CHARACTERIZATION OF BIOACTIVE ALKALOID CONTENT VERSUS SOIL CHEMISTRY IN GOLDENSEAL *(HYDRASTIS CANADENSIS)*

A thesis presented to the faculty of the Graduate School
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ABSTRACT

CHARACTERIZATION OF BIOACTIVE ALKALOID CONTENT VERSUS SOIL CHEMISTRY IN GOLDENSEAL *(HYDRASTIS CANADENSIS)*

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Goldenseal *(Hydrastis canadensis)* is widely used as a dietary supplement due to the biological activity of the isoquinoline alkaloids berberine and hydrastine. Differences in location and growing conditions often lead to variations in alkaloid content in goldenseal. These variations in chemical composition of plants are directly affected by soil conditions in which they are grown. Soil metal concentration is only one factor affecting the bioavailability of metals to plants. Bioavailability is dramatically affected by soil pH, cation exchange capacity, and total organic carbon concentrations. Metals within the soil can enhance or inhibit pathways that produce alkaloids. Copper, zinc, manganese, iron, magnesium, and calcium are all essential metals to plant health. However, copper, zinc manganese, and iron can be harmful in high concentrations causing oxidative stress. Low concentrations of these and other nutrients can lead to deficiencies that cause stress to the plant. Production of secondary metabolites like alkaloids is increased by plants in response to stressors such as oxidative stress and nutrient deficiency.

In this project, the concentrations of the alkaloids in leaf and root extracts of goldenseal samples grown in different locations were quantified. In addition, variations in metal concentrations of plants grown in each location were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES). Soil parameters were all studied through soil tests to get a complete picture of conditions that may be related

to alkaloid variations within the sample set. Alkaloid concentrations were determined using high pressure liquid chromatography (HPLC). Plant alkaloid concentrations were compared to soil metal concentrations to determine if any correlation exists.

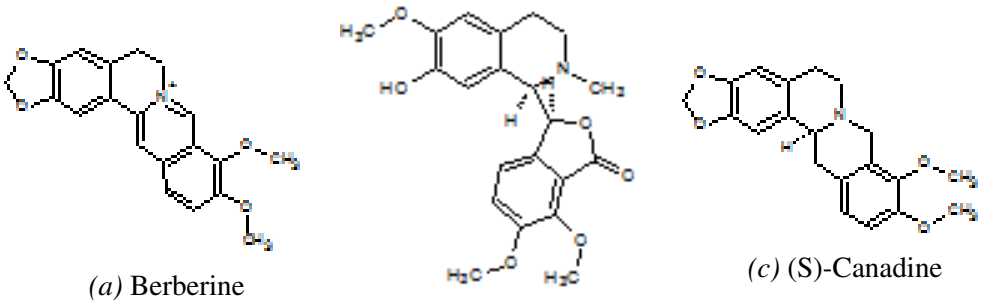
Results showed that there were no strong correlations between metal concentrations (R^2 above .8) and alkaloid content. However, weaker correlations of 0.37 and 0.38 between iron and aluminum in the root show negative relationships between iron and aluminum and berberine in goldenseal roots. Overall trends appearing within the data suggest positive relationships between berberine production and magnesium, calcium, and manganese concentrations. Trends in the data also suggest that some of the samples with high berberine concentrations may have experienced nutrient deficiency due to soil conditions before harvesting.

CHAPTER 1

INTRODUCTION

1.1 Uses of Goldenseal

Goldenseal *(Hydrastis canadensis)* has been cultivated for medicinal purposes for centuries and is still a popular herbal remedy. Biological activity in the plant is ascribed to the three main benzylisoquinoline alkaloids: berberine, canadine, and hydrastine (Figure 1.1c). Plant extracts are sold for the treatment of a variety of ailments includ-



(a) Berberine *(b)* Hydrastine *(c)* (S)-Canadine

Figure 1.1: Alkaloids in Goldenseal

ing: skin conditions, viruses, and bacterial infections.

Many studies on the effectiveness of goldenseal alkaloids against bacteria and viruses have been conducted in recent years. In a study performed by Cecil et al., [3] berberine was shown to inhibit growth of influenza A. The exact mechanism is unknown, but it is suspected that berberine prevents virus proteins from unfolding. Berberine has also been shown to inhibit the production of compounds in influenza that cause cytokeine inflammatory responses in the body.[3] In a similar study, Junio et al. [4] found that berberine inhibits growth of *Staphylococcus aureus*.

1.2 Commercial and Environmental Concerns

Goldenseal is a perennial herb in Ranunculaceae family. It grows for 2 to 5 years before flowering. Since alkaloids are most abundant in the plant when flowering, plants are not harvested until at least two years after planting.[5].

Market prices for the main alkaloid, berberine, vary widely from year to year. Price fluctuations make business planning difficult for growers. It is difficult to predict prices five years in advance and low prices can force growers to sell extracts at a loss. For this reason, most producers of goldenseal extracts harvest wild plants instead of investing the capital to cultivate their own [6].

The highest alkaloid content is in the roots with only minimal content in the leaves. Harvesting involves excavating and destroying the entire plant. Harvesting of wild plants has lead to a decrease in the population of wild goldenseal. As a result, goldenseal has been placed on the Convention on International Trade in Endangered Species (CITES) list. Increasing alkaloid concentrations in goldenseal plants may encourage more extract producers to grow rather than harvest wild goldenseal.

1.3 Biosynthesis of Benzylisoquinoline Alkaloids

Like all alkaloids, the isoquinoline alkaloids found in goldenseal are produced in the shikimic acid pathway. This pathway converts shikimic acid to aromatic amino acids like L-tryptophan, L-phenylalanine, and L-tyrosine[2]. Berberine, canadine, and hydrastine are all benzyltetrahydroisoquinoline alkaloids formed from the precursor (S)-reticuline. Although there are over 2,500 known alkaloids in the tetrahydroisoquinoline alkaloid group, they are mainly produced by only five families of plants, the Papaveraceae, Fumariaceae, Berberidaceae, Ranunculaceae, and Menispermaceae families. Goldenseal (*Hydrastis cadensis*) is a member of the Ranunculaceae family [2].

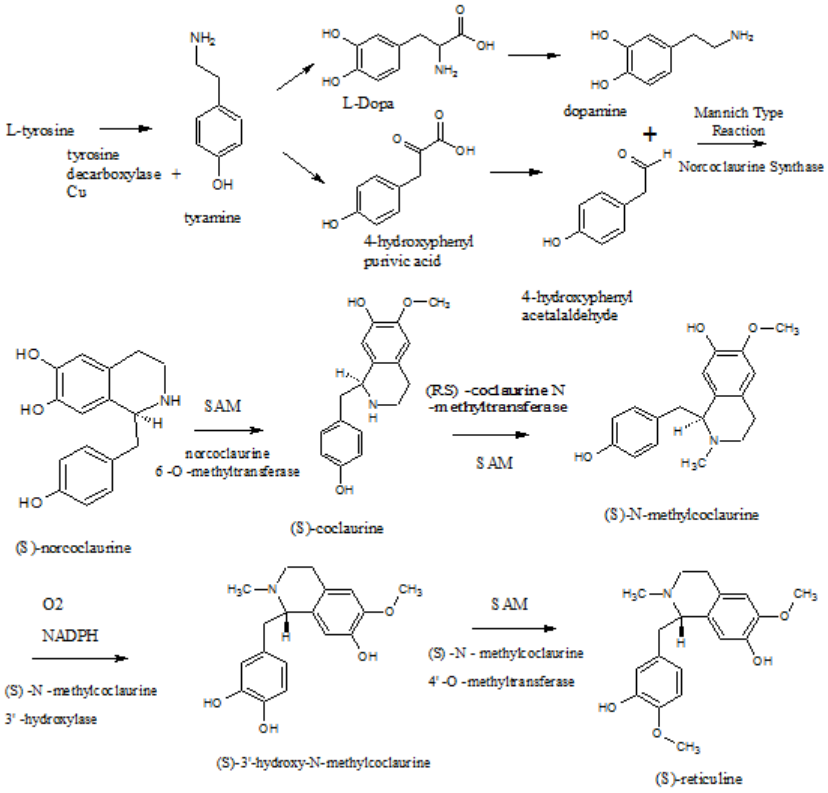


Figure 1.2: Biosynthesis of (S)-reticulne from L-DOPA [2]

(S)-reticuline comes from a pathway involving L-tyrosine. Manganese is needed for the production of L-tyrosine [7]. Figure 1.2 shows the process used by plants to convert L-tyrosine into (S)-reticuline. L-tyrosine is converted (decarboxylated by tyrosyne decarboxylase-enzyme catalysed by copper) to form tyramine which is then converted to L-DOPA by phenol oxidase. L-Dopa is decarboxylated by pyridoxal phosphate (PLP) to form dopamine. 4-hydroxy phenyl acetalaldehyde, from a different reaction with L-tyrosine that also involves tyrosine decarboxylaase, adds to dopamine. A mannich type reaction takes place to close the ring and eliminate water, producing (S)-norcoclaurine. (S)-norcoclaurine is then methylated by S-Adenosyl Methionine (SAM) to produce (S)-coclaurine. Berberine, canadine, and hydrastine differ from other tetrahydroisoquinoline alkaloids in the respect that they have an additional carbon

added to the nitrogen on the tetrahydroxy skeleton of (S)-coclaurine through methylation by SAM. This carbon is called the berberine bridge.[2] The N-methylation is followed by a hydroxylation step to produce (S)-3'-hydroxy-N-methylcoclaurine. After an additional methylation by SAM, (S)-reticuline is produced.

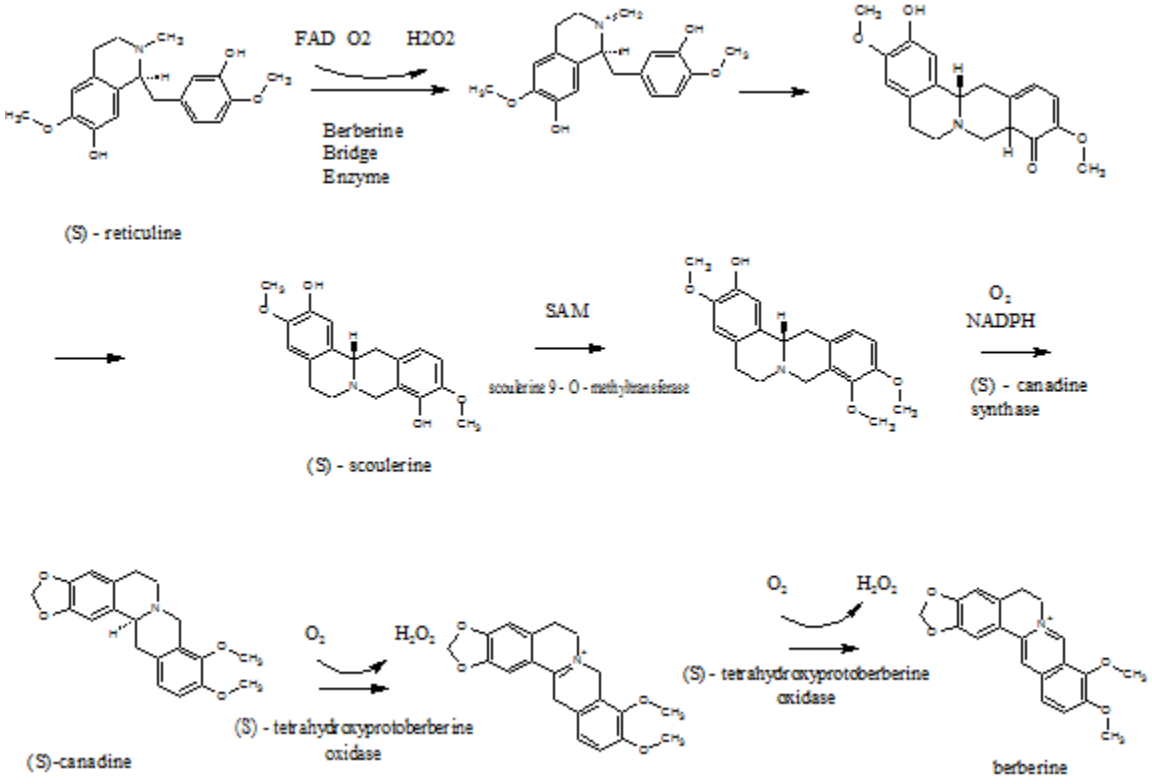


Figure 1.3: Formation of Canadine and Berberine from (S)-Reticuline [2]

Canadine is a precursor to berberine and is often found in plants that produce berberine. To form canadine, the tertiary amine in (S)-reticuline is oxidized to an iminium ion by an enzyme (berberine bridge enzyme) that uses flavin as a cofactor. This triggers a Mannich-like reaction eliminating the hydrogen on the nearby OH group, pushing electrons from the ring toward the nitrogen to form a single bond(see Figure 1.3). Next, the carbonly group tautomerizes to restore the aromatic ring forming (S)-scoulerine. The OH group that was reformed is then methylated by SAM. A methylene dioxy ring is formed in an oxidation to produce canadine. (S)-tetrahydroxyprotoberberine oxidase

(which in some species of the *Renunculales* family conatains iron) catalyzes two oxidation steps reforming the iminium cation and removing two hydrogens from the ring to create an aromatic ring. In other members of the same plant family these last two reactions are catalyzed by a flavoprotein and generate two peroxide rather than two water molecules.[8] Although most ions are unstable, berberine is more stable in the iminium ion form. To neutralize the charge on the nitrogen, the double bond with its neighboring carbon would have to be reduced to a single bond which would eliminate the aromaticity of the ring, raising the energy of the system[2].

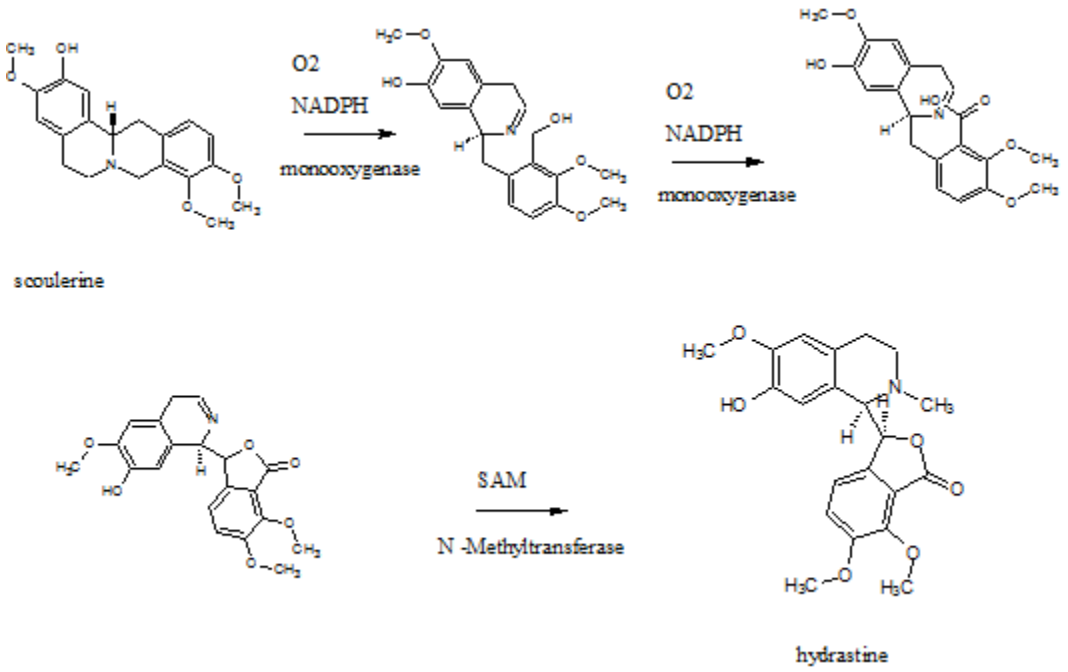


Figure 1.4: Formation of Hydrastine from Scoulerine [2]

Hydrastine is formed by a ring opening of scoulerine next to the nitrogen. The opening occurs through a reaction with a monooxygenase that hydroxylates the ring breaking the carbon-nitrogen bond (see Figure 1.4). Monooxygenases are enzymes that contain iron. They perform electrophilic substitution reactions on saturated carbons with retention of sterochemistry (the OH group is added on the same side as the H that is removed). A new ring is formed by an oxygen carbon bond. The nitrogen is then

methylated by a reaction involving SAM and an N-methyltransferase.

1.3.1 Studies of Alkaloid Production in Plants

Secondary metabolites, like alkaloids, are often produced in plants as a response to stress[9]. Cell culture studies on goldenseal performed by Bhojwani and Razdan have proven that under conditions optimal for growth, cells produce very low levels of secondary metabolites. Alkaloid production did increase in later stages of the experiment when nutrients in the growth medium have been depleted. Bhojwani and Razden noted that this lack of nutrients caused an increase in the enzymes involved in alkaloid pathways followed by a sharp increase in alkaloid production.[9] In other cell culture studies, Bhowani and Razdan obtained the highest alkaloid production by creating a two stage growing process for cells. First cells were grown in a medium optimal for growth resulting in the largest possible biomass. After a few days, cells were removed to a medium that inhibited growth and caused cells to increase production of alkaloids. Most experiments that attempted to increase alkaloid production in only one phase resulted in either high biomass and low alkaloid content or high alkaloid content and low biomass[9].

The only successful experiment achieving both high biomass and increased berberine production in one phase involved increasing copper concentrations. Increasing copper by ten percent (enough to increase berberine without reducing biomass) in the first or growth phase increased berberine by twenty to thirty percent. Some conditions in this second phase that showed the greatest increase in berberine production included, reducing phytohormones, adding bacteria, and reducing phosphates needed for cell nutrition [9].

