QUANTIFICATION AND CHARACTERIZATION OF BIOLOGICALLY ACTIVE COMPONENTS OF *ACTAEA RACEMOSA* L. (BLACK COHOSH) FOR IDENTIFYING DESIRABLE PLANTS FOR CULTIVATION

A thesis presented to the faculty of the Graduate School of
Western Carolina University in partial fulfillment of the
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LIST OF ABBREVIATIONS

HPLC……High Pressure Liquid Chromatography
UV……..……Ultraviolet
ELSD……..…….Evaporative Light Scattering Detection
MS……...Mass Spectrometry
SLM……....Standard Liter Minute
mL……Milliliter
ANOVA……...Analysis of Variance
ppm……..Parts per million
g……...Grams
mg…….Milligrams
µg…….……...….Micrograms
µL…….…….....Mircoliters
δ……...Signal
MW……Molecule Weight
MeOH…….Methanol
ACN.……...…….....Acetonitrile
$CHCl_3$…….….Chloroform
CH_2Cl_2…….....Dichloromethane
TFA……...Trifluoroacetic Acid
FA……..Formic Acid
NMR……..….Nuclear Magnetic Resonance
LCMS……..Liquid Chromatography Mass Spectrometry
TLC……...Thin-layer Chromatography
m/z……Mass to Charge ratio
ESI-MS…….Electrospray Ionization Mass Spectrometry
PMT……..…Photomultiplier tube
BCGR……...Bent Creek Germplasm Repository, Asheville, NC

ABSTRACT

QUANTIFICATION AND CHARACTERIZATION OF BIOLOGICALLY ACTIVE COMPONENTS OF *ACTAEA RACEMOSA* L. (BLACK COHOSH) FOR IDENTIFYING DESIRABLE PLANTS FOR CULTIVATION

Patrick Michael Looney

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Actaea racemosa, more commonly known as black cohosh, is a medicinal plant used for the suppression of menopausal symptoms. The growing popularity of black cohosh extract is leading to extensive wild harvesting of the perennial plant. The main purpose of this research is to use high-pressure liquid chromatography evaporative light scattering detection analysis of 20 accessions of black cohosh from Bent Creek Germplasm Repository to quantify the compounds with desirable biological activities. Plants identified as having desirable properties may be used to produce a superior hybrid plant. Cultivation of the hybrid plant may help black cohosh's sustainability. This research showed that there were significant differences in phytochemical concentrations among the accessions.

INTRODUCTION

Actaea racemosa, more commonly known as black cohosh, is a medicinal plant used for the suppression of menopausal symptoms.[1] Black cohosh is a plant native to the eastern United States and can be found as far south as Georgia, west into parts of Missouri, and even into Ontario, Canada. Black cohosh is found along moist creek banks, meadows, forest, and ravines and can be found locally in the Blue Ridge Parkway corridor and the Great Smoky Mountains.[1,2] The growing popularity of black cohosh extract is leading to extensive wild harvesting of the perennial plant. As with all natural resources, once the natural supply is gone, it is gone forever. To help preserve these natural products, cultivars of superior black cohosh plants could be established by local farmers.

The research project was funded by the North Carolina Biotechnology Center. The overall goal of the grant is to develop a regional cultivar of hybrid black cohosh plants to stimulate economic development in Western North Carolina. This grant funds a collaboration of multiple groups working on specific aspects of the overall project. Our collaborators at Bent Creek Germplasm Repository (BCGR) are responsible for collection, harvesting and measurements of growth characteristics for the plant accessions. Our collaborators in the Western Carolina University Biology Department, Cullowhee, NC are performing genetic marker analysis and genetic studies. The main purpose of this master's research is to use high-pressure liquid chromatography with evaporative light scattering detection analysis of 20 accessions of black cohosh from Bent Creek Germplasm Repository to quantify compounds with desirable biological activities

in hopes to produce a superior hybrid plant to decrease wild harvesting. The secondary purpose of this research is to isolate and characterize novel compounds from black cohosh plants from BCGR.

To accomplish the above task, standards must be isolated and characterized from black cohosh. The chromatography methods used in this research were modified from published articles. Once the methods were optimized, calibration standards were made and analyzed in triplicate. The calibration response was a second-degree polynomial because the polynomial gave the best coefficient of correlation. All of the raw sample data was transferred to Microsoft Excel for ANOVA analysis. The hypothesis for this research is that in the BCGR collection there will be significant chemical differences between accessions.

BACKGROUND

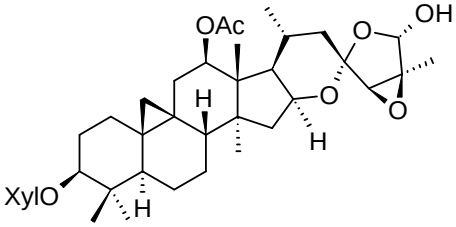
Menopause, as defined by the World Health Organization, is the permanent cessation of menstruation due to the loss of ovarian follicular activity.[3] The result of this loss of ovarian follicular activity causes hormone levels to fluctuate.[4] During menopause, the level of estrogen in the body decreases, due to the ovaries gradually stopping the production of estrogen as the egg supply decreases.[5] Symptoms associated with decreasing estrogen levels in the body include hot flashes, mood swings, depression, sleep disturbances, bone loss and loss of libido.[1]

Compounds found in black cohosh can help suppress some of the symptoms associated with low estrogen levels. The exact mechanism by which black cohosh provides menopausal relief has not been fully elucidated. However, available research

supports black cohosh as an effective treatment for the suppression of some menopausal symptoms, especially hot flashes.[1] Other studies show black cohosh having an inhibitory effect on the hypothalamus.[6] The hypothalamus plays a role in the regulation of the temperature of the body, as a result, inhibiting parts of the hypothalamus could alleviate hot flashes. Other published studies demonstrated that black cohosh affects the estrogen receptors (ER). [7] Estrogen receptors are sites where estrogen binds in a biological system. Recent studies have shown that there are correlations between therapeutic activity and the presence of the cycloartane triterpene glycosides (saponins) and estrogenic activities associated with the presence of the aromatic acids.[26] Also, the aromatic acids have been reported to have anti-inflammatory properties associated with rheumatism brought upon by menopause.[1, 27, 28]

Black cohosh contains many classes of compounds, but the five types of compounds of interest are: cycloartane triterpene glycosides,[1, 2] aromatic acids, isoflavones, dopamine-type derivatives, and cimipronidine-type alkaloids.[9] The two types of natural products that are the focus of the current study are the triterpene glycosides and the aromatic acids. It is unclear if isoflavones are actually present in black cohosh. Isoflavones have been reported by Jarry et al.,[8] however, the presence of isoflavones has not been confirmed in other published studies.[11,12] Examples of cycloartane triterpene glycosides found in black cohosh are actein (**1**), 23-*epi*-26-deoxyactein (**2**), cimiracemoside A (**3**), cimiracemoside C (**4**), acetyl shengmanol xyloside (**5),** and 26-deoxycimicifugoside (**6**). Examples of the aromatic acids are caffeic acid (**7**), isoferulic acid (**8**), and ferulic acid (**9**).[10] An example of an isoflavone is formononetin (**10**). An interesting note about formononetin is that although it has been reported to have

estrogenic activity, [11] it has not been identified in black cohosh samples from the United States.[11,12]



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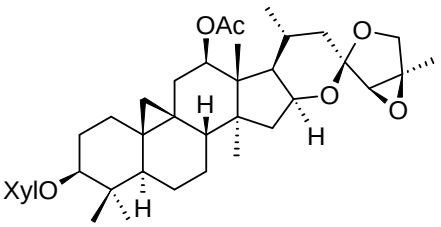

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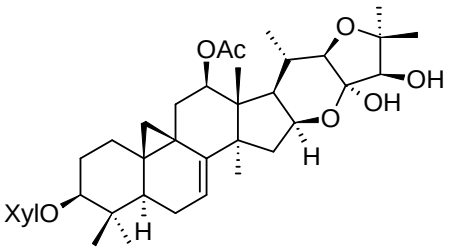

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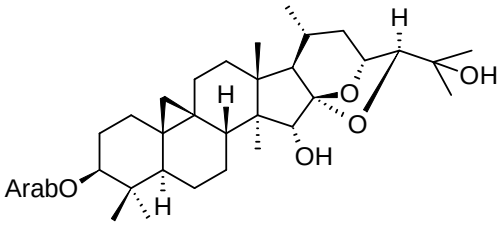

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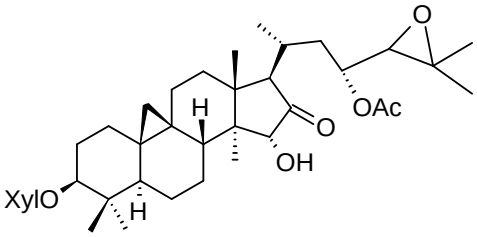

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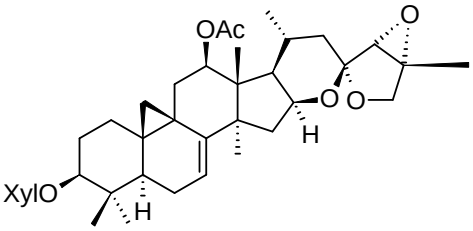
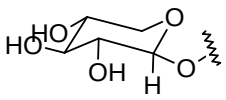
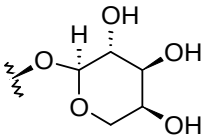


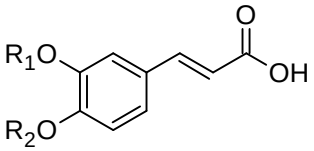
6

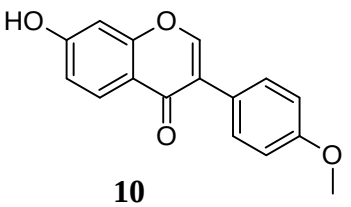
XylO=



ArabO=







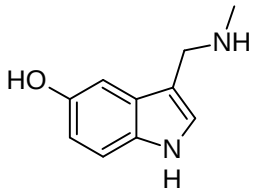
10

7: R_1=R_2=H

8: R_1= H; R_2=CH_3

9: R_1= CH_3; R_2=H

Previous studies show that alcoholic extracts of black cohosh have been successful in treating hot flashes.[13] Other studies show that actein, a triterpene glycoside, could act as selective estrogen receptor modulator (SERMs), which might explain how actein could have estrogen-modulating effects in the hypothalamus, but not in the uterus.[14] The hypothalamus is the area of the brain that produces hormones for the regulation of body temperature, sex drive, sleep, thirst hunger, moods, and release of hormones from other glands.[15] The Rhyu et al.[16] study stated that alcoholic extracts of black cohosh had constituents with an affinity for the human opiate receptors (HMOR). This is also noteworthy because the opiate receptor system affects several aspects of female reproductive neuroendocrinology, such as the control of sex hormones. Bodinet et al.[17] showed that growth of human breast adenocarcinoma cells (MCF-7) was significantly inhibited by black cohosh extract added to tamoxifen indicating that black cohosh could be used to suppress menopausal symptoms associated with breast cancer. Powell et al.[18] have identified *N*ω-methylserotonin (**11**), which is a known 5-HT agonist, as a potential active constituent of black cohosh extract.



11

Historically, black cohosh has been used medicinally to treat many illnesses. Native North Americans used black cohosh for malaria, rheumatism, abnormalities in kidney function, sore throat, menstrual complaints, tuberculosis, as an analgesic, as a

sedative, and as a stimulant.[1] Black cohosh has also been used in pain relief associated with aching muscles, arthritis, and child birth.[1] Finding out information about the mechanism would be exciting because then the plant could potentially be used in cancer research. Black cohosh is classified by the German Health Authority (Commission E) as a nonprescription drug to treat neurovegetative symptoms associated with menopause (i.e. hot flashes).[1] Neurovegetative refers to the section of the nervous system that controls involuntary functions.

Black cohosh has been heavily wild-harvested for the rhizome material. Wild harvesting of black cohosh makes up 90% of material used in trade.[1] There are many problems associated with this practice, such as adulteration with potentially toxic species and high chemical variation among harvests. The largest suppliers of wild harvested black cohosh rhizome material are located in the southeastern United States.[1] There are other species in the genus *Actaea* or *Cimicifuga,* which are similar to black cohosh and are common adulterants found in commercial black cohosh. These other species of plants, *A. pachypoda*, *A. podocarpa,* and *A. rubifolia,* have different chemical compositions and biological properties.[1]

Black cohosh is ranked among the top ten best-selling dietary supplements in the United States.[19] Western North Carolina would be a suitable environment for the growth of black cohosh crops, providing high economic returns per acre. The possible identification of plant accessions that contain high levels of biologically active compounds will help in identifying plants that can be developed into a superior hybrid that could be grown in Western North Carolina. This would help to reduce or stop wild

harvesting of the plant, which would assist in making black cohosh production more sustainable.

There have been previous studies researching the phytochemical variations among different accessions of black cohosh using high-pressure liquid chromatography evaporative light scattering detection.[20] One study by Al-Amier et al. only had a small number of plants with little geographic diversity. In contrast, this study has twenty accessions of plants from thirteen different states. Studying more accessions over a wide geographic range may increase the chances of finding plants with superior properties. The Al-Amier et al. study did not have closely-controlled growing conditions. The plants in this study were grown under strictly controlled conditions. The novel aspect in comparison to others is that no one has ever studied a large collection of black cohosh grown under identical conditions. The large number of accessions may show many chemical differences which could lead to the development of a superior hybrid plant for cultivation.

INSTRUMENT BACKGROUND

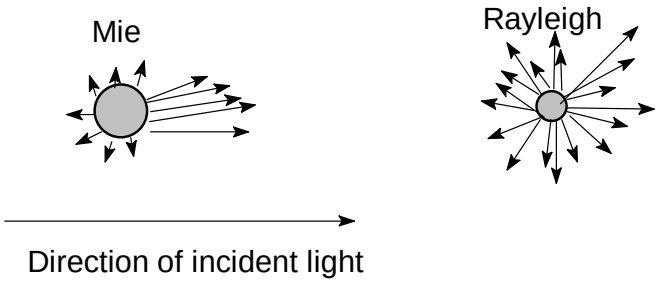
High-pressure liquid chromatography (HPLC) is the main analytical technique used in this phytochemical study. HPLC is a column chromatography technique that is widely used. The HPLC system is a Dionex Ultimate 3000. The Dionex system has solvent reservoirs, a pump (which contains an in-line degasser and mixing station), refrigerated autosampler, temperature controlled column compartment, a 4-channel ultraviolet detector, and an evaporative light scattering detector. The entire system is managed by Dionex Chromeleon software.

Ultraviolet and evaporative light scattering detectors were used. The Dionex UV detector is a multiple channel detector, collecting data from four different analytical wavelengths ranging from the visible to ultraviolet. The detector measures the attenuation of light, at a specific wavelength, by the compounds eluting from the column. This follows Beer's Law ($A = \varepsilon bc$) where A is the absorbance, ε is the extinction coefficient, c is the concentration and b is the pathlink. A point worth noting about the UV detector is that it is non-destructive and gives a linear response. However, at high concentrations the UV response can be nonlinear. Triterpene glycoside compounds do not absorb well in the ultraviolet range because they have poor chromophores. Therefore, for the purpose of this research another means of detection is needed.

The evaporative light scattering detector (ELSD) is the detector of choice for triterpene glycosides. The ELSD that was used for this research is a Varian 380 LC. The eluent from the UV detector is carried to the ELSD nebulizer. In the nebulizer, the eluent is mixed with a nitrogen carrier gas. The nitrogen gas and the eluent mixture form an aerosol plume of uniform droplets, which moves on to the evaporator. Larger droplets will collect in the nebulizer waste chamber, which empties into a collection bottle. The atomized spray is carried through the evaporation tube towards the laser light source with the help of the dry nitrogen flow. Any residual solvent is evaporated off, leaving a stream of dry particles of the analyte. Laser radiation then impinges on the streaming particles. The scattered radiation by the analyte is detected at a right angle to the flow by a silicon photodiode. Scattering caused by solvent is taken as a background signal at the start of the analysis.

The intensity of scattered light is dependent on the number of analyte particles and the analyte particle size. The scattering depends on the radius of the analyte particle (r) compared to the wavelength (λ) of incident light. Mie scattering occurs when r/λ is greater than $5x10^{-2}$. Rayleigh scattering is predominant when r/λ is less than $5x10^{-2}$.[34] In Mie scattering most of the scattered light is projected in the direction of incident light, whereas in Rayleigh scattering the scattering is in all directions. Generally, the amount of light scattered is proportional to the power 4 (Mie) or 6 (Rayleigh) of the radius of the analyte particle.[33] Mie scattering is dominant; however both Mie and Rayleigh can occur within the ELSD. Figure 1 (below) is a depiction of the two scattering mechanisms.

Figure1: Scattering mechanisms[34]



ELSD response to concentration is sample dependent,

$$A = a\, m^{\,b} \qquad \text{(Eq.1)}$$

where *A* is the peak area, *a* is the response factor unique to the analyte, *m* is the analyte concentration, and *b* is an exponent ranging from 4 for Mie scattering, to 6 for Rayleigh scattering. Recent articles have shown that in practice the power is closer to 5,[12] most likely the result of both Mie and Rayleigh scattering contributing to the response. For convenience Eq. 1 can be written as [29-32]

$$\log A = b \log m + \log a$$

(Eq.2)

When log *A* is graphed as a function of log *m*, the slope is equal to the exponent (*b*) in Eq. 1. However, since response is sample dependent, some samples can be better modeled as a polynomial function.[40, 12] The literature suggests that the response of the detector depends on the parameters of that specific detector.[40, 12] ELSD data appears to follow a sigmoidal regression, however at low concentrations a second-degree polynomial or a power function can be used to model the regression. In the present study, the coefficient of determination is slightly better with a second-degree polynomial than a power function, therefore the second-degree polynomial was used. The major advantage to using this detector is that it can be used to detect a wide range of non-UV absorbing compounds. There are downsides to this detection: it is a destructive technique, it requires a nitrogen carrier gas, and variation of response depends on mobile phase composition.

EXPERIMENTAL

Instrumentation.

The HPLC system that was used for the quantitative analysis was a Dionex Ultimate 3000 equipped with both a Dionex 4-channel UV detector and Varian evaporative light scattering detector (380-LC). The column used was a Phenomenex Kinetex (4.6 x 100mm, 2.6µm particle size, 100 Å, C18).

The isolation half of the project used the same HPLC system mentioned above for sample checks. A JEOL 300 MHz NMR was used to collect all of the carbon and proton spectra for the isolation of standards. A Perkin Elmer HPLC pump was used for both preparative and semi-preparative HPLC. The semi-preparative HPLC column that was used was a Varian Dynamax (250 x 10 mm, Microsorb 100-5, C-18) equipped with an Agilent Dynamax guard column (Microsorb 100-5, C-18). The preparative column is a Varian Dynamax (250 x 21.4 mm, Microsorb 100-5, C-18) equipped with a Varian guard column (1 inch, Microsorb 100-5, C-18). A Dionex Ultimate 3000 coupled with a ThermoFinnigan LTQ Linear Ion Trap mass spectrometer with an electrospray ionization source (ESI) was used to collect low resolution mass spectrometric data.

CHEMICALS

Chemicals used in this project are listed, along with the manufacturer, in Table 1.

Table 1: Chemicals Used During Analysis

Chemical	Manufacturer
HPLC Methanol	Pharmco-Aaper
HPLC Dichloromethane	Pharmco-Aaper
HPLC Chloroform	Pharmco-Aaper
d_5- Pyridine	Cambridge Isotope Laboratories Inc.
d-Chloroform	Acros Organics
Trifluoroacetic acid	Fisher Scientific
Formic Acid	Fisher Scientific
HPLC Hexanes	Fisher Scientific
HPLC Acetonitrile	Pharmco-Aaper
Nitrogen	Andy-oxy

PLANT PROPAGATION

The plants in this study were grown from wild-harvested rhizome material from twenty accessions of plants from 13 states (AR, DE, IN, KY, MD, MO, NC, NY, PA, SC, TN, VA, WV). The term “accession” refers to plants collected at a particular geographic location. The rhizome material was planted and allowed to grow at the BCGR for three years under identical environmental conditions with USDA barcode labels to maintain sample identity at BCGR. Once the plants matured, they were transplanted to 7’× 7’× 20’ controlled-pollination screen cages from Redwood Empire Awning Company. All pertinent information about the accessions is available from the Germplasm Resource Information Network (GRIN) database.[21] All of all the above work was performed by Joe-Ann McCoy and collaborators at BCGR.

