A COMPARISON OF A TANNIC ACID BIOPESTICIDE AND A COMMERCIAL FUNGICIDE USED FOR CROP PROTECTION AGAINST *FUSARIUM* HEAD BLIGHT

A thesis presented to the faculty of the Graduate School of
Western Carolina University in partial fulfillment of the
requirements for the degree of Masters of Science in Chemistry.

By

Brooke Bien

Advisor: Dr. Cynthia Atterholt
Associate Professor of Chemistry
Department of Chemistry & Physics

Committee Members: Dr. Arthur Salido, Chemistry & Physics
Dr. Scott Huffman, Chemistry & Physics

November 2016

ACKNOWLEDGMENTS

I would like to acknowledge past students that contributed to the tannic biopesticide research, Shea Connelly and Crystal Noel for researching formulations to achieve a stable tannic acid emulsion. Also Catherine Murray for researching various emulsifiers for the tannic acid emulsion. Also thanks to Dr. Christina Cowger, Small Grains Pathologist, USDA-ARS, Department of Plant Pathology, North Carolina State University, Raleigh, NC for performing field trials using the fungicide and biopesticide.

Thank you to my thesis committee members, Arthur Salido and Scott Huffman, for their assistance. A special thanks to my advisor, Cynthia Atterholt for our many discussions, her helpful advice, and continuous encouragement. I also extend thanks to Western Carolina University Department of Chemistry & Physics for funding this project.

I am thankful to God for my determination and calmness during this process as well as for this opportunity. Lastly, I thank my wonderful husband and my awesome parents for all of their support and encouragement.

TABLE OF CONTENTS

LIST OF TABLES v
LIST OF FIGURES vii
LIST OF ABBREVIATIONS viii
ABSTRACT ix
CHAPTER ONE: INTRODUCTION 1
1.1 Biopesticides 1
1.2 *Fusarium* Head Blight 2
1.3 Chromatography 3
CHAPTER TWO: TANNIC ACID BIOPESTICIDE 5
2.1 Tannic Acid 5
2.2 Wax Emulsion 6
2.2.1 Wax 7
2.2.2 Emulsifier 8
2.2.3 Guar Gum 8
2.2.4 Preparation 8
CHAPTER THREE: COMMERCIAL FUNGICIDE 11
3.1 Prosaro™ 11
3.1.1 Prothioconazole 11
3.1.2 Tebuconazole 13
3.2 Mode of Action 13
3.3 Tolerances 14
CHAPTER FOUR: APPLICATION STUDIES 15
4.1 Barley Plot Trials 15
4.2 Longevity of Biopesticide After Application 15
CHAPTER FIVE: EXPERIMENTAL DESIGN 19
5.1 QuEChERS Extraction 19
5.1.1 Procedure 19
5.1.2 Homogenization 20
5.1.3 Partitioning and Buffer Salts 20
5.1.4 D-SPE 21
5.2 Operations of HPLC 21
5.2.1 Solvent Delivery System 21
5.2.2 Injection System 22
5.2.3 Separation 23
5.2.4 Column Information 23
5.2.5 Detector Description 23
CHAPTER SIX: METHOD DEVELOPMENT FOR TANNIC ACID ANALYSIS 24
6.1 Optimization of Extraction Method 24

6.1.1 Partitioning and Buffer Salts 24
6.1.2 D-SPE 26
6.1.3 Optimum Extraction Procedure 27
6.2 Instrumental Analysis 27
6.2.1 Gradient 28
6.2.2 Flow Rate 28
6.2.3 Detection Wavelength 31
6.2.4 Temperature 31
6.2.5 Final Instrument Parameters 31
CHAPTER SEVEN: METHOD DEVELOPMENT FOR PROTHIOCONAZOLE AND TEBUCONAZOLE ANALYSIS 33
7.1 Optimization of Extraction Method 33
7.1.1 Partitioning and Buffer Salts 33
7.1.2 D-SPE 34
7.1.3 Optimum Extraction Procedure 35
7.2 Instrumental Analysis 37
7.2.1 Gradient 38
7.2.2 Flow Rate 39
7.2.3 Mobile Phases 41
7.2.4 Detection Wavelength 41
7.2.5 Temperature 42
7.2.6 Final Instrument Parameters 42
CHAPTER EIGHT: RESULTS 43
8.1 Sprayability and Plant Coverage 43
8.2 Barley Plot Trials 46
8.2.1 Effectiveness Data 46
8.2.2 Tannic Acid Residue Extracts 52
8.2.3 Fungicide Residue Extracts 53
8.3 Longetivity of Biopesticide After Application 53
8.3.1 *Euonymus alata* 54
8.3.2 *Liriope muscari* 54
CHAPTER NINE: CONCLUSION 58
CHAPTER TEN: FUTURE RESEARCH 60
REFERENCES 61

LIST OF TABLES

Table 1. The physicochemical properties of tannic acid.[1] 7
Table 2. Percentage of materials to prepare a stable 5% (w/w) tannic acid wax emulsion. 7
Table 3. The physicochemical properties of prothioconazole.[2] 12
Table 4. The physicochemical properties of tebuconazole.[2] 13
Table 5. The tolerances of prothioconazole and tebuconazole residues on barley in parts per million (ppm) 14
Table 6. Barley cultivars used in this research.[4] 15
Table 7. Treatments for barley application. 16
Table 8. Weather data during sampling time.[3] 18
Table 9. The modifications of partitioning and buffering salts studied for the extraction of tannic acid. 25
Table 10. The average percent recoveries of tannic acid for trials described in Table 9 25
Table 11. The modifications of sorbents studied for the extraction of tannic acid 26
Table 12. The average percent recoveries of tannic acid for trials described in Table 11. 27
Table 13. The modifications of partitioning and buffering salts studied for the extraction of prothioconazole and tebuconazole. 34
Table 14. The average percent recoveries of prothioconazole and tebuconazole for trials described in Table 13. 35
Table 15. The modifications of sorbents studied for the extraction of prothioconazole and tebuconazole 36
Table 16. The average percent recoveries of prothioconazole and tebuconazole for trials described in Table 15. 36
Table 17. Formulations of tannic acid emulsions for sprayability and plant coverage comparison. 43
Table 18. Concentration of DON in ppm for Inwood field after treatment with 1% and 3% emulsions in 2015.[4] 47
Table 19. SAS type III tests of fixed effects for Inwood field. 47
Table 20. SAS least square means for Inwood field. 47
Table 21. Concentration of DON in ppm for Caswell field after treatment with 1% and 3% emulsions in 2015.[4] 48
Table 22. SAS type III tests of fixed effects for Caswell field. 49
Table 23. SAS least square means for Caswell field. 49
Table 24. Concentration of DON in ppm after treatment of greenhouse plants with 1% and 3% emulsions in 2015.[4] 49
Table 25. Concentration of DON in ppm after treatment with 5% emulsion in 2016.[4] 50
Table 26. SAS type III tests of fixed effects for 2016 field trials. 51

Table 27. SAS least square means for 2016 field trials. 51
Table 28. Tannic acid residuals for *Euonymus alata* over a period of three weeks. ... 55
Table 29. Tannic acid residuals for *Liriope muscari* over a period of four weeks. 57

LIST OF FIGURES

Figure 1. A barley spike fully blighted with FHB after inoculation with *Fusarium* spores.[4] 2
Figure 2. The chemical structure of deoxynivalenol (DON). 3
Figure 3. The chemical structure of tannic acid. 6
Figure 4. The chemical structure of TEA sterate. 9
Figure 5. The chemical structure of guar gum. 9
Figure 6. The chemical structure of prothioconazole. 12
Figure 7. The chemical structure of tebuconazole. 13
Figure 8. Plots of barley used for field trials at NCSU. 16
Figure 9. Inoculation of barley plots with infected corn kernels. 16
Figure 10. Barley grain before (a) and after (b) grinding. 20
Figure 11. Description of the HPLC injection system 22
Figure 12. A scheme of the optimum extraction procedure for tannic acid. 27
Figure 13. Tannic acid HPLC method development: initial measurement 29
Figure 14. Tannic acid HPLC method development: gradient altered. 30
Figure 15. Tannic acid HPLC final method 32
Figure 16. A scheme of the optimum extraction procedure for prothioconazole and tebuconazole. 37
Figure 17. Fungicide HPLC method development: initial measurement 38
Figure 18. Fungicide HPLC method development: gradient altered. 39
Figure 19. Fungicide HPLC method development: flow rate decreased from 1 mL min^{-1} to 0.800 mL min^{-1} 40
Figure 20. Fungicide HPLC method development: formic acid removed from mobile phases. 41
Figure 21. Fungicide HPLC final method 42
Figure 22. Comparison of sprayability and plant coverage of emulsions 1 and 2 described in Table 17. 44
Figure 23. Comparison of sprayability and plant coverage of emulsions 3 and 4 described in Table 17. 45
Figure 24. Chromatogram of tannic acid calibration standards. 52
Figure 25. Chromatogram of prothioconazole and tebuconazole calibration standards. 53
Figure 26. Tannic acid treated *Euonymus alata*. 55
Figure 27. Tannic acid treated *Euonymus alata* results 56
Figure 28. Tannic acid treated *Liriope muscari*. 56
Figure 29. Tannic acid treated *Liriope muscari* results 57

LIST OF ABBREVIATIONS

ACE	acetone
BZ	benzene
CAS RN	Chemical Abstracts Service Registry Number
CHL	chloroform
CTC	carbon tetrachloride
DMI	demethylation-inhibitor
DON	deoxynivalenol
d-SPE	dispersive-solid phase extraction
EPA	Environmental Protection Agency
ETH	ethane
EtOH	ethanol
FFDCA	Federal Food, Drug, and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FQPA	Food Quality Protection Act of 1996
FHB	*Fusarium* head blight
FRAC	Fungicide Resistance Action Committee
FTIR	Fourier transform infrared
GC-MS	gas chromatography mass spectroscopy
GCB	graphitized carbon black
HPLC	high performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
MRL	maximum residue limit
NCSU	North Carolina State University
NOAA	National Oceanic and Atmospheric Administration
PSA	primary secondary amine
SAS	statistical analysis software
SIM	selected ion monitoring
TEA	triethanolamine
V/V	volume/volume
VS	very soluble
VWD	variable wavelength detector
W/W	weight/weight

ABSTRACT

A COMPARISON OF A TANNIC ACID BIOPESTICIDE AND A COMMERCIAL FUNGICIDE USED FOR CROP PROTECTION AGAINST *FUSARIUM* HEAD BLIGHT

Brooke Bien, Masters of Science in Chemistry

Western Carolina University (November 2016)

Advisor: Dr. Cynthia Atterholt

Fungicides are typically used on crops to prevent pathogenic diseases. However, there are biopesticides that can inhibit the same pathogens as several fungicides. In particular, tannic acid can inhibit the growth of *Fusarium graminearum*, a fungus that causes *Fusarium* head blight. *Fusarium* head blight is a disease that affects gramineous hosts.[5] Fungicides containing tebuconazole and prothioconazole, such as Prosaro™, are commonly used to prevent the disease. As a safer alternative, tannic acid wax emulsions have been formulated as a treatment method. Analytical techniques consisting of QuEChERS extractions and HPLC have been developed to measure the residual amounts of each treatment. Determining the residual amounts will indicate whether the tannic acid formulation is adhering to the sample long enough to prevent the crop disease, as well as if it is rainfast.

After developing methods to extract and analyze tannic acid, prothioconazole, and tebuconazole residues, samples of barley grain were analyzed. Field trials of barley plots were used to examine the effectiveness of the tannic acid biopesticide. The effectiveness of the tannic acid biopesticide was determined by analysis of DON, deoxynivalenol, on the grain samples. DON is produced from the *Fusarium* fungus. Therefore, a low DON concentration means the sample was slightly infected, whereas a high DON concentration means the sample was more infected. An outside party performed these analyses. The average DON concentra-

tions of the treatments seem to confirm that tannic acid treated barley is less infected than untreated barley. However, SAS analysis confirms that there is no significant difference in DON concentration of tannic acid treated barley and untreated barley. Grain samples from these plots were also analyzed for tannic acid or prothioconazole and tebuconazole residue. It was hypothesized that since treatment occurred prior to grain formation, that little to no residue would be present on the grain at harvest. After analysis, it was determined that this hypothesis was correct.

How long the biopesticide would remain on the plant was also examined. Tannic acid was present on *Euonymus alata* up to three weeks and on *Liriope muscari* for up to four weeks. This time period is ideal considering the plant only needs to be protected during a specific growth stage lasting approximately 10 days. Additionally, there would be little to no residue present on the grain at harvest. Overall, this research found that tannic acid could be effectively used to protect crops from FHB.

CHAPTER ONE: INTRODUCTION

1.1 Biopesticides

The Environmental Protection Agency (EPA) defines biopesticides as "certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals."[6] Three categories of biopesticides include biochemical, plant-incorporated protectancts, and biocontrol organisms. Biopesticides are naturally occurring compounds that are target specific and have low toxicity to nontarget organisms.[7] Even though biopesticides are not harmful to farmworkers and are ideal in organic agriculture, they are "only 3 % of the global annual market for pesticides, but currently increasing."[7]

Pesticides are registered by federally mandated statutes such as the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA). The Food Quality Protection Act of 1996 (FQPA) changed the EPA regulation standards of pesticides.[8] Pesticides are heavily regulated to prevent harm to human health and to the environment, as well as to develop specific levels that pesticides cannot exceed.[8] Some biopesticides are considered "minimum risk pesticides" and are exempt from FIFRA registration.[6]

Organically grown crops are not treated with traditional pesticides and are instead treated with non-toxic biological pesticides, or biopesticides, that are derived from natural sources. Biopesticides are not as harmful to humans and the environment as traditional pesticides. Conventional pesticides contain compounds that are toxic to humans through ingestion or inhalation. Even though these compounds are harmful when ingested, they are used as pesticides. "Most applied pesticides find their way as residue in the environment into the terrestrial and aquatic food chains where they undergo concentration and exert potential, long term, adverse health effects."[9]

1.2 *Fusarium* Head Blight

Fusarium Head Blight (FHB), also called ear blight or scab, is a fungal disease that affects gramineous hosts such as wheat, barley, and other cereal grains. "F. *graminearum* is the predominant FHB pathogen in much of the world,"[10] perhaps because it produces several different mycotoxins and the negative impact that it has on crop quality and yield.[11] The pathogen affects the gramineous host after coming into contact with the head of the plant. The pathogen spore will germinate there if conditions are suitable.[10] Once crops are infected with the disease, the head and surrounding tissues are infected and turn brown in color, as shown in Figure 1.

Figure 1. A barley spike fully blighted with FHB after inoculation with *Fusarium* spores.[4]

FHB produces mycotoxins, with deoxynivalenol (DON) being one of the most prevalent.[10] The chemical structure of DON is shown below in Figure 2. Other mycotoxins produced by FHB include zearalenone, nivalenol, and fusarenone x. These compounds, as well as DON, are trichothecenes, compounds with a tricyclic 12,13-epoxytrichothec-9-ene ring

structure.[12,13] DON is unsafe for human consumption. If consumed, DON causes nausea, vomiting, diarrhea, abdominal pain, headache, dizziness, and fever. FHB, while producing high levels of DON in the crop, also significantly decreases the quality of the crop and the crop yield.[10] Fungicide treatments are typically used to manage and prevent *Fusarium* head blight.[14] The most common treatment for FHB is Prosaro™, a fungicide containing tebuconazole and prothioconazole.