Auxins are plant growth hormones. Zinc is involved in synthesis of auxins.[10] and manganese activates IAA by acting on IAAoxidase [7]. In cellular studies, auxins cause increased growth in plants. Increasing the auxin indole-3-acetic acid (IAA) caused in-

creased cell growth but low berberine content, whereas auxins 1-Napthaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP) had the opposite effect. The highest berberine concentrations in cell studies using auxins in the growth medium were from adding IAA to growth phase followed by NAA and BAP in production phase culture[9].

1.3.2 The Effect of Metals on Alkaloid Content

Growing conditions can have significant effect on the biochemical composition of a plant. Exposure to metals in the soil can alter the concentrations of molecules found within plant extracts. Recent studies have been performed on *Catharanthus roseus* plants, commonly known as Madagascar periwinkle, to determine the correlation between metal content in soil and indole alkaloid production. Indole alkaloids are produced from the amino acid L-tryptophan. Several cellular studies showed that adding vanadyl sulphate cultures caused plant cells to increase synthesis of indole alkaloids [11].

A study performed by Lovkova et al. [12] studied results in seedlings grown for 4-6 days at increasing metal concentrations. The authors showed that cobalt, nickel, tungsten, and manganese increased indole alkaloid production. They proposed that cobalt activates an enzyme that is responsible for aromatic amino acid production. Tryptophan is only one of many molecules in the indole alkaloid biosynthesis pathway.

Lovkova et al. noted that increase in alkaloid content of the plant peaked at 0.1mM concentration of cobalt and nickel and 0.001mM concentrations of tungsten and manganese, then decreased.[12] This indicates that the content of metals in soil can be optimized in order to increase alkaloid content in plants and that concentrations of metals that are too high have a negative effect on the plant.

Chromium, copper, molybdate, zinc, and borate were shown to first decrease then increase alkaloid content in seedlings. The authors concluded that these metals must act by suppressing phosphotases thus eventually increasing the phosphoric acid esters

that act as precursors to amino acids that are in turn precursors to alkaloids. Zinc, which activates over a hundred different enzymes, is also thought to increase alkaloid production by involvement with enzymes at a later stage of this pathway, after tryptophan production. Zinc also increases the production of growth hormones in plants. The growth hormone IAA was also thought to stimulate alkaloid production, though cell culture studies with berberine showed the opposite effect[12].

Srivastava and Srivastava [11] doped soil in which *Catharanthus roseus* plants were growing with cadmium, nickel, manganese, and lead 15 days before harvesting them. The plants were harvested when flowering because this is the time when alkaloid content is the highest. They found that all four of the metals increased the alkaloid content of the roots while manganese, lead, and nickel decreased the alkaloid content of the leaves slightly[11]. Although these studies focused on indole alkaloid not benzylisoquinoline alkaloid production, indole alkaloids are produced in the same shikimic acid pathway.

Isoquinoline alkaloids are produced from the amino acid L-Tyrosine rather than L-tryptophan. Metals like cobalt that affect the production of L-tryptophan are likely to affect the production of L-tyrosine. Even though zinc was suspected of activating enzymes after the production of tryptophan, it may play a role in goldenseal alkaloid production. Enzymes affected by zinc concentrations are likely to play a role in oxidation and hydroxylation steps involved in the production of goldenseal alkaloids. However, not all metals should have similar affects. Calcium has also been shown to increase some alkaloids like indole alkaloids and decrease others like tropane alkaloids in plants [13].

No studies were found that investigated the relationship between metal exposure in whole plants and isoquinoline alkaloids like those found in goldenseal. If a correlation can be found between metal content in the soil and increased alkaloid production in goldenseal, growing conditions might be changed to improve yields. Higher alkaloid

content, especially in plant leaves, could lead to the harvesting of fewer plants.

1.3.3 Soil Conditions

The studies that were previously cited all reduced the effects of soil dynamics in order to control the bioavailability of metals. Cell cultural studies obviously do not include soil dynamics. Other studies involved growing seeds and plants in either solutions or silica sands washed in acid before planting. The reason for these simplified design aspects is that naturally occurring soils contain numerous variables in equilibrium with each other. Some of these variables include: total organic carbon (TOC) content, pH, cation exchange capacity (CEC), and total metal concentrations. These variables all affect the solubility and therefore bioavailability of metals.

Soil characteristics exist in a complex dynamic equilibrium. Increasing total organic carbon (TOC) content can increase cation exchange capacity (CEC), but it can also decrease soil pH which decreases CEC. The concentration of aluminum can reduce bioavailability of metals like zinc at soil pH above 6 because aluminum at that pH forms hydoxides that adsorb zinc species. Reducing pH can increase the number of free ions of both species, but also leads to competition for binding sites on soils. Since aluminum adsorbs more strongly, it is more likely to bind, leaving zinc in soil solution. However zinc is more likely to chelate with organic molecules in solution making it less bioavailable than aluminum in the same solution[14]. Soils also contain much higher concentrations of aluminum than zinc and reducing pH will result in higher aluminum concentrations in soil solutions than zinc.

There are many tests available to determine these parameters within soils, but test results are inconsistent and there is no consensus on which soil tests offer the best predictions. For example, one test involving exchanging cations with ammonium acetate and measuring the quantity of metals in the resulting acetate solution is one of the most accepted ways to determine total CEC. The problem with this test is that generally only

easily exchanged ions like magnesium, potassium, and calcium are exchanged for ammonia. The ammonium acetate test proves to be a reliable predictor for soils with pH close to or above neutral, but at a lower soil pH, aluminum and hydrogen occupy some of the negatively charged sites and are not exchanged with ammonia. To accurately determine CEC in acidic soils, an additional test using KCl is required[15].

Another problem stems from the fact that metals behave differently in soil matrices than in water solutions. For example, copper has a high affinity for organic compounds within the soil. Organo-copper complexes are not very pH dependent. This means that soils with a high TOC show less pH dependence on copper bioavailability. Carboxylic acid groups on large organic molecules in soil form strong bonds with most metals. Due to the size of these organic molecules, they are not absorbed by plant roots, making the metals bound to these molecules unavailable to plants. Zinc and copper compete for these sites. Since copper has a higher affinity for organic material binding sites, the total organic matter content plays a larger role in copper bioavailability than pH, whereas pH plays a larger role in the bioavailability of zinc[14].

Copper adsorbs strongly to clay and in calcerous soils to $CaCO_3$. Zinc adsorbs to clay, $CaCO_3$, and metal oxides. While copper forms complexes to organic materials, zinc tends to adsorb to soil exchange sites as well as metal oxides. Many of the zinc species in the soil are soluble at varying pH values.

Manganese, iron, and aluminum form insoluble hydroxides at soil pH above seven. Not only is there a strong correlation between pH and availability of these metals, but at higher soil pH these hydroxides lose protons to basic elements in the soil making them negatively charged exchange sites for other metal complexes. As pH decreases, metal hydroxides dissolve releasing both metal ions formerly bound to the hydroxyl group and metal complexes adsorbed to them. For this reason, zinc bioavialability increases as manganese, iron, aluminum bioavailability increases[15]. In acid soils, manganese and zinc free ions compete with aluminum and hydrogen ions for binding sites. These

sites have higher affinities for hydrogen and aluminum and thus zinc and manganese ions are in higher concentrations in the soil solution than aluminum[14].

Manganese differs from copper and zinc in that it is capable of having many oxidation states in soil, Mn(II), Mn(III), Mn(IV), and Mn(VI). Only the Mn(II) species are available for uptake by plants. Redox conditions like flooding can reduce higher oxidation states of manganese to Mn(II) even at higher pH. Organic carbon content also affects manganese solubility. Like copper, manganese binds to organic compounds in the soil. These compounds are not available to be taken up by the plant, but H^+ released by plants into the soil can exchange with Mn on these compounds releasing the bioavailable Mn^{2+}.[7]

Another soil test to determine CEC involves shaking soil solutions in water. There are many variations concerning the length of shaking time ranging from 15 minutes to 24 hours. In studies, water solutions correlated well to Al concentrations in the plant, but not to Zn, Cu, or Mn concentrations. The stronger correlation to aluminum is largely due to the fact that Al in solutions is often in free ion form which is bioavailable, whereas Zn, Cu, and Mn in water solutions are likely to be in the form of organometallic compounds that are not available for uptake in the plant. While some studies show that plants like goldenseal cannot take up organometallic chelates, the formation of these chelates does not preclude metal uptake.

pH may not be a good measure of bioavailability of metals. Most plants release protons or organic acids into the soil around the root system reducing the pH to improve metal availability. Reducing the pH releases metal ions from organic ligands allowing these ions to enter the root system. Unfortunately, measuring the pH of soils does not accurately predict the pH directly in contact with roots which can be as much as 2.5 pH units lower[14].

The affect that releasing acids has on pH is also dependent on the type of soil. Soils with high clay content have higher buffering capacities. The buffering capacity of the

soil is directly related to the cation exchange capacity (CEC). Clay soils which have the highest buffering capacity also have the highest CEC. Clay has numerous negatively charged binding sites that adsorb cations. These cations can be released to replace protons removed in increasing pH. In decreasing pH these binding sites can adsorb additional protons or Lewis acids[15].

In this study, the soil CEC was measured by the conventional ammonium acetate test as well as the water solution test to gauge soil buffering capacity, soil aluminum availability, as well as soil capacity for ca and mg exchange. pH, total organic carbon, and total soil metal concentration were also measured. These parameters were used to search for links between plant root and plant leaf/stem uptake as well as correlations between soil metal concentration ratios and plant metal concentration ratios.

The complex nature of soil metal biovavailability makes controlling plant uptake too difficult. Most researchers opt instead to use soil solutions for plant studies. The problem with studies in which plants are grown in solution is that they do not accurately model nature. Increasing one metal in solution does not equate to adding the same metal to natural soils. The challenge of this study is to determine the correlation between metal and alkaloid concentrations in light of the interference of so many other variables.

CHAPTER 2

PROJECT DESIGN

This project was designed to determine whether or not soil metal concentrations have any effect on alkaloid content in goldenseal roots and leaves grown organically in native soils. Samples of goldenseal and soil were donated by BotaniPharm LLC, an organic farming cooperative, from locations in the Western Carolina mountain region, Canada, Alabama, Georgia, and Oregon. Samples represent crops from both private and commercial growers. All samples were grown without the use of pesticides.

Inductively coupled plasma optical emission spectrometry (ICP-OES) was used to determine the metal in plant materials as well as the metal concentrations in the soil where the plant was grown. The ICP-OES instrument that was used for this experiment is a Perkin Elmer 3100 using Argon as the carrier gas. Inductively coupled plasma torches use radio frequency to excite the molecules of the carrier gas producing a plasma. Samples are nebulized and carried into the plasma where they collide with the plasma particles as well as each other. These collisions cause atoms to lose and regain electrons. As atoms regain electrons, they release a photon at a wavelength specific to their element. The concentration of each element in a sample is determined by the number of photons that strike the detector. For this project, we digested plant and soil samples in nitric acid to remove all organic matter. Samples were then diluted and placed into the autosampler of ICP-OES.

High pressure liquid chromatography (HPLC) was used to determine the alkaloid content in extracts from roots and leaves of goldenseal. The HPLC instrument that was be used for this experiment is an Agilent 1220 infinity with a variable wavelength detector. A Zorbax eclipse XDB-C_{18} 4.6 $\times$ 150mm, 3.5 μm particle size, 80 $\mathring{A}$ pore size column was used for separation. Standards of concentrations from 10-250 μg/mL were used for calibration. Peak size was measured at 230 nm and used to develop calibration

curves. Roots and leaves were ground separately and extracted with an water/ethanol solution. Concentrations of alkaloids were extrapolated from the calibration data using peak sizes from each sample.

Plant metal concentrations were compared with soil metal concentrations to calculate the percentage of soil metals that were absorbed by the plant. Plant metal concentrations were also plotted against alkaloid concentrations to determine whether or not a linear relationship exists.

2.1 Experimental

2.1.1 Plant Propagation

The plant materials used in this project were grown in various locations in North Carolina (Boone, Cullowhee), Alabama (Mentone), Georgia (Blairesville, Fayetteville, Cummings, Dalton), Canada, and Eugene,Oregon. Plant material from the location in Cullowhee, NC was only collected for the year 2013. Plant materials were harvested in both September of 2012 and September of 2013 from all other locations. [1] Leaf/stem materials were obtained for all locations, while roots were only obtained from Cullowhee, Canada, and Eugene. Plant materials were washed with water and allowed to dry. Soil was also collected in October, 2013 from all locations.

2.1.2 Plant Extracts

Root samples harvested in September 2012, were received as ground, dried material. Root material was ground to approximately 80 mesh in a Retsch Ultra Centrifugal Mill (Newton, PA) and homogenized prior to shipment. Powdered raw material samples were tested as-is. Plant material from Cullowhee, harvested in October 2013, was received in marked plastic bags with the soil in which the plant was grown. Stems

[1] It should be noted that 2013 was an usually wet year in the mountain region with over double the usual annual rainfall. There may be differences in the plant material due to the abnormal weather conditions.

and leaves were separated from roots and tubers. Plant material was gently rinsed, not scrubbed, and all materials were allowed to dry in the open air. Dried plant materials were ground into a fine mash using a using a Willie mini-mill.

All ground materials were extracted in the same manner. Ground plant matter, 250 $\pm$ 20 mg were soaked in 20 mL extraction solvent (50% water, 50% ethanol) in a 50mL conical tube. Extraction solvent was prepared in 500 mL HPLC bottle using 100 mL graduated cylinders. Samples were vortexed for 10 seconds then sonicated for 10 minutes at a setting of 20. After sonication, samples were again votexed for 10 seconds then centrifuged at 5000 rpm for 15 minutes. Supernatant was removed to a 20mL scintillation vial. To prepare vials for HPLC analysis, samples were filtered using a 0.45μm syringe tip filter and a glass syringe. The syringe was rinsed between samples with hot water followed by nanopure water then roughly 2mL of extraction solvent. A syringe filter was screwed onto the syringe. Supernatant(0.5mL) was placed into the syringe. The plunger was then placed into the syringe expelling the supernatant into a 15mL conical tube. The remaining liquid in the syringe was poured back into the original sample container. The solution was then diluted to 10mL. A labeled low actinic glass HPLC vial was filled below the neck with diluted filtrate.

2.1.3 Alkaloid Standards

Preparation of standard solutions - Berberine and Hydrastine stock solutions
Berberine chloride dihydrate and (1R,9S)-(-)- β -hydrastine were obtained from ChromaDex (Santa Ana, CA). Palmatine chloride and tetrahydroberberine (canadine) reference standards, which were used for identification and system suitability purposes, were provided as solutions at concentrations of approximately 1000 g/mL each in wateracetonitrile (90 + 10, v/v).

Stock solutions were prepared by AOAC standards. [1] 500 μ g/mL solutions of standards were prepared by dissolving approximately 6.87mg of berberine chloride di-

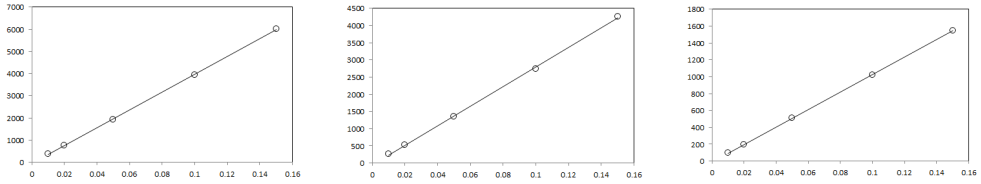
hydrate (73.1% purity berberine) and 5.05mg (1R,9S)-(-)- β -hydrastine (99.0% pure), each in exactly 10mL MeCN/H_2O 10% solutions. Solutions were mixed on a Vortex at room temperature until fully dissolved and stored at $\pm 4^{\circ}$ C and protected from light. To prepare solutions of only berberine serial dilution of the above stock solution was performed using mechanical pipettes and the MeCN/H_2O solution. To prepare a mixed solution of alkaloids, 250 μ g/mL each of berberine and hydrastine from stock solutions were added to 125 μ g/mL concentration of stock canadine solution as shown in Table 2.1. These solutions were stored in same manner as stock solutions.

Table 2.1: Mixed Standards of Alkaloids [1]

Standard Number	Volume (μL) of mixed stock solution	Volume(μL) of ethanol/water solution	Expected concentration in μg/mL of Hydrastine and Berberine	Expected concentration in μg/mL Canadine
1	100 Mix Std 4	900	10	5
2	100 Mix Std 6	900	20	10
3	200	800	50	25
4	400	600	100	50
5	600	400	150	75
6	800	200	200	100

2.1.4 Quantitative Analysis of Alkaloid Content

Calibration Curves and Concentration Calculations were performed according to the AOAC official method. [1]



(a) Berberine 012014 *(b)* Hydrastine 012014 *(c)* Canadine 012014

Figure 2.1: Alkaloid Calibration Curves 012014

The equations of the lines are Berberine Concentration($\frac{\mu g}{\mu L}$) $= 40383peak - 56.21$ with and R^2 of 0.9999, Hydrastine Concentration($\frac{\mu g}{\mu L}$) $= 28559peak - 54.539$ with an R^2 of 0.9995, and Canadine Concentration($\frac{\mu g}{\mu L}$) $= 10354peak - 9.4273$ with an R^2 of 1.[2]

Buffer Solution:

Buffer Solutions were prepared according to protocol designed by Hendrickson Lab at the University of Arkansas for Medical Sciences. Ammonium Acetate (3.87g) was dissolved in about 300mL of nanopure water in a 500mL volumetric flask. The solution was mixed for ten minutes. Glacial Acetic Acid (2mL) was added to give a pH of about 4. Nanopure water was added to volume and the solution was mixed for an additional ten minutes. Buffer solution was made using 510mL of nanopure water, 310mL Acetonitrile, and 100mL of Ammonium Nitrate solution.

