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Separation and Identification of Bioactive Compounds from Oplopanax horridus

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SEPARATION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM *OPLOPANAX HORRIDUS*

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ABSTRACT

For centuries, natural products, such as plants, have been used for the prevention and treatment of diseases and ailments. Modern science is now working to identify the beneficial compounds from these sources to implement into pharmaceutical drugs, vitamins or supplements in an area of study called nutraceuticals. The plant *Oplopanax horridus*, or Devil's Club is a member of the ginseng family and has over 30 documented uses for spiritual and medicinal purposes. The few studies that have been carried out on this plant are limited to the volatile chemicals present. Few studies have determined the plant possesses antifungal, antioxidant and antimicrobial properties but without confirmation of the chemical compounds responsible for these properties.

The purpose of this study was to fractionate compounds from a crude sample of *Oplopanax horridus* by solid phase extraction (SPE) with the assistance of high pressure liquid chromatography (HPLC). These fractions were subjected to antioxidant testing where bioactivity guided further fractionation and analysis by mass spectrometry (MS). The data collected from the mass spectrophotometer was used to propose the chemical compounds present in the antioxidant active samples.

Successful separation of natural products was completed by Soxhlet extraction, liquid-liquid extraction, SPE, and HPLC. Three sample sub-fractions were found to be bioactive after the assessment of antioxidant activity with two variations of assays. Mass spectrum data produced ion chromatograms that were useful in the prediction of chemical structures.

DEDICATION

I would like to dedicate herein to my family for their unconditional love and support throughout my life. To my parents, David and Linda Bailey, for instilling in me the values of education, determination and hard-work; without these traits I would not be where I am today. Mom and dad you have always encouraged me to achieve my dreams and you've always stood by me as I've made my way through life. To my sister, Stephanie Hiett, for paving the way for me to become a scientist and for your wisdom of lessons and advice learned through your experiences. You have always been my sister but through the years you have become my friend. THANKFUL.

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CHAPTER ONE

LITERATURE REVIEW

Oplopanax horridus

The plant *Oplopanax horridus*, which is more commonly known as Devil's Club in North America, is a dense shrub found in the forests along the Alaskan coast with its presence found in the Canadian Rockies and reaches as far south as Oregon and east to Idaho.[1] Due to the far reaches of the plant over 38 linguistic groups of indigenous people in the Pacific Northwest of North America have made use of this plant for upwards of 34 different ailments and for various spiritual purposes.[2] In fact, few other plants have been consistently used for medicinal purposes within the geographical range of the foliage.[3] For traditional medical conditions, the plant has been used for, but not limited to, ailments such as cough, cold, fever, headaches, tuberculosis[4] and even diabetes[5]. Depending on the illness being treated, different parts of the plant has been prepared and utilized in various ways. The most generic preparations for medicinal purposes were infusion or decoction of the plant parts, generally the bark, and sometimes mixtures of its essential oils or other plant extracts.[1] The essential oils, which can be extracted from the bark of both the stem and root of Devil's Club[6] has exhibited anticancer, antioxidant, antibacterial and anti-inflammatory properties.[7]

The family of Devil's Club (*Araliaceae*) is also known to contain the plants of Asian and American Ginsengs, both of which are known for health benefits like improving stamina and cognitive function, along with assistance in managing diabetes[8] and the ability to stimulate immune cells.[9] American ginseng has also shown benefits in a

number of biological systems.[10] With the knowledge of the health benefits from other ginsengs, Devil's Club has grown in commercial popularity as an herbal medicine.[1]

Natural products

Beginning in approximately 3000BC the Ayurvedic Indian and Chinese cultures have been exploring medicines derived from plants.[11,12] The Ayurveda or knowledge of life,[11] was among the first written books recording roughly 2,000 plant species as sources as medicines and their potential side effects.[12] At the same time traditional Chinese medicine history has mainly been practiced through clinical trials and a theory has been devised containing the prevention and treatment of diseases, which is generally a mixture of multiple herbs.[13] Herbal medicines have played an important role in Chinese health care by choosing plants or animals similar to humans in chemical composition to be easily absorbed and help recreate a balance within the body.[14] While Ayurvedic and Chinese were frontrunners in bio-prospecting,[12] many individuals throughout history continued to document plant and animal extracts that could be used for medicines.[11]

Although botanicals have long been used for medicinal purposes but only recently has modern science been able to determine the beneficial active compounds. Incorporating these nutrients (active compounds) into pharmaceutical products has generated a new branch of study called nutraceuticals.[15] Nutraceuticals can be dietary supplements, pharmaceutical pills, capsules or tablets, that deliver a concentration of a bioactive agent with the purpose of enhancing one's health.[16] The success of nutraceutical studies has led to over 40% of currently marketed pharmaceutical drugs derived from natural products,[11] which can be fruit, vegetables or any part of the plant. The reason that natural products are useful in drug discovery is that they are structurally diverse, have relatively

small molecular weights and possess "drug-like" properties meaning they can be absorbed and metabolized within the human body.[11] For example, many natural products that show signs of antibacterial or antioxidant behavior fall into the category of polyphenols.[15]

Polyphenols

Polyphenols are compounds comprised of at least two hydroxyl groups while single phenolic compound consists of an aromatic ring bearing at least one hydroxyl group.[17] These compounds are derived from plants' secondary metabolism,[18] which generally have no vital role in the building or maintaining of plant cells but may play a role as plant growth regulators, gene expression modulators, signal transduction and the potential for medicinal purposes.[19] Polyphenols are the most abundant antioxidants present in the human diet[19] and it is the antioxidants that may aid in the prevention of several chronic diseases.[20] Studies have not only indicated that phenolic compounds are dominant antioxidant components but also that antioxidant activity is linearly related to the phenolic content present in various traditional medicinal plants.[13]

Antioxidants

The accepted definition of antioxidant is "any substance that delays or inhibits, oxidative damage to a target molecule."[21] Oxidative damage or stress can arise in humans when there is an imbalance between reactive oxygen species (ROS) and antioxidant defense and repair mechanisms.[22] All molecules within the body of a living organism can be targets for oxidative damage including but not limited to lipids, proteins, nucleic acids and carbohydrates.[21] Damage caused by reactive oxygen species (ROS) or reactive nitrogen species (RNS) include ailments such as cancer, cardiovascular diseases,

neurodegenerative disorders and immune dysfunctions.[23] Antioxidants protect these molecules through various mechanisms and pathways: 1)scavenging oxygen-derived species, 2)minimizing the formation of oxygen-derived species, 3)binding metals to convert reactive species, 4)repairing target damage, and 5)destroying badly damaged targets and replacing them.[21] Phenolics are suitable antioxidants as they possess at least one hydroxyl group that has the potential to quench a free radical by forming phenoxyl, radicals that are resonance-stabilized.[24] The reduction-oxidation properties of polyphenols are shown to contribute to antioxidant activity.[25]

Antioxidant testing

Antioxidant activity reflects a capacity of an antioxidant to capture free radical,[25] which can be determined by various methods based on one of two types of assays that indicate the effectiveness of the antioxidant to hinder the oxidation of substances under specific conditions.[22] First, a hydrogen atom transfer assay applies a competitive reaction scheme between an antioxidant and substrate for peroxyl radicals that are thermally generated. Second, an electron transfer assay measures an antioxidants' capacity to reduce an oxidant which will change color once reduced.[24] Performing one single method generally is not adequate as multiple types of measurements will provide information on various possible actions of the antioxidant.[24] Factors that can affect the results of antioxidant capacity are pH, polarity, and the ability of the solvent to accept or donate hydrogen atoms.[25] The two most common assays for their reproducibility and straightforward analysis are DPPH and ABTS assays.[24] These two assays in many cases have shown close correlations with phenolic content.[26]

In 1958, Blois described an assay involving the free radical scavenger diphenylpicrylhydrazine (DPPH) that interacted with hydrogen donating compounds causing the DPPH radical to be reduced, losing its deep violet color[27] that can be analyzed by a spectrophotometer at a wavelength between 515 to 520nm[24,27,28] The DPPH assay is useful in light of its high reproducibility, easy measurements taken at ambient temperature without risk of thermal degradation, and obeying (or in compliance with) the Lambert-Beer law.[27] Besides, this method has a broad solvent compatibility with aqueous and polar or nonpolar organic solvents which is a major advantage over other assays.[25] Unfortunately the DPPH method can neither show partitioning of antioxidants in emulsions nor is it useful for determining the antioxidant activity of plasma as proteins tend to precipitate in the medium.[27]

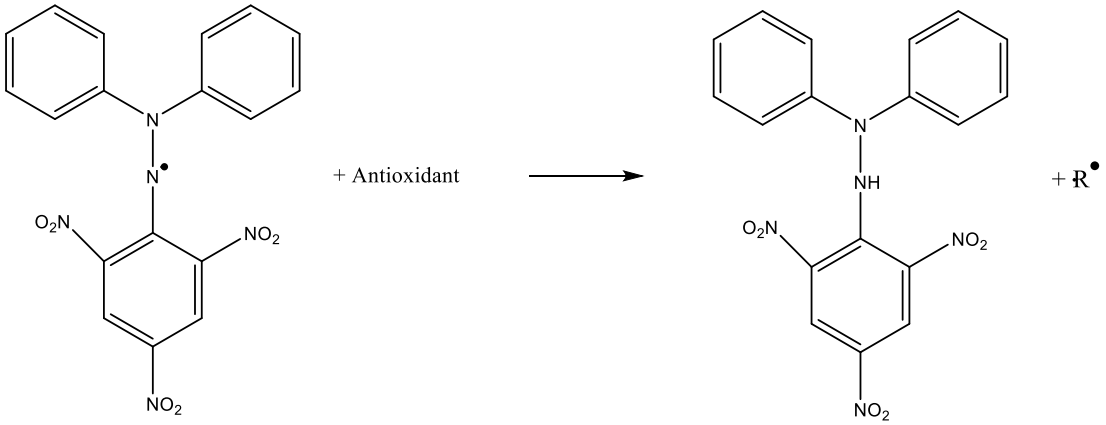

Figure1.1: DPPH radical reaction with antioxidant compound

Lambert and Beer's law of spectrophotometry tells us that there is a direct linear correlation between absorbance with analyte concentration and the cell path length. The general accepted equation is $A = \varepsilon \ell c$ where A is the absorbance (recorded from a spectrophotometer based on the power of the incident and transmitted radiant power),[29] c

is the analyte concentration, ℓ is the cell path length and ε is the molar absorptivity or sometimes called extinction coefficient, that is unique for each analyte.[30] In addition, it is important to remember that the absorptivity for an analyte is dependent on the refractive index of the medium.[29] The extinction coefficient of the DPPH radical and standard is used to calculate the antioxidant activity for an analyte.[27]

In 1994 Rice-Evans and Miller developed the 2,2'-azinobis(3-ethylbenzothiaziline-6-sulfonate) (ABTS) method that was later modified by Re et al.[28] The ABTS radical cation is generated when the ABTS is oxidized with potassium persulfate.[28,49] The cation is fairly stable until it reacts energetically with a hydrogen atom[31] donated by an antioxidant, generally a phenolic compound, which reduces the ABTS and decolorizes the initial blue/green solution.[26] Spectrophotometrically, the assay is generally measured at 734nm[22,24,26] although the ABTS cation has a strong absorption between 600-750nm.[31] Most commonly, Trolox, a vitamin E analog, is used as a positive control to determine antioxidant activity that is reported in a Trolox- equivalent capacity.[28] The Trolox equivalent antioxidant capacity (TEAC) is the number of ABTS radicals consumed by one molecule of antioxidant.[31] Just like the DPPH assay, ABTS is relatively simple and it can easily be done routinely in a laboratory setting but the reaction with ABTS occurs slowly so the TEAC value is dependent on the incubation time of and analyte to ABTS radical concentration.[31] The ABTS assay can be performed in water or organic solvents and the cation, which is more reactive than the DPPH radical, can interact with both hydrophilic or lipophilic antioxidants but the ABTS cation radical is non-physiologically relevant and is a disadvantage to the assay.[25]

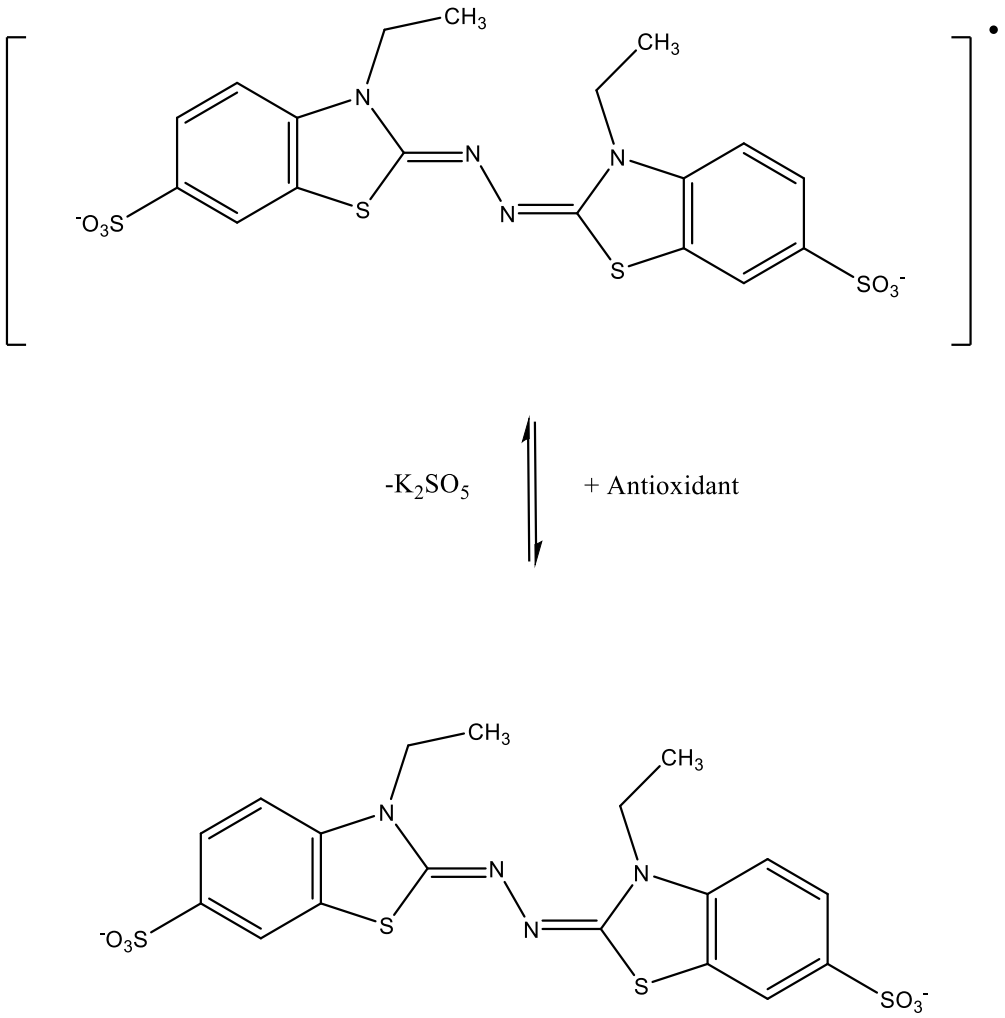

Figure 1.2: ABTS radical reaction with antioxidant compound

Sample preparation

Sample preparation is the first step in the process of studying natural products and their antioxidant properties. For this, it is important to select a sample preparation technique that avoids oxidation, enzymatic reactions and any other chemical changes that could hinder the target compounds.[32] For the recovery and isolation of bioactive compounds, extraction techniques such as liquid-liquid and solid-liquid extractions are among the most common for their ease of use, efficiency and wide applicability.[17]

Soxhlet extraction

Soxhlet extraction, generally known as solid-liquid extraction, is a conventional method for solid samples, which should more accurately be referred to as leaching.[33] This hybrid continuous-discontinuous extraction method is carried out when a thimble containing the sample is slowly filled with fresh solvent. Once the solvent approaches the overflow level, a siphon effect comes into play as the solute from the thimble is aspirated into the distillation flask containing the desired extracted analytes.[33] This process continues until the extraction is complete. The solute is then separated from the solvent by evaporation of the solvent. Antioxidants can be transferred from solid material into a liquid phase based on affinity for the selected solvent and by increasing the solid to liquid ratio the extraction yield can be improved.[32]

This extraction method can be time consuming, with extractions lasting upwards to twelve hours. The extraction typically uses a large amount of solvent[34] and can potentially thermally degrade the sample.[33] However, the Soxhlet extraction has the advantages of continuously bringing fresh solvent into contact with the solid sample, maintaining a relatively high temperature. In addition, no filtration step is required after the extraction is complete,[35] but it does require an evaporation/concentration follow-up step.[33] Other extraction methods that have been used for phytochemistry include maceration, which operates on the principle of separating or decomposing grounded solid material by steeping in water or solvent possibly with shaking or stirring. In maceration, the method is to homogenize the mixture to facilitate the transfer of compounds into the solvent. After a period of time the mixture is filtered or centrifuged and the extraction is repeated on the residue. This method is not difficult and requires no specific equipment

but it is time consuming and consumes a large amount of solvent.[32] The Soxhlet extraction uses less solvent than maceration and fresh solvent is always being introduced into the thimble and interacting with the material and has the potential to extract more from a sample.[33]

Liquid-liquid extraction

In liquid-liquid extraction (LLE), solvents are used depending on the type of compounds wished to be partitioned. Alcohols, acetone and ethyl acetate are common solvents for extracting polar compounds. Less polar compounds can be extracted by mixtures of alcohol-water as well as non-polar solvents such as chloroform or hexane.[17] Other factors to consider for liquid-liquid extraction are pH, temperature, solvent volume to sample ratio and the time interval for an extraction step.[17]

Solid phase extraction

After the initial extraction of the natural products, the next important step is the clean-up or isolation stage, where compounds of interest are fractionated into clean extracts.[17] Solid phase extraction (SPE) utilizes chromatographic stationary phase to isolate analytes of interest from a sample.[36] The first disposable cartridges for SPE were introduced in 1978 followed by syringe-format cartridges in 1979 and in the early 1980s pre-columns for an on-line format with high pressure liquid chromatography were developed. Nevertheless, LLE has remained as a popular sample preparation technique because it can quickly and on a larger scale separates polar and apolar compounds, although initial or preliminary screenings of SPE perform the same task of LLE.

The use of solid phase extraction (SPE) is a growing practice in light of its advantages of using low amount of solvents, the flexible selectivity of sorbent[37] and the

ability of high-throughput.[38] The greatest advantage of SPE is that it can be combined with high performance liquid chromatography in an on-line format, where a "crude" extract can be directly injected into a system.[17] On the other hand, a disadvantage of SPE is attributed to the method development; in terms of the choice of sorbent, sample volume and the solvent choice for clean-up and desorption solutions.[38]

The partitioning of SPE is determined by the interactions of the analyte, the solid phase and the chosen solvent.[37] Depending on the sorbent used, various chemical mechanisms, such as Van der Waals or π-π interactions and ion exchange are involved[37]

The sorbent or solid-phase selection is critical for any solid phase extraction process.[38] Today there are various types of sorbents geared toward the selective recovery and separation of selected classes of compounds. The more selective the sorbent is the more sensitivity the SPE will achieve.[38] The most frequently used sorbent is C_{18} bonded silica based on reverse phase interactions,[17] but in recent years new phases have been developed including cross-linked polymers, graphitized carbons and n-alkylsilicas.[38] These improvements are being researched in hopes of improving recovery and selectivity especially in mixed mode.[37] Mixed mode sorbents are becoming popular as well, especially in drug extraction as the sorbent contains both non-polar and strong ion exchange groups based on the reverse phase silica with residual silanols.[38] A new popular phase for the use of biological extractions is hydrophilic-lipophilic balanced sorbent (HLB). This copolymer sorbent has been shown to possess the retention comparable to C_{18} silicas.[38] Both acids and bases of ionic nature can be extracted via ion-exchange sorbents based on ion-exchange chromatography where cation exchangers contain sulfonic acid groups or weak carboxylic acids. Anion exchangers are composed

of amino groups, primary and secondary for weak exchangers and quaternary groups for strong exchangers.[38] Not only is the sorbent selection important, the pore diameter should also be taken into account. Most sorbents are made into sizes of approximately 60Å in diameter, but 125Å diameter is also available with a larger surface area as well as a smaller pore size.[37] This can lead to better overall separation.

After collecting the eluted fractions from SPE, samples are subsequently cleaned, dried and reconstituted, then they are subject to analysis by various analytical instruments such as gas chromatography (GC), mass spectrometry (MS) and high performance liquid chromatography (HPLC). Because the majority of plant secondary metabolites are not very volatile, HPLC is preferred for further separation. HPLC, generally in tandem with MS, is the common choice for simultaneous qualitative and quantitative determination.[39]

High pressure liquid chromatography

By its basic definition, HPLC is a system that uses high pressure to force solvent through a column that contains fine particles that interacts with the sample to yield high resolution separations.[36] The instrumentation of an HPLC consists of a solvent delivery system (pump), an injection valve, a column (typically in an oven) and a detector. The success of HPLC separations and detections is due in part to the selection of mobile phases (solvent), column type, oven temperature and type of detector. All of these variables change depending on the type of compounds trying to be identified.

The elution process is based on adsorption chromatography where solvent competes with solute for sites on the stationary phase. Elution occurs when the solvent successfully displaces the solute from the stationary phase. Eluent strength can be increased by making the used solvent more like the stationary phase of the column; i.e.

polar solvents are more useful with normal phase and more non-polar solvents have a greater eluent strength in reverse-phase columns.[36]

When attempting to elute compounds, either an isocratic or gradient elution must be selected. The former uses one solvent at a fixed flow rate throughout the entire elution process. This is simple and enough for some separations, but for more complex solutes, it may be necessary to use a gradient elution, which uses two or more different solvents of varying elution strengths. Over time the solvent composition is changed by pulling set percentages of the different solvents to change the eluent strength. This idea is similar to a gradient temperature program for gas chromatography.[36]

The injection valve is important as it possess a sample loop that holds the sample at atmospheric pressure until injected onto the column and comes in contact with the solvent. The injection loop ranges in size from 2 to 1,000 μL for analytical and preparative scales, respectively.

HPLC columns are typically packed columns with a silica based backbone with particle sizes between 1.7 and 5.0μm.[36] The smaller the particle size is, the higher the plate number and higher pressure can be resulted in, which can lead to shorter running time and lower detection levels.[36] In addition smaller particle size achieves a more uniform flow through the column and ultimately gives better resolution. Small particles tend to decrease plate height which is not sensitive to increased flow.[36] The following van Deemter plot equation shows the correlation between important variables to HPLC.

$$H = A + \frac{B}{u_x} + Cu_x$$

Where A is related to the multiple flow paths through the column that leads to band broadening. B is the rate of diffusion of the solute in the mobile phase. C is the rate of mass transfer between the stationary and mobile phase. The variable u_x is the linear flow rate. The three factors add up to the theoretical plate height, H, of which a larger number results in better resolution of the chromatographic separation.

A HPLC column is typically housed in an oven to maintain constant and/or decrease viscosity of solvent, decrease pressure and retention times because a column oven set above room temperature will improve reproducibility.[36]

The stationary phase of a column can be porous silica itself or a bonded phase attached to the silica surface. The latter can be polar (normal phase) or nonpolar (reverse phase) which can vary in the number of carbon chain length. Many of the different column types are limited to constraints of the pH value of the mobile phase. For instance pure silica cannot be used above pH 8 because it is subjected to be dissolve.[36]

The final important piece of an HPLC system is the detector. Ideally, a detector should be able to analyze low concentrations of solutes, provide linear response, not give broaden peaks and be insensitive to temperature or solvent changes[36] as well as respond independently of the mobile phase and be nondestructive to the solute of interest.[40] The most widely used detector is the ultraviolet (UV) detector because many solutes absorb wavelengths within the UV spectrum but it only works with non-absorbing solvents.[36] The UV radiation from the lamp, which could be mercury or deuterium, shines through the solvent/analyte mixture then striking a photocell that generates a current and signal proportional to the light intensity.[40] The diode-array detector (DAD) operates by the same principle but offers spectral comparison for easier confirmation of data results.[41]

An evaporating light-scattering detector (ELSD) responds to laser lights that is scattered by nebulized droplets of sample. An electrochemical detector (ECD) is used to detect solutes, such as aromatics, ketones, aldehydes, etc., that can be reduced or oxidized. The reduction or oxidation takes place at a working electrode where current is measured with respect to a reference electrode, usually Ag|AgCl.

When developing a method for HPLC there are three import overall goals in regards of adequate resolution of analytes, a short running time, and a rugged process that is not affected by small variations.[36] The stationary phase should be chosen based on the compounds present in the sample. For example, reverse phase works best for low molecular mass molecules as well as neutral or organic compounds. To determine the best solvent, there is a HPLC method development triangle. First it is best to try the three corners of the triangle individually (see Figure 1.3). If desired separation is not achieved, it is best to try mixtures of two solvents at a time. The final solvent trial is a mixture of all three solvents generally in a 1:1:1 ratio. It is also important to keep in mind that a gradient can be generated with solvent and water. To establish a useful gradient the quickest way is to run a broad gradient and then make changes based on the chromatogram, eliminate portions of the gradient where no peaks elute and length the time of a gradient percentage where multiple peaks elute.[36]

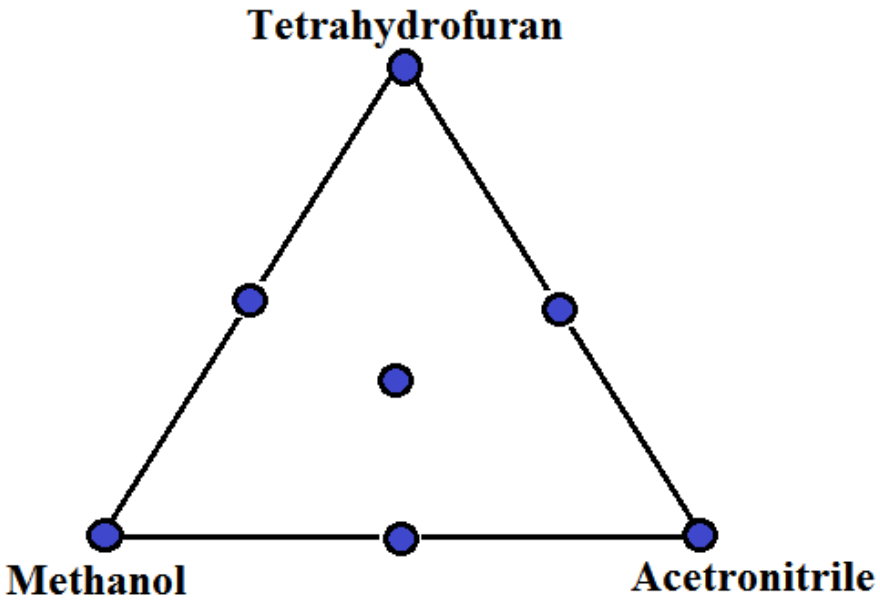

Figure 1.3: Solvent triangle used to determine best gradient and elution process

Application of HPLC has increased in recent years for the analysis of plant-derived matrices as it is useful in the detection of phenolic compounds along with the wide range of available columns and the possibility of combining two columns.[17] Monolithic columns are of interest for biological analysis but many studies have yet to be carried out.[17] For plant extracts, the choice of column is of the utmost important as a crude sample could decrease the life time of a column, which could be protected by the addition of a guard column. If one column does not produce high quality separations of the complex plant samples it is possible and may be advantageous to employ two-dimensional liquid chromatography.[17]

For phenolic compounds, isocratic or gradient elution can be used depending on the number of compounds and the matrix of the sample. The most common mobile phases of choice are acetonitrile and methanol. Methanol has a slight advantage because of its non-toxic property and a higher percentage that can be used in the mobile phase without

harming the column.[17] Small amounts of acid can be added to the mobile phase solvents to adjust the pH range when necessary. Most phenolics can be detected at many wavelengths in the ultraviolet and visible spectrum. For plant extracts it is more beneficial to monitor multiple wavelengths as it helps in the identification of multiple compounds in the complex plant mixtures.[17]

Mass spectrometry

Another important analytical instrument is the mass spectrometer (MS) as it is the most sensitive and versatile detector.[42] It is used to aid in the identification of compounds based on the characteristic MS spectrum of respective compounds. Once a sample enters the spectrometer, the molecules are vaporized and then ionized.

$$M \rightarrow M^{\bullet +} + e^-$$

The ionization process removes an electron from the molecule by bombardment of high energy and this in turn generates a molecular ion. When high energy is injected, most molecular ions continue to break apart into fragment ions, which are analyzed by the detector. The abundance of each fragment ion is plotted against its mass to charge ratio (*m/z*), which is referred to as a mass spectrum.[43] The retention time and the ion fragmentation pattern in a mass spectrum is unique for each compound, facilitating the identification of analytes.[42] Recently, many software programs contain a library of known fragmentation spectra to aid in chemical identification.

One common ionization technique is electrospray ionization (ESI), which is a soft ionization technique, and capable of generating ions for more than 90% of analytes including larger molecules like proteins and nucleic acids.[29,44] This method is also known to cause adduct formations when in the positive mode. These adducts take on the

ion form of $[M+X]^+$ where X are common cations such as NH_4, Na, K, or H.[42] Therefore, it is important to remember which mode the instrument operates, either positive or negative, when analyzing spectra and determining the mass of the molecular ion. Negative mode separates molecules based on mass in the same fashion as the positive mode except deprotonated molecular ions are formed, $[M-H]^-$.[45] ESI operates at atmospheric temperature and pressure with the sample passing through a steel capillary needle that is maintained with a charge in kilovolts and as the sample passes through, a spray of fine droplets is generated that then pass through a de-solvating capillary where the solvent is evaporated and a charge is attached to the analyte.[29] After ions are formed, the droplets are pulled into a vacuum and propelled through a tube towards a detector. With the evaporation of solvent, the charged droplets become smaller and the surface tension can no longer support the charge and the droplet breaks into smaller droplets leaving multiply charged analyte ions.[29]

When MS is coupled with liquid chromatography, the compounds that pass through the HPLC column and are successfully separated will be detected by the MS as that mentioned above. Mass spectrometry requires successful separation prior to injection, as then will then improve the reliability of results due to the decrease in matrix suppression.[47] On-line mass spectrometry, coupled to liquid chromatography, can reduce the possibility of undetected co-eluting peaks.[48] However, the best solvent used for the chromatography portion may not be suitable for the MS such as solvents with high concentrations of non-volatile inorganic salts[42] as they may damage parts of the MS instrument.