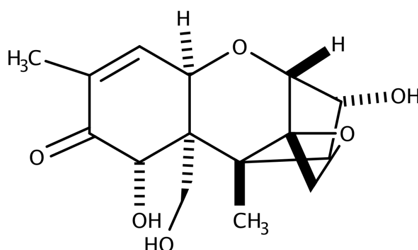


Figure 2. The chemical structure of deoxynivalenol (DON).

1.3 Chromatography

Chromatography is often used for pesticide residue analysis. Chromatography is a method of separating individual components of a complex mixture in order to identify closely related compounds. In chromatography, the sample is dissolved in a mobile phase that flows through a column with a fixed stationary phase. These phases are chosen to allow the compounds to separate and exit the column individually.[15,16] Compounds that interact with the stationary phase strongly move slower than compounds that are weakly retained by the stationary phase. This partitioning between the mobile phase and stationary phase determines the order in which the compounds elute. The weakly retained, faster compounds elute from the column first, followed by the slower eluting compounds.[16]

In chromatography the mobile phase can be gas, liquid, or a supercritical fluid. However, liquid chromatography (LC) "is the most widely used of all of the analytical separation

techniques."[16] This is because of its sensitivity, automation, quantitative determination, and its almost universal applicability. Further information will be discussed in section 5.2, including HPLC operations and specifics about the instrument used for this research.

CHAPTER TWO: TANNIC ACID BIOPESTICIDE

2.1 Tannic Acid

Tannins, including tannic acid, are plant polyphenols that contribute to the color and taste of food and beverages.[17] Polyphenols are secondary metabolites of plants and are often found in foods, such as fruits, vegetables, and cereals, as well as beverages such as wine, coffee, and tea.[18] Polyphenols are "involved in defense against ultraviolet radiation or aggression by pathogens, parasites and predators."[19] Tannins are only one type of polyphenols; other types include phenolic acids, flavonoids, stilbenes, and lignans. Overall there are more than 8,000 polyphenols currently known.[19]

Polyphenols exist in free and bound forms. Flavanols compose the majority of free phenolics, and phenolic acids are the main bound phenolics.[20] Bound phenolics are esterified to cell-wall polymers, whereas free forms are not.[21] It is suggested that phenolic acids are absorbed when consumed and have antioxidant activity that may reduce the risk of diseases, cancers, and aging.[20] Polyphenols, free and bound, that naturally occur in cereal grains exist primarily in the outer layers (husk, pericarp, testa, and aleurone cells). However, some also exist in the endosperm and germ.[21,22] Since tannic acid naturally occurs in cereal grains, it supports the fact that a tannic acid biopesticide is a safer alternative treatment for FHB prevention. It also suggests that residues remaining on the grain are safe for human consumption.

Hans-Rudolf[5] suggested that tannic acid, a naturally occurring compound, inhibits the growth of the *Fusarium* pathogen. As early as 1913, Knudson reported tannic acid to be toxic to many fungi, even at low concentrations.[5] This discovery began with the idea that botanicals are used for medicinal purposes and have high tannin and phenolic content, which have antioxidant and antimicrobial activity.[5] Based on this research, tannic acid wax

emulsions have been formulated to apply to crops, specifically barley, as an alternative treatment method for FHB. The physicochemical properties of tannic acid are included in Table 1. The chemical structure of tannic acid is shown below in Figure 3.

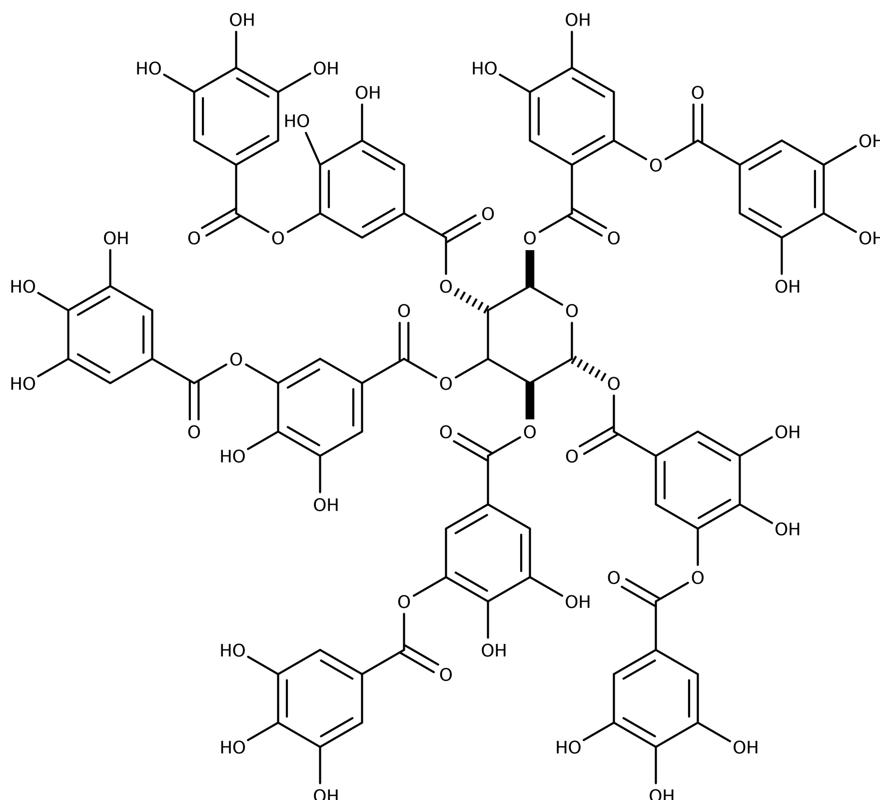


Figure 3. The chemical structure of tannic acid.

2.2 Wax Emulsion

In order for tannic acid to remain on the crop for a lengthy period of time, it is incorporated into a wax emulsion of food grade materials. To prepare an emulsion, the following materials were required: water, vegetable oil, tannic acid, an emulsifier, soy wax, and guar gum. The following sections describe the purpose of each material. Table 2 shows the percentages of each material needed to prepare a stable 5% (w/w) tannic acid wax emulsion. Past

Table 1. The physicochemical properties of tannic acid.[1]

Property	**Tannic Acid**
CAS RN	1401-55-4
Molecular Weight	1701.198 g
Molecular Formula	$C_{76}H_{52}O_{46}$
Melting Point	210 °C
Solubility	vs EtOH, ace; i bz, chl, eth, ctc
Form	yellow-brown amorphous powder

research has been done to determine the percentages of each material to create the most stable emulsion, as well as the most efficient emulsifier.

Table 2. Percentage of materials to prepare a stable 5% (w/w) tannic acid wax emulsion.

Ingredient	**Percent**
Soy wax	3
Vegetable oil	6
TEA stearate	3
Tannic acid	5
Guar gum	0.5
Water	82.5

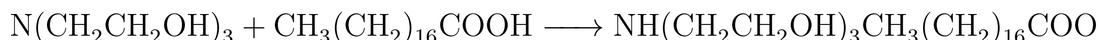
2.2.1 Wax

Wax was incorporated into the emulsion to hold the tannic acid on the crop long enough to protect the crop from the pathogen. Natural waxes are used in applications in which the wax comes into contact with humans and food. These waxes are renewable, biodegradable, and non-toxic.[23] It has been determined that soy wax creates a more stable emulsion when compared to paraffin wax. Soy wax is hydrogenated soybean oil consisting of fatty acids containing 16 to 20 carbons.[23] The soy wax used in this research is Golden Brands, LLC 415 Soy Wax. Soy wax, at room temperature, is a solid with a melting point between 48 °C and 52 °C. The density of soy wax is approximately 0.9 g/cm^3.[24]

2.2.2 Emulsifier

An emulsifier is used to stabilize the emulsion by preventing the components of the mixture from separating. Span60, sorbitan monostearate, was used as the emulsifier for the tannic acid emulsion used in the 2015 field trials. For the 2016 emulsions, triethanolamine (TEA) stearate was used as the emulsifier. TEA stearate has a molecular weight of 433.67 g/mol and a molecular formula of $C_{24}H_{51}NO_5$.[24] The chemical structure of TEA stearate is shown in Figure 4.

TEA stearate was synthesized from TEA and stearic acid according to the following equation:



$$N(CH_2CH_2OH)_3 + CH_3(CH_2)_{16}COOH \longrightarrow NH(CH_2CH_2OH)_3CH_3(CH_2)_{16}COO$$

A stoichiometric amount of stearic acid was added to a beaker and heated at 140 °C until completely melted. While stirring, TEA was added dropwise and the temperature was slightly increased to form an isotropic solution. The properties of an isotropic solution are uniform in all directions. The isotropic solution was transferred to an aluminum boat where it cooled and hardened overnight. TEA stearate is a white solid that can be ground to a powder using a mortar and pestle and stored in a glass jar until use as the emulsifier of the emulsion.[24]

2.2.3 Guar Gum

Guar gum is a food grade non-ionic hydrophilic biopolymer that is used as a thickener and stabilizer in the emulsion.[25] The chemical structure of guar gum is shown in Figure 5.

2.2.4 Preparation

To prepare the tannic acid wax emulsion, appropriate amounts of soy wax, vegetable oil, and TEA stearate were weighed into a beaker containing a stir bar and heated to 140 °C while

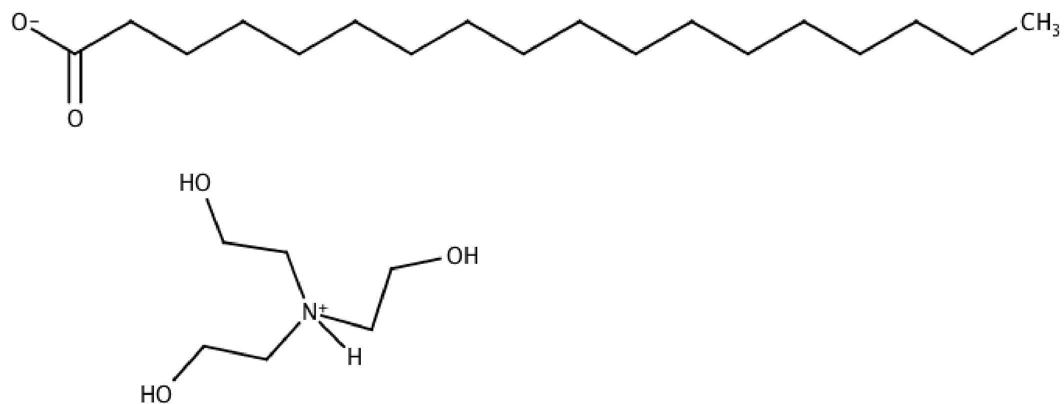


Figure 4. The chemical structure of TEA sterate.

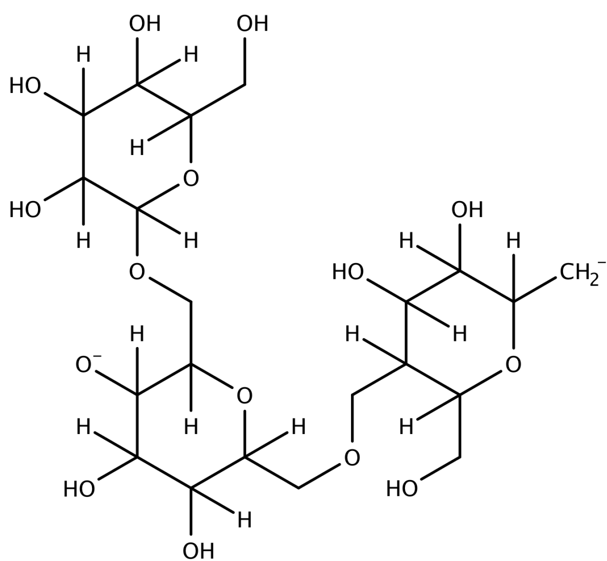


Figure 5. The chemical structure of guar gum.

stirring at a slow speed. The tannic acid was measured in a separate beaker. Enough water was added to the tannic acid to dissolve the solid, and the remaining water was added to the wax mixture. Once the water was added, the stir rate was increased to medium speed, and the tannic acid solution was added. The stir rate was then increased to high speed and the appropriate amount of guar gum was added very slowly. The mixture was removed from the hot plate and homogenized immediately using a high shear homogenizer. The emulsion was homogenized every 20 min until it reached room temperature; it was then stored in the refrigerator at 4 °C until use.

CHAPTER THREE: COMMERCIAL FUNGICIDE

3.1 Prosaro™

Prosaro™ is commonly used on gramineous crops to manage and prevent FHB. Gramineous crops are cereal grasses such as wheat, barley, rye, and oats. The Prosaro™ fungicide is considered a demethylation-inhibiting (DMI) fungicide, or a group 3 fungicide in FRAC grouping. FRAC, or Fungicide Resistance Action Committee, provides guidelines to manage resistance and effectiveness of fungicides.[26] Group 3 fungicides prevent sterol formation, which is crucial in fungal cell walls. DMI fungicides are transported through the plant by the transpiration stream after penetrating into the plant cuticle. DMIs can negatively effect the growth of the crop by slowing cell growth and should not be applied repeatedly.[26]

Prosaro™ contains tebuconazole and prothioconazole as its active ingredients.[27] For this research, Prosaro™ 421 SC Fungicide manufactured by Bayer CropScience was used. This product contains an average of 19% prothioconazole and 19% tebuconazole by weight. Personal protection is required when handling Prosaro™. Prosaro™ is an off-white liquid suspension with a pH of 7.5 - 7.9. The density of the suspension is approximately 1.112 g/cm^3 at 20 °C (MSDS). When applying Prosaro™ to the crops, a minimum of 10 gallons of spray solution per acre is recommended.

3.1.1 Prothioconazole

Prothioconazole's IUPAC name is 2-[(2RS)-2-(1-chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl] 2H-1,2,4-triazole-3(4H)-thione. The physicochemical properties of prothioconazole are included in Table 3.[2] The chemical structure of prothioconazole is shown below in Figure 6.

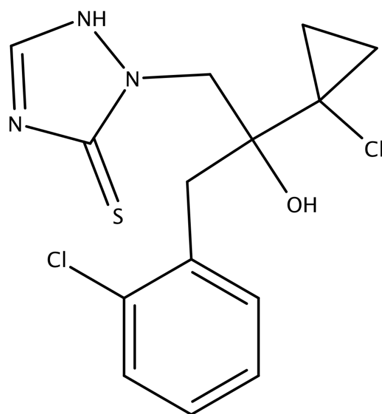


Figure 6. The chemical structure of prothioconazole.

Table 3. The physicochemical properties of prothioconazole.[2]

Property	**Prothioconazole**
CAS RN	178928-70-6
Molecular Weight	344.3 g
Molecular Formula	$C_{14}H_{15}Cl_2N_3OS$
Melting Point	139.1-144.5 °C
Boiling Point	487 °C
Form	White to light beige crystalline powder

3.1.2 Tebuconazole

Tebuconazole's IUPAC name is (RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl) pentan-3-ol. The physicochemical properties of tebuconazole are included in Table 4.[2] The chemical structure of tebuconazole is shown below in Figure 7.