Samples were analysized on a Agilent HPLC using a Zorbax eclipse XDB-C_{18} 4.6 $\times$ 150mm, 3.5 μm particle size, 80 $\mathring{A}$ pore size column at 30° C using injection volumes of either 5 or 10 μL and a mobile phase of H_2O:MeCN:Buffer Solution 51:31:10 v:v:v at a flow rate of 1.0 mL per min with UV detection at 230nm.

After equilibrating the column with the mobile phase for at least 10 minutes to obtain a stable baseline, single 10 μL injections of Mixed Standard Numbers 1-5 were made, see Table 2.1. Calculations for the slope, y-intercept, and R^2 value for each calibration curve of canadine, hydrastine and berberine were made made, see Figure 2.1.

Injections (10μL) of each extract solution were made. The following equation was used to calculate the amount of each alkaloid in percent weight:

$$\frac{P_0 - b_0}{m_0} \times \frac{V}{W} \times D \times 100\% \tag{2.1}$$

where P_0 is the area of the peak for berberine or hydrastine, b_0 is the calculated y-intercept, m_0 is the calculated slope, V is the volume of test solution number 1 in mL, D is the dilution factor, and W is the weight of the sample in mg.

[2]Calibration performed on January 20, 2014

2.1.5 Digestion of Plant Materials

All reagents were certified ACS grade. NOTE: All safety precautions were taken, including the use of acid resistant gloves, safety goggles, and lab coats. Powdered goldenseal (0.2 g) was measured and transferred to 15 mL test tubes. Each goldenseal sample was prepared in duplicate. A triplicate blank was also prepared. NIST Standard Reference Material 1515 " Apple Leaves " was analyzed in triplicate. To digest samples, concentrated HNO_3 (3.0 mL) was added to each sample and allowed to sit at room temperature overnight. The samples were then heated at 175°C for 2 hours in a lab-designed heating mantel. After heating, the samples were cooled to room temperature. When cool, 30% H_2O_2 (1 mL) was added to each test tube to further oxidize the samples. The samples were allowed to sit for another 2 hours. The samples were heated again at 175°C for 2 hours in the same manner and allowed to cool.

Table 2.2: Unique Wavelengths of Metals Being Analyzed

Element Symbol (Name)	Wavelength (nm)
Ag (Silver)	328.066
Al (Aluminum)	308.213
As(Arsenic)	188.979
Ba(Barium)	233.525
Ba(Beryllium)	313.102
Bi(Bismuth)	223.058
Ca(Calcium)	317.930
Cd(Cadmium)	228.801
Co(Cobalt)	228.614
Cr(Chromium)	267.706
Cu(Copper)	327.393
Fe(Iron)	238.201
Mg(Magnesium)	285.211
Na(Sodium)	330.237
Ni (Nickel)	231.601
Pb(Lead)	220.352
Sr(Strontium)	232.235
V(Vandium)	290.880
Zn(Zinc)	206.198

After cooling, the digested material was transferred to a 15 mL plastic vial and di-

Analyte	Intercept	Slope	Curvature	Corr. Coef
Al 308.215	81.1	10210	0.00000	0.9999
As 188.979	6.7	223.7	0.00000	0.9999
Bi 223.061	36.4	2406	0.00000	0.9999
V 290.880	932.8	31860	0.00000	0.9998
Cd 228.802	284.3	8395	0.00000	0.9998
Pb 220.353	26.1	1378	0.00000	0.9999
Ag 328.068	1688.0	61080	0.00000	0.9998
Co 228.616	160.4	6124	0.00000	0.9998
Cu 327.393	1024.2	65720	0.00000	0.9999
Fe 238.204	502.3	14050	0.00000	0.9998
Mn 257.610	5824.7	167600	0.00000	0.9998
Zn 206.200	117.8	3755	0.00000	0.9998
Ni 231.604	204.0	8814	0.00000	0.9999
Cr 267.716	620.3	20420	0.00000	0.9998
Be 313.107	11882.8	435300	0.00000	0.9998
Na 330.237	164.9	181.7	0.00000	0.9612
Mg 285.213	1455.9	143700	0.00000	0.9999
Ba 233.527	796.9	23870	0.00000	0.9998
Sr 232.235	13.0	390.3	0.00000	0.9999
Ca 317.933	888.4	24680	0.00000	0.9998

Figure 2.2: Calibration Summary.

luted to 15 mL with nanopure water. For diluted samples, 1.5 mL of the samples were removed to another 15 mL conical tube and nanopure water was added to volume.

2.1.6 Quantitative Analysis of Metal Content

Prepared and diluted samples were analyzed by Inductively Coupled Plasma Optical Emission Spectrometry (Perkin Elmer 3100 ICPOES).

The elements in Table 2.2 were analyzed at the manufacturers recommended wavelengths.

Instrument parameters were set for the following:

1. Plasma support gas (argon) flow: 15 L/min
2. Auxiliary gas (argon) flow: 0.2 L/min
3. Nebulizer gas (argon) flow: 0.8 L/min
4. Radiofrequency Power: 1500W

5. Axial view
6. Sample flow rate: 1.5 mL/min
7. Wash time: 30s
8. Background correction: Near-line, 2-pt
9. 7-pts per peak integration

Calibration was linear and included 5 standards ranging from 0.0 to 10 mg/L solution concentration. Typical standard concentrations were: 0.0, 0.2, 1.0, 2.0, and 10.0 mg/L. The R^2 coefficient for V, Cd, Ag, Co, Fe, Mn, Zn, Cr, Be, Ba, and Ca was 0.9998. The R^2 for Na was 0.9612. Sodium emissions appear in the visible spectrum which is not monitored by the instrument used in this experiment. All of the rest of the metals had correlation coefficients of 0.9999.

2.1.7 pH of Soils

Samples of dried soil (10 $\pm$ 0.25 g) were weighed and placed in a 25mL beaker. Nanopure water (10 mL) was added. The mixture was stirred and allowed to stand for 30 minutes. The pH was measured with a pH probe.

2.1.8 Cation Exchange Capacity of Soils

Cation exchange capacity was measure using two methods, water and ammonium acetate.

Water Method:

Samples of dried soil (1.00 $\pm$ 0.20 g) was weighed and placed into a 50 mL conical tube and 25 mL of nanopure water was added. Each soil sample was measured in duplicate. Mixture was shaken for 15 minutes then filtered into a 15 mL conical tube. Excess solution and soil were discarded. Samples were analyzed on the Perkin-Elmer

3100 ICP-OES by the same procedure as plant metal concentrations above. The cation exchange capacity is equal to the sum of the metal concentrations in the soil.

Ammonium Acetate Method:

Two of each dried soil sample (2.00 $\pm$ 0.25 g) were weighed and placed into a 15 mL conical tube. To the first, 1M $C_2H_3O_2NH_4$ was added to volume. To the second, 0.1M $C_2H_3O_2NH_4$ was added to volume. Solutions were sonicated for 10 minutes then centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and diluted to 100 mL. Fifteen milliliters of diluted samples were transferred to a 15mL conical tube and analyzed on the Perkin-Elmer 3100 ICP-OES by the same procedure as plant metal concentrations above. The cation exchange capacity of the soil is equal to the sum of the metal concentrations.

2.1.9 Total Organic Carbon Content of Soil

Total organic carbon content was determined by thermal decomposition. Crucibles and lids were cleaned with soap and hot water then soaked overnight in a 10% nitric acid bath and finally rinsed with nanopure water and thoroughly dried. Dried soil samples (5.00 $\pm$ 0.25 g) were weighed and placed in a cleaned preweighed ceramic crucible. Samples were analyzed in duplicate. Lids were placed loosely on each crucible, allowing a small amount of air flow. The crucibles with lids were placed in an oven set to 550 °C and heated for one hour. Crucibles were allowed to cool in the oven then were reweighed. The difference in soil mass equates to the mass of organic species of carbon in the soil. [16]

CHAPTER 3

RESULTS

3.1 Alkaloid Concentrations

Analysis was performed on twenty-seven leaf/stem samples from ten different locations. Plant samples arrived dried, ground, and labeled according to the BotaniPharms LLC. cooperative member who supplied them. Two of the samples, Cullowhee 1 and Cullowhee 2 were harvested at Dr. Steve Henson's farm directly and dried and ground in our lab. Samples were all analyzed for alkaloid and metal concentrations to determine if any correlations existed. Tables 3.1 and 3.2 show all of the alkaloid concentrations for leaf/stem materials along with percent relative standard deviations. 2 replicates of each sample were extracted for HPLC analysis of alkaloid concentrations. Note that the samples are arranged by location.

Table 3.1: Alkaloid Concentrations in Leaf/Stem Samples in mmol/kg.

Sample	Location	Berberine	RSD[1]%	Hydrastine	RSD%	Canadine	RSD %
14	Cummings	7.4	7.6	5.0	8.5	0.30	1.4
28	Cummings	4.8	24.9	3.6	16.5	0.30	0.0
Average		6.1	16.2	4.3	12.4	0.30	0.7
29	Mentone	5.7	4.1	4.4	5.1	0.40	0.6
33	Mentone	4.4	6.1	3.7	7.2	0.30	2.7
Average		5.1	5.1	4.0	6.2	0.30	1.7
18	Dalton	4.9	2.2	4.4	4.1	0.20	NA[2]
19	Dalton	5.0	3.7	3.5	4.4	0.30	0.4
32	Dalton	5.0	5.7	5.0	1.8	0.30	1.0
20	Dalton	4.5	19.9	3.1	16.2	0.30	0.6
Average		4.9	7.9	4.0	6.6	0.30	35.8
17	Blairesville	4.9	0.5	3.5	2.5	0.0	0.0
16	Blairesville	3.6	6.7	2.8	8.5	0.0	0.0
Average		4.3	3.6	3.1	5.6	0.0	0.0
22	Boone	4.1	15.3	3.3	11.8	0.30	0.7
Average		4.1	15.3	3.3	11.8	0.30	0.7
30	Fayetteville	3.5	6.7	3.0	7.8	0.20	NA[2]
15	Fayetteville	3.3	5.2	2.8	6.1	0.0	0.0
Average		3.4	5.9	2.9	7.0	0.10	70.7

[1] Relative standard deviation(RSD) are reported in percent of concentration.
[2] Not Available

Table 3.1 shows samples from locations for which no root material was supplied. The RSD for the canadine concentrations of Dalton (sample 18) and Fayetteville (sam-

ple 30) could not be calculated. These two locations had samples in which one sample for the location had a concentration below detection limit and the other sample for that location had a concentration around 3.5 mmol/kg.

Table 3.2 shows samples from locations from which root materials were also supplied. Sample 31 was only extracted one time due to the small quantity of the sample provided. There is no RSD to report. The lack of RSD for sample 31 brings down the RSD for the unknown location when all of the sample RSD's for this location are averaged.

Ranking sample locations from highest to lowest berberine concentration gives: Cummings $>$ Eugene $>$ Mentone $>$ Dalton $>$ Canada $>$ Blairesville $>$ Cullowhee $>$ unknown = Boone $>$ Fayetteville. Hydrastine concentrations descend in same order for the samples in Table 3.1, but notice in table 3.2, the hydrastine concentrations do not vary with the berberine concentrations. Ranking hydrastine concentrations from highest to lowest gives: Cullowhee $>$ Cummings $>$ Mentone = Dalton $>$ Boone $>$ Blairesville = Canada = unknown $>$ Eugene $>$ Fayetteville. Canadine concentrations for Cummings, Mentone, Dalton, Boone, Eugene, Canada, and Cullowhee are the same (0.30 mmol/kg) while Fayetteville had a lower canadine concentration of 0.10 mmol/kg and Blairesville's canadine concentration was too low to determine.

The range of the berberine concentrations in the leaf/stem materials is 3.3 - 7.4 mmol/kg with a mean of 4.778 mol/kg for all samples. This equates to 1.1006 - 2.4947 g/kg with a mean of 1.60880 g/kg. The standard deviation is 8.4 mmol/kg for all of the samples. The first Cummings sample shows high concentrations compared to the rest of the samples (3.1190 standard deviations above the mean). Sample 14 was removed from figures and calculations as an outlier. The standard deviation of all of the remaining leaf/stem samples is 6.7 mmol/kg. The difference between the second highest berberine concentration (5.7 mmol/kg) and the lowest (3.3 mmol/kg) is about 4 standard deviations ($0.00067 \times 3.69346 = 0.00247$).

Table 3.2: Alkaloid Concentrations in Leaf/Stem Samples in mmol/kg.

Sample	Location	Berberine	RSD[1]%	Hydrastine	RSD%	Canadine	RSD %
4	Eugene	5.7	13.2	2.9	10.2	0.30	0.6
2	Eugene	5.6	7.8	3.1	6.4	0.30	1.0
24	Eugene	5.5	3.9	3.2	3.3	0.30	4.9
3	Eugene	5.3	16.8	2.8	17.1	0.30	1.3
25	Eugene	4.9	4.5	2.9	4.1	0.30	3.7
23	Eugene	4.9	4.5	3.1	7.3	0.30	1.9
Average		5.3	8.5	3.0	8.1	0.30	2.2
9	Canada	4.9	11.3	3.0	11.1	0.30	2.1
8	Canada	5.3	0.3	3.5	0.6	0.30	0.3
6	Canada	4.9	1.5	3.2	2.1	0.30	0.5
7	Canada	4.2	1.2	2.6	0.7	0.30	0.6
Average		4.8	3.6	3.1	3.6	0.30	0.9
21	unk	4.2	8.5	2.9	9.1	0.30	0.0
31	unk	4.1	0.0	3.2	0.0	0.0	0.0
Average		4.1	4.2	3.1	4.5	0.20	0.0
34	Cullowhee1	4.0	4.1	4.8	9.7	0.30	3.0
36	Cullowhee2	4.4	1.9	4.6	0.3	0.30	0.9
Average		4.2	3.0	4.7	5.0	0.30	2.0

[1] Relative standard deviation(RSD) are reported in percent of concentration.

The range of the hydrastine concentrations is 2.6 - 5.0 mmol/kg or 1.0750 - 1.9243 g/kg. The mean and standard deviation of this sample set are 3.4 and 6.7 mmol/kg respectively. The difference between the highest concentration of hydrastine and the lowest is 2.4 mmol/kg (3.5821 standard deviations). The range of the canadine concentrations is 0 - 3.0 mmol/kg with a mean of 0.28 mmol/kg. This equates to 0 - 0.1182 g/kg with a mean of 0.0945 g/kg. The standard deviation of the canadine concentrations is 0.126 mmol/kg. The difference between the highest and lowest canadine concentrations is 0.3 mmol/kg which represents 2.7481 standard deviations. This shows that berberine has the most variation and canadine the least amongst the leaf/stem samples.

Table 3.3: Alkaloid Concentrations in Roots in mmol/kg.

Sample	Location	Berberine	RSD%	Hydrastine	RSD %	Canadine	RSD %
26	Eugene	24.9	7.9	10.7	7.9	0.4	4.2
Average		24.9	7.9	10.7	7.9	0.4	4.2
13	unk	23.7	2.5	8.2	3.3	0.40	1.4
10	unk	23.6	14.3	10.0	14.7	0.40	0.0
12	unk	21.9	24.6	7.7	22.9	0.50	1.0
11	unk	21.4	21.2	7.4	19.6	0.40	5.0
Average		22.6	15.6	8.3	15.1	0.40	1.9
1	Canada	16.4	5.0	7.6	4.1	0.40	0.2
5	Canada	13.6	1.2	8.3	0.3	0.40	0.6
27	Canada	11.9	29.7	7.7	26.4	0.30	5.6
Average		14.0	12.0	7.9	10.3	0.40	2.1
37	Cullowhee2	11.3	18.1	7.9	19.8	0.40	4.8
35	Cullowhee1	9.1	3.2	9.1	5.0	0.30	1.3
Average		10.2	10.0	8.5	10.0	0.40	2.8

Table 3.3 shows the root alkaloid concentrations for locations in Table 3.2. Berberine concentrations in roots range from 9.1 - 24.9 mmol/kg which equates to 3.06 - 8.37 g/kg. The average berberine concentration in mmol/kg is 17.8 with a standard deviation of 6.0 mmol/kg. There are 2.6425 standard deviations between the highest and lowest concentrations.

The range of the hydrastine concentrations is 9.1 - 10.7 mmol/kg which equates to 3.5034 - 4.0878 g/kg. The average hydrastine concentration of the root samples in mmol/kg is 8.5 with a standard deviation of 1.1 mmol/kg. The range of the hydrastine concentrations equates to 1.3898 standard deviations. We can see that hydrastine has less variation than berberine with about half the range and one sixth the precision of data points. The canadine concentrations in both the roots and leaves did not vary much. The concentration in the roots was slightly higher (0.40 roots - 0.30 mmol/kg leaf/stem).

Ranking locations by berberine concentration gives: Eugene $>$ unknown $>$ Canada $>$ Cullowhee. This agrees with the ranking for the leaf/stem samples except for the unkown samples. It is possible these samples come from more than one location. Ranking locations by hydrastine concentration gives: Eugene $>$ unknown $>$ Cullowhee $>$ Canada compared with the ranking Cullowhee $>$ Canada = unknown $>$ Eugene for the leaves. This shows that high berberine concentrations in the roots correlates to high concentrations in the leaves, but high concentrations of hydrastine in the roots does not translate to high hydrastine concentrations in the leaves.