Research Objectives

The purpose for my research is to separate and propose identification of bioactive compounds from the plant Devil's Club. After the initial liquid-liquid extraction, there were five fractions that were extracted by water; butanol, ethyl acetate, chloroform and hexane respectively. My research has been focusing on improving the separation of compounds in the ethyl acetate fraction with the aid of solid phase extraction (SPE) and HPLC. The separated fractions will be subjected to an antioxidant test with the use of a spectrophotometer where positive results will be further analyzed by MS for compound identification. A systematic flow chart gives a graphical representation of the overall process of separation and identification process, which is shown in Figure 1.4 below.

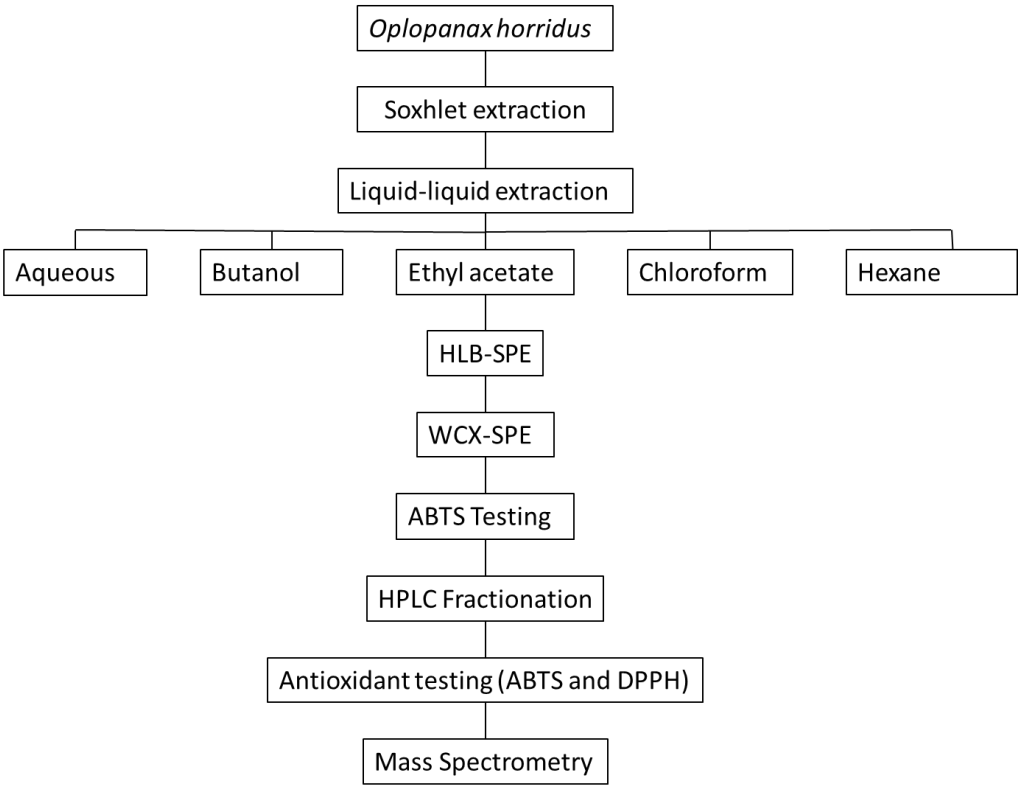

Figure 1.4: Systematic flow chart for separation and identification of bioactive compounds from *Oplopanax horridus.*

As aforementioned, important factors for a successful chemical separation relies on the appropriate selections of sorbent for SPE and HPLC columns, and solvents of the mobile phase. More than one SPE phase may be the most efficacious. Polar basic compounds can be difficult to be separated on reverse phase and therefore it is beneficial to use a cation exchanger[46] particularly it is useful for polyphenols. In order to facilitate the chemical identification and antioxidant test, the eluted fractions will be collected, dried and reconstituted. The isolation and identification of the potential bioactive compounds could lead to novel drug discovery, where this prototype is synthesized along with various analogs for further tests of effectiveness and possible side effects.[43]

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CHAPTER TWO

SAMPLE PREPARATION

Introduction

Sample preparation is the most important step for chemical identification. In the study of natural products the first and mandatory steps are isolation and purification.[8] Without proper extraction and separation of the analytes, any analytical methods utilized will yield inaccurate results and will lower probability of correct compound identification; therefore a good selection of various techniques is essential.[12] When a solid natural product such as a plant is analyzed, the first extraction step is to use either Soxhlet, maceration or a similar technique that will efficiently extract the desirable analytes of interest while avoiding oxidation to the sample or any possible side reactions.[1] The semi-continuous method of Soxhlet extraction is a popular choice for solid-liquid extraction as fresh solvent is constantly introduced to the sample and but filtration step is needed upon completion of extraction.[2] Although it is typically time consuming, the Soxhlet extraction method has the advantages in light of being semi-continuous, operating at a high constant temperature, using less, but sometimes environmentally unfriendly solvents and having more extraction potential than maceration.[3,9] The Soxhlet method employed here was scaled up to a preparative level to obtain large amounts of extract for further extraction, separation and analysis.

Once the initial extract from the solid plant is acquired, liquid-liquid extraction (LLE) can be performed to partition compounds based on their chemical polarity and fractionate complex mixtures.[17] Solvents such as water, alcohol and acetone are generally used to attract polar compounds while chloroform and hexane will withdraw

non-polar compounds[4] with aqueous mixture with methanol, ethyl acetate and acetone are among the most common solvents.[13] For chemicals with in-between polarities, water-alcohol mixtures can be exercised or solvents like ethyl acetate can be used. Ethyl acetate (EtOAc) is a useful medium as it has a low probability of causing side reactions.

Once crude extracts are acquired a "clean-up" stage should be utilized, in order to improve separation of the thousands of compounds that are present in the extract, as the small volume of chromatographic stationary phase removes much of the sample matrix that then simplifies analysis.[7] Solid phase extraction (SPE) is a common practice for this process. Although SPE might be time consuming even utilizing a vacuum manifold for off-line separation, it uses less solvent than other methods thus resulting in a short concentration step.[16] Screening for an optimized method often relies on varying the chromatographic stationary phase and elution solvents. Most common sorbent or stationary phase is silica based C_{18} but advances in recent years have led to the development of more sensitive and selective phases.[5,14] Among the most promising of these is the hydrophilic-lipophilic balanced (HLB) sorbent as its retention times of analytes are comparable to those of the C_{18} silicas.[5] This phase has been becoming widely popular in drug discovery and biological extractions. HLB is being called a universal sorbent as it will extract polar, non-polar or neutral compounds depending on the solvent chosen, therefore it is used as a good starting point for separation from initial fractions. Once polar and non-polar are segregated; acidic and basic compounds can be further separated by the use of ion-exchange sorbents. Cation exchangers that contain sulfonic or carboxylic groups will retain positively charged compounds while bases or negative species will be washed away. Analytes can be targeted by separation of basic,

acidic and neutral compounds with the use of mixed-mode ion exchangers and thus decreasing extract complexity.[10] As many natural products, known to be antioxidants contain ionizable groups, ion exchanges are chosen as sufficient stationary phases in chromatography.[15]

Experimental

Sample Information

The root bark of *Oplopanax horridus* was purchased from Pacific Botanicals (Grants Pass, OR) where the plant sample was harvested and collected between June and August 2012. The bark was air dried until <10% moisture before shipping to Clemson, SC. Ahead of initial extraction, the bark was ground with a Thomas-Wiley laboratory mill model 4 with a screen size of 2.0mm (Thomas-Wiley, Swedesboro, NJ). Then using a Wonder Mill, a second grinding was performed until sample were able to pass through a 500μm screening sieve (a No. 35 sieve). All samples were stored in air tight vacuumed sealed polyester bags in the dark at -20°C until used in the extraction process.

Chemicals

Commercially purchased chemicals include ACS and HPLC grade methanol (MeOH), ACS grade hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), n-butanol (BuOH), and acetonitrile (ACN) from Fisher Scientific. Formic acid 99% (FA) was purchased from Acros Organics. Ammonium hydroxide (NH_4OH) was bought from Mallinkrodt AR Select.

Equipment

Preparative scale Soxhlet extraction was performed with the custom designed extractors manufactured by Kimble Chase Life Sciences (Rockwood, TN). The cellulose

thimbles used in the extractors were purchased from Aqualab Supplies (Barcelona, Spain). To heat the solvent in the extract apparatus, twelve liter heating mantles (1500W) from Briskheat were used (Columbus, OH) and were controlled by Glas-Col PL312 Minitrol 1500W temperature controllers (Terre Haute, IN).

Disposable Oasis HLB (20cc / 1g) and WCX (6mL / 500mg / 60μm) solid phase extraction (SPE) single use cartridges were purchased from Waters Inc. along with the vacuum manifold used in conjunction with the cartridges. Disposable centrifuge tubes (15mL) with flat cap were purchased from Fisher Scientific. Rotoevaporator apparatuses used include Yamato RE400 (Santa Clara, CA) and Buchi Brinkman Rotavapor (New Castle, DE).

Sample Preparation

Preparative Soxhlet Extraction

The extraction began with six liters of MeOH in the 12L round bottom flask and then situated in the heating mantle. Dried, ground sample of *O. horridus* was weighed to 350 grams and packed in the cellulose thimble that was then placed in the Soxhlet extractor which was connected with a condenser. After the heating mantle was turned on, the extraction process lasted for 10 hours. Then the mantles were turned off and the system was allowed to be cooled down for one hour. The crude methanol extract was concentrated by using a rotary evaporator in a 3L round bottom flask. After the concentration, the liquid sample was placed in a 2L screw top bottle and stored at -20°C until liquid-liquid extraction.

Liquid-liquid Extraction

Concentrated crude MeOH was removed from the freeze, where it was being stored, at which point a 200 gram aliquot that was re-suspended in 700mL distilled water. This mixture was shaken vigorously for 2min to ensure complete mixing. This suspension was placed in a 2L separatory funnel where 1L of hexane was added. With stop-cock closed and glass stopper in place, the funnel was shaken for 3min then placed in a holder where layers were able to separate. The top layer (hexane) was removed into a 2L bottle. This process was repeated two more times. Again, similar extraction was repeated with three other individual solvents, i.e., chloroform, ethyl acetate and butanol. After the last layer of butanol was removed the aqueous fraction left in the separatory funnel was also retained. All five fractions were concentrated in 3L round bottom flasks by rotary evaporators. Dried fractions were stored in -20°C.

Fractionation of Ethyl Acetate Extract

The concentrated ethyl acetate fraction from the liquid-liquid extraction was re-suspended in 100% MeOH to achieve a concentration of 400mg/mL and filtered through a 0.45μm filter membrane. This sample was further fractionated using a semi-preparative system consisting of Varian Prostar HPLC pumps (Santa Clara, CA) with Rheodyne (Rohnert Park, CA) injection valves, loops and a Rainin Dynamax (Oakland, CA) ultra violet detector. A high pressure solvent gradient consisting of 0.5% formic acid in water and 0.5% formic acid in methanol (ACS grade) at a flow rate of 20mL/min was used. From here eleven fractions were collected and dried down by rotoevaporation and re-suspended in varying percentages of MeOH in water.

The eleven fractions were acidified by the addition of 0.2% FA in 20% MeOH, and then each fraction was separated by a HLB-SPE cartridge into three washes and three

elutes. A sample would be loaded onto the cartridge column after the condition and equilibration steps; the contents from these two steps were collected in a beaker while the solvent waste was disposed. Once the cartridge conditioning/equilibration steps began it was important to continue with sample loading, washing and eluting as the sorbent should never dry out. The washes and elutes were collected in 15mL vials, capped and stored until further processing.

A total number of 66 fractions from the HLB separation were collected and dried down to determine yield percentage and reconstituted in acidified methanol, i.e., 0.2%FA in 20%MeOH. Those samples were subjected to another SPE separation using a weak cation exchanger (WCX) cartridge separating the samples further into one wash and two more elutes.

Subfractions were collected in 15mL vials and capped until further processing. After this second SPE separation, samples were dried by rotoevaporation and reconstituted in 500µL 100% MeOH then transferred to a 2mL capped vial and stored at -20°C until further analysis.

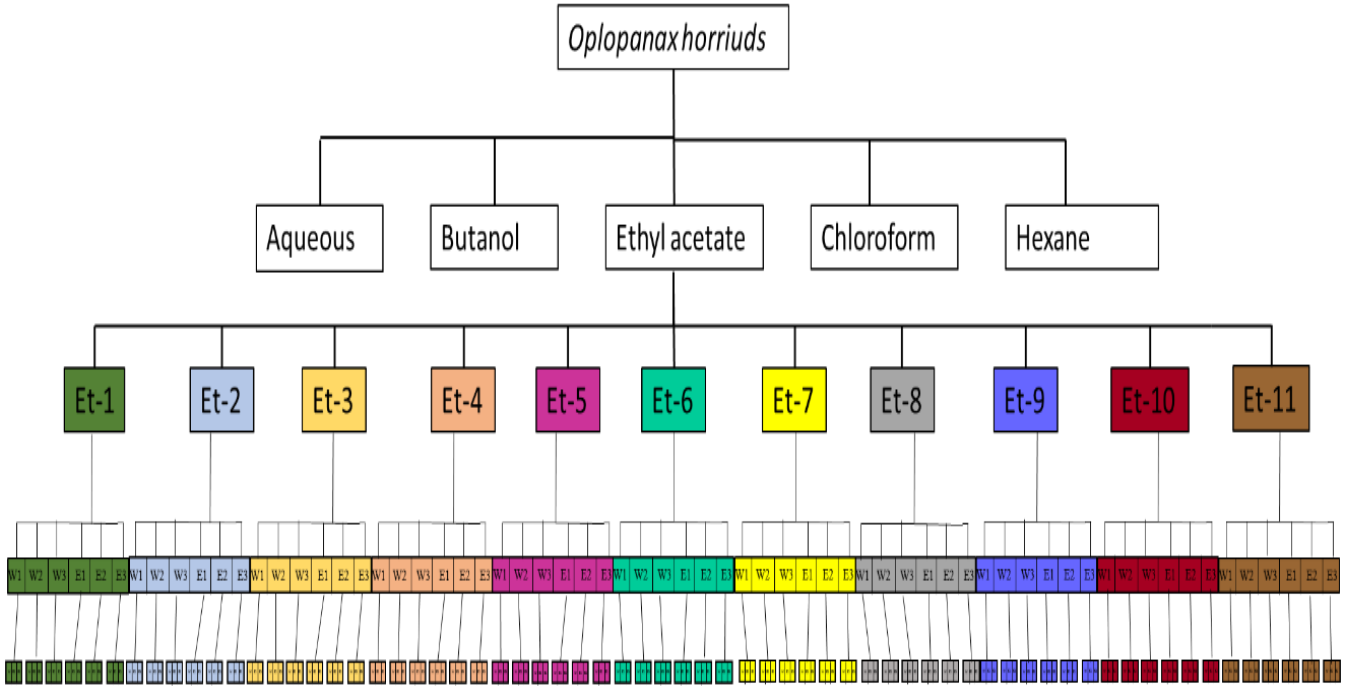

Figure 2.1: Graphic representation of fractionation by LLE, HLB-SPE, and WCX-SPE.

Results

Table 2.1: LLE fractionation of crude methanolic extract.

LLE Extraction	Weight per 200g LLE separation (g)	Percentage of crude methanol extract (w/w)
Hexane	99	49.5
Chloroform	80.8	40.4
Ethyl Acetate	2	1
n-Butanol	10.2	5.1
Aqueous	8	4

Table 2.2: Fraction concentrations from crude ethyl acetate extract.

Fraction	Empty Flask (g)	Dried Sample (g)	Re-suspended	Concentration (g/mL)
1	14.2040	14.2639	4mL 10% MeOH	0.015
2	17.1104	17.1401	4mL 10% MeOH	0.007
3	11.5246	11.5581	4mL 10% MeOH	0.008
4	17.0314	17.0886	4mL 20% MeOH	0.014
5	11.4973	11.5924	4mL 10% MeOH	0.024
6	11.6215	11.8551	4mL 50% MeOH	0.058
7	12.8948	13.0888	4mL 50% MeOH	0.049
8	10.7994	10.9999	4mL 50% MeOH	0.050
9	42.9136	43.2587	4mL 50% MeOH	0.086
10	44.7976	45.1874	4mL 50% and 4mL 100% MeOH	0.049
11	21.3310	21.7898	4mL 50% and 4mL 100% MeOH	0.057

Table 2.3: Separation method used for HLB-SPE cartridge.

HLB Method	
Fraction	**Solvent**
Condition	100% MeOH
Equilibration	DI Water
Wash 1	5% MeOH
Wash 2	2% FA in 20% MeOH
Wash 3	2% FA in 50% MeOH
Elute 1	2% NH4OH in 20%MeOH
Elute 2	2% NH4OH in 50%MeOH
Elute 3	5% NH4OH in 60/40 ACN/MeOH

Table 2.4: Separation method used for WCX-SPE cartridge.

WCX Method	
Fraction	**Solvent**
Condition	100% MeOH
Equilibration	DI Water
Wash	5% NH_4OH in 100% DI Water
Elute 1	100% MeOH
Elute 2	2% FA in 60/40 ACN/MeOH

Table 2.5: SPE fractionation of EtOAc fraction 1 (Et-1).

	HLB				WCX			
EtOAc Crude Fraction #	Frac #	Solvent/Title	Weight	Percentage	Frac #	Solvent/Title	Weight	Percentage
1	1	200uL 0.2%FA 20% MeOH 300uL MeOH	0.035g	9.15%	1	400uL 10% MeOH	0.027g	78.00%
					2	200uL 10% + 200uL 100%	0.004g	12.29%
					3	400uL 10% + 200uL 100%	0.003g	9.71%
	2	200uL 0.2%FA 20% MeOH 300uL MeOH	0.019g	4.99%	1	400uL 10%	0.005g	23.56%
					2	400uL 10% + 200uL 100%	0.010g	51.83%
					3	400uL 10%	0.005g	24.61%
	3	200uL 0.2%FA 20% MeOH 300uL MeOH	0.049g	12.89%	1	400uL 10%	0.027g	55.17%
					2	400uL 10%	0.011g	22.52%
					3	400uL 10%	0.011g	22.31%
	4	200uL 0.2%FA 20% MeOH 300uL MeOH	0.064g	16.73%	1	400uL 10%	0.048g	74.22%
					2	400uL 10%	0.000g	0.00%
					3	400uL 10%	0.017g	25.78%
	5	200uL 0.2%FA 20% MeOH 300uL MeOH	0.073g	18.98%	1	400uL 10%	0.044g	60.47%
					2	400uL 10%	0.007g	9.64%
					3	400uL 10%	0.022g	29.89%
	6	200uL 0.2%FA 20% MeOH 300uL MeOH	0.142g	37.25%	1	400uL 10%	0.105g	73.61%
					2	400uL 10%	0.011g	7.37%
					3	400uL 10%	0.027g	19.02%

Table 2.6: SPE fractionation of EtOAc fraction 2 (Et-2).

	HLB				WCX			
EtOAc Crude Fraction #	Frac #	Solvent/Title	Weight	Percentage	Frac #	Solvent/Title	Weight	Percentage
2	1	200uL 0.2%FA 20% MeOH 300uL MeOH	0.057g	10.19%	1	400uL 10% MeOH	0.031g	54.82%
					2	400uL 10% MeOH	0.011g	19.09%
					3	400uL 10% MeOH	0.015g	26.09%
	2	200uL 0.2%FA 20% MeOH 300uL MeOH	0.027g	4.82%	1	400uL 10% MeOH	0.008g	29.63%
					2	400uL 10% MeOH	0.006g	20.37%
					3	400uL 10% MeOH	0.014g	50.00%
	3	200uL 0.2%FA 20% MeOH 300uL MeOH	0.040g	7.15%	1	400uL 10% + 400uL 100%	0.019g	46.38%
					2	400uL 10%	0.005g	11.72%
					3	400uL 10%	0.017g	41.90%
	4	200uL 0.2%FA 20% MeOH 300uL MeOH	0.146g	25.96%	1	400uL 10% + 800uL 100%	0.038g	26.32%
					2	400uL 10%	0.058g	40.07%
					3	400uL 10%	0.049g	33.61%
	5	200uL 0.2%FA 20% MeOH 300uL MeOH	0.035g	6.19%	1	400uL 10%	0.030g	87.32%
					2	400uL 10%	0.001g	3.46%
					3	400uL 10%	0.003g	9.22%
	6	200uL 0.2%FA 20% MeOH 300uL MeOH	0.256g	45.69%	1	400uL 10%	0.186g	72.67%
					2	400uL 10%	0.033g	12.73%
					3	400uL 10%	0.037g	14.60%

Table 2.7: SPE fractionation of EtOAc fraction 3 (Et-3).

	HLB				WCX			
EtOAc Crude Fraction #	Frac #	Solvent/Title	Weight	Percentage	Frac #	Solvent/Title	Weight	Percentage
3	1	200uL 0.2%FA 20% MeOH 300uL MeOH	0.057g	9.01%	1	400uL 10% MeOH	0.018g	31.24%
					2	400uL 10% MeOH	0.028g	49.04%
					3	400uL 10% MeOH	0.011g	19.72%
	2	200uL 0.2%FA 20% MeOH 300uL MeOH	0.070g	11.00%	1	400uL 10% MeOH	0.028g	39.29%
					2	400uL 10% MeOH	0.016g	23.00%
					3	400uL 10% MeOH	0.026g	37.71%
	3	200uL 0.2%FA 20% MeOH 300uL MeOH	0.045g	7.01%	1	400uL 10% + 400uL 100%	0.005g	11.21%
					2	400uL 10%	0.022g	49.33%
					3	400uL 10%	0.018g	39.46%
	4	200uL 0.2%FA 20% MeOH 300uL MeOH	0.107g	16.78%	1	400uL 10%	0.072g	67.60%
					2	400uL 10%	0.014g	13.01%
					3	400uL 10%	0.021g	19.38%
	5	200uL 0.2%FA 20% MeOH 300uL MeOH	0.126g	19.80%	1	400uL 10%	0.059g	46.67%
					2	400uL 10% + 200uL 100%	0.057g	45.40%
					3	400uL 10%	0.010g	7.94%
	6	200uL 0.2%FA 20% MeOH 300uL MeOH	0.232g	36.40%	1	400uL 10% + 800uL 100%	0.184g	79.49%
					2	400uL 10% + 400uL 100%	0.025g	10.97%
					3	400uL 10% + 400uL 100%	0.022g	9.54%

Table 2.8: SPE fractionation of EtOAc fraction 4 (Et-4).

	HLB				WCX			
EtOAc Crude Fraction #	Frac #	Solvent/Title	Weight	Percentage	Frac #	Solvent/Title	Weight	Percentage
4	1	200uL 0.2%FA 20% MeOH 300uL MeOH	0.044g	5.50%	1	400uL 10% MeOH	0.028g	63.49%
					2	400uL 10%	0.003g	6.35%
					3	400uL 10%	0.013g	30.16%
	2	200uL 0.2%FA 20% MeOH 300uL MeOH	0.116g	14.52%	1	400uL 10%	0.004g	3.10%
					2	400uL 10%	0.094g	80.65%
					3	400uL 10%	0.019g	16.25%
	3	200uL 0.2%FA 20% MeOH 300uL MeOH	0.096g	12.03%	1	400uL 10%	0.016g	16.18%
					2	400uL 10%	0.019g	19.81%
					3	400uL 10%	0.062g	64.00%
	4	200uL 0.2%FA 20% MeOH 300uL MeOH	0.121g	15.05%	1	400uL 10%	0.068g	56.72%
					2	400uL 10%	0.022g	17.91%
					3	400uL 10%	0.031g	25.37%
	5	200uL 0.2%FA 20% MeOH 300uL MeOH	0.112g	14.02%	1	400uL 10%	0.038g	33.39%
					2	400uL 10%	0.046g	40.61%
					3	400uL 10%	0.029g	26.00%
	6	200uL 0.2%FA 20% MeOH 300uL MeOH	0.311g	38.87%	1	400uL 10% + 400uL 100%	0.233g	74.76%
					2	400uL 10% + 400uL 100%	0.046g	14.87%
					3	400uL 10%	0.032g	10.37%

Table 2.9: SPE fractionation of EtOAc fraction 5 (Et-5).

	HLB				WCX			
EtOAc Crude Fraction #	Frac #	Solvent/Title	Weight	Percentage	Frac #	Solvent/Title	Weight	Percentage
5	1	200uL 0.2%FA 20% MeOH 300uL MeOH	0.098g	10.10%	1	400uL 10% + 600uL 100%	0.059g	60.22%
					2	400uL 10%	0.020g	19.94%
					3	400uL 10%	0.019g	19.84%
	2	200uL 0.2%FA 20% MeOH 300uL MeOH	0.093g	9.56%	1	400uL 10%	0.024g	26.24%
					2	400uL 10%	0.044g	47.41%
					3	400uL 10%	0.024g	26.35%
	3	200uL 0.2%FA 20% MeOH 300uL MeOH	0.301g	31.97%	1	400uL 10% + 400uL 100%	0.254g	81.89%
					2	400uL 10%	0.015g	4.88%
					3	400uL 10%	0.041g	13.24%
	4	200uL 0.2%FA 20% MeOH 300uL MeOH	0.073g	7.50%	1	400uL 10%	0.036g	49.45%
					2	400uL 10%	0.008g	10.47%
					3	400uL 10%	0.029g	40.08%
	5	200uL 0.2%FA 20% MeOH 300uL MeOH	0.086g	8.90%	1	400uL 10%	0.045g	52.32%
					2	400uL 10%	0.006g	7.08%
					3	400uL 10%	0.035g	40.60%
	6	200uL 0.2%FA 20% MeOH 300uL MeOH	0.301g	31.97%	1	400uL 10% + 400uL 100%	0.254g	81.89%
					2	400uL 10% + 400uL 100%	0.015g	4.88%
					3	400uL 10%	0.041g	13.24%

Table 2.10: SPE fractionation of EtOAc fraction 6 (Et-6).

	HLB				WCX			
EtOAc Crude Fraction #	Frac #	Solvent/Title	Weight	Percentage	Frac #	Solvent/Title	Weight	Percentage
6	1	200uL 0.2%FA 20% MeOH 300uL MeOH	0.075g	6.37%	1	400uL 10% + 400uL 100%	0.032g	42.51%
					2	200uL 10% + 200uL 100%	0.002g	2.67%
					3	400uL 10% + 200uL 100%	0.041g	54.81%
	2	200uL 0.2%FA 20% MeOH 300uL MeOH	0.051g	4.36%	1	400uL 10%	0.008g	15.23%
					2	400uL 10%	0.016g	32.03%
					3	400uL 10%	0.027g	52.73%
	3	600uL 0.2%FA 20% MeOH 200uL MeOH	0.185g	15.75%	1	400uL 10% + 200uL 100%	0.093g	50.51%
					2	400uL 10%	0.031g	16.60%
					3	400uL 10%	0.061g	32.88%
	4	200uL 0.2%FA 20% MeOH 300uL MeOH	0.289g	24.63%	1	400uL 10% + 400uL 100%	0.184g	63.54%
					2	400uL 10% + 200uL 100%	0.063g	21.65%
					3	400uL 10%	0.043g	14.80%
	5	200uL 0.2%FA 20% MeOH 300uL MeOH	0.299g	25.47%	1	400uL 10% + 400uL 100%	0.179g	59.83%
					2	400uL 10%	0.012g	3.88%
					3	400uL 10%	0.109g	36.29%
	6	200uL 0.2%FA 20% MeOH 300uL MeOH	0.275g	23.42%	1	800uL 100%	0.220g	80.14%
					2	400uL 10% + 400uL 100%	0.029g	10.59%
					3	400uL 10%	0.026g	9.28%

Table 2.11: SPE fractionation of EtOAc fraction 7 (Et-7).

	HLB				WCX			
EtOAc Crude Fraction #	Frac #	Solvent/Title	Weight	Percentage	Frac #	Solvent/Title	Weight	Percentage
7	1	200uL 0.2%FA 20% MeOH 300uL MeOH	0.101g	9.76%	1	400uL 10% MeOH	0.022g	21.86%
					2	400uL 10%	0.036g	35.71%
					3	400uL 10%	0.043g	42.43%
	2	200uL 0.2%FA 20% MeOH 300uL MeOH	0.069g	6.67%	1	400uL 10%	0.028g	39.94%
					2	400uL 10%	0.002g	2.75%
					3	400uL 10%	0.040g	57.31%
	3	200uL 0.2%FA 20% MeOH 300uL MeOH	0.073g	7.08%	1	400uL 10% + 400uL 100%	0.044g	60.57%
					2	400uL 10%	0.008g	10.37%
					3	400uL 10%	0.021g	29.06%
	4	200uL 0.2%FA 20% MeOH 300uL MeOH	0.189g	18.21%	1	400uL 10% + 400uL 100%	0.136g	72.07%
					2	400uL 10% + 200uL 100%	0.023g	11.98%
					3	400uL 10%	0.030g	15.95%
	5	200uL 0.2%FA 20% MeOH 300uL MeOH	0.182g	17.60%	1	400uL 10% + 400uL 100%	0.087g	47.61%
					2	400uL 10%	0.055g	30.28%
					3	400uL 10%	0.040g	22.11%
	6	200uL 0.2%FA 20% MeOH 400uL MeOH	0.422g	40.69%	1	400uL 10% + 400uL 100%	0.317g	75.18%
					2	400uL 10% + 400uL 100%	0.052g	12.43%
					3	400uL 10%	0.052g	12.38%

Table 2.12: SPE fractionation of EtOAc fraction 8 (Et-8).