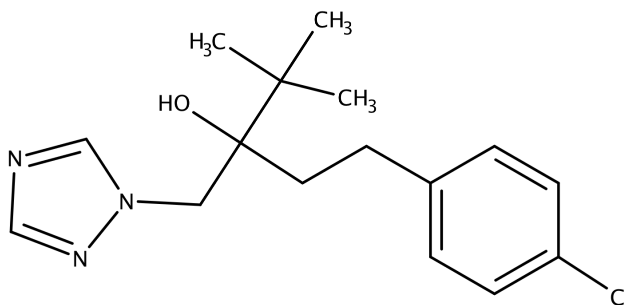


Figure 7. The chemical structure of tebuconazole.

Table 4. The physicochemical properties of tebuconazole.[2]

Property	**Tebuconazole**
CAS RN	107534-96-3
Molecular Weight	307.8 g
Molecular Formula	$C_{16}H_{22}ClN_3O$
Melting Point	105 °C
Form	Colorless crystals

3.2 Mode of Action

Prothioconazole and tebuconazole have similar protective modes of action.[27] Both compounds are members of the DMI (demethylation-inhibitor) fungicide group. The mode of action for these compounds is inhibiting the synthesis of sterols. Once used as treatment, the active ingredients are absorbed by the root and leaf tissue and continue moving into the growing plant, being protective and curative.[28]

3.3 Tolerances

Tolerances, also called maximum residue levels (MRL), must be safe, meaning "provide a reasonable certainty that no harm will result from aggregate (dietary, water, and household) exposure."[29] The tolerances established in EPA's Code of Federal Regulations for residues of prothioconazole and tebuconazole on barley are included in Table 5.

Table 5. The tolerances of prothioconazole and tebuconazole residues on barley in parts per million (ppm). The abbreviation NR represents data not reported.

Tolerances of prothioconazole and tebuconazole on barley in ppm

Barley	**Prothioconazole**	**Tebuconazole**
Grain	0.35	0.30
Hay	8.00	7.0
Straw	NR	3.5

CHAPTER FOUR: APPLICATION STUDIES

4.1 Barley Plot Trials

Plots of organic barley, shown in Figure 8, were grown at North Carolina State University (NCSU) in spring 2016 to study the effectiveness of the tannic acid biopesticide. Four cultivars of barley were grown, including Atlantic, Endeavor, Nomini, and Thoroughbred.[4] Information about each cultivar is provided in Table 6. Plots were treated with either the tannic acid biopesticide or the Prosaro^{TM} fungicide. The various treatments are included in Table 7. After treatment, the barley was exposed to the *Fusarium* fungus through contact with infected corn kernels, shown in Figure 9. The effectiveness of each treatment was studied. After harvest the barley grain was analyzed for tannic acid residue or prothioconazole and tebuconazole residues. Calibration standards were made and measured prior to all sample measurements. All standards and samples were measured in duplicate. Results are included in Section 8.2.

Table 6. Barley cultivars used in this research.[4]

Cultivar	**Barley Type**	**Expected response to *Fusarium* head blight**
Atlantic	Feed	Susceptible
Endeavor	Malting	Moderately resistant
Nomini	Feed	Moderately resistant
Thoroughbred	Feed	Susceptible

4.2 Longevity of Biopesticide After Application

To determine the length of time the tannic acid biopesticide adheres to the plant surface, various plants were treated and analyzed over a period of three or four weeks. The plants included *Euonymus alata* and *Liriope muscari*.[30] The common names are winged euonymus,

Table 7. Treatments for barley application.

Abbreviation	**Treatment Application**
T1	Tannic acid once (when spikes have just fully emerged)
T2	Tannic acid twice (when spikes have fully emerged AND 6 days later)
P1	Prosaro^{TM} (when spikes have just fully emerged)
P2	Prosaro^{TM} (6 days after spikes have just fully emerged)
U	Untreated (no application)

(a)

(b)

Figure 8. Plots of barley used for field trials at NCSU.

Figure 9. Inoculation of barley plots with infected corn kernels.

also known as burning bush, and monkey grass. Weather data was recorded in order to determine how temperature and precipitation affect the biopesticide. Weather data was collected by the National Oceanic and Atmospheric Administration (NOAA) at a weather station in Cullowhee, North Carolina. The high temperature, low temperature, and precipitation for days during the sampling time are listed in Table 8.

The procedure involved randomly collecting five gram samples from each plant in triplicate. Samples were collected the day of treatment and several days over a period of three or four weeks. Tannic acid was extracted from the plant surface using a surface extraction method. Preliminary tests were conducted to determine the adequate amount of solvent and appropriate extraction time for 5 g samples. A treated plant sample was soaked in acetonitrile for 10 min, then removed and placed into a different beaker of solvent for an additional 10 min. This was repeated to get extracts of the same sample at 10, 20, 30, 45, and 60 minutes. Each extract was analyzed to determine how long it took to extract all the tannic acid from the plant surface. It was determined that all tannic acid was extracted from the surface when stirred in 100 mL of acetonitrile for one hour. Calibration standards were made and measured prior to sample measurements. All samples were measured in duplicate. Results are included in Section 8.3.

Table 8. Weather data during sampling time.[3]

Date	High Temp. (°C)	Low Temp. (°C)	Precipitation (in)
Aug. 27	92	64	0.00
Aug. 28	90	64	0.00
Aug. 29	87	58	0.02
Aug. 30	89	58	0.00
Aug. 31	87	59	0.00
Sept. 1	89	60	1.09
Sept. 2	87	63	0.09
Sept. 3	75	56	0.01
Sept. 4	82	57	0.00
Sept. 5	83	58	0.00
Sept. 6	86	58	0.00
Sept. 7	87	56	0.00
Sept. 8	89	56	0.00
Sept. 9	89	56	0.00
Sept. 10	88	58	0.00
Sept. 11	86	60	0.12
Sept. 12	85	57	0.00
Sept. 13	87	57	0.00
Sept. 14	87	57	0.00
Sept. 15	88	59	0.00
Sept. 16	89	59	0.00
Sept. 17	89	61	0.02
Sept. 18	82	60	0.00
Sept. 19	73	62	0.21
Sept. 20	82	58	0.00
Sept. 21	84	55	0.00
Sept. 22	87	55	0.00
Sept. 23	86	58	0.00
Sept. 24	86	56	0.00
Sept. 25	88	56	0.00
Sept. 26	90	58	0.00
Sept. 27	87	63	0.22
Sept. 28	79	59	0.28
Sept. 29	82	47	0.00
Sept. 30	75	47	0.00
Oct. 1	73	44	0.00
Oct. 2	72	45	0.00

CHAPTER FIVE: EXPERIMENTAL DESIGN

5.1 QuEChERS Extraction

The QuEChERS extraction method was used for analysis of residual tannic acid or the fungicide compounds, prothioconazole and tebuconazole. QuEChERS is an acronym for quick, easy, cheap, effective, rugged, and safe.[31] The QuEChERS method was developed in 2003 by Anastassiades as a multipesticide extraction method that removes matrix compounds. This method was used to shorten the process of extraction and cleanup.[32,33] The QuEChERS method uses very little solvent and time.

5.1.1 Procedure

The original extraction method developed by Anastassiades[33] includes the sample being homogenized and mixed with acetonitrile. Magnesium sulfate and sodium chloride are added for a salting out step and the removal of water. The sample is then vortexed and centrifuged. After centrifugation, an aliquot is mixed with primary secondary amine (PSA) bonded silica for a dispersive-solid phase extraction (d-SPE) step. After centrifugation, an aliquot is analyzed.[33]

Many variations of this method now exist. There are variations using different solvents, salts, and sorbents. Also many modifications of the original method have been made to accommodate different types of samples. For this research, a modified method for samples with little to no water content was used. This method includes adding water to the sample during the process of homogenization.

5.1.2 Homogenization

Recommendations from literature for homogenizing the sample include air-drying or freeze-drying the sample prior to homogenization.[19] Multiple techniques have previously been used to homogenize the sample, such as using a blender, mortar and pestle, grinder, or high sheer homogenizer. For analysis of barley grain samples, a coffee grinder was used to mill the samples prior to extraction. Figure 10a shows the barley grain prior to grinding, and Figure 10b shows the grain after it was milled.

(a) (b)

Figure 10. Barley grain before (a) and after (b) grinding.

5.1.3 Partitioning and Buffer Salts

Magnesium sulfate, sodium citrate dibasic sesquihydrate, sodium citrate dihydrate, sodium acetate, and sodium chloride have been used in the salting out step. The addition of excess salts and buffers cause a liquid-liquid partition and after centrifugation, the acetonitrile layer is collected. Modifications of the amounts and various combinations of the salts were studied to achieve the optimum extraction procedure for the biopesticide and fungicide. Recoveries of 80-120% are an acceptable recovery range. This study is further discussed in sections 6.1.1

and 7.1.1.

5.1.4 D-SPE

The next step is a dispersive-solid phase extraction (d-SPE). In this step, sorbents such as primary secondary amine (PSA) bonded silica, C18, and graphitized carbon black (GCB) are used to remove fatty acids and sugars from the sample.[31] The PSA is used to remove polar components of the matrix, such as sugars and organic acids.[33] C18 removes lipid material that has been coextracted from the matrix.[34] GCB is used to remove pigment from the matrix. Modifications of the amounts and various combinations of the sorbents were studied to achieve the optimum procedure for the cleanup step for both the biopesticide and the fungicide. Recoveries of 80-120% are an acceptable recovery range. This study is further discussed in sections 6.1.2 and 7.1.2.

5.2 Operations of HPLC

For this research, analysis was performed with an Agilent 1220 Infinity HPLC featuring a dual channel gradient pump, autosampler, and variable wavelength detector to analyze tannic acid, prothioconazole, and tebuconazole. The LC was equipped with a 250 mm i.d., 5 µm C_{18} column. As mentioned in section 1.3, chromatography is used to separate components in a mixture by forcing a mobile phase through an immiscible stationary phase fixed inside the column.[16] The following sections further discuss how an HPLC operates.

5.2.1 Solvent Delivery System

The solvent is delivered from the solvent bottles, through the injector, column, and detector by pumps operated by pistons made of sapphire. The solvents are degassed by passing through an online vacuum degasser.[35,36]

5.2.2 Injection System

The HPLC used for this research is equipped with an autosampler that performs the sampling sequence automatically by retrieving the vial, drawing the sample, and injecting the sample into the column. Before the injection sequence begins, the injection valve is in the mainpass position (Figure 11a) in which the mobile phase flows through the sample loop and needle; this ensures the system is flushed with solvent. When the sequence begins, the injection valve shifts to the bypass position (Figure 11b) in which solvent now flows to the column. The needle is then inserted into the vial and the specified volume of sample is drawn into the sample loop (Figure 11c). The needle lowers into the needle seat and the injection valve switches back into the mainpass position, flushing the sample onto the column (Figure 11d).[36]

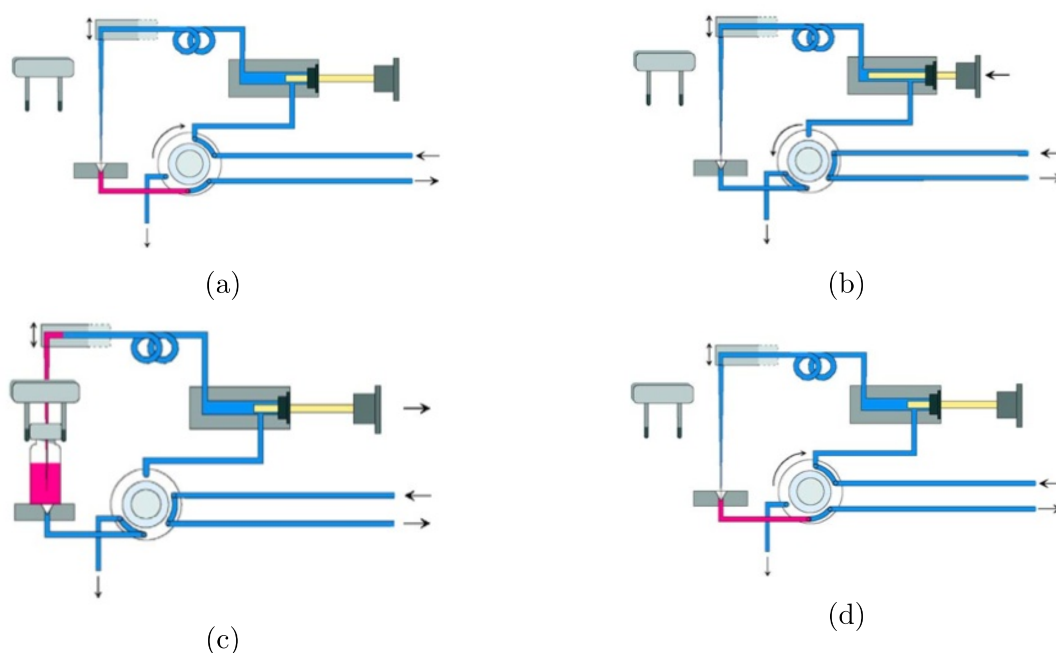


Figure 11. Description of the HPLC injection system, (a) mainpass position, (b) bypass position, (c) drawing the sample, (d) sample injection.[36]

5.2.3 Separation

Separation in the column occurs by the interaction, or partitioning, of the analyte between the mobile phase and stationary phase. The polar phase attracts polar compounds and the nonpolar phase attracts nonpolar compounds. If the stationary phase is polar, nonpolar compounds will elute first followed by the polar compounds, and vice versa.[35]

5.2.4 Column Information

Analytical columns for HPLC are typically 5 to 25 cm in length with a diameter of 3 to 5 mm. The packing material of the column is usually 3 or 5 µm. The packing material is often alumina, a synthetic resin, an ion-exchange resin, or silica which is the most common.[16] The Agilent 1220 Infinity LC used in this research is equipped with a 250 mm C_{18} column with 5 µm packing. A C_{18} column is an octadecyl column coated with a long-chain, saturated hydrocarbon consisting of 18 carbons.[35]

5.2.5 Detector Description

There are many types of detectors used with liquid chromatography. Some of the most common include absorbance, fluorescence, electrochemical, refractive index, FTIR, and mass spectrometry.[16] The Agilent 1220 Infinity LC used in this research is equipped with a variable wavelength detector (VWD) that covers the wavelength range from 195 to 650 nm.

CHAPTER SIX: METHOD DEVELOPMENT FOR TANNIC ACID ANALYSIS

6.1 Optimization of Extraction Method

As mentioned above in section 5.1.1, the extraction procedure has been modified to achieve the method with the highest percent recovery for the compounds of interest. The following sections discuss in detail the modifications to each step of the procedure to achieve the highest percent recovery of tannic acid from the sample.

6.1.1 Partitioning and Buffer Salts

Modifications of the original QuEChERS method studied in this research included various combinations and amounts of magnesium sulfate, sodium citrate dibasic sesquihydrate, sodium citrate dihydrate, sodium acetate, and sodium chloride in the extraction step. In each trial, 5 mL of a 250 ppm standard diluted with ethanol was added to a 50 mL conical tube and mixed with 10 mL of ultrapure water and 10 mL acetonitrile. The salts and buffers, according to Table 9, were then added, the tube was immediately shaken and vortexed for one minute. After centrifugation the solution was analyzed to determine the percent recovery for this step. Each trial was repeated twice and each extract was measured in duplicate.