3.2 Metals not Considered

Samples were analyzed using ICP-OES for concentrations of aluminum, vanadium, cadmium, lead, silver, cobalt, copper, iron, manganese, zinc, nickel, chromium, sodium, magnesium, barium, strontium, and calcium. Some of these metals were not present in the majority of samples. Table 3.4 shows the leaf/stem samples which con-

Table 3.4: Metal Concentrations in Leaf/Stem Materials in mmol/kg

Samples	Vanadium	Cadmium	Lead	Silver	Cobalt	Nickel	Chromium
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00394
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00124
9	0.00	0.00	0.00	0.00	0.00	0.00415	0.0757
14	2.44	0.00	0.00460	0.00	0.0310	0.0420	1.72
15	0.00233	0.000443	0.00172	0.000618	0.00145	0.000508	0.00190
20	0.00	0.00	0.00	0.00	0.00	0.00	0.00276
21	2.73	0.00	0.00	0.00	0.00	0.00	0.00
36	0.00	0.00	0.00	0.00	0.00	0.00407	0.00

tained vanadium, cadmium, lead, silver, cobalt, nickel, or chromium. Only a small number (less than five) of the leaf/stem samples contain Co, Cd, V, Pb, Ag, Ni,or Cr and the concentrations in those that do are very small. Table 3.5 shows the root samples that contained lead or vanadium. All other root concentrations of V, Pb, Ag, Co, Cd, Cr,and Ni were below the detection limit of the ICP.

Table 3.5: Metal Concentrations in Root Materials in mmol/kg

Sample	Lead	Vanadium
11	0.00498	0.00
35	0.00	0.0166

Table 3.6 shows soil samples that contained lead, cobalt, nickel or chromium. All samples not shown in Tables 3.4, 3.5, and 3.6 did not contain Cd,Pb,Ag,Co,Ni, and Cr.

Table 3.6: Soil Concentrations in Root Materials in mmol/kg

Sample	Lead	Cobalt	Nickel	Chromium
Cullowhee1	0.00845	0.0341	0.0175	0.072
Cullowhee2	0.00725	0.0279	0.0287	0.00857
Cummings	0.00	0.00	0.00	0.0339
Mentone	0.00	0.00	0.00	0.000211

Sodium is difficult to analyze because it is ubiquitous in the environment and water supply. Careful measures were taken to avoid sodium contamination i.e. soaking glassware in an acid wash before use and using nanopure water in sample digestion and dilution, however sodium contamination is still possible. Sodium concentrations were also unreliable because sodium emissions appear in the visible spectrum which is not sensitive in the ICP instrument used in this experiment. Thus sodium was not

considered in the remainder of the thesis either. There were no correlations between barium and alkaloid concentrations and strontium and alkaloid concentrations. There is no reported function of either of these metals in plant metabolism. The data collected for strontium and barium was not reported in this thesis.

3.3 Aluminum and Iron

Tables 3.7 and 3.8 show the aluminum and iron concentrations in the leaf/stem materials. The range of aluminum is 6.7 - 1886.5 mmol/kg or 0.1816 - 50.8990 g/kg. Sample fourteen (1886.5 mmol/kg) from Cummings, Ga is more than three times the second highest concentration of aluminum (512.0 mmol/kg). The mean without sample fourteen is 141.3 mmol/kg with a standard deviation of 143.3 mmol/kg. Sample fourteen is 12.1766 standard deviations away from the mean and as such was removed from graphs as an outlier. In a normal population, 99.7 % of the sample population falls within three standard deviations from the mean. A sample with a value that is over 12 standard deviations from the mean is likely not from the same population as the rest of the samples.

The range of iron concentrations in the leaf/stem materials is 1.2 - 715.4 mmol/kg which equates to 0.0666 - 39.9541 g/kg. Sample fourteen from Cummings, Ga (715.4 mmol/kg) is 4.2664 times higher than the second highest iron concentration (167.7 mmol/kg). The mean without this sample is 48.8 mmol/kg with a standard deviation of 49.4 mmol/kg. Sample fourteen is only 1.2137 standard deviations away from the mean and therefore not an outlier.

Ranking the leaf samples by aluminum concentration gives: Cummings $>$ unknown $>$ Cullowhee $>$ Eugene $>$ Canada $>$ Blairesville $>$ Dalton $>$ Boone $>$ Fayetteville $>$ Mentone. The rankings for the iron concentration are the same except that Fayetteville and Mentone have the same iron concentration.

Comparing these rankings to the berberine concentrations in Tables 3.1 and 3.2,

Table 3.7: Aluminum and Iron Concentrations in Leaf/Stem Samples in mmol/kg.

Sample	Location	Aluminum(mmol/kg)	RSD[1]%	Iron(mmol/kg)	RSD %
14	Cummings	1886.5	0.5	715.4	0.4
28	Cummings	31.0	3.8	10.5	3.5
Average		958.8	2.2	363.0	2.0
29	Mentone	10.5	4.1	2.6	4.2
33	Mentone	9.9	4.9	2.9	4.1
Average		10.2	4.5	2.8	4.2
18	Dalton	65.3	0.4	26.5	0.7
19	Dalton	12.2	4.2	3.4	4.0
32	Dalton	16.9	4.8	4.3	4.1
20	Dalton	12.8	3.9	3.6	3.6
Average		26.8	3.3	9.4	3.1
17	Blairesville	128.8	1.0	39.9	0.4
16	Blairesville	17.9	4.7	4.5	4.0
Average		73.4	2.9	22.2	2.2
22	Boone	24.5	3.8	10.5	3.2
Average		24.5	3.8	10.5	3.2
30	Fayetteville	22.2	4.0	4.5	4.5
15	Fayetteville	6.7	0.1	1.2	0.0
Average		14.5	2.1	2.8	2.3

[1] Relative standard deviation(RSD) are reported in percent of concentration.

Cummings has the highest berberine and aluminum and iron concentrations and Fayetteville the lowest. The order of berberine concentrations highest to lowest does not match the order of aluminum or iron concentrations for the rest of the samples. Hydrastine rankings show Cullowhee had the most hydrastine followed by Cummings. Again Fayetteville had the lowest concentrations of hydrastine as well.

Table 3.8: Aluminum and Iron Concentrations in Leaf/Stem Samples in mmol/kg.

Sample	Location	Aluminum(mmol/kg)	RSD[1]%	Iron(mmol/kg)	RSD %
4	Eugene	236.9	1.6	108.7	0.5
2	Eugene	302.2	0.9	133.1	0.4
24	Eugene	88.0	3.6	31.0	4.3
3	Eugene	301.8	2.2	131.9	0.9
25	Eugene	84.8	4.1	31.3	3.3
23	Eugene	78.8	3.8	27.9	3.0
Average		182.1	2.7	77.3	2.1
8	Canada	211.1	0.7	89.6	0.5
9	Canada	199.9	0.4	70.2	0.2
6	Canada	183.4	1.3	53.3	0.3
7	Canada	117.9	1.6	40.3	0.5
Average		178.1	1.0	63.4	0.38
21	Unknown	519.7	6.2	167.7	4.4
31	Unknown	305.8	4.7	98.1	4.0
Average		412.8	5.5	132.9	4.3
36	Cullowhee 2	438.8	4.6	105.3	3.7
34	Cullowhee 1	246.7	4.9	65.4	3.2
Average		342.8	4.8	85.3	3.5

[1] Relative standard deviation(RSD) are reported in percent of concentration.

Table 3.9 shows the concentrations in the root samples from the locations shown in Table 3.8. Ranking root samples by aluminum concentrations gives: Cullowhee > Murphy > unknown > Canada > Eugene. The root samples have the same ranking for iron. This is interesting because it does not agree with the above order for aluminum and iron rankings.

Table 3.9: Aluminum and Iron Concentrations in Root Samples in mmol/kg.

Sample	Location	Aluminum(mmol/kg)	RSD[1]%	Iron(mmol/kg)	RSD %
26	Eugene	9.2	4.72	3.3	4.80
Average		9.2	4.72	3.3	4.80
1	Canada	30.9	1.60	12.2	2.32
5	Canada	107.1	1.21	37.9	0.41
27	Canada	35.4	3.42	9.1	3.17
Average		57.8	2.08	19.7	1.97
13	Unknown	106.9	1.53	43.2	0.53
10	Unknown	34.8	1.14	12.1	0.46
12	Unknown	98.0	0.48	45.5	0.47
11	Unknown	15.5	2.99	7.0	0.17
Average		63.8	1.5	27.0	0.4
37	Cullowhee2	289.0	5.3	92.5	4.2
35	Cullowhee1	929.0	3.8	219.5	2.5
Average		609.0	4.6	156.0	3.3
44	Murphy	132.7	3.6	66.1	2.8
Average		132.7	3.6	66.1	2.8

[1] Relative standard deviation(RSD) are reported in percent of concentration.

Table 3.10 shows the aluminum and iron concentrations in soils provided in the Fall of 2013 from Mentone, Alabama and Spring of 2014 from Dalton and Cummings, Georgia. The Cullowhee 1 and 2 Samples were harvested directly with the plants growing in them. The Murphy sample was received in Spring 2014 with a root sample grown in the soil. Notice that the Cullowhee samples had the second and third highest aluminum and iron concentrations. This agrees with the Cullowhee 1 and Cullowhee 2 root samples from these soils which had aluminum values 2.7206 and 0.43993 standard deviations above the mean respectively and iron values 2.5920 and 0.6705 standard deviations above the mean respectively. The aluminum and iron concentrations for the leaf samples from these two plants were both above average. The sample from Cullowhee 1 had an aluminum concentration that was 0.7356 standard deviations above

the mean and the sample from Cullowhee 2 was 2.0759 standard deviations above the mean. These two samples were closer to the mean for iron concentrations with values 0.3362 and 1.144 standard deviations above the mean respectively.

What is interesting is that Cullowhee 1 has higher root concentrations of iron and aluminum than Cullowhee 2, yet Cullowhee 2 has higher concentrations than Cullowhee 1 in the soil and leaf/stem materials. Cummings has the highest aluminum and iron concentration compared to the other three samples. The leaf/stem sample number one that was excluded as an outlier for high iron and aluminum concentrations was from the Cummings, Ga location. Ranking the samples by aluminum concentrations gives: Cullowhee > Cummings > Murphy > Mentone > Dalton. Again, this does not agree with rankings for the leaf/stem samples. Cullowhee is the only location for which we have both soil and roots. The ratio of aluminum and iron concentrations in the soil to that in the roots is 6.91 and 3.96 respectively.

Table 3.10: Aluminum and Iron Concentrations in Soil Samples in mmol/kg.

Sample	Location	Aluminum(mmol/kg)	RSD[1]%	Iron(mmol/kg)	RSD %
41	Murphy	863.8	3.5	211.3	1.9
42	Cummings	691.1	3.6	343.5	1.1
43	Dalton	546.4	3.4	151.8	2.3
38	Cullowhee 1	2693.7	4.5	525.4	1.3
39	Cullowhee 2	3156.3	5.1	511.6	1.5
40	Mentone	616.7	3.5	145.3	3.1

[1] Relative standard deviation(RSD) are reported in percent of concentration.

3.4 Copper and Zinc

Table 3.11 and Table 3.12 show copper and zinc concentrations in leaf/stem materials.

The range of copper concentrations in the leaf/stem materials is 0.0 - 5.8 mmol/kg with an average of 0.90 mmol/kg and a standard deviation of 1.5 mmol/kg. Ranking sample locations based on copper concentrations gives: Cummings> Canada > unknown > Eugene > Blairesville > Dalton > Cullowhee > Boone = Fayetteville =

Table 3.11: Copper and Zinc Concentrations in Leaf/Stem Samples in mmol/kg.

Sample	Location	Copper(mmol/kg)	RSD[1]%	Zinc(mmol/kg)	RSD %
14	Cummings	5.8	1.72	10.1	0.70
28	Cummings	0.0	37.85	0.50	4.16
Average		2.9	19.78	5.3	2.43
29	Mentone	0.0	13.98	0.20	8.32
33	Mentone	0.0	18.61	0.20	6.17
Average		0.0	16.30	0.20	7.2
18	Dalton	1.0	0.78	2.2	1.69
19	Dalton	0.0	47.95	0.40	3.17
32	Dalton	0.0	13.83	0.20	8.82
20	Dalton	0.0	41.01	0.40	4.62
Average		0.30	25.89	0.80	4.58
17	Blairesville	1.1	1.11	2.4	0.94
16	Blairesville	0.0	29.56	0.20	5.97
Average		0.60	15.33	1.3	3.46
22	Boone	0.0	40.70	0.40	5.68
Average		0.0	40.70	0.40	5.68
30	Fayetteville	0.0	4.35	0.10	6.91
15	Fayetteville	0.0	0.35	0.0	0.24
Average		0.0	2.35	0.10	3.57

[1] Relative standard deviation(RSD) are reported in percent of concentration.

Mentone = 0.

The range of zinc concentrations in the leaf/stem materials is 0.0 - 10.1 mmol/kg with an average of 1.5 mmol/kg with a standard deviation of 2.1 mmol/kg. Ranking samples based on zinc concentrations gives: Cummings $>$ Canada $>$ unknown $>$ Blairesville $>$ Eugene $>$ Dalton $>$ Cullowhee $>$ Boone $>$ Mentone $>$ Fayetteville which is similar to the copper rankings.

The Cummings sample(14) had copper and zinc concentrations 3.3422 and 4.1960 standard deviations higher than average respectively. The other Cummings sample had a copper concentration below the detection limit of the ICP and a zinc concentration about one third the average. The Eugene samples to have been grown in two separate locations. Samples 2,3, and 4 had copper concentrations of 1.6, 1.6, and m1.3 mol/kg while samples 23, 24, and 25 had copper concentrations of only 0.10 mmol/kg. The Canadian sample 12 had copper and zinc concentrations that were 2.8753 and 0.9164 standard deviations above the average respectively.

The range of the copper concentrations for the root samples was 0.10 - 2.2 mmol/kg with an average of 1.3 mmol/kg and standard deviation of 0.70 mmol/kg. Ranking the

Table 3.12: Copper and Zinc Concentrations in Leaf/Stem Samples in mmol/kg.

Sample	Location	Copper(mmol/kg)	RSD[1]%	Zinc(mmol/kg)	RSD %
4	Eugene	1.3	0.58	2.0	1.56
2	Eugene	1.6	0.55	2.4	1.82
24	Eugene	0.10	16.50	0.30	8.03
3	Eugene	1.6	1.14	2.2	1.58
25	Eugene	0.10	13.25	0.30	5.17
23	Eugene	0.10	17.64	0.30	4.78
Average		0.80	8.28	1.2	3.83
8	Canada	1.8	0.38	2.9	0.92
9	Canada	1.5	0.99	3.2	1.04
6	Canada	1.2	0.81	2.5	1.45
7	Canada	5.1	1.29	3.4	1.65
Average		2.4	0.87	3.0	1.27
21	Unknown	0.60	7.05	1.0	5.77
31	Unknown	1.3	29.56	2.3	5.97
Average		0.90	18.30	1.6	5.87
36	Cullowhee 2	0.10	10.44	0.40	6.75
34	Cullowhee 1	0.0	111.08	0.50	5.07
Average		0.10	60.76	0.50	5.91

[1] Relative standard deviation(RSD) are reported in percent of concentration.

locations based on copper concentration gives: unknown $>$ Canada $>$ Cullowhee $>$ Eugene $>$ Murphy compared to the leaf/stem materials of the same locations Canada $>$ unknown $>$ Eugene $>$ Cullowhee. Cullowhee had copper concentrations ten times higher in roots. Eugene almost twice as much in the roots compared to the leaf/stem material. The Canadian and unknown samples showed more copper in the leaf/stem material than the roots. Canada had copper concentrations of 1.4 in roots and 2.4 mmol/kg in the leaf/stem. Eugene had copper concentrations of 0.10 mmol/kg in the roots and 0.80 mmol/kg in the leaf/stem materials.

The range of the zinc concentrations in the root samples was 0.20 - 200.6 mmol/kg with an average of 3.7 mmol/kg and standard deviation of 3.2 mmol/kg. Ranking the sample locations by zinc concentrations gives: Murphy $>$ unknown $>$ Canada $>$ Cullowhee $>$ Eugene compared to leaf/stem concentration rankings of Canada $>$ unknown $>$ Eugene $>$ Cullowhee. The Murphy root sample had concentrations for zinc over twenty to over five hundred times higher than the averages of other root samples. The Murphy sample is also the only root sample containing copper concentrations below the detection limit of the ICP. Again Cullowhee showed a higher concentration in the

roots compared to the leaf/stem when it comes to zinc. The zinc concentrations in the Cullowhee roots (0.90 mmol/kg) is only about twice the zinc concentration in the leaf/stem materials (0.50 mmol/kg). Canada, however showed a higher zinc concentration in the roots than in the leaves. Eugene had a zinc concentration of 0.40 mmol/kg in the root compared to 1.2 mmol/kg in the leaf/stem materials. The unknown samples showed almost four times higher concentrations of zinc in the root than in the leaf/stem materials.

Table 3.13: Copper and Zinc Concentrations in Root Samples in mmol/kg.