RHIZOME PREPARATION

After collection, the rhizomes where pressure sprayed with water to ensure that the rhizomes were clean. After washing, the rhizomes were placed in a drawer with a screened bottom in a cabinet containing a dehumidifier to dry. The material was then cut with pruning shears into 3-4 cm pieces and separated into different populations before milling in a Thomas Wiley Mini-Mill. The ground material was weighed both before and after drying. The mill was cleaned after each plant sample was milled. The mill was vacuumed and blown out via an air compressor. The glass front was cleaned with alcohol to ensure no plant material was left. The above work was performed by Sara Pate at BCGR and WCU.

ANALYTICAL METHODS

SAPONINS

Method 1.

Method 1 was the first of two methods for detecting the triterpene saponins (**1**, **2, 3, 4, 5, 6**). The flow rate for method 1 was 1.0 ml/min and the injected amount of sample was 20 µl. The mobile phase is composed of a gradient of acetonitrile (ACN) and 0.1% formic acid (FA) aqueous (Time: 0 min, 30% ACN, 70% of 0.1% FA aqueous, 15 min, 40% ACN, 60% of 0.1% FA aqueous, 30 min, 60% ACN, 40% of 0.1% FA aqueous, 45 min, 30% ACN, 70 % of 0.1% FA aqueous). The ELSD is the means of detecting the triterpene glycosides (**1, 2)**. ELSD parameters: Nebulizer temperature 55^{o}C, Evaporator temperature 90^{o}C, PMT gain of 5 and gas flow of 1.60 SLM. The run time is 55 minutes. The column used is a Phenomenex Kinetex at a controlled temperature of 25 ^{o}C.

Method 1 was used primarily for the isolation of the standards. Method 1 served as a way to check that the desirable compounds was present in a sample and used to determine relative purity of the sample. The raw rhizome material, provided by BCGR was extracted and analyzed via method 1. The analysis showed that there were co-eluting peaks around the retention time of two of the compounds of interest. Method 2 was developed to resolve the co-eluting peaks. Method 2 is like the original method with a sharp initial gradient from 10% ACN to 30 % ACN over 10 minutes.

Method 2.

Method 2 was used for detecting the triterpene saponins (**1**, **2, 3, 4, 5, 6**). The flow rate for method 2 was 1.0 ml/min. The injected amount of sample was 20 µl. The mobile phase is a gradient of acetonitrile (ACN) and 0.1% formic acid aqueous (FA) (Time: 0 min, 10% ACN, 90% of 0.1% FA aqueous, 10 min, 30% ACN, 70% of 0.1% FA aqueous, 30 min, 40% ACN, 60% of 0.1% FA aqueous, 45 min, 60% ACN, 40% 0.1% FA aqueous, 46 min, 10 %ACN, 90% of 0.1% FA aqueous, 55 min, 10% ACN, 90% of 0.1% FA aqueous). The ELSD is the means of detecting the triterpene glycosides. ELSD parameters: Nebulizer temperature 55^{o}C, Evaporator temperature 110^{o}C, PMT gain of 5 and gas flow of 1.60 SLM. The run time is 55 minutes. The column used is a Phenomenex Kinetex at a controlled temperature of 25 ^{o}C.

Method 2 was used for the quantitative analysis part of this project. This method was developed because of a peak co-eluting peak with 23-*epi*-26-deoxyactein. Method 2 was needed due to extraction differences probably from solvent changes in comparison to the literature. The flow rate was increased to 1 ml/min from the original 0.5 ml/min. The major change to the method was the addition of the initial 10% ACN to 30% ACN. Lowering the starting acetonitrile concentration then ramping up to 30% acetonitrile over ten minutes separated the co-eluting peaks.

PHENOLIC ACIDS

The phenolic acids method was used for quantifying the aromatic acids (**7**, **8**, and **9**). The solvent flow rate is 1.0 ml/min. The mobile phase is composed of acetonitrile

(ACN) and 0.05% trifluoroacetic acid (TFA) in water. The solvent system for the phenolic acids compounds was a gradient (0 min, 5% ACN, 95% of 0.05% TFA aqueous, 5 min, 10% ACN, 90% of 0.05% TFA aqueous, 15 min, 30 % ACN, 70% of 0.05% TFA aqueous, 16 min, 100% ACN, 21 min, 100% ACN, 22 min, 5% ACN, 95% of 0.05% TFA aqueous, 32 min, 5% ACN, 95% TFA). The means of detection was the ultraviolet detector set at 254 nm. The run time of the method is 32 minutes and the injected amount is 10µl. The column used was a Phenomenex Kinetex.

The phenolic acids method, as in the saponin method, was also optimized.[12] Initially, peak shapes of the phenolic acids compounds were not sharp. The injection volume was cut to 10 µl and the peak shape sharpened. The first phenolic acids method was an isocratic method. Since the gradient method worked for the saponins a gradient method was implemented here. However, the use of a gradient method increased the analysis time but the resolution and sharpness of the peaks increased as well. In comparison to other published methods these methods gave the best resolution relative to other methods attempted.[12]

ANALYTICAL SAMPLE PREPARATION

The plant material samples were all individually wrapped and labeled, 54 in total. From each sample, 1.00 ± 0.01 g was placed into a 50 ml centrifuge tube. To the tube, 40 ml of a 1:1 ethanol:water (v/v) solution was added. The extraction mixture was allowed to shake on an orbital shaker for 24 hours. The sample was filtered through a Whatman 0.45µm PTFE filter and placed into a glass HPLC sample vial before analysis.

ANALYTICAL STANDARD PREPARATION

Calibration standards were needed to quantify the concentrations of the aromatic acids and the triterpene glycosides. Standard solutions were made up in varying concentrations of 10, 20, 40, 60, 80 and 100µg/ml. All of the standards were analyzed by both the saponin and phenolic acids methods. All of the standard solutions were analyzed in triplicate. The information obtained from all of the runs were placed into a excel spreadsheet where calibration equations and R^2 were calculated. The "y" in the polynomial equation refers to average peak area and "x" refers to concentration (ppm) area. All calibration information is listed in Table 2.

Table 2: Calibration Information of all of the Standards

Compound	Equation	R^2
Actein	$y = 0.003x^2 + 0.147x - 1.534$	0.9991
23-*epi*-26-Deoxyactein	$y = 0.005x^2 + 0.415x - 2.968$	0.9997
Cimiracemoside C	$y = 0.005x^2 + 0.058x - 0.340$	0.9997
Cimiracemoside A	$y = 0.010x^2 + 0.136x - 0.985$	0.9993
Deoxycimicifugoside	$y = 0.007x^2 + 0.353x - 6.869$	0.9998
Acetyl Shengmanol Xyloside	$y = 0.003x^2 + 0.083x - 1.438$	0.9990
Caffeic Acid	$y = 0.343x + 0.744$	0.9960
Ferulic Acid	$y = 0.396x – 2.147$	0.9991
Isoferulic Acid	$y = 0.410x – 3.222$	0.9997

RECOVERY ANALYSIS

From wild harvested black cohosh material from BCGR, (106A169A) 1.003 g of crude rhizome material was weighed out and placed into a 50 mL centrifuge tube. The rhizome material was extracted with 40 ml of a 1:1 ethanol:water (v/v) solution and sonicated for 30 minutes. After sonication, the extract was filtered and HPLC-UV-ELSD analysis was performed. The above procedure was repeated until no peaks were seen in the UV and ELSD chromatograms.

The exhaustively-extracted rhizome material was split into two samples for analysis. Half of the material (0.432 g) was used for a blank. The other 0.501 grams of rhizome material contained 1 ml of a working standard solution of 23-*epi*-26-deoxyactein (510 ppm) and ferulic acid (810 ppm). The rhizome material was dried under argon, re-extracted, and analyzed via HPLC-UV-ELSD by both the saponin and phenolic acid methods. Both of the samples were analyzed in triplicate.

LIMIT OF DETECTION AND QUANTIFICATION

The limit of detection was determined by triangulation of the lowest concentration to determine the area.[39] The triangulation formula is Area= ½ x Base x Height. The base is the mean peak width of the lowest concentration standard. The height is equal to the noise of a blank, which was determined by analysis of the blank in triplicate, at the specific time of the eluting standard multiplied by 3.[39] The noise level was calculated by the Chromeleon software. This area was used to calculate the limit of detection for each of the standards respectively[39]. The limit of quantification was calculated as mentioned above with the height equaling 10 x noise.

STATISTICS

To determine if there are any statistical differences in the concentrations of the saponins and phenolic acids between different accessions of plants, a single factor ANOVA analysis was performed. Before ANOVA analysis all the data for each compound by accession was converted to weight percentages and averaged in Microsoft Excel.

ISOLATION OF STANDARDS

Crude black cohosh rhizome material was collected and processed by Bent Creek Germplasm Repository. The rhizome powder (219.100 g) (106A066A) rhizome powder was extracted with 500 mL of a 1:1 dichloromethane:methanol (v/v) mixture for 24 hours at room temperature. This solution was filtered and 500 mL of methanol was added to the powder. The methanol extraction was sonicated for 30 minutes and the solution was dried under vacuum to yield 14.104 grams of extract (106A066C).

The crude extract was dissolved in 900 mL of 60% aqueous methanol, which was washed with 3 portions of hexanes (300 mL) to yield a hexane fraction (106A067B, 1.685 g). To the initial 60% methanol aqueous layer 180 mL of water was added to dilute the methanol concentration to 50%. The 50% methanol aqueous solution was then washed with 3 portions of chloroform (360 mL) to yield the chloroform fraction (106A067C, 5.464 g). The remaining 50% methanol aqueous solution was dried under vacuum to remove the methanol. The remaining water solution (540 mL) was washed with 3 portions of 1-butanol (180 mL) to yield a 1-butanol fraction (106A067E, 4.402 g). The water layer was freeze dried to yield a water fraction (106A067D, 2.448g).

ISOLATION OF CIMIRACEMOSIDE A

The chloroform fraction (106A067C) was fractionated further by an open C_{18} step gradient column. The solvent system for the column was 50%-100% methanol followed by 100% chloroform. The column was a glass filter frit column with dimensions 9.5 cm tall by 4.5 cm wide. The column was packed with 220 grams of C_{18} packing material.

The step gradient gave seven fractions (106A069A-G). After checking all samples by LCMS, fraction 106A069C (615.33 mg) was chosen for further purification.

To further purify fraction 106A069C (1.218g), a silica gel step gradient open column technique was used. The solvent system consisted of a step gradient using 19:1, 9:1, 4:1, 2:1 chloroform:methanol (v/v) mixtures followed by a 100% methanol flush (400 mL fractions). This step gradient gave 5 fractions (106A072A-E). After LCMS analysis, fraction 106A072B had the compound of interest. The next step in the isolation was an HP20 open column. The HP20 (80 grams) separation gave nine fractions (106A080A-I). The solvent system was a step gradient from 20%-100% methanol (330 mL each fraction). The column was 4.5 cm wide by 11 cm high and the sample was dry loaded. Dry loading refers to the sample absorbed to the column packing material before elution. The 106A080A-I series of fractions were analyzed by HPLC-ELSD. The analysis showed that fractions 106A080D-I were similar. The fractions were recombined (106A080J) for further purification.

Fraction 106A080J was purified by preparative HPLC. The peak of interest eluted in 20 minutes. The ELSD detector was used in the detection of cimiracemoside A, therefore a splitter was used to split the flow in two directions. The splitter setup was a three way splitter with a 2 cm piece of orange tubing (high flow) and a 20 cm piece of black tubing (low flow to detector). The solvent system was 30% acetonitrile and 70% 0.1% formic acid aqueous, the sample concentrations was 250 mg/mL, the flow rate was 10 mL/min, the injected volume was 25µL (total mass injected 320 mg), and the ELSD settings were nebulizer: 55°C, evaporator: 110°C, gas flow: 1.60 SLM, and the gain was set to 1. This preparative isolation was performed twice and gave 12 fractions

(106A088A-F and 106A090A-F). The purity of the sample was checked via the Phenomenex Kinetex analytical column on the Dionex HPLC, LCMS, carbon NMR, and proton NMR.

All of the fractions were analyzed by LCMS. Fraction 106A088A and 106A090A had the compound of interest. Fraction 106A090A was further purified by non-end capped C_{18} reverse phase semi-preparative HPLC to yield 8.15 mg of cimiracemoside A (106A113C) and 5.04 mg of cimiracemoside F (106A113B).[35] The solvent system was an isocratic 37% acetonitrile 63% of a 0.1% formic acid aqueous, the flow rate was 2 mL/min, the sample concentrations was 14 mg/mL, the injected volume was 2µL (total mass injected 29.61mg), and the ELSD settings were nebulizer: 55°C, evaporator: 110°C, gas flow: 1.60 SLM, and the gain was set to 5.

Fraction 106A088A was further purified by C_{18} reverse phase preparative HPLC. The solvent system was 37% acetonitrile 63% 0.1% formic acid aqueous, the sample concentrations was 14 mg/mL, the flow rate was 10 mL/min, the injected volume was 25µL (total mass injected 44.06 mg), and the ELSD settings were nebulizer: 55°C, evaporator: 110°C, gas flow: 1.60 SLM, and the gain was set to 5. Preparative HPLC gave 1.93 mg of cimiracemoside A. The purity of the same was checked via the analytical column on the Dionex HPLC, LCMS, carbon NMR, and proton NMR.

Additional cimiracemoside A was purified from fraction 106A069A (504.97 mg). To further purify 106A069A a silica gel step gradient open column technique was used. The solvent system for this column was a 19:1, 9:1, 4:1, 2:1 chloroform: methanol mixture followed by a 100% methanol flush (192 mL each fraction). This step gradient gave 5 fractions (106A104A-E). After LCMS analysis, fraction 106A104B had the

compound of interest. Fraction, 106A104B, was further purified by C_{18} reverse phase preparative HPLC. The solvent system was 40% acetonitrile 60% 0.1% formic acid aqueous, the sample concentrations was 250 mg/mL, the flow rate was 10 mL/min, the injected volume was 25µL (total mass injected 76.94 mg), and the ELSD settings were nebulizer: 55°C, evaporator: 110°C, gas flow: 1.60 SLM, and the gain was set to 1. Preparative HPLC separation was preformed twice to give nine fractions (106A106A-D and 106A107A-D).

Fractions 106A106C and 106A107C were combined for semi-preparative HPLC purification (106A107E). This sample was further purified by non-end capped C_{18} reverse phase semi-preparative HPLC to yield 1.48 mg of cimiracemoside A (106A108C). The solvent system was an isocratic 37% acetonitrile 63% 0.1% formic acid aqueous, the flow rate is 2 mL/min, the sample concentrations was 14 mg/mL, the injected volume was 2µL (total mass injected 6.39 mg), and the ELSD settings were nebulizer: 55°C, evaporator: 110°C, gas flow: 1.60 SLM, and the gain was set to 5.

ISOLATION OF CIMIRACEMOSIDE C AND CIMIGENOL XYLOSIDE

To further purify fraction 106A069D (708.91 mg), a silica gel (35 grams) step gradient open column technique was used. The solvent system for this column was a step gradient using 19:1, 9:1, 4:1, 2:1 chloroform:methanol (v/v) mixture followed by a 100% methanol flush (380 mL fractions). The column dimensions are 3.5 cm wide by 12.5 cm high. This step gradient gave 5 fractions (106A111A-E). After LCMS analysis, fraction 106A111B had the compounds of interest. Fraction106A111B was further purified by C_{18} reverse phase preparative HPLC. The solvent system was 35% acetonitrile 65% 0.1%

formic acid aqueous, the sample concentration was 250 mg/mL, the flow rate was 10 mL/min, the injected volume was 25µL (total mass injected 376.27 mg) , and the ELSD settings were nebulizer: 55°C, evaporator: 110°C, gas flow: 1.60 SLM, and the gain was set to 1. The separation gave three fractions (106A114A-C).

Fraction 106A114A (197.59 mg) was further purified again by C_{18} reverse phase preparative HPLC. The solvent system was 40% acetonitrile 60% of 0.1% formic acid in water, the sample concentrations was 250 mg/mL, the flow rate was 10 mL/min, the injected volume was 25µL (total mass injected 197.59 mg), and the ELSD settings were nebulizer: 55°C, evaporator: 110°C, gas flow: 1.60 SLM, and the gain was set to 1. The separation gave two fractions (106A117A-C). Fraction 106A117C (10 mg) was confirmed to be cimiracemoside C, and fraction 106A117D (9.62 mg) was confirmed to be cimigenol xyloside by LCMS, proton NMR, and by carbon NMR.

ISOLATION OF ACETYL SHENGMANOL XYLOSIDE

To further purify fraction 106A069B (615.33 mg), a silica gel step gradient open column technique was used. The solvent system for this column was a step gradient using 19:1, 9:1, 4:1, 2:1 chloroform:methanol (v/v) mixture followed by a 100% methanol flush (240 mL fractions). This step gradient gave 5 fractions (106A092A-E). After LCMS analysis, fraction 106A092B had the compound of interest. Fraction 106A092B, was further purified by C_{18} reverse phase preparative HPLC. The solvent system was 35% acetonitrile 65% 0.1% formic acid in water, the sample concentrations was 250 mg/mL, the flow rate was 10 mL/min, the injected volume was 25µL (total mass injected 170.96 mg), and the ELSD settings were nebulizer: 55°C, evaporator: 110°C, gas flow: 1.60

SLM, and the gain was set to 1. The prep-HPLC separation gave two fractions (106A093A-B). Fraction 106A093B (13.12 mg) was confirmed to be acetyl shengmanol xyloside by LCMS, proton NMR, and by carbon NMR.

ISOLATION: RESULTS AND DISCUSSION

CIMIRACEMOSIDE A

Cimiracemoside A gave a base peak signal of 659.18 m/z ([M+H-H_2O]) in positive mode low resolution ESI-MS. To further confirm the identity of cimiracemoside A both proton and carbon NMR techniques were used. Since the compound is not soluble in chloroform, all of the NMR analysis was performed using d_5 pyridine as the solvent. Table 5 is the tabulated data for the NMR analysis in comparison to published data for the compound. The carbon spectrum showed the expected signals. The spectrum also has the acetate ester signal at δ 170.95 ppm and the double bond signals at δ 148.00 and δ 114.21 ppm. The data also shows signals for the oxygenated carbons between δ 67-88 ppm. All experimental carbon NMR data corresponds well to that found in the literature. Those results are found in Table 3.

CIMIRACEMOSIDE C

Cimiracemoside C showed after positive mode ESI-MS analysis a peak at 621.36 m/z which is the molecule plus hydrogen. The mass to charge ratio matches that of the Shao data for cimiracemoside C. Carbon NMR analysis reveals a good correlation between the experimental data and the published data. The carbon data suggest that there are oxygenated carbons with the signals between δ59.3-90 ppm. The only double bond in the compound shows up at δ 107.82 ppm and δ 112.35 ppm, which is agrees with the

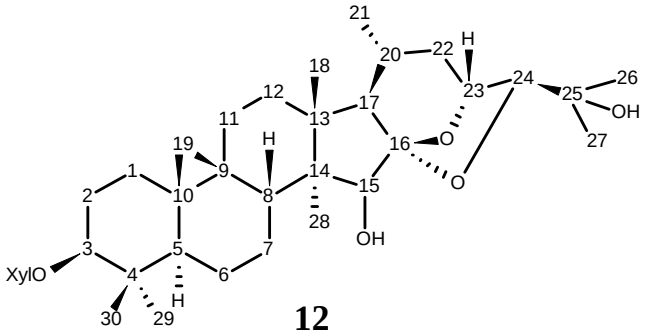
Shao data. All experimental carbon NMR data corresponds well to that found in the literature. Those results are found in Table 3.