Table 9 lists the combinations and amounts of salts and buffers used to determine the procedure with the highest percent recovery. The average percent recoveries are included in Table 10. The original extraction procedure from Anastassiades developed method was first tested.[33] Trials 1 through 8 are modifications from other literature.

The optimum method for this step was determined to be trial 1. The percent recovery for this trial was 82.62%, which is within the acceptable range. This trial used 4 g of magnesium sulfate, 0.5 g of sodium citrate dibasic sesquihydrate, 1 g sodium citrate dihydrate and 1 g sodium chloride.

Table 9. The modifications of partitioning and buffering salts studied for the extraction of tannic acid. The original method is from Anastassiades.[33] The optimum method is trial 1*.

Mass (g) of salts for each trial

	Original[33]	**1***	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Magnesium sulfate	4	4	4	3	4	4	5	4	4
Sodium citrate sesquihydrate	—	0.5	—	—	—	—	—	—	—
Sodium citrate dihydrate	—	1	1.5	1	—	1.5	—	—	—
Sodium acetate	—	—	—	1.7	1.5	—	—	1.5	1.5
Sodium chloride	1	1	1	—	—	—	—	—	1

Table 10. The average percent recoveries of tannic acid for trials described in Table 9. The abbreviation ND indicates tannic acid was not detected.

Trial	**Average Percent Recovery**
Original	ND
1	82.62
2	ND
3	ND
4	ND
5	13.54
6	ND
7	ND
8	ND

6.1.2 D-SPE

Following the extraction step is a dispersive-solid phase extraction in which sorbents are added to the extract to remove pigment, lipid material, fatty acids, sugars, and other polar components of the matrix.[31]

For this study, 5 mL of a 250 ppm standard diluted with ethanol was added to a 15 mL conical tube containing the specified amount of sorbents. The tube was immediately shaken and vortexed for one minute. After centrifugation an aliquot was analyzed to determine the percent recovery for this step. Multiple trials were studied using various combinations and amounts of sorbents, specifically magnesium sulfate, primary secondary amine (PSA), and C18. Table 11 lists the combinations and amounts of sorbents used for each trial. Each trial was repeated twice and each extract was measured in duplicate. The average percent recovery for tannic acid is shown in Table 12. The original trial, as well as trials 1 and 2 have such a low percent recovery because PSA is used as a sorbent in those trials. PSA removes polar components from the extract, and since tannic acid is polar, it is also being removed in those trials.

The optimum method for the cleanup step was determined to be trial 3 with a percent recovery of 95.14%. This method used 0.6 g magnesium sulfate and 0.3 g C18. Even though the percent recovery is slightly higher for trial 4, it was not chosen because 0.6 g of magnesium sulfate would remove the excess water added before the extraction.

Table 11. The modifications of sorbents studied for the extraction of tannic acid. The original method is from Anastassiades.[33] The optimum method was trial 3.*

Mass (g) of sorbents for each trial

	Original[33]	**1**	**2**	**3***	**4**
Magnesium sulfate	0.6	0.9	0.3	0.6	0.3
PSA	0.3	0.3	0.3	—	—
C18	—	0.3	0.3	0.3	0.3

Table 12. The average percent recoveries of tannic acid for trials described in Table 11.

Trial	Average Percent Recovery
Original	5.849
1	2.632
2	4.527
3	95.14
4	95.74

6.1.3 Optimum Extraction Procedure

The trial that gave the recoveries closest to 100% for the extraction step was trial 1: 4 g magnesium sulfate, 0.5 g of sodium citrate dibasic sesquihydrate, 1 g sodium citrate dihydrate and 1 g sodium chloride. Trial 4 gave the best recoveries for the d-SPE step in which 0.3 g magnesium sulfate and 0.3 g C18 was used. However, to ensure that all water and coextracted matrix components are eliminated, trial 3 was used. Therefore 0.6 g magnesium sulfate and 0.3 g C18 was used for the cleanup step. An overall scheme of the optimized extraction method is included in Figure 12.

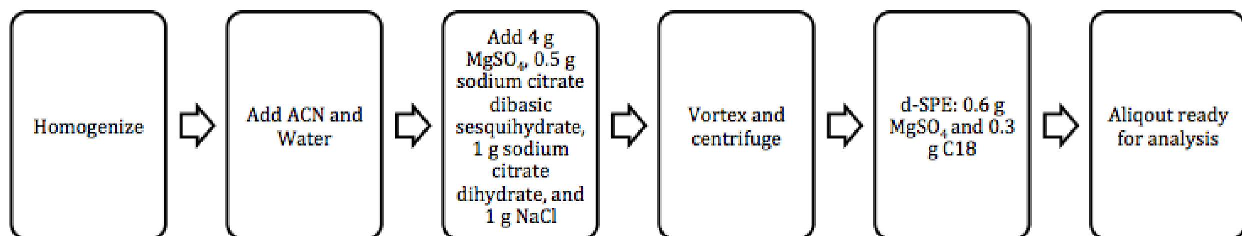


Figure 12. A scheme of the optimum extraction procedure for tannic acid.

6.2 Instrumental Analysis

A method to analyze tannic acid using HPLC was developed based on an HPLC method from the literature to analyze polyphenols in cranberry products.[37] A univariate method development approach was taken, in which one instrumental variable at a time was altered.

These variables include the mobile phase gradient, flow rate, detection wavelength, and temperature. A 400 ppm standard of tannic acid in acetonitrile was used for the stages of instrumental method development.

For the initial method, mobile phase A was 2.5% (v/v) acetic acid in water and mobile phase B was 70% (v/v) methanol in water. The solvent gradient elution program was as follows: 10% to 26% B (v/v) in 10 min, to 70% B at 20 min and finally to 90% B at 25 to 31 min. The solvent composition was equilibrated back to the starting composition of 10% B for 2 min. The flow rate and temperature used in the literature was 1.00 mL min^{-1} and 25 °C. The detection wavelength was 280 nm. The chromatogram of the initial measurement is included in Figure 13. The composition of the mobile phase consists mostly of mobile phase B after 20 min, producing large broad peaks.

6.2.1 Gradient

The first variable altered in the method development was the gradient. The original method increased the composition of mobile phase B to a maximum of 90%. The gradient was altered to increase the composition of mobile phase B to a maximum of 70% instead. The chromatogram for this change in the gradient is shown in Figure 14. There is not much difference in the initial chromatogram and the altered gradient chromatogram. The broad peaks after 20 min are still present even though the composition of mobile phase B was decreased from 90% to 70%. For this reason, an isocratic method was examined.

6.2.2 Flow Rate

The flow rate was programmed to 1 mL min^{-1} and was not altered during HPLC method development.

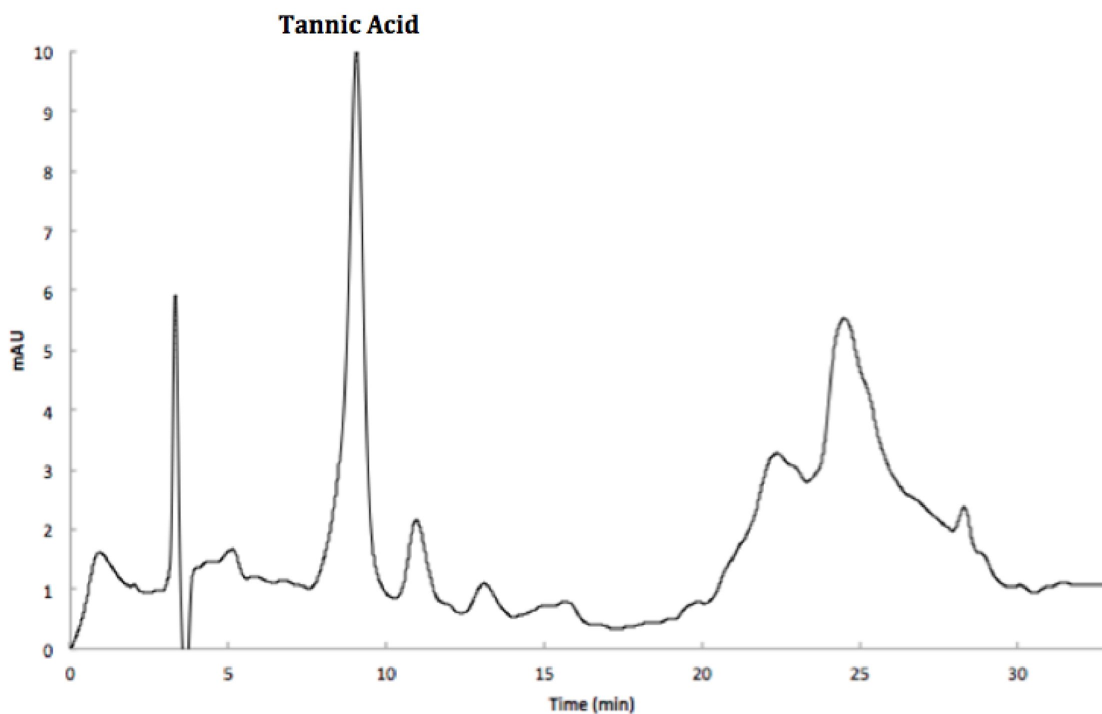


Figure 13. Tannic acid HPLC method development: initial measurement of 400 ppm tannic acid in acetonitrile. The mobile phase gradient with 2.5% (v/v) acetic acid in water (A) and 70% (v/v) methanol in water was as follows: 10% to 26% B (v/v) in 10 min, to 70% B at 20 min and to 90% B at 25 to 31 min and equilibrated back to 10% B for 2 min. The flow rate and temperature was 1.00 mL min^{-1} and 25 °C. The detection wavelength was 280 nm.

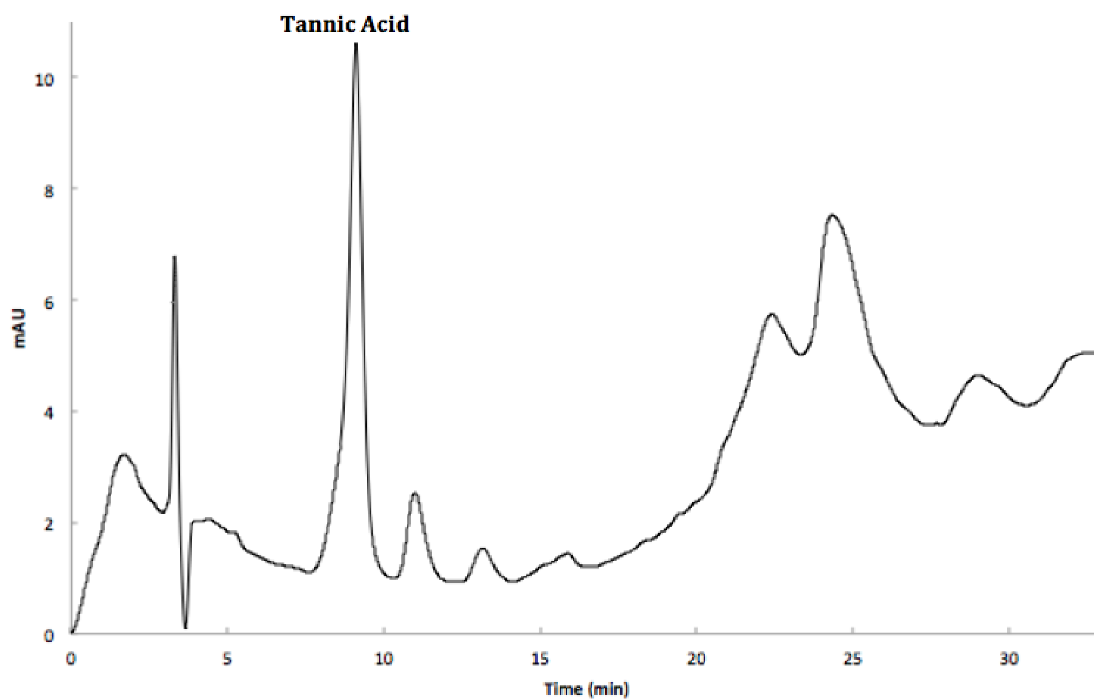


Figure 14. Tannic acid HPLC method development: gradient altered to measure 400 ppm tannic acid in acetonitrile. The mobile phase gradient with 2.5% (v/v) acetic acid in water (A) and 70% (v/v) methanol in water was as follows: 10% to 26% B (v/v) in 10 min, to 70% B over the next 10 min. The composition was held at 70% B from 20 to 31 min and equilibrated back to 10% B for 2 min. The flow rate and temperature was 1.00 mL min^{-1} and 25 °C. The detection wavelength was 280 nm.

6.2.3 Detection Wavelength

The detection wavelength was programmed to 280 nm and was not altered during HPLC method development.

6.2.4 Temperature

The temperature was programmed to 25 °C and was not altered during HPLC method development.

6.2.5 Final Instrument Parameters

After determining the most efficient gradient, flow rate, detection wavelength, and temperature for the detection and quantification of tannic acid, the optimum method was developed. An isocratic method of 20% mobile phase B was examined in order to achieve a clean chromatogram. This mobile phase composition resulted in a chromatogram without the broad peak at 20 min. The following paragraph discusses the final instrumental parameters in detail.

An isocratic elution was used with mobile phase A as 2.5% (v/v) acetic acid in water and mobile phase B as 70% (v/v) methanol in water. The composition of the mobile phases were 80% A and 20% B for 15 min. The injection volume and flow rate were 10 µL and 1.00 mL min^{-1}. The column temperature was maintained at 25 °C. Tannic acid analysis was performed at a wavelength of 280 nm. The retention time of tannic acid was determined to be 7.9 min. Figure 15 shows a chromatogram of tannic acid analyzed with the final instrument parameters. The unlabeled peaks may be contaminants in the tannic acid.

