhibitor for PPO from three different sources. Again, the novel inhibitor from this study showed lower K_i values than any shown using diethyldithiocarbamic acid. The lowest K_i value calculated for diethyldithiocarbamic acid was when using it as an inhibitor for mushroom PPO with 4-methylcatechol as a substrate for the enzyme. The K_i value was 0.015 mM, which is greater than the maximum K_i calculated in this study using the novel inhibitor, which was 0.0138 mM (Dogan and others 2008).

Another study investigated a number of substrate/inhibitor pairings, using yacon PPO. When chlorogenic acid was the substrate for PPO, *p*-coumaric and cinnamic acids had K_i values of 0.017 and 0.011 mM, respectively (Neves and Silva 2007). In that enzyme-substrate-inhibitor system, the cinnamic acid achieved up to 95% inhibition, an inhibitory ability comparable to the value documented in this study for the novel inhibitor and apple PPO.

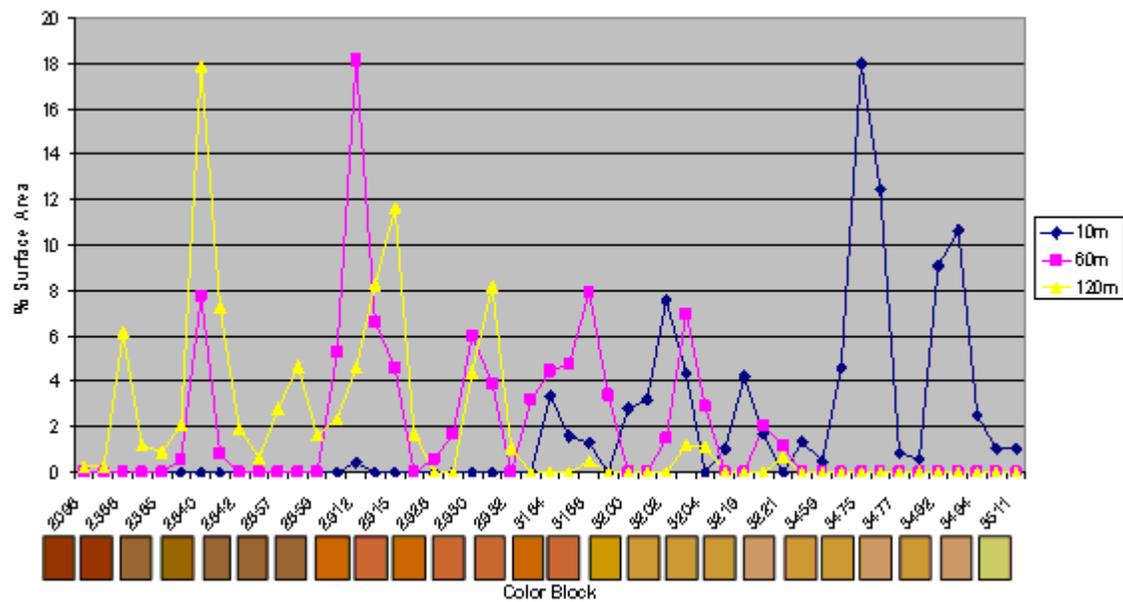
Table 4-1. Kinetic parameters for apple, avocado, banana, and potato.

Parameter	Apple	Avocado	Banana	Potato
Km (Mm)	40.47	10.26	25.52	8.322
Vmax (Absorbance)	0.5247	0.3997	0.5376	0.4049
IC50 (Mm)	0.2177 +/- 0.0009	0.2253 +/- 0.0224	0.2853 +/- 0.03552	0.1962 +/- 0.0008
Ki (Mm)	0.0138 +/- 7.774×10^{-5}	0.01004 +/- 0.0018	0.0038 +/- 5.324×10^{-4}	0.0027 +/- 1.552×10^{-5}

Note: Km and Vmax determined by non-linear regression fit to Michaelis-Menten model. Ki calculated from Km and average IC50 using the Cheng-Prusoff equation (Cheng and Prusoff 1973). Error is given as one standard deviation.

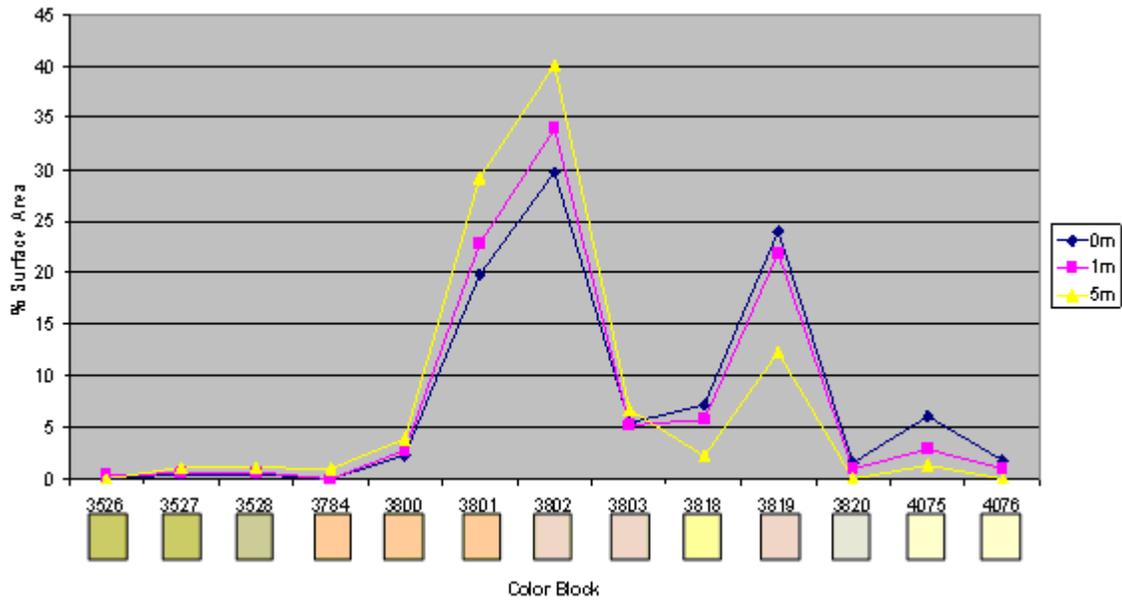


A

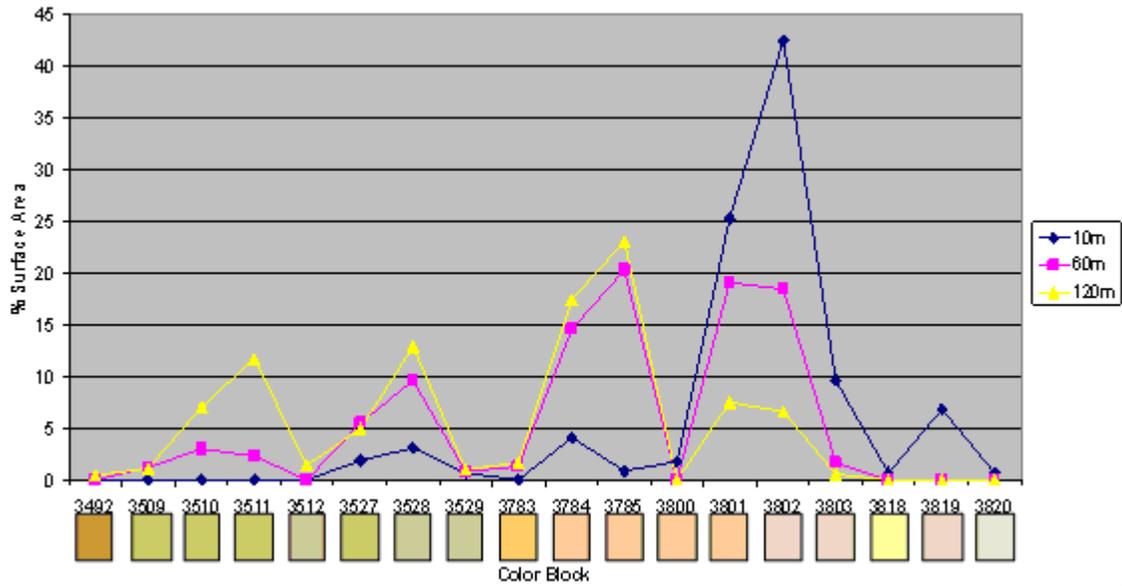


B

Figure 4-1. Untreated samples in the first apple trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas. A) Time points 0-5 minutes. B) Time points 10-120 minutes.

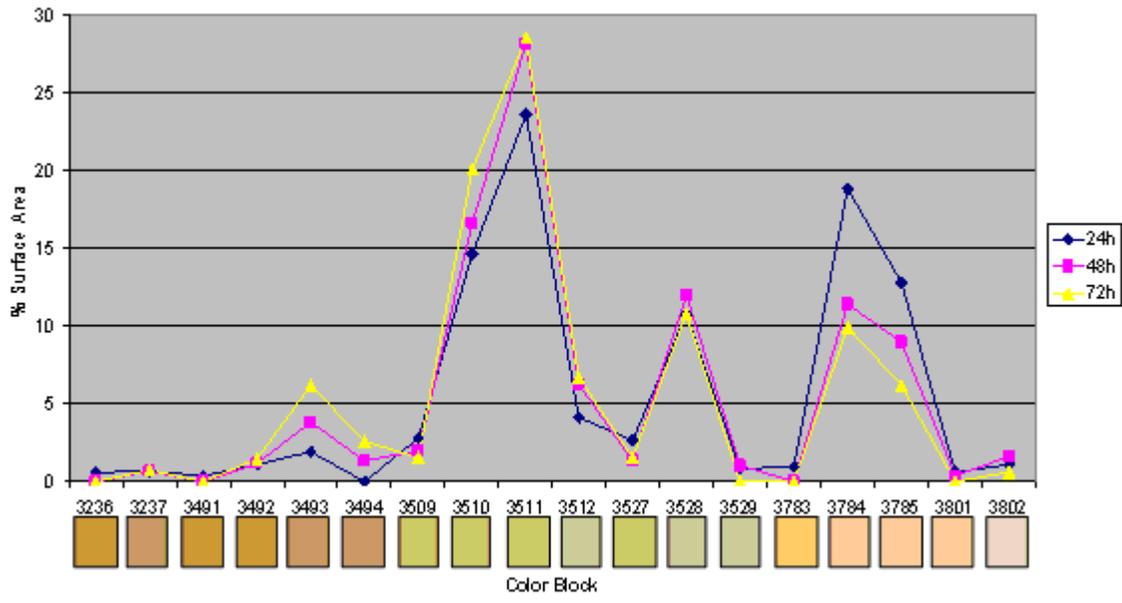


A

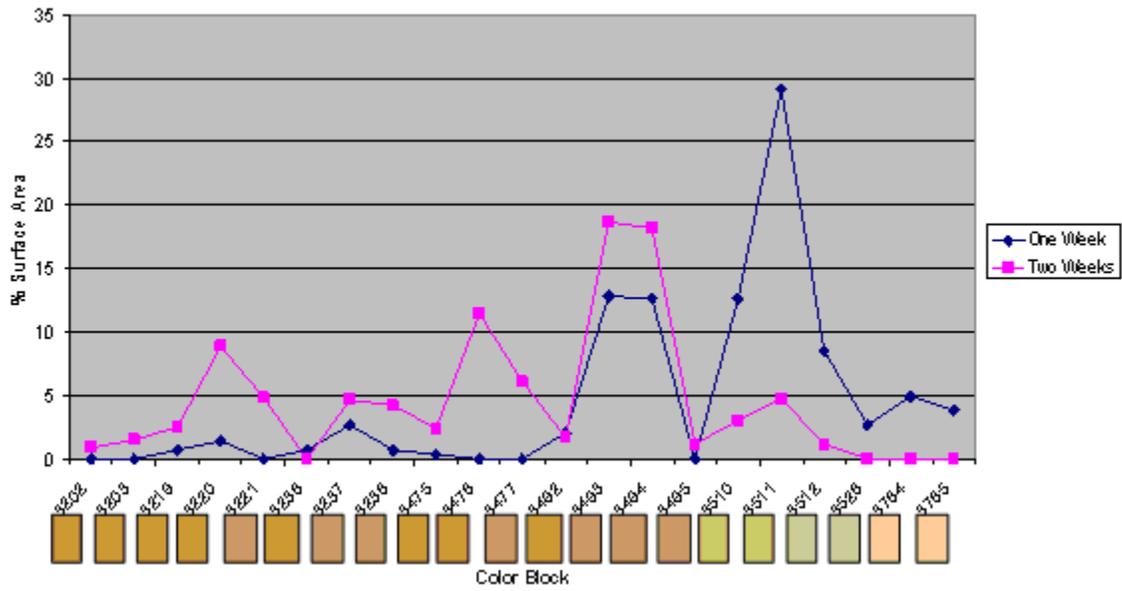


B

Figure 4-3. Treated samples in the first apple trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas. A) Time points 0-5 minutes. B) Time points 10-120 minutes.

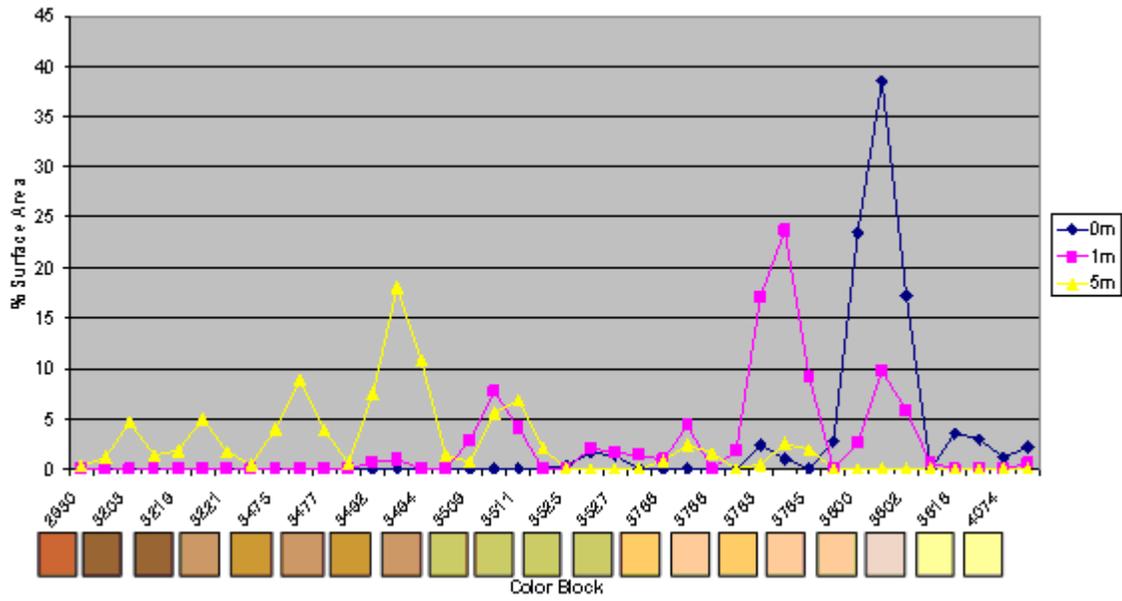


A

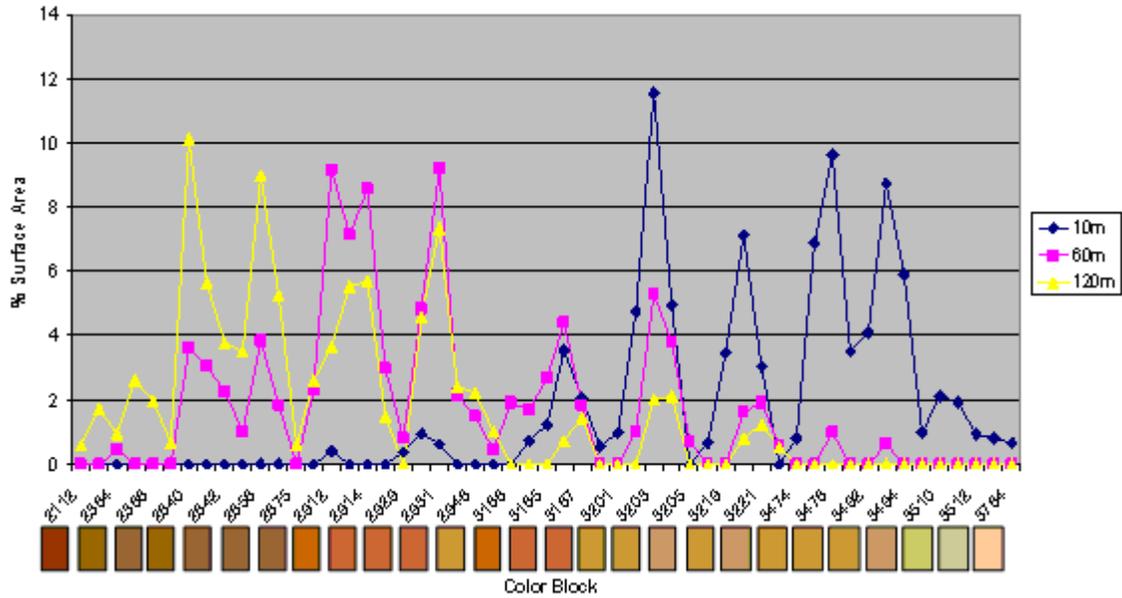


B

Figure 4-4. Treated samples in the first apple trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas. A) Time points 24-72 hours. B) Time points 1-2 weeks.

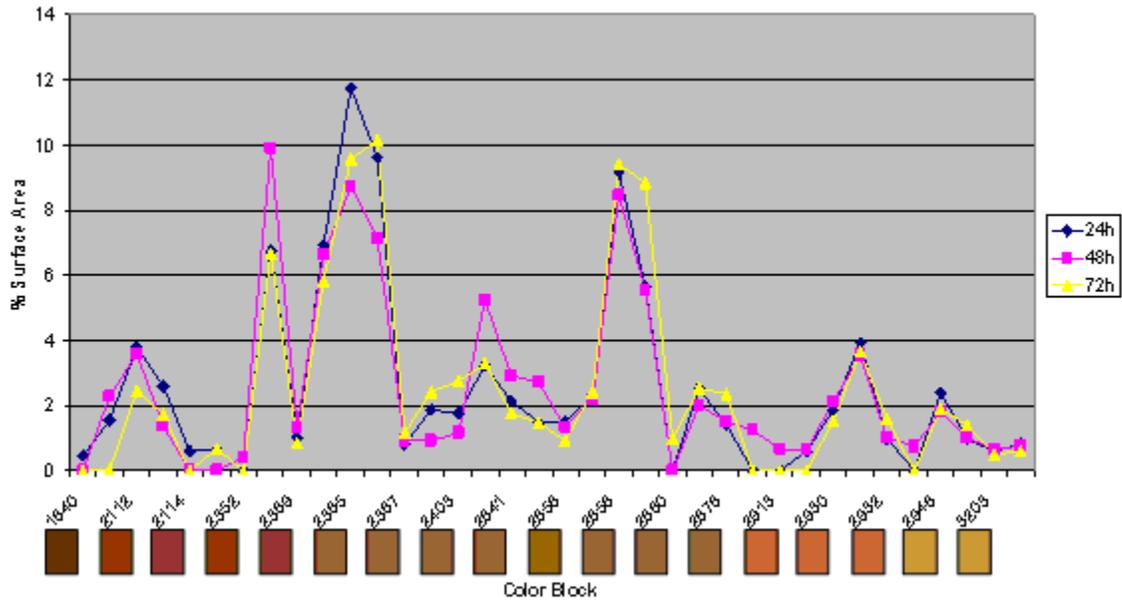


A

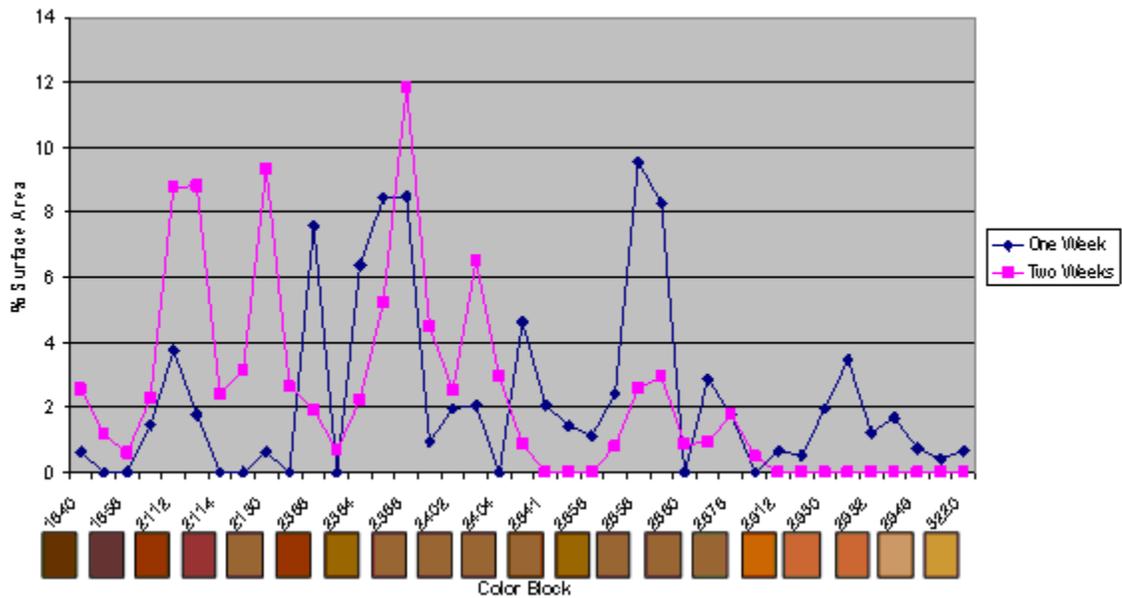


B

Figure 4-5. Untreated samples in the second apple trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas. A) Time points 0-5 minutes. B) Time points 10-120 minutes.