Table 3: Carbon NMR data for Cimiracemoside A and Cimiracemoside C

	3			**4**	
Position	δ_C [a] (Shao)	δ_C [a] (exp)	Position	δ_C [a] (Shao)	δ_C [a] (exp)
1	30.50	30.50	1	32.70	32.80
2	29.80	29.84	2	30.30	30.44
3	88.20	88.21	3	88.90	88.96
4	40.70	40.75	4	41.60	41.71
5	42.70	42.78	5	47.90	47.96
6	22.10	22.10	6	21.40	24.46
7	114.20	114.21	7	26.60	26.81
8	148.00	148.00	8	48.90	48.99
9	21.50	21.95	9	20.30	21.44
10	28.60	28.59	10	26.90	27.57
11	37.00	37.04	11	26.70	27.03
12	77.00	77.01	12	34.40	34.44
13	49.10	49.09	13	42.10	42.23
14	51.30	51.31	14	47.60	47.66
15	42.30	42.29	15	80.50	80.58
16	72.60	72.60	16	112.20	112.34
17	53.60	53.31	17	59.80	59.93
18	15.40	15.38	18	19.90	20.37
19	29.00	29.03	19	31.20	31.24
20	34.60	34.58	20	24.40	25.78
21	17.90	17.89	21	19.80	19.95
22	87.00	87.01	22	38.40	38.52
23	106.00	106.05	23	72.10	72.19
24	83.60	83.63	24	90.40	90.55
25	84.00	83.99	25	71.20	71.31
26	28.30	28.32	26	25.70	26.11
27	25.20	25.25	27	26.00	26.72
28	27.00	27.04	28	12.10	12.17
29	26.00	26.06	29	27.50	30.34
30	14.50	14.58	30	15.70	19.88
1’	107.80	107.83	1’	107.70	107.82
2’	75.90	75.98	2’	73.20	73.32
3’	79.00	79.01	3’	74.90	75.01
4’	71.60	71.60	4’	69.70	69.88
5’	67.50	67.49	5’	67.00	67.10

CIMIGENOL XYLOSIDE

Cimigenol xyloside (**12)** gave in ESI-MS a signal at 621.13 m/z ([M+H]). The mass spectrum also appears to show sodium adduct at 643.47 ([M+23]). The experimental carbon NMR data shows 35 total carbons with 9 oxygenated carbons (δ 67 ppm- δ 90.5 ppm) which match the Jamroz data. All experimental carbon NMR data corresponds well to that found in the literature. Those results are found in Table 4.



12

ACETYL SHENGMANOL XYLOSIDE

ESI-MS analysis of acetyl shengmanol xyloside revealed a peak at 645.45 m/z which correlates to the molecule losing a water molecule ([M+H-H_2O]). The loss of water in ESI-MS is commonly observed with these types of compounds.[38] The experimental carbon NMR data shows 37 carbons, agrees with Kusano's data. The experimental carbon data suggest that there are 2 carbonyl carbons (δ 170.98 ppm & δ 220.34 ppm) and 8 oxygenated carbons all together, which match the Kusano reference. All experimental carbon NMR data corresponds well to that found in the literature. Those results are found in Table 4.

Table 4: Cimigenol Xyloside and Acetyl Shengmanol Xyloside Carbon data

12			**5**		
Position	δ_C[a (Jamroz)]	δ_C[a (exp)]	Position	δ_C[a (Kusano)]	δ_C[a (exp)]
1	32.70	32.82	1	32.20	32.60
2	30.30	30.50	2	30.00	30.45
3	88.90	88.60	3	88.40	88.77
4	41.60	41.75	4	41.30	41.70
5	47.90	48.00	5	47.50	47.83
6	21.40	21.45	6	21.00	21.32
7	26.60	26.76	7	26.70	27.14
8	48.90	49.02	8	48.20	48.61
9	20.30	20.40	9	20.10	20.45
10	26.90	27.06	10	26.80	28.31
11	26.70	26.80	11	26.00	27.04
12	34.40	34.45	12	33.00	33.40
13	42.10	42.26	13	41.50	41.90
14	47.60	47.68	14	46.10	46.45
15	80.50	80.60	15	82.90	83.29
16	112.20	112.37	16	219.58	220.34
17	59.80	59.96	17	60.00	60.31
18	19.90	19.96	18	19.80	20.15
19	31.20	31.27	19	30.50	30.84
20	24.40	24.48	20	28.00	30.33
21	19.80	19.90	21	20.30	20.68
22	38.40	38.55	22	37.00	37.33
23	72.10	72.23	23	72.10	72.45
24	90.40	90.50	24	65.20	65.51
25	71.20	71.64	25	58.50	58.89
26	25.70	25.80	26	24.70	26.03
27	26.00	26.12	27	19.30	19.69
28	12.10	12.20	28	12.00	12.33
29	27.50	27.58	29	25.70	26.33
30	15.70	15.83	30	15.40	15.80
1'	107.70	107.98	$\underline{CO}CH_3$	170.50	170.98
2'	73.20	75.98	$CO\underline{CH_3}$	21.53	25.05
3'	74.90	79.01	1’	107.40	107.93
4'	69.70	71.34	2’	74.60	78.98
5'	67.00	67.52	3’	72.90	75.93
			4’	69.50	71.61
			5'	65.70	67.48

QUANTITATIVE ANALYSIS

The main purpose of the quantitative analysis was to identify desirable black cohosh plant accessions for cultivation. A desirable plant is one that has the highest concentrations of the triterpene saponins. All 54 samples were analyzed via both the saponin and phenolic acid methods. The extraction procedure was the same for all of the samples. The samples were analyzed in triplicate. The peak areas were transferred to Microsoft Excel for data analysis. In Excel all the data was converted to weight percent and averaged.

RECOVERY ANALYSIS

The two sets of recovery samples were analyzed via HPLC-UV-ELSD by both the saponin and phenolic acids methods. Both of the samples were analyzed in triplicate. The average recovery that was observed for 23-*epi*-26-deoxyactein was 98.79%, and for ferulic acid the recovery was 91.06%. This data shows that the extraction method and the analysis methods are accurately measuring saponin and phenolic acid concentrations.

LIMIT OF DETECTION AND QUANTIFICATION

The limit of detection and quantification for each of the standards are shown in table 8 below.

Table 5: Limit of Detection & Quantification

Compound	LOD (ng)	LOQ (ng)
Ferulic Acid	109.00	109.40
Isoferulic Acid	158.60	162.20
Caffeic Acid	43.60	44.40
Actein	237.40	349.20
23-*epi*-26-Deoxyactein	171.60	254.20
Cimiracemoside A	130.00	182.00
Cimiracemoside C	168.80	302.00
Acetyl Shengmanol Xyloside	280.60	319.80
Deoxycimicifugoside	321.40	351.00

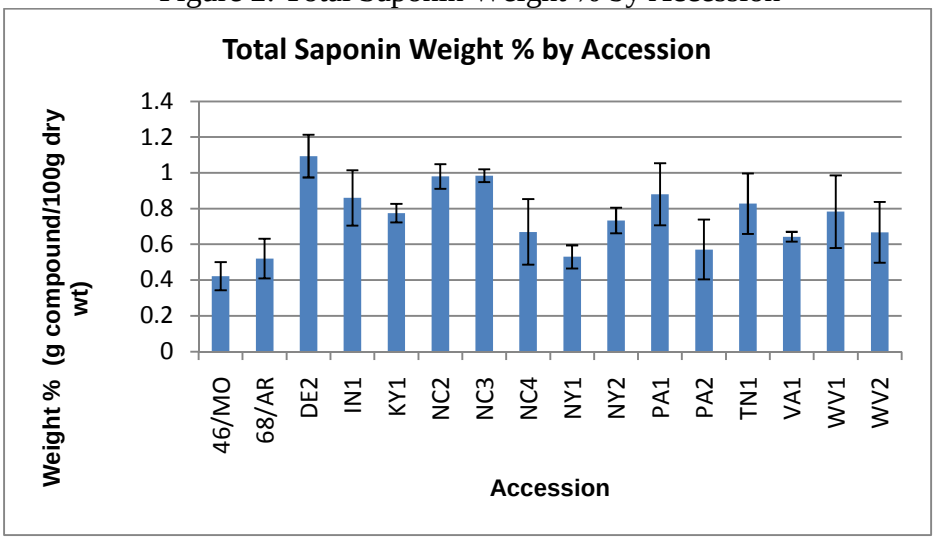
ANOVA ANALYSIS

The null hypothesis for the ANOVA was that there will not be chemical differences between plant accessions. A significant difference occurs when $F > F_{crit}$ with a p-value less than 0.05. If a significant difference is observed, then the data will be further investigated. If no difference is seen then no further action is required because the data for the compounds concentrations are similar. For the analysis, all of the data was organized by compound, total measured saponin, total measured phenolic acids, and actein plus 23-*epi*-26-deoxyactein. The ANOVA analysis rejected the null hypothesis for the total saponins and failed to reject the null hypothesis for the total phenolic acids. The ANOVA data only includes the 16 accessions that contained 3 rhizome samples that were randomly collected for conformity.

TOTAL SAPONIN

The total saponin ANOVA was performed on the sum of all the saponins measured for each of the 16 accessions. This was done for each accession and is presented in Figure 2. The ANOVA analysis showed that among accessions, the total saponin concentrations are significantly different. The F value (6.34) was higher than F_{crit} (1.99) with a p value of 6.16E-06. The total saponin ANOVA revealed that the null hypothesis was rejected. The error bars in Figure 2 represent +/- one standard deviation.

Figure 2: Total Saponin Weight % by Accession

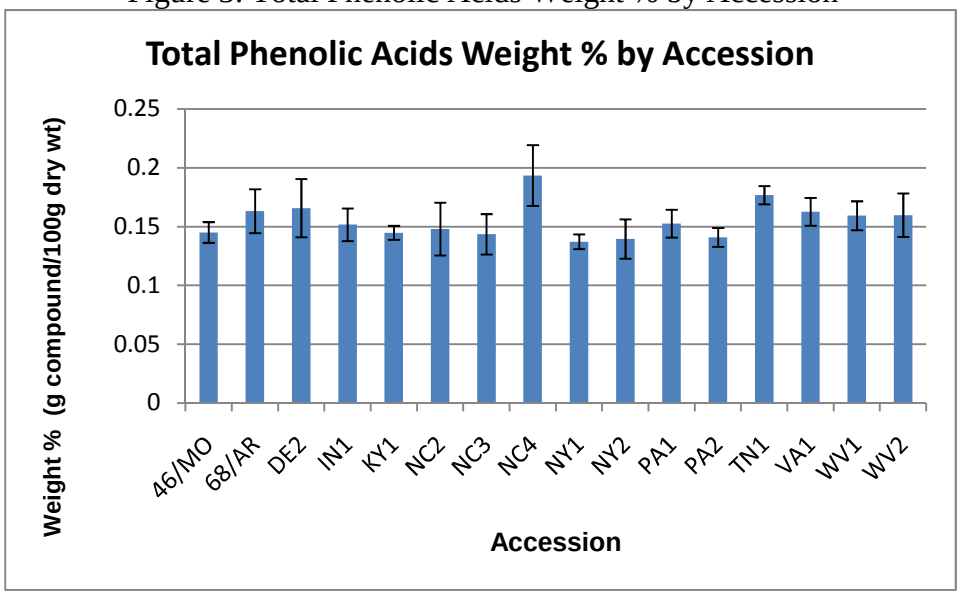


TOTAL PHENOLIC ACIDS

The ANOVA analysis showed that among the accessions, total phenolic acid concentrations are significantly different. The F value (2.7551) was greater than F_{crit} (1.992) with a p value of 0.0079. The total phenolic acids ANOVA revealed that the null hypothesis was rejected. However, leaving out accession NC4 and performing another ANOVA analysis revealed that there is not a significant difference between the total

phenolic acids. The ANOVA results show F value (1.8054) was less than F_{crit} (2.0374) with a p value of 0.0855. NC4 has the highest weight percent of the phenolic acids as compared to the other accessions. If the NC4 accession is taken out of the ANOVA analysis then the concentrations are similar between accessions. The error bars in Figure 3 represent +/- one standard deviation.

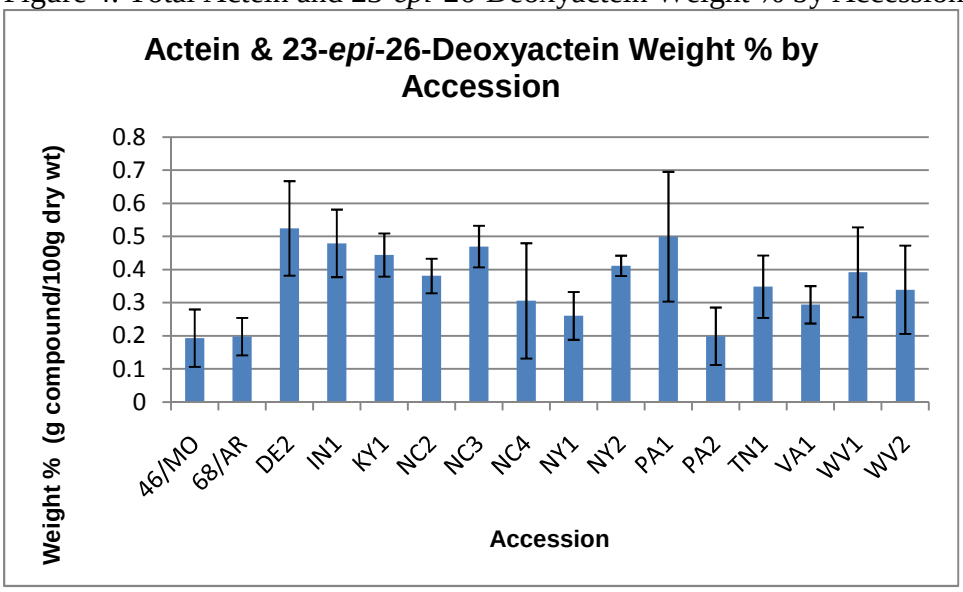
Figure 3: Total Phenolic Acids Weight % by Accession



ACTEIN AND 23-*EPI*-26-DEOXYACTEIN

According to Gaia Herbs,[36] the triterpene saponins actein and 23-*epi*-26-deoxyactein are used to standardize black cohosh preparations. Table 13, in the appendix, is the combined data for actein and 23-*epi*-26-deoxyactein. The ANOVA analysis showed that for actein and 23-*epi*-26-deoxyactein there are significant differences across the accessions. The F value (3.177) was higher than F_{crit} (1.99) with a p value of 0.0029. The Actein and 23-*epi*-26-deoxyactein ANOVA revealed that the null hypothesis was rejected. The error bars in Figure 4 represent +/- one standard deviation.

Figure 4: Total Actein and 23-*epi*-26-Deoxyactein Weight % by Accession



Since industry uses actein and 23-*epi*-26- deoxyactein concentrations to standardize black cohosh products, an accession with the highest levels of actein and 23-*epi*-26-deoxactein would be desirable for producing a cultivar. DE2 has the highest total concentration of actein and 23-*epi*-26-deoxyactein. After ANOVA analysis of a subset (Table 33 in appendix) of accessions DE2, IN1, PA1, NY2, NC3, KY1, it is apparent that there are no differences. Statistically speaking there is no superior accession with high concentrations of actein and 23-*epi*-26-deoxyactein. Figure 4 shows all of the data for both actein and 23-*epi*-26-deoxyactein as weight percent's and the DE2 plants have the highest percent, by weight, of both compounds on average. The error bars in Figure 4 represent +/- one standard deviation.

This is the first study to present data looking at both the triterpene saponins and the phenolic acids concentrations for plant accessions in hopes to find a superior accession or accessions for potential of developing a region cultivar. The Al-Amier reference only refers to a small collection of plants from only five states.[20] They only

reported data for the phenolic acids (ferulic, isoferulic, caffeic acids). There HPLC method was not optimized for the phenolic acid compounds. Instead of having an 80 minute method they could have modified it to 32 or even 13 minute analysis. The novel aspect of this research is that no one has ever studied a large collection of black cohosh grown under identical conditions.

CONCLUSION

The purpose of this study was to run evaporative light scattering detection analysis of plants from 20 accessions of black cohosh to quantify the compounds with desirable biological activities in hopes of producing a superior hybrid plant. The hypothesis that there will be phytochemical differences between accessions was confirmed. Although there are significant differences across the collection, there are no significant differences in a subset of the collection (IN1, PA1, NY2, NC3, KY1, DE2) in terms of actein and 23-*epi*-26-deoxyactein. The NC4 accession has the highest weight percent of the phenolic acids. Growth characteristics and phytochemical concentrations will be used to identify candidates for breeding studies. The breeding studies have the potential to produce a hybrid plants that could produce higher concentrations of compounds which then could be grown by local farmers. The plants grown by local farmers could be harvested and sold to stimulate the local economy. Hybrid cultivars of black cohosh could dramatically change the use of black cohosh in the clinic. These plants with higher concentrations of the saponins could lead to a more potent and effective supplement.

FUTURE STUDIES

Further studies will include developing a regional cultivar of the superior black cohosh plants in Western North Carolina. Screening of black cohosh extracts for their bioactivity is currently being conducted. A future goal is to isolate and purify desirable compounds for industry for analytical reference standards. Future studies also involve isolating novel biologically active compounds from black cohosh. Once a desirable accession has been identified based on growth studies and phytochemical concentrations Bent Creek Germplasm Repository plans to implement a breeding program to make a hybrid plant that maintains the desirable properties.

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APPENDIX

Table 6: Total Actein Content for Each Accession

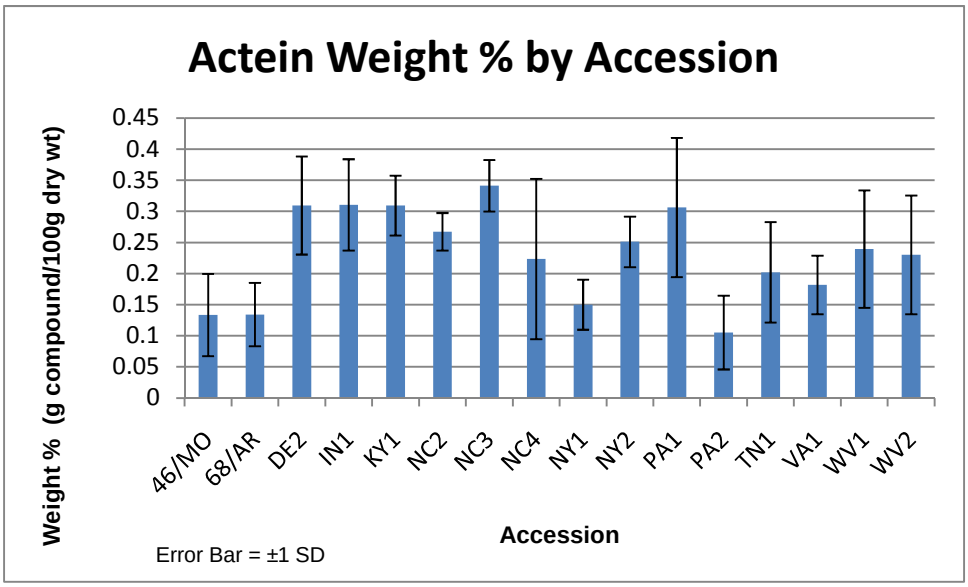


Table 7: Actein Raw Values (Weight %)

Accession	1st	2nd	3rd	Average	Standard Deviation
TN1	0.243	0.109	0.254	0.202	0.080
46/MO	0.121	0.204	0.074	0.133	0.066
VA1	0.174	0.138	0.232	0.181	0.047
PA2	0.066	0.173	0.075	0.105	0.059
68/AR	0.105	0.192	0.104	0.134	0.050
DE2	0.341	0.367	0.219	0.309	0.078
NY1	0.191	0.111	0.147	0.150	0.040
NY2	0.284	0.263	0.205	0.251	0.040
WV1	0.207	0.164	0.345	0.239	0.094
NC2	0.294	0.234	0.273	0.267	0.030
KY1	0.265	0.360	0.301	0.309	0.048
IN1	0.318	0.233	0.379	0.310	0.073
NC3	0.371	0.294	0.358	0.341	0.041
WV2	0.140	0.330	0.219	0.230	0.095
PA1	0.313	0.191	0.414	0.306	0.111
NC4	0.156	0.372	0.141	0.223	0.128

Table 8: ANOVA Analysis Data for Actein

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.248	15	0.017	3.073	3.73E-03	1.991
Within Groups	0.172	32	5.38E-03			
Total	0.420	47				

Table 9: Total 23-epi-26-Deoxyactein Content for Each Accession

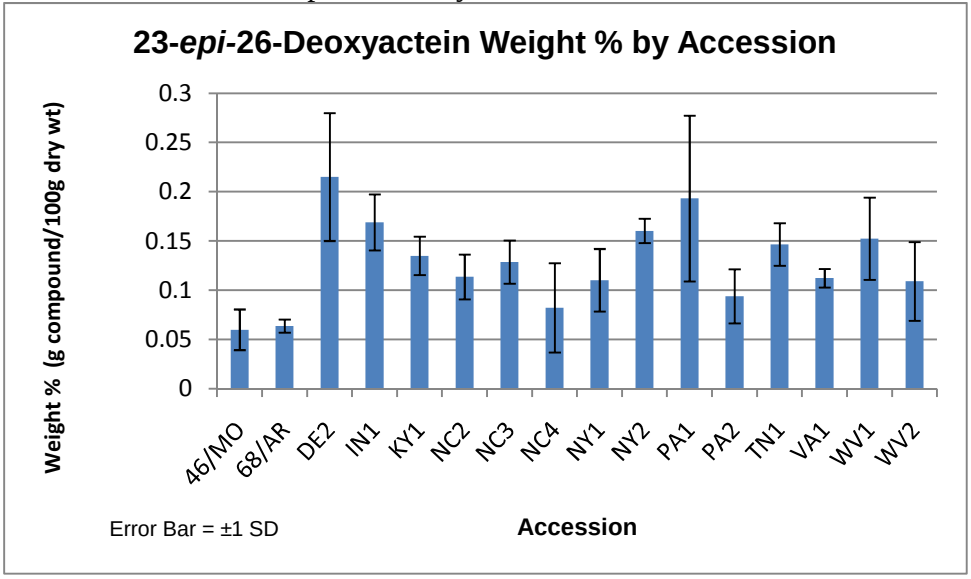


Table 10: 23-epi-26-Deoxyactein Raw Values (Weight %)

