Combining QuEChERS and Multi-Dimensional Liquid Chromatography for the Analysis of Fungicides in Produce Items

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Abstract

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The broad term 'pesticide' encompasses a variety of chemicals belonging to subcategories including insecticides, herbicides, and fungicides.[1] The latter, fungicides, is the type of pesticide studied in this thesis research. Despite the beneficial properties of pesticides for regulating crop pests, they have also been shown to threaten the health of human organs and systems.[2] The health concerns of pesticides are widespread and can be both acute and chronic.[2] Acute health effects include irritation and burning of the skin, mouth or nose, and more urgent responses such as impaired cognition and seizures.[1] Potential chronic repercussions include detriment to reproductive, pulmonary, and neurological health.[1] In recent years, pyrimethanil (PYRI), fludioxonil (FLU), boscalid (BOS), and pyraclostrobin (PYRA) were commonly detected in produce items by the Environmental Working Group (EWG).[2]

This research focuses on developing a two-dimensional liquid chromatographic (2D-LC) method for the separation, detection, and quantification of PYRI, FLU, BOS and PYRA in produce items. With this goal in mind, a 2D-LC heart-cutting method was developed, which produced properly resolved, symmetrical and sharp peaks in both separation dimensions. Interday coefficients of variation (CV) for retention times and peak areas ranged from 0.093% – 7.352 % in both dimensions. A modified Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS)

clean-up procedure was employed for the clean-up of produce samples.[5] Second dimension (^{2}D) recoveries were determined to range from 51.3% ± 1.4% to 75.3% ± 2.1% for all analytes. Internal standard calibration was completed for all fungicides using phenanthrene as the internal standard. The coefficient of determination (R^2) for all four calibration curves ranged from 0.990 – 0.992. For ^{2}D analysis, the limits of detection (LOD) ranged from 0.043 – 0.131 mg/mL, while the limits of quantification (LOQ) ranged from 0.143 – 0.436 mg/mL. Using the optimized method, a Nova Scotian and a Peruvian blueberry sample were analyzed.

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List of Abbreviations

PYRI	Pyrimethanil
FLU	Fludioxonil
BOS	Boscalid
PYRA	Pyraclostrobin
IS	Internal Standard
EWG	Environmental Working Group
USDA	United States Department of Agriculture
FDA	Food and Drug Administration
2D-LC	Two-Dimensional Liquid Chromatography
CV	Coefficient of Variation
%RSD	Percent Relative Standard Deviation
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
^{1}D	First Dimension
^{2}D	Second Dimension
R^2	Coefficient of Determination
LOD	Limit of Detection
LOQ	Limit of Quantification
ADI	Acceptable Daily Intake
MRL	Maximum Residue Limit
HPLC	High Performance Liquid Chromatography
UHPLC	Ultra High Performance Liquid Chromatography
mLC-LC	Multiple Heart-Cutting Two-Dimensional Liquid Chromatography
AOAC	Association of Official Agricultural Chemists
ACN	Acetonitrile
MeOH	Methanol
H_2O	Water
FA	Formic Acid
CEN	European Committee for Standardization

d-SPE	Dispersive Solid Phase Extraction
PSA	Primary Secondary Amine
GCB	Graphitized Carbon Black
C18	Octadecyl Silica
SERS	Surface-Enhanced Raman Spectroscopy
LC	Liquid Chromatography
GC	Gas Chromatography
SFC	Supercritical Fluid Chromatography
NP-LC	Normal Phase Liquid Chromatography
RP-LC	Reversed Phase Liquid Chromatography
t_R	Retention Time
t_0	Zero Retention Time
K_C	Distribution Constant
a_A	Activity of Species A
k_a	Retention Factor
t_s	Retention Time of Solute
α	Selectivity Factor
R_s	Resolution
A_s	Peak Asymmetry
N	Theoretical Chromatographic Plates
H	Theoretical Chromatographic Plate Height
w	Peak Width
1D-LC	One-Dimensional Liquid Chromatography
η_c	Peak Capacity
UV-vis	Ultraviolet-Visible Spectroscopy
PTFE	Polytetrafluoroethylene
ME	Matrix Effects
rcf	Relative Centrifugal Force
RRT	Relative Retention Time

Chapter 1: Introduction

1.1: Preamble

Pesticide is a term used to categorize a large group of chemicals employed as plant protection products.[3] Despite the long-standing history of pesticide use by humans dating back to the eighth century BC, there has been a large increase in the number and volume of pesticides introduced in the last 50 years.[1,4] New pesticides are continuously being discovered, and pesticides that are no longer in use can be found in soil or water.[5] The broad term 'pesticide' encompasses a variety of chemicals belonging to subcategories including insecticides, herbicides, and fungicides.[3] The latter, fungicides, is the type of pesticide studied in this thesis.

Despite the beneficial properties of pesticides for regulating crop pests, they have also been shown to act on parts of the human body, sometimes threatening the health of the involved organs or systems.[6] The European Environment Agency assessed this issue in a 2023 report, discussing the various health conditions that can be directly caused by pesticide exposure, and urged for a significant decrease in their use to prevent further contamination.[7] The degree to which a pesticide affects human health depends on several factors including its molecular structure and the sensitivity and immunity of the individual involved. Vulnerable individuals including children, pregnant women, and the elderly are at an increased risk of these deleterious effects.[6,7] The health concerns of pesticides are widespread and can be both acute and chronic.[6] Acute health effects include irritation and burning of the skin, mouth or nose, and more urgent responses such as impaired cognition and seizures. Potential chronic repercussions include detriment to reproductive, pulmonary, and neurological health.[6] Humans can be exposed to pesticides by several exposure

media and biological routes.[8] Potential exposure media include, but are not limited to: soil, water, and food. Biologically, pesticides commonly interact with human physiological systems through inhalation, dermal absorption, and ingestion.

The Environmental Working Group (EWG) is an American non-profit association focused on research and advocacy in environmental sciences and is active in researching the negative effects of pesticide contamination in the environment.[2] The EWG *Shopper's Guide to Pesticides in Produce* is released annually, outlining produce items with high pesticide contamination, using data collected by the United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA).[2,9]As a part of this report, a list titled the Dirty Dozen is created, highlighting the 12 studied produce items with the highest pesticide contamination.[2] Despite the hundreds of pesticides detected among these 12 produce items, four out of five of the most frequently detected pesticides were fungicides, including pyrimethanil (PYRI), fludioxonil (FLU), boscalid (BOS) and pyraclostrobin (PYRA). Previously, these pesticides have been linked to negative human health effects. For example, FLU, BOS, and PYRI have been shown to be endocrine disrupting compounds, while PYRA is associated with apoptosis of HepG2 cells, causing damage to the liver.[10–14]

As a result of research advancements related to health disturbances that pesticides can cause, acceptable daily intake (ADI) and maximum residue limit (MRL) values are commonly established for pest control products. In 2018, Health Canada determined the ADI for FLU to be 0.037 mg/kg bw/day.[15] Similarly, the ADI for BOS and PYRI were determined to be 0.14 mg/kg bw/day and 0.17 mg/kg bw/day, respectively.[16,17] The ADI of pyraclostrobin has not yet been determined in Canada.[18] MRL values are different for every pesticide and for every crop for which they are applied.[17,19] For example, the MRL for PYRI, FLU, BOS and PYRA on highbush

blueberries are 8.0 ppm, 2.0 ppm, 6.0 ppm, and 3.5 ppm, respectively. According to data published by the USDA in 2022, all four fungicides were commonly detected in blueberry, grape, and pear samples, in concentrations ranging from 0.003 ppm – 10 ppm.[20]

As PYRI, FLU, BOS and PYRA are often detected at low concentrations in produce items with complex matrices, the analysis and quantitation of such species requires a highly sensitive and selective separation technique. Previously, PYRI, FLU, BOS and PYRA have been studied by a variety of analytical methods including fluorescence spectroscopy and surface-enhanced Raman spectroscopy.[21,22] These methods are not separation techniques, which poses an inherent challenge for the analysis of complex samples. Alternatively, high-performance liquid chromatography (HPLC) and ultra high-performance liquid chromatography (UHPLC) have gained significant attention for similar analyses, as the complex mixtures can be separated, identified and quantified, in a high-throughput method.[23–25] Although 1D liquid chromatographic methods are advantageous for their separation capabilities, they can have some inherent disadvantages, such as the difficulty of adequately resolving all components of a complex mixture in a timely manner.[26]

Two-dimensional liquid chromatography (2D-LC) is an advanced separation technique that offers a second dimension of chromatographic separation, making it ideal for improved peak resolution in complex mixtures.[26] In multiple heart-cutting mode, the sample is analyzed using a first dimension (^{1}D) column, and then select portions of the ^{1}D effluent are redirected onto a chemically distinct second-dimension (^{2}D) column to be further separated. As a result of the two-dimensional analysis of a single sample, peak capacity is significantly enhanced compared to 1D-LC. This increased peak capacity makes 2D-LC ideal for the analysis of complex mixtures with difficult-to-resolve peaks.[26] In this study, multiple heartcutting mode (mLC-LC) is used, where a typical separation can occur in a ^{1}D column followed by a second separation of select ^{1}D peaks in

a ^{2}D column.[27] Previous reports outline the use of mLC-LC for the analysis of analytes in various complex mixtures including canned food items, pharmaceuticals, and dairy products.[27–29] Previously, Zeng et al. reported the detection of triazole fungicides by 2D-LC, performing a reversed-phase separation in the first-dimension and an enantiomeric separation in the second-dimension.[30] To the best of our knowledge, a method for 2D-LC separation of FLU, PYRA, BOS, and PYRI in produce has not yet been reported in the literature.

The Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method is a sample clean-up procedure which has become increasingly popular for its rapid sample clean up time, decreased solvent volumes, and its ease of use.[31] QuEChERS was first introduced in 2003 by Anastassiades et al. for the analysis of a broad spectrum of pesticide residues in fruits and vegetables.[32] Today, this technique is used for several sample categories including meat, cheese, and biological fluids. All QuEChERS methods are based on two steps: a salting-out extraction step, followed by a dispersive solid-phase extraction (d-SPE) step. A modified version of the method validated by the Association of Official Agricultural Chemists (AOAC) was used for this thesis research.[33,34]

In this work, an mLC-LC method was developed for the analysis of PYRI, FLU, BOS and PYRA in produce items. Once the method was fully developed, QuEChERS clean-up was used for produce samples prior to 2D-LC analysis. Internal standard calibration using phenanthrene was employed for the quantitation of all analytes.

1.1 Research goals

As previously discussed, PYRI, FLU, BOS and PYRA are all highly prevalent fungicides, with potential associated health concerns, such as endocrine-disrupting activity. It is hypothesized that an optimized 2D-LC method would offer a highly sensitive detection and separation technique for analysis of fungicides of interest. Using this method, all four fungicides can be separated from complex mixtures, such as produce items, and can be accurately quantified, ensuring that they are found at concentrations safe for human consumption. This result would allow produce items to be routinely monitored for the presence of select fungicides, reducing distribution and consumption of products with high fungicide content.

This research project started with optimization of a 2D-LC method for the separation of PYRI, FLU, BOS and PYRA. During method development, fungicide standard solutions prepared in acetonitrile (ACN) were used. Optimization of the 2D-LC method is a crucial step in ensuring that the fungicides can be accurately detected and quantified. In addition to the fungicides of interest, phenanthrene was added to all standard solutions during method development as an internal standard for later quantification. The method was then validated by completing reproducibility and recovery tests. Once the method was validated, internal standard calibration curves were constructed, which were used to determine the limit of detection (LOD) and the limit of quantification (LOQ) for all analytes. The developed 2D-LC method was then used for the analysis of local and international blueberry samples.

1.2 Scope of this thesis

This thesis includes 8 chapters. Chapter 1 offers an introduction to the fungicides studied in this research, and the 2D-LC methodology employed. This chapter also outlines the research goals and the scope of this thesis. Chapter 2 includes a detailed literature and theory overview for concepts related to this research. Chapter 3 outlines all experimental procedures followed to obtain the results presented in this thesis. Chapter 4 includes all significant results and a discussion of these findings. Chapter 5 summarizes the main conclusions of this work. Chapter 6 details future work that could be considered to expand on the results of this research. Chapter 7 includes all references consulted for this work. Chapter 8 includes supplementary data as well as copyright permissions.

Chapter 2: Background

2.1 Literature review

2.1.1. Pesticides

Pesticides are chemical compounds used to control a variety of organisms such as insects, fungi, parasites, and rodents that may jeopardize the growth or yield of crops or livestock.[23] Pesticides are vital in ensuring there is enough food to feed the world's population by maintaining crop quality and output.[35] Pesticides may be derived from natural sources or can be synthetically produced. Due to the increasing demand for pesticides globally, the number of current-use pesticides is consistently growing. Additionally, pesticides with long-standing use, or those that have been banned, continue to be present as residues in soil and water.[5] The application of pesticides to crops at varying stages of growth and harvest inevitably results in pesticide residues being found in almost all food items, and most notably, fruits and vegetables.[36]

2.1.2 Pesticide use and health concerns

Although pesticides are ideal substances for killing or harming pests present on crops, they can also pose harm to humans and non-target organisms.[6] Due to the many human exposure pathways to pesticides, to be outlined in this thesis, pesticides can readily act on various parts of the human body, sometimes causing deleterious effects. Notably, it is important to consider that pesticide toxicity to humans can vary between individuals, depending on their sensitivity, immunity, or vulnerability (i.e. children, pregnant women, and the elderly).[6,7] The European Environment Agency's 2023 briefing on pesticides outlines how pesticides can cause several diseases in humans, and urges societies to reduce and more highly regulate pesticide use.[7] The

health concerns of pesticide exposure are widespread and include both acute and chronic health effects.[37] Acute effects include irritation and burning of the skin, mouth or nose, and more urgent responses such as impaired cognition and seizures.[8] Potential chronic repercussions include detriment to reproductive, pulmonary, and neurological health.[6]

2.1.3 Human exposure to pesticides

Due to the increasing prevalence of pesticides, humans can be exposed to these compounds during a variety of activities, and via multiple pathways.[8] Exposure can vary greatly, involving different exposure media and biological pathways, which all play a role in determining the degree of toxicity of the pesticides of concern. Exposure media of pesticides include but are not limited to soil, potable water, and food. Tudi et al. have previously highlighted three main biological routes for human exposure to pesticides including inhalation, dermal contact, and ingestion, outlined in more detail below. [8]

Human exposure to pesticides through inhalation occurs primarily for the volatile components of pesticides. This is a common occupational health concern during the application of pesticides, especially for those working without proper respiratory protective equipment.[38] As for non-volatile pesticides, this exposure pathway can still be relevant when the product is applied to crops in a form where they can be inhaled (i.e. as an aerosol). Although only about 10% of human pesticide exposure occurs by inhalation, it has been shown that tissues of the nose, throat, and lungs can be significantly affected when large quantities of pesticides are inhaled from air, water, or soil.[8,38,39]

Dermal exposure to pesticides is considered the most common form of exposure for those working directly with pesticide-containing products.[38,40] This form of exposure usually occurs as

a result of spillage, splashing, or aerosol drift of pesticides during the application process.[8] When pesticides come in direct contact with the skin, they may be absorbed into the body through the layers of the epidermis, depending on the properties and formulation of the pesticide.[41] In addition to adverse human health effects that may be caused by dermal exposure to pesticides, skin can act as a reservoir for pesticides after absorption, increasing the probability of further pesticide contamination.

Finally, human exposure to pesticides by ingestion is known to be the most common poisoning route and can have serious effects on human health.[8] If a person's skin has been contaminated after pesticide application, pesticide ingestion could occur when the individual consumes food. In addition, the general population may regularly ingest pesticides by consumption of pesticide-contaminated foods. Common pesticide-contaminated foods include pesticide-treated produce items.

2.1.4 The Environmental Working Group and the Dirty Dozen

As discussed, the consumption of produce items by humans serves as a significant exposure pathway for pesticides. The Environmental Working Group (EWG), an American non-profit association focused on research and advocacy in environmental sciences, is just one of many active contributors to this field of research.[2] Using data contributed by the United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA), the EWG's Shopper's Guide to Pesticides in Produce is released annually, highlighting produce items with high pesticide contamination.[9,20]

As a part of the EWG's Shopper's Guide to Pesticides in Produce, the top 12 fruits and vegetables with the most pesticide contamination are listed, which as a whole is referred to as the

"Dirty Dozen".[2] To compile this list, 46 fruits and vegetables were tested by the USDA for a wide range of pesticides, and classified by the EWG using the following six measures of pesticide contamination:

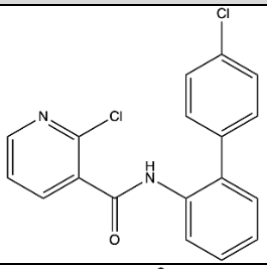
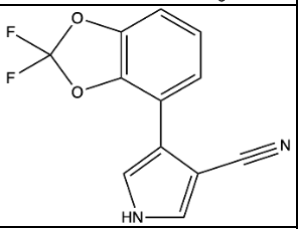
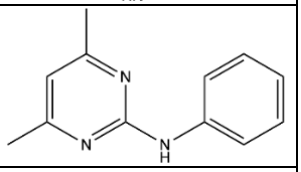
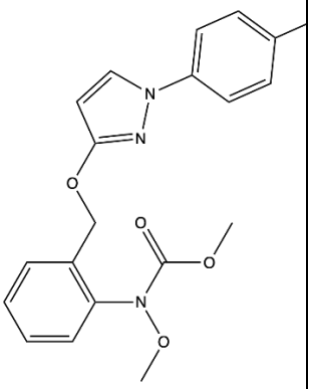
- Percent of samples tested with detectable pesticides
- Percent of samples with two or more detectable pesticides
- Average number of pesticides found in a single sample
- Average amount of pesticides found, in parts per million (ppm)
- Maximum number of pesticides found on a single sample
- Total number of pesticides found on the crop

Notably, the samples utilized for this testing included domestically grown produce as well as imported produce. Unless otherwise noted, the country of origin did not significantly affect the sample's classification.

2.1.4.1 Frequently detected fungicides of the Dirty Dozen

A fungicide is defined as a pesticide used to kill or inhibit the growth of fungal species.[42] Of the many pesticides listed in the "Dirty Dozen", four of the five most frequently detected pesticides were fungicides.[2] These compounds included pyrimethanil (PYRI), fludioxonil (FLU), boscalid (BOS) and pyraclostrobin (PYRA), all of which have been shown to have potential risks for human health. Table 1 outlines the fungicides of interest for this study, as well as some of their chemical properties.[43–46] All four of these fungicides are registered for use in Canada.

Table 2.1. Chemical information for fungicide analytes including formal and common names, molar mass, and structure.

Compound	Common name	Molar mass (g/mol)	Structure
2-chloro-N-(4'-chloro(1,1'-biphenyl)-2-yl)	Boscalid	343.21	
4-(2,2-difluoro-1,3-benzodioxol-4-yl)pyrrole-3-carbonitrile	Fludioxonil	248.19	
4,6-dimethyl-N-phenyl-2-pyrimidinamine	Pyrimethanil	199.25	
Methyl N-{2-[1-(4-chlorophenyl)-1H-pyrazol-3-yl] oxymethyl]phenyl}(N-methoxy)carbamate	Pyraclostrobin	387.82	

2.1.4.1.1 Pyrimethanil

Pyrimethanil (PYRI), first marketed in 1993, is a synthetic aminopyrimidine fungicide, known for its high stability and resistance to hydrolytic degradation.[47] Consequently, PYRI residues are often detected in waterways. PYRI acts by impeding the organism's ability to

synthesize methionine, inhibiting hydrolytic enzymes secreted by the fungus.[48] PYRI has been previously reported to be an endocrine-disrupting chemical, with anti-androgenic properties.[14] PYRI has more recently gained attention for its potential role in the development of neurodegenerative diseases, including Alzheimer's disease.[49]

2.1.4.1.2 Fludioxonil

Fludioxonil (FLU) is a fluorinated fungicide, originally derived synthetically from pyrrolnitrin, an antifungal compound produced by select *Pseudomonas* strains.[50] Since its registration in 1990, FLU has been used for the control of a wide variety of fungi, through a mechanism which involves the inhibition of protein kinases, ultimately impairing the viability of fungal organisms. Studies have shown that FLU may have the potential to mimic estrogen in the body, causing an increase in the proliferation of breast cancer cells in women.[10] Alternatively, Lee et al. found that fludioxonil can induce apoptosis and cell cycle arrest in T and B lymphocytes.[51]

2.1.4.1.3 Boscalid

Boscalid (BOS) is a fungicide belonging to the nicotinamide class and is widely used in agriculture for broad-spectrum applications.[52] As a result of the high stability of BOS in soil, it is one of the most frequently detected fungicides in the United States. Boscalid acts by inhibiting the electron transfer from succinate dehydrogenase to ubiquinone, hindering the production of energy in fungal cells.[53] Previous research suggests that after exposure, boscalid may be an endocrine-disrupting compound, having the potential to negatively affect fertility in humans.[13] D'Hose et al. showed that boscalid may induce mitochondrial dysfunction in human cells after short-term exposure.[54]

2.1.4.1.4 Pyraclostrobin

Pyraclostrobin (PYRA) is a broad-spectrum fungicide belonging to the strobilurin fungicide family, which is recommended for control of several foliar and soilborne diseases in crops.[55] PYRA acts on fungi by inhibiting mitochondrial respiration. This inhibition occurs from the blockage of electrons being transported from ubihydroquinone to cytochrome c. Previously, PYRA has been linked to the apoptosis of HepG2 cells in humans as a result of DNA damage and mitochondrial dysfunction within the cells.[11,12]

2.1.5 Fungicide regulations and concentrations

As a result of the growing research related to the negative health effects that pesticides can have, acceptable daily intake (ADI) values are generally established for every pesticide. The ADI of a pesticide describes the maximum amount of the pesticide of interest that a person could consume every day for a lifetime without any associated negative health effects.[19] Based on the ADI of a pesticide, a maximum residue limit (MRL) can be established, defined as the legal and enforceable limit of pesticide residue that can be found in or on a given produce item when a pesticide is applied according to its label instructions. For a given pesticide, the MRL will differ for every crop for which it is used.

In 2018, Health Canada determined the ADI for FLU to be 0.037 mg/kg bw/day.[15] Similarly, the ADI for BOS and PYRI were determined to be 0.14 mg/kg bw/day and 0.17 mg/kg bw/day, respectively.[16,17] The ADI of PYRA has not yet been determined in Canada.[18] MRL values are different for every pesticide and for every crop for which they are applied.[17,19] Table 2.2 outlines the MRL of the fungicides of interest on select produce items.

Table 1.2. MRL of FLU, BOS, PYRI and PYRA in blueberries, grapes, and pears.[56]

	FLU	BOS	PYRI	PYRA
MRL for blueberries (ppm)	2.0 (Highbush) 3.0 (Lowbush)	6.0 (Highbush) 11 (Lowbush)	8.0 (Highbush) 8.0 (Lowbush)	3.5 (Highbush or Lowbush)
MRL for grapes (ppm)	2.0	3.5	5.0	2.0
MRL for pears (ppm)	5.0	3.0	15	1.5

According to data published by the USDA in 2022, FLU was detected in 50.1% of tested pear samples, ranging from 0.010-1.6 ppm.[20] FLU was also commonly detected in 32.7 % of grape samples, in concentrations from 0.010-0.45 ppm. In this same study, PYRA was detected in 27.5% of all frozen blueberry samples and 20.3% of all fresh blueberry samples, with concentrations ranging from 0.004-0.047 ppm and 0.003-0.33 ppm, respectively. Similarly, BOS was frequently detected in blueberries, including 49% of all frozen blueberry samples, ranging from 0.003 ppm to 0.42 ppm, and 37.3% of fresh blueberries, ranging from 0.003-3.0 ppm. Finally, PYRI was notably prevalent in pear samples, detected in 63.7% of all samples, in concentrations from 0.004-9.5 ppm. Although PYRI, FLU, BOS and PYRA are commonly found in blueberries, grapes, and pears, only blueberries are used as a sample matrix for analyses in this work due to time constraints.

