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# Optimization of Microwave Accelerated Extraction of Resveratrol from Tree Bark

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# OPTIMIZATION OF MICROWAVE ACCELERATED EXTRACTION OF RESVERATROL FROM TREE BARK

by

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## Abstract

Resveratrol is a compound with various properties of interest. Traditionally it is extracted from tree bark via a Soxhlet extraction. Due to the high energy cost of Soxhlet extraction, this project studied a possible alternative method known as microwave accelerated extraction. Microwave trials were run testing solvents and times of bark extraction and compared to a traditional Soxhlet extraction on the same tree sample. Preliminary results suggest that microwave extraction may be more efficient than Soxhlet extraction. A microwave extraction using 0.5 g of Norway spruce bark, 5 mL of ethanol, run for 5 minutes yielded 14.74±0.79 μg/g of bark. A traditional Soxhlet extraction required 5g of bark in 250 mL of ethanol and a run time of 6 hours. It extracted 12.55 μg/g of bark.



**Keywords:** resveratrol, microwave accelerated extraction, Soxhlet extraction

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## Introduction

Wood has found numerous uses in human society from an energy rich and direct source of fuel to the source of celluloses and hemicelluloses. Cellulose especially, is the parent compound to many products we use. These can range from food additives to textiles as well as the traditional uses namely, paper and building materials. Trees, apart from being the source of that woody biomass can also serve as a source of some chemicals. The lesser known component of woody biomass called extractives have various chemical properties. Compounds that are considered extractives fall into such categories as fatty acids, flavonoids, and stilbenes to name a few. The properties of extractives as well as methods of extraction are the subjects of many studies.

### Resveratrol

Resveratrol (Figure 1) is a compound of interest in human health. It has been found to have healthful effects in regards to cardiovascular health, cancer treatment, and prevention of inflammation. It became well known as a result of an attempt to explain what is known as the "French paradox" (Rimando *et al*. 2009). The idea behind this paradox is that the French generally have a diet high in fat content. However it was noted that despite this diet they had an unexpectedly low occurrence of cardiovascular disease. This was attributed to the presence of resveratrol in red wine, which led to many studies on the health effects of red wine and grapes and resveratrol.

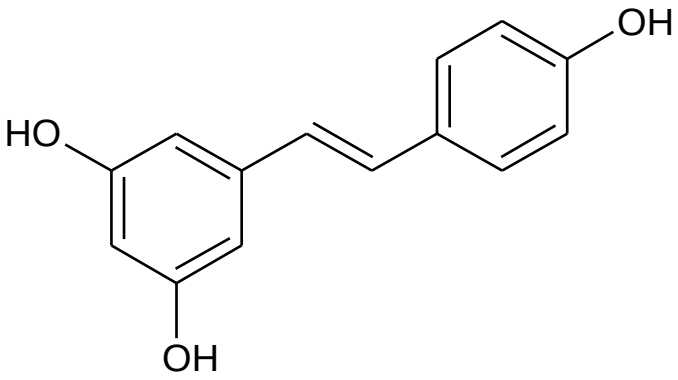



Figure 1: *trans*-resveratrol

Chemopreventive activity

Resveratrol is an extractive of woody biomass under the category of stilbenes. It has various health properties of interest to scientists. Among its potential health benefits is the possibility that it could be used as a drug against cancer. Cancer therapy is highly dependent on cell cycle arrest and induction of apoptosis. Resveratrol has the ability to induce apoptosis in some cancer cells in a cell type and concentration dependent manner (Kraft *et al*. 2009).

Weng *et al.* (2009) studied the apoptotic effects of resveratrol on lung carcinoma cells. They observed that resveratrol could induce apoptosis (programmed cell death) in which the carcinoma cells are induced to self destruct. Resveratrol and other polyphenolics could individually cause damage to the cancer cells or completely prevent them from growing depending on the cancer cell line. Resveratrol in particular, had the ability to induce apoptosis in cancerous cells. In essence, they found that resveratrol and related polyphenolics had the potential to be a viable alternative to chemotherapy with respect to lung cancer.

Antioxidant activity

Resveratrol is an antioxidant. This means that it has the ability to protect living cells from what are called free radicals. These are compounds created from unused oxygen in our bodies that have the ability to damage or kill cells and have been linked to various diseases. For this reason and others, antioxidants such as resveratrol are of particular interest to the scientific community though antioxidant activity is currently a point of controversy in regards to health benefits.

Rimando *et al.* (2009) provide an account of the content of resveratrol in various berry species. They describe some of the purported benefits of resveratrol as well as some other antioxidants of interest. Resveratrol, pterostilbene, and piceatannol are compounds found in nature under the category of stilbenes and more specifically phenolics. They can be found in grapes and wine among other places. It has shown anti-cancer activity in all three stages of cancer (initiation, promotion, and progression) *in vitro* (Rimando *et al.* 2004). Such possible benefits certainly explain the interest taken in compounds such as resveratrol. Part of that interest is in locating sources of resveratrol in nature other than grapes as a means of studying its effects. It may also make waste products such as tree bark become a new source of revenue if resveratrol can be extracted from it.

Dani *et al.* (2008) examined the antioxidant effects of resveratrol. They observed that resveratrol protected the eukaryotic microorganism *Saccharomyces cerevisia* (a species of baking yeast) against oxidative stress (Dani *et al*. 2008). The oxidative stress was induced by hydrogen peroxide, carbon tetrachloride, and cadmium. It was found that resveratrol and another antioxidant, catechin, increased the ability of the organism to survive oxidative stress particularly when subjected to the stress induced by contact with

peroxide. This kind of interaction can happen with cells in the human body. The benefit is that in protecting against oxidative stress, they protect against several acute chronic disorders (Dani *et al*. 2008).

Pezzuto (2008) studied grapes and their health benefits presumably dependant on their content of resveratrol. Pezzuto gives a basic summation of the purported benefits and possible correlations between health and grape consumption. Grapes are currently one of the greatest sources of resveratrol (Pezzuto 2008). It is, therefore, worth studying grapes for their content of resveratrol to see if they could have positive effects on human health. Numerous studies suggest that the cardiovascular system benefits from grape consumption. There is some anticancer activity as well, particularly in regards to skin cancer.

The resveratrol content of grapes has led to studies on the health benefits of consuming grapes. That said, there are supplements of antioxidants currently on the market so that consumption of grapes is not the only option for obtaining resveratrol. The idea of creating a resveratrol supplement is a useful one simply because the amount of resveratrol one obtains from each grape may not necessarily be consistent. Often the amount of resveratrol obtained is rather small and the supplements are often impure. Whether obtaining resveratrol through supplements is better than through food is a question for future studies.

## Extraction

The properties of resveratrol and other extractives led to the necessity to study extraction methods. Various methods exist and have been tested. These methods include, but are certainly not limited to, Soxhlet extraction, microwave accelerated extraction, and even a combination of the two.

Traditionally it involves the use of a large amount of solvent and has some advantages such as enhanced displacement of the analyte from the matrix. The main issue, however, consists of the amount of time taken to perform this extraction. They used a traditional Soxhlet apparatus coupled with a microwave source to irradiate the sample solvent condensed around the solid sample. Garcia-Ayuso *et al*. (1998) found that by applying the microwave irradiation to the Soxhlet extraction they could reduce what was an 8 hour extraction to one taking between 50 and 60 minutes. They also found that microwave extraction allowed them to recycle their solvent, reducing overall environmental impact of the Soxhlet process. If there is a way to improve the rate of extraction and keep the advantages of Soxhlet extraction, it is of great use to chemistry. Microwave accelerated chemistry is of interest specifically because of its potential ability to accelerate reactions and potentially replace the Soxhlet extraction, reducing both solvent volume and reaction time.

**Microwave Accelerated Chemistry**

Microwave assisted chemistry has been around for some time now. The first organic synthesis assisted by microwaves was reported in 1986 in the journal *Tetrahedron Letters* by Gedye *et al*. (1986). It was noted that an advantage in rate of reaction existed as a result of the use of the microwave. Shorter reaction times were found for common organic processes including esterification, hydrolysis, etherification, addition, and rearrangement.

Originally chemists used conventional microwave ovens for experimentation. Such an appliance lacked real scientific controls. This made it difficult to get consistent and reproducible results. It was clear that it was potentially advantageous to use microwaves in chemistry and it wasn't long before safer methods of applying microwaves were found. This included solvent free reactions as well as scientific controls on temperature and pressure that could help prevent explosions when using volatile solvents. Once the method had been made safe it was found that with little energy applied to the reaction, one could often obtain higher yields as well as purer products in less time. It was also found that microwave chemistry allowed for the use of less toxic and environmentally destructive reagents and solvents (Larhed *et al*. 2002). As more came to be learned about microwave chemistry, two variations of it evolved: open vessel and closed vessel microwave chemistry (Roberts and Strauss 2005). Depending on the reaction, one variation would be preferred over the other.

The full range of microwave frequencies goes from 0.3 to 300 GHz. For the purposes of heating substances a frequency of 2.45GHz is conventionally used. The

particular advantage of microwaves is the ability to achieve heating *in situ*. That is to say that heating of the reaction mixture happens directly without needing to heat the walls of the vessel first. Figure 2 provides a comparison of *in situ* and classic wall heating.

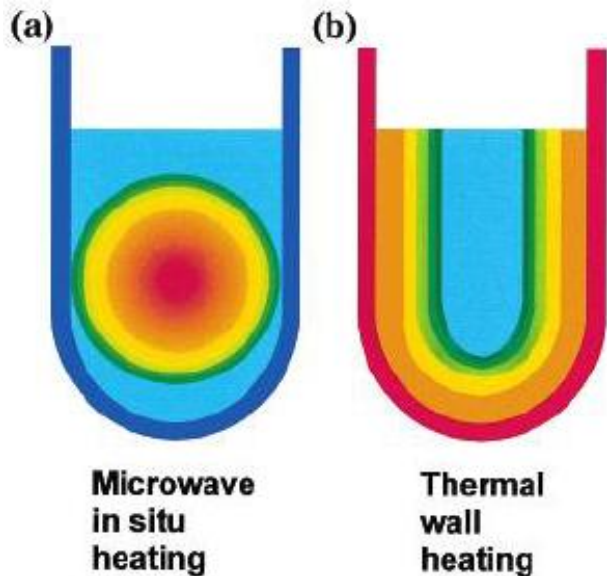



Figure 2. (a) Schematic illustration of microwave *in situ* heating. The heat is transferred directly into reaction mixture. (b) Classic wall heating. The heat energy must be transferred via the vessel wall. The darkest color indicates the most intense heat. (Larhed *et al*. 2002).