Acession	1st	2nd	3rd	Average	Standard Deviation
TN1	0.133	0.134	0.171	0.146	0.021
46/MO	0.057	0.081	0.040	0.059	0.020
VA1	0.109	0.104	0.122	0.112	0.009
PA2	0.080	0.125	0.075	0.093	0.027
68/AR	0.064	0.070	0.056	0.063	0.006
DE2	0.256	0.248	0.140	0.215	0.064
NY1	0.143	0.080	0.106	0.110	0.031
NY2	0.156	0.149	0.173	0.160	0.012
WV1	0.129	0.127	0.200	0.152	0.041
NC2	0.127	0.087	0.125	0.113	0.022
KY1	0.128	0.156	0.119	0.134	0.019
IN1	0.174	0.137	0.193	0.168	0.028
NC3	0.138	0.103	0.144	0.128	0.021
WV2	0.083	0.155	0.088	0.109	0.039
PA1	0.197	0.107	0.275	0.193	0.084
NC4	0.060	0.134	0.052	0.082	0.045

Table 11: ANOVA Analysis Data for 23-epi-26-Deoxyactein

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.086	15	5.73E-03	4.207	3.15E-04	1.991
Within Groups	0.043	32	1.36E-03			
Total	0.129	47				

Table 12: Total Cimiracemoside A Content for Each Accession

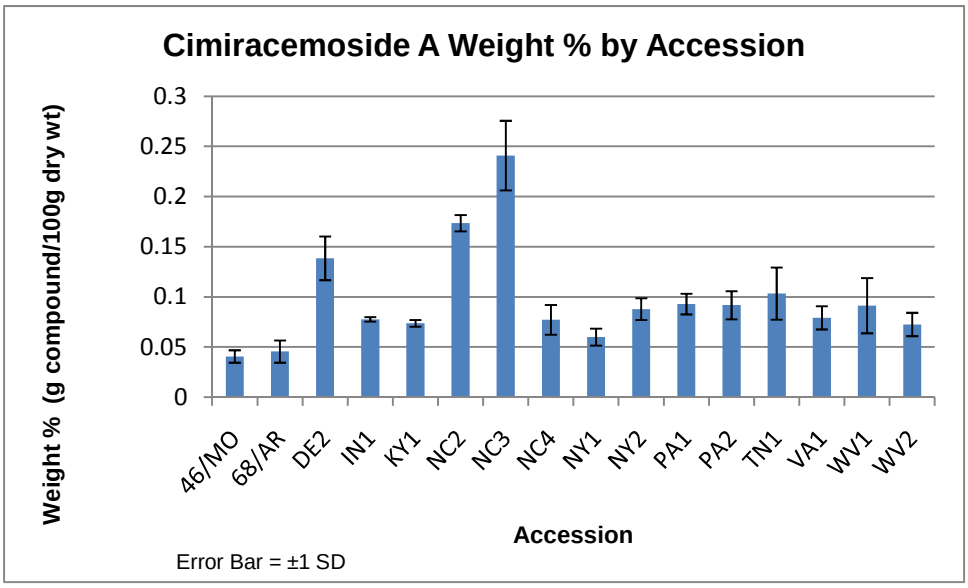


Table 13: Cimiracemoside A Raw Values (Weight %)

Accession	1st	2nd	3rd	Average	Standard Deviation
TN1	0.122	0.073	0.114	0.103	0.026
46/MO	0.038	0.047	0.036	0.040	0.006
VA1	0.073	0.071	0.092	0.079	0.011
PA2	0.099	0.100	0.075	0.091	0.013
68/AR	0.040	0.037	0.058	0.045	0.011
DE2	0.118	0.161	0.135	0.138	0.021
NY1	0.050	0.065	0.064	0.059	0.009
NY2	0.077	0.086	0.099	0.087	0.010
WV1	0.064	0.119	0.090	0.091	0.027
NC2	0.180	0.176	0.164	0.173	0.008
KY1	0.073	0.070	0.077	0.073	0.003
IN1	0.079	0.077	0.075	0.077	0.002
NC3	0.280	0.218	0.222	0.240	0.034
WV2	0.062	0.069	0.085	0.072	0.011
PA1	0.090	0.084	0.104	0.092	0.010
NC4	0.086	0.085	0.060	0.077	0.014

Table 14: ANOVA Analysis Data for Cimiracemoside A

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.112	15	7.50E-03	27.941	3.59E-14	1.991
Within Groups	0.008	32	2.69E-04			
Total	0.121	47				

Table 15: Total Deoxycimicifugoside Content for Each Accession

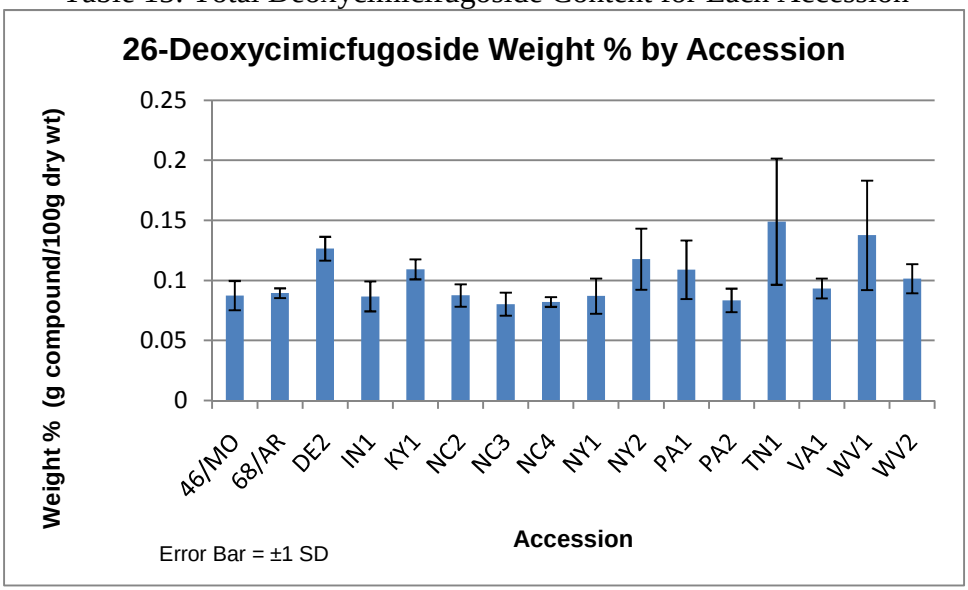


Table 16: 26-Deoxycimicifugoside Raw Values (Weight %)

Accession	1st	2nd	3rd	Average	Standard Deviation
TN1	0.146	0.097	0.202	0.148	0.052
46/MO	0.096	0.092	0.073	0.087	0.012
VA1	0.100	0.095	0.084	0.093	0.008
PA2	0.093	0.074	0.082	0.083	0.009
68/AR	0.087	0.086	0.094	0.089	0.004
DE2	0.130	0.133	0.115	0.126	0.009
NY1	0.103	0.079	0.077	0.087	0.010
NY2	0.142	0.118	0.091	0.117	0.025
WV1	0.092	0.137	0.183	0.137	0.045
NC2	0.097	0.078	0.086	0.087	0.009
KY1	0.099	0.112	0.115	0.109	0.008
IN1	0.075	0.084	0.099	0.086	0.012
NC3	0.087	0.069	0.083	0.080	0.009
WV2	0.093	0.095	0.115	0.101	0.012
PA1	0.122	0.080	0.123	0.108	0.024
NC4	0.083	0.085	0.077	0.082	0.004

Table 17: ANOVA Analysis Data for 26-Deoxycimicifugoside

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.020	15	1.34E-03	2.960	0.005	1.991
Within Groups	0.014	32	4.55E-05			
Total	0.034	47				

Table 18: Total Acetyl Shengmanol Xyloside Content for Each Accession

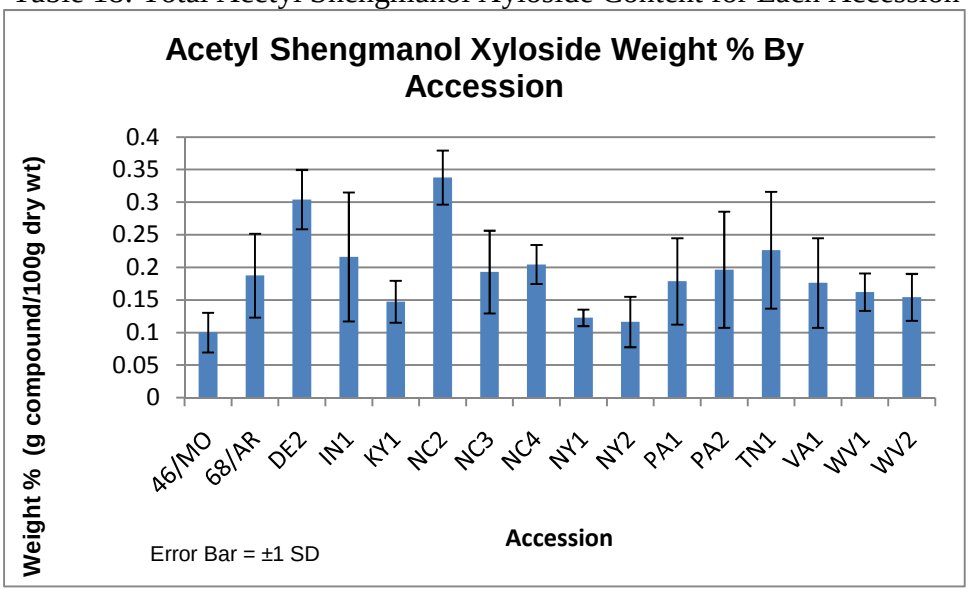


Table 19: Acetyl Shengmanol Xyloside Raw Values (Weight %)

Accession	1st	2nd	3rd	Average	Standard Deviation
TN1	0.317	0.222	0.138	0.226	0.089
46/MO	0.081	0.083	0.135	0.100	0.030
VA1	0.216	0.215	0.096	0.176	0.068
PA2	0.111	0.289	0.189	0.196	0.089
68/AR	0.128	0.255	0.178	0.187	0.064
DE2	0.266	0.291	0.354	0.304	0.045
NY1	0.110	0.135	0.121	0.122	0.012
NY2	0.112	0.156	0.079	0.116	0.038
WV1	0.146	0.144	0.195	0.162	0.028
NC2	0.304	0.325	0.384	0.337	0.041
KY1	0.148	0.114	0.178	0.147	0.032
IN1	0.329	0.150	0.167	0.216	0.098
NC3	0.143	0.264	0.170	0.192	0.063
WV2	0.125	0.194	0.141	0.154	0.036
PA1	0.210	0.223	0.102	0.178	0.066
NC4	0.174	0.205	0.234	0.204	0.030

Table 20: ANOVA Analysis Data for Acetyl Shengmanol Xyloside

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.178	15	1.18E-01	3.554	0.001	1.991
Within Groups	0.106	32	3.34E-03			
Total	0.285	47				

Table 21: Total Caffeic Acid Content for Each Accession

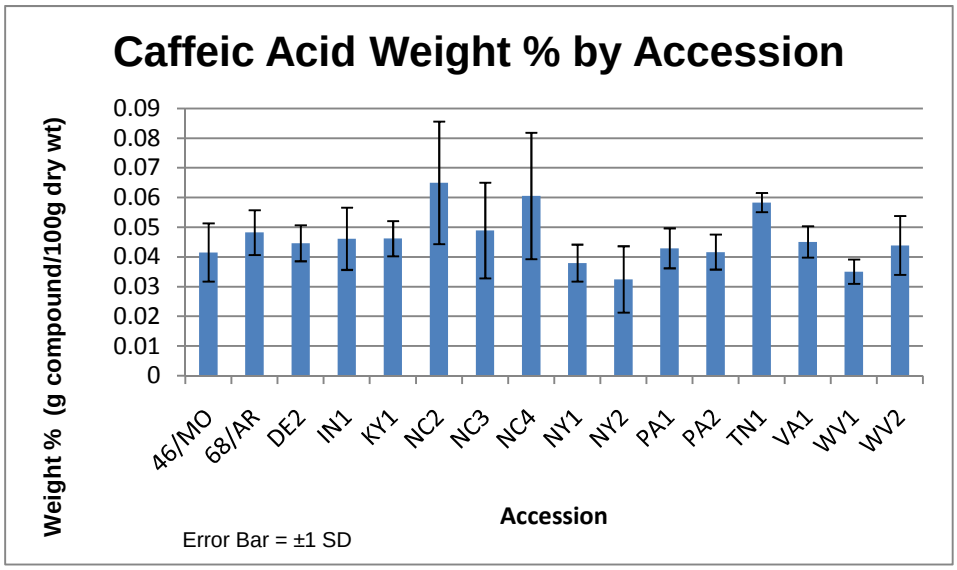


Table 22: Caffeic Acid Raw Values (Weight %)

Accession	1st	2nd	3rd	Average	Standard Deviation
TN1	0.061	0.057	0.055	0.058	0.003
46/MO	0.037	0.052	0.034	0.041	0.009
VA1	0.044	0.050	0.039	0.045	0.005
PA2	0.036	0.047	0.040	0.041	0.005
68/AR	0.045	0.042	0.056	0.048	0.007
DE2	0.044	0.050	0.038	0.044	0.006
NY1	0.032	0.036	0.044	0.037	0.006
NY2	0.022	0.044	0.030	0.032	0.011
WV1	0.033	0.039	0.031	0.035	0.004
NC2	0.088	0.055	0.050	0.064	0.020
KY1	0.039	0.040	0.049	0.046	0.005
IN1	0.040	0.058	0.039	0.046	0.010
NC3	0.037	0.041	0.067	0.048	0.016
WV2	0.032	0.052	0.046	0.043	0.009
PA1	0.050	0.040	0.037	0.042	0.006
NC4	0.055	0.042	0.083	0.060	0.021

Table 23: ANOVA Analysis Data for Caffeic Acid

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.003	15	2.32E-04	1.985	0.050	1.991
Within Groups	0.004	32	1.17E-04			
Total	0.007	47				

Table 24: Total Ferulic Acid Content for Each Accession

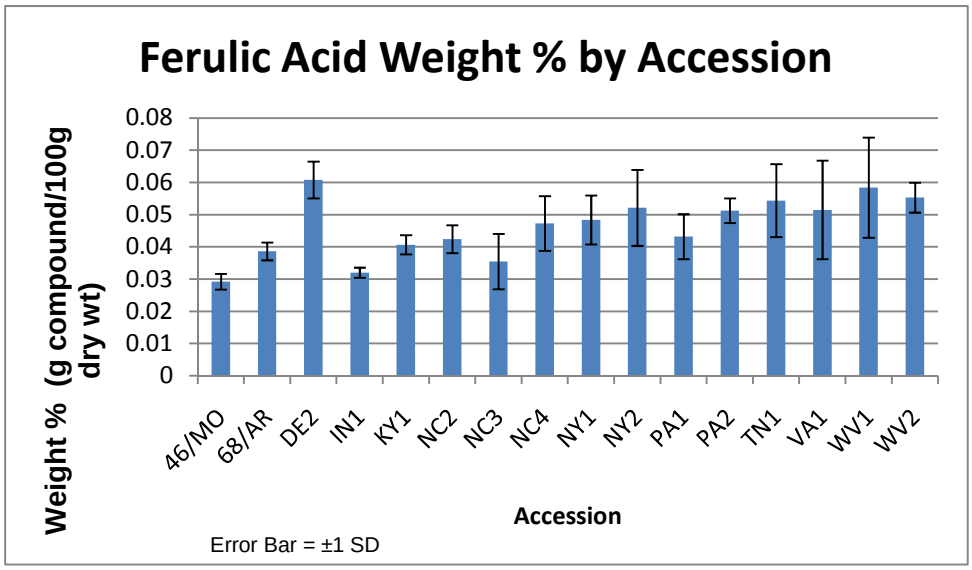


Table 25: Ferulic Acid Raw Values (Weight %)

Accession	1st	2nd	3rd	Average	Standard Deviation
TN1	0.052	0.066	0.043	0.054	0.011
46/MO	0.031	0.029	0.026	0.029	0.002
VA1	0.069	0.044	0.041	0.051	0.015
PA2	0.054	0.047	0.052	0.051	0.003
68/AR	0.041	0.037	0.036	0.038	0.002
DE2	0.054	0.065	0.062	0.060	0.005
NY1	0.039	0.054	0.050	0.048	0.007
NY2	0.064	0.051	0.040	0.052	0.011
WV1	0.041	0.062	0.071	0.058	0.015
NC2	0.047	0.041	0.038	0.042	0.004
KY1	0.043	0.040	0.037	0.040	0.002
IN1	0.030	0.031	0.033	0.031	0.001
NC3	0.030	0.030	0.045	0.035	0.008
WV2	0.058	0.057	0.049	0.055	0.004
PA1	0.041	0.037	0.050	0.043	0.006
NC4	0.041	0.056	0.043	0.047	0.008

Table 26: ANOVA Analysis Data for Ferulic Acid

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.004	15	2.65E-04	3.822	7.06E04	1.991
Within Groups	0.002	32	6.92E-05			
Total	0.006	47				

Table 27: Total Isoferulic Acid Content for Each Accession

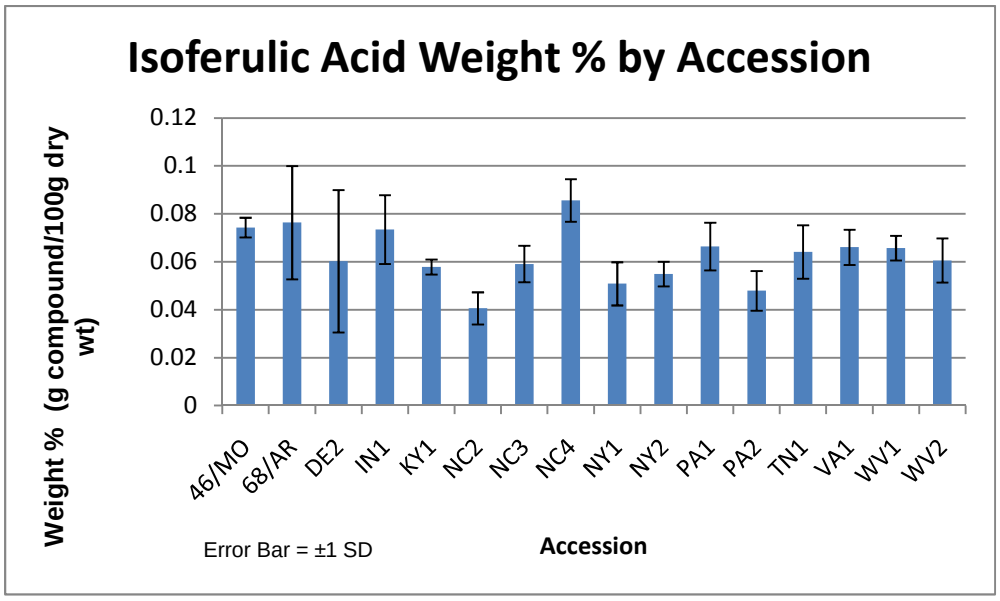


Table 28: Isoferulic Acid Raw Values (Weight %)

Accession	1st	2nd	3rd	Average	Standard Deviation
TN1	0.071	0.0512	0.070	0.064	0.011
46/MO	0.071	0.0729	0.078	0.074	0.004
VA1	0.057	0.0720	0.068	0.066	0.007
PA2	0.054	0.0500	0.038	0.047	0.008
68/AR	0.059	0.1033	0.066	0.076	0.023
DE2	0.051	0.0361	0.093	0.060	0.029
NY1	0.060	0.0428	0.049	0.050	0.009
NY2	0.051	0.0608	0.052	0.054	0.005
WV1	0.070	0.0605	0.065	0.065	0.005
NC2	0.037	0.0355	0.048	0.040	0.006
KY1	0.056	0.0615	0.056	0.057	0.003
IN1	0.065	0.0653	0.090	0.073	0.014
NC3	0.065	0.0615	0.050	0.059	0.007
WV2	0.050	0.0692	0.061	0.060	0.009
PA1	0.074	0.0692	0.055	0.066	0.009
NC4	0.081	0.0790	0.095	0.085	0.008

