Extraction and Purification of (E)-Resveratrol from the Bark of Maine's Native Spruces

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EXTRACTION AND PURIFICATION OF (E)-RESVERATROL FROM THE
BARK OF MAINE'S NATIVE SPRUCES

By
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A DISSERTATION
Submitted in Partial Fulfillment of the
Requirements for the Degree of
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Resveratrol is a plant secondary metabolite which protects plants from pathogenic invasions and acts as a remedial agent against injury, stress, and tissue damage by UV radiation. With its characteristic stilbene structure, resveratrol adopts \((E)\) and \((Z)\) isomeric forms. However, the \((E)\) isomer is the most biologically active and abundant form of resveratrol in nature.

\((E)\)- Resveratrol confers a variety of benefits to human health and well-being. Besides its antioxidant activity, the role of \((E)\)-resveratrol as an anti-inflammatory, anti-aging, cardioprotection, and anticancer agent has been a widely explored research interest. Many plant species including mulberry, peanut, and grape are sources of the compound, while Japanese knotweed \((Polygonum cuspidatum)\), an invasive herbaceous plant species, is currently the prime source for the commercial production of \((E)\)-resveratrol.

Previous research findings of our group and other published work have shown that spruces native to Maine are rich sources of \((E)\)-resveratrol, and the compound is located
primarily in the bark of the trees. Bark is typically a waste material generated from the forest bioproducts industries in the state. Hence, development of a method to isolate \((E)\)-resveratrol from Maine’s native conifer species would find a rewarding use for bark and open up industrial avenues that are highly relevant to the state of Maine. However, spruces inherently contain a variety of polyphenolic compounds with similar structural and functional properties. Hence the isolation of \((E)\)-resveratrol from spruce bark extracts poses a challenging research problem.

In this study, a solvent extraction and column chromatography-based purification method was developed to isolate \((E)\)-resveratrol from black spruce \((Picea mariana)\) bark in high yield. Various chromatographic and other separation techniques have been explored in this work, and the concept of E-Z isomerization of resveratrol is discussed. Based on HPLC-MS and HPLC-UV analyses, the proposed purification method isolates the compound in 84\% yield and 99\% purity. The overall yield is \(279.9 \pm 4.9 \, \mu g\) \((E)\)-resveratrol per dry gram of bark. Since the biosynthetic pathways of plants that produce a variety of polyphenolic compounds are related, it is anticipated that this purification method is applicable for isolating \((E)\)-resveratrol from other plants as well.
DEDICATION

To my beloved mom, dad, and my dearest wife, Anushka!
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CHAPTER 1
INTRODUCTION

(E)-Resveratrol (5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol) is a naturally occurring polyphenolic compound of plant origin. It belongs to the category of stilbenes, which are plant secondary metabolites with a “stilbene skeleton” comprising two aromatic rings bridged by an alkene (Figure 1).

![E-stilbene skeleton](left) and chemical structure of (E)-resveratrol (right).

The presence of (E)-resveratrol in red wine was first discovered by Cornell University researchers (Siemann and Creasy, 1992). This discovery linked the compound to the famous “French paradox”, a term derived from epidemiology research that showed the mortalities related to coronary heart diseases are significantly lower in the French population compared to other European countries despite their higher consumption of dietary cholesterol and saturated animal fat (Richard et al. 1981). Further investigations suggested that the lower coronary heart disease risks could be attributed to the high red wine consumption habits among the French (Chadwick and Goode, 1998). The ability of (E)-resveratrol to modulate lipid metabolism was already known by then from the work of Arichi et al. (1982), which led to the belief that (E)-resveratrol was the substance in red wine that confers the beneficial health effects to reduce heart diseases. Since then,
many studies have been conducted investigating the health benefits of \((E)\)-resveratrol, and isolating it from natural sources to formulate supplements and potential medications.

1.1. Medicinal Value of \((E)\)-Resveratrol

The potent biological activity of natural products from plants has earned its place in traditional medical practices of many cultures, tribes, and civilizations for centuries. Among those naturally occurring compounds, \((E)\)-resveratrol particularly has been shown to confer desirable effects to human health and well-being by modulating a variety of different metabolic and enzymatic pathways in the body.

1.1.1. Radical-Scavenging (Antioxidant) Activity

Perhaps the most widely known health benefit of \((E)\)-resveratrol is its ability to act as an antioxidant. Metabolic reactions occurring in biological systems are prone to produce free radicals such as superoxide radical and hydroperoxyl radical, which start oxidative modifications to the cellular macromolecules such as DNA, lipids, and proteins to induce harmful mutations and trigger cellular apoptosis (Hancock et al. 2001). Since these radicals are derived from molecular oxygen, they are collectively known as reactive oxygen species (ROS). Cells inherently contain a variety of mechanisms to neutralize ROS. However, exogenous factors that include smoking, drugs, and pollutants can elevate the ROS generation to a level such that the cellular mechanisms are inadequate to neutralize them. Natural products such as polyphenols and tocopherols help to control ROS generation by scavenging them (Chanvitayapongs et al. 1997).

Being a polyphenolic compound, \((E)\)-resveratrol has been shown to act as a radical scavenger itself, and also as a promoter of cellular antioxidant enzymes such as
glutathione peroxidase, glutathione S-transferase, and glutathione reductase to induce neutralization of peroxo radicals (Lastra and Villegas, 2007). Based on in-vitro study, Shang et al. (2009) proposed a reaction mechanism for the antioxidant activity of (E)-resveratrol, using galvinoxyl (GO') and 2,2-diphenyl-1-picrylhydrazyl (DPPH') radicals (Figure 2).

Figure 2: Radical scavenging reaction mechanism by (E)-resveratrol in ethanol (Shang et al. 2009).

This study suggested that the antioxidant activity of (E)-resveratrol towards the radicals proceeded via a hydrogen atom transfer (HAT) mechanism or sequential proton-loss electron transfer (SPLET) mechanism. However, the energy cost for abstracting an electron from hydrogen-bonded phenolic OH is higher than that from a phenolic ring.
Hence, the more plausible mechanism is to abstract an electron from the phenolic ring prior to the loss of a proton. The dimer product formation was evident from the NMR studies.

Other in-vitro studies that mimic physiological systems (Murcia et al. 2001), and biological systems such as rat pheochromocytoma cell lines (Chanvitayapongs et al. 1997) have shown that (E)-resveratrol is a potent antioxidant compared to a number of typical food additives such as α-tocopherol (vitamin E), BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and vanillin. With the research findings that suggest ROS is linked to cardiovascular diseases, cancers, diabetes, and periodontal diseases (Sarangarajan et al. 2017), the antioxidant activity of (E)-resveratrol makes it a potential therapeutic agent for those disease conditions.

1.1.2. Prevention of Atherosclerosis and Inflammation

The National Institute of Health defines atherosclerosis as a disease condition where blood vessels in the body, arteries particularly, start to harden and narrow due to the deposition of plaques consisting of fat, cholesterol, calcium, and other related substances in the blood. This condition weakens the circulation of oxygen-rich blood in the body, leading to an array of serious complications such as cardiovascular disease.

Many scientific investigations suggest that (E)-resveratrol has the ability to reduce the risk of atherosclerosis by modulating the lipid metabolism in the body. The work published by Arichi et al. (1982) has shown that oral and intra-peritoneal ingestions of (E)-resveratrol to the mouse models fed with high cholesterol diet, resulted in reduced deposition of cholesterol and triglycerides in the liver and blood. These observations
were further supported by a recent study where a significant reduction in atherosclerotic lesions and lower aortic calcium deposition was seen in mouse models treated with a diet supplement containing (E)-resveratrol (Tomayko et al. 2013).

Studies aimed at the mechanism of action of (E)-resveratrol attributes these anti-atherosclerotic effects to the ability of the compound to upregulate lipid metabolism related gene expression. In-vivo studies conducted with mouse models (Ahn et al. 2008) and in-vitro studies conducted with human hepatic cell lines (Curtin et al. 2008) have demonstrated that (E)-resveratrol induces the expression of paraoxonase 1 (PON1) gene to produce paraoxonase enzymes. These enzymes have a detoxification role in the body by hydrolyzing organophosphate insecticides. However, they are also found to be binding to HDL and exerting anti-atherosclerotic functions as well (Khalil and Berrougui, 2009). In addition, the work of Cho et al. (2008) has shown that (E)-resveratrol causes a significant decrease in cholesterol ester transport protein (CETP) levels, which in turn results in an increase of high-density lipoprotein cholesterol (HDL-C) in the body. HDL-C transports cholesterol from the cells and tissues to the liver, where it is metabolized. Hence, it helps to reduce the risk of coronary heart disease.

Inflammation, the body’s response to exogenous toxins and infections, is also triggered by ROS and could result in tissue injury and atherosclerosis (Mittal et al. 2014). The antioxidant activity of (E)-resveratrol helps to reduce inflammatory responses by scavenging ROS. However, some studies suggest that (E)-resveratrol is also capable of regulating inflammatory responses by disrupting the associated biochemical pathways. One such pathway involves activation of a transcription factor known as NF-κB, which induces the expression of cell signaling proteins (cytokines) to initiate the inflammatory
response in target tissues. \((E)\)-Resveratrol has been shown to inhibit the pathways of NF-κB activation, which results in down-regulation of the inflammatory signal (Soleas et al. 2001, Tian et al. 2016). An in-vitro study conducted with pulmonary artery endothelial cell cultures has proposed a mechanism for the anti-inflammatory response of \((E)\)-resveratrol in humans (Yang et al. 2011). The study identified eotaxin-1 as a vital component of the inflammatory signaling pathway, and its expression in the cells is induced by cytokines such as IL-13 and TNF-α. Their findings indicated that \((E)\)-resveratrol suppresses this signal transduction pathway at multiple stages, as shown in Figure 3.

![Figure 3: Proposed mechanistic scheme illustrating induction of eotaxin-1 gene transcription by IL-13 and TNF-α, and modulation by \((E)\)-resveratrol (Yang et al. 2011).](image-url)
The above mechanism suggests that (E)-resveratrol suppresses the IL-13 and TNF-α mediated transcription signals for the expression of eotaxin-1. It proposes that the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is also involved in the eotaxin-1 expression, and (E)-resveratrol is capable of suppressing that pathway as well. The involvement of NF-κB in the expression of eotaxin-1 is also suggested in the proposed mechanism. As discussed previously, the ability of (E)-resveratrol to inhibit NF-κB is already known. Hence the compound is useful in down-regulating the inflammatory responses triggered by various signals.

1.1.3. Anti-Carcinogenic Activity

The anti-cancer activity of (E)-resveratrol has been a thoroughly studied area for the past decade. Many research findings suggest that the compound has a pleiotropic functionality against cancers. Cancers are abnormal proliferations of cells due to unfavorable mutations in DNA. As discussed previously, the antioxidant and anti-inflammatory actions of (E)-resveratrol contribute to preventing such damage to the genetic material. In addition, many studies have shown that the compound plays an essential role in regulating multiple signal transduction pathways to inhibit mutations and cancer cell progression.

Epidermal growth factor (EGF) and its receptor (EGF-R) are components of a cellular signal transduction pathway that promotes cell growth and proliferation. Genetic mutations cause over-expression of EGF and EGF-R, resulting in an uncontrolled growth and progression of the mutated (cancer) cells (Kubota et al. 2003). A recent study conducted with human ovarian cancer cell lines has shown that (E)-resveratrol significantly reduces the EGF-R activation, suppressing the cancer cell proliferation
Further studies suggest that \((E)\)-resveratrol also inhibits the cancer cell proliferation by suppressing the cell cycle at G1 (the gap phase where metabolic changes prepare the cell for division) and S (the phase where DNA synthesis replicates the genetic material) phases, forcing cell apoptosis. The work of Yu et al. (2013) has shown the ability of the compound to cause cell cycle arrest by regulating the expression of transcriptional factors involved in the S phase of cell cycle, even in very aggressive malignancies such as anaplastic thyroid carcinoma and colon cancer which are least responsive to the conventional methods of treatment. Additionally, the research findings of Shankar et al. in 2007 have indicated that \((E)\)-resveratrol is capable of inducing the expression of proteins associated with the cell apoptotic signaling pathways to cause programmed cell death in human prostate cancer cell lines. These findings were further confirmed by the observations of similar activity of \((E)\)-resveratrol in fibrosarcoma cells, nasopharyngeal cancer cells, breast cancer cells, and non-small cell lung cancer cells. The results also indicated that the compound has a minimal effect on healthy cell lines for inducing apoptosis (Varoni et al. 2016). Hence, \((E)\)-resveratrol has the potential to be used as a selective chemotherapeutic agent for cancers.

1.1.4. Anti-Aging Effects

Perhaps one of the most sought-after properties of any chemical supplement is its ability to rejuvenate cells and tissues to extend their lifespan. Some of the early evidence for the ability of \((E)\)-resveratrol to extend cellular lifespan comes from the work of Howitz et al. (2003), in which they demonstrated that \((E)\)-resveratrol in yeast \((Saccharomyces cerevisiae)\) cells is capable of increasing the stability of the genomic DNA through various signal transduction pathways, resulting in an overall extension of the lifespan by
70%. These research findings led to further explorations of the anti-aging activity of the compound, and its suitability to treat aging-related disorders such as dementia and Alzheimer's disease.

A study conducted by Kumar et al. (2016) discovered that (E)-resveratrol in 20 mg/kg (body weight) supplements has the ability to regenerate the depleted areas of the hippocampus tissue in the brain of aged rats (Figure 4). They further observed that the compound upregulates the proteins associated with the survival of neurons and elongation of the cellular lifespan. These results were consistent with the in-vitro studies performed on cultured cell lines of human neural progenitor cells. Further investigations conducted with normal human epithelial cells, human metastatic breast cancer cells, human lung cancer cells, and rat glioma cells revealed that the tissue regeneration effects of (E)-resveratrol are specific to neural cells. These research findings have brought (E)-resveratrol into the limelight, reiterating its potential to be developed as a therapeutic agent for age-related neurodegenerative diseases.

Despite the many health benefits of (E)-resveratrol and the attraction to achieving those benefits by drinking red wine, wine is a poor source of the compound. A recent study has estimated that a daily average of 111 glasses of wine (11.1 liters) has to be consumed in order to reach the minimum dose that would exert therapeutic effects, if wine is the only dietary source of (E)-resveratrol (Lachenmeier et al. 2014). Hence, the need to extract and purify the compound from other sources to prepare (E)-resveratrol formulations for research and medicinal purposes is apparent.
Figure 4: Neuron regeneration in the presence of (E)-resveratrol in the sub-granular zone (SGZ) and hilus area of the hippocampus tissue in the brain of aged rats (Kumar et al. 2016).