2.1.6 QuEChERS sample clean-up

The Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method is a sample clean-up method that has become exceedingly popular due to its fast clean up time, decreased solvent volumes, and ease-of-use.[31] QuEChERS was first introduced by Anastassiades et al. for the analysis of a broad spectrum of pesticide residues on fruits and vegetables.[32] Following the publication of the original method, two validated methods were introduced, including the AOAC official method 2007.01, and the European Committee for Standardization (CEN) Standard

Method EN 15662, both of which employ buffering systems for stabilization of pH-dependant analytes.[56,57] All QuEChERS methods are based on two steps: a salting-out extraction step and a dispersive solid-phase extraction (d-SPE) step.[31] In the first step, an organic solvent (usually acetonitrile) and select salts are added to a homogenized sample, which promotes an equilibrium between the aqueous and the organic layer. A portion of the organic layer containing the pesticide(s) of interest is transferred to a centrifuge tube containing various d-SPE sorbents. These sorbents include magnesium sulfate for the removal of excess water, primary secondary amine (PSA) for the removal of organic acids, graphitized carbon black (GCB) for the removal of pigments and chlorophyll, and octadecyl silica (C18) for the removal or fats and lipids. Some or all of these sorbents may be present, depending on the analytes and the matrix being analyzed. After d-SPE clean-up, the solution can be directly analyzed by several techniques including LC-DAD, LC-MS-MS, and GC-MS. For this work, the AOAC official method 2007.01, as well as a modified version of this method were employed, which both involve an acetate-buffered extraction step.[33,34] These conditions promote buffering at pH 4.8.[56] The modified AOAC QuEChERS procedure proposed by Zhao et al. is designed to improve extraction recoveries for planar analytes.[33] Maximum recovery values for planar analytes may be low using the original AOAC 2007.01 method due to adsorption of planar analytes onto the GCB in the d-SPE step.[33]

2.1.7 Analytical methods for the detection of pesticides

Fungicides studied in this work have previously been detected and quantified by a variety of analytical techniques. Shicai et al. used fluorescence spectroscopy to detect BOS in grape juice samples.[21] Despite the accurate and precise quantification of boscalid using this technique, fluorescence spectroscopy does not have an inherent separation step. As a result, a preliminary sample clean-up step is required for complex produce samples. Alternatively, Mandrile et al.

studied PYRI residues on various pome fruits by Surface-Enhanced Raman Spectroscopy (SERS).[22] This method allowed for detection with very limited sample preparation. It should be noted that Raman scattering is an inherently weak spectroscopic method, which limits the sensitivity of associated analyses. Seemingly, the most common method for the detection of the studied fungicides in produce items is high-performance liquid chromatography (HPLC). This technique is beneficial for these purposes as it involves the separation of the produce sample, as well as the detection of analytes of interest.[58] Several publications report the separation and detection of one, or several, of the fungicides of interest in produce samples.[23,24,59] This work aims to create a two-dimensional liquid chromatographic (2D-LC) method for the detection of PYRI, FLU, BOS and PYRA in produce samples. Produce samples will be prepared for analysis using a QuEChERS clean-up procedure, which is commonly employed for the analysis of pesticides in produce.[31] As a result of the two-dimensional separation of a single sample, peak capacity can be significantly enhanced compared to conventional HPLC. In the past, mLC-LC has been used for the analysis of several pesticides in food samples.[60–63] Additionally, Zeng et al. reported a 2D-LC method for the analysis of triazole fungicides in fruits. These researchers used a reversed-phase separation in the first dimension and an enantiomeric separation in the second dimension.[30] Notably, there have been no reports of 2D-LC being used for the analysis of any of the fungicides studied in this thesis work.

2.2 Theory

2.2.1 Liquid chromatography (LC)

Chromatography is a fundamental separation technique whereby an analyte, contained in a mobile phase, is transported through a stationary phase.[64] The stationary phase in chromatography is fixed in a column or on a planer surface, while the mobile phase can be a liquid (LC), a gas (GC), or a supercritical fluid (SFC).[65] Depending on the chemical characteristics of the selected analyte, it may interact more or less strongly with the stationary phase, affecting its migration rate.[64] Based on the preferential association of a mixture's components with the mobile phase or the stationary phase, a separation will occur.

Most chromatographic methods can be divided into two basic categories: column chromatography and planar chromatography.[65] In column chromatography, the stationary phase is held within a metal or glass cylinder. Together, the cylinder and the stationary phase are called a column. The mobile phase containing the sample is forced through the column, which allows partitioning and separation to occur. Alternatively, in planar chromatography, the stationary phase is supported on a flat surface, often glass or aluminum. Similarly, the mobile phase can move through the stationary phase by either capillary action, or under the influence of gravity. For this research, the focus will be on column chromatography, and specifically liquid chromatography (LC).

In LC, there are normal phase (NP-LC), and reversed phase (RP-LC) modes. In NP-LC, the separation occurs using a polar stationary phase and a non-polar mobile phase. Alternatively, RP-LC uses a non-polar stationary phase and a relatively polar mobile phase. For this work, ultra-high-performance liquid chromatography (UHPLC) was used, which takes advantage of ≤ 2 μm

particle packing, increasing the pressure limit of the system. Using this technique, resolution of sample components can be significantly enhanced.[66]

2.2.1.1 General chromatographic parameters

To analyze chromatographic data, signal intensity can be plotted as a function of time, producing a series of peaks (a chromatogram), representing the analyzed mixture's components. A chromatogram is a powerful representation of chromatographic data, as it provides qualitative information in the form of retention time, as well as quantitative data in the form of peak area. Peak area is proportional to the concentration of the species represented by the peak of interest. Retention time (t_R) represents the time at which a component will elute from a column during a chromatographic separation.[64] In addition to the retention times of the components of the analyzed sample, a zero retention time (t_0) is defined (dead time, void time), representing the time at which an unretained species exits the column. The peak width is also measured, which can be calculated by taking the difference between the two intersections of the tangent lines on each side of the peak, with the baseline. Figure 2.1 is included to illustrate these concepts.

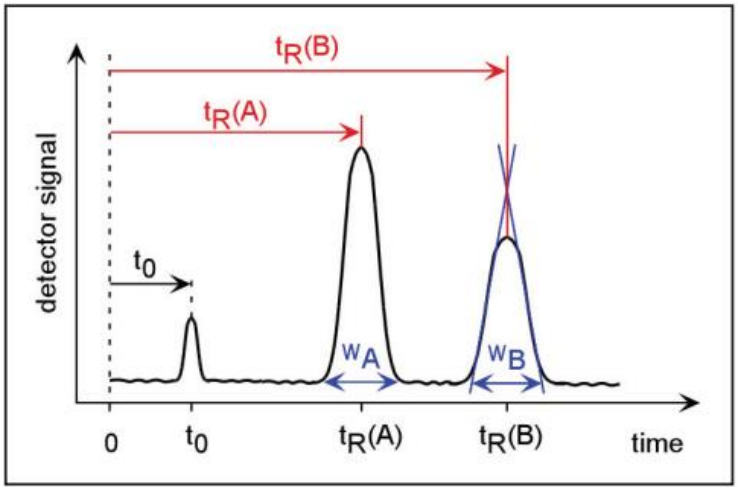


Figure 2.1. Schematic illustrating a chromatogram. Retention time of the mobile phase, retention times of analytes are included, and peak widths are included. This image was reproduced with permission from Manz et al. 2015.[64]

In liquid chromatography, the main goal is to separate peaks in a chromatogram in the least time possible, and with the highest possible resolution.[65] This means that in an ideal situation, peaks in a chromatogram would be Gaussian in shape, as well as properly separated from other components. To achieve and monitor the quality of a chromatographic separation, several additional parameters can be derived. As discussed, when an analyte is introduced onto a column, while being carried in a mobile phase, there is an equilibrium established between the time the analyte spends in the mobile phase, and the time spent in the stationary phase. This equilibrium is represented by its equilibrium constant, which is referred to as the distribution constant of the species (K_C). In Equation 2.2.1, K_c is the distribution constant, $(a_A)_S$ represents the activity of a certain analyte A in the stationary phase, and $(a_A)_M$ represents the activity of the same compound in the mobile phase. The difference between distribution constants of components in a mixture is the basis of chromatographic separation.

$$K_c = \frac{(a_A)_S}{(a_A)_M} \quad (2.2.1)$$

Similarly, the retention factor (k_A) of a solute is the ratio between the time it spends in the stationary phase and the time it spends in the mobile phase. The retention factor for a solute A is defined as Equation 2.2.2, where t_s is the time the solute spends in the stationary phase. This parameter can be easily calculated from a chromatogram. The ideal retention factor for an analyte is between 1 and 5.

$$k_A = \frac{t_R - t_0}{t_0} = \frac{t_s}{t_0} \ (2.2.2)$$

The selectivity factor (α) relates the distribution constant of a more strongly retained species B, and a less strongly retained species A. Using these conditions, the selectivity factor can be defined by Equation 2.2.3, where its value is always larger than 1. This parameter quantifies the ability for a chromatographic method to differentiate between two species in a mixture. The ideal selectivity factor for two species is between 2 and 5.

$$\alpha = \frac{K_B}{K_A} \ (2.2.3)$$

Resolution (R_s) is an important parameter describing separation which accounts for both the retention factor and the average peak width of two peaks. For a mixture with species of interest A and B, the resolution can be calculated as follows:

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_B}{1 + k_B}\right) \ (2.2.4)$$

As previously mentioned, the theoretical shape of a chromatographic peak follows a Gaussian distribution, meaning that it would be symmetrical along the vertical axis intersecting its maximum.[67] In practice, peaks often do not exhibit an ideal shape, and rather, they can show features such as fronting or tailing. When a peak is fronting, it is being drawn out towards earlier retention times, while the opposite occurs for peak tailing. Peak fronting often suggests that the injection volume is too high (column is overloaded), while tailing results from inconsistencies in the distribution constant of an analyte throughout a chromatographic separation. Figure 2.2 illustrates these common features of chromatographic peaks.

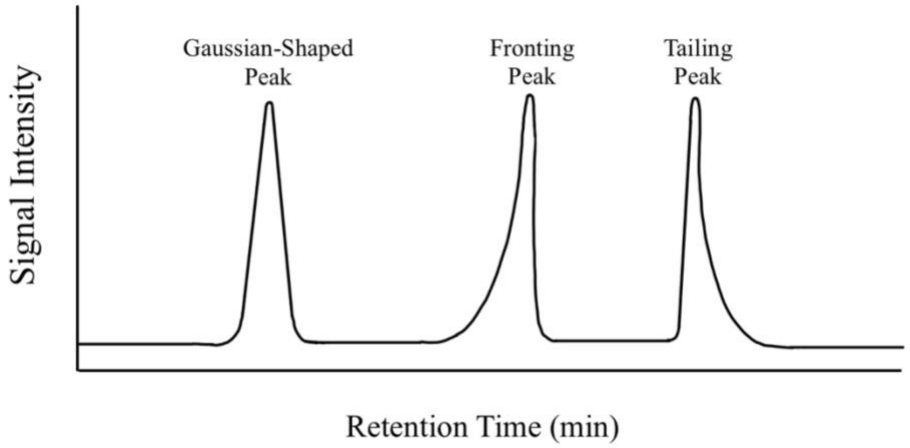


Figure 2.2. Representation of fronting and tailing in chromatographic peaks.

Based on these peak features, peak asymmetry (A_s) can be calculated.[68] Peak asymmetry can be described as the ratio of (a) and (b), where they are the two widths at half maximum of sections created when the peak is divided by a vertical line drawn from the maximum to the baseline. In Equation 2.2.5, (a) is the width of the leading edge of the peak, and (b) is the width of the tailing edge.

$$A_s = \frac{b}{a} \quad (2.2.5)$$

Based on Equation 2.2.5, peak asymmetry will be larger than 1 if tailing is present and will be smaller than 1 if the peak of interest is fronting. Peak asymmetry values between 0.75-1.25 are considered acceptable.[68] Figure 2.3 illustrates the values necessary to calculate peak asymmetry, in addition to (a') and (b') which represent the corresponding widths of the two divisions of the peak at the baseline.

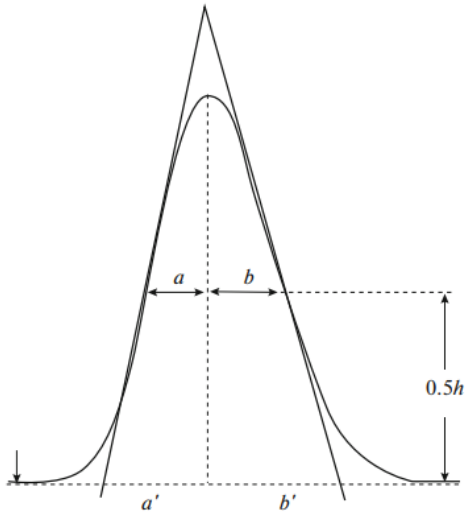


Figure 2.3. Representation of peak parameters used for the calculation of asymmetry. This image was reproduced with permission from Zenkevich et al. 2017.[68]

2.2.1.2 Plate theory of chromatography

As previously discussed, chromatographic separation of a mixture depends on the association of an analyte with the mobile phase relative to its association with the stationary phase.[69] Therefore, the assumption is made that a solute is in continuous equilibrium between the mobile phase and the stationary phase, as described by the previously outlined distribution

constant. Plate theory was introduced to explain how this equilibrium occurs, despite the constant movement of the solute contained in the mobile phase.

In plate theory, a chromatographic column is considered to be divided into many theoretical plates (N), where species will reside for only a finite amount of time. The height of each plate is established so that the solute has adequate time to reach equilibrium. Consequently, a column with a smaller plate height (H), and more theoretical plates, will require less time for a solute to establish equilibrium with the mobile phase and the stationary phase, and is therefore deemed to have a high column efficiency. The number of theoretical plates and the plate height are related, and can be deduced, based Equations 2.2.6 and 2.2.7, where L is the length of the column.

$$N = 16\left(\frac{t_R}{w}\right)^2 \quad (2.2.6) \qquad H = \frac{L}{N} \quad (2.2.7)$$

Plate theory has become a fundamental concept in chromatography as it explains the differential rates of migration between solutes in a mixture. It also describes the physical processes which result in the formation of a Gaussian-shaped peak in a chromatogram.

2.2.1.3 Rate theory of chromatography

Although plate theory has been extremely important in understanding the chemistry underlying chromatographic separations, it fails to account for band broadening, which is commonly observed in a chromatographic separation.[65] Band broadening describes the widening of an analyte peak that can occur throughout a separation procedure.[70] To complement plate theory, rate theory was developed, which is defined by the Van Deemter equation (2.2.8).

$$H = \frac{B}{u} + C_s u + C_M \quad (2.2.8)$$

The first term of this equation, $\frac{B}{u}$ (the longitudinal diffusion term), accounts for the diffusion of solutes from the most concentrated center of a band to the more dilute outer regions of the band. The constant B (longitudinal diffusion) is inversely proportional to the flow rate (u). The second term, $C_S u$ (stationary phase mass-transfer term), describes the fact that it takes time for the solute to establish an equilibrium between the mobile and stationary phases. Finally, the last term, C_M (Multipath or Eddy diffusion term), accounts for the multitude of paths that a solute could take as it moves through the stationary phase, being carried by the mobile phase. By summing these terms, the theoretical plate height for the column can be determined and an approximation of the ideal flow rate can be identified. The Van Deemter plot graphically illustrates the summation of these terms, and indicates the ideal flow rate of the system, where the plate height is near minimum, as shown as Figure 2.4.

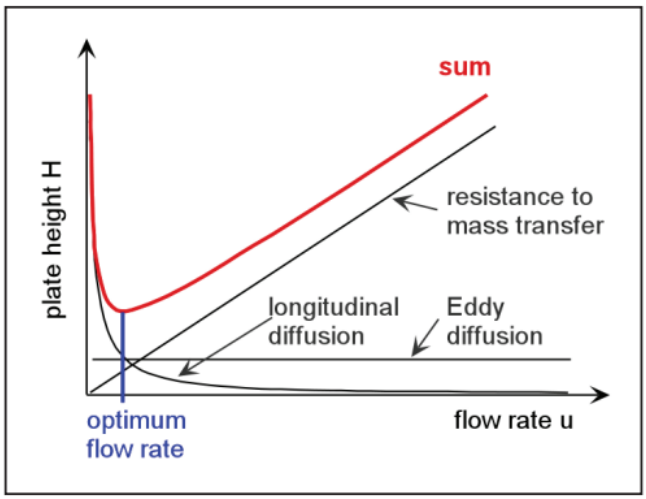


Figure 2.4. Van Deemter plot and its application for the determination of ideal flow rate. This image was reproduced with permission from Manz et al. 2015.[64]

2.2.1.4 pH control in liquid chromatography

Variations in the pH of a mobile phase can have significant impacts in LC, including the promotion of changes in peak shape, retention, and resolution.[71] The effect of pH is most relevant for highly polar or ionizable compounds. Importantly, ion-suppressed analytes will have better retention in the column as opposed to ionized solutes. For this reason, it is common practice to modify the mobile phase pH when separating a mixture ionizable solutes. This study utilizes these concepts to optimize the employed chromatographic methods.

2.2.2 Two-dimensional liquid chromatography

Two-dimensional liquid chromatography (2D-LC) is a sophisticated separation technique, tailored for the rapid and high-quality separation of complex mixtures that would otherwise be very difficult to separate by conventional chromatographic methods.[72] The mixture is analyzed in a ^{1}D column, then select portions of the ^{1}D effluent are redirected into a chemically distinct ^{2}D column to be further separated. As a result of the two-dimensional analysis of a single sample, peak resolution can be significantly enhanced compared to 1D liquid chromatography (1D-LC).

2.2.2.1 2D-LC modes

Several 2D-LC modes have been developed to produce relevant results depending on specific samples or research interests. Comprehensive 2D-LC involves sending all ^{1}D effluent to the ^{2}D system, creating a thorough qualitative picture of the components contained within the sample of interest. This method is optimal for qualitative analysis of complex mixtures but produces an immense amount of data that can be computationally challenging to interpret. Alternatively, high-resolution 2D-LC is best suited for highly accurate quantitation of a compound within the sample of interest. This mode involves choosing a peak from the ^{1}D chromatogram, and then dividing the peak into a series of segments of equal width. Each segment is separately sent to

the ^{2}D column, where further separation can occur. The analyte peak area from each of the multiple ^{2}D chromatograms is added together to obtain the total area attributed to the species. Despite the advantages that come with this mode, it can be time-consuming. Finally, heart-cutting modes (single and multiple heart-cutting) allow users to select one or multiple peaks for ^{2}D analysis. The instrument will select a band, with a predefined width, at the most intense point of the peak, which will be sent as a fraction to the ^{2}D column for analysis. Heart-cutting 2D-LC is very commonly employed as it is fast, allows for the study of multiple peaks in the ^{2}D, and can be quantitative if ^{1}D peaks are sharp enough to fit in the heart-cutting band width. For this reason, heart-cutting mode was used for this study.

2.2.2.2 2D-LC instrumentation

2D-LC system can be conceptualized as two complete 1D-LC systems, which operate sequentially, each with its own pump, column, and detector. The box diagram for a 2D-LC system is included as Figure 2.5.

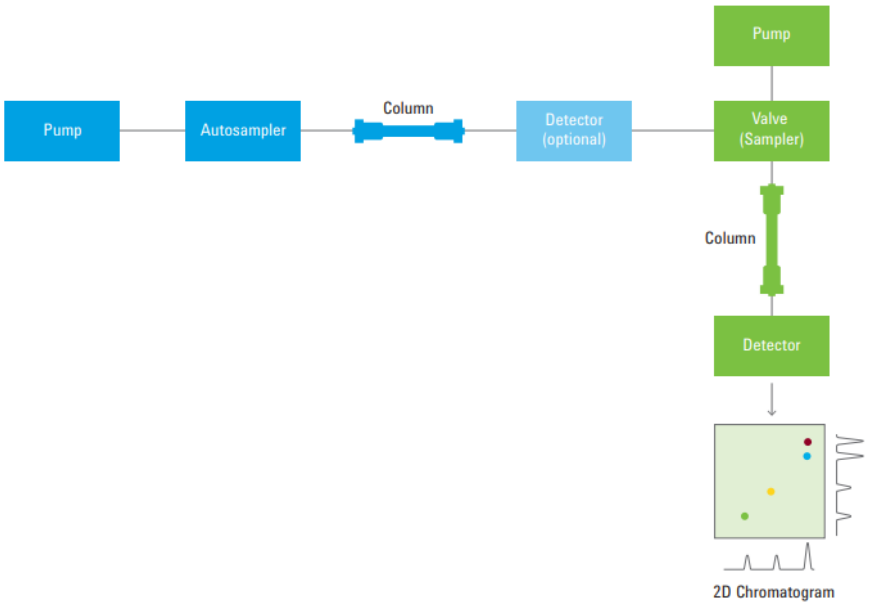


Figure 2.4. Box diagram of a two-dimensional liquid chromatograph. This image was reproduced with permission from Carr et al. 2015.[72]

These two systems are connected by a state-of-the-art 2D-LC switching valve, as shown in Figure 2.6. During 2D-LC analysis, select portions of the ^{1}D effluent (cuts) are collected and stored in the sample loop within the valve, until the 2D column is ready to commence the second separation. This valve contains several samples loops so that multiple 1D cuts can be stored at the same time, in the case that they all elute in a short period of time.

In Figure 2.6, a first valve position is depicted, in which 1D effluent flows into the sample loops, and then a second valve position, where a cut from a sample loop is allowed to flow into the ^{2}D column. The 2D-LC instrument used for this study contains two decks with 5 sample loops each.

