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Study of the secondary metabolites of black Aspergilli from Canadian vineyards

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Chemistry

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STUDY OF THE SECONDARY METABOLITES OF BLACK ASPERGILLI
FROM CANADIAN VINEYARDS

(Thesis format: Monograph)

by

Tianyu Qi

Graduate Program in Chemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Ochratoxin A (OTA) and fumonisin are mycotoxins produced by black *Aspergilli* that contaminate wine in many countries around the world. Both OTA and fumonisins are possible carcinogens to humans. There is limited data about the occurrence of OTA and fumonisins in Canadian vineyards. Therefore, a survey of the black *Aspergilli* from 2012-2014 has been conducted. In the mycotoxin study, high performance liquid chromatography-mass spectrometry was used to screen and quantify OTA and fumonisins from both black *Aspergilli* isolated on artificial media and grape samples. OTA was only detected from one fungal isolate and not on any grapes. Fumonisin B₂, B₄, and B₆ were detected from both isolates and grapes, but in very low concentrations. Therefore, the risk of wine grapes contamination with OTA and fumonisins is low. In the metabolomics study, the principal component analysis and the Kruskal-Wallis test was carried out on the metabolomics data obtained from the black *Aspergillus* extracts. Thirty-three potential metabolite markers were found to discriminate black *Aspergillus* species isolated from Canadian vineyards.

Keywords: ochratoxin A, fumonisin, mycotoxin, black *Aspergilli*, Canadian vineyards, high performance liquid chromatography-mass spectrometry, species-specific metabolite markers, metabolomics

Co-Authorship Statement

All experimental work and data analysis in the thesis was performed by the author except the followings: Samples were collected by Dr. Mark Sumarah and lab technician Tim McDowell. Sample plating and DNA extraction of black *Aspergillus* isolates from 2013 were done by the co-op student Janet Dimmers.

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List of Symbols and Abbreviations

°C	degree Celsius
AAFC	Agriculture and Agri-food Canada
AGC	automatic gain control
ANOVA	analysis of variance
Asp	Aspergillus
BLAST	Basic Local Alignment Search Tool
CID	collision-induced dissociation
CR	charged residue
CS	commercial site
CYA	Czapek yeast autolysate
DC	direct current
DG18	dichloran 18% glycerol
DRBC	dichloran rose bengal chloramphenicol
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization source
et al.	and others
EU	European Union
FB ₁	fumonisin B ₁
FB ₂	fumonisin B ₂
FB ₄	fumonisin B ₄
FB ₆	fumonisin B ₆
FDR	false discovery rate
FWHM	peak at full width at half maximum
GC	gas chromatography
GRS	Grape Research Station
h	hour
HCD	higher collision energy dissociation
HESI	heated electrospray
HPLC	high-performance liquid chromatography
HRAM	high-resolution accurate mass

HRMS	high-resolution mass spectrometry
i.d.	inner diameter
IARC	International Agency for Research on Cancer
IE	ion evaporation
IUPAC	International Union of Pure and Applied Chemistry
JRS	Jordon Research Station
KRS	Kentville Research Station
kV	kilovolts (10^3 volts)
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
m/z	mass-to-charge ratio
MEGA	Molecular Evolutionary Genetics Analysis
mL	milliliter (10^{-3} liter)
MS	mass spectroscopy
ms	millisecond (10^{-3} second)
MS2	tandem mass spectrometry
NMR	nuclear magnetic resonance
NTP	deoxynucleotide
OTA	ochratoxin A
PARC	Pacific Agri-Food Research Centre
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
PLS	partial least squares
RF	radio frequency
RRHD	Rapid Resolution High Definition
RSD	relative standard deviation
RT	retention time
SPE	solid phase extraction
TAE	Tris-acetate-EDTA
TCE	tricarballic ester
UV	ultraviolet
VQA	Vintners Quality Alliance

μg	microgram (10^{-6} gram)
μL	microliter (10^{-6} liter)
μm	micrometer (10^{-6} meter)
μM	micromolar (10^{-6} mol/L)

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Chapter 1 – Introduction

1.1 Mycotoxins in Grapes

1.1.1 Fungi

Fungi are a group of eukaryotic and heterotrophic microorganisms, including moulds, yeasts and mushrooms. They belong to the Kingdom Fungi which is separated from animals, plants and bacteria. Fungi have been widely used for fermentation of food products, such as wine and beer. In addition, organic acid, antibiotics, and enzymes produced by fungi are commonly used in the food and pharmaceutical industries. However, some fungi are known to be pathogenic and responsible for food spoilage. For example, *Aspergillus* bunch rot in grapes is caused by *Aspergillus* (abbreviated to *Asp.*).

1.1.2 Secondary metabolites and mycotoxins

There are an estimated 1.5 million species in the Kingdom Fungi, but only about 80,000 species are named and studied [1]. The diversity of fungi is reflected by the diversity of secondary metabolites. Fungal secondary metabolites are a wide range of relatively low-molecular-weight natural products biosynthesized in fungi. Unlike primary metabolites, secondary metabolites are usually not essential for fungi growth, development and proliferation. The reason why fungi produce secondary metabolites is not clear.

Many filamentous fungi, including *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria*, are responsible for food spoilage and severe yield loss. More importantly, they have the ability to produce mycotoxins. Mycotoxins are toxic secondary metabolites produced by filamentous fungi that can cause acute or chronic toxic effects in animals and humans, even when consumed at very low concentration [2]. The presence of mycotoxins in food products is not necessarily linked to food with a mouldy appearance. Likewise, seemingly healthy-looking food which has been contaminated with mycotoxins could be mistakenly consumed by humans or animals.

There are more than 300 mycotoxins produced by 350 species of fungi [3], among which aflatoxins, ochratoxin A (OTA), fumonisins, deoxynivalenol, T-2 toxin and zearalenone are well-known due to their severe toxicity to humans and animals. For example, in the United Kingdom, thousands of turkey deaths in the 1960s from Turkey-X disease was later traced to aflatoxins produced by *Asp. flavus* and *Asp. parasiticus* in their nut meals

[4]. Most mycotoxins are thermostable molecules, and thus the toxicity is difficult to be reduced or eliminated by traditional food processing procedures [5]. For instance, OTA is stable up to 180°C [6]. Therefore, it is of vital importance to regulate and monitor the presence of mycotoxins in crops.

1.1.3 Black *Aspergilli*

The filamentous fungal genus *Aspergillus* is able to produce a wide range of mycotoxins that are responsible for food spoilage. Aflatoxins produced by *Asp. flavus* and *Asp. parasiticus* are the major mycotoxins found in figs and dates [7]. OTA produced by *Asp. carbonarius*, and fumonisin B₂ (FB₂) produced by *Asp. niger* and *Asp. welwitschiae* are the major mycotoxins detected in grape and grape-derived products, such as wine, grape juice and raisin [8-10]. Information on OTA and fumonisin in grape and grape-derived products are to be presented in Section 1.1.4 and 1.1.5 respectively.

Asp. carbonarius, *Asp. niger* and *Asp. welwitschiae* belong to *Aspergillus* section Nigri or “black *Aspergilli*”. Soil beneath the grapes in vineyard is the primary habitat for black *Aspergilli* [11]. The infection of grapes with air-borne black *Aspergilli* spores often occurs on damaged berry skins caused by many factors including pests (grape berry moth or mites) and the environment (wind, rain or hail) [9].

Currently, it was reported that up to 25 species comprise the black *Aspergilli* [12]. Besides the mycotoxin producers *Asp. carbonarius*, *Asp. niger* and *Asp. welwitschiae*, other species such as *Asp. tubingensis*, *Asp. brasiliensis*, *Asp. uvarum*, *Asp. acidus*, and *Asp. ibericus* are also commonly isolated from grapes [13]. Many species of black *Aspergilli* are morphologically indistinguishable and were treated as *Asp. niger* ‘aggregate’. With the on-going research on black *Aspergilli*, misidentification and misclassification have been gradually discovered and corrected. For example, *Asp. awamori* has been renamed as *Asp. welwitschiae*, since it is identical to a strain isolated from genus *Welwitschia* but not related to fungi involved in awamori koji (a type of black fungus) fermentation [14]. *Asp. welwitschiae* used to be regarded as a synonym of *Asp. niger*. However, it is considered as a phylogenetically distinct species based on its seven fixed differences in nucleotides sequences from *Asp. niger* [15].

A major challenge is the identification of black *Aspergilli* to the species level, since most of the strains cannot be differentiated by morphology. Gene-based analyses, such as

β -tubulin gene sequencing and calmodulin gene sequencing, are commonly used for *Aspergillus* species identification, especially with the advantage that DNA sequence databases have become more robust. However, gene sequencing requires laborious sample preparation procedures including DNA extraction, polymerase chain reaction (PCR), gel electrophoresis and DNA cleanup. A more efficient means to differentiate black *Aspergilli* to the species level is needed.

1.1.4 OTA in grapes and grape-derived products

OTA is a mycotoxin known for its nephrotoxic, carcinogenic and immunosuppressive effects in animals [16]. A number of studies have suggested that OTA is responsible for Balkan endemic nephropathy, which is a human kidney disease found in the Danube Region [17]. OTA was classified as group 2B (possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC). It is also difficult to eliminate out of the human body (half-life 35 days) because OTA has high affinity toward human serum albumin [18].

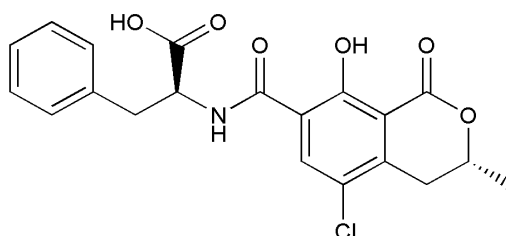


Figure 1.1 Chemical structure of OTA.

The chemical structure of OTA is shown in Figure 1.1. It is a very stable compound that only can be fully hydrolyzed by heating under reflux for 48 h in 6 mol/L acetic acid [19]. OTA has weak acidic properties. The pKa values of the carboxyl group of the phenylalanine moiety is in the ranges 4.2–4.4, and that of the phenolic hydroxyl group of the isocoumarin part is 7.0–7.3 [20]. OTA is soluble in polar solvents such as methanol, but not very soluble in water.

OTA has been detected in many agriculture products, such as cereal, wine, cocoa and onion [21-23]. It was first detected in wine by Zimmerli and Dick in 1996 [24]. Since then, a number of studies reported that OTA was found in grape-based beverages, including wine and grape juice in Europe [25, 26]. Besides Europe, OTA has been

detected in wine from many countries around the world including Australia, Brazil, Israel and South Africa [27-30]. Considering its toxicity and that wine is the second largest source of daily intake of OTA after cereal in Europe [31], the European Commission established a maximum level for OTA contamination at 2.0 µg/kg (2 ppb) for wine and grape juice and 10.0 µg/kg (10 ppb) for dried vine fruits (Commission Regulation 2005).

There were limited data showing whether OTA is present in the Vintners Quality Alliance (VQA) wine, which is produced by 100% Canadian grown grapes. In contrast, the presence of OTA in Canadian wheat is well documented and controlled [32]. Health Canada regulates an allowable OTA limit in raw cereal grain at 5 µg/kg (Health Canada, 2009), but to date, they have not set limits for OTA in Canadian wine.

OTA was first described as a secondary metabolite of *Asp. ochraceus* [19]. Subsequently, it was reported that it was produced by several *Aspergillus* and *Penicillium* species. *Penicillium verrucosum* is a storage fungus and is responsible for OTA production in Canadian wheat and grain [32, 33]. The main source of OTA on wine grapes or remained in wine is the infection of grapes by *Asp. carbonarius* and to a lesser extent by *Asp. niger* [34]. It has been reported that 70-100% of *Asp. carbonarius* strains are known to produce OTA [35], and by comparison, only 0-10% of *Asp. niger* strains are OTA producers, and the amount of OTA produced is 10-1000 times lower [29, 36, 37].

Therefore, to better understand the risk of OTA contamination in Canadian wine, a survey needs to be carried out to evaluate OTA contamination in Canadian wine grapes and to study which black *Aspergillus* species are responsible for the contamination.

1.1.5 Fumonisin in grapes and grape-derived products

Fumonisin are cardiotoxic, hepatotoxic, nephrotoxic, which are responsible for pulmonary edema in pigs [38] and possible esophageal cancer in humans [39]. Fumonisin are major concern in maize, and the European Union (EU) has set maximum levels of 0.2-2 mg/kg (0.2 ppm-2 ppm) for fumonisin [40, 41]. They have been known to be produced by *Fusarium* species since the 1980s, but surprisingly, full genome sequencing of *Asp. niger* in 2006 showed that the genome included a putative Fum gene cluster [42], meaning *Asp. niger* could also produce fumonisin. FB₂ was found in some industrial strains of *Asp. niger* by Frisvad *et al.* (2008) [43]. Subsequently, Frisvad *et al.* (2009) reported fumonisin B₄ (FB₄) production by *Asp. niger* in coffee beans [44].

Fumonisin B₆ (FB₆), a positional isomer of fumonisin B₁ (FB₁), was also reported from *Asp. niger* by the same group in 2010 [45]. Mogensen *et al.* (2010) reported the occurrence of FB₂ and FB₄ by *Asp. niger* on grapes and raisins with 77% of their *Asp. niger* strains produces fumonisins [41]. Therefore, in addition to OTA, the fumonisin contamination in Canadian wine grapes should also be investigated.

The chemical structures of the fumonisins containing two tricarballic acid groups and with hydroxyl substitution positions are shown in Figure 1.2. The pKa values for tricarballic acid group are 3.49 and 4.56. It also has one primary amine group, which would have a pKa higher than 9. Fumonisin are high polarity compounds, which are solubilized both in water and polar organic solvent.

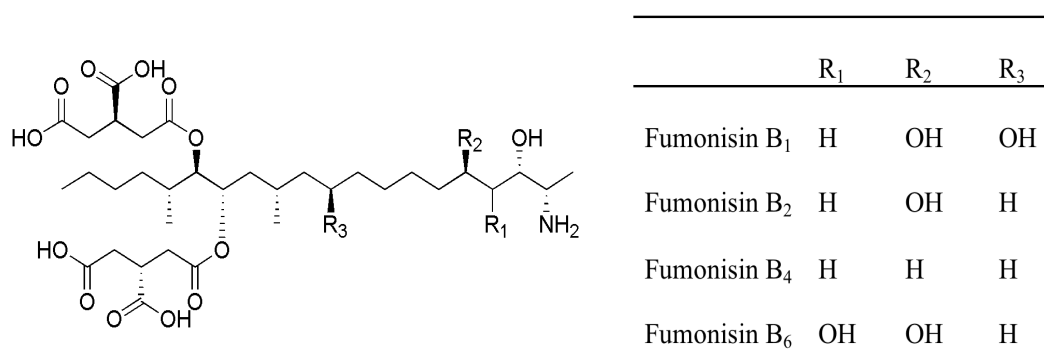


Figure 1.2 Chemical structures of fumonisin B₁, B₂, B₄, and B₆.

1.1.6 Research aims of the mycotoxin study (Chapter 2)

Since there is limited data on OTA and fumonisin occurrence in Canadian vineyards, a three-year survey has been carried out to achieve the following three goals, which will be discussed in Chapter 2:

- Monitor the distribution of black *Aspergillus* species in Canadian vineyard
- Screen for and quantify OTA and fumonisins from black *Aspergilli* isolated from Canadian vineyards
- Screen for and quantify OTA and fumonisins from grape samples

1.2 Mass Spectrometry-based Metabolomics

1.2.1 Metabolomics

Metabolomics is the global analysis of metabolites found in organisms. It is a relatively new ‘omics’ science after genomics, transcriptomics and proteomics. The ‘metabolome’ was first described by Oliver and co-workers (1998) as being the set of all of low-molecular-mass chemical compounds (<1kDa) synthesized by an organism [46].

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are two well-developed analytical techniques used for metabolomics. NMR spectroscopy offers non-destructive analysis, but is limited by having low sensitivity. On the contrary, the major strength of MS is high sensitivity. Various metabolites in a complex sample usually cause significant ion suppression if they are directly injected into the MS. Therefore, separation techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC), are usually coupled to the MS for metabolomic analysis. The theory and instrumentation of LC-MS will be discussed in Section 1.3.

Metabolomics is widely used in many fields, such as cancer research [47, 48], food safety studies [49], and environmental studies [50]. Metabolomics has been increasingly applied to biomarker discovery [51, 52]. For instance, by comparing the relative abundance of metabolites between patient cohorts and controls in cancer research, biomarkers could be discovered.

There are three major approaches in metabolomics: targeted analysis, metabolite profiling, and metabolic fingerprinting [53].

Targeted analysis is a quantitative approach to analyze one or a few known compounds. It is the most developed approach in metabolomic studies. It requires standard compounds with known concentrations. In the targeted analysis, we are not interested in any other metabolites besides the target. OTA and FB₂ detection and quantification in Chapter 2 is an example of targeted analysis.

Metabolite profiling is the relative quantification of a number of metabolites, usually a class of metabolites, in biological samples. Quantification is usually carried out relative to controls [54].

Metabolic fingerprinting does not aim to quantify known compounds or identify unknown compounds specifically, but instead aims to classify metabolites based on the biological origin (e.g. produced by which organism). It can be used for biomarker discovery.

1.2.2 MS-based metabolomics data analysis

Large quantities of data are usually generated in MS-based metabolomics. Processing and analysis of data requires mathematical and statistical tools [55]. The main procedures involved in a MS-based metabolomics study are as follows: sample preparation, data acquisition, data pre-processing, statistical analysis, and metabolite identification. The last three procedures will be discussed in this section.

1.2.2.1 Data pre-processing

After data acquisition, the raw data usually requires pre-processing prior to further downstream data processing. An advantage of data pre-processing in metabolomics is to convert raw data from different samples into an entire dataset that can be used to compare variables across samples, especially when there are hundreds to thousands of samples to analyze.

MZmine [56] and XCMS [57] are two of the most popular free software programs for data pre-processing in metabolomics. There are many parameters required to set for a data pre-processing procedure, which have been observed to affect data quality [58]. Typical pre-processing flow includes data conversion, peak detection, and peak alignment, which are briefly discussed as follows. More detailed explanation can be found in a review by Sugimoto *et al.* (2012) [59].

Data conversion → Data need to be converted from the MS vendor raw data format to an open format that can be read by software. For example, Thermo ‘raw’ file needs to be converted to ‘mzXML’ format that can be read by MZmine.

Peak detection → Peak detection is to convert the continuous spectral data into a discrete peak list so that each peak is represented by a mass-to-charge ratio (m/z), RT, and peak area/height [60]. A purpose of peak detection is to remove background peaks in the continuous spectral data. A certain peak area threshold should be set up. Peaks above this threshold are considered to be true peaks and are selected for integration. Peaks below this threshold are regarded as baseline peaks and are discarded.

Peak alignment → Corresponding peaks are aligned across multiple samples within a few seconds retention time (RT) and mass tolerance. The same ion in different samples could have a slightly different RT because of co-eluting peaks, pressure change, or other factors. The aligned peak list is exported to a table for further statistical analyses. This peak list contains all m/z and RT detected, along with their relative abundance (peak area or height) in every sample.

1.2.2.2 Statistical analysis

Statistical analysis methods can be classified into multivariate and univariate analysis. Multivariate statistical analysis, such as principal component analysis (PCA) and partial least squares regression (PLS regression), is usually carried out in metabolomics to visualize similarities and differences between groups, considering all the variables in the dataset. Univariate statistical analysis, such as the t-test, the analysis of variance (ANOVA), and the Kruskal–Wallis test, is commonly used in metabolomics to compare differences for single metabolites between two or more groups. The t-test and ANOVA should be applied to data that are normally distributed. The Kruskal–Wallis test should be used to the data when a normal distribution cannot be assumed. This section will only discuss the commonly used multivariate statistical analysis PCA in detail.

When there are many variables to analyze in a dataset, it becomes complicated to obtain meaningful information from the data. PCA works by reducing (without the loss of any information) the number of variables in the data allowing visual representation. It transforms multivariate data to a new coordinate system, where new coordinates are defined as principal components. The origin of the new coordinate system is based on the centered mean value of all of the variables. By definition, the largest variation in the data occurs along principal component 1 (PC1), and the second largest variation along principal component 2 (PC2), which is perpendicular to PC1. This process is repeated until principal component k (PC k) where no more components can be found. Figure 1.3 is a schematic explanation of how PCA reduces variables. Table 1.1 shows an LC-MS peak list in the form of a $m \times n$ matrix containing n variables in each of the m samples, and reduced to $m \times k$ variables by new coordinate systems. In metabolomics, “feature” is defined as a potential metabolite (m/z and RT) [61].

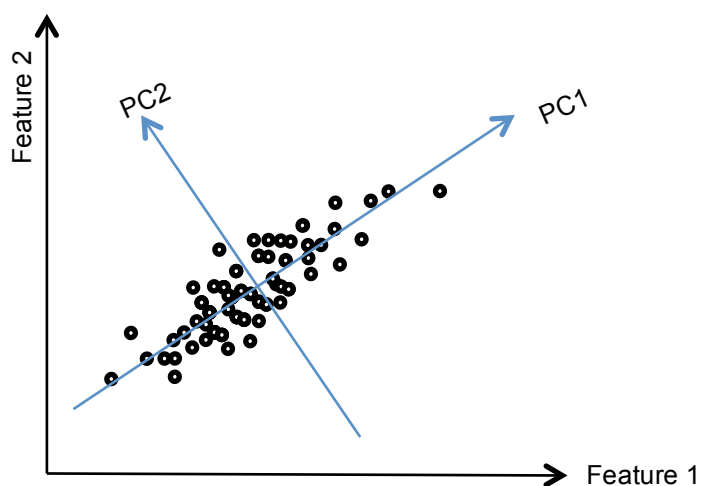


Figure 1.3 Schematic explanation of how PCA reduces variables. Each point stands for each sample. PC1 has the largest variation across data, and PC2 has the second largest variation across data. PC1 and PC2 should be perpendicular to each other.