Table 29: ANOVA Analysis Data for Isoferulic Acid

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.006	15	0.0004	2.582	0.012	1.991
Within Groups	0.004	32	0.0002			
Total	0.011	47				

Table 30: ANOVA Data for Subset (IN1, PA1, NY2, NC3, KY1, DE2) Analysis of Actein and 23-*epi*-26-deoxyactein

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.024	5	0.0048	0.368	0.860	3.105
Within Groups	0.156	12	0.0130			
Total	0.180	17				

Figure 5: ESI(+ Mode) Mass Spectrum of Cimiracemoside A

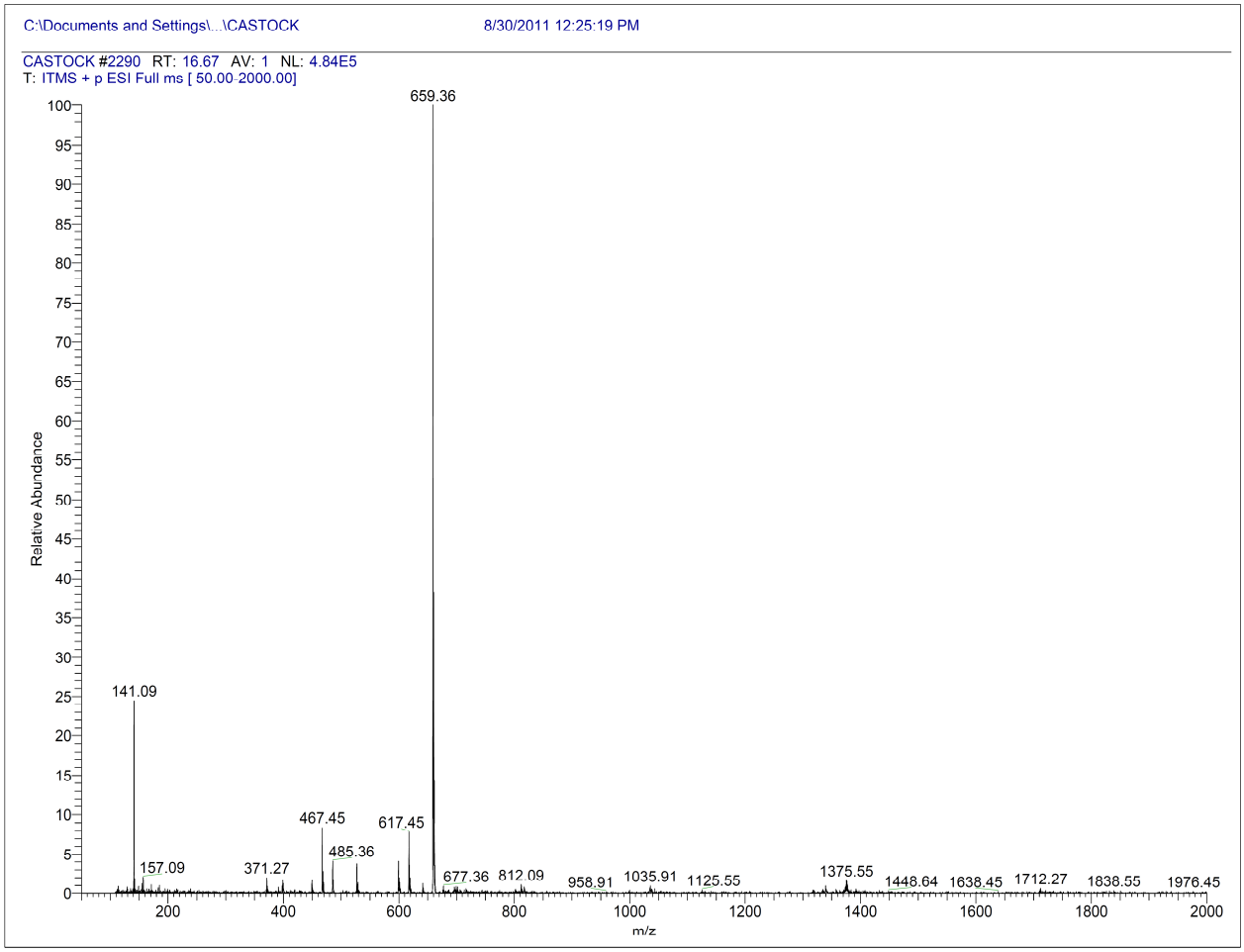


Figure 6: Proton NMR Spectrum of Cimiracemoside A

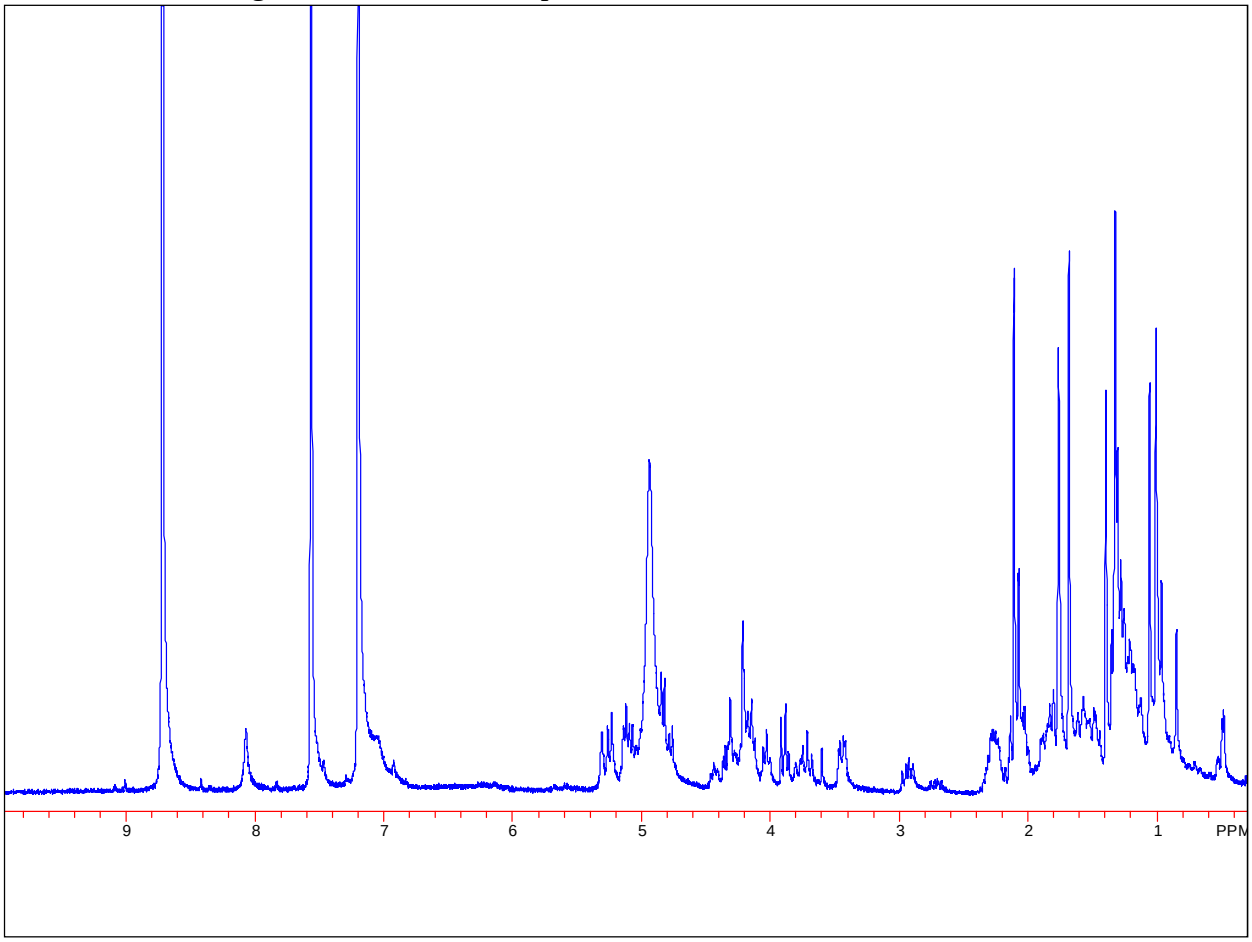


Figure 7: Carbon NMR Spectrum of Cimiracemoside A

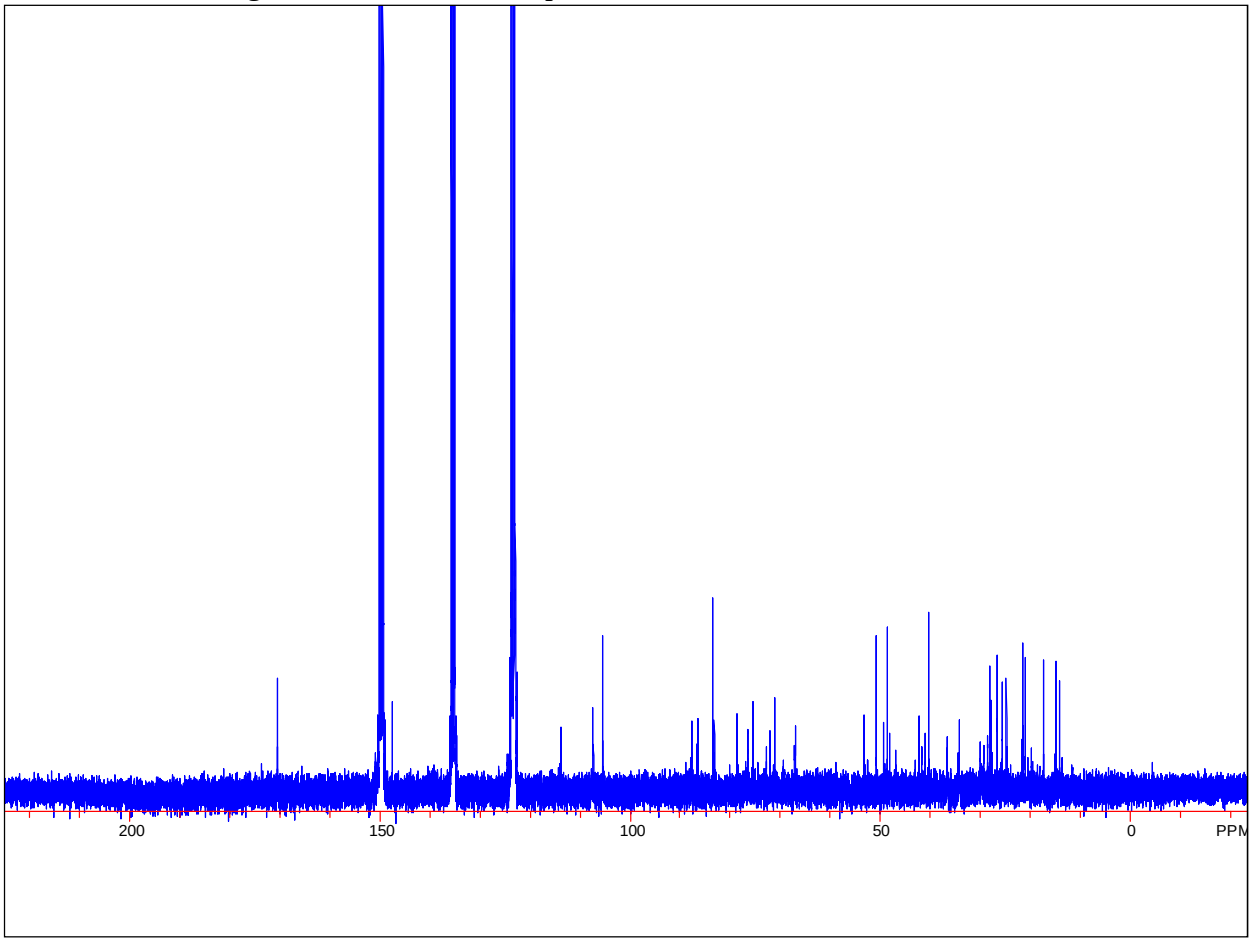


Figure 8: ESL (+ Mode) Mass Spectrum of Cimiracemoside C

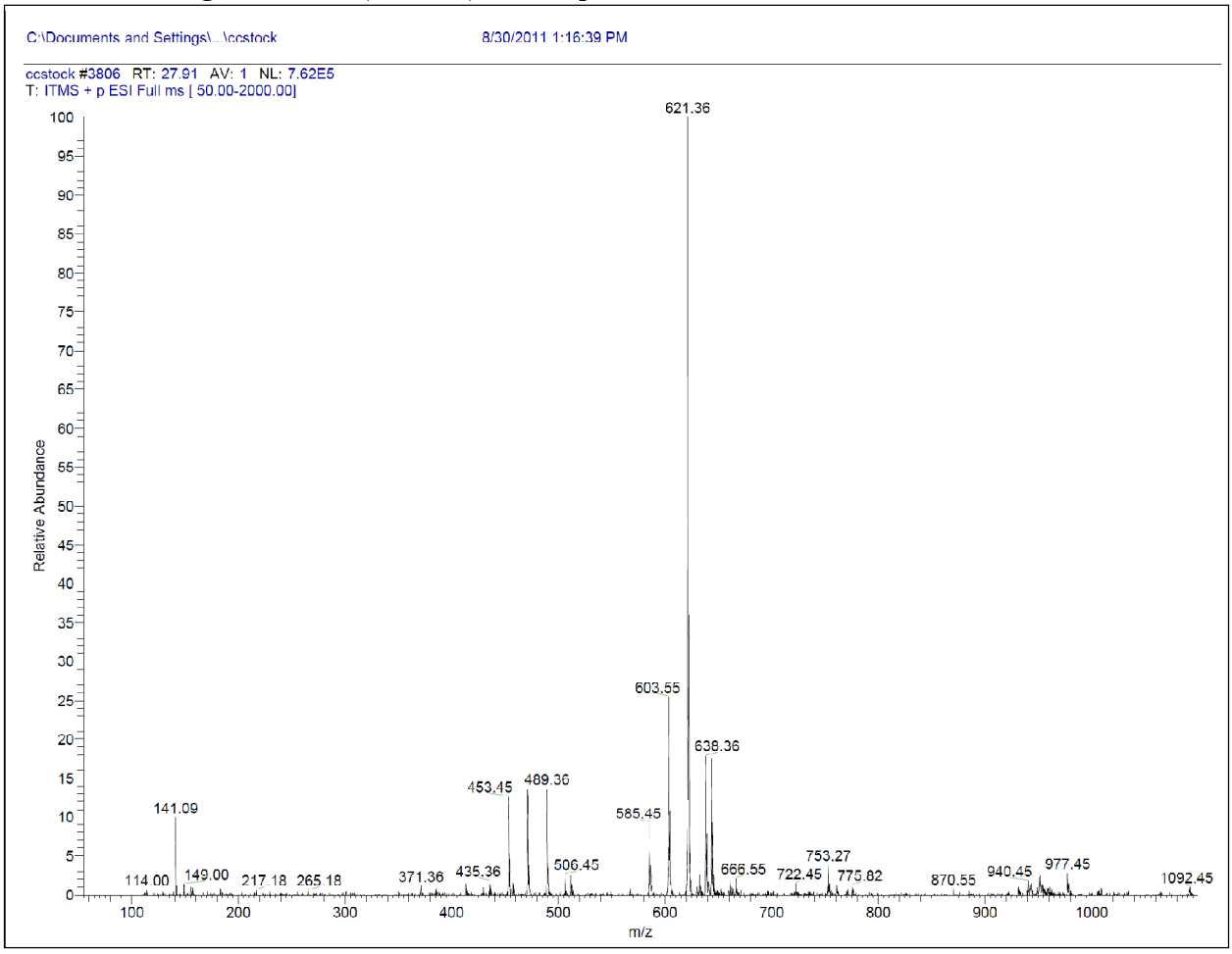


Figure 9: Proton NMR Spectrum of Cimiracemoside C

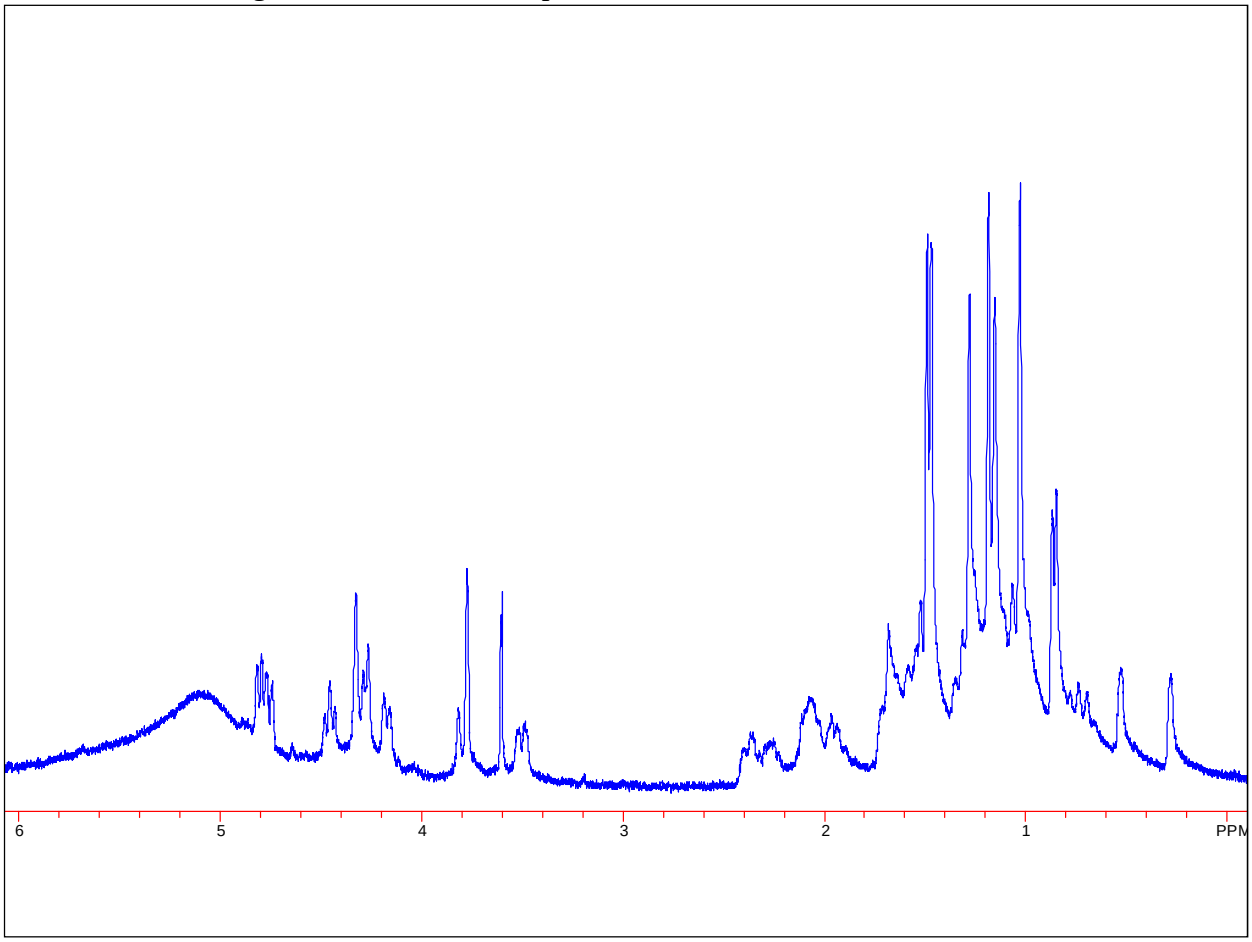


Figure 10: Carbon NMR Spectrum of Cimiracemoside C

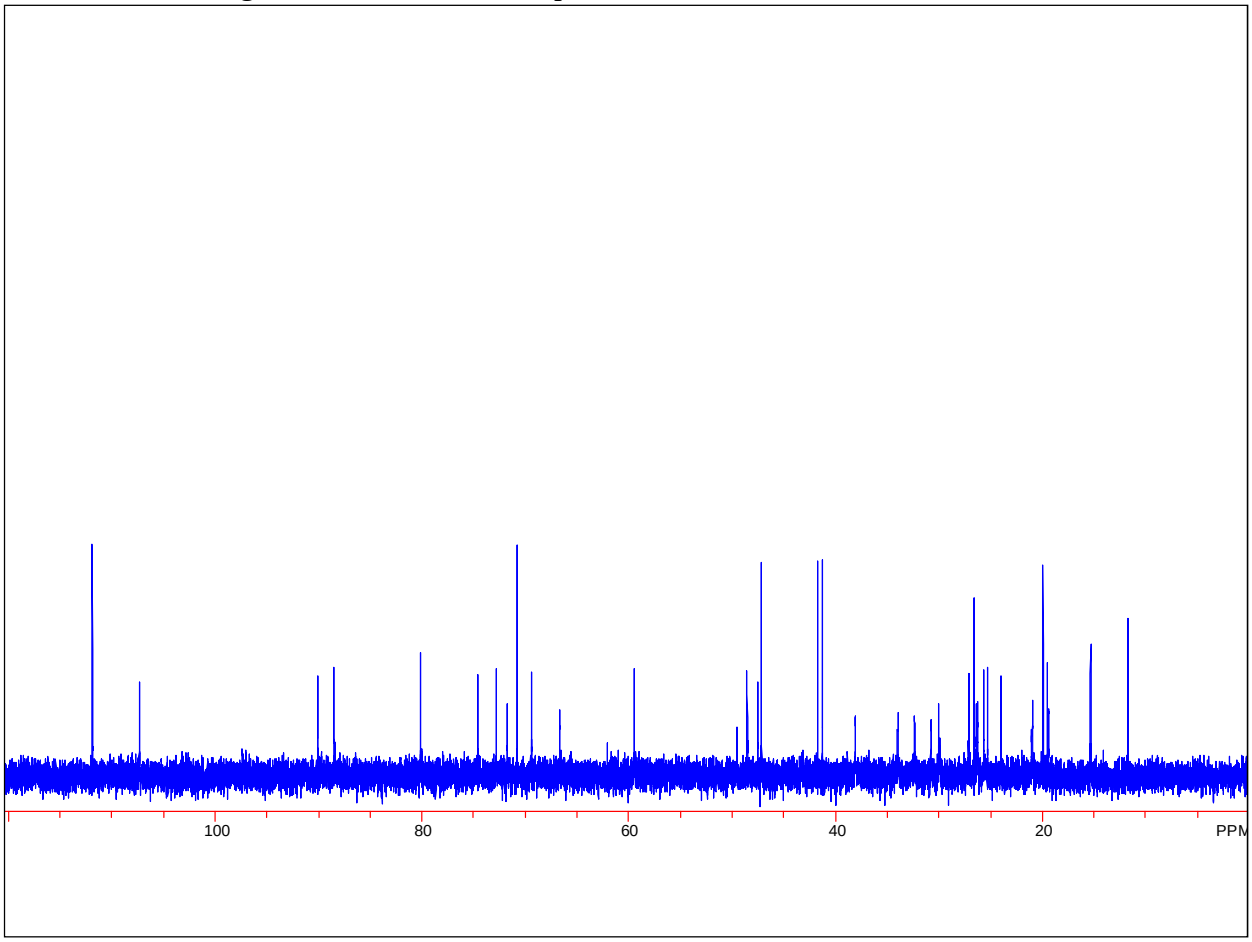


Figure 11: ESI (+ Mode) Mass Spectrum of Cimigenol Xyloside

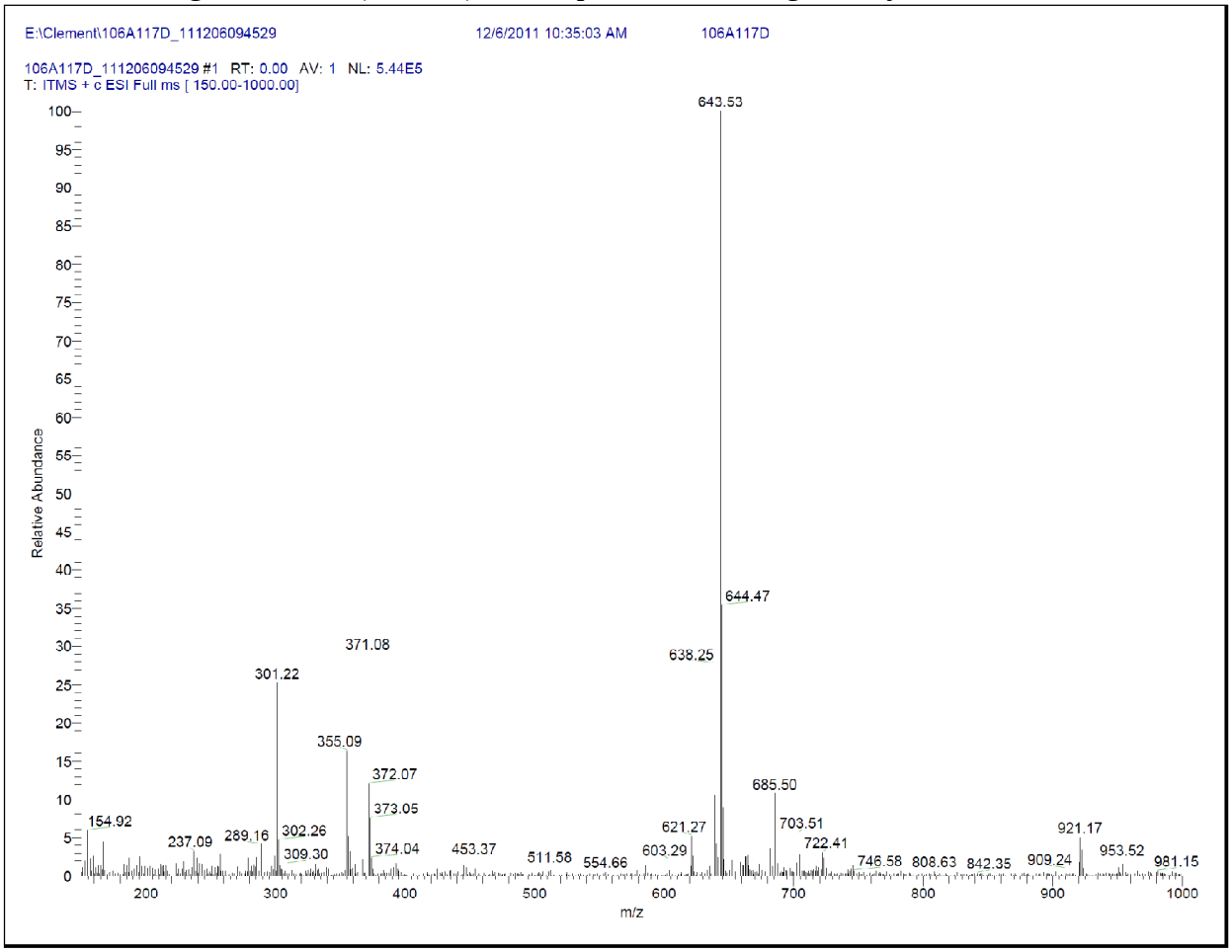


Figure 12: Proton NMR Spectrum of Cimigenol Xyloside

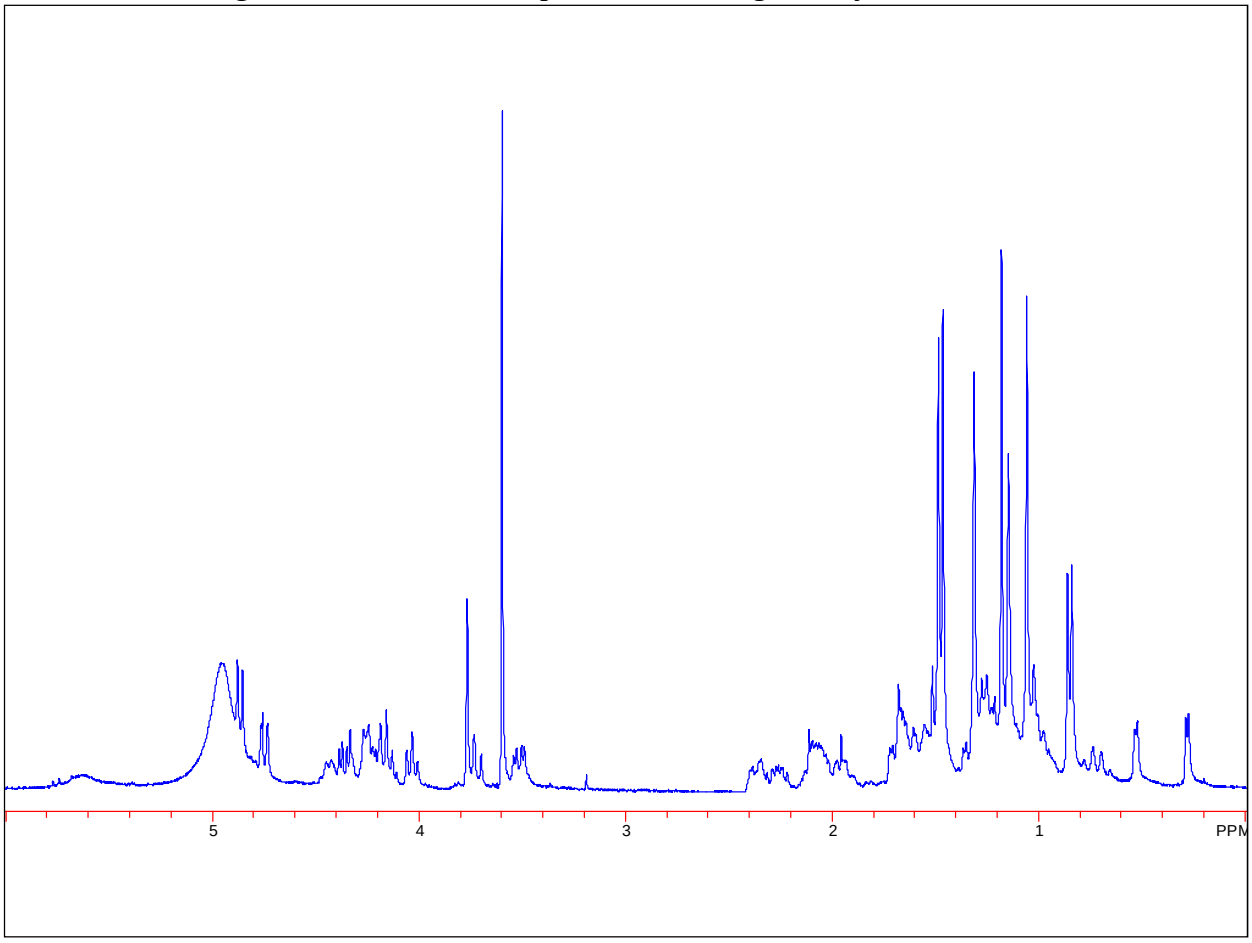


Figure 13: Carbon NMR Spectrum of Cimigenol Xyloside

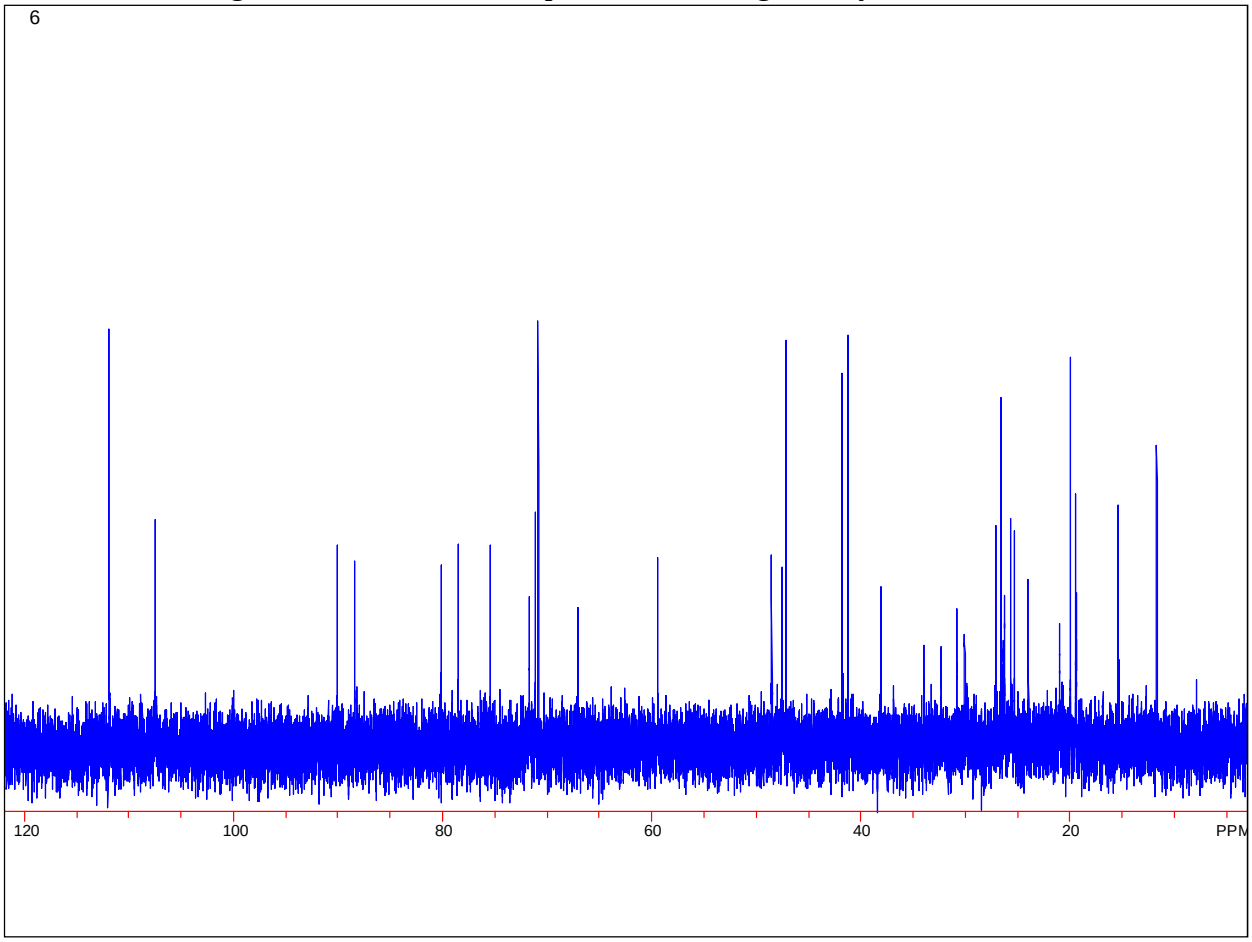