1.2. (E)-Resveratrol in Plants

(E)-Resveratrol is found in terrestrial plant species including peanut, grape and some herbaceous species such as Japanese knotweed (Mei et al. 2015). It is produced as a secondary metabolite in response to a wide variety of environmental factors such as oxidative stress, UV radiation damage to the tissues, and pathogenic infections. (Bru et al. 2006, Shiraishi et al. 2010). However, secondary metabolites are not essential for the growth and development of plants, and their function is mostly limited to improving the survival fitness of plants in the environment. Hence, many plants produce them in minute
concentrations only, which makes the choices for viable sources of (E)-resveratrol limited (Burns et al. 2002). Therefore, the prime source for the commercial production of (E)-resveratrol remains Japanese knotweed (*Polygonum cuspidatum*), a resveratrol-rich weed species that is identified as an invasive plant (Murrell et al. 2011).

Many of the commercial preparations of (E)-resveratrol are Japanese knotweed root extracts, and they range widely in purity. Some studies suggest that additional compounds such as emodin present in unpurified or partially purified Japanese knotweed root extracts may have laxative effects (Srinivas et al. 2007). Since the plant thrives even in heavily polluted environments and soils contaminated with heavy metals, cellular uptake of those contaminants by the root tissues poses further concerns regarding the safety of the commercial preparations of (E)-resveratrol made from root extracts (Berchová-Bímová et al. 2014).

### 1.2.1. Spruces as a Source of (E)-Resveratrol

The stilbene biosynthetic pathway in spruces (*Picea sp.*) leading to the production of resveratrol and other polyphenolic compounds has been a focus in some recently published studies (Hammerbacher et al. 2011, Kiselev et al. 2016). Their findings indicate that spruces are a source of (E)-resveratrol, and they inherently contain a large number of other closely related polyphenolic compounds as well. According to the plant physiological studies on the biosynthetic pathways, spruces such as Norway spruce (*Picea abies*), white spruce (*Picea glauca*), and Sitka spruce (*Picea sitchensis*) contain two major genes known as STS1 and STS2 that encode stilbene synthase (STS) enzyme for the biosynthesis of (E)-resveratrol, as shown in Figure 5.
Figure 5: Potential intermediates, products, and by-products of stilbene biosynthesis in spruce (Hammerbacher et al. 2011).

Further investigations into the biosynthetic pathway shown above reveal that the immediate precursors of resveratrol are p-coumaroyl CoA and malonyl CoA, and the stilbene compounds such as pinosylvin, resveratrol, piceatannol, and isorhapontigenin are synthesized by stilbene synthase mediated condensation between those two precursors. It also suggests that the stilbene compounds may react further to form their glycosides with sugars (Hammerbacher et al. 2011). However, the biosynthetic pathway produces the E-stilbene skeleton explicitly. Hence, the naturally occurring isomer of resveratrol in spruces is (E)-resveratrol, which is also the most bioactive form.
In spruces, (E)-resveratrol and other secondary metabolites locate primarily in the bark since they are required in the outer tissues of the plant for providing defense against pathogenic infections (Jyske et al. 2016). In northern American states like Maine, spruce bark is a waste material generated from wood processing and forest bioproducts industries. The 2016 Maine wood processor report indicates that the annual processing of spruces and fir in the state is 556,104 green tons, which accounts for a large quantity of bark produced as waste (Figure 6).

![Figure 6](Source: www.maine.gov/dacf/mfs/publications/annual_reports.html)

The bark is used mostly as a source of fuel for producing heat and energy. However, this demand for wood bark as a fuel is set to decline with the implementation of novel strategies aimed at the adoption of green energy alternatives to minimize the environmental pollution caused by conventional fuels.
Previous work of our group has shown that the bark of Norway spruce (Picea abies) and black spruce (Picea mariana), native to Maine, contain (E)-resveratrol in comparable amounts to Japanese knotweed root extracts (Figure 7). Hence they are potential commercial sources for the production of (E)-resveratrol.

Figure 7: (E)-Resveratrol in wood bark extracts (LeBlanc, 2010) in comparison to Japanese knotweed root reported by Burns et al. (2002).

Since wood bark is abundant and inexpensive, an extraction and purification process aimed at isolating (E)-resveratrol from it would find a rewarding use for the material and promote new industrial avenues in the state. However, as shown in Figure 8, published work on wood bark extractives suggest a high complexity and presence of a large number of closely related compounds that interfere with methods of isolating (E)-resveratrol.

Hence, development of a commercially viable method to extract and purify (E)-resveratrol from spruce bark in high yield is a challenging research problem.
Figure 8: Major stilbenes and flavonoids in spruce bark extracts (Pietarinen et al. 2006).
1.2.2. Isomerization of (E)-Resveratrol

As discussed in the previous section, stilbene biosynthetic pathways specifically synthesize E-stilbene compounds, hence the naturally available isomer of resveratrol in plants is (E)-resveratrol. However, the coexistence of E and Z isomers of resveratrol in plant extracts and wine has been reported in a number of research communications (Gambini et al. 2015). The Z-isomer is considered the bio-inactive form of resveratrol, and its presence is attributed to isomerization of (E)-resveratrol under various environmental conditions (Koga et al. 2016).

The work of Mallory et al. (1964) has demonstrated that photochemical reactions of stilbenes occur rapidly under radiation, leading to formation of dimers by [2+2] cycloaddition or phenanthrene rings. The resulting dihydrophenanthrenes from cyclizations relax back to their precursor, unless they are trapped by an oxidant such as iodine or oxygen (Figure 9).

![Photocyclization of stilbenes - the Mallory reaction. (Jørgensen, 2010)](image-url)
These research findings provided the first insights into the studies of E-Z isomerization of resveratrol. This phenomenon has been studied in the work of Figueiras et al. (2011), in which they suggested that the photochemical activation energy for the isomerization of $(E)$-resveratrol to its $Z$ isomer was as low as $3.7 \pm 0.3$ Kcal/mol, as opposed to the thermal activation energy of 67 Kcal/mol reported by Deak and Falk (2003). Other related studies have shown that the activation energy for the E-Z isomerization of stilbene has only a marginal change in different solvents (Courtney and Fleming, 1985). Hence, isomerization of resveratrol occurs readily upon exposure to UV radiation.

1.3. Potential Techniques and Approaches for the Purification Method Development

A number of techniques have been investigated for the isolation of $(E)$-resveratrol from complex plant extracts. Among them, some of the recent and relevant work are briefly described below.

1.3.1. Previous Work on Isolating $(E)$-Resveratrol from Spruce Bark

An HPLC-based method to isolate $(E)$-resveratrol from the bark extracts of black spruce has been proposed by García-Pérez et al. (2012), signifying the use of black spruce as a rich and alternative source of $(E)$-resveratrol to Japanese knotweed. Their method incorporated an initial hot water extraction of spruce bark, partitioning with ethyl acetate and separating the compound from the matrix by silica gel column chromatography followed by HPLC with a gradient solvent system. The low solubility of $(E)$-resveratrol in water largely affected the overall yield, and the final purified product of $(E)$-resveratrol obtained after tedious column chromatography and HPLC-based separations contained an impurity, mearnsetin.
The work of Regan LeBlanc of our group has shown that multiple column chromatographic separations of ethyl acetate extract of black spruce bark resulted in a column fraction containing mostly \((E)\)-resveratrol and catechin (Figure 10). This was a promising approach for isolating \((E)\)-resveratrol, since the removal of catechin might be achieved by borate complex extraction (described in the next section). However, poor yield due to a large number of chromatographic steps posed a challenge for the scaling-up of this process.

Figure 10: GC-MS chromatogram of fraction 65 from silica gel column chromatography of black spruce bark (LeBlanc, 2010).

1.3.2. Borate Complex Extraction

Borate complex extraction was proposed by Tsuchiya et al. (1998) as a method to extract vicinal diols from complex mixtures selectively. Vicinal diols react with Lewis acids such as diphenylborate-ethanolamine to form anionic complexes which can be made soluble in non-polar organic solvents by pairing up with organic cations, as shown in Figure 11. Since \((E)\)-resveratrol is not a vicinal diol, this approach might be useful for the
passive purification of plant extracts containing vicinal diols such as catechin and piceatannol, that are mixed with \((E)\)-resveratrol.

As discussed previously, spruce bark extracts contain many polyphenolic compounds that do not belong to the category of vicinal diols, and hence are unresponsive towards borate complex extraction. Therefore, this extraction technique alone is not sufficient for the purification of \((E)\)-resveratrol from complex mixtures.

Figure 11: Schematic diagram of borate complex extraction of vicinal diols (Tsuchiya et al. 1998).

1.3.3. Molecularly Imprinted Polymer Based Purification

Molecularly Imprinted Polymer (MIP) based extraction is a relatively new concept attempted on the Japanese knotweed rootstocks, with the focus on selectively isolating \((E)\)-resveratrol and emodin. The technique relies on the synthesis of a cross-linked polymer with pores complimentary to the shape and size of an analyte of interest. During
the imprinting process, the functional monomers are first arranged around a template that represents the analyte, and then polymerized and locked in their 3D structure by cross-linking. When the template is removed, the porous polymer exposes multiple binding sites that confer high selectivity and affinity for the analyte of interest. These polymers are useful for the selective extraction of compounds from complex mixtures (Ma et al. 2007).

In the study reported, the polymer was synthesized using 4-vinylpyridine as the functional monomer to imprint on \((E)\)-resveratrol, isobutyronitrile as the initiator, and ethylene glycol dimethacrylate as the cross-linker in the presence of a porogenic solvent. Once the synthesis was complete, the polymer contained binding sites complimentary to the size and shape of \((E)\)-resveratrol. It was then used as the column packing material in HPLC, and the plant extract was passed through the column, allowing the active sites of the imprinted polymer to trap the compound. The trapped \((E)\)-resveratrol was desorbed into another solvent in order to obtain a pure sample (Ma et al. 2008). HPLC analysis of the purified sample revealed that high purity and high yield was achieved (Figure 12). Hence the researchers claimed that this approach is highly effective for isolating compounds from complex mixtures, despite its dependence on HPLC for the operation.
Figure 12: Chromatograms of Japanese knotweed extract (A), resveratrol fraction (B), and emodin fraction (C) from MIP extraction (Ma et al. 2008). Peaks a: (E)-resveratrol, b: emodin.

1.3.4. Column Chromatography with Specialized Column Materials

Specialized column materials such as Sephadex LH-20 have been widely used in recent natural products purification studies (Sun et al. 2015). Sephadex LH-20 possesses a vast number of hydroxyl groups in its polymer structure that allows a high degree of interaction with the compounds in complex mixtures (Figure 13).
The work of Güder et al. (2014) has demonstrated that (E)-resveratrol could be isolated in pure form from grape extracts by Sephadex LH-20 column chromatography. The suggested purification method involves an initial silica gel column chromatographic step followed by Sephadex LH-20 column chromatography to obtain a pure fraction of (E)-resveratrol. Despite the need to collect a large number of column fractions prior to the elution of the target compound, this approach yields (E)-resveratrol in high purity and high yield from the crude grape extracts.

Many published works on extraction and purification method development have used Japanese knotweed as the raw material since it is a widely available invasive plant that thrives in almost any terrestrial environment (Murrell et al. 2011). As discussed previously, spruces inherently contain a vast diversity in their extractives profile, while
those extractives share a high degree of similarity in certain intrinsic properties such as solubility. Hence, the applicability of the techniques and methods described in literature, for the isolation of (E)-resveratrol from spruce bark is yet to be explored. Therefore, the ultimate goal of this project was to establish a commercially viable and environmentally friendly method to accomplish isolation of (E)-resveratrol in high purity and high yield from spruce bark.
CHAPTER 2
MATERIALS AND EXPERIMENTAL METHODS

2.1. Materials

2.1.1. Spruce Bark Samples

A healthy, 90-year-old black spruce (*Picea mariana*) tree was identified in the University forest and harvested on September 10, 2013 by the University of Maine research forest management group. The branches were removed and the trunk was cut into 4-foot logs, which were then debarked by hand. The bark samples were air dried for 72 hours and ground into a powder using a Wiley mill (Thomas Scientific, Swedesboro, NJ). Powdered bark samples were stored in a freezer at -23 °C until further use. Similarly processed bark from a healthy, 12-year-old Norway spruce (*Picea abies*) tree, was obtained from the same source on July 20, 2010.

2.1.2. Chemicals and Supplies

HPLC grade n-butanol, chloroform, dichloromethane, diethyl ether, ethyl acetate, hexanes, methanol, toluene, and ACS grade glacial acetic acid and hydrochloric acid were purchased from Fisher Scientific (Waltham, MA). Acetic anhydride (≥99%), diphenylborate-ethanolamine (≥98%), formic acid (≥95%), sodium bicarbonate (≥99.7%), sodium hydroxide (≥97%), 4-phenylphenol (≥97%), pyridine, and 1.0 M tetra-n-butylammonium fluoride in THF were purchased from Sigma-Aldrich (St. Louis, MO), and (+)-catechin (≥97%) was purchased from TCI (Portland, OR). The (E)-resveratrol standard was purchased from Mega Resveratrol (Danbury, CT). A Milli-Q water purification system (EMD Millipore, Billerica, MA) was used to obtain ultra-pure water.
Ceramic 30 mm (I.D.) x 95 mm (L) Soxhlet thimbles were obtained from the Chemical Engineering Department of the University of Maine. Disposable Pyrex 5 μL micropipettes and glass backed thin layer chromatography plates of 20 cm (L) x 20 mm (W) containing 60 Å silica gel coating of 250 μm layer thickness and a fluorescent indicator were purchased from Fisher Scientific (Waltham, MA). Silica gel (60 Å, 40μm - 63μm particle size, 230-400 mesh ASTM), Dowex 2X8, and Amberlite IRA-400 Cl column materials were purchased from Sigma-Aldrich (St. Louis, MO). Sephadex LH-20 column material was purchased from GE Healthcare Bio-Sciences (Pittsburgh, PA), and Cosmosil 75C18-OPN reversed phase column material was purchased from Nacalai Inc. (San Diego, CA).

2.2. Extraction of Spruce Bark

A Soxhlet extraction apparatus equipped with a ceramic thimble was used for the extraction of black spruce bark. A 12.5 g sample was extracted with 150 mL of methanol in the Soxhlet apparatus for 12 hours (overnight). The same procedure was followed for the extraction of Norway spruce bark. All crude methanol extracts were stored in the refrigerator until further use.

2.3. Purification Method Development

2.3.1. Liquid-Liquid Extractions

Crude extracts of Norway spruce bark and black spruce bark resulting from the Soxhlet extraction process were used in the experiments described below.

2.3.1.1. Solvent Extraction. A 150.0 mL sample of the crude extract was concentrated six-fold by rotary evaporation to near dryness, and re-dissolution in methanol. The final
volume of the sample was set to 25.0 mL, and it was partitioned between 5 mL of deionized water and 35 mL of hexanes in a separatory funnel. The aqueous methanol layer was recovered and further extracted with two 35.0 mL portions of hexanes. All three hexane fractions were combined, and rotary evaporated to an estimated volume of 35 mL.