Table 1.1 (a) LC-MS peak list containing variables in ‘n’ features in each of the ‘m’ samples. **(b)** Variables are reduced from $m \times n$ to $m \times k$ ($k < n$) in a new coordinate system. A ‘feature’ is a potential metabolite (m/z and RT). Variables are peak areas or heights of each feature in each sample

(a)

	Sample 1	Sample 2	Sample 3	Sample 4	...	Sample m
Feature 1	$m \times n$ variables					
Feature 2						
Feature 3						
Feature 4						
...						
Feature n						

(b)

	Sample 1	Sample 2	Sample 3	Sample 4	...	Sample m
PC 1	$m \times k$ variables ($k < n$)					
PC 2						
PC 3						
PC 4						
...						
PC k						

Before PCA is performed on the dataset, some transformation, mean centering, and scaling of data may be helpful for a better visualization. Transformation such as logarithmic transformation or square root transformation can be used to modify data distribution or stabilize variances [62]. Variables usually need to be mean-centred before scaling. Each variable is centered by subtracting the mean value of all of the variables in one feature (the mean value is the average of variables in a certain row in Table 1.1a). Scaling is used to adjust the weight of variables, especially variables with large magnitudes of difference. Autoscaling and pareto scaling are the commonly used scaling methods in metabolomics. Autoscaling scales each variable by its mean-centred value divided by the standard deviation. Pareto scaling scales each variable by its mean-centered value divided by the square root of standard deviation. Pareto scaling reduces the magnitude difference of variables but does not completely remove the difference, which often provides better results in LC-MS data compared to autoscaling [63]. The procedures for PCA were done in the statistical computing language R (<http://www.r-project.org>) for Chapter 3.

1.2.2.3 Metabolite identification

The most challenging task in MS-based metabolomics is the metabolite identification. The identification procedure relies on accurate masses in the mass spectra and tandem mass spectrometry (MS²) spectra for fragment interpretation. These concepts will be discussed in Section 1.3.2. Below describes the commonly used procedure of metabolite identification in MS-based metabolomics.

Firstly but not always necessary, the accurate mass of a metabolite of interest (the m/z in the mass spectrum) can be used to determine, or at least narrow down, the possibility of the chemical formula. The isotopic pattern is also helpful to identify compound containing sulfur (S) or chlorine (Cl). Seven golden rules developed by Kind and Fiehn can be referred to for chemical formulae filtering [64].

Secondly, the elemental formula or the accurate mass (if skip the first step) is to search against database, such as METLIN metabolomics database [65], AntiBase 2013 and KNApSAcK [66], to find the compound candidates. Compound candidates from the same genus as the sample are more likely to be the right compound than metabolites produced from other Kingdom. For example, a secondary metabolite of *Asp. uvarum* is more likely to be shared a metabolite with other *Aspergillus* species than with a species of bacteria.

Thirdly, the MS² spectrum is used to identify the right compound from compound candidates. The MS² spectrum of metabolite of interest needs to be compared against MS² spectra of compound candidates in the literature or database, such as METLIN metabolomics database [65] and Massbank [67]. Two MS² spectra are comparable only when they are acquired using a similar instrument and settings [68]. For example, a different setting in collision energy may result in a different MS² spectrum, which may result in a false negative result.

Accurate mass and MS² spectrum alone can only be used for tentative metabolite identification. For further verification, standards need to be analyzed under the identical conditions with the sample. If the *m/z* and RT, fragments information matches that of the standard, the tentative identification of the metabolite is verified. For novel uncharacterized metabolites, LC-isolated eluents need to be collected for structure elucidation by NMR spectroscopy.

1.2.3 Research aim of the metabolomics study (Chapter 3)

The main goal for the metabolomics study in this work is to discover and identify potential species-specific metabolite markers to effectively discriminate black *Aspergillus* species isolated from Canadian vineyards. This work will be discussed in Chapter 3.

1.3 Analytical Techniques

High-performance liquid chromatography-mass spectroscopy (HPLC-MS) is the main analytical techniques used in both the mycotoxin study (Chapter 2) and the metabolomics study (Chapter 3). This section will introduce the principles and instrumentation of HPLC-MS.

1.3.1 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a technique used to separate, qualify and quantify components in a mixture.

Separation

A sample mixture is injected into a stream of mobile phase flowing through a column where the separation takes place. The mobile phase needs to be pushed through by a

pump under high pressure to get a reasonable flow rate. The sample is placed in an autosampler and instructed by the data system for injection. The column is packed with small-size (usually 1.5 to 5 μm) particle stationary phase, which gives a surface area for interactions between the stationary phase and the components flowing past it. Components get separation because of their different chemical structures and polarity, the time of interaction with the solid phase is therefore different. After the separated components pass through the column, they reach the detector designed to be able to recognize some physicochemical characteristics of the component, such as ultraviolet absorbance (UV detector), fluorescence (fluorescence detector), and mass-to-charge ratio (mass spectrometer). The signal from the detector are translated by the data system as a series of peaks - each one representing a component in the mixture passing through the detector at a specific RT, providing a 'chromatogram' with qualitative and quantitative data.

The most common HPLC is reversed-phase HPLC, where the mobile phase is more polar than the stationary phase. The mobile phase includes water and an organic solvent that is used to lower the polarity of the mobile phase for the purpose of varying the retention of components in a mixture. Typical reversed-phase stationary phases are hydrophobic and chemically bonded (such as C4, C8, C18 bonded) to the surface of a silica support particle. Less polar compounds will have more interaction with stationary phase than with mobile phase so that get a better separation, while polar compounds elute early in the analysis. Therefore, reversed-phase HPLC is good at separating neutral polar and less polar compounds.

Qualification

HPLC can be used to determine the presence of an analyte in a sample. When the analyte is a known compound in a sample mixture and an authentic standard of the compound is available, the sample and the standard can be injected onto the column under the identical chromatographic conditions. The analyte peak in the chromatogram of the sample can be assigned based on the RT of the standard, since the same analyte in the sample and the standard will have the same RT under the same LC conditions. In addition, the spectrum recorded from a detector (e.g. UV spectrum or mass spectrum) at the eluting time of an analyte can also be used to compare with that of the standard for verification. When the analyte is completely unknown or no reference standards available, the mass-to-charge

ratio information can be obtained by MS, and the LC-isolated fraction of the analyte should be collected for NMR characterization.

Quantification

Often, we are not only interested in identifying which compound is in a sample, but also the quantity of the compound. The signal response (peak area or height) in the chromatogram is used to determine the concentration of a compound in the mixture by comparing it to the response of an internal or external standard. The external standard calibration procedure is the most common quantification technique where both standards and unknown samples are analyzed under the identical instrumental conditions. A series of different standard concentrations are prepared and injected from low to high concentrations. A calibration curve is determined by plotting the linear region of the signal response as a function of the concentration. The amount of analyte in the sample will be determined using the calibration curve and the signal response measured. When necessary, appropriate correction factors for a recovery test, and signal suppression/enhancement are also applied.

1.3.2 Mass spectrometry

Mass spectrometry (MS) is a powerful analytical technique used to identify compounds by measuring their mass-to-charge ratio and fragmentation chemistries of their gas phase ions [69]. The three main components of a MS are: ionizer, mass analyzer and detector. In the ionization region, compounds are ionized to charged molecules, then moved to a mass analyzer where the ions are separated based on their mass-to-charge ratio, and finally detected. MS is usually coupled to chromatographic separation technique like GC or HPLC.

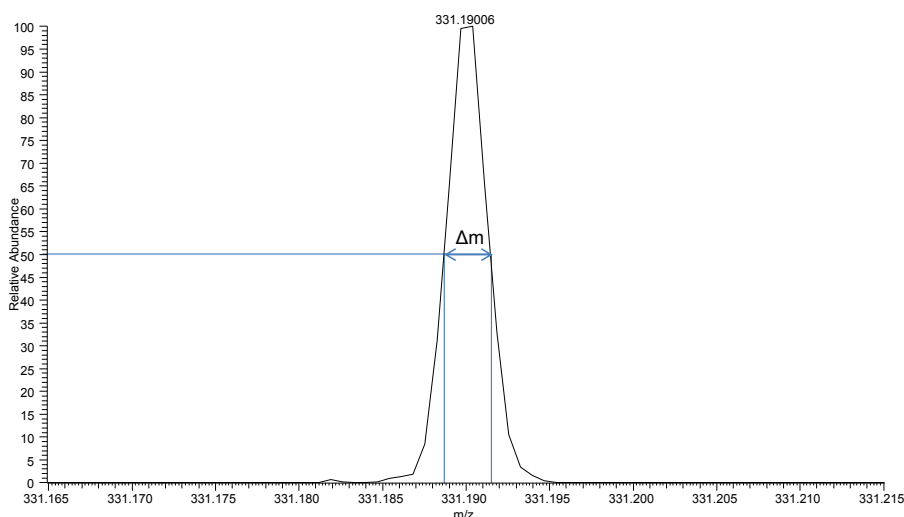
1.3.2.1 Mass accuracy and mass resolving power

Two important parameters in MS are the mass accuracy and mass resolution.

Mass accuracy refers to mass measurement error. It is defined as the difference between the experimentally measured mass (accurate mass) and the theoretical mass (exact mass), divided by the theoretical mass. Deviations specified in parts per million (ppm).

$$\text{Mass accuracy} = \frac{\text{Experimental mass} - \text{Theoretical mass}}{\text{Theoretical mass}} \times 10^6 (\text{ppm})$$

There are different definitions of mass resolution. One of the commonly used meanings as defined by the International Union of Pure and Applied Chemistry (IUPAC) as follows: for a single peak made up of singly charged ions at mass m in a mass spectrum, the resolution may be expressed as $m/\Delta m$, where m is the ion mass, and Δm is the width of the peak at full width at half maximum (FWHM). Figure 1.4 is an example of mass resolution in a high-resolution mass spectrometry (HRMS). The sharper and narrower of the peak, the higher the mass resolution will be. The mass resolution reflects the ability to discriminate ions with slightly m/z differences. A similar term “resolving power” describes the capability of the instrument to separate ions with a close m/z .



$$R = \frac{m}{\Delta m} = \frac{331.19006}{331.1915 - 331.1886} = 114\,203$$

Figure 1.4 Mass resolution in high-resolution mass spectrometry.

1.3.2.2 Orbitrap LC-MS

All the LC-MS and LC-MS² analysis in the thesis were carried out on an Agilent 1290 Infinity LC system (Agilent Technologies Inc., USA) coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, USA) with a heated electrospray (HESI) ionization source. This instrument will be termed ‘Orbitrap LC-MS’ in the following sections of the thesis. The mass accuracy of the Orbitrap MS is typically

<3 ppm for externally calibrated mass spectra and <1 ppm for internally calibrated mass spectra [70]. The maximum resolving power of the Orbitrap MS is 140 000 at m/z 200. The Orbitrap LC-MS offers high-resolution accurate mass (HRAM) measurements, which are necessary for tentative compound identification.

This section will focus on the instrumentation of main components of the Orbitrap LC-MS. Figure 1.5 shows the schematic of the Q-Exactive provided by the vendor.

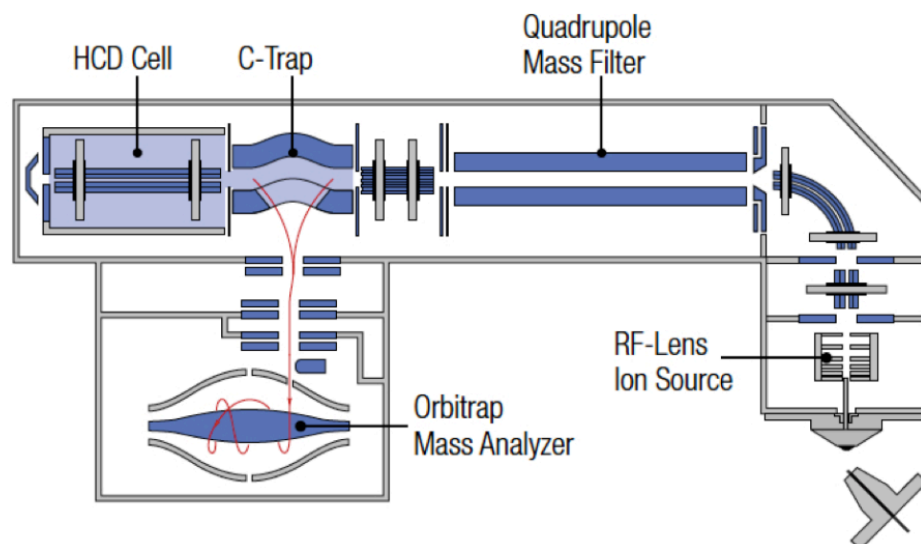


Figure 1.5 Schematic of the Thermo Scientific Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer.

Ion source - ESI

In 2002, John B. Fenn was rewarded with the Nobel Prize in Chemistry for his role in the development of electrospray ionization (ESI) [71]. LC-ESI-MS is widely used for routine and metabolomics analysis. Small liquid droplets eluting from the capillary are dispersed into a fine aerosol in the presence of a strong electric field. The aerosol contains either excess positive or negative charge generated from oxidation or reduction reactions at the liquid capillary interface [72] as well as electrophoretic mobility of ions within the droplet itself [73, 74]. As the droplet evaporates in the source region where high pressure and heat are provided. The typical solvents for ESI are prepared by mixing water with volatile organic compounds such as acetonitrile or methanol. Proton sources like acetic acid, formic acid and trifluoroacetic acid could also be added to the solution to facilitate the ionization process. Two major theories, the charged residue (CR) model and the ion evaporation (IE) model, explain how the desolvated ions are formed. In the CR model,

the droplets shrink as the solvent evaporates. Droplets undergo Coulombic explosion, and the smaller and charged progeny droplets are generated. Progeny droplets undergo shrinkage cycles until a charged analyte remains [75]. In the IE model, the droplets shrink while the solvent evaporates, but charged analytes escape directly from the surface of droplets into gas-phase ions as the droplets reach a certain radius [76]. The gas-phase ions move towards to the mass analyzer.

Electrospray ionization is a soft ionization technique, which generates relatively few fragments ions in comparison the electron impact ionization. The intact molecular ion is usually observed, which is advantageous to determine the m/z of the precursor ion. However, to further get the structure information of the compound, the instrument with an ESI source needs to couple to tandem MS.

Mass Filter - Quadrupole

The Quadrupole mass filter is used to filter through ions based on mass-to-charge ratio. Quadrupoles can also be used as the mass analyzer in some configurations (triple quadrupole MS). The quadrupole consists of four parallel metal rods, two horizontal ones and two vertical ones. Between one pair of rod, both a constant direct current (DC) voltage and an oscillating radio frequency (RF) voltage are applied. Detailed descriptions of the behaviour of trapped ions in quadrupoles can be found in a review by Raymond E. March [77]. The Q-Exactive mass spectrometer uses the quadrupole to isolate an analyte of interest from a complex mixture. It can be sent directly into the mass analyzer or also to a collision cell for tandem mass spectrometry (MS^2).

Mass analyzer - Orbitrap

The mass analyzer is the ‘heart’ of MS where mass measurements occur. The roots of the Orbitrap mass analyzer go back to 1923 when Kingdon put forward the theory of orbital trapping, which uses a wire stretched inside an enclosed metal cylinder to trap electrons [78]. Based on the idea of the Kingdon trap, the initial description of Orbitrap was published by Alexander Makarov in 2000 [79], and commercialized by Thermo Fisher Scientific in 2005.

The Orbitrap is a Fourier transform, ion trap mass analyzer delivering a high mass accuracy measurement and a high resolving power. It is composed of a barrel-shaped

outer electrode surrounding a spindle-shaped inner electrode [50]. Ions rotate around the inner rod and oscillate along the axial at the same time [80]. The frequency of the axial oscillation (ω_z) only depends on the m/z and field curvature [50]. The formula is as follows:

$$\omega_z = \sqrt{\frac{k}{(m/z)}}$$

The angular frequency of the axial oscillation of an ion is inversely proportional to the square root of its mass to charge ratio. k is the force constant of the potential.

The image current of trapped ions are detected through a differential amplifier connected to both halves of the outer electrode, then transformed into a frequency domain signal by Fourier transform, finally converted to mass spectra [49].

Before ions are analyzed by the Orbitrap, a bent RF-only ion trap called the C-trap is applied for serving as ion cooling, accumulation and storage. Ions need to be cooled by collision with nitrogen for the purpose of resting ions to the middle of C-trap [81]. The ions are then forced to a funnel shape and injected into the Orbitrap.

Fragmentation Cell - HCD

Higher collision energy dissociation (HCD) is a collision-induced dissociation (CID) technique in the Orbitrap MS. It should be noted that “higher energy” does not refer to higher collision energy in the HCD cell, but higher RF voltage in the C-trap. Ions are injected from C-trap to HCD for fragmentation. Ions are activated to high kinetic energy using an electrical potential in HCD. The collision between ions and neutral gas convert some kinetic energy to internal energy that results in breaking chemical bonds of the molecular ions. Those fragment ions return to the C-trap and are themselves injected to the Orbitrap for mass measurement.

1.3.2.3 Orbitrap LC-MS in Metabolomics

Targeted Analysis of Metabolites

Targeted analysis is often used to detect and quantify known metabolites in a targeted MS^2 (t- MS^2) experiment. For the Orbitrap LC-MS, targeted precursor ions are isolated by

a quadrupole, and fragmented in the HCD. Fragment ions are focused into the C-trap and injected into the Orbitrap for mass measurement. To identify a metabolite in a sample mixture, the standard solution of that metabolite should be analyzed under the identical conditions. The m/z , RT and MS² fragments should match those obtained from the standards. To quantify a metabolite in a sample mixture, a series of standards with known concentrations and the sample are analyzed under the identical LC conditions. Samples are quantified based on the calibration curve built from the series of standards.

The limit of detection (LOD) is the lowest amount or concentration of an analyte in a sample that can be reliably detected (99% confidence). The limit of quantification (LOQ) is the lowest amount or concentration of an analyte in a sample, which can be reliably quantified with an acceptable level of precision and trueness. The LOQ is the lowest concentration of a series where relative standard deviation (RSD) of several measurements is below 25%. It should be noted that, in the Orbitrap MS, there is no noise, thus a signal to noise ratio is not appropriate measures of the LOD and the LOQ.

Non-targeted Analysis of metabolites

Non-targeted analysis of metabolites using LC-MS and LC-MS² is a way to putatively identify metabolites. A full MS-ddMS² experiment is usually carried out. A full MS survey scan is taken and a pre-defined number of peaks are chosen based on intensity. These peaks are selected sequentially for MS² scans after which the process is repeated. The obtained MS² data can be used for tentative metabolite identification discussed in Section 1.2.2.3.

1.4 Thesis Overview

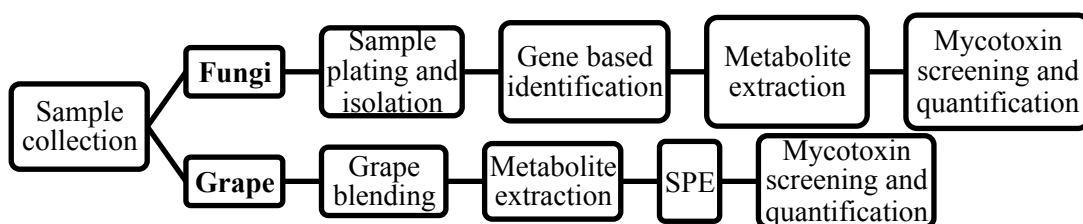
Black *Aspergilli* are responsible for the contamination of grape or grape-derived products with OTA and fumonisins. However, there is limited data on the occurrence of OTA and fumonisins on Canadian grapes. The primary objective of this work is to study the distribution of black *Aspergillus* species in Canadian vineyards, and to screen for and quantify OTA and fumonisins from both black *Aspergilli* isolated on artificial media and grape samples from Canadian vineyards (Chapter 2). The second objective is to establish species-specific metabolite markers that can effectively differentiate black *Aspergilli* isolated (Chapter 3).

Chapter 2 – OTA and Fumonisin Contamination in Canadian Vineyards

2.1 Introduction

It is known that black *Aspergilli* are responsible for wine contamination with OTA and fumonisins. However, there is limited data on the occurrence of black *Aspergilli* and its associated risk of OTA and fumonisin contamination in Canadian vineyards. Health Canada (2004) reported the presence of OTA in ‘Canadian wine’ [82], but it was later determined that the wine tested was not VQA wine, meaning that it was a blend of Canadian grapes with grapes from other countries.

Mycotoxin analysis on both black *Aspergilli* isolated from Canadian vineyards and grape samples has been carried out. Scheme 2.1 shows the overall workflow of this chapter. For the mycotoxin analysis of fungi, black *Aspergilli* were primarily isolated from grape or soil samples and identified to the species level (Section 2.3.1). In addition, individual species were screened (Section 2.3.2) and quantified (Section 2.3.3 and Section 2.3.4) for OTA and fumonisins produced on artificial media. For mycotoxin analysis on grapes, a solid phase extraction (SPE) method for OTA and FB₂ cleanup was optimized (Section 2.3.5), and samples were then screened and quantified for OTA and fumonisins (Section 2.3.6).



Scheme 2.1 The overall workflow of Chapter 2.