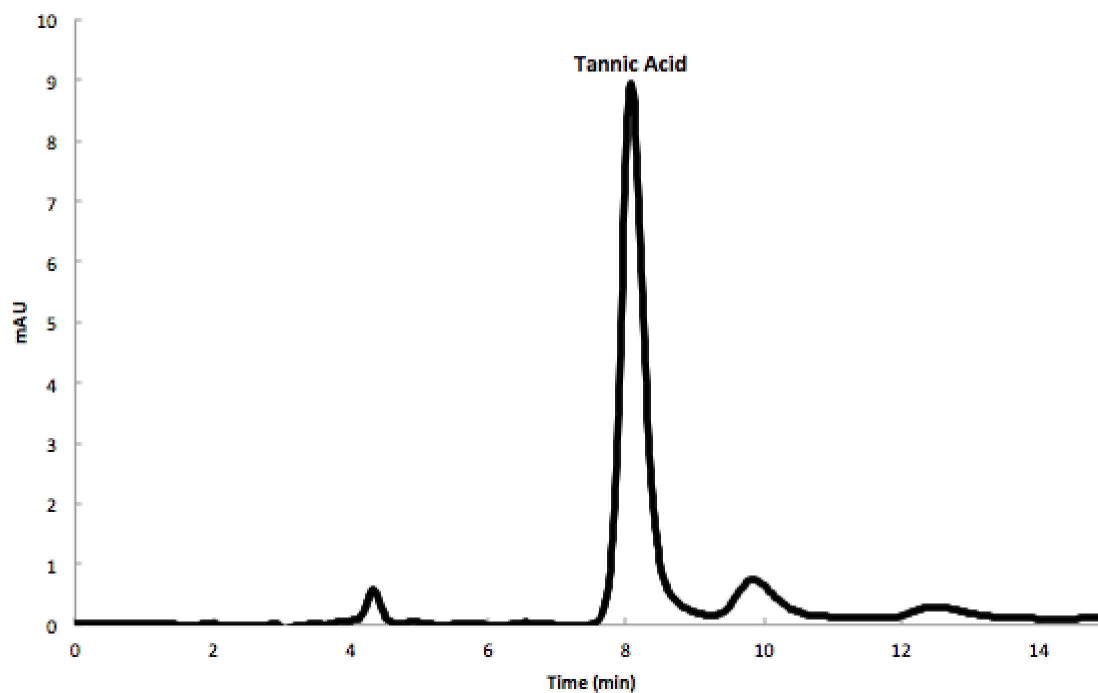


Figure 15. Tannic acid HPLC final method: isocratic elution of 80% A, 2.5% (v/v) acetic acid in water, and 20% B, 70% (v/v) methanol in water, with an injection volume and flow rate of 10 µL and 1.00 mL min^{-1}. The column temperature was 25 °C and detector wavelength was 280 nm.

CHAPTER SEVEN: METHOD DEVELOPMENT FOR PROTHIOCONAZOLE AND TEBUCONAZOLE ANALYSIS

7.1 Optimization of Extraction Method

The extraction procedure has also been modified to achieve the method with the highest percent recovery for prothioconazole and tebuconazole. The following sections discuss the modifications to each step of the procedure in detail. When determining the optimum method for each step of the procedure, the percent recoveries for both compounds were examined because it is more efficient to have one procedure to extract both compounds. Also the compounds will exist in the same sample, so in order to analyze both compounds the procedure must be effective for both.

7.1.1 Partitioning and Buffer Salts

Modifications of the original QuEChERS method studied in this research included various combinations and amounts of magnesium sulfate, sodium citrate dibasic sesquihydrate, sodium citrate dihydrate, sodium acetate, and sodium chloride in the extraction step. The following trials involved analyzing pure prothioconazole and tebuconazole standards diluted with acetonitrile. In each trial, 5 mL of a 250 ppm standard of prothioconazole and tebuconazole diluted with acetonitrile was added to a 50 mL conical tube and mixed with 10 mL of ultrapure water and 10 mL acetonitrile. The same trials used with tannic acid were used for the fungicide compounds and these amounts are restated in Table 13. Once the salts and buffers were added, the tube was immediately shaken and vortexed for one minute. After centrifugation the solution was analyzed to determine the percent recovery for this step. Each trial was repeated twice and each extract was measured in duplicate.

Table 13 lists the combinations and amounts of salts and buffers used to determine

the procedure with the highest percent recovery. The original extraction procedure from Anastassiades was first tested.[33] Trials 1 through 8 are modifications from other literature. The average percent recoveries for prothioconazole and tebuconazole are included in Table 14. A percent recovery greater than 100% may have been caused by evaporation of solvent and instrumental error, such as peaks overlapping from sample and background.

The optimum method for this step was determined to be trial 5. The percent recoveries for this trial were 101.2% for prothioconazole and 99.06% for tebuconazole, both are within the acceptable range. This trial used 4 g of magnesium sulfate and 1.5 g of sodium citrate dihydrate.

Table 13. The modifications of partitioning and buffering salts studied for the extraction of prothioconazole and tebuconazole. The original method is from Anastassiades.[33] The optimum method is trial 5*.

Mass (g) of salts for each trial

	Original[33]	**1**	**2**	**3**	**4**	**5***	**6**	**7**	**8**
Magnesium sulfate	4	4	4	3	4	4	5	4	4
Sodium citrate sesquihydrate	—	0.5	—	—	—	—	—	—	—
Sodium citrate dihydrate	—	1	1.5	1	—	1.5	—	—	—
Sodium acetate	—	—	—	1.7	1.5	—	—	1.5	1.5
Sodium chloride	1	1	1	—	—	—	—	—	1

7.1.2 D-SPE

The cleanup step of the QuEChERS extraction is a dispersive solid phase extraction (d-SPE) in which sorbents are added to the extract to remove pigment, lipid material, fatty acids, sugars, and other polar components of the matrix.[31]

For this study, 5 mL of a 250 ppm standard of prothioconazole and tebuconazole diluted with acetonitrile was added to a 15 mL conical tube containing the specifed amount of sorbents. The tube was immediately shaken and vortexed for one minute. After centrifugation,

Table 14. The average percent recoveries of prothioconazole and tebuconazole for trials described in Table 13. The abbreviation ND indicates not detected.

Average Percent Recoveries

Trial	**Prothioconazole**	**Tebuconazole**
Original	104.6	152.9
1	ND	ND
2	88.66	82.91
3	103.7	99.79
4	104.6	104.1
5	101.2	99.06
6	92.33	100.5
7	91.30	97.70
8	101.0	113.1

an aliquot was analyzed to determine the percent recovery for this step. Multiple trials were studied using various combinations and amounts of sorbents, specifically magnesium sulfate, PSA, and C18. Table 15 lists the combinations and amounts of sorbents used for each trial. Each trial was repeated twice and each extract was measured in duplicate. The average percent recoveries for prothioconazole and tebuconazole are shown in Table 16.

The optimum method for the cleanup step was determined to be trial 3. The percent recoveries were 107.3% for prothioconazole and 91.41% for tebuconazole. This trial used 0.6 g magnesium sulfate and 0.3 g C18. Even though the percent recoveries for trial 4 were closer to 100%, it was not chosen because 0.6 g of magnesium sulfate would remove the excess water added before the extraction.

7.1.3 Optimum Extraction Procedure

The trial that gave the best recoveries for the extraction step was trial 5: 4 g magnesium sulfate and 1.5 g sodium citrate dihydrate. Trial 4 gave recoveries closest to 100% for the d-SPE step, however trial 3 will be used to ensure all water and coextracted matrix components are removed. Trial 3 uses 0.6 g magnesium sulfate and 0.3 g C18. An overall scheme of the

Table 15. The modifications of sorbents studied for the extraction of prothioconazole and tebuconazole. The original method is from Anastassiades.[33] The optimum method was trial 3.*

Mass (g) of sorbents for each trial

	Original[33]	**1**	**2**	**3***	**4**
Magnesium sulfate	0.6	0.9	0.3	0.6	0.3
PSA	0.3	0.3	0.3	—	—
C18	—	0.3	0.3	0.3	0.3

Table 16. The average percent recoveries of prothioconazole and tebuconazole for trials described in Table 15.

Average Percent Recoveries

Trial	**Prothioconazole**	**Tebuconazole**
Original	0.5205	88.81
1	2.320	82.48
2	1.866	92.96
3	107.3	91.41
4	107.1	94.14

optimized extraction method is included in Figure 16.

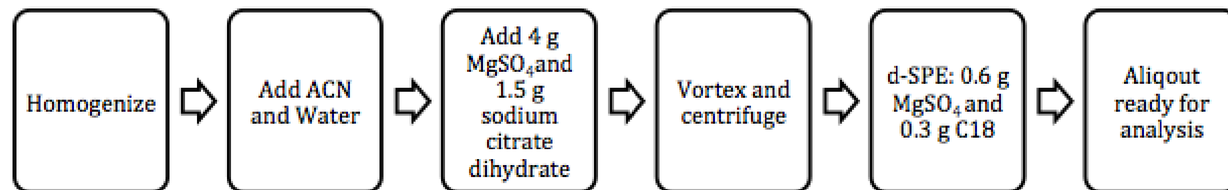


Figure 16. A scheme of the optimum extraction procedure for prothioconazole and tebuconazole.

7.2 Instrumental Analysis

A gas chromatography method was first developed to analyze Prosaro™ residue containing prothioconazole and tebuconazole. GC-MS analysis of the fungicide extracts were performed with an Agilent 7890A Gas Chromatograph coupled to a 5975C Mass Spectrometer. The GC was equipped with a 30 m x 0.25 mm i.d., 0.25 µm film, HP-5MS column. A 2 µL sample was injected in splitless mode. The injection temperature was maintained at 250 °C. Helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. The oven temperature was maintained at 150 °C for 1 min after the injection and then programmed to linearly increase 60 °C min^{-1} to 270 °C, then to linearly increase 30 °C min^{-1} to 300 °C, and was held at 300 °C for 5 min for a total run time of 14 min. The MS source temperature was maintained at 230 °C. Due to low detection limits of prothioconazole, a method using selected ion monitoring (SIM) mode was also used to measure prothioconazole.

It was determined that prothioconazole had a poor detection limit on the GC-MS, therefore an HPLC method was also developed to analyze prothioconazole and tebuconazole residues. This method was developed by a univariate method development approach. A 250 ppm standard of prothioconazole and tebuconazole in acetonitrile was used for all measurements. An initial measurement included using a method from the literature.[38] Mobile phase A was 10% (v/v) acetonitrile in water and mobile phase B was acetonitrile, both containing

0.1% formic acid. A linear gradient was used from 20% B to 100% B in 10 min and held for 5 min, then lowered to 20% B in 5 min and re-equilibrated for 5 min. The flow rate used in the literature was 0.2 mL min^{-1}, however for the instrument used in this research the flow rate was programmed to 1.00 mL min^{-1}. The chromatogram of the initial measurement is included in Figure 17.

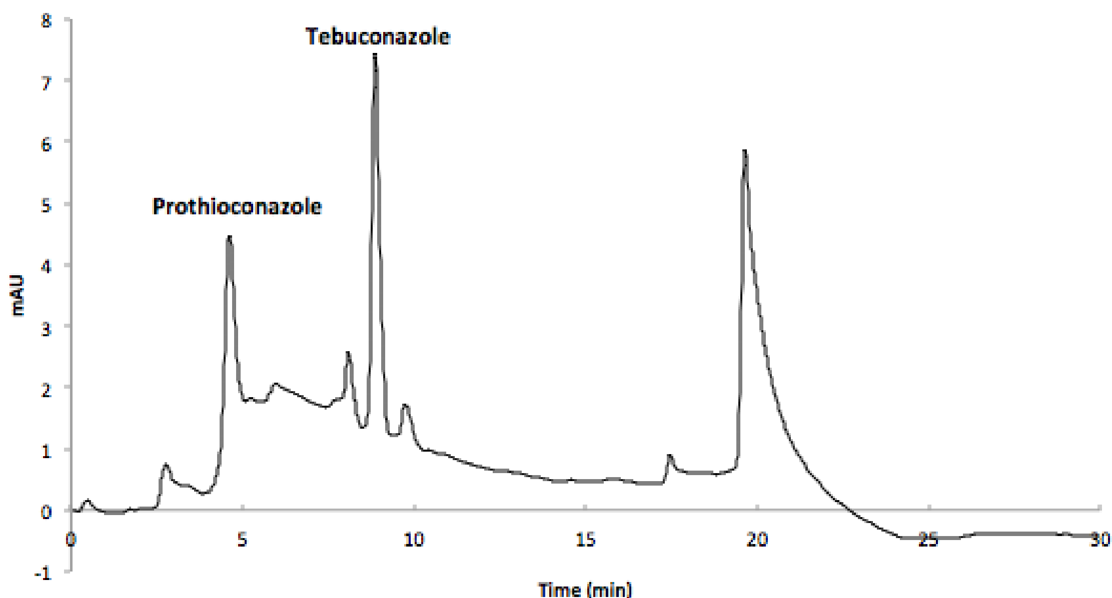


Figure 17. Fungicide HPLC method development: initial measurement of 250 ppm standard of prothioconazole and tebuconazole in acetonitrile. A linear gradient with 10% (v/v) acetonitrile in water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) was used beginning with 20% B to 100% B in 10 min and held for 5 min, then lowered to 20% B in 5 min and re-equilibrated for 5 min. The flow rate was 1.00 mL min^{-1}. The column temperature was 25 °C and detector wavelength was 280 nm.

7.2.1 Gradient

The first variable altered in the method development stages was the gradient. The initial gradient was 20% B to 100% B in 10 min and held for 5 min, then lowered to 20% B in 5 min and re-equilibrated for 5 min. The gradient was altered to allow 5 additional minutes of 100% B, holding 10 min rather than 5 min. The altered gradient was 20% B to 100% B

in 10 min and held for 10 min, then lowered to 20% B in 5 min and re-equilibrated for 5 min. Increasing the hold time improved the chromatogram, Figure 18, by lowering the baseline as well as better separation of components.

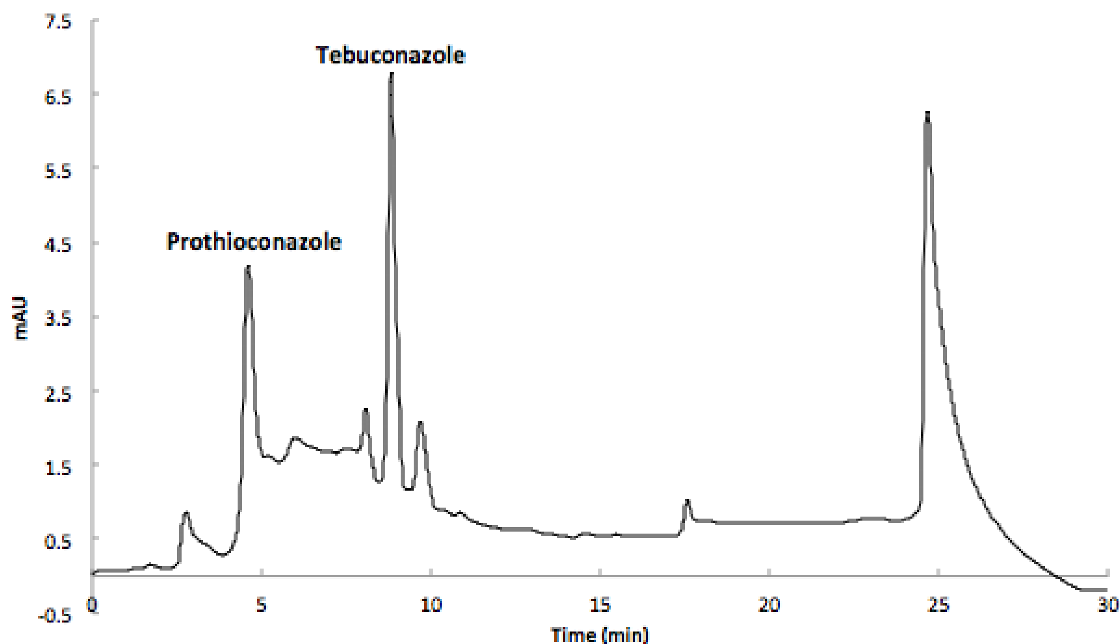


Figure 18. Fungicide HPLC method development: gradient altered to additional 5 min hold time of 100% B from 10 to 20 min to measure 250 ppm standard of prothioconazole and tebuconazole in acetonitrile. A gradient with 10% (v/v) acetonitrile in water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) was used beginning with 20% B to 100% B in 10 min and held for 10 min, then lowered to 20% B in 5 min and re-equilibrated for 5 min. The flow rate was 1.00 mL min^{-1}. The column temperature was 25 °C and detector wavelength was 280 nm.