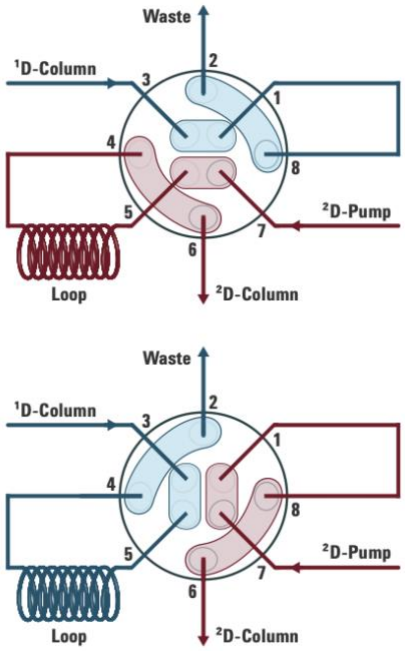


Figure 2.5. Switching valve used in 2D-LC instruments. This switching valve is equipped with 8 ports, 2 valves, and 1 sampling loop. Image reproduced with permission from Carr et al. 2015.[72]

2.2.2.3 Peak capacity

Peak capacity, (η_c), is an extremely important theoretical concept in LC and partially defines the increased resolving power of a 2D-LC system. By definition, peak capacity is the maximum number of chromatographic peaks that can be fully resolved in a given separation window. The separation window is the time difference between the last eluting peak and the first eluting peak. This concept assumes that all peaks are equally resolved, which deviates from what would be observed experimentally. In 2D-LC analysis, the peak capacity of the combined

chromatographic set-up ($\eta_{c,tot}$) can be estimated by the product of the first-dimension peak capacity ($^1\eta_c$) and the second-dimension peak capacity ($^2\eta_c$) (Equation 2.2.9):

$$\eta_{c,tot} = {}^1\eta_c \; x \; {}^2\eta_c \; (2.2.9)$$

To achieve this ideal peak capacity in a ^{2}D separation, there are specific conditions that must be met. Peaks must be resolved depending on at least two different physiochemical properties, the resolved components must occupy the entire two-dimensional space, and finally, the fractions must not undergo any remixing when introduced to the ^{2}D column.[73]

2.2.2.4 Orthogonality

Orthogonality is an ideal property of a 2D-LC method, which involves using two entirely different chromatographic conditions in the first and second dimensions. The dissimilarity between the two dimensions promotes optimal separation, and maximal use of the entire 2D separation space. Although there are several possible combinations of methods to be used in 2D-LC, some may have higher orthogonality. For example, RP x NP would have higher orthogonality than RP x RP, as the separation in the two dimensions would work by opposing mechanisms. Notably, despite the high orthogonality of the RP x NP system, it is often difficult to realize, in practice. This incompatibility stems from the immiscibility of the common solvents that would be used for these methods. Carr et al. previously proposed a system to rank mode modifications based on vital factors including orthogonality, peak capacity, solvent compatibility, and applicability.[72] By this system, the RP x RP mode combination was determined to be the best suited for 2D-LC analysis, achieving a score of 9 out of 10. For this thesis research, RP x RP 2D-LC was employed.

Chapter 3: Experimental

3.1 Reagents and materials

All glassware used for experimentation was soaked for a minimum of 1 hour in an acid bath containing neat sulfuric acid and subsequently rinsed using Millipore water (≥ 18.2 MΩ cm) produced using a Milli-Q plus system (Millipore, CA, USA). Fludioxonil (≥ 98%), Pyraclostrobin (≥ 98%), Boscalid (≥ 98%), Pyrimethanil (≥ 98%), water (HPLC grade), and disposable microcentrifuge tubes (1.5 mL) were purchased from Sigma Aldrich (St. Louis, MO, USA). Phenanthrene (≥ 95%) was purchased from BDH Chemicals (Poole, England). Formic acid (≥ 88%) and glacial acetic acid (99.5%) were purchased from ACP (Montreal, Quebec, Canada). Methanol (99.9%, HPLC grade), acetonitrile (99.8%, HPLC grade), toluene (≥ 99.8%, HPLC grade) and PTFE 0.2 µm syringe filters (13 mm, 13-1001-14) were purchased from Fischer Scientific (Ottawa, Ontario, Canada). QuEChERS extraction kits (AOAC 2007.01 method, containing 6 g $MgSO_4$, 1.5 g $NaC_2H_3O_2$ and 50 mL centrifuge tubes) and dispersive solid-phase extraction kits for pigmented fruits and vegetables (AOAC 2007.01 method, containing 150 mg $MgSO_4$, 50 mg GCB and 50 mg PSA in 2 mL centrifuge tubes), HPLC vials (amber, 2 mL), bonded screw caps with PTFE septa, 250 µL pulled point glass HPLC vial inserts, a Zorbax Eclipse C18 Plus column (3.0 mm x 50 mm x 1.8 µm) and a Zorbax Bonus RP column (2.1 mm x 50 mm x 1.8 µm) were all purchased from Agilent Technologies (Santa Clara, CA, USA). Argon gas (99.999%) was purchased from Air Liquide Canada Incorporated (Montreal, QC, Canada). The Nova Scotia blueberry sample was picked at a U-Pick in the Annapolis Valley, NS. The Peruvian blueberry sample was purchased at a local grocery store.

3.2 Ultraviolet-visible spectroscopy (UV-vis)

UV-vis spectra were acquired for all analytes in order to qualitatively determine the lambda max (λ_{max}) for each species. Individual standard solutions were prepared for every fungicide. These solutions were made by adding an appropriate mass of each fungicide to a volumetric flask and then adding HPLC acetonitrile (ACN), to attain a concentration of 10.00 mg/L. 200 µL of this solution was transferred into a quartz cuvette with dimensions of 45 mm x 10 mm x 1 mm. After blanking the spectrometer using HPLC ACN, a UV-vis spectrum was acquired using a Cary 60 UV-vis spectrometer (Agilent Technologies, CA, USA). The UV-vis spectrum for each analyte is shown in Figure 3.1. The λ_{max} for FLU, BOS, PYRI, and PYRA was 266 nm, 233 nm, 269 nm, and 272 nm, respectively. The UV-vis spectra are included in the appendix as Figure A1.

3.3 QuEChERS of Blueberry samples

3.3.1 Solution preparation for QuEChERS clean-up

A solution of 1% glacial acetic acid in HPLC acetonitrile was prepared by first adding approximately 10 mL of HPLC acetonitrile to a 50 mL volumetric flask. 500 µL of glacial acetic acid was pipetted into the flask. The flask was filled to the meniscus using HPLC acetonitrile. This solution is used for the salting-out extraction of the QuEChERS process.

All produce samples analyzed in this study were spiked with a phenanthrene IS solution. The phenanthrene IS solution was prepared by adding 0.0060 g of phenanthrene to a 2 mL volumetric flask. This volumetric flask was filled to the meniscus with a solution of 1% acetic acid in acetonitrile, for which its preparation was previously outlined, resulting in a final phenanthrene

concentration of 3000 mg/L. A second standard solution containing FLU, BOS, PYRA, PYRI, and phenanthrene was prepared for recovery studies. This solution had a concentration of 3000 mg/L of each compound.

3.3.2 AOAC QuEChERS procedure for clean-up of produce samples

The first QuEChERS method attempted for the clean-up of produce samples was the AOAC method reported in 2007.[34] This method involves an acetate-buffered extraction step. The first of two steps in the QuEChERS clean-up method is the salting-out extraction. Using an analytical balance, a 50 mL centrifuge tube in a beaker was tared, and 15 g of homogenized produce sample was directly weighed in the tube. 50 µL of the 3000 mg/L phenanthrene internal standard (IS) solution or the 3000 mg/L mixed standard solution was added. This produced a final concentration of each analyte or IS of 10 mg/L in the acetonitrile layer post-extraction. The centrifuge tube containing the produce sample was mixed for 30 seconds using a VWR Mini Vortexer VM-3000 (VWR, PA, USA). 15 mL of 1% acetic acid in HPLC acetonitrile solution was pipetted into the centrifuge tube. The contents of one extraction packet from the purchased QuEChERS extraction kit was added to the tube. The centrifuge tube containing the produce sample mixture was mixed for 1 minute using the vortex mixer. The mixture was centrifuged using a Thermo IEC-Multi RF Refrigerated Benchtop Centrifuge for 1 minute at a relative centrifugal force (rcf) of 2000. This completes the salting-out extraction step.

To commence the dispersive solid-phase extraction (d-SPE), 1 mL of the organic layer from the salting-out extraction centrifuge tube was transferred to a 2 mL centrifuge tube, pre-filled with d-SPE sorbents, purchased as a part of the QuEChERS clean-up kit. This transfer was repeated for a second pre-filled d-SPE tube. 375 µL of HPLC toluene was added to each centrifuge tube. The

contents of the tubes were mixed for 30 seconds using a vortex mixer. The mixtures were centrifuged for 2 minutes at 2000 rcf (Labnet PRISM microcentrifuge, Edison, NJ, USA). The supernatant was removed from both tubes and transferred to a glass vial using a Pasteur pipette. This final extract was filtered through a 0.2 µm PTFE filter into a HPLC vial fitted with a 250 µL pulled point glass vial insert. This filtrate was then injected onto the 2D-LC for analysis.

3.3.3 Modified QuEChERS procedure for clean-up of produce samples

The QuEChERS method ultimately used for the clean-up of produce samples has been previously reported by Zhao et al.[33] This method is designed to improve clean-up recoveries for planar pesticides. This modified procedure was used as the phenanthrene internal standard used in this study is planar, and certain fungicides of interest have large planer regions.

To start, the procedure follows the same salting-out extraction method as the AOAC method, as outlined in Section 3.3.2. As with the AOAC method, to commence the dispersive solid-phase extraction (d-SPE), 1 mL of the organic layer from the salting-out extraction centrifuge tube was transferred to two 2 mL centrifuge tube pre-filled with d-SPE sorbents, purchased as a part of the QuEChERS clean-up kit. Using this modified procedure, 375 µL of HPLC-grade toluene was added to each centrifuge tube. The solution was mixed using the vortex mixer for 30 seconds. The mixture was centrifuged for 2 minutes at 2000 rcf (Labnet PRISM microcentrifuge, Edison, NJ, USA). The solution was removed from both tubes and transferred to a glass vial using a Pasteur pipette and 825 µL of this extract was transferred to a new microcentrifuge tube. The extract solvent was completely evaporated under argon gas. The evaporation was completed by allowing a stream of argon gas to blow over the centrifuge containing the extract for approximately 30 minutes. The solid was reconstituted by the addition of 600 µL of HPLC ACN. This final extract

was filtered through a 0.2 µm PTFE filter into an HPLC vial fitted with a 250 µL pulled point glass vial insert. The filtrate was then injected into the 2D-LC for analysis.

3.4 Two-dimensional liquid chromatography (2D-LC)

3.4.1 Fungicide standard solutions for method development

To develop a 2D-LC method for fungicide analysis, standard solutions of PYRI, FLU, BOS and PYRA were prepared. To prepare working standard solutions of all fungicides, stock solutions were first prepared. Serial dilution was then used to obtain the desired concentration of each analyte to be analyzed in the 2D-LC. The mixed stock solution utilized for this study contained 500 mg/L of each fungicide standard and the internal standard (PYRI, FLU, BOS, PYRA and phenanthrene).

To create the working standard which was injected onto the chromatograph, 200 µL of the desired standard stock solution (mixed standard or individual standard) was pipetted into a 2 mL volumetric flask. All stock solutions and working solutions were stored at -20°C for up to 8 weeks. For 2D-LC experimentation, approximately 0.5 mL of working standard solution was transferred to an amber HPLC vial.

3.4.2 2D-LC Instrumentation

The two-dimensional liquid chromatograph used for this study was an Agilent 1290 Infinity II series 2D-UPLC system (Agilent Technologies, CA, USA). This system is equipped with an autosampler, one high-speed quaternary pump in the ^{1}D, one high-speed binary pump in the ^{2}D, a multi-column thermostat, a diode array detector for each dimension, and a fraction collector. Additionally, OpenLab CDS software (Agilent Technologies, CA, USA) is coupled to the 2D-LC,

allowing for on-site data processing and review. Data analysis was completed using Origin 2020b software (OriginLab Corporation, MA, USA).

3.4.3 Optimized 2D-LC method

An optimized 2D-LC method for the separation and analysis of PYRI, FLU, BOS, PYRA and the internal standard phenanthrene was developed. Phenanthrene was chosen as the internal standard for this study as it has been previously reported as an internal standard for HPLC analysis of fungicides.[23] When employing this optimized method, the following steps were taken. The optimized ^{1}D dimension mobile phase consisted of two acidified solvents: 0.1% formic acid in HPLC water and 0.1% formic acid in HPLC acetonitrile. 500 mL of each solution was transferred into its respective solvent bottle and connected to the 2D-LC pump. Similarly, solvents for the ^{2}D were transferred, including 0.1% formic acid in HPLC water and 0.1% formic acid in methanol. The optimized 2D-LC parameters were input into the OpenLab CDS software in preparation for the chromatographic run. These parameters are outlined in detail in Tables 3.1 and 3.2. In addition to these parameters, ^{1}D peaks of interest were chosen to be sent to the ^{2}D chromatograph for further analysis. A minimum absorbance of 2 mAU was set for all ^{1}D peaks for their transfer to the ^{2}D to be initiated.

Throughout the method development process, a non-acidified combination of mobile phase solvents was tested. The mobile phase for these preliminary trials was HPLC water and HPLC acetonitrile but without the addition of formic acid. The effects of changes to the mobile phase composition are later discussed in Chapter 4.

Table 2.1. Optimized 1D method parameters for the analysis of FLU, BOS, PYRI and PYRA.

^{1}D Parameters	
Column	Zorbax Eclipse C18 Plus (2.1mm x 50mm x 1.8µm)
Column temperature	30 ºC
Injection Volume	2.5µL
Solvent A	Water
Solvent B	Acetonitrile
Flow Rate	0.7 mL/min
Gradient	30% B at 0 min 62% B at 0.5 min 65% B at 1.5 min 85% B from 2 to 3.5 min 30% B from 4 to 5 min
^{1}D Run time	5 min
Post run time	5 min
Detection wavelength	270 nm
Reference wavelength	360 nm

Table 3.2. Optimized 2D method parameters for the analysis of FLU, BOS, PYRI and PYRA.

^{2}D Parameters	
Column	Zorbax Eclipse Bonus RP (2.1mm x 50mm x 1.8µm)
Column temperature	30 ºC
Injection Volume	2.5µL
Solvent A	Water
Solvent B	Methanol
Flow Rate	1.0 mL/min
Gradient	10% B at 0 min 70% B at 0.5 min 100% B at 1.5 min
^{2}D Run time	2 min
Detection wavelength	270 nm
Reference wavelength	360 nm

3.5 Optimized QuEChERS-2D-LC method validation

3.5.1 Reproducibility validation of the 2D-LC method

Reproducibility studies are designed to determine the degree of precision between chromatographic runs using a given method. For this study, the reproducibility of the optimized method was quantified by calculating the percent relative standard deviation (%RSD), otherwise referred to as the coefficient of variation (CV). This calculation was completed for retention times and peak areas for all analytes in both dimensions. To evaluate the reproducibility of the proposed 2D-LC method, intraday and interday reproducibility trials were performed. Intraday reproducibility validation involved analyzing the prepared 50 mg/L mixed standard and phenanthrene solution three separate times on the same day. Alternatively, running the same standard solution 5 times, on 5 consecutive days, allowed interday reproducibility to be assessed.

3.5.2 Recovery validation of both QuEChERS-2D-LC methods

To validate the recovery for the entire 2D-LC process, including the QuEChERS clean-up procedure and the 2D-LC separation process, a local blueberry sample was spiked with a mixed fungicide solution and analyzed. This process began with the homogenization of fresh Nova Scotian blueberries (Country Magic blueberries, product of NS, Canada). The sample was prepared using the non-modified or modified QuEChERS procedure previously outlined in Sections 3.3.2 and 3.3.3, using a spiking solution containing 3000 mg/L ppm of all fungicides and phenanthrene. After the clean-up procedure, the extract was filtered into an HPLC vial and injected onto the 2D-LC using the optimized separation method. This process was repeated with the same blueberry sample spiked with only internal standards to ensure that the sample did not already contain

significant concentrations of the fungicides of interest. A 10 mg/L mixed standard solution containing the fungicides of interest and the internal standard was also run on the 2D-LC using the optimized method. Peak areas corresponding to the species of interest in the resulting chromatograms for all runs were compared, allowing recoveries to be calculated for each analyte. Equation 3.5.1 was used to calculate the total recovery for each analyte studied:

$$\%\ Recovery = \frac{Peak\ area\ of\ analyte\ in\ extract}{Peak\ area\ of\ analyte\ in\ standard} \quad (3.5.1)$$

3.5.3 Recovery validation of the QuEChERS method alone using the Matuszewski method[74]

As stated, the recovery study previously outlined in Section 3.5.2 encompasses the entire QuEChERS-2D-LC method. Using this method, there are several sources of error which contribute to the final recovery values calculated. These sources of error include the QuEChERS procedure, 2D-LC instrumentation, matrix effects (ME), and more. The Matuszewski method aims to deconvolute these sources of error, and provides a way to determine the recovery of the QuEChERS procedure alone.[75] To determine the QuEChERS procedure recovery, a Nova Scotian blueberry sample was used. This sample was homogenized and prepared using the QuEChERS procedure, as outlined in Section 3.3.3, using the 3000 mg/L mixed fungicide solution to spike the sample after it had been initially added to the 50 mL centrifuge tube. This sample was analyzed in duplicate using the optimized 2D-LC method. The QuEChERS clean-up procedure was repeated for more blueberries from the same Nova Scotia blueberry carton. However, this time, the sample was not spiked at the beginning of the QuEChERS procedure, but rather at the end, creating a post-clean-up spiked sample. To create this sample, the QuEChERS procedure was followed exactly as performed for pre-clean-up spiked samples, up until the 2-minute centrifugation at 2000 rcf had been completed. At this stage, 825 µL of the supernatant was transferred to an Eppendorf tube.

This solution was spiked with 2 µL of the 3000 mg/L spiking solution. The spiked solution was allowed to evaporate under argon as normal. The remainder of the QuEChERS procedure was completed exactly as previously outlined. The post-clean-up spiked sample was analyzed in duplicate using the optimized 2D-LC method. The analyte and IS peak ^{1}D areas were determined for the pre-clean-up spiked sample and the post-clean-up spiked sample. Using these peak areas, given analyte A, the recovery can be calculated using Equation 3.5.2.

$$\frac{Average\ peak\ area\ of\ A\ in\ pre\ clean\ up\ spiked\ sample\ (N=3)}{Average\ peak\ area\ of\ A\ in\ post\ clean\ up\ spiked\ sample\ (N=3)}\ x\ 100\% \quad (3.5.2)$$

Using this method, the pre-clean-up sample is subject to error from the QuEChERS method itself. Alternatively, the post-clean-up sample is not subject to error from the QuEChERS method. Notably, this method assumes that there is no error associated with the evaporation or reconstitution processes. As both samples were analyzed in the same matrix, using the same instrument, it is assumed that when the ratio of peak areas is calculated, the resulting percent recovery value is representative of the QuEChERS error alone.

3.5.4 Quantification of Matrix Effects using the Matuszewski method

The Matuszewski method can be extended to quantify the significance of ME during the analysis of a sample.[75] The quantification of ME allows for the identification of peak enhancement or suppression due to the matrix in which it is being analyzed. To quantify ME, the data from the post-clean-up spiked sample outlined in section 3.5.4 was used. Additionally, a 10 mg/L standard solution of PYRI, FLU, BOS, PYRA and the IS in ACN was analyzed in duplicate using the optimized 2D-LC method. This standard solution is analyzed to mimic the concentration of all

analytes that should be in the final spiked QuEChERS extract, but without the potentially interfering blueberry matrix. The peak areas were determined for each analyte in the post-clean-up spiked blueberry sample and the 10 mg/L mixed standard solution. Using these values, given analyte A, percent ME can be calculated using the following Equation 3.5.3.

$$\%ME = 1 - \left[\frac{Peak\ area\ of\ A\ in\ post\ clean\ up\ spiked\ sample}{Peak\ area\ of\ 10\frac{mg}{L}\ mixed\ standard\ solution}\right] x\ 100\% \quad (3.5.3)$$

The resulting value for each analyte describes the significance of ME, where the sign of the value indicates whether the peak is being enhanced or suppressed. The peak is enhanced if the percent ME value is negative, and the peak is suppressed if the percent ME value is positive.

3.6 Internal Standard Calibration

To quantify the fungicides of interest in the analyzed produce samples, the method of internal standard calibration was chosen. Phenanthrene was selected as the internal standard as it had previously been reported as an internal standard for HPLC analysis of fungicides.[23] Internal standard calibration is employed in this work as it allows sample loss to be monitored throughout the chromatographic analysis.[27] Calibration standard solutions containing PYRI, FLU, BOS, PYRA, and the IS were prepared in concentrations ranging from 0.5 mg/L to 15 mg/L. For all calibration standard mixtures, the concentration of phenanthrene remained constant at 10 mg/L. Following the addition of appropriate volumes of standard stock solutions to 10.00 mL volumetric flasks, the mixture was diluted using HPLC ACN to achieve the desired calibration concentration. Each calibration standard was run on the 2D-LC in triplicate, except the 0.1 mg/L calibration standard which was run 10 times. When analyzing produce samples for quantitative analysis of the

fungicides of interest, the samples were spiked with phenanthrene standard before the salting-out extraction of the QuEChERS clean-up procedure. Table 3.3 outlines the concentration of PYRI, FLU, BOS, PYRA and IS in each calibration standard, as well as the concentration of IS in spiked samples. Internal standard calibration curves were made for each analyte by plotting $\frac{Peak\ Area_{fungicide}}{Peak\ Area_{IS}}$ against the concentration of the corresponding fungicides in the calibration solution. Least squares regression analysis was performed for the resulting data. Using the constructed calibration curves, the limit of detection (LOD) and limit of quantification (LOQ) was calculated for each analyte. Calibration mix C6 was the lowest concentration calibration standard for which all analytes were visible in the resulting ^{2}D chromatograms. Therefore, this calibration solution was run 10 times on the 2D-LC using the optimized method and analyzed as a spiked blank. The integration results of these runs and the slopes of the previously determined calibration curves were used to calculate the LOD and the LOQ of each analyte using Equations 3.6.1 and 3.6.2.

$$LOD = \frac{3\sigma_{Blank}}{Slope} \quad (3.6.1) \,, LOQ = \frac{10\sigma_{Blank}}{Slope} \quad (3.6.2)$$

Table 3.3. Composition of internal standard calibration standards used to construct calibration curves, and the concentration of internal standard added to produce samples.

	Mix C1 (mg/L)	Mix C2 (mg/L)	Mix C3 (mg/L)	Mix C4 (mg/L)	Mix C5 (mg/L)	Mix C6 (mg/L)	Produce samples (mg/L)
FLU	15.0	10.0	5.0	3.0	1.0	0.5	-
BOS	15.0	10.0	5.0	3.0	1.0	0.5	-
PYRA	15.0	10.0	5.0	3.0	1.0	0.5	-
PYRI	15.0	10.0	5.0	3.0	1.0	0.5	-
IS	10.0	10.0	10.0	10.0	10.0	10.0	10.0

Chapter 4: Results and Discussion

4.1 2D-LC method development for the analysis of fungicides

4.1.1 ^{1}D method development

To start, a first dimension (^{1}D) optimized method had to be established for the separation of PYRI, FLU, BOS, PYRA and IS. For all method optimization trials, a 50 mg/L mixed standard solution of all fungicides and the IS in ACN was used.

4.1.1.1 Column

In this study, the ZORBAX Eclipse C18 (2.1 mm x 50 mm x 1.8 µm) column was used for ^{1}D analysis of the analytes of interest. This column was made by the manufacturer using a packing consisting of dimethyl-n-octadecylsilane bound to a high purity ZORBAX Rx-SIL sporous silica support, as a dense and uniform monolayer.[76] The bonded silane was doubly end-capped using proprietary reagents, minimizing silanol interactions and ensuring the long-term stability of the silica support. All these enhanced features made the column ideal for analysis of a wide range of compounds with varying pH (pH 2-9). Considering the potential acidity/basicity of the target analytes, the ZORBAX Eclipse C18 column was ideal for achieving the optimized method reported in this thesis. The stationary phase used had a pore size of 95 Å, and the column itself could withstand pressures up to 1300 bar.