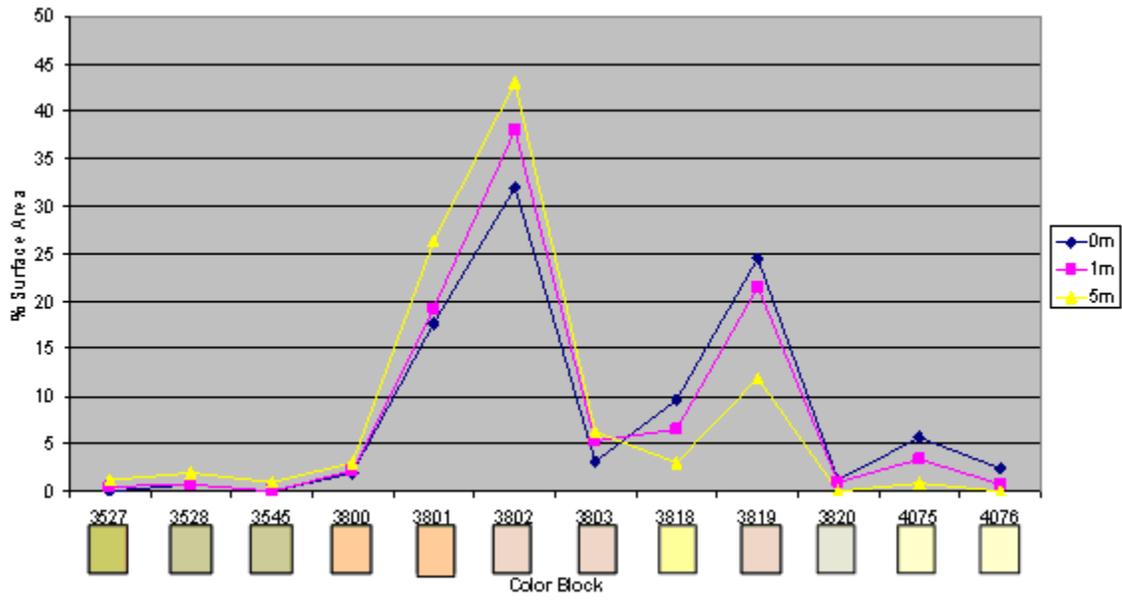


A

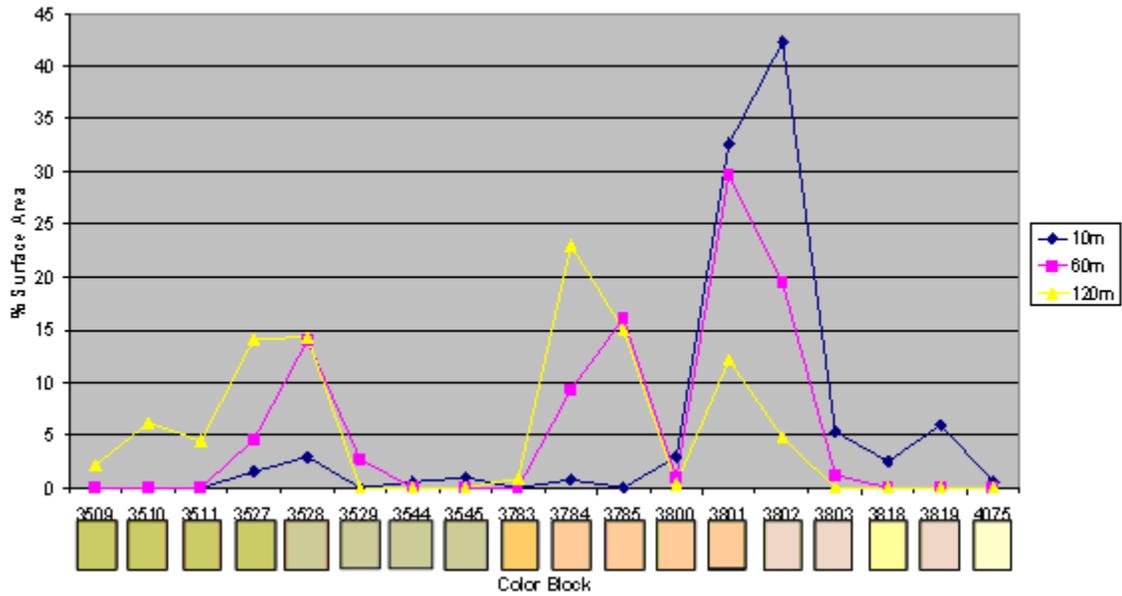


B

Figure 4-6. Untreated samples in the second apple trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas. A) Time points 24-72 hours. B) Time points 1-2 weeks.

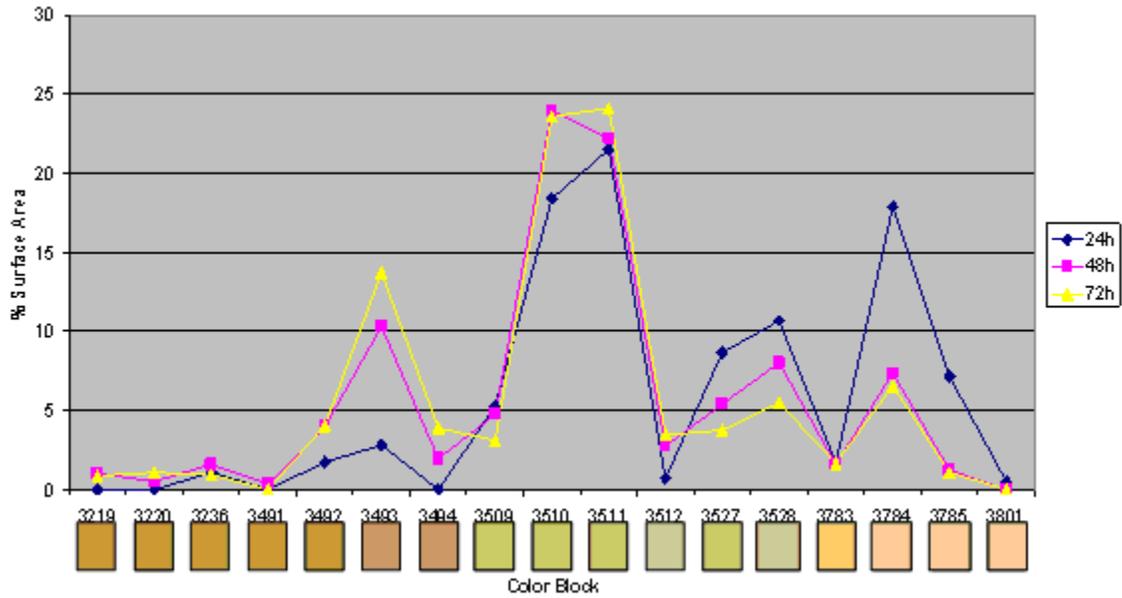


A

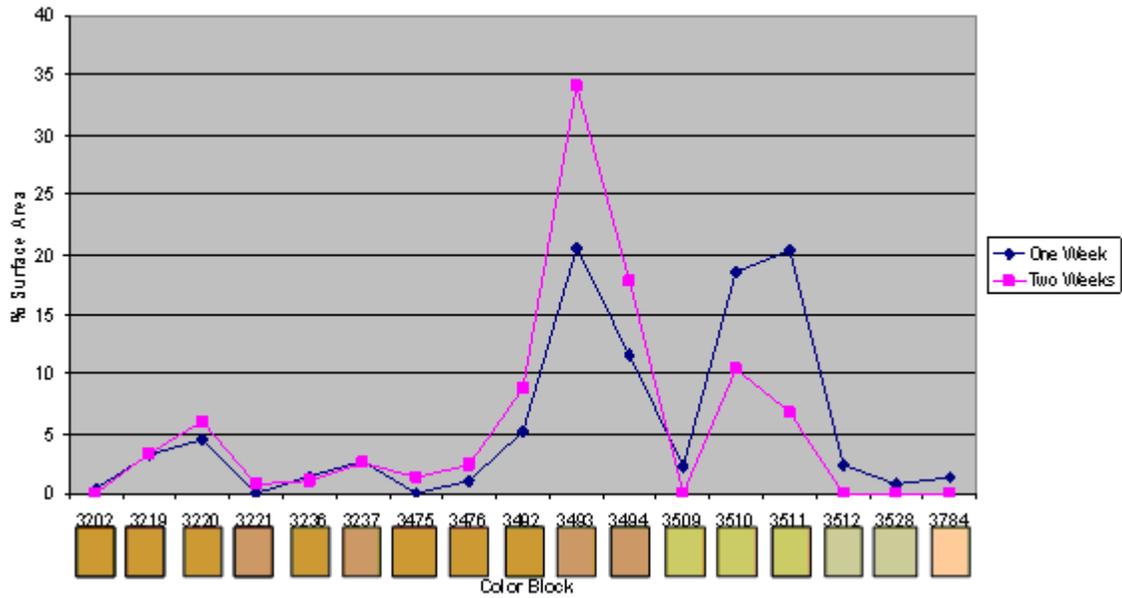


B

Figure 4-7. Treated samples in the second apple trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas. A) Time points 0-5 minutes. B) Time points 10-120 minutes.

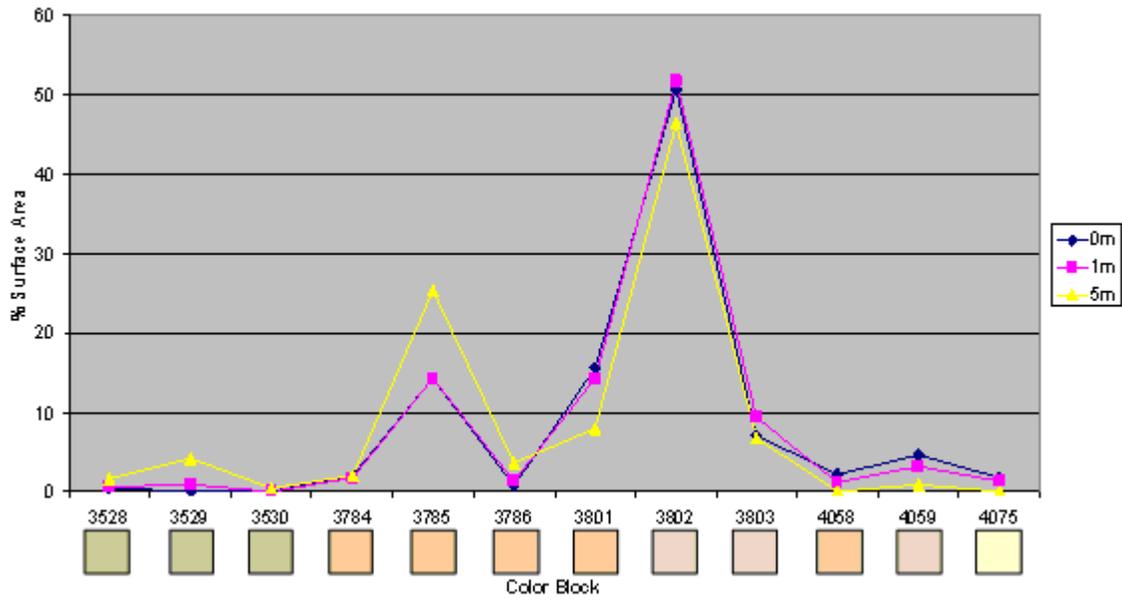


A

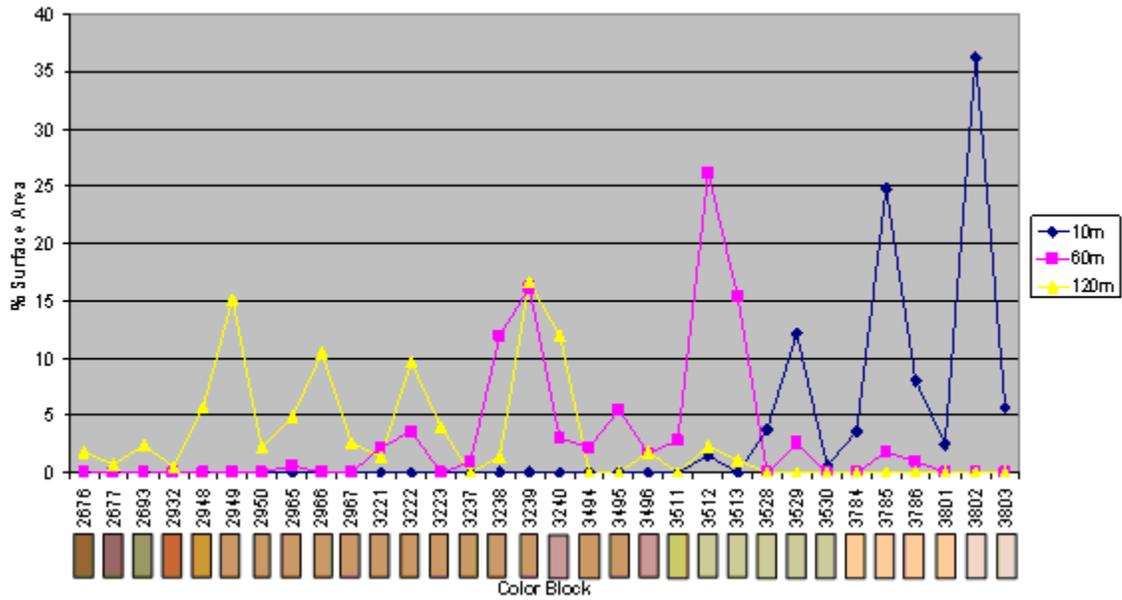


B

Figure 4-8. Treated samples in the second apple trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas. A) Time points 24-72 hours. B) Time points 1-2 weeks.

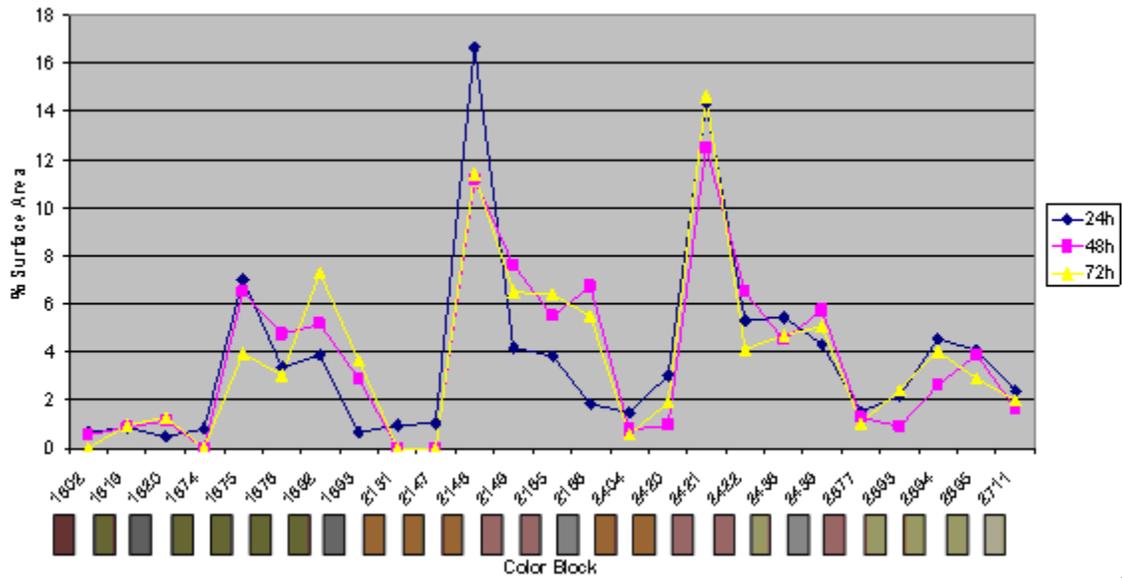


A

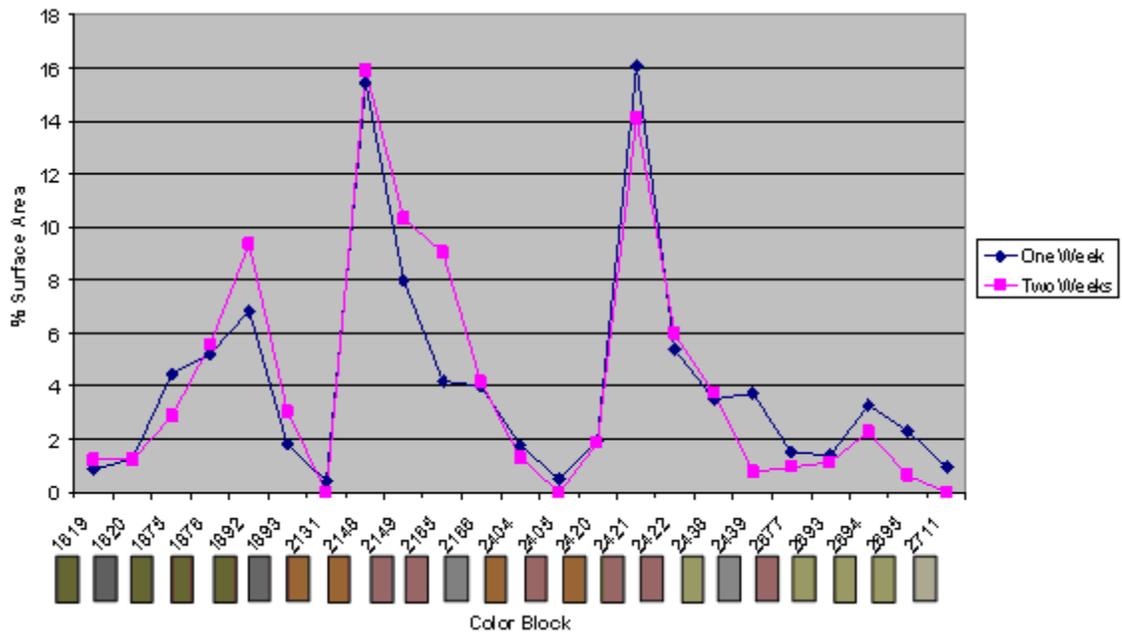


B

Figure 4-9. Untreated samples in the first banana trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas. A) Time points 0-5 minutes. B) Time points 10-120 minutes.