	HLB				WCX			
EtOAc Crude Fraction #	Frac #	Solvent/Title	Weight	Percentage	Frac #	Solvent/Title	Weight	Percentage
8	1	200uL 0.2%FA 20% MeOH 300uL MeOH	0.095g	11.62%	1	400uL 10% MeOH	0.030g	31.72%
					2	400uL 10%	0.034g	35.30%
					3	400uL 10%	0.031g	32.98%
	2	200uL 0.2%FA 20% MeOH 300uL MeOH	0.076g	9.30%	1	400uL 10%	0.021g	27.63%
					2	400uL 10%	0.022g	28.82%
					3	400uL 10%	0.033g	43.55%
	3	200uL 0.2%FA 20% MeOH 300uL MeOH	0.112g	13.71%	1	400uL 10%	0.025g	22.32%
					2	400uL 10%	0.059g	52.68%
					3	400uL 10%	0.028g	25.00%
	4	200uL 0.2%FA 20% MeOH 300uL MeOH	0.110g	13.44%	1	400uL 10% + 400uL 100%	0.085g	77.23%
					2	400uL 10%	0.002g	1.73%
					3	400uL 10%	0.023g	21.04%
	5	200uL 0.2%FA 20% MeOH 300uL MeOH	0.128g	15.67%	1	400uL 10% + 800uL 100%	0.079g	61.56%
					2	400uL 10% + 400uL 100%	0.019g	14.77%
					3	400uL 10%	0.030g	23.67%
	6	500uL 0.2%FA 20% MeOH 1mL MeOH	0.296g	36.26%	1	400uL 10% + 400uL 100%	0.188g	63.57%
					2	400uL 10%	0.072g	24.27%
					3	400uL 10%	0.036g	12.15%

Table 2.13: SPE fractionation of EtOAc fraction 9 (Et-9).

	HLB				WCX			
EtOAc Crude Fraction #	Frac #	Solvent/Title	Weight	Percentage	Frac #	Solvent/Title	Weight	Percentage
9	1	400uL 0.2%FA 20% MeOH 100uL MeOH	0.087g	7.71%	1	400uL 10% + 800uL 100%	0.033g	37.59%
					2	400uL 10%	0.026g	29.43%
					3	400uL 10%	0.029g	32.99%
	2	200uL 0.2%FA 20% MeOH 300uL MeOH	0.062g	5.50%	1	400uL 10% + 200uL 100%	0.004g	7.09%
					2	400uL 10%	0.024g	38.97%
					3	400uL 10%	0.034g	53.95%
	3	200uL 0.2%FA 20% MeOH 300uL MeOH	0.090g	8.00%	1	400uL 10% + 400uL 100%	0.040g	44.19%
					2	400uL 10%	0.021g	22.81%
					3	400uL 10%	0.030g	33.00%
	4	200uL 0.2%FA 20% MeOH 300uL MeOH	0.355g	31.44%	1	400uL 10% + 400uL 100%	0.099g	27.76%
					2	400uL 10% + 400uL 100%	0.171g	48.28%
					3	400uL 10%	0.085g	23.96%
	5	200uL 0.2%FA 20% MeOH 300uL MeOH	0.215g	19.01%	1	400uL 10% + 1200uL 100%	0.114g	53.01%
					2	400uL 10% + 400uL 100%	0.023g	10.86%
					3	400uL 10%	0.078g	36.13%
	6	200uL 0.2%FA 20% MeOH 1mL MeOH	0.320g	28.33%	1	1400uL 100%	0.253g	79.07%
					2	400uL 10% + 800uL 100%	0.035g	10.79%
					3	400uL 10%	0.032g	10.13%

Table 2.14: SPE fractionation of EtOAc fraction 10 (Et-10).

	HLB				WCX			
EtOAc Crude Fraction #	Frac #	Solvent/Title	Weight	Percentage	Frac #	Solvent/Title	Weight	Percentage
10	1	200uL 0.2%FA 20% MeOH 300uL MeOH	0.116g	15.03%	1	400uL 10% MeOH	0.021g	18.06%
					2	400uL 10%	0.065g	56.44%
					3	400uL 10%	0.030g	25.50%
	2	200uL 0.2%FA 20% MeOH 300uL MeOH	0.075g	9.78%	1	400uL 10%	0.026g	34.66%
					2	400uL 10%	0.008g	10.76%
					3	400uL 10%	0.041g	54.58%
	3	400uL 0.2%FA 20% MeOH 100uL MeOH	0.092g	11.99%	1	400uL 10% + 400uL 100%	0.014g	14.84%
					2	200uL 10% + 200uL 100%	0.046g	49.40%
					3	400uL 10%	0.033g	35.75%
	4	200uL 0.2%FA 20% MeOH 300uL MeOH	0.041g	5.29%	1	400uL 10% + 400uL 100%	0.007g	17.69%
					2	400uL 10% + 400uL 100%	0.004g	10.32%
					3	400uL 10%	0.029g	71.99%
	5	200uL 0.2%FA 20% MeOH 300uL MeOH	0.128g	16.68%	1	400uL 10% + 400uL 100%	0.067g	52.41%
					2	400uL 10% + 400uL 100%	0.035g	27.10%
					3	400uL 10%	0.026g	20.48%
	6	400uL 0.2%FA 20% MeOH 1mL MeOH	0.317g	41.22%	1	400uL 10% + 800uL 100%	0.240g	75.57%
					2	400uL 10% + 400uL 100%	0.020g	6.34%
					3	400uL 10%	0.057g	18.10%

Table 2.15: SPE fractionation of EtOAc fraction 11 (Et-11).

	HLB				WCX			
EtOAc Crude Fraction #	Frac #	Solvent/Title	Weight	Percentage	Frac #	Solvent/Title	Weight	Percentage
11	1	400uL 0.2%FA 20% MeOH 600uL MeOH	0.192g	22.41%	1	400uL 10% + 1200uL 100%	0.058g	30.39%
					2	400uL 10% + 200uL 100%	0.087g	45.17%
					3	400uL 10%	0.047g	24.44%
	2	200uL 0.2%FA 20% MeOH 300uL MeOH	0.112g	13.06%	1	400uL 10% + 200uL 100%	0.016g	13.98%
					2	400uL 10%	0.049g	43.64%
					3	400uL 10%	0.047g	42.38%
	3	200uL 0.2%FA 20% MeOH 300uL MeOH	0.166g	19.45%	1	400uL 10%	0.023g	13.54%
					2	400uL 10%	0.087g	52.05%
					3	400uL 10%	0.057g	34.42%
	4	200uL 0.2%FA 20% MeOH 300uL MeOH	0.065g	7.65%	1	400uL 10%	0.042g	63.46%
					2	400uL 10%	0.002g	3.21%
					3	400uL 10%	0.022g	33.33%
	5	200uL 0.2%FA 20% MeOH 300uL MeOH	0.113g	13.21%	1	400uL 10% + 800uL 100%	0.026g	22.67%
					2	400uL 10%	0.051g	45.35%
					3	400uL 10%	0.036g	31.98%
	6	400uL 0.2%FA 20% MeOH 2mL MeOH	0.207g	24.22%	1	1200uL 100%	0.129g	62.46%
					2	1000uL 100	0.043g	20.92%
					3	400uL 10%	0.034g	16.62%

Discussion

Figure 2.1 is a visual representation of all three extraction methods that were employed. From the starting point, the *Oplopanax horridus* underwent the Soxhlet extraction followed by liquid-liquid extraction which five crude extracts were obtained, including hexane, chloroform, ethyl acetate, butanol and aqueous extracts. The ethyl acetate fraction was then divided in to eleven arbitrary fractions as a starting point for compound separation. The eleven fractions were subdivided by HLB-SPE to three washes (coded as W1, W2 and W3), that were washed by 5% MeOH, 2% FA in 20% MeOH and 2% FA in 50% MeOH respectively, and three elutes (coded as E1, E2 and E3), that were eluted using 2% NH_4OH in 20% MeOH, 2% NH_4OH in 50% MeOH and 5% NH_4OH in 60/40 ACN/MeOH respectively. The now 66 fractions were further subdivided by WCX-SPE to one more wash (W) with 5% NH_4OH in 100% water and two elutes (E1 and E2), that were eluted with 100% MeOH and 2%FA in 60/40 ACN/MeOH respectively. Yielding 18 fractionated samples per each ethyl acetate fraction, or a total number of 198 subfractions from the EtOAc fraction. Samples were coded by the following notation, Et-# HLB-WCX, i.e. Et-1 W1-E1 is from the EtOAc fraction 1, it is the first wash from the HLB and the first elute from the WCX.

Soxhlet Extraction

The optimization of the preparative scale Soxhlet extraction was completed in previous works. It was found that a 12%(w/w) yield of the MeOH crude extract was obtained, which may seem low but in fact, the Soxhlet extraction gives better yield than other techniques because it operates at such high temperatures the diffusivity of the solvent is increased.[6] After the 10 hour extraction period the extraction yield did not

increase so the extraction was then stopped. Using methanol as a solvent proved to be more successful than hexane or ethanol. In addition, no filtration step was needed by this method. The crude extract was able to be concentrated and then prepared for further extraction.

Liquid-liquid Extraction

Table 2.1 shows how the crude methanolic extract from the Soxhlet extraction was divided into the other fractions by three other solvents of varying polarity after the LLE. As hexane is very non-polar and chloroform, also non-polar, these fractions are of little interest for the prospect of compounds with antioxidant properties based on the previous experiments and experience. The very polar aqueous phase, although it is promising of valuable compounds, would be difficult to work with partially due to its high boiling point. When heating an aqueous sample in a water bath of a rotoevapotor apparatus, the high heat could destroy beneficial bioactive compounds. In comparison, ethyl acetate is a polar aprotic solvent that should extract the weakly basic compounds, flavonoids and glycosides which are often the types of bioactive compounds with potential antioxidant capacity. Therefore, the EtOAc fraction was prepared (Table 2.1), separated into 11 arbitrary fraction (Table 2.2), and collected for further chemical analyses.

Solid Phase Extraction

The hydrophilic-lipophilic balanced (HLB) SPE was selected as the first step in the "clean-up" process as it was a quick way to separate the acids from the neutral and basic compounds. Optimization of this method (as seen in Table 2.3) occurred after performing the suggested generic method provided by Waters Inc. with the purchase of

the HLB-SPE cartridges. When optimizing the separation method important conditions to consider is the composition and volume of washing and eluting solvents.[11] The strategy for HLB optimization, as recommended by Waters Inc. is as follows; (1) conditioning the column with 100% MeOH, then (2) equilibrate the column with distilled water, at which point the sample is to be loaded onto the column, (3) washing first with 5% MeOH, (4) washing second with 2% NH_4OH in methanol/water, and (5) finally eluting the column with 2% acetic acid in methanol/water. This method contained two washes (i.e, steps 3 and 4) and one elute (i.e., step 5). During the washing and eluting period some visible separated bands could be observed in the sorbent. Since the initial recommended solvents increased polarity too quickly this resulted in the co-elution of the bands. In this context, the method was modified to include an additional wash step as well as changing the second wash step from ammonium hydroxide to formic acid in methanol. Two more eluting solvents were added with varying strengths. At first, the acetic acid was substituted for NH_4OH in methanol and second an ACN/MeOH mixture was added. This proved successful and became the method of choice (Table 2.3).

Before selecting the weak cation exchanger as the second SPE, a comparison was made between the WCX and the mixed-mode cation exchanger (MCX). Waters Inc. provides a generic protocol (or a 2x4 method) used by both the WCX and MCX SPEs for the crude EtOAc as the sample. Both SPEs were conditioned with MeOH and equilibrated with distilled water. For the MCX, it was washed with 2% acetic acid, eluted at first with MeOH and then eluted by 5% NH_4OH in methanol. In comparison, the WCX cartridge, was washed with 5% NH_4OH, eluted at first by 100% MeOH and then eluted by 2% acetic acid in methanol. These protocols and the subsequent HPLC

analyses (described in more details in chapter 4) demonstrated that the MCX possessed excessive retention of analytes and had poor recovery. Therefore, the weak cation exchanger (WCX) was chosen based on its successful recovery of basic compounds. Table 2.4 illustrates the final method that was employed with the weak cation exchanger (WCX).

Once the condition and equilibration steps were performed, it was important to quickly add the acidified sample and subsequent eluting solvents before the sorbent dries out. It is also imperative that the sample be acidified to achieve a pH<2.7 by adjusting the degree of protonation and increasing the desired interactions between sorbent, sample and solvent. Basic analytes should be protonated to maximized ionic interactions with the cation exchange sorbent.[10] It is also worthy of mention that the loaded sample amounts were to be no more than 10% of the cartridge sorbent bed weight. All volumes of solvent used in SPE separations were approximately 5mL or 5 column volumes. For some elutes, more solvent might be needed for a successful and complete separation. In this context, an additional 5 or 10mL of solvent was added as needed.

Table 2.2 illustrates the dried concentrations of the initial eleven fractions from the crude ethyl acetate (i.e., the EtOAc fractions of 1 to 11). These eleven samples were subjected to HLB-SPE, where each sample produced three washes (**W1,W2,W3**) and three elutes (**E1,E2,E3**) for a total of 66 fractions. Once dried and reconstituted with acidified methanol, these samples underwent the further separation by WCX-SPE where each sample was divided into another wash (**W**) and two more elutes (**E1,E2**) for a total of 198 samples. The fractionation of the eleven crude fractions are listed in Tables 2.5 – 2.15; with the tables depicting the reconstitution solvents and determined concentration

for each sample after each separation as well as percentage of distribution. Within the tables, the fraction numbers under the HLB heading correspond to the washes and elutes in the following way; 1=Wash 1, 2=Wash 2, 3=Wash 3, 4=Elute 1, 5=Elute 2, 6=Elute 3. Also within those tables, the fraction numbers listed under the WCX heading correspond to the wash and elute accordingly; 1=Wash, 2=Elute 1 and 3=Elute 2.

The reconstituted solvents depicted in Table 2.5-2.15 for the HLB fractions showed that most of the dried samples were easily dissolved in 200µL of acidified 20% methanol with the addition of 300µL of 100% methanol. Some samples needed an excess of methanol with upwards of 600µL, 1mL or 2mL for complete dissolution. The addition of the 100% methanol to the samples aided in the reconstitution by fully re-suspending the dried particulates, due to the increased dipole interactions between sample and solvent change.

When reconstituting the dried samples after the SPE fractionation, the majority of all samples were dissolved in 400µL of 10% methanol but again, 100% of methanol was used to fully reconstitute some of the samples that required a higher organic concentration for complete dissolution. This time, the 10% methanol was not acidified as the sample would not be loaded onto another SPE sorbent, therefore the only solvent precaution taken was to use HPLC methanol as some samples would eventually be injected onto an HPLC column.

All 198 samples were stored in 2mL vials at -20°C until further analysis.

All samples would be tested for antioxidant properties with the most active set being investigated further by the analytical techniques of high pressure liquid chromatography and mass spectrometry. As the compounds of interest (antioxidants)

tend to be basic and with knowledge of extraction principles, it was predicted that Et-# W2-E1 and Et-# W3-E1 fractions might possibly be containing the weak bases while the Et-# W2-E2 and Et-# W3-E2 fractions might possibly contain the strong bases. This process of extraction and separation before antioxidant testing has not been researched with *Oplopanax horridus* as a way to begin chemical identification, which will be described in details in Chapter 3 and Chapter 4.

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CHAPTER THREE

ANTIOXIDANT TESTING

Introduction

Antioxidants are compounds that delay, inhibit or prevent the oxidation of matter and effectively diminish oxidative stress.[1] Oxidative stress occurs when there is an imbalance between oxidants and antioxidants, favoring an oxidant's concentration that is greater than needed for normal cell function, and resulting in multiple diseases.[1,2,10] To be a successful antioxidant, a compound must not only delay, inhibit or prevent oxidation, but a resulting radical formed must be stable.[3] Antioxidant assays operate on the notion of a pre-formed radical, scavenging a hydrogen donor through a variety of mechanisms.[4,11] The most common of these methods utilize chromophores, or a color radical, where the reduction, scavenging ability, is monitored by spectrophotometer.[5,19] For their ease and rapidity, the 2,2'-azino-bis-3-ethylbenzthiazoline -6-sulphonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays are widely used and accepted.[12,16] Both of these assays utilize a dark colored radical that is lightened once a hydrogen has been scavenged to satisfy the unstable radical and this reaction can occur within a matter of minutes. It has been shown that the ABTS and DPPH assays exhibit a strong correlation to phenolic content.[5]

The basic structure of a phenol is a six member carbon ring with a hydroxyl group attached (Figure 3.1). Polyphenols can contain multiple rings and more than one hydroxyl group. Studies have demonstrated the strong antioxidant activity of phenolic phytochemicals[7], which are fervently present in natural sources, for example, over 8,000 polyphenols have been found as secondary metabolites from plants.[1] These natural

antioxidants are being integrated into the nutraceutical industry[2] with global market value of $117 billion dollars, as of 2013.[9] As there are many documented cases of Devil's Club being used for ailments and diseases,[17,18] it can be thought that antioxidants are present and most likely as the plant's secondary metabolites.

Polyphenols, known for their antioxidant effects and potential role in disease prevention,[13] can be divided into various groups based on their chemical structures with flavonoids being the largest group of plant polyphenolics.[1] Flavonoids (Figure 3.2) are important compounds for their natural functions within the plant,[6] such as protection against pathogens, herbivores and ultraviolet radiation.[15] And physiologically in humans, flavonoids, inhibit autoxidation and scavenge free radicals, acting as an antioxidant.[6,14] As these compounds are effective antioxidants due to their hydrogen donating capability, the overall activity depends on the number of hydroxyl groups present in the molecule. Flavonoids can further be subdivided into classifications based on the addition of hydroxyl or carbonyl groups.

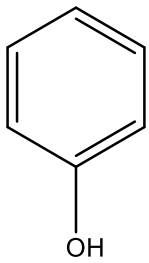

Figure 3.1: Simple phenol structure

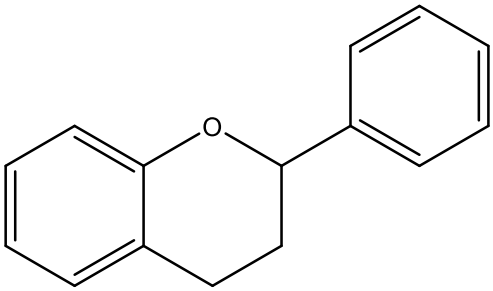

Figure 3.2: Flavonoid base structure

The two antioxidant tests that were chosen for this study were the ABTS and DPPH assays. ABTS was selected for its ability to be used in organic or aqueous solvents. It uses a specific absorbance wavelength (734nm) and requires a short reaction time.[7] A second assay is often selected as multiple tests can provide information on various possible actions of the antioxidants.[2] The DPPH method can also be studied using ultraviolet/visible spectroscopy and has shown to be more selective than the ABTS assay.[8] These assays generally use Trolox as a standard to create a calibration curve of varying concentrations. Trolox is a water soluble analog of vitamin E and in replacement of this, butylated hydroxytoluene (BHT) was used as it is also an analog of vitamin E and as a phenol derivative produces antioxidant capabilities.[20,21]

One hundred and ninety eight fractions obtained after the SPE separation (described in Chapter 2) were tested for their antioxidant capacity at first with the ABTS assay. The most active ethyl acetate fraction was then further fractionated using an HPLC fraction collector. These sub-fractions were then tested for their antioxidant activity and their BHT equivalency was calculated. Statistical analysis was executed using computer software JMP to analyze the absorbance readings and determine significant differences.

Experimental

Sample Information

Samples were eluted after the second solid phase extraction and dried by rotoevaporation and reconstituted in various amounts and strengths of methanol. The most active EtOAc fraction was further fractionated using a Shimadzu HPLC with fraction collector. Either 1μL or 10μL of sample was used per well depending on the samples being used either initial fractions or subfractions from HPLC.

Chemicals

Chemical standards including ACS grade methanol (MeOH), potassium persulfate ($K_2S_2O_8$) and anhydrous ethanol (EtOH) were commercially purchased from Fisher Scientific (Waltham, MA). ABTS and DPPH radicals as well as butylated hydroxytoluene (BHT) were bought from Sigma-Aldrich (St. Louis, MO)

Equipment

A μQuant microplate reader from BioTek (Winooski, VT) with ultraviolet/visible light reading capabilities was used to analyze samples in 96 well plates, which have flat bottoms with lids, are tissue culture treated, and made of non-pyrogenic polystyrene that are obtained from Corning Inc. (Corning, NY).

Antioxidant Testing

BHT Standards

A stock solution of 15mM BHT in methanol was made to prepare all of the subsequent concentrations. Serial dilution of the stock solution created the following concentrations, 3, 5, 7.5 and 10mM for the ABTS testing. The DPPH method required

higher concentrated samples and therefore 60mM BHT was prepare and serially diluted for 12, 20, 30, and 40mM.

ABTS Assay

The radical solution was first prepared by creating a 7mM ABTS solution in distilled water and a 2.45mM solution of $K_2S_2O_8$ also in water. These two solutions were then mixed in even parts to generate a solution that was allowed to sit undisturbed in the dark for sixteen hours. This dark green solution was then diluted with 80% EtOH to obtain an absorbance reading of approximately 0.7; this was done by diluting 1mL of the radical solution with 12mL of the ethanol. At all times, the radical solutions were kept wrapped in aluminum foil to decrease any photo-bleaching as the ABTS radical is very light sensitive.

To the 96 well plate, 1μL (or 10μL for subfractions) of each sample was added in five replicates where each plate had a positive and negative control. The positive control was the highest concentration of the BHT solutions used for the calibration curve and the negative control was the diluted ABTS solution. To the wells with samples, 100μL of the diluted ABTS solution was added and the reaction allowed to proceed with the complete reaction taking no longer than five minutes.

The plate was then placed into the μQuant plate reader where the absorbance at 734nm was recorded and exported to an Excel file. The same process was followed using the five BHT standards to generate a calibration curve but ten replicates were made instead of five.

DPPH Testing of Subfractions

The DPPH radical was put into a $6x10^{-5}$ M solution with methanol and also covered in aluminum foil to reduce the amount of light interaction. This generated a dark purple solution that did not need to sit for sixteen hours nor did it need to be diluted.

To the 96 well plate, 8µL of sample was added in five replicates with 100µL of DPPH solution and again a positive control of BHT standard and negative control of just solution was present. Once the radical was added, the well plate was covered in aluminum foil and allowed to sit for twenty minutes for the reaction to occur. Once the reaction was complete, the plate was placed into the µQuant plate reader with the absorbance being recorded at 517nm and values being exported to an Excel file. This same process was carried out for the five different concentrations of BHT standards to generate a calibration curve.

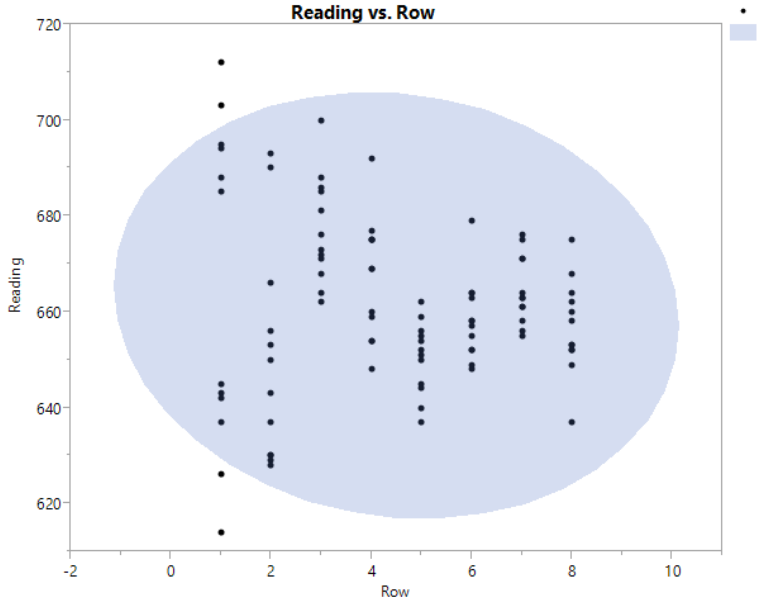

Figure 3.3: JMP generated plot of microplate reading of standard solution as distributed across all rows with overlaying 95% bivariate density map.

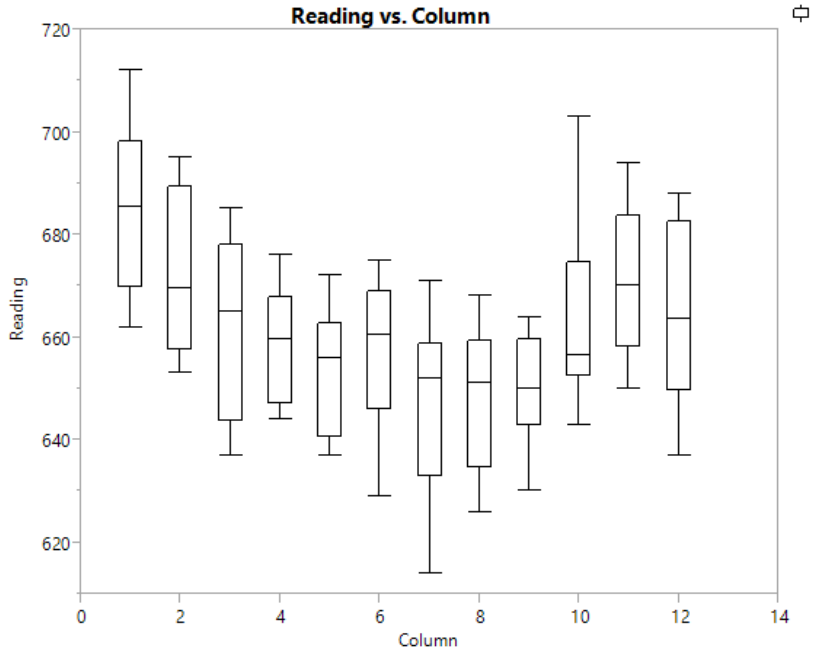

Figure 3.4: JMP generated plot of quartile ranges with outlier bars for absorption means of each column.

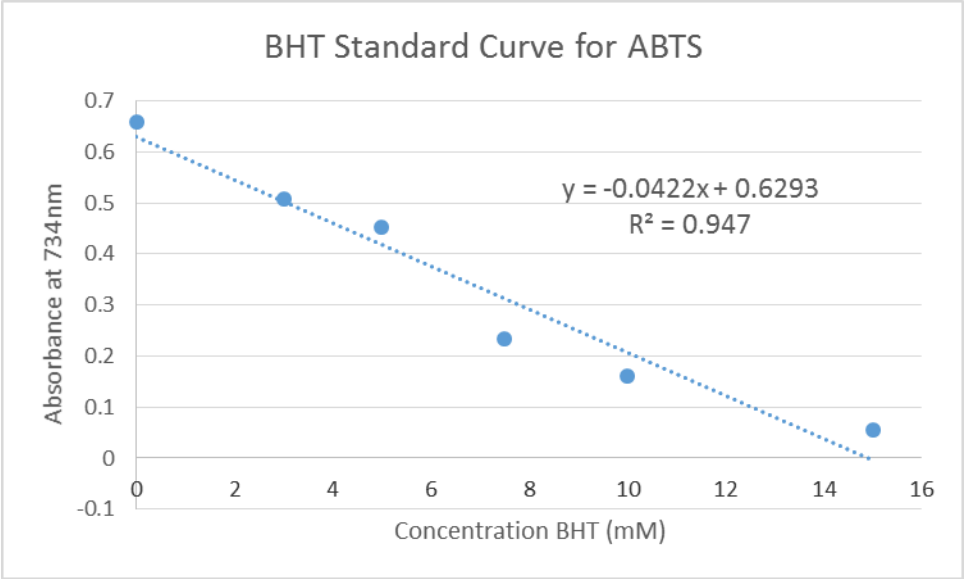

Figure 3.5: Calibration curve of BHT standards using ABTS assay.

Level	Least Sq Mean	Std Error
+ Control	0.08902353	0.00470373
-Control	0.66560000	0.00470373

Table 3.1: Least square mean and standard error calculated for both controls across all plates (from JMP software with p-value<0.0001).

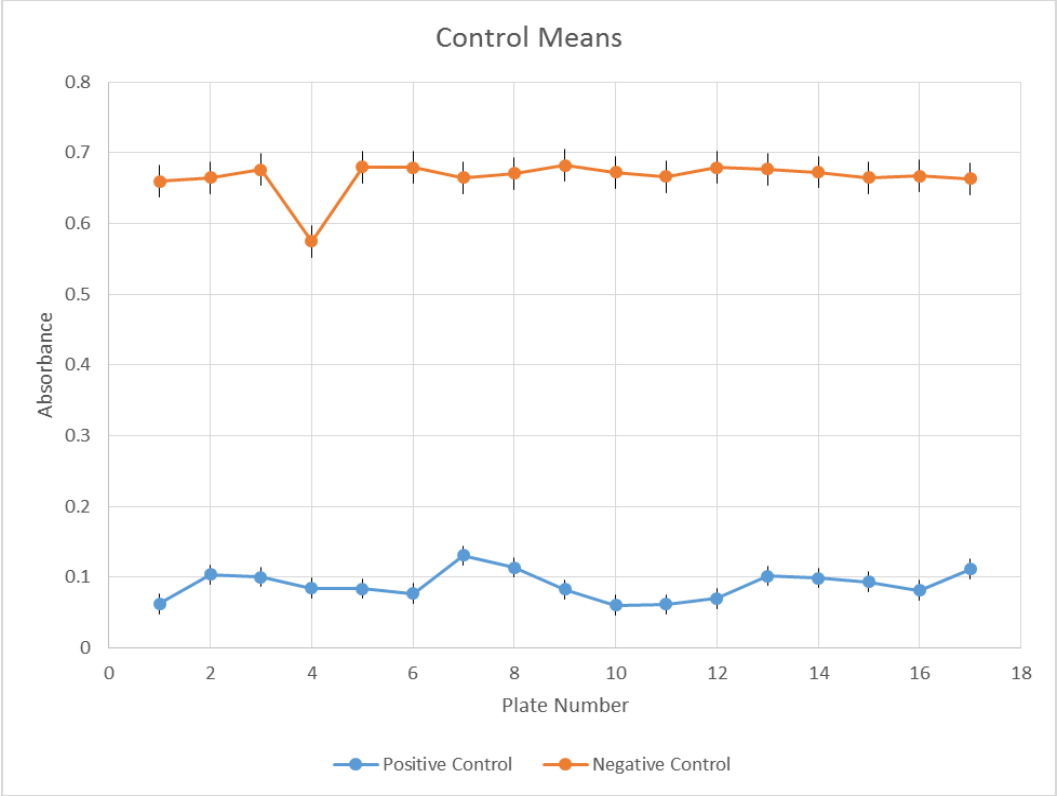

Figure 3.6: Plotted means with standard error bars for negative and positive controls that vary by well plate from ABTS testing.