7.2.2 Flow Rate

The next variable altered was the flow rate. The flow rate was decreased from 1 mL min^{-1} to 0.800 mL min^{-1}. Decreasing the flow rate simply shifted everything to the right, slightly increasing retention times.

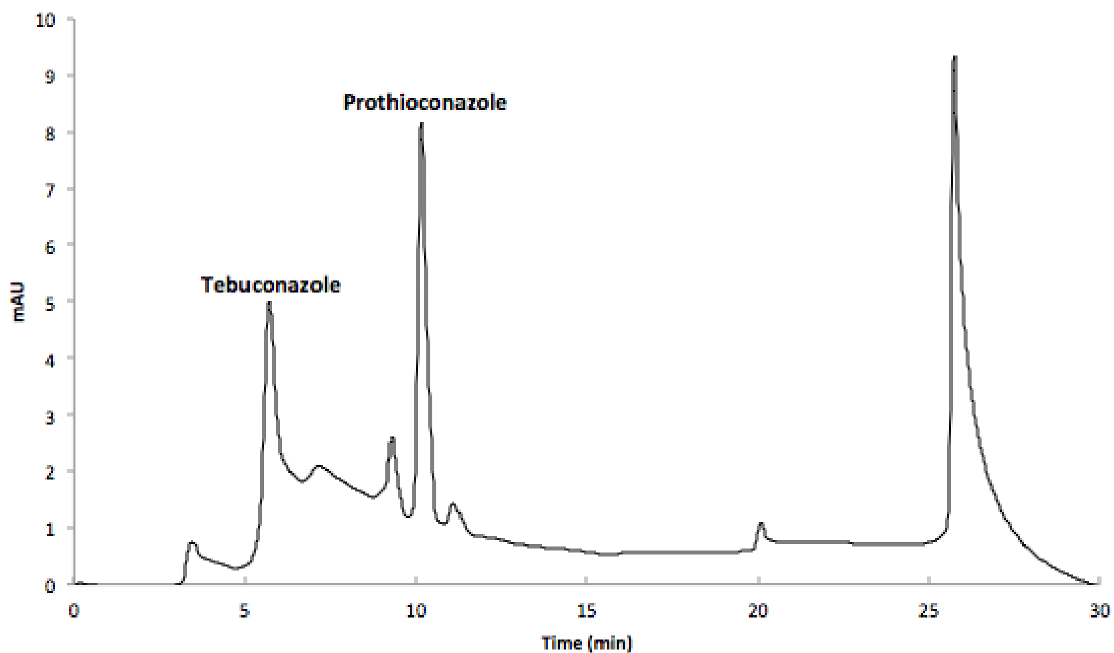


Figure 19. Fungicide HPLC method development: flow rate decreased from 1 mL min^{-1} to 0.800 mL min^{-1} to measure 250 ppm standard of prothioconazole and tebuconazole in acetonitrile. A gradient with 10% (v/v) acetonitrile in water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) was used beginning with 20% B to 100% B in 10 min and held for 10 min, then lowered to 20% B in 5 min and re-equilibrated for 5 min. The flow rate was 0.800 mL min^{-1}. The column temperature was 25 °C and detector wavelength was 280 nm.

7.2.3 Mobile Phases

The mobile phases were changed from 10% (v/v) acetonitrile in water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) to water (A) and acetonitrile (B). Figure 20 shows the chromatogram of a 250 ppm prothioconazole and tebuconazole standard diluted in acetonitrile analyzed using mobile phases without formic acid.

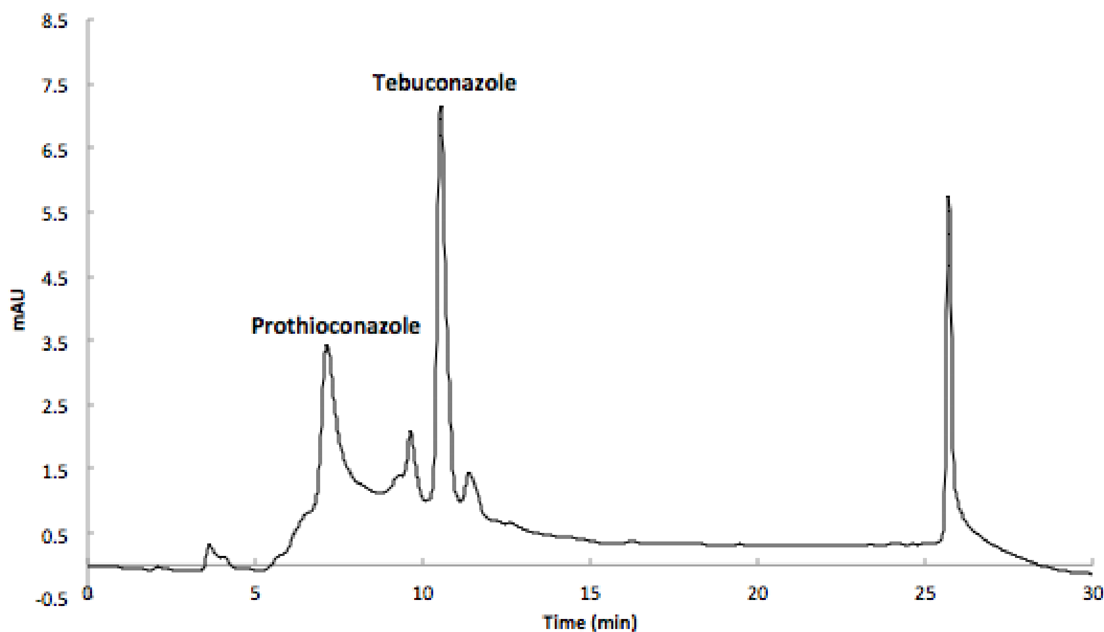


Figure 20. Fungicide HPLC method development: formic acid removed from mobile phases to measure 250 ppm standard of prothioconazole and tebuconazole in acetonitrile. A gradient with water (A) and acetonitrile (B) was used beginning with 20% B to 100% B in 10 min and held for 10 min, then lowered to 20% B in 5 min and re-equilibrated for 5 min. The flow rate was 0.800 mL min^{-1}. The column temperature was 25 °C and detector wavelength was 280 nm.

7.2.4 Detection Wavelength

The detection wavelength was programmed to 280 nm and was not altered during HPLC method development.

7.2.5 Temperature

The temperature was programmed to 40 °C and was not altered during HPLC method development.

7.2.6 Final Instrument Parameters

An isocratic elution was used with mobile phase A as water and mobile phase B as acetonitrile. The composition of the mobile phases were 20% A and 80% B for 10 min. The injection volume and flow rate were 10 µL and 0.800 mL min^{-1}. The column temperature was maintained at 40 °C.The detector was programmed to a wavelength of 280 nm. A chromatogram of prothioconazole and tebuconazole standards in acetonitrile analyzed by this final method is included in Figure 21.

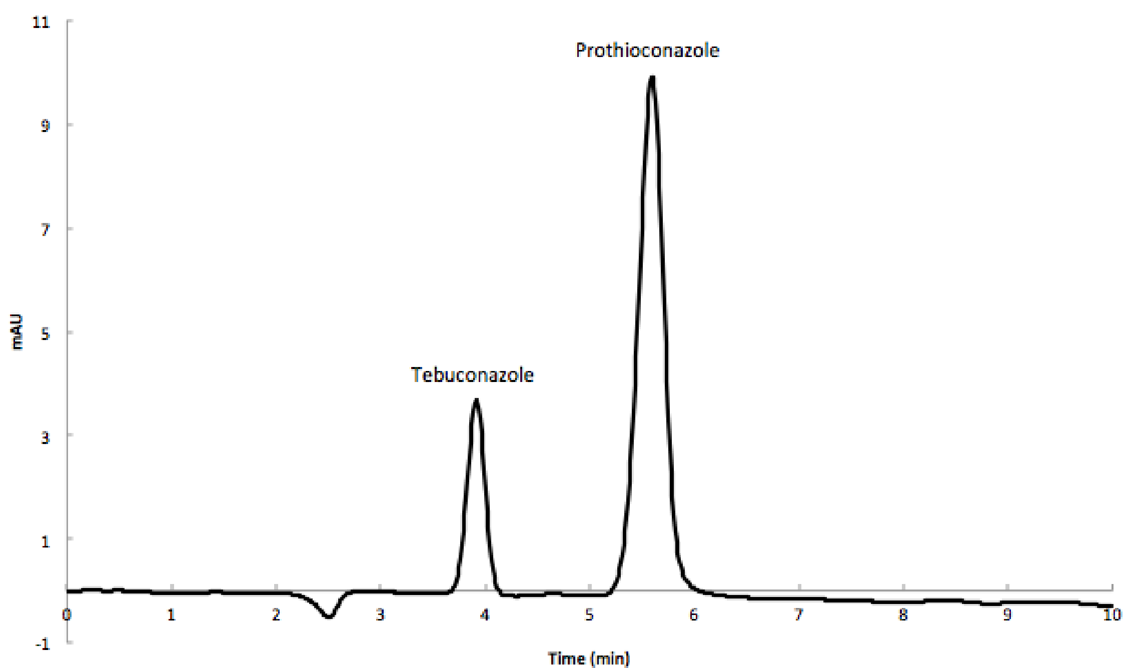


Figure 21. Fungicide HPLC final method: isocratic elution of 20% water and 80% acetonitrile with an injection volume and flow rate of 10 µL and 0.800 mL min^{-1}. A column temperature of 40 °C and detector wavelength of 280 nm.

CHAPTER EIGHT: RESULTS

8.1 Sprayability and Plant Coverage

Sprayability of the stable emulsion was tested and compared to other 5% tannic acid wax emulsions by visual examination. The formulations for the emulsions varied in their percentage of soy wax and/or vegetable oil. The ideal emulsion will spray through a nozzle to achieve good coverage but not immediately drip off of the plant. A mist spray results in optimum coverage of the plants, whereas a stream spray results in minimum coverage. Table 17 list the formulations of each emulsion used for sprayability and plant coverage comparison, as well as the formulation used as treatment for 2015 field trials. The emulsion used for 2015 treatments used Span60 as the emulsifier instead of TEA stearate. This emulsion also differed from the 2016 emulsion in percentage of soy wax, vegetable oil, tannic acid, and guar gum. Figures 22 and 23 show each of the 2016 emulsions being sprayed through a spray nozzle, as well as the plant coverage for each. Emulsion 4 was the only formulation able to achieve a mist spray for optimum plant coverage. This formulation was used for the 2016 barley plot trials, as well as the studies to determine the length of time that tannic acid remains on the surface of the plant.

Table 17. Formulations of tannic acid emulsions for sprayability and plant coverage comparison. Emulsion 4 was used for 2016 field trials.

Percent Composition

2015		**2016**	**1**	**2**	**3**	**4**
Soy wax	1	Soy Wax	3	5	5	3
Vegetable Oil	5	Vegetable Oil	2.5	6	2.5	6
Span60	2	TEA Stearate	3	3	3	3
Tannic Acid	1 or 3	Tannic Acid	5	5	5	5
Guar Gum	0.75	Guar Gum	0.5	0.5	0.5	0.5

(a) Emulsion 1

(b) Emulsion 1

(c) Emulsion 2

(d) Emulsion 2

Figure 22. Comparison of sprayability and plant coverage of emulsions 1 and 2 described in Table 17.

(a) Emulsion 3

(b) Emulsion 3

(c) Emulsion 4

(d) Emulsion 4

Figure 23. Comparison of sprayability and plant coverage of emulsions 3 and 4 described in Table 17.

8.2 Barley Plot Trials

8.2.1 Effectiveness Data

For each plot, all of the grain was harvested, weighed, and sub-sampled for mycotoxin determinations to examine the effectiveness of the biopesticide. An outside party performed DON analysis. The lower the concentration of DON in the sample, or the less infected the sample, the more effective the treatment was. In 2015, three trials were conducted, including a greenhouse trial and two field trials, Inwood and Caswell. Endeavor and Nomini were grown in the Inwood field and Endeavor, Nomini, Atlantic, and Thoroughbred were grown in the Caswell field. In all trials barley was treated with either a 1% or 3% tannic acid emulsion. The formulations are included in Table 17.

The average concentration of DON for each treatment for the Inwood field is included is Table 18. The data were also analyzed by statistical analysis software, SAS, with DON as the dependent variable. Table 19 includes the type III tests of fixed effects, which include hypothesis tests for the significance of each of the fixed effects. Num DF is the number of degrees of freedom of the effect, variety and treatment. Den DF is the number of degrees of freedom associated with model errors. The F value is the statistic for the given predictor and text statistic. The Pr > F value is the p-value associated with the F statistic. There is a significant difference in DON concentration among the varieties since the p-value is less than 0.05. However, there is no significant difference in DON concentration among the treatments. The high p-value for variety*treatment means that there is no significant difference in treatment across varieties. Table 20 includes the least square means for each effect. The variety Endeavor has a very low DON concentration, estimate, compared to Nomini, meaning that Endeavor was less infected. The untreated barley has the highest DON concentration, making it appear that it was more infected than the treated barley. However there is no significant difference between the treatments, shown in Table 19.

Table 18. Concentration of DON in ppm for Inwood field after treatment with 1% and 3% emulsions in 2015.[4] The uncertainty is the standard deviation of averages of DON concentrations for each treatment.

Inwood: DON Concentration (ppm)

Cultivar	Untreated Control	1% Treatment	3% Treatment
Endeavor	0.34 ± 0.021	0.62 ± 0.30	0.64 ± 0.40
Nomini	7.1 ± 1.4	5.6 ± 2.2	4.4 ± 1.3

Table 19. SAS type III tests of fixed effects for Inwood field.

Inwood: Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F value	Pr > F
Variety	1	11	70.58	<.0001
Treatment	2	11	1.22	0.3315
Variety*Treatment	2	11	1.93	0.1914

Table 20. SAS least square means for Inwood field. The estimates for variety include all treatments and the estimates for treatment include all varieties.

Inwood: Least Square Means

Effect	Variety	Treatment	Estimate
variety	Endeavor		0.5306
variety	Nomini		5.6889
treatment		1%	2.5200
treatment		3%	3.0917
treatment		untreated	3.7175

The average concentration of DON for each treatment, by variety, for the Caswell field is included is Table 21. Table 22 includes the type III tests of fixed effects, which include hypothesis tests for the significance of each of the fixed effects. There is a significant difference in DON concentration among the varieties since the p-value, 0.0006, is less than 0.05. However, there is no significant difference in DON concentration among the treatments. The high p-value for variety*treatment means that there is no interaction, or significant difference, in treatment across varieties. Table 23 includes the least square means for each effect. The estimate is the average DON concentration in ppm. The variety Endeavor is the least infected, followed by Thoroughbred, Atlantic, and lastly Nomini. There is no significant difference in DON concentration between Endeavor and Thoroughbred and between Atlantic and Nomini. Like the Inwood field, the untreated barley has the highest DON concentration of the treatments, making it seem that the treated barley was less infected than the untreated. However there is no significant difference between the three treatments, shown in Table 22.