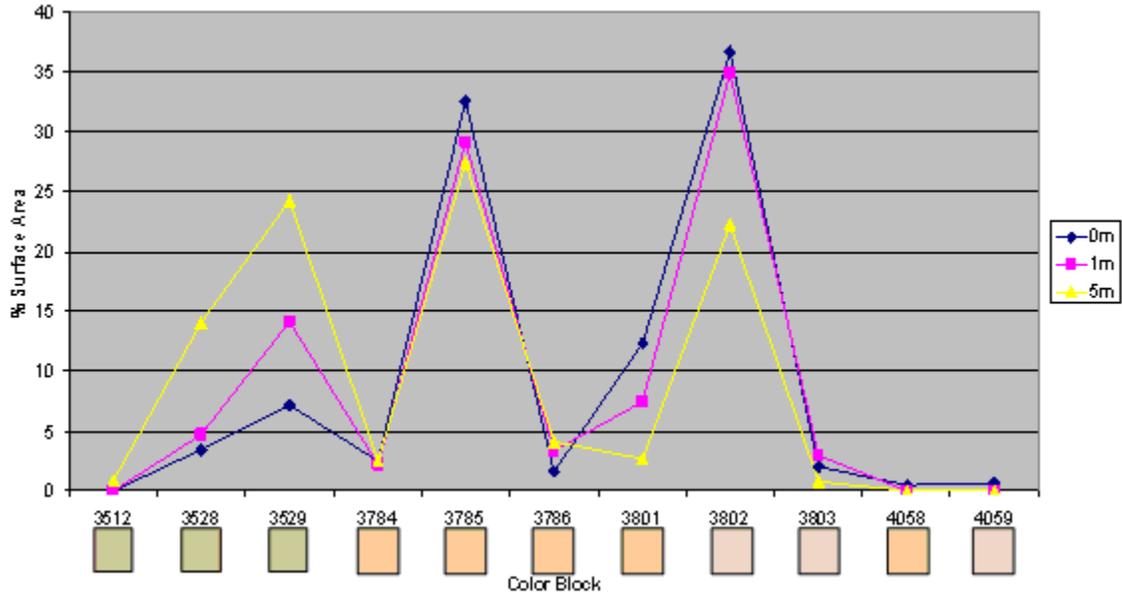


A

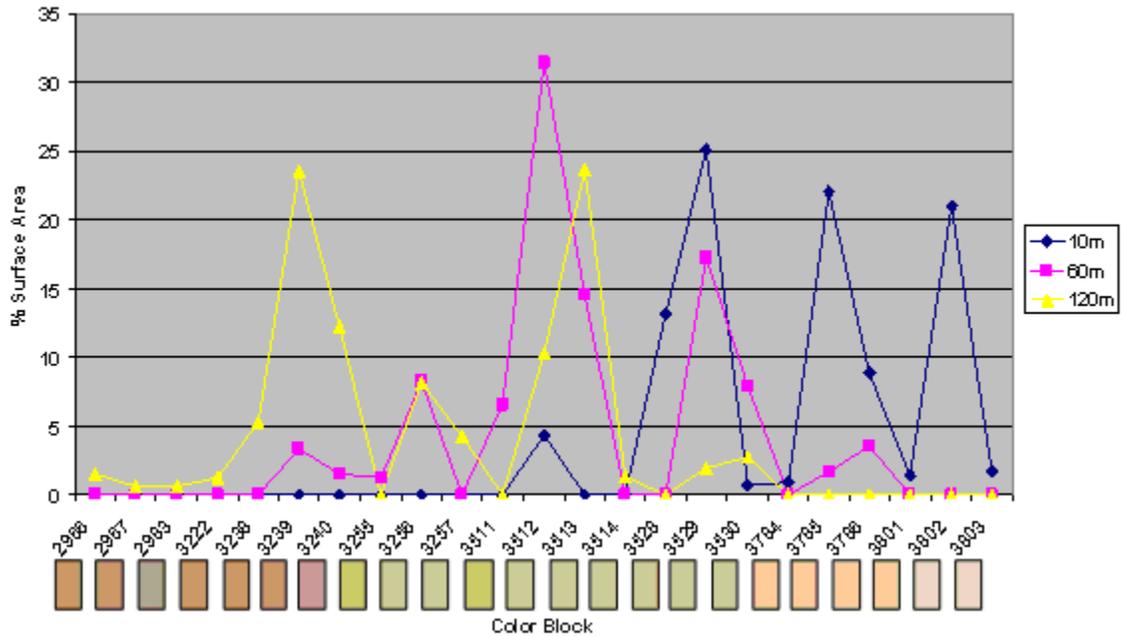


B

Figure 4-10. Untreated samples in the first banana trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas. A) Time points 24-72 hours. B) Time point 1-2 weeks.

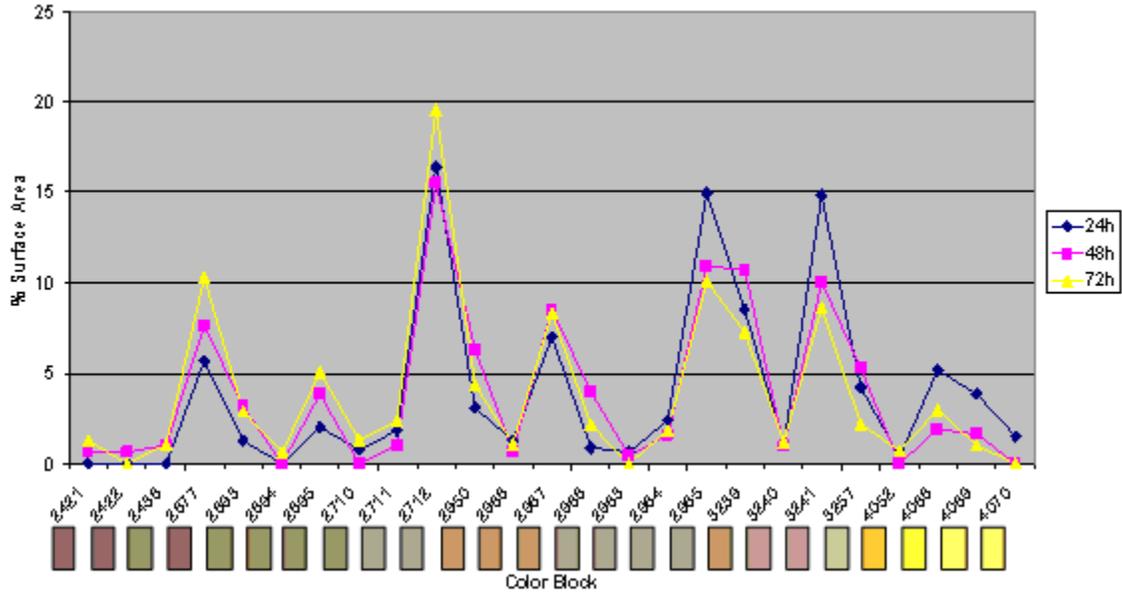


A

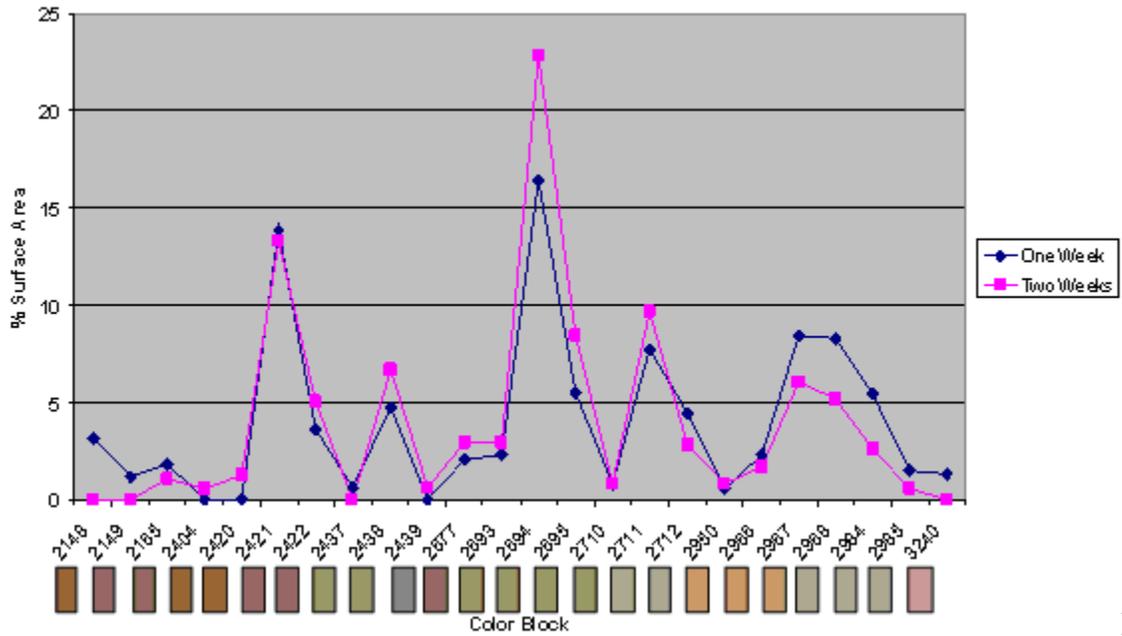


B

Figure 4-11. Treated samples in the first banana trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas. A) Time points 0-5 minutes. B) Time points 10-120 minutes.

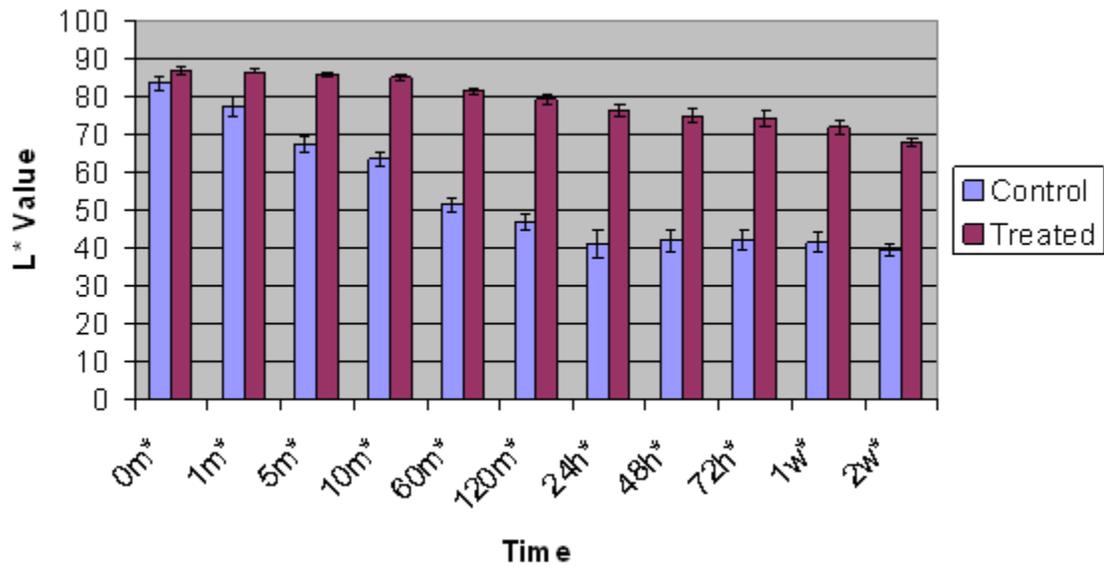


A

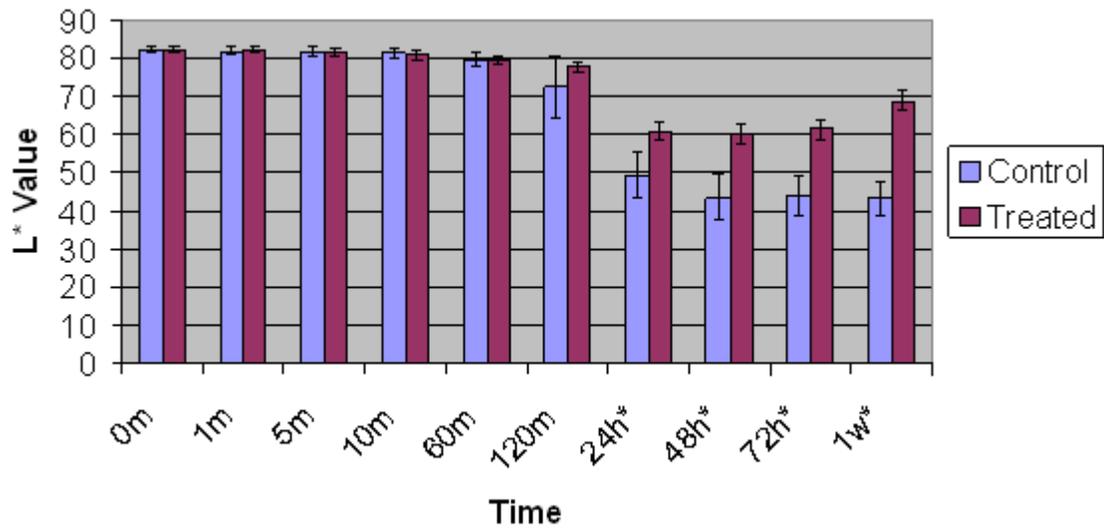


B

Figure 4-12. Treated samples in the first banana trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas. A) Time points 24-72 hours. B) Time point 1-2 weeks.

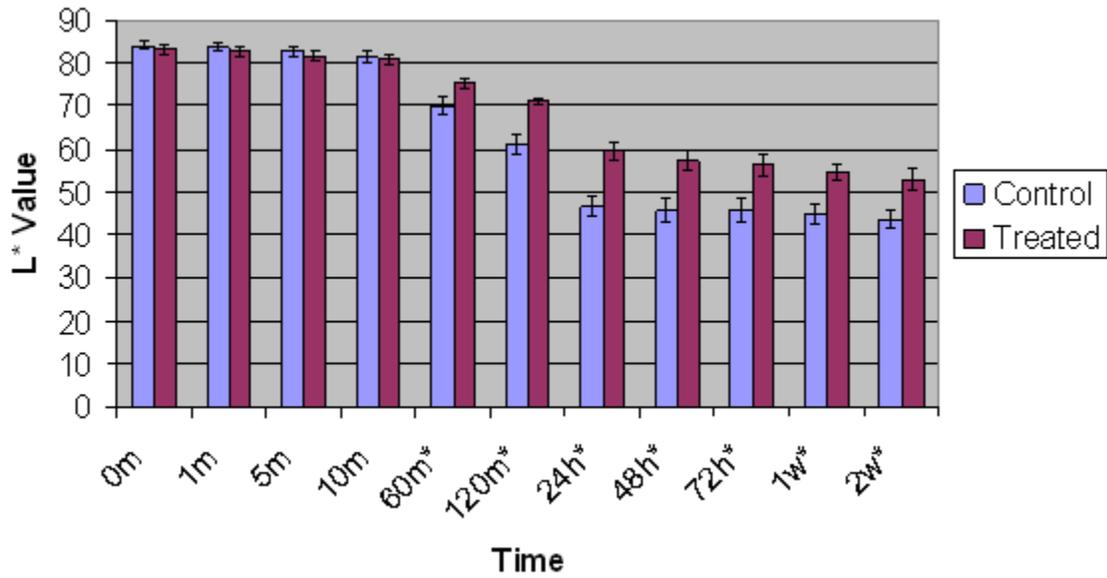


A

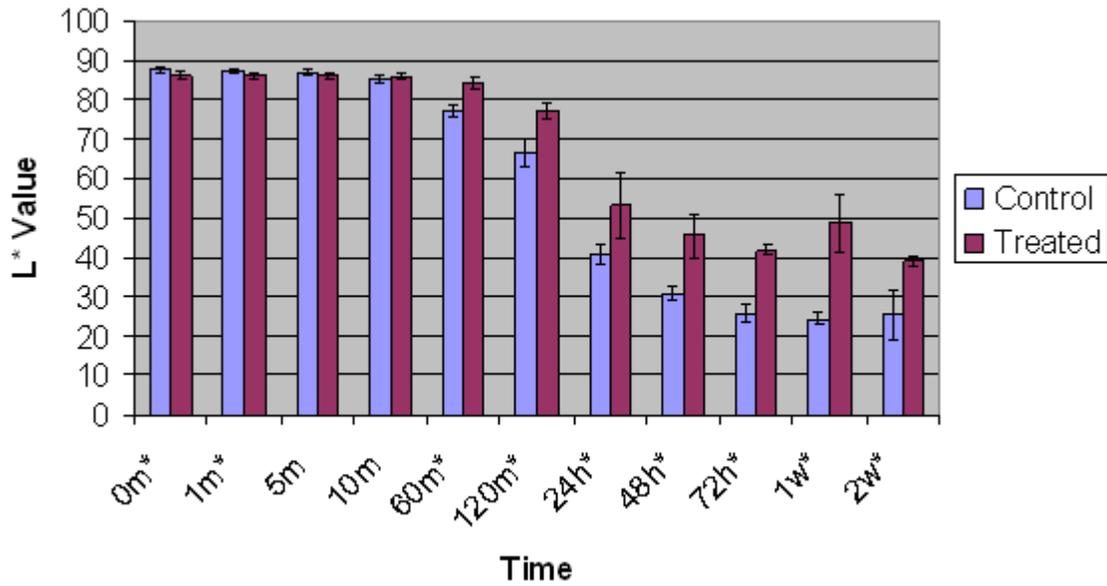


B

Figure 4-13. Apple and avocado: comparison between control and treated L* values at each time point. Time point labels followed by an asterisk indicate a significant difference between control and treated samples at that time point ($p < 0.05$). Error bars show one standard deviation below and above average. A) Values for apple. B) Values for avocado.



A



B

Figure 4-14. Banana and potato: comparison between control and treated L* values at each time point. Time point labels followed by an asterisk indicate a significant difference between control and treated samples at that time point ($p < 0.05$). Error bars show one standard deviation below and above average. A) Values for banana. B) Values for potato.