2.2 Experimental

2.2.1 Sample collection

Grapes and soil samples were collected from August and September in 2012 (22 soil samples and 11 grape samples), August to November in 2013 (90 soil samples and 90 grape samples) and September to November in 2014 (16 soil samples and 78 grape samples) from Agriculture and Agri-food Canada's Jordon Research Station (AAFC-JRS) and the University of Guelph's Grape Research Station (GRS) in the Niagara region of Ontario. In 2014, sample collection was also extended to other Canadian vineyards including: 9 soil samples and 9 grape samples from commercial sites (CS) in Niagara, 4 soil samples and 4 grape samples were collected from AAFC's Kentville Research Station (KRS) in Nova Scotia, and 2 soil samples were collected from AAFC's Pacific Agri-Food Research Centre (PARC) in British Columbia (Courtesy of Dr. Ian Scott, AAFC).

Grape samples were collected from vineyards and placed into Ziploc bags. Soil samples were collected to a depth of 5 cm from vineyards, and placed into a 50 mL falcon tube. Grape and soil samples were kept on ice during transportation, and stored in a freezer at -20°C until sample preparation.



Figure 2.1 Grape sample (left) and soil sample (right).

2.2.2 Analysis of mycotoxins produced by black *Aspergilli* isolated from grape and soil samples

In this section, I will discuss isolation of black *Aspergilli* from grape and soil samples, identification of black *Aspergillus* species using a gene-based approach, and LC-MS²

analysis of OTA and fumonisin from black *Aspergilli* isolates.

2.2.2.1 Sample plating and black *Aspergilli* isolation

The objective of this section was to isolate black *Aspergilli* from infected grapes and soil. Three grapes from each sample bag were cut into two pieces by a sterilized knife in a bio cabinet, and half pieces were plated on dichloran rose bengal chloramphenicol (DRBC) agar, the other half pieces of the same sample on dichloran 18% glycerol (DG18) agar. Media recipe was listed in Appendix A. Grape samples were plated out in triplicates onto DRBC agar and DG18 agar (6 plates total per sample). Five grams of soil was weighed out and suspended into 45 mL sterile water with 0.2 % peptone as the stock solution in a milk dilution bottle. 10^{-1} dilution bottle was made by transferring 5 mL stock solution into a milk bottle with 45 mL sterile 0.2% peptone water. Similarly, 10^{-2} , 10^{-3} , 10^{-4} dilution bottles were made [83]. One drop of Tween-20 was added to stock solution and each dilution bottle. Figure 2.2 shows one stock bottle and four dilution bottles. 0.1 mL from each dilution bottle was aseptically transferred in duplicate onto DRBC agar and DG18 agar by sterile pipets (16 plates total per sample).



Figure 2.2 Soil dilution bottles (one stock bottle and four dilution bottles) for soil sample plating.

The grape and soil plates were then incubated at 30 °C until black *Aspergilli* were observed by morphology. Since other microorganisms in grape or soil could grow into colonies on agar, in order to isolate black *Aspergilli* from other microorganisms, spores from black *Aspergilli* were transferred onto fresh Czapek yeast autolysate (CYA) agar plates by a sterile needle. Fungal plates were incubated at 30 °C for 7 days to get fungal isolates of black *Aspergilli*. A fungal isolate or simply ‘isolate’ is defined as an isolated

fungal strain on a medium. 'Fungal isolate from 2012' indicates that the original sample was collected from the sampling year 2012. Figure 2.3 shows black *Aspergillus* isolated from grape and soil sample on DRBC or DG18 agar, and the fungal isolate on CYA agar after transferring spores from the original grape or soil plate.

The reason why I use different agar media and soil dilution technique were explained as follows: DRBC and DG18 were used to plate grapes and soil due to their anti-bacteria properties as well as inhibiting of rapid-growing fungi, whereas CYA supports the abundant growth of *Aspergillus* species. The reason to plate soil in different dilution is that there are other microorganisms in soil, such as bacteria, yeast and other fungi, which could interfere with isolation of black *Aspergilli*. Soil dilution increases the possibility plating black *Aspergilli* out.

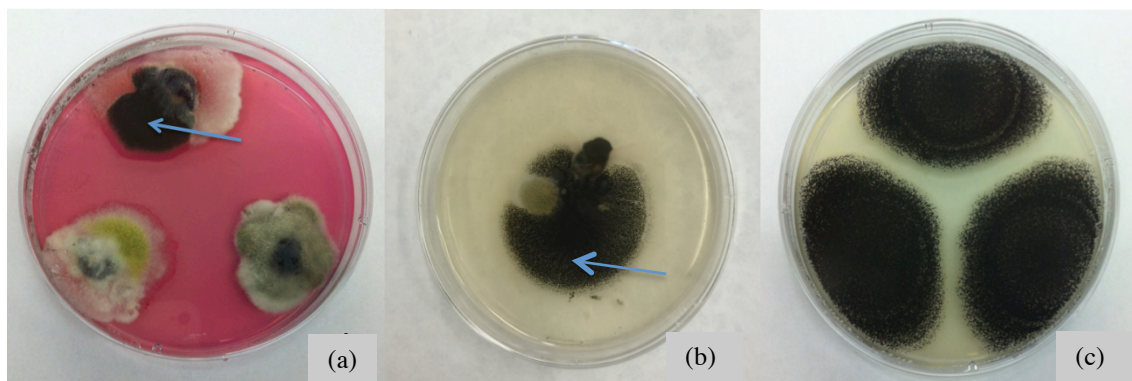


Figure 2.3 (a, b) Black *Aspergillus* colony (arrow) surrounded by other microorganisms on DRBC agar plated grape sample and on DG18 agar plated soil sample respectively. **(c)** Fungal isolate on CYA agar.

2.2.2.2 Gene-based fungal identification

The goal of this section was to identify black *Aspergilli* to the species level. Because some of the species in black *Aspergilli* are morphologically indistinguishable [84], gene-based analyses, β -tubulin gene sequencing and PCR amplification of the calmodulin gene using species-specific primers, were carried out. The theory of PCR techniques is explained in Appendix B.

DNA of fungal isolates (2012-2014) was extracted using a MoBio Ultraclean Microbial DNA Extraction Fungal Kit (MoBio Laboratories, Solana Beach, CA, USA), according to

manufacturer's protocol. The DNA was amplified by PCR with the forward β -tubulin 2a primer (Bt2a) and the reverse β -tubulin 2b primer (Bt2b). DNA sequences of β -tubulin primers are listed in Appendix C. Each PCR reaction included 22.5 μ L of Platinum PCR SuperMix (containing Mg^{2+} , dNTPs, and recombinant Taq DNA polymerase), 0.75 μ L of Bt2a primer (10 μ M), 0.75 μ L of Bt2b primer (10 μ M), and 1.0 μ L of DNA product. The PCR reaction was carried out on a thermo cycler (Eppendorf Mastercycler Nexus Gradient). The PCR conditions were as follows: 3 min at 94 °C; 35 cycles of amplification for 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C; and 5 min at 72 °C. The PCR amplification was confirmed by agarose gel electrophoresis made with 1.2% agarose gel in Tris-acetate-EDTA (TAE) buffer.

PCR products were cleaned up using exoSAP-IT (Affymetrix, Santa Clara, CA, USA) to consume unused dNTPs and primers remaining in the PCR product. Each reaction contains 4.0 μ L of exoSAP-IT and 10.0 μ L of PCR product. The PCR conditions were 15 min at 37°C, and 15 min at 80°C. After cleanup, 4.0 μ L of purified PCR product with 2.0 μ L of Bt2a primer, and 4.0 μ L of purified PCR product with Bt2b primer were sent to Roberts Research Institution of Western University for sequencing.

Forward and reverse sequences from each strain were aligned using SeqMan software [85]. The sequences of our strains and type strains of known *Aspergillus* species published in NCBI GenBank were used to carry out phylogenetic analysis using MEGA (Molecular Evolutionary Genetics Analysis) 6.0 [86]. Strains that grouped with a type strain in the same branch in a phylogenetic tree are considered to be the same species as the type strain. Strains that did not group with any type strains could be potentially identified using NCBI nucleotide BLAST (Basic Local Alignment Search Tool) [87].

Palumbo *et al.* (2015) designed species-specific primers An and Aw based on species-conserved differences in calmodulin gene sequence for *Asp. niger* and *Asp. welwitschiae* differentiation [88]. DNA sequences of An and Aw primers are listed in Appendix C. Slight modifications to the PCR conditions with An primers are as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 62.3°C for 30 s, 72°C for 30 s; and 72°C for 5 min. Slight modifications to the PCR conditions with Aw primers are as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 61.1°C for 30 s, 72°C for 30 s; and 72°C for 5 min. PCR products were analyzed by gel electrophoresis. All of the strains sequenced to be *Asp. niger* and *Asp. welwitschiae* were amplified with Aw and An primers for the purpose of verification.

Therefore, all the fungal isolates were identified to the species level using sequence analysis of β -tubulin gene. Strains sequenced to be *Asp. niger* and *Asp. welwitschiae* were verified by PCR amplification using species-specific An and Aw primers.

2.2.2.3 Metabolites extraction

Fungal isolates from 2012-2014 were extracted using the agar plug method [89] with some modifications described below. Six agar plugs from each fungal isolate were cut by a sterilized cork borer (diameter 3.5 mm), and were put into a 7 mL glass vial. 1.5 mL of 75 % methanol in water was added to the vial. The vials were sonicated for one hour at room temperature to extract the metabolites. The solution was transferred to a 1.5 mL Eppendorf tube, and was centrifuged (Sorvall Legend Micro 17 microcentrifuge, Thermo Scientific) for 2 min at 13,000g to remove cell debris. The supernatant was passed through a 0.45 μ m syringe filter (Acrodisc CR 13 mm syringe filter with 0.45 μ m PTFE membrane, Life Sciences) into an amber HPLC glass vial. The solution in the HPLC glass vial is defined as the agar plug extract.

2.2.2.4 OTA, FB₂, FB₄ and FB₆ detection

All of the following LC-MS² analyses were performed on an Agilent 1290 Infinity LC system (Agilent Technologies Inc., USA) coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, USA) shown in Figure 2.4. The purpose was to screen for OTA, FB₂, FB₄ and FB₆ from the agar plug extract using LC-MS².

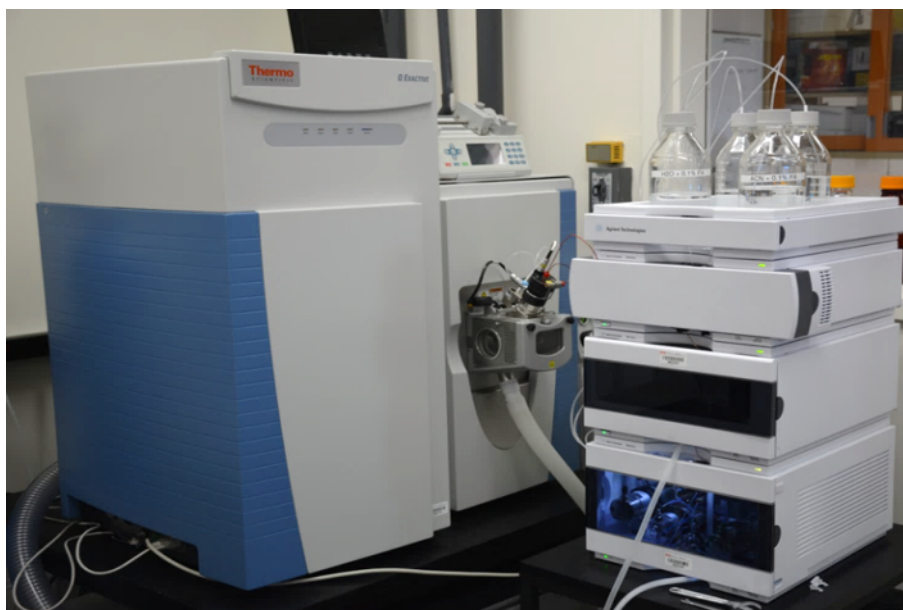


Figure 2.4 An Agilent 1290 Infinity LC system coupled to the Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer

2.0 μL of each sample was injected onto an Agilent ZORBAX Rapid Resolution High Definition (RRHD) Eclipse Plus C18 threaded column, 2.1 mm (i.d.) \times 50 mm (length) packed with a high performance 1.8 μm microparticulate maintained at 35 $^{\circ}\text{C}$. Separation was achieved using a flow rate of 0.3 mL/min with a mobile phase of LC-MS grade water with 0.1 % formic acid (solvent A), and acetonitrile with 0.1 % formic acid (solvent B). The gradient conditions were as follows: solvent B was held at 0 % for 0.5 min, and was linearly increased to 100 % over 3 min, held constant for 1 min, and was decreased linearly from 100 % to 0 % over 0.5 min. The ionization conditions used for HESI were: capillary temperature, 400 $^{\circ}\text{C}$; sheath gas, 17.00 units; auxiliary gas, 8.00 units; probe heater temperature, 450 $^{\circ}\text{C}$; S-Lens RF level, 45.00. A capillary voltage was 3.9 kV.

Agar plug extracts were screened for OTA, FB₂, FB₄ and FB₆ using a t-MS² experiment in positive ion mode. The settings were as follows: resolution of 17 500, automatic gain control (AGC) target of 5×10^5 , a maximum injection time of 100 ms, and a 1.0 m/z isolation window. The targeted ions are listed in Table 2.1.

Table 2.1 Targeted ions in a t-MS² experiment for OTA, FB₂, FB₄ and FB₆ detection and quantification. Retention time tolerance was set to 0.20 min.

Compound	RT (min)	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	NCE
OTA	3.40-3.60	404.08954	358.08368	25
FB ₂	2.82-3.02	706.40083	336.32581	28
FB ₄	2.93-3.13	690.40592	320.33118	28
FB ₆	2.77-2.97	722.39575	370.33157	28

2.2.2.5 OTA and FB₂ quantification

After the detection of OTA, FB₂, FB₄ and FB₆ from agar plug extracts, the next goal was to quantify them. Since standards of FB₄ and FB₆ were not commercially available, the goal was to quantify OTA and FB₂.

Two agar plugs were cut by a sterilized cork borer (diameter 5.5 mm) from each fungal isolate where OTA and/or FB₂ were detected. Agar plugs were macerated with 25 mL sterile Milli-Q water using a polytron blender. 2.5 mL of the agar plug solution was inoculated into individual Erlenmeyer flask containing 50 mL sterile CYA liquid medium, which was then incubated at 30 °C. Time series experiments were carried out by taking 0.5 mL aliquots out periodically from day 1 to day 27 after incubation. The aliquots were diluted with 0.5 mL Milli-Q water, syringe filtered into a HPLC vial, and analyzed by LC-MS² to monitor for mycotoxin production.

Ten standard solutions (concentration from 0.0001 µg/mL to 10 µg/mL) of OTA and FB₂ were injected five times onto the column. Each sample was injected twice, followed by a single acetonitrile blank. All standards, samples and blanks were injected 2.0 µL onto the column. The LC-MS² method was set up as described above in Section 2.2.2.4.

After data acquisition, OTA and FB₂ were identified based on the *m/z* of their fragment ions along with specific RTs in comparison to their standards. Samples were quantified based on their signal response measured and calibration curves generated from OTA and FB₂ standards.

2.2.3 Analysis of mycotoxins in grape samples

Isolated black *Aspergilli* and any mycotoxins detected on artificial media does not mean the presence of mycotoxins on grape samples. While it is important to test the occurrence of mycotoxins on grape samples, there are two challenges. Firstly, the grape is a complex matrix compared to CYA medium. Natural compounds in grapes may cause significant ion suppression over a certain mycotoxin during ionization when they elute at the same time. The second challenge is that the concentration of mycotoxins on grapes could be below the LOD of the LC-MS instrument. This means even if there were mycotoxins on grapes, they may not be detected. To overcome these problems, solid phase extraction (SPE) was used for sample cleanup and concentration. The theory of SPE techniques is explained in Appendix D.

2.2.3.1 Optimization of SPE cleanup

OTA and fumonisins can use reverse phase SPE cartridges for cleanup. Bond Elut Plexa cartridges (Agilent) were pre-equilibrated with 2×5 mL methanol and 2×5 mL water (pH 7). 100 μ L of 1 μ g/mL OTA and FB₂ standards were spiked into 5 mL of water (pH 7) in a 7 mL scintillation vial. The same amount of OTA and FB₂ standard solutions was spiked into another 5 mL of water (pH 7) and kept as the control, which was not subjected to an SPE cleanup. The sample was then loaded onto the cartridge at the speed of one drop per second. The cartridge was washed with 5 mL of methanol/formic acid/water (10:1:89), followed by 5mL of methanol/formic acid/water (25:1:74), 5mL of methanol/formic acid/water (50:1:49), 5mL of methanol/formic acid/water (75:1:24), and finally 5mL of methanol/formic acid (99:1). All of the SPE flow-through solutions were collected into 7 mL scintillation vials. Another SPE cleanup experiment was carried out as described above, except using pH 3 water for sample preparation and cartridge pre-equilibration.

Anion-exchange SPE cartridges can be used for OTA and fumonisins cleanup, because they both have carboxyl groups to be deprotonated in a neutral or basic environment. The following procedure was modified from Hibino *et al.* (1999) [90]. Bond Elut SAX cartridges (Agilent) were pre-equilibrated with 2×3 mL of methanol and 2×3 mL of 75% methanol in water. 100 μ L of 1 μ g/mL OTA and FB₂ standards were spiked into 5 mL of water. 15 mg of EDTA was added to water for the purpose of reducing the reactivity of metals in the SPE cartridge, and the pH was adjusted to 10. The control group contains

the same amount of OTA and FB₂ as the sample, but without SPE cleanup. After the sample was loaded to the SPE cartridge, the cartridge was washed with 2 × 3 mL of 75% methanol in water, then with 2 × 3 mL of 100% methanol, and finally with 2 × 3 mL of methanol/acetic acid (99:1). All of the SPE flow-through solutions were collected into 7 mL scintillation vials. An additional experiment was performed using the pH 7 H₂O for sample preparation, and SPE was carried out under the same procedures as above. Figure 2.5 shows SPE procedures using a Bond Elut Plexa cartridge and Bond Elut SAX cartridges.



Figure 2.5 SPE procedures using a Bond Elut Plexa cartridge and a Bond Elut SAX cartridge. All of the SPE flow-through solutions were collected into separate scintillation vials. The solutions were to be concentrated for LC-MS² analysis.

OTA and fumonisins can also use cation-exchange SPE cartridges for cleanup, since they both have one amine group to be protonated in a neutral or acidic environment. The following procedure was performed according to the manufacturer's recommended protocol, but with some modifications. Oasis MCX cartridges (Waters) were pre-equilibrated with 2 × 5 mL of methanol, 2 × 5 mL of water and 2 × 5 mL of water/formic acid (98:2). 100 µL of 1 µg/mL OTA and FB₂ standards were spiked into 5 mL water. 15 mg of ethylenediaminetetraacetic acid (EDTA) was added for the purpose of reducing the reactivity of metals, and the pH was adjusted to 7. The same amount of standard solution as the sample was kept as the control group. The cartridge was loaded with the sample, washed with 2 × 3 mL of methanol/formic acid (99:1), and then 2 × 3 mL of methanol/ammonia hydroxide (95:5). All of the SPE flow-through solutions were

collected into 7 mL scintillation vials. A parallel experiment was carried out at pH 4 for sample preparation.

In the above three SPE procedures, the collected SPE flow-through solutions and controls were dried under a gentle stream of nitrogen stream, resolubilized in 0.5 mL of 75% methanol in water, and analyzed by LC-MS² with the same method as described in Section 2.2.2.4. The recovery was calculated by dividing the ion intensity in the experimental samples (post SPE) by the ion intensity in the controls (pre SPE) for OTA and FB₂ separately.

2.2.3.2 Method development of grape extraction and SPE cleanup

Spiking of OTA and FB₂ standards in water may not be representative of OTA and FB₂ on grapes because of the matrix effect. Therefore, the OTA-positive and FB₂-positive fungal isolates were inoculated onto commercial grapes bought from a local store in London Ontario for method development.

Two hundred grams of commercial grapes were placed inside sterile 2 L Erlenmeyer flasks containing filter paper, and then capped with foam plugs. Inoculant was prepared by macerating a 3 x 3 cm portion of *Asp. carbonarius* isolate in 20 mL sterile water with a polytron blender, and then was added to the flask. Similarly, *Asp. welwitschiae* inoculant was added to another flask. Grapes inoculated with *Asp. carbonarius* and *Asp. welwitschiae* and were incubated at 30°C for 14 days.

After 14 days, grapes were blended with 100 mL of water using a polytron blender (became ‘grape must’ after blending). In order to find the best solvent for extraction, five grams of grape must were measured and extracted by shaking with 20 mL of the following different solvents in a 50 mL Falcon tube: methanol/formic acid (99:1), methanol/water/formic acid (75:24:1), acetonitrile/water/formic acid (79:20:1), acetonitrile/water/acetic acid (79:20:1) [91], acetonitrile/water/formic acid (59:40:1) , acetonitrile/water/acetic acid (59:40:1), and acetonitrile/methanol/water (1:1:2) [92]. After the extraction, the Falcon tube was centrifuged at 2000 rpm for 2 min to precipitate the grape must. Twenty mL of supernatant was transferred to the parallel evaporator (Buchi Multivapor P-12 Parallel Evaporator) and samples were evaporated to dryness. Samples were resolubilized with 0.5 mL of 75% methanol in water and syringe filtered into HPLC vials. Grape extractions with different solvents were analyzed using LC-MS².

Ion intensities of OTA and fumonisins were compared between different solvents.

After finding the appropriate solvent, samples were divided into four parts (A, B, C, D) and each part was transferred into a 7 mL scintillation vial. 5 mL of mili-Q water were added to each vial. Part-A was the pre-SPE as the control group. Parts-B, C, and D were loaded on a Bond Elut Plexa cartridge, a Bond Elut SAX cartridge, and an Oasis MCX cartridge respectively using the same procedure described in Section 2.2.3.1.