Table 21. Concentration of DON in ppm for Caswell field after treatment with 1% and 3% emulsions in 2015.[4] The uncertainty is the standard deviation of averages of DON concentrations for each treatment.

Caswell: DON Concentration (ppm)

Cultivar	**Untreated Control**	**1% Treatment**	**3% Treatment**
Atlantic	7.0 ± 2.6	4.9 ± 2.7	5.2 ± 1.6
Endeavor	$2.3 \pm$ NA	1.5 ± 1.6	1.0 ± 0.53
Nomini	8.4 ± 2.7	8.3 ± 1.8	7.6 ± 1.9
Thoroughbred	$2.5 \pm$ NA	2.4 ± 0.071	1.6 ± 0.21

The greenhouse results from 2015 are included in Table 24. The average concentration of DON appears to decrease with the use of tannic acid. However, when considering the standard deviation, there is no significant difference in DON concentration between the treatments.

Table 22. SAS type III tests of fixed effects for Caswell field.

Caswell: Type III Tests of Fixed Effects

Effect	**Num DF**	**Den DF**	**F value**	**Pr > F**
Variety	3	15	10.43	0.0006
Treatment	2	15	0.15	0.8593
Variety*Treatment	6	15	0.78	0.5975

Table 23. SAS least square means for Caswell field. The estimates for variety include all treatments and the estimates for treatment include all varieties.

Caswell: Least Square Means

Effect	**Variety**	**Treatment**	**Estimate**
variety	Atlantic		5.7000
variety	Endeavor		1.6217
variety	Nomini		7.1667
variety	Thoroughbred		2.1333
treatment		1%	4.2808
treatment		3%	3.8437
treatment		untreated	4.3417

Table 24. Concentration of DON in ppm after treatment of greenhouse plants with 1% and 3% emulsions in 2015.[4] The uncertainty is the standard deviation of averages of DON concentrations for each treatment.

Treatment	**DON concentration (ppm)**
Untreated Control	30.7 ± 24.5
1%	30.4 ± 24.1
3%	17.7 ± 8.24

Field trials were also conducted in 2016 with Endeavor, Atlantic, Nomini, and Thoroughbred barley. Four treatments were compared, as well as untreated. These treatments included 5% tannic acid treated once (T1) and twice (T2), as well as Prosaro™ treated on time (P1) and late (P2). The average concentration of DON for each treatment is included is Table 25. Table 26 includes the type III tests of fixed effects, which include hypothesis tests for the significance of each of the fixed effects. There is a significant difference in DON concentration among the varieties since the Pr > F value, 0.0390, is less than 0.05. There is also a significant difference in DON concentration among the treatments, however this value includes the Prosaro™ results. The high p-value for variety*treatment means that there is no interaction, or significant difference, in treatment across varieties. Table 27 includes the least square means for each effect. The estimate is the average DON concentration in ppm. The variety Endeavor is the least infected, followed by Nomini, Thoroughbred and lastly Atlantic. There is a significant difference in DON concentration between Endeavor, the least infected, and Atlantic, the most infected. Prosaro™ treated barley was the least infected followed by the tannic acid treatments and untreated. The only significant difference between treatments was P2, it was significantly different than both tannic acid treatments and the untreated barley. The tannic acid treated barley is not significantly different than the untreated.

Table 25. Concentration of DON in ppm after treatment with 5% emulsion in 2016.[4] The uncertainty is the standard deviation of averages of DON concentrations for each treatment.

DON Concentration (ppm)

Cultivar	**Untreated Control**	**T1**	**T2**	**P1**	**P2**
Atlantic	2.7 ± 0.54	2.5 ± 1.1	3.4 ± 1.4	1.8 ± 0.91	1.5 ± 0.57
Endeavor	1.3 ± 0.59	1.5 ± 0.55	1.2 ± 0.49	1.3 ± 0.27	0.58 ± 0.27
Nomini	1.8 ± 0.76	1.5 ± 0.57	1.8 ± 1.2	1.6 ± 0.52	1.2 ± 0.62
Thoroughbred	1.7 ± 0.31	1.9 ± 1.4	1.9 ± 1.2	1.6 ± 1.2	0.90 ± 0.11

Table 26. SAS type III tests of fixed effects for 2016 field trials.

2016: Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F value	Pr > F
Variety	3	9	10.43	0.0390
Treatment	4	15	48	0.0012
Variety*Treatment	12	48	0.79	0.6607

Table 27. SAS least square means for 2016 field trials. The estimates for variety include all treatments and the estimates for treatment include all varieties.

2016: Least Square Means

Effect	Variety	Treatment	Estimate
variety	Atlantic		2.3815
variety	Endeavor		1.1585
variety	Nomini		1.5565
variety	Thoroughbred		1.5890
treatment		P1	1.5594
treatment		P2	1.0612
treatment		T1	1.8150
treatment		T2	2.0650
treatment		untreated	1.8562

8.2.2 Tannic Acid Residue Extracts

Barley grain samples were analyzed for tannic acid residues or prothioconazole and tebuconazole residues. It was hypothesized that little to no residue would be present on the grain since the treatment occurred before the head of the barley was fully emerged.

The previously developed optimum QuEChERS extraction method for tannic acid was used on the barley samples treated with the tannic acid biopesticide and the extracts were analyzed by the HPLC method for tannic acid. Each analysis began with a calibration of fresh standards. Figure 24 shows chromatograms of tannic acid calibration standards ranging from 0.75 ppm to 100 ppm. After analysis of all samples treated with tannic acid, it was determined that no tannic acid residue was present on the barley grain. A small peak with the same retention time of tannic acid was present in all samples, including the untreated samples. This peak may be contributed from tannic acid that naturally occurs in the barley grain.

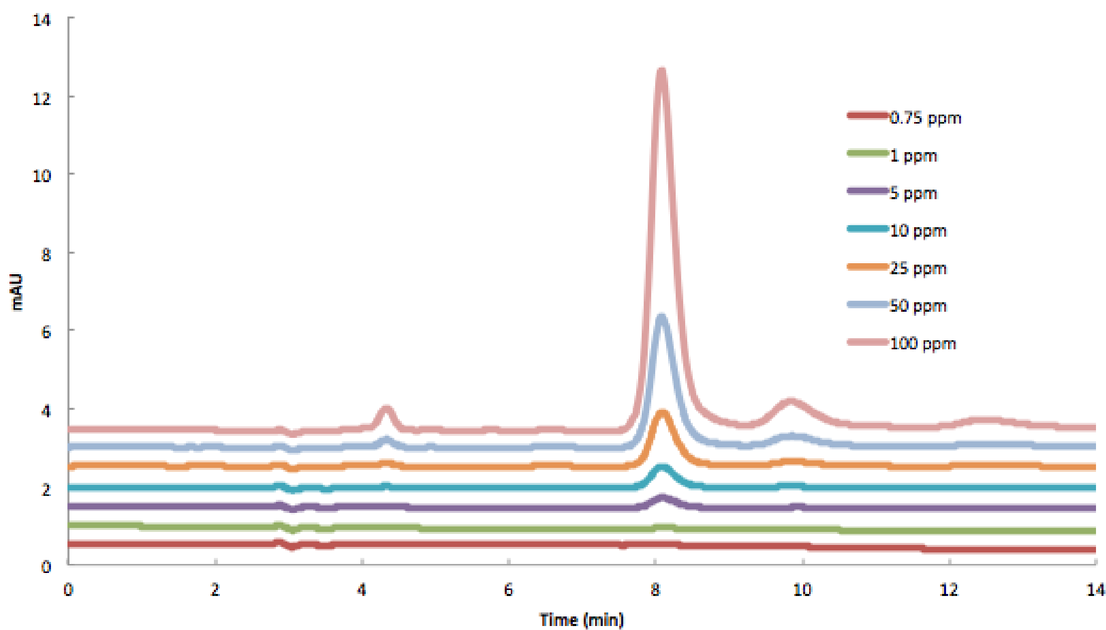


Figure 24. Chromatogram of tannic acid calibration standards offset by 0.5 mAU.

8.2.3 Fungicide Residue Extracts

The previously developed optimum QuEChERS method for prothioconazole and tebuconazole was used on the barley samples treated with the Prosaro fungicide. Extracts were analyzed by the HPLC method for prothioconazole and tebuconazole analysis. Each analysis began with a calibration of fresh standards. Figure 25 shows chromatograms of prothioconazole and tebuconazole calibration standards ranging from 15.625 ppm to 250 ppm. The results supported the hypothesis that little to no fungicide residue was detected on the grain.

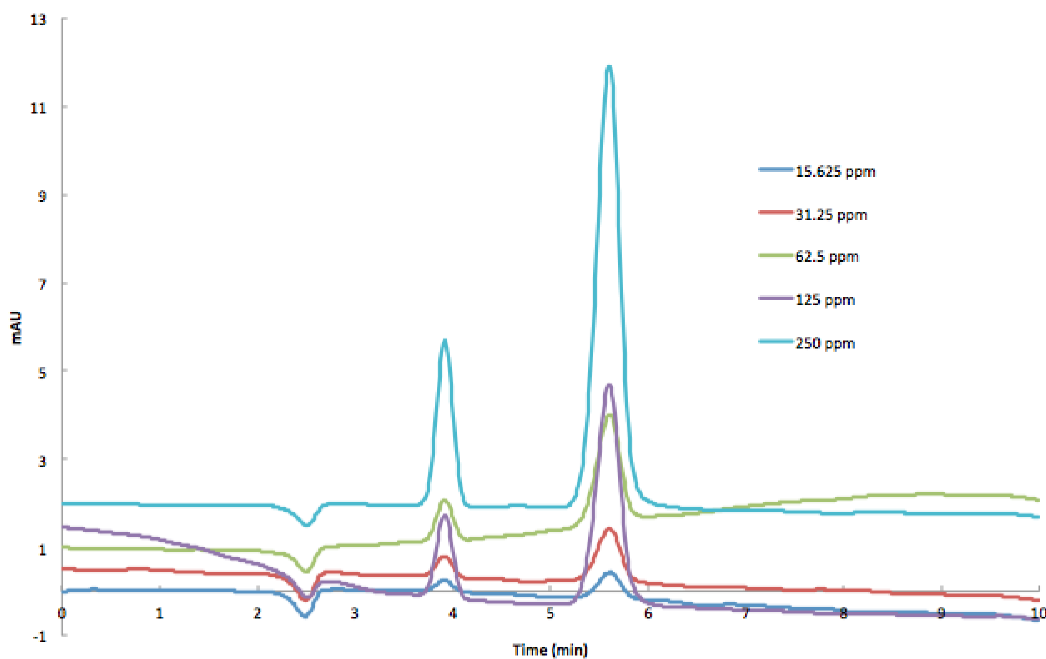


Figure 25. Chromatogram of prothioconazole and tebuconazole calibration standards offset by 0.5 mAU. The standard contains the reported concentration of both compounds.

8.3 Longetivity of Biopesticide After Application

After treatment with the tannic acid biopesticide, samples were taken from *Euonymus alata* and *Liriope muscari* over a period of three or four weeks to determine how long the biopesticide adheres to the plant surface, and how the weather affects it, including whether or

not the biopesticide is rainfast. The following sections include the results from each plant. Samples were taken in triplicate, and each sample measured in duplicate. The areas were averaged and the concentration was calculated using the equation from the calibration curve. The concentration was multiplied by 100 mL to determine the mass of tannic acid in the 100 mL of solvent. This value was divided by the total mass of the sample to determine the micrograms of tannic acid per gram of plant material. The results for the three samples were averaged and are reported in the following sections.

8.3.1 *Euonymus alata*

Figure 26 shows the treated *Euonymus alata* on the day of treatment. The results for *Euonymus alata* are listed in Table 28 as residuals remaining on plant surface after specified days since treatment. A graph of the data is included in Figure 27. Tannic acid decreases linearly during the first week, and then plateaus during the second and third weeks. The decrease in residual from day 5 to day 7 is most likely caused by the 1.09 in of rain on day 6. The tannic acid was present on the surface of the treated plant up to 3 weeks. This time period is ideal, considering the plant only needs to be protected during a specific growth stage lasting approximately 10 days. There would be little to no tannic acid present on the grain at harvest.

8.3.2 *Liriope muscari*

Figure 29 shows the treated *Liriope muscari* on the day of treatment. The results for *Liriope muscari* are listed in Table 29. A graph of the data is included in Figure 29. The significant decrease between the day of treatment and day one is most likely due to the 1.09 in of rain on the day of treatment. After the initial decrease caused by rain, the presence of tannic acid decreases linearly from day 1 to 28. After 4 weeks tannic acid was still present on the plant surface.

Figure 26. Tannic acid treated *Euonymus alata*.

Table 28. Tannic acid residuals for *Euonymus alata* over a period of three weeks. The uncertainty is the standard deviation of averages of μg TA/g plant from triplicate samples.

Date	**Days Since Treatment**	**μg/g**
Aug. 26	0	12660 ± 1756
Aug. 27	1	10350 ± 1813
Aug. 29	3	6557 ± 1411
Aug. 31	5	6093 ± 915.7
Sept. 2	7	158.9 ± 9.267
Sept. 6	11	40.16 ± 16.84
Sept. 9	14	232.7 ± 23.98
Sept. 16	21	26.16 ± 45.31

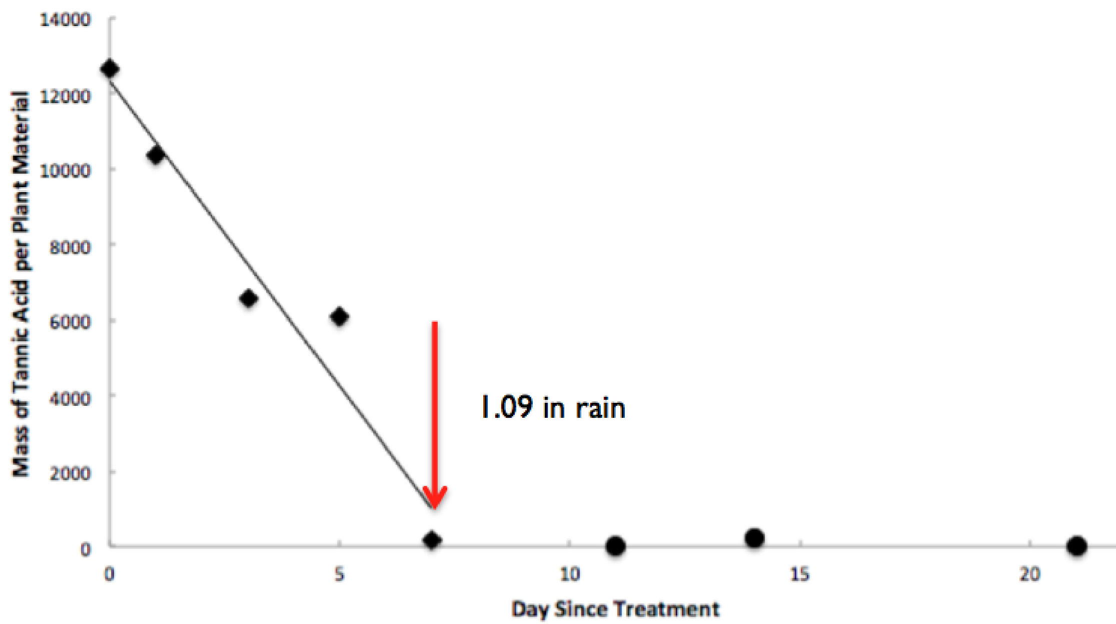


Figure 27. Tannic acid treated *Euonymus alata* results with a coefficient of determination of 0.94 for days 0 through 7.