The aqueous methanol layer was then rotary evaporated to an estimated volume of 10 mL and partitioned between 25 mL of deionized water and 25 mL of ethyl acetate in a separatory funnel. The ethyl acetate layer was recovered, and the aqueous layer was further extracted with two 25 mL portions of ethyl acetate. All ethyl acetate fractions were combined, rotary evaporated to dryness, and re-dissolved in 4.0 mL of methanol.

The crude extract, hexane layer, methanol layer, and the remaining aqueous layer were analyzed by TLC against (E)-resveratrol and (+)-catechin, employing a variety of mobile phases that are described in section 2.4.1. The aqueous layer was then acidified with eight drops of concentrated HCl, stirred for 5 minutes and extracted with three 10.0 mL portions of ethyl acetate to recover (E)-resveratrol from its bound forms to sugars (resvaterol-3-β-mono-D-glucoside, commonly known as polydatin). The ethyl acetate fractions were combined, and rotary evaporated to an estimated volume of 4 mL. This sample was analyzed by TLC with a mobile phase consisting of chloroform, ethyl acetate and formic acid in 25:10:1 ratio, for the presence of (E)-resveratrol, and (+)-catechin. Figure 14 illustrates the layers resulting from the above liquid-liquid extraction procedure.
2.3.1.2. Aqueous Sodium Bicarbonate Extraction. The crude extract of black spruce bark (150 mL) was rotary evaporated to dryness and re-dissolved in 100 mL of diethyl ether by sonication for 20 minutes. It was then extracted with five 35.0 mL portions of 5% (w/v) aqueous NaHCO₃ solution. The diethyl ether layer was recovered, rotary evaporated to dryness and re-dissolved in 35.0 mL of methanol. Aliquots of this sample
were analyzed by high-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV) at 306 nm, as described in section 2.4.2.

2.3.2. Silica Gel Column Chromatography

Ethyl acetate fraction 1 of Norway spruce bark crude extract and black spruce bark crude extract were used in this series of experiments. They were prepared from the bark extracts following the procedure described in the previous section, with a two-fold scale up. The following trials were performed to evaluate the ability of each chromatographic system to provide a separation of compounds leading to pure (E)-resveratrol.

2.3.2.1. Column Chromatography with Chloroform and Methanol. Silica gel was packed in a 1 cm diameter glass column to a 15 cm height bed with a solvent mixture consisting of chloroform and methanol in 5:1 ratio. The Norway spruce bark sample (ethyl acetate fraction 1 re-dissolved in 4.0 mL of methanol) was loaded onto the column and eluted with a mobile phase consisting of chloroform and methanol in 5:1 ratio. Ten 4 mL fractions were collected. All fractions were analyzed by TLC for the presence of (E)-resveratrol and (+)-catechin, following the procedure described in section 2.4.1.

2.3.2.2. Column Chromatography with Chloroform, Ethyl acetate and Formic acid. Another separation was attempted on a silica gel bed of 16 cm height packed in a glass column of 1 cm internal diameter, with a solvent mixture consisting of chloroform, ethyl acetate and formic acid in 25:10:1 ratio. The Norway spruce bark sample (ethyl acetate fraction 1) was re-dissolved in 4.0 mL of the chloroform-ethyl acetate-formic acid solvent mixture prior loading onto the column, and eluted with a mobile phase which has the same composition (chloroform, ethyl acetate and formic acid in 25:10:1 ratio). A total
of 20 fractions, each 4 mL, were collected and analyzed by TLC following the procedure described in section 2.4.1. Fractions 3 to 20 were combined, and rotary evaporated to an estimated volume of 5 mL, which was again chromatographed on a silica gel column of 1 cm internal diameter and 18 cm height, with a mobile phase of the aforementioned composition. A total of twenty-five fractions, each 4 mL, were collected and analyzed by TLC, and HPLC-UV at 306 nm. This procedure was repeated with the ethyl acetate fraction 1 of black spruce bark sample as well.

2.3.2.3. Column Chromatography with Multiple Mobile Phases. The chromatographic separation was performed on a silica gel bed of 11 cm height packed in a glass column of 1 cm internal diameter, with an initial solvent system consisting of toluene and methanol in 9:1 ratio. The Norway spruce bark sample (ethyl acetate fraction 1 re-dissolved in 4 mL of methanol) was loaded onto the column and eluted with a mobile phase consisting of toluene and methanol in 9:1 ratio. Eight fractions of 4 mL each were collected. Then the mobile phase was changed to chloroform, ethyl acetate and formic acid in 25:10:1 ratio to collect 24 more fractions each 4 mL. All fractions were analyzed by TLC following the procedure described in section 2.4.1. Fractions collected with chloroform, ethyl acetate and formic acid solvent mixture were then combined, rotary evaporated to an estimated volume of 4 mL and analyzed again by TLC to estimate the purity of (E)-resveratrol eluted from the column.

2.3.2.4. Flash Chromatography. A flash chromatographic separation of black spruce bark extract (bicarbonate-extracted, as described in section 2.3.1.2.) was conducted on a silica gel bed of 14 cm height packed with dichloromethane in a glass column of 1 cm internal diameter. A 5.0 mL aliquot of the sample was rotary evaporated to an estimated
volume of 2 mL with 0.5 g silica gel in it and was flushed with air until complete dryness, prior to dry loading onto the column. The first fraction was collected with 100 mL of dichloromethane, which was followed by three 50 mL fractions resulting from the elution of the sample with 10% (v/v) methanol in dichloromethane. All fractions were rotary evaporated to dryness, and the final volume of each was set to 5.0 mL with methanol before they were analyzed by HPLC-UV at 306 nm.

2.3.3. Borate Complex Extraction

2.3.3.1. Pilot Experiments with (+)-Catechin Standard. An aqueous solution of 1.0 mg/mL (+)-catechin was prepared in 0.01 N HCl. A 10 mM solution of tetra-n-butylammonium fluoride (TBAF) in a solvent mixture of heptanol and hexane in 1:4 ratio was prepared using a standard solution of 1.0 M TBAF in tetrahydrofuran. Aqueous solutions of 0.5 M KH₂PO₄ and Na₂HPO₄ were prepared to formulate a buffer of pH 8.21 by mixing the two solutions in a volume ratio determined by the following equation. This buffer was then used as the medium to prepare a 0.15 (w/v) solution of diphenylborate-ethanolamine.

\[
\begin{align*}
\text{H}_2\text{PO}_4^{-1}(\text{aq}) & \rightleftharpoons \text{H}^+ (\text{aq}) + \text{HPO}_4^{2-} (\text{aq}) \\
K_a &= \frac{[\text{HPO}_4^{2-}(\text{aq})]}{[\text{H}_2\text{PO}_4^{-1}(\text{aq})]} + [\text{H}^+ (\text{aq})] \\
pK_a &= -\log \frac{[\text{HPO}_4^{2-}(\text{aq})]}{[\text{H}_2\text{PO}_4^{-1}(\text{aq})]} + \text{pH} \\
pH &= pK_a + \log \frac{[\text{HPO}_4^{2-}(\text{aq})]}{[\text{H}_2\text{PO}_4^{-1}(\text{aq})]}
\end{align*}
\]
For a buffer of pH = 8.21,

\[
8.21 = 7.21 + \log \frac{[\text{HPO}_4^{2-}(aq)]}{[\text{H}_2\text{PO}_4^{1-}(aq)]}
\]

\[
\log \frac{[\text{HPO}_4^{2-}(aq)]}{[\text{H}_2\text{PO}_4^{1-}(aq)]} = 1.0
\]

Hence, \( \frac{[\text{HPO}_4^{2-}(aq)]}{[\text{H}_2\text{PO}_4^{1-}(aq)]} = 10 \)

An aliquot of 0.5 mL from the aqueous (+)-catechin standard was mixed with 1.5 mL of 0.15 % (w/v) diphenylborate-buffer solution and vortexed until a color change occurred from pale yellow to pale orange. The entire mixture was then mixed with 4.0 mL of TBAF solution and vortexed for another 3 minutes. It was kept undisturbed for a few minutes until the layer separation was complete. The bottom aqueous layer was recovered and analyzed by HPLC-UV at 280 nm for the presence of (+)-catechin.

This procedure was repeated with all reagents (except TBAF solution) prepared in 5% (v/v) ethanol in water solutions, and 5% (v/v) methanol in water solutions, in order to evaluate the success of the reaction in an aqueous-alcoholic medium. The poor solubility of buffers (and spruce bark extracts) in 5% alcoholic solutions led to a few changes in the procedure which include the preparation of all reagents in methanol (except tetra-n-butylammonium fluoride solution), and the discontinuation of the attempts to control the pH. A complete removal of (+)-catechin from the aqueous-methanol layer was evident from the experiments performed with 1.5 mL of 0.4% (w/v) diphenylborate-ethanolamine in methanol, 0.5 mL of 10 mg/mL (+)-catechin, 4 mL of 20 mM TBAF solution, and a few drops (4-8) of pH 8.21 buffer to facilitate the layer separation.
2.3.3.2. **Experiments with Black Spruce Bark Extracts.** Fractions of black spruce bark extracts resulting from the silica gel column chromatographic purification (described in section 2.3.2.2.) were used in further separation trials. Initial trials were conducted with 0.5 mL aliquots of the 3rd fraction from the silica gel column, which contains the highest amount of (E)-resveratrol. The sample size was increased in the subsequent trials by combining the first 14 fractions (4 mL each). The combined fractions were rotary evaporated, and re-dissolved in methanol to a final volume of 10.0 mL. An aliquot of 0.5 mL from this concentrated sample was mixed with 8.0 mL of 0.8 % (w/v) diphenylborate-ethanolamine in methanol and was vortexed until a color change occurred from pale yellow to pale orange. The entire mixture was then mixed with 8.0 mL of 35 mM tetra-n-butylammonium fluoride solution and vortexed for 3 minutes. It was added to 8.0 mL of hexane and a few drops (4-8) of pH 8.21 buffer to facilitate the layer separation. After vortexing for an additional 1 minute, the reaction mixture was left undisturbed until the layer separation was complete. The bottom aqueous-methanol layer was recovered, volume was measured, and analyzed in HPLC-UV at 306 nm against a sample of unreacted black spruce bark extract at the same dilution.

2.3.4. **Ion Exchange Chromatography**

Dowex 2X8 and Amberlite IRA-400 Cl ion exchange resins were used in this series of experiments. Each resin was activated by mixing it gently in an aqueous solution of 6 M HCl overnight. It was then packed in a glass column of 1 cm diameter to make a bed of 18 cm height. The column was flushed successively with aqueous solutions of 6 M HCl, 2 M CH₃COOH, and 2 M CH₃COOK prior to the loading of the sample. The 3rd fraction of black spruce bark extract resulting from the silica gel column chromatographic
puriﬁcation (described in section 2.3.2.2.) was used in the initial trials. Further trials were conducted with samples obtained by re-dissolving the initial black spruce bark extract in 100 mL of diethyl ether, and also with the samples resulting from borate complex extraction.

To prepare for loading on the anion exchange column, a 10.0 mL aliquot of the sample was rotary evaporated, and dissolved in 3.0 mL solution of 8 M NaOH and methanol in 1:19 ratio. The sample was loaded, and 50 mL of pH 11.9 NaOH solution was initially passed through the column. The effluent from the column loading solution was neutralized with 0.5 M HCl and extracted with 10 mL of diethyl ether. The diethyl ether layer was analyzed by HPLC-UV at 306 nm to conﬁrm no (E)-resveratrol has escaped the column. The sample was eluted with mobile phases of various compositions as shown in Table 1. All fractions collected were rotary evaporated to an estimated volume of 3 mL, and the total volume of each was set to 5 mL with methanol prior to analysis by HPLC-UV at 306 nm.

Table 1. Sample elution information for ion exchange chromatography.

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Volume</th>
<th>Mobile Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 mL</td>
<td>Methanol and 2M aqueous CH₃COOH in 3:1 ratio</td>
</tr>
<tr>
<td>2-5</td>
<td>50 mL</td>
<td>0.5 M CH₃COOH in methanol</td>
</tr>
<tr>
<td>6</td>
<td>100 mL</td>
<td>0.75 M CH₃COOH in methanol</td>
</tr>
<tr>
<td>7</td>
<td>100 mL</td>
<td>1 M CH₃COOH in methanol</td>
</tr>
<tr>
<td>8</td>
<td>100 mL</td>
<td>1 M CH₃COOH in 98% (v/v) methanol</td>
</tr>
<tr>
<td>9</td>
<td>50 mL</td>
<td>0.25 M HCl in 98% (v/v) methanol</td>
</tr>
<tr>
<td>10-12</td>
<td>100 mL</td>
<td>0.5 M HCl in 98% (v/v) methanol</td>
</tr>
</tbody>
</table>
Subsequent trials were conducted with 25% (v/v) CH$_3$COOH (3.5 M total concentration) in methanol as the mobile phase, and 5% to 15% (v/v) HCOOH in methanol as the mobile phase. In each trial, 11 fractions each 50 mL were collected. The chromatographic separation was performed under gravity, and also under pressurized air (flash chromatography) to compare the yield and purity of the resulting fractions containing (E)-resveratrol.

This procedure was repeated with a 0.1 mg/mL solution of (E)-resveratrol standard which was prepared in a solvent consisting of 8 M NaOH and methanol in 1:19 ratio.

2.3.5. Column Chromatography with Sephadex LH-20

This set of experiments was initially performed for the black spruce bark extract samples resulting from the silica gel flash column (section 2.3.2.4.). Sephadex LH-20 column material was mixed with dichloromethane and kept undisturbed overnight to allow the swelling of the beads to complete. It was then packed with dichloromethane in a glass column of 1 cm internal diameter, to make a bed of 14 cm height. A glass wool plug was used to prevent the column material from floating in the solvent. Fractions containing (E)-resveratrol from the silica gel flash chromatography of 10.0 mL of black spruce bark extract with methanol and dichloromethane, were combined and dried with 0.35 g of Sephadex LH-20 column material to prepare it for dry loading onto the column. Once loaded, another glass wool plug was applied to prevent the material from floating in the solvent. The sample was first eluted with dichloromethane to collect a fraction of 150 mL. Then the mobile phase was changed to a mixture of methanol and dichloromethane in 1:9 ratio to collect 12 fractions each amounting to 25 mL in volume. All fractions were
rotary evaporated to an estimated volume of 5 mL, and the total volume of each was set to 10 mL with methanol for the analysis by HPLC-UV and HPLC-Ion trap MS.

This procedure was then adopted in the chromatography of direct samples of black spruce bark extract resulting from the NaHCO₃ wash (section 2.3.1.). The flow rate of the mobile phase was maintained approximately at 70 µL per minute, and the fractions were collected by gravity flow until (E)-resveratrol was completely eluted.