Sample	Location	Copper(mmol/kg)	RSD[1]%	Zinc(mmol/kg)	RSD %
26	Eugene	0.10	10.01	0.40	7.12
Average		0.10	10.01	0.40	7.12
1	Canada	1.7	0.69	3.2	0.86
5	Canada	2.2	0.75	17.1	0.25
27	Canada	0.20	5.75	1.4	3.92
Average		1.4	2.40	4.1	1.68
13	Unknown	1.7	0.53	8.2	0.56
10	Unknown	1.7	0.38	2.8	1.29
12	Unknown	1.7	0.95	8.0	0.38
11	Unknown	1.2	0.66	3.7	0.85
Average		1.6	0.63	5.7	0.77
37	Cullowhee2	1.1	5.17	0.90	5.75
35	Cullowhee1	1.3	4.03	0.90	4.43
Average		1.2	4.60	0.90	5.09
44	Murphy	0.0	26.81	200.6	4.26
Average		0.0	26.81	200.6	4.26

[1] Relative standard deviation(RSD) are reported in percent of concentration.

Table 3.14: Copper and Zinc Concentrations in Soil Samples in mmol/kg.

Sample	Location	Copper(mmol/kg)	RSD[1]%	Zinc(mmol/kg)	RSD %
41	Murphy	0.0	1.52	0.50	8.67
42	Cummings	1.0	7.12	0.30	12.97
43	Dalton	0.0	0.97	0.0	7.39
38	Cullowhee 1	1.3	5.60	1.8	4.67
39	Cullowhee 2	0.80	6.70	2.1	5.65
40	Mentone	0.0	1.11	0.10	22.29

[1] Relative standard deviation(RSD) are reported in percent of concentration.

The range of copper concentrations in the soil was 0.0 - 1.0 mmol/kg with an average of 0.50 mmol/kg and a standard deviation of 0.60 mmol/kg. Ranking the soil samples by copper concentration gives: Cullowhee 1 $>$ Cummings $>$ Cullowhee 2 $>$

Murphy = Dalton = Mentone = zero. Other Murphy and Mentone samples also showed zero copper concentrations. Of the four Dalton, Ga samples, three showed copper concentrations below the detection limit of the ICP, but one had a copper concentration in the leaf/stem materials of 1.0 mmol/kg. Copper may not be evenly distributed in the growing area in Dalton. The soil received likely came from a portion of the property in which one or more of the plants with copper concentrations below the detection limit were grown. Cullowhee 1 which had the highest soil copper concentration had copper concentrations below the detection limit of the ICP in the leaf/stem materials and 1.3 mmol/kg in the roots of the sample harvested from that soil. Cullowhee 2 had a slightly higher copper concentration in the roots (1.1 mmol/kg) than the soil (0.80 mmol/kg) and a low concentration in the leaf/stem materials(0.10 mmol/kg). Cummings had one leaf/stem sample with the highest copper concentration (5.8 mmol/kg) and one with copper concentrations below detection limits.

The range of the zinc concentrations in the soil was 0.0 - 2.1 mmol/kg with an average of 0.80 mmol/kg and 0.90 mmol/kg. Ranking the locations by soil zinc concentrations gives: Cullowhee 2 > Cullowhee 1 > Murphy > Cummings > Mentone > Dalton =0. The Cullowhee soils had zinc concentrations around twice the root concentrations which in turn was about twice the leaf/stem concentrations. Their soil concentrations were slightly different even though their root concentrations were equal. This shows that total soil concentrations are not the only factor in plant uptake. Dalton had zinc concentrations below detection limits in the soil. All four Dalton plant samples had zinc concentrations above detection limits. It is possible that zinc was either washed from the soil in the year between the harvesting of the plants and the collection of the soil. The area received record rainfall in 2013. Mentone soil samples contained half the zinc concentration of the leaf/stem materials. Murphy soil samples did not have the highest zinc concentration, but the highest root zinc concentration.

The Murphy soil concentrations were 3 standard deviations lower than average

while the root samples from roots harvested with the soil were 62 standard deviations higher than the average. The Cummings soil samples had lower concentrations than the leaf/stem samples from both the sample with very high concentrations of other metals and the sample closer to the average concentrations of other metals. The soil samples from Cummings help explain why the first Cummings sample (14) had such high concentrations of copper, but not zinc. It may be that the soil on this property has a very uneven distribution of metals.

Cullowhee 1 and 2 were harvested from two locations on the same property along with the soils in which the plant was growing. We can see that there is some variation in concentrations between these samples, but not as much variation as the Cummings data.

Table 3.15: Manganese Concentrations in Leaf/Stem Samples in mmol/kg.

Sample	Location	Manganese(mmol/kg)	RSD[1]%
14	Cummings	41.1	0.43
28	Cummings	1.8	3.32
Average		21.5	1.88
29	Mentone	2.0	3.66
33	Mentone	2.8	3.38
Average		2.4	3.52
18	Dalton	23.0	0.45
19	Dalton	1.6	3.59
32	Dalton	4.0	3.56
20	Dalton	1.7	3.41
Average		7.6	2.75
17	Blairesville	11.6	0.29
16	Blairesville	1.5	4.12
Average		6.6	2.21
22	Boone	1.0	3.79
Average		1.0	3.79
30	Fayetteville	4.5	3.74
15	Fayetteville	1.2	0.17
Average		2.8	1.95

[1] Relative standard deviation(RSD) are reported in percent of concentration.

3.5 Manganese

Table 3.15 shows the concentration of manganese in leaf/stem materials from locations that did not supply root samples. Table 3.16 shows leaf/stem manganese concentrations in samples for which

both leaf/stem and root materials were supplied. The range of the manganese concentrations is 0.200 - 41.1 mmol/kg with an average of 73.0 mmol/kg and a standard deviation of 9.30 mmol/kg.

Ranking the sample locations by manganese concentrations gives: Cummings > Canada > unknown > Dalton > Blairesville > Cullowhee > Fayetteville > Eugene > Mentone > Boone. The Cummings sample 14 was 3.6443 standard deviations above the average while the other Cummings sample was 0.5897 standard deviations below the average.

The range of manganese concentrations in the root samples is 4.00 - 17.1 mmol/kg with an average of 6.30 mmol/kg and a standard deviation of 4.50 mmol/kg. Ranking the locations by manganese concentration gives: Murphy > Canada > unknown > Cullowhee > Eugene which is the same as the leaf/stem rankings. The leaf/stem concentrations are higher than the root concentrations in all samples but Cullowhee.

Table 3.16: Manganese Concentrations in Leaf/Stem Samples in mmol/kg.

Sample	Location	Manganese(mmol/kg)	RSD[1]%
4	Eugene	4.0	0.53
2	Eugene	5.1	0.52
24	Eugene	0.80	5.25
3	Eugene	4.7	1.03
25	Eugene	0.90	4.31
23	Eugene	0.80	3.55
Average		2.7	2.53
8	Canada	19.6	0.53
9	Canada	13.3	0.26
6	Canada	14.4	0.33
7	Canada	10.7	0.51
Average		14.5	0.41
21	Unknown	4.6	5.14
31	Unknown	17.0	4.12
Average		10.8	4.63
36	Cullowhee 2	3.4	4.04
34	Cullowhee 1	3.2	3.81
Average		3.3	3.92

[1] Relative standard deviation(RSD) are reported in percent of concentration.

The range of manganese concentrations in the soil samples is 4.80 - 34.5 mmol/kg with an average of 17.1 mmol/kg and a standard deviation of 10.8 mmol/kg. Ranking the locations according to manganese concentration gives: Cullowhee 2 > Cullowhee 1

Table 3.17: Manganese Concentrations in Root Samples in mmol/kg.

Sample	Location	Manganese(mmol/kg)	RSD[1]%
26	Eugene	0.40	6.06
Average		0.40	6.06
1	Canada	3.2	0.42
5	Canada	17.1	0.41
27	Canada	3.6	3.17
Average		7.9	1.33
13	Unknown	8.4	0.42
10	Unknown	3.8	0.34
12	Unknown	8.3	0.54
11	Unknown	5.8	0.29
Average		6.6	0.40
37	Cullowhee2	4.8	4.48
35	Cullowhee1	7.4	3.19
Average		6.1	3.83
44	Murphy	119.4	3.88
Average		119.4	3.88

[1] Relative standard deviation(RSD) are reported in percent of concentration.

Table 3.18: Manganese Concentrations in Soil Samples in mmol/kg.

Sample	Location	Manganese(mmol/kg)	RSD[1]%
41	Murphy	7.8	2.99
42	Cummings	14.9	3.28
43	Dalton	17.5	2.56
38	Cullowhee 1	23.0	3.97
39	Cullowhee 2	34.5	4.17
40	Mentone	4.8	3.25

[1] Relative standard deviation(RSD) are reported in percent of concentration.

$>$ Dalton $>$ Cummings $>$ Murphy $>$ Mentone. This is almost the opposite order of the leaf/stem rankings Cummings $>$ Dalton $>$ Cullowhee $>$ Mentone. In the Cullowhee samples, the manganese concentration is highest in the soil followed by the root and then the leaf/stem material. In the Murphy sample, the manganese concentration is significantly higher in the root (119.4 mmol/kg) than the soil (7.80 mmol/kg). The manganese concentrations in the leaf/stem materials of the Cummings sample 14 (41.1 mmol/kg) are also higher than the soil concentrations (7.80 mmol/kg) for that location. The Mentone and Dalton samples had higher concentrations of manganese in the soil than in the roots or leaves.

3.6 Magnesium and Calcium

Table 3.19: Magnesium and Calcium Concentrations in Leaf/Stem Samples in mmol/kg.

Sample	Location	Magnesium(mmol/kg)	RSD[1]%	Calcium(mmol/kg)	RSD %
14	Cummings	899.9	0.29	465.5	0.44
28	Cummings	226.2	1.95	494.8	2.81
Average		563.1	1.12	480.2	1.63
29	Mentone	264.1	1.83	624.7	2.66
33	Mentone	306.8	1.90	644.8	2.99
Average		285.4	1.87	634.7	2.82
18	Dalton	1785.4	0.23	3722.1	0.1641
19	Dalton	226.5	1.93	441.9	2.5604
32	Dalton	299.0	1.79	0.0	2.75
20	Dalton	230.8	1.92	462.7	2.92
Average		635.4	1.47	1156.7	2.10
17	Blairesville	1228.9	0.30	2355.1	0.81
16	Blairesville	226.3	2.59	463.8	3.20
Average		727.6	1.44	1409.5	2.01
22	Boone	254.2	1.98	685.4	2.35
Average		254.2	1.98	685.4	2.35
30	Fayetteville	215.1	2.20	561.5	2.37
15	Fayetteville	18.0	0.08	43.0	0.05
Average		116.5	1.14	302.2	1.21

[1] Relative standard deviation(RSD) are reported in percent of concentration.

Tables 3.19 and 3.20 show the magnesium and calcium concentrations in the leaf/stem materials. The range of magnesium concentrations in the leaf/stem materials is 18.0 - 1785.4 mmol/kg with and average of 570.3 mmol/kg with a standard deviation of 466.2 mmol/kg. Ranking the locations by leaf/stem magnesium concentrations gives: Canada $>$ unknown $>$ Blairesville $>$ Dalton $>$ Eugene $>$ Cummings $>$ Mentone $>$ Boone $>$ Cullowhee $>$ Fayetteville.

The range of calcium concentrations in the leaf/stem materials is 43.0 - 4340.2 mmol/kg with an average of 1424.4 mmol/kg with a standard deviation of 1250.1 mmol/kg. Ranking the locations by leaf/stem calcium gives: Eugene $>$ unknown $>$ Canada $>$ Blairesville $>$ Dalton $>$ Cullowhee $>$ Boone $>$ Mentone $>$ Cummings $>$ Fayetteville.

The range of magnesium concentrations in the roots is 137.4 - 916.8 mmol/kg with an average of 585.5 mmol/kg with a standard deviation of 372.4 mmol/kg. Ranking the locations by magnesium concentrations gives: Murphy $>$ unknown $>$ Canada $>$

Table 3.20: Magnesium and Calcium Concentrations in Leaf/Stem Samples in mmol/kg.

Sample	Location	Magnesium(mmol/kg)	RSD[1]%	Calcium(mmol/kg)	RSD %
4	Eugene	973.7	0.41	3803.5	3.20
2	Eugene	1074.3	0.20	4340.2	0.36
24	Eugene	205.1	2.68	922.2	3.26
3	Eugene	981.8	0.81	3968.2	0.53
25	Eugene	192.0	2.28	858.5	2.58
23	Eugene	178.4	1.64	794.2	2.07
Average		600.9	1.34	2447.8	2.00
8	Canada	894.5	0.66	1684.5	0.41
9	Canada	975.1	0.31	1831.3	0.61
6	Canada	852.5	0.46	1788.0	0.49
7	Canada	856.6	0.22	1680.5	0.22
Average		894.7	0.41	1746.1	0.43
21	Unknown	217.2	3.29	938.2	4.05
31	Unknown	1342.5	2.59	2722.2	3.20
Average		779.9	2.94	1830.2	3.63
36	Cullowhee 2	281.8	2.21	694.7	3.49
34	Cullowhee 1	191.1	2.71	693.5	2.84
Average		236.4	2.46	694.1	3.16

[1] Relative standard deviation(RSD) are reported in percent of concentration.

Eugene > Cullowhee. This is similar to leaf/stem concentration ranking with only unknown and Canada switching order. The magnesium concentrations were higher in the leaf/stem samples than the root samples in all but the unknown samples.

The range of calcium is 77.1 - 527.4 mmol/kg with an average of 301.4 mmol/kg with a standard deviation of 190.5 mmol/kg. Ranking the locations by root calcium concentrations gives : Murphy > unknown > Canada > Cullowhee > Eugene compared to Eugene > Canada > unknown > Cullowhee in the leaf/stem rankings. The root samples had lower concentrations of calcium than the leaf/stem samples from the same location.

The range of magnesium concentrations in the soil is 47.1 - 142.6 mmol/kg with an average of 86.2 mmol/kg and a standard deviation of 37.8 mmol/kg. Ranking the locations by magnesium soil concentrations gives: Cullowhee > Cummings > Murphy > Mentone > Dalton. The order from highest to lowest magnesium concentration of the leaf/stem samples from these locations is Dalton > Cummings > Mentone > Cullowhee.

The range of calcium concentrations in the soil is 18.5 - 65.0 mmol/kg with an

Table 3.21: Magnesium and Calcium Concentrations in Root Samples in mmol/kg.

Sample	Location	Magnesium(mmol/kg)	RSD[1]%	Calcium(mmol/kg)	RSD %
26	Eugene	156.7	2.80	80.2	3.16
Average		156.7	2.80	80.2	3.16
1	Canada	916.8	0.05	421.1	0.30
5	Canada	840.2	0.30	426.9	0.46
27	Canada	149.0	2.18	084.3	2.55
Average		635.3	0.84	310.7	1.10
13	Unknown	853.6	0.46	527.4	0.11
10	Unknown	895.2	0.66	409.6	0.76
12	Unknown	845.6	0.20	20.4	0.47
11	Unknown	888.5	0.15	356.2	0.30
Average		870.7	0.37	453.4	0.41
37	Cullowhee2	171.3	3.09	110.6	4.06
35	Cullowhee1	137.4	2.67	77.1	2.84
Average		154.3	2.88	93.8	3.45
44	Murphy	3103.6	1.92	2448.1	2.46
Average		3103.6	1.92	2448.1	2.46

[1] Relative standard deviation(RSD) are reported in percent of concentration.

average of 44.7 mmol/kg and a standard deviation of 18.3 mmol/kg. Ranking the locations by soil calcium concentrations gives: Mentone $>$ Dalton $>$ Cullowhee $>$ Murphy $>$ Cummings. Leaf/stem concentrations were in similar order, but Mentone was third instead of first. Leaf/stem calcium concentrations are much larger than soil calcium concentrations from the same location.

Table 3.22: Magnesium and Calcium Concentrations in Soil Samples in mmol/kg.

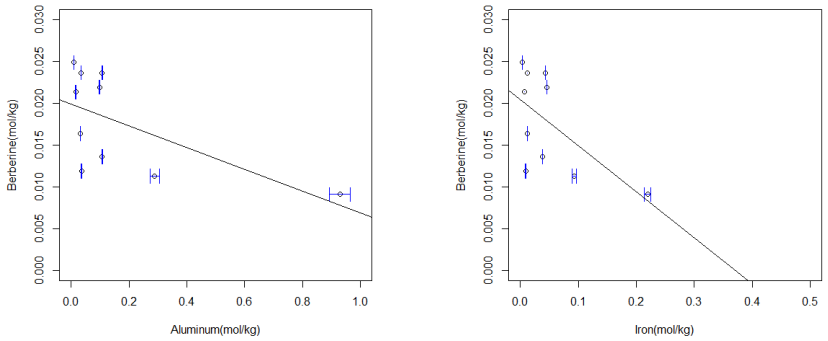
Sample	Location	Magnesium(mmol/kg)	RSD[1]%	Calcium(mmol/kg)	RSD %
41	Murphy	60.9	2.85	40.6	2.33
42	Cummings	95.8	2.87	18.5	3.39
43	Dalton	47.1	2.84	51.2	2.31
38	Cullowhee 1	114.4	4.01	30.4	3.06
39	Cullowhee 2	142.6	4.27	62.5	4.12
40	Mentone	56.5	2.95	65.0	3.04

[1] Relative standard deviation(RSD) are reported in percent of concentration.