Figure 14: ESI (+ Mode) Mass Spectrum of Acetyl Shengmanol Xyloside

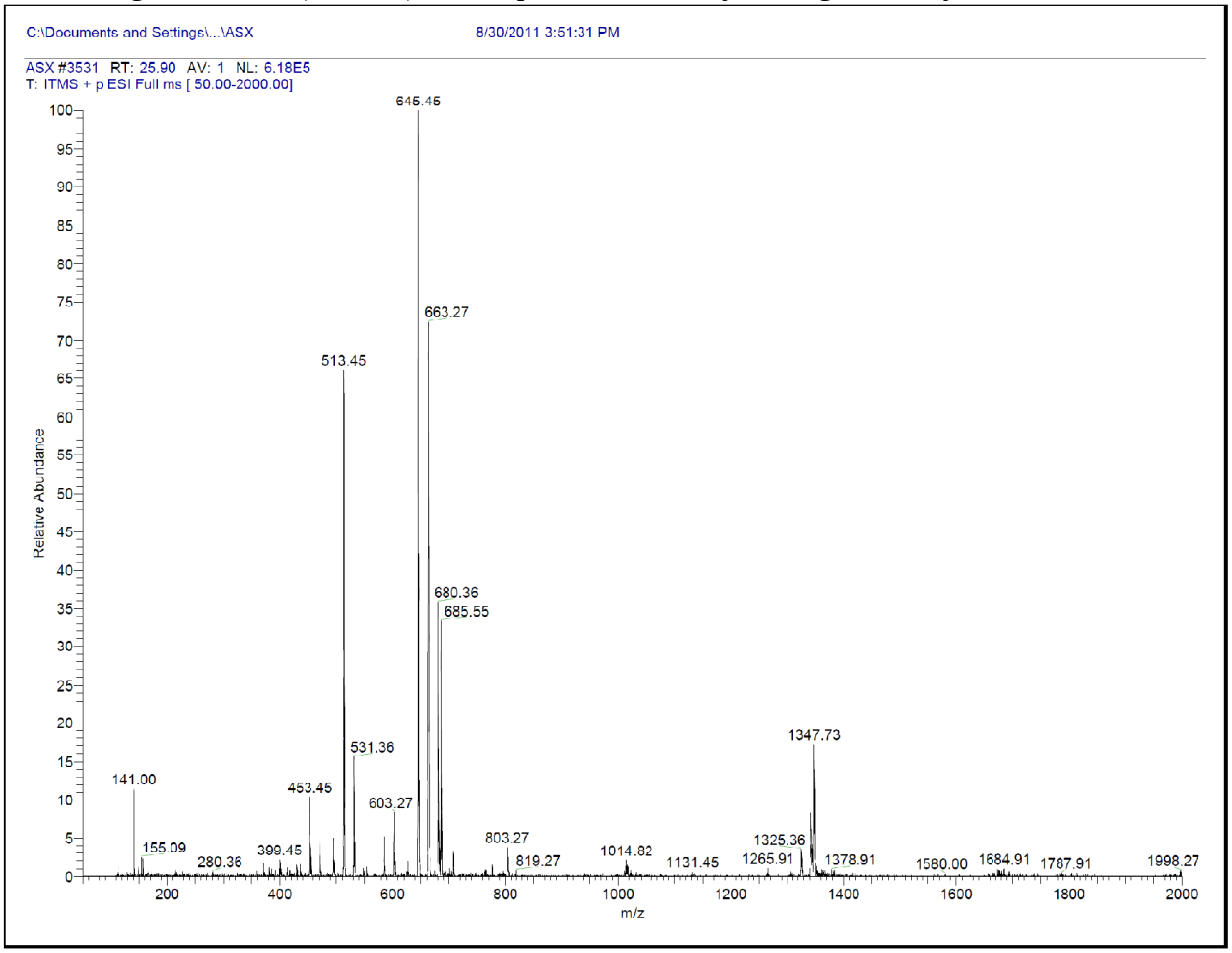


Figure 15: Proton NMR Spectrum of Acetyl Shengmanol Xyloside

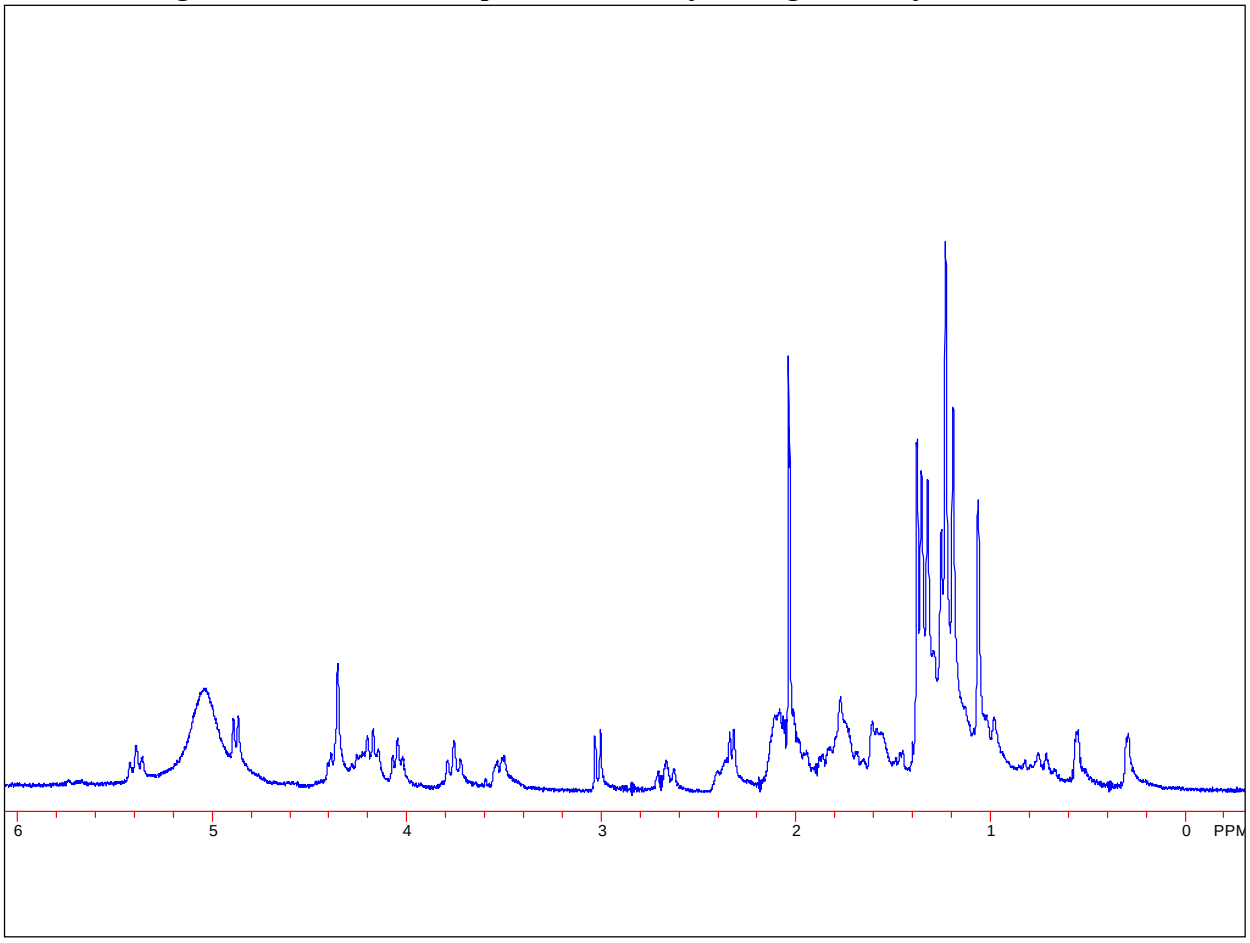


Figure 16: Carbon NMR Spectrum of Acetyl Shengmanol Xyloside

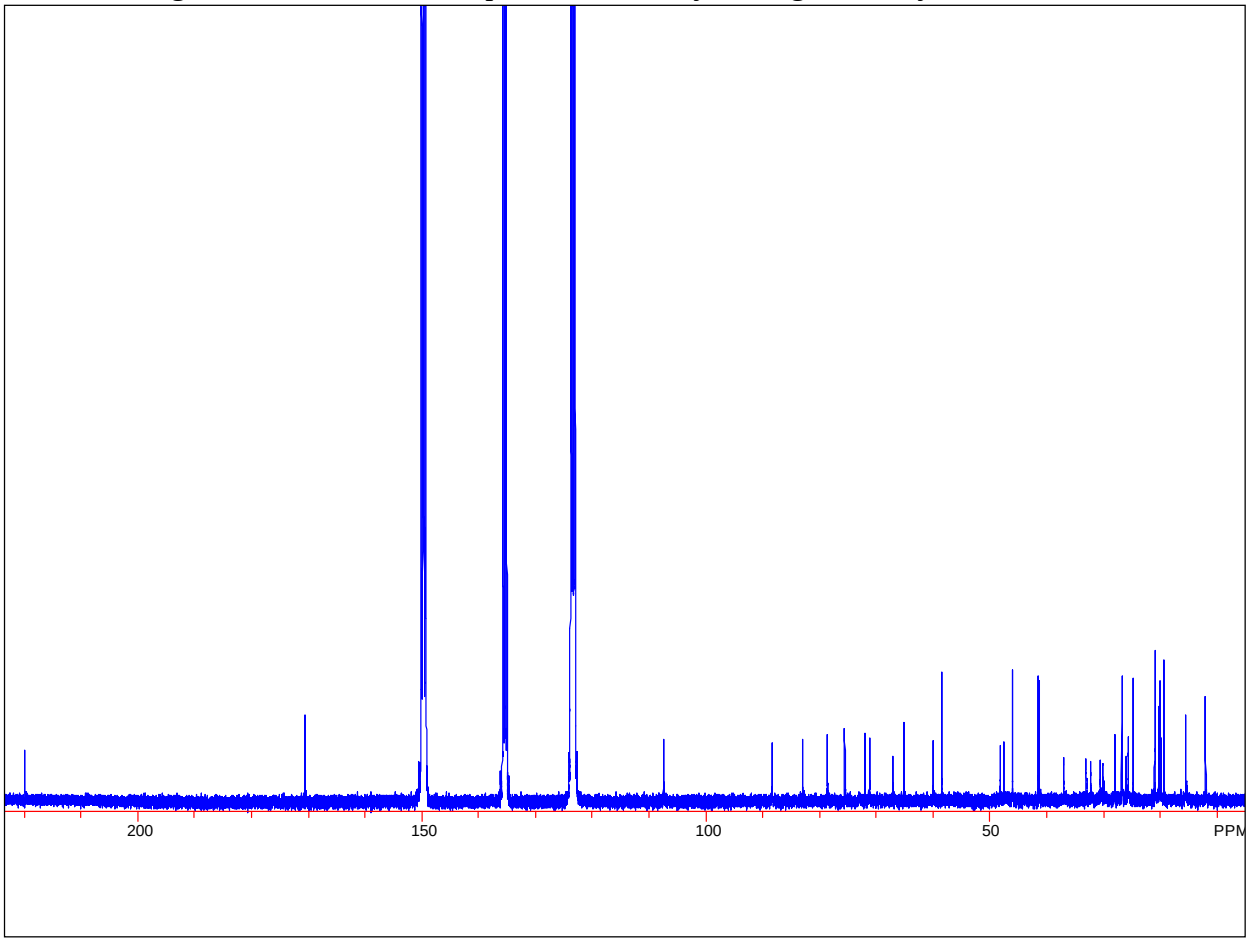


Figure 17: Sample Saponin HPLC-ELSD Chromatogram

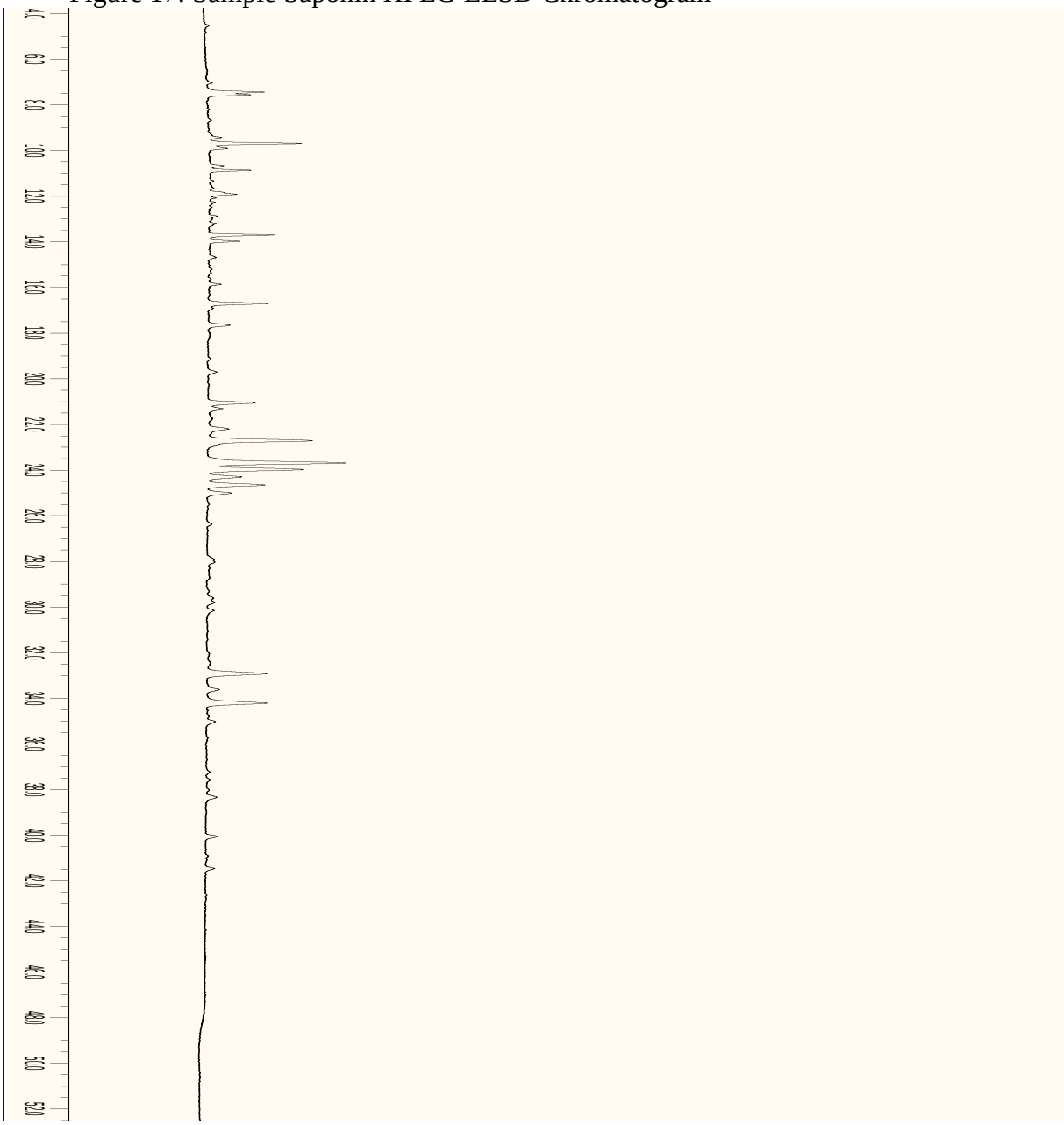


Figure 18: Zoom in of Sample Saponin Chromatogram

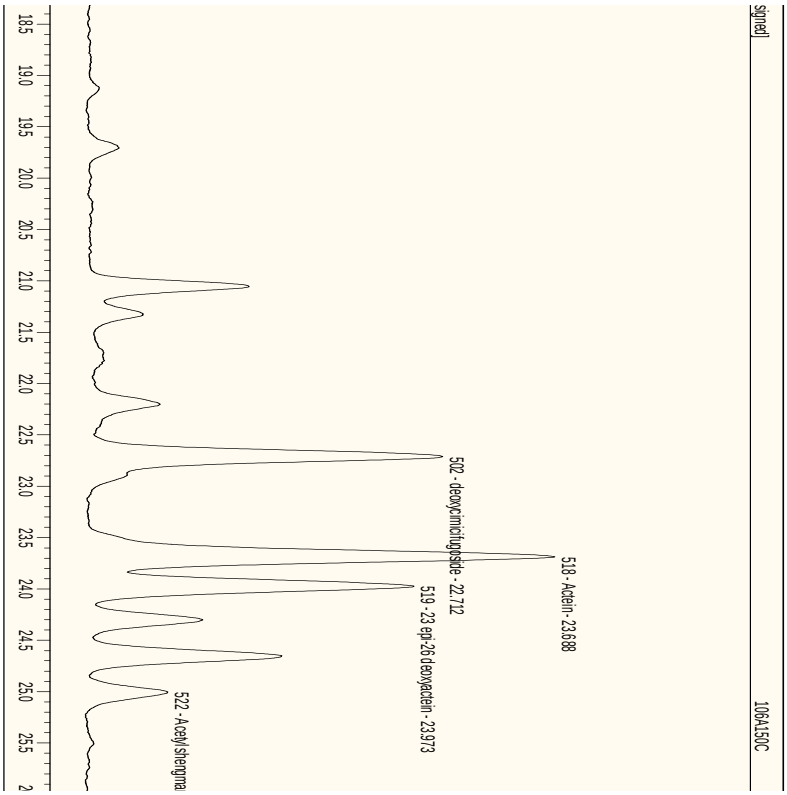


Figure 19: Sample Phenolic Acids Chromatogram

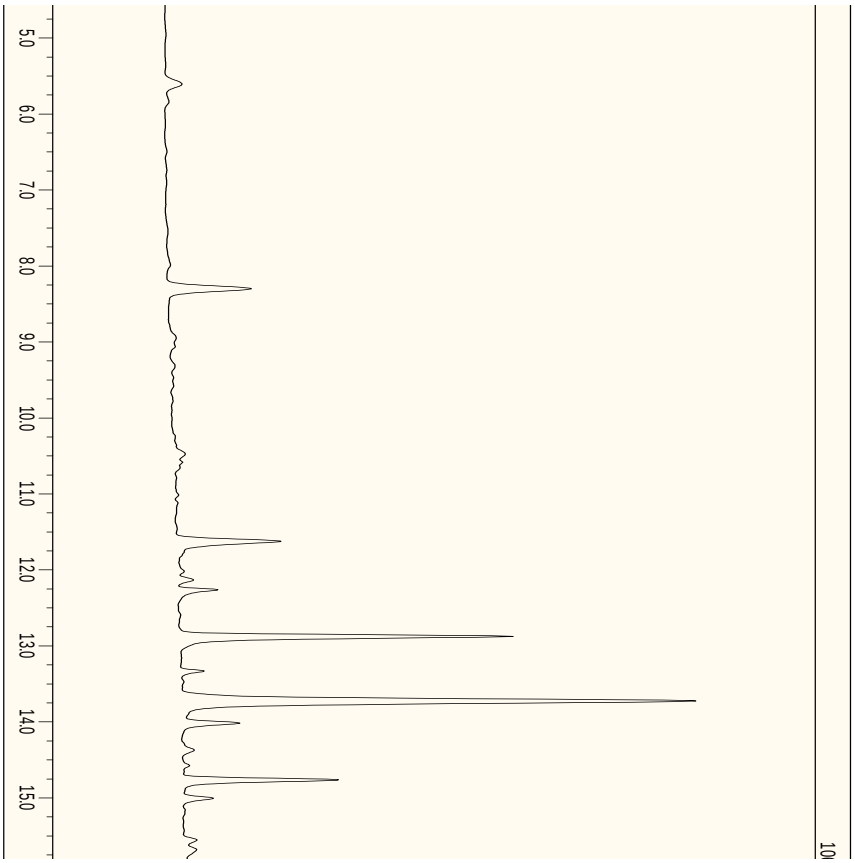


Figure 20: Zoom in of Sample Phenolic Acids Chromatogram

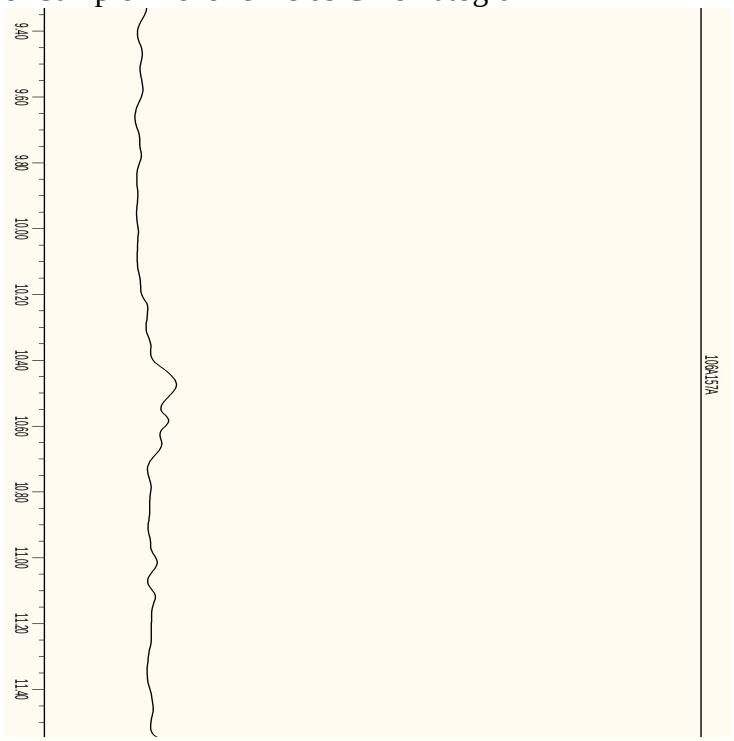
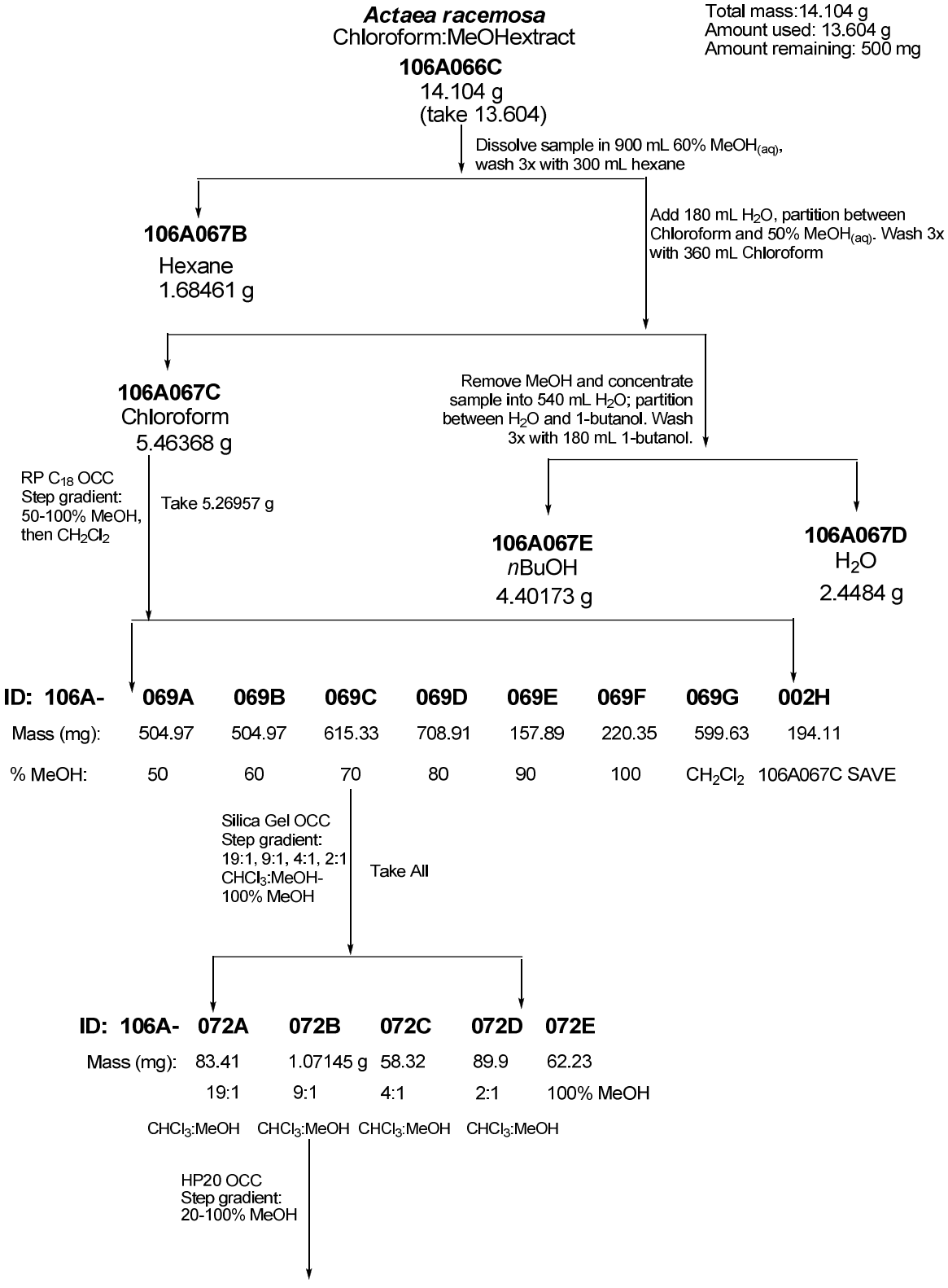
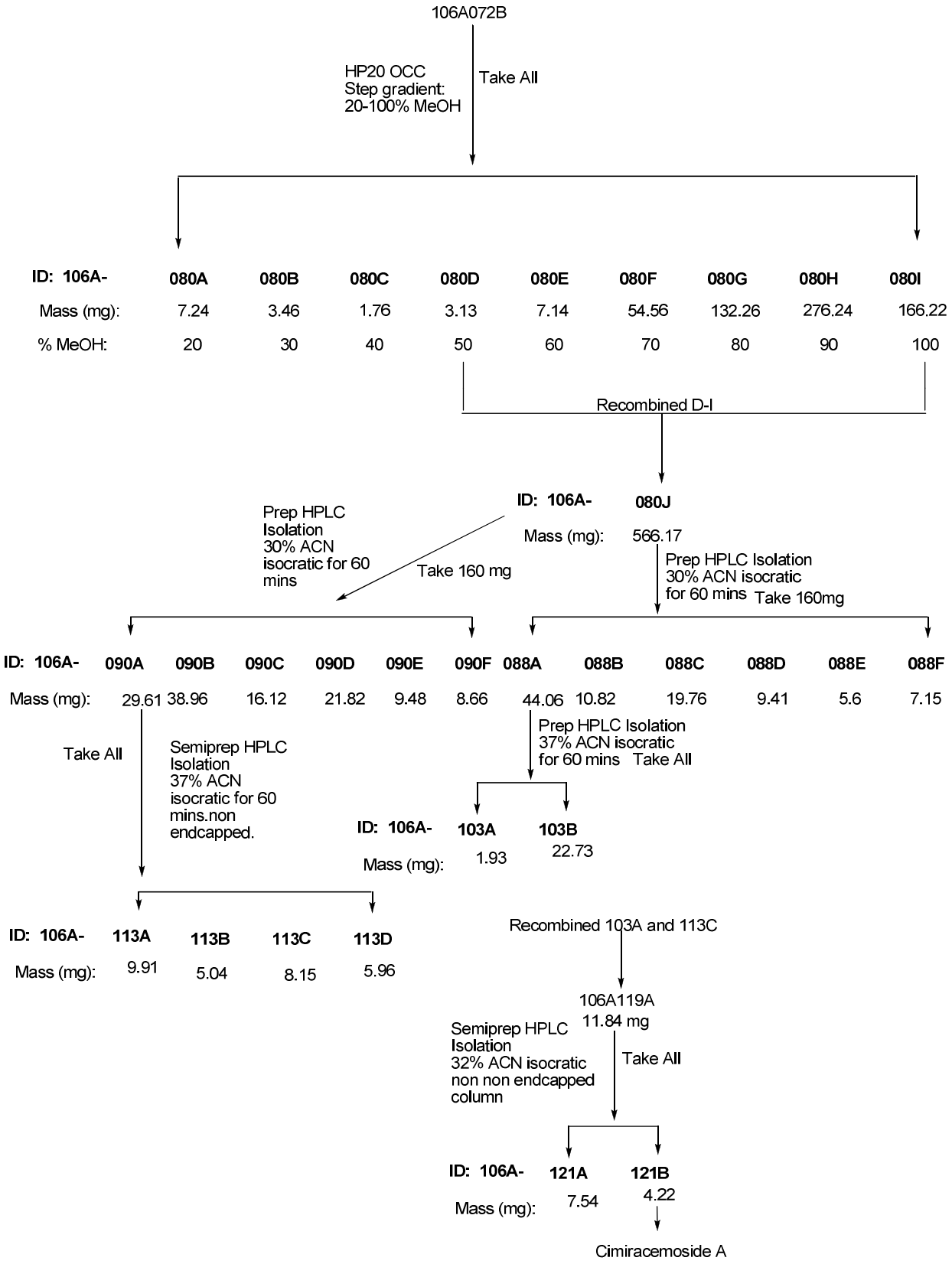
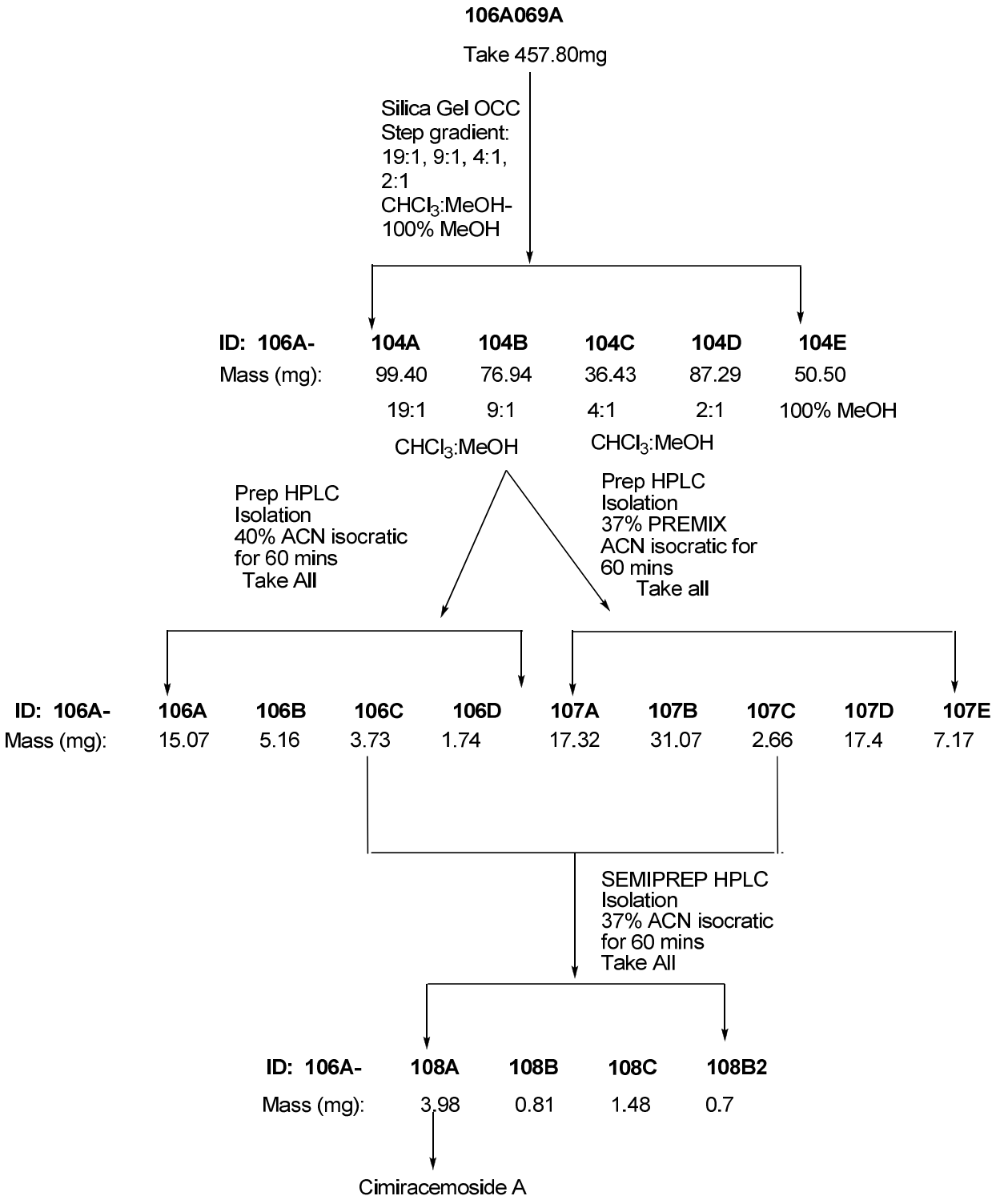
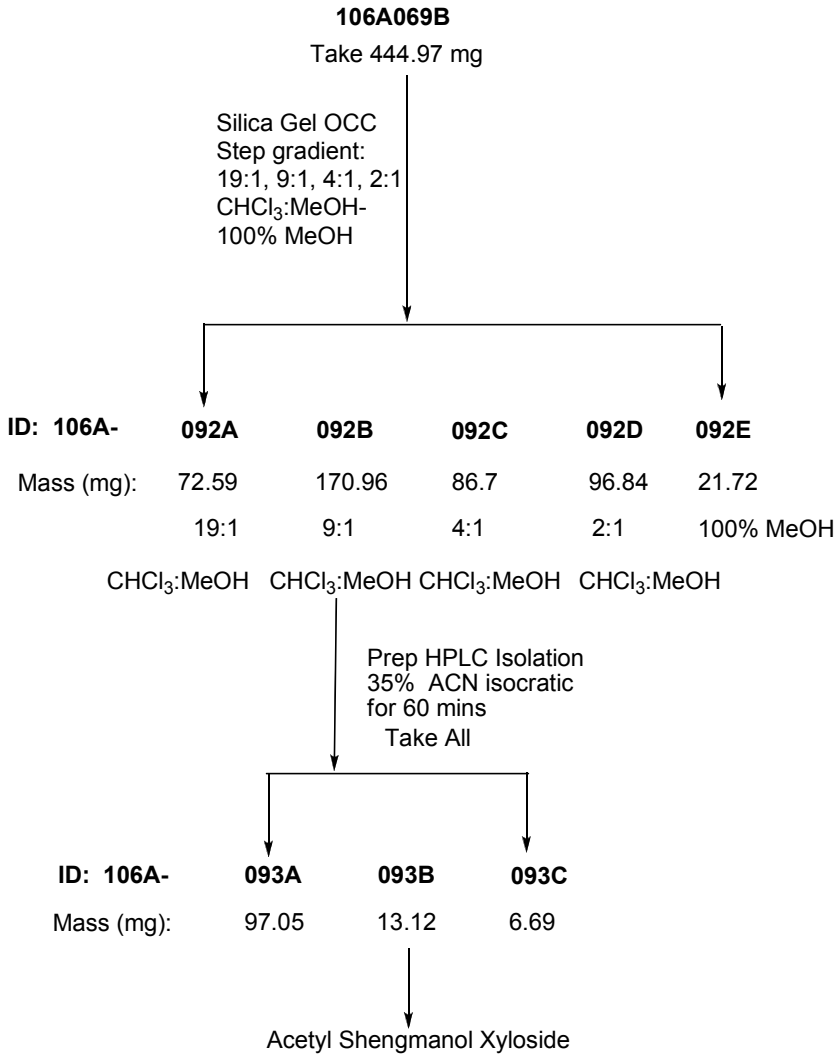


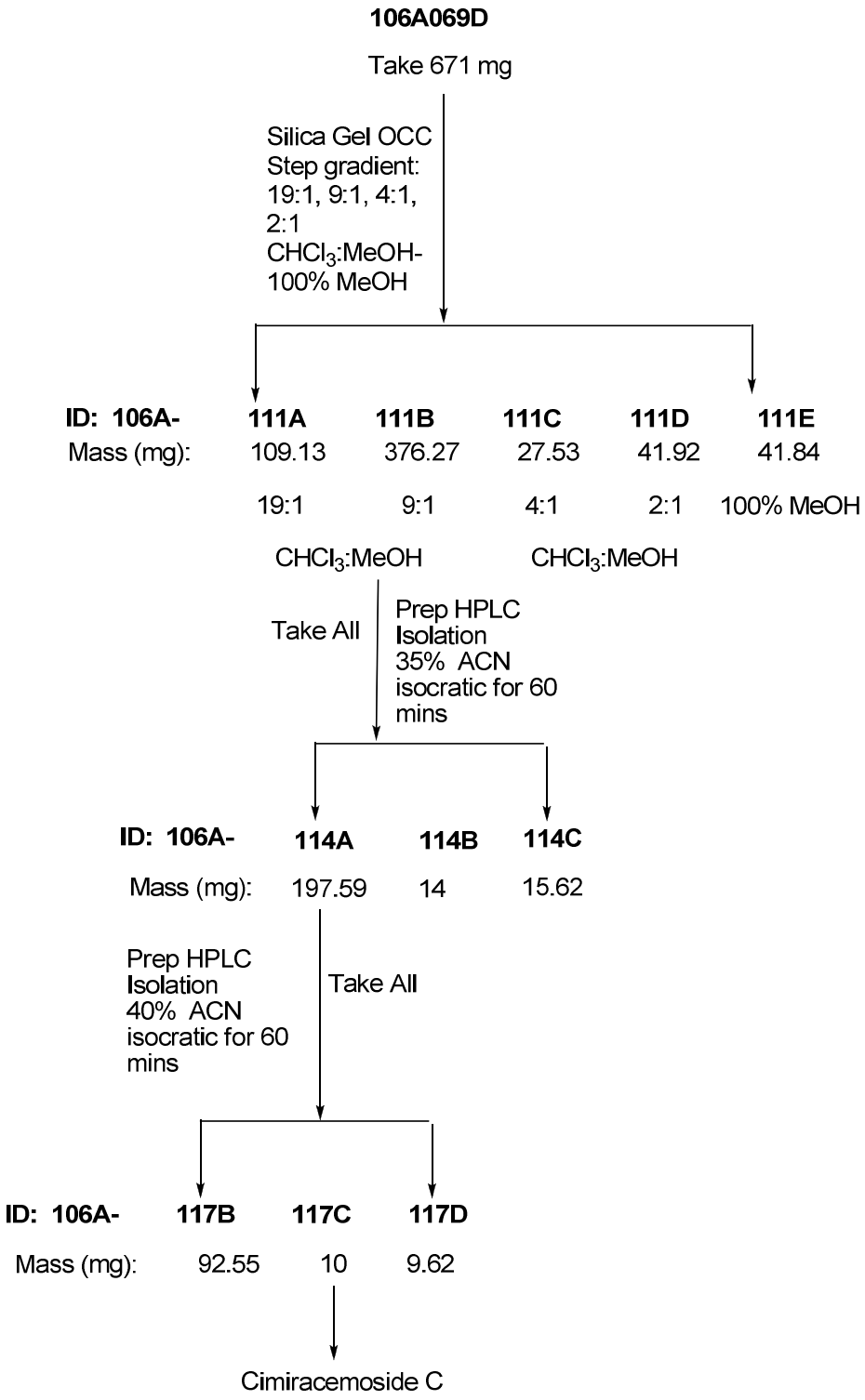
Figure 21: Fractionation Tree for Black Cohosh

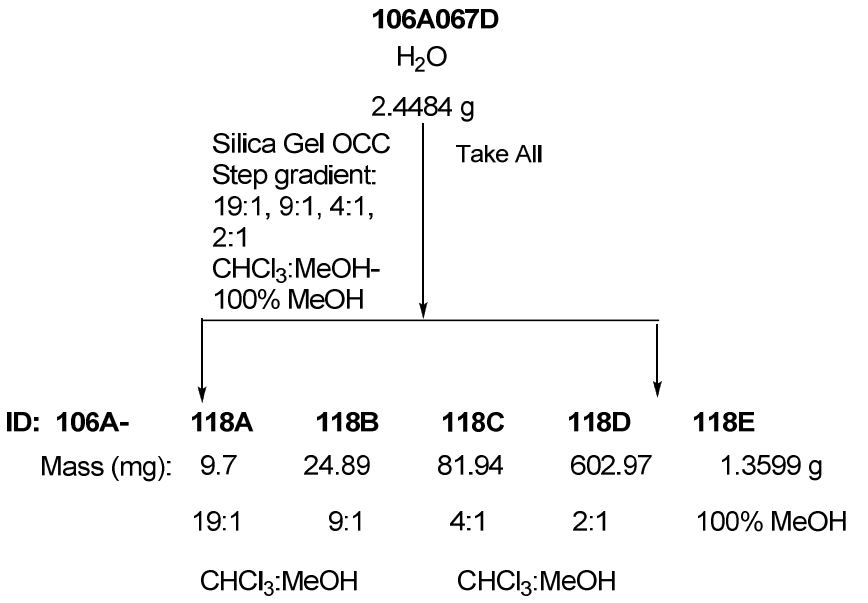


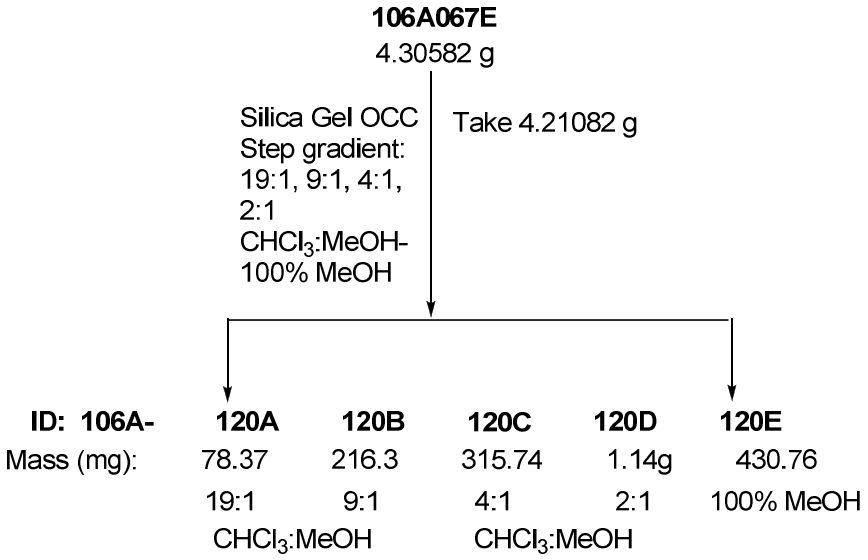

106A072B
HP20 OCC
Step gradient:
20-100% MeOH
Take All
ID: 106A- 080A 080B 080C 080D 080E 080F 080G 080H 080I
Mass (mg): 7.24 3.46 1.76 3.13 7.14 54.56 132.26 276.24 166.22
% MeOH: 20 30 40 50 60 70 80 90 100
Recombined D-I
ID: 106A- 080J
Mass (mg): 566.17