Ethyl Acetate Fraction	Fraction Name	Average Response	Average BHT equivalence
Et-1	W1-W	0.634	-0.102
	W1-E1	0.662	-0.765
	W1-E2	0.657	-0.647
	W2-W	0.662	-0.780
	W2-E1	0.655	-0.614
	W2-E2	0.657	-0.661
	W3-W	0.584	1.073
	W3-E1	0.638	-0.197
	W3-E2	0.666	-0.860
	E1-W	0.648	-0.448
	E1-E1	0.666	-0.860
	E1-E2	0.663	-0.799
	E2-W	0.656	-0.637
	E2-E1	0.647	-0.415
	E2-E2	0.660	-0.727
	E3-W	0.598	0.746
	E3-E1	0.625	0.107
	E3-E2	0.644	-0.358

Table 3.2: Ethyl acetate fraction 1 with average UV/Vis response and BHT equivalence from ABTS testing.

Ethyl Acetate Fraction	Fraction Name	Average Response	Average BHT equivalence
Et-2	W1-W	0.616	0.315
	W1-E1	0.651	-0.514
	W1-E2	0.66	-0.727
	W2-W	0.66	-0.727
	W2-E1	0.664	-0.822
	W2-E2	0.657	-0.656
	W3-W	0.533	2.282
	W3-E1	0.574	1.310
	W3-E2	0.658	-0.680
	E1-W	0.623	0.149
	E1-E1	0.619	0.244
	E1-E2	0.665	-0.846
	E2-W	0.452	4.201
	E2-E1	0.582	1.121
	E2-E2	0.665	-0.846
	E3-W	0.57	1.405
	E3-E1	0.597	0.765
	E3-E2	0.665	-0.846

Table 3.3: Ethyl acetate fraction 2 with average UV/Vis response and BHT equivalence from ABTS testing.

Ethyl Acetate Fraction	Fraction Name	Average Response	Average BHT equivalence
Et-3	W1-W	0.642	-0.301
	W1-E1	0.66	-0.727
	W1-E2	0.664	-0.822
	W2-W	0.654	-0.585
	W2-E1	0.668	-0.917
	W2-E2	0.661	-0.751
	W3-W	0.519	2.614
	W3-E1	0.5	3.064
	W3-E2	0.668	-0.917
	E1-W	0.482	3.491
	E1-E1	0.532	2.306
	E1-E2	0.659	-0.704
	E2-W	0.387	5.742
	E2-E1	0.412	5.149
	E2-E2	0.65	-0.491
	E3-W	0.378	5.955
	E3-E1	0.433	4.652
	E3-E2	0.661	-0.751

Table 3.4: Ethyl acetate fraction 3 with average UV/Vis response and BHT equivalence from ABTS testing.

Ethyl Acetate Fraction	Fraction Name	Average Response	Average BHT equivalence
Et-4	W1-W	0.598	0.742
	W1-E1	0.658	-0.680
	W1-E2	0.656	-0.633
	W2-W	0.64	-0.254
	W2-E1	0.638	-0.206
	W2-E2	0.66	-0.727
	W3-W	0.448	4.296
	W3-E1	0.075	13.135
	W3-E2	0.642	-0.301
	E1-W	0.483	3.467
	E1-E1	0.225	9.581
	E1-E2	0.659	-0.704
	E2-W	0.076	13.111
	E2-E1	0.28	8.277
	E2-E2	0.647	-0.419
	E3-W	0.109	12.329
	E3-E1	0.275	8.396
	E3-E2	0.606	0.552

Table 3.5: Ethyl acetate fraction 4 with average UV/Vis response and BHT equivalence from ABTS testing.

Ethyl Acetate Fraction	Fraction Name	Average Response	Average BHT equivalence
Et-5	W1-W	0.628	0.031
	W1-E1	0.64	-0.254
	W1-E2	0.631	-0.040
	W2-W	0.662	-0.775
	W2-E1	0.659	-0.704
	W2-E2	0.657	-0.656
	W3-W	0.466	3.870
	W3-E1	0.352	6.571
	W3-E2	0.557	1.713
	E1-W	0.252	8.941
	E1-E1	0.387	5.742
	E1-E2	0.622	0.173
	E2-W	0.064	13.396
	E2-E1	0.178	10.694
	E2-E2	0.614	0.363
	E3-W	0.118	12.116
	E3-E1	0.266	8.609
	E3-E2	0.606	0.552

Table 3.6: Ethyl acetate fraction 5 with average UV/Vis response and BHT equivalence from ABTS testing.

Ethyl Acetate Fraction	Fraction Name	Average Response	Average BHT equivalence
Et-6	W1-W	0.555	1.761
	W1-E1	0.389	5.694
	W1-E2	0.631	-0.040
	W2-W	0.654	-0.585
	W2-E1	0.578	1.216
	W2-E2	0.66	-0.727
	W3-W	0.056	13.585
	W3-E1	0.055	13.609
	W3-E2	0.584	1.073
	E1-W	0.091	12.756
	E1-E1	0.344	6.761
	E1-E2	0.635	-0.135
	E2-W	0.198	10.220
	E2-E1	0.393	5.600
	E2-E2	0.602	0.647
	E3-W	0.058	13.538
	E3-E1	0.326	7.187
	E3-E2	0.605	0.576

Table 3.7: Ethyl acetate fraction 6 with average UV/Vis response and BHT equivalence from ABTS testing.

Ethyl Acetate Fraction	Fraction Name	Average Response	Average BHT equivalence
Et-7	W1-W	0.628	0.031
	W1-E1	0.625	0.102
	W1-E2	0.666	-0.870
	W2-W	0.661	-0.751
	W2-E1	0.657	-0.656
	W2-E2	0.665	-0.846
	W3-W	0.113	12.235
	W3-E1	0.287	8.111
	W3-E2	0.602	0.647
	E1-W	0.137	11.666
	E1-E1	0.351	6.595
	E1-E2	0.613	0.386
	E2-W	0.173	10.813
	E2-E1	0.436	4.581
	E2-E2	0.628	0.031
	E3-W	0.066	13.348
	E3-E1	0.132	11.784
	E3-E2	0.59	0.931

Table 3.8: Ethyl acetate fraction 7 with average UV/Vis response and BHT equivalence from ABTS testing.

Ethyl Acetate Fraction	Fraction Name	Average Response	Average BHT equivalence
Et-8	W1-W	0.557	1.713
	W1-E1	0.611	0.434
	W1-E2	0.655	-0.609
	W2-W	0.66	-0.727
	W2-E1	0.662	-0.775
	W2-E2	0.668	-0.917
	W3-W	0.484	3.443
	W3-E1	0.312	7.519
	W3-E2	0.626	0.078
	E1-W	0.341	6.832
	E1-E1	0.331	7.069
	E1-E2	0.638	-0.206
	E2-W	0.062	13.443
	E2-E1	0.384	5.813
	E2-E2	0.624	0.126
	E3-W	0.112	12.258
	E3-E1	0.242	9.178
	E3-E2	0.599	0.718

Table 3.9: Ethyl acetate fraction 8 with average UV/Vis response and BHT equivalence from ABTS testing.

Ethyl Acetate Fraction	Fraction Name	Average Response	Average BHT equivalence
Et-9	W1-W	0.569	1.429
	W1-E1	0.53	2.353
	W1-E2	0.654	-0.585
	W2-W	0.638	-0.206
	W2-E1	0.622	0.173
	W2-E2	0.667	-0.893
	W3-W	0.244	9.130
	W3-E1	0.203	10.102
	W3-E2	0.625	0.102
	E1-W	0.411	5.173
	E1-E1	0.397	5.505
	E1-E2	0.639	-0.230
	E2-W	0.142	11.547
	E2-E1	0.213	9.865
	E2-E2	0.63	-0.017
	E3-W	0.048	13.775
	E3-E1	0.127	11.903
	E3-E2	0.594	0.836

Table 3.10: Ethyl acetate fraction 9 with average UV/Vis response and BHT equivalence from ABTS testing.

Ethyl Acetate Fraction	Fraction Name	Average Response	Average BHT equivalence
Et-10	W1-W	0.401	5.410
	W1-E1	0.472	3.727
	W1-E2	0.644	-0.348
	W2-W	0.628	0.031
	W2-E1	0.643	-0.325
	W2-E2	0.666	-0.870
	W3-W	0.319	7.353
	W3-E1	0.248	9.036
	W3-E2	0.636	-0.159
	E1-W	0.468	3.822
	E1-E1	0.466	3.870
	E1-E2	0.652	-0.538
	E2-W	0.111	12.282
	E2-E1	0.364	6.287
	E2-E2	0.615	0.339
	E3-W	0.048	13.775
	E3-E1	0.212	9.889
	E3-E2	0.607	0.528

Table 3.11: Ethyl acetate fraction 10 with average UV/Vis response and BHT equivalence from ABTS testing.

Ethyl Acetate Fraction	Fraction Name	Average Response	Average BHT equivalence
Et-11	W1-W	0.135	11.713
	W1-E1	0.228	9.509
	W1-E2	0.634	-0.111
	W2-W	0.647	-0.419
	W2-E1	0.643	-0.325
	W2-E2	0.621	0.197
	W3-W	0.578	1.216
	W3-E1	0.622	0.173
	W3-E2	0.654	-0.585
	E1-W	0.656	-0.633
	E1-E1	0.645	-0.372
	E1-E2	0.667	-0.893
	E2-W	0.284	8.182
	E2-E1	0.483	3.467
	E2-E2	0.64	-0.254
	E3-W	0.316	7.424
	E3-E1	0.197	10.244
	E3-E2	0.643	-0.325

Table 3.12: Ethyl acetate fraction 11 with average UV/Vis response and BHT equivalence from ABTS Testing.

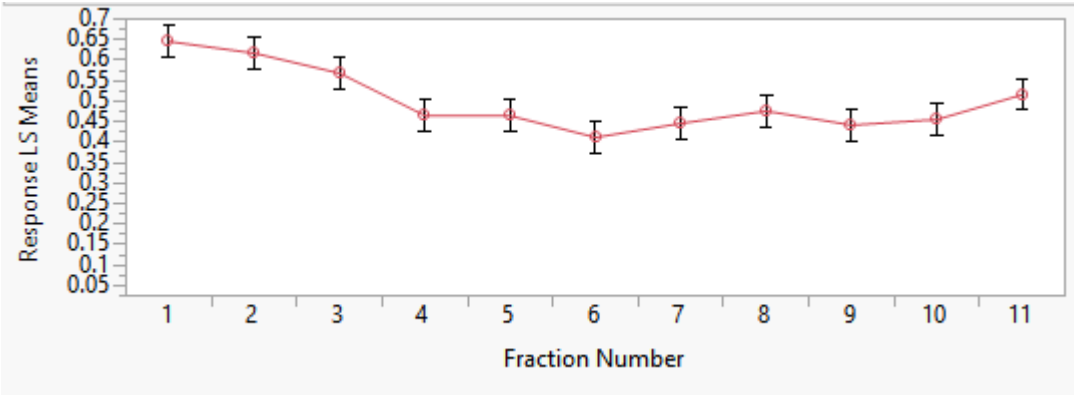

Figure 3.7: Plotted mean with standard error for ethyl acetate fractions (from software JMP with p-value<0.0001).

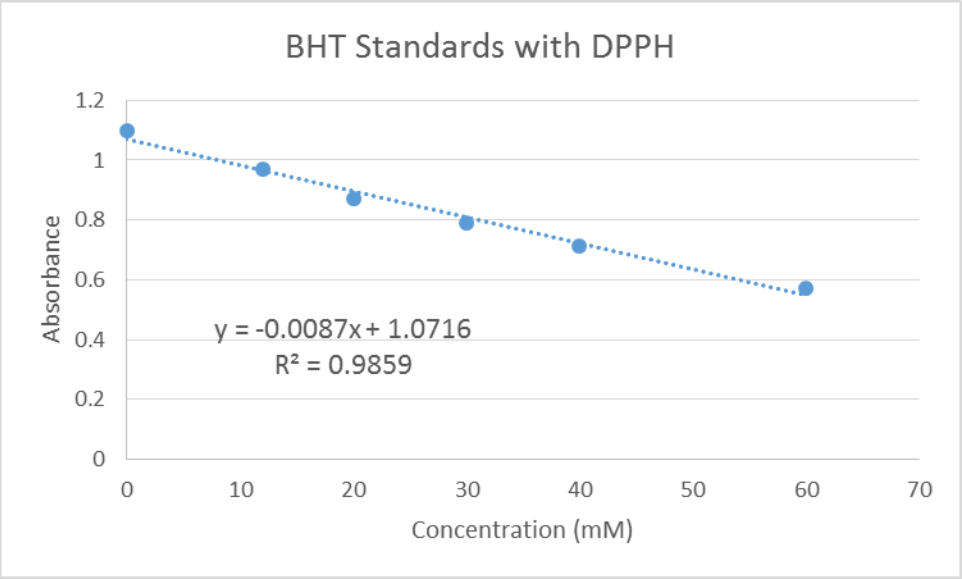

Figure 3.8: Calibration curve of BHT standard with DPPH antioxidant testing.

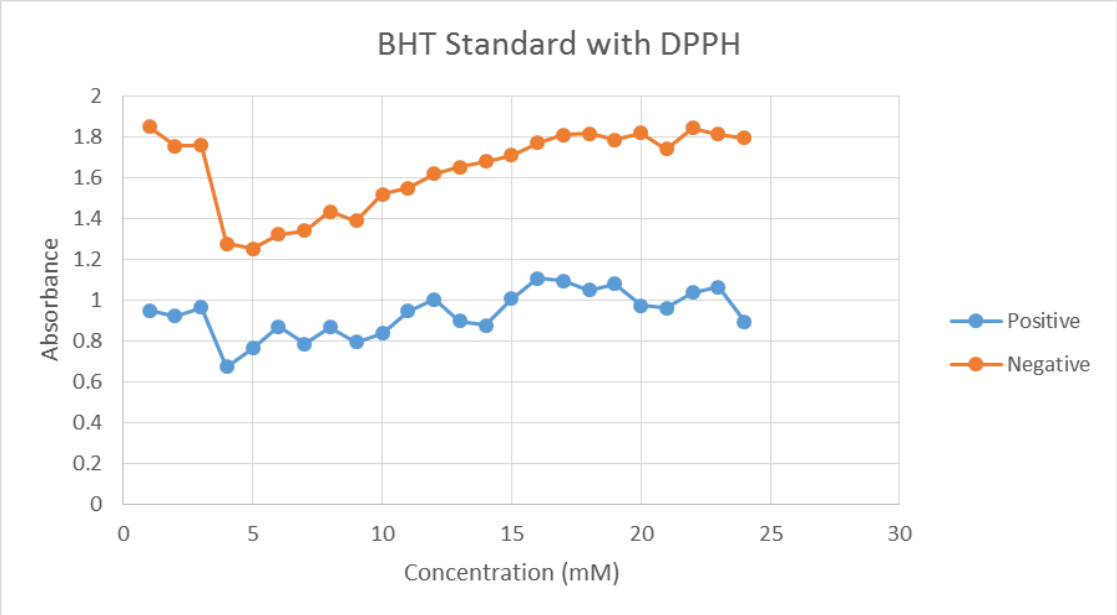

Figure 3.9: Plotted means for positive and negative controls across all well plates with DPPH antioxidant testing.

Level	Std Error	Mean
NEGATIVE	0.01567789	1.63671
POSITIVE	0.01567789	0.93419

Table 3.13: Mean and standard deviation for all positive and negative controls from the DPPH antioxidant testing.

ABTS Testing			
Fraction Name	**Subfraction Name**	**Average Response**	**Average BHT Equivalence (mM)**
ET-6 W1-E1	A5	0.041	13.941
	A6	0.180	10.642
	A14	0.040	13.964
ET-6 E2-W	J1	0.042	13.912

Table 3.14: Subfractions with significant difference from negative control with ABTS antioxidant testing.

DPPH Testing			
Fraction Name	**Subfraction Name**	**Average Response**	**Average BHT Equivalence (mM)**
ET-6 W1-E1	A5	0.443	72.299
	A14	0.979	10.670
ET-6 E2-W	J1	0.157	105.103

Table 3.15: Subfractions with positive results from DPPH antioxidant testing.

Discussion

ABTS antioxidant testing was first performed on the 198 samples that were prepared and described in Chapter 2. The ABTS radical solution was formulated by combining 7mM ABTS with 2.45mM $K_2S_2O_8$ in equal amounts and placed in the dark for 16 hours. The solution was diluted to achieve a 0.7 absorbance, this was done by diluting 1mL of ABTS solution with 12 mL of 80% EtOH. Before the testing of actual samples, the variability of the microplate reader was assessed by adding 100µL of the prepared ABTS solution to all 96 wells and the absorbance was recorded for each well at 734nm. It was found that the first row along with the first column had the most variance.

Figure 3.3 depicts the bivariate density map with 95% coverage for the mean absorbance readings of ABTS solution in each well, grouped by row. Row 1 has four points outside of the coverage area as well as a clear divide of means and therefore it was decided that Row 1 would not be used for data analysis. Figure 3.4 depicts that the quartile range of Column 1 is outside of the range for the column with the lowest mean (Column 9) and therefore Column 1 was determined to not be used in further analysis. For the ease of analysis a second column (Column 12) was to not be used either. In this case, there would be ten usable columns allowing for two samples in five replicates per row available for samples. With a positive and negative control in each plate, a total of 12 samples could be tested for antioxidants.

Most ABTS methods use Trolox, a water soluble vitamin E analog with potent antioxidant activity,[11] as a standard to create a calibration curve. The results for samples reported are Trolox equivalent absorption capacity (TEAC). For the work listed within this chapter, butylated hydroxytoluene (BHT) was chosen as the standard as it is also a vitamin E analog that has shown antioxidant activity, and results are listed as BHT equivalents as determined by the calibration curve with linear regression as depicted in Figure 3.5. A stock solution of 15mM BHT in methanol was prepared, followed by serial dilutions to obtain 10, 7.5, 5 and 3mM solutions. A solution of 1.5mM of BHT was also made but the BHT was in such low concentration it did not produce viable results. In ten replicates, 1μL of each sample was added to a well with 100μL of ABTS solution and allowed to react for approximately 5 minutes or until the wells with the highest concentration contained a clear or near clear solution.

A positive and negative control was utilized in each plate to monitor variance that may occur. One μL of the 15mM BHT solution was used as the positive control while the ABTS solution was used as the negative control. Table 3.1 lists the means with standard errors for the controls across all plates combined and Figure 3.6 has the plotted means with error bars for the mean of each plate and each control. The negative control appears to be stable across all plates except for Plate 4, which could be caused by a pipetting error when diluting the ABTS solution or when delivering sample to the wells. The positive control varies as well but this could be related to the time the plate was allowed to react. Each plate was allowed to stand for approximately five minutes for the reaction to reach completion but if it was visible that the positive control had reacted by becoming a clear solution then the plate was analyzed by the microplate reader.

Table 3.2 – Table 3.12 list the average absorbance response for each of the 198 samples obtained after the SPE fractionation as described in Chapter 2 along with the average BHT equivalence. Fraction 6 of the EtOAc fraction showed the most potent antioxidant activity as seen when all mean values of antioxidant activities of fractions are compared. From this, Et-6 was chosen to be further analyzed and separated by HPLC, of which details can be found in Chapter 4. The subfractions obtained from the fraction collector were dried down and reconstituted in 500μL MeOH, all of which were subjected to both ABTS and DPPH antioxidant testing. The ABTS assay for the subfractions were performed in the same manner as with the 198 samples, except 10μL of sample was placed in the well with 100μL of ABTS solution due to the lower concentration of samples.

Following the ABTS assay, the DPPH method was conducted with a $6x10^{-5}$M DPPH radical solution being prepared in ethanol. This solution did not need to be incubated so it was made fresh the day of testing and stored at -20°C when not being used. Based on literature it was determined to use 8µL of sample or standard and 100µL of DPPH solution. Again to control for plate variability, a positive (60mM BHT) and negative (DPPH solution) control was utilized with each plate. As with the ABTS assay, DPPH studies also use the vitamin E analog, Trolox to report equivalency, and once again butylated hydroxytoluene (BHT) was chosen as the standard. The standard amounts of BHT used in the ABTS assay were not concentrated enough to yield a reaction with the DPPH radical solution and therefore the strengths were increased four times; making a stock solution of 60mM and then serially diluted to 40, 30, 20 and 12mM. The calibration curve obtained from using these standards can be seen in Figure 3.8 with the linear regression that was used to determine the BHT equivalence for the subfraction samples. To analyze the plate variance, the positive and negative control means are plotted in Figure 3.9 and the overall mean for each control is listed with the standard error in Table 3.13. The variance of the negative control could be due to temperature fluctuations because the DPPH solution was frequently removed from and placed in the freezer. Although never mentioned in literature, once the radical solution was prepared it might need to equilibrate before use. The positive control also varied and this could be in part to the incubation time. The reaction was allowed to proceed for 20 minutes while covered in aluminum foil, but the plate was not always analyzed by the plate reader at the end of the incubation time as pipetting of samples in other plates was being completed.

Results from the ABTS and DPPH assays were compared to determine the most active subfractions. Most samples during the ABTS testing showed slight antioxidant activity but the most active, the ones with the highest BHT equivalency values are listed in Table 3.14. When the samples were subjected to the DPPH test, all subfraction samples but three showed no activity. The positive samples with BHT equivalence are listed in Table 3.15.

Upon comparison fractions 5 and 14 of Et-6 W1-E1 and fraction 1 of Et-6 E2-W were found to have activity under both test conditions. Fraction 6 that was positive with ABTS and not DPPH is most likely because DPPH is a more selective assay. The ethyl acetate (EtOAc) fraction #6 showed the most potent antioxidant activity. However its sub-fractions exhibited very little antioxidant activity possibly due to the low concentration of compounds. Most samples from the ABTS assay did possess a small BHT equivalency and no equivalency with DPPH because of the selectivity.

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CHAPTER FOUR

ANALYTICAL METHODS

Introduction

Separation and identification of chemical compounds can only be achieved with the assistance of analytical methods, such as high pressure liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS). Phytochemicals extracted from a natural source (e.g., plant) is often comprised of a complex organic compounds that contain cyclic and linear molecules with relative low molecular mass. With this, conventional gas chromatography is not suitable and sufficient for non-volatile compound separation.[10] Therefore, the application of HPLC for isolation and purification of natural products has increased in the past few years to become the method of choice for phytochemicals including polyphenols.[9,13] It is also common to connect HPLC with MS to facilitate the chemical separation and identification.[1,9]

HPLC operates on the principle of using high pressure to force the flow of solvent and sample through a packed column to separate analytes based on interactions with the column particles.[2] Elution of analytes is determined by the notion of adsorption chromatography where solvent and solute compete for biding sites within the stationary phase of the packed column. Solutes can be dislodged from the binding sites by altering the solvent polarity, which is done easily by using a gradient elution process. Most common gradients employ a binary solvent system where generally one solvent is aqueous and the second employs a type of organic solvent, such as most commonly methanol, acetone and ethyl acetate, etc..[14] A gradient program allows for the separation

of a larger range of molecules.[11] In this process, an aqueous and methanol phase were selected with 0.05% formic acid for pH adjustment.

The selection of stationary phase is of the utmost importance for a successful chemical separation. The analytes should be able to interact with the stationary phase but not bind so much as that no solvent is able to compete with and displace the solute. Therefore, it is necessary to determine whether normal phase (polar) or reverse phase (nonpolar) is best for separating the compounds in the sample. Generally, reverse phase is chosen for separations due to its high capacity, recovery, reproducibility and resolution.[12] With the previous experience on separating natural products and knowledge of working with organics, a reverse phase hexyl-phenyl (C6P) column was chosen for the Devil's Club extract.

Detection of phytochemicals is carried out by the analysis of light absorption by the chemicals present. Diode array detection (DAD) is a common choice for detecting phenols as simple phenolic compounds have one absorption band between 240nm and 290nm while complex phenolic compounds has another absorption band in the range of 300-350nm.[16]

The purpose of exercising HPLC was two-fold; first by monitoring the success of solid phase extraction methods on preliminary separation of phytochemicals; and second by analyzing fractioned samples along with further fractionation by an automated fraction collector.

A second analytical technique useful in chemical identification is mass spectrometry (MS),[15] of which the principle operation is vaporization and ionization of molecules. With developments over the years there are many ionization techniques, with

electrospray ionization (ESI) being among the more common soft ionization techniques. The sample travels through a needle and into a chamber, with the needle kept at a high voltage and a de-solvation gas, the liquid sample is converted into an aerosol spray of fine particles. A capillary pulls the particles and with its high temperature, evaporates excess solvent and particles pass through an electrostatic lens.[17] The ions then travel through a magnetic quadrupole, resulting in the detection of molecular parent ions and smaller fragmented ions. Ions generated are complexes generally with the addition of a hydrogen atom to create a positively charged ion. The ions detected are recorded as a mass to charge ratio (*m/*z). Based on the mass to charge ratio of detected ions, the MS spectra is used to assist in the identification of chemical compounds.

Experimental

Sample Information

The samples that were dried by rotoevaporation and reconstituted after solid phase extraction were subjected to analysis by using a Shimadzu HPLC and Agilent LC-MS system.

Chemicals

Distilled water, commercially purchased formic acid 99% (FA) from Acros Organics and methanol (HPLC grade) from Fisher Scientific.

Instrumentation

Shimadzu high pressure liquid chromatography

The Shimadzu HPLC system purchased from Shimadzu Scientific Instruments (Columbia, MD) comprised of a system controller (CBM-20A), auto sampler (SIL-

20AHT), binary pump (LC-20AT), online degasser (DGU-20A5), column oven (CTO-20A), diode array detector (SPD-M20A) with a semi-micro 5mm path length flow cell and fraction collector (FRC-10A). Acquisition and analysis of data was performed using Class-VP version 7.4 software.

Shimadzu HPLC system parameters

A binary mobile phase gradient was exercised with 0.5% formic acid in aqueous solution (A) and 0.5% formic acid in HPLC methanol (B) with a flow rate of 2mL/min. The gradient started at 13%B and gradually increased over the 120 minute time program accumulating to a ten minute step of 100%B, to ensure a clean column followed by a five minute equilibration step at which the percent of methanol returned to the starting value. The column oven that was held at a constant temperature of 32°C housed a Phenomex kinetex 5µ Phenyl-Hexyl column (100Å 250x4.6mm).

Agilent mass spectrometry

The mass spectrometry instrumentation employed was an Agilent LC-MS system (Agilent Technologies, Santa Clara, CA) consisting of a degasser (G1379B), binary pump (G1312A), auto sampler (G1329A), temperature controlled column compartment (G1316A), UV/Vis wavelength detector (G1314B), and a single quadrupole mass spectrometer with electrospray ion source.

Agilent LC-MS parameters

The binary gradient program from the optimized HPLC method was applied to the Agilent system. The flow rate and column temperature were also transferred from the Shimadzu to the Agilent instrument. Furthermore, the same Phenomex Kinetex Phenyl-Hexyl column (250 x 4.6mm) was used. A full scan mode was selected for 110-1500 *m/z*

with the fragmentor set to 300 and a capillary voltage of 3.5kV. The de-solvation gas was set to a temperature of 350°C and a flow rate of 10mL/min.