3.7 Correlations Between Metals and Alkaloids

Metal concentrations were plotted against berberine, hydrastine, and canadine concentrations in leaf/stem and root materials. The Pearson correlation coefficient, reported as R^2, shows how much of the variance in alkaloid concentrations is related to

variance in the metal concentrations in the plot. Berberine showed the strongest correlations with the metals. Figure 3.1 shows the correlations between berberine and iron and berberine and aluminum in the root materials. The p-values for the plots shown are both 0.06, which indicates a 6% probability that the correlations are random. Note that the correlations are negative. Plots show a peak in berberine concentration close to zero. The peak for alkaloid concentrations for iron occurred around 10 mmolar concentrations. In goldenseal, the highest concentration of berberine occurred between 30 and 40 mmol/kg of iron and near 100 mmol/kg of aluminum. The canadine correlation with iron in the roots is positive, but again the canadine concentration peaks at concentrations near 45 mmol/kg of iron. The correlation between canadine and aluminum is negative with a peak canadine concentration around 100 mmol/kg.



(a) Berberine vs Aluminum in Roots *(b)* Berberine vs Iron in Roots

Figure 3.1: Berberine vs. Aluminum and Iron
The equations of the lines are $Berberine = -0.0131Al + 0.01994$ with an R^2 of 0.3773 and $Berberine = -0.05520Fe + 0.0204$ with an R^2 of 0.3729. The blue error bars represent the relative standard deviations of metal concentrations of replicates.[1]

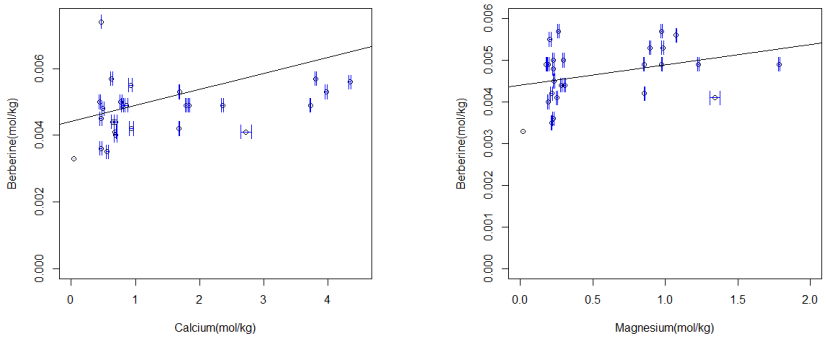
The canadine correlations were weaker and more likely to be random. There were no correlations between iron and aluminum and hydrastine. Table 3.25 shows all of the correlations between the metals and root materials. All of the other metals had positive

[1]Figures created in R

or no correlations to the alkaloids in the root materials.

Figure 3.2 shows the correlations between berberine and calcium and magnesium in the leaf/stem materials. The p-value of the correlation between berberine and calcium is 0.009 (indicates a 0.9% probability that the correlation is random and not from actual relationship between Calcium and Berberine). There is a trend in the plot of increasing berberine with increasing calcium. The relationship may not be linear. There is a slight trough in the region of higher of calcium concentrations. With less interference from other variables, a parabolic relationship might appear between berberine and calcium.

The p-value of the correlation between berberine and magnesium is 0.0894 (indicates a 9% probability that the correlation is random and not from actual relationship between Magnesium and Berberine). Here a parabola can already be seen. Berberine appears to peak when magnesium concentrations are around 1 mol/kg and then decline.

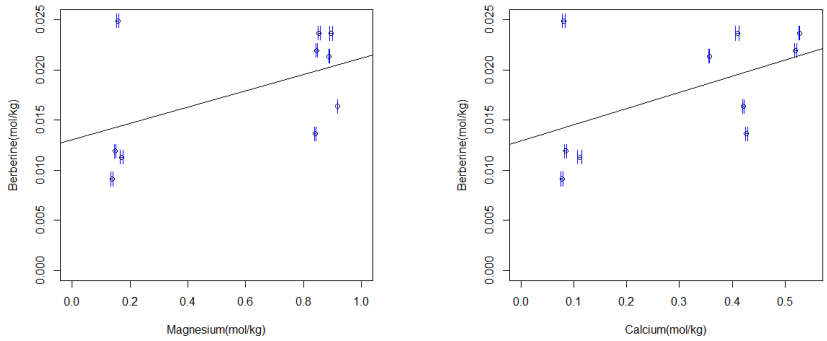


(a) Berberine vs. Calcium *(b)* Berberine vs. Magnesium

Figure 3.2: Berberine vs. Calcium and Magnesium in Leaves
The equations of the lines shown is $Berberine = 0.0003Ca + 0.0043$ with an R^2 value of 0.2472, $Berberine = 0.0005Mg + 0.0044$ with an R^2 value of 0.1155.The blue error bars represent the relative standard deviations of metal concentrations of replicates.[2]

The berberine and calcium plot shown in Figure 3.3 results in an in an R^2 of 0.2649 and p-value of 0.128. It is interesting to note that these points appear in two clusters

[2]Figures created in R



(a) Berberine vs Magnesium in Roots *(b)* Berberine vs Calcium in Roots

Figure 3.3: Berberine vs. Magnesium and Calcium in Roots
The equations of the lines are $Berberine = 0.008122Mg + 0.013020$ with an R^2 of 0.2565 and $Berberine = 0.016132Ca + 0.012913$ with an R^2 of 0.2649. The blue error bars represent the relative standard deviations of metal concentrations of replicates.[3]

around 100 and 45 mmol/kg of calcium with little in between. This may have something to do with the uptake of calcium into the shoots and leaves of the plant. The magnesium and berberine points show a similar clustering around 200 and 900 mmol/kg of magnesium. The p-value for the correlation between berberine and magnesium in the graph is 0.1352 (this indicates an 14% probability that the correlation between magnesium and berberine is random). The relationshps between berberine and calcium and berberine and magnesium appear stronger in the leaf/stem materials with visible nonlinear relationships. The relationships may be more noticeable because there are more leaf/stem samples to plot. With a larger sample size, root samples may show this same parabolic relationship. The clustering on the left and right could be connected.

Due to the large number of graphs, the remaining figures will not be shown here. The pearson correlation coefficients from each plot are shown in Tables 3.23, 3.24, and 3.25. The p-values for the hypothesis test of each correlation are also listed.

In correlations with berberine only magnesium and calcium have correlations that

[3]Figures created in R

Table 3.23: Correlations between Berberine and Metals in Leaf/Stem Materials

Metal	Correlations with Berberine	pvalue[1]	Correlation with Hydrastine	p-value
Al	0.0118	0.60	0.0013	0.86
Fe	0.0657	0.21	0.0283	0.41
Cu	0.0201	0.49	0.1254	0.76
Zn	0.0991	0.12	0.0697	0.19
Mn	0.0226	0.46	0.0070	0.68
Mg	0.1155	0.09	0.0016	0.85
Ca	0.2472	0.01	0.0231	0.46

[1] p-values are probabilities that the correlations were arrived at randomly.

may be valid. However, magnesium has a p-value that is slightly outside the acceptable range. Even if this correlation were considered valid, only about 12% of the variation in berberine is related to variation in magnesium. None of the metal-hydrastine correlations pass the hypothesis test.

Table 3.24 shows the correlations between the metals and canadine in goldenseal leaf/stem materials. None of the metals pass the hypothesis test. The variation of the each of the metals are related to less than 5% of the canadine concentrations.

Table 3.24: Correlations between Canadine and Metals in Leaf/Stem Materials

Metal	Correlations with Canadine with Sample 1	pvalue[1]
Al	0.0133	0.57
Fe	0.0153	0.54
Cu	0.0067	0.68
Zn	0.0024	0.81
Mn	0.0051	0.72
Mg	0.0415	0.31
Ca	0.0027	0.80

[1] p-values are probabilities of the correlations were arrived at randomly.

Table 3.25 shows the correlations between the alkaloids and the metals in the root samples. The berberine-metal correlations are higher in the roots than in the leaf/stem samples. Aluminum and iron show the strongest correlations and magnesium and calcium also show noteworthy correlations. The hydrastine-metal correlations are higher in the root samples for most metals except for calcium and magnesium. Although the correlations for calcium and magnesium are higher, they do not pass the hypothesis test and the correlations are too low to determine whether or not a real correlation exists. The results would be likely be more conclusive more than ten root samples were provided. Overall the canadine-metal correlations are stronger in the roots than in the leaf/stem material. The correlations between calcium and magnesium and canadine are significant (0.5823 and 0.5062 respectively). Zinc also shows a valid correlation to canadine (0.3726).

Table 3.25: Correlations between Alkaloids and Metals in Root Materials

Metal	Correlations with Berberine	pvalue[1]	Correlation with Hydrastine	p-value	Correlation with Canadine	p-value
Al	0.3773	0.06	0.0166	0.72	0.2426	0.15
Fe	0.3729	0.06	0.0012	0.84	0.1943	0.20
Cu	0.0015	0.92	0.0832	0.42	0.1282	0.31
Zn	0.1067	0.36	0.1441	0.28	0.3726	0.06
Mn	0.0629	0.49	0.0972	0.38	0.0004	0.96
Mg	0.2565	0.14	0.0967	0.38	0.5062	0.02
Ca	0.2649	0.13	0.1059	0.36	0.5823	0.01

[1] p-values are probabilities that the correlations were arrived at randomly.

3.8 Soil Analysis

Metal concentrations in soil are measures of both soluble and insoluble species of metal. The majority of soil metals are in insoluble forms that are not available for uptake into plants root systems. There are many factors that determine the bioavailability of metals contained in the soil. A major factor is metal solubility which is a function of soil pH, cation exchange capacity, and total organic carbon content of the soil. CEC is

measured in either milliequivalents of charge per 100 grams of soil or centimoles per kilogram. Centimoles per kilogram is the official SI unit, but these units are equal and can be compared directly.

Table 3.26: Cation Exchange Capacity (mol/kg).

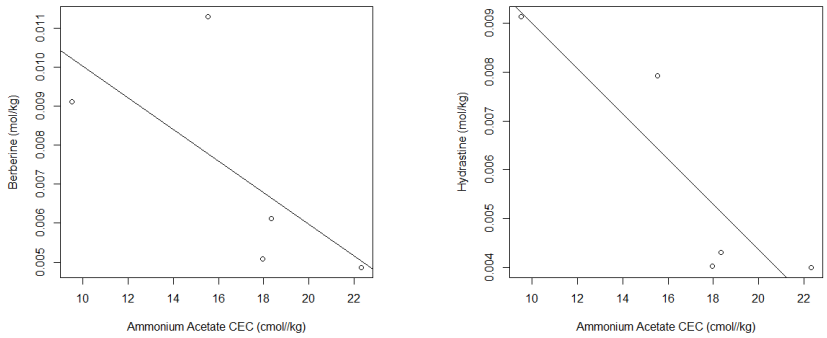
	Water	NH_3Ac	Estimated
Sample	CEC (cmol/kg)	CEC (cmol/kg)	Soil Type
Dalton	5.99	22.32	Clay-Clay Loam
Cullowhee1	1.54	9.53	Sandy Loam
Cullowhee 2	2.25	15.55	Loam
Murphy	1.06	9.74	Loam
Cummings	2.05	18.34	Clay-Clay Loam
Mentone	1.61	17.96	Clay-Clay Loam

Soil textures were estimated by the exchange capacities reported with all of the samples have CEC similar to either loam soils or clay-clay loam soils. These categories are really a measure of the ratios of clay in the soil with loam having a 1:1 ratio between clay and organic matter and clay soils having a 2:1 ratio[15]. At the soil pH and soil types determined for our soil samples, it is likely that 60-80 % of the exchange sites in the soil are taken up by Ca, K, or Mg and the remaining sites are adsorbing Al or H.[17].

There are a number of ways of measuring cation exchange capacity. We compare two, the total number of ions that are soluble in water and the total number of ions that will undergo substitution reactions with another ion, ammonia in this case. After shaking soil samples in either water or ammonium acetate, the supernatant was analyzed using ICP.

In water samples, most samples only contained aluminum and calcium with one sample also containing iron and magnesium and one sample also containing sodium. There were no manganese, zinc, or copper concentrations in the water solutions. It may be that these metals formed chelates to organic materials that were not small enough to pass through the filter paper. It may also be an indication that shaking soil solutions for only 15 minutes is not long enough to extract all soluble ions.

Total CEC was considerably lower in water solutions than in amonium acetate solutions. Only magnesium and calcium were exchanged with ammonium in the acetate solutions. This suggests that aluminum and iron were bound to exchange sites, but were not easily exchangeable in the soil. Aluminum is not typically found using ammonium acetate, likely because trivalent aluminum has a higher affinity for soil exchange sites than monovalent ammonia and thus is not displaced. In acidic soils, where aluminum is present in ionic form, aluminum is usually measured separately by extraction with KCl and then concentrations are added to the total CEC. This experiment was not performed so it is not possible to assess the base concentration in the soil (percentage of exchangeable ions that are either magnesium or calcium).



(a) Berberine vs. CEC *(b)* Hydrastine vs. CEC

Figure 3.4: Alkaloid Concentration in Leaf/Stem Samples vs. Cation Exchange Capacity in Soils.
The equations of the lines shown is $Berberine = -0.0004CEC + 0.0141$ with an R^2 value of 0.4845, $Hydrastine = -0.0005 + 0.0137$ with an R^2 value of 0.7889.[4]

Comparing Table 3.26 and Table 3.27 you can see that cation exchange capacity measured using water did not correlate to berberine or hydrastine concentrations. The CEC determined using ammonium acetate shown in Figure 3.4 showed a 0.4645 correlation with berberine, a 0.7889 correlation with hydrastine, and a 0.3713 correlation with canadine. The berbeine regression has a high p-value(0.2051) indicating a 20 % chance that the correlation was random. The hydrastine regression has a much

lower p-value (0.04411). This is within the standard 5 % margin that is considered acceptable for hypothesis testing. The canadine regression has p-value of 0.2753. Again this is too high to be considered a conclusive relationship.

From Figure 3.4, we can conclude that a definite relationship exists between the exchange capacity of the soil and hydrastine and that this relationship is negative (as seen in the negative slope). The relationships between berberine and canadine were not as conclusive, but also exhibited negative slopes. This indicates that higher retention in the soil of magnesium and calcium may reduce production of the alkaloids.

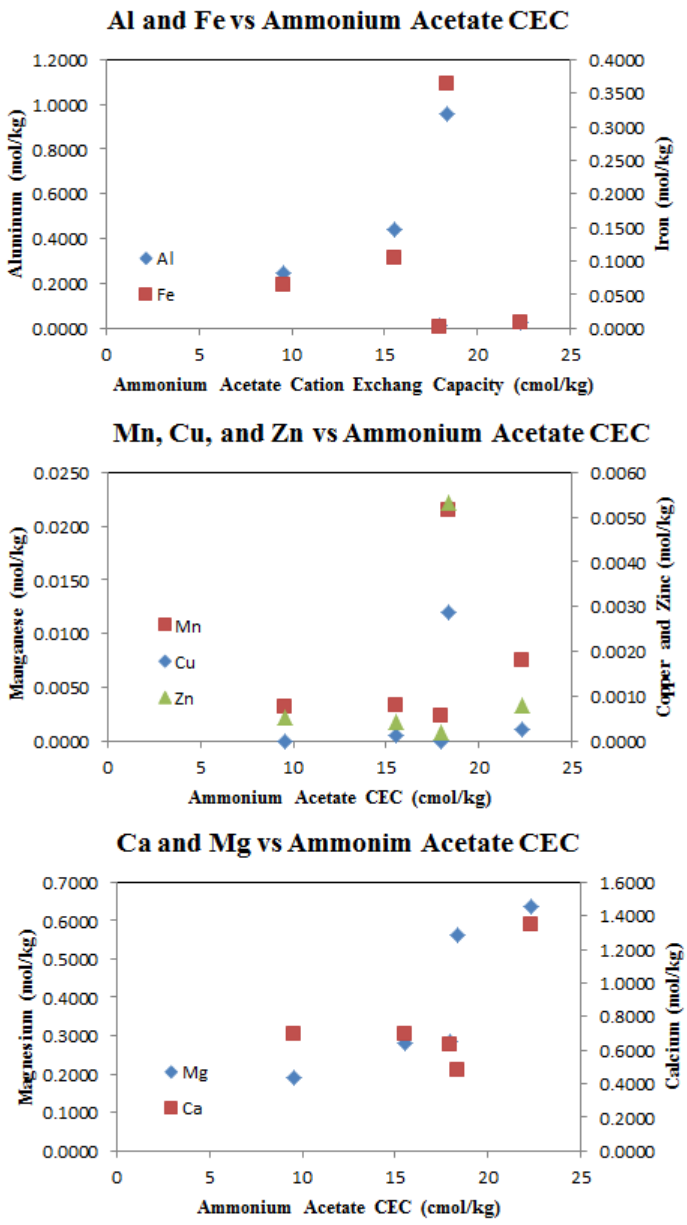


Figure 3.5: Leaf/Stem Metal Concentrations vs CEC

In Figure 3.5, it can be seen that a nonlinear relationships exist between cation exchange capacity and aluminum, iron, calcium, and magnesium. Iron and aluminum concentrations appear to increase exponentially as cation exchange capacity increases

[4]CEC measured with Ammonium Acetate

while magnesium and calcium appear to slowly decline. Dalton and Mentone, seen as the points with high CEC and very low iron and aluminum do not follow this trend.

Dalton has the highest total organic carbon content and Mentone the least (see Table 3.28). These two soils also had the lowest and highest pH respectively. Dalton is also the highest calcium point. There does not appear to be any relationship between CEC and manganese, copper, and zinc concentrations in the leaves. With only six samples for comparison, it is hard to say if these trends are accurate.

Soil pH is another important factor in plant health and metal bioavailability. As pH dips below neutral, metals hydroxides begin to dissolve. Each metal has its own pH dependent solubility, but in general as pH decreases metal solubility increases. Table 3.27 shows there is no apparent connection between soil pH and berberine or hydrastine concentrations. With only 6 soil samples, its perhaps too little data to determine.