The ability to heat *in situ* provides both faster as well as more efficient heating of the reaction mixture. This allows for a faster reaction and overall reduced energy usage. *In situ* heating has the added advantage of minimizing wall effects, and therefore seed formation which prevents precipitation, meaning that superheating can occur (Larhed *et al*. 2002).

As with anything, there are some disadvantages to microwave chemistry. One of the greater disadvantages is in how much reaction mixture can be processed at once. It can be difficult to scale up or even scale down the process. To solve this problem continuous flow reactors have been developed. They allow a mixture to flow through the microwave zone. By the time the mixture has arrived at the other end of the microwave

zone the intended reaction is complete. It has been reported that commercial systems have had up to 120 mL within the zone and could be operated at a flow rate of up to 100 mL/min allowing for scaling up or down (Roberts and Strauss 2005). Figure 3 shows a schematic of a CMR (continuous microwave reactor).

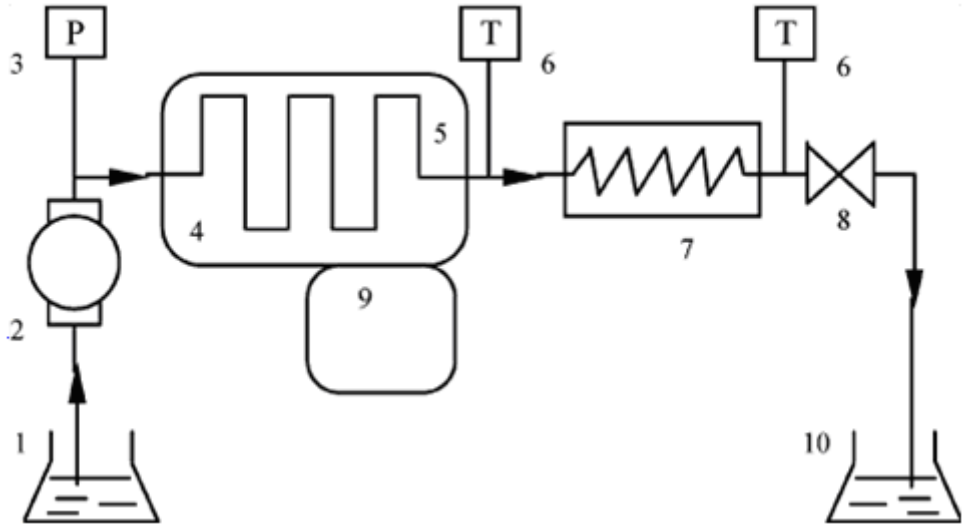


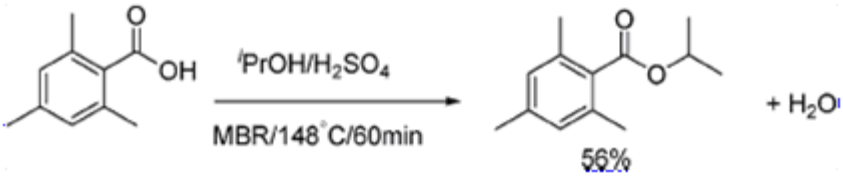

Figure 3 is a schematic diagram of the CMR. 1, reaction mixture; 2,metering pump; 3, pressure transducer; 4, microwave cavity;, 5, reaction coil; 6, temperature sensor; 7, heat exchanger; 8, pressure regulator; 9, microprocessor controller; and 10, product vessel. (Roberts and Strauss 2005).

## Applications of Microwave Chemistry

Improved rates of reaction and yield

Microwave reaction has found many homes within various fields of chemistry, particularly in organic chemistry. Roberts and Strauss (2005) saw the ability to reduce the environmental impact of chemistry as a particularly useful advantage. The heating ability provided by microwave chemistry reduces the need for what are often toxic catalysts as well as some especially toxic reaction solvents. Its greatest advantage is the faster reaction times it allows. Reactions involving highly sterically hindered molecules such as

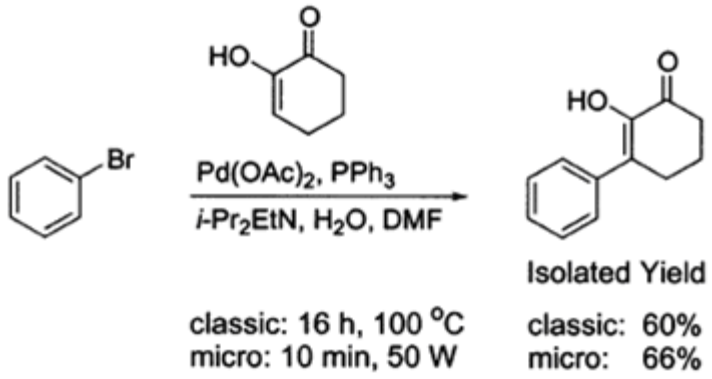
2,4,6-trimethylbenzoic acid in an esterification (Scheme 1) could be reduced from a matter of days of reflux to a period of 1 hour at high temperatures.




Scheme 1. Esterification of 2,4,6-trimethylbenzoic acid (Roberts and Strauss 2005).
Similarly 2,4,6-trichlorophenol could proceed through methylation in 54 minutes as opposed to 192 hours by conventional heating methods (Roberts and Strauss 2005).

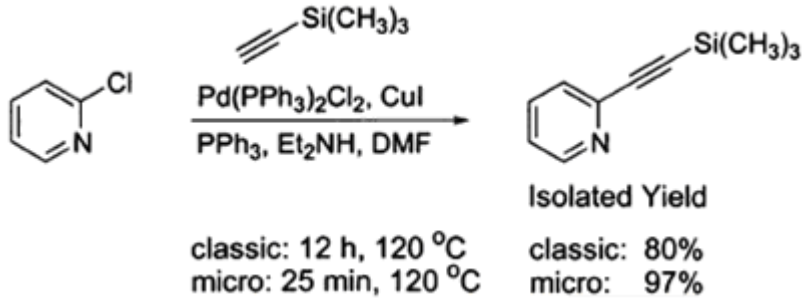
There are plenty of other examples of the heating advantages of microwave reactors.

Larhed *et al*. used microwave chemistry to accelerate homogeneous catalysis in organic chemistry. Four types of homogeneous catalysis reactions are most often reported as assisted by use of microwave chemistry. They are Heck reactions, Sonogashira reactions, cross-coupling reactions, and allylic substitutions. Particular to Heck reactions is the Heck arylation of olefins. Such reactions often require times ranging from hours to days even when using a catalyst, and reaction temperatures ranging from 60 to 120˚C (using conventional heating) above which the chemistry would fail. The following equation (Scheme 2) gives an example of a Heck reaction under both classical heating and microwave heating conditions.




Scheme 2. Heck arylation involving 1,2-cyclohexanedione with an aryl bromide. (Larhed *et al*. 2002).

There is a great decrease in the time needed to complete the reaction through microwave heating and the microwave reaction increased the product yield by 6%. It has been found that microwave heating can reduce the times, in many cases, down to a matter of minutes. Sonogashira coupling reactions are closely related to Heck reactions. One particular example involved 2-cholropyridine in reaction (Scheme 3).

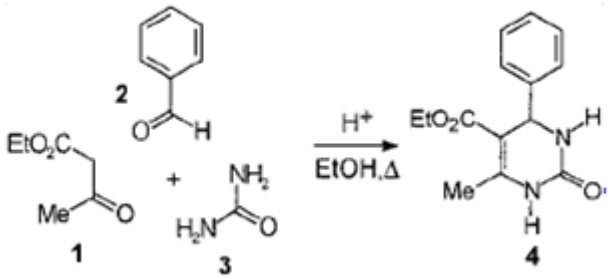



Scheme 3. 2-chloropyridine reacted with an alkyne in Sonogashira coupling reaction. (Larhed *et al*. 2002).

The reaction proceeded in 25 minutes by microwave irradiation versus the reported very slow reaction time of 12 hours for this particular compound in traditional heating. There was even a notable increase in product yield. Cross coupling reactions can be broken into Suzuki reactions and Stille reactions. One example involved phenyl-boronic acid being coupled with 4-methylphenylbromide in dimethoxyethane/water in less than 4 minutes through the use of microwaves. The Stille reaction, which involves coupling to

organopalladium intermediates, had similar results. Reactions at 80˚C could take 1 day conventionally but by microwave chemistry could be reduced to 2 or less minutes. Highly enantioselective palladium catalyzed allylic substitutions have also been shown to gain from the use of microwave irradiation (Larhed *et al*. 2002).

Kappe (2000) applied the use of microwave chemistry to the Biginelli dihydropyrimidine synthesis. This reaction is a multi-component reaction. Such reactions generally involve three or more reactants in the vessel at one time. In the case of Biginelli, three reactants were used.




Scheme 4 Biginelli dihydropyrimidine synthesis (Kappe 2000).

One issue with this process was the low yield encountered with the use of some reactants. Kappe reported that yield and rate enhancements were significant when microwave irradiation was applied to this synthesis especially when coupled with neat polyphosphate ester as a reaction mediator. It could be performed on the 1-50 mmol scale in an average domestic microwave with reaction times running less than 2 minutes (Kappe 2000)

Horvath (2010) described a type of chemistry known as fluorous biphase chemistry. Its purpose was derived from the miscibility of perfluoroalkanes, perfluorodialkyl ethers, and perfluorotrialkyl amines. The term fluorous indicated that the chemical transformation was mostly controlled by a catalyst that was designed to dissolve preferentially in the fluorous phase. The fluorous phase was simply the phase of the reaction during which any of the above perfluoro compounds were particularly abundant.