2.3.6. Acetylation-Deacetylation Reactions

2.3.6.1. Acetylation of Black Spruce Bark Extracts. Derivatization by acetylation was conducted on samples resulting from,

(a). Solvent extraction (ethyl acetate layer 1)

(b). Borate complex extraction

(c). Ion exchange chromatography (samples and standard)

(d). Column chromatography with Sephadex LH-20

The published work of Lugemwa et al. (2013) has suggested a procedure which uses acetic anhydride and NaHCO₃ in ethyl acetate to acetylate phenols with >93% yield in 24-hours. To shorten this reaction time, acetylation of spruce bark samples was conducted at an elevated temperature, in the presence of acetic anhydride, NaHCO₃, and pyridine (Figure 15). These reagents were used in an estimated excess, since the full composition of the spruce bark extracts was unknown.
Figure 15: Acetylation of (E)-resveratrol.

Black spruce bark extract samples resulting from (b), (c) and (d) were rotary evaporated to dryness and re-dissolved in 20.0 mL ethyl acetate before the reaction. Samples resulting from (a) were directly used in the reaction. The 20.0 mL sample was mixed with 2.5 mL acetic anhydride, 0.5 g sodium bicarbonate and ten drops of pyridine, and was stirred at 220 rpm at 75 °C overnight. At the end of the reaction period, the sample was partitioned between 10 mL ethyl acetate and 20 mL water in a separatory funnel. The top ethyl acetate layer was recovered for the column chromatographic purification.

The above acetylation procedure was repeated with a 20 mL solution of 75 µg/mL (E)-resveratrol standard prepared in ethyl acetate.

**2.3.6.2. Column Chromatographic Purification.** The ethyl acetate layer resulting from the previous step was rotary evaporated to an estimated volume of 3 mL. It was then transferred onto a silica gel column of 10 cm height and 1 cm diameter, packed in a mobile phase consisting of chloroform and ethyl acetate in 7:3 ratio. A 30 mL fraction was collected, and rotary evaporated to an estimated volume of 2 mL prior to the loading onto a silica gel column of 11 cm height and 1 cm diameter, prepared in a mobile phase consisting of chloroform and dichloromethane in 1:1 ratio. Three fractions, each 25 mL were collected, rotary evaporated to an estimated volume of 5 mL and analyzed by HPLC-UV at 306 nm and GCMS.
In subsequent trials, the ethyl acetate layer resulting from the acetylation step was dry loaded directly onto a silica gel column of 11 cm height and 1 cm diameter prepared in a mobile phase consisting of chloroform and dichloromethane in 1:1 ratio. Three fractions of 25 mL each were collected for the analysis by HPLC-UV and GCMS.

2.3.6.3. Deacetylation. Base-catalyzed hydrolysis of ester bonds was initially attempted on acetylated samples. A 5 mL sample from the previous step was rotary evaporated to dryness and dissolved in 10 mL of 80% aqueous ethanol. It was added to 200 mg of NaOH and refluxed for an hour. The reaction mixture was then acidified with 0.1 M HCl, and was partitioned between 10 mL of diethyl ether and 10 mL of water. The ethyl acetate layer was recovered, rotary evaporated to dryness, and re-dissolved in methanol to bring the final volume to 5 mL prior to analysis by HPLC-UV and HPLC-Ion trap MS.

Acid-catalyzed hydrolysis with HCl was employed to replace the above procedure in subsequent experiments (Figure 16). A 5.0 mL sample from the previous step was rotary evaporated to dryness and dissolved in 5.0 mL of 80% (v/v) ethanol. It was added to 5.0 mL of 0.1 M HCl prepared in 80% (v/v) ethanol, and refluxed for 2 hours. The resultant mixture was rotary evaporated to an estimated volume of 5 mL and partitioned between 15 mL diethyl ether and 15 mL water in a separatory funnel. The diethyl ether layer was recovered, rotary evaporated to dryness, and re-dissolved in methanol, and the final volume was set to 5.0 mL. It was then analyzed by HPLC-UV and HPLC-Ion trap MS.
Figure 16: Deacetylation of (E)-resveratrol.

2.3.7. Reversed-Phase Column Chromatography with C18 Coated Silica

Reversed-phase column chromatography with C-18 coated silica was attempted as an alternative to acetylation-deacetylation reactions based purification. The objective was to develop a column chromatographic system that would provide a separation similar to that of HPLC columns used in the qualitative and quantitative analysis of samples.

The experiments was aimed at final purification of black spruce bark extract samples resulting from the Sephadex LH-20 column chromatography (section 2.3.5). Fractions collected from the Sephadex LH-20 column chromatography of 10.0 mL of the black spruce bark extract (in methanol, after NaHCO$_3$ wash) that contain (E)-resveratrol were combined, rotary evaporated, and the final volume was set to 10.0 mL with methanol, prior using them in this chromatographic separation. An 11 cm height and 1 cm diameter Cosmosil 75C18-OPN reversed phase column was prepared in acetonitrile and water 1:3 mixture. A 2.0 mL aliquot of the sample was dry-loaded onto the column and eluted with the same solvent to collect 10 fractions of 20 mL each. Each fraction was partitioned between 10 mL of diethyl ether and 10 mL of water in a separatory funnel, and the diethyl ether layer was recovered and rotary evaporated to an estimated volume of 2 mL before the HPLC-UV and HPLC-Ion trap MS analysis. This procedure was repeated with a mobile phase consisting of dichloromethane and methanol in 9:1 volume ratio. Since
water was not incorporated in this mobile phase, partitioning each fraction between diethyl ether and water was not required. Four fractions each 20 mL were collected, rotary evaporated to an estimated volume of 2 mL and analyzed by HPLC-UV and HPLC-Ion trap MS.

Separation of impurities from (E)-resveratrol in the samples was evident on a column prepared with dichloromethane. The sample was dry-loaded, and the first 20 mL fraction was collected with dichloromethane. Three more fractions of 20 mL each were collected with a solvent mixture consisting of dichloromethane and ethyl acetate in 9:1 volume ratio. All fractions were rotary evaporated to an estimated volume of 2 mL prior to the HPLC-UV and HPLC-Ion trap MS analysis.

2.3.8. Method Optimization and Further Improvements

All purification steps including liquid-liquid extractions, borate complex extraction, and chromatographic separations were evaluated employing the analytical techniques described in the next section, for their ability to minimize the loss and maximize the purity. With the aim of minimizing the number of steps in the purification procedure, Soxhlet extraction solvent was changed from methanol to diethyl ether. Bark samples were subjected to an initial extraction with a 125 mL volume of diethyl ether for 12 hours followed by an additional 12-hour extraction with a fresh 125 mL volume of the same solvent. Borate complex extraction, ion exchange chromatography, flash chromatography on silica gel, and acetylation-deacetylation reactions were removed from the final purification procedure. Glass columns used for the reversed phase chromatographic separation were silanized with dichlorodimethylsilane to deactivate the active sites on the glass surface (see Appendix C for the silanization procedure).
2.4. Analytical Methods and Techniques

2.4.1. Thin Layer Chromatographic Analysis

All samples and column fractions collected from the procedures described in section 2.3.2. were analyzed by TLC against (E)-resveratrol, and (+)-catechin standards prepared in 10 mg/mL concentration in methanol. Fractions collected from the silica gel columns eluted with toluene-methanol mobile phase and subsequently with chloroform-ethyl acetate-formic acid mobile phase (section 2.3.2.3.) were analyzed against 5 mg/mL piceatannol standard prepared in methanol as well.

A few mobile phases consisting of n-butanol, pyridine, ultra-pure water and glacial acetic acid in 3:2:2:0.6 ratio, toluene and methanol in 9:1 ratio, chloroform, ethyl acetate and formic acid in 5:4:1 ratio, and chloroform, ethyl acetate and formic acid in 25:10:1 ratio were initially tested, and the latter was employed to continue with the TLC analysis. Samples were spotted on a baseline marked 2 cm above the bottom of the TLC plate. The end-line was marked 3 cm below the top of the plate. The mobile phase was added to the developing chambers, which were then kept closed for approximately 30 minutes prior to the insertion of the TLC plates spotted with the samples. Once developed and dried, the spots were observed under 254 nm UV light, and the $R_f$ values were determined.

2.4.2. HPLC-UV Analysis

Crude extracts of Norway spruce bark and black spruce bark, and samples resulting from the procedures described in sections 2.3.2. to 2.3.7. were analyzed by HPLC-UV. An Agilent 1100 series HPLC system with an automatic liquid sampler and a variable wavelength (VW) detector (Agilent, Waldbronn, Germany) was used for the analyses. The system was equipped with a Phenomenex Kinetex 5μm C18 4.6 x 100 mm column
(Phenomenex, Torrance, CA). Ultra-pure water and acetonitrile were employed as the chromatographic solvents. Table 2 summarizes the HPLC conditions used for the qualitative analysis of samples resulting from the procedure described in section 2.3.2.2.

Table 2. HPLC conditions for silica gel column chromatographic sample analysis.

<table>
<thead>
<tr>
<th>Detector Wavelength</th>
<th>306 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Volume</td>
<td>8 μL</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>2 mL/min</td>
</tr>
<tr>
<td>Total Run Time</td>
<td>9 mins</td>
</tr>
<tr>
<td>Gradient Profile</td>
<td></td>
</tr>
<tr>
<td>Time (min.)</td>
<td>% H₂O</td>
</tr>
<tr>
<td>0.0</td>
<td>90</td>
</tr>
<tr>
<td>6.0</td>
<td>40</td>
</tr>
<tr>
<td>8.0</td>
<td>90</td>
</tr>
</tbody>
</table>

A modified gradient profile from the above that provides improved separation of peaks was employed for the analysis of samples resulting from ion exchange chromatography, and borate complex extractions (Table 3).

Table 3. HPLC conditions for ion exchange chromatographic sample analysis.

<table>
<thead>
<tr>
<th>Detector Wavelength</th>
<th>306 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Volume</td>
<td>8 μL</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>2 mL/min</td>
</tr>
<tr>
<td>Total Run Time</td>
<td>11.8 mins</td>
</tr>
<tr>
<td>Gradient Profile</td>
<td></td>
</tr>
<tr>
<td>Time (min.)</td>
<td>% H₂O</td>
</tr>
<tr>
<td>0.0</td>
<td>90</td>
</tr>
<tr>
<td>9.5</td>
<td>60</td>
</tr>
<tr>
<td>9.7</td>
<td>60</td>
</tr>
<tr>
<td>10.5</td>
<td>90</td>
</tr>
</tbody>
</table>
The progress of borate complex extractions conducted with (+)-catechin (as described in section 2.3.3.1.) was evaluated employing the HPLC conditions shown in Table 4.

Table 4. HPLC conditions for borate complex extractions with (+)-catechin.

<table>
<thead>
<tr>
<th>Detector Wavelength</th>
<th>280 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Volume</td>
<td>8 μL</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>2 mL/min</td>
</tr>
<tr>
<td>Total Run Time</td>
<td>8 mins</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gradient Profile</th>
<th>Time (min.)</th>
<th>% H₂O</th>
<th>% CH₃CN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>7.6</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Samples resulting from the acetylation reactions described in section 2.3.6.1 were analyzed for the acetylated form of (E)-resveratrol, with the HPLC conditions given in Table 5.

Table 5. HPLC conditions for acetylated sample analysis.

<table>
<thead>
<tr>
<th>Detector Wavelength</th>
<th>306 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Volume</td>
<td>8 μL</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1.5 mL/min</td>
</tr>
<tr>
<td>Total Run Time</td>
<td>11 mins</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gradient Profile</th>
<th>Time (min.)</th>
<th>% H₂O</th>
<th>% CH₃CN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

The HPLC conditions were modified to improve the separation of peaks further, for the qualitative analysis of samples resulting from flash chromatography, Sephadex LH-20
chromatography, reversed phase column chromatography, and deacetylation reactions.

Table 6 provides a summary of those conditions.

Table 6. Improved HPLC conditions for qualitative analysis of samples.

<table>
<thead>
<tr>
<th>Detector Wavelength</th>
<th>306 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Volume</td>
<td>8 μL</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1.5 mL/min</td>
</tr>
<tr>
<td>Total Run Time</td>
<td>16.5 mins</td>
</tr>
<tr>
<td>Gradient Profile</td>
<td>Time (min.)</td>
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<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>15.5</td>
</tr>
</tbody>
</table>

For the quantitative analysis, vanillin, trans-stilbene, and 4-phenylphenol were evaluated for their stability and ease of use as external standards. The calibration curve (Figure 17) generated with (E)-resveratrol standard and 4-phenylphenol as the external standard, was used in the quantifications.

Figure 17: Calibration curve for the quantitative analysis of bark samples.
All samples were analyzed at 306 nm for \((E)\)-resveratrol and 260 nm for 4-phenylphenol. The mobile phase consisted of methanol: formic acid: water in 10:1:89 volume ratio (solvent A), and acetonitrile (solvent B). A gradient profile modified from Marshall et al. (2012) was employed, and is summarized in Table 7. All determinations were performed in triplicate and expressed in \(\mu g\) per g dry bark units.

Table 7. HPLC conditions for quantitative analysis of samples.

<table>
<thead>
<tr>
<th>Injection Volume</th>
<th>8 (\mu L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Rate</td>
<td>0.3 mL/min</td>
</tr>
<tr>
<td>Total Run Time</td>
<td>40 mins</td>
</tr>
<tr>
<td>Gradient Profile</td>
<td>Time (min.)</td>
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<td></td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>23.0</td>
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<td></td>
<td>38.0</td>
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<td>Detector Wavelength Setup</td>
<td>Time (min.)</td>
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<tr>
<td></td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td>37.0</td>
</tr>
</tbody>
</table>

2.4.3. HPLC Ion-Trap MS Analysis

Further confirmation of the identity and purity of \((E)\)-resveratrol in samples was performed using an Agilent 1100 series HPLC system equipped with a diode array detector (DAD) coupled to an Agilent 1100 series ion trap mass spectrometer with an electrospray ionization (ESI) source (Waldbonn, Germany). Chromatographic separation was performed employing the same column and gradient profile (given in Table 7) as described in the previous section. Total ion chromatogram and mass spectrum of the sample were obtained and compared with the \((E)\)-resveratrol standard. The ion-trap MS was operated in negative ion mode at 350 \(^\circ\)C drying gas temperature, 8.97 L/min drying
gas flow, 49.9 psi nebulizer pressure, 3500 V capillary voltage and 7.0 kV dynode voltage at 44.2 trap drive level. The target mass of the ion trap was set to 227 m/z, and the detection range was 50-500 m/z. Further confirmation was accomplished by comparison of the mass fragments of the final purified sample with that of (E)-resveratrol standard.