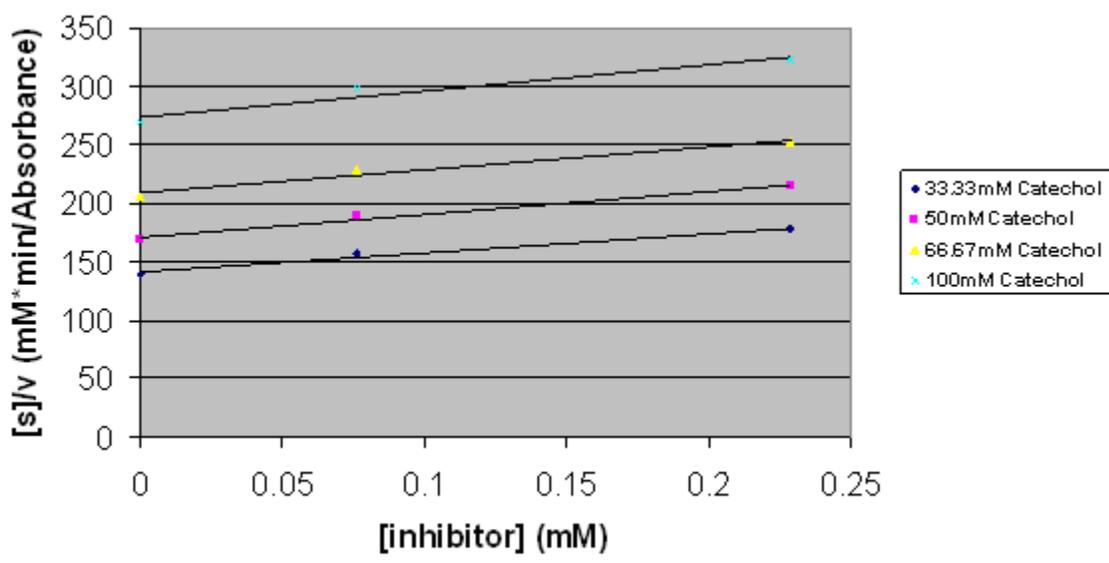
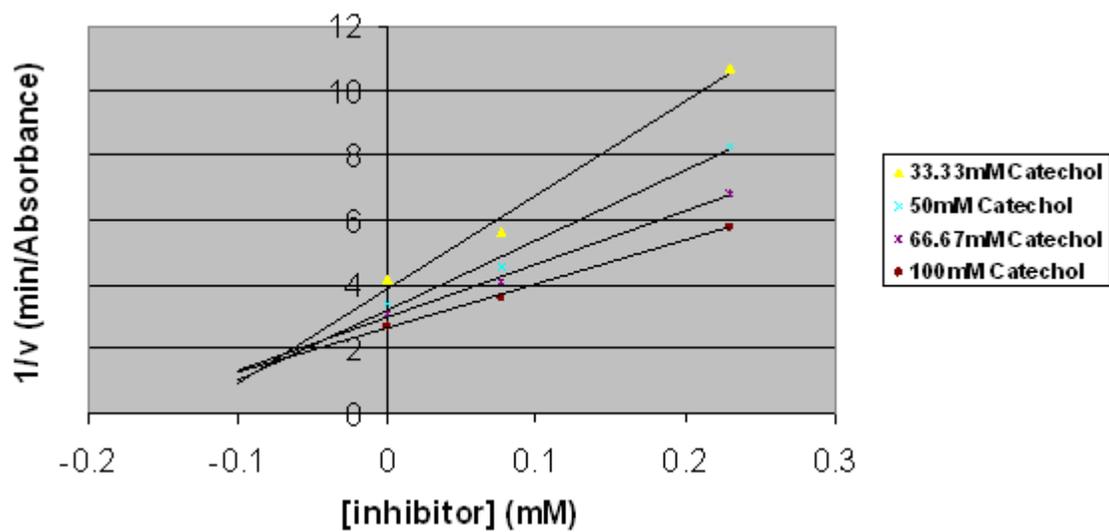
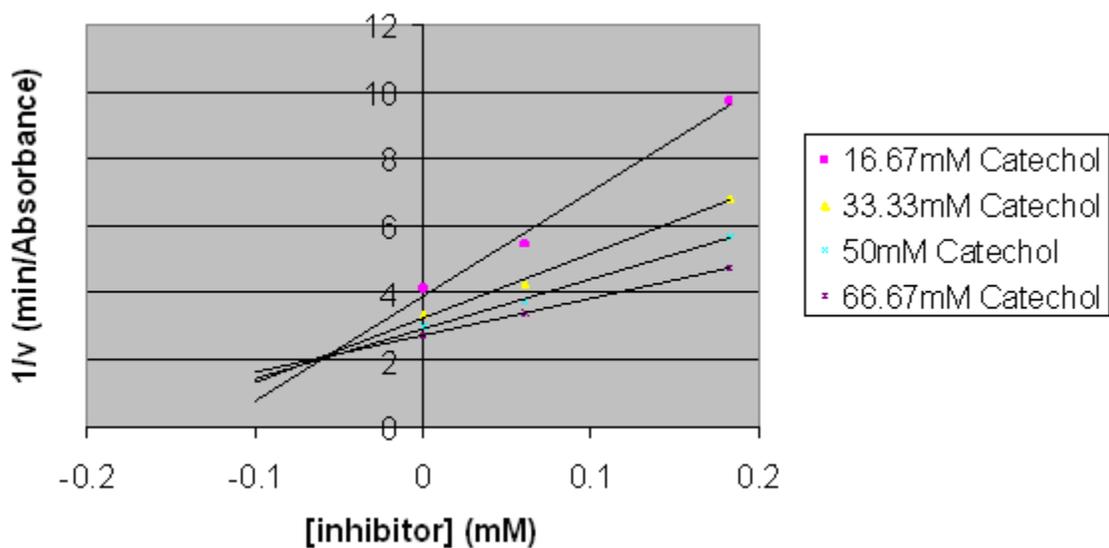
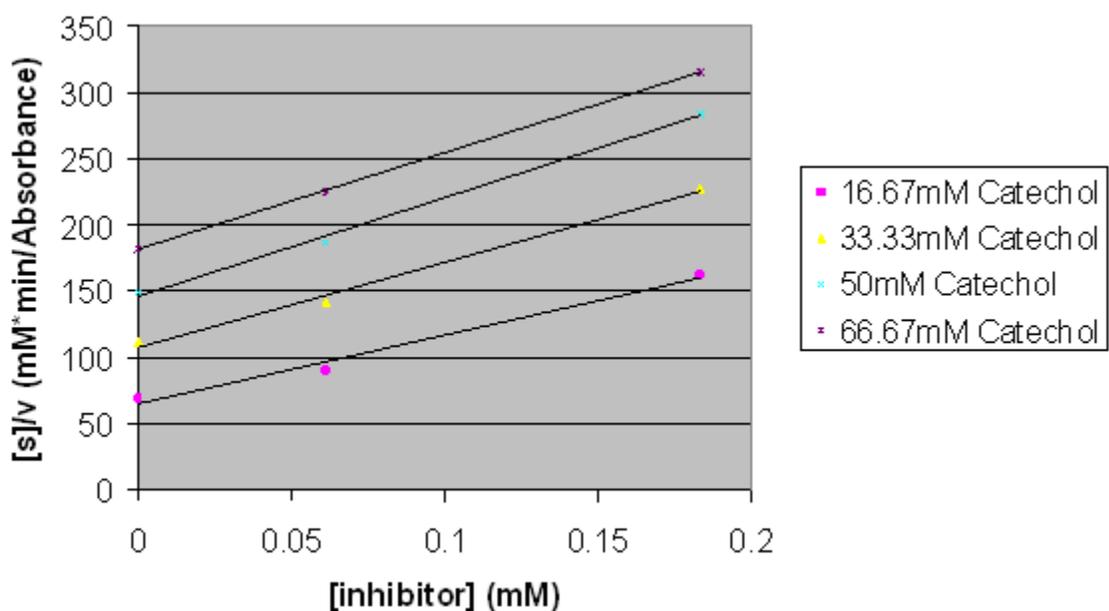


Figure 4-15. Apple kinetics plots. These two plots, in conjunction, show that the inhibitor is competitive with regard to apple polyphenol oxidase. A) Dixon and B) Cornish-Bowden plots for apple kinetic data (Cornish-Bowden 1974).

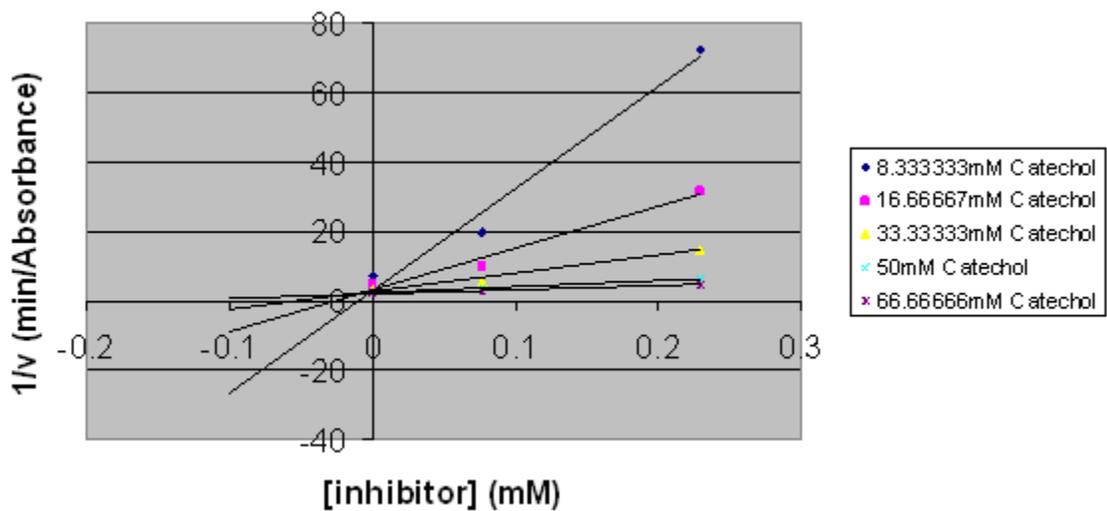


A

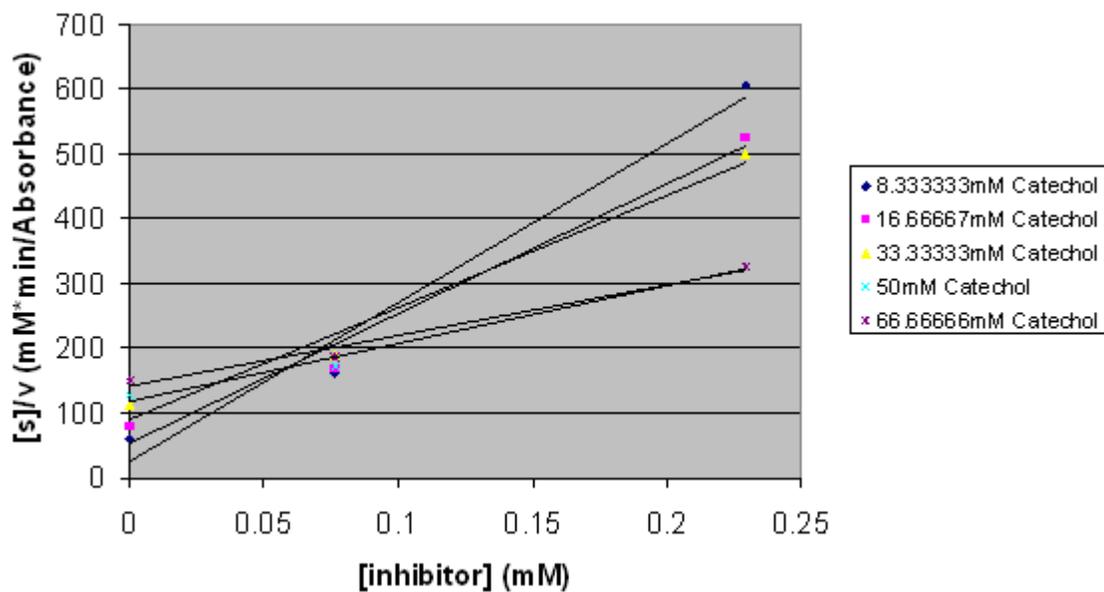


B

Figure 4-16. Avocado kinetics plots. These two plots, in conjunction, show that the inhibitor is competitive with regard to avocado polyphenol oxidase. A) Dixon and B) Cornish-Bowden plots for avocado kinetic data (Cornish-Bowden 1974).

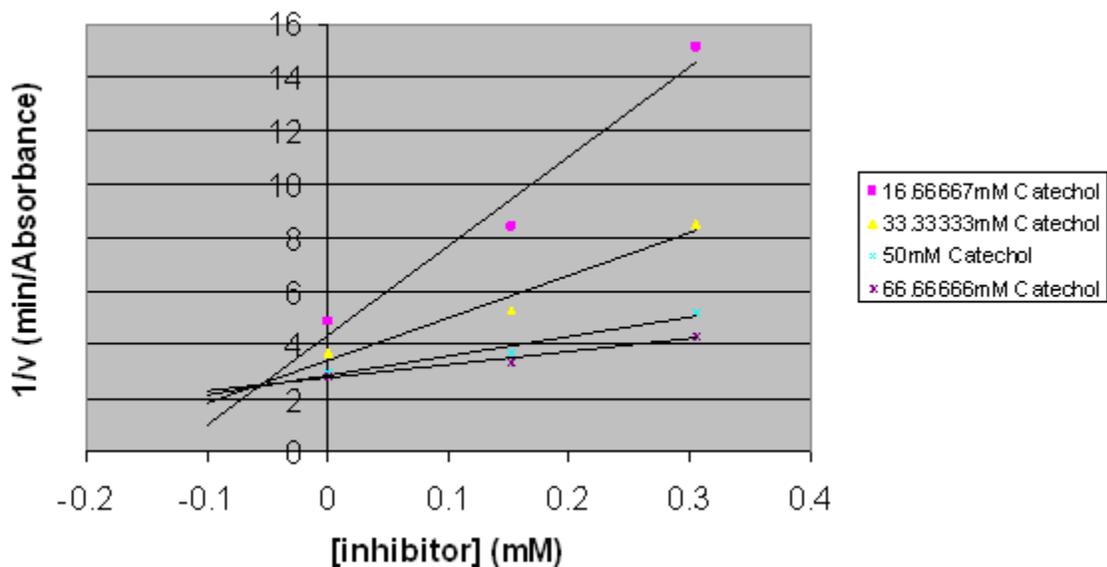


A

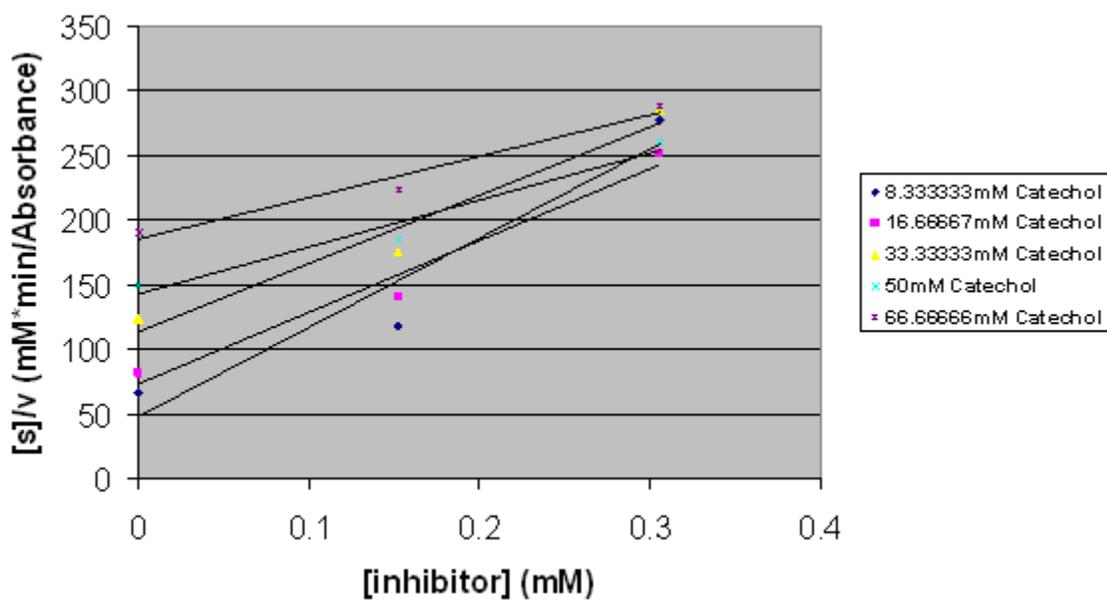


B

Figure 4-17. Banana kinetics plots. These two plots, in conjunction, are inconclusive but indicate either competitive or mixed inhibition. A) Dixon and B) Cornish-Bowden plots for banana kinetic data (Cornish-Bowden 1974).



A



B

Figure 4-18. Potato kinetics plots. These two plots, in conjunction, are inconclusive but indicate either competitive or mixed inhibition. A) Dixon and B) Cornish-Bowden plots for potato kinetic data (Cornish-Bowden 1974).

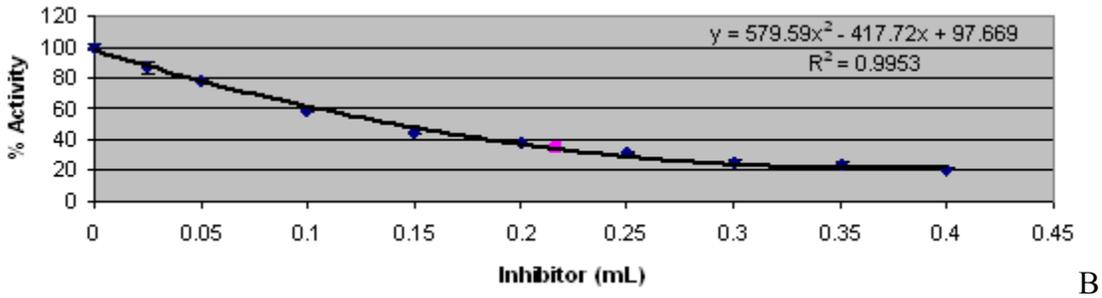
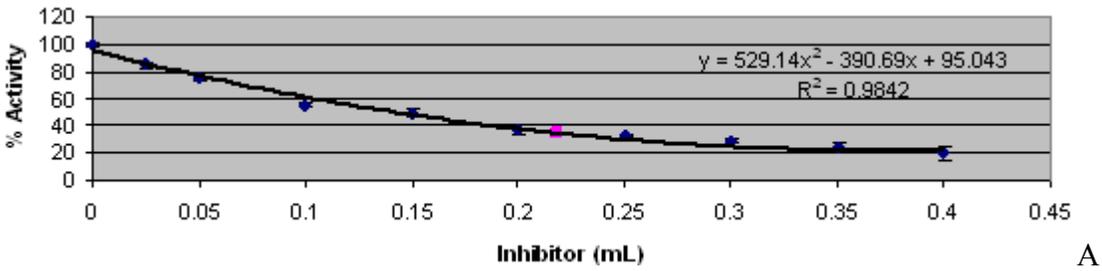


Figure 4-19. Apple: plots of percent activity vs. inhibitor concentration to determine IC50. The replicate experiment (B) is shown for this crop but not for the others, as variance between trials was small. IC50 for trials one and two are 0.21832 mM and 0.217066 mM, respectively. IC50s are indicated by points marked in pink.

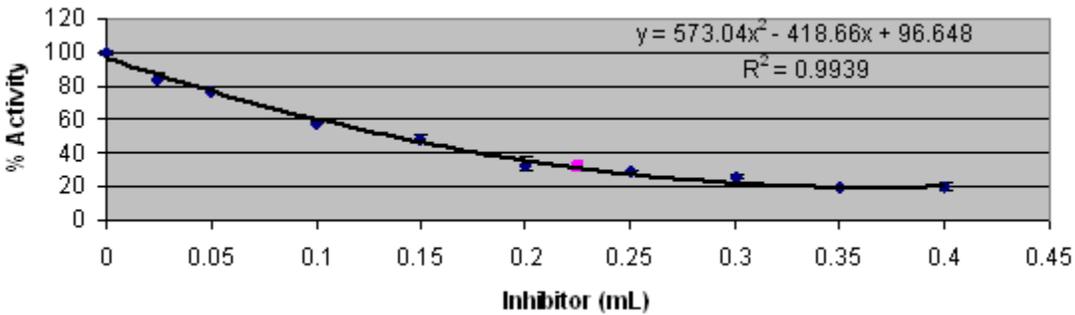


Figure 4-20. Avocado: plot of percent activity vs. inhibitor concentration to determine IC50. IC50 for trials one and two are 0.209467 mM and 0.241135 mM, respectively. Average IC50 is indicated by point marked in pink.

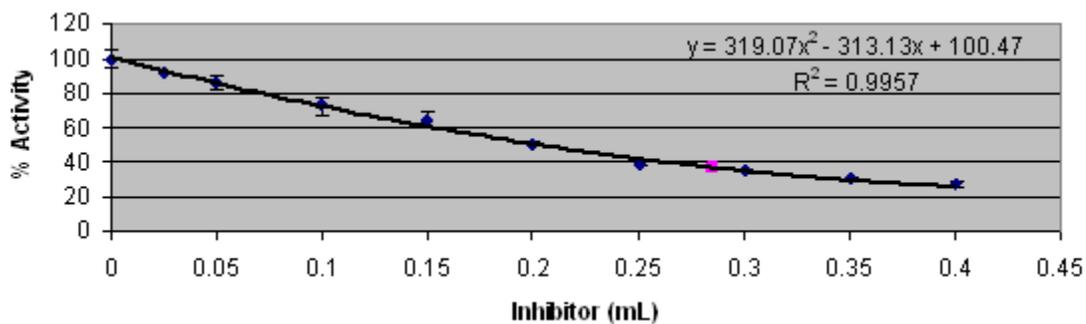


Figure 4-21. Banana: plot of percent activity vs. inhibitor concentration to determine IC50. IC50 for trials one and two are 0.310414 mM and 0.260183 mM, respectively. Average IC50 is indicated by point marked in pink.