The biphase system was thus one in which the fluorous phase existed along with the second product phase which used an organic or inorganic solvent that was not soluble with the fluorous phase. Fluorous chemistry was useful for its high selectivity. For example it could separate products and catalysts at rather mild conditions. Some of its thermal reactions can be rather slow however. In particular a fluorous tin reagent was used in a cross coupling Stille reaction at 80˚ C. Instead of taking the usual day to react, it was completed in 90 to 120 seconds when run under microwave irradiation.

Polshettiwar and Varma (2008) wrote on their recent activity in applying microwave technology to environmentally friendly or "green" chemistry. The ability of microwave technology to help make chemistry greener came from its ability to employ the use of benign reaction media, use solvent free conditions, and use solid supported reusable catalysts. It has, therefore, been very useful in making organic synthesis reactions more environmentally friendly. One example of the use of microwave technology that they wrote about was in the synthesis of heterocycles. Microwave flash heating was described as bringing the reaction times down from a matter of days and hours to minutes and seconds. Microwave technology was useful in nitrogen-containing heterocycles, oxygen-containing heterocycles, and heterocyclic hydrazones.

**Application to Extraction**

Kwon *et al*. (2003) performed a study on the efficacy of microwave assisted chemistry in the extraction of components of the health food ginseng. The particular component being sought after was saponin. Kwon reports that having gone through the extraction process and analyzed the various trials it was found that microwave extraction

did not increase the yield. That was dependent on solvent. However, the use of the microwave as the heat source did serve the purpose of greatly increasing the speed of the reaction. In the conclusion it was noted that future research might be done to study the time of extraction with variations of microwave power.

Vasquez *et al*. (1997) described a method for extracting methyl mercury from aquatic sediments that incorporated the use of microwave assisted extraction. The purpose of the study was simply to develop a quick method for the extraction of methylmercury from freeze dried aquatic sediments. The microwave extraction was performed with the assistance of hydrochloric acid and toluene as the organic solvent. Due to the potential for the solvent to affect extraction efficiency, many trials were performed. The primary utility of the microwave was its ability to extract many samples at one time and to do so in much less time than normally required by conventional heating. The microwave extraction set was completed in less than 30 minutes whereas conventional extraction methods required 2-3 hours. It was also found that the microwave extraction procedure allowed for the recovery of greater amounts of methyl mercury than the conventional method.

Raman and Gaikar (2002) studied the extraction of piperine from powdered black pepper. The interest in piperine resides in its uses in medicine as a bioavailability enhancer in the production of certain drugs. Some groups of piperine may also be applied to use in insecticides. The conventional method for extracting piperine is via solvent extraction using aliphatic and chlorinated hydrocarbons. The extraction times can vary within 16 to 24 hours and the process is rather inefficient in its selectivity. This experiment used the *P. nigrum* species of pepper fruit. The results showed that extraction

of piperine that had not been irradiated in the microwave both slow and low yielding 20% in one hour. In the case where microwave irradiation was used for 2 minutes there was an 80% increase in the recovery of piperine. Raman and Gaikar concluded that microwave assisted extraction was effective for rapid and selective extraction of piperine.

Pylypiw *et al*. (1997) studied the use of microwave assisted extraction for multi-residue pesticide analysis. They systematically studied the ability of microwave assisted extraction in testing various crops for pesticide content. They used five crops and seven pesticides. The conventional means of extraction is by blender. They found that after determining the parameters for efficient microwave extraction they were able to get reproducible results that were equal to or better than the results of blender extraction.

According to Guzman Mar *et al*. (2009) rice is known to have various concentrations of arsenic (As) and selenium (Se). Arsenic is a well known toxin to humans and selenium is good for the body only within a minimal range of ingestion. Guzman Mar *et al*. developed a method for using microwave assisted enzymatic extraction as a means to analyze the content of selenium and arsenic in rice. In trying to find the conditions for maximum extraction of selenium and arsenic based compounds they found that microwave assisted extraction in combination with enzymatic treatment was the best way to extract optimal amounts of As and Se from the rice flour.

Mattina *et al*. (1997) employed microwave extraction in studying the removal of taxanes (specifically palitaxel) from Taxus needles. In employing microwave extraction they also searched for the optimal conditions for the extraction. Through the experimentation and optimization they found parameters for which microwave assisted extraction showed distinct advantages over traditional extraction methods. They found

that they could change solvent from methanol to 95% ethanol which is less expensive. The amount of solvent used per extraction was also reduced. For 5 g of fresh needles 10 ml of 95% ethanol was used for a 9-10 minute extraction by microwave at 85˚C. Under these conditions 90% of taxanes were recovered. The traditional method required 100 mL of methanol and an overnight shake of the biomass in solvent. The fact that microwave assisted extraction could be optimized to achieve 90% of product in such a relatively short time with less solvent is quite an achievement and is a testament to the effectiveness of microwave chemistry.

As we have seen, microwave extraction has a prominent place in organic chemistry. With that in mind it is no surprise that we would consider its use for the extraction of resveratrol. The main extraction technique used to analyze the various portions of trees for resveratrol has been the Soxhlet extraction. In our research group we want to find out if microwave extraction can be optimized for the extraction of resveratrol from trees. The microwave extraction parameters that have been used so far don't give results comparable to the Soxhlet extraction. The goal of this project therefore is to determine if the parameters exist for efficient microwave extraction of resveratrol from tree bark. The variables that will be tested are solvent, temperature, and extraction time.

## Methodology

### Sample Collection and Preparation

Bark samples were obtained from healthy trees within the University of Maine research forest. Identification of the tree samples was made with the assistance of Francis Avery and the University of Maine School of Forestry. The ages of the trees were determined by the growth rings. The species and ages are as follows: *Picea abies* (Norway spruce), 12 years old; *Picea abies* (Norway spruce) 18 years old; *Larix laricina* (larch), 8 years old; *Larix laricina* (larch), 12 years old; and *Picea mariana* (black spruce), 27 years old.

The trees were cut down and collected in July 2008 (black spruce 27 years old), July 2010 (Norway spruce 12 years old), November 2010 (larch 8 years old and larch 12 years old), and March 2012 (Norway spruce 18 years old). The branches and foliage were removed from the trees and the trunks were debarked manually using a draw knife. The strips of bark were cut into smaller pieces and allowed to air dry for a minimum of 72 hours. The bark was then ground into a meal using a Wiley mill. The 20-40 mesh fraction was collected. The samples were labeled and stored in the freezer at -18 ˚C.

### Materials

Resveratrol standard and heneicosanoic acid were purchased from Sigma-Aldrich. The sylilating agent N,O-bis(trimethylsilyltrifluoroacetamide) (BSTFA) was purchased from UCT. Solvents including ethanol, methanol, ethyl acetate, and hexanes were all HPLC grade and were purchased from Fisher Scientific.

**Moisture Content**

Moisture content of the bark was needed to determine the dry weight of the bark to be used in the quantification of extractives. About 0.5000 g of sample was weighed out into three pre-weighed sample vials. These samples were dried in an oven at 100 ˚C for a minimum of 24 hours. The samples were then reweighed and returned to the oven for another 24 hours minimum. Once more, the samples were weighed and that weight was compared to the previous weight after 24 hours in the oven. If the change was statistically negligible the bark was considered dry and the moisture content was calculated. If the difference was not negligible, then the samples were returned to the oven for another 24 hours. The process was repeated until the change in weight from one drying to the next was negligible. The moisture content was calculated based on the change from the original bark mass to the dry bark mass using the equation $\frac{AirDriedMass - OvenDriedMass}{AirDriedMass} \times 100\%$. The final moisture content was taken as the average of the three trials. Table 1 shows a sample moisture content experiment for the 12 year old Norway spruce.

Table 1: Moisture content determination of bark from the 12 year old Norway spruce

| Moisture Content | | | | |
|---|---|---|---|---|
| Sample # | Vial Weight (g) | Initial Bark Weight (g) | Final Bark Weight (g) | % Moisture |
| N.S. Sample 1 | 13.2731 | 0.5096 | 0.4233 | 16.41 |
| N.S. Sample 2 | 13.1045 | 0.4956 | 0.4160 | 16.06 |
| N.S. Sample 3 | 13.2680 | 0.4548 | 0.3812 | 16.18 |
| Average Moisture Content | | | | 16.21±0.18 |

For quantification of resveratrol in the bark, the dry weight of the bark is necessary. The dry weight of any extracted sample was calculated as follows: mass of sample = air dried mass * $(1-\frac{MoistureContent}{100})$.

Example: For a 2.0000g (air dried) sample,

the dry weight = $2.0000*(1-\frac{16.21}{100}) = 1.6758$g.

**Extraction Methods**

Microwave Extraction

Microwave extractions were carried out using a Biotage Initiator 2.0 Microwave reactor (Figure 4). Samples extracted using this microwave were weighed out to either 0.5000 g or 2.0000 g. The samples were then extracted using 5.00 mL and 20.00 mL of solvent, respectively. The reactor functions by heating the reaction mixture with high-frequency microwaves (2.45 MHz). Extraction parameters that could be altered were solvent, temperature, and length of extraction. Initial parameters were determined based on prior work done by Regan LeBlanc (LeBlanc, 2010) and Sara Knowles (Knowles, 2010). Extractions were performed using various solvents for various lengths of time. The temperature used was always 65 ˚C. A stir bar was included in all microwave vials for stirring. Pre-stirring prior to heating was set for 30 seconds and stirring would then continue throughout the extraction period.

Figure 4: Biotage® Initiator 2.0 Microwave Synthesizer

## Soxhlet Extraction

As a method of comparison, Soxhlet extractions were also performed on the various bark species. Soxhlet extraction (like microwave extraction) is a solid-liquid extraction method. It is often used to remove extractives from biomass material. For this particular type of extraction, ethanol was exclusively used as the solvent. The apparatus of a Soxhlet extractor is shown in Figure 5. For our experiments 5.0000 g of bark sample was weighed into the cellulose thimble placed in the center of the apparatus. The ethanol (250 mL) was measured into the round bottom flask with a few boiling stones and connected to the Soxhlet glassware. At the top of the Soxhlet apparatus was placed a condenser. The ethanol was heated to boiling so that the vapors would rise through the side arm to the condenser. The condensed ethanol dripped back into the chamber with the bark sample. The solvent collected around the bark up to a certain volume at which point the solvent was automatically siphoned out of the chamber and back into the round bottom flask carrying with it any extractives that dissolved into it. This process was carried out for 6 hours.