2.2.3.3 OTA and FB₂ quantification from grapes

Based on the initial agar plug screenings from sampling sites where fumonisins and OTA were detected, grape samples were then assessed to confirm the presence of OTA and FB₂. Grape musts were prepared by blending 50 grams of grape samples with 25 mL of water using a polytron blender. Five grams of grape must were measured out and extracted with 20 mL of acetonitrile/water/acetic acid (79:20:1) by shaking at 300 rpm for 1 hour. After centrifuging at 2000 rpm for 2 min, 20 mL of supernatant was dried down using the parallel evaporator (Buchi Multivapor P-12 Parallel Evaporator). Figure 2.6 shows extracts from the grape extraction procedure, and sample evaporation.

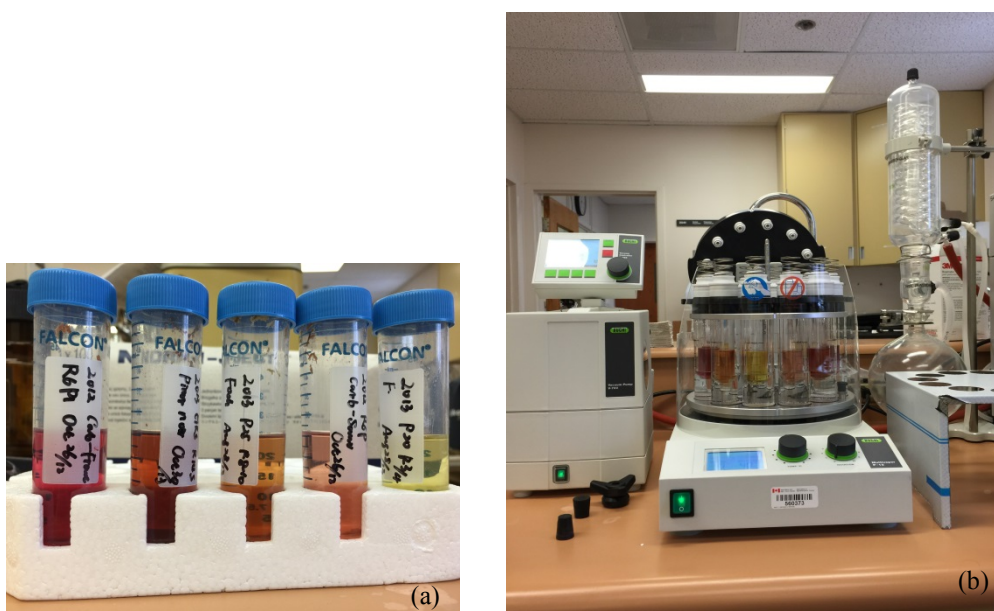


Figure 2.6 (a) Grape extracts. (b) Sample evaporation using the Buchi parallel evaporator.

The sample was re-suspended with 10 mL of 5% methanol in water, and adjusted pH 7 for Bond Elut Plexa cartridge. The cartridges were pre-equilibrated with 2 × 5 mL methanol and 2 × 5 mL water (pH 7), followed by the sample being loaded onto the cartridge. It was washed with 5 mL of methanol/formic acid/water (50:1:49), then with 5mL of methanol/formic acid/water (75:1:24), and finally with 5mL of methanol/formic acid (99:1). All of the SPE flow-through solutions were collected, dried under a stream of nitrogen, re-suspended with 2 mL of 75% methanol in water, and finally analyzed by LC-MS². The settings for LC-MS² were similar to Section 2.2.2.4, except that 10 µL of each standard and sample were injected five times onto the column. The settings of HESI ionization source were optimized for FB₂ analysis as follows: capillary temperature, 240°C; sheath gas, 22.00 units; auxiliary gas, 10.00 units; probe heater temperature, 410 °C; S-Lens RF level, 60.00. A capillary voltage was 3.8 kV.

2.3 Results and Discussion

2.3.1 Identification of black *Aspergilli* to the species level

Grape samples (n=192) and soil samples (n=143) were collected from Canadian vineyards over three years (2012–2014). Samples were mainly collected from AAFC's JRS, the University of Guelph's GRS in the Niagara region of Ontario; a few samples were collected from commercial sites in Niagara, AAFC's KRS in Nova Scotia, and AAFC's PARC in British Columbia. Each grape sample was plated in triplicate onto DRBC and DG18 agar media. Four different dilutions of each soil sample were pipetted in duplicate onto DRBC and DG18 agar media. Therefore, in total, there were 1152 grape plates and 2288 soil plates. These plates were incubated at 30 °C for 7 days. Black *Aspergilli* were isolated from these plates by transferring spores onto fresh CYA agar plate. There were 567 black *Aspergillus* isolates over these three years. The following work was to identify black *Aspergilli* to the species level.

Gene-based tools were used to discriminate species within black *Aspergilli*. Sequence analysis of β-tubulin gene was primarily carried out over those 567 black *Aspergillus* fungal isolates. A phylogenetic tree was generated from β-tubulin gene sequences of all of the samples and known black *Aspergillus* type strains published in GenBank [93]. Since the dataset is large (567 strains), a subset of 2013 fungal isolates (50 strains) is shown to be representative of the whole dataset in a phylogenetic tree in Figure 2.7.

Phylogenetic trees or evolutionary trees show relationships among strains. The closer the strains are to each other on each branch on the tree, the more similarity in gene sequences and the more closely they are related. Strains that grouped with a type strain in the same branch are considered to be the same species as the type strain. The confidence of the observed branch is represented by bootstrap values (numbers near branches). The bigger the number is, the higher the probability of getting the same samples and relationships within that branch when rebuilding the tree using the same dataset. A bootstrap value of 70 is often regarded as a threshold value for good confidence [94].

Phylogenetic analysis results show the following five main black *Aspergilli* species were present in the investigated Canadian vineyards (Figure 2.7): *Asp. welwitschiae*, *Asp. uvarum*, *Asp. brasiliensis*, *Asp. tubingensis*, and *Asp. niger*. *Asp. uvarum*, *Asp. brasiliensis*, and *Asp. tubingensis* had good separations supported by high bootstrap values (bootstrap values ≥ 98). *Asp. niger* and *Asp. welwitschiae* strains were closely related species. In Figure 2.7, they had little separation (only MWS 063 and MWS 239 were grouped with *Asp. niger* CBS 12049 type strain) supported by bootstrap values below 65, which lowers the confidence of differentiation between *Asp. niger* and *Asp. welwitschiae* strains using β -tubulin gene sequencing.

To verify β -tubulin gene sequencing results for *Asp. niger* and *Asp. welwitschiae* differentiation, PCR amplification was performed using *Asp. niger* and *Asp. welwitschiae* specific primers (An and Aw) respectively [95]. PCR reactions were carried out in all of the DNA extracts of *Asp. niger* or *Asp. welwitschiae* strains based on β -tubulin gene sequence analysis results. PCR products with a UV-sensitive DNA-binding dye were analyzed by gel electrophoresis. If there is a DNA band visualized under UV, the sample was successfully amplified using the An/Aw primer, suggesting that it was identified to be either *Asp. niger* or *Asp. welwitschiae*. Figure 2.8 shows the gel image of PCR products using both the An and Aw primers for each strain sequenced to be *Asp. niger* or *Asp. welwitschiae* in Figure 2.7 (from MWS 071 to MWS 239, but not the exact order shown in the tree). For instance, the PCR product amplified using Aw primer was loaded in well 1 row 1, and the same PCR product which was amplified using An primer was loaded in well 1 row 2. In Figure 2.8, both PCR products in lane 7 (MWS 063) and lane 19 (MWS 239) were amplified successfully using An primers. As a result, MWS 063 and MWS 239 were identified to be *Asp. niger*. The other strains were amplified using Aw primers successfully, and they were therefore determined to be *Asp. welwitschiae*. These

results agreed with β -tubulin gene sequencing as shown in Figure 2.7. Using this method, all of the strains sequenced to be *Asp. niger* or *Asp. welwitschiae* were verified by PCR amplification using species-specific primers.

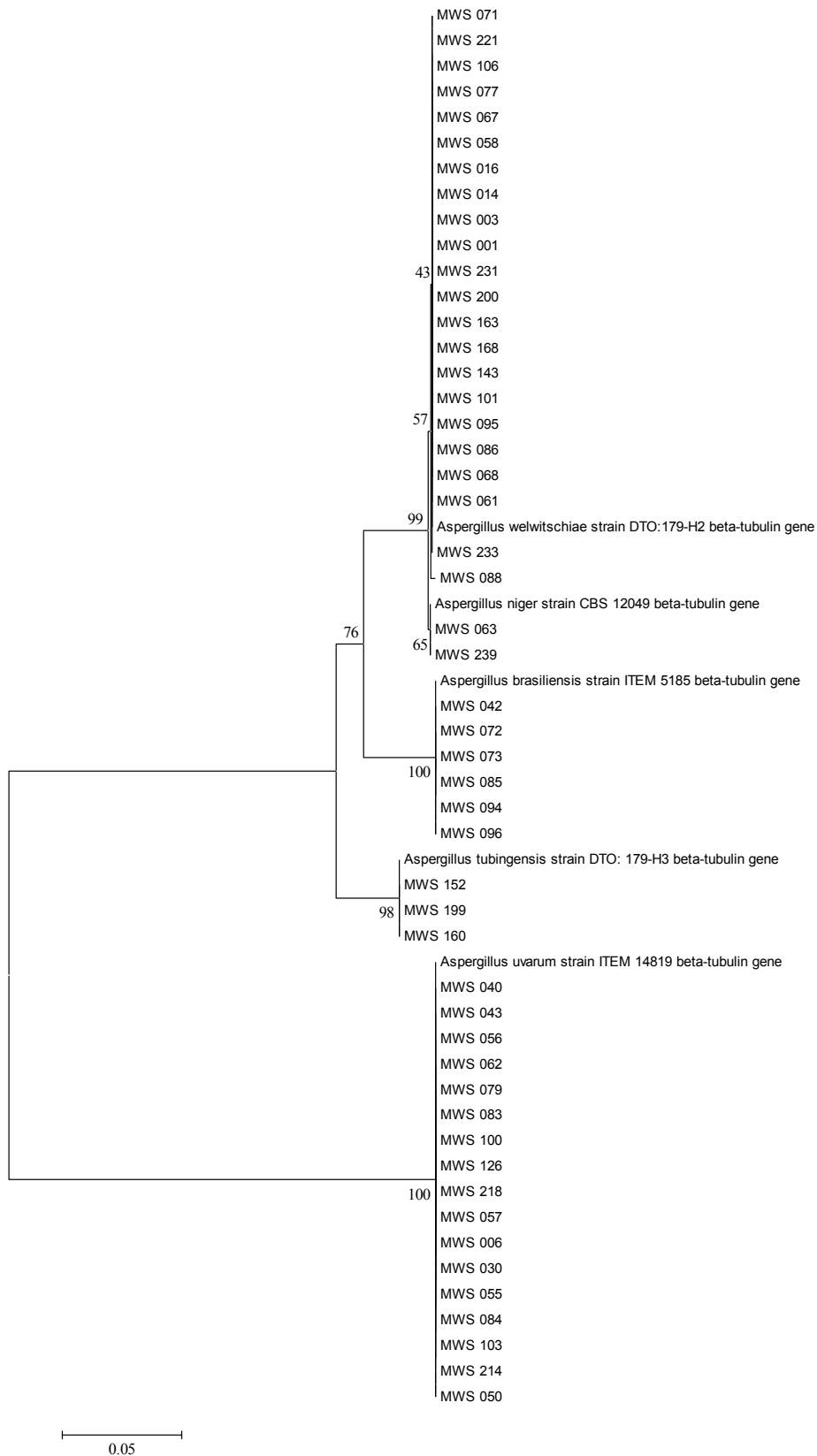


Figure 2.7 Phylogenetic tree of a subset of 2013 isolates sequenced using β -tubulin primers. Numbers near branches are bootstrap values based on 1000 replications.

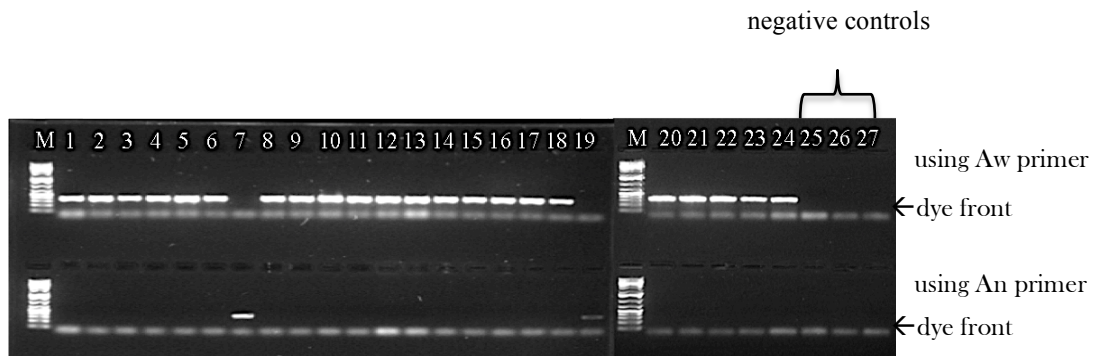


Figure 2.8 Gel image of PCR products using both An and Aw primers for each strain sequenced to be either *Asp. niger* or *Asp. welwitschiae* in Figure 2.7. The PCR product amplified using Aw primer and An primer for the same strain was loaded in the same lane but row one and row two respectively. PCR products of 24 strains were loaded in lane 1-24. MWS 040 (*Asp. uvarum*), MWS 042 (*Asp. brasiliensis*), and MWS 160 (*Asp. tubingensis*) were loaded in lane 25-27 as negative controls. Lane M = DNA ladder.

After the gene-based identification for the 567 black *Aspergillus* isolates, the information about isolates (e.g. sampling site, sampling date) were inputted into Microsoft Access for a custom database. Strains which were isolated from the same sampling site, sampling row, sampling date, source (grape/soil), and sequenced to be the same species were considered to be replicates, and were removed from the following statistics.

There were 253 black *Aspergillus* strains (replicates removed) isolated from Canadian vineyards from 2012-2014. The species distribution was as follows: *Asp. welwitschiae* (43%), *Asp. uvarum* (32%), *Asp. brasiliensis* (11%), *Asp. tubingensis* (9%), and *Asp. niger* (4%). The percentage of each species is calculated based on the number of identified strains in a certain species divided by the total number of black *Aspergillus* strains (253 strains). *Asp. carbonarius*, the main producer of OTA on grapes in other parts of the world, was isolated only once from a grape sample collected from AAFC-JRS of Niagara in 2014. It was identified by β -tubulin gene sequencing, verified by PCR amplification using *Asp. carbonarius* specific primers (DNA sequences are listed in Appendix C) [96], and confirmed by comparison to a type strain published in GenBank: *Aspergillus carbonarius* strain CICC41254. This data suggests that the occurrence of *Asp. carbonarius* infected grapes in Canadian vineyards (0.4%) was lower than that in Italian (24%) and French (26%-48%) vineyards as reported by Oliveri *et al.* (2011) [97] and Bejaoui *et al.* (2006) [98] respectively. Figure 2.9 shows the stacked bar diagram indicating the number of different black *Aspergillus* species isolated from 2012 to 2014.

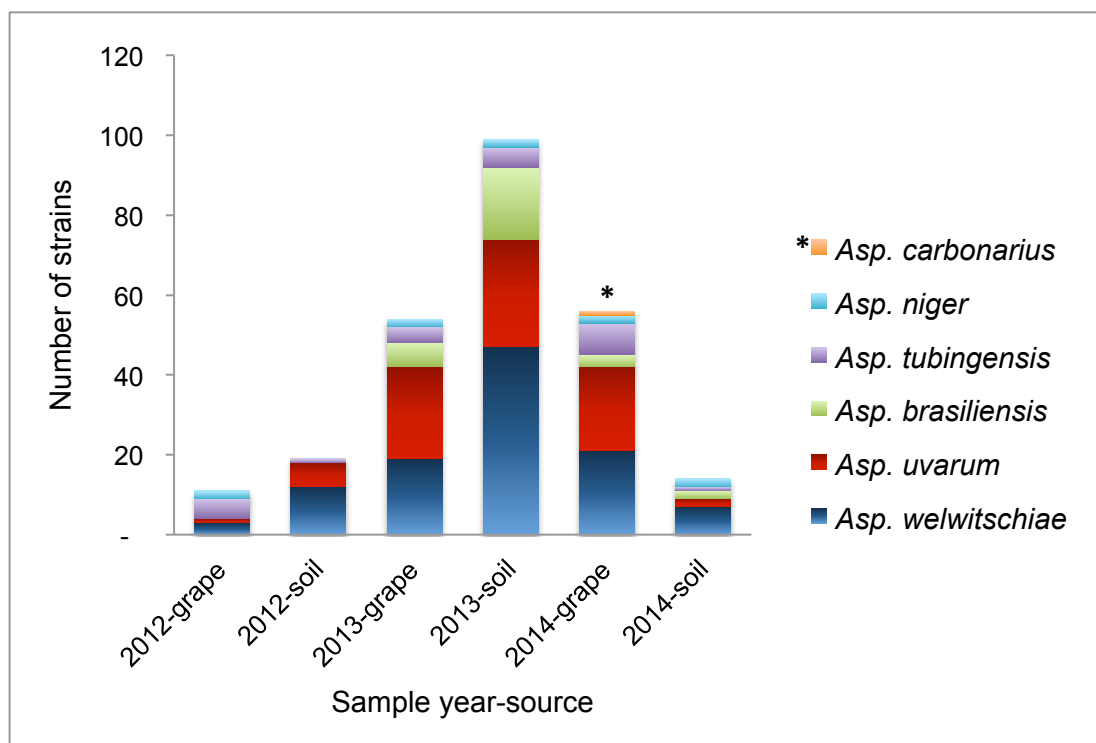


Figure 2.9 Total black *Aspergillus* (n=253) strains isolated over a three-year period.

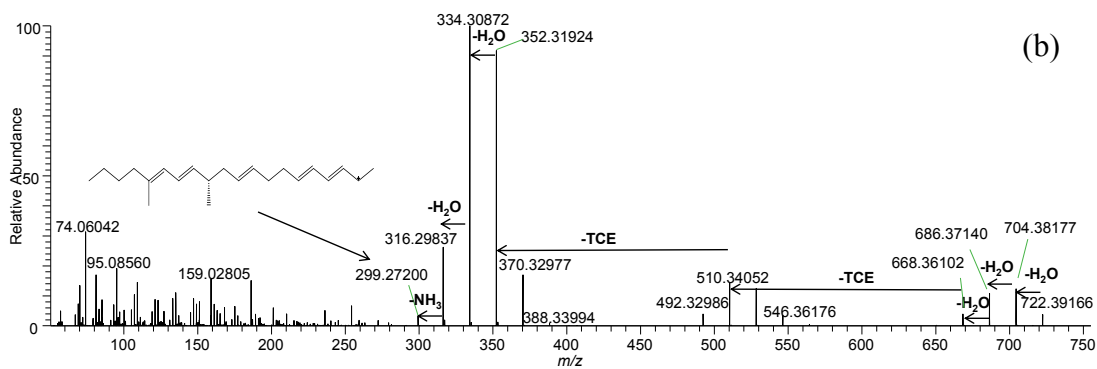
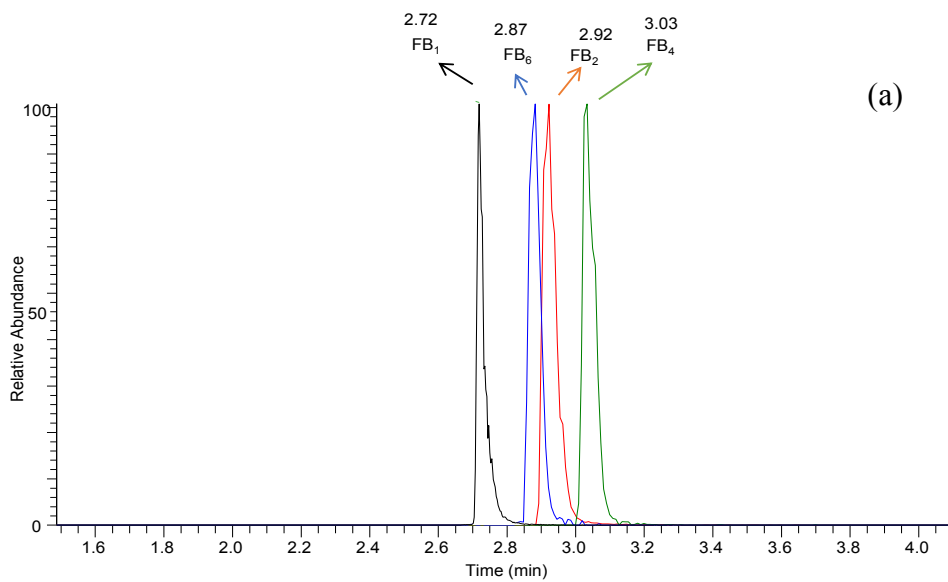
2.3.2 Occurrence of OTA and fumonisins in black *Aspergilli*

Black *Aspergilli* are responsible for wine contamination with OTA and fumonisins. The next goal was to screen for OTA and fumonisins from fungal isolates. Agar plugs were cut from all of the fungal isolates, extracted with 75% methanol in water, and screened for the presence of OTA, FB₂, FB₄ and FB₆ using a t-MS² experiment. OTA and FB₂ positive strains were determined by matching the accurate mass, RT and MS² fragmentation with that in authentic standards. Standards of FB₄ and FB₆ are not commercially available, and as such spectra were compared to data in Frisvad *et al.* (2010) [45].