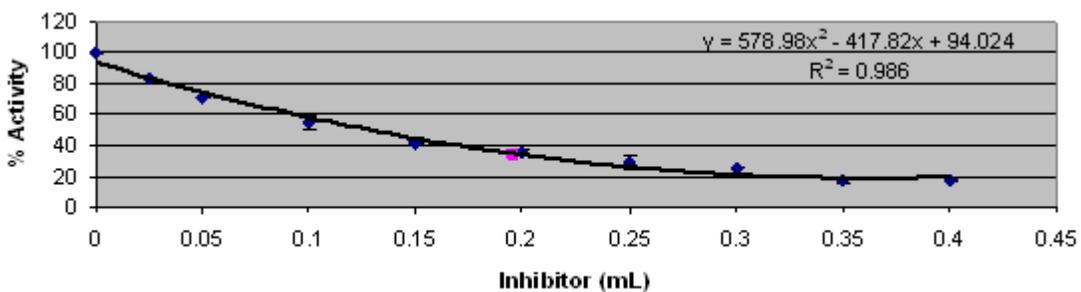


Figure 4-22. Potato: plot of percent activity vs. inhibitor concentration to determine IC50. IC50 for trials one and two are 0.195615 mM and 0.196749 mM, respectively. Average IC50 is indicated by point marked in pink.

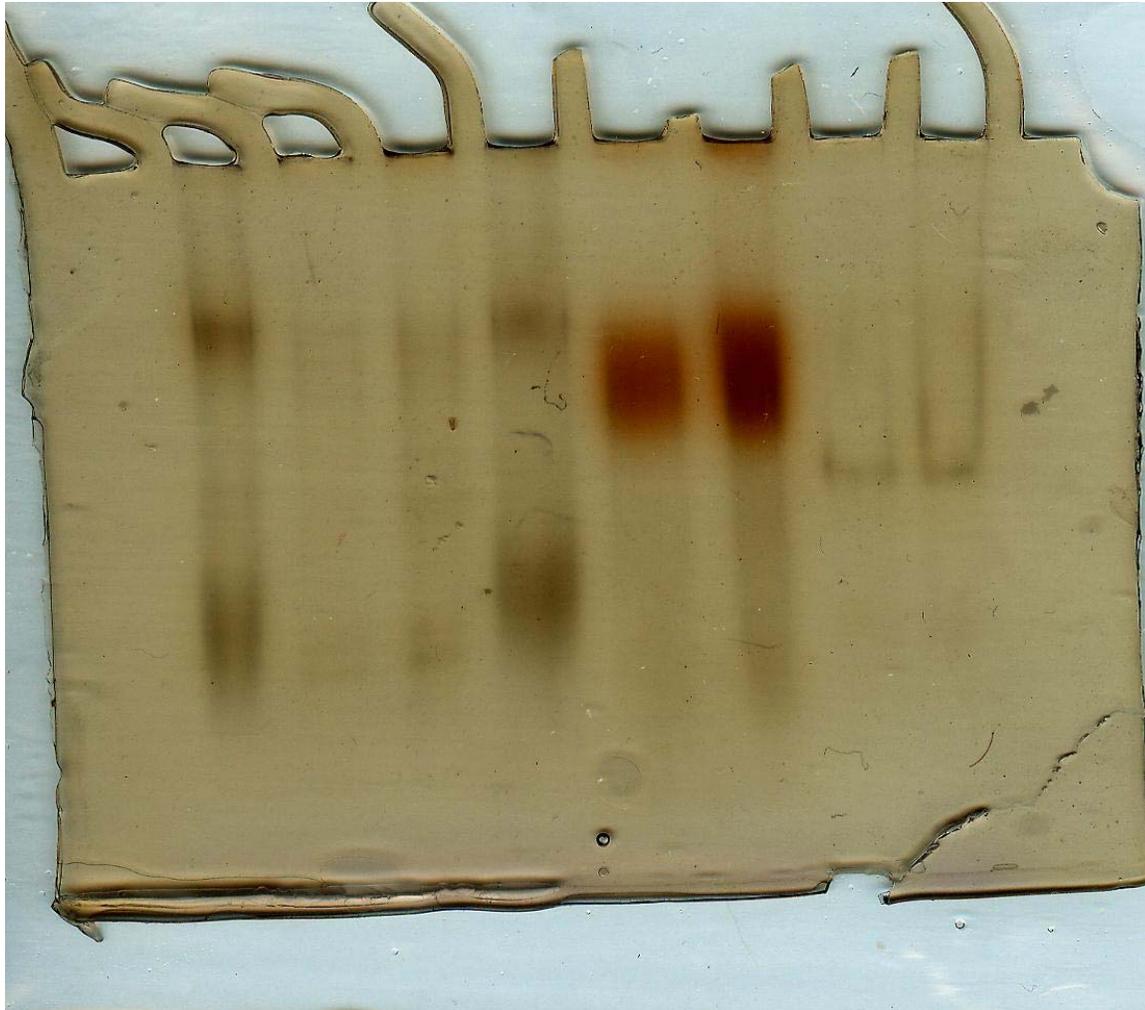


Figure 4-23. Results of native gel electrophoretic separation of crude PPO isolates. From left to right, the lanes were loaded as follows 25ug banana PPO, 25ug potato PPO, 45ug potato PPO, 45ug banana PPO, 25ug avocado PPO, 45ug avocado PPO, 25ug apple PPO, and 45ug apple PPO. All volumes include 1:2 dilution of PPO extract with running buffer.

CHAPTER 5 CONCLUSIONS

A novel polyphenol oxidase inhibitor was extracted from blue mussel (*Mytilus edulis*), identified as hypotaurine, and characterized with regards to its inhibitory properties in avocado, potato, banana, and apple and the modes of inhibition displayed in these four species of crops. Over a storage period of two weeks, all samples showed significant decreases in browning when exposed to the inhibitor compound. Significant differences in browning appearance between control and experimental samples were observed to begin from one minute after the start of the experiment to one day after the start of the experiment, depending on the species of the specific sample.

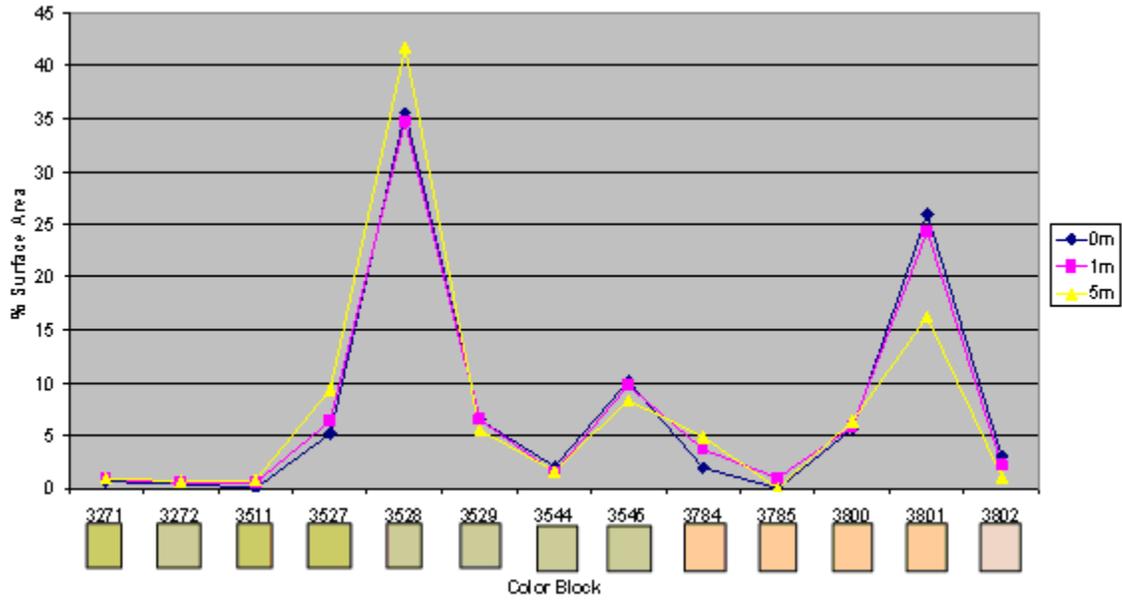
The inhibitor was also shown to exhibit both competitive and mixed-type inhibition, depending upon the species of crop on which it was tested. Problems arose when attempting to graphically determine the types of inhibition in the cases of banana and potato. It is believed that these results are due to the presence of more than one PPO isozyme in the enzyme extract from the fruits that each exhibit different types of inhibition.

In order to further elucidate the benefits of this novel enzyme inhibitor, the Machine Vision experiments should be conducted again using a purified form of the inhibitor. Closely controlling the concentration of the inhibitor in the samples would greatly benefit the ability to compare the effect of the inhibitor between various crop species. There are clearly differences in how well the compound is able to inhibit the action of PPO in different crops, but without being able to exactly control the inhibitor concentration it is hard to compare the samples accurately side by side. In addition, it would be beneficial to be able to perform the enzyme kinetics experiments again using purified PPO extracts, as opposed to the crude ones used in this study.

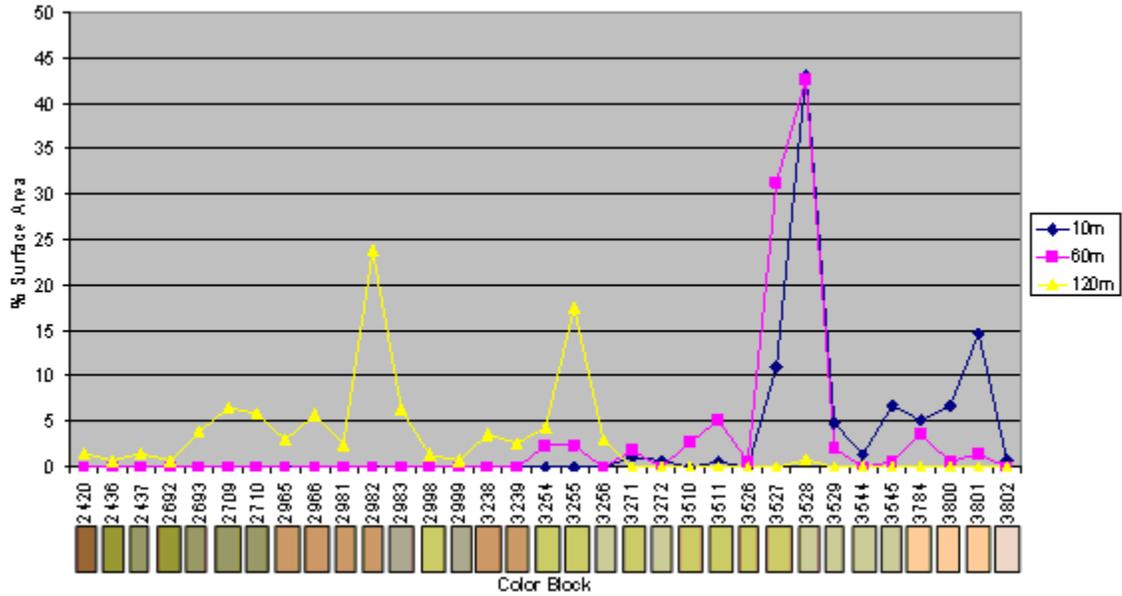
By utilizing only one PPO isozyme at a time and the purified hypotaurine as the inhibitor, clearer results may be obtained.

Finally, further work should be done in order to allow the purification of hypotaurine at a lower cost. One major hurdle for this inhibitor with regard to the commercial market is its excessive price. The inhibitor has many benefits over conventional PPO inhibitors. It is widely found in nature, colorless, odorless, tasteless, and is able to provide very high levels of PPO inhibition at relatively low concentrations. However, it is most likely cost-prohibitive at this time for commercial use in most industries. By making the isolation process more efficient, costs to supply the inhibitor could be reduced and would make the prospect of commercial use of the inhibitor more likely.

APPENDIX
ADDITIONAL DATA

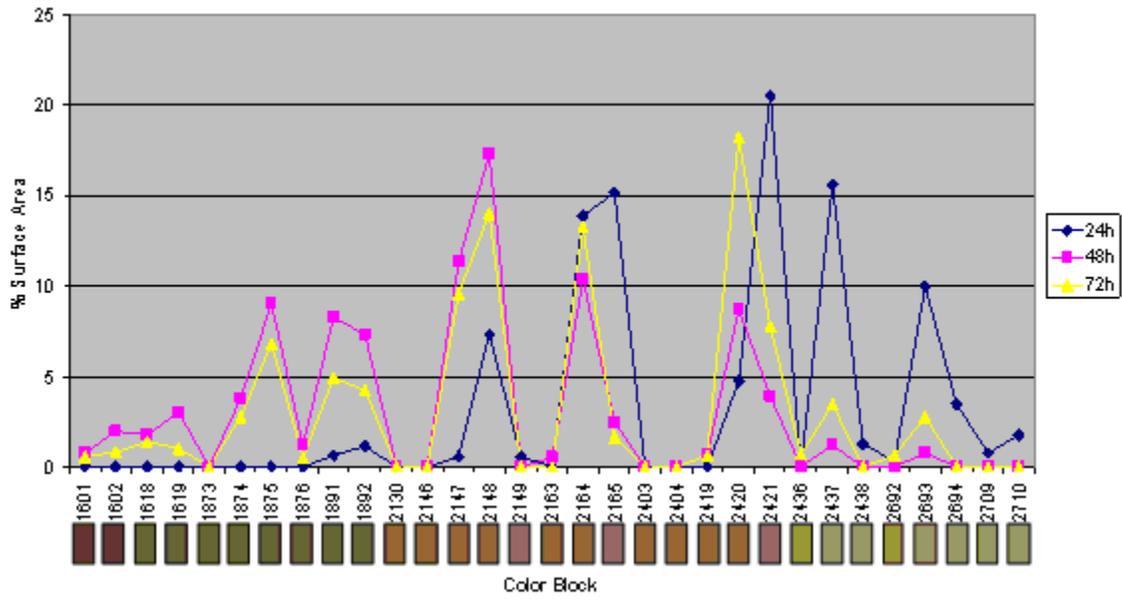


A

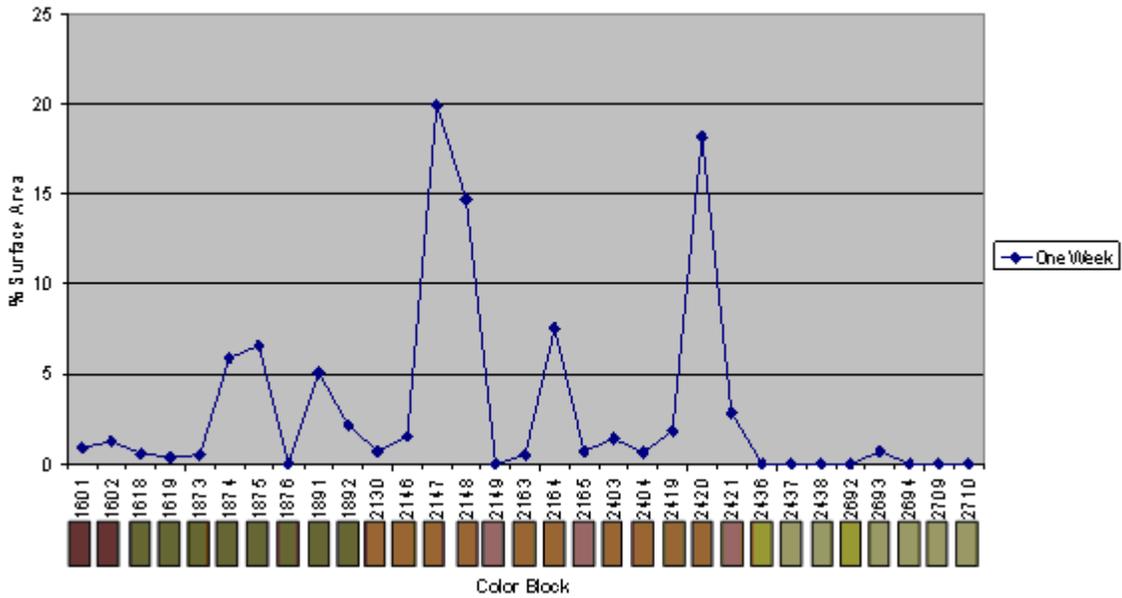


B

Figure A-1. Untreated samples in the first avocado trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas. A) Time points 0-5 minutes. B) Time points 10-120 minutes.

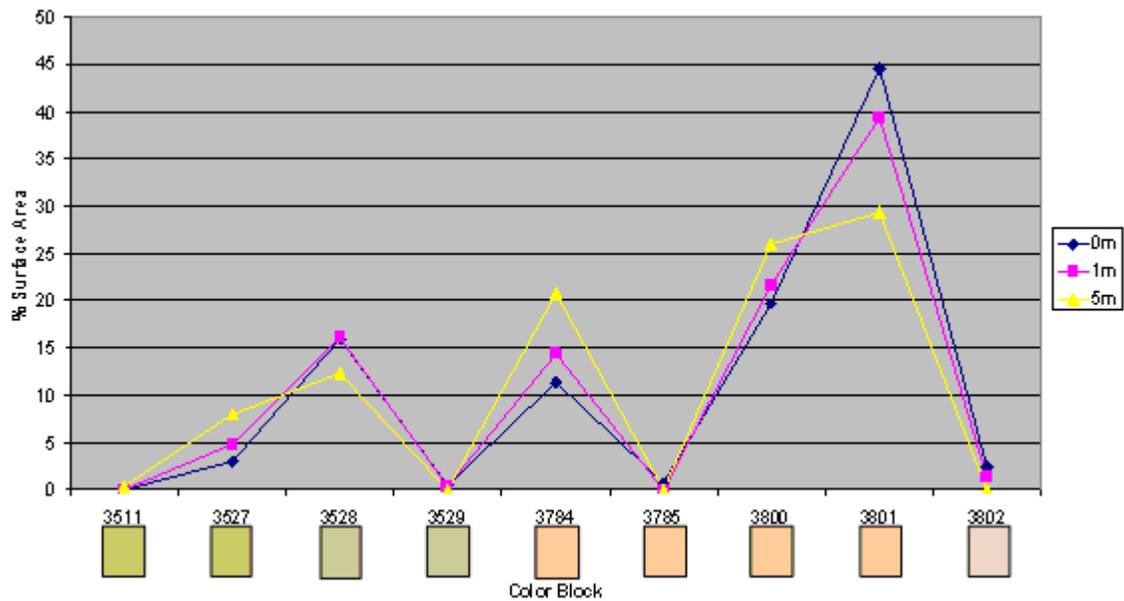


A

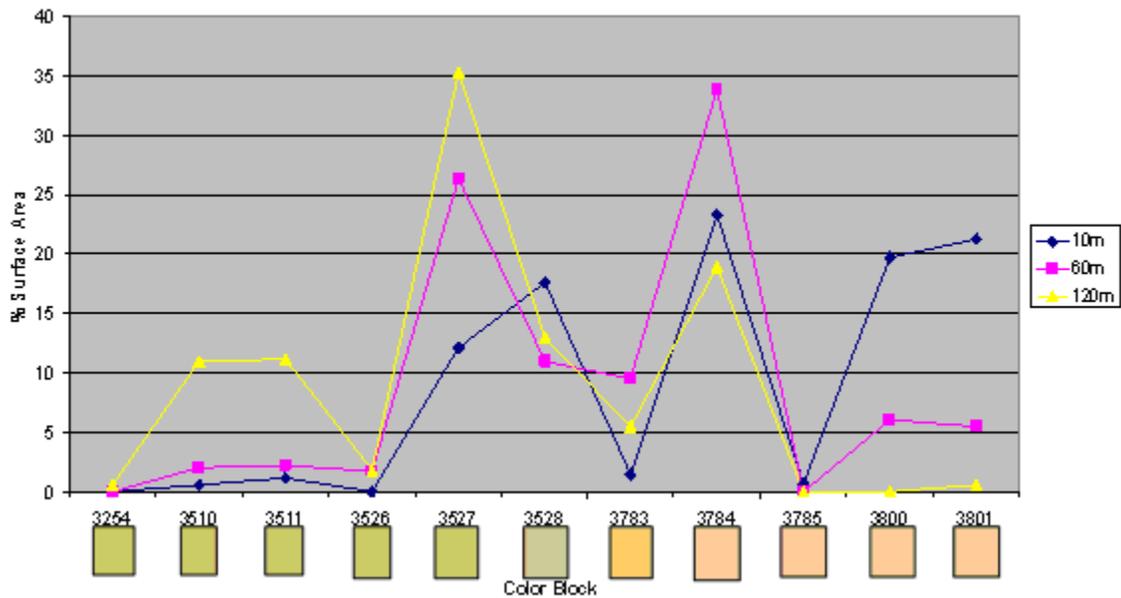


B

Figure A-2. Untreated samples in the first avocado trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas. A) Time points 24-72 hours. B) Time point one week.