4.1.1.2 Mobile phase solvents

In liquid chromatography, mobile phase solvents play a significant role in the final separation of analytes in the chromatographic column. Throughout the ^{1}D method development, three sets of mobile phase solvents were explored for the first dimension separation. The first set

of solvents (#1) used for 2D-LC separation involved the use of water and MeOH. The second set (#2) utilized a combination of water and ACN for the first dimension. Alternatively, the third set of solvents (#3) was similar to those of the second set, however, they were acidified using formic acid (FA). The acidified mobile phase solvent was 0.1% FA in water and 0.1% FA in ACN. The described mobile phase solvent sets are outlined in Table 4.1.

Table 4.1. 1D mobile phase solvent sets used during 2D-LC method development.

Mobile Phase Solvent Sets	
Set Number	^{1}D Mobile Phase Solvents
1	Water and MeOH
2	Water and ACN
3	Water + 0.1% FA and ACN + 0.1% FA

The first step in the ^{1}D method development process was to compare two starting solvent sets, including solvent sets 1 and 2. These sets were chosen as preliminary conditions as they are commonly used for the separation of complex mixtures by RP 2D-LC and do not involve the use of mobile phase additives such as acids.[77] Although solvent set 1 has previously been reported as a ^{1}D mobile phase set for 2D-LC separation, ACN-based mobile phases are known to produce sharp chromatographic peaks due to ACN's low viscosity.[27,78]

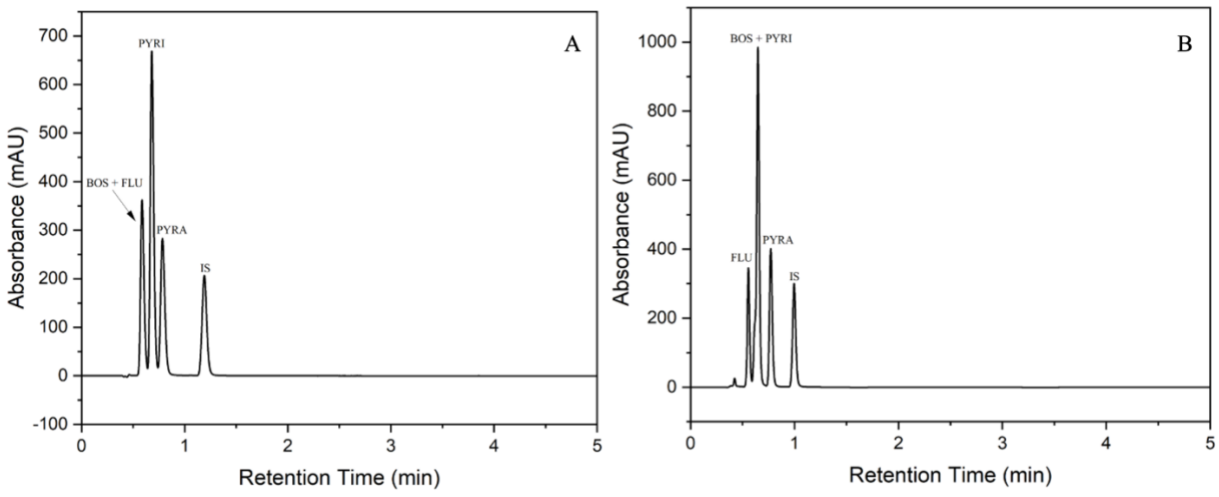


Figure 4.1. 1D chromatograms of FLU, BOS, PYRI, PYRA and IS. Analytes were eluted at 0.5 mL/min. (A) Isocratic elution: 86% MeOH for 5 min. (B) Isocratic elution: 86% ACN for 5 min.

Figure 4.1A shows the isocratic elution of all fungicides and the IS with a mobile phase composition of 86% MeOH for 5 minutes. The balance of the mobile phase at all stages was water. Using this method, BOS, and FLU completely coeluted at approximately 0.6 minutes. The peak associated with BOS and FLU was not properly resolved from the peak for PYRI. Additionally, the peak for PYRI was not resolved from the peak for PYRA. Figure 4.1B shows the separation of all fungicides and the IS using the same elution gradient described for Figure 4.1A, but with the substitution of ACN for MeOH. This variation to the mobile phase caused BOS to elute later in the chromatographic run. BOS and PYRI coelute into a single peak at approximately 0.7 minutes. The use of ACN provided better resolution of PYRA from the peaks for BOS and PYRI. The variation in retention times and the better resolution between certain peaks shows that ACN is a better ^{1}D organic solvent for this separation. Additionally, all peaks appear sharper using ACN compared to the same method using MeOH. The flow rate for both methods was 0.5 mL/min. To

improve the separation efficiency, gradient elution, wherein the mobile phase composition changes as a function of the separation, was explored.

4.1.1.3 Mobile phase gradient

In addition to the solvents used in the mobile phase, the mobile phase gradient can play a significant role in the quality of separation observed in resulting chromatograms. Specifically, switching from an isocratic elution (where the mobile phase composition stays constant throughout the run) to a gradient elution (where the mobile phase composition changes throughout the run) can aid in overcoming the general elution problem. After determining that solvent set 2 was ideal compared to solvent set 1, varying the mobile phase composition was explored in an attempt to obtain a better separation of all fungicides in the standard solution.

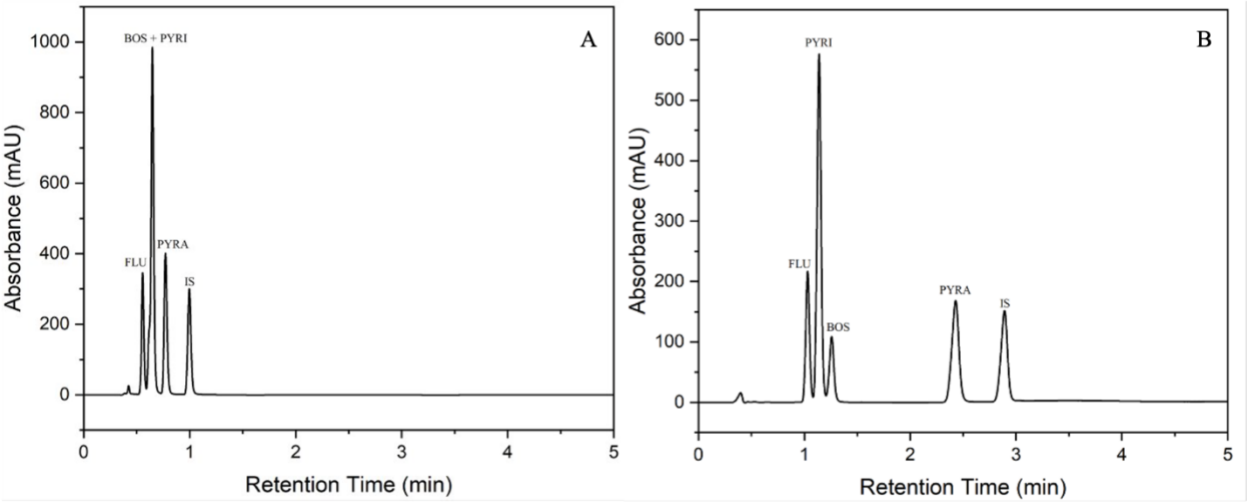


Figure 4.2. 1D chromatograms of FLU, BOS, PYRI, PYRA and IS. Analytes were eluted at 0.5 mL/min. (A) Isocratic elution: 86% ACN for 5 min. (B) Gradient: 62% ACN at 0 min, 65% ACN at 1.5 min, 85% ACN from 2-4 min and 62% ACN from 4.5-5 min.

Figure 4.2A shows the isocratic elution of all fungicides and IS with a mobile phase of 86% ACN for 5 minutes. Using this mobile phase, it can be observed that all analytes are eluting very

early in the run (before or at 1 minute). Additionally, BOS and PYRI are nearly completely coeluting, with only one very intense peak observed for both analytes. FLU is also not completely resolved from the peak representative of BOS and PYRI. Figure 4.2B shows the elution of all fungicides and the IS using a gradient elution of 62% ACN at 0 min, 65% ACN at 1.5 min, 85% ACN from 2-4 min, and 62% ACN from 4.5-5 min. The balance of the mobile phase at all stages was water. Using this gradient elution, FLU, PYRI and BOS separated into three distinguishable peaks. PYRA and the IS are also eluting later in the chromatographic run, compared to the previously outlined isocratic elution. Although FLU, PYRI, and BOS are not completely resolved using this gradient elution, it did improve the separation of BOS and PYRI. Based on this data, it was determined that gradient elution would be ideal for separating the fungicides studied in this research.

4.1.1.4 pH modification of the mobile phase

As discussed previously in section 2.2.1.4 of this thesis, the pH of the mobile phase during a chromatographic separation can have a significant impact on the shape, retention time, and resolution of peaks being separated.[71] This effect is most relevant for highly polar or ionizable compounds. After many attempts to separate the fungicides and IS using non-acidified solvents, mobile phase solvent set 3 was used. This solvent set contains water and ACN acidified with FA.

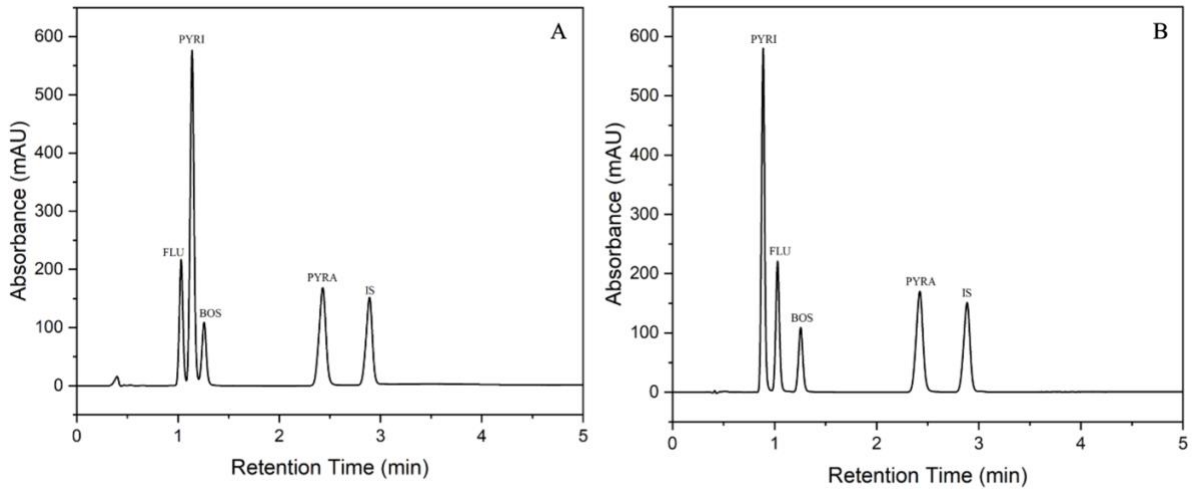


Figure 4.3. 1D chromatograms of FLU, BOS, PYRI, PYRA and IS. Analytes were eluted at 0.5 mL/min. (A) Gradient: 62% ACN at 0 min, 65% ACN at 1.5 min, 85% ACN from 2-4 min and 62% ACN from 4.5-5 min. (B) Gradient: 62% ACN + 0.1% FA at 0 min, 65% ACN + 0.1% FA at 1.5 min, 85% ACN + 0.1% FA from 2-4 min and 62% ACN + 0.1% FA from 4.5-5 min.

Figure 4.3A shows the elution of all fungicides and the IS using a gradient elution of 62% ACN at 0 min, 65% ACN at 1.5 min, 85% ACN from 2-4 min, and 62% ACN from 4.5-5 min. The balance of the mobile phase at all stages was water. Using this method, there are distinct peaks present for all compounds being separated. FLU, PYRI, and BOS are not completely resolved. Figure 4.3B shows the elution of all fungicides and the IS using the same gradient as described for Figure 4.3A, but with ACN being substituted for ACN + 0.1% formic acid (FA). The balance of the mobile phase at all stages is water + 0.1% FA. The acidification of the mobile phase provided complete separation of all compounds.

The complete separation of all analytes using the acidified mobile phase can be attributed to the change in the retention time of PYRI. PYRI is a weak base, containing a secondary amine that can be protonated at pH values below 5.[79] Therefore, when the mobile phase is acidified, PYRI is protonated to form its conjugate acid, which associates more strongly with the acidified mobile

phase. This change in PYRI's affinity for the mobile phase causes it to elute faster compared to the non-acidified mobile phase.

4.1.1.5 Flow rate

As described in section 2.2.1.3, the rate theory of chromatography outlines how flow rate can influence several phenomena that occur during a chromatographic run. Namely, as the flow rate increases, there is less time for band diffusion to occur, often resulting in desirably sharp peaks. On the other hand, as the flow rate is increased, the less time there is for solutes to establish an equilibrium between the mobile and stationary phase. Therefore, a flow rate that is too fast can lead to improper separation of analytes. Considering these potential effects of the chosen flow rate, several flow rates were explored during ^{1}D method development.

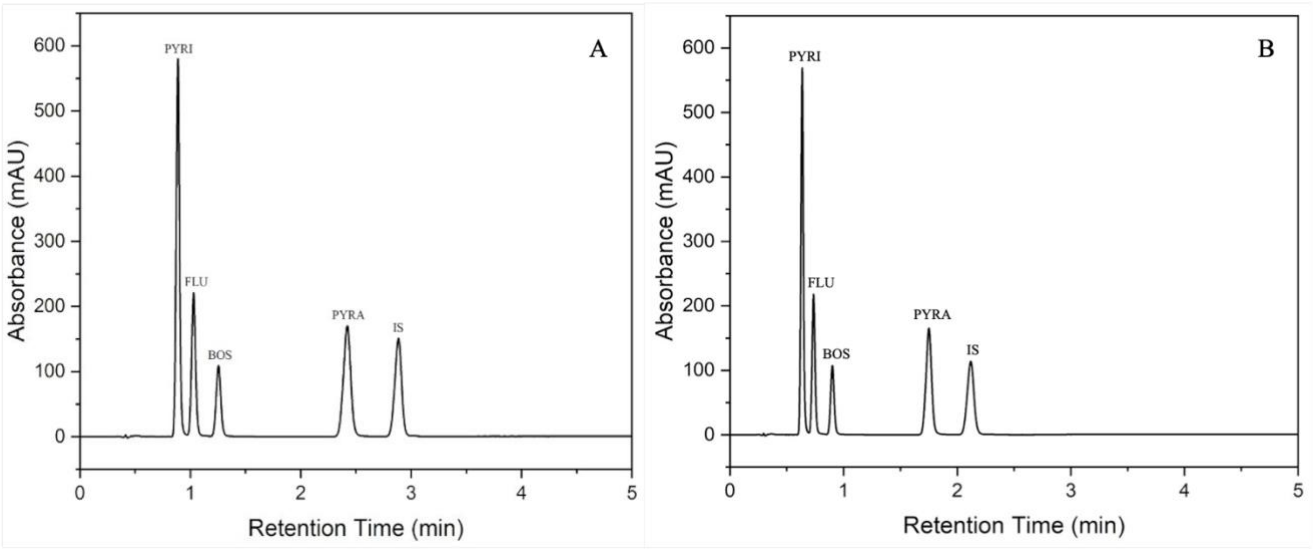


Figure 4.4. 1D chromatograms of FLU, BOS, PYRI, PYRA and IS. Analytes were eluted at 0.5 ml/min in (A) and 0.7 mL/min in (B). (A) and (B) Gradient: 62% ACN + 0.1% FA at 0 min, 65% ACN + 0.1% FA at 1.5 min, 85% ACN + 0.1% FA from 2-4 min and 62% ACN + 0.1% FA from 4.5-5 min.

Figure 4.4A shows the elution of all fungicides and the IS using a gradient elution of 62% ACN + 0.1% FA at 0 min, 65% ACN + 0.1% FA at 1.5 min, 85% ACN + 0.1% FA from 2-4 min, and 62% ACN + 0.1% FA from 4.5-5 min. Figure 4.4B shows the elution of all analytes using the same gradient and mobile phase described in Figure 4.4A, but with an increased flow rate of 0.7 mL/min. When comparing these two chromatograms, it is evident that the increased flow rate slightly sharpens all chromatographic peaks. The increased flow rate also allows all analytes to elute in less time compared to the separation presented in Figure 4.4A. Since sharp peaks were important for ensuring highly accurate and precise quantification of analytes during the analysis of real produce samples, 0.7 mL/min was deemed the optimal flow rate for the separation of these fungicides.

4.1.2 ^{2}D method development

Once the method for ^{1}D separation of all four fungicides was completed, the optimization process for ^{2}D separation could begin. With ^{2}D optimization, the goal was to ensure all peaks were symmetrical and sharp in the second dimension to ensure low ^{2}D limits of detection and limits of quantification. Additionally, proper symmetry of all peaks would ensure that the analytes would be adequately resolved from other components that could be present in produce samples.

4.1.2.1 Column

In this study, the ZORBAX Bonus RP (2.1 mm x 50 mm x 1.8 µm) column was used for ^{2}D analysis of the analytes of interest. This column was made by the manufacturer using a packing consisting of sterically protected diisopropyl-C14 chains bound to a ZORBAX Rx-SIL porous silica support, as a dense and uniform monolayer.[80] This column is best suited for pH values of 2-

9 and is often used for orthogonal separations, like those performed in this study. The stationary phase had a pore size of 80 Å, and the column could withstand pressures up to 1300 bar.

4.1.2.2 Mobile phase solvents

As discussed in the theory section of this thesis, two-dimensional separations should be designed so that the ^{2}D conditions are orthogonal to the ^{1}D conditions. To optimize the orthogonality of the 2D-LC separation, ^{2}D mobile phase solvents had to be chosen so that they were chemically distinct from the ^{1}D solvents, while still being miscible with them. With this in mind, the mobile phase solvents for ^{2}D separations included water + 0.1% FA and MeOH + 0.1 % FA. Notably, these solvents were also acidified with formic acid, as acidification provided the best analyte separation and peak shape in the ^{1}D.

4.1.2.3 Flow rate

As the ^{2}D flow rate is independent of the ^{1}D flow rate, it is a parameter that could be optimized for the ^{2}D separation of PYRI, FLU, BOS, PYRA, and the IS. In ^{2}D analysis, the flow rate is typically much higher than the flow rate in the ^{1}D to maximize the throughput of the entire 2D-LC method[81]. As described in section 4.1.1.5, an increased flow rate can result in sharper peaks, which are ideal for any chromatographic analysis.

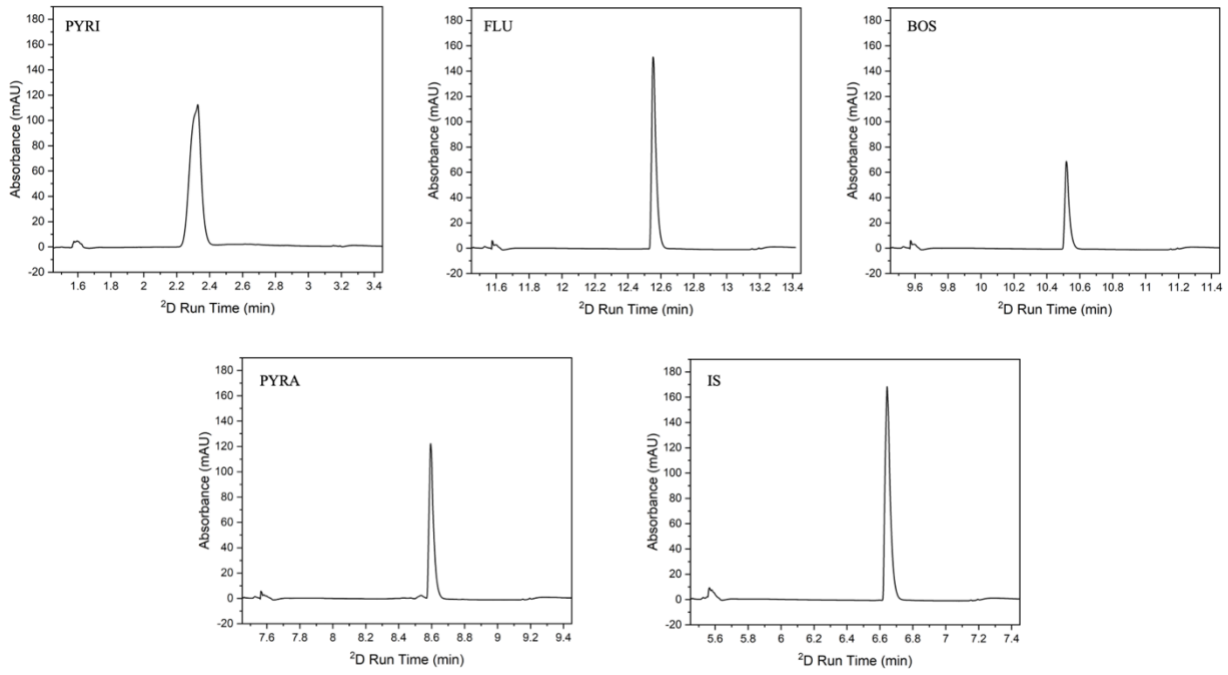


Figure 4.5. 2D chromatograms of FLU, BOS, PYRI, PYRA and IS. Analytes were eluted at 0.7 mL/min. Gradient: 20% MeOH + 0.1% FA from 0-0.2 min and 90% MeOH + 0.1% FA from 1.0-2.0 min. All analytes were separated in the ^{1}D using the optimized method prior to being analyzed in the ^{2}D.

Figure 4.5 shows the ^{2}D elution of all fungicides and the IS using a gradient elution of 20% MeOH + 0.1% FA from 0-0.2 min and 90% MeOH + 0.1% FA from 1.0-2.0 min. The balance of the mobile phase at all stages is water + 0.1% FA. The flow rate for this method was 0.7 mL/min. Using this method and flow rate, a very broad peak for PYRI was produced, with a peak width of 0.063. Additionally, all peaks were asymmetrical with asymmetry values ranging from 0.535 to 2.160. Based on the rate theory of chromatography, longitudinal diffusion will decrease with increased flow rate, therefore sharpening chromatographic peaks.[69] With this in mind, and based on 2D-LC methods previously reported in the literature, the flow rate was increased to 1.0 mL/min in the ^{2}D.[77,82]

4.1.2.4 Mobile phase composition

With ^{2}D method development, the final optimized method must produce sharp and symmetrical peaks for all analytes. These two factors are important as high peak symmetry and sharpness will ensure that the analyte peaks will not be affected by the presence of peaks derived from the sample matrix.