Table 3.27: pH of Soil Samples Compared to Averaged Berberine (B) and Hydrastine (H) Concentrations (mol/kg).

Sample	pH	Leaf B	Leaf H	Root B	Root H
Dalton	5.31	0.0049	0.0040	ND[1]	ND
Cullowhee1	5.39	0.0040	0.0048	0.0091	0.0091
Cullowhee2	6.22	0.0044	0.0046	0.0113	0.0079
Murphy	6.09	ND	ND	ND	ND
Cummings	6.15	0.0061	0.0043	ND	ND
Mentone	6.86	0.0051	0.0040	ND	ND

[1] Not Determined. Samples for these locations were not taken.

There also does not appear to be a correlation between pH and iron and aluminum concentrations in the leaf/stem samples. Cummings had the highest iron and aluminum concentrations, but not the lowest pH as expected. pH is only one factor in the bioavailability of metals. Total organic carbon (TOC) and cation exchange capacity also affect the solubility of metals. The biggest factor is probably the total pool of aluminum and iron in the soil. Cummings had the highest aluminum and iron soil concentrations.

No roots were supplied from Dalton, Cummings, or Mentone, but a comparison can be made between the pH, CEC, and TOC to the metal concentrations in the leaf/stem

samples, see Table 3.29.

Table 3.28 shows the total organic carbon in percent mass of the sample. Organic carbon compounds are usually a mix of recently deposited plant materials and decomposed plant materials (humus). Humus can exist as anything from simple sugars, carbohydrates, and proteins to organic acids. Organic carbon compounds have the ability to bind form complexes with metal ions and hydroxides that are soluble in water. In this way TOC content can increase the solubility of metals within the soil.[18]

Table 3.28: Total Organic Carbon Content (TOC) of Soil Samples.

Sample	TOC %
Dalton	22.7477
Cullowhee1	14.5492
Cullowhee2	10.6985
Murphy	12.1646
Cummings	10.1814
Mentone	6.1207

Comparing Table 3.28 to leaf/stem alkaloid concentrations for samples from the same location we see that TOC percentages alone do not appear to affect berberine and hydrastine concentrations. TOC percentages also appear to have no relationship to soil CEC determined with ammonium acetate, but CEC determined with water appears to rise then fall as the percentage of organic carbon increases. This may be because the aluminum and iron were not exchangeable. There does appear to be a direct relationship between pH and TOC. Figure 3.6 shows a negative relationship between total organic carbon and pH. The equation of the line shown in Figure 3.6 is $pH = -0.0924TOC + 7.18$ with an R^2 of 0.8058. This shows that is an 80 % correlation between the TOC and the reduction of pH of the soil. It is not surprising that compounds like organic acids would reduce soil pH.

Aluminum and Iron appear to increase then decrease with increasing pH while Cu, Mn, and Zn decrease as pH increases. The Cummings sample with highest concentrations of all the metals seems to be an outlier just as it is in the leaf samples. Without the outlier, using only the other Cummings sample, the patterns are clearer. Ca and Mg

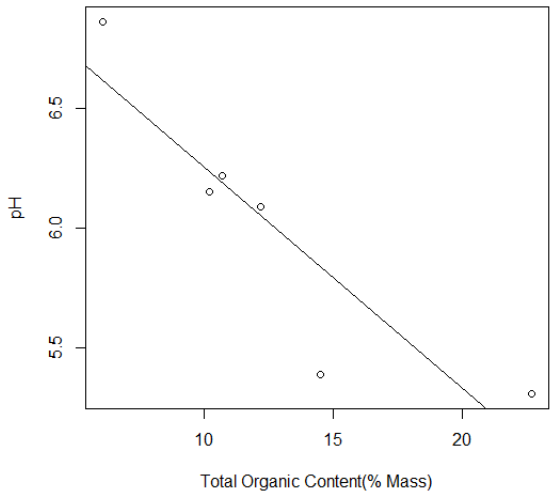


Figure 3.6: pH vs. Total Organic Carbon Percent by Mass.
The black line is the line of best fit. The equation of the line is $pH = -0.0924TOC + 7.1800$. The correlation coefficient is 0.8058.[5]

Table 3.29: Soil Parameters and Leaf/Stem Metal Concentrations (mol/kg)

Location	pH	TOC [1]%	NH_3AC CEC [2]	H_2O CEC [3]
Dalton	5.31	22.7	22.32	5.99
Cullowhee1	5.39	14.5	9.53	1.54
Cullowhee2	6.22	10.7	15.55	2.25
Cummings	6.15	10.2	18.34	2.05
Mentone	6.86	6.1	17.96	1.61

Location	Al	Cu	Fe	Mn	Zn	Mg	Ca
Dalton	0.0315	0.0003	0.0114	0.0095	0.0009	0.7703	1.6461
Cullowhee 1	0.2467	0.0000	0.0654	0.0032	0.0005	0.1911	0.6935
Cullowhee 2	0.4388	0.0001	0.1053	0.0034	0.0004	0.2818	0.6947
Cummings	0.9588	0.0029	0.3630	0.0215	0.0053	0.5631	0.4802
Mentone	0.0102	0.0000	0.0028	0.0024	0.0002	0.2854	0.6347

[1] Total organic carbon in percent mass
[2] Cation exchange capacity in cmol/kg measured using ammonium acetate solution
[3] Cation exchange capacity in cmol/kg measured using ions soluble in water

do not appear to be affected by soil pH.

Manganese, Copper, and Zinc all increase with an increase in TOC. Aluminum appears to decrease with an increase in TOC if we discount the Cummings sample with high concentrations. Iron doesn't really appear to have a relationship with total organic carbon percentage. Magnesium appears to decrease slightly with TOC then increase greatly with TOC values above 15%. Calcium appears to increase slightly with in-

[5]The p-value for this correlation was 0.0387

creasing TOC then increase dramatically with TOC percentages above 15%.

Aluminum and iron have negative correlations to CEC determined using water but a poitive apparently exponential relationship to the CEC determined using ammonium acetate. Mentone and Dalton had the highest water CEC, but only 20 and 71 percent of the ions in the water solutions in these samples came from aluminum. In the other samples almost one hundred percent of the ions in the water solution were aluminum. There appears to be an inverse relationship between aluminum percent and total CEC value. In other words, samples with higher CEC values had larger concentrations of calcium ions in the water solution. This indicates that plants with higher soluble calcium levels had lower aluminum and iron concentrations in their leaves and shoots. Copper, Manganese, and Zinc do not have a clear relationship to CEC determined with ammonium acetate, but show a positive correlation to CEC determined using water. Magnesium and Calcium appear to incease with H_2O CEC with a stronger correlation than to NH_3Ac. Fe, Al, Mg, and Ca were the ions present in water solutions, while only Mg and Ca appeared in the ammonium acetate solution. It is interesting that aluminum and iron show stronger correlations to the ammonium acetate solution and calcium and magnesium to the water solutions.

CHAPTER 4
DISCUSSION

4.1 Uptake of Metals by Plants

Metals are taken into plants and transported within the plant by different mechanisms. All metals come into the plants in soil solutions, therefore how metals enter plants is determined by metal solubility in soil conditions. How metals are transported depends on a number of factors such as charge of the metal, function of the metal within the plant, and concentration of the metal already within in the plant. Information about metal uptake and transport is needed to understand relationships between metal concentrations in the root and leaf/stem materials in goldenseal.

Both iron and aluminum form insoluble precipitates in neutral soils, but become soluble trivalent species in soils with pH below 5. Aluminum is typically found as $Al(H_2O)^{3+}$[19] and iron is usually found as Fe_2O_3. Both can also be found in an insoluble hydroxide or phosphate form. When soluble, iron and aluminum are taken into the roots through diffusion where they attach to cell walls in the root by bonding with carboxyl groups contained in the walls.

In studies performed on tea plants [20], addition of aluminum to the nutrient matrix resulted in reduced iron concentrations within the plant. Aluminum appeared to replace iron in the root hair zone of the rhyzomes. Iron is taken up by the plant and transported from this location [20]. The metal-carboxylate bonds are broken through a reduction reaction with reductases on the cell membrane. Other studies have shown that iron accumulates on cell walls until an iron deficiency occurs in the leaves, indicating that the reductases are activated by a signaling process.

Once reduced, iron and aluminum are transported to the xylem [21]. In a study by Grillet et al., researchers were able to isolate iron chelates from the xylem and char-

acterize them. They discovered that most of the iron within the xylem was a trivalent species chelated to citrate and malate molecules ($Fe(III)_3Cit_2Mal_2$, $Fe(III)_3Cit_3Mal$, and $Fe(III)Cit_2$) [22]. A very small portion of the iron existed as a divalent species bound to nicotinanamine(NA). NA is an aminopropyl polymer synthesized through a condensation reaction of S-adenosylmethionine found all plants. Citrate is known in all plants to transport both iron and aluminum through the xylem, while malate and oxalate have only been identified in certain species to transport iron.

Citrate is a tricarboxylate with a negative three charge while malate is a dicarboxylate with a negative two charge. Citrate bonds to aluminum more strongly than malate does [23]. Aluminum-citrate complexes have a formation constant of 9.6, while aluminum-malate complexes have a formation constant of 5.7. Iron complexes with citrate and malate have higher formation constants.

Ana Flor Lopez-Millan, Fermin Morales, Anunciacion Abadia and Javier Abadia used computer software to predict iron complexes with organic acids in the xylem of iron deficient sugar beets determined that likely iron-citrate complexes would include $[FeCit]^0$, $[FeCitH]^{1+}$, $[FeCitOH]^{1-}$, $[FeCit_2]^{3-}$, and $[Fe2Cit_2(OH)_2]^{2+}$. They reported the formation constants of these chelates to be 13.13, 14.43, 10.11, 20.13, and 24.51 respectively at the pH and ionic conditions within the xylem. The iron-malate species determined to be in the same plant, $[FeMal]^{1+}$, $[Fe_2Mal_2(OH)_2]^0$, $[Fe_2Mal_3(OH)_2]^{2-}$, and $[Fe_3Mal_3(OH)_6]_{1-}$ were calculated have formation constants of be 8.39, 15.32, 20.33, and 27.75, respectively [24].

It is possible that although aluminum displaces iron in root cell walls, accumulating at a higher rate in the roots than iron, that iron is more easily transported because it forms stronger bonds to citrate and malate than aluminum. Once transported through the xylem, iron and aluminum are reduced to divalent species and a ligand swap takes place.

NA which has a charge of $^+2$, has been identified as a transporter molecule that car-

ries iron to other locations within the leaves. Studies on plants with low levels of NA have resulted in plants with high iron concentrations in roots and low concentrations in leaves proving that without NA, iron can be transported into the xylem, but not taken into leaf tissue[21]. NA has high affinity for iron, copper, and zinc.[22]. It is assumed that aluminum must also undergo a ligand exchange and bond to NA in order to leave the xylem and be transported into leaf tissue, but there is no literature on the affinity of NA for aluminum.

Copper and zinc are transported in plants in a similar manner to iron. Iron-regulated transporter proteins bind to zinc and iron and allow them to cross the plasma membrane of the plant. Excess of zinc or iron causes plants to reduce production of these transporters. Since, these transporters have high affinities for both zinc and iron, there is a correlation between zinc and iron concentrations in leaf/stem materials. Metals like copper and zinc are kept from freely entering the plant in the same way as metals like iron and aluminum, they are attracted to and bound to cell walls in the root hairs. Transporters carry these metals across the plasma membrane where they are chelated as discussed previously. These chelates, often organic acids, carry zinc and copper through the root system and into the xylem to be carried into the leaves.

Nicotianamine (NA) also has a high affinity for zinc, copper, and manganese[23]. An Australian study showed that copper mostly binds to amino acids rather than chelating to organic acids as zinc, aluminum, and iron in the xylem. This study also showed a correlation between increases in copper concentrations and increases in NA in the plant. This correlation suggests that copper is more likely to be found in leaf/stem material than root material[14]. The data in Tables 3.11, 3.12, and 3.13 shows that in fact there is more copper in goldenseal leaf materials than root materials for most samples.

Like other metals, manganese is chelated by compounds in the plant in order to prevent free ions from reacting with cells. Manganese is chelated by phenolic compounds and taken into vacuoles. Manganese is also chelated by oxalic acid. Manganese can

be transported by NA and organic acids, but was found to exist mainly as a free ion in the xylem sap[14]. Some proteins that transport calcium and iron, including calcium transporters that remove excess calcium from cells, will transport manganese. High manganese concentrations can interfere with the transport of calcium. Manganese can be precipitated in the roots by forming precipitates with phosphates. This reaction can create the potential for phosphate deficiencies. Manganese has been shown to accumulate more in shoots than roots [7]. This agrees with all of our samples except the Cullowhee location samples. The root concentrations in the Cullowhee samples are about half that of leaf/stem concentrations. It is possible that the Cullowhee roots have higher concentrations of phosphates to precipitate manganese and keep it in the roots as a solid.

4.2 Covariance of Metals

Figure 4.1 shows the covariance of all of the metals studied in the leaf/stem materials of goldenseal. The lower left triangle of the matrix shows the scatter plots of metals in each column with metals in each row. The upper right triangle of the matrix shows the Pearson's correlation coefficient for each metal pair. The size of the font for each correlation coefficient corresponds to the size of the coefficient.

The largest correlations exist between metals with similar transport mechanisms. For example aluminum and iron have a correlation of 0.95. These two metals enter plants as trivalent ions and bind most strongly to cell walls in the root hairs. They are transported by the same organic acids through the roots into the xylem. Copper and zinc also have a correlation coefficient close to 1. These two metals enter the root hairs as divalent ions. They compete to bind to carboxylic acid groups on cells, but make weaker bonds to these functional groups than aluminum and iron. Copper is transported by amino acids as well as the same organic acids that transport zinc, iron and aluminum. The correlations between copper and zinc with iron or aluminum are

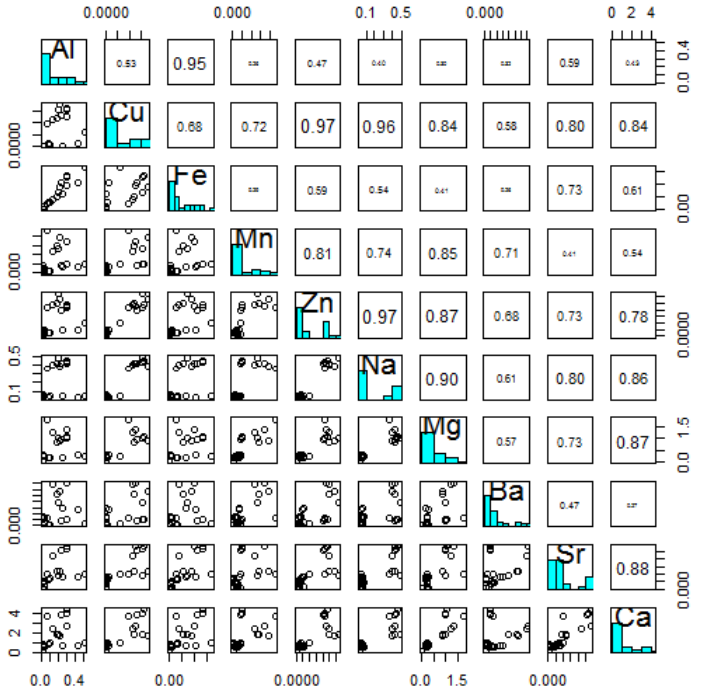


Figure 4.1: Covariance Matrix of Metals in Leaf/Stem

much lower than those of copper with zinc. Calcium and magnesium also have a high correlation coefficient (0.87).

These strong correlations make it impossible to determine whether correlations between each metal and each alkaloid comes from the metal itself or a correlation that metal has with another metal. For example, iron and aluminum showed very similar correlations to berberine in the root materials (0.38 and 0.37 respectively). It is possible that only iron has a relationship to berberine and that the aluminum correlation is merely the result of aluminum's correlation to iron. It is also possible that one of these metals increases berberine while the other decreases it damaging the correlation of both with berberine concentrations. Figure 4.1 shows the need to hold concentrations of all but one metal constant to determine the relationships of each metal with alkaloid concentrations without interference.

4.3 Correlations Between Metals and Alkaloids

The strongest correlations with berberine come from iron and aluminum. Iron is one of the the metals that play roles in the biosynthesis pathway of berberine. Iron is part of the enzyme that helps convert canadine to berberine. It appears that aluminum and iron affect berberine concentrations more strongly than canadine. For iron, this makes sense because the enzyme that is part of the reaction transforming canadine into berberine contains iron. Increasing iron concentrations would be expected to active more of this enzyme. However, the correlations with iron and aluminum in this study were actually negative. Plots show a peak in berberine concentration close to zero. This agrees with Previous studies by L.M. Ya. Lovkova, G. N. Buzuk, S. M. Sokolova, and L. N. Buzuk's study of seedlings. Although they did not test aluminum, but iron in their study first decreased then increased alkaloid concentrations to a peak and decreased alkaloid concentrations above this peak. The peak for alkaloid concentrations for iron occurred around 10 mmolar concentrations. In goldenseal, the highest concentration of berberine occurred between 30 and 40 mmol/kg of iron and mol/kg of aluminum. The canadine correlation with iron in the roots is positive, but again the canadine concentration peaks at concentrations near 45 mmol/kg of iron. The correlation between canadine and aluminum is negative with a peak canadine concentration around 100 mmol/kg

There is no evidence that aluminum is needed by any organism. It has no known role in biosynthethic pathways of primary or secondary metabolites[25]. Aluminum is only likely to increase alkaloid production by causing oxidative stress to the plant. It is possible that the correlation is stronger because iron and aluminum are so strongly correlated that it is affecting aluminum's correlation to berberine. Magnesium and Calcium appear to affect the production of canadine more strongly than that of berberine. Magnesium and Calcium are not involved directly in alkaloid synthesis, but are impor-

tant nutrients. Perhaps plant health plays a larger role in canadine production.