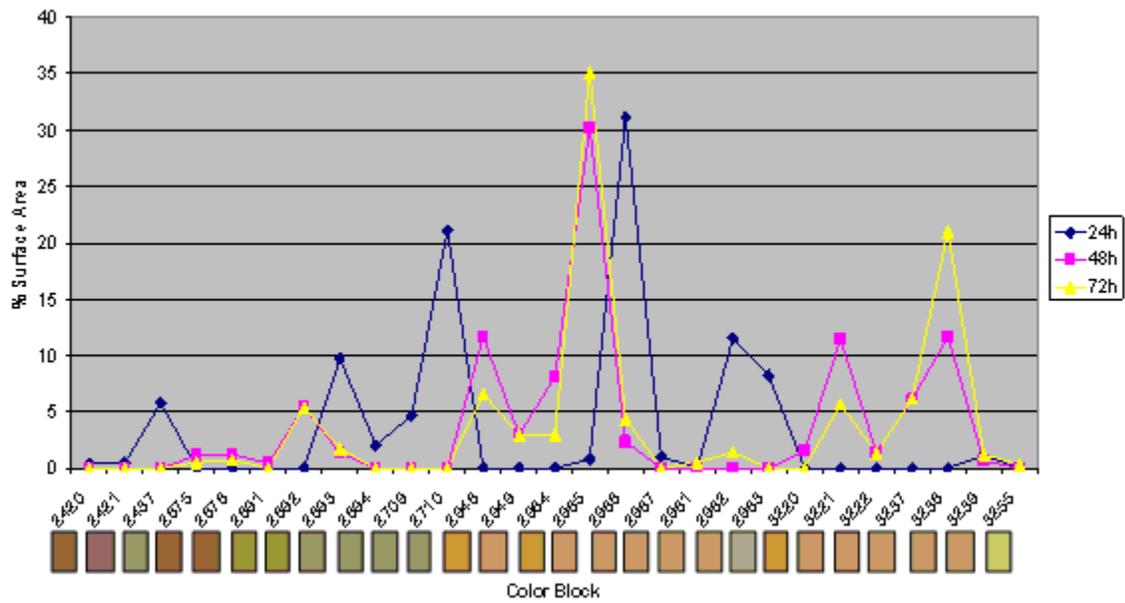


A

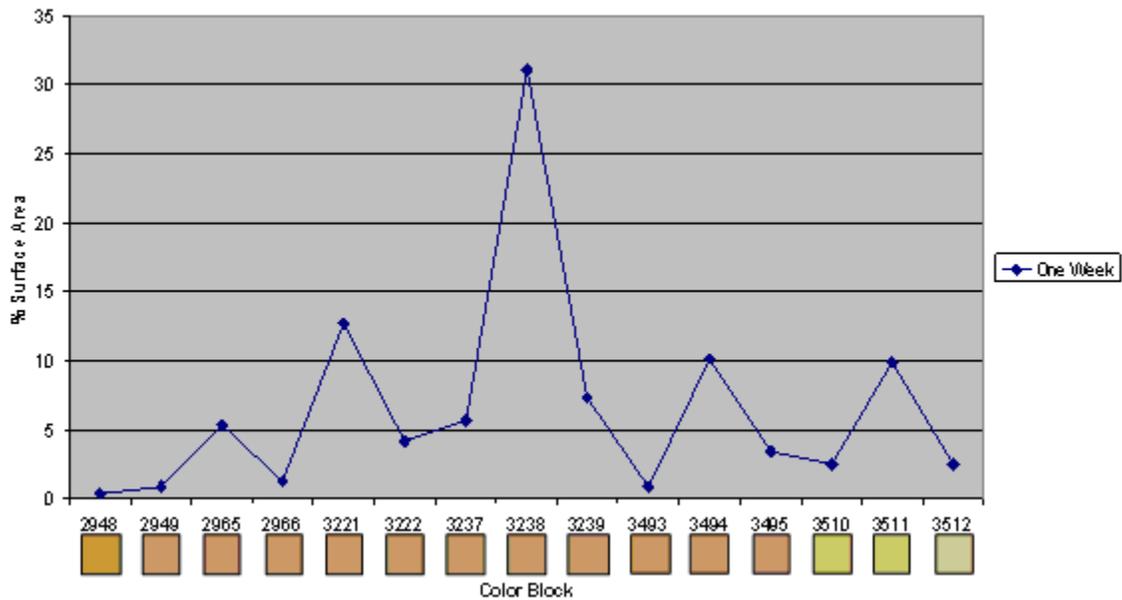


B

Figure A-3. Treated samples in the first avocado trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas. A) Time points 0-5 minutes. B) Time points 10-120 minutes.

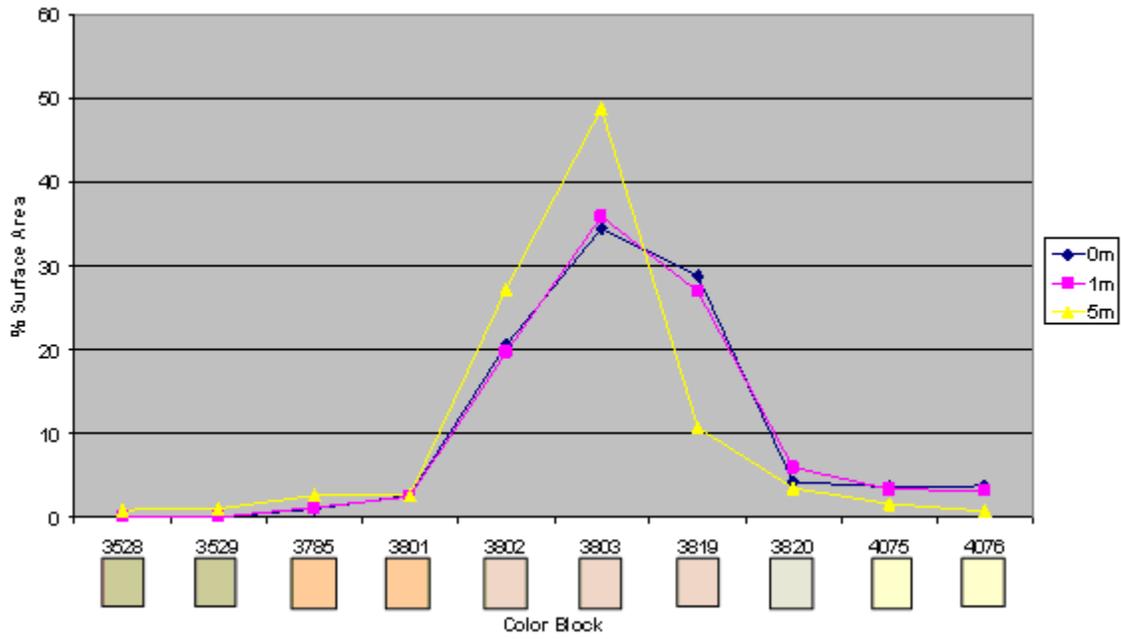


A

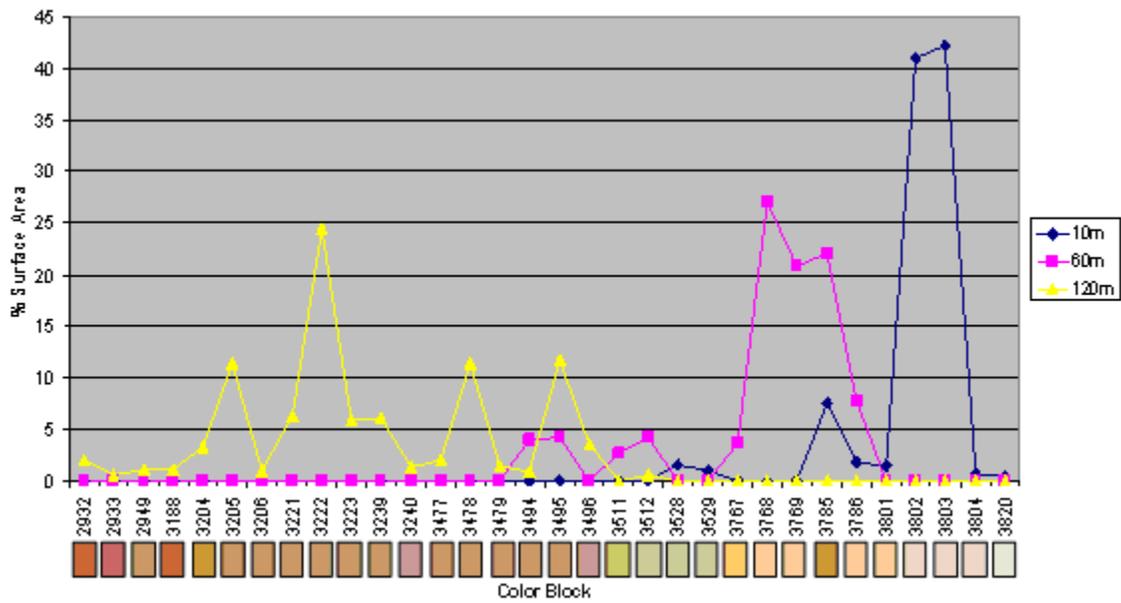


B

Figure A-4. Treated samples in the first avocado trial; 24-72 hours and one week; color blocks and percentage surface areas. A) Time points 24-72 hours. B) Time point one week.

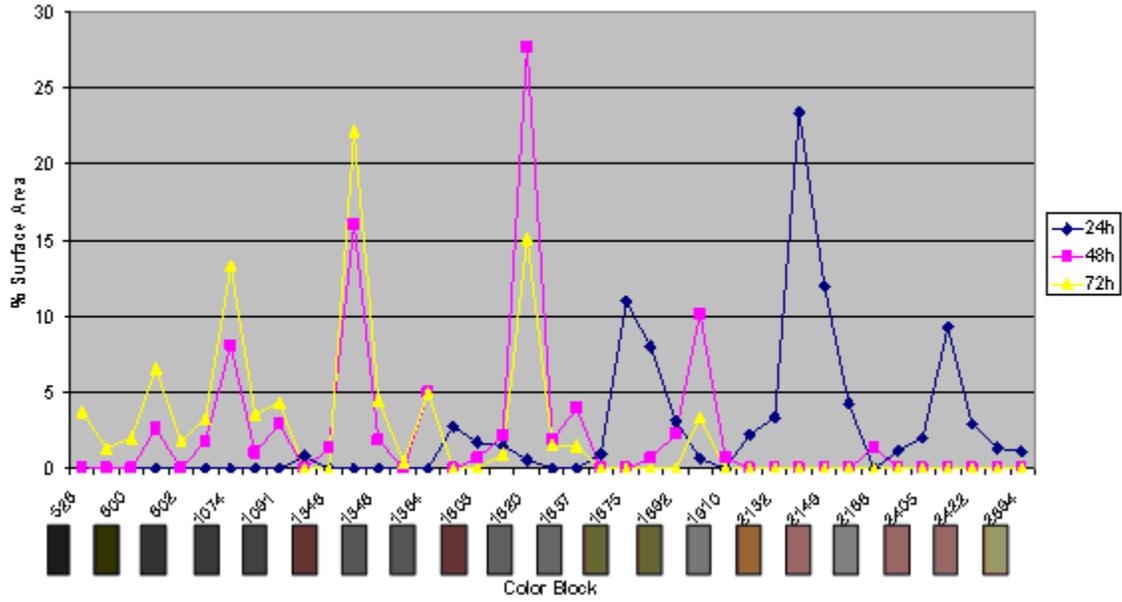


A

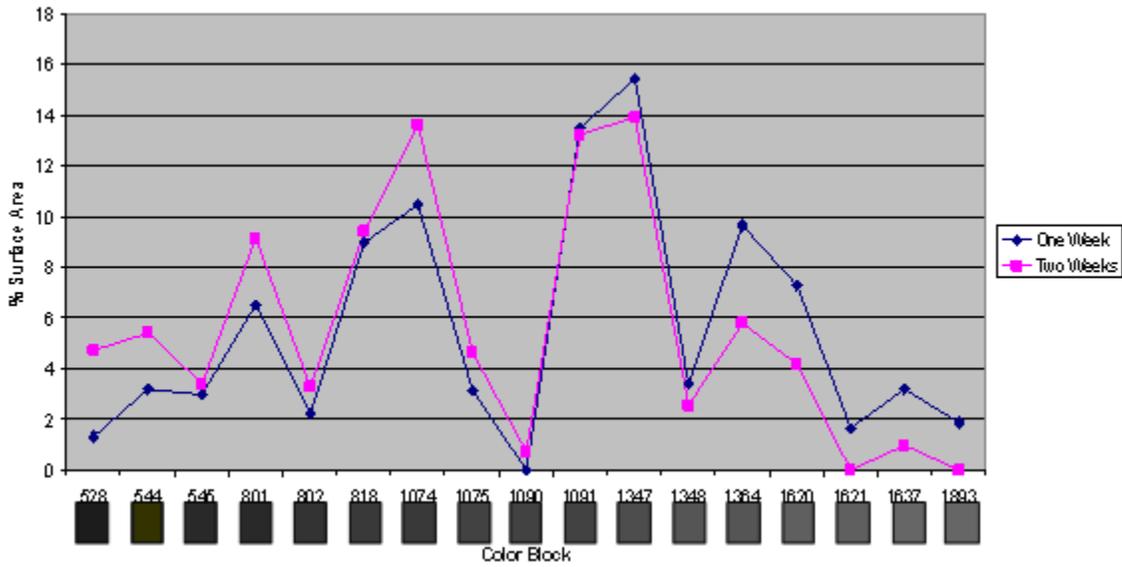


B

Figure A-5. Untreated samples in the first potato trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas. A) Time points 0-5 minutes. B) Time points 10-120 minutes.

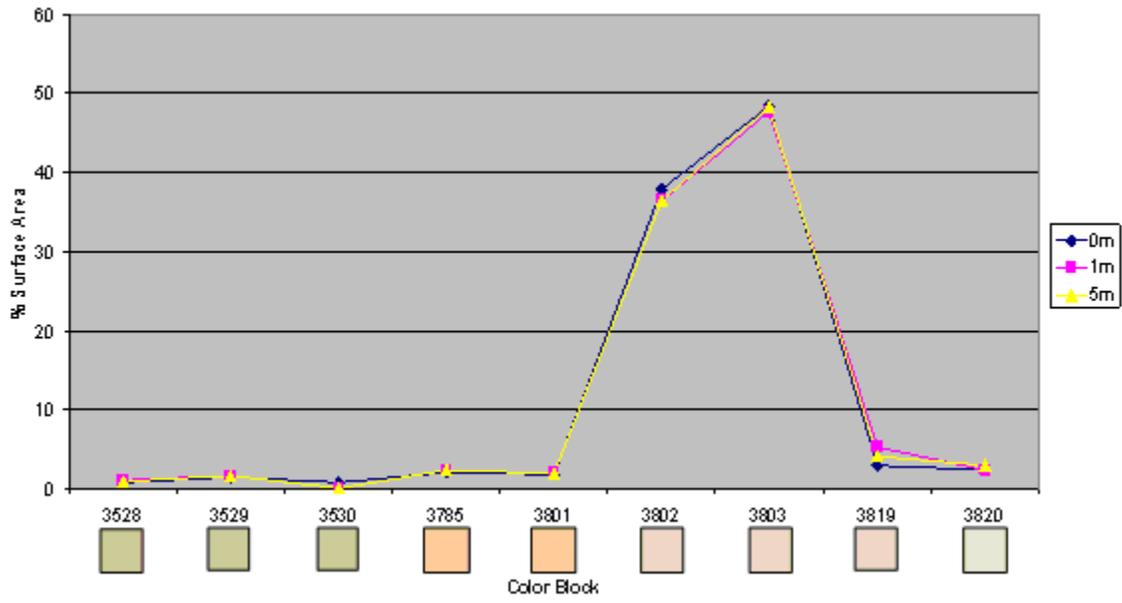


A

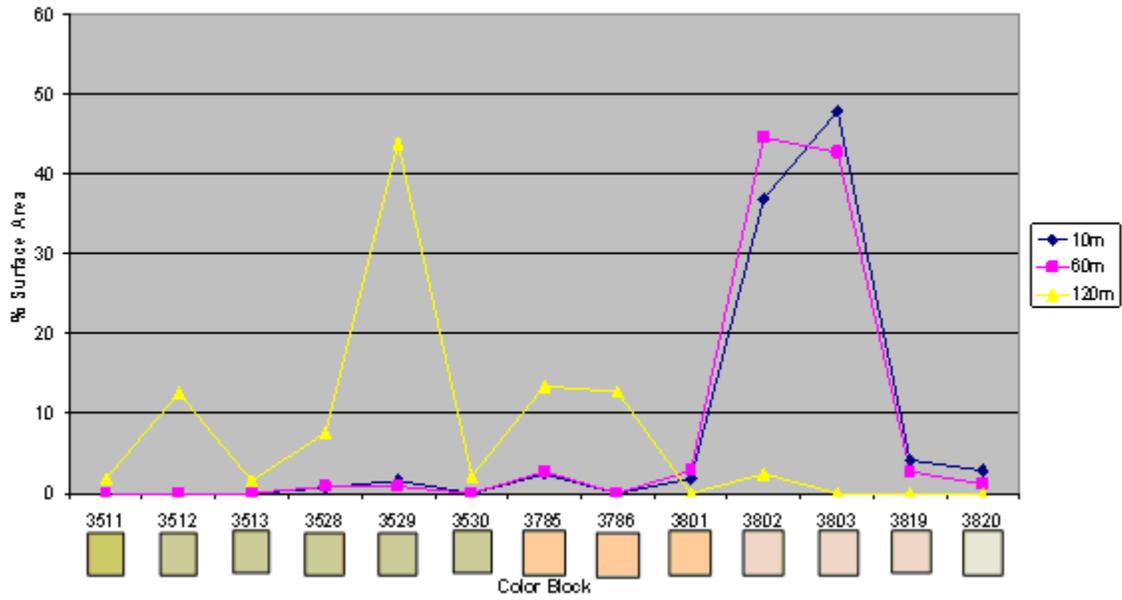


B

Figure A-6. Untreated samples in the first potato trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas. A) Time points 24-72 hours. B) Time point one week.