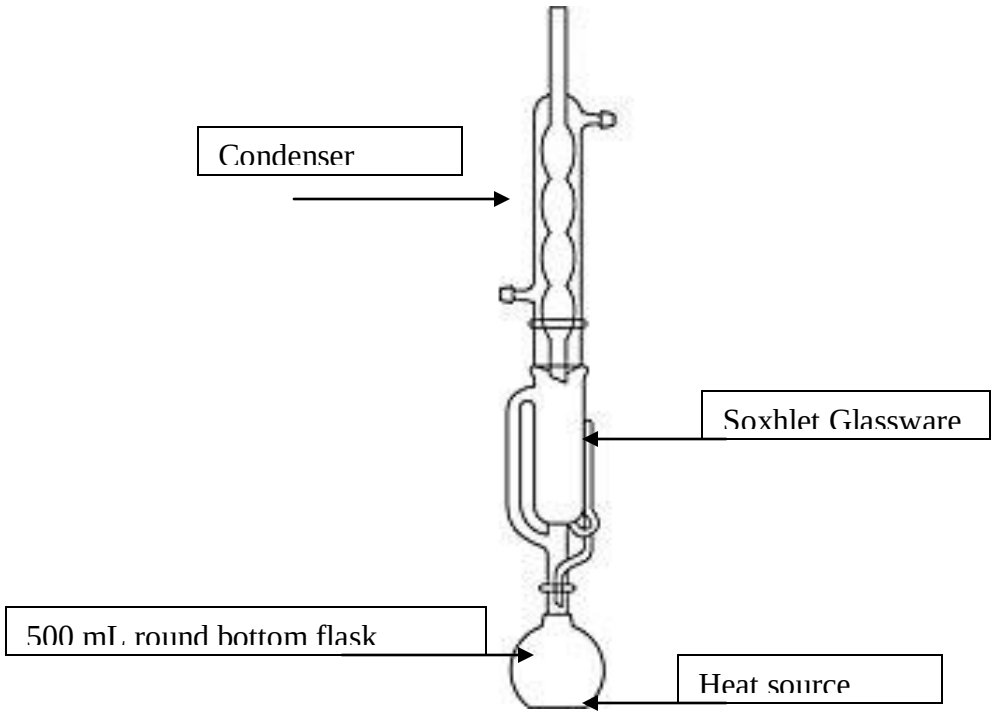



Figure 5: Schematic of a Soxhlet apparatus (Grainger Industrial Supply)

Separation Procedure

The microwave extraction and Soxhlet extraction separation procedures differ slightly. In the case of the microwave extraction the vial cap was removed and the extract was separated from the bark via vacuum filtration. The vial and bark were rinsed until the solvent ran clear and the bark sample was dry. The extract was collected into a sample vial and the solvent was evaporated by running nitrogen gas over the sample as it sat in an approximately 90 ˚C water bath. The residue was then dissolved in 15.00 mL of nanopure® water and sonicated for 20 minutes. Resveratrol is not very soluble in water. Its solubility is about 0.3mg/mL in water which leads to the necessity for relatively large volumes of water. The aqueous solution was then put through liquid-liquid extractions in order to separate the resveratrol from most of the other extractives that came from the bark. For the microwave sample size, the 15.00 mL of aqueous solution were poured into

a separatory funnel containing 15.00 mL of hexane. The solution was mixed so as to wash the aqueous layer of any hexane soluble materials. This was performed at least twice for the microwave extracts or until the hexane layer remained clear. The hexane layer did not remove the resveratrol from the water and could be discarded. The aqueous layer was saved for the next set of liquid-liquid extractions

The hexane washed aqueous layer was subsequently washed a minimum of three times using 25.00 mL of ethyl acetate to extract the resveratrol from the water. Generally it took longer for the ethyl acetate layer to separate from the water layer than for hexane. The combined ethyl acetate solution was dried down using a rotary evaporator set to 40 ˚C. The residue was dissolved in 15.00 mL of methanol and stored in the refrigerator until further analysis could be performed.

In the case of the Soxhlet extraction the extract was evaporated using a rotary evaporator after removing the extract to another round bottomed flask to remove the boiling stones. The dried residue was then dissolved in about 100.00 mL or more of Nanopure® water and sonicated for 1 hour. This solution was then washed a minimum of three times with 30.00 mL of hexane. The aqueous layer was kept and then washed a minimum of three times using 50.00 mL of ethyl acetate each time. The combined ethyl acetate layer was then dried down and re-dissolved in 50.00 mL of methanol until further analysis. From this point the procedures for preparing the extractions from microwave and Soxhlet are identical.

Sample Preparation for GC/MS analysis

The properties of resveratrol render it difficult to analyze via gas chromatography coupled with a mass spectrometer (GC/MS) without first derivatizing it so that it may more easily volatilize under high temperature. In order to quantify the resveratrol based on its peak area it must also be combined with an internal standard during analysis. To do this a 1.000 mL aliquot of the sample that was dissolved in methanol was placed into a sample vial. To it was added 0.500 mL of heneicosanoic acid solution. The concentration of the heneicosanoic acid solution was 200.00 μg/mL. The mixture was dried down with nitrogen gas then re-dissolved in 1.000 mL of ethyl acetate and 200 microliters (μL) of the silylating agent BSTFA (Figure 6).

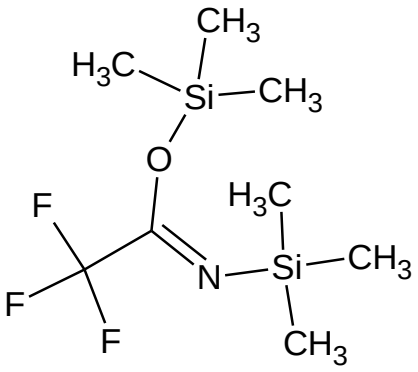
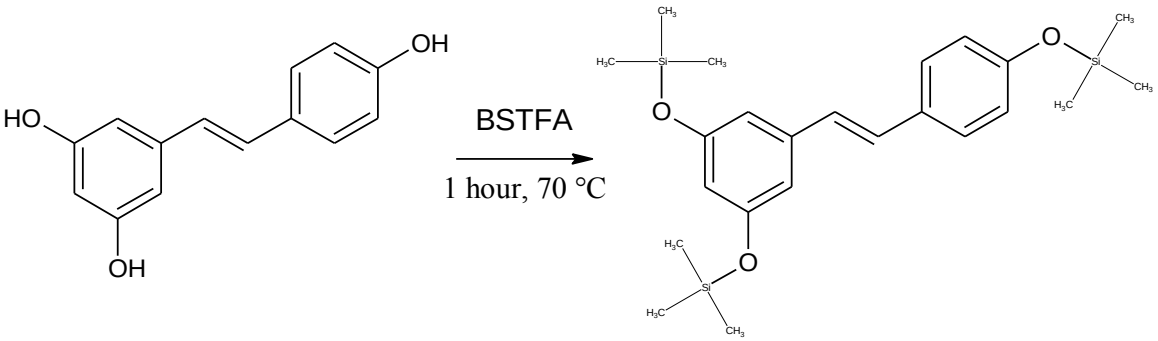



Figure 6: BSTFA

After the addition of ethyl acetate and BSTFA the solution was heated in a 70 ˚C water bath for 1 hour to ensure complete derivatization. The BSTFA reacted with resveratrol by replacing the hydroxyl groups of resveratrol, which eliminated the hydrogen bonding and consequently increased the volatility of the resveratrol. The reaction is shown in Scheme 5. After silylation the solution was transferred to a GC vial.




Scheme 5: Silylation of *trans*-resveratrol

GC/MS analysis

After silylation the samples were ready for GC analysis. Samples were analyzed using an Agilent 6850 Network GC system. An Agilent 5% Phenyl Methyl Siloxane capillary column, (30.0m x 250µm x 0.25µm) was used. Helium was used as the carrier gas at 11.05 psi. Over the course of the project, three GC methods were used, each an improvement over its predecessor. The improved method simply changed the ramping rate such that over all the method took 15 minutes. Later on the ramping was slightly modified to increase separation of peaks. This increased the time of the method to 18 minutes (henceforth the methods shall be referred to by their overall run time). In the 45 minute method the *trans*-resveratrol peak had a retention time of about 33.5 minutes and the heneicosanoic acid showed up around 30 minutes. *Cis*-resveratrol's retention time could not be determined. In the 15 minute method the *trans* isomer of resveratrol was visible at 10.45 minutes, the heneicosanoic acid at 9.57 minutes, and the *cis*-resveratrol was visible at 9.07 minutes. In the 18 minute method the retention times shifted slightly which helped resolve the peaks better. The *trans*-resveratrol was visible at 12.66 minutes, the HA was visible at 11.68 minutes, and the *cis*-resveratrol was seen at about 10.99 minutes, if any was present. The chromatograms were analyzed by determining the peak

areas of the internal standard and the resveratrol isomers (if both were present), then calculating the ratio i.e. the area of resveratrol / area of internal standard. The peaks that were used for calculations were determined from the selected ion monitoring chromatogram (SIM) a function of the software on the GC/MS. Resveratrol was identified by a characteristic m/z peak of 444 (in the selective ion chromatogram) in its mass spectrum along with the its determined retention time for the method that was used.