Results

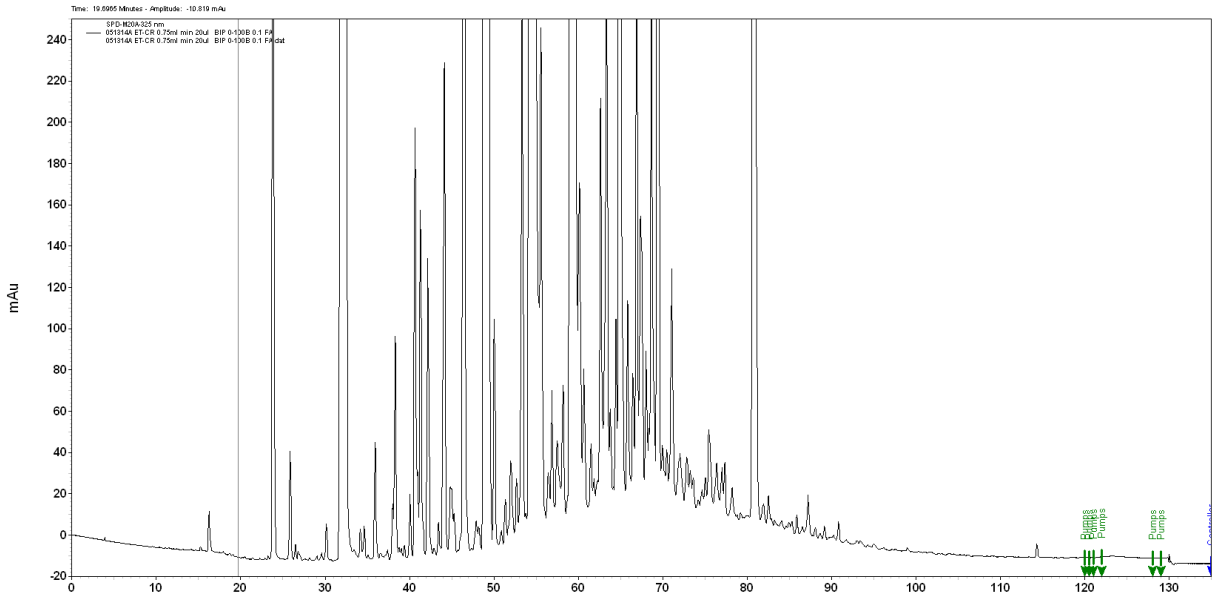

Figure 4.1: HPLC spectra of ethyl acetate crude fraction

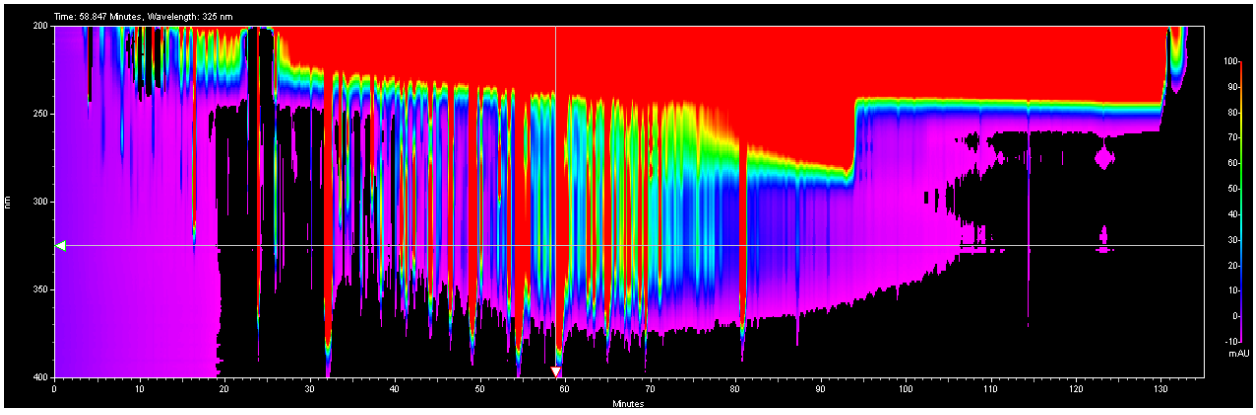

Figure 4.2: DAD output for ethyl acetate crude fraction

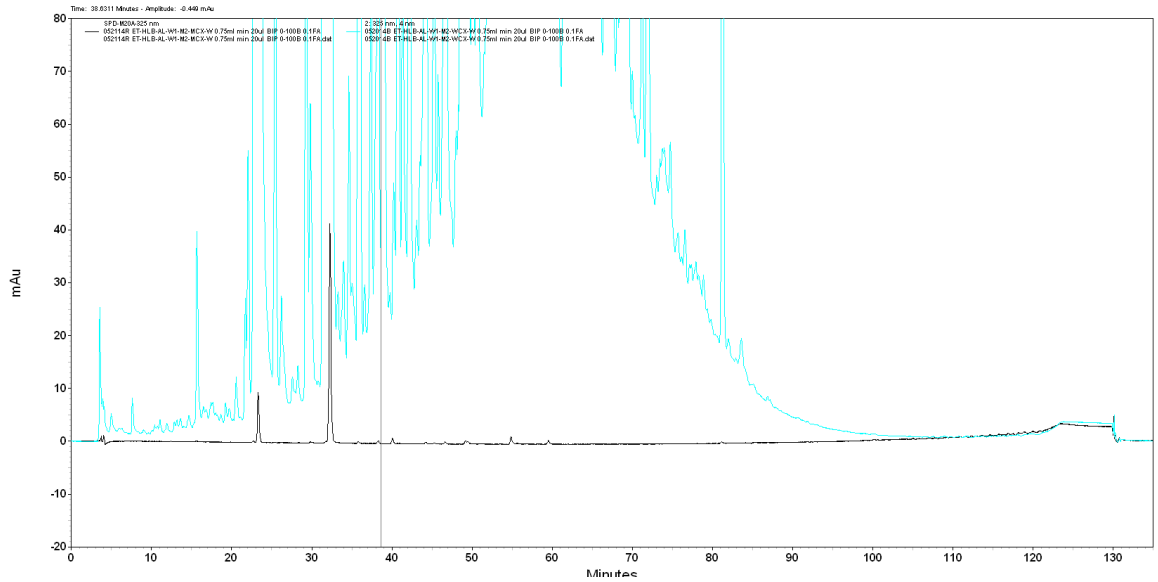

Figure 4.3: Comparison of MCX (black) and WCX (blue) wash 1 of HLB-SPE wash 1

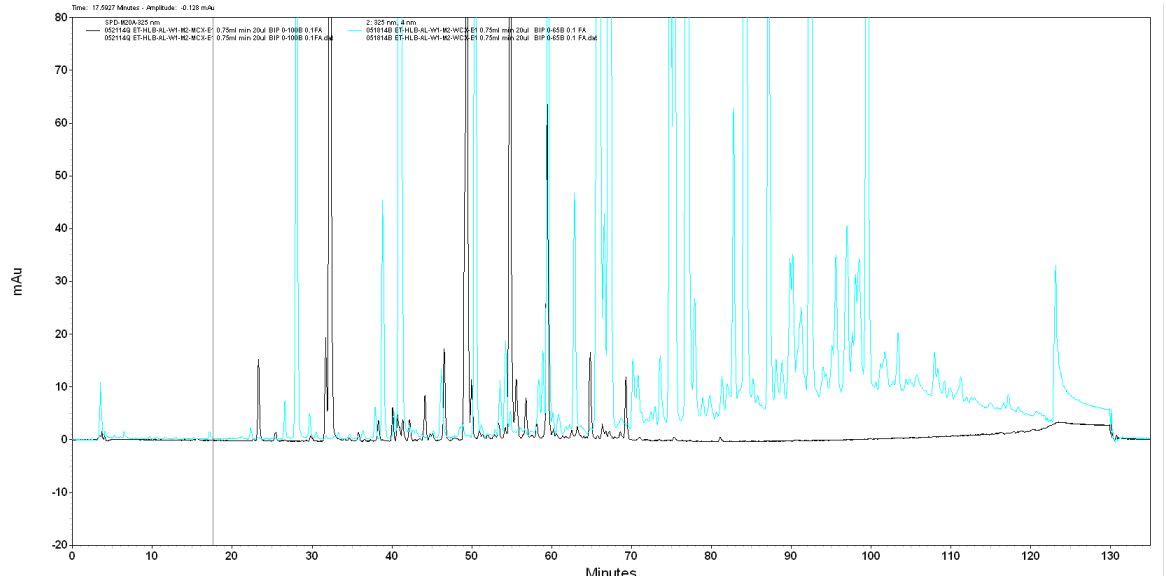

Figure 4.4: Comparison of MCX (black) and WCX (blue) elute 1 of HLB-SPE wash 1

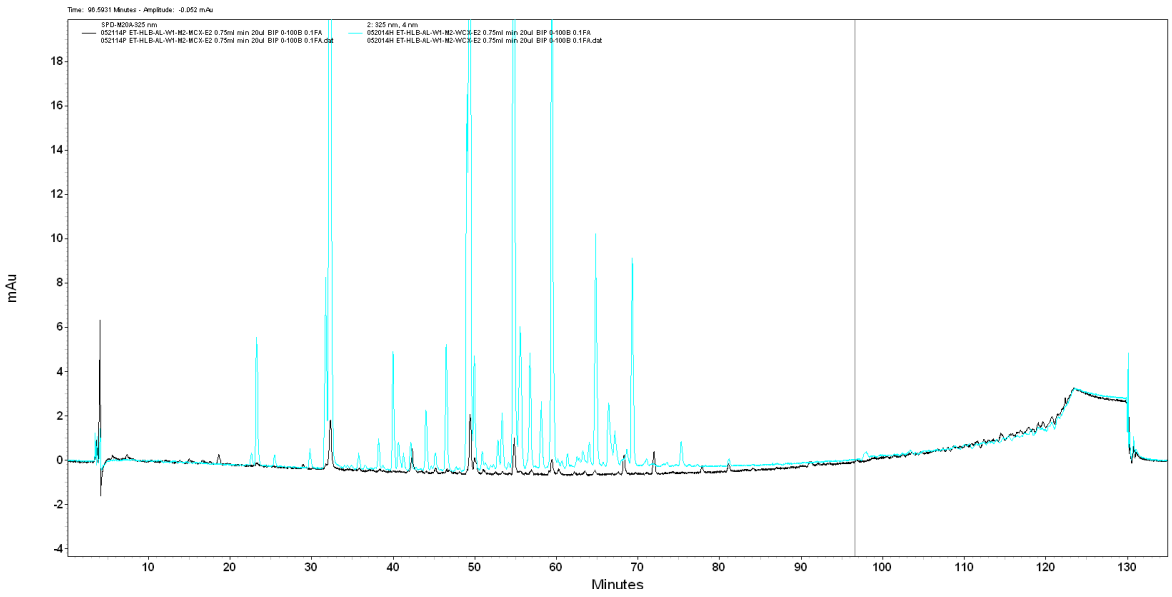

Figure 4.5: Comparison of MCX (black) and WCX (blue) elute 2 of HLB-SPE wash 1

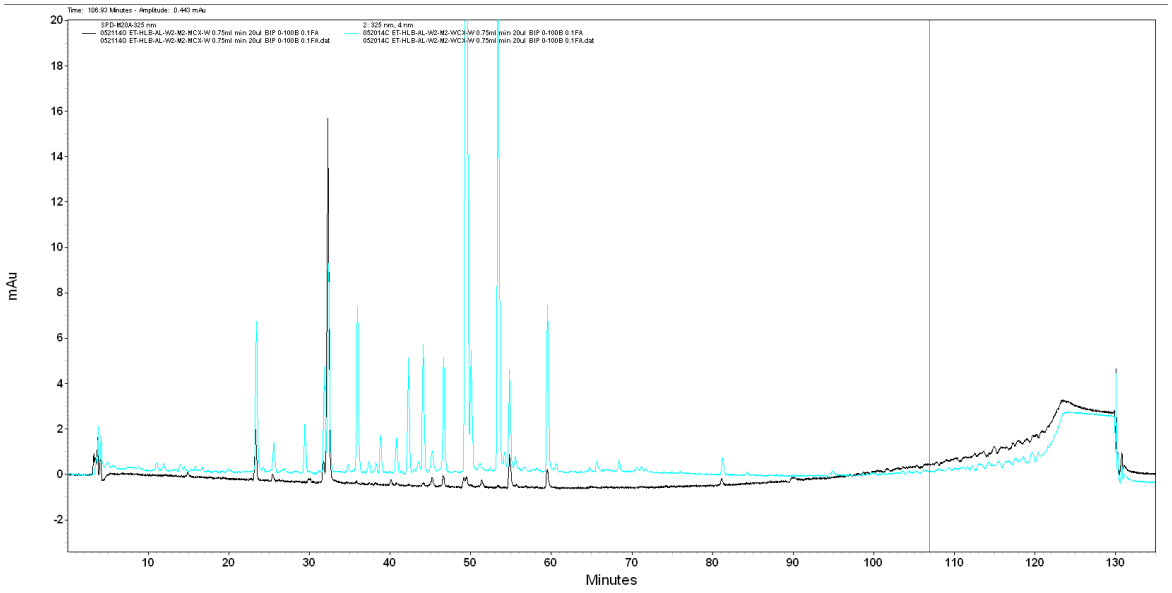

Figure 4.6 Comparison of MCX (black) and WCX (blue) wash of HLB-SPE wash 2

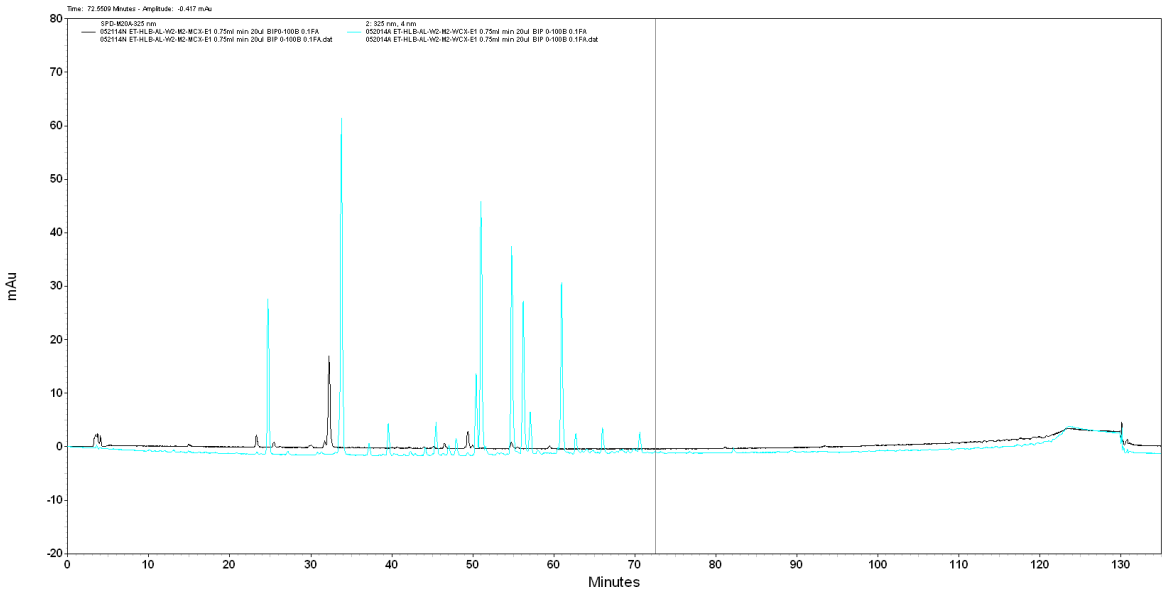

Figure 4.7: Comparison of MCX (black) and WCX (blue) elute 1 of HLB-SPE wash 2

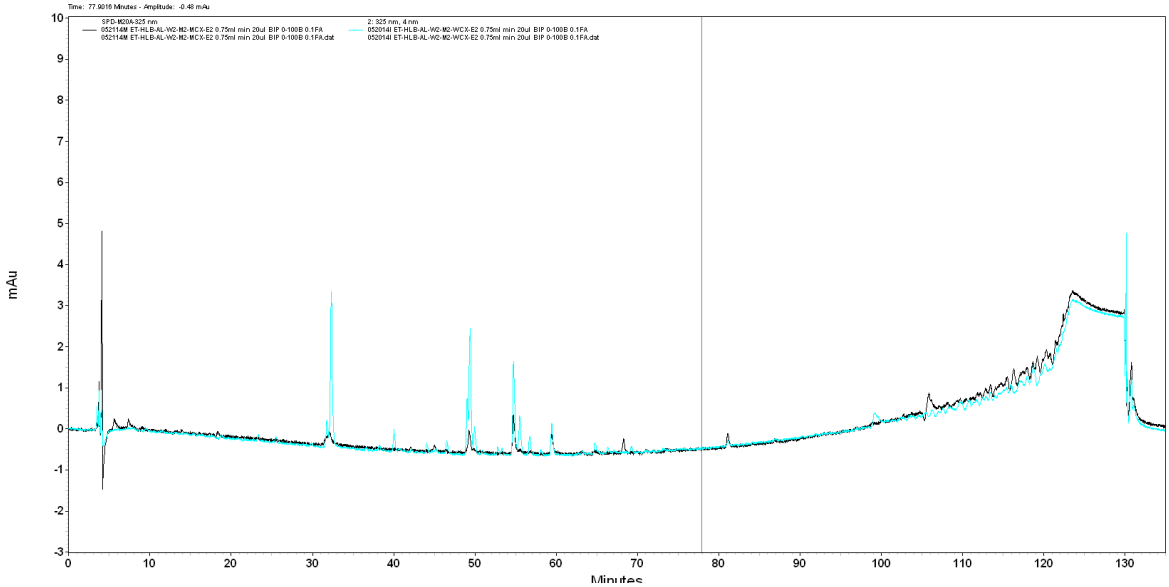

Figure 4.8: Comparison of MCX (black) and WCX (blue) elute 2 of HLB-SPE wash 2

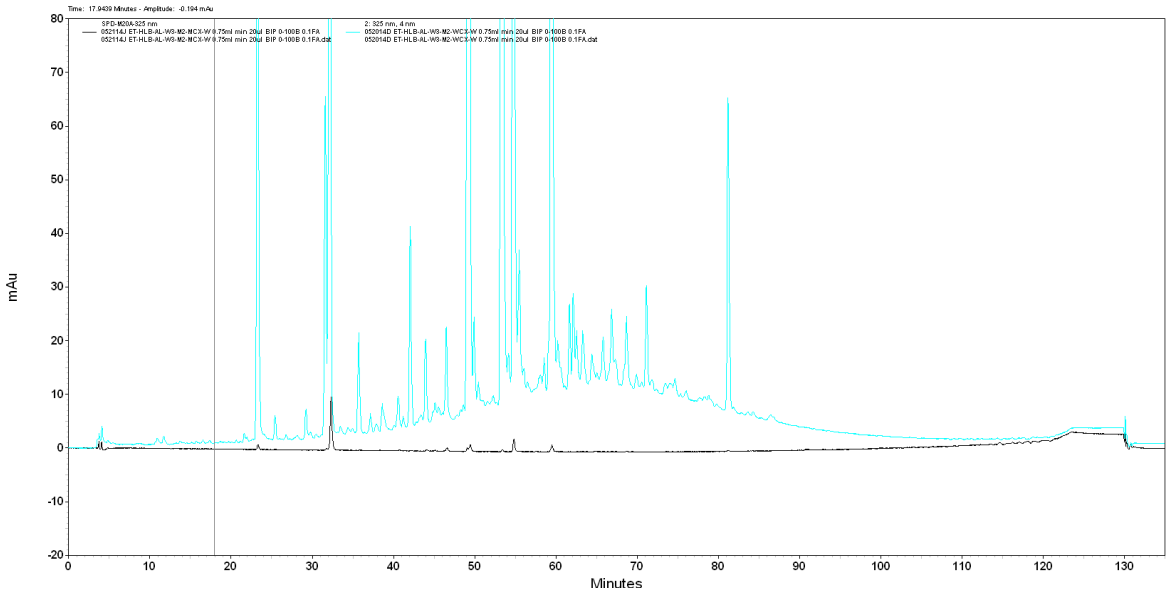

Figure 4.9: Comparison of MCX (black) and WCX (blue) wash of HLB-SPE wash 3

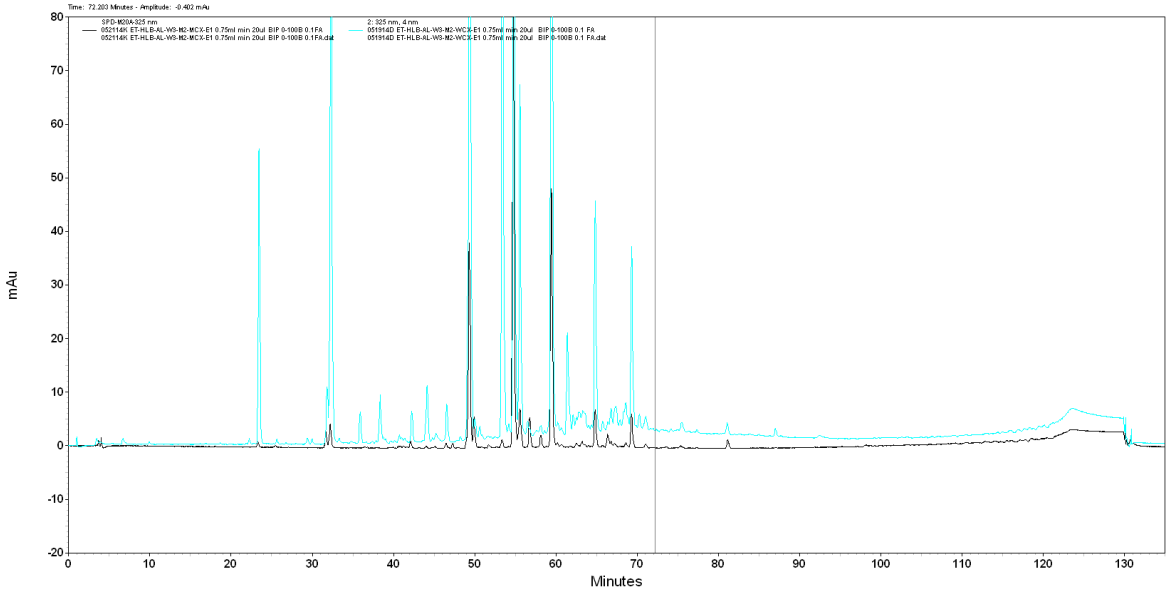

Figure 4.10: Comparison of MCX (black) and WCX (blue) elute 1 of HLB-SPE wash 3

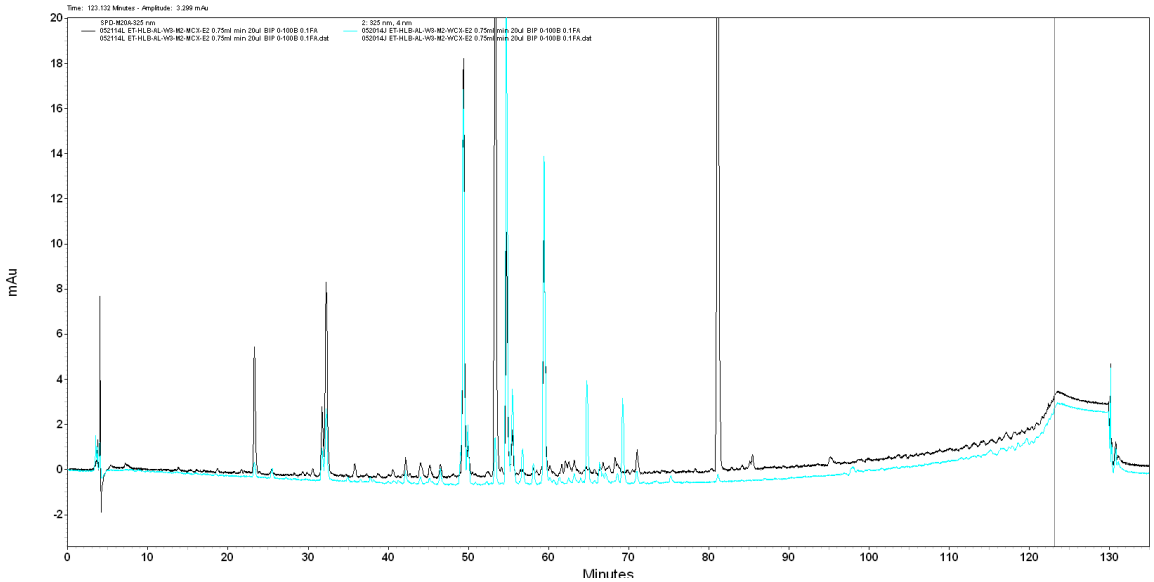

Figure 4.11: Comparison of MCX (black) and WCX (blue) elute 2 of HLB-SPE wash 3

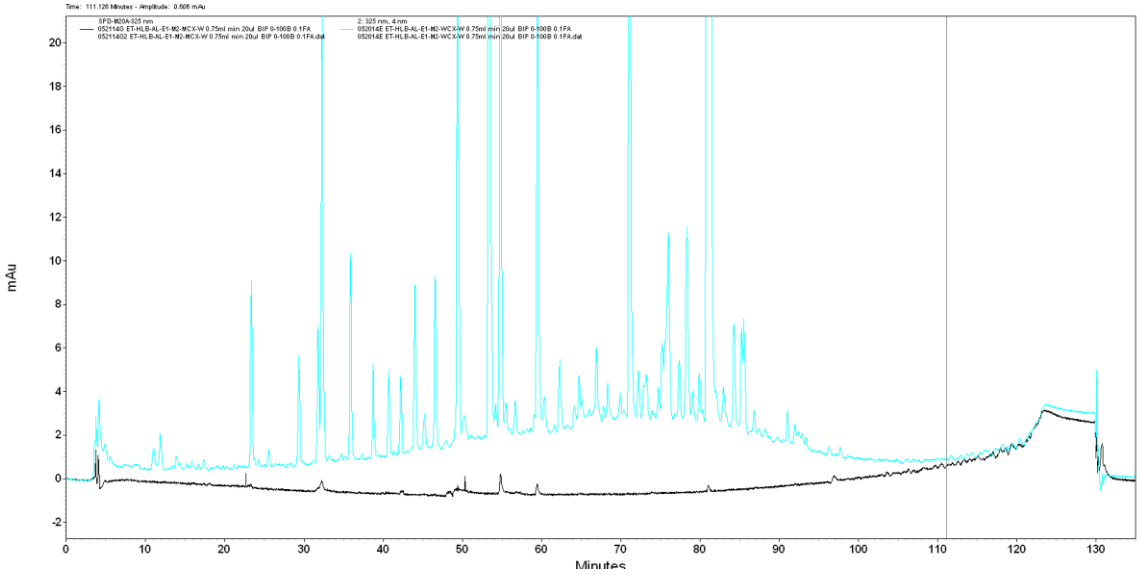

Figure 4.12: Comparison of MCX (black) and WCX (blue) wash of HLB-SPE elute 1

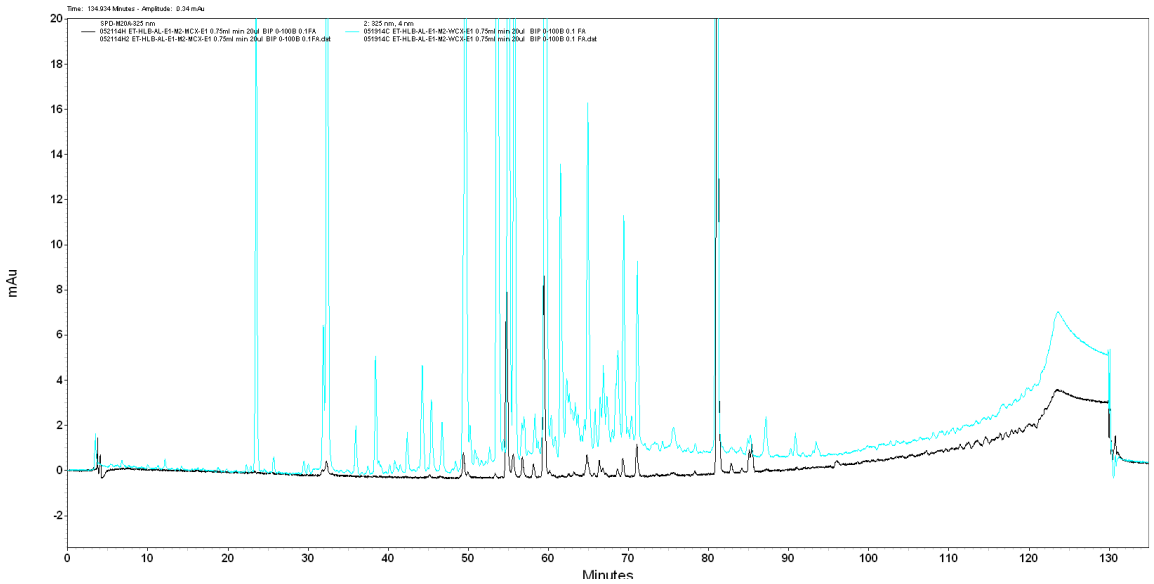

Figure 4.13: Comparison of MCX (black) and WCX (blue) elute 1 of HLB-SPE elute 1

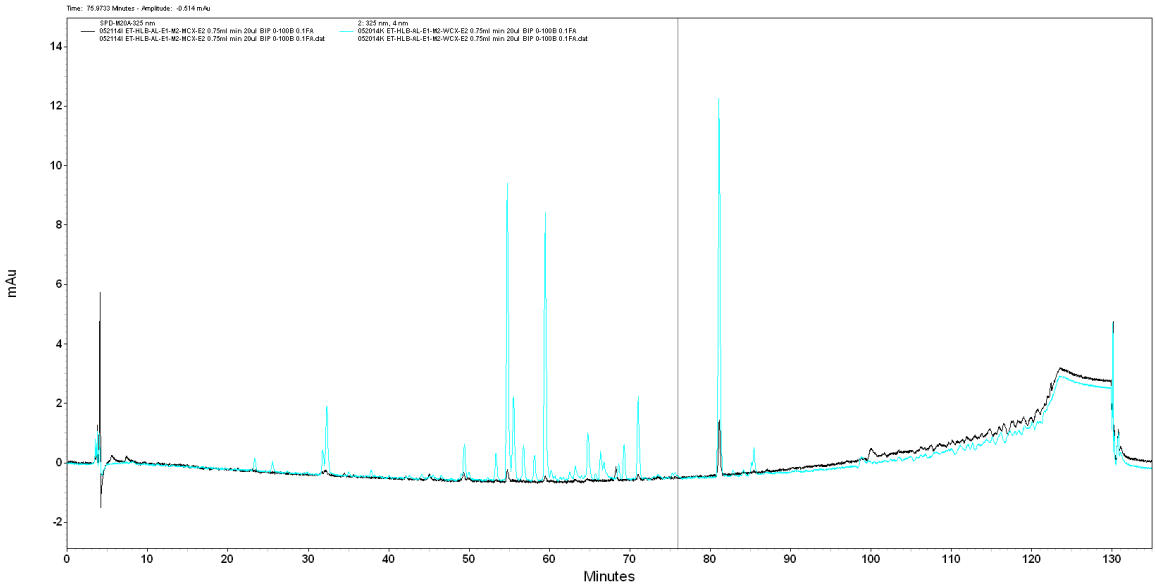

Figure 4.14: Comparison of MCX (black) and WCX (blue) elute 2 of HLB-SPE elute 1