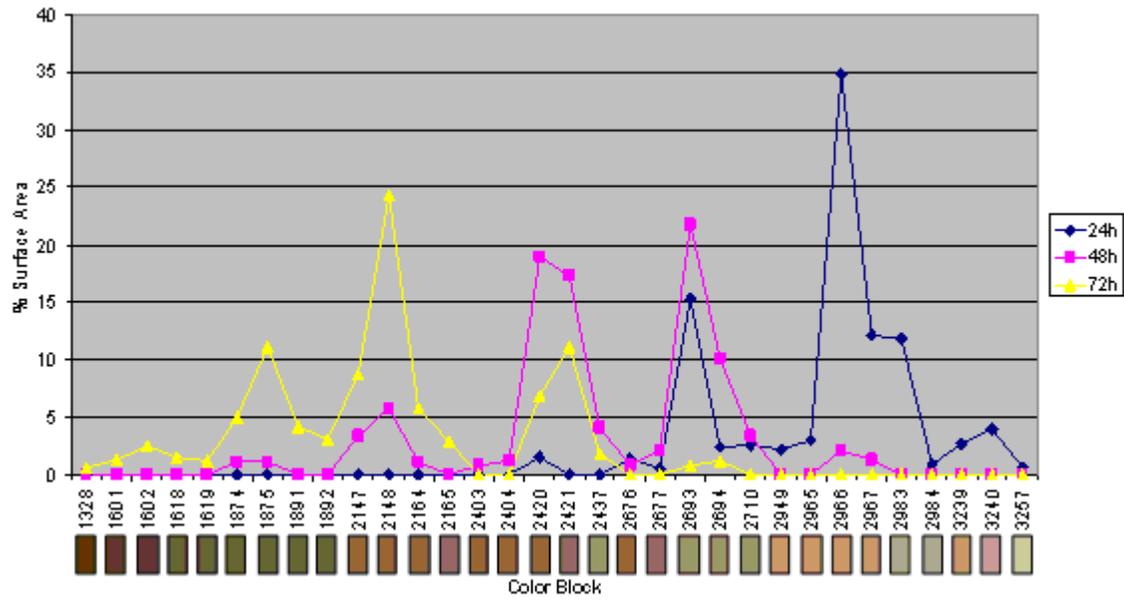


A

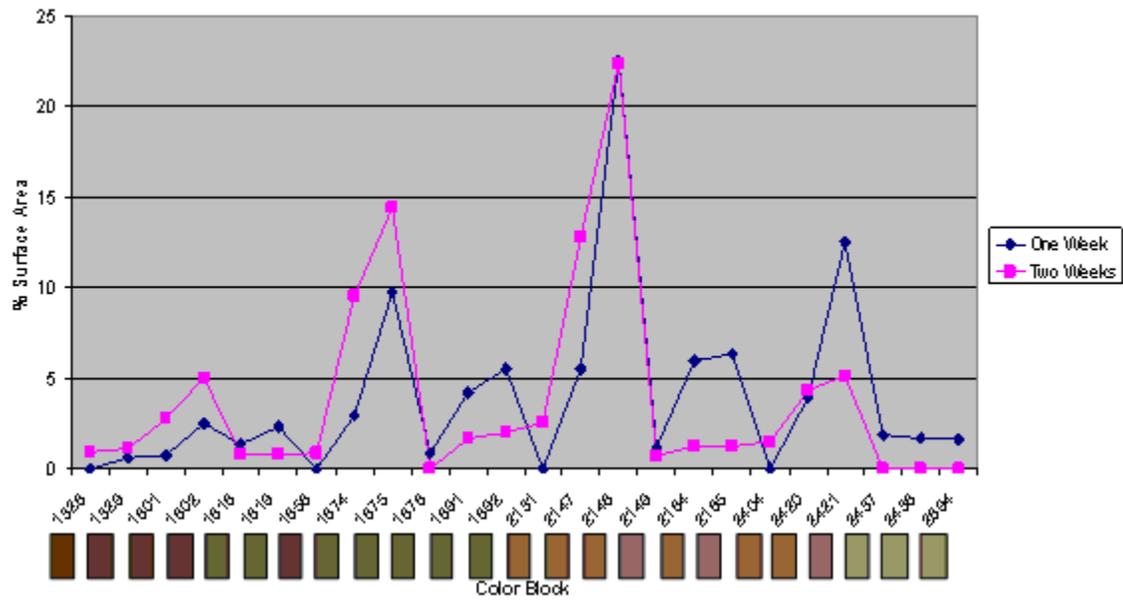


B

Figure A-7. Treated samples in the first potato trial; 0-5 minutes and 10-120 minutes; color blocks and percentage surface areas. A) Time points 0-5 minutes. B) Time points 10-120 minutes.



A



B

Figure A-8. Treated samples in the first potato trial; 24-72 hours and 1-2 weeks; color blocks and percentage surface areas. A) Time points 24-72 hours. B) Time point one week.

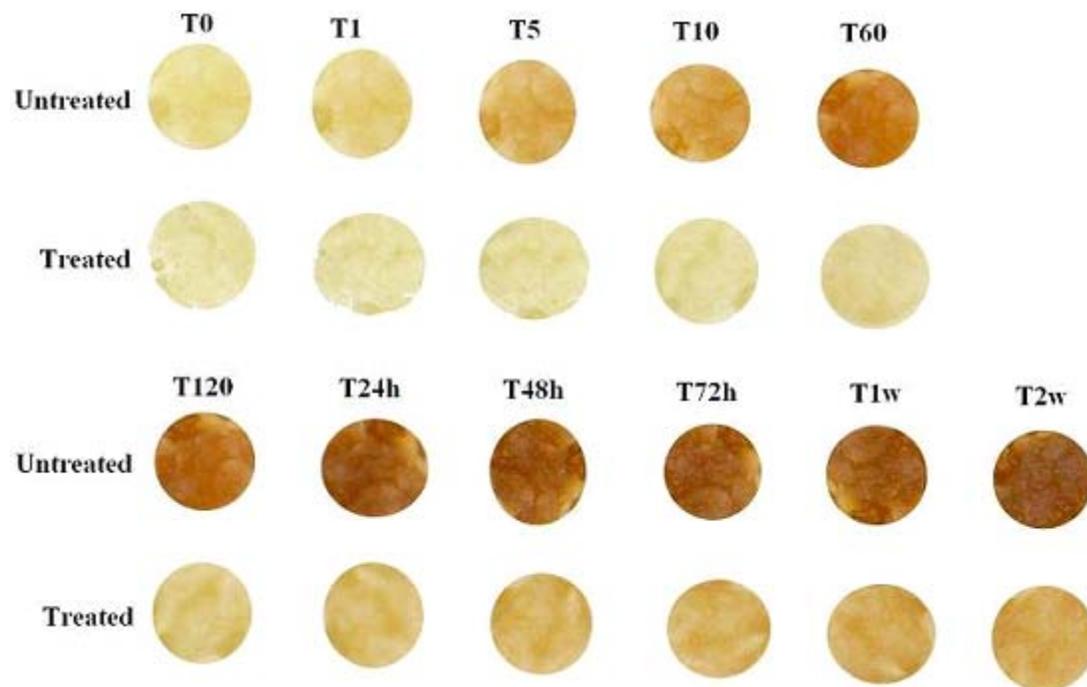


Figure A-9. Apple: browning progression in treated and untreated samples during Machine Vision experiment.

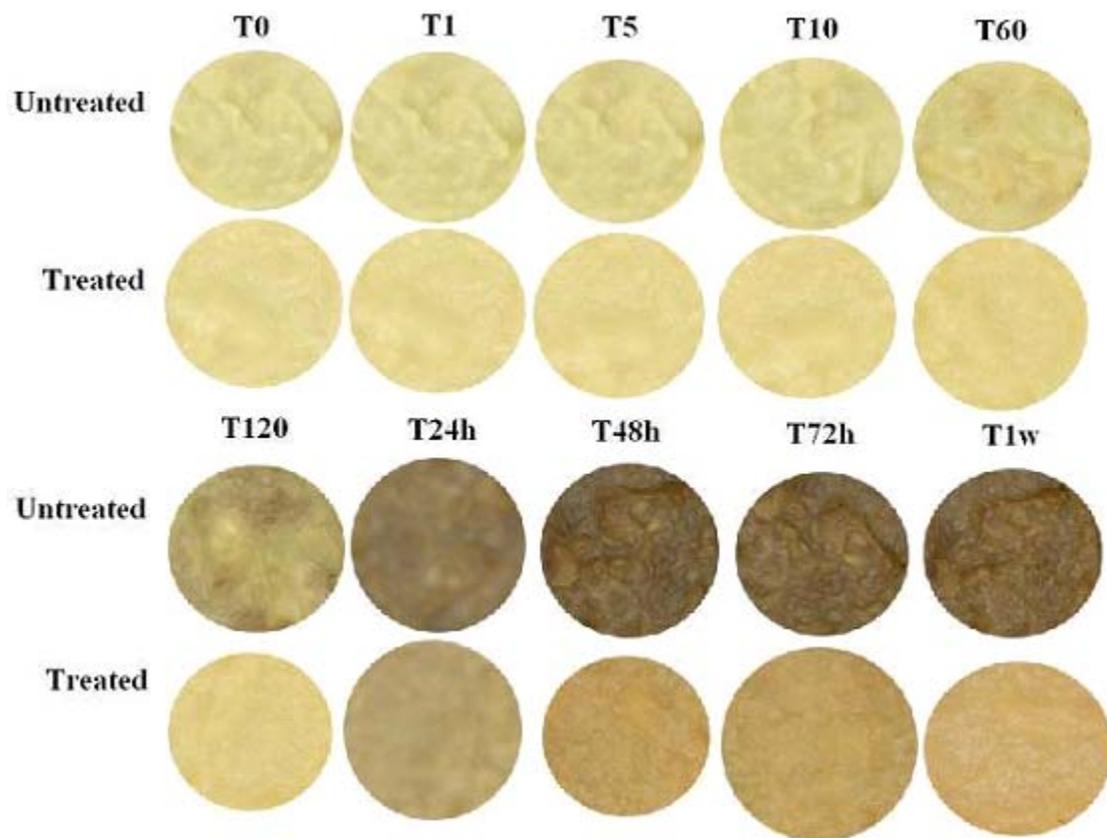


Figure A-10. Avocado: browning progression in treated and untreated samples during Machine Vision experiment.



Figure A-11. Banana: browning progression in treated and untreated samples during Machine Vision experiment.



Figure A-12. Potato: browning progression in treated and untreated samples during Machine Vision experiment.

----- storage=0 -----

The ANOVA Procedure

Dependent Variable: l

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	34.51020833	34.51020833	16.60	0.0022
Error	10	20.79041667	2.07904167		
Corrected Total	11	55.30062500			

R-Square	Coeff Var	Root MSE	l Mean
0.624047	1.693798	1.441888	85.12750

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	1	34.51020833	34.51020833	16.60	0.0022

Figure A-13. Time zero SAS output for apple machine vision L* data.

----- storage=24 -----

The ANOVA Procedure

Dependent Variable: l

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	3717.472008	3717.472008	523.33	<.0001
Error	10	71.035083	7.103508		
Corrected Total	11	3788.507092			

R-Square	Coeff Var	Root MSE	l Mean
0.981250	4.532273	2.665241	58.80583

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	1	3717.472008	3717.472008	523.33	<.0001

Figure A-14. Time 24 hours SAS output for apple machine vision L* data.

LIST OF REFERENCES

- Akissoe N, Mestres C, Hounhouigan J, Nago M. 2005. Biochemical origin of browning during the processing of fresh yam (*Dioscorea* spp.) into Dried Product. *J Agric Food Chem* 53(7):2552-2557.
- Arias E, Gonzalez J, Oria R, Lopez-Buesa P. 2007. Ascorbic Acid and 4-Hexylresorcinol Effects on Pear PPO and PPO Catalyzed Browning Reaction. *Journal of Food Science* 72(8):C422-C429.
- Aubourg SP, Losada V, Prado M, Miranda JM, Barros-Velázquez J. 2007. Improvement of the commercial quality of chilled Norway lobster (*Nephrops norvegicus*) stored in slurry ice: Effects of a preliminary treatment with an antimelanolic agent on enzymatic browning. *Food Chem* 103(3):741-748.
- Bahadur RP, Zacharias M. 2007. The interface of protein-protein complexes: Analysis of contacts and prediction of interactions. *Cell Mol Life Sci* 65:1059-1072.
- Bartolo I, Brik EO. 1998. Some factors affecting Norway lobster (*Nephrops norvegicus*) cuticle polyphenol oxidase activity and blackspot development. *International Journal of Food Science and Technology* 33(3):329-336.
- Batten T. 2008. Blue Mussel. <http://www.ocean.udel.edu/mas/seafood/bluemussel.html>. University of Delaware. Newark, DE.
- Boss PK, Gardner RC, Janssen BJ, Ross GS. 1995. An apple polyphenol oxidase cDNA is up regulated in wounded tissues. *Plant Molecular Biology* 27(2):429-433.
- Brandelli A, Lopes CHGL. 2005. Polyphenoloxidase activity, browning potential and phenolic content of peaches during postharvest ripening. *Journal of Food Biochemistry* 29(6): 624-637.
- Castro SM, Saraiva JA, Lopes-da-Silva JA, Delgadillo I, Loey AV, Smout C, Hendrickx M. 2008. Effect of thermal blanching and of high pressure treatments on sweet green and red bell pepper fruits (*Capsicum annuum* L.) *Food Chem* 107(4):1436-1449.
- Chase MR, Raina K, Bruno J, Sugumaran M. 2000. Purification, characterization and molecular cloning of prophenoloxidases from *Sarcophaga bullata*. *Insect Biochem Mol Biol* 30:953-967.
- Chen JS, Wei CI, Marshall MR. 1991a. Inhibition mechanism of kojic acid on polyphenol oxidase. *Journal of Agricultural and Food Chemistry* 39(11):1898-1901.
- Chen JS, Wei CI, Rolle RS, Otwell WS, Balaban MO, Marshall MR. 1991b. Inhibitory effect of kojic acid on some plant and crustacean polyphenol oxidases. *Journal of Agricultural and Food Chemistry* 39(8):1396-1401.

- Cheng Y and Prusoff WH. 1973. Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochemical Pharmacology* 22(23):3099-3108.
- Cheriot SC, Billaud C, Nicolas J. 2006. Use of Experimental Design Methodology To Prepare Maillard Reaction Products from Glucose and Cysteine Inhibitors of Polyphenol Oxidase from Eggplant (*Solanum melongena*). *Journal of Agricultural and Food Chemistry* 54(14):5120-5126.
- Chevalier T, Rigal D, Mbeguie-A-Mbeguie D, Gaillard F, Richard-Forget F, Fils-Lycaon BR. 1999. Molecular cloning and characterization of apricot fruit polyphenol oxidase. *Plant Physiology* 119:(4)1261-1269.
- Chisari M, Barbagallo RN, Spagna G. 2007. Characterization of Polyphenol Oxidase and Peroxidase and Influence on Browning of Cold Stored Strawberry Fruit. *J. Agric. Food Chem* 55(9):3469-3476.
- Cipollini DF Jr, Redman AM. 1999. Age-dependent effects of jasmonic acid treatment and wind exposure on foliar oxidase activity and insect resistance in tomato. *Journal of Chemical Ecology* 25(2):271-281.
- Cornish-Bowden A. 1974. A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. *Biochemical Journal* 137:143-4.
- Dahchour AD and De Witte P. 2000. Ethanol and amino acids in the central nervous system: assessment of the pharmacological actions of acamprosate. *Prog Neurobiol* 60:343-362.
- Diaz-Tenorio LM, Garcia-Carreno FL, Pacheco-Aguilar R. 2007. Comparison of freezing and thawing treatments on muscle properties of whiteleg shrimp (*Litopenaeus vannamei*). *Journal of Food Biochemistry* 31(5):563-576.
- Dogan S, Turan P, Dogan M, Alkan M, Oktay A. 2007. Inhibition Kinetic of *Ocimum basilicum* L. Polyphenol Oxidase. *International Journal of Chemical Reactor Engineering* 5:A46.
- Dogan S, Turan P, Dogan M, Alkan M, Oktay A. 2008. Inhibition kinetic and mechanism of polyphenol oxidase from various sources by diethyldithiocarbamic acid. *International Journal of Food Science & Technology* 43(7):1316-1322.
- Dogan S, Turan Y, Erturk H, Arslan O. 2005. Characterization and purification of polyphenol oxidase from artichoke (*Cynara scolymus* L.). *Journal of Agricultural and Food Chemistry* 53(3):776-785.
- Double KL, Riederer P, and Gerlach M. 1999. Significance of Neuromelanin for Neurodegeneration in Parkinson's Disease. *Drug News and Perspectives* 12(6):333.

- Duangmal K, Apenten RKO. 1999. A comparative study of polyphenoloxidases from taro (*Colocasia esculenta*) and potato (*Solanum tuberosum* var. Romano). *Food Chemistry* 64:351-359.
- Duffey SS, Felton GW. 1991. Enzymatic antinutritive defences of the tomato plant against insects. In: Hedin PA editor. *Naturally Occurring Pest Bioregulators*. ACS Symp. Ser. 449. Dallas, TX. p. 167-197.
- Duffey SS, Stout MJ. 1996. Antinutritive and toxic components of plant defense against insects. *Archives of Insect Biochemistry and Physiology* 32(1):3-37.
- Eidhin DMN, Murphy E, O'Beirne D. 2006. Polyphenol oxidase from apple (*Malus domestica* Borkh. cv. Bramley's seedling): purification strategies and characterization. *Journal of Food Science* 71(1):C51-C58.
- Eskin NAM, Henderson HM, Townsend RJ. 1971. Browning reactions in foods. In: Eskin NA, Michael H, Townsend RJ, editors. *Biochemistry of foods*. New York: Academic Press. p. 69-83.
- Eskin NAM, Robinson DS. 2000. Effects of Sulfur Dioxide on Food Quality. In: Eskin NAM, Robinson DS, editors. *Food Shelf Life Stability: Chemical, Biochemical, and Microbiological Changes*. Boca Raton, FL: CRC Press. p. 283.
- Farmer ASD. 1975. Synopsis of biological data on the Norway lobster *Nephrops norvegicus* (Linnaeus, 1758). *FAO Fisheries Synopsis* no. 112. Rome: FAO.
- Felton GW, Donato K, Del Vecchio RJ, Duffey SS. 1989. Activation of plant polyphenol oxidases by insect feeding reduces the nutritive quality of foliage for noctuid herbivores. *J Chem Ecol* 15:2667-2694.
- Felton GW, Donato KK, Broadway RM, Duffey SS. 1992. Impact of oxidized plant phenolics on the nutritional quality of dietary protein to a noctuid herbivore. *J Insect Physiol* 38: 277-285.
- Ferrer OJ, Koburger JA, Simpson BK, Gleeson RA, Marshall MR. 1989. Phenoloxidase levels in Florida spiny lobster (*Panulirus argus*): relationship to season and molting stage. *Comparative Biochemistry and Physiology* 93B:595-599.
- Flurkey WH. 1986. Polyphenoloxidase in higher plants. *Plant Physiol* 81:614-618.
- Font L, Miquel M, Aragon CMG. 2001. Behavioral consequences of the hypotaurine-ethanol interaction. *Pharmacology, Biochemistry and Behavior* 70:333-339.
- Fontana M, Pecci L, Dupre S, Cavallini D. 2004. Antioxidant Properties of Sulfinates: Protective Effect of Hypotaurine on Peroxynitrite-Dependent Damage. *Neurochemical Research* 29(1):111-116.

- Food and Agriculture Organization of the United Nations. 2008. Cultured Aquatic Species Information Programme: *Mytilus edulis*.
http://www.fao.org/fishery/culturedspecies/Mytilus_edulis. Accessed: 11/23/08.
- Fraignier M, Marques L, Fleuriot A, Macheix J. 1995. Biochemical and immunochemical characteristics of polyphenol oxidase from different fruits of *Prunus*. *J Agric Food Chem* 43:2375-2380.
- Gacche RN, Shete AM, Dhole NA, Ghole VS. 2006. Reversible inhibition of polyphenol oxidase from apple using L-cysteine. *Indian Journal of Chemical Technology* 13(5):459-463.
- Galeazzi MAM, Sgarbieri VC. 1981. Substrate Specificity and Inhibition of Polyphenoloxidase (PPO) From a Dwarf Variety of Banana (*Muss Cavendishii*, L.) *Journal of Food Science* 46(5):1404–1406.
- Gandía-Herrero F, Escribano J, García-Carmona F. 2005. Betaxanthins as substrates for tyrosinase. An approach to the role of tyrosinase in the biosynthetic pathway of betalains. *Plant Physiology* 138:421-432.
- Gomez-Lopez VM. 2002a. Some biochemical properties of polyphenol oxidase from two varieties of avocado. *Food Chemistry* 77:163–169.
- Gomen-Lopez VM. 2002b. Inhibition of surface browning, cut avocado. *Journal of Food Quality* 25(4):369-379.
- Gooding PS, Bird C, Robinson SP. 2001. Molecular cloning and characterisation of banana fruit polyphenol oxidase. *Planta* 213(5):748-757.
- Government of Ontario. 2006. Minimally processed fruits and vegetables information bundle: industry overview.
[http://www.gov.on.ca/ont/portal/!ut/p/.cmd/cs/.ce/7_0_A/.s/7_0_252/_s.7_0_A/70\)252/_/1/en?docid=053076](http://www.gov.on.ca/ont/portal/!ut/p/.cmd/cs/.ce/7_0_A/.s/7_0_252/_s.7_0_A/70)252/_/1/en?docid=053076). Government of Ontario. Ontario, Canada.
- Government of Saskatchewan. 2005. Fruit overview 2005.
<http://www.agr.gov.sk.ca/docs/processing/fruit/Fruit.asp>. Government of Saskatchewan. Saskatchewan, Canada.
- Green TR, Fellman JH, Eicher AL, Pratt KL. 1991. Antioxidant role and subcellular location of hypotaurine and taurine in human neutrophils. *Biochimica et biophysica acta* 1073(1):91-97
- Harvey DJ. 2004. U.S. Seafood Market Shifts to Aquaculture.
<http://www.ers.usda.gov/amberwaves/April04/pdf/findingsMarketsTrade.pdf>. United States Department of Agriculture: Economic Research Service. Washington, DC.