### Resveratrol Standard

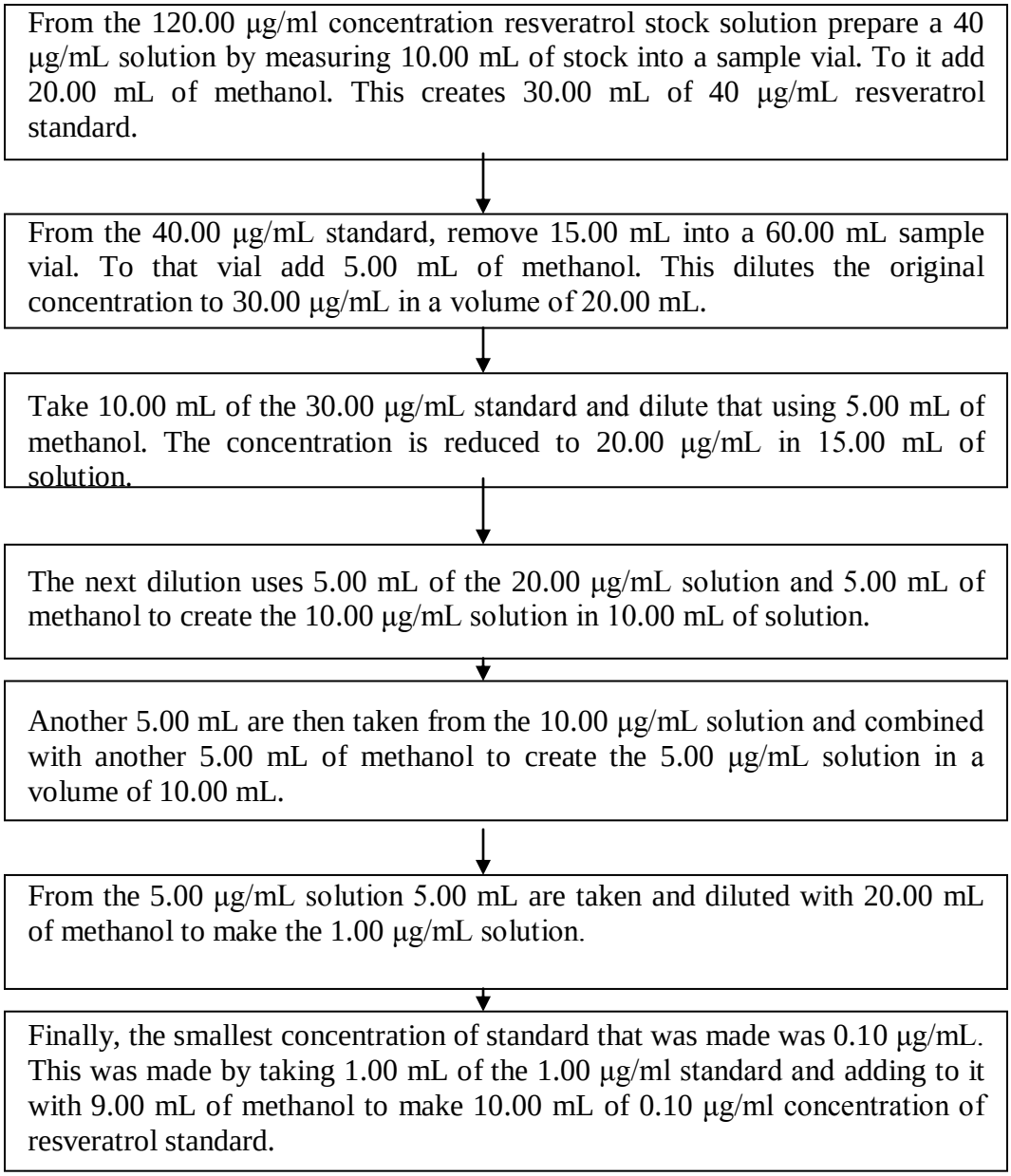
A stock solution of concentration 120.00 μg/mL was prepared by weighing out 0.0520 g of resveratrol standard and dissolving it in 443.00 mL of methanol.

### Heneicosanoic Standard

Heneicosanoic acid standard solution was prepared by dissolving 0.0200g of heneicosanoic acid (HA) in 100.00 mL of methanol. The HA was allowed to sit for some time before being used as it took a while for all of the HA to completely dissolve in the methanol. The concentration prepared was 200.00 μg/mL.

### Standard Curve Preparation

To be able to quantify the amount of resveratrol obtained from unknown samples, a standard curve of resveratrol was created. Standard dilutions were made from a portion of the stock solution of resveratrol. The procedure for making the standard dilutions is shown in Scheme 6.




Scheme 6: Procedure for standard dilutions of resveratrol

The standard curve was created by preparing samples as if they were unknowns. Aliquots from each concentration of standard were placed into separate sample vials. To these vials was added 0.500 mL of HA. The mixtures were dried down by nitrogen then re-dissolved in 1mL of ethyl acetate and 200 μL of BSTFA. The samples were silylated for 1 hour then analyzed on the GC/MS. Samples were prepared in triplicate and all area

ratios were taken from the SIM chromatogram. Table 2 shows the results of the standard curve analysis.

Table 2: Standard Curve Data

| Standard Curve Data | | | |
|---|---|---|---|
| Concentration | Resveratrol Area | HA Area | Ratio Resv/HA |
| 0.10 μ/mL | 186979 | 21073940 | 0.008872522 |
| | 93128 | 17053170 | 0.005461037 |
| | 88580 | 19113536 | 0.004634412 |
| 1.00 μ/mL | 1372736 | 22440667 | 0.0611718 |
| | 1321934 | 24221731 | 0.054576364 |
| | 1471762 | 22848172 | 0.064414869 |
| 5.00 μ/mL | 6834015 | 19235172 | 0.355287439 |
| | 7635051 | 22317056 | 0.342117303 |
| | 7874474 | 22606413 | 0.348329211 |
| 10.00 μ/mL | 14680222 | 23151480 | 0.634094321 |
| | 14439700 | 21897809 | 0.659413003 |
| | 14870295 | 21312464 | 0.69772763 |
| 20.00 μ/mL | 25547028 | 21838182 | 1.169833093 |
| | 26465016 | 23111168 | 1.145118066 |
| | 26305770 | 21735402 | 1.210272992 |
| 30.00 μ/mL | 40037880 | 23948680 | 1.671819908 |
| | 39975974 | 21650040 | 1.8464619 |
| | 41215488 | 24081193 | 1.71152185 |
| 40.00 μ/mL | 46143429 | 22803655 | 2.023510222 |
| | 49652474 | 22602128 | 2.196805274 |
| | 48932812 | 20878444 | 2.343700134 |

The resveratrol area is the sum of the peak areas of the two stereoisomers of resveratrol. *Trans*-resveratrol was the predominant isomer but *cis*-resveratrol could also be found in the chromatograms of the standards and so was included in the total resveratrol content of the standards. Each concentration was analyzed in triplicate and the average ratio was taken to create the standard curve. The standard was fit slightly better by a polynomial than a linear fit so the polynomial fit was used. In theory, the fit would be linear however, the differences were small. The standard curve is shown in the results and discussion section. The equation of the line is $y = 1.7302x^2 + 14.456x - 0.0319$ with an $R^2$ of 0.9994.

Quantification of Resveratrol from Chromatograms

The SIM chromatograms were used to analyze the data from all samples. Our goal in this project was to determine if microwave extraction could become a more efficient method of extracting resveratrol from tree bark. Therefore, it was not unusual to see peaks other than those of resveratrol and heneicosanoic acid in the chromatograms since complete separation of resveratrol from all other extractives was not the focus. To quantify the resveratrol, the peak areas for the heneicosanoic acid, *trans*-resveratrol, and *cis*-resveratrol (if it was present) were recorded and the ratio of total resveratrol to internal standard was determined. That ratio was entered into the equation of the standard curve which gave the concentration of resveratrol in the solution that was analyzed. That concentration was then multiplied by the volume of the original methanol solution (15.00 mL for microwave samples 50.00 mL for Soxhlet extractions) and then divided by the dry weight of the bark sample used for the extraction (based on moisture content). That calculation resulted in a value with the units of micrograms of resveratrol per gram of dry bark (μg/g of dry weight) which are the units often used to report resveratrol content in the literature. The following is a sample calculation for a sample of bark weighed out as 2.0000 g. Its final extract was dissolved in 15.00 mL of methanol and the moisture content of the bark was 16.00%. The ratio of resveratrol to internal standard is 0.500 for the purposes of this calculation. First the ratio is entered as the value of "x" in the equation for the standard curve:

$y = 1.7302(0.500)^2 + 14.456(0.500) - 0.0319$

$y = 7.629$ μg/mL = concentration of resveratrol in the GC vial.

This calculated concentration is then multiplied by 15.00 mL (the volume of the methanol solution). That gives a value of 7.629 μg/mL x 15.00mL = 114.4 μg of resveratrol. This value is then divided by the dry weight of the bark which is 2.000 g – (2.000 g x 16.00%) = 1.680 g. The final calculation is 114.4 μg resveratrol / 1.680 g of bark
= 68.09 μg resv./g of dry bark which is the number to be reported and compared to other values of resveratrol content.

## Results and Discussion

The goal of this project was to determine if microwave accelerated extraction could be an efficient method of extracting resveratrol from tree bark. The expectation was that microwave accelerated extraction (MAE) should be able to extract resveratrol as effectively as the Soxhlet extraction in a much shorter period of time. Various trees were tested over the course of this project for resveratrol content. It was necessary that the resveratrol content be detectable even in an un-optimized microwave extraction. Finding such a tree proved difficult and in conjunction with inconsistencies with the gas chromatography mass spectrometer, the goal of determining the effectiveness of MAE in regards to extracting resveratrol from bark was not reached. Below are the data that were collected through the course of this project.

### Standard Curve

A lot of time was spent making standard curves. In the beginning it was spent refining the method of creating a standard curve. Towards the end of the project, however, the standard curve was often reanalyzed so that it was always current with any changes that occurred with regards to the GC/MS. The following (Figure 7) is the last standard curve that was made and the one that was used for the majority of calculations.