Several studies indicated that in black *Aspergilli*, OTA is commonly produced by *Asp. carbonarius* [35], to a lesser extent produced by *Asp. niger* [29, 36, 37], and occasionally produced by *Asp. welwitschiae* [14]. However, from our strains, OTA was only detected from the *Asp. carbonarius* strain. OTA was not detected from any of our *Asp. niger* or *Asp. welwitschiae* strains.

Some conflicting reports in literature have suggested that FB₁ is produced by *Asp. niger* [99, 100]. FB₁, a positional isomer of FB₆, was also analyzed under the same conditions to confirm that its RT and MS² fragmentation was different. Figure 2.10 (a) compares the RT of a FB₁ standard with FB₂, FB₄, and FB₆ detected from our strains. Under these conditions, FB₁ elutes 0.15 min earlier than FB₆, and the two isomeric compounds are fully resolved. In the screening of 253 black *Aspergillus* isolates, none produced FB₁.

Figure 2.10 (b-e) also shows the ESI (+) MS² spectrum and representative dissociation pathway of protonated FB₁, FB₂, FB₄ and FB₆ respectively. Due to the presence of an amine group, fumonisins are of sufficient ion intensity to trigger MS² in positive mode. Protonated fumonisins dissociate by losing backbone hydroxy groups as water and form double bonds, and losing both tricarballic esters (TCE) and form double bonds. This is usually followed by the neutral loss of the protonated amine as ammonia, forming a characteristic alkyl chain cation with a m/z which is indicative of the length of the fumonisin backbone, and the number of hydroxy and tricarballic esters that were present. Figure 2.10 also shows the representative structure of the characteristic product ion for each fumonisin. FB₁ and FB₆ both possess three backbone hydroxy groups and have a characteristic product ion of m/z 299.2736. Since FB₂ has two hydroxyl groups, one fewer double bond is formed during the dissociation process and the characteristic product ion is m/z 301.2891. Similarly, FB₄ has a single hydroxy group and a characteristic ion of m/z 303.3046.



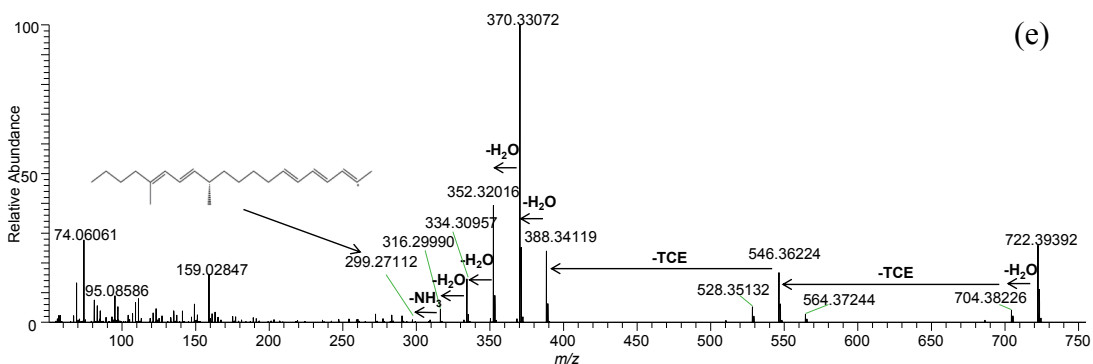
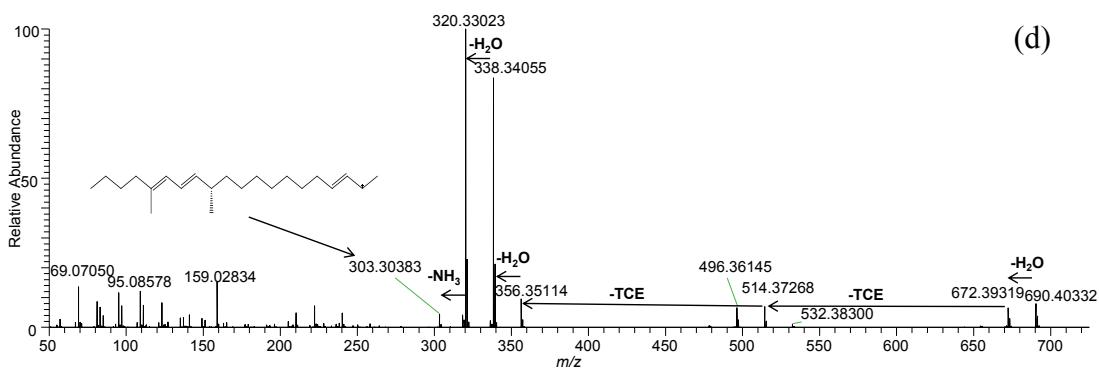
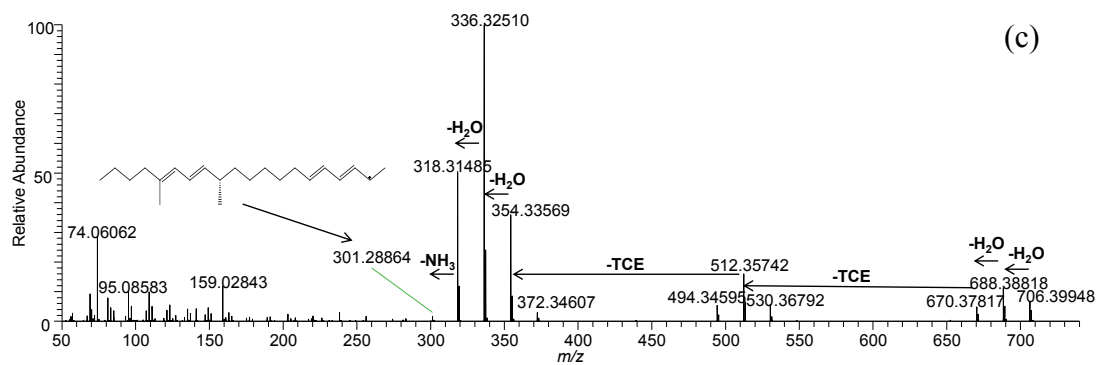


Figure 2.10 (a) Elution profiles of FB₁ standard, FB₂, FB₄, FB₆ detected from our strain MWS 200. (b-e) the ESI (+) MS² spectrum and representative dissociation pathway of protonated FB₁, FB₂, FB₄, and FB₆ respectively. The characteristic product ions were labeled beside the arrow.

It is now understood that *Asp. niger* strains have the capacity to produce FB₂, FB₄, and FB₆. *Asp. welwitschiae* strains isolated from maize and onion were also reported to be capable of producing FB₂ [101, 102]. Our study shows fumonisins were only detected from *Asp. niger* and *Asp. welwitschiae* strains. Specifically, 10/10 (100%) of the *Asp. niger* strains were FB₂, FB₄ and FB₆ producers. 28/109 (26%) of the *Asp. welwitschiae* strains produced FB₂, 27/109 (25%) produced FB₂ and FB₄, 23/109 (21%) produced FB₂, FB₄ and FB₆. Table 2.2 shows the occurrence of OTA and fumonisins from potential mycotoxin-producing strains over three years. These data agree with the study of maize kernels from the USA and Italy [101] that shown a higher percentage of *Asp. niger* isolates (72.0%) produced FB₂ compared to *Asp. welwitschiae* (36.6%). This study is the first to report the occurrence of FB₄ and FB₆ from *Asp. welwitschiae*.

Table 2.2 Occurrence of OTA and fumonisins in potential mycotoxin-producing strains over three years. ‘Not detected’ was abbreviated to ‘nd’.

Year	Source	Potential mycotoxin-producing strains	Mycotoxin-producing strains (Frequency)			
			OTA	FB ₂	FB ₄	FB ₆
2012	Grape	<i>Asp. welwitschiae</i> (n=3)	nd	1 (33%)	1 (33%)	1 (33%)
		<i>Asp. niger</i> (n=2)	nd	2 (100%)	2 (100%)	2 (100%)
	Soil	<i>Asp. welwitschiae</i> (n=12)	nd	3 (25%)	3 (25%)	3 (25%)
2013	Grape	<i>Asp. welwitschiae</i> (n=19)	nd	4 (21%)	4 (21%)	4 (21%)
		<i>Asp. niger</i> (n=2)	nd	2 (100%)	2 (100%)	2 (100%)
	Soil	<i>Asp. welwitschiae</i> (n=47)	nd	2 (4%)	2 (4%)	2 (4%)
		<i>Asp. niger</i> (n=2)	nd	2 (100%)	2 (100%)	2 (100%)
2014	Grape	<i>Asp. welwitschiae</i> (n=21)	nd	12 (57%)	11 (52%)	7 (33%)
		<i>Asp. niger</i> (n=2)	nd	2 (100%)	2 (100%)	2 (100%)
		<i>Asp. carbonarius</i> (n=1)	1 (100%)	nd	nd	nd
	Soil	<i>Asp. welwitschiae</i> (n=7)	nd	6 (86%)	6 (86%)	6 (86%)
		<i>Asp. niger</i> (n=2)	nd	2 (100%)	2 (100%)	2 (100%)

It was found that the ability to produce fumonisins varies among different *Asp. welwitschiae* strains. A study indicated the reason why some *Asp. welwitschiae* strains produce fumonisin but some do not, based on genetic analysis [10]. The fumonisin-producer of *Asp. welwitschiae* strains has all the genes related to fumonisin biosynthesis (‘fum’ genes), but the fumonisin non-producers lack eight fum genes [10]. On the contrary, both the fumonisin-producer and the non-producer of *Asp. niger* strains have all the fum genes for fumonisin biosynthesis. The reason why *Asp. niger* strains have the all the fum genes but not to produce fumonisin is unknown. Interestingly, in our strains, some of *Asp. welwitschiae* strains are fumonisin producers and some are not, but all of the *Asp. niger* strains are fumonisin producers. However, there are only ten *Asp.*

niger strains isolated over these three years, which is not sufficient to draw a conclusion.

2.3.3 FB₂ production by *Asp. niger* and *Asp. welwitschiae*

The next goal was to quantify FB₂ from fungal isolates detected FB₂. Based on the results of agar plug extract, there were 38 isolates detected FB₂ in 2012-2014. Agar plugs cut from those 38 strains were inoculated in CYA liquid medium individually and incubated at 30°C. From those 38 strains, 11 strains were monitored for FB₂ production during a 27-day period. The goal of this time series experiment was to determine the time period required for maximum FB₂ production. The other 27 strains were incubated until that time period for FB₂ analysis.

For time series experiment, aliquots were taken out from every other day, diluted twice, and quantified based on the calibration curve generated from five injections of each FB₂ standard solution (0.0001 µg/mL-10 µg/mL). FB₂ was consistently detected at 5 ng/mL and the signal response was below 25% RSD. Therefore, both the LOD and the LOQ was 5 ng/mL. The response was linear over five orders of magnitude. Time series results are shown as the following three representative samples. Figure 2.11 illustrates a low-level FB₂ producer *Asp. welwitschiae* MWS 231 (maximum production in a range of 0.0-0.2 µg/mL), a mid-level FB₂ producer *Asp. niger* MWS M225 (maximum production in a range of 0.2-2.0 µg/mL), and a high-level FB₂ producer *Asp. welwitschiae* MWS 200 (maximum production ≥ 2.0 µg/mL). The level is referred to the regulatory limit (0.2-2 µg/g) for FB₂ on maize set by European Commission [40]. It should be pointed out that this regulatory limit should not be used as the regulatory level for FB₂ production of black *Aspergilli in vitro*, but it was used here for determination of groups (low, medium and high). From the results of time series experiment, day 15 was selected as the incubation time for FB₂ production, since most of the strains have the maximum production at day 15. Therefore, the rest of the 27 strains were incubated until day 15 for FB₂ analysis. Table 2.3 listed FB₂ production by *Asp. niger* and *Asp. welwitschiae* strains over three years. There were 38 fumonisin-producing strains over these three years based on the agar plug extract results. For only one sample MWS N089, FB₂ was detected from agar plug extract, but not from CYA liquid medium. For those 37 strains, most of the strains (86%) have the FB₂ production in a range of 0.0-2.0 µg/mL.

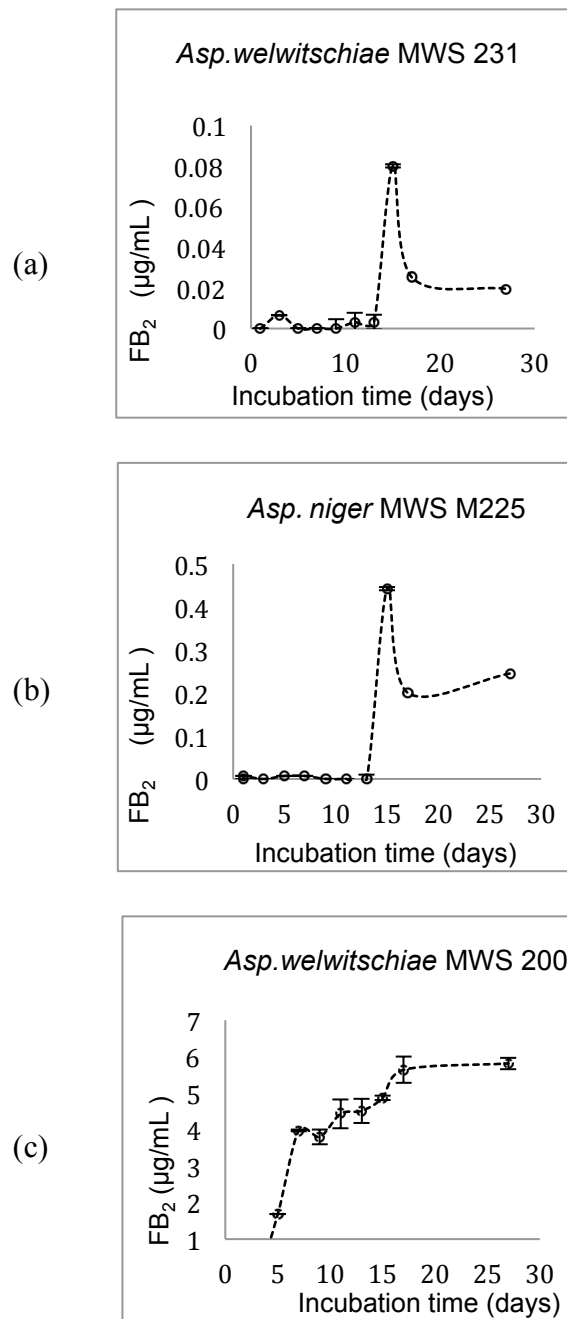


Figure 2.11 Concentrations of FB₂ monitored in three FB₂-producing strains during a 27-day incubation period at 30°C. **(a)** A low-level FB₂ producer (maximum production in a range of 0.0-0.2 µg/mL). **(b)** A mid-level FB₂ producer (maximum production in a range of 0.2-2.0 µg/mL). **(c)** A high-level FB₂ producer (maximum production ≥2.0 µg/mL).

Table 2.3 FB₂ production by *Asp. niger* and *Asp. welwitschiae* strains from 2012-2014 after 15 days growth at 30°C in CYA liquid media.

Isolate #	Species	Sampling	Site	Source	Variety	FB ₂ (mg/L)
MWS 200	<i>Asp. welwitschiae</i>	Sep-13	Niagara-JRS	Grape	Foch	4.87 ± 0.06
MWS N033	<i>Asp. welwitschiae</i>	Oct-14	Niagara-CS4	Grape	Cab. Franc	4.36 ± 0.09
MWS N121	<i>Asp. welwitschiae</i>	Oct-14	Niagara-JRS	Soil	Concord	4.2 ± 0.1
MWS M189	<i>Asp. welwitschiae</i>	Sep-13	Niagara-JRS	Soil	Foch	3.33 ± 0.08
MWS N101	<i>Asp. welwitschiae</i>	Sep-14	NS-KRS	Soil	Valiant	2.10 ± 0.06
MWS L035	<i>Asp. niger</i>	Oct-12	Niagara-JRS	Grape	Cab. Sauv	1.388 ± 0.001
MWS N124	<i>Asp. welwitschiae</i>	Oct-14	Niagara-JRS	Soil	Concord	0.935 ± 0.006
MWS 239	<i>Asp. niger</i>	Oct-13	Niagara-GRS	Soil	Pinot Noir	0.74 ± 0.05
MWS N110	<i>Asp. niger</i>	Oct-14	Niagara-CS4	Soil	Cab. Franc	0.70 ± 0.01
MWS M225	<i>Asp. niger</i>	Aug-13	Niagara-JRS	Soil	Riesling	0.44 ± 0.01
MWS L041	<i>Asp. niger</i>	Sep-12	Niagara-JRS	Grape	Concord	0.39 ± 0.01
MWS N108	<i>Asp. niger</i>	Oct-14	Niagara-CS4	Soil	Cab. Franc	0.39 ± 0.01
MWS M194	<i>Asp. welwitschiae</i>	Aug-13	Niagara-JRS	Grape	Riesling	0.319 ± 0.003
MWS N073	<i>Asp. niger</i>	Oct-14	Niagara-JRS	Grape	Concord	0.197 ± 0.003
MWS N081	<i>Asp. welwitschiae</i>	Oct-14	Niagara-GRS	Grape	Cab. Franc	0.118 ± 0.002
MWS N102	<i>Asp. welwitschiae</i>	Aug-14	NS-KRS	Soil	Foch	0.091 ± 0.002
MWS N100	<i>Asp. welwitschiae</i>	Aug-14	NS-KRS	Soil	Leon Millot	0.087 ± 0.003
MWS 063	<i>Asp. niger</i>	Sep-13	Niagara-JRS	Grape	Vidal	0.083 ± 0.001
MWS 231	<i>Asp. welwitschiae</i>	Oct-13	Niagara-JRS	Grape	Riesling	0.080 ± 0.004
MWS 012	<i>Asp. welwitschiae</i>	Aug-13	Niagara-JRS	Grape	Foch	0.056 ± 0.001
MWS N082	<i>Asp. welwitschiae</i>	Oct-14	Niagara-GRS	Grape	Cab. Sauv	0.049 ± 0.001
MWS 048	<i>Asp. welwitschiae</i>	Sep-13	Niagara-JRS	Soil	Riesling	0.037 ± 0.001
MWS L003	<i>Asp. welwitschiae</i>	Oct-12	Niagara-JRS	Soil	Riesling	0.036 ± 0.001
MWS N104	<i>Asp. welwitschiae</i>	Oct-14	Niagara-CS1	Soil	Cab. Franc	0.028 ± 0.000
MWS M219	<i>Asp. welwitschiae</i>	Sep-13	Niagara-JRS	Soil	Foch	0.027 ± 0.001
MWS N011	<i>Asp. welwitschiae</i>	Oct-14	Niagara-CS1	Grape	Cab. Franc	0.026 ± 0.000
MWS M159	<i>Asp. niger</i>	Oct-13	Niagara-GRS	Grape	Pinot Noir	0.026 ± 0.002
MWS N084	<i>Asp. welwitschiae</i>	Oct-14	Niagara-GRS	Grape	Pinot Noir	0.022 ± 0.001
MWS L016	<i>Asp. welwitschiae</i>	Oct-12	Niagara-JRS	Soil	Riesling	0.018 ± 0.002
MWS N075	<i>Asp. welwitschiae</i>	Sep-14	Niagara-GRS	Grape	Pinot Noir	0.016 ± 0.000
MWS N052	<i>Asp. welwitschiae</i>	Oct-14	Niagara-JRS	Grape	Riesling	0.012 ± 0.000
MWS N113	<i>Asp. welwitschiae</i>	Oct-14	Niagara-CS2	Soil	Cab. Franc	0.012 ± 0.008
MWS L025	<i>Asp. welwitschiae</i>	Oct-12	Niagara-JRS	Soil	Chardonnay	0.011 ± 0.008
MWS N069	<i>Asp. welwitschiae</i>	Oct-14	Niagara-JRS	Grape	Concord	0.011 ± 0.007
MWS N066	<i>Asp. niger</i>	Oct-14	Niagara-JRS	Grape	Concord	0.011 ± 0.000
MWS N036	<i>Asp. welwitschiae</i>	Oct-14	Niagara-CS5	Grape	Cab. Franc	0.010 ± 0.001
MWS N005	<i>Asp. welwitschiae</i>	Oct-14	Niagara-CS1	Grape	Cab. Franc	0.010 ± 0.000

Note: JRS=Jordon Research Station, CS=commercial site, NS-KRS=Nova Scotia Kentville Research Station, GRS=Grape Research Station; Cab. Franc=Cabernet Franc, Cab. Sauv=Cabernet Sauvignon. Sampling rows were not listed in this table.

2.3.4 OTA production by *Asp. carbonarius*

The goal was to quantify OTA from the only *Asp. carbonarius* strain isolated. Agar plugs cut from *Asp. carbonarius* isolate MWS N095 were inoculated in CYA liquid medium. It was incubated at 30°C and monitored for OTA production during a 27-day incubation period.

OTA standards in a range between 0.0001 µg/mL and 10 µg/mL were injected five times onto the HPLC column. OTA was consistently detected at 1 ng/mL and the signal response was below 25% RSD. Therefore, both the LOD and LOQ was 1 ng/mL. The response was linear over five orders of magnitude. Figure 2.12 shows the OTA production by the *Asp. carbonarius* strain during a 27-day incubation period. MWS N095 has the maximum OTA production at day 25 (18.1 ± 0.2 µg/mL).