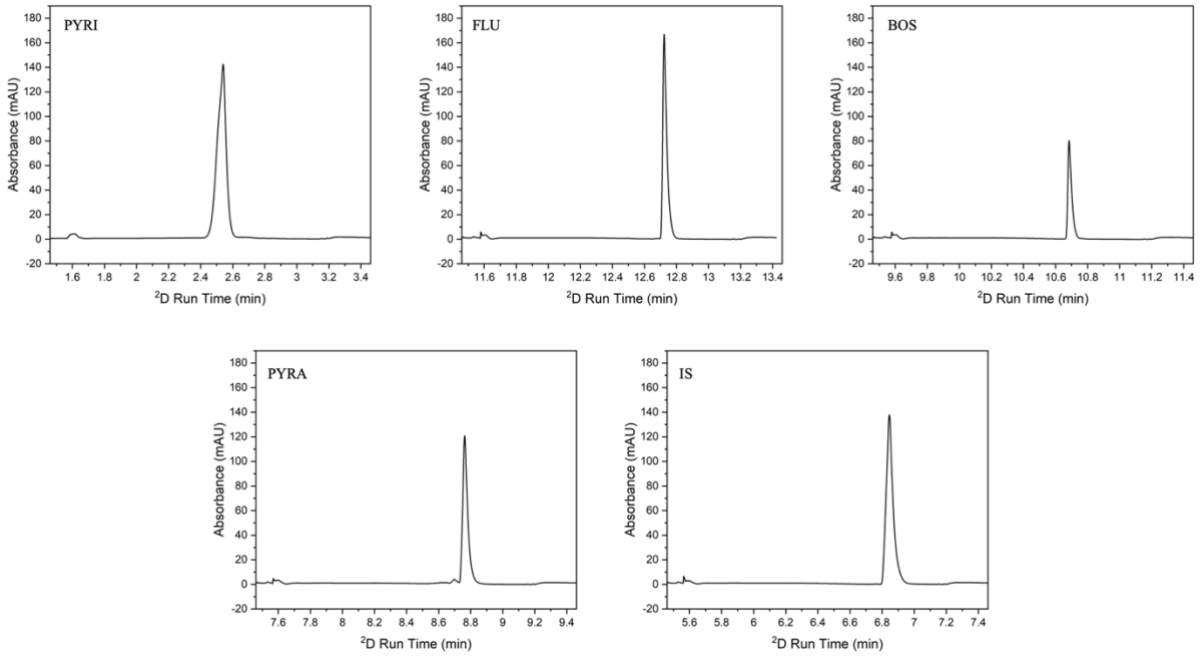


Figure 4.6. 2D chromatograms of FLU, BOS, PYRI, PYRA, and IS. Analytes were eluted at 1 mL/min. Gradient: 20% MeOH + 0.1% FA from 0-0.5 min, 85% MeOH + 0.1% FA from 1.0-1.5 min, and 20% MeOH + 0.1% FA at 2.0 mins. All analytes were separated in the ^{1}D using the optimized method prior to being analyzed in the ^{2}D.

Figure 4.6 shows the ^{2}D elution of all fungicides and the IS using a gradient elution of 20% MeOH + 0.1% FA from 0-0.5 min and 80% MeOH + 0.1% FA from 1.0-2.0 min. The balance of the mobile phase at all stages is water + 0.1% FA. Using this ^{2}D method, peaks for all analytes and the IS did not have acceptable symmetry and peak width values. More specifically, all ^{2}D peaks

using this method had asymmetry values outside of the 0.75-1.25 range generally considered acceptable. Additionally, the peaks for PYRI and the IS had large peak widths of 0.051 and 0.041, respectively. As this method produced peaks that were both asymmetrical and wide, ^{2}D optimization continued.

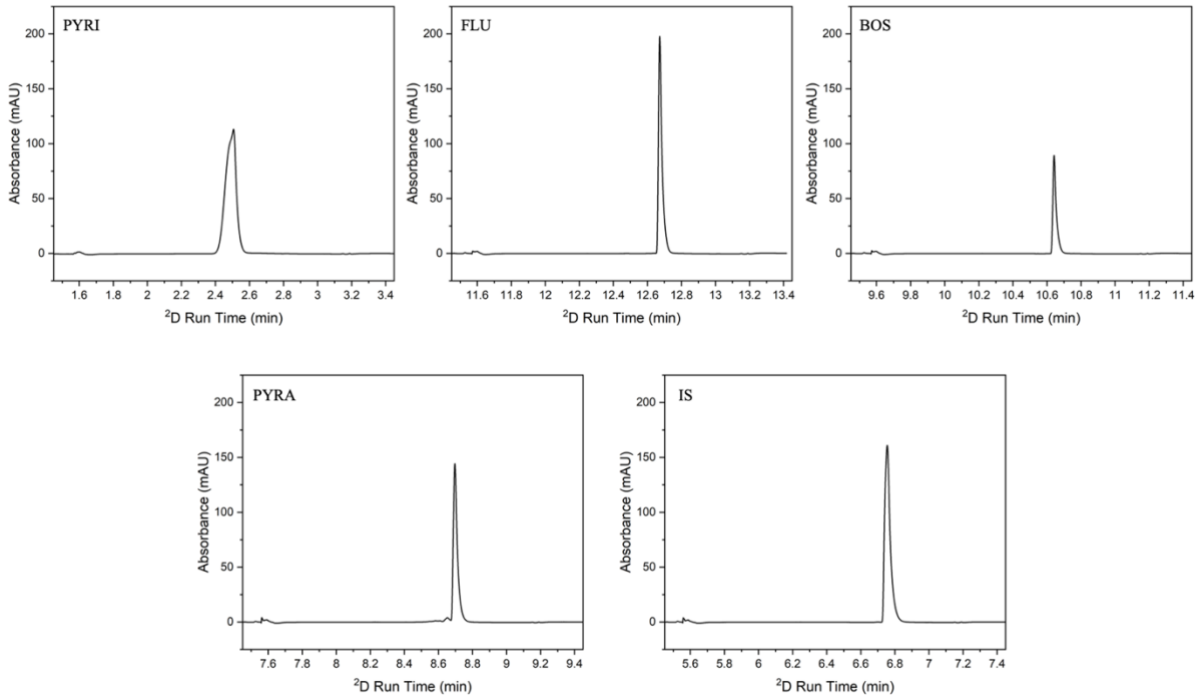


Figure 4.7. ^{2}D chromatograms of FLU, BOS, PYRI, PYRA and IS. Analytes were eluted at 1 mL/min. Gradient: 20% MeOH + 0.1% FA from 0-0.5 min, 85% MeOH + 0.1% FA from 1.0-1.5 min, and 20% MeOH + 0.1% FA at 2.0 mins. All analytes were separated in the ^{1}D using the optimized method prior to being analyzed in the ^{2}D.

Figure 4.7 shows the ^{2}D elution of all fungicides and the IS using a gradient elution of 20% MeOH + 0.1% FA from 0-0.5 min, 85% MeOH + 0.1% FA from 1.0-1.5 min, and 20% MeOH + 0.1% FA at 2.0 mins. The balance of the mobile phase at all stages is water + 0.1% FA. In the ^{1}D, analytes were eluted using the optimized ^{1}D method. Using this method, the peak for PYRI was

very broad, with a peak width of 0.056. Additionally, some fronting can be observed for PYRI, leading to non-deal peak symmetry. The ^{2}D peaks for FLU, BOS, PYRA, and the IS were all very sharp using this method, with peak widths ranging from 0.021 to 0.037. Although these peaks were all relatively sharp, which would increase the LOD and the LOQ of this method, they had poor symmetry. The ^{2}D peaks for FLU, BOS, PYRA, and the IS had low asymmetry values ranging from 1.38 to 2.02. As poor symmetry values can lead to inaccurate peak integration in a complex matrix, ^{2}D method optimization continued in an effort to improve peak widths and shapes.

Throughout the ^{2}D optimization process, the mobile phase gradient was changed many times to optimize the peak width and symmetry for all fungicides being analyzed. Notably, as peaks became more symmetrical, they often became broader. Similarly, as the peaks became sharper, they were often less symmetrical. The final optimization represents a compromise between these two parameters.

4.1.3 Optimized 2D-LC method

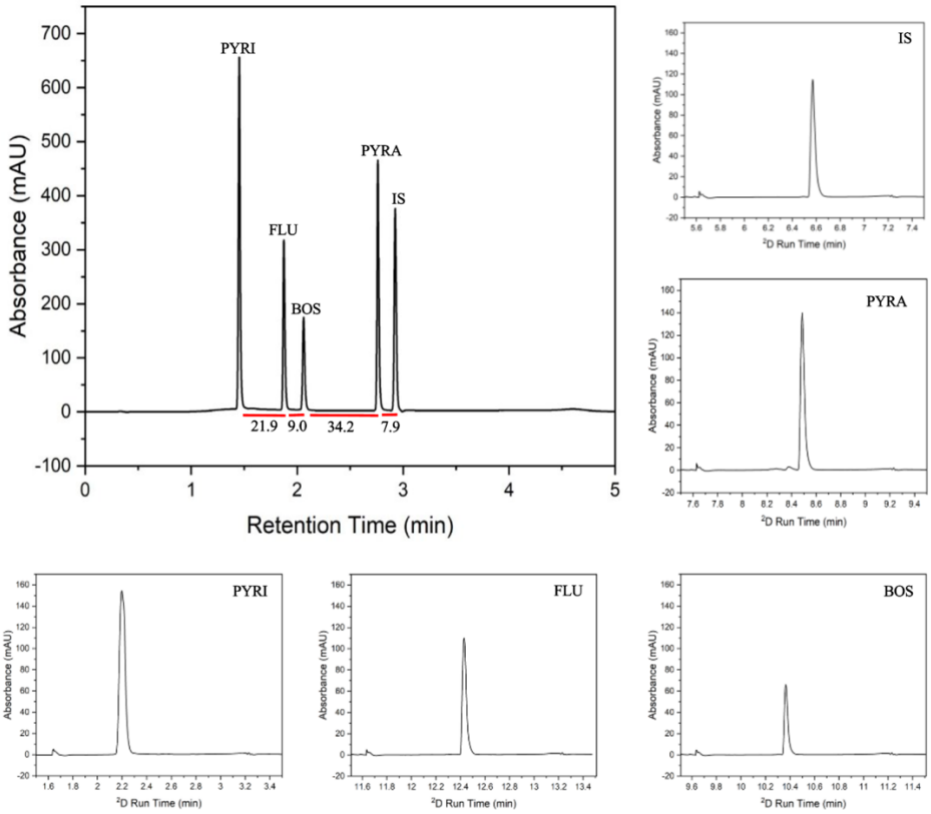


Figure 4.8. Optimized 2D-LC method for the separation of FLU, BOS, PYRI and PYRA. In the first dimension the gradient was as follows: 30% ACN + 0.1% FA at 0 min, 62% ACN + 0.1% FA at 0.5 min, 65% ACN + 0.1% FA at 1.5 min, 85% ACN + 0.1% FA from 2-3.5 min, and 30% ACN + 0.1% FA from 4-5 min. The flow rate was 0.7 mL/min. In the second dimension the gradient was as follows: 10% MeOH + 0.1% FA at 0 min, 70% MeOH + 0.1% FA at 0.5 min, 100% MeOH + 0.1% FA at 1.5 min. The total run time was 2 min, and the flow rate was 1.0 mL/min. The resolutions between peaks are included under the red lines in the ^{1}D chromatogram.

Figure 4.8 shows the optimized method for the separation of all fungicides and the IS. In the first dimension, compounds were separated using a gradient elution of 30% ACN + 0.1% FA at 0 min, 62% ACN + 0.1% FA at 0.5 min, 65% ACN + 0.1% FA at 1.5 min, 85% ACN + 0.1% FA from 2-3.5 min, and 30% ACN + 0.1% FA from 4-5 min. The flow rate was 0.7 mL/min. In the second dimension, compounds were separated by a gradient elution of 10% MeOH + 0.1% FA at

0 min, 70% MeOH + 0.1% FA at 0.5 min, and 100% MeOH + 0.1% FA at 1.5 min. The total run time was 2 mins. The flow rate was 1.0 mL/min. The balance of the mobile phase at all stages in both dimensions was water + 0.1% FA. The resolution values between each peak in the ^{1}D chromatogram was calculated and are included under the red lines on the ^{1}D chromatogram. The resolution values for the ^{1}D separation span from 7.9 to 34.2, which are all found above the accepted minimum of 1.5.[83] Resolution calculations did not apply to ^{2}D chromatograms, as only one peak eluted for all ^{2}D separations.

As previously mentioned, when developing this 2D-LC method, a primary goal was to obtain symmetrical and sharp peaks for all analytes in both dimensions. Chromatographic parameters for the ^{1}D and ^{2}D optimized methods are outlined in detail in Tables A1 and A2. For the ^{1}D separations of the fungicides of interest, the asymmetry values of the peaks raged from 0.81 to 0.93. The peaks in the ^{1}D chromatogram were also very sharp, with peak widths ranging from 0.020 to 0.024. The highly symmetrical and sharp nature of the analyte peaks in the ^{1}D ensured that all peaks were properly resolved and could be sent to the ^{2}D using the pre-determined sampling time interval. For the ^{2}D separation, peak asymmetries were calculated to range from 0.60 to 1.28. The peak widths for all analyte peaks ranged from 0.027 to 0.038. Although most ^{2}D peaks had acceptable symmetry and peak width values, some had parameters that fell outside the acceptable range. Chromatographic parameters such as symmetry and peak width can be more difficult to optimize due to the limited run time of ^{2}D separations. Despite these deviations from ideal parameter ranges, the separation of analytes in the ^{2}D was not affected.

4.2 Optimized 2D-LC method validation

To ensure high performance of the optimized 2D-LC method, validation experiments were performed. Reproducibility validation experiments are designed to quantify the precision of the method, while recovery validation experiments are designed to quantify the accuracy of the method.

4.2.1 Reproducibility validation

Reproducibility validation was completed for the optimized 2D-LC method to ensure that the method was highly precise. Specifically, intraday reproducibility determines the degree to which chromatographic runs using the optimized method are similar to each other when performed several times throughout a single day. Interday reproducibility determines the degree to which chromatographic runs using the optimized method are similar to each other when performed several times on consecutive days (one run per day).

4.2.1.1 Intraday Validation

The 2D-LC method developed was validated by intraday reproducibility studies, which involved running a standard solution of 50 ppm FLU, BOS, PYRI, PYRA, and phenanthrene 3 times throughout a single day. An overlay of the three chromatograms used to determine intraday reproducibility is displayed in Figure 4.9.

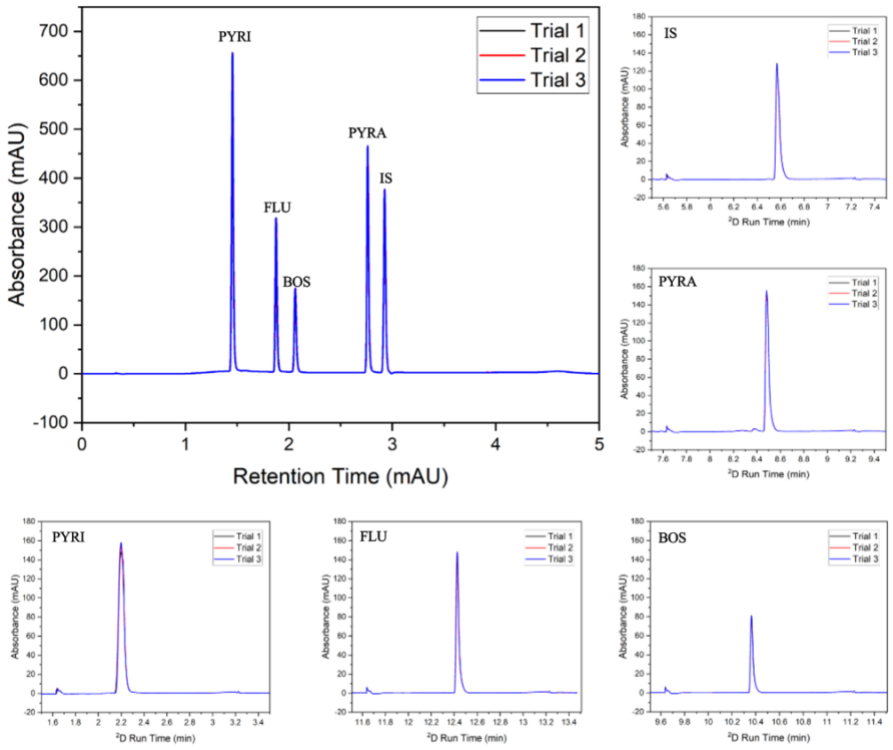


Figure 4.9. Overlay of intraday reproducibility chromatograms for the separation of FLU, BOS, PYRI, PYRA, and the IS using the optimized 2D-LC method. (N=3)

The reproducibility of these chromatographic runs was evaluated by calculating the coefficient of variation (%RSD). The %RSD for peak areas and retention times in both dimensions are provided in Table 4.2. The data used to calculate RSD values for intraday validation are outlined in Table A3. The %RSD values for all peak areas ranged from 0.026% - 0.991%, while %RSD values for all retention times ranged from 0.000% - 0.484%. Based on guidelines provided by the European Commission, the %RSD for reported results should not exceed 30% when the sample concentration is significantly higher than the LOD. The determined %RSD values for peak areas and retention times are well below the limit reported by the European Commission, suggesting high intraday reproducibility of the developed 2D-LC method.

Table 4.2. Intraday %RSD values for retention times and peak areas. Data were collected in triplicate on the same day (N=3).

Coefficient of Variation (%RSD) for Intraday Validation				
	^{1}D Retention Time / min	**^{1}D Peak Area**	**^{2}D Retention Time / min**	**^{2}D Peak Area**
Pyrimethanil	0.032	0.188	0.068	0.600
Fludioxonil	0.067	0.119	0.185	1.252
Boscalid	0.040	0.201	0.055	2.199
Pyraclostrobin	0.017	0.037	0.048	0.809
Phenanthrene	0.000	0.016	0.089	1.190

4.2.1.2 Interday Validation

Interday reproducibility testing of the proposed 2D-LC method is accomplished by running the same standard solution as previously described for intraday validation, but 5 times, over 5 consecutive days. Using this process, the coefficient of variation (%RSD) between experimentation days for retention times and peak areas in both dimensions was determined. The 5 runs collected for this validation were overlayed for comparison, which is included in Figure 4.10.

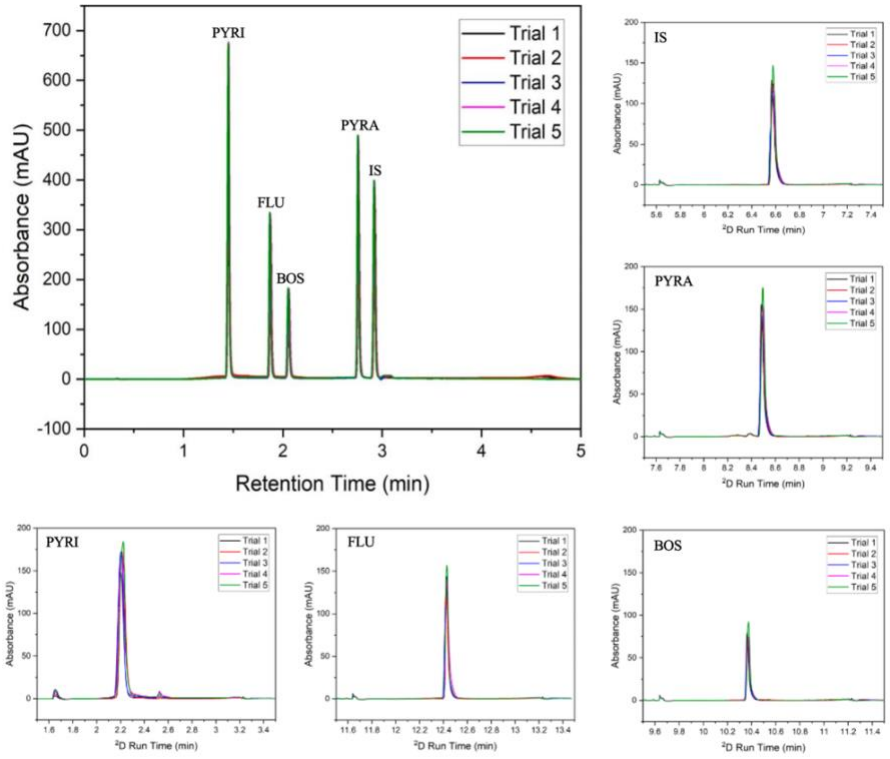


Figure 4.10. Overlay of interday reproducibility chromatograms for the separation of FLU, BOS, PYRI, PYRA and the IS using the optimized 2D-LC method (N=5).

The interday RSD values are presented in Table 4.3. The data used to calculate RSD values for interday validation are outlined in Table A4. Based on guidelines provided by the European Commission, the %RSD for reported results should not exceed 30% when the sample concentration is significantly higher than the LOD. The coefficients of variation calculated for all peak areas ranged from 1.032%–7.827%, while the coefficients of variation for all retention times ranged from 0.088%-1.212%. The interday repeatability %RSD values fall well below the accepted limit.

Table 4.3. Interday %RSD values for retention times and peaks areas. Data were collected once per day for 5 consecutive days (N=5).

Coefficient of Variation (%RSD) for Interday Validation				
	^{1}D Retention Time / min	**^{1}D Peak Area**	**^{2}D Retention Time / min**	**^{2}D Peak Area**
Pyrimethanil	0.128	3.435	1.212	7.352
Fludioxonil	0.133	1.577	0.288	4.577
Boscalid	0.119	1.032	0.403	6.363
Pyraclostrobin	0.093	3.617	0.419	5.469
Phenanthrene	0.088	1.742	0.415	7.827

4.2.2 Recovery validation

4.2.2.1 Recovery validation: AOAC QuEChERS-2D-LC method

As previously noted, the first QuEChERS method evaluated for sample preparation in this study was the AOAC-validated QuEChERS method.[34] Using this method, a produce sample can be spiked with a known concentration of mixed fungicide solution to determine the recovery of the entire method, including errors from the clean-up method and the 2D-LC separation. To initiate the determination of the AOAC QuEChERS-2D-LC method, a Nova Scotian blueberry sample was prepared using the AOAC QuEChERS method previously outlined in section 3.3.2. The spiking solution for this experiment was a 3000 mg/L mixed fungicide and internal standard solution. After the sample was prepared using the QuEChERS method, it was analyzed by the optimized 2D-LC method. Peak areas of ^{2}D analyte peaks were determined from the resulting data. The percent recovery for each analyte was analyte was calculated using equation 3.5.1. Table 4.4 outlines the recovery values calculated for each analyte using the AOAC QuEChERS method.

Table 4.4. Mean recoveries for analytes using the AOAC QuEChERS-2D-LC method (N=2).

	PYRI	FLU	BOS	PYRA	IS
Recovery (%)	19.4 ± 12.1	17.5 ± 5.7	20.8 ± 2.5	11.2 ± 15.8	18.7 ± 14.4

The recovery values for all fungicides and the IS were very low using this method. Additionally, standard deviations were very high, indicating that the method had very low precision. To reliably detect and quantify the fungicides of interest in produce items, a clean-up method with acceptable recovery was required. Therefore, a modified QuEChERS method was explored to increase the recoveries for all analytes.

4.2.2.2 Recovery validation: Modified QuEChERS-2D-LC method

Considering the overall poor recovery of the AOAC QuEChERS-2D-LC method, a modified QuEChERS presented by Zhao et al. was explored.[33] This method was reported to improve recoveries for planar compounds, that could adsorb to GCB during the d-SPE step of the clean-up procedure. The method for recovery determination was completed exactly as outlined for the AOAC QuEChERS method but using the modified QuEChERS method outlined in section 3.3.3. The produce sample for this recovery study was the same Nova Scotian blueberry sample used for the determination of the recoveries for the AOAC QuEChERS-2D-LC method. Table 4.5 outlines the recovery values calculated for each analyte using the modified QuEChERS method reported by Zhao et al.[33]

Table 4.5. Mean recoveries for analytes using the modified QuEChERS-2D-LC method (N=2).