Prep HPLC Isolation 30% ACN isocratic for 60 mins
Take 160 mg
Prep HPLC Isolation 30% ACN isocratic for 60 mins Take 160mg
ID: 106A- 090A 090B 090C 090D 090E 090F 088A 088B 088C 088D 088E 088F
Mass (mg): 29.61 38.96 16.12 21.82 9.48 8.66 44.06 10.82 19.76 9.41 5.6 7.15
Take All
Semiprep HPLC Isolation 37% ACN isocratic for 60 mins.non endcapped.
Prep HPLC Isolation 37% ACN isocratic for 60 mins Take All
ID: 106A- 103A 103B
Mass (mg): 1.93 22.73
ID: 106A- 113A 113B 113C 113D
Mass (mg): 9.91 5.04 8.15 5.96
Recombined 103A and 113C
106A119A
11.84 mg
Semiprep HPLC Isolation 32% ACN isocratic non non endcapped column
Take All
ID: 106A- 121A 121B
Mass (mg): 7.54 4.22
Cimiracemoside A


106A069A
Take 457.80mg
Silica Gel OCC
Step gradient:
19:1, 9:1, 4:1,
2:1
CHCl3:MeOH-
100% MeOH
ID: 106A- 104A 104B 104C 104D 104E
Mass (mg): 99.40 76.94 36.43 87.29 50.50
19:1 9:1 4:1 2:1 100% MeOH
CHCl3:MeOH
CHCl3:MeOH
Prep HPLC
Isolation
40% ACN isocratic
for 60 mins
Take All
Prep HPLC
Isolation
37% PREMIX
ACN isocratic for
60 mins
Take all
ID: 106A- 106A 106B 106C 106D 107A 107B 107C 107D 107E
Mass (mg): 15.07 5.16 3.73 1.74 17.32 31.07 2.66 17.4 7.17
SEMIPREP HPLC
Isolation
37% ACN isocratic
for 60 mins
Take All
ID: 106A- 108A 108B 108C 108B2
Mass (mg): 3.98 0.81 1.48 0.7
Cimiracemoside A


106A069B
Take 444.97 mg