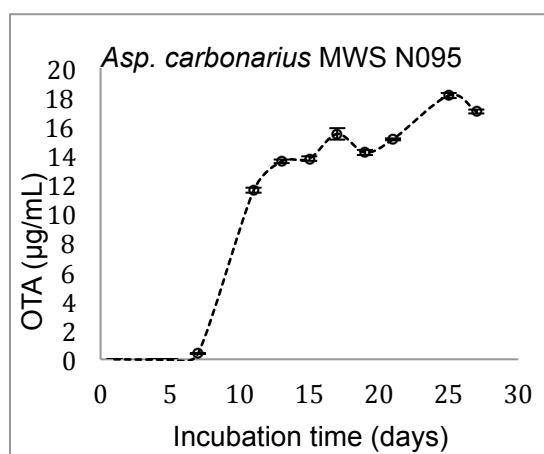


Figure 2.12 Concentration of OTA monitored in the *Asp. carbonarius* strain during a 27-day incubation period at 30°C.

2.3.5 Results of SPE optimization

Reversed-phase SPE, anion-exchange SPE (targeted to the carboxyl group), and cation-exchange SPE (targeted to the amine group) were primarily optimized for OTA and FB₂ cleanup by spiking standards in water.

In Section 2.2.3.1, all of the solutions that went through cartridges were collected,

concentrated, and analyzed by LC-MS². By using Bond Elut Plexa cartridge, the results of OTA and FB₂ elution are as follows: FB₂ eluted with methanol/formic acid/water (75:1:24), and OTA eluted with methanol/formic acid (99:1). The relative recovery of OTA and FB₂ with the sample adjusted to pH 7 was higher than when the sample was adjusted to pH 3. By using the Bond Elut SAX cartridge, both OTA and FB₂ eluted with methanol/acetic acid (99:1). When the sample was adjusted to pH 10, the OTA and FB₂ recovery was higher than when the sample was adjusted to pH 7. By using Oasis MCX cartridge, OTA eluted with methanol/formic acid (99:1), and FB₂ eluted with methanol/ammonium hydroxide (95:5). When the sample was adjusted to pH 7, there was a higher OTA and FB₂ recovery than when the sample was adjusted to pH 4. The recovery of different SPE methods is shown in Table 2.4.

Table 2.4 OTA and FB₂ recoveries in three SPE methods by spiking OTA, FB₂ standards in water.

SPE Cartridge	Spike recovery for OTA	Spike recovery for FB ₂
Bond Elut Plexa (sample pH 7)	101%	107%
Bond Elut SAX (sample pH 10)	121%	81%
Waters Oasis MCX (sample pH 7)	96%	80%

However, spiking of OTA and FB₂ standards in water could not be representative of OTA and FB₂ on grapes. Commercial grapes inoculated with our *Asp. carbonarius* isolate and *Asp. welwitschiae* isolate were used for the method development of grape extraction and SPE cleanup. Different solvents were tried for the grape extraction, and were analyzed by LC-MS² for comparison of OTA and FB₂ ion intensities. Acetonitrile/water/acetic acid (79:20:1) was found to be the best extraction solvent for OTA and FB₂ analysis. SPE purification was carried out using the optimized method based on the spiking of OTA and FB₂ standards in water. The recovery of OTA was 101% and that of FB₂ was 108% using Bond Elut Plexa cartridge (reversed-phase SPE). The recovery of OTA using Bond Elut SAX cartridge (anion-exchange SPE) and Oasis MCX cartridge (cation-exchange SPE) was 81% and 85% respectively. However, by using Bond Elut SAX cartridge, a very low recovery (30%) of FB₂ was observed, which was a very different result from spiking of

FB₂ standard in water. The reason could be the high content of organic acids, which salt-out FB₂ explained by Mogensen *et al.* (2010) [41]. As for Oasis MCX cartridge, the recovery of FB₂ was 65%, which was lower than that of spiking FB₂ standard in water. Therefore, only Bond Elut Plexa SPE was selected for grape sample cleanup.

2.3.6 OTA and FB₂ quantification from grapes

Based on initial agar plug screenings, twenty-two grape samples were analyzed for OTA and fumonisins from sites where mycotoxin producers were isolated (potential contaminated grape samples). Grape samples were blended, extracted with acetonitrile/water/acetic acid (79:20:1), and purified using the Bond Elut Plexa cartridge. FB₂ and OTA eluents were both analyzed by LC-MS². LC-MS² method in Section 2.2.2.4 was firstly applied to this study. However, fumonisin was detected but below LOQ in some samples. Therefore, an optimized method described below was set up for trace analysis. Ten µL of each sample and standard solution were injected onto the HPLC column five times using the same LC-MS² method as described in Section 2.2.2.4, but using optimized HESI settings for FB₂ trace analysis. The instrument LOQ for FB₂ analysis was improved to be 1 ng/mL in comparison with 5 ng/mL in Section 2.3.3. The instrument LOQ for OTA analysis was 1 ng/mL. The method LOQ was defined as the lowest concentration of the original sample that can be reliably quantified by the instrument. It was calculated based on experimental procedures (such as dilutions and SPE recoveries). The method LOQ for FB₂ and OTA analysis in this experiment was 0.37 µg/kg and 0.40 µg/kg respectively. Therefore, samples were quantifiable if their FB₂ and OTA concentrations were greater than 0.37 µg/kg, and 0.40 µg/kg respectively.

FB₂ was detected in 7/22 (32%) grape samples, but OTA was not detected in any of the grape samples tested. The concentration of FB₂ in the positive samples is listed in Table 2.5. Since standards of FB₄ and FB₆ are not commercially available, they cannot be quantified, however, their presences are also reported in Table 2.5. There is no regulatory limit for FB₂ on grapes, but it was quantified to be about 200 times lower than the regulatory limit (0.2-2 µg/g) for FB₂ on maize set by European Commission.

Table 2.5 The concentration of FB₂ and OTA detected on grape samples, and the presence of FB₄ and FB₆ on grape samples. FB₂-negative samples tested are not shown.

Sampling month-year	Site	Variety	OTA	FB ₂ (µg/kg)	FB ₄	FB ₆
Oct-14	Niagara-GRS	Cabernet Sauvignon	-	14.6 ± 1.1	+	+
Nov-14	Niagara-JRS	Foch	-	9.0 ± 0.8	+	+
Oct-14	Niagara-CS1	Cabernet Franc	-	1.8 ± 0.1	+	-
Oct-14	Niagara-JRS	Concord	-	1.2 ± 0.1	+	-
Sep-12	Niagara-JRS	Cabernet Franc	-	1.03 ± 0.09	+	-
Oct-13	Niagara-GRS	Pinot Noir	-	0.93 ± 0.04	+	-
Oct-14	Niagara-GRS	Pinot Noir	-	0.81 ± 0.08	+	-

2.4 Conclusions and Future Work

A three-year survey of the black *Aspergilli* isolated from Canadian vineyards has been conducted to study their species distribution in Canada and to assess the risk of OTA and fumonisin contamination of wine grapes.

In 2012-2014, in the investigated Canadian vineyards, the predominant black *Aspergillus* species were *Asp. welwitschiae* and *Asp. uvarum* (75% of all the strains isolated). *Asp. brasiliensis*, *Asp. tubingensis*, and *Asp. niger* were also present but with lower prevalence. *Asp. carbonarius* was isolated only once, where OTA was detected. It was reported that *Asp. niger* and *Asp. welwitschiae* also have the ability to produce OTA [29, 37, 103]. However, OTA was not detected (LOQ=0.4 µg/kg) in any of the *Asp. niger* or *Asp. welwitschiae* strains. These results agreed with a survey conducted in cooler grape-growing regions of Northern Italy [104]. Potentially contaminated grape samples were also screened for OTA, but no OTA was detected. Therefore, during the three sampling years, the wine grapes in the investigated Canadian vineyards have a very low risk of OTA contamination caused by black *Aspergilli*.

FB₂, FB₄, and FB₆ (not FB₁) were detected from some of the *Asp. welwitschiae* strains and *Asp. niger* strains. Trace amounts of FB₂ (0.73 to 15.7 µg/kg), FB₄, and FB₆ were also detected from the tested grapes. The presence of fumonisin-producing fungi and fumonisins on grapes indicates that the risk of fumonisin contamination exists in the investigated Canadian vineyards in 2012-2014, but is low.

However, to understand the risk of OTA and fumonisin contamination of Canadian VQA wine, wine analysis needs to be carried out in the future.

Chapter 3 – Mass Spectrometry-based Metabolomics
Study of Black *Aspergilli* from Canadian Vineyards

3.1 Introduction

As introduced in Section 1.2, mass spectrometry-based metabolomics can be used for the global analysis of metabolites produced by black *Aspergilli*. Using the state-of-the-art Orbitrap LC-MS, a MS-based metabolomics study of black *Aspergilli* isolated from Canadian vineyards was carried out to discover novel metabolite markers for individual black *Aspergilli* species discrimination.

The typical metabolomics procedures discussed in Section 1.2.2 were carried out in this chapter as follows: sample preparation (Section 3.2.1), data acquisition (Section 3.2.2), data pre-processing (Section 3.3.3). To determine the most abundant secondary metabolites produced by Canadian strains, known secondary metabolites produced by our black *Aspergillus* species were studied and compared with these in the literature (Section 3.3.1). PCA was also performed to visualize the metabolome similarities and differences among samples (Section 3.3.2). The background of PCA and related data transformation was previously discussed in Section 1.2.2.2. The potential species-specific metabolite markers were discovered using the Kruskal-Wallis test [105] (Section 3.3.3). The Kruskal-Wallis test was used to evaluate statistical significance of single metabolites between more than two groups, when a normal distribution in the dataset is not known. The metabolite with a high prevalence in one group but with no or extremely low prevalence in any other groups can be considered as a metabolite marker candidate. The statistical significance is measured by the p-value. For example, a p-value of 0.05 means that 5% of all tests results in a false positive. A false discovery rate (FDR) adjusted p-value introduced by Benjamini Hochberg is used for p-value correction in order to reduce the number of false positives for multiple testing [106]. Finally, potential species-specific metabolite markers were putatively identified based on accurate masses and MS² spectra (Section 3.3.4).

3.2 Experimental

3.2.1 Metabolite extraction

Agar plug extracts prepared in Section 2.2.2.3 were stored at -20°C until LC-MS sample analysis. A total of 260 fungal extracts from Canadian vineyards were carried out for the metabolomics study, including thirty-one *Asp. brasiliensis* samples, one *Asp. carbonarius*

sample, eight *Asp. niger* samples, eighteen *Asp. tubingensis* samples, sixty-eight *Asp. uvarum* samples and one hundred and thirty-four *Asp. welwitschiae* samples.

3.2.2 LC-MS and LC-MS² analysis

LC-MS and LC-MS² analysis was performed on a Thermo Scientific Q-Exactive Quadrupole Orbitrap Mass Spectrometer, coupled to an Agilent 1290 HPLC system with a HESI ionization source. 2.0 μ L of each sample was injected onto a Zorbax Eclipse Plus RRHD C18 column (2.1 x 50 mm, 1.8 μ m; Agilent) maintained at 35°C. Samples were separated using a flow rate of 0.3 mL/min with a mobile phase of LC grade water with 0.1% formic acid (mobile phase A), and acetonitrile with 0.1% formic acid (mobile phase B). Mobile phase A was held at 100% for 30 seconds, and decreased to 0% over three minutes. Mobile phase B was then held at 100% for 1 minute before decreasing to 0% B in 30 seconds. The following conditions were used for the HESI ionization source: capillary temperature, 400°C; sheath gas, 17.00 units; auxiliary gas, 8.00 units; probe heater temperature, 450 °C; S-Lens RF level, 45.00. A capillary voltage was 3.9 kV and 3.7 kV for positive and negative mode respectively.

Full MS scans were carried out on all of the samples in both positive and negative ion mode at a 140 000 resolution using a 6 minute method. The scan range was from 100–1200 m/z , AGC target was 1×10^6 , and maximum injection time was 512 ms. Full MS was acquired at a resolution of 17 500 and 100–1200 m/z of scan range. AGC target and maximum injection time was set to 5×10^5 and 64 ms respectively. Top 12 signals of the previous full MS scan were selected to do ddMS² experiments using a 1.0 m/z isolation window, a resolution of 17 500, 1×10^5 of AGC target, 64 ms of maximum injection time, and 30 units of normalized collision energy.

To ensure quality control, a sample was injected at the beginning and end of every sample plate, and assessed for instrument drift. To limit carryover, 2.0 μ L of acetonitrile was injected onto the column between adjacent samples as a blank injection. 260 samples with blanks in full MS scans and some of the samples in ddMS² experiments were running for five consecutive days.

3.2.3 LC-MS data pre-processing in MZmine 2.0

After data acquisition, Thermo raw files were converted to mzXML format using

ProteoWizard 3.0. After importing the mzXML files into MZmine 2.0, data acquired in positive mode and negative mode were processed separately for peak detection, chromatogram builder, and isotopic peaks grouper with the following settings. Peaks were detected at the 5×10^5 intensity threshold. For the chromatogram builder, the minimum time span was set to 0.10 min, the minimum peak height was 5×10^5 , and the mass tolerance was 0.010 m/z or 8 ppm. For the isotopic peaks grouper, the m/z tolerance was set to 0.001 m/z or 1 ppm, the RT tolerance was 0.01 min, the maximum charge was 3, and the representative isotope was lowest m/z .

After the deisotoped chromatogram was built for every sample, corresponding peaks are aligned across multiple samples with the following settings: the m/z tolerance was 0.010 m/z or 8 ppm, RT tolerance was 0.10 min, and the weight for m/z and RT was 2 and 0.5 respectively. The aligned peak list containing all m/z detected along with their peak area in every sample is exported to a peak list table for further statistical analysis.

3.2.4 Statistic analysis in R

PCA and the Kruskal-Wallis test were performed in R (programming language). To accomplish this, the peak areas generated by MZmine were first logarithm base 2 transformed, specifically using $\log_2(x+1)$ (x =peak area, when the peak area is 0, the transformed data equals 0). Pareto scaling was then implemented using the MetabolAnalyze R package, which scales the data by mean-centered value divided by square root of standard deviation [107]. Basic information about the samples (species, sample year, sample site etc. termed “factors”) was stored in a table and processed into R. PCA scoreplots were generated using the FactoMineR R package [108], and coloured according to the above factors. Coloured PCA scoreplots provided insight into the variables that may be affecting the clustering of the metabolites [109].

The Kruskal-Wallis test was performed to discover metabolite markers specific to each species of black *Aspergillus*. Samples were first grouped by fungal species. The Kruskal-Wallis test was then used to identify features that were significantly different in any one of the groups (species). For each feature (m/z and RT), a p-value and a FDR corrected p-value were calculated. The prevalence of each feature in each species was also calculated (the proportion of samples that have peak area greater than 5×10^5 threshold in samples of the same species).

3.3 Results and Discussion

3.3.1 Known secondary metabolites produced by Canadian black *Aspergillus* species

After the peak list described in Section 3.2.3 was generated, the most intense chromatographic peaks for each species were studied. Most metabolites were identified by database searches based on their high mass accuracy, and comparing to known metabolites produced by black *Aspergilli* in Europe as reported in a review paper by Nielsen *et al.* (2009) [36]. The MS² spectra and authentic standards (when available) were used for verification.

The occurrence of secondary metabolites detected in Canadian black *Aspergillus* species is summarized in Table 3.1. The percentage was the ratio of specific metabolite positive strains (peak area greater than 5×10^5 threshold) to the number of samples. Because there was only one *Asp. carbonarius* strain, it was not included in Table 3.1.

By comparing our Canadian strains with European strains reported by Nielsen *et al.* (2009) [36], Varga *et al.* (2007) [110], and Perrone *et al.* (2008) [111], similarities and differences in secondary metabolite production were found. It should be noted that *Asp. welwitschiae* was considered as a synonym of *Asp. niger* in that review paper [36]. Aurasperone B, C, E, F, malformins and tensidol B were mainly found in *Asp. brasiliensis*, *Asp. niger*, *Asp. tubingensis*, and *Asp. welwitschiae*, which agreed with the study of Nielsen *et al.* [36] and Varga *et al.* [110]. However, low prevalence of these metabolites in *Asp. uvarum* was also detected in our strains. Funalenone was not only found in *Asp. niger*, *Asp. tubingensis*, and *Asp. brasiliensis* as reported by Nielsen *et al.* [36], it also was also found in *Asp. uvarum*. Pyranonigrins, mainly as pyranonigrin A, were primarily detected in *Asp. niger*, *Asp. tubingensis*, and *Asp. welwitschiae*, but low occurrence was also found in *Asp. brasiliensis* and *Asp. uvarum*.

Nigragillin was found in every strain of the different species. In contrast, Nielsen *et al.* [36] reported that nigragillin was found in *Asp. niger* and *Asp. tubingensis*. The nigragillin analogue reported by Nielsen *et al.* [36] was identified to be demethylated nigragillin by its accurate mass and MS² spectrum. Similar to nigragillin, demethylated nigragillin was also found in all of those five species. Nigerazine A or B was mainly

produced by *Asp. uvarum* (its chromatographic peak is usually the most intense one in *Asp. uvarum* strains), but low abundance in other four species. However, there was no prior data showing that nigragillin and nigerazine A or B were also found in *Asp. brasiliensis* and *Asp. uvarum*.

Asperazine was mainly found in *Asp. tubingensis*, which agreed with Nielsen *et al.* that it could be used as a metabolite marker to differentiate *Asp. tubingensis* from other black *Aspergilli* [36]. Kotanin was primarily produced by *Asp. niger* but not other species. Similarly, secalonin acid D or F was mostly produced by *Asp. uvarum*. They could be potential metabolite markers.

Perrone *et al.* (2008) reported that *Asp. uvarum* produced secalonin acid D, F, geodin, erdin, dihydrogeodin and asterolic acid [111]. However, the LC-MS² results revealed that only secalonin acid D and F were present in some of our strains.

Table 3.1 The occurrence of known secondary metabolites in black *Aspergillus* species isolated from Canadian vineyards (the ratio of specific metabolite positive strains to the number of samples). Isomers such as secalonin acid D/F could not be identified by MS² data alone.

	<i>Asp. brasiliensis</i> (n=31)	<i>Asp. niger</i> (n=8)	<i>Asp. tubingensis</i> (n=18)	<i>Asp. uvarum</i> (n=68)	<i>Asp. welwitschiae</i> (n=134)
Asperazine	0%	0%	67%	1%	2%
Aurasperone B	97%	88%	78%	16%	93%
Aurasperone C	84%	88%	89%	10%	86%
Aurasperone E	84%	88%	67%	7%	88%
Aurasperone F	71%	88%	83%	6%	79%
Demethylated Nigragillin	68%	100%	94%	87%	98%
Flavasperone	65%	100%	61%	76%	92%
Funalenone	65%	100%	89%	99%	97%
Kotanin	0%	75%	0%	1%	3%
Malformin A ₁ /B ₁ /B ₃ /B ₅ /C	81%	75%	83%	35%	88%
Malformin B ₂	74%	50%	72%	4%	73%
Nigerazine A/B	52%	50%	39%	93%	55%
Nigragillin	100%	100%	100%	100%	100%
Pyranonigrin A	32%	88%	94%	37%	84%
Pyranonigrin B/C	10%	25%	33%	1%	43%
Secalonin acid D/F	0%	0%	6%	47%	1%
Tensidol B	100%	88%	56%	37%	91%

3.3.2 Principle component analysis

There were 1660 features detected in positive ion mode, 1879 features detected in

negative ion mode. Similarities and differences within data can be visualized from PCA scoreplots. Figure 3.1(a) shows a PCA scoreplot generated by samples analyzed in positive ion mode, (b) shows a PCA scoreplot generated by samples analyzed in negative ion mode. Each point in a PCA scoreplot represents a fungal extract sample. Positions of the points display similarities and differences in the metabolome. Samples clustered together may share metabolites, and samples furthest from one another are most dissimilar. In Figure 3.1, the different colours are representative of the different species in the PCA plot. In both PCA scoreplots of Figure 3.1, *Asp. brasiliensis*, *Asp. welwitschiae*, and *Asp. uvarum* samples group into three different clusters, suggesting each species may produce some unique metabolites to differentiate them from other species. In addition, *Asp. niger* and *Asp. tubingensis* samples appeared to group with *Asp. welwitschiae* samples, suggesting that they likely share similar metabolites. The only *Asp. carbonarius* isolate was not clearly separated from other species (near the edge of *Asp. welwitschiae* cluster and coloured in red on Figure 3.1). However, one isolate cannot represent the entire metabolome of *Asp. carbonarius* species; it has been therefore removed from the Kruskal-Wallis test.

The data points on the PCA scoreplot were also coloured according to sample year, sample month, site, source (group or soil), and grape variety information. However, there was no clear separation based on these factors (data not shown). Therefore, “species” was found to be the major factor affecting the metabolite clustering.