2.4.4. GCMS Analysis

GCMS analysis was performed on an Agilent 6850 network GC system with an automatic injector coupled to an Agilent 5975B VL mass spectrometric detector (Waldbronn, Germany). Samples resulting from the acetylation reactions (described in section 2.3.6.1.) were used in the analyses. The sample volume was 1 µL, and the injection mode was splitless. The inlet temperature was maintained at 290 °C, and the column gas flow was set to 1.1 mL/min at 10.55 psi inlet pressure. The instrument was equipped with a Zebron ZB5-MS capillary column of 0.25 µm film thickness, 250 µm diameter and 30 m length (Phenomenex, Torrance, CA). The oven temperature profile was set to start at 80 °C with a hold time of 0.5 mins, and to ramp up at 15 °C/min rate until the final temperature of 300 °C was reached. The total run time was 25.17 minutes. The mass spectrometric detector was set to a mass detection range of 50-550 m/z and a solvent delay of 3 minutes. The electron multiplier (EM) voltage was 1188 V, while the MS source and quadrupole temperatures were maintained at 230 °C and 150 °C respectively. The analysis method was configured to generate total ion chromatogram, selective ion chromatogram and the mass fragments graph simultaneously.
CHAPTER 3
RESULTS AND DISCUSSION

3.1. Extraction of Spruce Bark

The primary objective of this project was to develop a method to extract and purify ($E$)-resveratrol from conifer bark in high yield and high purity. Since bark is a waste material generated in large quantities from wood processing and forest bioproducts industries, it is potentially an inexpensive source of ($E$)-resveratrol.

A previous study conducted by our group using balsam fir bark suggests that Soxhlet extraction with polar organic solvents such as methanol provides the highest percentage of extractives, compared to other common extraction techniques such as sonication and microwave extraction (Figure 18). Hence, Soxhlet extraction was used in the extraction of ($E$)-resveratrol from spruce bark.

Figure 18: Percent extractives of balsam fir from various extraction techniques and solvents (LeBlanc, 2010).
From the quantitative analysis, it was evident that overnight extraction of black spruce bark with methanol yields the maximum amount of (E)-resveratrol per dry gram of bark. With relatively nonpolar solvents such as diethyl ether in which (E)-resveratrol has a moderate solubility, the extraction time and solvent volume had to be increased by two-fold to obtain comparable results.

3.2. Initial Purification Attempts

3.2.1. Liquid-Liquid Extractions

Purification of the Soxhlet extract of spruce bark was first attempted with the liquid-liquid extractions. Hexane was employed to separate resins, fatty acids, sterols and other largely non-polar compounds from the crude extracts of spruce bark (Soxhlet extract in methanol). Partitioning of the resulting spruce bark extract from hexane extraction, between ethyl acetate and water, was intended to remove highly water-soluble phenolic compounds and their glycosides. Polydatin, the glycoside of (E)-resveratrol (resveratrol-3-β-monο-D-glucoside), was assumed to migrate into the aqueous layer during this step. Hence, acidification and back-extraction of the aqueous layer resulting from the above ethyl acetate-water extraction were aimed at hydrolyzing the sugar-phenol ether bond of polydatin and recovering (E)-resveratrol.

The TLC analysis revealed that this liquid-liquid extraction scheme was able to generate an (E)-resveratrol rich sample from the crude extracts of spruce bark. Furthermore, TLC analysis indicated the presence of (+)-catechin in the bark extracts. However, the results did not provide any evidence for the presence of (E)-resveratrol glycosides in the bark. From the mobile phases tested in the TLC analyses, the solvent mixture consisting of
chloroform, ethyl acetate and formic acid in 25:10:1 ratio provided the best separation of compounds (see Appendix A for details).

3.2.2. Silica Gel Column Chromatography

Column chromatography was employed to attempt isolation of \((E)\)-resveratrol from the spruce bark extracts, which were partially purified by the liquid-liquid extraction scheme mentioned in the previous section. The stationary phase (silica gel) and mobile phases were chosen based on the separation of compounds observed on silica gel TLC plates. None of the chromatographic systems and mobile phase combinations including chloroform-methanol, toluene-methanol, and chloroform-ethyl acetate-formic acid, and flash chromatography with dichloromethane-methanol were successful in yielding a pure sample of \((E)\)-resveratrol from the bark extracts. Hence, the need for strategies aimed at further purification was evident. However, silica gel column chromatography with chloroform-ethyl acetate-formic acid mobile phase was shown to separate and remove \((+)-\text{catechin}\) from the bark extracts (see Appendix A for more details).

3.2.3. Borate Complex Extraction

The purpose of borate complex extraction was to remove vicinal diols from black spruce bark extracts resulting from the liquid-liquid extractions and silica gel column chromatography. Vicinal diols react with Lewis acids such as diphenylborate-ethanolamine to form anionic complexes which could be made soluble in non-polar organic solvents by combining with organic cation compounds such as TBAF. The reaction scheme is shown in Figure 19.
As discussed previously, silica gel column chromatography was shown to remove some vicinal diols such as (+)-catechin from the bark extracts. The borate complex extraction was employed to remove any remaining vicinal diols. Since the exact amount of vicinal diols present in the bark extracts was unknown, excess amounts of reagents were required to conduct the extraction.

The HPLC-UV analysis of the samples resulting from borate complex extraction revealed that a moderate purification was achieved. However, peak area comparison of (E)-resveratrol in the chromatograms indicated a sample loss of 31% during the process. Since bark extracts require non-aqueous solvents for the maximum solubility, this sample loss was attributed to the partial miscibility of solvents employed in the back-extraction of diol-ion pair complexes from the extracts (see Appendix A for more details). The poor sample recovery and the need to use excess amounts of reagents greatly discouraged the adoption of borate complex extraction in the development of a purification method.
3.2.4. Acetylation-Deacetylation Reactions

3.2.4.1. Acetylation and Column Chromatographic Purification. This experimental approach was aimed at modifying the interactions between the analytes and the silica gel column material by acetylating the polyphenolic compounds in bark extracts (Figure 20), and attempting separation of them on a column with relatively non-polar solvent systems to obtain a pure sample of (E)-resveratrol in its acetylated form.

![Figure 20: Esterification of (E)-resveratrol by acetylation.](image)

Acetylation followed by silica gel column chromatographic separation with chloroform-ethyl acetate, and chloroform-dichloromethane was conducted on black spruce bark extracts resulting directly from the liquid-liquid extractions, and also on samples resulting from the borate complex extraction. The column eluate revealed only one peak corresponding to (E)-resveratrol triacetate, in the HPLC-UV chromatogram obtained at 306 nm. However, the peak area comparisons revealed that the incorporation of multiple silica gel column chromatographic separation steps caused a substantial reduction in the yield (see Appendix A for more details).

Further analysis by GCMS was conducted to obtain a full estimation of the purity of the samples, and the results are shown in Figure 21. The presence of multiple peaks in the GCMS total ion chromatograms suggested that the acetylation followed by column chromatographic separation does not purify (E)-resveratrol from the bark extracts.
Figure 21: Comparison of the purity of acetylated black spruce bark extracts.
(The above chromatograms represent acetylated black spruce bark extracts that were in different dilutions. Hence, the peak heights or areas are not directly comparable)
The large sample loss, the need for excess reagents and multiple chromatographic steps, and the results indicating an incomplete purification led to the abandonment of this approach. However, acetylation emerged as a derivatization method to prepare spruce bark samples for the GCMS analysis, which was widely applied in the anion exchange chromatography experiments discussed in section 3.2.6.

3.2.4.2. Deacetylation Reactions. The purpose of conducting the deacetylation reactions was to generate \((E)\)-resveratrol back from its acetylated form by cleaving the phenylacetate ester bond, with a minimal sample loss. From the results, it was evident that refluxing the acetylated sample with 0.05 M HCl in 80% ethanol was sufficient to produce \((E)\)-resveratrol back from its triacetate form in one-step (See Appendix A for more details on deacetylation reactions). Since acetylation followed by silica gel column chromatographic separation of black spruce bark extracts resulting from liquid-liquid extractions and borate complex extractions (discussed previously) was unable to provide the expected purification, the deacetylation step was only needed in the experiments conducted with Sephadex LH-20, which is discussed in section 3.3.2.

3.2.5. Isomerization of \((E)\)-Resveratrol

The GCMS analysis of acetylated \((E)\)-resveratrol standard solution revealed a peak for \((Z)\)-resveratrol triacetate in the total ion chromatogram (Figure 22). This peak was observed in all acetylated black spruce bark samples as well, and its identity was confirmed by the comparison of mass fragments with those of \((E)\)-resveratrol triacetate.
As discussed in Chapter 1, studies published on the biosynthetic pathways of stilbene compounds in plants indicate that the Z-isomer of resveratrol is not synthesized along with the E-isomer. Furthermore, (Z)-resveratrol was not observed in the HPLC-UV chromatograms recorded at 280 nm and 306 nm for the (E)-resveratrol standard solution. Hence the presence of (Z)-resveratrol could be attributed to the occurrence of isomerization events either by acetylation conditions, or exposure to light (photo-induced isomerization).

As described in the introduction, the Mallory reaction (Mallory et al. 1964) provides the mechanistic insights to the E-Z isomerization occurring in resveratrol. Mallory’s work suggests that irradiation by UV reversibly converts (E)-stilbenes to (Z)-stilbenes which would further react and cyclize to phenanthrene rings (in the presence of an oxidant), or
form dimers by [2+2] cycloaddition. The isomerization and cyclization events probable in resveratrol are illustrated in Figure 23.

![Figure 23: E-Z isomerization, cyclization, and cycloaddition of resveratrol.](image)

This phenomenon was studied using resveratrol in the undergraduate thesis work of Thomas Williams (2016). His work was able to demonstrate the photo-induced isomerization of (E)-resveratrol, and the ability of heat, strong bases such as sodium ethoxide, and air (oxygen) to catalyze the conversion of (Z)-resveratrol back to (E)-resveratrol.

The theoretical calculations performed by Dr. Raymond C. Fort of the University of Maine, with density functional theory (DFT) at B3LYP/6-311+G* basis set predicted that the activation energy and the enthalpy change for the E-Z isomerization of resveratrol are
below 40 kcal/mol and 10 kcal/mol, respectively (Table 8). Hence it is probable that the
acetylation reaction conditions provided the energy required to generate the Z-isomer of
resveratrol in the extracts and also in the standard solutions. In addition, these low energy
barriers for the E-Z isomerization of resveratrol explain the occurrence of the Z-isomer in
very small amounts in the spruce bark extracts while they were being subjected to column
chromatographic purification. This knowledge was instrumental in the later stages of the
purification method development, to carefully design the steps to minimize the
isomerization events.

Table 8. Predicted energy cost for E-Z isomerization of resveratrol (DFT at B3LYP/6-311+G* basis set).

<table>
<thead>
<tr>
<th>Method</th>
<th>Activation Energy (Kcal/mol)</th>
<th>Enthalpy Change (Kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncatalyzed isomerization</td>
<td>39.3</td>
<td>5.1</td>
</tr>
<tr>
<td>Base-catalyzed isomerization</td>
<td>28.8</td>
<td>8.6</td>
</tr>
<tr>
<td>Radical (O₂) isomerization</td>
<td>22.4</td>
<td>6.5</td>
</tr>
</tbody>
</table>

3.2.6. Ion Exchange Chromatography

A study conducted by Lo Péz-Nicolas et al. (2008) determined the experimental pKₐ
values of (E)-resveratrol (Table 9), which provided useful information to attempt anion
exchange chromatography for the purification of bark extracts. Black spruce bark extracts
resulting from silica gel column chromatography, re-dissolution of the Soxhlet extracts in
diethyl ether, and borate complex extraction, were subjected to anion exchange
chromatography with the aim of obtaining a purified sample of (E)-resveratrol from
them. With the pKₐ values ranging from 8.8 to 11.4, a strongly basic solvent such as
sodium ethoxide in ethanol was mandatory to deprotonate and load (E)-resveratrol onto
the anion exchange column. Desorption of the compound from the ion exchange resin required re-protonation of \((E)\)-resveratrol into its neutral form and sufficient solubility in the medium. Hence, mobile phases consisting of acids and alcohols, which include acetic acid-methanol and formic acid-methanol were necessary for the chromatography. These requirements demanded the use of anion exchange resins such as Amberlite IRA-400 and Dowex 2X8, which are capable of operating in a wide pH range, preferably 1 to 14.

Table 9. The pK\(_a\) values of \((E)\)-resveratrol.

<table>
<thead>
<tr>
<th>Structure</th>
<th>pK(_{a1})</th>
<th>pK(_{a2})</th>
<th>pK(_{a3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Structure 1]</td>
<td>8.8</td>
<td>![Structure 2]</td>
<td>9.8</td>
</tr>
</tbody>
</table>

The HPLC-UV analysis of the samples resulting from anion exchange chromatography revealed a poor sample recovery and an inadequate purification. The maximum yield obtained was only 21\%, and the GCMS analysis indicated that the anion exchange resins introduce more impurities into the sample (Figure 24). These impurities were released
from the resin material when it was subjected to a rapid pH change, from highly alkaline column loading solution to an acidic mobile phase.

Figure 24: Dowex 2X8 anion exchange column eluate of (a) black spruce bark crude sample, and (b) \((E)\)-resveratrol standard solution.
In addition, the strong affinity of deprotonated \((E)\)-resveratrol to the resin material posed difficulties in desorbing the compound even in the presence of strong acids in the mobile phase (See Appendix A for more details). Hence, the attempts to purify spruce bark extracts by this method were abandoned after several unsuccessful trials.

3.3. Further Experiments on Purification Method Development

3.3.1. Aqueous Sodium Bicarbonate Extraction

The aqueous sodium bicarbonate extraction was aimed at removing phenolic acids from black spruce bark extracts. Carboxylic acids react with sodium bicarbonate to generate sodium salts of them that are highly soluble in water, hence extractable into aqueous solutions (Figure 25). This reactivity was used as an approach to purify Soxhlet extracts of black spruce bark by removing any phenolic acids present in them.

![Chemical reaction](Image)

**Figure 25:** Reaction of phenolic acids with sodium bicarbonate to form water-soluble salts.

Published work on similar studies has suggested that 5% (w/v) aqueous solution of sodium bicarbonate is suitable for the extraction of phenolic acids (Wang et al. 2013). This extraction was attempted on direct Soxhlet extracts of black spruce bark, that were pre-dissolved in diethyl ether to facilitate the layer separation. A visual difference of color from greenish dark yellow to light yellow in the Soxhlet extract was evident from the aqueous sodium bicarbonate extraction, indicating a possible improvement in purity. This inference was further confirmed by the results of HPLC-UV analysis at 306 nm, as
shown in Figure 26. Based on these results, it was decided to incorporate this extraction step in the purification method development.

Figure 26: Soxhlet extract of black spruce bark (a) before, and (b) after aq. NaHCO₃ extraction.

3.3.2. Column Chromatography with Specialized Stationery Phases

3.3.2.1. Sephadex LH-20. Column chromatography with Sephadex LH-20 is reported in the published work of Güder et al. (2014) for the isolation of resveratrol from fox grapes (*Vitis labrusca*). Being a cross-linked polysaccharide network prepared by the hydroxypropylation of dextran, the Sephadex LH-20 column material contains a vast number of active sites in it to interact with the analytes in complex mixtures such as spruce bark extracts.
The Sephadex LH-20 column chromatography with dichloromethane-methanol mobile phase of sodium bicarbonate extracted black spruce bark extract yielded column fractions containing a sizeable \((E)\)-resveratrol peak and only one unknown peak in the HPLC-UV chromatogram obtained at 306 nm (Figure 27). The quantitative analysis indicated a sample recovery of 96% at this level of purity. However, Sephadex LH-20 column chromatographic separation of bark extracts directly resulting from the Soxhlet extraction did not display a comparable purity, suggesting that the aqueous sodium bicarbonate extraction is a vital step in the purification process (see Appendix A for more details).