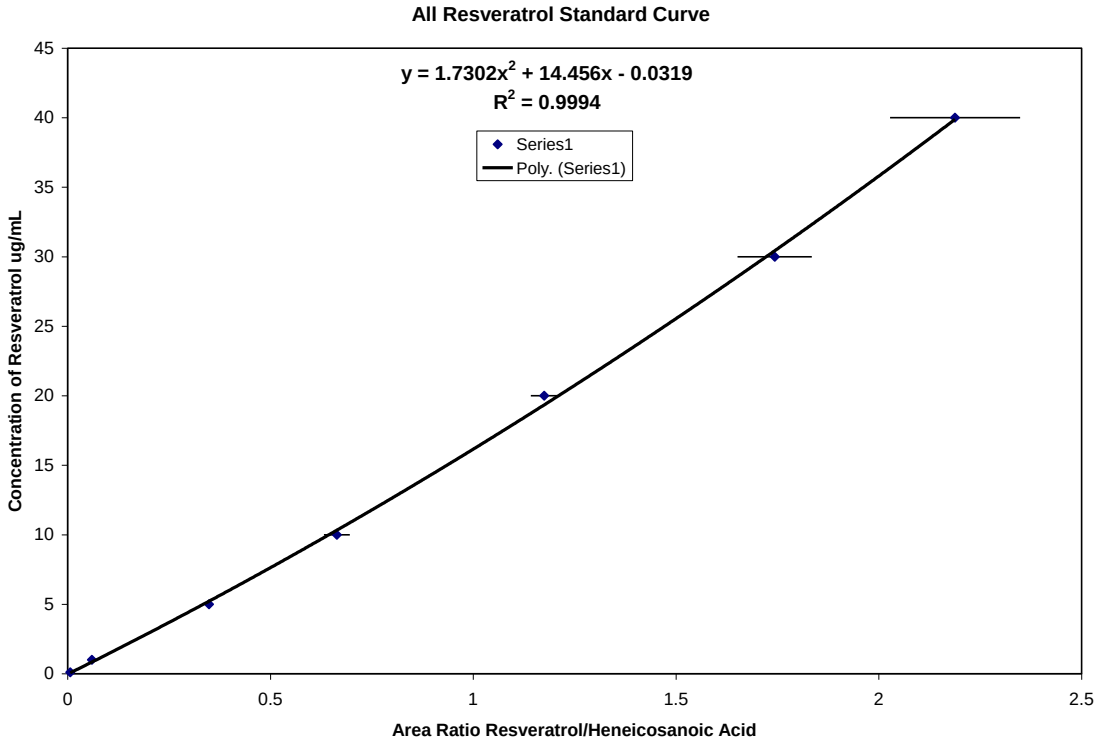



Figure 7: Resveratrol Standard Curve.

The standard curve is a plot of resveratrol concentration (μg/mL) verses the ratio of resveratrol peak area to heneicosanoic acid peak area. The $R^2$ value indicates the fit of the data points to each other. An $R^2$ value of 0.9994 indicates very good fit and allows for more reliable calculations based on the standard curve. The standard deviation for each concentration value is shown by the horizontal bars crossing each point. This standard curve was made using the 15 minute GC/MS method. There was no need to change standard curves when the 18 minute GC/MS method was created since the sensitivity of detection did not change from one method to the next in that particular case. An example of a standard chromatogram is shown in Figure 8. It was created using the 15 minute method.

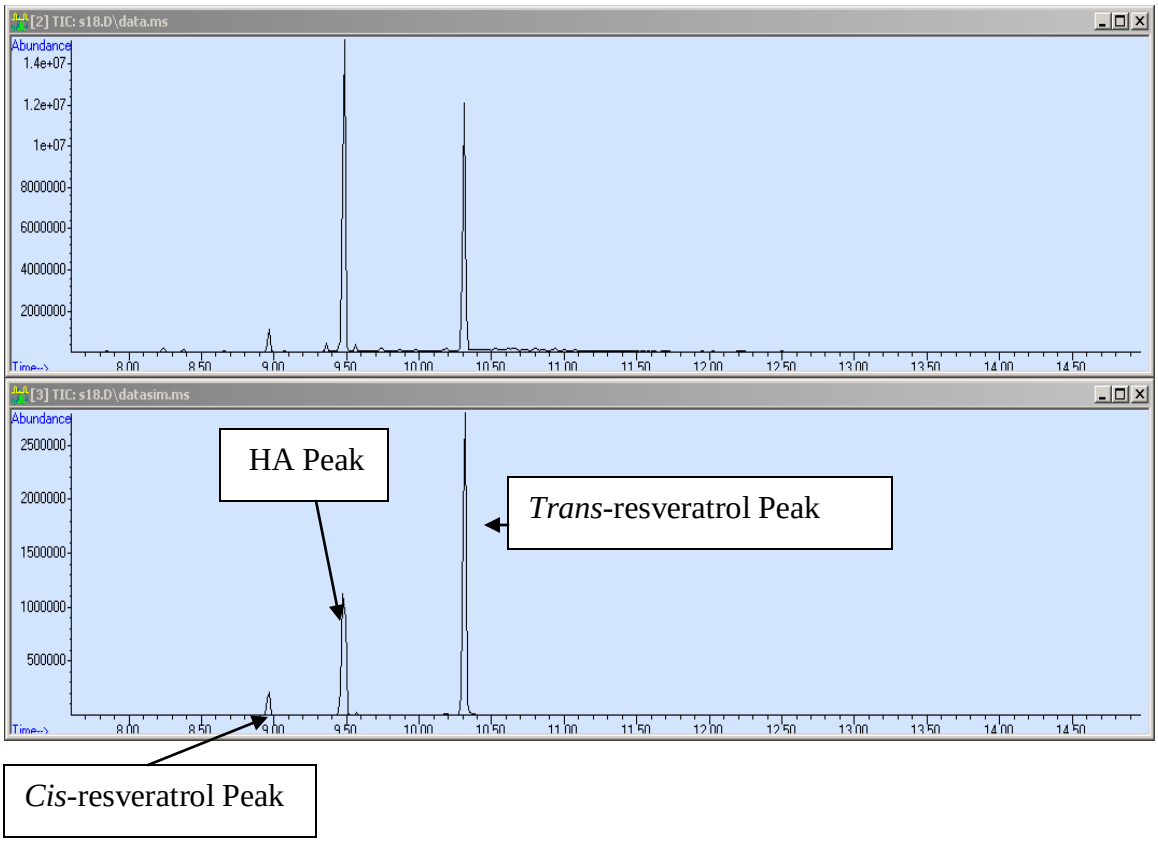



Figure 8: GC chromatogram of the 40 µg/mL concentration resveratrol standard. The bottom chromatogram is the selected ion chromatogram.

Larch 8 years old (*Larix Laricina*)

In my first experiment with larch bark I neglected to keep track of the age of the tree. Consequently a new set of experiments was done keeping track of the age of the tree from which the bark was taken. The moisture content of the bark from the 8 year old larch tree is shown in Table 3.

Table 3: Moisture content of 8 year old larch tree bark

| Moisture Content | | | | |
|---|---|---|---|---|
| Sample # | Vial Weight (g) | Initial Bark Weight (g) | Final Bark Weight (g) | % Moisture |
| Larch Sample 1 | 13.7141 | 0.4969 | 0.4412 | 12.46 |
| Larch Sample 2 | 13.7893 | 0.6133 | 0.5471 | 10.79 |
| Larch Sample 3 | 14.0212 | 0.5167 | 0.4603 | 10.92 |
| Average Moisture Content | | | | 11.39±0.93 |

A Soxhlet extraction was run on this bark sample but the results were inconclusive. The chromatogram from this larch Soxhlet is shown in Figure 9.

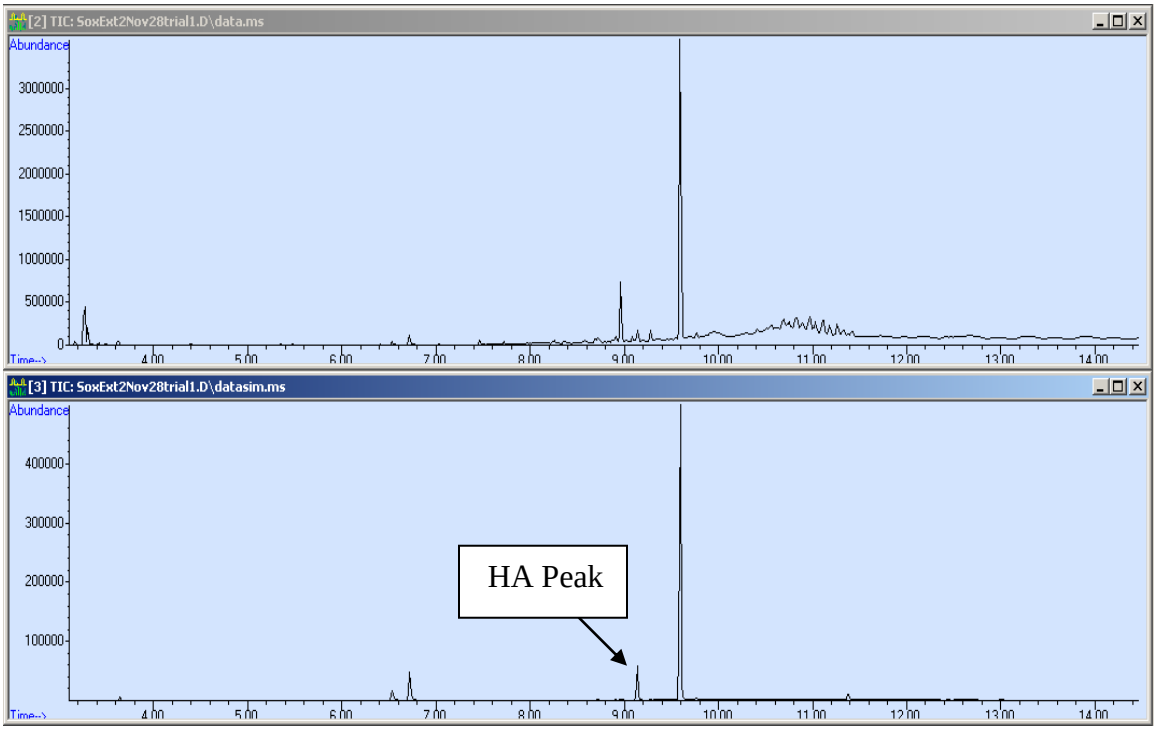



Figure 9: Chromatogram of Soxhlet extract from 8 yr old Larch

Despite changes to the method on the GC/MS (which required a new standard curve) the *trans*- and *cis*-resveratrol peaks were difficult to resolve. In the end it was determined

that the resveratrol content of this particular larch tree bark was too low to work with. A different sample of bark became the new subject of study.