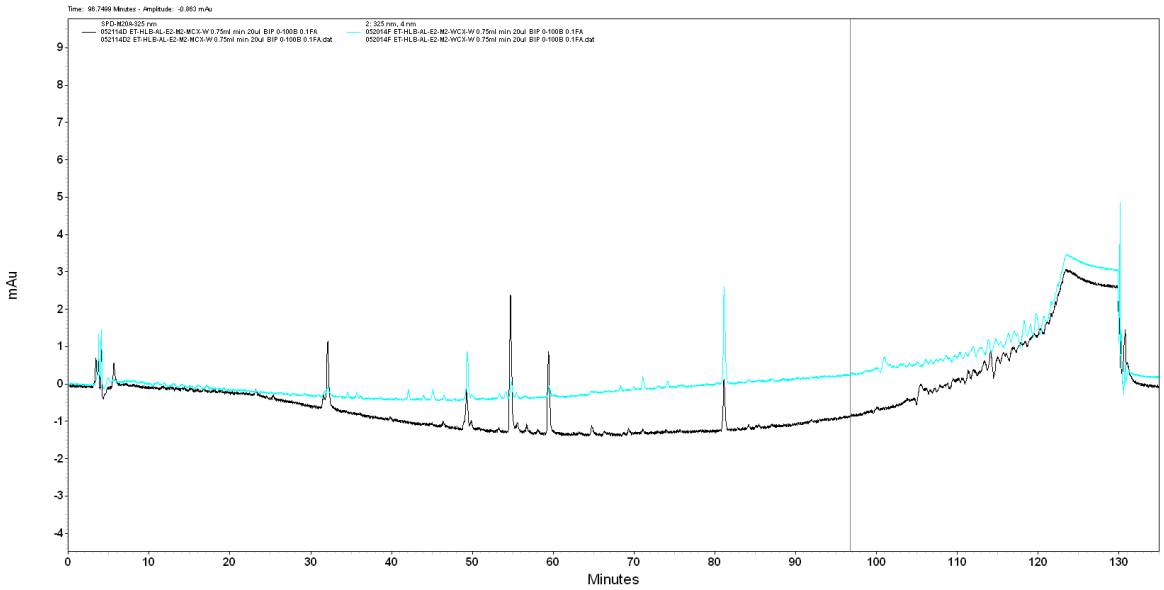

Figure 4.15: Comparison of MCX (black) and WCX (blue) wash of HLB-SPE elute 2

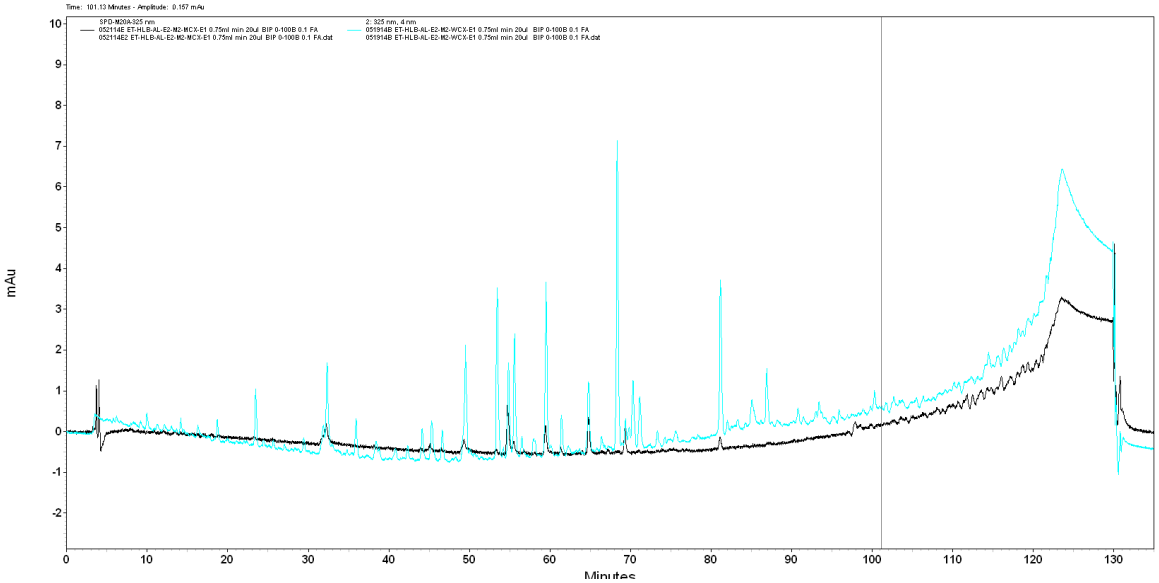

Figure 4.16: Comparison of MCX (black) and WCX (blue) elute 1 of HLB-SPE elute 2

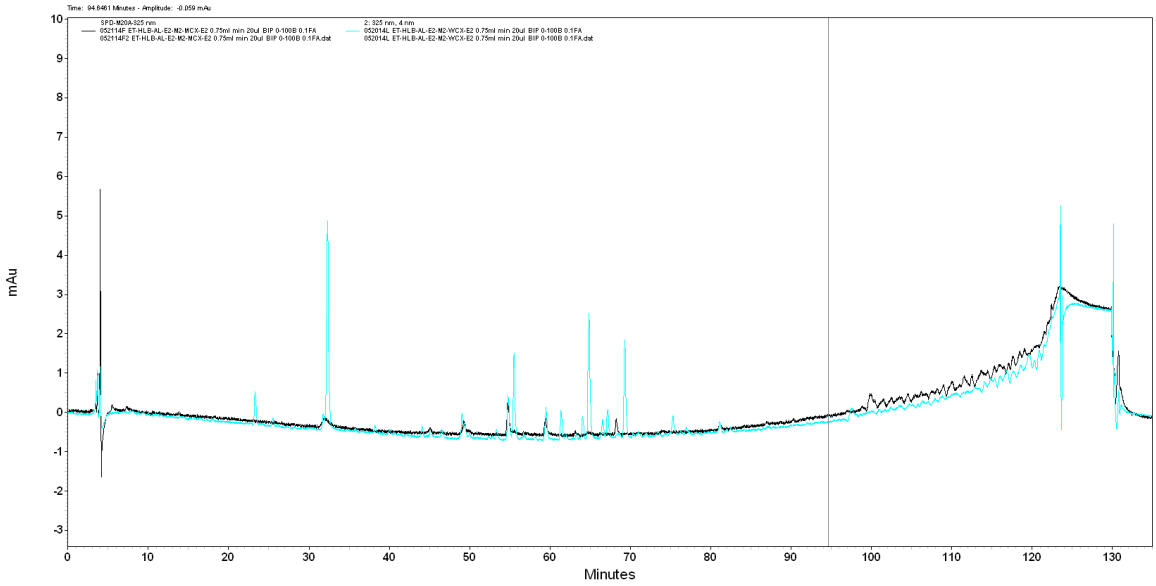

Figure 4.17: Comparison of MCX (black) and WCX (blue) elute 2 of HLB-SPE elute 2

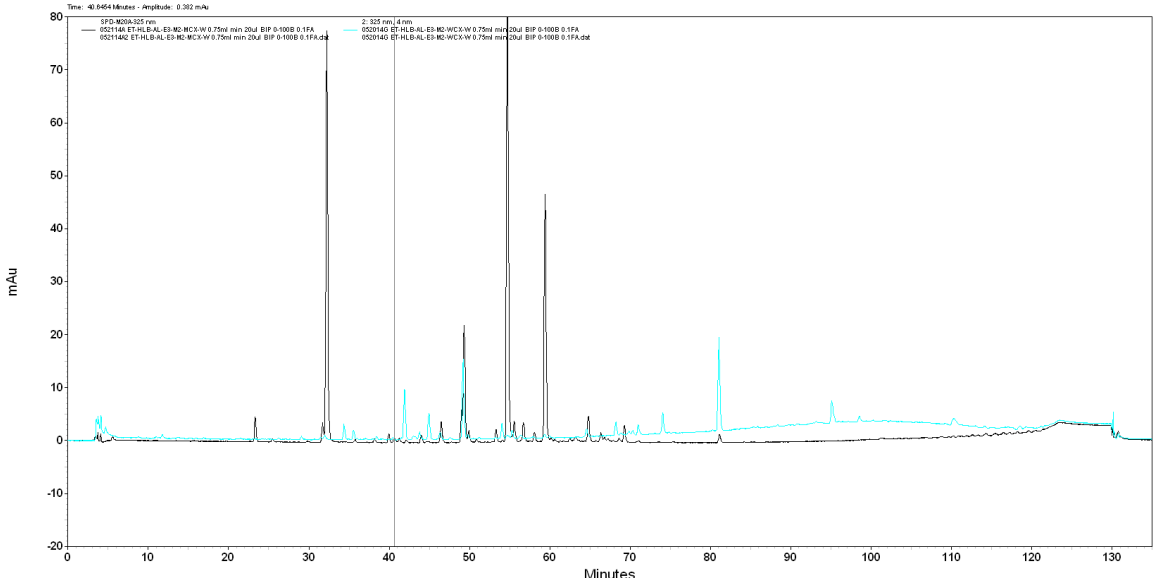

Figure 4.18: Comparison of MCX (black) and WCX (blue) wash of HLB-SPE elute 3

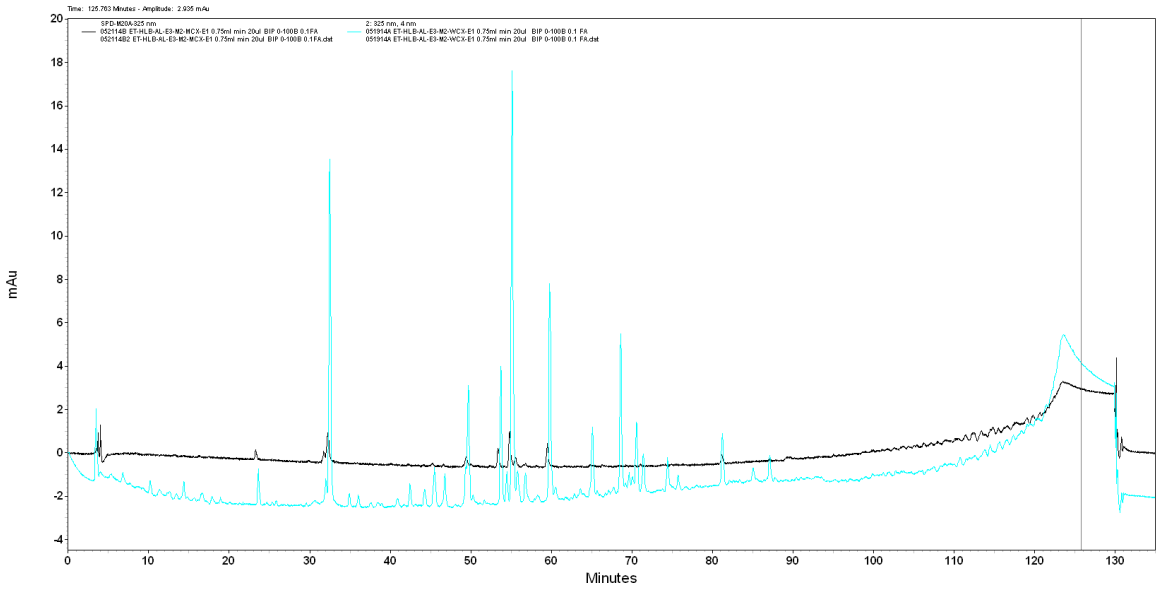

Figure 4.19: Comparison of MCX (black) and WCX (blue) elute 1 of HLB-SPE elute 3

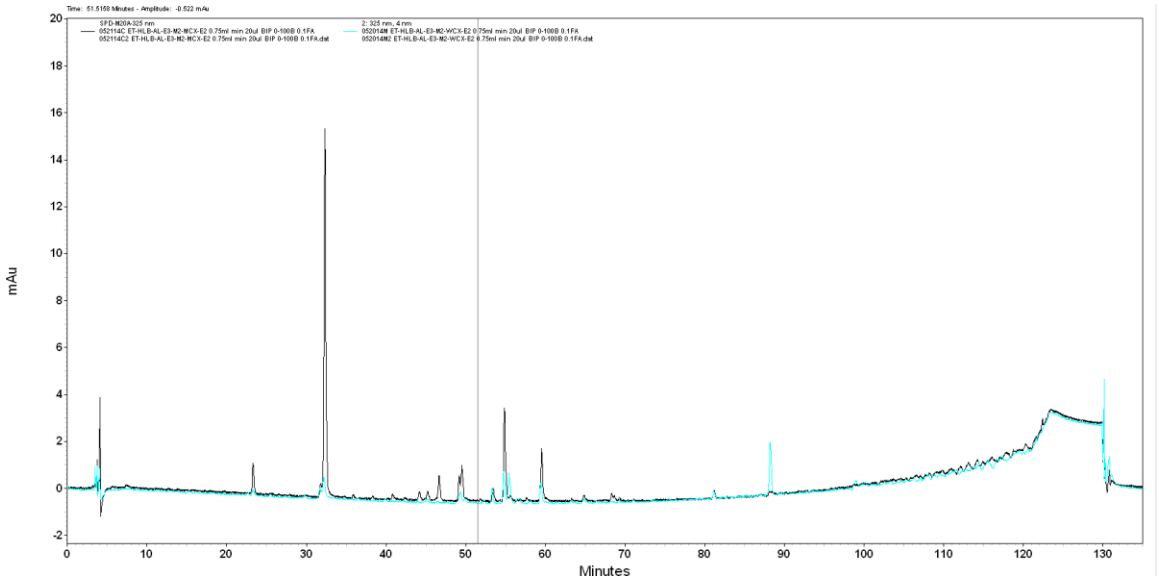

Figure 4.20: Comparison of MCX (black) and WCX (blue) elute 2 of HLB-SPE elute 3

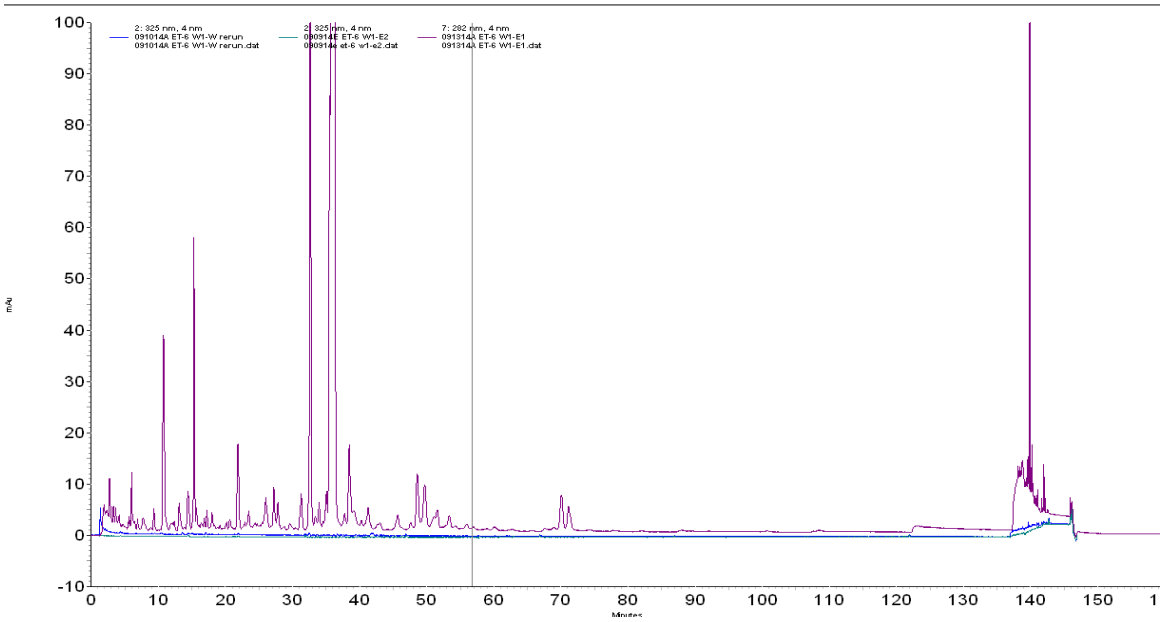

Figure 4.21: Combined spectra of Et-6 W1-W (blue), E1 (purple), E2 (green)

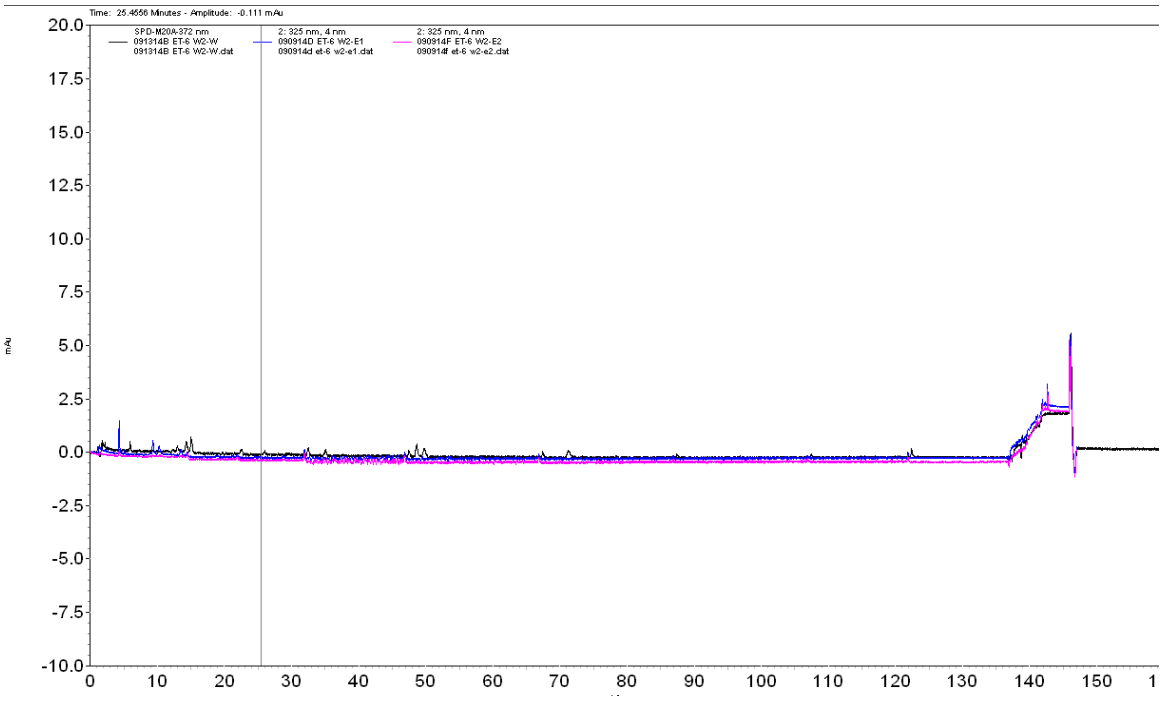

Figure 4.22: Combined spectra of Et-6 W2-W (black), E1 (blue), E2 (purple)

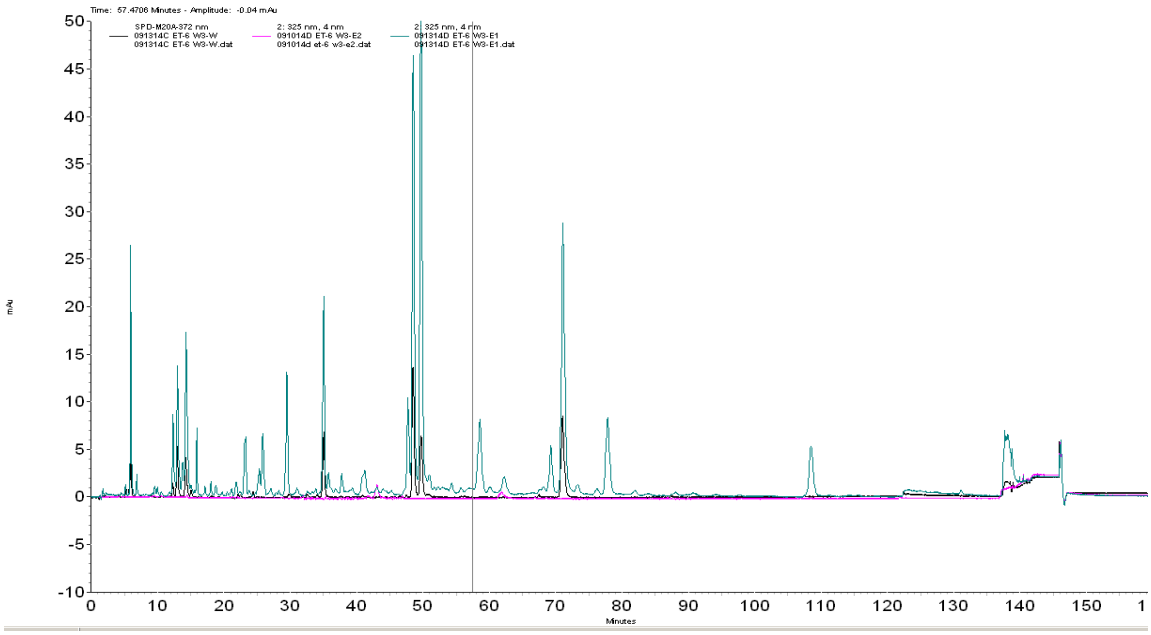

Figure 4.23: Combined spectra of Et-6 W3-W (black), E1 (green), E2 (purple)

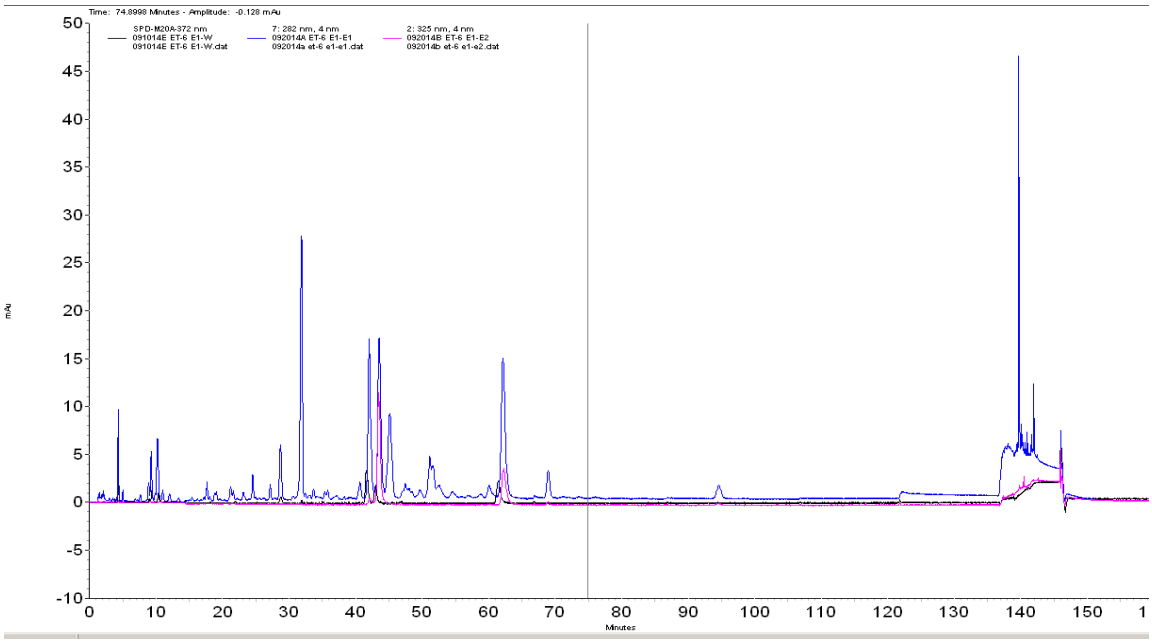

Figure 4.24: Combined spectra of Et-6 E1-W (black), E1 (blue), E2 (purple)

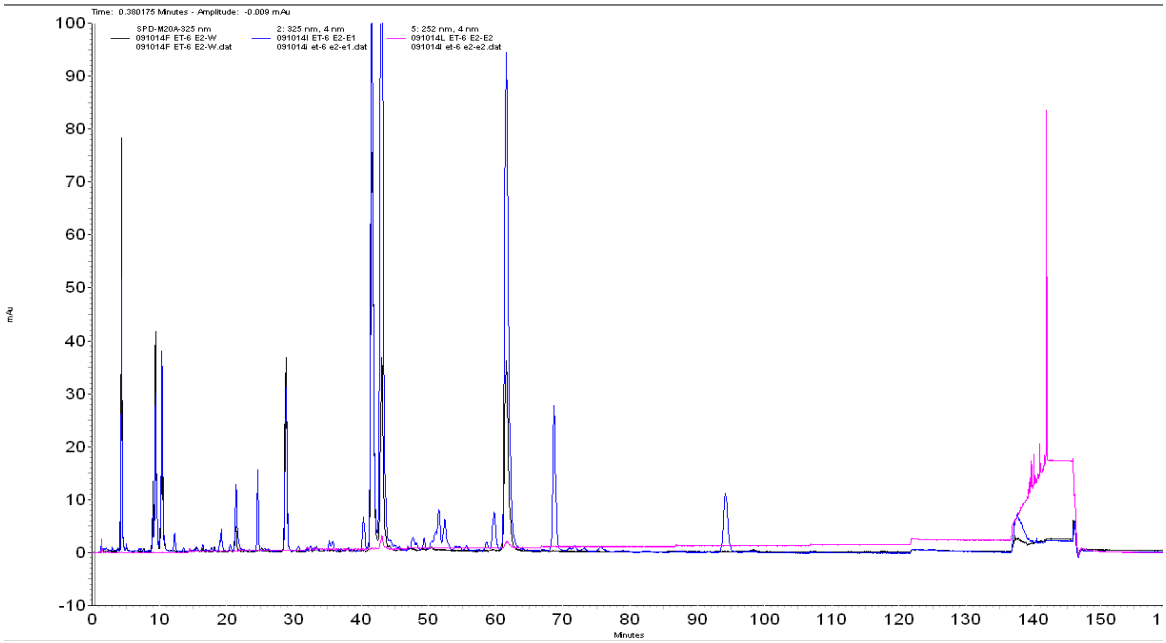

Figure 4.25: Combined spectra of Et-6 E2-W (black), E1 (blue), E2 (purple)

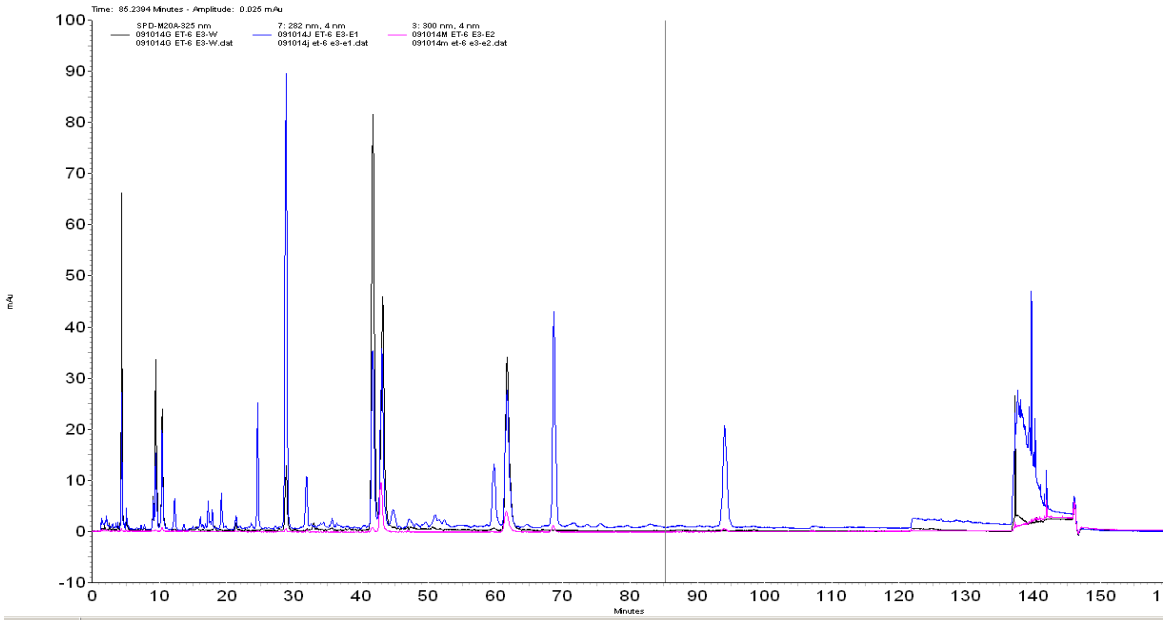

Figure 4.26: Combined spectra of Et-6 E3-W (black), E1 (blue), E2 (purple)

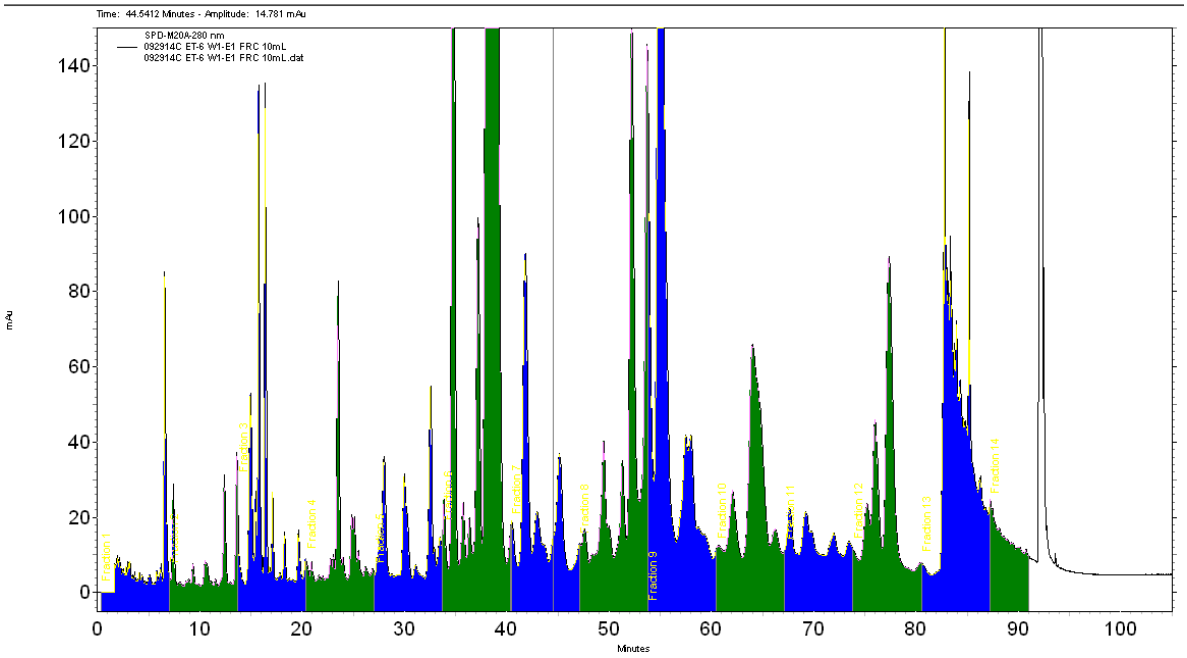

Figure 4.27: Fraction collection of Et-6 W1-E1 (Subfraction A)

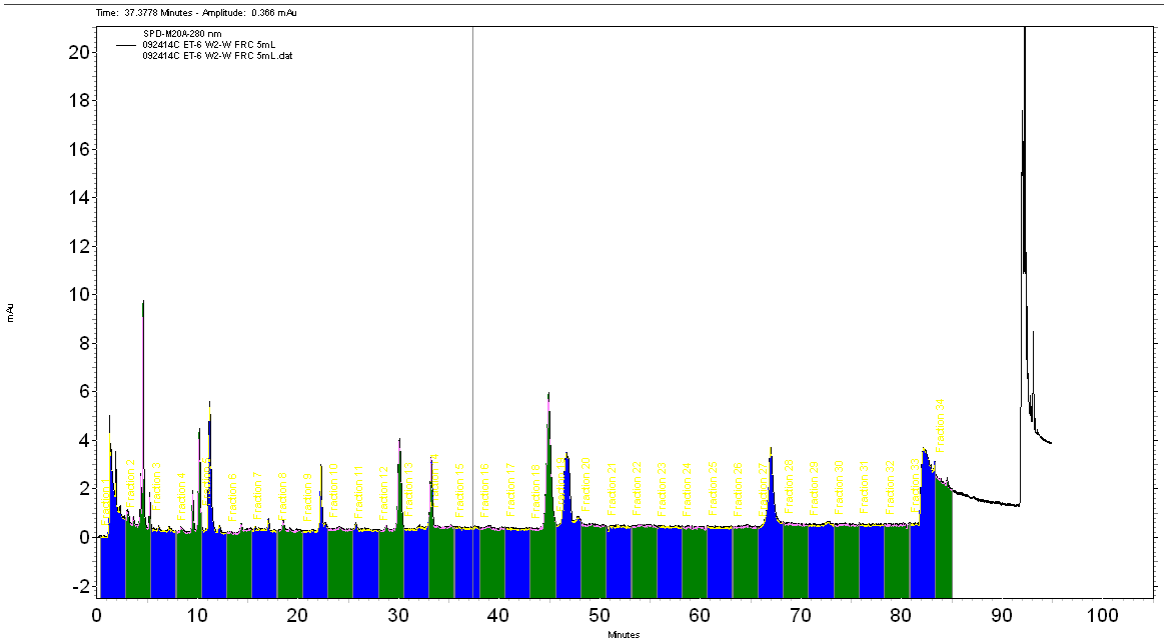

Figure 4.28: Fraction collection of Et-6 W2-W (Subfraction B)