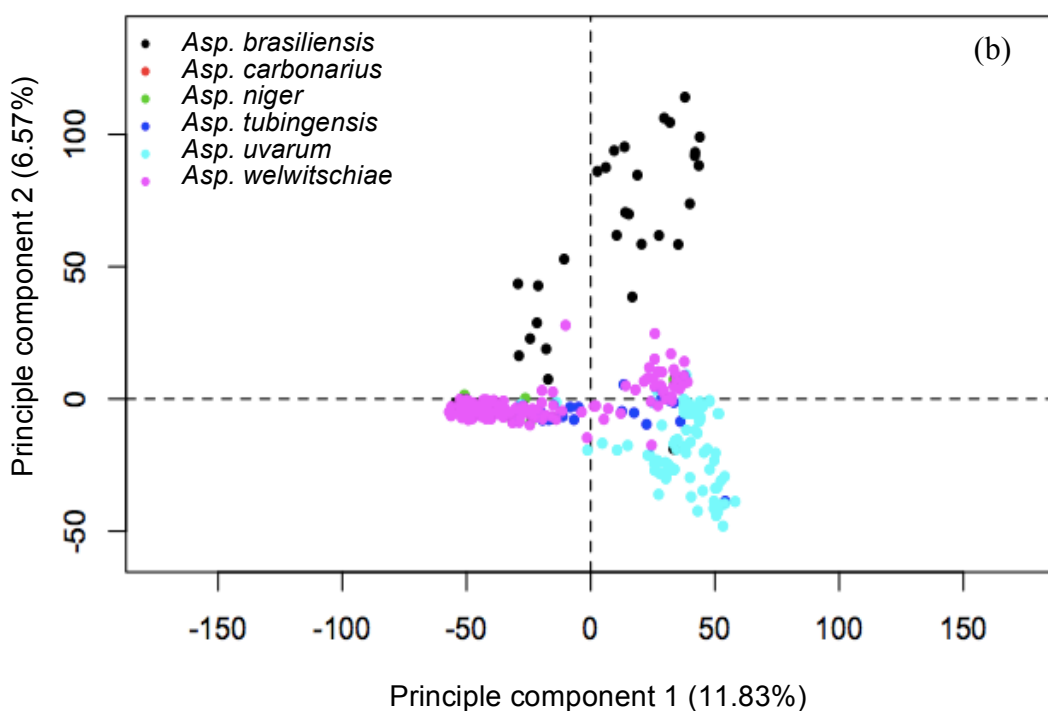
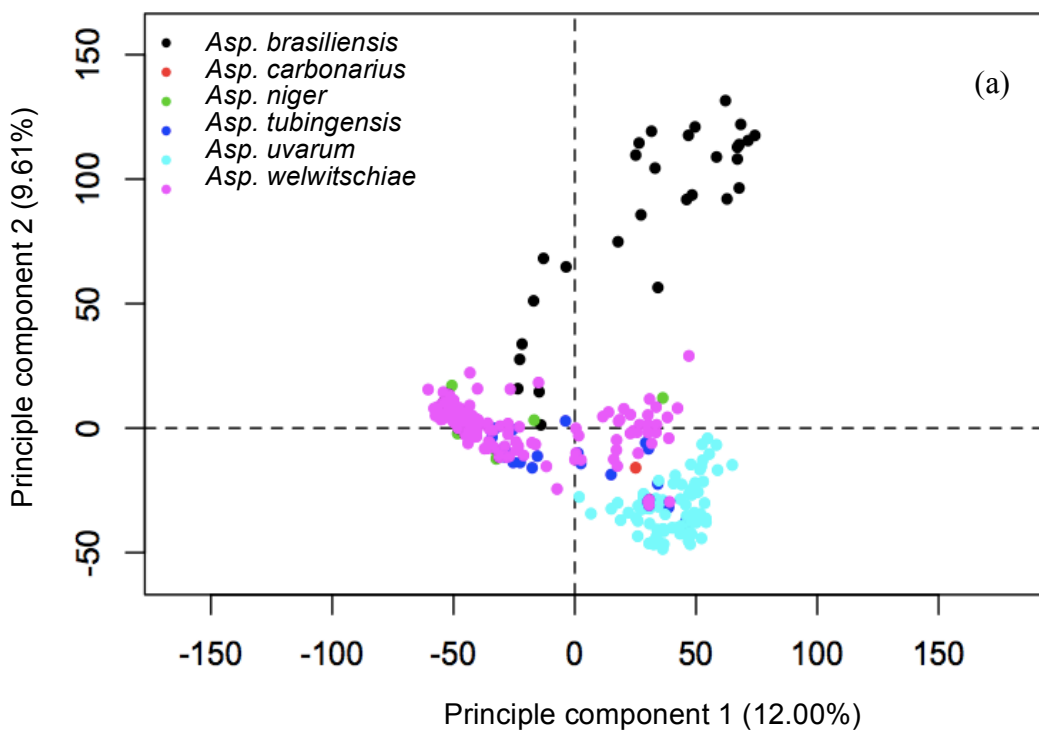


Figure 3.1 (a) PCA scoreplot of 260 fungal extracts analyzed in positive ion mode. (b) PCA scoreplot of 260 fungal extracts analyzed in negative ion mode. Each point in a PCA scoreplot represents a sample. Positions of the points display similarities and differences in the metabolome of samples. Samples clustered together may share metabolites, and samples furthest from one another are most dissimilar. Samples were coloured by black *Aspergillus* species they belong to. (a) and (b) explain 21.61% and 18.4% of the total variation respectively.

In Figure 3.1, some variations were found along PC1 for *Asp. welwitschiae* samples. It has been discussed in Chapter 2 that both fumonisin-producing and fumonisin non-producing *Asp. welwitschiae* strains were isolated. Therefore, I was interested in whether it was the fumonisin production that caused the separation along PC1. Therefore, PCA was performed on *Asp. welwitschiae* samples. Figure 3.2 shows PCA scoreplot coloured by FB₂ production of the strains. As the PCA scoreplot shows in Figure 3.2, fumonisin production is unlikely the reason for the separation of *Asp. welwitschiae* samples. In the future, the factors causing metabolite differences within *Asp. welwitschiae* samples will be studied.

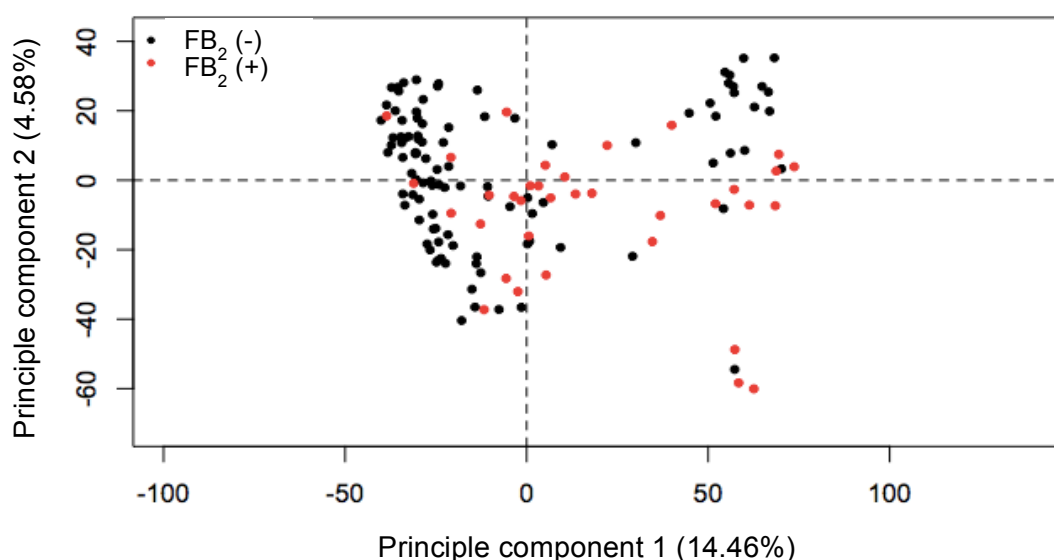


Figure 3.2 PCA scoreplot of *Asp. welwitschiae* coloured by FB₂ producer and FB₂ non-producer (data generated in negative ion mode).

3.3.3 Species-specific metabolite marker discovery

It has been reported that the various species of black *Aspergilli* are difficult to differentiate by morphology [84]. DNA sequencing is effective at differentiating between black *Aspergillus* species, but the preparation procedures (DNA extraction, PCR, gel electrophoresis and PCR cleanup) are time-consuming, especially when analyzing large sample sets. The goal was to find species-specific metabolite markers which can rapidly differentiate between isolated black *Aspergillus* strains. The metabolite marker should have a high prevalence in a certain species, but no or very low occurrence in other

species.

The Kruskal-Wallis test [105] was executed in R to evaluate the statistical significance of the differences observed between metabolites for each of the five groups: *Asp. brasiliensis*, *Asp. welwitschiae*, *Asp. uvarum*, *Asp. niger* and *Asp. tubingensis*. The p-value and FDR adjusted p-value for each feature (*m/z* and RT) were calculated, and features were sorted based on these values from smallest to highest (Table 3.2). The smaller the FDR adjusted p-value, the smaller chance of getting false positive results. FDR adjusted p-values lower than 0.05 were considered to be a statistically significant for each feature among the groups. The prevalence of each feature in each species was also calculated (the number of positive strains divided by the number of samples in a certain species).

Metabolite marker candidates were found by selecting features that were unique to a single species and had a high prevalence across that species. Table 3.2 shows a subset of the ESI (-) data and how to select metabolite marker candidates. Any feature with prevalence higher than 65% in a certain species, and no greater than 5% in any other species was selected as a potential metabolite marker. In addition, the RT of the potential metabolite marker should be from 0.5 min to 5 min to avoid pre- and post-running LC conditions. The selected metabolite markers and their related species were highlighted in the same color. For instance, *m/z* 329.1749 was selected as a metabolite marker candidate for *Asp. brasiliensis* (highlighted in red), because it was present in 81% of *Asp. brasiliensis* strains, but was not detected in any of the other four species.

Table 3.2 The Kruskal-Wallis test results across five species. The six features with the lowest p values in ESI negative mode are shown. 0% was hidden for a better visualization.

Feature		Prevalence					p-value	FDR adjusted p-value
<i>m/z</i>	RT (min)	<i>Asp. brasiliensis</i>	<i>Asp. niger</i>	<i>Asp. tubingensis</i>	<i>Asp. uvarum</i>	<i>Asp. welwitschiae</i>		
329.1749	4.44	81%					7.40×10^{-45}	2.73×10^{-41}
417.0791	2.14	90%			3%	1%	3.58×10^{-44}	6.62×10^{-41}
409.0912	2.90		75%			1%	5.05×10^{-36}	1.87×10^{-33}
283.0600	3.39	3%			71%	1%	1.82×10^{-32}	4.22×10^{-30}
663.2703	3.02			67%		2%	3.84×10^{-30}	5.46×10^{-28}

It is noteworthy that the metabolite markers should be the actual metabolites, not some adduct or fragment ions. The adduct ions or fragment ions from the same precursor ions should be removed by examining the MS and MS² spectra. Table 3.3 summarizes the potential metabolite markers for *Asp. brasiliensis*, *Asp. niger*, *Asp. uvarum*, and *Asp. tubingensis* differentiation. Interestingly, potential metabolite markers were found to differentiate *Asp. niger* from *Asp. welwitschiae*, two closely related species in phylogeny (discussed in Figure 2.7).

Table 3.3 Potential species-specific metabolite markers for individual *Aspergillus* species in (a) positive ion mode, (b) negative ion mode. In both positive and negative ion mode, ‘+’ means occurrence above 65%, ‘-’ means occurrence below ‘5%’.

(a)

[M+H ⁺]	RT (min)	<i>Asp. brasiliensis</i>	<i>Asp. niger</i>	<i>Asp. uvarum</i>	<i>Asp. tubingensis</i>	<i>Asp. welwitschiae</i>
375.1539	4.44	+	-	-	-	-
467.1624	4.44	+	-	-	-	-
331.1907	4.44	+	-	-	-	-
313.2158	4.94	+	-	-	-	-
323.2002	4.28	+	-	-	-	-
352.1879	3.36	+	-	-	-	-
357.2073	4.57	+	-	-	-	-
349.1983	2.91	+	-	-	-	-
403.1853	4.66	+	-	-	-	-
359.2249	4.65	+	-	-	-	-
439.1372	3.45	-	+	-	-	-
425.1216	3.13	-	+	-	-	-
411.1060	2.90	-	+	-	-	-
899.2487	3.46	-	+	-	-	-
477.0930	3.46	-	+	-	-	-
871.2177	3.14	-	+	-	-	-
288.0582	3.46	-	+	-	-	-
484.1950	3.46	-	+	-	-	-
571.3006	3.54	-	-	+	-	-
345.0963	3.27	-	-	+	-	-
331.0803	3.27	-	-	+	-	-
741.4161	3.25	-	-	+	-	-
755.4319	3.35	-	-	+	-	-
665.2858	3.03	-	-	-	+	-

(b)

[M-H ⁻]	Rt (min)	<i>Asp. brasiliensis</i>	<i>Asp. niger</i>	<i>Asp. uvarum</i>	<i>Asp. tubingensis</i>	<i>Asp. welwitschiae</i>
417.0791	2.14	+	-	-	-	-
397.1621	4.43	+	-	-	-	-
347.1642	3.97	+	-	-	-	-
409.0912	2.90	-	+	-	-	-
423.1069	3.14	-	+	-	-	-
477.0783	2.91	-	+	-	-	-
283.0600	3.39	-	-	+	-	-
331.0799	3.09	-	-	+	-	-
345.0966	3.34	-	-	+	-	-

3.3.4 Tentative identification of potential species-specific metabolite markers

Tentative identification of potential metabolite markers using accurate masses and MS²

spectra is a time-consuming procedure. As discussed in Section 1.2.2.3, the accurate mass of a potential metabolite marker was searched against Antibase 2013 and KNApSAcK [66] within a 5 ppm tolerance. The MS² spectrum of the potential metabolite marker was compared with MS² spectra of compound candidates after the database search. Potential metabolite markers with related accurate masses and similar MS² fragmentation could narrow the scope of locating right compounds, because fungi usually produce a family of compounds via a certain biosynthetic pathway.

Table 3.4 shows the tentatively identified potential species-specific metabolite markers in black *Aspergilli*. The only potential metabolite markers [M+H⁺] 665.2858 (compound A) for *Asp. tubingensis* was identified to be asperazine, which agreed with Nielsen *et al.* (2009) [36]. One of the potential metabolite marker [M+H⁺] 439.1372 (compound B) for *Asp. niger* was identified to be kotanin by its accurate mass, and verified by authentic standards. Six potential metabolite markers for *Asp. uvarum* were putatively identified by their accurate masses and MS² spectra (compounds C to H).

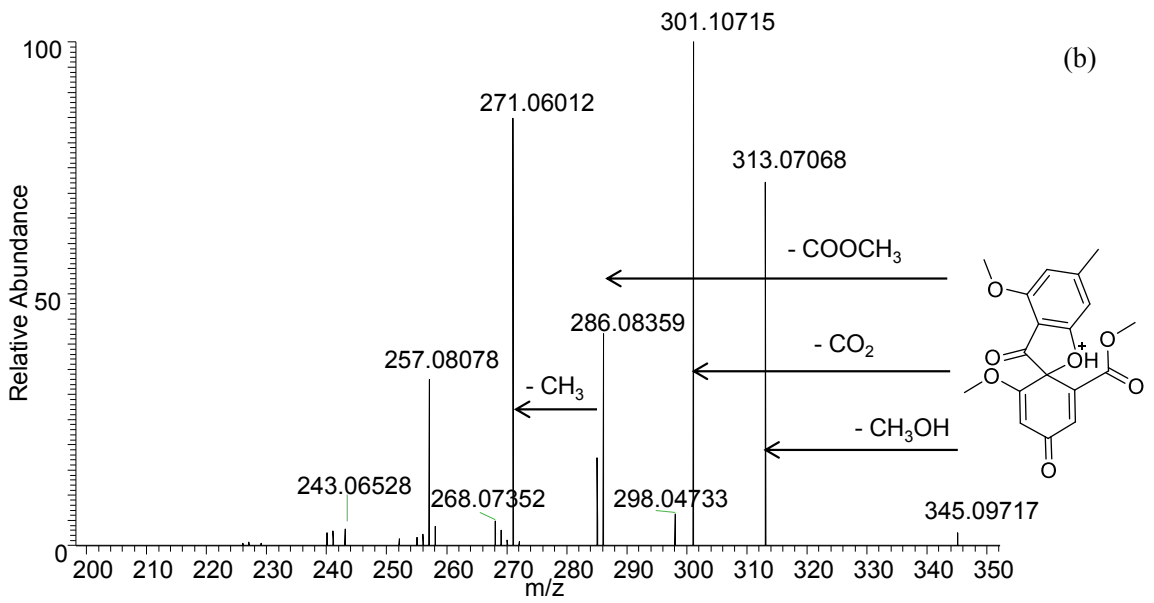
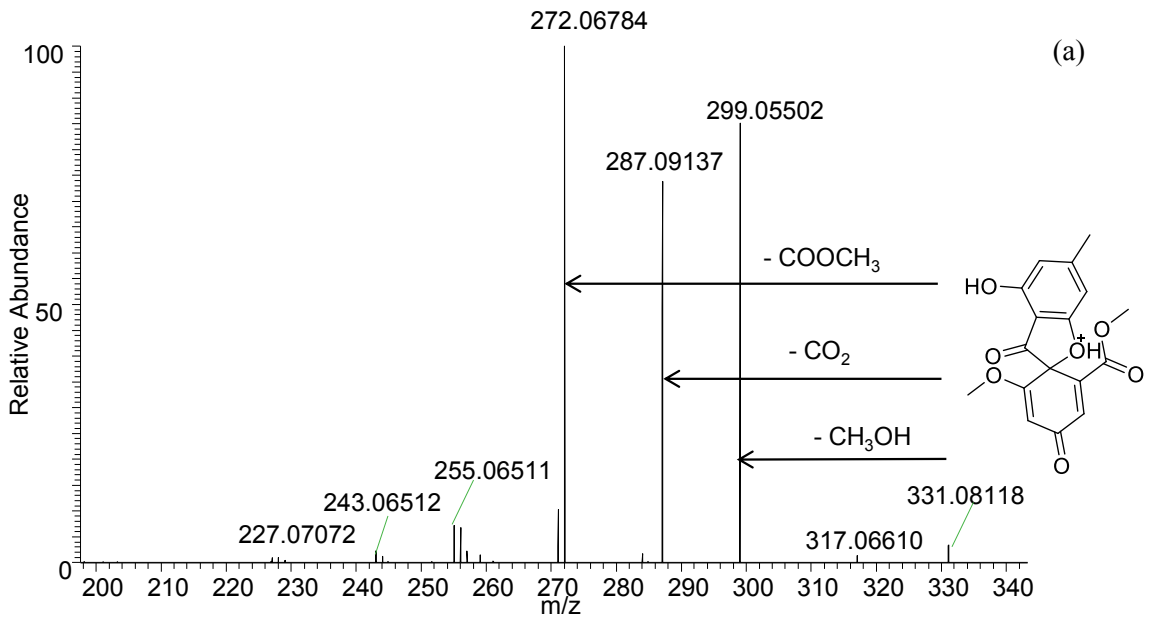
For compounds C to F, the similarity between their accurate masses and MS² spectra narrowed down the choice of the right compounds. The MS² spectra of these four metabolite markers and their representative dissociation pathways are shown in Figure 3.3. For compound C (Figure 3.3a), MS² spectrum of bisdechlorogeodin was not found in the literature. However, the related compound geodin ([M+H]⁺ 399.0057) has major product ions of 366.9813 (elimination of CH₃OH), 355.0138 (elimination of CO₂), 339.9911 (elimination of COOCH₃) reported by Bizukojc *et al.* (2013) [112], which supports our identification of compound C. Compound D (Figure 3.3b) share a similar fragmentation with compound C, but has an additional methylene group. Therefore, compound D can be putatively identified based on compound C. For compound E (Figure 3.3c), its major product ion *m/z* 209.0445 agreed with that of sulochrin reported by Varga *et al.* (2013) [113]. Compound F (Figure 3.3d) has additional one methylene group more than compound E, and its fragmentation is close to that of compound E. Thus, compound F can be tentatively identified based on compound E.

Trypacidin (compound D) was reported as a toxin from *Asp. fumigatus* (not black *Aspergillus* species) with cytotoxicity to lung cells [114]. Sulochrin (compound E) and monomethylsulochrin (compound F) are components of the biosynthetic pathway for trypacidin [114]. Therefore, *Asp. uvarum* could possess a similar biosynthetic pathway based on the metabolites I identified. Perrone *et al.* (2008) reported that *Asp. uvarum*

produced geodin, erdin, dihydrogeodin and asteric acid [111]. Interestingly, bisdechlorogeodin (compound C) is a non-chlorinated compound related to the chlorinated compound geodin. Therefore, it seems that *Asp. uvarum* strains studied by Perrone group could possess a chlorinated pathway, that is, a geodin biosynthetic pathway (MetaCyc online database, <http://solcyc.solgenomics.net/>). By contrast, our *Asp. uvarum* strains appear to possess non-chlorinated pathways, that is, an asterrate pathway (MetaCyc online database, <http://solcyc.solgenomics.net/>) and a trypacidin pathway similar in *Asp. fumigatus* [114].