Black Spruce (*Picea mariana*)

Due to difficulties in detecting useful amounts of resveratrol in the larch species we decided that it was worth attempting to work with a bark sample from a tree that was known to have resveratrol based on past work by Regan LeBlanc and Sara Knowles. Black spruce was determined in their works to have usable amounts of resveratrol on the order of 401.4±45.8 μg/g dry bark to 438.7±35.2 μg/g of dry bark (Knowles, 2010). The moisture content for the black spruce is shown in Table 4.

Table 4: Black spruce moisture content

| Moisture Content | | | | |
|---|---|---|---|---|
| Sample # | Vial Weight (g) | Initial Bark Weight (g) | Final Bark Weight (g) | % Moisture |
| B.S. Sample 1 | 13.1445 | 0.5005 | 0.3373 | 32.61 |
| B.S. Sample 2 | 13.2978 | 0.4962 | 0.3473 | 30.01 |
| B.S. Sample 3 | 13.2715 | 0.5136 | 0.3640 | 29.13 |
| Average Moisture Content | | | | 30.58±1.81 |

A Soxhlet extraction was performed on the black spruce in the hopes of finding a similar resveratrol content. The black spruce Soxhlet chromatogram is shown in Figure 10.

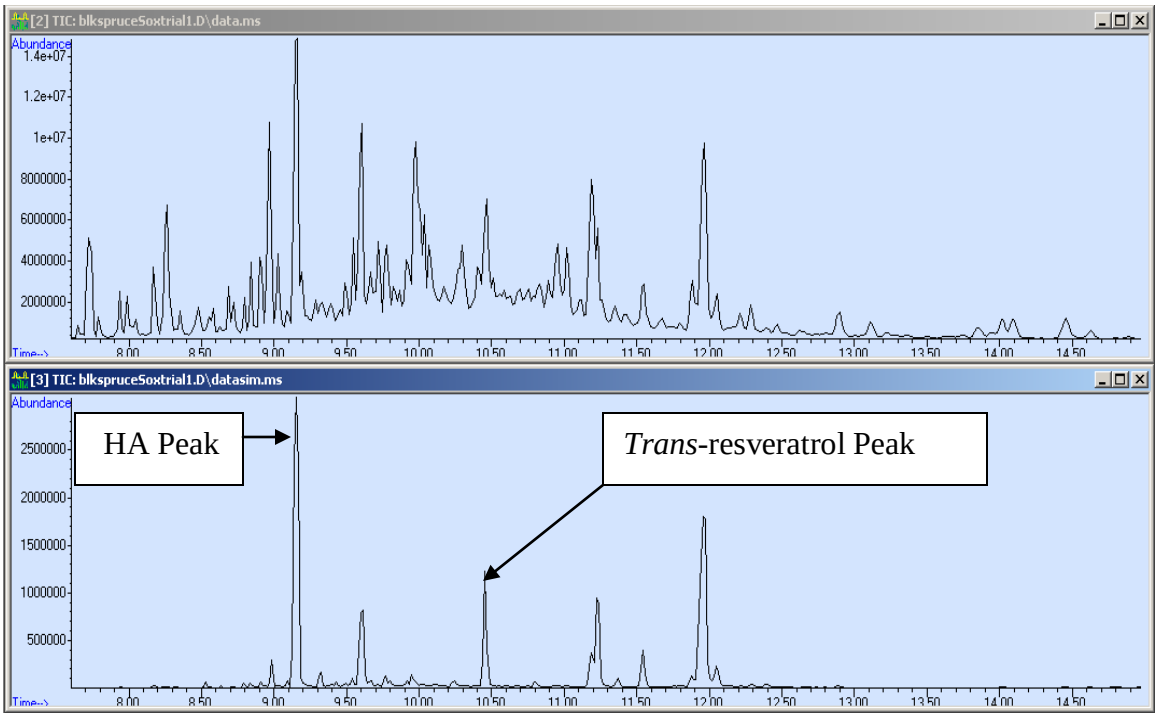



Figure 10: Black spruce Soxhlet chromatogram

Qualitatively the black spruce showed a larger peak area than the larch but it was never quantified because during analysis of the black spruce it was noted that the column on the GC had lost sensitivity. A new standard curve had to be made and analysis of the black spruce was no longer a priority. It was eventually noted that there would likely not be enough black spruce bark to last through the microwave extraction trials. A new plan was hatched to obtain a fresh Norway spruce (another species that was found to contain reasonable amounts of resveratrol) to ensure that there would be plenty of bark sample for all necessary experiments.

Norway spruce 18 years old (*Picea abies*)

The 18 year old Norway spruce was cut, debranched, and debarked. The bark was air dried for 72 hours then ground into a bark meal. The moisture content of that bark is shown in Table 5.

Table 5: Moisture content of 18 year old Norway spruce bark

| Moisture Content | | | | |
|---|---|---|---|---|
| Sample # | Vial Weight (g) | Initial Bark Weight (g) | Final Bark Weight (g) | % Moisture |
| N.S. Sample 1 | 13.2562 | 0.5160 | 0.4594 | 10.96 |
| N.S. Sample 2 | 13.3985 | 0.4554 | 0.4010 | 11.95 |
| N.S. Sample 3 | 13.2812 | 0.5372 | 0.4767 | 11.26 |
| Average Moisture Content | | | | 11.19±0.51 |

Immediately upon acquisition of the bark meal, microwave experiments were performed. Table 6 shows the results of those experiments which focused on varying the solvent used for extraction. The mass of bark used in these experiments was around 0.500g.

Table 6: Results of the effects of various solvents on resveratrol yield

| Microwave Extraction Solvent Tests | | | | |
|---|---|---|---|---|
| Solvent | Temperature (˚C) | Run Time (minutes) | Standard Curve Calculated Concentration (µg/mL) | Averaged value µg/g dry bark |
| Ethanol | 65 | 5 | 0.4301 | 14.74±0.79 |
| Ethyl Acetate | 65 | 5 | 0.4372 | 14.26±7.27 |
| Water | 65 | 5 | 0.5594 | 17.82±3.56 |
| Methanol | 65 | 5 | 0.0936 | 3.045±0.50 |

The resveratrol level for these trials were below 1.00 µg/mL and though a further dilution was added to expand the standard curve, the effect of error was likely too great for these

values to be very reliable. In case the low values were a result of the small sample size, a Soxhlet extraction was performed on a bigger amount (~2.0000 g) of 18 year old Norway spruce bark. The chromatograms are shown in Figure 11 and the results are shown in Table 7.

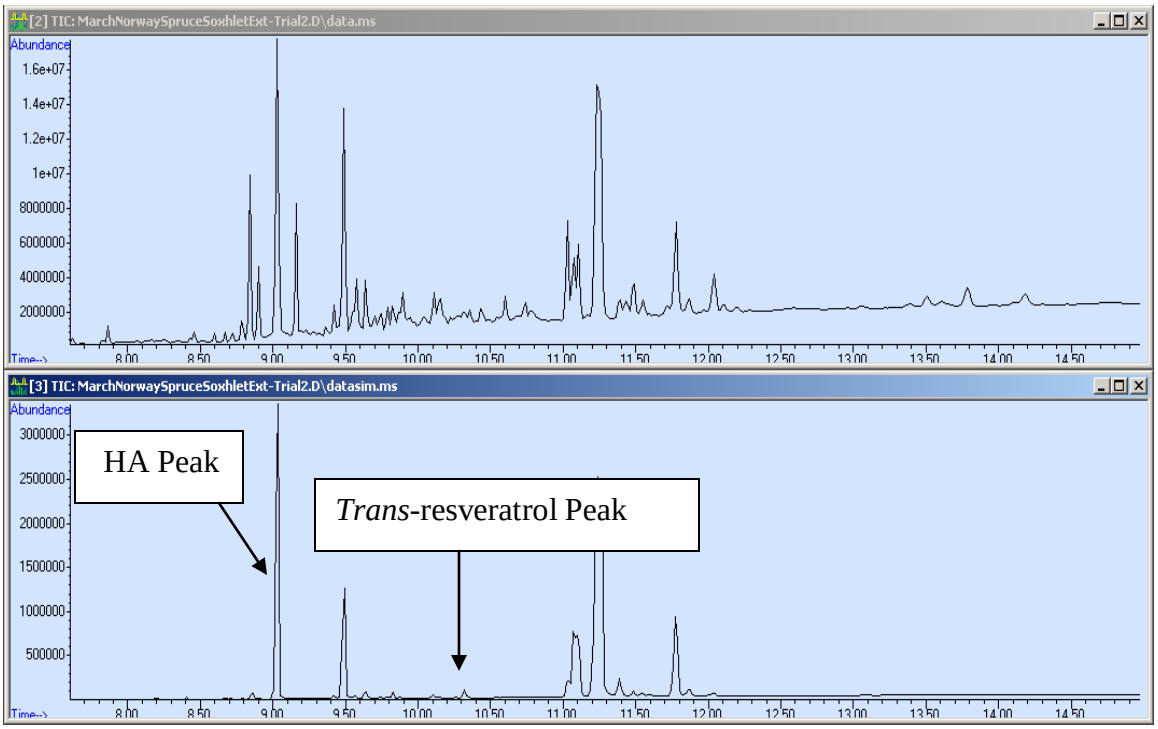



Figure 11: Soxhlet extraction chromatogram for 18 year old Norway spruce

Table 7: 18 year old Norway spruce bark Soxhlet experiment

| Soxhlet Results | | | | |
|---|---|---|---|---|
| Solvent | Temperature (˚C) | Run Time (minutes) | Resveratrol Concentration (µg/mL) | Resveratrol Content µg/g dry bark |
| Ethanol | High | 360 | 1.135 | 12.55 |

The result of the Soxhlet experiment (not run in triplicate for lack of time) was comparable to the microwave experiments. With resveratrol concentrations often near of below 1µg/mL in solution it was determined that this particular tree did not have enough resveratrol in its bark to be worth experimenting with. A comparison of the results of the

two methods is shown in Table 8. Though not necessarily conclusive, the comparison is useful on the level of preliminary results.