Silica Gel OCC
Step gradient:
19:1, 9:1, 4:1, 2:1
CHCl3:MeOH-
100% MeOH
ID: 106A- 092A 092B 092C 092D 092E
Mass (mg): 72.59 170.96 86.7 96.84 21.72
19:1 9:1 4:1 2:1 100% MeOH
CHCl3:MeOH CHCl3:MeOH CHCl3:MeOH CHCl3:MeOH
Prep HPLC Isolation
35% ACN isocratic
for 60 mins
Take All
ID: 106A- 093A 093B 093C
Mass (mg): 97.05 13.12 6.69
Acetyl Shengmanol Xyloside


106A069D
Take 671 mg
Silica Gel OCC
Step gradient:
19:1, 9:1, 4:1,
2:1
CHCl3:MeOH-
100% MeOH
ID: 106A- 111A 111B 111C 111D 111E
Mass (mg): 109.13 376.27 27.53 41.92 41.84
19:1 9:1 4:1 2:1 100% MeOH
CHCl3:MeOH CHCl3:MeOH
Take All
Prep HPLC
Isolation
35% ACN
isocratic for 60
mins
ID: 106A- 114A 114B 114C
Mass (mg): 197.59 14 15.62
Prep HPLC
Isolation
40% ACN
isocratic for 60
mins
Take All
ID: 106A- 117B 117C 117D
Mass (mg): 92.55 10 9.62
Cimiracemoside C


106A067D
H_2O
2.4484 g
Silica Gel OCC
Step gradient:
19:1, 9:1, 4:1,
2:1
$CHCl_3$:MeOH-
100% MeOH
Take All
ID: 106A- 118A 118B 118C 118D 118E
Mass (mg): 9.7 24.89 81.94 602.97 1.3599 g
19:1 9:1 4:1 2:1 100% MeOH
$CHCl_3$:MeOH $CHCl_3$:MeOH


106A067E
4.30582 g
Silica Gel OCC
Step gradient:
19:1, 9:1, 4:1,
2:1
$CHCl_3$:MeOH-
100% MeOH
Take 4.21082 g
ID: 106A- 120A 120B 120C 120D 120E
Mass (mg): 78.37 216.3 315.74 1.14g 430.76
19:1 9:1 4:1 2:1 100% MeOH
$CHCl_3$:MeOH $CHCl_3$:MeOH