Figure 27: Sodium bicarbonate washed black spruce bark extract (a) before, and (b) after Sephadex LH-20 column chromatographic purification.
(The chromatograms shown above were obtained at different dilutions of the samples. Hence, the peak heights or areas are not directly comparable)
Further analysis of sodium bicarbonate extracted and Sephadex LH-20 column chromatographed black spruce bark extract by HPLC-Ion trap MS revealed that the unknown peak observed in the HPLC-UV chromatogram above corresponds to 3 major unknown compounds that were co-eluting from the column, as shown in Figure 28. A weak molecular ion peak representing (Z)-resveratrol was also detected at this retention time, indicating the occurrence of isomerization events in the extract.

![Figure 28](image)

**Figure 28:** Sephadex LH-20 column eluate of sodium bicarbonate washed black spruce bark extract, with the molecular ion peaks of the impurities shown.

Acetylation followed by silica gel column chromatographic separation and deacetylation enabled the removal of these unknown compounds from the black spruce bark extract, resulting in a clean sample of \((E)-\)resveratrol. However, the acetylation-deacetylation reaction conditions generated a significant amount of \((Z)-\)resveratrol by isomerization, as shown in Figure 29.
Figure 29: Total ion chromatogram (a) and averaged mass spectrum (b) of Sephadex LH-20 column eluate after acetylation, column chromatographic separation, and deacetylation.

These isomerization events, and multiple steps incorporated in the acetylation-deacetylation reactions caused a sample loss of 35%, making the entire process less encouraging for a commercial adaptation.

3.3.2.2. C18 Coated Silica. Reversed-phase column chromatography with C-18 coated silica was attempted as an alternative to acetylation-deacetylation reactions based purification of Sephadex LH-20 column eluates of black spruce bark extract. The objective was to develop a column chromatographic system that would provide a separation similar to that of HPLC columns used in the qualitative and quantitative analysis of samples in this work.
The reversed-phase column chromatography of black spruce bark extracts resulting from Sephadex LH-20 column chromatography, with C-18 coated silica and dichloromethane-ethyl acetate mobile phase generated a pure column fraction of \((E)\)-resveratrol. However, peak area comparisons indicated that a sample loss of 44% has occurred during this chromatographic separation, as shown in the HPLC-UV chromatograms obtained at 306 nm (Figure 30).

Figure 30: Sephadex LH-20 column chromatographed black spruce bark extract (a) before, and (b) after reversed-phase C18 column chromatography.
The primary reason for this sample loss was the affinity of the target compound to the polar glass surface, due to the largely non-polar nature of the chromatographic system. Deactivating the glass surfaces by silanization of glass containers and columns improved the sample recovery.

The purity of the sample resulting from reversed-phase column chromatography was further confirmed by HPLC-Ion trap MS analysis (Figure 31). Besides the sizeable peak for \((E)\)-resveratrol, a negligible amount of \(Z\)-isomer was present in the total ion chromatogram, which indicates that the occurrence of isomerization events has been markedly reduced compared to the acetylation-deacetylation reactions. The sample recovery from this chromatographic step was quantified as 87%.

Figure 31: HPLC-UV\(_{306}\) nm chromatogram, HPLC-MS total ion chromatogram, and averaged mass spectrum of purified \((E)\)-resveratrol from black spruce bark.
3.3.3. Method Optimization and Further Improvements

The Sephadex LH-20 and C-18 coated silica (reversed-phase) column chromatography demonstrated the ability to purify (E)-resveratrol from black spruce bark, with a sample recovery of 96% and 87%, respectively. However, re-dissolution of the initial Soxhlet extract in diethyl ether caused a sample loss of 32%, decreasing the overall yield to 57%.

The substitution of Soxhlet extraction solvent with diethyl ether rather than methanol prevented this loss. However, the Soxhlet extraction time and volume of the solvent had to be increased by two-fold to obtain comparable results to the extractions conducted with methanol, due to the moderate solubility of (E)-resveratrol in diethyl ether. Even with those modifications, diethyl ether only extracted 332.9 ± 0.4 µg of (E)-resveratrol per dry gram of bark, while methanol extracted 397.9 ± 3.6 µg of the compound per dry gram of bark. Nevertheless, use of diethyl ether for the Soxhlet extraction enhanced the overall yield of the purification process to 84%, by obviating an additional re-dissolution step prior to the liquid-liquid extraction with aqueous sodium bicarbonate.

Many of the commercial producers of (E)-resveratrol including Sigma-Aldrich and Mega-Resveratrol state the purity of their product as “99% (HPLC)”.

Figure 32 lists the HPLC-UV chromatograms obtained at 306 nm (the absorption maximum for E isomer of resveratrol), 286 nm (the absorption maximum for Z isomers of resveratrol), and 260 nm (a common absorption maximum for many other polyphenolic compounds) for (E)-resveratrol resulting from the purification of black spruce bark. The results indicate a clean peak of (E)-resveratrol, and a small (Z)-resveratrol peak (identity confirmed by mass fragment comparison) which has an area averaging to 1%. Hence, (E)-resveratrol
resulting from the proposed purification method is shown to meet the purity level of the commercially available products.

![HPLC-UV analysis](image)

**Figure 32**: HPLC-UV analysis of purified (E)-resveratrol from black spruce bark at 306, 260, and 286 nm wavelengths.

The finalized method for the extraction and purification of (E)-resveratrol from the spruce bark is shown in Figure 33. The overall yield was quantified as $279.9 \pm 4.9 \mu g$ (E)-resveratrol per dry gram of bark, with 99% purity (HPLC).
Figure 33: Schematic diagram of the proposed purification procedure.

Black spruce bark extract in diethyl ether

Extraction with NaHCO₃

Sephadex LH-20 column chromatography with CH₂Cl₂ and CH₃OH

Reversed phase column chromatography with CH₂Cl₂ and CH₃COOC₂H₅

Final product
CHAPTER 4
CONCLUSIONS AND SUGGESTED WORK

4.1. Conclusions

The work presented in this dissertation suggests that the bark of conifer species native to Maine are potential sources of (E)-resveratrol, a plant polyphenolic compound that has been shown to demonstrate a variety of health benefits. The primary candidates for the extraction are spruces, especially black spruce (Picea mariana) and Norway spruce (Picea abies), since their bark contain (E)-resveratrol in comparable amounts to Japanese knotweed (Polygonum cuspidatum), which is the current prime commercial source of the compound.

In northern American states like Maine, spruce bark is a waste generated in large quantities from wood processing and forest bioproducts industries. It is mostly used as one of the fuels for producing heat and energy for the mills. However, the demand for bark as a fuel is set to decline with the increasing adoption of green energy alternatives. Since spruce bark is abundant and potentially inexpensive, the extraction and purification of (E)-resveratrol from it would find a rewarding use for the material, and promote new industrial avenues. In addition, bark would be a good alternative to the invasive plant Japanese knotweed. Furthermore, the bark could still be usable as a fuel after the extraction of resveratrol.

As discussed in the previous chapter, (E)-resveratrol could be extracted from spruce bark with common organic solvents such as methanol. However, bark extracts inherently contain a large number of polyphenolic compounds that introduce an added complexity for the isolation of the target compound. The proposed purification method in this
research produces \((E)\)-resveratrol from black spruce bark, in 99% purity with an overall yield reaching 84%. The amount of \((E)\)-resveratrol generated from this process is 279.9 ± 4.9 µg per dry gram of bark.

Previous studies have suggested that Soxhlet extraction has a high efficiency compared to common extraction techniques such as microwave extraction and sonication. The use of diethyl ether instead of methanol for the Soxhlet extraction reduces the co-extraction of other polyphenolic compounds, which results in an increase in the overall yield of the target compound from the purification process due to the comparatively less intricacy of the extract. In addition, the boiling point of 34.6 °C of diethyl ether maintains a low energy demand for the Soxhlet extraction even though the moderate solubility of \((E)\)-resveratrol in it requires a two-fold increase in solvent volume and extraction time to yield comparable amounts to methanol-based extractions.

As shown in the results, E-Z isomerization of resveratrol was observed in the spruce bark extracts at various steps. The occurrence of Z-isomer was apparent in the samples resulting from the acetylation-deacetylation reactions, where the reaction conditions involved heat and high pH. The theoretical (DFT) calculations predicted that the activation energy is lower than 40 kcal/mol for even an uncatalyzed E-Z isomerization of resveratrol. Hence, the occurrence of isomerization events was able to be minimized by the introduction of column chromatography to replace the steps in the procedure that require external energy (heat), such as the acetylation-deacetylation reactions. Use of a low-boiling solvent (diethyl ether) for the Soxhlet extraction served this purpose as well.
The use of specialized column materials that include Sephadex LH-20 and C-18 coated silica have shown to separate and purify (E)-resveratrol from the extract with a yield of 96% and 87%, respectively. These column chromatographic separation steps require considerable amounts of organic solvents in various compositions for the purification of the compound. However, the impact to the environment from them could be minimized by recycling and reusing them in the purification process.

4.2. Suggested Work

An extraction and purification method to isolate (E)-resveratrol from black spruce bark has been established in this dissertation work. Recommendations for future work are stated below.

Scaling up the purification method: The experiments reported in this work were conducted in laboratory scale. Scaled up trials are required to evaluate the cost-effectiveness, environmental impact, and feasibility of the proposed purification method for an industrial adaptation.

Regeneration of the column materials: The two column materials used in this study (Sephadex LH-20 and C-18 coated silica) are specially formulated, and expensive. Hence, investigating the ability to regenerate the column materials for reuse would help to reduce the operational cost of the proposed purification process.

Experiments on other rich sources of (E)-resveratrol: The purification process reported here was applied to black spruce bark samples. Since the biosynthetic pathways of plants that give rise to a variety of polyphenolic compounds are related, it is anticipated that the proposed method is capable of purifying (E)-resveratrol from the
crude extracts of other plants as well. However, further studies are required to evaluate
the feasibility of adopting this purification method to produce \((E)\)-resveratrol from other
potential sources in high purity and high yield.

**Identification of other bioactive compounds in bark extracts:** The primary focus of
this research was to develop a method for isolating \((E)\)-resveratrol from spruce bark
extracts. However, it was observed that the liquid-liquid extractions and column
chromatographic purifications involved in this process are capable of separating other
unknown compounds from the crude extract as well. No experiments were conducted to
identify those constituents in the samples. Such attempts would provide useful
information to determine the composition of bark extracts and discover other potentially
bioactive compounds.

**Induce the expression of stilbene synthase gene in spruce seedlings:** Biosynthesis of
plant secondary metabolites including \((E)\)-resveratrol are largely regulated by exogenous
influences such as pathogenic infections and tissue damage. An alternative approach to
enhance the production of resveratrol in plants is the induction of the expression of
stilbene synthase gene in seedlings. A dedicated study on this aspect would help to
maximize the availability of \((E)\)-resveratrol in spruce bark for extraction.
CHAPTER 5

(E)-RESVERATROL CONTENT IN MAINE GROWN COLD HARDY GRAPES

5.1. Abstract

(E)-Resveratrol is a polyphenolic compound of plant origin, which confers a variety of benefits to human health and well-being. Grapes are one of the earliest known sources of the compound, with wide variability in the amount among different varieties. In this study, thirteen varieties of cold-hardy grapes grown in Maine were extracted and analyzed for their (E)-resveratrol content. The highest amount observed is 21.21 ± 0.23 µg (E)-resveratrol per dry gram of grapes (for Prairie Star). The results suggest that the grape color and (E)-resveratrol content has no direct correlation.

5.2. Introduction

Grapes are undoubtedly one of the most economically important agricultural fruits due to their use in highly sought-after commercial products such as wine, juice, raisins, and jam. Natural habitats of the best winemaking grapes are the regions with a temperate climate, and the average temperature and soil conditions play a vital role in grape ripening and flavor development (LaMar 2011, Cheng et al. 2014). Hence, cultivation of commercially important grapes in the areas susceptible to extreme weather such as northern New England has been a challenge until the development of cold-hardy grape varieties. Most of the cold-hardy varieties are hybrids of Mediterranean and European grapes (Vitis vinifera) with native North American grapes such as fox grapes (Vitis labrusca) and frost grapes (Vitis riparia). They confer tolerance to extreme winter conditions and resistance to diseases, which brings stability to winemaking industries in northern New England (Pedneault et al. 2013). However, wines from cold-hardy grapes have a low tannin
content, a distinct taste, and light color compared to wines from *Vitis vinifera* varieties (Rice et al. 2017).

Grapes contain a complex mixture of natural products that include stilbenes, phenolic acids, anthocyanins, and flavonoids. (Xia et al. 2010). As shown in Figure 34, those compounds are distributed in the skin, flesh, and seeds of the fruit. Anthocyanins are the dominant class of compounds that determine the color of the fruit, and flavonoids (and tannins) are responsible for the bitterness and astringent properties (Conde et al. 2008).

While these compounds confer a variety of benefits to human health and well-being, their concentration has a high dependency on environmental factors, besides the genetic variation between species (Teixeira et al. 2013).

Figure 34: Schematic diagram of natural products distribution in ripe grape berry. (Teixeira et al. 2013)
Challenging environmental conditions such as water stress affect the size, maturity level and sugar content of grapes, and also increases the flavonoid and tannin content affecting the taste, which are all crucial factors for winemaking (Downey et al. 2006). In addition, some studies have indicated that exposure to UV radiation particularly enhances the stilbene content in grapes, as they are involved in the repair mechanisms against tissue damage by radiation (Carbonell-Bejerano et al. 2014).

(E)-Resveratrol is one of the stilbene compounds present in grapes, that has drawn much attention due to its putative health benefits. Being a polyphenolic compound, (E)-resveratrol is known for its antioxidant activity (Shang et al. 2009). The ability of (E)-resveratrol to modulate lipid metabolism in the human body has found its place in the famous “French paradox”, an epidemiological study that showed coronary heart disease-related mortality is significantly lower among the French, due to their habit of high red wine consumption (Chadwick and Goode, 1998). In addition, the compound’s claimed ability to reduce obesity and hyperglycemia, and to demonstrate anti-aging, anti-inflammatory, and anti-cancer properties has been investigated and proved by many in-vivo and in-vitro studies (Ulrich et al. 2005, Holme et al. 2007, Szkudelska et al. 2010, Zhang et al. 2010). A recent study has shown that (E)-resveratrol possesses the capability of inducing neurogenesis, suggesting its potential to be developed as a therapeutic agent against age-related neurodegenerative diseases (Kumar et al., 2016). With these benefits to human health and well-being, (E)-resveratrol continues to gain ever-increasing attention from the pharmaceutical, food and supplements industries, and the demand for the compound is expected to grow even further with time. Hence, a study on the content of (E)-resveratrol in cold-hardy grape varieties grown in Maine would provide valuable
information for farmers and other stakeholders who are interested in establishing this crop and developing value-added products from it.