Figure 28. Tannic acid treated *Liriope muscari*.

Table 29. Tannic acid residuals for *Liriope muscari* over a period of four weeks. The uncertainty is the standard deviation of averages of μg TA/g plant from triplicate samples.

Date	**Days Since Treatment**	**μg TA/g plant**
Sept. 1	0	11450 ± 791.6
Sept. 2	1	1646 ± 33.04
Sept. 6	5	2007 ± 302.4
Sept. 8	7	1515 ± 240.5
Sept. 15	14	924.6 ± 102.4
Sept. 22	21	206.2 ± 131.9
Sept. 29	28	124.7 ± 24.13

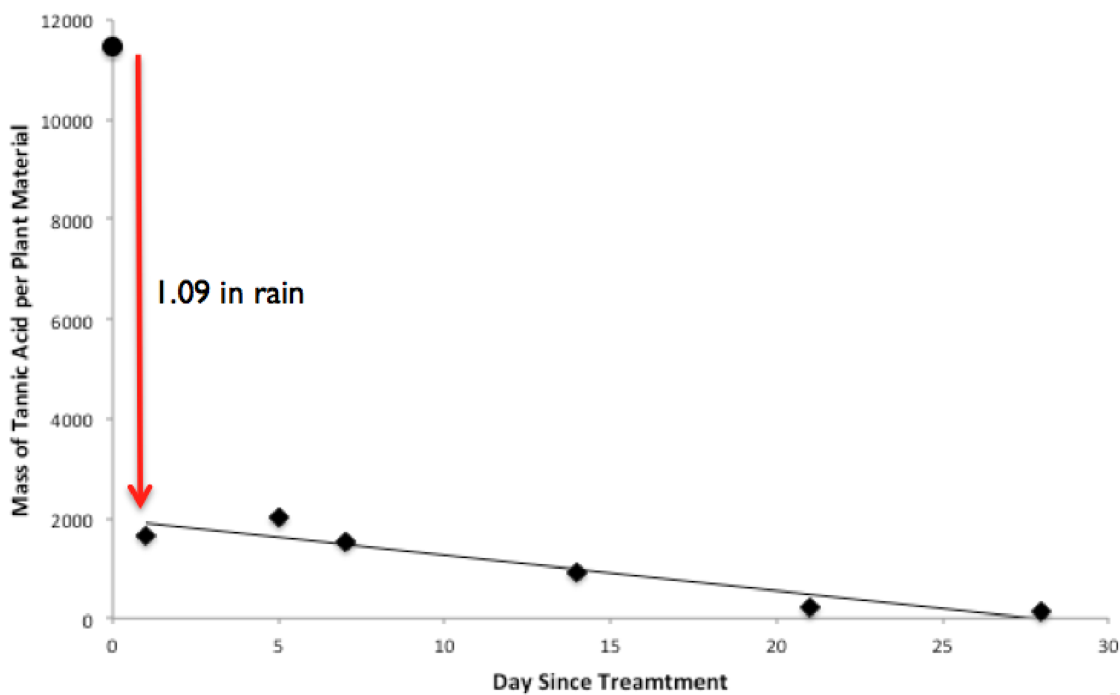


Figure 29. Tannic acid treated *Liriope muscari* results with a coefficient of determination of 0.97 for days 1 through 28.

CHAPTER NINE: CONCLUSION

The purpose of this research was to compare a tannic acid biopesticide with a commercial fungicide that is used for crop protection against *Fusarium* head blight. The commercial fungicide examined was Prosaro^{TM}, containing prothioconazole and tebuconazole. Analytical methods were developed to extract and analyze tannic acid or prothioconazole and tebuconazole residue. The original QuEChERS method was modified to achieve the highest percent recovery for the tannic acid and the commercial fungicide compounds. HPLC was used for analysis of the residue extracts. Optimum instrumental parameters were developed by altering one parameter at a time until a clean chromatogram was achieved.

Using the developed methods, the application and effectiveness of the tannic acid biopesticide were examined. Barley plots were treated with either the tannic acid biopesticide or the commercial fungicide, and the grain was harvested and analyzed for residuals. The barley was treated once the heads were just fully emerged, but before grain formation. Since the grain was not treated directly, it was hypothesized that little to no residue would be present on the grain. After the grain was analyzed, this hypothesis was supported. There was little to no residue of tannic acid or prothioconazole and tebuconazole present on the grain samples from each plot.

The effectiveness of the tannic acid biopesticide was determined by analysis of DON on the grain samples. Deoxynivalenol, DON, is produced from the *Fusarium* fungus. Therefore, a low DON concentration means the sample was slightly infected, whereas a high DON concentration means the sample was more infected. An outside party performed these analyses. The average DON concentrations of the treatments seem to confirm that tannic acid treated barley is less infected than untreated barley. However, SAS analysis confirms that there is no significant difference in DON concentration of tannic acid treated barley and untreated barley.

Plants were also treated to determine how long the wax emulsion holds the tannic acid on the surface of the plant. *Euonymus alata* and *Liriope muscari* were the plants treated for this study. Tannic acid was extracted from the surface of the plants on the day of treatment and over a period of three or four weeks. Tannic acid was present on *Euonymus alata* up to three weeks and on *Liriope muscari* for up to four weeks. The difference in time may have been caused by exposure to weather, such as as temperature and precipitation. This time period is ideal since the plant only needs to be protected during a specific growth period lasting approximately ten days. This study also determined that the emulsion is not completely rainfast. After one inch of rainfall the amount of tannic acid on the plant significantly decreased. Overall, this research suggests promising results for tannic acid to be effective in inhibiting *Fusarium* head blight, however, much further research is required.

CHAPTER TEN: FUTURE RESEARCH

There are several areas of interest that could be further researched. The tannic acid wax emulsion could be further explored to achieve a stable formulation with a higher concentration of tannic acid as well as an emulsion that is more rainfast and able to hold the tannic acid to the crop longer. Also, determining the concentration of tannic acid needed, as well as the coverage needed, on the crop to prevent FHB could be researched. The previous research examined the sprayability of various emulsions with a simple household spray nozzle, however further research on application methods would be useful. Application methods, such as a pressurized spray nozzle, would apply better coverage on the crop. This type of nozzle would also be able to achieve a mist spray with a thicker emulsion containing more wax, allowing the treatment to be more rainfast.

Continued research on optimizing the extraction method could also be conducted. This research determined that the use of sodium citrate dibasic sesquihydrate in the partitioning and buffer extraction step significantly increased the percent recoveries of tannic acid, tebuconazole, and prothioconazole. Further trials can be examined with the use of sodium citrate dibasic sesquihydrate to achieve a better extraction procedure. Longevity studies should be conducted on barley samples to determine how long tannic acid is held to the surface.

REFERENCES

[1] Haynes, W. M., Ed. *Handbook of Chemistry and Physics*, 97th ed.; CRC Press, 2016.

[2] MacBean, C., Ed. *The Pesticide Manual*, sixteenth ed.; BCPC: Alton, 2012; pp 956–958, 1063–1065.

[3] *Record of Climatological Observations*; 2016.

[4] Cowger, C. personal communication.

[5] Forrer, H.-R.; Musa, T.; Schwab, F.; Jenny, E.; Bucheli, T.; Wettstein, F.; Vogelgsang, S. Fusarium head blight control and prevention of mycotoxin contamination in wheat with botanicals and tannic acid. *Toxins* **2014**, *6*, 830–849.

[6] EPA, What are biopesticides? 2016; https://www.epa.gov/ingredients-used-pesticide-products/what-are-biopesticides.

[7] Seiber, J. N.; Coats, J.; Duke, S. O.; Gross, A. D. Biopesticides: state of the art and future opportunities. *Journal of Agricultural and Food Chemistry* **2014**, *62*, 11613–11619.

[8] Cantrell, C. L.; Dayan, F. E.; Duke, S. O. Natural products as sources for new pesticides. *Journal of Natural Products* **2012**, *75*, 1231–1242.

[9] Kaushik, G.; Satya, S.; Naik, S. N. Food processing a tool to pesticide residue dissipation - A review. *Food Research International* **2009**, *42*, 26–40.

[10] Osborne, L. E.; Stein, J. M. Epidemiology of Fusarium head blight on small-grain cereals. *International Journal of Food Microbiology* **2007**, *119*, 103–108.

[11] Grafenhan, T.; Patrick, S. K.; Roscoe, M.; Trelka, R.; Gaba, D.; Chan, J. M.; Mckendry, T.; Clear, R. M.; Tittlemier, S. A. Fusarium damage in cereal grains from western Canada. 1. Phylogenetic analysis of moniliformin-producing Fusarium species and their natural occurrence in mycotoxin-contaminated wheat, oats, and rye. *Journal of Agricultural and Food Chemistry* **2013**, *61*, 5425–5437.

[12] Tittlemier, S. A.; Roscoe, M.; Trelka, R.; Gaba, D.; Chan, J. M.; Patrick, S. K.; Sulyok, M.; Krska, R.; Mckendry, T.; Gra, T. Fusarium damage in small cereal grains from western Canada. 2. Occurrence of Fusarium toxins and their source organisms in durum wheat harvested in 2010 . *Journal of Agricultural and Food Chemistry* **2013**, *61*, 5438–5448.

[13] Lin, T.; Wang, G.; Zhou, Y.; Zeng, D.; Liu, X.; Ding, R.; Jiang, X.; Zhu, D.; Shan, W.; Chen, H. Structure elucidation and biological activity of two new trichothecenes from an endophyte, myrothecium roridum. *Journal of Agricultural and Food Chemistry* **2014**, *62*, 5993–6000.

[14] Sobrova, P.; Adam, V.; Vasatkova, A.; Beklova, M.; Zeman, L.; Kizek, R. Deoxynivalenol and its toxicity. *Interdisciplinary Toxicology* **2010**, *3*, 94–99.

[15] Wilson, C. L., Wilson, D. W., Eds. *Comprehensive Analytical Chemistry*; Elsevier Publishing Company: Amsterdam, 1968.

[16] Skoog, D. A.; Holler, F. J.; Crouch, S. R. *Principles of Instrumental Analysis*, sixth ed.; Cenage Learning: Andover, 2007.

[17] Vernhet, A.; Dubascoux, S.; Cabane, B.; Fulcrand, H.; Dubreucq, E.; Poncet-Legrand, C. Characterization of oxidized tannins: comparison of depolymerization methods, asymmetric flow field-flow fractionation and small-angle x-ray scattering. *Analytical and Bioanalytical Chemistry* **2011**, *401*, 1559–1569.

[18] Pérez-Jiménez, J.; Neveu, V.; Vos, F.; Scalbert, A. Systematic analysis of the content of 502 polyphenols in 452 foods and beverages: an application of the phenol-explorer database. *Journal of Agricultural and Food Chemistry* **2010**, *58*, 4959–4969.

[19] Dai, J.; Mumper, R. J. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules* **2010**, *15*, 7313–7352.

[20] Holtekjølen, A. K.; Kinitz, C.; Knutsen, S. H. Flavanol and bound phenolic acid contents in different barley varieties. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 2253–2260.

[21] Kandil, A.; Li, J.; Vasanthan, T.; Bressler, D. C. Phenolic acids in some cereal grains and their inhibitory effect on starch liquefaction and saccharification. *Journal of Agricultural and Food Chemistry* **2012**, *60*, 8444–8449.

[22] Yu, J.; Vasanthan, T.; Temelli, F. Analysis of phenolic acids in barley by high-performance liquid chromatography. *Journal of agricultural and food chemistry* **2001**, *49*, 4352–4358.

[23] Yao, L.; Lio, J.; Wang, T.; Jarboe, D. H. Synthesis and characterization of acetylated and stearylyzed soy wax. *J. Am. Oil Chem. Soc.* **2013**, *90*, 1063–1071.

[24] Ballew, S. D. Rheological characteristics of aqueous wax emulsions used for the controlled release of pheromones as an alternative to the use of pesticides for insect pest managment. M.Sc. thesis, Western Carolina University, Cullowhee, NC USA, 2011.

[25] Furukawa, Y.; Reed, A. H. Effect of organic matter on estuarine flocculation: a laboratory study using montmorillonite, humic acid, xanthan gum, guar gum and natural estuarine flocs. *Geochemical Transactions* **2014**, *15*.

[26] Pscheidt, J. W. Fungicide resistance and fungicide groups. 2016; http://pnwhandbooks.org/plantdisease/pesticide-articles/fungicide-resistance-and-fungicide-groups.

[27] Andersen, K. F.; Morris, L.; Derksen, R. D.; Madden, L. V.; Paul, P. A. Rainfastness of prothioconazole + tebuconazole for Fusarium head blight and deoxynivalenol management in soft red winter wheat. *Plant Disease* **2014**, *98*, 1398–1406.

[28] Navarro, S.; Pérez, G.; Navarro, G.; Mena, L.; Vela, N. Influence of fungicide residues on the primary fermentation of young lager beer. *Journal of Agricultural and Food Chemistry* **2007**, *55*, 1295–1300.

[29] Wheeler, W. Role of research and regulation in 50 years of pest management in agriculture. *J. Agric. Food Chem* **2002**, *50*, 4151–4155.

[30] Mathews, K. personal communication.

[31] Walorczyk, S.; Drozdzyriski, D.; Kierzek, R. Determination of pesticide residues in samples of green minor crops by gas chromatography and ultra performance liquid chromatography coupled to tandem quadrupole mass spectrometry. *Talanta* **2015**, *132*, 197–204.

[32] Takatori, S.; Okihashi, M.; Kitagawa, Y.; Fukui, N. Rapid and easy multiresidue method for determination of pesticide residues in foods using gas or liquid chromatography tandem mass spectrometry. *Pesticides - Strategies for Pesticide Analysis* **2005**, *2003*, 19 pp.

[33] Anastassiades, M.; Lehotay, S. J. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and dispersive solid-phase extraction for the determination of pesticide residues in produce. *Journal of AOAC International* **2003**, *86*, 412–431.

[34] Kowalski, J.; Lupo, S.; Cochran, J. Mitigating matrix effects: examination of dilution, QuEChERS, and calibration strategies for LC-MS/MS analysis of pesticide residues in diverse food types. *Restek* **2014**, 1–22.

[35] McMaster, M. C. *HPLC: A Practical User's Guide*, 2nd ed.; John Wiley & Sons, Inc.: Hoboken, 2007.

[36] Agilent Technologies, Agilent 1220 Infinity LC User Manual. 2015.

[37] Biswas, N.; Balac, P.; Narlakanti, S. K.; Haque, E.; Hassan, M. Identification of phenolic compounds in processed cranberries by HPLC method. *Journal of Nutrition & Food Sciences* **2013**, *3*, 1–6.

[38] Lehoczki-krsjak, S. Translocation and degradation of tebuconazole and prothioconazole in wheat following fungicide treatment at flowering. *Pest Management Science* **2013**, *69*, 1216–1224.