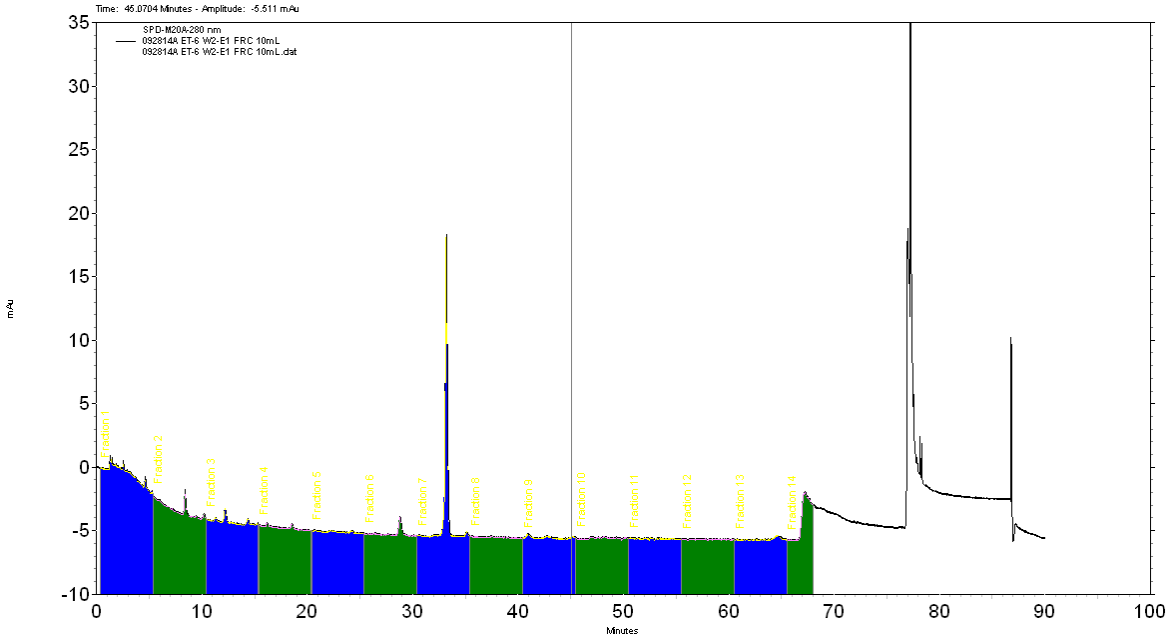

Figure 4.29: Fraction collection of Et-6 W2-E1 (Subfraction C)

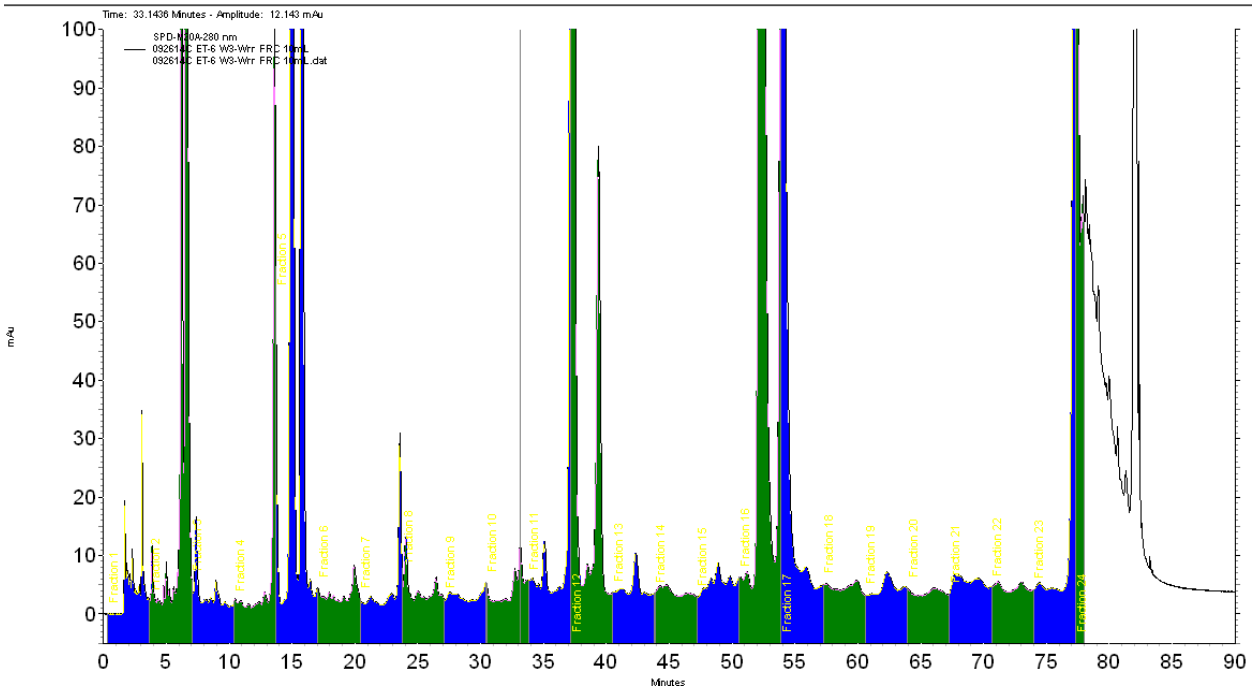

Figure 4.30: Fraction collection of Et-6 W3-W (Subfraction D)

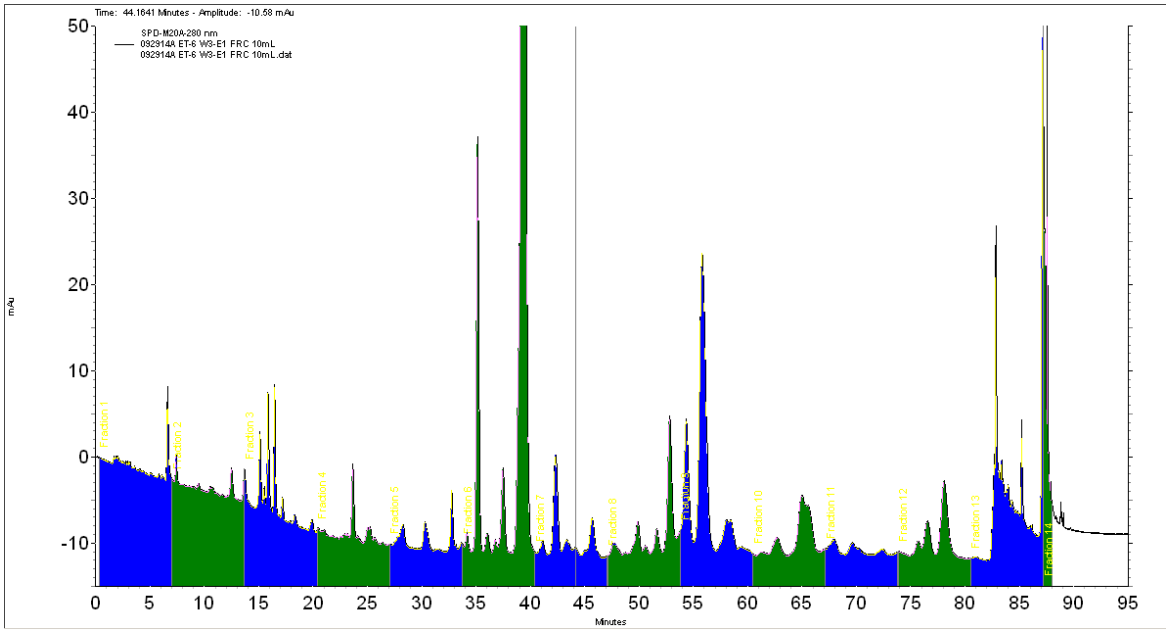

Figure 4.31: Fraction collection of Et-6 W3-E1 (Subfraction F)

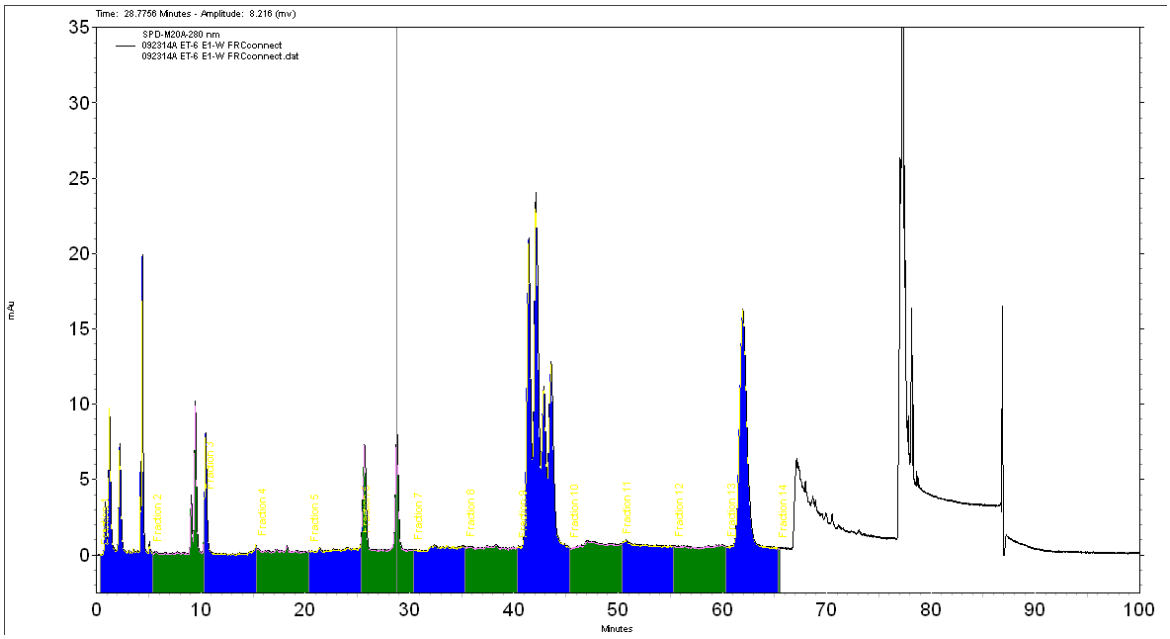

Figure 4.32: Fraction collection of Et-6 E1-W (Subfraction G)

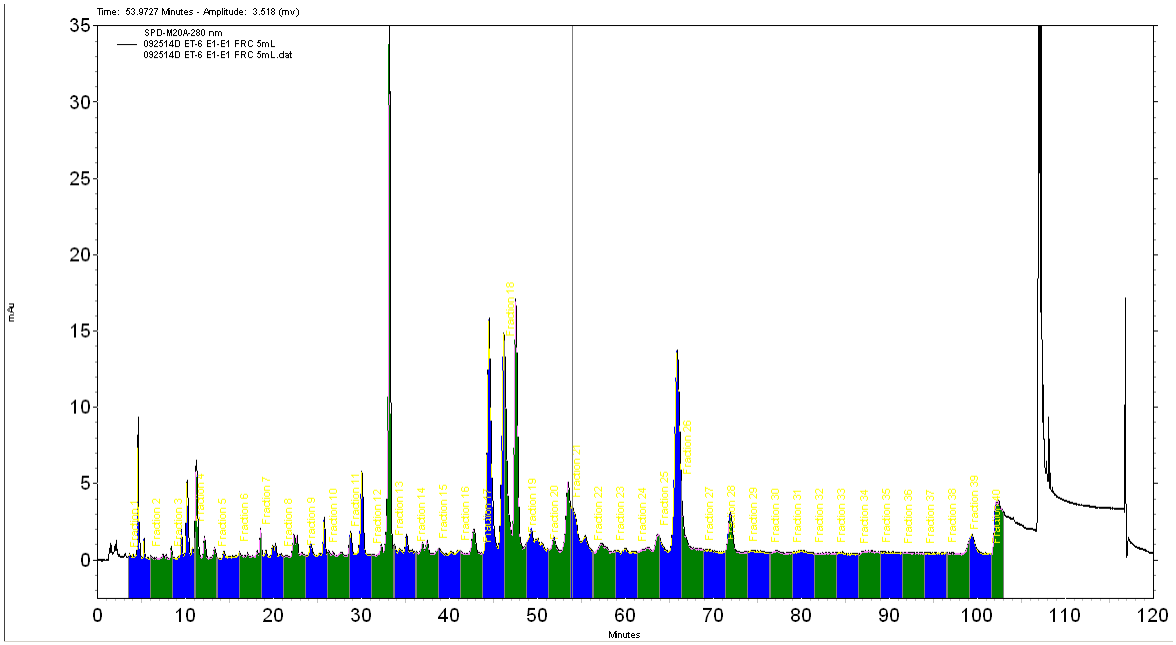

Figure 4.33: Fraction collection of Et-6 E1-E1 (Subfraction H)

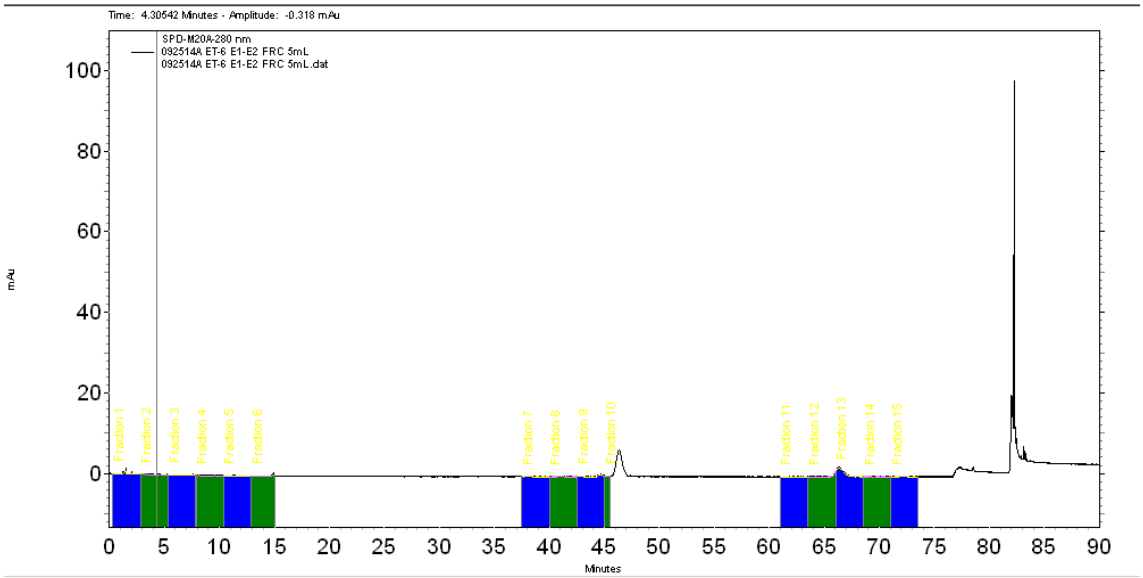

Figure 4.34: Fraction collection of Et-6 E1-E2 (Subfraction I)

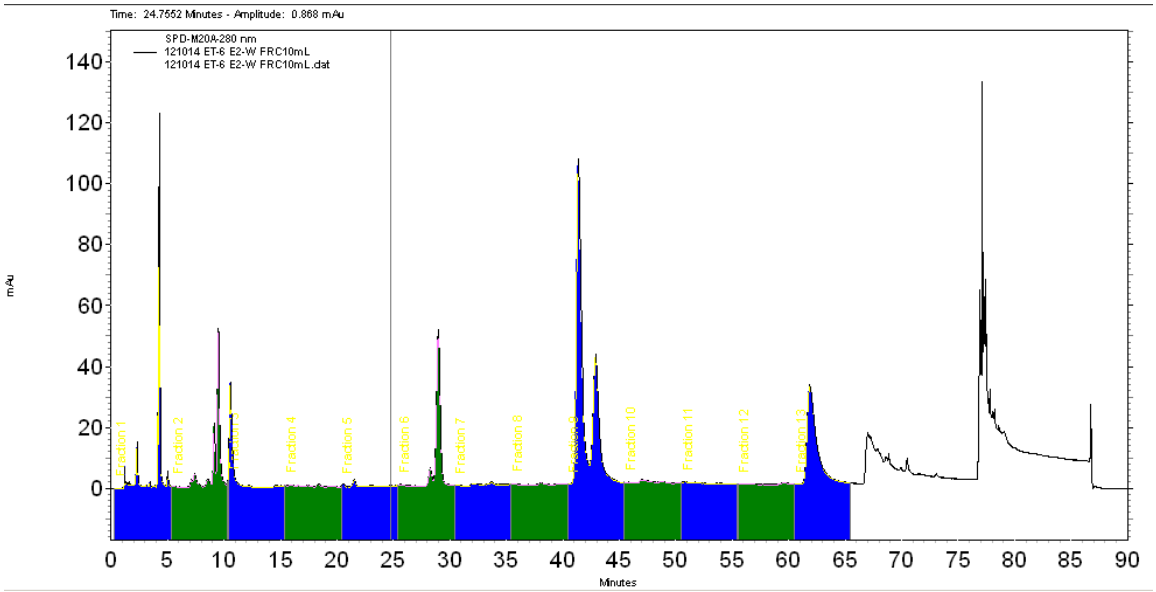

Figure 4.35: Fraction collection of Et-6 E2-W (Subfraction J)

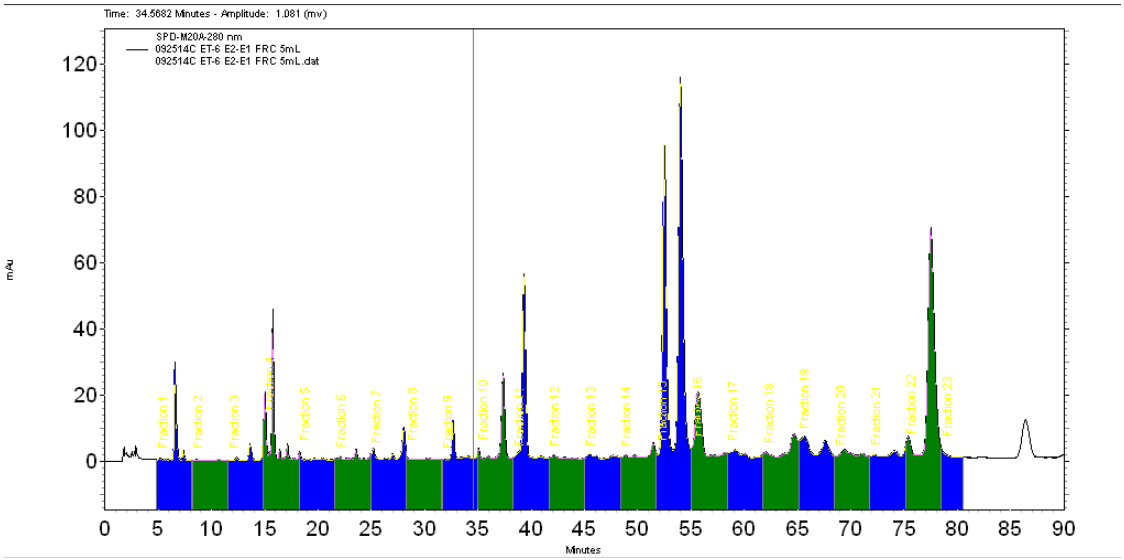

Figure 4.36: Fraction collection of Et-6 E2-E1 (Subfraction K)

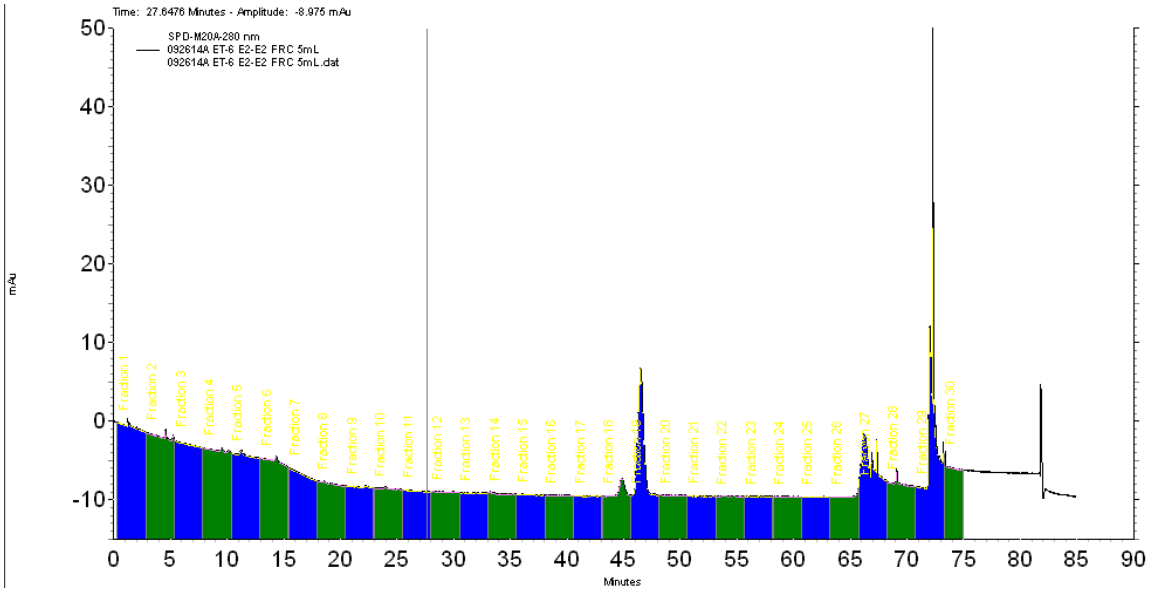

Figure 4.37: Fraction collection of Et-6 E2-E2 (Subfraction L)

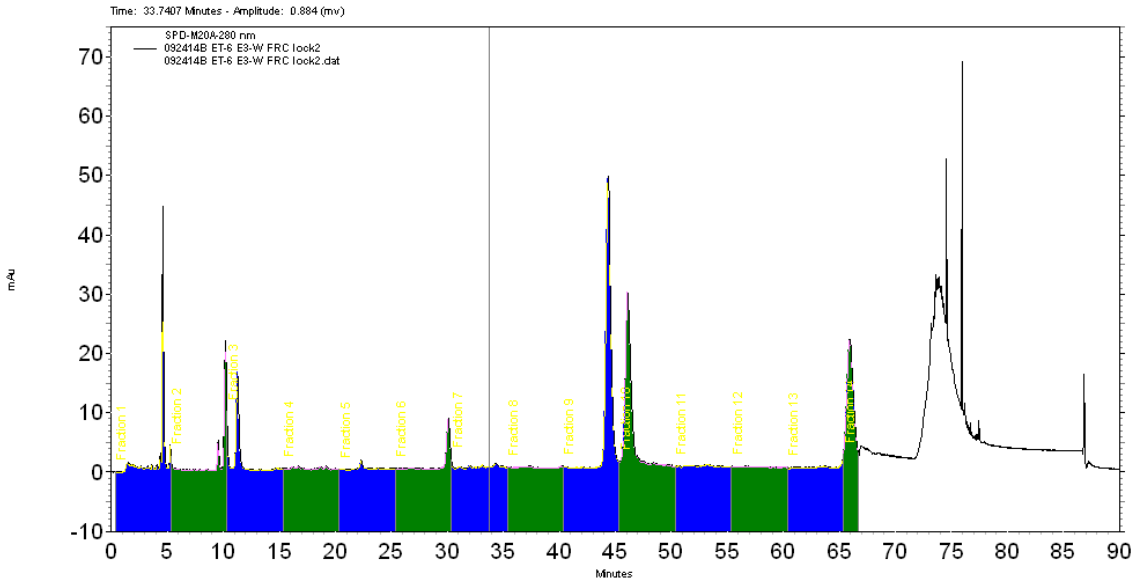

Figure 4.38: Fraction collection of Et-6 E3-W (Subfraction M)

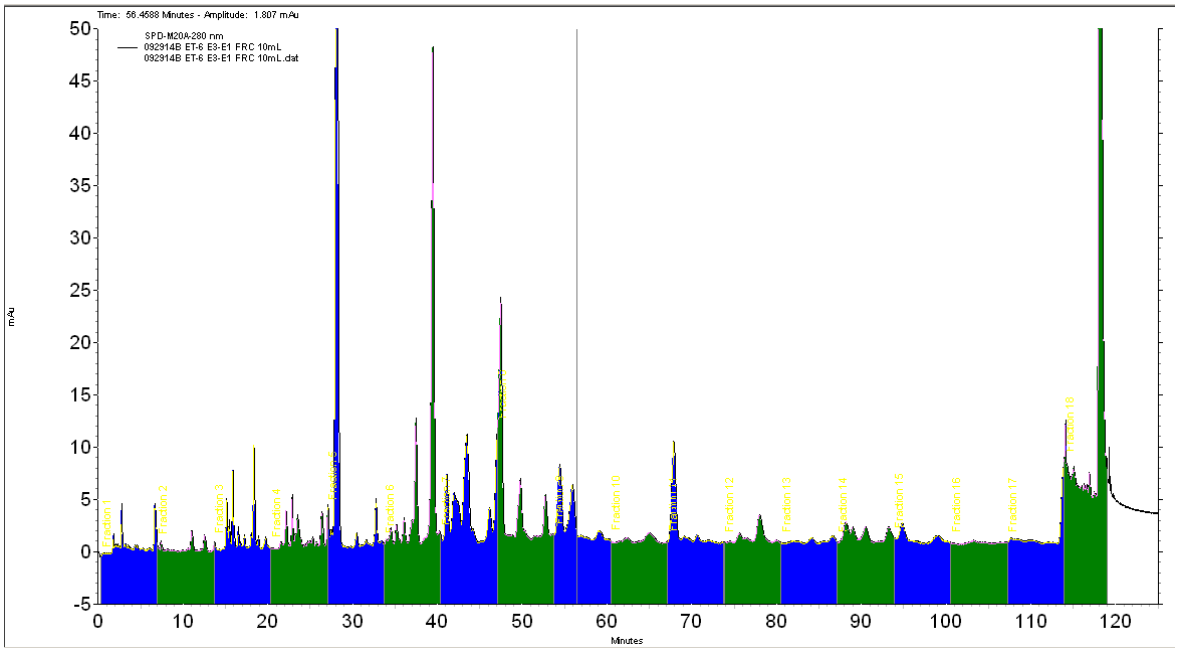

Figure 4.39: Fraction collection of Et-6 E3-E1 (Subfraction N)

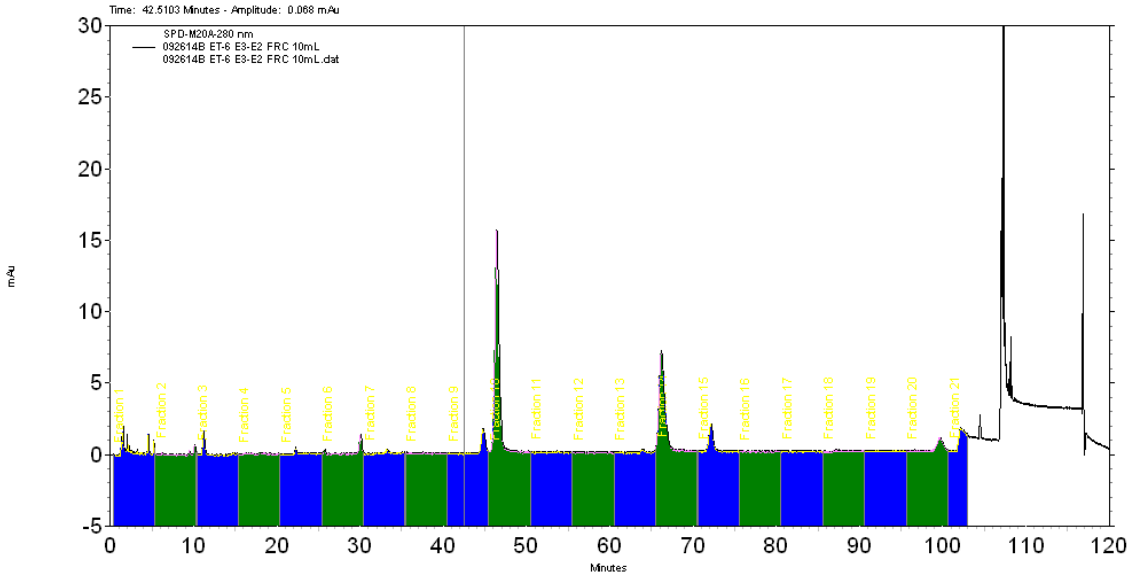

Figure 4.40: Fraction collection of Et-6 E3-E2 (Subfraction O)