Table 3.4 Tentatively identified potential species-specific metabolite markers in black *Aspergilli*

Compound	[M+H] ⁺	Chemical formula	Tentative identification	Potential metabolite marker for which species
A	665.2858	C ₄₀ H ₃₆ N ₆ O ₄	Asperazine	<i>Asp. tubingensis</i>
B	439.1372	C ₂₄ H ₂₂ O ₈	Kotanin	<i>Asp. niger</i>
C	331.0813	C ₁₇ H ₁₄ O ₇	Bisdechlorogeodin	<i>Asp. uvarum</i>
D	345.0969	C ₁₈ H ₁₆ O ₇	Trypacidin	<i>Asp. uvarum</i>
E	333.0970	C ₁₇ H ₁₆ O ₇	Sulochrin	<i>Asp. uvarum</i>
F	347.1127	C ₁₈ H ₁₈ O ₇	Monomethylsulochrin	<i>Asp. uvarum</i>
G	741.4161	C ₃₈ H ₅₆ N ₆ O ₉	Aspergillicin A	<i>Asp. uvarum</i>
H	755.4319	C ₃₉ H ₅₈ N ₆ O ₉	Aspergillicin E	<i>Asp. uvarum</i>



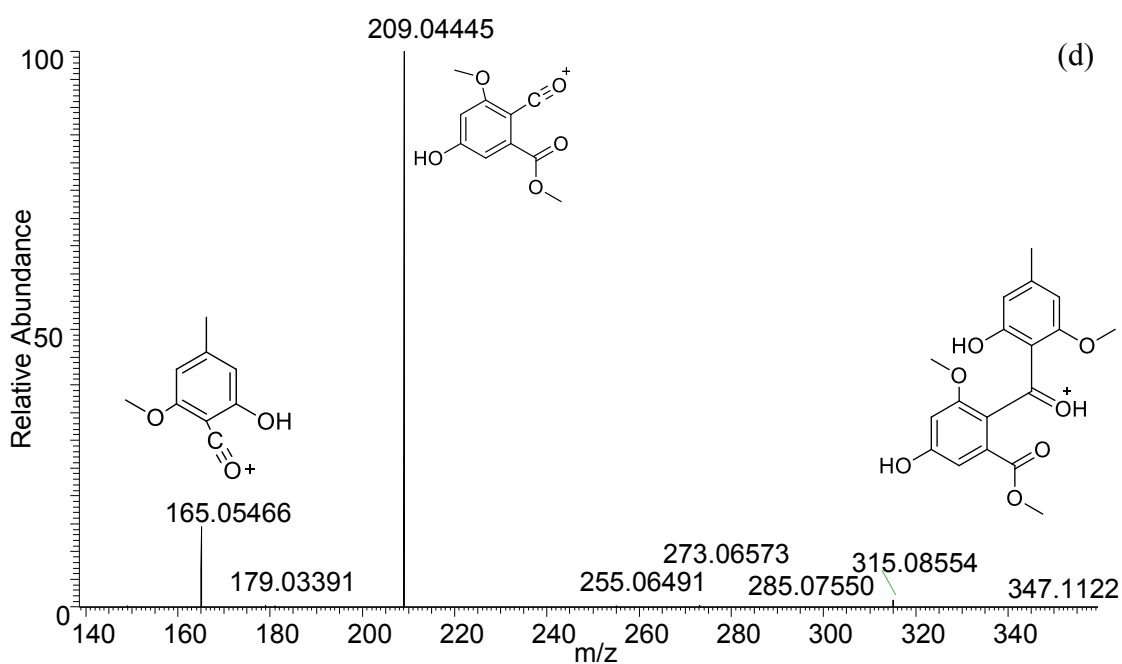
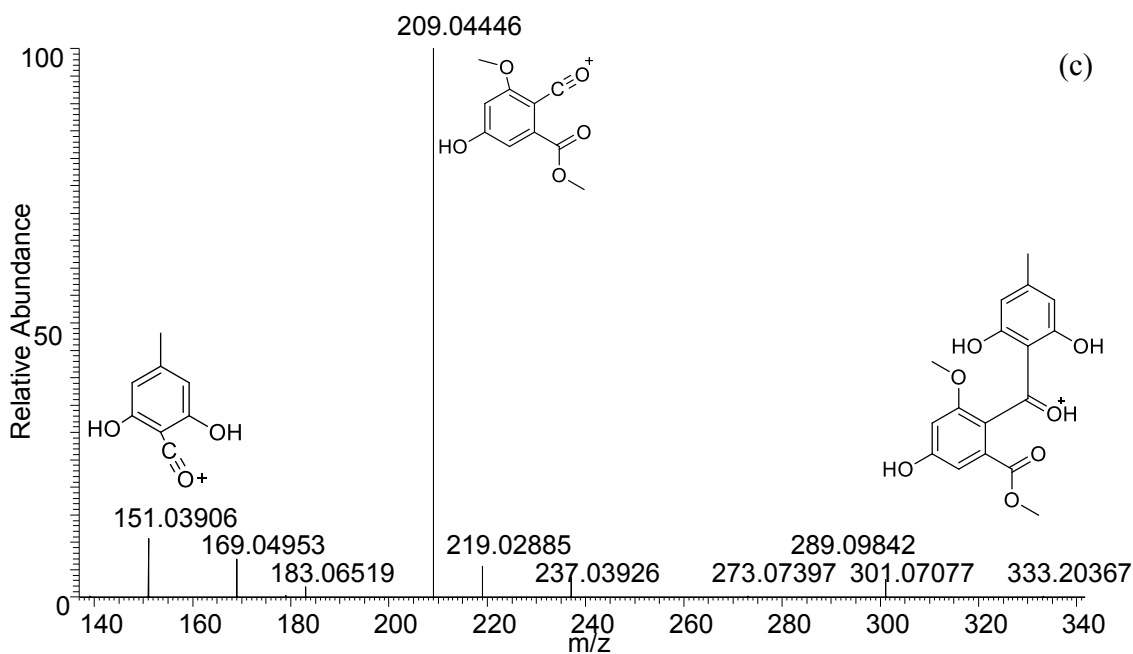


Figure 3.3 (a-d) MS² spectra of compound C, D, E, F with their possible representative dissociation pathways. The possible structures of precursor ion and fragment ion were labeled beside their *m/z*. Compounds E and F were selected as potential metabolite markers in ESI (-). In order to compare the published ESI (+) MS² spectrum of sulochrin in Varga *et al.* (2013) [113], ESI (+) MS² spectra of compounds E and F are shown.

For compound G and H, their accurate masses were searched against AntiBase 2013. There was only one hit for each compound within 5 ppm mass accuracy. Compound G and H are tentatively identified to be aspergillicin A and aspergillicin E respectively. Aspergillicin A and E are depsipeptides produced by the marine-derived fungus, *Asp. carneus* [115]. Aspergillicin B, C, D were also detected from our *Asp. uvarum* strains. However, the low abundance and low prevalence prevent them from being species-specific metabolite markers. Structures of aspergillicin families are shown in Figure 3.4. There was no prior reports suggested that aspergillicin families were found in any species of black *Aspergilli*.

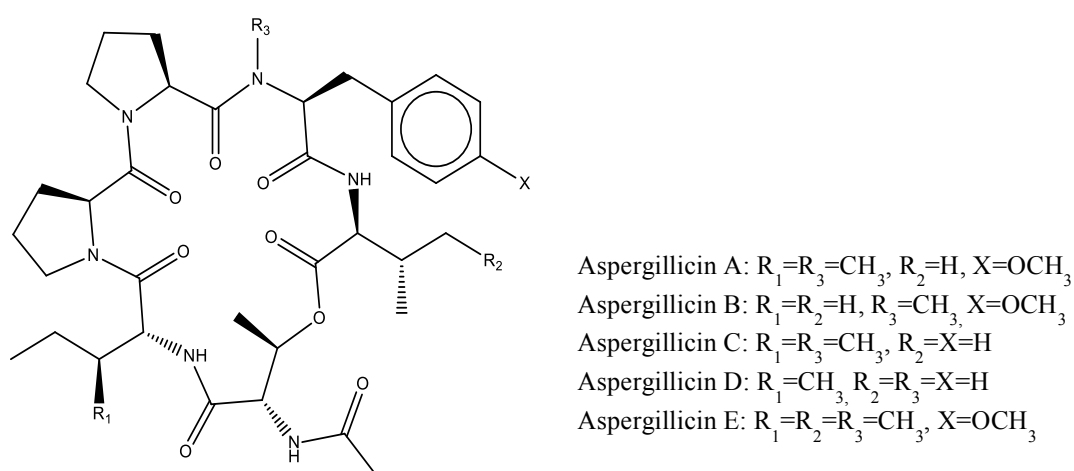


Figure 3.4 Structures of aspergillicin A to E.

However, accurate masses and MS^2 spectra can only be used for tentative identification. Compound A to F needs to be verified by authentic standards. Compound G and H need to collect LC-isolated fractions and characterize by the NMR spectroscopy, since standards are not available.

The potential metabolite markers I found are limited in *Asp. brasiliensis*, *Asp. niger*, *Asp. uvarum*, *Asp. tubingensis* and *Asp. welwitschiae*, until more information about the black *Aspergillus* strains becomes available.

3.4 Conclusions and Future Work

Five species of black *Aspergilli* isolated from Canadian vineyards can be distinguished from one another using a metabolomics approach. Thirty-three potential metabolite markers were discovered for *Asp. brasiliensis*, *Asp. niger*, *Asp. uvarum*, *Asp. tubingensis* identification. Eight of them have been tentatively identified by HRMS and MS² (Table 3.4). These identification results need to be verified by authentic standards if available. In addition, the remaining potential metabolite markers in Table 3.3 need to be tentatively identified, followed by authentic standards verification or LC-isolated fractions for structure elucidation by NMR spectroscopy. Besides, larger sample sizes for *Asp. nigers* and *Asp. tubingensis* should be obtained in the future, because these species probably did not have enough samples to determine if they were metabolically distinct from the rest or to identify reliable metabolite markers.

Chapter 4 – Conclusions and Future Work

4.1 Conclusions and Future Work

OTA and fumonisins produced by black *Aspergilli* are known contaminants on grapes and in wine [9]. However, there was limited data suggesting the presence of OTA and fumonisins on Canadian wine grapes and in Canadian wine. Therefore, a three-year survey has been conducted to study the occurrence of black *Aspergilli* in Canada and to assess the risk of OTA and fumonisin contamination in Canadian vineyards (Chapter 2). To my knowledge, this study is the first report on the OTA and fumonisin contamination in Canadian vineyards.

OTA and fumonisin quantitative analyses were carried out using the state-of-the-art Orbitrap LC-MS. The LOQ for OTA and FB₂ is 0.4 µg/kg and 0.37 µg/kg respectively, which is below the regulatory limit for OTA in wine at 2.0 µg/kg set up by EU, and is three orders of magnitude lower than the 0.2-2.0 µg/g regulatory limit of EU for FB₂ contamination in maize. Therefore, the LC-MS² results of mycotoxin analysis presented in the thesis are reliable.

In the investigated Canadian vineyards, OTA was only detected once from the only *Asp. carbonarius* strain isolated, and was not detected from any of grape samples tested. This indicates the risk of OTA contamination in the investigated Canadian vineyards is very low. The reason could be that the relatively low temperature in Canada is not appropriate for the growth and reproduction of *Asp. carbonarius*, the main OTA producers in black *Aspergilli*. These results agreed with a survey conducted in cooler grape-growing regions of Northern Italy [104].

The predominant black *Aspergilli* in the investigated Canadian vineyards were *Asp. welwitschiae* strains. Fumonisins (B₂, B₄ and B₆) were detected from 21% of *Asp. welwitschiae* strains. Another fumonisin producer isolated were *Asp. niger* (10 strains). Fumonisins were detected from both artificial media where *Asp. welwitschiae* and *Asp. niger* are isolated and from grape samples, but in low concentrations. These results indicate that the risk exists for fumonisin contamination in Canadian vineyards, but is low.

To study the risk of OTA and fumonisin contamination in wine, wine analysis needs to be performed. The LC-MS² and SPE methods for OTA and fumonisin quantification and cleanup developed for grape analysis should be the starting point for wine analysis.

In the metabolomics study (Chapter 3), thirty-three potential species-specific metabolite markers were found to distinguish black *Aspergillus* species isolated from Canadian vineyards (*Asp. brasiliensis*, *Asp. niger*, *Asp. uvarum*, *Asp. tubingensis*, and *Asp. welwitschiae*). Eight of them (Table 3.4) were tentatively identified by accurate masses and MS² spectra. They need to be verified by authentic standards, or isolated by LC for NMR spectroscopic characterization. The rest of the potential species-specific metabolite markers in Table 3.3 need to be identified and verified using the same method discussed above. These potential species-specific metabolite markers will be evaluated using unknown black *Aspergillus* species by comparing the results of species identification with results using traditional DNA sequencing.

The advantage of using species-specific metabolite markers is that it is a rapid tool for black *Aspergilli* identification, since it requires a simple sample preparation procedure and it takes only several minutes per sample to screen for several species-specific metabolite markers using LC-MS². On the contrary, traditional DNA sequencing usually requires the following procedures: DNA extraction, PCR, gel electrophoresis, PCR products cleanup, sequencing, and sequence alignment. The limitation of the current study is that only five main species were isolated from Canadian vineyards, therefore the metabolite markers cannot be used to discriminate all the black *Aspergilli* until other species of black *Aspergilli* are isolated. More species could be isolated from other sources, such as onion, maize and peanuts [116]. The results of metabolite marker discovery will be updated using the same metabolomics approach discussed in Chapter 3, after more species are isolated.

Interestingly, the potential metabolite markers for *Asp. uvarum* identification, bisdechlorogeodin, trypacidin, sulochrin, monomethylsulochrin, aspergillicin A and E, have not been found to be produced by any black *Aspergillus* species, to my knowledge. These results are related but different from those produced by *Asp. uvarum* isolated from grapes in Europe [111]. In the future, European *Asp. uvarum* strains will be studied to better understand what causes metabolite differences between European and Canadian strains.

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Appendices

Appendix A: Media used

1. Dichloran Rose Bengal Chloramphenicol (DRBC) (50 mg/L) Agar (1 L)
 - 31.5 g Dichloran Rose Bengal Agar
 - 984.25 mL RO water
 - 781 μ L chloramphenicol
2. Dichloran Glycerol(DG18) Agar (1 L)
 - 31.6 g Dichloran Glycerol Agar
 - 220 g glycerol
 - 984.20 mL RO water
3. Czapek Yeast Agar (CYA) (1 L)
 - 35 g Czapek Dox Broth (Fluka Analytical)
 - 3% sucrose (30g)
 - 2% agar (20g)
 - 10 g yeast extract
 - 952.50 mL RO water
4. Czapek Yeast Autolysate (CYA) Liquid (1 L)
 - 35 g Czapek Dox Broth (Fluka Analytical)
 - 5 g yeast extract
 - 980 mL RO water

Appendix B: Polymerase chain reaction

To identify *Aspergillus* to species level, fungal DNA is extracted using a fungal DNA kit according to the protocol provided by the manufacturer. PCR is used to amplify a specific DNA fragment of interest in small quantity to a million or billion copies, which makes DNA detection possible. PCR products with a UV-sensitive DNA-binding dye were analyzed by gel electrophoresis.

A PCR reaction needs template DNA, primers, deoxynucleotides (dNTPs), Taq DNA polymerase, MgCl₂, and PCR buffer. A primer is a single strand DNA sequence designed to be complementary to the DNA fragment of interest. Forward primer is to locate the beginning of one strand of the target DNA fragment. Reverse primer is to locate the end of the complementary strand of the target DNA fragment. dNTPs are building blocks of new DNA strands. Taq DNA polymerase is an enzyme that helps the polymerization of the dNTPs into a DNA strand. MgCl₂ is an essential factor for the function of Taq DNA polymerase. PCR buffer creates an optimum environment for the Taq DNA polymerase.

PCR relies on thermal cycling, containing repeated heating for DNA melting and cooling for DNA replication. The following PCR settings in Chapter 2 are used as an example to explain the principle of PCR procedures. PCR includes the following five steps. The first step is to heat sample to 95°C for 5 min for the purpose of Taq DNA polymerase activation. The second step “denaturation” is to heat the sample to 95°C for 30 s in order to denature double-stranded DNA to open up to single-stranded DNA. In the third step “annealing”, hydrogen bonds are formed between template DNA and primers by setting the temperature 5°C -10°C below the melting temperature of the primer for 30 s. The annealing temperature is unique for a specific gene amplification. The fourth step “extension” is to extend the new DNA strand using dNTPs with the help of Taq DNA polymerase at 72°C for 30 s. Step 2, 3, 4 are repeated 30 to 40 times to amplify a billion copies of target DNA. The last step is for final extension for 5 min at 72°C so as to ensure the single strands are fully extended.

By running gel electrophoresis, PCR product stained by a nucleic acid dye could be visualized under UV, and confirm whether the target DNA fragment is being successfully amplified.

Appendix C: PCR Primers used

1. β -tubulin primer sequence (5' to 3') [1]:

Bt2a: GGTAACCAAATCGGTGCTGCTTTC

Bt2b: ACCCTCAGTGTAGTGACCCTTGGC

2. *Asp. carbonarius* specific primer sequence (5' to 3') [2]:

CarbF: GTGAAGTCTGAGTCGATTGTT

CarbR: GGAAAAAAGGTTGGAGTT

3. *Asp. niger* specific primer sequence (5' to 3') [3]:

AnF: GGATTTTCGACAGCATTTCAGAACG

AnR: GATAAAACCATTGTTGTCGCGGTCG

4. *Asp. welwitschiae* specific primer sequence (5' to 3') [3]:

AwF: GGGATTTTCGACAGCATTTCAGAAATT

AwR: GATAAAACCATTGTTGTCGCGGTCA

Note: 'F' means 'forward primer', 'R' means 'reverse primer'.

Appendix D: Solid phase extraction

Samples in a complex matrices often needs to be cleaned up, because matrix effect could cause significant ion suppression in LC-MS analysis. Quantitative results with a high level of ion suppression and higher limits of detection are not as reliable.. In addition, samples with low amount of analytes of interest need to concentrate so that the compound could be above the LOD of the MS. The grape samples for mycotoxin analysis (Chapter 2) needs to be purified and concentrated by SPE, since grape is a complex matrix and mycotoxin levels on grapes could be very low.

Solid phase extraction (SPE) is a sample preparation technique for sample purification and concentration. Reversed-phase SPE and ion exchange SPE are two commonly used types of SPE. Reversed-phase SPE separates analytes by polarity. Ion exchange SPE separates charged analytes based on their electrostatic attraction to charged functional groups on the solid phase. The following three SPE cartridges are used in Chapter 2.

Bond Elut Plexa (Agilent) is a non-polar divinylbenzene-based neutral polymeric sorbent. It can be applied for non-polar and less polar compound purification.

Bond Elut SAX cartridge (Agilent) is an anion exchange SPE cartridge. The sorbent is quaternary amine group bonded to the silica surface. The sorbent is positively charged at all pH because its pKa is greater than 14. Acidic compounds will bind to the sorbent when the matrix pH is at least 2 units greater than its pKa. Some basic impurities can be washed off at an appropriate pH. Elution is based on pH adjustment. Acidic compounds elute with 1-5% acetic acid in methanol.

Oasis MCX cartridge (Waters) is a cation exchange SPE cartridge. Silica with aliphatic sulfonic acid groups is the sorbent. The sorbent is negatively charged at almost all pH because it is strongly acidic with pKa less than 1. Basic compounds will electrostatically interact with the sorbent when the matrix pH is at least 2 units smaller than its pKa. A solvent can be used to wash off acidic impurities at an appropriate pH. A solvent containing 1-5% ammonium hydroxide in methanol disrupts the electrostatic interaction between basic compounds and sorbent for elution to occur.

The general experimental procedure of SPE can be described as follows (Figure S1): SPE cartridge is first pre-equilibrated with a solvent. A liquid sample with analyte of interest passes though the sorbent where analyte of interest and some impurities get absorbed.

The cartridge is washed with a solvent, where some impurities can be washed off, and analyte of interest remained in the cartridge. Finally, analyte of interest will elute with another solvent. By this way, the analyte of interest is purified.

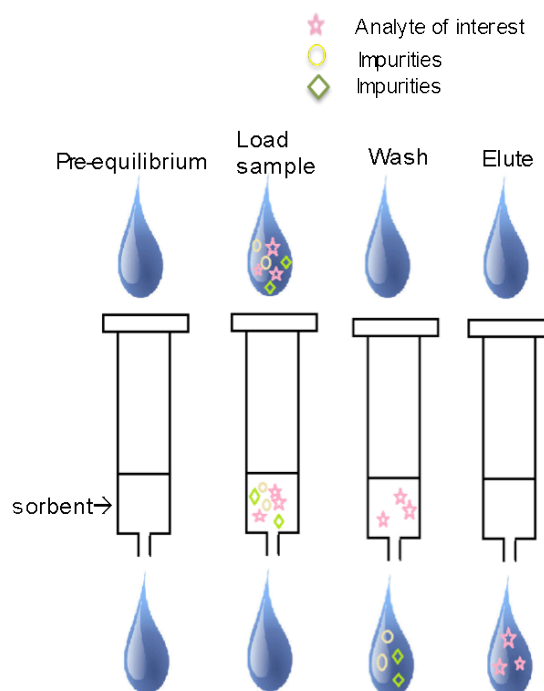


Figure S1 Schematic diagram of SPE procedure

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Curriculum Vitae

Tianyu Qi

Education

- ✧ M.Sc. in Analytical Chemistry, University of Western Ontario, 2013-2015
Thesis title: Study of the secondary metabolites of black *Aspergilli* from Canadian vineyards
Supervisor: Dr. Ken K.-C. Yeung and Dr. Mark W. Sumarah
- ✧ B.Sc. in Pharmaceutical Sciences, Capital Medical University (China), 2009-2013

Honors and Awards

- ✧ Recipient of 2015 Lipson-Baines Award, graduate seminar presentation “Spiroindolones and Imidazolopiperazines: Potential New Weapons to Defeat Malaria”

Related Work Experience

- ✧ Graduate research assistant, Dr. Mark W. Sumarah lab, Agriculture and Agri-food Canada (AAFC), 2014-2015
- ✧ Graduate teaching assistant, Department of Chemistry, University of Western Ontario, 2013 – 2015

Publications

- ✧ Justin Renaud, Megan J. Kelman, **Tianyu Qi**, Keith A. Seifert, and Mark W. Sumarah (2015) Rapid Communications in Mass Spectrometry Product ion filtering with rapid polarity switching for the detection of all fumonisins and AAL-toxins. *Rapid Communications in Mass Spectrometry*. (Accepted)

Presentations

- ✧ Gordon Research Conference on Mycotoxins & Phycotoxins, June 2015, Easton, MA, USA
“Study of black *Aspergilli* from Canadian vineyards and the associated risk of ochratoxin A and fumonisins contamination of wine grapes” **Tianyu Qi**, Justin B. Renaud, Tim McDowell, Keith A. Seifert, Ken K.-C. Yeung and Mark W. Sumarah