	PYRI	FLU	BOS	PYRA	IS
Recovery (%)	74.6 ± 6.0	68.2 ± 1.6	54.1± 4.3	101.1 ± 4.5	59.4 ± 7.5

Notably, the ^{2}D peak for BOS slightly co-eluted with an interfering component from the clean-up procedure. The blank ^{1}D and ^{2}D chromatograms for the modified QuEChERS clean-up procedure can be found in Figure A2 of the appendix. For this reason, manual peak de-convolution and integration was performed to calculate recoveries. This manual procedure may have introduced additional error compared to the automatic integration completed for all other analytes. Although the modified QuEChERS method provides better recoveries for certain pesticides, it introduces additional potentially interfering peaks compared to the AOAC QuEChERS method. This is an inherent limitation of this modified clean-up procedure.

When comparing the recoveries obtained for the AOAC QuEChERS 2D-LC method and the modified QuEChERS 2D-LC method, it is evident that the latter provides significantly improved recoveries for all fungicides and for the internal standard. Therefore, the increased recoveries for all analytes are consistent with the results reported for different pesticide analytes by Zhao et al.[33] The authors of this report suggest that the added toluene may reduce the affinity of the pesticides for the GCB d-SPE sorbent, or may participate in the back-extraction of the pesticides from the GCB.[33] Since the addition of toluene significantly increases recoveries, the modified QuEChERS was deemed the best method for use in this research, as compared to the AOAC QuEChERS-2D-LC method.

4.2.2.3 Recovery validation: Modified QuEChERS method alone

Although the recovery determination procedure outlined in section 4.2.2.1 allows for an informative look at the total recovery of the QuEChERS-2D-LC method, knowing the recovery of the clean-up method alone can be important for understanding data. As previously discussed, this

study employed the Matuszewski method, which allows for the calculations of recovery independent from errors not associated with the clean-up procedure. This method involved the analysis of a Nova Scotian blueberry sample two times with two replicates per analysis. Specifically, the sample was analyzed by 2D-LC once where it was spiked with the mixed fungicide standard at the beginning of the QuEChERS method (directly in homogenized blueberry), and once where it was spiked at the end of the QuEChERS procedure (before evaporation and reconstitution). Equation 3.5.1 was used to calculate the recovery for each analyte, using ^{1}D peak areas, which is attributed to the QuEChERS procedure alone. The calculated recovery values using this method are outlined in Table 4.6.

Table 4.6. Calculated QuEChERS recoveries for each analyte using the Matuszewski method (N=2).

	PYRI	**FLU**	**BOS**	**PYRA**	**IS**
Recovery (%)	59.3 ± 2.0	57.1 ± 3.3	75.3 ± 2.1	67.3 ± 1.9	51.3 ± 1.4

Notably, as with the other recovery methods, the ^{2}D peak for BOS slightly co-eluted with a component from the QuEChERS clean-up. For this reason, manual peak de-convolution and integration was performed to calculate recoveries. This manual procedure may have introduced additional error compared to the automatic integration completed for all other analytes.

Based on standards put in place by the European Union for the analysis of pesticide residues, recovery values between 60-140% are generally acceptable.[84] Additionally, in cases where accuracy is low, but precision between replicates is high, these methods may be considered acceptable. Therefore, with error considered, the percent recoveries for all analytes except for the internal standard are considered acceptable. Even though the QuEChERS method used in this thesis may be accepted, future work should involve optimizing the method for the specific

fungicides studied to increase the overall recovery of the clean-up procedure. In future studies, an alternative internal standard for this may be considered to increase IS recovery. Notably, Zhao et al. determined the recovery of this method for 16 pesticides, none of which are studied in this thesis. Recoveries for the 16 pesticides using this method ranged from 20.4% - 109.1%, with recoveries generally increasing as compared to the recoveries determined for the original AOAC QuEChERS method.

4.2.2.4 Recovery validation: Effect of matrix effects

When analyzing samples with complex matrices, such as produce samples, it is important to understand how the matrix is affecting the accuracy and precision of the analysis. Using the Matuszewski method, ME can be quantified, providing an understanding of how chromatographic peaks of analytes are potentially enhanced or suppressed because of the matrix in which they are being analyzed. Quantifying the ME present in a Nova Scotian blueberry sample involved analyzing the sample where it was spiked with mixed fungicide standard at the beginning of the QuEChERS method (directly in homogenized blueberry). Additionally, a 10 mg/L mixed standard solution containing all fungicides of interest was analyzed using the optimized 2D-LC method. Percent ME was calculated for each analyte of interest using Equation 3.5.2. The results of these calculations are outlined in Table 4.7.

Table 4.7. Quantification of matrix effects for every analyte using the Matuszewski method (N=2).

	PYRI	**FLU**	**BOS**	**PYRA**	**IS**
% Matrix Effects	-4.0 ± 4.7	-82.5 ± 19.3	19.1± 1.3	-11.8 ±14.1	-9.2 ± 10.6

Notably, as with the recovery methods, the ^{2}D peak for BOS slightly co-eluted with a component from the QuEChERS clean-up. For this reason, manual peak de-convolution and

integration was performed to calculate recoveries. This manual procedure may have introduced additional error compared to the automatic integration completed for all other analytes.

As previously discussed, a negative percent ME value signifies that the analyte peak is being enhanced due to the matrix in which it is being analyzed. This is the case for PYRI, FLU, PYRA, and the IS. Specifically, FLU has a significant peak enhancement, with a percent ME of -65.5%. Alternatively, BOS shows a positive percent ME value, which means that the peak for BOS is being suppressed by the matrix. These values are consistent with the increase in recovery observed for PYRI, FLU, BOS, and the IS using the Matuszewski method (clean-up recovery alone), compared to the recovery calculated for the modified QuEChERS-2D-LC method. Similarly, the results are consistent with the decrease in recovery for BOS using the Matuszewski method (clean-up recovery alone), compared to the recovery calculated for the modified QuEChERS-2D-LC method. To decrease the interference of the produce matrix on the peaks being analyzed, future work should focus on optimizing the QuEChERS clean-up procedure to remove additional matrix components that could be interfering with sample analysis.

4.3 Internal standard calibration

Internal standard calibration is a method that is often employed in chromatographic analysis for the monitoring and compensation of many random and systematic errors.[67] The internal standard is chosen assuming it will react similarly to all analytes in response to errors that could occur during analysis, such as sample loss, temperature changes, etc.. For this study, the internal standard was chosen to be phenanthrene, as it has previously been reported as an IS for the fungicides studied in this thesis.[23]

To accurately quantify fungicides that could be found in the analyzed produce samples, internal standard calibration was employed. The process started with preparing calibration standards with an IS (phenanthrene) concentration of 10 mg/L and varying concentrations of the fungicides of interest (0.5 mg/L to 15 mg/L). Notably, the calibration curve for PYRI only has 5 calibration points, as PYRI concentrations below 1.0 mg/L produced peaks that were too weak to be properly integrated. The compositions of these calibration solutions are outlined in Table 3.3 in section 3.6. Each calibration solution was run in triplicate on the 2D-LC using the optimized method. For each fungicide peak at a given concentration, the ^{2}D peak area was divided by the ^{2}D peak area of the IS in the same run. This ratio is called the signal response. The average signal response at each concentration (with its standard deviation) was plotted against its respective analyte concentration to produce ^{2}D internal standard calibration curves for each analyte. A linear regression was performed for each plot to determine the line of best fit, as well as the coefficient of determination (R^2). These ^{2}D calibration curves are included in Figure 4.11. ^{1}D peak areas were used to produce ^{1}D internal standard calibration curves for each fungicide. These ^{1}D calibration curves are available in the appendix as Figure A3. The error in regression for the ^{2}D and ^{1}D calibration curves are present in Table A5 of the appendix.

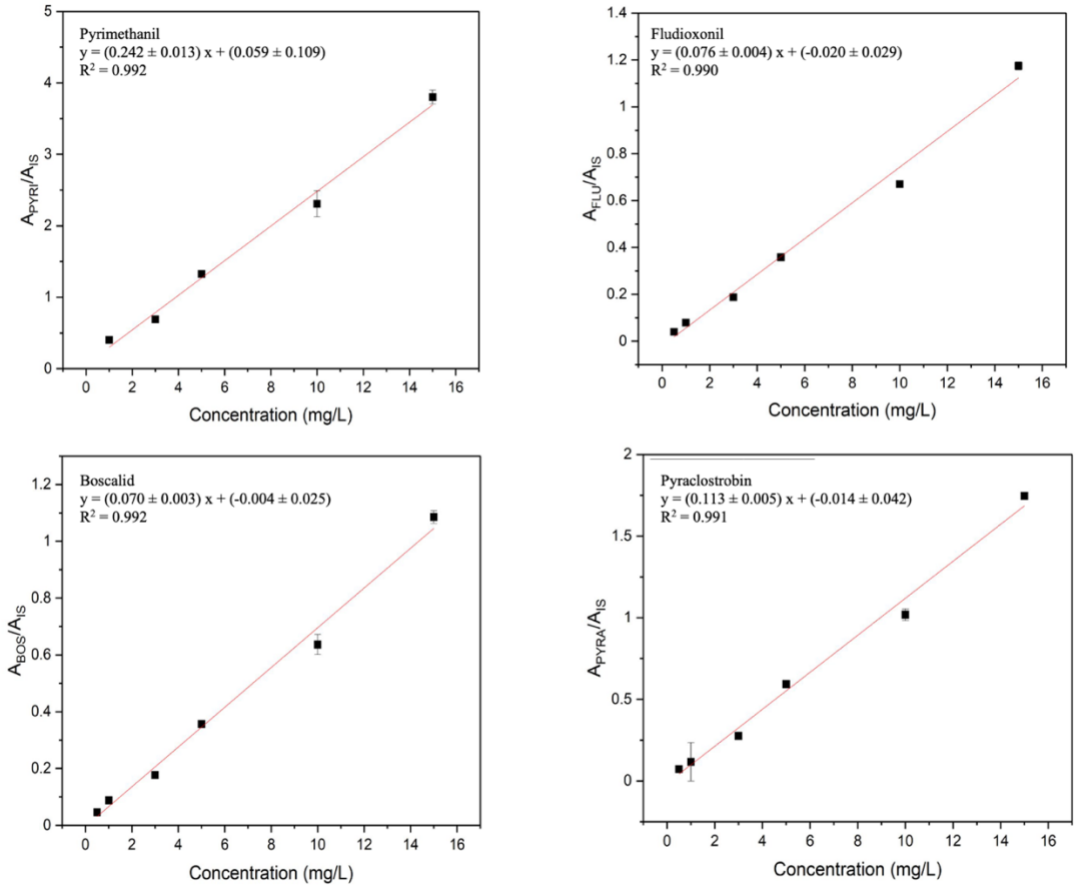


Figure 4.11. ^{2}D internal standard calibration curves for PYRI, FLU, BOS and PYRA. All plots include the equation of the line of best fit and the coefficient of determination (R^2).

For all analytes, the 0.5 mg/L mixed calibration standard was the lowest concentration at which the analyte peak could be accurately integrated by the software in the ^{2}D. For this reason, the analyte peaks at this concentration were considered to be spiked blanks and used for the calculation of ^{2}D limits of detection (LOD) and ^{2}D limits of quantification (LOQ) for each analyte. To calculate these values, the 0.5 mg/L was analyzed on the 2D-LC using the optimized method 10 consecutive times. The signal response was calculated for each trial using the peak area of the IS. Using this data, the average signal response was determined. Using Equations 3.61 and 3.62,

the ^{1}D and ^{2}D, LOD and LOQ values were determined for all analytes. These values are outlined in Table 4.8. This process was repeated using ^{1}D peak areas to determine the ^{1}D LOD and LOQ values for each analyte.[72]

Table 4.8. LOD and LOQ values for PYRI, FLU, BOS and PYRA using the optimized 2D-LC method.

	First Dimension				Second Dimension			
	PYRI	FLU	BOS	PYRA	PYRI	FLU	BOS	PYRA
LOD (mg/L)	0.051	0.014	0.029	0.070	0.043	0.095	0.096	0.131
LOQ (mg/L)	0.171	0.048	0.097	0.234	0.143	0.317	0.321	0.436

For most analytes (except PYRI), the LOD and LOQ increased in the ^{2}D, indicating that the ^{2}D separation has lower sensitivity than the ^{1}D separation. Decreased sensitivity in the ^{2}D is an inherent limitation to 2D-LC and contributes to the trade-off between sensitivity and peak capacity. This decrease in sensitivity is primarily related to the dilution of the ^{1}D effluent as a cut is transferred to the ^{2}D column. Despite the decreased sensitivity of the ^{2}D quantification, the 2D-LC method remains an ideal separation method for the analysis of complex samples due to its increased peak capacity and its ability to avoid false positives.

The LODs and LOQs for this method, in both dimensions, are below the Canadian MRL of blueberries, grapes, and pears, for all fungicides of interest.[19] Therefore, using this method, fungicides that are above the acceptable MRL for their respective matrix will be able to be detected and quantified. Therefore, the sensitivity of the optimized 2D-LC method far surpasses the sensitivity needed for the scope of this thesis.

4.4 Produce sample analysis

The optimized 2D-LC method described in this thesis was designed to analyze produce items for the presence of the fungicides of interest, including PYRI, FLU, BOS and PYRA. For proof of concept and applicability, blueberries were chosen as a model produce sample, as the recovery experiments were conducted using blueberry samples. In this work, it was of interest to investigate how the country of origin of produce samples affects the presence and concentrations of PYRI, FLU, BOS and PYRA in the sample. Comparing produce samples from different countries is interesting, as different methods for the application of pesticides could be used, or different regulations could be in place for their concentration limits. For this reason, two blueberry samples were analyzed in this study; one from Canada and one from Peru.

When analyzing analytes in a complex matrix, such as blueberries, a shift in the retention times of the analytes of interest may occur due to matrix effects. To overcome this inconsistency, relative retention time (RRT) can be calculated. As all produce samples were spiked with the chosen IS (phenanthrene) prior to sample clean-up, its retention time can be used to accurately compare the retention time of an analyte in a complex matrix to its standard retention time in HPLC solvent. The RRT for an analyte is calculated using Equation 4.4.1:

$$RRT = \frac{R_T\ (analyte)}{R_T\ (internal\ standard)} \quad (4.4.1)$$

The RRT is calculated for the analyte in the sample matrix, and once again a standard solution. According to the European Commission, two retention times are deemed to be the same if the RRT of the analyte in the standard solution is equal to the RRT of the analyte in the sample matrix, with a margin of error of 2.5% of the sample RRT.[85]

4.4.1 Analysis of blueberry sample #1

To start, a Nova Scotian blueberry sample was analyzed using the optimized QuEChERS-2D-LC method. These blueberries were picked at a U-Pick in Centreville, Nova Scotia. They were stored in a freezer for 4 months before being analyzed. Before analysis, the blueberry sample was prepared using the modified QuEChERS method, where it was spiked with phenanthrene spiking solution. Figure 4.12A shows the ^{1}D chromatogram of the analyzed blueberry sample. In this chromatogram, a strong peak, boxed in red, elutes at 2.050 minutes. This peak is eluting during the sampling period for cut 3, which is designated for the fraction transfer of BOS, which has a retention time of 2.051 minutes. Additionally, the IS which was added to the blueberry sample is boxed in blue. In the ^{1}D, the peak boxed in red has a retention time within the margin of error for the retention time of BOS (2.058 ± 0.002), it could be concluded that this peak is BOS using just the ^{1}D. However, using the 2D-LC, more in-depth ^{2}D analysis can be completed.

Figures 4.12B and 4.12C show the ^{2}D chromatograms for cut 3 and cut 5 (IS), respectively. The peak in Figure 4.12B eluted at a ^{2}D run time of 0.856 minutes compared to standard BOS which elutes at a ^{2}D run time of 0.892 minutes. Using equation 4.4.1, RRT values were calculated for the sample peak, and the BOS standard peak. The RRT values for the sample peak and the BOS standard peak were 0.742 ± 0.019 and 0.813, respectively. Since the RRT for BOS is not within the margin of error for cut 3, it can be concluded that these peaks are not the same compound.

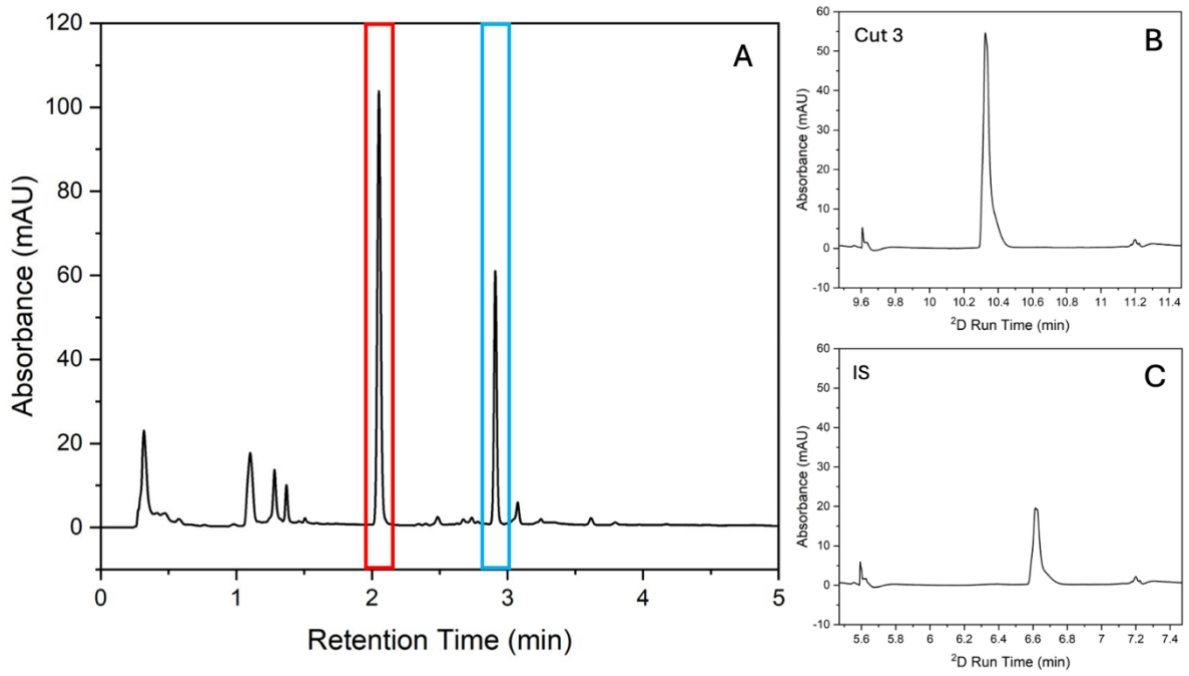


Figure 4.12. (A) [1]D chromatogram for the analysis of a Nova Scotian blueberry sample using the optimized QuEChERS-2D-LC method. A peak eluted during the sampling time of cut 3 is boxed in red. The peak for the phenanthrene IS is boxed in blue. (B) [2]D chromatogram of cut 3 from the [1]D separation. (B) [2]D chromatogram of the phenanthrene IS from the [1]D separation.

Using the developed QuEChERS-2D-LC, none of the fungicides of interest were detected in the Nova Scotian blueberry sample analyzed. However, this analysis serves as an ideal example of how false positives may occur in conventional 1D chromatographic analysis. As outlined, if the sample had not been further analyzed in the [2]D, it would have appeared that BOS had been detected in very high concentrations, as a peak appeared with a nearly identical retention time to the retention time of BOS. Using the power of [2]D separation, the sample was able to be further analyzed. Using this additional data, it was determined that the peak of interest was not BOS.

As mentioned for recovery experiments, the modified AOAC QuEChERS method introduced a component that partially co-eluted with the [2]D peak for BOS. The interfering peak eluted at a [2]D run time of 0.825 and had an RRT of 0.729 ± 0.018. As stated above, the strong peak

present in blueberry sample #1 elutes at a ^{2}D run time of 0.742 ± 0.019. A peak with an RRT within this margin of error also appears in chromatograms for blueberry samples analyzed for recovery experiments. This further suggests that the ^{2}D peak present in cut 3 for blueberry sample #1 is not BOS but is likely the same compound that is introduced during the modified QuEChERS method.

4.4.2 Analysis of blueberry sample #2

With the goal of analyzing an international blueberry sample using the optimized QuEChERS 2D-LC method, blueberries which had been grown in Peru, as distributed from the USA, were purchased at a local grocery store. Peruvian blueberries were chosen to be analyzed, as Peru is known to have poor farming practices, including misuse of pesticides.[86] In fact, the National Service of Agrarian Health reports (2011-2019) note that more than 10% of Peruvian produce samples tested exceeded the local MRLs.[87–95] The Peruvian blueberries were prepared using the modified QuEChERS method and analyzed using the optimized 2D-LC method. Figure 4.13A shows the ^{1}D chromatogram of the analyzed blueberry sample. In this chromatogram, a fairly weak peak, boxed in red, elutes at 2.050 minutes. This peak is eluting during the sampling period for cut 3, which is designated for the fraction transfer of BOS, which has a retention time of 2.051 minutes. Additionally, a weak peak, boxed in green, is eluting at 2.734 minutes. The internal standard, phenanthrene, is eluting as a peak which is boxed in blue. In the ^{1}D, the peak boxed in red has a retention time within the margin of error for the retention time of BOS (2.058 ± 0.002). Similarly, the peak boxed in green has a retention time within the margin of error for the retention time of PYRA (2.759 ± 0.003). Therefore, it could be concluded that these peaks are BOS and PYRA using just the ^{1}D. However, using the 2D-LC, more in-depth ^{2}D analysis can be completed.

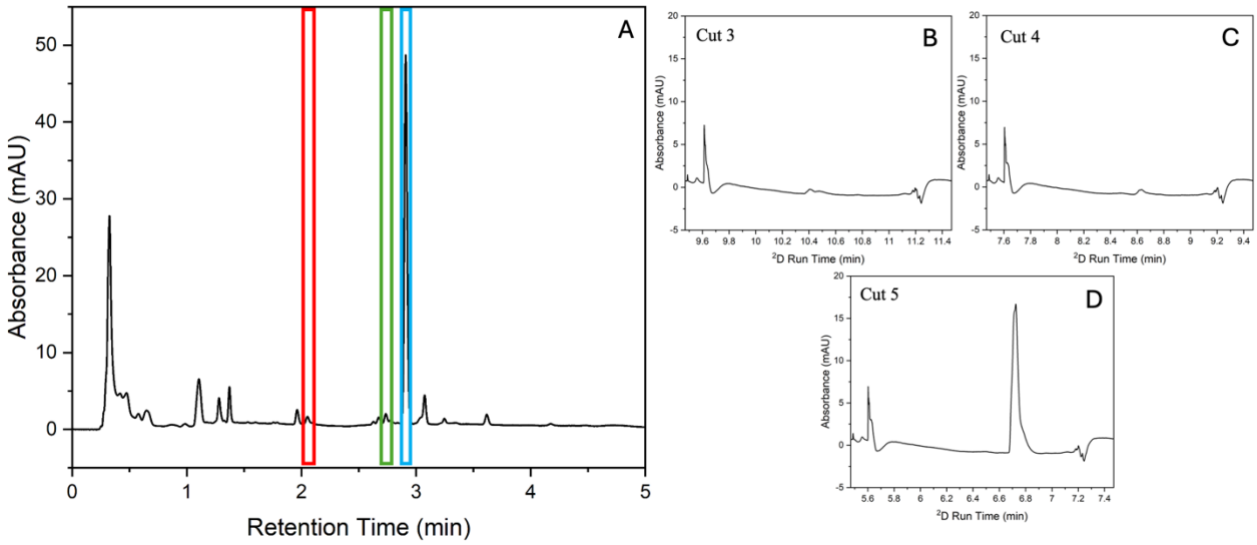


Figure 4.13. (A) ^{1}D chromatogram for the analysis of a Peruvian blueberry sample using the optimized QuEChERS-2D-LC method. A peak eluted during the sampling time of cut 3 (boxed in red) and during the sampling time of cut 4 (boxed in green). The peak for the phenanthrene IS is boxed in blue. (B) ^{2}D chromatograms of cut 3 and cut 4 from the ^{1}D separation. (B) ^{2}D chromatogram of the phenanthrene IS from the ^{1}D separation.