Table 8: Comparison of microwave extraction and Soxhlet extraction in ethanol of 18yr N.S.

| Soxhlet Extraction versus Microwave Extraction | | |
|---|---|---|
| | Soxhlet Extraction | Microwave Extraction |
| Mass of bark | 5.1037 g | 0.4940±.0015 g |
| Volume of solvent | 250 mL | 5 mL |
| Time of extraction | 360 minutes | 5 minutes |
| Extraction solvent | Ethanol | Ethanol |
| Temperature of Extraction | Warm | 65 ˚C |
| Yield of resveratrol | 12.55 μg/mL | 14.74±0.79 μg/mL |

The preliminary results suggested that microwave extraction was comparable to Soxhlet extraction in yield of resveratrol. Additionally the microwave extraction required much less solvent, time, and sample. Due to the very small amounts of resveratrol however, I moved on to a different tree in the hopes that it would have higher resveratrol content. Higher resveratrol content would allow the results from that bark to be more conclusive. Another member of our group was working with bark from a different Norway spruce tree (12 years old) and was finding usable amounts of resveratrol. Thus this other bark sample was used for all remaining experiments.

Norway spruce 12 years old (*Picea abies*)

The moisture content for this Norway spruce sample is shown in Table 9.

Table 9: Moisture content of 12 year old Norway spruce bark

| Moisture Content | | | | |
|---|---|---|---|---|
| Sample # | Vial Weight (g) | Initial Bark Weight (g) | Final Bark Weight (g) | % Moisture |
| N.S. Sample 1 | 13.2731 | 0.5096 | 0.4233 | 16.41 |
| N.S. Sample 2 | 13.6001 | 0.4956 | 0.4160 | 16.06 |
| N.S. Sample 3 | 13.7228 | 0.4548 | 0.3812 | 16.18 |
| Average Moisture Content | | | | 16.21±0.18 |

For this tree sample instead of changing the solvent variable, the heating period was changed and the solvent was kept constant as ethanol. Ethanol had been determined to be an effective solvent in work by Sara Knowles (Knowles, 2010) and Regan LeBlanc (LeBlanc, 2010). In an effort to try to ensure concentrations above 1.00 μg/mL, the sample size for these experiments was increased from a mass of around 0.5000 g to a mass of near 2.0000 g per sample. The extraction times varied from 5 minutes to 40 minutes. The chromatogram for the 20 minute extraction is shown in Figure 12 and the results of these experiments are shown in Table 10.

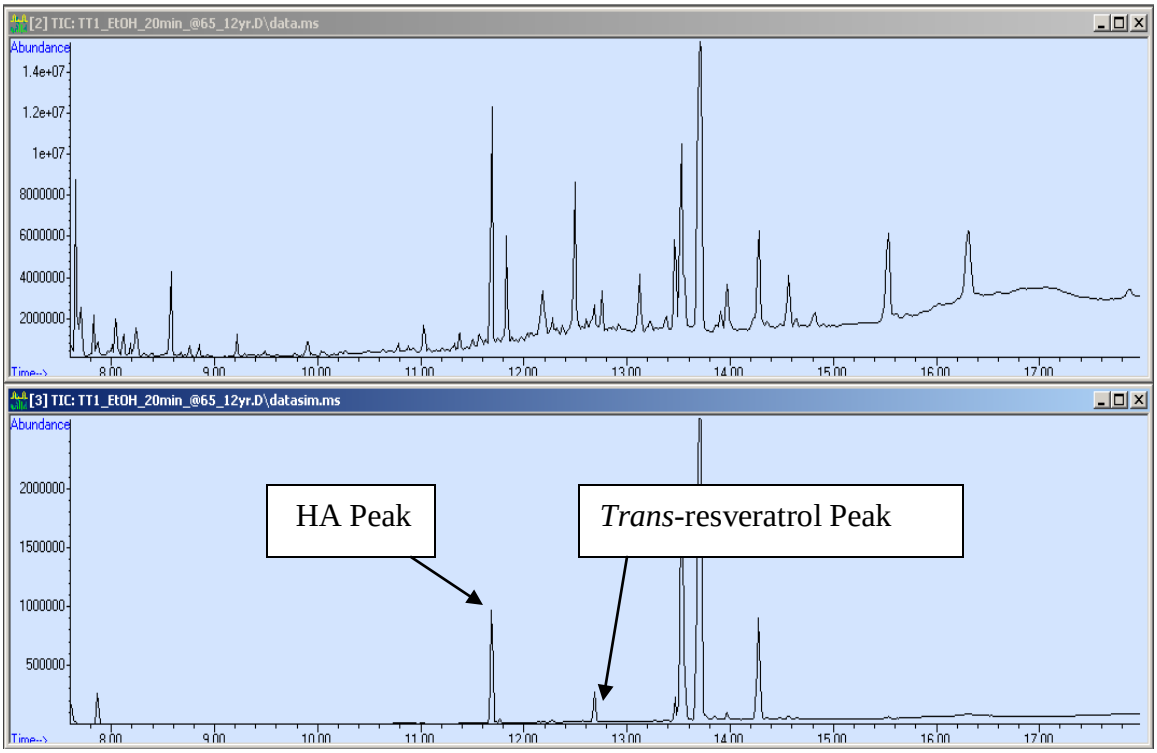



Figure 12: Chromatogram of a 20 minute microwave extraction of the 12 year old Norway spruce

Table 10: Time trial microwave experiments for 12 year old Norway spruce bark.

| Microwave Extraction Time Tests | | | | |
|---|---|---|---|---|
| Solvent | Temperature (˚C) | Run Time (minutes) | Standard Curve Calculated Concentration (µg/mL) | Resveratrol µg/g dry bark |
| Ethanol | 65 | 5 | 2.817 | 17.01 |
| Ethanol | 65 | 10 | 2.135 | 17.91 |
| Ethanol | 65 | 20 | 3.717 | 31.05 |
| Ethanol | 65 | 30 | 2.443 | 21.74 |
| Ethanol | 65 | 40 | 2.156 | 17.57 |

The results of this set of singlet experiments show that the bark from this particular tree has more resveratrol per gram of dry bark and the concentrations of resveratrol in the sample solution were all above 1.00 µg/mL making them more reliable. The point of highest interest however, is in the effect of extraction time on yield of resveratrol. The best yield was gained at an extraction time of 20 minutes suggesting this as an optimum

time value. The results of these extractions implied a loss of free resveratrol at extraction periods beyond 20 minutes. This inspired a set of experiments to study the effects of the microwave heating over time, on standard resveratrol. A sample chromatogram from a microwave extraction is shown for this bark sample.

Resveratrol degradation studies

In an effort to determine if resveratrol actually degraded in the microwave over an extended period of time (beyond 20 minutes) a set of singlet degradation experiments were performed with a known concentration of standard resveratrol. A volume of 5.00 mL of 40.00 μg/mL concentration of resveratrol in standard (in methanol) was pipetted into 5 separate microwave vials. These vials were given stir bars, capped and run through the microwave reactor. Each sample was heated for a different period of time. The results are shown in Table 11.

Table 11: Results of the tests on standard resveratrol placed under microwave conditions for varying time periods.

| Microwave Extraction Degradation Tests | | | |
|---|---|---|---|
| Solvent | Temperature (˚C) | Run Time (minutes) | Resveratrol Concentration (μg/mL) |
| Methanol | 65 | 5 | 41.50 |
| Methanol | 65 | 10 | 35.03 |
| Methanol | 65 | 20 | 34.34 |
| Methanol | 65 | 30 | 37.20 |
| Methanol | 65 | 40 | 38.00 |

In this case the solvent is methanol instead of ethanol simply because I neglected to dry the 5.00 mL aliquots down and re-dissolve them in ethanol before heating the samples in the microwave reactor. This provided an inconsistency between experiments. Despite the change in solvent, based on the lack of a downward trend throughout the data it was

concluded that there was no degradation of resveratrol in any of the trials. This was unexpected due to the result of the 20 minute extraction on the 12 year old Norway spruce sample. It is possible that the value given by the 20 minute extraction was an inconsistency. These bark extractions were not run in triplicate for lack of time. A simple test of using pH paper and a solution of the Norway spruce bark in water showed the mixture to be acidic in nature. This allows for the possibility that within the mixture of extractives, reactions were occurring that caused some of the free resveratrol to be lost over an extended period of time under microwave heating. At this point it is impossible to tell without replicated trials on the bark extractions to verify that 20 minutes is the optimal extraction time and that there actually is a drop in yield at extraction times other than 20 minutes.

## Conclusions and Future Work

Currently tree bark is an unused product of the tree in the pulp and paper industry. If enough resveratrol could be extracted from that bark efficiently, it could become another source of revenue.

This project was an attempt at determining the optimal parameters to extract resveratrol from the bark by microwave accelerated chemistry. Unexpectedly, the research turned out to be much slower than anticipated so that not much was determined in terms of optimal parameters. What can be concluded is that 40.00 μg/mL standard resveratrol in methanol does not degrade in the microwave reactor when heated to 65 ˚C for up to 40 minutes.

Future work will focus on optimizing the extraction of resveratrol by microwave accelerated extraction. Conclusive results on solvents and extraction time are still needed as are studies about the temperature and time that are optimal. This project served the purpose of finding possible roadblocks. Future work can hopefully avoid many of the pitfalls encountered during this project.

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## Author's Biography

Dustin Niedt was born in Toulon, France on January 31, 1990. He traveled many times throughout his childhood and adolescence before settling down in Maine. He graduated from South Portland High School in 2008. Dustin is majoring in chemistry.

Upon graduation, Dustin plans to search for a job implementing what he has learned from his time spent at the University of Maine.