5.3. Materials and Methods

5.3.1. Chemicals

Methanol and acetonitrile were purchased from Fisher Scientific (Waltham, MA), and formic acid and 4-phenylphenol were purchased from Sigma-Aldrich (St. Louis, MO). (E)-Resveratrol standard was purchased from Mega Resveratrol (Danbury, CT). Ultrapure water was obtained from a Millipore water system (EMD Millipore, Billerica, MA) installed in the laboratory.

5.3.2. Sampling and Processing of Grapes

Thirteen varieties of cold-hardy grapes that include Alpenglow, Brianna, Chontay, Frontenac Gris, Hungarian, Marechal Foch, Marquette, Prairie Star, Reliance, Sabrevois, St. Croix, St. Pepin, and Vanessa were harvested in 2015 at Highmoor Farm in Monmouth, ME. The suitability for harvesting was determined by the visual ripeness of the fruit. All grapes were stored at 4°C until processing. They were de-stemmed, cleaned to remove debris and damaged fruits, and freeze-dried prior to storing at -80°C until further processing. All grapes were processed within 15 days of their harvest date.

Freeze dried grapes were ground to a powder using an electric blender (Waring, New Hartford, CT). Each ground grape variety was extracted for 6 hours in a Soxhlet apparatus equipped with 22 x 80 mm glass fiber thimble. The extraction solvent mixture was comprised of methanol, formic acid and water in 97:2:1 volume ratio. The final volume of the extract was set to 100.0 mL using methanol prior to the HPLC analysis.
Samples were protected from sunlight in order to prevent possible isomerization of (E)-resveratrol.

5.3.3. Quantitative Analysis

HPLC analysis was performed on an Agilent 1100 series HPLC system with an automatic liquid sampler and a variable wavelength (VW) detector (Agilent, Waldbronn, Germany). The system was equipped with a Phenomenex Kinetex 5µm C18 4.6 x 100 mm column (Phenomenex, Torrance, CA). The calibration curve (Figure 35) was generated with (E)-resveratrol standard and 4-phenylphenol as the external standard. All samples were analyzed at 306 nm for (E)-resveratrol and 260 nm for 4-phenylphenol (external standard).

![Figure 35: Calibration curve for the quantitative analysis of grapes.](image)

The HPLC conditions are listed in Table 10. A gradient profile modified from Marshall et al. (2012) was employed to obtain a good resolution. The mobile phase consisted of methanol: formic acid: water in 10:1:89 volume ratio (solvent A), and acetonitrile (solvent B).
Table 10. HPLC conditions for quantitative analysis of grapes.

<table>
<thead>
<tr>
<th>Injection Volume</th>
<th>8 μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Rate</td>
<td>0.3 mL/min</td>
</tr>
<tr>
<td>Total Run Time</td>
<td>40 mins</td>
</tr>
<tr>
<td>Gradient Profile</td>
<td>Time (min.)</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>38.0</td>
</tr>
<tr>
<td>Detector Wavelength Setup</td>
<td>Time (min.)</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td>37.0</td>
</tr>
</tbody>
</table>

Further confirmation of the identity of (E)-resveratrol in grape extracts was performed using an Agilent 1100 series HPLC system equipped with a diode array detector (DAD) coupled to an Agilent 1100 series ion trap mass spectrometer with electrospray ionization (ESI) source (Waldbronn, Germany). Chromatographic separation was performed employing the same column and gradient profile described above. Mass spectra of samples were obtained and compared with the (E)-resveratrol standard. The ion-trap MS was operated in negative ion mode at 350 °C, 8.97 L/min drying gas flow, 49.9 psi nebulizer pressure, 3500 V capillary voltage and 7.0 kV dynode voltage at 44.2 trap drive level. The target mass of the ion trap was set to 227 m/z, and the detection range was 50-500 m/z.

5.4. Results and Discussion

Previous work on the analysis of total resveratrol content in grape and cranberry juice has indicated that grapes inherently contain both the glycoside (reservatrol-3-β-monoglucoside, also known as polydatin) and the free form of (E)-resveratrol (Wang et al.)
Since the sugar-phenol ether bond of polydatin is acid-labile, an acidified solvent mixture was employed in the extraction process. It was assumed that the powdered form of grapes would yield more resveratrol due to high surface area, and increased accessibility of the solvent into the sample. Further confirmation to this approach was provided by the results of a similar work conducted by Soural et al. (2015) suggesting that powdered grapes generate a higher yield of \((E)\)-resveratrol compared to grape pieces, and Soxhlet extraction has a high extraction efficiency compared to simple mixing or refluxing with solvents. Hence, the powdered form of grapes was used in the Soxhlet extraction.

The HPLC-UV chromatograms obtained at 306 nm revealed many unknown peaks alongside \((E)\)-resveratrol, suggesting that the extracts are complex mixtures of compounds (Figure 36). However, the \((E)\)-resveratrol peak was well separated from other compounds in the chromatograms, providing a reliable quantification.

Figure 36: Representative chromatogram from the HPLC-UV analysis of grape extracts.
As shown in Figure 37, Prairie Star contains the highest (E)-resveratrol content. No detectable amount of the compound was observed in Hungarian and St. Croix varieties.

Figure 37: (E)-resveratrol content in cold-hardy grape varieties grown in Maine.

The above results were compared with the published data from a study (Ratnasooriya et al. 2010) conducted on cold-hardy grape varieties grown in Nova Scotia (Eastern Canada) which included Marechal Foch and Reliance, and their (E)-resveratrol contents are in agreement with this study (Table 11).
Table 11. (E)-Resveratrol content in grapes grown in Nova Scotia (Ratnasooriya et al. 2010), with a comparison to two Maine grown varieties.

<table>
<thead>
<tr>
<th>Grape Category</th>
<th>Cultivar</th>
<th>(E)-resveratrol (mg/100g)</th>
<th>(E)-resveratrol glucoside (mg/100g)</th>
<th>Total (E)-resveratrol (mg/100g)</th>
<th>Results from our study (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wine grapes</td>
<td>Lucie Kuhlman</td>
<td>0.43</td>
<td>0.85</td>
<td>1.28</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Castel</td>
<td>0.28</td>
<td>0.80</td>
<td>1.08</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Leon Millot</td>
<td>0.43</td>
<td>0.90</td>
<td>1.33</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Baco Noir</td>
<td>0.45</td>
<td>0.98</td>
<td>1.43</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Marechal Foch</td>
<td>0.47</td>
<td>0.78</td>
<td>1.25</td>
<td>1.26 ± 0.02</td>
</tr>
<tr>
<td>Table grapes</td>
<td>Suffolk Red</td>
<td>0.37</td>
<td>0.68</td>
<td>1.05</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Van Buren</td>
<td>0.20</td>
<td>0.53</td>
<td>0.73</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Sovereign Coronation</td>
<td>0.23</td>
<td>0.65</td>
<td>0.88</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Reliance</td>
<td>0.23</td>
<td>0.43</td>
<td>0.66</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Swenson Red</td>
<td>0.27</td>
<td>0.45</td>
<td>0.72</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

However, some studies indicate that the average (E)-resveratrol content in European red wine grapes (Vitis Vinifera sp.) and Asian variants of them are in the range of 9.4 mg/100g and 1.1 mg/100g, respectively, while native North American varieties are not good sources of the compound (Zhu et al. 2012). Hence, cold-hardy varieties, which are hybrids of Vitis Vinifera and native North American grapes, seem to confer better health benefits than native North American grape varieties alone. In addition, the highest (E)-resveratrol content among the cold-hardy varieties studied was found in a green grape (Prairie Star), while none was detected in some bluish red varieties (Hungarian and St. Croix), suggesting that the skin color of cold-hardy grapes has no direct correlation with the (E)-resveratrol content.
5.5. Conclusions and Future Work

The (E)-resveratrol content of 13 cold-hardy grape varieties grown in Maine was determined in this study. The cold-hardy grapes grown in other Northern American regions such as Nova Scotia has a comparable amount of (E)-resveratrol in them, while European grapes have much higher amounts of the compound.

A study aimed at the total phenolic content (TPC) of cold-hardy grapes would be informative to identify any correlations between TPC and (E)-resveratrol content. Investigation of methods to induce the expression of stilbene synthase gene in grape seedlings would help to enhance (E)-resveratrol biosynthesis, and hence the health benefits of them.
REFERENCES


Liquid-Liquid Extractions and TLC Analysis

Partial miscibility of methanol in other solvents such as hexane and ethyl acetate affected the layer separation in liquid-liquid extractions. Results of the TLC analysis of samples resulted from the liquid-liquid extractions are listed in Table 12.

Table 12. TLC analysis of Norway spruce bark liquid-liquid extracts.

<table>
<thead>
<tr>
<th>TLC Mobile Phase</th>
<th>Ethyl acetate layer 1</th>
<th>Ethyl acetate layer 2</th>
<th>Hexane layer</th>
<th>Aqueous Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butanol, pyridine, water and glacial acetic acid in 30:20:20:6 ratio</td>
<td>(E)-resveratrol (R&lt;sub&gt;f&lt;/sub&gt; 0.85) (+)-catechin (R&lt;sub&gt;f&lt;/sub&gt; 0.80) Multiple unknown spots</td>
<td>No spots matching the R&lt;sub&gt;f&lt;/sub&gt; of (E)-resveratrol or (+)-catechin</td>
<td>No spots</td>
<td>Pale spots with unknown R&lt;sub&gt;f&lt;/sub&gt; values</td>
</tr>
<tr>
<td>Chloroform, ethyl acetate, formic acid in 5:4:1 ratio</td>
<td>(E)-resveratrol (R&lt;sub&gt;f&lt;/sub&gt; 0.80) (+)-catechin (R&lt;sub&gt;f&lt;/sub&gt; 0.51) Multiple unknown spots</td>
<td>No spots matching the R&lt;sub&gt;f&lt;/sub&gt; of (E)-resveratrol or (+)-catechin</td>
<td>Pale spots with unknown R&lt;sub&gt;f&lt;/sub&gt; values (mostly unresolved)</td>
<td>Pale spots with unknown R&lt;sub&gt;f&lt;/sub&gt; values</td>
</tr>
<tr>
<td>Toluene and methanol in 9:1 ratio</td>
<td>(E)-resveratrol (R&lt;sub&gt;f&lt;/sub&gt; 0.10) No spot for (+)-catechin</td>
<td>No spots</td>
<td>Pale spots with unknown R&lt;sub&gt;f&lt;/sub&gt; values (mostly unresolved)</td>
<td>No spots</td>
</tr>
<tr>
<td>Chloroform, ethyl acetate and formic acid in 25:10:1 ratio</td>
<td>(E)-resveratrol (R&lt;sub&gt;f&lt;/sub&gt; 0.64) (+)-catechin (R&lt;sub&gt;f&lt;/sub&gt; 0.14) Multiple unknown spots</td>
<td>No spots matching the R&lt;sub&gt;f&lt;/sub&gt; of (E)-resveratrol or (+)-catechin</td>
<td>Pale spots with unknown R&lt;sub&gt;f&lt;/sub&gt; values (mostly unresolved)</td>
<td>Pale spots with unknown R&lt;sub&gt;f&lt;/sub&gt; values</td>
</tr>
</tbody>
</table>
As shown in the table above, the mobile phase consisting of chloroform, ethyl acetate and formic acid in 25:10:1 ratio, which is suggested in published work by Babu et al. (2005), provided the best separation of compounds.

**Silica Gel Column Chromatography**

Silica gel column chromatography with a variety of mobile phases was attempted on the ethyl acetate layer 1 (resulting from the procedure described in section 2.3.1), to separate (E)-resveratrol from the complex mixture of other compounds. None of the mobile phases were able to provide clean fractions of (E)-resveratrol. However, the mobile phase consisting of chloroform, ethyl acetate and formic acid in 25:10:1 ratio separated (E)-resveratrol from (+)-catechin in both Norway spruce and black spruce bark samples. The HPLC-UV chromatograms indicated the presence of the highest amount of (E)-resveratrol in the 3rd fraction eluted from the above column (Figure 38), while fractions 4 to 19 contained decreasing amounts of the compound.

![Figure 38. HPLC-UV chromatogram of fraction 3 eluted from the silica gel column with chloroform, ethyl acetate and formic acid in 25:10:1 ratio.](image)
A similar approach to the flash chromatography attempted in this work, has been published by Guder et al. (2014) for the isolation of resveratrol from grapes. It was observed that the column bed is prone to cracking due to the difference of viscosities between methanol and dichloromethane. Hence elution of the sample had to be carried out with careful application of pressure to minimize such events. The HPLC-UV analysis revealed that fractions 2 to 4 contain (E)-resveratrol. However, the chromatograms recorded at 306 nm detector wavelength (Figure 39) did not show a significant difference between the initial sample and the column eluate, implying that the chromatographic separation is unable to provide the desired purification.

Figure 39. Black spruce bark extract (a) before, and (b) after flash chromatographic separation.
Borate Complex Extraction

Published work by Tsuchiya et al. (1998) suggests that the diol-diphenylborate anionic complex formation proceeds efficiently at pH values between 8 and 9. Hence, a phosphate buffer solution of pH 8.2 was employed in the initial experiments conducted with aqueous solutions of (+)-catechin to test the concept. The HPLC-UV analysis of the aqueous phase resulting from the above experiments suggested that (+)-catechin is extracted into the organic phase, presumably by forming an ion pair with TBAF in the hexane-heptanol medium. However, it was necessary to investigate the feasibility of this reaction in aqueous-alcoholic and pure alcoholic reagents since the spruce bark extracts have minimal solubility in water. Attempts to use such reagents caused precipitation of phosphate buffers, as well as spruce bark extracts. Hence, the procedure was modified to use pure methanol instead of an aqueous medium to form the diol-borate anionic complex, with no attempts to control the pH. Only a few drops of pH 8.21 buffer was used at the addition step of TBAF (prepared in hexane-heptanol), to facilitate the layer separation. The HPLC-UV analysis of the methanol layer resulting from the borate complex extraction revealed a diminished peak for (+)-catechin, implying that the extraction is feasible under these modified conditions.

The HPLC-UV analysis (Figure 40) of black spruce bark sample resulting from the borate complex extraction revealed a less number of peaks in comparison to the sample before extraction, suggesting that the borate complex extraction is capable of removing the vicinal diols in bark extracts.
The primary reason for the sample loss of 31%, as seen from the chromatograms above, is the incomplete separation of layers between methanol and hexane-heptanol, at the ion pair extraction step. Methanol is miscible in hexane, and the addition of an aqueous solution to facilitate the layer separation reduces the solubility of (E)-resveratrol in the methanol layer. The need to use excess amounts of reagents for the reaction further complicates this problem, while affecting the viability of this purification technique in a commercial adaptation.