Figures 4.13B, 20C, and 20D show the ^{2}D chromatograms for cut 3, cut 4, and cut 5 (IS), respectively. The peak in Figure 4.13B eluted at a ^{2}D run time of 0.884 minutes compared to standard BOS which elutes at a ^{2}D run time of 0.866 minutes. Using Equation 4.4.1, RRT values were calculated for the sample peak, and the BOS standard peak. The RRT values for the sample peak and the BOS standard peak were 0.743 ± 0.019 and 0.809, respectively. Since the RRT for BOS is not within the margin of error for cut 3, it can be concluded that these peaks are not representative of the same compound. Notably, an interfering peak from the QuEChERS clean-up procedure elutes at a ^{2}D run time of 0.825 and had an RRT of 0.729 ± 0.018. Therefore, the peak eluting during the sampling time for cut 3 in Figure 4.13A can be attributed to the interfering compound from the QuEChERS clean-up procedure. Similarly, the peak in Figure 4.13C eluted at

a ^{2}D run time of 1.098 minutes compared to standard PYRA that elutes at a ^{2}D run time of 0.986 minutes. The RRT values for the sample peak and the PYRA standard peak were 0.923 ± 0.023 and 0.919, respectively. Since the RRT for PYRA falls within the margin of error for the RRT of the sample peak, it can be concluded that the small peak boxed in green in Figure 4.13A is PYRA. The ^{2}D peak was determined to be PYRA and the IS were integrated in the ^{2}D and were found to have peak areas of 0.55 and 25.44. When the area of the sample peak was divided by the peak area of the IS, the resulting ratio was 0.02. As this ratio value falls below the minimum ratio for the ^{2}D PYRA calibration curve constructed, the compound cannot be quantified, and PYRA is therefore not analytically detectable.

Using the developed QuEChERS-2D-LC, none of the fungicides of interest were detected in the Peruvian blueberry sample analyzed. However, in addition to the analysis of the Nova Scotian blueberry sample, this analysis serves as an ideal example of how false positives may occur in conventional 1D chromatographic analysis. Without the added power of ^{2}D separation, fungicides may have been falsely detected in the studied blueberry samples.

Chapter 5: Conclusions

This thesis reports a sensitive and selective 2D-LC method for the separation, detection, and quantification of PYRI, FLU, BOS, and PYRA in produce items. As a model matrix, blueberry samples were used to test the accuracy and reproducibility of the method. The 2D-LC method was optimized using standard solutions of all fungicides of interest. The final method provided sharp and resolved peaks for the fungicides in both dimensions. In the ^{1}D, resolution values for all peaks ranged from 7.9 to 34.2. Intraday and intraday validation studies were performed for the optimized method to test its reproducibility and precision. ^{2}D intraday coefficients of variation (CV) for retention times and peak areas ranged from 0.048% – 2.199% in both dimensions. ^{2}D interday coefficients of variation (CV) for retention times and peak areas ranged from 0.093% – 7.352% in both dimensions. A modified QuEChERS procedure was employed for the clean-up of produce samples.[5] ^{2}D recoveries were determined to range from 51.3% ± 1.4% to 75.3% ± 2.1% for all analytes. The recovery of this method is less than ideal for most analytes and should be optimized in future work. Internal standard calibration was completed for all fungicides using phenanthrene as the internal standard. The coefficient of determination for all four calibration curves ranged from 0.990 to 0.992. For ^{2}D analysis, the limit of detection (LOD) ranged from 0.043 – 0.131 mg/mL, while the limit of quantification (LOQ) ranged from 0.143 – 0.436 mg/mL. Using the optimized method, a Nova Scotian and a Peruvian blueberry sample were analyzed. The fungicides of interest were not detected in the analyzed samples using this method. Therefore, if present in the blueberry samples, the fungicides of interest are at a concentration below their respective MRLs.

Overall, the 2D-LC method reported in this work serves as a promising pathway for the detection of PYRI, FLU, BOS, and PYRA in produce items. Notably, the QuEChERS method used in this study produced non-ideal recovery values, which may have impacted the sensitivity of detection in the 2D-LC. Additionally, recovery studies were conducted using blueberry as a sample matrix. The recovery results reported in this thesis may differ if a different produce matrix was used. Therefore, the combination of low clean-up recoveries, and the inherent sample dilution that occurs during the transfer of an analyte to the ^{2}D, could have impacted the sensitivity of the entire QuEChERS-2D-LC method. Despite the decrease in sensitivity that the ^{2}D can introduce, the ^{2}D separation proved to be vital in avoiding false positives that commonly occur in 1D-LC analyses.

Chapter 6: Future Work

Although this 2D-LC method allowed for relatively precise and accurate separation and quantification of PYRI, FLU, BOS and PYRA, some improvements could be made to improve the performance and reliability of the method. First, the modified QuEChERS used for the clean-up of produce samples in this study produced relatively low recovery values of 51.3 ± 1.4% to 75.3 ± 2.1%. To improve the recovery of analytes during sample preparation, the QuEChERS method should be further optimized to increase the recovery of PYRI, FLU, BOS, and PYRA in several produce matrices. Further work could include repeating the recovery experiments completed in this thesis but using a different produce sample, such as grapes or pears.

Additionally, although the LODs and LOQs of this method are sufficient for the fungicides of interest, considering their respective MRLs, the sensitivity of the 2D-LC could be improved in future work. This goal could be attained by increasing the sharpness of analyte peaks, or by working to reduce baselines interferences. Decreasing the LODs and LOQs of this method would allow the analytes to be successfully quantified, even when their concentrations do not surpass their concentration limits. With this increased sensitivity, the optimized 2D-LC method could be used for assessing preventatively produce items for the presence of fungicides, before the MRL is surpassed.

Finally, future work should involve analyzing additional produce items such as grapes or pears for the presence of PYRI, FLU, BOS, and PYRA. Analyzing different produce samples would expand the scope of this method and allow for the determination of the method's performance when subjected to different produce matrices.

Chapter 7: References

(1) National Research Council. *Pesticides in the Diets of Infants and Children*; National Academies Press (US), 1993,13-22. DOI: 10.17226/2126

(2) Environmental Working Group. *Dirty Dozen™ Fruits and Vegetables with the Most Pesticides*. https://www.ewg.org/foodnews/dirty-dozen.php (accessed 2024-05-15).

(3) Kiljanek, T.; Niewiadowska, A.; Semeniuk, S.; Gaweł, M.; Borzęcka, M.; Posyniak, A. Multi-Residue Method for the Determination of Pesticides and Pesticide Metabolites in Honeybees by Liquid and Gas Chromatography Coupled with Tandem Mass Spectrometry—Honeybee Poisoning Incidents. *J. Chromatogr. A* **2016**, *1435*, 100–114. DOI:10.1016/j.chroma.2016.01.045.

(4) Eddleston, M. Pesticides. *Medicine (Baltimore)* **2016**, *44* (3), 193–196. DOI:10.1016/j.mpmed.2015.12.005.

(5) Kalyabina, V. P.; Esimbekova, E. N.; Kopylova, K. V.; Kratasyuk, V. A. Pesticides: Formulants, Distribution Pathways and Effects on Human Health – a Review. *Toxicol. Rep.* **2021**, *8*, 1179–1192. DOI:10.1016/j.toxrep.2021.06.004.

(6) Debnath, M.; Khan, M. S. Health Concerns of Pesticides. In *Pesticide Residue in Foods: Sources, Management, and Control*; Khan, M. S., Rahman, M. S., Eds.; Springer International Publishing: Cham, 2017, 103–118. DOI:10.1007/978-3-319-52683-6_6.

(7) European Environment Agency. *More action needed in the EU to reduce the impacts of chemical pesticides*, last revised April 2023. https://www.eea.europa.eu/en/newsroom/news/more-action-needed-in-the-eu (accessed 2024-09-27).

(8) Tudi, M.; Li, H.; Li, H.; Wang, L.; Lyu, J.; Yang, L.; Tong, S.; Yu, Q. J.; Ruan, H. D.; Atabila, A.; Phung, D. T.; Sadler, R.; Connell, D. Exposure Routes and Health Risks Associated with Pesticide Application. *Toxics* **2022**, *10* (6), 335. DOI:10.3390/toxics10060335.

(9) Center for Food Safety and Applied Nutrition. *Pesticide Residue Monitoring Report and Data for FY 2021*, last revised October 2023. https://www.fda.gov/food/pesticides/pesticide-residue-monitoring-report-and-data-fy-2021 (accessed 2024-09-25).

(10) Stone, N.; Kendall, C.; Shepherd, N.; Crow, P.; Barr, H. Near-Infrared Raman Spectroscopy for the Classification of Epithelial Pre-Cancers and Cancers. *J. Raman Spectrosc.* **2002**, *33* (7), 564–573. DOI:10.1002/jrs.882.

(11) Wu, M.; Bian, J.; Han, S.; Zhang, C.; Xu, W.; Tao, L.; Li, Z.; Zhang, Y. Characterization of Hepatotoxic Effects Induced by Pyraclostrobin in Human HepG2 Cells and Zebrafish Larvae. *Chemosphere* **2023**, *340*, 139732. DOI:10.1016/j.chemosphere.2023.139732.Selected Fungicides

(12) Luz, A. L.; Kassotis, C. D.; Stapleton, H. M.; Meyer, J. N. The High-Production Volume Fungicide Pyraclostrobin Induces Triglyceride Accumulation Associated with Mitochondrial Dysfunction, and Promotes Adipocyte Differentiation Independent of PPARγ Activation, in 3T3-L1 Cells. *Toxicol.* **2018**, *393*, 150–159. DOI:10.1016/j.tox.2017.11.010.

(13) Jabłońska-Trypuć, A.; Wydro, U.; Wołejko, E.; Makuła, M.; Krętowski, R.; Naumowicz, M.; Sokołowska, G.; Serra-Majem, L.; Cechowska-Pasko, M.; Łozowicka, B.; Kaczyński, P.; Wiater, J. Selected Fungicides as Potential EDC Estrogenic Micropollutants in the Environment. *Molecules* **2023**, *28* (21), 7437. DOI:10.3390/molecules28217437.

(14) Kugathas, S.; Audouze, K.; Ermler, S.; Orton, F.; Rosivatz, E.; Scholze, M.; Kortenkamp, A. Effects of Common Pesticides on Prostaglandin D2 (PGD2) Inhibition in SC5 Mouse Sertoli Cells, Evidence of Binding at the COX-2 Active Site, and Implications for Endocrine Disruption. *Environ. Health Perspect.* **2016**, *124* (4), 452–459. DOI:10.1289/ehp.1409544.

(15) Government of Canada. *Proposed Maximum Residue Limit PMRL2023-34, Fludioxonil,* last revised June 2023. https://www.canada.ca/en/health-canada/services/consumer-product-safety/pesticides-pest-management/public/consultations/proposed-maximum-residue-limit/2023/fludioxonil-sugar-beet-roots/document.html (accessed 2024-05-13).

(16) Pest Management Regulatory Agency. *Boscalid/BAS 510*. Health Canada, 2004. https://publications.gc.ca/collections/Collection/H113-7-2004-2E.pdf (accessed 2024-05-13).

(17) Government of Canada. *Proposed Maximum Residue Limit PMRL2016-38, Pyrimethanil*, last revised August 2016. https://www.canada.ca/content/dam/hc-sc/migration/hc-sc/cps-spc/alt_formats/pdf/pest/part/consultations/pmrl2016-38/pmrl2016-38-eng.pdf (accessed 2024-05-13).

(18) Pest Management Regulatory Agency. *Pyraclostrobin Headline EC Cabrio EG*. Health Canada, 2003. https://publications.gc.ca/collections/Collection/H113-7-2003-6E.pdf (accessed 2024-05-13)

(19) Maximum Residue Limits, Human Health, and Food Safety, 2023. https://www.canada.ca/en/health-canada/services/consumer-product-safety/pesticides-pest-management/public/protecting-your-health-environment/pesticides-food/maximum-residue-limits-pesticides.html (accessed 2024-09-29).

(20) U.S. Department of Agriculture. *Pesticide Data Program Annual Summary: Calendar Year 2022*, last revised January 2024. https://www.ams.usda.gov/sites/default/files/media/2022PDPAnnualSummary.pdf (accessed 2024-05-20).

(21) Ma, S.; Ji, R.; Wang, X.; Yu, C.; Yu, Y.; Yang, X. Fluorescence Detection of Boscalid Pesticide Residues in Grape Juice. *Optik* **2019**, *180*, 236–239. DOI:10.1016/j.ijleo.2018.11.106.

(22) Mandrile, L.; Giovannozzi, A. M.; Durbiano, F.; Martra, G.; Rossi, A. M. Rapid and Sensitive Detection of Pyrimethanil Residues on Pome Fruits by Surface Enhanced Raman Scattering. *Food Chem.* **2018**, *244*, 16–24. DOI:10.1016/j.foodchem.2017.10.003.

(23) Dost, K.; Öksüz, M.; Cittan, M.; Mutlu, B.; Tural, B. Determination of Boscalid, Pyraclostrobin and Trifloxystrobin in Dried Grape and Apricot by HPLC/UV Method. *J. Food Compos. Anal.* **2023**, *115*, 104926-. DOI:10.1016/j.jfca.2022.104926.

(24) Lv, L.; Su, Y.; Dong, B.; Lu, W.; Hu, J.; Liu, X. Dissipation Residue Behaviors and Dietary Risk Assessment of Boscalid and Pyraclostrobin in Watermelon by HPLC-MS/MS. *Molecules*. **2022**, *27* (14). DOI: 10.3390/molecules27144410

(25) Xiaoxin, C.; Sheng, H.; Yimeng, G.; Yecheng, M.; Jiye, H.; Xiaolu, L. Dissipation Behavior, Residue Distribution and Dietary Risk Assessment of Field-Incurred Boscalid and Pyraclostrobin in Grape and Grape Field Soil via MWCNTs-Based QuEChERS Using an RRLC-QqQ-MS/MS Technique - ScienceDirect. *Food Chem.* **2019**, *274*. DOI:10.1016/j.foodchem.2018.08.136.

(26) Stoll, D. R.; Carr, P. W. Two-Dimensional Liquid Chromatography: A State of the Art Tutorial. *Anal. Chem.* **2017**, *89* (1), 519–531. DOI:10.1021/acs.analchem.6b03506.

(27) Chotoye, S. A. B.; Granados, N. P.; Brosseau, C. L. Harnessing the Resolution Power of Two-Dimensional Liquid Chromatography (2D-LC) for the Screening of Bisphenol Contaminants in Canned Food Items. *J. Chromatogr. A* **2024**, *1738*, 465502. DOI:10.1016/j.chroma.2024.465502.

(28) Wang, L.; Yang, B.; Zhang, X.; Zheng, H. Novel Two-Dimensional Liquid Chromatography–Tandem Mass Spectrometry for the Analysis of Twenty Antibiotics Residues in Dairy Products. *Food Anal. Methods* **2017**, *10* (6), 2001–2010. DOI:10.1007/s12161-016-0763-4.

(29) Petersson, P.; Haselmann, K.; Buckenmaier, S. Multiple Heart-Cutting Two Dimensional Liquid Chromatography Mass Spectrometry: Towards Real Time Determination of Related Impurities of Bio-Pharmaceuticals in Salt Based Separation Methods. *J. Chromatogr. A* **2016**, *1468*, 95–101. DOI:10.1016/j.chroma.2016.09.023.

(30) Zeng, H.; Xie, X.; Huang, Y.; Chen, J.; Liu, Y.; Zhang, Y.; Mai, X.; Deng, J.; Fan, H.; Zhang, W. Enantioseparation and Determination of Triazole Fungicides in Vegetables and Fruits by Aqueous Two-Phase Extraction Coupled with Online Heart-Cutting Two-Dimensional Liquid Chromatography. *Food Chem.* **2019**, *301*, 125265. DOI:10.1016/j.foodchem.2019.125265.

(31) Perestrelo, R.; Silva, P.; Porto-Figueira, P.; Pereira, J. A. M.; Silva, C.; Medina, S.; Câmara, J. S. QuEChERS - Fundamentals, Relevant Improvements, Applications and Future Trends. *Anal. Chim. Acta* **2019**, *1070*, 1–28. DOI:10.1016/j.aca.2019.02.036.

(32) Anastassiades, M.; Lehotay, S. J.; Stajnbaher, D.; Schenck, F. J. Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce. *J. AOAC Int.* **2003**, *86* (2), 412–431.

(33) Zhao, L.; Stevens, J. *Optimizing Recoveries of Planar Pesticides in Spinach Using Toluene and Agilent Bond Elut QuEChERS AOAC Kits with Graphitized Carbon.* Agilent (41)

Technologies, 2012. https://www.agilent.com/cs/library/applications/5990-4247EN.pdf (accessed 2024-10-05).

(34) *AOAC Official Method 2007.01 Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate*. AOAC International, 2007. https://nucleus.iaea.org/sites/fcris/Shared%20Documents/SOP/AOAC_2007_01.pdf (accessed 2025-07-04).

(35) Government of Canada. *Pesticides and food safety*, last revised November 2022. https://www.canada.ca/en/health-canada/services/about-pesticides/pesticides-food-safety.html (accessed 2024-11-26).

(36) Hajou, R. M. K.; Afifi, F. U.; Battah, A. H. Comparative Determination of Multi-Pesticide Residues in *Pimpinella Anisum* Using Two Different AOAC Methods. *Food Chem.* **2004**, *88* (3), 469–478. DOI:10.1016/j.foodchem.2004.03.051.

(37) Hu, R.; Huang, X.; Huang, J.; Li, Y.; Zhang, C.; Yin, Y.; Chen, Z.; Jin, Y.; Cai, J.; Cui, F. Long- and Short-Term Health Effects of Pesticide Exposure: A Cohort Study from China. *PLoS ONE* **2015**, *10* (6), e0128766. DOI:10.1371/journal.pone.0128766.

(38) Ye, M.; Beach, J.; Martin, J. W.; Senthilselvan, A. Occupational Pesticide Exposures and Respiratory Health. *Int. J. Environ. Res. Public. Health* **2013**, *10* (12), 6442–6471. DOI:10.3390/ijerph10126442.

(39) Yoshida, T.; Mimura, M.; Sakon, N. Estimating Household Exposure to Pyrethroids and the Relative Contribution of Inhalation Pathway in a Sample of Japanese Children. *Environ. Sci. Pollut. Res.* **2021**, *28* (15), 19310–19324. DOI:10.1007/s11356-020-12060-9.

(40) Schneider, T.; Vermeulen, R.; Brouwer, D. H.; Cherrie, J. W.; Kromhout, H.; Fogh, C. L. Conceptual Model for Assessment of Dermal Exposure. *Occup. Environ. Med.* **1999**, *56* (11), 765–773. DOI:10.1136/oem.56.11.765.

(41) MacFarlane, E.; Carey, R.; Keegel, T.; El-Zaemay, S.; Fritschi, L. Dermal Exposure Associated with Occupational End Use of Pesticides and the Role of Protective Measures. *Saf. Health Work* **2013**, *4* (3), 136–141. DOI:10.1016/j.shaw.2013.07.004.

(42) Smart, N. *Encyclopedia of Food Sciences and Nutrition,* 2nd ed.; Academic Press, 2003; pp 2832-2942. DOI:10.1016/B0-12-227055-X/00543-5

(43) Bartholomaeus, A. *Fludioxonil.* Therapeutic Goods Administration, 2004.

(44) *Pesticide Fact Sheet - Boscalid*. United States Environmental Protection Agency, 2003. https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/fs_PC-128008_01-Jul-03.pdf (accessed 2024-09-25).

(45) *Pyrimethanil Technical EPA Registration.* United States Environmental Protection Agency, 2009. https://www3.epa.gov/pesticides/chem_search/ppls/000264-00704-20080808.pdf (accessed 2024-09-25).

(46) *Pyraclostrobin (210)*. Food and Agriculture Organization, 2003. https://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation04/Pyraclostrobinaf.pdf (accessed 2024-09-25).

(47) Meng, Y.; Zhong, K.; Xiao, J.; Huang, Y.; Wei, Y.; Tang, L.; Chen, S.; Wu, J.; Ma, J.; Cao, Z.; Liao, X.; Lu, H. Exposure to Pyrimethanil Induces Developmental Toxicity and Cardiotoxicity in Zebrafish. *Chemosphere* **2020**, *255*, 126889. DOI: 10.1016/j.chemosphere.2020.126889.

(48) Aleksić, M.; Stanisavljević, D.; Smiljković, M.; Vasiljević, P.; Stevanović, M.; Soković, M.; Stojković, D. Pyrimethanil: Between Efficient Fungicide against Aspergillus Rot on Cherry Tomato and Cytotoxic Agent on Human Cell Lines. *Ann. Appl. Biol.* **2019**, *175* (2), 228–235. DOI: 10.1111/aab.12532.

(49) Lafon, P.; Wang, Y.; Arango-Lievano, M.; Torrent, J.; Salvador-Prince,; Mansuy, M.; Mestre- Frances, N.; Givalois, L.; Liu, J.; Mercader, J.; Jeanneteau, F.; Desrumaux, C., Perrier, V. Fungicide Residues Exposure and β-amyloid Aggregation in a Mouse Model of Alzheimer's Disease. *Environ. Health Perspect.* **2020**, 128 (1). DOI: 10.1289/EHP5550

(50) Alexandrino, D. A. M.; Mucha, A. P.; Almeida, C. M. R.; Carvalho, M. F. Microbial Degradation of Two Highly Persistent Fluorinated Fungicides - Epoxiconazole and Fludioxonil. *J. Hazard. Mater.* **2020**, *394*, 122545. DOI:10.1016/j.jhazmat.2020.122545.

(51) Lee, G.-H.; Hwang, K.-A.; Choi, K.-C. Effects of Fludioxonil on the Cell Growth and Apoptosis in T and B Lymphocytes. *Biomolecules* **2019**, *9* (9), 500. DOI:10.3390/biom9090500.

(52) Wang, H.; Meng, Z.; Liu, F.; Zhou, L.; Su, M.; Meng, Y.; Zhang, S.; Liao, X.; Cao, Z.; Lu, H. Characterization of Boscalid-Induced Oxidative Stress and Neurodevelopmental Toxicity in Zebrafish Embryos. *Chemosphere Oxf.* **2020**, *238*, 124753-. DOI:10.1016/j.chemosphere.2019.124753.

(53) Li, P.; Yin, X.; Yang, Y.; Yu, Y.; Fang, A.; Tian, B.; Bi, C. Field Resistance to Boscalid and Molecular Mechanisms of Strawberry Botrytis Cinerea in Chongqing, China. *Australas. Plant Pathol.* **2024**, *53* (1), 103–113. DOI:10.1007/s13313-023-00955-3.