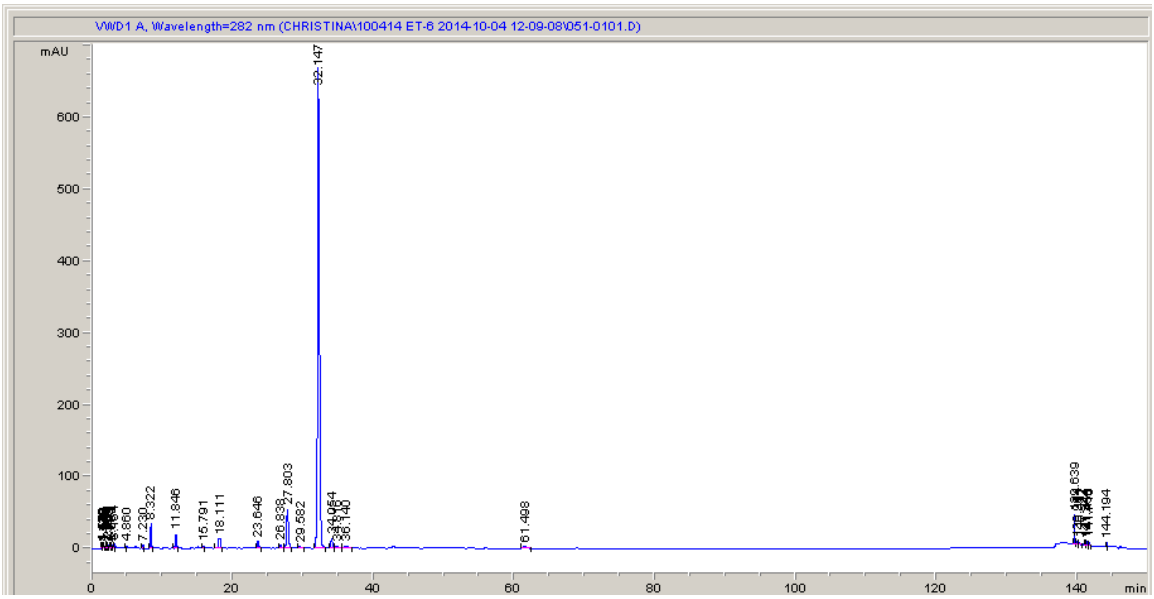

Figure 4.41: UV spectra (282nm) from LC-MS of Et-6 W1-E1

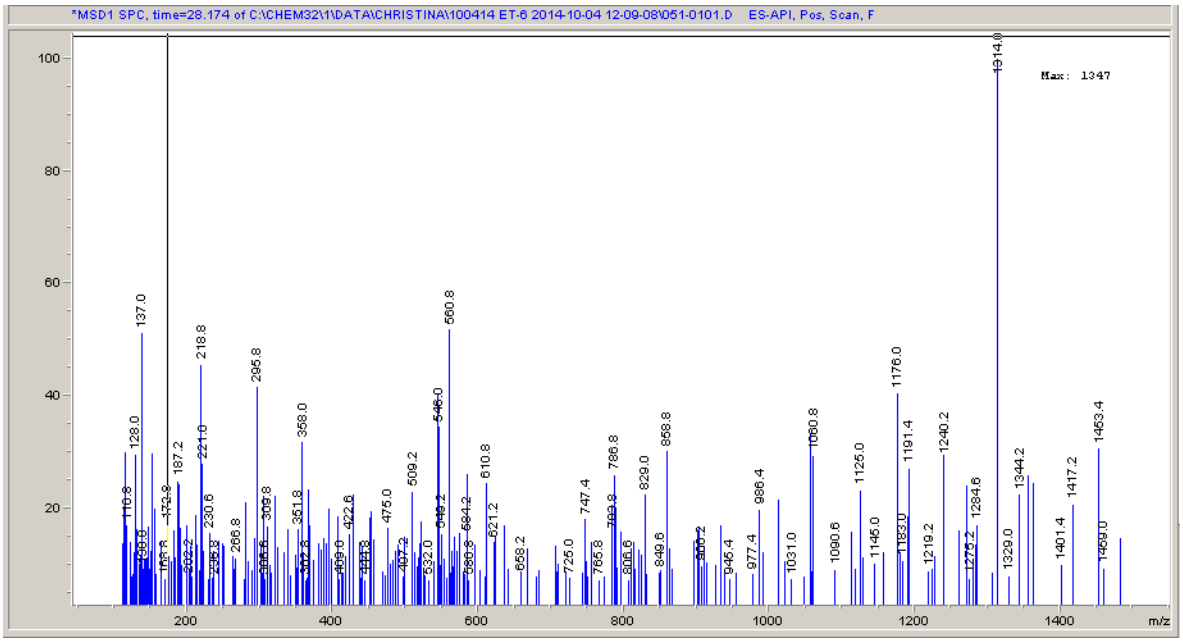

Figure 4.42: Extracted ion chromatogram from LC-MS of Et-6 W1-E1 at retention time 27.803 minutes corresponding to sub-fraction **A5**

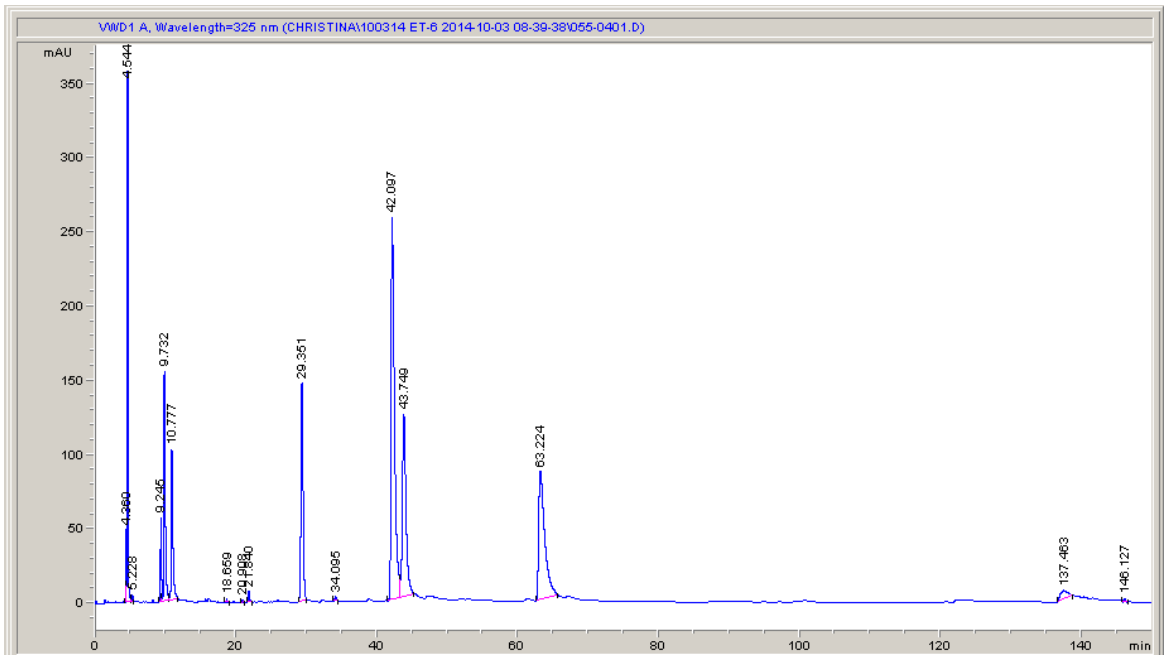

Figure 4.43: UV spectra (325nm) from LC-MS of Et-6 E2-W

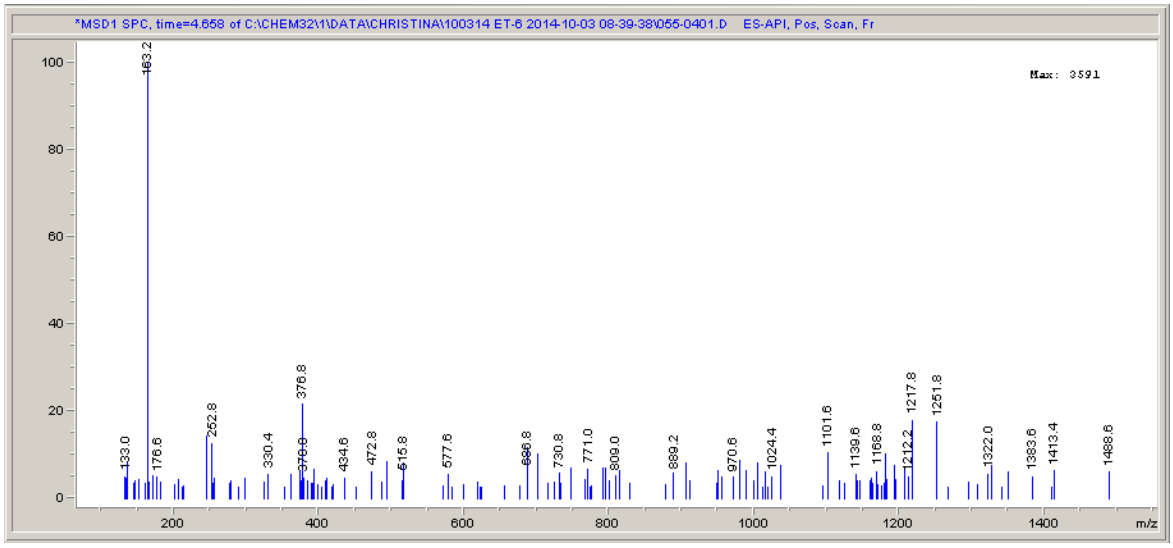

Figure 4.44: Extracted ion chromatogram from LC-MS of Et-6 E2-W at retention time 4.658 minutes corresponding to sub-fraction **J1**

Discussion

After the procurement of the crude ethyl acetate fraction from the liquid-liquid extraction, a sample was prepared to concentration of 40 mg/mL and injected into the Shimadzu HPLC system with a simple 0-100%B gradient. The HPLC chromatogram that was achieved is presented in Figure 4.1, which shows a large number of compounds present. The large mAu responses of the detected compounds are indicative of a concentrated sample, which is suitable for further separation by solid phase extraction (SPE). On the other hand, of the diode array detector (DAD) was set to scan the detected chemicals in a range of wavelength from 200 to 400nm, which is favorable as many plant chromophores that exhibit antioxidant properties absorb at the following seven specific wavelengths within that range, they are 252, 266, 270, 282, 300, 325, and 350nm. The output of the DAD for the crude extract is shown in Figure 4.2 which depicts the absorbance detected across the 200 to 400 nm range for the duration of the time program. The more colorful the DAD spectra appears, the more compounds are being detected and the large red portion near the top of the spectra corresponds to co-eluting points at the lower wavelengths.

In regards of selecting the second sorbent type of SPE, a comparison of the MCX and WCX sorbents was made since both types of SPEs, would separate the acidic from the basic compounds. Figures 4.3 to Figure 4.20 shows the comparison of MCX and WCX fractions. In each spectrum, the black line corresponds to the MCX fraction whereas the blue spectrum represents the WCX. The trend across all of the fractions indicated that the MCX SPE had displayed more retention of the chemicals, which was

evidenced by those spectra that show a less number of peaks. In comparison, any peaks that correspond with the WCX sorbent are generally of lesser intensity.

After the ABTS antioxidant testing of the 198 SPE prepared samples, fraction 6 revealed to be the most active fraction and therefore was further analyzed by HPLC. The eighteen SPE samples of fraction 6 can be seen in Figure 4.21 to Figure 4.26; where spectra are combined by grouping the WCX fractions for each HLB fraction (i.e. the WCX's wash, elute 1 and elute 2 for each HLB wash 1, wash 2, wash 3, elute 1, elute 2 and elute 3). In each figure, the wash for each spectra corresponds to the black line, blue or green is elute 1 and the purple represents the elute 2.

For the majority of the HLB fractions, the elute 1 (E1) shows the most response signifying that the greatest number of compounds was eluted out of the WCX-SPE. As this eluting solvent is 100% methanol, it is not a surprise to elute a large amount of compounds since methanol is among the most polar organic solvents. Few peaks that correspond to the WCX fractions of HLB wash 2 are shown in Figure 4.22 but this does not mean there are no compounds present. In most cases, it means there are just no compounds that absorb between 200 and 400nm. On the other hand, several of the 18 samples from the fraction 6 visibly bear a brown color, which means these compounds absorb in the visible spectrum of wavelengths.

To continue the investigation of bioactive compounds, the Et-6 fractions were separated further by using the Shimadzu fraction collector. Four of the fractions were chosen not to be further analyzed as their DAD spectra showed little to no response. Therefore, the remaining 14 fractions were divided into subfractions. It was found that the easiest operation of the fraction collector was to collect either 5 or 10mL intervals for

the duration of the HPLC separations. The way in which the collector partitioned the samples is shown in Figure 4.27 to Figure 4.40. The amount of each sample collected depended on the response from previous spectra; spectra with few and separated peaks deemed acceptable to collect 10mL (Figures 4.27, 4.29, 4.31, 4.32, 4.35, 4.38, 4.39 and 4.40). Where spectra with numerous peaks had 5mL subfractions collected (Figures 4.28, 4.30, 4.33, 4.34, 4.36 and 4.37). The fraction collector was told to not collect fractions if there was a large period of time in the spectra where no peaks were present this was done with fraction Et-6 E1-E2, seen in Figure 4.34.

The collected subfractions were then subjected to the antioxidant testing by the ABTS and DPPH methods. A description of these methods and the assay results were described in detail in Chapter 3. The fractions that confirmed their antioxidant activity in both assays (W1-E1 and E2-W) were analyzed by mass spectrometry in attempt to identify the bioactive compounds. The larger fraction instead of the subfractions was analyzed so comparisons could be made between the HPLC spectra and the spectra obtained from the LC-MS.

The fraction Et-6 W1-E1, also called **A** to refer to the subfractions, had two subfractions that were positive for their antioxidant properties in the ABTS and DPPH tests, referred to as **A5** and **A14**. The DAD spectra for the first subfraction **A5**, has multiple peaks with retention times of approximately 27, 30 and 33 minutes. When analyzing the HPLC data, the subfraction **A14** collected the tail of a peak which could explain the antioxidant activity. The spectrum of the W1-E1 fraction from the LCMS is shown in Figure 4.41 with prominent peaks at 27.8 and 32.1 minutes that fall in line with the subfraction **A5.** The latter peak did not ionize but the extracted ion chromatogram of

the 27.8 minute is shown in Figure 4.42. Using the fragmentation data based on the mass to charge ratio (*m/z*) the chemical compound was temporarily to be identified based on the online chemical database ChemSpider, which aided the compound search by inputting intrinsic properties such as the parent ion fragment (1314.0 *m/z*). Based on the online search, the database generated possible compound structures with molecular weights of 1314.0 ±1.0 g/mol. The list of potential compounds could be narrowed down by determining which elements may and may not be present. Viewing the probable structures, further analysis of one was carried out; L-Arginyl-L-α-aspartyl-L-tyrosyl-L-leucyl-L-prolyl-L-tyrosyl-L-tyrosyl-L-prolyl-L-leucyl-L-aspartic acid has a chemical formula of $C_{63}H_{87}N_{13}O_{18}$ and a molecular weight of 1314.44 g/mol. This compound can be seen in Figure 4.45 below.

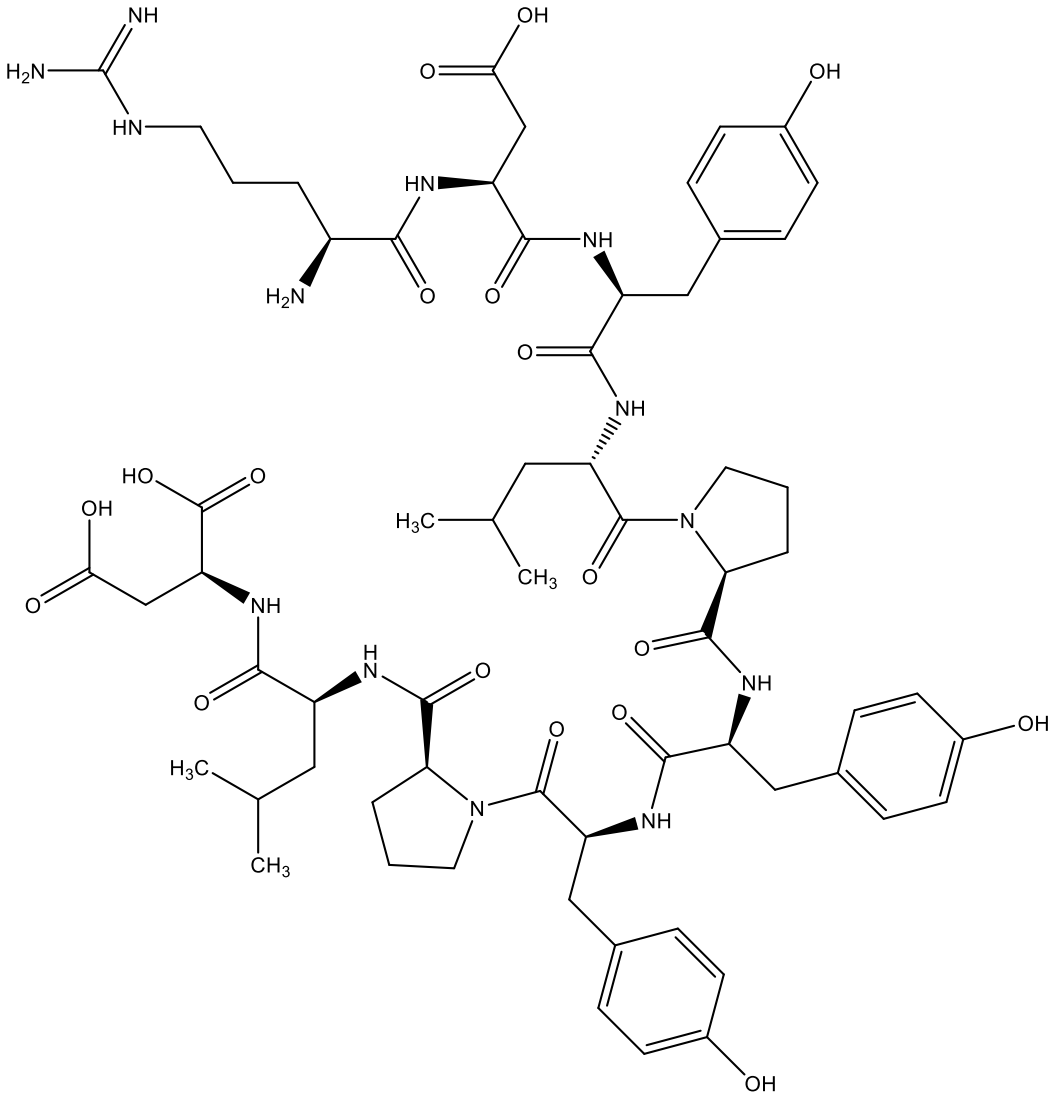

Figure 4.45: L-Arginyl-L-α-aspartyl-L-tyrosyl-L-leucyl-L-prolyl-L-tyrosyl-L-tyrosyl-L-prolyl-L-leucyl-L-aspartic acid

The suggested peptide might be present in the sample. Since it has multiple hydroxyl groups attached to cyclic carbon rings, it could participate in radical scavenging and yield the positive results from the antioxidant testing. Peptides or a composition of a sequence of amino acids, have been studied from medicinal plants, as well as fruits and vegetables.[3] Previous studies have measured the antioxidant activity of various peptides.[4] Some peptides that contained tyrosine showed antioxidant properties due to the existence

of its phenolic structure.[5] The proposed peptide chain is comprised of two aspartic acid residues and three tyrosine rings that most likely contribute to the antioxidant activity of this fraction (**A5**). The nitrogen containing arginine residue could possibly add to the activity.

A larger fragment ion with a *m/z* of 560.8 is approximately the loss of the arginyl, aspartyl, tyrosyl, leucyl and prolyl. The fragment ion with 137.0 *m/z* is approximately the aspartic acid based on the MS spectrum.

Other compounds were also proposed instead of the peptide, for their molecular weights of approximately 1314, the same as the parent ion from the extraction ion chromatogram in Figure 4.42. One such of these compounds is (6E, 10E, 14E, 18E, 22E, 26E, 30E, 34E, 38E, 42E, 46E, 50E, 54E, 58E, 62E, 66E, 70E)- 3, 7, 11, 15, 19, 23, 27, 31, 35, 39, 43, 47, 51, 55, 59, 63, 67, 71, 75- Nonadecamethyl-6, 10, 14, 18, 22, 26, 30, 34, 38, 42, 46, 50, 54, 58, 62, 66, 70, 74-hexaheptacontaoctadecean-1-ol. This structure is shown in Figure 4.46 below.

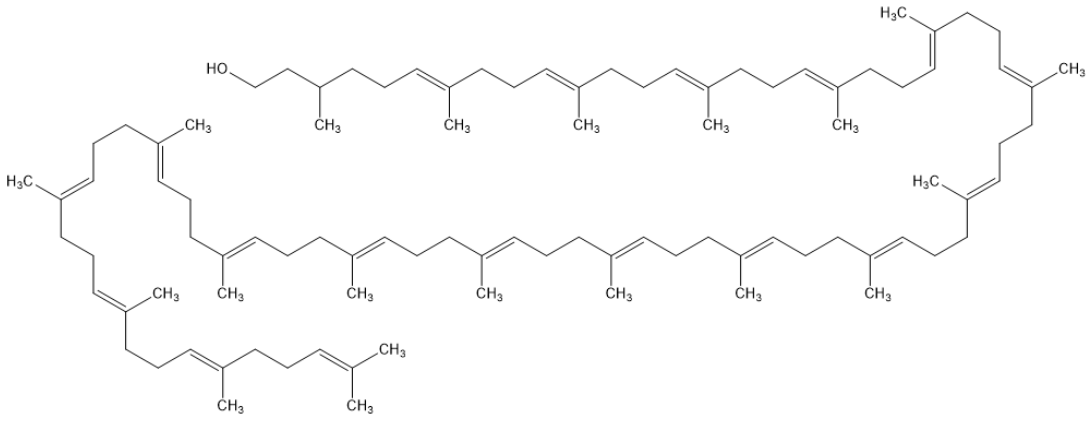

Figure 4.46: Proposed structure (#2) for compound present in subfraction **A5**.

The above chemical compound is classified as a monoterpenoid, that tends to be a constituent of plant essential oils, produces a sweet scent and has been found to have antiseptic activity seven times stronger than phenol.[8] Fragmentation of approximately nine hydrocarbon groups yields a mass of 1176 which corresponds to a fragment of the ion chromatogram. Fragmenting 50 hydrocarbon groups yields a 560 *m/z.* If everything is fragmented except the hydroxyl group and seven hydrocarbon groups the approximately mass to charge ration is 137, also corresponding to a fragment ion.

Another hydrocarbon chain with hydroxyl groups present with the same parent ion ratio is the isoprenoid zeaxanthin diglucoside diester which has been suggested in the following figure.

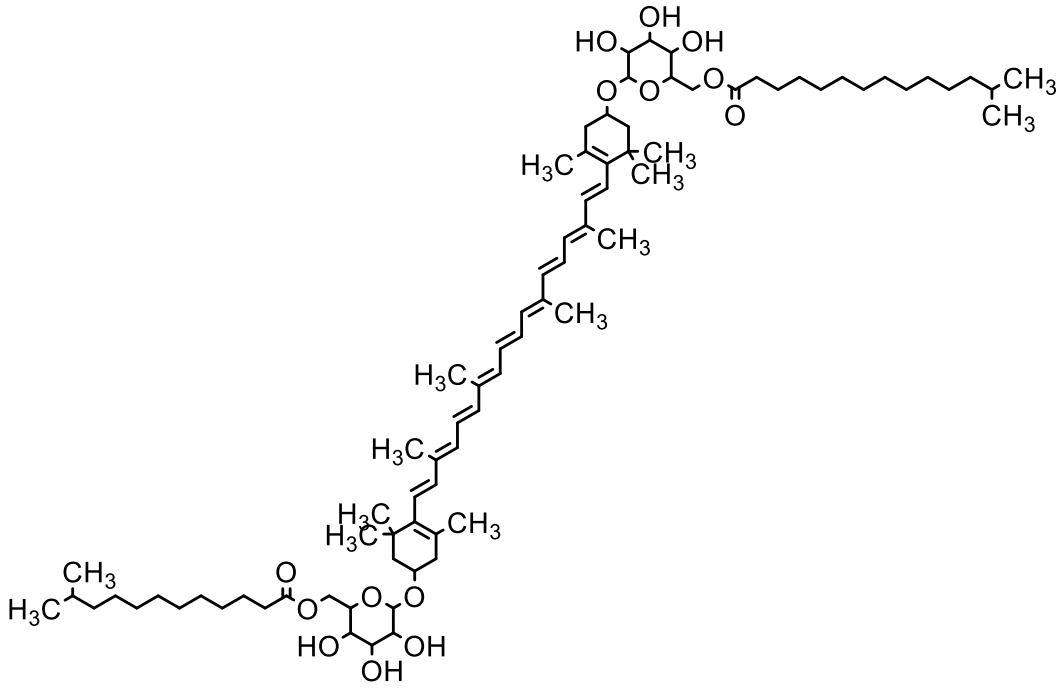

Figure 4.47: Proposed structure (#3) for compound present in subfraction **A5**.

The preceding structure is not the most likely compound as the fragmentation of this compound does not match that of the extracted ion chromatogram. If the bottom hydrocarbon chain is fragmented, a mass to charge ratio of 1145 is achieved which is a present fragment in the chromatogram but it is not prominent. In regards to the antioxidant activity, there are multiple hydroxyl groups that could scavenge radicals and donate hydrogen atoms.

A final structure proposed for subfraction **A5** is N-{[(1S, 3R, 5R, 6S, 8R, 10R, 11S, 13R, 15R, 16S, 18R, 20R, 21S, 23R, 25R, 26S, 28R, 30R, 31S, 33R, 35R, 36R, 37R, 38R, 39R, 40R, 41R, 42R, 43R, 44R, 45R, 46R, 47R, 48R, 49R)-36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 – Tetradecahydroxy- 10, 15, 20, 25, 30, 35- hexakis (hydroxymethyl)- 2, 4, 7, 9, 12, 14, 17, 19, 22, 24, 27, 29, 32, 34-tetradecaoxaoctacyclo[31.2.2.2^{3,6}.2^{8,11}.2^{13,16}.2^{18,21}.2^{23,26}.2^{28,31}] nonatectracont-5-yl] methyl}-3-(5-methyl-2, 4-dioxo-3, 4-dihydro-1(2H)-pyrimidinyl) propan. Figure 4.48 depicts this structure below.

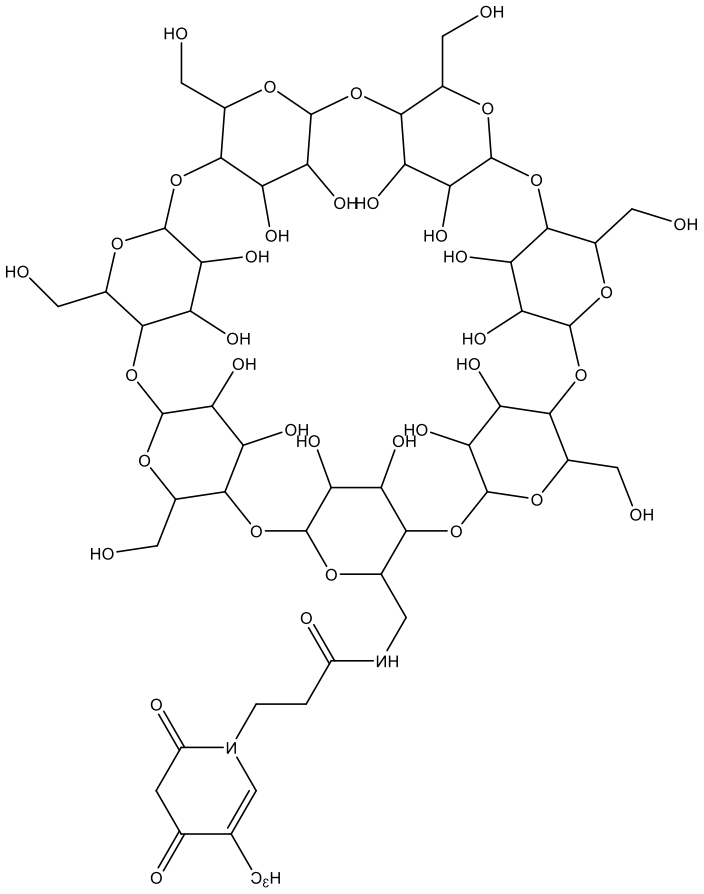

Figure 4.48: Proposed structure (#4) for compound present in subfraction **A5.**

The compound above is another proposition for the subfraction **A5** as its molecular weight is approximately 1314 with a chemical formula of $C_{50}H_{79}N_3O_{37}$. If the six member ring and two hydrocarbon groups are fragmented then the compound would obtain a 1176 *m/z*. If the entire side chain and all hydroxyl groups are fragmented (although unlikely) the compound would then have a 766 *m/z* which is a fragment that appears in the ion chromatogram but in low abundance.

The fraction Et-6 E2-W, given the code **J** for the subfractions, had one subfraction (**J1**) that tested positive with both antioxidant tests. Therefore the Et-6 E2-W fraction was analyzed by LCMS. Its liquid chromatography spectrum was shown in

Figure 4.43 with a large response at retention time of 4.54 minutes. This peak is present in the first subfraction and the compound was ionized to produce an ion chromatogram that was shown in the Figure 4.44. Again, using the ChemSpider database and the prominent ion fragment of 163.2 *m/z,* a compound was suggested to be 3-cyano-4-hydroxybenzoic acid which is visible in Figure 4.46 below.

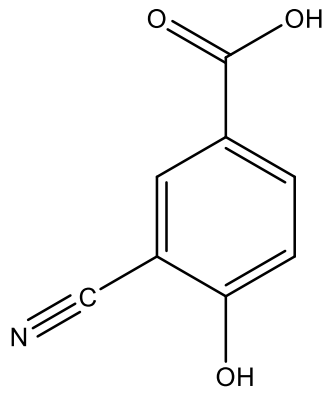

Figure 4.49: 3-cyano-4-hydroxybenzoic acid

I propose the presence of this molecule because it has the approximate mass of the prominent fragment ion, as well as the fragment with a value of 133.0 *m/z* that is equivalent to the loss of the triple bonded carbon and nitrogen. It is documented that benzoic acids have antioxidant activity and these acids can polymerize to yield large molecules such as albicidin that is known to be a plant produced phytotoxin with antibacterial properties.

As a result of the limited amount of fragmentation, chemical identification with complete accuracy is rather difficult. The little fragmentation was a result of the soft ionization from the electrospray source, ESI derived spectra contain quasi molecular ions with no fragmentation.[6] The ionization parameters that were set on the instrument did not generate enough energy to fully ionize more than the one characteristic ion. This would

also be why some peaks from the UV spectra appear to have no ionization within the mass spectra.

A soft ionization source, such as ESI, is common in the application of tandem mass spectrometry or mass spectrometry-mass spectrometry (MS/MS). This method produces ions in the first mass analyzer, then a specific ion is selected to be sent to the second mass analyzer and ion detector.[7] This method is often chosen to achieve a better idea of present compounds and to increase the probability of correctly identifying said compounds as more fragmentation of a selected ion occurs.

Further investigation to achieve compound identification would rely on nuclear magnetic resonance (NMR), which is exercised to obtain a working notion of a present compound. This modern type of spectroscopy perturbs a sample with magnetization, when a radio frequency response travels through a coil that is sensed by a detector and information is converted into spectrum. NMR techniques can identify chemical constituents as well as their relation to one another based on the principle that each nuclei has spin and its own angular momentum. When perturbed by magnetization these spins produce unique radio frequency as they relax back to a zero magnetic state. The interpretation of the radio frequency assists in compound identification.

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