Zinc and copper showed no correlation to any of the alkaloids. Zinc activates enzymes involved in oxydation steps in the biosynthetic pathway that produces alkaloids. Copper activates an enzyme in the beginning steps of berberine production that transforms tyrosine into tyramine so that L-Dopa can be produced. However, copper concentrations do not appear to be as important as iron concentrations to berberine production. This may be because a number or our samples contained copper concentrations below the detection limit of the ICP. There was also no correlation found between manganese and alkaloid concentrations. Manganese takes part in the pathway to produce tyrosine, the amino acid from which all three alkaloids studied are produced. It is probable that berberine is affected by factors that were not held constant in this experiment. Nutritional content and metal concentrations were not controlled. These factors may be interfering with the correlations between berberine and copper, manganese, and zinc.

None of the metal-hydrastine correlations in the leaf/stem materials pass the hypothesis test. Zinc comes the closest with a p-value of 0.19, but has a very weak correlation of 0.07. The branch point between hydrastine and berberine production is (S)-scoulerine. (S)-scoulerine can either be methylated to produce canadine or oxidized to cause a ring opening produce hydrastine. Zinc actives a number of enzymes involved in oxidation steps in plant biochemistry. It is possible that increasing zinc increases hydrastine production through enzyme actions. Additional studies in which some of the variables like concentrations of other metals, plant nutrition, and soil conditions are better controlled might lead to more conclusive results.

In the root samples, the iron and aluminum show reliable correlations with berberine (0.3773 and 0.3729 respectively). Calcium and magnesium also had correlations around 0.25, but had around a 14 % probabilities of being random. Copper, zinc, and manganese did not appear to affect berberine production in the root. The correlations to hydrastine and canadine are generally worse than the correlations with berberine. None

of the metals showed reliable correlations to hydrastine in the root samples.

Many of the metals have noteworthy correlations with canadine in the root samples. Zinc, magnesium, and calcium have correlations with canadine of 0.3726, 0.5067, and 0.5823 with a p-values of 0.0609, 0.02094 and 0.01024 respectively. Zinc is barely significant, but can be used to identify a trend that increasing zinc increases canadine somewhat. Magnesium and Calcium have stronger correlations and a similar trend. All of the other metals had correlations too small to be conclusive.

It appears that aluminum and iron affect berberine concentrations more strongly than canadine. For iron, this makes sense because the enzyme that is part of the reaction transforming canadine into berberine contains iron. Increasing iron concentrations may activate more of this enzyme.

Aluminum is only likely to increase alkaloid production by causing oxidative stress to the plant. It is possible that the relationship is stronger either because goldenseal produces more berberine than canadine or because iron and aluminum are so strongly correlated to each other that it is affecting aluminum's correlation to berberine.

Magnesium and Calcium appear to affect the production of canadine more strongly than that of berberine. Magnesium and Calcium are not involved directly in alkaloid synthesis, but are important nutrients. Perhaps plant health plays a larger role in production of canadine than the production of berberine. Larger, healthier plants produce more alkaloids in general. Since canadine concentrations are small, the increase in nutrition may affect the canadine numbers more strongly.

Trends that are noteworthy in the data are a positive relationship between aluminum and iron with berberine in most samples. The Cummings sample with abnormally high concentrations of both of these metals also had abnormally high concentrations of berberine. Ratios of calcium and aluminum indicate that the Cummings leaf/stem and Cullowhee root samples may have been suffering from aluminum toxicity which can stunt plant growth. However, the higher concentrations of metals in the leaf/stem mate-

rial compared to the root materials suggest that goldenseal may be a hyperaccumulator. Hyperaccumulators have higher tolerances for metals than non-hyperaccumulators and it is possible that the high aluminum concentration did not negatively affect this plant's growth. The dry-mass of the plant samples were not provided by the growers to answer questions about the plant's size.

4.4 Connection Between Individual Sample Metal Concentrations and Alkaloid Concentrations

Previous studies by L.M. Ya. Lovkova, G. N. Buzuk, S. M. Sokolova, and L. N. Buzuk showed that manganese and zinc increased indole alkaloids in Madagascar periwinkle seedlings. Lovkova et al. also reported that there were optimum concentrations for the metals in their study around 0.001mM for manganese and 0.1mM for zinc. Concentrations above this peak caused decreasing concentrations. The whole plant study performed by L. N. K. Srivastava and A. K. Srivastava on the same plant showed that 5mM manganese caused decreases in alkaloid concentrations. This decrease was likely due to the fact that 5mM is much higher than the optimum concentration of 0.001 mM. At 5 millimolar concentrations the plant may experience some manganese toxicity.

In the goldenseal plant materials studied for this project, manganese concentrations ranged between 0.2 and 41.1 mmol/kg of plant material. It is impossible to directly compare the concentrations with Lovkova et al.'s research because we do not know the biomass of the Madagascar perwinkle seedlings or the volume of the solutions with which each seedling was doped. L.M. Ya. Lovkova, G. N. Buzuk, S. M. Sokolova, and L. N. Buzuk only reported the mass of alkaloids in microgram per seedling, but not the seedling mass itself. Lovkova et al. also did not report the exact volume of metal chlorides used to dope seedlings. They doped ashless ribbon until moist. It is not clear how many moles of metal entered each seedling. L. N. K. Srivastava and A. K. Srivastava did report biomass of plants studied and volumes of each metal given. They reported

doping plants with 250mL of the 5mM solutions daily for 6 days. Assuming all of the plants absorbed the entire metal concentrations, that would equate to 7.5 mmoles of each metal. For the plant mass reported, that would give a concentration of 3.191 mol/kg for each plant. This is much higher than the highest goldenseal concentrations, 41.1 mmol/kg manganese in the leaves and 17.1 mmol/kg in the root materials.

Assuming goldenseal has a similar response to manganese as Madagascar periwinkle, alkaloid production would be expected to be elevated, but not optimal for the manganese concentrations determined. No correlations were found between manganese and any of the alkaloids in either the leaf/stem materials or the roots, but several of the plants with above average berberine concentrations in the leaf/stem material had above average manganese concentrations.

Figure 4.2 shows the correlation between berberine and the total concentration of magnesium, calcium, and manganese in leaf/stem samples. The concentrations shown represent the average concentrations of each of the ten locations. While the correlation between calcium and berberine and magnesium and berberine were only 0.25 and 0.12 respectively, the correlation between the sum of calcium, manganese, and magnesium with berberine is much higher at 0.49. It should be noted that the plot of the sum of only magnesium and calcium is similar because manganese concentrations are several orders of magnitude lower than calcium and magnesium concentrations.

Figure 4.2 shows no significant pattern among samples with the highest berberine concentrations. The R^2 of 0.49 and close proximity to the line of best fit shows that equation of the line is a good approximation of the relationship between berberine and these three metals. The slope, however, is extremely small, only 0.0004. This shows that increasing (Mg+Ca+Mn) by one mole only increases berberine by about 0.0004 moles. This suggests that there is no real correlation.

The lack of correlation may stem from two different mechanisms at work, one in which magnesium, calcium, and manganese increase berberine, and one in which nu-

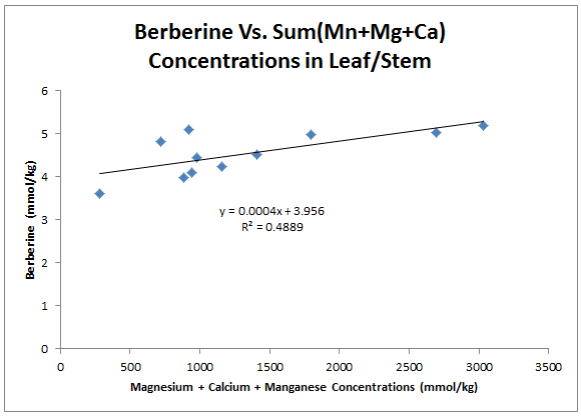


Figure 4.2: Berberine vs Mn, Mg, and Ca.

tritional deficiencies trigger increased berberine production. Most samples with above average berberine concentrations in the leaf/stem material also contained above average concentrations of magnesium, calcium, and manganese. Canada and Dalton. which had above average berberine concentrations, had above average concentrations of manganese, magnesium, and calcium. Blairesville showed average berberine concentrations and average manganese concentrations with above average magnesium and calcium concentrations. These three samples show a possible connection between concentrations of manganese, magnesium, and calcium and berberine.

Cummings and Mentone were the exceptions. Cummings and Mentone both have above average alkaloid concentrations, but below average concentrations of all of the metals. Soil conditions for Mentone samples showed the highest pH of all the samples (6.86) and the lowest total organic carbon content (6.1%). Cummings had a pH of 6.15 and the second lowest TOC (10.2%). These two samples also had zinc concentrations below detection in the soil. Mentone had copper concentrations below detection in the soil. These samples may have been grown in poor soil conditions and suffered nutritional deficiencies. Cullowhee samples also showed slightly below average berberine and manganese, magnesium, and calcium in the leaf/stem material.

In the root, samples from the Cullowhee 1 location show below average berberine,

but above average manganese. The unknown samples showed above average alkaloid and above average manganese, magnesium, and calcium. Eugene samples showed above average alkaloid concentrations with above average magnesium and calcium in the leaf/stem, but below average concentrations of all metals in the roots. There were no soil samples provided for this location, but based on the metal concentrations in the root, these samples fit the low nutrient pattern of the Mentone and Cummings locations.

All of the samples showed higher manganese concentrations in the leaf/stem material than root material, except the two Cullowhee locations. Manganese enters the root as a free ion and binds to phosphates in the roots creating insoluble precipitates that remain in the root material. The fact that Cullowhee samples have higher root concentrations than leaf/stem concentrations of manganese may indicate that Cullowhee plants had higher phosphate concentrations. If Cullowhee had higher phosphate concentrations, the higher berberine concentrations in Dalton, Blairesville, and Canadian samples may be the result of phosphate deficiencies rather than increased manganese concentrations.

4.5 Soil Conditions

Cation exchange capacity measures the total concentration of negatively charged sites on soil surfaces that can attract positive ions. Clay soils and organic materials usually make up most of the soils exchange sites with iron and manganese hydroxides also providing binding sites. The number of exchange sites on organic matter and metal oxides are pH dependent with a higher number of sites at pH's above 7. The pH dependence is due to the loss of protons in basic conditions resulting in negatively charged groups.

Cations adsorb to soil sites with different adsorption strengths depending on their charge and hydrated radius. For example, aluminum with plus three charge has a stronger adsorption than calcium and magnesium with plus two charges. Calcium has a

smaller hydrated radius (0.96) than magnesium (1.08) and thus calcium has a stronger adsorption because it can effectively get closer to the site.

Cation exchange capacity also gives us an idea of how well soils retain nutrients. If metals have soil sites to adsorb to, they are not as likely to wash away in water diffused through the soil by rains. The amount of clay in the soil is important to the buffering capacity and water retention of the soil. Clay has more adsorption sites than sand and therefore can retain acidic elements like H^+ and Al^{3+} that can be released to replace H^+ removed from soil solutions. Clay also retains water better than sandy soils.

Increases in cation exchange capacity in this study correlated to exponential increases in aluminum and iron concentrations in the plant and decreases in magnesium and calcium concentrations. There appears to be an inverse relationship between the percent of the CEC from aluminum ions and total CEC value. In other words, samples with higher CEC values had larger concentrations of calcium ions in the water solution and little or no soluble aluminum. This indicates that plants with higher soluble calcium levels had lower aluminum and iron concentrations in their leaves and shoots. Samples with higher cation exchange capacity had lower concentrations of berberine and hydrastine. A strong correlation (0.78) existed between hydrastine and cation exchange capacity with a low p-value (0.044). The correlation between berberine and CEC was weaker (0.37). Since CEC is a measure of the soil's ability to retain nutrients, higher CEC thus higher retention of nutrients in the soil appears to lead to lower concentrations of hydrastine and berberine. This agrees with the cellular studies that showed that plants produce more berberine when nutrients are less available.

There are no identifiable relationships in the data between the alkaloid concentrations and the soil pH or total organic carbon content. Total organic content showed a strong negative correlation with pH. Organic materials degrade into organic acids in the soil which would naturally decrease the soil pH. The two soil samples, Mentone and Cummings, which had below average concentrations of all metals had the two lowest

total organic. Mentone had a pH close to neutral (6.86) and Cummings had a pH of 6.15. Since pH and TOC are strongly correlated, it not possible to say whether high pH or low TOC caused the low concentrations of metals in the plants. The soils themselves also were low in concentrations of each metal with copper concentrations below detection limits. If a soil sample had been provided for each plant sample, stronger correlations between TOC and metal concentrations may have emerged.

CHAPTER 5

SUMMARY

The goal of our experiment was to determine what, if any, correlations exist between alkaloid concentrations and metal concentrations within soil and plant materials. The strongest correlations with berberine come from iron and aluminum. The correlations to iron and aluminum are both negative, but show peaks at concentrations close to zero (near 35mmol/kg for iron and 100 mmol/kg for aluminum). Iron is one of the three metals that play roles in the biosynthesis pathway of berberine. Iron is part of the enzyme that helps convert canadine to berberine. Aluminum has no known role in the biosynthesis of alkaloids. Aluminum may increase berberine concentrations by creating oxidative stress on the plant. Excess aluminum and iron may cause toxicity in the plant.

Calcium and magnesium showed the highest correlations to berberine production (R^2 = 0.12 and 0.25 respectively) in the leaf/stem materials. The p-value for calcium was above the standard of 5% and may not be reliable. Calcium and magnesium do not play direct roles in the biosynthesis of alkaloids, but may be an indication of plant nutrition which affects the pathway indirectly.

There does appear to be a pattern in the samples with above average berberine concentrations. These samples can be split into two groups. The first group (Eugene, Mentone, and Cummings) had low concentrations of all metals. This group may have suffered from nutrient deficiencies at some point in the growing cycle. Mentone and Cummings showed low total organic carbon and low copper and zinc concentrations in the soil. The metals within the soil may also have been unavailable to plants in these locations. The soil pH values for Mentone and Cummings were 6.86 and 6.15 respectively. Metals in the soil have low solubility pH values near 7. The second group (Dalton, Blairesville, and Canada) showed a connection between elevated manganese, magne-

sium, and calcium and elevated berberine concentrations. Cullowhee samples showed above average manganese concentrations, but below average berberine concentrations. Cullowhee samples were the only samples to have higher manganese concentrations in the roots than the leaf/stem materials. Since manganese binds to phosphates and becomes trapped as an insoluble precipitate in the roots, it is possible that the Cullowhee samples had higher phosphate concentrations in the root materials. If Cullowhee had higher phosphate concentrations, the above average berberine concentrations may be the result of a phosphate deficiency rather than an increase in manganese concentrations.

The metal variables in this study were not independent. A covariance matrix of all of the metals shows strong correlations between many of the metals. Since iron is strongly correlated to aluminum ($R^2 = 0.95$), it is not possible to prove that the correlation between iron and berberine comes from iron's relationship to berberine and not iron's relationship to aluminum. The same problem arises in the calcium and magnesium ($R^2 = 0.87$). With other strong correlations between metals in the study it is also not possible to determine whether or not some of these metals would have shown correlations to the alkaloid concentrations if all other metals had been held constant.

Holding metal variables constant was not the only problem encountered in this study. The plants studied were grown in different soil and nutrient conditions. Cation exchange capacity, total organic carbon content, and soil pH all play a role in determining the availability of soil metals to the plant. With only 5 soil samples, a complete picture of soil parameters is not possible. There appears to be a connection between alkaloid concentrations and cation exchange capacity. All of the alkaloids showed negative correlations between cation exchange capacity and alkaloid concentration ($R^2 =$ 0.47, 0.79, and 0.37 for berberine, hydrastine, and canadine respectively). The general negative relationship with ammonium acetate CEC and alkaloids indicates negative relationship between soil retention of Mg and Ca and alkaloids. These correlations could

have been stronger or weaker if soil samples were available for all locations.

There were also variables that may affect alkaloid concentrations that were not studied. Cellular studies of goldenseal showed that nutritional deficiencies triggered increased berberine production. Phosphate and nitrate concentrations in soil and plant materials were not measured. The negative correlations between alkaloids and cation exchange capacity and pattern in samples with higher manganese concentrations in the leaf/stem materials than root materials suggest that plant nutrition may play a larger role than metal concentrations in alkaloid production. Nutritional variables should be studied in future work.

The results of this study were inconclusive. Correlations between iron and aluminum with berberine and magnesium and calcium with berberine were low. Manganese showed no correlation to berberine, but a pattern in the data suggests there may be a connection between manganese concentrations and berberine production. It is possible that this connection is related to phosphate availability in the root system of the plant. There were no control groups in this study and all the variables were allowed to change in the growing conditions of the samples and some variables like phosphates and nitrates were not studied.

Future studies should address these problems. Plants should be grown in a controlled environment in homoginized soil with the same nutrient content. Experimental groups should hold all but one variable constant. The effects of copper, zinc, manganese, iron, aluminum, calcium, magnesium, phosphate, and nitrate concentrations on both alkaloid concentrations and plant biomass should be studied. Such studies may find stronger correlations between nutrition and alkaloid production.

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