(54) d'Hose, D.; Isenborghs, P.; Brusa, D.; Jordan, B. F.; Gallez, B. The Short-Term Exposure to SDHI Fungicides Boscalid and Bixafen Induces a Mitochondrial Dysfunction in Selective Human Cell Lines. *Molecules* **2021**, *26* (19), 5842. DOI:10.3390/molecules26195842.

(55) Li, B.; Wang, W.; Zhang, X.; Zhang, D.; Ren, Y.; Gao, Y.; Mu, W.; Liu, F. Using Coordination Assembly as the Microencapsulation Strategy to Promote the Efficacy and Environmental Safety of Pyraclostrobin. *Adv. Funct. Mater.* **2017**, *27* (29), 1701841. DOI:10.1002/adfm.201701841.

(56) Lehotay, S. J.; Mastovska, K.; Lightfield, A. R. Use of Buffering and Other Means to Improve Results of Problematic Pesticides in a Fast and Easy Method for Residue Analysis of Fruits and Vegetables. *J. AOAC Int.***2005**, 88 (2), 615-629. DOI: 10.1093/jaoac/88.2.615

(57) Anastassiades, M.; Scherbaum, E.; Tasdelen, B.; Stajnbaher, D. Recent Developments in QuEChERS Methodology for Pesticide Multiresidue Analysis. *Pestic. Chem. Crop Prot. Public Health Environ. Saf.* **2007**, 439–458. DOI: 10.1002/9783527611249.ch46

(58) Sharma, K.; Mullangi, R. A Concise Review of HPLC, LC-MS and LC-MS/MS Methods for Determination of Azithromycin in Various Biological Matrices. *Biomed. Chromatogr.* **2013**, *27* (10), 1243–1258. DOI: 10.1002/bmc.2898.

(59) Khalid, A.; Ali, S. N.; Qayoom, A.; Iqbal, S.; Ansari, S.; Awan, Z. ul H.; Kishwar, F.; Daniel, P. High Performance Liquid Chromatography Ultraviolet Method for the Determination of Fludioxonil Fungicide Residues: Application on Rice Grains Cultivated in Pakistan. *Arab. J. Chem.* **2022**, *15* (4), 103692-. DOI: 10.1016/j.arabjc.2022.103692.

(60) Muehlwald, S.; Meyburg, N.; Rohn, S.; Buchner, N. A Comparison between a Two-Dimensional Liquid Chromatography System and a Traditional QuEChERS-LC Method with Regard to Matrix Removal and Matrix Effects in Pesticide Analysis Using Time-of-Flight Mass Spectrometry. *J. Agric. Food Chem.* **2021**, *69* (49), 15005–15019. DOI: 10.1021/acs.jafc.1c05199.

(61) Kittlaus, S.; Kempe, G.; Speer, K. Evaluation of Matrix Effects in Different Multipesticide Residue Analysis Methods Using Liquid Chromatography–Tandem Mass Spectrometry, Including an Automated Two-Dimensional Cleanup Approach. *J. Sep. Sci.* **2013**, *36* (13), 2185–2195. DOI: 10.1002/jssc.201300044.

(62) Urban, M.; Hann, S.; Rost, H. Simultaneous Determination of Pesticides, Mycotoxins, Tropane Alkaloids, Growth Regulators, and Pyrrolizidine Alkaloids in Oats and Whole Wheat Grains after Online Clean-up via Two-Dimensional Liquid Chromatography Tandem Mass Spectrometry. *J. Environ. Sci. Health Part B* **2019**, *54* (2), 98–111. DOI: 10.1080/03601234.2018.1531662.

(63) Muehlwald, S.; Rohn, S.; Buchner, N. Evaluating the Applicability of a Two-Dimensional Liquid Chromatography System for a Pesticide Multi-Screening Method. *J. Chromatogr. A* **2019**, *1599*, 95–107. DOI: 10.1016/j.chroma.2019.04.003.

(64) Manz, A.; Dittrich, P. S.; Pamme, N.; Iossifidis, D. *Bioanalytical Chemistry*, 2nd ed, Imperial College Press, 2015.

(65) Skoog, D. A.; West, D. M.; Holler, F. J.; Crouch, S. R. *Fundamentals of Analytical Chemistry*, 10th ed, Cengage, 2021.

(66) Xu, Q. *Ultra-High Performance Liquid Chromatography and its Applications*; John Wiley & Sons, Ltd, 2013. DOI: 10.1002/9781118533956.fmatter.

(67) Skoog, D. A.; Holler, F. J.; Crouch, S. R. *Principles of Instrumental Analysis*, 7th ed, Cengage, 2016.

(68) Zenkevich, I. G.; Makarov, A. A.; Pavlovskii, A. A. New Approaches to the Calculation and Interpretation of Asymmetry Factors of Chromatographic Peaks. *J. Anal. Chem.* **2017**, *72* (7), 710–718. DOI: 10.1134/S1061934817070164

(69) Cazes, J.; Scott, R. P. W. *Chromatography Theory*, CRC Press, 2002.

(70) Zhong, Z.; Geng, M. L. Microscopic Origins of Band Broadening in Chromatography. Polarity Distribution in C18 Stationary Phase Probed by Confocal Ratiometric Imaging of Nile Red. *Anal. Chem.* **2007**, *79* (17), 6709–6717. DOI: 10.1021/ac071272o.

(71) *Control pH During Method Development for Better Chromatography.* Agilent Technologies, 2015. https://www.agilent.com/cs/library/technicaloverviews/Public/5990-9984EN.pdf (accessed 2024-06-28).

(72) Carr, P., Stoll, D. *Two-Dimenisonal Liquid Chromatography - Primer.* Agilent Technologies, 2015. https://www.agilent.com/Library/primers/Public/5991-2359EN.pdf (accessed 2024-06-28).

(73) Cortes, H. J. *Multidimensional Chromatography: Techniques and Applications*; CRC Press: Boca Raton, 1990.

(74) Williams, M. L.; Olomukoro, A. A.; Emmons, R. V.; Godage, N. H.; Gionfriddo, E. Matrix Effects Demystified: Strategies for Resolving Challenges in Analytical Separations of Complex Samples. *J. Sep. Sci.* **2023**, *46* (23), 2300571. DOI: 10.1002/jssc.202300571.

(75) Williams, M. L.; Olomukoro, A. A.; Emmons, R. V.; Godage, N. H.; Gionfriddo, E. Matrix Effects Demystified: Strategies for Resolving Challenges in Analytical Separations of Complex Samples. *J. Sep. Sci.* **2023**, *46* (23), e2300571. DOI: 10.1002/jssc.202300571.

(76) *Agilent ZORBAX Eclipse C18 Data Sheet*. Agilent Technologies, 2006. https://www.agilent.com/cs/library/specifications/Public/820114-002.pdf (accessed 2024-08-09).

(77) Chotoye, S. A. B. Two-Dimensional Liquid Chromatography (2D-LC) : A Novel Technique for the Analysis of Bisphenol Contaminants in Canned Food Items. Honours Thesis, Saint Mary's University, Halifax, NS, 2023.

(78) *Why is Acetonitrile Used in HPLC?*. KBV Research. https://www.kbvresearch.com/blog/why-is-acetonitrile-used-in-hplc/ (accessed 2025-01-06).

(79) Gajanayake, R. *PUBLIC RELEASE SUMMARY of the Evaluation by the NRA of the New Active Constituent: Pyrimethanil in the Product: SCALA 400 SC FUNGICIDE.* National Registration Authority for Agricultural and Veterinary Chemicals, 1996.

(80) *KB005940: Agilent ZORBAX Bonus-RP Column Specifications and Options | Agilent*. https://www.agilent.com/en/support/liquid-chromatography/kb005940 (accessed 2025-01-19).

(81) Papatheocharidou, C.; Samanidou, V. Two-Dimensional High-Performance Liquid Chromatography as a Powerful Tool for Bioanalysis: The Paradigm of Antibiotics. *Molecules* **2023**, *28* (13), 5056. DOI: 10.3390/molecules28135056.

(82) Eisnor, M. M.; Biton, M.; Granados, P.; Reader, H.; Brosseau, C. L. Coupling Multidimensional Chromatography with Plasmonic Sensing: An Exploration of Electrochemical SERS as a Detection Modality for 2D-LC. *Can. J. Chem.* **2024**, *102* (11), 724–733. DOI: 10.1139/cjc-2023-0172.

(83) Lane, J. *Resolution Too Much, Too Little, or Just Right.* Agilent Technologies, 2019. https://www.agilent.com/cs/library/eseminars/public/Resolution_Too_Much_Too_LIttle_Just_Right_Jan_2019.pdf (accessed 2025-02-03).

(84) European Commission. *Control Procedures for Pesticide Residues Analysis,* last revised 1999. https://www.eurl-pesticides.eu/library/docs/allcrl/AqcGuidance_Sanco_2000_3103.pdf (accessed 2024-05-20).

(85) European Union. *Decision - 2002/657 - EN - EUR-Lex*, last revised 2002. https://eur-lex.europa.eu/eli/dec/2002/657/oj/eng (accessed 2025-02-03).

(86) Galagarza, O. A.; Ramirez-Hernandez, A.; Oliver, H. F.; Álvarez Rodríguez, M. V.; Valdez Ortiz, M. del C.; Pachari Vera, E.; Cereceda, Y.; Diaz-Valencia, Y. K.; Deering, A. J. Occurrence of Chemical Contaminants in Peruvian Produce: A Food-Safety Perspective. *Foods* **2021**, *10* (7), 1461. DOI: 10.3390/foods10071461.

(87) Servicio Nacional de Sanidad Agraria SENASA. *Informe Del Plan Anual de Monitoreo de Contaminantes Quimicos En Alimentos Agropecuarios Primarios Ano 2011.* https://cdn.www.gob.pe/uploads/document/file/2242216/Informe%20del%20plan%20anual%20de%20monitoreo%20de%20contaminantes%202011.pdf.pdf (accessed 2025-03-06).

(88) Servicio Nacional de Sanidad Agraria SENASA. *Informe Del Plan Anual de Monitoreo de Contaminantes Quimicos En Alimentos Agropecuarios Primarios Ano 2012.* https://cdn.www.gob.pe/uploads/document/file/2242213/Informe%20del%20Monitoreo%20de%20Residuos%20Químicos%20y%20otros%20Contaminantes%20en%20Alimentos%20Agropecuarios%20Primarios%20año%202012.pdf.pdf (accessed 2025-03-06).

(89) Servicio Nacional de Sanidad Agraria SENASA. *Informe Del Plan Anual de Monitoreo de Contaminantes Quimicos En Alimentos Agropecuarios Primarios Ano 2013.* https://cdn.www.gob.pe/uploads/document/file/2242209/Informe%20del%20Monitoreo%20de%20Residuos%20Químicos%20y%20otros%20Contaminantes%20en%20Alimentos%20Agropecuarios%20Primarios%20año%202013.pdf.pdf (accessed 2025-03-06).

(90) Servicio Nacional de Sanidad Agraria SENASA. *Informe Del Plan Anual de Monitoreo de Contaminantes Quimicos En Alimentos Agropecuarios Primarios Ano 2014.* https://cdn.www.gob.pe/uploads/document/file/2242207/Informe%20del%20plan%20anual%20de%20monitoreo%20de%20contaminantes%202014.pdf.pdf (accessed 2025-03-06).

(91) Servicio Nacional de Sanidad Agraria SENASA. *Informe Del Plan Anual de Monitoreo de Contaminantes Quimicos En Alimentos Agropecuarios Primarios Ano 2015.* https://cdn.www.gob.pe/uploads/document/file/2242204/Informe%20del%20Monitoreo%20de%

(92) Servicio Nacional de Sanidad Agraria SENASA. *Informe Del Plan Anual de Monitoreo de Contaminantes Quimicos En Alimentos Agropecuarios Primarios Ano 2016.* https://www.senasa.gob.pe/senasa/descargasarchivos/2016/08/Informe-de-monitoreo-2016.pdf (accessed 2025-03-06).

(93) Servicio Nacional de Sanidad Agraria SENASA. *Informe Del Plan Anual de Monitoreo de Contaminantes Quimicos En Alimentos Agropecuarios Primarios Ano 2017.* https://www.senasa.gob.pe/senasa/descargasarchivos/2018/12/INFORME-FINAL-DEL-PLAN-DE-MONITOREO-2017-1.pdf (accessed 2025-03-06).

(94) Servicio Nacional de Sanidad Agraria SENASA. *Informe Del Plan Anual de Monitoreo de Contaminantes Quimicos En Alimentos Agropecuarios Primarios Ano 2018.* https://cdn.www.gob.pe/uploads/document/file/2242195/Informe%20del%20plan%20anual%20de%20monitoreo%20de%20contaminantes%202018.pdf.pdf (accessed 2025-03-06).

(95) Servicio Nacional de Sanidad Agraria SENASA. *Informe Del Plan Anual de Monitoreo de Contaminantes Quimicos En Alimentos Agropecuarios Primarios Ano 2019.* https://cdn.www.gob.pe/uploads/document/file/1516765/Informe%20del%20plan%20anual%20de%20monitoreo%20de%20contaminantes%202019.pdf (accessed 2025-03-06).

Chapter 8: Appendix

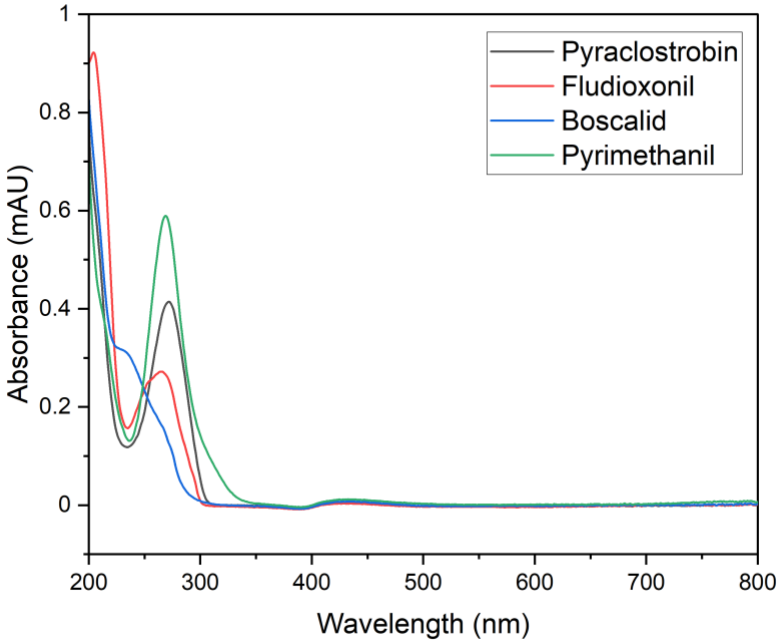


Figure A1. UV-vis spectra for FLU, BOS, PYRI, and PYRA.

Table A1. Chromatographic parameters for the optimized first dimension method.

	Retention Time (R_t)[n=5]	Width (W)	Retention Factor (k')	Asymmetry (A_s)
PYRI	1.452 ± 0.002	0.021	3.538	1.078
FLU	1.873 ± 0.002	0.020	4.850	1.232
BOS	2.058 ± 0.002	0.024	5.431	1.195
PYRA	2.759 ± 0.003	0.023	7.622	1.202
IS	2.922 ± 0.003	0.024	8.131	1.148

Table A2. Chromatographic parameters for the optimized second dimension method.

	Retention Time (R_t)[n=5]	Width (W)	Retention Factor (k')	Asymmetry (A_s)
PYRI	0.706 ± 0.009	0.038	4.883	0.784
FLU	0.922 ± 0.003	0.029	6.683	1.656
BOS	0.866 ± 0.003	0.027	6.217	1.610
PYRA	0.986 ± 0.004	0.033	7.217	1.350
IS	1.072 ± 0.004	0.038	7.933	1.218

Table A3. Mean and standard deviation data for retention times and peak areas used for intraday validation.

	Mean ^{1}D Retention Time ± Standard Deviation/ min	Mean ^{1}D Peak Area ± Standard Deviation/ min	Mean ^{2}D Retention Time ± Standard Deviation/ min	Mean ^{2}D Peak Area ± Standard Deviation/ min
Pyrimethanil	1.454 ± 0.000	848.4 ± 1.6	0.694 ± 0.000	520.9 ± 3.1
Fludioxonil	1.875 ± 0.001	490.2 ± 0.5	0.920 ± 0.002	256.3 ± 3.2
Boscalid	2.061 ± 0.001	246.7 ± 0.5	0.859 ± 0.000	133.7 ± 2.9
Pyraclostrobin	2.762 ± 0.000	578.5 ± 0.2	0.978 ± 0.000	318.2 ± 2.6
Phenanthrene	2.925 ± 0.000	524.3 ± 0.1	1.062 ± 0.001	291.5 ± 3.5

Table A4. Mean and standard deviation data for retention times and peak areas used for interday validation.

	Mean ^{1}D Retention Time ± Standard Deviation/ min	Mean ^{1}D Peak Area ± Standard Deviation/ min	Mean ^{2}D Retention Time ± Standard Deviation/ min	Mean ^{2}D Peak Area ± Standard Deviation/ min
Pyrimethanil	1.452 ± 0.002	843.3 ± 29.0	0.706 ± 0.009	595.1 ± 43.8
Fludioxonil	1.873 ± 0.002	400.2 ± 6.3	0.922 ± 0.003	276.2 ± 12.6
Boscalid	2.058 ± 0.002	252.0 ± 2.6	0.866 ± 0.003	147.2 ± 9.4
Pyraclostrobin	2.759 ± 0.003	607.1 ± 22.0	0.986 ± 0.004	350.6 ± 19.2
Phenanthrene	2.922 ± 0.003	540.4 ± 9.4	1.072 ± 0.004	330.2 ± 25.8

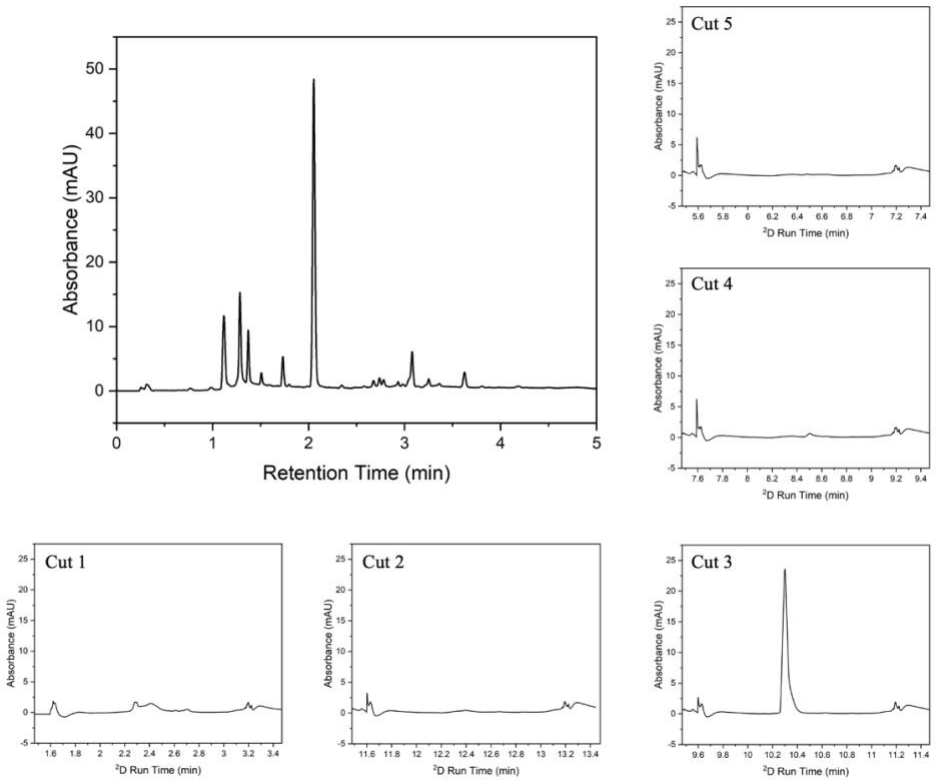


Figure A2. ^{1}D and ^{2}D blank chromatograms using the modified QuEChERS procedure and the optimized 2D-LC method.

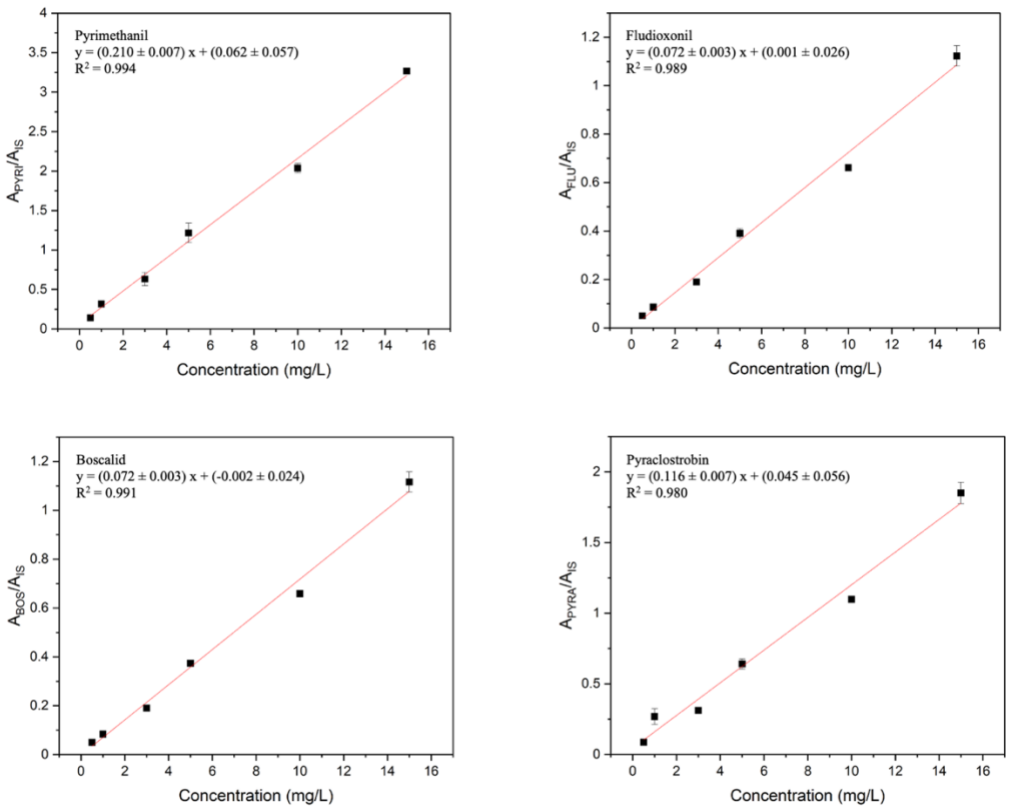


Figure A3. [1]D internal standard calibration curves for PYRI, FLU, BOS and PYRA. All plots include the equation of the line of best fit and the coefficient of determination (R^2).

Table A5. Error in regression (S_R) values for [1]D and [2]D calibration curves for PYRI, FLU, BOS, and PYRA.

	[1]D Calibration Curves				**[2]D Calibration Curves**			
Analyte	PYRI	FLU	BOS	PYRA	PYRI	FLU	BOS	PYRA
Error in Regression (S_R)	0.094	0.042	0.040	0.092	0.147	0.047	0.041	0.069

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