- Jaenicke E, Decker H. 2003. Tyrosinases from crustaceans form hexamers. *Biochem J* 371(Pt 2):515-23.
- Jiang Y, Fu J, Zauberman G, Fuchs Y. 1999. Purification of polyphenol oxidase and the browning control of litchi fruit by glutathione and citric acid. *Journal of the Science of Food and Agriculture* 79(7):950-954.
- Kahn V. 1985. Effect of proteins, protein hydrolyzates and amino acids on o dihydroxyphenolase activity of polyphenol oxidase of mushroom, avocado, and banana. *Journal of Food Science* 50(1):111-115.
- Kim J, Marshall MR, Wei C. 2000. Polyphenoloxidase. In: Haard NF, Simpson BK, editors. *Seafood enzymes utilization and influence on postharvest seafood quality*. New York: Marcel Dekker. p. 271-315.
- Kipe S. 2004. An Economic Overview of Horticultural Products in the United States. [http://www.fas.usda.gov/htp/Presentations/2004/An%20Economic%20Overview%20of%20HTP%20-%20\(08-04\).pdf](http://www.fas.usda.gov/htp/Presentations/2004/An%20Economic%20Overview%20of%20HTP%20-%20(08-04).pdf). United States Department of Agriculture: Economic Research Service. Washington, DC.
- Kowalski SP, Eannetta, NT, Hirzel AT, Steffens JC. 1992. Purification and characterization of polyphenol oxidase from glandular trichomes of *Solanum berthaultii*. *Plant Physiology* 100(2):677-684.
- Kurachi M, Yochihara K, Aihara H. 1983. Effect of taurine on depolarization induced L-glutamate and other excitatory amino acids in the isolated spinal cord of frog. *Jpn J Pharmacol* 33:1247-1254.
- Laveda F, Nunez-Delicado E, Garcia-Carmona F, Sanchez-Ferrer A. 2000. Reversible Sodium Dodecyl Sulfate Activation of Latent Peach Polyphenol Oxidase by Cyclodextrins. *Archives of Biochemistry and Biophysics* 379(1):1-6.
- Lax AR, Vaughn KC, Templeton GE. 1984. Nuclear inheritance of polyphenol oxidase in *Nicotiana*. *J Heredity* 75:285-287.
- Leatherbarrow RJ. 1990. Use of nonlinear regression to analyze enzyme kinetic data: application to situations of substrate contamination and background subtraction. *Anal Biochem* 184(2):274-8.
- Lerch K. 1983. *Neurospora* tyrosinase: structural, spectroscopic and catalytic properties. *Mol Cell Biochem* 52:125-138.
- Lee CY, Whitaker JR. 1995. *Enzymatic Browning and Its Prevention*, ACS Symposium Series 600. Washington, DC: American Chemical Society. 352p.

- Lee-Kim MS, Hwang ES, Kim KH. 1997. Inhibition studies on burdock polyphenol oxidase (PPO) activity. *Journal of Food Processing and Preservation* 21(6):485-494.
- Lerner HR, Mayer AM, Harel E. 1972. Evidence for conformational changes in grape catechol oxidase. *Phytochemistry* 11:2415-21.
- Lineweaver H, Burk D. 1934. The determination of enzyme dissociation constants. *J Am Chem Soc* 56:658-666
- López-Caballero ME, Martínez-Alvarez O, Gómez-Guillén MC, Montero P. 2007. Quality of thawed deepwater pink shrimp (*Parapenaeus longirostris*) treated with melanosis inhibiting formulations during chilled storage. *International Journal of Food Science and Technology* 42(9):1029–1038.
- Luzuriaga DA, Balaban MO, Yeralan S. 1997. Analysis of Visual Quality Attributes of White Shrimp by Machine Vision. *Journal of Food Science* 62(1):113-118.
- Macheix J, Fleuriet A, Billiot J. 1990. Phenolic compounds in fruit processing. In: Macheix J, Fleuriet A, Billiot J, editors. *Fruit Phenolics*. CRC Press, Boca Raton, Florida. p. 239-312.
- Marshall MR, Jeongmok K, Cheng-I W. 2000. Enzymatic browning in fruits, vegetables and seafoods. <http://www.fao.org/ag/ags/agsi/ENZYMFINAL/Enzymatic%20Browning.html>. Food and Agriculture Organization of the United Nations. Rome, Italy.
- Martinez MV, Whitaker JR. 1995. The biochemistry and control of enzymatic browning. *Trends Food Sci Technol* 6:195–200.
- Matheis G, Whitaker JR. 1984. Modification of proteins by polyphenol oxidase and peroxidase and their products. *J Food Biochem* 8:137-162.
- Mayer AM, Harel E. 1979. Polyphenol oxidases in plants. *Phytochemistry* 18:193-215.
- Mayer AM. 2006. Polyphenol oxidases in plants and fungi: Going places? A review. *Phytochemistry* 67(21):2318–2331.
- McEvily AJ, Iyengar R, Otwell WS. 1992. Inhibition of enzymatic browning in foods and beverages. *Crit Rev Food Sci Nutr* 32:253-273.
- Murata M, Tsurutani M, Hagiwara S, Homma S, Kaneko K. 1995. Relationship between apple ripening and browning: Changes in polyphenol content and polyphenol oxidase. *J Agric Food Chem* 43:1115-1121.
- Neves VA and Silva MAD. 2007. Polyphenol Oxidase from Yacon Roots (*Smallanthus sonchifolius*). *J Agric Food Chem* 55(6):2424-2430.

- Newell RIE. 1989. Species Profiles: Life Histories and Environmental Requirements of Coastal Fishes and Invertebrates (mid-Atlantic): Blue Mussel. U.S. Fish and Wildlife Service Biological Report 82(11.102). U.S. Army Corps of Engineers TR EL-82-4.
- Noci F, Riener J, Walkling-Ribeiro M, Cronin DA, Morgan DJ, Lyng JG. 2008. Ultraviolet irradiation and pulsed electric fields (PEF) in a hurdle strategy for the preservation of fresh apple juice. *Journal of Food Engineering* 85(1):141-146.
- Ogawa M, Perdigao NB, Santiago ME, Kozima TT. 1984. On physiological aspects of blackspot appearance in shrimp. *Nippon Suisan Gakkaishi* 50:1763-1769.
- Ogawa M. 1987. Blackspot occurrence in lobsters and shrimp. *Infotish Marketing Digest* 1:43-44.
- Oktay A and Dogan S. 2005. Inhibition of polyphenol oxidase obtained from various sources by 2,3-diaminopropionic acid. *Journal of the Science of Food and Agriculture* 85(9):1499-1504.
- Ophardt CE. 2003. Denaturation of Proteins in *Virtual Chembook*. <http://www.elmhurst.edu/~chm/vchembook/568denaturation.html>. Elmhurst College. Elmhurst, IL.
- Opoku-Gyamfua A, Simpson BK, Squires EJ. 1992. Comparative studies on the polyphenol oxidase fraction from lobster and tyrosinase. *Journal of Agricultural and Food Chemistry* 40(5):772-775.
- Partington JC, Smith C, Bolwell GP. 1999. Changes in the location of polyphenol oxidase in potato (*Solanum tuberosum* L.) tuber during cell death in response to impact injury: comparison with wound tissue. *Planta* 207(3):449-460.
- Pierpoint WS. 1969. o-Quinones formed in plant extracts. Their reaction with bovine serum albumin. *Biochem J* 112:619-629.
- Porata R, Weissa B, Cohena L, Dausa A, Biton A. 2005. Effects of polyethylene wax content and composition on taste, quality, and emission of off-flavor volatiles in 'Mor' mandarins. *Postharvest Biology and Technology* 38(3):262-268.
- Prigent SVE, Voragen AGJ, Visser AJWG, Koningsveld GA, Gruppen H. 2007. Covalent interactions between proteins and oxidation products of caffeoylquinic acid (chlorogenic acid). *Journal of the Science of Food and Agriculture* 87(13):2502-2510.
- Quinn MR and Harris CL. 1995. Taurine allosterically inhibits binding of (3S)-tbutylbicyclophosphorothionate (TBPS) to rat brain synaptic membranes. *Neuropharmacology* 34:1607-1613.

- Rawel HM, Kroll J, Rohn S. 2001. Reactions of phenolic substances with lysozyme physicochemical characterisation and proteolytic digestion of the derivatives. *Food Chem* 72:59-71.
- Rawel HM, Rohn S, Kruse HP, Kroll J. 2002. Structural changes induced in bovine serum albumin by covalent attachment of chlorogenic acid. *Food Chem* 78:443-455.
- Ray H, Hammerschmidt R. 1998. Responses of potato tuber to infection by *Fusarium sambucinum*. *Physiological and Molecular Plant Pathology*. 53:81-92.
- Richard-Forget FC, Goupy PM, Nicholas JJ. 1992. Cysteine as an inhibitor of enzymatic browning. 2. Kinetic studies. *J Agric Food Chem* 40:2108–2114.
- Robinson SP, Dry IB. 1992. Broad bean leaf polyphenol oxidase is a 60 Kilodalton protein susceptible to proteolytic cleavage. *Plant Physiol* 99:317-323.
- Sanchez-Ferrer A, Levada F, Garcia-Carmona F. 1993. Substrate-dependent activation of latent potato leaf polyphenol oxidase by anionic surfactants. *J Agric Food Chem* 41:1583-1586.
- Scalbert A. 1991. Antimicrobial properties of tannins. *Phytochemistry* 30:3875-3883.
- Schmitz GE, Sullivan ML, Hatfield RD. 2008. Three Polyphenol Oxidases from Red Clover (*Trifolium pratense*) Differ in Enzymatic Activities and Activation Properties. *J Agric Food Chem* 56(1):272-280.
- Sapers GM. 1993. Browning of foods: control by sulfites, antioxidants, and other means. *Food Technol* 47(10):75-84.
- Saransaari P, Oja SS. 1993. Uptake and release of beta-alanine in cerebellar granule cells in primary culture: regulation of release by glutamatergic and GABAergic receptors. *Neuroscience* 53(2):475-81.
- Serradell MDLA, Rozenfeld PA, Martinez GA, Civello PM, Chaves AR, Anon MC. 2000. Polyphenoloxidase activity from strawberry fruit (*Fragaria x ananassa*, Duch., cv Selva) characterization and partial purification. *J Sci Food Agric* 80:1421-1427.
- Shahar T, Hennig N, Guttinger T, Hareven D, Lifschitz E. 1992. The tomato 66.3-kD polyphenol oxidase gene: molecular identification and developmental expression. *Plant Cell* 4:135-147.
- Shaw J, Chao L, Chen M. 1991. Isoenzymes of papaya polyphenol oxidase. *Bot Bull Acad Sinica* 32: 259-263.
- Sherman TD, Vaughn KC, Duke SO. 1991. A limited survey of the phylogenetic distribution of polyphenol oxidase. *Phytochemistry* 30:2499-2506.
- Schulbach K. 2008. Personal communication.

- Simpson BK, Gagne N, Ashie INA, Noroozi E. 1997. Utilization of chitosan for preservation of raw shrimp (*Pandalus borealis*). *Food Biotechnology* 11(1):25-44.
- Sims C. 2008. Personal communication.
- Slatery SL, Williams DJ, Cusack A. 1995. A sulphite-free treatment inhibits blackspot formation in prawns. *Food Australia: Official Journal of CAFTA and AIFST* 47(11):509-514.
- Smith DL, Stommel JR, Fung RWM, Wang CY, Whitaker BD. 2006. Influence of cultivar and harvest method on postharvest storage quality of pepper (*Capsicum annuum* L.) fruit. *Postharvest Biology and Technology* 42(3):243-247.
- Sojo MM, Nunez-Delicado E, Garcia-Carmona F, Sanchez-Ferrer A. 1999. Cyclodextrins as activator and inhibitor of latent banana pulp polyphenol oxidase. *Journal of Agricultural and Food Chemistry* 47(2):518-523.
- Soliva-Fortuny RC, Elez-Martinez P, Sebastian-Caldero M, Martin-Belloso O. 2002. Kinetics of polyphenol oxidase activity inhibition and browning of avocado puree preserved by combined methods. *Journal of Food Engineering* 55(2):131-137.
- Spagna G, Barbagallo RN, Chisari M, Branca F. 2005. Characterization of a tomato polyphenol oxidase and its role in browning and lycopene content. *J Agric Food Chem* 53(6):2032-2038.
- Steffens JC, Harel E, Hunt MD. 1994. Polyphenol oxidase. In: Ellis BE, Kuroki GW, Stafford HA, editors. *Recent Advances in Phytochemistry, Vol. 28. Genetic Engineering of Plant Secondary Metabolism*. New York: Plenum Press. p. 275-312.
- Stevenson JR 1985. Dynamics of the integument. In: Bliss DE, Mantel LH, editors. *The Biology of the Crustacea: Integument, Pigments and Hormonal Processes*. New York: Academic Press. p. 1-44.
- Storey RMJ, Davies HV. 1992. Tuber quality. In: Harris P, editor. *The potato crop*. London: Chapman and Hall. p. 507-569.
- Teixeira GHA, Durigan JF, Alves RE, O'Hare TJ. 2007. Use of modified atmosphere to extend shelf life of fresh-cut carambola (*Averrhoa carambola* L. cv. Fwang Tung). *Postharvest Biology and Technology* 44(1):80-85.
- Teixeira, GHA, Durigan JF, Alves RE, O'Hare TJ. 2008. Response of minimally processed carambola to chemical treatments and low-oxygen atmospheres. *Postharvest Biology and Technology* 8(3):415-421.
- Thomas TR, Matthews MA, Shackel KA. 2006. Direct in situ measurement of cell turgor in grape (*Vitis vinifera* L.) berries during development and in response to plant water deficits. *Plant, Cell and Environment* 29(5):993-1001.

- Thipyapong P, Hunt MD, Steffens JC. 1995. Systemic wound induction of potato (*Solanum tuberosum*) polyphenol oxidase. *Phytochemistry* 40(3):673-676.
- Tipton KF, Dixon HBF. 1983. Effects of pH on enzymes. In: Purich, DL, editor. *Contemporary enzyme kinetics and mechanism*. New York: Academic Press. p. 97-148.
- Toivonen PMA. 2004. Postharvest storage procedures and oxidative stress. *HortScience* 39:938-942.
- Toivonen PMA, Brummell DA. 2008. Biochemical bases of appearance and texture changes in fresh-cut fruit and vegetables. *Postharvest Biology and Technology* 48(1):1-14.
- Tolbert NE. 1973. Activation of polyphenol oxidase of chloroplasts. *Plant Physiology* 51:234-244.
- Tricand de la Goutte J, Kahn JA, Vulfson EN. 2001. Identification of novel polyphenol oxidase inhibitors by enzymatic one-pot synthesis and deconvolution of combinatorial libraries. *Biotechnology and Bioengineering* 75(1):93-99.
- Unal UM. 2007. Properties of polyphenol oxidase from Anamur banana (*Musa cavendishii*). *Food Chemistry* 100(3):909-913.
- USDA Economic Research Service. 2007a. Apples: U.S. import-eligible countries; world production and exports. <http://www.ers.usda.gov/Data/fruitvegphyto/Data/fr-apples.xls>. United States Department of Agriculture: Economic Research Service. Washington, DC.
- USDA Economic Research Service. 2007b. Bananas: U.S. import-eligible countries; world production and exports. <http://www.ers.usda.gov/Data/fruitvegphyto/Data/fr-bananas.xls>. United States Department of Agriculture: Economic Research Service. Washington, DC.
- USDA Economic Research Service. 2007c. Avocados: U.S. import-eligible countries; world production and exports. <http://www.ers.usda.gov/data/fruitvegphyto/Data/fr-avocados.xls>. United States Department of Agriculture: Economic Research Service. Washington, DC.
- USDA Economic Research Service. 2007d. Potato: U.S. import-eligible countries; world production and exports. <http://www.ers.usda.gov/data/fruitvegphyto/Data/veg-potato.xls>. United States Department of Agriculture: Economic Research Service. Washington, DC.
- Vamos-Vigyazo L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. *Crit Rev Food Sci Nutr* 15:49-127.
- Van Gelder CWG, Flurkey WH, Wichers HJ. 1997. Sequence and structural features of plant and fungal tyrosinases. *Phytochem* 45:1309-1323.

- Vaughn KC, Lax AR, Duke SO. 1988. Polyphenol oxidase: The chloroplast oxidase with no established function. *Physiol Plant* 72: 659-665.
- Vijayanand P, Chand N, Eipeson WE. 1995. Optimization of osmotic dehydration of cauliflower. *Journal of Food Processing and Preservation* 19(4):229-242.
- Wakayama T. 1995. Polyphenol oxidase activity in Japanese apples. In: Lee CY, Whitaker JR, editors. *Enzymatic Browning and Its Prevention*, ACS Symposium Series 600. Washington, DC: American Chemical Society. p. 251-266.
- Wang Z, Taylor KDA, Yan X. 1994. Further studies on the role of proteases in the activation of phenolase from Norway lobster (*Nephrops norvegicus*). *Food Chemistry* 51:99-103.
- Whitaker JR. 1994. *Principles of enzymology for the food sciences*. 2nd ed. New York: Marcel Dekker. 625 p.
- Yan X, Taylor KDA. 1991. Studies of the mechanism of phenolase activation in Norway lobster (*Nephrops norvegicus*). *Food Chemistry* 41:11-21.
- Yang C, Fujita S, Ashrafuzzaman M, Nakamura N, Hayashi N. 2000. Purification and characterization of polyphenol oxidase from banana (*Musa sapientum L.*) pulp. *J Agric Food Chem* 48:2732-2735.
- Yang C, Nong Z, Lu J, Lu L, Xu J, Han Y, Li Y, Fujita S. 2004. Banana polyphenol oxidase: occurrence and change of polyphenol oxidase activity in some banana cultivars during fruit development. *Food Science and Technology Research* 10(1):75-78.
- Yerliturk FU, Arslan O, Sinan S, Gencer N, Ozensoy O. 2008. Characterization of Polyphenoloxidase from Wild Pear (*Pyrus elaeagnifolia*). *Journal of Food Biochemistry* 32(3):368-383.
- Yoruk R, Hogsette JA, Rolle RS, Marshall MR. 2003. Apple polyphenol oxidase inhibitor(s) from common housefly (*Musca domestica L.*). *Journal of Food Science* 68(6):1942-1947.
- Yoruk R, Marshall MR. 2003. Physicochemical properties and function of plant polyphenol oxidase: a review. *Journal of Food Biochemistry* 27(5):361-422.
- Yueming J, Litao P, Jianrong L. 2004. Use of citric acid for shelf life and quality maintenance of fresh-cut Chinese water chestnut. *Journal of Food Engineering* 63(3):325-328.
- Zawistowski J, Biliaderis CG, Eskin NAM. 1991. Polyphenol oxidase. In: Robinson DS, Eskin NAM, editors. *Oxidative Enzymes in Foods*. New York: Elsevier Science Publishing. p. 217-273
- Zotos A, Taylor KDA. 1996. Partial purification and characterization of proteases from Norway lobster (*Nephrops norvegicus*) and their role in the phenolase activation process. *Food Chemistry* 56:61-68.

BIOGRAPHICAL SKETCH

Robert Schwartz Bent was born in Takoma Park, MD to a biotechnology patent lawyer and a zoological neuroscientist. He has one brother three years older than himself. He attended Montgomery Blair high school in Silver Spring, MD and participated in the math and science magnet program there. After high school, Rob moved to Florida to attend the University of Florida. After graduating with a bachelor's degree in food science, he began pursuing a master's degree in food science with Dr. Marshall at the University of Florida.

Outside of school, Rob enjoys powerlifting, building cars, and training in mixed martial arts. After graduation, Rob hopes to pursue a career in food product development.