**Acetylation-Deacetylation Reactions**

Liquid-liquid extraction with water, and silica gel column chromatography with chloroform-ethyl acetate mobile phase was needed for the removal of excess reagents.
from the acetylation of spruce bark sample. The eluate from the silica gel column with chloroform-ethyl acetate mobile phase contained several peaks relating to unknown compounds, in the HPLC-UV chromatogram at 306 nm. Hence, a subsequent silica gel column chromatographic purification step was needed. From the many compositions of chloroform-dichloromethane mobile phases tested, the 1:1 ratio mixture was able to generate a column fraction of (E)-resveratrol triacetate with no other peaks present in the HPLC-UV chromatogram at 306 nm (Figure 41).

![HPLC-UV chromatogram](Image)

Figure 41. Ethyl acetate layer of black spruce bark extract after (a) acetylation, and (b) silica gel column chromatographic purification with chloroform and dichloromethane.
However, incorporation of multiple silica gel column chromatographic separation steps affected the yield of the compound, as shown by the peak area comparison above. The need to use excess reagents in the acetylation process posed concerns on the scale-up of this process. Above all, GCMS total ion chromatograms revealed the presence of many impurities in the sample.

Reaction conditions for the deacetylation of (E)-resveratrol triacetate were established for the use in column chromatographic experiments conducted with Sephadex LH-20. In the initial trials, NaOH in 80% ethanol was employed to hydrolyze the ester bonds which was followed by an acidification step with HCl to protonate the phenoxide anion. An ethanol-based medium was required to prevent the compounds from precipitation. These reaction conditions were later replaced with HCl in 80% ethanol to reduce the number of steps in the process. A liquid-liquid extraction step with diethyl ether was required to separate and recover deacetylated products from the reaction mixture.

**Ion Exchange Chromatography**

Dowex 2X8 and Amberlite IRA-400 Cl ion exchange resins were used in the chromatography (Figure 42).

![Figure 42](image_url)

**Dowex® 2X8**: Dimethylethanolbenzyl ammonium functional group on styrene-divinylbenzene matrix  
**Amberlite IRA 400**: Trimethyl ammonium functional group on styrene-divinylbenzene matrix

Figure 42. Chemical structure of the active sites of the anion exchange resins.
The HPLC-UV analysis of the column eluates of black spruce bark sample revealed that fractions 2-7 largely contain (E)-resveratrol with minor amounts of other compounds. These observations were the same for both anion exchange resins used. Being a stronger acid than acetic acid, formic acid in the mobile phase resulted in a higher yield of (E)-resveratrol in the column eluate. However, a sample loss in the range of 79% was observed from the HPLC-UV results, and the use of even stronger acids such as HCl in the mobile phase was unable to improve the sample recovery any further. Figure 43 illustrates the purification achieved (as determined from the HPLC-UV analysis at 306 nm) from the Amberlite IRA-400 anion exchange chromatography with formic acid-methanol mobile phase.

Figure 43. Black spruce crude sample (a) before loading on the column, and (b) after the column elution with formic acid-methanol.
The objective of conducting GCMS analysis was to obtain a full estimation of the purity of column fractions resulting from this chromatographic technique. The anion exchange column eluates of black spruce bark sample containing (E)-resveratrol were derivatized by acetylation for the GCMS analysis. The total ion chromatogram obtained was compared with a total ion chromatogram of an anion exchange column elute of 0.1 mg/mL (E)-resveratrol standard solution.

The results indicated that anion exchange column resin introduced more impurities into the sample. This was evident from the chromatogram obtained for the (E)-resveratrol standard solution eluted from the anion exchange column, since a standard solution of acetylated (E)-resveratrol generates a clean total ion chromatogram. Several washes of the ion exchange resin with the mobile phase prior to its re-activation, significantly reduced the levels of these impurities. However, it also reduced the capacity of the resin to provide the necessary separation of compounds in the spruce bark samples. Hence, the attempts to purify spruce bark extracts by anion exchange chromatography were unsuccessful.

**Column Chromatography with Sephadex LH-20**

The Sephadex LH-20 column material poses a degree of swelling that varies with the composition of the solvent. Hence, it was required to soak the material in the mobile phase for an extended period (typically overnight) before packing it onto a column. The mobile phase, dichloromethane, was denser than the column material. Therefore, glass wool plugs were used to prevent the material from floating in the column.
The initial experiments were performed with the black spruce bark samples resulting from the silica gel flash chromatography. Samples resulting from the sodium bicarbonate extractions were directly used in the subsequent experiments with Sephadex LH-20, when it was determined that the flash chromatography has the least effect on the purification.

It was required to elute the sample first with an adequate amount of dichloromethane to remove any non-polar compounds in it. Since (E)-resveratrol has negligible solubility in dichloromethane (and dry loading removes the interferences from methanol), this elution did not cause any noticeable losses of the target compound. The change of mobile phase from dichloromethane to a mixture of dichloromethane and methanol in 9:1 ratio reduced the flow rate to an approximate value of 70 µL per minute, due to further swelling of the stationary phase in the presence of methanol. From the 12 fractions eluted with dichloromethane-methanol mobile phase, fractions 5 to 9 contained a sizeable peak for (E)-resveratrol and one unknown peak, in the HPLC-UV chromatogram obtained at 306 nm. The HPLC-Ion trap MS analysis of those column fractions revealed the presence of 3 unknown compounds in them, in addition to (E)-resveratrol and a small amount of its Z-isomer (identities were confirmed by comparison to a standard solution of (E)-resveratrol that has been deliberately exposed to light and heat to generate the Z-isomer).

**Column Chromatography with C18 Coated Silica**

Fractions resulting from Sephadex LH-20 column chromatography were directly used in these experiments, and were dry loaded to minimize the interference from solvents. Initial experiments were conducted with a mobile phase consisting of acetonitrile and water in 1:3 ratio, which was later replaced with a mixture of dichloromethane and methanol in
9:1 ratio, due to the poor separation of compounds. The viscosity difference between dichloromethane and methanol resulted in cracking of the column bed, affecting the separation and sample recovery. Therefore, a solvent system consisting of dichloromethane and ethyl acetate was employed as the mobile phase. From the many compositions tested, a 9:1 ratio mixture of dichloromethane and ethyl acetate was able to provide the desired separation. First column fraction was eluted with pure dichloromethane to remove any soluble impurities, and second and third fractions were eluted with dichloromethane-ethyl acetate mobile phase which resulted in a clean sample of (E)-resveratrol.

The observed sample loss of 44% was unrecoverable, however, despite the attempts made with changes to the mobile phase. Incorporation of any polar solvents to the mobile phase affected the separation, while marginally improving the yield. Hence, silanization of the glass surfaces of the glassware and columns used in the experiments was required to improve the sample recovery.
Quantitative Analysis of Black Spruce Bark

External standard: 4-Phenylphenol 1400 µg/mL in methanol, 100 µL per 1.0 mL.

Sample: 12.5 g black spruce bark, extracted and final volume set to 35.0 mL.

Table 13. HPLC data for calibration curve.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>[Resveratrol] µg/mL</th>
<th>Ratio between peak area for Resv. and ES</th>
<th>Average peak area ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
<td>Run 3</td>
</tr>
<tr>
<td>1</td>
<td>200</td>
<td>1.6740</td>
<td>1.6745</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>0.8876</td>
<td>0.8874</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0.4440</td>
<td>0.4437</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>0.2210</td>
<td>0.2210</td>
</tr>
<tr>
<td>5</td>
<td>12.5</td>
<td>0.1110</td>
<td>0.1072</td>
</tr>
</tbody>
</table>

Figure 44. Calibration curve for the quantitative analysis of (E)-resveratrol in spruce bark.
Figure 45. Representative chromatograms from the quantitative analysis of \((E)\)-resveratrol in black spruce bark extract at each purification step. 
(a) Crude diethyl ether extract after NaHCO₃ wash, (b) Sephadex LH-20 column chromatographed sample, (c) RP-silica column chromatographed sample.
Table 14. HPLC average peak area ratio and calculated \((E)\)-resveratrol content at each step of the purification procedure.

<table>
<thead>
<tr>
<th>Trial</th>
<th>(A_{\text{Resv}} / A_{\text{ES}})</th>
<th>Trial</th>
<th>(A_{\text{Resv}} / A_{\text{ES}})</th>
<th>Trial</th>
<th>(A_{\text{Resv}} / A_{\text{ES}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0193</td>
<td>1</td>
<td>0.9828</td>
<td>1</td>
<td>0.8454</td>
</tr>
<tr>
<td>2</td>
<td>1.0186</td>
<td>2</td>
<td>0.9800</td>
<td>2</td>
<td>0.8426</td>
</tr>
<tr>
<td>3</td>
<td>1.0155</td>
<td>3</td>
<td>0.9701</td>
<td>3</td>
<td>0.8883</td>
</tr>
</tbody>
</table>

\((E)\)-resveratrol 332.9 ± 0.4 µg/g (\((E)\)-resveratrol 319.6 ± 1.3 µg/g (\((E)\)-resveratrol 279.9 ± 4.9 µg/g

Figure 46. Mass fragmentation pattern of \((E)\)-resveratrol and piceatannol (Stella et al. 2008).
Figure 47. Comparison of GCMS mass fragments of Sephadex LH-20 column chromatographed and acetylated black spruce bark extract with (E)-resveratrol triacetate standard.
Figure 48. Comparison of LCMS mass fragments of purified black spruce bark extract with (E)-resveratrol standard.

Quantitative Analysis of Maine Grown Cold Hardy Grapes

External standard: 4-Phenylphenol 260 µg/mL in methanol, 100µL per 1.0 mL sample.

Table 15. HPLC data for calibration curve (Analysis of grapes).

<table>
<thead>
<tr>
<th>[Resveratrol] mg/mL</th>
<th>Ratio between peak area for Resv. and ES</th>
<th>Average peak area ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
</tr>
<tr>
<td>120</td>
<td>5.8670</td>
<td>5.8632</td>
</tr>
<tr>
<td>60</td>
<td>2.9486</td>
<td>2.9452</td>
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<tr>
<td>30</td>
<td>1.5164</td>
<td>1.5158</td>
</tr>
<tr>
<td>15</td>
<td>0.7236</td>
<td>0.7236</td>
</tr>
<tr>
<td>7.5</td>
<td>0.3577</td>
<td>0.3580</td>
</tr>
<tr>
<td>3.75</td>
<td>0.1790</td>
<td>0.1787</td>
</tr>
<tr>
<td>1.875</td>
<td>0.0854</td>
<td>0.0855</td>
</tr>
</tbody>
</table>
Figure 49. Calibration curve for the quantitative analysis of grapes.

Table 16. (E)-resveratrool content in cold-hardy varieties of grapes.

<table>
<thead>
<tr>
<th>Code</th>
<th>Variety</th>
<th>Date picked</th>
<th>Date processed</th>
<th>Date extracted</th>
<th>Amount of (E)-resveratrool µg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL-1027</td>
<td>Alpenglow</td>
<td>10/27/2014</td>
<td>10/31/2014</td>
<td>3/14/2016</td>
<td>4.87 ± 0.08</td>
</tr>
<tr>
<td>BR-106</td>
<td>Brianna</td>
<td>10/6/2014</td>
<td>10/10/2014</td>
<td>2/26/2016</td>
<td>9.79 ± 0.02</td>
</tr>
<tr>
<td>CH-1015</td>
<td>Chontey</td>
<td>10/15/2014</td>
<td>10/20/2014</td>
<td>3/1/2016</td>
<td>6.02 ± 0.02</td>
</tr>
<tr>
<td>FG-1014</td>
<td>Frontenac Finis</td>
<td>10/14/2014</td>
<td>10/20/2014</td>
<td>3/4/2016</td>
<td>10.15 ± 0.01</td>
</tr>
<tr>
<td>HU-1029</td>
<td>Hungarian</td>
<td>10/29/2014</td>
<td>10/31/2014</td>
<td>2/26/2016</td>
<td>Not detected</td>
</tr>
<tr>
<td>MF-106</td>
<td>Marechal Foch</td>
<td>10/6/2014</td>
<td>10/10/2014</td>
<td>2/29/2016</td>
<td>12.56 ± 0.23</td>
</tr>
<tr>
<td>MA-107</td>
<td>Marquette</td>
<td>10/7/2014</td>
<td>10/10/2014</td>
<td>1/28/2016</td>
<td>19.93 ± 0.18</td>
</tr>
<tr>
<td>PS-1020</td>
<td>Prairie Star</td>
<td>10/20/2014</td>
<td>10/31/2014</td>
<td>3/7/2016</td>
<td>21.21 ± 0.23</td>
</tr>
<tr>
<td>RE-106</td>
<td>Reliance</td>
<td>10/6/2014</td>
<td>10/10/2014</td>
<td>3/10/2016</td>
<td>7.65 ± 0.17</td>
</tr>
<tr>
<td>SA-1014</td>
<td>Sabrevois</td>
<td>10/14/2014</td>
<td>10/20/2014</td>
<td>2/23/2016</td>
<td>13.82 ± 0.27</td>
</tr>
<tr>
<td>SP-1020</td>
<td>St. Pepin</td>
<td>10/20/2014</td>
<td>10/31/2014</td>
<td>3/8/2016</td>
<td>10.90 ± 0.10</td>
</tr>
</tbody>
</table>
Figure 50. LCMS total ion chromatogram and mass spectrum of (a) Prairie Star grape extract, and (b) standard solution of (E)-resveratrol.
APPENDIX C - GLASS SILANIZATION PROCEDURE

All glassware were carefully cleaned with Piranha solution (concentrated H$_2$SO$_4$ and 30% H$_2$O$_2$ in 3:1 volume ratio) followed by distilled water, and thoroughly dried, before silanization. A 5% (v/v) solution of dimethyldichlorosilane (DMDCS) was prepared in toluene and the clean and dry glassware were soaked in it for about 15 minutes. Then they were rinsed twice with toluene and once with methanol, prior to the air-drying for 24 hours.
BIOGRAPHY OF THE AUTHOR

Panduka Siribandara Piyaratne was born in Sri Lanka on February 18, 1984. After completing his high school education at Dharmaraja College, Kandy, Panduka entered University of Colombo, Sri Lanka, to pursue a Bachelor’s degree in Science. In 2010, he graduated from University of Colombo with an honors degree in Chemical Biology. After completing a one-month internship at the Industrial Technology Institute of Sri Lanka, and a three-month internship at HayCarb PLC in Sri Lanka, Panduka joined the National Dangerous Drugs Control Board of Sri Lanka in 2011, as a Scientific Officer. In 2012, he joined Sri Lanka Customs as an Assistant Superintendent in the Narcotics Control Division. He entered the graduate program of the Department of Chemistry at the University of Maine, in the fall of 2012. Panduka has been a Teaching Assistant for the Organic Chemistry and Analytical Chemistry programs of the department from 2012 to 2018, and he is the recipient of 2017 Corell Graduate Fellowship, and Graduate Student Excellence in Teaching award from the College of Liberal Arts and Sciences.

Panduka is a candidate for the Doctor of Philosophy degree in Chemistry from the University of Maine in May 2018.